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Collaborative Study to Establish a World Health Organization International Standard for Hepatitis B e Antigen (HBeAg)

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**** See Appendix 1***

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*

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1. Collaborative Study report

1.1 Summary

A standard for Hepatitis B e antigen (HBeAg) can be used in the standardization and calibration of quantitative and qualitative diagnostic HBeAg assay kits and for quality control purposes by manufacturers, control authorities and users. A WHO collaborative study was undertaken to assess the suitability of a candidate material for HBeAg in diagnostic assays. The candidate HBeAg International Standard (sample A1, code 129097/12) is a lyophilized preparation of the current Paul-Ehrlich-Institut (PEI) HBeAg standard (sample A2, HBe-Referenzantigen 82, PEI 82). In the study A1 and A2 were compared and the potency of the candidate International HBeAg standard was assessed relative to A2. In addition, further HBeAg positive samples from Hepatitis B virus (HBV) infected carriers were selected and included in the study: (A3) high titer HBeAg serum sample (high positive for HBsAg), (A4) medium titer HBeAg serum sample (high positive for HBsAg), and (AB5) low titer HBeAg serum sample (high positive for HBsAg and positive for anti-HBe).

Nineteen laboratories from 12 countries tested the materials using 14 different HBeAg assays. The dilution range of the candidate material A1 was selected to cover the dynamic measuring ranges of the assays. The endpoint titers equivalent to the assays' cut-offs ranged from 312 to 3160. Since the PEI HBe-Referenzantigen 82 (sample A2, 100 PEI U/ml) is well-characterized and has been used by many manufacturers for the standardization of HBeAg assays since 1982, it was used as the source material for the candidate A1 and the potency of the candidate A1 was expressed relative to the PEI HBe-Referenzantigen 82. The overall potency of the candidate A1 was 95.1 U/ml. Thus, the difference from the source material is approximately 5 U/ml. This difference lies within the normal intra-assay variation of HBeAg tests and is therefore considered negligible.

The HBeAg high, medium and low titer samples A3, A4 and AB5, were included in the study to evaluate commutability of the candidate standard A1. Samples A3 and A4 were detected as positive by almost all assays, but S/CO ratios differed substantially according to the diverse measuring ranges of the HBeAg assays. The low positive sample AB5 was detected positive by nine HBeAg assays, but was missed by five assays. Those kits that failed to detect samples A4 and AB5 as positive or were low positive only had the lowest endpoint titers of the candidate material A1 and thus the lowest analytical sensitivity. This correlation between sample recovery and analytical sensitivity of the assays was statistically significant. Samples A1 and A2 had comparable intra-assay and inter-laboratory variation and it can therefore be assumed that the material is homogenous.

The accelerated stability study on sample A1 revealed a stable HBeAg recovery at stress temperatures up to 45°C for one week which may predict long term stability. The long term stability study at the recommended storage temperature is ongoing.

In summary, the candidate HBeAg material (code 129097/12) is proposed to be established as 1st International Standard for HBeAg with an assigned potency of 100 IU/ml. The standard is intended for use in the standardization and calibration of HBeAg assays or for assessment of the analytical sensitivity of HBeAg assays.

1.2 Introduction

Hepatitis B virus (HBV), a small packaged partially double-stranded DNA virus, represents an enormous global health care burden. Despite an effective vaccine, HBV is endemic in many countries and affects two billion people worldwide [1, 2]. The spectrum of HBV disease varies greatly from asymptomatic infection to clinically manifest liver disease and even fulminant hepatitis. In general, infection resolves in 90% of immunocompetent individuals while especially children and immunocompromised persons may develop chronic disease. The chain of HBV transmission is maintained partly by chronically infected HBV carriers with 350 million people infected worldwide [1, 3, 4].

The diagnosis of acute Hepatitis B virus (HBV) infection comprises the detection of HBsAg and anti-HBc and if positive, further markers such as anti-HBc IgM, HBeAg and anti-HBe are determined. Chronic Hepatitis B is diagnosed using a combination of biochemical, histological and virological markers including the determination of HBV DNA. Amongst these, determination of the presence of HBeAg is crucial for deciding whether and when to start and when to stop antiviral therapy. Recently new diagnostic tests emerged for the quantification of HBeAg for the assessment of the efficacy of antiviral therapy. Treatment with antiviral medication is continued until HBeAg seroconversion (and loss of HBeAg reactivity) has been achieved. This is commonly considered to be favorable for therapeutic success. HBeAg is associated with HBV replication, indicating high infectivity. HBeAg appears shortly after HBsAg and is detectable for a few days to several weeks. In chronic HBV infections, elevated levels of HBeAg may be detected for years. In some patients HBeAg cannot be detected although the virus replicates persistently. Here, HBeAg seroconversion might evolve into the selection of precore mutants in some patients and therefore has to be tightly monitored [5, 6].

So far, an International HBeAg standard is not available and many diagnostic test manufacturers globally as well as authorities have been using the PEI HBeAg standard which was developed in 1982 (HBe-Referenzantigen 82, PEI 82). Thus, the sensitivity of many HBeAg assays is expressed as PEI Units/ml, a unitage that is traceable to the PEI HBeAg standard. This material has also been used for the control of the analytical sensitivity of HBeAg assays, for the calibration of sub-standards to be used in manufacturers' quality control for final product release testing and for official batch release testing of HBeAg assays by national authorities. Apart from that, the standard was used to compare and standardize assays (7, 8, 12) and to develop protocols for the quantitative determination of HBeAg in qualitative assays (9-14).

At the meeting of the Expert Committee for Biological Standardization (ECBS) at the World Health Organization (WHO) in October 2011, a project proposed by the PEI was endorsed to establish a WHO International HBeAg Standard. The proposed standard would be formulated with the same source material used to produce the current PEI HBeAg standard and lyophilized to ensure its long-term stability. In addition, it was proposed to include further HBeAg positive materials from HBV infected individuals in the study in order to see whether the results from the candidate material are commutable with the results from other clinical samples.

The aims of the collaborative study therefore were to (i) establish the 1st International Standard for HBeAg, (ii) to define its potency in international units (IU) and (iii) to demonstrate similar performance to other HBeAg positive high, medium and low titer samples.

An international conventional reference measurement procedure for HBeAg currently does not exist and this measurand is not traceable to International System of Units (SI) of quantity.

1.3 Materials

1.3.1 Sample A1: Candidate HBeAg Material, code 129097/12

The candidate HBeAg material is a freeze-dried preparation of the PEI HBeAg standard material (HBe-Referenzantigen 82). The material is HBeAg positive serum that was collected in the early 1980s and stored liquid at -70°C. Aliquots of the material were thawed on ice and then pooled to give a final volume of 1.05 liter. No stabilizers or preservatives were added. Five hundred microliter aliquots were filled into neutral amber glass ampoules and freeze-dried at Greiner Diagnostic AG, Switzerland. A total number of 2003 ampoules were obtained. The freeze-dried material was characterized as follows: high positive for HBsAg, anti-HBc IgG and HBV DNA (20.000 IU/ml by Cobas TaqMan). The material tested negative for anti-HBs, anti-HBc IgM and anti-HBe. It was found positive for antibodies to HCV. HCV RNA was tested weak positive (< 30 IU/ml using ABBOTT RealTime HCV assay). HCV core antigen, anti-HIV-1/2 and anti-HDV were negative. The residual moisture content was 1.3% and the fill coefficient of variation (CV) 0.9% (wet fill weight). Participants of the collaborative study had been requested to reconstitute this material in 0.5 ml distilled water.

1.3.2 Sample A2: PEI HBeAg Standard (HBe-Referenzantigen 82, PEI 82)

This serum was collected by PEI in 1982 and was stored liquid at -70°C in glass ampoules in 0.5 ml aliquots. Each ampoule has an assigned unitage of 100 PEI U/ ml (expressed as 100U/ml for the purpose of this study). The material is high positive for HBV DNA, HBsAg and anti-HBc. HCV RNA and anti-HCV were tested positive. The material tested negative for markers anti-HBs, anti-HBc IgM, anti-HBe, HCV core antigen, anti-HIV-1/2 and anti-HDV.

1.3.3 Sample A3:

This material was purchased from Trina Bioreactives (Nänikon, Switzerland) and is HBeAg positive human plasma obtained from a 45 year-old female Asian donor (draw date 01.02.2009). Since the material was high positive for HBeAg, it was diluted 1:250 (vol/vol) in anti-HBe free normal human serum to bring the concentration of HBeAg down to within the linear range of most assays. The serological profile of the diluted material is high positive for HBeAg and HBsAg and negative for anti-HBe, anti-HIV 1/2 and anti-HCV. The material is liquid and stored frozen at -70°C without preservative. Corrigendum: In contrast to the study plan sent out to the participants, the material does not contain anti-HBe.

1.3.4 Sample A4:

This material was purchased from Trina Bioreactives (Nänikon, Switzerland) and is HBeAg positive human plasma from a 45 year-old Asian female donor (draw date 13.08.2009, donor not identical to sample A3). Since the material was high positive for HBeAg, it was diluted 1:1000 (vol/vol) in anti-HBe free normal human serum to bring down the concentration of HBeAg to the linear range of the assays. The serological profile of the diluted material is HBeAg positive and HBsAg high positive. The sample is negative for anti-HBe, anti-HIV 1/2 and anti-HCV. The material is liquid and stored frozen at -70°C without preservative.

1.3.5 Sample AB5:

This material was purchased from Trina Bioreactives (Nänikon, Switzerland) and consists of undiluted HBeAg positive human serum from a 38 year-old African male donor. The serological profile of AB5 is low positive for HBeAg, high positive for HBsAg and high positive for anti-

HBe. The sample is negative for anti-HIV 1/2 and anti-HCV. Corrigendum: In contrast to the study plan sent out to the participants, the material did not consist of plasma.

1.4 Design of the collaborative 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 results for the dilution matrix. The study plan included the following:

- From each of the samples A1 and A2 seven 2-fold dilutions should be prepared and tested in the range as shown in the result's sheets, i.e. A1 and A2 from 1:50 to 1:6400, which were the dilution ranges found adequate by a former feasibility study. Samples A1 and A2 were requested to be tested in each HBeAg assay in triplicate independently on 3 three different days by using a fresh ampoule on each day. The feasibility study showed that one freeze/thaw cycle using the reconstituted material was possible without loss of HBeAg, so that some laboratories chose to analyze the dilutions post thaw (laboratory 1 with kit 1, 9 and 11, and laboratory 19 with kit 14).
- The dilution matrix normally in use in the participant's laboratory should be used. Normal human serum (NHS) negative for anti-HBe was considered appropriate. If NHS was not available, fetal calf serum could be used. The dilution matrix should be tested in triplicate as a control.
- Samples A3-AB5 should be tested neat in triplicates on one single day only.
- All samples should be centrifuged for 10-15 minutes at 3000*g* prior to testing in the case that particulate matter appears.

1.4.1 Participants

Twenty laboratories were provided with study material and 19 returned data. The participants were from 12 countries: Canada (1), Brazil (1), China (1), France (2), Germany (3), Japan (2), Korea (1), Netherlands (1), Russia (2), Thailand (1), UK (2) and USA (2). Participating laboratories are listed in Appendix 1 and were assigned a laboratory code number (1-14) in random order.

