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Development of Cultivo™ Ultra Plus Total Biodecontamination Procedure

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Abstract

The effectiveness of the H_2O_2 biodecontamination procedure for the Cultivo™ Ultra Plus CO_2 incubator was tested with a goal of achieving a complete kill of contaminating microorganisms (vs. a log reduction). Chemical indicators were used to determine the optimal concentration (6%, 10% or 15%) of H_2O_2 required for biodecontamination, and biological indicators were used to determine whether the procedure at the optimal concentration effectively decontaminated the unit. It was determined that a 15% concentration of H_2O_2 effectively decontaminated the incubator using the procedures herein.

Introduction

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

The biodecontamination protocol for the Cultivo™ Ultra Plus CO_2 incubator was designed and tested for its ability to achieve a complete kill of a wide variety of contaminating microorganisms.

The incubator is configured with a vaporized hydrogen peroxide (H_2O_2) package to aid the researcher in decontaminating the incubator. The package includes a tested, pre-programmed biodecontamination procedure. 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 a biodecontamination procedure followed. The CIs are commonly used for sterility assurance in clean room decontamination and were repurposed for use in this test. The CIs are sensitive to the presence of H_2O_2, containing a chemical that changes color (from purple to yellow) upon exposure to an amount of H_2O_2 that has been determined by the manufacturer (see *Materials and Equipment*) to be sufficient for decontamination. The chemical will only react to exposure to H_2O_2, not the temperature of the environment. 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) (in triplicate) were placed 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 H_2O_2 but not affected by the increased heat. This species was also selected because, as a spore, it is challenging to kill. Following exposure to H_2O_2, the BIs were placed in new media and incubated. The failure of the BIs to produce growth during this incubation would indicate a complete kill of the spores. Because each BI is in triplicate, if at least two tests in a single location are negative for growth, the entire location is considered negative.

Easy-to-follow instructions on the incubator touchscreen guide users through setting up for the initiation of the cycle and show the progress of the various phases that constitute the procedure. A biodecontamination kit is also available from Baker that contains all the materials needed for the procedure, each clearly labeled with a letter. The kit contains sufficient stabilized H_2O_2 to use in the biodecontamination process outlined below. All proper PPE was used to handle this highly concentrated material, and all company and laboratory protocols were followed.

Materials and Equipment

	Materials	Serial / Lot #	Exp. Date	Catalogue #	Supplier
1	Steraffirm VH_2O_2 Process Indicator Chemical Indicator Strip	0243181	31 May 2014	PCC051	STERIS Corporation Mentor, OH
2	Apex Biological Indicator Disc Packets (triplicates)	H0994	31 Jan 2015	HMV-091T	Mesa Laboratories Bozeman, MT
3	Releasat® Culture Media (Count)	PM-146	11 Dec 2014	PM/100	Mesa Laboratories Bozeman, MT
4	Cultivo™ CO_2 Incubator	110961	n/a	INCPILOT	The Baker Company Sanford, ME
5	Dometic Biomedical Refrigerator	1231747	n/a	LR 490 G	The Baker Company Sanford, ME
6	WFI Quality Cell Culture Grade H_2O	25055522	30 Apr 2016	25-055	Corning Cellgro MediaTech, Inc. Manassas, VA
7	Hydrogen Peroxide Biodecontamination Kit	n/a	n/a	TBD	The Baker Company Sanford, ME
	A - Removal Syringe (100 cc)			32-514	Cotran Portsmouth, RI
	B - Waste Container (500 mL)			69031	United States Plastic Corporation, Lima, OH
	C - 2 Amber Bottles			69031	United States Plastic Corporation, Lima, OH
	C - 50% H_2O_2 (inside amber bottles above)			516813-500mL	Sigma Aldrich St. Louis, MO
	D - Evacuation System (Pump)			T10060	TAC Outdoor Products Antioch, TN
	E - Lockout Tape			n/a	The Baker Company Sanford, ME
	F - Thick Disposable Towels			000584-3400P	C. W. Hayden Auburn, ME
	Instructions			n/a	The Baker Company Sanford, ME

Procedure

Chemical Indicator Test

The below process was repeated for 6%, 10% and 15% concentrations of H_2O_2 to determine which was optimal for biodecontamination.

