World Health Organization

WHO/BS/09.2121
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 19 to 23 October 2009

Collaborative Study to Establish a World Health Organization International Genotype Panel for Hepatitis B Virus Nucleic Acid Amplification Technique (NAT) – Based Assays

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Summary
The aim of the collaborative study was to evaluate a panel of lyophilized plasma samples containing different genotypes of hepatitis B virus (HBV) for use in nucleic acid amplification technique-(NAT)-based assays. The HBV genotype panel (PEI code number 5086/08) comprises 15 different members, which represent genotypes A (3), B (3), C (3), D (3), E (1), F (1), and G (1). Each laboratory analyzed the panel samples in parallel to the 2nd WHO International Standard (IS) for HBV DNA (NIBSC code 97/750) representing HBV genotype A2. The study was performed on 3 separate occasions by quantitative NATs or on 4 separate occasions by qualitative NATs. The data were collated and analyzed at the Paul-Ehrlich-Institut (PEI). Seventeen laboratories from 12 countries participated in the study. A total of 19 sets of data were returned; 16 from quantitative NAT assays, 2 from qualitative NAT assays. One laboratory performed sequence and genotype analysis. The majority of NAT assays used were commercially available and based on real-time polymerase chain reaction (PCR). The results showed that the genotypes A–G were detected consistently by the majority of participants, although a small number of assays detected genotypes F and G less efficiently or not at all. Only few genotype B, C, and E samples were underquantified by two used methods. The finding that some NAT assays had reduced detection efficiency with some of the non-A2 genotypes proves the necessity of a well-characterized genotype panel in addition to the WHO IS. Residual moisture content was determined to be 0.82% in the final container. This indicates that the panel of lyophilized HBV positive plasma samples is very stable under normal conditions of storage, i.e., at -20ºC or below and is therefore suitable for long term use. On-going real-time stability studies of the panel members are in progress. 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 NAT-based assays. No unitage is assigned to individual panel members.

Introduction
Hepatitis B is a potentially life-threatening liver infection caused by HBV. It is a major global health problem and the most prevalent cause of liver cirrhosis and hepatocellular cancer. The virus is preferentially transmitted through contact with the blood or other body fluids of an infected person. 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 acute or chronic consequences of hepatitis B (1,2). Sensitive screening and accurate diagnostic assays play a crucial role for the prevention and in the management of the disease. The current WHO IS materials for HBV DNA and HBsAg were generated from genotype A2/HBsAg subtype *adw2*. These materials are widely used for standardization of diagnostic assays and for traceability of test results. This HBV genotype is mainly prevalent in Western Europe and in North America and represents only 1% of the worldwide HBV-infected population. The majority of the HBV-infected people living in or coming from the Mediterranean area, Africa and Asia have the genotypes A1, B, C, D, and E, whereas F and H originate from the Americas. The origin of genotype G is not clarified yet. 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 (3,4,5). The PEI, as one of the 3 WHO Collaborating Centres involved in the Biological Standardization Programme, proposed projects to establish WHO International Biological Reference Preparations for HBV DNA and for HBsAg representing different genotypes of HBV. The projects were endorsed and assigned as a high priority by the WHO Expert Committee on Biological Standardization (ECBS) in October 2005.

Comparison of the nucleotide sequence of the different HBV genotypes reveals various genome regions with high homology. As a consequence, the currently used NAT assays target different regions of the HBV genome, e. g. the preS-, S-, core- or X-gene region. In spite of the highly conserved target sequences for primers and probes, not all assays were equally good in previous collaborative studies (6).

The proposed HBV genotype panel intended for use with HBV NAT assays consists of 15 samples and covers the most prevalent HBV genotypes (A-G) collected worldwide. The collaborative study was designed to test the panel samples (15 lyophilized preparations) concurrently with the WHO 2nd IS (97/750). The study addressed the question of commutability of the current IS preparation in relation to the other genotypes. Where possible, laboratories were encouraged to use quantitative methods, reporting the results in International Units (IU) HBV DNA/ml. If results are reported in copies/ml, the assay-specific conversion factor copies to IU should be provided. However, data from qualitative assays were also of interest. One invited laboratory offered to perform sequence and genotype analysis of each panel member.

Preparation of the HBV genotype panel

Characterization of the candidate materials

HBsAg and HBV DNA high titre plasma units were collected worldwide. Two hundred and fifteen potential candidate materials were characterized performing the following analysis:

- quantitative HBV DNA and HBsAg determination
- sequencing of the entire S open reading frame
- HBV genotyping and HBsAg subtyping
- other serological hepatitis markers (anti-HBc, HBeAg, anti-HBe and anti-HDV)
- HIV-1 RNA and HCV RNA

Briefly, for sequence analysis, HBV DNA was extracted from 600 µl plasma or serum using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany). HBV DNA was eluted from the silica columns with 75 µl ultrapure water. Ten µl were subjected to real time PCR in the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using 10 mM primers (PreS1 sense primer(2816-2835): 5'GTCACCATATTCTTGGGAAC 3', and S6 antisense primer (997-973):5'CKTTGACADACTTTCCAATCAATAG 3'). Pre-S/S-gene products were purified by agarose gel electrophoresis and sequenced by GATC Biochem. Sequence data and the phylogenetic tree aligned with other sequences with the same subgenotype are available under the web-site http://www.pei.de/cln_116/nn_159176/DE/institut/who-cc/who-pei-aktivitaeten.html.

Samples were selected to represent typical examples of different subgenotypes. Samples with known S gene escape mutations or ambiguous sequences were not considered as candidate material for the panel. Fifteen candidate materials representing genotypes A to G were chosen as HBV panel members. Unfortunately, at that time no appropriate genotype H sample was available. Table 1a summarizes the data of the characterization of the panel members.

Preparation of bulk materials and freeze-drying

The HBV DNA concentration (Table 1) of the samples based on the quantification by 4 different NAT assays, Cobas AmpliPrep / Cobas TaqMan HBV Test, Cobas Amplicor HBV Test (Roche Diagnostics GmbH, Mannheim, Germany), Abbott RealTime HBV assay (Abbott, Wiesbaden, Germany), and *artus* HBV LC PCR Kit (Qiagen GmbH, Hilden, Germany). The dilution factors for the preparation of the bulk materials were determined by using the arithmetic mean of $\log_{10}$ IU/ml values of the concentrations. Dilutions were prepared with a plasma pool which had tested

negative tested for the following markers: HAV RNA, HIV-1 RNA, HCV RNA, HBV DNA, HBsAg, anti-HBs, anti-HBc (IgG and IgM), anti-HIV-1/2, and anti-HCV. To take in consideration any potential loss of HBV DNA during the processing and freeze-drying of about 10% each panel sample was diluted to an estimated final HBV DNA concentration of approximately 6.04 $\log_{10}$ IU/ml in a volume of 1.2 litres, with the exception of panel samples 6/B, 14/F, and 15/G. The original concentration and/or quantity of these three samples allowed only a dilution to a final concentration of 4.04 $\log_{10}$ IU/ml, 5.04 $\log_{10}$ IU/ml, and 4.04 $\log_{10}$ IU/ml, respectively. The details of sample processing are summarized in Table 2. The bulk preparations were stored at -20°C until further processing. A certified Swiss company performed the 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 they had just thawed. After thoroughly mixing, the materials were stored at 2°C -8°C and 0.5 ml volumes were dispended 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% (samples 7/C, 11/D, 12/D), 0.8% (sample 15/G), 0.9% (samples 1/A, 2/B, 5/B, 8/C, 13/E, 14/F), 1% (samples 3/A, 9/C, 10/D), and 1.1% (samples 4/B and 6/B). Overall 5 batches of lyophilisation with 2,000 vials each of the panel members were produced. Additionally, 144 vials filled with 0.5 ml of negative plasma pool were randomly distributed on the trays of the 5 freeze-dried batches. These vials were later used for the determination of the residual moisture content. After the freeze-drying procedure the vials were removed from the freeze-drier trays, sealed with screw-caps and stored at -20°C. All 30,000 vials were produced in October 2008. The lyophilized vials are stored at -20°C at PEI with constant temperature monitoring.

All manufacturing records are held by PEI and are available on request by the ECBS.

Studies on the final product

The HBV genotype panel has been prepared as lyophilized material and is recommended to be stored at ≤ -20°C. A programme to investigate the stability of the panel members was introduced. Recent results, obtained so far, demonstrate that the panel members are very stable under normal conditions of storage i.e. at -20ºC or below. Comparable results with the previous and current IS preparations for HBV DNA indicate that these preparations are suitable for long term use. An interims report of real-time stability study results of the stability will be submitted to the ECBS in 2010.

Preliminary results from real-time stability studies showed stability of the panel of HBV Samples 1-15 under recommended storage conditions, i.e. at -20ºC or below. Comparable results with the previous and current IS preparations for HBV DNA indicate that these preparations are suitable for long term use. The assessment of the stability of the HBV genotype panel is on-going and it is planned that an interims report reviewing the real-time stability of the panel members will be submitted to ECBS in 2010. The material is supplied lyophilized and should be stored at or below -20ºC. Each vial contains the equivalent of 0.5 ml of plasma material. The panel members should be reconstituted in 0.5 ml of sterile nuclease-free water. If the material is not used completely, laboratories may aliquot the remaining reconstituted material into suitable volumes which should be stored at or below -70°C.

Due to the infectious nature 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-dried batches and underwent the same processing conditions as all other vials. The residual moisture content was investigated at PEI

used an accredited method according to the European Pharmacopoeia (7). The water content was determined to be 0.82% (standard deviation ± 0.03%) which complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards (8).

Collaborative study

Participants, samples and study design

The collaborative study included 17 laboratories, from 12 countries. The laboratories were requested to analyze the 15 panel samples concurrently with the 2nd WHO IS for HBV DNA (97/750). The participants in the collaborative study are listed in Table 3. 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 laboratory has been referred to by a code number allocated at random and not representing the order of listing in Table 3. Where a laboratory performed more than one assay method, the results from the different methods were analyzed independently, as if from separate laboratories, and coded, for example, laboratory 3A and laboratory 3B (Table 4). The samples analysed in the study were labelled as Sample 1, Sample 2, Sample 3 to Sample 15, which correspond to respective genotypes A to G (see Table 2). Participants did not know the corresponding genotype during the study. The participants were requested to perform 3 separate assay runs when performing quantitative tests and 4 separate assay runs when performing qualitative tests, as detailed in the study protocol. The types of assays used by participants are recorded in Table 4. The assays cover a range of commercially available tests, and some in-house tests were also included. Where laboratories performed quantitative tests, they were requested to report results in IU/ml. In the case of qualitative assays, participants were requested to assay each panel member by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For 3 subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point estimated in their first assay. For further characterization of the panel samples one laboratory was invited to perform sequence and genotype analysis. For this purposes the laboratory received one set of the panel.

Statistical Methods

Qualitative Assays

The results from the 4 assays were pooled to give a series of number positive out of number tested at each dilution. The pooled results of the single assays were evaluated with a probit analysis to estimate the concentration at which 63% of the samples tested were positive (i.e. the dilution at which on average one single copy per sample tested could be expected under the assumption of an underlying Poisson distribution).The calculated end-point was used to give estimates expressed in NAT detectable units/ml after correcting for an equivalent volume of the test sample.

Quantitative Assays

For this evaluation no single values were removed from the analysis. Due to the high variability in the data no reasonable specification for identifying outliers could be defined. For comparison of laboratories the replicate results of each laboratory, corrected for the dilution factor, were combined as arithmetic mean of $\log_{10}$ IU/ml values. These estimates were then combined across assays to obtain an overall laboratory mean value of $\log_{10}$ IU/ml.

An additional evaluation was performed with data weighted by the IS results in the following way:

$$y_{ij}' = y_{ij} - y_{i,IS} + 6.0$$

with y_{ij}' weighted mean value for participant *i* and sample *j* in $\log_{10}$ IU/ml, y_{ij} unweighted mean value, $y_{i,IS}$ mean value for the IS sample and 6.0 $\log_{10}$ IU/ml the assigned unitage of the IS sample (i=1,…,16 laboratories; j=1,…,15 samples). The mean values obtained from this weighting were denoted as *"mean estimates relatively to concurrent tested IS assay"*.

Furthermore overall mean estimates were calculated for each sample with 95%-confidence intervals, standard deviation, minimum, maximum and range. This was performed for the absolute mean estimates as well as for the estimates relative to IS.

To evaluate the uncertainty of the estimation and the variability between laboratories and within replicated assays, a mixed linear model was applied to the unweighted data with random factors laboratory and quantitative assay. As measure for the uncertainty as well as intra- and inter-assay precision a coefficient of variation was calculated. Variation between the different assay methods could not be estimated because of too few replications of the methods (13 different methods tested by 16 participants).

The statistical analysis was performed with SAS®/STAT software, version 9.2, SAS System for Windows. Estimation of end-point dilution was done with CombiStats Software, version 4.0, from EDQM / Council of Europe.

Data Received

Data were received from a total of 17 participating laboratories. Fourteen laboratories (code numbers 1-14) used quantitative assays, 2 laboratories (code numbers 15 and 16) used qualitative assays, and one laboratory (code number 17) performed sequence and genotype analysis. Laboratories 3 and 7 performed the testing using 2 different quantitative assays. These have been analysed independently, and are referred to as 3A and 3B, and 7A and 7B, respectively. In total there were 16 data sets for quantitative assays, 2 data sets for qualitative assays, and one data set for the sequence and genotype analysis.

Each participant, who performed a quantitative test, provided results of 3 assays for duplicate testing of each of the 16 samples (Sample 1 – Sample15 and sample IS). Laboratory 15 performed a qualitative assay and provided results of one assay performed in $\log_{10}$ dilutions with single determinations and results of 3 assays with 0.5 $\log_{10}$ dilutions in single determinations. Laboratory 16 also used a qualitative assay and provided results from the 1st assay performed in $\log_{10}$ dilutions with duplicate determinations and three further assays with 0.5 $\log_{10}$ dilutions in duplicates.

From laboratories 6 and 8, no data could be generated by the assays for Sample 14. Therefore, the calculation of the absolute overall mean estimates of Sample 14 based on 14 data sets for absolute estimates (only quantitative assays) and 16 data sets for relative estimates (quantitative and qualitative assays). The other data sets were complete except some single missing values due to e. g. invalid results.

Laboratory 9 provided results expressed in copies/ml. Because the results for the IS corresponded well with the assigned concentration of 6.0 $\log_{10}$ IU/ml, the results for all tested samples by this laboratory were included in the calculation of the mean absolute estimates, expressed in IU/ml.

Results

Quantitative Assay Results

The laboratory mean absolute estimates for the Samples 1 -15 and IS expressed in IU/ml (log_{10}) are shown in histogram form in Figures 1a-15a and 16. Each box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. The individual laboratory means are given in Table 5.

The overall means from all laboratories for the quantitative assays are shown in Table 6 including additional statistical data (95%-confidence intervals, standard deviation, min and max values and range). The overall mean of 6.01 log_{10} IU/ml (95%-confidence intervals of 5.92 – 6.10) of the IS is very close to its assigned potency of 6.0 log_{10} IU/ml. Whereas the confidence intervals show a very precise estimation of the sample mean, the range between minimum and maximum individual results mostly reaches 1 log_{10} and sometimes even exceeds this value. To compare the results between laboratories, the overall mean estimates were separately depicted for each sample. For most samples the results show a good agreement between laboratories. Results outside a range of the overall mean of ± 0.5 log_{10} IU/ml were regarded as outliers (this value corresponds to approximately two times the average standard deviation of inter-laboratory variation). Laboratory 8 reported deviating results compared to the overall mean estimates for Samples 4, 5, 6, 7, 8 and 15 (0.68, 0.72, 1.36, 0.64, 0.83, and 0.86 log_{10} IU/ml, respectively), representing the genotypes B, C, and G. Laboratory 11 showed a lower result for Samples 6, 9 and 13 (0.53, 0.66, and 0.55 log_{10} IU/ml, respectively. Laboratories 4 and 6 represented also deviating results for Sample 15 compared to the overall mean estimate (differences of 1.21 and 0.99 log_{10} IU/ml, respectively). This genotype G sample was drastically underquantified by laboratories 6 and 8, and surprisingly overquantified by laboratory 4. The reason for the underestimation of Sample 15/G by laboratories 6 and 8 of about 1.0 log_{10} IU/ml is unclear, because both assays used the X- and the S-gene region, respectively, as the target region for amplification and detection. Therefore, the region with the 36 base-pair insert characteristic for genotype G is not affected by the assays. Additionally, Sample 14 (genotype F) was not detected by the methods used by laboratories 6 and 8 and drastically underquantified (1.24 log_{10} IU/ml) by laboratory 2. The shortcomings of some NAT assays in efficient detection of the genotype F sample (Sample 14) could be explained by the fact that this genotype shows the most distant sequence of all HBV genotypes compared to the sequence of IS, which represents genotype A2 (see also Figures 18a and 18b). An additional evaluation of the overall sample means was performed excluding the outlying results (Table 6 and 8, in italics).

The relative high variation of individual laboratory mean absolute estimates for some samples is also illustrated by the box-and-whisker-plot (Figure 17a). The effect of excluding outlying results from laboratories 2, 4, 6, 8 and 11 for some of the samples results in a more precise estimation of the unitage of the respective samples with considerable smaller confidence intervals and ranges (Table 6, printed in italics; Figure 17b), rather than greater changes in the laboratory overall mean estimates.

Laboratories 5 and 14, 3A and 13, as well as 3B and 12 used the same quantitative NAT assays, respectively. The mean absolute estimates of laboratories 5 and 14, or 3B and 12, agreed very well. In contrast, the results obtained from laboratory 13 were continuously higher for all tested samples compared with the values obtained from laboratory 3A. A potential explanation could be due to the performance of the nucleic acid preparation method which is based on a manual procedure. The preparation kit is not an essential part of the amplification/detection kit, and differences in the efficiency of nucleic acid extraction could be assumed between lots.

Qualitative Assay Results

The main focus of the collaborative study was to investigate the panel samples by different quantitative NAT assays, preferentially calibrated against the IS. Nevertheless, the inclusion of 2 participants which used qualitative assays will provide interesting results about the efficiency of these assays in detection of different HBV genotypes in relation to the analytical test sensitivity. This plays an important role when such tests are used for the screening of blood donations.

The laboratory mean absolute estimates for the Samples 1 -15 and IS expressed in NAT detectable units/ml (log_{10}) are shown in Table 5 (rows in grey). The majority of the results of the 2 laboratories using the qualitative assay were very close to each other and showed the same range as those of the quantitative assays. Notable, laboratory 15 achieved significant higher mean absolute estimates (log_{10} NAT detectable units/ml) of the Samples 5, 10, 11, and 12 compared to the results of laboratory 16. The NAT technology of both assays is very different; laboratory 15 used real-time PCR (TaqMan technology) whereas the other laboratory used the transcription-mediated amplification (TMA) method based on isothermal amplification. It seems that the TaqMan assay is more sensitive to detect HBV genotype D samples. No differences could be found in the ability to detect the genotypes F and G.

