Use of Optical Density Detection Times To Assess the Effect of Acetic Acid on Single-Cell Kinetics[∇]

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The growth of *Listeria innocua* at different acetic acid concentrations (0 to 2,000 ppm) was monitored by optical density measurements in a Bioscreen (LabSystems, Vantaa, Finland). The generated populations came from low inocula that were obtained by serial dilution. A new method to estimate both the growth rate and the lag time of single cells from the detection times (time to reach an optical density of 0.11) was developed. It assumes that the single-cell lag times follow a gamma distribution and takes into account the randomness of the inoculation level. (The initial cell number per well was assumed to follow a Poisson distribution.) In this way, relatively small numbers of replicates are sufficient to obtain a robust estimation of the distribution of single-cell lag times. The results were validated with plate count experiments. It was found that logarithms of both the growth rates and of population lag times increased linearly with the acetic acid concentration. The logarithm of the scale parameter of the gamma distribution of the single-cell lag times also increased linearly with the acetic acid concentration irrespective of the phase of the inoculum.

Single-cell kinetics has recently become a focus of research in predictive microbiology studies (2, 3, 8, 9, 13, 17, 20, 21, 23, 27, 30, 32, 33). Beyond the scientific interest in understanding the behavior of single cells, the advent of quantitative microbial risk assessment in food safety has underlined the need for quantitative assessments of cell-to-cell kinetics parameter variability. Indeed, under the food processing conditions of the Western world, the contamination of food by pathogens usually occurs with low numbers and a low probability, and the chance that their multiplication results in an infectious level at a later stage depends greatly on the kinetics of the contaminating cells as well as whether they are able to grow.

If the statistical distribution of single-cell division times is known at the various stages of growth, then the growth of the generated subpopulation can be accurately predicted (23). Unfortunately, it is not easy to measure the division times of sufficient single cells to derive statistically robust distributions for a whole population. Microscopic observations can be used (12, 25, 33, 34), but the experimental conditions are limited. Indeed, if the variability of the lag times is great, then the cells with shortest lag times will overgrow the cells with a long lag time. One possibility is to use the flow chamber technique which flushes away the daughter cells as they divide, and successive generation times of the attached mother cell can be observed (8). However, this system only works with cells that attach well to a surface (e.g., with *Escherichia coli* but not with *Listeria*).

Alternatively, the kinetics of populations originating from single cells can be followed by optical density (OD) measurements. A convenient automated tool to measure detection times is the Bioscreen C (LabSystems, Vantaa, Finland), where growth within up to 200 wells can be followed by monitoring

their turbidity. If the specific growth rate is the same for each well and the inoculum in a well is exactly one cell, then the detection time, the time to reach a given detection level characteristic of the population size, is a shifted value of the lag time of the initial single cell. This technique has been applied by several researchers in recent years (9, 13, 21, 27, 30, 34).

The most important kinetics parameters describing the growth of a microbial population are the growth rate and the lag time. The lag time concept has traditionally been defined as the intersection of the initial level with the extension of the linear phase of growth on the logarithmic scale (24). Because the lag time from this definition is a geometrical concept, rather than having a microbiological significance, a few authors have recently introduced different concepts such as the “physiological state” (6), the “work to be done” (28), or the “relative lag times” (29), which all characterize the lag as an adjustment process. These quantities depend both on the amount of work to be carried out during the adjustment process and on the rate at which this work is carried out.

It has been shown experimentally (1, 27) and theoretically (3) that the lag time depends on the inoculum level as the inoculum decreases to less than 100 cells per ml. This may be due in part to the fact that not all of the cells are able to divide, so what we observe is an “apparent lag” as opposed to the “true lag” (24). Even under environmental conditions such that all of the cells divide, there is in addition a statistical effect due to the low number of cells; the population lag time is determined by the shorter single-cell lag times, and this effect is more apparent at low initial counts (3). Studies in recent years have underlined the difficulties of defining and measuring the end of a lag period for a single cell (22, 33). The time to first division is the sum of the “physiological” lag time and the first generation time. No phenotypic event, however, allows the direct observation of the end of the lag period. In what follows, we define the lag time as the “geometrical lag,” the time when the linear extension to the exponential phase of the $y(t)$ curve crosses the time axis (denoted by L_g in Fig. 1) irrespective of

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FIG. 1. The distribution of the T_{det} detection times is a shifted version of that of the “geometrical” single-cell lag times. The shift is determined by the maximum specific growth rate, μ , as long as the detection level is in the exponential phase.

the inoculum level. This lag definition does not correspond exactly to the “time to the first division” or a “physiological” lag time, but this is a lag time that we can estimate from the optical density experiments. Indeed, let $y(t) = \ln N(t)$ describe the time variation of a bacterial population in a well, where $N(t)$ is the number of cells at time t and y is the natural logarithm of N .

