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Development of an Onboard Hydrogen Peroxide Vapor Biodecontamination Procedure for the ReCO$_2$ver™ Plus CO$_2$ Incubator

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ABSTRACT

Incubators are standard laboratory equipment but are constantly prone to internal contamination by bacteria, water molds, fungi and mycoplasmas, and require a simple yet effective method for decontamination. Heating to high temperatures is a common method, however this takes 12+ hours to complete. Here we develop an onboard biodecontamination using hydrogen peroxide that will achieve a greater than 6-log reduction (>10^6-fold decrease) as measured by biological indicators in under 4 hours.

INTRODUCTION

Standard equipment in almost all biological laboratories will include an incubator to provide the proper growing temperature for the desired biological entities being studied. Often, these are warm, humid and gas controlled boxes. As they are designed to provide the ideal growing environment for cultures, they will subsequently also grow undesired contaminants such as water molds, fungi, bacteria, and mycoplasmas. Common practice is to periodically biodecontaminate the incubator. This can be achieved through a few different methods. The first and simplest method is a surface decontamination where the shelves and walls are wiped with a disinfectant. This will remove and kill the contaminants from the surfaces, but will not get all hidden areas, plenums or unexposed areas of the incubator. This is also only as effective as the user's wiping skills.

The second-most common method would be high heat, where all cultures are removed from the incubator and the temperature is increased to 90+℃ to effectively autoclave the interior. This will kill off anything within the heated area of the incubator, but this method often takes a long time, and is usually done overnight. The cultures will have to find another home in the meantime and can cause logistic issues.

A third method is using a gaseous based disinfectant, mainly hydrogen peroxide vapor. This method will allow for the disinfecting agent to penetrate all areas of airflow within the incubator including plenums, the fan, and the HEPA filter ensuring all areas of the incubator are effectively decontaminated. This procedure will often take only a few hours, which allows laboratories to get back to standard work faster. This is the method to be tested in the $ReCO_2ver$™ Plus Rapid Recovery CO_2 incubator.

In order to test the effectiveness of the biodecontamination procedure, chemical indicators (CIs) are used to determine presence of H_2O_2 via a chemical color change reaction and biological indicators (BIs) are used to quantify the reduction in biological load represented in a logarithmic scale. The gold standard for an effective decontamination will achieve a 6 log reduction, whereby the biological sample will be reduced in quantity by 10^6-fold. For example, a sample containing 2,000,000 bacterial spores that is reduced to 2 spores after decontamination will have a 6-log reduction. Since some BIs will die in high heat environments, the Geobacillus stearothermophius spores are used for H_2O_2 as these are resistant to heat and is challenging to kill. Using these tools, a quick and effective protocol for providing a biodecontamination of the $ReCO_2ver$™ Plus Rapid Recovery Incubator can be developed.

MATERIALS AND EQUIPMENT

	MATERIALS	CATALOGUE #	SUPPLIER
1	$ReCO_2ver$™ Plus CO_2 Incubator	REC-602 Plus	The Baker Company Sanford, ME
2	Steraffirm Vaporized VH2O2 Type 1 Process Indicator Chemical Indicator	PCC051	STERIS Corporation Mentor, OH
3	Apex Biological Indicator Discs	HMV-091	Mesa Laboratories Bozeman, MT
4	Releasat® Culture Media	PM/100	Mesa Laboratories Bozeman, MT
5	Hydrogen Peroxide, Humidity and Temperature probe	HPP272	Vaisala Inc. Woburn, MA
6	Hydrogen Peroxide Biodecontamination Kit	673A700	The Baker Company Sanford, ME
	a. Removal Syringe	32-514	Cotran Portsmouth, RI
	b. Hand pump	T10060	TAC Outdoor Products Antioch, TN
	c. Lock out tape Warning label	45317	The Baker Company Sanford, ME
	d. 2 Amber bottles of 50% H_2O_2	516813-500mL	Sigma Aldrich St. Louis, MO

Biodecontamination Cycle

The $ReCO_2ver$™ Plus CO_2 incubator onboard biodecontamination cycle goes through several phases: Phase 1 – Setup, Phase 2 – Biodecontamination, and Phase 3 – Cool Down as shown in **Figure 1**.

