Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61788R2
Full Title:	Identification of EGFR and RAS inhibitors using Caenorhabditis elegans
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Additional Information:	
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TITLE:

Identification of EGFR and RAS inhibitors using *Caenorhabditis elegans*

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KEYWORDS:

Caenorhabditis elegans, LET-60, LET-23, K-RAS, EGFR, GFP

SUMMARY:

The genetically tractable nematode *Caenorhabditis elegans* can be used as a simple and inexpensive model for drug discovery. Described here is a protocol to identify anticancer therapeutics that inhibit the downstream signaling of RAS and EGFR proteins.

ABSTRACT:

The changes in the plasma membrane localization of the Epidermal Growth Factor Receptor (EGFR) and its downstream effector RAS have been implicated in several diseases including cancer. The free-living nematode *C. elegans* possesses an evolutionary and functionally conserved EGFR-RAS-ERK MAP signal cascade which is central for the development of the vulva. Gain of function mutations in RAS homolog LET-60 and EGFR homolog LET-23 induce the generation of visible nonfunctional ectopic pseudovulva along the ventral body wall of these worms. Previously, the multivulval (Muv) phenotype in these worms has been shown to be inhibited by small chemical molecules. Here we describe a protocol for using the worm in a liquid-based assay to identify inhibitors that abolish the activities of EGFR and RAS proteins. Using this assay, we show R-fendiline, an indirect inhibitor of K-RAS, suppresses the Muv phenotype expressed in the *let-60(n1046)* and *let-23(sa62)* mutant worms. The assay is simple, inexpensive, is not time consuming to setup, and can be used as an initial platform for the discovery of anticancer therapeutics.

INTRODUCTION:

The cellular pathways that regulate developmental events within organisms are highly conserved among all metazoans. One such pathway is the EGFR-RAS-ERK mitogen activated protein kinase (MAPK) signaling cascade which is a critical pathway that governs cell proliferation, differentiation, migration and survival[1,2]. Defects in this signaling pathway can lead to pathological or disease states such as cancer. The epidermal growth factor receptor (EGFR) has shown to be highly expressed in human tumors, including 50% of oral squamous cell carcinomas (OSCCs), and contributes to the development of malignant tumors[3-5]. Whereas mutations in the three RAS isoforms H-, K- and N-RAS are major drivers for malignant transformation in multiple human cancers. Amongst these three RAS isoforms, oncogenic mutations in K-RAS are most prevalent[6-8]. For EGFR and RAS to function, they must localize to the plasma membrane (PM). Preventing the localization of these molecules to the PM can completely abrogate the biological activity of this signal pathway[9,10]. Hence the inhibition of the localization of these proteins to the PM is a therapeutic strategy to block the downstream signaling and the resulting adverse outcomes. Using a high-content screening assay, fendiline, an L-type calcium channel blocker, was identified as an inhibitor of K-RAS activity[11]. Nanoclustering of K-RAS to the inner leaflet of the PM is significantly reduced in the presence of fendiline. Furthermore, K-RAS is redistributed from the plasma membrane to the endoplasmic reticulum (ER), Golgi apparatus, endosomes, and cytosol. More importantly, the proliferation of pancreatic, colon, lung, and endometrial cancer cell lines expressing oncogenic mutant K-RAS is blocked by the inhibition of downstream signaling by fendiline[11]. These data suggest fendiline functions as a specific K-RAS anticancer therapeutic that causes the mis-localization of the RAS protein to the plasma membrane.

The nematode *Caenorhabditis elegans* has been extensively studied in the context of development. Many of the signal pathways that govern development in the worm are evolutionary and functionally conserved. For example, the EGFR mediated activation of RAS and the subsequent activation of the ERK MAPK signal cascade is conserved in the worm[12]. The cascade is represented by the following proteins: LET-23 > LET-60 > LIN-45 > MEK-2 > MPK-1. LET-60 is homologous to RAS, while LET-23 is homologous to EGFR. In the worm, this pathway regulates the development of the vulva[13]. The vulva is an epithelial aperture on the ventral body wall of the worm that allows fertilized eggs to be laid. The formation of the vulva in the worm is dependent on the exposure of the vulval precursor cells (VPC) to a gradient of activation of the EGFR-RAS-MAPK signal cascade. During the normal development, the proximal VPCs receive strong signals from the gonadal anchor cells to differentiate into 1˚ and 2˚ cell fates which give rise to a functional vulva[12]. Whereas distal VPCs differentiate into 3˚ cell fates that fuse to the hypodermal syncytium and do not form vulva due to depleted signaling. In the absence of signaling, all VPCs differentiate into 3˚ cell fates resulting in the formation of no vulva. However, constitutive signaling leads to the formation one or more non-functional vulva due to the induction of all VPCs to assume a 1˚ and 2˚ cell fates.

Mutations that cause defective or excessive vulval induction have been identified for many of the genes that encode for proteins representing this pathway. Defective vulval induction results in a vulvaless (Vul) phenotype, while excessive vulval induction results in a multivulva (Muv) phenotype that is represented by the development of numerous nonfunctional ectopic

pseudovulvae throughout the ventral body wall. The Muv phenotype expressed by the *let-60(n1046)* strain is due to a gain of function mutation in RAS, while in the *let-23(sa62)* strain it is due an activating mutation in EGFR[14,15]. The strong Muv phenotype in these mutant strains has been shown to be perturbed by pharmacological interventions as demonstrated by the treatment of *let-60(n1046)* worms with the MEK-1 inhibitor U0126[16,17]. Interestingly, we have shown that R-fendiline and inhibitors that affect sphingomyelin metabolism suppress the Muv phenotype in the worm[18]. To demonstrate these inhibitors block *let-60* signaling at the level of RAS, the *lin-1* null strain has been utilized[17]. Lin-1 is an Ets-like inhibitory transcription factor that functions as a repressor in the development of the vulva[19]. Strong reversion of the Muv phenotype in *let-60(n1046)* worms and no effect on *lin-1* null worms suggest that these inhibitions occur at the level of RAS.

