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Collaborative Study to Establish a World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Technique (NAT)-Based Assays

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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 Dr David Wood at email: woodd@who.int

Summary

This report describes the World Health Organization (WHO) project to develop an international standard for hepatitis D virus (HDV) RNA for use with nucleic acid amplification technique (NAT)-based assays. The candidate standard is a lyophilized preparation of HDV genotype 1 strain, obtained from a clinical plasma specimen, diluted in negative human plasma. Fifteen laboratories from nine countries participated in a collaborative study to evaluate with their routine HDV NAT the candidate preparation (sample 1 and sample 2) alongside the corresponding liquid-frozen bulk material (sample 3) and a liquid frozen neat HDV RNA positive plasma specimen (sample 4). The results of the study indicate the suitability of the candidate material (sample1 and sample 2, HDV genotype 1) as the proposed 1st WHO standard for HDV RNA. It is therefore proposed that the candidate material (PEI code 7657/12) is established as the 1st WHO International Standard for HDV RNA for NAT-based assays with an assigned potency of 5.75×10^5 International Units per mL (IU/mL) when reconstituted in 0.5 mL of nuclease-free water. On-going real-time and accelerated stability studies of the proposed International Standard indicate that the preparation is stable and suitable for long-term use at the proposed storage conditions.

Introduction

The human pathogenic hepatitis delta virus (HDV), a member of the genus *Deltavirus*, is a defective virus, which needs the hepatitis B virus (HBV) for its replication. HDV causes the most severe forms of acute and chronic viral hepatitis.[1,2] It is transmitted via the same routes as HBV either by simultaneous coinfection with HBV or by superinfection of an already HBV infected individual, i.e. of hepatitis B surface antigen (HBsAg) carriers. Worldwide about 5% HBV carriers are anti-HDV positive (10-15 million people) and the mortality rate lies between 2 and 20% which is ten times higher than HBV alone.

HDV is a small, spherical virus with a 36 nm diameter. Its outer coat contains host lipid and the three HBsAg forms, the large, medium, and small HBs. The ribonucleoprotein is composed of the single, negative stranded, circular RNA of 1.7 kb encapsidated by 70 to 200 molecules of the delta antigen (HDAg), the only HDV-encoded protein. Eight major genotypes (HDV-1 to HDV-8) are known and the genetic variability ranges from 20 to 35% between the genotypes. HDV-1 is the most widely distributed genotype throughout the world and is predominant in Europe, the Middle East, North Africa and North America. It is associated with a broad spectrum of chronic HDV disease. HDV-2 is frequent in the Far East, whereas genotype 3 was exclusively observed in South America. HDV-4 is found in Taiwan and Japan. HDV-5 to HDV-8 are found in West and central Africa, HDV-8 was also recently found in Brazil.[3,4] The therapeutic strategy for chronic HDV infection is difficult due to the unique HDV replication mechanism and because HDV is associated with HBV infection. Currently interferon is the only established therapy for chronic HDV disease.[5]

Nowadays nucleic acid amplification techniques (NATs) to detect HDV RNA are the most sensitive tools for HDV diagnosis. The main clinical utility for the quantification of HDV RNA is to monitor antiviral therapy of patients. Additionally, individuals with an active HDV infection can be identified by NAT testing and will provide a more real picture of the HDV prevalence in the HBsAg positive population. Due to the high sequence diversity, primers and probe(s) of NAT assays have to be selected from highly conserved regions to cover all HDV genotypes. The majority of NAT assays are developed in-house and based on real-time polymerase chain reaction (PCR) technology. The quantification of HDV RNA is usually based on internal standards of different origin, either *in vitro* transcribed HDV RNA or plasmid DNA containing HDV target sequences (rDNA). Due to their nature these standards are often amplified and detected without the sample preparation step (nucleic acid extraction). Naked RNA can easily be degraded by ubiquitous RNases, and external DNA calibrators do not accurately reflect RNA isolation and reverse transcription steps. Armored RNA technology* was developed as a direct response to these inherent weaknesses. Such material performs well during the whole NAT procedure including the extraction step.

