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Collaborative Study to Establish a World Health Organization International Hepatitis B Virus Genotype Panel for HBsAg Assays

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Summary

This report describes the World Health Organization (WHO) project to develop an international reference panel for hepatitis B virus (HBV) genotypes. The panel has been designed for the determination of the detection efficiency of hepatitis B surface antigen-(HBsAg)-based diagnostic kits in relation to different HBV-genotypes.

Panel manufacturing. The HBV genotype panel (PEI code number 6100/09) comprises 15 different members, which represent subgenotypes A1 (2), A2, B1, B2, C2 (3), D1, D2, D3, E, F2 (2), and H. The amount of infectious virus particles in the HBV positive plasma samples was significantly reduced by an ultracentrifugation step prior to dilution and lyophilisation of the panel members. This step resulted in a virus removal of >97%, with the exception of Sample 14 with 80 % elimination and Sample 10 without ultracentrifugation due to the limited volume. The determination of HBsAg concentration by three different methodologies (chemiluminescent immunoassay (CLIA), quantitative immunoelectrophoresis (QIE) and antigen purification) demonstrated that the corresponding different reported HBsAg unitages, international unit (IU), Paul-Ehrlich-Institut unit (PEI-U) and nanogram (ng), respectively, yielded for most of the HBV genotype samples similar results but the differences exceeded for some samples the standard deviation caused by technical limitations.

Panel stability. Residual water content in the final vials containing lyophilised plasma was determined as 0.70 ± 0.11%, predicting long-term stability for the recommended storage condition (-20°C or below). On-going real-time stability studies are in progress.
Collaborative study. The aim of the collaborative study with the candidate WHO reference panel was the evaluation of lyophilised plasma samples containing different HBV subgenotypes for the detection efficiency by HBsAg-based diagnostic assays. Each laboratory analysed the panel samples in parallel to the 2nd WHO International Standard (IS) for HBsAg (NIBSC code 00/588) representing HBV subgenotype A2. The study was performed with 3 independent runs. The data were collated and the statistical analysis performed at the Paul-Ehrlich-Institut (PEI).

Study outcome. In total 22 qualitative data sets (18 different HBsAg tests) and 6 quantitative data sets (2 different HBsAg tests) from 15 laboratories were used in the evaluation. Overall, the results demonstrated quite consistent detection of HBV genotypes A-F and H by the majority of the test kits investigated, with few assays showing genotype-dependent effects on detection efficiency.

Conclusion. Based on the results of the collaborative study, it is proposed that the panel should be established as the 1st International Reference Panel for HBV Genotypes for HBsAg-based assays (PEI code number 6100/09). No unitage is assigned to the individual panel members. However, the statistical data for each panel member from the outcome of the collaborative study will be provided. The panel would be helpful for IVD manufacturers and IVD users to check the relative detection efficiency of HBsAg diagnostic test kits in relation to HBV-genotypes. Furthermore it will support regulatory authorities to assess HBsAg assays for the detection of HBsAg in relation HBV genotypes prevalent in their regions.

Introduction

HBV infection is a major global health problem and the most prevalent cause of liver cirrhosis and hepatocellular cancer. About 2 billion people worldwide have been infected with the virus and about 350 million live with chronic infection. An estimated 600 000 persons die each year due to the chronic consequences of hepatitis B (1, 2). HBV is preferentially transmitted through contact with blood or other body fluids of an infected person. Sensitive screening and accurate diagnostic assays play a crucial role for the prevention and in the management of the disease. The current WHO IS material for HBsAg was generated from HBV subgenotype A2 and has the HBsAg subtype *adw2*. This HBV subgenotype is predominant in Western and Central Europe and in North America, but it represents only 1% of the worldwide HBV-infected population. Nevertheless, it is used for standardization of diagnostic assays and for traceability of test results worldwide. The majority of the HBV-infected people living in or coming from the Mediterranean area, Africa and Asia have the (sub)genotypes A1, B, C, D, and E, whereas F and H originate from the indigenous Americans. The origin of genotype G is not clarified yet and epidemiological data are limited. It occurs very often as co-infection with other HBV genotypes. The putative genotype I was first described in Vietnam as a recombinant form of the genotypes A, C, and G (3) and later found in Laos, China, India and France. Although a revised evaluation based on the group scan method points at aseparate genotype, it is not decided whether this is an independent genotype or a complex HBV recombinant (4). The proposed genotype J sequence does not show evidence of recombination with any of the other human HBV genotypes; however, until now it was found only in one individual (5).

During the ‘WHO Consultation on Global Measurement Standards and their use in the in vitro Biological Diagnostic Field’ in June 2004 concern was raised that HBsAg test kits and NAT test kits might be less efficient for some HBV genotypes other than A2 represented by the current IS preparations (6,7). The Paul-Ehrlich-Institut (PEI) proposed projects to establish WHO International Biological Reference Preparations for HBV DNA and for HBsAg representing different HBV subgenotypes. The HBV genotype reference panel for nucleic acid amplification technique (NAT)-based assays (PEI No. 5086/08) was established by the WHO Expert Committee on Biological Standardization (ECBS) in October 2009 (8).

HBsAg is the most important screening and diagnostic marker in acute and chronic hepatitis B infection. Due to the separate S open reading frame for the three co-carboxyterminal HBV surface proteins and separate mRNAs, its synthesis in liver cells is principally independent from the HBV replication if the episomal form of HBV DNA is present. HBsAg is formed by the smallest of the three HBV surface proteins. It is the main component of the viral envelope and carries epitopes recognized by neutralizing antibodies. Most produced HBsAg is secreted as empty 20 nm spherical or filamentous subviral particles by the infected liver cells. This explains the HBsAg excess (typically about 3000 fold and more) over the virions (Dane particles).

Since many years the mandatory HBsAg screening of blood donations has been implemented in transfusion services worldwide. Yet sensitivity of HBsAg assays vary considerably and may need careful selection (9,10). Improvement of HBsAg sensitivity appears to be still possible and desirable (11). Moreover, HBsAg quantification comes to be a valuable tool in monitoring of the infection and prediction of the long-term success of treatment (12). With the introduction of HBsAg testing in the 1970s, several national HBsAg standards were established. Due to different source materials and test platforms used, these standards in terms of their assigned unitages were not comparable. For uniform HBsAg determination the development of an IS was an irrefutable requirement. The current 2nd IS 00/588 with an assigned concentration of 33 international units (IU) per vial was heat inactivated to 100°C before lyophilisation. The calibration of this preparation in a WHO international collaborative study showed its suitability as an international reference material (7). Furthermore, the preparation has been characterized in regard to its biochemical features (13).

Reduced sensitivity of some HBsAg assays was occasionally reported due to genetic diversity of HBV genotypes (10,14).This study addressed these questions of commutability and traceability of the current IS preparation in relation to the native HBsAg protein and to other HBV genotypes. The detection efficiency of HBsAg tests for escape mutants was not addressed within this study.

The proposed HBV genotype panel is intended to examine the analytical sensitivity of HBsAg assays for different HBV subgenotypes. The panel consists of 15 members and covers the most prevalent HBV subgenotypes (A1-F2 and H) collected worldwide. The collaborative study was designed to test the panel samples (15 lyophilised preparations) concurrently with the WHO 2nd IS (00/588).

Composition, characterization and preparation of the HBV genotype panel

Characterization of the candidate materials

HBsAg positive plasma units, preferentially with high titres, were collected worldwide. About two hundred potential candidate materials were characterized performing the following analysis:

- Quantitative HBsAg determination (Abbott ARCHITECT Quantitative, expressed in IU/ml)
- Sequencing of the entire S open reading frame and HBV genotyping/ HBsAg subtyping
- Quantitative HBV DNA determination
- Other serological hepatitis markers (anti-HBc, HBeAg, anti-HBe and anti-HDV)
- HIV-1 RNA and HCV RNA

Sequence analysis was performed as described in the report WHO/BS/09.2121 (8). Samples were selected to represent typical examples of different subgenotypes. Samples with known surface gene escape mutations or ambiguous sequences were not considered as candidate material for the panel. Fifteen candidate materials representing the HBV

subgenotypes A1 (2), A2, B1, B2, C2 (3), D1, D2, D3, E, F2 (2), and H were chosen as HBV panel members. Due to the low HBsAg concentration in genotype G samples without coinfection by genotype A, this genotype could not be included into the panel. The corresponding HBsAg subtype of the panel members is described in Table 1. The panel members 1-8 and 13-15 belonging to the HBV genotypes A, B, C, F and H represent the HBsAg subtype group *ad*, whereas the panel members 9-12 (HBV genotypes D and E) hold the HBsAg subtype group *ay*. The panel contains 12 HBV samples with the same source as the WHO HBV genotype panel for NAT assays, PEI Code No 5086/08 (8). Sequence data and the phylogenetic tree aligned with other sequences with the same subgenotype are available by the link http://www.pei.de/cln_116/nn_159176/DE/institut/who-cc/who-pei-aktivitaeten.html.

Figure1 illustrates the chosen sample processing procedure. The HBsAg concentration of the 15 candidate materials included into this reference panel was determined by three different methodologies:

1. Quantitative chemiluminescent immunoassay (**CLIA**, ARCHITECT Quantitative, Abbott). HBsAg concentration is reported as IU/ml, traceable to the 2nd IS.
   Lab Giessen: The original plasmas were diluted stepwise 1:10, 1:100, 1:1000, 1:3162 and 1:10,000 and the 3 last dilutions were tested in comparison to the internal standard of the test. Values between 5 and 50 IU/ml (reflecting the linear measuring range of the assay) were used for determination of arithmetic mean values. Lab PEI: Based on the mean values from Lab Giessen the original plasma samples were diluted to 10 IU/ml, 1 IU/ml, and 0.1 IU/ml and tested in replicates with the quantitative ARCHITECT test. From the results the final arithmetic mean values for the undiluted samples were calculated. These HBsAg results were the numerical basis for the further processing of the study samples.
2. Quantitative immunoelectrophoresis (**QIE**) according Laurell (15). HBsAg concentration was expressed in PEI-units (U)/ml based on the first PEI HBsAg standards ad and ay (native, non-inactivated preparations) from 1975 containing 50,000 PEI-U/ml (16).
3. Biochemical purification of native, empty HBsAg particles and filaments (**HBsAg**). The HBsAg concentration was expressed in ng/ml. The detailed procedure will be described elsewhere (Schüttler et al., in preparation). The method used is a modification of the method described by Gerlich and Thomssen 1975 (16). Briefly, 1ml of plasma was subjected to sucrose gradient rate zonal sedimentation. HBsAg positive fractions were combined, adjusted to high density with caesium chloride and HBsAg was flotated through a caesium chloride gradient to its buoyant density around 1.20 g/ml. Purity and protein composition of the HBsAg in the caesium chloride gradient fraction was confirmed by SDS gel electrophoresis with subsequent silver stain and Western blot analysis. HBsAg was quantitated by UV photometry at 280 nm assuming a specific optical density of 4.3 for 1mg/ml pure HBsAg protein. After correction for losses in the side fractions the yield of pure HBsAg in the caesium chloride fractions was assumed to be 100 %, and the amount of HBsAg in the original native plasma sample was expressed in ng/ml. This method is relatively accurate as shown for the internal reference plasma with 100,000 PEI-U/ml. In three purification runs this HBsAg was determined as 96,800 ± 6,800 ng/ml. However,

with lower HBsAg concentrations (<12,000 PEI-U/ml) this method is less accurate. Therefore, the source materials for the panel members 10 and 13 were not evaluated by using this method.

Table 1 summarizes the HBsAg data for the 15 candidate materials used for processing the panel members. To reduce the infectivity of the HBV plasma samples, 14 panel materials were purified by ultracentrifugation to eliminate the infectious virus particles (Dane particles). A 2x1/2 inch tube was filled with layers of 0.6 ml 20 % (w/w) sucrose in TNE buffer, 0.6 ml 10 % sucrose, 2.4 ml plasma and 0.6 ml TNE buffer. Centrifugation was performed for 2 h at 40,000 rpm and 10 °C in a SW60Ti rotor (Beckman). The top 0.2 ml containing lipid was discarded, the following 3.5 ml were collected as HBsAg sample, and the bottom fraction with the virus pellet was collected separately. Determination of the HBV DNA concentration before and after centrifugation revealed a virus removal of >97% (median 99.6 %) except for Sample 14 with a removal rate of 80 % (Table 2). However, this material had a low virus load of only 1000 IU/ml after centrifugation. One material (panel sample 10/D) was not ultracentrifuged due to the limited volume. The highest virus load before centrifugation was $1.1x10^9$ IU/ml (median of all $2.7x10^8$ IU/ml), after centrifugation $2.4x10^7$ IU/ml (median $1.0x10^6$ IU/ml).

Preparation of bulk materials and freeze-drying

Each of the 15 samples was further processed by diluting with negative plasma to a final concentration of 100 IU/ml in a total volume of 1.1 litres. This concentration corresponds to the 3fold concentration of the 2nd IS 00/588. This reference panel was designed to be also suitable for the performance evaluation of rapid diagnostic test kits. The dilution factors for the preparation of the bulk materials were determined by using the arithmetic mean IU/ml values of the CLIA HBsAg concentrations (Abbott ARCHITECT Quantitative). Dilutions were prepared with a plasma pool which had tested negative for the following markers: HAV RNA (artus HAV LC RT-PCR Kit, Qiagen, Hilden), HIV-1 RNA, HCV RNA, HBV DNA (Procleix Ultrio Assay, Novartis Diagnostics, Emeryville, CA, USA), HBsAg, anti-HBs, total anti-HBc (PRISM HBsAg, AxSYM, and PRISM HBc, Abbott GmbH & Co. KG, Wiesbaden), anti-HIV-1/2, and anti-HCV (AxSYM HIV1/2 gO and AxSYM HCV 3.0, Abbott GmbH & Co. KG, Wiesbaden).

The bulk preparations were stored at -20°C until further processing. The certified (EN ISO: 9001:2000; EN ISO: 13485:2003) company Greiner Diagnostic AG, Langenthal, Switzerland, was subcontracted for filling and lyophilisation. For these procedures the bulk preparations were removed from storage at -20°C and thawed at 37°C in a water bath with constant agitation until thawing. After thorough mixing, the materials were stored at 2°C to 8°C and 0.5 ml volumes were dispensed in 4-ml screw-cap glass vials. Rubber seals were then placed on top of the filled vials before loading into the freeze-drier (Instrument CHRIST Epsilon 2-25 D; LPC-16/NT process documentation). The coefficient of variation of the fill volume was 0.7% (sample 12/E), 0.8% (samples 4/B, 6/C, 15/H), 0.9% (samples 1/A, 2/B, 5/B, 11/D, 13/F, 14/F), 1% (samples 7/C, 8/C, 9/D), and 1.1% (samples 2/A, 3/A and 10/D). Overall 30,000 vials (2,000 vials each of the panel members) were lyophilised in 5 runs in October/November 2009. Additionally, 90

vials filled with 0.5 ml of negative plasma pool were randomly distributed on the trays of the 5 freeze-drying runs. These vials were later used for the determination of the residual moisture content. After freeze-drying the vials were sealed with screw-caps and stored at -20°C. The lyophilised vials are stored at -20 °C with regular temperature monitoring at PEI. All manufacturing records are held at PEI and are available on request by the ECBS. The HBV genotype reference panel has the PEI code number 6100/09.

Studies on the final product

The HBsAg concentration of the panel members was determined before and after lyophilisation by CLIA ARCHITECT Quantitative (IU/ml). Assuming the same reduction factor for CLIA and QIE, the loss of CLIA reactive antigen in per cent was also translated into the HBsAg values determined by the QIE method. The absolute HBsAg protein quantity is not influenced by the freeze-drying procedure, therefore the HBsAg protein values (ng/ml) were not changed for the panel members (see Table 3). To prove the traceability of the HBsAg values (IU/ml) of enzyme-immunoassay CLIA (calibrated with the WHO IS) to a physicochemical determination of the HBsAg unitage (expressed as ng), the relationship of HBsAg unitages IU and ng were determined (Table 3). The deduced values are in part different between the study samples and also between samples from one genotype, esp. for the genotypes C and D.

On average, 1 IU corresponded to 0.98+/-0.36 ng HBsAg protein and to 0.71+/-0.25 PEI-U. Three samples showed particularly large deviations: Sample 6 with genotype C2 had 2.06 ng per IU, samples 4 (B1) and 14 (F2) 0.54 ng per IU. The previous collaborative study to assess the suitability of a candidate replacement IS for HBsAg (genotype A2) had revealed a relationship of 1 IU = 0.58 ng (7). Interestingly, we found 0.93 ng per IU for panel member 3 with the same genotype.

The HBV DNA concentration of the panel members was determined before and after lyophilisation using the quantitative Cobas AmpliPrep/Cobas TaqMan HBV Test, v2.0 (Roche Diagnostics). The results confirm previous studies showing no significant effect of freeze-drying procedure on the HBV DNA integrity (Table 4).

A programme to investigate the stability of the panel members was initiated covering different temperatures (-20°C, 4°C, room temperature, 37°C). Preliminary results from real-time stability studies prove the stability of HBsAg in the panel members 1-15 under recommended storage conditions, i.e. at -20ºC or below. Analogous results obtained of the previous and the current IS preparations for HBsAg indicate that these preparations are suitable for long term use. The material is supplied lyophilised and should be stored at or below -20ºC. Each vial contains the equivalent of 0.5 ml of human plasma. The panel members should be reconstituted in 0.5 ml of distilled water. If the material is not used completely, laboratories may aliquot the residual reconstituted material into suitable portions to be stored at or below -70°C.

Due to the potential residual infectivity of the preparations the residual moisture content has been determined from the freeze-dried vials filled with negative plasma pool. These

vials were randomly distributed on the trays of the 5 freeze-drying runs and underwent the same processing conditions as all other vials. The residual moisture content was determined at PEI using an accredited method according to the European Pharmacopoeia (17). The water content was determined to be 0.70 ± 0.11% (standard deviation) and therefore is compliant with the WHO recommendations for the preparation, characterization and establishment of international and other biological reference standards (18).

