EMSL

www.emsl.pnl.gov

Environmental Molecular Sciences Laboratory
902 Battelle Boulevard • P.O. Box 999 • Richland, WA 99352
Tel: (509) 371-6585 ● Fax: (509) 371-6555 ● ljiljana.pasatolic@pnnl.gov

Aug 10, 2020

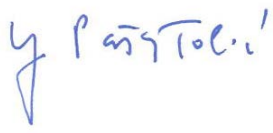
Dear Vineeta,

We are submitting our revised manuscript titled "Isolation of Histones from Sorghum Leaf Tissue for Top-Down Mass Spectrometry Profiling of Potential Epigenetic Markers". We appreciate all the critical comments from reviewers to help improve our manuscript, especially some of the technical comments from reviewer 3. We also reorganized some of the text to improve clarity.

Both reviewer 2 and 3 requested additional analysis to extract biologically significant information from the results. We are glad that they found the technique interesting and potentially useful to address important biological questions. However, the focus of this manuscript is the protocol itself, and not the specific biological question. In addition, the reviewer 2 was questioning the reproducibility of our protocol. To address these concerns, we have now added Figure 3 from our published manuscript describing quantitative comparison between control and drought samples (https://doi.org/10.1016/j.ymeth.2019.10.007). We believe this inclusion better demonstrates that the protocol can generate histones for LC-MS analysis reproducibly in a large-scale study setting. Figure 3 also addresses the reviewer 3 comments regarding quantitative analysis of the data, and we thank both of the reviewers for this improvement of the manuscript.

Detailed responses are attached below in this letter. We believe we have addressed all of the reviewers' concerns and that the manuscript is now ready for publication. We are looking forward to hearing from you soon.

Sincerely,



Ljiljana Paša-Tolić, Ph.D.
Laboratory Fellow and Deputy for Technology
W.R. Wiley Environmental Molecular Sciences Laboratory
Pacific Northwest National Laboratory

Response to comments

Original comments are shown in gray Calibri. Responses are in black Arial font.

Editorial comments:

NOTE: Please read this entire email before making edits to your manuscript. Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Protocol Detail: 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 ensure that all specific details (e.g. button clicks for software actions, numerical values for settings, etc) have been added 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.

1) 1.1: Mention plant growth conditions and environment. Mention age at plucking.

The protocol is generally agnostic to growth conditions. Details are now added in step 1.

• Protocol Numbering: Add a one-line space between each protocol step.

Added.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

Updated as requested.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We believe the discussion already addressed all of these points. We have modified the text to expand on a few points the reviewers asked about.

• Figures: Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Updated as requested.

• References:

1) Please spell out journal names.

2) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For less than 6 authors, list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).]

Updated as requested.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are BioRex70, Amicon, Phenomenex , Phenomenex Aeris,

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

All commercial sounding language has been removed from the main text.

• Table of Materials: Sort the list alphabetically.

Updated as requested.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form

of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All figures in the submitted manuscript were new, generated from previously unpublished data. In response to the reviewer comments, we added a published figure as Figure 3 in the revised manuscript and uploaded the re-print permission.

Reviewers' comments:

Reviewer #1:

The authors describe a protocol to isolate the nuclei and purify histones from sorghum leaf tissue and analyze the intact histones by top-down LC-MS. The manuscript is well written.

Reviewer #2:

Manuscript Summary:

This paper reports the extraction of histone from sorghum leaves and its application in the post-translational modification of histone. The manuscript does report some data that may be considered important and interesting, but it needs substantial improvement before it can be published. First of all, English is often incorrect. Many sentences have logical errors that make the manuscript difficult to understand. Professional language editors must be consulted before submitting revisions. The following suggestions are put forward:

We have done our best to revise the text and we hope it now addressed this concern.

Major Concerns:

1st major point

The experimental materials must be clear, such as sorghum varieties, planting environment and sampling time. Due to different periods and different states of sampling, histone extraction and analysis will be affected to some extent. Therefore, it is necessary to make clear all kinds of information about sorghum materials.

We agree that sample condition can affect the histone extraction efficiency. We added the information in the note to step 1.1 to describe the condition of the sample(s) we used to generate the data shown:

“NOTE: The sorghum plants were grown in soil in the field in Parlier, CA. Leaf tissue was collected by tearing off the third and fourth fully emerged leaf from the primary tiller. More details of field condition, sample growth, and collection can be found in published report.[18] The example data shown in Figure 1 and Figure 2 were from sorghum leaf collected at week 2 after planting. Although variation of yield is expected, we believe this protocol is generally agnostic to specific sample conditions. We have successfully used the same protocol for sorghum plant leaf tissue collected 2, 3, 5, 8, 9, and 10 weeks after planting.”

2nd major point

From the perspective of operational steps, it is not seen that this experiment has been repeated for three times to ensure the accuracy and scientific nature of the experiment.