Because the Samples 1-15 were analyzed concurrently with the 2nd WHO IS for HBV DNA (97/750) it was possible to calculate the conversion factor between NAT detectable unit to IU for each assay based on the calculation of the potencies of IS in NAT detectable units/ml. For the TaqMan assay 1 IU correspond to 2.04 NAT detectable units, whereas for the TMA assay the conversion factor was determined to be 1 IU to 1.82 NAT detectable units.

Determination of Overall Laboratory Means – Combined Qualitative and Quantitative Results

The values of the Sample 1-15 calculated relative to the concurrently tested IS are shown in Figures 1b-15b in histogram form, and are presented in Table 7. Results from both, quantitative and qualitative assays can be compared in the same figures since the units have been expressed in IU/ml (log_{10}) in all cases. In general, the results from the 2 qualitative assays are in good agreement with those of the quantitative assays. It should be kept in mind that the potencies calculated for laboratories 15 and 16 based on data from end-point dilution, where the precision is depending from the amount of replicates tested per dilution per sample. Weighting of the data (relatively to IS 97/750) shifts the participant results from the quantitative assays only a little bit more together. Thus there seems not to be a general shift in the results from laboratory to laboratory (Figures 1b – 15b). The re-evaluation of the overall sample means was performed excluding the outlying results. In addition to the analysis with the unweighted results laboratory 7B showed a lower result for Sample 15 (difference of 0.52 log_{10} IU/ml to the overall mean). Weighting of data apparently has only a minor effect on the overall mean estimates for the samples (Tables 6 and 8). Again, the effect of excluding outlying results from laboratories for some of the samples does not have a great influence on the laboratory overall mean estimates (IU/ml). The outlier procedure results in a more precise estimation of the unitage of the respective samples with considerable smaller confidence intervals and ranges.

Table 9 shows the variation in terms of total uncertainty (as coefficient of variation (CV%)), inter-laboratory variation, inter-assay variation and intra-assay variation. These data are based on the analysis of the quantitative results of overall replicates tested per sample. This analysis was not performed of the data obtained from the 2 qualitative assays, because the statistical calculation of the concentration of a sample is based on end point dilution. Except for Samples 6 and 15 the uncertainty is (sometimes clearly) below 10% which indicates a very good

reproducibility, if accounting for the large number of participants and variety of assays applied. The CV% values are below 10% for all samples when excluding the few outlying results from laboratories 2, 4, 6, 8 and 11 for some of the samples. The precision between laboratories accounts for the main portion of variation (56% to 92% of the total variation; data not shown) whereas the inter-assay variation (i.e. the variation between the 3 independent runs performed by each laboratory) is for the most samples below 1%. The CV for reproducibility (intra-assay variation; estimated from the residual variation) is mostly below 5%. The re-evaluation of the analysis without the outlying results as defined above provides CV% values below 3% for the intra-laboratory precision and CV% values below 6% for the inter-laboratory precision for Samples 1 - 14. CV% values were estimated below 4% for the intra-laboratory precision and CV% values below 7% for the inter-laboratory precision for all samples.

HBV Sequencing and Genotyping Results

Vials were reconstituted following the instructions of the Study Protocol. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Two nested PCRs were performed to amplify the small S gene and the preS region of the viral genome. Amplicons were purified and sequenced using an ABI DNA sequencer. The obtained sequences from the panel were aligned with ClustalX v1.83 software (9) and edited with Bioedit v7.0.9.0 software (10) along with different HBV genotypes downloaded from GenBank. Phylogenetic relationships were evaluated using maximum likelihood method (ML). ML trees were generated with PAUP* v4b10 software package (11) and an appropriate nucleotide substitution model estimated using Modeltest v3.7 (12) according the Akaike Information Criterion. Robustness of the phylogenetic grouping was evaluated by bootstrap analysis using ML method (1000 replicates) with PhyML v2.4.4 (13). The results of the phylogentic analysis of the HBV Samples 1-15 and IS are shown in Figures 18a and b. Table 1b summarizes the results of the determination of the HBsAg subtypes. Assignment of subtypes was performed according to Norder et al. (14). All pre-determined HBV genotypes and HBsAg subtypes could be confirmed. Sequence data are available under the web-site http://www.pei.de/cln_116/nn_159176/DE/institut/who-cc/who-pei-aktivitaeten.html.

All raw data for the collaborative study are held by PEI and are available on request by the ECBS.

Conclusions

In this study, a wide range of quantitative HBV NAT assays (11 different commercial tests and 2 in-house developed tests) and 2 commercial qualitative tests were used to evaluate the HBV genotype panel in parallel with the WHO IS. The panel consists of 15 lyophilized HBV positive plasma samples and covers the most prevalent HBV genotypes: Samples 1-3 (genotype A), Samples 4-6 (genotype B). Samples 7-9 (genotype C), Samples 10-12 (genotype D), Sample 13 (genotype E), Sample 14 (genotype F), and Sample 15 (genotype G).

The data of this study confirm the validity of the assigned value of 97/750, the 2nd IS for HBV DNA, i.e. 10^6 IU/ml. Only few HBV NAT assays showed some deficiencies in the detection of some HBV genotypes, other than genotype A2 (IS). Two assays (laboratories 8 and 11) showed lower potencies for more than one sample from the panel representing genotypes B, C, E and G. Additionally, the genotype F sample could not be detected by laboratory 8. The method used by laboratory 6 was also not able to detect genotype F and in addition underquantified the Sample 15/G. Laboratory 4 represented a significant higher result for Sample 15/G compared to the overall mean potencies. One further NAT assay (laboratory 2) significant underquantified the genotype F sample (Sample 14). The results of the collaborative study clearly demonstrate that

the majority of the used quantitative assays and both qualitative assays are able to detect HBV genotypes A to G consistently. Therefore, the present 2nd IS for HBV DNA, which represents genotype A2, seems to be commutable to the other genotypes. Nevertheless, the availability of a well characterized HBV genotype panel is an essential prerequisite for the evaluation and validation of HBV NAT assays. Furthermore such a panel allows the comparison of different NAT assays on a standardized basis and can be used for the quality control of NAT tests by national control laboratories as well as by kit manufacturers. It is known that the genotype H shows a variability of the nucleotide sequences comparable to genotype F. The prevalence of this genotype is currently restricted to Central America, mainly in Nicaragua and Mexico, and some cases in California. Very recently new HBV strains were identified in Laos and Vietnam which phylogenetically clustered in a new group proposed as genotype I which is in fact a recombinant of several genotypes. Based on the availability of improved molecular diagnostic tools and carrying out of epidemiological studies worldwide new subgenotypes and recombinant forms may be identified in the future. The global distribution of genotypes may also change in the future and therefore updating the HBV genotype reference panel, to reflect the changing global epidemiological trend of HBV genotypes, may be necessary.

The overall results of the collaborative study demonstrate that the range of the concentrations of the Samples 1-15 are very close to the HBV DNA concentrations which were chosen for the final preparation of the panel samples: approximately 10^6 IU/ml (Samples 1 – 5, 7 – 13), approximately 10^5 IU/ml (Sample 14), and approximately 10^4 IU/ml (Samples 6 and 15). The freeze-drying procedure of the HBV positive plasma samples had no influence on the viral DNA integrity. In view of different estimates for the panel samples, assignment of IU/ml for each panel sample may be inappropriate. Nevertheless, the use of this HBV genotype panel for the validation of NAT tests will provide information of possible deficiencies in detecting of HBV genotypes other than A2.

Proposals

Based upon the results of the collaborative study, it is proposed that the panel should be established as the 1st International Reference Panel for HBV DNA Genotypes for NAT-based assays (PEI code number 5086/08). The panel consists of 15 lyophilized HBV positive plasma samples and covers the most prevalent HBV genotypes: Samples 1-3 (genotype A), Samples 4-6 (genotype B). Samples 7-9 (genotype C), Samples 10-12 (genotype D), Sample 13 (genotype E), Sample 14 (genotype F), and Sample 15 (genotype G). No unitage is assigned to individual panel members. However, the statistical data for each panel member from the outcome of the collaborative study are provided, for information only (overall arithmetic mean estimates of $\log_{10}$ IU/ml values, confidence intervals, standard deviation, minimum and maximum values, and ranges). The HBV Genotype Reference Panel (PEI Code number 5086/08) would provide a valuable set of well characterised HBV reagents for NAT for use in all regions of the world.

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. The participants agreed with the proposal that the HBV genotype panel (PEI Code number 5086/08) should be established as the 1st International Reference Panel for HBV DNA Genotypes for NAT-based assays. They agreed with the proposal that the individual panel members will not have an assigned value in IU. Laboratory 17 provided additional data for subgenotyping, esp. of genotype F samples.

Acknowledgements

We are grateful to all the laboratories and institutes who are kindly provided the candidate materials: Prof Gerlich, Institute of Medical Virology, Justus Liebig University Giessen, Germany; Profs Yoshizawa and Tanaka, Department of Epidemiology, Infectious Disease Control and Prevention, Hiroshima University, Japan; Prof Zhibourt, Federal Blood Center, Moscow, Russia; Drs Sabino and Otani, Fundação Pró-Sangue Hemocentro de São Paulo, Brazil; Dr Schmidt, Institute of Transfusion Medicine and Immunohematology, German Red Cross, Frankfurt/Main, Germany; Dr Cheraghali and Dr Abolghasemi, Iranian Blood Transfusion Organization, Tehran, Iran; and Mrs Sykes and Mr Watts, South African National Blood Service, Weltevreden Park, South Africa. We would like to thank all the participants in the collaborative study without whom this exercise could not have been undertaken. We also wish to thank Dr Padilla from WHO for her continuous support of this project.

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Figure 1a: Mean absolute estimates of Sample 1 (quantitative assays)

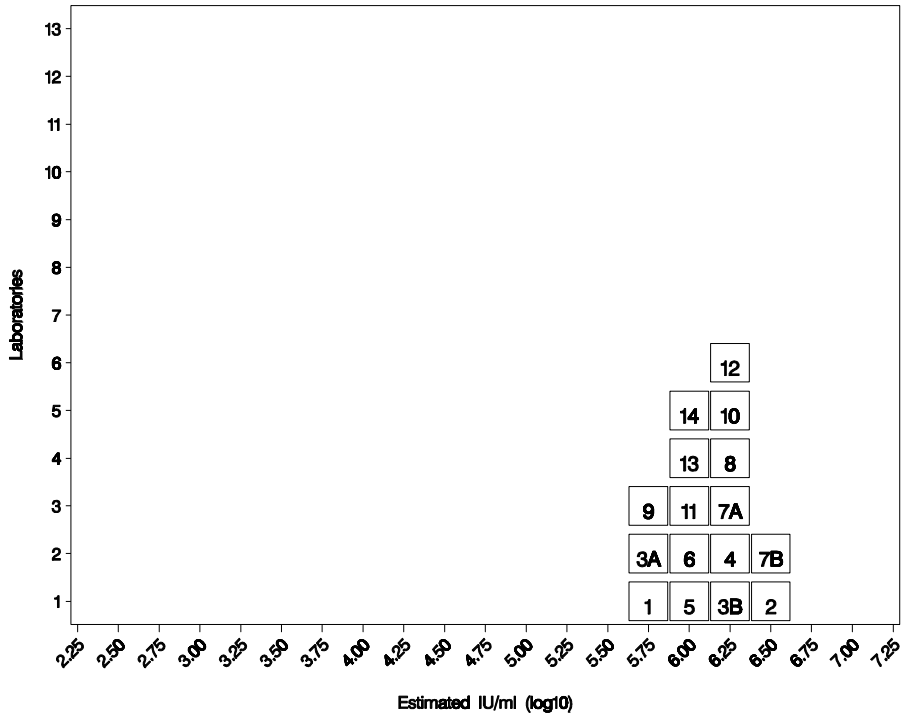


Figure 1b: Mean estimates of Sample 1 relative to 97/750

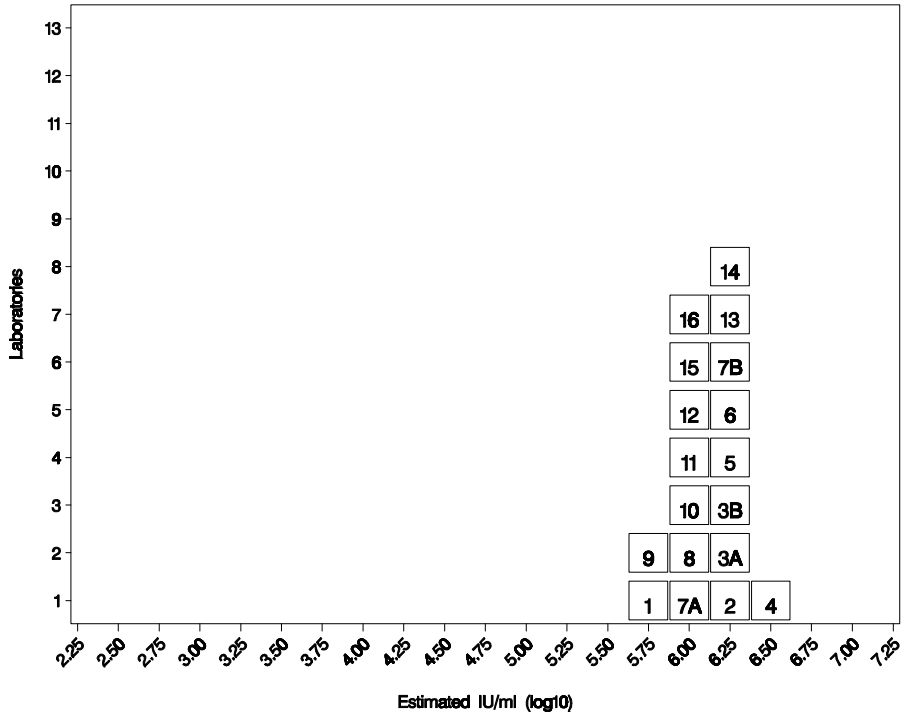


Figure 2a: Mean absolute estimates of Sample 2 (quantitative assays)

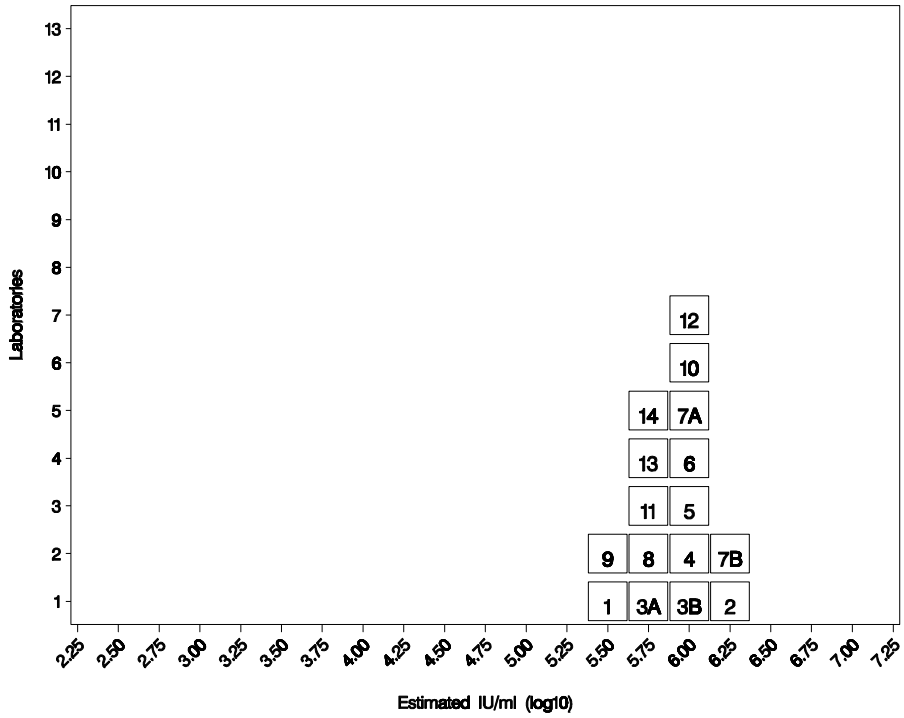


Figure 2b: Mean estimates of Sample 2 relative to 97/750

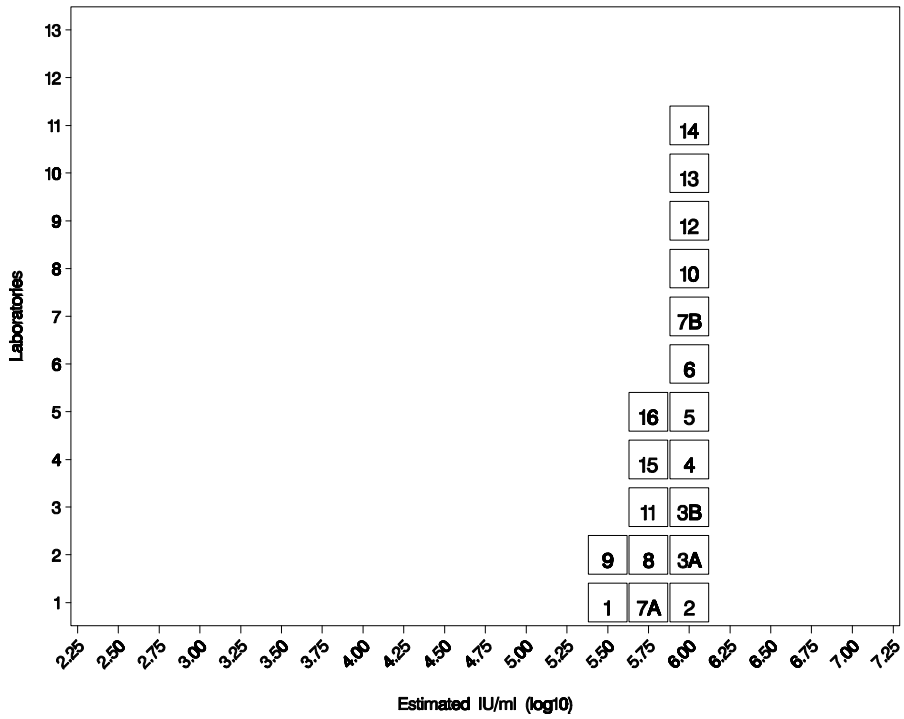


Figure 3a: Mean absolute estimates of Sample 3 (quantitative assays)