In this protocol, we demonstrate the use of *C. elegans* as a model to identify inhibitors of RAS and EGFR proteins. Using a liquid-based assay, we demonstrate the inhibitory effects of R-fendiline by suppressing the Muv phenotypes in *the let-60(n1046)* and *let-23(sa62)* mutant strains of *C. elegans*. This assay validates the use of *C. elegans* as tool in the initial phase of drug discovery for anticancer therapeutics.

PROTOCOL:

1. Nematode growth medium (NGM) plate preparation

1.1. Add 2.5 g of peptone and 3 g of NaCl to 970 mL of deionized water contained in a 2 L Erlenmeyer flask. Stir contents using a magnetic stir bar. Thereafter, add 20 g of agar to the flask. Autoclave the contents of the flask at 121 °C and a pressure of 15 lb/in^2 for 30 min. After sterilization, place the flask on a stir plate and allow the medium to cool until the temperature reaches 50 ˚C.

1.2. To prepare the NGM plates add the following reagents to the cooled medium: 25 mL of 1 M potassium phosphate buffer (pH = 6.0), 1 mL of 1 M $MgSO_4$, 1 mL of 1 M $CaCl_2$, 1 mL of (5 mg/mL in 95% ethanol) cholesterol, 1 mL of (10% v/w in ethanol) nystatin, and 1 mL of 25 mg/mL streptomycin.

1.3. Under a laminar flow, pour the cooled medium into 60 mm x 15 mm sterile Petri dishes. Let the plates solidify for 2 h. These plates can be kept for 1 month at 4 °C.

2. Propagation of *C. elegans*

2.1. Spot 50 µL of overnight grown *E. coli* OP50 onto the center of each NGM plate and allow the plates to dry for 24 h in a laminar hood. Subsequently, the plates can be stored in polystyrene container.

NOTE: The *E. coli* OP50 culture is grown in Luria-Bertani (LB) media at 37 ˚C media prior to seeding of the plates. The culture can be stored at 4 ˚C for 1–2 months and used for periodic seeding of plates.

2.2. Using a sterile worm pick, gather 10–12 gravid adult worms from a previously grown plate under a dissecting microscope. Transfer these worms to a fresh NGM plate seeded with *E. coli* OP50 and incubate the plate for 24 h at 20 ˚C.

2.3. After 24 h, using a sterile worm-pick remove adult worms from the plates. Incubate the plates at 20 °C for ~ 3 days. Embryos will develop into gravid adult worms.

3. Preparation of a synchronous *C. elegans* culture

3.1. Collect gravid adult worms into a 15 mL conical tube by washing 2–4 plates with M9W.

3.1.1. Preparation of M9W: Dissolve 5 g of NaCl, 6 g of Na_2HPO_4, and 3 g of KH_2PO_4 in deionized water to a final volume of 1 L. Autoclave the solution at 121 °C at a pressure of 15 lb/in^2 for 30 min. Add 1 mL of 1 M $MgSO_4$ to the cooled solution.

3.2. Pellet the worms by centrifuging the tube at 450 x *g* for 1 min. Decant the supernatant without disturbing the worm pellet.

3.3. Prepare worm lysis solution by combining 400 µL of 8.25% sodium hypochlorite (household bleach) and 100 µL of 5 N NaOH. Add this solution to the worm pellet and flick the tube to mix contents of the tube. Prevent overbleaching of the embryos in the tube by observing the lysis of the worms under a dissecting microscope.

3.4. Add 10 mL of M9W to the conical tube to dilute the lysis mix when 70% of the adult worms have lysed.

3.5. Centrifuge the tube at 450 x *g* for 1 min. Replace the supernatant with 10 mL of M9W.

3.6. Repeat step 3.5 two times.

3.7. After completing the washing steps, add 3–5 mL of M9W to resuspend the egg pellet. Rotate the tube overnight at a speed of 18 rpm on a tube rotator at room temperature (RT).

3.8. After overnight incubation, remove the tube from the rotator, pellet the L1 larvae by centrifuging the tube at 450 x *g* for 1 min. Aspirate the M9W until 250 µL is left in the tube.

3.9. Shake the tube to resuspend the larvae. Add three 5 µL drops containing the larvae onto a Petri dish lid. Using a dissecting microscope count the number of worms in each drop and determine the number of worms in 1 µL.

4. Preparation of drug assays

NOTE: The steps in this assay are shown in **Figure 1**.

4.1. Grow 30 mL of *E. coli* OP50 in a 50 mL conical tube at 37 °C overnight in an orbital shaker at 150 rpm.

4.2. Spin overnight grown *E. coli* OP50 culture at 4,000 x *g* for 10 min to pellet the cells. Remove the supernatant and resuspend the pellet in 3 mL of M9W to concentrate the culture.

4.3. Prior to preparing the working solutions for the drug assay, add 0.1 mL of (5 mg/mL in 95% ethanol) cholesterol into 100 mL of M9W.

