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TITLE:

Measurement of Specific Mycobacterial Mistranslation Rates with Gain-of-function Reporter Systems

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SUMMARY:

In this article, we present two complementary methods to measure specific rates of translational error and mistranslation in the model mycobacterium, *Mycobacterium smegmatis*, using gain-of-function reporter systems. The methods can be employed to measure accurate error rates in low throughput or relative error rates in a more high-throughput setting.

ABSTRACT:

The translation of genes into proteins is prone to errors. Although the average rate of translational error in model systems is estimated to be 1/10,000 per codon, the actual error rates vary widely, depending on the species, environment, and codons being studied. We have previously shown that mycobacteria use a two-step pathway for the generation of aminoacylated glutamine and asparagine tRNAs and that this is specifically associated with relatively high error rates due to the modulation of mistranslation rates by an essential component of the pathway, the amidotransferase GatCAB. We modified a previously employed *Renilla*-Firefly dual-luciferase system that had been used to measure mistranslation rates in *Escherichia coli* for use in mycobacteria to measure specific mistranslation rates of glutamate at glutamine codons and aspartate for asparagine codons. Although this reporter system was suitable for the accurate estimation of specific error rates, lack of sensitivity and requirements for excessive manipulation steps made it unsuitable for high-throughput applications. Therefore, we developed a second gain-of-function reporter system, using Nluc luciferase and green fluorescent protein (GFP), which is more amenable to medium/high-throughput settings. We used this system to identify kasugamycin as a small molecule that can decrease mycobacterial mistranslation. Although the reporters that we describe here have been used to measure specific types

of mycobacterial mistranslation, they may be modified to measure other types of mistranslation in a number of model systems.

INTRODUCTION:

The flow of information in molecular biology requires the translation of genetic information to functional proteins. As with all biological systems, gene translation also involves measurable errors. Estimates of the rates of error in translation are typically quoted as approximately 1/10,000 per codon (reviewed by Ribas de Pouplana et al.[1]). However, error rates vary widely, from fewer than 10^{-5} to more than 0.05/codon[1–5]. The wide range of error rates, spanning more than three orders of magnitude, is due to the fact that errors can arise from multiple steps in the translation pathway: from stochastic, mutational, or stress-induced errors in aminoacylation[6–10], physiological misacylation of asparaginyl- and glutaminyl-tRNAs[5], or ribosomal decoding errors[2,3,11]. Measurably high error rates, representing over 0.01/codon, suggested that translational errors may perform physiological functions[1,12] and that mistranslation may be context-specific[13].

We and others have shown that naturally occurring errors in gene translation may be adaptive, especially during environmental stress[1,5,12,14–18]. In mycobacteria, errors generated by the two-step indirect glutamine/asparagine tRNA aminoacylation pathway[19,20] result in a remarkably increased tolerance for the first-line antituberculosis antibiotic rifampicin[5]. Therefore, we speculated that decreasing mycobacterial mistranslation with a small molecule may potentiate killing by rifampicin. We screened for and identified the naturally occurring aminoglycoside kasugamycin as a compound that could decrease mycobacterial mistranslation, potentiate rifampicin-mediated killing of mycobacteria both in vitro and in vivo[21], and limit the emergence of rifampicin resistance[21], which threatens the global control of tuberculosis[22]—the world's most deadly pathogen.

To study translational error, methods for the measurement of mistranslation have to be employed. There are multiple methods that have been developed for the measurement of mistranslation, each with advantages and disadvantages. Briefly, precision mass spectrometry-based methods have several advantages, the most important of which is that with newer algorithms for the detection of multiple types of translational error, a relatively unbiased measurement of mistranslation can be performed[18]. However, mass spectrometry is not very suitable for the measurement of deamidation mistranslation events—precisely the type of mistranslation that occurs in mycobacteria due to the error-prone indirect tRNA aminoacylation pathway. This is because of high-frequency nonenzymatic deamidation that occurs in the processing of samples for mass spectrometry[23], resulting in an extremely high background signal. Therefore, for the detection of errors in this pathway, genetic gain-of-function reporters offer distinct advantages. Specifically, suitable gain-of-function reporters can have extremely low background rates, allowing the measurement of very low error rates[11].

