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Measuring microbial mutation rates with the fluctuation assay

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Dear Vineeta,

Thank you very much for enabling us to submit a revised version of our manuscript now titled "Measuring microbial mutation rates with the fluctuation assay" for publication as an invited methods article in JoVE.

We were glad to see the positive and constructive comments of the reviewers. We have now addressed all their comments and criticisms, as laid out in detail in the response to reviewers document. This involves a substantial amount of new text, new main-text figure and a new co-author.

This manuscript remains a work of a broad appeal. Described protocol is required to study microbial mutation rates and their environmental dependences, with a particular relevance to antimicrobial resistance, evolutionary biology, microbiology, genetics and medical sciences as well as a wide popular audience.

On behalf of all the co-authors, thank you for your time and consideration in assessing the suitability of this work for publication in JoVE, we look forward to your comments.

Yours Sincerely,

Rok Krašovec Postdoctoral Research Associate

Christopher Knight Senior Lecturer

TITLE:

Measuring Microbial Mutation Rates with the Fluctuation Assay

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SUMMARY:

Here, a protocol is presented to perform a fluctuation assay and estimate microbial mutation rate using phenotypic markers. This protocol will enable researchers to assay mutations in diverse microbes and environments, determining how genotype and ecological context affect spontaneous mutation rates.

ABSTRACT:

Fluctuation assays are widely used for estimating mutation rates in microbes growing in liquid environments. Many cultures are each inoculated with a few thousand cells, each sensitive to a selective marker that can be assayed phenotypically. These parallel cultures grow for many generations in the absence of the phenotypic marker. A subset of cultures is used to estimate the total number of cells at risk of mutations (i.e., the population size at the end of the growth period, or N_t). The remaining cultures are plated onto the selective agar. The distribution of observed resistant mutants among parallel cultures is then used to estimate the expected number of mutational events, m, using a mathematical model. Dividing m by N_t gives the

estimate of the mutation rate per locus per generation. The assay has three critical aspects: the chosen phenotypic marker, the chosen volume of parallel cultures, and ensuring that the surface on the selective agar is completely dry before the incubation. The assay is relatively inexpensive and only needs standard laboratory equipment. It is also less laborious than alternative approaches, such as mutation accumulation and single-cell assays. The assay works on organisms that go through many generations rapidly and it depends on assumptions about the fitness effects of markers and cell death. However, recently developed tools and theoretical studies mean these issues can now be addressed analytically. The assay allows mutation rate estimation of different phenotypic markers in cells with different genotypes growing in isolation or in a community. By conducting multiple assays in parallel, assays can be used to study how an organism's environmental context affects spontaneous mutation rate, which is crucial for understanding antimicrobial resistance, carcinogenesis, aging, and evolution.

INTRODUCTION:

In 1901 the Dutch botanist Hugo de Vries coined the term mutation[1]. Twenty-six years later, when Hermann Joseph Muller discovered the mutagenic action of X-rays[2], mutations were already perceived as one of the driving forces of evolution. However, the nature of mutations was not clear. To answer the fundamental question of whether mutations emerge spontaneously (i.e., a spontaneous mutation) or in response to selection (i.e., an induced mutation), a method was needed to observe mutational events. Such a method would measure the expected number of mutations per cell division or what was already known as a mutation rate[3,4].

Salvador Luria and Max Delbrück in 1943 provided an ingenious solution to this problem with the fluctuation assay[5] (see **Figure 1**). The assay starts with multiple populations (named parallel cultures) that are initiated with a small number of microbial cells (**Figure 1A**,**B**). After growth in a benign, non-selective environment (**Figure 1C**), parallel cultures are transferred on plates containing a selective marker (phages, antibiotics, etc.), where only cells with a resistance mutation survive and can produce a colony (**Figure 1D**). The main expectation was that if resistance mutations are induced, the number of cells that carry a mutation should be distributed among different populations with the mean equal to the variance. What Luria and Delbrück found with the fluctuation assay is that the number of mutants fluctuated drastically and that the variance in the number of mutants among different populations was considerably greater than the mean. Luria and Delbrück thereby demonstrated that mutations are spontaneous. They showed that mutations spontaneously emerge whenever the DNA is replicated, and the number of mutants depends on when the mutation occurs during the growth of the population. See **Figure 1C**, where six populations, each initiated with a microbial cell (in blue), experience none, 1, or 2 single mutations. Populations A1, E1, and F1 experienced one single mutation (first red cell), but because a single mutation spontaneously emerges at various time points during a culture cycle, populations ended up with a very different number of observed mutants (four, two, and one, respectively). On the other hand, populations C1 and G1 experienced two single mutations, again at different time points, and ended up with the same number of mutants, two and four. Populations E1 and A1 experienced only one mutation. The fluctuation of observed mutants among populations not only gave the assay the name, but also showed that a mutant frequency (i.e., the proportion of mutant cells) is an inadequate indicator of the mutation rate.

The overall goal of the fluctuation assay is to estimate the spontaneous mutation rate of a particular genotype of bacteria or other single-celled organism growing in a particular liquid environment. The fluctuation assay remains the most appropriate tool for studying environmental dependency of microbial mutation rates and allows rapid and inexpensive mutation rate estimation. Alternative approaches to mutation rate estimation, such as maximum-depth sequencing[6], population sequencing[7], mutation accumulation experiments[8], or comparing genome sequences of an offspring to those of the parents[9] are much more laborious, and thus poorly suited to potentially detecting environmental dependencies. However, dynamic aspects of a mutation's generation and repair are largely inaccessible to a fluctuation assay or to any of the methods of assaying a mutation rate listed above. To study how the number of mutations changes in time, space, or among individual cells within a population, single cell approaches[11,12] are necessary, which, in addition to being more laborious than fluctuation assays, require highly specialized skills and equipment.

In practice, a fluctuation assay is counting cells gaining a phenotypic marker due to a mutation that occurs in an environment lacking selection for that marker. The meta-analysis of hundreds of published assays[10] shows that at least 39 different phenotypic markers have been used since the assay's inception in 1943. The fluctuation assay can be used to compare averages and environmental dependency of mutation rates among laboratory, clinical, nonmutator, and mutator strains growing in permissive environments. The assay allows for the mutation rate estimation in cells with different genetic backgrounds growing in either minimal or rich environments. The assay is suitable not only for populations growing as a monoculture but can also be used for studying the effects of cell-cell interactions on mutation rates[11]. When the strain of interest is cocultured with a second strain, and a neutral marker is used to distinguish the strains, mutation rates can be assayed for two strains in the same tube at the same time.

Fluctuation assays have revealed that the spontaneous mutation rate depends upon both a cell's genotype and its environment[12] and is a trait that is itself subjected to evolution[13]. Whenever the mutation rate of one particular genotype changes with the environment, it is described as mutation-rate plasticity[11]. Plastic mutation rates have been most thoroughly addressed for stress-induced mutagenesis (SIM)[14]. In addition, using fluctuation assays, it has been recently shown that the density to which a population of cells grows (typically a batch culture at carrying capacity) is closely associated with mutation rates across bacteria and unicellular eukaryotes. The mutation rate per genome per generation decreases in dense populations by as much as 23-fold[10,11]. This density-associated mutation rate plasticity (DAMP) can depend on a quorum-sensing system[15] and act independently of SIM[16].

Here, a detailed protocol is presented for the fluctuation assay used to study *Escherichia coli* strain K-12 gaining resistance to the antibiotic rifampicin in a glucose minimal media environment. However, this protocol should be viewed as a basic template that can be utilized to study a wide variety of microbes by simply modifying the culture conditions and phenotypic markers of mutation. The protocol has evolved from its inception[5,17-29] through its use on a wide range of microbes and even cancer cells[30] and has been modified to increase the throughput,

which was essential for properly testing environmental dependencies of microbial mutation rates[10,11,16]. The protocol described here does not cover all the methodological and analytical issues of the fluctuation assay that have been already well discussed in the literature, particularly fitness effects of resistant mutations[31], phenotypic delay[32], cell death[33], and the suitability of various algorithms available to estimate mutation rates[26,34]. This can be important, for instance, when the environmental dependence of fitness effects can give rise to erroneous variation in the mutation rate estimates[35]. However, we note that the analytical tools that we use here can deal with the variation in mutant fitness and cell death. As addressed in the notes and discussion, it is also recommended that multiple phenotypic markers that are unlikely to have the same environmentally dependent fitness effects be considered. This protocol will enable people to routinely assay environmental dependencies of mutation rates in the diversity of microbial strains and environments. Assaying mutations in different environments has not yet been thoroughly tested and once population density is considered, fluctuation assays can give a more precise estimate of mutation rate[10]. This protocol will enable more fluctuation assays to be performed, as is necessary for understanding the mechanisms underpinning mutation rates, which in turn is vital for understanding evolution, carcinogenesis, aging, and antimicrobial resistance.

PROTOCOL:

1. Day 1: Inoculation and acclimation of cultures

1.1. Inoculate 3 mL of liquid lysogeny broth (LB, see **Supplementary Table S1**) with a scrape of ice from the *E. coli* MG1655 glycerol stock (18% glycerol, -80 °C). Shake the LB culture at 120 rpm for ~7 h at 37 °C.

NOTE: In this experiment, *E. coli* K12 MG1655 growing in LB is used, but this assay can be performed with any *E. coli* strain or any other culturable microbial species. Incubation temperature, incubation times, and nutrient level of the growth media may all be subject to variation by species or strain.

1.2. Dilute the culture 2,000-fold using the saline solution. Add 100 µL of the diluted solution to three 50 mL screw cap conical bottom polymer tubes (50 mL tubes) with 10 mL of liquid Davis minimal medium (DM, see the **Supplementary Table S1**), respectively containing 80 mg/L, 125 mg/L, or 250 mg/L of glucose. This is the same medium (i.e., environment) in which the mutation rate will be estimated. Shake the cultures at 120 rpm overnight at 37 °C.

NOTE: The choice of media is subject to variation by species, strain, or research question.

2. Day 2: Generation of mutants in parallel cultures

2.1. First, prepare the environments in which the bacteria will be cultured. Always prepare 10% more than required (i.e., twenty 1 ml cultures require 22 mL). Prepare 22 mL of 1) DM with 80 mg/L of glucose, 2) DM with 125 mg/L of glucose, 3) DM with 250 mg/L of glucose, 4) DM with

80 mg/L of glucose, and 5) DM with 250 mg/L of glucose in five 50 mL tubes. Label them as GLC-80A, GLC-125, GLC-250A, GLC-80B, and GLC-250B, respectively.

2.2. Prepare inocula for the environments. Ensure that the inoculum for 1 mL of the environment contains 1,000–5,000 cells. Do this by following the steps below.

2.2.1. Measure the optical density (OD) of overnight cultures (from step 1.2) at 600 nm.

2.2.2. Dilute the overnight culture using saline solution and add between 22,000–110,000 cells to the 22 mL of the environment prepared in step 2.1. These are enough cells to have an inoculum size of between 1,000–5,000 cells per mL of DM with glucose.

2.3. Prepare parallel cultures.

NOTE: This protocol is written for one 96 deep well plate, performing five fluctuation assays (the maximum reasonable number on one 96 well plate), using three environments. With experience, multiple deep-well plates may be run in parallel.

