

1 **Overcoming the Anaerobic Hurdle in Phenotypic Microarrays: Generation and**
2 **Visualization of Growth Curve Data for *Desulfovibrio vulgaris* Hildenborough**

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1 **Abstract**

2 Growing anaerobic microorganisms in phenotypic microarrays (PM) and 96-well microtiter
3 plates is an emerging technology that allows a high throughput survey of the growth and
4 physiology and/or phenotype of cultivable microorganisms. For non-model bacteria, a swift
5 method for phenotypic analysis is invaluable, not only to serve as a starting point for further
6 evaluation, but also to provide a broad understanding of the physiology of an uncharacterized
7 wild-type organism or the physiology/phenotype of a newly created mutant of that organism.
8 Given recent advances in genetic characterization and targeted mutations to elucidate genetic
9 networks and metabolic pathways, high-throughput methods for determining phenotypic
10 differences are essential. Here we outline challenges presented in studying the physiology
11 and phenotype of a sulfate-reducing anaerobic delta proteobacterium, *Desulfovibrio vulgaris*
12 Hildenborough. Modifications of the commercially available OmniLog™ system (Hayward,
13 CA) for experimental setup, and configuration, as well as considerations in PM data analysis
14 are presented. Also highlighted here is data viewing software that enables users to view and
15 compare multiple PM data sets. The PM method promises to be a valuable strategy in our
16 systems biology approach to *D. vulgaris* studies and is readily applicable to other anaerobic
17 and aerobic bacteria.

18

1 **1.0 Introduction**

2 *Desulfovibrio vulgaris* Hildenborough, a sulfate-reducing anaerobic, delta
3 proteobacterium, has been identified as a model organism in many types of sulfate-reducing
4 environments, especially those related to metal-contaminated sites (Caumette 1993; Telang,
5 Voordouw et al. 1994; Noguera, Brusseau et al. 1998; Cottrell and Cary 1999; Wind, Stubner
6 et al. 1999; Heidelberg, Seshadri et al. 2004). Detailed studies on growth and metabolism of
7 this organism are necessary to understand metal reduction processes under a variety of
8 environmental conditions. Conducting a growth curve to determine lag time, growth rate, and
9 maximum cell densities often is necessary to understand the physiology or phenotype and the
10 specific effect of environmental stressors on the organism. Culturing in batch cultures has the
11 disadvantage of large amounts of media with single or multiple component differences, of
12 large numbers of tubes or flasks, and of the inconvenience of manually monitoring optical
13 densities of large numbers of cultures. In addition, growth times often are difficult to predict
14 and key experimental data may be lost if sampling is not sufficiently frequent (Sani, Peyton et
15 al. 2003). Increased convenience and quantity of the data can be obtained by automation of
16 the growth curve measurements in 96-well microtiter plates (hereafter referred to as plates).
17 After preparation, plates can be incubated and growth monitored by recording opacity changes
18 by automation at discrete intervals, continuously over several days. The opacity response was
19 recorded as a positive integer which we have named OmniLogTM units (OL units) which were
20 calibrated to standard microbiological techniques. The high throughput 96-well plate format
21 also facilitates replication so that growth from either technical or biological replicates can be
22 compared simultaneously on a single plate.

23 Biolog (Hayward, CA) has developed a microarray for rapid characterization of an
24 organism based on phenotypic response to substrate utilization. The instrument, called the
25 OmniLogTM (OL), is designed to simultaneously measure phenotypic responses from a

1 standardized inoculum to almost 2000 substrates, grouped into arrays. The Phenotype
2 MicroArray (PM) panels, designed for use in the OL, consist of twenty pre-prepared 96-well
3 plates that include substrates to test carbon, nitrogen, sulfate and phosphate utilization, as well
4 as ionic, osmotic, pH and chemical sensitivity assays. In addition to the PM plate assays, the
5 OL can also be used to visualize growth in standard empty plates with user-defined media
6 components.

7 The focus of this study was the development of methods for surveying and visualizing
8 the growth of the anaerobic sulfate reducing bacteria, *D. vulgaris*, using the OmnilogTM
9 workflow. While an evaluation of the complete phenotype of *D. vulgaris* is beyond the scope
10 of this study, phenotypic data recorded using these methods is included in the supplementary
11 data.

12

13 **Previous Work: Anaerobic Growth on Microtiter plates**

14 Microtiter plate readers can rapidly measure turbidity or color development in the
15 wells of a plate, and most instruments can both incubate plates and log the data. Typical
16 growth using plates is quantified by measuring absorbance of a tetrazolium dye at a specific
17 wavelength or by pixel intensity measurement from a flat bed scanner or camera system
18 (Bhupathiraju, Hernandez et al. 1999; Gabrielson, Hart et al. 2002; Bochner 2003). Originally
19 developed for aerobic microbes, it was recognized that with appropriate modifications, that
20 this platform would be valuable in the characterization of sulfate reducers and other anaerobic
21 bacteria. Microbes that are pathogenic or virulent to human, animal stock or agriculture are
22 historically the most well studied anaerobic bacteria. These and clinical variants of these
23 strains constitute a critical group of microbes that continue to be studied. Recent research
24 emphasis on environmental anaerobic microbial systems has further broadened the interest in
25 rapid phenotypic profiling of bacteria found in the environment. Examples include

1 *Clostridium spp* for biofuels research (Demain, Newcomb et al. 2005), *Dehalobacter sp* and
2 *Dehalococcoides spp* for bioremediation (Grostern and Edwards 2006), and sulfate- and
3 metal-reducing bacteria such as *Desulfovibrio spp* and *Geobacter spp* for heavy metal
4 biocontainment (Chang and Kim 2007; Martinez, Beazley et al. 2007; Neculita, Zagury et al.
5 2007; Yi, Tan et al. 2007).

6 Previous researchers have adapted the plate method for studying facultative anaerobes.
7 In a study on growth inhibition, *Salmonella enteritidis*, *Escherichia coli*, and *Paracoccus*
8 *denitrificans* were grown on plates in a variety of media under anaerobic conditions (Brewster
9 2003; Koutny and Zaoralkova 2005), The lids were sealed onto plates with silicone and then
10 flushed with nitrogen to remove oxygen. Problems with condensation during incubation were
11 overcome with the use of anti-fogging agents on the lids. This study observed anaerobic
12 growth by measuring turbidity on a microplate reader at 550 nm which was calibrated with
13 direct cell counts. Other toxicity tests of facultative anaerobes in plates, also with the silicon
14 sealant method, were used to observed growth of natural and engineered luminescent bacteria
15 *Vibrio fischeri* and *Pseudomana putida* (Schmitz, Eisentrager et al. 1999; Gellert 2000) for
16 determination of 50% and 20% inhibitory concentrations. The authors of these studies
17 concluded that to calculate toxicity as measured by the assay, both integral (area under the
18 curve) calculation and endpoint calculation methods can demonstrate different toxicity levels.
19 Schmitz et al. (1999) also proposed using kinetic measurements rather than the maximum
20 absorbance value obtained from the growth curve for calculation of growth inhibition because
21 some compounds increased the absolute values, due to abiotic or background absorbance
22 effects, but not the rate of growth. To determine Minimum Inhibitory Concentrations (MIC),
23 dose-response relationships were developed by plotting growth retardation vs. log
24 concentration using the intercept to compare retardation amounts. This method was shown to
25 be effective in quantifying differences in growth (Schmitz, Kretkowski et al. 1999) and was

1 useful for interpreting data in which the toxic compounds being tested affected turbidity
2 thereby making lag time, maximum optical density (OD), and growth rate difficult to
3 quantify. It was observed that for some tested compounds abiotic reactions in the medium
4 may increase or decrease background, so care must be taken in comparing growth curves in
5 different media compositions.

6 During *D. vulgaris* growth, sulfate is reduced to hydrogen sulfide, which reacts with
7 metals, mainly Fe^{+2} , in defined lactate sulfate medium, forming black metal/iron sulfide
8 precipitates (Postgate and Campbell 1966). The increased opacity, caused by increase in cell
9 numbers and precipitation of metal sulfides, can be used in most growth conditions as an
10 analog for growth. Use of tetrazolium dyes have been successful with some anaerobic systems
11 (Bhupathiraju, Hernandez et al. 1999); however, abiotic reduction of the tetrazolium salt
12 occurs in the lactate sulfate medium when the reducing agent (titanium citrate) is added to
13 reduce the medium, making the use of these dyes ineffective.

14 In this paper, techniques are described for observing anaerobic growth of the sulfate-
15 reducing bacterium, *D. vulgaris*, in PM plates using increased opacity as a growth indicator.
16 The development of PM technology for the characterization of this anaerobic sulfate-reducing
17 bacterium was made possible through chemical, engineering, and mechanical modifications to
18 the standard PM protocol (Biolog, Hayward, CA) and represents a novel and important new
19 tool for screening of anaerobic bacterial phenotypes. Described here are methods for
20 preparation of cells for general growth curve measurement, as well as stress and media
21 component testing along with precautions taken during interpretation of PM results. Key to
22 analysis and interpretation of data is processing and visualization. Included in this paper is
23 the description of a Web-based software package for visualizing growth curves. These
24 software tools allow for more complete understanding of PM data and ease viewing and
25 sharing of large data sets with collaborators.

