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A Disinfectant Efficacy Study on the Practical Cleanroom Disinfectants of Hydrogen Peroxide, Peracetic Acid, Isopropyl Alcohol, and Sodium Hypochlorite to Qualify a Sporicide

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ABSTRACT
In-house and commercial disinfection products have been studied through disinfectant efficacy testing (DET) with a suspension test and a surface test. A neutralizer has been employed in the study whereas the neutralizer efficacy is established before each DET. Vegetative cells and spore formers are challenged, including strains from the ATCC cultures and Altaire's cleanroom environmental isolates. Hard surfaces sampled from the cleanroom construction materials and equipment are employed for the surface test. It is generally found that the recovery and the log reduction in the surface test are more challenging than those in the suspension test studied at 5 minutes and 10 minutes contact times, interpreted as the physicochemical interactions between the microorganism cells and the hard surfaces, and the spatial "barrier" effect against the disinfection during the surface test. Isopropyl Alcohol (IPA) 70% is qualified as a general-purpose disinfectant. Both Spor-Klenz® and Sodium Hypochlorite (NaClO) 5.25% are consistent with the sporicidal claim. However, it is surprisingly found that Hydrogen Peroxide (H_2O_2)6% is incapable of inactivating bacterial spore formers, explained by an insufficient concentration and/or an inadequate contact time for $H_2O_2$to take the full function. The rate-limiting step for disinfection is discussed to be associated with the diffusion of the chemical agent into the membrane structure of the cell wall. One successful approach to enhance $H_2O_2$6% by adding a more-efficient-diffusion oxidizer (e.g., Peracetic Acid (CH_3CO-OOH), 0.05%) enables the product to be qualified as a sporicide by passing the USP <1072> acceptance criteria.

Keywords: disinfectant efficacy testing (DET), neutralizer efficacy, suspension test and surface test, spore former and sporicide, hydrogen peroxide, sodium hypochlorite, isopropyl alcohol, peracetic acid

I. INTRODUCTION

In support of aseptic processing in pharmaceutical manufacturing, chemical disinfectants are used to eliminate bio-contaminants (e.g. bacteria and fungi) on stainless steel tanks, filling machines, walls/windows/floors, which are within the controlled area or Cleanroom.[1] Such disinfection is usually performed on the hard surfaces in a liquid form of the disinfectant (e.g. via spraying). In a modern pharmaceutical cleanroom with the highest grade – ISO-5 or Grade A, the bio-contamination shall be controlled at the minimum level (e.g. approximate to zeros in cfu or colony-forming unit), and the contamination recovery rate is recommended to be <1% according to the USP<1116>.[2]

There are several disinfectants available on the market, basically falling into the categories of alcohols, peroxides, quaternary ammonium compounds, etc.[3] The most popular disinfectants used in a pharmaceutical cleanroom include Isopropyl Alcohol (IPA) (e.g. 70%),[4-6] Hydrogen Peroxide solution (e.g. 3%, 6%),[7-11] Peracetic Acid (0.1%),[12-13] and Sodium Hypochlorite solution (e.g. 0.5%, 5.25%).[14-15] Such chemical disinfectants either function on the cell wall, or damage the nucleic acids(DNA/ RNA) and the proteins, and therefore disrupt the reproduction/ replication of the microorganisms.[16] In manufacturing the cleanroom disinfectants (in-house prepared or commercial brands), a sterilization process (e.g. via 0.22 μm membrane filtration or γ-irradiation) must be employed to ensure the sterility of the disinfectants

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themselves. Each disinfectant has its advantages and limitations. For example, Sodium Hypochlorite solution can be used on the wall and the floor (e.g. regarded as the "dirtiest") but it is too harsh or corrosive for stainless steel(SS) equipment; IPA 70% can be used on stainless steel surfaces with the easiness of evaporation but it is not so powerful in eliminating all microorganisms (e.g. including spore formers).

The microorganisms in Cleanroom typically are represented by bacteria and fungi (yeast and molds).The resistance of one microorganism towards a disinfectant could be significantly different from one to another. The most challenging microorganisms to destroy in the pharmaceutical cleanroom are the endospores (or spore-forming organisms), especially the bacterial spore formers. Therefore, a disinfectant capable of destroying bacterial and fungal spores is called "sporicidal" (e.g. Sodium Hypochlorite5.25% is a sporicide).In comparison, IPA 70% is a general-purpose disinfectant, capable of only destroying vegetative cells. There are many disinfectants (e.g. Hydrogen Peroxide solution or the relevant) with the "killing power" in-between the sporicidal Sodium Hypochlorite 5.25% and the general-purpose IPA 70%, and thus misunderstanding/ mislabeling could occur in such a gray area. In particular, a sporicidal disinfectant can be further termed as a sterilant (e.g. Peracetic Acid solution), which is closely related to establishing a validated cold sterilization cycle (or low-temperature sterilization) for routine production use. The "killing power" of a disinfectant correlates with the concentration and the exposure time (or contact time) towards the microorganisms.

In evaluating a disinfectant in terms of the "killing power", a disinfectant efficacy testing (DET) validation study shall be performed.[16-19] The DET study is very important in qualifying an in-house prepared disinfectant to be used in the pharmaceutical cleanroom. The commercial disinfectant brands should be verified to demonstrate consistency with the product labeling by the end user. Typical DET includes a suspension test and a surface test.[20] The suspension test is conducted by suspending the challenge microorganism cells in the disinfectant for a certain contact time and checking the recovery/ survival post-exposure. The surface test is performed by applying the disinfectant (e.g. via spraying) onto a microorganism pre-inoculated hard surface for a contact time and then checking the recovery/ survival. The population recovery of microorganism cells post-exposure can be performed with either a membrane filtration or a plate-count method based on serial dilution (e.g. so-called pop count).[21] The disinfectant efficacy is determined based on the log reduction of the challenge microorganism cells initially against the survival cells post-exposure. Presumably, a greater log reduction demonstrates a higher "killing power" for the disinfectant under the same experimental conditions. According to the USP <1072>[16], the acceptance criteria of log reduction is at least 2 for bacterial spores and at least 3 for vegetative bacteria during a contact time of 10 minutes or above. According to the PDA Technical Report#70,[17] the acceptance criteria of log reduction is at least 1 for the various disinfectants and sporicides within the recommended contact time of 1 -5 minutes. Regardless of the minor difference in acceptance criteria, the sterile cleanroom disinfectant is designed to be used for supporting the aseptic processing within a controlled area containing a limited Bioburden (e.g. the Bioburden is usually <1 – 100 cfu depending on the classification of Cleanroom). The selected microorganisms for the DET study shall be selective and representative, e.g., including both the ATCC cultures and the cleanroom environmental isolates. The chosen microorganisms shall cover gram-positive bacteria (e.g. GPC, GPB), spore formers, gram-negative bacteria (e.g. GNB), yeast, and molds. The selected hard surfaces shall be sampled from the typical construction materials of the cleanroom (e.g. surface coupons of walls, floors, and windows), and materials used for manufacturing equipment and utensils (e.g. stainless steel 316 and polypropylene).

One important factor for a successful DET is the use of a neutralizer. A chemical disinfectant can become inhibitory to the studied microorganism under the diluted condition, e.g., leading to a state in which the microorganism is not completely killed(e.g. "dormancy"); the consequence is that a fake zero survivor will be reported in the DET. Note: the plate count method relies on the visual observation of the microbial colonies; in other words, technically there is no difference between a plate with all dead (killed) cells and a plate with all inhibited (not-fully-grown) cells. Microorganisms are living bodies and are capable of adjusting to the rough environment for survival.[22] Such inhibitory effect due to the residue in the diluted disinfectant

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shall be countered with a neutralizer. For example, an oxidizing-type disinfectant can be neutralized with a reducing agent and therefore the disinfectant will lose the ability to destroy the microorganisms at the desired DET stages. With the satisfactory neutralizer efficacy established, the final count of the recovered cells in the DET study reflects the true number of survival cells and hence the validity of the log reduction calculation is supported.

In this study, we will present DET data for five disinfectants, including one commercial disinfectant – Spor-Klenz® RTU (Ready to Use) (Hydrogen Peroxide 1% and Peracetic Acid 0.05%) manufactured by Steris[23] and four in-house disinfectants– Sodium Hypochlorite 5.25%, Isopropyl Alcohol (IPA) 70%, Hydrogen Peroxide 6%, and Hydrogen Peroxide 6% enhanced with Peracetic Acid 0.05%. A few typical microorganisms have been investigated – including both the ATCC panel organisms and the environmental-monitoring (EM) isolates collected from Altaire's Cleanroom. The chosen EM isolates have been associated with the availability in the QC-microbiology lab library contemporarily. Those studied microorganisms have covered gram-positive bacteria (including spore formers), gram-negative bacteria, yeast and molds. A few hard surfaces (e.g. 2 x 2 inch^2 coupons) of the materials used in Cleanroom have been adopted for the surface test. Both contact times of 5 minutes and 10 minutes have been used in the suspension test and the surface test (the conformance to the USP <1072>must be demonstrated for the data points at 10 minutes). Finally, a neutralizer efficacy test has been seriously employed before the suspension test and the surface test, in support of the yielded log reduction results.

EQUIPMENT AND SUPPLIERS

Disinfectants

The studied disinfectants are listed with the chemical name and the concentration:
Sodium Hypochlorite 5.25% (in-house manufactured)
Isopropyl Alcohol 70% (in-house manufactured)
Hydrogen Peroxide 6% (in-house manufactured)
Hydrogen Peroxide 6% and Peracetic Acid 0.05% (in-house manufactured)
Hydrogen Peroxide 1% and Peracetic Acid 0.08% (Spor-Klenz® RTU, manufactured by Steris)

Note: Altaire's in-house disinfectants are prepared fresh routinely for the Cleanroom use (e.g. utilized within four weeks or 28 days). Spor-Klenz® RTU manufactured by Steris has a label expiration date of 12 months.

