Response to Reviewers

We would like to thank the reviewers and the editor for the constructive comments and helpful suggestions.

Editorial comments

1.

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript and corrected spelling and grammar errors.

2.

Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. ″This figure has been modified from [citation].″

We have obtained copyright permission to reuse figures from our previous publication. We have also cited reused figures in the Figure Legend.

3.

Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient.

We removed the titles and Figure Legends from the figures.

'4.

Figure 1: Please change ″ng/ml″ to ″ng/mL″.

We corrected the Figure 1.

5.

Figures 1, 2, and 6: Please note that there are no panel labels A and B in the figure. Please add these labels.

We added panel labels A and B in the Figures 1, 2, 5, 6, and 8.

6.

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 and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Ultralink, sepharose, Ribo-Zero, AMPure, NEBNext, Spin X, SuperScript®, etc.

We removed company names and commercial languages from the manuscript.

7.

Please revise the protocol to contain only action items that direct the reader to do something (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." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We revised the protocol to ensure that the protocol contains only action items.

8.

1.1: Listing approximate volumes to prepare would be helpful.

We added approximate volumes of reagents listed in section 1.1.

9.

Please ensure that conditions and primers are listed for all PCR procedures.

Conditions and primers used in this study are provided in the table of materials.

10.

Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We combined shorter steps with the previous ones.

11.

After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less 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.

We highlighted just over 2 pages of the Protocol for the video.

12.

Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

We highlighted complete sentences that contain at least one action for the video.

13.

Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.

We highlighted complete sections to include all relevant details for the video.

14.

Because data for FACS and immunoprecipitation efficiency test are presented in the Results section, please consider include their procedures in the Protocol.

We added Notes about the FACS to check the homogeneity of the cell cycle in the Protocol section. However, we did not include detailed steps of preparing FACS samples because these steps are standard procedure and including them would disrupt the flow of our protocol.

15.

Discussion: Please discuss any limitations of the technique.

We added a paragraph in the Discussion section on limitations of formaldehyde crosslinking that it can decrease immunoprecipitation efficiency and increase in background because it can crosslink protein-protein complexes in addition to protein-RNA complexes.

16.

Table of Materials: Please use SI abbreviations for all units (L, mL, µL) and include a space between all numerical values and their corresponding units (15 mL, 37 ° C, etc.). Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

We corrected the table of materials accordingly to use SI abbreviations for all units, include a space between all numerical values and their corresponding units, removed trademark, and registered symbols. We have also sorted the items in alphabetical order.

17.

References: Please do not abbreviate journal titles

We corrected the references to show the full journal titles.

Reviewer #1

1.

There is a problem of non-specific binding of RNAs to a constant part of IgGs and/or to the Protein A sepharose. This should be at least discussed. For example, negative controls could be used: (i) Hela cell lysates with knockdown of PKR proteins, (ii) control IgG antibody.

We thank the reviewer for the constructive comment. In the revised manuscript, we provided additional data that includes negative controls. In Figure 7, we show that mitochondrial RNAs are not enriched when rabbit IgG control antibody was used for immunoprecipitation. We also present data from using DGCR8 antibody as a negative control because DGCR8 is a nuclear protein that is physically separated from mitochondrial RNAs.

2.

Figure 3: No significant enrichment of the PKR protein was observed after immunoprecipitation with Millipore antibodies, as compared to D7F7 antibodies, indicating that Millipore antibodies might not work at all in immunoprecipitations.

It would be also preferable if the authors could show that D7F7 antibodies recognize only PKR protein and have no other non-specific binding. This could be done with Hela cell lysates and Western blot using D7F7 antibodies.

Following the reviewer's suggestion, we showed whole blot western data for both PKR IP and total HeLa cell lysates (Figures 5A and 5B). We observe only one strong band corresponding to the size of PKR protein, which shows that the D7F7 antibody is highly specific in recognizing PKR.

3.

1.1.2 Preparation of paraformaldehyde solutions usually requires adjustment of pH to 7.4 using NaOH or HCl. Does pH need to be adjusted in 0.1% paraformaldehyde preparation?

When preparing paraformaldehyde solution, we use PBS buffer with pH 7.4~7.6 to dissolve paraformaldehyde. We do not adjust pH in 0.1% paraformaldehyde preparation, but do check that pH is about 7.

4.

Line 135: (section 1.1.6) an abbreviation "TDW" is used here for the first time and needs to be explained. The explanation may be removed later in line 270 (section 2.4.10).

We provided the full name for the abbreviation TDW.

5.

Line 147: Indicate for how long after seeding one should grow Hela cells before the 2mM Thymidine treatment.

We grow HeLa cells for 24 h after seeding before treating them with thymidine. This information is now provided in 1.2.1 of the Protocol section.

Reviewer #2.

1.

In their introduction, the authors stated the advantages of formaldehyde crosslinking assay but also that the use of UV crosslinking is recommended no longer. Considering that there are many researchers still using UV crosslinking for RNP assays, the authors may need to tone down the disadvantages of UV crosslinking analysis.

We thank for the reviewer for this critical comment. We amended the text accordingly to tone down the disadvantages of UV crosslinking and focused on advantages of using formaldehyde crosslinking.

2.

In step 1.1.1, the authors should provide information of any antibiotics added in the culture media.

We do not add antibiotics in the culture media. This information is now provided in 1.1.1 of the Protocol section.

3.

In step 1.1.6, they did not provide abbreviation of TDW for the first time.

We provided the full name for the abbreviation TDW.

4.

In Figure 3, it is recommended to provide the % input used in the western blot

We used 2% input in the western blot shown in Figure 3. We revised the figure to provide the information on the % input.