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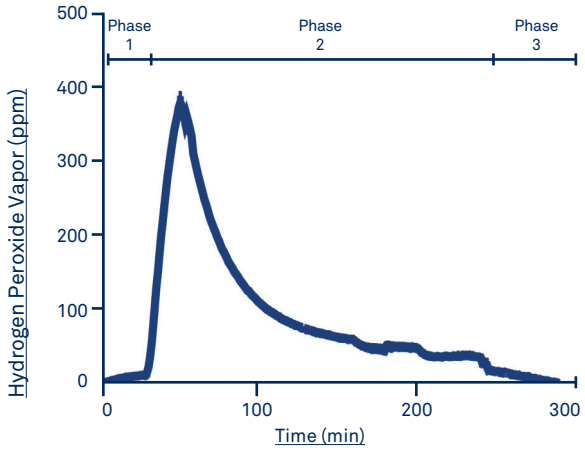
FIGURE 1.

Average hydrogen peroxide concentration (ppm) over time throughout the biodecontamination protocol highlighting the different phases: Phase 1 - Setup, Phase 2 - Biodecontamination, and Phase 3 - Cool Down.



Phase 1 – Setup is user driven, whereby the user will need to enter the Service wrench icon on the Home screen, then the Decon program button, and follow all instructions on screen. This includes removing all cultures from the incubator, removing some water from the internal water reservoir with the provided removal syringe, adding 50% H_2O_2 to the internal water reservoir, mixing the solution with the removal syringe, and sealing the door with the Lock out tape warning label.

Phase 2 – Biodecontamination is completely controlled by the $ReCO_2ver$™ Plus. The internal temperature will increase to 45˚C. The nebulizer will engage for 20 minutes, creating an aerosol of hydrogen peroxide. The internal fan will circulate the H_2O_2 throughout the entire incubator chamber. The nebulizer will stop, and the fan will disperse the H_2O_2 vapor evenly for 30 minutes. Then, the UV light will switch on to start the catalyzation of H_2O_2 to oxygen and water.

Phase 3 – Cool Down returns the $ReCO_2ver$™ back to operation. The UV light and fan turn off, allowing the remaining H_2O_2 vapor to settle on surfaces and finish catalyzing to oxygen and water. The heater turns off to return to the original setpoint temperature. The touchscreen prompts the user to remove the lock out warning label and remove the remaining H_2O_2 from the internal reservoir. This can be achieved by using the provided hand pump or a vacuum pump and using the easy access drain port on the front righthand side of the $ReCO_2ver$™. Once acknowledging the removal of the remaining H_2O_2, the $ReCO_2ver$™ will enter back into operation and the water pump will automatically refill the internal water reservoir. After achieving setpoint, the user may begin regular usage again. Always use caution when opening the inner glass door after a biodecontamination. Gloves should always be worn to prevent coming in contact with damp surfaces in case there is residual H_2O_2 present.

This procedure is outlined in detail in the Vaporized Hydrogen Peroxide Biodecontamination Procedure provided with your Biodecontamination Kit (1).

Chemical Indicators (CIs)

Chemical indicators were placed in all trial locations throughout the $ReCO_2ver$™ incubator to determine if hydrogen peroxide vapor was reaching all areas in the internal chamber, including the HEPA filter. The CIs were placed with the purple chemical reaction dot facing the center of the chamber or face up on the HEPA filter (facing upstream). After completion of the bio-decontamination cycle, the CIs were harvested, and the color change observed. Purple dictated insufficient contact with H_2O_2, pink or peach indicated some contact with H_2O_2, and yellow indicated a sufficient amount of contact with H_2O_2.