Hard Surfaces

The studied hard surfaces are sampled from Altaire's cleanroom construction materials and equipment, e.g. by cutting to form a 2 x 2 inch^2 coupon, including:
Stainless Steel (SS) 316
Glass
Plastic vinyl (Curtain)
Terrazzo tiles (Floor)
Epoxy-coated gypsum (Wall)
Polypropylene

Selected Microorganisms

The studied microorganisms are selected from the typical ATCC cultures, e.g. used for the USP <51> antimicrobial effectiveness testing[24], as well as isolates recovered from Altaire's Cleanroom during executing the environmental monitoring (EM) and the personnel monitoring (PM).

ATCC Organisms:
Pseudomonas aeruginosa (GNB) ATCC 9027
Candida albicans (Yeast) ATCC 10231
Aspergillus brasiliensis (Mold) ATCC 16404
Bacillus subtilis spores (sGPB) ATCC 6633
Those ATCC microorganisms are purchased from ATCC Cultures, growth-promoted, and harvested to form microorganism suspensions at the QC-microbiology lab; *Bacillus subtilis* spore suspension (10^6 – 10^8 cfu/mL) is purchased from Crosstex.

EM Isolates:
Staphylococcus epidermidis (GPC)
Micrococcus luteus (GPC)
Fusarium oxysporum (Mold)
Chaetomium globosum (Mold)
Aspergillus versicolor/ sydowii (Mold)
Bacillus horneckiae spores (sGPB)

The EM isolates are sourced from Altaire's EM/PM program and their identifications are performed by Charles River Laboratory (e.g. Accugenix laboratory, Newark, Delaware). The EM isolates are growth-promoted, and harvested to form the microorganism suspensions at the QC-microbiology lab.

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Note: The populations of all the ATCC and EM isolate suspensions are verified using serial dilution and plate-count technique each time when a DET is scheduled and performed.

Culture Media

Tryptic Soy Agar (TSA)
Sabouraud Dextrose Agar (SDA)
Tryptic Soy Broth (TSB)
AK Agar #2 (Sporulating Agar)

All media are purchased from Sigma-Millipore, prepared by dissolving the corresponding amount of dry powder in Water for Injection, heating to boiling, followed by steam-sterilizing (e.g. 121.1°C for 15 minutes)

Neutralizer – Dey-Engley (D/E) Broth

The neutralizer – D/E broth is prepared by dissolving 39 grams of the D/E broth powder (purchased from Sigma-Aldrich) in Water for Injection (q.s. 1 L), with the final composition of:

Casein Enzymic Hydrolysate 5.0 g/L
Yeast extract2.5 g/L
Dextrose 10.0 g/L
Sodium thiosulfate6.0 g/L
Sodium thioglycollate1.0 g/L
Sodium bisulfite2.5 g/L
Lecithin 7.0 g/L
Polysorbate 80 5.0 g/L
Bromo Cresol Purple 0.02 g/L

The Other Equipment and Supplies

Sterile test tubes, pipettes, flasks, specimen cups, syringes, Petri dishes, sterile loops/spreaders, sterile cell scraper, sterile jar and or/ autoclavable polypropylene jar (500 mL)
Autoclave – steam sterilizer (Steris AMSCO 250LS)
Water Bath (45°C)
Incubators (20-25°C and 30-35°C)
Biosafety Hood equipped with HEPA filter with laminar flow (Liberty Industries)

II. METHODOLOGY

Neutralization - As mentioned in the Introduction, the observation of a fake zero survivor during DET should be avoided in the DET. Therefore, the utilization of a neutralizer is involved in each phase of the DET.

Neutralizer Toxicity

The neutralizer is combined with a microorganism, side-by-side with a control. The recovery should be at least 70% in the plate count.

For example, *Staphylococcus epidermis* (EM isolate) strains from a prepared suspension were inoculated into the neutralizer – D/E broth, and into the control – saline 0.9%, resulting in 10 – 100 cfu/mL approximately. The plate count results are indicated in Figure 1 and summarized in Table 1.

Table 1. Toxicity Study of the Neutralizer of D/E Broth for *Staphylococcus epidermis*

Test	Plate 1	Plate 2	Plate 3	Average	Recovery
Saline Control	84	92	100	92	N/A
D/E broth	87	89	90	89	96.7%
Negative Control	0	0	0	0	N/A

$$\text{Recovery\%} = \frac{\text{pop count\_neutralizer}}{\text{pop count\_control}} \times 100\% = \frac{89}{92} \times 100\% = 96.7\%$$

Therefore, the recovery of 96.7% for D/E broth with respect to the saline control met the requirement of at least 70% recovery. In other words, the neutralizer - D/E broth is nontoxic toward *Staphylococcus epidermis*.

Neutralizer Efficacy

The neutralizer is combined with a disinfectant at three different dilution levels (e.g. the disinfectant concentration at the 10^{-1}, 10^{-2}, and 10^{-3} dilutions), followed by adding the challenge microorganism individually. The recovery of at least 70% with respect to the control demonstrates the acceptable neutralizer efficacy.

For example, the disinfectant of Hydrogen Peroxide (H_2O_2) 6% was ten-fold diluted into 10^{-1}, 10^{-2}, and 10^{-3} strengths with the diluent of the neutralizer – D/E broth. Diluted H_2O_2 6% solutions

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(10^{-1}, 10^{-2}, and 10^{-3}) were individually inoculated with *Staphylococcus epidermis* (e.g. 10 – 100 cfu/mL). Side-by-side, D/E broth alone was inoculated (positive control in the table below).The plate count results are indicated in Figure 1 and summarized in Table 2.

Table 2. Neutralizer Efficacy of D/E Broth for $H_2O_2$6%/ *Staphylococcus epidermis*

Test		Plate 1	Plate 2	Plate 3	Average	Recovery
Dilution Level	10^{-1}	0	0	0	0	0%
	10^{-2}	61	63	64	63	70.8%
	10^{-3}	80	85	101	89	100.0%
Positive Control		87	89	90	89	N/A
Negative Control		0	0	0	0	N/A

10^{-3} dilution:

$$\text{Recovery\%} = \frac{\text{pop count\_at } 10^{-3}\text{dilution}}{\text{pop count\_control}} \times 100\% = \frac{89}{89} \times 100\% = 100\%$$

Therefore, the recovery from the neutralization of H_2O_2 6% at the dilution factors of 10^{-2} and $10^{-3}$with D/E broth met the requirement of at least 70% recovered. In other words, the neutralizer of D/E broth is effective in neutralizing H_2O_2 6% for the organism of *Staphylococcus epidermis* when the disinfectant is diluted down to the 10^{-2} or 10^{-3} level with D/E broth.

The **neutralization mechanism** is as follows: 1. the oxidation-reduction (redox) reaction between D/E broth and Hydrogen Peroxide (H_2O_2) converts the latter into water (H_2O), 2. the series of dilutions of Hydrogen Peroxide (H_2O_2) 6%bring the strength of Hydrogen Peroxide down dramatically (e.g. theoretically equivalent to 0.006% or 0.06 mg/mL or 60 ppm Hydrogen Peroxide at the dilution of 10^{-3} if not considering the redox reaction). Three ingredients in the formula of D/E broth play the role of reductants, e.g. Sodium thiosulfate (6.0 g/L), Sodium thioglycollate (1.0 g/L), and Sodium bisulfite (2.5 g/L), whereas other ingredients (including the organism nutrients) provide a friendly chemical environment(nontoxicity) of the broth towards the subject microorganism. The combination of the redox reaction and the dilution (e.g. 10^{-3}) reduces the concentration of the Hydrogen Peroxide (H_2O_2) residue to a negligible level (e.g. close to 0), which is not inhibitory to *Staphylococcus epidermis* anymore.

Disinfectant Efficacy Test (DET): According to the AOAC guidance,[20] the DET typically include suspension test and surface test. Suspension test is more or less like a quick screening-like test in the current study. Surface test is more important in reflecting the real picture of disinfection/ sterilization on the hard surfaces in the pharmaceutical cleanroom.

Suspension Test

The suspension test is performed by directly exposing the microorganism to the disinfectant over a certain contact time (e.g. kill time). Thereafter, the survival cells are recovered with serial dilution using the neutralizer – D/E broth. Note: such serial dilution will also dilute the disinfectant and cause it to lose the disinfecting ability according to the neutralizer efficacy study shown above.

For example, one mL of *Staphylococcus epidermis* suspension (>10^9 CFU/mL) was added into ninety nine mL of H_2O_2 6% solution (e.g. 1:100 dilution),and side-by-side, D/E broth was inoculated (positive control). Once the contact time of5 or 10 minutes passed, the serial dilutions of the aliquots with the neutralizer – D/E broth were performed immediately. The plate count results are demonstrated in Figure 2 and summarized in Table 3.