1.4.2 Assay methods

Fourteen 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 with the code 1-14 used for the statistical analysis. The HBeAg assays that were used were all sandwich test formats with different conjugate antibodies. The assays 12 and 14 allowed quantitative determination of HBeAg concentration with assay 14 referring to PEI 82. All other assays gave qualitative interpretations. The assays were conducted manually or by automation (assay 10 was conducted on ETI-Max in laboratory 14 whereas it was conducted manually in laboratories 13 and 15). Assay 5 is a modification of assay 4 run on a different instrument. The participants returned signal to cut-off (S/CO) values except for laboratories 14, 15 and 16, where optical density (OD) values were given. Participant 19 also returned quantitative results in addition to the S/CO values. S/CO values $\geq$1.0 were considered reactive, whereas values <1.0 were considered negative. One exception was kit 2 with borderline results ranging from 0.8 S/CO to 1.20 S/CO that were then considered equivocal.

1.4.3 Statistical methods

Statistical analysis was performed at PEI based on the raw data provided by the participants. The detection limits with the diluted materials sample A1 (lyophilized candidate material) and sample A2 (PEI 82) were calculated by linear interpolation at the intercept of the dilutions series (titers) with the assay's cut-off.

Geometric mean values (GMV) including their 95% confidence intervals (CI) were used to describe each assay. The geometric coefficient of variation (GCV = sqrt(exp(SD*SD)-1), where SD is the standard deviation of the ln-transformed data) was used to describe the intra-assay and inter-laboratory variation. Only values > 0 were considered in the calculations.

In order to estimate the potency of sample A1 relative to sample A2 two approaches were used:

1. For each assay (within the different laboratories) the potency was estimated by the ratio of the GMV for this assay.
2. The potency was also estimated by means of a parallel line model, PLA [15], using either ln-transformed response data or a four parametric logistic function. The assumption of parallelism was shown for 86% of the assays, linearity was shown for 79% of the assays and both parallelism and linearity was shown for 64% of the assays.

Spearman's rank correlation coefficient was used to assess the correlation between analytical sensitivity and HBeAg recovery in samples A3, A4 and AB5 [16].

1.5 Results and Discussion

1.5.1 Data received

All data received were subjected to analysis. The majority of participants followed the study plan with the following exceptions: laboratory 11 and 12 tested 1 replicate only for each of the 3 days using assay 9. For laboratory 14, run 1 had to be deleted from the calculation due to deviating values leading to a lack of intercept with the cut-off. All laboratories used either serum or plasma as diluent except for laboratories 14 and 16, which used 0.9% NaCl supplemented with 10% fetal calf serum (FCS) and phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), respectively.

1.5.2 Comparison of candidate material A1 (code 129097/12) and sample A2 (PEI 82, 100 U/ml) titers at cut-off

The GMV of the endpoint titers for each assay were calculated for the two materials are shown in **Table 2.** The confidence intervals for the GMV's are also shown **(Table 2)**. The dilution ranges for samples A1 and A2 were in the dynamic measuring range for all assays included in the study. Titers of A1 varied from 312 to 3160 due to differences in analytical sensitivity of the HBeAg assays. The majority of HBeAg assays detected the analyte at titers between 400 and 800. Those laboratories investigating the same assay gave very comparable titers except for kit 10 with an outlier value in laboratory 14. This might be explained by the different testing procedures used (manual vs. automated) and/or different diluents used (serum/plasma vs. NaCl+FCS). Highest titers were reached by kits 1, 4 and 6. When the titers at cut-off of sample A1 were plotted against titers of sample A2, comparable dose responses were obtained (**Figure 1**), i.e. the data points closely lined up along the angle bisector which represents the 100% identity line. This indicates that lyophilization resulted in no or only little loss of HBeAg of the candidate material A1 in comparison to PEI 82 (A2).

1.5.3 Potency calculations

All potency calculations of the candidate material A1 were performed relative to sample A2 (PEI 82) since this was the only material available as a reference for potency determinations. The potency calculation for the candidate material A1 was performed by calculating the ratios of the geometric mean values (GMV) of the different test runs of sample A1 (candidate material code 129097/12) to the GMV of sample A2 multiplied by 100 (**Tables 3a and 3b**). Most assays grouped around the 100 U/ml value with minimum and maximum values of 70.0 for kit 3 and 129.6 for kit 6. Calculating the ratio for all assays by GMV, the overall potency of the candidate 129097/12 was 95.1 U/ml (95%-CI: 90.7 – 99.8). When calculating the potency of the candidate material A1 for all assays by parallel line assay (PLA), the overall potency was 95.8 U/ml (95%-CI: 91.9 – 99.8) again with minimum values for kit 3 (85.0) and maximum values for kit 6 (130.3) indicating very similar results for both calculation methods (**Table 3a**). Nevertheless, the results from the confidence intervals indicated that the recovery of sample A1 was significantly lower compared to the reference 100 U/ml in sample A2. The potencies were also presented in a histogram (**Figure 2**). Each box represents the laboratory potency for a certain assay with the boxes individually labeled by laboratory and kit code number. The graph shows a Gaussian curve-like distribution of all assays around the 100 U/ml value of sample A2 with most assays located in the 90-100 U/ml sector. The potency comparison in **Figure 3** shows that all assays group along the angle bisector, reflecting a near 100% recovery of the candidate material A1 relative to A2. Minimum and maximum potencies were represented by laboratory 4 with kit 3 and laboratory 6 with kit 6. One out of these outliers was due to lower GMV ratios because of low single run values in the respective assays (kit 3, run 3; see **Table 3b**).

1.5.4 Correlation of assay analytical sensitivity relative to sample A1 with recovery of supplemental samples A3, A4 and AB 5

In this approach, additional HBeAg high, medium and low titer samples were included in the collaborative study to investigate whether the candidate material A1 performed similar to further HBeAg positive samples. At first, a qualitative analysis was performed by calculating S/CO ratios due to the qualitative design of most HBeAg assays of the study. Kits were ranked according to their analytical sensitivity relative to A1 presented as units in a top-down manner (**Table 4)** starting with the most sensitive assay. The high positive sample A3 was detected positive by all assays and the medium positive sample A4 was detected positive by most assays (12 out of 14). One assay (kit 11) which was performed by 2 laboratories yielded borderline results with one result just above and one result just below cut-off. One test was negative. The low positive sample AB5 was detected positive by nine assays, but was missed by five assays. In general, the tests with the borderline and the negative results on samples A4 and AB5 had the lowest endpoint titers of the candidate material A1 and clearly showed lowest analytical sensitivity relative to A1. Statistical analysis of the correlation of S/CO values in the samples A3, A4 and AB5 to analytical sensitivity of the assays revealed p-values $p = 0.0167$, $p = 0.0014$ and $p = 0.0407$, respectively, and were therefore significant. Interestingly, kit 9 and kit 12 did not appear to follow the overall close correlation of dose response and analytical sensitivity.

Since S/CO signal levels of the samples in the different assays differed substantially, the relative potencies of test samples were determined using the serial dilutions of A1 as a standard curve assuming a potency of 100 U/mL (**Table 4,** dark shaded areas). Determination of potencies of the samples relative to A1 yielded comparable values each for samples A3 and A4 in all assays. Values for sample A3 ranged from 0.92 U/ml to 1.99 U/ml and for sample A4 from 0.20 U/ml to 0.60 U/ml. Here it should be mentioned that for some assays the relative potency of sample A3 could not be determined due to values beyond the standard curves (nine laboratories had results above the upper limit 2 U/ml, denoted > 2). For those assays that detected sample AB5 as positive relative

potencies ranged from 0.11 U/ml to 0.88 U/ml and therefore showed higher variation. This effect might be due to the low positive nature of the sample and/or the presence of both HBeAg and anti-HBe which could lead to complex formation. The potencies of the samples A3, A4 and AB5 were also presented in a histogram (**Figure 4**, right). Each box represents the laboratory potency of the samples relative to A1 with the boxes individually labeled by laboratory and kit code number. These potency distributions were then compared to the distributions of S/CO values (**Figure 4**, left). For all three samples, it could be shown that edge-values grouped closer to the majority of values when transformed to U/ml (e.g. laboratory L1K1 in sample A3 and A4 and laboratory L18K13 in sample AB5). For sample A3, nine laboratories that gave values > 2 U/ml could not be grouped and were therefore shown as one column designated "> 2", although it is worth noticing that 4 out of these 9 laboratories might display values just beyond 2 U/ml (laboratory L13K10, laboratory L7K7, L10K7 and L19K7, see Table 4). For sample AB5, seven laboratories gave negative values which grouped together in one column (see histogram "AB5 [U/ml]"). Also, the laboratory value L14K10 was grouped here which was due to an outlier value (see also section 4.2.1 for more details).

Next, it was investigated whether the relative potencies of the samples using dilutions of A1 as a standard curve were comparable to relative potencies using dilutions of A2 as a standard curve (**Figure 5**). Overall, the samples lined up very closely to the angle bisector (dotted line) in all assays, indicating no significant difference in the standard curves of A1 relative to the standard curve of A2. One deviation could be observed in kit 10 which was due to low values for A3 in laboratory 14 (not shown).

Generally, the results on the additional samples provide some indication that the candidate standard material A1 behaves similar to further HBeAg positive clinical samples. Ranking of the sample signals according to the standard potency gave similar results. Since the data on the additional samples was somewhat limited, further studies were initiated to show comparability of the results of the candidate HBeAg standard A1 to results from representative patient samples. A range of five HBeAg tests representative of current serological methods and technologies using HBV seroconversion and longitudinal panels which include a HBeAg positive phase. These panels are well-characterized and include negative as well as low and high titer clinical samples. By this the analytical sensitivity can be correlated to the diagnostic sensitivity in terms of first HBeAg detection and signal height. The study outcome is summarized and included in Part 2 of the report.

1.5.5 Intra-assay and inter-laboratory variability

Intra-assay and inter-laboratory variation (% GCV) with samples A1 and A2 are shown in **Table 5**. Sample A1 intra-assay variability at the assay's cut-off was mostly < 16% GCV. Kit 8 gave a higher CV of 31%, which could be traced back to a run outlier (run 1 was very low). Sample A2 intra-assay variability gave similar results with CV values below 16%. Three laboratories, however, had higher values with 23% and 22% CV in kit 10 and kit 8, respectively. Inter-laboratory (between labs using the same assay) variability was investigated for kits 2, 7, 9, 10 and 11. These kits used automated procedures except for kit 10, which was performed both automated (laboratory 14) and manually (laboratories 13 and 15). Values for A1 were 5.1 for kit 2, 10.5 for kit 7, 15.5 for kit 9 and 21.3 for kit 11. For kit 10, inter-laboratory variability was 67.9%. The values were in general in an acceptable range. One exception was kit 10, where the values from laboratory 14 were unexpectedly high. These high values were found for both A1 and A2 and might be explained by the different procedures used by the 3 laboratories (manual vs. automated kit performance) and/or the different diluents used (serum/plasma vs. NaCl+FCS).

Overall, it appeared that samples A1 and A2 had comparable intra-assay and inter-laboratory GCV values and it can therefore be assumed that the candidate material is still homogenous after the lyophilization step.

1.5.6 Preliminary stability study

Ampoules of sample A1 were incubated at PEI from 03/11/2013 to 03/18/2013 each at 4°C, 25°C, 37°C and 45°C. At the end of the respective temperature treatment, each ampoule was reconstituted immediately and then frozen at -20°C until analysis. The serial dilutions were tested using kit 7 in 2 replicates for each run. As a baseline, the activity of freshly reconstituted sample was used. This sample had been continuously stored at -20°C (recommended storage temperature). The titers at cut-off are presented in **Table 6**. There was no loss of HBeAg for the ampoules observed at any of the temperatures and days investigated so far suggesting that the material might be stable under long-term storage at the recommended temperature (-20°C). Further stability studies are ongoing.

