Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Evaluation and validation of Biolog OmniLog® system for antibacterial activity assays

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Significance and Impact of the Study: The determination of minimal inhibitory concentration of drugs and screening of novel antimicrobial compounds are common practices in clinical and research settings. In this work, the OmniLog® system, developed for the identification and metabolic fingerprinting of micro-organisms, was evaluated and validated for antibacterial assay performance. For the three antibiotics tested, OmniLog showed similar results when compared, in parallel, to the standard methodology defined by the Clinical Laboratory Standards Institute. OmniLog offers an option of a flexible, walk-away and label-free system, ideal for increasing the throughput of screening compound libraries for potential antimicrobial activity.

Keywords
antibiotics, antimicrobials, quality control, pharmaceuticals, Biolog OmniLog®.

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2020/2407: received 9 November 2020, revised 22 December 2020 and accepted 23 December 2020

doi:10.1111/lam.13450

Abstract

Minimal inhibitory concentration of antimicrobials, determined by the broth microdilution method, requires visual assessment or absorbance measurement using a spectrophotometer. Both procedures are usually performed manually, requiring the presence of an operator to assess the plates at specific time point. To increase the throughput of antimicrobial susceptibility testing, and concurrently convert into an automatic assay, the Biolog OmniLog® system was validated for a new, label-free application using standard 96-well microplates. OmniLog was evaluated for its signal strength to ensure that the signal intensity, detected and measured by the system's camera, was satisfactory. Variability due to the plate location inside the OmniLog incubator, as well as variation between wells, was investigated. Then the system was validated by determining the minimal inhibitory concentration of ciprofloxacin, piperacillin and linezolid against a selected Gram-negative and Gram-positive strains. No significant difference was observed in relation to position of the plates within the system. Plate edge effects were noticeable, thus the edge wells were not included in further experiments. Minimal inhibitory concentration results were comparable to those obtained by conventional protocol as well as to values defined by the Clinical Laboratory Standards Institute or published in the literature.

Introduction

Methodologies that are utilized for antibacterial activity assessment of compounds typically rely upon conventional microbiological assays, based on broth macro- or micro-dilution or agar disc diffusion approaches. These procedures are defined by the Clinical and Laboratory Standards Institute guidelines (CLSI 2012; CLSI 2015) and they remain as current standards in research and clinical settings. The interpretation and conclusion derived from antibacterial assay data are done by visual assessment, absorbance reading using a spectrophotometer, or manual measurement of inhibition zones. An operator, who must be present and assess the results at specific times, performs all these procedures. Automated systems available in the market, such as the Sensititre Antimicrobial Susceptibility Testing System (Thermo Fisher Scientific; Chapin and Musgnug 2004) are suitable for determining minimal inhibitory concentration (MIC) of standard antibiotics with most clinical relevance by

providing pre-configured plates. However, they do not offer much flexibility to study novel compounds for antibacterial properties.

The Biolog OmniLog® instrument (Fig. 1a) is a fully automated platform for micro-organism identification and phenotypic analysis of microbial and mammalian cells, and thus a walk-away instrument (https://www.biolog.com/). OmniLog is typically used with pre-configured plates, which is the basis for the patented redox technology (i.e. reduction of tetrazolium dye present in the wells and formation of a strong colour). Image-based readings of colour intensity are taken every 5 to 15 min, and data reported as OmniLog units. In laboratory research settings, the OmniLog is used for a range of assays in vision of the versatility of pre-configured plates available (Sandle *et al.* 2013; Blanco *et al.* 2018; Dunkley *et al.* 2019), thus illustrating its accessibility for researchers. The instrument allows for up to 50 plates to be assessed in parallel, thus it also provides an increase in the throughput generated within a day's work. MIC is a key indicator of an antimicrobial agent's potency, and it is defined as the concentration at which growth of bacteria is prevented (Wiegand *et al.* 2008). Thus, with these premises in mind, the OmniLog system was evaluated and validated for a new, label-free application for antibacterial activity assessment by the determination of MICs, using standard microtiter plates.

Results and discussion

Signal strength (SS) assessment

Four strains were tested for SS, and assays' quality parameters Signal to Noise (S/N), Signal to Background (S/B), and Z factor (Z') were calculated (Table 1). S/N and S/B were all ≥2, which falls within acceptance range (Inglese *et al.* 2007). Z' was also satisfactory for the majority of the strains tested (i.e. $Z' \geq 0{\cdot}4$; Iversen *et al.* 2004), except for *Enterococcus faecalis*. Enterococci have a slow growth pattern in Cation-adjusted Mueller-Hinton broth (CAMHB), which directly affects mostly Z' values. Thus, further testings for strains belonging to this genus were performed using Brain Heart Infusion (BHI) instead, previously shown to provide optimum growth (Wiegand *et al.* 2008).