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Table 3. Suspension Test for H_2O_2 6% Against *Staphylococcus epidermis*

Test		Dilution Factor of H_2O_2 6%	Serial Dilution of *S. epidermis*	Plate 1	Plate 2	Plate 3	Average	Sample Population	Log Reduction
H_2O_2 6%	5 min	~~10^{-1}~~	~~10^{-1}~~	~~0~~	~~0~~	~~0~~	~~0~~	$<10^2$ CFU/ mL	>5.0
		10^{-2}	10^{-2}	0	0	0	0		
		10^{-3}	10^{-3}	0	0	0	0		
	10 min	~~10^{-1}~~	~~10^{-1}~~	~~0~~	~~0~~	~~0~~	~~0~~	$<10^2$ CFU/ mL	>5.0
		10^{-2}	10^{-2}	0	0	0	0		
		10^{-3}	10^{-3}	0	0	0	0		
Positive Control		10^{-4}	10^{-4}	TNTC	TNTC	TNTC	TNTC	9.9×10^6 CFU/mL	N/A
		10^{-5}	10^{-5}	90	98	110	99		
		10^{-6}	10^{-6}	9	10	16	12		
Negative Control				0	0	0	0	N/A	N/A

Since the neutralizer efficacy has been established at the dilution factor of 10^{-2}, the plate count readings of zeros at the dilution factor of 10^{-1} were not reliable and therefore disregarded (e.g. strikethrough). The recovered cells for the test sample were reported to be $<10^2$cfu/mL based on the nearest effective dilution, e.g. the 10^{-2} dilution. The side-by-side positive control yielded the cells within the countable range at the 10^{-5} dilution (e.g. average = 99cfu/mL) and therefore the log reduction was calculated to be:

$$\text{LR} = \log_{10}\frac{\text{control pop count}}{\text{survival pop count}} > \log_{10}\frac{9.9 \times 10^6/\text{mL}}{10^2/\text{mL}} = 5.0$$

This result (e.g. LR >5.0) met the acceptance criteria of at least 3 log for vegetative cells according to the USP <1072>. In other words, the disinfectant of H_2O_2 6% is found to be effective in destroying *Staphylococcus epidermis* in the suspension test.

Surface Test

A surface test is slightly more complicated than the suspension test wherein a hard surface coupon is used as a carrier for the microorganisms. The hard surface is first inoculated with the studied microorganism, a disinfectant is then applied (e.g. via spraying) and the contact time is started; side-by-side, a positive control is performed without applying the disinfectant. Once the contact time is over, the neutralizer of D/E broth is added to stop the function of the disinfectant. Serial dilution is then immediately performed for recovering survival cells with the plate count method.

For example, two 2x2 $inch^2$ stainless steel (SS) 316 test coupons were individually inoculated with 0.1 mL of *Staphylococcus epidermis* suspension($>10^9$ CFU/mL). The inoculum was left open under the laminar flow until dryness (e.g. about 20 -30 minutes depending on the room relative humidity).H_2O_2 6% was then applied onto one of the inoculated SS coupons via spraying (~10 mL) and started timing. After 5 or 10 minutes, 90 mL D/E broth was immediately added to neutralize the disinfectant of H_2O_2 6%. The survival cells were recovered from the SS coupon by vortexing and with the aid of a cell-scraper. A serial dilution was then performed for plating. For the other inoculated SS coupon without applyingH_2O_2 6% (positive control), the inoculum cells were recovered using a similar serial dilution. The plate count results are demonstrated in Figure 3 and summarized in Table 4.

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Table 4. Surface Test on SS Coupon for H_2O_2 6%/ *Staphylococcus epidermis*

Test		Dilution Factor of H_2O_2 6%	Serial Dilution of *S. epidermis*	Plate 1	Plate 2	Plate 3	Average	Sample Population per mL Rinse	Sample Population per Coupon	Log Reduction
Surface Challenge	5 min	~~10^{-1}~~	~~10^{0}~~	~~0~~	~~0~~	~~0~~	~~0~~	$<10^1$ CFU/mL	$<10^3$ CFU/ coupon	>5.4
		10^{-2}	10^{-1}	0	0	0	0			
		10^{-3}	10^{-2}	0	0	0	0			
		10^{-4}	10^{-3}	0	0	0	0			
		10^{-5}	10^{-4}	0	0	0	0			
	10 min	~~10^{-1}~~	~~10^{0}~~	~~0~~	~~0~~	~~0~~	~~0~~	$<10^1$ CFU/mL	$<10^3$ CFU/ coupon	>5.4
		10^{-2}	10^{-1}	0	0	0	0			
		10^{-3}	10^{-2}	0	0	0	0			
		10^{-4}	10^{-3}	0	0	0	0			
		10^{-5}	10^{-4}	0	0	0	0			
Positive Control		N/A	10^{-2}	TNTC	TNTC	TNTC	TNTC	2.6×10^6 CFU/mL	2.6×10^8 CFU/ coupon	N/A
		N/A	10^{-3}	TNTC	TNTC	TNTC	TNTC			
		N/A	10^{-4}	TNTC	TNTC	TNTC	TNTC			
		N/A	10^{-5}	28	27	24	26			
Negative Control				0	0	0	0	N/A	N/A	N/A

Similar to the suspension test, because the neutralizer efficacy is established at the dilution factor of 10^{-2}, the plate count readings at the dilution of 10^{-1}relative to the disinfectant (H_2O_2 6%) were disregarded (e.g. strikethrough). Note: the dilution factors for the disinfectant and the microorganism were different herein. The recovered cells for the test sample were reported to be $<10^1$cfu/mL based on the nearest effective dilution, e.g. the 10^{-2} dilution for the disinfectant but the 10^{-1} dilution with respect to *Staphylococcus epidermis*; the corresponding recovery per coupon was estimated to be $<10^3$cfu/ coupon. The side-by-side positive control yielded the cells within the countable range (e.g. 26cfu) at the 10^{-5} dilution for *Staphylococcus epidermis*, resulting in 2.6×10^6cfu/mL and 2.6×10^8cfu/coupon. Therefore, the log reduction was calculated to be:

$$\mathrm{LR} = \log_{10}\frac{\text{control pop count}}{\text{survival pop count}} > \log_{10}\frac{2.6 \times 10^8/\text{coupon}}{10^3/\text{coupon}} = 5.4$$

This result (e.g. LR >5.4) met the acceptance criteria of at least 3 log for vegetative cells according to the USP <1072>. In other words, the disinfectant of H_2O_2 6% is found to be effective in destroying *Staphylococcus epidermis* in the surface test (SS).

Note: As the DET involves the combination of various disinfectants, microorganisms, hard surfaces, and contact times, the following Results section will present the summary of the final results for each disinfectant in tabular format, rather than individual recoveries of the microorganisms in cfu (e.g. shown in Table 1-4). The supportive neutralizer toxicity and efficacy tests are included in the respective tables. The rationale of selecting the interested microorganisms for a particular disinfectant is based on the general belief and purpose of use (e.g. general-purpose or sporicidal disinfectant)

III. RESULTS

Commercial Disinfectant – Spor Klenz® RTU

Spor-Klenz® RTU manufactured by Steris consists of Hydrogen Peroxide 1% and Peracetic Acid 0.08%. It is generally accepted as a sporicidal disinfectant and widely used in the pharmaceutical cleanroom. Plenty of DET data have been already generated by Steris and the end users. Therefore, the DET study presented herein focused on the most-difficult-to-kill microorganisms - bacterial spore formers, represented by one ATCC culture (e.g. *Bacillus subtilis* spore ATCC 6633) and one EM isolate (*Bacillus horneckiae* spore). *Bacillus subtilis*

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spore ATCC 6633 suspension was purchased from Crosstex as ready-to-use (RTU). *Bacillus horneckiae* spore suspension was prepared in-house using the sporulating AK#2 agar based on the recovered Cleanroom EM isolate. Both spore suspensions had a cell population level of $>10^9$cfu/mL according to the population verification (note: the original plan has been to validate Spor-Klenz ® RTU as a cold Sterilant, for which a six (6) log reduction of the bacterial spore former is desired. The DET results for Spor-Klenz® RTU are summarized in Table 5.

Table 5. Summary of DET Data for the Disinfectant of Spor-Klenz® RTU (Hydrogen Peroxide (H_2O_2) 1% and Peracetic Acid 0.08%)

Test Type		ATCC Panel			EM Isolate		
		***Bacillus subtilis* spore (6633)**			***Bacillus horneckiae* spore**		
Neutralization	Neutralizer Toxicity	110.3%			102.2%		
	Neutralizer Efficacy	10^{-1}	62.5%		10^{-1}	80.4%	
		10^{-2}	96.9%		10^{-2}	91.3%	
		10^{-3}	96.9%		10^{-3}	97.8%	
DET		Contact time	Log reduction	Survival Cells	Contact time	Log reduction	Survival Cells
Suspension Test (Kill Time)	Test#1	5 min	>6 log	No	5 min	**3.3 log**	Yes
		10 min	>6 log	No	10 m	**4.5 log**	Yes
	Test#2	Not Performed			2.5 min	**3.2 log**	Yes
					5 min	**3.9 log**	Yes
					10 min	**4.8 log**	Yes
					15 min	>6.2 log	No
	Test#3				10 min	**4.6 log**	Yes
					15 min	**5.3 log**	Yes
Surface Test (Stainless Steel 316)	Test#1	5 min	**5.0 log**	Yes	5 min	**3.9 log**	Yes
		10 min	**5.5 log**	Yes	10 min	>6.2 log	No
	Test#2	5 min	>6.1 log	No	10 min	**5.2 log**	Yes
		10 min	>6.1 log	No	15 min	>6.1 log	No
	Test#3	5 min	**5.5 log**	Yes	Not Performed		
		10 min	**6.2 log**	Yes			
	Test#4	5 min	**5.0 log**	Yes			
		10 min	**5.0 log**	Yes			
	Test#5	5 min	**5.2 log**	Yes			
		10 min	**4.4 log**	Yes			

For both spore formers, the neutralizer of D/E broth was found to be non-toxic. The neutralizer efficacy of D/E broth for Spor-Klenz® RTU was also established for both spore formers.

