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A Collaborative Study to Evaluate the Candidate 4th WHO International Standard for Hepatitis B Surface Antigen

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** Details provided in Appendix 1*

NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposal(s) contained therein. Written comments on the proposal(s) MUST be received in English by **25 September 2026** and should be addressed to:

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Summary

A WHO international collaborative study was undertaken to characterise and assess the suitability of a candidate 4th International Standard (IS) for hepatitis B Surface Antigen (HBsAg) to replace the current IS (NIBSC Code 12/226) for use in the standardisation of HBsAg serological assays. The candidate is prepared from the same source material as the 3rd IS; a non-adjuvanted, plasma-derived HBsAg vaccine bulk formulated in human serum at a target concentration of ~35 International Units (IU)/mL. The formulated bulk was then dispensed into ampoules in 0.5 mL volumes and lyophilised for long-term stability. The candidate antigen is Hepatitis B virus (HBV) genotype B4 with a heterogeneous HBsAg subtype population of *ayw1* and *adw2*.

Thirteen laboratories from ten countries participated in the collaborative study to evaluate the replacement standard. The lyophilised candidate IS was evaluated alongside the 3rd WHO IS for HBsAg (NIBSC code: 12/226). Additional blinded samples were provided for testing in the collaborative study. These included three clinical samples, a recombinant HBsAg protein and a liquid preparation of the candidate IS. A range of commercial qualitative and quantitative HBsAg tests were used to evaluate the candidate.

The results of the collaborative study indicate the suitability of the candidate to serve as a replacement IS and it is proposed that 25/120 is established as the 4th WHO IS for HBsAg with an assigned potency of 17.5 IU/ampoule (35 IU/mL when reconstituted in 0.5 mL of deionised water).

1. Introduction

In 2022, the World Health Organization (WHO) estimated that 254 million people globally were living with chronic hepatitis B virus (HBV), with chronic infection accounting for an estimated 1.1 million deaths. This represents a significant rise in mortality relative to estimates in 2019 of 550,000 [1], reiterating the continued burden of liver cirrhosis and hepatocellular carcinoma, resulting from chronic HBV infection.

Despite the availability of effective prophylactic vaccines, HBV transmission persists, primarily through exposure to infected blood and bodily fluids, with many infected individuals remaining undiagnosed or untreated. WHO data indicates that only 13.4% of people chronically infected with HBV are diagnosed, and just 2.6% receive antiviral treatment, underscoring substantial gaps in the global cascade of care [2].

Diagnosis of HBV infection relies predominantly on sensitive serological in-vitro diagnostic assays, including enzyme-linked immunosorbent assays (ELISA) and chemiluminescent immunoassays (CLIA), which detect the hepatitis B surface antigen (HBsAg). Quantitative HBsAg assays additionally enable clinicians to monitor disease progression, assess therapeutic response, and help distinguish between clinical phases of chronic HBV infection [3]. Given the global circulation of multiple HBV genotypes and subtypes, HBsAg assays are designed to target conserved antigenic regions to ensure reliable detection across diverse viral strains.

To support consistent diagnostic performance, the European Commission requires that first-line HBsAg assays meet a minimum analytical sensitivity of <0.130 IU/mL, as specified in the Common Specifications for class D in vitro diagnostic devices, under the EU IVD Regulation (EU) 2022/1107 [4]. These criteria ensure adequate sensitivity for both clinical and blood screening contexts. The WHO International Standard (IS) for HBsAg, first established

in 1985 (NIBSC code 80/549), is used extensively by in-vitro diagnostic manufacturers for over 40 years for assay calibration and validation, and by clinical and transfusion laboratories for establishing secondary reference materials. The long-term availability of such standards has contributed to the high level of sensitivity and harmonisation observed across modern HBsAg assay platforms.

Stocks of the 3rd WHO IS for HBsAg (NIBSC code: 12/226, with an assigned potency of 47.3 IU per ampoule) are nearing depletion. Following review and endorsement by the WHO Expert Committee on Biological Standardization (ECBS) in October 2024, development of a replacement material was initiated. This report presents the candidate preparation and global collaborative evaluation of the proposed 4th WHO IS for HBsAg, intended to support harmonisation and long-term consistency in HBsAg assay performance worldwide.

Aims of the Study

The aims of this collaborative study were to:

- Assess the suitability of the freeze-dried HBsAg preparation, formulated in human serum, to serve as the 4th WHO IS for HBsAg for use in the standardisation of HBsAg serology assays, including an assessment of its long-term stability
- Evaluate the candidate 4th IS relative to the 3rd WHO IS for HBsAg (NIBSC code: 12/226) and assign a unitage in International Units (IU).
- Assess the candidate's potency i.e. reactivity in a range of typical assays performed in different laboratories.
- To provide a limited assessment of commutability i.e. to establish the extent to which the candidate standard performs relative to clinical samples in different assay systems.

2. Bulk material

The HBsAg source material is a non-adjuvanted plasma-derived HBsAg vaccine bulk (Lot HB-PL01) that was previously used to prepare the 3rd IS for HBsAg (NIBSC code: 12/226). There were sufficient stocks remaining to prepare a candidate for the 4th IS for HBsAg. The material was kindly donated in 2012 by Professor Nguyen Thu Van, VABIOTECH, Hanoi, Vietnam. The HBsAg stock material was derived from pooled human plasma obtained from asymptomatic carriers with high titres. The HBsAg stock material was purified and inactivated at VABIOTECH using validated methods for the manufacture of plasma-derived Hepatitis B vaccine [5]. The HBsAg bulk material was tested at MHRA-NIBSC and found negative for antibodies to HIV and HCV RNA. The inactivated HBsAg material is positive for HBV DNA
.

Molecular and antigenic characterisation of source HBsAg bulk

The plasma-derived HBsAg vaccine bulk was characterised in 2012 at the National Reference Centre for Hepatitis B and D Viruses, Justus-Liebig University Giessen (Germany) [6]. HBV DNA sequence analysis of the S gene was performed to determine genotype and subtype composition, while antigenic characterisation of the HBsAg preparation was assessed using immunological methods, including Western blot analysis with antibodies specific for S, preS1 and preS2 regions. These analyses provided information on the genetic composition and protein profile of the source material.

3. Processing of the candidate WHO IS

Preparation, filling and freeze-drying of the candidate 4th IS (25/120)

The HBsAg concentration of the candidate was determined prior to filling, using parallel line analysis (CombiStats) of prepared dilutions of the HBsAg bulk material relative to the 3rd IS for HBsAg using the DiaSorin LIAISON® XL – Murex HBsAgQ assay (REF 310250, DiaSorin S.p.A, Italy; a test selected based on availability of a LIAISON® XL instrument at MHRA-NIBSC).

Using this data the HBsAg vaccine bulk (HB-PL01) was diluted 1:1000 in pooled human serum (supplied by the NHS Blood and Transplant, Colindale UK), which had been shown to be negative for anti-HCV, anti-HIV 1+2, HBsAg, anti-HBs, and Syphilis. Bronidox was added as a preservative (0.05% final concentration). Filling and lyophilisation of the prepared bulk was undertaken by the Market Analysis, Manufacturing and Logistics (MML) team at MHRA-NIBSC, Potters Bar, UK, in May 2025.

The bulk material (held at ambient temperature and stirred gently) was dispensed into 2.5 mL glass ampoules in 0.5 mL volumes, using a Bausch & Strobel (Ilshofen, Germany) filling machine AVF5090. The homogeneity of the fill volume was determined by on-line check-weighing, giving a coefficient of variation (%CV) of 0.39%. Trays of ampoules were loaded onto the shelves at 4 ºC, and the base plates removed. The shelf temperature was then dropped over 120 minutes to –50 ºC and held at this temperature for 2 hours. A vacuum was applied to 200 µb over 1 hour, followed by a ramping to 100 µb over 1 hour. The temperature was then raised to -35 ºC over 75 minutes, and the vacuum maintained at this temperature for 40 hours. The shelves were ramped to 25 ºC over 10 hours and the vacuum applied to 30µb, this was held for 30 hours before releasing the vacuum and backfilling the vials with nitrogen. The ampoules were then stoppered in the dryer, removed and flame sealed on the AVF5090 filling machine. The sealed ampoules are stored at -20 ºC at MHRA-NIBSC under continuous temperature monitoring for the lifetime of the product (MHRA-NIBSC to act as custodian and worldwide distributor). The material was labelled as the candidate 4th WHO IS for HBsAg (NIBSC product code: 25/120). A total of 9515 ampoules were produced (Table 1).

Ampoule integrity and residual moisture were assessed in a subset of twelve randomly selected ampoules using frequency modulated spectroscopy (FMS). Headspace oxygen content was measured non-invasively using an FMS-760 headspace analyser (Lighthouse Instruments, Charlottesville, VA, USA) against traceable oxygen standards prepared in the same ampoule type. Water activity, as an indicator of residual moisture, was determined using an FMS-1400H analyser (Lighthouse Instruments) and expressed as equilibrium relative humidity against saturated salt standards.

These analyses demonstrated a mean residual moisture content of 0.29% and a residual oxygen content of 0.34%, both within acceptable limits for WHO reference materials [7] and consistent with similar serum/plasma-based preparations produced at MHRA-NIBSC.

The homogeneity of HBsAg content across the batch was evaluated by testing ampoules taken from the beginning (A), middle (B), and end (C) of the filling process. Ampoules were reconstituted with 0.5 mL deionised water, allowed to stand for at least 20 minutes with intermittent mixing, and analysed as dilution series within the working ranges of the respective assays. Samples were tested in parallel with the 3rd WHO IS for HBsAg (NIBSC code: 12/226).

Two assay platforms were used: the Roche Elecsys® HBsAg II assay was applied to ampoules from positions A–C, while the DiaSorin LIAISON® XL Murex HBsAgQ assay was used for ampoules from positions A and C only. Position B was not assessed using the LIAISON® XL assay due to insufficient remaining volume following initial neat testing. Across both assays, dilution series demonstrated consistent responses between ampoules, with no evidence of position-dependent variability among the positions tested (Tables 2 and 3).

Calibration of ampoule contents against the 3rd WHO IS using homogeneity testing data yielded closely aligned potency estimates across fill positions. The LIAISON® XL assay gave values ranging from 36.06 to 37.09 IU/mL, while the Roche Elecsys® assay produced estimates between 36.05 and 36.67 IU/mL. Based on these results the estimated relative uncertainly of measurement due to ampoule heterogeneity was low (0.4%). These results confirm the absence of measurable differences in HBsAg content between ampoules and demonstrate that the batch is homogeneous with respect to antigen content.

4. Stability studies

The stability of the lyophilised preparation (NIBSC code 25/120) is being assessed in an ongoing accelerated thermal degradation (ATD) study to predict long-term stability when stored at the recommended temperature of −20 °C. Ampoules of the lyophilised material are stored at −20 °C, +4 °C, +20 °C, +37 °C, and +45 °C, and are removed for testing at defined time points (3, 6, and 12 months, with additional time points planned throughout the lifetime of the material).

At each time point, samples are tested in duplicate using the DiaSorin LIAISON® XL Murex HBsAgQ assay at doubling dilutions (1:20 to 1:1280), and potency is expressed relative to ampoules stored at −20 °C. Following 3, and 6 months of storage, relative potency estimates remained close to 1 for samples stored at +4 °C and +20 °C, indicating minimal loss of activity under these conditions. In contrast, more pronounced reductions in potency were observed in samples stored at +37 °C and +45 °C, particularly after 3 months. Samples stored at +45 °C for longer than 6 months could not be reconstituted and were therefore not tested.

The 12-month data were used to model thermal degradation using the Arrhenius equation, assuming first-order decay, relating degradation rate to absolute temperature [8]. From this analysis, the predicted loss of activity for the candidate IS when stored at −20 °C was estimated to be 0.001 % per year.

5. Collaborative study (study code: CS752)

Study design

The collaborative study was designed to assess the potency of the candidate IS when tested against the WHO 3rd IS for HBsAg (NIBSC code 12/226; Sample F) within the dynamic range

of representative serological assays. The pre-lyophilised liquid bulk sample was included to evaluate the loss in potency after lyophilisation. Three HBV-positive plasma samples were included to provide a limited assessment of commutability.

Lastly, a recombinant HBsAg sample was included to assess performance relative to the candidate and clinical samples as an alternative source material for future replacement standards.

Study Samples

The study comprised a total of seven different samples that were anonymised and labelled as samples A – G (Table 5). Study samples included two lyophilised preparations (3rd IS for HBsAg ~ Sample F & candidate IS ~ Sample E) and five liquid samples. Liquid samples included the pre-lyophilised liquid bulk of the candidate IS (sample A), a recombinant HBsAg preparation (sample G), and three clinical HBV-positive samples (Samples B, C and D).

Samples B, C and D were selected from a larger collection of clinical HBV positive plasma donations sourced from NHS Blood and Transplant (NHSBT). These samples were selected based on low qPCR titre and serological response. Selected samples were further screened and found negative for Anti-HBs.

Sample G comprised a lyophilised HBsAg vaccine preparation, of recombinant origin, which was reconstituted and subsequently diluted extensively in negative human serum (1:150,000) to bring the antigen concentration within the working range of routine HBsAg immunoassays.

Participants received all 7 samples, with the exception of two laboratories that were not able to test the three clinical samples under high containment.

Study samples were shipped to participants by courier on dry ice. Upon delivery participants were instructed to store the lyophilised samples at -20 ºC and the liquid preparations at -80 ºC.

Characterisation and preparation of clinical samples

HBV clinical samples were sourced from NHS Blood and Transplant (NHSBT, UK). Donations with low viral loads (<500 IU/mL), as determined by quantitative PCR (qPCR), were selected for initial evaluation. Samples were screened for HBsAg reactivity using the DiaSorin Murex HBsAg Version 3 assay to establish appropriate testing dilutions within the dynamic range. Based on these assessments, three samples were selected for inclusion in the collaborative study. Aliquots were prepared by dispensing material from the original donations into 2 mL Sarstedt tubes and stored at –80 °C until use.

