Growth Rates Made Easy

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Abstract

In the 1960s–1980s, determination of bacterial growth rates was an important tool in microbial genetics, biochemistry, molecular biology, and microbial physiology. The exciting technical developments of the 1990s and the 2000s eclipsed that tool; as a result, many investigators today lack experience with growth rate measurements. Recently, investigators in a number of areas have started to use measurements of bacterial growth rates for a variety of purposes. Those measurements have been greatly facilitated by the availability of microwell plate readers that permit the simultaneous measurements on up to 384 different cultures. Only the exponential (logarithmic) portions of the resulting growth curves are useful for determining growth rates, and manual determination of that portion and calculation of growth rates can be tedious for high-throughput purposes. Here, we introduce the program GrowthRates that uses plate reader output files to automatically determine the exponential portion of the curve and to automatically calculate the growth rate, the maximum culture density, and the duration of the growth lag phase. GrowthRates is freely available for Macintosh, Windows, and Linux.

We discuss the effects of culture volume, the classical bacterial growth curve, and the differences between determinations in rich media and minimal (mineral salts) media. This protocol covers calibration of the plate reader, growth of culture inocula for both rich and minimal media, and experimental setup. As a guide to reliability, we report typical day-to-day variation in growth rates and variation within experiments with respect to position of wells within the plates.

Key words: **growth rates, fitness, adaptation.**

Protocol

Background

Growth rates have long been used in microbiology to quantify phenotypic properties. Experimental evolution studies have often used growth rates as a measure of fitness (Hall 1978; Dykhuizen and Dean 1990). In this century, determination of growth rates fell out of common use as exciting, high-throughput molecular tools for characterizing bacteria were developed. Classical methods of growth rate determinations are both tedious and labor intensive. The introduction of automated microtiter plate readers has brought high-throughput analysis for growth rate measurements and has resulted in a renewed use of growth rate data in a variety of areas including regulation of gene expression (Swint-Kruse L, personal commununication) and diverse areas of microbiology including antibacterial activities of biological fluids (Feher et al. 2012), quantifying phenotypes in environmental studies (Warringer and Blomberg 2003), desulfurization of mucin by *Pseudomonas* in the lungs of cystic fibrosis patients (Robinson et al. 2012), inhibition of biofilm formation (de la Fuente-Nunez et al. 2012), and characterization of *E. coli* isolated from cystic fibrosis patients (Cremet et al. 2013). The application of most interest to evolutionary biologists is likely a measure of fitness, including fitness cost of streptomycin resistance (Paulander et al. 2009), the fitness cost of rifampicin resistance (Hall et al. 2011), the likelihood of adaptation to rapid environmental changes (Lindsey et al. 2013), and adaptive landscapes related to antibiotic resistance (ongoing study in the laboratory of M.B.).

Before the advent of plate readers, cultures were grown in Erlenmeyer flasks or tubes, in constant temperature rooms or in water baths, and on shakers or rotating drums to provide sufficient aeration. At intervals of a few minutes, a sample would be sterilely removed, transferred to a cuvette in a spectrophotometer, and the apparent absorption (optical density [OD]) of the culture would be measured and recorded. Typically, the OD versus time points would be plotted on a semilog graph paper, and those points that appeared to fall along a straight line would be used to calculate the growth rate. At best, one could measure the growth rates of about 20 cultures at a time, and those measurements would require the full-time attention of an investigator over a period of most of the day. Following the collection of data, calculation of the growth rates—whether manually or by entering all the data into a computer—was tedious and required extra time.

Now, the investigator inoculates cultures into wells in a microtiter plate, puts the plate into a reader, enters a few commands, and returns a few hours later to find all of the data neatly recorded in an electronic file. In the meantime, the plate reader has measured the OD in each of the wells at frequent intervals. All that remains is to use that data to

obtain the desired growth rates. It is at this final stage that problems begin to arise, and many investigators are unsure how to calculate growth rates and unsure which of the time points should be used. A new program, GrowthRates, that automatically calculates growth rates from the correct subset of points is now freely available for Macintosh OS X, Windows, and Linux platforms at http://bellinghamresearchinstitute.com/software/index.html (last accessed October 21, 2013). Effective use of that program, however, requires understanding the important aspects and limitations of growth rate measurements.