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2.0 Materials and Methods

A schematic of the sample preparation techniques is shown in Figure 1. *D. vulgaris* was grown from a -80°C preserved stock culture (ATCC 29579, Manassas, VA) in a defined lactate-sulfate medium (LS4D). LS4D medium contains 50 mM NaSO₄, 60 mM sodium lactate, 8 mM MgCl, 20 mM NH₄Cl, 2.2 mM KPO₄, 0.6 mM CaCl₂, 30 mM PIPES buffer, 0.016 Resazurin, 10 mM NaOH, 1ml/L Thauers vitamins, 12.5 ml/L trace minerals, and 5ml/L titanium citrate. The trace minerals stock contains 50 mM nitrilotriacetic acid, 5 mM FeCl₂*4H₂O, 2.5 mM MnCl₂*4H₂O, 1.3 mM CoCl₂*6H₂O, 1.5 mM ZnCl₂, 210 μM Na₂MoO₄*4H₂O, 320 μM H₃BO₃, 380 μM NiSO₄*6H₂O, 10 μM CuCl₂*2H₂O, 30 μM Na₂SeO₃, and 20 μM Na₂WO₄*2H₂O. The Thauers vitamins stock contains 82 μM d-biotin, 45 μM folic acid, 490 μM pyridoxine hydrochloride, 150 μM thiamine hydrochloride, riboflavin, 410 μM nicotinic acid, 210 μM pantothenic acid, 310 μM p-aminobenzoic acid, 240 μM thioctic acid (lipoic acid), 14 μM choline chloride, and 7.4 μM vitamin B12 (Mukhopadhyay, He et al. 2006).

D. vulgaris was grown at 30°C in an anaerobic atmosphere consisting of 5% CO₂, 5% H₂, balance N₂ (Airgas, Concord, CA) to mid-log phase. Cell inoculum was standardized to ensure repeatable conditions between experiments. Mid-log phase cells, with density of 1 x 10⁸ cells/ml, were pelleted at 6000 g for 15 minutes. After removal of the supernatant, the pellet was resuspended and homogenized by pipetting into plate-specific medium (see Table 1 and description below) to achieve a final concentration of 1 x 10⁷ cells/ml, corresponding to approximately 10 OL units. The resuspended cells were then pipetted onto the 96-well plates at a volume of 100 μl /well. Plates used with both OL PM arrays and user-prepared media are standard, clear, half area plates with flat bottoms and are designed to hold 100 μL volume per well.

1 Engineering modifications were made for the anaerobic incubation of the inoculated
2 plates. When the sealant method was attempted, poor or no growth was observed in the wells
3 closest to the edge of the plate indicating oxygen leakage or potentially toxic effects from the
4 sealant. Instead, prior to removal from the anaerobic chamber, inoculated plates were placed
5 into 18 oz Whirl-Pak® Long-Term Sample Retention Bags (Nasco, Fort Atkinson,
6 Wisconsin) and the open end was sealed with a 2 mm wide heat sealer (American International
7 Electric, Newport News, Virginia). The Nasco bags are 2.5 μm thick and have a reported
8 oxygen permeability of 0.125 ml/645 cm^2 /24 h and a water vapor transmission rate of 0.48
9 gms/645 cm^2 /24 h at 22.8°C (information provided by manufacturer). After sealing, the bag
10 surface area is 284 cm^2 (including the top and bottom surfaces) and volume is 180 cm^3 . With
11 an incubation time of 60 hours and this bag geometry, at 22.8°C the calculated water loss
12 would be 0.528 g and the oxygen entering the bag would be 0.1375 ml, or 5.5% of the initial
13 volume lost and a final oxygen concentration of 0.076%. While this is relatively minor, losses
14 at higher temperatures or longer incubation times may be significant and should be taken into
15 consideration. To verify water loss in our system, plates were prepared with 100 μL of media
16 per well and incubated for 48 hours at 30°C which resulted in measured water loss of 0.018
17 g/645 cm^2 /24 hrs. Our lower observed value is most likely due to the geometry of the plate,
18 the low volume of water overall, and because the plates, except when reading, are housed
19 between two metal plates which may reduce water vapor transmission. Oxygen was
20 monitored both by indicator strips and by the presence of resazurin in the media. It was
21 established that with this method anaerobic conditions lasted for at least 80 h in the OL at
22 30°C without problems of fogging or toxicity from silicon or other sealants.

23 While these bags were effective in maintaining anaerobic conditions, it was found that
24 the plastic bags frequently jammed in the instrument due to the physical size and mechanical
25 motion of the plate reader. Metal clamps were added to properly seat the bagged microtiter

1 plate into the tray to reduce friction from the bag and the possibility of jamming during plate
2 reading, as well as to hold the plates horizontally in the trays so that the detector could gather
3 non-distorted images.

4 To start incubating, bagged and sealed plates were removed from the anaerobic
5 chamber, clamped in trays and incubated at 30°C in the OL until a maximum OL value was
6 reached. The plate was automatically scanned by the instrument and opacity (by pixilation
7 intensity) was recorded every 15 minutes. Cell growth was observed in both user prepared
8 96-well plates and Biolog-prepared PM plates PM1-PM20 (Biolog, Hayward, CA). A full
9 listing of all PM array well substrates can be found on the Biolog website
10 (<http://www.biolog.com/pmMicrobialCells.html>) and in the supplementary materials.

11 The scanning technology used by the OL records the increased in well opacity as
12 digital OL units. These OL units were given a microbiological value by calibrating to OD at
13 600nm and cell count data (see Results section below). The cell count calibration was
14 achieved by sacrificing plates and sampling cells from the 96-well plates at various stages of
15 growth representing a range of OL units and quantified using acridine orange (Sigma-Aldrich,
16 St. Louis, MO) direct cell counts (AODC) (Francisco, Mah et al. 1973) and measured at
17 OD₆₀₀ using a standard spectrophotometer (Perkin Elmer, Waltham, Massachusetts).

18 To accommodate the various plate-specific phenotype testing, LS4D medium was
19 modified for each type of PM plate (Table 1). In PM 1 and 2, carbon substrate utilization is
20 evaluated; therefore, medium was made without addition of the LS4D carbon source lactate.
21 Nitrogen was removed by omitting the medium component NH₄Cl for PM 3 (N sources), 6, 7,
22 and 8 (peptide N sources) plates, which assay a variety of nitrogen substrates and amino acids.
23 In addition, because *D. vulgaris* contains *nif* genes on its native plasmid that support growth
24 on N₂ (Heidelberg, Seshadri et al. 2004) PM 3, 6, 7, and 8 plates were prepared in an Ar
25 atmosphere. For PM 4, which assays P and S utilization pathways, P was removed from

1 LS4D for rows A-E, and S was removed for rows F-G. Plates PM 9 and 10 evaluate the
2 effects of osmotic and pH stress, and plates PM 11-20 contain a wide variety of inhibitors.
3 For PM 9-20 standard LS4D medium was used. For the PM1-8 plates, which use media
4 without a critical nutrient or substrate, the plates contain control wells to monitor growth in
5 the inoculated media without the added component. Growth in control wells indicate faulty or
6 over-inoculation, so that the data from the entire plate could be rejected.

7

8 **3.0 PMViewer Software**

9 Because of the potentially large number of growth curves produced in a phenotype
10 microarray experiment and the need to easily share results, we have developed a number of
11 Web-based interfaces to display growth curve data. BiologTM provides software for data
12 viewing with the OL instrument, but it is not Web based and requires installation on
13 individual computers for viewing PM data and the transfer of data files between computers.
14 The PMViewer software that we developed has a database backend and provides different
15 views of the data using Web-based interfaces.

16 The main data viewer for the results is PMRowView. This interface allows the user to
17 select one or more PM datasets, a single plate type, and a row on that plate. For the purpose
18 of the data viewers, a PM dataset consists of growth curve data from a phenotype microarray
19 experiment involving a single strain of an organism and a set of distinct PM plates run on the
20 same day. Growth curves are plotted twelve to a (Web) page in a three-column, four-row
21 format (Figure 2a). Each plot is labeled with the chemical name, plate type, and well id.
22 Because some chemicals are used to treat four consecutive wells (using different doses) on a
23 plate, the plots are placed on the page so that when a chemical is repeated, growth curves for
24 all four doses appear in one column to facilitate comparing dose responses. If the user clicks
25 on one of the twelve small plots, a new window will open that shows a larger version of that

1 plot (example shown in Figure 2b). In the new window, the user has the option to download
2 the data as a tab-delimited file for the curve(s) shown in the plot.

3 One additional feature of the PMRowView is that if the user selects a single dataset to
4 view, then in addition to the plot of the growth curve, approximate accumulation times are
5 displayed. The accumulation time is approximated as follows. A four-point centered
6 averaging method is applied first in order to smooth the curve. The time at which the
7 maximum value, v_{max} , of the curve is attained is determined, and following that, the times at
8 which points on the curve equal 10%, 20%, 30%,.... 90% of the maximum value are
9 determined. Four approximate accumulation times, AT, are calculated from $AT(t_a, t_b) = t_b -$
10 $t_a / 3.3 \log_{10}(v_b/v_a)$ using the times and values corresponding to the 10% and 90% points, 20%
11 and 80% points, 30% and 70% points, and 40% and 60% points. For example, for the 10%
12 and 90% points, $v_b(t_b) = 0.9v_{max}$ and $v_a(t_a) = 0.1v_{max}$. In cases where there is no response, or
13 the curve does not have a leading rising edge (i.e., the maximum value occurs earlier than the
14 minimum value), the accumulation time is not approximated.

15 In this workflow, the OL unit is a measure of increased opacity due to cell growth
16 processes, principally FeS precipitation, therefore the accumulation time determined above,
17 though affected by growth rate, does not directly measure growth rate.

