Antimicrobial Preservative Efficacy Tests on a 384PillarPlate

1. Scope

This standard operating procedure (SOP) outlines the experimental steps for the maintenance, storage, verification requirements, and documentation of microbial cultures. It also details the assessment of the effectiveness of various liquid formulations against microbials on a 384PillarPlate platform, utilizing single and double challenge methods with a high-throughput cell viability assay.

2. Basic Principle

ATCC (or equivalent) strain cultures are obtained in a stabilized form from an accredited culture collection agency or from an accredited reference strain vendor. The authenticity of each lot is verified upon receipt. ATCC (or equivalent) culture strains used for testing should not exceed five transfers from the master seed lot culture. Proper record keeping (traceability) is an essential component of proper culture maintenance. For a single challenge, microbials in 3% agarose are loaded on the 384PillarPlate, sandwiched with the 384DeepWellPlate containing test samples for 7 days, viability assessed with BacTiter-Glo luminescence assay, and log-reduction calculated. For a double challenge, the 384PillarPlate with fresh microbials is sandwiched with the 384DeepWellPlate used for the single challenge and incubated for additional 7, 14, and 21 days.

3. Safety Requirements

A. General laboratory safety practices should always be followed while performing this method.
B. Assure safety assessment of all chemicals used is completed and documented prior to implementing this procedure (including review of chemical storage requirements and MSDS).
C. Safety glasses, laboratory coats and the proper Personal Protective Equipment should be worn throughout all steps.
    i. Disposable impermeable gloves must be worn when handling infectious material when open cuts or lesions are present on the hands.
    ii. Gloves are disposed of when contaminated by placing in a biohazard bag, removed when work with infectious material is completed and not worn outside of the laboratory. Mouth pipetting is prohibited. Automatic pipettors should be used.
D. Waste solvent disposal should be carried out in accordance with proper safety and environmental regulations.

4. Equipment and Plasticware Necessary

A. Refrigerator (FFHI1832TS0, Frigidaire)
B. Vortex mixer (02215365, Fisher Scientific)
C. Temperature-controlled shaker (Z765686, Sigma-Aldrich)
D. Three temperature-controlled incubators (IMC18 50125590, Thermo Scientific)
E. Plate warmer (HP88850100, Thermo Scientific)
F. Sterile disposable pipettes – 10 mL and 25 mL (1367610J, 1367610K, Fisher Scientific)
G. Sterile loops or sterile swabs (131045, Fisher Scientific)
H. Sterile petri dishes (FB0875711, Fisher Scientific)
I. Sterile test tubes with screw caps (2110085, VWR)
J. Single pan balance, accurate to 0.1 g minimum (ALF104, Fisher Scientific)
K. 384PillarPlate (Bioprinting Laboratories Inc.)
L. 384DeepWellPlate (Bioprinting Laboratories Inc.)

M. LoadingPlate (Bioprinting Laboratories Inc.)
N. Opaque 384-well plate (165195, ThermoFisher)
O. Microplate reader (Synergy H1, BioTek)
P. Spectrophotometer (Biomate 3, Thermo Electron Corporation)
Q. Orbital shaker (13687704, Fisher Scientific)
R. Aluminum foil
S. Nunc™ Square BioAssay Dishes (240835, ThermoFisher)

5. Microbials, Media, and Reagents Used

A. Microbials including *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), *Burkholderia cepacia* (ATCC 10856), and *Enterobacter cloacae* (PLS237)
B. Tryptic Soy Broth (TSB) (R08944, ThermoFisher)
C. Tryptone-Azolectin-Tween (TAT) broth (R112611, ThermoFisher)
D. Modified Letheen Agar (MLA) (R453722, ThermoFisher)
E. Low temperature gelling agarose (A2576, Sigma-Aldrich)
F. BacTiter-Glo microbial cell viability kit (G8230, Promega)
G. Sterile distilled water
H. 0.85% (w/v) saline solution by dissolving 8.5 g NaCl in 1000 mL distilled water

6. Definitions

Source culture – Original source of organism, i.e., ATCC (or equivalent culture bank). Cultures from ATCC (or equivalent) are considered the master seed lot culture.

