David S. Libich, Ph.D.
Assistant Professor, Biochemistry and Structural Biology
St. Baldrick's Foundation Scholar
Email: libich@uthscsa.edu

July 30, 2021.

Amit Krishnan, Ph.D.
JoVE Review Editor
1 Alewife Center, Suite 200
Cambridge, MA 02140

Re: Authors Response to Reviewers of JoVE 63057.

Dear Dr. Krishnan,

Thank you for the opportunity to respond to the comments that the reviewers made regarding our recent JoVE submission "Paramagnetic Relaxation Enhancement for Detecting and Characterizing Self-Associations of Intrinsically Disordered Proteins." We have had a chance to review the comments and suggestions raised by the reviewers and have made the appropriate changes to the text and figures. We have also corrected the editorial comments noted by JoVE.

We sincerely thank the anonymous reviewers for spending their time and considerable effort reading and commenting on our manuscript. Incorporation of their suggestions has produced a clearer, more detailed, and most importantly, more accurate protocol. We have responded in detail to each point from each of the three reviewers in the appended pages.

Sincerely,

David S. Libich, Ph.D.

Assistant Professor, St. Baldrick's Foundation Scholar
Department of Biochemistry and Structural Biology
Greehey Children's Cancer Research Institute
University of Texas Health San Antonio

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The overview by Johnson and Libich, presents an outline of a general protocol for the use of NMR paramagnetic relaxation enhancement (PRE) experiments to study intrinsically disordered proteins (IPSs). NMR is well-suited to the study of IDPs, as it is amenable to working in solution conditions and can provide information on several conformationally-relevant timescales which often involves very weak molecular interactions. Here, a protocol for the use of PREs to characterize conformational changes and intermolecular association is presented, which ostensibly can detect transient associations in the single-digit percentage population range. The protocol is detailed, although there is substantial room for the authors to add their own insights to help future readers. Additionally, while the representative results provide an example of the data analysis and processing, they do not guide the reader through the types of thorough quantitative analysis that is suggested in the introduction and discussion sections.

We appreciate the reviewer's time and effort in constructively criticizing our manuscript. We have addressed all of the points raised by the reviewer and made significant clarifications in the text. We welcome that the manuscript is clearer and more focused after these corrections. We address the reviewer's specific comments below.

Major Concerns:

Need to go over the types of thorough quantitative analysis that is suggested in the introduction and discussion sections.

We have clarified that we intended to position this protocol as an introductory method for casual and novice users to be able to obtain measurements of PREs on their intrinsically disordered protein (IDP) of interest. It is intended to provide the initial steps necessary to begin these types of experiments with a specific focus on IDPs. The reviewer is entirely correct that quantitative analysis of these types of data have been developed by several groups, but we note (and now describe) that the quantitative interpretation of IDP interactions using PREs can become complicated very quickly. We have made the decision to make the reader aware of the different approaches for analysis and have provided appropriate references to support further exploration of these analysis but have decided to limit the protocol to qualitative interpretations. We discuss some of the confounding factors that complicate qualitative interpretation to provide context to the complexity of these measurements. We have clarified these points in the Introduction, Protocol, Representative Results and Discussion sections.

Minor Concerns:

1. Line 57: "ridged" may be a typo for "rigid"

Corrected

2. Line 71-72: "detection" and "identification" are not quantifiable

Corrected – removed "to quantify"

3. Line 79: The PRE effect is also observed for systems having an anisotropic g-tensor; it is merely confounded by the presence of PCS and RDC that may make analysis more difficult (see ref 13 in this work).

We agree and thank you for pointing out this important point. We have clarified the text by adding: "*While nuclei with anisotropic g-tensors also produce a PRE effect, analysis of these systems is more difficult due to confounding effects contributed by the pseudo-contact shifts (PCS) or residual dipolar coupling (RDC)*."

