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Title: Practical Aspects of Sample Preparation and Setup of ^{1}H $R_{1\rho}$ Relaxation Dispersion Experiments of RNA

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **NO**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **YES. DONE.**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes (for mentioned b-roll)**

   If **Yes**, how far apart are the locations? 500 meters

Current Protocol Length

Number of Steps: 12
Number of Shots: 39

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hannes Feyrer:** This protocol describes RNA sample preparation and NMR setup for proton R1rho relaxation dispersion measurements to examine conformational exchange in RNA molecules.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-Roll: 2.8.2 for "NMR set up"*

1.2. **Hannes Feyrer:** Since protons are probed, the method does not need isotopic labelling and can access structural features like shifted base-pairing directly.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Hannes Feyrer:** The pipeline is quite modular, in the sense that R1rho relaxation dispersion can be measured even if the sample is produced with a different method.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-Roll: 2.7.4 for "sample is produced"*

Protocol

2. NMR Sample Preparation

2.1. Begin by preparing the plasmid sample and setting up an *in vitro* transcription and cleavage reaction as described in the text manuscript **[1]**. Incubate the reaction at 37 Celsius for 1 hour **[2]**, then dilute 1 microliter of the sample 10-fold in loading solution **[3]** and run 1 microliter on a denaturing PAGE gel **[4]**.

2.1.1. WIDE: Establishing shot of talent adding reaction components. **TEXT: Refer to text for details**

2.1.2. Talent removing reaction tubes from an incubator.

2.1.3. Talent diluting the sample.

2.1.4. Talent runs the sample on the gel and proceeds for imaging.

2.2. If the cleavage reaction is successful, scale the reaction to the desired volume and run it overnight, then run another denaturing PAGE gel **[1]** and assess whether the cleavage reaction has been completed **[added 2]**.

2.2.1. Talent placing the gel in the imager

2.2.2. Added shot: Talent observing the resulting gel image at the computer

2.3. In case of incomplete cleavage, heat the reaction solution in a conventional microwave at 450 watts for 15 seconds **[1]**. Then, cool the solution slowly to 37 degrees Celsius for 40 minutes seconds to reanneal the RNA and the cleavage guide **[2]** and note the formation of a new precipitate **[3]**.

2.3.1. Talent placing reaction solution in microwave and setting time. *Videographer: This step is important!*

2.3.2. Talent taking out solution from microwave and placing the solution in a water bath.

2.3.3. Shot of precipitate being formed.

2.4. Add more inorganic pyrophosphatase and RNase H **[1]**, incubate the reaction for 1 to 3 hours at 37 degrees Celsius **[2]**, then confirm completion of the cleavage reaction with denaturing PAGE **[3]**.

2.4.1. Talent adding IPPAse and RNase to reaction mixture.

2.4.2. Talent putting the reaction in the incubator. Videographer NOTE: Use 2.3.2

2.4.3. Talent adding sample to PAGE. Author NOTE: Use 2.1.4, without the part 'cleaning of wells with the syringe'

2.5. When the RNase H cleavage reaction is completed **[1]**, quench the reaction by adding EDTA to a 50 millimolar final concentration **[2]** and vortex thoroughly **[3]**. Filter the solution through a 0.2-micron syringe filter **[4]** and concentrate the solution to a volume injectable into an HPLC system for purification **[5]**.

2.5.1. Talent removing sample. Autho NOTE: Use 2.3.3, removing the part showing talent with the tube in hand

2.5.2. Talent adding EDTA. Videographer NOTE: Slated as 2.5.1

2.5.3. Talent vortexing the solution. Author NOTE: 2.5.2 and 2.5.3 are filmed as a single shot: 2.5.1

2.5.3.1. Added shot: Talent filters the solution using the syringe filter

2.5.4. Talent injecting the sample into HPLC system.

2.6. Before adding the concentrated and purified sample to the NMR tube, clean the tube by flushing with abundant water **[1]**, then RNase decontamination reagent, water, and 95% ethanol **[2]**. After the final rinse with water, leave it to dry **[2]**.

