BioPhorum

ISOLATOR GOOD OPERATING PRACTICE

Industry guidance for vapor phase hydrogen peroxide cycle development

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About BioPhorum

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Since its inception in 2004, BioPhorum has become the open and trusted environment where senior leaders of the biopharmaceutical industry come together to openly share and discuss the emerging trends and challenges facing their industry.

Growing from an end-user group in 2008, BioPhorum's membership now comprises top leaders and subject matter experts from global biopharmaceutical manufacturers and suppliers, working in both long-established and new Phorums. They articulate the industry's technology roadmap, define the supply partner practices of the future, and develop and adopt best practices in drug substance, fill finish, process development and manufacturing IT.

In each of these Phorums, BioPhorum facilitators bring leaders together to create future visions, mobilize teams of experts on the opportunities, create partnerships that enable change and provide the quickest route to implementation, so that the industry shares, learns and builds the best solutions together.

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Abstract

Vapor phase hydrogen peroxide (VPHP) has been in use for decades as a surface decontaminant in pharmaceutical applications, particularly in filling isolators, sterility test isolators, pass-through chambers and room-fogging applications. However, there is surprisingly little industry guidance on what should be evaluated during the process of establishing successful bio-decontamination parameters – a process described as cycle development. This gap was recognized by the BioPhorum Isolator Good Operating Practices Team and included as a focused workstream to promote a best practice approach across the pharmaceutical industry.

This paper outlines the BioPhorum Isolator Good Operating Practices Team's recommendations for VPHP cycle development. It includes the applications of various new technologies to support this activity, as well as a brief description of activities leading up to and following cycle development.

Any reference to specific technologies or brands in this paper does not constitute an endorsement or recommendation by BioPhorum. Any references to equipment or vendors reflect the original input from authors and are included solely for reflection on current common practices. BioPhorum does not promote, favor or discourage the use of any particular supplier or technology.

1.0

Introduction

The BioPhorum Isolator Good Operating Practices Team is an industry working group whose aim is to share best practices for the operation of isolators and develop common solutions to issues that impact isolator operations. The team has worked together to recommend a set of parameters to follow when developing VPHP bio-decontamination cycles.

The objectives of this work are:

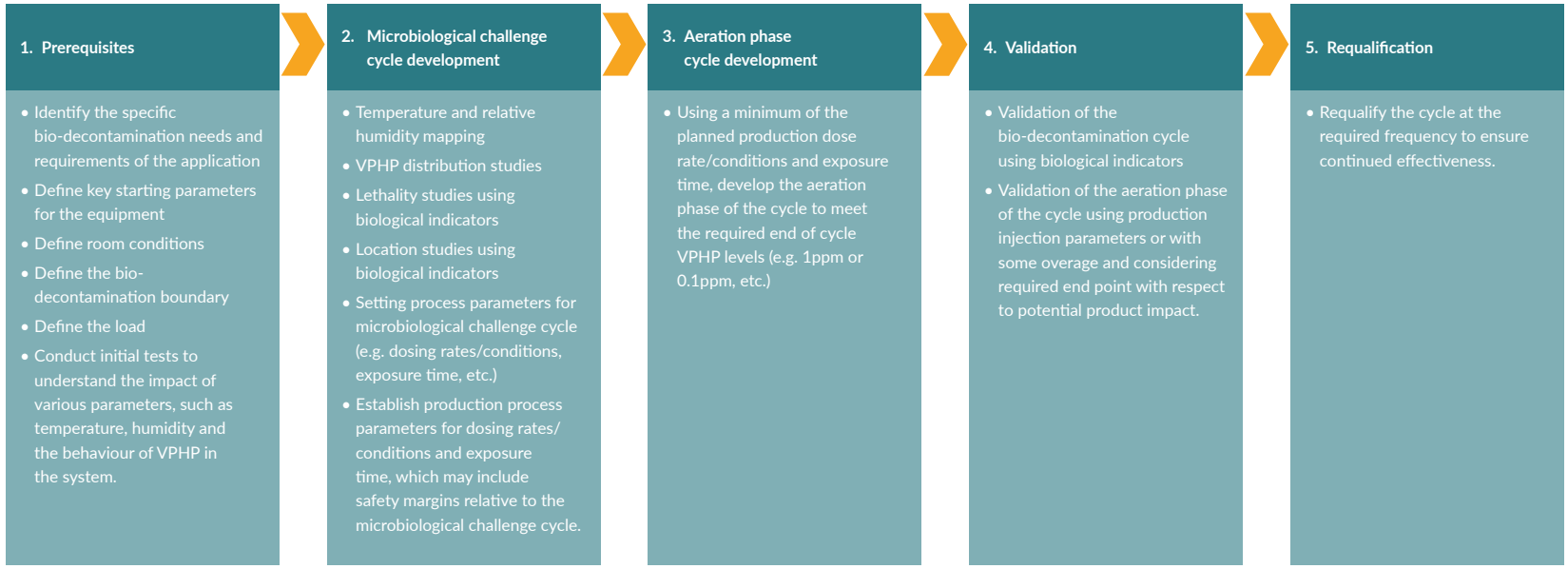
- To increase the robustness and reliability of VPHP cycles through a better understanding of the key steps
- To recommend industry-standardized operational practices appropriate to the bio-decontamination technology being used
- To increase the efficiency of validation processes for VPHP cycles and to assist with training new staff.

Where an isolator is used to achieve segregation of an aseptic process, the isolator consists of a main chamber that encloses the equipment where the aseptic process will take place. The VPHP process decontaminates the isolator chamber, materials and equipment inside the chamber. Similar bio-decontamination processes are required for sterility test isolators and any pass-through chambers, and the processes described in this document should be applied to these (room-fogging applications are out of scope of this paper).