18 The PMRowView interface is useful for directly comparing the response of different
19 strains of organisms for a given plate type. As many PM arrays are done in replicates, an
20 extension of the PMRowView interface (not shown) plots *averaged* growth curve data, and
21 markers showing plus or minus one standard deviation from the average value, from replicate
22 runs of phenotype microarray experiments using the same organism and plate. This extension
23 of the PMRowView interface is valuable both for assessing the reproducibility of replicate
24 runs and for comparing the responses of different strains when there are replicate runs.

1 A second interface, the PMColorMap, was developed to facilitate the display of
2 growth curve data for many plates or many experiments on a single Web page. The
3 PMColorMap interface displays a growth curve as a thin horizontal line in which magnitude is
4 represented by color (Figure 2c). Using this technique, and by vertically stacking the
5 horizontal lines representing the growth curves, it is possible to display all 96 curves for a
6 single PM plate in a relatively small area. In the 'preview' mode of this interface, it is possible
7 to display either color images representing all of the plates in a single dataset, or color images
8 for a single plate type for multiple datasets. The first option shows at a glance plates for
9 which there is poor growth overall, anomalies resulting from equipment malfunctions, or
10 chemical reactions between the growth medium and the chemical treatment in the well
11 (Figures 3 and 8). The second option allows the user to make gross comparisons of the
12 responses of different strains of an organism to the treatments on a single plate.

13 If the user clicks on a color image in the preview mode, a new window will open
14 showing a larger version of the color image, in which the color lines representing each growth
15 curve are distinct (Figure 2d). Slowly moving the computer mouse over a color line displays
16 the plate type, well id, chemical name, and mode of action. Clicking on a color line will open
17 a new window showing the growth curve corresponding to the color line, i.e., corresponding
18 to a single well (Figure 2b). Clicking on the row label will open a new window in which the
19 growth curves for the twelve wells in that row will be plotted as previously described for the
20 PMRowView interface. The PMColorMap interface provides a quick look at both the
21 responses of different strains of an organism and the quality of data for a phenotype
22 microarray experiment. Examples of how the PMColorMap interface is used to check data
23 quality are described in the following section.

24 The PMRowView and PMColorMap interfaces are Perl-CGI scripts that use the Perl
25 module GD, available from the Comprehensive Perl Archive Network (CPAN), for creating

1 the growth curve plots and the color images. Plots of growth curves (growth vs. time) are
2 generated on the fly, but the color map images, which are more computationally intensive to
3 produce, are generated once and stored on the Web server. The approximate accumulation
4 times also are calculated and stored on the Web server. The PMRowView and PMColorMap
5 interfaces provide convenient and flexible ways of viewing phenotype microarray data
6 (Jacobsen, Joyner et al. 2007).

7

8 **4.0 Results and Discussion**

9 *4.1 Inoculum Standardization and Calibration of OL value*

10 The PM plate analyses function by the addition of a key substrate to a deficient
11 medium, or by adding an inhibitory chemical to limit growth. The goal is to inoculate wells
12 uniformly to be able to visualize differential growth. Successful growth of *D. vulgaris* in
13 plates was found to be a straightforward technique, however reproducible and representative
14 growth in the PM array presented a methods development challenge. Several steps outlined in
15 the methods section have detailed important modifications that are necessary for successful
16 growth and testing of the *D. vulgaris* phenotype. As with any cell culture process,
17 development of precise and consistent culture and inoculation techniques are very important
18 to the production of high quality PM data from the OL system. Recognizing when
19 representative growth has been visualized by the OL will prevent misinterpretation of results
20 and will facilitate the generation of an accurate phenotype for an organism.

21 Figure 3 demonstrates the three basic growth patterns that can be found after adding a
22 new or unknown strain in a PM plate. Under-inoculation results in no growth in all of the
23 wells as shown in Figure 3a. Alternatively, over-inoculation of the plate or failure to remove
24 residual medium and dead cells from the inoculum (through centrifugation) often resulted in
25 either cryptic or unmodified growth of *D. vulgaris* in the plates as shown in Figure 3b. This

1 growth is independent of the well contents and does not represent a valid phenotype. It was
2 found that for *D. vulgaris*, if as little as 10% residual LS4D medium was transferred with the
3 inoculum cells to the plates, growth on this carryover medium often superseded the limited
4 growth in the PM well. Figure 3c demonstrates differential growth due to proper inoculation
5 and removal of carryover medium and represents a valid assessment of phenotype. To assure
6 that the observed plate results were valid, all results were confirmed in triplicate with
7 biological replicates.

8 When different strains or mutants of a strain are compared, differences in growth rates
9 or yields will have to be taken into account. To compare strains, their growth must be
10 standardized or normalized to the parent strain, and if yields are sufficiently different between
11 strains to make direct comparison difficult, it may be necessary to report differences as a
12 percent change of normal growth in each strain rather than the absolute OL values.

13 Figure 4a shows the results of standardized growth for *D. vulgaris* inoculated into
14 empty, (MT), plates, showing an average of three biological replicates. Error bars represent
15 measured standard deviations between the replicates, each run as 96 technical replicates. This
16 growth of *D. vulgaris*, as described above in the methods section, represents an initial
17 concentration of 1×10^{-7} cells/ml of mid-log phase cells in LS4D medium. In practice, this
18 standardization of the inoculum concentration proved to be pivotal in producing representative
19 growth, because adding lower concentrations of cells resulted in limited or no growth, and
20 higher concentration showed abnormal growth curves. Figure 4b compares the observed OL
21 data to a biological replicate measured in a hungate tube at 600 nm and to a literature reported
22 growth curve, also measured at 600 nm (Mukhopadhyay, He et al. 2006) demonstrating that
23 the OL measurement of opacity and measured FeS accumulation rates are equivalent to OD_{600}
24 measurements in standard LS4D (see Table 2).

1 The observed increase in opacity was calibrated to standard cell enumeration
2 techniques: cell counts and spectrophotometric measurement of turbidity at 600 nm (OD₆₀₀)
3 (see Figure 5). Calibration was accomplished by preparing a wide range of cells
4 concentrations and measuring by all three techniques. The calibration demonstrated the
5 following relationships between OL units and cell density:

6 Log cell density = 1.89 * ln (OL units) + 10.5
7 (r² = 0.9329), or on the order of 1 x 10⁸ cells/ml for an observed value of 120 OL units (see
8 Figure 5), and for OD 600 nm:

9 OD 600 nm = 8.4 x 10⁻³ (OL units) + 0.03
10 (r² = 0.9332).

11 These calibrations of opacity measurement was necessary in order to further validate
12 that increase in opacity with time measured by the OL using the darkening color, both from
13 FeS precipitate and other cell products, indeed serves as an analog for increase in cell density
14 over time. Similar to OD₆₀₀ measurements, OL data does not measure exponential growth,
15 but rather reflects the increased opacity due to accumulation of cells (as a function of the
16 increase in number of cells) and metal sulfide precipitation within a defined range, which for
17 OL units is 0 to 250. As can be seen by the relationship between cell counts and OL units, the
18 relationship is non-linear and lacks sensitivity at both the low and high cell counts. While cell
19 counting remains as one of the most robust methods for monitoring growth, it is not possible
20 to assay cell density in nearly 2000 substrates every 15 minutes. In contrast, the PM
21 represents a high throughput tool to determine overall phenotype. Therefore, if an interesting
22 phenotype is detected, additional validation by cell count assays must be obtained when using
23 MT plates and batch reactors.

24

25 4.2 *Artifacts associated with growth of D. vulgaris in microtiter plates*

1 During long incubation times OL units will eventually reach a plateau value when the
2 FeS accumulation has reached a maximum value, both due to depletion of Fe in the media and
3 cells reaching stationary phase. Due to changes in FeS precipitate, or cell clumping, cell lysis,
4 or changes in chemistry in the wells, the plateau level may steadily decrease, increase, or
5 show fluctuation. In addition some wells show biphasic or hump in growth curve as
6 compared to batch experiments. This may be due to the complex nature of the FeS
7 precipitation reaction and the combined effect of many co-occurring chemical or biological
8 processes, or perhaps phage lysis that may affect well opacity. The differences observed in
9 the plates as compared to standard batch experiments can be attributed to difference in culture
10 geometry including size and shaking (mixing) of culture. Batch cultures are closed systems,
11 whereas PM plates sealed in a bag are open systems and release of H₂S from other wells on
12 the plate may change the chemistry of the medium during growth as compared to a closed
13 batch. These are general features associated with growth in the OL, which have to be
14 accounted for when interpreting results and comparing PM plates to other growth
15 measurement techniques.

16 Because of the wide range of growth parameters that can be tested there is a possibility
17 of mixotrophic growth occurring, where cells continue to grow but not via sulfate reduction, a
18 possibility when alternate carbon source and yeast extract are present (Postgate 1984).
19 Therefore, as stated earlier, observed growth patterns should be confirmed by secondary
20 methods such as cell counts or protein assays.

21 The use of opacity to visualize growth also presents some challenges for data
22 interpretation. The observation that addition of medium in the absence of cells causes an
23 immediate reaction in some wells is an artifact of the PM plates. Some wells components
24 may also abiotically reduce sulfate to sulfide and cause formation of precipitate. It was found
25 that several wells consistently have abiotic reactions that preclude the measurement of any

1 opacity change due to cell growth and cannot be used to evaluate the *D. vulgaris* phenotype.
2 Using the PMColorMap viewer (Figure 8b), a list of these wells was identified and is given in
3 Table 3.

