|  |
| --- |
|  |
| Janssen Vaccines & Prevention B.V. Archimedesweg 6  2333CN Leiden  The Netherlands |
|  |

July 2, 2018

Dear Dr. Nguyen,

Please find our revised manuscript entitled **“***In vitro* assay for studying the aggregation of tau protein and drug screening” [JoVE58570] that we hope is now acceptable for publication in Jove. We have taken into account the reviewers’ comments and have added the details requested for the protocol and provided clarifications in the text where requested.

A point-by-point response to the specific comments of the reviewers follows.

Sincerely Yours,

Adrian Apetri, Ph.D.

Janssen Prevention Center, Janssen Pharmaceutical Companies of Johnson & Johnson

**Response to reviewers’ comments**

**Editorial comments:**  
Changes to be made by the Author(s):  
1*. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

We have proofread the manuscript.

*2. Figure 1: Please label the ladder (kDa, Da?).*

We have now specified the unit of the ladder (kDA) in the legend of Figure 1.

*3. Please tone down the title: Delete “Highly Reproducible”*

Thank you for your suggestion. Although the assay has a very high reproducibility in the variety of formats we have tested it is indeed conceivable that some non-voluntary alteration in different lab settings would still induce variability. We have eliminated the “highly reproducible” part from the title of the manuscript.

*4. 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: Synergy Neo, Eppendorf, etc.*

We have removed all commercial language and used generic terms instead. The commercial products are listed in the Table of materials and reagents.

*5. 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.*

We have improved the protocol by including more experimental details.

*6. 1.2.3: What happens after spinning? Resuspension?*

We have adjusted the text to indicate that the specific spin is to remove air bubbles.

*7. 1.3.3: Adjust to what concentration?*

We have added the exact ThT concentration (i.e. 500 M)

*8. 2: Continues Mode? Please revise for clarity.*

We have added “automatic measurements” to clarify what we mean by “continuous mode”

*9. 2.4.7: How many times for the up and down pipetting? How much is added and what is the desired seed percentage?*

We added the missing details to the revised version of the manuscript

*10. Please do not abbreviate journal titles.*

We removed journal abbreviations from the manuscript. The references were inserted using the Jove template which comes with abbreviations. Please clarify if the comment refers to abbreviation of journal names in the reference list or if it is limited to the manuscript.

**Reviewers' comments:**  
  
**Reviewer #1:**  
*Manuscript Summary:  
Aggregation of tau is intrinsic to Alzheimer's disease and other neurodegenerative diseases. Study of the aggregation of tau using recombinant proteins has been hampered by variable results, both in reaction kinetics and the fibrillarity of the end products. The authors describe a highly reproducible way to generate recombinant fibrillar tau aggregates. This is a very useful method that can be employed for study of tau aggregation per se, as well as for the study of tau seeding in cells or animals.*  
  
*Major Concerns:  
- The authors do not directly compare their protocol to other protocols, which makes it difficult to assess for other scientists how they could improve their own protocols. A clearer description/discussion of what makes this protocol so robust would be helpful; is it the tau protein, the mutations, the heparin concentration for example.*

We thank to the reviewer for the comment and for encouraging us to further stress the most important factors in the assay optimization process. We have elaborated in the revised version and we hope that this strengthens the manuscript. We focus in the first three paragraphs of the discussion section on the importance of protein quality, its monomer content and the ability to keep it in a state where disulfide bridging is not possible. We also discuss the importance of accurate protein concentration assessment, the adjustment we made to the tau protein and the optimization of heparin concentration and instrument settings. Furthermore, figure 1 shows the high purity and monomer content of tau, one of the most important quality attributes in our assay.

*- The authors use tau with mutated cysteines to prevent disulfide bridge formation, but they still use a reducing agent in their reactions. This should be discussed as a particular advantage of their system appears to be that this can be omitted?*

The use of TCEP is due to its required presence when aggregation employing wild type Tau containing cysteines is performed. In our optimization efforts we compared side by side different forms of tau and for a fair comparison we decided to use the same buffer. It is conceivable that for this particular tau variant, the presence of TCEP will only lower the pH of PBS from 7.4 to pH 6.7 but will not affect the conversion. We added a sentence to the revised version of the manuscript to explain.

*- Sonication is very different between apparatuses, would it be possible for the benefit of the scientific community to make a generalized addition to this part of the methodology so that it becomes easier to implement? Perhaps of how the optimal settings were determined for this machine?*

We added a sentence to the revised manuscript related to the parameters of our sonicator and also a statement as how we optimized sonication to achieve a homogeneous population of short fibers of 20-50 nm in length.

- *A claim is made in the discussion that the optimal tau : heparin ratio was identified. In the manuscript only 2 concentrations of heparin were used, apparently without affecting the tau aggregation. This should be clarified and if there are data on which this is based it would be good to show or at least discuss. In addition, what determined the reaction being optimal? lag phase? fibrillarity? etc.*

We thank the reviewer for the observation and we clarified in the revised version of the manuscript how optimization was performed including a reference to previous studies.