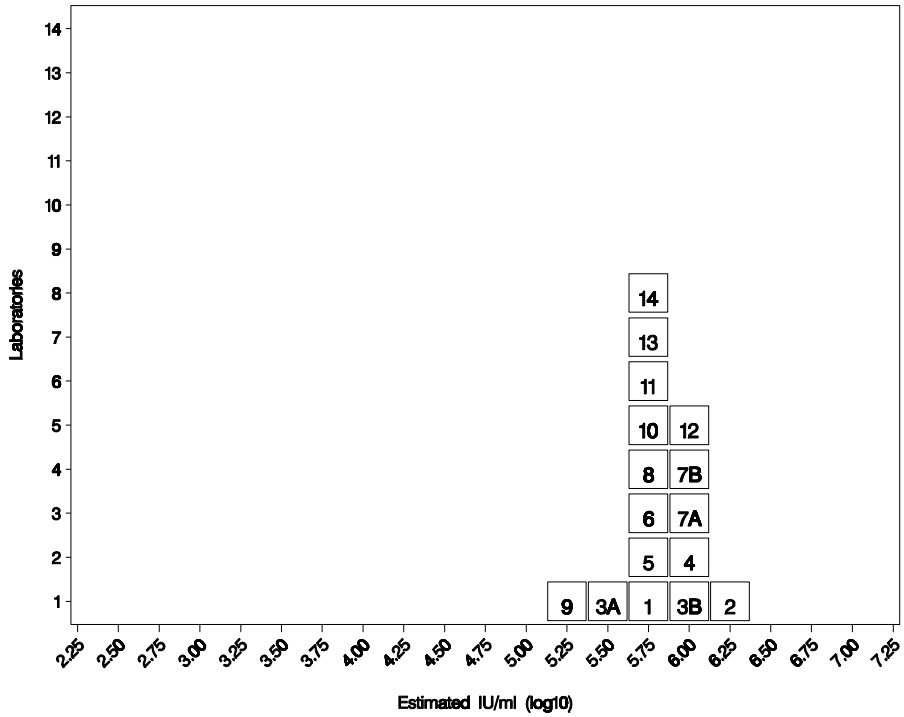


Figure 3b: Mean estimates of Sample 3 relative to 97/750

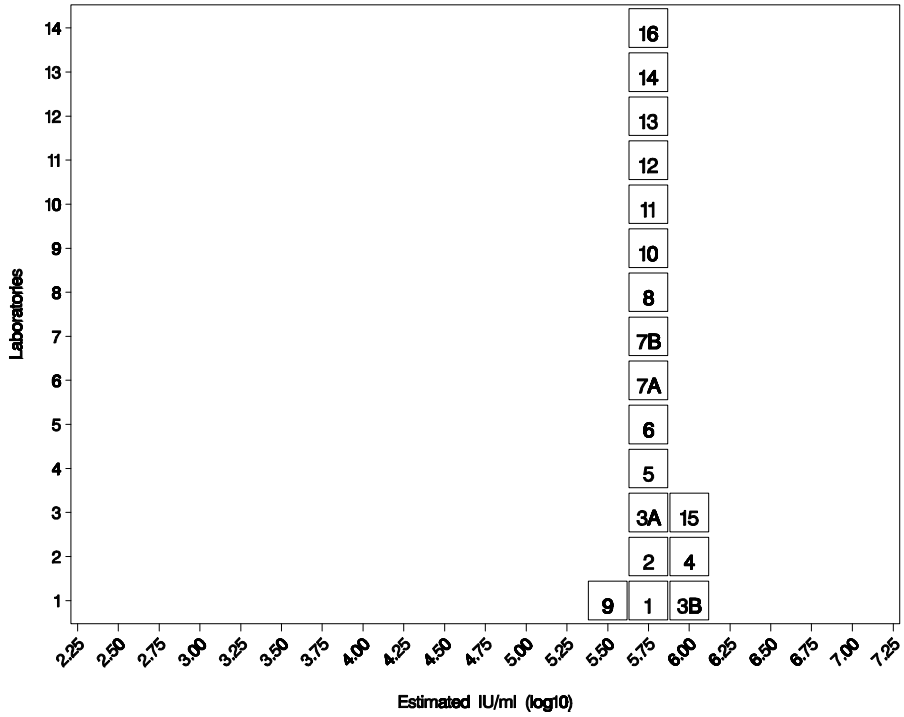


Figure 4a: Mean absolute estimates of Sample 4 (quantitative assays)

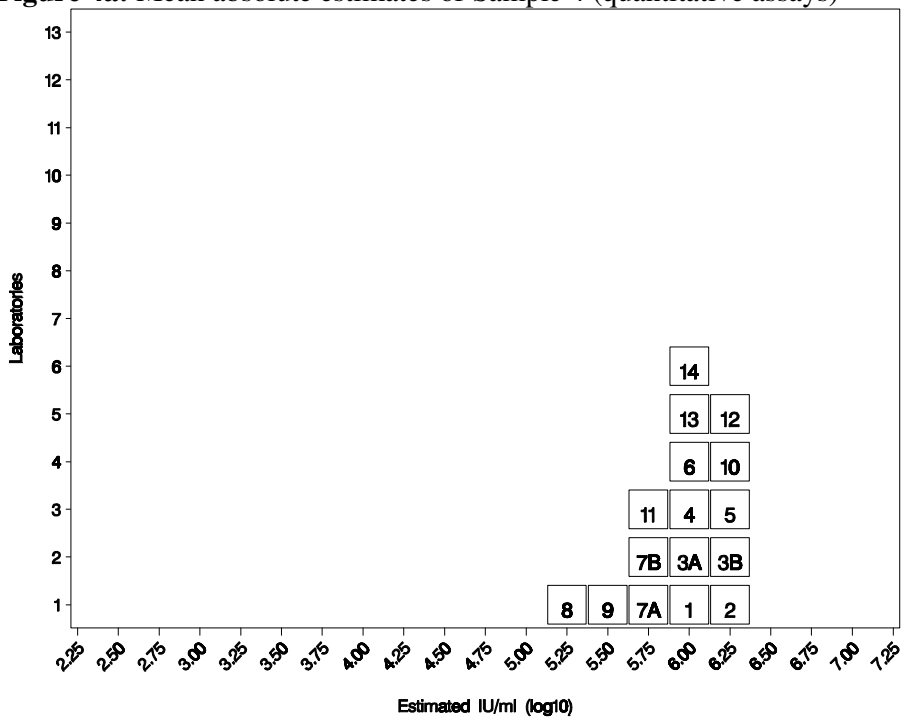


Figure 4b: Mean estimates of Sample 4 relative to 97/750

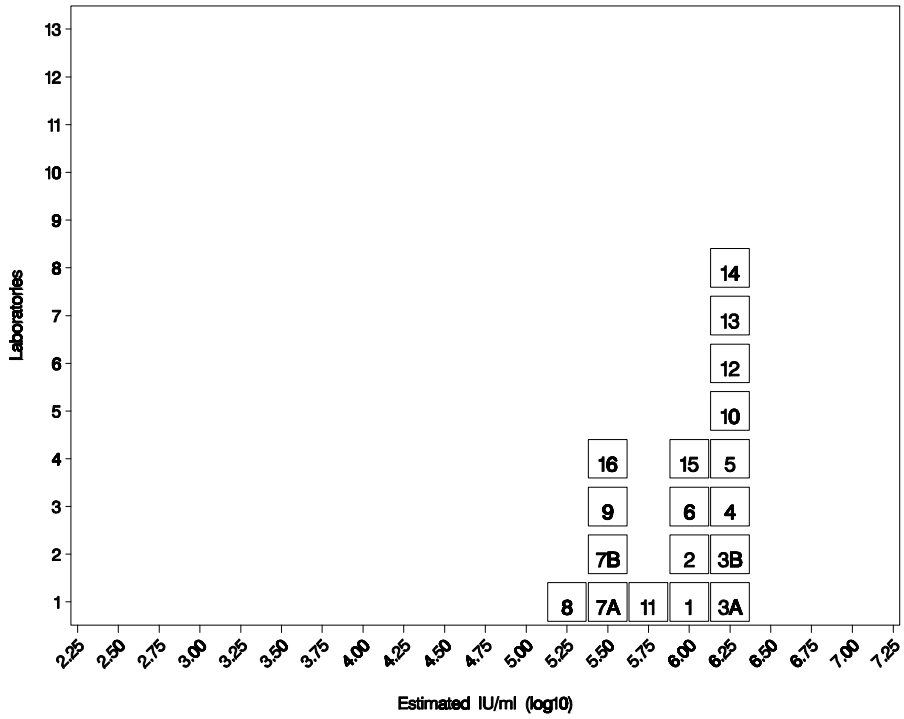


Figure 5a: Mean absolute estimates of Sample 5 (quantitative assays)

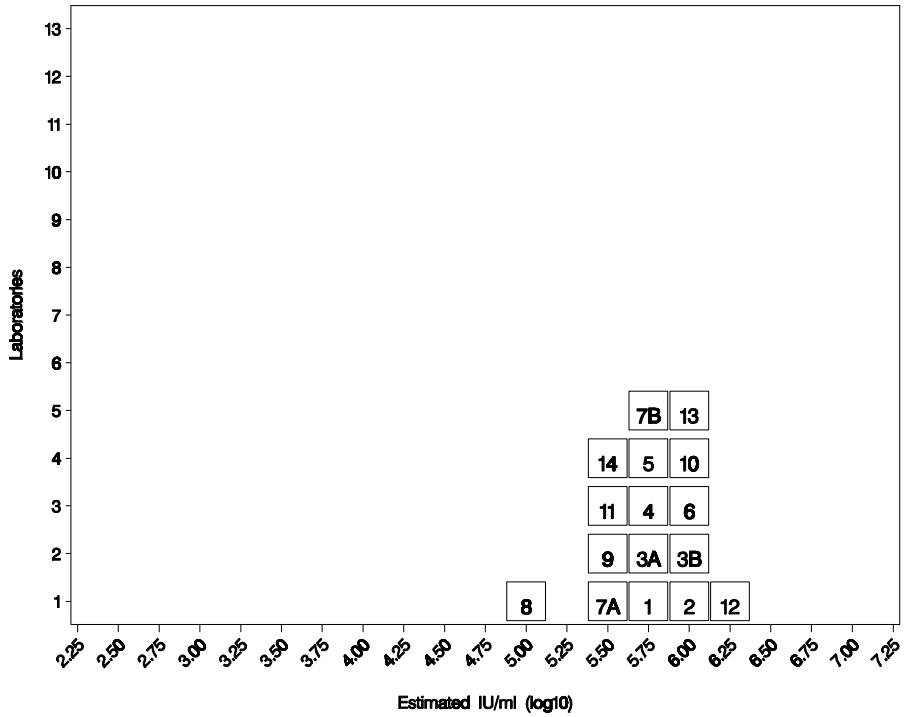


Figure 5b: Mean estimates of Sample 5 relative to 97/750

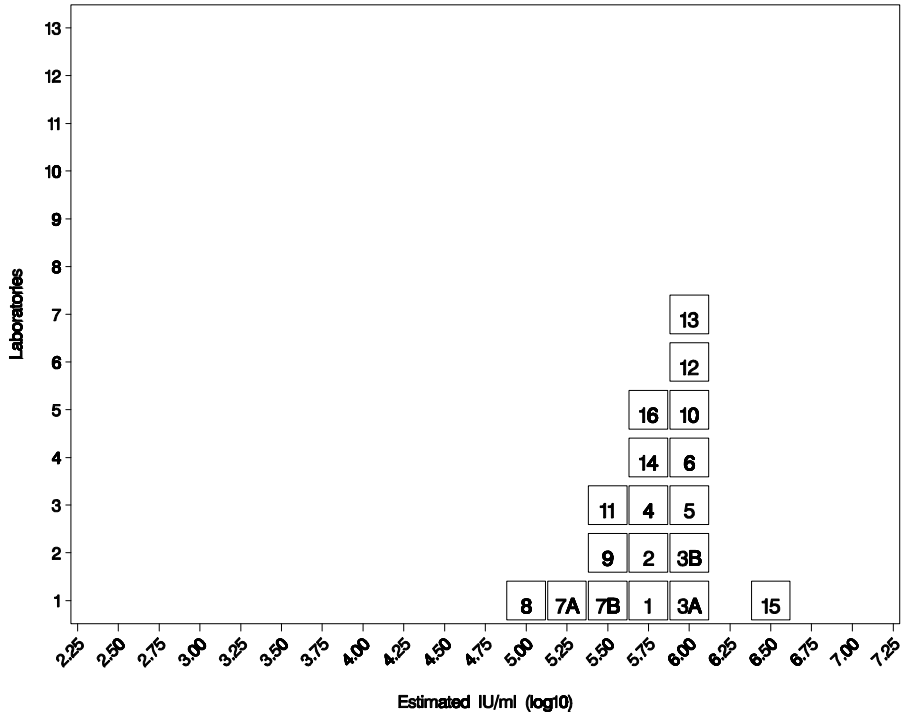


Figure 6a: Mean absolute estimates of Sample 6 (quantitative assays)

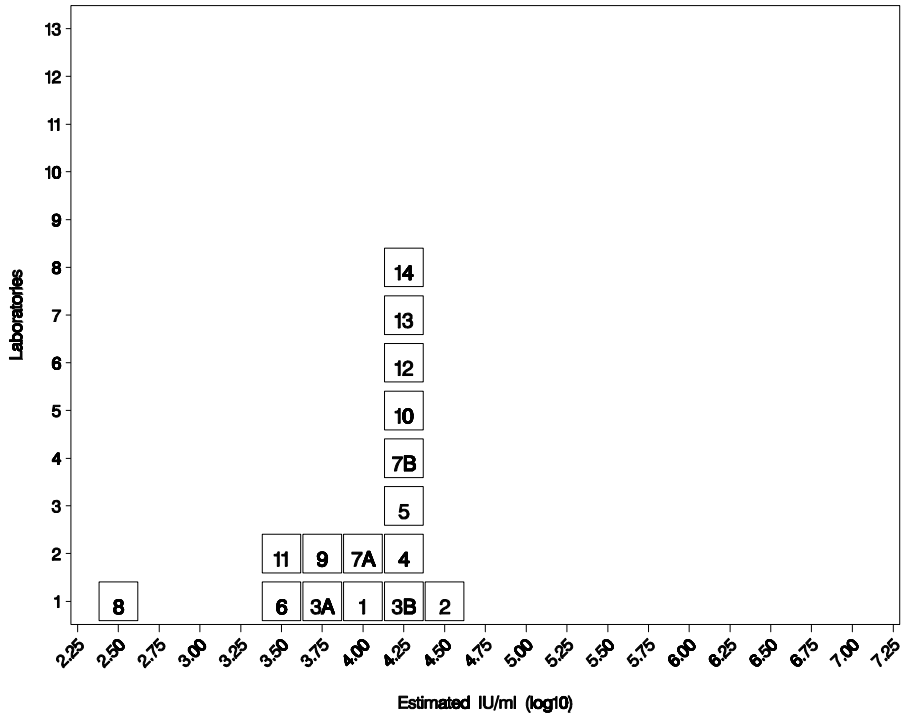


Figure 6b: Mean estimates of Sample 6 relative to 97/750

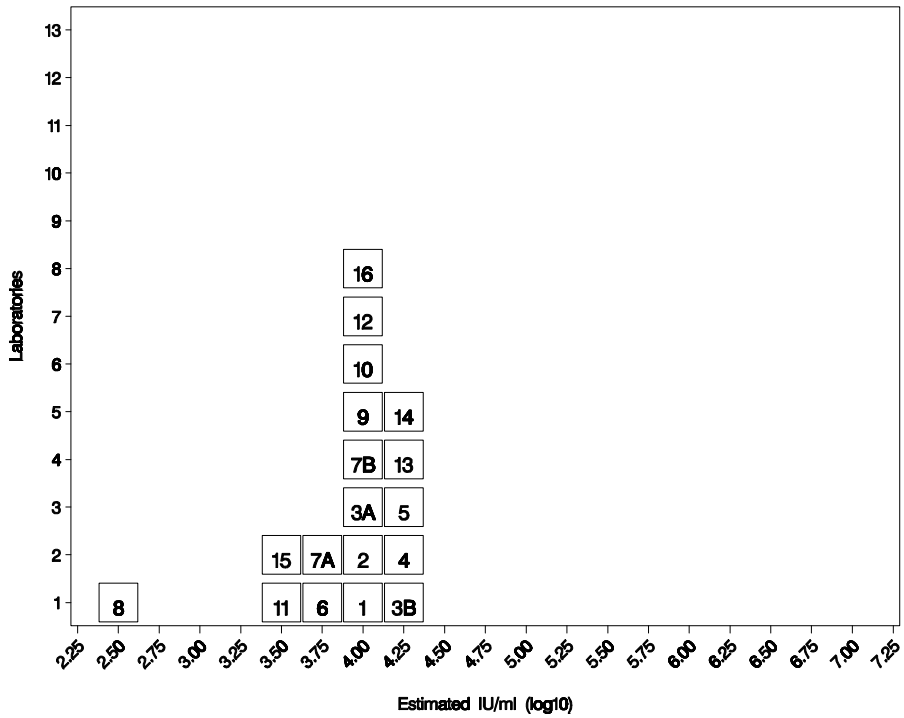


Figure 7a: Mean absolute estimates of Sample 7 (quantitative assays)