2.3.1. Create a random layout of parallel cultures for one 96 deep well plate. The **Supplementary R script LayoutGenerator.R** (see the layout in **Figure 1B**) can be used for this. Position each parallel culture on the 96 deep well plate according to the layout.

NOTE: Running the LayoutGenerator.R will ensure that the first assay has 20 parallel cultures, and that the second, third, fourth, and fifth assays have 19 parallel cultures each.

2.3.2. Transfer 1 mL of inoculated media into each well of a 96 deep well plate according to the randomized layout.

2.3.3. Fix the lid of the deep well plate with the tape. Do not fix the lid tightly, because the culture growth is sensitive to the amount of aeration.

2.3.4. Weigh the entire plate with the lid and the tape and shake the plate at 250 rpm for 24 h at 37 °C. Place 2 L of distilled water in the incubator to stabilize the amount of evaporation among experimental sets.

2.4. Determine the inoculum size by plating 10 µL of each of the inoculated media on the non-selective Tetrazolium (TA) agar plate (see **Supplementary Table S1**). Use a sterile L-shaped spreader until the agar surface is dry. Incubate TA agar plates lid down overnight at 37 °C.

NOTE: TA agar is a rich agar that contains the sugar L-arabinose and the water-soluble dye 2,3,5-triphenyltetrazolium chloride, which is colorless in its oxidized form. When bacteria reduce the dye, it turns red due to the formation of formazan. Colonies on TA agar that cannot utilize L-arabinose are dark red. Other strains, such as MG1655, are pinkish. Use of TA agar rather than standard LB agar is recommended because colored bacterial colonies are easier to spot, which

makes colony counting more reliable and quicker.

2.5. Prepare selective TA agar containing rifampicin in 6 well plates. Pipette 5 mL of the selective TA agar into each well of the 6 well plates. Prepare the antibiotic rifampicin just before it is added to the TA agar.

NOTE: When cycloserine is used as a marker, use Davis minimal medium with 250 mg/L of glucose supplemented with agar, or L-arabinose and 2,3,5-triphenyltetrazolium chloride as a selective agar. Namely, TA agar does not select only cells that are resistant to cycloserine, because tryptone and yeast extract (both the essential components of TA agar) contain the amino acid D-alanine. This amino acid antagonizes the antimicrobial effect of the cycloserine and allows *all* cells to make a colony.

2.5.1. Leave the selective and remaining non-selective plates in the dark (e.g., in a box) at room temperature and incubate for 24 h.

3. Day 3: Plating cultures on selective and non-selective agar plates

3.1. Count colony forming units (CFU) on the non-selective agar plates and determine the size of the inocula by multiplying CFU by 100. This is a ratio between a volume of the parallel culture (1 mL = 1,000 µL) and the plated volume (10 µL).

NOTE: If non-selective plates are stored refrigerated the CFU can be counted later.

3.2. After 24 h of incubation, weigh the entire deep well plate to determine the amount of evaporation. This is likely to be around 10%.

3.3. Calculate the average volume of a parallel culture after 24 h of incubation ($V[24h]$) in microliters using a starting volume $V[0h]$ = 1,000 µl and the plate's weight converted into microliters, where the density of the growth medium is measured in mg/µl. This study uses a density of 1 mg/µl:

$$V[24h] = V[0h] - \frac{1}{density} \times \frac{(weight\ [0h] - weight[24h])}{96}$$

3.4. Transfer the three randomly chosen cultures per assay (15 in total, highlighted with a black circle in the layout in **Figure 1B**) into labeled microcentrifuge tubes. Leave them on the bench to be used later (see step 3.6) for determining the final population size (or N_t).

3.5. Plate the remaining 81 parallel cultures from the deep well plate onto the selective TA agar containing rifampicin. Pipette one entire parallel culture from the 96 deep well plate into one well of a 6-well plate. Ensure that any 6 well plate contains parallel cultures from more than one fluctuation assay.

3.5.1. Remove the lids from the selective agar plates, and leave uncovered in sterile conditions. Dry out all the liquid on the surface of the selective TA agar.

NOTE: The agar being completely dry is critical. However, do not overdry the selective TA agar, because it must not be allowed to crack.

3.6. While the selective TA agar plates are drying, which can take up to several hours, determine N_t of the 15 cultures prepared in step 3.4 using colony forming units (CFU).

3.6.1. Determine CFU by diluting the cultures from the microcentrifuge tubes. Use five 10-fold dilution steps, mixing and vortexing 900 µL of saline solution with 100 µL of the culture on each step. Plate 40 µL of the last dilution (with a dilution factor of 10^5) on the non-selective TA agar and incubate plates lid down overnight at 37 °C.

NOTE: The dilution series in 96 well plates can be performed with a multichannel pipette, which can increase the speed of this step.

3.7. Once all the wells on a 6 well plate are free of the culture liquid, put the lid back on and place the 6 well plate with the lid down on the bench until all 6 well plates are dry. Once all are dry, incubate the plates lid down at 37 °C for 44–48 h.

NOTE: For other markers (nalidixic acid, cycloserine, hygromycin B, or 5-FOA) incubate the plates for 68–72 hours. Make sure that the humidity in the incubator is high. It is critical that the selective agar plates not dry out during the incubation period.

4. Day 4: Determination of the number of the cells in the cultures

4.1. Count the CFU on the non-selective agar plates. Estimate the number of viable cells in the culture by multiplying the CFU with the dilution factor (10^5) and ratio between the calculated average volume $V[24h]$ in microliters (see step 3.3) and plated volume (40 µl):

$$Nt = CFU * \text{dilution factor} * \frac{V[24h]}{40}$$

4.2. N_t of a particular genotype growing in a particular environment is the mean of these values from the three cultures.

5. Day 5: Estimation of the mutation rate

5.1. Count the number of colonies resistant to the antibiotic on the selective TA agar plates (i.e., the numbers of resistant cells that arose through spontaneous mutation in the deep-well plate on days 2−3). Record the distribution among parallel cultures of the observed number of mutants for a particular assay (e.g., 16 or 17 values, including zero counts).

NOTE: If selective plates are stored refrigerated, colonies resistant to the antibiotic can be counted later.

5.1.1. Use each distribution to estimate the number of mutational events, *m*, using the R-package flan[36]. NOTE: There is also the R-package rSalvador with similar functionality[29].

5.1.2. Save the distribution of the observed mutants for one assay in a text file as a single column.

5.2. Use Shinyflan software (http://shinyflan.its.manchester.ac.uk/) to estimate *m* as detailed below.

5.2.1.1. In the tab **Hypothesis Testing** leave values as defaults (i.e., **Unknown Fitness** ticked, **Estimation Method** = Maximum Likelihood (ML), **Distribution of Mutant Lifetime** = Exponential (LD model), **Winsor Parameter** = 1,024, **Mutation Number and Fitness** = 1, **Confidence Level** = 0.95, **Number of Class and Maximal Value** = 100).

5.2.1.2. Click **Browse** and select the text file with the distribution of observed mutants. Try first with the Supplementary Data file.

5.2.1.3. After uploading the file, click on **Perform Test**. On the right side under the **Result of the Test**, under the **One Sample ML-Test** (LD model), find the **Mutation Number**. This is *m*, the expected number of mutational events.

5.2.1.4. Under the **95 Percent Confidence Interval for the Mutation Number** find the upper and lower bound of *m*.

5.3. Once *m* and N_t (determined by CFU) are available, estimate the mutation rate of a particular genotype in a particular environment as

$$\frac{m}{Nt}$$

NOTE: Results are presented as the mutation rate per *rpoB* locus per generation. Mutation rate per base pair is generated by dividing the mutation rate per *rpoB* locus by 79, which is our current knowledge of how many point mutations within an *rpoB* gene confer resistance to rifampicin[37]. Multiplying the mutation rate per nucleotide by the size of the chromosome (*E. coli* K-12 MG1655 = 4,639,675 bp), gives the mutation rate per genome.

5.4. Repeat steps 5.1–5.3 with the remaining four fluctuation assays.

REPRESENTATIVE RESULTS:

Results were gathered with the reported protocol in four different weeks by three different individual researchers, where each week a 96 deep well plate was used to generate five mutation rate estimates. Altogether three 96 deep well plates generated 15 mutation rate estimates (± 95% confidence interval, CI) in *E. coli* K-12 MG1655 (**Figure 2A**, as used in the protocol), while one 96 deep well plate was used for five estimates (± 95% CI) of *E. coli* K-12 BW25113 Δ*mutT* mutant (**Figure 2B**). In **Figure 2** the median precision with interquartile range of estimating *m* is 17.5% (1.00%−28.9%, n = 20). Precision is a coefficient of the variation of the expected number of mutational events (*m*) calculated as σ_m / *m* x 100% (where σ is calculated as shown previously[38]). Raw data for **Figure 2** is available in the **Supplementary Data File** "Krasovec_etal_JoVE_data.csv". The **Supplementary Data File** is accompanied by an R script to generate **Figure 2**. See also **Supplementary Table S2** for more details on bacterial strains, and **Supplementary Table S3**, where columns in the data file are explained. Data points acquired with rifampicin and nalidixic acid were previously published in Krašovec et al.[10] and have the same IDs here as when first published.

In **Figure 2A** the MG1655 mutation rates of three different phenotypic markers, cycloserine, rifampicin, and nalidixic acid are presented. Here, mutation rates were assessed in Davis minimal medium with 80, 125, and 250 mg/L of glucose, as explained in the protocol. In one case 1,000 mg/L of glucose was used (**Figure 1B**). In practice, any initial glucose concentration can be used. As expected, mutation rates were higher for cycloserine resistance, lowest for nalidixic acid resistance, and the rate of rifampicin resistance was in the middle. This is in accordance with the known target sizes for these three resistances, where the largest is for the cycloserine and the smallest for the nalidixic acid. The discussion and **Figure 3** give details on how to exploit the target sizes, volumes of parallel cultures, and level of nutrients in the environment to optimize the measuring of microbial mutation rates with the fluctuation assay.

The fluctuation assay clearly shows which strain is a constitutive mutator and which has normal mutation rates. The Δ*mutT* strain had an approximately 50x higher mutation rate to nalidixic acid resistance (**Figure 2B**) as MG1655 (**Figure 2A**). However, to determine the mutation rate of a particular genotype in different environments, doing at least five replicates per genotype per environment using only one phenotypic marker is recommended. For a detailed outline of the statistical methods previously used to analyze this type of experiment, see the supplementary information in Krašovec et al.[10].