For $t > L_g$, the natural logarithm of the population in the exponential phase can be expressed as

$$y(t) = \ln N_0 + \mu \cdot (t - L_g) \quad (1a)$$

with L_g the “geometrical lag time” (Fig. 1).

Suppose that the initial number of cells per well, N_0 , and the population generated from N_0 can be detected when the cell number reaches N_{det} (i.e., $y_{det} = \ln N_{det}$ is the detection level at the detection time, T_{det}). Then

$$y_{det} = \ln N_0 + \mu \cdot (T_{det} - L_g) \quad (1b)$$

It has been shown that after the lag time, the cells do not enter the exponential phase at once, but it is a gradual continuous process (16, 22, 23). The first division times have a larger variance than when in the exponential phase. As a consequence, the geometrical lag time variability as defined here not only depends on the lag time of the single cell’s variability but also on the variability of the first few generation times. However, what is relevant in terms of risk assessment is the concentration of an infectious level; so for modeling purposes, the geometrical lag time variability is the most useful.

To obtain the distribution of single-cell lag times, the wells of the Bioscreen should be inoculated with exactly one cell. In practice, a flow cytometer can be used to sort the cells (30), but it has been shown that cell size is linked to its stage in the division process (22). Consequently, a large cell which is close to division is likely to be discarded in the sorting process, introducing a bias against cells with short lag times. A simpler method to obtain approximately one cell in each well is by

serial dilution. Assuming that all of the cells are able to grow, the initial number of cells follows a Poisson distribution, the parameter of which can be estimated from the proportion of wells in which no growth occurred.

From equation 1b, the distribution of the lag time can be estimated from the distribution of the detection times. However, as the initial cell number per well is random, the detection time distribution is a convolution of the distribution of the initial cell numbers, N_0 , and of the distribution of single-cell lag times.

In addition, the lag time calculation is very sensitive to the specific growth rate of the population. Indeed, as the detection level is around 10^7 cells per ml, approximately 24 generations are necessary to reach a measurable turbidity from one cell. Equation 1b is still valid if the cells are near the stationary phase at the detection level, but then the rate μ' to be used in the calculation would be smaller than the maximum specific rate, μ (Fig. 1). The specific growth rate is traditionally estimated by plate counts. Its relative accuracy (i.e., standard error of the estimate divided by the estimate itself) is about 5% under optimal growth conditions (21). Under less favorable conditions, the error increases significantly, to 10% or more. This is not sufficiently accurate to estimate lag time values from OD measurements. OD measurements can be converted into the logarithm of cell concentration via calibration curves (10), but in our experiments, we found this to be even less accurate than the plate count method and dependent on the environmental conditions.

Finally, to calculate the lag times from the detection times, the cell concentration at the detection level needs to be accurately known under all conditions.

In this paper, we present a method to estimate the kinetics parameters (including the growth rate) of single cells from the distribution of detection times assuming that the single-cell lag times follow a gamma distribution with a fixed shape parameter. The method takes into account the fact that the number of cells inoculated into each well is random, with an average ideally between 1 and 3. We then apply the new method to study the effect of acetic acid on the distribution of single-cell lag times.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *Listeria innocua*, strain NCTC 11288 (serotype 6a) was stored on trypticase soy agar (TSA; Oxoid) slopes at 3°C. A subculture in Trypticase soy broth (TSB; Oxoid) was incubated at 30°C for 24 h and inoculated into TSYGB (TSB plus 0.3% yeast extract plus 1% glucose), pH 7.0. This culture was incubated at 22°C for 24 h to late exponential phase or 48 h to stationary phase for inocula for the OD experiments.

Preparation of media. Acetic acid was added to TSYGB at concentrations of 500, 1,000, and 2,000 ppm. After addition of the acid, the pH was adjusted to 5.5 using 5 M NaOH. Broths were sterilized by filtration (Millipore). The acetic acid concentrations were selected so that the acid would cause some growth rate inhibition at pH 5.5 (equivalent to 1.3, 2.6, or 5.1 mM undissociated acid) but would also allow sufficient growth to be detected by turbidity measurements.