4. Line 88: The conditions for detection of transient states by PRE should have a citation; eg: Anthis and Clore, Q. Rev. Biophys. 2015, 48(1), 35-116.

Citation was added

5. Line 105: The authors neglect other forms of metal chelated tags. If no room to list, then I'd suggest stating that there are a number of options for metal tags.

This is an astute comment, and we appreciate the work of many groups that have contributed to the development and characterization of tags useful for PRE and PCS measurements. We now note that there are many options for both the choice of the paramagnetic tags as well as for ligation chemistries. We chose to limit the discussion to nitroxide-based tags for their relative ease of use over metal-chelation tags since for example, ascorbic acid readily produces the diamagnetic condition while with metal-chelation tags, two samples are needed. We agree with the reviewer that there are multiple, good choices for PRE tags, and that the best choice for a particular protein is dependent on specific experimental considerations as well as the characteristics of the protein of interest. We have clarified this by adding: "*Several types of*

molecules have been proposed for use as spin labels including metal chelating (EDTA-based) and free-radical (nitroxide-based)." And have included additional references to direct the reader to alternatives.

6. Line 106-107: In addition to chemical linkage differences, nitroxide spin labels can be made more rigid through the introduction of bulky groups or the presence of a second linker.
Thanks to the reviewer for raising this important point. Certainly, one of the major challenges when deriving restraint information from PREs is accounting for the anisotropic motions of the paramagnetic tag. The complexity of this problem may be significantly reduced by using more rigid tags. We note that our protocol was intended for the specific case of detecting transient interactions between IDPs, and because of other unknowns introduced by flexible polypeptide chains, and the qualitative analysis presented, the rigidity of the tag is of lesser concern. We have noted this by adding: "*Inherent flexibility of the tag or of the linker may be problematic for certain analyses and in these situations, different strategies have been proposed to limit the motion of the tag such as by adding bulky chemical groups or the use of a second linker to anchor the tag to the protein (two site attachment).*" to the introduction and have included appropriate references.

7. Line 110-111: The choice of isotropic g-tensor also eliminates confounding RDCs in addition to PCS
Changed to "…has an isotropic *g*-tensor, no PCS or RDCs are induced…"

8. Line 115: move the comma to before "and"
Corrected

9. Section 1.1.1: Adding citations to the potential importance of native cysteines, or the deleterious effects of removing disulfide bridges would be helpful for future readers
This section was moved to the Discussion and expanded to describe how to deal with native cysteines as well as what might happen if native cysteines that participate in disulfide bridges are disrupted. Appropriate references were added.

10. Section 1.1.2: Why it is advantageous to introduce spin labels at multiple sites should be stated
This section was moved to the discussion and the explanation was expanded.

11. Sections 1.2.1 and 1.2.2 make more sense inverted, so that the note on expression system follows more closely to 1.2
Sections were re-ordered.

12. Section 1.2.3: This is protein, buffer, pH, and temperature dependent, and reducing agents may not be required at some or even all steps during purification depending on the system and even the engineered site within a protein. A citation and explanation on why this would be a requirement for IDPs would be useful.
The reviewer is correct to point out that need for including a reducing agent during all steps of the purification are dependent on several physiochemical factors as well as the characteristics of the protein of interest. We have found, that in the case of many self-associating IDPs, it is practical to always have reducing agent present. The text "*Preforming all purification steps in the presence of 1 mM reducing agent (DTT or TCEP) to prevent reaction of the free cysteine and formation of intermolecular disulfide bonds is recommended for IDPs although some systems may be more tolerant due to non-reducing conditions depending on the specific characteristics of the protein, as well as the pH, temperature and of buffer system chosen for purification.*" was added as well as a reference describing these points in more detail (Rabdano, et al., (2017) *Sci Rep*. **7**:11161)

13. Section 1.2.4: This should have an explanation for the case where no cysteine is present, and probably a citation as well
This was renumbered to section 1.2.3. We recommend removal of the purification tag in all situations, even if the tag is relatively simple and seems innocuous, (e.g. hexa-His tag) since the effect on the protein remains unknown in most situations. For example, for EWS 171-264, we realized that the presence of a hexahistidine tag alters the self-associative properties of the protein. The suggestion that the purification tag may contain a reactive cysteine was for a tag like GST or thioredoxin. The text has been revised to reflect this recommendation more clearly.

