Adaptation of a bacterial growth detection assay on the VICTOR Nivo multimode plate reader for measurement of antibiotic effects.

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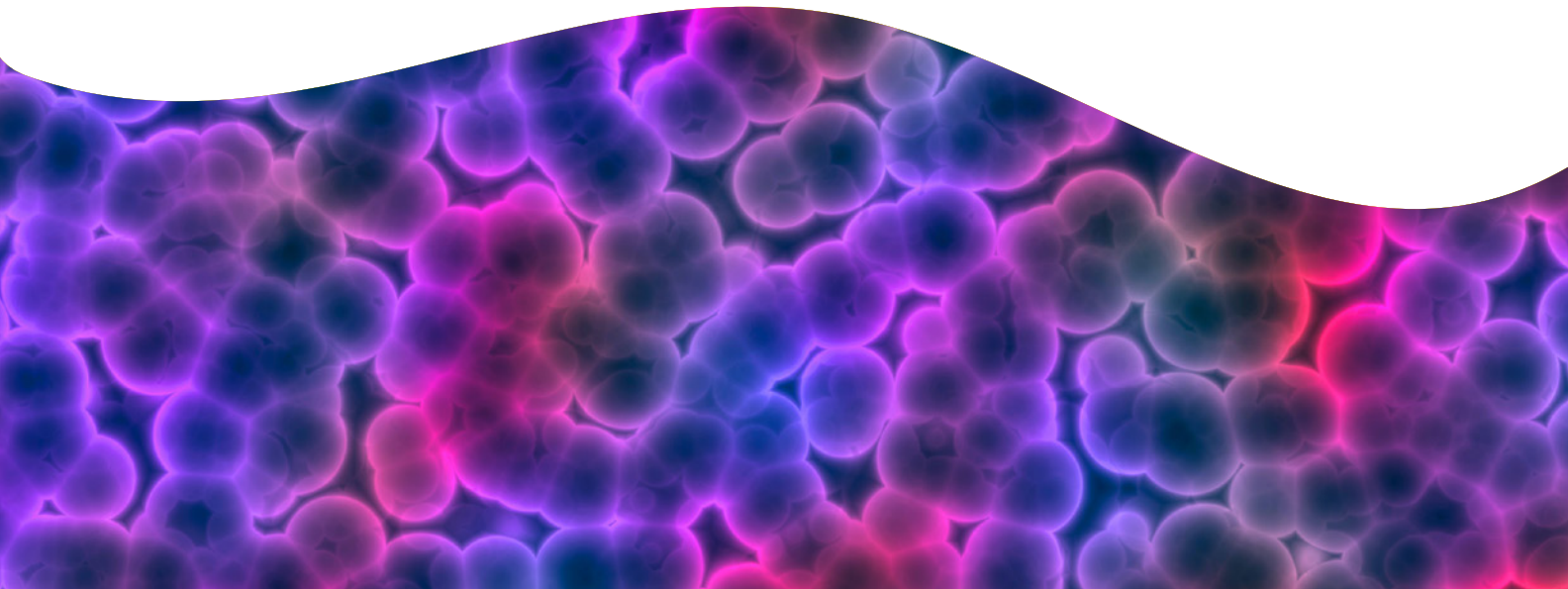Hamburg, Germany

Introduction

Monitoring of bacterial growth is a fundamental technique in microbiology. The most widely used method for enumerating bacteria is plate-based counting, which requires serial dilutions of the bacterial stock culture and manual counting of colonies after plating. The procedure is very time consuming and cost intensive, as plates must be prepared, inoculated and counted manually. Any serial dilutions with too many colonies for manual counting must be discarded. Assays also often require several replicates, rapidly increasing the amount of material and reagents. Running the assay in a microplate format and using a plate reader to measure bacterial growth greatly reduces consumables and waste. Automating sample preparation with an automated liquid handler helps to further reduce hands-on time and improve precision.

Here we present the adaptation of a microplate-based assay on the VICTOR® Nivo™ Multimode Reader for automated detection of bacterial growth assay, using the readout for optical density (OD)/turbidity. The integrated temperature control and shaking function of VICTOR Nivo allows the realization of controlled assay conditions, helping to prevent common issues such as condensation under the plate lid, and allowing continuous detection of changes in turbidity over time.

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Bacterial growth conditions

The E. coli strain Escherichia coli K12 (DSMZ, No. 498) was kindly provided by the School of Life Science Hamburg GmbH. Bacteria were grown overnight in Luria Bertani (LB) medium at 37°C and plated on non-selective agar plates.

