Microbial Viability Assay on Pillar Plate

1. Scope

This Standard Operating Procedure (SOP) describes viability assessment of microorganisms encapsulated on a 384PillarPlate using a high-throughput ATP-based luminescence assay (BacTiter-Glo). When required, the SOP also includes a workflow for microbial recovery and colony-forming unit (CFU) enumeration. The luminescence workflow may be used for post-exposure log reduction analysis in single- or repeated-challenge formulation studies, or for direct relative viability and dose-response analysis where post-exposure signal amplification is not required. The optional CFU workflow may be used when conventional viable count recovery is required. This SOP shall be used with 384PillarPlates prepared according to the SOP titled “3D Microbial Culture in Agarose on Pillar Plate.” When microorganisms have been exposed to formulations or other test articles, the relevant application-specific exposure SOP shall define the exposure conditions, controls, and plate layout.

2. Basic Principle

Microbial viability is assessed using the BacTiter-Glo Luminescent Cell Viability Assay, which quantifies intracellular ATP as an indicator of viable cell number. When applicable, background-corrected luminescence signals may be converted to optical density (OD) values and subsequently to estimated cell counts using pre-established calibration curves. Depending on the study objective, a post-exposure recovery and incubation step may be included to amplify microbial signal prior to luminescence measurement, or omitted for direct dose-response or relative viability analysis when sufficient signal is already present. As an optional alternative to luminescence-based analysis, microorganisms may be recovered from individual pillars by controlled removal or breakage of the pillar tip. The excised pillar is then sonicated in an appropriate recovery or neutralization medium to detach and disperse the encapsulated cells. Recovered microorganisms may subsequently be quantified using standard microbiological methods, including serial dilution and CFU enumeration.

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. Media, and Reagents

A. Tryptic Soy Broth (TSB) (R08944, ThermoFisher)
B. Tryptone-Azolectin-Tween (TAT) broth (R112611, ThermoFisher)
C. Modified Letheen Agar (MLA) (R453722, ThermoFisher)
D. BacTiter-Glo microbial cell viability kit (G8230, Promega)
E. Sterile distilled water
F. 0.85% (w/v) saline solution by dissolving 8.5 g NaCl in 1000 mL distilled water

6. Definitions

Untreated / 100% live control: A pillar plate prepared identically to the test plates but not exposed to a test article, used as the reference condition representing 100% viability.
Background control: A control used to measure non-microbial background signal, such as agarose-only or matrix-only conditions, as appropriate.
Recovery control: A control processed through the recovery, dissociation, and plating workflow without exposure to a test article, used to verify viable recovery and suitability for CFU-based analysis.

7. Experimental Protocols

A. Media and Reagent Preparation

Tryptic Soy Broth (TSB)

TSB is used for preparation of microbial cultures and as a diluent throughout this SOP.

1. Prepare TSB according to the manufacturer's instructions.
2. Store at room temperature for up to 7 days. For longer storage, refrigerate at 4°C.

Modified Letheen Agar (MLA)

MLA is used for colony counting and working culture isolation.

3. Suspend 59.1 g of MLA powder in 1000 mL of deionized water.
4. Heat to boiling with agitation until completely dissolved.
5. Sterilize by autoclaving at 121°C for 15 minutes.
6. Allow to cool slightly, then dispense 25 mL per sterile petri dish.
7. Let solidify at room temperature before use. Store plates inverted at 4°C.

Tryptone-Azolectin-Tween (TAT) Broth

TAT broth is used in the rinsing and viability incubation steps of the assay.

8. Dispense 45 mL aliquots of TAT broth into screw-capped bottles.
9. Autoclave at 121°C for 15 minutes.
10. Cool to room temperature before use.

0.85% Saline Solution

Saline is used for inoculum serial dilutions and colony counting.

11. Dissolve 8.5 g NaCl in 1000 mL distilled water.
12. If required, adjust to pH 6 - 7 using 1N NaOH or 1N HCl.

13. Dispense 9 mL into screw-capped tubes and autoclave at 121°C for 15 minutes.
14. Cool to room temperature before use. Store at room temperature for up to 7 days; refrigerate for longer storage.