Participants

Thirteen laboratories from nine different counties were invited to take part in the collaborative study. Participant details are summarised in Appendix 1. Participants were selected for their previous experience in participating in WHO collaborative studies, geographic distribution and representative range of HBsAg assays in routine use. Participating organisations included IVD manufacturers, OMCL and reference laboratories. Each laboratory was randomly assigned a numeric lab code. For laboratories that evaluated the panel in more than one assay platform, a letter code (i.e. A, B, C etc) was designated after the numerically assigned lab code (Table 6).

Study protocol

Participants were asked to test the study samples in three independent assays using their routine HBsAg testing method as described in Appendix 2 All lyophilised preparations were reconstituted with deionised, nuclease-free, molecular biology-grade water (Sample E, 0.5 mL & Sample F, 1.0 mL) and left for at least 20 minutes with occasional agitation before use. Participants were requested to dilute samples in negative human serum (confirmed as anti HBs negative). For samples A and E–G, an initial dilution was prepared as specified in the protocol, followed by testing of a predefined series of two-fold (doubling) serial dilutions.
Additional dilutions were performed, as specified in the protocol, where an endpoint was not reached. For samples B, C and D participants were provided approximate dilutions to test, with the aim of obtaining a reactive sample within the linear range of the assay. Participants were requested to record results and essential assay information on the results sheet provided (Appendix 3) and return to MHRA-NIBSC for analysis.

Statistical methods

All assay data were analysed at MHRA-NIBSC using R software packages [9]. Where possible, potency estimates for each assay were calculated relative to the nominated reference using a minimum of three dilutions taken from the linear section of the assay response range for both samples. Assay responses were $\log_{10}$ transformed to obtain a linear dose-response relationship and potencies estimated using a parallel line model [10].

Potency estimates for each sample within a laboratory and assay method were calculated as the unweighted geometric mean (GM) of valid assay estimates. Summary potency estimates across laboratories or across assay methods were calculated as the unweighted geometric mean of laboratory or assay method potency estimates, along with 95% confidence limits. Additional summary measures, the median and Huber's robust GM (calculated using R package 'WRS2') [11], which mitigate the effects of more extreme or outlying results, were also calculated.

Where applicable, data were assessed for outliers using Grubbs' Test [12], and the implications of those outliers assessed by looking at summary results both with and without the outlying data included.

Variability in results between assays within laboratories and between laboratories or assay methods was assessed using geometric coefficients of variation (GCV = $\{10^{s}\text{-}1\} \times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed results).

Validity criteria

Relative potency estimates where the response ranges of the test sample and the reference sample did not overlap were excluded from further analysis. Linearity was assessed visually and by confirming a high R^2 value. Samples with an R^2 a value below 0.95 were excluded from further analysis.

A threshold of 0.80 – 1.25 was used as an acceptable slope ratio range for concluding parallelism. Results outside of this range were excluded from further calculations.

Commutability

Commutability of the candidate IS (25/120) with test samples B, C and D was assessed by a "calibration effectiveness" approach [13]. Study median values for the test samples, calculated relative to both the 3rd IS and the candidate IS, were calculated on a $\log_{10}$ scale before being transformed back to the original scale of measurement (IU/mL). Laboratory GM estimates for samples B, C and D, calculated relative to both the 3rd IS and the candidate IS, were expressed as a % of the corresponding study median estimate for the sample to assess the extent to which laboratories agreed with consensus estimates within this study. A value of 100% indicates complete agreement with the study sample consensus value, so the relative activity of test sample and standard is consistent with other labs and therefore the standard is commutable in such cases.

In order to derive an acceptable range (for analysis of this study only) for concluding commutability, the standard deviation of the $\log_{10}$-transformed values for samples calculated relative to the 3rd IS was calculated within each laboratory, and a median value, s_M, was calculated across all laboratories. The acceptance range was then set as $\pm 2 s_M$.

Results and data analysis

Data received

All thirteen participating laboratories returned data, with ten laboratories submitting results generated using two or more different assay systems. In total, 30 datasets were received, representing 22 distinct assay methodologies. These comprised eight quantitative automated assays (36%), nine qualitative automated assays (41%), and five qualitative or semi-manual assays (23%), employing a range of capture and detection technologies. One laboratory-developed assay was included (Laboratory 4a), and a small subset of methodologies were region-specific.

A summary of the assay methodologies and the number of datasets contributed by each system is provided in Table 7.

Exclusions

All data generated by Laboratory 4a were excluded from the statistical analysis, including assignment of potency estimate for the candidate IS. The assay used by this laboratory was an in-house developed ELISA, and review of the submitted results indicated substantial inconsistency across all three assay runs. In the first assay, valid parallelism with the 3rd IS could not be demonstrated, with evidence of non-linear and non-parallel dose–response behaviour across multiple study samples. Although parallelism criteria were met in the subsequent two assays, the resulting potency estimates differed markedly between runs and remained widely discrepant from those reported by other laboratories for the same samples. Given also the laboratory's own assessment of assay performance, it was concluded that the results from Laboratory 4a would have a disproportionate influence on overall assigned estimates.

Data generated by Laboratory 12b were also excluded from the statistical analysis. This dataset was obtained using a confirmatory assay. While several qualitative assays included in the study generated numerical outputs (e.g. signal-to-cut-off values) that could be used for relative potency estimation, the confirmatory assay is designed solely to verify HBsAg positivity. In

this case, all samples were reported as positive across the dilution series, precluding determination of a meaningful dose–response relationship or calculation of relative potency.

Although the confirmatory assay was not suitable for quantitative calibration, these results confirm that all study samples, including the clinical samples, were HBsAg positive. On this basis, the data were not included in the statistical analysis.

Intra-laboratory variability

Table 8 summarises intra-laboratory variability, expressed as geometric coefficients of variation (%GCV), calculated from replicate potency estimates for samples A to E and G, each expressed relative to sample F (3rd IS). Laboratories contributing fewer than three valid estimates for a given sample were excluded from %GCV calculations, and %GCV values were not calculated where samples were not tested or results were deemed invalid.

Overall, laboratories demonstrated a good level of within-laboratory precision across the majority of assay systems. For the candidate IS 25/120 (sample E), 22 out of 27 valid assay datasets yielded %GCV values 10% or below, with the remaining values ranging between 10% and 18%. Comparable results were obtained for sample A (candidate liquid bulk), with most laboratories reporting %GCV values below 10% and only four valid datasets exceeding this threshold. Sample G (recombinant HBsAg) showed a similar pattern, with %GCV values largely within the same range as samples A and E across most assay systems.

For the clinical samples (B, C and D), most laboratories also reported low intra-laboratory variability, with %GCVs typically below 10%. However, a small number of laboratories showed markedly higher %GCV values for these samples, with Laboratories 1E, 4B and 5A generating %GCVs ranging from 42% to 80%, predominantly for samples C and D.

Comparison of assay-reported values and estimates relative to the 3rd IS

Assay calibration performance was assessed using values reported directly by participating laboratories for quantitative assays, expressed in IU/mL (Table 9), with summary calculations in Table 10. Corresponding potency estimates calculated relative to the current IS are shown in Table 11 and 12.

When presented “as reported” by individual assays, substantial inter-laboratory variability was observed across all study samples. This variability is evident when compared with relative potency estimates against Sample F, which has an expected potency of 47.3 IU/mL, providing an indication of assay calibration performance. For the candidate preparation Sample E), reported values showed a broad range between laboratories, with corresponding %GCV values of 43%. This dispersion is illustrated by the broad distribution of laboratory estimates observed in the histogram of reported values (Figure 1). Similar findings were observed for the liquid bulk preparation (Sample A) and the recombinant protein (Sample G), with %GCV values of 40 & 45% respectively when presented as reported.

For clinical samples, variability was also pronounced, with %GCV values in the range of 41–85% when presented “as reported”, indicating that agreement between assay systems is suboptimal when relying on manufacturer calibration alone.

Recalculation of the HBsAg estimates in IU/mL relative to the current IS resulted in a substantial reduction in inter-laboratory variability (Table 12 – Grouping: Quantitative). For the candidate preparation, %GCV was reduced to 7% when expressed relative to the current IS (Figure 2). A similar improvement was observed for the liquid bulk (Sample A) and recombinant protein (Sample G), where %GCV values were reduced from 40% to 7% and 45% to 17% following recalibration.

In contrast, the reduction in variability for clinical samples was more limited, with %GCV values in the range of 20–80% (Table 12 – Grouping: Quantitative), when expressed relative to the current IS. Comparison of reported values with those expressed relative to the IS did not show a consistent reduction in variability across all clinical samples, which may reflect differences in dataset composition and exclusion criteria between analyses.

Relative potency of 25/120

Laboratory GM potency estimates (IU/mL) for the candidate IS (sample E), calculated relative to the 3rd WHO IS (sample F), are presented for individual laboratories in Table 11, with corresponding graphical representations shown in Figure 3 and overall summary statistics provided in Table 12. Potency estimates derived from individual assays are presented in Appendix 4.

Across the range of assay methodologies, estimates for the candidate were generally in good agreement, forming a near-normal distribution (Figure 3), with values ranging from 29.65 to 41.17 IU/mL. An overall estimate across laboratories yielded a value of 35.46 IU/mL, which is consistent with the target concentration of 35 IU/mL.

As some assay methods were used by more than one participating laboratory including Roche Elecsys® HBsAg II and two distinct DiaSorin MUREX HBsAg assays (manual MUREX HBsAg Version 3 and the automated LIAISON® XL MUREX HBsAg Quant assay), an additional analysis was performed in which results from laboratories using the same assay methodology were first combined to avoid over-representation of individual assay platforms.

Potency estimates were therefore summarised by assay method (Table 13), with corresponding summary statistics shown in Table 12 (Assay methods). The GM, median, and robust mean all yielded consistent estimates, which were in close agreement with those obtained from the laboratory-level analysis, supporting an overall potency of 35 IU/mL (corresponding to 17.5 IU per ampoule) with an inter-method %GCV of 7%.

Continuity of Candidate Standard 25/120 Relative to the 3rd International Standard 12/226

To assess continuity of the candidate standard relative to the 3rd IS, the candidate was assigned its proposed potency of 35 IU/mL. Potency estimates for all other study samples, including the 3rd IS, were then expressed relative to the candidate (Table 14), and compared with estimates calculated when the 3rd IS was used for calibration (Table 12, assay methods).

When data from the study were analysed relative to the candidate, estimates for the 3rd IS and other study samples were comparable to those obtained when the 3rd IS was used as the

reference for calibration. Across study samples, geometric mean estimates were largely unchanged between the two approaches, with similar GCVs observed (Tables 12 and 14).

The estimate for the 3rd IS relative to the candidate was approximately 47.3 IU/mL (Table 14), corresponding closely to its assigned potency. This comparability is further reflected in the distribution of laboratory estimates shown in Figure 4, where the histogram for the 3rd IS relative to the candidate demonstrates a unimodal distribution that fits a similar profile to that observed for the candidate relative to the 3rd IS (Figure 3).

Liquid bulk material (Sample A) and Lyophilisation assessment

Laboratory mean potency estimates for the liquid bulk preparation (sample A), calculated relative to sample F, are presented in Table 11 and Figure 5, with summary statistics with exclusions shown in Table 12. Potency estimates obtained from individual assay systems are provided in Appendix 4.

As observed for the lyophilised candidate IS (sample E), potency estimates for the liquid bulk preparation were generally consistent across different assay methodologies (Figure 5), with values ranging from 34.34 to 42.87 IU/mL. Following application of the same exclusion criteria used for the lyophilised candidate, and after combining datasets from laboratories using the same assay methodology to avoid over-representation, an overall estimate was derived using a robust mean. This analysis yielded a potency estimate of 37.69 IU/mL for the liquid bulk, with an inter-method GCV of 6%, indicating good agreement between methods.

The effect of lyophilisation on the candidate preparation was assessed by comparing the relative potency of the lyophilised material (sample E) to that of the liquid bulk (sample A), with results summarised in Table 15. Relative potency estimates showed values ranging from 0.88 to 1.01 indicating a reduction in potency following lyophilisation of between 0% and 12%, with an overall robust mean loss of 7% across methods.

One assay, the LIAISON® XL HBsAg qualitative method, showed a marginal increase in relative potency of 1%.

Results for Clinical Samples

Participating laboratories were requested to test three HBV clinical samples (samples B, C and D) within the linear range of their routine assay systems. Assay method GM potency estimates relative to the 3rd IS are presented in Table 11, with corresponding summary statistics for the 3rd IS and candidate shown in Tables 12 and 16. Histograms representing clinical samples relative to the 3rd IS are shown in Figures 6, 7 and 8).

The three clinical samples spanned a wide range of HBsAg concentrations. When expressed relative to the 3rd IS, sample B yielded a low GM estimate of 1.070 IU/mL, whereas sample C and sample D produced substantially higher estimates of 3306 IU/mL and 978.2 IU/mL, respectively.

Agreement between methods was generally consistent relative to the concentration of each clinical sample. However, samples C and D showed substantially higher estimates in Laboratory 4B using the FUJIREBIO HBsAg HQ assay. For this assay, potency estimates of

9,825 IU/mL for sample C and 3,839 IU/mL for sample D were reported, markedly exceeding the corresponding mean estimates in this study. No similar divergence was observed for sample B.

When visualised on the corresponding histograms, samples B (Figure 6) and C (Figure 7) displayed a broader dispersion of estimates across assay systems, whereas sample D (Figure 8) showed comparatively closer agreement. This is reflected in the calculated GCVs relative to the 3rd IS of 61% for sample B, 49% for sample C, and 23% for sample D (Table 12, Grouping – Lab 4a & outliers excluded). Similarly, when expressed relative to the candidate (Table 16), the GCVs were comparable (64%, 54% and 24%, respectively).

To align with the methodology used for candidate potency assignment, %GCV values for the clinical samples were recalculated by combining results by assay method, thereby removing potential over-representation of individual platforms (Table 12). Using this approach, a marked reduction in variability was observed for Sample B, with %GCV decreasing from 61% to 30%. In contrast, only small changes were observed for Samples C and D, with %GCV values for Sample C remaining broadly similar (49% to 51%) and those for Sample D showing a modest decrease (23% to 18%). Further exclusion of Laboratory 4b results had minimal additional impact on %GCV estimates, indicating that the primary reduction in variability arose from harmonisation by assay method rather than removal of a single laboratory. Notably, the value for Sample D from Laboratory 4b was identified as an outlier by Grubbs' test within the assay method analysis, consistent with its limited influence on overall variability estimates.

