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Research Article

Development and Preliminary Evaluation of a Lyophilized HBsAg Internal Quality Control Material for ELFA

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A B S T R A C T

Hepatitis B surface antigen (HBsAg) testing is essential for the screening, diagnosis, and monitoring of hepatitis B virus infection, and the reliability of test results depends on stable internal quality control (IQC) materials with consistent analytical performance. Commercial IQC materials are generally reliable but relatively expensive, import-dependent, and not always sustainably available. Lyophilization offers an effective preservation method that maintains antigen stability and extends shelf life. This study aimed to develop a next-generation lyophilized HBsAg IQC material and to evaluate its homogeneity, stability, and analytical performance using the Enzyme-Linked Fluorescent Assay (ELFA) method. HBsAg-positive donor sera with moderate reactivity were pooled, supplemented with cryoprotectants, lyophilized, and reconstituted prior to testing. Homogeneity assessment showed excellent vial-to-vial consistency, with coefficients of variation (CVs) below 5%. The lyophilized control remained reactive (index >1.0) throughout 10 days of observation, with all storage conditions showing good precision (CV 5.92–10.77%). The best performance was found in samples reconstituted with distilled water and refrozen, yielding a mean HBsAg index of 9.874, CV of 5.92%, bias of 0.94%, and no significant trend over time ($p = 0.982$). Room-temperature storage and NaCl reconstitution showed significant declines ($p < 0.05$), whereas refrigerated storage at 2–8°C remained stable (CV 7.13%; $p = 0.435$). The developed lyophilized HBsAg IQC material exhibited excellent homogeneity, good short- and long-term stability, and analytical performance comparable to commercial IQC materials on the ELFA platform, indicating its potential as a reliable, stable, and cost-effective internal quality control for routine HBsAg testing.

Keywords: *Enzyme-Linked Fluorescent Assay (ELFA); Hepatitis B surface antigen (HBsAg); Internal quality control; Lyophilization; Post-reconstitution stability*

1. Introduction

Hepatitis B remains a major public health issue worldwide. According to 2022 estimates from the World Health Organization (WHO), approximately 254 million people worldwide are living with chronic hepatitis B virus (HBV) infection. In addition, viral hepatitis is the second-leading cause of death from infectious diseases globally. In the screening and diagnosis of HBV infection, the detection of hepatitis B surface antigen (HBsAg) plays a key role, as it is the earliest serological marker to appear and the most widely used for identifying HBV infection. (*World Health Organization*, 2024).

Various immunoassay platforms have been developed to detect HBsAg; one of these is the Enzyme-Linked Fluorescent Assay (ELFA), which offers high sensitivity and reproducibility and is therefore frequently used as the reference method (Putri et al., 2024). However, the performance and reliability of an immunoassay method are determined not only by its analytical characteristics, but also by the availability of stable internal quality control (IQC) materials and the implementation of quality control protocols underpinned by a robust statistical approach (Peng et al., 2021). Commercial IQC materials generally offer a good level of reliability, but

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they are relatively expensive to use and their availability is not always guaranteed on a long-term basis. As an alternative, control materials obtained through pooling or produced in-house are reported to offer precision and stability comparable to that of commercial control materials, at a lower cost (Kulkarni et al., 2020). In line with this, a number of studies have begun to explore the use of in-house-produced freeze-dried quality control (QC) materials as a more cost-effective alternative. One such application is the use of freeze-dried plasma as a control material in rapid HIV diagnostic tests, which has been reported to remain stable for six months under refrigerated storage conditions (Jomjunyoung et al., 2026).

Freeze-drying, which comprises the freezing, primary drying and secondary drying stages, is a well-established process that adheres to the principles of Quality by Design to maintain the stability of biopharmaceuticals and biological materials during storage (Pardeshi et al., 2023). The use of appropriate lyoprotectants, such as trehalose or sucrose, can help preserve the native conformation and antigenic epitopes of proteins, including HBsAg, through water replacement and vitrification mechanisms (Li et al., 2024). Although this principle has been widely applied, there are still few publications on locally developed lyophilized HBsAg Internal Quality Control (IQC) materials validated on the ELFA platform. Therefore, this study aims to develop a new generation of lyophilized HBsAg IQC materials and evaluate their homogeneity, stability, and analytical performance using the ELFA method.