To perform the growth assay, the initial bacterial number was determined. E. coli was inoculated from an overnight culture and incubated for 3 h at 37°C. At the end of the incubation time, 100 µL of bacterial suspension was transferred to a 96-well clear-bottom plate (ViewPlate-96 Black, Revvity, #6005185). The OD_{615} was measured to be 0.36. The suspension was diluted 1:5, resulting in an OD_{615} of 0.17 (background 0.085). This dilution was further diluted 1:20, serially diluted and plated in non-selective agar plates. 20 µL of each serial dilution was plated for calculation of the cfu/mL concentration (colony forming units/milliliter). Plating of the first dilution step at 1,000 cfu/mL resulted in overgrown colonies. The fourth dilution step resulted in formation of a countable number of colonies. 14 single colonies were observed, which represent $1.12x10^8$ cfu/mL (at an OD_{615} of 0.17). An OD_{615} of 0.01 means app. $1.3x10^7$ cfu/mL. Bacterial growth assays are typically run at a concentration of about $1X10^6$ cfu/mL, therefore the dilution of bacterial suspension used for the assay was adapted to reach these values.[1]

Bacterial growth assay

Bacterial growth and the effects of known antibiotics on the growth rate were observed under changing temperature and shaking conditions. Incubation at room temperature (22°C; RT) was compared to incubation at 37°C, as well as the growth under shaking condition to bacterial culture at rest, mixed for 1 min directly before measurement. The OD_{615} of an overnight culture inoculate was measured and the dilution was adapted to reach $1X10^6$ - $5x10^6$ cfu/mL. 200 µL of the bacterial suspension were transferred to a 96-well clear-bottom plate (ViewPlate-96 Black, Revvity, #6005185) and incubated for 24 h. In parallel, the number of cfu/mL was determined as described previously (Table 1). Amoxillin (VWR, #SAFF31586) and Levofloxacin (VWR, #J66943.03) were added to wells using the Echo® 550 Liquid Handling System (Labcyte, Inc.) before plating bacteria.
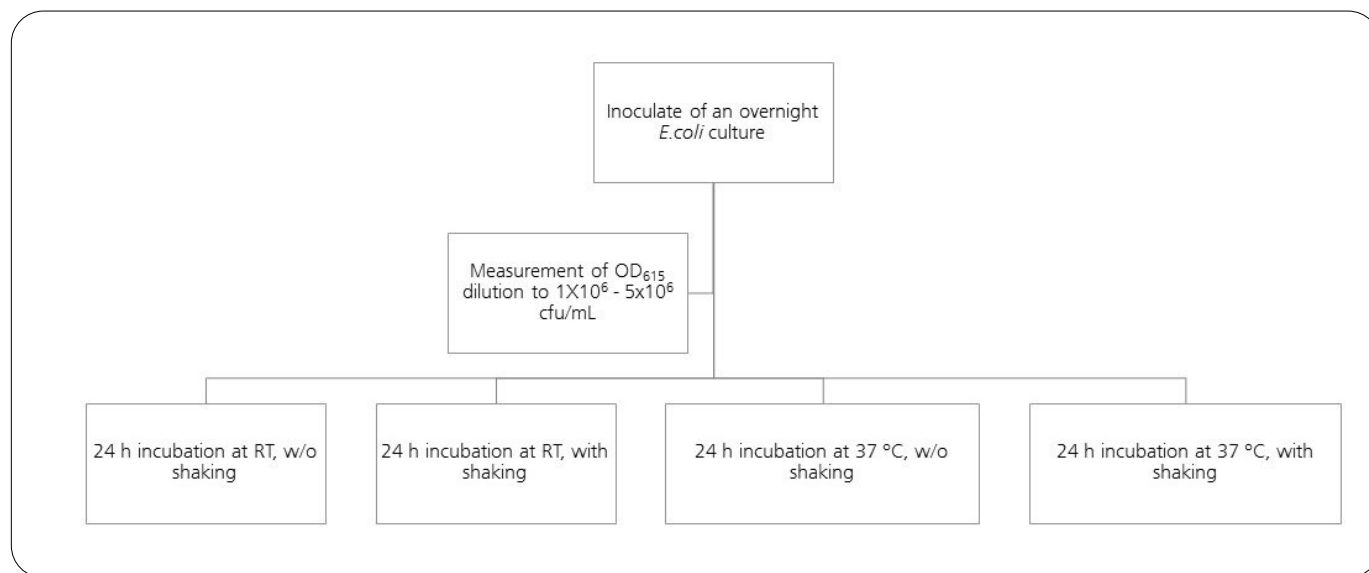

