**Response to editor and referees**

Your manuscript, JoVE58996 "Site-directed mutagenesis for in vitro and in vivo experiments exemplified with RNA interactions in Escherichia coli," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

*Response: We are happy to submit a revised manuscript and we are grateful for the referee comments. We have addressed all the concerns raised by the reviewers and rewritten parts of the manuscript to improve clarity. Below is a detailed response to each editorial and referee comment.*

**Editorial comments:**  
Changes to be made by the author(s) regarding the written manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
Response: The manuscript has been thoroughly proofread and large parts of the manuscript was rewritten.

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].”

Response: Links to publisher’s editorial policy is provided in a separate file.

3. Figure 2: Please explain different lanes in the figure legend.

Response: Added for fig. 2A+B

4. Figure 4: Please describe different panels in the figure legend.

Response: Added to the figure legend

5. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Response: Trademark (™) and registered (®) symbols are now removed from the table.

6. Please expand the Long Abstract (150-300 words). It should include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate.

Response: The long abstract has been modified

7. Please define all abbreviations before use.

Response: All abbreviations are now defined before use.

8. Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Response: Alternative method has been described with reference to the literature. The advantage of this method has also been described. A statement regarding the applications of the method has been added.

9. 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: GenerulerTM, Fermentas, etc.

Response: Commercial names are now removed from the manuscript.

10. 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.

Response: Use of language not written in imperative tense is removed and changed in section 2.2.1 and 2.3.1. In addition, we have included a comment on safety precautions when working with radioactivity.

11. 1, 2, and sub-steps: Please note that these vague design steps cannot be adequately filmed. Please provide specific actions being performed, otherwise please consider un-highlighting them.   
12. 3.1, 4.1.1, 4.1.5, 4.2.1, 4.2.5, etc.: Please specify PCR conditions throughout.

Response: Added, see table 1.

13. 3.2: Please describe how to perform agarose gel electrophoresis.

Response: Added, see section 3.2.1

14. 3.3: Please describe how to purify the PCR product and measure DNA concentration.

Response: Added, see section 3.3

15. Much of the protocol is very abstract. We cannot film a generalized protocol; we need more specifics (such as strains and specific conditions used in step 6) in order to film.

Response: Specifics has been added.

16. 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.

Response: Highlighting has been updating.

17. 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.

Response: Highlighting has been updating.

18. 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.

Response: Highlighting has been updating.

19. References: Please do not abbreviate journal titles.

Response: Journal titles have been extended   
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
  
Manuscript Summary:  
The authors described the general molecular technique, site-directed mutagenesis based on PCR tool which approaches for protein-RNA and RNA-RNA interaction studies. This manuscript is well written and arranged in suitable organization; however, some scientific concerns were raised and more suitable references must be added.  
Response: We thank the reviewer for their comments on the manuscript and tried to address all the points raised by the reviewer.

Overall Concerns:  
  
Methods  
Section 1.1 A good vector should be concerned for an appropriated regulation based on promoter activity (high toxic protein requires tightly regulation or gaining high yield required strong promoter).

Response: Upon revision of the manuscript, we have removed the part regarding choice of vector as it extends beyond the scope of this method. The method can be applied to any vector, which is now stated instead.  
Section 2.2 (Both .1 and .2) Most of the criteria were varies depending on the polymerase enzyme efficiency and template characteristics.

Response: We thank the reviewer for this observation. As general oligo design is not a part of this protocol we have removed the section. However, we have kept the details of primer design introducing specific point mutations.

Section 3.3/4.1.3/4.1.7/4.2.3/4.2.7 please briefly describe or give references how to purify the PCR product and measure the concentration

Response: Description on PCR purification has been added, see section 3.3/4.1.3/4.1.7/4.2.3/4.2.7

Section 3.4/4.1.4/4.1.8/4.2.4/4.2.8/5.5 Store at 4C or -20C depending on the elution of PCR products and a time kept. If 4C, it should be kept in DW but if -20C, it should be kept in TE buffer or cryoprotecting agent. Response: Solvents added, see section 3.4/4.1.4/4.1.8/4.2.4/4.2.8/5.5

Section 5.3 Treat RE-digested PCR product with AP enzyme can be allowed only using blunt-end generated RE, not common in sticky-end generated RE.

Response: It is correct that phosphatase treatment of a RE-digested product PCR product is not necessary for sticky ends. However, AP treatment catalyzes the release of 5’ and 3’ phosphate groups on sticky DAN ends as well. We routinely dephosphorylate our cloning vector DNA to eliminate recircularization during ligation.

