

Bio-Decontamination Cycle Development Study: Comparative Evaluation of Nebulized Hydrogen Peroxide (NHP) and Vaporized Hydrogen Peroxide (VHP) for Pharmaceutical Isolator Decontamination

Esco Lifesciences Healthcare Business Unit In partnership with Protak Scientific

Executive Summary

This white paper presents the findings of a comparative bio-decontamination cycle development study conducted by Esco Technologies Inc. in collaboration with Protak Scientific. The study evaluated Nebulized Hydrogen Peroxide (NHP) and Vaporized Hydrogen Peroxide (VHP) technologies across 12 parametric cycle configurations for each technology. Performance was assessed using Biological Indicators (BI) and Enzyme Indicators (EI) positioned at 20 representative locations within an empty pharmaceutical isolator.

The primary acceptance criterion was complete BI inactivation at all 20 monitored locations following a 7-day incubation period. EI log reduction values were reported as a supplementary performance metric. The final dataset includes complete Day 7 incubation results for all 24 test cycles. For NHP testing, hydrogen peroxide (H₂O₂) injection weights were recorded for every cycle using a calibrated balance to characterize the actual delivered dose; all cycle performance assessments in this study are based on these recorded actual injection weights.

Key conclusions:

The fastest NHP and VHP cycles achieving complete BI inactivation are summarized below. Both cycles demonstrated no BI growth at any of the 20 monitored locations.

Parameter	NHP – Cycle 10	VHP – Cycle 5
Injection configuration	High-dose group; dual-point injection (Transfer Chamber + Isolator Chamber)	High-dose group; single-point injection
Dwell time	Short dwell (within tested range)	Short dwell (shortest tested)
Total cycle time	28.12 minutes (shortest observed across either dataset)	36 minutes
All BI negative	Yes, all 20 locations	Yes, all 20 locations
Avg EI log reduction	8.20	9.31
Min EI log reduction	6.48	5.22 (Loc 14 EI anomaly; BI negative)
Chamber access	After aeration completes	After aeration completes

- Nine EI/BI divergence anomalies were identified across both technologies:
 - NHP Cycle 11: Locations 4 and 19
 - VHP Cycle 2: Locations 14 and 20
 - VHP Cycle 4: Locations 14 and 20
 - VHP Cycles 5–7: Location 14
- In all cases, BI results were negative (no growth) while EI results underreported performance. These locations have been flagged for confirmation through a three-BI retest protocol.
- Certain low-dose, short-dwell cycle configurations did not achieve acceptable biological inactivation performance and should not be considered for use. Specifically:
 - VHP: Low-dose injection group cycles
 - NHP: low-dose group cycles run at the two shorter dwell-time settings tested
- Both configurations demonstrated widespread BI growth and failed to meet the study acceptance criteria.

1. Introduction: Why Bio-decontamination Matters

1.1 Background

Aseptic pharmaceutical manufacturing requires robust and validated bio-decontamination processes to achieve the sterility assurance levels mandated by regulatory standards, including EU GMP Annex 1 (2022), PIC/S PE 009, and the WHO Technical Report Series. Vaporized Hydrogen Peroxide (VHP) is a widely adopted and well-established sporicidal technology that has become the industry standard for pharmaceutical isolator decontamination.

Nebulized Hydrogen Peroxide (NHP) offers an alternative hydrogen peroxide-based decontamination approach and has been implemented across various pharmaceutical and healthcare applications. However, customer acceptance of NHP for aseptic isolator systems often depends on demonstrating decontamination performance comparable to the widely accepted VHP technology.

To support customer confidence and provide evidence-based performance data, Esco Lifesciences, in collaboration with Protak Scientific, conducted a comparative cycle development study evaluating NHP and VHP under equivalent test conditions. The objective of this study was to assess and compare the sporicidal efficacy of both technologies using biological and enzyme indicator challenges within a pharmaceutical isolator, and to demonstrate that NHP can achieve decontamination performance comparable to VHP when appropriately developed and validated for aseptic processing applications.

This white paper presents the methodology, results, and key findings from the study, providing scientific evidence to support the use of NHP as an effective bio-decontamination solution for Esco aseptic isolators.

1.2 Technology Overview

Nebulized Hydrogen Peroxide (NHP): NHP generates a fine hydrogen peroxide (H_2O_2) aerosol using a vibrating piezoelectric crystal that forces liquid H_2O_2 through a fine mesh without the use of compressed air. The resulting aerosol disperses throughout the isolator chamber volume while remaining within the chamber and not crossing the HEPA filter boundary.

Following injection, the H_2O_2 aerosol is retained within the chamber for a defined dwell period, during which microbial reduction occurs. Dwell time was evaluated as a study variable across the tested cycle configurations. Upon completion of the dwell phase, the chamber enters aeration mode, where air is circulated through catalytic converters that facilitate the decomposition of hydrogen peroxide into water and oxygen, reducing residual H_2O_2 concentrations to safe levels before chamber access is permitted.

For this study, NHP was delivered through simultaneous injection into both the Transfer Chamber (TC1) and Isolator Chamber (IC1), reflecting the configuration of the test isolator used during cycle development.

Vaporized Hydrogen Peroxide (VHP): VHP delivers hydrogen peroxide in a dry, sub-condensation vapor phase through a single injection process. Prior to H_2O_2 injection, the chamber undergoes a conditioning phase during which environmental conditions are adjusted to predefined setpoints to promote consistent vapor distribution and decontamination efficacy. For this study, the chamber operated at ambient room temperatures of approximately 22–25°C and was conditioned to a relative humidity of 20% before vapor injection.

Following injection, the vapor is maintained within the chamber for a defined dwell period to achieve microbial reduction. As with NHP, the hydrogen peroxide agent remains within the chamber and does not cross the HEPA filter boundary.

The areas of the isolator most distal from the injection point, geometrically restricted, or located at the boundary of the H₂O₂ distribution zone represent potential critical locations where sporicidal challenge is greatest. In this study, several such locations were monitored, including the HEPA filter face positions and the sterility test pump fluid path (Location 14). Among these, Location 14 consistently exhibited the lowest EI log reduction values across VHP cycles and was therefore designated the most challenging location for VHP penetration. This position presents a distinct geometric challenge due to restricted fluid pathways that can limit hydrogen peroxide access regardless of the delivery technology used. Location 14 was included in the VHP monitoring layout but was not monitored during NHP testing; this exclusion represents a limitation on the direct comparability of worst-case location findings between the two technologies, and assessment of the fluid-path position under NHP conditions is recommended as part of future cycle development work. Upon completion of the dwell phase, the chamber enters aeration mode, where air is circulated through catalytic converters that decompose hydrogen peroxide into water and oxygen until residual concentrations are reduced to safe levels for chamber access.