Stock culture – The culture propagated from the source culture used for making working cultures.

Working culture - The culture propagated from the stock culture used for making transfers for laboratory testing.

Test culture – The culture transferred from the working culture to be used in the test.

Passage – A subculture is a passage. One passage is defined as the transfer of organisms from an established culture to fresh medium. All transfers are counted toward the maximum.

7. Experimental Protocols

A. Verification of Cultures

i. Verify the correct organism and strain on the vendor's certificate.
ii. The first passage from a source or stock culture must be cultivated prior to the manufacturer's expiration date. ATCC do not guarantee viability past expiration.
iii. Prior to use in testing, the Gram-stain, morphological characteristics, and/or identification of each bacterial culture serves as a verification of the authenticity of the organism as compared to the vendor's certificate.
iv. If the first culture does not match the expected identification or morphology start a new working culture or stock culture and repeat the verification. If the organism still does not match, contact the vendor.
v. When a culture is transferred from one medium to another, aseptic technique shall be used. The culture is to be visually inspected and compared to the source after the transferred culture has grown and documented as typical or atypical, and if determined necessary by the microbiologist, identification should be performed. If the purity or identification of the culture is in question:
    - Perform a Gram stain on the culture as per your company requirement
    - Verify culture identification as per your company requirement.

- Verification should correlate to the original identification performed.

B. Media/Reagent Preparation

1. Tryptic Soy Broth (TSB) will be used for the preparation of bacterial and yeast cultures. TSB may be stored at room temperature for up to 7 days. If the TSB is stored for a longer period, it must be refrigerated.
2. Prepare Modified Letheen Agar (MLA) according to the manufacturer's instructions, autoclave, and cool at room temperature. MLA will be used for plating during the test.
   i. Suspend 59.1 g of MLA powder in 1000 mL of deionized water.
   ii. Heat to boiling temperature with agitation to completely dissolve.
   iii. Sterilize by autoclaving at 121°C for 15 minutes.
   iv. Dispense 25 mL in each petri dish and cool to room temperature.
3. Dispense TAT broth aliquots (9 mL) into screw-capped test tubes and autoclave it at 121°C for 15 minutes. Cool to room temperature before use.

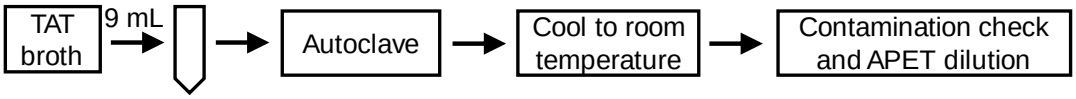


Figure 1. Preparation of TAT broth.

4. Prepare 0.85% saline solution by dissolving 8.5 g NaCl in 1000 mL of distilled water. If required, adjust to pH 6 - 7 using 1N NaOH or 1N HCl. Dispense 9 mL into screw-capped tubes, autoclave at 121°C for 15 minutes, and cool to room temperature before use. Use for inoculum counts. Saline may be stored at room temperature for up to 7 days. If the saline is stored for a longer period, then it must be refrigerated.

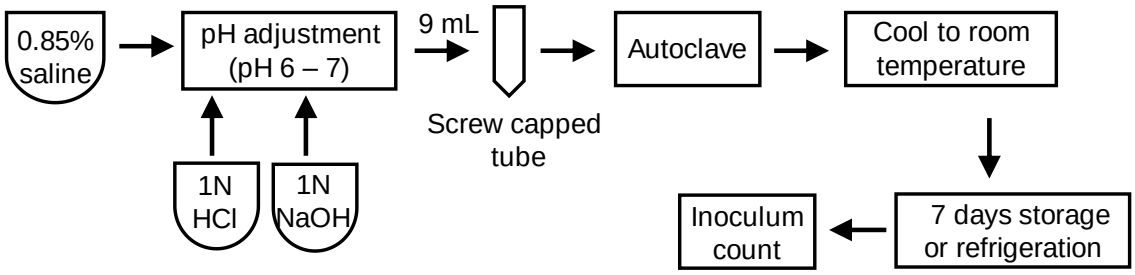


Figure 2. Preparation and storage of a saline solution.

