

**Pharmaceutical Grade Vial Stopper Test
Utilizing SixLog's iHP[®] (ionized Hydrogen Peroxide)
Decontamination Technology**

A. Introduction

SixLog developed a hydrogen peroxide based mist technology that has proven effectiveness in reducing bioburden to below detectable levels for various microorganisms. Surface structures on the microorganisms (Proteins, carbohydrates and lipids) are destroyed by the reactive oxygen species (ROS) and reactive nitrogen species produced when a fine mist of hydrogen peroxide and isopropyl alcohol is passed through a cold plasma arc.

B. Purpose

The purpose of this study was to determine the efficacy of the iHP[®] generator with the iHP[®] solution (7.5% Hydrogen Peroxide Ready-To-Use solution) against *Staphylococcus aureus* inoculated onto siliconized rubber vial stoppers supplied by the Pharmaceutical Manufacturer.

C. Apparatus

- a. Serological pipettes (sterile)
- b. 500 ml media bottles
- c. Scale
- d. Weigh boats
- e. Autoclave
- f. Autoclave bags
- g. Laminar flow hood
- h. Biohood
- i. 60 mm glass petri dishes
- j. 50 mL falcon tubes
- k. 1.7 mL eppendorf tubes
- l. Rubber vial stoppers (stoppers)
- m. Test tube racks
- n. Sterile inoculating loop (sterile)
- o. Sterile forceps
- p. Micropipette
- q. Micropipette tips (sterile)
- r. Vortex mixer
- s. Certified timer
- t. 70% ethyl alcohol
- u. Multitube vortexer
- v. Ethanol burner

D. Media and Reagents

1. Trypticase Soy Broth
 - a. Dissolve 15.0 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal and 5.0 g NaCl in 1 L deionized water. Adjust pH to 7.3 ± 0.2 .
 - b. Autoclave for 20 min at 121 C.
 - c. Store at 4 C.
2. Trypticase Soy Broth with 0.04% Bromocresol purple
 - a. Dissolve 15.0 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal, 5.0 g NaCl and 4.0 g Bromocresol purple in 1 L deionized water. Adjust pH to 7.3 ± 0.2 .
 - b. Autoclave for 20 min at 121 C.
 - c. Dispense in 5 ml aliquots in sterile 50 mL falcon tubes.
 - d. Store at 4 C.
3. Trypticase Soy Agar
 - a. Dissolve 15.0 g pancreatic digest of casein, 5.0 g papaic digest of soybean meal, 5.0 g NaCl and 15 grams of agar in 1 L deionized water. Adjust pH to 7.3 ± 0.2 .
 - b. Autoclave for 20 min at 121 C.
 - c. Dispense in 20 ml aliquots in 150 mm sterile petri dishes and let solidify.
 - d. Store at 4 C.
4. Sterile Deionized Water
5. iHP[®] Solution, 7.5% Ready-To-Use.

E. Test Organisms

1. *Staphylococcus aureus*, ATCC 6538
 - a. Replace every 6 months directly from ATCC.
 - b. Reconstitute in trypticase soy broth and streak onto trypticase soy agar slants.
 - c. Incubate both for 18-24 hours in 37 C. Store working slants at 2-5 C.
 - d. Examine broth for contamination by inoculating TSA. Incubate over night at 37 C.
 - e. Examine plates for purity and colonial morphology (round, shiny and yellow, 1-2 mm in diameter).
 - f. If colonies are not uniformly yellow and shiny, pick a colony that is and streak onto TSA. Repeat if necessary. If still unsuccessful, obtain new culture from ATCC.
 - g. Perform gram stain from TSA. Gram stain should show gram-positive cocci fitting morphology in Bergey's Manual.
 - h. If uncontaminated, continue storage of working slants up to 30 days at 2-5 C. Also, select well-isolated colonies from TSA and stab inoculate trypticase soy agar butts. Incubate 18-24 hours at 37 C. Store agar butt stock cultures at 2-5 C.

F. Operating Technique

1. Carrier Preparation
 - a. Visually screen rubber stoppers for scratches, chips or cracks. Discard those which are damaged or defective.
 - b. Rinse stoppers once with deionized water, rinse 3 times with 70% ethyl alcohol and finally rinse 3 times with deionized water.
 - c. Allow stoppers to dry.

- d. Place 3 stoppers each in a 60 mm glass Petri dish for a total of 32 dishes to be used for testing. Place 1 stopper each in a 60 mm glass Petri dish for a total of 16 dishes to be used for controls.
- e. Steam sterilize 30 min at 121°C with a 30 min dry cycle or sterilize for 2 hr in hot air oven at 180 C. Cool.

2. Stopper Inoculation

- a. Stoppers are inoculated in groups of nine for each experimental condition to complete the validation study. Note: The bacterial culture is vortex mixed frequently to ensure uniform distribution of bacteria and stoppers are inoculated in the Class II A/ B laminar flow hood to minimize contamination.