Plate uniformity (PU) assessment

A scatter plot can reveal patterns of drift, edge effects and other sources of variability. The response is plotted against well number, where the wells are ordered by row first, then by column. In this study, edge effect was observed equally in all plates, independently of the bacterial strain tested or the position in which the plate was located inside the OmniLog incubator (Fig. 1). Edge effects are characterized by an increase in the variability within replicate wells, thus affecting the performance of the assay. One of the factors that contributes to edge effects is the unequal evaporation rate from outer wells, typically observed when assays are performed over long period of time (e.g. 24 h), high temperatures (e.g. 37°C) and low volumes (e.g. 200 μl). This phenomenon has been described a long time ago, in different types of assays using microplates (Kricka *et al.* 1980; Oliver *et al.* 1981). Some techniques and specialized plates with an outer moat insulate zone have shown to decrease this effect (Lundholt *et al.* 2003). Still, a common practice is to refrain from the use of outer wells. The latter approach was chosen for further assays (i.e. MIC determination).

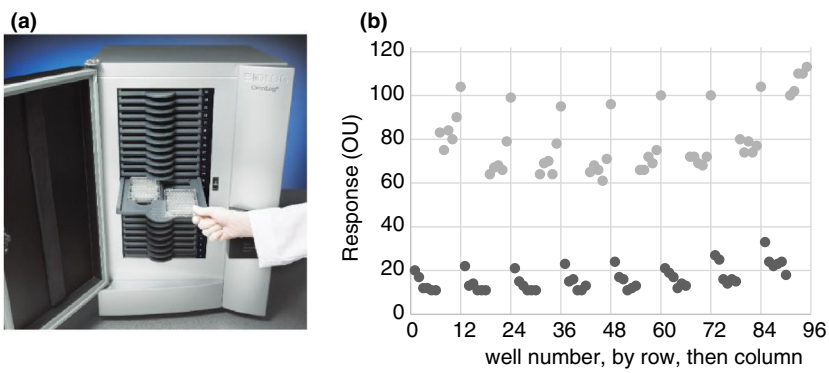

Figure 1 (a) Interior of OmniLog® system, illustrating 25 trays (rows) and two positions (columns). Reproduced by permission from Biolog, Inc. (b) Representative scatter plot showing edge effect trends on *Escherichia coli* ATCC 25922 plate at position 1a. Response is provided as OmniLog Units (OU). The concave trend (bowl-shape) of the max values shows that the outer rows of the assay plate displayed the highest signals, with decreasing intensity moving towards the center rows. Linear edge effect, with the highest signal observed in the last column. (●) MAX: maximum signal (i.e. bacterial growth) and (●) MIN: minimum signal (i.e. media). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Average of quality parameters in the signal strength assessment assays

	Quality parameters* (±SD)		
Strain	S/B	S/N	Z'
Escherichia coli 25922	4·5 (±0·2)	9·0 (±0·5)	0·5 (±0)
Enterococcus faecalis 29212	3·3 (±0·2)	5. 3(±0·5)	0·2 (±0·1)
Pseudomonas aeruginosa 27853	9·5 (±0·6)	19·7 (±1·3)	0·8 (±0)
Staphylococcus aureus 29213	3·5 (±0·1)	5·9 (±0·3)	0·4 (±0)

S/B: signal to background; S/N: signal to noise; Z': Z' factor; SD: standard deviation.
*Calculations followed the formulas of Inglese *et al.* (2007). Experiments were performed once with six replicate plates per strain tested.

In this study, six replicate plates were placed in different trays and columns to better assess any positional variability. All results complied with the criteria defined by Iversen *et al.* (2004) of coefficient of variation of the mean (CV) <20% within rows, columns and within plates (Table 2), when excluding edge wells from calculations. Therefore, it was concluded that plate position within the OmniLog incubator did not affect the performance of the assay.