Generally, these values contrast with the lower %GCVs observed for the candidate and liquid preparation and indicate greater inter-assay variability for the clinical materials, particularly for samples B and C.

Commutability of candidate IS (NIBSC code 25/120)

Individual laboratory estimates for samples B, C and D expressed relative to both the current IS (Table 17) and the candidate IS (Table 18) were expressed as percentages of the study median of each sample. Acceptable limits for commutability, calculated as described in the statistical analysis section, ranged from 72% to 139%, with values for individual laboratory estimates outside this range indicated in the tables.

The results show that the majority of laboratory estimates for the clinical samples fell within the acceptance limits. The proportion of values outside this range when calculated relative to the current IS and the candidate were equivalent (17 out of 69 in both cases), indicating that commutability is maintained at the same level with the proposed candidate IS.

Results for Recombinant Protein

Sample G, a recombinant HBsAg preparation, was included to evaluate its performance across assay platforms and to explore its potential suitability as a source material for future reference standards.

The distribution of potency estimates for sample G relative to the 3rd IS across participating laboratories is shown in Figure 9. Across laboratories, Sample G yielded a robust mean potency estimate of 1.373 IU/mL (Table 12, assay methods), with an inter-laboratory %GCV of 29%

relative to the 3rd IS. This variability is higher than that observed for the candidate preparation (7%) and comparable to the average %GCV observed for the clinical samples.

When potency estimates for study samples were instead expressed relative to Sample G (Table 20, Assay Methods), a marked increase in variability was observed across all materials. In particular, %GCV values increased substantially for samples B and C (≥50%), with marked increases for samples A, D and E (23–31%). This indicates that the use of the recombinant preparation as the reference introduced greater between-assay variability compared with calibration against the 3rd IS.

The commutability of Sample G with Samples B – D was also assessed using the same approach as described above and is summarised in Table 19. Overall, results showed a similar spread compared with those obtained using the current and candidate IS (Table 17 & 18), with values spanning 24–357% of the study median. While many laboratory estimates remained within the predefined acceptance limits, a greater number of values fell outside this range (24 out of 67), indicating increased variability in assay response when results were expressed relative to Sample G.

6. Discussion, Conclusions and Proposal

The source material for the HBsAg candidate (NIBSC code 25/120), comprising a non-adjuvanted HBsAg vaccine bulk derived from human plasma obtained from viraemic carriers, was provided to MHRA-NIBSC in 2012. During manufacture, the purified bulk was rendered non-infectious by heat treatment and formaldehyde inactivation [6]. It was initially used to formulate the current 3rd IS, with remaining material used to prepare the replacement candidate in 2025.

The bulk was diluted in anti-HBs and HBsAg-negative human serum to approximately 35 IU/mL, dispensed in 0.5 mL volumes, and freeze-dried. Reducing titre and fill volume relative to the 3rd IS allowed generation of a substantially larger batch, which is expected to last for at least 25 years based on recent sales of the 3rd IS. Despite this, the material remains sufficiently concentrated that extensive dilution is required to assess it within the dynamic range of modern HBsAg assays.

The precision of the fill of the candidate preparation was good (CV 0.39%), and evaluation using dilution series demonstrated excellent agreement between ampoules collected from different positions within the fill, supporting the homogeneity of the candidate preparation. Furthermore, data following accelerated thermal degradation of the candidate IS at 12 months showed a minimal predicted loss of 0.001% a year upon storage at –20 °C, with no significant losses in potency at +4 °C. This prediction is likely to improve with data from testing at longer timepoints.

In this international collaborative study, a broad range of qualitative and quantitative HBsAg immunoassays were used to evaluate the suitability of the candidate (NIBSC code: 25/120) to serve as the 4th WHO IS for HBsAg, relative to the current 3rd IS (NIBSC code 12/226). In addition to the primary study materials, blinded samples were included to assess the effects of lyophilisation and to explore the commutability of the candidate preparation across different assay platforms.

Comparison of the lyophilised candidate IS (sample E) with the corresponding liquid bulk preparation (sample A) indicated that the losses associated with lyophilisation were modest and within an expected range. These observations are consistent with the behaviour of other protein-based biological reference materials during freeze-drying and are generally considered acceptable where improved long-term stability of the preparation is achieved.

Having established the stability and consistency of the candidate preparation, its behaviour was further evaluated in the context of clinical samples to assess commutability across assay platforms. Serology kit manufacturers primarily target highly conserved antigenic regions of the HBsAg protein, most notably the "a" determinant located within the major hydrophilic region (MHR), which is present across all known HBV subtypes. This design strategy underpins broad subtype sensitivity in commercial assays. While mutations within the "a" determinant have been reported to alter antigenicity and may affect sensitivity [14], the limited scope of the present study does not allow this aspect to be evaluated.

To address commutability more generally, three HBV clinical samples were included to assess how clinical specimen behaviour compares with that of the candidate preparation and the current IS across multiple assay platforms. An inherent challenge is that clinical specimens often contain HBsAg at concentrations exceeding assay working ranges, necessitating substantial dilution (as for Samples C and D). While analytically required, this alters the native matrix and may influence antigen presentation, complicating direct assessment of commutability.

Despite this limitation, the inclusion of clinical samples provided insight into differences in assay response that are not apparent when testing reference-like preparations alone. Samples B and C showed a wider dispersion of potency estimates across assays, whereas Sample D generally exhibited closer agreement between methods relative to the current IS and the candidate replacement. However, the small number of clinical samples included in this study represents a recognised limitation and does not allow a comprehensive assessment of commutability. The differences observed therefore provide only an indication of sample-dependent assay behaviour and highlight the potential for variability between individual clinical specimens.

A particularly marked effect was observed for Samples C and D when measured using the FUJIREBIO HBsAg-HQ assay, which returned substantially higher values than conventional immunoassays. The assay incorporates a pre-analytical treatment step designed to disrupt HBV virions and subviral particles and employs antibodies recognising both external and internal epitopes of the HBsAg molecule. This combination is likely to enhance detection by reducing the impact of HBsAg–anti-HBs interactions and by increasing accessibility to internal antigenic sites, even when externally exposed epitopes, are partially masked, resulting in a reported sensitivity of <0.005 IU/mL.

Previous studies have demonstrated that HBsAg can be underestimated or undetectable using standard assay formats but becomes measurable following pretreatment and broader epitope targeting, consistent with immune-complex dissociation and additional antibody binding to internal HBsAg regions not obstructed by anti-HBs [15, 16, 17]. The selective enhancement observed for Samples C and D, suggests that such effects may be sample-specific and reflect underlying biological differences in antigen structure or accessibility rather than general assay bias.

In contrast, the remaining samples, including the recombinant protein, and Sample B, behaved similarly across assay platforms and showed no marked divergence between conventional assays and the HBsAg-HQ method. This indicates that, for these materials, the majority of HBsAg is already presented in a readily detectable form in these materials.
The improved agreement observed when results were expressed relative to the IS highlights the importance of appropriate calibration in achieving harmonisation across quantitative HBsAg assays.

However, the variability observed in assay-reported IU/mL values indicates that calibration is not consistently aligned across assay platforms. This is illustrated by the behaviour of certain assays in this study, including the LIAISON® XL Murex HBsAgQ, INNODX – HBsAg and Abbott Alinity I - HBsAg Quantitative platform, which produced estimates approximately two-fold the assigned values for both the current IS and candidate. Similar discrepancies in calibration and quantitative agreement between assays have been reported in independent evaluations of HBsAg assays [18].

Review of manufacturer documentation (Table 7) suggests that some quantitative assays remain calibrated against earlier HBsAg International Standards, while others referencing the current standard do not achieve full alignment in practice. Similarly, for qualitative assays, approximately half reference older standards; however, although the International Standard may be cited, it is not used for calibration but instead serves to assess analytical sensitivity (e.g. limit of detection). These observations emphasise the importance of ongoing alignment and periodic reassessment of assay calibration, or performance relative to the current IS where applicable, to ensure consistency. While recalibration relative to the IS substantially improves agreement for reference materials, the effect appears more variable for clinical samples. However, different exclusion criteria were applied in the analyses, and therefore the results are not directly comparable and should be interpreted with caution.

The inter-assay variability observed for the clinical samples is reflected in both the %GCV and commutability analyses. Samples B and C exhibited higher %GCV values and broader distributions relative to the study median, whereas Sample D showed lower variability by both measures. Despite this, most results fell within predefined commutability acceptance limits, with only a limited number of outliers. Comparable profiles relative to both the current and candidate IS indicate that this variability is not attributable to differences in calibration between the two materials.

These findings are consistent with those reported in the collaborative evaluation of the 3rd WHO IS for HBsAg [19], in which inter-laboratory %GCV values ranged from 18% to 34% depending on the material tested. In that study, the duplicate of the candidate 3rd IS showed particularly good agreement between laboratories, with %GCV values of approximately 10%, consistent with the levels of variability observed for the candidate liquid preparation in the present study. A notable difference, however, is the nature of the materials evaluated, as the earlier study utilised well-characterised reference preparations, whereas the present study includes true clinical specimens. Importantly, the candidate preparation demonstrated commutability characteristics comparable to those of the current IS, indicating that it provides an equivalent basis for harmonisation across assay systems.

Finally, the behaviour of the recombinant preparation (Sample G) observed across assay platforms highlights important considerations when interpreting its performance relative to native HBsAg preparations. Native HBsAg particles depend on host cell processing and

higher-order organisation that are not fully replicated in recombinant expression systems [20]. Consequently, differences in epitope presentation can arise, influencing antibody binding and leading to assay-dependent variation in measured responses.

Unlike the plasma-derived preparations used for the current and candidate IS, which more closely reflect native antigen presentation, Sample G represents a modified preparation lacking the full structural and compositional complexity of clinical or vaccine-derived HBsAg. This difference in antigen format is likely to contribute to the increased variability observed when Sample G is used as a reference across diverse assay platforms.

These findings indicate that although Sample G can be measured reproducibly within individual assays, it does not harmonise results across different assay platforms as effectively as the candidate and current IS. This is reflected in the higher inter-assay variability observed when results are expressed relative to Sample G, with increased %GCV values across multiple samples. Similarly, commutability assessment shows a broader distribution and a greater number of results outside predefined acceptance limits, consistent with the increased variability captured by the %GCV analysis. Overall, these findings suggest that, in its current form, the recombinant preparation would be unsuitable as a replacement IS without further characterisation and optimisation.

The results of the collaborative evaluation demonstrate consistent performance of the candidate preparation across assay platforms, with acceptable inter-laboratory variability and comparable behaviour to the current IS. On the basis of these findings, a consensus potency estimate of 35 IU/mL, derived from laboratory geometric mean (GM) estimates across different assay methodologies (Table 11), was obtained for the candidate (NIBSC code 25/120).

To minimise potential bias arising from over-representation of individual assay platforms, results from multiple laboratories using the same assay method were combined prior to calculation of the overall estimate. This adjustment, however, had no significant impact on the final value, with laboratory-level and method-based analyses yielding consistent results, supporting the robustness of the potency estimate. Continuity between the candidate standard and the 3rd IS was shown to be good, consistent with both materials being derived from the same source.

Proposal

It is proposed that the candidate IS, NIBSC code 25/120, is established as the 4th WHO IS for HBsAg, with an assigned potency of 17.5 IU/ampoule (35 IU/mL when reconstituted in 0.5mL deionised water). The standard should be stored at -20 °C or below until use. The total number of ampoules available for distribution is 9384.

Proposed Instructions for Use (IFU) for the standard are included in Appendix 5. The custodian laboratory is the NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG UK.

7. Comments Received from Collaborative Study Participants

Eight participating laboratories responded to the report. There were no disagreements with the suitability of the candidate standard to serve as the WHO International Standard for HBsAg.

A number of comments were received relating to clarity, presentation, and minor inaccuracies in the report. Revisions were made to improve the description and interpretation of homogeneity testing using the LIAISON® XL assay, including clarification of ampoule position definitions and the exclusion of position B from analysis due to insufficient volume.

Text and tables were updated to clarify the use of the WHO International Standard in qualitative assays, emphasising its role as a reference material for assessing analytical sensitivity rather than for calibration. Minor corrections were also made throughout the report, including updates to the list of participants, correction of the cited WHO International Standard for the Abbott Alinity i HBsAg assay in Table 7, and resolution of shading inconsistencies in Table 19.

In addition, figure formatting has been improved (Figures 3–9) to enhance readability.

8. Acknowledgements

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10. Tables and Figures

Table 1: Summary characteristics of candidate WHO IS

General material characteristics		
Product code	25/120	
Presentation (liquid or lyophilized)	Lyophilized	
Appearance	Robust opaque cake	
Container type	2.5mL Ampoules	
Number of containers filled	9515	
Number of containers available for distribution	9384	
Date of fill	15th May 2025	
Storage temperature	-20°C	
Microbiological findings	Negative for Bacterial/Mould/Yeast colony counts (Cfu/mL)	
	Target value (where applicable)	**Result**
All presentations		
Mean fill mass (CV%)[a]		0.5150 (0.3915)
Additionally for lyophilized material		
Mean dry weight (CV%)		0.04394 (0.77)
Mean residual moisture (CV%)	<1%	0.29 (10.70)
Mean oxygen headspace (CV%)	<1.14%	0.34 (48.42)

[a] CV% = coefficient of variation (%)

Table 2: Comparison of tested dilutions used for homogeneity assessment from an ampoule taken from the beginning (A), and end (C) of the filling process using the LIAISON® XL HBsAgQ assay with estimates relative to the 3rd IS – 12/226.

	IU/mL				
	Dilution	R1	R2	Mean	Estimate (95% CI)
25/120 (A)	1/15	4.5	4.4	4.45	37.09 (36.06 – 38.16)
	1/30	2.1	2.1	2.1	
	1/60	1.1	1.1	1.1	
	1/120	0.51	0.53	0.52	
	1/240	0.26	0.25	0.26	
	1/480	0.14	0.13	0.14	
25/120 (B)*	Not Tested (insufficient volume)				
25/120 (C)	1/15	4.3	4.3	4.3	36.06 (35.01 – 37.15)
	1/30	2.1	2.1	2.1	
	1/60	1.0	1.0	1.0	
	1/120	0.51		0.51	
	1/240	0.26		0.26	
	1/480	0.14	0.12	0.13	

* Position B was not included in the homogeneity assessment because only neat testing was performed initially (data not shown), leaving insufficient remaining volume for replicate dilution analysis.