Collaborative study

Participants, samples and study design

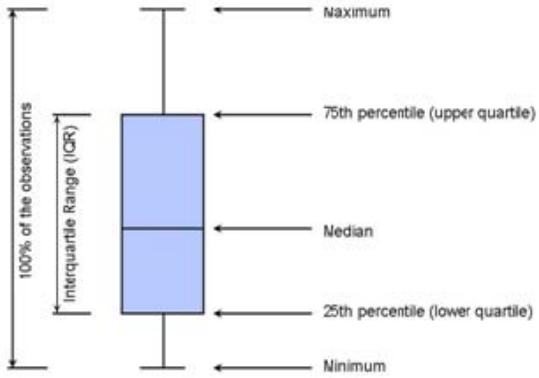
Initially, nineteen laboratories from 10 countries had agreed to participate in the collaborative study and received the study materials. The laboratories were requested to analyse the 15 panel members concurrently with the 2nd WHO IS for HBsAg (00/588). The protocol distributed to the study participants is attached in Appendix 1. Data sheets and a method form were provided to ensure that all relevant information was recorded. For the purposes of data analysis, each test method has been referred to a number representing a ranking referred to the results of the analytical sensitivity calculated by titrating of the IS 00/588. Test no 1 is the HBsAg kit with the highest analytical sensitivity. The laboratory has been referred to by a capital letter allocated at random and not representing the order of Table 5. Same HBsAg assays performed by different laboratories have the same number. When a laboratory performed more than one assay method, the results from the different methods were analysed independently, and coded, for example, test 1A and test 2A (Table 6a and 6b). The samples analysed in the study were labelled as Sample 1, Sample 2, Sample 3 to Sample 15, which correspond to respective subgenotypes A1 to H. Participants did not know the corresponding subgenotypes. The participants were requested to perform 3 separate assay runs with qualitative or quantitative tests as detailed in the study protocol. The HBsAg test kits used by the participants cover a broad range of commercially available enzyme immunoassays as well CLIA kits (Table 6a). Results from qualitative tests should be reported in sample/cut-off (S/CO) values. Where laboratories performed quantitative tests, they were requested to report results in IU/ml.

Statistical Methods

Dilutional sensitivity (Table 7) was obtained by linear interpolation using the 2 dilutions which revealed values below and above the assay´s cut-off (1.00 S/CO) in the dilution series. The sensitivity scores (table 10) were then calculated dividing the above cut-off dilutions by the WHO IS cut-off dilution of the corresponding participant. Sensitivity in terms of the different HBsAg tests (IU/ml, PEI-U/ml or ng/ml respectively; table 9) was determined using the dilution sensitivity multiplied with the values of the diluted panel members (Table 3).

The log-transformed data from qualitative and quantitative assays were evaluated for each participant and each run separately with a parallel line assay model (according European Pharmacopoeia, 5.3. Statistical analysis of results of biological assays and tests,

01/2008:50300) in order to estimate the potency (IU/ml) relative to the Standard IS 00/588 (assigned potency: 33 IU/ml). The logarithmic-transformation was necessary, as the prescribed dilution range was chosen to cover the sample cut-off rather than the linear range of the dose response curve. Due to the high precision of most assays (regarding the variability of the duplicate values of each dilution) and the combined evaluation of 15 samples relative to the reference, the assays often occurred to be significantly non-linear or non-parallel. Thus, as an alternative, all data were evaluated with a model not formally verifying the linearity and localising the linear range on a visual basis (deviations from linearity). Furthermore mean estimates per assay and participant and overall mean estimates per sample were calculated by means of an Analysis of Variance (ANOVA) model. The estimation of uncertainty, inter-laboratory, inter-assay, and intra-laboratory precision was done using a mixed linear model with random factors participant and assay. The statistical analysis was performed with SAS®/STAT software, version 9.2, SAS System for Windows. Estimation of relative potency was done with CombiStats Software, version 4.0, from EDQM / Council of Europe. The box-and-whisker-plots show the distribution of the data. The box itself contains the middle 50 per cent of the results (interquartile range, IQR) and the median as horizontal line. The ends of the whiskers denote the minimum and maximum values.



Data Received

No study results were obtained from three laboratories. One of these laboratories was not able to perform the study due to associated costs and work load. From the other two laboratories PEI did not receive any response. Data were received from 16 laboratories. One laboratory sent results generated by an HBV NAT assay instead of HBsAg tests. In total 15 laboratories sent 24 qualitative data sets obtained with 21 different HBsAg tests and 6 quantitative data sets from 2 different HBsAg tests for the evaluation (Table 5). One laboratory performed a quantitative HBsAg test and reported results both qualitatively in S/CO values (12H1) and quantitatively in IU/ml (12H). Another laboratory performed the study with two qualitative assays, GS HBsAg EIA 3.0 (Bio-Rad) and Abbott PRISM HBsAg. The results were excluded from the statistical analysis due to inconsistency of the HBsAg S/CO values within the dilution series of the samples. A mean S/CO value equal to or higher than 1.00 is considered reactive in each qualitative HBsAg test. The used HBsAg-negative matrix for the dilution of the study samples is

listed in Table 6b. All tests were performed according the test kit manufacturer's instruction.

Results

From former feasibility studies it was known that the dilution ranges for the Samples 1 to 15 given in the result´s sheet should be within the detection range of all assays and should include the endpoint titer (intercept with the cut-off of the assays). Data sets received from the testing with the qualitative HBsAg tests, including the S/CO data of 12H1, were used for the analysis. The data from participant F with test 7 (F7) were not included in the calculation of the sensitivity because no cut-off was estimable.

Analytical sensitivity related to the IS

The analytical sensitivity was calculated using the values obtained with the IS 00/588 by linear interpolation and expressed in IU/ml. (Tables 7 and 8, and Figure 2). The most sensitive assay in this study was the PRISM test followed by Enzygnost HBsAg 6.0, Monolisa HBsAg Ultra and the prototype ARCHITECT HBsAg Improved. The calculated sensitivity for these test kits was determined to be 0.0163, 0.0169, 0.0172, and 0.0174 IU/ml, respectively. Eight further HBsAg tests showed HBsAg values between 0.02 and 0.04 IU/ml. For only six test kits an analytical sensitivity data of >0.04 IU/ml was obtained. Formally all HBsAg test kits used in the collaborative study fulfilled the criteria for the analytical sensitivity of <0.13 IU/ml defined by the Common Technical Specification (CTS) of the European Directive 98/79/EC on in vitro diagnostic medical devices (19). Because the analytical sensitivity for HBsAg usually is correlated with the diagnostic sensitivity it is a useful tool for the estimation of the overall test kit sensitivity. The striking differences of the results from 7A and 7C (AxSYM) are partially explained by the use of different diluent matrices (negative human plasma from different vendors) for the study samples known as a potentially critical issue for the test performance. Furthermore laboratory F used a protein-free matrix (PBS) for the dilution of the samples which resulted in a non-definable endpoint when using the AxSYM. But with the parallel line method the relative potencies could be calculated to the Samples 1-15 for data set 7F.

Diagnostic sensitivity

The results from this collaborative study will gain insight into the efficacy of the HBsAg tests to detect different HBV genotypes by using two different approaches:

- Dilution sensitivities of the Samples 1 to 15 at S/CO of 1.00 (Table 7) are traced to the different HBsAg unitages IU, PEI-U and ng per ml (Table 3; post-lyophilisation values)
- Dilution sensitivities of the Samples 1 to 15 are compared to the dilution sensitivity of the IS 00/588.

The first approach is independent from the results of the IS. Table 9 reveals that most test kits, in particular the more sensitive ones (nos. 1 -10 and 12) reacted quite homogeneously with all samples irrespective of which unitage (IU, PEI-U or ng) was used. In contrast test no 11 showed a very different reactivity of various samples with detection limits between 0.029 and 0.146.

Due to the titration in parallel between the panel members and the IS the study results allow statements regarding the commutability of the IS 00/588 in relation to other HBV genotypes when using different HBsAg tests.

Comparison of the starting HBsAg concentration (IU/mL post-lyo in Table 3) with the overall mean potencies (IU/ml) in Table 14 shows that there was close agreement (range in factors 0.93 to 1.21) between the 2nd HBsAg IS and the genotype samples in the panel. In general, the HBsAg content ("post-lyo") in the panel, as quantified by the ARCHITECT HBsAg Quantitative, was reproduced with the other HBsAg assays in the collaborative study. With panel members A/2, 6/C, 7/C, 8/C, 11/D, and 12/E there was on average a slightly higher overall recovery compared to the ARCHITECT HBsAg Quantitative (factor 1.1 to 1.2) which can be seen across most HBsAg test of the study (Figure 3). With panel member 13/F there was a slightly lower recovery (factor 0.93). In addition, the pattern of HBsAg detection in the panel varies dependent on the particular HBsAg test kit used as displayed in Table 9 and Figure 3. One test kit (no 11, Advia Centaur HBsAg) generated results stronger deviating from the overall picture. It detected the IS with 0.030 IU/ml and Sample 9 with 0.029 IU/ml, but all other samples were detected less sensitive, 4 of with >0.100 IU/ml. Test no 13 showed the opposite pattern. Sensitivity of the IS was 0.050, but most samples of the panels were detected more sensitive with values between 0.024and 0.050 IU/ml.

Calculated sensitivity scores for each study sample related to the IS were compared between HBsAg tests and laboratories (Table 10 and Figure 4). If all tests recognized the different genotypes with same efficiency as the IS, there would be a narrow sensitivity score distribution for the genotypes with all tests (Fig. 4). . As an example, if a study sample was provided with a pre-determined HBsAg concentration around 100 IU/ml a sensitivity score of about 3 can be assumed for this sample. Higher score values reveal that test kits recognize the sample with a higher sensitivity compared to the IS and vice versa. Although the IS 00/588 belongs to subgenotype A2, the applied HBsAg tests detected the genotype A samples (Samples 1-3) more heterogeneously compared to the IS than expected. A similar picture was found for the HBV genotypes C (Samples 6-8), D (Samples 9-11), and E (Sample 12). Furthermore the results from this collaborative study showed that the Advia Centaur HBsAg test (test no 11C) had the lowest detection efficiency for the HBV samples from the genotype samples 3/A, 8/C, 9D and 11/D compared to the IS 00/588. In contrast the Immulite HBsAg assay (test no 17) seems to be relatively more efficient in the detection of the HBV genotype samples 11/D and 12/E. The samples 12/E, 13/F and 14/F were less efficient detected by test no 14. Less variation was identified for Samples 13/F and 15/H and for the two genotype B samples (Samples 4 and 5) since all tests detected this genotype with comparable efficiency related to the IS sample.

Quantitative assay results

Two different quantitative HBsAg kits were used in the study, 6 participants performed the ARCHITECT Quantitative (12A, 12C, 12H, 12J, 12K, 12L) and one participant performed the HISCL HBsAg Assay (19N) with an analytical sensitivity of 0.03 IU/ml (data provided from the participant). The results from all participants performing the

HBsAg test no 12 showed higher mean concentrations for each dilution obtained with the IS 00/588 as its assigned potency of 33 IU/ml, whereas the test no 19 had significant lower recovery values for the IS (Tables 11 and 12, Figure 5). Overall a mean potency of 49.7 IU/ml and 26.9 IU/ml was determined by test kits no 12 and 19, respectively.

Relative potency of Samples 1-15

The HBsAg values (IU/ml) of the Samples 1-15 calculated relative to the IS 00/588 are presented in Table 13 and in Figure 6 (histogram). The results confirm the observed trends of the calculated sensitivity scores with the study samples by using different qualitative HBsAg tests. Additionally this analysis also included the datasets obtained from the quantitative tests. Due to the parallel line assay evaluation method for the determination of the potency both data sets (qualitative and quantitative results) of laboratory H for test 12 resulted in the same potency and the study participant for this assay is referred as 12H. The quantitative assay 19N showed higher values for the Samples 1-15 compared to the results with the ARCHITECT Quantitative (12A, 12C, 12H, 12J, 12K, 12L). The overall mean relative potency of the Samples 1-15 calculated for all 28 data sets (including the results from qualitative and quantitative assays) are shown in Table 14 and illustrated as a Box Plot in Figure 7. The overall mean potencies for all test kits were in close agreement with the amount quantified for the ampoules ("post-lyo", Table 3). Nevertheless, the variability of the potencies for the various panel members against the current IS amounted to 20 to 50% (mean 36%) which is greater than for the 2nd IS (7). Panel members 6-12 showed the highest variability. Additionally the Figures 4, 5, 6 and 7 show that the results obtained for panel members 6-12 were more scattered than those for the other panel members.

Precision between participants and methods

Table 15 shows the geometric coefficients of variation based on the estimated relative potencies) for all test kits and participants in terms of total uncertainty, inter-laboratory precision, inter-method precision, and intra-assay precision. Intra-lab variability for all test kits in all labs was within an imprecision range inherent to the technical precision of immunoassays. Also inter-lab variability did not indicate for an unusual assay imprecision. There was one case of higher inter-lab variability with assay no 7 (AxSYM) between labs A and C, most probably due to a matrix effect as discussed above. Also with 2B compared with 2C and 8G compared with 8H there were differences in the analytical sensitivity which may partly be due to the matrix used in the study. Nevertheless, the within assay variability for the 2nd HBsAg IS (00/588) in this study was similar to the results presented in the study report WHO/BS/03.1987 (7). In conclusion, the precision was in a range consistent with the variability inherent with the test kit and performancet of the test indicating no significant variation due to the panel.

Conclusions

In this study, a wide range of HBsAg tests (21 qualitative and two quantitative assays) were used to evaluate the HBV genotype panel in parallel to the current WHO IS 00/588. The panel consists of 15 lyophilised, processed HBV positive plasma samples and covers the most prevalent HBV genotypes: Samples 1-3 (genotype A), Samples 4-5 (genotype B), Samples 6-8 (genotype C), Samples 9-11 (genotype D), Sample 12 (genotype E), Samples 13-14 (genotype F), and Sample 15 (genotype H).
The collaborative study aimed at the crucial question of commutability and traceability of the current reference material in relation to native HBsAg protein and to other HBV genotypes. Additionally the results of the study will give evidence about the efficacy of HBsAg tests to detect different HBV genotypes. Analytical sensitivity determination for the HBV genotypes was carried out in parallel with the 2nd WHO IS for HBsAg (code no 00/588). The analytical sensitivity found for the HBV genotypes in the panel was overall in close agreement with the HBsAg concentration assigned to the 2nd HBsAg IS. Some test kits seemed to detect the IS better than the native samples of the panel, others reacted weaker with the IS although not completely consistent for all 15 panel members and by all test kits. Therefore, a unitage (IU/ml) will not be assigned to the panel members. .

The study results show that the candidate reference panel was suitable for determination of the analytical HBsAg sensitivity for different HBV subgenotypes and that, for some test kits, significant differences in the detection of different genotypes exist. The panel will be of high value for IVD manufacturers and users to assess the relative detection efficiency of their assay in relation to HBV genotypes. The comparative data generated in the collaborative study may allow an evaluation of the current “state of the art” sensitivity of assays in regard to the most prevalent HBV genotypes. Furthermore, regulatory authorities in countries with HBV genotype prevalence different from A2 (as represented in the WHO IS for HBsAg) will have an important tool to assess relative sensitivities of HBsAg assays for the genotypes more prevalent in their region. Thus, the panel is proposed to be established as the 1st International Reference Panel for HBV Genotypes for HBsAg Assays.

Comments from participants

A copy of the draft report was sent for comments to all laboratories participating in the collaborative study. So far, all comments of the participants have now been addressed and appropriate corrections were performed.

Lab A. Test number 4 (listed in the report as ARCHITECT HBsAg Improved) is now CE marked and commercially available as "ARCHITECT HBsAg Qualitative II", product list number 2G22. This is the same assay as was used in the collaborative study. Food note of Table 6a was revised. *The diluents in Table 6b for assays 7A and & 7C are described as different matrices, although they were both negative human plasma. We do agree that different diluent matrices can affect results, were these two diluents really different?* Although both diluents were negative human plasma, the manufacturer/vendor was different. Statement was added on page 10.

Lab B. The comment refers to a slight, but significant discrepancy in the results obtained by 2B and 2C. The analytical sensitivity calculated from the results of lab C was roughly 20 to 25% lower than in lab B. Lab C did not adhere to the recommendations described in the package insert for the Enzygnost HBsAg 6.0 assay and used their own dilution medium (FCS) which might have led to an underestimation of titers in the dilution experiments. The same explanation is given for the striking differences of the results from 7A and 7C (AxSYM).

Lab C. Comment to add in the precision part of the report (p12): *Also with 2B compared with 2C and 8G compared with 8H there were differences in the analytical sensitivity which may partly be due to the matrix used in the study. Nevertheless, the within assay variability for the 2nd HBsAg IS (00/588) in this study was similar to the results presented in the study report WHO/BS/03.1987 (7).*
Lab D. Specification of the diluent matrix used for the study, negative human serum. Table 6B was revised.

The participants agreed with the proposal that the HBV genotype panel (PEI Code number 6100/09) should be established as the 1st International Reference Panel for HBV Genotypes for HBsAg-based assays. There was an overall agreement that the individual panel members will not have an assigned value in IU.