BacTiter-Glo Reagent

15. Thaw the BacTiter-Glo™ Buffer and equilibrate to room temperature. This solution may be held at room temperature for up to 48 hours.
16. Equilibrate the lyophilized BacTiter-Glo™ Substrate to room temperature.
17. Transfer the Buffer into the amber bottle containing the Substrate to reconstitute the enzyme/substrate mixture.
18. Mix by gentle vortexing, swirling, or inversion until homogeneous (< 1 minute).
19. Equilibrate the reconstituted reagent at room temperature for at least 1.5 hours before use.
    Note: *For long-term storage, the lyophilized substrate and buffer should be stored at −20°C. Use the reconstituted reagent within 8 hours at room temperature, or store at 4°C for up to 4 days, −20°C for 1 week, and −80°C for 1 month (< 20% activity loss).*

B. Luminescence-Based Viability Assessment

Assessment of 100% Live Control (Untreated Microbials)

1. Set aside one 384PillarPlate containing microorganism-laden agarose and prepared according to the SOP titled "3D Microbial Culture in Agarose on Pillar Plate" for use as the 100% live control.
2. Dispense 70 µL of 0.85% (w/v) saline solution into each well of a 384DeepWellPlate.
3. Sandwich the control 384PillarPlate with the saline plate and incubate for 1 hour.
   Note: *This rinse removes unbound microbials and residual agarose components that could interfere with luminescence readings.*
4. Immediately proceed to the luminescence measurement steps described below to record baseline luminescence.

Assessment of Log Reduction, Requiring Signal Amplification

1. Prepare a 384DeepWellPlate containing 70 µL of 0.85% (w/v) saline solution in each well.
2. Rinse the 384PillarPlate by sandwiching it with the saline plate for 1 hour to remove residual test samples.
   Note: *Additional rinses may be required if test samples are viscous and difficult to remove.*
3. Prepare a 384DeepWellPlate containing 70 µL of TAT broth in each well.
4. Sandwich the rinsed 384PillarPlate with the TAT broth plate and incubate at 30°C for 8 - 24 hours (or as determined by the microorganism's doubling time and signal intensity) to allow sufficient microbial growth and signal generation (**Fig. 1**).
   Note: *This step is required for the microbial plates exposed to test samples to obtain luminescence intensity above the detection limit, particularly for calculating Log Reduction. However, it is not necessary for generating dose-response curves.*
5. Prepare an opaque 384-well assay plate containing 40 µL of viability reagent per well. When using BacTiter-Glo, dilute the reagent with TSB at a 1:1 ratio, unless otherwise specified by the validated method.
6. Sandwich the 384PillarPlate with the opaque assay plate and incubate for 30 minutes at room temperature, protected from light.
7. Detach the opaque assay plate and measure luminescence using a compatible plate reader. Instrument settings shall be defined and recorded for the local plate reader and kept consistent within a study.
8. Save all raw data for analysis and record retention.

