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Collaborative Study to Evaluate the Candidate 3rd WHO International Standard for Hepatitis B Surface Antigen

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **4 October 2014** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Jongwon Kim** at email: kimjon@who.int.

Summary

A WHO international collaborative study was undertaken to characterize and assess the suitability of a candidate replacement WHO International Standard (IS) for HBsAg for use in the standardization of HBsAg assays. The candidate is a non-adjuvanted, plasma-derived HBsAg vaccine bulk formulated to give ~ 50 International Units (IU)/mL in thrombinized and declotted plasma, dispensed into ampoules in 1.0 mL aliquots and freeze-dried for long-term stability. The candidate antigen is hepatitis B virus (HBV) genotype B4 with a heterogeneous HBsAg subtype population of *ayw1* and *adw2*.

Twelve laboratories from 9 countries participated in the collaborative study to evaluate the fitness for purpose and potency of the candidate using their routine assays for HBsAg. The freeze-dried candidate 3rd WHO IS (NIBSC code 12/226) was evaluated alongside the 2nd WHO IS for HBsAg (NIBSC code 00/588) and the 1st WHO IS for HBsAg (NIBSC code 80/549). Blinded study samples were also provided for testing in the collaborative study. These included a duplicate of the candidate 3rd IS, a negative control and samples representing different HBV genotypes.

In this collaborative study, a range of commercial qualitative and quantitative HBsAg tests were used to evaluate the candidate IS. The overall geometric mean potency of the candidate, plus its coded duplicate, relative to the concurrently tested 2nd IS for HBsAg was 47.3 IU/mL, a value that is very close the target 50 IU/mL Laboratories showed a generally good level of reproducibility for each kit. Intra-laboratory agreement generally was better than the inter-laboratory agreement. Some HBV genotype-dependent effects on the inter-laboratory variability were observed with variability between laboratories being greatest for samples more distantly related to the reference standard. The results obtained from accelerated thermal degradation studies at ~1 year indicate that the candidate is stable and suitable for long-term use.

The results of the collaborative study indicate the suitability of the candidate to serve as an IS and it is proposed that 12/226 is established as the 3rd WHO IS for HBsAg with an assigned potency of 50 IU when reconstituted in 1 mL water.

Introduction

It is estimated that >2 billion people world-wide have been infected with HBV. Of these, approximately 600,000 people die annually from acute infections or cirrhosis and hepatocellular carcinoma caused by chronic infection. In spite of the availability of safe and effective prophylactic vaccines, HBV infection remains a major public health problem worldwide, with chronic infection serving as the main reservoir for the transmission of new infections.
The virus is highly contagious and is transmitted by percutaneous and permucosal exposure to infected blood and other body fluids [1, 2]. Laboratory diagnosis of hepatitis B infection centers on the detection of the hepatitis B surface antigen HBsAg. Sensitive screening and accurate diagnostic HBsAg assays are therefore essential for the prevention and the management of HBV disease.

The WHO's Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO ISs are recognized as the highest order of references for biological substances and are assigned potencies in International Units. Their primary purpose is to calibrate secondary references used in routine laboratory assays in terms of the IU thereby providing a uniform results-reporting system and traceability of measurements independent of the method used. International Standards are prepared in accordance with published WHO recommendations [3].

The HBsAg IS is used by clinical laboratories, blood transfusion laboratories, manufacturers of blood products and in vitro diagnostic kit manufacturers to calibrate secondary reference materials for HBsAg and in the validation of HBsAg assays. Stocks of the 2nd WHO IS for HBsAg (NIBSC code 00/588) are now depleted. The proposal to establish its replacement was endorsed by ECBS in October, 2012. This report describes the development and worldwide collaborative study evaluation of a candidate 3rd WHO IS for HBsAg for use in the standardization of HBsAg assays.

Aims of study

The aims of this collaborative study were to

- assess the suitability of the freeze-dried HBsAg, formulated in thrombinzed and de-clotted plasma, to serve as the 3rd WHO IS for HBsAg for use in the standardisation of HBsAg serology assays.
- evaluate the candidate 3rd IS relative to the 2nd WHO IS for HBsAg (NIBSC code 00/588) and assign unitage in International Units (IU) per ampoule. There is no international conventional reference measurement procedure for HBsAg in serology and the IU is not readily traceable to the International System of Units (SI) of quantity.
- assess the candidate's potency i.e. reactivity in a range of typical assays performed in different laboratories.
- assess commutability i.e. to establish the extent to which the reference standard is suitable to serve as a standard for different samples being assayed.

Materials and Methods

Table 1 lists the collaborative study samples. All study samples were stored at -20°C or below until dispatched on dry ice to participants. Codes for the quantitative and qualitative assays used by participants in the collaborative study are summarised in Table 2.

Candidate material

Source HBsAg bulk material

This is a non-adjuvanted plasma-derived HBsAg vaccine bulk (Lot HB-PL01) kindly donated by Professor Nguyen Thu Van, VABIOTECH, Hanoi, Vietnam. The HBsAg was derived from human plasma obtained from asymptomatic carriers with high titers. The HBsAg bulk was purified and inactivated at VABIOTECH using validated methods for the manufacture of plasma-derived Hepatitis B vaccine (http://whqlibdoc.who.int/trs/WHO_TRS_858.pdf) [4]. The HBsAg bulk has been tested at NIBSC and found negative for antibodies to HIV and HCV RNA. The inactivated HBsAg bulk is positive for HBV DNA.

Molecular and antigenic characterisation of source HBsAg bulk lot HB-PL01

The plasma-derived HBsAg vaccine bulk was sent to the National Reference Centre for Hepatitis B and D Viruses, Institute of Medical Virology, Justus-Liebig University Giessen (JLU),

Germany for additional characterisation. For determination of the HBV genotype and HBsAg subtype, HBV DNA was extracted and purified from the bulk (High Pure Viral Nucleic Acid Kit, Roche) and amplified using a proofreading Pfu polymerase (Agilent Technologies) and primers flanking the S gene which encodes the major protein of HBsAg. The sequence of the purified PCR product was analysed using MegAlign and ClustalW alignment software (DNASTAR). To assess the population of HBV surface proteins, Western blot analysis was performed on the HBsAg bulk using specific monoclonal antibodies against the epitope in the preS1 domain in the L-HBs protein (MA18/7) [5], the glycosylated preS2 domain of the M-HBs protein (Q19/10) [6] or a sequential epitope of the S-HBs protein (HB1) [7].

Preparation, filling and freeze-drying of the candidate 3rd IS

To obtain a target concentration of 50 IU/mL, the HBsAg vaccine bulk was diluted 1:728 in thrombinized and declotted plasma which had been shown to be negative for anti-HCV, anti-HIV 1+2, HBsAg, anti-HBs, HCV RNA, HBV DNA and HIV RNA. Bronidox was included as preservative (0.05% final concentration). In November 2012, the formulation was prepared and filled in 1ml aliquots into 5mL DIN ampoules and freeze-dried at NIBSC in the Standards Processing Division using standard operating procedures. The material is labelled as the candidate 3rd WHO IS for HBsAg (NIBSC product code 12/226).

Post-fill testing of freeze-dried 12/226

The HBsAg concentration of 12/226 was determined using different quantitative and qualitative assays at the Testing Laboratory for IVD, Paul-Ehrlich-Institut (PEI), Federal Institute for Vaccines and Biomedicines, Germany. The analytical sensitivity was calculated at PEI using the values obtained for serial dilutions of the 2nd IS and candidate 3rd IS by linear interpolation and expressed in IU/mL. The potency of 12/226, expressed relative to the 2nd IS (NIBSC 00/588) was calculated at NIBSC using the same methods as described for the main collaborative study analysis (see Statistical Methods).

Additional study samples

2nd WHO IS for HBsAg (NIBSC code 00/588)

This is the current IS for HBsAg with an assigned potency of 33 IU/vial. 00/588 is a freeze-dried preparation of plasma-derived, purified and inactivated HBsAg diluted in re-calcified plasma [8]. The antigen has been shown to be HBV genotype A2, HBsAg subtype *adw2* [9].

1st WHO IS for HBsAg (80/549)

This is a former IS for HBsAg (subtype group *ad*) with an assigned potency of 100 IU/vial. 80/549 is freeze-dried, heat-treated serum obtained from a clinically healthy, HBsAg-positive donor [10, 11].

Blinded study samples

***Sample A*:** This is a member of the WHO HBsAg reference panel (NIBSC product code 03/262). The material is a freeze-dried preparation of a 1:16 dilution of the bulk material used to make the 2nd IS and has an approximate unitage of 2 IU/vial [8].

