Single-Exposure Antimicrobial Challenge Using 3D Microbial Culture

1. Scope

This application SOP describes a single-exposure antimicrobial challenge workflow for evaluating antimicrobial agents, including preservatives, disinfectants, cleaners, cosmetics, home-care products, and other test formulations, against 3D microbial cultures encapsulated in agarose on a pillar plate. Post-exposure microbial viability is determined using ATP-based luminescence assays, with optional colony-forming unit (CFU)-based recovery when required. This SOP shall therefore be used together with the SOPs titled "**3D Microbial Culture in Agarose on Pillar Plate**" for microbial inoculum preparation and agarose encapsulation on a 384PillarPlate, and "**Microbial Viability Assay on Pillar Plate**" for luminescence-based and CFU-based viability assessment. This SOP is intended for broad antimicrobial formulation screening and comparative testing using a single microbial inoculation event applied to one challenged formulation condition. For general screening applications, the workflow may be conducted using a single predefined exposure interval. For **antimicrobial preservative efficacy testing (APET)**-style studies, a single microbial challenge is applied at day 0, and surviving microorganisms are evaluated at predefined post-challenge intervals, typically days 7, 14, 21, and 28, unless otherwise specified in the approved study design. This timing framework reflects the conventional single-challenge preservation design used in traditional APET methodologies. This workflow is intended as a platform-adapted starting point for antimicrobial evaluation using the 384PillarPlate system. Users shall define microorganism-specific culture conditions, control strategies, exposure durations, monitoring intervals, recovery procedures, and any required neutralization methods appropriate for the intended application.

This SOP does not replace compendial preservative effectiveness tests, regulatory disinfectant standards, or direct bulk-product APET methodologies. Studies involving repeated microbial re-inoculation, multiple challenge cycles, short contact-time disinfection studies, or regulatory compliance testing shall be conducted under separate application-specific SOPs or approved study protocols. Conventional APET methods are based on direct bulk-product inoculation followed by serial recovery from the challenged product over time, whereas this SOP utilizes a 384PillarPlate-based 3D microbial culture platform. Accordingly, this method shall be regarded as a platform-adapted screening method unless independently validated for equivalence to applicable regulatory or compendial standards.

2. Basic Principle

A 384PillarPlate containing agarose-encapsulated microorganisms is sandwiched onto a complementary 384DeepWellPlate containing antimicrobial formulations and appropriate controls, thereby exposing the microorganisms to the test formulations during a defined single-challenge event. Following exposure, the pillar plate is separated and, when required, rinsed or neutralized to remove residual antimicrobial formulations. Microbial viability is subsequently quantified using ATP-based luminescence according to the SOP titled "**Microbial Viability Assay on Pillar Plate**." When conventional viable enumeration is required, microorganisms may be recovered from individual pillars by sonication and quantified by serial dilution and colony counting according to the same assay SOP. For broad antimicrobial formulation screening, the challenged pillar plate may be evaluated after a single predefined exposure interval. For APET-like single-challenge preservation studies, microbial survival within the challenged formulation condition is monitored at multiple predefined post-challenge intervals, typically days 7, 14, 21, and 28 following a single initial inoculation event, unless otherwise specified in the approved study design.

3. Safety Requirements

- General laboratory safety practices shall be followed throughout the procedure.
- All work involving microorganisms shall be conducted using appropriate biosafety practices and aseptic techniques in accordance with institutional requirements.
- Safety glasses, laboratory coat, gloves, and any other required personal protective equipment (PPE) should be worn throughout all steps.

- All microbial waste, contaminated disposables, and chemical waste shall be discarded according to institutional biohazard and chemical safety procedures.
- Safety Data Sheets (SDS) for all chemicals, reagents, and test articles shall be reviewed prior to use.