Alternative Resources for Calculating Growth Rates When Using Microtiter Plate Readers

This protocol and our program GrowthRates are intended for laboratories that use standard plate readers such as the BioTek and Spectra-Max readers. There are dedicated instruments such as the Bioscreen reader that are designed specifically for measuring growth rates. Those considerably more expensive instruments incorporate their own proprietary software.

Others have discussed the issue of analyzing the growth data from microtiter plate readers and have written programs to facilitate that analysis (Holowachuk et al. 2003; Novak et al. 2009), but those programs do not appear to be publicly available. An approach similar to ours was described (Breidt et al. 1994), but the program no longer appears to be available. An approach that uses a Microsoft Excel spreadsheet with an embedded macro has been described (Warringer and Blomberg 2003) and is available upon request to the authors. This approach includes the nonlinear portion of the ln(OD) versus time curve and attempts to correct for the nonlinear relationship between ln(OD) and cell number at high cell densities. This correction, however, will depend upon the geometry of the detector and on the particle size and so may not be applicable to different instruments or to larger microorganisms such as yeast.

None of the earlier articles, however, provides an explicit, step-by-step protocol for determination of bacterial growth rates.

Toussaint and Conconi (2006) provide an explicit protocol for measuring growth rates in yeast; however, much of that protocol is very specific to yeast and is less generally applicable than is the protocol mentioned later. They describe an algorithm to calculate growth rates and lag times, but details of the basis of calculating growth rates and lag times are not provided. A perl script to implement their algorithm is available upon request, but the utility of that script depends on knowing how to use perl, whereas the compiled program GrowthRates requires no special computer skills.

What Is Actually Being Measured?

The purpose of a growth rate measurement is to determine the rate of change in the number of cells in a culture per unit time. This requires estimating the cell density at a series of time points. Whether done by a modern plate reader or by the classical shake flask and spectrophotometer approach, the number of cells per milliliter of culture is estimated from the turbidity of the culture that is measured by the plate reader or spectrophotometer and is estimated as OD. Ideally, the actual culture density (cells/ml) can be inferred directly from OD.

Spectrophotometers measure the fraction of light that is absorbed by a solution and report that as absorbance units. The absorption of a solution depends on the wavelength of the light, so the typical unit is reported as A_{600}, where the subscript indicates the wavelength in nanometers and A is the negative log of transmittance (the fraction of incident light that is detected). Beer's law tells us that A is proportional to the concentration of a solution. However, a bacterial culture is not a solution; it is a suspension of bacterial cell particles. Light is not as much absorbed as it is scattered, and there is only a limited range over which the measured absorbance is proportional to the number of cells per milliliter. For that reason, it is preferable to refer to OD rather than to absorbance.

Typically, OD is determined at a wavelength of 600 nm, in which case, we measure OD_{600}. If we want to infer the true culture density from OD_{600}, it is essential to determine the range over which OD measurements are directly proportional to culture density. For most purposes, however, the absolute culture density is unimportant.

Volume of Culture in the Wells of a Plate Reader Matters

When OD is measured in a classical spectrophotometer, the sample is put into a cuvette with a fixed light path, typically 1.0 cm. When OD is measured in a plate reader, the length of the light path within which light scattering occurs is determined by the volume in the well. Thus, the same culture that gives an OD of 0.2 for a 150 µl sample will give an OD of 0.4 for a 300 µl sample. Typically, any volume between 100 and 300 µl can be used, but it is important to use a consistent and accurate culture volume in the wells of a plate reader, especially if you want to infer cells/ml from OD.

The Growth Curve

Ideally, bacterial cultures grow exponentially and the OD increases as a function of ln(OD), not of OD itself. The growth rate is the change in the number of cells per minute, which we estimate as the change in OD per minute. But the instantaneous change is a function of the number of cells that are present at any given moment:

$$\frac{dN}{dt} = \alpha N, \quad (1)$$

where N is the number of cells at time t and α is the first-order growth rate constant; that is, the growth rate. α is in reciprocal time units, so if t is in minutes, the growth rate is α min^{-1}.

$$\frac{dN}{N} = \alpha t, \quad (2)$$

When that is integrated from $t = 0$ to $t = t$, the relationship is

$$\ln \frac{N_t}{N_0} = \alpha(t - t_0), \quad (3)$$

and α is the slope of ln N versus t. In practice, we use α = the slope of ln OD versus t.