1. Prior to the start of the biodecontamination cycle, the incubator reservoir may contain an unknown volume of water. The process to create the diluted concentration of H_2O_2 within the incubator is the same regardless of initial water volume, because the incubator is programmed to automatically fill the reservoir to a volume of 1.15 L prior to beginning the biodecontamination protocol. To create the diluted concentration of H_2O_2, a volume of water must first be evacuated from the incubator, and then the same volume of 50% H_2O_2 must be added back in. The following formula was used to calculate the volumes of water to evacuate / 50% H_2O_2 to add to create the desired test concentrations:

   $C_1V_1 = C_2V_2$

   $(50\%)(V_1) = (6\%)(1.15\ L)$
   $V_1 = 138$ mL for a 6% solution

   $(50\%)(V_1) = (10\%)(1.15\ L)$
   $V_1 = 230$ mL for a 10% solution

   $(50\%)(V_1) = (15\%)(1.15\ L)$
   $V_1 = 345$ mL for a 15% solution

2. The vaporized H_2O_2 biodecontamination kit was located and checked to ensure that all components were present.
3. All biological instruments and other material that should not be exposed to H_2O_2 were removed from the incubator. All shelves and internal incubator parts remained in the unit and the cable port plug was installed.
4. CIs were placed inside the incubator in various locations.
5. Both the interior and exterior doors of the incubator were closed.
6. The below three-phase process, provided by Baker, was followed.

   a. 1st Phase – Setup

   i. Touch "Service" on the incubator touchscreen.

   ii. Touch "Decon" on the next screen. This will open the vaporized H_2O_2 biodecontamination initiation screen. It also stops the CO_2 input and turns the nebulizer off to allow the steps outlined below.

   iii. Using the syringe (A), evacuate the calculated volume of water from Step 1 from the reservoir and empty it into the waste container (B). Dispose of this water according to company or laboratory protocol.

   iv. Pour the same volume of 50% H_2O_2 to the remaining volume of water in the reservoir to create the concentration required (6%, 10% or 15%). For the 15% solution, the contents of both amber bottles (C) from the biodecontamination kit (345 mL) were poured into the reservoir.

   v. Close the doors and place the lockout tape (E) across the exterior door of the incubator so the warning is visible and the area indicating signature is accessible.

   vi. Follow the prompts on the screen to continue the biodecontamination process.

   b. 2nd Phase – Biodecontamination

   i. The incubator achieves temperature (45°C). This stage takes approximately 40 minutes, but varies depending on the temperature of the incubator at the start of the process.

   ii. The nebulizer engages and runs for approximately 20 minutes to achieve recommended suspension and density of vaporized H_2O_2.

   iii. The nebulizer stops and the UV light is switched on to catalyze the breakdown of the H_2O_2 into oxygen and water. This stage takes approximately 90 minutes.

   iv. The UV light is switched off.

   c. 3rd Phase – Cool Down

   i. The density of H_2O_2 is greatly reduced, but to allow any suspended reactive H_2O_2 to settle, the fan is switched off.

   ii. The temperature of the incubator is reduced slowly for approximately 60 minutes.

   iii. Once the touchscreen prompt indicates that it is time to open the incubator, remove the lockout tape from the door.

7. After following the above process, the door was opened slowly to a gap of approximately 1 inch to allow any remaining H_2O_2 to dissipate from the chamber. After 5 seconds, the door was slowly opened all the way. All damp surfaces potentially contained H_2O_2, so gloves were used and precautions followed for handling H_2O_2.

8. CIs were removed from the incubator and observed for color changes.
9. Thick towels provided in the kit (F) were used to wipe all surfaces.
10. Using the evacuation pump (D), reservoir contents were removed and the reservoir wiped. Because the reservoir held the remaining H_2O_2, the contents were disposed of in a proper manner using company and laboratory procedures. There was no residue to re-clean, as in ClO_2 decontamination.

Biological Indicator Test

The below process was conducted using 15% H_2O_2.