We have used this protocol to extract histones from ~50 samples in previously published work (reference 29) and from over 100 field samples in a follow-up large-scale study (manuscript in preparation). Current manuscript is focused on the detailed protocol for histone extraction. We revised the last part of the abstract to emphasize that this protocol has been used for a larger study before:

“We have applied this protocol previously to profile histone PTMs from sorghum leaf tissue collected from a large-scale field study, aimed at identifying epigenetic markers of drought resistance. The protocol could potentially be adapted and optimized for chromatin immunoprecipitation – sequencing (ChIP-seq), or for studying histone PTMs in similar plants.”

3rd major point

It is not rigorous to determine the integrity of nucleus only by observing the color change of chloroplast during filtration. We should use some qualitative and quantitative data to ensure the accuracy of the experiment.

This protocol originated from another protocol initially developed for the ChIP-seq workflow. Initial optimization was performed with more rigorous testing. The color change is mentioned in the notes to help troubleshoot any potential issues. With the conditions already sorted out, we don’t feel it is necessary to check the integrity of the nuclei in every step. We agree it would be valuable to check the integrity of the nuclei at various points in the protocol, especially for the first time user, but this would be inefficient for large number of samples (as often encounter is such studies). We mentioned in the discussion that checking integrity of nucleus is advised, especially when transferring the protocol to other systems. We reworded the sentence to avoid confusion (highlighted in red):

“Optimal detergent concentration for other tissue or organisms may be different and need to be experimentally determined. Color change of the supernatant during the filtration process could indicate potential issues such as inefficient release of chloroplast or insufficient grinding of leaf. If possible, use a microscope (e.g. with methylene blue stain) to check for lysis of chloroplasts and retention of intact nuclei after each step to further optimize the protocol (especially if modifying the protocol for other tissues or plants).”

Additional details are added in NOTES for 3.1.4 and 3.2.2:

“NOTE: Both the filtrate and the filtered debris should be green. If tracking using a microscope, you should be able to see intact nuclei and intact chloroplasts in the filtrate at this point. The majority of large debris should be absent/depleted. Mix dyes such as methylene blue with sample. Nuclei are easily observable as ~3-5 µm diameter dark blue/aquamarine spheres when visualized using a using a 20X, 40X and/or 100X objective. Relative to nuclei, chloroplasts are similar in size, but greenish in color and often more oval in shape. Vacuoles are also similar to nuclei in size and shape, but they will not readily take up the Methylene blue dye.”

“NOTE: The detergent concentration needs to be optimized to preferentially lyse intact cells and chloroplasts but not nuclei. The amount required can vary among organisms. It is recommended to check for lysis of chloroplasts and retention of intact nuclei under microscope.”

4th major point

According to the author's operation steps, histone can be extracted successfully, but the output of histone is low. It is suggested to change reagents or carry out experiments at low temperature to reduce the degradation and unnecessary loss of histone.

We agree the protocol may be further optimized to improve yield. However, this is out of the scope of our current study. The current protocol on average yields sufficient material for the downstream mass spectrometry analysis as outlined in the manuscript. We revised discussion to note there is still room for improvement, especially regarding the enzyme inhibitors (edited text in red):

“We have used this protocol to consistently extract high purity histones for subsequent LC-MS analyses from over 150 samples. On average we were able to obtain higher yield without the use of “additional inhibitors” (unpublished data). Therefore, it is advised to cautiously test the new inhibitors when modifying or adapting this protocol for other purposes. If phosphorylation is not of interest, the phosphatase inhibitors can be omitted in the extraction buffers.”

5th major point

The key word "drought" given by the author is not involved in the experiment, but the prospect of histone modification of sorghum under drought condition is put forward. It would be more convincing if the author could carry out experiments on Sorghum leaves under both drought and normal conditions at the same time.

We have previously performed and published the comparison study (reference 29). To avoid confusion, we now added Figure 3 and a paragraph in the result section citing the published data for quantitative comparison of 48 samples. This manuscript focuses on the detailed protocol for histone extraction. We encourage the reader to check our published manuscript for more details.

6th major point

It is suggested that in the introduction part, the whole content written by the author should be divided into clear paragraphs according to a certain logical order, so that the readers can read more clearly.

We thank reviewer for this comment and have now divided the introduction into smaller paragraphs.

Minor Concerns:

Line 106, line 108, line 123, etc.

Loss of ℃ in 4 ℃.

Corrected.

Reviewer #3:

Manuscript Summary:

The manuscript of Zhou et al. presents a protocol for purification of histone proteoforms for top-down proteomics. The topic is definitely of high interest as there is a necessity to integrate data from various approaches to extend knowledge in the field of plant histones. Generally, the methods presented seem to be appropriate and feasible. However, the authors should specify / clarify / correct several points.

Major Concerns:

1) The authors should comment on the purity of isolated histones and provide the graph showing the proportion of histones within all identified proteins.

We added a comment in the result section. Based on our previous experience we usually see ribosomal proteins as contamination, but they can be separated by LC and generally do not interfere with histone analysis.

“Following the protocol, the histones can be extracted and identified using the LC-MS analysis. Based on the TopPIC results from the representative sample, we identified 303 histone proteoforms (106 H2A, 72 H2B, 103 H3, and 22 H4 proteoforms). Co-purified ribosomal proteoforms have also been detected, typically eluting early in the LC. They usually represent

~20% of the identified proteoforms, but do not overlap with the histone proteoforms eluting in the later stage of the LC gradient."

