Chapter 3

Measuring Mutation Rates Using the Luria-Delbrück Fluctuation Assay

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Abstract

The Luria-Delbrück fluctuation assay is one of the most commonly used methods for measuring the mutation rate in microorganisms. Specifically, it is used to measure the mutation rate at a particular locus or loci at which mutations give rise to a selectable phenotype. Here, I outline the essential features of performing Luria-Delbrück fluctuation assays as well as common missteps and tips for improving the accuracy of mutation rate estimates. In addition, I provide tools for analyzing data from fluctuation assays. This 96-well plate protocol has been optimized for use in yeast but should perform equally well for a range of microorganisms using standard microbiological methods.

Key words Mutation rate, Fluctuation test, Poisson distribution

1 Introduction

1.1 Principle of the Fluctuation Assay

The principle of the fluctuation assay (first introduced by Salvador Luria and Max Delbrück in their classic 1943 *Genetics* paper [1]) is simple, and if implemented properly, provides a powerful way to measure phenotypic mutation rates. The key to understanding the logic of the Luria-Delbrück experiment is to grasp the distinction between the distribution of the number of *mutation events* per culture and the distribution of the number of *mutant cells* per culture. Fig. 1 shows three hypothetical cultures that each start from a single cell and proceed through four generations of growth to produce sixteen cells. In each of these cultures exactly two mutation events occur, but the number of mutant cells differs depending upon when during the growth of the culture those mutations arose.

The distribution of the number of mutation events per culture follows the Poisson distribution and the distribution of the number

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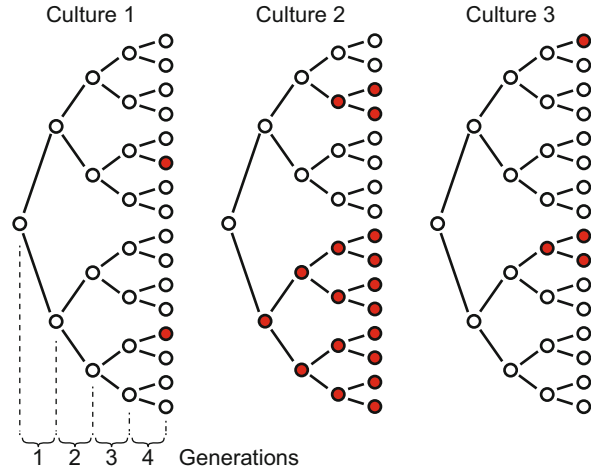

Fig. 1 Cartoon illustrating the principle of the Luria-Delbrück fluctuation assay. During the growth of a culture the number of mutation events will follow the Poisson distribution; however, the number of mutants per culture will have a larger variance because early arising mutations produce more mutant cells. For example, each of the three cultures had two mutation events during growth; however, the number of mutant cells differs depending upon when during growth the mutations arose

of mutant cells per culture follows the Luria-Delbrück distribution (Fig. 2). Both these distributions are described by a single parameter m, the expected number of mutation events per culture. Notice that for all values of m, the zero class (p_0) is the same for both the Poisson and the Luria-Delbrück distributions. This is because a culture will have zero mutant cells if, and only if, zero mutation events occurred. A single mutation event could produce just one mutant cell (if it arose in the last generation) but could also lead to a "jackpot" of mutant cells if it arose early in the growth of the culture. These "jackpots" are rare. Their rarity can be explained by looking retrospectively at the growth of a culture, recognizing that half of the mutation events occurred in the last generation, one quarter in the generation previous to that, etc. The "jackpot" principle was the key observation in the Luria Delbrück 1943 *Genetics* paper that proved that bacterial resistance to bacteriophage occurred through genetic mutation and not to acquired immunity following exposure to the bacteriophage at the time of plating.

1.2 Assumptions of the Luria-Delbrück Distribution

The formulation of the Luria-Delbrück distribution in Fig. 2 was derived by Ma, Sandri, and Sarkar [2]. In order for the number of mutant cells per culture to follow this distribution, several biological assumptions must be met. When performing fluctuation assays it is important to be aware of these assumptions because deviations could affect the accuracy of mutation rate estimates.