MIC determination

To validate the OmniLog system for the determination of antibacterial activity, MIC assays were performed. For ciprofloxacin, equal MICs were obtained for all the strains ($n = 4$) tested simultaneously using two different incubation systems and signal measurements (Table 3). Moreover the assay performance, determined by the calculation of Z', was also acceptable ($Z' \geq 0{\cdot}4$). Most of the remaining strains (6/7), only tested using the OmniLog system, further confirmed matching MICs when compared to previous results obtained in our group. *Enterococcus faecium* 35667 MIC was 2-fold lower when using OmniLog system. Piperacillin's MICs were also similar between the methods, albeit 3/11 strains presented a 2-fold lower MIC when using the OmniLog system (Table 4). The variability of 2-fold in MIC value do not account for significant difference and fall within expected intra-laboratory variability, as previously described by Mouton *et al.* (2018).

OmniLog system provides a good separation band between highest and lowest assay readouts, allowing for distinction between bacterial growth and inhibition, without the need of using pre-configured plates or dyes. Moreover OmniLog incubator's uniformity within and between plates is also satisfactory, albeit exclusion of edge wells is necessary. Edge effect is also phenomenon commonly observed in cell-based assays, using conventional incubators and spectrophotometers as detection instruments. OmniLog capacity of 50 plates is ideal for high-throughput performance. The flexibility to use standard microtiter plates also provides the user with an open and economic method. A possible limitation of the system is interference when testing strongly coloured compounds. In this scenario, visual inspection for MIC determination is required. In conclusion, the OmniLog system can be used as a platform with fully automated plate incubation and signal readings for antibacterial assays.

Materials and methods

Bacterial strains and reagents

Clinical control strains belonging to the ESKAPE (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,

Table 2 Average of maximum signal (OmniLog units) of bacterial growth and coefficient of variation (%)

	Signal (OU)									
	Columns				Rows					
Plate position	A		B		1		12		24	
Strain	Max*	CV†	Max	CV	Max	CV	Max	CV	Max	CV
Escherichia coli 25922	71·1	1·5	69·8	0·4	70·2	0·0	71·0	2·5	70·2	1·2
Enterococcus faecalis 29212	48·1	2·6	47·7	1·5	48·0	0·7	47·0	0·4	48·7	2·3
Pseudomonas aeruginosa 27853	135·8	0·5	134·1	0·4	134·3	0·8	135·1	1·1	135·4	0·8
Staphylococcus aureus 29213	51·2	1·5	50·8	2·5	50·7	1·0	50·5	3·1	51·8	0·2

*Max: maximum signal (i.e. bacterial growth) in OmniLog units (OU).
†Coefficient of variation (CV) calculation was done according to Iversen *et al.* (2004). Experiment was performed once with six replicate plates per tested strains.

Table 3 MIC values in μg ml^{-1} (Z') of ciprofloxacin and linezolid

Strain	Reference range	*In-house**	Visual reading	Absorbance†	OmniLog
Acinetobacter baumannii 19606	1[a]	1[g]	1	–	1 (0·9)
Enterobacter aerogenes 13048	0·125–0.5[b]	0·031[g]	0·031	–	0·031(0·8)
Escherichia coli 25922	0·004–0·016[c,d]	0·016[g]	0·016	0·016 (0·9)	0·016 (0·8)
Enterococcus faecalis 29212	0·25–2[c,d]	1[h]	1	1 (0·8)	1 (0·6)
VR *E. faecalis* 51575	0·25–2[d]	0.5[h]	0·5	–	0·5 (0·7)
Enterococcus faecium 35667‡	2–4[d]	4[h]	2	–	2 (0·6)
VR *E. faecium* 700221‡	2–4[d]	2[h]	2	–	2 (0·6)
Klebsiella pneumoniae 700603	0.5[e]	0.5[g]	0·5	–	0·5 (0·8)
Pseudomonas aeruginosa 27853	0·25–1[c,d]	0.5[g]	0·5	0·5 (0·9)	0·5 (0·8)
Staphylococcus aureus 29213	0·125–0.5[c,d]	0.5[h]	0·5	0·5 (0·7)	0·5 (0·6)
MR *S. aureus* 43300	0.5[f]	0.5[h]	0·5	–	0·5 (0·6)

VR: vancomycin-resistant; MR: methicillin-resistant.

[a]Hamouda and Amyes (2006); [b]Thiolas *et al*. (2005); [c]CLSI (2015); [d]EUCAST (2020); [e]Rasheed *et al*. (2000); [f]Masadeh *et al*. (2016); [g]Tiz *et al*. (2019); [h]Cruz *et al*. (2018).

*Minimal inhibitory concentration previously determined by our group.

†Values determined concurrently with OmniLog. Z'-factor (Z') was calculated according to Inglese *et al*. (2007).