	Test Samples			
Dilution	Control 10 µL	10 µl + BIT	Control 50 µL	50 µL + BIT
Undiluted	ABC	ABC	ABC	ABC
-1	ABC	ABC	ABC	ABC
-2	ABC	ABC	ABC	ABC
-3	ABC	ABC	ABC	ABC

- b. Transfer the sample to the stopper with a micropipette using mist barrier tips and a positive displacement pipette. Due to the droplet surface tension and the hydrophobic nature of the stopper, the sample will resist spreading and will remain as a single drop on the stopper.
- c. Transfer the Petri dish covered from the laminar flow hood to the SixLog test chamber immediately and treat to minimize loss of viable cells.

3. Test Procedure Overview

- a. 12 stoppers will be used for each test dilution according to the matrix below. Positive controls are run in triplicate at lower dilutions to permit accurate enumeration of the actual bacterial count applied to the test stoppers.

Sample, Volume on Stopper	Dilution
10 µl + BIT	U, -1, -2, -3
50 µL + BIT	U, -1, -2, -3
10 µl - BIT	-4,-5,-6
50 µL - BIT	-4,-5,-6

- b. For the test sample dishes, place Petri dish on the middle platform of the chamber, uncover the dish and close the hood door. Turn the unit on, turn the arc on and spray inoculated stopper for 5 minutes. After 5 minutes, turn the unit off and allow for 5 minutes of dwell time.
- c. Transfer each stopper to a 50ml Falcon tube with 5 ml trypticase soy broth with bromocresol purple. Using the multitube vortexer in medium setting, vortex for 30 sec, then allow for 30 minutes growth/ recovery time in the media.

- d. Take 100 µL of the sample and use undiluted or dilute in 900 µL of sterile DI water. Serially dilute the tubes according to the matrix above.
- e. Plate 100 µL from the tubes on trypticase soy agar plate. Plate in triplicate per tube.
- f. Incubate tubes and plates for 24 hours at 37 C.
- g. Read tubes for positive (+) growth with color change (indicating acid production) from purple to yellow with turbidity and negative (-) growth with no color change, no turbidity. Count the number of CFUs per control plate and record.

G. Results

Starting Bacterial Culture Concentration

-6	TNTC
-7	177,167,183
-8	11,16,6

CFU Counts from Undiluted Cell Suspension = 1.8×10^{10}

Cell Suspension Concentration Inoculated on Stoppers

10 µl	1.8×10^8
50 µL	9×10^8

Control CFU Counts from Stoppers

Dilution	Sample Volume and CFU's	
	10 µl	50 µL
-4	65, 61, 67	483, 500, 493
-5	11, 11, 11	34, 34, 37
-6	1, 1, 1	1, 3, 3
CFU Recovered from Stoppers per mL		
	6.4×10^6	4.9×10^7
CFU log reduction per mL		
	0.72	0.94

Quantity of SixLog Solution Used to Treat Chamber

Sample	Weight Start/ End	Volume (ml)
10 µl	5.840/ 5.678	147.3
50 µL	5.518/ 5.348	154.5

Test Results

Dilution	Stopper	Control 10 µL	10 µl + BIT	Control 50 µL	50 µl + BIT
Undiluted	A	+	+	+	+
	B	6.4×10^6	+	4.9×10^7	+
	C		+		+
-1	A	+	-	+	-
	B	6.4×10^5	-	4.9×10^6	-
	C		-		-
-2	A	+	-	+	-
	B	6.4×10^4	-	4.9×10^5	-
	C		-		-
-3	A	+	-	+	-
	B	6.4×10^3	-	4.9×10^4	-
	C		-		-

Notes:

- + positive growth with color change (indicating acid production) from purple to yellow with turbidity
- negative growth with no color change, no turbidity

H. Conclusions

The initial concentration of the *S. aureus* was 1.8×10^{10} CFU. The stopper loaded with 10 µl initial concentration was 1.8×10^8 CFU while the stopper loaded with 50 µl was 9.0×10^8 CFU. Less than 1 log loss in CFU was observed following recovery.

The control results produced a turbid yellow color media indicating acid production and positive growth. Our tests show that when *S. aureus* is cultured in trypticase soy broth with bromocresol purple indicator, the sensitivity is about 5 CFU per ml and can be used as a quick method to enumerate log kill +/- 1 log.

These data show iHP® 7.5% Ready-To-Use was effective in reducing the bacterial load to below detectable levels with cell suspension concentrations $\leq 4.9 \times 10^6$ for the 50 µl inoculations and below detectable levels with cell suspensions $\leq 6.4 \times 10^5$ for the 10 µl inoculations. Based upon the concentration of cells where we see growth (10 ul sample at 6.4×10^6) and the highest determined number of cells to be killed (50 ul sample at 4.9×10^6), and the 1 log loss during transfer time spent on the stopper, we can reasonably say that iHP® is capable of killing 9.8×10^6 CFU per ml.

The surface of the stopper is hydrophobic. This prevents the sample from spreading evenly across the stopper and focuses the bacterial cells on a small area. Despite the focus of a high local concentration of cells, a 5 min iHP® exposure is > 99.9999% effective at killing the *S. aureus* inocula



of 1.8×10^{10} per ml when applied as small droplets. Longer treatment would be expected to increase the effectiveness of the BIT process.

Contamination of surfaces can arise from contact with skin or even from coughs or sneezes. Considering human saliva contains about 3×10^5 CFU per ml¹, contamination on surfaces can be reasonably expected to have been generated by inocula significantly less than 1.8×10^{10} per ml.