Figure 1. Flowchart of performed bacterial growth assay. After measurement of the OD_{615} of an E. coli overnight culture inoculate and adaptation to the required dilution 200 µL of the bacterial suspension were analyzed at one of the displayed conditions.
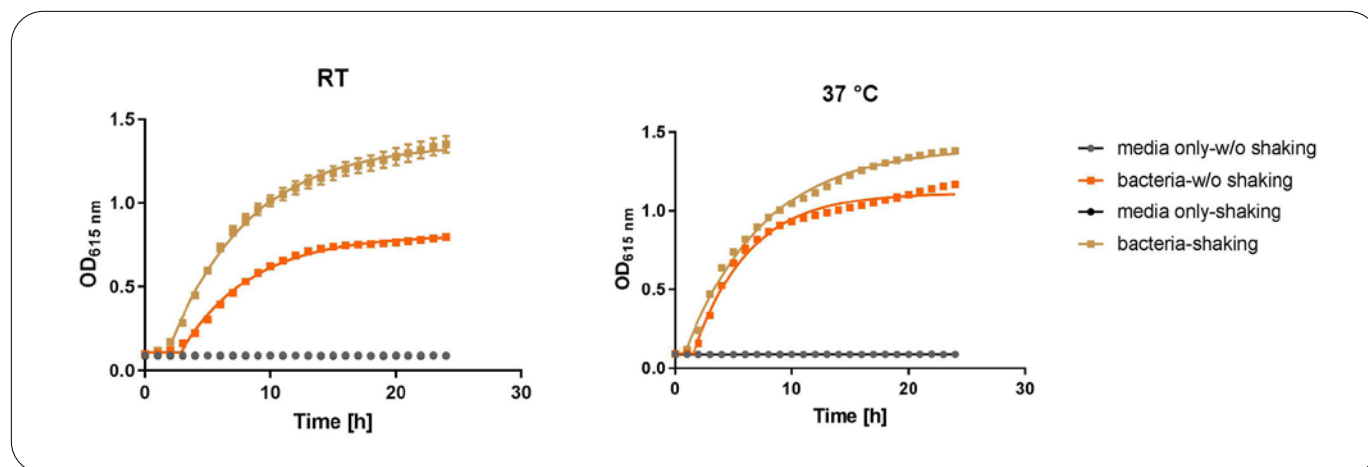
Figure 2. Diagram of bacterial growth at room temperature or 37°C with or without shaking conditions.

Table 1. Determined bacterial stock concentration (cfu/mL) for the growth assay at room temperature or 37°C with or without shaking.

Assay Conditions	cfu/mL
RT; w/o shaking	2.00E+06
RT; shaking	4.00E+06
37°C; w/o shaking	7.50E+06
37°C; shaking	2.00E+06

The obtained curves show the typical three phase growth progression of a bacterial culture with an initial lag-phase, followed by exponential/log growth and a stationary phase. The increase of temperature to 37°C and shaking shorten the lag-phase, reducing the time until the start of the log-phase, and increased the overall growth, observed as the increase of detected OD_{615} span between t_0 and plateau (Figure 2, Table 2). The slightly increased rate constant k without shaking indicates a putative faster growth, but the overall growth according to detected OD values is lower. The observation can be due to improved nutrients and oxygen distribution and the reduction of temperature gradient allowing for continued growth. The growth maximum could be reached faster without shaking, which stopped the growth earlier due to resource limitations.
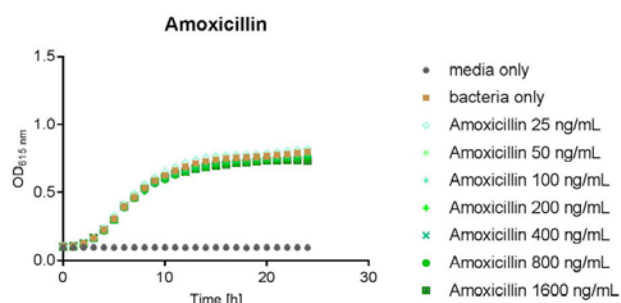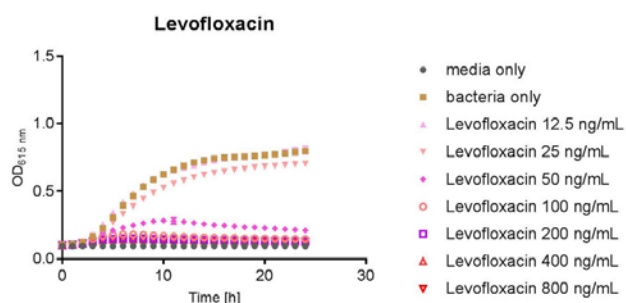
Levofloxacin and Amoxillin were used to test the performance of the assay and to observe antibiotic related effect on bacterial growth. Levofloxacin is a broad-spectrum antibiotic of the fluoroquinolones group used for treatment of pneumonia, urinary tract infections, and chronic prostatitis. The bactericide function is due to the inhibition of the DNA gyrase and topoisomerase II and IV of gram-positive and gram-negative bacteria. Amoxillin is a broad-spectrum antibiotic of the beta-lactam family used for treatment of the pneumonia, skin infections and urinary tract infections. It is active against gram positive as well as some gram-negative bacteria.

