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Dear Dr. Vidhya Iyer,

Thank you for your email regarding the above manuscript. We are grateful for the critical and helpful reviews made by the editors and reviewers, and have attempted to satisfy the points raised, which are listed as follows.

Sincerely yours,

Zebo Huang Ph.D.
Professor, Institute for Food Nutrition and Human Health

Editorial comments

Comment 1

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: We have carefully checked the manuscript again and defined all abbreviations.

Comment 2

2. Please provide an email address for each author.

Response: The emails of all authors were provided when the manuscript was submitted to the submission website. They are now also added at the first page of the manuscript (the text in red); if this is not the case, they can be deleted.

Comment 3

3. Please provide a Summary (before the abstract) to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Response: As required, a Summary is added before the Abstract (Lines 19-22).

Comment 4

4. Please revise the following lines to avoid overlap with previously published work: 33-35, 45-49, 66-68, 274-275.

Response: We have modified relevant sentences as required (the text in red: Lines 32-35, 44-47, 63-66, and 290-292).

Comment 5

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: ImageXpress Pico (Molecular Devices, San Jose, CA, USA); MetaXpress software; Mshot MF31-LED fluorescence microscope etc.
Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper. Comparison of your equipment or software to other commercially available ones is allowed in the discussion, but without unnecessary repetition of these names and only for scientific discussion.

Response: Thanks for the detailed instructions. We have removed all commercial language from the text (Lines 142, 144, 153, 195, and 236 in the current version) and added a table of Materials (Table 2) as suggested.

Comment 6

6. 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 you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: We have examined each step of the protocols and also added references to published materials when necessary, e.g. Lines 81, 110, 137, 180, 199 and 214 (the text in red).

Comment 7

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

Response: We have formatted the manuscript as instructed. Selected text from the protocols Part 2 and Part 3 (lines highlighted in yellow) can be included in the video section.

Comment 8

8. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: We have revised the Discussion section as instructed, including critical steps, potential limitations, methodological significance and future applications. We have also added citations where necessary.

Comment 9

9. Consider calling the current Table of Materials Table 1; it will contain the composition of different media and buffers.

Response: The title of Table 1 is now changed to "Solution Recipes", which contains the composition of media and buffers as in the first version.

Comment 10

10. Please create another table of essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: Table 2 ("Materials") is now added to list strains, reagents, equipment and software used.

Reviewer #1

General Comment

Summary:

The authors described protocols to investigate protective effects of bioactive molecules against PolyQ induced toxicity: PolyQ-aggregation assay (Number of PolyQ aggregates in muscle) and PolyQ-mediated neurotoxicity assays (ASH neuronal survival and Chemosensory avoidance assays). The text is well organised and well described, and will be helpful to the scientific community. I recommend the publication after minor revision.

Response: Many thanks!

Comment 1

Major concerns:

The authors should include in Part 1 the preparation of OP50 bacterial culture.

Response: We have added a step for OP50 preparation in Part 1 as suggested (Lines 82-87).

Comment 2

Line 83 (also in line 181): What was the reference used for temperature conditions of HA759 maintenance? Why 15°C instead of 20°C? I did not find this information in GCG.

Response: Thanks for reminding us this important information. The *pqe-1* (polyglutamine enhancer-1) background in HA759 strain (*pqe-1;Htn-Q150*) accelerates polyQ-mediated toxicity, leading to the death of most ASH neurons within three days. Therefore, this strain is grown at 15°C for neuronal survival assay as previously reported (e.g. Varma et al. 2007; Voisine et al. 2007). We have added references to the protocol (Lines 81 and 180 in the current version) and also briefly discussed this point in Discussion (Lines 326-329).

References:

Varma, H., Cheng, R., Voisine, C., Hart, A. C., Stockwell, B. R. Inhibitors of metabolism rescue cell death in Huntington's disease models. *Proceedings of the National Academy of Sciences of the United States of America*. **104** (36), 14525–14530 (2007).

