APPLICATION NOTE

# Advanced kinetic analysis of a bacterial growth assay

**Michel Hoogenkamp** | Research Technician | Academic Centre for Dentistry Amsterdam (ACTA)
**Cathleen Salomo** | Field Applications Scientist | Molecular Devices

## Benefits

- **Save time with simultaneous detection of both cell density and fluorescent protein kinetic traces using the Workflow Editor**
- **Simplify data report generation with preconfigured and customizable data analysis options**
- **Easily consolidate desired data output parameters into table or plate formats and graph as scatter plots or bar graphs**

## Introduction

Difficult-to-kill bacteria have become a problem in hospital-acquired infections. Identifying compounds which kill these bacteria is of interest to many pharmaceutical companies. Developing and optimizing assays to screen for the efficacy of these compounds is a challenge faced by many microbiologists.

Here, we describe the setup of a bacterial growth assay using SoftMax® Pro 7.0 (or higher) Data Acquisition and Analysis Software. Both cell density and GFP signals were recorded over a time course using the Workflow Editor acquisition function. Various data transformation steps in the software are discussed, such as normalizing the GFP signal to cell density, as well as extracting growth rates or other kinetic relevant information.

## Materials

- *Enterococcus faecalis* strain OG1RF, containing:
  - Plasmid pMV158-GFP (green fluorescent protein)
- 96-well black-walled µClear microplate (Greiner Bio-One cat. #655096)
- qPCR seal (Eurogentec cat. #RT-OPSL)
- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3X) with:
  - SoftMax Pro 7.0 (or higher) Software

## Methods

### Data acquisition using the Workflow Editor

Bacterial growth data were captured using the SpectraMax® i3x Multi-Mode Microplate Reader at the Department of Preventive Dentistry at the Academic Centre for Dentistry (ACTA) in the Netherlands. Here, *Enterococcus faecalis* strain OG1RF containing plasmid pMV158-GFP was used at various growth conditions. GFP (green fluorescent protein) expression is heterologous, and the strain is described in the publication of Hoogenkamp et al[1], where GFP was studied as a viability marker for the difficult-to-stain species *E. faecalis*. 150 µl of *E. faecalis* in PBS, and PBS as background control, were pipetted into a 96-well black-walled µclear plate. To prevent evaporation, the microplate was

**Figure 1. SoftMax Pro 7 Workflow Editor for a dual read mode kinetic reading.** The drag and drop feature is easy to configure and enables a customized workflow to be set up quickly.

covered with a qPCR seal. The microplate was then placed in a SpectraMax i3x reader and incubated at 37°C for the duration of the kinetic measurement.

Using the SoftMax Pro Workflow Editor, a kinetic cycle containing an absorbance read (PlateOD600) and a fluorescence read (PlateGFPBottom) with a 5-second linear plate shake between reads was created (Figure 1). Absorbance was measured at 600 nm ($OD_{600}$) to determine the bacterial growth. GFP expression, an indicator of viability, was monitored using bottom-read fluorescence detection with excitation at 485 nm (bandwidth 9 nm) and emission at 515 nm (bandwidth 15 nm). Both data traces (absorbance and fluorescence) were recorded in two independent plate sections. The kinetic cycle was set to repeat once every 15 minutes for a total of 18 hours. Optional software features allow the user to pause and resume a kinetic read, enabling the addition of reagents during the run (not used in this experiment). The resulting dual read mode kinetic data were analyzed using SoftMax Pro Software.

## Data analysis options using the reduction settings dialogue

As an initial step of data analysis, the user must decide whether or not to define a blank. The blank well location was set using the template editor, which offers the choice of a group blank or plate blank. A plate blank is subtracted from the raw data of all sample wells in the plate at each timepoint, whereas a group blank is subtracted from associated sample wells only. In this experimental setup, buffer background traces were captured as a control for possible contamination with bacteria or other species. The buffer was not interfering with either the $OD_{600}$ or the GFP measurement, therefore no subtraction was required. If media or buffer components interfere with the $OD_{600}$ or fluoresce, a subtraction of the blank is recommended to retrieve the true signal values for either the absorbance or fluorescence channel. Also, this allows comparability of measurements.

Advanced kinetic data analysis is offered through the reduction settings dialogue. Figure 2 shows the reduction settings for the bacterial growth experiment. The menu is split into two sections: raw data and data reduction steps.