Biological Indicators (BIs)

The Geobacillus stearothermophius BIs contained spores on steel discs inside a Tyvek pouch. These BIs were placed in triplicate in all trial locations throughout the $ReCO_2ver$™ incubator. The BIs were placed with the printed side of the Tyvek pouch facing outward, or downstream of the airflow. After BIs have been exposed to H_2O_2, they were then placed into growth medium and incubated at 55˚C. The presence (positive, turbid yellow media color change) or absence (negative, clear purple media) of bacterial growth is then determined after 48 hours. The most common way to show effectiveness is to determine the log fold reduction in biological load. A 1-log fold reduction would mean that if there were 10 bacterial spores to begin with and 1 remained after the decon. A 6-log reduction would be 1 remaining spore from an initial 1,000,000 (or 10^6). The log fold reduction in biological load can be calculated by the following equation as previously described (2):

Equation 1:

$SLR = Log_{10} N_0 – Log_{10} (ln\ n/r)$

Where SLR = spore log reduction, N_0 = the initial spore concentration, n = number of replicates at each location, and r = the number of growth negative BIs at each location.

For example, a BI with 1.6×10^6 spores, in triplicate, with 2 negative samples and 1 positive, would have a 6.6-log reduction. This method was used to calculate the log reduction for each location in the $ReCO_2ver$™.

Measuring the concentration of hydrogen peroxide vapor

The internal concentration of hydrogen peroxide vapor that is created throughout the biodecontamination cycle is critical to ensure sufficient levels have been achieved and can be monitored through the use of the H_2O_2 probe (Vaisala). This probe will log the data over time, as well as read live data, as measured in parts per million (ppm). The probe was placed in the center of the incubator chamber on a ring stand and logged on an external computer.

Calculating final H_2O_2 concentration

The final working concentration of hydrogen peroxide was determined by calculating the required amount of 50% H_2O_2 to be added to the internal reservoir of water which holds 600mL. The basic volume/concentration equation (Equation 2) can be used.

Equation 2: $C_1V_1 = C_2V_2$

Therefore, the following amounts were required to reach the 6, 10, and 15% final concentrations of H_2O_2 **(Table 1)**.

FINAL CONCENTRATION	AMOUNT OF 50% H_2O_2 TO BE ADDED
6%	72 mL
10%	120 mL
15%	180 mL

Table 1: Volumes of 50% H_2O_2 to be added to the internal water reservoir for desired final decontamination concentrations.

Determining the effective concentration of hydrogen peroxide

Separate biodecontamination cycles were run in triplicate using 6, 10 and 15% final concentration H_2O_2 in the internal water reservoir, complete with CIs, BIs, and the H_2O_2 probe. As shown in **Figure 2a**, there was a drastic increase in the internal concentration of H_2O_2 vapor with 15% H_2O_2. All three concentrations produced enough H_2O_2 vapor to turn all of the CIs (data not shown), however, only the 15% H_2O_2 was sufficient to kill the BIs (**Figure 2b**). It was then determined to use 15% H_2O_2 for all future studies.

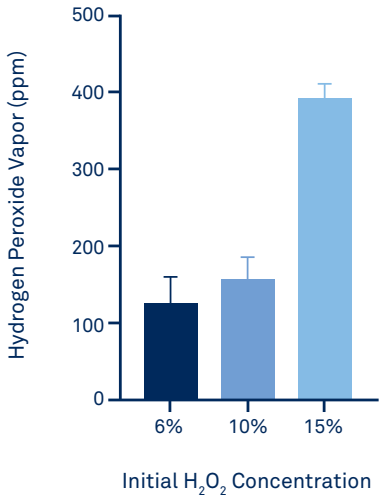
FIGURE 2.

Determining the effective dose of H_2O_2 for successful biodecontamination.

2a. Peak hydrogen peroxide concentration shown for 6%, 10%, and 15% H_2O_2 (ppm).

2b. Triplicate biological indicator results. Negative means no spore growth and a good kill, positive means spore growth and a poor kill.