Currently, standardization of HDV NAT assays has not been targeted and HDV RNA quantification is unreliable. Results are not comparable between assays and the development of treatment guidelines with defined HDV RNA concentrations is not possible. The establishment of an international standard for HDV RNA is therefore an urgent need.[6,7]

The development of a reference material based on armored RNA technology was not favoured due to the disadvantage of the limited size of the RNA insert of up to one kilo base, which will not comprise all viral target regions. According to the recognized strategy used for the establishment of previous international standards of World Health Organization (WHO) for blood-borne viruses for use with NAT-based tests, the development of an HDV RNA reference material should be based on whole virus material in plasma.

The proposal by the Paul-Ehrlich-Institut (PEI) to develop the 1st WHO International Standard for HDV RNA for use with NAT-based assays was endorsed by the Expert Committee on Biological Standardization (ECBS) in 2009 (WHO/BS/09.2126). The viral strain being developed as standard belongs to genotype 1 which is the most frequent HDV genotype globally. The aim of the collaborative study was to demonstrate the suitability of the candidate standard for its use, to evaluate its potency and assign an internationally agreed unitage.

* Armored RNA technology stabilizes and protects RNA transcripts from nuclease degradation by packaging them in a protective protein coat. Armored Technology™ is available for licensing from Asuragen, Inc., Austin, TX USA.

Characterization and preparation of bulk material

Characterization of the candidate materials

Seven plasma samples derived from chronically HDV infected patients were evaluated.

All materials with a minimum volume of 200 mL each were provided by the Institute of Hepatology, Ankara University, Turkey. These preinvestigated[8] samples were further evaluated by performing the following analysis:

- Quantitative HDV RNA (determination by an alternative NAT method)
- Anti-HDV antibodies
- Quantification of HBsAg and HBV DNA
- Other serological hepatitis markers (HBeAg, anti-HBc and anti-HBe antibodies)
- Anti-HIV-1/2 and anti-HCV antibodies

All specimens represented HDV genotype 1 and were anti-HDV positive. The HBV DNA concentration was rather low compared to the concentration of HDV RNA. Interestingly, there is no correlation between the concentration levels of HDV RNA, HBsAg and HBV DNA, as it has been also described by other authors.[9,10] Anti-HCV and anti-HIV-1/2 antibodies were not detected in these materials (Tables 1 and 2, Figure 1). The two materials N6357 and N6360 representing the highest HDV RNA concentrations determined by the RoboGene assay were further analyzed in a feasibility study included different HDV NAT assay and laboratories. In conclusion, the material N6357 with a predetermined HDV RNA concentration of >7 $\log_{10}$ copies/mL was chosen as the potential candidate material for the WHO standard (data not shown). The complete HDV sequence is accessible in GenBank under accession number HQ005371.

Preparation of bulk material and freeze-drying

For the preparation of the candidate WHO bulk standard, 44 mL of the HDV plasma material N6357 were mixed with 2156 mL of human plasma. This 1:50 dilution was prepared with a plasma pool which had been tested negative for the following markers: HBV DNA, HCV RNA, HDV RNA, HIV-1 RNA, HBsAg, anti-HDV, anti-HCV, and anti-HIV-1/2. The filling and lyophilization was performed by an ISO 13485:2007 accredited Swiss company. For these procedures the bulk preparation was removed from storage at -20°C and thawed at 37°C in a water bath with constant agitation until it had just thawed. After thoroughly mixing, the material was stored at 2 -8°C and 0.5 ml volumes were dispensed in 4-ml screw-cap glass vials. The coefficient of variation of the fill volume was 0.8%. Rubber seals were then placed on top of the filled vials before loading into the freeze-drier (Instrument CHRIST Epsilon 2-25 D; LPC-16/NT process documentation). After freeze-drying the vials were sealed and stored at -20°C with constant temperature monitoring. Overall 4,010 vials were produced in March 2012. All

manufacturing records are held by PEI and are available on request by the ECBS. The lyophilized preparation has the PEI code no 7657/12.