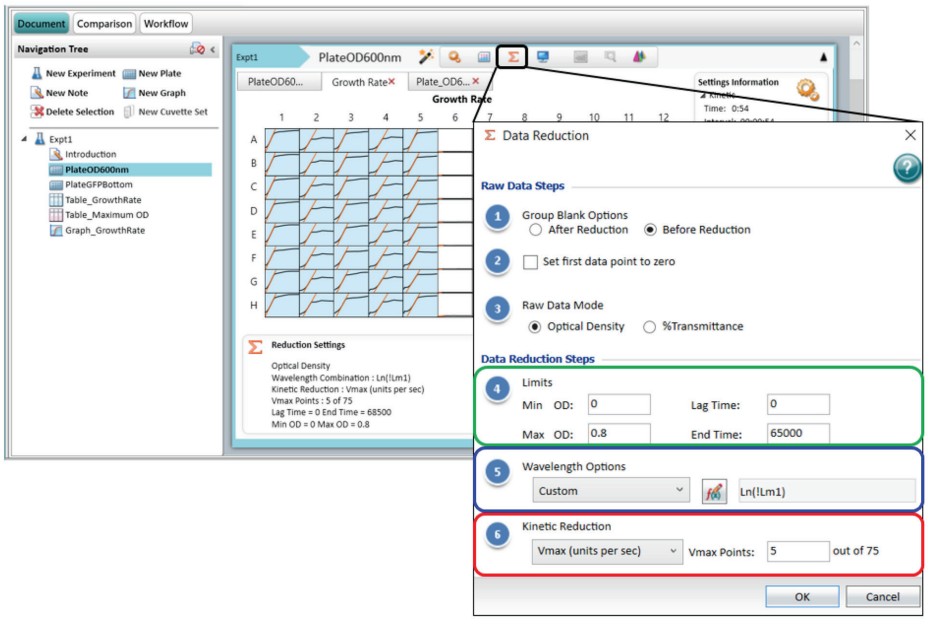


**Figure 2. Data analysis options in the Reduction Settings menu.** This example shows the logarithmic transformation of the optical density data in plate section 'PlateOD600nm' and the subsequent reduction to the Vmax rate value with 5 Vmax points to retrieve the steepest part of the curve.

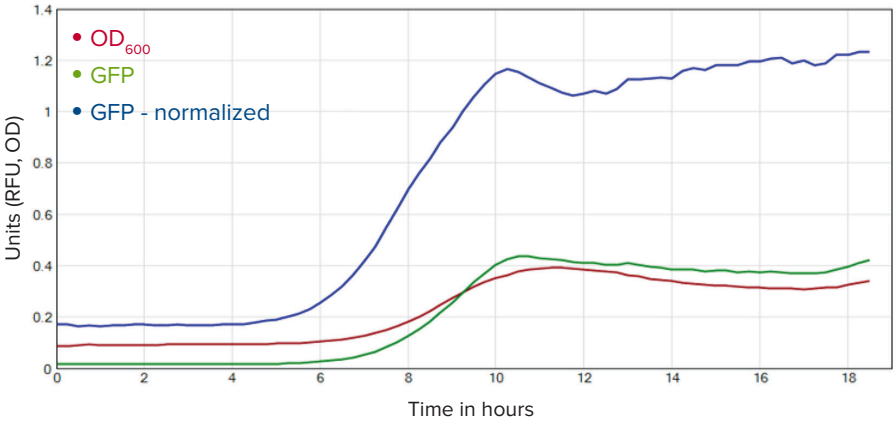


**Figure 3. GFP Signal normalized to cell density at $OD_{600}$.**

Raw data steps include the option to set the first data point for all kinetic traces to zero. Furthermore, a blank calculation can be included before or after the kinetic reduction. When 'before reduction' is selected, the averaged blank well(s) kinetic data trace is subtracted at each timepoint from all raw data sets individually. Choosing 'after reduction' subtracts the average of the reduced blank well data, such as Vmax rate, from the sample well data.

The data reduction steps and data output types comprise the following options (Figure 2):

- Limits: If required, limits restrict the range of kinetic data that is included in the data reduction analysis. Limits can be set for the signal-axis (OD, RLU, RFU) and/or the time-axis (seconds). The same analysis range is applied to all wells (Figure 2, green box).
- Wavelength Options: This transforms the signal-axis within the selected limits. By default, the first wavelength measured (named as !Lm1) is selected. Two examples of signal-axis transformation are shown here:
  - Normalization: If the two wavelength data traces are captured within the same plate section, the dropdown menu lists preconfigured selections such as ratio (Wavelength 1/ Wavelength 2 = !Lm1/!Lm2). In the example data described in this application note, the data traces were acquired in two separate plate sections, so a custom formula was required for ratiometric signal normalization. To normalize the GFP signal to the bacteria cell density, we entered a formula as follows: !KinPlot@PlateGFPBottom/!KinPlot@ PlateOD600. This was applied in either of the two plate sections. The formula !KinPlot extracted the kinetic data set of a specified plate section referenced with the symbol '@' and the corresponding name of the plate section. A comparison view of the raw data traces versus the transformed (normalized) data trace is shown in Figure 3.
  - Logarithmic Scaling: To linearize the exponential growth phase of the $OD_{600}$ data trace, a logarithmic transformation was applied in the