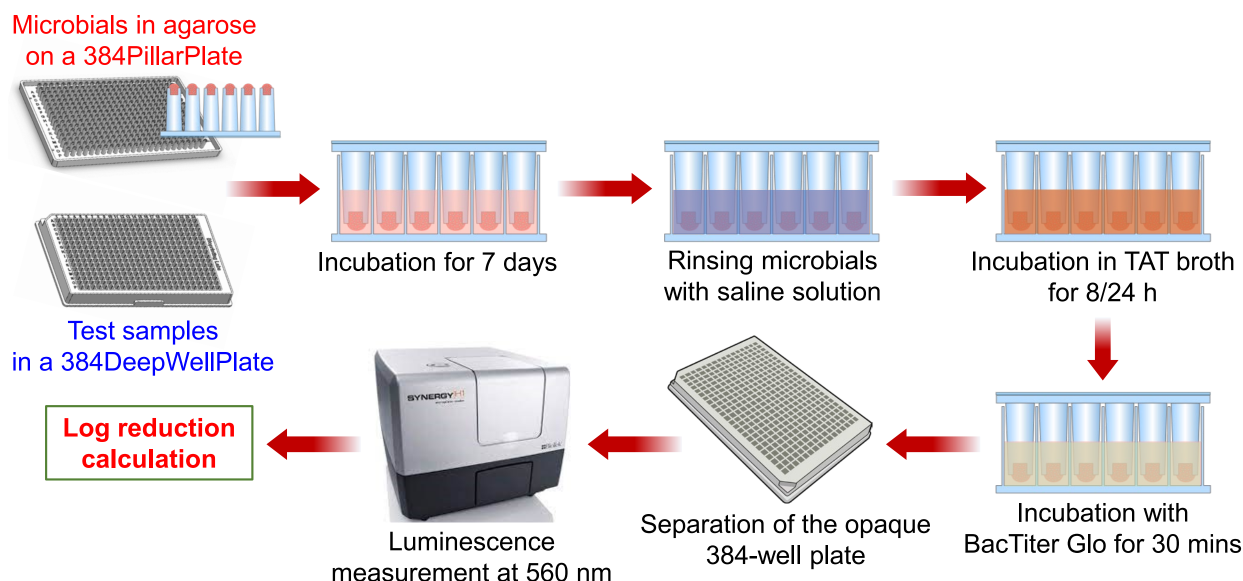

Figure 1. Experimental procedures for antimicrobial preservatives efficacy test on a pillar plate.

Assessment of Relative Viability or Dose-Response

1. When post-exposure signal amplification is not required for relative viability or dose-response assessment, rinse the 384PillarPlate by sandwiching it with a saline plate for 1 hour to remove residual test samples.
2. After the rinsing step, sandwich the 384PillarPlate directly with an opaque 384-well assay plate containing 40 µL of viability reagent per well. When using BacTiter-Glo, dilute the reagent 1:1 with TSB unless otherwise specified in the validated method.
3. Incubate for 30 minutes at room temperature protected from light, then detach the opaque assay plate and measure luminescence using a compatible plate reader. Instrument settings shall be defined, documented, and maintained consistently throughput the study for the selected plate reader.
4. Document the rationale for omitting the signal amplification step and retain all raw data for analysis and record retention.

C. CFU-Based Viability Assessment by Microbial-Agarose Dissociation and Colony Counting

1. Prepare a sterile 96-well plate, sterile Eppendorf tube, or equivalent recovery vessel containing 200 µL of recovery or neutralization medium per sample.
   Note: *TAT broth may be used as the default recovery medium unless a different neutralizer or recovery broth is required by the test article or study design.*
2. Following test sample treatment and any required rinse or neutralization steps, remove individual pillars or pillar tips containing the microbial-agarose dome using sterile needle-nose pliers, sterile cutters, or another controlled removal method appropriate for the pillar plate design. Grasp the pillar near its base and gently rock until the pillar tip separates without splashing or cross-contaminating adjacent pillars.
3. Transfer one recovered pillar or pillar tip into each well or tube containing 200 µL of recovery medium. Use one vessel per pillar unless pooling is intentionally defined by the study design.
4. Seal or cover the recovery vessel and place it in a bath sonicator. Sonicate on high power for 30 ± 5 minutes using a dry insert tray, floating plate holder, or sealed secondary container so that the recovery vessel is not directly flooded by the sonicator bath.
   Note: *This step is intended to detach and dissociate the microbial-agarose dome. Ensure that the water temperature in the bath sonicator remains below 37°C, as prolonged sonication may*

increase the bath temperature. Thus, excessive sonication may reduce cell viability, whereas insufficient sonication may result in incomplete recovery and underestimation of viable counts.