1.3 Indicator Methodology

Biological Indicators (BIs), consisting of *Geobacillus stearothermophilus* spores presented on Tyvek-sealed discs and supplied by Mesa Labs, were used as the definitive measure of cycle efficacy. Following exposure, all BI samples were incubated for seven days and monitored daily for microbial growth. BI results served as the primary pass/fail criterion for the study, consistent with the principles outlined in EU GMP Annex 1 and ISO 22441:2022.

Enzyme Indicators (EI) were used as a supplementary tool to provide quantitative log reduction (LR) data based on a different measurement principle than BIs. The EI utilized in this study contains thermostable adenylate kinase (tAK), an enzyme originally isolated from *Sulfolobus acidocaldarius*, a thermophilic microorganism known for its high resistance to heat and oxidative stress. During H₂O₂ exposure, the tAK enzyme is progressively inactivated in proportion to the sporicidal dose received.

Immediately following cycle completion, residual tAK activity is measured using a luciferin/luciferase bioluminescence assay. An ADP reagent is added to the exposed indicator, and ATP generated by any remaining active tAK is quantified as a relative light unit (RLU) value using a luminometer. The measured RLU is subsequently converted to a log reduction value. Higher H₂O₂ exposure results in greater enzyme inactivation, lower RLU readings, and therefore higher calculated log reduction values.

A key advantage of EI technology is the rapid availability of results, typically within minutes of cycle completion, compared with the seven-day incubation period required for BI analysis. This makes EI particularly valuable during cycle development and optimization activities. A published study by McLeod et al. [9] demonstrated a strong correlation between tAK-based EI responses and BI log kill across replicate VHP decontamination cycles, supporting the use of EI as a surrogate process indicator.

However, EI and BI results should be considered together with the physical conditions at each monitoring location. Restricted pathways or enclosed spaces can reduce hydrogen peroxide exposure to both indicators, so a low EI result may identify a location that received less exposure. In this study, however, the observed divergence occurred when an EI result was below the expected threshold while the co-located BI showed no growth. This pattern does not, by itself, prove either inadequate exposure or indicator error. McLeod et al. reported different levels of within-cycle variability for BIs and tAK indicators. Therefore, divergent results should be investigated using the location geometry, cycle data, indicator handling records, and repeat testing rather than being attributed to BI variability alone.

For these reasons, BI results remain the definitive acceptance criterion throughout this study. Where BI and EI results diverge (BI negative with EI log reduction < 6), the cycle is classified as a BI pass, with the EI result recorded as an anomaly. Such anomalies are discussed in Section 6 and are subject to planned three-BI confirmation retesting.

1.4 Study Objectives

The objectives of this study were to:

1. Identify the fastest cycle configuration achieving complete BI inactivation for both NHP and VHP technologies.
2. Characterize and document the performance envelope across all 24 evaluated cycle configurations.
3. Record and verify actual hydrogen peroxide (H₂O₂) injection weights delivered during NHP cycles through gravimetric measurement of the H₂O₂ reservoir before and after each cycle.
4. Document BI/EI divergence anomalies and identify the most challenging monitoring locations within the isolator.

2. Materials and Methods

2.1 Test System

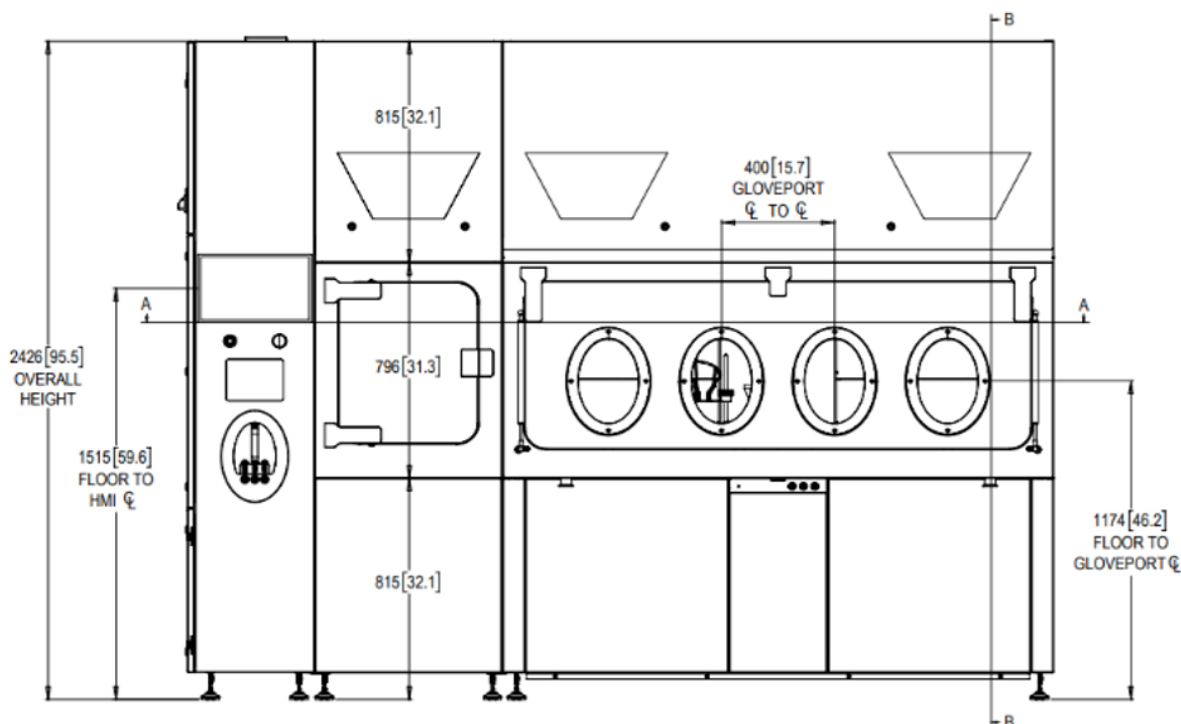


Figure 1: Overall dimensions of the test isolator

An empty Esco pharmaceutical isolator was used throughout the study. The isolator comprised two chambers: the Transfer Chamber (TC1) with an internal volume of approximately 0.483 m³ and the Isolator Chamber (IC1) with an internal volume of approximately 2.257 m³. The isolator was equipped with Esco's Nebulized Hydrogen Peroxide (NHP) diffuser system. For VHP testing, hydrogen peroxide vapor was introduced into the chamber using a third-party VHP generation system. In both cases, hydrogen peroxide was distributed within the sealed chamber volume and did not cross the HEPA filter boundary.