Since performing experiments with pathogenic *Mycobacterium tuberculosis* requires specialized facilities and extra precautions, we perform most experiments in the nonpathogenic mycobacterium *M. smegmatis*—and have shown earlier that results between the two species are broadly comparable[5,21]. To measure mistranslation rates in mycobacteria generated by the indirect tRNA aminoacylation pathway, we modified a *Renilla*-Firefly dual-luciferase system that had been previously developed to measure ribosomal decoding errors in *E. coli*[11] for use in mycobacteria. We made three specific modifications: the original reporter did not express efficiently in mycobacteria, and therefore, the sequence was codon-optimized, and the C-terminal three amino acids of the firefly luciferase, serine-lysine-leucine, which has been annotated as a trafficking signal in some systems[24], was modified to isoleucine-alanine-valine[25]. The original reporter had a critical lysine residue in the firefly luciferase mutated. Instead, we mutated either a critically conserved aspartate (D120) or glutamate (E144) residue in the *Renilla* luciferase to asparagine and glutamine, respectively[25] (**Figure 1**). The reporter was subcloned into the episomal tetracycline-inducible plasmid pUV-tetOR (see the **Table of Materials**). Gain-of-function reporters mutate critically conserved functional residues in enzymes/fluorescent proteins that renders them nonfunctional[11,26]. Translational (or theoretically, transcriptional) errors that synthesize the functional variant of the protein would result in measurable enzyme activity in a subset of translated proteins. To correct for variation in protein abundance, the mutated reporter is coexpressed with a functional protein that acts as a benchmark and allows for accurate quantification of gain-of-function[11]. Whilst the *Renilla*-Firefly dual-luciferase reporter allowed for the accurate measurement of specific mycobacterial mistranslation rates[5,25] (**Figure 2** and section 1 of the protocol), we quickly realized that it is not suitable for medium/high-throughput screening of molecules that would alter the mistranslation rate. This is due mainly to two reasons, namely, a) the relative lack of potency of *Renilla* luciferase meant that a minimum of 1 mL of mycobacterial culture/sample was required to measure mistranslation rates, and b) the requirement for lysis of the cells prior to enzyme activity measurement required excessive manual handling: mycobacterial cells have a thick and multi-layered cell wall and envelope that is relatively resistant to lysis. Therefore, we looked to develop a new gain-of-function reporter system that could be used with small volumes (e.g., in a 96-well plate system) and did not require cell-lysis for measurements. We used the highly potent Nluc luciferase and identified a critical aspartate residue that, when mutated to asparagine, resulted in 2 logs loss of function (**Figure 1**). Furthermore, the small size of Nluc allowed it to have an N-terminal secretion signal tag—from antigen 85A, a major secreted antigen in mycobacteria[27]—that would allow Nluc to be secreted into culture supernatant and circumvent the requirement for cell lysis. The benchmark protein, GFP, was expressed from the same promoter as the mutated Nluc, but from an integrated vector (see the **Table of Materials**), and could be measured in intact cells[21] (**Figure 3**). Despite these advantages, the Nluc/GFP reporter (section 2 of the protocol) also has disadvantages: the relatively modest reduction in Nluc activity (100-fold) with the D140N mutation would not permit the measurement of extremely low mistranslation rates, making the reporter more suitable as a screening tool than for the accurate measurements of translational error. Furthermore, Nluc has no critical glutamate

residues; therefore, only asparagine-to-aspartate error rates could be measured. The general principles described in this work should allow researchers to either use these reporters as we have done or modify reporters as appropriate for an accurate and/or facile measurement of other specific translational error rates in their model system of choice.

PROTOCOL:

1. *Renilla*-Firefly dual-luciferase reporter

NOTE: For a visual representation of this method, see **Figure 2**.

1.1. Inoculate mycobacterial reporter strains from -80 °C stock with 2 mL of 7H9 medium. Wild-type dual-luciferase, as well as mutated *Renilla* (reporter) strains, needs to be used to allow the calculation of mistranslation rates (see step 1.7). Shake at 37 °C for 1 to 2 days till OD_{600nm} reaches the stationary phase (OD >3).

1.2. Aliquot and dilute to OD_{600nm} around 0.1–0.5. For typical experiments, use three independent biological replicate cultures. Anhydrotetracycline (ATC), a tetracycline analog inducer of reporter expression (which is controlled by a tetracycline-inducible promoter) to a final concentration of 50 ng/mL.

1.3. To measure effects of kasugamycin on mistranslation rates[21], add different doses of kasugamycin to the culture (see **Figure 4** for the indicated doses) at the same time (at the tested doses, kasugamycin has no antimicrobial activity). Note that it is important to include at least one non-induced control for each reporter. Culture-induce cultures for 4–6 h at 37 °C with shaking.

1.4. Transfer the bacterial cultures to a 2 mL tube, and centrifuge at 3,220 x *g* for 5 min at room temperature to pellet down the bacteria. Discard the supernatant.

1.5. Disrupt the bacteria by adding 40 µL of 1x passive lysis buffer (supplied by a dual-luciferase kit), which has been diluted (1:1) in double-distilled water. Transfer the resuspended bacterial lysate to a white 96-well plate, one well per sample, and shake at room temperature for 20 min.

CAUTION: Do not over incubate the bacteria in lysis buffer (for more than 30 min).

1.6. Add 80 µL of firefly substrate to each well, shake for 15 s, and measure the luminescence by luminometer with 1,000 ms as integration time. Use either an automated injector or a multichannel pipette to avoid pipetting errors.

1.7. Add 80 µL of *Renilla* substrate to each well, shake for 15 s, and measure the luminescence by luminometer with 1,000 ms as integration time.

1.8. Subtract the background luminescence—measured using either wild-type *M. smegmatis* (i.e., not containing reporters) or uninduced reporter lysate—from the measured values. Use the corrected values to calculate the mistranslation rates of each condition by using the following equation[11,25], where DN refers to the activity in the mutated reporter strain:

$$Mistranslation\ rate = \frac{\left(\frac{|Renilla|}{|Firefly|}\right)DN}{\left(\frac{|Renilla|}{|Firefly|}\right)WT}$$

2. Nluc/GFP reporter

NOTE: For a visual representation of this method, see **Figure 3**.