1.6 Conclusions and Proposals

In the collaborative study sample A1, the HBeAg candidate International Standard (code 129097/12), was assessed relative to sample A2 (HBe-Referenzantigen 82, PEI 82) which has already been used for assay standardization by manufacturers and has an assigned unitage of 100 PEI units per ml. In addition, PEI 82 was the source material for A1 and served already as a calibrator for kit 14. The overall potency of the candidate material A1 relative to PEI 82 was 95.1 U/ml as determined by the geometric mean value of all results obtained or 95.8 U/ml when calculated by parallel line assay. Overall, the deviation of the potency of A1 from that of A2 is lower than the inter-assay variability of HBeAg tests and within the usual intra-assay variability of single HBeAg test kits. It is noted that A1 will be the 1st International HBeAg Standard and not a replacement of a previous standard, hence it is reasonable to assign a unitage of 100 IU/ml to sample A1.

Intra-assay and inter-laboratory variation for the candidate material A1 showed homogeneity of the material and thus did not indicate inappropriate sample conditions after lyophilization. The dilution capacity of the candidate material A1 is high enough to cover the dynamic measuring ranges of the HBeAg assays of the study indicating that it could serve as a calibrator also for other HBeAg assays. Suitable stability of the material has been demonstrated in a preliminary study applying heat stress conditions. The candidate International HBeAg Standard A1 will be very useful for the standardization of qualitative and quantitative HBeAg tests that gain increasing importance in HBV therapy and disease monitoring as new therapy options arise, for the determination of the analytical sensitivity of HBeAg tests and for the calibration of secondary standards to be used for quality control purposes, e.g. in batch release testing.

To address how the candidate HBeAg standard A1 and clinical HBeAg positive samples compare in different HBeAg tests, additional samples were included in the study. If values of samples A3, A4 and AB5 were above cut-off and fell in the dilution range of A1 selected for the study, the results showed that the performance was in general similar to that of sample A1. Therefore, there is already some indication that the proposed International HBeAg Standard may be commutable. This assumption is supported by scientific studies relating to sample A2 (PEI 82) which was the source material (9 to 14).

In summary, sample A1 is proposed to be established as the 1st International Standard for HBeAg with an assigned unitage of 100 International Units per milliliter. The proposed unitage does not carry an uncertainty associated with its calibration. The only uncertainty is therefore derived from the variability of the wet fill weight of the ampoule content which had a coefficient of variation of 0.9%.

1.7 Comments from participants

All participants responded to the request for comments on the report. All comments from the participants were addressed and, where necessary, respective corrections were made to the report. Overall, the commutability of the proposed 1st International HBeAg standard was discussed in depth and therefore, it was decided to add supporting data to the report. This data would be created by the investigation of follow-up clinical samples from acute and recovered HBV infection by HBeAg tests in comparison to sample A1. These clinical samples would include negative as well as low, medium and high titer HBeAg positive specimens and thus are considered representative of typical clinical samples. Selected HBeAg tests comprise commonly used current serological methods.

2. Complementary characterization of the proposed HBeAg standard

2.1. Purpose of the complementary investigation

The outcome of the Collaborative Study and the results of the three additional HBeAg positive clinical samples suggested that the candidate 1st HBeAg International standard (IS) is commutable. However, the quantity of data is limited. Therefore, it was decided to further address commutability of the candidate 1st International HBeAg standard in an additional study performed at the Paul-Ehrlich-Institut. Currently there is no golden standard method for the assessment of commutability and approaches taken depend on the nature of the measurand, availability and features of testing methods and also on the expected use of a reference material (17, 18). One potential use of the candidate 1st HBeAg IS will be determination of the analytical sensitivity of HBeAg tests. The approach chosen for this study was therefore to determine the analytical sensitivities of different HBeAg tests by measuring serial dilutions of the candidate 1st International HBeAg standard. Analytical sensitivity is defined as the concentration of HBeAg (in U/ml) that correlates to the cut-off of a HBeAg test. In addition, clinical samples from different stages of HBV disease were selected and measured in parallel to the dilutions of the candidate standard to determine clinical sensitivity of the HBeAg tests. Hence the assumption is that if the analytical and the clinical sensitivity (ability to detect HBeAg in samples from HBV infection) of the HBeAg tests correlate well, the HBeAg standard is commutable.

2.2. Materials and methods

2.2.1. Test kits

Five HBeAg tests were selected for this additional investigation. Selected HBeAg tests represented widely used current serological methods. The tests exhibited a qualitative assay format and different technologies comprising ELISA, microparticle enzyme immunoassays, chemiluminescence immunoassays and electro-chemiluminescence immunoassays **(Table 7)**. Test features are described in **Table 1** of the Collaborative Study report. A further reason for the choice of the HBeAg tests was that these tests are routinely used in our laboratory and the laboratory staff was well-trained and familiar with assay performance.

2.2.2. Clinical HBV samples

Nine commercial HBV seroconversion (SC) and longitudinal (LT) panels were obtained from either Biomex GmbH (Siemensstraße 38, 69123 Heidelberg Germany) or from ZeptoMetrix Corporation (872 Main Street Buffalo, NY 14202, USA). SC and LT panels comprise sequential plasma samples from one individual drawn during a certain time frame. Samples were collected at acute symptomatic and at asymptomatic or resolved HBV infection and the panels are well-characterized for further markers of HBV infection **(Table 8)**. The panels enclosed specimens containing different amounts of HBeAg and are considered representative of typical clinical samples. The HBV LT panels include 12 sequential panel members each, out of which only those members were tested comparatively in the five HBeAg assays that most likely represented the HBeAg positive samples. The selection of the samples for testing was based on the information provided by the panel vendor and the results of a pre-screening of all samples of the 9 panels using the ADVIA Centaur HBeAg test. The members of the three HBV SC panels varied from 3 to 20 samples. Again suitable HBeAg containing samples were selected taking into account the vendor´s data sheets and the ADVIA HBeAg test results. In total, 51 clinical samples could be investigated comparatively by all five tests.

2.3. Results of the complementary study

Analytical sensitivity was determined using serial dilutions of the candidate WHO 1st International HBeAg standard (sample A1). To avoid an influence from the choice of the dilution matrix as far as possible, serial dilutions were prepared in normal human plasma which was the specimen type of the clinical samples. Mean analytical sensitivities (detection of antigen at limiting dilutions) of the HBeAg tests were between 0.059 U/ml for the test with the highest analytical sensitivity (Kit 1) and 0.855 U/ml for the test with the lowest sensitivity (Kit 11). Kit 7 detected 0.376 U/ml as positive, Kit 8 0.412 U/ml and Kit 9 0.553 U/ml. Results are shown in detail in **Table 9** and **Figure 6**.

When using the clinical samples, Kit 1 showed highest sensitivity and detected 41 samples as HBeAg positive and 9 samples as HBeAg negative. One sample (SCP-HBV-001 RP-009-01) did not yield a result. This sample could not be retested due to insufficient volume left. The outcomes for the four further HBeAg kits were 32 HBeAg positive results and 10 negative results for Kit 7, 28 HBeAg positive and 21 negative results for Kit 8, 27 HBeAg positive results and 24 negative results for Kit 9 and 23 HBeAg positive results and 28 negative results for Kit 11. Sample SCP-HBV-001 RP-009-01 was reactive by Kit 8, but excluded from the calculation, since the result was neither obtained with any other HBeAg test nor was the result "confirmed" by a positive result of the direct sequential sample or by the vendor´s data. Moreover, the sample was exhausted and could not be retested. Results of the investigation are found in detail below **(Table 10, Figure 6)**.

2.4. Summary and conclusions of the complementary study

In the complementary study, analytical sensitivities of five HBeAg tests determined using the proposed 1st International HBeAg standard were compared to clinical sensitivities of the same tests determined by investigating samples from different stages of HBV infection and containing different quantities of HBeAg. Kit 1 showed the highest analytical sensitivity and also the highest sensitivity using the clinical samples. Kit 11 showed the lowest analytical sensitivity and also the lowest sensitivity when testing the clinical samples. When ranking the test kits according to their analytical sensitivity or their clinical sensitivity, they clustered exactly at the same position (Kit 1 > Kit 7 > Kit > Kit 9 > Kit 11). Thus correlation between the determination of analytical sensitivity using the proposed HBeAg standard and determination of sensitivity using clinical samples was very high **(Figure 7)** suggesting that the proposed 1st International HBeAg standard is suitable for its purpose and may serve as a standard for the variety of different samples being assayed.

3. Acknowledgements

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4. References

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Table 1: HBeAg Assays[1)] used in the Collaborative Study.

Assay Code	Product name	ID-No.	Manufacturer	Assay procedure	Capture Antibody	Conjugate	Measu-ring	Analyt. sens. [2)]	No. of labs using kit
1.	ADVIA Centaur HBeAg	01512127	Siemens Healthcare Diagn.	Centaur	Mab	AcrE-Mab	CMIA	≤0.1	1
2.	VITROS HBeAg	8211880	Ortho-Clinical Diagnostics	VITROS	Mab	HRP-Mab	ChLEIA	-	2
3.	RIAKEY HBeAg IRMA	RH05	Shinjin Medics	Manually	Mab	^{125}I-Mab	IRMA	-	1
4.	Lumipulse G HBeAg	RAX3111	Fujirebio Inc.	LUMIPULSE G1200	Mab	AP-Mab	CLEIA	-	1
5.	Lumipulse Presto HBeAg	RO3041	Fujirebio Inc.	LUMIPULSE Presto	Mab	AP-Mab	CLEIA	-	1
6.	VectoHBe-antigen	D-0576	Vector Best	Manually	Mab	HRP-Mab	ELISA	-	1
7.	Architect HBeAg	6C32	Abbott	Architect	Mab	AcrE-Mab	CMIA	<0.5	5
8.	AxSYM HBe 2.0	7D52	Abbott	AxSYM	Mab	AP-Mab	MEIA	≤1.0	1
9.	Elecsys HBeAg	11876376	Roche Diagnostics	Elecsys	Mab	Ru-Mab	ECLIA	≤0.3	3
10.	ETI-EBK Plus HBeAg EIA	N0140	DiaSorin	Manually/ETI-Max	Mab	HRP-Mab	EIA	-	3
11.	Enzygnost HBe monoclonal	OQDM	Siemens Healthcare Diagn.	BEP	Mab	HRP-Mab	EIA	≤1.5	2
12.	ST AIA-Pack HBeAg	0025273	Tosoh	TOSOH AIA	Mab	AP-Mab	MEIA	-	1
13.	DS-EIA HBeAg	B-951	RPC Diagnostic Systems	Manually	Mab	HRP-Mab	EIA	-	1
14.	AutoLumoA2000 HBeAg	CM.03.12	Autobio Diagnostics	AutoLumo A2000	Mab	HRP-Mab	CMIA	≤0.1	1