‡Linezolid was used for these strains instead of ciprofloxacin

Acinetobacter baumannii, *Pseudomonas aeruginosa* and *Enterobacter* spp.) panel and additional strains were tested. *A. baumannii* American Type Culture Collection (ATCC) 19606, *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *E. faecalis* ATCC 29212, vancomycin-resistant *E. faecalis* ATCC 51575, *E. faecium* ATCC 35667, vancomycin-resistant *E. faecium* ATCC 700221, *K. pneumoniae* ATCC 700603, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213, methicillin-resistant *S. aureus* 43300 were obtained from Microbiologics Inc. (St. Cloud, MN). The ESKAPE panel is a group of bacterial pathogens which exhibits multidrug resistance and are highly virulent. Piperacillin sodium salt and linezolid was obtained from Sigma-Aldrich (St. Louis, MO). Ciprofloxacin HCl was purchased from ICN Biomedicals Inc. (Irvine, CA). CAMHB, Mueller-Hinton agar (MHA), Lysogeny broth agar and BHI were purchased from Labema (BD). Antibiotics were dissolved in sterile ultrapure water, filter-sterilized with PES 0·2 μm filter (VWR International, Radnor, PA) and stored at −20°C.

OmniLog system

The OmniLog system (Biolog, Hayward, CA) includes an incubator and a charged-coupled device camera system. The instrument has a test capacity of 50 plates, which are placed in 25 trays, numbered 1 to 25, and two columns (i.e. a and b; Fig. 1a). The incubation temperature range is from 22° to 45°C, with a temperature consistency of ±2°C.

The OmniLog digital camera measures the colour level of each well in OmniLog Units (OU), a proprietary scale that measures light transmission. An OU of 0 represents a 100% of light transmission, while an OU of 500 represents a 0% transmission. OU are comparable to optical density (OD), and can be calculated as 500 times the OD.

Inoculum preparation

Overnight cultures were prepared on MHA plates. Briefly, few colonies were taken from overnight agar culture, inoculated into 0·9% saline solution and vortexed to ensure that the bacterial suspension was homogeneous. Bacterial suspensions were measured using a densitometer (DEN-1, BioSan, USA) and adjusted to 1×10^6 CFU per ml by diluting with CAMHB or BHI (CLSI 2015).

SS and PU assessment

Signal strength and PU assays were performed to ensure that the signal obtained using the OmniLog system was adequate to detect antibacterial activity (i.e. growth or no growth) and whether significant variability within replicate plates and wells occur. To do so, four representative strains (*E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213) were selected and tested in clear flat bottom 96-well Nunc plates (Thermo Fisher Scientific; six plates/strain). Half of the assay plates (i.e. 48 wells) contained 200 μl per well of CAMHB (min wells) and the other half contained 100 μl per well of CAMHB and 100 μl per well of 1×10^6 cells per ml bacterial suspension (max wells). Plates were placed in the centre, top, and bottom trays located inside incubation chamber (Fig. 1a), thus

Table 4 MIC values in μg ml^{-1} (Z') of piperacillin

Strain	Reference range	Visual reading	Absorbance*	OmniLog
Acinetobacter baumannii 19606	32[a]	32	32 (1·0)	32 (0·9)
Enterobacter aerogenes 13048	4[b]	8	8 (1·0)	4 (0·8)
Escherichia coli 25922	1–4[c]	4	4 (1·0)	2 (0·8)
Enterococcus faecalis 29212	1–4[c]	2	2 (0·9)	2 (0·6)
VR *E. faecalis* 51575	–	4	4 (0·9)	4 (0·5)
Enterococcus faecium 35666	–	4	4 (0·8)	4 (0·6)
VR *E. faecium* 700221	–	>128	>128 (0·9)	>128 (0·6)
Klebsiella pneumoniae 700603	>128[d]	128	128 (1·0)	128 (0·7)
Pseudomonas aeruginosa 27853	1–8[c]	8	8 (1·0)	4 (0·8)
Staphylococcus aureus 29213	1–4[c]	2	2 (0·9)	2 (0·9)
MR *S. aureus* 43300	–	64	64 (0·9)	64 (0·8)

VR: vancomycin-resistant; MR: methicillin-resistant; –: information not found.

[a]Malone and Kwon (2013), [b]Yigit et al. (2002), [c]CLSI (2015), [d]Rasheed et al. (2000).

*Values determined concurrently with OmniLog. Z'-factor (Z') was calculated according to Inglese *et al.* (2007).

distributing the plates throughout the incubator (i.e. positions 1A, 1B, 12A, 12B, 24A and 24B). Plates were incubated for 24 h at 37°C. Prior to performing the assay, the OmniLog system was calibrated for the plate type used.