2.1. Inoculate the bacterial reporter strain from -80 °C stock with 2 mL of 7H9 medium. Shake at 37 °C for 1 to 2 days till OD_{600nm} reaches the stationary phase (OD >3).

2.2. Subculture to 50 mL of 7H9 medium and grow till OD_{600nm} reaches the late stationary phase (>4).

2.3. Before aliquoting the bacteria to a 96-well plate, add ATC at a final concentration of 50 ng/mL and mix well. Induction of the bulk culture ensures that all wells contain the same amount of inducer and that the induction of the reporter is synchronized. Aliquot the bacteria to a clear, round-bottomed 96-well plate, with 100 µL of volume in each well.

2.4. To screen for small molecules affecting mistranslation rates, add the compound at the indicated concentration to select wells. For the purposes of this protocol, use kasugamycin (at doses indicated in **Figure 5**) as an illustration. Add different doses of kasugamycin to select wells (each experimental group should contain at least two biological replicates).

2.5. Shake and induce the samples at 37 °C for 16–20 h.

NOTE: It is necessary to seal the plate with film. Also, all the edge wells need to be filled with at least 200 µL of sterile water to limit evaporation from the test wells.

2.6. Take 80 µL from each well, using a multichannel pipette, and transfer the samples to a black 96-well plate (which maximizes the fluorescence signal measurement). Measure the GFP signal by luminometer with 20 ms as integration time.

2.7. After measuring the GFP signal, centrifuge the plate at 3,220 x *g* for 10 min. Transfer 50 µL of the supernatant to a white-bottomed 96-well plate (which maximizes the

luminescence signal measurement), add 50 µL of Nluc substrate to each well, mix them well, and measure the luminescence by luminometer with 1,000 ms as integration time.

2.8. Determine the Nluc/GFP ratio by dividing the corrected Nluc luminescence values by GFP fluorescence: this measure (in arbitrary units) is a relative measure of aspartate for asparagine mistranslation.

REPRESENTATIVE RESULTS:

A cartoon illustrating the general outline of the two reporter systems used in this work are shown in **Figure 1**. An overview of section 1 of the protocol is shown in **Figure 2** and an overview of section 2 in **Figure 3**. The effect of kasugamycin on mycobacterial mistranslation is shown in **Figure 4**, as measured by the *Renilla*-Firefly reporter system. To show the specificity of the kasugamycin action, the reporter was also expressed in a strain of *M. smegmatis* in which *ksgA* was deleted (ΔksgA). KsgA is an rRNA dimethyl transferase, and *ksgA*-deleted strains are relatively resistant to kasugamycin. Kasugamycin action on mistranslation was also measured using the Nluc/GFP reporter, as shown in **Figure 5**.

FIGURE LEGENDS:

Figure 1: Cartoon illustrating the two gain-of-function reporter systems. (**A**) The *Renilla*-Firefly reporter system is comprised of two luciferase enzymes expressed as a fusion protein and under the control of a tetracycline-inducible promoter. Expression of the wild-type dual enzyme results in high measurable activity of both *Renilla* and firefly luciferases (top). Mutation of a critical aspartate residue, D120, in *Renilla* to asparagine renders the *Renilla* enzyme (D120N) inactive, but firefly luciferase is still active. Mistranslation of asparagine to aspartate (in this case, from physiologically misacylated Asp-tRNA^{Asn} tRNA^{5,21}) results in a small proportion of the translated *Renilla* proteins gaining activity, which can be measured. Comparison of the *Renilla*/Firefly activity of the mutated reporter compared with the wild-type dual enzyme allows the calculation of the asparagine to an aspartate mistranslation rate. (**B**) The Nluc/GFP reporter system operates on a similar principle. The mutated Nluc (D140N) gene has an N-terminal secretion signal derived from antigen 85A, a major secreted mycobacterial protein. Both the *nluc* and *gfp* genes are expressed from identical tetracycline-inducible promoters, to allow the use of GFP as a relative expression benchmark. Note the lack of wild-type Nluc control strain: this reporter is primarily used in screening, where measurements of relative mistranslation rates are sufficient for the primary screen.

Figure 2: Outline of measuring the mistranslation rate using *Renilla*-Firefly reporter (section 1 of the protocol). Fresh cultures of *M. smegmatis*, transformed with a plasmid expressing the dual-luciferase reporters, are grown. The reporter expression is induced, and investigational compounds or conditions are tested. Following 4–6 h of reporter expression, the cultures are pelleted and lysed and the lysates are tested for dual-luciferase activity.

Figure 3: Outline of measuring the mistranslation rate Nluc-GFP reporter (section 2 of the protocol). A fresh culture of *M. smegmatis*, transformed with the Nluc and GFP reporters, is grown to a sufficient volume for all the plates to be tested (assuming ~10 mL/96-well plate). Immediately prior to pipetting the bacterial culture into each well, induce the expression of the reporters with ATC added to the bulk culture. Incubate the wells by shaking them overnight. To measure the relative mistranslation rates, transfer the cultures to a black plate (to increase the sensitivity of fluorescence detection), measure the GFP fluorescence, and then, spin the plates down to pellet the bacteria. Transfer the supernatant to a white plate for Nluc activity measurement.