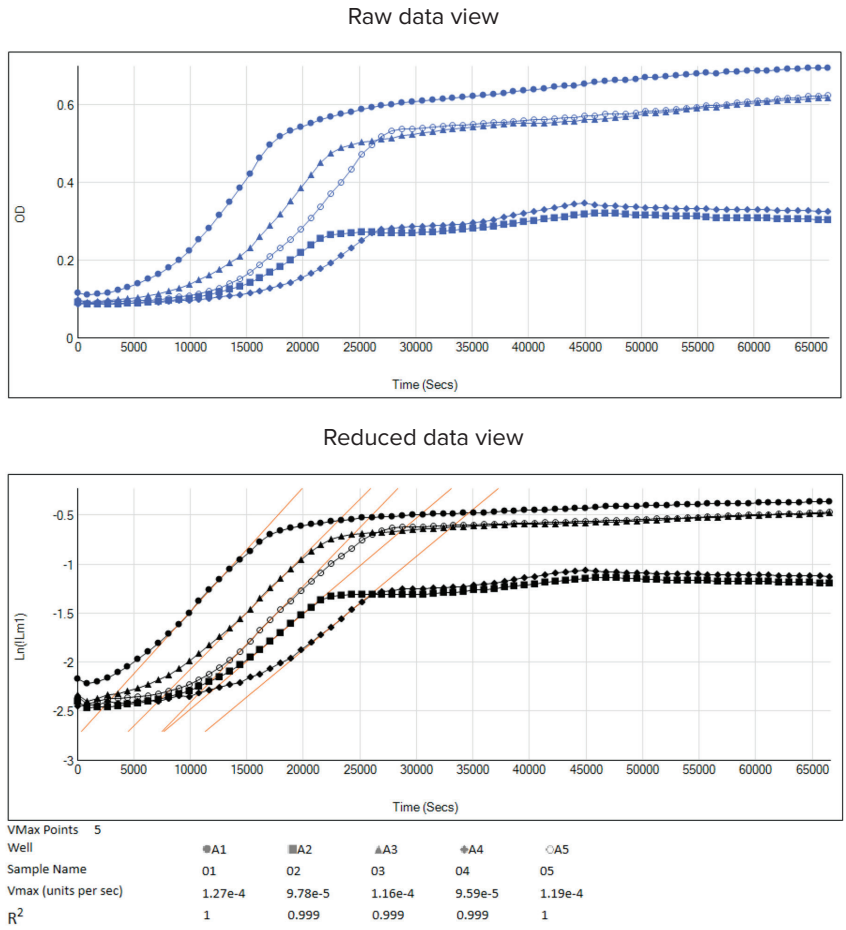


**Figure 4. Raw and reduced data view with logarithmic scaling applied to the $OD_{600}$ data.** Wells A1 to A5 are used as an example for each different experimental condition. **Top:** raw data of $OD_{600}$ kinetic data traces (blue lines). **Bottom:** transformed raw data with logarithmic scaling (black lines) and the determination of the growth rate in the exponential phase by applying a Vmax data reduction (orange line). Corresponding reduction settings are shown in Figure 2.

| Growth Condition | Vmax (units/second) | Growth rate (units/h) | Growth Rate StDev | Doubling time (h) |
|---|---|---|---|---|
| 1 | 1.23e-4 | 0.443 | 0.016 | 1.564 |
| 2 | 9.73e-5 | 0.350 | 0.004 | 1.979 |
| 3 | 1.10e-4 | 0.396 | 0.015 | 1.749 |
| 4 | 9.21e-5 | 0.332 | 0.010 | 2.090 |
| 5 | 1.01e-4 | 0.364 | 0.031 | 1.906 |

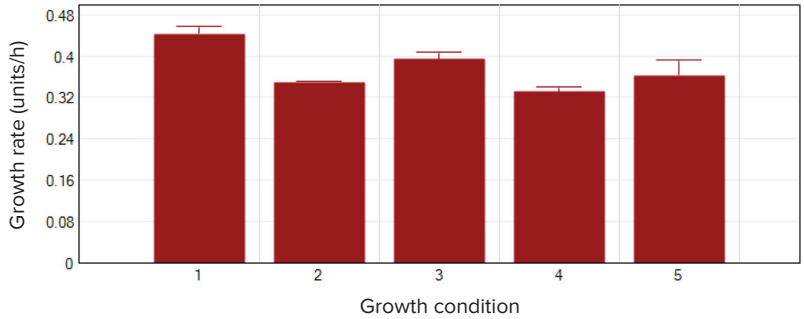


**Figure 5. Results display options.** The reduced data display of the plate section and results of each experimental condition of *E. faecalis* (Column 1 to 5, n=8) are summarized in the results table as wells as a bar graph. Vmax rate was used to retrieve both the growth rate (k=Vmax*3600) and doubling time (g=ln2/k).

