3D Microbial Culture in Agarose on Pillar Plate

1. Scope

This Standard Operating Procedure (SOP) describes the methodology for preparing three-dimensional (3D) microbial culture on a 384PillarPlate by encapsulating microorganisms in 3% agarose. The SOP includes procedures for media and reagent preparation, microbial inoculum preparation, agarose-based encapsulation, and pillar plate loading. This SOP is designed for broad applicability across pillar plate-based studies involving live microbial encapsulation and is primarily validated for microbial studies using the pillar plate format. Application-specific exposure studies, such as antimicrobial efficacy, preservative testing, disinfectant validation, or repeated-challenge experiments, shall be conducted under separate application SOPs or approved study-specific protocols. Viability assessment, including ATP-based luminescence and optional colony-forming unit (CFU)-based recovery, is described separately in the SOP titled “Microbial Viability Assay on Pillar Plate.”

2. Basic Principle

Microorganisms are cultured from verified stock, resuspended to a defined optical density, and mixed with warm 3% agarose at a 1:9 (v/v) ratio. The microorganism-agarose mixture is then deposited onto a 384PillarPlate *via* stamping, forming hemispherical gel domes on each pillar that physically encapsulate the cells. Following loading, the pillar plate may be assembled with a complementary deep well plate containing growth medium, recovery medium, neutralization medium, or test formulations, depending on the application-specific SOP. The loaded 384PillarPlate may then be transferred directly into an application-specific exposure SOP or into the SOP titled “Microbial Viability Assay on Pillar Plate,” depending on the 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. Spectrophotometer (Biomate 3, Thermo Electron Corporation)

O. Orbital shaker (13687704, Fisher Scientific)
P. Aluminum foil
Q. Nunc™ Square BioAssay Dishes (240835, ThermoFisher)

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. Modified Letheen Agar (MLA) (R453722, ThermoFisher)
D. Low temperature gelling agarose (A2576, Sigma-Aldrich)
E. Sterile distilled water

6. Definitions

Source culture: Original culture obtained from ATCC or an equivalent accredited source.
Stock culture: Culture propagated from the source culture and retained for preparation of working cultures.
Working culture: Culture propagated from the stock culture and used to prepare inoculum for testing.
Test inoculum: Standardized microbial suspension prepared for encapsulation on the 384PillarPlate.
Passage: A transfer of microorganisms from an established culture to fresh medium.

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.

B. Microbial Inoculum Preparation

1. Prepare the microbial inoculum according to a user-defined or internally approved culture method appropriate for the test microorganism(s).
2. Use suitable growth media, incubation conditions, and culture age to generate a viable inoculum for the intended assay application.
3. Standardize the inoculum to the required assay input concentration using a qualified measurement method, such as optical density (OD), colony-forming unit (CFU) enumeration, luminescence, or equivalent.
4. Where applicable, confirm inoculum suitability by assessing viability, purity, and/or concentration prior to encapsulation in agarose.
5. For mixed-culture studies, prepare and standardize each microorganism individually prior to pooling at the intended ratio.
6. Use the inoculum within an appropriate, user-defined time period after preparation.
   Note: *Microorganism-specific procedures for stock recovery, subculture, passage tracking, colony isolation, broth expansion, and inoculum count verification are outside the scope of this SOP and shall be established by the user. If an alternative inoculum preparation method is used,*

it shall produce a viable microbial suspension compatible with agarose encapsulation and the target assay seeding density.

C. Microbial Encapsulation in Agarose on a 384PillarPlate

Preparing the Agarose Solution

1. Dissolve 0.6 g of low-temperature gelling agarose in 20 mL deionized water (final 3% w/v).
2. Autoclave the agarose solution.
3. Transfer the hot agarose to a 37°C incubator and equilibrate for at least 1 hour before use.
   Note: *Mixing microorganisms with agarose above 37°C significantly reduces viability. Confirm that agarose has cooled to 37°C before proceeding.*

Preparing the Microorganism-Agarose Mixture

4. Centrifuge the microorganism inoculum at 1800 × g and discard the supernatant.
5. Resuspend the cell pellet in fresh, sterile TSB, and adjust to OD_{600} = 1.0 (equivalent to ~1 × 10^9 cells/mL).
   Note: *This step maintains high viability and standardizes the seeding density. If OD > 1.0, dilute as needed.*
6. For standard single-microorganism tests, dilute the OD 1.0 suspension 10-fold with warm 3% agarose to reach a final OD of 0.1.
   For pooled-microorganism tests (e.g., mixed microbial inoculum), mix equal volumes (e.g., 50 µL each) of each OD 1.0 suspension, then dilute 10-fold with agarose. Ensure each species is at OD 1.0 in TSB before pooling.