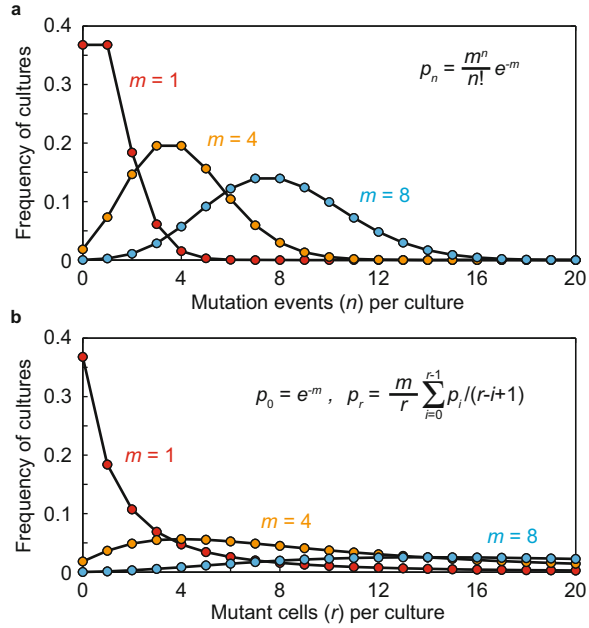

Fig. 2 Distributions for the number of mutation events per culture and the number of mutant cells per culture for three values of the parameter *m*, the expected number of mutation events per culture. (**a**) The distribution of the number of mutation events per culture follows the Poisson distribution with an average number of mutation events per culture equal to m. (**b**) The distribution of mutant cells per culture follows the Luria-Delbrück distribution. Note that p0 is the same for both distributions; in other words, a culture will have no mutant cells if, and only if, there were no mutation events

I have listed these assumptions in the order in which they are likely to be violated in a typical fluctuation assay.

1. *Growth of each culture starts with a single cell.* This assumption will almost always be violated, but as long as the initial inoculum size is negligible relative to the final number of cells per culture, this is not a concern. This is because the number of cell divisions is equal to the number final cell number minus the inoculum size.
2. *Mutant and non-mutant cells have the same growth rate.* This will skew the distribution of the number of mutant cells that result from each mutation event. This will have less of an effect on mutation rate estimates using the p_0 method than the MSS-maximum-likelihood method (*see* Subheading 4), since differential growth will not affect the zero class.
3. *No post-plating mutations.* In the case where the selection does not effectively kill or arrest non-mutant cells, additional cell divisions will produce additional mutant cells. These post-plating mutants will be Poisson-distributed.

4. *All mutants are detected.* If plating efficiency is less than 100% or if there is protein perdurance (for instance in the case of counter-selectable markers such as *CAN1* or *URA3*), the number of true mutant cells will be undercounted.
5. *No change in mutation rate during growth.* Changes in mutation rate in response to changes in growth conditions will skew the distribution of mutant cells per culture. An elevated mutation rate early would lead to more "jackpot" cultures; elevated mutation rate late in growth would increase the proportion of the lower classes of the distribution.
6. *Same final number of cells in replicate populations.* This is typically a safe assumption, though complications with the experimental setup (such as uneven evaporation across the 96-well plate) could result in this assumption being violated.
7. *No reverse mutations.* Under most circumstances, this is a safe assumption. Though it is possible, for example if a large class of suppressor mutations exists for the phenotype being assayed, that this assumption will not always hold.

1.3 Common Missteps in Performing Fluctuation Assays

From my experience performing fluctuation assays and advising others on this method, I have come to recognize several common missteps that should be avoided.