***Sample B*:** This is freeze-dried, re-calcified plasma supplied as a negative control for panel 03/262 [8].

***Sample C*:** This is a blinded duplicate of candidate 12/226.

***Sample D*:** PEI Sample N4222 is the unprocessed source material used in the preparation of a member of the 1st WHO International Reference Panel for HBV genotypes for HBsAg assays (PEI product code 6100/09) [12]. N4222 is genotype B2 HBsAg subtype *adw2* with a concentration of 95300 IU/mL as measured by the ARCHITECT HBsAg quantitative assay (Abbott Diagnostics). For use as sample D in the collaborative study, N4222 was diluted 1:1200 in thrombinized and declotted plasma. For dispatch to participants, 250uL aliquots were dispensed into screw-capped tubes and stored at -20°C.

***Sample E*:** PEI sample N4879 is the unprocessed source material used in the preparation of another member of PEI 6100/09 [12]. N4879 is HBV genotype A2, HBsAg subtype *adw2* with a concentration of 74300 IU/mL as determined by the ARCHITECT HBsAg quantitative assay. For use as sample E in the collaborative study, N4879 was diluted 1:1000 in thrombinized and declotted plasma. For dispatch to participants, 250uL aliquots were dispensed into screw-capped tubes and frozen at -20°C.

Participants

Study samples were sent to 13 laboratories who accepted the invitation to participate in the study. Twelve laboratories completed the study by returning results. The participants were from 9 countries: Australia (1), France (1), Germany (2), India (1), Italy (2), Japan (1), UK (1), USA (2) and Vietnam (1). Participating organisations included assay kit manufacturers, research laboratories and national control or assay evaluation laboratories. Participating laboratories are listed in Appendix 1.

Study protocol

The final version of the study protocol, as provided to the participants, is given in Appendix 2. In brief, participants were requested to test the samples using their routine assay(s) for HBsAg. Participants were asked to perform 3 independent assays on different days. An Excel reporting sheet was provided with suggested dilutions for assaying each study sample. For each assay, participants were requested to make 2 independent series of dilutions of the study samples, and assay all samples concurrently. If multiple plates were required for testing all study samples, participants were instructed to include the candidate standard on each plate. Participants were requested to record in the reporting sheet all essential information including the raw data from each assay.

Statistical methods

All laboratories are referred to by code number allocated at random and not representing the order of listing in Appendix 1. Where a laboratory returned data using different assay methods, the results were assayed separately for each method and are referred to according to the lab number and assay code e.g. 3 Eql and 3 Mv3.

Individual assays were analyzed as multiple parallel-line assays, expressing the potency of the study samples relative to the different references. All study samples were included in the analysis of each assay. In all cases, a log transformation of the response and subtraction of the plate's average blank well were used to achieve linearity and parallelism. The EDQM statistical analysis package CombiStats was used [13]. Where assays were spread over different plates, the data from individual plates were analyzed independently, where the relevant reference was included on the plate. Potency estimates were then combined across plates, to give single estimated potencies for each sample for each assay.

Potency estimates were combined using unweighted geometric means, to give laboratory mean potency estimates for the study samples against the appropriate reference. Overall means for the study were calculated as unweighted geometric means of the individual laboratory means. Variability within (intra-laboratory) and between (inter-laboratory) laboratories was expressed as a geometric coefficient of variation (%GCV) [14].

Agreement between the potency estimates of the duplicate samples (12/226 and Sample C) for each laboratory and method was assessed by calculating the square root of the mean squared difference in log potency estimates across assays, expressed as a percentage.

Differences in potency estimates between different kits were assessed using a multiple comparison Analysis of Variance (ANOVA) with Tukey's correction for multiple comparisons [15].

12/226 stability assessment

An accelerated degradation study was performed in order to predict the long-term stability of the candidate 3rd IS when stored at the recommended temperature of -20˚C. Ampoules of 12/226 were stored at -70˚C, -20˚C, +4˚C, 20˚C, 37˚C, 45 ˚C and 56 ˚C and then removed after 343 days and held at -80˚C until assayed. Samples from each temperature were assayed concurrently in 3 independent assays using the Murex v3 assay kit, Diasorin Ltd. The potencies of the samples were calculated relative to the sample stored at -70˚C using the same methods as described for the main collaborative study analysis. The stability of the candidate standard was assessed using the Arrhenius model for accelerated degradation studies using weighted and semi-weighted data. [15]

Results and data analysis

Molecular and antigenic characterization of the plasma-derived HBsAg vaccine bulk Lot HB-PL01

Characterization of the source HBsAg bulk was performed at JLU. Sequence analysis of PCR products amplified from the HBsAg bulk and covering the S gene revealed the wild-type sequences of at least two different HBV strains, both with genotype B4, typical for Vietnam where the plasmas were collected and purified. The HBsAg subtype is heterogeneous with *ayw1* and *adw2*. Western analysis of the HBsAg bulk indicated that the material contains mainly S-HBs protein with only traces of preS1 and preS2 antigens (results will be published elsewhere).

Production QC, validation and stability assessment of 12/226

Filling and freeze-drying

The product summary for the freeze-dried candidate standard (NIBSC code 12/226) is shown in Table 3. The % coefficient of variation (CV) of the fill mass (0.28%) and mean residual moisture (0.40 %) are within acceptable limits for a WHO IS [3]. Residual oxygen content (0.3%) is within the NIBSC working limit of 1.1%.

Validation

Estimations of assay sensitivity of 12/226 compared to the 2nd IS were determined at PEI using three different methods as shown in Table 4. The analytical sensitivities estimated using 12/226 were in close agreement with those obtained for the 2nd IS. The values obtained for both the 2nd IS and 12/226 are in line with those published for those methods used in the collaborative study evaluating a HBV genotype panel for HBsAg assays [12].

Post-fill validation also included estimating the potency in IU/mL of 12/226 relative to the 2nd IS which has an assigned potency of 33 IU/mL (Table 5). Two independent assays were performed for each of the 5 assay methods. The 1st IS was also included in the assays to serve as a benchmark. With the exception of results using the ADVIA Centaur HBsAg II method, potencies for the 1st IS and the candidate 3rd IS were close to the expected values of 100 IU/mL and 50 IU/mL, respectively (Table 5). The overall GMs for the 1st IS and 12/226 across the 5 assay methods were 111 IU/mL and 58 IU/mL, respectively. The level of inter-method variability for both samples was high with overall GCVs of ~ 45%. The ADVIA Centaur method gave potencies ~2-fold greater than the expected values. The reason for this discrepancy is not known. When the results of the ADVIA Centaur assay were excluded from the validation, the inter-

method variability was improved, giving an overall GCV of 19% for the 1st IS and 31% for candidate 12/226. The overall GM for the remaining 4 methods was 95 IU/mL for the 1st IS and 50 IU/mL for 12/226.
Overall, this validation assessment indicated that the antigenicity, analytical sensitivity and potency of 12/226 were acceptable and that the candidate was suitable for further evaluation in the international collaborative study.

Stability

Accelerated degradation studies were undertaken on the candidate 3rd IS. Samples of the candidate were stored at varying temperatures of -70°C, -20ºC, +4ºC, +20ºC, +37ºC, +45ºC and +56ºC for a period of ~ 1 year. The samples stored at +56ºC could not be reconstituted and were therefore not assayed. The potency of samples of the candidate IS 12/226 stored at the elevated temperatures were expressed relative to the material stored at –70ºC. The results of individual assays are summarized in Table 6.
The long-term stability of the candidate IS 12/226 was predicted using the Arrhenius model, using all temperatures up to +45°C, which gave a statistically good fit to the data. The predicted percentage loss per month, or loss per year, at the different temperatures of storage, is also shown in Table 6. The predictions are dependent on the estimated potencies at +37ºC and above being reliable, and the apparent drop in potency not being affected by problems of reconstitution. This was seen with +45ºC and the sample cannot be assigned a percentage predicted loss due to the difficulty in reconstituting the material. It is not possible to obtain reliable predictions from the data for +4ºC and +20ºC alone, as insufficient degradation has occurred which shows the candidate is stable at +20ºC for up to 1 year. From these data, 12/226 appears to be adequately stable to serve as an IS, and is suitable for ambient transportation up to +37ºC as the loss is only 2% after 1 month. Further studies are being conducted to assay the stability of the reconstituted material at +4ºC and to assess the freeze/thaw effect.

Collaborative Study

Data received

Twenty-two data sets were received from 12 laboratories using a variety of quantitative and qualitative methods. Five participants performed more than one assay method. In the majority of cases, laboratories returned the requested three assays with each method that they used. Laboratory 8 only performed one assay with each of three different methods. Laboratory 12 used multiple plates per assay so not all samples comparisons could be made against the 1st, 2nd or candidate 3rd IS Standard for each assay.

Assay Validity:

Linearity of the log-transformed dose-response curves was assessed visually after plotting all assay data. In a few cases, responses at the extremes of the dose-response were excluded to improve the linearity. Parallelism of the dose-response lines for the different samples was assessed using an equivalence testing approach using the CombiStats package [13]. Samples where the ratio of the slope of the dose-response for the sample to the slope of the dose-response to the reference fell outside an 80-125% range were excluded. Overall, only five assays had individual samples excluded by this criterion. Unless stated otherwise, there were no other exclusions of data from the analysis.

