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Dr. Nilanjana Saha
Review Editor
Journal of Visualized Experiments

Dear Dr. Saha:

I am submitting a revised version of our manuscript (JoVE63014) entitled "A reporter based cellular assay for monitoring splicing efficiency" for your consideration.

We sincerely appreciate the editor and the reviewers for their constructive feedback. In response to their comments, we have revised the manuscript to carefully address each of the comments. The changes to the manuscript are tracked to identify the edits and content for videography is highlighted. The details of our response and the edits to the manuscript are attached.

We hope that with these improvements, the manuscript is now acceptable for publication in *Journal of Visualized Experiments*. Please let me know if you need more information.

Thank you for your consideration!

Sincerely,

Shalini Sharma, PhD
Associate Professor

We thank the editors and reviewers for their careful reading of the manuscript. In response to their helpful suggestions, we have revised the text and figures, and trust that the revisions clarify and enhance the revised manuscript. The specific changes and our responses to the points raised in the critique are tracked in the revised manuscript and detailed below:

Editorial comments:
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.**
   We have proofread the manuscript to check for spelling or grammar and would willingly correct any further errors.

2. **Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, a protocol is presented to …"**
   We have revised the abstract, reducing the size 50 words.

3. **The current Abstract is over the 150-300 word limit. Please rephrase the Abstract to more clearly state the goal of the protocol.**
   We checked and found that the abstract is 291 words long, and therefore, did not change it.

4. **Please ensure that abbreviations are defined at first usage.**
   We have gone through the protocol and defined abbreviations at first usage on pages 1 (line 38 and 40) and 2 (line 82) of the revised manuscript.

5. **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 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. There should be enough detail in each Step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**
   We have gone through the protocol to check that we are explaining how each step is performed. However, if any of the Steps need further explanation, we would be happy to provide it.

6. **Please add more details to your protocol steps:**
   Step 1.1: What the (*) symbol denotes here?
   This was an error; the symbol has been deleted.

   Step 1.2/1.3/1.4/1.5: Please mention the filtration membrane size and material.
   A NOTE has been added to page 2 that includes membrane size and material information.

   Step 2.10/8.1: Please mention the centrifugation speed and temperature.

The centrifugation speed and temperature have been added to Steps 2.11 (2.10 in the original manuscript) and 8.1 which are on page 4 and page 10, respectively, of the revised manuscript.

Step 2.12: Please elaborate on this step in case this needs to be filmed.

Step 2.13 (2.12 in the original manuscript) is described in detail in Steps 2.13.1 – 2.13.11 on page 5 of the revised manuscript.

Step 2.13: How long can the RNA be stored?
RNA can be stored for 6-12 months. This information has been added to Step 2.14 (2.13 in the original manuscript) on page 5, line 203 of the revised manuscript.

Step 3.3: Please provide a detailed explanation for this step in case this needs to be filmed.
Step 3.3 has been elaborated further and appears in Steps 3.3 and 3.4 on pages 5 of the revised manuscript.

Step 3.4: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). Also, please mention the temperature.
In Step 3.5 (Step 3.4 in the original manuscript), centrifugation speed has been converted to centrifugal force and temperature has been added.

Step 3.7/7.6: What is the centrifugation temperature?
Centrifugation temperature has been added to Steps 3.8 (Step 3.7 in the original manuscript) and 7.6 on pages 6 and page 9, respectively of the revised manuscript.

Step 4.8: Please explain how the step is done.
An equation for the calculation of % exon 2 inclusion has been added to Step 4.8 on page 6 of the revised manuscript.

7. **Please include a one-line space between each protocol step and then 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. Also, please ensure that it is in line with the title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will shall be available to the reader.**
   Protocol steps are separated by one-line space. The steps that need to be visualized have been highlighted.

8. **As we are a methods journal, please revise the Discussion to explicitly cover 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
To cover the above points, a paragraph has been added to the Discussion on page 12 of the revised manuscript.

9. **Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).**
   Figure have been prepared as .ai files.

10. **Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.**
    All tables have been removed from the manuscript and prepared separately as an .xlsx file. Table titles and descriptions have been added after the Representative Results on pages 11-12 of the revised manuscript.

11. **Please spell out the journal titles in the References.**
    References have been formatted in the JoVE output style using Endnote20.

Reviewer #1:
Major Concerns:

1. **In this study, the author designed the splicing site mutations associated with Usher syndrome and designed the corresponding U1 snRNA to restore the splicing abnormalities caused by this mutation. Then, in different minigenes or different types of mutations, whether a U1 snRNA can be designed to restore the effects of mutations?**
   The reviewer is correct, minigenes carrying other 5´-splice site mutations can be used to design U1 snRNA carrying compensatory sequence changes to their 5´-region for rescue of splicing.

2. **The author believes that the exogenous variant snRNA may be sufficient to splice the reporter gene precursor mRNA. Please provide direct evidence to confirm it.**
   We appreciate the reviewer for bringing this up. Actually, the ability of the U1-5a snRNA to rescue exon 2 inclusion to ~95% is the evidence that the variant U1-5a snRNA levels are sufficient for splicing of the Dup51p transcript. We have edited the text to include this explanation on page 11, line 464-467 of the revised manuscript.

Reviewer #2:
Minor Concerns:
I have only a few questions concerning the method and reagents described:

1. **item 1.2, please indicate the concentration of components used to prepare DMEM or the amount of each packet of DMEM (in grams) - although many labs will use DMEM from the same manufacturer, other media might also be used; also please indicate the final penicillin/streptomycin concentration.**
   The amount of DMEM powder (in grams), and the final penicillin and streptomycin concentrations have been added to Step 1.2 on pages 2-3 of the revised manuscript.

2. **item 1.5, please indicate trypsin molarity to prepare the 10X solution.**
   The 10x solution has 2.5% trypsin and this information has been added to Step 1.5 on page 3 of the revised manuscript.

3. **item 1.6, please indicate the final volume of the formamide DNA/RNA loading dye.**
   The final volume of the formamide dye has been added to Step 1.6 on page 3 of the revised manuscript.

4. **item 2.1, is the number of passages important for this experiment? This might be important to note because different labs might have HeLa cells with variable number of passages.**
   The number of HeLa cell passages in not important for these experiments.

5. **item 2.7, the medium used here is still DMEM? With or without serum?**
   In Step 2.7, the medium used is DMEM with serum and the text has been edited on page 4 of the revised manuscript.

6. **item 2.12, does the RNA needs to be precipitated after extraction? If so, please specify.**
   Yes, the RNA needs to be precipitated. The steps involved in extraction and precipitation have been elaborated on page 5 of the revised manuscript.

7. **item 3.3, please specify the recommended exclusion size of the beads.**
   The beads have a molecular weigth cut off of 25 kDa. This information has been added to Step 3.3 on page 5 of the revised manuscript.

8. **item 8, is there a recommended length for the Urea-PAGE gels?**
   Length of the urea-PAGE gels has been added to Steps 4.7 and 6.6 of the revised manuscript on pages 6 and 8, respectively.

9. **Tables 1-5: please specify the enzymes concentrations (T4 PNK or RT), in U.**
   The enzyme concentration has been added in units to Tables 1-5.