4.4. Prepare working solutions of each experimental drug by diluting each drug plus vehicle (dimethyl sulfoxide; DMSO) in 4.8 mL M9W supplemented with cholesterol to obtain the appropriate concentrations. Dissolve DMSO in 4.8 mL of M9W supplemented with cholesterol to prepare the vehicle control.

4.5. Add 200 µL of concentrated *E. coli* OP50 culture to each tube containing the vehicle control or drugs and vortex tubes to ensure they are mixed.

4.6. To perform the experiment, add 2 mL 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.

4.7. Add ~100 L1 larvae per well (see synchronous *C. elegans* culture steps) using a sterile micropipette. Limit the volume of worms to 10 mL.

4.8. Incubate the plates at 20 °C.

4.9. Supplement wells with 50 mL of 10x concentrated *E. coli* OP50 on day 3 of the assay if needed.

5. Agarose pad preparation for microscopy

5.1. Prepare a 2% w/v solution of agarose by dissolving 0.1 g of agarose in 5 mL of deionized water. Heat the contents in a microwave to dissolve the agarose. 20 slides can be prepared from 5 mL of agarose solution.

5.2. Place strips of lab tape along two glass slides. The tape will act as spacers limiting the thickness of the agarose pads. Thereafter, place a third clean glass slide between the taped slides.

5.3. To make an agarose pad, spot 100 µL of molten agarose onto the center of the clean slide. Place another clean glass slide across and on top of the agarose and gently press down to form a pad. Remove the top slide when the pad has solidified.

6. Observation of the Muv phenotype in the *let-60, let-23 and lin-1* strains

NOTE: Only candidate drugs that suppress the Muv phenotypes in *let-23* and *let-60* strains will be assayed using the *lin-1* strain to determine if the inhibition occurs at the level of RAS or EGFR.

6.1. When the appropriate stage of the life cycle is reached (3 days for the *let-60* and *let-23* strains, 5 days for the *lin-1* strain), remove the plates from the incubator. and collect the worms in 15 mL conical tubes using a pipette.

6.2. Centrifuge the tubes at 450 x *g* for 1 min. Remove M9W without disturbing the worm pellet and add 5 mL of fresh M9W.

6.3. Repeat step 6.2 two times.

6.4. Without disturbing the worm pellet, remove all remaining M9W by aspiration. Thereafter, add 500 µL of 2 mM sodium azide or 2 mM tetramisole hydrochloride. Allow tubes to incubate at RT for 15 min. 2 mM sodium azide or 2 mM tetramisole hydrochloride will anesthetize the worms for imaging.

CAUTION: Make sure to wear personal protective equipment (PPE) when handling sodium azide. The solution must be prepared under a chemical hood.

6.5. Add 10 µL of the anesthetized worm suspension onto the center of an agarose pad. Place a #1.5 coverslip gently over the worm suspension. If needed, fix the coverslip with some nail polish to prevent drying out.

6.6. Use a DIC microscope to observe the *let-60(n1046)*, *let-23(sa62)* and *lin-1(sy254)* strains. Image the worms at the 10x and 20x magnifications.

6.7. For *let-60(n1046)* and *let-23(sa62),* score the adult worms based on the presence or absence of the Muv phenotype. While for the *lin-1* strain, count the number VPCs that adopted 1˚ or 2˚ cell fates on the ventral side of L4 larvae using a high resolution DIC microscope.

6.8. After scoring the micrographs for each strain and drug treatment, perform Student's *t*-test to determine the statistical differences between the treatments.

REPRESENTATIVE RESULTS:

We first demonstrate that R-fendiline is able to suppress the Muv phenotype in the *let-60(n1046)* mutant strain compared to the DMSO treated worms. Our data shows that R-fendiline is able to block the Muv phenotype in the *let-60(n1046)* in a dose-dependent manner (**Figure 2A,B**).

However, non-reversal of the Muv phenotype was observed in the *lin-1* null mutant strain in response to increasing concentrations of R-fendiline (**Figure 2B**). The data suggests that R-fendiline blocks activated *let-60* signaling at the level of RAS in *C. elegans*. Similarly, we observed the Muv phenotype was significantly reduced in the *let-23(sa62)* strain in response to 3, 10 and 30 µM R-fendiline treatment relative to the DMSO treated worms (**Figure 2C,D**). In all experiments, Students *t*-test was used to determine the statistical significance.

FIGURE AND TABLE LEGENDS:

Figure 1: Flowchart representing the steps involved in preparing the drug assays using *let-60(n1046)*, *let-23(sa62)* and *lin-1(sy254)* strains.

Figure 2: R-fendiline alters *let-60* and *let-23* function in *C. elegans* in a dose-dependent manner. (**A**) Representative images of *let-60(n1046)* worms in the presence of vehicle (DMSO) or 30µM R-fendiline. (**B**) Quantification of Muv phenotype in *let-60(n1046)* worms treated in the presence of DMSO, 3, 10 and 30 µM R-fendiline, or 30 µM U0126. (**C)** Representative images of *let-23(sa62)* worms in the presence of vehicle (DMSO) or 30 µM R-fendiline. **(D)** Quantification of Muv phenotype in *let-23(sa62)* worms treated in the presence of DMSO, 3, 10 and 30 µM R-fendiline. In all images the pseudovulva are indicated by white arrows and normal vulva by white asterisks. A total of 60 worms were imaged for each treatment. The experiment was repeated 3 times. (*** $P<0.001$ and ** $P<0.01$ were considered significant)

DISCUSSION:

The assays we describe using the worm are simple and inexpensive to identify inhibitors of EGFR and RAS function. *C. elegans* is an attractive model for drug discovery because it is easy to grow in the lab due to the short life cycle (3 days at 20 ˚C) and the ability to generate large numbers of larvae. More importantly, the EGFR-RAS-ERK MAPK pathway is evolutionarily and functionally conserved with mammals providing a genetically tractable system to analyze the effects of EGFR and RAS inhibitors. Further, the transparent nature of the worms enables an investigator to visualize distinct structures and the localization of Green fluorescent Protein (GFP) or other fluorophore fused to proteins of interest by DIC and fluorescent microscopy.