14. Section 1.2.5: This reference sample does not have a clear reference in the rest of the text.
This was renumbered to section 1.2.4. This is a recommended control sample to assess the occurrence of non-specific interactions between the tag and the protein of interest. Its utility is now described in the Discussion.

15. Line 257: I am a bit confused why you'd suggest a second delay estimate to be 1.2 times 15N T2. In most cases thew

15N T2 would be much longer than the 1H T2 (about 5x). Therefore 1.2 times 15N T2 delay in a proton T2 expt would result in signal that would be less than one percent of the original signal.
The reviewer is absolutely correct on this point and the approach to selecting an appropriate value for T_b was clarified to suggest starting at 1x the ^{1}H T_2 and optimizing from there by trading off S/N for accuracy in the T_2 measurement. Since many of the proteins we deal with are aggregation prone, they cannot be maintained at very high sample concentrations and thus it is important in these cases to optimize S/N for more reproducible sample-to-sample measurements. In general, PRE experiments are performed at low sample concentrations (< 300 μM) to limit the contribution from the solvent PRE effect.

16. Line 259-261: The 40-50% residual signal for high quality data is a bit misleading. This is a compromise between S/N and being able to sample longer T2 delay. The longer T2 delay will lead to more accurate T2 determination albeit with lower S/N. There is an optimal sampling delay.
We agree with the reviewer's point, and for the 2-point measurement approach, there is an optimal delay that accurately measures T_2 within a few percent of the T_2 values determined with a fully sampled evolution curve. In the qualitative case presented here, the absolute accuracy of the measured T_2 is less important than S/N for sample-to-sample reproducibility, thus the recommendation is to choose delays that do not lead to significant signal decay. Further, we note that there really is not an optimal T_2 delay for measuring both the paramagnetic and diamagnetic sample, thus common practice is to optimize S/N in the paramagnetic sample. We accept the reviewer's point and have included a clarification in section 5.1.

17. Section 2.2: Please provide a reference for not agitating during the labeling reaction, as this is commonly performed.
As the reviewer succinctly pointed out earlier, the exact incubation conditions are sample dependent, and since it is important not to introduce oxygen at this step vigorous agitation should be avoided. Gentle nutation or rocking may (or may not) improve the labeling reaction. The text has been clarified to reflect that nutation at this step may be helpful for the labeling reaction.

18. Line 216: "primary considerations" should be changed to "the primary consideration"
Corrected

19. Section 3.2.2. While the ratio of unlabeled and labeled protein should be optimized, this would strongly benefit by having a set of previously useful ratios from the authors, and the considerations they used for optimization.
Typical ^{14}N-labeled to ^{15}N-unlabeled protein concentrations were included as well as points to consider for optimization of the ratios are now included along with an appropriate reference (Fawzi et al., (2010) *PNAS*. **107**:1379).

20. Section 6.4: Define "significantly different" in the context of pulses
We have defined significantly different pulse lengths to be greater than a 0.5 μs difference on ^{1}H since differences larger than that will begin to manifest as errors when comparing peak intensities.

21. Section 7.1: It should be made clearer when certain programs substitute for others (eg: Sparky vs CCPN)
Thank you for pointing out this potential source of confusion. For the description of the protocol, we have decided to mention only Sparky to reduce the potential confusion of the reader. We now include a paragraph in the Discussion that details the many software packages available that can perform the analysis as described, their overlapping capabilities and now clarify where one package may be substituted for one another.