4 Some wells have more minor abiotic background reactions which affect visualization
5 of growth of *D. vulgaris*. For example, the PM 10 plate evaluates pH effects, and the first row
6 contains reagents to control the pH in a range of 4.6 – 8.5 (this represents the actual measured
7 pH range in LS4D media; PM 10 reported range given by Biolog is 3.5 – 10). The data
8 (Figure 6a) show poor or no growth below pH 6.9 and consistent growth from pH 6.9 – pH
9 7.9. At pH 8.2 and above, there are abiotic reactions with the medium raising the initial OL
10 unit. Background abiotic reactions are about 80 OL units for pH 7.9 and 130 OL units for pH
11 8.2. The background values may be due to precipitation or other reactions by well components
12 with LS4D, poor mixing, or changes in aggregation patterns of the cells. While these
13 background values make absolute determination of the effect of pH on growth difficult,
14 comparisons between conditions or strains using the same medium still would be possible.
15 The growth curves also can be corrected by subtraction of the background value (Figure 6b).
16 For this assay of pH effects, comparing absolute minimum or maximum OL units is not
17 informative in determining the effects of pH. However, both the generation time, lag time and
18 the difference between the maximum and the minimum OD might be used in evaluating
19 effects of pH in a comparative study. An additional consideration is that depending on the
20 buffer capacity and chemical composition of the growth media, the pH adjustment indicated
21 for a OL well may not be accurate and should be measured independently.

22 Plates PM 1 and PM 2 are designed to evaluate carbon utilization patterns. The carbon
23 source in the wells is ~20mM, which is too low to elicit robust growth of *D. vulgaris*. In this
24 case, the addition of 10 mM Fe-NTA to the medium increased the FeS precipitation and as a
25 result, the opacity. However, while the additional Fe-NTA boosted the intensity for

1 visualization, a growth curve was not obtained, and the result can be used only as an
2 indication of carbon utilization capabilities, rather than as a kinetic assay. Using PM 1 and
3 PM 2, *D. vulgaris* was shown to have growth on lactate and pyruvate and formic acid, as is
4 reported in the literature (Heidelberg, Seshadri et al. 2004). These C utilization results were
5 further confirmed by measuring cell density and *D. vulgaris* purity was confirmed with PCR
6 probes.

7 PM 4 is a split plate, the first five rows evaluate P sources and the remainder evaluates
8 S sources. It was found that the S concentration in these wells were insufficient to support
9 growth of *D. vulgaris* so the S phenotype could not be determined.

10 While each plate and individual wells are labeled by basic description, BiologTM has
11 not yet released the detailed component description of each prepared well. Hence a well
12 description such as 5% (wt/vol) NaCl refers to the primary component in the well, but the well
13 may contain other components that may affect cell growth. For example, growth of *D.*
14 *vulgaris* on plate PM 9 row A is shown in Figure 7. This plate assays growth from 1 to 10%
15 (0.56 – 5.6 M) NaCl and measures the effect of increasing NaCl concentration on growth, and
16 *D. vulgaris* demonstrates positive growth at the 1 and 2% levels. Previous results have
17 shown the minimum inhibitory concentration of NaCl to *D. vulgaris* in LS4D to be and
18 addition of 0.44% NaCl (0.250 M) to Na salts already present, namely sodium lactate and
19 sodium sulfate (Mukhopadhyay, 2006). The increased resistance to NaCl shown in Figure 7 is
20 potentially due to the 0.2% yeast extract (YE) present in the plates, added by Biolog as a
21 fixing agent. YE is known to contain components such as glutamate, proline and glycine
22 betaine (Dulaney, Dulaney et al. 1968) that may be used by *D. vulgaris* as an osmoprotectant
23 (Mukhopadhyay, 2006). Limited growth of *D. vulgaris* was observed in a user prepared plate
24 with 2% NaCl with 0.2% YE added, and was only observed up until 1% NaCl (0.56 M)
25 without YE (data not shown). This reemphasizes that the OL serves best as a tool to survey

1 phenotype, while the precise determination of inhibitory concentrations, antibiotic resistance
2 patterns, etc., in a specific media require experiments in that defined medium either in user-
3 prepared plates or flasks. In other words, results from the PM array, although inoculated with
4 LS4D, actually reflect growth of LS4D plus PM media, which will be different than pure
5 LS4D in most wells since the wells on many of the other plates also contain stabilizers or
6 undefined buffers.

7 Further examples of growth visualization problems are shown in Figure 8. Partial
8 plate under-inoculation is clearly seen in Figure 8a, with the lower third of the plate
9 demonstrating no growth. In Figure 8b yellow and orange bands indicate abiotic reactions
10 between LS4D and PM well contents. Figure 8c is a visualization of an OL instrument
11 malfunction that caused periodic spikes in the data for this plate. These examples show that
12 PMColorMap plots are valuable in rapid screening of plate validity, which may not be
13 apparent from looking at individual growth curves.

14

15 **5.0 Conclusions**

16 The small well size and manufacturing process of PM plates lead to certain differences
17 between known measurements for several of the observed results. Evaporation, transfer of
18 headspace gases between wells, presences of osmolytes in fixing agents (such as the presence
19 of yeast extracts in PM 9), and pH changes will occur during growth. Monitoring growth in
20 the plates using FeS precipitate and cell accumulation can be correlated well with cell counts
21 and OD₆₀₀ nm measurements traditionally used to monitor growth. Results should be used
22 with some reservations when evaluating kinetic data because abiotic or other chemical
23 reactions in the wells can affect measured values for some wells. Despite the existence of
24 unusual abiotic reactions among medium components and/or metabolic products, the growth
25 information can still be useful as long as the abiotic features are recognized. Observed growth

1 and interesting phenotypic changes should always be confirmed by cell counts, user prepared
2 MT plates, or subsequent larger scale batch studies. Looking at a suite of growth parameters,
3 both kinetic and absolute metrics, including generation time, the overall change in opacity as
4 measured by OL units, and the lag time, will provide a clearer picture of the growth
5 characteristics and overall phenotype. For increased confidence in observed phenotypes,
6 biological triplicates and confirmation of observed growth in user prepared plates or in batch
7 culture is recommended. Also important is monitoring of control wells to observe over-
8 inoculation and normalizing the inoculum of new or unknown strains for consistent growth.
9 A phenotype survey of *D. vulgaris* can be used as a basis for comparison of a variety of
10 growth conditions, effects of stressors, or for the evaluation of knock out mutants. The PM
11 array can provide a shotgun approach for screening, allowing the researcher to find potentially
12 interesting growth changes that were not previously considered. However, despite these
13 limitations the OL system is a valuable tool for broad screening of phenotypes for *D. vulgaris*
14 and other such environmentally important sulfate / metal reducing bacteria.

15 Development of the PMViewer Web-based data viewers facilitated the sharing of data
16 among project participants, assessment of the overall quality of phenotype surveys, and the
17 identification of substrates that resulted in abiotic reactions. Using a color mapping technique
18 to represent growth curves as thin color images, we were able to display large numbers of
19 growth curves in a space-efficient way. We added to this the ability to filter views of the data
20 and to zoom in on data from plates, rows on plates, or individual wells of interest. The
21 PMViewer software tools have proven valuable for viewing the large amounts of data
22 necessary for development of phenotype microarrays as a screening tool for *D. vulgaris*
23 growth.

24 The above discussion highlights some observations for the PM survey for *D. vulgaris*
25 across the various carbon requirements in addition to salt, osmotic and pH stress. This

1 complete dataset for *D. vulgaris* is available in the supplementary data. The focus of this
2 study is the optimization of the OL workflow for *D. vulgaris*, and this data set merits a more
3 comprehensive analysis, which would constitute a separate study.

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14 **6.0 References**

- 15 Bhupathiraju, V. K., M. Hernandez, D. Landfear and L. Alvarez-Cohen (1999). "Application
16 of a tetrazolium dye as an indicator of viability in anaerobic bacteria." Journal of
17 Microbiological Methods **37**(3): 231-243.
- 18 Bochner, B. (2003). "New technologies to assess genotype-phenotype relationships." Nature
19 reviews/Genetics **4**: 309-314.
- 20 Brewster, J. D. (2003). "A simple micro-growth assay for enumerating bacteria." Journal of
21 Microbiological Methods **53**: 77-86.
- 22 Caumette, P. (1993). "Ecology and Physiology of Phototrophic Bacteria and Sulfate-Reducing
23 Bacteria in Marine Salterns." Experientia **49**(6-7): 473-481.
- 24 Chang, I. S. and B. H. Kim (2007). "Effect of sulfate reduction activity on biological
25 treatment of hexavalent chromium [Cr(VI)] contaminated electroplating wastewater
26 under sulfate-rich condition." Chemosphere **68**(2): 218-226.
- 27 Cottrell, M. T. and S. C. Cary (1999). "Diversity of dissimilatory bisulfite reductase genes of
28 bacteria associated with the deep-sea hydrothermal vent polychaete annelid *Alvinella*
29 *pompejana*." Appl. Environ. Microbiol. **65**(3): 1127-1132.
- 30 CPAN Comprehensive Perl Archive Network. Perl modules, including the GD module, may
31 be downloaded from the CPAN Web site at www.cpan.org.
- 32 Demain, A. L., M. Newcomb and J. H. Wu (2005). "Cellulase, clostridia, and ethanol." Microbiol Mol Biol Rev **69**: 124-154.
- 34 Dulaney, E. L., D. D. Dulaney and E. L. Rickes (1968). "Factors in yeast extract which relieve
35 growth inhibition of bacteria in defined medium of high osmolarity." Dev. Ind.
36 Microbiol **9**: 260-269.