Potencies

The individual laboratory geometric mean potencies expressed relative to the 2nd IS along with the intra-laboratory %GCVs are shown in Table 7 for the 1st IS, the candidate 3rd IS and blinded samples A-E. For each sample, the overall geometric mean potency across laboratories, excluding the result from laboratory3 SD3, is also shown, along with the inter-laboratory %GCV, which measures between-laboratory variability. The results for laboratory 3 SD3 are

from a single assay and are much higher than all other laboratories and methods. Because of this discrepancy, the results from laboratory 3 SD3 assay were excluded from the calculations of overall geometric means and between laboratory %GCVs.
As expected, HBsAg is not detected in the negative control sample B by any of the assay methods.
With an overall geometric mean of 98.7 IU/mL, the potency of the 1st IS is in excellent agreement with its assigned potency of 100 IU/mL. The between-laboratory agreement for the 1st IS, with a %GCV of 28%, is close to the published %GCV of 30% which was determined in the collaborative study that established the 2nd IS [8]. These results indicate that the continuity of the 2nd IS with respect to the 1st IS has been maintained since its establishment by ECBS in 2003.
At 48.6 IU/mL, the potency of the candidate 3rd IS, 12/226, comes out very close to the target of 50 IU/mL. The potency of sample C, the duplicate of 12/226, is also close to the target (46.2 IU/mL). The difference between the potency of 12/226 and sample C is not statistically significant and combining the data across the two samples gives an overall geometric mean potency of 47.3 IU/mL (Table 8).
The potency of sample A (1.8 IU/mL), a freeze-dried preparation of a 1:16 dilution of the bulk material used to make the 2nd IS, is in excellent agreement with its target potency of 2 IU/mL) [8].
For this collaborative study, the source materials for sample D (PEI N4222 genotype B2, subtype *adw2*) and E (PEI N4879 genotype A2, subtype *adw2*) were pre-diluted to potencies of ~79 IU/mL and ~74 IU/mL, respectively. The overall geometric mean potency for sample D, at 80.9 IU/mL, comes out very close to the target potency. The potency for sample E, at 105.2 IU/mL, is however somewhat higher than the expected value based on the potency of the source material (N4879) determined by the ARCHITECT HBsAg quantitative assay (see additional study samples). The results obtained for the two samples, however, confirm the findings of the recent WHO collaborative study which showed a greater variability of HBV genotype A2 compared to genotype B2 [12]. Potencies notwithstanding, samples D and E, representing diluted human HBV-positive plasma specimens with different HBV genotypes, can be used to assess the inter-laboratory variability of potencies as well as to address aspects of the commutability of the IS candidate material.

Intra-laboratory variability

The individual laboratory %GCVs for potencies expressed relative to the 2nd IS are shown for the study samples in Table 7. Generally, laboratories show a good level of reproducibility for each kit. For candidate 12/226 pooled with its duplicate sample C, the majority of laboratories have a %GCV below 8%, with the highest %GCV being 14.9% (Table 8). Similarly, for the 1st IS, the majority of laboratories have a %GCV of 10% or below, with the highest being 11.5% (Table 7). For samples A, D and E, most laboratories have a %GCV of 10% or below, with the highest being 21.2%, 22.9% and 15.7%, respectively.
The agreement between laboratory estimates of the candidate 3rd IS and its duplicate, sample C, is shown in Table 9 as an average percentage difference (see statistical methods). For the majority of laboratories there is good agreement between estimates. However, for some laboratories, the discrepancies are higher than would be expected with this type of assay. For example, laboratories 8 HeB and 9 Mv3 have average percentage differences of more than 30% between the duplicate samples. These discrepancies are reflected in the different overall means obtained for the candidate 3rd IS and sample C by these laboratories (Table 7).

Inter-laboratory variability

In general, the inter-laboratory variation is greater than the intra-laboratory variation, although some individual laboratories have relatively high intra-laboratory variation e.g. laboratory 6 Eql. There were no significant differences in potency estimates between quantitative and qualitative methods (as shaded in Figures 1-4)

Comparing the %GCV values for inter-laboratory variability in Table 7, there is excellent agreement between laboratories for the candidate 3rd IS against the 2nd IS (15% GCV). This is an improvement on the 1st IS against the 2nd IS which gives a higher %GCV of 28%. The candidate and its duplicate Sample C against the 2nd IS shows similar levels of inter-laboratory variability giving a combined overall %GCV of 13% (Table 8).

The overall mean potencies of samples A, C, D and E relative to the 2nd IS, the candidate 3rd IS and the 1st IS are shown in Table 10 along with the inter-laboratory %GCVs. The results are also shown in histogram form in Figures 1-4. Assays of samples A, C, D and E give inter-laboratory %GCVs in the range of 11%-39%, which is consistent with inter-laboratory variability reported for similar collaborative studies [8, 12].

The best between-laboratory agreement is obtained when the potency of a sample is expressed relative to a reference that is most like itself. For example, sample A is composed of the same source material (genotype A2 subtype *adw2*) used to prepare the 2nd IS. The between-laboratory agreement for sample A, when expressed relative to the 2nd IS, is excellent giving a %GCV of 11%. The between-laboratory comparability is somewhat reduced, but still acceptable, when potencies for sample A are expressed relative to the candidate 3rd (18% GCV) or the 1st IS (29% GCV) (Table 10 and Figure 1).

Similarly, between-laboratory comparability of sample C (B4 *ayw1/adw2*) is best when potencies are expressed relative to its duplicate, the candidate 3rd IS (10% GCV). When expressed against the 2nd IS or the 1st IS (subtype *ad*), the between-laboratory %GCV for sample C increases to 15% and 37%, respectively.

The inter-laboratory %GCV values for Sample D (B2 *adw2*), relative to the 2ndIS, candidate 3rd IS or 1st IS are 24%, 23% and 44%, respectively. For sample E (A2 *adw2*), the inter-laboratory %GCVs are 29%, 34% and 39% respectively. While the variability between laboratories is greatest for these samples, the overall %GCVs for samples D and E is typical for samples tested in HBsAg assays [12].

Discussion and conclusions

The candidate 3rd WHO IS for HBsAg (NIBSC code 12/226) is a batch of ampoules containing the freeze-dried equivalent of 1.0 mL HBsAg formulated in thrombinized and declotted plasma. The material used to produce 12/226 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. At NIBSC, the HBsAg bulk was formulated to give a target HBsAg content of 50 IU/mL, then filled and freeze-dried. Three-thousand two-hundred ampoules of 12/226 are available to WHO.

Sequence analysis of the 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*. The candidate material has a HBV genotype, HBsAg subtype which differs to the previous International Standards which are genotype A, subtype *adw2*. The HBV genotype A2 is found in Europe and the USA and is typical for regions of low prevalence while the genotype

of the candidate 3rd IS (B4) is representative of a region with high prevalence. Western analysis of the HBsAg bulk indicates that the material contains mainly S HBs protein with only traces of preS1 and preS2 antigens (results to be published elsewhere).

The molecular and antigenic characteristics of the candidate 3rd IS could have consequences for some diagnostic kits, where detection of HBsAg can be different for different HBV genotypes [8, 12]. Also, HBsAg detection could potentially be affected by the heterogeneous population of HBsAg subtypes *ayw1* and *adw2* present in the candidate. Additionally, purification and/or inactivation steps could alter the structure of the HBsAg protein or subviral particles. Such structural changes could have an effect on the detectability of HBsAg. Validation studies on the freeze-dried product indicated however that antigenicity, analytical sensitivity and potency of 12/226 are acceptable and that the candidate was suitable for further evaluation and calibration in the international collaborative study.

In this international collaborative study, a range of qualitative and quantitative HBsAg tests were used to evaluate the candidate 3rd IS (12/226) in parallel to the current, 2nd WHO IS (00/588). Serving as an additional benchmark, the 1st WHO IS (80/549) was also included in the study. Blinded study samples from individuals infected with different HBV genotypes were also included for assay.

When assayed against the 2nd IS, the candidate combined with its coded duplicate (12/226 plus sample C), had an overall geometric mean of 47.3 IU/mL, a value very close the target 50 IU/mL (Tables 7 & 8). The between-laboratory agreement for 12/226 plus sample C was very good giving an overall %GCV of 13% (Table 8).

The HBV subgenotype is an important consideration for HBsAg detection assays [8, 12]. In this collaborative study, some genotype-dependent effects on the inter-laboratory variability were observed. For example, the %GCV among laboratories for the duplicate (sample C) is 10% when potencies are expressed relative to itself (12/226). This inter-laboratory variability increases to 15% and 37%, respectively, when potencies are expressed relative to the 2nd IS and 1st IS, which are both HBV genotype A (Table 10 and Figure 2). Similarly, the inter-laboratory comparability is best for sample A, which consists of the same material as the 2nd IS, when potencies were expressed relative to the 2nd IS (% GCV, 11%) (Table 10 and Figure 1). The inter-laboratory variability is greatest with the more distantly related sample D (B2 *adw2*) and sample E (A2 *adw2*) (Table 10 and Figures 3-4). It should be noted that, while the variability between laboratories is greatest for samples that are more distantly related to the reference standard, the overall %GCVs for samples expressed against the candidate 3rd IS are in line with overall %GCVs observed in similar studies [8, 12].