5. BacTiter-Glo reagent can be made by the following steps prior to use.
   i. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature before use. For convenience the BacTiter-Glo™ Buffer may be thawed and stored at room temperature for up to 48 hours before use.
   ii. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature.
   iii. Transfer the BacTiter-Glo™ Buffer into the amber bottle containing BacTiter-Glo™ Substrate to reconstitute the lyophilized enzyme/substrate mixture. This forms the BacTiter-Glo™ Reagent.
   iv. Mix by gently vortexing, swirling or by inverting the bottle to obtain a homogeneous solution. The BacTiter-Glo substrate should dissolve easily, in less than one minute.
   v. Equilibrate reagent at room temperature for at least 1 and half hours before use. ***Note:*** *For long-term storage, the lyophilized BacTiter-Glo™ Substrate and BacTiter-Glo™ Buffer should be stored at –20°C. For frequent use, the BacTiter-Glo™ Buffer can be stored at 4°C or at room temperature for 48 hours without loss of activity. For optimal performance, reconstituted BacTiter-Glo™ Reagent*

(buffer plus substrate) should be used within eight hours when the reagent is kept at room temperature. The reconstituted BacTiter-Glo™ Reagent can be stored at 4°C for four days, at –20°C for one week or at –70°C for one month with less than 20% loss of activity.

C. Stock Cultures from ATCC (or equivalent)

i. Bacteria Stock Cultures

a. If applicable, verify the passage number on culture certificate upon receipt. ATCC (or equivalent culture bank) – the passage number should be zero (0).
b. Expand cultures using the media and incubation method as described by source recommendations (i.e. ATCC).
c. Prepare aliquots of bacteria stock culture in cryovials and label each vial appropriately.
d. This is the stock culture isolation and should be stored -80ºC or per source recommendations.
e. Working culture plates are prepared from the stock culture isolate as needed.

D. Preparation of Microbial Inoculum (Pre day 0 and day 0) and Colony Counting

Bacteria Working Culture

1. Pour 25 mL of the MLA into a sterile petri dish and wait until MLA is solidified. This petri dish will be referred to as an MLA plate and will be used for cell counting and colony isolation. Store this plate in a 4°C refrigerator for future use.
2. Obtain the frozen bacteria stock culture.
3. Using a 10 µL disposable sterile loop scrape some bacteria off the top from the frozen bacteria stock culture and streak onto the MLA plate. Label as working culture with the appropriate passage number (i.e., If the stock culture is T1, the working culture is T2).
4. Record the source transfer in the culture maintenance record.
5. Incubate bacterial cultures at 30-35°C for 24-48 hours
6. After incubation, the working culture should be stored inverted at 4°C for a maximum of 6 weeks. ***Note:*** *B. cepacia is stored for a maximum of 4 weeks at 22.5°C.*