FIGURE LEGENDS:

Figure 1: Schematic illustration of how to perform the fluctuation assay with a microbial strain in a 96 deep well plate. (**A**) Inoculate and acclimate cells in 50 mL tubes containing five different environments ('red', 'blue', 'green', 'purple', and 'orange' assays). (**B**) Prepare parallel cultures with a small number of sensitive cells in a 96 deep well plate. The 'red' assay has 20 parallel cultures, whereas the 'blue', 'green', 'purple', and 'orange' assays all have 19 parallel cultures. The positions of the parallel cultures on the 96 deep well plate are random. Randomization can be done with the **Supplementary LayoutGenerator.R script** or by some other tool. The layout on the top right is the randomization result. (**C**) Incubate the 96 deep well plate and allow the cells

to divide and spontaneously mutate. Six cultures from deep wells A1, B1, C1, E1, F1, and G1 show how the number of mutants fluctuates: 4, 0, 2, 2, 1, and 4 red cells after the third cell division, respectively. The number of mutants differs not only because of the different number of spontaneous mutations (0, 1, or 2 as shown by the first red cell), but also because it is important when during a culture cycle a resistance mutation spontaneously emerges (cell division 1, 2, or 3). (**D**) After the incubation of the 96 deep well plate the number of mutants is determined by plating 81 parallel cultures. On the layout these are circles without bolded edges. The entire parallel culture is plated on one well of a 6 well plate containing a selective agar. (**E**) The remaining 15 cultures are diluted and plated on the non-selective agar to determine the average number of cells (N_t). On the layout these are labelled as Ntwells and have bolded edges. For each assay N_t is averaged over three parallel cultures. On the bottom right is a Petri dish containing a non-selective agar plate with 25 CFUs of a diluted culture grown in a deep well D1 (part of a 'green' assay). (**F**) After incubation of the selective 6 well plates the number of observed mutants were counted and the expected number of mutational events, m, was estimated using a Maximum Likelihood estimator. (**G**) Knowing both the number of mutations, m, and the number of cells per assay, N_t, the mutation rate was estimated as m/N_t.

Figure 2: Representative figure of mutation rates estimated by the fluctuation assay in *Escherichia coli* populations. (**A**) The mutation rate as determined by the reported protocol in wild type MG1655 (circles). The figure shows the mutation rates in the presence of rifampicin (light blue circles), nalidixic acid (red circles), and cycloserine (dark blue circles) resistance. For nalidixic acid, larger culture volumes of 10 mL were used (see **Supplementary Data File**). Data for rifampicin and nalidixic acid is replotted from Figure 2a in Krašovec et al.[10]. (**B**) The mutation rate to nalidixic acid resistance in the Keio[39] Δ*mutT* mutant (red triangles). Note the logarithmic scale on the mutation rate axis. Data is replotted from Figure 4a in Krašovec et al.[10].

Figure 3: **A flowchart for troubleshooting the fluctuation assay.** The flowchart is to be followed from the top, addressing the three questions in the yellow diamonds in turn, adjusting the protocol according to the contents of the resulting green boxes, and implementing the protocol as indicated in the red ovals. See the first three paragraphs of the discussion for more details on how to troubleshoot.

Supplementary Table S1: Formulas for the media used in the protocol.

Supplementary Table S2: Bacterial strains.

Supplementary Table S3: Detailed description of the columns in the raw data file Krasovec_etal_JoVE_data.csv.

DISCUSSION:

Any estimate of a mutation rate needs to maximize the precision achieved in order to ensure repeatability and reproducibility within and between studies[34]. For a fluctuation assay, there are three critical considerations. The first two have been set in the protocol given but will need troubleshooting (see **Figure 3**) if the protocol is adapted to work with different strains or

environments. First is to choose the appropriate phenotypic marker. For bacteria, it is recommended to estimate rates at one of two marker loci, *rpoB* or *gyrA*, conferring resistance to antibiotics rifampicin and nalidixic acid, respectively. The target size for mutations to antibiotic resistance at these two loci is different. There are 79 and 20 unique mutations conferring resistance to rifampicin[37] and nalidixic acid[40] respectively. In practice, this means that on average, rifampicin resistant mutants are more frequently observed. So, the first question (Q1 in **Figure 3**) that needs to be answered is whether or not the strain is a mutator. When constitutive mutators are studied, where many observed mutant colonies are expected, it is better to use a marker with a smaller target size (e.g., nalidixic acid). See **Figure 2B**, where mutation rates of the constitutive mutator *E. coli* K-12 BW25113 Δ*mutT* were estimated using nalidixic acid as a marker. When working with nonmutator bacterial strains that have a wild type (i.e., normal) mutation rate, rifampicin is a better choice (see **Figure 2A**). If for some reason more observed mutants are needed, a relevant marker is resistance to a cycloserine. For this marker, resistance mutations can emerge in more than ten genes[41], meaning that the target size is even larger than for rifampicin. When studying yeast and archaea it is recommended to estimate mutation rates in 25S ribosomal proteins and URA3, conferring resistance to hygromycin B and 5-fluoro-orotic acid (5-FOA), respectively.

The second critical consideration is the volume of parallel cultures. Which volume to use depends on the actual number of observed mutants. The expected number of observed mutants is affected by the target size for the chosen phenotypic marker, the strain's capacity to repair and avoid mutations (the average mutation rate), and the carrying capacity of the environment, which is affected both by the media used and the culture volume. If no parallel cultures contain mutant colonies resistant to rifampicin, then cycloserine should be used or the number of plated cells should be increased. This can be achieved by adding more glucose to a minimal medium or by growing cells in a richer (or complete) environment. However, in many cases, such an increase in population density is associated with a reduction in mutation rate, resulting in limited, if any, increase in the number of mutant colonies observed[10]. If increasing the nutrients is not a solution, then increasing the number of cells by increasing the volume of each parallel culture is an option. When the environment includes minimal salts with sugar as the only source of carbon and energy (i.e., the answer to Q2 in **Figure 3** is "minimal medium"), then volumes between 0.5 and 1.5 mL should be used. If the environment is rich, then volumes of parallel cultures should be between 0.35−1 mL. The last question concerns the median number of resistant colonies. If too few mutant colonies are observed (i.e., the answer to Q3 in **Figure 3** is 0) and the environment is not to be modified, then the volume of parallel cultures should be increased or the antibiotic with a larger target size (e.g., cycloserine) should be used. On the other hand, if a lot of mutant colonies are observed on all selective plates (more than ~150 per plate, see **Figure 3**), then the number of plated cells should be decreased, which usually means using a lower volume or switching to an antibiotic with a smaller target size (e.g., nalidixic acid).

Once the volume is chosen, it is best that all parallel cultures on one 96 deep well plate have the same volume. That allows more precise determination of the actual volume of parallel cultures from the weight of the plate. When mutation rates of a particular genotype are compared between different environments, it is again best to use the same volume of parallel cultures in

all environments. If nalidixic acid is used for estimating wild type (i.e., normal) mutation rates or some other phenotypic marker is used that has an even smaller target size than nalidixic acid, the volume must be increased even more. One option is to make parallel cultures in 50 mL tubes with volumes of up to 15 mL. For instance, 10 mL parallel cultures were prepared in 50 mL tubes when estimating the *E. coli* K-12 MG1655 mutation rate to nalidixic acid (see **Figure 2A**). The parallel 10 mL cultures were then plated on the selective TA agar and poured into large 150 mm plates instead of standard 90 mm Petri dishes. The downside of preparing parallel cultures in 50 mL tubes is that the throughput is considerably lower compared to assaying mutation rates in a 96 deep well plate. One solution is to decrease the number of parallel cultures. However, this will affect the precision for the estimate of *m*, which depends on the expected number of mutational events and the number of parallel cultures[26]. Obtaining a distribution of observed mutants with 14−17 parallel cultures (as was done in **Figure 2**), is a good balance between a solid throughput and an acceptable precision level[26] of 20%. A median precision level of 17.5% is similar to the median precision with an interquartile range of 16.4% (5.7%−38.9%, n = 580) calculated from a much larger data set[10]. Thus, it is recommended that when preparing parallel cultures in 96 deep well plates or 50 mL tubes the distribution of observed mutants is obtained with at least 14 parallel cultures. When mutation rates are estimated in different environments it is recommended to test precision levels by doing a multiplate experiment, where all 96 parallel cultures on one plate are grown in the same environment. In addition, when preparing parallel cultures, it is critical that inocula contain a low number of cells, because it reduces the chances of any resistant cells being present in the inoculum. Preexisting resistant mutants are not wanted in the inoculum, because they will increase in numbers and create a lawn on selective plates and estimation of the mutation rate will not be possible. For instance, in most nonmutator *E. coli* populations, mutation rate to rifampicin resistance is in the order of ~10^{-8}. Thus, to avoid inoculating the culture with a preexisting resistant mutant, one must inoculate with fewer than 10^8 cells (e.g., 10^3–10^4 cells). The final critical step is to ensure that before selective agar plates are incubated, the surface on the selective agar is completely dry. Spreaders cannot be used if 6-well plates are used and the initial volume of a parallel culture is 1 mL, for example. Plates must be left uncovered in sterile conditions to let the surface liquid dry out. The time this takes can be highly variable, dependent on ambient conditions and the condition of the plates. This time should be minimized but can be up to several hours.

The fluctuation assay has inherent constraints. It assays phenotypic markers of mutation only in a small subset of the genome. The assay thus requires large populations that go through a sufficient number of generations to observe enough mutations to estimate a rate at all. This means that fluctuation assays can only be used on organisms that are capable of going through a large number of generations rapidly, like bacteria, baker's yeast[42], or liquid-culture mammalian cells[30]. Also, mutations are rare events occurring in the specific biochemical circumstances of a particular cell. The fact that fluctuation assays look across large populations of cells over time means that those circumstances can differ substantially. Using this assay, it is thus difficult to study the progression of mutation rates of a particular population from the lag phase to early and late exponential phase and finally to a stationary phase. Any differentiation of mutation rates among single cells within the population are completely hidden from the fluctuation assay. Single-cell mutation dynamics can be studied with a single-molecule tracking of DNA repair

protein MutS[43] or by counting foci of accumulated MutL proteins[44]. Recent advances in high-throughput sequencing have also made it possible to directly estimate mutation rates from parent-offspring trios[9,45] and multigeneration pedigrees[46]. Such methodological advances are beginning to permit the direct counting of mutations occurring within a single generation. However, this direct approach needs expensive and state-of-the-art technologies like fluorescence microscopy, microfluidics, or whole-genome sequencing. On the other hand, the fluctuation assay is relatively inexpensive and only standard laboratory equipment is needed. Doing more fluctuation assays will also facilitate the generation of novel hypotheses that may be tested with more direct single-cell approaches.

There is a long-standing interest in the study of mutations, so the fluctuation assay will likely remain a widely used method. The number of citations of the seminal paper by Luria and Delbrück[5] in the last 4 years (2015-2018) have all been among the top five for citations of this paper. However, due to a large amount of precise manual work needed for properly carrying out a fluctuation assay, most studies only conduct a handful of fluctuation assays. This, however, is insufficient to reveal the environmental dependencies of the mutation rate. By streamlining fluctuation assays using multiwell plates, as explained in this paper, the current maximum throughput possible is 11 deep well plates (55 fluctuation assays) in parallel, as described here. Running two sets of fluctuation assays staggered by a day in parallel, allows carrying out up to 110 assays per week. Another step change in throughput may yet be possible by automating various steps of the fluctuation assays from the purely manual protocol given. Also, for studying environmental dependencies of the mutation rate, population density needs to be taken into account. Previous results[10] show that when known factors that affect mutation rate are accounted for, controlling for population density can reduce variation in mutation-rate estimates by more than 90%. To control the density, we recommend that N_t (used for estimating mutation rate) be determined independently from the method used to determine population density. In bacteria, N_t can be determined by CFU and density, for instance, with an ATP-based luminescence assay[10].