The novelty of this study lies in the development and preliminary characterization of a locally produced, plasma-based, lyophilized HBsAg internal quality control (IQC) material formulated with 5% (w/v) trehalose for enzyme-linked fluorescent assay (ELFA). The study evaluates between-vial homogeneity and systematically compares two reconstitution diluents and three post-reconstitution storage conditions in terms of analytical precision and signal stability over 10 days. The findings provide preliminary evidence to support local IQC development and identify conditions associated with better signal preservation, while highlighting the need for further evaluation of long-term stability, between-batch consistency, and commutability.

2. Methods

2.1 Materials and Instruments

One voluntary blood donor was selected as the source of approximately 150 mL of residual HBsAg-reactive plasma obtained through routine blood donation and screening at the Indonesian Red Cross (PMI). The donor met routine blood donation eligibility criteria, and the plasma showed an initial HBsAg reactivity of 10.76 S/CO using the VIDAS® HBs Ag Ultra assay (bioMérieux). The plasma had adequate volume and acceptable physical quality, without marked hemolysis, lipemia, or icterus, and was non-reactive for HIV, HCV, and syphilis. Samples were excluded for insufficient volume, poor physical quality, inappropriate storage, repeated freeze–thaw cycles, incomplete screening information, reactivity for other relevant infectious markers, or unsuitable HBsAg reactivity.

Donor consent for the use of residual non-transfusion plasma was obtained by PMI as part of routine blood donation. No additional donor intervention or blood collection was performed. The use of residual plasma was conducted under an established collaboration between PMI and the research institution and was reviewed and approved by the relevant Health Research Ethics Committee. The specimen was de-identified and coded before transfer. Because HBsAg-reactive plasma was considered potentially infectious, all specimens were handled by trained personnel using appropriate PPE and containment according to institutional biosafety procedures. Contaminated materials were decontaminated and disposed of as infectious/B3 laboratory waste, with access restricted to authorized personnel (WHO, *Laboratory Biosafety Manual*, 4th ed., 2020).

The plasma was titrated to obtain a low-positive HBsAg reactivity of 1.5–2.0 S/CO, followed by addition of trehalose to a final concentration of 5% (w/v). The formulation was homogenized and filled at 1 mL per vial. Lyophilization was performed using a LYO60B-1T benchtop freeze dryer with a −60 °C condenser. The formulation was frozen to −40 °C at approximately 0.5 °C/min and held for 60 min, followed by primary drying at approximately 100 mTorr (13 Pa), with the shelf temperature initially at −25 °C and gradually increased to −10 °C. Secondary drying was performed at +20 to +25 °C for 4–6 h under reduced pressure. The endpoint was defined by stabilization of product temperature and chamber pressure and confirmation of adequate drying, with residual moisture acceptance of ≤3%. The vials were then stoppered under controlled conditions and stored at 2–8 °C until characterization.

The lyophilized HBsAg IQC material was reconstituted by adding 3,000 µL of 0.9% NaCl to 1,000 µL of lyophilized material (1:3, v/v). After gentle mixing until completely dissolved, the material was allowed to

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stand at room temperature for 30 min for rehydration and equilibration, followed by gentle homogenization before analysis using the VIDAS® HBs Ag Ultra assay.

For homogeneity assessment, 14 vials (approximately 10% of the 137-vial batch) were randomly selected. Each vial was reconstituted as described above and tested individually for HBsAg reactivity. The resulting S/CO values were assessed for between-vial homogeneity in accordance with ISO 33405:2024, Reference materials—Approaches for characterization and assessment of homogeneity and stability. For post-reconstitution stability assessment, distilled water (aquadest) and 0.9% NaCl were evaluated under three storage conditions: freeze–thaw, room temperature, and 2–8 °C. For the freeze–thaw condition, reconstructed material underwent 10 cycles, each consisting of 12 h at −20 °C followed by 12 h at room temperature. After the cycles, the material was returned to the respective storage condition according to the study schedule. HBsAg reactivity was measured daily for 10 consecutive days using the VIDAS® HBs Ag Ultra assay. Changes in S/CO over time were evaluated to assess potential analytical drift and compare stability across diluent and storage conditions.