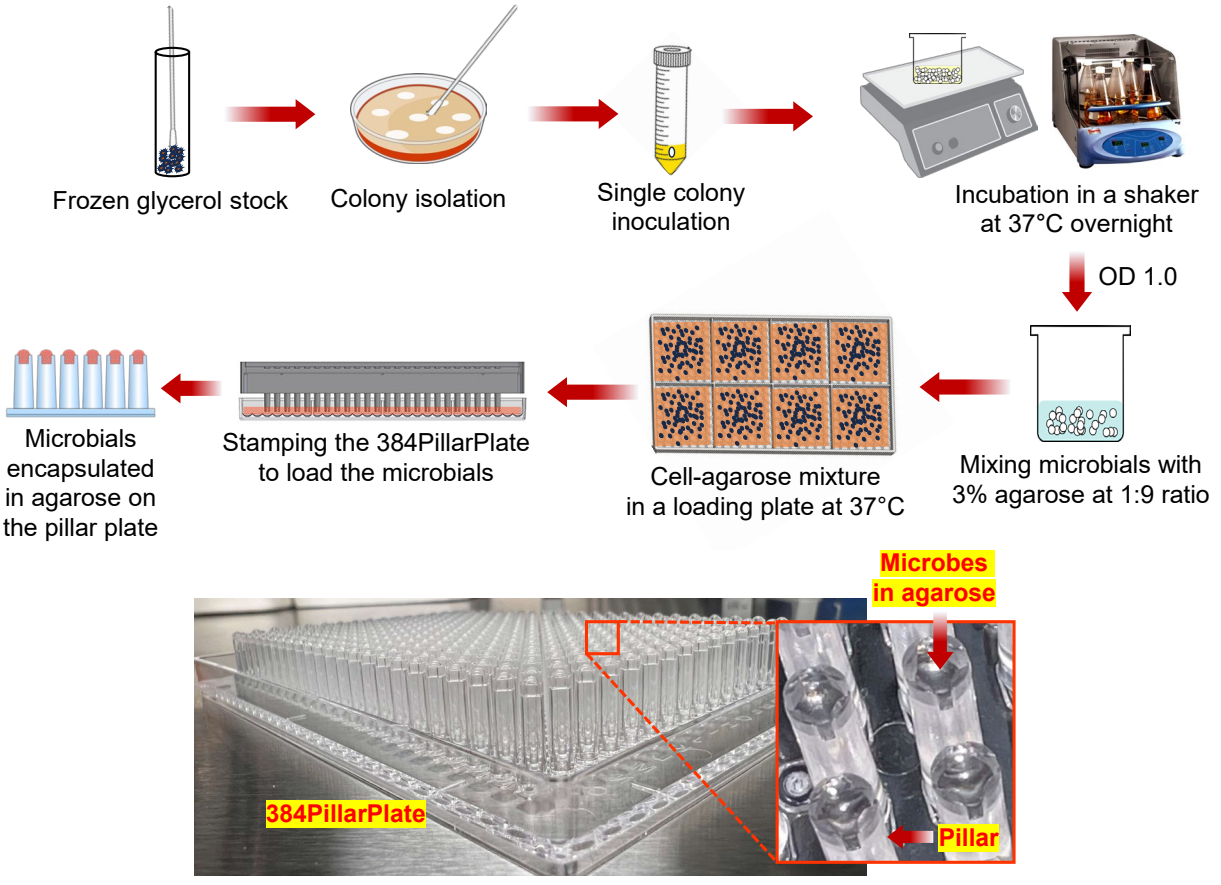

Figure 1. Encapsulation of microbials in agarose on the 384PillarPlate**.**

Loading on the 384PillarPlate

7. Mix 2 mL of microbial suspension (at OD 1.0 in TSB) with 18 mL of warm 3% agarose by vortexing. The final seeding density will be approximately 1×10^8 cells/mL.
8. While maintaining good cell suspension by gentle pipetting, pour the microorganism-agarose mixture into a sterile LoadingPlate (or 384-well plate lid).
9. Place the LoadingPlate on a heating block set to 37°C to prevent premature gelation.
10. Stamp a blank 384PillarPlate onto the LoadingPlate, ensuring all pillars contact the mixture.
    Note: *Complete stamping within ~1 hour while the microorganism-agarose mixture is still fluid. Maintain gentle suspension throughout to ensure uniform cell loading.*
11. Separate the 384PillarPlate from the LoadingPlate and place it in a biosafety cabinet.
12. Allow microorganism-agarose domes to solidify at room temperature for 30 minutes.
13. Visually inspect the 384PillarPlate to confirm that all pillars carry a hemispherical gel dome (**Fig. 1**).
    Note: *Once loading is complete and the agarose domes have passed visual inspection, use the prepared 384PillarPlate according to the relevant application SOP or proceed to the SOP titled "Microbial Viability Assay on Pillar Plate," as appropriate for the study design.*

D. Documentation

1. Record microorganism identity and inoculum preparation details.
2. Record matrix type, preparation conditions, and loading conditions.
3. Archive study records according to internal document control requirements.

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