The protocols we used to propagation and maintain the various *C. elegans* used in this study were previously established[20,21]. However, in the preparation of NG plates we incorporated streptomycin and nystatin to prevent bacterial and fungal contamination. The addition of these antimicrobial agents did not impede the development of the worms and the induction of Muv phenotype.

There are several advantages for obtaining L1 larvae by the worm synchronization protocol. Many larvae can be obtained from 2 or more plates containing gravid adults and the larvae collected are all age synchronized. This ensures development of the worms is consistent within the population. Some mutant strains displaying the Muv phenotype are poor egg layers resulting in low yields of larvae as seen in the null mutation harbored in the Ets family transcription factor

lin-1[19]. Bleach treatment of the *lin-1* gravid adults will significantly increase the number of larvae required for the assay.

It is vital to observe the lysis of the worms during the preparation of a synchronous *C. elegans* culture. The thick eggshell partially protects the embryos from the action of the bleach-sodium hydroxide mix even as the cuticle of the larvae and adults dissolve. However, prolonged exposure to the lysis solution will penetrate this protective casing leading to the death of the embryos. Hence it is important to stop the action of the bleach-sodium hydroxide mix when 70% of the adult worms have lysed. This is achieved by diluting the lysis solution with M9W. The maintenance of the arrested L1 larvae is another step to consider in the synchronous *C. elegans* culture protocol. Prolonged incubation of the L1 arrested larvae in M9W can cause them to transform into the dauer stage due to accumulation of the dauer pheromone. To avoid the formation of the dauer stage, it is suggested to use the L1 arrested larvae within 1–2 days of collecting the embryos.

The basal Muv phenotype is 60%–90% and 90% for the *let-60(n1046)* and *let-23(sa62)* worms, respectively. This suggests the *let-60(n1046)* worms are subject to phenotypic drift and, therefore, it is important to report the basal levels of expression of the Muv phenotype. Treatment of *let-60(n1046)* and *let-23(sa62)* worms with R-fendiline and other K-RAS inhibitors reduce the percentage of worms expressing the Muv phenotype. However, it is also reported that some inhibitors of the EGFR-RAS-ERK MAPK pathway may reduce the number of pseudovulvae per worm alone or may affect both the expression and the number pseudovulvae[17]. Hence it is important to count the number of pseudovulvae in the Muv worms in both drug and DMSO treated worms. The Muv phenotype expressed in *let-60(n1046)* and *let-23(sa62)* adult worms is clearly visible under a dissecting microscope. However, the *lin-1* (null) strain is relatively unhealthy, developmentally impaired and the vulval protrusions are poorly distinguished in the adult worms. Therefore, instead of the ectopic vulval protrusions, in *lin-1* (null) worms, VPCs that adopt a adopted 1˚ or 2˚ cell fates on the ventral side in the L4 stage, can be counted using a high resolution DIC microscope.

The assay is inexpensive, easy, and not time consuming to setup. To further improve the processing time, the worms can be anesthetized to a final concentration of 2 mM sodium azide within the wells of tissue culture plate and imaged using dissecting microscope equipped with a camera. Another modification of the assay would be to use heat killed *E. coli* OP50 instead of live bacteria[23]. It has been shown that bacteria can metabolize certain small molecules leading to reduced bioactivities[24].

Previous studies have shown that the induction of the vulva in the worm is dependent on certain environmental cues. The vulvaless phenotypes of *lin-3(n378)* and *let-23(n1045)* have shown to be partially suppressed by starvation and exiting the dauer stage[14]. Furthermore, a study by Moghal et al., showed that vulvaless *lin-3(n378), let-23(sy1)* and *let-60(sy95dn)* mutants grown in M9 buffer had a higher number of VPCs assuming vulval cell fates compared with animals grown on standard NG plates[25]. The data suggests worms grown in a liquid environment

influences vulval induction. However, in our studies we did not observed the suppression of Muv phenotype in a liquid environment.

In this protocol we demonstrate the use of *C. elegans* to evaluate the anti-RAS properties of fendiline. In a previous study, we have shown that multiple acid sphingomyelinase inhibitors, including tricyclic antidepressants such as desipramine, imipramine, and amitriptyline inhibit the Muv phenotype[18]. Furthermore, inhibitors of the sphingomyelin and ceramide biosynthetic pathways suppress the Muv phenotype expressed in the *let-60(n1046)* worms. These findings using the worm were validated in mammalian cell lines.

In conclusion, we demonstrate the use of *C. elegans* to identify inhibitors of EGFR and RAS activity in a liquid-based assay. Furthermore, the worm provides another system to identify and characterize the mechanism of action of anti-RAS and EGFR therapeutics.

