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Research Article

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Posted Date: September 30th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7643728/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at World Journal of Microbiology and Biotechnology on February 27th, 2026. See the published version at <https://doi.org/10.1007/s11274-026-04805-4>.

High-Throughput Antimicrobial Efficacy Tests on a 384PillarPlate

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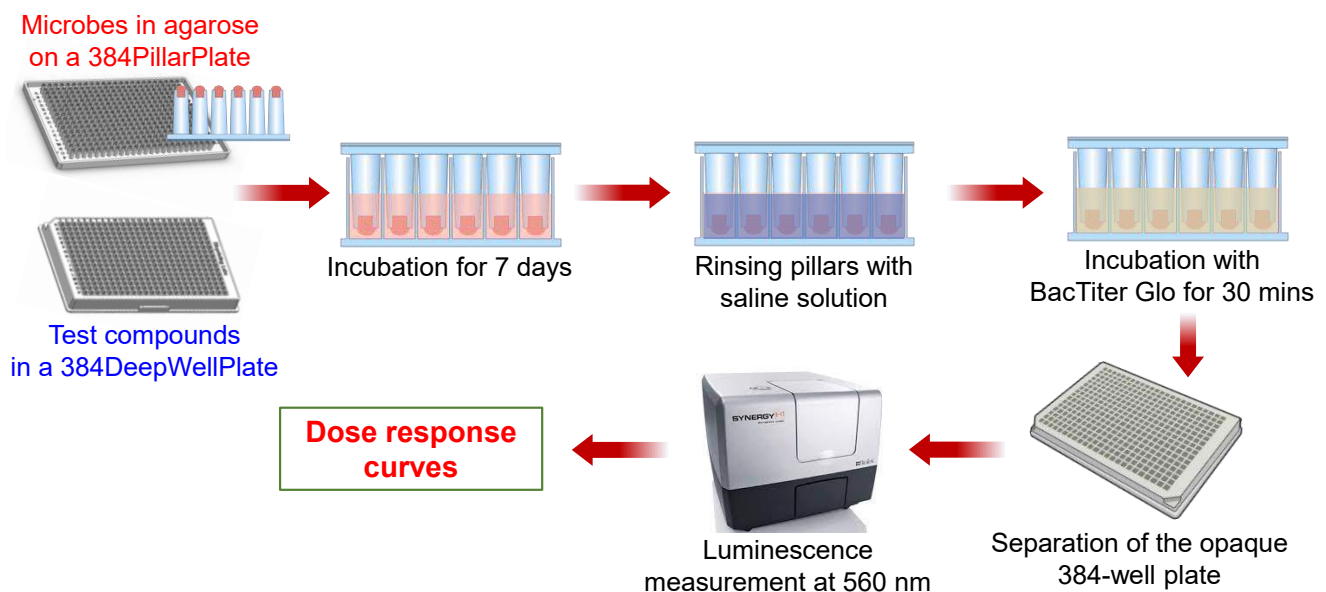
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Graphical abstract. High-throughput antimicrobial efficacy tests on a 384PillarPlate.

Abstract

Preservatives, including antimicrobial compounds, are routinely added to home and personal care products such as dishwashing detergents to prevent microbial contamination during repeated consumer use. Traditional antimicrobial efficacy testing (T-AET) relies on colony counting methods, where microbial cocktails are inoculated into preservative-containing products and plated on agar to measure microbial survival. While considered a gold standard, T-AET is time-consuming, labor-intensive, costly, and prone to variability and error due to manual handling. To address these limitations, we developed a high-throughput, miniature, ATP-based on-chip AET (O-AET) using a 384PillarPlate platform. *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Staphylococcus aureus* were encapsulated in 3% agarose and exposed to test compounds and detergent formulations. ATP levels in viable microbes were quantified using the BacTiter-Glo™ luminescence assay to generate dose–response curves, IC₅₀ values, and log reduction measurements. Two industrial antimicrobials, Acticide LG and Acticide MBR2, were evaluated, showing CV values below 8%. Nine detergent formulations from Colgate-Palmolive, including fresh and 4-week aged samples, were tested. Log reduction data from O-AET showed strong correlation with T-AET. Using a 3-log reduction cutoff, O-AET achieved 98% sensitivity, 44% specificity, and 71% overall predictivity. The lower specificity was partly attributed to limited true negative samples and nutrient deficiencies. The O-AET enables significant reduction in reagent volume, rapid turnaround, and compatibility with standard plate readers, making it cost-effective and scalable. This ATP bioluminescence-based platform provides a robust, reproducible, and high-throughput alternative to T-AET, with high potential to streamline product development and preservative screening in consumer care applications.

Keywords: 384PillarPlate, high-throughput microbial assay, preservative, antimicrobial efficacy, consumer product testing

Introduction

The antimicrobial efficacy test (AET), also known as the preservative efficacy test (PET), is a critical quality control assay designed to evaluate the effectiveness of antimicrobial preservatives in inhibiting the growth of microorganisms that may be introduced during storage or product use. This assessment is essential to ensure that consumer and pharmaceutical products remain microbiologically stable and safe for long-term use. Antimicrobial agents that are active against bacteria, fungi, and yeast help mitigate residual contamination and improve the reliability and shelf-life of the final product (Booth 2014). However, preservative efficacy is not solely determined by antimicrobial potency. Several extrinsic and intrinsic factors, including pH, concentration of the preservative, temperature, formulation matrix, presence of interfering substances, and potential interactions with packaging materials, can significantly influence the efficacy of the preservative *in situ* (Russell 2003; Kramer et al. 2008). Therefore, these parameters must be carefully evaluated when selecting and validating preservatives or antimicrobial agents for a specific formulation.

Traditional antimicrobial efficacy testing (T-AET) has been performed using colony counting methods, which involve inoculating the product with standard microbial strains and monitoring colony formation on agar plates over time (Russell 2003; Booth 2014). Various pharmacopeial and industrial guidelines, including the European Pharmacopeia (EP), British Pharmacopeia (BP), United States Pharmacopeia (USP), and the Cosmetic, Toiletry, and Fragrance Association (CTFA), describe different protocols that vary by microbial strains, inoculum size, and incubation time, but are unified in their reliance on colony formation as a proxy for microbial survival (Farrington et al. 1994; Russell 2003; Kramer et al. 2008). Typically, a microbial suspension is added to the product and plated on modified letheen agar (MLA) and incubated for one to three weeks. The number of surviving colonies is compared to control plates without preservative exposure to determine preservative efficacy (Russell 2003). Other culture-based approaches, such as membrane filtration and disc diffusion methods, are also commonly used to quantify microbial survival (Balouiri et al. 2016). In membrane filtration assays, the product-microbial mixture is passed through a sterile membrane, which is then transferred onto tryptic soy agar (TSA) plates and incubated at 30 - 35°C for 3 - 5 days. Log reduction is calculated by comparing colony counts from treated and untreated samples (Booth 2014; Balouiri et al. 2016). In disc diffusion assays, the diameter of the zone of inhibition around discs impregnated with preservatives is used as a measure of antimicrobial potency. For example, Pasquet et al. employed this method to evaluate the efficacy of zinc oxide as a preservative agent (Pasquet et al. 2014). While these traditional methods are widely accepted, they have significant limitations. They are labor-intensive, time-consuming, and require large reagent volumes, making them cost-inefficient for high-throughput testing. Additionally, they are prone to variability and may yield inaccurate or inconsistent results depending on

the microbial strain, operator technique, and experimental conditions (Kramer et al. 2008). Highly skilled personnel are required to perform and interpret the tests, which further restricts their accessibility and scalability in modern formulation screening pipelines.