The paper outlines how cycle development fits into the validation lifecycle, what constitutes a standard process for cycle development for all VPHP bio-decontamination processes and how the use of new technologies (e.g. enzyme indicators) could potentially increase the efficiency and robustness of the cycle development process. The information is provided as a summary that covers the important points in each section, with some including an accompanying list of suggested resources to provide more detailed information.

This guidance was developed through engagement with a number of end-users from multiple companies and several equipment manufacturers, and provides a shared understanding of the current processes for VPHP cycle development.

Figure 1: Standard bio-decontamination cycle process flow



2.0

The technologies used for VPHP bio-decontamination

In commercial applications, bio-decontamination using hydrogen peroxide (H_2O_2) is achieved using one of the following techniques: vaporized H_2O_2 (hot-plate vaporization), aerosolized H_2O_2 (fogging) and ionized H_2O_2 (passing H_2O_2 through an ionized plasma field). In this document, we focus on the first two techniques as these are the processes currently used by BioPhorum member companies.

Vaporized H_2O_2 (hot-plate method)

This uses heat to transform injected, liquid aqueous H_2O_2 solution into vapor, which is then distributed in the chamber to be decontaminated.

Mechanism: A metering system within the isolator pumps a specific amount of H_2O_2 liquid on to vaporizing plates located in the isolator at a temperature high enough to result in flash evaporation. Once heated, the resulting vapor (VPHP) is distributed into the chamber by a carrier gas (which may be preheated), such as filtered compressed process air or HEPA filtered air, and reaches surfaces for contact bio-decontamination. The heated vapor can be introduced directly to the isolator environment or through a HEPA filter.

Wet process: The intent is to minimally condense the vaporized H_2O_2 on the surface to deliver 'kill', primarily in the liquid phase.

Dry process: The intent is to avoid condensation of the H_2O_2 on surfaces and effect 'kill' in the gas phase.

Combination process: Recognizing that condensation is unavoidable, the object is to keep the chamber well mixed so that the phases in contact with surfaces are as uniform as possible.

Aerosolized H_2O_2 (fogging)

This is based on the generation of H_2O_2 microdroplets with a large surface area, which facilitates vaporization without the need for additional heating. Droplets are usually generated using nozzles or nebulizers. Depending on the generated droplet size, fog is termed 'dry' or 'wet' by industry users.

Wet fog: These are aerosols with droplet sizes larger than approximately 15–20µm.

Mechanism: These particles settle and attach more easily to surfaces, and bio-decontamination is achieved using a combination of vapor-liquid deposited H_2O_2.

Dry fog: These are aerosols with droplet sizes below 15µm.

Mechanism: Smaller droplets have minimal sedimentation, evaporate quickly and tend to bounce off without leaving liquid deposits, and hence bio-decontamination is achieved by adsorption of VPHP on surfaces. The aerosolized vapor is either directly injected into the chamber or injected into the air stream upstream of the HEPA filter.

Supporting information

Detailed information on the mechanism of action for VPHP and the technology used is available in the documents listed below:

Hultman, C., Hill, A., and McDonnell, G. *The Physical Chemistry of Decontamination with Gaseous Hydrogen Peroxide.* Pharmaceutical Engineering. January/February 2007. Vol. 27 No. 1. **https://ispe.org/pharmaceutical-engineering/january-february-2007**

Imai, K., et al. *A New Approach to Vapor Hydrogen Peroxide Decontamination of Isolators and Cleanrooms.* Pharmaceutical Engineering. May/June 2006. **https://static1.squarespace.com/static/52d6d893e4b0edcb252bf2af/t/52deedf3e4b0322091776349/1390341619677/New+Approach+to+VHP+Decon+of+Isolators+and+Cleanrooms.pdf**

Selecting Decontamination Technology: Aerosolized versus Vaporized Hydrogen Peroxide. Steris Life Sciences. [Accessed 25 June 2024]. **Selecting Decontamination Technology: Aerosolized versus Vaporized Hydrogen Peroxide Steris White Paper**

Parenteral Drug Association. *Points to Consider for the Aseptic Processing of Sterile Pharmaceutical Product in Isolators.* June 2020. **www.pda.org/bookstore/product-detail/5699-points-to-consider-isolators**

3.0

The role of VPHP as a sterilant

Hydrogen peroxide can be used as a sterilant under specific operational circumstances. As with other gaseous sterilization processes, using VPHP as a sterilant requires a vacuum. ISO 22441[1] and ISO 11138[2] describe in detail the requirements for VPHP sterilization processes, and it is evident that within the typical operating settings for a commercial isolator, commercial restricted access barrier system (RABS) or cleanroom, VPHP processes will not meet the acceptance criteria required for sterilization.

The scope of this document is to support the use of VPHP in bio-decontamination processes used in commercial isolators only. Sterilization applications are outside the scope and should follow recommendations in ISO 22441[1] and ISO 11138[2].

Supporting information

The resources below contain further information on the role of VPHP:

1 International Organization for Standardization. *ISO 22441:2022 Sterilization of health care products — Low temperature vaporized hydrogen peroxide — Requirements for the development, validation and routine control of a sterilization process for medical devices.* Edition 1, 2022. **www.iso.org/standard/73214.html**

2 International Organization for Standardization. ISO 11138-1:2017, "Sterilization of health care products— Sterilization of health care products—**Biological indicators**" Part 1: General requirement. **https://www.iso.org/standard/66442.html**

Defining VHP Sterilization and Biodecontamination – Common Denominators and Differences. American Pharmaceutical Review. 1 December 2024. **https://www.americanpharmaceuticalreview.com/Featured-Articles/616787-Defining-VHP-Sterilization-and-Biodecontamination-Common-Denominators-and-Differences/**

4.0

The VPHP bio-decontamination process in commercial isolators

A VPHP bio-decontamination cycle has multiple phases and, while the names of the cycle phases differ across equipment vendors, the following terms are commonly used.