$OD_{600}$ plate section. A custom formula was added using the natural logarithm by entering Ln(!Lm1) as shown in Figure 2 (blue box). The data comparison of raw and logarithmic scaling data is shown Figure 4.

- Kinetic Reduction: This step further transforms each data trace to a reduced single value such as Vmax rate or Onset Time. A full list and details of available preconfigured kinetic reduction parameters are listed and described in Table 1. The logarithmic scaling example above is used to describe kinetic reduction:
  - As a subsequent step of the logarithmic transformation of the $OD_{600}$ data trace, the maximum growth rate can be retrieved by extracting the Vmax rate as shown in Figure 2 (red box). Vmax rate offers the advantage for users to be able to adjust Vmax points that define the maximum size of the line segment that is used to determine the slope. The Vmax is shown as an orange line in the kinetic reduced plot (Figure 4, Reduced data view). To best compare reduced kinetic graphs of all wells in the plate section view, go to Display and select 'Reduced Data' with the option 'Plot'.

    For users to best compare different growth conditions as numeric results in a tabular view, the template editor tool enables assignment of wells to sample groups, and then presents the reduced data (Vmax rate) in a table as shown in Figure 5 (upper). Through subsequent calculation steps, both growth rate (k) and doubling time (g) are obtained. For better data visualisation, the results can be shown as a bar graph (Figure 5, lower) to more easily evaluate growth conditions or treatment effects.

| Kinetic reduction | Details |
|---|---|
| Vmax | Maximum slope of the kinetic trace either as milli-units per minute or units per second. The number of Vmax points defines the maximum size of the line segment used to determine the slope. |
| Time to Vmax | Time to Vmax elapsed time data is useful for applications including coagulation chemistry where the changing concentration of the reagents does not change Vmax, but rather changes the time at which the reaction reaches the maximum rate. |
| Slope | Is the same as Vmax rate (units per second) using all available datapoints within the reduction limits to determine the slope. |
| Onset time | Onset time is a method for analyzing non-linear Kinetic reactions. Onset time reports the time required for a kinetic reaction to reach a specified OD or RFU/RLU (onset OD/RFU/RLU). Useful for cascade reactions such as clot formation in endotoxin testing. |
| Time at minimum or maximum | This setting reports the time at the minimum or maximum OD, RFU/RLU, or %T that falls within the reduction limits. |
| Time at 1/2 maximum | This setting reports the time at half of the maximum OD, RFU/RLU, or %T that falls within the reduction limits. |
| Area under curve | This reduction estimates the area under the curve as defined by the data plots within the reduction limits. The data plots are treated as a series of trapezoids with vertices at successive data points and at the X-axis coordinates of the data points. The areas defined by each of the trapezoids are then computed and summed. |
| Minimum or maximum | This reports the minimum OD, RFU/RLU, or %T that falls within the reduction limits. |
| Max-Min | This reports the maximum subtracted from the minimum OD, RFU/RLU, or %T that falls within the reduction limits. |
| Mean | This reports the mean OD, RFU/RLU, or %T that falls within the reduction limits. |

**Table 1. Preconfigured kinetic reduction options.**

## Conclusion

The Workflow Editor in SoftMax Pro 7 Software, together with Molecular Devices multi-mode microplate readers including the SpectraMax i3x reader, offers the flexibility to record dual read mode kinetic growth data with optical density and fluorescence protein expression at the same time. Analysis is built into SoftMax Pro Software and offers a variety of data analysis options and transformation for bacterial growth data, such as data normalization or logarithmic scaling adjustment. A variety of kinetic data reduction parameters, including Vmax rate or Onset time, are preconfigured in the software. The desired data output parameters can be easily consolidated in table or plate format and graphed as bar graphs or scatter plots to support the evaluation of various experimental conditions.

### Reference


1. Hoogenkamp MA, Crielaard W, Krom BP. Uses and limitations of green fluorescent protein as a viability marker in Enterococcus faecalis: An observational investigation. *J. Microbiol. Methods* (2015) Aug;115:57-63.



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