The data showed that Amoxicillin did not inhibit the growth of E. coli at all tested conditions and concentrations. This is in line with literature values and served as an internal control for contaminations while the assay was performed. Levofloxacin showed differences in activity depending on the incubation temperature and shaking conditions (Figure 3). The increase of temperature to 37°C seemed to reduce the sensitivity of the bacteria to Levofloxacin, and shaking resulted in slight increase of inhibition potency (Figure 4). This is due to mproved growth and increased metabolic activity at 37°C, meaning that more cells must be inhibited, and these cells contain more targets. The obtained EC_{50} of 40-67 ng/mL values are in line with reported MIC_{50} of 64 ng/mL, both values comparing the minimum inhibitory concentration where 50% of isolates are inhibited.[2]
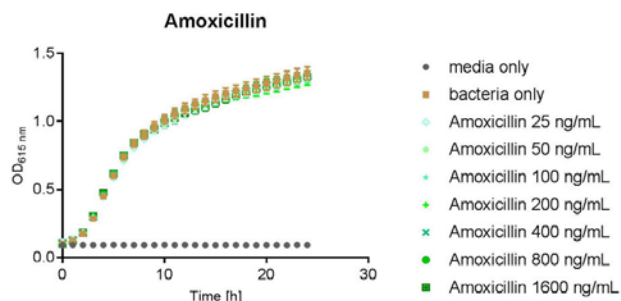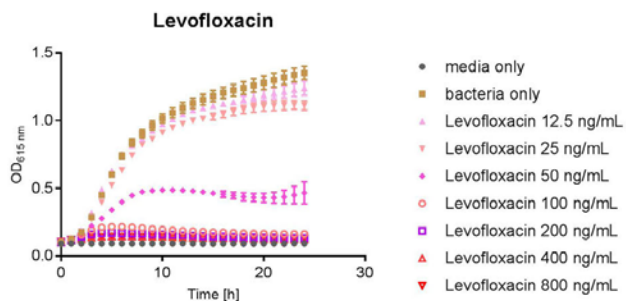
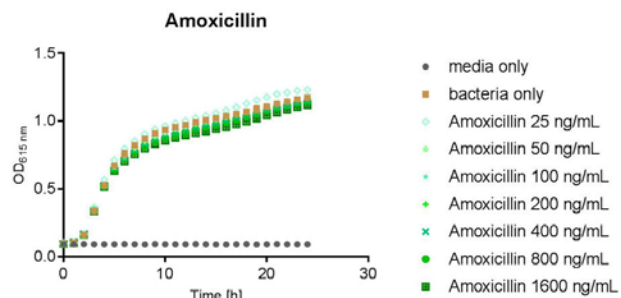**Table 2. Calculated key values of E. coli culture growth at RT, 37°C with or without shaking.**