5. Immediately after sonication, vortex each sample for 5 - 10 seconds or pipette-mix thoroughly 10 times to improve recovery uniformity.
6. Prepare a 10-fold serial dilution using sterile 0.85% saline solution. A recommended starting scheme is to add 180 µL saline to each dilution well or tube, transfer 20 µL of the sonicated recovery suspension into the first dilution well, mix thoroughly, and repeat sequentially as needed to obtain countable colonies on agar plates.
7. Plate suitable dilutions onto MLA or another validated agar medium. As a recommended starting approach, spot-plate 10 µL of each selected dilution in duplicate or triplicate onto pre-dried agar plates, or spread-plate 100 µL when a full plate count method is preferred.
8. Incubate plates under microorganism-appropriate conditions until discrete colonies are visible. For routine microbial workflows using the microorganisms listed in this SOP, incubation at 30 - 37°C for approximately 18 - 48 hours may be used as a starting condition unless otherwise defined by the microorganism-specific method.
9. Count colonies from countable spots or plates and calculate CFU per recovered pillar, CFU per mL of recovery suspension, log10 CFU, log reduction relative to the untreated control, or another user-defined viable count metric, as appropriate. When using spread plates, a typical countable range is 30 - 300 colonies per plate. When using 10 µL spot plating, users should define and apply a countable range appropriate for the local method.
10. Include untreated recovery controls and any additional controls needed to assess recovery efficiency, neutralizer suitability, contamination, and background carryover in the same workflow.
    Note: *The recovery workflow described above is provided as an initial operational method for conventional viable counting on the pillar plate. Key parameters, including sonication conditions, recovery volume, diluent composition, plating medium, dilution depth, and incubation conditions, may require optimization during method qualification, depending on the target microorganism and assay format.*

D. Data analysis

1. Collect and review all raw assay data, including signals or counts from treated samples, untreated controls, background controls, recovery controls, and any additional controls required by the study design.
2. Subtract background signal from all readings using the appropriate blank or matrix-only control. Where no application-specific background control is defined, agarose-only pillars or matrix-only wells without microorganisms may be used as the default background control, as appropriate for the assay format.
3. Normalize treated sample values to the untreated control to determine relative microbial viability or reduction.
4. Results may be reported as percent viability, percent reduction, CFU per pillar, CFU per sample, log10 CFU, log reduction, or other user-defined response metrics appropriate to the assay readout.
5. The following general calculations may be applied as appropriate:
    - **Percent Viability = (Treated Signal / Untreated Control Signal) × 100**
    - **Percent Reduction = [1 − (Treated Signal / Untreated Control Signal)] × 100**
    - **Log Reduction = log10 (Control / Treated)**
6. Where assay signals are converted to OD, cell number, CFU, or other derived values, conversion shall be based on a user-defined or internally approved calibration method appropriate for the microorganism and assay format. For exploratory, screening, or relative-comparison studies, background-corrected luminescence values may be used directly as the primary response metric until organism-specific calibration curves are established.
7. Any normalization factor or correction applied to the data shall be documented and justified.

8. Replicate data shall be summarized using appropriate statistical descriptors as required by the study protocol.
9. All raw data, processed data, calculations, exclusions, and deviations shall be retained in the study record.

E. Quality Requirements / Acceptance Checks

1. Untreated control signals or recovery-control counts shall be within the internally established assay range for the specific microorganism and assay configuration.
2. Background signals shall remain acceptably low relative to untreated control.
   Note: *For CFU-based workflows, recovery controls shall produce countable colonies within the working range of the selected plating method, and the dilution scheme shall bracket at least one countable spot or plate whenever growth is present.*
3. Any deviations from the defined procedure shall be documented and reviewed.
4. A replicate scheme appropriate to the study objective shall be defined before testing. Triplicate or greater is recommended for exploratory studies unless otherwise justified.

F. Documentation

1. Record assay reagent preparation, plate reader settings, recovery vessel type and volume, sonication conditions if used, plating medium, dilution scheme, incubation conditions, colony counting approach, and raw output data.
2. Record all calculations, normalized values, and final reported results.
3. Archive study records according to internal document control requirements.