José-Antonio Daròs
IBMCP (CSIC-UPV)
Campus Universidad Politécnica de Valencia
Avenida de los Naranjos s/n
46022 Valencia, SPAIN
Tel.: (+34) 963877893; Fax: (+34) 963877859
E-mail: jadaros@ibmcp.upv.es
http://personales.upv.es/jadaros/

Valencia, 26 July 2018

Dear Dr. Cao:

We are submitting a revised version of our manuscript "Production of large amounts of recombinant RNAs on a circular scaffold using a viroid-derived system in Escherichia coli" (JoVE58472) in which we have addressed all editorial comments as well as concerns and comments raised by the three reviewers. We have used the MS Word revision tools to highlight changes in our manuscript. In addition, below you can find a detailed point-by-point response to reviewers.

We hope our work is now acceptable for JoVE. We trust publication in your innovative journal will highly increase the visibility of our method.

Sincerely,

José-Antonio Daròs

Point-by-point response to editor and reviewers

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. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

--- Ok.

2. Figure 3: Please line up the panels better. Please apply the same vertical dimension for A and B if possible.

--- Done.

3. Figure 5: Change “ml” to “mL”.

--- Changed.

4. Keywords: Please provide at least 6 keywords or phrases.

--- We added one more.

5. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

--- Rephrased.

6. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

--- After a first introductory sentence, the goal of the protocol is clearly stated in the second sentence of Long Abstract.

7. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

--- The overall goal of the method is stated at the end of Introduction.

8. Please define all abbreviations before use (SOC, etc.).

--- Done.

9. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

--- Done.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

--- Converted.

11. 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.

For example: Thermo Scientific, Zymoclean Gel DNA Recovery, NanoDrop, New England BioLabs, DNA Clean & Concentrator, Zymo Research, Eporator, Eppendorf, NucloSpin Plasmid, Macherey-Nagel, Sarstedt, GE Healthcare Life Sciences, etc.

--- Removed.

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

--- Done.

13. 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.

--- Done.

14. 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.

--- Done.

15. 1.1: Please list the PCR conditions.

--- Listed.

16. 1.2: Please provide the composition of buffer G. What volume of buffer G is used, and what container is used in this step?

--- Provided.

17. 1.3: Please break up into sub-steps and add more details. For instance, what wavelength is measured? What is the elution solution? Please specify throughout.

--- Done.

18. 1.5: What volume of liquid medium is used?

--- Kit instructions must be followed.

19. 2.3: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

--- Done.

20. 3.3: After centrifugation, is supernatant discarded? Please specify.

--- Specified now.

21. 3.7: Please break up into sub-steps.

--- Done.

22. 3.8: Please write the text in the imperative tense.

--- Done.

23. 4.2: What is used to heat?

--- Clarified now.

24. There is a 2.75 page limit for filmable content. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

--- Done.

25. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. 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.

--- Done.

26. Please discuss critical steps within the protocol and any limitations of the technique in the Discussion.

--- Done.

27. JoVE article does not have a Conclusion section. Please move information in the Conclusion section to Results or Discussion section.

--- Removed.

28. Please disclose your patent for the method in the Disclosure section.

--- Disclosed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Manuscript describes a good method for mass-production of RNA through a circular viroid. Embedded RNA will be a part of circular RNA.

Major Concerns:

Major question is that the self-splicing ribozyme is known to cut and ligate RNA (reversible reaction) at the specific site. Only magnesium ion is required. Did the authors test their system without the tRNA ligase? If this is the case, the technique becomes much simpler and more useful. Authors mentioned that for "the system to work", the tRNA ligase is required. No proof.

--- Ribozyme-mediated self-ligation is extremely inefficient. Substantial ligation in *E. coli* only occurs in the presence of co-expressed eggplant tRNA ligase. This has been largely demonstrated in our previous works (see references 12 and 19).

Minor Concerns:

Line 282: recombinant plasmids usually migrate "faster" (it should be slower). Fig. 3: I do not see the cellular tRNAs (about 80-100nt). There is no evidence for the comment (lines 400-401). Pointing to Fig. 1 does not make it real!

--- The reason why recombinant plasmids usually migrate faster is because the polylinker that is released during the cloning process is usually larger than the cDNAs corresponding to the RNAs of interest. In Fig. 3, E. coli tRNAs run out of the gel (note the position of the 100 nt RNA marker). In discussion, we speculate that RNA of interest must protrude from the viroid scaffold and point to the schematic representation in Fig. 1 for the readers to understand what we mean (not to demonstrate the speculation). However, in our opinion this speculation is rather likely based on the extremely stable secondary structure of the viroid scaffold and the experimental observation with the Spinach aptamer that folds properly and efficiently emits green fluorescence (see Reference 12).