Table 3: Comparison of tested dilutions used for homogeneity assessment from an ampoule taken from the beginning (A), middle (B) and end (C) of the filling process using the Cobas Pure – HBsAg II assay, with estimates relative to the 3rd IS – 12/226.

		COI			IU/mL
	Dilution	R1	R2	Mean	Estimate (95% CI)
25/120 (A)	1:20	43.7	44.7	44.20	36.67 (36.13 – 37.20)
	1:40	22.2	22.3	22.25	
	1:80	11.3	11.3	11.30	
	1:160	5.82	5.75	5.79	
	1:320	3.05	3.01	3.03	
	1:640	1.58	1.68	1.63	
	1:1280	0.93	0.948	0.94	
25/120 (B)	1:20	43.6	43.6	43.60	36.05 (35.52 – 36.58)
	1:40	22.3	21.7	22.00	
	1:80	11.1	11.1	11.10	
	1:160	5.71	5.63	5.67	
	1:320	2.93	3.05	2.99	
	1:640	1.60	1.59	1.60	
	1:1280	0.897	0.883	0.890	
25/120 (C)	1:20	43.7	44.4	44.05	36.37 (35.84 – 36.90)
	1:40	22.1	22.0	22.05	
	1:80	11.3	11.3	11.30	
	1:160	5.83	5.70	5.77	
	1:320	2.97	3.11	3.04	
	1:640	1.56	1.59	1.58	
	1:1280	0.961	0.992	0.98	

Table 4: Accelerated degradation relative potencies and predicted loss for the candidate IS (NIBSC code 25/120)

	Geometric Mean Potency Relative to –20 °C (95% CI)			Predicted % loss per year
Temp	3 months	6 months	12 months	
-20°C				0.001
4°C	1.03 (0.99 – 1.07)	1.02 (0.99 – 1.05)	0.96 (0.94 – 0.98)	0.193
20°C	0.99 (0.94 – 1.03)	0.98 (0.95 – 1.01)	0.92 (0.90 – 0.94)	3.991
37°C	0.82 (0.79 – 0.86)	0.69 (0.66 – 0.72)	0.48 (0.47 – 0.49)	52.18
45°C	0.51 (0.49 – 0.53)	NT	NT	

NT - Not Tested

Table 5. Collaborative study samples (CS752).

Sample Code	Sample Name	Description	Presentation
Sample A	Candidate 4th WHO IS for HBsAg - Liquid Bulk	Non-adjuvanted plasma-derived HBsAg vaccine bulk. HBV genotype B4, HBsAg subtypes *ayw1/adw2*	Liquid Screw-capped tube
Sample B	Clinical Sample 1 (502 IU – NAT)	National blood service UK derived positive HBV plasma pack	Liquid Screw-capped tube
Sample C	Clinical Sample 2 (38 IU – NAT)	National blood service UK derived positive HBV plasma pack	Liquid Screw-capped tube
Sample D	Clinical Sample 3 (<2 IU – NAT)	National blood service UK derived positive HBV plasma pack	Liquid Screw-capped tube
Sample E	Candidate 4th WHO IS for HBsAg (25/120)	Non-adjuvanted plasma-derived HBsAg vaccine bulk. HBV genotype B4, HBsAg subtypes *ayw1/adw2*	Freeze-dried Ampoule
Sample F	3rd IS for HBsAg (12/226)	Non-adjuvanted plasma-derived HBsAg vaccine bulk. HBV genotype B4, HBsAg subtypes *ayw1/adw2*	Freeze-dried Ampoule
Sample G	Recombinant HBsAg Protein	Diluted recombinant HBsAg produced from rDNA in yeast	Liquid Screw-capped tube

Table 6: Participant assays details and codes

Lab Code	Manufacturer	Platform	Assay Methods
1A	Roche	e411, & e601	Elecsys® HBsAg II
1B	Roche	e801	Elecsys® HBsAg II
1C	INNODX	Wan200+	HBsAg (CMIA)
1D	MAGLUMI®	MAGLUMI X8	HBsAg (CLIA)
1E	Abbott	Alinity i	HBsAg Quantitative
1F	Mindray	Mindray CL-6000i	HBsAg II Hepatitis B Surface Antigen (CLIA)
2	DiaSorin	LIAISON® XL	Murex HBsAg Quant
3A	DiaSorin	LIAISON® XL	Murex HBsAg Quant
3B	DiaSorin	LIAISON® XL	Murex HBsAg
4A	-	Manual	in-house ELISA
4B	FUJIREBIO	LUMIPULSE® G1200 Plus	HBsAg HQ
5A	DiaSorin	LIAISON® XL	Murex HBsAg Quant
5B	DiaSorin	Manual	Murex HBsAg Version 3.0
6	Roche	e411	Elecsys® HBsAg II
7A	Roche	Cobas Pro integrated solutions	Elecsys® HBsAg II
7B	Roche	Cobas Pro integrated solutions	Elecsys® HBsAg II Quant II
8A	Siemens Healthcare	ADVIA Centaur XP	HBsAgII
8B	Siemens Healthcare	Atellica IM	HBsII
9A	Abbott	ARCHITECT	HBsAg Qualitative II
9B	Abbott	ARCHITECT	HBsAg Next Qualitative
9C	Abbott	Alinity S	HBsAg
9D	Abbott	ARCHITECT	HBsAg Quantitative
10A	Biomerieux	VIDAS®	HBs Ag Ultra (HBS) – HBS Protocol
10B	Biomerieux	VIDAS®	HBs Ag Ultra (HBS) – HBL Protocol
11	DiaPro	Manual	HBsAg One ULTRA
12A	Bio-Rad	Manual	Monolisa HBs Ag ULTRA
12B	Bio-Rad	Manual	Monolisa HBs Ag ULTRA Confirmatory
13A	DiaSorin	LIAISON® XL	Murex HBsAg Quant
13B	DiaSorin	Manual	Murex HBsAg Version 3.0
13C	Roche	e402	Elecsys® HBsAg II

Table 7. Collaborative study assay methods.

Quantitative automated assays			
Assay	**Manufacturer**	**Calibrated to**	**No. of data sets**
ARCHITECT HBsAg (Quant)	Abbott	80/549	1
Elecsys® HBsAg II Quant II	Roche	00/588	1
LUMIPULSE® HBsAg HQ	Fujirebio	unknown	1
LIAISON® XL MUREX HBsAg Quant	DiaSorin	12/226	4
HBsAg CMIA	INNODX	00/588	1
HBsAg (CLIA)	MAGLUMI®	unknown	1
Alinity I HBs Ag	Abbott	80/549	1
HBsAg II Hepatitis B Surface Antigen (CLIA)	Mindray	unknown	1
Qualitative automated assays			
Assay	**Manufacturer**	**LOD assessment**	**No. of data sets**
Elecsys® HBsAg II	Roche	00/588	4
LIAISON® XL Murex HBsAg	DiaSorin	12/226	1
Atellica® IM Hepatitis B surface Antigen II (HBsII)	Siemens Healthcare Diagnostics Products GmbH	12/226	1
ADVIA Centaur Hepatitis B surface Antigen II (HBsII)	Siemens Healthcare Diagnostics Products GmbH	12/226	1
ARCHITECT HBsAg Qualitative II	Abbott	00/588	1
ARCHITECT HBsAg NEXT Qualitative	Abbott	00/588	1
ALINITY s HBsAg	Abbott	00/588	1
VIDAS Ag HBs ULTRA (HBS)	BioMérieux	12/226	1
VIDAS Ag HBs ULTRA (HBL)	BioMérieux	12/226	1
Qualitative (semi) manual assays			
Assay	**Manufacturer**	**LOD assessment**	**No. of data sets**
HBsAg one Ultra	DiaPro	00/588	1
Monolisa *HBs Ag ULTRA*	Bio Rad	00/588	1
Monolisa *HBs Ag ULTRA* confirmatory	Bio Rad	00/588	1
In-house ELISA	N/A	N/A	1
Murex HBsAg Version 3.0	DiaSorin	12/226	2

Table 8 – Intra-Laboratory variability, measured by GCV

Lab	A	B	C	D	E	G
1a	4%	1%	2%	3%	3%	6%
1b	2%	1%	3%	1%	2%	1%
1c	6%	3%	4%	7%	1%	5%
1d	0%	5%	2%	2%	4%	2%
1e	8%	19%	42%	21%	14%	25%
1f	0%	4%	1%	3%	2%	1%
2	6%	4%	11%	12%	2%	8%
3a						
3b						
4a						
4b	5%	19%		67%	10%	5%
5a	14%	12%	80%	53%	4%	22%
5b	7%	6%		6%	4%	
6	7%				12%	16%
7a	5%	3%	2%	4%	4%	1%
7b	2%	2%	7%	8%	1%	5%
8a	2%	6%	5%	5%	2%	2%
8b	3%	4%	5%	3%	2%	3%
9a	1%		3%	4%	6%	1%
9b	12%		14%	20%	2%	7%
9c	1%		8%	12%	5%	1%
9d	4%		13%	11%	9%	10%
10a	20%	15%	24%		18%	20%
10b	3%	4%	11%		7%	5%
11	19%		6%	22%	9%	
12a	5%	2%	7%	9%	3%	4%
13a	0%				1%	2%
13b	3%		6%		3%	5%
13c	4%				2%	0%

Table 9 - Laboratory Reported Potency Estimates (in IU/mL)

Lab	A	B	C	D	E	F	G
1c	62.54	1.814	1233	1979	60.45	86.18	2.349
1d	36.51	1.365	2709	1021	35.41	46.73	1.297
1e	71.42	1.705	6867	1666	73.72	81.28	2.589
1f	50.47	0.9276	2996	1570	46.22	62.91	1.937
2	77.74	0.6971	2960	1424	68.20	84.34	2.618
3a	56.23	0.7189	4049	1445	51.95	69.77	2.008
4b	39.33	1.093	12803	3850	36.00	51.31	1.095
5a	92.26	0.7799	5013	2731	91.99	113.3	3.327
7b	30.58	1.364	3354	1050	27.39	34.04	1.082
9d	59.16	1.059	4232	1055	51.09	68.22	2.275
13a	60.84				57.31	75.13	2.082

Table 10 - Summary Calculations on Reported Potency Estimates (in IU/mL)

Grouping	Measure	A	B	C	D	E	F	G
Quantitative (Outliers excluded)	GM	55.16	1.092	3868	1623	51.57	67.04	1.944
	Median	59.16	1.076	3685	1506	51.95	69.77	2.082
	Robust Mean	57.14	1.139	3851	1617	53.80	69.54	2.030
	GCV	40%	41%	85%	54%	43%	39%	45%
	n	11	10	10	10	11	11	11

Table 11 – Laboratory Geometric Mean Potency Estimates Relative to 3rd IS (Assigned 47.3 IU/mL)

Lab	A	B	C	D	E	G
1a	37.92	1.740	4023	1100	35.78	1.615
1b	37.85	1.818	4030	1097	35.58	1.649
1c	34.34	1.012	671.8*	1099	33.28	1.282
1d	36.59	1.387	2762	1038	35.08	1.288
1e	42.31	1.053	4459	1023	40.57	1.664
1f	37.95	0.698	2272	1190	34.71	1.446
2	41.45	0.388	1691	812.8	38.15	1.432
3a	38.13		2809	995.7	35.20	1.332
3b	35.73			1073	36.06	1.602
4b	36.22	1.034	9825	3839*	33.00	0.962
5a	38.71	0.311	2407	1307	36.86	1.262
5b	41.49	1.227	3474	843.7	39.69	1.557
6	42.59				41.17	1.769
7a	36.42	1.588	4038	1162	33.76	1.501
7b	42.87	1.626	3903	1286	38.92	1.733
8a	36.70	1.507	3379	997.2	34.02	0.971
8b	37.29	1.597	3663	1050	34.96	0.972
9a	35.49	0.803	2254	773.9	33.21	1.353
9b	38.41		3662	1162	35.25	1.878
9c	38.27		2020	779.7	36.82	1.495
9d	39.54		2839	785.3	34.92	1.553
10a	37.05	1.205	2349	826.2	32.50	1.279
10b	37.42	1.332	3033	997.7	35.03	1.320
11	40.48	1.131	6158	994.1	34.14	0.936
12a	34.73	1.202	5336	996.6	29.65	0.730
13a	38.33				36.08	1.308
13b	41.39	0.712	2304	522.2**	37.63	2.330
13c	37.81				35.66	1.567

* Outlier detected by Grubbs' Test

** Outlier detected by Grubbs' Test only if Lab 4b result excluded

Table 12 - Laboratory Geometric Mean Summary Calculations relative to 3rd IS (Assigned 47.3 IU/mL)

Grouping	Measure	A	B	C	D	E	G
Lab 4a excluded Outliers excluded	GM	38.27	1.070	3306	978.2	35.55	1.382
	Median	37.93	1.203	3379	1010	35.22	1.439
	Robust Mean	38.16	1.187	3273	1001	35.46	1.431
	GCV	6%	61%	49%	23%	7%	28%
	n	28	20	23	24	28	28
Assay Methods (Lab 4a excluded, Outliers excluded)	GM	37.84	1.178	3396	985.0	35.02	1.329
	Median	37.42	1.202	3379	1008	35.03	1.332
	Robust Mean	37.69	1.216	3263	1005	35.15	1.373
	GCV	6%	30%	51%	18%	7%	29%
	n	19	15	17	18	19	19
Assay Methods (Lab 4a excluded, Outliers excluded, Lab 4b, B, C & D excluded)	GM	37.84	1.189	3178	998.2	35.02	1.329
	Median	37.42	1.203	3201	1008	35.03	1.332
	Robust Mean	37.69	1.232	3204	1007	35.15	1.373
	GCV	6%	31%	38%	15%	7%	29%
	n	19	14	16	18	19	19
Quantitative (Outliers excluded)	GM	38.69	0.8224	3196	1045	36.00	1.372
	Median	38.33	1.023	2809	1038	35.20	1.332
	Robust Mean	38.78	0.9337	3002	1060	35.94	1.386
	GCV	7%	80%	66%	20%	7%	17%
	n	11	8	9	9	11	11