To overcome these challenges, alternative methods such as impedance microbiology, direct epifluorescence, polymerase chain reaction (PCR), and ATP bioluminescence have been explored (Connolly et al. 1994; Elizaquível et al. 2013; Balouiri et al. 2016; Trinh and Lee 2022). These methods typically offer faster turnaround times and reduced material consumption. For instance, impedance microbiology measures electrical conductivity changes associated with microbial metabolism. Connolly et al. demonstrated that impedance measurements using a bactometer correlated with CFU counts for various preservatives, including chlorhexidine and methylparaben. However, the method exhibited limited sensitivity, detecting microbial concentrations only above 10^5 - 10^6 CFU/mL and with delayed signal detection (Connolly et al. 1994). The direct epifluorescence method uses fluorescent dyes to distinguish viable and non-viable cells by targeting RNA (viable cells, red) and DNA (non-viable cells, green). However, RNA from non-viable cells can also stain red, leading to false positives. To overcome this, a modified epifluorescence microcolony method was developed using nucleopore membrane filtration and image-based quantification (Trinh and Lee 2022). Similarly, PCR techniques, especially when combined with DNA-intercalating agents like propidium monoazide (PMA), can differentiate viable from dead cells. Yet, the accuracy of PCR may be compromised in the presence of high microbial loads or interfering substances like detergents, which can inhibit PCR amplification and result in erroneous microbial quantification (Elizaquível et al. 2013; Trinh and Lee 2022).

To address these methodological gaps, we developed a high-throughput, ATP bioluminescence-based, on-chip AET (O-AET) using a 384PillarPlate platform - a novel miniaturized assay platform that allows rapid quantification of microbial viability through ATP detection (**Figures 1 and 2**). In this system, microorganisms are encapsulated in agarose and loaded onto the pillar plate by stamping. Following treatment with test compounds or detergent formulations, the ATP content of surviving microorganisms is measured using a BacTiter-Glo™ assay kit (Promega), which generates luminescence proportional to viable cell counts. This assay provides a broad detection window (10^{-4} to 1.0 OD) with high reproducibility and can be conducted entirely within the pillar plate format using standard microplate readers.

To demonstrate the utility and predictive power of our O-AET assay, we tested three microbial species commonly recommended in regulatory guidance: *Staphylococcus aureus* (gram-positive), *Pseudomonas aeruginosa*, and *Enterobacter cloacae* (both gram-negative). Dose response curves were generated for two industrial antimicrobial agents, Acticide LG and Acticide MBR2, and corresponding half-maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC)

values were calculated. In addition, log reduction values were determined with detergent formulations using our O-AET assay, which were compared with the results from the T-AET. Our results demonstrate that the O-AET is not only highly predictive of traditional methods but also offers significant advantages in speed, throughput, reproducibility, and cost-effectiveness. The 384PillarPlate allows hundreds-fold reduction in assay volume, reduced incubation time, and greater compatibility with automated screening workflows. Given these benefits, we propose that the ATP bioluminescence-based O-AET has high potential to replace conventional colony-counting-based methods in both research and industrial settings.

Materials and Methods

Materials

Microorganisms, including *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 1542), were purchased from ATCC, while *Enterobacter cloacae* (PLS237) was obtained from ThermoFisher. Tryptic soy broth (TSB) (R08944), Tryptone-Azolectin-Tween (TAT) broth (R112611), and modified letheen agar (MLA) (R453722) were also sourced from ThermoFisher. Low temperature gelling agarose (A2576) was purchased from Sigma-Aldrich. The 384PillarPlate (384-01-00), LoadingPlate (384-03-00), and 384DeepWellPlate (384-02-00) were obtained from Bioprinting Laboratories Inc. for microbial encapsulation and culture. The BacTiter-Glo microbial cell viability kit (G8230) was acquired from Promega and diluted with a saline solution at a 1:1 ratio. Two industrial antimicrobial compounds, Acticide LG and Acticide MBR2, along with nine fresh and aged dishwashing detergent samples with confidential formulations, were provided by Colgate Palmolive. Aged samples were prepared by incubating the fresh samples at 40°C for 8 weeks to replicate shelf-life conditions.

Preparation of microbial inoculum and colony counting

The modified letheen agar (MLA) plates were prepared in advance by pouring 25 mL of 6% (w/v) autoclaved MLA into sterile petri dishes, allowing it to solidify, and storing them at 4°C. The working culture was prepared by streaking frozen bacterial stock in glycerol onto MLA plates using a sterile 10 µL loop. The streaked plates were incubated at 30 - 35°C for 24 - 48 hours and stored inverted at 4°C for up to 6 weeks. Colony inoculation was conducted by transferring a single colony into 10 mL of tryptic soy broth (TSB), followed by incubation at 37°C for 8 - 20 hours, depending on the microbial growth rates. For scale-up culture, an aliquot of TSB containing microorganisms was transferred into 50 mL of TSB in a tube and incubated for 8 - 20 hours, producing the final inoculum used for agarose encapsulation and compound testing (**Figure 1**). The inoculum was vortexed and used within one hour.

For *P. aeruginosa*, surface pellicles were carefully removed prior to vortexing to avoid uneven cell distribution.

Viable cell counting was performed by serially diluting the final inoculum at 10^{-4} - 10^{-8} folds in 0.85% sterile saline solution, streaking 10 μ L of each dilution onto MLA plates, and incubating them inverted at 30°C for 48 hours. Colony-forming units (CFUs) were recorded to verify the number of viable cells in the final inoculum prior to encapsulation. To rapidly quantify the number of viable bacteria with luminescence measurement, two calibration curves were established. First, an optical density (OD) vs. CFU calibration curve was generated to find the correlation between OD measured at 600 nm (OD_{600}) with the diluted final inoculum and the number of colonies formed on MLA plates (**Supplementary Fig. 1**). The OD vs. CFU calibration curves were generated for each microbial in triplicates with three different MLA plates per OD. Second, a luminescence vs. OD calibration curve was generated to correlate luminescence intensity from live microorganisms measured with the BacTiter-Glo microbial cell viability kit to OD_{600} (**Supplementary Fig. 2**). The luminescence vs. OD calibration curves were generated for each microbial in triplicates. The two calibration curves were used to determine the number of live microorganisms by measuring luminescence and calculating log reduction after sample treatment.

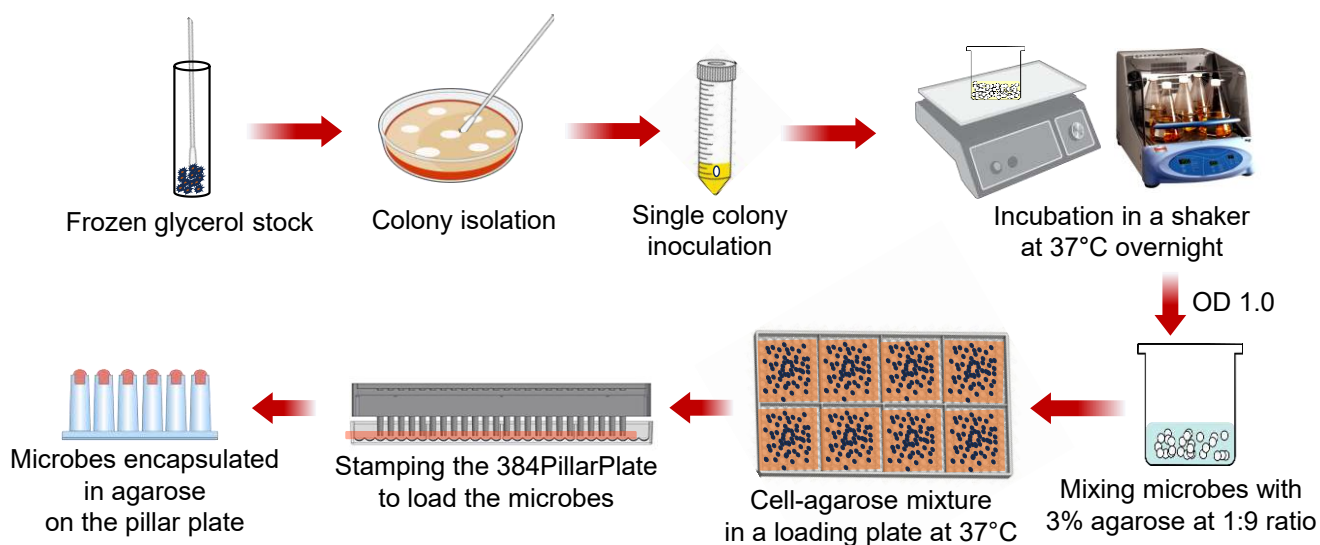


Figure 1. Experimental procedures for microbial cell loading and encapsulation in agarose on the 384PillarPlate for high-throughput antimicrobial efficacy test (AET).

