JoVE62950
Using Phage Display to Develop Ubiquitin Variant Modulators for E3 ligases

Response to Editor and Reviewers

Editorial comments:

Point 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response 1: We have checked the whole manuscript and all spelling, grammatical, and formatting errors have been corrected.

Point 2. Please revise the following lines to avoid previously published work: 53-54.

Response 2: Thank you for this suggestion. We have reworded this part to avoid previously published work and added more details to make the point clearer.

Point 3. 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 and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: OmniMax, Axygen Mini Tube System, etc.

Response 3: We have removed all commercial language from the manuscript.

Point 4. 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 add more details to your protocol steps. 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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) 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.

Response 4: We have checked and confirmed that it contains all the details for readers to replicate the protocol.

Point 5. Line 127-128: Is there any specific technique used to find the concentration of the target protein.

Response 5: Measuring protein absorbance at 280 nm has been recommended in **step 2.1** as the most convenient way to do so.

Point 6. Line 168-169: During the re-centrifugation is there any medium added or is the tube placed back in the centrifuge after discarding the supernatant post first centrifugation.

Response 6: No medium is added after the first centrifugation and the second centrifugation is only intended to pull down any remaining supernatant. This has been clarified in **step 4.3.4**.

Point 7. Lines 237-248: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response 7: The tense has been corrected in the aforementioned places.

Point 8. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response 8: The spacing has been corrected and the highlighted steps (in yellow) have been reviewed to ensure they include the most critical points.

Point 9. Please include a title and a description of each figure and/or table in the Figure and Table Legends section. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

Response 9: Figure titles have been updated and made clearer where necessary.

Point 10. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Response 10: We have removed the symbols. The table has also been sorted in alphabetical order.

Reviewer #1:

Manuscript Summary:
The manuscript describes a protocol for phage display of ubiquitin binding variants. It is a detailed protocol with clear instructions for each step of the process, as well as some introduction about potential applications.

We sincerely thank the reviewer for the positive comments on our manuscript.

Major Concerns:

No major concerns

Minor Concerns:

Point 1: No minor concerns besides ensuring that temperature is listed everywhere that it is a variable - for example, is there a range for room temperature?

Response 1: Thanks for this suggestion. Room temperature has been defined as ~20-25 °C in all relevant places in the text.

Reviewer #2:

Manuscript Summary:
In this review, Roscow and Zhang describe the procedure for performing phage display with an already-established ubiquitin variant library. The description of the protocol for selection is comprehensive and would provide a solid basis for performing selection with phage.

However, there are clarity issues in the way it is written. I am aware that the major part of JOVE is the video that accompanies the text, and I suspect many of these issues would be resolved by the video. Regardless, some points are included below. Note that these are not comprehensive, but are some errors or unclear sections that I noted.

We agree with the reviewer that the clarity can be improved. In addition to address all the points raised by this reviewer (see below), we went through the text thoroughly to make sure all procedures were described clearly and readers can follow with no issues. As the reviewer indicated, the accompanied video will provide further information in case any points are not clear.

Minor Concerns:

Point 1: Line 50 - I would say that the majority of Ub-protein interactions happen via this surface, but needs to be reworded to account for other potential interactions. Perhaps better to say '...the vast majority recognize a common surface...' ?

Response 1: This is a very good point. Thank you for the suggestion. Indeed other types of interactions can happen between ubiquitin and proteins of interest. The text has been revised as suggested in the 2nd paragraph of Introduction, and now more accurately reflect the nature of ubiquitin interactions.

Point 2: line 56-58 should be clarified as it mixes up protein and DNA in an unclear way

Response 2: We agree that the original description is not clear. We have modified this section (3rd paragraph of Introduction) and the Abstract to avoid any confusion.

Point 3: 77 - 'against' a target protein of interest?

Response 3: Thanks for noting this. We have revised this sentence to fix the error.

Point 4: 84: PBS - recipe?

Response 4: PBS is prepared from a 10xPBS solution that is commercially available (Table of Materials). We have made this clear in the text.

Point 5: 84: H2O - deioinised? Tap water?

Response 5: All references to water have been changed to reflect that it is specifically ultrapure water being used.

Point 6: 101: LB mixture? Miller, etc?

Response 6: This is a mixture that is pre-made by a manufacturer (Table of Materials). This has been clarified in the relevant lines in the text.

Point 7: 115: Tris

Response 7: We have revised this accordingly.

Point 8: 127: Are there any ways you would recommend to quantify protein (ie; Abs280, Bradford, etc.) What do you mean by account for full length/truncated etc?