2) Figure 1: The authors should describe the proteoforms in the figure properly, or explain in the legend or in the text in more details what's behind. It should be mentioned that acetylations in Figure 1d represent both N-terminal and lysine acetylation. Similarly, methylations presented in Figure 1e represent me1, me2, and me3. No acetylation is indicated either in Figure 1e or 2, however, identification of acetylated forms of H3 is mentioned in the text. This should be clarified.

We thank the reviewer for this comment and have revised the discussion to clarify the labeling as follows:

"However, three methylation groups (14*3 Da) have the equal nominal mass to one acetylation (42 Da). Because these PTMs cannot be easily resolved at intact protein level, they are referred to as "methyl equivalents" (i.e. multiples of 14 Da; one acetylation equals three methyl equivalents). In Figure 1e, H3 proteoforms are labeled in the form of methyl equivalents based on their intact mass."

3) The authors admit that many proteoforms cannot be identified due to co-elution (especially proteoforms of H3 - lines 425-432; 509-516). On the other hand, they could identify altogether 303 proteoforms which is extremely high number. With this respect it would be useful to add the list of all identified proteoforms into supplementary data. This would be really interesting information not only from analytical but also from biological point of view.

Many of the proteoforms are truncated histones. At this point, we don't know the exact origin of these truncated proteoforms. Some of them may be biologically relevant, but some may be result of degradation during the sample processing steps. The number of identifications is directly taken from TopPIC output without extensive manual examination and filtering. We now included these results in the deposited data.

4) The authors should comment on quantification issue. The number of unambiguously identified proteoforms which could have been reliably quantified should be added in the text. In addition, those proteoforms together with co-eluting proteoforms should be marked in the list in supplementary data. The authors should discuss if it's feasible to estimate the quantity of individual sequential variants and calculate their ratios (e.g., H3.1 : H3.3 ratio). This would be useful and interesting information for plant biologists.

We have performed intensity-based label free quantitation in a previous study (reference 29) using the same protocol. We were able to observe significant differences in abundance for several proteoforms. The data is now included as Figure 3 to better demonstrate the quantitative aspects of the study, i.e. without having to look up the published study.

We agree with the reviewer that this is an interesting aspect to estimate the histone homologs/variants. However, our current manuscript is focused on the protocol for histone extraction. The example data is to show what a successful extraction looks like. The raw data

including different biological conditions for the previous study are available for the readers who are interested in researching these aspects.

5) The authors should deposit all MS data into PRIDE.

Example dataset is now uploaded at:
https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=dedbebf3dc124315b3de47af05607b3c
The original data for the comparative study (reference 29) are also available online as described in the manuscript.

Minor Concerns:

1) Line 95: Change „4 g of cryo-ground leaf" for „5 g of cryo-ground leaf" in order the amount of starting material is consistent with information in line 150.

Thanks for pointing out the inconsistency. For this data set the amount was 4 g.

2) Line 112: The reviewer recommends to change "Sodium Orthovanadate" for "activated Sodium Orthovanadate". The authors should add the information that Sodium Orthovanadate has to be activated - depolymerized before use to enhance the ability to inhibit phosphatases (see Gordon JA. Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzymol. 1991;201:477-82. DOI:10.1016/0076-6879(91)01043-2). Alternatively, the activated liquid form of Sodium Orthovanadate is also available (5086050004 Sigma-Aldrich).

We thank the reviewer for this comment. We only used sodium orthovanadate without intentionally depolymerize it. We have now added the note in step 2.4.

3) Line 113: Sodium butyrate is an HDAC inhibitor, not phosphatase inhibitor. Make a correction in the text and in Table 1 accordingly.

Corrected.

4) Line 121-125: The text in 2.7 and 2.8 is confusing. The reviewer recommends to merge 2.7 with 2.8 as both paragraphs refer to Gdn buffer preparation. Change "Prepare 5% Guanidine buffer pH7" for "Prepare 5% Guanidine in phosphate buffer, pH7". Change "Adjust pH to 7 by checking with pH paper" for "Check pH with pH paper and adjust to 7 using ….". More precisely, Potassium phosphate monobasic and dibasic should be mixed to adjust pH7 while keeping desired ion concentration.

Corrected.

5) Tables 1 and 2:

Change "Tris pH8" for "Tris-HCl pH8".

All inhibitors of enzymatic activity have to be added freshly just before use - add this information into 2.10. Transfer "Make the Nuclei Lysis Buffer (NLB) based on Table 2." from 2.10 to 2.11.

Add "overall volume" into Table 2 - similarly as it is in Table 1.

Corrected.

6) Line 143: Change "steps 1-3" for "steps 3.1-3.3".

Corrected.

7) Line 150: Change "leaf material" for "ground leaf powder".

Corrected.

8) Line 152: Add volume of EB1. Apparently, EB1 is prepared for all following steps - altogether 24 ml are need. Adjust the amount of PI accordingly.

Lines 175, 188: Add volume of EB2A and EB2B, respectively. Adjust the amount of PI accordingly.

Line 198: Add information that PI has to be added into NLB.

Corrected.