Under the operating conditions used in this study, H₂O₂ was retained within the chamber volume and did not pass beyond the HEPA filter boundary. This has two important consequences: first, surfaces immediately facing the filter face are at the boundary of the H₂O₂ distribution zone and receive lower sporidical contact than surfaces in the open chamber volume; second, the filter face locations were identified as critical zones and were therefore included as monitored locations in this study.

A sterility test pump assembly, including an internal fluid path and tri-clamp pass-through connection (Location 14), was installed within the IC1 chamber to serve as a representative worst-case geometric challenge location.

2.2 Biological and Enzyme Indicators

Biological Indicators (BIs) consisting of *Geobacillus stearothermophilus* spores on Tyvek-sealed discs were supplied by Mesa Labs. Growth media was supplied by True Indicating. Enzyme Indicators (EIs) were supplied by Protak Scientific.

Following exposure, all BI samples were incubated for seven days and monitored daily for evidence of microbial growth. EI samples were analyzed immediately following cycle completion using a luminometer to obtain Relative Light Unit (RLU) measurements, which were subsequently converted to log reduction (LR) values. Positive and negative controls were included in each test run. BI results served as the definitive pass/fail criterion for cycle efficacy, while EI results were used as a supplementary performance metric.

2.3 NHP Injection Weight Recording

For NHP cycles, hydrogen peroxide (H₂O₂) injection weights were recorded gravimetrically for each cycle. NHP dosing was performed through simultaneous injection into both the Transfer Chamber (TC1) and Isolator Chamber (IC1). Injection weights were determined by measuring the H₂O₂ reservoir mass before and after each cycle using a calibrated balance and calculating the corresponding mass loss. Complete injection weight records were obtained for all 12 NHP cycles.

2.4 Monitoring Locations

Prior to the parametric cycle study, a Worst-Case Location (WCL) assessment was performed to identify the areas within the isolator that presented the greatest decontamination challenge. The 20 monitoring locations selected for this study were based on the results of the WCL assessment and represented geometric extremes, equipment interfaces, enclosed spaces, and areas of restricted hydrogen peroxide circulation across both chambers. Locations 1–7 were located within the Transfer Chamber (TC1), while Locations 8–20 were located within the Isolator Chamber (IC1). Monitoring location positions are illustrated in Appendix A.

2.5 Cycle Parameter Matrices

NHP Cycles: Twelve NHP cycle configurations were evaluated, spanning three injection-weight groups (low, mid, and high) and four dwell times (1, 4, 7, and 10 minutes). Injection weights for each cycle were recorded gravimetrically, as described in Section 2.3. Table 1 summarizes the complete cycle matrix, including the recorded injection weights for TC1 and IC1.

Table 1. NHP Cycle Parameter Matrix (Relative Presentation)

Cycle	Dose Group	Dwell Tier	Relative Total Cycle Time
1	Low	Minimal	(fastest)
2	Low	Short	
3	Low	Moderate	
4	Low	Extended	
5	Mid	Minimal	
6	Mid	Short	
7	Mid	Moderate	(slowest)
8	Mid	Extended	
9	High	Minimal	
10	High	Short	
11	High	Moderate	
12	High	Extended	

NHP dosing used simultaneous dual-point injection (Transfer Chamber + Isolator Chamber) for every cycle. Injection weight increased progressively from the Low to High dose groups; dwell tiers are ordered Minimal < Short < Moderate < Extended. Bars show total cycle time relative to the fastest and slowest cycles in this dataset; exact injection weights, dwell minutes, and cycle-time minutes are omitted from this public edition.

VHP Cycles: Twelve VHP cycle configurations were evaluated using three injection-dose groups consisting of low, standard, and high, and four dwell-time settings, ranging from a short dwell up to a maximum dwell. Table 2 summarizes the VHP cycle matrix in relative terms, without disclosing the underlying quantitative recipe.

Table 2. VHP Cycle Parameter Matrix (Relative Presentation)

Cycle	Dose Group	Dwell Tier	Relative Total Cycle Time
1	Standard	Short	(fastest)
2	Standard	Moderate	
3	Standard	Extended	
4	Standard	Maximum	
5	High	Short	
6	High	Moderate	
7	High	Extended	
8	High	Maximum	(slowest)
9	Low	Short	
10	Low	Moderate	
11	Low	Extended	
12	Low	Maximum	

VHP dosing used a single-point injection for every cycle. Injection dose increased progressively from the Low to High dose groups; dwell tiers are ordered Short < Moderate < Extended < Maximum. Bars show total cycle time relative to the fastest and slowest cycles in this dataset; exact injection weights, dwell minutes, and cycle-time minutes are omitted from this public edition.

3. NHP Injection Weight Verification

Prior to the parametric cycle study, a Worst-Case Location (WCL) assessment was performed to identify the areas For NHP cycles, hydrogen peroxide injection weights were verified for every cycle by measuring the H₂O₂ reservoir mass before and after each run, as described in Section 2.3. Table 3 summarizes the general trend in recorded TC1 and IC1 injection weights across the low-, mid-, and high-dose configurations used for cycle development.

Table 3. NHP Recorded Injection Weights by Group (Relative Presentation)

Group	Relative TC1 Dose	Relative IC1 Dose
Low	Baseline (lowest tested)	Baseline (lowest tested)
Mid	Increased relative to Low	Increased relative to Low
High	Increased relative to Mid (highest tested)	Increased relative to Mid (highest tested)

Injection weight increased progressively and proportionally from the low-dose to the high-dose group in both chambers, with some run-to-run variability observed within each group, consistent with the manual weighing method used. Exact TC1 and IC1 injection weights are omitted from this public edition.

All cycle performance assessments reported in this study, including the BI pass/fail determinations in Sections 4 and 7, are based on these recorded injection weights.

Continued gravimetric verification of injection weight is recommended as a standard practice during future NHP cycle development, optimization, and validation activities, to confirm that the intended dose is delivered consistently.

4. NHP Results

The results for all 12 NHP cycle configurations are summarized in Table 4. Complete BI inactivation across all 20 monitoring locations was achieved in four cycles: Cycles 4, 7, 10, and 11. Of these, Cycle 10 achieved complete BI inactivation with the shortest total cycle time among the four, 28.12 minutes, and is therefore identified as the minimum effective NHP cycle evaluated in this study.