2a



2b

H_2O_2	BIOLOGICAL INDICATOR GROWTH		
6%	+	+	+
10%	+	+	+
15%	-	-	-

Full internal coverage of H_2O_2 exposure

In order to assure that the H_2O_2 vapor will reach all areas within the incubator chamber, HEPA filter, plenums and fan, CIs and triplicate BIs were placed throughout the incubator as shown in **Figure 3**. Locations included: 1) Control at the USB port, 2) center of the upstream side of the HEPA filter, 3) back corner of the upstream side of the HEPA filter near the fan, 4) inside center of the inner glass door, 5) center of the center shelf, 6) center of the left side wall.

All of these locations were chosen as difficult and critical to decontaminate areas. The results are shown in **Table 2**. All sample location CIs demonstrated positivity for H_2O_2 exposure. Most of the sample locations were negative for spore growth with the BIs. A few locations had a single BI exhibit growth out of the triplicate. Using **Equation 1**, the overall Log Reduction can be calculated, which are shown the final column of **Table 2**. All locations showed a better than 6-log reduction in biological load.

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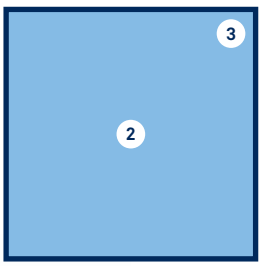
FIGURE 3.

Biological and Chemical Indicator placement within the ReCO$_2$ver™.

1. Control, outside chamber on USB port
2. Center of the HEPA filter
3. Back right corner of HEPA filter
4. Inside inner glass door
5. Center shelf
6. Left side wall.



HEPA filter

LOCATION	CHEMICAL INDICATOR	BIOLOGICAL INDICATOR GROWTH			LOG REDUCTION
1 - Ctrl	-	+	+	+	0
2	+	-	-	-	100%
3	+	-	-	-	100%
4	+	-	+	-	6.96
5	+	-	-	+	6.75
6	+	-	-	+	6.75

Table 2: Chemical and Biological Indicator data based on locations described in Figure 3. Positive CIs indicated exposure to sufficient hydrogen peroxide. Negative BIs indicated no growth and sufficient kill. Log reduction was calculated according to Reference 2.

H_2O_2 effectiveness

In **Figure 1**, the overall trace of H_2O_2 vapor concentration is shown. There is a sharp increase in concentration and a gradual decrease, and then finally returning to 0 ppm where it is safe to open the incubator door and begin work again. This peak of H_2O_2 vapor concentration is critical to ensure that all intended contaminating microorganisms are killed. As shown in **Figure 4**, the $ReCO_2ver$™ more than satisfies the concentrations required to kill bacteria, fungus and mycoplasma.

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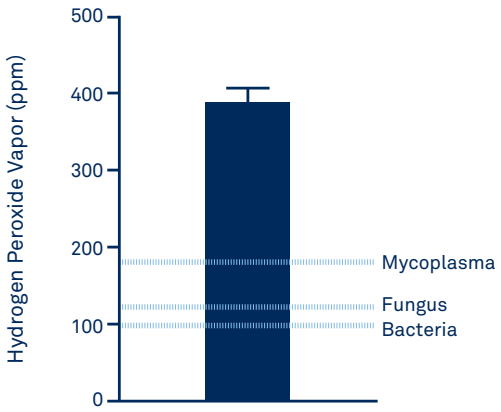
FIGURE 4.

Average peak hydrogen peroxide concentration (ppm) shown with required concentrations to kill bacteria, fungus and mycoplasma.



CONCLUSIONS

The $ReCO_2ver$™ Plus onboard hydrogen peroxide biodecontamination protocol has been shown to effectively kill industry standard biological indicators at greater than 6-log reductions, and often a full kill of those bacterial spores. By monitoring the internal H_2O_2 vapor concentration, it is also possible to show that this is an effective biodecontamination protocol for common contaminants seen in the laboratory, such as bacteria, fungus and mycoplasma. It was also shown that 15% H_2O_2 is required in the internal water reservoir to have a sufficient decontamination.

REFERENCES

1. Vaporized Hydrogen Peroxide Biodecontamination Procedure, Baker, 2021.
2. Krushefski, G. “Using replicate BIs to evaluate biodecontamination cycles in isolators” SporeNews biological indicators newsletter, Volume 9, No. 4.