Microbial encapsulation in agarose on the 384PillarPlate

After inoculating and culturing a single colony, microbial cells were grown in a flask, centrifuged at 1,800 g, and resuspended in fresh sterile TSB to achieve an optical density (OD_{600}) of 1.0,

corresponding to approximately 1×10^9 CFU/mL. For encapsulation, 2 mL of the microbial suspension was combined with 18 mL of 3% (w/v) low-temperature gelling agarose at 37°C, resulting in a final OD₆₀₀ of 0.1. The agarose solution was pre-prepared by dissolving 0.6 g of low-temperature gelling agarose in 20 mL of deionized water, followed by autoclaving and cooling at 37°C for at least 1 hour to prevent thermal damage to the microorganisms during encapsulation. To maintain a homogeneous suspension and prevent premature gelation, the microorganism-agarose mixture was kept at 37°C on a heated block and gently agitated by pipetting. The mixture was then transferred into a sterile LoadingPlate. A blank 384PillarPlate was carefully stamped onto the microorganism-agarose mixture in the LoadingPlate, ensuring uniform wetting of all pillars (**Figure 1**). The pillar plate was subsequently separated and placed in a biosafety cabinet at room temperature for 30 minutes to allow for agarose gelation. Visual inspection confirmed the formation of a hemispherical dome on each pillar. The 384PillarPlate, with encapsulated microorganisms, was then sandwiched with a 384DeepWellPlate containing either growth medium or test compounds for microbial culture and analysis (**Figure 2**).

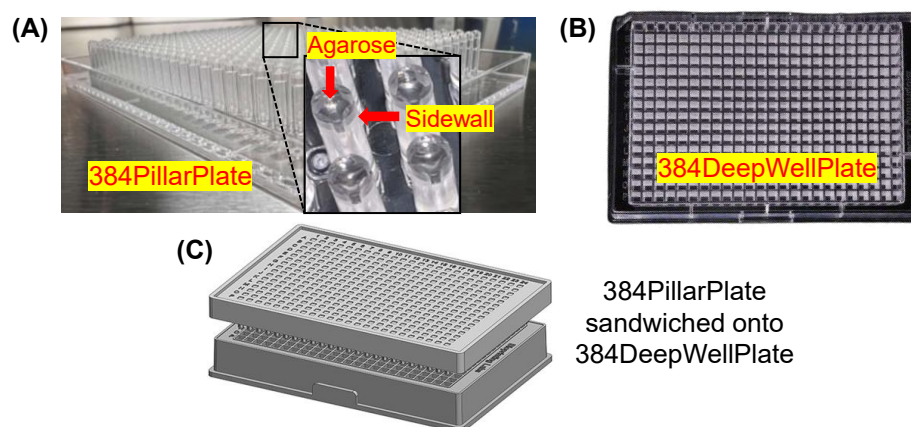


Figure 2. The combination of (A) a 384PillarPlate and (B) a 384DeepWellPlate for high-throughput microbial cell culture and analysis. Sidewalls and slits on the pillars are designed for microbial cell loading and encapsulation in agarose. (C) The 384PillarPlate loaded with microorganisms are sandwiched onto the 384DeepWellPlate with test samples for high-throughput AET.

Compound exposure and cell viability measurement to obtain dose response curves

Stock solutions of the industrial antimicrobial compounds Acticide LG and Acticide MBR2, both at 1,000 mg/mL, were diluted 100-fold in saline to obtain initial working solutions at a concentration of 10 mg/mL. A no-compound control and eight concentrations of each antimicrobial compound, ranging from 10 mg/mL to 2.5 µg/mL, were prepared *via* four-fold serial dilution in saline and dispensed at 80 µL per well into a 384DeepWellPlate. The 384PillarPlate, containing microorganisms encapsulated in agarose,

was carefully positioned onto the 384DeepWellPlate containing the diluted antimicrobial compounds, ensuring proper contact between the plates. The assembled plates were then incubated at room temperature for seven days. Following incubation, the 384PillarPlate was rinsed in a 384DeepWellPlate containing saline to remove any residual antimicrobial compounds.

To assess microbial viability, BacTiter-Glo reagent was diluted 1:1 with saline and dispensed at 40 μ L per well into an opaque 384-well plate. The rinsed 384PillarPlate was then placed onto the reagent-containing plate, ensuring proper contact, and shaken on an orbital shaker. The assembly was incubated in the dark at room temperature for 30 minutes to facilitate luminescence development. Following incubation, the 384PillarPlate was removed, and luminescence was measured at 560 nm using a Synergy H1 microplate reader (BioTek Instruments, Winooski, VT, USA). Luminescence intensities obtained from the no-compound control and eight concentrations of each antimicrobial compound were used to calculate the minimum inhibitory concentration (MIC) and half-maximal inhibitory concentration (IC_{50}) values (**Figure 3**).

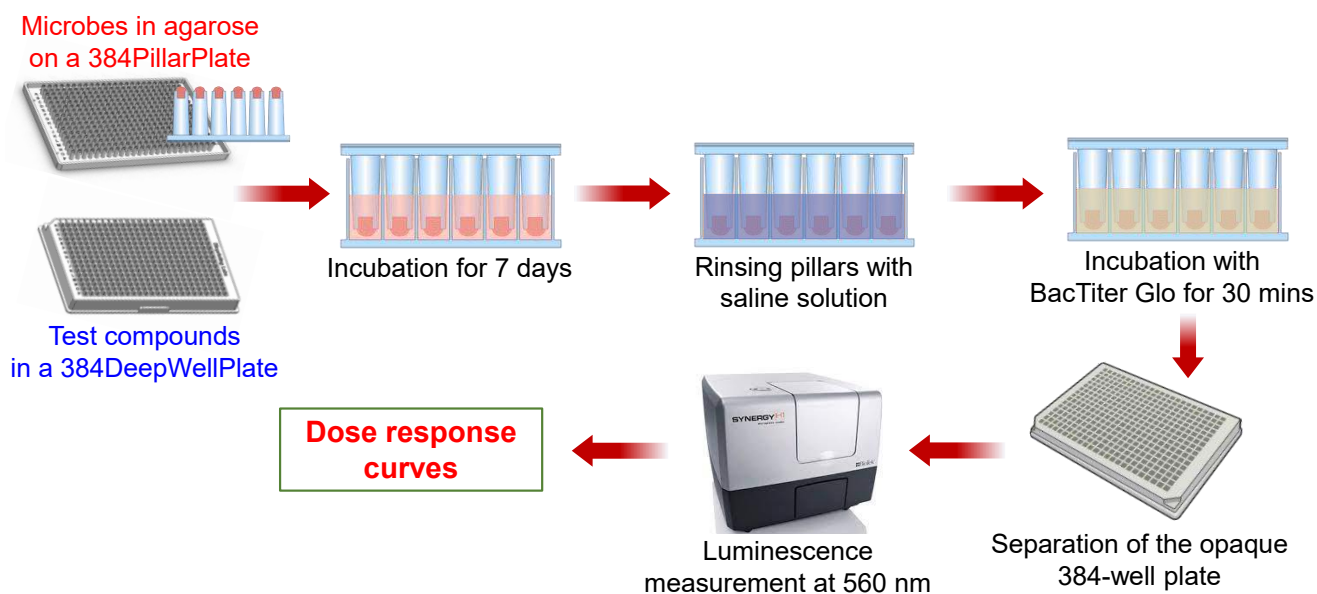


Figure 3. Experimental procedures for obtaining dose response curves of antimicrobial compounds using the 384PillarPlate and the 384DeepWellPlate. Luminescence reading was used to plot the dose response curves of antimicrobial compounds and determine IC_{50} and minimum inhibitory concentration (MIC) values.