Bacteria Inoculum

1. Pick up a colony from the MLA plate using a sterile loop and transfer into 10 mL of Tryptic Soy Broth (TSB) and incubate at 37°C for 8-20 hours on a shaker.
   Note: *S. aureus, P. aeruginosa, and E. cloacae are incubated for 8 hours whereas B. cepacia is incubated for 20 hours. Measure the OD/luminescence of microbials from the first transfer to check the growth.*
2. Remove a loopful of culture from the first transfer and place into a 10 mL of TSB. This second transfer is performed 8-20 hours prior to the inoculation of the samples. Incubate at 37°C on a shaker.
   Note: *Each subsequent transfer (working or test cultures) is plus one (+1) passage from the plate. The final transfer number used for testing shall not exceed T5. When working culture T3 is used for the first transfer into broth for the APET testing, the first broth transfer represents T4 and second broth transfer representsT5.*
3. Vortex the tube of the second transfer. This is the bacteria inoculum that will be used for the APET. Pooled inoculum must be used within one hour.
   Note: *Prior to vortexing, carefully remove the pellicle from the P. aeruginosa and B. cepacia. To perform this step, either carefully decant the organisms into a sterile tube (leaving the pellicle behind) or by carefully removing the pellicle by using a pipette.*

Verification of Inoculum Counts

1. Prepare serially diluted bacteria suspensions (ranging from 10^{-4} to 10^{-8}) by using the bacteria inoculum and 0.85% sterile saline solution.
2. Streak 10 µL of different concentrations of bacterial suspensions in MLA plates using sterilized loops. Incubate inverted MLA plates at 30°C for a minimum of 48 hours.
3. To determine the number of viable cells in the bacteria inoculum, count the number of colonies on the MLA plates and record CFUs at different dilution ratios.
4. The sample testing will continue with the remaining original bacteria inoculum.

E. Encapsulation of Microbials in Agarose on a 384PillarPlate

1. Centrifuge the remaining original bacteria inoculum at 1800 g and remove the supernatant.
2. Resuspend the cell pellet with fresh, sterile Tryptic Soy Broth (TSB) and prepare a fresh bacteria inoculum with optical density (OD) of 1.0 at 600 nm (approximately 1 x 10^9 cells/mL). ***Note:*** *This step is necessary to maintain the high viability of bacteria in agarose for a longer period and/or adjust the seeding density of bacteria greater than OD 1.0 to enhance ATP signal intensity.*
3. For the liquid sample test, dilute the bacteria suspension 10-fold resulting in OD of 0.1 with 3% agarose. ***Note:*** *OD of 0.1 is required to achieve high seeding density for higher log-reduction.*
4. For dishwashing liquid test against pool of bacteria, mix the bacteria in equal volume (i.e., 50 µL each) with a final seeding density of 0.1 after 10-fold dilution in agarose. ***Note:*** *Each bacterium should have an OD of 1 in TSB so that they can be mixed at an equal volume*.
5. Prepare 20 mL of 3% (w/v) agarose solution by mixing 0.6 g of low temperature gelling agarose with 20 mL of deionized water.
6. Pour the agarose solution into a clean glass bottle and autoclave the solution.
7. Incubate the hot agarose solution in an incubator with 37°C temperature setting for at least 1 hour to cool down the agarose solution to 37°C. ***Note:*** *The viability of microbials will be significantly reduced when mixed with the hot agarose solution. Be sure to incubate the hot agarose solution long enough to cool down to 37°C.*
8. Once the agarose solution cools down to 37°C, mix 2 mL of the bacteria inoculum with 18 mL of warm 3% agarose *via* vertexing. ***Note:*** *When the bacteria inoculum with OD of 1.0 is used, the final seeding density will be approximately 1 x 10^8 cells/mL due to the 10-fold dilution with the agarose solution. The bacteria seeding density in agarose is extremely important to minimize cell loading errors.*
9. While maintaining good suspension of bacteria in agarose by gentle pipetting, pour the warm mixture of bacteria and agarose to a sterile LoadingPlate (or 384-well plate lid) and place the LoadingPlate on a heating block with 37°C temperature setting. ***Note:*** *Make sure to maintain the temperature of the bacteria-agarose mixture at 37°C on the heating block to avoid premature gelation of agarose. It is very important to maintain good suspension of bacteria in agarose to increase accuracy and reproducibility of cell loading on the 384PillarPlate via stamping.*
10. Stamp a blank 384PillarPlate onto the LoadingPlate containing the mixture of bacteria and agarose. ***Note:*** *Stamping must be completed while maintaining good cell suspension. Since 3% agarose is viscous, microbials can maintain good suspension for approximately 1 hour. Make sure all pillars to contact the bacteria-agarose mixture.*
11. Separate the 384PillarPlate and let the bacteria-agarose mixture on pillars form a gel in a biosafety cabinet at room temperature for 30 minutes. ***Note:*** *Visually inspect the*

384PillarPlate to make sure that all pillars have a hemispherical dome.