1. *Not plating the whole culture.* The principle of the Luria-Delbrück fluctuation is based on accurately measuring the distribution of the numbers of mutant cells per culture. This is best determined by plating the entirety of the culture. For this reason, culture volumes should typically be kept small. If the entire volume of the culture is not plated, it is necessary to correct for this by adjusting for the dilution factor before calculating m from the data. When you have a large number of mutant cells per culture and subsampling is required, it becomes more difficult to determine how well (or how poorly) the data fit to the Luria-Delbrück distribution, which flattens out at high m.
2. *Not having a zero class.* There are two reasons why I think it is important to have a zero class. First, it allows the p_0 method to be used to calculate mutation rate in addition to the MSS-maximum-likelihood (*see* Subheading 4). Second, the maximum likelihood fitting method is most sensitive to the lower end of the distribution of the data.
3. *Using too few populations.* There is no specific number of populations required, but the accuracy of the fluctuation assays (if performed properly) increases as the square root of the number of cultures [3]. Fig. 1 in [4] can be used as a guide for selecting an appropriate number. I routinely use 72 cultures to estimate m, though I have used as little as 36 and as many as 720.

2 Materials

1. 96-well plates.
2. Aluminum plate seals.
3. Whatman filter paper circles (Grade 3, Cat. No. 1003-090).
4. Replica-plating block.
5. Replica-plating velvets, sterile.
6. Liquid nonselective growth medium (*see* **Note 1**).
7. Agar plates containing selective growth medium (*see* **Note 2**).
8. Beckman Coulter particle counter (*see* **Note 3**).
9. Vials for Beckman Coulter particle counter.
10. ISOTON II Diluent for Beckman Coulter particle counter.
11. Sonicator.

3 Performing Fluctuation Assays

3.1 Overdrying Plates

1. Set up a replica-plating block with a sterile velvet.
2. Using sterilized forceps, place a sterile 90 mm Whatman filter paper circle on the velvet (*see* **Note 4**).
3. Press plate onto the replica-plating block to transfer the filter to the plate (*see* **Note 5**).
4. Allow the filter to sit on the plate for at least 30 min, up to overnight, to allow the filter to pull water out of the plate.
5. Repeat for each plate.

3.2 Set Up 96 Parallel Cultures

1. Grow a single overnight culture of the strain to be tested (*see* **Note 6**).
2. Dilute culture 1:10,000 into 40 ml of nonselective (*see* **Note 7**).
3. Add the appropriate volume of culture to each well of a 96-well plate (*see* **Note 8**).
4. Cover the plate with a plate seal to avoid evaporation.
5. Incubate the 96-well plate at 30 °C (or other appropriate temperature) until cultures reach saturation (*see* **Note 9**).

3.3 Determining the Average Number of Cells Per Culture (N)

1. For each 96-well plate, pool 24 of the cultures, and set the remaining 72 cultures aside to be plated on selective medium (*see* **Note 10**).
2. If necessary, sonicate the pooled cultures to break up cell clumps for 1–2 min in a bath sonicator or for 10 half-second pulses using a probe sonicator.
3. Make three independent dilutions of each culture into filtered ISOTON II Diluent (*see* **Note 11**).

4. Count each vial three times (*see* **Note 12**).
5. Calculate the average number of cells per culture (N) as the median-of-the-median cell counts, correcting for the initial dilution and culture volume (*see* **Note 13**).

   Example data:

	Count 1	Count 2	Count 3	Median
Dilution 1	33,284	32,912	33,518	33,284
Dilution 2	33,268	33,126	32,766	33,126
Dilution 3	33,972	33,578	33,440	33,578
Median				**33,284**

3.4 Determining the Number of Mutant Cells Per Culture

1. For the remaining 72 cultures, bring the volume up to 100 μl by adding water to each well using a multichannel pipette.
2. Plate the entire volume of each of the 72 cultures onto the overdried plates by spot-plating nine cultures per plate (*see* Fig. 3 and **Note 14**).
3. Allow the plates to sit at room temperature for at least a half hour or until all of the liquid is absorbed (*see* **Note 15**).
4. Incubate plates for 1–2 days at 30 °C until colonies are large enough to count.
5. Count the number of mutant cells per culture (*see* **Note 16**).