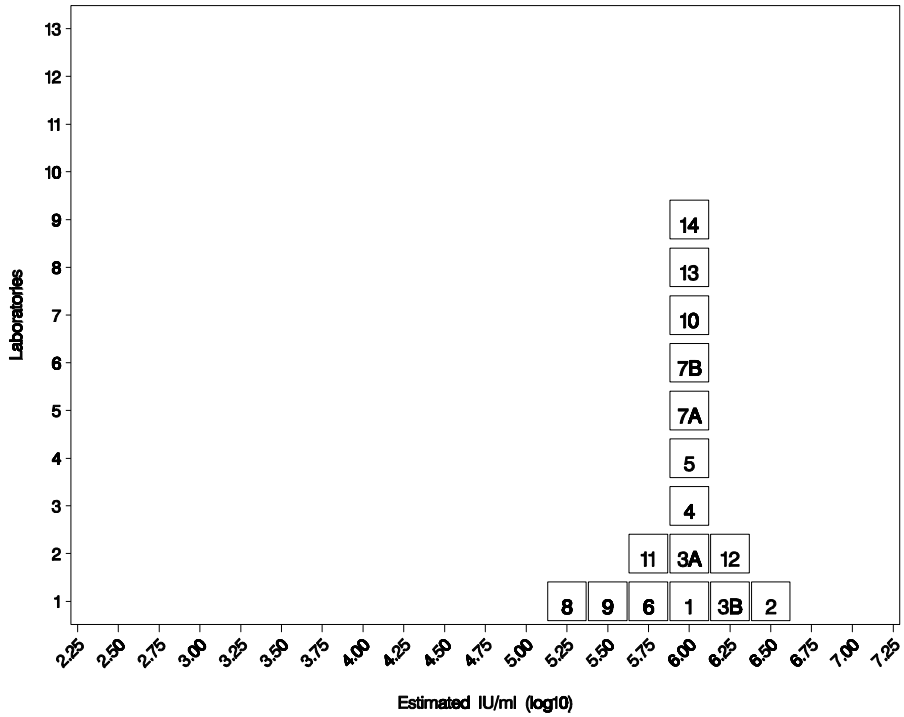


Figure 7b: Mean estimates of Sample 7 relative to 97/750

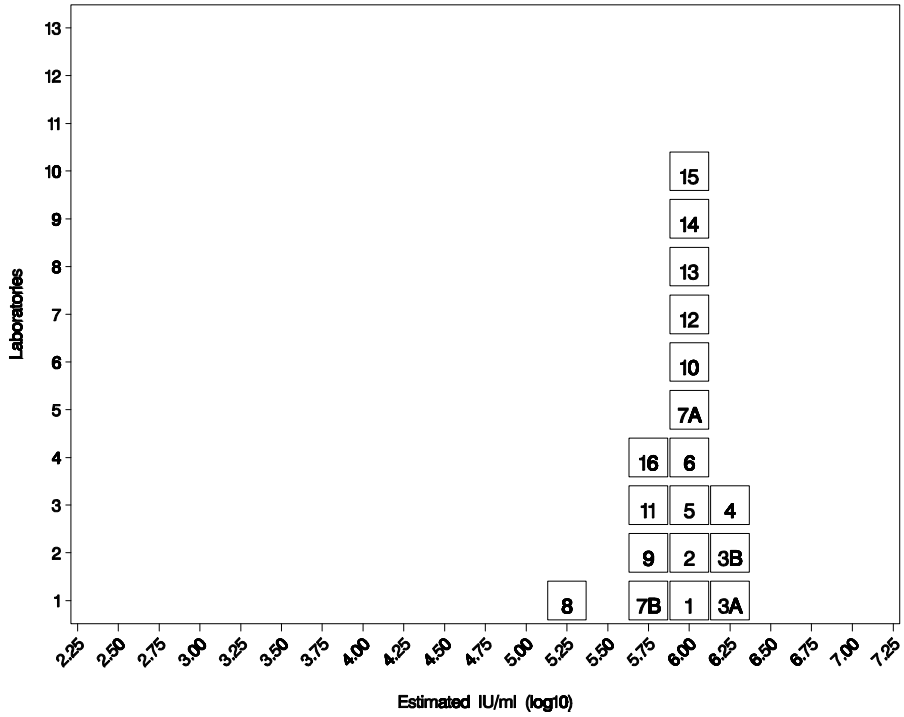


Figure 8a: Mean absolute estimates of Sample 8 (quantitative assays)

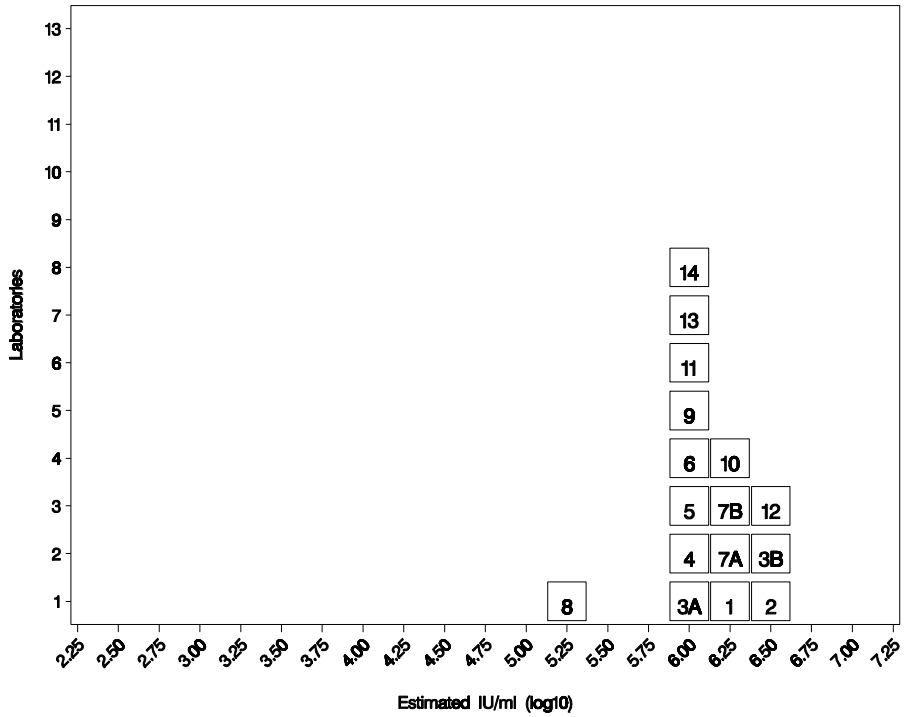


Figure 8b: Mean estimates of Sample 8 relative to 97/750

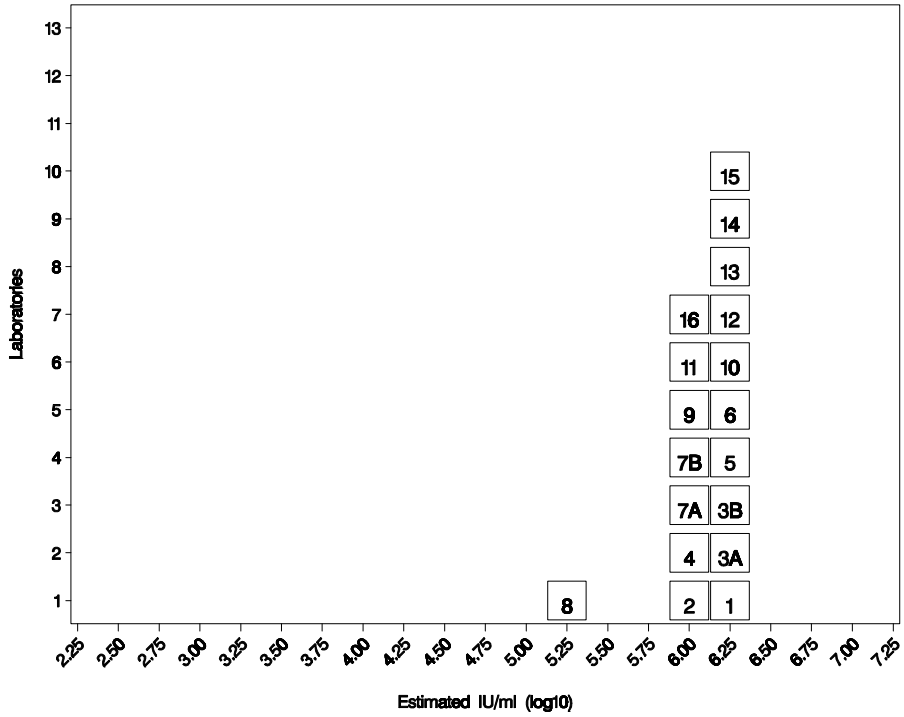


Figure 9a: Mean absolute estimates of Sample 9 (quantitative assays)

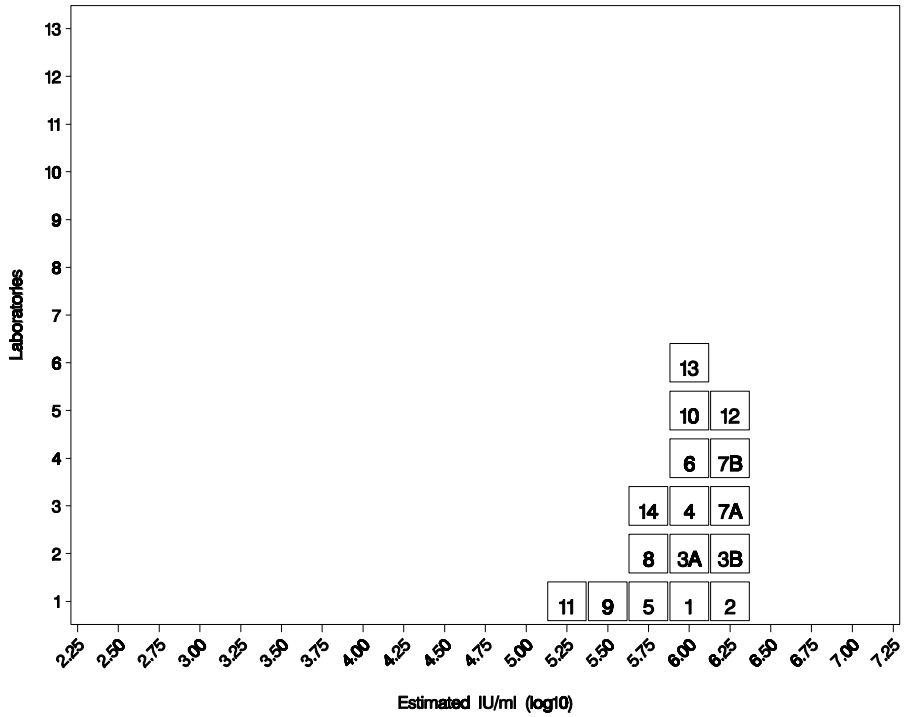


Figure 9b: Mean estimates of Sample 9 relative to 97/750

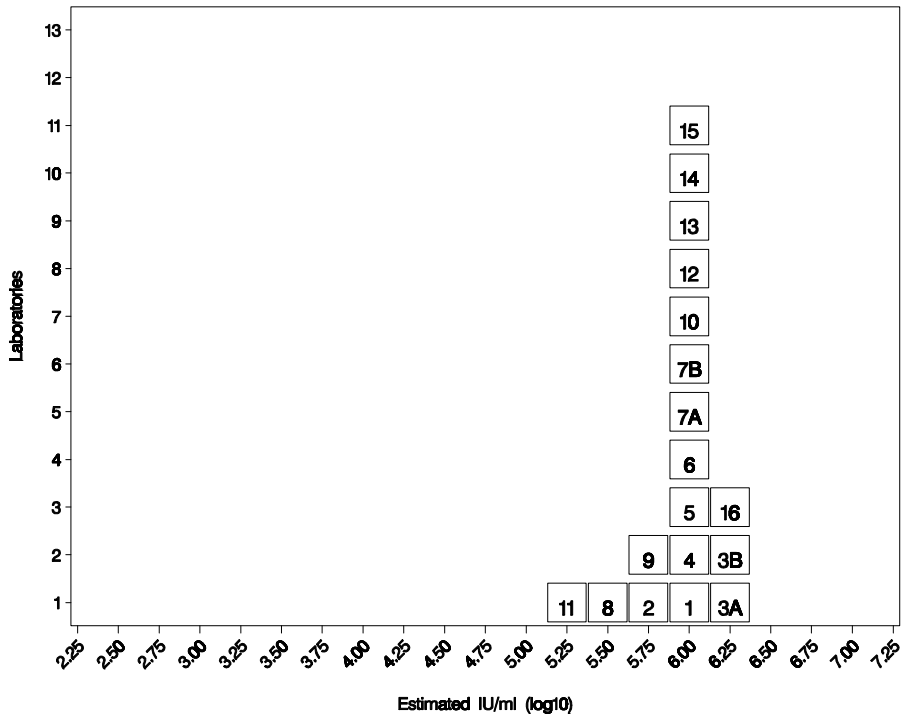


Figure 10a: Mean absolute estimates of Sample 10 (quantitative assays)

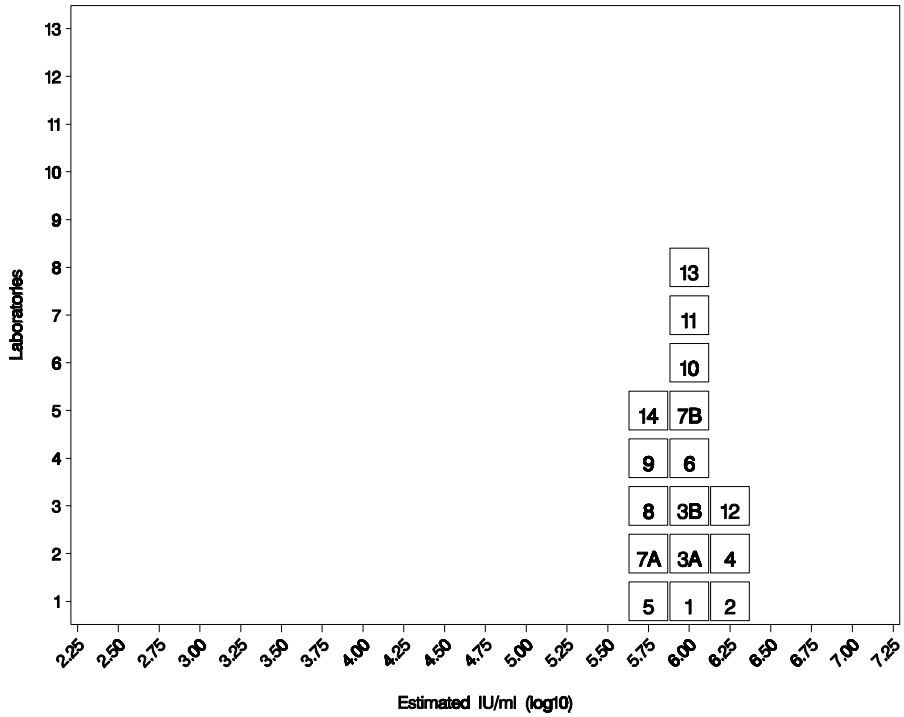


Figure 10b: Mean relative estimates of Sample 10 relative to 97/750

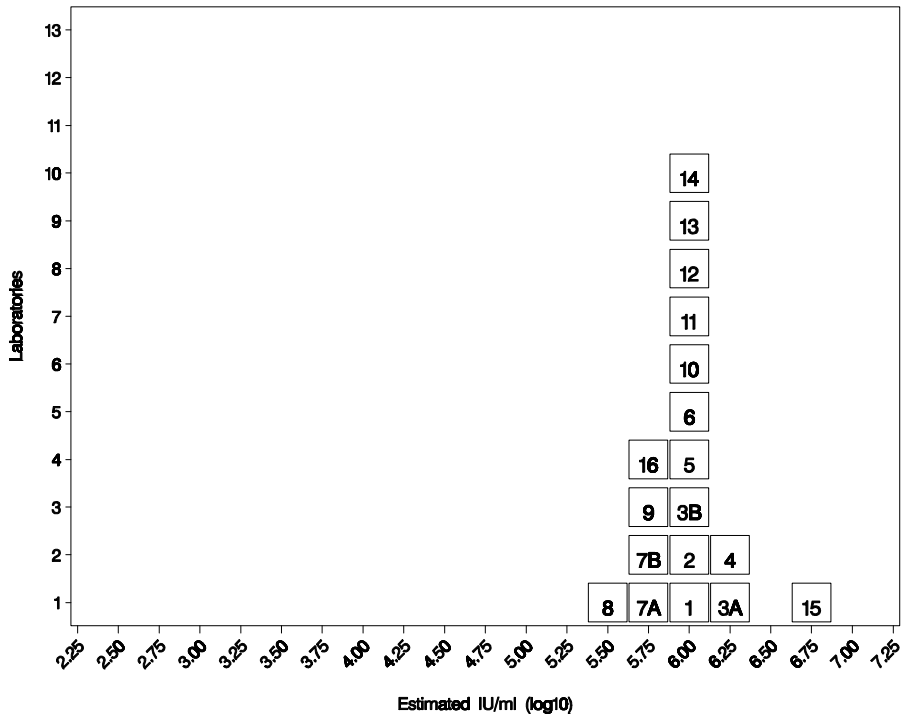


Figure 11a: Mean absolute estimates of Sample 11 (quantitative assays)

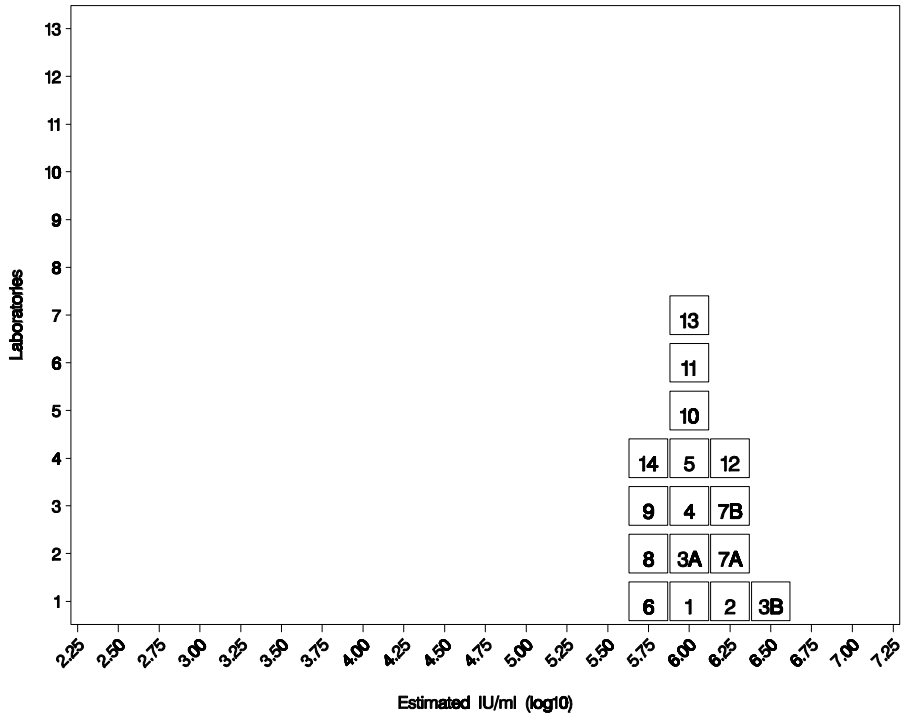


Figure 11b: Mean estimates of Sample 11 relative to 97/750

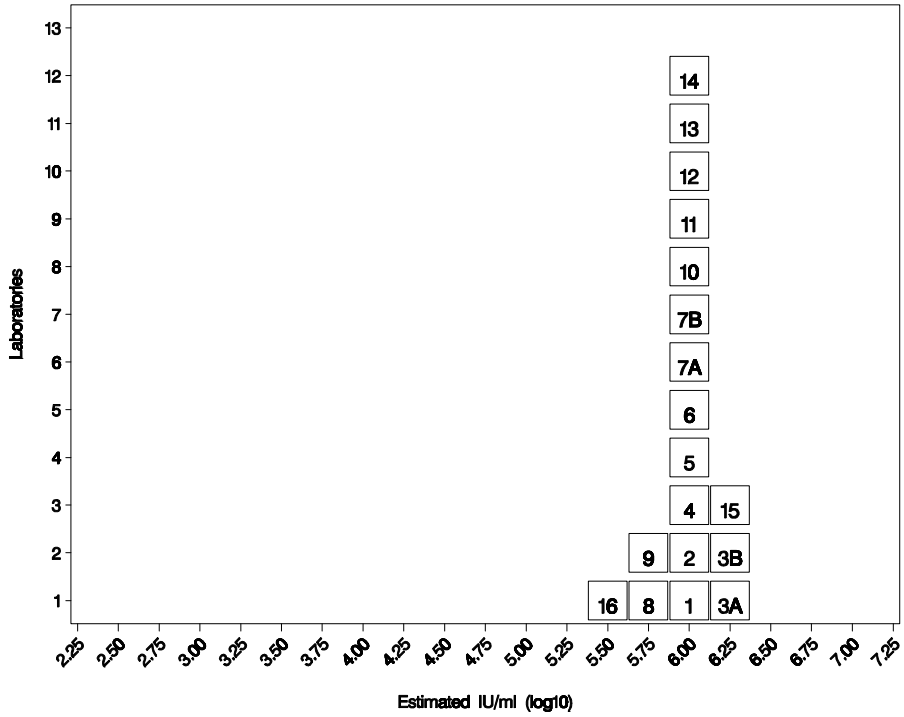


Figure 12a: Mean absolute estimates of Sample 12 (quantitative assays)