Figure 4: Representative result of using *Renilla*-Firefly reporter measuring the mistranslation rate in the presence of kasugamycin in different strains. Mistranslation rates of aspartate for asparagine in (**A**) wild-type *M. smegmatis* and (**B**) a strain deleted for *ksgA* (ΔksgA) following treatment with kasugamycin (ksg) as measured by the *Renilla*-Firefly dual reporter. The cultures were treated with kasugamycin at indicated doses (in micrograms/milliliter) for 6 h prior to cell lysis and measurements. The corrected Ren/FF ratios (y-axes) are indicative of the aspartate for asparagine mistranslation rates. The deletion of *ksgA* results in a higher baseline mistranslation rate, which is more resistant to modulation by kasugamycin. The bars indicate the mean values, with standard deviation as error.

Figure 5: Representative result of using Nluc-GFP reporter measuring the mistranslation rate in the presence of kasugamycin. Relative mistranslation rates of aspartate for asparagine in wild-type *M. smegmatis* as measured by the Nluc/GFP reporter, following treatment with kasugamycin (ksg) overnight (16 h). The bars indicate the mean values, with standard deviation as error.

DISCUSSION:

The protocol described here can be adapted for the measurement of mistranslation rates in a wide variety of organisms. There are a number of considerations that should be kept in mind when adapting the protocol to other systems. First, the purpose of the measurement should be considered. For the accurate measurement of mistranslation rates using gain-of-function reporters, what is needed is (a) a robust readout assay (e.g., enzymatic function [in this case, luciferase activity] with a broad linear range). Also, look for (b) a loss of function mutation in a critical residue of the reporter protein that results in a very significant loss of function, below the expected rate of mistranslation. For example, if the expected mistranslation rate to be measured is approximately 10^{-3}/codon, the loss of function mutation needs to be greater than 1,000-fold; otherwise, the lower range of mistranslation events will not be sensitively detected. Lastly, for the accurate measurement of mistranslation rates using gain-of-function reporters, (c) a robust benchmark reporter is needed—in this case, firefly luciferase for section 1 of the protocol. Ideally, the benchmark reporter should be fused to the mutated enzymatic reporter, guaranteeing (for all intents and purposes) that they are both expressed in equimolar

ratios. The benchmark (and primary reporter) readout should be robust to perturbations to exposed environments. For example, the fluorescence of wild-type GFP is highly susceptible to perturbation by changes in pH[28], making it unsuitable as a benchmark for the measurement of mistranslation rates in phagocytosed mycobacteria in macrophages, which reside in phagosomes that are below pH 7[29]. On the other hand, for applications such as small molecule screening, the most important considerations for a primary screen will be the reproducibility of the measurements (i.e., precision, as opposed to accuracy), the minimization of manual handling, and small culture volumes. The accurate measurements of mistranslation rates are less important and can be performed as secondary assays. Finally, it should be noted that the reporters described in this protocol, based on the enzymatic function of luciferase enzymes, will only measure mean mistranslation rates of a bacterial population but cannot give information about single-cell heterogeneity variation in mistranslation rates. Given the importance of single-cell variability in adaptive phenotypes[30–32], fluorescent reporters for the measurement of mistranslation events have been developed[12,33], including for the measurement of mistranslation in mycobacteria[5].

Having considered the requirements for the measurement of mistranslation, it should be noted that the genetic gain-of-function reporters such as described in this protocol can only measure one type of mistranslation (i.e., one amino acid substitution for one codon) per reporter. Therefore, for the measurement of different mistranslation events, multiple reporters are required. For example, for the measurement of mistranslation by ribosomal decoding errors of near-cognate codons by lysyl-tRNA at one position, at least 16 reporters are required[2,3,11]. High-precision mass spectrometry and bioinformatics allow for the potential identification of many different types of mistranslation events simultaneously[18]; however, these methods also come with caveats. In general, they are less accurate than gain-of-function reporters. Furthermore, as stated earlier, they are less suitable for the detection of certain types of mistranslation, namely those that could be conflated with nonenzymatic deamidation[23]. Finally, mass spectrometry is unsuitable as a read-out for medium- or high-throughput screening.

For the adaptation of these reporters and protocols for the measurement of mistranslation in other systems, further considerations include a robust expression of the reporter in the desired model system. We initially attempted to use the dual-luciferase system developed by Farabaugh and colleagues in our mycobacterial system without modifications but had no success, due to a lack of reporter expression. Extensive troubleshooting identified that both codon-optimization and a minor sequence modification of the reporters were required to allow for robust expression[25] (and see above). It is conceivable that similar adaptations of the reporters would be required for use in other systems.

Finally, consideration should be given to the type of mistranslation event being measured. For example, if there is a need to measure stop-codon readthrough (nonsense suppression), the stop codon's loss of function mutation needs to be introduced within a

noncritical region of the primary reporter protein[34]. Otherwise, only specific nonsense suppression events that regain the critical residue (and, hence, reporter function) will be measured by the assay, which would potentially lead to a substantial underestimation of the true mistranslation rates. There is increasing evidence that translational error plays a possible adaptive role in a large number of organisms[1,12,13,35]. However, the measurement of mistranslation events is still limited to a small, albeit growing number of model species. The adaptation of sensitive methods to measure mistranslation has the potential to further increase scientists' understanding of the role of translation error in physiology and pathology.

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DISCLOSURES:

The authors have nothing to disclose.