Detergent sample exposure and incubation in TAT broth to obtain log reduction values

In addition to testing individual antimicrobial compounds, nine fresh detergent samples and nine aged samples provided by Colgate-Palmolive were evaluated with each microbial species using the

384PillarPlate and 384DeepWellPlate to calculate log reduction in microbial growth. The aged detergent samples were prepared by incubating the fresh detergent samples for 8 weeks at 40°C. Briefly, 90 µL of each detergent sample was dispensed into two columns per sample (32 replicates per sample) in the 384DeepWellPlate (**Supplementary Fig. 3**). The 384PillarPlate, containing agarose-encapsulated microorganisms, was inserted into the 384DeepWellPlate to facilitate microbial exposure to the detergent samples. The sandwiched plates were incubated for seven days at 22 - 27°C in a humidified chamber to prevent evaporation. Following incubation, the 384PillarPlate was separated and rinsed twice by sandwiching it with a 384DeepWellPlate containing 80 µL of saline per well for one hour. Subsequently, the 384PillarPlate was sandwiched with a 384DeepWellPlate containing 80 µL of TAT broth per well and incubated at 30°C for 8 - 24 hours, depending on the microbial doubling time. This step was crucial for enhancing signal intensity from viable microorganisms post-detergent exposure. Microbial viability was then assessed by sandwiching the pillar plate with an opaque 384-well plate containing 40 µL of BacTiter-Glo reagent diluted 1:1 with TSB. After shaking on an orbital shaker at room temperature for 30 minutes in the dark, luminescence was measured at 560 nm using the Synergy H1 microplate reader to quantify viable cells (**Figure 4**).

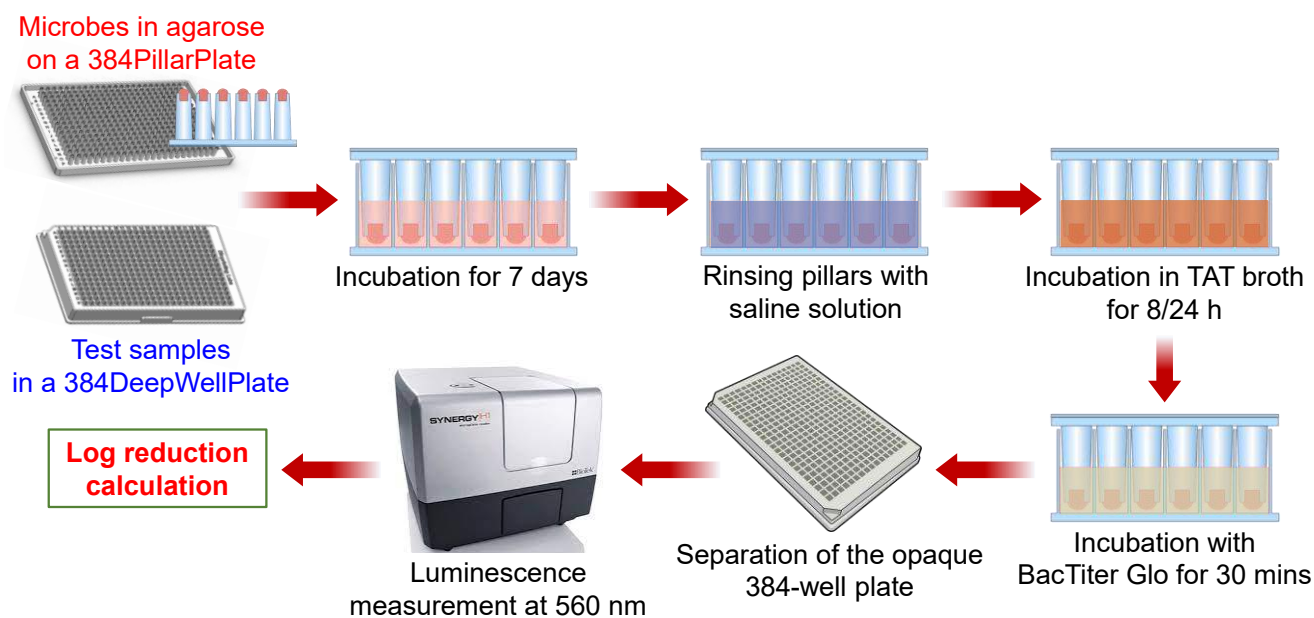


Figure 4. Experimental procedures for an antimicrobial efficacy test on the 384PillarPlate. Luminescence reading was used to calculate the log-reduction of microorganisms by the test samples.

Calculation of log reduction and determination of predictivity

Before calculating log reduction, background luminescence from agarose-only pillars was subtracted from all measured luminescence values. Luminescence intensities obtained from the pillar plate were first converted to optical density (OD) and subsequently to cell numbers using the two calibration curves specific to each microbial species. Since these cell numbers were determined after 8 - 24 hours of incubation to amplify signal intensity, initial cell numbers at time zero were estimated based on the microbial doubling time. Normalized log reduction for each microbial was then calculated using the following equation:

$$\text{Log reduction} = \log_{10} \left(\frac{X}{X_i} \right) \times \text{NF}$$

where X represents the cell number at time zero in the absence of detergent treatment (control), X_i denotes the estimated cell number at time zero following detergent treatment, and NF is a normalization factor accounting for differences in seeding density between traditional and on-chip AET assays (**Figure 5**).

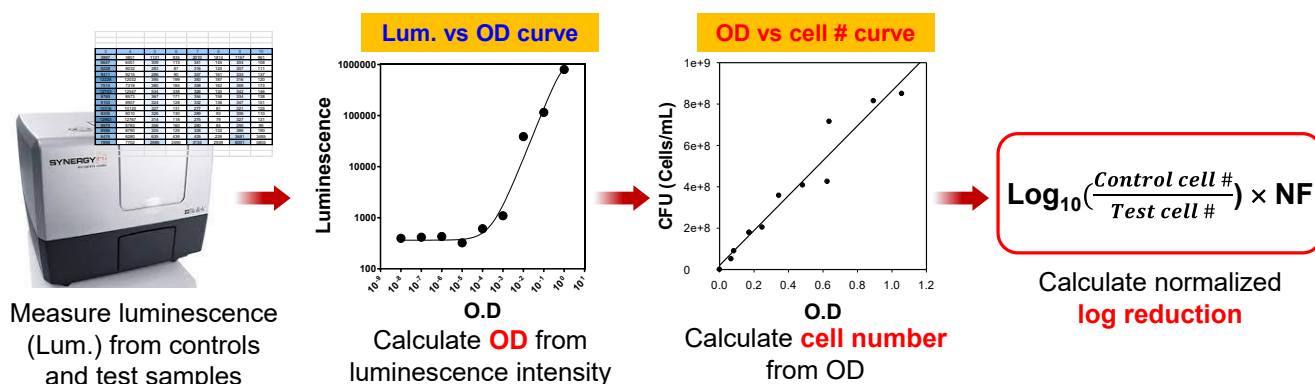


Figure 5. Analytical procedures for calculating normalized log reduction of microorganisms using luminescence data obtained from the 384PillarPlate. Normalization Factor (NF) indicates the maximum log reduction from traditional AET divided by the maximum log reduction from O-AET. NF was introduced to normalize the difference in cell seeding density in the two AET assays, which is typically 6/5 – 6/7.

To evaluate the sensitivity, specificity, and overall predictivity of O-AET compared to T-AET, a log reduction cutoff of 3 was applied as the threshold for determining antimicrobial effectiveness. A detergent sample was classified as effective if it achieved a log reduction ≥ 3 , whereas a log reduction < 3 indicated non-effectiveness. The results from O-AET were compared to those from T-AET to categorize outcomes into true positive (TP), false negative (FN), true negative (TN), and false positive (FP) using the following criteria:

- **True Positive (TP):** A sample classified as effective by both T-AET and O-AET
- **False Negative (FN):** A sample classified as effective by T-AET but non-effective by O-AET
- **True Negative (TN):** A sample classified as non-effective by both T-AET and O-AET
- **False Positive (FP):** A sample classified as non-effective by T-AET but effective by O-AET

Using the counts of TP, FN, TN, and FP, the following calculations were performed:

- **Sensitivity** = $TP / (TP + FN) \times 100$
Represents the probability that O-AET correctly identifies effective preservatives
- **Specificity** = $TN / (TN + FP) \times 100$
Represents the probability that O-AET correctly identifies non-effective preservatives
- **Overall Predictivity** = $(\text{Sensitivity} + \text{Specificity}) / 2$
Represents the average of sensitivity and specificity, reflecting balanced performance

Calculation of coefficient of variation (CV) and Z' factor

The coefficient of variation (CV) was calculated to assess the accuracy and reliability of the assay using luminescence readings from control groups that were not exposed to compounds or detergents. CV is defined as the ratio of the standard deviation (σ) to the mean (μ) and is expressed as a percentage:

$$CV = \frac{\sigma}{\mu} \times 100$$

To evaluate assay robustness and suitability for high-throughput screening (HTS), the Z' factor was calculated using luminescence data from positive controls (100% live cells, no treatment) and negative controls (100% dead cells, maximum treatment):

$$Z' = 1 - \frac{3(\sigma_p + \sigma_n)}{\mu_p - \mu_n}$$

where μ_p and μ_n are the means, and σ_p and σ_n are the standard deviations of the positive and negative control groups, respectively. A Z' factor between 0.5 and 1.0 indicates a highly robust assay suitable for HTS applications.