Studies on the final product

The HDV RNA standard has been prepared as lyophilized material and is recommended to be stored at ≤ -20°C. The material should be reconstituted in 0.5 mL of sterile nuclease-free water (molecular biological grade). If the material is not used completely, laboratories may aliquot the remaining reconstituted material into suitable volumes which should be stored at or below -70°C.

Stability of the candidate WHO standard is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate WHO standard have been stored at -20°C (the normal storage temperature; and to provide a baseline if there is any indication of instability at higher temperatures). For the accelerated stability study, vials have been incubated at +4°C, +20°C, and +37°C for up to 1 year. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 mL of nuclease free water and analyzed by real-time PCR. Aliquots of the corresponding frozen liquid bulk materials storage at -80°C were analyzed in parallel.

Due to the high variation of the absolute quantitative values from batch to batch the threshold cycle (Ct)[†] values of the real-time assay were used to demonstrate the stability results. The results from stability studies of the first year showed that the candidate WHO standard is stable under the recommended storage conditions (Figure 2). It was also demonstrated that the freeze-drying procedure did not lead to a significant decrease of the HDV RNA concentration compared to the corresponding liquid bulk material.

Due to the infectious nature of the preparations the residual moisture content has been determined from the freeze-dried vials filled with negative plasma pool. These vials were randomly distributed on the trays of the freeze-drier and underwent the same processing conditions as the vials filled with the standard. The residual moisture content was investigated at PEI used an accredited method according to the European Pharmacopoeia.[11] The water content was determined to be 0.89% (standard deviation ± 0.07%) which complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards.[12]

Collaborative study

Participants, samples and study design

A total of 20 laboratories from 10 countries were invited to participate in the study. The potential participants have been selected because of their recognized expertise in the

[†] Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

HDV diagnostic field. Nineteen laboratories agreed to participate in the collaborative study and received the study materials. Four samples had to be analyzed in the study. Sample 1 (S1) and sample 2 (S2) were replicates of the candidate WHO standard (freeze dried preparation), sample 3 (S3) was the corresponding frozen liquid bulk material and sample 4 (S4) was a neat clinical specimen (N6359; HDV positive human plasma, genotype 1).

The laboratories were asked to test the panel using their routine assay for HDV RNA. The study protocol was divided into two phases (Phase 1 and Phase 2).
Phase 1: Participants were requested to test S1–S3 each by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. Results should be reported as positive or negative independent from the assay type used, qualitative or quantitative assay, and the corresponding Ct values should also be provided. S4 should be tested at least undiluted and results should be reported in copies/ml (quantitative assay) or as positive/negative (qualitative assays).

Phase 2: Based on the results of Phase 1 the participants were asked to perform the end-point dilution (ED) procedure or to follow the quantitative protocol.
According the ED protocol, participants were requested to assay a minimum of 5 half-$\log_{10}$ dilutions around the Phase 1 estimated end-point for S1-S3 in three independent runs (Table 4). Results should be reported as positive or negative and the corresponding Ct values should be provided. S4 should be assayed in three runs undiluted and at least at one further 10-fold dilution. Results should be reported in copies/ml (quantitative assay) or as positive or negative (qualitative assays).

Participants selected for the quantitative protocol were requested to test S1-S4 in three separate runs using a minimum of two dilutions which should be within the linear range of the assay. Testing of S4 should start with neat material. The results should be reported in copies/mL and the corresponding Ct values should be provided.

A fresh vial of each sample should be used for each run independent from the used study protocol. Data sheets and a method form were provided for both phases to ensure that all relevant information was recorded.