ACKNOWLEDGMENTS:
We thank Dr. Swathi Arur (MD Anderson Cancer Center) for providing the *let-60(n1046)*. We also thank Dr. David Reiner (Texas A&M Health Science Center Institute of Biosciences & Technology in Houston) for the *lin-1* strain. Finally, we thank Dr. Danielle Garsin and her lab (The University of Texas, McGovern Medical School) for providing some of the reagents. Some worm strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). This Research was supported by the Cancer Prevention and Research Institute of Texas (CPRIT) grant RP200047 to JF Hancock.

DISCLOSURES:
The authors declare no competing financial interests.

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Figure 1

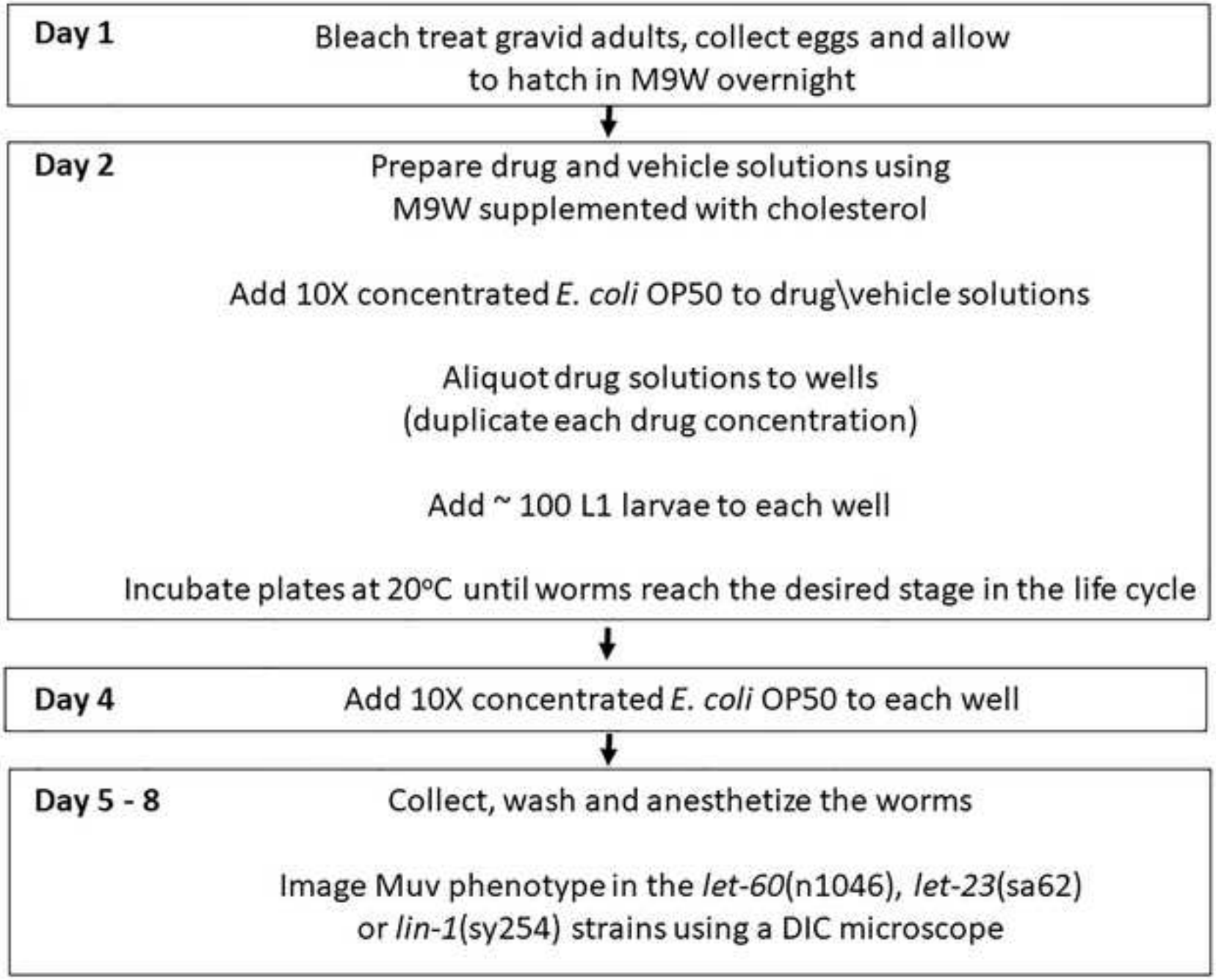

Figure 2

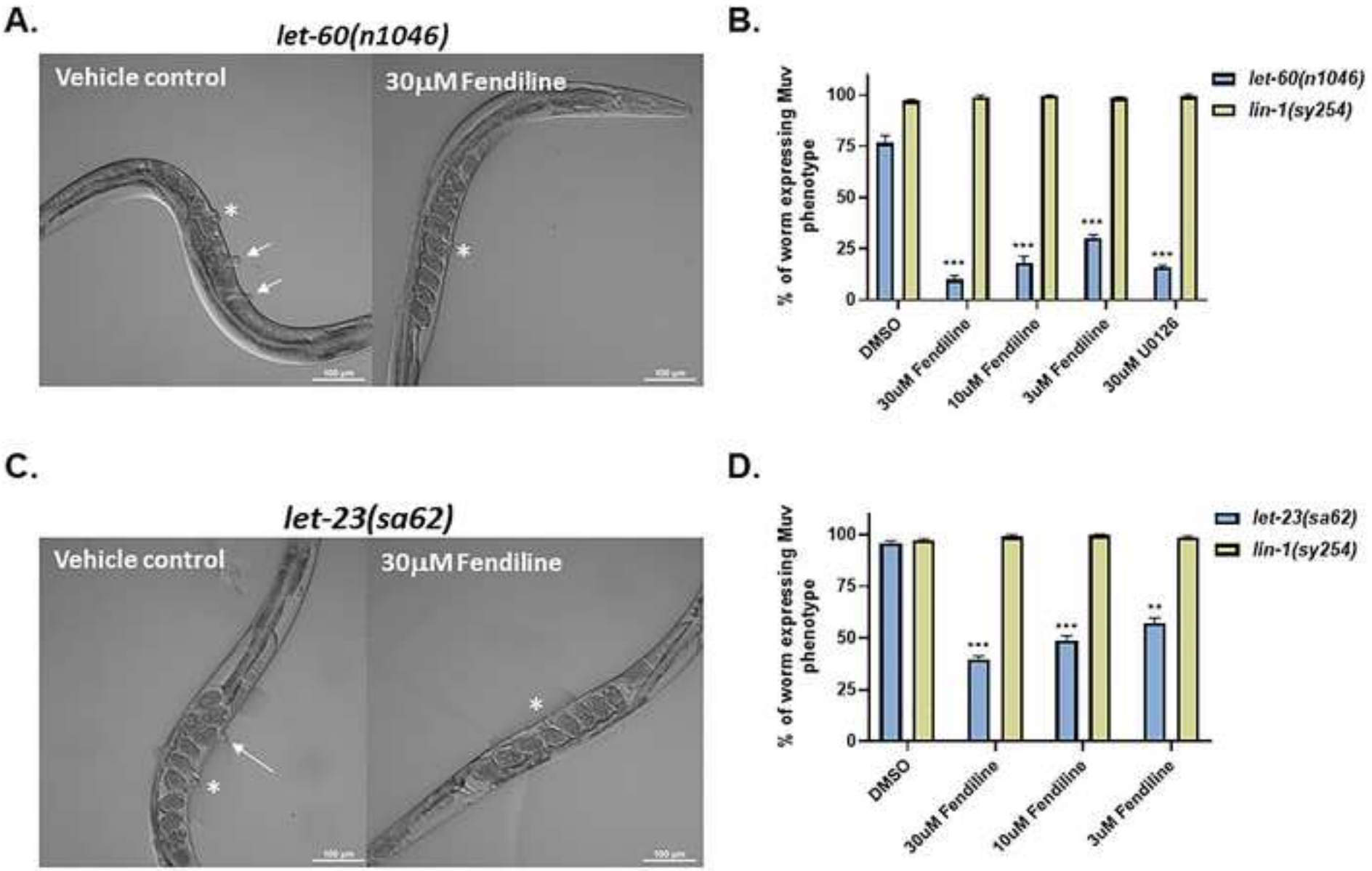

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Media and chemicals		
Agarose	Millipore Sigma	A9539-50G
Bacto Peptone	Fisher Scientific	DF0118-17-0
BD Difco Agar	Fisher Scientific	DF0145-17-0
BD Difco LB Broth	Fisher Scientific	DF0446-17-3
Calcium Chloride	Fisher Scientific	BP510-500
Carbenicillin	Fisher Scientific	BP26481
Cholesterol	Fisher Scientific	ICN10138201
let-23(sa62) II.	Fisher Scientific	MP21021012
Magnesium Sulfate	Fisher Scientific	BP213-1
Nystatin	Acros organics	AC455500050