Results

Correlation between luminescence from ATP measurement and microbial cell number

Two calibration curves were established to enable accurate quantification of viable microbial cells before and after treatment (**Supplementary Figs. 1 and 2**). The first calibration curve correlated optical density (OD₆₀₀) with colony-forming unit (CFU, cells/mL) on agar plates (**Supplementary Fig. 1**). Microbial suspensions of varying concentrations were measured for OD at 600 nm and plated on MLA to determine CFU counts. This OD vs. CFU calibration enabled efficient estimation of microbial seeding

density based on OD measurements, eliminating the need for repeated CFU determinations. Since OD reflects light scattering by particles in suspension, and scattering increases proportionally with microbial concentration, absorbance readings provide an indirect measure of viable cell numbers within an OD of 1.0. Beyond this threshold, the response becomes nonlinear. Absorbance values for each microbial species were linearly proportional to CFU counts, as shown in the figures. While slopes of the calibration curves varied slightly among species due to differences in microbial particle size, all values fell within a similar range ($\sim 10^9$ viable cells/mL at OD = 1.0). Specifically, an OD of 1.0 corresponded to 9.1×10^8 viable cells/mL for *E. cloacae*, 8.1×10^8 cells/mL for *P. aeruginosa*, and 8.3×10^8 cells/mL for *S. aureus*.

The second calibration curve correlated luminescence intensity from the BacTiter-Glo microbial cell viability assay with OD values for microbial suspensions used in agarose encapsulation on the 384PillarPlate (**Supplementary Fig. 2**). This luminescence vs. OD calibration facilitated efficient OD estimation using luminescence, enabling subsequent calculation of viable cell numbers on the pillar plate before and after treatment. Luminescence intensity exhibited a nonlinear relationship with OD, with detectable upper and lower limits influenced by extreme cell seeding densities. These limits varied among microbial species, likely due to differences in intracellular ATP levels. Specifically, the lower and upper limits of luminescence for accurate OD correlation were 212 and 9.7×10^5 for *S. aureus*, 175 and 1.2×10^5 for *P. aeruginosa*, and 322 and 5.0×10^4 for *E. cloacae*, respectively. These calibration curves and their associated equations were essential for determining viable cell counts and log reduction throughout the study.

Effectiveness of antimicrobial compounds against microbial species

Dose response curves were generated using microorganisms encapsulated in 3% agarose on the 384PillarPlate and exposed to two industrial antimicrobial compounds, Acticide LG and Acticide MBR2, in the 384DeepWellPlate to determine their IC_{50} values. Each microbial species was subjected to Acticide LG and Acticide MBR2 for seven days, after which luminescence measurements were obtained to construct dose response curves (**Figure 6**). Using these curves, IC_{50} values - representing the concentration required to inhibit 50% of microbial growth - and minimum inhibitory concentration (MIC) values - the lowest concentration at which microbial growth is completely suppressed - were calculated across three independent experiments (**Table 1**). For *E. cloacae*, compounds were diluted in saline, whereas *S. aureus* and *P. aeruginosa* required tryptic soy broth (TSB) as a diluent due to poor microbial viability in saline over the seven-day incubation period (data not shown). This species-specific media requirement reflects established nutritional dependencies: *S. aureus* and *P. aeruginosa* cannot sustain prolonged viability in saline due to the absence of essential nutrients (Parfentjev and Catelli

1964; LaBauve and Wargo 2012; Missiakas and Schneewind 2013). In contrast, *E. cloacae* remained viable throughout the seven-day incubation in saline.

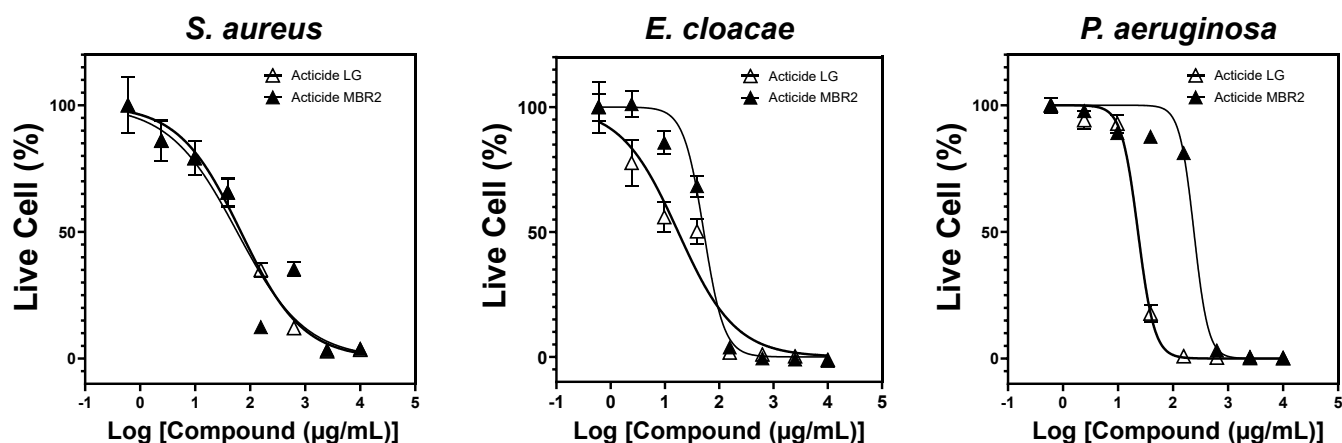


Figure 6. Dose response curves of Acticide LG and Acticide MBR2 for *S. aureus*, *E. cloacae*, and *P. aeruginosa*. The microorganisms on the 384PillarPlate were treated with the two antimicrobial compounds for 7 days at room temperature, and cell viability was measured with the BacTiter-Glo assay kit.

Table 1. The IC₅₀ and MIC values of Acticide LG and Acticide MBR2 for the microorganisms tested. The compound tests with the microorganisms were performed in triplicates, and the IC₅₀ values obtained were reproducible and within the same range for the three microorganisms tested.