Population growth curve experiments. Growth curves were made in all of the acid concentrations tested. Growth media were prepared in 100-ml volumes, preincubated at 22°C, and then inoculated with an overnight culture of *L. innocua* to give approximately 1,000 cells per ml. Samples were taken at intervals depending on the acid concentration (five times a day for 0.05% acetic acid, two or three times a day for other concentrations), diluted (serial 10-fold dilutions) in maximum recovery diluent (MRD), and plated onto triplicate TSA plates. Plates were incubated at 30°C for 2 days.

OD calibration curves. Calibration curves were prepared for TSYGB, pH 5.5, with no acetic acid and with 2,000 ppm acetic acid. An overnight culture of *L. innocua* was diluted in the test medium to give around 10^6 cells per ml. A microwell plate was filled with 400 μ l of diluted culture per well and incubated in the Bioscreen at 22°C, and the OD was measured at a wavelength of 600 nm. Samples (100 μ l) were removed from wells into 9 ml MRD, further diluted, and plated onto TSA for viable counts. The OD of the well was recorded immediately before the sample was taken. OD values were plotted against viable count for each test medium to determine the cell concentration at the OD corresponding to the detection level, 0.11.

Optical density measurements. Exponential- and stationary-phase cultures were diluted to give approximately 20 cells per ml. The initial serial 10-fold dilutions were in MRD, but the final four dilutions were done in the test growth medium so that the inoculum would not change the pH and acid concentration in the wells. To obtain on average one cell per well, each of 200 wells was inoculated with 50 μ l of diluted culture. Wells were then filled with 350 μ l of the test growth medium. Microwell plates were incubated at 22°C in the Bioscreen for up to 2 weeks. Growth was determined by measuring the OD at 10-min intervals. From plots of OD against time, the detection time for each well (the time at which the OD reached 0.11) was calculated.

Estimation of the single-cell kinetics parameters. In the following, we assume that the detection level is in the exponential phase and that the specific growth rate, μ , is the same for all the wells ($i = 1 \dots W$).

The physiological state. To make the link between population and single-cell modeling, Baranyi has introduced the concept of the physiological state (3). For an inoculum consisting of N_0 cells, the physiological state is defined by

$$\alpha(N_0) = \exp[-\mu L_g(N_0)] \quad (2)$$

The $L_g(N_0)$ notation indicates that the lag time, L_g , depends on the initial number of cells per well, N_0 .

The physiological state variable has been shown to be a convenient variable as the physiological state of the culture is equal to the arithmetic mean of the individual physiological states so

$$\alpha(N_0) = \sum_{j=1}^{N_0} \alpha_j(1)/N_0 \quad (3)$$

Let S_α be the sum of the physiological state values of the cells in a well. From equation 1b and the definition of the physiological state (equations 2 and 3), we can calculate $S_\alpha(N_0)$ in each well:

$$S_\alpha(N_0) = N_{\text{det}} e^{-\mu T_{\text{det}}} = N_0 e^{-\mu L_g(N_0)} = \sum_{j=1}^{N_0} \alpha_j(1) \quad (4)$$

If we know μ and N_{det} , the expected value and variance of S_α can be estimated from the average and variance of the transformed T_{det} detection times obtained experimentally. The cell concentration N_{det} at the detection level in a well (OD = 0.11) was determined experimentally.

The gamma distribution assumption. We assume that the single-cell lag times, $L_g(1)$, follow a gamma distribution (16) with scale parameter θ and shape parameter β . $L_g(1)$ can be conceived as a sum of β -independent exponentially distributed intervals for subtasks to be carried out by a cell during the lag time, each having expected value θ . The mean and variance of $L_g(1)$ are $\beta\theta$ and $\beta\theta^2$, respectively.