- 1 Francisco, D., R. A. Mah and A. Rabin (1973). "Acridine orange epifluorescence technique
2 for counting bacteria in natural waters." Transactions of the American Microscopical
3 Society **92**(3): 416-421.
- 4 Gabrielson, J., M. Hart, A. Jarelov, I. Kuhn, D. McKenzie and R. Mollby (2002). "Evaluation
5 of redox indicators and the use of digital scanners and spectrophotometer for
6 quantification of microbial growth in microplates." Journal of Microbiological
7 Methods **50**: 63-73.
- 8 Gellert, G. (2000). "Sensitivity and Significance of Luminescent Bacteria in Chronic Toxicity
9 Testing Based on Growth and Bioluminescence." Exotoxicology and Environmental
10 Safety **45**: 87-91.
- 11 Grostern, A. and E. A. Edwards (2006). "Growth of Dehalobacter and Dehalococcoides spp.
12 during Degradation of Chlorinated Ethanes." Appl. Environ. Microbiol. **72**: 428-436.
- 13 Heidelberg, J. F., R. Seshadri, S. A. Haveman, C. L. Hemme, I. T. Paulsen, J. F. Kolonay, J.
14 A. Eisen, N. Ward, B. Methe, L. M. Brinkac, S. C. Daugherty, R. T. Deboy, R. J.
15 Dodson, A. S. Durkin, R. Madupu, W. C. Nelson, S. A. Sullivan, D. Fouts, D. H. Haft,
16 J. Selengut, J. D. Peterson, T. M. Davidsen, N. Zafar, L. W. Zhou, D. Radune, G.
17 Dimitrov, M. Hance, K. Tran, H. Khouri, J. Gill, T. R. Utterback, T. V. Feldblyum, J.
18 D. Wall, G. Voordouw and C. M. Fraser (2004). "The genome sequence of the
19 anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough." Nature
20 Biotechnology **22**(5): 554-559.
- 21 Jacobsen, J. S., D. C. Joyner, S. E. Borglin, T. C. Hazen, A. P. Arkin and E. W. Bethel (2007).
22 Visualization of Growth Curve Data from Phenotype Microarray Experiments. 11th
23 International Conference on Information Visualization IV, Zürich, Switzerland, IEEE
24 Computer Society Press.
- 25 Koutny, M. and L. Zaoralkova (2005). "Miniaturized kinetic growth inhibition assay with
26 denitrifying bacteria *Paracoccus denitrificans*." Chemosphere **30**: 49-54.
- 27 Martinez, R. J., M. J. Beazley, M. Taillefert, A. K. Arakaki, J. Skolnick and P. A. Sobecky
28 (2007). "Aerobic uranium (VI) bioprecipitation by metal-resistant bacteria isolated
29 from radionuclide- and metal-contaminated subsurface soils." Environmental
30 Microbiology **9**(12): 3122-3133.
- 31 Mukhopadhyay, A., Z. L. He, E. J. Alm, A. P. Arkin, E. E. Baidoo, S. C. Borglin, W. Q.
32 Chen, T. C. Hazen, Q. He, H. Y. Holman, K. Huang, R. Huang, D. C. Joyner, N. Katz,
33 M. Keller, P. Oeller, A. Redding, J. Sun, J. Wall, J. Wei, Z. M. Yang, H. C. Yen, J. Z.
34 Zhou and J. D. Keasling (2006). "Salt stress in *Desulfovibrio vulgaris* Hildenborough:
35 An integrated genomics approach." Journal of Bacteriology **188**(11): 4068-4078.
- 36 Neculita, C. M., G. J. Zagury and B. Bussiere (2007). "Passive treatment of acid mine
37 drainage in bioreactors using sulfate-reducing bacteria: Critical review and research
38 needs." Journal of Environmental Quality **36**(1): 1-16.
- 39 Noguera, D. R., G. A. Brusseau, B. E. Rittmann and D. A. Stahl (1998). "Unified model
40 describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different
41 environmental conditions." Biotechnology and Bioengineering **59**(6): 732-746.
- 42 Postgate, J. (1984). The Sulphate-Reducing Bacteria, 2nd Edition. Cambridge, MA,
43 Cambridge University Press.
- 44 Postgate, J. R. and L. L. Campbell (1966). "Classification of *Desulfovibrio* Species
45 Nonsporulating Sulfate-Reducing Bacteria." Bacteriological Reviews **30**(4): 732-&.
- 46 Sani, R. K., B. A. Peyton and M. Jandhyala (2003). "Toxicity of lead in aqueous medium to
47 *Desulfovibrio desulfuricans* G20." Environ. Toxicol. Chem. **22**(2): 252-260.
- 48 Schmitz, R., A. Eisentrager and W. Dott (1999). "Agonistic and antagonistic toxic effects
49 observed with miniaturized growth and luminescence inhibition assays " Chemosphere
50 **38**(1): 79-95.

- 1 Schmitz, R., C. Kretkowski, A. Eisentrager and W. Dott (1999). "Ecotoxicological testing
2 with new kinetic *Photorhabdus luminescens* growth and luminescence inhibition
3 assays in microtitration scale." Chemosphere **38**(1): 67-78.
- 4 Telang, A. J., G. Voordouw, S. Ebert, N. Sifeldeen, J. M. Foght, P. M. Fedorak and D. W. S.
5 Westlake (1994). "Characterization of the Diversity of Sulfate-Reducing Bacteria in
6 Soil and Mining Waste-Water Environments by Nucleic-Acid Hybridization
7 Techniques." Canadian Journal of Microbiology **40**(11): 955-964.
- 8 Wind, T., S. Stubner and R. Conrad (1999). "Sulfate-reducing bacteria in rice field soil and on
9 rice roots." Systematic and Applied Microbiology **22**(2): 269-279.
- 10 Yi, Z. J., K. X. Tan, A. L. Tan, Z. X. Yu and S. Q. Wang (2007). "Influence of environmental
11 factors on reductive bioprecipitation of uranium by sulfate reducing bacteria."
12 International Biodeterioration & Biodegradation **60**(4): 258-266.
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Table 1. LS4D media preparation guidelines.

LS4D type	PM Plates	LS4D (mL)	Culture (mL)	Vitamins (μL)	μL TC*	Atm
No Lactate	1, 2	30	3	30	150	
No P	4 (rows A-E)	10	1	10	50	↑
No S	4 (rows F-G)	10	1	10	50	5% CO ₂ , 5% H ₂ , balance N ₂
No vitamins	5	10	1	0	50	↓
Standard	9 – 20	150	15	150	750	
No N	3, 6, 7, 8	60	6	60	300	Argon

*TC: Titanium citrate reducing agent.

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Table 2. Calculated accumulation rates (AR) of FeS precipitates from observed growth curves (see Figure 4).

OmniLog™				
t_a (hrs)	t_b (hrs)	v_a (OL units)	v_b (OL units)	$AR = (t_b - t_a)/(3.3*\log(v_b/v_a))$
17	37.5	17.52	116.48	7.551
21	37.5	35.46	116.48	9.680
24	37.5	41.74	116.48	9.179
17	27.5	17.52	67.48	5.433
21	27.5	35.46	67.48	7.049
24	27.5	41.74	67.48	5.083
AVERAGE				7.3 ± 1.9

Hungate tubes				
t_a (hrs)	t_b (hrs)	v_a (OD 600 nm)	v_b (OD 600 nm)	$AR = (t_b - t_a)/(3.3*\log(v_b/v_a))$
17	37.5	0.177	0.892	7.550
21	37.5	0.292	0.892	8.033
24	37.5	0.362	0.892	8.317
17	27.5	0.177	0.524	6.750
21	27.5	0.292	0.524	6.984
24	27.5	0.362	0.524	7.075
AVERAGE				7.5 ± 0.6

Mukhopadhyay, 2006				
t_a (hrs)	t_b (hrs)	v_a (OD 600 nm)	v_b (OD 600 nm)	$AR = (t_b - t_a)/(3.3*\log(v_b/v_a))$
17	37.5	0.174	0.959	8.380
21	37.5	0.268	0.959	9.030
24	37.5	0.337	0.959	9.007
17	27.5	0.174	0.586	6.034
21	27.5	0.268	0.586	5.797
24	27.5	0.337	0.586	4.414
AVERAGE				7.1 ± 1.9