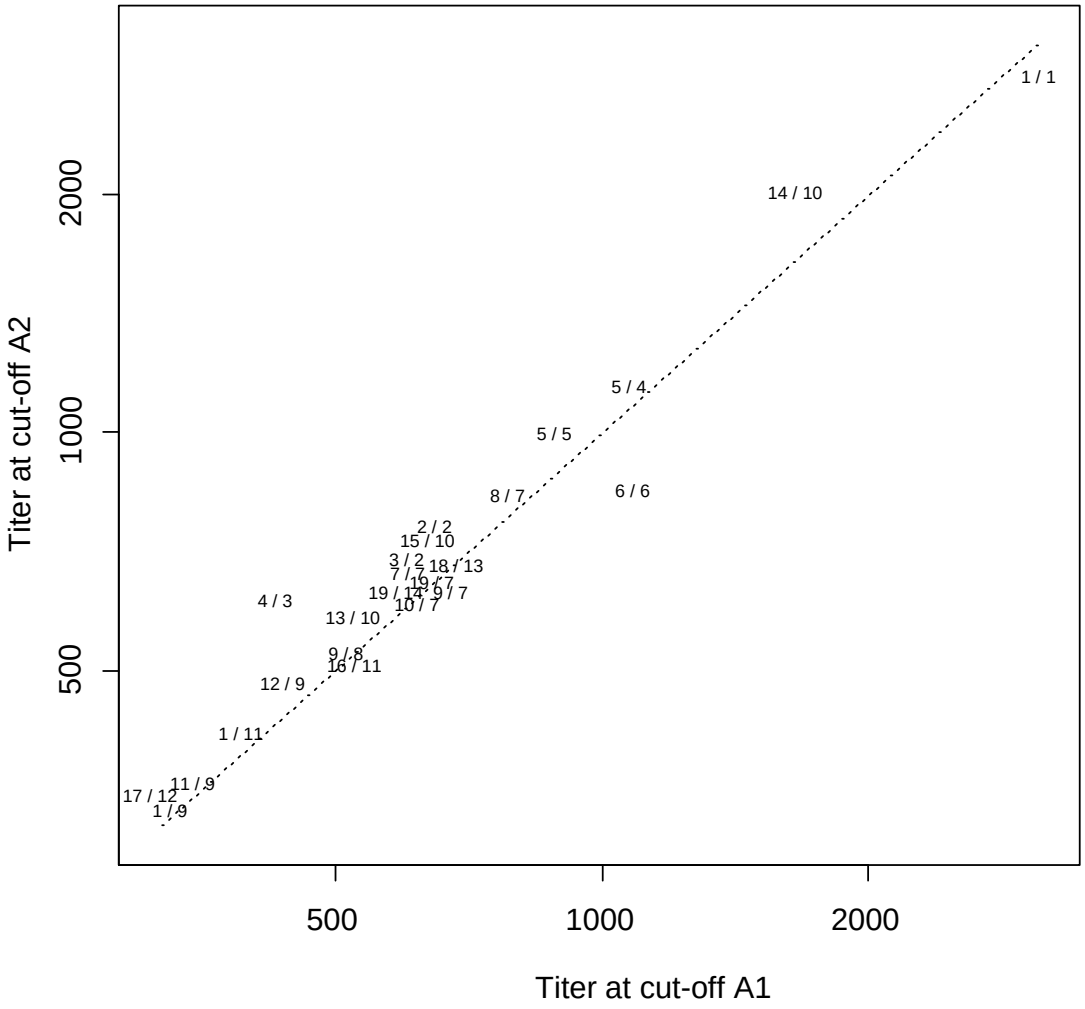
Footnotes:
[1)] All assays follow the sandwich format. No pre-treatment of sample was necessary for any of the assays. Assays 12 and 14 are quantitative tests; all other assays measure the analyte qualitative.
[2)] The analytical sensitivity of the analyte [PEI-U/ml] as of the package insert of the assay.
AcrE=Acridiniumester; AP= Alkaline Phosphatase; HRP=Horseradish Peroxidase; Ru=Ruthenium-Komplex; Ig = immunoglobulin; Mab = monoclonal antibody; PAb = polyclonal antibody; DTT = dithiothreitol; ECLIA = electrochemiluminescence immunoassay; EIA = Enzyme immunoassay; MEIA = microparticle enzyme immunoassay; ELISA = enzyme linked immunosorbent assay; CMIA = chemiluminescence microparticle immunoassay; ChLIA = chemiluminescence immunoassay; CLEIA = chemiluminescence enzyme immunoassay.

Table 2: Mean Endpoint Titers of samples A1 (candidate material) and A2 (PEI 82, assigned unitage of 100 U/ml).

		Sample A1 (candidate material, code 129097/12)		Sample A2 (PEI 82)	
Kit code	**Laboratory code**	**Titer GMV [1)]**	**95%-CI[2)]**	**Titer GMV [1)]**	**95%-CI[2)]**
1	**1**	**3160**	2557 - 3904	**2837**	2245 - 3585
2	**2**	**657**	536 - 804	**756**	565 - 1010
2	**3**	**611**	508 - 735	**696**	595 - 814
3	**4**	**434**	370 - 508	**619**	431 - 889
4	**5**	**1092**	978 - 1219	**1153**	1039 - 1281
5	**5**	**896**	823 - 976	**1005**	809 - 1249
6	**6**	**1102**	970 - 1252	**850**	677 - 1068
7	**7**	**614**	492 - 767	**660**	606 - 718
7	**8**	**796**	697 - 909	**839**	664 - 1060
7	**9**	**638**	538 - 757	**632**	610 - 655
7	**10**	**631**	498 - 798	**626**	578 - 677
7	**19**	**654**	518 - 826	**649**	571 - 738
8	**9**	**523**	248 - 1104	**530**	312 - 902
9	**1**	**332**	298 - 370	**342**	329 - 355
9	**11**	**351**	311 - 396	**363**	294 - 448
9	**12**	**443**	395 - 498	**486**	433 - 544
10	**13**	**535**	389 - 734	**589**	334 - 1040
10	**14**	**1689**	1051 - 2713	**2031**	1781 - 2317
10	**15**	**649**	575 - 732	**736**	420 - 1290
11	**1**	**399**	270 - 591	**419**	327 - 536
11	**16**	**537**	498 - 579	**518**	397 - 675
12	**17**	**312**	237 - 410	**347**	292 - 413
13	**18**	**693**	663 - 725	**667**	460 - 968
14	**19**	**618**	555 - 687	**631**	564 - 707

Footnotes:
[1)] GMV = geometric mean value of titer
[2)] CI = confidence interval

Figure 1. Titer at cut-off comparison: candidate material A1 against sample A2 (PEI 82, assigned unitage of 100 U/ml).



Footnotes:
Each code/code represents one laboratory performing the analysis with a specific kit. The first code represents the laboratory, the second code represents the kit. The angle bisector indicates 100% identity between both materials, A1 and A2, according to titer at cut-off determination. Please note that the data are presented in logarithmic scale for identification of single lab results.

Table 3a: Potency estimates of sample A1 (candidate material) relative to sample A2 (PEI 82, assigned unitage of 100 U/ml). Values were depicted as overall potency as calculated from runs 1, 2 and 3. The potency calculation for the candidate material A1 was performed by setting the ratio of sample A1 relative to sample A2 multiplied by 100. For potency of individual runs, see Table 3b.

		Potency based on GMV[1] Ratio		Potency based on PLA[2]	
Assay	Laboratory	potency	95%-CI[3]	Potency	95%-CI[3]
1	1	**111.4**	77.5 - 160.0	**112.9**	78.6 - 162.2
2	2	86.9	69.5 - 108.7	84.7	65.1 - 110.1
	3	87.8	80.8 - 95.4	86.4	79.8 - 93.7
	Total	**87.4**	82.1 - 93.0	**85.6**	75.2 - 97.4
3	4	**70.0**	52.0 - 94.3	**85.0**	78.7 - 91.7
4	5	**94.6**	93.0 - 96.4	**92.6**	90.6 - 94.6
5	5	**89.1**	77.5 - 102.5	**89.2**	78.6 - 101.4
6	6	**129.6**	108.3 - 155.0	**130.3**	106.4 - 159.5
7	7	93.0	71.4 - 121.2	93.0	71.9 - 120.5
	8	94.9	66.0 - 136.5	93.2	65.0 - 132.7
	9	101.0	87.9 - 116.1	101.3	92.2 - 111.4
	10	100.8	85.8 - 118.4	101.1	86.3 - 118.5
	19	100.8	86.3 - 117.7	101.6	85.4 - 120.8
	Total	**98.0**	93.4 - 103.0	**98.0**	92.5 - 103.8
8	9	**98.7**	79.5 - 122.4	**100.0**	93.2 - 107.3
9	1	97.1	89.8 - 105.0	97.5	89.9 - 105.8
	11	96.7	78.1 - 119.8	95.5	79.4 - 114.7
	12	91.3	78.4 - 106.4	91.8	78.5 - 107.3
	Total	**95.0**	87.2 - 103.5	**94.9**	87.9 - 102.5
10	13	90.8	69.7 - 118.1	91.6	75.3 - 111.5
	14	83.2	45.4 - 152.4	84.3	52.7 - 134.9
	15	88.2	54.6 - 142.5	98.0	65.6 - 146.4
	Total	**87.3**	78.2 - 97.5	**91.1**	75.5 - 109.9
11	1	95.3	82.2 - 110.5	92.5	80.3 - 106.5
	16	103.8	82.4 - 130.7	101.1	79.7 - 128.1
	Total	**99.5**	57.9 - 170.8	**96.7**	55.0 - 169.8
12	17	**89.9**	75.2 - 107.4	**87.9**	84.6 - 91.3
13	18	**103.9**	71.8 - 150.5	**99.0**	74.4 - 131.6
14	19	**97.8**	96.9 - 98.8	**98.7**	97.7 - 99.7
Total		**95.1**	90.7 - 99.8	**95.8**	91.9 - 99.8

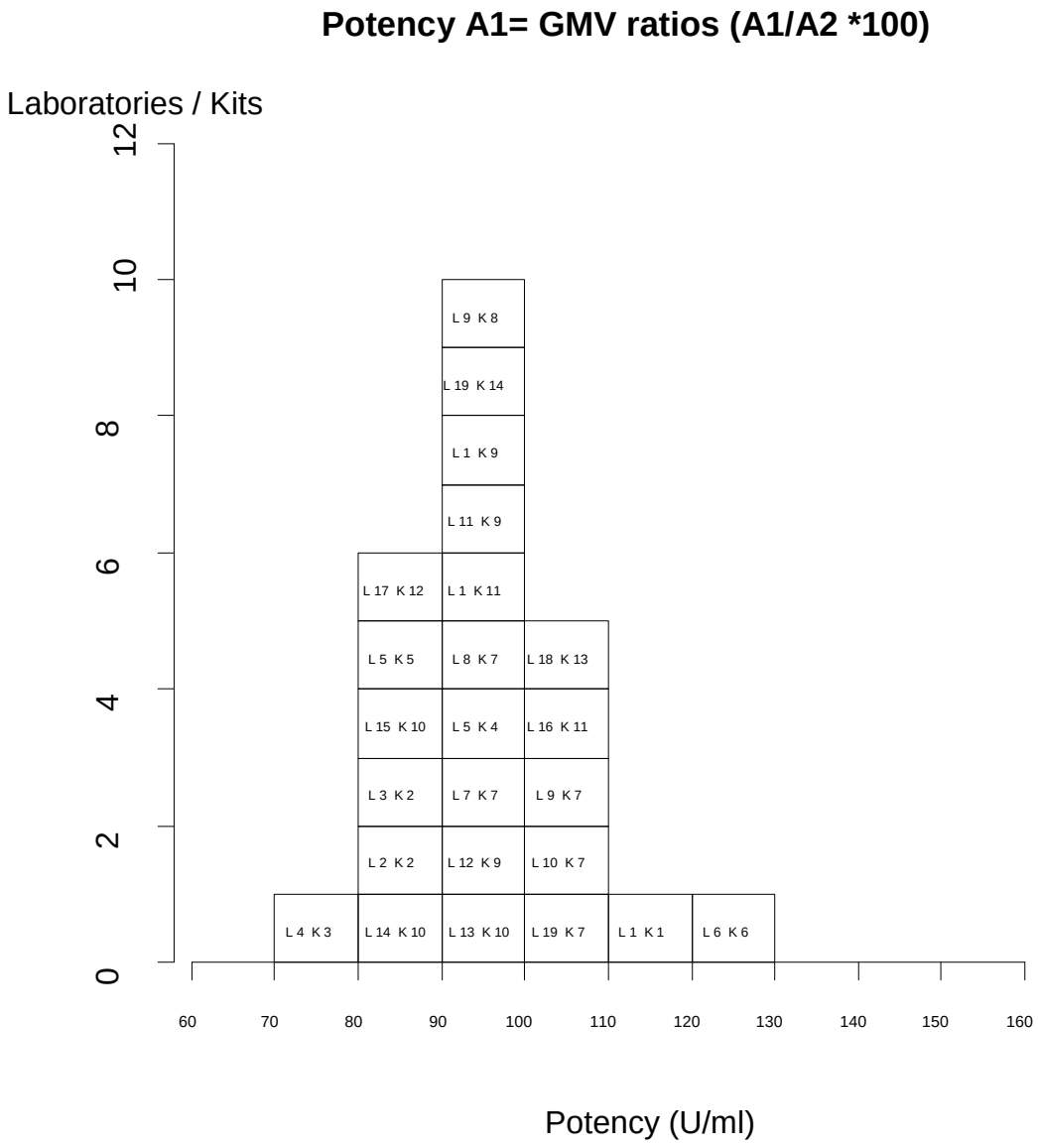
Footnotes:
[1] GMV = geometric mean value
[2] PLA = parallel line assay
[3] CI = confidence interval

Table 3b: Potency estimates of sample A1 (candidate material) relative to sample A2 (PEI 82, assigned unitage of 100 PEI units/ml). Values were shown itemized into run 1, 2 and 3.