High throughput and controlling density are both essential when studying how an organism's ecological context affects spontaneous mutation rate. Knowing the existence of mutation rate plasticity is important, but understanding its causes and effects are key challenges that need to be met if mutation rate plasticity is to be incorporated into a wider biological context. The fluctuation assay is a great tool that can be used to test many hypotheses, because results are obtained rapidly, and assays are inexpensive relative to other methods. The stage is set, for instance, to study environmental dependencies of mutation rate in bacterial communities and microbiomes. Adapting the fluctuation assay to cocultures can test the hypothesis that strains influence each other's mutation rates via small molecules. Doing thousands of fluctuation assays with cocultures can determine if strains vary both in their ability to modify each other's mutation rates and in their susceptibility to have their mutation rate modified by others. Perhaps variation among strains in susceptibility to mutation rate manipulation is attributable to specific genetic variation. This may transform our views on how evolution works in complex communities, not least in examples of wide importance such as how antimicrobial resistance emerges.

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DISCLOSURES:

The authors have nothing to disclose.

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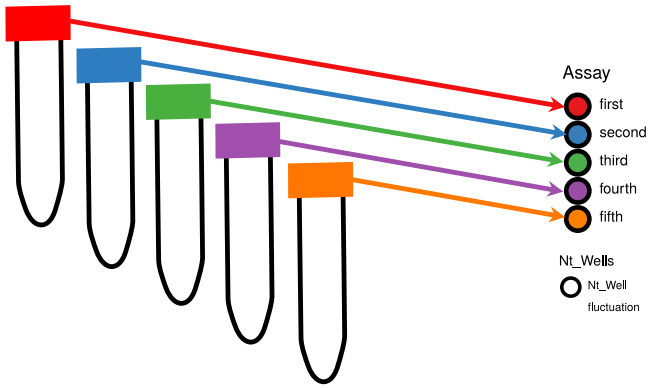
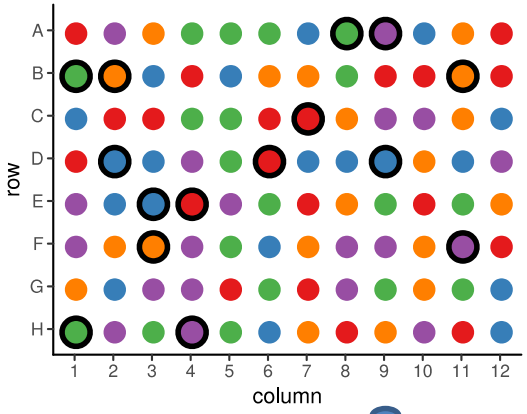
Figure 1

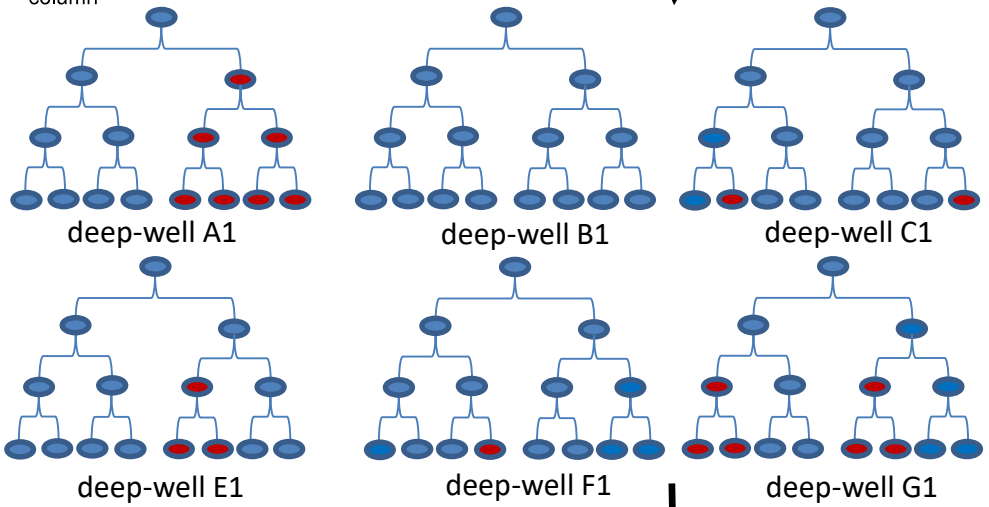
A. Inoculate and acclimate cultures



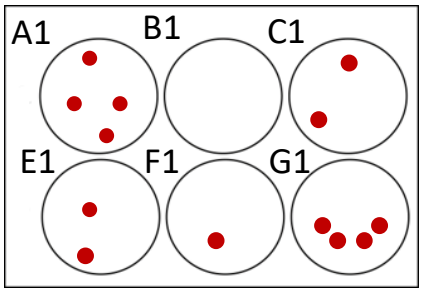
B. Prepare parallel cultures in a 96 deep-well plate



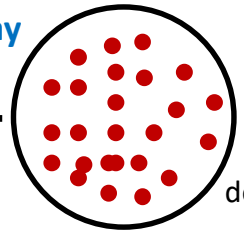
C. Incubate plate with cultures and allow cells to spontaneously mutate



D. Plate cultures on 6-well plates containing selective agar



E. Determine number of cells per assay



F. Count observed mutant colonies and calculate expected number of mutational events; visit http://shinyflan.its.manchester.ac.uk/