Preservatives	<i>S. aureus</i>		<i>E. cloacae</i>		<i>P. aeruginosa</i>	
	IC ₅₀	MIC	IC ₅₀	MIC	IC ₅₀	MIC
Acticide LG	68 µg/mL	2500 µg/mL	18 µg/mL	156 µg/mL	23 µg/mL	156 µg/mL
Acticide MBR2	58 µg/mL	2500 µg/mL	52 µg/mL	156 µg/mL	240 µg/mL	625 µg/mL

P. aeruginosa exhibited greater sensitivity to Acticide LG, with lower IC₅₀ (23 µg/mL) and MIC (156 µg/mL) values compared to Acticide MBR2, which had an IC₅₀ of 240 µg/mL and MIC of 625 µg/mL. In contrast, both Acticide LG and MBR2 had similar effects on *S. aureus*, with IC₅₀ values of 68 µg/mL and 58 µg/mL, respectively, and a MIC of 2500 µg/mL for both compounds. Similarly, *E. cloacae* responded comparably to Acticide LG and MBR2, with IC₅₀ values of 18 µg/mL and 52 µg/mL, respectively, and a MIC of 156 µg/mL. Among the tested species, *S. aureus* displayed the highest resistance, requiring a substantially higher concentration (2500 µg/mL) of Acticide LG and MBR2 for complete inhibition. This resistance is likely due to well-documented mechanisms in *S. aureus*, including the expression of antimicrobial-modifying enzymes and multiple efflux pumps

(Balasubramanian et al. 2017a). In contrast, *E. cloacae*, a gram-negative bacterium, exhibited relatively low resistance to both compounds. The observed antimicrobial activity can be attributed to the shared active ingredients in Acticide LG and MBR2. Acticide LG contains 5-chloro-2-methyl-4-isothiazolin-3-one (CIT) and 2-methyl-4-isothiazolin-3-one (MIT), while Acticide MBR2 contains 1,2-benzisothiazolin-3-one (BIT) and MIT. These isothiazolinone-based biocides disrupt microbial viability by penetrating cell membranes and reacting with nucleophilic cellular components such as thiols through their electron-deficient N–S bonds. This process leads to enzymatic inhibition, interference with oxygen consumption and ATP synthesis, and disruption of the Krebs cycle, ultimately resulting in cell death (Collier et al. 1990; Silva et al. 2020).

Reproducibility and robustness of the high-throughput antimicrobial screening assay

All experiments were conducted at least three times to ensure reliability, and coefficient of variation (CV) values and Z' factors were calculated to assess assay reproducibility and robustness. The CV value represents variability relative to the average signal strength, serving as the inverse of the signal-to-noise ratio (Yu et al. 2018a). To evaluate day-to-day assay variability on the 384PillarPlate, CV values were measured on three separate dates. The CV values for assays across three microbial samples remained below 8%, demonstrating high reproducibility (**Figure 7**). Similarly, assay robustness on the 384PillarPlate was determined by calculating Z' factors (**Table 2**). Z' factors for assays across three microbial samples ranged from 0.5 to 1, confirming the robustness of the high-throughput antimicrobial screening assay developed on the 384PillarPlate (Sui and Wu 2007).

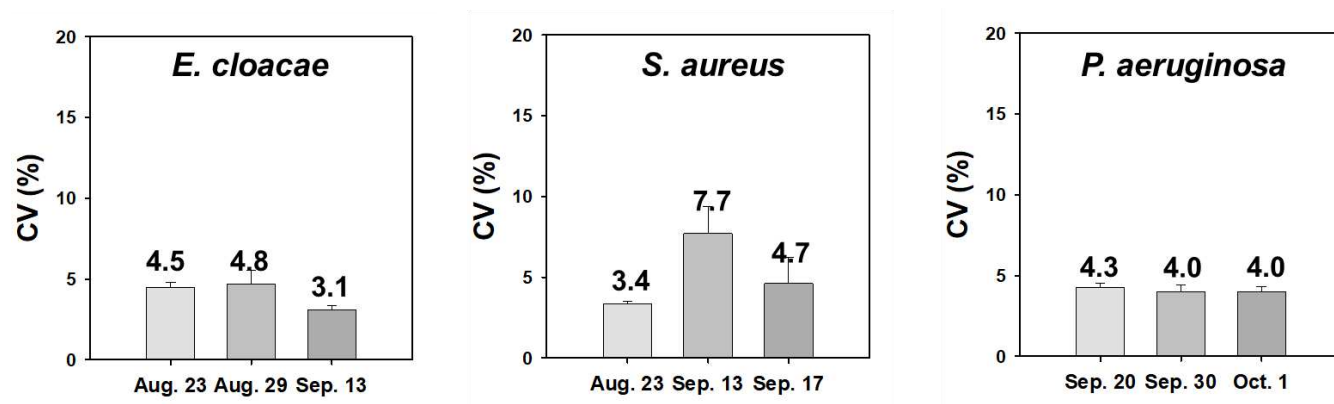


Figure 7. Reproducibility and robustness of the antimicrobial compound screening assay determined by calculating the coefficient of variation (CV). The CV values of the assay from three microorganisms were below 8%, indicating high reproducibility of the data from the 384PillarPlate.

Table 2. Robustness of the antimicrobial compound screening assay determined by calculating Z' factor. Z' factors between 0.5 - 1 indicated the robustness of the assay.

Microbials	Acticide LG	Acticide MBR2
<i>E. cloacae</i>	0.66	0.84
<i>S. aureus</i>	0.89	0.88
<i>P. aeruginosa</i>	0.79	0.75

Effectiveness of the detergent samples against microorganisms

Traditional colony counting (T-AET), performed by Colgate Palmolive, and on-chip ATP measurement (O-AET), conducted by our group, were compared to evaluate the antimicrobial efficacy of detergent solutions containing preservatives. For both assessments, nine fresh detergent samples and nine aged samples, stored for eight weeks at 40°C to simulate shelf-life conditions, were tested against three representative microorganisms, including *S. aureus*, *E. cloacae*, and *P. aeruginosa*. The log reduction data from T-AET and O-AET are summarized in **Table 3**. We marked the samples that became more toxic due to aging in red and identified false-negative and false-positive samples with a red box.

Overall, the log reduction values obtained using the O-AET method were comparable to or slightly higher than those obtained with the T-AET method across all detergent samples and microorganisms. For *S. aureus*, fresh and aged detergent samples were highly effective in killing the microorganism, as determined by the O-AET method, which correlated well with the results from the T-AET method. For *E. cloacae*, the log reduction values from both methods were well correlated, except for one false-negative result from aged sample #1. Unlike the findings for sample #6, the O-AET method did not detect changes in efficacy due to aging in sample #1. For *P. aeruginosa*, fresh and aged detergent samples were highly effective in killing the microorganism, with results from the O-AET method moderately correlating with those of the T-AET method. Out of 18 detergent samples tested, five false-positive results were observed using the O-AET method. This discrepancy is potentially due to species-specific media requirements. Some microorganisms, such as *S. aureus* and *P. aeruginosa*, are highly sensitive to nutrient deficiency (Parfentjev and Catelli 1964; LaBauve and Wargo 2012; Missiakas and Schneewind 2013), making them more susceptible to antimicrobial compounds.

Table 3. Comparison of log reduction values at day 7 between traditional AET (T-AET) and on-chip AET (O-AET). The aged samples were prepared by incubating the fresh samples for 8 weeks at 40°C.

Detergent samples tested	<i>S. aureus</i>				<i>E. cloacae</i>				<i>P. aeruginosa</i>			
	Fresh samples		Aged samples		Fresh samples		Aged samples		Fresh samples		Aged samples	
	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET
Sample 1	6.0	5.9	5.8	5.3	2.3	2.0	5.9	1.9	2.1	5.7	5.9	5.7
Sample 2	6.0	5.9	5.8	5.3	6.4	5.9	5.9	5.9	6.0	5.7	5.9	5.7
Sample 3	6.0	5.9	5.8	5.3	6.4	5.9	5.9	5.9	6.0	5.7	5.9	5.7
Sample 4	6.0	5.9	5.8	5.3	6.4	5.9	5.9	5.9	6.0	5.7	5.9	5.7
Sample 5	6.0	5.9	5.8	4.4	0.8	2.3	1.9	2.3	1.1	5.7	1.5	5.7
Sample 6	6.0	5.9	5.8	5.3	0.6	2.4	5.9	5.9	3.1	5.7	2.3	5.7
Sample 7	6.0	5.9	5.8	5.3	6.4	5.9	5.9	5.9	6.0	5.7	5.9	5.7
Sample 8	6.0	5.9	5.8	5.3	6.4	5.9	5.9	5.9	6.0	5.7	5.9	5.7
Sample 9	6.0	5.9	5.8	5.3	6.4	5.9	4.1	5.9	1.3	5.7	5.9	5.7