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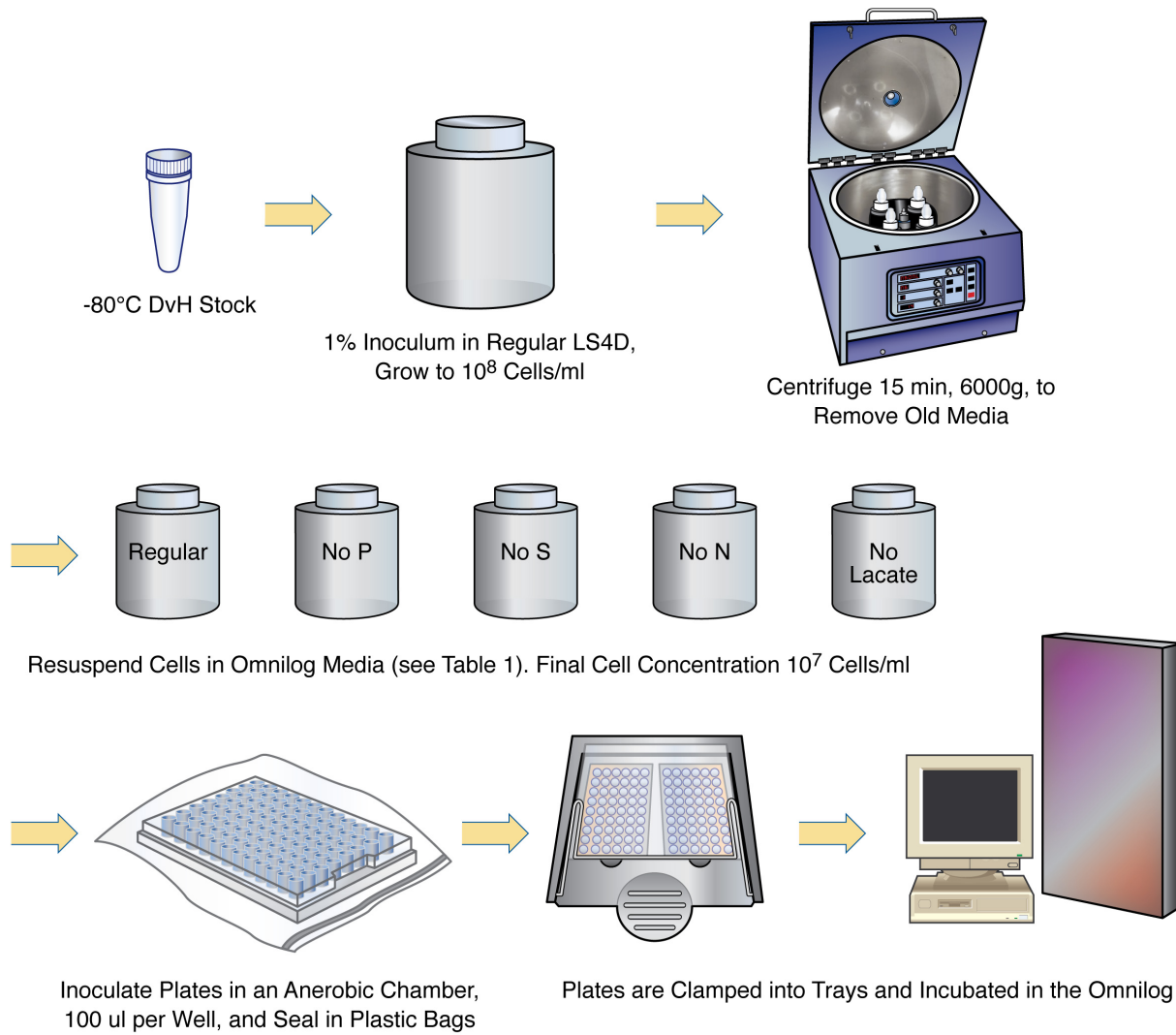
Table 3. PM wells with unworkable abiotic reactions with *D. vulgaris* and LS4D media

PM Microplate	Well position	Well Contents
1	C4, H6	D-Ribose, L-Lyxose
2	E6, F9, H9, E12	2-Hydroxybenzoic acid, Sorbic Acid, Dihydroxyacetone, 2-Oxovaleric acid
4	D10, E10	Uridine 5'-Monophosphate, Thymidine 5'-Monophosphate
10	F3, F7, D9	pH 9.5 + L-Proline, pH 9.5 + L-Tyrosine, pH 4.5 + 5-hydroxytryptophan
13	A11-12, H1-4	Nickel chloride, Cupric chloride
15	D11-12	Nordihydroquaiaretic acid
16	E11-12; G9	Rifamycin SV, L-Glutamic acid g-hydroxamate
17	E4, F9-12, G11	Niaproof, Tannic acid, Cefoperazone
18	G11,12	Myricetin
19	A5-8, D6-8	Harmane, Iodonitro tetrazolium violet
19	E9-12, H4	Lawson, Hexammincobalt (III) Chloride
20	A9-12, E1-4	Benserazide, Crystal Violet

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Figure 1. Schematic of the preparation steps for the PM microplates for *D. vulgaris*.

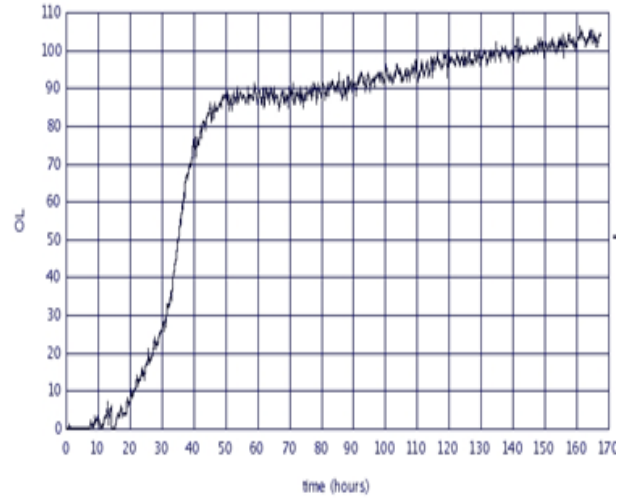
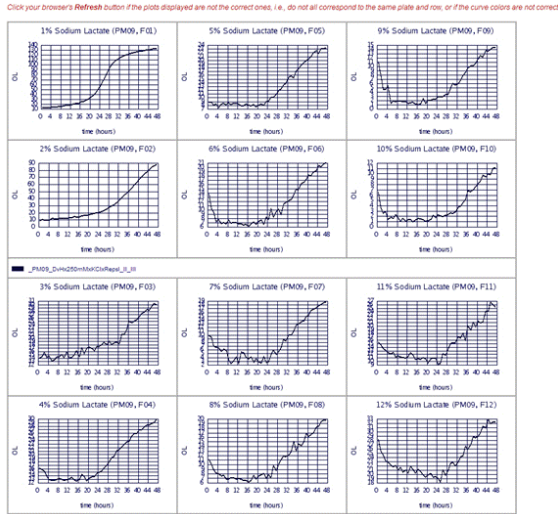


ESD08-003

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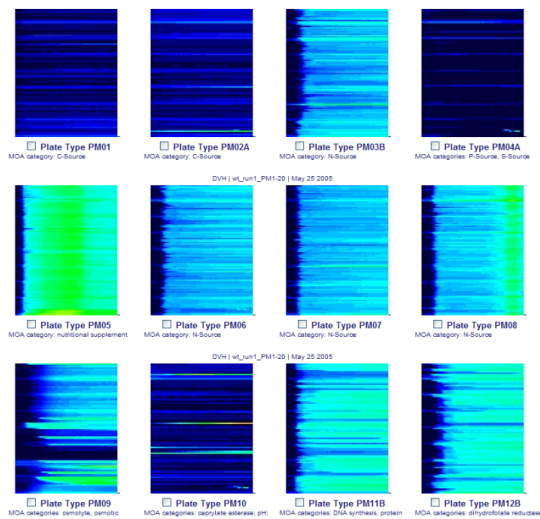
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Figure 2. Screenshot of **PMRowView** Web-based data viewer is shown in (a). This view shows twelve plots corresponding to the twelve wells on a single row of a PM microplate. Selecting a plot will open a new window that displays the plot on a larger scale (full page) and that has a link to download the data as a CSV file as shown in (b). Plot (c) groups all available PM microplates for a single run in a preview mode in the **PMColorView** Web data viewer. Each plot, when selected will produce the plot for a single plate as shown in (d). Selecting a color band (i.e., a microwell) in (c) will produce a full-size plot (b) for the selected microwell, as well as a link to download the raw data. This data is shown to illustrate the interface only and should not be used for phenotype interpretation.

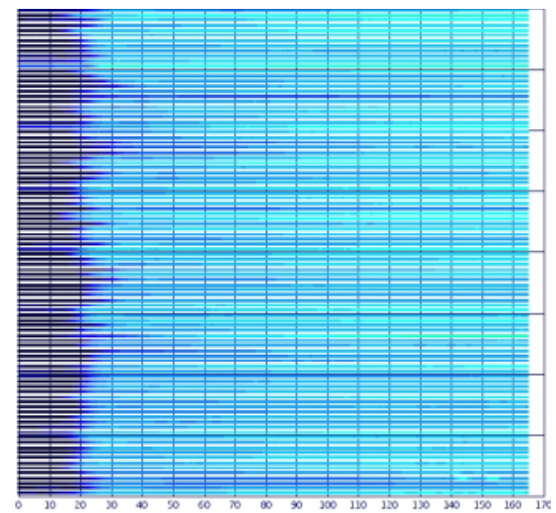


(a)

(b)



(c)