Table 4. NHP Cycle Results Summary (BI-Definitive, Relative Presentation)

Cycle	Group	Dwell Tier	Group	All BI Pass	Avg EI LR	Min EI LR	Notes
1	Low	Minimal		NO	3.25	0.02	11 locs with BI growth
2	Low	Short		NO	6.36	0.58	Loc 19 (glove sleeve) growth
3	Low	Moderate		NO	7.96	4.32	Loc 3 growth
4	Low	Extended		YES	8.22	6.77	All BI negative
5	Mid	Minimal		NO	6.54	1.39	Locs 15, 20 growth
6	Mid	Short		NO	8.06	4.21	Loc 10 (top back right corner) growth
7	Mid	Moderate		YES	8.52	7.60	All BI negative
8	Mid	Extended		NO	9.18	7.64	Loc 19 (glove sleeve) growth
9	High	Minimal		NO	6.41	0	6 locs with BI growth
10	High	Short		YES	8.20	6.48	★ Best: fastest full-kill cycle
11	High	Moderate		YES	7.79	1.94	All BI negative; EI anomaly Locs 4, 19
12	High	Extended		NO	8.40	2.31	Loc 19 (glove sleeve) growth

Relative Time bars are scaled within the NHP dataset only (fastest = most empty, slowest = most filled) and omit exact cycle-time minutes.

Note: the specific locations behind these results are identified in Sections 4.4 and 6, with chamber positions illustrated in Appendix A.

4.1 Low-Dose Group (Cycles 1–4)

The low-dose group demonstrated a clear improvement in decontamination performance with increasing dwell time. Cycle 1 (minimal dwell tier) exhibited widespread BI growth across 11 monitoring locations, indicating insufficient exposure. Moving to the short dwell tier reduced BI growth to a single location (Location 19), while Cycle 3 (moderate dwell tier) showed growth at only one location (Location 3).

Complete BI inactivation was first achieved in Cycle 4 using the extended dwell tier, resulting in successful decontamination at all 20 monitoring locations. Injection weights for this group were the lowest tested; Cycle 4 itself used the lower end of the low-dose range.

4.2 Mid-Dose Group (Cycles 5–8)

The mid-dose group also demonstrated improved efficacy with increasing dwell time. Cycle 5 exhibited BI growth at two locations, while Cycle 6 showed growth at a single location. Complete BI inactivation was achieved in Cycle 7 using the moderate dwell tier, producing an average EI log reduction of 8.52.

Unexpectedly, Cycle 8 did not achieve complete BI inactivation despite a longer dwell time and strong EI performance. BI growth was observed at Location 19, suggesting either localized variability or an anomalous result. As discussed in Section 6, this result has been identified as a candidate for confirmation through additional BI retesting.

IC1 injection weights for this group fell within the mid-dose range.

4.3 High-Dose Group (Cycles 9–12)

Cycle 9, which utilized the shortest dwell time within the high-dose group, showed BI growth at six monitoring locations, demonstrating that increased hydrogen peroxide dosage alone was insufficient to achieve complete decontamination without adequate dwell time.

Cycle 10 achieved complete BI inactivation at all 20 monitoring locations with a short dwell time and the shortest total cycle time recorded for NHP in this study, at 28.12 minutes. This represents the fastest successful NHP cycle evaluated during the study and is considered the recommended minimum effective cycle. Recorded injection weights for this cycle fell within the high-dose range.

Cycle 11 also achieved complete BI inactivation; however, EI anomalies were observed at Locations 4 and 19 and are discussed further in Section 6. Cycle 12 showed BI growth at Location 19 despite strong overall EI performance, reinforcing the observation that this location represents a particularly challenging decontamination site.

4.4 Most Challenging NHP Locations

Location 19 (right middle glove sleeve) was the most challenging monitoring location identified during NHP testing. BI growth was observed at this location in Cycles 2, 8, and 12, despite otherwise strong cycle performance across the chamber. The repeated occurrence of growth at this location suggests that local geometry and airflow patterns may reduce hydrogen peroxide exposure relative to other areas within the chamber. It is noted that the equivalent position in the VHP monitoring layout (Location 20) did not emerge as a worst-case location during VHP testing, indicating that the two technologies distribute hydrogen peroxide differently within the chamber volume and that worst-case locations are not necessarily consistent across technologies.

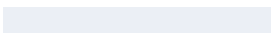
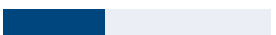
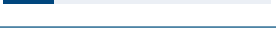
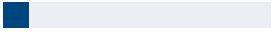
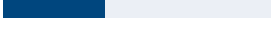
Additional challenging locations included the HEPA filter face and filter-adjacent positions (Locations 15, 17, and 18), which exhibited BI growth during lower-dose or shorter-dwell cycles. Because hydrogen peroxide remains within the chamber volume and does not cross the HEPA filter boundary, effective decontamination of these locations depends entirely on chamber airflow dynamics, hydrogen peroxide distribution, and sufficient dwell time.

5. VHP Results

The results for all 12 VHP cycle configurations are summarized in Table 5. Complete BI inactivation across all 20 monitoring locations was achieved in six cycles: Cycles 2, 4, 5, 6, 7, and 8. Among these, Cycle 5 achieved complete BI inactivation with the shortest total cycle time among the six, 36 minutes, and is therefore identified as the minimum effective VHP cycle evaluated in this study.

In contrast, none of the four cycles in the low-dose group achieved complete BI inactivation, indicating that this dose level is insufficient for effective decontamination of the test isolator under the evaluated conditions.

Table 5. VHP Cycle Results Summary (BI-Definitive, Relative Presentation)

Cycle	Group	Dwell Tier	Group	All BI Pass	Avg EI LR	Min EI LR	Notes
1	Standard	Short		NO	7.30	1.88	BI growth Loc 14 (fluid path)
2	Standard	Moderate		YES	7.67	2.80	All BI negative; EI anomaly Locs 14, 20
3	Standard	Extended		NO	7.23	4.10	BI growth Loc 14 (fluid path)
4	Standard	Maximum		YES	6.86	3.68	All BI negative; EI anomaly Locs 14, 20
5	High	Short		YES	9.31	5.22	★Best: fastest full-kill; EI anomaly Loc 14
6	High	Moderate		YES	9.96	5.62	All BI negative; EI anomaly Loc 14
7	High	Extended		YES	9.60	4.88	All BI negative; EI anomaly Loc 14
8	High	Maximum		YES	9.90	6.20	All BI negative; highest avg EI LR
9	Low	Short		NO	4.83	0	11 locs with BI growth
10	Low	Moderate		NO	5.29	0.66	11 locs with BI growth
11	Low	Extended		NO	4.32	2.00	13 locs (ST pump panel) with BI growth
12	Low	Maximum		NO	4.53	1.47	13 locs with BI growth

Relative Time bars are scaled within the VHP dataset only (fastest = most empty, slowest = most filled) and omit exact cycle-time minutes.

Note: the specific locations behind these results are described qualitatively in Sections 5.4 and 6, and positions illustrated in Appendix A.

