#4 Development of Cultivo® Ultra Plus Total Biodecontamination Procedure

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ABSTRACT

Objectives:
1. Biosafety officers typically view biological safety cabinets as the primary engineering control that needs to be assessed, but CO_2 incubators are another point of control that must be considered in any safety protocol.
2. A CO_2 incubator can be a system that invites contamination; therefore, it requires a decontamination protocol that is understood and assessed by biosafety officers, because the protocol may involve the use of potentially hazardous materials.
3. To evaluate the safety and effectiveness of one method of decontamination of a CO_2 incubator by vaporized hydrogen peroxide (H_2O_2).

Method: The effectiveness of an H_2O_2 biodecontamination procedure for a CO_2 incubator was tested with a goal of safely achieving a complete kill of contaminating microorganisms (vs. a log reduction). Chemical indicators (CIs) were used to determine the optimal concentration (6%, 10%, or 15%) of H_2O_2 required for biodecontamination, and biological indicators (BIs) were used to determine whether the procedure at the optimal concentration effectively decontaminated the unit. A heterologous solution in exponential growth phase was applied in the same test areas as the CIs and BIs to test the unit's ability to effectively decontaminate a spill or splatter. A H_2O_2 sensor was also used to measure the concentration of H_2O_2 to determine when the incubator could be safely (per the Permissible Exposure Limit that OSHA requires) opened following the biodecontamination cycle.

Results: At a 15% concentration of H_2O_2, the following results were observed: 1. The CIs showed that the overall presence of H_2O_2 was sufficient to evenly cover the entire interior of the incubator. 2. Following decontamination, the BIs showed no growth after either 24 hours or one week, proving sufficient exposure of the BIs to the H_2O_2 suspended in the system. 3. Swabs of areas contaminated by the heterologous solution showed no growth after 72-84 hours of incubation, showing that the protocol produces a complete kill of a contaminating spill or splatter. 4. OSHA PEL safety requirements were attained at the end of the decontamination cycle.

Conclusion: It was determined that a 15% concentration of H_2O_2 safely and effectively decontaminated the CO_2 incubator using the procedures herein. Outcomes: A safe and effective method for decontaminating a CO_2 incubator with vaporized H_2O_2 was developed, which can be an evaluation tool for a biosafety officer in developing safety protocols for CO_2 incubators.

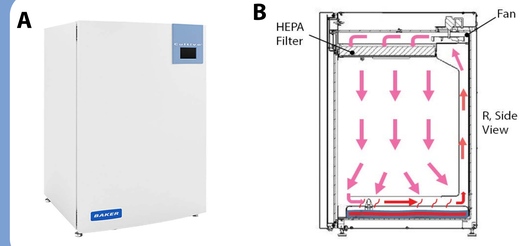

Figure 1:
(**A**) Baker's Cultivo® Ultra Plus incubator. It regulates temperature, CO_2 and relative humidity, with a built in H_2O_2 biodecontamination cycle. (**B**) The airflow pattern unique to Cultivo that provides better-than-ISO Class 4 clean air for cultures.

INTRODUCTION

When decontaminating CO_2 incubators, the effectiveness of a given protocol is often assessed by calculating a log reduction in the number of microorganisms. Typically, a six-log reduction is considered sufficient to have decontaminated the incubator. A log reduction in numbers (even by a six log fold) may not result in a **complete kill** of all contaminants (See **Table 1**). Some microbes may be more resistant than others, posing a more serious risk of re-contaminating cultures in the incubator.

The biodecontamination protocol for Cultivo® Ultra Plus CO_2 incubator **(Figure 1A)** was designed with a vaporized hydrogen peroxide (H_2O_2) system and tested for its ability to achieve a **complete kill** of contaminating microorganisms. Cultivo has a unique HEPA-filtered airflow system that provides better-than-ISO Class 4 clean air to cultures. Coupled with the H_2O_2 biodecontamination procedure, this HEPA system leaves the cultures in an optimal environment.

To discover the optimal concentration of H_2O_2 for total biodecontamination, as well as the H_2O_2 vapor's ability to penetrate all areas of the chamber, chemical indicators (CIs) were placed at various locations inside the chamber and the biodecontamination procedure followed. The CIs, commonly used for clean room assurance, are sensitive to the presence of H_2O_2, containing a chemical that changes color (from purple to yellow) upon exposure to an adequate amount of H_2O_2 that has been determined by the manufacturer (STERIS) to be sufficient for decontamination. The chemical will only react to exposure to H_2O_2, not to temperature or humidity. If all CIs change color completely from purple to yellow, this would indicate a sufficient amount of H_2O_2 for biodecontamination has reached all surfaces of the chamber.