1. The vaporized H_2O_2 biodecontamination kit was located and checked to ensure that all components were present.
2. All biological instruments and other material that should not be exposed to H_2O_2 were removed from the incubator. All shelves and internal incubator parts remained in the unit and the cable port plug was installed.
3. BIs were placed in 16 different locations inside the incubator. One BI was placed outside the incubator to test for any seepage of H_2O_2 from the chamber.
4. Both the interior and exterior doors of the incubator were closed.
5. The below three-phase process, provided by Baker, was followed.
    - **a. 1st Phase – Setup**
        - i. Touch "Service" on the incubator touchscreen.
        - ii. Touch "Decon" on the next screen. This will open the vaporized H_2O_2 biodecontamination initiation screen. It also stops the CO_2 input and turns the nebulizer off to allow the steps outlined below.
        - iii. Using the syringe (A), evacuate exactly 345 mL of water from the reservoir and empty it into the waste container (B). Dispose of this water according to company or laboratory protocol.
        - iv. Pour the contents of both amber bottles (C) from the biodecontamination kit into the reservoir. The addition of 50% H_2O_2 creates a 15% H_2O_2 solution in the reservoir.
        - v. Close both doors and place the lockout tape (E) across the exterior door of the incubator so the warning is visible and the area indicating signature is accessible.
        - vi. Follow the prompts on the screen to continue the biodecontamination process.
    - **b. 2nd Phase – Biodecontamination**
        - i. The incubator achieves temperature (45°C). This stage takes approximately 40 minutes, but varies depending on the temperature of the incubator at the start of the process.
        - ii. The nebulizer engages and runs for approximately 20 minutes to achieve recommended suspension and density of vaporized H_2O_2.
        - iii. The nebulizer stops and the UV light is switched on to catalyze the breakdown of the H_2O_2 into oxygen and water. This stage takes approximately 90 minutes.
        - iv. The UV light is switched off.
    - **c. 3rd Phase – Cool Down**
        - i. The density of the 15% H_2O_2 is greatly reduced, but to allow any suspended reactive H_2O_2 to settle, the fan is switched off.
        - ii. The temperature of the incubator is reduced slowly for approximately 60 minutes.
        - iii. Once the touchscreen prompt indicates that it is time to open the incubator, remove the lockout tape from the door.
6. After following the above process, the door was opened slowly to a gap of approximately 1 inch to allow any remaining H_2O_2 to dissipate from the chamber. After 5 seconds, the door was slowly opened all the way. All damp surfaces potentially contained H_2O_2, so gloves were used and precautions followed for handling H_2O_2.
7. BIs were removed from the incubator and the enclosed coupons were placed in tubes containing media with a sensitive pH indicator and allowed to incubate in a 57°C incubator for a minimum of 24 hours. Following incubation, the media was observed for turbidity and the pH indicator was observed for color changes.
8. Thick towels provided in the kit were used to wipe all surfaces.
9. Using the evacuation pump (D), reservoir contents were removed and the reservoir wiped. Because the reservoir held the remaining H_2O_2, the contents were disposed of in a proper manner using company and laboratory procedures. There was no residue to re-clean, as in ClO_2 decontamination.

Results

Chemical Indicators

Figure 1 shows representative color changes from the CIs. At 6% concentration of H_2O_2, the color of the CIs was light purple, and there was some variation in the color changes. At 10%, there was less color variation, and the CIs turned a pale peach color. At 15%, the color of the CIs changed to yellow and there was no color variation.