Figure 1 shows a typical growth curve. A lag phase where growth is absent is followed by an acceleration phase during which the growth rate increases until a constant growth rate is achieved during the exponential phase. Eventually, the growth rate begins to decline during the deceleration phase and eventually growth ceases due to resource exhaustion and/or waste accumulation during the saturation phase. Most of the time, we are only interested in the growth rate during the exponential phase, which is the rate reported by the GrowthRates program. In some cases, we may also be interested in the maximum OD that is reached or in the duration of the lag phase. GrowthRates reports both of those parameters, but it reports the lag phase by extrapolating the slope of the exponential phase back to the initial OD; that is, it treats the lag as though no growth occurred until the end of the lag phase at which point growth becomes instantly exponential. Although unrealistic, this approach allows comparisons of relative lag times without having to account for details of the acceleration phase.

Protocol

The plate reader should be capable of maintaining a constant temperature and should be able to shake the plate between readings. The software should permit exporting the results either as a text (ASCII) or as a Microsoft Excel file.

Step 1. Calibrate the Plate Reader

Step 1 only needs to be done once, but it is important to calibrate your system.

Step 1.1. Determine the relationship between OD and cells/ml. This step is needed only if it is important to relate OD to absolute cell density. If you use both rich media (typically yellow or brown in color) and minimal media (typically colorless), this step should be performed for cultures in both media.

Step 1.1.1. Grow a culture to midexponential phase to ensure maximum cell viability.

Step 1.1.2. Concentrate the cells 10× by centrifugation.

Step 1.1.3. Make a serial 1.25× dilution series from the concentrated cells, making 20 dilutions. This covers an almost 100 × range of culture densities. However, 1.25× dilutions give a finer scale than traditional 2× serial dilutions.

Step 1.1.4. Using a well that contains the same volume and medium as you will subsequently use in all experiments, set the absorbance to read zero.

Step 1.1.5. Determine the OD of each dilution in the plate reader using the same volume as you will subsequently use in all experiments. This requires only one reading, not a time series.

Step 1.1.6. Determine the number of cells/ml in each dilution by serial dilution and plating.

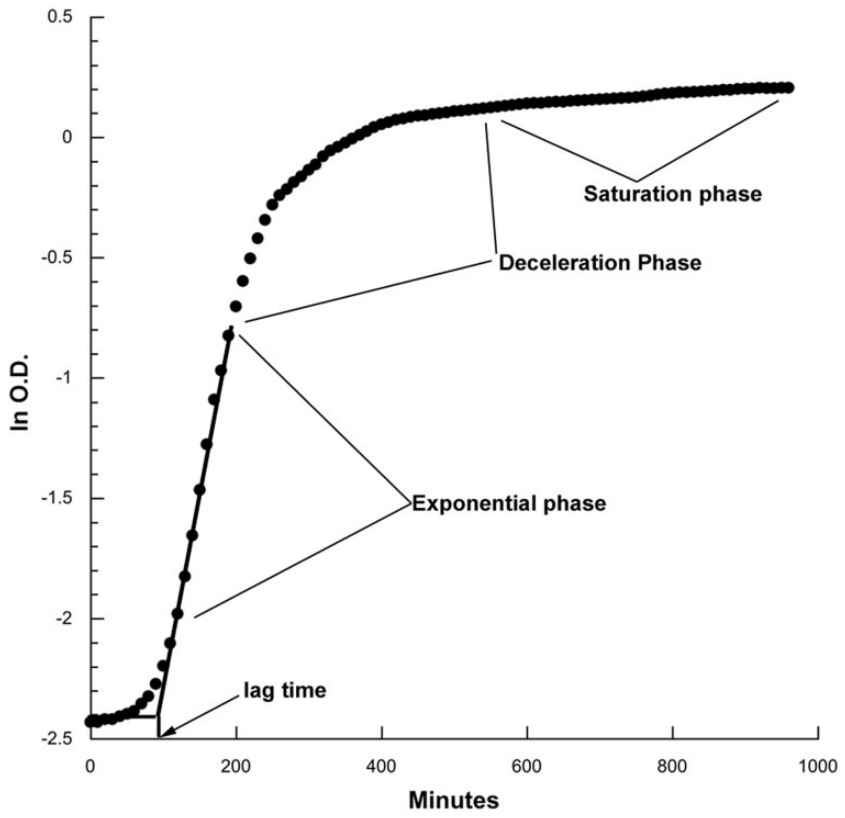

FIG. 1. Typical bacterial growth curve: ln OD is plotted versus time. The points that fall along the straight line represent the exponential growth phase. Here, the lag time is shown as the time to enter exponential phase and really includes the time during which growth accelerates.