Analytical Run Structure and Monitoring of Analytical Drift. For homogeneity assessment, a stratified random sampling approach was used to select 14 vials (approximately 10% of 137 vials), comprising four vials from trays 1–4, six from trays 5–10, and four from trays 11–14. Each vial was independently reconstructed and analyzed in duplicate within the same analytical run, with all 14 vials tested in the same run to minimize between-run variation. All analyses were performed using the VIDAS® automated immunoanalyzer (model IVD 3004041), with the VIDAS® HBs Ag Ultra reagent kit, reagent lot number 1011772160. For post-reconstitution stability testing, six experimental conditions were evaluated daily for 10 consecutive days, resulting in 10 analytical runs. All six conditions were analyzed within the same run whenever possible. An independent IQC material was included in each run to monitor assay and instrument performance. Runs were accepted according to predefined QC criteria, while instrument status, reagent lot, QC results, and relevant maintenance or calibration records were documented throughout the study. Potential analytical drift was assessed by comparing daily HBsAg S/CO values with the Day 0 baseline, including absolute and percentage changes and longitudinal trends across the six conditions (ISO 33405:2024).

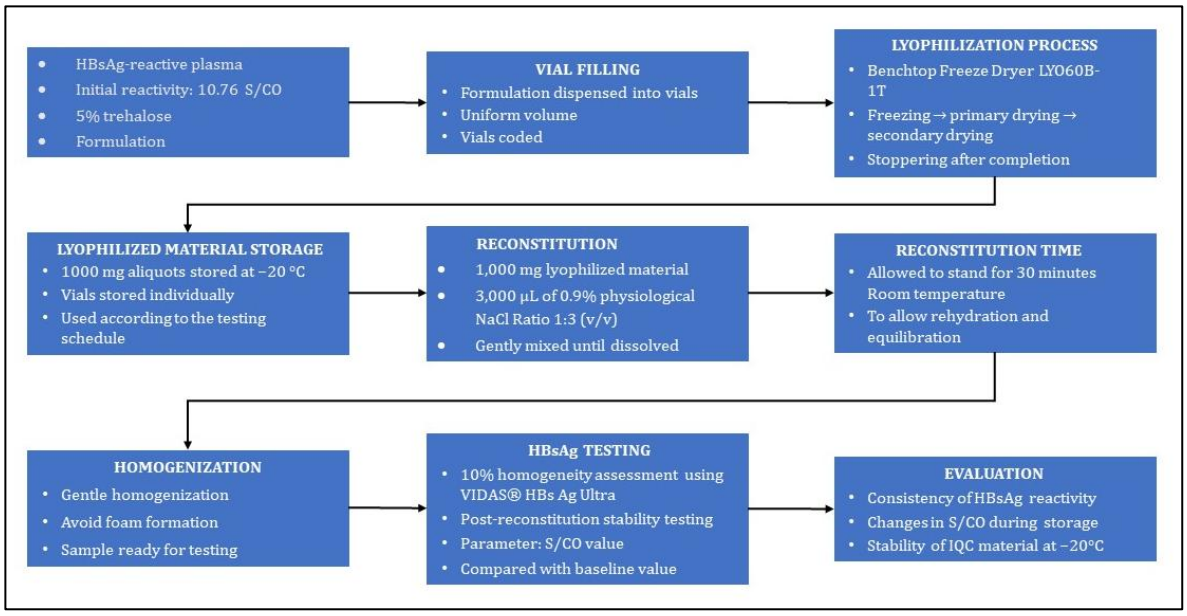


Figure 1. Flow diagram of the experimental procedure

3. Results and Discussion

3.1 Homogeneity data

Table 1. Homogeneity data

Sample	Result 1	Result 2
1	11.45	10.28
2	10.26	9.78
3	10.43	10.54
4	10.21	9.89
5	10.67	11.59
6	10.32	10.52
7	10.73	11.56
8	9.21	10.23
9	10.87	10.54
10	10.71	10.81
11	10.36	10.22
12	11.22	10.41
13	10.33	10.21
14	10.31	10.64
Mean	10.50571429	10.51571429
SD	0.507862668	0.507792341
CV	4.834156483	4.828890619
Bias	-0.023632501	-0.022703133
Target	10.76	10.76
TEa	20	20

Table 2. Homogeneity assessment results of the lyophilized HBsAg internal quality control material