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References

1. World Health Organization, Hepatitis B, Fact sheet N°204, http://www.who.int/mediacentre/factsheets/fs204/en/index.html
2. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004, **11**:97-107
3. Hannoun C, Norder H, Lindh M. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam.. *J Gen Virol* 2000, 8**1**:2267-2272
4. Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS. Molecular and phylogenetic analysis suggest an additional hepatitis B virus genotype I. *Plos One* 2010, **5**:e9297
5. Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T, Nakayoshi T, Wakuta M, Miyakawa Y, Mizokami M. A genetic variant of hepatitis B virus

divergent from known human and ape genotypes isolated from a Japanes patient and provisionally assigned to new genotype J. *J Virol* 2009, **83**:10538-10547

6. WHO Consultation on Global Measurement Standards and their use in the *in vitro* Biological Diagnostic Field. Geneva, Switzerland (June 2004) http://www.who.int/bloodproducts/publications/en/Minutes-220804.pdf
7. Ferguson M, Heath A, Lelie N, Nübling M, Nick S, Gerlich W, Decker R, Padilla A. Report of a collaborative study to 1) assess the suitability of a candidate replacement International Standard for HBsAg and a reference panel for HBsAg and 2) to calibrate the candidate standard in IU. WHO/BS/03.1987. http://www.who.int/bloodproducts/cs/en/031987.pdf
8. Chudy M, Hanschmann KM, Kreß J, Gerlich W, Nübling CM. Collaborative study to establish a World Health Organization international genotype panel for hepatitis B virus nucleic acid amplification technique (NAT)-based assays. WHO/BS/09.2121. http://whqlibdoc.who.int/hq/2009/WHO_BS_09.2121_eng.pdf
9. Nick S, Scheiblauer H. Sensitivities of CE-marked HIV, HCV, and HBsAg assays. *J Med Virol* 2007, **79**:S59-S64
10. Scheiblauer H, El-Nageh M, Diaz S, Nick S, Zeichhardt H, Grunert HP, Prince A. Performance evaluation of 70 hepatitis B virus (HBV) surface antigen (HBsAg) assays from around the world by a geographically diverse panel with an array of HBV genotypes and HBsAg subtypes. *Vox Sang* 2010; **98**:403-414
11. Matsubara N, Kusano O, Sugamata Y, Itoh T, Mizuii M, Tanaka J, Yoshizawa H. A novel hepatitis B virus surface antigen immunoassay as sensitive as hepatitis B virus nucleic acid testing in detecting early infection. *Transfusion* 2009, **49**:585-595
12. Brunetto MR. A new role for an old marker, HBsAg. *J Hepatol* 2010, **52**:475-477
13. Schüttler CG, Wend UC, Faupel FM, Lelie PN, Gerlich WH. Antigenic and physicochemical characterization of the 2nd International Standard for hepatitis b virus surface antigen (HBsAg). *J Clin Virol* 2010, **47**:238-242
14. Mizuochi T, Okada Y, Umemori K. Evaluation of 10 commercial diagnostic kits for in vitro expressed hepatitis B virus (HBV) surface antigens encoded by HBV of genotypes A to H. *J Virol Methods* 2006; **136**:254–256
15. Gerlich WH, Wend U, Glebe D. Quantitative assay of hepatitis B surface antigen in serum or plasma using Laurell electrophoresis. 2004. In: Lau J, Hamatake R. Hepatitis B Virus protocols book, p. 57-63, Humana press, Totowa, N.J.
16. Gerlich WH, Thomssen R. Standardized detection of hepatitis B surface antigen: determination of its serum concentration in weight units per volume. Develop. biol. standard. 1975 Vol 30, 78-87 (S Karger, Basel)
17. Water: Micro determination. Methods of analysis EP 2.5.32; in *European Pharmacopoeia*, 6th Edition. Strasbourg, France, Council of Europe, 2008
18. Recommendations for the preparation, characterization and establishment of international and other biological reference standards. W HO Expert Committee on Biological Standardization. Fifty-fifth report, 2004. (WHO Technical Report Series, No. 932)
19. Official Journal of the European Union **L 318**, 4.12.2009, p. 25.

Table 1. Characterization of the panel members

Panel Member	**Origin**	**HBsAg subtype[1]**	**HBV Sub-genotype[1]**	**CLIA HBsAg[2] (IU/ml)**	**QIE HBsAg[3] (PEI-U/ml)**	**HBsAg[4] (ng/ml)**
1/A	South Africa	*adw2*	A1	80,130	48,680	77,470
2/A	Brazil	*adw2*	A1	57,737	40,400	53,500
3/A	Germany	*adw2*	A2	50,710	33,770	38,050
4/B	Japan	*adw2*	B1	45,280	20,510	15,370
5/B	Japan	*adw2*	B2	63,120	27,140	43,590
6/C	Japan	*adr*	C2	34,585	47,030	62,420
7/C	Japan	*adr*	C2	27,446	23,500	27,820
8/C	Russia	*adr*	C2	29,384	21,700	27,100
9/D	Germany	*ayw2*	D1	94,090	53,660	73,580
10/D[5]	Russia	*ayw3*	D2	8,413	8,900	n.a.
11/D	South Africa	*ayw2*	D3	42,949	28,650	26,030
12/E	West Africa	*ayw4*	E	66,204	47,030	53,800
13/F	Brazil	*adw4*	F2	22,258	11,400	n.a.
14/F	Brazil	*adw4*	F2	23,928	13,400	12,290
15/H	Germany	*adw4*	H	139,508	59,930	83,740

[1]Sequencing; [2]CLIA, chemiluminescent immunoassay ARCHITECT Quantitative, Abbott, performed by PEI lab; [3]QIE, quantitative immunoelectrophoresis; [4]purified HBsAg; n.a., not available; Results from EIA and QIE were obtained from the samples after ultracentrifugation; [5]no ultracentrifugation. HBsAg values after ultracentrifugation.

Table 2. HBV DNA determination of the panel members before and after ultracentrifugation

	HBV DNA (log_{10} IU/ml)[1]		
Panel Member	**Before UC**	**After UC**	**reduction factor**
1/A	8.75	7.21	1.54
2/A	8.78	5.27	3.31
3/A	8.83	4.79	4.04
4/B	8,13	6.44	1.49
5/B	8.42	6.82	1.60
6/C	8.56	5.14	3.42
7/C	8.06	6.53	1.53
8/C	8.43	6.08	2.35
9/D	9.03	5.40	3.63
10/D[2]	4.03		
11/D	7.98	5.94	2.04
12/E	8.94	7.07	1.87
13/F	7.01	5.20	1,81
14/F	2.93	2.24	0.69
15/H	9,01	7.38	1.63

[1]In-house LC HBV NAT (Samples 1-9; 11-15) and Cobas AmpliPrep/CobasTaqMan HBV Test, v1.0, Roche Diagnostics (Sample 10); [2]no UC; no ultracentrifugation.

Table 3. HBsAg determined in diluted panel members (Target dilution to 100 IU HBsAg/ml[1])

	Pre-Lyo		Post-Lyo					
Panel Member	**HBsAg[2] (IU/ml)**	**HBsAg[3] (PEI-U/ml)**	Loss by Lyo (%)	**HBsAg[2] (IU/ml)**	**HBsAg[3,4] (PEI-U/ml)**	**HBsAg[5] (ng/ml)**	**1 IU = x PEI-U**	**1 IU = x ng**
1/A	107,9	60,8	*11,6*	95,4	53,7	96,68	0,56	1,01
2/A	100,6	70,0	*12,8*	87,7	61,0	92,66	0,70	1,06
3/A	94,5	66,6	*15,0*	80,3	56,6	75,03	0,70	0,93
4/B	74,9	45,3	*15,5*	63,3	38,3	33,94	0,61	0,54
5/B	88,7	43,0	*15,8*	74,7	36,2	69,06	0,48	0,92
6/C	98,5	136,0	*11,0*	87,7	121,0	180,48	1,38	2,06
7/C	101,0	85,6	*16,6*	84,2	71,4	101,36	0,85	1,20
8/C	100,6	73,9	*10,5*	90,0	66,1	92,23	0,73	1,02
9/D	100,9	57,0	*17,4*	83,3	47,1	78,20	0,57	0,94
10/D	91,1	105,8	*3,2*	88,2	102,4	n.a.	1,16	n.a.
11/D	88,4	66,7	*18,1*	72,4	54,6	60,61	0,75	0,84
12/E	96,1	71,0	*11,0*	85,5	63,2	81,26	0,74	0,95
13/F	100,8	51,2	*20,2*	80,4	40,9	n.a.	0,51	n.a.
14/F	113,1	56,0	*16,0*	95,0	47,0	51,36	0,49	0,54
15/H	102,7	43,0	*13,8*	88,5	37,1	60,03	0,42	0,68
					Arithmetic mean		0.71	0.98
					Geometric mean		0.67	0.92
					Median		0.70	0.76
					Standard deviation		0.25	0.36

[1]Dilution based on mean CLIA values (see Table 1); [2]CLIA, chemiluminescent immunoassay ARCHITECT Quantitative, Abbott; [3]QIE, quantitative immunoelectrophoresis; [4]assumed loss (%) as measured by the EIA; [5]purified HBsAg; n.a., not available;

Table 4. HBV DNA determination of the panel members before and after lyophilisation[1]

	HBV DNA (IU/ml)	
Panel Member	**Pre-Lyo**	**Post-Lyo**
1/A	26,700	26,100
2/A	140	107
3/A	97	<20
4/B	13,900	15,900
5/B	15,500	11,000
6/C	693	237
7/C	15,900	21,700
8/C	8,010	10,400
9/D	433	221
10/D	135	62
11/D	4,020	2,160
12/E	304,000	165,000
13/F	383	341
14/F	<20	<20
15/H	31,900	64,400

[1]Cobas Ampliprep/ Cobas TaqMan HBV Test, v2.0 (Roche Diagnostics)

Table 5. List of participants (alphabetic order)

Scientist	Affiliation
Dr R.M. Biswas / S. Kerby	Center for Biologics Evaluation and Research/ Food and Drug Administration, Bethesda, MD, USA
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Dr M. Koot	Sanquin, Amsterdam, The Netherlands
Dr I. Krueger	Roche Diagnostics, Penzberg, Germany
M. C. Kuhns, PhD,	Abbott Diagnostics, Abbott Park, USA
Dr S. Laperche / Dr Servant-Delmas	Centre National Ref. Hép. B, INTS, Paris, France
T. Mizuochi, PhD	National Institute of Infectious Diseases Tokyo, Japan
Dr S. Lin Ngui	Health Protection Agency, London, UK
Dr N.S. Cho, MD, PhD	Korean Red Cross, Seoul, Korea
Dr M. Rapicetta / Dr A.R. Ciccaglione	Istituto Superiore di Sanita Rome, Italy
Dr E. Sabino	Fundação Pró-Sangue, Homocentro de São Paulo Sao Paulo, Brazil
Dr H. Scheiblauer	Paul-Ehrlich-Institut, Langen, Germany
Dr W. Stoeckigt	bioMérieux, Nürtingen, Germany
Dr M. Weik	Siemens, Marburg, Germany

Table 6a. List of test kits used in the order of end point titer with the 2nd IS

Test No	HBsAg test	Kit Manufacturer	Product No	Laboratory code
	Qual			
1	ABBOTT PRISM HBsAg	Abbott Diagnostics	6D19	1A
2	Enzygnost HBsAg 6.0	Siemens Healthcare Diagnostics Products	OPFM05	2B/2C
3	Monolisa HBsAg ULTRA	Bio-Rad Laboratories	72346-72348	3D
4	ARCHITECT HBsAg Improved	Abbott Diagnostics	2G22*	4A
5	ARCHITECT HBsAg	Abbott Diagnostics Division	1L80	5A
6	VIDAS HBsAg ULTRA	bioMeriéux	30315	6E
7	AxSYM HBsAg	Abbott Diagnostics Division	9B01 (7A) 7A40 (7C, 7F)	7A/7C/7F
8	Elecsys HBsAg II	Roche Diagnostics	04687787190	8G / 8H
9	ARCHITECT HBsAg Qualitative	Abbott Diagnostics Division	1P97	9A
10	ETI-MAK 4	Dia-Sorin	N0019	10I
11	Advia Centaur HBsAg	Siemens Healthcare Diagnostics	3393362	11C
12	ARCHITECT HBsAg Quantitative	Abbott Diagnostics Division	6C36	12H1
13	DS-EIA-HBsAg-0.01	RPC Diagnostic System, Ltd.	B1255, B1256	13C
14	HBsAg ELISA	Human GmbH	51048	14C
15	Vitros HBsAg ES	OrthoClinical Diagnostics	6802131/ 6802132	15M
16	Vitros HBsAg	OrthoClinical Diagnostics	8435307/ 1421932	16M
17	Immulite HBsAg	Siemens Healthcare Diagnostics	L2KHB2	17C
18	Bioelisa HBsAg 3.0	Biokit S.A.	3000-1158, 3000-1159	18C
	Quant			
12	ARCHITECT HBsAg Quantitative	Abbott Diagnostics Division	6C36	12A/12C/12H/ 12J/12K/12L
19	HISCL HBsAg Assay Kit	Sysmex Corporation		19N

*This assay was recently CE-marked and is commercially available as ARCHITECT HBsAg Qualitative II..

Table 6b. Diluents used for the study by the participants

Lab Code	HBsAg Test	Diluent used for sample dilution
A	1A/4A/5A/7A/9A/12A	Recalcified normal human plasma, negative for HBsAg and anti-HBs
B	2B	Negative HBsAg control serum of the Kit
C	2C/7C/11C/12C/13C/14C/17C/18C	Negative human plasma tested negative for anti-HBs (7C, 14C), Fetal Calf Serum, Gibco, Ref 10270106 (2C, 11C, 12/C, 13C,17C, 18C)
D	3D	Negative human serum
E	6E	VIDAS HBsAg ULTRA confirmation diluent
F	7F	Phosphate buffer saline 1X
G	8G	HBsAg negative serum
H	8H/12H	Abbott HAVAB diluent
I	10I	Human plasma negative for HBsAg and anti-HBs
J	12J	Normal human plasma (NHP_4/5/10U0010)
K	12K	Negative human plasma
L	12L	Architect HBsAg diluent
M	15M/16M	Anti-HBs negative human plasma
N	19N	Triethanolamine buffer with 1% BSA and 0.1% sodium azide (pH7.5)

Table 7. Dilution sensitivity (1:y) at S/CO of 1.00

	Sample no in the panel															
Assay	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	IS
1A	7446	6917	6627	4194	4303	7845	6566	8838	6840	6861	7541	7031	4551	6279	5264	2022
2B	5851	6387	5577	3497	3517	8261	7407	8572	5241	6036	5462	6067	4240	5008	4169	1947
2C	5334	5265	4616	2603	3022	6225	5475	7618	4903	5398	4811	5257	3403	4186	3723	1477
3D	5158	5331	5342	2838	3525	7859	5594	6658	4842	8477	5434	9863	4306	6361	4676	1919
4A	8417	7410	5167	5496	7086	7240	7954	6241	5128	6077	7372	12452	4782	6170	5844	1898
5A	5559	4981	3714	3219	3720	3909	3972	3701	4188	4856	5187	5656	4896	6535	4778	1551
6E	4238	4211	3603	2429	2770	6514	5567	5957	3358	3402	3063	3593	3023	3468	3439	1519
7A	3474	2974	2837	2778	2957	4043	4075	3774	2945	3283	3301	3739	3500	4525	4146	1291
7C	922	849	624	647	750	896	983	881	590	709	663	949	861	974	923	405
8G	3053	3018	2143	1942	2282	3135	3118	2756	1683	2291	2018	2370	1875	2291	2224	1225
8H	3348	3477	2534	1877	2190	3263	3345	3457	2793	2466	3010	2578	1881	2199	2185	829
9A	3770	3525	2814	2609	3197	3605	3749	3730	2262	2568	3133	3985	3197	4167	3273	1161
10I	3169	3089	2577	1768	1975	2714	2737	3640	2036	1834	2036	2216	1677	2026	1977	1122
11C	2150	1713	753	1627	1686	1292	1337	862	570	1307	705	2899	2030	2282	2066	1098
12H1	3391	3082	2335	2051	2399	3017	3027	2641	2397	2749	2500	2577	2302	2484	2994	1020
13C	3377	3110	3014	1739	1717	3197	2705	3715	2984	2434	2543	3124	1396	1882	1916	650
14C	1176	935	847	670	753	1271	1017	1201	1274	1297	1396	882	633	585	1068	575
15M	1637	1793	1553	914	1084	2496	2062	2613	1691	1946	1868	2128	1435	1693	1519	520
16M	1557	1736	1443	781	900	2721	2190	2858	1665	2123	1919	2118	1346	1577	1450	444
17C	1521	1536	1323	777	865	1900	1364	1555	1962	1924	2104	2407	1042	1306	1341	354
18C	972	1598	970	859	700	1301	1516	1445	1605	1245	1167	902	653	838	567	321

Data from 7F not included

Table 8. Analytical sensitivity of HBsAg tests obtained with the 2nd IS (00/588)

Assay	IU/ml	Assay	IU/ml	Assay	IU/ml
1A	0.0163	7A	0.0256	12H1	0.0323
2B	0.0169	7C	0.0815	13C	0.0507
2C	0.0223	8G	0.0269	14C	0.0574
3D	0.0172	8H	0.0398	15M	0.0635
4A	0.0174	9A	0.0284	16M	0.0743
5A	0.0213	10I	0.0294	17C	0.0931
6E	0.0217	11C	0.0301	18C	0.1029

Data from 7F not included

Table 9. Efficacy of HBsAg tests to detect different HBV genotypes. Dilution sensitivities (Table 7) are traced to the different HBsAg unitages IU, PEI-U and ng per ml (post-lyophilisation values; Table 3)

		Sample														
Assay	**HBsAg**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1A	IU/ml	0.013	0.013	0.012	0.015	0.017	0.011	0.013	0.010	0.012	0.013	0.010	0.012	0.018	0.015	0.017
	PEI-U/ml	0.007	0.009	0.009	0.009	0.008	0.015	0.011	0.007	0.007	0.015	0.007	0.009	0.009	0.007	0.007
	ng/ml	0.013	0.013	0.011	0.008	0.016	0.023	0.015	0.010	0.011	n.a.	0.008	0.012	n.a.	0.008	0.011
2B	IU/ml	0.016	0.014	0.014	0.018	0.021	0.011	0.011	0.010	0.016	0.015	0.013	0.014	0.019	0.019	0.021
	PEI-U/ml	0.009	0.010	0.010	0.011	0.010	0.015	0.010	0.008	0.009	0.017	0.010	0.010	0.010	0.009	0.009
	ng/ml	0.017	0.015	0.013	0.010	0.020	0.022	0.014	0.011	0.015	n.a.	0.011	0.013	n.a.	0.010	0.014
2C	IU/ml	0.018	0.017	0.017	0.024	0.025	0.014	0.015	0.012	0.017	0.016	0.015	0.016	0.024	0.023	0.024
	PEI-U/ml	0.010	0.012	0.012	0.015	0.012	0.019	0.013	0.009	0.010	0.019	0.011	0.012	0.012	0.011	0.010
	ng/ml	0.018	0.018	0.016	0.013	0.023	0.029	0.019	0.012	0.016	n.a.	0.013	0.015	n.a.	0.012	0.016
3D	IU/ml	0.018	0.016	0.015	0.022	0.021	0.011	0.015	0.014	0.017	0.010	0.013	0.009	0.019	0.015	0.019
	PEI-U/ml	0.010	0.011	0.011	0.013	0.010	0.015	0.013	0.010	0.010	0.012	0.010	0.006	0.009	0.007	0.008
	ng/ml	0.019	0.017	0.014	0.012	0.020	0.023	0.018	0.014	0.016	n.a.	0.011	0.008	n.a.	0.008	0.013
4A	IU/ml	0.011	0.012	0.016	0.012	0.011	0.012	0.011	0.014	0.016	0.015	0.010	0.007	0.017	0.015	0.015
	PEI-U/ml	0.006	0.008	0.011	0.007	0.005	0.017	0.009	0.011	0.009	0.017	0.007	0.005	0.009	0.008	0.006
	ng/ml	0.011	0.013	0.015	0.006	0.010	0.025	0.013	0.015	0.015	n.a.	0.008	0.007	n.a.	0.008	0.010
5A	IU/ml	0.017	0.018	0.022	0.020	0.020	0.022	0.021	0.024	0.020	0.018	0.014	0.015	0.016	0.015	0.019
	PEI-U/ml	0.010	0.012	0.015	0.012	0.010	0.031	0.018	0.018	0.011	0.021	0.011	0.011	0.008	0.007	0.008
	ng/ml	0.017	0.019	0.020	0.011	0.019	0.046	0.026	0.025	0.019	n.a.	0.012	0.014	n.a.	0.008	0.013
6E	IU/ml	0.023	0.021	0.022	0.026	0.027	0.013	0.015	0.015	0.025	0.026	0.024	0.024	0.027	0.027	0.026
	PEI-U/ml	0.013	0.014	0.016	0.016	0.013	0.019	0.013	0.011	0.014	0.030	0.018	0.018	0.014	0.014	0.011
	ng/ml	0.023	0.022	0.021	0.014	0.025	0.028	0.018	0.015	0.023	n.a.	0.020	0.023	n.a.	0.015	0.017
7A	IU/ml	0.027	0.029	0.028	0.023	0.025	0.022	0.021	0.024	0.028	0.027	0.022	0.023	0.023	0.021	0.021
	PEI-U/ml	0.015	0.021	0.020	0.014	0.012	0.030	0.018	0.018	0.016	0.031	0.017	0.017	0.012	0.010	0.009
	ng/ml	0.028	0.031	0.026	0.012	0.023	0.045	0.025	0.024	0.027	n.a.	0.018	0.022	n.a.	0.011	0.014
7C	IU/ml	0.103	0.103	0.129	0.098	0.100	0.098	0.086	0.102	0.141	0.124	0.109	0.090	0.093	0.097	0.096
	PEI-U/ml	0.058	0.072	0.091	0.059	0.048	0.135	0.073	0.075	0.080	0.144	0.082	0.067	0.047	0.048	0.040
	ng/ml	0.105	0.109	0.120	0.052	0.092	0.201	0.103	0.105	0.133	n.a.	0.091	0.086	n.a.	0.053	0.065