During the suspension test, *Bacillus subtilis* spores were completely killed with zero survivors recovered at both contact times of 5 minutes and 10 minutes, yielding more than 6 log reduction which met the USP <1072> requirements (e.g. at least 2 log for spore formers). Three suspension tests have been performed on *Bacillus horneckiae* spores; for each test, survival cells were recovered at those contact times of 2.5 minutes, 5 minutes, 10 minutes, and 15 minutes, suggesting that *Bacillus horneckiae* spores

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are probably more difficult to kill than *Bacillus subtilis* spores with Spor-Klenz® RTU; however, the yielded log reduction values were found to be all greater than the USP <1072>required 2 log. Therefore, Spor-Klenz® RTU is found to be effective in destroying both *Bacillus subtilis* spores and *Bacillus horneckiae* spores in the suspension test.

During the surface test using stainless steel (SS) 316coupon, survival cells were generally recovered for both *Bacillus subtilis* and *Bacillus horneckiae* spores with few cases wherein complete kills were observed. The contact of the microorganism cells with the disinfectant is different and less efficient in the surface test than in the suspension test. One side of the microorganism cells is attached to the hard surface, and therefore such side is somehow protected from the direct exposure to the disinfectant (this effect leads to more survival opportunities for the microorganism cells underneath). However, all the yielded log reduction values supported the efficiency of Spor-Klenz® RTU in destroying both spore formers by meeting the USP <1072> requirements of at least 2 log for spore formers. Once again, the EM isolate - *Bacillus horneckiae* spore was found to be possibly more resistant than the panel Bacillus subtilis spore (ATCC 6633), and thus even a longer contact time such as 15 minutes was investigated herein.

During the various phases of DET, the presence of survival cells has been realized to be an indicator to evaluate the performance of a disinfectant. Therefore, in the following DET studies on the other disinfectants, any log reduction values based on the counts of survival cells will be bolded; such data points are not necessary to indicate a DET failure but rather an alert or an issue.

Sodium Hypochlorite (NaClO) 5.25%

Sodium Hypochlorite solution or bleach has been generally accepted as a sporicidal disinfectant (e.g. also used at 0.5% commercially). However, Sodium Hypochlorite solution is corrosive and is used only for sanitizing and disinfecting inert surfaces such as floors and walls. For Sodium Hypochlorite solution at the strength of 5.25%, only the difficult-to-kill spore formers are considered in the current study, including two ATCC cultures (a bacterial spore former - Bacillus subtilis spore ATCC 6633 and a fungal spore former - *Aspergillus brasiliensis* ATCC 16404), and two EM isolates (a bacterial spore former - *Bacillus horneckiae* spore and a fungal spore former – Fusarium oxysporum). In support of qualifying Sodium Hypochlorite 5.25% as a sporicide which requires at least 2 log reduction for spore formers according to the USP <1072>, the employed suspensions of bacterial spore formers had a cell population of 10^7 - 10^9cfu/mL, and those of fungal spore formers had a cell population of 10^6 - 10^7cfu/mL. The DET results for Sodium Hypochlorite 5.25% solution are summarized in Table 6.

Table 6. Summary of DET Data for the Disinfectant of Sodium Hypochlorite (NaClO) 5.25%

Test Type		**ATCC Panel**				**EM Isolates**			
		***Aspergillus brasiliensis* (16404)**		***Bacillus subtilis* spore (6633)**		***Fusarium oxysporum***		***Bacillus horneckiae* spore**	
Neutralization	Neutralizer Toxicity	109.1%		100.0%		106.7%		102.6%	
	Neutralizer Efficacy	10^{-1}	95.8%	10^{-1}	72.7%	10^{-1}	0%	10^{-1}	89.7%
		10^{-2}	100%	10^{-2}	103.0%	10^{-2}	102.1%	10^{-2}	98.7%
		10^{-3}	100%	10^{-3}	103.0%	10^{-3}	104.2%	10^{-3}	98.7%

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Table 6. Summary of DET Data for the Disinfectant of Sodium Hypochlorite (NaClO) 5.25%									
Test Type		**ATCC Panel**				**EM Isolates**			
		***Aspergillus brasiliensis* (16404)**		***Bacillus subtilis* spore (6633)**		***Fusarium oxysporum***		***Bacillus horneckiae* spore**	
DET		Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction
Suspension Test (Kill Time)		5 min	>2.7 log	5 min	>2.0 log	5 min	>2.1 log	5 min	>2.6 log
		10 min	>2.7 log	10 min	>2.0 log	10 min	>2.1 log	10 min	>2.6 log
Surface Test	Terrazzo tiles	5 min	>3.2 log	5 min	>2.8 log	5 min	>2.2 log	5 min	>3.2 log
		10 min	>3.2 log	10 min	>2.8 log	10 min	>2.2 log	10 min	>3.2 log
	Epoxy-coated gypsum	5 min	>3.3 log	5 min	>3.1 log	5 min	>2.4 log	5 min	**2.04 log**
		10 min	>3.3 log	10 min	>3.1 log	10 min	>2.4 log	10 min	>3.7 log
	Glass	5 min	>3.4 log	5 min	>3.0 log	5 min	>2.1 log	5 min	**1.5 log**
		10 min	>3.4 log	10 min	>3.0 log	10 min	>2.1 log	10 min	>3.1 log
	Plastic vinyl	5 min	>3.5 log	5 min	>3.0 log	5 min	>2.5 log	5 min	**1.7 log**
		10 min	>3.5 log	10 min	>3.0 log	10 min	>2.5 log	10 min	>3.2 log

For all those spore formers, the neutralizer of D/E broth was found again to be non-toxic. The neutralizer efficacy of D/E broth for Sodium Hypochlorite 5.25% was also established for those spore formers. *Fusarium oxysporum* was found to be the most vulnerable because it was the only one that the disinfectant must be diluted down to another level (e.g. 10^{-2}) with the neutralizer to demonstrate acceptable efficacy.

During the suspension test, all the spore formers were completely killed with zero survivors recovered at both contact times of 5 minutes and 10 minutes, conforming to the log reduction requirement of at least 2 log for spore formers according to the USP <1072>.

During the surface test using four coupons made of cleanroom materials or the equivalent (e.g. Terrazzo tiles, Epoxy-coated gypsum, Glass, and Plastic vinyl), all the challenge spore formers were completely killed (e.g. zero survivors) at both contact times of 5 minutes and 10 minutes, except three cases for the bacterial spore former - *Bacillus horneckiae* spore, all at the 5 minutes contact time (highlighted in bold). This observation again suggests that the *Bacillus horneckiae* spore is the most difficult to kill microorganism among those spore formers studied. Two out of the three cases (all at 5 minutes) with the recovered survival cells resulted in the log reduction failing to pass the required 2 log for spore formers according to the USP <1072> (e.g. Glass and Plastic vinyl surfaces). However, the corresponding surface test at the 10 minutes contact time did pass the required 2 log for both cases. All the other cases for the combinations of the four spore formers (*Bacillus*

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subtilis spore, *Aspergillus brasiliensis*, *Bacillus horneckiae* spore, and *Fusarium oxysporum*), the four surfaces (Terrazzo tiles, Epoxy-coated gypsum, Glass, and Plastic vinyl), and both contact times (e.g. 5 minutes and 10 minutes) also passed the required 2 log under the USP <1072>, indicating that Sodium Hypochlorite 5.25% is effective in destroying those studied bacterial and fungal spore formers.

Isopropyl Alcohol (IPA) 70%

Isopropyl Alcohol 70% is a general-purpose disinfectant and is popularly utilized in cleanrooms. IPA 70% is easy to evaporate in the atmosphere and therefore user-friendly. The drawback of IPA 70% is its weakness in disinfecting the spore formers. Therefore, only the vegetative cells are considered for IPA 70% in the current study, including two ATCC cultures (e.g. *Pseudomonas aeruginosa*, ATCC 9027 and *Candida albicans*, ATCC 10231) and two EM isolates (e.g. *Staphylococcus epidermidis* and *Micrococcus luteus*). In support of qualifying IPA 70% as a general-purpose disinfectant which requires at least 3 log reduction for the vegetative cells according to the USP <1072>, the employed suspensions of those vegetative microorganisms had a cell population of 10^8 - 10^{10}cfu/mL. The DET results for IPA 70% solution are summarized in Table 7.

Table 7. Summary of DET Data for the Disinfectant of Isopropyl Alcohol (IPA) 70%

Test Type		ATCC Panel				EM Isolates			
		***Pseudomonas aeruginosa* (9027)**		***Candida Albicans* (10231)**		***Staphylococcus epidermidis***		***Micrococcus luteus***	
Neutralization	Neutralizer Toxicity	105.0%		96.7%		91.8%		110.0%	
	Neutralizer Efficacy	10^{-1}	100%	10^{-1}	100.0%	10^{-1}	91.1%	10^{-1}	100.0%
		10^{-2}	104.8%	10^{-2}	105.1%	10^{-2}	91.1%	10^{-2}	110.9%
		10^{-3}	104.8%	10^{-3}	106.8%	10^{-3}	100.0%	10^{-3}	112.7%
DET		Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction
Suspension Test (Kill Time)		5 min.	>4.3 log	5 min	>4.5 log	5 min	>6.0 log	5 min	>4.7 log
		10 min.	>4.3 log	10 min	>4.5 log	10 min	>6.0 log	10 min	>4.7 log
Surface Test	Stainless Steel 316	5 min.	>6.0 log	5 min	>4.2 log	5 min	**5.8 log***	5 min	>4.6 log
		10 min.	>6.0 log	10 min	>4.2 log	10 min	>6.4log	10 min	>4.6 log
	Terrazzo tiles	5 min	>4.5 log	5 min	>3 log	5 min	>4.7 log	5 min	>4.5 log
		10 min	>4.5 log	10 min	>3 log	10 min	>4.7 log	10 min	>4.5 log
	Epoxy-coated gypsum	5 min	>4.8 log	5 min	>4.6 log	5 min	>4.8 log	5 min	>4.7 log
		10 min	>4.8 log	10 min	>4.6 log	10 min	>4.8 log	10 min	>4.7 log
	Glass	5 min	>5.0 log	5 min	>3.8 log	5 min	>3.1 log	5 min	**5.1 log****
		10 min	>5.0 log	10 min	>3.8 log	10 min	>3.1 log	10 min	>4.7log

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Table 7. Summary of DET Data for the Disinfectant of Isopropyl Alcohol (IPA) 70%

Test Type		ATCC Panel				EM Isolates			
		***Pseudomonas aeruginosa* (9027)**		***Candida Albicans* (10231)**		***Staphylococcus epidermidis***		***Micrococcus luteus***	
	Plastic vinyl	Not Performed		5 min	>4.0 log	Not Performed			
				10 min	>4.0 log				
	Polypro-pylene	5 min	**4.5 log *****	5 min	>4.5 log	5 min	>4.5 log	5 min	>4.3 log
		10 min	>5.5 log	10 min	>4.5 log	10 min	>4.5 log	10 min	>4.3 log

* Three plates at the 10^0 level demonstrated 3, 4, 5 cfu, respectively, likely demonstrating the resistance of *Staphylococcus epidermidis*

** Only one out of the three (3) plates at the 10^0 level demonstrated 1 cfu, could be due to a cross-contamination during the experiment.