Variation Component	SS	df	MS	F	p-value
Between-vial	4,5979	13	0,35368	1,887	**0,1258**
Within-vial	2,6237	14	0,18741		
Total	7,2216	27			
SD within-vial	0,43291 S/CO				
SD between-vial	0,28834 S/CO				
CV within-vial	4,12%				
CV between-vial	2,74%				
SD total	0,51717 S/CO				

One-way ANOVA was performed to evaluate between-vial and within-vial variability using 14 randomly selected vials, with two independent measurements per vial. The grand mean was 10.511. The between-vial sum of squares was 4.598 (df = 13), with a mean square of 0.354, whereas the within-vial sum of squares was 2.624 (df = 14), with a mean square of 0.187. The resulting F-statistic was 1.887 with a p-value of 0.126. Since the p-value was greater than 0.05, no statistically significant difference was observed among the vial means. These findings indicate that the lyophilized material demonstrated no significant between-vial variation under the conditions tested and was considered statistically homogeneous based on the one-way ANOVA.

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3.2 Stability Data

Table 3. Stability assessment results of the lyophilized HBsAg internal quality control material under different storage and post-reconstitution conditions

	Reconstitution with distilled water			Reconstitution with NaCl 0.9%		
Day	**Freeze–thaw**	**Room Temperature (20-25)**	**Refrigerator (2-8)**	**Freeze–thaw**	**Room Temperature (20-25)**	**Refrigerator (2-8)**
1	10.42	9.93	9.56	11.58	11.87	11.35
2	11.32	10.45	10.21	12.32	11.33	11.66
3	11.26	10.89	9.34	11.43	11.02	10.49
4	12.65	9.16	9.69	11.34	10.61	9.65
5	12.49	9.82	11.21	10.33	10.45	10.26
6	13.20	9.4	9.22	11.24	9.96	10.98
7	12.37	9.11	10.11	12.3	10.2	9.57
8	11.40	9.38	10.21	12.12	10.57	9.17
9	11.23	9.23	9.54	11.83	9.54	10.22
10	11.85	9.06	9.65	13.43	9.77	9.72
Mean	11.819	9.643	9.874	11.792	10.532	10.307
SD	0.55438615	0.589576967	0.799605528	0.776325318	0.684219263	0.784306063
CV	4.69063497	6.114040931	8.098091227	6.583491506	6.496574849	7.609450497
Target		10.5	10.5	10.5	10.5	10.5
Bias	12.5619048	-8.161904762	-5.961904762	12.3047619	0.304761905	1.838095238

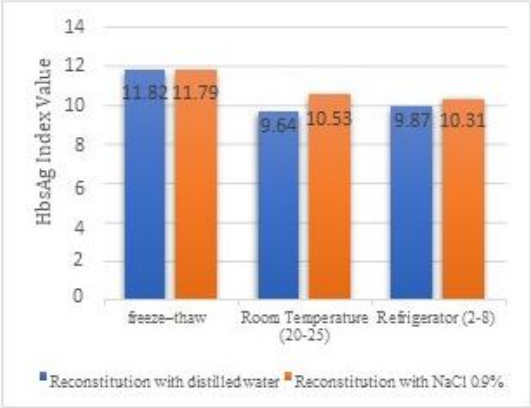


Figure 2. The results of the stability evaluation under six experimental conditions using two reconstitution solvents, distilled water and 0.9% NaCl, showed that the HBsAg control remained reactive (index > 1.0) throughout the observation period. The mean reactivity index ranged from 9.64 under storage at 25°C to 11.84 after repeated freeze–thaw cycles

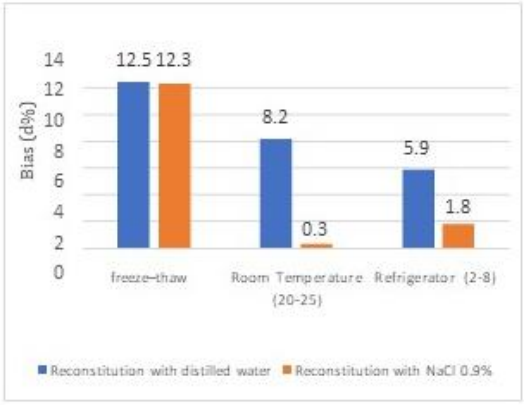


Figure 3. Accuracy comparison based on percentage bias relative to the target value using two reconstitution solvents. Bias values ranged from 0.3% to 1.8% when 0.9% NaCl was used under storage conditions at 2–8°C and 25°C, whereas bias values exceeded 5% when distilled water was used across all evaluated storage conditions