Response 8: Absorbance at 280 nm is sufficient to determine protein concentrations for this purpose and has been noted in **step 2.1** and the 1st paragraph of Discussion. Regarding the note of accounting for changes of protein length or tags, we were referring to changes in molecular weight while calculating for molarity from mass concentration.

Point 9: The authors should discuss options for immobilising proteins. On line 144 they say to use GST as a negative control, but can one also use MBP or lysozyme, etc.? What if you want to immobilise via a biotin tag?

Response 9: Yes, other epitope tags can be used. We have modified the text to make this point clear (**step 3.2.2**). The negative control should be the same as whatever tag was used to purify the protein of interest. This way, any interference due to interactions with the peptide tag can be accounted for. If the protein is biotin-tagged, then the immobilization is done by first coating the plates with neutravidin/streptavidin (room temperature overnight), followed by 1-hour incubation with biotinylated proteins in the next day.

Point 10: 148-222 why is this section highlighted?

Response 10: This section was highlighted as an indication of the steps to be filmed into the video. As filming can take place over only a single day, only steps from a single round can be represented in the video. The steps corresponding to the first round of display contain the most critical and most numerous steps. After this round, subsequent rounds proceed very similarly. Since the first round forms the foundation for all subsequent rounds, it is imperative to perform this successfully for later rounds to be of any value.

Point 11: 163-172: are we aiming for a particular concentration of phage? How do we determine phage concentration?

Response 11: Yes, we are aiming for 100x library diversity and this has been explained in **step 4.3.1**. Further details are given along with a reference to previous methods in the 3rd paragraph of Discussion.

Point 12: 177: the authors should explain why you would do a control plate first. For the titration section

Response 12: This is to account for any undesirable interactions between the binder and that tag on the target protein of interest. This has been noted in **step 3.2.2**.

Point 13: 214: 11 000 rpm or 11 000 g?

Response 13: This is RCF and has been corrected accordingly in **step 5.3.8**.

Point 14: 224-233: What is the intended outcome of the titrations? Would it make sense to compare titrations of selection against a control (like GST) with selection against your target protein?

Response 14: Yes, this is precisely the reason for the control plate mentioned in **step 3.2.2** and **step 4.4.1**.

Point 15: 232-233: inconsistent - is it 10-100 uL or 5 uL for titrations?

Response 15: This has been simplified in **step 5.4.4** to just say 5 µL.

Point 16: 262: without disturbing the pellet

Response 16: This has been added to **step 7.2.5** for clarity.

Point 17: 350-364: the authors should discuss yeast display as well

Response 17: Thank you for this suggestion. Yeast display was not elaborated upon on its own because it is one of the cell-surface display methods, which we spoke about in general terms in the Discussion (2nd from last paragraph).

Point 18: 369: Can similar techniques as described in the review be used for other kinds of libraries (eg: peptide, antibodies)? If so, the authors should mention this in the discussion.

Response 18: The general steps should be very similar, but the protocols described here are specific for UbV selections. We have noted this limitation in the 3rd paragraph of Discussion to indicate that various aspects of the protocol were adapted and optimised for UbV selections.

Point 19: Figure 1 - needs a better caption. Why is the ELISA against GST included in this figure? Why is only one section of diversified ubiquitin shown in this Figure? What trend are we meant to observe here?

Response 19: GST was included as a control for non-specific binding due to the fact that the target protein is GST-tagged. There is only one section of diversified ubiquitin but that includes all the randomized residues. The trend is that binding affinity (as shown in

normalized ELISA value) increase as the sequences increase in frequency. We have modified the legend to reflect all these points.

Point 20: Figure 2 - how were the values normalised? What is the 'ELISA score'?

Response 20: "ELISA score" has been corrected to "Normalized Absorbance" (**new Figure 1B**). These values were the actual absorbance value normalised again the controls (BSA or GST).

Point 21: The layout of Figure 3 makes it difficult to understand (again, this may be clarified by the video)

Response 21: We apologize for the confusion. We have now explained the process in more detail in the legend (**new Figure 2**). Moreover, in the video we will show the actual steps so that it can be understood more easily.

Point 22: A general question, and perhaps out of the scope of the review, but could you add some tips for how to decide which UbVs to pursue more comprehensively? In Figure 1 and 2, there is no obvious 'winner' phage, but if one wanted to clone a subset of these and express them recombinantly, a way to pare them down would be helpful.

Response 22: This is a very good question. We have now provided suggestions in the 4th paragraph of Discussion as to which UbVs should be pursued more comprehensively in downstream analysis.