Leak test

This is performed periodically for most commercial systems. It will be done for every cycle to ensure that the environment within the isolator is safely enclosed and poses no risk to either operator safety or isolator aseptic conditions from leaking H_2O_2. The leak test must pass within acceptable limits, and the methods and frequency will vary based on company policy, application, etc. The calculation described by Coles[1] is based on the volume of the isolator and the surrounding room, and can be used to determine an acceptable leak rate.

Pre-conditioning/conditioning

The main purpose of this phase is to ensure consistent starting conditions for the cycle. The environmental conditions in the chamber are stabilized before temperature- and humidity-controlled air is supplied to the entire enclosure space and any load inside the enclosure. This step may involve dehumidification to reduce water/moisture particles in the environment, allowing for a higher relative saturation with H_2O_2 particles through the injection and bio-decontamination phases.

Injection and bio-decontamination

This is where H_2O_2 is supplied into the enclosure at a controlled rate to maintain a concentration of VPHP for a determined amount of time, and assure the correct time-dosing effect for surface bio-decontamination.

One method is to start with a high H_2O_2 injection rate and decrease to maintain a plateau. Others use different approaches to increase injection rates as the chamber warms up during the injection time to saturate the environment over time (i.e. multiple injection rate changes with increasing rates over time). Pulsed injection and other methods are also possible. Regardless, the minimum H_2O_2 level is set to achieve the assurance level, or spore log reduction, specified in the user's contamination control strategy.

Aeration

This is when residual H_2O_2 is removed from the enclosure to ensure safe levels are attained for personnel if the enclosure is opened, and for products if needed. Safety levels differ from country to country, but below 1.0ppm or 0.5ppm are typically required for personnel safety. For a system where oxidation-sensitive products are filled, aeration to lower parts per billion (PPB) levels may be required.

Supporting information

1 Coles, T. *Leak rate measurement for pharmaceutical isolators.* Clean Air and Containment Review, July 2012. Issue 11, p 8-12. **CACR11_Final.pdf**

2 International Organization for Standardization. *ISO14644-7:2004 – Cleanrooms and associated controlled environments.* 2004. **https://www.iso.org/standard/38264.html**

3 US Food and Drug Administration. *Toxic and hazardous substances.* May 2019. eCFR :: 29 CFR Part 1910 Subpart Z—Toxic and Hazardous Substances **https://www.ecfr.gov/current/title-29/subtitle-B/chapter-XVII/part-1910/subpart-Z**

5.0

Factors to consider during the design and qualification (IQ/OQ) stages to deliver successful bio-decontamination

Design

The definition of the key parameters for new equipment begins at the design stage. It is important at this point to understand the overarching quality assurance policies impacting the bio-decontamination process and the contamination control strategy for the process being defined. A technical specification or data sheet (i.e. user requirements specification) is produced that includes many performance requirements, including parameters that define the bio-decontamination cycle, such as:

- Maximum acceptable cycle duration
- Materials of construction and their resistance to H_2O_2.

Commonly, these documents are created internally but may include input from equipment vendors.

The design phase usually results in the creation of a line 'mock-up' or design prototype. This prototype allows the users to determine key design factors, such as the locations for equipment inside the chamber, the number and location of isolator doors and associated glove ports, and the loading and unloading mechanisms. This design prototype allows important criteria to be defined and optimized before building. It ensures that all manual operations required can be aseptically and ergonomically executed, including those that impact the bio-decontamination process.

At this point, the bio-decontamination boundary for the chamber needs to be fully understood, along with the definition of any area requiring manual disinfection. This could be done based on theoretical and vendor experience or based on the results of the qualification tests. It is also important to understand the key requirements that will define the bio-decontamination process, like the spore log reduction values required and the aeration end point, before moving into cycle development activities.

Commissioning

The purpose of the commissioning phase is to demonstrate that the equipment built by the vendor functions as specified and that the functionality described in the user requirements specification is correct. This phase is usually comprised of testing at the factory (factory acceptance tests) and then testing on site (site acceptance tests).

The role of each phase and the type of tests performed are summarized below.

Factory acceptance tests: This phase confirms that the equipment works and is carried out before shipment by the vendor, typically with company representatives present to confirm the results. At this stage, companies may decide to perform pre-cycle development activities by running cycles using chemical, enzyme and/or biological indicators (BIs).

Site acceptance tests: This phase confirms that the equipment works when on site after delivery. Some portion of factory acceptance testing will be repeated and will usually include any of the pre-cycle development activities that were not fully met during factory acceptance tests.

The order and sequence of testing may vary between different vendors and there may also be an overlap between these phases.

Qualification

The qualification phase tests the installation and equipment functionalities, including those that could impact bio-decontamination cycle parameters. This phase is typically divided into installation qualification (IQ) and then operation qualification (OQ).

6.0

Bio-decontamination cycle development

The purpose of this phase is to define the critical bio-decontamination cycle parameters by evaluating conditions within the chamber and challenging the cycles with suitable BIs to demonstrate an acceptable level of spore log reduction.

This ensures that the bio-decontamination cycle is repeatable and that it delivers bio-decontamination to the standard required for the specific process, filling isolator, sterility test isolator or pass-through chambers.

The cycle development approach is agreed in collaboration with the equipment supplier during the design phase of the project. Many of the parameters critical to success will depend on the design of the equipment and the VPHP technology used and, depending on the technology, slight differences to the principal concepts we outline may be required.

In this document, we use the term 'process performance qualification' (PPQ) to refer to the **complete** process. Other terms sometimes used in practice for this phase are performance qualification or microbiological qualification.

7.0

Activities performed before starting cycle development

Defining the key starting parameters for the equipment

Starting parameters such as hot-plate temperatures, starting H_2O_2 injection rates, etc., are defined before starting cycle development. These parameters are equipment- and technology-dependent and should be identified in collaboration with the vendor. The parameters need to be developed during the early qualification phases and before starting any cycle development activities.

Defining room conditions

The key parameters for the room where the isolator is located need to be defined before cycle development begins. These include the room temperature, the room differential pressures and the relative humidity ranges.