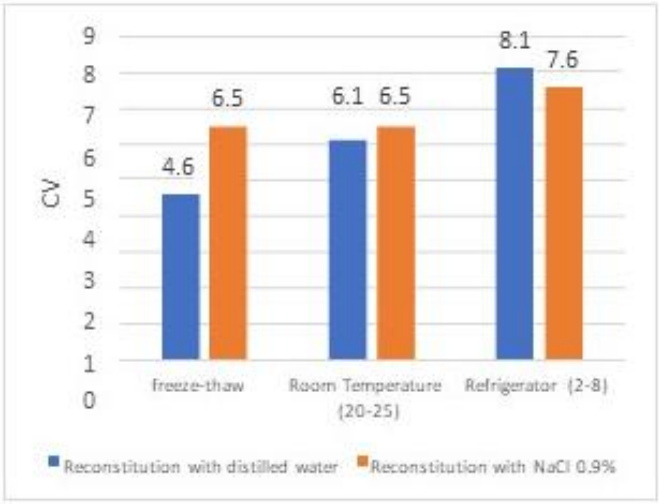


Figure 4. Precision, expressed as the coefficient of variation (CV%), ranged from 4.6% to 8.1%. Distilled water showed higher sensitivity to storage conditions (CV ranging from 4.6% to 8.1%), while 0.9% NaCl exhibited a more stable CV profile across temperature changes (ranging from 6.5% to 7.6%)

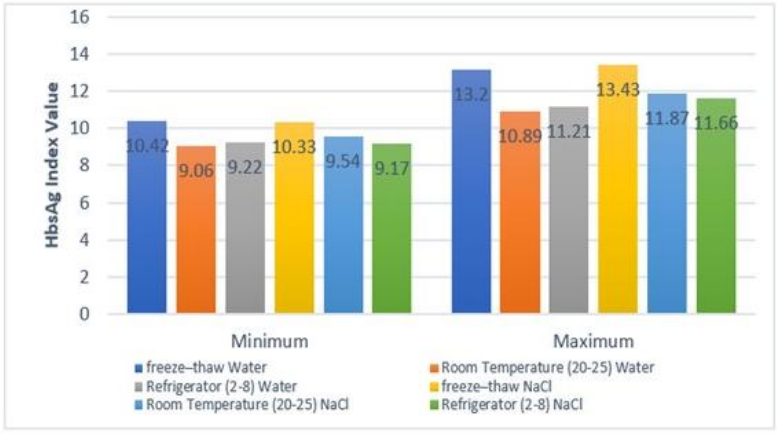


Figure 5. Peak Index: Freeze–thaw produced the highest HBsAg index values overall, reaching up to 13.43 with NaCl. Lowest Index: Room temperature (water) yielded the lowest values across both minimum (9.06) and maximum (10.89) levels. Diluent Effect: 0.9% NaCl consistently resulted in higher maximum index values than distilled water across all storage conditions