Step 1.1.7. Plot cells/ml versus OD so that you can infer culture density from OD.

Step 1.2. Determine the reproducibility of growth rates across wells. Some people have observed that cultures in the outer (edge) wells grow slightly faster than those in the inner (core) wells. This property may depend upon the particular plate reader, the volumes in the wells, etc, but it is important to know about the uniformity across the plates in your setup. If you find significant differences between edge and core wells, you may not want to use the edge wells.

Inoculate identical cultures into all wells (except a control well that contain uninoculated medium) and determine the growth rates. Repeating the experiment on three different days provides a good measure of the variation with respect to well position as well as a good measure of the day-to-day reproducibility of absolute growth rates.

Step 2. Grow Cultures to Use As Inocula for Growth Rate Experiments

The duration of the lag phase and the shape of the curve during the acceleration phase depend on the physiological state of the cells in the inoculum, the fraction of viable cells in the inoculum, and the handling of the inoculum cultures. Especially if you intend to monitor and interpret the duration of the lag phase, it is important to grow the inoculum cultures consistently. Inocula should be grown at the same temperature as the temperature at which the growth rate will be measured.

Step 2.1. Rich medium, overnight cultures. Inoculate rich medium (LB, Mueller-Hinton broth, 2xYT [all available from Difco] or other undefined medium) and grow the culture overnight to saturation. This approach can result in a high proportion of inviable cells and viable cells that are in a physiological state that requires considerable adaptation before the culture starts to grow. The result tend to be long with variable lag times.

Step 2.2. Rich medium, overnight oxygen-limited cultures. Inoculate 10 ml of rich medium in a tightly sealed 15 ml centrifuge tube. Allow the culture to stand overnight without shaking. The culture density will be limited by the available dissolved oxygen, and as a result, there will be fewer dead cells, and viable cells will be in a physiological state that allows rapid resumption of growth. The result is shorter and more consistent lag times.

Step 2.3. Minimal medium, saturated overnight cultures. Minimal, or defined, media such as M9 (Difco) include only the resources required for the particular strain to grow. Typically, this means a buffered solution containing a carbon source, a nitrogen source, inorganic phosphorus, trace elements, and any required amino acids, nucleosides, and so forth. Undefined supplements, such as yeast extract, tryptone, casamino acids, and so forth, are not included in minimal media. If those resources are provided in excess, cultures will grow to saturation. For members of the *Enterobacteriaceae*, excess carbon means $\geq$0.2% w/v carbon source. Care should be taken, when possible, to avoid dramatic shifts in carbon sources to avoid diauxic growth due to catabolite repression. For instance, *E. coli* inocula grown in glucose will experience a longer lag when shifted to glycerol or lactose medium than will cells grown in the poorer carbon sources glycerol or succinate. As with rich media, saturated cultures will result in longer, more variable lag times.

Step 2.4. Minimal medium, nutrient-limited cultures. A growth-limiting concentration of the carbon source or of a required amino acid produces a culture that stops growing abruptly in early exponential phase rather than reaching saturation phase. The abrupt cessation of growth prevents many of the physiological changes associated with saturation phase and allow rapid resumption of exponential growth upon restoration of an excess of the limiting nutrient. For members of the *Enterobacteriaceae*, limiting carbon means 0.01% (w/v) glucose or 0.01% (v/v) glycerol concentrations that limit cultures to about 10^8 cells/ml. Nutrient-limited inocula results in short, reproducible lag periods.

Step 3. Do the Growth Experiment

Step 3.1. Program the plate reader. Follow the plate reader manufacturer's instructions for programming. Four critical parameters will certainly need to be set:

1) Temperature.
2) Shaking. Shaking serves two purposes: 1) to ensure that the cells are uniformly suspended before the OD reading and 2) to keep the culture aerated and thus provide adequate oxygen for growth. At a minimum, you need to ensure that the plate is shaken just before each reading, and you probably want to provide continuous shaking to maintain good aeration. The decision to shake continuously will depend upon your experimental design, the organism, the nutrient source, and so forth.
3) Reading interval. The time interval between readings should be set so that at least six readings are made during the doubling of the OD of the fastest growing wells. This will require some preliminary data, but it is better to read too frequently than to read too infrequently. Reading slow-growing cultures more frequently just results in more data points, which is not a problem when using the GrowthRates program to analyze the results.
4) Run time. Again, experience will dictate the total run time that is needed, but it is usually wise to run long enough that all cultures will have reached a plateau in OD.