4. Equipment and Plasticware Necessary

A. Refrigerator (FFHI1832TS0, Frigidaire)
B. Vortex mixer (02215365, Fisher Scientific)
C. Temperature-controlled shaker (Z765686, Sigma-Aldrich)
D. Temperature-controlled incubator (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 (384-01-00, Bioprinting Laboratories Inc.)
L. 384DeepWellPlate (384-02-00, Bioprinting Laboratories Inc.)
M. LoadingPlate (384-03-00, 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)
T. Bath sonicator

5. Microbials, Media, and Reagents

A. Microbials including *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 15442), and *Enterobacter cloacae* (PLS237, ThermoFisher)
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. Controls and Plate Layout Guidance

- Include at least one untreated control condition representing 100% viable microorganisms for each microorganism and exposure day.
- Include formulation blanks or matrix-only controls when test formulations may generate background signal, interfere with luminescence measurements, contribute reagent carryover, or otherwise require interpretation relative to a non-microbial baseline.
- Include background controls, such as agarose-only pillars or matrix-only wells without microorganisms, when no application-specific blank condition has been defined.
- When microbial recovery or neutralization procedures are required, include appropriate user-defined recovery controls, neutralizer suitability controls, and recovery-efficiency assessments appropriate for the formulation class and intended application.

- For APET-like preservation studies, users should additionally confirm that test formulations are suitable for microbial challenge testing and are not materially pre-contaminated prior to study initiation. Conventional APET methodologies typically include pre-test contamination assessment and neutralization suitability evaluation as part of method suitability qualification.
- Use a minimum of three technical replicates per condition. For screening applications, six or more technical replicates are recommended when plate capacity permits.
- Use one microorganism per pillar plate unless a mixed-culture study design has been intentionally defined, justified, and documented before study initiation.
- Define and document plate maps, control placement, replicate numbers, excluded edge wells if applicable, scheduled monitoring intervals, formulation-specific handling requirements, and any recovery or neutralization procedures prior to study initiation.

7. Experimental Protocols

A. Preparation of a Pillar Plate with Agarose-Encapsulated Microorganisms

Prepare the microbial inoculum and encapsulate microorganisms in agarose on the 384PillarPlate according to the SOP titled "**3D Microbial Culture in Agarose on Pillar Plate**." This application SOP begins after preparation of the pillar plate containing agarose-encapsulated microorganisms has been completed in accordance with that SOP.

Before proceeding, confirm that microorganism-containing agarose domes are uniformly deposited on the pillar plate and are free of major defects, including incomplete loading, dome detachment, excessive bubble formation, or visible cross-contamination.

Preparation of untreated controls, ATP-based luminescence viability measurements, and optional CFU-based viable recovery procedures shall be performed according to the SOP titled "**Microbial Viability Assay on Pillar Plate**," unless otherwise specified in this application SOP.

B. Preparation of a 384DeepWellPlate with Antimicrobial Test Formulations

1. Prepare a complementary 384DeepWellPlate by dispensing user-defined volumes of antimicrobial test formulations, control samples, and any required blank or neutralization controls into designated wells according to a predefined plate map.
   Note: *Confirm that test formulations are prepared in a microbial culture medium, diluent, or vehicle appropriate for the intended study objective and compatible with microbial survival during the defined exposure period where applicable. Control conditions shall support measurable microbial viability when required for data interpretation. If microorganisms do not maintain acceptable viability in the relevant untreated or vehicle control conditions, study results may be misleading and the assay may be considered invalid or unsuitable for interpretation.*
2. For antimicrobial formulation screening using the 384DeepWellPlate, a loading volume of **70 µL per well** may be used as a practical starting condition unless another volume has been justified based on the formulation properties, exposure requirements, or paired pillar/deep-well plate geometry.
   Note: *Excessive loading volumes may increase the risk of overflow, cross-contamination, incomplete plate separation following sandwiching, or unintended liquid bridging between wells and should therefore be avoided.*
3. Arrange antimicrobial test formulations, controls, blanks, and replicate conditions according to a predefined plate map that enables clear identification and traceability of each condition and its associated replicate set throughout the study.
4. When multiple microorganisms are evaluated within the same study, prepare a separate 384DeepWellPlate for each microorganism unless an alternative study design, including mixed-culture conditions, has been explicitly justified, validated, and documented before study initiation.
5. When extended monitoring periods are required, maintain the assembled or stored plates within a humidified container, evaporation-control chamber, or other appropriately controlled

environment to minimize well-volume loss, edge effects, and unintended concentration changes during the study period.