The matter of commutability of the candidate 3rd HBsAg IS for clinical samples has not been specifically assessed in this study. Commutability is determined by a range of factors including the sample matrix (i.e. plasma vs serum) and molecular and antigenic variants of the analyte (i.e. differences in genotype or vaccine escape mutants). An aspect of commutability was addressed in this study by including some samples representing different HBV genotypes, It was not feasible, however, to examine further HBV genotypes and HBsAg subtypes or mutants within the context of this collaborative study, in part, because of the work load required by the participants.

The commutability of the standard can also be affected by processing steps such as purification, heat inactivation and formaldehyde treatment which can aggregate the HBsAg or alter epitopes

necessary for HBsAg detection by assays. The source HBsAg bulk contains mainly S-HBs protein with only traces of preS1 and preS2 antigens. It is likely that the loss of these preS antigens occurred in the course of its manufacture into vaccine bulk material. In a recent study it was shown that the removal of HBV particles and HBsAg filaments do not have a great impact on the HBsAg detection as none of the tests uses anti-preS1 antibodies [12].

The results of this collaborative study demonstrate that the performance of the candidate 3rd IS is comparable to that of the 2nd WHO IS in harmonising HBsAg assays. Along with the WHO HBV genotype panel for HBsAg assays (PEI product 6100/09) [12] and the WHO reference panel, HBsAg subtype adw2, genotype A (NIBSC product 03/262) [8], the implementation and use of 12/226 as calibrator, will facilitate the characterization and standardisation of the factors that contribute to assay sensitivity and variability and assist in the development of uniform management strategies for HBV-associated disease.

The results obtained from the accelerated thermal degradation study at ~ 1 year indicate that the candidate 12/226 is sufficiently stable for long-term storage at -20°C and shipment at ambient temperatures.
In summary, this study demonstrates the suitability and stability of 12/226 to serve as the 3rd WHO IS for HBsAg.

Proposal

It is proposed that the candidate standard (NIBSC code 12/226) is established as the 3rd WHO International Standard for HBsAg (HBV genotype B4, HBsAg subtypes *ayw1*/*adw2*) with an assigned potency of 50 IU when reconstituted in 1 mL of distilled water. The proposed standard is intended to be used by clinical laboratories, blood transfusion laboratories, blood products manufacturers and in vitro diagnostic kit manufacturers for the determination of analytical sensitivity of HBsAg assays and for the calibration of secondary reference materials for HBsAg. The proposed Instructions-for-Use (IFU) for 12/226 is included in Appendix 3.

Comments from participants

Six participating laboratories responded to the report. There were no disagreements with the suitability of the candidate (NIBSC code 12/226) to serve as the 3rd WHO IS HBsAg. Some respondents had queries or suggestions for editorial changes and these have been addressed.

Acknowledgements

We gratefully acknowledge the important contributions of the collaborative study participants. We would also like to thank NIBSC Standards Production and Dispatch for filling, freeze-drying and distribution of the candidate material. We also thank Professor Nguyen Thu Van, VABIOTECH, Hanoi, Vietnam, for the kind donation of the HBsAg bulk material.

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Table 1. Collaborative study samples.

Sample Code	Sample Name	Description	Presentation
12/226	Candidate 3rd WHO IS for HBsAg	Non-adjuvanted plasma-derived HBsAg vaccine bulk. HBV genotype B4, HBsAg subtypes *ayw1/adw2*	Freeze-dried Ampoule
00/588	00/588	2nd WHO IS for HBsAg genotype A2 subtype *adw2* (33 IU/mL)	Freeze-dried Vial
80/549	80/549	1st WHO IS for HBsAg subtype group *ad* (100 IU/mL)	Freeze-dried Vial
HBsAg 3rd IS CS **sample A**	01/402	Reference panel member HBsAg genotype A2 subtype *adw2* (~ 2 IU/mL)	Freeze-dried Vial
HBsAg 3rd IS CS **sample B**	00/616	Negative reference panel member normal re-calcified plasma	Freeze-dried Vial
HBsAg 3rd IS CS **sample C**	12/226	Duplicate candidate 3rd WHO IS for HBsAg	Freeze-dried Ampoule
HBsAg 3rd IS CS **sample D**	PEI Sample N4222	HBV genotype B2 HBsAg subtype *adw2* Unitage not assigned Pre-diluted to ~79 IU/mL)	Liquid Screw-capped tube
HBsAg 3rd IS CS **sample E**	PEI sample N4879	HBV genotype A2 HBsAg subtype *adw2* Unitage not assigned Pre-diluted to ~ 74 IU/mL)	Liquid Screw-capped tube

Table 2. Collaborative study assay methods and codes.

Quantitative automated assays		
Assay Code	**Assay**	**No. of data sets**
Aqn	ARCHITECT HBsAg, Abbott Diagnostics	2
Eqn	Elecsys HBsAg II, Roche Diagnostics	2
LxL	LIAISON XL murex HBsAg Quant., Diasorin S.p.A.	1
Qualitative automated assays		
Assay Code	**Assay**	**No. of data sets**
ADq	ADVIA Centaur HBsAg, Siemens Healthcare Diagnostics Inc. (product number 03393362)	1
Aql	ARCHITECT HBsAg Qualitative II, Abbott Diagnostics	1
Eql	Elecsys HBsAg II, Roche Diagnostics	2
LDi	LIAISON HBsAg, Diasorin S.p.A.	1
PRq	PRISM HBsAg, Abbott Diagnostics	3
VBi	VIDAS HBsAg ULTRA ELFA, BioMérieux	1
Qualitative (semi)manual assays		
Assay Code	**Assay**	**No. of data sets**
En6	Enzygnost HBsAg 6.0, Siemens Healthcare Diagnostics Products GmbH	2
DPr	HBsAg one, Dia. Pro	1
HeB	Hepanostika HBsAg Ultra, BioMérieux	1
MiS	Microscreen HBsAg ELISA, Span Diagnostics	1
Mv3	Murex Version 3, Diasorin Ltd.	2
SD3	SD HBsAg ELISA 3.0, Standard Diagnostics	1

Table 3. Production summary for the candidate standard.

Address of production facility	NIBSC South Mimms, Potters Bar, Herts., EN6 3QG, UK
NIBSC code	12/226
Product name	Candidate 3rd WHO International Standard for HBsAg
Presentation	Freeze-dried serum in 5 ml DIN ampoule
Appearance	Robust cake
Filling machine	Bausch and Strobel AFV5090
Date of filling	8 November 2012
No. of ampoules filled	3558
Mean fill weight (g)	1.0091 n=6
CV of fill weight (%)	0.28
Freeze dryer	Serail CS100
Date of completion of lyophilization	12 November 2012
Mean dry weight (g)	0.0793 n=6
CV of dry weight (%)	0.2604
Mean residual moisture (%)	0.4045 n=12
CV of residual moisture (%)	25.98
Mean oxygen content (%)	0.30 n=12
CV of oxygen content (%)	34.18
No. of ampoules available to WHO	3200
Custodian	NIBSC

Table 4. Post-fill validation testing. Estimations of analytical sensitivities (IU/mL) using the 2nd IS and candidate 3rd IS.

Sample		Analytical sensitivity (IU/mL)		
	Assay	Aqn	Eql	Mv3
2nd IS (00/588)	Assay 1	0.027	0.032	0.036
	Assay 2	0.021	0.025	0.043
Candidate 3rd IS (12/226)	Assay 1	0.021	0.029	0.023
	Assay 2	0.016	0.018	0.026

Abbreviations: Aqn = ARCHITECT HBsAg (quantitative), Abbott Diagnostics; Eql = Elecsys HBsAg II (qualitative), Roche Diagnostics; Mv3 = Murex HBsAg Version, Diasorin Ltd.

Table 5. Post-fill validation testing. Estimations of potency (IU/mL) of the 1st IS and the candidate 3rd IS (12/226) relative to the 2nd IS.

		1st IS (100 IU/mL)		Candidate 3rd IS (12/226) (target ~50 IU/mL)	
Assay		**Potency (IU/mL)**	**95% CI**	**Potency (IU/mL)**	**95% CI**
code	**No.**				
ADqII	1	196	187-206	93	89-98
	2	218	207-230	108	102-113
Aql	1	75	73-76	56	55-57
	2	79	78-80	64	63-65
LxL	1	104	101-107	60	58-61
	2	109	102-117	70	66-75
PRq	1	95	91-100	45	43-47
	2	82	78-86	47	45-50
EN6	1	108	104-112	30	29-32
	2	119	112-127	43	41-46
Overall GM (%GCV)		**111 (43)**		**58 (46)**	
Overall GM excluding ADq (%GCV)		**95 (19)**		**50 (31)**	

Abbreviations: 95% CI = 95% Confidence Interval for the potency result; GM = Geometric mean; ; %GCV = geometric coefficient of variation.
ADqII = ADVIA Centaur HBsAg II, Siemens Healthcare Diagnostics Inc. (product number 10492138); Aql = ARCHITECT HBsAg Qualitative II, Abbott Diagnostics; LxL = LIAISON XL murex HBsAg Quant., Diasorin S.p.A.; PRq = PRISM HBsAg, Abbott Diagnostics; EN6 = Enzygnost HBsAg 6.0, Siemens Healthcare Diagnostics Products GmbH.