Voisine, C., Varma, H., Walker, N., Bates, E. A., Stockwell, B. R., Hart, A. C. Identification of potential therapeutic drugs for Huntington's disease using *Caenorhabditis elegans*. *PLoS One*. **2** (6), e504 (2007).

Comment 3

Minor concerns:

Line 58: I suggest change "symptoms" to "features" or similar.

Response: We have changed "symptoms" to "features" as suggested (Line 55 in current

version). Thanks!

Comment 4

Line 100: Please spell EP

Response: We have changed “EP” to “Eppendorf” in the manuscript (text in red: Lines 104, 116 and 130).

Comment 5

Line 123: "for the indicated times". Please indicate the times here.

Response: As suggested, we have modified the text to indicate the times (text in red: Lines 128-129).

Comment 6

Line 137: "Add 20 mM of sodium azide in each well". Is it the final concentration of azide? Or, what is the volume that should be added?

Response: A final concentration of about 20 mM will work here. We have modified the text to clarify the confusion (Line 140). Thanks!

Comment 7

Line 251: Please correct "neurprotective" to "neurOprotective"

Response: We have corrected the mistake (Line 260) and also checked the manuscript for other typo errors. Thanks!

Reviewer #2

General Comment

Manuscript Summary:

This manuscript describes protocols for assessment of neuroprotective capacities of test compounds using transgenic polyQ C. elegans models. The protocols provide detailed assays in terms of polyQ aggregation as well as polyQ-mediated neuronal death and behavioral dysfunction. These details are generally easy to follow and certainly convenient for researchers to screen for anti-polyQ compounds. However, as a method manuscript, I have a few concerns for the authors to address prior to acceptance for publication.

Response: Many thanks! We have attempted to address the concerns as follows.

Comment 1

Major Concerns:

1. Protocol: Part 1, Section 2, Step 5. More details are needed for preparation of OP50 bacterial culture. This is important especially for beginners.

Response: We have added information for preparation of OP50 bacterial culture as suggested (highlighted: Lines 87-92).

Response: We have added a specific step for preparation of OP50 bacterial culture in Part 1 (Lines 82-87) as also suggested by Reviewer 1 (Comment 1). Thanks!

Comment 2

2. Protocol: Part 3, Section 4, Step 6-8. I understand from Step 3, ~200 nematodes are used for each plate, but how many replicate plates usually needed for this chemosensory avoidance assay in each group?

Response: We have added more details to clarify these points (Lines 209 and 216-217).

Comment 3

3. Figure 4: More details are needed for generating the radar chart, e.g. the software used.

Response: To address this point, we have added "Part 4. Creating a radar chart" in the Protocol section (Lines 221-231). The legend of Figure 4 is also modified accordingly (text in red: Lines 295-297). Thanks!

Reviewer #3

General Comment

Manuscript Summary:

C. elegans has proven to be an attractive model organism for prioritizing lead compounds in the early stages of drug development for human diseases. C. elegans possess many characteristics that make it ideal for drug testing including its short lifecycle, small size, ease of culturing in liquid, simple nervous system and plethora of well characterized behavioral assays. In the manuscript entitled "Caenorhabditis elegans as a Model System for Discovering Bioactive Compounds against Polyglutamine-mediated Neurotoxicity", Wang et al. outline multiple protocols to assay the impact of small molecules on polyQ aggregation and polyQ-related neurotoxicity using two different C. elegans models. To assess aggregation, the authors employ a transgenic line expressing 40 glutamines fused to YFP in the body wall muscle (AM141). To evaluate neurotoxicity, the authors use a second transgenic line expressing a

huntingtin protein fragment with 150 glutamines expressed in a subset of neurons (HA759). Using AM141, the authors provide a high through method for counting the number of aggregates in worms treated with the polysaccharide astragalin. Using HA759, the authors describe two protocols for assessing neurotoxicity after treatment with astragalan, one measuring ASH neuronal cell death and a separate protocol for measuring ASH neuron function. This reviewer agrees that protocols for using C. elegans models for drug screening are important for developing treatments for neurodegenerative diseases. However, additional clarification in the steps of the protocols and additional representative datasets are needed before publication.