C. Single-Challenge Exposure with Antimicrobial Test Formulations

6. Sandwich the 384PillarPlate containing agarose-encapsulated microorganisms onto the complementary 384DeepWellPlate containing antimicrobial test formulations, controls, and any required blank or neutralization conditions, ensuring full, stable, and uniform pillar-to-well contact across the plate assembly.
7. Incubate the assembled plate system for the predefined single-challenge exposure interval under temperature and environmental conditions appropriate for the intended study objective and microorganism.
   Note: *For general antimicrobial formulation screening, a single predefined exposure duration may be used and documented as the primary study endpoint interval.*
8. For **APET-like single-challenge preservation studies**, apply one initial microbial challenge event and monitor surviving microorganisms at predefined post-challenge intervals, typically **days 7, 14, 21, and 28**, unless an alternative schedule has been justified and approved in the study design. The challenged formulation condition shall remain associated with its intended monitoring schedule throughout the study period. This approach reflects the conventional APET single-challenge framework, in which one initial inoculation event is followed by serial monitoring over time rather than repeated microbial re-inoculation.
   Note: *Unless otherwise justified, ambient incubation conditions of approximately 22°C to 27°C may be used as a practical starting condition for APET-like preservation monitoring studies.*
9. At each designated monitoring interval, aseptically detach the pillar plate from the formulation plate, or retrieve the corresponding challenged pillar plate designated for that interval, according to the predefined study design and plate-handling workflow.
10. Retain or discard the formulation plate in accordance with the study design, documentation requirements, biosafety procedures, and laboratory waste-handling policies.

D. Post-Exposure Recovery and Microbial Viability Measurement

After completion of the single-challenge exposure interval or scheduled post-challenge monitoring time point, perform any required post-exposure recovery or neutralization procedures, ATP-based luminescence viability assessment, and optional CFU-based viability measurement according to the SOP titled "**Microbial Viability Assay on Pillar Plate**."

For typical antimicrobial log-reduction workflows, recovery or neutralization using a compatible 384DeepWellPlate containing an appropriate recovery medium or neutralization solution may be used as a practical starting condition prior to luminescence-based viability assessment, as specified in the assay SOP and appropriate for the formulation class being evaluated.

When APET-like single-challenge monitoring is performed, conduct the viability assay at each predefined post-challenge interval and document the corresponding study day, exposure duration, recovery conditions, and assay time point as part of the study record.

E. Optional Conventional Microbial Viability Assessment by Colony Counting

When conventional viable microbial enumeration is required, perform sonication-assisted dissociation of agarose dome for microorganism recovery, serial dilution, and colony counting according to the SOP titled "**Microbial Viability Assay on Pillar Plate**."

When APET-like preservation assessment is intended, CFU-based viable counting may serve as the primary conventional microbiological endpoint when required by the study design, customer requirements, or application-specific evaluation criteria. Document dilution schemes, plating methods, incubation conditions, countable ranges, colony enumeration procedures, and all calculated CFU-based results in accordance with laboratory documentation practices and the referenced assay SOP.

F. Data Analysis

11. Collect and review all raw assay data, including measurements obtained from treated samples, untreated controls, background controls, recovery controls, neutralization controls, and any additional control conditions defined by the study design.
12. Subtract background signal from all applicable assay readings using the appropriate blank, matrix-only, or non-microbial control condition. When no application-specific background control has been defined, agarose-only pillars or matrix-only wells without microorganisms may be used as default background controls, as appropriate for the assay format and detection method.
13. Normalize treated sample values relative to the corresponding untreated control condition to determine microbial viability, antimicrobial response, or microbial reduction.
14. Results may be reported as percent viability, percent reduction, CFU per pillar, CFU per sample, log10 CFU, log reduction, relative luminescence units (RLU), or other user-defined response metrics appropriate for the assay readout and study objective.
15. The following general calculations may be applied as appropriate:
    - **Percent Viability = (Treated Signal / Untreated Control Signal) x 100**
    - **Percent Reduction = [1 - (Treated Signal / Untreated Control Signal)] x 100**
    - **Log Reduction = log10 (Control / Treated)**
16. When assay signals are converted to OD, estimated cell number, CFU, or other derived biological values, the conversion shall be based on a user-defined, validated, or internally approved calibration method appropriate for the microorganism, assay format, and detection platform. For exploratory, screening, or relative-comparison studies, background-corrected luminescence values may be used directly as the primary response metric until microorganism-specific calibration models or standard curves have been established.
17. For CFU-based workflows, viable recovery may be reported as CFU per pillar, CFU per sample, log10 CFU, or log reduction relative to untreated controls, as appropriate for the study objective. When no colonies are observed at the lowest plated dilution, results shall be reported using the user-defined lower limit of detection (LOD) established for the plating and recovery method.
18. For APET-like single-challenge studies, results shall be assigned to the corresponding scheduled monitoring interval, typically days 7, 14, 21, or 28 following the initial microbial challenge event, unless an alternative monitoring schedule has been predefined and documented in the approved study design.
19. Any normalization factor, correction factor, calibration adjustment, excluded data point, or data-processing modification applied during analysis shall be documented and scientifically justified (**Fig. 1**).
20. Replicate measurements shall be summarized using appropriate statistical descriptors, which may include mean, standard deviation, coefficient of variation, confidence intervals, or other study-defined statistical parameters, as required by the study protocol.
21. All raw data, processed data, calculations, calibration records, exclusions, deviations, and analytical outputs shall be retained as part of the permanent study record in accordance with laboratory documentation and record-retention procedures.

