JIS Z 2801

AddMaster
Additive Masterbatch Solutions

MD/105c

METHOD FOR DETERMINING EFFICACY OF ANTIMICROBIALS INCORPORATED INTO MANUFACTURED ARTICLES
"FILM CONTACT METHOD"

1. OBJECTIVE:

This test procedure is used to determine the log reduction of bacterial suspensions in contact with solid substrata. References for the procedures in this test method are listed in Appendix III.

2. SAMPLE REQUIREMENTS:

The plastic sample submitted for testing should have a flat and smooth surface to facilitate a close contact with the cover film. The surface of the plastic sample may be either hydrophilic or hydrophobic and the sample itself should not readily absorb water. A control sample of the same resin and additives, except for the antimicrobial compound, must be included in the study.

3. SAMPLE PREPARATION:

Samples to be used in this test may arrive in a variety of forms.

3.1 Cover Film

Stomacher Paddle Blender Bags or Cryovac Food Grade Film 5506 are cut into approximately 1.5 x 2.5cm pieces and placed in a sterile petri dish. The dish is exposed to UV light for at least 24 hours to sterilize the film before the individual film pieces are to be used.

3.2 Preparation of Samples

Test samples are made by cutting plaques into 3.5 x 3.5 cm pieces. Each piece is an individual test sample. Typically, three separate pieces of each specimen are prepared for each bacterial strain to be evaluated.

Test samples are not generally disinfected or sterilised prior to testing. The level of natural contamination of the samples is so low compared to the number of cells exposed to the samples during the test that sterilization is not necessary.

When necessary, samples may be sterilized prior to testing by means that will not affect the integrity of the sample or it's additives. Appropriate methods of disinfection include, but are not limited to, autoclaving at 121°C for 20 minutes, or dipping in 70% isopropyl or ethyl alcohol and drying.

4. TEST ORGANISMS:

Strain	Optimal growth conditions
***Escherichia coli* (ATCC #25922)**	**37°C, 24 hour, Tryptic Soy Broth (TSB)**
Escherichia coli (ATCC #8739)	37°C, 24 hour, TSB
Staphylococcus aureus (ATCC #6538)	37°C, 48 hour, TSB
***Staphylococcus aureus* (ATCC #6538P)**	**37°C, 24 hour, TSB**

* Currently, the organisms in bold are the ones most commonly tested. The choice of organism(s) will depend on the end-use application.

4.1 Microorganism Stock Cultures:

Stock cultures are purchased from ATCC (American Type Culture Collection, Manassas, VA; phone 1-800-638-6597). A stock culture of the above single organism culture is maintained by inoculating one loop onto a Tryptic Soy Agar slant (or equivalent medium) and incubating at 37°C for 48 hours before storing at 4-10°C. For long-term storage, prepare freezer stocks by adding viable cells from a fresh culture to a solution of 15-30% glycerol (final concentration), then place in a –20 to –80°C freezer. Before using the culture in a test, allow the culture to grow at least 24 hours in fresh nutrient broth or agar.

5. PREPARATION OF BACTERIAL CELL SUSPENSIONS FOR EXPOSURE

1. From a stock culture, inoculate a sterile flask containing Tryptic Soy Broth (TSB) or equivalent medium and incubate for 18 hours at 37°C while shaking.
2. From this culture, remove 0.2 ml and disperse in 20 ml of sterile potassium, sodium, phosphate buffer (50 m*M*, pH 7.0); vortex well.
3. Using a spectrophotometer zeroed with phosphate buffer at 660nm, read the Optical Density (OD) of the bacterial solution in step #2. Compare this OD value to a previously developed standard curve of OD vs. number of viable cells/ml to obtain the approximate number of viable cells. A standard curve of (OD) vs colony forming units (CFU) should be developed for each bacterial strain (Appendix III). Please remember that OD measures total cells, not just viable cells. However, under these optimal growth conditions, most of the cells in the culture are viable.
4. Adjust the bacterial solution by diluting into phosphate buffer to yield ca. **5×10^5 viable cells/ml**. This will be the solution used to expose to the samples. See Section 6 (Exposure Viability) to determine the actual number of viable cells in the exposure solution.

6. VIABILITY OF CELL SUSPENSION USED FOR EXPOSURE

To determine the actual number of viable organisms in the exposure solution, make six (6) serial 1:10 dilutions of the cell suspension in the previous section. Plate 0.5 ml of the last three dilutions: 10^{-4}, 10^{-5}, 10^{-6}. Incubate inverted for the time and temperature required by the organism. Following incubation, count the colony formation units (CFU) and calculate the CFU/ml. This number is the actual number of viable cells/ml exposed to the samples.

7. EXPOSURE OF SAMPLES TO CELL SUSPENSION:

Three (3) plaque samples in individual sterile petri dishes make one test. Apply 200 or 400ul of the adjusted bacterial suspension to the plaque sample, depending on the size of the sample. Using sterile forceps cover the bacterial suspension with a sterilized piece of film and carefully press the film down to ensure that the liquid spreads to the edges of the cover film without overflowing the edges of the cover film or the plaque. Incubate the exposed samples in 90 to 100% relative humidity at 25 to 37°C depending on the bacterial or test requirements. Exposure time is dependent on the experiment, but usually is 18 to 24 hours. The standard test is incubation at 37°C for 18 hrs.

8. EXPOSURE EFFICACY

To determine the number of bacteria killed from the exposure to the antimicrobial compounds incorporated into the plastic plaque, wash the plaque and film 'sandwich' with 10 ml of TSB. Lift up the cover film with forceps or the pipette tip and pipette the wash medium repeatedly over the plaque and cover film to suspend as many cells as possible. Plate 0.5 ml of this solution onto a TSA plate (10^0 dilution) and make three (3) serial 1:10 dilutions from this solution, plating 0.5 ml of each dilution. Repeat this for all replicates of the test. Incubate the inoculated TSA plates for each sample, inverted, at 37°C (or other selected temperature) for the time required for the organism (<24 hrs for most test m/o).

Following incubation, count the plates for CFU. Only CFU between 25 and 250/plate are considered accurate.

Within each replicated experiment, use the median, not the average, to determine the number of viable bacteria washed from the plaque sample. Use of the median helps with occasional “outlier” data within triplicate samples.

Calculate the number of viable cells/ml in the exposure solution using the following formulae as a guideline:

CFU/ml

= Counts * Dilution factor

= Counts * (1/ml on plate) * (ml wash medium/ml exposure solution) * $10^{\#\text{dilution tube}}$

= Counts * (1/0.5) * (10/0.4) * $10^{\#\text{dilution tube}}$

= Counts * 2 * 25 * $10^{\#\text{dilution tube}}$

To measure the effect of an antimicrobial compound, compute the log reduction between the control sample and the test sample: **Log Reduction = Log Control (CFU/ml) - Log Test Sample(CFU/ml)**

To measure any inherent biocidal effect of the control sample (no antimicrobials added to sample), compute the log reduction between the viability counts and the control sample:

Log Reduction = Log Viability (CFU/ml) - Log Control Sample (CFU/ml)

Note: The measurement constraints of this method are a minimum of $5x10^1$ cells/ml (for a 400 ul inoculum volume, $1x10^2$ for 200ul inoculum volume) and a maximum determined by the concentration of cells in the inoculum (usually $5x10^5$ cells/ml) plus or minus any deviations from growth or morbidity in the untreated control during the exposure period.