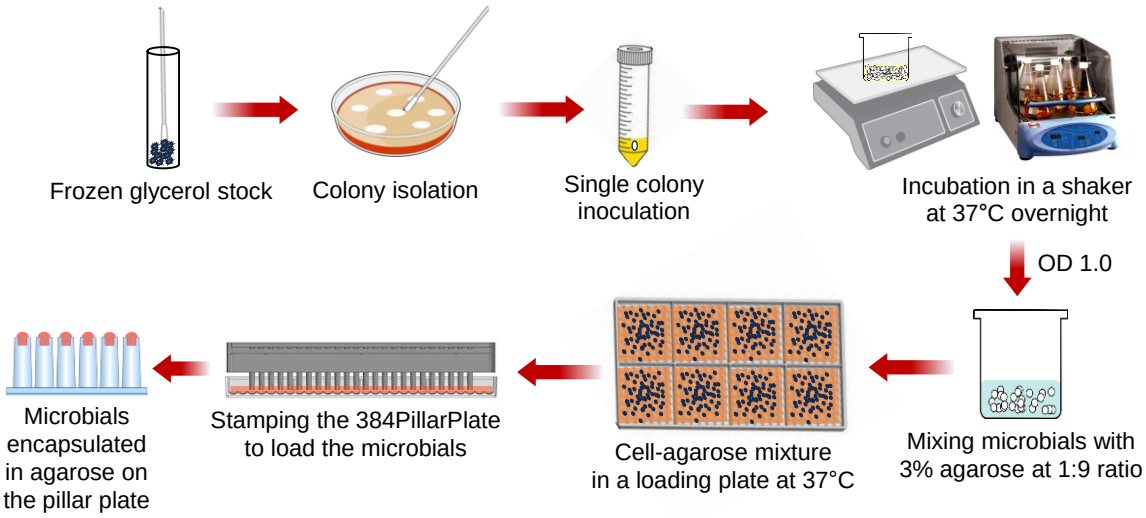


Figure 3. Encapsulation of microbials in agarose on the 384PillarPlate**.**

For luminescence measurement of untreated microbials (100% live control)

1. Set aside the 384PillarPlate with bacteria encapsulated in 3% agarose from **step E.11** for measuring luminescence from untreated microbials (100% live control). ***Note:*** *This control condition is required to calculate % reduction in cell viability.*
2. Prepare a 384-well plate with TAT broth by dispensing 45 µL of TAT broth in each well.
3. Rinse the 384PillarPlate by sandwiching with the 384-well plate containing TAT broth for 1 hour.
   Note: *This rinsing step is necessary to remove residual samples on the 384PillarPlate that might interfere with luminescence measurement. It is required to remove any unbound microbials.*
4. Immediately measure bacteria viability by following **steps 5-8** in **Section H**.

F. Single Challenge of Microbials against Liquid Samples

6. Prepare three 384DeepWellPlates, each plate containing nine test samples by dispensing 90 µL of each sample in two columns (32 replicates, starting from columns 4 to 21), using a 30-300 µL multichannel pipette.
   Note: *Each microbial requires three 384DeepWellPlates with test samples throughout the testing period for both single and double challenges.*
7. Insert the three 384PillarPlates with one type of bacterium encapsulated in 3% agarose from **step E.11** into the three 384DeepWellPlates with the test samples.
8. Incubate the sandwiched plates in a humid container (bioassay dishes filled with water) in an incubator at an ambient temperature (22°C to 27°C) for 7 days.
   Note: *Incubation in a humid container is necessary to prevent water evaporation.*
9. On day 7, detach the 384PillarPlates from the 384DeepWellPlates. Set aside the 384DeepWellPlates with test samples for double challenge.
10. Measure bacteria viability on the 384PillarPlate by following the steps in **Section H**.