G. Estimate mutation rate

Figure 2

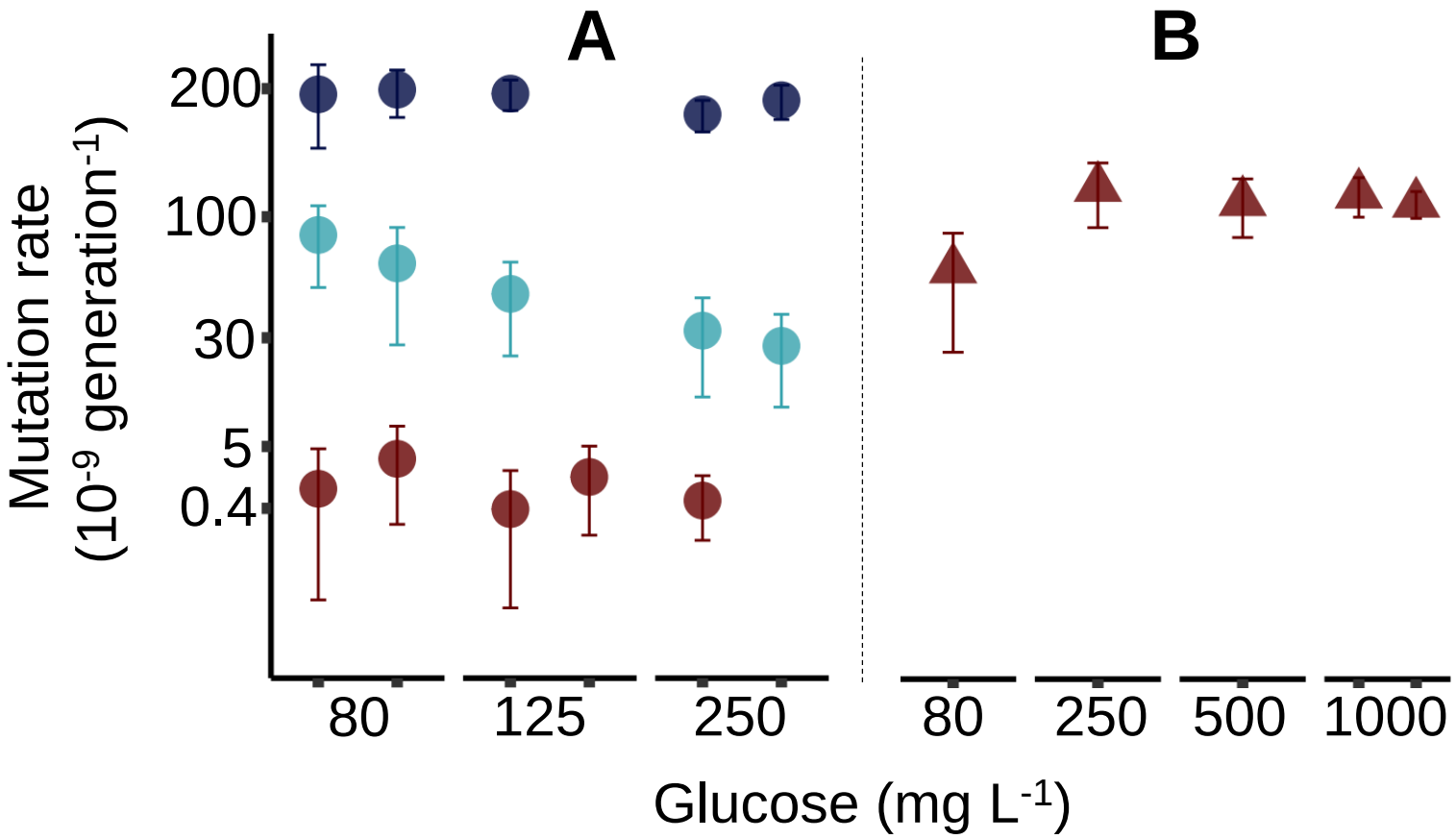


Figure 3

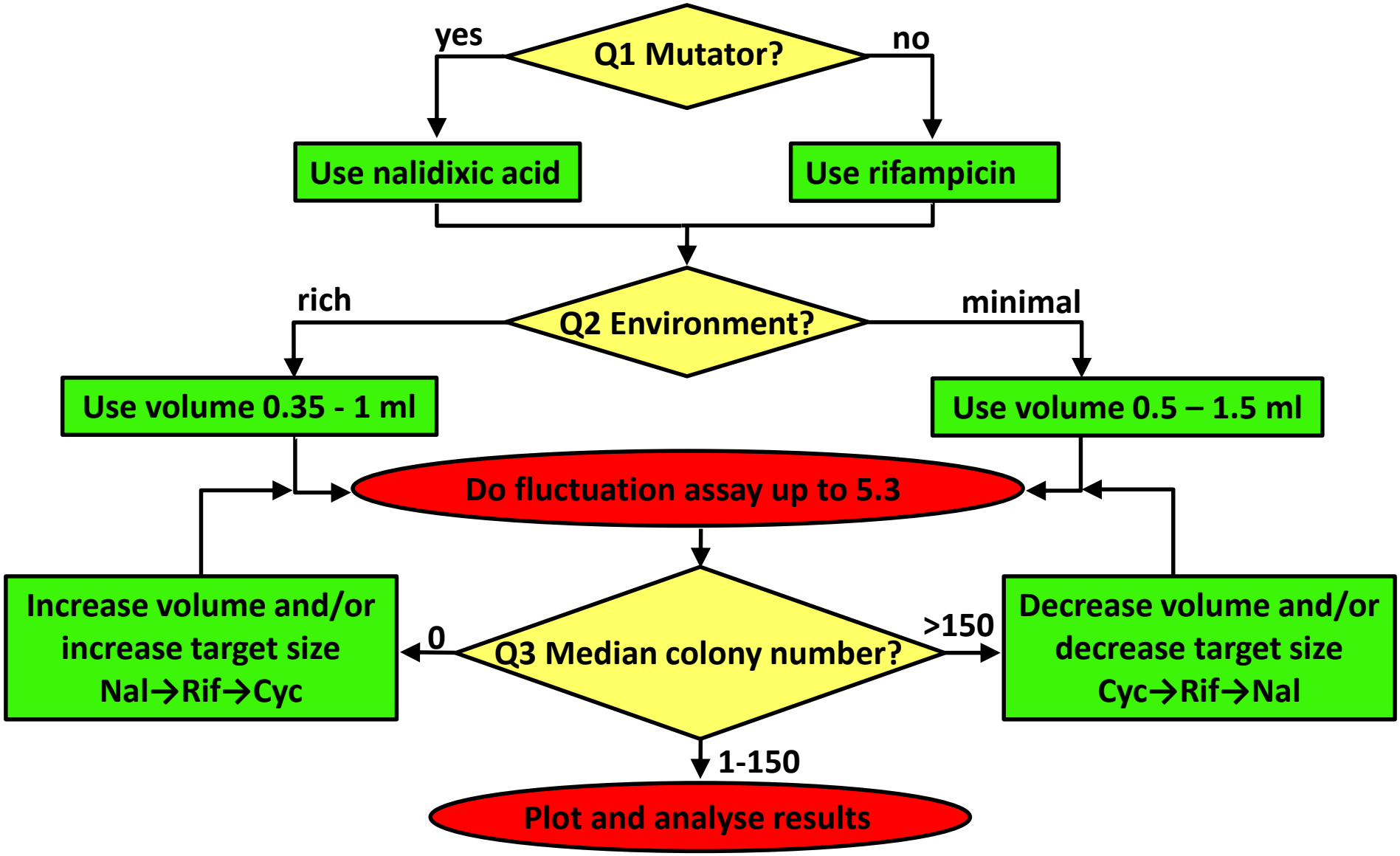


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 ml Microcentrifuge tubes	Starlab International GmbH	S1615-5550	
2,3,5-Triphenyltetrazolium chloride	Sigma-Aldrich	T8877-10g	
6-well plates	Greiner Bio-One	REF 657102	
90mm Petri Dishes Triple Vented	ThermoFisher Scientific	REF 120189	
96 deep-well plate (Masterblock 2 ml)	Greiner Bio-One	REF 780270	
Ammonium sulfate	Fisher Chemical	A/6440/53	
Bacto Agar	Becton, Dickinson and Company	REF 214010	
Bacto yeast extract	Becton, Dickinson and Company	REF 212750	
Cycloserine	Sigma-Aldrich	1158005-250MG	Only for assaying an alternative phen
D-Glucose anhydrous	Fisher Chemical	G/0500/61	
50 ml Centrifuge Tube	Corning	REF 430828	
L-(+)-Arabinose	Sigma-Aldrich	A3256-500g	
Magnesium sulfate heptahydrate	Fisher Chemical	M/1050/53	
Nalidixic acid	Sigma-Aldrich	N8878-5G	Only for assaying an alternative phen
Potassium phosphate dibasic trihydrate	Sigma-Aldrich	P5504-500g	
Potassium phosphate monobasic	Sigma-Aldrich	P0662-500g	
Rifampicin	EMD Millipore Corp, USA	557303-1GM	
Sodium chloride	Fisher Chemical	S/3160/60	
Spectophotometer	Jenway	6320D	
Thiamine hydrochloride	Sigma-Aldrich	T4625-25g	
Trisodium citrate dihydrate	Sigma-Aldrich	S1804-500g	
Tryptone	Fisher Chemical	1278-7099	

otypic marker

otypic marker

Dear Editor,

Thank you for the constructive comments. Please find our reply to each comment (colored in blue) colored in red.

We are pleased that Reviewer 1 found our protocol "of significant value to the field" and that Reviewer 2 described it as "a broadly useful tool for the research and medical communities".

Editorial comments:

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. Please use American English throughout.
Done.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
Done, it now has 40 words.

3. We cannot have punctuation marks in the title of the manuscript. Please reword to remove colon and aligning it with the protocol presented.
We modified the title to "Measuring microbial mutation rates with the fluctuation assay".

4. Please ensure the Introduction contains all of the following:
a) A clear statement of the overall goal of this method
Statement is the first sentence of the second paragraph in the introduction.

b) The rationale behind the development and/or use of this technique
The rationale behind the assay is now part of the first paragraph. We also created new Figure 1, which illustrates the fluctuation assay. When to use the assay is explained in the third paragraph (introduction).

c) The advantages over alternative techniques with applicable references to previous studies
Advantages of the assay are listed in the paragraph two in the introduction.

d) A description of the context of the technique in the wider body of literature
In the first paragraph (introduction) we explain the rationale of the assay. In the penultimate and last paragraph (of the introduction) the wider body of literature is then cited.

e) Information to help readers to determine whether the method is appropriate for their application
Paragraph three in the introduction contains enough information to help the reader to decide if a fluctuation assay is the appropriate method.

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points throughout.
Done.

6. 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: Falcon tubes,
Done, instead of Falcon tubes we are now using “50 ml screw cap conical bottom polymer tubes” or simply 50-ml tubes.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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.”
Done.

8. 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. We moved rational for the necessity of a small inoculum in the discussion (third paragraph).

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Done.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
Protocol frequently contains a basic microbiology technique and we believe (together with the Reviewer 1) that the description of how to carry out such a basic step (for instance, how to transfer a scrape of ice, Reviewer’s 1 Comment 11) will over-complicate the protocol. However, e.g. in step 2.3.4. we have added detail about stabilizing the evaporation.

11. Lines 118-128: We cannot have paragraph of text in the protocol section. Details about antibiotic additions can be moved as note whereever applicable.
Done, useful parts of this paragraph (as noted by the reviewer’s 1 comment 10) are now in NOTEs.

12. 1.1.: What is the importance of using MG1655? Can you use any bacterial strain in this case?
We added a NOTE after step 1.1 where we explicitly state that any *E. coli* strain or culturable bacterial species can be used in the assay.

13. Lines 166-169, 178-181: These notes can be moved to the discussion as it is not directly related to the steps above.
We moved the part about precision (178-181) in the discussion. However, we removed the "cocultures" aspect (166-169) from the protocol, to address concerns from Reviewer 1. We discuss precision in more details in the third paragraph in the discussion. We also calculated precision of the estimate of *m* for our data.

14. 2.3.1: Needs more clarity on what is being done.
Done, we rephrased the step and it is now more clear.

15. 2.3.2: Please check the volume -1mL in 96 well plate doesn't sounds correct.
It is a deep-well plate that has a capacity of up to 2 ml medium per well. 1 ml is thus correct.

16. 2.4: Reasons for using TA agar? What is the use of non-selective plates in this case? 2.5 and 2.4 needs more clarity.
Done, please see the NOTE to 2.4, where we explain why TA agar is more appropriate.

17. 3.5: Again 81 culture volumes = one well of 6 well plate? Reason for selection in this case?
We ensured that this step is now more clearly described.

18. 234-238: What is DAMP and how it is done? Reason for introducing this here.
Thank you, we moved the NOTE explaining the necessity for the independent estimations of the number of cells in the discussion.

19. 5.1: When at which step is the antibiotic resistance added to the bacteria?
Antibiotic resistance is a result of *de novo* mutation that emerges spontaneously when cellular DNA is replicated. A resistant mutant then makes a colony on the selective agar plate and these mutant colonies are counted in this step. This has now been clarified.

20. 286: is the software commercial? Also the link seem to be broken. If commercial please move the link to the table of materials and use generic terms here referring to the table.
Software is non-commercial and open source and we have made sure that the link is now working. The author of the shinyflan tool is now a co-author on the paper and shinyflan is long-term hosted by the University of Manchester.

21. Please introduce the terminologies used in the protocol section, e.g. parallel culture, inoculated environments, GLCs, etc somewhere in the introduction in a paragraph style (maybe briefly describe the experiment and discuss what each one represents and its importance in the experiment described) to have an idea as to what each term represent.
Done, we introduced the terminology in the first paragraph and explain terms in more details in the legend of the new Figure 1.

Also please provide a schematic illustration of what is being done in the protocol starting from the beginning to the end.
Thank you, we created a new figure 1 that is a schematic illustration of what is being done in the protocol. Also Figure 1 tries to explain the rationale behind the assay, as wanted by the

reviewer 1. For instance, six lineages on the middle-right of a figure showing the importance of the mutation timing during a culture cycle.

22. There is a 10-page limit for the Protocol, but 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.
Done.

What does the inoculum environment represent with respect to the steps mentioned previously?
We changed inoculated environments into inoculated media, we hope it is now more clear.

23. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
Done.

24. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.
Done.

25. 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]."
Mutation rates to rifampicin and nalidixic acid resistance shown in the Figure 2 were published in our PLOS Biology paper (reference 10 in the JoVE manuscript). PLOS Biology paper was published under a CC BY license that allows free and unrestricted use. On the other hand, mutation rate to D-cycloserine is previously unpublished and thus an original data. For more about the license, please find the PLOS policy on this link
https://journals.plos.org/plosbiology/s/licenses-and-copyright

26. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Please include the legends for tables as well.
Done.

Please separate the recipe table from the table of materials and number it as table 1.
Done, recipe table is now a Supplementary table S1 in the file Supplementary Tables.

27. Figure1: Please reformat the unit to mg/L instead of mgL-1. Please use this style throughout the manuscript.
Done.

28. Figure 3: This is not relevant, and the figure can be removed.
Done.

29. 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
In the first three paragraphs we describe steps that are critical for the assay.

b) Any modifications and troubleshooting of the technique
We discuss designing of a fluctuation assay in details in the first three (Discussion) paragraphs, where we follow and support Figure 3 (that is created to troubleshoot the assay).

c) Any limitations of the technique
We discuss limitations of the fluctuation assay in the paragraph four of the discussion. The paragraph starts by "The fluctuation assay has inherent constraints."

d) The significance with respect to existing methods
We discuss the significance in the last paragraph. For instance, "fluctuation assay is a great tool that can be used to test many hypotheses, because results are obtained rapidly and assays are cheap relative to other methods."

e) Any future applications of the technique
We discuss the potential future applications in the last paragraph. For instance, to study environmental dependencies of mutation rate in bacterial communities and microbiomes.

30. Our UK ALA has changed, please sign the attached ALA and upload to the editorial manager account.
Done.

Reviewer #1:

[Although outside the scope of this review, an online web-based tool for calculating mutation rates using raw data from fluctuation assays would be extremely valuable. If this is something JoVE could accomplish and put on their website, then maybe that is a win-win and achieves the goals of JoVE and the authors. Despite its clear advantages, sadly, not every lab has someone that knows how to use an R-package.]
Nice idea, thank you. Adrien Mazoyer, the author of the FLAN R package, also created a web-app version called Shinyflan that does not require any user expertise in R. While JoVE declined to host Shinyflan app on its website, we are happy to say that the University of Manchester decided to long-term host a Shinyflan (http://shinyflan.its.manchester.ac.uk/). Adrien Mazoyer is now listed as a co-author of this manuscript.