8G	IU/ml	0.031	0.029	0.037	0.033	0.033	0.028	0.027	0.033	0.050	0.039	0.036	0.036	0.043	0.041	0.040
	PEI-U/ml	0.018	0.020	0.026	0.020	0.016	0.039	0.023	0.024	0.028	0.045	0.027	0.027	0.022	0.021	0.017
	ng/ml	0.032	0.031	0.035	0.017	0.030	0.058	0.033	0.033	0.046	n.a.	0.030	0.034	n.a.	0.022	0.027
8H	IU/ml	0.028	0.025	0.032	0.034	0.034	0.027	0.025	0.026	0.030	0.036	0.024	0.033	0.043	0.043	0.041
	PEI-U/ml	0.016	0.018	0.022	0.020	0.017	0.037	0.021	0.019	0.017	0.042	0.018	0.025	0.022	0.021	0.017
	ng/ml	0.029	0.027	0.030	0.018	0.032	0.055	0.030	0.027	0.028	n.a.	0.020	0.032	n.a.	0.023	0.027
9A	IU/ml	0.025	0.025	0.029	0.024	0.023	0.024	0.022	0.024	0.037	0.034	0.023	0.021	0.025	0.023	0.027
	PEI-U/ml	0.014	0.017	0.020	0.015	0.011	0.034	0.019	0.018	0.021	0.040	0.017	0.016	0.013	0.011	0.011
	ng/ml	0.026	0.026	0.027	0.013	0.022	0.050	0.027	0.025	0.035	n.a.	0.019	0.020	n.a.	0.012	0.018
10I	IU/ml	0.030	0.028	0.031	0.036	0.038	0.032	0.031	0.025	0.041	0.048	0.036	0.039	0.048	0.047	0.045
	PEI-U/ml	0.017	0.020	0.022	0.022	0.018	0.045	0.026	0.018	0.023	0.056	0.027	0.029	0.024	0.023	0.019
	ng/ml	0.031	0.030	0.029	0.019	0.035	0.066	0.037	0.025	0.038	n.a.	0.030	0.037	n.a.	0.025	0.030
11C	IU/ml	0.044	0.051	0.107	0.039	0.044	0.068	0.063	0.104	0.146	0.068	0.103	0.029	0.040	0.042	0.043
	PEI-U/ml	0.025	0.036	0.075	0.024	0.021	0.094	0.053	0.077	0.083	0.078	0.077	0.022	0.020	0.021	0.018
	ng/ml	0.045	0.054	0.100	0.021	0.041	0.140	0.076	0.107	0.137	n.a.	0.086	0.028	n.a.	0.023	0.029
12H1	IU/ml	0.028	0.028	0.034	0.031	0.031	0.029	0.028	0.034	0.035	0.032	0.029	0.033	0.035	0.038	0.030
	PEI-U/ml	0.016	0.020	0.024	0.019	0.015	0.040	0.024	0.025	0.020	0.037	0.022	0.025	0.018	0.019	0.012
	ng/ml	0.029	0.030	0.032	0.017	0.029	0.060	0.033	0.035	0.033	n.a.	0.024	0.032	n.a.	0.021	0.020
13C	IU/ml	0.028	0.028	0.027	0.036	0.043	0.027	0.031	0.024	0.028	0.036	0.028	0.027	0.058	0.050	0.046
	PEI-U/ml	0.016	0.020	0.019	0.022	0.021	0.038	0.026	0.018	0.016	0.042	0.021	0.020	0.029	0.025	0.019
	ng/ml	0.029	0.030	0.025	0.020	0.040	0.056	0.037	0.025	0.026	n.a.	0.024	0.026	n.a.	0.027	0.031
14C	IU/ml	0.081	0.094	0.095	0.094	0.099	0.069	0.083	0.075	0.065	0.068	0.052	0.097	0.127	0.162	0.083
	PEI-U/ml	0.046	0.065	0.067	0.057	0.048	0.095	0.070	0.055	0.037	0.079	0.039	0.072	0.065	0.080	0.035
	ng/ml	0.082	0.099	0.089	0.051	0.092	0.142	0.100	0.077	0.061	n.a.	0.043	0.092	n.a.	0.088	0.056
15M	IU/ml	0.058	0.049	0.052	0.069	0.069	0.035	0.041	0.034	0.049	0.045	0.039	0.040	0.056	0.056	0.058
	PEI-U/ml	0.033	0.034	0.036	0.042	0.033	0.048	0.035	0.025	0.028	0.053	0.029	0.030	0.028	0.028	0.024
	ng/ml	0.059	0.052	0.048	0.037	0.064	0.072	0.049	0.035	0.046	n.a.	0.032	0.038	n.a.	0.030	0.040
16M	IU/ml	0.061	0.051	0.056	0.081	0.083	0.032	0.038	0.031	0.050	0.042	0.038	0.040	0.060	0.060	0.061
	PEI-U/ml	0.034	0.035	0.039	0.049	0.040	0.044	0.033	0.023	0.028	0.048	0.028	0.030	0.030	0.030	0.026
	ng/ml	0.062	0.053	0.052	0.043	0.077	0.066	0.046	0.032	0.047	n.a.	0.032	0.038	n.a.	0.033	0.041
17C	IU/ml	0.063	0.057	0.061	0.081	0.086	0.046	0.062	0.058	0.042	0.046	0.034	0.036	0.077	0.073	0.066
	PEI-U/ml	0.035	0.040	0.043	0.049	0.042	0.064	0.052	0.043	0.024	0.053	0.026	0.026	0.039	0.036	0.028

	ng/ml	0.064	0.060	0.057	0.044	0.080	0.095	0.074	0.059	0.040	n.a.	0.029	0.034	n.a.	0.039	0.045
18C	IU/ml	0.098	0.055	0.083	0.074	0.107	0.067	0.056	0.062	0.052	0.071	0.062	0.095	0.123	0.113	0.156
	PEI-U/ml	0.055	0.038	0.058	0.045	0.052	0.093	0.047	0.046	0.029	0.082	0.047	0.070	0.063	0.056	0.065
	ng/ml	0.099	0.058	0.077	0.039	0.099	0.139	0.067	0.064	0.049	n.a.	0.052	0.090	n.a.	0.061	0.106

Table 10. Sensitivity scores (ratio of the S/CO for the sample over S/CO for the IS 00/588)

	Sample														
Assay	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1A	3.68	3.42	3.28	2.07	2.13	3.88	3.25	4.37	3.38	3.39	3.73	3.48	2.25	3.11	2.60
2B	3.00	3.28	2.86	1.80	1.81	4.24	3.80	4.40	2.69	3.10	2.81	3.12	2.18	2.57	2.14
2C	3.61	3.57	3.13	1.76	2.05	4.22	3.71	5.16	3.32	3.66	3.26	3.56	2.30	2.84	2.52
3D	2.69	2.78	2.78	1.48	1.84	4.09	2.91	3.47	2.52	4.42	2.83	5.14	2.24	3.31	2.44
4A	4.43	3.90	2.72	2.90	3.73	3.81	4.19	3.29	2.70	3.20	3.88	6.56	2.52	3.25	3.08
5A	3.58	3.21	2.39	2.08	2.40	2.52	2.56	2.39	2.70	3.13	3.34	3.65	3.16	4.21	3.08
6E	2.79	2.77	2.37	1.60	1.82	4.29	3.67	3.92	2.21	2.24	2.02	2.37	1.99	2.28	2.26
7A	2.69	2.30	2.20	2.15	2.29	3.13	3.16	2.92	2.28	2.54	2.56	2.90	2.71	3.50	3.21
7C	2.28	2.10	1.54	1.60	1.85	2.21	2.43	2.17	1.46	1.75	1.64	2.34	2.13	2.41	2.28
8G	2.49	2.46	1.75	1.59	1.86	2.56	2.55	2.25	1.37	1.87	1.65	1.94	1.53	1.87	1.82
8H	4.04	4.19	3.06	2.26	2.64	3.94	4.04	4.17	3.37	2.98	3.63	3.11	2.27	2.65	2.64
9A	3.25	3.04	2.42	2.25	2.75	3.11	3.23	3.21	1.95	2.21	2.70	3.43	2.75	3.59	2.82
10I	2.82	2.75	2.30	1.58	1.76	2.42	2.44	3.25	1.81	1.64	1.81	1.98	1.50	1.81	1.76
11C	1.96	1.56	0.69	1.48	1.54	1.18	1.22	0.79	0.52	1.19	0.64	2.64	1.85	2.08	1.88
12H1	3.32	3.02	2.29	2.01	2.35	2.96	2.97	2.59	2.35	2.69	2.45	2.53	2.26	2.44	2.93
13C	5.19	4.78	4.63	2.67	2.64	4.91	4.16	5.71	4.59	3.74	3.91	4.80	2.15	2.89	2.95
14C	2.05	1.63	1.47	1.17	1.31	2.21	1.77	2.09	2.22	2.26	2.43	1.54	1.10	1.02	1.86
15M	3.15	3.45	2.99	1.76	2.09	4.80	3.97	5.03	3.26	3.74	3.59	4.10	2.76	3.26	2.92
16M	3.51	3.91	3.25	1.76	2.03	6.13	4.93	6.44	3.75	4.78	4.32	4.77	3.03	3.55	3.27
17C	4.29	4.33	3.73	2.19	2.44	5.36	3.85	4.39	5.54	5.43	5.94	6.79	2.94	3.68	3.78
18C	3.03	4.98	3.03	2.68	2.18	4.06	4.73	4.51	5.01	3.88	3.64	2.81	2.04	2.61	1.77

Data from 7F not included

Table 11. Recovery for IS 00/588 (assigned potency 33 IU/ml) for quantitative assays

Method[1]	Assay	Dose	N	Mean	sd[2]	cv[3]	Min	Median	Max	95%-CI[4]		R%[5]	95%-CI[6]	
A	12A	33	6	45.2	2.8	6.2	41.6	46.2	48.5	42.2	48.1	137%	128%	146%
		165	6	50.1	4.5	9.0	44.6	50.3	56.1	45.3	54.8	152%	137%	166%
		825	6	50.9	3.4	6.6	49.5	49.5	57.8	47.3	54.4	154%	143%	165%
	12H	6.6	6	42.0	2.0	4.7	38.7	42.6	44.0	39.9	44.1	127%	121%	134%
		33	6	43.9	2.3	5.3	40.6	44.4	47.2	41.4	46.3	133%	126%	140%
		165	6	47.0	3.1	6.6	42.9	47.0	51.2	43.8	50.3	143%	133%	152%
		825	6	50.9	6.2	12.2	41.3	49.5	57.8	44.4	57.4	154%	134%	174%
	12J	6.6	6	43.4	6.3	14.5	37.9	40.7	54.9	36.8	50.0	131%	111%	151%
		33	6	46.0	5.9	12.9	40.3	44.6	55.8	39.8	52.2	139%	121%	158%
		165	6	46.8	4.5	9.6	41.3	47.0	52.8	42.0	51.5	142%	127%	156%
		825	6	56.4	9.6	17.1	49.5	53.6	74.3	46.3	66.5	171%	140%	202%
	12K	6.6	6	41.1	2.0	4.9	37.9	41.0	43.4	39.0	43.2	124%	118%	131%
		33	6	43.6	4.9	11.3	37.0	46.5	47.2	38.5	48.8	132%	117%	148%
		165	6	46.5	6.8	14.6	36.3	49.5	52.8	39.3	53.6	141%	119%	162%
		825	6	50.9	8.1	15.9	41.3	53.6	57.8	42.4	59.4	154%	128%	180%
	12L	6.6	6	41.7	1.4	3.5	40.2	41.2	44.4	40.2	43.2	126%	122%	131%
		33	6	44.7	2.1	4.7	40.9	45.4	46.5	42.5	46.9	135%	129%	142%
		165	6	52.3	2.0	3.8	51.2	51.2	56.1	50.2	54.3	158%	152%	165%
		825	6	53.6	4.5	8.4	49.5	53.6	57.8	48.9	58.4	163%	148%	177%
	12C	16.5	6	40.3	1.7	4.2	38.9	39.4	42.9	38.6	42.1	122%	117%	128%
		33	6	41.0	2.3	5.5	38.9	40.1	44.9	38.7	43.4	124%	117%	132%
		165	6	41.8	3.1	7.3	38.0	41.3	46.2	38.6	45.0	127%	117%	136%
		825	6	48.1	6.2	12.9	41.3	49.5	57.8	41.6	54.6	146%	126%	166%
B	19N	6.6	6	26.9	0.4	1.6	26.2	26.9	27.5	26.4	27.3	81%	80%	83%
		33	6	27.5	0.7	2.7	26.7	27.6	28.4	26.7	28.3	83%	81%	86%
		165	6	28.6	1.3	4.7	26.4	28.9	29.7	27.2	30.0	87%	82%	91%
		825	6	24.8	0.0	0.0	24.8	24.8	24.8			75%		

[1]A, Abbott Architect HBsAg Quantitative; B, HISCL HBsAg Assay; [2]standard deviation; [3]Coefficient of variation; [4]95%-Confidence Interval for the Mean; [5]Mean recovery (%); [6]95%-Confidence Interval for the Recovery (%).

Table 12. Recovery for IS 00/588 (assigned potency 33 IU/ml) for quantitative assays (mean value for all dilutions)

Method[1]	Assay	N	Mean	sd[2]	cv[3]	Min	Median	Max	95%-CI[4]		R%[5]	95%-CI[6]	
A	12H	24	45.9	4.9	10.7	38.7	44.6	57.8	43.9	48.0	139%	133%	146%
	12A	18	48.7	4.3	8.8	41.6	49.0	57.8	46.6	50.8	148%	141%	154%
	12C	24	42.8	4.7	11.0	38.0	41.3	57.8	40.8	44.8	130%	124%	136%
	12J	24	48.1	8.1	16.9	37.9	47.9	74.3	44.7	51.6	146%	135%	156%
	12K	24	45.5	6.7	14.6	36.3	44.8	57.8	42.7	48.3	138%	129%	146%
	12L	24	48.1	5.7	11.9	40.2	48.0	57.8	45.6	50.5	146%	138%	153%
	all	138	46.4	6.2	13.3	36.3	45.9	74.3	45.4	47.5	141%	138%	144%
B	19N	24	26.9	1.6	6.0	24.8	26.9	29.7	26.2	27.6	82%	80%	84%

[1]A, Abbott Architect HBsAg Quantitative; B, HISCL HBsAg Assay; [2]standard deviation; [3]Coefficient of variation; [4]95%-Confidence Interval for the Mean; [5]Mean recovery (%); [6]95%-Confidence Interval for the Recovery (%).