Table 6. Thermal degradation assessment of 12/226. Potencies (IU/mL) are expressed relative to the material stored at -70ºC. The % loss per month or year was predicted using the Arrhenius model.

Temperature (°C)	Day	Potency (IU/mL)	Combined Potency (IU/mL)	Predicted % Monthly Loss	Predicted % Yearly Loss
-20	1	1.00	1.05	<0.01	<0.01
	2	1.12			
	3	0.99			
+4	1	1.02	1.02	<0.01	0.03
	2	1.04			
	3	1.00			
+20	1	1.00	0.99	0.07	0.84
	2	0.99			
	3	0.89			
+37	1	0.89	0.78	2.00	21.52
	2	0.75			
	3	0.73			
+45	1	0.33	0.35	NC	NC
	2	0.38			
	3	0.40			

Abbreviation: NC = not calculated.

Table 7. Potencies (IU/mL) relative to 2nd IS.

	1st IS (100 IU/mL)			Candidate 3rd IS (12/226) (~50 IU/mL)			Sample A (01/402) (~2 IU/mL)			Sample B (00/616) Negative Control	
Lab no. Assay code	**N**	**GM**	**GCV**	**N**	**GM**	**GCV**	**N**	**GM**	**GCV**	**N**	**GM**
1 VBi	3	168.9	2.4	3	56.0	1.7	3	2.25	1.5	3	ND
2 PRq	3	82.5	7.8	3	45.2	5.9	3	1.76	0.9	3	ND
3 Eql	3	97.2	6.3	3	43.9	2.5	3	1.84	8.5	3	ND
3 Mv3	3	132.7	10.0	3	53.4	6.8	3	1.72	11.9	3	ND
4 LDi	3	132.3	8.4	3	42.3	4.7	3	1.80	6.7	3	ND
4 LxL	3	91.0	2.7	3	62.2	6.2	3	2.02	7.6	3	ND
5 Eqn	9	104.9	4.2	9	42.0	2.3	9	1.84	5.2	9	ND
6 Eql	3	95.5	10.9	3	45.3	3.9	2	1.44	21.2	3	ND
7 Aql	3	74.5	1.8	3	63.2	1.8	3	1.77	1.3	3	ND
7 Aqn	3	86.5	2.8	3	48.5	5.0	3	1.72	7.3	3	ND
7 PRq	3	97.7	4.7	3	49.8	3.8	3	1.87	5.1	3	ND
8 HeB	1	116.2	NA	1	50.6	NA	1	1.45	NA	1	ND
8 MiS	0	IV	NA	1	47.7	NA	0	1.90	NA	1	ND
8 SD3	0	IV	NA	0	IV	NA	0	IV	NA	1	ND
9 Mv3	4	118.6	11.5	4	62.6	7.3	4	2.05	10.5	4	ND
10 ADq	3	66.7	0.2	3	43.7	1.3	3	1.88	3.0	2	ND
10 Aqn	3	91.4	0.7	3	49.3	8.4	3	1.84	7.8	3	ND
10 Eqn	3	93.9	2.3	3	43.8	1.9	3	1.90	3.2	0	NT
10 En6	3	115.5	5.7	3	43.4	6.6	2	1.98	4.5	0	NT
10 PRq	3	88.3	5.9	3	50.6	3.7	0	IV	NA	2	ND
11 EN6	3	120.4	4.2	3	39.5	4.4	3	1.90	2.9	3	ND
12 DPr	1	57.9	NA	3	46.1	11.5	1	1.73	NA	3	ND
Overall GM	**98.7**			**48.6**			**1.8**			**Not Detected**	
Overall % GCV	**28**			**15**			**11**			**NA**	

Abbreviations: GM = Geometric mean; %GCV = geometric coefficient of variation;
ND = not detected; NA = not applicable; NT = sample not tested; IV = all assays invalid for this sample.

Table 7 cont. Potencies (IU/mL) relative to 2nd IS.

	Sample C (12/226) (~50 IU/mL)			Sample D (PEI N4222) (~79 IU/mL)			Sample E (PEI N4879) (~74 IU/mL)		
Lab no. Assay code	**N**	**GM**	**GCV**	**N**	**GM**	**GCV**	**N**	**GM**	**GCV**
1 VBi	3	53.5	1.3	3	90.4	2.0	3	138.3	0.5
2PRq	3	45.2	2.6	3	73.5	4.8	3	100.5	3.0
3 Eql	3	45.0	1.5	3	73.1	3.3	3	96.4	3.0
3 Mv3	3	47.4	14.9	3	60.0	8.7	3	105.4	15.7
4 LDi	3	45.5	7.5	3	103.6	6.5	3	165.3	2.1
4 LxL	3	62.0	8.1	3	99.1	0.7	3	138.0	5.2
5 Eqn	9	41.1	2.8	9	71.2	3.6	9	118.1	2.8
6 Eql	3	39.0	17.1	3	75.8	22.9	3	89.3	12.3
7 Aql	3	61.7	1.3	3	122.4	1.9	3	103.1	9.5
7 Aqn	3	47.0	5.0	3	80.0	2.1	3	107.9	4.8
7 PRq	3	49.1	2.6	3	84.6	4.1	3	119.7	4.3
8 HeB	1	38.1	NA	0	NT	NA	1	128.8	NA
8 MiS	1	47.5	NA	1	127.2	NA	0	NT	NA
8 SD3	1	~~141.5~~*	NA	0	NT	NA	1	~~89.2~~*	NA
9 Mv3	4	48.7	6.5	4	70.1	5.3	4	66.2	11.2
10 ADq	3	41.4	4.2	3	72.2	2.6	3	75.5	3.0
10 Aqn	3	46.3	5.0	3	69.4	2.3	3	69.0	4.5
10 Eqn	3	42.0	3.3	3	69.1	3.2	3	81.9	4.8
10 En6	3	41.2	6.9	3	62.2	1.3	3	90.2	1.4
10 PRq	3	42.0	6.5	0	NT	NA	0	NT	NA
11 EN6	3	39.5	5.8	3	69.0	4.2	3	120.6	10.0
12 DPr	1	57.1	NA	1	101.1	NA	3	145.6	7.4
Overall GM	**46.2**			**80.9**			**105.2**		
Overall % GCV	**15**			**24**			**29**		

Abbreviations: GM = Geometric mean; %GCV = Geometric coefficient of variation; ND = not detected; NA = not applicable; NT = assay not performed.

* Value not included in calculation of overall GM and overall %GCV.

Table 8. Potencies of both 12/226 and coded C against 2nd IS.

	Candidate 3rd IS and Sample C (12/226) (~50 IU/mL)		
Lab Code	N	GM	GCV
1 VBi	3	54.7	1.4
2PRq	3	45.2	3.3
3 Eql	3	44.5	0.8
3 Mv3	3	50.3	6.1
4 LDi	3	43.8	2.0
4 LxL	3	62.1	7.0
5 Eqn	9	41.5	2.4
6 Eql	3	42.1	8.6
7 Aql	3	62.5	1.6
7 Aqn	3	47.7	3.8
7 PRq	3	49.5	3.2
8 HeB	1	43.9	NA
8 MiS	1	47.6	NA
8 SD3	1	~~141.5~~*	NA
9 Mv3	4	55.3	2.1
10 ADq	3	42.5	2.7
10 Aqn	3	47.8	6.6
10 Eqn	3	42.9	2.6
10 En6	3	42.3	5.0
10 PRq	3	46.1	1.8
11 EN6	3	39.5	5.0
12 DPr	4	48.7	14.9
Overall GM		47.3	
Overall %GCV			13

Abbreviations: GM = Geometric mean; %GCV = geometric coefficient of variation; NA = not applicable. * Value not included in calculation of overall GM and overall %GCV.

Table 9. Intra-Assay agreement between potency estimates of 12/226 and coded duplicate C against 2nd IS.

	Average (RMS) % Difference in potency estimate between Candidate 3rd IS(12/226) and Sample C
Lab Code	
1 VBi	4.8
2 PRq	5.0
3 Eql	4.0
3 Mv3	21.0
4 LDi	12.3
4 LxL	2.9
5 Eqn	2.9
6 Eql	22.0
7 Aql	2.5
7 Aqn	6.2
7 PRq	1.9
8 HeB	32.9
8 MiS	0.3
8 SD3	NC
9 Mv3	31.5
10 ADq	6.2
10 Aqn	7.4
10 Eqn	4.3
10 En6	9.2
10 PRq	22.4
11 EN6	1.6
12 DPr	9.7

Abbreviations: RMS = Root mean square; NC = Not calculated.