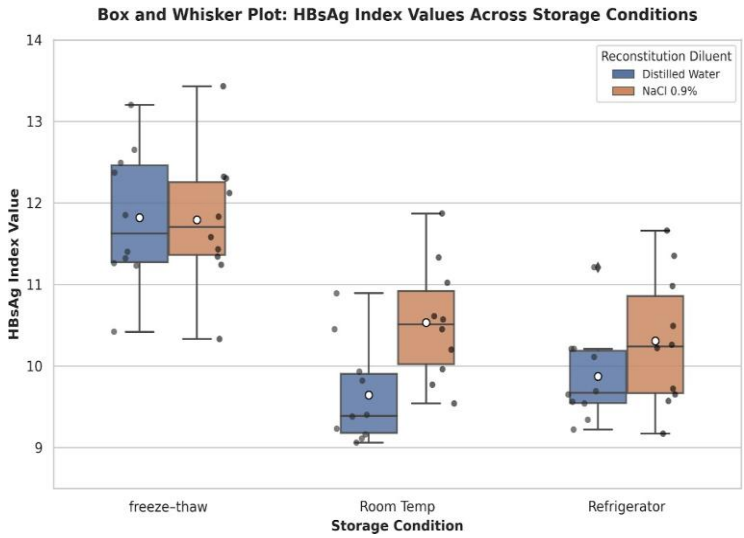


Figure 6. Box and whisker plot of HBsAg index values over 10 days across three storage conditions (*freeze–thaw*, room temperature [20–25°C], and refrigerator [2–8°C]) following reconstitution with distilled water vs. 0.9% NaCl. Boxes indicate the interquartile range (Q1–Q3), center horizontal lines mark the median, white circles represent the mean, and whiskers extend to the minimum and maximum values

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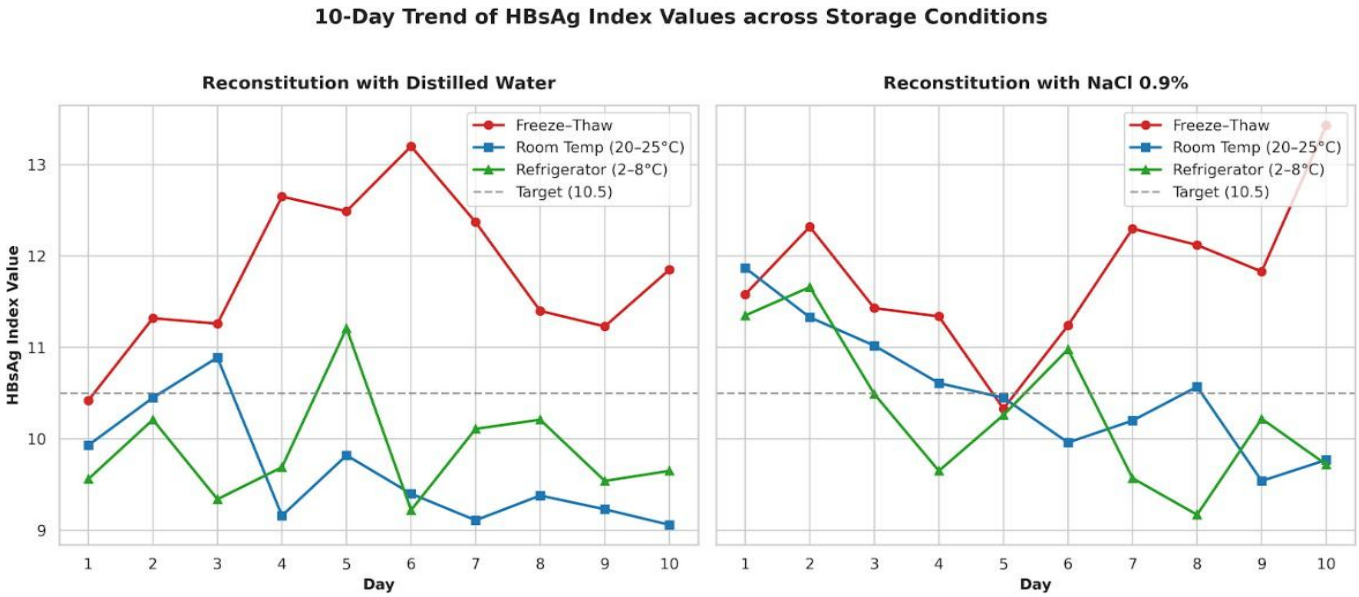


Figure 8. Over the 10-day observation period, stability trends differed among storage conditions. Significant Downward Trends: Room Temperature storage for both diluents and Refrigerator storage for 0.9%NaCl show statistically significant daily declines ($p < 0.05$). Strongest Linearity: NaCl 0.9% at Room Temperature exhibits the strongest linear decrease ($R^2 = 0.8112$, $p = 0.0004$). Stable Conditions: Freeze–thaw cycles show slight positive slopes but no statistically significant daily trend ($p > 0.05$)