Step 3.2. Zero the plate reader. Before starting the run, it is essential to zero the absorbance reading using a blank well. A blank well is not an empty well. Instead, it is a well that contains the same volume of the same culture medium as the experimental wells. Zeroing the absorbance with a blank well ensures that the ODs that are reported have any contribution from the medium subtracted. For some long experiments where there is the possibility of instrument drift over time, GrowthRates offers the option of including a blank well during the experiments and subtracting the reading in that blank well from the readings in experimental wells.

Step 3.3. Inoculate the cultures in the plate wells. Determine the OD of each inoculum by reading the same volume that you will use in growth rate determinations. Based on that OD, dilute the inoculum to a calculated OD roughly between 0.05 and 0.1 in fresh growth medium. Check the ODs of the diluted cultures.

An initial OD between 0.05 and 0.1 is not critical, but we have found that it generally works well. If the initial OD is too high and the cells grow quickly, there may be an insufficient number of readings before the culture reaches the point where OD becomes nonlinear with respect to cell number, or growth rate actually begins to decline as the culture starts to enter stationary phase. During initial calibration of the instrument, the threshold above which OD is proportional to cell number should have been determined. The initial OD should always be above that threshold.

Step 3.4. Distribute the diluted cultures to wells and run the experiment.

Step 4. Analyze the Results with GrowthRates

The GrowthRates package for Windows, Mac, and Linux platforms reduces the time needed to calculate growth rates from the plate reader output from hours to seconds.

At the end of the experiment, the plate reader software will save the results in an electronic file. Typically, that file can be exported in a variety of formats to accommodate downstream analysis. The program GrowthRates requires an input file in a very specific format (see fig. 2 and step 4.1 below). The input file is a tab-delimited text file. The plate reader may be able to export the data as a tab-delimited text file that can be edited manually to the correct format, but this manual editing is likely to be tedious. Most plate reader software can also export the file as a Microsoft Excel spread sheet.

Time	A1	A2	A3	A4
0	0.088	0.088	0.088	0.088
10	0.088	0.088	0.088	0.087
20	0.088	0.088	0.089	0.088
30	0.089	0.089	0.089	0.088
40	0.089	0.089	0.09	0.089
50	0.091	0.09	0.091	0.089
60	0.092	0.092	0.092	0.091
70	0.095	0.095	0.095	0.093
80	0.098	0.098	0.098	0.096
90	0.104	0.103	0.103	0.101

FIG. 2. Part of a correctly formatted input file for GrowthRates.

Step 4.1. Format the output for input into GrowthRates. The program FormatGR, which is included in the GrowthRates package, formats exported Excel files correctly for input into GrowthRates and requires virtually no effort by the investigator.

The input file for GrowthRates is a tab-delimited text file. Files written for word processing programs, such as Word by Word or WordPerfect, will not work. Notice (fig. 2) that times must be given in minutes, not in hours:minutes:seconds.

Step 4.1.1. Export the Excel file as tab-delimited text. From Excel's File menu choose Save as . . . and in the resulting dialog set Format to tab-delimited text (.txt).

Step 4.1.2. Run FormatGR as described in Appendix II of the GrowthRates documentation. It will write a correctly text file that includes the word "_formatted"; that is, if the text file exported by Excel was named "MyRun.txt," the correctly formatted file will be named "MyRun_formatted.txt." At this time, FormatGR only formats the output from BioTek and VersaMax plate readers. Readers who use other plate readers are asked to contact BGH (barryghall@gmail.com) so that FormatGR can be updated to include their machines.

Step 4.2. Run GrowthRates as described in its documentation, using the MyRun_formatted.txt file as the input. GrowthRates write two output files, both with the same name as the input file but with different extensions.

MyRun_formatted.results provides a description of the growth in each well as shown in figure 3.

MyRun_formatted.summary lists the growth rate, lag time, and maximum OD for each well in a way that makes it easy to paste that information into a spreadsheet, graphing, or statistics program for further analysis (fig. 4).