Defining the bio-decontamination boundary

Full consideration should be given to understanding and defining the boundaries that need to be challenged to ensure effective bio-decontamination. This would include assessing the return locations, any indirect product-contact part locations, vacuum lines, etc. and deciding which present high or low contamination risks. This assessment should be formalized in either a specific assessment for the equipment or a broader risk assessment. It will play a key role in the decisions on the placement of chemical indicators (CIs) and BIs in the next phase of the bio-decontamination cycle development process.

Defining the load

For filling isolators, non-product-contact parts for the operation are assembled in the enclosure before starting the bio-decontamination cycle. The chamber may also contain tools for adjusting process equipment, sterile wipes, sterile bags and other required materials.

To ensure satisfactory bio-decontamination, some basic principles need to be followed:

1. Loaded items should not overlap each other to facilitate contact with VPHP. Surfaces should be exposed to the environment in the enclosure without touching other surfaces to prevent occlusion of areas.
2. All parts designed to be in motion during the filling process need to move slowly to ensure all equipment surfaces are exposed to VPHP
3. Materials that will allow penetration of H_2O_2—such as steam-sterilized items in Tyvek/SteriBags and any items that absorb VPHP (e.g. silicone and PVC tubing) and then outgas during aeration—should be assessed with respect to cycle time requirements
4. Using fixed locations/orientations, similar to an autoclave load definition, with fixed hanging hooks, e.g. s-hooks should be considered. Hanging baskets with occluded surfaces should be avoided.

Before starting cycle development activities, it is important to define the worst-case load(s). This characterization includes understanding the requirements for holding the items suspended in the enclosure and the equipment/material orientation within the chamber. Cycle development cycles are usually carried out with the maximum quantity of materials present that form the worst-case load.

All the materials, equipment and wrapping need to be compatible with VPHP to ensure proper bio-decontamination of material in the chamber.

The purpose of defining the load in the enclosure is to create a set of loading rules for the cycle that define the positions of materials and items positioned inside the enclosure. Detailed descriptions and photographs are important to maintain consistency of the load pattern.

8.0

Cycle development

The following four steps form the basic requirements for a successful cycle development program and include studies to optimize the cycle to ensure that distribution of VPHP throughout the chamber and load will result in adequate bio-decontamination. Cycle development activities are usually performed with a fully loaded enclosure with all equipment turned on and in operation.

Temperature and relative humidity mapping

Mapping the temperature and/or humidity throughout the chamber is performed to plot and document the distribution of these characteristics throughout the enclosure. Temperature sensors and/or data loggers are used to record air and surface temperatures, and to look for any hot and cold spots in the chamber. The temperatures achieved within the enclosure are equipment- and process-dependent, and should be established by working with the equipment vendor during the earlier qualification stages.

Depending on the system design, target injection rates are determined through temperature and/or humidity testing to determine the point where H_2O_2 condensation occurs for the system in question. The injection rate can then be adjusted to achieve the desired conditions in the enclosure. Temperature and humidity should be within a range established during earlier studies. Where variations in the temperature and humidity are observed, consideration should be given to making appropriate system changes to improve the distribution (e.g. fan adjustments in different returns, enclosure air system recirculation rates).

Mapping studies are carried out with a chamber fully loaded. Companies may also decide to perform studies with an empty chamber for each isolator chamber configuration, depending on the volume of the chamber. At least one run for each isolator chamber configuration needs to be tested when different VPHP recipes are present (e.g. a combined cycle for a production line (including main and side chamber if applicable) and independent cycles for a material transfer chamber with different loads).

Comprehensive mapping studies should be performed to find any surfaces that are hotter than the air temperature during the bio-decontamination cycle, as these may adversely impact the bio-decontamination performance. It is important to find the worst-case conditions during operation, so that residual heat from recently running motors, scales, sensors, etc. (inside or below the filling deck of the isolator) can be identified, as well as the impact of adjacent systems like dry heat tunnels, lyophilizers, etc.

The results from these mapping studies are used to define which hot and cold spots are selected for further studies with CIs and BIs during the next phase of cycle development.

VPHP distribution studies using chemical indicators

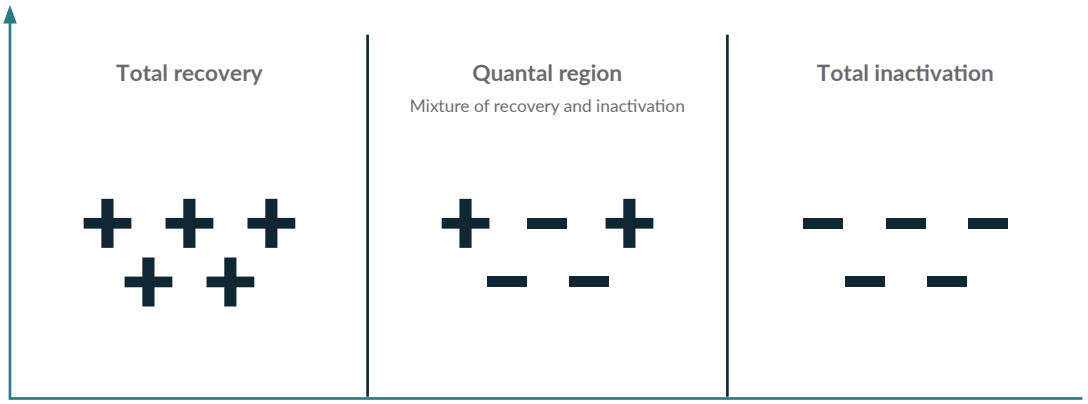
Chemical indicators for VPHP bio-decontamination processes are defined in ISO 11140:2014[1] and have historically been used for VPHP visualization according to Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S)[2]. Chemical indicator mapping studies are typically performed on at least one cycle before proceeding with any BI studies.