		Potency based on GMV[1] Ratio			Potency based on PLA[2]		
Assay	Laboratory	run 1	run 2	run 3	run 1	run 2	run3
1	1	118.9	123.3	94.2	114.9	129.4	96.8
2	2	78.4	92.2	90.9	75.1	91.7	88.2
	3	90.5	84.7	88.2	89.0	83.5	87.0
3	4	76.2	73.8	61.0	84.4	87.8	82.7
4	5	94.8	93.9	95.2	93.2	92.9	91.6
5	5	89.3	94.2	84.2	89.4	84.7	93.9
6	6	132.7	137.2	119.5	131.6	140.6	119.6
7	7	82.5	101.2	96.4	82.7	100.8	96.6
	8	80.2	104.6	102.0	79.2	99.1	103.1
	9	96.6	99.2	107.6	99.3	98.9	105.9
	10	94.5	100.8	107.6	94.0	103.9	105.9
	19	100.4	107.4	94.8	100.9	109.3	95.1
8	9	89.3	103.1	104.4	98.3	103.3	98.4
9	1	100.1	97.3	94.0	101.2	96.5	95.0
	11	88.5	97.2	105.1	88.2	96.6	102.1
	12	94.5	94.7	85.1	94.2	96.0	85.4
10	13	82.5	101.8	89.0	86.8	100.3	88.4
	14		79.3	87.2		81.2	87.5
	15	72.6	106.9	88.3	84.0	116.0	96.6
11	1	101.5	94.6	90.2	98.8	89.6	89.5
	16	115.4	99.7	97.2	112.8	94.9	96.4
12	17	88.7	84.3	97.1	89.0	88.3	86.4
13	18	123.0	98.3	92.8	112.9	91.3	94.1
14	19	97.8	97.5	98.2	98.3	98.7	99.1

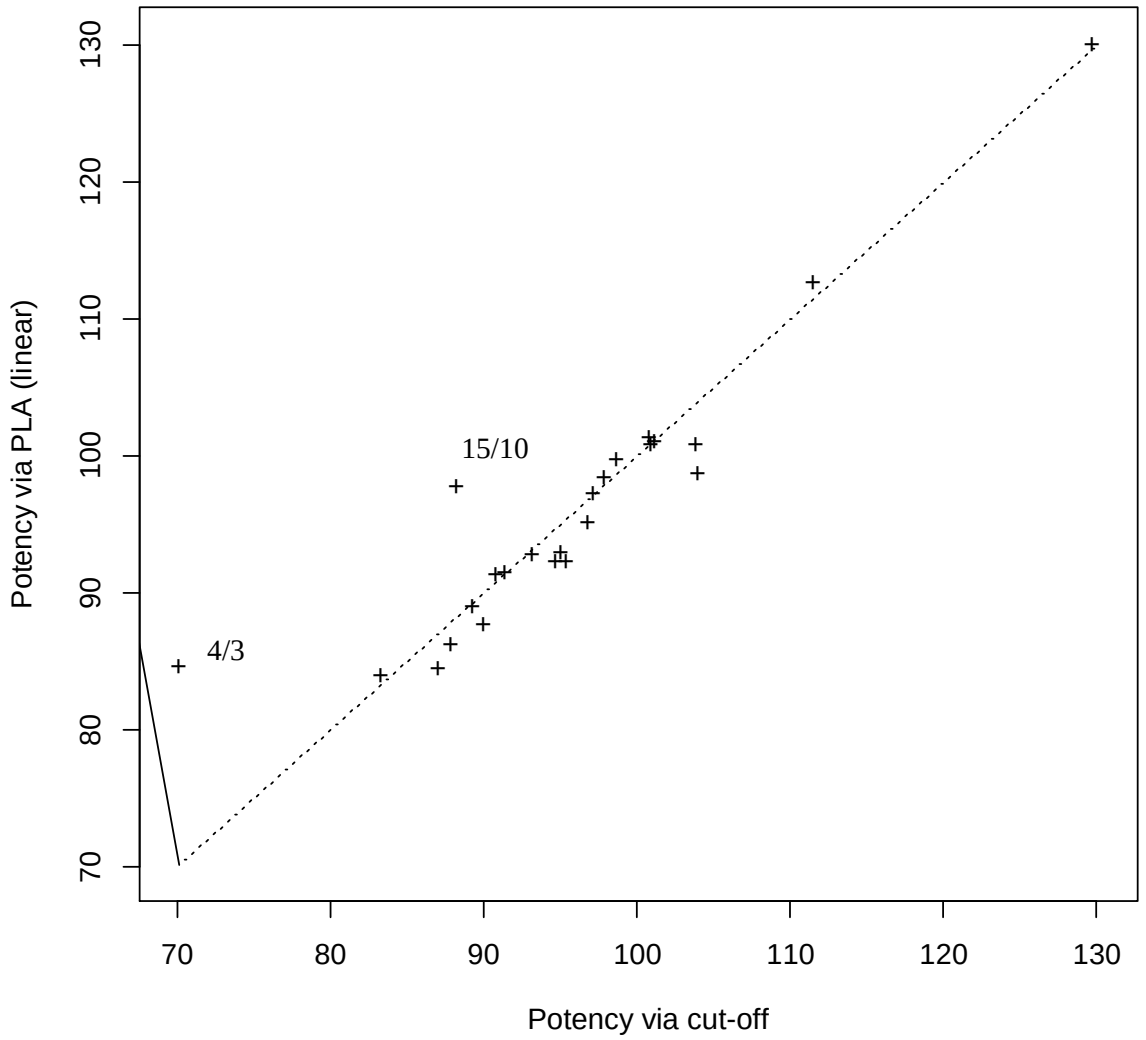
Footnotes:
[1] GMV = geometric mean value
[2] PLA = parallel line assay

Figure 2. Potency of candidate material A1 (GMV ratios relative to A2, PEI-Standard 82, assigned unitage of 100 PEI units/ml)



Footnotes:
Each box represents the laboratory potency estimate relative to sample A2 for an individual kit.
The boxes are labeled with the laboratory (=L) code number, and a code representing the kit used (= K)

Figure 3. Potency comparison: GMV ratios against PLA ratios



Footnotes:
Each cross represents the laboratory potency estimate for an individual kit. The first code represents the laboratory, the second code represents the kit used. The angle bisector indicates 100% identity between both potency analyses, via cut-off and via parallel line assay.

Table 4: Correlation of analytical sensitivity with recovery of samples A3, A4 and AB5.

Analytical Sensitivity[1]					A3	A4	AB5	A3	A4	AB5
U/ml			Kit No.	Lab No.	[S/CO]			[U/ml]		
	Mean values	Lab values								
high		0.032	1	1	50.09	5.50	3.31	1.96	0.20	0.11
		0.091	6	6	8.28	2.12	2.84	1.20	0.24	0.36
		0.092	4	5	14.99	3.30	2.93	1.72	0.34	0.30
		0.112	5	5	11.83	2.37	1.57	1.63	0.29	0.19
		0.187	10	13	11.79	2.75	2.01	> 2.00[2]	0.52	0.38
	0.119	0.059	10	14	16.86	2.41	1.11	1.37	0.16	0.08
		0.154	10	15	13.66	2.58	2.70	1.99	0.32	0.35
		0.144	13	18	10.70	3.01	4.55	> 2.00	0.56	0.88
		0.163	7	7	11.14	2.30	3.30	> 2.00	0.44	0.65
		0.126	7	8	8.81	2.04	2.88	1.71	0.34	0.51
	0.151	0.157	7	9	8.42	2.12	2.92	1.77	0.41	0.59
		0.159	7	10	11.55	2.43	3.44	> 2.00	0.47	0.71
		0.153	7	19	9.31	2.11	2.86	> 2.00	0.40	0.57
	0.158	0.152	2	2	20.10	2.63	0.33	1.38	0.30	0.06
		0.164	2	3	19.99	2.58	0.19	1.35	0.30	0.05
		0.162	14	19	8.35	1.48	0.50	0.94	0.22	0.09
		0.191	8	9	7.70	1.86	0.76	1.91	0.41	0.13
	0.216	0.251	11	1	6.01	1.20	0.43	1.44	0.30	0.09
		0.186	11	16	3.14	0.95	0.66	0.92	0.17	0.08
		0.231	3	4	4.96	0.72	0.71	1.12	0.14	0.13
		0.301	9	1	10.60	2.23	2.02	> 2.00	0.71	0.64
	0.269	0.285	9	11	9.12	1.94	1.75	> 2.00	0.60	0.55
		0.226	9	12	9.91	2.26	1.90	> 2.00	0.55	0.45
low		0.321	12	17	8.37	1.63	2.46	> 2.00	0.55	0.83
Total GMV[3]					10.56	2.11	1.53	> 1.64	0.34	0.26

Footnotes:
[1] Analytical sensitivity was determined using the candidate standard A1. Light shaded areas represent positive samples. Dark shaded areas represent quantitative results using serial dilutions of A1 (starting with 2 U/ml) as standard curve.

[2] the OD values were beyond the A1 standard curve

[3] GMV= Geometric mean value

Figure 4: Individual laboratory mean estimates for sample A3 (a), A4 (b) and AB5 (c).
On the left, S/CO values were depicted, on the right, qualitative values were transformed to U/ml using serial dilutions of the candidate standard A1 as a standard curve.