-There is not an adequate description of the fundamental principle behind the assay. Please see comment #5 below. This deserves a dedicated figure and an entire paragraph explaining how and why the fluctuation assay can ultimately estimate a mutation rate.
Thank you, we re-wrote the first paragraph in the introduction and introduce a new Figure 1. Please read also our reply to the reviewer's 1 comment 5.

-It is overly complex and too detailed in some parts. The 'co-culture' aspect should be removed entirely. The part about linear mixed effects modeling can also be removed. This will help streamline the protocol for the reader. You can supply references for these aspects instead.
Thank you, we removed both the ‘co-culture’ aspect and modelling from the protocol, and we listed appropriate references.

-The writing and organization throughout the manuscript needs to be improved. I have offered some suggestions in the comments, but this is a global issue throughout the manuscript.
Editorial comments and both reviews were very constructive and their suggestions have improved the paper quality.

-The Abstract should be shortened by taking out extraneous details and focusing on the bigger picture (see comment #8).
Agreed, we rephrased the Abstract and finish it with the statement of what we see as the future of the assay (mutation rate in communities) and what is the wider applicability of learning about mutations with fluctuation assays (antimicrobial resistance, carcinogenesis, ageing and evolution).

The list of necessary reagents and equipment is so comprehensive that it is completely unusable. This must be reduced to the list of items that is specific to the protocol and not an entire laboratory.
Done, documents Materials now contains 23 items, where the original one contained 59.

No quantitative comparison of this 'high-throughput' method is made to a more traditional 'low-throughput' method.
The 'high-throughput’ sentiment is not explicit anymore, however, in the discussion we state that high-throughput is necessary for studying, using fluctuation assays, environmental dependence of mutation rates. Please also read our reasoning (response to the next comment 1 about the title) why we consider our protocol as a high-throughput. We also discuss the balance between throughput and precision in the third paragraph in the discussion.

1. Title. Fluctuation assays are commonly performed in 96-deep-well plate format. Although this assay is more space-saving (i.e. compact) than using culture tubes, using the term "high-throughput" gives the title a hint of novelty and speed that is not warranted. The major rate-limiting step of these assays is plating and counting. I think a title that avoids use of the term "high-throughput" would be more appropriate.
We consider our method as a “high-throughput” because doing 110 fluctuation assays (the maximum number that we have done in a week so far) is 23% of all the assays that we found to be published from 1943 to 2017 (Figure 1 in our PLOS Biology paper, reference 10 in this manuscript). Also, Reviewer 2 pointed out that “Many people still use much larger volumes in single tubes for fluctuation assays which creates major limitations for throughput.” And Reviewer 2 puts it: “This throughput argument for using 96-well plates is important and should be raised in the introduction”.

It was not our intention to highlight our protocol as a novelty but simply to point out that it is actually possible to do a lot of (for many still extremely tedious) fluctuation assays in a short period of time. However, we agree that “high-throughput” might be misleading. So we changed the title to “Measuring microbial mutation rates with the fluctuation assay” and we expect that readers will realize that by following our protocol they can carry out many fluctuation assays in a relatively short period of time.

2. Recommended media. The protocol recommends specific media (i.e. LB, TA, DM), however, this aspect of the assay is quite flexible. Given this, it is specifically not clear to me why tetrazolium agar (TA) plates are recommended in this protocol since the strains used for the protocol are not Ara+/Ara- and do not require this differential indicator media. Presumably, this is for the mentioned 'co-culture' version of the assay, but this is never expounded upon and so I think this will be very confusing for most readers. I would favor just keeping it simple with LB and removing all discussion of 'co-culture' experiments.
Thank you, we now state that other media (not just LB or DM) can be used (see please NOTEs 1.1. and 1.2.). We also removed the co-cultures from the protocol. Nonetheless, we have retained the TA agar plates – *E. coli* colonies on TA agar are easier to spot and counting is thus quicker and more accurate, we therefore use it as a standard part of our fluctuation assays regardless of Ara markers. The rational for TA agar plates is now in NOTE 2.4.

3. Line 58: "evolves among organisms" is a bit vague. Perhaps try, "is a trait that is itself subject to evolution."
Done.

4. Introduction: Would suggest providing a sentence (maybe even opening up the paragraph, line 47) that describes the importance of mutational phenomena. e.g.: Mutations are the source of heritable variation for evolution.
Done, in the second sentence of the first introduction paragraph we included the sentence “…mutations were already perceived as one of the driving forces of evolution…”

5. Line 67: "In practice, ….". Instead of leading off with a practical description, I think it would be much better to first describe the fundamental principle of the fluctuation assay. In fact, I think this is so important that you should devote a figure to help the reader get the concept. The figure schematic would show a growing 'tree' with a single cell dividing into two, then four, then eight, and so on… You would use this figure to show how a random mutation at one part of the tree results in a different number of cells containing that mutation compared to a different random mutation in a different part of the tree. This is the basis of the observed fluctuation and the math behind the mutation rate calculator programs you reference. Luria and Delbruck's 'ingenious' assay method must be described well in this methods piece. If this publication is successful, you will be initiating a new group of scientists into this method, so they need to be educated on the fundamentals. In terms of the economy of this pieces word count (and considering that it would be published in JoVE), I think a fundamental description of the assay is much more important than describing the 'co-culture' method with multiple strains.
Thank you, we prepared a new Figure 1, which not only explain the protocol but also explains the fundamental principle of the fluctuation assay. We also re-wrote the introduction and cite four panels of Figure 1 in the text.

6. Line 95: "However, we believe that this protocol is extensible to most culturable microbes…mutation." Would change this sentence. For example:
"This protocol should be viewed as a basic template that can be utilized to study a wide variety of microbes by simply modifying the culture conditions and phenotypic markers of mutation.
Done.

7. Line 111: Remove the words 'than previously believed' and just the end the sentence directly after '…mutation rate.'
Done.

8. Lines 107-114. This should be re-written to be more succinct. Also, if the "main goal of this protocol is to enable people to routinely assay environmental dependencies of mutation rates in the diversity of microbial strains and environments", then this sentiment should be highlighted in the 'Abstract' and also be present in the 'Introduction'.
We slightly re-wrote the Abstract and make it more clear that testing environmental dependencies is important. The Abstract ends by "…, it can be used to study how an organism's environmental context affects spontaneous mutation rate". We also re-wrote the Introduction and now in the second paragraph we are trying to catch the right "environmental dependency" sentiment.

9. Line 118-119: "Before starting…order the list of materials…make sure you have all the equipment..." This is a given and does not need to be explicitly stated. Remove this sentence.
Done.

10. Lines 118-128. I'm not sure this paragraph is necessary at all. Would delete it in its entirety. It is largely redundant or contains seemingly unnecessary information like "always label on the lid and side of the plate." The only useful part is lines 123-125: "This protocol is written for…environments." I would keep that sentence, but nothing else in the paragraph.
Done, "useful parts" of this paragraph are now in protocol's NOTEs.

11. Line 132-133. You do not need to describe basics, such as how one should scrape a glycerol stock. If the researcher's using this protocol do not understand sterile procedure and how to work with their own organism, then, well, good luck performing a fluctuation assay. Cut out these extraneous details. Brevity is your friend.
Done.

12. Line 136: Delete the words within the parentheses, as they are redundant.
Done.

13. Line 152-155. I agree this is an important and most critical point. Given that your "main goal" is to make this assay more generalizable, I would expand upon this by more specifically defining what a "low number of cells" is in terms of actual numbers. This way, the reader will get the concept and be able to apply this concept to their own situation. Just use E. coli as an example: Rifampicin mutation frequencies in most non-mutator E. coli

cultures is on the order of ~10^-6. Thus, to avoid inoculating the culture with a pre-existing resistant mutant, one must inoculate with much less than 10^6 cells (e.g. 10^3-10^4 cells) to avoid the problem you mention. You give approximate numbers later in the protocol, but an explanation of how and way one arrives at those numbers would be very useful.
Thank you, excellent point. We explain the criticality of a small inoculum at the end of the third paragraph in the discussion.

14. Line 157: OD measurements. OD600 measurements should be obtained in the linear range of the instrument (typically OD values of ~0.1-1.0), especially if you are using the values to perform calculations. Typically, this requires diluting overnight cultures by a factor of ~10 prior to measurement. The protocol does not state to do this. That being said, I think this level of detail is probably not warranted anyway. I'd favor removing this entire discussion from the protocol and just telling the reader to estimate their inoculum sizes using OD600 (i.e. you can remove the equation from Line 160).
Done, step 2.2. with 2.2.1 and 2.2.2 are all re-written and the equation is removed

15. Line 166-169: Would remove co-culture discussion from the manuscript.
Done.

16. Lines 173-176: R program for random plate layout. Not every lab has someone that knows how to use R. Furthermore, it's not clear to me that plate well randomization is even, in principle, the correct method here. If you feel that the distribution of specific well conditions across the plate must be equal across sample-types to minimize 'edge-effects', then I would create a non-random plate layout that reflects that concept, as random plate layouts may randomly put one sample type disproportionately near the edges of the plate. Alternatively, a rational plate layout that is specifically designed to give approximately equal proportions of edge-wells across sample types may actually be a better method to address the underlying issue of 'edge-effects'. So, this may not only be easier and accessible to more labs, but also better even in principle. One could also just not use the edge wells on the plate.
It is an important point, but we find that plate spatial effects are not consistent enough and are too changeable across the incubator to be well accounted for by a rational layout. For us it makes more sense to use random layout with multiple replicate environments on multiple plates over multiple fluctuation tests and then account for the plate effects statistically. For instance, linear mixed-effects models, used in our three main publications (PLOS Biology, Nature Communications and ISME Journal), have a random factor of plates nested within an experimental block. The text has been modified to make it clear that the R script is just an optional facilitator for obtaining a random layout.

17. Lines 178-181. I assume the point of this is to determine the number of parallel cultures necessary to obtain the desired precision. Please state this explicitly. I would also include estimates of precision from your lab, so that others know roughly what to expect.
We discuss precision in details in the third paragraph in the discussion, where we state precision of 20% to be acceptable. We also calculated precision levels of the estimate of *m* in our data and report this in the part where we describe our results. We now have precision as “precison_m” in the Supplementary Data file, which will give a clear indication of the precision that can be expected from our protocol. In the penultimate paragraph of

the discussion we also point out that controlling for the population density will greatly decrease the variance (>90%) and thereby increase precision.

Figure 1 should have the confidence intervals plotted.
Done.

18. Line 186: Plate weight. It would be good to clue the reader in at this time that media evaporation is a major issue during shaking incubation and one needs to address it. These assays are also sensitive to the amount of aeration in the culture, so clueing the reader in as to why you recommend, "Do not fix the lid tightly!", would be more useful than simply demanding they do so with the exclamation mark. I would not use the exclamation mark.
Very sensible, thank you. We made two separate steps in the protocol, 2.3.3 and 2.3.4, where we explain the need for a proper aeration and for the monitoring of evaporation, respectively. We also removed this and other exclamation marks.

19. Lines 190 and 193: What is the point of using TA agar here? Why not just use LB? As far as I can tell, this protocol only has one type of bacterial strain and so does not require a differential indicator agar.
We added a NOTE to 2.4 where we explain the advantage of using TA agar plates.