Table 13. Mean potencies (IU/ml) relatively to IS 00/588 (The lower 6 rows represent the results from the quantitative HBsAg assays)

Sample / Assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1A	105.0	101.3	93.7	67.1	70.5	117.1	94.6	112.5	93.1	94.8	102.1	93.4	71.2	94.1	77.7
2B	102.1	109.2	97.1	62.3	63.8	137.0	125.4	147.4	91.1	104.0	93.8	103.2	75.2	87.4	73.1
2C	112.2	112.6	100.5	57.4	66.9	134.2	119.9	160.0	105.9	116.5	105.4	114.1	77.1	92.8	81.2
3D	88.1	93.0	93.2	53.3	61.8	130.5	97.3	114.7	82.6	138.8	94.2	155.1	75.5	108.1	81.5
4A	122.4	111.5	77.6	82.0	102.7	104.7	113.0	94.4	76.3	89.8	99.0	151.0	76.9	96.1	86.9
5A	82.8	73.3	63.4	57.2	66.3	66.7	72.7	57.6	62.4	72.0	64.3	80.0	73.6	90.8	80.8
6E	94.3	100.3	88.8	58.4	67.6	145.4	126.2	140.4	77.5	79.7	74.0	84.6	72.7	85.6	82.3
7A	74.8	65.6	60.3	55.4	68.1	75.1	78.8	72.1	58.1	66.8	57.7	73.4	73.1	90.9	83.2
7C	75.1	69.2	48.1	50.2	60.7	71.4	83.8	65.1	44.1	57.9	54.7	77.6	68.3	78.7	76.3
8G	81.7	81.3	58.7	48.9	60.9	84.0	86.9	79.4	47.6	64.6	56.3	70.7	54.0	63.8	60.3
8H	109.3	108.3	78.4	63.0	73.4	120.0	116.8	106.2	76.0	87.2	81.6	97.2	67.3	80.0	80.4
9A	89.7	82.6	68.4	63.2	78.0	85.5	85.5	85.0	56.0	61.4	69.8	88.8	77.2	95.8	77.1
10I	93.7	96.5	81.0	51.9	59.2	86.2	82.0	107.4	59.8	57.9	62.0	67.1	52.3	63.8	61.8
11C	54.9	40.8	19.9	44.7	48.0	35.5	37.6	23.4	15.1	35.5	19.4	82.2	58.2	66.1	58.6
12H1	102.6	98.5	81.5	67.3	78.2	95.3	95.8	92.4	82.7	88.0	82.6	87.9	77.7	89.8	98.3
13C	187.4	174.2	166.2	96.3	97.4	170.4	141.5	216.8	160.2	143.7	146.2	170.0	83.4	100.1	111.0
14C	79.1	65.1	57.6	42.6	49.9	76.1	64.6	77.8	76.9	81.5	85.0	51.1	34.1	37.4	59.3
15M	104.0	115.6	98.7	54.2	64.4	164.2	133.0	165.5	107.9	123.6	119.4	137.1	90.9	106.3	97.0
16M	115.6	127.7	104.8	52.7	64.5	195.9	161.9	204.3	124.5	151.7	140.0	161.4	98.6	115.2	107.0
17C	148.2	149.9	135.3	79.0	90.9	186.3	136.6	156.6	184.0	193.4	224.1	238.4	113.6	136.0	130.0
18C	118.8	155.0	121.3	88.7	82.3	154.5	152.9	154.1	129.8	136.0	119.2	96.6	79.5	84.9	64.5
7F	85.0	75.9	57.2	59.9	70.8	70.8	84.0	68.3	53.1	66.7	56.8	84.6	75.5	88.5	85.2
12A	93.1	85.5	80.7	62.2	70.0	91.9	88.4	93.0	81.5	82.1	83.4	86.2	82.3	99.9	86.8
12C	81.4	78.2	69.5	62.4	72.2	78.1	84.7	81.2	64.0	73.4	72.5	83.2	76.0	90.7	86.8
12J	70.0	63.6	56.1	50.5	61.9	66.5	62.3	66.2	51.5	54.6	52.6	62.1	67.8	80.7	76.8
12K	66.4	61.4	52.0	48.3	58.2	56.2	58.2	55.7	47.5	60.1	47.9	60.9	62.7	75.9	70.2
12L	80.4	78.3	69.8	56.1	64.6	71.6	68.2	70.8	59.7	67.0	65.8	72.1	69.9	83.1	72.1
19N	155.1	149.3	122.4	101.2	111.8	174.0	163.2	162.4	111.7	123.4	130.8	190.5	118.7	154.6	164.2

Table 14. Overall laboratory mean relative potencies (IU/ml) for quantitative and qualitative HBsAg assays (28 data sets)

Sample	**Mean**[1]	95%-CI[2]		Min	Max
1	**99.0**	87.4	110.7	54.9	187.4
2	**97.3**	84.2	110.3	40.8	174.2
3	**82.2**	69.9	94.5	19.9	166.2
4	**62.0**	56.0	68.1	42.6	101.2
5	**70.9**	64.9	76.9	48.0	111.8
6	**109.1**	89.3	129.0	35.5	195.9
7	**100.6**	87.2	114.0	37.6	163.2
8	**108.4**	87.3	129.5	23.4	216.8
9	**81.5**	66.5	96.4	15.1	184.0
10	**91.5**	75.9	107.1	35.5	193.4
11	**87.9**	71.5	104.2	19.4	224.1
12	**104.3**	86.0	122.6	51.1	238.4
13	**75.1**	68.2	82.0	34.1	118.7
14	**90.6**	81.6	99.6	37.4	154.6
15	**84.7**	75.5	93.8	58.6	164.2

[1]mean estimated relative potency; [2]95%-confidence interval for mean potency.

Table 15. Geometric coefficients of Variation (GCV) for quantitative and qualitative assays (estimated from relative potencies) as percentage for overall uncertainty, inter-laboratory precision (i.e. variance between participants), inter-method precision (i.e. variance between different assay methods), and intra-assay precision (or repeatability / reproducibility; i.e. precision, if same method in same laboratory was repeated)

Sample	Uncertainty (GCV%)	Inter-Laboratory Precision	Inter-Method Precision	Intra-Assay Precision
1	21.8%	7.7%	18.2%	8.9%
2	26.4%	7.7%	22.7%	10.6%
3	33.4%	8.9%	30.4%	9.9%
4	18.8%	4.2%	14.8%	10.7%
5	17.7%	n.e.	13.3%	11.5%
6	32.2%	9.5%	28.8%	10.1%
7	27.7%	9.7%	23.4%	10.8%
8	37.9%	9.0%	34.7%	11.3%
9	41.3%	13.0%	38.2%	6.9%
10	30.7%	10.1%	27.9%	7.0%
11	37.9%	12.5%	34.8%	6.4%
12	29.3%	10.0%	26.7%	5.9%
13	20.0%	3.5%	18.1%	7.6%
14	21.5%	5.1%	20.0%	5.8%
15	19.3%	7.9%	16.9%	4.7%

n.e., not estimable.

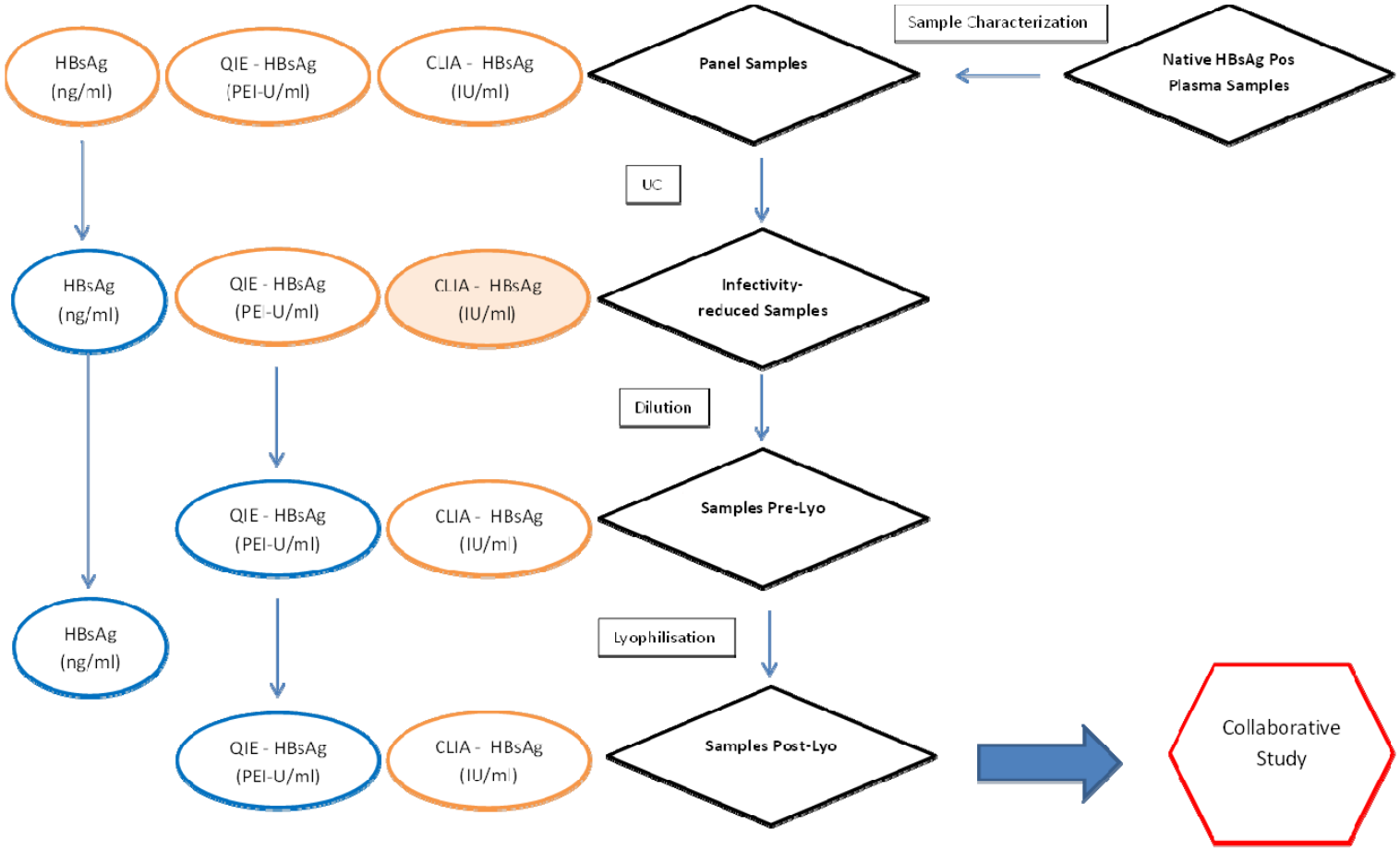


Figure 1. Flowchart of sample processing.
CLIA-HBsAg, quantitative chemiluminescent immunoassay (ARCHITECT Quantitative, Abbott); QIE-HBsAg, quantitative immunoelectrophoresis (Laurell); HBsAg, biochemical purification of HBsAg; UC, ultracentrifugation; Lyo, Lyophilisation; Blue oval symbols, HBsAg values based on assumptions and calculation from the dilution step.

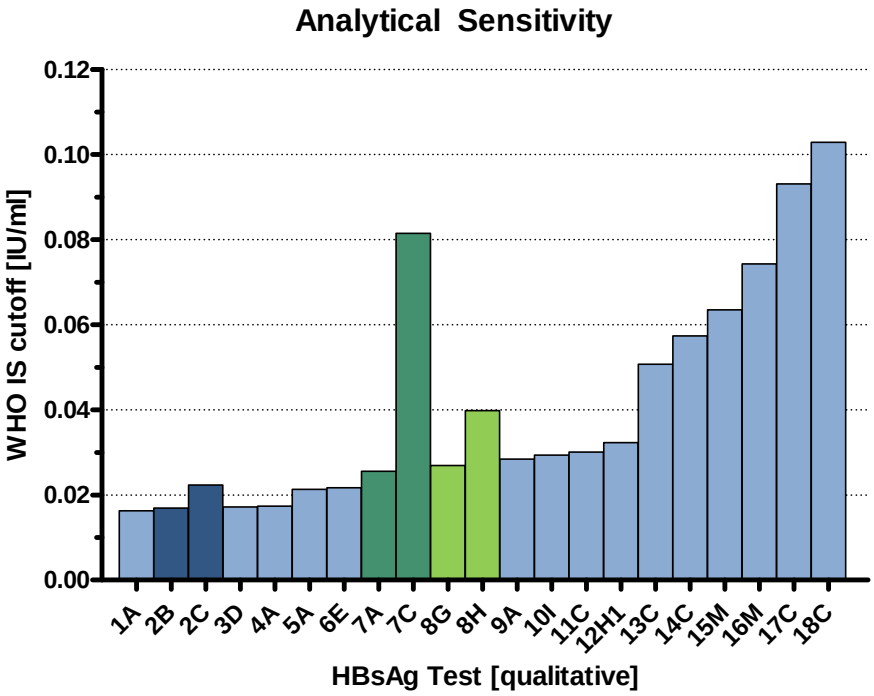


Figure 2. Analytical sensitivity (IU/ml)

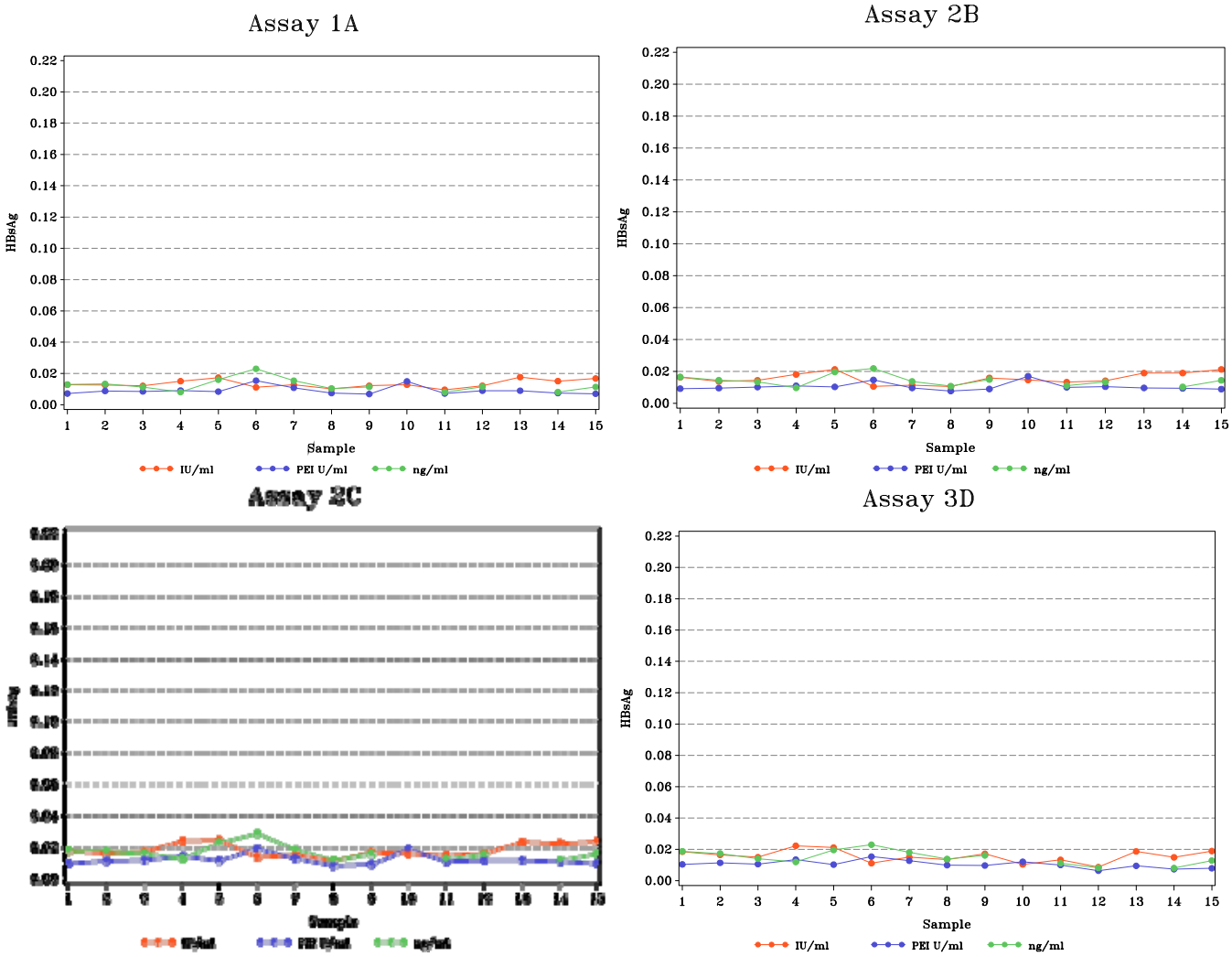
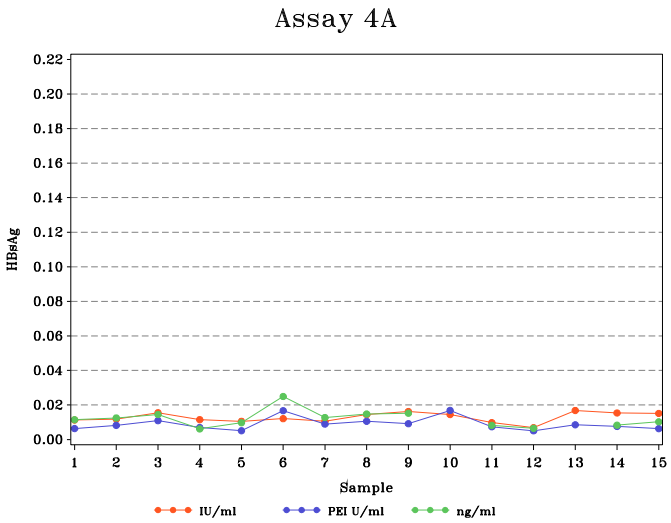
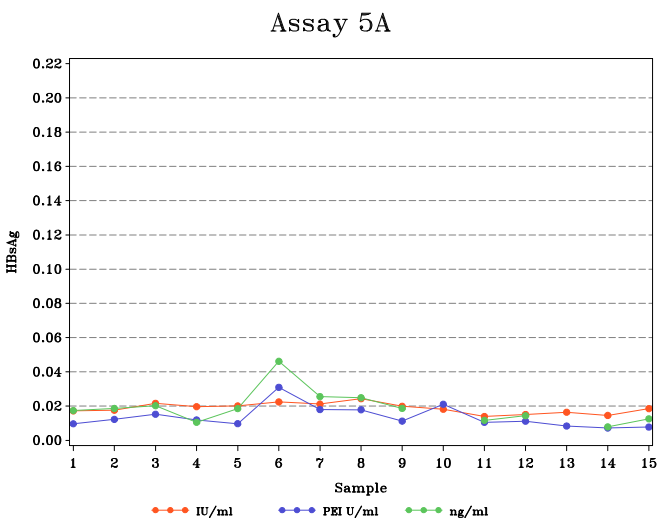
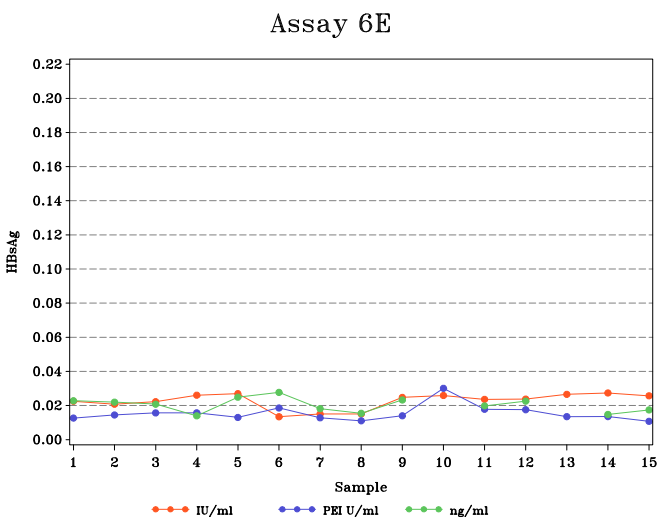


Figure 3. Efficacy of HBsAg tests to detect different HBV genotypes (Figures from Table 9).