This study evaluated the development, homogeneity, and post-reconstitution stability of an in-house lyophilized Hepatitis B surface antigen (HBsAg) internal quality control (IQC) material evaluated on the Enzyme-Linked Fluorescent Assay (ELFA) platform. As commercial quality control materials pose substantial long-term financial burdens for clinical laboratories, particularly in low- and middle-income settings, locally prepared lyophilized controls offer a viable, cost-effective alternative to ensure analytical reliability in routine HBV screening. The cut-off value for the VIDAS reagent is 0.13 when the short protocol (HBS) is used and 0.10 when the long protocol (HBL) is used. In this study, the HBsAg IQC material was characterized using its assay index value, which cannot be directly converted into a cut-off value because both represent assay index values, whereas the cut-off for VIDAS HBs Ag Ultra is established according to the protocol and the reagent instructions for use (IFU). The HBsAg IQC material exhibited an index value of 10.76, approximately 82.77 times the assay cut-off of 0.13, indicating a reactive material with moderate-to-high reactivity.

The use of lyophilized material as an internal quality control (IQC) material requires it to have appropriate characteristics for monitoring the quality of testing, including meeting several requirements, namely stability, homogeneity, precision, safety for use, and compliance with established acceptance criteria (CLSI, 2023). Based on the homogeneity, stability, precision, and other evaluation data, lyophilized material may be used as an alternative internal quality control (IQC) material for HBsAg testing, provided that it meets the established acceptance criteria and is suitable for the intended analytical method.

3.3 Batch Homogeneity

Assessment of the 137-vial batch demonstrated high batch uniformity. One-way ANOVA revealed no statistically significant between-vial variation ($F = 1.887, p = 0.126$), meeting the criteria established by ISO 33405:2024 for reference materials. The between-vial coefficient of variation (CV) was 2.74%, and the within-vial CV was 4.12%, yielding a total standard deviation of 0.517 S/CO. These metrics confirm that the formulation, automated filling (1 mL/vial), and lyophilization cycles with 5% (w/v) trehalose produced a statistically homogeneous product. The protective role of trehalose as a lyoprotectant effectively prevented protein denaturation during the freeze-drying process by maintaining the structural integrity of HBsAg epitopes. There are no universal CV and bias acceptance limits applicable to all methods and reactivity levels in infectious disease immunoassays. However, for this study, the internal acceptance criteria were established based on a strict target of CV < 5% for reactive IQC materials, while the acceptable relative bias against the target value was set at ≤ ±11.58%, based on the inter-assay precision reported for the VIDAS HBs Ag Ultra short protocol (HBS).

3.4 Post-Reconstitution Stability and Temperature Dynamics

Across all six experimental conditions evaluated over 10 consecutive days, the reconstituted HBsAg control material consistently maintained S/CO values well above the reactivity threshold (> 1.0 S/CO), confirming sustained analytical reactivity. However, distinct longitudinal trends and precision profiles emerged depending on the storage temperature and choice of reconstitution diluent:

1. Room Temperature Storage (20--25◦C): Post-reconstitution storage at room temperature (20–25°C) demonstrated promising performance of the lyophilized HBsAg material, particularly when reconstituted with 0.9% NaCl. The mean S/CO values were 9.643 for distilled water and 10.532 for 0.9% NaCl, with corresponding CVs of 6.11% and 6.50%. Relative to the target value of 10.5 S/CO, the biases were −8.16% and +0.30%, respectively. Notably, the NaCl-reconstituted material maintained a mean reactivity close to the target, supporting its potential suitability for further development as an in-house IQC material. Although both groups exceeded the predefined CV criterion of less than 5%, these findings provide useful preliminary information for optimizing the reconstitution protocol. Further investigation is needed to determine the contribution of storage conditions, diluent composition, and analytical variation to the observed results before establishing a validated post-reconstitution storage period.
2. Refrigerated Storage (2--8◦C): At 2–8°C, mean S/CO values were 9.874 for distilled water and 10.307 for 0.9% NaCl, with CVs of 8.10% and 7.61% and biases of −5.96% and −1.84%, respectively. The NaCl group showed mean reactivity closer to the target and a slightly lower CV. Although both groups exceeded the 5% CV criterion, these results support further evaluation of NaCl as a reconstitution medium under refrigerated conditions. Studies using independent aliquots, controlled measurements, and longer observation periods are needed to establish storage duration and performance limits.
3. Freeze–Thaw Cycles: Repeated freeze–thaw cycles over 10 days, consisting of freezing at −20°C for 12 hours followed by thawing at room temperature, resulted in an overall increase in S/CO values, with maximum values of 13.20 S/CO in distilled water and 13.43 S/CO in 0.9% NaCl. Although the daily linear trend was not statistically significant ($p > 0.05$), the increase in S/CO values during freeze–thaw cycling may be associated with concentration effects caused by freezing, small-scale protein aggregation, or structural changes that expose additional antigenic epitopes, making them more readily recognized by monoclonal antibodies in the ELFA assay. These proposed mechanisms require further investigation for confirmation.
4. Diluent Comparison : Under freeze–thaw conditions, mean S/CO values were comparable for distilled water (11.819; CV 4.69%) and 0.9% NaCl (11.792; CV 6.58%). Only the distilled-water group met the predefined CV criterion of less than 5%. Under room-temperature and refrigerated storage, the NaCl group showed mean values closer to the target of 10.5 S/CO, suggesting that the reconstitution medium may influence post-reconstitution performance.