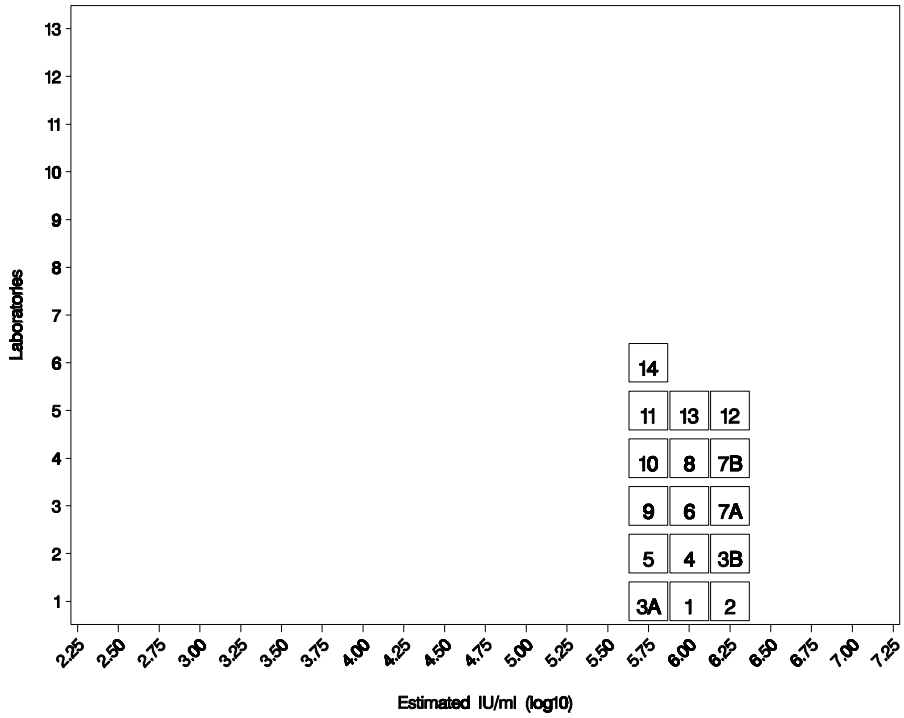


Figure 12b: Mean estimates of Sample 12 relative to 97/750

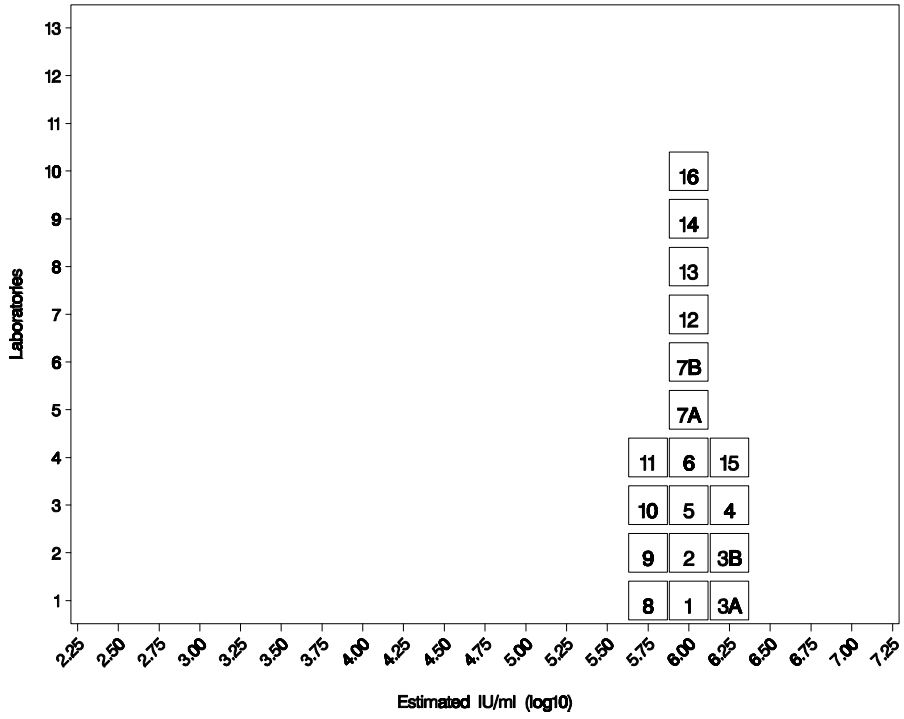


Figure 13a: Mean absolute estimates of Sample 13 (quantitative assays)

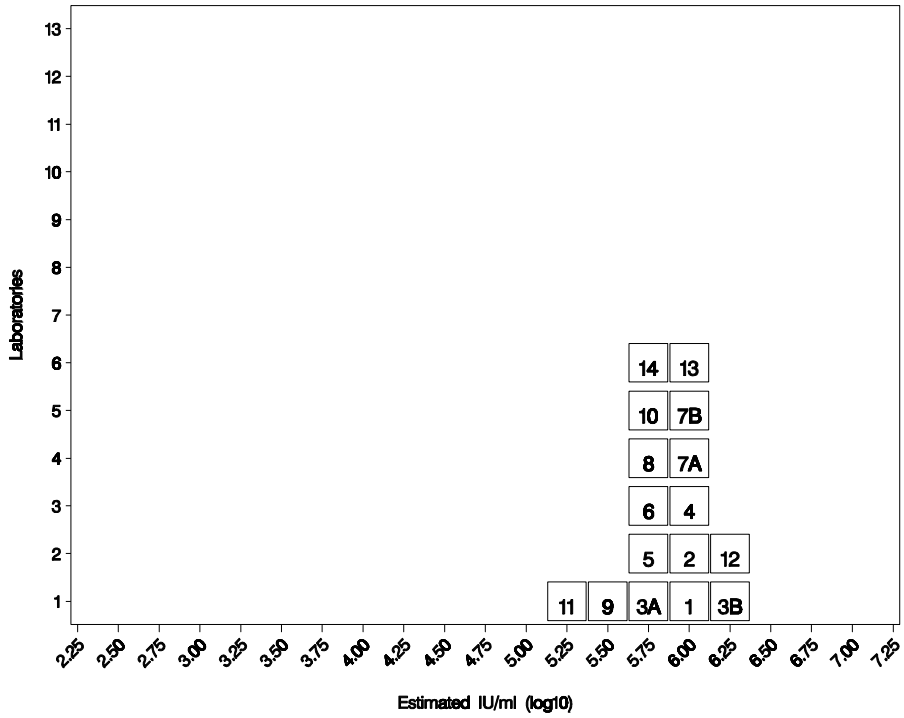


Figure 13b: Mean estimates of Sample 13 relative to 97/750

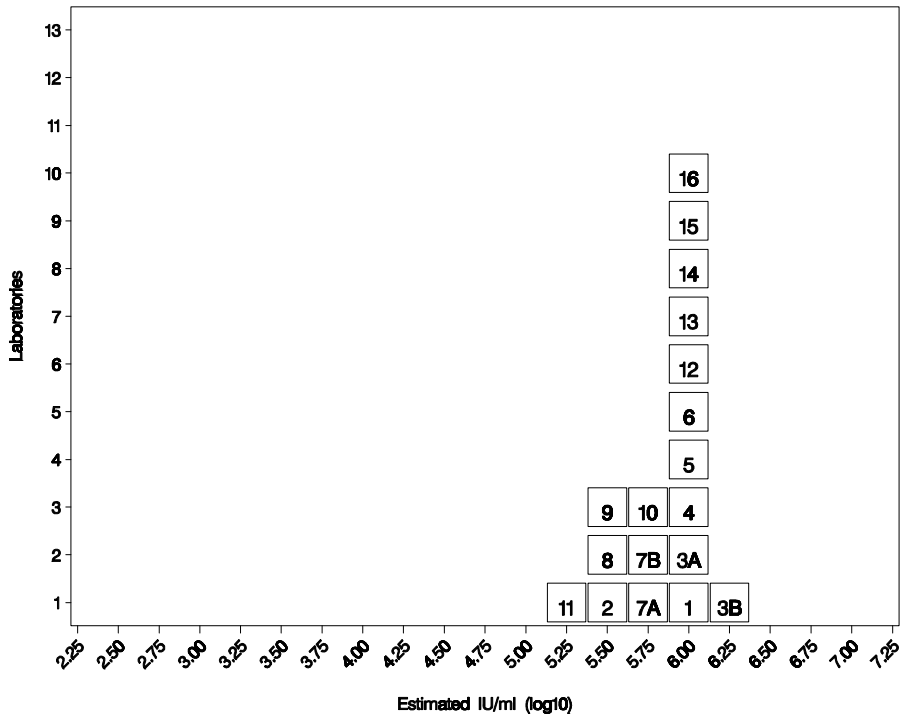


Figure 14a: Mean absolute estimates of Sample 14 (quantitative assays)

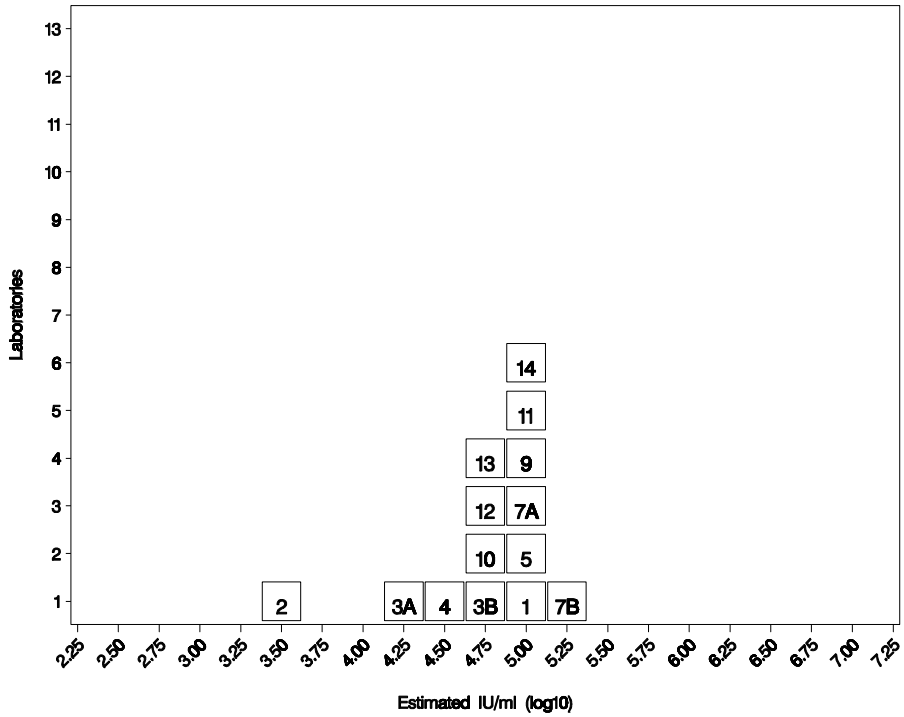


Figure 14b: Mean estimates of Sample 14 relative to 97/750

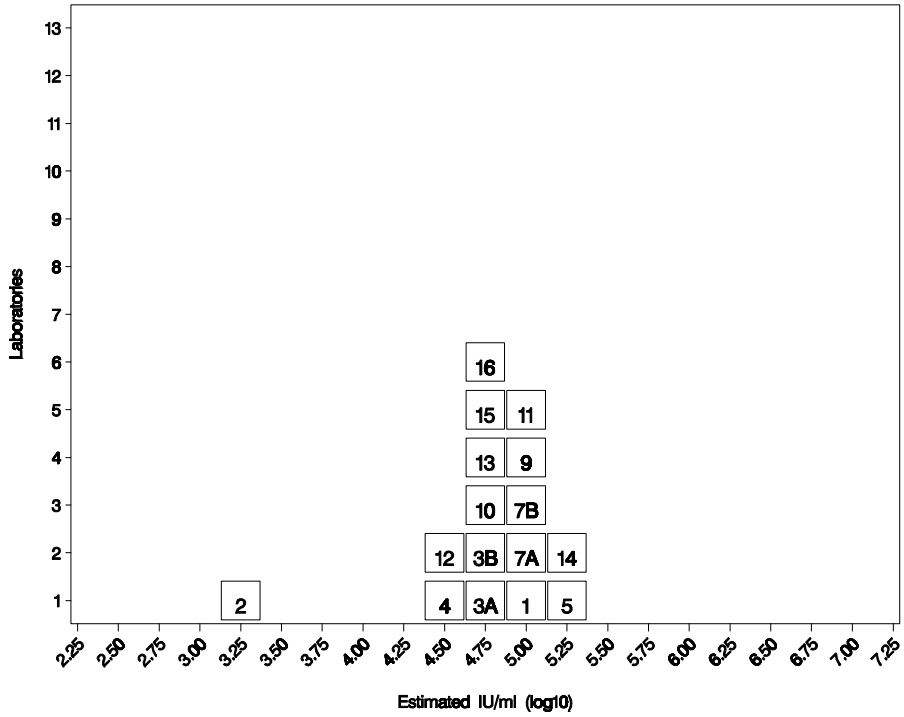


Figure 15a: Mean absolute estimates of Sample 15 (quantitative assays)

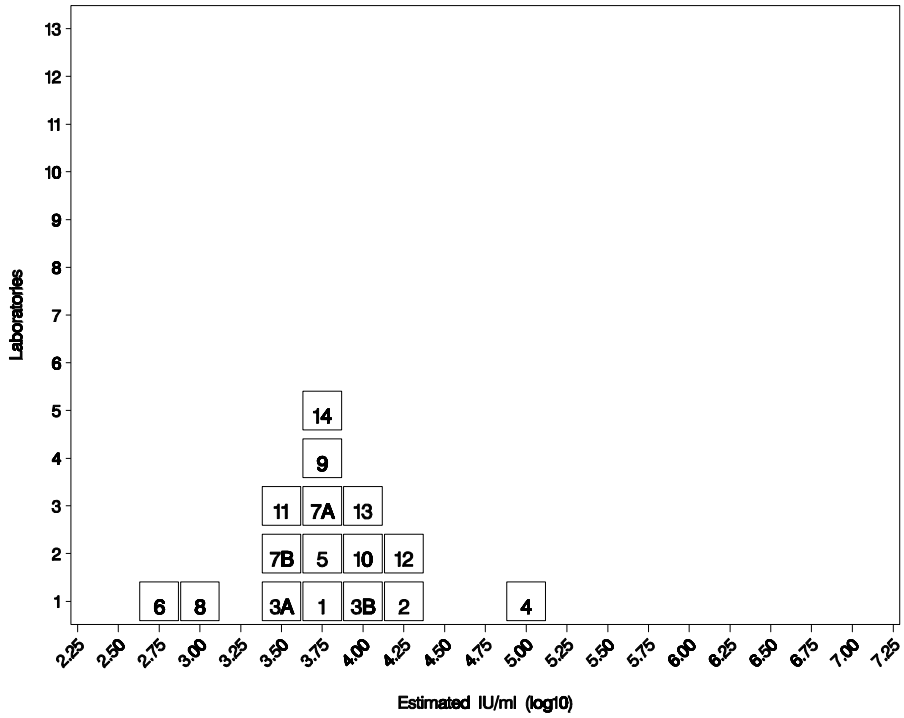


Figure 15b: Mean estimates of Sample 15 relative to 97/750

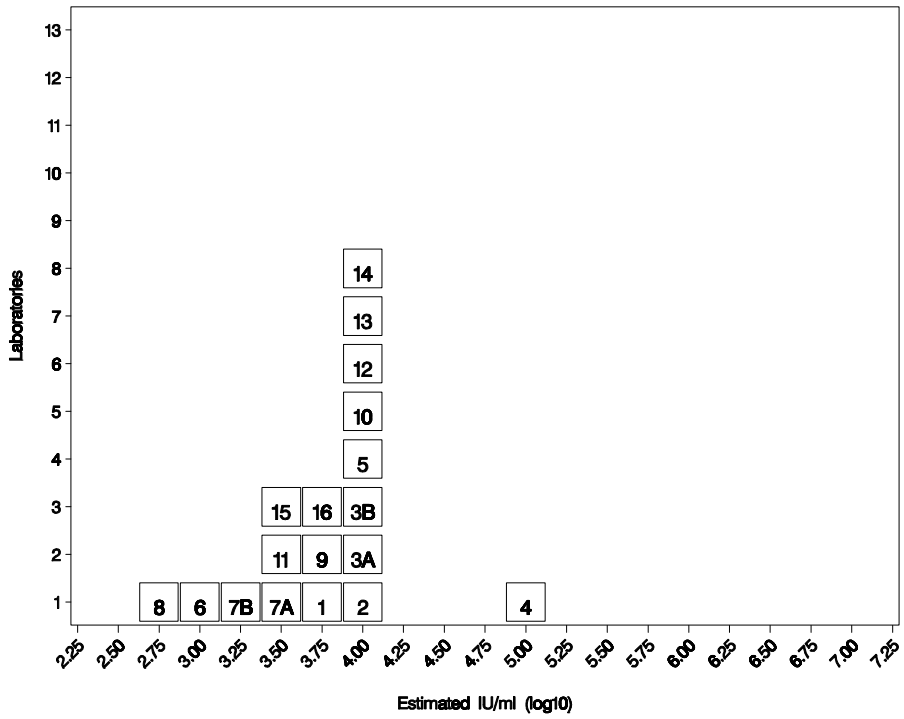


Figure 16: Mean absolute estimates of 97/750 (quantitative assays)

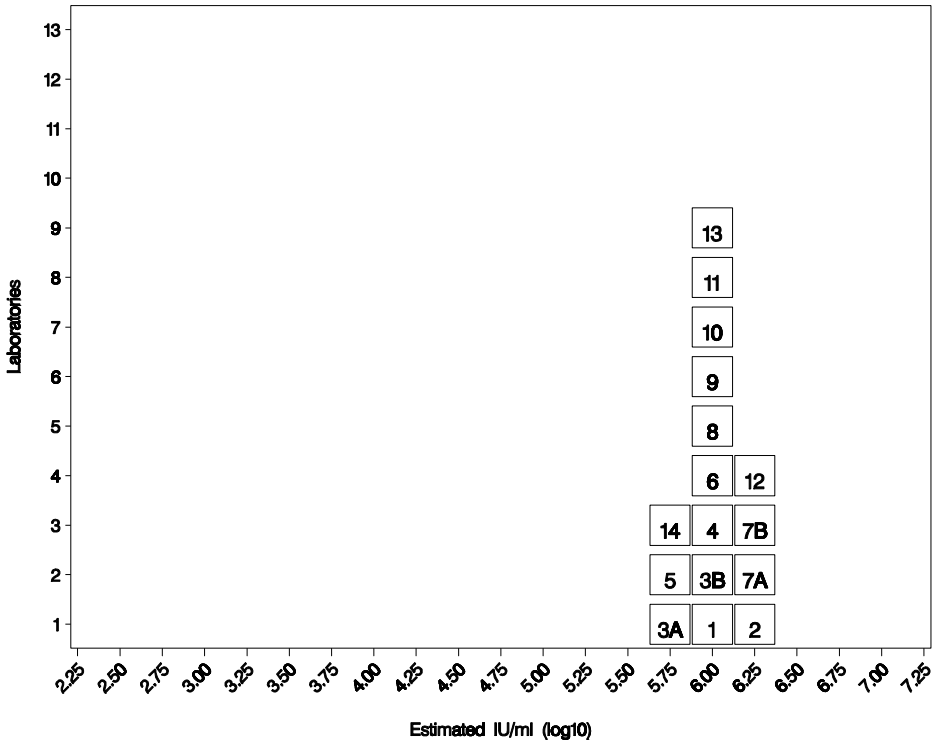


Figure 17a: Box-Plot for all samples (quantitative assays)

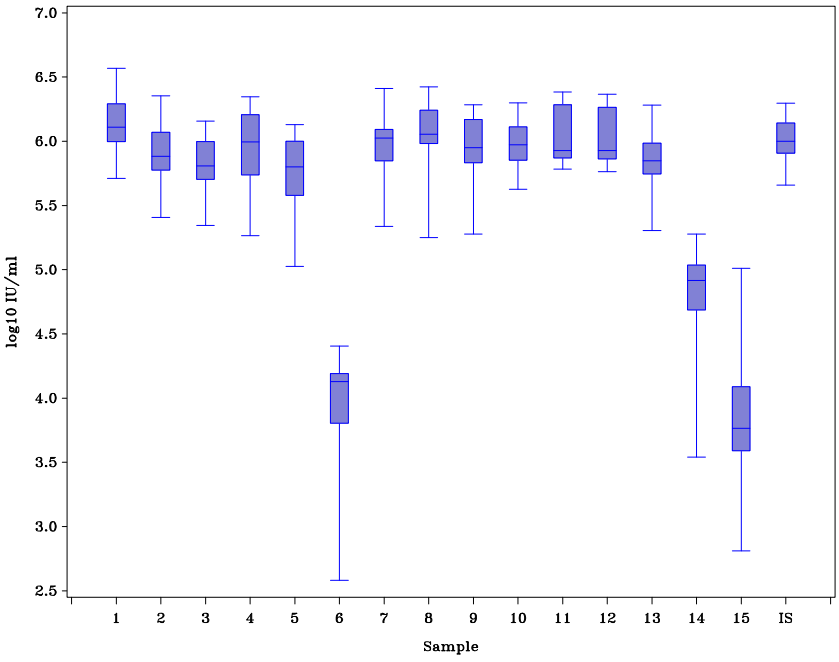
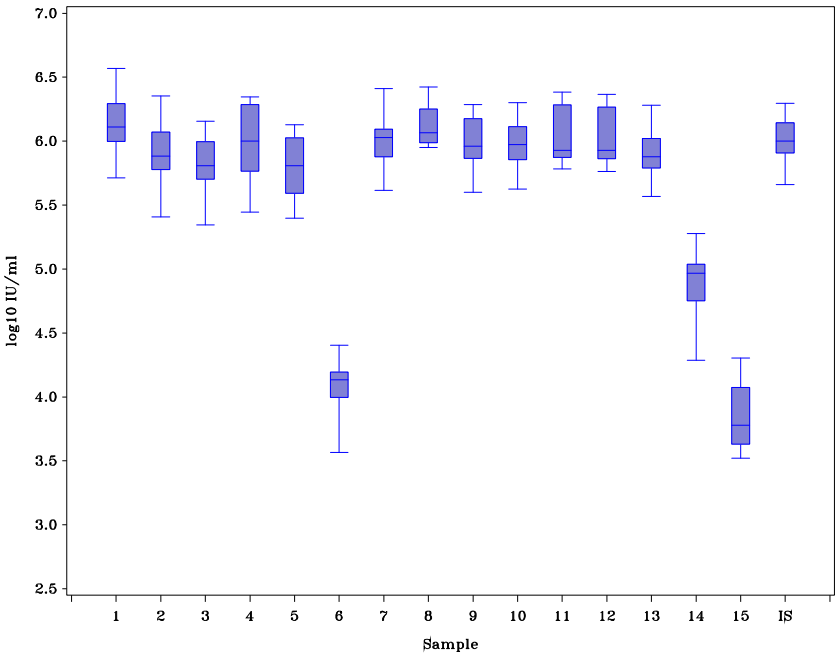
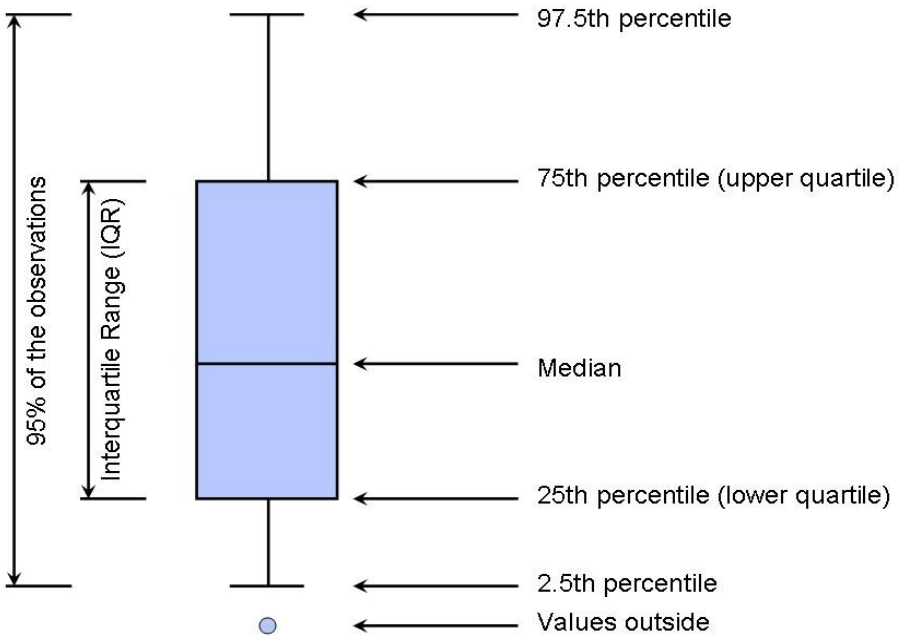


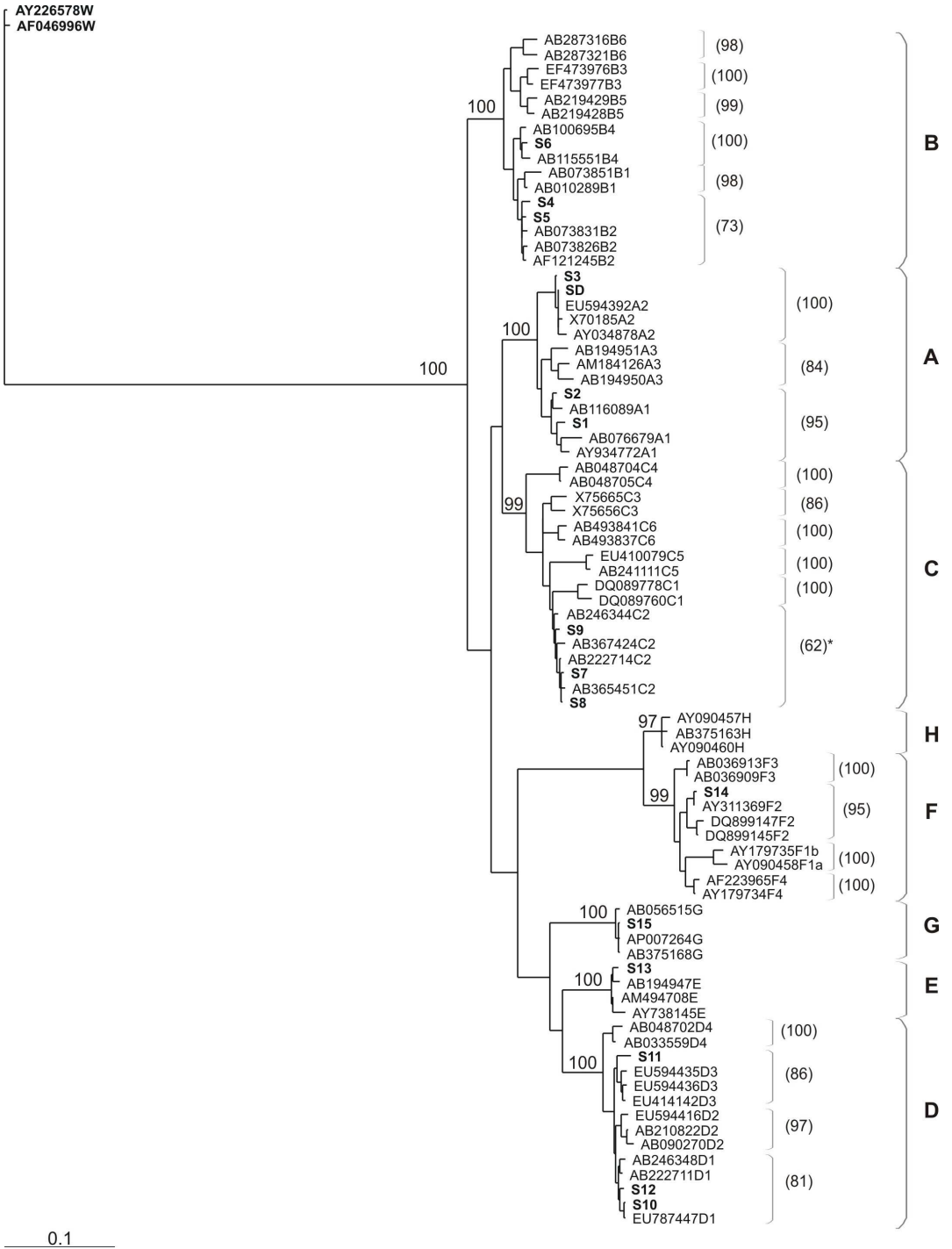
Figure 17b: Box-Plot for all samples (quantitative assays), excluding results from laboratory 8 (Samples 4-8, 15), laboratory 11 (Samples 6, 9, 13), laboratory 2 (Sample 14) and laboratories 4 and 6 (Sample 15)





Legend Box-Plot
The box-and-whisker-plots show the distribution of the data. The boxes itself contain the middle 50 per cent of the results (interquartile range, IQR) and the median as horizontal line. Between the whiskers lie 95% of the observations and the upper and lower outlying 2.5% of the observations are denoted by dots.

Figure 18a: Phylogenetic tree constructed on concatenated 1128 bp belonging to the preS region (nucleotide positions 2762 to 177) and the S gene (nucleotide positions 248 to 738) of HBV reference sequences (retrieved from GenBank) reflecting genotypes A-H, with bootstrap values shown for main branches. HBV sequences from the panel indicate as S1-S15, and SD (IS 97/750). Woolly-Monkey HBV sequences were used as outgroups.



*this bootstrap support increased to 95 when bootstrapping was performed by Neighbor Joining method (10000 replicates)

Figure 18b: Phylogenetic tree for HBV genotype F constructed on concatenated 1128 bp belonging to the preS region (nucleotide positions 2762 to 177) and the S gene (nucleotide positions 248 to 738) of different HBV genotype F sequences (retrieved from GenBank). reflecting genotypes A-H, with bootstrap values shown for main branches. HBV Sample 14 from the panel is in bold and indicated as S14. HBV genotype H sequences were used as outgroups.

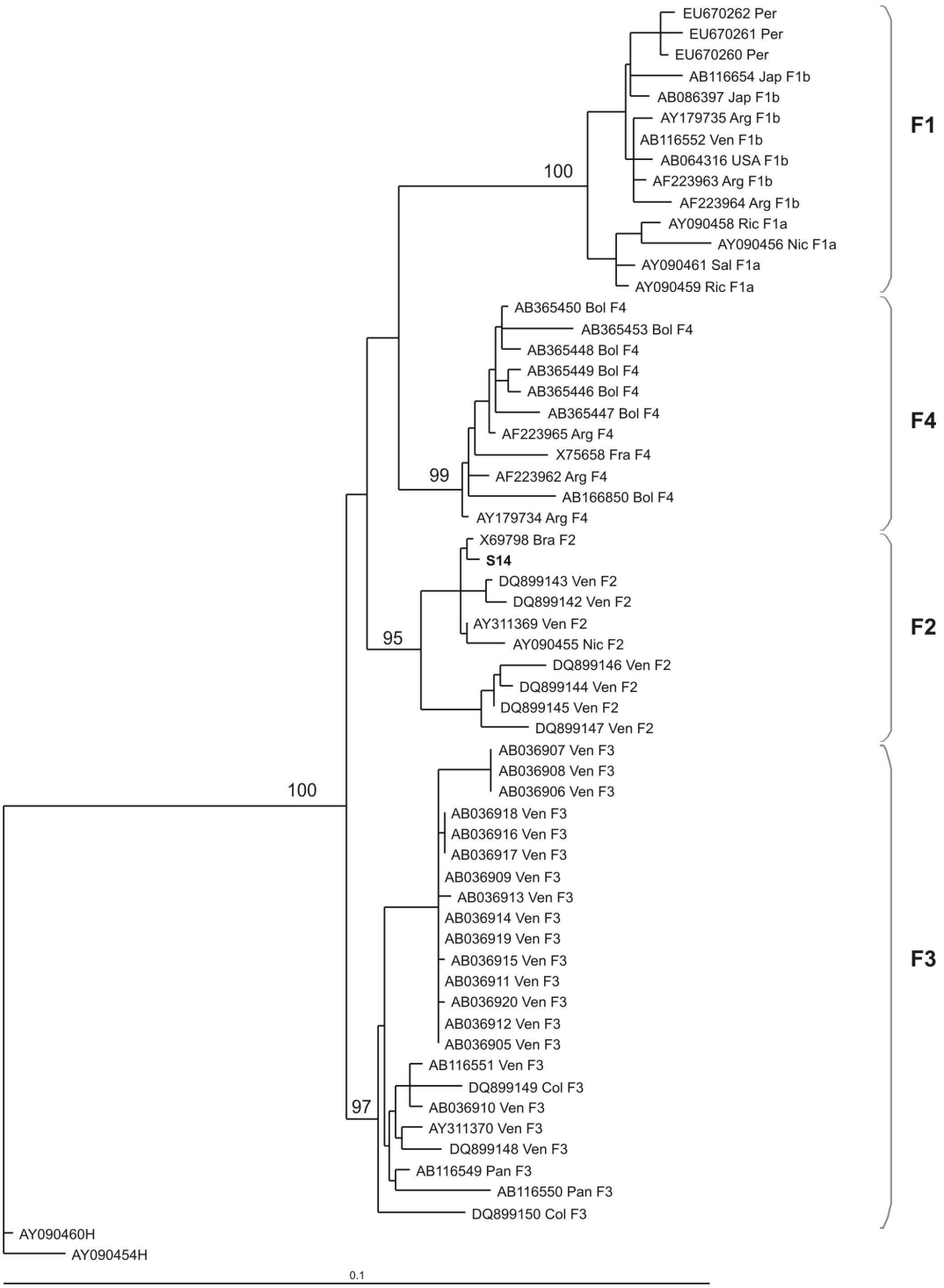


Table 1a. Characterization of the panel members

Sample No	Origin	HBsAg Subtype	HBV Genotype[1]	HBV Genotype[2]	HBV DNA* (IU/mL)[3]	HBsAg (KIU/mL)[4]	Anti-HBc[5]	HBeAg[6]	Anti-HBe[7]	Anti-HDV[8]	HIV1/HCV RNA[9]
1	South Africa	*adw2*	**A**	A1	6,08E+08	131,9	pos	pos	neg	neg	neg
2	Brazil	*adw2*	**A**	A1	6,53E+08	94,0	pos	pos	neg	neg	neg
3	Germany	*adw2*	**A**	A2	6,87E+08	74,3	pos	pos	neg	neg	neg
4	Japan	*adw2*	**B**	B2	1,48E+08	51,4	pos	pos	neg	neg	neg
5	Japan	*adw2*	**B**	B2	2,84E+08	95,3	pos	pos	neg	neg	neg
6	Viet Nam	*ayw1*	**B**	B4	6,29E+06	4,6	pos	pos	neg	neg	neg
7	Japan	*adr*	**C**	C2 (Ce)	3,99E+08	70,2	pos	pos	neg	neg	neg
8	Japan	*adr*	**C**	C2 (Ce)	1,25E+08	47,0	pos	pos	neg	neg	neg
9	Russia	*adr*	**C**	C2 (Ce)	2,92E+08	54,4	pos	pos	neg	neg	neg
10	Germany	*ayw2*	**D**	D1	1,17E+09	130,4	pos	pos	neg	neg	neg
11	South Africa	*ayw2*	**D**	D3	1,04E+08	63,8	pos	pos	neg	neg	neg
12	Iran	*ayw2*	**D**	D1	1,00E+08	17,7	pos	pos	neg	neg	neg
13	West Africa	*ayw4*	**E**	E	9,45E+08	82,6	pos	pos	neg	neg	neg
14	Brazil	*adw4*	**F**	F2	1,10E+07	32,2	pos	neg	pos	neg	neg
15	Germany	*adw2*	**G**	G	1,40E+07	0,9	pos	neg	neg	neg	neg

[1]INNO-LiPA; [2]Sequencing; [3]mean value of 4 different qNATs; [4]Architect; [5]Architect; [6]Elecsys; [7]Architect; [8]Murex Anti-Delta; [9]Procleix HIV-1/HCV Assay.

Table 1b. Assignment of HBsAg subtypes according to the amino acids analysis of the S gene

Sample No	Origin	Amino acid position				HBsAg Subtype
		122	127	134	160	
1	South Africa	Lys	Pro	Phe	Lys	*adw2*
2	Brazil	Lys	Pro	Phe	Lys	*adw2*
3	Germany	Lys	Pro	Phe	Lys	*adw2*
4	Japan	Lys	Pro	Phe	Lys	*adw2*
5	Japan	Lys	Pro	Phe	Lys	*adw2*
6	Viet Nam	Arg	Pro	Phe	Lys	*ayw1*[1]
7	Japan	Lys	Thr	Phe	Arg	*adr*
8	Japan	Lys	Pro	Phe	Arg	*adr*
9	Russia	Lys	Pro	Phe	Arg	*adr*
10	Germany	Arg	Pro	Tyr	Lys	*ayw2*
11	South Africa	Arg	Pro	Tyr	Lys	*ayw2*
12	Iran	Arg	Pro	Tyr	Lys	*ayw2*
13	West Africa	Arg	Leu	Phe	Lys	*ayw4*
14	Brazil	Lys	Leu	Phe	Lys	*adw4*
15	Germany	Lys	Pro	Tyr	Lys	*adw2*
IS	#97/750	Lys	Pro	Phe	Lys	*adw2*

[1]Sample 6 showed Ala^{159} also required for *w1* specificity.

Table 2. Panel 5086/08 - Processing of 1.2 litre bulk preparations (Sample 1 to Sample 15)

		HBV DNA (log_{10} IU/ml)		Dilution Volume (ml)	
Sample	**Genotype**	**Origin[1]**	**Panel**	**Sample Origin**	**Neg Plasma**
1	**A**	8.784	6.04	2.170	1197.830
2	**A**	8.812	6.04	2.023	1197.977
3	**A**	8.837	6.04	1.923	1198.077
4	**B**	8.171	6.04	8.895	1191.105
5	**B**	8.454	6.04	4.640	1195.360
6	**B**	6.799	4.04	2.098	1197.902
7	**C**	8.601	6.04	3.308	1196.692
8	**C**	8.098	6.04	10.544	1189.456
9	**C**	8.466	6.04	4.519	1195.481
10	**D**	9.069	6.04	1.128	1198.872
11	**D**	8.018	6.04	12.668	1187.332
12	**D**	8.001	6.04	13.160	1186.840
13	**E**	8.975	6.04	1.320	1198.680
14	**F**	7.043	5.04	11.957	1188.043
15	**G**	7.146	4.04	0.700	1199.300

[1]geometric mean values based on the results obtained by 4 different NAT tests..

Table 3. List of participants

Scientist	Affiliation
Dr Rodolfo Campos / Dr Viviana Mbayed / Dr Carolina Torres	School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina
Jennifer Chen / Linda Wong	Siemens Clinical Laboratory Berkeley, CA, USA
Dr Michael Chudy / Dr Micha Nübling	Paul Ehrlich Institute Langen, Germany
Thomas Grewing / Maren Rudolph	Qiagen GmbH Hamburg, Germany
Dr Otto Hsu / Yen Shih-Chieh	General Biologicals Corp., HsinChu, Taiwan, R.O.C.
Dr Birger Jansson / Inger Bokliden	Cepheid AB Bromma, Sweden
Dr Rosendo Jardi	Universitary Hospital Vall Hebron, Barcelona, Spain
Stephen Kerby	Center for Biologics Evaluation and Research/ Food and Drug Administration Bethesda, MD, USA
Prof Anna Kramvis / Chien-Yu Chen	University of the Witwatersrand Johannesburg, South Africa
Dr Hermann Leying / Dr Florian Boehl	Roche Diagnostics AG Rotkreuz, Switzerland
Dr Jeffrey M. Linnen	Gen-Probe Incorporated San Diego, CA, USA
Dr Saeko Mizusawa / Fumihiko Ban	National Institute of Infectious Diseases Tokyo, Japan / BML, Inc., Saitama, Japan
Dr Carolyn R Mullen	AbbottMolecular Des Plaines, IL, USA
Dr Maria Rapicetta	Istituto Superiore di Sanita Rome, Italy
Dr Ester Sabino / Dr Marcia Otani	Fundação Pró-Sangue, Hemocentro de São Paulo Sao Paulo, Brazil
Dr W. R. Willems / Prof Wolfram Gerlich	Institut für Medizinische Virologie, Justus-Liebig-Universität Gießen Gießen, Germany
Hyun-Sook Yim	Biosewoom, Inc. Seoul, Korea

Table 4. Assay protocols used by participants

Laboratory code	**Assay type (quantitative or qualitative)**	**Extraction/assay method**	**Region of HBV genome amplified**
1	quantitative	Cobas Amplicor HBV Monitor Test	pre-C / C
2	quantitative	Real-Q HBV Quantification Kit	S
3A 3B	quantitative quantitative	Cobas TaqMan HBV Test for Use with the High Pure System Cobas AmpliPrep / Cobas TaqMan HBV Test, v2.0	pre-C / C pre-C / C
4	quantitative	Cobas AmpliPrep / Cobas TaqMan HBV Test	pre-C / C
5	quantitative	Abbott RealTime HBV	S
6	quantitative	QIAamp MinElute Virus spin / HBV RealQuant PCR	X
7A 7B	quantitative quantitative	affigene HBV trender Smart HBV	n.a.
8	quantitative	In house TaqMan PCR	S
9	quantitative	In house TaqMan PCR	S
10	quantitative	Versant HBV DNA 3.0 Assay (bDNA)	n.a.
11	quantitative	QIAamp DSP Virus Kit / *artus* HBV RG PCR Kit	C
12	quantitative	Cobas AmpliPrep / Cobas TaqMan HBV Test, v2.0	pre-C / C
13	quantitative	Cobas TaqMan HBV Test for Use with the High Pure System	pre-C / C
14	quantitative	Abbott RealTime HBV	S
15	qualitative	cobas TaqScreen MPX Test	n.a.
16	qualitative	Procleix Ultrio Plus assay	n.a.
17	qualitative	QIAamp DNA Mini Kit / In house PCR/Amplicon sequencing/ Phylogenetic analysis	pre-S / S

n.a.: not available

Table 5. Laboratory mean absolute estimates for quantitative assays (log_{10} IU/ml) and for qualitative assays (log_{10} NAT detectable units/ml; rows in grey)

Laboratory	Assay Type	Sample															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	IS
1	quantitative	5.73	5.41	5.80	5.99	5.84	4.00	6.03	6.15	5.94	5.97	6.01	5.92	5.93	5.11	3.72	5.99
2	quantitative	6.57	6.35	6.16	6.31	6.02	4.41	6.41	6.42	6.16	6.30	6.31	6.37	5.91	3.54	4.30	6.30
3A	quantitative	5.85	5.63	5.42	5.88	5.74	3.74	5.88	5.95	5.88	5.94	5.90	5.79	5.70	4.29	3.62	5.66
3B	quantitative	6.32	6.06	5.99	6.34	6.03	4.15	6.28	6.38	6.28	6.12	6.38	6.35	6.28	4.69	4.10	6.01
4	quantitative	6.30	6.03	5.96	6.05	5.79	4.19	6.09	6.04	5.99	6.22	5.89	6.12	5.95	4.54	5.01	5.92
5	quantitative	6.07	5.89	5.71	6.13	5.81	4.13	5.98	6.03	5.87	5.84	5.88	5.84	5.81	5.00	3.78	5.87
6	quantitative	6.09	5.88	5.70	5.96	5.98	3.57	5.80	6.07	5.91	5.97	5.87	5.90	5.81	n.d.	2.81	5.90
7A	quantitative	6.29	6.08	6.01	5.76	5.57	4.05	6.09	6.23	6.17	5.87	6.29	6.23	6.02	5.12	3.63	6.21
7B	quantitative	6.39	6.15	6.02	5.68	5.69	4.20	6.08	6.25	6.20	6.10	6.33	6.33	6.03	5.28	3.52	6.26
8	quantitative	6.13	5.86	5.81	5.27	5.03	2.58	5.34	5.25	5.70	5.68	5.78	5.94	5.68	n.d.	2.94	6.09
9	quantitative	5.71	5.50	5.35	5.45	5.40	3.87	5.61	5.96	5.60	5.63	5.80	5.76	5.57	4.97	3.75	5.96
10	quantitative	6.17	5.97	5.81	6.29	6.05	4.13	6.07	6.20	5.98	5.99	5.94	5.87	5.82	4.86	4.07	6.05
11	quantitative	6.01	5.80	5.82	5.71	5.49	3.41	5.82	5.99	5.28	5.95	5.92	5.87	5.31	5.04	3.56	6.01
12	quantitative	6.26	6.09	6.02	6.35	6.13	4.27	6.22	6.39	6.25	6.27	6.28	6.30	6.22	4.75	4.17	6.20
13	quantitative	6.05	5.86	5.73	6.06	5.89	4.19	6.02	6.05	5.96	5.99	5.99	5.96	5.88	4.78	3.99	5.91
14	quantitative	5.98	5.75	5.64	6.00	5.59	4.14	5.89	5.98	5.80	5.74	5.81	5.86	5.79	4.97	3.85	5.84
15	qualitative	6.31	5.97	6.27	6.31	6.80	3.88	6.31	6.61	6.31	6.95	6.61	6.61	6.31	4.95	3.88	6.31
16	qualitative	6.34	6.00	5.95	5.68	5.95	4.20	6.07	6.37	6.40	6.03	5.86	6.20	6.19	5.06	4.00	6.26

n.d.: not detected.

Table 6. Overall laboratory mean estimates for quantitative assays (log_{10} IU/ml) and qualitative assays (log_{10} NAT detectable units/ml; column in grey)

Sample	N	Overall Mean (log_{10} IU/ml)	95%-Confidence Intervals (log_{10} IU/ml)		Standard Deviation	Min	Max	Range	Mean of qualitative assays[6]
1	16	6.12	6.00	6.25	0.24	5.71	6.57	0.86	6.33
2	16	5.89	5.76	6.03	0.24	5.41	6.35	0.95	5.99
3	16	5.81	5.69	5.93	0.22	5.35	6.16	0.81	6.11
4	16	5.95	5.78	6.12	0.32	5.27	6.35	1.08	5.99
	15[1]	*6.00*	*5.85*	*6.14*	*0.27*	*5.45*	*6.35*	*0.90*	
5	16	5.75	5.60	5.91	0.29	5.03	6.13	1.10	6.38
	15[1]	*5.80*	*5.68*	*5.92*	*0.22*	*5.40*	*6.13*	*0.73*	
6	16	3.94	3.70	4.18	0.45	2.58	4.41	1.82	4.04
	14[2]	*4.07*	*3.95*	*4.20*	*0.22*	*3.57*	*4.41*	*0.84*	
7	16	5.98	5.84	6.11	0.26	5.34	6.41	1.08	6.19
	15[1]	*6.02*	*5.91*	*6.13*	*0.20*	*5.61*	*6.41*	*0.80*	
8	16	6.08	5.94	6.23	0.27	5.25	6.42	1.17	6.49
	15[1]	*6.14*	*6.05*	*6.23*	*0.16*	*5.95*	*6.42*	*0.47*	
9	16	5.94	5.80	6.08	0.26	5.28	6.28	1.01	6.36
	15[3]	*5.98*	*5.87*	*6.09*	*0.20*	*5.60*	*6.28*	*0.68*	
10	16	5.97	5.87	6.08	0.20	5.63	6.30	0.67	6.49
11	16	6.02	5.91	6.14	0.21	5.78	6.38	0.60	6.24
12	16	6.03	5.91	6.14	0.22	5.76	6.37	0.60	6.40
13	16	5.86	5.73	5.98	0.24	5.31	6.28	0.98	6.25
	15[3]	*5.89*	*5.79*	*6.00*	*0.19*	*5.57*	*6.28*	*0.71*	
14	14	4.78	4.53	5.03	0.44	3.54	5.28	1.74	5.01
	13[4]	*4.88*	*4.71*	*5.04*	*0.27*	*4.29*	*5.28*	*0.99*	
15	16	3.80	3.53	4.08	0.52	2.81	5.01	2.20	3.94
	13[5]	*3.85*	*3.70*	*4.01*	*0.25*	*3.52*	*4.30*	*0.78*	
IS	16	6.01	5.92	6.10	0.17	5.66	6.30	0.64	6.29

[1]excluding laboratory 8; [2]excluding laboratory 8 and 11; [3]excluding laboratory 11; [4]excluding laboratory 2; [5]excluding laboratories 4, 6 and 8; [6]2 estimates (laboratory 15 and 16).

Table 7. Laboratory mean estimates (log_{10} IU/ml) relatively to concurrent tested 97/750

Laboratory	Assay Type	Sample														
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
1	quantitative	5.74	5.42	5.81	6.00	5.85	4.01	6.04	6.16	5.95	5.98	6.02	5.93	5.94	5.12	3.73
2	quantitative	6.27	6.05	5.86	6.01	5.72	4.11	6.11	6.12	5.86	6.00	6.01	6.07	5.61	3.24	4.00
3A	quantitative	6.19	5.97	5.76	6.22	6.08	4.08	6.22	6.29	6.22	6.28	6.24	6.13	6.04	4.63	3.96
3B	quantitative	6.31	6.05	5.98	6.33	6.02	4.14	6.27	6.37	6.27	6.11	6.37	6.34	6.27	4.68	4.09
4	quantitative	6.38	6.11	6.04	6.13	5.87	4.27	6.17	6.12	6.07	6.30	5.97	6.20	6.03	4.62	5.09
5	quantitative	6.20	6.02	5.84	6.26	5.94	4.26	6.11	6.16	6.00	5.97	6.01	5.97	5.94	5.13	3.91
6	quantitative	6.19	5.98	5.80	6.06	6.08	3.67	5.90	6.17	6.01	6.07	5.97	6.00	5.91	n.d.	2.91
7A	quantitative	6.08	5.87	5.80	5.55	5.36	3.84	5.88	6.02	5.96	5.66	6.08	6.02	5.81	4.91	3.42
7B	quantitative	6.13	5.89	5.76	5.42	5.43	3.94	5.82	5.99	5.94	5.84	6.07	6.07	5.77	5.02	3.26
8	quantitative	6.04	5.77	5.72	5.18	4.94	2.49	5.25	5.16	5.61	5.59	5.69	5.85	5.59	n.d.	2.85
9	quantitative	5.75	5.54	5.39	5.49	5.44	3.91	5.65	6.00	5.64	5.67	5.84	5.80	5.61	5.01	3.79
10	quantitative	6.12	5.92	5.76	6.24	6.00	4.08	6.02	6.15	5.93	5.94	5.89	5.82	5.77	4.81	4.02
11	quantitative	6.00	5.79	5.81	5.70	5.48	3.40	5.81	5.98	5.27	5.94	5.91	5.86	5.30	5.03	3.55
12	quantitative	6.06	5.89	5.82	6.15	5.93	4.07	6.02	6.19	6.05	6.07	6.08	6.10	6.02	4.55	3.97
13	quantitative	6.14	5.95	5.82	6.15	5.98	4.28	6.11	6.14	6.05	6.08	6.08	6.05	5.97	4.87	4.08
14	quantitative	6.14	5.91	5.80	6.16	5.75	4.30	6.05	6.14	5.96	5.90	5.97	6.02	5.95	5.13	4.01
15	qualitative	6.00	5.66	5.96	6.00	6.49	3.57	6.00	6.30	6.00	6.64	6.30	6.30	6.00	4.64	3.57
16	qualitative	6.08	5.74	5.69	5.42	5.69	3.94	5.81	6.11	6.14	5.77	5.60	5.94	5.93	4.80	3.74

n.d.: not detected.

Table 8. Overall mean estimates (log_{10} IU/ml) relative to concurrently tested WHO IS (97/750)

Sample	N	Overall Mean (log_{10} IU/ml)	95%-Confidence Intervals (log_{10} IU/ml)		Standard Deviation	Min	Max	Range
1	18	6.10	6.02	6.18	0.16	5.74	6.38	0.64
2	18	5.86	5.77	5.95	0.18	5.42	6.11	0.69
3	18	5.80	5.73	5.87	0.14	5.39	6.04	0.65
4	18	5.92	5.74	6.09	0.35	5.18	6.33	1.15
	17[1]	*5.96*	*5.80*	*6.12*	*0.31*	*5.42*	*6.33*	*0.91*
5	18	5.78	5.61	5.96	0.35	4.94	6.49	1.55
	17[1]	*5.83*	*5.68*	*5.98*	*0.29*	*5.36*	*6.49*	*1.13*
6	18	3.91	3.69	4.12	0.43	2.49	4.30	1.81
	16[2]	*4.03*	*3.92*	*4.14*	*0.21*	*3.57*	*4.30*	*0.73*
7	18	5.96	5.84	6.08	0.24	5.25	6.27	1.02
	17[1]	*6.00*	*5.91*	*6.08*	*0.17*	*5.65*	*6.27*	*0.62*
8	18	6.09	5.96	6.21	0.25	5.16	6.37	1.21
	17[1]	*6.14*	*6.09*	*6.20*	*0.11*	*5.98*	*6.37*	*0.39*
9	18	5.94	5.82	6.06	0.23	5.27	6.27	1.00
	17[3]	*5.98*	*5.89*	*6.07*	*0.17*	*5.61*	*6.27*	*0.66*
10	18	5.99	5.86	6.12	0.25	5.59	6.64	1.05
11	18	6.01	5.91	6.10	0.19	5.60	6.37	0.77
12	18	6.03	5.95	6.10	0.15	5.80	6.34	0.54
13	18	5.86	5.75	5.97	0.22	5.30	6.27	0.97
	17[3]	*5.89*	*5.80*	*5.98*	*0.18*	*5.59*	*6.27*	*0.68*
14	16	4.76	4.52	5.00	0.45	3.24	5.13	1.89
	15[4]	*4.86*	*4.75*	*4.98*	*0.20*	*4.55*	*5.13*	*0.58*
15	18	3.78	3.53	4.02	0.50	2.85	5.09	2.24
	14[5]	*3.85*	*3.72*	*3.97*	*0.21*	*3.42*	*4.09*	*0.67*

Combined results from quantitative and qualitative assays.
[1]excluding laboratory 8; [2]excluding laboratory 8 and 11; [3]excluding laboratory 11;
[4]excluding laboratory 2; [5]excluding laboratories 4, 6, 7B and 8.

Table 9. Coefficients of Variation (CV) for quantitative assays as percentage for overall uncertainty, inter-laboratory precision, inter-assay precision and intra-assay precision

Sample	Uncertainty (CV%)	Inter-Laboratory Precision (CV%)	Inter-Assay Precision (CV%)	Intra-Laboratory Precision (CV%)
1	4.5%	3.7%	0.6%	2.4%
2	4.7%	4.0%	0.3%	2.3%
3	4.1%	3.7%	n.a.	1.8%
4	5.7%	5.2%	0.7%	2.2%
4[1]	*4.9%*	*4.4%*	*0.6%*	*2.2%*
5	5.2%	5.0%	n.a.	1.5%
5[1]	*4.1%*	*3.8%*	*0.2%*	*1.6%*
6	11.8%	11.3%	n.a.	3.5%
6[2]	*5.9%*	*5.3%*	*0.3%*	*2.5%*
7	4.6%	4.3%	n.a.	1.5%
7[1]	*3.6%*	*3.3%*	*n.a.*	*1.6%*
8	4.8%	4.4%	0.1%	1.8%
8[1]	*3.1%*	*2.6%*	*n.a.*	*1.8%*
9	4.6%	4.4%	n.a.	1.3%
9[3]	*3.6%*	*3.3%*	*<0.1%*	*1.3%*
10	4.2%	3.1%	0.2%	2.7%
11	3.9%	3.5%	n.a.	1.7%
12	3.9%	3.6%	0.3%	1.4%
13	4.3%	4.0%	0.4%	1.6%
13[3]	*3.5%*	*3.2%*	*0.2%*	*1.4%*
14	9.6%	9.1%	1.2%	2.7%
14[4]	*6.0%*	*5.4%*	*1.0%*	*2.5%*
15	14.4%	13.4%	1.0%	5.4%
15[5]	*7.4%*	*6.4%*	*0.1%*	*3.7%*
IS	3.1%	2.7%	0.1%	1.4%

[1]excluding laboratory 8; [2]excluding laboratory 8 and 11; [3]excluding laboratory 11; [4]excluding laboratory 2; [5]excluding laboratories 4, 6 and 8.
n.a.: not assessable.

Appendix 1: Collaborative Study Protocol

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

- STUDY PROTOCOL -

Objective

The purpose of the Collaborative Study is to evaluate a plasma panel of different Hepatitis B Virus (HBV) genotypes using nucleic acid amplification technique (NAT) assays. The study includes the parallel testing of the 2nd International Standard for HBV DNA (97/750).

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, which is represented by the current WHO International Standard. 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 genotypes of HBV. 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 HBV NAT assays has now been prepared (lyophilized material). It consists of 15 members and covers the most prevalent HBV genotypes (A-G).
The study is designed to test the panel samples (15 lyophilized preparations) concurrently with the WHO International Standard (97/750). Where possible we would encourage laboratories to use quantitative methods, reporting results in IU/ml of HBV DNA. If results are reported in copies/ml, the assay-specific conversion factor copies to IU should be provided. However, data from qualitative assays is acceptable.

Materials

Fifteen HBV positive lyophilized plasma preparations, representing HBV genotypes A – G, and the 2nd WHO International Standard for HBV RNA (97/750), previously assigned a unitage of 1 x 10^6 IU/ml. 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 (quantitative assays) or four vials (qualitative assays) of each study sample preparation. All samples should be stored frozen at -20 ºC on receipt.

Samples 1 to 15 are lyophilized preparations in rubber stoppered, 4-ml screw-cap glass vials. The 2nd WHO International Standard 97/750 is a lyophilized preparation in a 2 ml glass vial with a tear off crimp seal.

Each vial should be reconstituted in 0.5 ml of deionised, nuclease-free, molecular biology grade water and left for a minimum of 20 minutes with occasional agitation before use.

Participants are requested to perform testing (and as appropriate, dilutions) of these reagents in three independent assay runs (quantitative assays) and four independent assay runs (qualitative assays), respectively (for details see below). A fresh vial of each reagent should be used in each assay run. All dilutions should be carried out in the diluent (e.g. HBV DNA negative plasma) normally used in the assay system and this should be recorded on the result form.

For quantitative assays

Where participants have access to quantitative HBV DNA assays, we suggest testing dilutions that fall within the linear range of the assay. The titre of the WHO IS 97/750 is 1 x 10^6 IU/ml. The expected concentration range of the Samples 1 to 5, and 7 to 13 is approximately 10^6 IU/ml; the expected concentration range of Sample 6, 14, and 15 is approximately 10^4 to 10^5 IU/ml. We propose testing of the materials using a common dilution factor (e. g. a dilution of 10^{-2}). Participants are requested to test each sample in duplicate in each assay run, reporting values for each single replicate performed (the complete NAT procedure for each replicate, including extraction through to amplification and detection).

For qualitative assays

In the first run participants should assay ten-fold dilutions of each preparation in order to determine the HBV DNA end-point. We suggest starting the testing with a dilution of 10^{-2} for all preparations.

In the three remaining runs, samples should be treated as follows: Participants are requested to assay a minimum of two half $\log_{10}$ dilutions either side of the pre-determined end-point. It is therefore not necessary to carry out more than five half $\log_{10}$ dilutions (centred around the estimated end-point) unless individual labs wish to do so. Whenever possible, samples should be tested in duplicate per dilution.

Evaluation of results

Results of each assay should be recorded on the appropriate results form included with this information sheet.

Please indicate on each 'Reporting Sheet' the Laboratory and Name of Investigator.

All completed forms should be returned preferably by email by **April 30th, 2009**:

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 be submitted to the WHO Expert Committee on Biological Standardization for adoption in July 2009.

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Attachment

- Instructions for Use for 97/750
- Important Notice – Storage Conditions of the Study Samples

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

Method Reporting Sheet

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

Short description of in-house NAT / Test Kit

Qualitative ☐ Quantitative ☐

Conversion factor IU vs copies:

Amplified region:

Volume of plasma used for nucleic acid extraction:

Elution volume of extracted nucleic acid:

Volume of extracted nucleic acid used for amplification:

Diluent used in the study:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 1 (Quantitative)

Laboratory:	Name of Investigator:

Test Run 1	Date of Assay:		
Reagent	Dilution(10^{-x})	Results (IU/ml copies/ml)*	
Sample 1			
Sample 2			
Sample 3			
Sample 4			
Sample 5			
Sample 6			
Sample 7			
Sample 8			
Sample 9			
Sample 10			
Sample 11			
Sample 12			
Sample 13			
Sample 14			
Sample 15			
97/750			

*Please indicate

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 2 (Quantitative)

Laboratory:	Name of Investigator:

Test Run 2	Date of Assay:		
Reagent	Dilution(10^{-x})	Results (IU/ml copies/ml)*	
Sample 1			
Sample 2			
Sample 3			
Sample 4			
Sample 5			
Sample 6			
Sample 7			
Sample 8			
Sample 9			
Sample 10			
Sample 11			
Sample 12			
Sample 13			
Sample 14			
Sample 15			
97/750			

*Please indicate

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 3 (Quantitative)

Laboratory:	Name of Investigator:

Test Run 3	Date of Assay:		
Reagent	Dilution(10^{-x})	Results (IU/ml copies/ml)*	
Sample 1			
Sample 2			
Sample 3			
Sample 4			
Sample 5			
Sample 6			
Sample 7			
Sample 8			
Sample 9			
Sample 10			
Sample 11			
Sample 12			
Sample 13			
Sample 14			
Sample 15			
97/750			

*Please indicate

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 1 (Qualitative)

Laboratory:	Name of Investigator:
Date of Assay:	

Test Run 1: Qualitative Test/Estimation of Putative End-Point (ten-fold dilutions; results pos or neg)

Material / Dilution							
Sample 1							
Sample 2							
Sample 3							
Sample 4							
Sample 5							
Sample 6							
Sample 7							
Sample 8							
Sample 9							
Sample 10							
Sample 11							
Sample 12							
Sample 13							
Sample 14							
Sample 15							
97/750							

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 2 (Qualitative)

Laboratory:	Name of Investigator:
Date of Assay:	

Test Run 2: Qualitative Test/ five half $\log_{10}$ dilutions (replicates; results pos or neg)

Material / Dilution					
Sample 1					
Sample 2					
Sample 3					
Sample 4					
Sample 5					
Sample 6					
Sample 7					
Sample 8					
Sample 9					
Sample 10					
Sample 11					
Sample 12					
Sample 13					
Sample 14					
Sample 15					
97/750					

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 3 (Qualitative)

Laboratory:	Name of Investigator:
Date of Assay:	

Test Run 3: Qualitative Test/ five half $\log_{10}$ dilutions (replicates; results pos or neg)

Material / Dilution					
Sample 1					
Sample 2					
Sample 3					
Sample 4					
Sample 5					
Sample 6					
Sample 7					
Sample 8					
Sample 9					
Sample 10					
Sample 11					
Sample 12					
Sample 13					
Sample 14					
Sample 15					
97/750					

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Data Reporting Sheet 4 (Qualitative)

Laboratory:	Name of Investigator:
Date of Assay:	

Test Run 4: Qualitative Test/ five half $\log_{10}$ dilutions (replicates; results pos or neg)

Material / Dilution					
Sample 1					
Sample 2					
Sample 3					
Sample 4					
Sample 5					
Sample 6					
Sample 7					
Sample 8					
Sample 9					
Sample 10					
Sample 11					
Sample 12					
Sample 13					
Sample 14					
Sample 15					
97/750					

Additional details and/or comments:

Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

PACKAGE RECEIPT FORM

Name of Investigator:

Laboratory:

Date of arrival of package:

Parcel damaged	Yes	No
Sample container broken	Yes	No

Additional comments:

Please send this form to Dr. Michael Chudy by email (chumi@pei.de)
or by fax +49 6103 77 1280.

Appendix 2: Draft "Instructions for Use"

Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
for Quality Assurance of Blood Products and
in vitro Diagnostic Devices

1st WHO International Reference Panel for Hepatitis B Virus Genotypes for Nucleic Acid Amplification Techniques -Based Assays
PEI code 5086/08
(Version 1.0, 29th July 2009)

1. **INTENDED USE**

The current WHO International Standard material for HBV DNA (97/750) was generated from genotype A2/HBsAg subtype *adw2*. This HBV genotype is mainly prevalent in Western Europe and in North America and represent only 1% of the worldwide HBV-infected population. The majority of the HBV-infected people living in or coming from the Mediterranean area, Africa and Asia have the genotypes A1, B, C, D, and E, whereas F and H originate from the Americas. The origin of genotype G is not yet known. There was a need to develop a reference panel of well characterized samples representing the different HBV genotypes to investigate the commutability of the current standard material (97/750) in relation to the other HBV genotypes.

The 1st WHO International Reference Panel for HBV Genotypes for nucleic acid amplification technique (NAT)-based assays (PEI code number 5086/08) was established by the Expert Committee on Biological Standardization of the WHO in October 2009. The reference panel consists of 15 lyophilized HBV positive plasma samples and covers the most prevalent HBV genotypes: Samples 1-3 (genotype A), Samples 4-6 (genotype B), Samples 7-9 (genotype C), Samples 10-12 (genotype D), Sample 13 (genotype E), Sample 14 (genotype F), and Sample 15 (genotype G). The panel has been evaluated in an international collaborative study with concurrent testing of the 2nd WHO International Standard for HBV DNA (97/750). The reference panel is intended for the control and/or for the validation of HBV NAT assays.

2. **UNITAGE**

No unitage is assigned to individual panel members.

3. **CONTENTS**

Each vial contains 0.5 ml of lyophilized plasma containing infectious HBV. Each of the high titre HBV positive plasma stocks have been diluted in a plasma pool negative tested for the following markers: HIV-1 RNA, HCV RNA, HBV DNA, HBsAg, anti-HBs, anti-HBc (IgG and IgM), anti-HIV-1/2, and anti-HCV. The Table (see page 3) provides the statistical data for each panel member from the outcome of the collaborative study (overall arithmetic mean estimates of $\log_{10}$ IU/ml values, confidence intervals, standard deviation, minimum and maximum values, and ranges). The values were obtained by comparison testing with the 2nd International Standard for HBV DNA (97/750). The results of the collaborative study based on 16 data sets for quantitative assays and 2 data sets for qualitative assays. Further details of the collaborative study are available in the report WHO/BS/YY.XXXX.

4. **CAUTION**

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains material of human origin, and contains infectious HBV. The reference materials has been diluted in human plasma negative for HIV-1 RNA, HCV RNA, HBV DNA, HBsAg, anti-HBs, anti-HBc (IgG and IgM), anti-HIV-1/2, and anti-HCV.

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 probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. **USE OF MATERIAL**

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

The material is supplied lyophilized and should be stored at or below-20ºC. The panel members should be reconstituted in 0.5 ml of sterile nuclease-free water. If all the material is not used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at or below -70ºC.

6. **STABILITY**

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended.

The reference materials are held at PEI within assured, temperature-controlled storage facilities. Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact PEI.

7. **REFERENCES**

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

WHO ECBS report. 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

Baylis SA, Heath AB, Chudy M, Pisani G, Klotz A, Kerby S, W. Gerlich W. An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid

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amplification technology-based assays. *Vox Sang* 2008, **94**:358-362

Collaborative Study to Establish a World Health Organization International Genotype Panel for HBV Nucleic Acid Amplification Technology (NAT) –Based Assays. WHO Report 2009, WHO/BS/YY.XXXX

8. ACKNOWLEDGEMENTS
We are very grateful to the Institute of Medical Virology, University Giessen, Germany; to the Department of Epidemiology, Infectious Disease Control and Prevention, Hiroshima University, Japan; to the Federal Blood Center, Moscow, Russia; to Fundação Pró-Sangue Homocentro de São Paulo, Brazil; to the Institute of Transfusion Medicine and Immunohematology, German Red Cross, Frankfurt/Main, Germany; to the Iranian Blood Transfusion Organization, Tehran, Iran; and the South African National Blood Service for supplying the candidate materials and to the participants in the collaborative study.

9. FURTHER INFORMATION
This material: whoccivd@pei.de
WHO Biological Reference Preparations:
http://www.who.int/biologicals/en/

10. 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 whoccivd@pei.de

11. CITATION
In any circumstance where the recipient publishes a reference to PEI materials, it is important that the title of the preparation and the PEI code number, and the name and address of PEI are cited correctly.

12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)		
Physical appearance	Lyophilized powder	
Fire hazard	None	
Chemical properties		
Stable	Yes	Corrosive:No
Hygroscopic	No	Oxidising:No
Flammable	No	Irritant: No
Other (specify) CONTAINS HUMAN PLASMA & INFECTIOUS HEPATITIS B VIRUS (HBV)		
Handling:	See caution, section 4	
Toxicological properties		
Effects of inhalation:	Avoid – *contains infectious HBV*	
Effects of ingestion:	Avoid – *contains infectious HBV*	
Effects of skin absorption:	Avoid – *contains infectious HBV*	
Suggested First Aid		
Inhalation	Seek medical advice - *contains infectious HBV*	
Ingestion	Seek medical advice - *contains infectious HBV*	
Contact with eyes	Wash thoroughly with water. Seek medical advice – *contains infectious HBV*	
Contact with skin	Wash thoroughly with water. Seek medical advice – *contains infectious HBV*	
Action on Spillage and Method of Disposal		
Spillage of vial 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.		

13. LIABILITY AND LOSS
Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependent on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

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Table Results from the Collaborative Study to Evaluate a Hepatitis B Virus Genotype Panel

Sample	N	Overall Mean (log_{10} IU/ml)	95%-Confidence Intervals (log_{10} IU/ml)		Standard Deviation	Min	Max	Range
1/A	**18**	**6.10**	**6.02**	**6.18**	**0.16**	**5.74**	**6.38**	**0.64**
2/A	**18**	**5.86**	**5.77**	**5.95**	**0.18**	**5.42**	**6.11**	**0.69**
3/A	**18**	**5.80**	**5.73**	**5.87**	**0.14**	**5.39**	**6.04**	**0.65**
4/B	**18**	**5.92**	**5.74**	**6.09**	**0.35**	**5.18**	**6.33**	**1.15**
5/B	**18**	**5.78**	**5.61**	**5.96**	**0.35**	**4.94**	**6.49**	**1.55**
	17[1]	*5.74*	*5.58*	*5.90*	*0.31*	*4.94*	*6.08*	*1.14*
6/B	**18**	**3.91**	**3.69**	**4.12**	**0.43**	**2.49**	**4.30**	**1.81**
	17[1]	*3.99*	*3.86*	*4.12*	*0.26*	*3.40*	*4.30*	*0.90*
7/C	**18**	**5.96**	**5.84**	**6.08**	**0.24**	**5.25**	**6.27**	**1.02**
8/C	**18**	**6.09**	**5.96**	**6.21**	**0.25**	**5.16**	**6.37**	**1.21**
	15[1]	*6.14*	*6.09*	*6.20*	*0.11*	*5.98*	*6.37*	*0.39*
9/C	**18**	**5.94**	**5.82**	**6.06**	**0.23**	**5.27**	**6.27**	**1.00**
10/D	**18**	**5.99**	**5.86**	**6.12**	**0.25**	**5.59**	**6.64**	**1.05**
	17[1]	*5.95*	*5.85*	*6.05*	*0.20*	*5.59*	*6.30*	*0.71*
11/D	**18**	**6.01**	**5.91**	**6.10**	**0.19**	**5.60**	**6.37**	**0.77**
	17[1]	*5.99*	*5.90*	*6.08*	*0.18*	*5.60*	*6.37*	*0.77*
12/D	**18**	**6.03**	**5.95**	**6.10**	**0.15**	**5.80**	**6.34**	**0.54**
	17[1]	*6.01*	*5.94*	*6.08*	*0.14*	*5.80*	*6.34*	*0.54*
13/E	**18**	**5.86**	**5.75**	**5.97**	**0.22**	**5.30**	**6.27**	**0.97**
14/F	**16**	**4.76**	**4.52**	**5.00**	**0.45**	**3.24**	**5.13**	**1.89**
	15[1]	*4.86*	*4.75*	*4.98*	*0.20*	*4.55*	*5.13*	*0.58*
15/G	**18**	**3.78**	**3.53**	**4.02**	**0.50**	**2.85**	**5.09**	**2.24**
	15[2]	*3.81*	*3.66*	*3.95*	*0.26*	*3.26*	*4.09*	*0.83*

Overall mean estimates (log_{10} IU/ml) relative to concurrently tested 2nd International Standard (97/750). Results of quantitative and qualitative assays combined. Further details of the collaborative study are available in the report WHO/BS/YY.XXXX.
[1] – excluding data from 1 laboratory; [2] – excluding data from 3 laboratories

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