Reviewer #2:

Manuscript Summary:

The authors describe a method for RNA production in large amounts. It is based on two E. coli plasmids: one carries a copy of a viroid (longer-than unit-length including two hammerhead ribozyme motifs) and the second a tRNA ligase. The viroid transcript is cleaved by the internal ribozymes into a unit-length molecule that is circularized by the co-expressed ligase. The viroid circles are produced in impressive amounts mainly due to their resistance against degradation. In addition the circularity of the RNA allows for easy purification. The viroid sequence (335 nt) is used as a scaffold containing an insert for the RNA of interest. The method is described for four different inserts: the aptamer Spinach (with 84 nt length, mimicking GFP), a Streptavidin-binding aptamer (45 nt?), an RNA hairpin (with 42 basepair double-stranded region), and a 3' cap-independent translation enhancer element. Features of these inserts and their relative yields are not described; such short additional descriptions might be helpful for the reader's judgment on possible further inserts. Fragmentation of the circular RNA and isolation of the insert are not described. The manuscript is based on a prior publication (Daros, Aragones & Cordero (2018) A viroid-derived system to produce large amounts of recombinant RNA in Escherichia coli. Sci. Rep. 8, 1904).

Minor Concerns:

I would change three minor things in the ``Long Abstract'':

1. ``... an ELVd precursor is efficiently transcribed and processed by the embedded hammerhead ribozymes ..." => ``... an ELVd precursor is efficiently transcribed by the E. coli RNA polymerase and processed by the embedded hammerhead ribozymes ..." (line 43--44)
This small addition avoids the impression that the precursor is transcribed by the RZ (or needs an additional polymerase).
2. ``, most probably based on formation of a stable ribonucleoprotein complex." (line 53)
I would delete this part of the sentence because the manuscript does not show any experimental hints for this hypothesis.
3. ``... facilitates the easy purification ..." => ``... facilitates the purification ..."(line 48)
IMHO, the `easy' is superfluous.
--- Changed.
Line 89: ``Viroids must account between the most stable RNAs ..." => ``Viroids must be ranked among the most stable RNAs ..." [Note that I am not a native speaker.]
--- Corrected.
Line 118: ``promote." => ``promoter."
--- Corrected.
Line 173: ``interst." => ``interest."
--- Corrected.
Line 318: ``... ribozymes and. "
Either the ``and" is unnecessary or some additional text is missing.
--- Corrected.
At several places the greek letter `micro' seems to be missing; for examples see lines 166, 189 (2X), 209, 210, 255 (2X), 261, etc.
--- This must be a problem with pdf conversion. It is okay in the original MS Word document.
The last three pages of the PDF show some random(?) text.
--- Same for this.

Reviewer #3:

Manuscript Summary:
The manuscript by Cordero et al. describes the production of recombinant RNA using a viroid-derived system. This manuscript follows up upon their detailed publication in Scientific Reports (2018), so it is not surprising that this work is detailed, well-written and almost ready for publication.
My comments are about the writing, since the scientific part is sound and well established.

Minor Concerns:

1) Given that this paper is an addition to the Sci Rep publication, I recommend trying to reduce the length of it, if possible.

--- We did our best to condense information.

2) The word amazing appears in six instances of the text. Please, reword the sentences or find suitable synonyms.

--- Good advice! We replaced all but one.

3) The sentence starting in line 112 (to 113) sounds odd to me. Please reword.

--- Reworded.

4) Line 118: "promoter".

--- Done.

5) Line 171: Consider advising the reader to use restriction mapping if electrophoretic migration of intact plasmids does not give clear results.

--- Done.

6) Line 219: Speeds are given in rpm. The g force exerted is dependent on the centrifuge used. Please change rpm to g in all instances.

--- Done.

7) Line 270, add "the" before first.

--- Done.

8) Line 286: have the authors had any problems with co-electroporation? In my hands, sometimes that did not work, so I had to transform in sequential order.

--- With the plasmids in this system, we have excellent results co-electroporating and we definitively recommend this strategy to save time.

9) Line 296: "dimensional" seems to be missing before PAGE.

--- Corrected.

10) Line 308: facilities?

--- Corrected to facilitates.

11) Line 318: revise the point in that sentence.

--- Revised.

12) Line 322: "to form" seems out of place.

--- Corrected.

13) Line 415: ...than large"r" "ones".?

--- Corrected.

14) Line 423: use etc. instead of "..."

--- Done.

15) Please revise in the Materials table, cell A71.

--- Thanks! This was a left over.