G. Quality Requirements / Acceptance Checks

1. Microbial loading on the pillar plate shall be visually uniform, adequately distributed across intended pillars, and free of major defects such as incomplete loading, dome detachment, excessive bubble formation, or visible cross-contamination.
2. Untreated control signals, recovery-control measurements, or viable recovery counts shall fall within the internally established acceptable assay range for the specific microorganism, assay format, and detection method used in the study.
3. Background signal levels shall remain acceptably low relative to untreated control conditions and shall not interfere with interpretation of antimicrobial response or viability measurements.

4. Replicate consistency shall meet internally defined assay acceptance criteria. For CFU-based workflows, serial dilution and plating results shall demonstrate reasonable internal consistency and shall be free of obvious technical artifacts or procedural failures, including uncontrolled lawn growth, complete absence of growth without scientific explanation, irregular dilution-response patterns, plating contamination, or failure of negative-control conditions.
   Note: *For CFU-based workflows, recovery controls shall generate countable colonies within the validated working range of the selected plating method. The dilution scheme should be designed to bracket at least one countable spot or plate whenever viable growth is present.*
5. No evidence of gross contamination, substantial evaporation, major liquid leakage, widespread pillar-loading failure, or other conditions that could compromise assay interpretation shall be present.
6. Any deviation from the approved procedure, predefined study design, or assay acceptance criteria shall be documented, reviewed, and evaluated for potential impact on study interpretation.
7. A replicate scheme appropriate for the intended study objective shall be predefined before study initiation. Unless otherwise justified, a minimum of triplicate technical replicates is recommended for exploratory or screening studies.

H. Documentation

1. Record microorganism identity, strain information where applicable, inoculum preparation details, culture conditions, and passage history or subculture information relevant to the study.
2. Record antimicrobial test formulation identity, concentration or neat-use status, formulation preparation details if applicable, plate map configuration, control placement, paired plate type, loaded volume per well, and any formulation-specific handling, recovery, or neutralization requirements.
3. Record exposure duration, incubation temperature, humidity-control or evaporation-control conditions, scheduled monitoring intervals, and all recovery, rinse, or neutralization procedures performed during the study.
4. Record assay reagent preparation details, reagent lot information when applicable, plate reader settings, instrument parameters, and all raw assay output data. When the optional CFU-based workflow is performed, additionally record recovery vessel type and recovery volume, sonication or dissociation conditions, plating medium, dilution scheme, incubation conditions, colony counting methodology, and raw colony count data.
5. Record all calculations, normalization methods, calibration approaches, processed data outputs, final reported results, deviations from the approved procedure, excluded data points, corrective actions, and study-specific observations relevant to data interpretation.
6. Archive all study records, raw data, processed data, plate maps, analytical outputs, calculations, deviations, and supporting documentation in accordance with internal document-control, data-retention, and laboratory record-management requirements.