***Three plates at the 10^0 level demonstrated 7, 9, 12 cfu, and at the 10^{-1} level 0, 1, 3 cfu respectively, very likely demonstrating the resistance of *Pseudomonas aeruginosa*.

For all those vegetative cells, the neutralizer of D/E broth was found again to be non-toxic. The neutralizer efficacy of D/E broth for IPA 70% was also established for those vegetative cells, all at the first level of dilution (e.g. 10^{-1}).

During the suspension test, all the vegetative cells were completely killed with zero survivors recovered at both contact times of 5 minutes and 10 minutes, conforming to the log reduction requirement of at least 3log for vegetative cells according to the USP <1072>.

During the surface test using six coupons made of cleanroom materials or the equivalent (e.g. Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, Plastic vinyl, and Polypropylene), all the challenge vegetative cells were completely killed (e.g. zero survivors) at both contact times of 5 minutes and 10 minutes, except three cases for different microorganisms on different surfaces but all at the contact time of 5 minutes (highlighted in bold). Such survival cells could demonstrate some sort of limited resistance of those microorganisms towards the disinfectant of IPA 70%, or just simply be due to cross-contaminations during the DET experiment. Nevertheless, all three cases with finding survival cells still passed the required log reduction of at least 3log for vegetative cells. All the other cases for the combinations of the four vegetative microorganisms (*Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus epidermidis*, and *Micrococcus luteus*), the six surfaces (Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, Plastic vinyl, and Polypropylene), and both contact times (e.g. 5 minutes and 10 minutes) passed the required 3 log according to the USP <1072>, indicating that IPA 70% is effective in destroying those studied vegetative cells.

Hydrogen Peroxide (H_2O_2) 6%

Hydrogen Peroxide solution is also widely used in the pharmaceutical cleanroom. In particular, Hydrogen Peroxide 6% is commercially produced, and is associated with sporicide and sterilant claim in some vendor' website. The advantage of Hydrogen Peroxide solution is that it is not as harsh as Sodium Hypochlorite solution during the oxidation reaction. However, it is much slower in evaporation than IPA 70% because of the considerable water content. In this study, a thorough DET has been originally planned for Hydrogen Peroxide 6% by using eight (8) microorganisms, including four ATCC cultures (*Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, and *Bacillus subtilis* spore, ATCC 6633) and four EM isolates (*Staphylococcus epidermidis*, *Micrococcus luteus*, *Fusarium oxysporum*, and *Bacillus horneckiae* spore).To support Hydrogen Peroxide 6% as a sporicidal disinfectant, the log reduction should be at least 2 log for spore formers and at least 3 log for vegetative cells according to the USP <1072>.The employed suspensions of those spore-forming and vegetative microorganisms had a cell population of 10^6 - 10^{10}cfu/mL. The DET results for Hydrogen Peroxide (H_2O_2) 6% are summarized in Table 8.

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Table 8. Summary of DET Data for the Disinfectant of Hydrogen Peroxide (H_2O_2) 6%

Test Type			ATCC Panel						EM Isolates					
			***Pseudomonas aeruginosa* (9027)**		***Candida albicans* (10231)**		***Bacillus subtilis* spore (6633)**		***Staphylococcus epidermidis***		***Micrococcus luteus***		***Bacillus horneckiae* spore**	
Neutralization	Toxicity		105.0%		82.4%		100.0%		96.7%		123.8%		102.2%	
	Neutralizer Efficacy		10^{-1}	0%	10^{-1}	0%	10^{-1}	0.0%	10^{-1}	0%	10^{-1}	0%	10^{-1}	0.7%
			10^{-2}	9.5%	10^{-2}	89.3%	10^{-2}	90.6%	10^{-2}	70.8%	10^{-2}	32.7%	10^{-2}	95.7%
			10^{-3}	85.7%	10^{-3}	100%	10^{-3}	95.3%	10^{-3}	100.0 %	10^{-3}	84.6%	10^{-3}	93.6%
DET			Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction
Suspension Test (Kill Time)	Test#1		5 min	>3.1 log	5 min	**2.9 log**	5 min	**0.26 log**	5 min	>5.0 log	5 min	>3.7 log	5 min	**0.65 log**
			10 min	>3.1 log	10 min	**3.9 log**	10 min	**0.28 log**	10 min	>5.0 log	10 min	>3.7 log	10 min	**0.95 log**
	Test#2		Not Performed				5 min	**0.03 log**	Not Performed					
							10 min	**0.03 log**						
Surface Test	Stainless Steel	Test #1	5 min	>3.8 log	5 min	**1.93 log**	Not Performed		5 min	>5.4 log	5 min	>3.9 log	Not Performed	
			10 min	>3.8 log	10 min	**1.43 log**			10 min	>5.4 log	10 min	>3.9 log		
		Test #2	n/a		5 min	**0.74 log**			n/a					
					10 min	**1.49 log**								
	Glass		5 min	>4.6 log	5 min	**2.04 log**			Not Performed		5 min	>4.0 log		
			10 min	>4.6 log	10 min	**2.00 log**					10 min	>4.0 log		
	Terrazzo tiles		5 min	>5.5 log	5 min	**2.06 log**			Not Performed					
			10 min	>5.5 log	10 min	**1.62 log**								
	Epoxy-coated gypsum		5 min	>4.3 log	5 min	**0.82 log**								
			10 min	>4.3 log	10 min	**1.67 log**								
	Polypropylene		5 min	>5.0 log	5 min	**0.07 log**								
			10 min	>5.0 log	10 min	**2.09 log**								

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Unexpected issues have been found for the DET of Hydrogen Peroxide 6%; in particular, rather poor log reduction values were found for the bacterial spore formers (*Bacillus subtilis* spore and *Bacillus horneckiae* spore). The scheduled DET study for the fungal spore formers (*Aspergillus brasiliensis* and Fusarium oxysporum) was therefore cancelled.

For all six studied microorganism cells, the neutralizer of D/E broth was found to be non-toxic. The neutralizer efficacy of D/E broth for Hydrogen Peroxide 6% was established for those six studied microorganism cells individually, mostly at the second level of dilution (e.g. 10^{-2}). *Pseudomonas aeruginosa* was found to be the most vulnerable and the disinfectant was required to dilute down to another level (e.g. 10^{-3}) with the neutralizer to demonstrate acceptable efficacy.

During the suspension test, the DET data demonstrated that the log reduction values for the four studied vegetative cells (*Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Micrococcus luteus*, and *Candida albicans*) passed the required at least 3 log according to the USP <1072> (e.g. based on the 10 minutes contact time). Complete kills with zero survivors were found for *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Micrococcus luteus* (both contact times of 5 minutes and 10 minutes).However, survival cells were recovered for *Candida albicans* at both contact times (corresponding log reduction = 2.9 and 3.9, highlighted in bold). Surprisingly, the log reduction for the two studied bacterial spore formers (Bacillus subtilis spore and *Bacillus horneckiae* spore) showed rather poor log reduction values (e.g. less than 1 log) in the suspension test, which failed to pass the required at least 2 log for spore formers according to the USP <1072>. Considerable survival cells (e.g. TNTC or too numerous to count) were identified from the test samples for both bacterial spore formers. A repetitive suspension test was performed on Bacillus subtilis spore with the same outcome. This result is strongly against the general belief that H_2O_2 6% could be a sporicide.

The surface test was only further conducted for those vegetative cells for which the log reduction passed the required 3 log during the suspension test. For the vegetative bacterial cells, sufficient log reduction values (e.g. at least 3 log) were achieved with complete kills from the currently obtained data at both contact times of 5 minutes and 10 minutes (note: since the disinfectant was unexpectedly found incapable of destroying the bacterial spore formers, some scheduled surface tests for the vegetative *Staphylococcus epidermidis* and *Micrococcus luteus* were also discontinued). The difficulty for the disinfectant of Hydrogen Peroxide 6% was found during disinfecting the vegetative yeast cells (e.g., *Candida albicans*) – survival cells were generally recovered from all the five surface coupons (e.g., Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, and Polypropylene). This resulted in all the log reduction values between 0.07 and 2.09(highlighted in bold), which failed to pass the required at least 3 log for vegetative cells. This is the other evidence against Hydrogen Peroxide 6% if claimed as "sporicidal" under the current experimental setup.

Enhanced Hydrogen Peroxide (H_2O_2) 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%)

As we have found that Hydrogen Peroxide 6% is poor in disinfecting the bacterial spore formers, Hydrogen Peroxide is enhanced with 0.05% Peracetic Acid, enlightened by the formula of Spor-Klenz® by using the combination of Hydrogen Peroxide 1% and Peracetic Acid 0.08% (see above in Table 5).