Table 13 – Assay Method Geometric Mean Potency Estimates relative to 3rd IS (Assigned 47.3 IU/mL)

Assay Method	A	B	C	D	E	G
Elecsys® HBsAg II	38.63	1.713	4030	1119	36.47	1.631
Elecsys® HBsAg II Quant II	42.87	1.626	3903	1286	38.92	1.733
HBs Ag Ultra (HBS) - HBL Protocol	37.42	1.332	3033	997.7	35.03	1.320
HBs Ag Ultra (HBS) - HBS Protocol	37.05	1.205	2349	826.2	32.50	1.279
HBsAg	38.27		2020	779.7	36.82	1.495
HBsAg (CLIA)	36.59	1.387	2762	1038	35.08	1.288
HBsAg (CMIA)	34.34	1.012		1099	33.28	1.282
HBsAg HQ	36.22	1.034	9825		33.00	0.962
HBsAg II Hepatitis B Surface Antigen (CLIA)	37.95	0.698	2272	1190	34.71	1.446
HBsAg Next Qualitative	38.41		3662	1162	35.25	1.878
HBsAg One ULTRA	40.48	1.131	6158	994.1	34.14	0.936
HBsAg Qualitative II	35.49	0.803	2254	773.9	33.21	1.353
HBsAg Quantitative	40.90	1.053	3558	896.1	37.64	1.608
HBsAgII	37.25	1.507	3379	997.2	34.83	1.233
HBsII	37.29	1.597	3663	1050	34.96	0.972
Monolisa HBs Ag ULTRA	34.73	1.202	5336	996.6	29.65	0.730
MUREX HBsAg	35.73			1073	36.06	1.602
MUREX HBsAg Quant	39.13	0.347*	2253	1019	36.56	1.332
Murex HBsAg Version 3.0	41.44	0.935	2829	663.7	38.64	1.905

* Outlier detected by Grubbs' Test

Table 14 – Laboratory Geometric Mean estimates, calculated relative to Candidate IS (if assigned potency of 35 IU/ml)

Lab	A	B	C	D	F	G
1a	37.11	1.715	3968	1081	46.27	1.577
1b	37.24	1.791	3971	1080	46.53	1.625
1c	36.15	1.060	707.7	1151	49.75	1.353
1d	36.53	1.393	2776	1041	47.19	1.279
1e	37.06	0.9049	3866	881.9	40.81	1.446
1f	38.27	0.7040	2287	1197	47.69	1.459
2	37.84	0.3570	1537	739.3	43.39	1.325
3a	37.91		2704	967.9	47.03	1.343
3b	34.69			1051	45.91	1.551
4b	38.36	1.103	10858	2946*	50.17	1.018
5a	36.73	0.2902	2250	1317	44.91	1.177
5b	36.56	1.097	3161	748.9	41.71	1.400
6	36.15				40.21	1.538
7a	37.69	1.575	3963	1168	49.04	1.578
7b	38.62	1.541	3736	1205	42.54	1.545
8a	37.74	1.551	3477	1028	48.66	1.001
8b	37.34	1.601	3675	1052	47.35	0.9707
9a	37.43	0.9027	2386	818.2	49.85	1.431
9b	38.15		3633	1152	46.96	1.865
9c	36.34		1901	729.9	44.97	1.400
9d	39.62		2837	784.6	47.42	1.558
10a	39.92	1.300	2534	947.6	50.94	1.379
10b	37.39	1.340	3051	944.9	47.26	1.313
11	40.46	1.086	6547	1021	48.49	0.9523
12a	41.02	1.420	6340	1178	55.83	0.8601
13a	37.18				45.89	1.269
13b	38.46	0.6766	2133	502.0	44.00	2.185
13c	37.12				46.42	1.540
Assay Method Summary	**A**	**B**	**C**	**D**	**F**	**G**
GM	37.78	1.193	3429	988.1	47.27	1.328
Median	37.43	1.300	3312	1034	47.26	1.379
Robust Mean	37.71	1.236	3251	1020	47.19	1.368
GCV	4%	30%	57%	20%	7%	23%
n	19	15	17	18	19	19

* Outlier detected by Grubbs' Test

Table 15 – Potency estimates of Candidate IS, calculated relative to A

Lab	Relative Potency	GCV	n
1a	0.9432	4%	3
1b	0.9399	3%	3
1c	0.9681	6%	3
1d	0.9581	4%	3
1e	0.9443	23%	3
1f	0.9145	2%	3
2	0.9250	4%	3
3a	0.9232	n/a	1
3b	1.009	n/a	1
4a	1.525*	n/a	2
4b	0.9125	6%	3
5a	0.9530	16%	3
5b	0.9573	4%	3
6	0.9682	5%	3
7a	0.9286	4%	3
7b	0.9063	2%	3
8a	0.9274	2%	3
8b	0.9372	2%	3
9a	0.9352	4%	3
9b	0.9174	11%	3
9c	0.9633	7%	3
9d	0.8834	6%	3
10a	0.8767	7%	3
10b	0.9360	6%	3
11	0.8651	13%	3
12a	0.8533	6%	3
13a	0.9415	1%	3
13b	0.9101	2%	3
13c	0.9429	2%	3
GM	0.9294		
Median	0.9356		
Robust Mean	0.9318		
GCV	4%		
n	28		

*** Excluded from summary calculations**

Table 16 – Laboratory Geometric Mean Summary Calculations (relative to Candidate IS)

Grouping	Measure	A	B	C	D	G
Lab 4a excluded Outliers excluded	GM	1.076	0.03045	93.81	27.72	0.03894
	Median	1.069	0.03422	90.33	29.55	0.04000
	Robust Mean	1.073	0.03421	92.11	28.62	0.03983
	GCV	4%	64%	54%	24%	23%
	n	28	20	23	24	28

Table 17 – Individual laboratory estimates for Samples B – D expressed as a percentage of the study median for each sample for results calculated relative to Current IS (Sample F)

Sample	1a	1b	1c	1d	1e	1f	2	3a	3b	4b	5a	5b	7a	7b	8a	8b	9a	9b	9c	9d	10a	10b	11	12a	13b
C	125%	126%	21%	86%	139%	71%	53%	88%		306%	75%	108%	126%	122%	105%	114%	70%	114%	63%	89%	73%	95%	192%	166%	72%
D	108%	107%	107%	102%	100%	116%	79%	97%	105%	375%	128%	82%	114%	126%	98%	103%	76%	114%	76%	77%	81%	98%	97%	97%	51%
B	145%	151%	84%	115%	87%	58%	32%			86%	26%	102%	132%	135%	125%	133%	67%				100%	111%	94%	100%	59%

Table 18 – Individual laboratory estimates for Samples B – D expressed as a percentage of the study median for each sample for results calculated relative to Candidate IS (Sample E)

Sample	1a	1b	1c	1d	1e	1f	2	3a	3b	4b	5a	5b	7a	7b	8a	8b	9a	9b	9c	9d	10a	10b	11	12a	13b
C	128%	128%	23%	89%	124%	74%	49%	87%		350%	72%	102%	128%	120%	112%	118%	77%	117%	61%	91%	82%	98%	211%	204%	69%
D	104%	104%	111%	100%	85%	115%	71%	93%	101%	283%	127%	72%	112%	116%	99%	101%	79%	111%	70%	75%	91%	91%	98%	113%	48%
B	143%	149%	88%	116%	75%	59%	30%			92%	24%	91%	131%	128%	129%	133%	75%				108%	111%	90%	118%	56%

Table 19 – Individual laboratory estimates for Samples B – D expressed as a percentage of the study median for each sample for results calculated relative to Sample G

Sample	1a	1b	1c	1d	1e	1f	2	3a	3b	4b	5a	5b	7a	7b	8a	8b	9a	9b	9c	9d	10a	10b	11	12a	13b
C	115%	113%	24%	100%	107%	71%	55%	98%			93%	107%	114%	105%	158%	171%	76%	89%	62%	83%	84%	103%	297%	357%	46%
D	98%	95%	119%	117%	88%	116%	81%	108%			155%	77%	105%	107%	145%	153%	81%	88%	73%	72%	100%	99%	153%	192%	32%
B	116%	117%	82%	114%	82%	51%	28%		55%	113%	26%	81%	103%	100%	161%	170%	63%				100%	105%	131%	169%	32%

Shaded cells are outside of the 72% to 139% acceptable range for concluding commutability, calculated as +/- 2 median SD of the results relative to Current IS

Table 20 – Laboratory Geometric Mean Summary Calculations (relative to Sample G)

Grouping	Measure	A	B	C	D	E
Lab 4a excluded Outliers excluded	GM	27.81	0.8061	2288	749.0	25.68
	Median	26.14	0.9603	2227	721.9	25.00
	Robust Mean	26.95	0.9107	2176	737.9	25.26
	GCV	26%	74%	61%	31%	23%
	n	28	21	22	22	28
Assay Methods (Lab 4a excluded, Outliers excluded)	GM	28.72	0.9249	2415	764.4	26.36
	Median	26.73	0.9839	2128	754.4	25.38
	Robust Mean	27.45	0.9634	2188	767.0	25.79
	GCV	28%	50%	66%	31%	23%
	n	19	16	16	17	19

Figure 1 - Laboratory-reported estimates (IU/mL) for the candidate preparation for quantitative assays

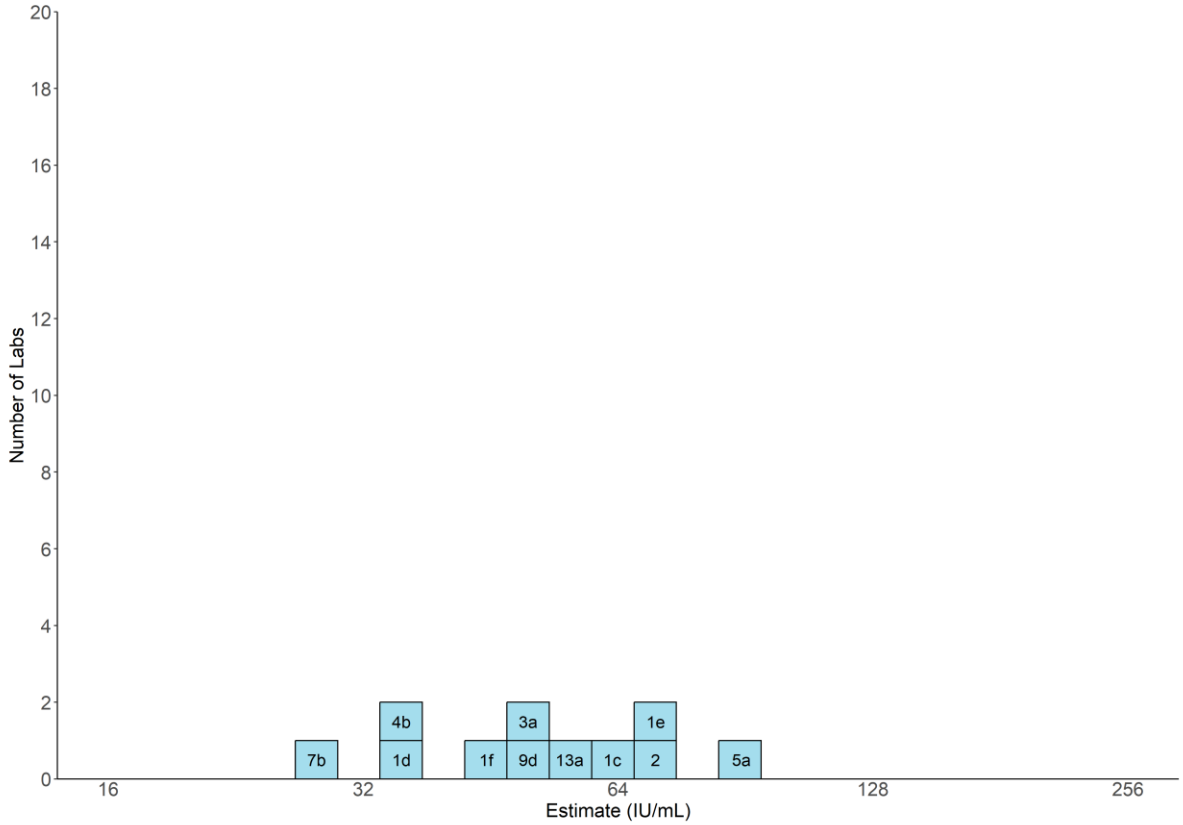


Figure 2 – Laboratory potency estimates for the candidate preparation expressed relative to the 3rd International Standard (assigned potency 47.3 IU/mL) for quantitative assays