20. Lines 219-221: Overnight storage is not advisable. It might work for you, but I wouldn't encourage others to do this, as their microbes and conditions may be different. Please remove this 'NOTE'.
Done. However, we did not remove NOTE to 3.1 and we added NOTE to 5.1, where we state that non-selective and selective plates can be stored in the fridge so that colonies can be counted later.

21. Line 223: Again, TA agar not necessary as far as I can tell.
See the rationale behind the usage of TA agar plates in the NOTE 2.4.

22. Line 245-247. Finally, we see the purpose of the TA agar in a 'NOTE'. I think for simplicity sake, I would remove all discussion of co-culture and remove TA agar from the protocol altogether.
We removed the cocultures but, as already stated, we would like to keep the TA agar plates. We also mention cocultures in the discussion, because we believe estimating mutation rates in co-cultures and communities could be a future use of this protocol.

23. Line 255: Please refrain from using exclamation points. Instead, you could recommend a value for the relative humidity level you think is required during the incubation to prevent the plates from drying out. Or, recommend a method to keep the humidity high enough in an incubator that does not have humidity control.
Exclamation point is removed and in 2.3.4 we explain how we stabilize the evaporation among experimental sets.

24. Lines 268-269: Recommend removing all discussion of 'co-culture' from the protocol to keep it simple (and shorter).

Done. However, we discuss the cocultures in the discussion, because we believe mutation rates in cocultures is an important research question and fluctuation assays are still the most suitable tool for getting an answer.

25. Lines 288-307. Why are these all separate steps with separate headings? Can this be collapsed into a single section?
We think it is more clear as it is. Having more text and more tasks in this section might be more confusing for the reader.

26. Line 312: Replace "total number of base pairs of the genotype (4,639,675)" with 'size of the chromosome (E. coli K-12 MG1655 = 4,639,675 bp)'.
Done.

27. Lines 339-360. Linear mixed effect model discussion. I would recommend removing this, as it is beyond the scope of this protocol and may not be appropriate for most users. This will save space and keep the protocol simple.
Agreed, we removed the paragraph about modelling and we are citing our PLOS Biology paper, where reader can learn about the statistical analysis.

28. Figure 1. Where are the confidence intervals? These should be plotted.
Done.

29. Lines 371-389. Discussion. The Discussion needs more focus. Perhaps you could explicitly center the discussion on how to use Figure 2? (see next comment).
Agreed, first two paragraphs in the discussion are now centered around Figure 3 (previously Figure 2).

30. Figure 2. Figure 2 is barely mentioned in the body of the text and is essentially unusable based on the minimal description in the Figure 2 legend.
Done, first two paragraphs are now centered around Figure 3 (previously Figure 2).

31. Figure 3. This figure doesn't add much to the protocol. It seems out of place. Would remove.
Done.

32. Table of materials. This is way too comprehensive to the point that it is completely unusable. Would reduce the size of this list substantially.
Document Materials now contains 23 items, previously it had 59.

Reviewer #2:

1. [L46] The way that the introduction is currently laid out makes it difficult to place the fluctuation test in the context of other approaches that are available. Some reorganization is needed so that the approach is placed in an appropriate context and the niche for this type of experiment is clearly defined. I would recommend introducing all approaches for mutation rate estimation much earlier, then clearly defining their limitations relative to the fluctuation test before focussing in on this approach.

Thank you, we reorganized the introduction, introduce all approaches in the second paragraph and after that we focus on the assay.

2. [L116] My biggest concern with the approach, as it is presented in this protocol, is that the use of five treatments on one plate, two of which are in duplicate, makes the whole approach considerably more difficult to follow and potentially less easily extendible to other experimental designs. I would like to see the method presented as one fluctuation assay on a 96-well plate, with a later discussion or notes sections devoted to running multiple treatments on one plate (similar to what is done for the co-culture extension).
We have to disagree here. While we could in principle describe a single fluctuation test on one plate, that would remove much of the point in obtaining a reasonable throughput. Our protocol is well balanced between a throughput and precision. Please read the third paragraph in the discussion where we discuss precision and show that doing 5 assays per plate will achieve the precision that will be below 20%, which is acceptable for the estimate of *m* (as stated in the reference 26 of this manuscript).

Beyond that, having several treatments together on one plate makes the approach *more* easily extendible to multiple plates because the assay, as described, can simply be repeated without further modification. This automatically confounds variation among replicates with variation among plates, significantly increasing the power of any statistical analysis.

It is not clear anywhere in the manuscript why performing five fluctuation assays using three environments is the most reasonable in a 96-well plate.

3. Related to the comment above, a discussion and some data about the effects of changing the number of wells per treatment is necessary. How did you come to determine that 19 or 20 wells per treatment was sufficient to obtain an accurate estimate of the mutation rate? What happens if you use fewer or more wells? Where would you recommend starting from if you are assaying a new strain? In the discussion the possibility of using 11 deep-well plates for 55 fluctuation assays and a total of 110 assays per week is raised. Could this be increased even further by using fewer wells or do those estimates just become too inaccurate?
Agreed, this is an important point that we now discuss in detail in the paragraph three in the discussion. The precision of the estimate of *m* depends on *m* and *C*, which is the number of parallel cultures (reference 26 in this manuscript). Precision is the coefficient of variation of *m*, calculated as $((\sigma_m / m) \times 100\%)$, where σ_m is a standard deviation of *m* calculated with Equation 1 from Stewart (1994); this is now the reference 41 in the manuscript. We explain this in the description of representative results.

Having 19 or 20 parallel cultures is experimentally determined number that is a good balance between throughput and precision. For instance, for the data published in PLOS Biology, the median precision (with interquartile range) is 16.4% (5.7%-38.9%, *N*=580) and the median number of parallel cultures (with interquartile range) is 16 (16-16). This is similar to values for the representative data in this manuscript (Figure 2), where precision is 17.5%(1.00%-28.9%, *N*=20) and the number of parallel cultures is 16 (15-16). We also added a column in the Supplementary data file named “precision_m”.

4. Throughout the protocol, it would be helpful to distinguish in some way between the components of the assay itself and the preparatory steps. I would recommend placing all aspects of the preparation like filling 6-well plates with TA agar in a separate section at the beginning of the protocol, labelled "Preparation of Materials" or something along those lines. If these have to be done at a specific time, like one day before they are used, this can just be noted at the end of the step. This will go a long way to increase the simplicity of the protocol.
Agreed, all the preparatory steps are now in NOTEs at the appropriate places in the protocol (1.1., 1.2., 2.4. and 2.5.).

5. Some approaches for analyzing the output of the results of a fluctuation assay are mentioned in the "Representative Results", but none of them are actually applied to the data. I would like to see some statistical analysis of the results to serve as a guideline for someone who uses this approach.
To increase the simplicity of the protocol we followed the reviewer's 1 comment 27 and thus excluded the statistical analysis from the protocol. Instead we cite our PLOS Biology paper, where guidelines for the analysis are explained in much more details as would be possible in this manuscript. Also, the amount of representative data in this manuscript is not enough to carry out a proper analysis.

Furthermore, there should be more discussion of the different approaches for determining mutation rates from a fluctuation assay and some of the software that is available online for doing so.
We believe this could over-complicate the manuscript. Just to say that we are citing all the seminal papers that has been crucial for the development of the fluctuation assay (these are references 5, 17-21, 23, 26, 29, 32, 34 and 36). We also mention an alternative R package rSalvador, but we are not citing FALCOR tool, because it seems to be not available anymore.

Minor Concerns:
1. [L51] Use expected number of mutations per cell division rather than referring to the lifetime of a cell.
Done.

2. [L54] Be more specific in this sentence about the fact that fluctuation tests are the go-to method for obtaining rapid and cheap estimates of mutation rates, given that other methods have become available that provide considerably more accurate estimates.
Done.

3. [L112] Some emphasis in this sentence should be placed on the fact that this protocol will enable fluctuation tests to be performed in a more high-throughput manner.
Reviewer 1 in his comment 1 argues that "high-throughput" might be misleading. Even if we do not completely agree with reviewer's view (please read our response), we decided to accept it. We thus changed the title of the paper, but we mention throughput in the discussion, where we discuss the balance between throughput and precision (paragraph three) and where we state our personal record of the number of assays conducted per week (penultimate paragraph in the discussion). We believe the reader will notice the high-

throughput potential of our protocol.

4. [L130] It would be helpful to have more specific titles for the different parts of the fluctuation assay, like "DAY 1: Inoculation and Acclimation of Cultures".
Wonderful idea, thank you, done.

5. [L132] Please provide storage conditions of the glycerol stock (ie. 15% glycerol, -80C).
Done.

6. [L134] It is worth noting that all incubation times may be subject to variation by species, strain, or condition.
Done.

7. [L179] It's not clear to me where the number 93 is coming from.
We rephrased the 2.3.1. NOTE and make it more clear.

8. [L227] Add a sentence to provide an estimate of how long drying can take - this is in the discussion but would be helpful to also have within the protocol.
Done.

9. [L240] Why not perform this dilution series in 96-well plates with a multi-channel pipette? It would increase the throughput of this step dramatically.
Done, we added the NOTE to 3.6.1.

10. [L418] This throughput argument for using 96-well plates is important and should be raised in the introduction. Many people still use much larger volumes in single tubes for fluctuation assays which creates major limitations for throughput.
We decided to remove “high-throughput” from the title, because for the Reviewer’s 1 view that this is not the feature of here presented protocol. However, we discuss throughput and precision in the third discussion paragraph. You can also read about our reasons why we still believe that our protocol allows a high-throughput in our response to the reviewer’s 1 comment 1.