Assay 4A
HBsAg
Sample
IU/ml
PEI U/ml
ng/ml


Assay 5A
HBsAg
Sample
IU/ml
PEI U/ml
ng/ml


Assay 6E
HBsAg
Sample
IU/ml
PEI U/ml
ng/ml

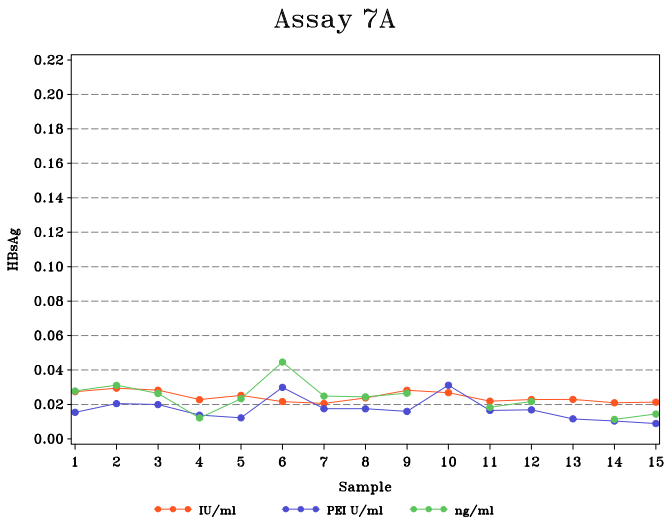

Assay 7A
HBsAg
Sample
IU/ml
PEI U/ml
ng/ml

Figure 3. (Cont'd.)

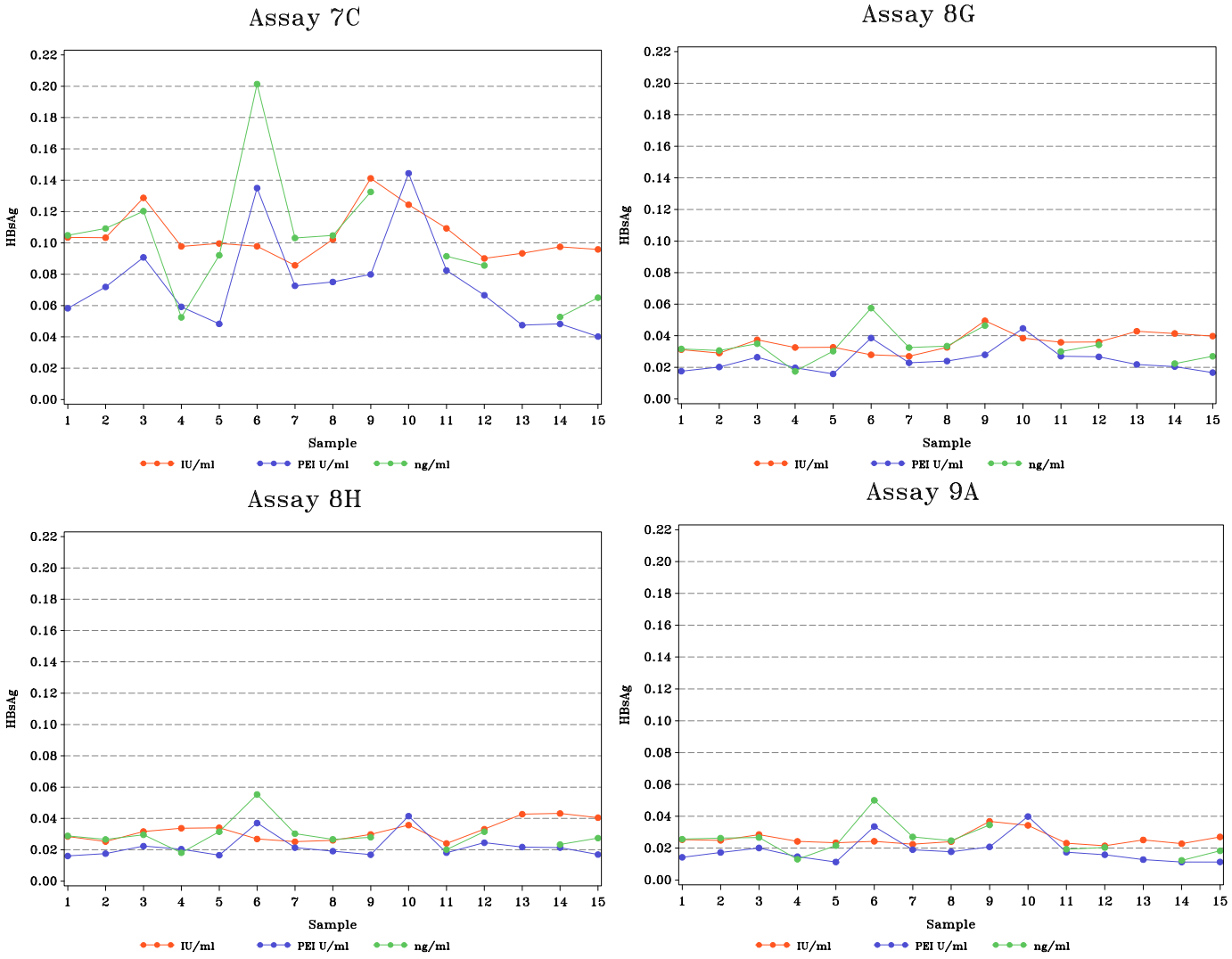
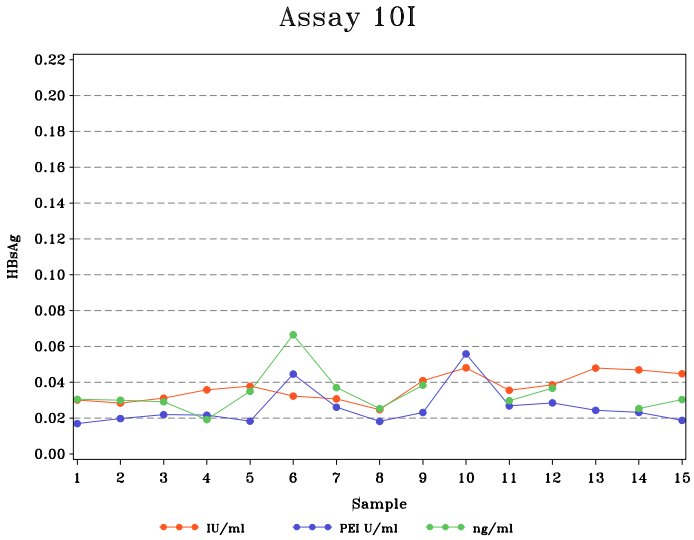
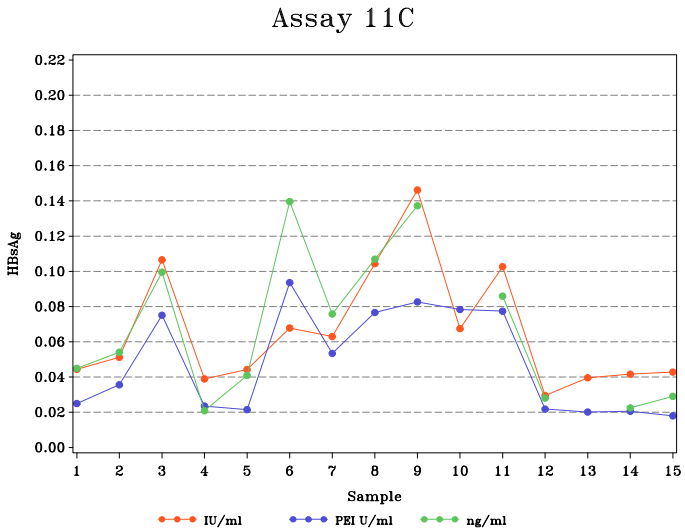
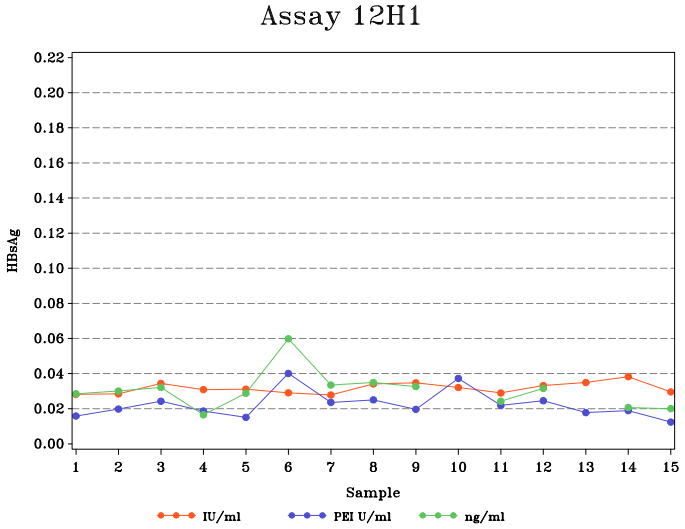


Figure 3. (Cont'd.)







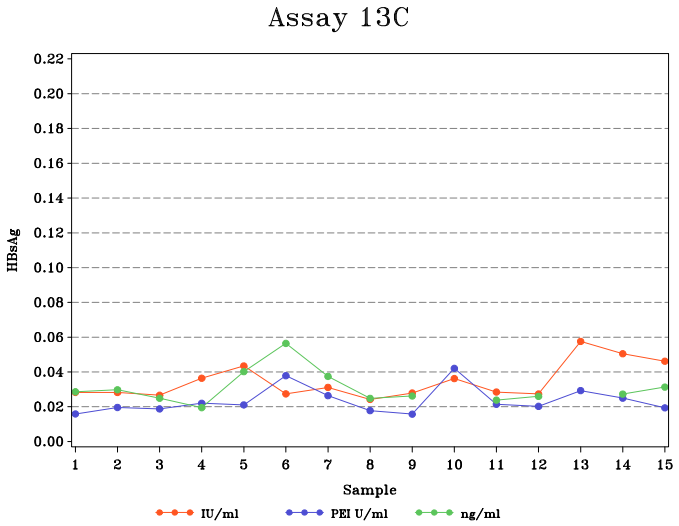


Figure 3. (Cont'd.)

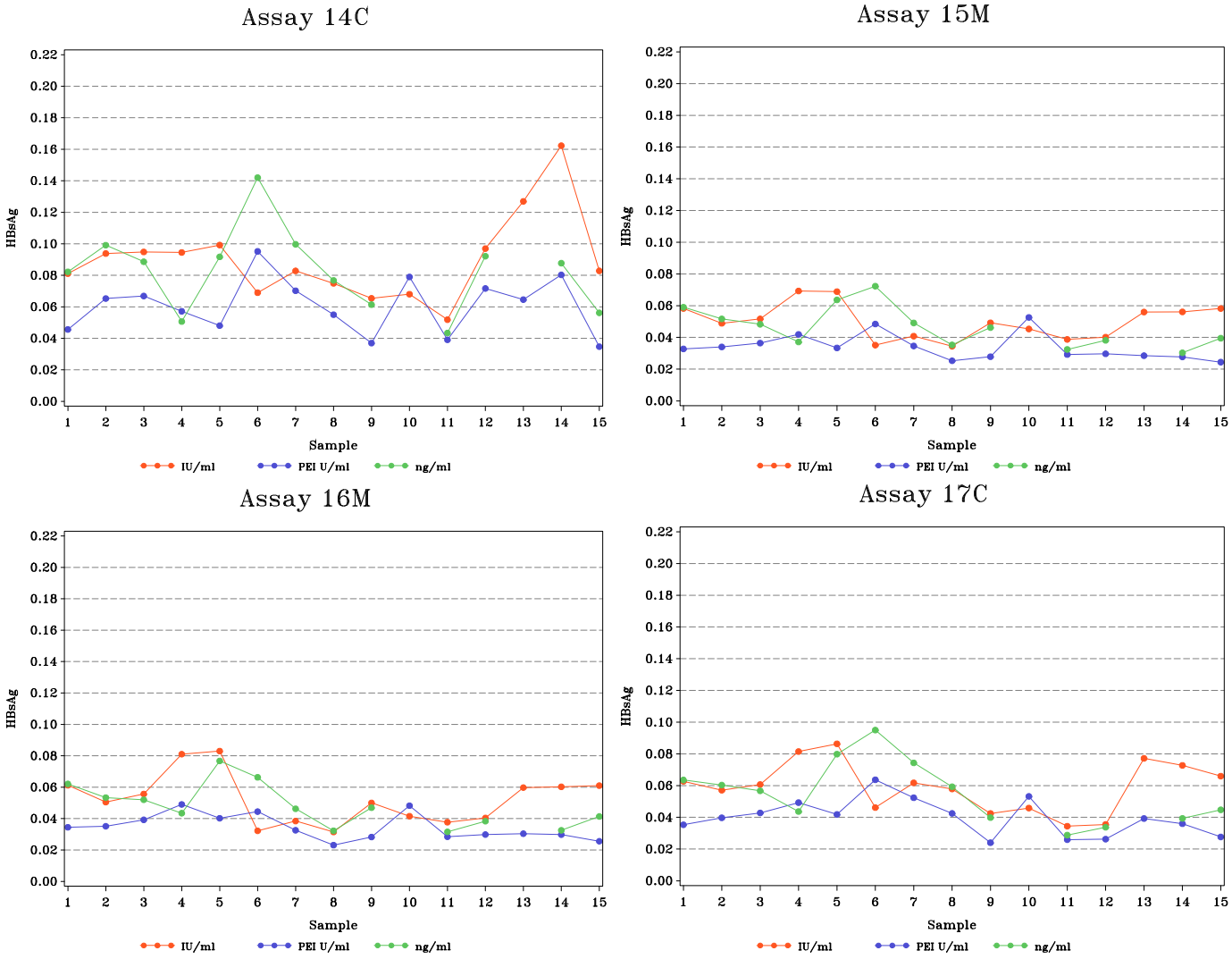


Figure 3. (Cont'd.)

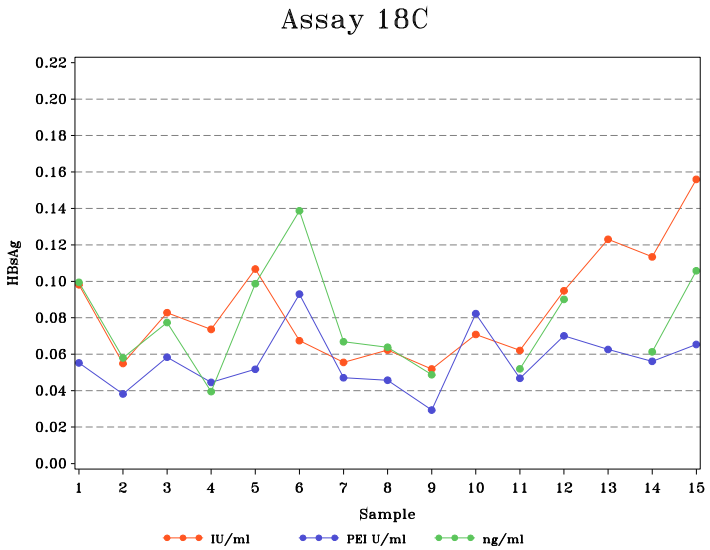


Figure 3. (Cont'd.)

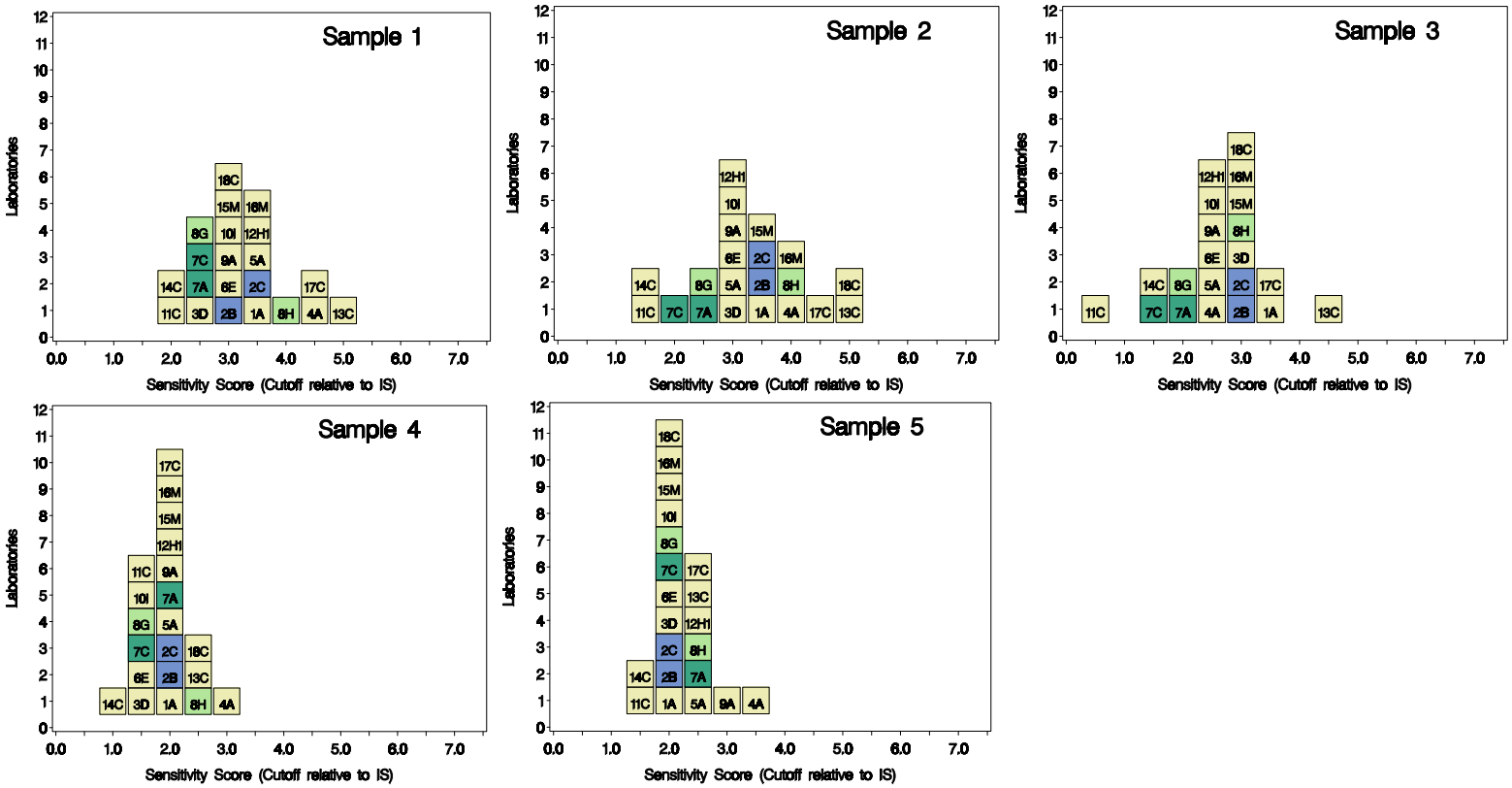


Figure 4. Dilution sensitivity score at S/CO 0f 1.00 of HBsAg tests for different HBV genotypes relatively to the IS 00/588