5.1 Standard-Dose Group (Cycles 1–4)

The standard-dose group demonstrated variable performance depending on dwell time. Cycle 1, using the shortest dwell tier, showed BI growth at Location 14, indicating insufficient decontamination at this challenging location. Complete BI inactivation was achieved in Cycle 2 at the moderate dwell tier and again in Cycle 4 at the maximum dwell tier.

Interestingly, Cycle 3 did not achieve complete BI inactivation despite employing a longer dwell time than Cycle 2, with BI growth again observed at Location 14. This result suggests variability associated with this location rather than a straightforward dwell-time dependency.

Cycles 2 and 4 both exhibited EI anomalies at locations 14 and 20, where BI results were negative but EI log reduction values remained below the expected threshold. These anomalies are discussed further in Section 6.

Overall, the results indicate that a standard-dose VHP configuration is capable of achieving complete BI inactivation; however, performance appears sensitive to location-specific factors, particularly at the Location 14.

5.2 High-Dose Group (Cycles 5–8)

The high-dose group demonstrated the most robust and consistent performance of all VHP conditions evaluated. All four cycles achieved complete BI inactivation across all 20 monitoring locations, regardless of dwell time.

Cycle 5 achieved complete BI inactivation with the shortest dwell tier evaluated in this group and the shortest total cycle duration among all successful VHP cycles, at 36 minutes, making it the fastest successful VHP cycle evaluated during the study and the recommended minimum effective VHP cycle.

Although complete BI inactivation was achieved in all four cycles, EI values at the fluid-path position remained consistently lower than the chamber average in Cycles 5–7. Only Cycle 8 achieved an EI log reduction above 6 at this location. Cycle 8 also produced the highest average EI log reduction of all VHP cycles evaluated, with an average LR of 9.90.

These results demonstrate that increasing the VHP dose from the standard-dose to the high-dose group significantly improves cycle robustness and reduces sensitivity to challenging chamber geometries.

5.3 Low-Dose Group (Cycles 9–12)

All four cycles in the low-dose group failed to achieve complete BI inactivation. Cycles 9 and 10 exhibited BI growth at 11 monitoring locations, while Cycles 11 and 12 exhibited growth at 13 locations. The poor performance of this group was also reflected in the EI results, which were consistently lower than those observed in the standard-dose and high-dose groups. Cycle 9 recorded an EI log reduction value of zero at Location 14, indicating that hydrogen peroxide exposure at this location was below the detectable threshold of the tAK assay. The results clearly demonstrate that a low-dose VHP configuration is insufficient for effective decontamination of the evaluated isolator and should not be considered for operational use on this platform.

5.4 Most Challenging VHP Location: The Fluid-Path Position

Location 14, located within the sterility test pump and representing an enclosed dead-leg geometry, was identified as the most challenging location during VHP testing. All BI failures within the standard-dose group occurred at this location, and EI values consistently underperformed relative to chamber averages across multiple successful cycles. At the high-dose level, complete BI inactivation was achieved at Location 14 in all four cycles; however, EI values remained lower than those observed at other monitoring locations. This recurring pattern suggests that the enclosed geometry restricts hydrogen peroxide access and may disproportionately affect EI performance while still permitting complete spore inactivation. The observed BI/EI divergence at this location is discussed further in Section 6.

6. EI/BI Divergence Anomalies

Nine instances of EI/BI divergence were identified within the final dataset: two during NHP testing (Cycle 11) and seven during VHP testing (Cycles 2, 4, 5, 6, and 7). In each case, the Biological Indicator (BI) demonstrated complete inactivation (negative growth result), while the corresponding Enzyme Indicator (EI) reported a log reduction (LR) value below 6. A summary of these anomalies is provided in Table 6.

Before interpreting these observations, it is important to consider the known performance characteristics and limitations of both indicator systems. McLeod et al., reported that BI inactivation during hydrogen peroxide decontamination follows a biphasic rather than linear response and demonstrated substantially greater variability compared with thermostable adenylate kinase (tAK)-based EI measurements. The study reported within-cycle variance of up to 96% for BIs, compared with approximately 19% for EI. The authors further noted that BIs are inherently challenging to manufacture with perfectly uniform spore distribution and that resistance characteristics (D-values) can vary significantly between commercial production batches.

These characteristics provide context for the nine instances of EI/BI divergence observed in this study. At each location, the BI showed no growth after incubation while the co-located EI result was below the expected threshold. Co-location indicates that the two indicators were exposed at the same nominal monitoring point, but it does not make their responses equivalent. The BI results support microbial inactivation at the sampled locations, while the lower EI values remain development signals that require investigation. The divergences were therefore treated as unresolved observations for follow-up testing rather than being attributed solely to geometry, BI variability, or a localised process deficit.

Consequently, all nine cases were classified as BI passes with EI anomalies recorded for investigation. The absence of BI growth remains the primary and definitive finding. To further strengthen confidence in these results, a planned three-BI repeatability study will be conducted at the affected locations to provide additional statistical verification of decontamination performance.

Table 6. EI/BI Divergence Anomaly Summary

Tech	Cycle	Location (See Appendix A for reference)	EI LR	BI Result	Interpretation
NHP	11	Loc 4	1.94	Negative	EI underreads; BI confirms kill. Flagged for 3× BI retest.
NHP	11	Loc 19	4.90	Negative	EI underreads; BI confirms kill. Flagged for 3× BI retest.
VHP	2	Loc 14	2.80	Negative	EI underreads; BI confirms kill. Structural dead-leg effect.
VHP	2	Loc 20	5.83	Negative	EI underreads; BI confirms kill.
VHP	4	Loc 14	3.68	Negative	EI underreads; BI confirms kill. Structural dead-leg effect.
VHP	4	Loc 20	5.94	Negative	EI underreads; BI confirms kill.
VHP	5	Loc 14	5.22	Negative	EI underreads; BI confirms kill. Flagged for 3× BI retest.
VHP	6	Loc 14	5.62	Negative	EI underreads; BI confirms kill. Flagged for 3× BI retest.
VHP	7	Loc 14	4.88	Negative	EI underreads; BI confirms kill. Flagged for 3× BI retest.

Note: the specific locations behind these results are illustrated in Appendix A.

6.1 VHP Fluid-Path Position – Systematic EI Underread

Location 14, situated within the sterility test pump fluid path and representing an enclosed dead-leg geometry, exhibited a recurring pattern of EI under-reading during VHP testing. Sub-threshold EI values were observed at this location in multiple BI-pass cycles (Cycles 2, 4, 5, 6, and 7), despite complete BI inactivation being confirmed in each case.

The consistency of this pattern suggests that the anomaly is location-specific rather than cycle-specific. A plausible explanation is that the enclosed geometry limits EI substrate exposure or recovery, resulting in lower measured EI values without preventing sufficient hydrogen peroxide penetration to achieve complete spore inactivation. This interpretation is supported by the fact that BI growth was not observed at Location 14 in any of the affected BI-pass cycles.