In the DET study of Enhanced Hydrogen Peroxide (H_2O_2) 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%),six (6) microorganisms, including four ATCC cultures (*Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404, and *Bacillus subtilis* spore, ATCC 6633) and two EM isolates (*Staphylococcus epidermidis* and *Aspergillus versicolor/sydowii* were utilized. Five hard surfaces (Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, and Polypropylene) were employed in the surface test. To support the enhanced Hydrogen Peroxide 6% as a sporicidal disinfectant, the log reduction should be at least 2 log for spore formers and at least 3 log for vegetative cells according to the USP <1072>. The employed suspensions of those spore-forming and vegetative microorganisms had a cell population of 10^6 - 10^{10}cfu/mL.

The DET results for the enhanced Hydrogen Peroxide (H_2O_2) 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%) are summarized in Table 9.

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Table 9. Summary of DET Data for the Disinfectant of Enhanced Hydrogen Peroxide (H_2O_2) 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%)

Test Type		ATCC Panel								EM Isolates			
		***Candida albicans* (10231)**		***Aspergillus brasiliensis* (16404)**		***Bacillus subtilis* spore (6633)**		***Pseudomonas aeruginosa* (9027)**		***Aspergillus versicolor/ sydowii***		***Staphylococcus epidermidis***	
Neutralization	Neutralizer Toxicity	97.8%		86.8%		101%		130.6%		102.0%		161%	
	Neutralizer Efficacy	10^{-1}	0%	10^{-1}	33.3%	10^{-1}	1.9%	10^{-1}	0%	10^{-1}	0%	10^{-1}	0%
		10^{-2}	97.8%	10^{-2}	118.2 %	10^{-2}	80.5%	10^{-2}	15.6%	10^{-2}	98.0%	10^{-2}	26%
		10^{-3}	100.0 %	10^{-3}	90.9%	10^{-3}	82.4%	10^{-3}	87.5%	10^{-3}	90.2%	10^{-3}	101%
DET		Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction	Contact Time	Log Reduction
Suspension Test (Kill Time)		5 min	>3.1 log	5 min	>2.5 log	5 min	>3.2 log	5 min	>5.6 log	5 min	>3.0 log	5 min	>4.4 log
		10 min	>3.1 log	10 min	>2.5 log	10 min	>3.2 log	10 min	>5.6 log	10 min	>3.0 log	10 min	>4.4 log
Surface Test	Stainless Steel	5 min	>4.0 log	5 min	>2.6 log	5 min.	>3.2 log	5 min	>4.8 log	5 min	>2.6 log	5 min	>3.4 log
		10 min	>4.0 log	10 min	>2.6 log	10 min	>3.2 log	10 min	>4.8 log	10 min	>2.6 log	10 min	>3.4 log
	Terrazzo tiles	5 min	>3.5 log	5 min	**1.6 log****	5 min	>3.0 log	5 min	>5.1 log	5 min.	>3.08 log	5 min	>3.3 log
		10 min	>3.5 log	10 min	>2.6 log	10 min	>3.0 log	10 min	>5.1 log	10 min	>3.08 log	10 min	>3.3 log
	Epoxy-coated gypsum	5 min	>3.2 log	5 min	**2.6 log****	5 min	**3.0 log *****	5 min	>5.2 log	5 min.	>2.28 log	5 min	>3.7 log
		10 min	>3.2 log	10 min	>2.6 log	10 min	>3.1 log	10 min	>5.2 log	10 min	>2.28 log	10 min	>3.7 log
	Glass	5 min	**3.5 log***	5 min.	**2.3 log****	5 min	>3.2 log	5 min	>4.8 log	5 min	>2.28 log	5 min	>3.8 log
		10 min	>3.0 log	10 min	>2.6 log	10 min	>3.2 log	10 min	>4.8 log	10 min	>2.28 log	10 min	>3.8 log
	Polypropy-lene	5 min	>3.1 log	5 min.	>2.8 log	5 min.	>2.9 log	5 min	>4.2 log	5 min.	>2.57 log	5 min	>4.3 log
		10 min	>3.1 log	10 min	>2.8 log	10 min	>2.9 log	10 min	>4.2 log	10 min	>2.57 log	10 min	>4.3 log

* could be due to cross-contamination - only one out of the three (3) plates at the 10^{-1} level demonstrated 1 cfu; the other two were zeros.

** demonstrating the resistance of *Aspergillus brasiliensis* three times (from top to bottom, three plate count readings at the serial dilution level of 10^{-1} were 9, 11, 12 cfu, 0, 1, 2 cfu,1, 2, 2 cfu, respectively).

*** could also be due to the microbial resistance – three plates at 10^{-1} level demonstrated 1, 1, 2 cfu.

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For all the studied microorganism cells (spore-forming and vegetative bacteria, yeast and molds), the neutralizer of D/E broth was found again to be non-toxic. The neutralizer efficacy of D/E broth for the disinfectant of Hydrogen Peroxide 6% and Peracetic Acid 0.05% was also established for those various microorganism cells, most at the first level of dilution (e.g. 10^{-1}) while *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were found to be more vulnerable and the disinfectant was needed to dilute down to another level (e.g. 10^{-2}) with the neutralizer for both organisms to demonstrate acceptable efficacy.

During the suspension test, all the studied microorganism cells were completely killed with zero survivors recovered at both contact times of 5 minutes and 10 minutes, conforming to the log reduction requirement of at least 2log for bacterial and fungal spore formers, and at least 3 log for vegetative cells according to the USP <1072>.

During the surface test using five coupons made of cleanroom materials or the equivalent (e.g. Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, and Polypropylene), all the challenge microorganism cells were completely killed (e.g. zero survivors) at both contact times of 5 minutes and 10 minutes, except a few cases for different microorganisms on different surfaces but all at the 5 minutes contact time (highlighted in bold). Such recovered survival cells could demonstrate the resistance of those microorganisms towards the disinfectant, or just simply be cross-contaminations for some cases during the DET experiment. All those cases with survival cells still passed the required log reduction of at least 2 log for the spore formers and at least 3 log for the vegetative cells except one case - *Aspergillus brasiliensis* on the Terrazzo tiles surface at the 5 minutes contact time wherein the log reduction was insufficient (e.g. 1.6) to pass the required at least 2 log because of those survival cells. All the other cases for the combinations of the six bacterial and fungal microorganisms (*Candida albicans*, *Aspergillus brasiliensis*, *Bacillus subtilis* spores, *Pseudomonas aeruginosa*, *Aspergillus versicolor/sydowii*, and *Staphylococcus epidermidis*), the five surfaces (Stainless Steel 316, Terrazzo tiles, Epoxy-coated gypsum, Glass, and Polypropylene), and both contact times (e.g. 5 minutes and 10 minutes) also passed the required at least 2 log for spore formers and at least 3 log for vegetative cells according to the USP <1072>, indicating that the enhanced Hydrogen Peroxide 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%) is effective in destroying those studied microorganism cells (spore-forming and vegetative bacteria, yeast and molds)at the required 10 minutes contact time. Therefore, the enhanced Hydrogen Peroxide 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%) has been qualified as a sporicidal disinfectant.

IV. DISCUSSION

As mentioned earlier, surface test is more challenging than suspension test because many additional factors have to be taken into account. For the positive control, a 100% full recovery would be generally achieved from the suspension test, but a reduced recovery would be obtained from the surface test. The first physicochemical parameter under consideration is the sticking probability of the microorganism cells onto the surface coupon, which would impact the number of cells to be recovered efficiently (e.g. supposing that some cells adhere tightly onto the surface). The roughness and porosity of the surface shall also be considered because some buried cells can also cause loss in recovery. For some vulnerable organisms (e.g. *Pseudomonas aeruginosa*), the drying process for the inoculum on the coupon (e.g. about 20 – 30 minutes under laminar flow) might lead to the death of cells and the missing of the expected positive-control counts. There have been situations (not shown here) that the cell population recovered in the positive control was lower than the theoretical expectation and therefore cannot demonstrate a sufficient log reduction (e.g. the greater-than value)even though all the test sample plates are zeros(complete kill), leading to an inconclusive result. In such a scenario, the surface test has to be restarted by using an inoculum with an even higher cell population to compensate for the unexpected low recovery.

The contact interface between the microorganism cells and the hard surface can behave as a “barrier” to prevent the disinfectant molecules from attacking the inoculum cells at one dimension (e.g. the surface coupon works like a shield). Of course, this spatial “barrier” effect will lead to the survival opportunities of the inside microorganism cells and a less log reduction in the surface test. In some senses, the surface testis more or less like a two-dimensional disinfecting process but the

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suspension test is three-dimensional. The spatial "barrier" effect for the surface test becomes more pronounced if there are large clumps in the challenge organism suspension. For example, the in-house *Bacillus horneckiae* spore suspension has been prepared in a laboratory condition (e.g. using the sporulating AK#2 agar), but the commercial *Bacillus subtilis* spore suspension purchased from Crosstex might undergo a different process control (for example, it contains 20% alcohol according to the product label), and therefore contain a finer distribution of the organism particles. In other words, the more difficulty found in disinfecting the in-house *Bacillus horneckiae* spores could be associated with the size of the organism cell clusters, rather than the true microorganism resistance (e.g. see data in Table 5 for Spore-Klenz® and Table 6 for Sodium Hypochlorite 5.25%).The other organism suspensions suspiciously containing clumps include the mold - *Aspergillus brasiliensis* suspension, for which an unusual low log reduction has been observed in a few cases of the surface test (e.g., Table 9 for the enhanced Hydrogen Peroxide 6%).The roughness and porosity of the surface can also play a role in influencing the log reduction as some microorganism cells can hide inside the porous holes and corners to avoid direct exposure to the disinfectant (e.g. more frequently found on the floor coupon). Generally, the surface test on the nonporous surface coupons such as stainless steel 316 is better controlled and works like a reference standard to guide the other surface tests.

One surprising finding in this study is the poor DET performance obtained for the disinfectant of Hydrogen Peroxide 6% against the bacterial spore formers (e.g. *Bacillus subtilis* spore, *Bacillus horneckiae* spore), which contradicts the general "sporicidal" claim for Hydrogen Peroxide solution as shown in some publications[3, 16]; this discrepancy can be interpreted as:

1. Both contact times of 5 minutes and 10 minutes employed in the current study could be too short for Hydrogen Peroxide 6% to take full effect. The diffusion rate of the active Hydrogen Peroxide molecules (H_2O_2) is limited by the surrounding overwhelming number of water molecules (H_2O) (e.g. Hydrogen Peroxide 6% corresponds to a molar/molecular ratio between H_2O and H_2O_2equivalent to 30:1). In disinfecting, the H_2O_2molecules have to penetrate the hydrophobic cell membrane/ wall. A rate-limited diffusion would cause the slow-down of the active Hydrogen Peroxide to build up the concentration and reach the killing threshold for the cell components. In some situations, a contact time of 30 minutes or even 60 minutes is required for Hydrogen peroxide to function as a sporicide.[3] For example, a 10v Hydrogen Peroxide solution demonstrated the lowest antifungal effect (e.g. against *Candida albicans* at the contact time of 10 minutes) among the studied disinfectants including vinegar.[25]
2. The concentration of the active Hydrogen Peroxide in the Hydrogen Peroxide solution is crucially important. A Hydrogen Peroxide solution with higher concentrations (e.g. 10%, 25%) might enable it to pass the acceptance criteria set in the USP <1072> and therefore is consistent with the sporicidal claim.
3. In an approach to enhancing Hydrogen Peroxide 6% solution, a fast-diffusion and stronger oxidizer, e.g. Peracetic Acid (CH_3COOOH),is added to the formula. The disinfectant efficacy of such enhanced Hydrogen Peroxide 6% (Hydrogen Peroxide 6% and Peracetic Acid 0.05%) has been successfully demonstrated in the current study. Peracetic Acid (CH_3COOOH) is generally accepted as a cold-sterilant[16]. It contains an acetyl group ($-CH_3$) which has a relatively high affinity towards the hydrophobic nature of the membrane structure of the cell wall, which would assist the molecule in penetrating into the wall efficiently. The rapid diffusion of the disinfectant molecules would lead to a quicker and more effective disinfection, e.g. via the oxidizing/destroying the membrane structure, the DNA/RNA, and the proteins inside the cell nucleus. Moreover, a synergetic effect between those two oxidizers (e.g. CH_3COOOH and H_2O_2) might exist; plenty of the H_2O_2 molecules can provide an oxidation state bank and keep the CH_3COOOH molecules at the peak working concentration for a considerable duration. Note: CH_3COOOH is a strong oxidizer but is prone to degeneration (e.g. limited stability); the availability of plenty of oxidation states from H_2O_2 will help to stabilize CH_3COOOH.

Regarding contact time, the DET data

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presented herein indicate the results at 5 minutes and 10 minutes are the same in most cases; the results at 10-minutes have demonstrated some sort of improvement for only a few cases. This suggests that the effective disinfection process could be short (e.g. much less than 5 minutes); this characteristic shares similarity to the chemical reactions in which the process can be spontaneous (e.g. completed in milliseconds) once the activation energy is satisfied. The rate-limiting step for disinfecting is associated with the diffusion through the cell wall and the build-up of an adequate concentration of the chemical agent within the cell. In this aspect, the PDA TR#70 recommends contact time for disinfectant efficacy to be 1-5 minutes while only 1 log reduction is required for the vegetative cells and spore formers; such acceptance criteria would be more practical and more scientific than the USP <1072> adopted in the current study.

So far, we have presented the DET data for five (5) disinfectants, including Spor-Klenz® RTU (Hydrogen Peroxide (H_2O_2) 1% and Peracetic Acid (CH_3COOOH) 0.05%), Sodium Hypochlorite (NaClO) 5.25%, Isopropyl Alcohol (IPA) 70%, Hydrogen Peroxide (H_2O_2) 6%, and the enhanced Hydrogen Peroxide (H_2O_2) 6% (Hydrogen Peroxide (H_2O_2) 6% and Peracetic Acid (CH_3COOOH) 0.05%). Based on what is generally accepted in the literature and the data obtained in the current study, it is summarized:

1. The commercial Spor Klenz® RTU is consistent with the label claim of a sporicide.
2. The in-house Sodium Hypochlorite (NaClO) 5.25% is qualified to be a sporicide.
3. The in-house Isopropyl Alcohol (IPA) 70% is qualified to be a general-purpose disinfectant
4. The in-house Hydrogen Peroxide (H_2O_2) 6% is found incapable of meeting the requirements for a sporicide according to the USP <1072>.
5. The in-house enhanced Hydrogen Peroxide (H_2O_2) 6% with Peracetic Acid (CH_3COOOH) 0.05% is qualified to be a sporicide.

V. CONCLUSION

A few in-house (e.g. Sodium Hypochlorite 5.25%, Isopropyl Alcohol 70%, Hydrogen Peroxide 6%, and enhanced Hydrogen Peroxide 6% with Peracetic Acid 0.05%) and commercial (e.g. Spor-Klenz® RTU - Hydrogen Peroxide 1% and Peracetic Acid 0.08%) disinfection products have been studied in terms of DET or disinfectant efficacy testing, including suspension test (kill time) and surface test. A neutralizer (e.g. D/E broth) is employed and a neutralizer efficacy is established to support the yielded log reduction calculations prior to the DET study. Both vegetative cells and spore-forming microorganisms are challenged, including strains from the ATCC cultures (e.g. *Candida albicans*, *Aspergillus brasiliensis*, *Bacillus subtilis* spores, and *Pseudomonas aeruginosa*) and from the EM isolates recovered from Altaire's cleanroom (e.g. *Staphylococcus epidermidis*, *Micrococcus luteus*, *Fusarium oxysporum*, *Chaetomium globosum*, *Aspergillus versicolor/ sydowii*, and *Bacillus horneckiae* spores). Hard surfaces sampled from the cleanroom construction materials (e.g. Terrazzo tiles, Epoxy-coated gypsum, Glass, and Plastic vinyl) and the equipment (e.g. Stainless Steel 316 and Polypropylene) are utilized for the surface test. It is generally found the recovery and the log reduction obtained in the surface test are more challenging than those in the suspension test, which is interpreted as the physicochemical interactions between the microorganism cells and the surface coupon, and the spatial "barrier" effect against the disinfectant during the surface test. The in-house Isopropyl Alcohol 70% is qualified as a general-purpose disinfectant. Both the commercial Spor Klenz® RTU and the in-house Sodium Hypochlorite 5.25% have passed the USP acceptance criteria for a sporicide. It is surprisingly found that the log reduction yielded from the in-house Hydrogen Peroxide 6% is not able to meet all the USP requirements, in particular, against the bacterial spore formers. It is interpreted that a longer contact time than the studied 5 minutes and 10 minutes shall be used for Hydrogen Peroxide to take the full disinfecting operation. The mechanism of disinfecting is correlated with the rate-limiting diffusion into the membrane structure of the cell wall to build up the local concentration. One approach to enhance Hydrogen Peroxide 6% by adding a more-efficient-diffusion oxidizer, e.g. Peracetic Acid 0.05%has resulted in the modified Hydrogen Peroxide 6% successfully qualified as a sporicide according to the USP <1072>.

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ABBREVIATIONS AND TERMS

CFU: Colony-Forming Unit or visible growth of microorganisms arising from a single or multiple cells, e.g. after growth on a medium upon incubation
Contact Time: The amount of time that a disinfectant or sporicide is left in complete contact with the microorganism and/or with the inoculated surface to be treated (e.g. in wet conditions)
Disinfectant: A chemical or physical agent that reduces or kills vegetative forms of microorganisms but not necessarily all bacterial and fungal spore formers. Besides this general term, there exist two additional terms frequently used ("sanitizer" and "sporicide") that distinguish "disinfectants" based on the effectiveness of their killing power.
Environmental Monitoring (EM): The processes and activities that need to take place to characterize and monitor the quality of the environment (e.g. Cleanroom), including non-viable particulate matters and viable particles (e.g. EM isolates).
EM Isolates: Microorganisms that are recovered from a facility (e.g. Cleanroom) during EM
GNB: Gram-negative bacilli
GPB: Gram-positive bacilli
sGPB: Spore-forming Gram-positive bacilli
GPC: Gram-positive cocci
HEPA: High efficient particulate air
In-house Disinfectant: A disinfectant that is prepared in-house, e.g. manufactured and utilized at Altaire's facility
Log Reduction (LR): defines as the first log being 90%, the second log being 9%, the third log being 0.9%, and so-on of the original population.
Neutralizer: A chemical compound used to counter the disinfecting function of a disinfectant
Neutralizer Toxicity: The viability of the test microorganism during the exposure to a neutralizer
Neutralizer Efficacy: The effectiveness of a neutralizer to stop the disinfecting function of a disinfectant through chemical reactions and/or dilution
PM: Personnel Monitoring
Suspension Test or Kill Time Test: The test microorganism is directly exposed to a certain volume of the disinfectant solution over a time limit (e.g. contact time).
Surface Test: The test surface is first inoculated with the microorganism cells and left for dry-out, and then the inoculated surface is exposed to the disinfectant via spraying over contact time (e.g. 5 and 10 minutes).
Sanitizer: Aproduct containing antimicrobial chemical agent(s) that reduces the number of vegetative microorganisms to a safe level, but does not destroy bacterial spores and may have limited effect on fungal spores.
Sporicide or **Sporicidal Disinfectant**: An agent that destroys bacterial and fungal spores when used in sufficient concentration for a specified contact time. It is expected to kill all vegetative organisms.
Sterilant: An agent that destroys all forms of microbial life including fungi, viruses, and all forms of bacteria and their spores
Surface Coupon: A piece of the example primary construction material used in the cleanroom to be cleaned and disinfected.
Spore Log Reduction (SLR): log reduction of spore formers, especially meaningful during sterilization validation using a biological indicator – the bacterial spore former with the highest resistance to the sterilization method.
TNTC: too numerous to count
USP: United States Pharmacopeia

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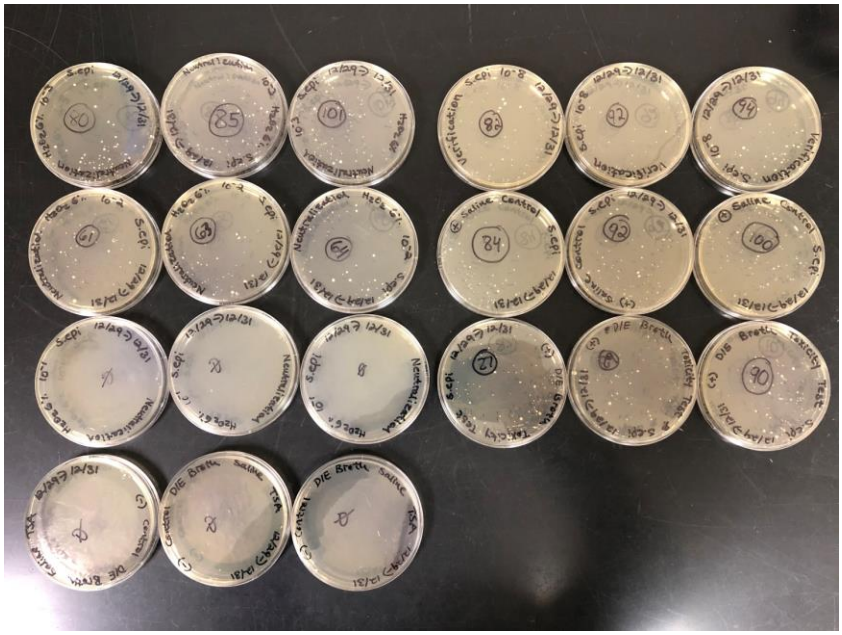
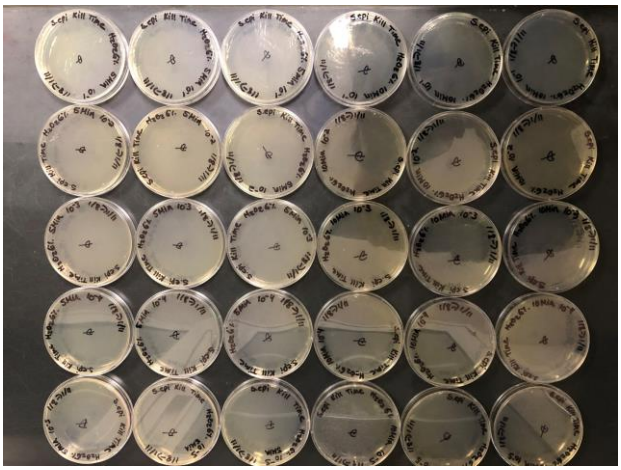


Figure 1. An example picture to show the neutralizer toxicity and the neutralizer efficacy test. *Staphylococcus* epidermidis at the population of 10 – 100 cfu was inoculated into 10 mL of saline control, D/E broth, and the diluted disinfectant (e.g. H_2O_2 6%) at 10^{-1}, 10^{-2}, and 10^{-3}. The test was performed in triplicate. Negative control and verification of the inoculum were also performed.



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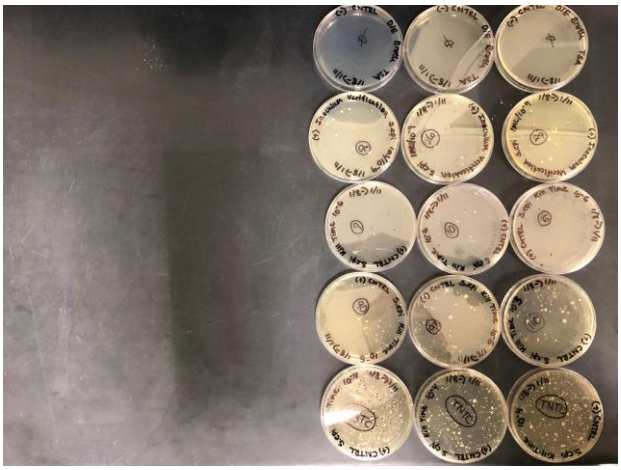

(Figure 2b)

Figure 2. Example pictures to show the suspension test or kill time study. One (1) mL of *Staphylococcus epidermidis* suspension at the population of $>10^9$cfu/mL was added into 99 mL of D/E broth and the disinfectant (e.g. H_2O_2 6%): a) plate count results of the disinfectant at contact times of 5 minutes and 10 minutes with serial dilution of 10^{-1} to 10^{-5};b) plate count results of the D/E broth (positive control) with serial dilution of 10^{-4} to 10^{-6}. The test was performed in triplicate. Negative control and verification of the inoculum were also performed.

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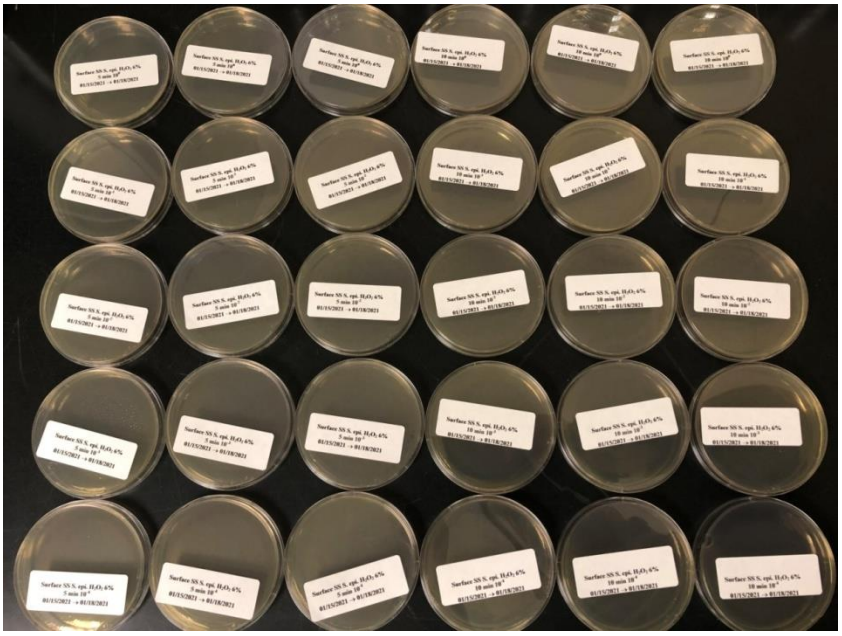
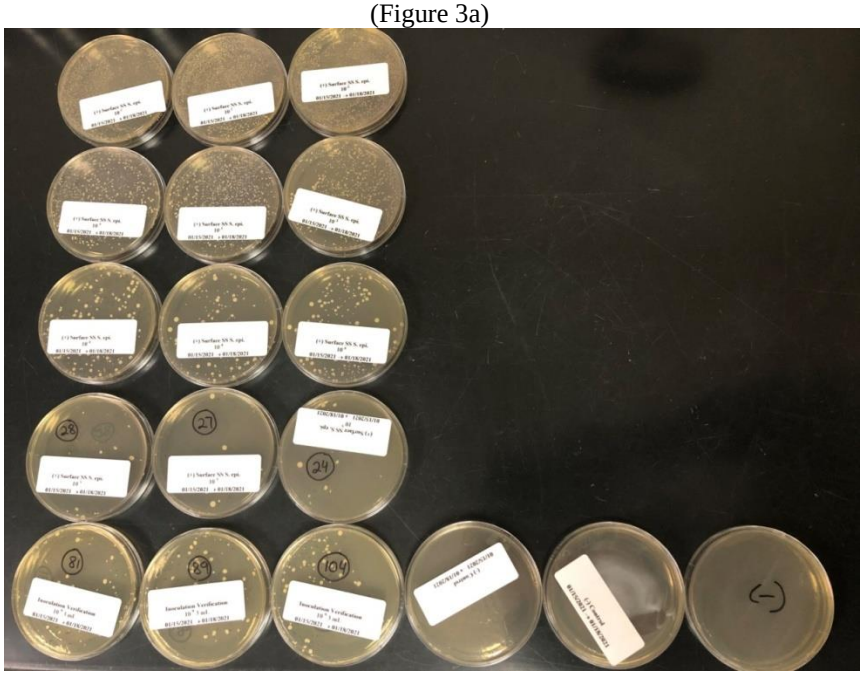


(Figure 3a)



(Figure 3b)

Figure 3. Example pictures to show the surface test. One tenth (0.1) mL of *Staphylococcus epidermidis* suspension at the population of $>10^9$cfu/mL was inoculated onto a 2x2 inch^2 SS coupon which was subject to surface challenge test against H_2O_2 6% upon dried: a) plate count results of the disinfectant at contact times of 5 minutes and 10 minutes with dilution factor from 10^0 to 10^{-4}; b) plate count results of the positive control (e.g. coupon without exposing to H_2O_2 6%) with dilution factor from 10^{-2} to 10^{-5}. The test was performed in triplicate. Negative control and verification of the inoculum were also performed.