Data received

Seventeen laboratories sent results only from Phase 1 of the study and 15 laboratories from nine countries sent complete results including data from Phases 1 and 2. All participants are referred to by a laboratory code number, allocated at random (Appendix 1, Tab. 3). Overall 16 complete data sets were received. Eleven data sets resulted from the ED protocol, whereas 5 laboratories performed the Quant protocol. One participant performed both protocols (6A and 6B). Tables 3 and 4 provide an overview of the HDV NAT assays used, the quantification standards used in case of quantitative assays and the results from Phase 1 of the study. All NAT tests are based on real-time PCR technology. Two participants (Laboratories 8 and 12) used commercial assays (Table 3). Laboratory 8 quantified S4 using a separate standard curve based on *in vitro* transcribed HDV RNA

comprising the target region (transcript was provided by the assay manufacturer on request). The quantification of the assay used by laboratory 9 was also based on *in vitro* transcribed HDV RNA, too. However, the participants pointed out that due to stability problems of the transcript the Ct values of Phase 2 were significantly higher (about 4 Ct values) compared to the values from Phase 1. This would lead to about one $\log_{10}$ lower quantitation results. Only the results from Phase 1 by laboratory 9 were evaluated for this study.

Statistical methods

End-point dilution procedure

The results from the overall 4 independent runs (Phase 1 and Phase 2) were pooled to give a series of number positive out of number tested at each dilution. The pooled results of the single assays were evaluated with a probit analysis to estimate the concentration at which 63% of the samples tested were positive (i.e. the dilution at which on average one single copy per sample tested could be expected under the assumption of an underlying Poisson distribution).The calculated end-point was used to give estimates expressed in NAT detectable units/ml after correcting for an equivalent volume of the test sample. Data of laboratories 2 and 15 were analysed by the Spearman-Kaerber method, because the slope of the curves could not be estimated by the probit method.

Quantitative procedure

Evaluation of the results followed the quantitative protocol was restricted to dilutions in the range where the assays of most participants seem to produce comparable data (linear range). For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as arithmetic mean of $\log_{10}$ copies/mL. Furthermore these estimates were combined to obtain an overall estimation for each sample by means of a mixed linear model with *laboratory* and *(log) dilution* as random factors.

Relative potencies

Evaluation of quantitative assays was performed without removing any outlying data. Assays giving Ct values and those giving copies were evaluated separately. Potencies of samples were estimated relative to the reference candidate S1 with an assigned value of 5.76 $\log_{10}$ International Units/mL (IU/mL) by parallel line assay on log transformed data (quantitative protocol) or probit transformed data (end-point dilution protocol).

The statistical analysis was performed with SAS®/STAT software, version 9.3, SAS System for Windows. Estimation of end-point dilution and relative potency were done with CombiStats Software, version 5.0, Release 2013, EDQM / Council of Europe.

Results

All 15 participants in the collaborative study used a HDV NAT based on real-time PCR technology. Twelve assays are intended for the quantification of HDV RNA, whereas 3 assays have been validated for the qualitative detection of HDV RNA. As the results from the pilot study with the samples N6357 and N6360 showed differences greater than 3 $\log_{10}$ steps in the concentrations quantified by different assays, one of the aims of the collaborative study was that most of the participants with quantitative NAT assays should follow a study procedure to estimate the concentration which is independent from the kit internal quantitation standards (end-point dilution). The results of Phase 1 of the study were the basis for the proposal of the procedure for Phase 2. Results and the corresponding proposals are shown in Table 4. Ten laboratories were requested to proceed in Phase 2 with the end-point dilution procedure, whereas 4 laboratories should perform the quantitative procedure. One laboratory was requested to perform both procedures. The assay performance in terms of the linear relationship between log dilution and mean Ct values of S1 and S2 (Phase 1) is demonstrated in Figure 3. The slope value with the optimal efficiency is -3.32.