Sensitivity, specificity, and overall predictivity of the O-AET

To calculate sensitivity, specificity, and overall predictivity, it is necessary to compare the log reduction values from the T-AET and O-AET methods against a log reduction cutoff. Although the log reduction cutoff can be varied to assess changes in sensitivity, specificity, and overall predictivity (Yu et al. 2018b) we elected to use a 3-log reduction cutoff, as it represents the midpoint of the maximum log reduction value. Accordingly, a detergent sample is classified as “effective” if its log reduction value exceeds 3, while a log reduction value below 3 classifies it as “non-effective”. A detergent sample is categorized as true positive (TP) if it is effective according to both T-AET and O-AET methods, whereas it is classified as false negative (FN) if deemed effective by T-AET but non-effective by O-AET. Similarly, a sample is classified as true negative (TN) if both methods categorize it as non-effective, while it is classified as false positive (FP) if T-AET identifies it as non-effective, but O-AET classifies it as effective. As a result, using the O-AET method, we obtained 44 true positives (TP), 1 false negative (FN), 4 true negatives (TN), and 5 false positives (FP). These classifications were derived from 54 log reduction values across 18 detergent samples and 3 microorganisms. Consequently, the O-AET method demonstrated 98% sensitivity, 44% specificity, and 71% overall predictivity. These findings indicate that O-AET is highly sensitive in correctly identifying detergent samples that are effective, as determined by T-AET. However, its specificity in correctly identifying non-effective detergent samples remains moderate. This limitation is likely attributed to nutrient deficiency during the 7-day incubation period, suggesting an opportunity for specificity improvement. Maintaining a consistent metabolic state in microorganisms during both O-AET and T-AET is essential for accurate predictivity assessment. Furthermore, the

disproportionate number of non-effective samples (9) compared to effective samples (45) may have introduced inaccuracies in specificity assessment.

Table 4. The number of true positive (TP), true negative (TN), false positive (FP), and false negative (FN) samples for log reduction cutoff of 3 to calculate sensitivity/specificity and predictivity.

Detergent samples tested Log reduction cutoff = 3	<i>S. aureus</i>				<i>E. cloacae</i>				<i>P. Aeruginosa</i>			
	Fresh samples		Aged samples		Fresh samples		Aged samples		Fresh samples		Aged samples	
	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET	T-AET	O-AET
Sample 1	TP	TP	TP	TP	TN	TN	TP	FN	TN	FP	TP	TP
Sample 2	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
Sample 3	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
Sample 4	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
Sample 5	TP	TP	TP	TP	TN	TN	TN	TN	TN	FP	TN	FP
Sample 6	TP	TP	TP	TP	TN	TN	TP	TP	TP	TP	TN	FP
Sample 7	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
Sample 8	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
Sample 9	TP	TP	TP	TP	TP	TP	TP	TP	TN	FP	TP	TP

Discussion

Traditional antimicrobial efficacy testing (T-AET) has historically relied on colony counting methods, wherein test samples are inoculated with microorganisms, streaked on agar plates, and incubated over several weeks to monitor colony formation (Russell 2003; Booth 2014). Although widely accepted, this approach is inherently time-consuming, labor-intensive, resource-intensive, and prone to variability and human error (Kramer et al. 2008). The limitations have driven the development of alternative methods to improve speed, accuracy, and scalability. Impedance-based methods, for example, suffer from low sensitivity due to the weak electrical conductivity of microorganisms (Connolly et al. 1994). Despite their promise, direct epifluorescence imaging techniques face challenges because non-viable cells may retain RNA, leading to staining similar to viable cells and confounding viability measurements (Trinh and Lee 2022). While PCR and flow cytometry methods offer speed and precision, they are highly susceptible to detergent interference and expensive for high-throughput applications (Elizaquível et al. 2013; Pan et al. 2014).

To address these challenges, we have developed a high-throughput, O-AET method using a 384PillarPlate platform, utilizing ATP-based luminescence for microbial viability assessment. This approach enables rapid, miniature assessment of antimicrobial activity against *S. aureus*, *E. cloacae*,

and *P. aeruginosa*, representative microorganisms recommended by USP, CTFA, and ASTM guidelines. These microorganisms were chosen for their clinical significance and varied physiological traits: *S. aureus*, a gram-positive coccus often found in the respiratory tract and skin (Missiakas and Schneewind 2013; Balasubramanian et al. 2017b); *E. cloacae*, a gram-negative facultative anaerobe linked to nosocomial infections (Liu et al. 2013; Davin-Regli and Pagès 2015); and *P. aeruginosa*, a robust gram-negative bacterium with high antimicrobial resistance and adaptability to low-oxygen environments at elevated temperatures (LaBauve and Wargo 2012; Farkas et al. 2024).

We successfully adapted the Bac-Titer Glo assay for use on the 384PillarPlate, encapsulating microorganisms in 3% agarose to enable compound and detergent exposure followed by viability assessment *via* ATP-based luminescence measurement. This system demonstrated a dynamic detection range between 10^{-4} to 1.0 OD, and the measured luminescence correlated well with viable cell numbers. The 384PillarPlate offered significant advantages for high-throughput applications: precise microbial loading by simple plate stamping, efficient washing, compatibility with conventional plate readers, and minimal reagent consumption. The platform enabled simultaneous measurement of 384 data points in a cost-effective manner. Furthermore, the coefficient of variation (CV) and Z'-factor values were within NIH-recommended ranges for high-throughput screening. To amplify low signals post-detergent treatment and extend the log reduction range, an overnight regrowth step in cell culture media was incorporated (**Figure 4**).

After establishing the microbial encapsulation protocol in agarose on the 384PillarPlate, we conducted initial validation using two industrial antimicrobial compounds, Acticide LG and Acticide MBR2, provided by Colgate Palmolive. Acticide LG, which contains 1.11% CIT and 0.37% MIT, is commonly used in aqueous-based products, whereas Acticide MBR2, composed of 2.45% BIT and 4.50% MIT, is suitable for applications in dishwashing detergents and soaps (Thor 2023a, b). The MIC values obtained for all three microorganisms were consistent with published ranges. For example, our MIC for *E. cloacae* was 156 $\mu\text{g/mL}$, aligning with previous reports ranging from 25 to 400 $\mu\text{g/mL}$ (Okoh et al. 2019). Reported MICs of CIT and MIT for *S. aureus* and *P. aeruginosa* in cosmetic formulations were in the sub-mg/mL range (Patrone et al. 2010) and our values for Acticide LG (156 $\mu\text{g/mL}$) and Acticide MBR2 (625 $\mu\text{g/mL}$) for *P. aeruginosa* fall within these boundaries. Notably, *S. aureus* demonstrated higher MICs (up to 2500 $\mu\text{g/mL}$), possibly reflecting intrinsic resistance mechanisms, such as expression of efflux pumps and enzymatic modification (Balasubramanian et al. 2017b).

Subsequently, log reduction values of fresh and aged detergent samples against *S. aureus*, *E. cloacae*, and *P. aeruginosa* were generated using our O-AET method, which were directly compared with those obtained through the T-AET method conducted by Colgate Palmolive. A 3-log reduction cutoff, representing the midpoint of the maximum log reduction value, was used to calculate sensitivity,

specificity, and overall predictivity. The O-AET method yielded 44 true positives (TP), 1 false negative (FN), 4 true negatives (TN), and 5 false positives (FP), resulting in 98% sensitivity, 44% specificity, and 71% overall predictivity. While the O-AET method demonstrated high sensitivity in correctly identifying effective detergent samples, it was less specific in identifying non-effective samples, particularly with *P. aeruginosa*. This discrepancy likely stems from the high vulnerability of *P. aeruginosa* to nutrient deficiency during the 7-day incubation period, suggesting a potential improvement in specificity through dilution of detergent samples in cell growth media. In this study, we were advised against diluting detergent samples with cell growth media due to concerns about changes in consumer use conditions. However, incorporating dilution of test samples in cell growth media may be essential to mitigate technical issues related to nutrient deficiency and to provide a nutrient-rich environment for microorganisms during testing, aligning more closely with the conditions of the T-AET method.