A CI is designed to have a distinct color change following prescribed exposure conditions. They therefore give qualitative and immediate feedback to the user, providing supplementary information to show if there are significant differences in VPHP distribution across the chamber being mapped. In locations where there is an incomplete color change for the CI, it can be indicative of marginal bio-decontamination performance, which may require remediation or further exploration before starting BI studies. Further information on the factors to consider when placing CIs can be found in the section on location studies using biological indicators.

Lethality studies using biological indicators

Lethality studies intend to find the cycle parameters that result in a series of BI inactivation conditions within the enclosure, and include assessing the following parameters for the BIs (see Figure 2):

Figure 2: Inactivation zones



Unlike sterilization modalities, and due to its two-phase nature, there is no industry-recognized D-value method for VPHP processes, and the biphasic nature of VPHP precludes the accurate determination of specific lethal conditions (USP <1229.5>[3] and <1229.11>[4]). Thus, D-values captured in vendor certificates of analysis are not transferable to other systems/isolators and end-users are left to define their unique BI resistance. For example, a vendor's certificate of analysis may indicate a D-value of 1.5 minutes; however, in-process testing may demonstrate that the system lethality is significantly more or less than this.

One approach to manage this is to establish a BI supplier or a consistent screening method and define an acceptable BI D-value range to use in cycle development studies and subsequent requalification activities.

Biological indicator positioning during an initial study is often in an easy-to-place or easy-to-reach location using gloves. The quantity of BIs in these locations can range from three to five per time point. BIs are either dropped into media inside the isolator or removed from the isolator every few minutes to incubate and determine the time at which growth stops.

As the reverse of this, studies can also be conducted with BIs that can be placed inside sealed containers, in an area accessible by gloves and removed from those containers at prescribed time points during exposure to help characterize biological inactivation within the system.

Location studies using biological indicators

Studies to find the worst-case locations for BI inactivation involve the use of many BIs placed in many different locations, requiring the use of hundreds of BIs. For all methods, the goal is to determine which locations are receiving a lesser concentration of VPHP, which would be indicated by BI recovery at those locations.

The selection of potential worst-case locations to place both CIs and BIs is developed from an understanding of the factors listed below, and is usually documented in a specific assessment. Factors to consider include:

1. Temperature and humidity mapping, which identifies hot and cold spots
2. Locations where vapor distribution may be difficult—these can be selected by working with the equipment vendor and/or through smoke generation inside the chamber to find the difficult locations where the smoke cannot reach easily or is not sufficiently mixed
3. Geometrically challenging locations in the load
4. Challenging locations identified from smoke studies and/or modeling using computational fluid dynamics
5. Criticality of the product, process and operations in the isolator
6. Filling zone and along the path that the primary packaging materials and product-filled containers flow
7. Distance from the VPHP injection point
8. Barriers to VPHP distribution, e.g. where airflow is substantially blocked by a glove port or piece of equipment
9. Heat sources
10. Corners or doors, and small or enclosed areas in the chamber
11. Gloves and glove fingers, which should be extended where possible but avoid contact with any other materials
12. Areas where movements or equipment have the potential to create occluded surfaces.

One approach to find the worst-case locations for inactivation is to use lots of BIs placed in many different locations, progressively increasing injection parameters until all BIs are killed and/or progressively decreasing injection parameters to determine worst-case locations, particularly if all BIs are killed in the initial test. Where possible, use triplicate BIs and find sites where all BIs are inactivated, and sites where some of the three BIs are not inactivated. The next step would be to increase injection time at the same injection rate iteratively until all BIs are inactivated. This will identify the worst-case sites with respect to BI inactivation.

The need to achieve and demonstrate consistent H_2O_2 vapor properties within the chamber means that it can take numerous test cycles before a suitable cycle is developed.

Performing these location studies allows the determination of a total inactivation time, the amount of VPHP and the length of cycle required to achieve the required spore log reduction. For a production isolator, these tests are performed with the equipment within the isolator chamber in operation, including a 'creep mode' with movement to expose areas of the moving parts to VPHP as they would be moving during actual filling.

The last step in cycle development would be a confirmation run demonstrating that all parameters are sufficient and can meet subsequent validation requirements. This could be one run or three, based on a risk assessment and a knowledge of the system.

All these parameters, including the strategy for location studies and the locations to be tested, should be defined by the user using a documented assessment.

Selecting the number of biological indicators for cycle development

The PDA Technical Report no. 34[5] recommends that 5–10 BIs per m^3 of enclosure volume is generally sufficient. The number and placement of BIs should always be supported by an assessment based on the chamber design, the bio-decontamination boundary and the required spore log reduction defined in the contamination control strategy.

Biological indicator placement: the advantages and disadvantages of placing one, two or three biological indicators at each location

Dating back as far as 2007, the use of one or multiple BIs can be found during qualification activities[2]. While a minimum of one BI per location is required during qualification, benchmarking data from the BioPhorum Isolator Good Operating Practices Team shows that some users choose to use three BIs at each location.

Placing one BI has the advantage of reducing the total number of BIs used and the time/resources required to support qualification activities (e.g. time to place/remove, media and incubation space). A disadvantage is that if one BI is found to be positive, this requires an additional investigational run with multiple BIs at that location to determine if the cause can be attributed to a change in the process or test method, BI placement, handling, storage, manufacturing, etc.

Placing three BIs is recommended in the PIC/S guidance, which says: "If there is only one BI in each position, and only growth/no growth is established, then the number of survivors is unknown and the size of the possible variation in the process cannot be estimated". Thus, by placing three BIs at a location, the spore log reduction value can be calculated using the Halvorson-Ziegler equation[6] (see Figure 3), which is a 'most probable number' (MPN) approach to assess surviving organisms. A disadvantage of this approach is spatial limitations as well as the increased time/resources required to support cycle development.

The Halvorson-Ziegler equation is: MPN = ln (n/r)

MPN = Most probable number of surviving spores; ln = natural log function; n = number of replicate BIs at each discrete test location; r = number of growth-negative BIs at each discrete test location

What happens if there are positive results from a biological indicator?