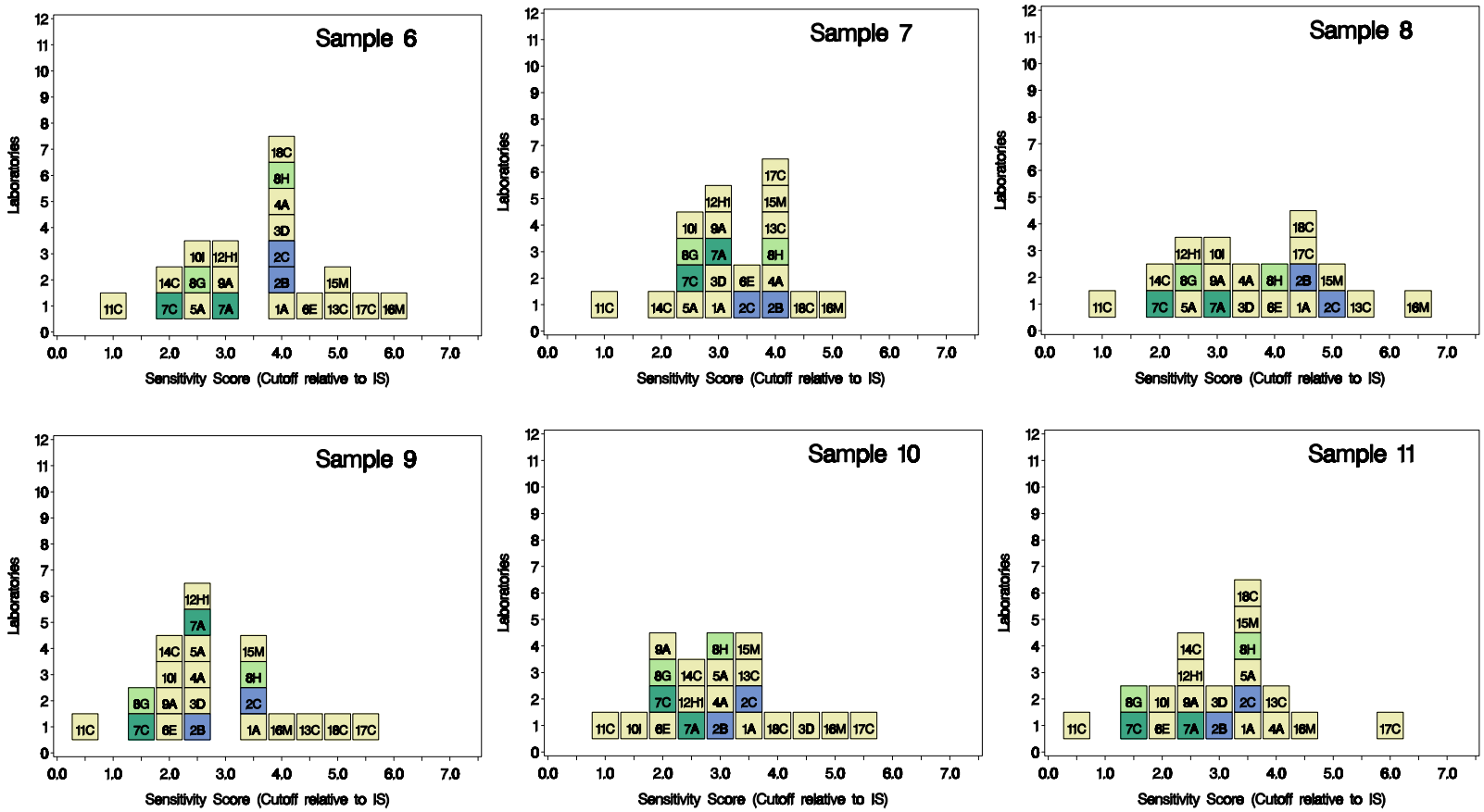


Figure 3. (Cont'd.)

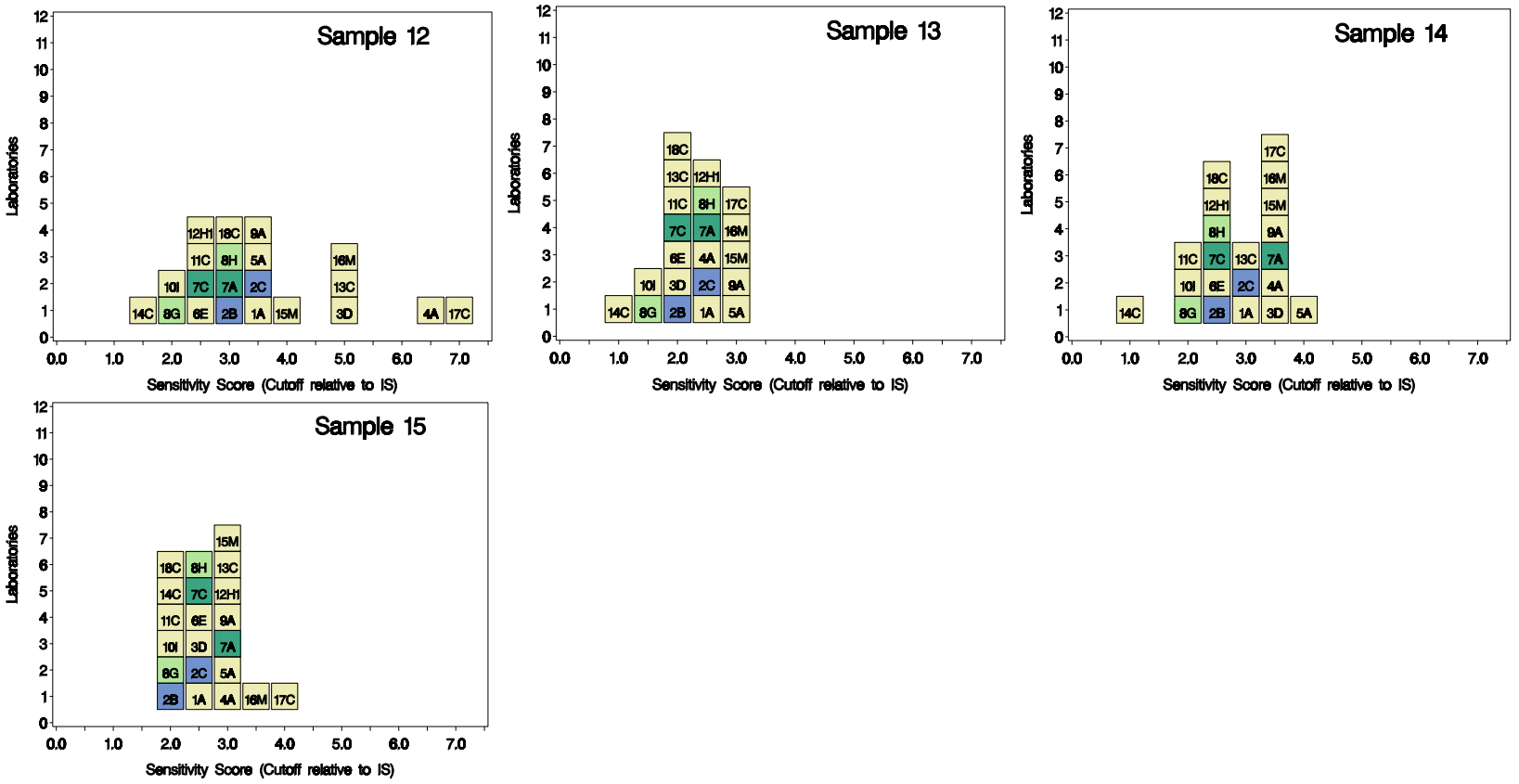


Figure 4. (Cont'd.)

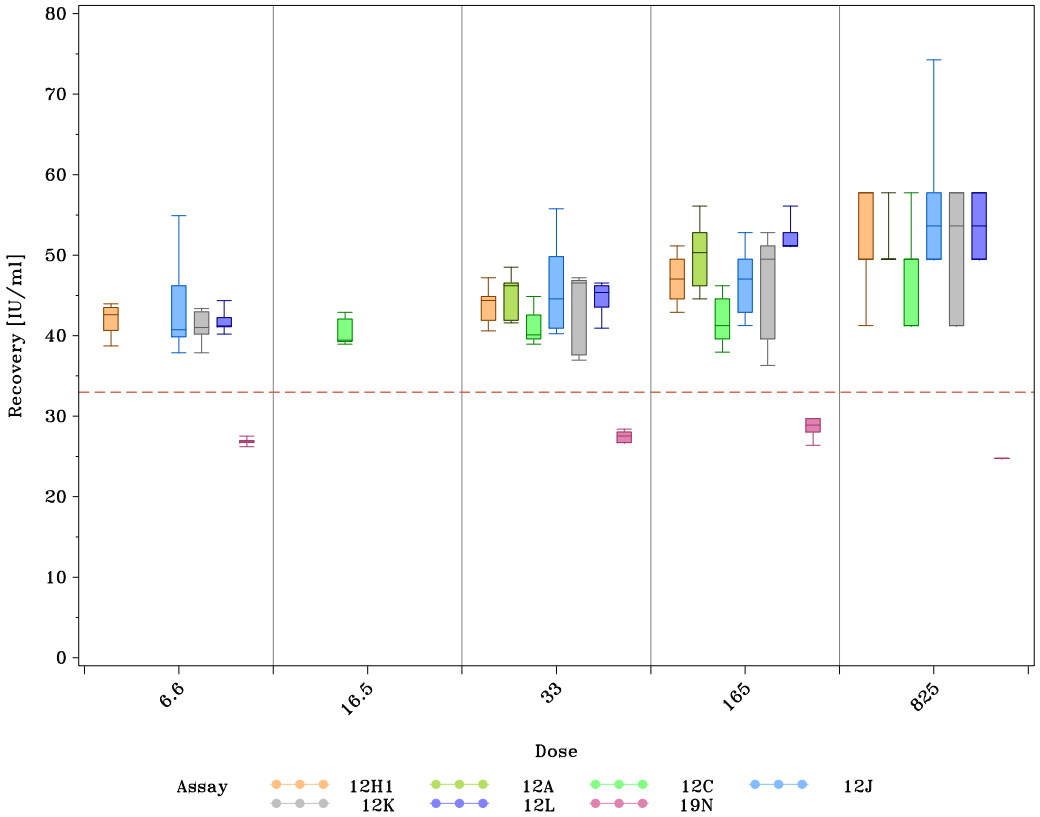


Figure 5. Box-Plot for recovery of 2nd IS HBsAg 00/588 (dashed line: assigned potency 33 IU/ml) depending upon assay kit and dose (quantitative assays; A=Abbott Architect HBsAg Quantitative, B= Sysmex Corporation / HISCL HBsAG Assay Kit)

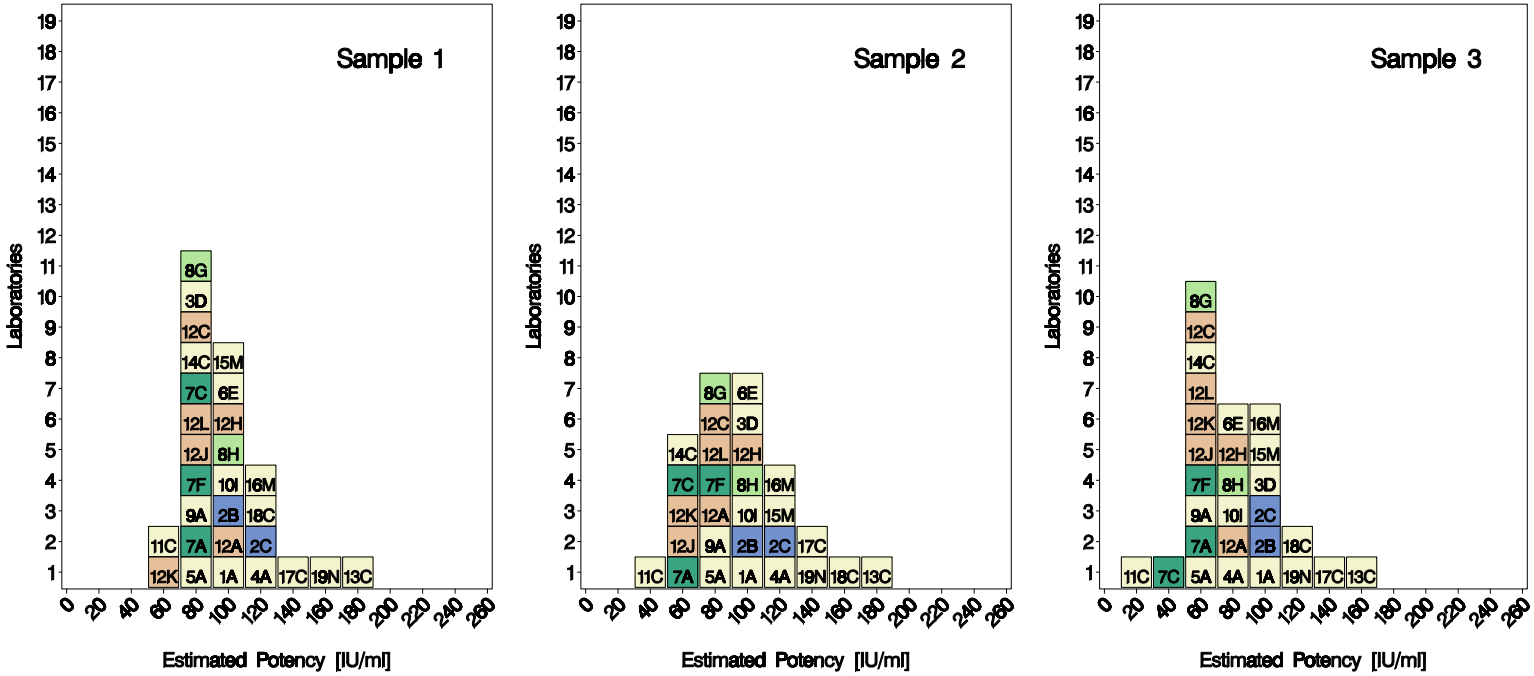
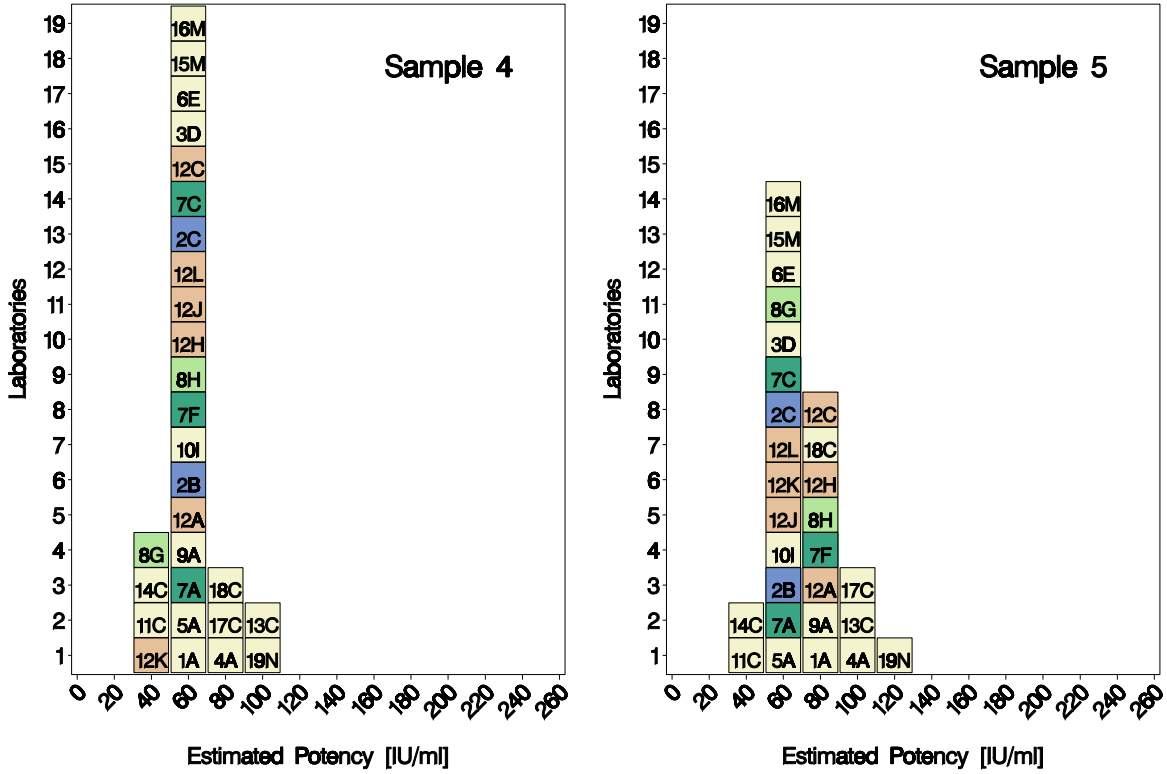