G. Double Challenge of Microbials against Dishwashing Liquid Samples

11. Prepare three 384PillarPlates with fresh bacteria encapsulated in 3% agarose by following the steps in **Section E**.

Note: *One additional 384PillarPlate is prepared for the 100% live control. Measure luminescence from untreated microbials.*

12. Insert the 384PillarPlate with bacteria into the 384DeepWellPlate containing the used test samples from single challenge.
13. Incubate the sandwiched plates in a humid container in an incubator at ambient temperature (22°C to 27°C) for additional 7, 14, and 21 days.
14. At days 7, 14, and 21, detach one 384PillarPlate from the 384DeepWellPlate and measure bacteria viability by following the steps in **Section H**.

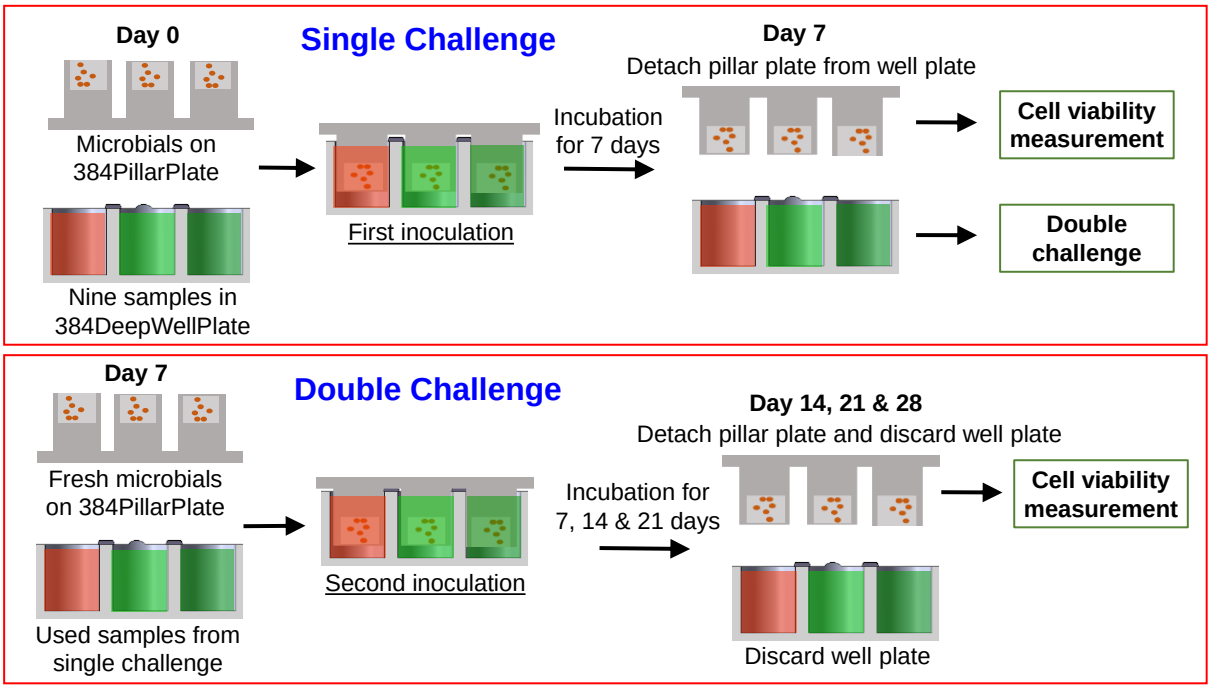


Figure 4. Single and double challenge of microbials against dishwashing liquid samples.