Results of the end-point dilution procedure for S1, S2 and S3

The HDV RNA concentration, expressed in NAT-detectable units/mL ($\log_{10}$), was calculated from the 11 data sets for the end-point dilution procedure. Results are shown in Table 5 and in histogram form in Figure 5 (each white box represents the mean estimate from an individual laboratory labelled with the code number). Supposed correlation of the calculated concentration and the dilution factor based on the NAT sample input volume could not be observed. Some assays (laboratories 1 and 12) with a small sample input volume compared to other tests (laboratories 10, 11 and 13) showed a very high sensitivity resulting in values of greater than 6 $\log_{10}$ NAT detectable units/mL for S1, S2 and S3. The overall mean estimates for all 11 assays are shown Tables 8a-c. No difference in the calculated values was observed for samples S1 and S2 demonstrating the uniformity of the material.

Results of the quantitative procedure for S1, S2 and S3

According the outcome and proposal from Phase 1 of the study overall five laboratories performed and sent data for the quantitative procedure (Table 6 and Figure 5). Laboratory 9 reported about problems in the stability of the transcript used for quantitation in Phase 2 of the study. A significant increase of up to four Ct values for the transcript used for the quantification was observed compared to the values from Phase 1. Only the results from Phase 1 by laboratory 9 were evaluated for this study.

Laboratory 6 performed both procedures (6A and 6B) and laboratory 12 sent additionally quantitative results of S1, S2 and S3 for the Phase 1 study. Comparison of the results of both procedures within one assay revealed that the calculated concentrations according the end-point dilution procedure were lower than the values based on the internal kit quantitation standards, but all results are in the same log range. The values ($\log_{10}$ per mL)

of S1/2 and S3 of 6A (end-point dilution procedure) were 5.19 and 5.06 compared to the estimates by 6B of 5.70 and 5.71, respectively. For laboratory 12 the estimates for S1/2 and S3 for the end-point dilution procedure (results from neat to dilution of E-04 are in the linear range of the assay with a slope of -3.22; Figure 3) and the quantitation results based on the internal standards were 6.31 and 6.62, and 6.39 and 6.79, respectively (data not shown).

Results of S4

This material was a neat HDV positive plasma specimen, which was not further processed. This sample should be tested in both phases of the study at least undiluted and in a dilution of 1:10 to address commutability. Taken the quantitation results from the preinvestigation (Table1, Figure 1) it would be supposed from the outcome of the collaborative study that S4 has a slightly lower concentration compared to the other study samples S1, S2 and S3. The estimated mean concentration of S4 from all 12 quantitative NAT assays is provided with Table 7 and Figure 5 (grey boxes). The overall mean concentration of S4 is 5.56 $\log_{10}$ copies/mL with a standard deviation of 0.64. Maximum and minimum values are 6.72 and 4.39 $\log_{10}$ copies/mL, respectively demonstrating a range of more than 2 $\log_{10}$. The main reason is that different test related quantitation standards are used. If the obtained concentrations resulted from the undiluted and the 1:10 diluted testing within a test are compared, the concentrations related to the neat material are not significantly different (data not shown). This underlies that no matrix effect was observed which could be have an influence on the quantitation. If the mean Ct values of S4 are compared with the corresponding mean Ct values of S1 and S2, 12 out of the 15 laboratories identified S4 with a lower concentration compared to S1/S2. Nevertheless, the laboratories 5, 6 and 13 determined a higher concentration of S4 with their NAT tests (Figure 4).