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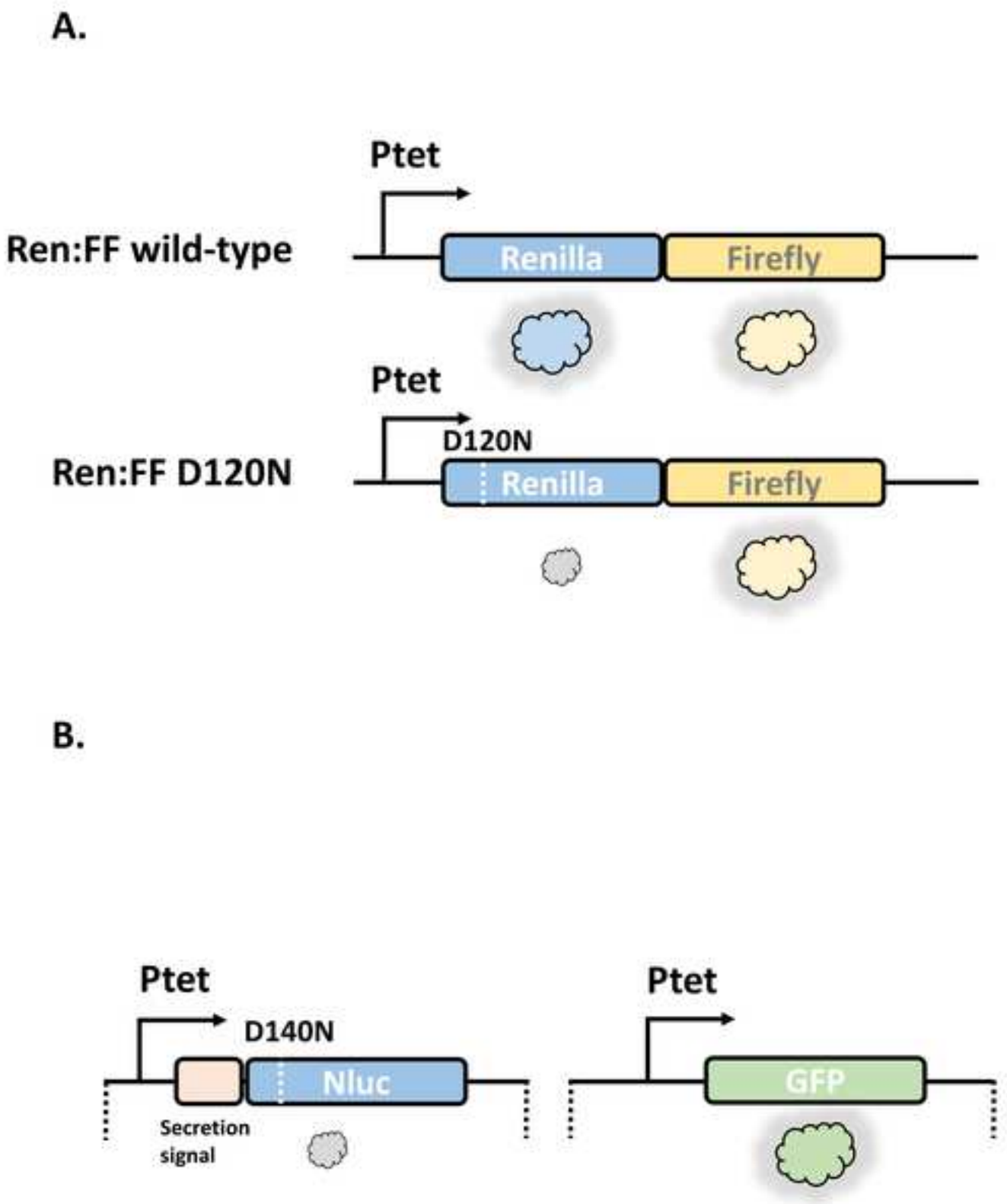