It has been shown that with this assumption, the expected value and variance of $\alpha(1)$ can be calculated analytically:

$$E[\alpha(1)] = (1 + \mu\theta)^{-\beta}, \quad \text{Var}[\alpha(1)] = (1 + 2\mu\theta)^{-\beta} - (1 + \mu\theta)^{-2\beta} \quad (5)$$

The distribution of N_0 . The number of cells in a well follows a Poisson distribution, i.e.,

$$P_k = P(N_0 = k) = \exp(-\rho) \frac{\rho^k}{k!} \quad (k = 0, 1, \dots)$$

with the expected value and variance $E(N_0) = \text{Var}(N_0) = \rho$. The fraction of empty wells can be used to estimate ρ by

$$\hat{\rho} = -\ln(W_0/W) \quad (6)$$

where W_0 is the number of empty wells (Poisson distribution for $k = 0$) and W is the total number of wells (for example, for the Bioscreen, $W = 200$).

However, growth occurs only in a fraction of wells, and the number of cells per positive well follows a truncated Poisson distribution. Its mean, ρ^+ , is given by

$$E(N_0|N_0 > 0) = \rho^+ = \rho/(1 - e^{-\rho}) \quad (7)$$

and the variance is

$$\text{Var}(N_0|N_0 > 0) = \rho^+ - [\rho^+]^2 e^{-\rho} \quad (8)$$

The mean and variance of the truncated Poisson distribution were estimated following the above formulae.

Estimation of the kinetics parameters. Equation 4 is a sum with a random number of terms. Its expected value and variance can be calculated analytically (14):

$$E(S_\alpha) = E\left(\sum_{j=1}^{N_0} \alpha_j(1)\right) = E(N_0) \cdot E(\alpha(1)) \quad (9)$$

$$\text{Var}(S_\alpha) = \text{Var}\left(\sum_{j=1}^{N_0} \alpha_j(1)\right) = \text{Var}(N_0) \cdot E[\alpha(1)]^2 + E(N_0) \cdot \text{Var}[\alpha(1)] \quad (10)$$

By replacing the expected value and variance of N_0 and the expected value and variance of the individual physiological state by their expressions (equations 7 and 8, respectively), we arrive at

$$E(S_\alpha) = \rho^+ \cdot (1 + \mu\theta)^{-\beta} \quad (11)$$

and

$$\begin{aligned} \text{Var}(S_\alpha) = \text{Var}\left(\sum_{j=1}^{N_0} \alpha_j(1)\right) &= (\rho^+ - [\rho^+]^2 e^{-\rho})(1 + \mu\theta)^{-2\beta} \\ &+ \rho^+((1 + 2\mu\theta)^{-\beta} - (1 + \mu\theta)^{-2\beta}) \end{aligned}$$

After simplification:

$$\text{Var}(S_\alpha) = \text{Var}\left(\sum_{j=1}^{N_0} \alpha_j(1)\right) = \rho^+(1 + 2\mu\theta)^{-\beta} - [\rho^+]^2 e^{-\rho}(1 + \mu\theta)^{-2\beta} \quad (12)$$

From equations 6 and 7, the values of the average of the Poisson distribution, ρ , and from the truncated distributions, ρ^+ , can be estimated from the number of empty wells, so they are known. The average and variance of S_α , the sum of the physiological state values of the cells in a well, can be expressed as a function of the detection times on one hand (equation 4) and as a function of the parameters of the gamma distributions, β and θ , on the other hand (equations 11 and 12) if the growth rate, μ , is known (e.g., from plate count measurements). Hence, β and θ can be identified from those two equations.

If μ is unknown, then the system is underdetermined as there are three unknown variables and two equations linking them. In this case, we need to fix one of the gamma parameters. The shape parameter, β , expresses the number of subtasks to be carried out before the cell starts the process of division. If there is a major task that would dominantly determine the work carried out during the lag (such as recovery from a stress), then β can be expected to be smaller than when there is no such task (25). Because $1/\beta$ is equal to the coefficient of variation (CV) of the gamma distribution, this is equivalent to saying that the CV is higher for stressed cells. (In comparison to their average, the detection times are more spread.) When the cells have reached an exponential phase, the CV of the division time is 25%, corresponding to $\beta = 16$ (22). However, because we expect smaller values for higher acid concentrations, but we would like β to remain fixed, we chose $\beta = 4$, which is equivalent to a relative deviation of 50% for the single-cell lag time.

Data analysis. All of the data analysis was carried out in Excel Spreadsheets (Microsoft Excel 2002).

The log concentration-versus-time curves of the plate count experiments were fitted with the Baranyi model (5) with the DMFit program (www.ifr.ac.uk/Safety/DMfit/default.html).