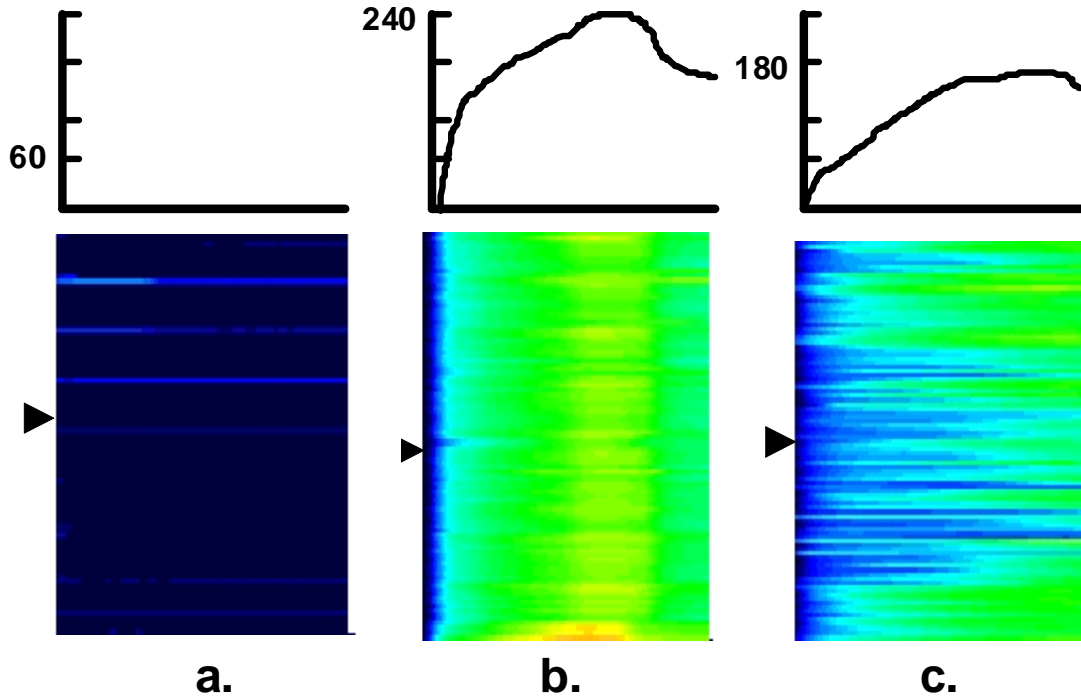


(d)



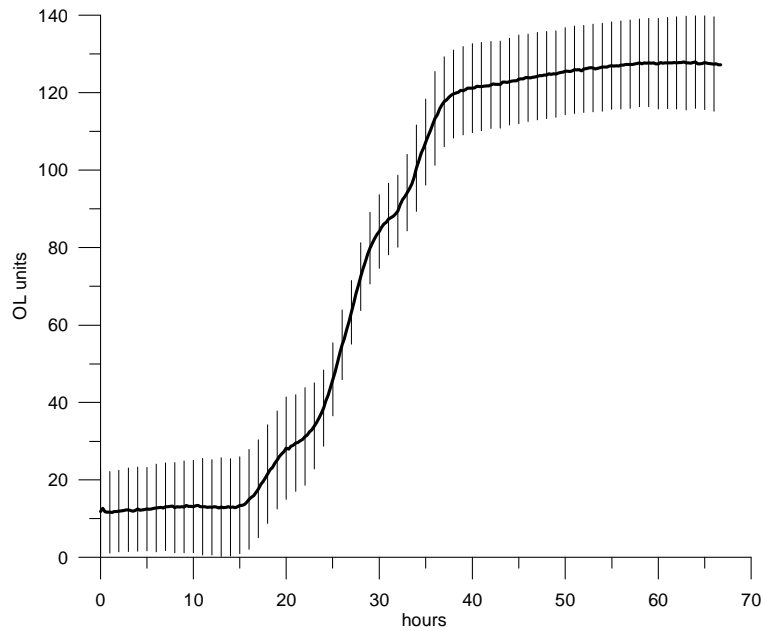
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Figure 3. False color images (see text) representing growth curves from 96-well microplates: (a) under inoculated microplate, (b) over inoculated microplate, (c) properly inoculated microplate. Arrows show location of wells whose growth curves are plotted above each color image. The color legend for these plots is shown in Figure 2.

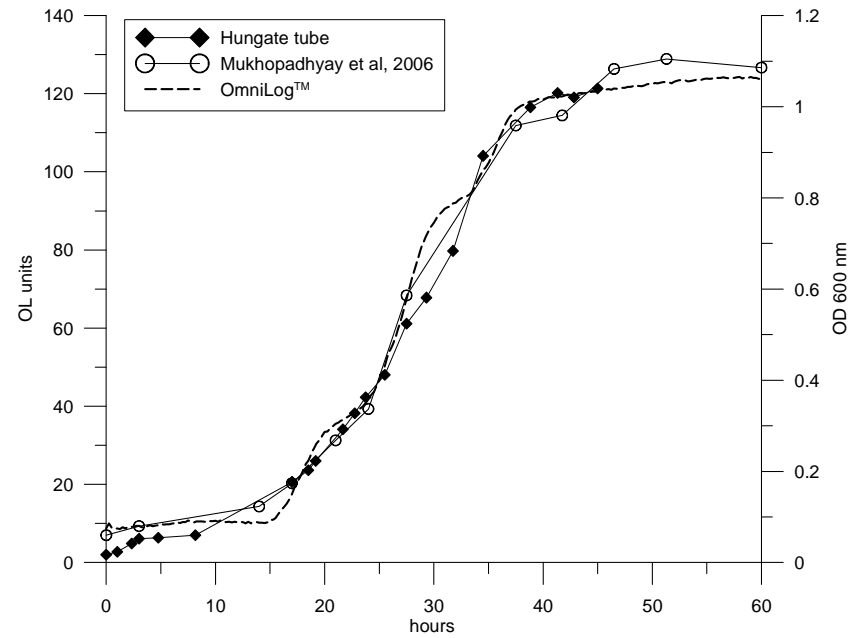


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Figure 4. (a) Representative growth curves from standardized inoculum. Shown are averages of three biological replicates of *D. vulgaris* growth in OL at 30°C in empty 96 well microtiter plates. Vertical lines represent one standard deviation. (b) Biological replicates of *D. vulgaris* growth in LS4D at 30°C assays on the OmniLog™, in hungate tubes and measured at 600 nm, and literature reported growth curve (Mukhopadhyay, 2006)



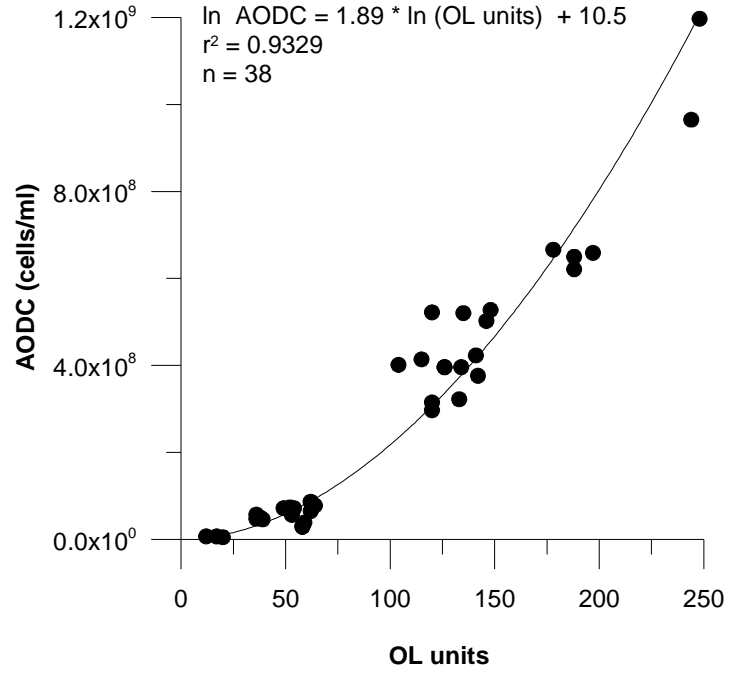
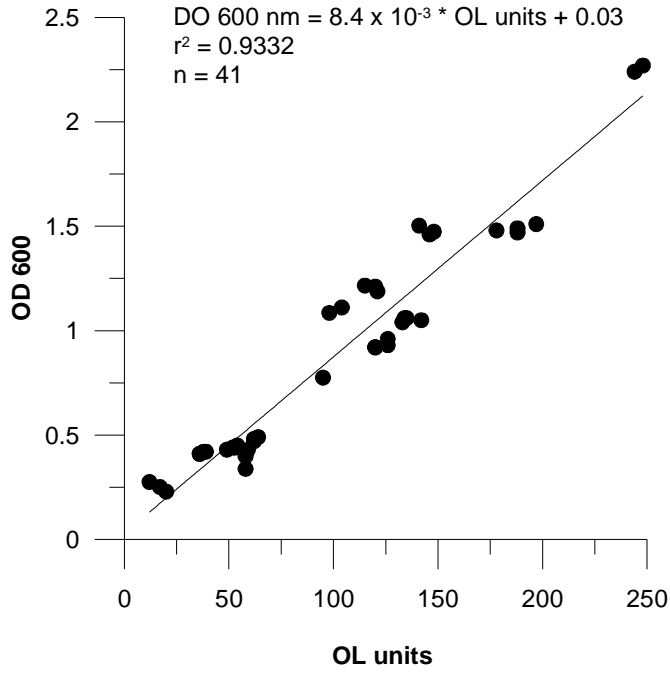
(a)



(b)

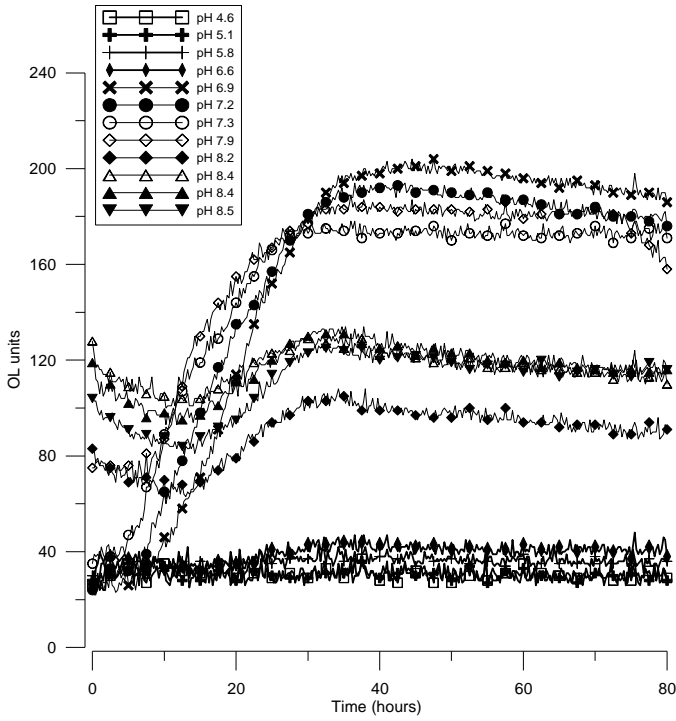
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Figure 5. Calibration of *D. vulgaris* with AODC (cell counts) and OD 600 nm. OD 600 nm values over 1.0 absorbance units were diluted prior to measurement.

