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Dear Editor,

We are submitting the revised manuscript entitled “**Combining wet and dry lab techniques to guide the crystallization of large coiled-coil containing proteins”** by Zalewski et al,for consideration for publication in JoVE.

We thank you for the opportunity to revise this document and hope we have addressed all of the concerns from the Editorial and Peer Review. Below we address each of the points in the order received. Please note that we have made all the indicated changes with “Track Changes” on and they should be clearly indicated in red.

**Editorial Comments:**

1) We have taken the opportunity to proof read the document thoroughly as suggested.

2) We have fixed the Figure4 figure legend, which was to a previous version of the figure. We apologize for the oversight.

3) We have added LB media to the materials list and defined it within the protocol. Autoinduction media was already present as the last item in the materials list.

4) We have removed all four instances of “you” or “your” within the document. We have also made corrected the uppercase I in imidazole, and place in the missing “buffer” in step 2.3.6.

5) Protein concentration is determined spectrophotometrically (*A280*) and this has been added. As has the Chromatography reference and a reference for the vapor diffusion method which we feel should help address the questions about how the trays are set up. Additional details have been added to address the magnification used on the microscope and the temperature of the trays.

6) The development of the initial domain boundaries step is far and away the most important step in the protocol and a short discussion of this is now included within the manuscript.

**Reviewer #1**

Major concerns

1) The reviewer comments on the desire to link information learned from the experiments as performed in Figure 5 & 6 with the outcomes in Figure 7. Additionally, it would be desirable to also link in the informatics analysis too, and could a quantitative score be attached that could aid the user towards the final answer quicker.

* We agree that it would be super to have such a score and had previously put quite a significant amount of time into asking whether a quantitative score could be obtained by sequence analysis alone with inconclusive results due to the lack of diversity within coiled-coil protein sequences. After applying this technique and its many variants on a variety of proteins, we feel that it is tool best utilized to quickly narrow down a very large set of possible domain boundaries down into a manageable set of fragments that can be tested quickly and cheaply. We were also worried that users new to structural analysis might rely too heavily upon the scoring function and by testing only the “best” fragment, might actually miss out on fragments that would actually work. We have added language into the discussion sections to address this important point.

2) The reviewer points out that there are a variety of Shrm and Rock constructs used throughout figures 5 and 6, and analyzed by a variety of assays and this mash up of proteins and techniques was confusing.

We agree that this section needed significant clarification as the important points in figures 5 and 6 were not readily apparent. We have made extensive changes to these figures including:

* Reordering figure 5 and making it panels A, B, and C. The point in panels 5A & B were to compare the behavior or purified proteins in limited proteolysis and how this assay was a good technique to improve crystallization outcomes.
* The new Figure 5C (the old 5A) describes the utility of this technique using Ruby-tagged protein. Since this is the type of protein that would be generated from the protocol, it was desirable to demonstrate that proteolysis could still be informative.
* Figure 6A now shows how this technique could be used with even point mutants of a protein fragment and the figure has been altered to make that clearer.
* Lastly, Figure 6B is designed to how that the technique can be applied successfully on protein complexes. In this case, the complexes all used the same human Shrm SD2 1427-1610 because we already knew it would crystallize on its own and it made the analysis in figure6 clearer. Thus the complexes only differed in what Rock fragment was used. While the complexes with the smaller two Rock fragments crystallized, the complex containing Rock 788-906 only formed crystals after many weeks and the protein within them had not remained intact. We did not analyze the specific breakdown products generated within the drops containing the 788-906 complex but did note that the last fragment generated crystals readily and quickly. To improve the clarity of this section we have included much that this discussion within the results and improved the figures for clarity.

3) Lastly, the reviewer states “Finally, in Figure 7, individual crystals are obtained, but we have no idea of the final constructs that were used, nor in how it was decided to try these specific constructs. Importantly - is this data supporting the authors evidence from the combined in silico, proteolysis and functional analyses? What constructs were used in the precipitated wells? Did those constructs 'fail' in the earlier analysis?”

* We apologize for any confusion, but the point in figure 7 was to provide the user a set of 12 common starting conditions that could be used with any protein and was not intended to demonstrate an analysis of the coiled-coil proteins discussed earlier. We have added some language within the results to address this.

Minor concerns:

* Protocol 1.1.4, the reviewer correctly notes that it should be Clustal-Omega. This has been changed.
* We have added labels as suggested by the reviewer and agree that it improves clarity.
* We have added MW markers as suggested.

**Reviewer #1**

There was some concern about why the full-length Shroom sequence was being split prior to analysis by PSIPRED and DISOPRED in step 1.2.1. This is a technical limitation of the DISOPRED web server and beyond our control. The reason we suggested using the C-terminal 1,000 amino acids is that the SD2 coiled-coil region is located at the extreme C-terminus of Shrm. This domain is annotated as ~300 amino acids in the literature, so this leaves about 700 amino acids of “buffering sequence”. We hoped this large buffer would make users confident that the results shouldn’t be influenced by the truncation. In practice, we have only noticed very subtle differences that occur within 1-2 amino acids of the truncation point. We agree that our wording wasn’t clear enough however, and altered that note accordingly.

**Minor concerns and additional notes:**

* We have added an entire section into the discussion section to elaborate on the limitations of the analysis (both biochemical and computational) as well as suggest alternative approaches.
* We have adjusted the background introduction of Shrm and Rock to appeal to a broader audience.
* We have adjusted “Autoinduction” in line 194 to match the earlier description.
* References have been updated and all now include DOIs, and the Duplicate “references” has been removed. We apologize for the oversight.
* We have included the relevant temperatures throughout.

We thank both the Editorial and Peer reviewers for their time and efforts. Their comments have been critical in improving the clarity and utility of this manuscript.

Thank you in advance for your time.

Sincerely,



Andrew P. VanDemark, Ph.D.

Associate Professor

Department of Biological Sciences