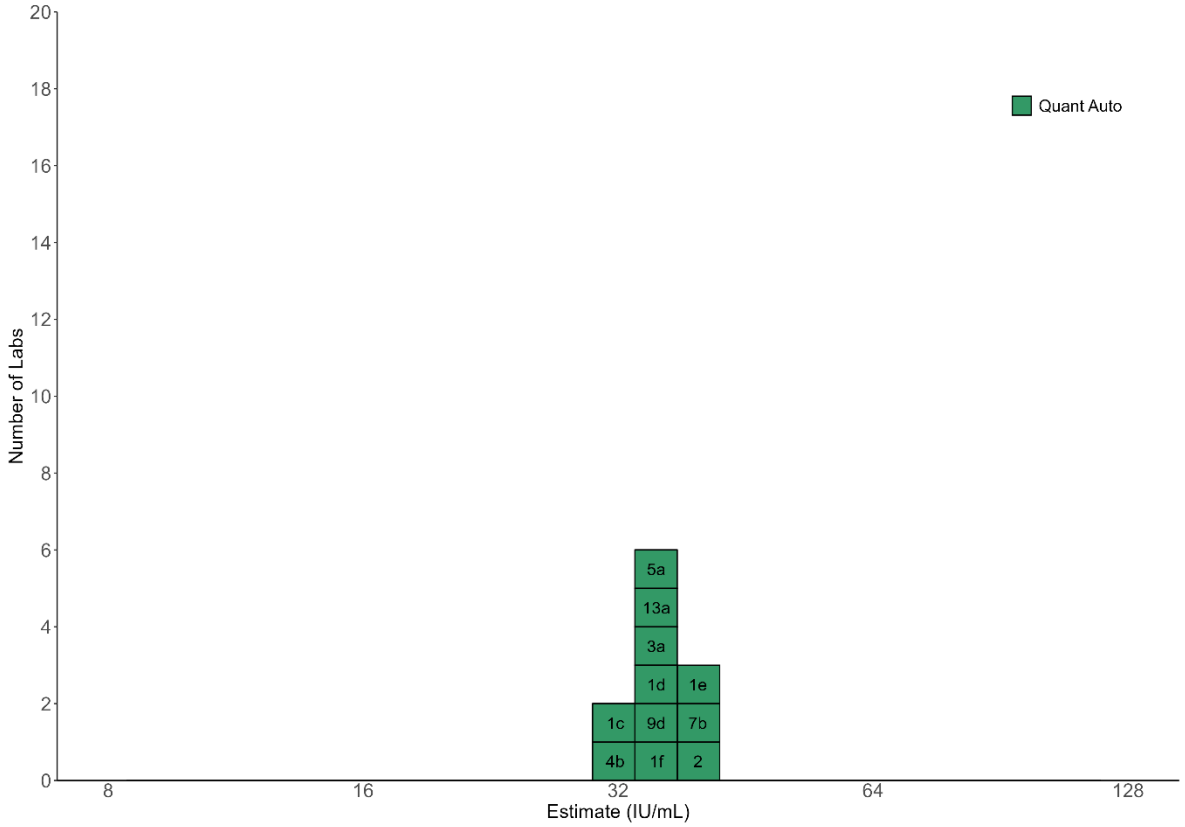


Figure 3 – Potency Estimates of Candidate IS, calculated relative to 3rd IS (assigned potency of 47.3 IU/mL)

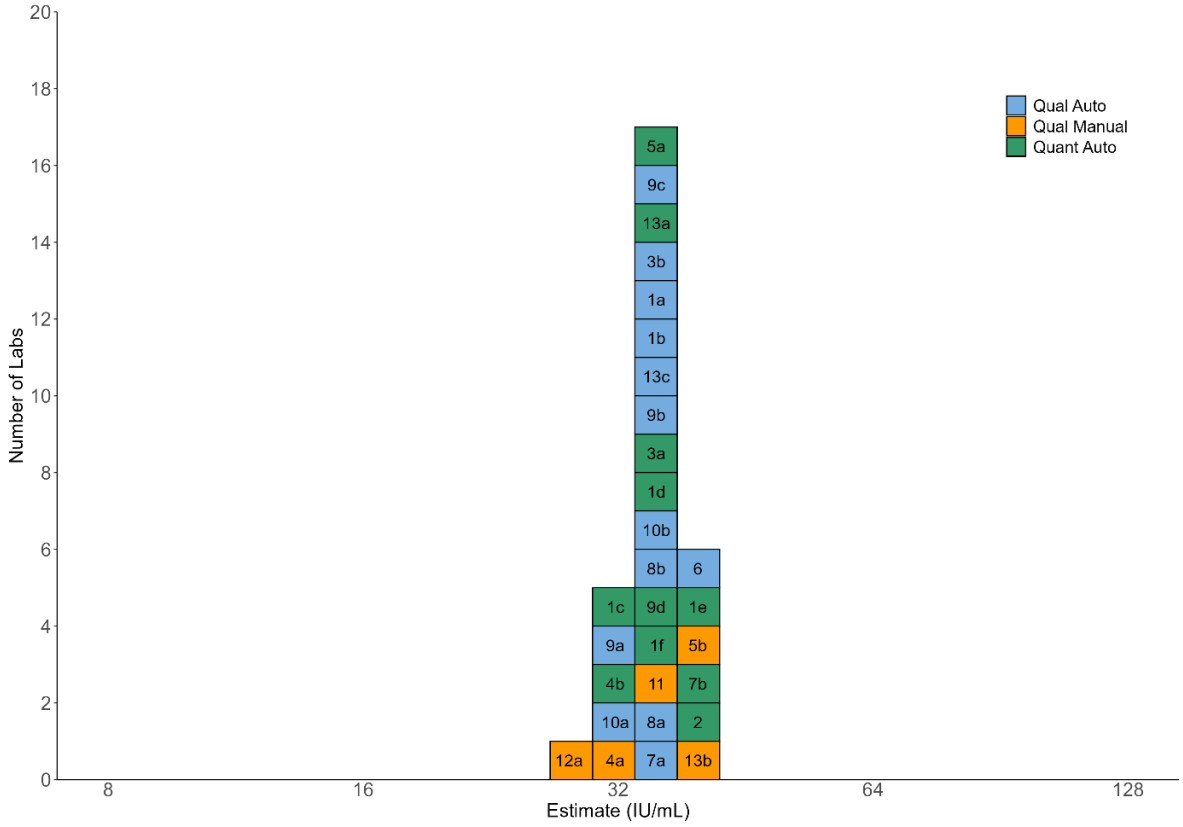


Figure 4 – Potency Estimates of 3rd IS, calculated relative to Candidate IS (assuming proposed potency of 35 IU/mL)

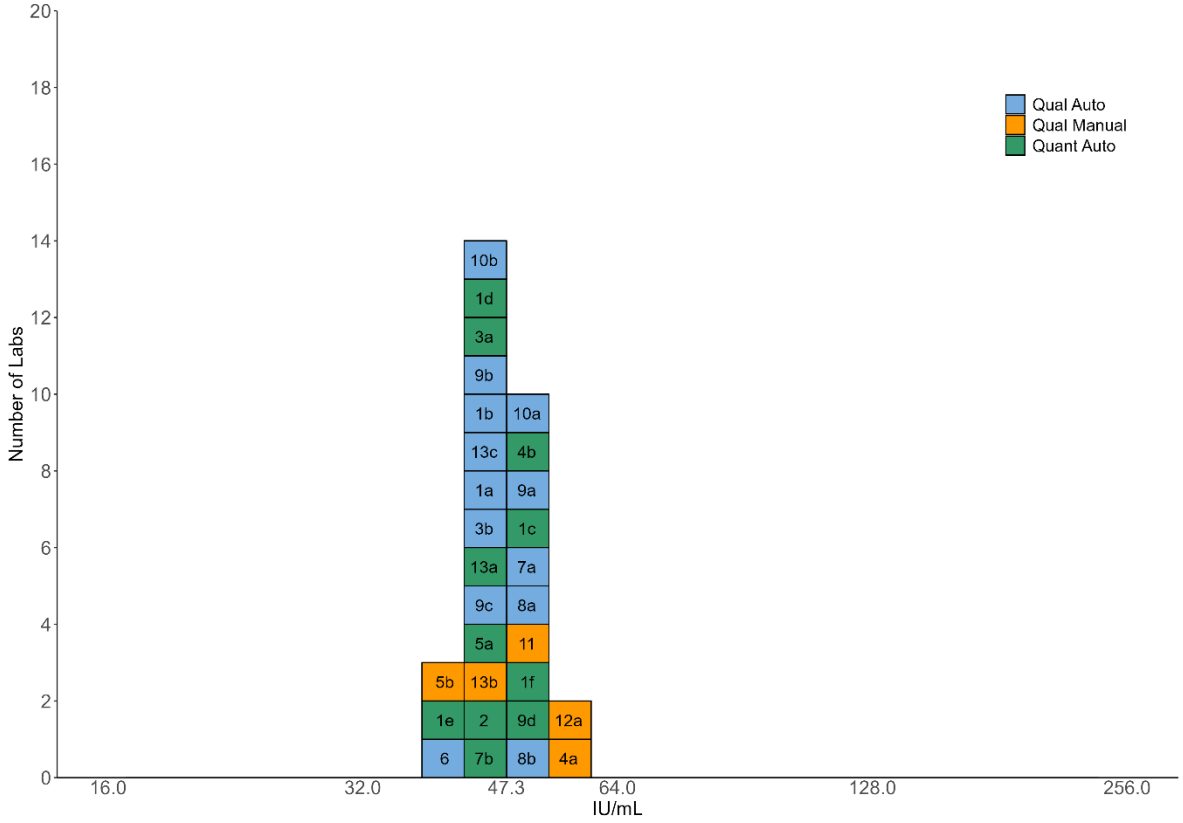


Figure 5 – Potency Estimates of Sample A, calculated relative to Candidate IS

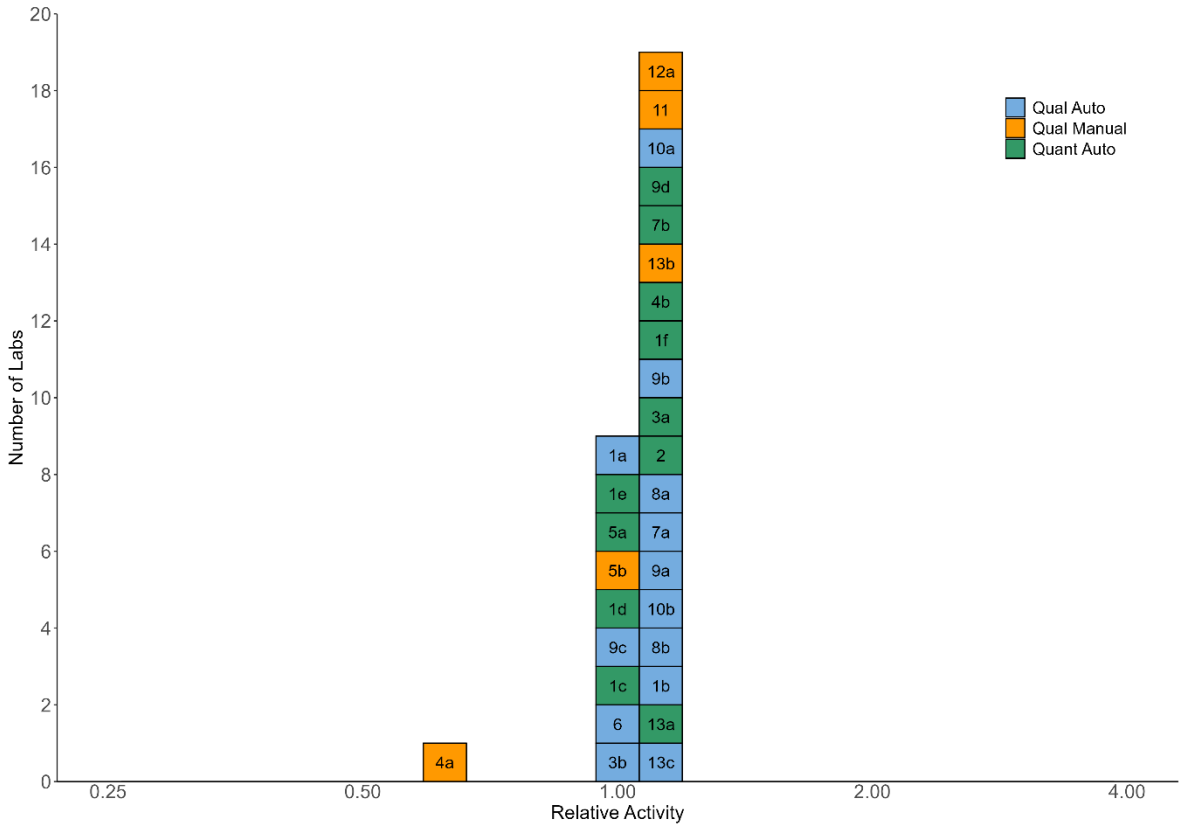


Figure 6 - Potency Estimates of Sample B, calculated relative to 3rd IS (assigned potency of 47.3 IU/mL)

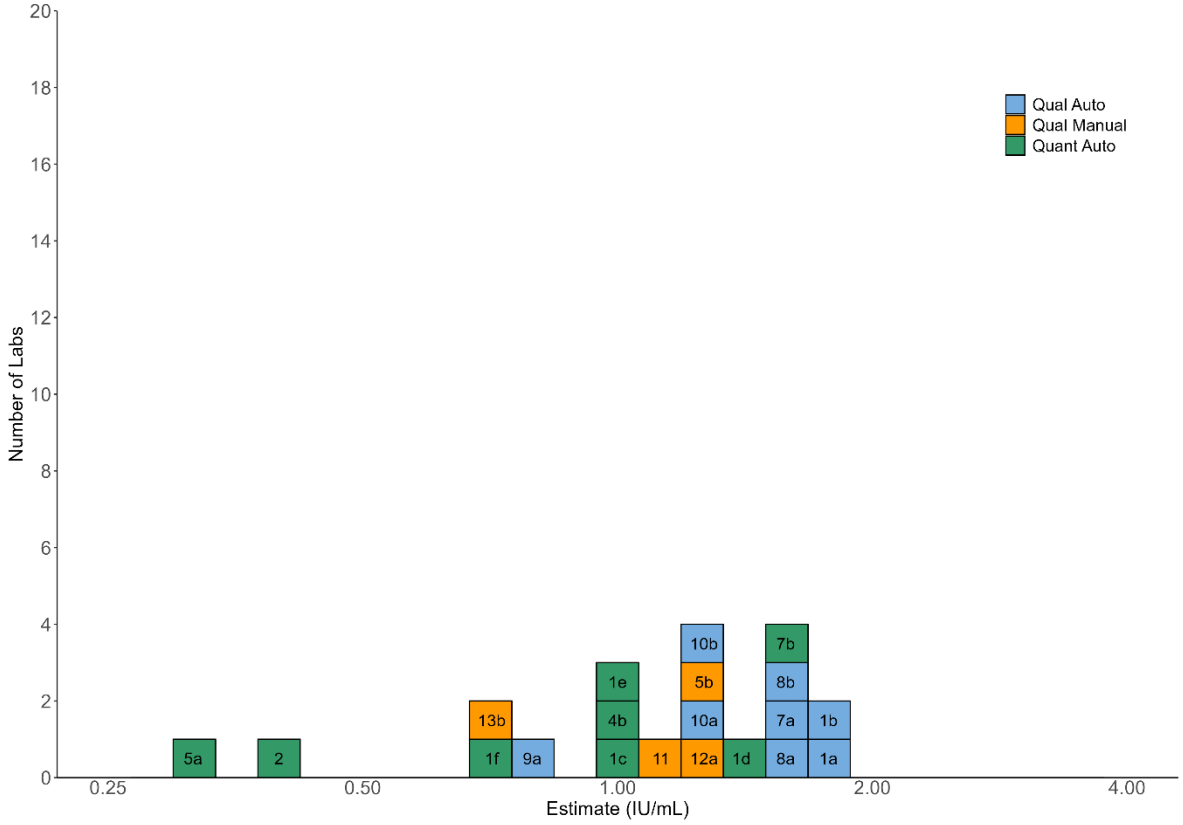


Figure 7 - Potency Estimates of Sample C, calculated relative to 3rd IS (assigned potency of 47.3 IU/mL)

