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Phillip Steindel, PhD
Review Editor
Journal of Visualized Experiments (JoVE)
Tel. 617-674-1888

Dear Dr. Steindel,

We thank you for the opportunity to resubmit our manuscript “Purification of H3 and H4 histone proteins and quantification of acetylated histone marks in cells and brain tissue” [JoVE58648R3]. We appreciate your constructive critiques. We are pleased to inform you that all issues have been addressed as follows:

1) The Protocol has been formatted according to JoVE guidelines and additional details required for filing have been added to the Protocol.
2) Figure 1 has been edited according to your recommendations and updated to include more details.
3) Statistical reporting has been revised throughout the entire document.
4) All questions brought up regarding manuscript presentation have been addressed.

Once again, thank you for your consideration of our manuscript and we look forward to a favorable review.

Sincerely,

Claude-Henry Volmar

Editor's comments:

1. 1.1.3: Please list what is in the Extraction buffer here. Also, assuming this is composed of the recipe given in the Results, what is the concentration of Tris-HCl and what is the protease inhibitor? The latter is not listed in the Table of Materials.

The Components of the Extraction Buffer have been listed in step 1.1.3 and the concentration of Tris-HCl has been added to the list of the Buffer components. Information regarding Protease Inhibitor has been added to the Table of Materials.

2. 3.1: Are the spin columns part of the purification kit listed in the Table of Materials? Please make this explicit if so.

Correct, the spin columns are part of the purification kit listed in the Table of Materials. This information has been added to the Table of Materials in the "Comments/Description" column.

3. 3.3.2/4.8: Does the 'this step' refer to the previous step (3.3.1/4.7, respectively)?

Correct, 'this step' in steps 3.3.2/4.8 refers to the previous steps, *i.e.* steps 3.3.1 and 4.7. The wording has been changed to clarify which step needs to be repeated.

4. 4.14-4.15/Figure 1: "Elute" seems to be the wrong word here; perhaps something like 'reconstitute'?

The word "elute" has been substituted with "resuspend" in steps 4.14. and 4.15.

5. 4.1.5: What do you mean by 'must be used for further quantification'?

The meaning of the sentence is that the final product of the extraction, *i.e.* purified and desalted histones, and not crude histones from previous steps should be used for histone quantification and analysis. The sentence has been reworded to convey the main point.

6. 6.6.1: There isn't really enough detail here for filming.

More details have been added to step 6.6.1. Please see steps 6.6.1 – 6.6.3 in the revised version of the protocol.

7. Formatted according to JoVE guidelines (spaces between all steps, all text aligned to the left margin), there is approximately 3.5 pages of the Protocol highlighted. Please shorted the highlighted portion to 2.75 pages or fewer, to fit within our limits for filming time and video length. Please do not highlight anything outside of the Protocol section (those are dealt with separately).

The Protocol has been formatted according to the recommendation and the highlighted portion of the manuscript (only within the Protocol section) has been shortened.

8. Results: In the discussion of Figure 6, you say that impurities are detected in the extract- how does Figure 6 show this?

Purified and desalted histone H3 and H4 are detected on a PVDF membrane as two fine bands at ~15kDa (Figure 7A, histone H4, top blot; Figure 8A, histone H3, top blot; Figures 7A and 8A, histones H3 and H4, bottom blots). Considering high specificity of the antibodies used, impurities present in the crude histone preparation are visible on the top and bottom blots in Figure 6A as the histone bands are not clearly defined.

9. Figure 1: There are some discrepancies between this and the protocol:
a) Crude extraction time is listed as 2 hours, but you include several options in the protocol.

The optimal extraction time is between 15 min and 24h. In the current protocol, we demonstrate results following 15 min, 2 h and 24 h of extraction. It is recommended that several extraction times be tested for cell lines other than the ones presented in the current protocol. This information is included in the manuscript (step 2.1. – "Note") and has been added to the legend of Figure 1.

b) Centrifugation happens before the optional stopping point in the protocol, but after here.

Centrifugation step (2.2.) must happen after the extraction step (2.1) and before optional stopping point (2.4). This information has been outlined correctly in Figure 1.

c) Histone precipitation is done overnight (so, potentially as little as 16 hours) in the protocol, but listed as a full 24 hours here.

It is recommended that histones are allowed to precipitate for 24 hours. This information has been clarified in the protocol (step 4.3.)

d) Histone centrifugation is done for 1 h 15 min in the protocol, but for 1 h here.

Centrifugation step is done for 1h 15min. This information has been outlined correctly in Figure 1.

e) Histone wash appears to take ~1 h in the protocol (6 washes of 10 minutes each), but 45 min here.

Histone wash steps last about 1h. This information has been outlined correctly in Figure 1.

NOTE: Figure 1 has been edited and updated to include more step-by-step protocol details.

10. Figures 2-8: All of these appear to have multiple replicates (3 for Figures 2-5 and 8 and 2 for Figures 6-7) that are not explained in the figure legends.

Sample size has been added to the legend of Figure 2-8. Statistical analysis and reporting has been revised throughout the manuscript.

11. Figures 6-7: n/N appears to have changed between drafts-they were 8 before.

The clerical error has been corrected. N= 4 per treatment group for Figures 6 and 7. N= 3 per treatment group for Figure 8.

12. Figures 6-8. If n/N are indeed 6, why are you using 6 degrees of freedom for your t-tests, then; shouldn't it be 4?

N= 4 per treatment group for Figures 6 and 7. There are two treatment groups, thus degrees of freedom for t – test equal 6. N= 3 per treatment group for Figure 8. There are two treatment groups, thus degrees of freedom for t – test equal 4.