Despite CVs exceeding 5% under room-temperature and refrigerated conditions, the findings identify opportunities to optimize reconstitution and storage protocols. Overall, the results support continued development of the lyophilized HBsAg preparation as a potential in-house IQC material for ELFA. However, further statistical analyses and studies involving independent batches, different storage durations, and controlled conditions are needed to confirm these findings and establish a reproducible protocol for routine internal quality control.

3.5 Practical Implications and Limitations

These findings indicate that while 0.9% NaCl offers predictable within-run precision, distilled water combined with refrigerated storage (2-8◦C) is the superior choice for minimizing analytical drift in daily post-reconstitution usage. To maintain optimal IQC accuracy, reconstituted controls should ideally be aliquoted and stored at 2-8◦Cor used within short timeframes to avoid room-temperature signal decay.

Limitations: This study represents a preliminary feasibility evaluation of a lyophilized HBsAg material as a potential alternative internal quality control (IQC) material. Only one production lot was prepared and evaluated. Although the findings provide initial evidence regarding homogeneity, stability, and precision, further evaluation involving multiple independent production lots is necessary to establish lot-to-lot consistency, assign target values and acceptance limits, assess metrological traceability where applicable,

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and demonstrate suitability for routine clinical laboratory use. Therefore, the material should not yet be considered fully validated for routine clinical implementation.

Another limitation is that IQC performance was evaluated using only one automated platform, VIDAS® ELFA, and post-reconstitution stability was monitored for 10 days. Furthermore, the commutability of the in-house lyophilized HBsAg IQC material was not evaluated; therefore, its equivalence to native patient specimens in terms of analytical behavior cannot be established. The homogeneity, stability, and precision demonstrated under the conditions investigated do not necessarily guarantee that the material behaves similarly to native patient specimens. Future studies should evaluate the long-term real-time shelf life of the un-reconstituted lyophilized vials, assess post-reconstitution stability to establish an acceptable storage period, and investigate commutability across alternative immunoassay platforms, such as chemiluminescence immunoassay (CLIA) and enzyme-linked immunosorbent assay (ELISA). Evaluation of multiple independent production lots is also necessary to strengthen the evidence of lot-to-lot consistency and support the validation of the material for routine IQC use.

4. Conclusion

The locally developed lyophilized HBsAg internal quality control material was successfully formulated using 5% trehalose and validated on the ELFA platform. The prepared material met ISO 33405:2024 statistical criteria for batch homogeneity (p = 0.126), demonstrating uniform between-vial quality. During 10 days of post-reconstitution monitoring, the control remained reactive across all experimental conditions. Reconstitution with distilled water stored under refrigerated conditions (2—8^{0}C) provided the most stable profile with virtually no analytical drift (p = 0.9821), whereas room-temperature storage caused significant degradation over time. This in-house lyophilized IQC material presents a robust, homogeneous, and cost-effective solution for routine laboratory quality assurance in HBV serological testing. The evaluation period of ten days does not establish long-term stability and that the study was limited to a single reactivity level. Therefore, further studies involving longer observation periods and materials with different reactivity levels, particularly those closer to the assay cut-off, are needed to establish the material's broader applicability as an IQC material. The limitations paragraph in the Discussion has been retained and the Abstract and Conclusion have been revised accordingly

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