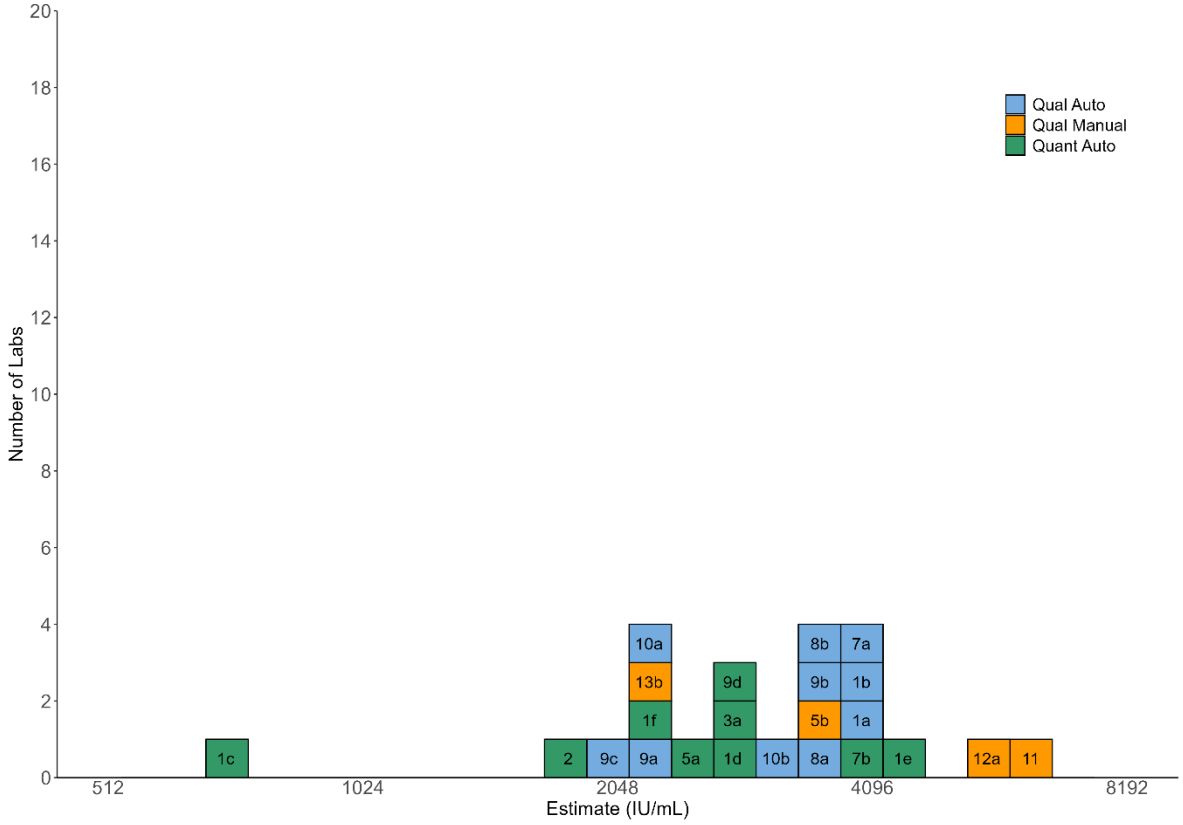


Figure 8 - Potency Estimates of Sample D, calculated relative to 3rd IS (assigned potency of 47.3 IU/mL)

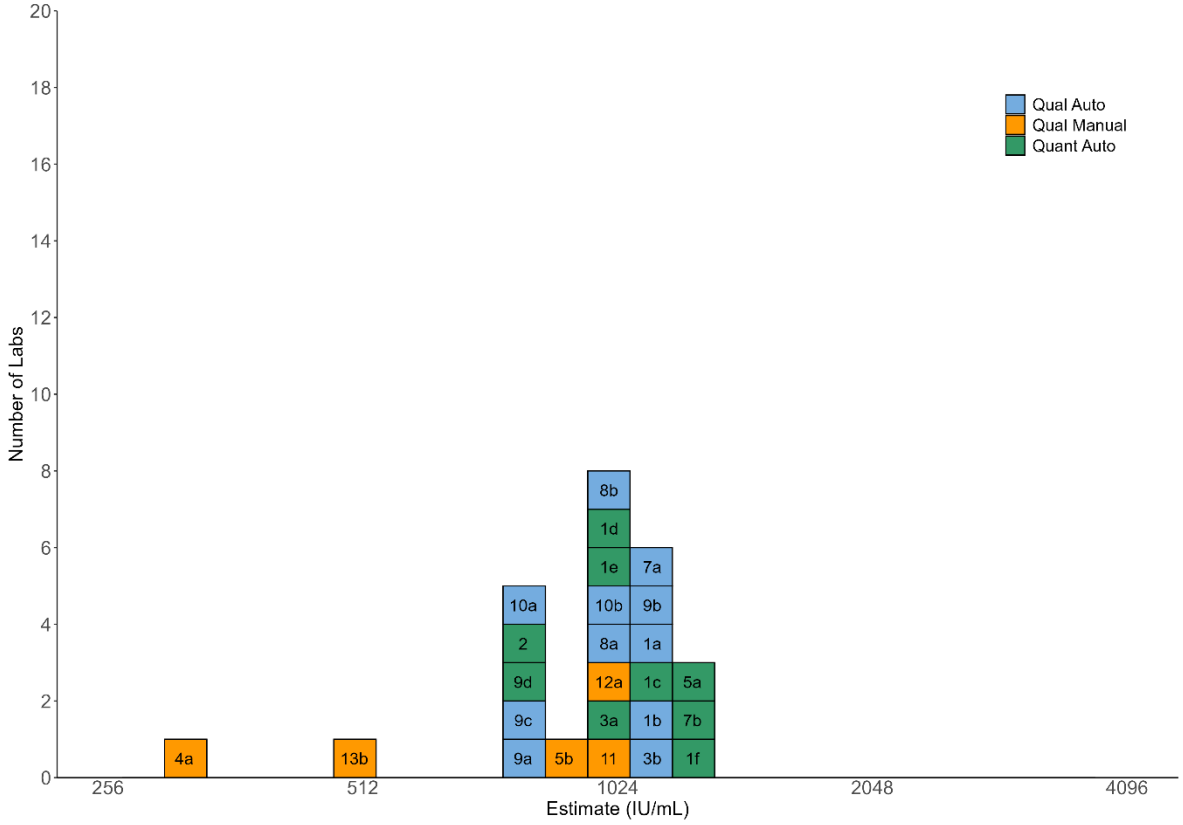
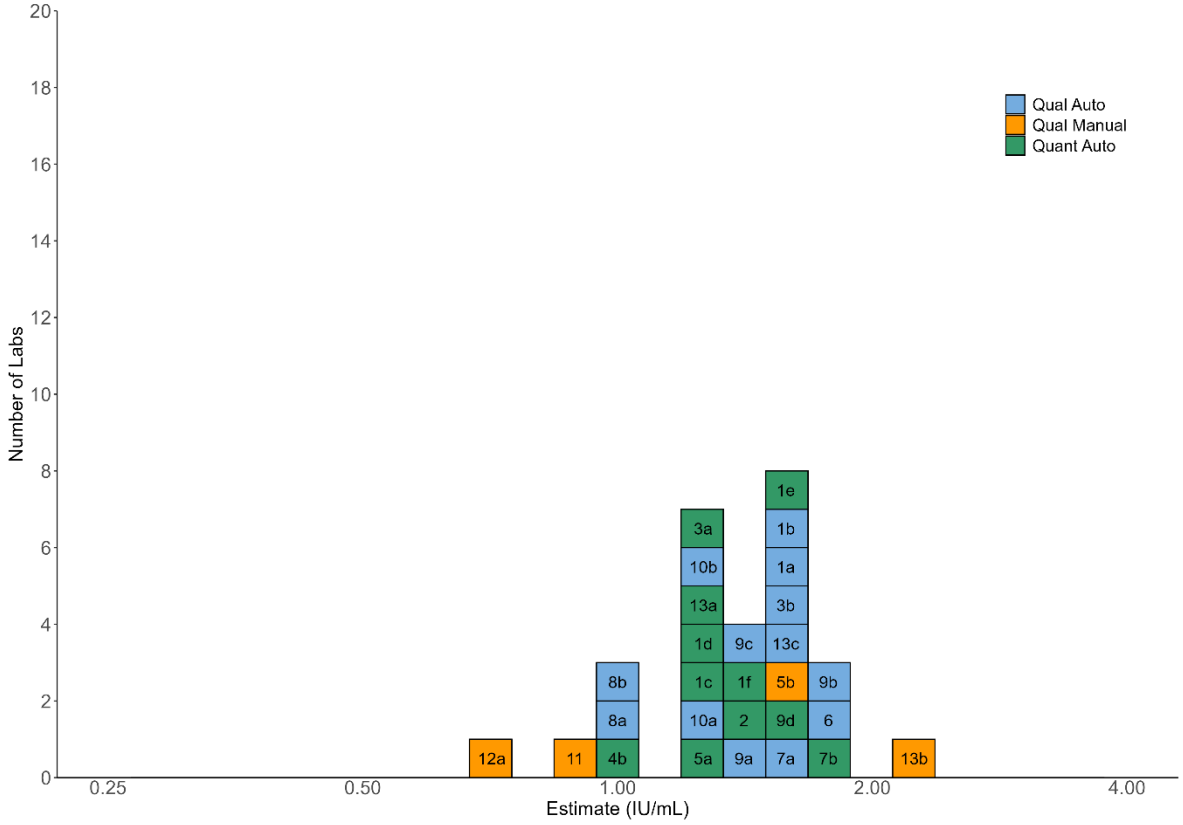


Figure 9 – Potency Estimates of Sample G, calculated relative to 3rd IS (assigned potency of 47.3 IU/mL)



11. Appendices

Appendix 1: List of participants

Participant	Organisation	Country
Mel Costello	Abbott	Ireland
Lucie Bertheault	Bio-Rad Laboratories	France
Nathalie Ripoll Auberger	BioMérieux	France
Stefania Simonelli	DiaPro Diagnostic BioProbes	Italy
Francesco Capuano	DiaSorin	Italy
Graham Prescott	NIBSC-MHRA	UK
Matteo Simeoni	National Centre for the Control and Evaluation of Medicines (CNCF)	Italy
Tomoko Kiyohara	National institute of Infectious Diseases (NIID)	Japan
Terri Sahin	National Reference Laboratory (NRL)	Australia
Alimire Abulikemu	NIFDC	China
Olivia Knauer	Paul-Ehrlich-Institut (PEI)	Germany
Martina Schaetzl	Roche Diagnostics	Germany
Sook in Shin	Siemens Healthcare Diagnostics	USA

Appendix 2: Collaborative Study Protocol

Collaborative Study to evaluate the performance of the proposed 4th International Standard for HBsAg

Background

The International Standard for HBsAg was first established in 1985 (NIBSC code 80/549) and aided in harmonising HBsAg assay systems. The standard has since been replaced, with the current 3rd International standard (12/226) now near depletion. The standard is still used to assess sensitivity and fulfil CTS requirements for IVDs in Europe. A replacement project was therefore proposed and approved by the WHO at the October 2024 ECBS meeting.

Objective

The aim of the study is to evaluate the performance of the proposed 4th International Standard for HBsAg against the 3rd International Standard (12/226) in a range of screening assays for the detection of HBsAg. Additional samples included will aid in accessing commutability of the candidate relative to clinical samples and assess other potential replacement formulations for future replacements.

Materials

Sample ID	A	B*	C*	D*	E	F	G
Storage Temp.	-70°C	-70°C	-70°C	-70°C	-20°C	-20°C	-70°C
Volume	0.5 mL	1mL			0.5mL	1mL	1mL
Formulation	Liquid				Lyophilised		Liquid
Vial Type	Sarstedt 2mL				Glass ampoule		Sarstedt 2mL

*** Samples B, C & D are HBV positive clinical samples please note that these may be infectious. These samples may be excluded if requested by the lab contact.**
Numbers supplied is dependent on the number of assays being performed. If volumes are too low, please contact NIBSC for advice on how to test samples.

Study Protocol

Participants will receive **Samples A to D & G** (liquid preparations) and **Samples E & F** (Lyophilised preparations). Liquid samples should be stored at -70°C (or below) & Lyophilised samples should be stored at -20°C on receipt.

Lyophilised preparations (Samples E and F) will be supplied in a sealed glass ampoule; care should be taken on opening the ampoules (Please refer to the IFU for full instructions). **Sample should be reconstituted with deionised (i.e. Sample E = 0.5mL and Sample F = 1mL), nuclease-free, molecular biology-grade water and left for a minimum of 20 minutes with occasional/gentle agitation before use. Check the content is completely reconstituted (and**

well vortexed) and then transfer into a sealed (labelled) tube prior to preparing a dilution series.
Study samples (A, E – G) contain human serum which have been screened and found negative for anti HIV1-/2, HBsAg and HCV RNA. Samples B, C and D are clinical HBV positive samples which should be considered infectious. All materials should be handled according to the laboratories Code of Practice for materials of human origin.
All dilutions should be carried out in negative human plasma/Serum (not supplied). This should be **confirmed negative for anti-HBs** prior to use and **negative for HBsAg**.