Well	Growth Rate	Max OD	lag time (minutes)
A1	0.01756	0.993	97.2
A2	0.01803	0.996	98.7
A3	0.01797	0.993	96.0
A4	0.01774	0.998	101.4
A5	0.01704	0.990	97.6
A6	0.01679	0.995	101.4
A7	0.01740	0.999	97.5
A8	0.01767	0.987	97.3
A9	0.01779	0.992	97.6
A10	0.01772	0.983	95.3
A11	0.01771	0.985	98.1
A12	0.01844	0.988	102.8

FIG. 4. Part of a .summary file.

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*******************************************************
          Well A1
6 points from 130 through 180 minutes were used to estimate the growth rate.
The growth rate +/- s.e. is 0.01756 +/-0.000475 per minute.
This is equivalent to a doubling time of 39.5 +/-1.07 minutes.
The correlation coefficient R is 0.99854
The maximum OD is 0.993
The lag time is 97.2 minutes
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FIG. 3. Description of growth in one well from a.esults file.

Table 1. Growth Rates in Edge versus Core Wells.

	Mean Growth Rate ± 95% Confidence Limit		
	All Wells	Core Wells	Edge Wells
Run 1	$0.00499 \pm 4.1 \times 10^{-5}\,min^{-1}$	$0.00497 \pm 5.0 \times 10^{-5}\,min^{-1}$	$0.00509 \pm 3.9 \times 10^{-5}\,min^{-1}$
Run 2	$0.00689 \pm 3.5 \times 10^{-5}\,min^{-1}$	$0.00691 \pm 3.9 \times 10^{-5}\,min^{-1}$	$0.00683 \pm 9.8 \times 10^{-5}\,min^{-1}$
Run 3	$0.00674 \pm 4.5 \times 10^{-5}\,min^{-1}$	$0.00675 \pm 5.4 \times 10^{-5}\,min^{-1}$	$0.00667 \pm 5.4 \times 10^{-5}\,min^{-1}$

Discussion

GrowthRates first converts OD values to ln OD. Starting at time zero, it considers a window of five time points (points 1–5) and calculates the slope of ln OD versus time and saves that value. It then moves one time point and considers the next window of five (points 2–6) and saves the slope. After it has calculated all possible five-point slopes, it uses the time points associated with slopes that are at least 95% of the maximum slope to determine the range of the points that are used to determine the final growth rate. The principle is that as points begin to fall away from the exponential growth line (fig. 1), the slopes over the five-point window begin to decrease below the maximum. The decrease in the estimated growth rate may result either from an actual decrease in rate or from nonlinearity of OD with respect to cell number at higher ODs.

The growth rate is reported as the first-order growth rate constant in units of $minutes^{-1}$. For those that are more comfortable thinking in doubling times, it is also reported as the doubling time. Growth rates are estimates based on at least five points, but as is always the case, interpreting growth rates requires some judgment. For instance, if there is no growth or if turbidity slowly decreases due to some cell lysis, random fluctuations in OD may lead to extremely slow or even negative growth rates. When that happens, there is usually a large standard error on the estimated rate and the correlation coefficient is low. Large standard error and low correlation coefficient can also result from linear, rather than exponential, growth. If the standard error is $>3\%$ of the growth or if the correlation coefficient is <0.995, the results may well not be reliable. A graphing program should be used to plot ln OD versus time, and the data should be examined visually.

The lag time is estimated by extrapolating the linear portion of ln OD versus time plot back to the initial OD as shown in figure 1.

Reproducibility

There has been some concern about systematic variation in growth rates resulting from the position of the wells in the plate; particularly, those wells at the edges of the plate. There is also concern about day-to-day variation in estimates. We estimated growth rates in all 384 wells using the same strain in identical media and repeated the experiment for 3 days using independent inocula. Table 1 compares the growth rates in "edge" wells with those in "core" wells and with the overall growth rate in all wells.

By *T*-tests, the only significant differences are from Run 1: the edge wells grow significantly faster than the core well or all wells. There are no significant differences for Runs 2 or 3. The growth rates in Run 1 were significantly lower than in Runs 2 or 3, while Run 2 did not differ significantly from Run 3.

These results do not suggest a reason to avoid using the edge wells, but there is a good reason to replicate experiments on different days. Growth rates can be estimated to be about ±2.5% using 6–12 replicate wells for each condition. We emphasize that our findings may not apply to all microtiter plate readers and all conditions (ambient temperature, effectiveness of temperature control, etc.) A similar set of experiments should be carried out by the user as part of the process of initial familiarization and calibration of the plate reader.

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