Stockholm, Sweden

August 14, 2018

To the review editor,

Please find our revised manuscript JoVE58670, “Using high content imaging to quantify target engagement in adherent cells”. We appreciate the constructive comments from both the reviewers and editors and have revised our manuscript to address these points. In the attached response file you will find the detailed adjustments we have made to the manuscript files and figures in order to thoroughly answer these questions. We believe that our clarifications, editing and experiments satisfactorily address the concerns posed by the reviewers and hope that the manuscript is now suitable for publication in *JoVE.*

Thank you for your time and consideration.

Best regards,

Brinton Seashore-Ludlow

**Editorial comments:**  
Changes to be made by the Author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

*We have taken time to proofread the manuscript.*

2. Please revise lines 325-327 and 331-333 to avoid previously published text.

*Thank you for pointing this out. We have changed these lines.*

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

*We have uploaded a copyright page from ACS regarding figures 2-4. Figure 1 is original to this work.*

4. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .psd, or .ai file.

*All figures have been uploaded as .ai files.*

5. Figures 2 and 3: Please line up the panels better. Some panels are off-set in Figure. Please ensure that the panels are of the same dimensions if possible.

*We have re-sized and aligned the panels in Figures 2 and 3 accordingly.*

6. Figures 2 and 4: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

*We have commented on the magnification used in the figure legend and added scale bars to these figures.*

7. Affiliation 3 is not assigned to any author. Please check.

*Thank you for pointing this out and the extra affiliation has been removed.*

8. Please spell out each abbreviation the first time it is used.

*We have spelled out each abbreviation in the revised text.*

9. 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 and Reagents. For example: Multidrop, Traceable, ImageXpress, Molecular Devices, MetaXpress, etc.

*In the revised manuscript we have removed commercial product names for instruments and reagents and have these listed in the Table of Materials and Reagents.*

10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

*We have revised our protocol text to reflect this request where applicable.*

11. Lines 85-86: This note may be deleted because such information is mentioned in step 1.1.

*Lines 85-86 have been removed from the text.*

12. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

*We have adjusted the language our protocol accordingly.*

13. Please add more details 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. 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.

*We have added significant detail to our protocol and hope the “how” for these steps is now clear.*

14. 1.1: Please specify the type and size of assay plates. What is used to drill holes? How large is the holes? How many holes are drilled per plate?'

*We have added more detail to the instructions for this step to clarify these points.*

15. 1.2: Please specify the conditions used to trypsinize the cells.

*We have added instructions for cell trypinization.*

16. 2.2: What concentration is considered to be appropriate?

*We have added discussion and instructions on compound concentrations.*

17. Lines 252-262: Please move this paragraph to the Discussion section.

*This paragraph has been moved to the discussion section.*

18. Discussion: Please also discuss any limitations of the technique.

*We have added several limitations including those brought up by the other reviewers.*

19. References: Please do not abbreviate journal titles.

*The reference section has been updated accordingly.*

20. Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

*The lot number of the antibody has been added to the Table.*

**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
The manuscript entitled "Using high content imaging to quantify target engagement in adherent cells" by Axelsson, Almqvist and Seashore-Ludlow describes the detailed steps of an adaptation of the previously reported CETSA format. The original paper with the data presented again here was a well needed adaptation of the CETSA protocol, allowing also studies of adherent cells without prior detachment. The potential to study adherent cells without detachment prior to detection is intriguing as the actual detachment could affect the cellular uptake and intracellular biology. Having a detailed protocol will certainly help spreading the use of the adaptation.  
The protocol is not too detailed but well enough for researchers with previous understanding of cell staining and fixation.  
  
Major Concerns:  
Rows 120-121 and 236-238. How does one construct a Tagg curve in a waterbath? This may be worth describing in better detail as it is not apparent from the protocol how that could be carried out. Again, this raises the question on how to approach targets with smaller shifts than p38a where the transfer from Tagg to ITDFRs is less forgiving?

*We have added detail in the representative results section on how to create a Tagg curve. We have also added the limitation of targets with small shifts to the discussion section.*

Minor Concerns:  
A few specific comments come to mind when reading the manuscript:  
1) There is an affiliation not belonging to neither of the authors in the author list (row 13)

*See above*

2) The statement on rows 62-63 could be clarified to underline that it is only the detection step that has required lysis, not that the incubation and heating steps of the original CETSA protocol requires lysed cell suspensions. In fact, the presented protocol also requires the permeabilization of cells to allow affinity reagents to reach the antigen, making it a somewhat lysed suspension. This is semantics.

*We have tried to clarify these steps in the introduction section.*

3) Row 73, something missing

*We have re-written this sentence.*

4) Rows 75-77. The study behind this protocol has already been published but maybe there could be room also in this detailed protocol for a discussion on how to approach targets with smaller shifts than p38a. Will there be challenges?