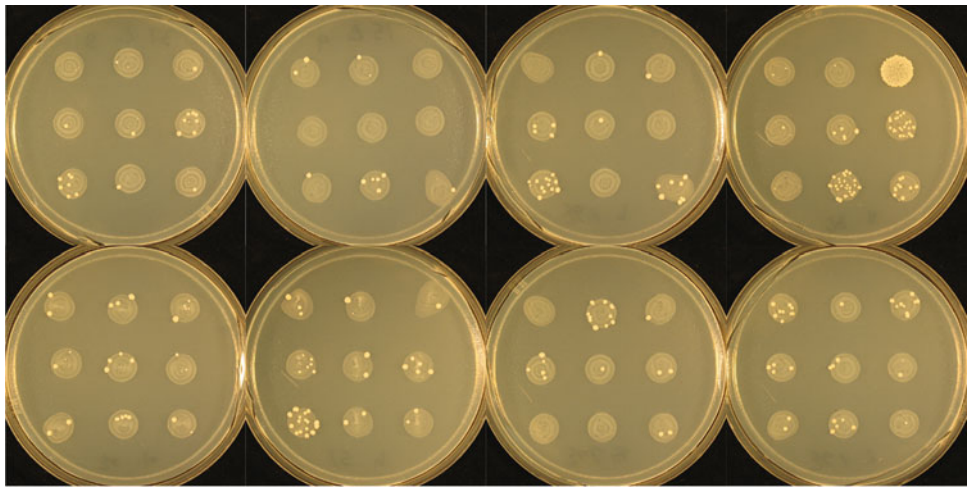

Fig. 3 Spot plating cultures onto overdryed plates. Example of a fluctuation assay on 10× canavanine medium from ref. [7]. Seventy-two 100 μl cultures were spot-plated onto eight canavanine plates. Colonies were counted after 2 days of growth. For larger culture volumes (~200 μl), only six cultures are spot plated onto each plate. For small volumes, cultures should be brought up to 100 μl.

4 Analyzing Data from Fluctuation Assays

Many methods exist to calculate m (the expected number of mutation events per culture) from fluctuation data (reviewed in [4, 5]). In practice, only two methods should be used: the p_0 method and the MSS-maximum likelihood method.

4.1 Calculate the Mutation Rate Using the p_0 Method

1. Determine the fraction of cultures with zero mutant cells. This value is p_0.
2. Calculate the expected number of mutation events per culture (m):
   $m = -\ln(p_0)$ (*see* **Note 17**).
3. Calculate mutation rate (μ):
   $\mu = \frac{m}{N}$.

4.2 Calculate the Mutation Rate Using the MSS-Maximum Likelihood Method

In 1992, Ma, Sandri, and Sarkar provided a solution to the Luria-Delbrück distribution for the single parameter m [2]. This made it possible to calculate the most likely value of m based on fluctuation assay data [6]. Stewart [3] shows that this method, known as the MSS-maximum-likelihood method, is the most accurate method for determining m, and provides a formula for calculating 95% confidence intervals for estimates of m calculated using this method.

1. Calculate the expected number of mutation events per culture (m) using the provided supplemental Matlab script "findMLm" (*see* **Note 18**) or Excel spreadsheet "fluctuationAssay_TEMPLATE.xls" (*see* **Note 19**).
2. Calculate 95% confidence intervals on m using the following equations:
   $m_{95-} = \ln(m) - 1.96\sigma(e^{1.96\sigma})^{0.315}$, and
   $m_{95+} = \ln(m) + 1.96\sigma(e^{1.96\sigma})^{-0.315}$,
   where $\sigma = \frac{1.255 m^{-0.315}}{\sqrt{C}}$, and C is the number of cultures used (*see* **Note 20**).
3. Calculate mutation rate (μ):
   $\mu = \frac{m}{N}$, where N is the average number of cells per culture.
4. Calculate 95% confidence intervals for the mutation rate:
   $\mu_{95-} = \frac{m_{95-}}{N}$, and
   $\mu_{95-} = \frac{m_{95-}}{N}$.