Once an optimal concentration of H_2O_2 was determined, the biodecontamination procedure itself was tested to determine its ability to completely kill spores. Biological indicators (BIs) were placed in triplicate inside the chamber in 17 different locations. To control for the effects of the increased heat required by the cycle, the BIs contained a known amount of *Geobacillus stearothermophilus* spores, which are killed by high levels of H_2O_2 but are not affected by the increased heat, and are known to be challenging to kill. Following exposure to H_2O_2, the BIs were placed in growth media and incubated. The failure of the BIs to produce growth during this incubation would indicate a complete kill of the spores.

Species	Class	Resistance	Prevalence	Brand X Log-fold Kill	Cultivo® Ultra Plus
G. Stearothermophilus	Bacterial Spores	Highest	Low	n/a	Complete Kill
Candida albicans	Fungus	Moderate	Common	6.4	
Mycoplasma orale	Bacteria	High	Very common*	6.2	
Staphylococcus aureus	Bacteria	Low	Common	6.6	

Table 1:
Sample common tissue culture contaminants, the class of microorganism, its known resistance to H_2O_2, prevalence in cultures, the reported log-fold decrease of a popular brand H_2O_2 decontamination incubator, and the Cultivo® kill rate. *Mycoplasma has been estimated to be present in 10-15% of all tissue cultures.

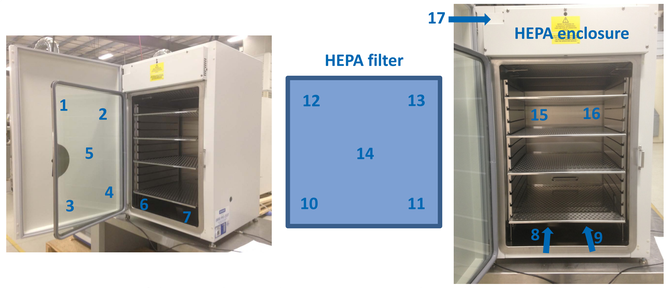

Figure 2: Positions of the CIs and BIs inside Cultivo®. Positons denoted by arrows and numbers. #17 is located outside the incubator, and serves as the negative control. Center is a schematic of the HEPA filter within the HEPA enclosure as shown in right picture.

METHODS

The effectiveness of an H_2O_2 biodecontamination procedure in Cultivo® Ultra Plus CO_2 incubator (**Figure 1A**) was tested using CIs (Steris Life Sciences) and BIs (MesaLabs). A H_2O_2 sensor (Analytical Technologies, Inc.) and sampling tubes (Dräger) were used to determine the maximum concentration of H_2O_2, and when the incubator would be safe to open (per the Permissible Exposure Limit required by OSHA, <1ppm) following the biodecontamination cycle.

CIs and BIs were placed throughout the incubator (**Figure 2**) and warmed 50% H_2O_2 was introduced into the incubator reservoir to achieve a total of 6%, 10% and 15% H_2O_2 in the bath as calculated below in **Equation 1**.

Equation 1: $C_1V_1=C_2V_2$ Reservoir bath = 1.15L

(50%)(V_1)=(6%)(1.15L) V_1= 138 mL for a 6% solution
(50%)(V_1)=(10%)(1.15L) V_1= 230 mL for a 10% solution
(50%)(V_1)=(15%)(1.15L) V_1= 435 mL for a 15% solution

The pre-programmed Cultivo® biodecontamination cycle was then initiated and the H_2O_2 sensor monitored through the various phases:

Setup: H_2O_2 is added to the reservoir and the program is initiated on the control panel.

Warm Up: The internal temperature rises to 45˚C.

Biodecontamination: The nebulizer turns on for 20 minutes to create the H_2O_2 vapor.

H_2O_2 Destruction: The nebulizer stops and UV light turns on to catalyze the breakdown of H_2O_2 into oxygen and water.

Cool Down: The fan switches off to allow suspended H_2O_2 to settle, and the internal temperature is reduced.

The CIs were checked for color change to illustrate an adequate exposure to H_2O_2. The BIs were placed in trypsin soy broth and allowed to grow for up to 7 days at 55˚C. Growth was determined by visual turbidity and microscopically.

The remaining H_2O_2 in the reservoir was pumped out, diluted, and discarded. The reservoir and internal surfaces of the incubator were all wiped clean and the incubator was allowed to refill the reservoir with fresh distilled water. Total procedure time was ~210 minutes (3.5 hours).