*This has been addressed in the discussion section.*

5) Row 86 and 90. The drilling of holes in plates does not resonate well with HTS screening. Are there no plates available that minimize the amount of air trapped?  
On that same note, regarding the edge effects (rows 254-255) - there are plates called "edge plates" with a reservoir surrounding the outer wells. Would such plates, without filling up the edge reservior minimize the amount of trapped air and at the same time reduce the heating surface of the edge wells?

*Interesting idea with reservoir plates and though applicable to cell culture, we are not aware of reservoir plates for imaging purposes. We feel that though these may address the edge wells, they will not influence air trapped under the plate.*

6) Row 111, please add an alternative for less equipped labs, similar to the step of dispensing PFA with a multidrop or multichannel pipet.

*We have added an alternative procedure.*

7) Row 260, variable?

*We have fixed this sentence.*

8) 291 and Figure 5. I do not see the need for this figure. Those reading this protocol should know how edge effects display.

*We have removed this figure.*

9) In the discussion part the authors discuss the need for good antibodies, this is of course imperative. Could this be a limiting factor for new targets where antibody availability is scarce (and where early screens are carried out). Could the use of a tagged protein be helpful?

*We have updated the discussion text to address this point.*

**Reviewer #2:**  
Manuscript Summary:  
The article entitled "Using high content imaging to quantify target engagement in adherent cells" proposes a new adaptation of the CETSA assay, in order to characterize deeply the interaction between a compound and its putative target. It is based on the high throughput measurements of drug-target interaction by using a specific antibody against the putative target followed by the incubation of a fluorescent secondary antibody. Then the interaction between the compound and its target is analysed by using a microplate-reader that permit a high throughput analysis. The new CETSA method is very interesting and offers important advances for a rapid screening of a library of new compounds especially if it is a big library.  
  
Major Concerns:  
Figure 1D .Could be interesting for the readers to see a comparison between a normal CETSA result (western blot analysis) and this new CETSA method, also for a single compound, to visualize the results between of both methods and evaluate if the sensitivity of the two methods is equal or different.

*We have data in the lab examining CETSA results for A-431 cells in suspension using both western blot and AlphaScreen technologies. These compare well. We have also published similar comparison data for K562 and HL-60 in reference 10 and compared AlphaScreen to the imaging assay in reference 12. Notably, comparisons between assay formats are challenging when the heating format is not the same, as the exact heating time of the sample for example in a water bath and in a PCR tube differs. In such comparisons right shifting of the potency for the sample that was heated to the temperature farthest from the Tagg is observed. Thus, for the true comparison we would want to compare adherent A-431 in imaging plates as detected by imaging and western blot. This poses a technical challenge as the amount of protein necessary for the western blot is difficult to obtain in the 384-plate format. Thus, we refer the reviewer to the already published comparisons*.

Minor Concerns:  
Authors should discuss some issues listed below:  
Step 2.3: This step proposes to incubate cells with the different compounds for 30 minutes at 37°C, but if the main goal of the method is to screen library of new compounds, how the Authors may exclude that the lack of interaction is not due to a reduced entry of the compound into the cells? In other terms they have never shown that a negative compound remain negative also after longer incubation.

*The length of compound treatment varies and certain molecules that need metabolic activation will need a longer incubation time. We have addressed this in the revised text.*

Steps 4.2/8.1/9.2/9.4: How many times did you wash the wells with PBS? Please provide the number of wash in this step since for our knowledge the 384 wells can maximum contain 100μL of a liquid. Therefore they mean 3 washes of 100μL or each wash with 300 μL, and if this is the case, how many washes are sufficient to further proceed in the method? Please add more details.

*We have provided more details for these steps to clarify these points in the protocol.*

Step 5.2: In this step Authors have written: "Alternatively, when appropriate, apply an antigen retrieval protocol…". According to your original protocol published in ACS Chemical Biology, the antigen retrieval step is useful for minimizing the number of false negative because it facilitates the detection of the target engagement. How new users can define when the antigen retrieval protocol is necessary? May the Authors give more hints about this important decision-step?  
  
*We believe this should be used as a counter-screening method and if target engagement is not observed for known binders. We have clarified this in the text.*

**Reviewer #3:**  
Manuscript Summary:  
The authors present a short, but well described protocol to perform an exciting new technique. The manuscript is well written and should serve as a great resource for the research field. The description of antibody limitations is quite helpful.  
  
Major Concerns:  
Perhaps the authors could expand upon the calculation of % stabilization from the raw data. It is difficult to follow how that relates to fluorescence intensity and thus why the drug treated curve in Figure 3A starts at 75% and ends at 50% while the control spans 100 - 0 %.

*We have added this to the protocol steps.*  
  
Minor Concerns:  
In Figure 5, the lookup table labels need to be fixed.

*This figure has been removed.*