A similar, though less frequent, pattern was observed at Location 20 (right middle glove sleeve) in Cycles 2 and 4. Additional confirmation testing is planned to further evaluate the reproducibility of these observations.

6.2 NHP Cycle 11 Anomalies

Two EI/BI divergence events were observed during NHP Cycle 11. EI values of 1.94 LR and 4.90 LR were recorded at Locations 4 and 19, respectively, while the corresponding BIs remained negative throughout the 7-day incubation period.

Unlike the VHP Location 14 observations, these anomalies were isolated to a single cycle and did not demonstrate a recurring location-specific pattern across the NHP dataset. Potential contributing factors include localized variability in hydrogen peroxide exposure, EI strip positioning, or handling-related effects. As no BI growth was observed, Cycle 11 remains classified as a successful full-BI-kill cycle.

6.3 Planned Follow-Up

To further investigate the identified EI/BI divergence events, additional confirmation testing is planned using a three-BI replicate approach at selected anomaly locations. The proposed retesting scope includes:

- NHP Cycle 11 – Locations 4 and 19
- VHP Cycles 5, 6, and 7 – Location 14

The objective of this follow-up testing is to confirm the reproducibility of BI inactivation at these locations and to determine whether the observed EI anomalies represent true performance limitations or are attributable to indicator-specific or geometry-related effects. Results from the retest program will be incorporated into the final validated cycle recommendations and future OQ documentation.

7. Comparative Analysis

7.1 Best Cycle Head-to-Head

Table 7. Best Cycle Comparison – NHP C10 vs. VHP C5 (Relative Presentation)

Parameter	NHP – Best Cycle (C10)	VHP – Best Cycle (C5)
Injection configuration	High-dose group; dual-point injection (TC1 + IC1)	High-dose group; single-point injection
Dwell time	Short dwell (within tested range)	Short dwell (shortest tested)
Aeration time	Equal duration for both technologies	Equal duration for both technologies
Total cycle time	28.12 minutes (shortest observed across either dataset)	36 minutes
BI outcome	All 20 negative	All 20 negative
Avg EI log reduction	8.20	9.31
Min EI log reduction	6.48	5.22 (Loc 14 - EI anomaly; BI negative)
EI/BI divergence	None	Loc 14 consistently; BI confirms kill at the high-dose level
Post-cycle access	Following completion of aeration and attainment of safe residual H ₂ O ₂ levels	Following completion of aeration and attainment of safe residual H ₂ O ₂ levels

Exact injection weights and dwell times are presented in relative/generalized form; the total cycle times shown above are for these two recommended minimum-effective cycles only.

7.2 Cycle Time and Operational Efficiency

The fastest successful NHP cycle (Cycle 10) achieved complete BI inactivation in 28.12 minutes using a short dwell time. The fastest successful VHP cycle (Cycle 5) achieved complete BI inactivation in 36 minutes using the shortest dwell time evaluated for that technology.

Under the conditions evaluated in this study, NHP achieved complete BI inactivation with a shorter total cycle duration than VHP. This difference may provide operational advantages in applications where rapid turnaround is desirable, such as sterility testing and aseptic processing environments. However, overall chamber availability remains dependent on achieving acceptable residual hydrogen peroxide levels before operator access is permitted.

7.3 Residue Profile

Both NHP and VHP technologies utilize hydrogen peroxide as the active sporicidal agent and therefore require reduction of residual H_2O_2 concentrations to safe levels before chamber access can be granted. In both systems, hydrogen peroxide is ultimately decomposed into water and oxygen through natural decay and/or catalytic conversion during the aeration phase.

The aeration time required to achieve acceptable residual H_2O_2 levels depends on several factors, including chamber configuration, hydrogen peroxide concentration, airflow characteristics, catalyst performance, and overall cycle parameters. In this study, the selected optimized cycles, NHP Cycle 10 and VHP Cycle 5, both achieved acceptable residual H_2O_2 levels following an aeration phase of equal duration for both technologies. However, because residual concentration profiles may vary depending on cycle conditions and equipment configuration, residual H_2O_2 monitoring and aeration verification remain important components of cycle development and validation for both technologies.

7.4 Technology Selection Guidance

Based on the results obtained in this study, both NHP and VHP demonstrated the ability to achieve complete BI inactivation when operated within validated cycle parameters.

NHP may be advantageous where:

- Rapid cycle completion is a priority.
- Short turnaround times are desired for routine operations.
- Simplified decontamination workflows are preferred.

VHP may be advantageous where:

- Existing facility infrastructure and operating procedures are already established around VHP technology.
- Customers prefer a decontamination technology with extensive historical use and regulatory familiarity in pharmaceutical isolator applications.
- Alignment with existing validation strategies is a key consideration.

Either technology may be appropriate where:

- Complete BI inactivation is the primary performance criterion.
- Validated cycle parameters are established and maintained.
- Appropriate residual hydrogen peroxide monitoring and aeration controls are implemented.

Continued verification of NHP injection weights using a calibrated balance, as described in Section 3, is recommended as part of ongoing cycle optimization and final validation activities to confirm consistent dose delivery.

8. Study Summary and Key Findings

The study was designed to generate comparative cycle development data for Nebulized Hydrogen Peroxide (NHP) and Vaporized Hydrogen Peroxide (VHP) technologies using Biological Indicators (BIs) and Enzyme Indicators (EIs) positioned throughout a pharmaceutical isolator. The results provide supporting evidence that both technologies can achieve complete biological inactivation when operated within appropriately developed cycle parameters.

The study addressed several key elements commonly considered during bio-decontamination cycle development:

1. **Sporicidal Efficacy** - Complete BI inactivation was demonstrated for both NHP and VHP under selected cycle conditions, with successful cycles achieving no BI growth across all 20 monitoring locations following 7-day incubation.

2. **Challenge Organism** - *Geobacillus stearothermophilus* Biological Indicators supplied by Mesa Labs were used as the primary efficacy challenge organism throughout the study. Growth media was supplied by True Indicating.
3. **Cycle Parameter Development** - The effects of hydrogen peroxide dose and dwell time were systematically evaluated across 24 cycle configurations, enabling identification of minimum effective cycle parameters for each technology.
4. **Worst-Case Location Assessment** - Challenging monitoring locations were identified and characterised through both the initial Worst-Case Location (WCL) assessment and subsequent cycle testing. Location 14 was identified as the most challenging location during VHP testing; Location 19 represented the most persistent challenge during NHP testing.
5. **Investigation of EI/BI Divergence** - Instances of BI/EI divergence were documented, evaluated, and incorporated into a follow-up testing plan. BI results were used as the primary biological acceptance measure, with EI results providing a supplementary measure of hydrogen peroxide exposure. Divergent results were retained for investigation and confirmatory testing.
6. **Indicator Controls** - Positive and negative controls were included throughout testing to verify BI viability, media suitability, and test integrity.