Table 10. Overall mean estimates (IU/mL) for potency and inter-laboratory variation (%GCV) for samples A-D relative to the 2nd IS, the candidate 3rd IS or the 1st IS.

Reference		2nd IS (33 IU/mL)		Candidate 3rd IS (50 IU/mL)		1st IS (100 IU/mL)	
Sample	No. of assays	Overall GM	Overall % GCV	Overall GM	Overall % GCV	Overall GM	Overall % GCV
A	56	1.8	11	1.9	18	1.9	29
C	63	46.2	15	47.2	10	46.8	37
D	60	80.9	24	83.1	23	80.2	44
E	63	105.2	29	107.9	34	106.2	39

Abbreviations: GM = Geometric mean; %GCV = Geometric coefficient of variation.

Figure legends

Figure 1. Relative potencies of sample A against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Different classes of assay methods are shaded in grey scale.

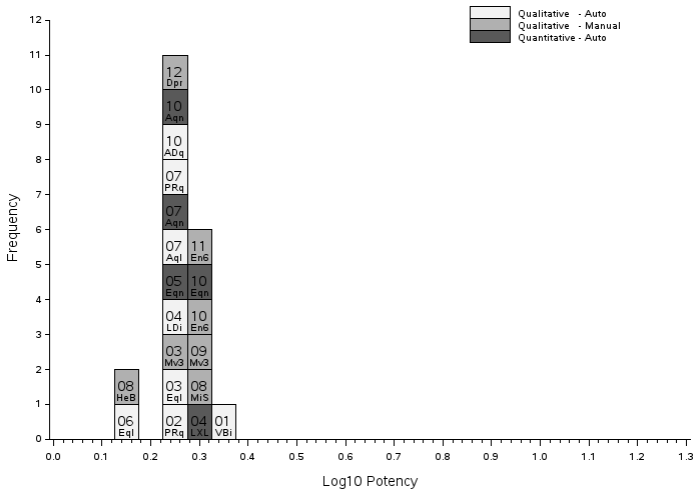
Figure 2. Relative potencies of sample C against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Different classes of assay methods are shaded in grey scale.

Figure 3. Relative potencies of sample D against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Different classes of assay methods are shaded in grey scale.

Figure 4. Relative potencies of sample E against the (a) 2nd IS, (b) candidate 3rd IS and (c) 1st IS. Each box in the histogram represents the laboratory geometric mean potency (log10 IU/mL) for a particular assay method, and is labelled with the laboratory and method code. Different classes of assay methods are shaded in grey scale.

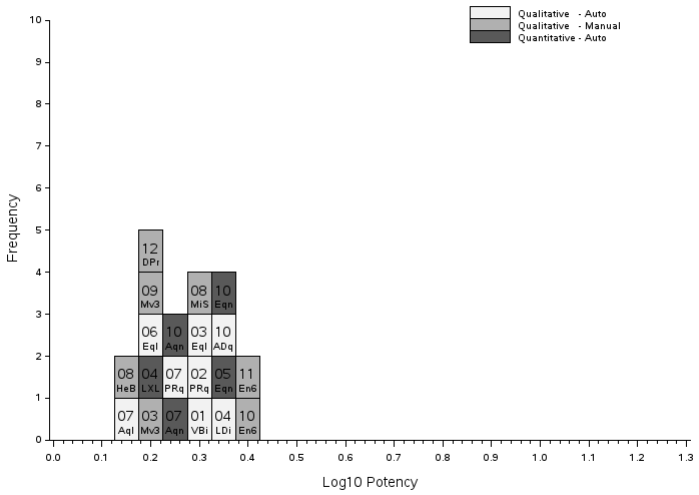
Figure 1. a)

Sample A relative to 2nd IS



b)

Sample A relative to candidate 3rd IS



c)

Sample A relative to 1st IS

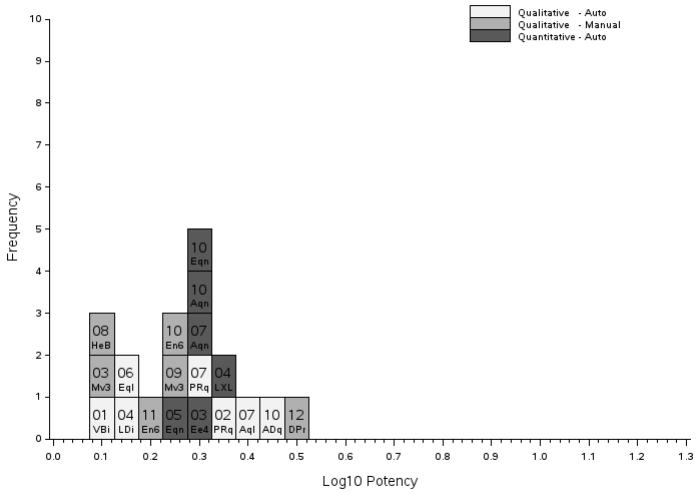
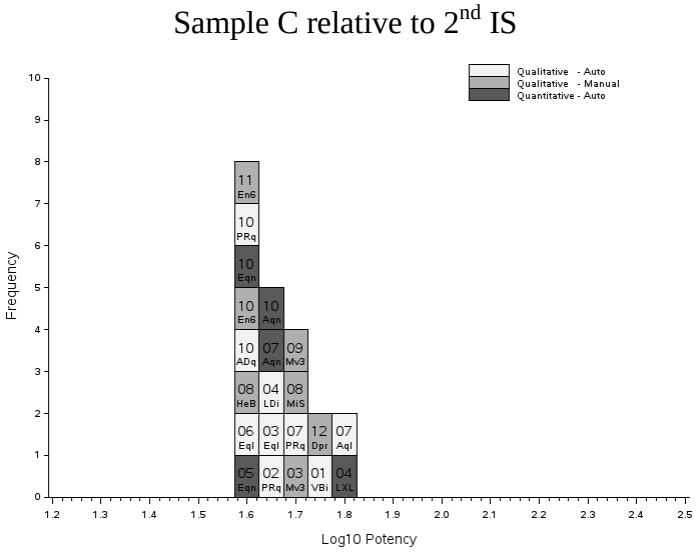
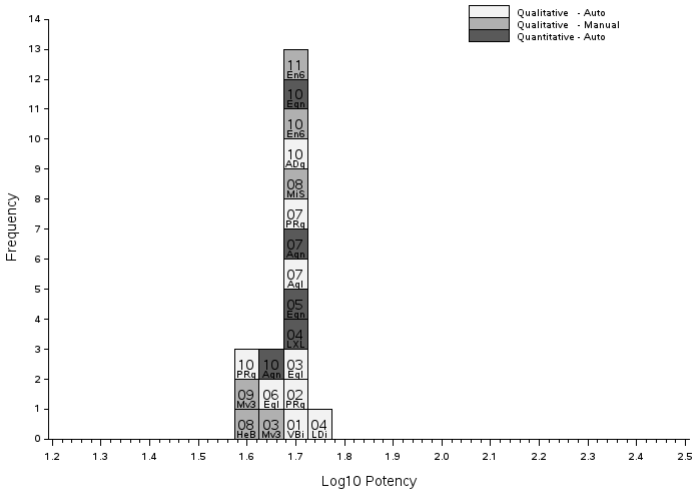


Figure 2.

a)


Sample C relative to 2nd IS
Qualitative - Auto
Qualitative - Manual
Quantitative - Auto
Frequency
Log10 Potency

b)


Sample C relative to candidate 3rd IS
Qualitative - Auto
Qualitative - Manual
Quantitative - Auto
Frequency
Log10 Potency

c)

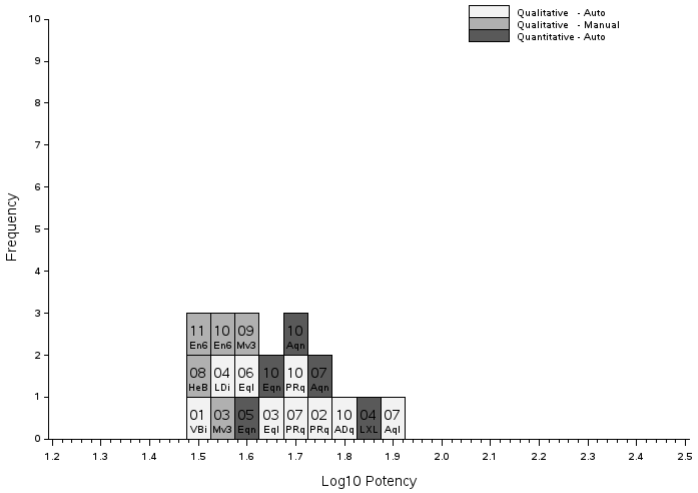
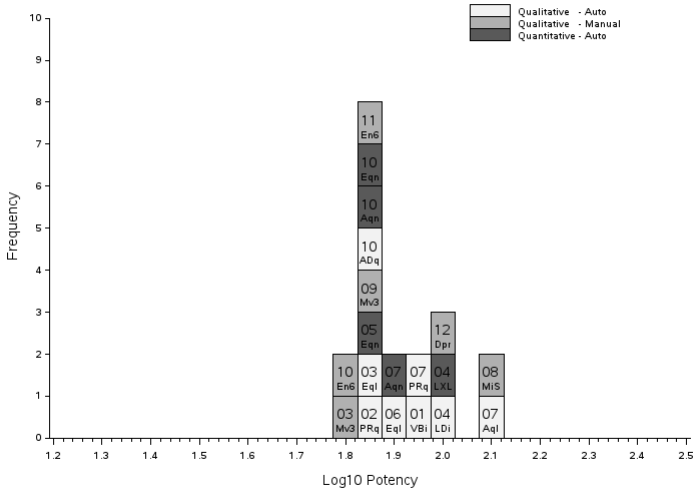

Sample C relative to 1st IS
Qualitative - Auto
Qualitative - Manual
Quantitative - Auto
Frequency
Log10 Potency

Figure 3.