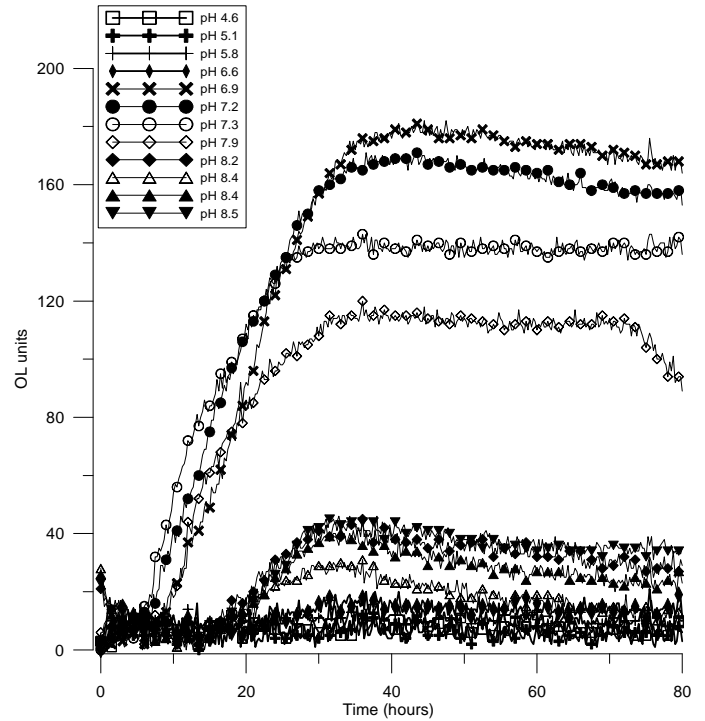


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Figure 6. PM microplate 10 row A (pH sensitivity). Plot (a) is raw data, plot (b) is the same data with the minimum value subtracted from each growth curve. Displayed pH is the measured value at time of inoculation.

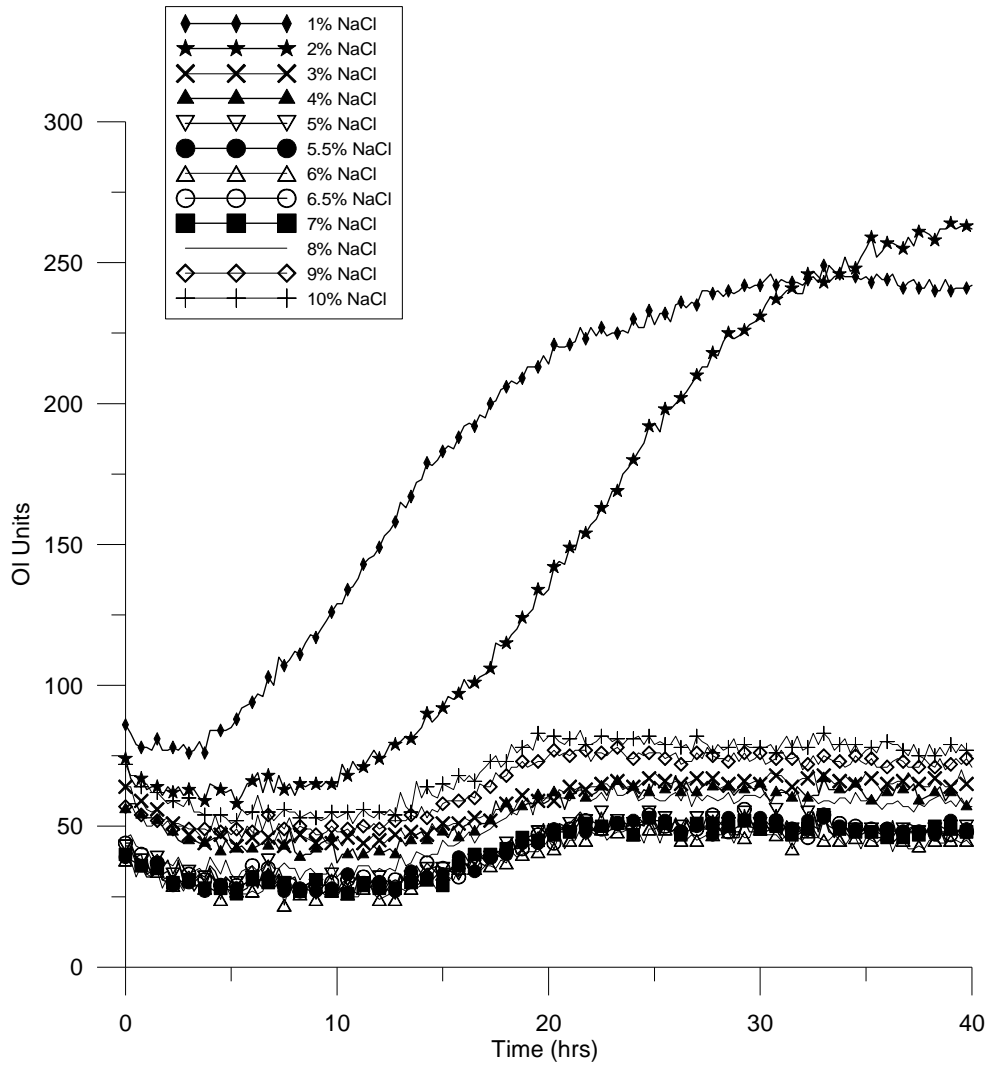


(a)



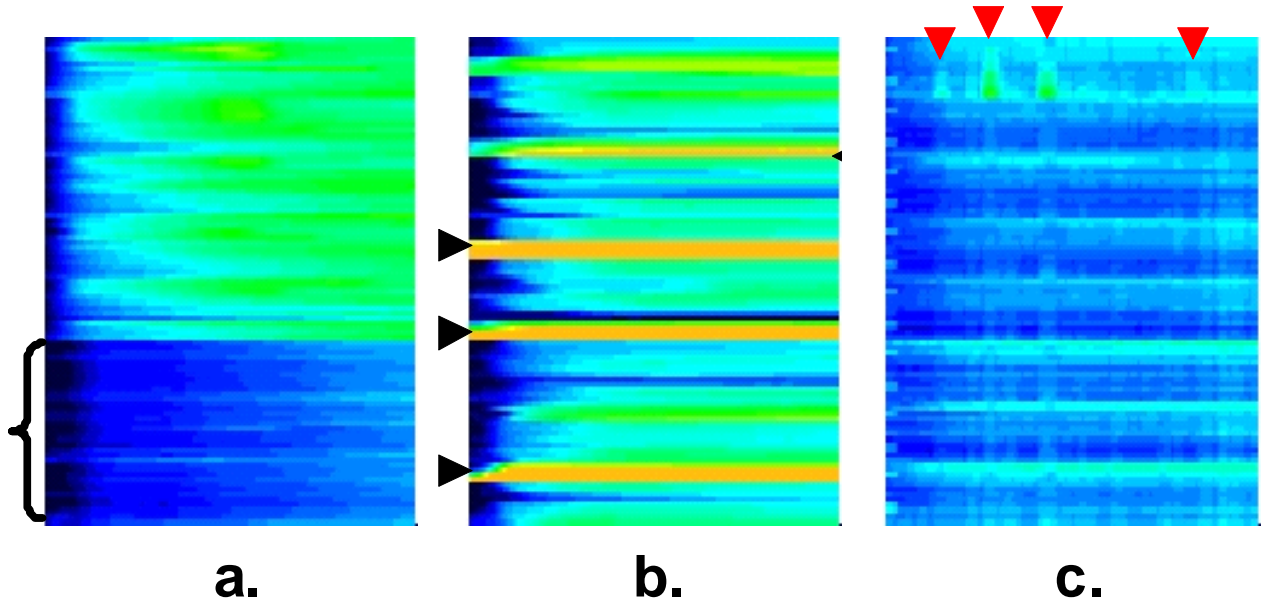
(b)

Figure 7. PM 9 row A, effects of increasing NaCl concentration on growth. Note that PM9 plate contains 0.2% Yeast extract (YE) as a fixing agent. YE may affect the ability of an organism to cope with salt stress (For instance, YE typically contains components such as glutamate, proline and glycine betaine known to serve as osmoprotectants for *D. vulgaris*).



1

Figure 8. False color images (see text) used to detect problem microplates:
(a) several rows of wells, indicated by left brace, are under inoculated,
(b) high turbidity indicated by black arrows and orange lines are due to chemical reactions between the growth medium and well treatment,
(c) spikes in signals indicated by red arrows and vertical banding are due to instrument malfunction.
The color legend for these plots is shown in Figure 2.



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