Signal calculations and plate acceptance criteria

The overall requirement was that the data generated and recorded by OmniLog had a significant separation between the max (maximum growth) and min (no growth) signals and that results from replicate plates and wells were similar. Average, standard deviation (SD) and CV for each signal (max and min), on each plate, were calculated (Iversen *et al.* 2004). The acceptance criterion was defined according to Iversen *et al.* (2004) where the average CV's of each signal should be ≤20%. Furthermore, S/N, S/B and Z' for each plate was also calculated (Inglese *et al.* 2007). S/N is a measure of the strength of the signal generated in the assay, thus indicating the degree of confidence with which a signal can be regarded as real, while S/B indicates if the level of the assay's signal is distinguishable from the level of the background. Nevertheless, these ratios cannot be used uniquely to measure the quality of the assay since neither of them take into account the variability within the sample and background measurements, and the signal dynamic range. Z' is the assay's performance indicator that measures the assay signal adjusted for assay variability. The recommended acceptance criteria for the above-mentioned assay quality parameters are: S/B and S/N ≥ 2, and $Z' \geq 0{\cdot}4$ (Iversen *et al.* 2004; Inglese *et al.* 2007). The following equations were used: $Z' = 1 - [(3SDs + 3SDb)/|Xs - Xb|]$, $S/B = Xs/Xb$ and $S/N = (Xs - Xb)/\sqrt{(SDs^2 + SDb^2)}$, where Xs represents the average of the signal obtained from samples exhibiting maximum signal and SDs the related standard deviation, and Xb and SDb represent the average and standard deviation of the signal obtained from min wells.

MIC determination

To establish the accuracy of OmniLog system in determining antibacterial activity, MIC values of standard antibiotics, ciprofloxacin (fluoroquinolone) and piperacillin (β-lactam) were determined against a selection of laboratory reference Gram-positive and Gram-negative strains. MIC values were determined according to the broth microdilution method in 96-well plate described in the CLSI guidelines (CLSI 2015), except for the incubation and signal acquisition procedures, which was done using the OmniLog incubator and its coupled device camera system respectively. Ciprofloxacin was tested for all the strains except for both *E. faecium* strains, where linezolid (oxazolidinone) was used. Piperacillin was tested for the full bacterial panel. For enterococci, assays were performed in BHI medium, due to insufficient growth observed when grown in CAMHB.

For four strains, *E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, two plates were equally prepared, for each strain, by adding 100 μl per well of bacterial suspension and 100 μl per well of 2-fold antibiotic serial dilutions. Antibiotic dilution range was selected according to CLSI guidelines (CLSI 2015). One plate was incubated at 37°C with shaking (500 rev min^{-1}) on Thermo-Shaker (PST-60HL-4, Biosan), following the standard methodology while the other was placed in the OmniLog incubator at the same temperature, both for 24 h.

Other strains were tested only using the OmniLog system and compared with previous MIC data obtained by our group (Cruz *et al.* 2018; Tiz *et al.* 2019). Piperacillin was tested using both methodologies for all strains mentioned above, since no previous MIC determinations were performed in our group with this antibiotic. For the

OmniLog approach, the distribution of media and diluted antibiotic in microplates was prepared using Biomek i7 automated Workstation (Beckman Coulter, Brea, CA), with only the addition of bacterial suspension performed manually. Edge wells were excluded (plate edges were filled with 200 μl media per well), based on the high variability observed during SS assessment.

In addition to visual inspection, the inhibition of bacterial growth was calculated based on absorbance measurements (OD_{620nm}) at 24 h using Multiskan Go plate reader (Thermo Fisher Scientific, Vantaa, Finland) for the conventional methodology. Absorbance measurements allow for the calculation of the quality assay parameters, as described above. Plates incubated in the OmniLog incubator were also visually assessed and values obtained from OmniLog readings (OU) were also converted into percentage of growth inhibition. The lowest concentration that resulted in ≥90% inhibition of bacterial growth was defined as the MIC. Two independent experiments in triplicate were performed.

Acknowledgements

This work was supported by the Academy of Finland for funding (Grant no. 319860). The authors thank the DDCB core facility supported by the University of Helsinki (HiLIFE) and Biocenter Finland. Special thanks to the Erasmus + Programme of the European Union for financial support for author PE.

Conflict of Interest

The authors declare that they have no conflict of interest.

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