H. High-throughput assessment of microbial viability with BacTiter Glo reagent

1. Prepare a 384-well plate with TAT broth by dispensing 45 µL of TAT broth in each well.
2. Rinse the 384PillarPlate by sandwiching with the 384-well plate containing TAT broth for 1 hour.
   Note: *This rinsing step is necessary to avoid residual sample carryover on the 384PillarPlate that might interfere with the viability. If the test samples are highly viscous, rinse multiple times to remove residual samples.*
3. Prepare a 384-well plate with TAT broth by dispensing 45 µL of TAT broth in each well.
4. Sandwich the 384PillarPlate from single and double challenge with the 384-well plate containing TAT broth and incubate at 30°C for 8 - 24 hours depending on the doubling time of microbials
   Note: *This step is necessary to obtain luminescence intensity above the detection limit.*
5. Dilute the BacTiter-Glo reagent with TSB at 1:1 ratio and dispense 40 µL of this solution into the wells of an opaque 384-well plate.
6. Sandwich the 384PillarPlate with the opaque 384-well plate containing the diluted BacTiter-Glo reagent.
7. Wrap the sandwiched plates completely using an aluminum foil and place it on the

Incu-Shaker for 30 minutes at room temperature.

8. Detach the opaque 384-well plate containing the diluted BacTiter-Glo reagent and record luminescence at 560 nm (gain at 135) and save the luminescence reading.

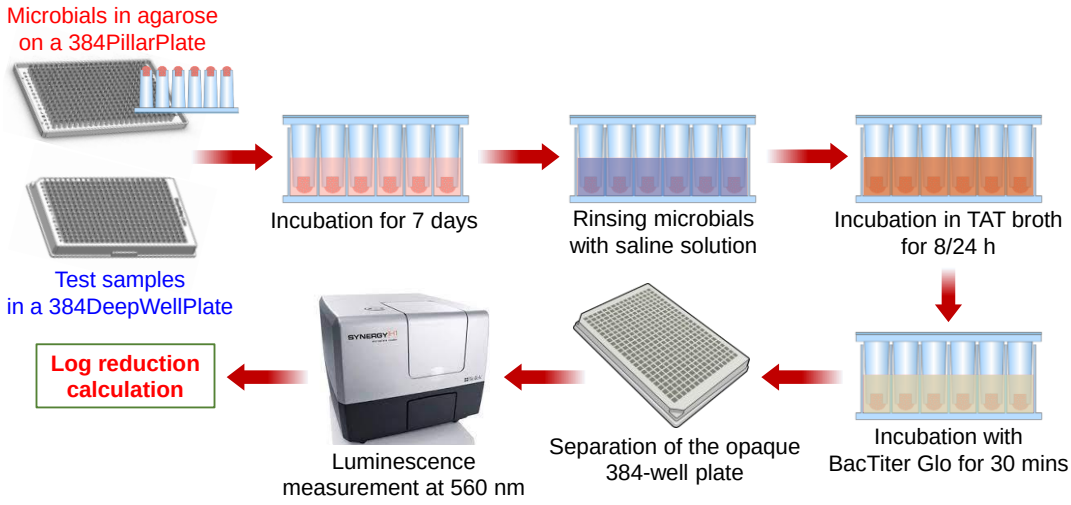
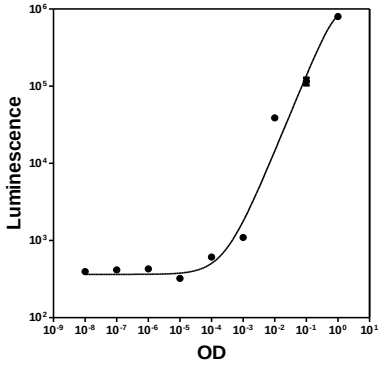


Figure 5. Experimental procedures for antimicrobial preservatives efficacy test (APET) on a 384PillarPlate.