Overall laboratory means

The overall mean estimates of S1, S2 and S3 from assays following the end-point dilution protocol are summarized in Table 8a, and the overall mean estimates of S1, S2, S3 and S4 for the 5 laboratories performed quantitation by the kit internal standards are shown in Table 8b. The means for both S1 and S2, replicates for the candidate WHO standard, demonstrate excellent agreement between the replicate samples by both end-point dilution and quantitative protocols. Assays following the end-point dilution protocol show 0.3 log10 higher mean estimates for S1, S2, and S3 than assays performed the quantitative procedure. The combined mean estimates of S1, S2 and S3 from all assays are shown in Table 8c. The mean Ct values of S1 and S2 (undiluted and 1:10 diluted) were compared with the mean Ct values of S3 resulted from the undiluted and 1:10 diluted testing (Figure 4). The relative concentration of S3 from the assays of all 15 laboratories were closed to the relative concentration of S1/S2 (< 1 Ct value), which demonstrated that the lyophilization had no effect on the integrity of HDV RNA.

Relative potencies

Based upon the data from both assay protocols, the candidate WHO standard was estimated to have a potency of 5.76 $\log_{10}$ units/mL (95% confidence limits 5.32 - 6.20). The potencies of the study samples S2, S3 and S4 were calculated relative to S1, taking the assigned potency of 5.76 $\log_{10}$ units/mL as the value of S1/S2. The relative potencies are shown in Tables 9a and 9b for the two assay protocols. Additionally, the data are plotted in histogram form (Figure 6). Due to different methods used for the calculation of the estimates of S1 (ED procedure) and S4 (quantitation procedure) by eleven laboratories, the relative potency for those laboratories was not determined. Nevertheless, laboratory assays with lower ED_{63} estimates for S1/S2 correspond with low quantitation results expressed in copies/ml of S4. Therefore, it can be assumed that excluding the estimates from laboratory 13 all the other laboratories would have relative potencies of S4 comparable to the relative potencies of S4 from laboratories 3, 9 and 14. The results demonstrated that the candidate standard (S1/S2) is commutable at least in 12 out of the 15 participating assays, which recognized this material with a comparable efficiency as the clinical sample (S4).

Conclusions

In this collaborative study a wide range of NAT assays have been used to evaluate the suitability of the candidate 1st WHO International Standard for HDV RNA for NAT-based assays and to determine its potency. All 15 different tests were exclusively based on the real-time PCR technology. Most of them are in-house developed assays validated for the quantification of HDV RNA underlying the broad linear range of the tests. No standard method or common quantitation standard material was used. The participants used plasmid DNA containing HDV sequences or *in vitro* transcribed HDV RNA as internal quantitation standards. Each participant also provided all threshold (Ct) values, which were also part of the evaluation of the study samples.

The candidate standard was prepared from an HDV RNA positive plasma sample belonging to genotype 1 which showed in a feasibility study the relative highest HDV RNA concentration compared to other HDV RNA positive plasma specimens. The candidate bulk was freeze-dried to ensure long term stability and to allow shipment conditions at ambient temperature globally.

In the collaborative study, the freeze-dried candidate preparation (S1 and S2) were evaluated alongside the corresponding frozen liquid bulk material (S3) and an individual clinical plasma sample (S4). Ten laboratories evaluated the study samples S1 – S3 according to the end-point dilution procedure, independent from the assay type, whereas 4 laboratories performed the quantitative procedure. One laboratory performed both procedures. S4 was tested qualitative or quantitative depending on the assay type. The calculation of the concentration of the study samples was analysed by Poisson distribution (ED procedure) or by quantification based on kit internal standards. Since there was no difference in the overall mean estimates for S1 and S2 observed, the

candidate standard (S1/S2) has been assigned a HDV RNA unitage of 575,000 IU/mL. The freeze-drying procedure had no effect on the integrity of the viral RNA as the comparison of the results of S1/S2 and S3 revealed. Unfortunately, due to the limited number of clinical samples with sufficient volume, the commutability of the candidate standard was addressed by comparison testing of one clinical HDV positive plasma sample (S4). Data from 12 out of the 15 laboratories demonstrated that the standard material and the clinical sample were detected with the same efficiency.