8. Notes and Limitations

1. This SOP is intended as a platform-adapted single-challenge workflow for evaluation of antimicrobial formulations using a 384PillarPlate-based 3D microbial culture system. It is not intended to replace formal preservative efficacy tests, disinfectant efficacy standards, compendial antimicrobial effectiveness tests, or regulatory compliance methodologies unless separately validated or conducted under an approved study-specific protocol.
2. This SOP does not directly replicate the bulk-product inoculation and serial recovery workflow used in conventional APET methodologies unless independently validated for equivalence. Instead, this method translates APET-like single-challenge preservation principles onto a 384PillarPlate-based 3D microbial assay platform for screening, comparative evaluation, and exploratory testing applications.

3. Formulations or study designs requiring short contact-time exposure, repeated microbial re-inoculation, multiple challenge cycles, specialized neutralization procedures, non-ambient storage or incubation conditions, direct bulk-product regulatory interpretation, or other application-specific handling requirements shall be evaluated using separate application-specific SOPs or approved study protocols.
4. Exposure duration, monitoring intervals, recovery procedures, neutralization strategy, incubation conditions, and control design may require optimization depending on formulation chemistry, microorganism susceptibility, intended product-use conditions, assay sensitivity, and overall study objective.
5. ATP-based luminescence measurements reflect metabolically active microbial signal and may not always directly correlate with conventional viable colony recovery under all formulation or stress conditions. When required by the study objective, confirmatory CFU-based viable recovery studies should be performed.
6. Recovery efficiency, neutralization suitability, and assay interference should be evaluated for formulation classes known to contain residual antimicrobial activity, high viscosity, strong coloration, surfactants, oxidizing agents, or other properties that may interfere with microbial recovery or assay signal interpretation.
7. Results generated using this platform-adapted workflow should be interpreted within the defined scope, validation status, and intended application of the study and should not be assumed to demonstrate regulatory equivalence to compendial APET or disinfectant efficacy methods unless formally established.

Appendix: Passage Number Tracking Reference

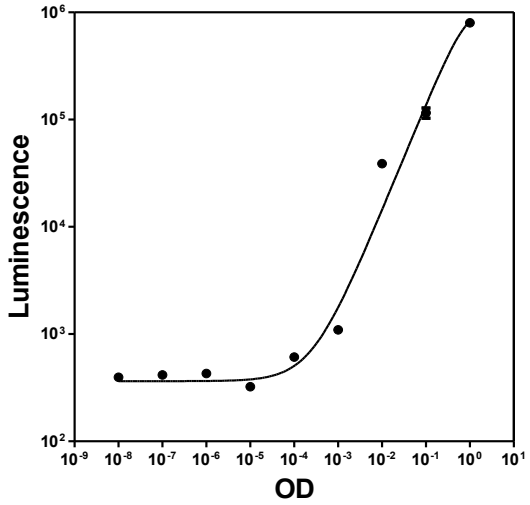
Proper passage tracking is essential to ensure biological reproducibility. Use the table below as a quick reference.

Culture stage	Label	Passage count	Notes
Source / master seed lot	T0	0	Obtained directly from ATCC
Stock culture	T1	1	Propagated from source
Working culture (MLA plate)	T2	2	Streaked from stock
First broth transfer	T3 - T4	3 - 4	From working culture plate
Second broth transfer (inoculum)	T4 - T5	4 - 5	Maximum T5 allowed for testing

Appendix: Calculation of Log Reduction

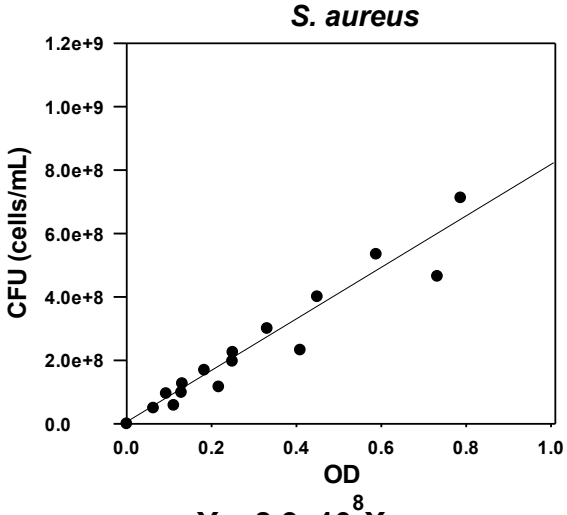
Reduction in microbial viability following antimicrobial sample treatment may be estimated by comparing microorganism levels in treated conditions with the corresponding untreated (100% viable) control condition.