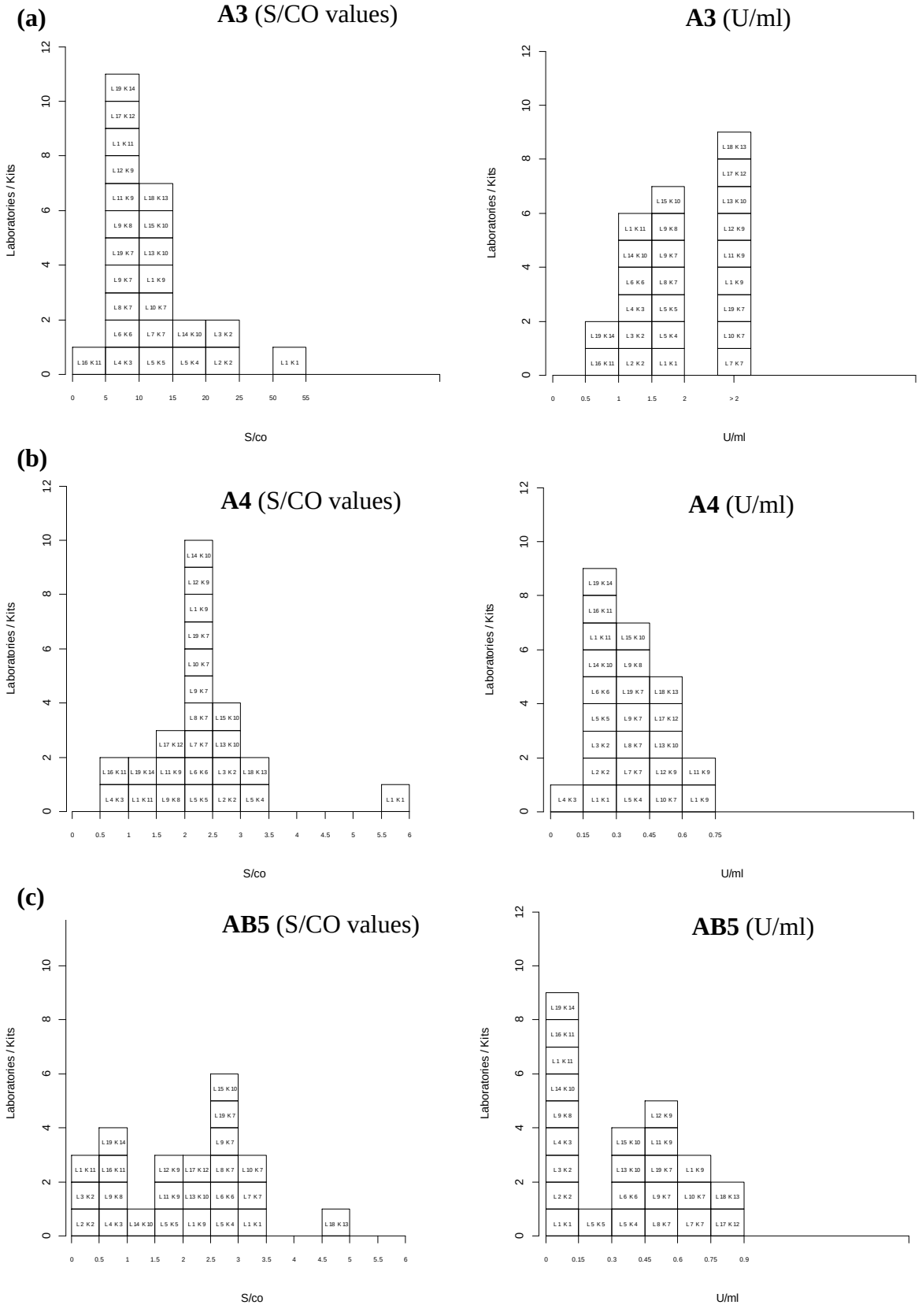
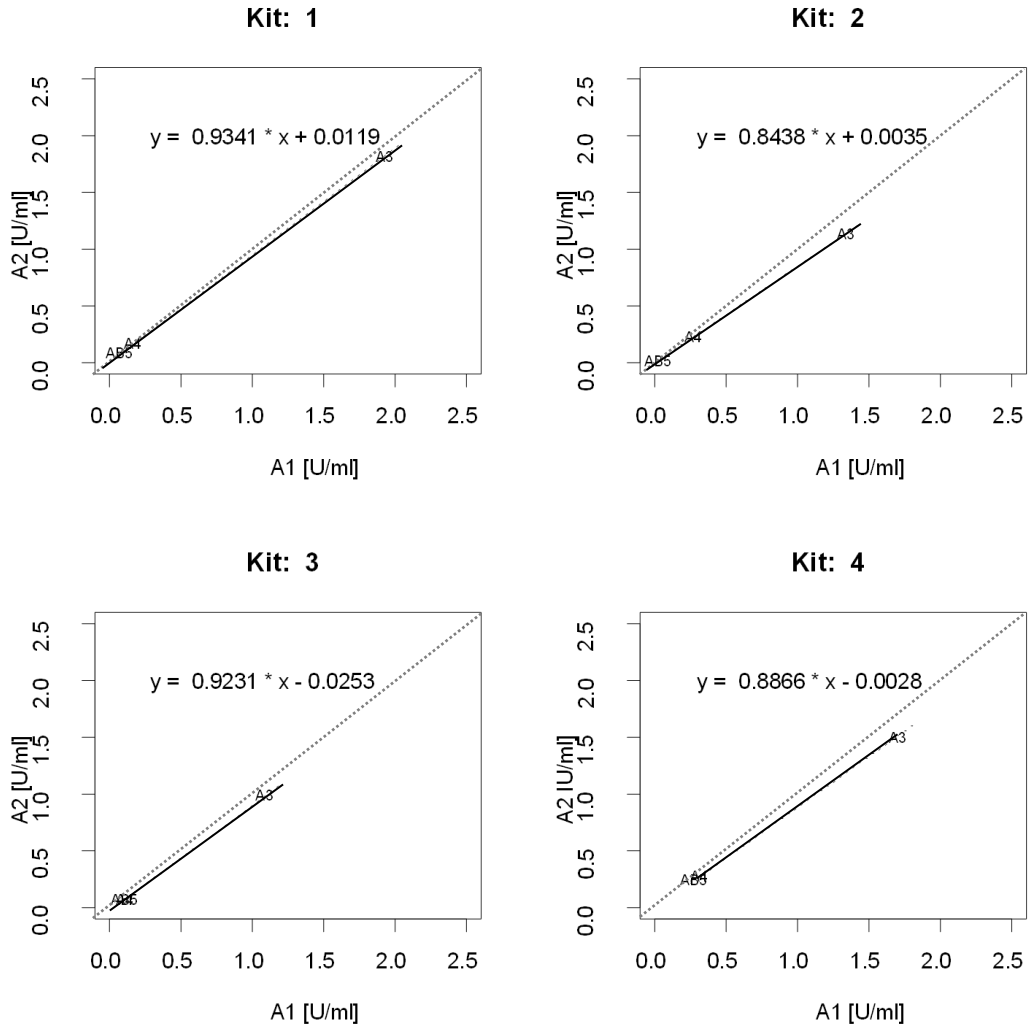
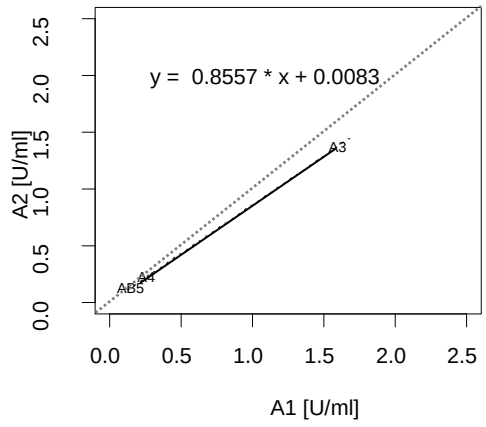


Figure 5: Scatterplots for supplemental samples A3, A4 and AB5 in U/ml using candidate standard A1 relative to A2 (PEI 82).



Kit: 5



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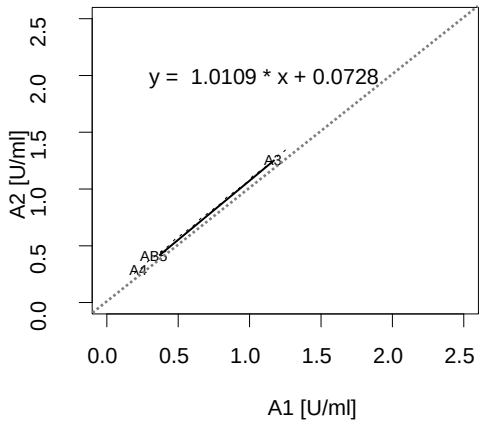
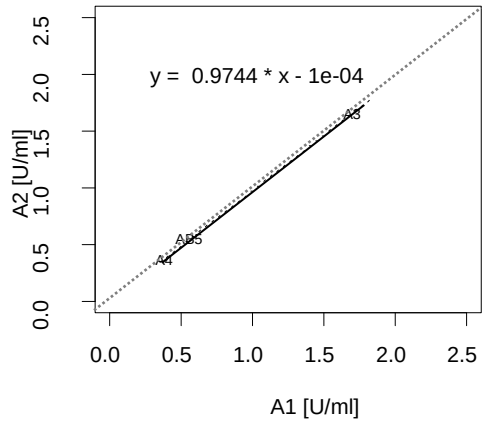
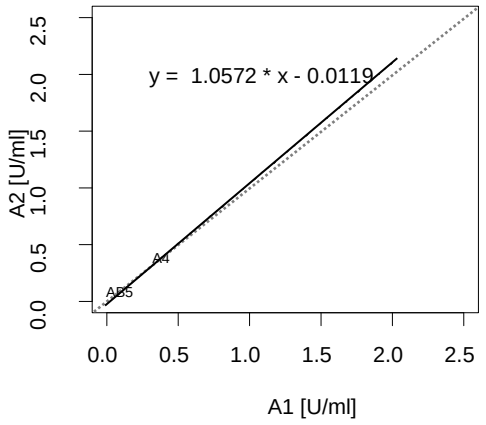
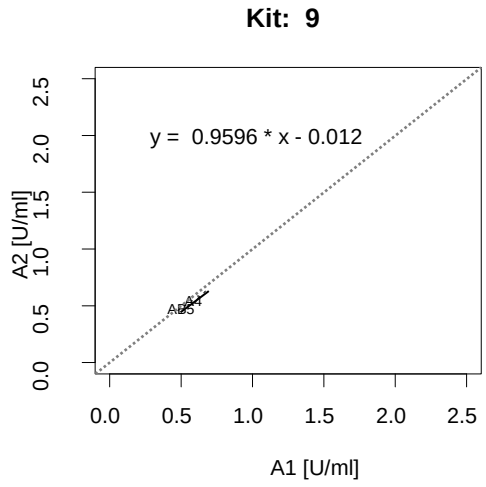


