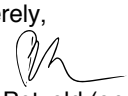
Revision for
"Practical aspects of sample preparation and setup of ^{1}H $R_{1\rho}$ relaxation dispersion experiments of RNA"

Please find below our changes are requested by the reviewers. We appreciate the time and input on our work and believe that we have addressed all concerns accordingly.

Sincerely,



Katja Petzold (on behalf of all the authors)

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.

2. Please provide an email address for each author.

ANSWER: We provided email addresses below the affiliation. The corresponding author has been underlined.

3. Include a single space between the quantity and its unit. E.g. "20 oC" instead of "20oC"

ANSWER: We used the notation for all quantities but "°C", and now changed all mentions to "20 °C".

4. Use "NOTE" instead of "Tip".

ANSWER: We changed all "Tip" to "NOTE"

5. 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. E.g. RNaseZap, etc.

ANSWER: We found no commercial language in the manuscript. If we missed any, please indicate the specific instance.

6. Please refrain from using bullets or dashes in the text.

ANSWER: Dashed were used only when enumerating items that did not require a specific order.

7. Maintain a 0-inch left indent throughout the text and indicate new paragraphs using single-line spacing.

ANSWER: Formatting was maintained.

8. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Do not use "& / and" in the author list of the references.

ANSWER: Changed all uses of "&" in the bibliography. Wrote all journal names in full length.

Reviewer #1:

In this manuscript, Petzold and co-workers give an overview of practical aspects of RNA sample preparation and the setup of R1ρ relaxation dispersion experiments. The manuscript is well written and covers most of the crucial aspects to run relaxation dispersion NMR experiments on RNA.
There are only a few minor points which should be addressed by the authors prior to publication:

1) * The authors should include a short note on the CEST experiment with high spin lock powers in the introduction section. This experiment could be a possibly more efficient alternative to the R1rho experiment as it can be run in 2D mode and not in a rather inefficient 1D data acquisition method.

Also add the reference:
(https://onlinelibrary.wiley.com/doi/full/10.1002/anie.202000493).

ANSWER: We thank the reviewer for the comment. We added the following sentence to line 59 and implemented the reference in line 53: "*It should be noted that recently a chemical exchange saturation transfer (CEST) experiment using higher spinlock (SL) powers, thereby shifting the applicability of the CEST experiment to faster exchange time scales, was published as a possible alternative to the $R_{1\rho}$ RD experiment for systems with one excited state.*"

2) * The authors should give an estimate of the RNA size limit (number of nucleotides, too much spectral crowding) in the SELOPE approach.

ANSWER: We thank the reviewer for the feedback. We added the following sentence to line 116:

"In general, we recommend using the SELOPE experiment for RNA samples of up to 50 nucleotides. For larger RNAs the overlap will be bigger, however, structurally interesting nucleotides often appear in chemical shift regions which are less overlapped and still might be accessible in even larger RNAs."

3) The authors should report the typical yields (nmol RNA) of their RNA synthesis procedure.

ANSWER: Following the reviewer's request, we implemented the following sentence into the discussion (line 720):

"Typical yields using this method range between 30 and 70 nmol RNA per 1 mL IVT. "

4) RNA folding: the authors should include a short discussion here: For the promotion of duplexes folding under thermodynamic control (i.e. heating, then slow cooling) should be used. For hairpin formation folding under kinetic control (heating, snap cooling) should be applied. The authors should also add a comment that a refolding procedure can be necessary - e.g. after 1 week in the spectrometer it might be necessary to do a heating, snap cooling procedure to again form the hairpin. This is especially true for RNA at high concentration, which tends to form homoduplexes.

ANSWER: We highly appreciate the reviewer's thoroughness for methodological detail here. Accordingly, we added the following paragraph to the discussion in line 774:

"*Highly concentrated hairpin samples folding under kinetic control (heating-and-snap-cooling) can form homodimers over time, which necessitates rigorous control of RNA folding before each NMR measurement. If the measured RNA is not a hairpin structure but an RNA duplex, slow folding under thermodynamic control should be applied. In this case, the cooling process after heating should be in the range of hours, while the RNA is used in its final volume and concentration of the NMR sample. "*

5) The authors should include a step between step 3.2 and 3.3 that it might be necessary to calibrate spin lock powers.

ANSWER: We agree with the necessity of proper spinlock calibration and added the following note between step 3.2 and 3.3:

"NOTE: If ^{1}H SL experiments are run for the first time, we recommend checking if the calculated SL powers correspond to the powers delivered to the sample by calibrating SL powers for each desired bandwidth. Detailed calibration steps are described in Steiner et al[10]. "

Reviewer #2:
The manuscript by Feyrer et al. describes a protocol for how to carry out NMR 1H R1ρ relaxation dispersion experiments to study RNA dynamics. Being intrinsically highly flexible, RNA molecules undergo distinct conformational changes during their biological functions. Recent development in NMR relaxation dispersion techniques has provided powerful tools for studying RNA dynamics at micro-to-millisecond timescales and at atomic resolution, a task that has been difficult if not impossible using conventional biophysical and biochemical techniques. In the present manuscript, the authors described a step-by-step protocol for sample preparation and

experimental setup for one of these powerful NMR techniques for RNA. The protocol is clearly presented and is easy to follow for readers interested in performing RNA dynamics measurements using NMR 1H R1ρ relaxation dispersion technique. In summary, with the expanding biological significance of RNA, the current manuscript provides a timely protocol to introduce recently developed RNA NMR techniques to the scientific community interested in studying RNA functional dynamics. Thus, the reviewer recommends publication of this excellent protocol with only minor suggestions and comments for the authors to consider.