Potassium Phosphate Dibasic	Fisher Scientific	BP363-500
Potassium pPhosphate Monobasic	Fisher Scientific	BP362-500
R-Fendiline		
Sodium Azide	Millipore Sigma	S2002-25G
Sodium chloride	Fisher Scientific	BP358-1
Sodium Hydroxide	Fisher Scientific	SS266-1
8.25% Sodium Hypochlorite		
Sodium Phosphate Dibasic	Fisher Scientific	BP332-500
Streptomycin Sulfate	Fisher Scientific	BP910-50
(−)-Tetramisole Hydrochloride	Millipore Sigma	L9756
UO126 (MEK inhibitor)	Millipore Sigma	19-147
Consumables		
15mL Conical Sterile Polypropylene Centrifuge Tubes	Fisher Scientific	12-565-269
50mL Conical Sterile Polypropylene Centrifuge Tubes	Fisher Scientific	12-565-271
Disposable Polystyrene Serological Pipettes 10mL	Fisher Scientific	07-200-574
Disposable Polystyrene Serological Pipettes 25mL	Fisher Scientific	07-200-575
No. 1.5 18 mm X 18 mm Cover Slips	Fisher Scientific	12-541A
Petri Dish with Clear Lid (60 x 15 mm)	Fisher Scientific	FB0875713A
Petri Dishes with Clear Lid (100X15mm)	Fisher Scientific	FB0875712
Plain Glass Microscope Slides (75 x 25 mm)	Fisher Scientific	12-544-4
12- Well Tissue Culture Plates	Fisher Scientific	50-197-4804

Software

Prism	Graphpad

Bacterial Strains

E. coli OP50

Worm Strains

Strain	Genotype	Transgene
MT2124	*let-60(n1046) IV.*	
MT7567	*lin-1(sy254) IV.*	
PS1839	*let-23(sa62) II.*	