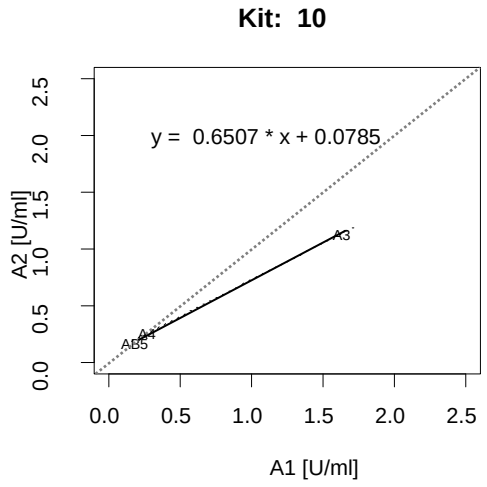
Kit: 7

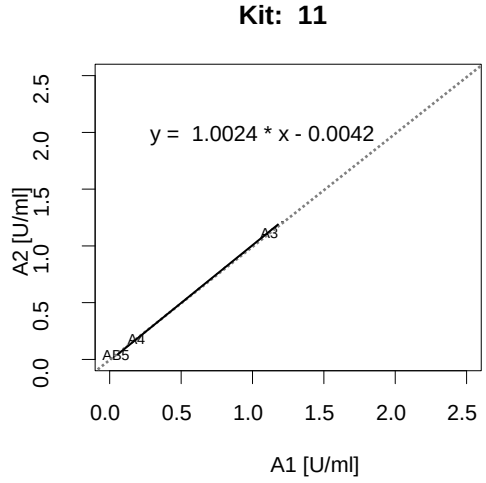


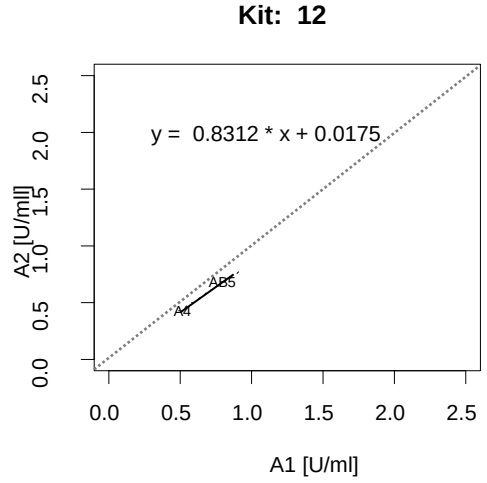
Kit: 8

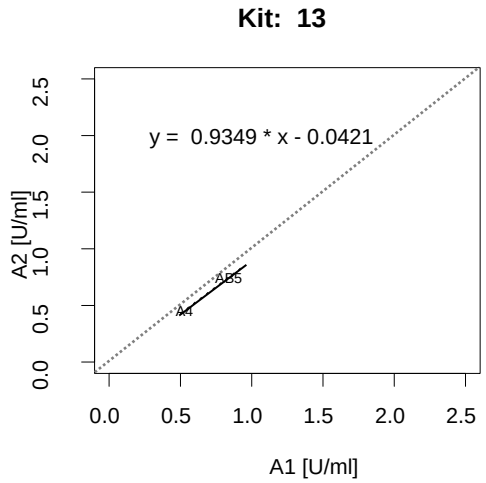


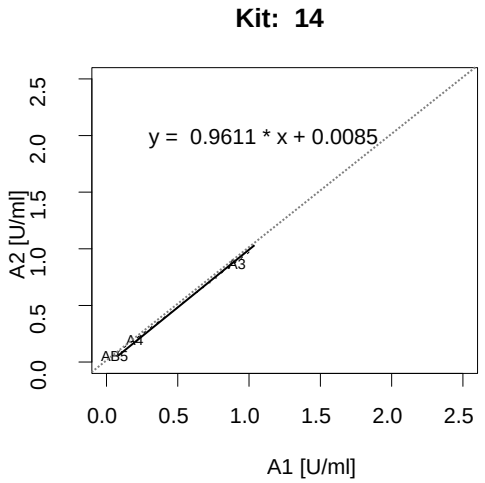












Footnotes:
Where A3 has not been depicted, quantification of A3 was not posible due to values beyond the standard curves (upper limit of standard curve: 2U/ml). The angle bisector indicates 100% identity between quantification using A1 and quantification using A2.

Table 5: Intra-assay and inter-laboratory variation of samples A1 (candidate standard) and A2 (PEI 82) at the assays' cut-offs.

		Titer at the assays' cut-off									
		A1					A2				
		Run			Geom.		Run			Geom.	
Kit	Lab.	1	2	3	mean	%GCV	Run 1	Run 2	Run 3	mean	%GCV
1	1	3442	3156	2903	3160	**8.5**	2895	2560	3081	2837	**9.4**
2	2	657	713	605	657	**8.2**	838	773	666	756	**11.7**
	3	662	603	571	611	**7.4**	731	711	648	696	**6.3**
	Inter-lab				633	**5.1**				725	**5.8**
3	4	455	403	444	434	**6.4**	598	547	727	619	**14.6**
4	5	1046	1088	1143	1092	**4.4**	1103	1159	1200	1153	**4.2**
5	5	914	862	914	896	**3.4**	1024	914	1086	1005	**8.8**
6	6	1165	1052	1092	1102	**5.2**	878	767	913	850	**9.2**
7	7	562	672	614	614	**9.0**	681	664	636	660	**3.4**
	8	750	833	808	796	**5.4**	935	796	792	839	**9.4**
	9	601	629	688	638	**6.9**	622	635	639	632	**1.4**
	10	570	640	688	631	**9.5**	603	635	639	626	**3.2**
	19	615	729	624	654	**9.4**	613	678	658	649	**5.2**
	Inter-lab				664	**10.5**				677	**12.2**
8	9	371	603	641	523	**30.7**	415	585	614	530	**21.6**
9	1	348	329	319	332	**4.3**	347	338	340	342	**1.5**
	11	354	333	367	351	**4.8**	400	343	349	363	**8.5**
	12	467	436	427	443	**4.7**	494	461	502	486	**4.6**
	Inter-lab				372	**15.5**				392	**19.0**
10	13	572	462	579	535	**12.8**	693	454	651	589	**23.2**
	14		1627	1753	1689	**5.3**		2052	2010	2031	**1.5**
	15	683	645	621	649	**4.8**	941	603	702	736	**22.9**
	Inter-lab				837	**67.9**				958	**73.8**
11	1	476	379	352	399	**15.9**	469	400	391	419	**10.0**
	16	528	556	527	537	**3.0**	458	558	542	518	**10.7**
	Inter-lab				463	**21.3**				465	**15.1**
12	17	284	303	352	312	**11.0**	320	360	363	347	**7.0**
13	18	691	707	682	693	**1.8**	561	719	735	667	**15.1**
14	19	593	646	614	618	**4.3**	607	663	625	631	**4.5**

Footnotes:
GCV= geometric coefficient of variation

Table 6: Preliminary stability study on sample A1 (candidate standard) using kit 7

	Titer at the assays' cut-off					
Temperature	baseline	2h	day 1	day 2	day 4	day 7
-20°C	756					
+4°C		678	635	651	647	661
+25°C		666	675	622	680	703
+37°C		685	670	713	678	658
+45°C		702	624	631	659	626

Table 7. HBeAg tests of the complementary study

Product name:	Manufacturer	Assay Code according toTable1
ADVIA Centaur HBeAg	Siemens	1
Architect HBeAg	Abbott	7
AxSYM HBeAg	Abbott	8
Elecsys HBeAg	Roche	9
Enzygnost HBeAg monoclonal	Siemens	11

Table 8. HBV seroconversion and longitudinal panels used

	Panel Catalogue-No.	Type	Panel vendor	Bleeds per panel	HBeAg positive samples (vendors´ information)	Samples tested
1	6509	Longitudinal panel (LT) from acute HBV infection	ZeptoMetrix	12	6509-01	6509-01 to 6509-03
2	6510	LT from acute HBV infection to recovery	ZeptoMetrix	12	6510-1 to 6510-05	6510-01 to 6510-05
3	6513	LT from symptomatic and asymptomatic HBV infection	ZeptoMetrix	12	6513-02 to 6513-11	6513-01 to 6513-12
4	6529	LT from asymptomatic HBV infection	ZeptoMetrix	12	6529-01	6529-01 to 6529-03
5	6534	LT from symptomatic to asymptomatic HBV infection	ZeptoMetrix	12	6534-01	6534-01 to 6534-03
6	6541	LT from symptomatic to asymptomatic HBV infection	ZeptoMetrix	12	6534-01	6534-01 to 6534-03
7	SCP-HBV-001 RP-009	Acute HBV infection	Biomex	20	RP-009-03 to RP-009-11	RP-009-01 to RP-009-14
8	SCP-HBV-007 RP-020	Acute HBV infection	Biomex	3	RP-020-01 to RP-020-03	RP-020-01 to RP-020-03
9	SCP-HBV-003 RP-028	Acute HBV infection	Biomex	9	RP-028-07 and RP-028-09	RP-028-06 to RP-028-09

Table 9. Analytical sensitivities of HBeAg assays relative to A1

A1 Candidate HBeAg IS (WHO)		Kit 1	Kit 7	Kit 8	Kit 9	Kit 11
Dilution steps	U/mL	S/CO*	S/CO	S/CO	S/CO	S/CO
1:10	10.000	307.84	36.02	24.02	32.05	24.41
1:20	5.000	121.50	16.98	11.69	14.75	10.20
1:50	2.000	41.08	5.96	3.99	5.04	3.08
1:100	1.000	18.11	2.54	2.01	2.08	1.35
1:200	0.500	8.87	1.26	1.23	0.87	0.50
1:400	0.250	4.37	0.74	0.58	0.42	0.25
1:800	0.125	2.05	0.54	0.50	0.24	0.13
1:1600	0.063	1.07	n.d.	n.d.	n.d.	n.d.
1:3200	0.031	0.55	n.d.	n.d.	n.d.	n.d.
Dilution matrix	0.000	0.00	0.39	0.40	0.11	0.01
Analytical sensitivity (U/ml)		0.059	0.376	0.412	0.553	0.855

* Mean of triple determinations.

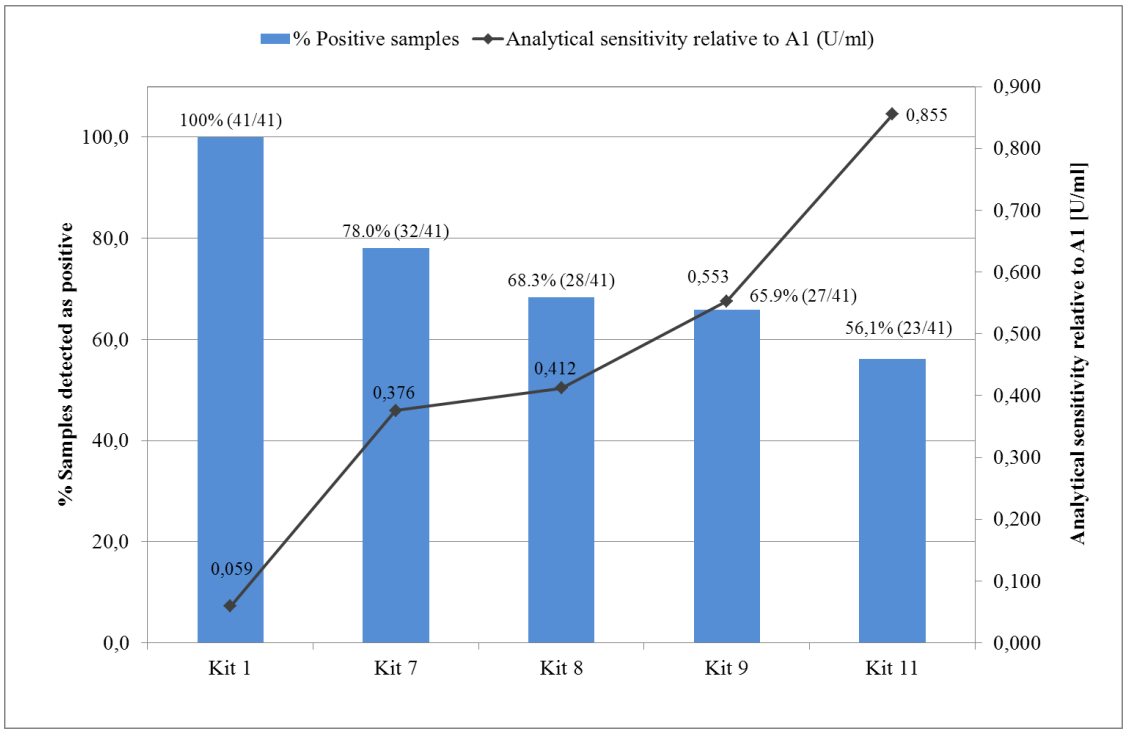
Table 10. Results using clinical HBeAg positive samples derived from commercial HBV seroconversion and HBV longitudinal panels

							HBeAg Assays	Kit 1	Kit 7	Kit 8	Kit 9	Kit 11
HBV seroconversion (SC) and longitudinal (LT)panels	Panel members tested	Date of Bleed	Day	HBsAg (data sheets)	Anti-HBc (data sheets)	Anti-HBe (ADVIA / or data sheets*)	HBeAg (vendors´data sheets)	S/CO	S/CO	S/CO	COI	S/CO
SCP-HBV-001	1	18.07.1995	0	positive	negative	negative	negative	n.d.	0.63	1.20*	0.40	0.35
RP-009	2	21.07.1995	3	positive	negative	negative	grey zone	0.39	0.64	0.58	0.39	0.11
	3	29.07.1995	11	positive	negative	negative	HBeAg pos	1.38	1.36	1.92	1.27	0.09
	4	31.07.1995	13	positive	negative	negative	HBeAg pos	2.34	2.07	158.97	1.85	1.16
	5	16.08.1995	29	positive	positive	negative	HBeAg pos	>1000.0	371.33	180.98	422.10	72.73
	6	18.08.1995	31	positive	positive	negative	HBeAg pos	>1000.0	578.28	193.02	676.10	72.73
	7	23.08.1995	36	positive	positive	negative	HBeAg pos	>1000.0	1347.56	191.97	1112.00	72.73
	8	30.08.1995	43	positive	positive	negative	HBeAg pos	>1000.0	1576.07	189.11	1010.00	72.73
	9	12.09.1995	56	positive	positive	negative	HBeAg pos	>1000.0	1234.29	110.6	858.60	72.73
	10	25.09.1995	69	positive	positive	negative	HBeAg pos	>1000.0	394.77	11.44	295.20	72.73
	11	07.10.1995	81	positive	positive	positive	HBeAg pos	278.64	27.75	0.57	20.72	6.49
	12	14.10.1995	88	positive	positive	positive	grey zone	4.80	0.88	0.40	0.58	0.18
	13	24.10.1995	98	positive	positive	positive	negative	1.34	0.61	0.39	0.31	0.11
	14	04.11.1995	109	positive	positive	positive	negative	0.42	0.47	n.d.**	0.17	0.09
SCP-HBV-007	1	07.01.1999	0	negative	negative	negative*	negative	0.00	0.37	0.49	0.07	0.13
RP-020	2	17.01.1999	10	positive	negative	negative*	HBeAg pos	3.56	3.57	2.47	2.53	1.16
	3	21.02.1999	35	positive	negative	negative*	HBeAg pos	40.92	17.06	10.11	11.99	5.89
SCP-HBV-003	6	07.11.1996	18	positive	negative	negative*	negative	0.55	0.64	0.78	0.38	0.29
RP-028	7	12.11.1996	23	positive	negative	negative*	HBeAg pos	1.80	1.46	1.39	1.27	0.91
	8	14.11.1996	25	positive	negative	negative*	grey zone	2.10	1.52	1.73	1.41	0.89
	9	20.11.1996	31	positive	negative	negative*	HBeAg pos	13.49	5.72	5.85	6.09	3.86
Longitudinal Panel	1	16.05.2001	0	positive	positive	positive	HBeAg pos	111.19	0.78	1.08	0.77	0.47
6509	2	28.05.2001	12	positive	positive	positive	negative	40.09	0.64	0.32	0.47	0.42
	3	13.06.2001	16	positive	positive	positive	negative	40.25	0.65	0.11	0.47	0.49
	4	27.06.2001	42	positive	positive	positive	negative	44.49	0.67	0.08	0.45	0.38
	5	11.07.2001	56	positive	positive	positive	negative	2.90	0.43	0.45	0.10	0.13
	6	25.07.2001	70	positive	positive	positive	negative	0.27	n.d.	n.d.	n.d.	n.d.

Longitudinal Panel	1	22.05.2001	0	positive	positive	positive	HBeAg pos	>1000.0	1443.73	185.45	1101.00	72.73
6510	2	05.06.2001	14	positive	positive	positive	HBeAg pos	>1000.0	750.63	160.23	701.90	72.73
	3	19.06.2001	28	positive	positive	positive	HBeAg pos	128.68	10.93	2.32	8.22	4.71
	4	03.07.2001	42	positive	positive	positive	HBeAg pos	72.29	7.32	2.7	4.57	3.53
	5	17.07.2001	56	positive	positive	positive	HBeAg pos	78.25	8.76	1.71	4.34	3.53
Longitudinal Panel	1	06.05.2001	0	positive	positive	positive	negative	120.54	1.52	2.1	0.55	0.16
6513	2	19.06.2001	44	positive	positive	positive	HBeAg pos	>1000.0	234.29	37.77	156.4	72.73
	3	04.07.2001	59	positive	positive	positive	HBeAg pos	>1000.0	240.01	47.15	180.7	61.58
	4	17.07.2001	72	positive	positive	positive	HBeAg pos	>1000.0	86.85	13.38	65.33	22.45
	5	31.07.2001	86	positive	positive	positive	HBeAg pos	842.49	52.67	8.13	30.78	11.44
	6	14.08.2001	100	positive	positive	positive	HBeAg pos	519.46	27.36	4.05	18.41	6.89
	7	28.08.2001	114	positive	positive	positive	HBeAg pos	408.22	18.68	3.18	12.37	5.69
	8	11.09.2001	128	positive	positive	positive	HBeAg pos	151.35	16.47	2.26	10.74	4.53
	9	25.09.2001	142	positive	positive	positive	HBeAg pos	70.13	1.70	0.42	0.92	0.24
	10	09.10.2001	156	positive	positive	positive	HBeAg pos	14.16	1.05	0.99	0.40	0.40
	11	23.10.2001	170	positive	positive	positive	HBeAg pos	25.09	1.16	0.55	0.45	0.22
	12	06.11.2001	184	positive	positive	positive	negative	0.86	0.36	0.73	0.07	0.15
Longitudinal Panel	1	10.08.2001	0	positive	positive	positive	HBeAg pos	0.71	0.67	0.72	0.45	0.30
6529	2	22.10.2001	-35	negative	positive	positive	negative	0.05	0.43	0.67	0.12	0.14
Longitudinal Panel	1	12.11.2001	0	positive	positive	positive	HBeAg pos	2.14	1.48	0.53	0.84	0.28
6534	2	26.11.2001	14	positive	positive	positive	negative	0.74	0.65	0.47	0.21	0.14
	3	11.12.2001	29	negative	positive	positive	negative	0.09	0.38	0.46	0.04	0.35
Longitudinal Panel	1	03.12.2001	0	positive	positive	positive	HBeAg pos	104.73	1.66	1.04	1.41	0.46
6541	2	17.12.2001	14	positive	positive	positive	negative	9.77	0.68	0.52	0.30	0.30
	3	31.12.2001	28	positive	positive	positive	negative	1.44	0.50	0.51	0.14	0.12

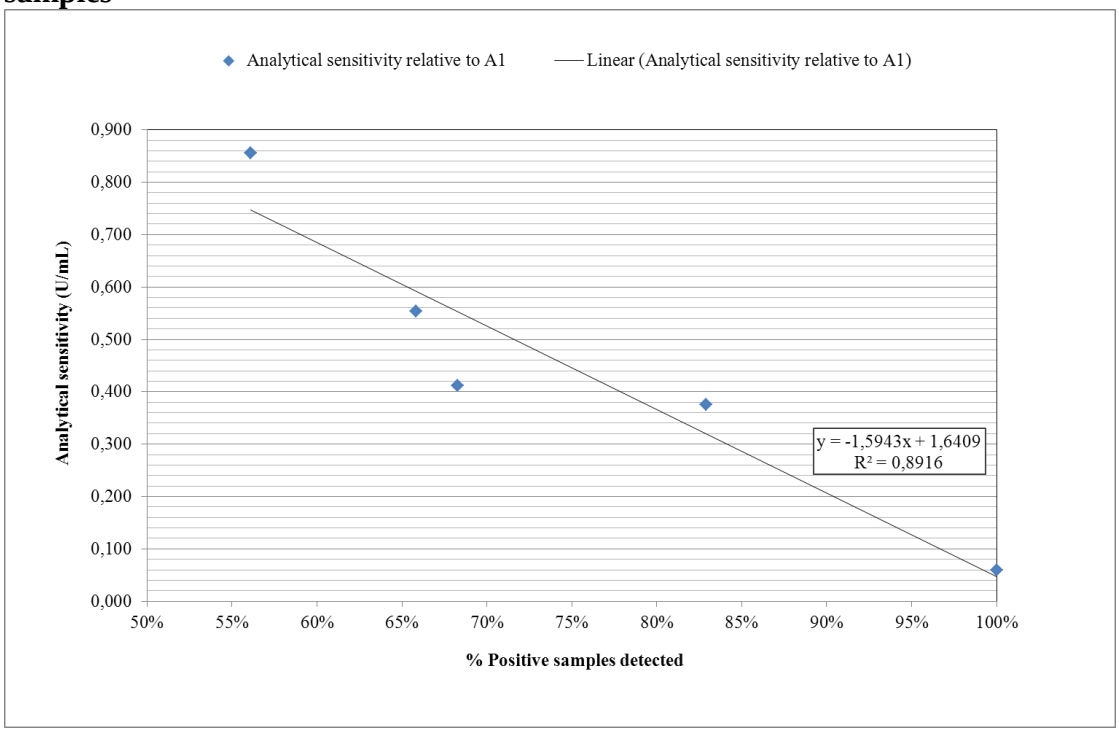
Grey zone: S/CO ratio > 0.90
* Repetition not possible due to insufficient sample volume
** n.d. not determined

Figure 6: Analytical sensitivities of HBeAg tests and detection of HBeAg clinical samples



Footnotes:
Numbers show clinical sensitivity (columns) and analytical sensitivity (line).

Figure 7. Correlation of analytical sensitivity and detection of HBeAg positive clinical samples



Appendix 1

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Appendix 2 Proposed Instructions For Use

See next page

1st World Health Organization International Standard for Hepatitis B Virus e Antigen (HBeAg)

PEI code 129097/12

(Version 1.0, dated)

1. INTENDED USE

The 1st World Health Organization (WHO) International Standard for hepatitis B virus (HBV) e Antigen is intended to be used in the standardization and calibration of quantitative and/or qualitative diagnostic HBeAg assays and for therapeutic and quality control purposes. The establishment of an international standard is an urgent need in the standardization, harmonization and quality control of serological tests and patient management (1). The standard represents a lyophilized preparation of the PEI HBe-Referenzantigen 82, deriving from HBV positive human serum. The material has been lyophilized in 0.5 ml aliquots and stored at -20°C. The material has been evaluated in an international collaborative study involving 19 laboratories performing 14 different HBeAg assays. Further details of the collaborative study are available in the report WHO/BS/2013.xxxx.

2. UNITAGE

This reagent has been assigned a unitage of 100 International Units/ml.

3. CONTENTS

Each vial contains 0.5 ml of lyophilized serum containing infectious HBV.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains material of human origin, and contains infectious HBV. It was characterized as follows: high positive for HBV DNA, HBsAg, anti-HBc, anti-HCV, and anti-HAV. HCV RNA was tested positive (<30 IU/ml). The material tested negative for markers anti-HBs, anti-HBc IgM, anti-HBe IgG, HCV core antigen, anti-HIV-1/2 and anti-HDV. This preparation should be regarded as 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. Each vial should be **reconstituted in 0.5 ml sterile ultrapure water**. The product should be reconstituted just prior to use, once reconstituted, multiple freeze thawing of the product is not recommended. If not all the material is used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at or below -20ºC.

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 the 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

Reissinger A., Volkers P., Scheiblauer H., Nick S. and the Collaborative Study Group.: Collaborative Study to Establish a World Health Organization International Standard for Hepatitis B e Antigen (HBeAg).
WHO Report, WHO/BS/2013.xxxx

8. ACKNOWLEDGEMENTS

We thank the participants and laboratories staff for their expertise and contribution.

9. FURTHER INFORMATION

Further information for this material can be obtained as follows: whoccivd@pei.de or pei-ivd@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 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 PEI 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 (specify): CONTAINS HUMAN SERUM & 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.

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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