Response: Many thanks for this general summary! We have modified the text and figures, aiming to clarify the protocols and address the following concerns.

Comment 1

Major Concerns:

1. For the protocol "Effect of astragalan on polyQ40 aggregation", the authors do not indicate whether the data in figure 1A shows the number of aggregates per worm. Please add that detail to the y axis. Also, the authors do not indicate the number of worms that are evaluated for each time point. What is the recommendation for the typical number of worms assayed and measured for the aggregate experiment at each time point? Is it ~500 in control and ~500 with compound?

Response: Thanks for these comments. (1) The data in Figure 1 are indeed the numbers of polyQ aggregates per worm, which is now added in the y axis. (2) The number of worms evaluated for each time point is 100-150 nematodes per treatment, i.e. 10-15 nematodes per well in 10 replicate wells in a 384-well plate (Lines 138-139). This is also the recommendation for a typical independent experiment, i.e. ~10 replicate wells at each time point and >100 nematodes in total for a treatment (text in red in Discussion: Lines 315-317). To clarify, we have modified the text in Protocol (text in red: Lines 124-127) and in Figure 1 legend (text in red: Lines 276-278) in addition to the text in Discussion. (3) To ensure the 10 replicate wells (100-150 nematodes in total) in the 384-well plates of Step 5 (Lines 138-139), the number of nematodes prepared in the 48-well plates of Step 1 (Lines 124-127) is no less than 300. We usually transfer up to 500 nematodes for ease of handling. (4) To better understand the experimental logic and images, we have now swapped panel A and B of Figure 1.

Comment 2

2. In figure 1B, the representative images are not helpful. It is difficult to see any changes in the number of aggregates over time. Please add magnified views of the images as insets within the figures for clarity. In addition, it would be important to include images of the AM141 strain with and without drug treatment. This reviewer is unclear if the representative images are on

AM141 or AM141 treated with the compound. Finally, a statistical analysis should be performed on the dataset.

Response: We have revised this figure as suggested, which is now Figure 1A in the current version as explained in the response to Comment 1. Specifically, images with and without drug treatment, together with insets showing magnified views of the aggregates, are now included. A description of data analysis is added in the figure legend (text in red: Lines 276-278).

Comment 3

3. In figure 2, the authors provide images representing a worm with two intact ASH neurons and an image with one ASH neuron demonstrating neuronal cell death. In addition to these images, the authors should provide a representative set of data quantifying ASH neuronal survival with and without the compound. As the title indicates "Effect of astragalan on Htn-Q150-mediated ASH neuronal death", this reviewer was anticipating a dataset demonstrating the neuroprotective effect of astragalan along with a statistical analysis.

Response: We agree with the reviewer and thus a representative result is now provided in Figure 2 C, which also includes *Poria cocos* glycan as a control in addition to astragalan. A description of data analysis is added in the figure legend (text in red: Lines 283-288).

Comment 4

4. In figure 3, it is unclear if the graph includes data from one osmotic avoidance assay with multiple technical replicates or three independent biological replicates to generate the standard deviation. Please indicate the recommended approach in the "Part 3: PolyQ-mediated neurotoxicity assays 4. Chemosensory avoidance assay" portion of the manuscript. To this point, this reviewer recommends changing the title of this section to "Osmotic avoidance assay"

Response: The results are from three replicate plates, representative of more than three independent experiments. To clarify this point, we have revised the text in Lines 209 and 216-217 (text in red). The title of Section 4 in Part 3 is changed to “Osmotic avoidance assay” (Line 204) as suggested. Thanks!

Comment 5

5. In figure 4, the authors do not provide a protocol or reference for building a radar chart to evaluate compounds. If readers are interested in integrating the three datasets into a radar chart, information on how to generate this presentation format would be helpful.

Response: Thanks for this suggestion. We have added “Part 4. Creating a radar chart” in the Protocol section (Lines 221-231) to address this concern. Relevant information is also provided in the table of Materials (Table 2), which is newly added in the current version.