	Comments/Description
Commercially Synthesized (Pharmaceutical grade)	
Bleach	

Source
CGC
CGC
CGC

UTHealth
The University of Texas
Health Science Center at Houston

School of Dentistry
Department of Diagnostic & Biomedical Sciences
Ransome van der Hoeven, PhD
Assistant Professor

20th August 2020

Vineeta Bajaj, Ph.D.
Review Editor
JoVE

Dear Dr. Vineeta Bajaj,

Thank you for giving us the opportunity to resubmit our manuscript titled “Identification of EGFR and RAS inhibitors using *Caenorhabditis elegans*”. As suggested by you and the reviewers, we have made the necessary changes to the text and figures in the manuscript. We greatly appreciate the comments and suggestions provided by the reviewers. We have provided our responses to the suggestions in italics. Please see below our responses.

Editorial comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.
The text has proofread for any errors.

• Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write lines 60-64, 102-104, 128-129, 151-153, 206-208, avoid this overlap.
We have made the necessary changes to the text.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
We have included the detail as required.

• Results: Mention statistical test used.
Student's t-test was included in the results section.

• References: Please spell out journal names.
We used endnote as the reference manager as well as the Jove style to insert references.
We have spelt out the journal names as requested.

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• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."
All figures and tables are original and have not been published previously.

Reviewer #1:
I have a few methodology questions rather than concerns. M9 solution is referred to M9W which has less NaCl (3 g vs 5 g) as compared to M9 solution described in Stiernagle, Maintenance of C. elegans, Wormbook.org
How does this change improve the drug screening and why the name M9W?
The weight of NaCl should be 5g and not 3g as described in wormbook.org. We made this error in our first submission.

For liquid culture with the drug, M9W is used rather than S-basal which contains cholesterol. Cholesterol, considered an important for optimal growth was not included in your liquid culture. Is there a reason for this? Does it affect vulva development or efficacy of drugs?
We had unintentionally omitted the supplementation of cholesterol to M9W in the text. Unlike the study conducted by Moghal et al., 2003, *we supplemented M9W with cholesterol. We did not use S-basal to ensure we used M9W as a working solution for both the synchronous protocol and for the drug assay.*

Also, do you rotate or shake your liquid culture to prevent the worms from sinking to the bottom and drowning?
We do not rotate or shake the liquid cultures. 2 ml of liquid culture is relatively shallow in the 12-well tissue culture plates and we did not observe any deaths or lag in development.

What is the ideal drug concentration for testing drugs in C. elegans vs. in cell culture? Do you need higher concentrations?
The concentrations of the drugs that we used in C. elegans are within in the range used in cell culture studies.

line 36. correct pseduovulva to pseudovulva
We have made the correction. Thank you for pointing it out.

line 77. and line 127. correct multivalve to multivulva
The corrections have been made.

line 209. let-60 allele is n1046, not n1060.
This error is made in several spots. These corrections have been made.

Line 209. Include allele name for lin-1 – The allele name has been included.
line 269. blead should be changed to bleach
Fig 2. B Correct Multivalva to Multivulva

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Fig 2. C Correct Multi-vulva to Multivulva
Fig 3. B Correct Multi-vulva to Multivulva
All corrections have been made in the figures.

Bacterial strain HT115 is listed as a reagent. Was this used in this protocol?
We incorrectly included this strain into the list of reagents.

Reviewer #2:
Specify "warm to handle" as approx. 50°C.

We have made the change as requested by reviewer 2.

2. 2.3 I don't see the point of removing adults from the plates at this stage-could it be omitted? Or if this step is needed (for example so that plates do not overgrow) explain it.
Yes, we removed the adults to ensure the worms weren't starved before they reached the gravid adult stage.

3. 6.1 specify the appropriate times for the different assays
We have included the times for the assays.

* The introduction as well as the discussion are accurate but an introduction for lin-1 is completely missing. Lin-1 is only mentioned later without introduction of its function. Explaining the role of lin-1 is essential to understand the results because the lin-1 Muv is MAPK independent.
We have included a description for lin-1 in the introduction.

* 294-298 they describe the Vulval induction Index as a common readout to assess the activity of the EGFR/RAS/MAPK pathway in C. elegans, but they fail to describe this method in an accurate manner.
This has been addressed in the discussion.

* 307-310 they claim that the same assay can be used to show misslocalization of EGFR homolog receptor by Fendiline treatment but no data to support this claim are shown. Several EGFR::GFP reporters are freely available in the community.
We have used the liquid-based assay to evaluate the localization of Let-23::GFP in the worms in response to Fendiline. We agree with the reviewer that this statement should be omitted since no data was provided.

* 288-290 confusing phrasing.
We have rephrased the sentences for lines 288 – 290.

* They do not explain why different effects on the Muv phenotype after Fendiline treatment were seen in let-60(n1046) vs. let-23(sa62) mutants.
Since the basal Muv phenotype varies for the let-60 mutant strain due to phenotypic drift the effects of Fendiline treatment will differ between the two strains.

Figure 1:
"collect eggs and allow to incubate" rephrase "collect eggs and allow to hatch"
We have made the change as requested.

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"each concentration is duplicated" -should be re-phrased to make clear that duplicates were used
We have made the change as requested.

Consistency: in the protocol 4.6 they seed approximately 50 L1 larvae in Figure 1 the workflow suggests to add approx. 100 larvae
We have the change to the protocol to reflect the workflow.