5 Notes

1. This is typically a rich medium or a synthetic complete medium, for example YPD or SC for yeast.

2. The exact medium used will depend on the organism and the particular locus or loci used for the fluctuation assay. For example, in yeast, 5FOA (1 mg/ml) and canavanine (0.6 mg/ml) is used to measure mutation rates at *URA3* and *CAN1*, respectively. In the case of canavanine, it is necessary to use higher drug concentrations than is necessary for counter-selection to prevent post-plating growth, typically 60 μg/ml [7].
3. Cells counts can also be performed using other methods such as a hemocytometer or dilution plating.
4. It is possible to sterilize the Whatman filters, but I have not found this to be necessary.
5. Press evenly so that the entire surface of the plate is in contact with the filter. The 90 mm filter circles will be slightly larger on the surface of the agar, which has diameter of ~85 mm for standard petri dishes.
6. This can be done in a nonselective medium or in a medium that selects against the mutants. For example, when selecting for 5FOA resistance, which occurs primarily through mutations at the *URA3* locus, I perform this overnight growth in medium lacking uracil.
7. Before beginning a set of experiments it is useful to pilot the experiment to identify the optimal culture conditions. To analyze the data using the p_0 method, ~20–80% of the cultures should have zero mutation events, thus zero mutants. An estimate of the mutation rate will help to select a culture volume to pilot. For example, when using 5FOA resistance ($\mu \sim 5 \times 10^{-8}$ per generation) as a selection, I typically use 200 μl cultures with 2% glucose; when using αF resistance ($\mu \sim 5 \times 10^{-6}$ per generation) as a selection, I typically use 10 μl cultures with only 0.2% glucose.
8. It saves time to use a multichannel pipette for this step.
9. It is not necessary to shake the plates. In fact, shaking could increase evaporation or introduce inconsistencies between wells. Side-by-side measurements of mutation rate without shaking and with shaking on a Titramax 1000 orbital shaker were indistinguishable.
10. I pool 24 wells along the diagonals, rather than two rows, to sample wells along the edge and in the center to control for possible edge effects such as uneven evaporation. This can be done without changing pipette tips. It is important that cells be resuspended well either by pipetting up and down several times or using a plate vortex such as the Titramax 1000 orbital shaker.
11. Filter ISOTON II Diluent with a 0.45 μm filter to remove particles. Use an automatic dispenser for the solution to

maintain uniformity in volume. After dispensing the diluent, let the vials sit for a few minutes before counting to avoid counting bubbles. I aim to dilute such that I count in the range of 30,000–90,000 events (For example, I dilute a 10^7 culture 1:2000, 10 μl into 20 ml).

12. Before beginning rinse and flush until the background is below 500 (ideally below 100, but I tolerate up to 1000. Sometimes this takes a while). Record the background before and after each run. It is not necessary to rinse and flush in between samples. If anything lodges in the aperture, rinse and flush, noting when this occurs. If the counts spike, stop and restart the count.
13. I do not subtract background. I use medians to ensure that these estimates are robust to non-normally distributed data.
14. If the volume is greater than 100 μl, it may not be possible to spot-plate nine cultures per plate. For yeast fluctuation assays on 5FOA, I use 200 μl cultures and spot-plate six cultures per plate. It is important that cells be resuspended well prior to plating. This can be accomplished by pipetting up and down several times. I have also used a Titramax 1000 orbital shaker to resuspend cells prior to plating.
15. Be careful not to disturb the plates. I usually allow an hour, but I have allowed plates to dry overnight before transferring them to the incubator.
16. It is possible to count unaided by eye; however, I prefer to use a dissection scope (10× magnification) and a gooseneck light source. I use threshold counting. For example, for fluctuation assays on canavanine, colonies smaller than 1 mm at 10× magnification are presumed to result from mutations that had occurred after the cells were plated and were counted separately.
17. This equation is derived from the Poisson distribution. Specifically, the probability of observing zero mutant cells (and thus, zero mutation events) is $p_0 = e^{-m}$.
18. The most likely value for m given fluctuation assay data can be calculated by executing the following Matlab script: **findMLm (*data*)**, where *data* is a column vector containing counts of mutant cells per culture. This program requires the following Matlab scripts to run: **scoreData** and **generateLD**. **scoreData** is a Matlab script that takes as its input data from a fluctuation assay and a value for m. This program outputs the *-log* probability of observing the *data* given m. This program requires the Matlab script **generateLD**. **generateLD** takes as its input a value of m and a maximum number of mutant cells per culture (*max*) for which to calculate the probability distribution. It outputs the Luria-Delbrück distribution from 0 to *max* with