I. Calculation of Log Reduction in Cell Viability

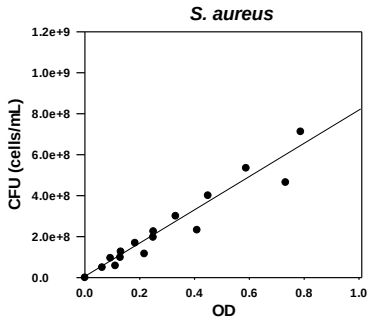
Reduction in cell viability after sample treatment is calculated by comparing the cell number from the sample treated conditions and 100% live control.

1. Subtract background luminescence (luminescence from agarose alone) from the luminescence of sample treated and control conditions.
2. Obtain the optical density (OD) from the respective luminescence using the OD vs Luminescence calibration curves for each microbial.
   Example: *OD vs luminescence calibration curve and the equation obtained for S. aureus, where Y is luminescence.*



$$\mathbf{OD} = \frac{-0.24 - \log(2.63x10^{6})}{(Y + 1.66x10^{6}) - 1}$$

3. Obtain the cell numbers from the respective OD using the OD vs cell number calibration curves for each microbial.
   Example: *OD vs cell number calibration curve and the equation obtained for S. aureus, where Y is cell numbers/mL and X is OD.*



$$Y = 8.3x10^{8}X$$

4. Calculate the cell numbers for the sample treated conditions at time **t = 0** using the cell doubling time equation.
   Example: *Cell doubling time equation of S. aureus.*

$$1 * (Log(Y) - Log(X)) = 8 * (LOG(2))$$

5. Calculate normalized log reduction in cell viability using the equation below.

$$\textbf{Log Reduction} = \log_{10}\left(\frac{X}{Xi}\right) \textbf{ x NF}$$

   where X= control cell number at time t=0, Xi= cell number after sample treatment calculated at time t=0, and NF is the normalization factor. Normalization factor indicates the maximum log reduction from traditional APET divided by the maximum log reduction from on-chip APET. NF is introduced to normalize the difference in cell seeding density in the two APET assays.

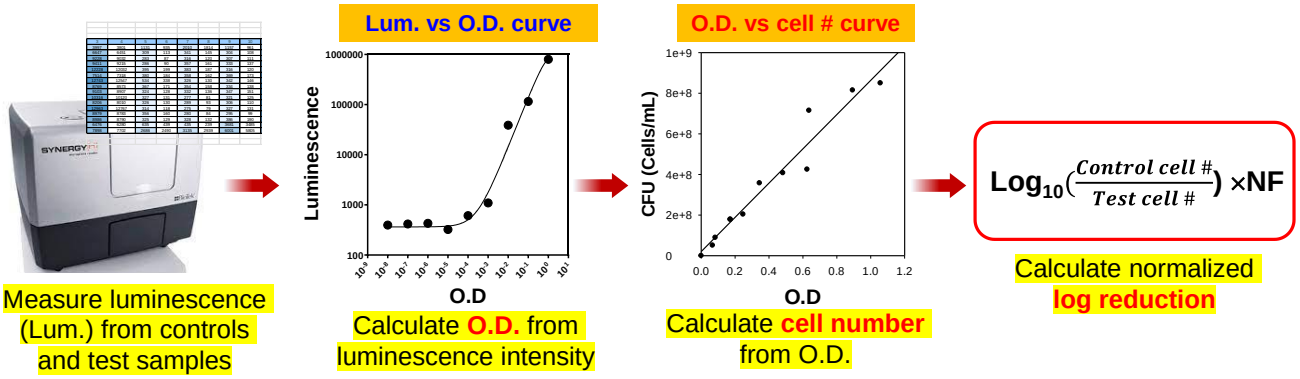


Figure 6. Analytical procedures for calculating normalized log reduction of microbials using luminescence data obtained from the 384PillarPlate.