The level of investigation required for BI positive results should be aligned with the phase of cycle development or qualification. During early cycle development – when process parameters, loading configurations and bio-decontamination conditions are still being established – BI positives may occur and are not unexpected. In this phase, positives primarily serve as diagnostic inputs for process optimization rather than indicators of process failure.

However, in the later stages of development – including final confirmation runs and subsequent qualification activities such as PPQ – BI inactivation should be consistently achieved. At this stage, any BI positive result is considered atypical and must trigger a documented investigation to determine root cause, assess impact and implement corrective actions as appropriate. This expectation reflects the requirement that the VPHP process demonstrates reliability, reproducibility and compliance with defined acceptance criteria before routine use.

The approach to positive results from a BI will depend on the number of indicators used and the location in which they are placed. Where single BIs are used, one approach is to repeat the test with multiple BIs.

In cases where three BIs are used, a common approach is to accept no more than 3–5% of the total number of BIs and no more than one in any location.

The strategy for investigating positive BIs should be defined in the contamination control strategy and the risk assessment for the specific cycle development program. At minimum the strategy should include a review of the VPHP process (e.g. trending of critical parameters) and test methods (e.g. BI handling, storage, placement, etc.) to determine if there was a change which could have contributed to the positive results.

Spore log reduction

The log reduction (sometimes referred to as 'log kill') is commonly required at a minimum of 6 log reductions for isolators. Historically, aseptic processing and sterility isolators have used a 6 log reduction requirement. However, only one industry guidance document references this, i.e. PIC/S *Isolators used for Aseptic Processing and Sterility Testing*[2], "A gaseous, vapour or liquid treatment applied to surfaces, using an agent that is recognized as capable of killing bacterial and fungal spores. The process is normally validated using BIs containing bacterial spores. The number of spore log reductions is not specified in this definition, but a target of six log reductions is often applied."

While the use of BIs with a population of $1x10^6$ to demonstrate a 6 log reduction has been an industry norm, some sources indicate that the use of 10^3 or 10^4 populations is more appropriate for applications claiming bio-decontamination and not sterilization. The spore log reduction required from any cycle should be defined in the contamination control strategy.

A note on definitions

To avoid confusion, this document does not contain specific terms for different location studies, as terminology can vary across equipment vendors. However, the most common terminology used within the industry is listed below.

Placing multiple BIs in easy-to-kill locations and removing some every few minutes throughout the cycle (or dropping in tryptic soy broth tubes inside the isolator) can be referred to as either 'fractional studies', 'system D-value' or 'lethality studies'.

Placing BIs in canisters before the start of the cycle, then opening to expose them every few minutes during a cycle, can be referred to as a 'reverse fractional study'.

Placing BIs all around the isolator in many locations, first at a low injection time to determine worst-case locations, then progressively increasing the injection time until all BIs are killed, can be referred to as 'worst-case location studies' or 'fractional studies'.

Supporting information

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9.0

Development of the aeration phase of the VPHP cycle

Routine process

To ensure that the aeration phase can consistently remove residual H_2O_2 from the enclosure, aeration studies are performed to measure residual VPHP. If the product is not sensitive to oxidation, electrochemical sensors (e.g. Draeger® tubes or similar measurement systems) may be used to confirm a routine production cycle end point for most systems; typically, aeration would be set to end at or below 1ppm.

The total length of the aeration phase will depend on the load and the volume of the enclosure. It is normal practice to incorporate an overage in the aeration cycle time for a time-based cycle. For routine systems, to end aeration based on real-time measurements, the end point level is typically checked through sensors, commonly integrated electrochemical sensors.

Aeration for oxidation sensitive products

Where products are oxidation sensitive (e.g. monoclonal antibodies (mAbs) and peptides), the aeration phase of the bio-decontamination cycle may be continued to remove VPHP residuals to PPB levels before introducing single-use parts or product into the system. Mapping studies must be performed to correlate the location used for routine VPHP measurements with the location where product will be exposed. In this case, a full aeration cycle development study must be performed after the production cycle for BI kill has been finalized.

Oxidation sensitivity for any product to be filled in the system being tested should be understood. One cycle development could be used to go to very low PPB levels, then the times calculated to reach higher levels for different products, where sensitivity may not be as much of a concern.

Measurements with a cavity ring-down spectroscopic instrument (Picarro®) should be used in developing cycles for oxidation-sensitive products. One of these instruments may be used to correlate points of potential product exposure within the system to the routine monitoring location (i.e. mapping studies), as well as to develop a cycle to consistently meet the required PPB levels for the system.

For routine monitoring, electrochemical sensors (such as a Draeger LC® calibrated with H_2O_2) could be used or a Picarro® instrument could be integrated to confirm each cycle end point instead of developing a time-based cycle with overage. Where low PPB level requirements are needed to protect product Draeger LC® sensors calibrated by other methods, such as using SO_2 gas, are not appropriate for monitoring during production mode due to the higher variability and reduced accuracy associated with the calibration. Sensors calibrated by other methods could be used for room monitoring or to end cycles at 1ppm for safety (not product) considerations.

Supporting information

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10.0

Validation (process performance qualification of the bio-decontamination cycle)

A well-developed validation and production cycle will use parameters where all the BIs are inactivated, i.e. in the 'Total inactivation' zone from Figure 2, with an additional safety margin (e.g. extended exposure time or higher dosing rate). This is to account for normal process variation and BI variation (e.g. population and resistance from lot to lot).

Validation is performed on the minimal effective cycle and will include a microbiological challenge performed at sub-minimal or minimally accepted cycle conditions and an aeration challenge performed at planned production parameters or slightly above. Loading conditions, parameter settings, BI quantity and position(s) should all be clearly defined.