Assuming that the shape parameter $\beta = 4$, the growth rate, μ , and the scale parameter of the distribution of the single-cell lag times, θ , were estimated as follows. (i) Assuming an initial value for the growth rate, μ , the average of S_α obtained experimentally from the detection times was estimated (equation 4). (ii) The value of the scale parameter, θ , was estimated from equation 11. (iii) The values of the growth rate, μ , and the scale parameter, θ , were adjusted with the Excel solver by minimizing the difference between the logarithm of the variance of S_α obtained with equation 4 and the logarithm of the variance of S_α obtained with equation 12.

FIG. 2. Natural logarithm of the specific growth rate as a function of undissociated acetic acid concentration. The rate estimated from the detection times assuming a gamma distribution (solid circles for experiments with an exponential inoculum and stars for experiments with a stationary inoculum) falls within the 95% confidence limit of the rates estimated by plate count (solid diamonds).

The lag time and specific growth rates obtained by plate counts were compared with those obtained from the optical density experiments.

RESULTS AND DISCUSSION

Estimation of the growth rate. The growth rates estimated by plate counts and from the Bioscreen experiments assuming a gamma distribution as a function of acetic acid concentration are shown in Fig. 2. The logarithm of the rate changed with the undissociated acid concentration as a linear function ($R^2 = 0.99$). The specific growth rates estimated from detection times by the new method are within the 95% confidence interval of the rates predicted by the model describing the effect of acid concentration, using plate count data.

Estimation of the single-cell lag times. The distributions of the single-cell lag times as well as the shifted detection times for the different acetic acid concentrations are shown in Fig. 3. The shifted detection times were calculated assuming that each well contains N_0 cells per well, the average of the truncated Poisson distribution parameter (equation 4), so they do not represent exactly the distribution of the single-cell lag times. The difference between the distributions is most obvious with higher average cell numbers per well: e.g., for the distributions with 2.1 mM of undissociated acid obtained with the inoculum in stationary phase, where the average number of cells per well was 3.6. Both the average and the spread of the single-cell lag times increased with acetic acid concentrations.

The scale parameter of the gamma distribution is shown in Fig. 4. The logarithm shows a linear trend with the concentration of undissociated acid. This linear relationship was similar for both the stationary and the exponential inocula.

Figure 5 shows the population lag times, λ , estimated from the equation of Baranyi and Pin (4)

$$\lambda = \frac{-\ln E[\alpha(1)]}{\mu} \quad (13)$$

compared to the population lag times obtained experimentally by plate count. The logarithm of the lag times also showed a linear trend as a function of undissociated acid ($R^2 = 0.88$). All

FIG. 3. Histograms of the lag times. The bars represent the shifted detection times (estimated with N_0 , the average cell numbers from the Poisson parameter), and the continuous lines represent the gamma distributions of lag times of single cells. On the left, the inoculum was in the exponential phase, and on the right, the inoculum was in the stationary phase. The acetic acid concentrations were, respectively, from top to bottom 0, 1.3, 2.6, and 5.1 mM undissociated acid.

of the lag times obtained from the Bioscreen detection times fall within the 95% confidence interval of the plate count experiments.

A new numerical method. The method presented in this article allows the estimation of both the growth rate and one parameter of the distribution or of the two parameters of the distribution of the single-cell lag times using an efficient experimental setup; the experiments do not need to be repeated to obtain sufficient data, and the readings are automated. The growth rate and the population lag times were shown to be in good agreement with estimates obtained by plate count, the reference in predictive microbiology.

To avoid having a random number of cells per well, it was proposed to dilute the inoculum further to obtain on average less than one cell per well to increase the chance of the growth in a well resulting from only one cell (31). However, this results in many wells being negative for growth and would produce insufficient data for robust statistical analysis, and the experiment would need to be repeated under the same conditions. Under unfavorable growth conditions, the repeatability of the

FIG. 4. Natural logarithm of the scale parameter of the gamma distribution (or time necessary for each task to be carried out as explained in the theory section) with a number of tasks equal to 4 (shape parameter) as a function of undissociated acid (solid circles for experiments with an exponential inoculum and stars for experiments with a stationary inoculum).

experiments can be poor (21). To overcome this problem, a “standardization procedure” of recentering the distributions of the detection times for each experiment has been proposed (13), but this results in more experimental work. In our experiments, the truncated Poisson distribution gave an average initial number of cells per well of between 1.8 and 3.6, corresponding to 146 to 194 of the wells out of 200 showing growth, so that two plates of 100 wells were sufficient to estimate the distribution of the single-cell lag times.