Assays

Perform 3 independent assays on separate occasions. A fresh vial for each sample should be used in each assay run. Similarly, if testing another assay system on the same day please use a fresh set of vials and dilutions.

If the participating lab has offered to perform testing using two or more assays, please use fresh vials and repeat as outlined above. A new results sheet can be used for each assay.

Samples

Participants are requested to test a dilutions series (as described in the worked examples below) in **duplicate**. Dilutions can be adjusted or extended to reach the assays end point in Assays 2 and 3 if required.

Sample A & E

Neat = Reconstitute sample E in 500µl Nuclease-free water

	Diluted Material		Negative diluent		Total Volume
*1:20	100µl	+	1900µl	=	2000µl
*1:2 = (1:40)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:80)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:160)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:320)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:640)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:1280)	1000µl	+	1000µl	=	2000µl

*TEST

Clinical Samples B, C and D

Note: If samples B, C & D fall out outside of sample E/F's results range, please test **further as required** in assays 2 and 3.

Sample B

	Diluted Material	Negative diluent	Total Volume
*NEAT:			
*1:4	250ul	+ 750ul =	1000ul

*TEST

Sample C

	Diluted Material	Negative diluent	Total Volume
Neat			
1:25	100µl	+ 2400µl =	2500µl
1:20 = (1:500)	100µl	+ 1900µl =	2000µl
1:2 = (1:1000)	1000µl	+ 1000µl =	2000µl
*1:2 = (1:2000)	1000µl	+ 1000µl =	2000µl
*1:4 = (1:8000)	250ul	+ 750ul =	1000ul

*TEST

Sample D

	Diluted Material	Negative diluent	Total Volume
Neat			
1:25	100µl	+ 2400µl =	2500µl
1:20 = (1:500)	100µl	+ 1900µl =	2000µl
1:2* = (1:1000)	1000µl	+ 1000µl =	2000µl
*1:4 = (1:4000)	250µl	+ 750µl =	1000µl

*TEST

Sample F

Neat = Reconstitute in 1000ul Nuclease-free water

	Diluted Material		Negative diluent		Total Volume
*1:25	100µl	+	2400µl	=	2500µl
*1:2 = (1:50)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:100)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:200)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:400)	1000ul	+	1000ul	=	2000ul
*1:2 = (1:800)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:1600)	1000µl	+	1000µl	=	2000µl

*Test

Sample G

Neat

	Diluted Material		Negative diluent		Total Volume
1:2	1000µl	+	1000µl	=	2000µl
1:2 = (1:4)	1000µl	+	1000µl	=	2000µl
1:2 = (1:8)	1000µl	+	1000µl	=	2000µl
1:2 = (1:16)	1000µl	+	1000µl	=	2000µl
1:2 = (1:32)	1000µl	+	1000µl	=	2000µl
*1:2 = (1:64)	1000µl	+	1000µl	=	2000µl

*TEST
Results

Results of each assay should be recorded on the appropriate result form included with this study protocol.

Results should be returned to NIBSC **as soon as possible or within 8 weeks of receiving your study samples** to allow sufficient time for statistical analysis and preparation of the final report for submission to the WHO Expert Committee for Biological Standardisation.

All completed forms should be returned to:

Graham Prescott
graham.prescott@mhra.gov.uk

Appendix 3: Collaborative Results sheet

Result form for 4th IS for HBsAg collaborative Study

Assay 1		
Date:	Lab number:	Operator:

Sample	Dilution	Positive/ Negative	OD	Cut off Value	Comments
Sample A (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample A (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample B (R1)	NEAT				
	1:4				
Sample B (R2)	NEAT				
	1:4				

Sample C (R1)	1:2000				
	1:8000				
Sample C (R1)	1:2000				
	1:8000				
Sample D (R1)	1:1000				
	1:4000				
Sample D (R1)	1:1000				
	1:4000				
Sample E (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample E (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				

Sample F (R1)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample F (R2)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample G (R1)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Sample G (R2)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Assay 2		
Date:	Lab number:	Operator:

Sample	Dilution	Positive/ Negative	OD	Cut off Value	Comments
Sample A (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample A (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample B (R1)	NEAT				
	1:4				
Sample B (R2)	NEAT				
	1:4				

Sample C (R1)	1:2000				
	1:8000				
Sample C (R1)	1:2000				
	1:8000				
Sample D (R1)	1:1000				
	1:4000				
Sample D (R1)	1:1000				
	1:4000				
Sample E (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample E (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				

Sample F (R1)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample F (R2)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample G (R1)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Sample G (R2)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Assay 3		
Date:	Lab number:	Operator:

Sample	Dilution	Positive/ Negative	OD	Cut off Value	Comments
Sample A (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample A (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample B (R1)	NEAT				
	1:4				
Sample B (R2)	NEAT				
	1:4				

Sample C (R1)	1:2000				
	1:8000				
Sample C (R1)	1:2000				
	1:8000				
Sample D (R1)	1:1000				
	1:4000				
Sample D (R1)	1:1000				
	1:4000				
Sample E (R1)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				
Sample E (R2)	1:20				
	1:40				
	1:80				
	1:160				
	1:320				
	1:640				
	1:1280				

Sample F (R1)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample F (R2)	1:25				
	1:50				
	1:100				
	1:200				
	1:400				
	1:800				
	1:1600				
Sample G (R1)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Sample G (R2)	1:2				
	1:4				
	1:8				
	1:16				
	1:32				
	1:64				

Method Details:

Assay	Assay Name	Product Code	Assay lot	Automated platform
1				
2				
3				

Appendix 4 – Estimates for results calculated relative to current IS

Lab	Assay	A	B	C	D	E	G
1a	1	38.11	1.731	4001	1106	34.74	1.582
1a	2	36.36	1.764	3969	1061	35.99	1.545
1a	3	39.34	1.726	4099	1134	36.63	1.722
1b	1	37.18	1.799	4046	1115	35.08	1.624
1b	2	37.76	1.824	3910	1085	36.37	1.671
1b	3	38.63	1.830	4137	1090	35.30	1.653
1c	1	34.06	0.9892	682.9	1110	33.77	1.313
1c	2	36.62	1.039	692.5	1171	33.14	1.323
1c	3	32.47	1.008	641.3	1022	32.93	1.212
1d	1	36.44	1.315	2702	1010	33.55	1.265
1d	2	36.54	1.405	2758	1049	36.26	1.277
1d	3	36.78	1.444	2828	1056	35.51	1.321
1e	1	39.23	1.032	4932	821.2	46.92	1.355
1e	2	42.23	1.269	5953	1114	38.91	1.613
1e	3	45.73	0.8905	3021	1170	36.57	2.110
1f	1	37.92	0.6976	2256	1159	33.89	1.443
1f	2	38.14	0.6712	2267	1189	35.54	1.458
1f	3	37.79	0.7271	2292	1223	34.72	1.436
2	1	39.10	0.3793	1518	717.6	37.67	1.509
2	2	44.22	0.4080	1880	901.8	38.97	1.476
2	3	41.18	0.3781	1694	829.8	37.84	1.317
3a	1	38.13	0.4882^{NP}	2809	995.7	35.20	1.332
3b	1	35.73	0.7360^{NP}	3747^{NP}	1073	36.06	1.602
4a	1	35.41^{NL}	4.322^{NP}	686.6^{NL}	938.6^{NL}	43.53^{NL}	136.7^{NP}
4a	2	24.15^{NP}	16.85^{NL}	381.3^{NL}	320.9	29.78	157.4
4a	3	17.69	13.96^{NL}	707.2^{NP}	384.0^{NP}	31.94	155.0
4b	1	35.08	1.247	11964	6544	29.71	1.017
4b	2	35.53	0.8832	22405^{RR}	3659	33.83	0.9370
4b	3	38.12	1.004	8069	2363	35.74	0.9333
5a	1	33.48	0.3251	1394	1089	37.29	1.029
5a	2	40.10	0.3385	4488	2119	37.93	1.529
5a	3	43.20	0.2726	2228	966.6	35.41	1.277
5b	1	40.26	1.225	3531	790.2	40.07	1.510
5b	2	39.70	1.154	3418	860.3	38.06	1.606
5b	3	44.67	1.307	6062^{NP}	883.2	40.99	1.647^{NP}
6	1	41.74	NT	NT	NT	39.34	1.641
6	2	40.22	NT	NT	NT	37.94	1.603
6	3	46.02	NT	NT	NT	46.74	2.104
7a	1	35.39	1.607	4030	1109	32.32	1.495
7a	2	38.68	1.630	4137	1195	34.69	1.521
7a	3	35.28	1.530	3950	1185	34.31	1.487
7b	1	43.84	1.660	4197	1404	39.29	1.823
7b	2	42.53	1.608	3774	1223	38.45	1.653

7b	3	42.26	1.611	3753	1239	39.02	1.727
8a	1	37.67	1.600	3535	1037	34.19	0.9729
8a	2	36.02	1.498	3406	1011	33.38	0.9922
8a	3	36.42	1.428	3203	945.4	34.51	0.9471
8b	1	38.36	1.666	3844	1074	35.06	0.9868
8b	2	36.36	1.530	3656	1060	34.38	0.9851
8b	3	37.18	1.597	3499	1016	35.45	0.9443
9a	1	35.07	0.8034	2306	744.4	31.22	1.344
9a	2	35.39	0.4936[NP]	2192	773.5	33.79	1.361
9a	3	36.01	0.5375[NP]	2266	805.1	34.71	1.353
9b	1	39.50	0.7928[NP]	3869	1361	35.83	2.003
9b	2	33.77	0.6825[NP]	3154	950.1	34.43	1.739
9b	3	42.47	0.8165[NP]	4026	1213	35.51	1.903
9c	1	37.67	0.4630[NP]	1908	886.0	38.50	1.505
9c	2	38.48	0.4133[NP]	2217	733.9	37.22	1.494
9c	3	38.65	0.4412[NP]	1948	729.0	34.83	1.486
9d	1	38.78	0.6896[NL]	2616	762.4	32.27	1.479
9d	2	38.71	0.7664[NP]	2677	721.3	34.62	1.462
9d	3	41.17	0.7476[NL]	3268	880.6	38.10	1.731
10a	1	36.70	1.294	2730	893.0	34.43	1.357
10a	2	31.06	1.028	1836	764.4	26.97	1.039
10a	3	44.63	1.314	2587	1643[NP]	36.96	1.483
10b	1	36.75	1.303	3428	2186[NP]	35.70	1.270
10b	2	38.59	1.401	2825	997.7	37.03	1.390
10b	3	36.93	1.297	2881	1612[NP]	32.52	1.302
11	1	38.37	1.117[NP]	6005	909.6	32.10	0.8761[NP]
11	2	35.05	1.396	5880	862.9	33.10	0.9257
11	3	49.33	0.9164	6613	1252	37.45	0.9458
12a	1	32.75	1.178	4950	916.6	29.66	0.7531
12a	2	35.41	1.194	5579	988.8	28.68	0.7433
12a	3	36.11	1.234	5501	1092	30.64	0.6953
12b	1	34.48	1.197	4996	917.0	31.46	0.6882
12b	2	32.21	1.018	4830	852.1	29.18	0.6023
12b	3	32.12	1.016	4919	925.3	29.66	0.6375
13a	1	38.23	NT	NT	NT	36.06	1.329
13a	2	38.54	NT	NT	NT	35.82	1.318
13a	3	38.21	NT	NT	NT	36.35	1.277
13b	1	40.49	0.6870	2416	530.5	36.56	2.296
13b	2	42.83	0.6771 [NL]	2333	506.6 [NL]	38.56	2.460
13b	3	40.87	0.7376	2169	514.0	37.79	2.240

NT = Not Tested
RR = Response ranges of reference and test samples not comparable
NL = Non-Linearity of test sample
NP = Non-Parallelism between reference and test samples

Appendix 5 – Proposed IFU for the candidate 4th International Standard for HBsAg

Medicines & Healthcare products Regulatory Agency

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WHO International Standard
Fourth International Standard for HBsAg
NIBSC code: 25/120
Instructions for use
(Version0.1, Dated 22/05/2026)

1. INTENDED USE

This preparation contains inactivated HBsAg (HBV genotype B4, HBsAg subtypes ayw1/adw2) and has been calibrated in International Units (IU) in an international collaborative study. It was calibrated against the 3rd international standard (IS) for HBsAg (NIBSC code: 12/226), B4, ayw1/adw2). The 4th WHO IS for HBsAg is intended to be used for the determination of analytical sensitivity of HBsAg assays and for the calibration of secondary references for HBsAg.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The 4th International Standard for HBsAg has an assigned unitage of 17.5 IU/ampoule

4. CONTENTS

Country of origin of biological material: United Kingdom.
Country of origin of biological material: Vietnam (HBsAg vaccine bulk) and UK (human serum) . Each ampoule contains the freeze-dried equivalent of 0.5 mL HBsAg in human serum containing 0.05% Bronidox as preservative. The international collaborative study for calibrating 25/120 indicated an overall geometric mean potency of 35 IU/ampoule. The source material used to produce 25/120 is a non-adjuvanted HBsAg vaccine bulk derived from human plasma obtained from viremic carriers. During its manufacture, the purified HBsAg vaccine bulk had been rendered non-infectious by heat treatment and formaldehyde inactivation steps. Sequence analysis of the source HBsAg bulk identified at least two different HBV strains, both with genotype B4. The B4 genotype is in good correlation to the prevalent HBV genotypes in Vietnam where the plasmas were collected and purified. As a consequence of pooling plasma from multiple donors, the candidate HBsAg subtype is a heterogeneous population of ayw1 and adw2.

5. STORAGE

25/120 should be stored at -20ºC on receipt.
Please note because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. The contents of the ampoule should be reconstituted with 0.5 mL deionised water and left for a minimum of 20 minutes with occasional agitation before use. The reconstituted material has a final concentration of 35 IU/mL.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

9. REFERENCES

TBC

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants. We also thank Professor Nguyen Thu Van, VABIOTECH, Hanoi, Vietnam, for the kind donation of the HBsAg bulk material

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG . T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

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13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified
https://nibsc.org/documents/ifu/msds/MSDS-25-120.pdf

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.5g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached**: False

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards https://www.who.int/publications/m/item/annex2-trs932(revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization