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Report of the WHO collaborative study to establish the First International Standard for detection of antibodies to hepatitis B virus e antigen (anti-HBe)

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Note:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **4 October 2013** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Department of Essential Medicines and Health Products (EMP). Comments may also be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: *padillaa@who.int* with a copy to David Wood at email: *woodd@who.int*

Summary

A WHO Collaborative Study was undertaken to assess the suitability of a candidate International Standard (B1) for detection of antibodies to hepatitis B virus e antigen (anti-HBe) in diagnostic assays involving 21 laboratories from 12 different countries with 16 different anti-HBe test kits. The potency was determined against the current Paul-Ehrlich-Institut (PEI) reference serum 82 anti-HBe IgG (B2).

The overall potency of the candidate International Standard B1 relative to sample B2 was 119.2 IU/ml in 14 test kits with a competitive test format. Two test kits with an indirect test format showed low dilutional sensitivity with the PEI reference material B2 so that the ratio relative to candidate standard B1 differed significantly.

Supplemental samples B3, B4, AB5, B6 and B7 were tested to demonstrate commutability with candidate standard B1. When converted in units relative to the candidate standard B1 the anti-HBe results, including the two test kits with indirect test format, were mainly within a range of factor ±2 which was more comparable than with the assay´s absolute values (s/co, co/s, inhibition). In addition, anti-HBe showed similar dose-response-characteristics across the 16 anti-HBe test kits.

A complementary investigation at PEI with five selected anti-HBe test kits including one anti-HBe test kit with indirect test format showed correlation of analytical sensitivity for candidate standard B1 with diagnostic sensitivity in terms of the total number of positive samples detected in 3 HBV seroconversion panels and 6 HBV longitudinal panels.

Intra-assay variability of candidate standard B1 at cut-off expressed by geometric coefficients of variation (GVC) was in most cases less than 20%. Inter-laboratory variability (same test kits in various laboratories) was GCV <33%.

Accelerated stability examination with the candidate standard B1 at up to 37°C until 3 months so far indicates long-term stability when stored at the recommended storage temperature -20°C.

In conclusion, the candidate material (B1) is proposed to be established as the 1st International Standard for anti-HBe with the code number 129095/12 and a proposed potency of 120 International Units per ml. The standard will be of value for determination of analytical assay sensitivity, for calibration of anti-HBe test kits, and for quality control.

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1 Introduction

Hepatitis B virus (HBV) infection is a serious global health problem affecting two billion people worldwide. It may lead to acute, fulminant or chronic hepatitis associated with liver cirrhosis or hepatocellular carcinoma. About one million people die every year due to the infection with the virus (1-2). The chain of HBV transmission is maintained partly by chronically infected HBV carriers with 350 million people worldwide. The diagnosis of HBV infection requires a combination of various assays. The first stage in the course of HBV infection is characterized by the presence of HBsAg, HBeAg, and anti-HBc IgM followed by anti-HBc IgG which may last for decades. In the intermediate stage, patients lose HBeAg, develop anti-HBe, and often enter into clinical remission. Finally, loss of HBsAg and rise of the anti-HBs indicates recovery from infection. Viraemia is highest during the HBeAg phase of infection, declines during the anti-HBe phase, and disappears at the anti-HBs phase. Also in the course of chronic HBV infection, loss of serum HBeAg and development of anti-HBe marks transition from the immune-active phase of disease to the inactive carrier state. As a result, anti-HBe seroconversion is an important therapeutic endpoint for patient treatment against HBV infection (3). In addition, detection of anti-HBe may be useful as evidence suggesting against a false-positive anti-HBc result. With regards to sensitivity for anti-HBe, switch from HBeAg to anti-HBe is expected to be reliably detected in recent infection. For chronic HBV infection it has been reported that anti-HBe test kits have low sensitivity (4).

A proposal by the Paul-Ehrlich-Institut (PEI) to prepare a standard for anti-HBe assays was endorsed by the WHO Expert Committee on Biological Standardization (ECBS) in October 2011. In an initial study at PEI, various anti-HBe positive plasma donations were selected for further development as a candidate International Standard for the WHO. The aim of the international collaborative study was to establish an international anti-HBe standard in terms of international units for analytical sensitivity determination of anti-HBe, for calibration of anti-HBe test kits and for quality control. Because the PEI anti-HBe reference serum 82 has already been used for long time by many manufacturers worldwide for calibration of their kits in PEI units/ml, the candidate standard was evaluated relative to this standard.

2 Materials

2.1 Sample B1: Candidate material anti-HBe, code 129095/12, for the 1st international anti-HBe standard

B1 is a freeze dried pool of anti-HBe positive plasma collected in 2009 from three Asian females (each donation 600 ml), code number 129095/12, purchased from Trina Bioreactives AG (8606 Nänikon, Switzerland). Each plasma unit was pre-characterized at PEI before pooling and found anti-HBe positive at least to a dilution of 1:1024 in six different anti-HBe assays. The plasma units were positive for HBsAg and total anti-HBc and negative for HBeAg, anti-HBc IgM, and anti-HBs. In addition, the material tested negative for anti-HIV 1/2, anti-HCV, HIV RNA and HCV RNA, and positive for HBV-DNA. In total 1.2 L plasma (200 ml plasma from each donor is held back as original) was pooled and filled in 3 ml ampoules (neutral amber glass with a 15.5 mm screw cap of polypropylene and a 14 mm freeze dry rubber stopper) as 0.5 ml aliquots subsequently freeze-dried at Greiner Diagnostics AG (4900 Langenthal, Switzerland) in 2012 following documented procedures. No stabilizers or preservatives were added. As a result, 2005 freeze-dried ampoules were produced and stored at -20°C. The ampoule has residual moisture of 1.7% and a coefficient of variation (CV) of 1.2% for the wet fill. Comparative testing of the pooled and freeze dried material revealed no significant differences in signal to cut-off ratios (s/co) values when tested with the same test kits as before (data not shown). Participants were requested to reconstitute the freeze dried material with 0.5 ml distilled water.

2.2 Sample B2: PEI reference serum 82 anti-HBe IgG

This serum was collected by the Paul-Ehrlich-Institut, Langen/Germany in 1982 from human blood donors and is stored as liquid at -70°C in glass ampoules. Each ampoule contains 0.5 ml material and has an assigned anti-HBe unitage of 100 PEI units per ml. The material is positive for HBsAg and anti-HBc IgG, and negative for anti-HBc IgM, anti-HBs and HBeAg. The material was also found negative for anti-HIV 1/2 and anti-HCV. Corrigendum: In contrast to the study plan sent out to the participants, the material is not positive for anti-HCV or HCV antigen.

2.3 Samples B3, B4, AB5, B6 and B7

These samples were used to compare the results of the candidate standard with other positive anti-HBe sample material. The samples were purchased from Trina Bioreactives AG and represent human plasma donated in 2010 from different male donors from Africa (each 50 ml). B3 and B4 are from one donor diluted 1:500 (B3) and 1:250 (B4), B6 and B7 are from a different donor also diluted 1:500 (B6) and 1:250 (B7). Both donations were diluted in normal human serum (NHS). Samples B3, B4, B6 and B7 are positive for HBsAg and HBc total, and negative for anti-HBc IgM, anti-HBs and HBeAg. Sample AB5 is a native anti-HBe low positive sample from another donor who was also positive for HBeAg. All samples were pre-tested at PEI with four different anti-HBe assays. All samples are negative for anti-HIV 1/2 and anti-HCV. The materials are liquid and stored frozen at -70°C (original material) or -20°C (working aliquots) without preservatives added.

3 Design of study

Participants received the samples and an accompanying study plan. They were asked to report the specifics of the assays performed, and to submit the raw data along with the corresponding cut-off value and the values for the dilution matrix. The study plan included the following:

- From each of the samples B1 and B2 eight dilutions should be prepared and tested in the range as shown in the result's sheets, i.e. for sample B1 from 1:32 to 1:4096 and for sample B2 from 1:50 to 1:6400 which were the dilution ranges found adequate by the previous feasibility study. Samples B1 and B2 were requested to be tested in each anti-HBe assay in triplicate independently on 3 three different days by using a fresh ampoule each day.
- The dilution matrix normally in use in the participant's laboratory should be used. Normal human serum negative for anti-HBe and HBeAg is appropriate. If NHS is not available, alternatively fetal calf serum (FCS) may be used. The dilution matrix should also be tested in triplicate in every independent run as a control.
- Samples B3, B4, AB5, B6 and B7 should be tested neat without any dilution in triplicates on one day.
- In the case that particulate matter appears, the samples should be centrifuged for 10-15 minutes at 3000 g prior to testing.
- All samples should be tested concurrently in each run.

3.1 Participants

Twenty-two laboratories were contacted to participate in the Collaborative Study. All laboratories answered and notified to participate. One laboratory received material but did not respond. All other laboratories returned results back. The participants were from 12 countries including Brazil (1), Canada (1), France (3), Germany (3), Japan (2), China (2), Korea (1), Netherlands (1), Russia (2), Thailand (1), UK (2) and USA (2). Participating laboratories are listed in **Appendix 1**.

3.2 Assays

Sixteen different test kits were used in the laboratories of the participants. The assays used are listed in **Table 1** together with the specific characteristics of the assays and coded for the test kit. The range of anti-HBe assays used includes, (i) competitive and indirect test formats, (ii) manual microplate format and instrument-based test kits. The two test kits (2 and 10) with the indirect test format detect anti-HBe IgG only (anti-human IgG conjugate), the other 14 anti-HBe assays are independent of the immunoglobulin class. One test kit was provided in a qualitative interpretation version (test kit 15) and a quantitative version (test kit 16). The majority of the competitive assays interpreted samples as positive with s/co ≤1, while test kit 8 was co/s ≥1 positive. Test kits 2 and 10 samples are positive when the s/co is ≥1, test kit 16 was positive at ≥0.33 units/ml and three test kits interpreted the results positive in terms of inhibition, ≥50% inhibition with test kits 7 and 11 and ≥60% inhibition with test kit 12.

3.3 Statistical methods

Statistical analysis was performed at PEI based on the raw data sent by the participants. The detection limits with the diluted material of sample B1 (code 129095/12) and sample B2 HBe reference serum 82 (anti-HBe IgG) were calculated by linear interpolation at the intersection of the dilutions series with the assay's cut-off. Geometric mean values (GMV) including their 95% confidence intervals (CI) of the several replicates were used to describe each assay. The geometric coefficient of variation (GCV) was used to describe the intra-assay and inter-laboratory variation. To assess the potency of the candidate standard B1 relative to sample B2 two approaches were applied: (i) estimation by the ratio of the GMV between B1 and B2, and (ii) estimation via a parallel line assay (PLA) (5). However, due to a lack of linearity and parallelism for many assays it was decided not to use the PLA results in first place. Potencies were expressed in units relative to sample B2 which has an assigned concentration of 100 PEI-U/ml. Analyses were performed using SAS software version 9.3 (6), R version 2.15.2 (7) and CombiStats version 5.0 (8).

4 Results and discussion

4.1 Data received

The majority of participants followed the study plan attached with the samples, with the following exceptions:

- Laboratory 13 with test kit 1 and laboratories 9 and 14 with assay 13 tested 1 replicate in each of the 3 runs.
- Laboratory 5 with test kit 4 tested the matrix only once in single-determination.
- Laboratory 16 with test kit 6 did not test the dilution matrix initially, but provided these results after retesting.

4.2 Candidate material B1 (code 129095/12) and sample B2 (PEI reference serum 82 anti-HBe IgG)

The GMV of the endpoint titres of each assay, equivalent to the assay's cut-off of candidate material B1 and sample B2 (100 PEI-U/ml) are shown in **Table 2**. Additionally, the detection limits at the assay´s cut-off expressed as U/ml are shown in **Table 2**. With sample B1 the majority of tests (n=15) had endpoint titres of 1:600 to 1:1000, 3 tests had endpoint titres of 1:400 to 1:600, 4 tests had endpoint titres of 1:1000 to 1:1600 and 4 tests had endpoint titres of 1:1900 to 1:2500. By contrast, dilution capacity with sample B2 was lower: most tests (n=18) being positive up to endpoint titres of 1:500 to ~1:1000, 4 tests had an endpoint titre of <1:500 and 4 assays found the intercept at 1:1200 to 1:2000. With regards to the detection limit referred to U/ml anti-HBe was detected in a range of 0.050–0.307 PEI-U/ml. Potency estimates of the candidate material B1 was calculated relative to sample B2 which has an

assigned unitage of 100 PEI U/ml (**Table 3**). The overall potency derived therefrom for all 16 test kits was 132.1 U/ml (95% CI 111.2–156.9 U/ml) and overall potency calculated by means of PLA was 130.0 U/ml (95%-CI 110.0–153.8 U/ml) indicating similar results. Remarkably, the two test kits that detect anti-HBe IgG only by their indirect test format (anti-human IgG) differed from the other 14 test kits by several magnitudes: test kit 2 had a potency of 448.5 U/ml and test kit 10 had a potency of 458.7 U/ml. As a result, these two test kits were not included in the final potency determination (see section 4.5 below). The final overall potency was so 119.2 U/ml (95% CI 108.1–131.5), with a range of 74.5-181.8. Again the potency by PLA was similar, i.e. 118.1 U/ml (range 106.4–131.0 U/ml). Different from the general pattern, test kits 3, 6 and 8 showed a lower potency for candidate material B1 (mean of 77.4, 78.4 and 86.8 U/ml). **Figure 1** shows the distribution of the potencies for B1 in all assays. Each data point represents the potency estimate relative to sample B2 for an individual kit and laboratory. Additionally, **Figure 2** shows the correlation in potency between B1 and B2 averaged for those test kits used in more than one laboratory.

4.3 Supplementary samples B3, B4, AB5, B6 and B7

These samples were used to study commutability with the candidate standard B1. Since the anti-HBe kits are all designed as qualitative assays (except version 16 of kit 15) the slope of the standard curve differed and quantification in terms of the assay specific absolute values in s/co, co/s or % inhibition was not comparable between the various test kits. Therefore, the results of samples B3, B4, AB5, B6 and B7 were converted in U/ml relative to B1 and B2 for each laboratory and test kit. The results are shown in **Table 4**. Relative to B1 the range for all assays was 0.26 to 1.10 IU/ml for sample B3, 0.35 IU/ml to 2.79 IU/ml for sample B4, 0.14 IU/ml to 2.33 IU/ml for sample AB5, 0.17 IU/ml to 1.64 IU/ml for sample B6 and 0.31 IU/ml to 0.94 IU/ml for sample B7. Relative to sample B2 the range for sample B3 was 0.34 IU/ml to 1.16, for sample B4 0.75 IU/ml to 1.86 IU/ml, for sample AB5 0.19 IU/ml to 1.72 IU/ml, for sample B6 0.18 IU/ml to 1.98 IU/ml and for sample B7 0.33 IU/ml to 0.8 IU/ml. The units for test kit 8 could not be evaluated because all samples were above the maximum measuring range. Also in test kit 2 with sample B7 relative to B1, and samples B4, AB5, B6 and B7 relative to B2 as well as in test kit 10 with sample AB5 and B7 relative to B2 and in test kit 14 with sample B4, the values were out of the measuring ranges of the respective kits. Apparently in these kits the slope of the signal curve was steep and/or the measuring range short. Overall as displayed in **Figure 3**, the results expressed in units/ml for each laboratory and test kit gather around one peak in the range of ± factor 2. For the neat supplemental sample AB5 the range was ± factor 4. This possibly reflects actual sensitivity differences between the kits, and potential interference with concurrent HBeAg in this sample. **Figure 4** shows the results in units/ml for supplemental samples B3 to B7 plotted in relation to B1 and B2. Ranking of the units for the supplemental samples B3 to B7 was similar, i.e. mostly with sample B6 at the lower side, followed by B3 or B7, B3 or AB5, and sample B4 or AB5 at the upper end. Thus overall, supplemental samples B3, B4, AB5, B6 and B7 supported suitability of the candidate standard B1.

4.4 Intra-laboratory and inter-laboratory variation

Intra-laboratory (within-assay) and inter-laboratory (between labs in the same assay) variation with samples B1 and B2 were calculated as GCV% at the assay´s cut-off as shown in **Table 5**. Intra-laboratory variability with sample B1 was mostly <20% GCV reflecting common test kit intra-assay imprecision. Test kits 1, 4, 6, 11 and 15 showed higher intra-assay variations in single laboratories: test kit 1 with GCV of 39.2% in one laboratory (code 4) is compared with the low GCV of 1.3% to 6.3 in the other four labs (codes 1, 6, 13, 20); test kit 4 with 48.9% and 29.9% GCV in laboratory 7 and 29.9% to 23,3% GCV in laboratory 17 respectively is compared with the low variability of 7.3% and 2.4% GCV in laboratory 5; test kit 6 showed

an intra-assay variation of 30.4% GCV with B1 but was 18.7% GCV with B2; test kit 11 had GCV 52.7% with B1 and GCV 30.3% with B2; test kit 15 had GCV of 47.2% with B2 and a lower GCV of 21.1% with B1. Since test kits 6 and 11 were used in one lab only a final explanation for the higher intra-assay variation with these test kits remains unclear.
Also the inter-laboratory variability was calculated for the same test kit used in different laboratories, i.e. test kits 1, 4, 9 and 13. Inter-laboratory variation for B1 ranged from 11.6% to 33.3% GCV and 16.7% to 38.8% GCV for B2. This range reflects known occurring inter-laboratory variability for antibody assays. The inter-laboratory variability of 33.0% and 38.8% GCV with test kit 3 may have partially been influenced by different dilution matrices used, i.e. phosphate buffered saline with bovine serum albumin in laboratory 3 and FCS in laboratory 19.
Overall, the precision ranges for intra-assays and inter-assay found in the study was mainly consistent with commonly occurring variability in serological assays. As can be seen from the single laboratories GCV% results other variability found was due to differences with the laboratories and different matrices used in the laboratories, and does not indicate deterioration of the quality of the candidate standard studied.

4.5 Special findings and results differing from the overall conclusion

As mentioned above, potency for candidate material B1 in test kits 2 and 10 (448.5 and 458.7 U/ml) differed by several magnitudes compared to the other assays (mean 119.2 U/ml). Because of this it was decided to exclude these two test kits from the final potency determination so that the final potency is 120 U/ml instead of 130 U/ml. Another aspect is that test kits 2 and 10 have an indirect test format with anti-human IgG conjugate compared to the competitive test format of the other 14 test kits independent from the immunoglobulin class (**Table 1**). The divergence with test kits 2 and 10 seems to be rather due to low dilutional sensitivity with the PEI reference serum B2. In addition test kits 2 and 10, relative to candidate standard B1, exhibited similar units with the supplemental samples B3 to B7 as the other test kits (**Figure 3** and section 4.3 above).

4.6 Stability

Ampoules of sample B1 have been stored at the recommended storage temperature of -20°C for the next five years. Additionally for accelerated stability examination ampoules of the candidate standard B1 were incubated at 4°C, 20°C (room temperature), 37°C and 45°C for 1, 2, 4 and 7 days and for 1 and 3 months (data for 3 months at 45°C are in process). Stability was furthermore tested for the reconstituted material after storage for two months at -20°C. All measurements were done with test kit 1 in duplicates. As shown in **Tables 6 and 7**, there was no activity loss for the candidate standard B1 compared to fresh reconstituted material stored at -20°C. Further studies are on-going to determine stability at 4°C, room temperature, 37°C and 45°C for 6 months and at 4°C, room temperature and 37°C for one year. Stability will also be tested for the reconstituted and diluted material B1 when stored for two weeks at +2 to 8°C, then frozen and stored for 200 days at -70°C, and eventually freeze/thawed 2 times.
Overall, the results of the incubated ampoules with standard B1 indicate high stability even at temperatures at 45°C. The candidate standard is therefore likely to have long-term adequate stability when stored at the recommended temperature of -20°C.

5 Conclusions and proposals

Based on the data of the collaborative study, the candidate anti-HBe standard B1 was estimated to have an overall potency of 120 U/ml relative to the current PEI anti-HBe standard B2 (PEI reference serum 82 anti-HBe IgG) which has an assigned unitage of 100 PEI U/ml. Two test kits (codes 2 and 10) showed low dilution capacity with B2 compared to the candidate standard B1 so that the potency was that higher compared to the other test kits that it was decided to exclude them from the overall potency calculation. Suitability of the candidate standard B1 was evaluated with supplemental samples (B3, B4, AB5, B6, B7). After transformation of the assay´s absolute values in units the results between the various test kits were more comparable. In addition, relative ranking of the supplemental samples expressed in units was similar across the 16 test kits.

Stability when stored under normal storage conditions at -20°C has been demonstrated so far for 3 months (in June 2013). Prediction of stability at elevated temperatures indicates that the standard is highly stable and suitable for long-term use when stored at the recommended temperature of -20°C.

The candidate material B1 is therefore proposed as the 1st international standard for anti-HBe. The standard will be of value for determination of analytical assay sensitivities, for calibration of anti-HBe test kits, and for quality control.

Each vial of the anti-HBe standard contains the lyophilized residue of 0.5 ml of anti-HBe positive plasma. The standard has been given the code number 129095/12; 1900 vials are available to the WHO and custodian laboratory is the Paul-Ehrlich-Institut.

6 Comments from participants

All participants were requested for comments on the report. Fourteen of the twenty-one responded. Thirteen of them agreed with the report and the conclusion for the candidate standard and had only minor corrections. One participant commented objections quoted as follows: *"The PEI82 material is the strongest candidate for developing into the IS"*, pointing out *"that any scientific issues that may be raised at ECBS for the candidate anti-HBeAg are the same as those for the HBeAg candidate"*. Reference is made to the corresponding comment in the initial report on the 1st IS for HBeAg which was submitted in parallel.

7 Investigations performed at PEI complementary to the collaborative study

Subsequently to the collaborative study, a complementary investigational study was performed at PEI in order: (i) to get a more comprehensive estimate about correlation of analytical sensitivity by candidate standard B1 with diagnostic sensitivity and (ii) to elucidate the different dilutional behaviour of PEI 82 reference serum B2 in the anti-HBe test kit with an indirect test format.

7.1 Diagnostic sensitivity in nine HBV panels and correlation with analytical sensitivity

Three HBV seroconversion panels and 6 HBV longitudinal panels which include an anti-HBe follow-up phase from different stages of infection and disease were tested as shown in **Table 8**. Eight panels were from Zeptometrix Corp. (878 Main Street, Buffalo NY 1420, USA) and one panel (RP-009) was from Biomex GmbH (Siemensstraße 38, 69123 Heidelberg, Germany). Five test kits were used including high sensitive and lower sensitive anti-HBe competitive assays (test kits 1, 3, 8 and 13) and one anti-HBe test kit with an indirect format (test kit 2). The aims of the study were, (i) to differentiate diagnostic sensitivity of the 5 anti-HBe test kits in the 9 HBV panels, and (ii) to compare the score in positive detection in the 9

panels with the rank order in analytical sensitivity as obtained in the collaborative study (**Table 2**). The results with the 9 panels are shown in **Table 8**. There were considerable differences in sensitivity between the 5 kits in the range of 110 total positives with test kit 2 to 62 positives with test kit 3. As shown in **Figure 5** the rank in anti-HBe positive panel score with the 5 test kits was essentially reflected in the rank order for analytical sensitivity with candidate standard B1. Test kits 1 and 13 were close to each other with respect to analytical sensitivity but differed by one rank position in the positive panel score (**Figure 5 A**). The mean value of test kit 1 for analytical sensitivity (0.112 U/ml) included one divergent result from one laboratory (0.068 U/ml) compared to the other 4 laboratories. Using the mean value of these 4 kits (0.124 U/ml) would give complete correlation between analytical and diagnostic sensitivity in the 5 test kits (**Figure 5 B**). By contrast, with PEI reference sample B2 correlation between analytical sensitivity and diagnostic sensitivity was not available (not shown).
In conclusion, the results are considered to support that the analytical sensitivity evaluated with candidate standard B1 in the collaborative study is adequate for evaluation of the level of diagnostic sensitivity including for different anti-HBe test kit formats.

7.2 Further characterisation of the candidate standard B1

An attempt was carried out to further characterize samples B1 and B2 by: (i) depletion of IgG (RF-Adsorbens, Siemens Healthcare Diagnostics, 35041 Marburg Germany); (ii) heat inactivation at 56°C for 30 minutes; (iii) avidity test (Serion avidity reagent B110 Avid, Institut Virion\Serion GmbH, 97076 Würzburg, Germany). Testing for (i) and (ii) was done in test kit 2 comparatively with test kit 1, and avidity (iii) was tested in test kit 2 only. The results are displayed in **Table 9**. Serum inactivation had no effect, anti-human IgG treatment decreased the signal to about 25% in both the indirect test format (test kit 2) and the competitive test format (test kit 1) as shown in **Table 9.1**. Avidity index in test kit 2 was 25% for both B1 and B2 (**Table 9.2**). Thus, neither an interference with complement, nor potential competition of anti-HBe-IgG with -IgM, or avidity of anti-HBe antibodies exhibited a significant different between B1 and B2. As already described above (2.1 and 2.2) there is also no concurrent HBeAg in B1 and B2 which may contribute to different detection of anti-HBe.
Overall, it is concluded that the different dilution capacity between candidate standard B1 and PEI reference serum B2 in test kits 2 and 10 does not compromise the selection for the candidate standard B1. The candidate standard therefore is deemed to be representative for anti-HBe in positive clinical samples.

In summary, the additional investigational examinations at PEI altogether supported that the candidate standard B1 is adequate for the international standard to develop.

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Table 1: Anti-HBe assays used in the Collaborative Study and characteristics of the anti-HBe assays

Test kit code	Catalogue no.	Product name	Manufacturer	Procedure/ instrument	Test format	Antigen	Conjugate	Ig-class	Measuring	Analytical sensitivity as in the mfct´s IFU
1	6C34	ARCHITECT Anti-HBe	Abbott GmbH & Co. KG	ARCHITECT	Competitive two-step	rHBeAg	MAb	IgG/IgM	CMIA	≤0.45 PEI-U/ml
2	B-851	DS-EIA-Anti-HBe	RPC Diagnostic Systems	Microplate/manual	Indirect	rHBeAg	MAb (human IgG)	IgG	EIA	0.1 PEI-U/ml
3	OQDM115	Enzygnost HBe/ Anti-HBe monoclonal	Siemens Healthcare Diagnostics GmbH	BEP	Competitive	rHBeAg	MAb	IgG/IgM	ELISA	≤1 PEI-U/ml
4	N0139 (EU) / P001929 (US)	ETI-AB-EBK Plus	DiaSorin S.p.A.	Manual	Competitive	rHBeAg	MAb	IgG/IgM	EIA	0.2 PEI-U/ml
5	7D27	AxSYM Anti-HBe 2.0	Abbott GmbH & Co. KG	AxSYM	Competitive	rHBeAg	MAb	IgG/IgM	MEIA	≤1 PEI-U/ml
6	RH 05	RIAKEY HBeAg IRMA/HBeAb RIA Tube	Shin Jin Medics Inc.	DREAM Gamma Counter	Competitive	rHBeAg	MAb	IgG/IgM	IRMA	Not specified
7	0025208	ST AIA-PACK HBeAb	Tosoh Bioscience Diagnostics	Tosoh AIA	Competitive	rHBeAg	MAb	IgG/IgM	EIA	<0.4 PEI-U/ml
8	03150214	ADVIA Anti-HBe	Siemens Healthcare Diagnostics GmbH	ADVIA Centaur	Competitive two-step	rHBeAg	MAb	IgM/IgG	CMIA	0.1 PEI-U/ml
9	886 4860	VITROS Anti-HBe test	Ortho Clinical Diagnostics	VITROS	Competitive	rHBeAg	MAb	IgG/IgM	CLIA	<0.25 PEI-U/ml
10	D-0578	VectoHBe-IgG	Vector Best	Microplate/manual	Indirect	rHBeAg	MAb (human IgG)	IgG	ELISA	Not specified
11	295304	Lumipulse G HBeAb-N	Fujirebio Diagnostics, Inc.	Lumipulse G1200	Competitive one-step	rHBeAg	MAb	IgG/IgM	CLEIA	Not specified
12	291481	Lumipulse Presto G HBeAb-N	Fujirebio Diagnostics, Inc.	Lumipulse Presto	Competitive one-step	rHBeAg	MAb	IgG/IgM	CLEIA	Not specified
13	11820613122	Anti-HBe	Roche Diagnostics	Elecsys 2010/ COBAS e	Competitive	rHBeAg	MAb	IgG/IgM	ECLIA	<0.2 PEI-U/ml
14	72396	Monolisa HBe Ag-Ab PLUS	Bio-Rad Laboratories GmbH	Microplate/manual	Competitive two-step	rHBeAg	MAb	IgM/IgG	EIA	Not specified
15/16	CM.03.13	AutoLumo A 2000 anti-HBe qualitative/ quantitative	Autobio Diagnostics Co., Ltd.	AutoLumo A 2000	Competitive	rHBeAg	MAb	IgM/IgG	CMIA	Not specified

Abbreviations:
Ig = immunoglobulin
Mfct = manufacturer
IFU = instructions for use
rHBeAg = recombinant HB e antigen
MAb = monoclonal antibody
CMIA = chemiluminescent microparticle immunoassay
EIA = enzyme immunoassay
ELISA = enzyme linked immunosorbent assay
MEIA = microparticle enzyme immunoassay
IRMA = radioimmunoassay
CLEIA = chemiluminescent enzyme immunoassay
ECLIA = electrochemiluminescence immunoassay
CLIA = chemiluminescence immunoassay.

Table 2: Mean endpoint titres and detection limits (Units/ml) of candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)

		Sample B1			Sample B2		
Test kit code	Laboratory code	U/ml	GMV Titre	95%-CI	U/ml	GMV Titre	95%-CI
1	1	0.119	840	747-944	0.146	686	665-708
1	4	0.068	1473	576-3769	0.081	1236	475-3216
1	6	0.127	787	688-901	0.145	687	453-1043
1	13	0.130	771	673-883	0.162	619	506-757
1	20	0.118	845	724-987	0.144	694	634-760
2	2	0.039	2564	1832-3587	0.175	572	422-775
3	3	0.229	436	370-515	0.184	543	435-677
3	19	0.146	687	420-1124	0.109	922	635-1337
4	5	0.052	1927	1607-2311	0.082	1218	1148-1294
4	7	0.064	1567	496-4947	0.097	1034	260-4110
4	17	0.063	1590	768-3288	0.114	874	493-1549
5	1	0.132	756	720-794	0.206	486	456-519
6	16	0.203	493	235-1032	0.159	628	396-995
7	8	0.149	670	559-802	0.242	413	343-497
8	19	0.115	870	795-953	0.100	1002	897-1119
9	10	0.142	705	658-755	0.178	561	513-613
9	18	0.112	892	785-1013	0.137	731	480-1112
10	11	0.067	1495	1014-2203	0.307	326	266-399
11	12	0.191	523	153-1786	0.292	342	164-714
12	12	0.137	731	536-996	0.158	631	571-698
13	9	0.128	779	547-1109	0.150	667	468-949
13	14	0.097	1031	925-1149	0.108	924	780-1095
13	19	0.158	632	581-688	0.158	634	602-667
14	15	0.117	855	646-1131	0.143	699	553-884
15	21	0.043	2305	1371-3875	0.051	1964	644-5989
16	21	0.043	2305	1523-3634	0.050	1985	671-5871

Abbreviations:
GMV = geometric mean value; CI = confidence interval; GCV = geometric coefficient of variation; U/ml = units per millilitre.

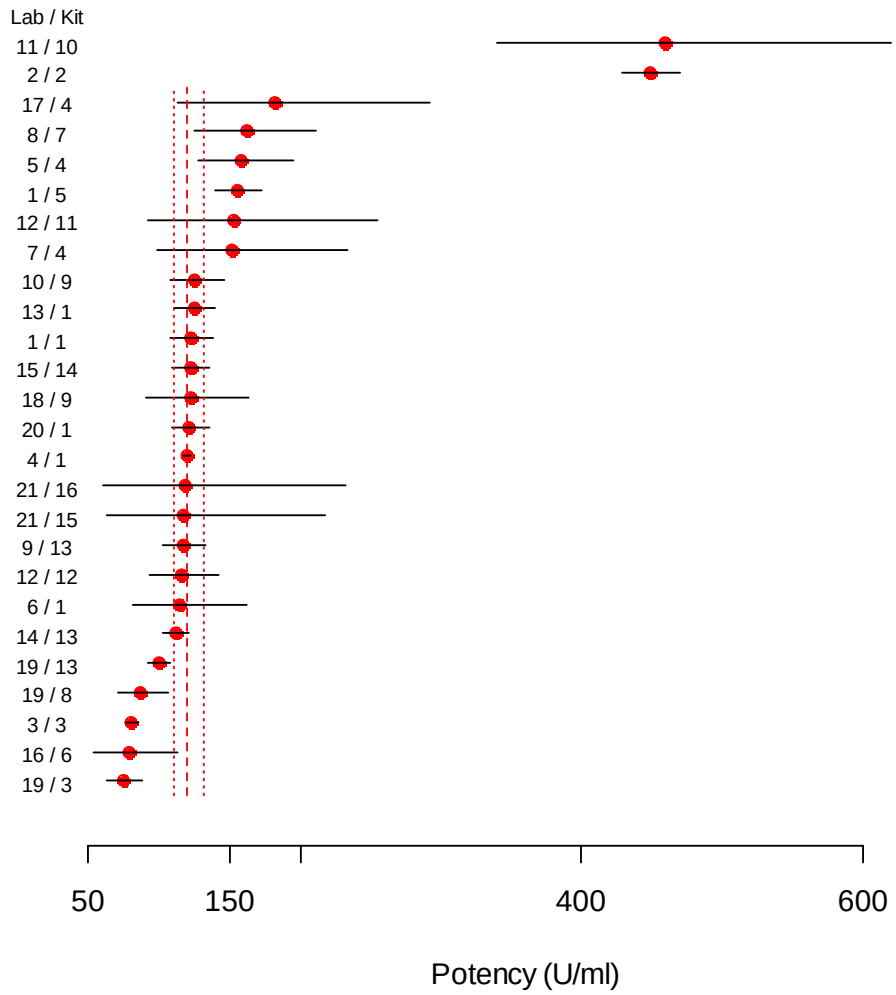
Table 3: Potency estimates of candidate material B1 (code 129095/12) relative to B2 (PEI reference serum 82 anti-HBe IgG)

		Sample B1 (candidate standard)					
		GMV			PLA		
Test kit code	Laboratory code	Potency	95%-CI	GCV	Potency	95%-CI	GCV
1	1	122.4	108.5 - 138.0	4.9	118.5	109.4 - 128.3	3.2
1	4	119.2	116.5 - 121.9	0.9	118.0	111.9 - 124.4	2.1
1	6	114.5	80.7 - 162.5	14.1	118.0	92.2 - 150.9	9.9
1	13	124.5	111.1 - 139.6	4.6	121.0	112.5 - 130.1	2.9
1	20	121.7	108.7 - 136.3	4.6	118.7	108.6 - 129.8	3.6
2	2	448.5	428.6 - 469.2	1.8	405.1	335.7 - 489.0	7.6
3	3	80.4	75.8 - 85.3	2.4	75.1	67.5 - 83.6	4.3
3	19	74.5	63.4 - 87.7	6.5	74.9	61.7 - 90.9	7.8
4	5	158.1	127.7 - 195.8	8.6	161.5	142.6 - 183.0	5.0
4	7	151.5	98.1 - 234.0	17.6	148.5	123.9 - 177.9	7.3
4	17	181.8	113.4 - 291.5	19.2	184.3	122.7 - 276.8	16.5
5	1	155.5	139.7 - 173.2	4.3	154.5	119.1 - 200.4	10.5
6	16	78.4	54.1 - 113.7	15.0	76.0	58.1 - 99.4	10.9
7	8	162.2	124.8 - 210.7	10.6	184.5	180.7 - 188.4	0.8
8	19	86.8	71.0 - 106.2	8.1	88.6	77.7 - 101.0	5.3
9	10	125.6	107.6 - 146.6	6.2	125.5	111.1 - 141.7	4.9
9	18	122.0	91.1 - 163.5	11.8	112.0	78.2 - 160.4	14.5
10	11	458.7	339.3 - 620.0	12.2	425.2	274.6 - 658.4	17.7
11	12	152.8	91.5 - 255.2	20.9	145.0	113.1 - 186.0	10.0
12	12	115.8	93.8 - 142.9	8.5	120.8	110.6 - 131.9	3.5
13	9	116.9	102.4 - 133.3	5.3	111.0	100.8 - 122.3	3.9
13	14	111.6	102.3 - 121.7	3.5	102.8	83.5 - 126.6	8.4
13	19	99.8	91.9 - 108.3	3.3	108.2	101.7 - 115.1	2.5
14	15	122.3	109.8 - 136.3	4.3	123.5	103.9 - 147.0	7.0
15	21	117.4	63.4 - 217.3	25.2	111.2	84.1 - 147.1	11.3
16	21	118.5	60.6 - 232.0	27.5	113.6	93.0 - 138.8	8.1
Total 1 [1)]		**119.2**	**108.1 - 131.5**	**23.5**	**118.1**	**106.4 - 131.0**	**25.0**
Total 2 [2)]		132.1	111.2 - 156.9	44.6	130.0	110.0 - 153.8	43.4

Footnotes:
[1)] Test kits 2 and 10 excluded
[2)] All test kits included

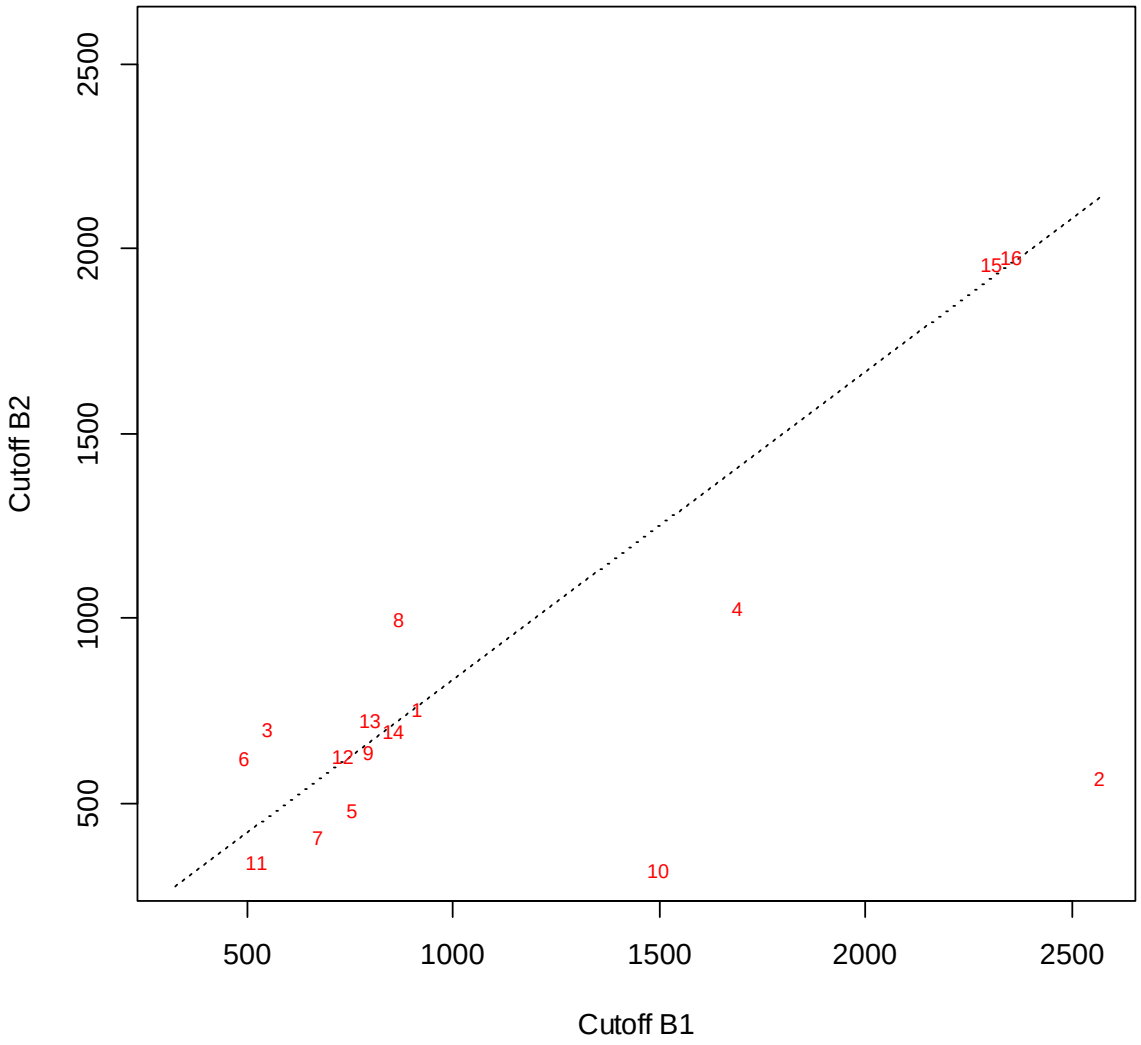
Abbreviations:
GMV = geometric mean value; CI = confidence interval; GCV = geometric coefficient of variation; PLA = parallel line assay.

Figure 1: Distribution of the geometric mean potencies of candidate standard B1 (code 129095/12) relative to B2 (PEI reference serum 82 anti-HBe IgG)



Legend:
Each point of the data represents the laboratory and test kit according to the code numbers in **Table 1**. The line with each point indicates the 95% confidence interval.
Y-axis: the codes represent the laboratory and kits.
X-axis: potency in U/ml.

Figure 2: Potency for candidate standard B1 (code 129095/12) relative to B2 (PEI reference serum 82 anti-HBe IgG)



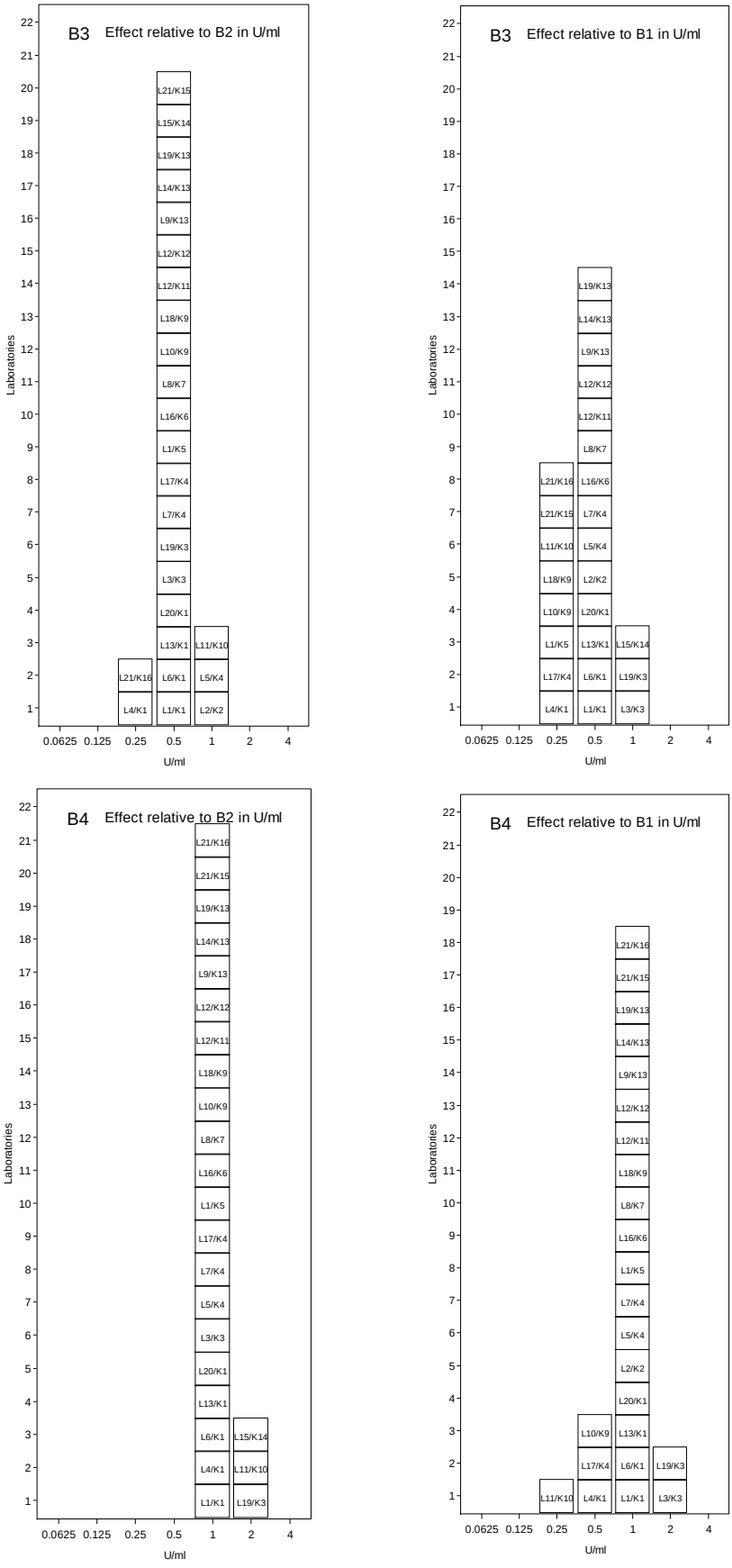
Legend:
The code numbers represent the kit numbers (see **Table 1**). Test kits used in more than one laboratory were averaged. The angle bisector indicates the potency for B1 of 120 U/ml relative to B2.

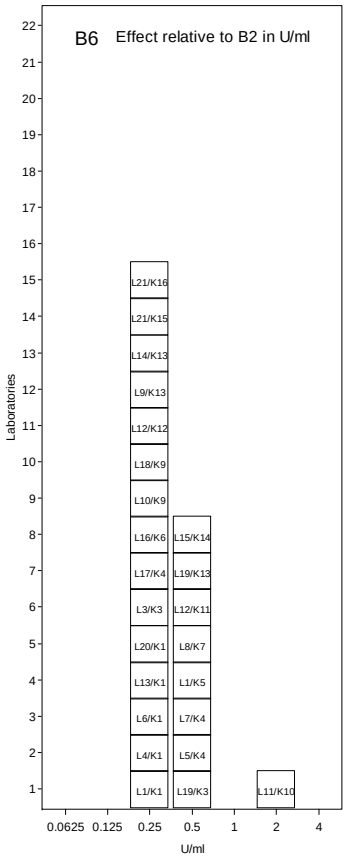
Table 4: Results for supplemental samples B3, B4, AB5, B6 and B7 in units relative to candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)

Test kit	Laboratory	Effect relative to B1 in U/ml					Effect relative to B2 in U/ml				
code number	code number	B3	B4	AB5	B6	B7	B3	B4	AB5	B6	B7
1	1	0.53	1.05	0.76	0.29	0.53	0.58	1.15	0.91	0.33	0.58
1	4	0.32	0.67	0.53	0.17	0.31	0.34	0.75	0.54	0.18	0.33
1	6	0.53	1.06	0.74	0.31	0.54	0.60	1.19	0.90	0.35	0.61
1	13	0.54	1.08	0.77	0.29	0.54	0.59	1.18	0.92	0.32	0.60
1	20	0.45	0.82	0.70	0.25	0.45	0.52	1.00	0.86	0.30	0.51
1	**Total**	**0.47**	**0.94**	**0.70**	**0.26**	**0.47**	**0.53**	**1.05**	**0.83**	**0.30**	**0.53**
2	2	0.46	1.03	2.33	1.64	ne	1.16	ne	ne	ne	ne
3	3	0.72	1.69	0.47	0.30	0.58	0.48	1.10	0.35	0.23	0.41
3	19	1.10	2.79	0.52	0.53	0.90	0.69	1.86	0.36	0.36	0.58
3	**Total**	0.91	2.24	0.50	0.41	0.74	0.59	1.48	0.36	0.29	0.50
4	5	0.50	0.81	0.18	0.27	0.57	0.73	1.25	0.33	0.43	0.80
4	7	0.36	0.78	0.14	0.28	0.38	0.48	1.27	0.19	0.38	0.56
4	17	0.33	0.65	0.14	0.19	0.33	0.49	0.94	0.22	0.35	0.49
4	**Total**	**0.40**	**0.75**	**0.15**	**0.25**	**0.43**	**0.57**	**1.15**	**0.25**	**0.39**	**0.62**
5	1	0.35	0.76	0.77	0.28	0.52	0.48	0.98	0.99	0.39	0.73
6	16	0.68	1.25	0.56	0.34	0.56	0.51	0.90	0.43	0.27	0.43
7	8	0.38	0.79	1.54	0.35	0.58	0.49	0.88	1.55	0.46	0.67
8	19	ne	ne	ne	ne	ne	ne	ne	ne	ne	ne
9	10	0.31	0.69	0.33	0.21	0.40	0.40	0.84	0.42	0.27	0.51
9	18	0.31	0.73	0.28	0.21	0.41	0.36	0.86	0.33	0.26	0.46
9	**Total**	**0.31**	**0.71**	**0.31**	**0.21**	**0.41**	**0.38**	**0.85**	**0.37**	**0.26**	**0.48**
10	11	0.26	0.35	1.50	0.44	0.56	1.16	1.65	ne	1.98	ne
11	12	0.37	0.95	2.01	0.28	0.51	0.46	0.92	1.72	0.38	0.57
12	12	0.38	0.78	0.73	0.29	0.53	0.42	0.90	0.80	0.29	0.48
13	9	0.44	0.84	0.65	0.33	0.56	0.41	0.83	0.58	0.32	0.47
13	14	0.46	0.94	0.68	0.35	0.59	0.40	0.81	0.56	0.32	0.47
13	19	0.61	1.25	0.73	0.41	0.69	0.47	0.93	0.61	0.36	0.55
13	**Total**	**0.50**	**1.00**	**0.70**	**0.36**	**0.62**	**0.43**	**0.86**	**0.58**	**0.33**	**0.50**
14	15	0.81	ne	0.15	0.33	0.94	0.61	1.57	0.19	0.38	0.66
15	21	0.33	0.74	0.71	0.22	0.40	0.37	0.84	0.80	0.24	0.46
16	21	0.31	0.71	0.68	0.22	0.40	0.34	0.77	0.74	0.24	0.43

Abbreviations: ne = not evaluable because above the measuring range of the test kit, calibration range respectively.

Figure 3: **Individual laboratory mean estimates for supplemental samples B3/B4, B5/B7 and AB5 relative to candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)**




B6 Effect relative to B2 in U/ml
Laboratories
U/ml
0.0625 0.125 0.25 0.5 1 2 4
L21/K16
L21/K15
L14/K13
L9/K13
L12/K12
L18/K9
L10/K9
L16/K6
L17/K4
L3/K3
L20/K1
L13/K1
L6/K1
L4/K1
L1/K1
L15/K14
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L8/K7
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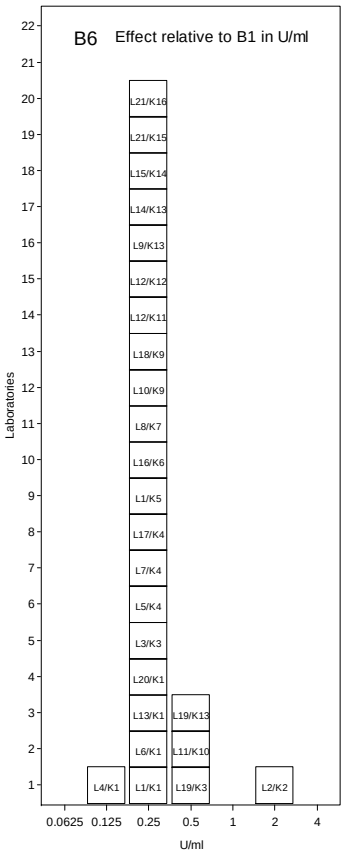
L7/K4
L5/K4
L19/K3
L11/K10


B6 Effect relative to B1 in U/ml
Laboratories
U/ml
0.0625 0.125 0.25 0.5 1 2 4
L21/K16
L21/K15
L15/K14
L14/K13
L9/K13
L12/K12
L12/K11
L18/K9
L10/K9
L8/K7
L16/K6
L1/K5
L17/K4
L7/K4
L5/K4
L3/K3
L20/K1
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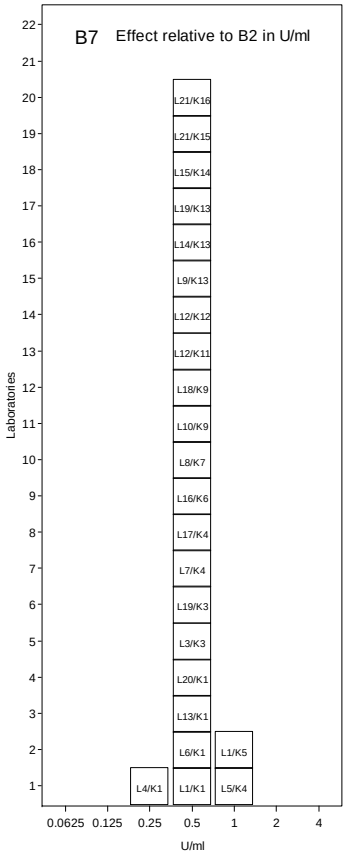
L19/K13
L11/K10
L19/K3
L2/K2


B7 Effect relative to B2 in U/ml
Laboratories
U/ml
0.0625 0.125 0.25 0.5 1 2 4
L21/K16
L21/K15
L15/K14
L19/K13
L14/K13
L9/K13
L12/K12
L12/K11
L18/K9
L10/K9
L8/K7
L16/K6
L17/K4
L7/K4
L19/K3
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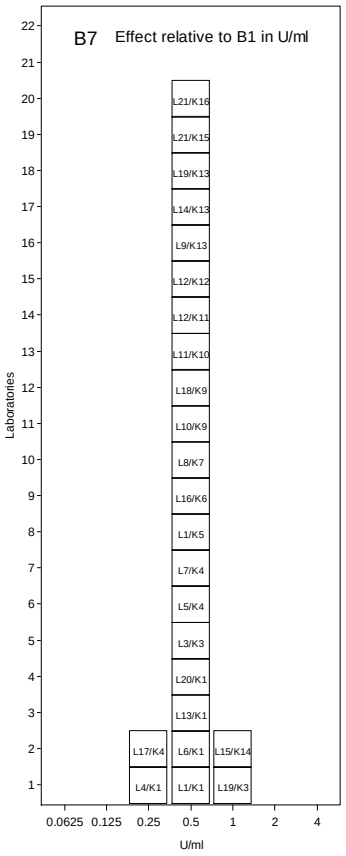
L13/K1
L6/K1
L1/K5
L4/K1
L1/K1
L5/K4


B7 Effect relative to B1 in U/ml
Laboratories
U/ml
0.0625 0.125 0.25 0.5 1 2 4
L21/K16
L21/K15
L19/K13
L14/K13
L9/K13
L12/K12
L12/K11
L11/K10
L18/K9
L10/K9
L8/K7
L16/K6
L1/K5
L7/K4
L5/K4
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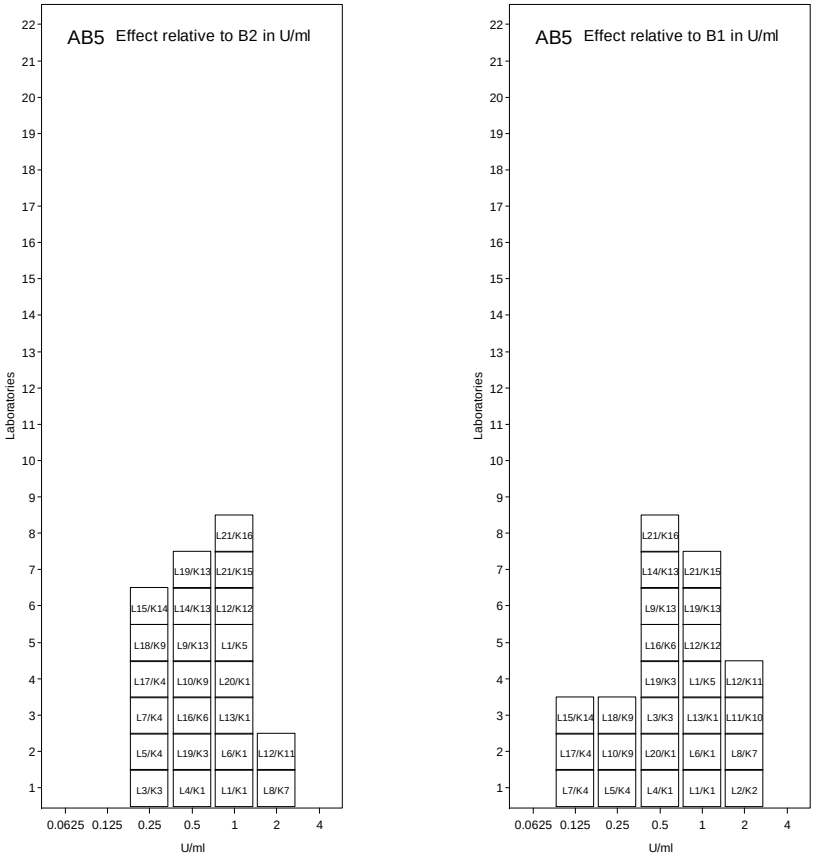
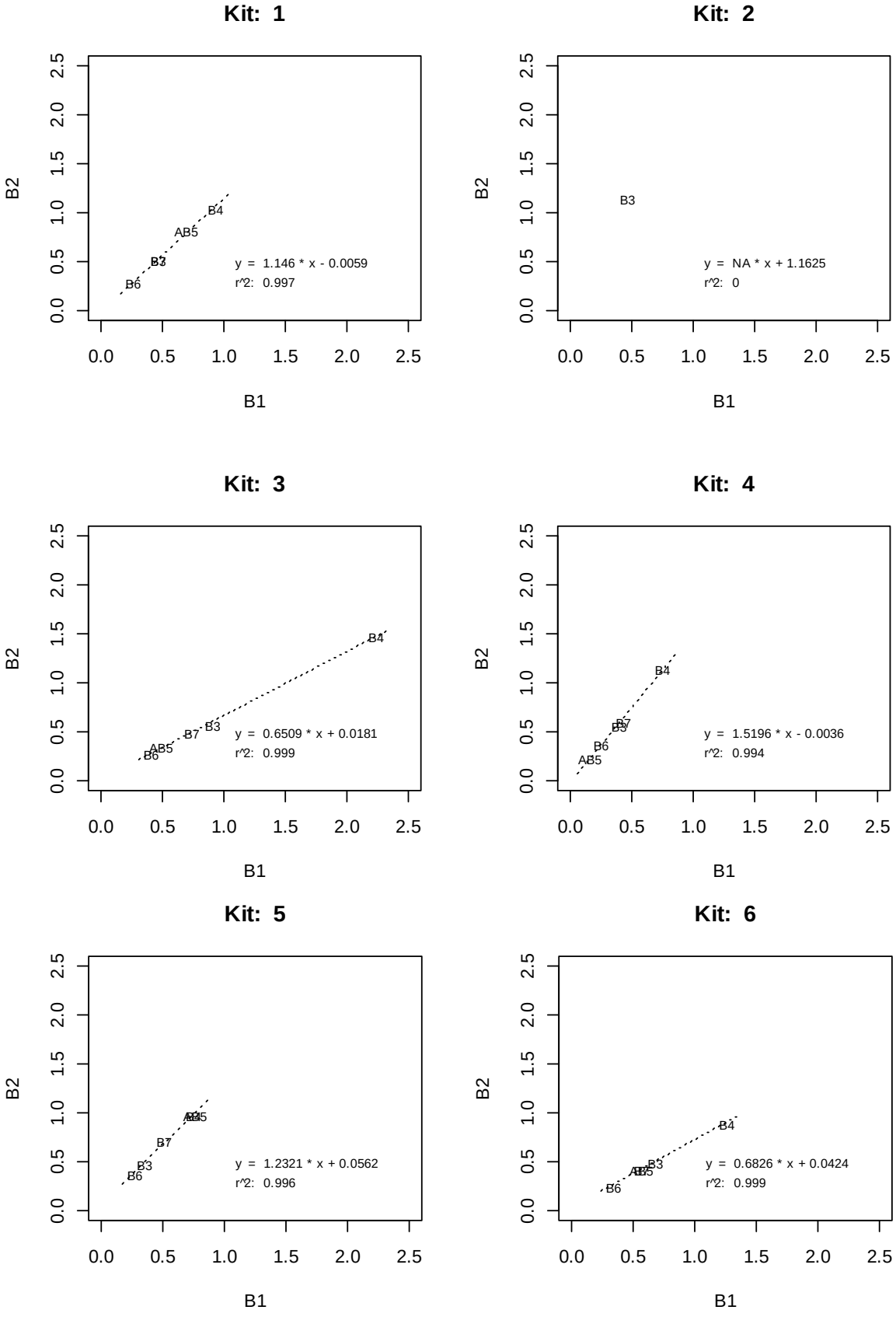
L15/K14
L4/K1
L1/K1
L19/K3



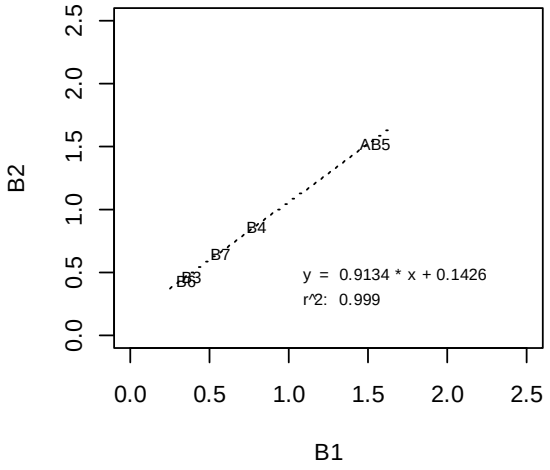
Legend:

Values from **Table 2** above; each box represents the value (U/ml) per Laboratory (L) and Kit (K). X-axis (U/ml) displayed in log scale (log_2).

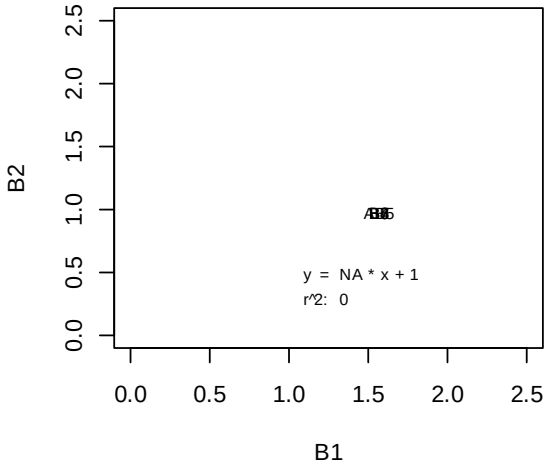
Figure 4: Scatterplots for supplemental samples B3, B4, AB5, B6 and B7 in U/ml relative to candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)



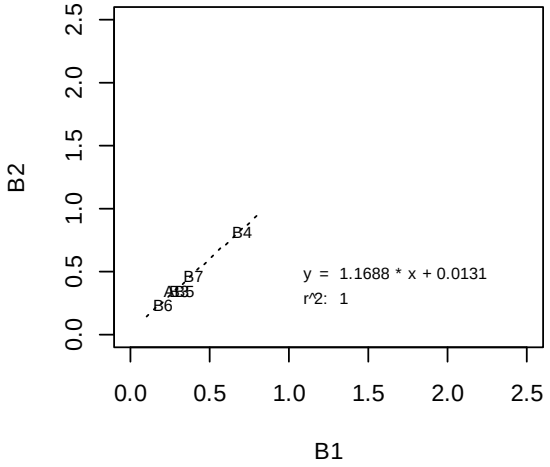
Kit: 7



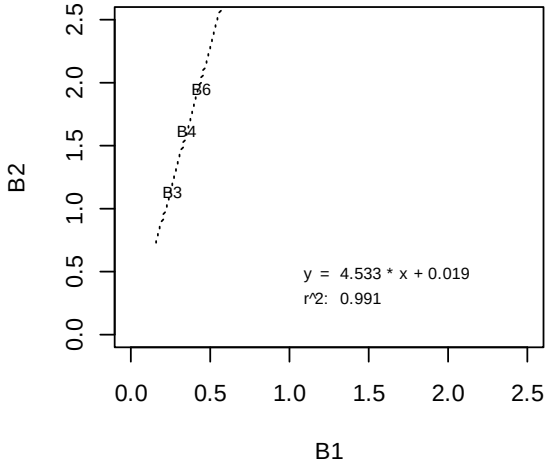
Kit: 8



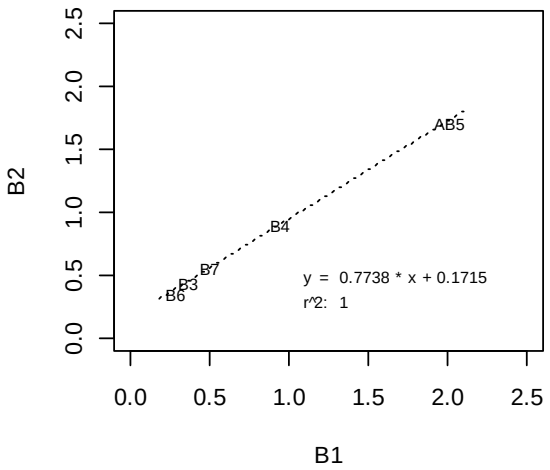
Kit: 9



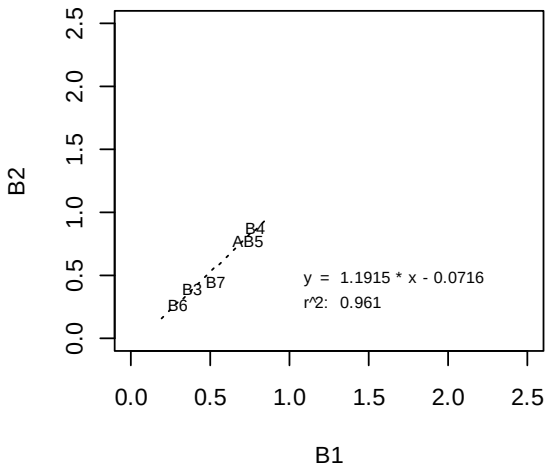
Kit: 10

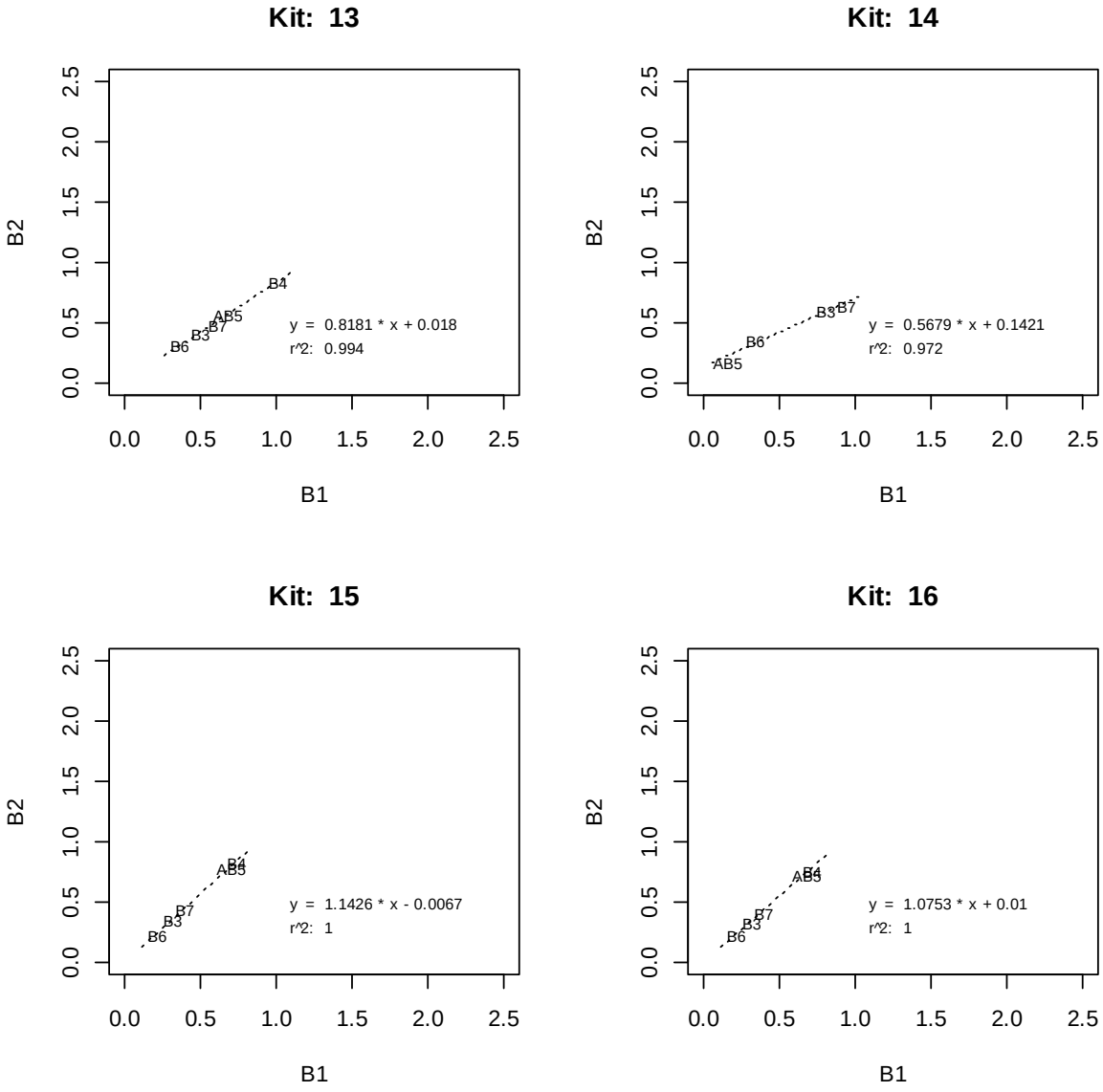


Kit: 11



Kit: 12





Legend:
Y-axis: sample B2 in U/ml
X-axis: sample B1 in U/ml

Table 5: Intra-and inter-laboratory variation (GCV%) with candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)

Test kit code number	Laboratory code number		Sample	
			B1	B2
			GCV%	
1	1	intra-lab	4.7	1.3
1	4	intra-lab	39.2	40.0
1	6	intra-lab	5.4	16.9
1	13	intra-lab	5.5	8.1
1	20	intra-lab	6.3	3.7
1	**inter-lab**		**27.5**	**28.3**
2	2	intra-lab	13.6	12.3
3	3	intra-lab	6.7	8.9
3	19	intra-lab	20.0	15.1
3	**inter-lab**		**33.0**	**38.8**
4	5	intra-lab	7.3	2.4
4	7	intra-lab	48.9	60.1
4	17	intra-lab	29.9	23.3
4	**inter-lab**		**11.6**	**16.7**
5	1	intra-lab	2.0	2.6
6	16	intra-lab	30.4	18.7
7	8	intra-lab	7.3	7.5
8	19	intra-lab	3.6	4.4
9	10	intra-lab	2.8	3.6
9	18	intra-lab	5.1	17.0
9	**inter-lab**		**16.8**	**18.9**
10	11	intra-lab	15.7	8.2
11	12	intra-lab	52.7	30.3
12	12	intra-lab	12.5	4.1
13	9	intra-lab	14.3	14.3
13	14	intra-lab	4.4	6.8
13	19	intra-lab	3.4	2.1
13	**inter-lab**		**24.9**	**20.7**
14	15	intra-lab	11.3	9.5
15	21	intra-lab	21.1	47.2
16	21	intra-lab	17.6	45.8

Abbreviations:
GCV = geometric coefficient of variation

Table 6: Stability of candidate standard B1 (code 129095/12) using kit 1

Day	Replicate	Endpoint titres (intercept at assay`s cut-off)				
		baseline (-20°C)	4°C	RT (20°C)	37°C	45°C
1	1	702.51	804.57	666.79	792.38	748.31
	2	735.18	755.81	725.33	747.24	736.00
2	1		774.92	761.76	819.20	804.57
	2		828.95	903.53	786.73	725.33
4	1		792.38	794.48	806.79	879.59
	2		762.98	786.73	800.00	867.56
7	1		695.79	842.32	915.39	792.38
	2		739.56	798.72	886.63	790.26
	Geomean	718.66	768.39	782.09	817.71	791.21

Months	Replicate	Endpoint titres (intercept at assay`s cut-off)				
		baseline (-20°C)	4°C	RT (20°C)	37°C	45°C
1	1	656.41	821.89	693.33	938.67	1008.00
	2	660.21	848.46	643.28	853.33	980.11
3	1		840.21	933.65	989.09	
	2		832.00	896.00	925.96	
	Geomean	658.29	835.58	781.55	925.47	993.43

Table 7: Stability studies of candidate standard B1 (code 129095/12) using kit 1 after reconstitution

Months	Replicate	Endpoint titres (intercept at assay`s cut-off)	
		baseline (-20°C)	reconstituted (stored at -20°C)
2	1	656.41	1455.16
	2	660.21	1494.49
	Geomean	658.29	1474.56

Abbreviations:
RT = room temperature

Table 8: Sensitivity for anti-HBe in 3 HBV seroconversion panels and 6 HBV longitudinal panels for five selected anti-HBe test kits

			Anti-HBe test kit code				
			2	**8**	**13**	**1**	**3**
	Date		s/co	s/co	COI	s/co	co/s
Panel #	of draw	Day	>=1 pos	>=1 pos	<=1 pos	<=1 pos	>=1 pos
6509-01*	16.05.01	0	0.00	>4.5	0.52	0.72	0.77
6509-02*	28.05.01	12	0.22	>4.5	0.70	0.91	0.84
6509-03*	13.06.01	28	0.27	>4.5	0.70	0.94	0.81
6509-04*	27.06.01	42	0.23	>4.5	0.66	0.88	0.80
6509-05	11.07.01	56	10.16	>4.5	0.13	0.15	1.36
6509-06	25.07.01	70	10.67	>4.5	0.14	0.17	1.51
6509-07	08.08.01	84	10.68	>4.5	0.11	0.13	1.70
6509-08	22.08.01	98	10.74	>4.5	0.10	0.13	1.81
6509-09	05.09.01	112	7.06	>4.5	0.17	0.19	1.45
6509-10	19.09.01	126	6.19	>4.5	0.15	0.18	1.58
6509-11	03.10.01	140	4.42	>4.5	0.21	0.23	1.48
6509-12	16.10.01	153	3.98	>4.5	0.20	0.20	1.26
6510-01	22.05.01	0	4.05	0.00	7.28	43.87	0.13
6510-02	05.06.01	14	10.45	0.00	4.15	31.90	0.13
6510-03	19.06.01	28	13.85	3.40	1.04	1.45	0.69
6510-04	03.07.01	42	13.05	3.14	1.07	1.59	0.79
6510-05	17.07.01	56	12.39	2.97	1.09	1.48	0.81
6510-06	31.07.01	70	12.50	>4.5	0.08	0.13	2.03
6510-07	14.08.01	84	9.32	>4.5	0.12	0.14	1.76
6510-08	28.08.01	98	8.44	>4.5	0.11	0.13	1.61
6510-09	11.09.01	112	5.27	>4.5	0.16	0.18	1.38
6510-10	25.09.01	126	4.37	>4.5	0.19	0.23	1.26
6510-11	09.10.01	140	4.15	>4.5	0.19	0.22	1.29
6510-12	22.10.01	153	4.10	>4.5	0.19	0.19	1.33
6513-01	06.05.01	0	18.92	>4.5	0.06	0.03	3.03
6513-02	19.06.01	44	18.53	3.18	0.88	3.31	0.13
6513-03	04.07.01	59	15.97	2.74	1.02	5.45	0.17
6513-04	17.07.01	72	16.50	>4.5	0.73	1.65	0.29
6513-05	31.07.01	86	16.41	>4.5	0.67	1.00	0.49
6513-06	14.08.01	100	16.89	>4.5	0.70	0.76	0.57
6513-07	28.08.01	114	18.14	>4.5	0.53	0.52	0.68
6513-08	11.09.01	128	17.25	>4.5	0.72	0.61	0.84
6513-09	25.09.01	142	18.51	>4.5	0.20	0.14	1.57
6513-10	09.10.01	156	18.10	>4.5	0.11	0.07	2.25
6513-11	23.10.01	170	18.07	>4.5	0.08	0.05	2.73
6513-12	06.11.01	184	18.26	>4.5	0.01	0.01	12.33
6529-01	10.08.01	0	1.05	>4.5	0.42	0.71	0.96
6529-02	22.10.01	73	2.69	>4.5	0.33	0.56	0.94
6529-03	05.11.01	87	7.91	>4.5	0.29	0.48	1.18
6529-04	19.11.01	101	12.64	>4.5	0.23	0.32	1.45
6529-05	03.12.01	115	17.03	>4.5	0.17	0.21	1.65
6529-06	18.12.01	130	17.91	>4.5	0.14	0.14	1.96

6529-07	01.01.02	144	19.16	>4.5	0.07	0.07	3.35
6529-08	14.01.02	157	18.27	>4.5	0.06	0.06	3.01
6529-09	28.01.02	171	18.97	>4.5	0.06	0.05	3.31
6529-10	11.02.02	185	19.60	>4.5	0.05	0.04	3.76
6529-11	25.02.02	199	19.09	>4.5	0.05	0.04	4.11
6529-12	09.03.02	211	19.10	>4.5	0.05	0.04	4.02
6534-01	12.11.01		2.02	>4.5	0.35	0.61	0.97
6534-02	26.11.01	14	6.02	>4.5	0.17	0.42	0.96
6534-03	11.12.01	29	6.79	>4.5	0.08	0.25	1.68
6534-04	25.12.01	43	16.17	>4.5	0.10	0.16	1.70
6534-05	08.01.02	57	17.42	>4.5	0.12	0.12	2.18
6534-06	22.01.02	71	18.02	>4.5	0.11	0.12	2.19
6534-07	05.02.02	85	18.32	>4.5	0.08	0.10	2.80
6534-08	19.02.02	99	18.12	>4.5	0.13	0.15	1.95
6534-09	05.03.02	113	18.85	>4.5	0.11	0.12	2.06
6534-10	19.03.02	127	18.33	>4.5	0.13	0.14	1.99
6534-11	02.04.02	141	18.56	>4.5	0.13	0.15	2.25
6534-12	16.04.02	155	18.35	>4.5	0.17	0.17	1.70
6541-01	03.12.01	0	3.16	>4.5	0.42	0.76	0.94
6541-02	17.12.01	14	9.00	>4.5	0.44	0.55	1.02
6541-03	31.12.01	28	10.51	>4.5	0.29	0.36	1.14
6541-04	14.01.02	42	14.22	>4.5	0.19	0.20	1.50
6541-05	28.01.02	56	15.86	>4.5	0.19	0.18	1.53
6541-06	11.02.02	70	15.55	>4.5	0.14	0.14	1.96
6541-07	25.02.02	84	16.25	>4.5	0.09	0.08	2.87
6541-08	11.03.02	98	16.57	>4.5	0.10	0.09	2.66
6541-09	25.03.02	112	14.68	>4.5	0.10	0.09	2.44
6541-10	08.04.02	126	15.44	>4.5	0.08	0.07	2.94
6541-11	22.04.02	140	15.54	>4.5	0.08	0.07	2.68
6541-12	06.05.02	154	12.49	>4.5	0.05	0.05	4.69
9092-01	20.12.05	0	0.03	0.35	1.74	1.91	0.52
9092-02	22.12.05	2	0.02	0.32	1.75	2.04	0.51
9092-03	28.12.05	8	0.03	0.33	1.75	1.95	0.49
9092-04	03.01.06	14	0.02	0.25	1.77	2.03	0.52
9092-05	05.01.06	16	0.03	0.26	1.68	1.94	0.54
9092-06	12.01.06	23	0.01	0.34	1.70	1.99	0.55
9092-07	17.01.06	28	0.02	0.36	1.75	1.93	0.52
9092-08	19.01.06	30	0.02	0.20	1.78	2.00	0.53
9092-09	24.01.06	35	0.02	0.34	1.75	1.92	0.54
9092-10	26.01.06	37	0.02	0.20	1.76	2.01	0.56
9092-11	31.01.06	42	0.02	0.28	1.74	2.06	0.50
9092-12	02.02.06	44	0.02	0.17	1.80	2.12	0.57
9092-13	07.02.06	49	0.03	0.10	1.77	2.01	0.58
9092-14	09.02.06	51	0.02	0.10	1.76	2.06	0.61
9092-15	22.02.06	64	0.03	0.25	1.80	2.79	0.42
9092-16	27.02.06	69	0.02	0.00	2.07	6.36	0.18
9092-17	06.03.06	76	0.02	0.00	4.70	32.99	0.14
9092-18	08.03.06	78	0.02	0.00	5.49	38.98	0.14

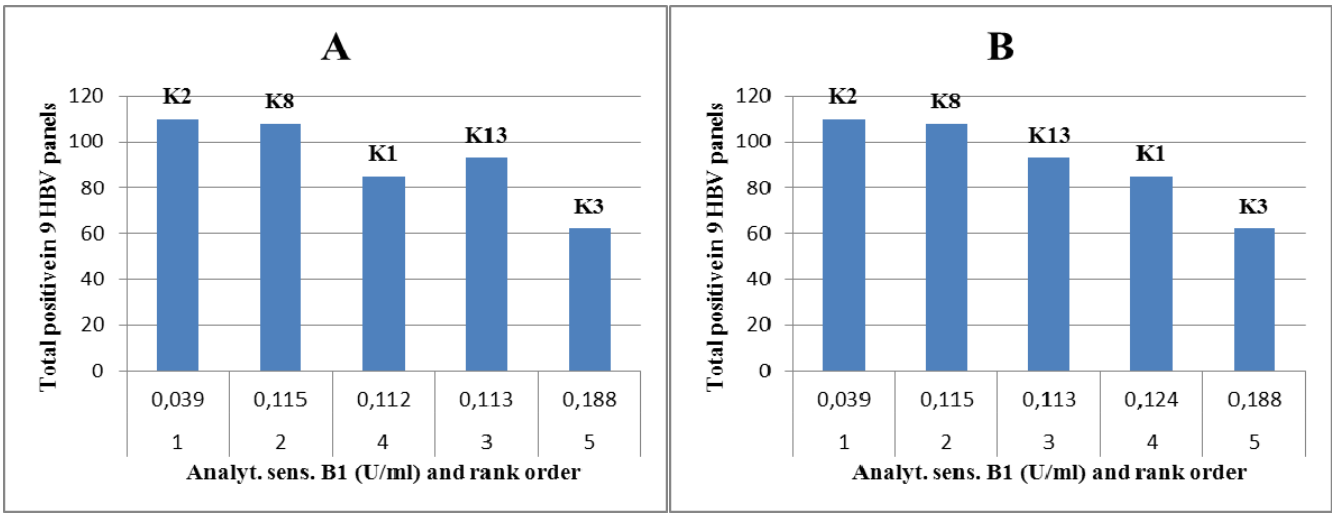
9092-19	15.03.06	85	0.03	0.00	6.47	45.07	0.14
9092-20	22.03.06	92	0.01	0.00	7.01	46.51	0.14
9092-21	29.03.06	99	0.03	0.00	4.68	45.72	0.14
9092-22	05.04.06	106	0.02	0.00	5.83	43.48	0.14
9092-23	19.04.06	120	0.84	0.00	3.99	32.97	0.14
9092-24	01.05.06	132	3.84	2.02	1.21	3.22	0.40
9092-25	03.05.06	134	5.40	3.01	1.06	2.79	0.46
9092-26	10.05.06	141	6.07	4.10	0.95	1.45	0.64
9092-27	17.05.06	148	5.90	>4.50	0.92	1.46	0.61
9092-28	31.05.06	162	9.56	>4.50	0.68	1.02	0.65
9092-29	06.06.06	168	5.20	>4.50	0.89	1.12	0.71
9092-30	08.06.06	170	7.79	>4.50	0.71	0.99	0.74
9092-31	13.06.06	175	4.23	>4.50	0.91	1.08	0.78
9092-32	15.06.06	177	6.74	>4.50	0.70	0.81	0.81
9092-33	20.06.06	182	5.42	>4.50	0.85	0.95	0.77
9092-34	22.06.06	184	4.24	>4.50	0.87	0.99	0.76
9092-35	27.06.06	189	4.37	>4.50	0.82	0.89	0.78
9092-36	29.06.06	191	4.04	>4.50	0.84	0.86	0.77
9092-37	06.07.06	198	4.16	>4.50	0.80	0.80	0.82
9093-01	28.06.07	0	0.02	0.17	1.73	1.99	0.75
9093-02	03.07.07	5	0.02	0.16	1.70	2.06	0.70
9093-03	05.07.07	7	0.03	0.12	1.03	1.87	0.70
9093-04	10.07.07	12	0.01	0.17	1.74	1.94	0.71
9093-05	12.07.07	14	0.02	0.13	1.77	2.08	0.73
9093-06	17.07.07	19	0.01	0.10	1.77	1.96	0.65
9093-07	19.07.07	21	0.02	0.06	1.75	1.91	0.70
9093-08	24.07.07	26	0.02	0.08	1.77	1.98	0.65
9093-09	02.08.07	35	0.02	0.00	2.02	5.70	0.24
9093-10	09.08.07	42	0.02	0.00	4.11	32.13	0.14
9093-11	16.08.07	49	0.02	0.00	5.84	45.84	0.14
9093-12	23.08.07	56	0.02	0.00	6.43	47.77	0.14
9093-13	10.09.07	74	0.03	0.00	5.27	42.69	0.14
9093-14	12.09.07	76	0.02	0.00	5.82	43.27	0.14
9093-15	11.10.07	105	6.36	0.00	3.82	33.10	0.14
9093-16	15.10.07	109	7.26	0.00	2.78	22.83	0.14
9093-17	22.10.07	116	11.01	0.88	1.39	5.96	0.26
9093-18	29.10.07	123	10.40	2.77	1.12	1.62	0.73
9093-19	01.11.07	126	9.13	2.97	1.15	1.50	0.78
9093-20	14.11.07	139	10.11	3.23	1.08	1.29	0.90
9093-21	27.11.07	152	10.04	3.36	1.06	1.14	0.96
9093-22	29.11.07	154	12.02	3.23	1.07	1.09	0.84
9093-23	04.12.07	159	10.05	3.25	1.07	1.14	0.80
9093-24	06.12.07	161	11.22	3.42	1.08	1.12	0.86
9093-25	11.12.07	166	13.83	3.74	1.02	1.06	0.87
9093-26	13.12.07	168	11.77	3.50	1.06	1.06	0.88
9093-27	18.12.07	173	12.87	>4.50	0.94	0.82	0.95
9093-28	27.12.07	182	14.60	>4.50	0.75	0.63	1.07
9093-29	31.12.07	186	13.99	>4.50	0.73	0.60	1.03
9093-30	03.01.08	189	13.42	>4.50	0.68	0.53	0.97

9093-31	07.01.08	193	13.79	>4.50	0.50	0.37	0.97
RP-009-01	18.07.95	0	0.02	0.16	nt	1.92	0.65
RP-009-02	21.07.95	3	0.02	nt	1.74	1.55	0.58
RP-009-03	29.07.95	3	0.02	nt	1.81	1.61	0.63
RP-009-04	31.07.95	11	0.03	nt	1.81	1.71	0.58
RP-009-05	16.08.95	13	0.02	0.00	3.24	17.53	0.14
RP-009-06	18.08.95	29	0.02	0.00	4.23	29.37	0.14
RP-009-07	23.08.95	31	0.02	nt	7.00	45.47	0.14
RP-009-08	30.08.95	36	0.02	0.00	7.14	49.61	0.14
RP-009-09	12.09.95	43	0.06	0.00	5.86	40.83	0.14
RP-009-10	25.09.95	56	3.75	0.68	1.32	15.87	0.14
RP-009-11	07.10.95	69	16.34	>4.5	0.26	1.41	0.72
RP-009-12	14.10.95	81	17.77	>4.5	0.12	0.22	1.31
RP-009-13	24.10.95	88	14.83	>4.5	0.17	0.23	1.14
RP-009-14	04.11.95	98	13.79	>4.5	0.16	0.31	1.23
RP-009-15	18.11.95	109	12.82	>4.5	0.15	0.20	1.36
RP-009-16	28.11.95	123	11.43	>4.5	0.18	0.21	1.24
RP-009-17	16.12.95	133	7.91	>4.5	0.24	0.33	1.40
RP-009-18	30.12.95	151	12.47	>4.5	0.33	0.33	1.37
RP-009-19	19.01.96	165	17.77	>4.5	0.43	0.47	1.29
RP-009-20	04.02.96	185	18.96	>4.5	0.40	0.30	1.30
N positive			110	108	93	85	62

Abbreviations: s/co = sample to cut-off ratio; co/s = cut-off to sample ratio; pos = positive; nt = not tested.

Legend:
Dark grey = positive; light grey = greyzone (for s/co = 1.00 – 0.90; for co/s = 1.00 – 1.10); Panel members 6509-01* - 6509-04*: patient medication documented which may have interfered with anti-HBe detection in test kits 2 and 3.

Figure 5: Correlation of the analytical sensitivity with candidate standard B1 (code 129095/12) to the diagnostic sensitivity in 9 HBV panels



Abbreviations:
K = kit code number; analyt. sens. = analytical sensitivity.

Legend:
Y-axis, total positives ranked in decreasing order according to **Table 8**;
X-axis, analytical sensitivity values from **Table 2** put in decreasing order of sensitivity.

Table 9: Further investigations for characterisation of candidate standard B1 (code 129095/12) and B2 (PEI reference serum 82 anti-HBe IgG)

1. Treatment with anti-human IgG and heat inactivation

		A	**B**		**C**	
		Untreated control	α-IgG		56°C, 30´	
Standard	Kit	Titre	Titre	**A/B**%	Titre	**A/C**%
B1	2	900	227.6		888.2	
	1	537.3	209.0	38.9%	638.3	118.8%
B2	2	191.5	91.6		185.9	
	1	487.4	131.1	26.9%	560.0	114.9%

2. Avidity treatment

		A	**B**	
		Untreated control	Avidity	
Standard	Kit	Titre	Titre	**A/B**%
B1	2	815.6	226.5	27.8%
B2	2	198.6	47.0	23.7%

APPENDIX 1:

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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

WHO 1st International Standard Antibodies to Hepatitis B Virus e Antigen (anti-HBe) code 129095/12

Instructions for use (Version 1, October 2013)

1. INTENDED USE

The International Standard was established for determination of the analytical sensitivity of anti-HBe assays. It may also serve for calibration of anti-HBe test kits and for quality control.

A WHO Collaborative Study organised by the Paul-Ehrlich-Institut (PEI) was undertaken to assess the suitability of a candidate international standard (code 129095/12) for antibodies to hepatitis B virus e antigen (anti-HBe) in diagnostic assays. Twenty-one laboratories from 12 countries tested the above described material using 16 different assays.

2. UNITAGE

This material is assigned a unitage of 120 IU/ml.

3. CONTENTS

Each vial contains 0.5 ml of freeze-dried anti-HBe positive human plasma.

4. CAUTION

This preparation is not for administration to humans. The preparation contains material of human origin, and is infectious for hepatitis B virus (HBV).

Testing for anti-HIV 1/2, anti-HCV, HIV RNA and HCV RNA was negative. The standard is also negative for HBeAg, anti-HBc IgM and anti-HBs. The material 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 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.
Each ampoule should be **reconstituted with 0.5 ml distilled water**.

6. STABILITY

The standard is supplied lyophilized and should be stored at or below -20°C. 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. Stability of the standard nevertheless is monitored by PEI at regular intervals. The results obtained so far indicate long-term stability at or below -20°C. Also remains of the reconstituted material may be stored at -20°C or below, provided the user determines stability under its own conditions for preparation of the material, storage and use. Multiple freeze/thaw cycles should be avoided.

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

7. REFERENCES

Knauer O., Volkers P., Nick S., Scheiblauer H.: Collaborative Study to establish a World Health Organization International Standard for detection of antibodies to hepatitis B virus e antigen (anti-HBe). WHO Report, WHO/BS/2013.xxxx.

8. ACKNOWLEDGEMENTS

We thank the participants of the collaborative study for their expertise and contribution.

9. FURTHER INFORMATION

Further information for this material can be obtained as follows: pei-ivd@pei.de or 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 or pei-ivd@pei.de

11. CITATION

In any circumstance where the recipient publishes a reference to PEI materials, it is important that the correct name of the preparation, the code number, the name and the 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: ONTAINS INFECTIOUS HEPATITIS B VIRUS (HBV) & HUMAN PLASMA	
Handling: See caution, section 4	
Toxicological properties	
Avoid inhalation , ingestion: or skin absorption – *contains infectious HBV*	
Suggested First Aid	
Inhalation and ingestion: Seek medical advice – *contains infectious HBV*	
Contact with eyes or 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. 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.

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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.