2.6.1. WIDE: Talent cleaning NMR tube with water.

2.6.2. Talent cleaning NMR tube with RNase decontamination reagent/ water/ 95% ethanol.

2.6.3. Shot of tube drying.

2.7. Rinse the plunger with water **[1]** and use a lint-free wipe to clean with RNase decontamination reagent and 95% ethanol **[2]**. After drying the tube, add 10% D_2O *(D-2-O)* to the NMR sample **[3]**. Use a large pipette tip to transfer the RNA sample into the NMR tube, flowing it along the side of the tube wall **[4]**.

2.7.1. Talent cleaning the plunger. Videographer NOTE: 2.7.1 and 2.7.2 are filmed as a single shot

2.7.2. Talent wiping the plunger.

2.7.3. Talent adding water to the sample.

2.7.4. Talent filling the sample into the tube.

2.8. Insert the plunger into the tube by pushing it down with a fast-twisting motion to remove air bubbles, then pull the plunger up slowly without creating new air bubbles **[1]** and fix it with paraffin wax film **[2]**.

2.8.1. Talent inserting, pushing the plunger and pulling it up. *Videographer: This step is important!*

2.8.2. Talent fixing the plunger with wax.

2.8.3. Added shot: Talent places the sample in the spectrometer. Author NOTE: Extra shot due to different location.

3. ^{1}H $R_{1\rho}$ Relaxation Dispersion

3.1. Create a new data set based on an aromatic proton-carbon-HSQC data set used on fully labeled RNA samples for RNA assignment **[1]**.

3.1.1. WIDE: Talent at the computer. *Videographer: Obtain a few shots of talent clicking the mouse and typing on the keyboard to use as b-roll throughout the video.*

3.2. Set the general parameters and RD-specific parameters **[1-TXT]**, then set proton spin lock power to 1.2 kilohertz for testing **[2]**. Generate a **test vd list** with only one entry, **0 milliseconds**, set **TDF1** to 1, and update **D30** to run a test spectrum **[3]**.

3.2.1. SCREEN: 62470_screenshot_1_321.mov. 0:00-0:53. Parameters being set. **TEXT: Refer to Table 2 in Manuscript**

3.2.2. SCREEN: 62470_screenshot_2_322.mov. 0:00-0:17. ^{1}H SL power being set.

3.2.3. SCREEN: 62470_screenshot_3_323.mov. 0:02-0:44. Test vd list being generated and D30 updated, then test spectrum being run.

3.3. After setting up a **test vd list [1]**, update D30 and **TDF1 [2-TXT]** and plot the intensity of the peak for different the spin lock lengths. Identify the spin lock length at which the intensity of the original peak decreases to 1/3 **[3]**.

3.3.1. SCREEN: 62470_screenshot_4_331.mov. 0:02-0:28. Setting up a test vd-list. **TEXT: six entries: 0 m, 5 m, 10 m, 20 m, 30 m, 40 m**

3.3.2. SCREEN: 62470_screenshot_5_332.mov. 0:00-0:15. D30 and TD[F1] being updated and experiment being run**. TEXT: Example, D30 = 42 ms and TDF1 = 6.**

3.3.3. SCREEN: 62470_screenshot_6_333.mov. 0:00-1:00. Intensity of the peak *vs.* SL length being plotted.

3.4. From the results of the test runs, create the final vd list to be used in the experiment **[1]**. Select the number of scans so that the weakest peak of the list has a signal-to-noise ratio of at least 10 **[2]**.

3.4.1. SCREEN: 62470_screenshot_7_341.mov. 0:00-0:48. Results being observed/ the final vd list being created.

3.4.2. SCREEN: 62470_screenshot_8_342.mov. 0:00-0:11. The number of scans being selected.

Results

4. Results: Analysis of produced sample and Two Different Constructs Based on an RNA Hairpin

4.1. The results of several cleavage reactions of tandem transcripts are shown here **[1]**. A 20-nucleotide target RNA was generated in a successful reaction **[2]**. Unsuccessful reactions resulted in incomplete **[3]** and failed cleavage of the sample **[4]**.