parameter *m*. All three scripts are provided as supplemental information and were originally published in ref. [7]. Alternatively, several web-based tools exist for analyzing fluctuation assays [8, 9].

19. Open the "fluctuationAssay_TEMPLATE.xls" spreadsheet, provided as supplemental information. Remove the example data in blue and enter your data here. These data will be in the form of the number of cultures with a given number of mutant cells. Check that the number "# of cult." value is the same as the number you expect. Select a value for *m*. The objective is to find the value for *m* that maximizes the value for "$-log\ P(data|m)$). Using the workspace provided pick several values of *m*: I typically start with values of *m* such as 0.1, 1, 2, 3, 4. Find the minimum value. Then determine if the actual minimum is greater or less than this value. For instance if 2 is the best value of *m* out of 0.1, 1, 2, and 3, I next check if 1.9 or 2.1 improves the fit. If *m* of 2.1 is better than 2.0, I would then test all values between 2 and 3 in increments of 0.1. I repeat this process until I find the best most likely value of *m* given the data to three significant digits.
20. The expected error of fluctuation assays was determined by Stewart [3] and assumes that the data truly fit the Luria-Delbrück distribution. If the quality of the data is poor, calculated values for *m* and the 95% confidence intervals on this value will be unreliable. For this reason it is useful to compare values for the mutation rate as determined by the P0 method and the MSS-maximum likelihood method. When performing fluctuation assays one needs to be cognizant of the assumptions that underlie Luria-Delbrück distribution and how violations of these assumptions could affect estimates of mutation rates. Some efforts have been made to account for deviations such as post-plating growth [7], plating efficiency [10], and differences in the growth rate of mutant and non-mutant cells [8, 11, 12].

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References

1. Luria S, Delbrück M (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511
2. Ma WT, Sandri GH, Sarkar S (1992) Analysis of the Luria-Delbrück distribution using discrete convolution powers. J App Prob 29:255–267

3. Stewart FM (1994) Fluctuation tests: how reliable are the estimates of mutation rates? Genetics 137(4):1139–1146
4. Rosche WA, Foster PL (2000) Determining mutation rates in bacterial populations. Methods 20(1):4–17
5. Foster PL (2006) Methods for determining spontaneous mutation rates. Methods Enzymol 409:195–213
6. Sarkar S, Ma WT, Sandri GH (1992) On fluctuation analysis: a new, simple and efficient method for computing the expected number of mutants. Genetica 85(2):173–179
7. Lang GI, Murray AW (2008) Estimating the per-base-pair mutation rate in the yeast *Saccharomyces cerevisiae*. Genetics 178(1):67–82
8. Gillet-Markowska A, Louvel G, Fischer G (2015) bz-rates: a web tool to estimate mutation rates from fluctuation analysis. G3 (Bethesda) 5(11):2323–2327
9. Hall BM et al (2009) Fluctuation analysis CalculatOR: a web tool for the determination of mutation rate using Luria-Delbrück fluctuation analysis. Bioinformatics 25 (12):1564–1565
10. Stewart FM (1991) Fluctuation analysis: the effect of plating efficiency. Genetica 84 (1):51–55
11. Stewart FM, Gordon DM, Levin BR (1990) Fluctuation analysis: the probability distribution of the number of mutants under different conditions. Genetics 124(1):175–185
12. Zheng Q (2005) New algorithms for Luria-Delbrück fluctuation analysis. Math Biosci 196(2):198–214