Cycles are usually carried out with the maximum quantity of materials present that form the worst-case load. This could be one load for a production isolator cycle; but for a material transfer chamber, it may be multiple loads (possibly one load with micro plates, a separate load with tooling for work in the isolator, etc.). If there is a maximum and minimum load, the minimum load should also be validated. Refer to previous sections for detailed information on BIs.

Validation will demonstrate the repeatability and reproducibility of the final parameters developed through cycle development activities.

11.0

Production cycle

Recipes for bio-decontamination cycles are created at the start of the cycle development phase. This starting recipe is modified throughout cycle development to form final recipes for validation and production that include:

- Injection parameters (derived from worst-case location/VPHP fractional studies)
- Aeration parameters
- Total cycle time.

For the production cycle, an overage may be added to the validation cycle to ensure routine cycles meet the required level of kill, allowing for potential cycle variables. The overage is customarily an extension to the cycle time, increasing the amount of H_2O_2 injected, not the rate of injection, which was optimized during cycle development. However, this is not always required if there is robust data from cycle development demonstrating the point of failure of the cycle. Safety margins for the validation and production recipe must also consider normal variation in BI resistance and population. Thus, it is common to develop a validation cycle that may be sufficient for an 8 logarithmic reduction and a production cycle that is capable of 10 or 12 logarithmic reductions.

Release criteria for the production cycle typically include the amount of H_2O_2 injected, injection rates over time, and temperature and humidity control of the system. High-level VPHP concentration is also typically monitored, but it is not a common practice to review cycle profiles or curves as part of release.

12.0

Requalification

Requalification is performed annually using a minimum of one confirmation cycle with worst-case load patterns following the same procedures and test methods used for the initial qualification. Aeration requalification may be performed for oxidation-sensitive product systems (where it is considered a critical attribute) at some periodic frequency, depending on the level of routine monitoring.

Where there are minor changes to loads in the chamber, incorporation into the routine requalification can be considered, but major changes will require a full qualification exercise. A common method of assessing the impact of changes to loads is to evaluate the increases or decreases in the surface area of the overall load pattern.

13.0

Conclusion

The purpose of the bio-decontamination cycle development process is to:

- Develop a cycle
- Ensure it is consistent
- Understand the factors that impact the cycle and the key parameters to measure and control.

It is a critical part of the aseptic assurance process, where process understandings can be gathered, realized and applied to develop a robust VPHP bio-decontamination process.

Appendix

Appendix A: Challenges with biological indicators during cycle development

Biological indicators (BIs) are used in cycle development to provide information on the ability of the cycle to decontaminate the enclosure. BIs are most commonly *G. stearothermophilus*, but *B. atrophaeus* can be used as detailed in the USP <1229.5>[1] and EP 5.1.2[2] monographs.

Using BIs effectively needs a determination of the BI D-value from BI characterization studies, as described in the section on lethality studies using BIs or through verification by a secondary company or internal system.

BIs used for cycle evaluation need to be stored under specific conditions, which can be provided by the vendor and may include temperature control and low relative humidity storage. The period between use, retrieval and testing in the laboratory (i.e. hold time) must be defined and it is important to equilibrate the indicators to the room conditions before use.

Orientation of the BI within the enclosure is important and, commonly, it is oriented either flat or slightly at an angle and slightly above the surface of the point being monitored as shown in Figure 3. Manufacturers recommend bending the BI flap so that it leaves the BI in the air to reduce the risk of human error in locating tape above the disc or not leaving the 'writing side' as the exposed side.

Indicators can be purchased in several different formats to aid handling, e.g. on stainless steel discs, contained within Tyvek envelopes or as naked strips. Each type of presentation has advantages and disadvantages, e.g. susceptibility to alcohol, cross-contamination from naked BIs, occluding the BI with the envelope, handling the inoculation point, ability to tape and place the BI, etc.

Figure 3: Examples of biological indicator placement and orientation

The following general recommendations are taken from *Ridding the World of 'Rogues': Improving Vapor-Phase H_2O_2 Sterilization and Bio-decontamination Processes*[3]:

- Using *G. stearothermophilus* strains to avoid false positives if a non-thermophilic spore to be used.

The ability to incubate at 55–60°C substantially reduces the potential for false positives.

- Using a fiberglass or other non-cellulose porous carrier substrate to allow for easier penetration of H_2O_2 and eliminate spore aggregation observed on planar surfaces
- Using primary packaging that is permeable to both gases and liquids is essential in what must always be considered a two-phase system. Elimination of primary packaging entirely would remove this as a factor in resistance variation.
- Using the same lot of recovery media for cycle development and validation of H_2O_2 vapor processes to eliminate the media as a resistance variable. This should be standard practice in any validation exercise.

Rogue biological indicators

A 'rogue' BI is resistant to bio-decontamination compared to others in the lot. Literature references to 'rogue BIs' can be found dating back to 2009, with the publication of *Biological indicators don't lie, but in sporicidal gassing disinfection cycles do they sometimes confuse the truth?*[4]. In this publication, the authors note the phenomenon described as 'rogue BIs' and their potential causes, such as manufacturing practices and increases in BI populations from 10^5 to 10^6, and a highly referenced figure of 0.3% occurrence for inoculated stainless steel carriers.

More recently, publication suggested quality deviations up to 1–3% rogue BI challenges have been experienced and managed by strategies (*Biological indicators (BIs) strategy to use in VHP/vH_2O_2 Bio-decontamination cycle development, performance qualification and ongoing re-qualification.* October 2025, EJPPS Volume 30 Issue 3).

Other publications include *Rogue Biological Indicators: Are They A Real Phenomenon?*[5], where the author goes into more detail about methods to proactively mitigate risks associated with rogue BIs. There is also *Ridding the World of 'Rogues': Improving Vapor-Phase H2O2 Sterilization and Bio-decontamination Processes*[3], with the author arguing that processes are not robust and the BI selection could play a role (e.g. using Tyvek as primary packaging, which is hydrophobic and does not allow H_2O_2 in a liquid phase to pass through).