On the specific rate and the population lag. To estimate the single-cell lag times, the growth rate needs to be known very accurately. For instance, with the higher acetic acid concentration studied here (2,000 ppm, 5.1 mM of the undissociated form at pH 5.5), plate count experiments gave a rate of 0.11 h^{-1} . With such a rate, the average of the single-cell lag times calculated from the Bioscreen detection times would have been -1 h with many negative values. With the new method, we found a rate of about 0.15 h^{-1} , which gave an average for the lag times of 41 h (ignoring the initial random number distribution effect). The latter result is in agreement with experiments carried out by Le Marc et al., who found a specific growth rate of about 0.15 h^{-1} under the same conditions (18).

In the range of acetic acid concentrations studied, both the logarithm of the rate and population lag times were found to be a linear function of undissociated acid. Le Marc et al. (18) found a similar link on a wider range of undissociated acetic acid concentrations, but with the square root of the specific rate.

The population lag time was much more scattered around the trend. It is well known from predictive microbiology literature that the lag time is more variable and uncertain than the growth rate (26).

On the gamma distribution assumption. This method is based on the assumption that the lag times follow a gamma distribution. This was proposed by Baranyi and Pin (4) following theoretical considerations. It has the advantages of allowing for an interpretation and also of having an analytical solution to evaluate the average and standard deviation of the

FIG. 5. Natural logarithm of the population lag times as a function of undissociated acetic acid concentration. The lag times estimated from the detection times assuming a gamma distribution (solid circles for experiments with an exponential inoculum and stars for experiments with a stationary inoculum) all fall within the 95% confidence limit of the lag times estimated by plate count (solid diamonds).

corresponding physiological states. In addition, it has been shown that it is a generally good approximation for the lag times (16). So far, in practice, the gamma distribution has proved to be satisfactory under many conditions, although not always having the best fit (7, 8, 13, 21, 30, 32, 34). In this particular study, it proved to be an appropriate assumption. In these experiments, no shift was assumed, which means that it is possible that some cells divide without a lag. This might not always be the case, especially after processing treatments cause damage to the cell, which needs to be repaired before the cell can divide again (19). The shape parameter of the gamma distribution was fixed at 4 and remained an acceptable constant in the acid concentrations tested. However, this is known to be an oversimplification imposed by the fact that we have only two equations (the expected value and variance of S_{α}) for three variables. If the stress is greater or of a different nature (e.g., after a starvation period or where fundamental functions of the cells have been disturbed), the coefficient of variation would probably increase. In fact, when we used the specific rates measured by plate counts in our two equations, so the two gamma parameters (β and the θ) were to be estimated instead of μ and θ , then the β estimates decreased from around 10 to around 2, as the acetic acid reached its highest concentration tested. The coefficient of variation of the lag times of individual cells measured by different methods reported in the literature lies between 0.2 and 1 (7, 8, 11, 12, 22).

Numerical studies showed that the estimation for the specific rate is not very sensitive to the choice of β . This is because the gamma distribution converges to a normal distribution as β increases. However the fixed β value may explain the slight deviation between the growth rate estimated from the Bioscreen experiments and that from the plate count experiments (Fig. 2).

With this method, method of the moments, we do not exploit the whole distribution of the detection times, only the mean and variance of S_{α} . An alternative method which takes into account the shape of the distribution is the maximum likelihood method (15). While this method allows more accurate determination of the shape parameter, maximum likeli-

hood methods are computationally demanding; to find the “most probable” values of the gamma parameters, it is necessary to generate many random numbers iteratively.

The estimation procedure introduced here is an easy-to-use algorithm that can be readily applied in commonly used spreadsheets like Microsoft Excel. As emphasized throughout the paper, this algorithm is useful in a scenario where there are a few initial cells in a well (ideally, on average between 1 and 3). The chance of obtaining negative wells would be only 1 in 1,000 if the average cell number was between 5 and 10. With no negative wells, it would be impossible to estimate the distribution of the initial cells per well. When the average is lower than 1, there are many unusable negative replicates.

As predictive microbiology studies turn to single-cell kinetics, there is a demand for robust but simple numerical procedures to estimate the parameters using cost- and time-effective measurement methods. The procedure we have presented here is an example of response to such demands.

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