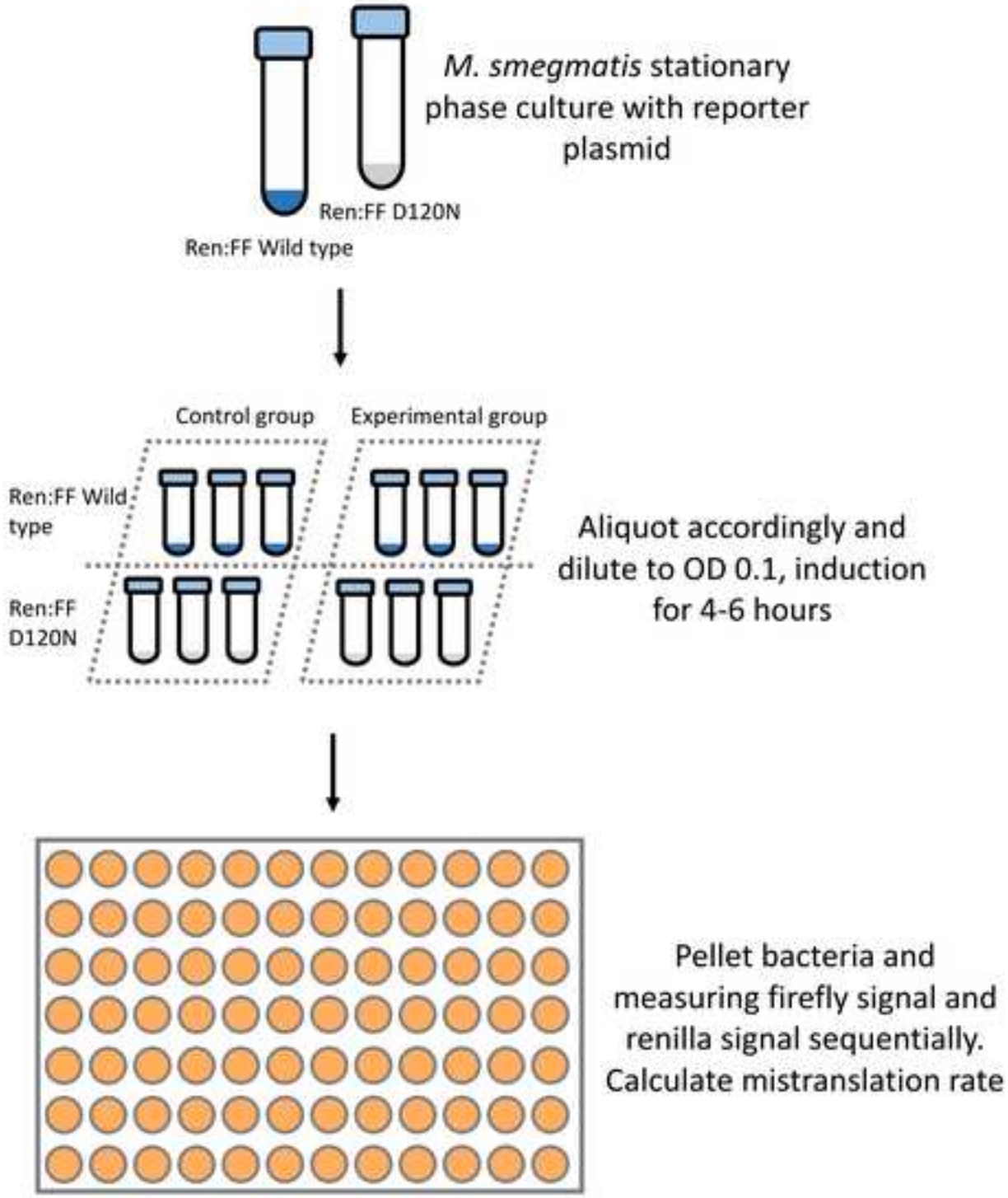
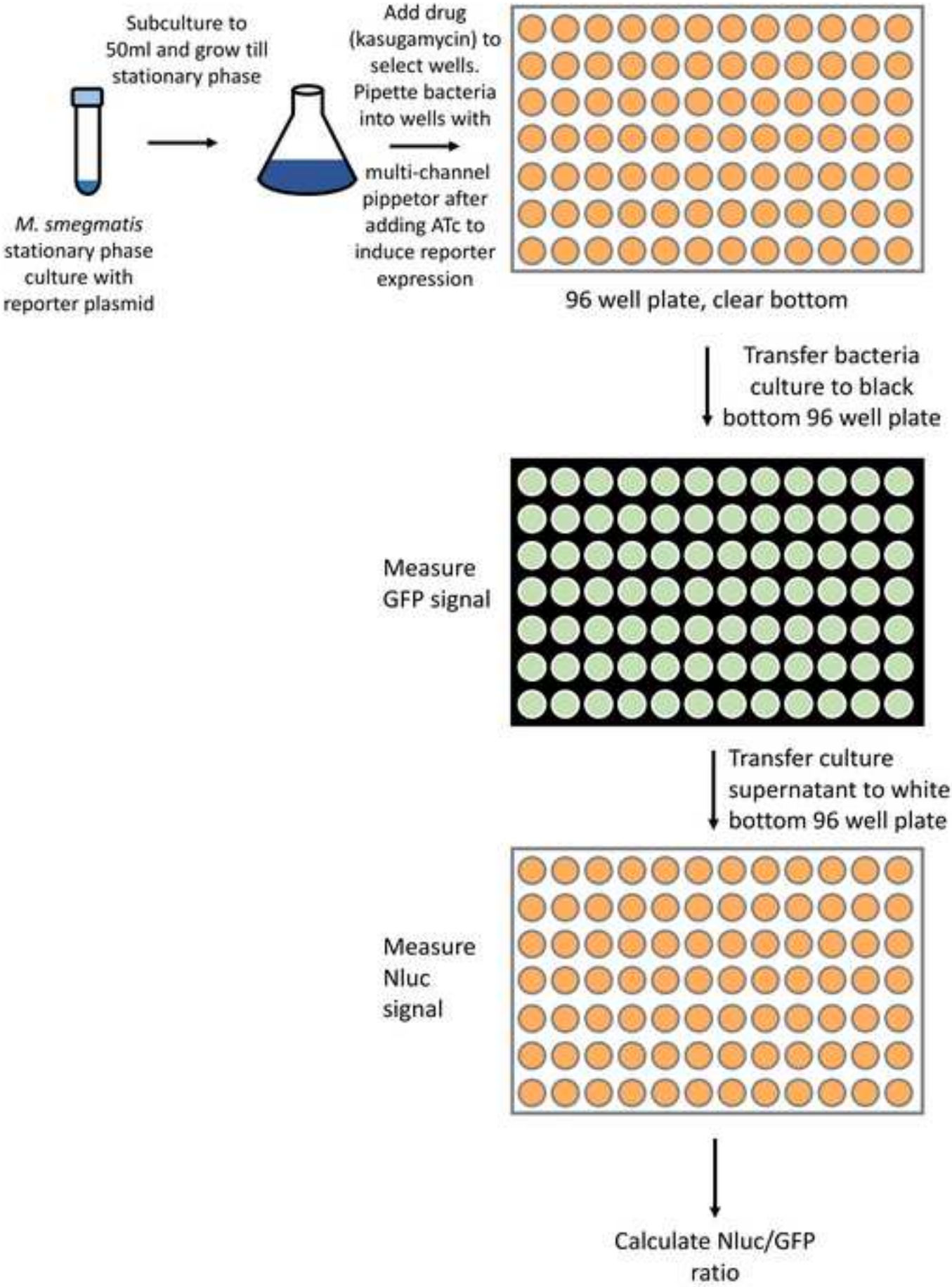
Figure