While the study successfully identified effective cycle parameters for both technologies, additional work is recommended before finalising cycle specifications. Continued gravimetric verification of NHP injection weights should be incorporated into future cycle optimisation activities to confirm consistent dose delivery. The planned three-BI confirmation runs for selected anomaly locations should also be completed before final cycle recommendations are issued.

9. Regulatory Context

This study was conducted as part of the process development activities preceding formal validation of the bio-decontamination cycle. It is not presented as evidence of a validated process. Rather, the results provide development data to support the definition and subsequent validation of cycle parameters.

The study was designed with reference to the following regulatory frameworks:

- **EU GMP Annex 1 (2022 revision) Section 4.22** — requires the interior bio-decontamination process for isolators to be automated, validated, and controlled within defined cycle parameters, using a sporicidal agent in a suitable form. It also states that the cleaning and sporicidal bio-decontamination methods should render the isolator's interior surfaces and critical zone free from viable microorganisms.
- **ISO 14937:2009** — which specifies general requirements for characterising a sterilizing agent and for developing, validating, and routinely controlling a sterilization process for medical devices. Although developed for medical-device sterilization, its general process-development and validation principles were used as a reference framework for the H₂O₂-based bio-decontamination cycle development described in this study.
- **ISO 22441:2022** — which specifies requirements for developing, validating, and routinely controlling low-temperature vaporized hydrogen peroxide sterilization processes for medical devices. The standard excludes hydrogen peroxide decontamination systems used in rooms, enclosures, or environmental spaces operating under ambient conditions. Its general process-development and validation principles were therefore used only as a reference framework for the VHP aspects of this bio-decontamination study, not as a directly applicable requirement for NHP or for isolator bio-decontamination.

10. Conclusions

This study compared Nebulized Hydrogen Peroxide (NHP) and Vaporized Hydrogen Peroxide (VHP) technologies across 24 cycle configurations in an Esco pharmaceutical isolator. Biological Indicators (BIs) and Enzyme Indicators (EIs) were placed at 20 representative monitoring locations. The objectives were to determine whether NHP could achieve sporicidal performance comparable to VHP and to identify effective cycle parameters for both technologies.

Both NHP and VHP achieved complete BI inactivation when operated using appropriately developed cycle parameters. Four NHP cycles and six VHP cycles achieved complete BI inactivation across all monitored locations, establishing an effective performance range for both technologies under the tested conditions.

For NHP, Cycle 10 was identified as the minimum effective cycle. It achieved complete BI inactivation in the high-dose group with a total cycle duration of 28.12 minutes, the shortest effective NHP cycle recorded in this study. No divergence between EI and BI results was observed, and all monitoring locations achieved EI log-reduction values greater than 6.

For VHP, Cycle 5 was identified as the minimum effective cycle. It achieved complete BI inactivation in the high-dose group with a total cycle duration of 36 minutes, the shortest effective VHP cycle recorded in this study. An EI under-read was observed at Location 14; however, the corresponding BI result remained negative, confirming successful biological inactivation at that location.

Nine instances of divergence between EI and BI results were recorded across the complete dataset: two during NHP cycles and seven during VHP cycles. In every instance, the BI showed no growth while the corresponding EI value was below the expected threshold. These divergent results should not be treated as evidence of decontamination failure or dismissed as indicator error without further investigation. Planned confirmatory testing using three BIs at selected locations will assess repeatability and support the final interpretation.

The study also established clear lower performance boundaries for both technologies. Low-dose VHP configurations consistently failed to achieve complete BI inactivation and are therefore not recommended for use on the evaluated platform. Similarly, low-dose NHP configurations with shorter dwell times did not meet the required performance criteria and should not be considered for operational use without an extended dwell time.

Overall, the results demonstrate that NHP can deliver sporicidal performance comparable to VHP while providing a shorter minimum effective cycle duration under the conditions evaluated. These findings support NHP as a viable bio-decontamination technology for pharmaceutical isolator applications and strengthen the internal evidence base for technology selection ahead of platform-level validation.

This study presents cycle-development data and does not constitute a validated process. The findings are intended to inform subsequent activities, including repeat cycle runs under loaded-chamber conditions, definition of aeration endpoints, and development of Installation Qualification (IQ), Operational Qualification (OQ), and Performance Qualification (PQ) protocols for the target production platform. These later activities will be governed by the applicable site validation and qualification procedures and regulatory requirements.

11. Considerations for Interpreting Cycle Time Claims

The cycle times reported in this study, 28.12 minutes for NHP Cycle 10 and 36 minutes for VHP Cycle 5, were generated under controlled cycle-development conditions. As with any vendor-quoted decontamination cycle time, these figures are most useful when read alongside the conditions and definitions behind them. The following considerations are provided to support informed interpretation and use of this data in future qualification and purchasing decisions.

Phase definition and clock start/stop

The total cycle times reported throughout this white paper include H₂O₂ injection/gassing, dwell, and the aeration phase required to reach safe residual H₂O₂ levels (a matched duration for both technologies' best cycles, per Section 7.3). Chamber access, as noted in Table 7, is granted only after aeration is complete. Readers comparing these figures against other vendor claims should confirm that the competing figure is measured on the same basis, since some quoted cycle times cover injection and dwell only and omit aeration, which is typically the longest phase of the cycle.

Chamber configuration and load

All results in this study were generated using an empty test isolator (Section 2.1). Actual production loads, stopper bowls, tubing, filling equipment, gloves, and other process materials, absorb and desorb hydrogen peroxide, and can meaningfully extend both the kill phase and aeration. The cycle times reported here should be reverified with a representative worst-case load and load pattern before being carried forward as production cycle specifications.

Run-to-run consistency

Each of the 24 cycle configurations evaluated in this study was demonstrated as a single run. This dataset characterises achievable performance under the tested conditions but does not, on its own, establish run-to-run repeatability. Repeat testing of the selected candidate cycles is recommended as part of continued internal cycle development to build confidence in cycle consistency.

Platform specificity

The cycle times and dose levels identified in this study are specific to the chamber geometry, volume, and H₂O₂ delivery configuration of the Esco test isolator used (Section 2.1). They should not be assumed to transfer directly to isolators of a different size or configuration without independent cycle development and verification on that platform.

Aeration endpoint definition

The residual H₂O₂ concentration threshold, sensor type, and sensor location used to confirm that aeration is complete should be documented as part of future validation protocols, since the aeration endpoint definition directly determines the reported total cycle time and the point at which chamber access is granted.