Figure 2:
* The resolution of the images in A is too low. One can barely see the protrusions.
* Indicate the genotypes of the worms shown in the images and in the graphs B vs C..
* The red bars in the graphs are unnecessary and confusing. The blue bars indicating the % Muv are sufficient.
* The X-axis is labelled as % Expression meaning in this specific case % of worms which show a specific phenotype. I think that % expression can be misleading since expression is more often used in context of gene expression rather than phenotype expression.
We have taken into consideration the comments suggested for the figures 2 and 3 and we have made the necessary changes.

* Compared to the Figure 5 of citation #18, 30µm Fendiline seems to reduce the Muv phenotype slightly less, but this could also be due to different number of worms scored in the two experiments.
Yes, the numbers varied for each treatment.

Figure 3: The same comments as for figure 2 apply.
We have made the changes as in Figure 2.

Media and Chemicals:
let-23(sas62) II is not a chemical or media.
We considered the strain as an important reagent.

Reviewer #3:
Because the main distinction I see between this and other protocols is that it is performed in liquid, I think that this should be addressed specifically in the document by indicating some of the strengths and some of the limitations of liquid growth. In particular for this pathway, it has been shown that it can be modulated by environmental signals including liquid growth (e.g., Moghal et al., 2003), and the let-60 genotype can exhibit significant phenotypic drift, so cautions about these features would seem appropriate in a methods paper.
We have addressed this comment by adding a paragraph to the discussion.

With respect to the protocol, I think it would be appropriate to extend the protocol (as in Figure 1) to include all the time-critical steps. In particular, the protocol directs to bleach gravid adults on day 1, but many will not appreciate what is needed before that step to get the animals ready. More detail on this portion of the experiment (especially how you can get all your different strains to be ready at the same time so that you can have your control animals and experimental animals available to test in parallel) would be helpful to new researchers.
This is possible with the let-23 and let-60 strains. However, since the lin-1 null strain is sickly it is impossible to perform the readouts for all strains on the same day.

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Performing the assay in different genotypes differently (line 221) does not seem particularly justified.
The Muv phenotype can be clear distinguished in the let-23 and let-60 strains using a DIC microscope. This fast and less tedious as opposed to counting the VPCs in the lin-1 strain. Only candidate drugs that suppress the Muv phenotypes in these strains will be assayed using the lin-1 strain to determine if the inhibition occurs at the level of RAS or EGFR. We have included this statement as a note in the protocol.

In abstract and introduction, the C. elegans convention for proteins is all caps, so should have LET-60 and LET-23.
We made this change throughout the text.

line 73. Multivulva (not Multivalve)
The change has been made.

Line 288+. There should be a reference for "It is also reported that some inhibitors...". It also seems that if the authors assert that it is important to count the numbers of pseudovulvae (for the reasons indicated), it is not clear why they have not done so and reported the data.
Treatment with fendiline resulted in the complete suppression of the pseudovulvae in the worms. However, it was reported in Reiner et al 2008 that some inhibitors can cause partial suppression of the Muv phenotype in the worms.

line 304, also should have a reference ("It has been shown...")
We have included a reference.

line 313, I assume that the let-60 allele should be n1046.
Yes, the change has been made throughout the text.

Figures 2 and 3 should be labelled on the figure more clearly. What are the genotypes in each panel, etc. It's also not clear why 2 and 3 are separate figures, rather than one altogether, or three figures.
We have made the changes.

Reviewer #4:
The article shows basic C. elegans protocol on maintenance or synchronization that are well known. Although it is ok to show their own recipes, they should mention other basic C. elegans articles as Stiernagle, 2006 ; or Porta-de-la-Riva et al 2012. In general, I miss more references...as example when they refer that E. coli as food source can be used as killed bacteria...etc.
We have included the appropriate references.

The manuscript is focused of Fendiline, but they should expand the discussion a bit more to guide in the use of C. elegans to use other drugs. As example, the concentration range is important. A what concentration fendiline would not rescue the Muv phenotype?
We have included a section with regards to other drugs that we have used in this assay.

Why they use nystatin and streptomycin in plates? this is not the standard procedure? Could these molecules interfere with drugs? Please develop this point.

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Nystatin and streptomycin are incorporated into the plates to prevent fungal and bacterial contamination during storage of the plates.

Check C. elegans genes that are not in italics. ex. lin-1 in line 238
We have made the change.

Reviewer #5:

- It is unclear how exactly the lin-1 strain was scored. The protocol mentions "While for the lin-1 strain, the Vulval Precursor Cells (VPC) and asymmetric invaginations were counted on the ventral side of L4 larvae using a high-resolution DIC microscope." What exactly is an asymmetric invagination? What exactly was scored using high resolution?"
  We have rewritten this step of the protocol.

- A basic description of what happens developmentally to generate a vulval invagination might be useful (i.e. the cellular process of vulval cell fate patterning).
  We have included a section in the introduction.

- There are many inconsistencies in applying correct C. elegans nomenclature for gene/protein names, e.g. in introduction and keywords (Let-60 instead of let-60 or LET-60).
  The changes have been made through the text.

- There are a quite few typos and grammatical errors in the text.
  The errors have been addressed.

- The microscopy images of the worms are of very poor quality - the Muv phenotype is not clearly visible in any of the images. They should be replaced.
  We have replaced these images with high resolution images.

We hope you will consider our manuscript for review and we look forward to your response.

Thank you.

Sincerely,

Ransome van der Hoeven, PhD

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