1. Subtract background luminescence, such as the signal obtained from agarose-only controls or other defined non-microbial background controls, from all treated and untreated sample readings.
2. Convert corrected luminescence values to estimated optical density (OD) values using microorganism-specific luminescence-versus-OD calibration curves established for the assay system.
   Example: *OD-versus-luminescence calibration curve established for Staphylococcus aureus, where Y represents luminescence signal.*



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

3. Convert OD values to estimated microorganism concentration or cell number using microorganism-specific OD-versus-cell-number calibration curves.
   Example: *OD-versus-cell-number calibration curve established for Staphylococcus aureus, where Y represents cell number per mL and X represents OD.*



4. When required for time-normalized comparisons, estimate microorganism concentration at time *t = 0* using a microorganism-specific growth or doubling-time equation appropriate for the assay conditions.
   Example: *Cell doubling-time equation established for Staphylococcus aureus.*

$$N_t = N_0 \times 2^{t/g}$$

   Where:
   - N_0 = initial cell number at time *t = 0* (This is *Xi* for log reduction calculation)
   - N_t = cell number at time *t* (10,000 cells at time *t = 8* hours. This is from *OD-versus-luminescence and OD-versus-cell-number calibration curves.*)
   - *t* = elapsed time (8-hour incubation for signal amplification)
   - *g* = doubling time of the microorganism (0.5 hour for *Staphylococcus aureus*)

5. Calculate normalized log reduction in microbial viability using the following general equation:

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

Where:

- *X* = estimated microorganism concentration or cell number in the untreated control condition at *t = 0*
- *Xi* = estimated microorganism concentration or cell number in the treated condition normalized to *t = 0*
- *NF* = normalization factor

The normalization factor (NF) may be applied to account for differences in microorganism loading density, assay geometry, or dynamic range between conventional APET methodologies and the on-chip 384PillarPlate-based workflow. In comparative studies, NF may be defined as the ratio of the maximum measurable log reduction obtained using a conventional reference APET method to the maximum measurable log reduction obtained using the on-chip APET workflow.

Note: *Use of normalization factors, calibration equations, and growth-correction models shall be scientifically justified, documented, and consistently applied within a given study.*

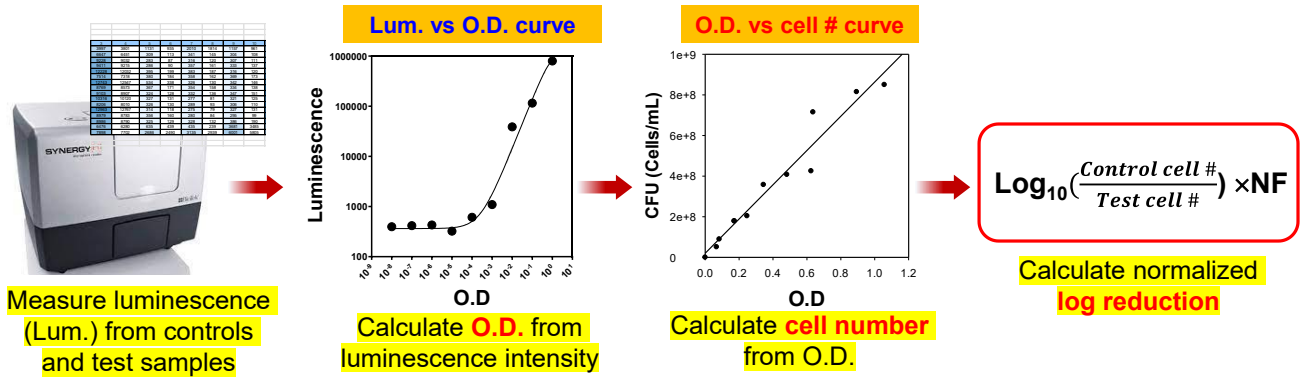


Figure 1. Analytical procedures for calculating normalized log reduction of microbials using luminescence data obtained from the 384PillarPlate.