22. Line 305: the phrase "can be optimized" appears to continue from the previous sentence
Corrected

23. Section 8.2 refers to an NMRPipe command, and so should probably come before section 8.1, which states that the following steps will use Sparky
Corrected

24. Line 325: Purposeful exclusion of peaks seems unlikely, perhaps this should be changed to "unintentionally exclude peaks"
Corrected

25. Section 9.1: This is phrased strangely. "spreadsheet" should probably be altered to "spreadsheet software" and

"computing interpretative language" should be altered to "preferred programming language" and examples (eg: Microsoft Excel for the former, and python or MATLAB for the latter, should be provided).
Corrected

26. Section 9.5: This equation for uncertainty depends on the peak intensity and SNR, but the uncertainty is redefined in section 9.6 in a way that depends only on the root-sum-squared of 1/SNR. The equation in Section 9.5 is likely redundant.
Corrected - thank you for pointing out this oversight.

27. Line 352: "Observed Γ2 is indicative of residues that are in close proximity to the spin label" is either redundant with the sentence on lines 355-356 or missing a phrase.
Corrected

28. Lines 353-354: Please provide an estimate of the range where "close proximity to the spin label" leads to undetectable broadening.
Corrected – proximity range is now given.

29. Lines 356-357: Please expand on how qualitative interpretation of the data indicates that the protein is more compact than an extended chain.
The Representative Results and Discussion sections have been revised to address this point and points 32, 33 and 34 below. We have clarified the description of the results and provided more detail about what constitutes the observed PREs as well as what might be giving rise to these observations.

30. Line 357: delete "is" in "what is remains unknown"
Corrected

31. Line 360: "qualitatively" should probably be changed to "quantitatively"
Corrected

32. Line 361: Change "position" to "positions." This statement should be added to the earlier methods description where it mentions to prepare for alternative sites. Also, please expand on how more tags provides a "more accurate interpretation of specific interactions."
A statement was added at the beginning of the protocol to indicate that multiple mutants will be required to introduce spin-labels at different position in the protein. The discussion of the utility of multiple tags for interpretation of observed PREs was included in the Representative Results section.

33. In general, the Representative Results section could use careful attention to phrasing. Additionally, the introduction section of the manuscript seems to indicate that this protocol can be used for quantitative interpretation, but no quantitative results are included in this section. There is no clear indication to future readers about how the use of further tag sites would be beneficial to them, and no sufficient explanation of how the use of intra- and inter-molecular PREs would be beneficial in the presented example.
We have clarified the introduction to indicate that the presented method is aimed at casual or novice users of NMR to obtain PREs of their protein of interest. Further, we use IDPs as an example which, for reasons discussed in the text, make quantitative interpretation difficult in many cases. The readers are directed to the appropriate references that can significantly expand on the undelaying theory as well as quantitative analysis approaches that will build upon the introductory method presented. We have also rewritten the Representative Results section and expanded the Discussion sections to address several of the comments of the reviewers.

34. The discussion is well-written, and only serves to highlight some of the omissions in the earlier portions of the manuscript, as it includes references to "complimentary information" available from "placing the spin label at different positions within the protein" and the ability to differentiate intra- and inter- molecular sources of the observed PRE. Both of these are not well explored in earlier sections of the text, and would be very helpful if included in detail in the representative results.
The Discussion and Representative Results sections have been reworked to address these and other concerns raised by the reviewers.

Reviewer #2:

Manuscript Summary:

In this manuscript Johnson and Libich provide a protocol for NMR-based analysis of disordered proteins using paramagnetic relaxation enhancements obtained from covalent tags. The protocol manuscript is well-described and will be useful for a plethora of applications.

We thank the reviewer for their time and effort in reviewing our manuscript. We appreciate the constructive comments and are appreciative of the improvements in the communication of our protocol.