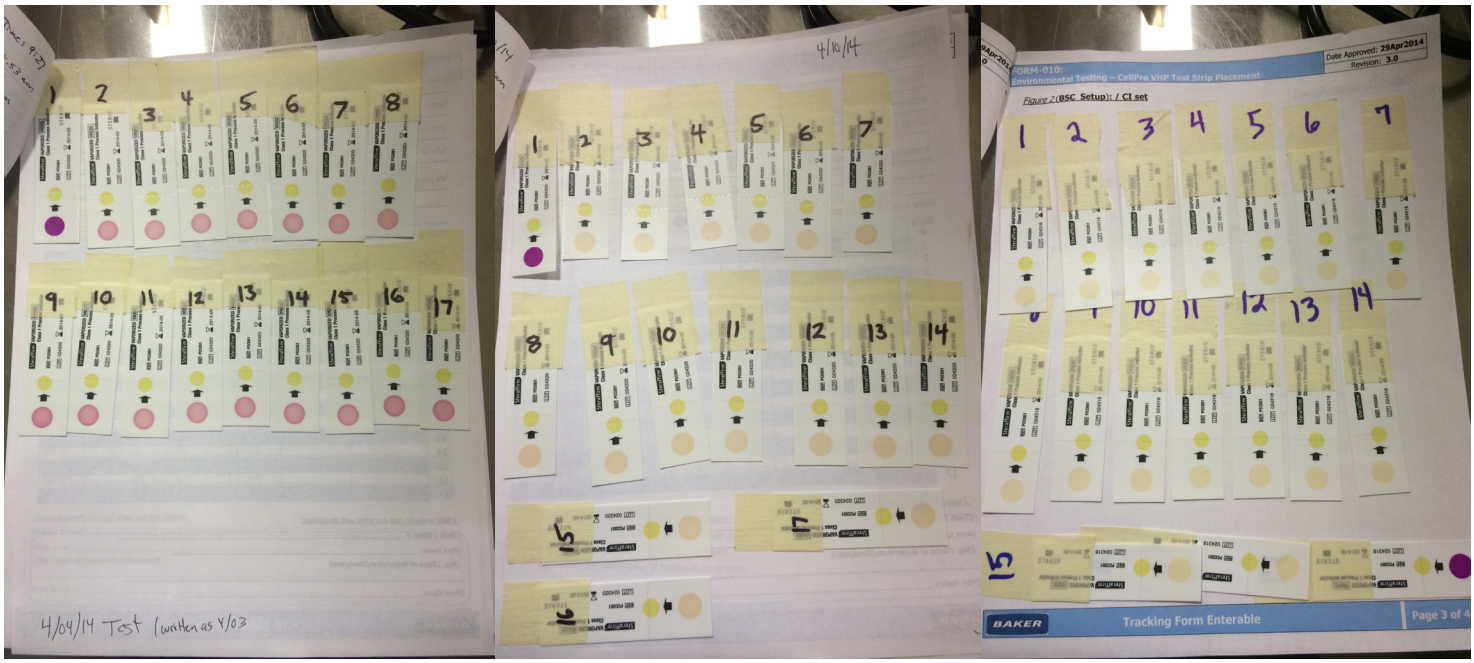

Figure 1: Chemical Indicators Color Changes. *From left to right, the results at 6%, 10% and 15% H_2O_2 concentrations. A concentration of 15% produces sufficient and consistent exposure to H_2O_2 throughout the chamber.*

Biological Indicators

Figure 2 on the next page shows sample results from one test of the biological indicators. Five such tests were conducted, with 16 triplicate BIs placed in different locations within the incubator interior. Triplicate tests are commonly accepted as a method to control for unknown variables in scientific experiments. If two tests in a triplicate produce a positive result and one is negative, the accepted interpretation of the entire triplicate test is that it is positive. This is because the one negative result out of the three may be explained by an unknown variable. However, if two out of three tests are negative, it is more likely that the tested variable is responsible for the negative results, and the entire test is considered negative. In other words, for any given triplicate test:

if A=positive, and B=positive, and C=positive, then the entire triplicate test is positive.
if A=positive, and B=positive, and C=negative, then the entire triplicate test is positive.
if A=positive, and B=negative, and C=negative, then the t entire riplicate test is negative.
If A=negative, and B=negative, and C=negative, then the entire triplicate test is negative.

Location 12 in Figure 2 on the next page is one example of a triplicate test that produced two negative growth results and one positive, with the result that the entire test for Location 12 is negative for growth.

A total of 80 triplicate BIs were used across the five biodecontamination tests. (This number excludes the BIs that were placed in Location 17 outside the incubator as a control). Of those 80 triplicate BIs, two produced positive results for their locations, meaning that 97.5% of the triplicate BIs were negative for growth after exposure to the biodecontamination protocol.

Location #	Result A / B / C	Comments
1	NG / NG / NG	
2	NG / NG / NG	
3	NG / NG / NG	
4	NG / NG / NG	
5	NG / NG / NG	
6	NG / NG / NG	
7	NG / NG / NG	
8	NG / NG / NG	
9	NG / NG / NG	
10	NG / NG / NG	
11	NG / NG / NG	
12	G / NG / NG	12A growth in tube does not match 12B and 12C; therefore, Location 12 is considered negative for growth.
13	NG / NG / NG	
14	NG / NG / NG	
15	NG / NG / NG	
16	NG / NG / NG	
17	G / G / G	The appearance of growth in these tubes is expected due to the BI's placement outside the incubator.

Figure 2: Sample Results From Biological Indicators Testing.
NG = No growth observed G = Growth observed.
Location 12 is considered negative for growth because only one of the three tests in that location produced growth. See Results section for an explanation of the use of triplicate tests.

Conclusion

The optimal vaporized H_2O_2 biodecontamination protocol for the Cultivo™ Ultra Plus CO_2 incubator uses a 15% concentration of H_2O_2 and achieves a total biodecontamination of the unit to completely kill a wide variety of microorganisms, including spores. Color changes in CIs exposed to 15% H_2O_2 indicated consistent exposure throughout the chamber interior at sufficient levels, according to the manufacturer of the CIs. The failure of the CIs to change color to yellow when exposed to concentrations of 6% and 10% H_2O_2 indicated that those levels were not strong enough for decontamination. In fact, proof of concept testing showed that BIs that had been exposed to the biodecontamination protocol at those lower concentrations of H_2O_2 produced growth after only one day of incubation. Therefore, concentrations of 6% and 10% H_2O_2 were not used in the Biological Indicator Test.

The lack of growth from the BIs that were incubated after exposure to the 15% H_2O_2 biodecontamination process indicated that 97.5% of the time, the protocol achieves a complete kill of *Geobacillus stearothermophilus* spores, rather than a log reduction. Although the results of decontamination are conventionally expressed as a log reduction, merely reducing the numbers of contaminants poses the risk of re-contamination of the incubator. The Cultivo Ultra Plus biodecontamination protocol produces a complete kill of a wide variety of contaminating microorganisms, beyond that which can be expressed as a log reduction. Following the protocol outlined herein, the use of 15% H_2O_2 should not result in contaminant regrowth.

To learn more about Cultivo™, the CO_2 incubator from Baker, please visit bakerco.com/products/cultivo

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