Applying this level of transparency consistently, both when presenting this study's results and when evaluating comparable claims from other suppliers, gives a more reliable basis for comparing decontamination technologies and for modeling real batch-to-batch turnaround time.

12. References and Standards

1. European Commission. *EudraLex Volume 4: Good Manufacturing Practice (GMP) Guidelines, Annex 1 – Manufacture of Sterile Medicinal Products*. European Commission; 2022.
2. International Organization for Standardization (ISO). *ISO 14644-4:2001 – Cleanrooms and Associated Controlled Environments – Part 4: Design, Construction and Start-up*. Geneva, Switzerland: ISO; 2001.
3. International Organization for Standardization (ISO). *ISO 14937:2009 – Sterilization of Health Care Products – General Requirements for Characterization of a Sterilizing Agent and the Development, Validation and Routine Control of a Sterilization Process for Medical Devices*. Geneva, Switzerland: ISO; 2009.
4. International Organization for Standardization (ISO). *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*. Geneva, Switzerland: ISO; 2022.
5. International Organization for Standardization (ISO). *ISO 11138-3:2017 – Sterilization of Health Care Products – Biological Indicators – Part 3: Biological Indicators for Moist Heat Sterilization Processes*. Geneva, Switzerland: ISO; 2017.
6. Pharmaceutical Inspection Co-operation Scheme (PIC/S). *PE 009-16: Guide to Good Manufacturing Practice for Medicinal Products*. PIC/S; 2022.
7. Protak Scientific. *Biological Indicator and Enzyme Indicator Performance Data for Hydrogen Peroxide Bio-Decontamination Applications*. Protak Scientific.
8. Mesa Labs. *Biological Indicator Product Specifications for Geobacillus stearothermophilus Spore Carriers*. Mesa Labs.
9. Esco Lifesciences. *Comparative Evaluation of Nebulized Hydrogen Peroxide (NHP) and Vaporized Hydrogen Peroxide (VHP) for Pharmaceutical Isolator Bio-Decontamination: Internal Cycle Development Study Report*. Healthcare Business Unit.
10. McLeod, N.P., Clifford, M., and Sutton, J.M. *Evaluation of Novel Process Indicators for Rapid Monitoring of Hydrogen Peroxide Decontamination Processes*. *PDA Journal of Pharmaceutical Science and Technology*. Published May 15, 2017. Licensed to Protak Scientific Ltd.
11. Cleanroom Technology / Protak Scientific. *How AstraZeneca Increased Isolator Productivity by Over 75% in 10 Days with Enzyme Indicators*. *Cleanroom Technology Magazine*. July 2024.
12. Esco Lifesciences. *Reading Between the Lines: How to Evaluate Decontamination Cycle Time Claims*. Internal white paper, 2026.

Appendix A: Monitoring Location Reference

NHP Locations

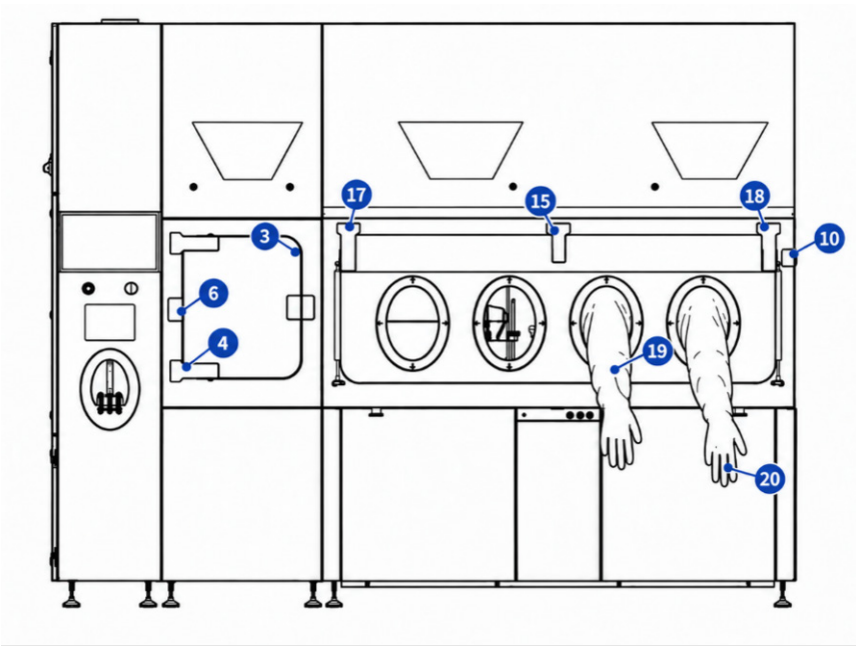


Figure A1: Monitoring Location Reference for NHP

VHP Locations

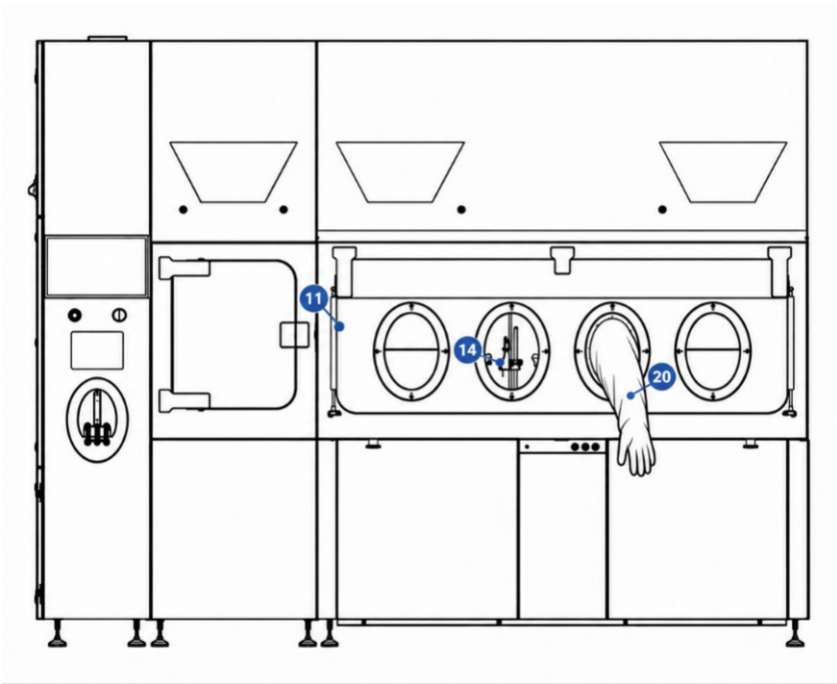


Figure A2: Monitoring Location Reference for VHP