11. [Figure 1] Please clarify in the legend where multiple circles of the same color come from.
Done.

```
ID	figure	mutation_rate	organism	strain	genotype
	marker	researcher	week	plate_ID	replicate	culture_volume
	glucose	Nt	m	SD_m	precision_m	m_lower	m_upper
	mutation_rate_lower	mutation_rate_upper	C_cultures	Culture_1
	Culture_2	Culture_3	Culture_4	Culture_5	Culture_6	Culture_7
	Culture_8	Culture_9	Culture_10	Culture_11	Culture_12	Culture_13
	Culture_14	Culture_15	Culture_16	Culture_17
HR033_01	2A	0.5346046828	Escherichia coli	MG1655	MG1655
	nalidixic_acid30	Huw Richards	2016_1	140	Glc80_1	10
	80	1366666667	0.7306264	0.3491615563	47.789342991
	0.1408801	1.3203728	0.103083	0.9661264388	15	0	2
	1	0	1	1	0	0	3	0	1	0	1	3
	3
HR033_04	2A	0.833056689	Escherichia coli	MG1655	MG1655
	nalidixic_acid30	Huw Richards	2016_1	140	Glc80_2	10
	80	1258333333	1.048263	0.3116322019	29.728436647
	0.397254	1.699272	0.3156985431	1.3504148348	15	0
	3	1	5	1	0	2	0	8	1	4	0	0
	6	3
HR033_02	2A	0.3957448182	Escherichia coli	MG1655	MG1655
	nalidixic_acid30	Huw Richards	2016_1	140	Glc125_1	10
	125	2200000000	0.8706386	0.3304011232	37.949284952
	0.2014815	1.5397958	0.0915825	0.6999071818	15	0	1
	2	1	0	2	1	0	6	2	0	0	3	1
	2
HR033_05	2A	0.6369383194	Escherichia coli	MG1655	MG1655
	nalidixic_acid30	Huw Richards	2016_1	140	Glc125_2	10
	125	1983333333	1.263261	0.2938462389	23.26092857	0.5332003
	1.9933214	0.2688404874	1.0050360002	15	4	0	0
	0	2	0	38	3	24	2	1	2	2	1	41

HR033_06	2A	0.4489906977	Escherichia coli	MG1655	MG1655
	nalidixic_acid30	Huw Richards	2016_1	140	Glc250_1	10
	250	3225000000	1.447995	0.2814808375	19.439351485
	0.8033851	2.0926043	0.2491116589	0.6488695504	15	0
	0	0	28	12	1	11	12	0	5	1	5	0
	7	53
HR035_06	2A	22.910831306	Escherichia coli	MG1655	MG1655
	rifampicin50	Huw Richards	2016_2	128	Glc80_1	1
	80	75126562.5	1.721212	0.2665646981	15.487034609
	0.7910238	2.6514002	10.529215948	35.292446663	15	6
	1	6	5	2	10	0	1	2	0	1	22	10
	2	5
HR035_09	2A	15.050883844	Escherichia coli	MG1655	MG1655
	rifampicin50	Huw Richards	2016_2	128	Glc80_2	1
	80	61393750	0.9240302	0.3242644341	35.092406511
	0.2762541	1.5718064	4.4997104754	25.602058841	15	2
	8	1	1	3	0	0	0	1	2	9	2	0
	0	1
HR035_07	2A	9.5760095977	Escherichia coli	MG1655	MG1655
	rifampicin50	Huw Richards	2016_2	128	Glc125_1	1
	125	127634375	1.222228	0.2969186704	24.293230919
	0.487436	1.95702	3.8190025219	15.333016674	15	3
```

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      3     1     1     4     0     16    0     10    0     0     1     3
      4     2
HR035_08    2A    5.5579781147      Escherichia coli MG1655      MG1655
      rifampicin50      Huw Richards      2016_2      128   Glc250_1    1
      250   231034375   1.284084    0.302597363 23.565231166      0.4806
      2.087568    2.0802099255      9.035746304 14    30    3     102   2
      1     1     0     1     0     0     1     6     6     2

HR035_10    2A    4.4352459683      Escherichia coli MG1655      MG1655
      rifampicin50      Huw Richards      2016_2      128   Glc250_2    1
      250   253653125   1.125014    0.2950946598      26.230310008
      0.4542684   1.7957592   1.7909040151      7.0795863445      16    2
      4     4     2     0     0     0     86    1     2     0     4     0
      2     8
RK129_11    2B    13.711710849      Escherichia coli BW25113     mutT
      nalidixic_acid30 Rok Krasovec      2016_3      420   Glc80_1     0.5
      80    52812760.42 0.7241533   0.3289008653      45.418679346
      0.213089    1.235218    4.0348014061      23.388627865      17    0
      3     0     0     1     1     0     0     0     0     2     28    8
      2     1
RK129_12    2B    45.652938403      Escherichia coli BW25113     mutT
      nalidixic_acid30 Rok Krasovec      2016_3      420   Glc250_1    0.5
      250   170216666.7 7.770891    0.1605379785      2.0658889497
      4.319681    11.222102   25.377544301      65.928338379      16    11
      11    25    22    33    16    15    18    62    33    22    45    26
      9     299
RK129_13    2B    37.021805052      Escherichia coli BW25113     mutT
      nalidixic_acid30 Rok Krasovec      2016_3      420   Glc500_1    0.5
      500   291040104.2 10.77483    0.144832762 1.3441767714      6.384198
      15.165461   21.935801657      52.107805011      16    34    22    38
      22    55    24    53    65    50    18    106   263   25    16    26

RK129_14    2B    41.365989739      Escherichia coli BW25113     mutT
      nalidixic_acid30 Rok Krasovec      2016_3      420   Glc1000_1   0.5
      1000  402364843.8 16.64422    0.1262926602      0.7587778835
      11.9231     21.36534    29.632559066      53.099420412      16    20
      72    56    262   80    1001  56    137   132   62    70    33    41
      65    39
RK129_15    2B    36.162559709      Escherichia coli BW25113     mutT
      nalidixic_acid30 Rok Krasovec      2016_3      420   Glc1000_2   0.5
      1000  377288281.3 13.64371    0.1344535787      0.9854620094
      10.97538    16.31204    29.090169359      43.234950059      16    32
      90    171   501   52    7     70    126   21    119   264   66    54
      108   25
GG6_01      2A    183.88405104      Escherichia coli MG1655      MG1655
      cycloserine4      Guillaume Gomez   2019_1      4     Glc80_1     1
      80    62700000    11.52953    0.1417768889      1.2296848955
      5.192748    17.866316   82.818947368      284.94921851 6     36    40
      33    38    31    35    30    19    26    41    89    31    36    88
      40    23
GG6_04      2A    197.00061538      Escherichia coli MG1655      MG1655
      cycloserine4      Guillaume Gomez   2019_1      4     Glc80_2     1
      80    65000000    12.80504    0.1330719841      1.0392156842
      8.489635    17.120435   130.60976923      263.39130769      17    15
```

```
      39    207   93    24    34    31    467   31    57    36    83    42
      75    42    48    28
GG6_02      2A    185.88064865      Escherichia coli MG1655      MG1655
      cycloserine4      Guillaume Gomez   2019_1      4     Glc125_1    1
      125   92500000    17.19396    0.1250065257      0.7270374347
      13.37114    21.01677    144.55286486      227.20832432      16    342
      55    54    34    15    75    53    334   87    40    183   98    101
      71    86    64
GG6_03      2A    136.71974619      Escherichia coli MG1655      MG1655
      cycloserine4      Guillaume Gomez   2019_1      4     Glc250_1    1
      250   197000000   26.93379    0.1085256497      0.4029349367
      20.75829    33.10928    105.37203046      168.06741117      16    166
      500   81    332   86    172   111   101   73    203   74    170   80
      40    149   164
GG6_05      2A    168.59912568      Escherichia coli MG1655      MG1655
      cycloserine4      Guillaume Gomez   2019_1      4     Glc250_2    1
      250   183000000   30.85364    0.1039787468      0.3370064175
      23.17952    38.52776    126.66404372      210.53420765      16    83
      215   107   10000 257   148   68    10000 108   240   207   103   83
      67    183   239
```

Media	Chemical	Batch
Davis minimal medium (DM)	Trisodium citrate dihydrate	Autoclave.
	Ammonium sulfate	
	Potassium phosphate monobasic	
	Potassium phosphate dibasic trihydrate	
		After autoclaving add filter and st
	Magnesium sulfate heptahydrate	2 g / 10 mL water
	Thiamine hydrochloride	110 mg / 5 mL water
Tetrazolium (TA) agar	Sodium chloride	Autoclave.
	Bacto yeast extract	
	Tryptone	
	Bacto Agar	
		After autoclaving add filter and st
	Arabinose	10 g / 50 mL water
	2,3,5-Triphenyltetrazolium chloride	500 mg / 10 mL water
D-Glucose anhydrous (filter sterilised)		10 g / 50 mL water
Antibiotic Rifampicin		50 mg / mL methanol
0.85% saline solution	Sodium chloride	Autoclave.
Liquid lysogeny broth (LB)	Sodium chloride	Autoclave.
	Bacto yeast extract	
	Tryptone	

Amount per L
0.5 g
1 g
2 g
7 g

erilize.

500 µL
200 µL

3.75 g
1 g
10 g
15 g

erilize.

15 mL
1 mL

1 mL

8.5 g

10 g
5 g
10 g

Strain	Genotype	Source or reference
E. coli K-12 MG1655		Reference 1
E. coli BW25113 JW0097-1	F-, Δ*(araD-araB)567* , *ΔlacZ4787* (::rrnB-3) , λ-, Δ*mutT790* ::kan, *rph-1* , Δ*(rhaD-rhaB)568* , *hsdR514*	Keio collection[2]

“ID"	A unique identifier for individual data points.
"figure"	The figure in which the row of data is used.
"mutation_rate"	The estimated mutation rate per genome per generation multiplied by 10^9.
"organism"	The organism used in the fluctuation assay.
"strain"	The strains used in the fluctuation assay are MG1655 and BW25113 (Keio). See the Supplementary table S2 for more details.
"genotype"	The genotype of the strain in which mutation rate was measured MG1655 and *mutT* corresponding to *E. coli* MG1655 and Δ*mutT* knockout from Keio collection. See the Supplementary table S2 for more details.
"marker"	Phenotypic marker used in the fluctuation assay.
"researcher"	The person who actually conducted the fluctuation assay.
"week"	The week in which the fluctuation assay was done.
"plate_ID"	A unique identifier of an individual 96-deep-well plate used in the fluctuation assay.
“replicate”	Assay with the same “genotype”, “glucose” and “marker”.
"culture_volume"	The initial volume of parallel cultures.
"glucose"	The initial concentration of glucose in the medium.
"Nt"	The population size at the end of the culture period estimated via colony forming units averaged over three parallel cultures.
"m"	the number of mutational events calculated with Ma-Sandri-Sarkar estimator[3] using tool FLAN[4] as explained in the protocol.
“SD_m”	Standard deviation of “m” calculated with the equation 1 from reference[5] using “m” and “C_cultures”.
“precision_m”	Coefficient of variation calculated as ((“SD_m”)/”m”)*100
“m_lower”	Lower bound of “m” (95%, CI)
“m_upper”	Upper bound of “m” (95%, CI)

“mutation_rate_lower”	Lower bound of the mutation rate is calculated as the “m_lower” divided by ”Nt”.
“mutation_rate_upper”	Upper bound of the mutation rate is calculated as “m_upper” divided by ”Nt”.
"C_cultures"	The number of parallel cultures used for estimating “m”.
“Culture_1”	Number of observed mutants in parallel culture 1
“Culture_2”	Number of observed mutants in parallel culture 2
“Culture_3”	Number of observed mutants in parallel culture 3
“Culture_4”	Number of observed mutants in parallel culture 4
“Culture_5”	Number of observed mutants in parallel culture 5
“Culture_6”	Number of observed mutants in parallel culture 6
“Culture_7”	Number of observed mutants in parallel culture 7
“Culture_8”	Number of observed mutants in parallel culture 8
“Culture_9”	Number of observed mutants in parallel culture 9
“Culture_10”	Number of observed mutants in parallel culture 10
“Culture_11”	Number of observed mutants in parallel culture 11
“Culture_12”	Number of observed mutants in parallel culture 12
“Culture_13”	Number of observed mutants in parallel culture 13
“Culture_14”	Number of observed mutants in parallel culture 14
“Culture_15”	Number of observed mutants in parallel culture 15
“Culture_16”	Number of observed mutants in parallel culture 16
“Culture_17”	Number of observed mutants in parallel culture 17

1 Hayashi, K. *et al.* Highly accurate genome sequences of Escherichia coli K-12 strains
2 Baba, T. *et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout
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Script to generate Figure 2

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Author(s): Kvasovec R., Richards H., Gomez G., Gifford D.R., Mazoyer A., Knight C.G.

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