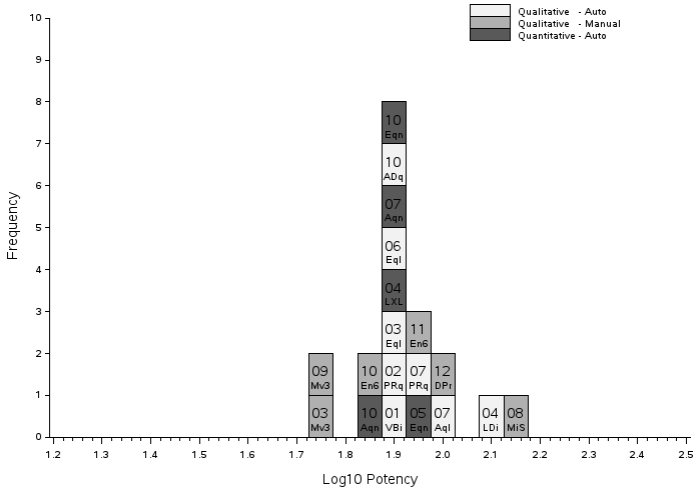
a)

Sample D relative to 2nd IS



b)

Sample D against relative to 3rd IS



c)

Sample D relative to 1st IS

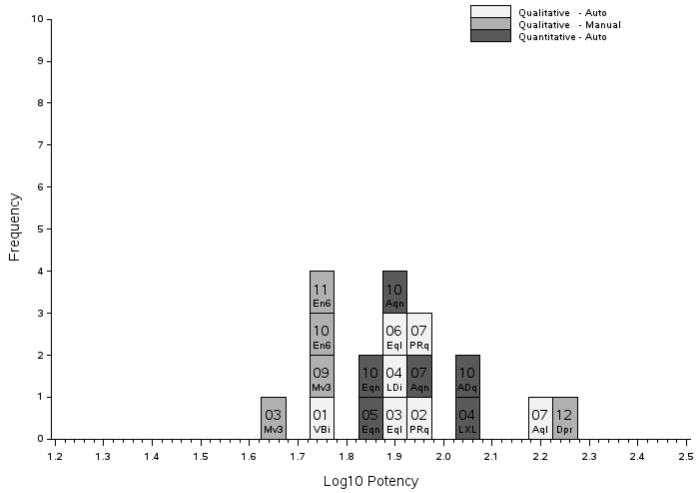
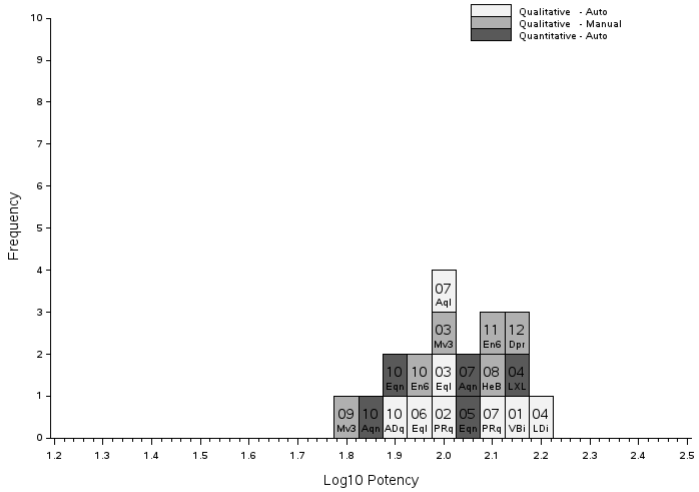


Figure 4.

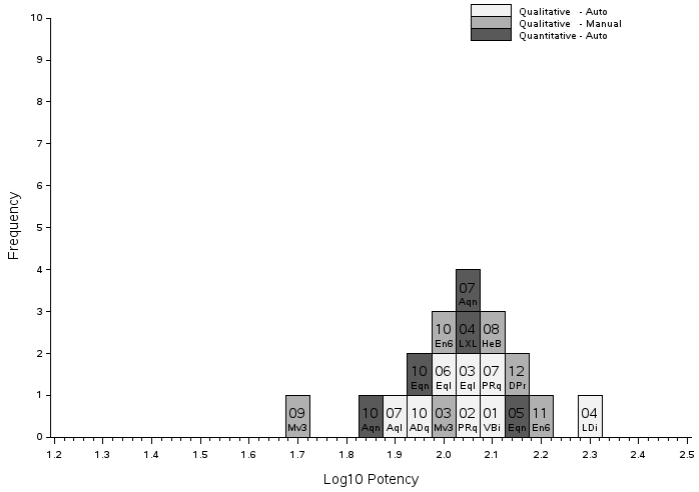
a)

Sample E relative to 2nd IS



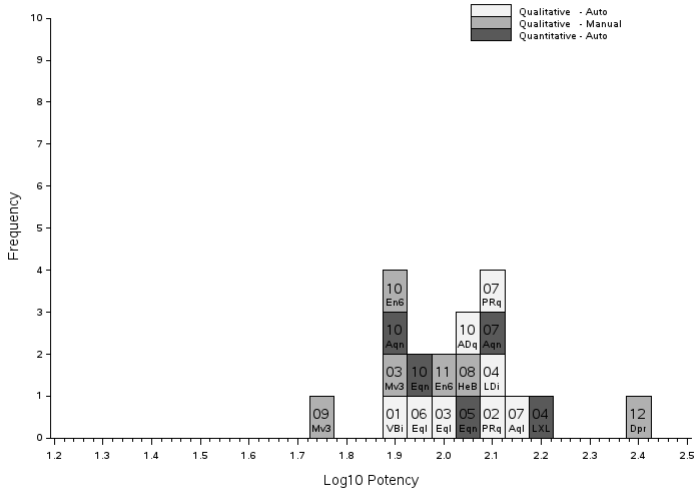
b)

Sample E relative to candidate 3rd IS



c)

Sample E relative to 1st IS



Appendix 1

Collaborative study participants

(In alphabetical order by country)

Name	Laboratory	Country
T McDonald	NRL, Fitzroy	Australia
V Lievre	L'Agence nationale de sécurité du médicament et des produits de santé – ANSM, Saint-Denis cedex	France
R Bäeuerlein and I Krüger	Roche Diagnostics GmbH, Penzberg	Germany
H Scheiblauer and S Nick	PRUEFLABOR (PEI-IVD) Paul-Ehrlich-Institut, Langen	Germany
R Chhabra	Immuno Diagnostic Kit Laboratory, National Institute of Biologicals, Ministry of Health and Family Welfare, NOIDA	India
L. Pallavicini and M. DeLuca	DiaSorin SpA, Saluggia (VC)	Italy
G Pisani and F Marino	Biologicals Unit, National Center for Immunobiologicals Research and Evaluation (CRIVIB), Istituto Superiore di Sanita, Rome	Italy
K Ishii	National Institute of Infectious Disease, Dept. of Virology II, Tokyo	Japan
C Morris	National Institute for Biological Standards and Control - NIBSC, South Mimms	UK
F Kori	U.S. Food and Drug Administration-FDA/CBER/OCBQ/DBSQC/LACBRP, Kensington	USA
M Kuhns	Abbott Diagnostics Division, Abbott Park	USA
N Thu Van	VABIOTECH, Ha Noi	Vietnam

Appendix 2

Collaborative Study Protocol

Protocol for the WHO collaborative study to assess the suitability of a candidate 3rd WHO IS for HBsAg

Introduction

Laboratories that have established assays for HBsAg are being invited to participate in an international collaborative study to assess the suitability of a candidate freeze-dried material to serve as the 3rd WHO IS for HBsAg. Participation in the study will involve the testing of the candidate International Standard alongside additional study samples in assay(s) that are in routine use in the participant's laboratory.