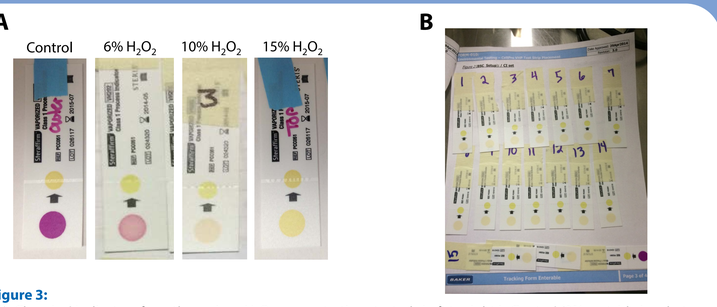

Figure 3:
(**A**) Chemical Indicators from the various H_2O_2 concentrations tested. Left to right is Control (0%, outside incubator door), 6%, 10% and 15% H_2O_2 respectively, exposed for the same amount of time, ~3.5 hours. (**B**) Indicators for each location of the 15% H_2O_2 decontamination cycle. The purple sample is the control.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17 (ctrl)
Rep 1																	
Rep 2																	
Rep 3																	

Figure 4:
Representation of the BI growth results. Orange = no growth, Blue = growth after 7 days of culture. Positions refer to **Figure 2**. The three replicates are shown. The growth in position 12 is negated due to the other 2 replicates having no growth.

RESULTS

CIs were run in triplicate in 16 positions within the incubator and one externally as shown in **Figure 2**. A location was marked positive if two or more of the three exhibited sufficient color change (yellow), meaning they had been exposed to prolonged H_2O_2 in a high enough concentration. Purple, pink and peach were all considered inadequate and deemed negative. As seen in **Figure 3A**, the 6% H_2O_2 exhibited uniform pink color changes, 10% yielded peach colors, while 15% gave sufficient yellow changes. Since 15% H_2O_2 was required for a successful run, as determined by the uniform yellow indicators throughout all location of the incubator (**Figure 3B**), the remaining experiments were done only using that concentration.

The maximum level of H_2O_2 as determined by the H_2O_2 sensor was 255 ± 20 ppm. (**Figure 5**) A sample tracing of the measured H_2O_2 throughout the decontamination cycle is show in **Figure 6**. The levels rose dramatically while the nebulizer was on in the Decontamination phase, then decreased as the UV light turned on in the Destruction phase. Once the fan turned off during the last 20 minutes, H_2O_2 levels dropped drastically until they were below 1 ppm, as determined by sampling tubes to ensure the door could be opened safely.

Triplicate BIs containing *G. stearothermophilus* were placed in the same 17 positions show in **Figure 2**. Again, if two or more of the three were negative for growth, the position was labelled "no growth." No growth was seen in any of the internal positions within the incubator, while the external sample which was not exposed to H_2O_2 did exhibit bacterial growth (**Figure 4**). The Cultivo® biodecontamination protocol provides a ***complete kill*** of all microorganisms, not a mere log-fold reduction (**Table 1**).

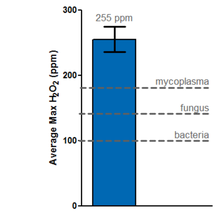

Figure 5:
Average maximum measured H_2O_2 during a 15% biodecontamination cycle by a H_2O_2 sensor. Dotted lines illustrate known levels of H_2O_2 required to kill common tissue culture contaminants.

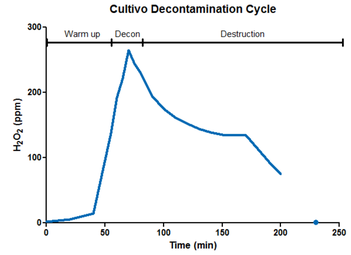

Figure 6:
H_2O_2 sensor measurements of H_2O_2 levels (ppm) during the biodecontamination cycle. Final reading was collected via Dräger sample tube.

CONCLUSIONS

Here we have shown the 15% H_2O_2 biodecontamination cycle of Cultivo® Ultra Plus CO_2 incubator to be extremely effective in achieving:

- A **complete kill** of microorganisms, rather than a log fold reduction as seen in most hydrogen peroxide based systems (**Figure 4**, **Table 1**).
- An internal level of H_2O_2 averaging **250 ppm** in a 45˚C, 90% humid environment, which has been shown to amplify the potency of hydrogen peroxide (**Figure 5**).
- **Complete coverage** of H_2O_2 throughout the entire area, made possible by our unique airflow design (**Figures 2 and 3B**).
- **Safety**. Once the cycle has finished, internal concentrations of H_2O_2 are below OSHA regulated levels, deeming it safe to open the incubator (**Figure 6**).
- **Fast**. The complete biodecontamination cycle takes less than 4 hours.

Using lower concentrations of H_2O_2 (6% and 10%) were shown to be unsuccessful in achieving an internal concentration of hydrogen peroxide vapor high enough to initiate biodecontamination (**Figure 3A**). Using a slightly higher initial concentration yields much stronger results, providing a much cleaner, safer environment for the laboratory user.

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