4.1.1. LAB MEDIA: Figure 3 A.

4.1.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the spot for 20 nt, Lane 1.*

4.1.3. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the lane 2a.*

4.1.4. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the lane 4.*

4.2. HPLC injection of the RNA sample **[1]** revealed a peak for pure target RNA at 38 minutes **[2]**, while longer and shorter products were well-separated from the peak of interest **[3]**.

4.2.1. LAB MEDIA: Figure 3 B.

4.2.2. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize the target RNA.*

4.2.3. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize abortive products, uncleaved repeats.*

4.3. Analysis of the RNA hairpin showed that the number of observed imino protons **[1]** matched the number of imino protons expected from a secondary structure simulation **[2]**.

4.3.1. LAB MEDIA: Figure 4 A and C. *Video Editor: Emphasize red and blue lines.*

4.3.2. LAB MEDIA: Figure 4 A and C. *Video Editor: Emphasize the grey panels and lines between letters in panel C.*

4.4. The proton-carbon-HSQC spectrum of the aromatic resonances of the RNA showed 4 signals after folding **[1]** and only 3 signals before folding **[2]**.

4.4.1. LAB MEDIA: Figure 4 B and D. *Video Editor: Emphasize red spots and blue A letters in 4D.*

4.4.2. LAB MEDIA: Figure 4 B and D. *Video Editor: Emphasize blue spots and blue A letters in 4D.*

4.5. In the representative on-resonance curves obtained for two different H8 atoms **[1]** in two different synthetic RNA hairpins **[2]**, the G6H8 experiences conformational exchange **[3]** while the A4H8 does not **[4]**. For G6H8 the colored spin lock powers were selected and off-resonance data was recorded **[5]**.

4.5.1. LAB MEDIA: Figure 5 A. *Video Editor: Emphasize highlighted G and A.*

4.5.2. LAB MEDIA: Figure 5 B - G.

4.5.3. LAB MEDIA: Figure 5 A, C, G. *Video Editor: Emphasize grey highlighted G in 5A.*

4.5.4. LAB MEDIA: Figure 5 A, B, F. *Video Editor: Emphasize grey highlighted A in 5A.*

4.5.5. LAB MEDIA: Figure 5 C, D, G, H. *Video Editor: Emphasize colored legends of spin lock strengths in 5C and 5G*

4.6. In the C-G construct, where the exchange is slow, the $R_{1\rho}$ *(r 1 rho')* values versus offset plot already displayed a slight asymmetry of the curve, indicating the sign of the chemical shift difference *('delta omega')* **[1-TXT]**. This becomes even more apparent in the R_2+R_{EX} *('r 2 plus r e x')* plot where the R_1 contribution is removed **[2]**.

4.6.1. LAB MEDIA: Figure 5 D. **TEXT: Chemical shift difference: $\Delta\omega$**

4.6.2. LAB MEDIA: Figure 5 E.

4.7. On the other hand, the G-C construct experiences faster exchange and the $R_{1\rho}$ *(r 1 rho')* values versus offset plot presented a broader curve where the extraction of delta omega becomes less obvious even in the R_2+R_{EX} *(r 2 plus r e x)* plot **[1].**

4.7.1. LAB MEDIA: Figure 5 H and I.

Conclusion

5. Conclusion Interview Statements

5.1. **Katja Petzold:** Once the RNA dynamics have been elucidated, the states can be characterized and trapped. These trapped states can for example be tested in biological assays.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

5.2. **Katja Petzold:** This method allows us to observe functionally relevant base pairing shifts in an active microRNA mRNA complex, loop rearrangement in ribosomal RNA and base-pair stability around single-nucleotide bulges.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-Roll: 3.4.2 for "to observe"*