Some suggestions:

* Introduction: a short discussion and references to application highlights from recent literature would be great

We have added examples (with references) of studies where PREs were used to characterize alpha-synuclein forming fibrils, the self-association of FUS, as well as the interaction of two bacterial signaling proteins.

* Protocol, part 1: please expand the discussion of how the choice for positioning of spin labels should be made. What would be a good strategy to ensure that mutations don't affect (transient) structure and interactions? Things to come to my mind are CSPs, solvent PREs, but maybe the authors have some more ideas along this line.

This is an important consideration raised by the reviewer. We have expanded in both the Protocol and Discussion sections strategies to use for positioning spin labels, suitable residues to mutate, monitoring the effects of mutations, and how to deal with proteins that contain multiple cysteines.

* Protocol, buffers: please add that some buffers, i.e. phosphate, are not compatible with TCEP (short half-life)

Corrected – added a sentence to clarify that compatibility between buffer components should be considered and that some combinations should be avoided outright. A reference was included to reinforce this point. (Burns et al., (1991) *J Org Chem.* **56**:2648).

* Line 194: completeness of SL incorporation of the 15N-labeled protein can also be monitored by looking at the HSQC cross peak of the tagged residue which should disappear completely if fully tagged.

We appreciate the excellent suggestion by the reviewer. As noted by other reviewers, the use of mass spectroscopy to judge label incorporation is problematic for several reasons. This suggestion has been incorporated.

* If there is space, the authors could mention determination of PREs using 13C/15N labeled samples. This can provide additional PREs of 13C-bound protons and allow recording of triple resonance experiments to reduce overlap (e.g. HNCO in combination with NUS). For IDPs this has been proven useful. Detection of 13C PREs using 13C direct detection has been reported as well.

Thank you to the reviewer for pointing these additional approached to measuring PREs. We are aware of the use of the HNCO experiment as a way of overcoming the dispersion problem for IDPs. As an aside, we have utilized the HNCO approach, and we found that in the case of proteins with repetitive sequences (like the example in the manuscript) the gains in resolving certain residues were not always consummate with the extra effort required for protein preparation for example. Thus, for this article that is aimed at for a casual user or novice audience, we opted to keep the approach as simple as possible. We suggest increasing acquisition time (where appropriate for an IDP) to help resolve partially overlapped peaks and describe a simplified quantitative analysis. We have added a paragraph in the Discussion section that describes the use of HNCO experiments and measuring ^{13}C PREs that would be useful in many cases.

* Line 244: we prefer to calibrate the 15N pulse using the same sample. This can be done using a HMQC-based experiment where the length of one of the 90° 15N pulses is varied (loss of signal -> 180°) to calibrate the 15N 90° pulse.

We agree with the reviewer that this is the most accurate way of calibrating the 90º ^{15}N hard pulse. We do note however, that for the two-point measurement of $^{1}H_N$ T_2 and qualitative analysis described in this manuscript, using an external standard provides an accurate enough calibration for the ^{15}N pulse length. We have included the reviewer's suggestion and added a sentence in the Protocol describing alternate approaches for calibrating nitrogen pulse lengths.

* Line 345: please check the error propagation formula. It should contain the partial derivatives of the formula shown in 9.3 in the peak intensities

Corrected - thank you for pointing out this oversight.

* Line 355: the PRE depends on the distance, population and the dynamics (tau_c) of the interaction vector connecting the electron and the nuclear spin.

This was clarified in the Representative Results section with the following sentence: "*In the case of EWSR1 171-264, attributing the source of the PRE effect is complicated since it may arise from a combination of inter and intra residue contacts, and is dependent on the distance from the nucleus to the paramagnetic center, the population of that conformation, and the dynamics of the vector connecting the electron and nuclear spins.*"

* The inter-molecular PRE depends on the protein concentration (-> population), whereas the intra-molecular PRE does not (assuming that correlation times don't change with concentration etc.). This might need to be included, together with a statement that the Kd and dynamics of the inter-molecular interactions affect the inter-molecular PRE.
Thank you to the reviewer for this comment. These are important points that needed clarification. Lines were added to both the Representative Results and Discussion sections to expand and reinforce these concepts.