The aims of the present collaborative study are to

- **Assess the suitability of freeze-dried serum to serve as the 3rd International Standard HBsAg with an assigned unitage in International Units per ampoule for use in immunoassay for the detection or quantitation of HBsAg. The unitage of the candidate will be determined by calibrating it against the 2nd IS for HBsAg (NIBSC code 00/588).**
- **Assess the candidate's reactivity in a range of typical assays performed in different laboratories.**
- **Assess commutability i.e. to establish the extent to which the reference standard is suitable to serve as a standard for the variety of different samples being assayed.**

Materials

All study samples should be stored at -20ºC or below. The study samples shall not be administered to humans or to animals and shall not be used for any reason whatsoever to conduct experiments on animals.

Candidate standard NIBSC code 12/226

The source material is a non-adjuvanted plasma-derived HBsAg vaccine bulk kindly donated by Professor Nguyen Thu Van, VABIOTECH, Hanoi,Vietnam. The HBsAg was derived from human plasma obtained from asymptomatic carriers with high titers. The HBsAg bulk was purified and inactivated at VABIOTECH using validated methods for the manufacture of plasma-derived Hepatitis B vaccine. The HBsAg bulk has been tested and found negative for antibodies to HIV and HCV RNA. The inactivated HBsAg bulk is positive for HBV DNA. Characterization of the HBsAg was performed at the National Reference Centre for Hepatitis B and D Viruses, Institute of Medical Virology, Justus-Liebig University Giessen, Germany. Sequence analysis of PCR products amplified from the HBsAg bulk and covering the S gene revealed the wild-type sequences of at least two different HBV strains, both with subgenotype B4. The HBsAg subtype is a heterogeneous mixture of ayw1 and adw2. The HBsAg of the candidate 3rd IS material contains only traces of preS antigens .

For formulating the candidate IS at NIBSC, the HBsAg bulk was diluted 1:728 in thrombinized and declotted plasma which had been shown to be negative for anti-HCV, anti-HIV 1+2, HBsAg and anti-HBs as well as negative for HCV RNA, HBV DNA and HIV RNA. Bronidox was included as preservative (0.05% final concentration). In November 2012, the formulation was filled in 1ml aliquots into 5mL DIN ampoules and freeze-dried. This has been labeled as the candidate 3rd WHO IS for HBsAg (NIBSC product code 12/226). 3500 ampoules are available.

Additional study samples

The 2nd WHO IS for HBsAg (NIBSC product code 00/588) will be included so that the HBsAg content of the candidate can be calculated in IU/mL relative to 00/588. The 1st WHO IS for HBsAg (80/549) will also be provided. To evaluate the ability of the candidate IS to assign unitage to test samples, additional study samples will be included in the study. These additional samples will be blinded (labeled Sample A-E 3rd IS HBsAg CS) and may include negative samples and samples positive for HBsAg of different genotypes and subtypes.

Assay methods

For testing the study samples, participants are requested to use the method(s) for the detection and quantitation of HBsAg in routine use in their laboratory. Qualitative and/or quantitative assays may be used. If multiple assay methods are used, participants may request multiple sets of study samples; although supplies may be limited.

Design of study

Participants are requested to:

- perform 3 independent assays on different days for HBsAg.
- for each independent assay, prepare and test a series of two-fold dilutions from the candidate standard and each of the study samples. Dilutions should be prepared in the diluent normally used in the testing laboratory. Two independent replicate series of dilutions (NOT two aliquots from a single dilution) should be prepared and assayed. Suggested dilutions are provided in the attached Excel reporting sheet. Dilutions may be adjusted as appropriate to the assay setup. The dilutions tested must be recorded in the reporting sheet. If feasible, test each independent dilution series in duplicate
- use the Excel reporting sheet to record for each dilution the readout from the instrumentation/plate reader (e.g. IU/mL, OD, S/CO, RLU). Our statistician will use the data readouts to perform the statistical analysis. The mean HBsAg content of the study samples will be calculated in IU/mL relative to the candidate 3rd WHO IS for HBsAg.
- include all study samples in each assay so that the concentration of HBsAg of study samples relative to the candidate standard may be calculated. If multiple plates are required for testing all study samples concurrently, then include the candidate standard on each plate. Include controls as appropriate to the specific assay setup.
- Reconstitute the freeze-dried samples as directed in the instructions for use. Use a freshly opened and reconstituted ampoule/vial or thawed sample for each independent assay.

Results and data analysis

Participants are requested to return their results to NIBSC no later than 13 Dec 2013. An excel spreadsheet is provided so that all essential information can be recorded including the methodology and raw data from each assay. The final version of the reporting spreadsheet will be e-mailed to each participant following shipment of study materials. The use of the reporting spreadsheet facilitates the analysis and interpretation of results. If multiple assay methods are used, separate worksheets should be used for each assay. Participants are requested to provide information in the reporting sheet regarding the assay system used. The confidentiality of each laboratory will be ensured with each participant being anonymous to the other laboratories. Analysis of the study will assess the potencies of each material relative to the candidate material, and the variation of the different assay methods.

Assay data will be analyzed at NIBSC by an experienced biometrician using standard statistical techniques and a draft study report will be sent to participants for comment prior to consideration by the WHO Expert Committee on Biological Standardization (ECBS). The finalised report will

then be submitted to the WHO ECBS who will decide on the suitability of the candidate standard to serve as the International Standard.

Participation in the WHO collaborative study is conducted under the following conditions:

- The data should not be published or cited before the formal establishment of the standard by WHO, without the expressed permission of the NIBSC Study organizer.
- It is normal practice to acknowledge participants as contributors of data rather than co-authors in publications describing the establishment of the standard.
- Individual participant's data will be coded and reported "blind" to other participants during the reparation of the study report, and also in subsequent publications.
- Participants will receive a copy of the report of the study and proposed conclusions and recommendations for comment before it is further distributed.
- The materials are provided for the purpose of the collaborative study only and not for independent use or research.
- Samples 12/226, 00/588, A, B and C are inactivated or non-infectious for Hepatitis B virus. Samples 80/549, D and E should be considered infectious for Hepatitis B virus. Participants accept responsibility for safe handling and disposal of the materials provided.

The launch of the collaborative study is set for 18 Oct 2013. Dispatch of samples will be conducted according to any individual participant's instructions.

Deadline for completed results spreadsheets is 13 December 2013.

All completed results spreadsheets should be returned electronically to:
Dianna.Wilkinson@nibsc.org
Alternatively, reports may be mailed or faxed to:
Dr. Dianna Wilkinson
Principal Scientist
Division of Virology
NIBSC
Blanche Lane
South Mimms
Potters Bar
Hertfordshire, EN6 3QG
UK
FAX +44(0)1707 641366

Appendix 3

Proposed instructions for use

WHO International Standard
Third International Standard (2014) for HBsAg (HBV genotype B4, HBsAg subtypes ayw1/adw2)
NIBSC code: 12/226
Instructions for use
(Version 1.00, Dated)

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 (1). It was calibrated against the 2nd international standard (IS) for HBsAg (A2, adw2) along with additional study samples representing different HBV genotypes.

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin. The source material is human plasma obtained from asymptomatic carriers positive for HBsAg. The HBsAg was purified and inactivated using validated methods for the manufacture of plasma-derived Hepatitis B vaccine.
(http://whqlibdoc.who.int/trs/WHO_TRS_858.pdf) (2).

The HBsAg bulk is negative for antibodies to HIV and HCV RNA. The inactivated HBsAg bulk is positive for HBV DNA. 12/226 was formulated by diluting the HBsAg vaccine bulk in thrombinized and declotted plasma which had been shown to be negative for anti-HCV, anti-HIV 1+2, HBsAg, anti-HBs, HCV RNA, HBV DNA and HIV 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 3rd International Standard for HBsAg has an assigned unitage of 50IU/vial (1).

4. CONTENTS

Country of origin of biological material: Vietnam

Each ampoule contains the freeze-dried equivalent of 1.0 mL HBsAg in thrombinized and declotted plasma. The source material used to produce 12/226 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. Bronidox has been included as a preservative (0.05% final concentration).

Sequence analysis of the 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

12/226 should be stored at –20ºC on receipt. The contents of the ampoule should be reconstituted with 1ml distilled water using safety precautions as described above.

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

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

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.
NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

1. WHO/BS/20XX.XXXX
2. WHO Expert Committee on Biological Standardization. Forty-fifth report. World Health Organ Tech Rep Ser, 1995. 858: p. 1-101.

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants. We would also like to thank NIBSC Standards Production and Dispatch for filling, freeze-drying and distributing the candidate material. 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/products/biological_reference_materials/frequently_
asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_qu estions.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

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 Physical and Chemical properties			
Physical appearance: Freeze-dried	Corrosive:	No	
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	Contains material of human origin. Contains 0.05% Bronidox.		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		
Effects of ingestion:	Not established, avoid ingestion		
Effects of skin absorption:	Not established, avoid contact with skin		
Suggested First Aid			
Inhalation:	Seek medical advice		
Ingestion:	Seek medical advice		
Contact with eyes:	Wash with copious		

	amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

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: 1g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached: No**