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.

713-486-2547
ransome.vanderhoeven@uth.tmc.edu
7500 Cambridge St, SOD 5362
Houston, Texas 77054

• 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

713-486-2547
ransome.vanderhoeven@uth.tmc.edu
7500 Cambridge St, SOD 5362
Houston, Texas 77054

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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ransome.vanderhoeven@uth.tmc.edu
7500 Cambridge St, SOD 5362
Houston, Texas 77054

"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.

713-486-2547
ransome.vanderhoeven@uth.tmc.edu
7500 Cambridge St, SOD 5362
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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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Houston, Texas 77054