Reviewer #3:
Manuscript Summary:
The protocol from Johnson and Libich provides some recipes for measuring PREs. The protocol reflects standard practices and advice on how to design the experiments, as well as a protocol using scripts from NMRpipe and Sparky.
Thank you to the reviewer for their time and effort in reviewing the manuscript. We appreciate their comments and critiques, and that the manuscript is strengthened because of their input.

Major Concerns:
The authors recommend 3-maleimido-PROXYL but in the list of reagents they just mention MTSL and the reference they provide (81213-52-7) is not the supplier reference but the CAS (Chemical Abstract Service) registry number.
Corrected

Figure 1 legend contains various errors: the structures depicted include radicals and diamagnetic molecules (e); a variety of derivatization chemistries, not just maleimido; entries (d) and (e) are pyrroline -with the unsaturated ring- and entries (a)-(c) are pyrrolidine -with the saturated ring- the rigidity of the ring is different. The substituted pyrrolidine has a chiral center and, presumably, the commercial product is racemic, which will lead to a mixture of diastereomerisomeric paramagnetic proteins. Not too serious an objection for the qualitative analysis described but, considering this is a protocol, the reader should be warned not just about the problem of pseudocontact shifts -not present- but also on the stereochemical heterogeneity, that would be present, although probably not noticed.
Thank you to the reviewer for pointing this out, and we agree that while these effects will be small for most cases, there is the potential for producing unintended results. The following text and references were added to clarify this point: "*Inherent flexibility of the tag or of the linker may be problematic for certain analyses and in these situations, different strategies have been proposed to limit the motion of the tag such as by adding bulky chemical groups or the use of a second linker to anchor the tag to the protein (two site attachment)* (Lindfors et al., (2008) *J Biomol NMR*. **3**:157). *Additionally, commercially available tags may contain diastereomeric proteins but generally this will not contribute to the observed PRE* (Bleicken et al., (2019) *ChemistryOpen.* **8**:1035)." The errors in the Figure 1 caption were corrected.

Minor Concerns:
The error analysis presented in part 9 is obscure. The same symbol (sigma) is used for inequivalent errors (peak intensities and relaxation rates). A clearer explanation is given in reference 22.
Corrected - thank you for pointing out this oversight.

The problem with using mass-spectrometry for quantification of the level of incorporation is more fundamental than the possible difficulties of some proteins to be ionized.
We agree with the reviewer and note that this was pointed out by other reviewers as well. We have removed the recommendation to monitor label incorporation by mass spectroscopy and replaced it with the more straight forward method of tracking labeled residue peak intensity. We note the limitation of this approach for some IDPs for example if the peak is overlapped, and thus also suggest the use of Ellman's reagent.

Some typing errors should be corrected (e.g. Line 394 Complementary)
Corrected

Editorial comments:

Editorial Changes

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.

Corrected

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

Corrected

3. 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.

For example: TopSpin, MilliporeSigma, Bruker, New Era, etc.

Corrected. We note that the instructions and pulseprograms described are written specifically for Bruker spectrometers running TopSpin version 3.2 or later.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Corrected

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Corrected

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Corrected

7. 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 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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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.

Corrected

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Appropriate protocol steps that should be visualized are highlighted.

9. As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Corrected

10. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
Corrected. Note that these errors arose from the JoVE EndNote style template that was downloaded from the JoVE website. We were using Endnote 19 and Mac OSX 10.15.X and the issue was replicated on multiple computers. Upon further inspection of the template file, the “&” modifier is included in the “*Bibliography:Author Lists*” field in the output style.

11. Please use uppercase letters to label the figures.
Corrected

12. Please sort the Table of Materials in alphabetical order.
Corrected