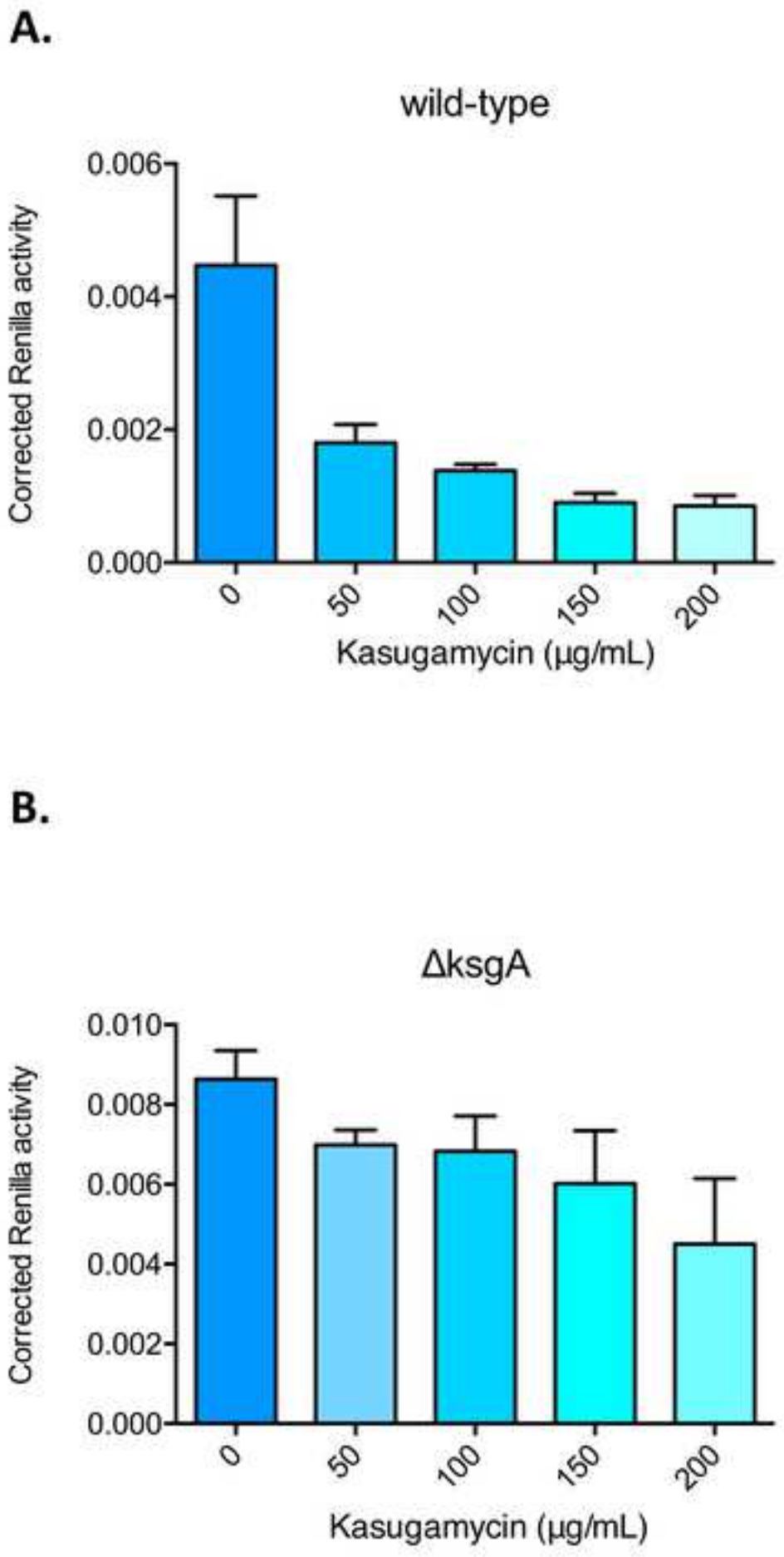


Figure



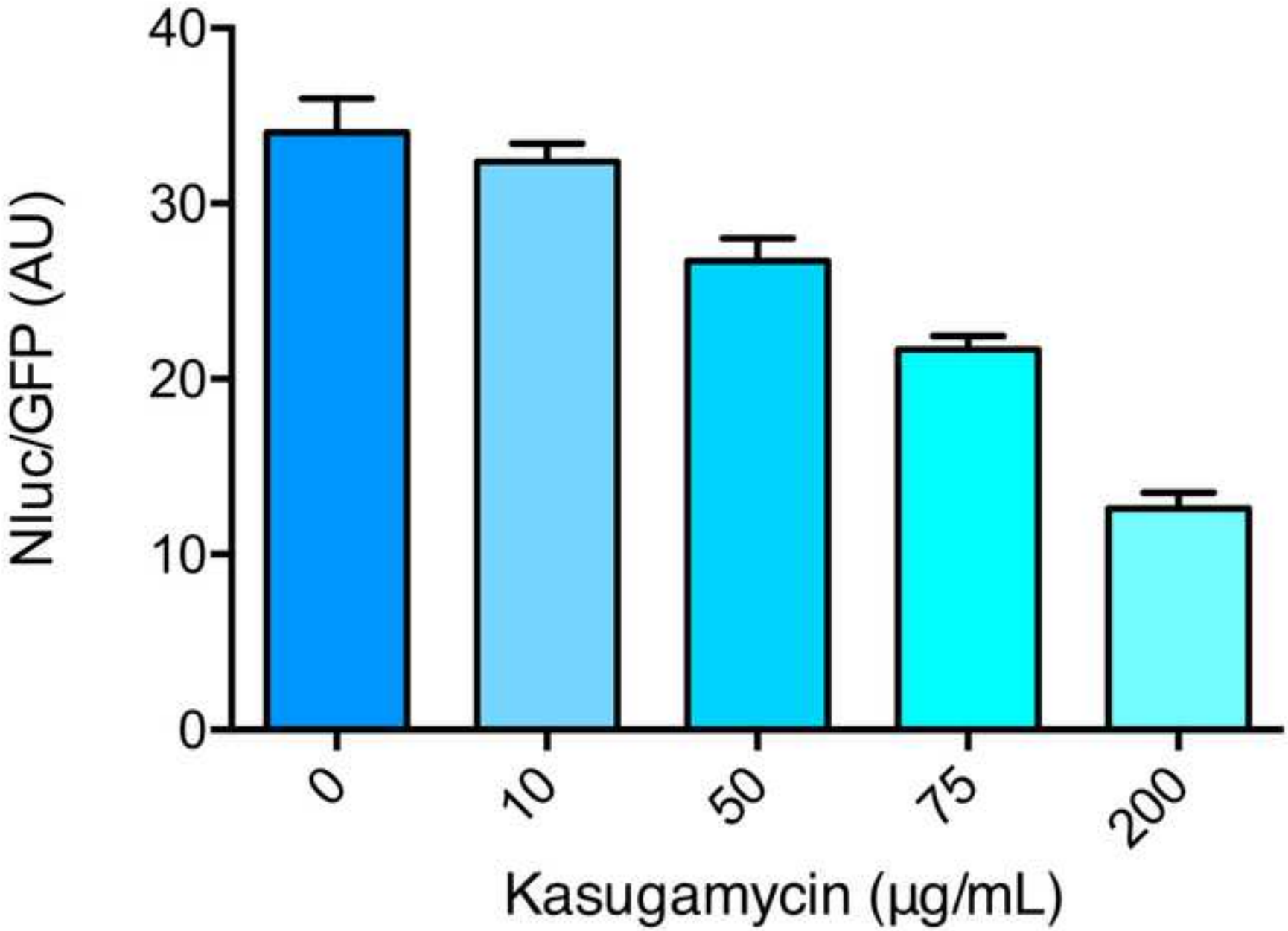
Figure





Figure

Click here to access/download;Figure;figure 5.jpg



Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
Middlebrook 7H9	BD Difco	271310	
anhydrotetracycline	Cayman Chemical	10009542	
kasugamycin	sigma	K4013	
Dual-luciferase reporter assay system	promega	E1960	
Nano-Glo luciferase assay	promega	N1120	
Fluoroskan Ascent FL luminometer	Thermo	/	
Assay Plate, 96 Well White, Flat Bottom High Binding, No Lid	Costar	3922	
Assay Plate, 96 Well Black, Flat Bottom High Biding, No Lid	Costar	3925	
96 Well Cell Culture Cluster, Flat Bottom with	Costar	3599	
Non-commercial reagents (plasmids)			
pUV-TetOR-RenFF	NA	NA	episomal shuttle plasmid that allows tetracycline-inducible expression of the wild-type dual
pUV-TetOR-Ren-D120N-FF			dual-luciferase reporter with mutated Renilla

pUV-TetOR-Ag85ASec-Nluc-D140N	NA	NA	episomal shuttle plasmid with a tetracyclin-inducible secretable version of mutated Nluc
pMC1S-GFP	NA	NA	Mycobacterial integrated (L5 site) vector for tetracycline-inducible expression of GFP

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Author(s): Yu Xing Chen, Miaomiao Pan, Yuming Chen & Sonbate Jamil

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CORRESPONDING AUTHOR

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Title:	Dr.
Signature:	
Date:	29/11/2018

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

10th January 2019

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.

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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