Section 5.4 Agarose gel usually cause a loss of DNA during experiment, using directly PCR purification column will give a better yield of DNA product (but only the contaminated DNAs are less than 100 bp). Response: While this is correct, our lab has experience with fewer colonies on the negative control plate when separating the digested vector on agarose gels as compared to purifying it directly.

Section 5.6 Ligation protocol could be described otherwise references must be stated.

Reponse: Ligation protocol added.

Section 5.7 Transformation protocol could be described otherwise references must be stated. Heat shock transformation is normally used for E. coli transformation.

Reponse: Heat shock protocol for transformation added

Section 5.8 Methods for checking the correct insertion must be listed i.e. PCR using primers one in vector and another one in an inserted DNA. Transforming an expression vector or an integration into chromosome must be concerned and using different techniques, please give a caution in this section.

Reponse: Method for checking the correction insertion is now mentioned in this section (5.8). Caution statement has been added. We use vector specific primers to check correct insertion and sequencing the entire fragments.

Section 6.1.2 Preparation of SDS-PAGE or native PAGE (which one is suitable?) should be mentioned in more details or give references in term of cell preparation (boiling or not) and gel electrophoresis set up. Reponse: SDS-PAGE preparation is now elaborated.

Section 6.1.4-6.1.10 Western blot technique is applied in these sections, it would be great if the author give in details otherwise references must be states.

Reponse: More details on the western blot technique are now added throughout this section.

Section 6.2.1 In vitro transcription, it would be great if the author give in details otherwise references must be states.

Reponse: More details on *in vitro* transcription are now added to this section.

Section 6.2.3-6.2.6 Radioactive labelling used in this step, the caution and information of the radioactive using regulation must be provided.

Reponse: Type of radioactive labelling is now elaborated. Caution on radioactive lab-work has been added. We cannot directly specify how to work with radioactivity as safety precautions vary between regions and countries. We have added a statement to follow the guidelines issued by the local radiation protection officer.

Results  
The results presented were clear and well described; however, description of band intensity analysis to construct the kinetic curve must be well written in the method with statistical analysis applied.

Reponse: A section shortly describing the method for analyzing the band intensities and determining dissociation constant values are now added.

Moreover, some statements below should be clarified:  
1. In Figure 2 legend, please state a final concentration of ethidium bromide and DNA ladder instead of volume.

Reponse: Corrected

2. In Figure 3 legend, fold of dilution using a full-stop symbol for a thousand unit may confuse some readers, please use comma sign instead, otherwise using text writing description.

Reponse: Corrected

3. In Figure 4, number showing the Hfq concentration with concentration unit must be presented in the figure.

Reponse: Concentrations (with concentration unit) are shown in the graph below the gel images and stated in the figure legend.   
  
Discussion  
The discussion presented were well written and organized. More details on pros/cons or comparison between this technique and other methods, and some successful applications in term of RNA interactions could help to improve this section.

Reponse: Pros/cons has been added to the discussion regarding alternative methods for site-directed mutagenesis. Many successful applications of RNA interactions are found in the literature. A statement about STM being the golden standard for RNA interactions studies has been added to the beginning of the discussion.   
  
  
**Reviewer #2:**  
  
Manuscript Summary:  
Andreassen et al. described a two and three step PCR technique to incorporate specific mutations into DNA and evaluate how target mRNA’s and small non-coding RNA’s interact throughout the PCR process. The authors also assessed how site-directed mutagenesis can be utilized to understand where the protein binding sites are in RNA. Although each technique was described in some detail, both techniques and the protocol need additional information in order for the work to be reproducible. This manuscript is valuable for others conducting PCR studies however, major revisions are needed to fully understand how this study was done and to be able to reproduce the results.  
Response: We thank the reviewer for the comments on the manuscript and we have tried to address all the points raised by the reviewer.  
  
Major Concerns:  
The authors seem to be hoping to appeal to a very broad audience but the protocol they are sharing is far too vague in its current form to be helpful to someone trying to follow it.  
Response: We have rewritten large parts of the manuscript and specified many protocol points.

- The authors present model data and specific primer sequences to perform these reactions without describing the parameters of their PCR reactions. For example, they do not mention duration, temperature, primer Tm, copy number of initial template, etc. and state only that the parameters can change. They reference other papers that have more details protocol but this tendency this would be unhelpful to anyone trying to use this paper as it is to perform the reactions they describe.

Response: Details has been added.  
  
As it is, the protocol is very often too vague by using phrases like “perform PCR”, “wash membrane”, “visualize with a method”, “identify transformants”, “ligate products” without describing any details on how to perform these steps. The authors do sometimes reference other papers that describe these details steps but these steps should be included in this text because in its current form it would require this paper and at least three or four papers that are referenced to successfully get through one of the reactions they describe.

Response: Details have been added to most steps throughout the manuscripts.   
  
They state that the 2 step PCR strategy is applicable if the mutation is <200bp from the end of the DNA of interest. However, in the 2 step PCR strategy in the protocol (line 51) - The authors state ("2 step is only for mutation >200bp from either end of DNA of interest"). Which one is correct? (Line 103).

Response: We thank the reviewer for noticing this error. <200 is correct, and the typo has been corrected in the manuscript.

-How do the authors plan to confirm that no errors were incorporated into the PCR products during the process? (Lines 60-66)

Response: Section 5.9 states that the sequence is validated by sanger sequencing  
  
- Although the authors say they have choosen an appropriate vector, they don't specific or give guidelines on how they or anyone would go about choosing a vector. Do these two PCR strategies not work for certain plasmids, what are the caveats for choosing a vector?(line 87).

Response: It has been clarified in the text that the approach is not dependent on a specific vector.   
  
There are other parts of the protocol that seem to be lacking details such as in Line 153 where the authors state "store purified DNA at -20oC or -4oC". Is one for long term storage compared to short term storage? What is the length of time the DNA can be stored at either temperature or does the DNA degrade and become unusable after a certain amount of time? More details are needed throughout the entirety of the protocol.

Response: It is now mentioned that -20°C is for long term storage and 4°C is for short term storage in section 3.4.  
  
-Again we are missing information about what type of label can be used and how is the DNA actually being purified? Are they using a specific kit or spin columns etc. (Line 270)

Response: The labeling method is now elaborated in the section (6.2.3)

The description of Figure 1 is explained well however, the layout of the actual diagram could be improved. For example, having panels that separate out each set of primers individually then adding step 1 and step 2 after those panels may provide clarity and be less confusing to readers (line 340, Figure 1).  
Response: We have changed the figure to improve clarity and reduce confusion.

Figure 1B: This figure is also a bit confusing and the layout/presentation of the information could be improved.  
Response: We have changed the figure to improve clarity and reduce confusion.

Figure 3: Should there be a control when running out the western blot? Answer..yes.. sloppy work not to automatically have one.

Response: We respectfully invite the reviewer to look at the figure again. The experiment examines a mutant *mcaS* allele in which we had disrupted the putative *csgD* binding site in the sRNA by changing four consecutive loop residues. In addition, we introduced compensatory mutations into the *csgD* 5′-UTR to restore interaction with the McaS42–45 mutant sRNA. As shown, regulation of CsgD63–66FLAG synthesis was lost with wild-type McaS but was restored with the McaS42–45 mutant. Collectively, the results indicate that McaS mediated downregulation of CsgD synthesis requires an antisense interaction between sRNA and the target RNA.

Included controls are the empty vector and induction of the individual mutants as well as probing GroEL as loading control. What control does the reviewer feel is missing?

-The authors state "it might be necessary to do more optimization of the PCR conditions.." what conditions did they use for each step of the experiment? Did they have to run optimizations; the specifics of each step of the PCR should be included (line 402).

Response: The specific PCR program used throughout the protocol is now added in section 3.1. Suggestions for optimizations has been added.  
  
How are some methods more costly than other? They seem to be very generalized/broad in terms of cost. Is there more planning or reagents required in other methods vs. PCR that was shown here? Is there a way to check that the correct targets were achieved with the 2 or 3 step PCR method without error?(Line 413-420).

Response: Cost has been added to the discussion regarding *de novo* synthesis being costlier than 2- or 3-step PCR. Sanger sequencing is mentioned in the protocol, which checks that the target was amplified without error.   
  
In Figure 2: It might be helpful to highlight the correct sized bands in each of the gels so the reader can identify those immediately.   
Response: Corrected

Lastly, is there a way for the formatting to be changed to allow the primer names to match up with their sequences? Again, this lack of detail reflects a sloppy presentation.

Response: We use a monospaced font for DNA sequences. This is, in general, used throughout the scientific community and across journals. When we write primer names, we consistently use the same format as the rest of the manuscript.