Supporting information

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Appendix B: New technology for VPHP cycles

Measuring H_2O_2 saturation and vaporization

The relative saturation level of H_2O_2 required in the enclosure depends on the methodology used by the vendor to generate the vapor and so saturation levels vary across different equipment. At present, saturation is established and indirectly managed through H_2O_2 injection rates, cycle time and injection amount.

Different technologies are currently used to measure saturation, with newer technologies becoming available.

Vaisala PEROXCAP®

This is a sensor for measuring vaporized H_2O_2. The sensors can be used for mapping and monitoring VPHP at high levels, relative saturation and humidity, temperature, etc. and are a valuable tool for investigating cycle parameters.

This technology enables measurement of multiple parameters during the bio-decontamination cycle and can replace the widely used and traditional Draeger HC® electrochemical sensor technology (note that it should not be used to replace Draeger LC® sensors).

Currently, the sensor is mostly used as an investigational tool, but it is gaining popularity as the ability to have multiple measurements during cycle developments is advantageous and some new isolator lines are now being offered with these PEROXCAP® sensors fitted to provide on-line measurements.

Draeger LC® sensors are typically used to measure residual VPHP to end aeration and, if required, to monitor VPHP during production. Picarro® cavity ring-down spectroscopic analyzers would be used to develop the cycle for oxidation-sensitive products, measuring from 100ppm to below 10ppb.

Indicator technology

Enzyme indicators

Enzyme indicators (EIs) are a relatively new type of VPHP indicator that were developed and evaluated for VPHP distribution mapping. They are strips of thermostable adenylate kinase (tAK), an enzyme that has been found to be inactivated by VPHP in a similar biphasic manner to BIs. Like BIs, EI inactivation by VPHP is dose- and time-dependent and, when exposed to VPHP, a quantitative response on enzyme deactivation is obtained, rather than the qualitative growth/no growth of a conventional BI. Further advantages compared to BIs include a rapid result (within minutes to read post-exposure) and the avoidance of viable organisms in the isolator, allowing their use within routine operations as a continuous process verification tool.

They allow the effectiveness of the cycle to be monitored more quickly than traditional BIs and have the advantage of providing quantifiable results rather than a binary pass/fail result. The supplier recommendations are to place EIs flat against the surface to better mimic the surface to be decontaminated.

An EI can be read in 2–3 minutes and means that, for a typical cycle development program, feedback will be available within a few days rather than 7–8 days for standard methodology.

At present, this technology is finding most use to increase process understanding via quantitative data:

- To optimize cycle parameters
- To identify worst-case locations and support reduction of locations to be mapped
- To support equivalency in terms of VPHP distribution and cycle efficacy between enclosure and loads (where applicable)
- To support microbiology failure investigations including investigation of rogue BIs by speeding up investigation of root cause and reducing downtime of equipment associated with the failure.

Use of EIs, in conjunction with BIs, may also be used during validation and re-qualification for trending of VPHP cycle performance since its initial validation, as well as supporting investigation of rogue BIs. This will facilitate regulatory approval of the use of EIs as an alternative to BIs for VPHP re-qualification, reducing time for overall activity. The technology promises future uses in routine monitoring of cycle performance.

To unlock EI full potential it is recommended:

- Plan time for training and new knowledge built: correct reading requires well-trained resources
- Consider using multiple EI readers if you have a high number of samples (x2 EI readers were used to read 150 samples in approx. 2hrs)
- Ensure data are analyzed by a statistician together with a subject matter expert (SME) of VPHP. This is key to extract valuable knowledge to drive informed decision-making.

Supporting information for enzyme indicators

McLeod, N. P., Clifford, M., Sutton, J. M. *Evaluation of Novel Process Indicators for Rapid Monitoring of Hydrogen Peroxide Bio-decontamination Processes.* PDA Journal of Pharmaceutical Science and Technology. September 2017. 71 (5), 393–404. **https://doi.org/10.5731/pdajpst.2016.007435**

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Bioquell® chemical indicators

These are CIs that give an indication of success to a 6 logarithmic reduction of a H_2O_2 vapor bio-decontamination cycle. A strip is printed on the CI using an ink that reacts by changing color when exposed to the H_2O_2 vapor, allowing visualization of the results as they occur.

Supporting information

Bioquell Isolator CI: Rapid Read 6 Log Chemical Indicators For Bioquell Bio-Decontamination Equipment and Rooms Less Than 10m^3. **Bioquell® data sheet**

Environmental monitoring

Biofluorescent particle counters

These are a new approach to environmental monitoring in isolators. Unlike traditional culture-based microbiological testing, biofluorescent particle counters offer continuous viable air monitoring without posing a risk to the manufacturing process. These devices use laser-induced fluorescence to provide real-time, automated detection of inert particles and microorganisms in the air. The TSI BioTrak® Real-Time Viable Particle Counter is an example of a biofluorescent particle counter.

Supporting information

BioTrak Real-Time Viable Particle Counter. **https://tsi.com/products/cleanroom-particle-counters/real-time-viable-particle-counter/biotrak-real-time-viable-particle-counter/**

Glossary

Term	Definition
BI	Biological indicator
CI	Chemical indicator
H_2O_2	Hydrogen peroxide
HEPA	High-efficiency particulate air

Term	Definition
PPB	Parts per billion
PPQ	Process performance qualification
VPHP	Vapor phase hydrogen peroxide

Supporting information

United States Pharmacopeia. *General Chapter <1208> Sterility testing—validation of isolator systems. USP-NF.* **https://doi.usp.org/USPNF/USPNF_M99927_02_01.html**

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Parenteral Drug Association. *PDA Technical Report No. 51: Biological Indicators for Gas and Vapor-Phase Decontamination Processes: Specification, Manufacture, Control and Use.* November 2010. **www.pda.org/bookstore/product-detail/1210-tr-51-biological-indicators-for-gas-vapor-phase**

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