Figure 6. Mean potencies (IU/ml) relatively to concurrent tested IS 00/588



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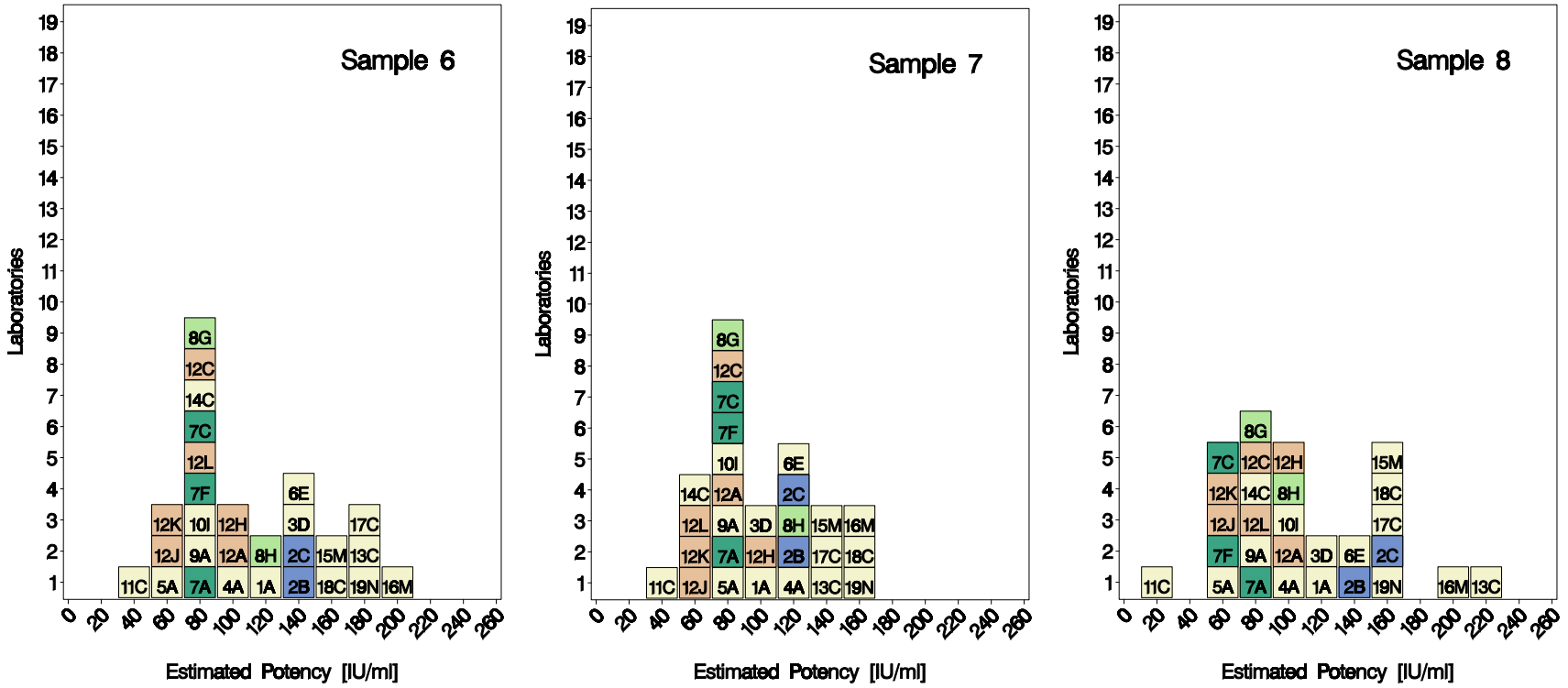
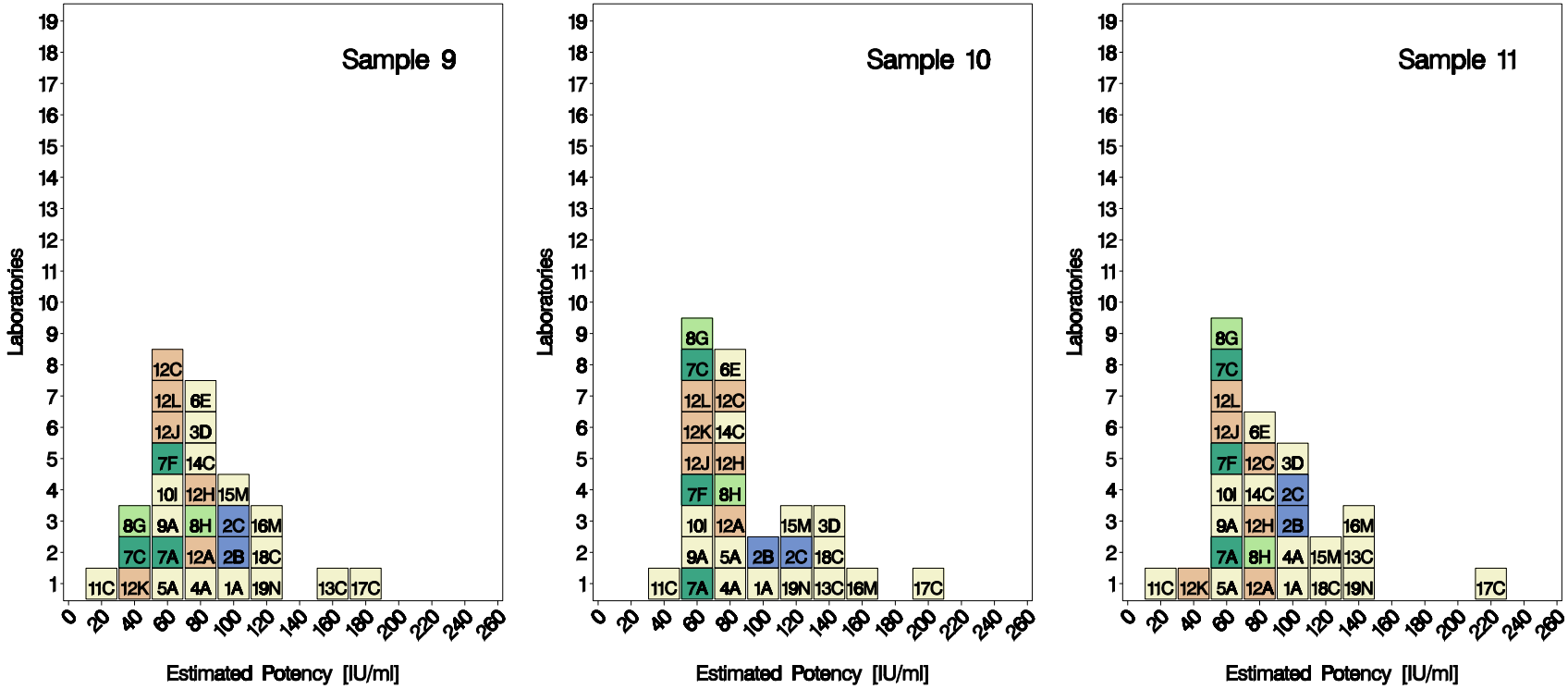


Figure 6. (Cont'd.)



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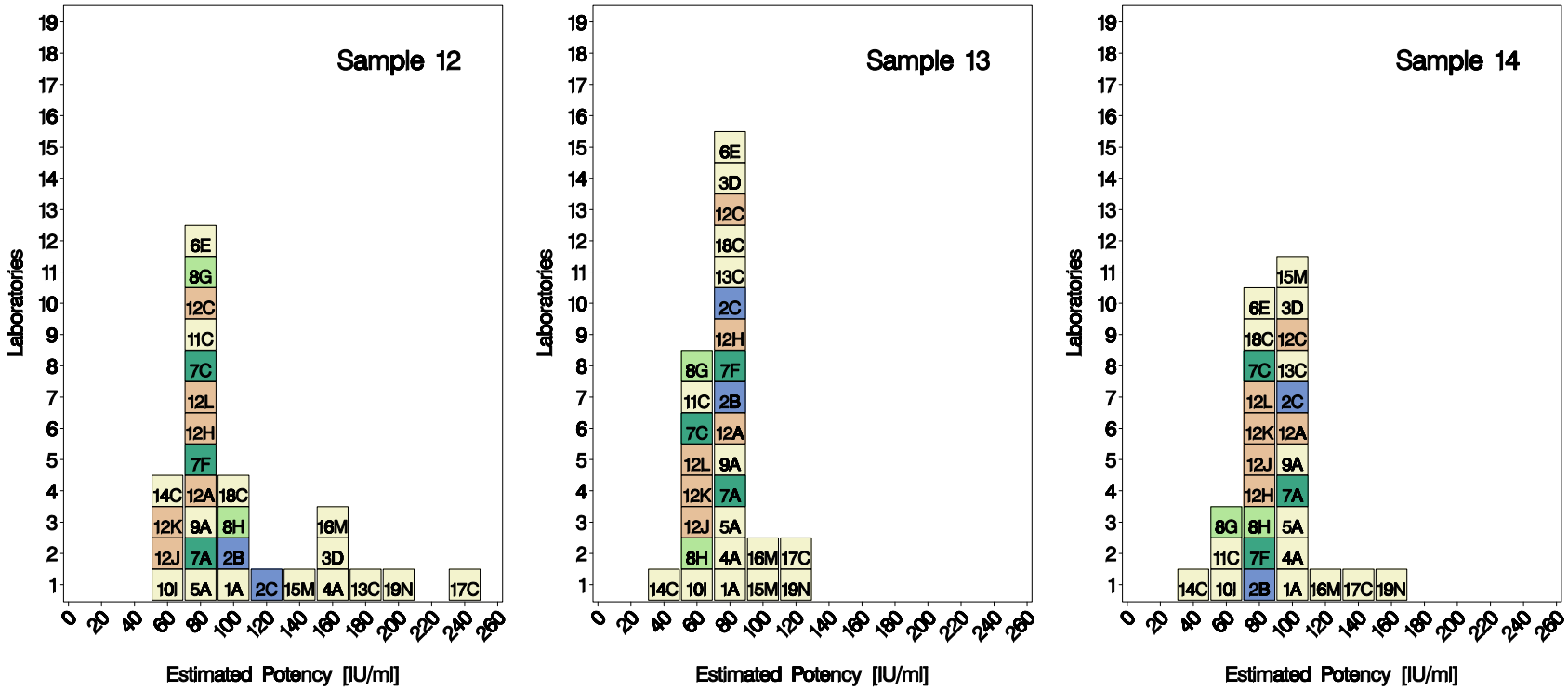

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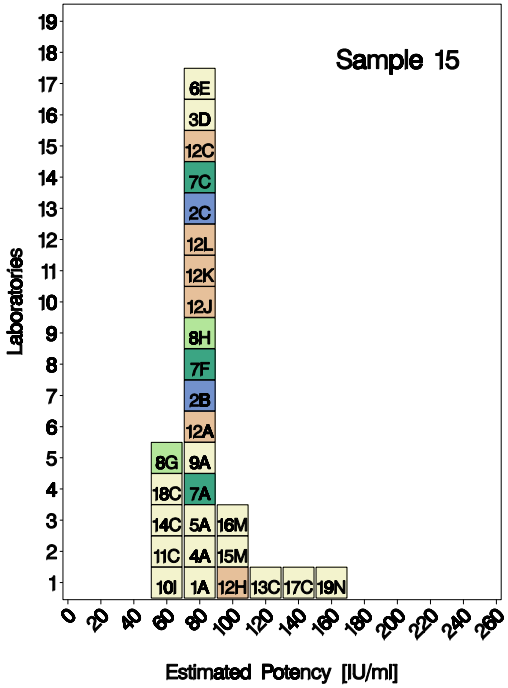

Figure 6. (Cont'd.)

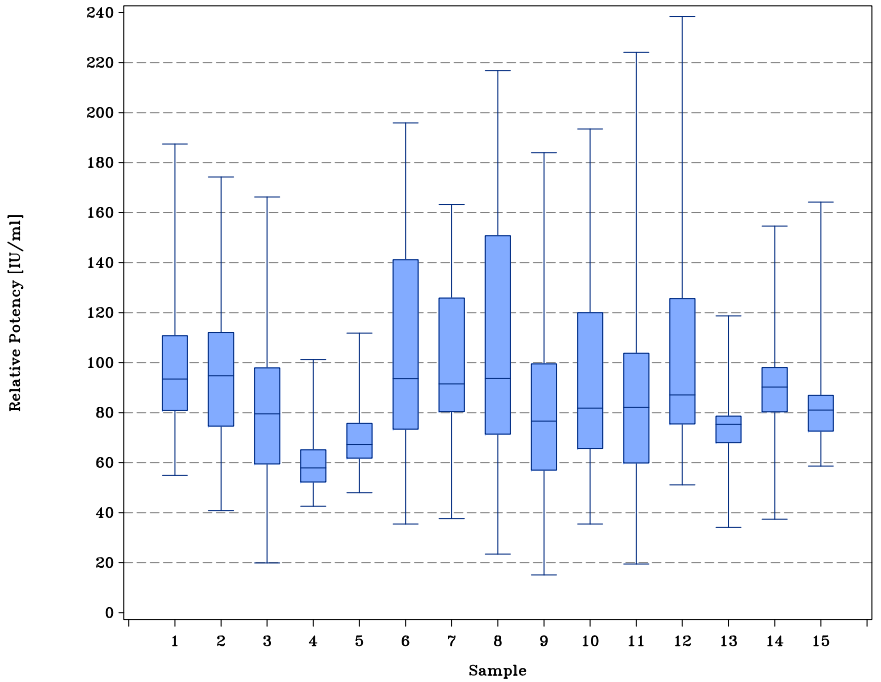

Figure 7. Box-plot of mean potencies (IU/ml) relatively to concurrent tested IS 00/588 for all panel samples

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Appendix 1 Collaborative Study Protocol

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg Tests)

- STUDY PROTOCOL -

Objective

The purpose of the Collaborative Study is to evaluate a plasma panel of different Hepatitis B Virus (HBV) genotypes using HBsAg tests. The study includes the parallel testing of the 2nd International Standard for HBsAg (00/588).

Background

During the 'WHO Consultation on Global Measurement Standards and their use in the in vitro Biological Diagnostic Field' in June 2004 concern was raised that HBsAg test kits and NAT test kits for the detection of HBV DNA might be less efficient for some HBV genotypes other than A2. HBV genotype A2 is the genotype represented by the current WHO International Standard both for HBsAg and for HBV DNA. The Paul-Ehrlich-Institut (PEI), as one of the three WHO Collaborating Centres involved in the Biological Standardization Programme, proposed projects to establish WHO International Reference Panels for HBV DNA and for HBsAg representing different HBV genotypes. The projects were endorsed and assigned as a high priority by the WHO Expert Committee on Biological Standardization in October 2005.

The candidate HBV genotype panel intended for use with HBsAg tests has now been prepared (lyophilised material). It consists of 15 members and covers the most prevalent HBV genotypes (A-F and H) and the respective HBsAg subtypes.

The study is designed to test the panel samples (15 lyophilised preparations) concurrently with the WHO International Standard for HBsAg (00/588).

Materials

Fifteen HBsAg positive lyophilised plasma preparations, representing HBV genotypes A – F and H, and the 2nd WHO International Standard for HBsAg (00/588), previously assigned a unitage of 33 IU/vial. The fifteen panel members have been coded Sample1 to Sample 15.

CAUTION

These preparations contain material of human origin and infectious HBV. These preparations should be regarded as potentially hazardous to health. They should be used and discarded according to your own laboratory safety procedures. Care should be exercised in opening vials to avoid cuts.

Study design

Participants will be sent three vials of each study sample preparation. All samples should be stored frozen at -20 ºC on receipt. Samples 1 to 15 are lyophilised preparations in rubber stoppered, 4-ml screw-cap glass vials. The 2nd WHO International Standard 00/588 is a lyophilised preparation (for details see attached package insert).

Each vial of the Samples 1 to 15 should be reconstituted with 0.5 ml of distilled water and left for a minimum of 20 minutes with occasional agitation before use. The International Standard 00/588 should be reconstituted with 1.0 ml distilled water.

The assay methods (as mentioned before in the questionnaire by the participating laboratories) should be performed according to the instructions for use of the respective manufacturer. If there is any deviation from the instructions in the laboratory, please justify and describe. Participants are requested to perform testing of these samples in three independent assay runs (for details see below). A fresh vial of each sample should be used in each assay run. All dilutions should be carried out in the diluent (dilution matrix) normally used in the assay system and this should be recorded on the result form (e. g. normal human serum negative for HBsAg and anti-HBs).

From a former feasibility study it is known that the dilution ranges for the Samples 1 to 15 given in the result´s sheet below should be within the detection range of all assays and

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should include the endpoint titer (intercept with the cut-off of the assays). Please recognize that samples 1-15 follow a common dilution scheme which is different from the scheme for the WHO IS HBsAg (00/588). The dilution matrix should be tested in parallel as a control. The dilutions of the study samples are requested to be tested in each HBsAg assay in duplicates independently on 3 three different days. However, if in Run 1 the end point will not be reached with the highest dilution of the Study Samples and/or the International Standard 00/588 the participants are requested to test a further 1:5 dilution (Samples 1-15: additional dilution 1:62,500; and for the WHO 00/588: additional dilution 1:20,625) in Run 2 and Run 3.

Evaluation of results

Test results should be recorded on the appropriate results form (see below pages 4 to 10). Assay response should be reported for qualitative tests in sample/cut-off values, and for quantitative tests in IU/ml.

Please indicate on each 'Reporting Sheet' the Laboratory and Name of Investigator. All completed forms should be returned preferably by email by May 7th, 2010.

The statistical evaluation will be performed by the PEI. A draft study report will be prepared and distributed to all participants for comments. The draft report will only be sent to the study participants. The final study report will have to be submitted to the WHO Expert Committee on Biological Standardization in July 2010.

Contact address

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Tel: +49 6103 77 3307; Fax: +49 6103 77 1280; Email: chumi@pei.de

Attachments

- Att 1 - Instructions for Use for 00/588
- Att 2 - Important Notice – Storage Conditions of the Study Samples
- Att 3 - Package Receipt Form

Important

PLEASE CONFIRM RECEIPT OF THE SAMPLES BY FAX OR EMAIL ON THE ENCLOSED "PACKAGE RECEIPT FORM".

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg tests)

Method Reporting Sheet

Laboratory: Name of Investigator: Address: Tel: Fax: E-Mail:

Assay kit name: (Manufacturer/kit name/version/cat. no.)	
Qual/Quant	
Date of test:	Run 1
	Run 2
	Run 3
Diluent used:	
Calculated cut-off value:	

Additional details and/or comments:

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Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg tests)

Data Reporting Sheet 1

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
Sample 1	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 2	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 3	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg tests)

Data Reporting Sheet 2

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
Sample 4	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 5	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 6	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						

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Data Reporting Sheet 3

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
Sample 7	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 8	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 9	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg tests)

Data Reporting Sheet 4

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
Sample 10	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 11	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 12	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						

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Data Reporting Sheet 5

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
Sample 13	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 14	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						
Sample 15	1:20						
	1:100						
	1:500						
	1:2,500						
	1:12,500						

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel (HBsAg tests)

Data Reporting Sheet 6

Laboratory:	Name of Investigator:

		Assay response: sample/cut-off (qual) or IU/ml (quant)					
Test Run		Run 1		Run 2		Run 3	
Reagent	Dilution						
IS 00/588	1:6.6						
	1:33						
	1:165						
	1:825						
	1:4,125						
Dil. matrix							
Cut-off value							

Comments: