Tsinghua University School of Medicine
Babak Javid MB BChir, PhD MRCP
Principal Investigator (Tenured)

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Bing Wu,
Editor, *JoVE*

Measurement of specific mycobacterial mistranslation rates with gain-of-function reporter systems

Dear Dr. Wu,

We thank you and the Reviewers for your comments and suggestions to improve our submission to *JoVE*. I have attached a revised manuscript and figures in response to these comments. Please find our detailed responses below.

Editorial comments:
We have addressed all the Editorial comments in our revised manuscript.

Reviewer #1
We thank the Reviewer for taking the time to review our manuscript and make suggestions for improvement. Specifically responding to the comments:

1. Regarding data, I have several doubts about the experiments and their interpretation. These are listed below.
-Were negative controls containing all the reaction components except the cell lysate used to correct background noise for each luciferase reaction?
Thank you for mentioning this. We used cell lysate from mycobacterial strains not containing the luciferase reporters or non-induced reporter-expressing strains as negative controls, which we believe is an even more stringent negative control for background noise generated from the luciferase reactions. Although this was detailed in the original, referenced studies, we apologise that this was omitted in the initial submission of this manuscript, now corrected.

2. The use of the tetracycline promoter in both reporters is questionable since this antibiotic is known to target bacterial translation.
There are very few options for regulated expression in mycobacteria, and tetracycline-inducible promoters are the most widely used. Do note that the relatively inactive tetracycline analogue, anhydrotetracycline (ATc), which has negligible anti-bacterial activity is used as an inducer. Finally, in our 2014 *PNAS* paper, we also validated the reporters with a different inducer (acetamide), with similar results, however, acetamide-inducible expression constructs suffer from much higher rates of "leaky" expression. For these reasons, we routinely use the tetracycline-inducible reporters and ATc.

3. -Did treatment with kasugamycin (ksg) caused cell viability loss?
As detailed in our 2018 *eLife* paper that examines kasugamycin in much greater detail, at the concentrations used, kasugamycin had no anti-microbial activity – now mentioned in this manuscript.

Laboratory 4303, Biotechnology Building
Tsinghua University, Haidian District, Beijing 100084, CHINA
Tel: +86-(0)10-6279-4201; e-mail: bjavid@gmail.com

4. Did the ksgA deleted and WT strains have similar growth rates? A decrease in growth rate may suggest repression of protein synthesis which could result in underestimation of error rate measurements.
This strain had similar growth rates to the wild-type parent. However, since the focus of this manuscript is on the methodology, these data are shown for illustrative purposes only, and the fundamental biology is not expanded on.

Reviewer #2
We thank the Reviewer for taking the time to review our manuscript and make suggestions for improvement. Specifically responding to the comments:

1. While the authors describe a few advantages of this construct, it is unclear as to why the authors do not use a WT Nluc control reporter to compare the rate of mistranslation as similarly described in approach 1. Comparing the arbitrary units of Nluc to GFP is difficult to interpret and correlate to the frequency of mistranslational errors in the system.
Thank you for this comment. The reasons we chose to use the arbritary measurement of Nluc/GFP instead of corrected values are two-fold and related to the development of the reporter for screening, which we allude to in the manuscript: a) as mentioned in the manuscript, the Nluc-D140N mutation only causes 2 logs loss of function, which means that very low error rates cannot be accurately measured. Since the primary utility of the reporter was in a screen to identify molecules that decrease mistranslation, the "signal" reverts to noise, and accurate calculation of mistranslation rates cannot be derived, b) for use in a screen, would require repeating the screen with the wild-type reporter, which would double the work-load, for limited additional information. Instead, we chose to validate all hits with the Renilla-firefly reporter (Protocol #1), which we believe was far more efficient.

2. The methods highlighted in this manuscript have previously been described in the author's recent publications. Additionally, I do not believe the nature of these methodologies would be further clarified with a visual aid and are easy to follow from the original publications.
Thank you for these comments. Our manuscript was an unsolicited commission by the Editors at *JoVE*. Furthermore, although we believe that experts in the translation field may find the protocols relatively straightforward, as the physiological/ adaptive roles of translational error become more apparent, more researchers that are new to the field may wish to follow/ adapt our research methodology, and we believe that our manuscript/ video may assist them to do so.

3. Addressing the advantages and disadvantages of each reporter is informative to the reader and does provide some merit for publishing this manuscript
The authors have clearly explored various loss-of-function mutations for these reporters. If the authors could provide a table with some of this information (additional to the D to N and E to Q substitutions), I believe it would add value to this report, allow for greater utilization of these techniques, and increase the visibility of this manuscript.
Thank you for mentioning some of the merits of our manuscript. Since the focus of the manuscript is a methods paper, and not a review of measuring mistranslation, we haven't included details of our loss-of-function mutations in these reporters. Although previously generated reporters developed by Farabaugh are mentioned and referenced in the introduction.

Reviewer #3
We thank the Reviewer for their thoughtful reading of our manuscript, highlighting the potential utility of the work. With regards to the specific comments raised:

1. Realizing that although this journal is method-focused, a brief statement on the relevance of M. smegmatis could be useful in the introduction. Is it an important human pathogen? Why is resistance to rifampicin an important problem?
Thank you for these comments. We have now briefly addressed them in the introduction.

2. Pg. 2 ln. 45. Typo: '...error rates ___ widely..' did you mean 'vary widely'?
Pg. 2 Ln. 49: 'physiological misacylation' is confusing, consider rewording (e.g., signaled amino acid misincorporation)
Fig. 4,5: x-axes are labelled in unusual format. Change categories to, e.g., 0, 50, 100,… with axis label 'Kasugamycin (µg/mL)'
Supplementary table: The Comments/Description column is empty and should be removed.
Thank you for these comments. We have corrected the typo, and relabelled the axes. We have chosen to retain the term "physiological misacylation" since it is used in the literature, but have hopefully improved clarity of the term.
In our revised Excel file, we now have comments, so have retained the column.

Reviewer #4
We thank the Reviewer for their positive assessment of our manuscript. With regards to specific comments raised:

1. It would be very helpful to have additional information about the reporter plasmids. Are they multi-copy episomal plasmids or integrating? If they integrate, where and by what mechanism? Related to this, it would be helpful to have a table containing the reporter plasmid names and key properties.
Thank you for these comments. We have now added this information in the Materials file.

2. The manuscript does not have a table of required materials and equipment as suggested in the reviewer guidelines.
We did included this table, and are not sure why the Reviewer did not receive.

3. The abstract states that typical mistranslation rates are estimated to be 1/10,000 per codon, while the introduction says 1/1000. Please reconcile.
Thank you for spotting this typo, now corrected.

4. In step 7 of protocol A, it would be helpful to define "DN" or use a more intuitive term.
We have retained the term but clarified its meaning.

5. In figure 3, it would be helpful to include the method used to cover the 96 well plates for overnight incubation, since finding a balance between aeration and evaporation can be a challenge in such experiments. Breathable film?

Also in Figure 3, "Transfer bacteria culture to white bottom 96 well plate" should read "Transfer culture supernatant to white bottom 96 well plate."
Thank you – now amended/ corrected.

Reviewer #5
We thank the Reviewer for their positive comments and suggestions. Specifically:

1. It would have been helpful to have more discussion of how the activities reported in Figures 4 and 5 are converted into rates of mistranslation at that codon.
Thank you for this comment. We have made minor modifications to Protocol 1 to improve clarity, as well as referencing the key primary literature on how these calculations were derived.

2. The legend to figure 1 is quite long and contains a lot of information that isn't included in the figure. The figure is actually quite simplistic. Could more downstream information be added to the figure or the legend be simplified?
We appreciate this comment, but despite several attempts to move the information, in the end we felt that legend would be where most readers would have a "quick look" to understand the cartoons and moving the information might reduce clarity for the casual reader.

3. In the abstract they have the word "average" in quotes and in line 69 "unbiased" is in quotes. I don't think either of these words should be in quotes.
Numbers and units should always have a space between them and the L in mL and other volumes is typically capitalized to reflect the abbreviation for liter (L).
In line 45, the word "vary" is missing at the end.
In the paragraph that begins with line 45, I think it would be helpful to pick one method of describing errors (either % or frequency) and be consistent throughout the paper (including responding to concern #1 above).
Thank for these comments: all amended/ corrected as appropriate.

4. The authors refer to clear, white, and black 96 well plates as well as one set being round-bottomed. It would be helpful to provide catalog numbers for the different plates and perhaps more of an explanation of why plate changes were needed.
Thank you – information added to the document, as well as explanatory notes in the Protocols.

I look forward to your response.

Yours faithfully,

Babak Javid