We have expanded in our revised manuscript the discussion on the importance of having high quality reagents, an optimal lag phase and that we aimed to set up an assay that mimics what is believed to be the *in vivo* tau aggregation process with the corresponding kinetic profile showing quantitative conversion, seeding activity and fibrillar morphology of aggregates. *Minor Concerns:  
- for tau and ThioT the extinction coefficients are in different units, please unify.*

We have listed in the revised version the extinction coefficient for tau protein in both mass and molar units.

*- it would be good to note whether ThT should be made fresh for every experiment or that simple concentration adjustment suffices.*

We clarified in the revised version of the manuscript the details regarding preparation, shelf life and storage of ThT.  
  
  
**Reviewer #2:**  
*Manuscript Summary:  
The manuscript "Highly reproducible in vitro assay for studying the aggregation of tau protein and drug screening" by Rosa Crespo et al. explains the method of producing highly reproducible tau aggregation triggered by heparin in vitro. With this method, the author claims to achieve reproducible tau aggregation between different wells, runs, and batches of protein. Thus, this method has huge implications in the field of tau pathogenesis. Although, the author has made attempt to explain details of methodology; however, there are some concerns that should be considered.  
  
Major Concerns:  
(1) The author should attempt to show the sensitivity of huTau441 aggregation using the lower amount of huTau441 aggregates up to 0.0001% (v/v) or less. The amount of huTau441 seed in 0.01% (v/v) is considerably high. At a high concentration, any unrelated protein aggregate could cross-seed huTau441 aggregation.*

We are know showing 10 different concentrations of seeds in the 0.0025- 1.25 % range (adjusted Figure 5).The concentration of seeds used in our assay is significantly lower than shown in other publications. Also, seeding displayed in our experiments is concentration dependent and reproducible (kinetic traces for all four replicates corresponding to each concentration of seeds are virtually indistinguishable) and leads to tau aggregates with clear fibrillar morphology. Furthermore, we are not aware of systematic studies showing seeding efficiency of “any unrelated protein aggregate” and we do not think that the concerns are justified.

*(2) The author should also test the specificity of huTau441 aggregation in this condition against other protein aggregates such as, alpha-Synuclein and amyloid beta.*

We thank the reviewer for the constructive suggestion. However, while we acknowledge the importance of these scientific questions, the suggested experiments are not germane to this article. Our intention is to report on a robust tau aggregation assay that mimics what is believed to be the mechanism of *in vivo* tau pathogenesis. The specificity of tau aggregates as seeds in the conversion is confirmed by multiple things:

* Concentration dependent seeding potential
* Seeding efficiency at extremely low seed concentrations
* Homogeneous populations of *de novo* generated tau fibrils

*(3) The lag phase of unseeded huTau441 aggregation is very short. It is possible that huTau441 preparation contains pre-aggregated seed or heparin concentration is too high. It is well known that these two factors highly modulate the aggregation of Tau. Author should attempt to centrifuge purified huTau441 at a high-speed 100,000Xg to remove the pre-aggregates rather than 12000Xg. On the other hand, if possible, the author should re-optimize the conditions with different concentrations of heparin, concentration of huTau441 protein, temperature and shaking speed.*

Previous reports focused on tau aggregation showed very short lag phases if any. The lag phase for the spontaneous conversion of huTau441 has been optimized in our assay set up efforts to 12-15 hours. We consider this lag phase as optimal since it allows a broad time window to study seeding while still avoiding protein degradation, non-specific aggregation and/or artifacts that could appear when intrinsically unfolded proteins such as tau are incubated for extended periods of time under physiological conditions.

We appreciate the concern of the reviewer regarding the possible presence of tau aggregates in the starting material. Purity, integrity and absence of aggregates are some of the crucial parameters we have focused on. Thus, we have dedicated a full figure (Figure 1) to show the quality attributes of our monomer tau preparations. Thus, SDS-PAGE shows that huTau441 preparations contain no degradation products and are virtually 100 % intact. Furthermore, SEC-MALS analysis shows that the huTau441 preparation is 100 % monomeric as emphasized by a single peak in the chromatographic profile and a MW of 51 kDa (theoretical MW is 49 kDa). These observations eliminate the potential presence of any detectable traces of aggregates in the starting material. Also, the fact that the final “aggregated” material does not contain fragments validates our optimized lag time as optimal for this assay.

Moreover, the high reproducibility of the assay in different experiments and while using different batches should fully eliminate concerns regarding the quality of the protein reagents used in this study.

In order to better explain our rational in optimizing the lag time, we included a short paragraph in the revised version of the manuscript.

*Minor Concerns:  
(1) It is unclear about the storage buffer of purified huTau441. It should be explained.*

For the protein purification we have referenced one of our previous publications. We also added the formulation buffer, PBS + 0.5 mM TCEP, pH 6.7 to the revised version of the manuscript.