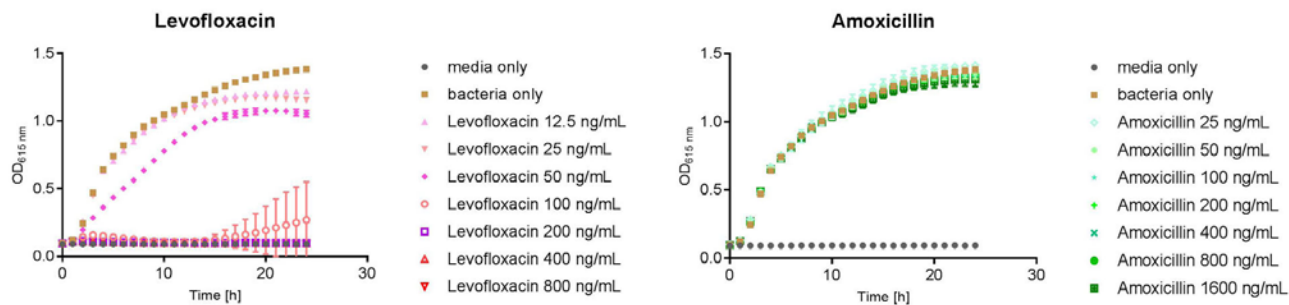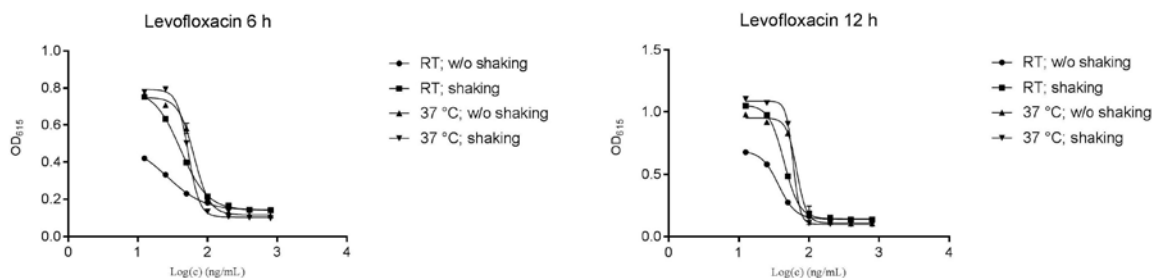|	RT; w/o shaking	RT; shaking	37°C; w/o shaking	37°C; shaking
t_0 log phase start (in h)	2.85	1.79	1.59	0.82
OD_{615} at t_0	0.11	0.11	0.1	0.1
Plateau	0.81	1.36	1.11	1.4
k	0.18	0.16	0.21	0.15
Span (OD_{615} plateau- OD_{615} t_0)	0.7	1.25	1.02	1.3
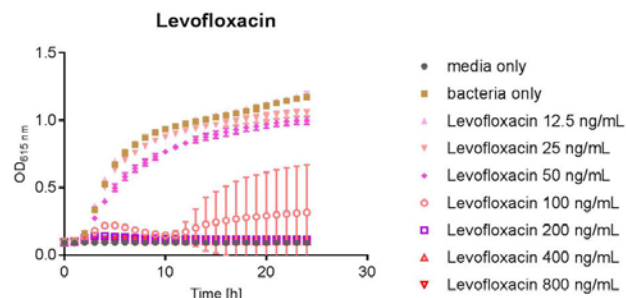
| Figure 3. Inhibition of bacterial growth at room temperature or 37°C with or without shaking conditions using Levofloxacin and Amoxicillin.

| Figure 4. Inhibition of bacterial growth at room temperature or 37°C with or without shaking conditions using Levofloxacin detected in the exponential/log phase (6 h) and beginning of the stationary phase (12 h). Data obtained at room temperature, w/o shaking were extrapolated, as the curve fit does not represent a complete sigmoidal shape.
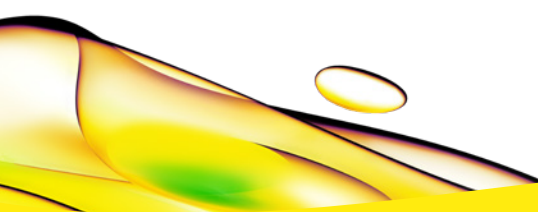
Conclusion

Analyzing of bacterial growth is often time- and material-intensive. Here we showed the adaptation of a microplate-based assay on the VICTOR Nivo™ Multimode Reader for analysis of bacterial growth and antibiotic related effects.

The integrated temperature control and shaking function allowed precise adaptation of conditions for bacterial growth. The control of temperature over the plate prevented the condensation and allowed a homogenous signal detection. Therefore, the kinetics of antibiotic effect on E. coli growth could be observed precisely. In summary the readout reported inhibition values for the antibiotic Levofloxacin that are in line with reported data and allowed a fast and precise quantitative analysis.

References

1. Campbell, J. (2011). High-throughput assessment of bacterial growth inhibition byoptical density measurement. Curr Protoc Chem Biol.

2. Madhusudhana K.T, C. C. (2003). Comparative *in vitro* Activity of Three Fluoroquinolones against Clinical Isolates by E Test. Chemotherapy, 184-188.

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