Conclusions

In this study, we successfully developed and validated a high-throughput, ATP bioluminescence-based antimicrobial efficacy test (AET) using the 384PillarPlate platform to assess the effectiveness of preservatives in home and personal care formulations. By encapsulating key microbial species, including *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Staphylococcus aureus*, in agarose on the 384PillarPlate and quantifying viability via ATP luminescence, we demonstrated a miniature, rapid, and reproducible alternative to traditional colony counting methods. Our approach enabled the generation of dose response curves, as well as IC₅₀ and MIC values for industrial preservatives such as Acticide LG and Acticide MBR2, while also allowing direct testing of fresh and aged detergent formulations. The O-AET method exhibited high sensitivity, acceptable specificity, and strong overall predictivity when benchmarked against conventional techniques. Furthermore, the substantial reductions in assay volume, time, and resource requirements underscore its potential for cost-effective implementation in formulation screening and preservative optimization workflows. Overall, our O-AET method based on the 384PillarPlate presents a scalable and practical solution for accelerating preservative efficacy testing in consumer and industrial product development. With further protocol optimization, particularly for nutrient deficiency-sensitive microorganisms, and broader sample validation with a complex mixture of microorganisms, this method could serve as a new standard for rapid and high-throughput preservative testing.

Acknowledgements

This study was financially supported by Colgate Palmolive.

Competing interests

M.Y.L. is the founder and CEO of Bioprinting Laboratories Inc., which manufactured and commercialized the 384PillarPlate platform.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Pranav Joshi, Prabha Acharya, and Oluwadara Fadeyibi. The first draft of the manuscript was written by Pranav Joshi and Prabha Acharya; and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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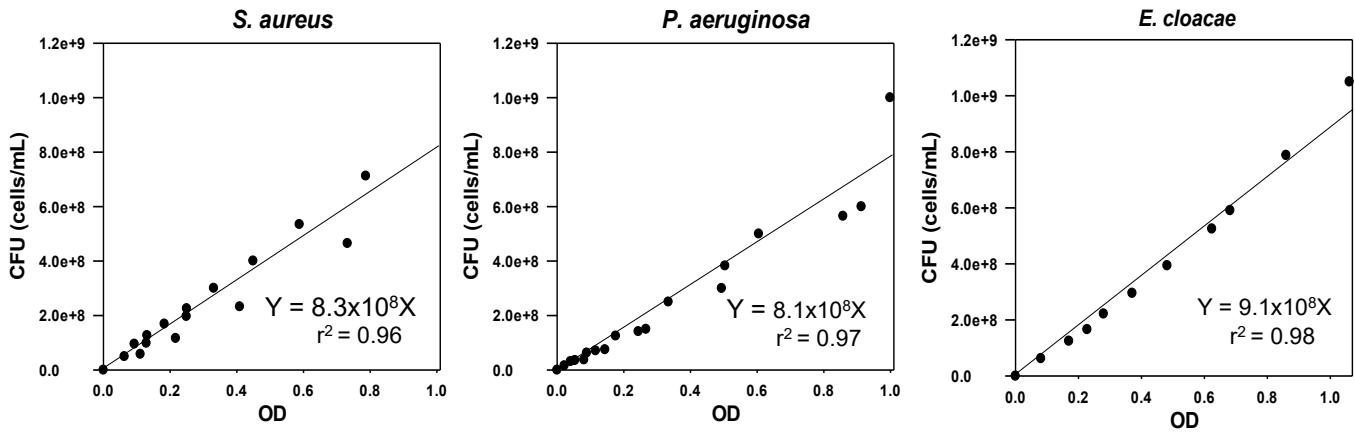
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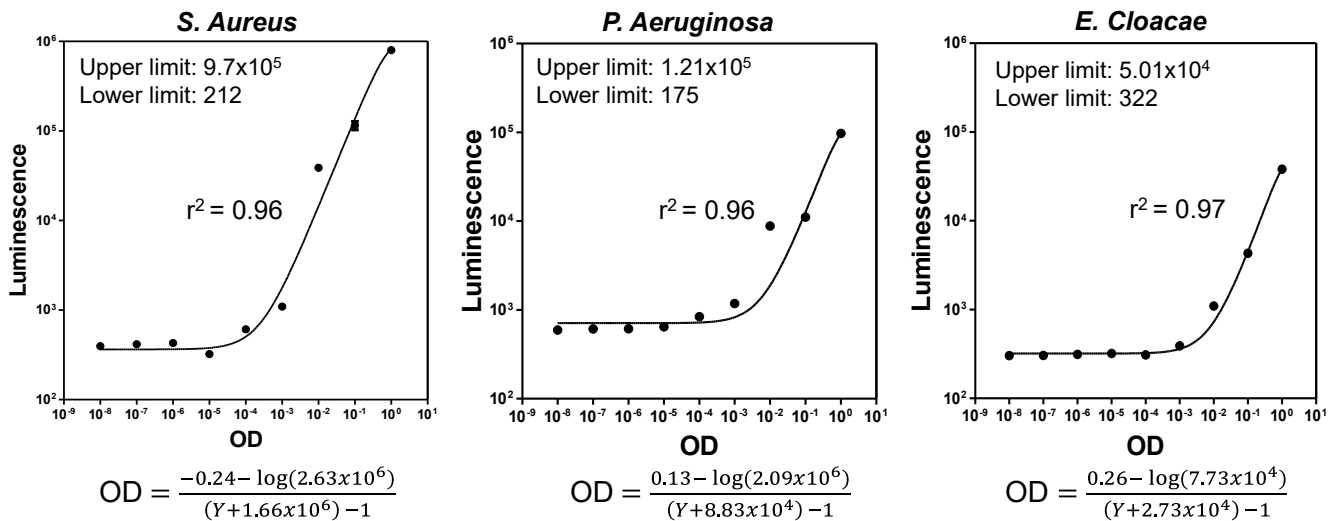
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Supplementary Data



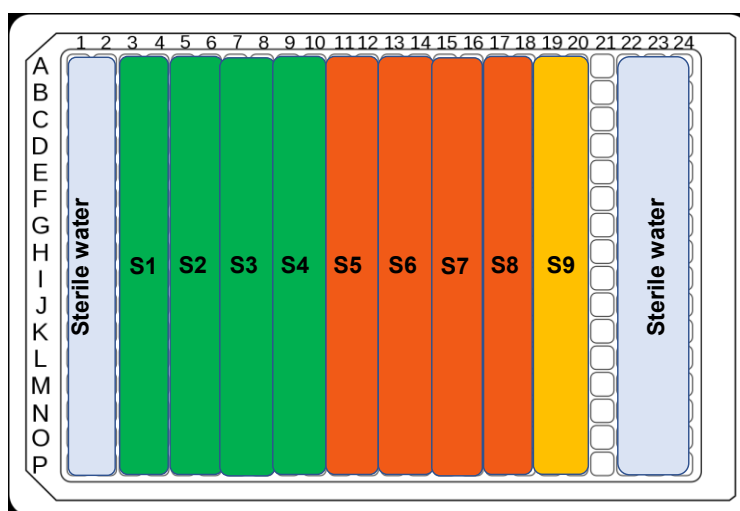
Supplementary Figure 1. Calibration curves correlating optical density (OD) with colony-forming unit (CFU). The equations obtained for *S. aureus*, *P. aeruginosa*, and *E. cloacae* were $Y = 8.3 \times 10^8 X$, $Y = 8.1 \times 10^8 X$, and $Y = 9.1 \times 10^8 X$, respectively, where Y and X represent CFU (cell numbers/mL) and optical density (OD).



Supplementary Figure 2. Calibration curves correlating luminescence with optical density (OD) for *S. aureus*, *P. aeruginosa*, and *E. cloacae*.

Legend

- Sample 1 (S1): Formulation A w/o preservative
- Sample 2 (S2): Formulation A w/ $\frac{1}{2}$ preservative
- Sample 3 (S3): Formulation A w/ full preservative
- Sample 4 (S4): Formulation A w/ full preservative & w/ fragrance
- Sample 5 (S5): Formulation B w/o preservative
- Sample 6 (S6): Formulation B w/ $\frac{1}{2}$ preservative
- Sample 7 (S7): Formulation B w/ full preservative
- Sample 8 (S8): Formulation B w/ full preservative & w/ fragrance
- Sample 9 (S9): Formulation C



Supplementary Figure 3. Layout of the 384DeepWellPlate dispensed with nine detergent samples from Colgate Palmolive for the high-throughput O-AET method.