1. The current title states "… R1ρ relaxation dispersion experiments of RNA". It would be more accurate to specify the technique described in the protocol as "… 1H R1ρ relaxation dispersion experiments of RNA".

ANSWER: Thank you for the good suggestion. We changed the title accordingly: *"Practical aspects of sample preparation and setup of ^{1}H $R_{1\rho}$ relaxation dispersion experiments of RNA."*

2. Lines 56-57, it was stated that "… R1ρ RD experiments also give the chemical shift difference of the excited state, …" This statement is not accurate. CPMG RD can also give the chemical shift difference of the excited state. In this regard, the property that distinguishes these two RD experiments is that CPMG RD only provides the absolute value of difference, while R1ρ RD also provides the direction of chemical shift changes. This statement needs to be reworded.

ANSWER: We agree with the assessment and changed the sentence of line 55 to the following:

"*While both can be used to extract population of and exchange rate and chemical shift difference to the minor state, $R_{1\rho}$ RD experiments also give the sign of the chemical shift difference of the excited state. This allows an inference on secondary structure, which strongly correlates to chemical shift in RNA structures[17].*"

3. Line 150, the term "(T7:" is confusing, as the following sequence includes both the T7 promoter and an initiation sequence. The authors might want to consider another term.

ANSWER: We understand that the double notation of T7 might be slightly confusion here. The initiation sequence is technically part of the T7 promoter, even though it is already RNA which is being transcribed and more variable than the 17nt dsDNA of the promoter. We use them together as one sequence, as it provides the start of transcription in high yield. We added the following sentence to line 150:

"*NOTE: Transcription will start at the nucleotide indicated with ^. The initiation sequence GGGAGA is variable, but strongly sequence-dependent and we recommend the use of this sequence.* "

4. Line 201 and Table 1, the authors need to specify pH conditions for reagents such as Tris-Cl and NTPs.

ANSWER: We thank the reviewer for their methodological thoroughness. We added the following parenthesis to step 1.3.1:

"Prepare stock solutions of Tris-Cl (pH 8.0), DTT, $MgCl_2$, Spermidine and NTPs/GMP (unbuffered). Mix reagents as shown in table 1. A master mix of these reagents can be prepared in advance, before addition of enzymes or nucleic acids. "

5. Line 328 and section 2.5 "Confirm folding by NMR." The authors presented an approach to evaluate sample folding by comparing NMR spectra of the sample to those of the properly folded reference sample. While this is a valid approach in principle, it highly depends on whether a user would have access to the properly folded reference sample. In case the RNA to be studied is a new system, there would not be a reference sample. Hence, the reviewer recommends the authors to include some biochemical methods for evaluating RNA folding in section 2.3 "Folding of RNA sample", which can further complement the presented spectroscopic approach in section 2.5.

ANSWER: We completely agree with the reviewer's assessment on the folding confirmation. Our protocol focusses on the dynamics measurements with a new sample. However, we omit the crucial step of resonance assignment and at least a secondary-structure determination, as mentioned in step 2.5 and line 714. As a crude approximation, we propose a count of expected imino signals (line 774) and added the following sentence as suggested by the reviewer to step 2.5:

"NOTE: Electrophoretic mobility shift assay (EMSA) can be a helpful indicator of RNA folding and serve as complementary data next to NMR experiments."

6. Line 440, the authors need to provide name(s) of the software used in data processing and analysis.

ANSWER: according to the reviewer's suggestion, we added software to steps 3.6.1 and 3.6.2.

3.6.1 "In TopSpin (we use Topspin 3.6.2) process each slice of each pseudo-2D data set using the same processing parameters (e.g. line broadening, phase) using command xf2 and split dataset into 1Ds using the Bruker au program split2D."

3.6.2 "Obtain signal intensities and volumes for each 1D slice. In practice we actually recommend to deconvolve the spectra. This allows to get rid of contributions from potentially overlapping peaks but also allows the usage of the Bruker au program multidcon, which conveniently summarizes the intensities or areas of the peaks of all slices in one experiment in the text file "decall.txt" which can then be read out easily with other programs (we use in-house written Python scripts, as e.g. described in Steiner et al[10]), in steps 3.6.3 and 3.6.4. "

7. Line 473, "± (3 or 4 Hz)*SL" should be "± (3 or 4)*SL".

ANSWER: We thank the reviewer for their detailed review. We changed line 473 accordingly.