Nevertheless, striking differences of the estimates for the study samples by the different real-time PCR assays were observed. All samples represented the same genotype. The expression of the potencies relative to S1 improved the agreement between the different laboratories and methods and demonstrates that the candidate material is suitable as a common standard. The proposed international standard may also contribute in the development of new sensitive HDV NAT tests. As the assigned unitage in IU is an arbitrary unitage the conversion factor between IU and copies is assay dependent. The more sensitive NAT tests show a factor of greater than 1 (1 IU corresponds to more than one copy).

The outcome of the collaborative study demonstrated that the candidate standard representing HDV genotype 1 is suitable as a reference preparation to standardize and harmonize HDV-NAT assays. The results from the accelerated and real-time stability studies indicate that the preparation is stable over a long period at the recommended storage condition, i.e., at -20°C or below and can be shipped at ambient temperature globally.

Proposal

It is proposed that the candidate standard, PEI code 7657/12, is established as the 1st WHO International Standard for HDV RNA for use in NAT-based assays, with an assigned potency of 575,000 IU/mL (5.76 $\log_{10}$ IU/mL), when reconstituted in 0.5 mL of nuclease-free water. The proposed standard is intended to be used to standardize and validate HDV NAT assays and to calibrate secondary reference materials. The proposed Instructions for Use (IFU) for the reference preparation are included in Appendix 2.

Comments from participants

The collaborative study report circulated to the participants. All comments of the participants were addressed and corrections were performed where appropriate. Additional comments are mentioned below (in italic). All laboratories who have replied have agreed that the candidate material is suitable for use as a WHO International Standard with the proposed assigned potency.

Lab 2: *Consider explaining why the variation was 'high' and why using Ct values instead of absolute values overcame this problem of high variability. Should this be 'run to run' instead of 'batch to batch'?* (Page 5 'Studies on the final product')

Indeed, considerable variations were observed from lot to lot (batch to batch) of both sample preparation kit as well of the amplification/detection kit. The use of Ct values for the stability studies is justified because the results from standard vials stored at -20°C are used as the baseline. The results of the collaborative study were generated with one lot each of the sample prep kit and of the amplification/detection kit.

Lab 2: *Consider providing further explanation here. How do we know that S4 was recognized with the same efficiency as the other samples?* (Page 9 'Relative potencies') Additionally, the comparison of the corresponding Ct values of S1/S2 and S4 (Figure 4) support this finding. Nevertheless, the wording was slightly modified: "…which recognized this material with a comparable efficiency as the clinical sample (S4)."

Lab 2: *The slope of -2.1 reported by Lab 10 represents a PCR efficiency of 199% which is physically impossible. The range of slopes which is generally considered acceptable in real-time qPCR assays is -3.6 to -3.1 which is equivalent to a PCR efficiency range of approximately 90% to 110%. Consider mentioning that several of the laboratories generated slopes that fell outside the normally acceptable range. Consider also including the R2 values of these slopes in view of the rather non-linear distribution of data points from Labs 13. Is there an argument for excluding such non-linear data and/or data from slopes which fall outside the 'acceptable' -3.6 to -3.1 range? Note that the Lab 13 results presented in Figure 4 appear somewhat abberant. Why does Lab 13, unlike all other laboratories, not include a data point for the undiluted sample and why does it have an extra data point half way between 1E-2 and 1E-3?* (Page 22, Figure 3)

We agree with the theoretical basics of real-time qPCR assays. Nevertheless, the intention of the collaborative study was not to disqualify any assay. As long as the assay data were consistent, these data were not excluded from the analysis. Each participant can draw their own conclusion from this evaluation. Lab 13 used an in-house assays validated for the qualitative detection of HDV RNA. This lab could only follow the ED procedure. Because the data of undiluted testing of S1/S2 were missing, additional data from Phase 2 (dilution 3.16E-03) were used to generate the curve.

Lab 9: *The Ct values for the 4 samples (S1-S4) were stable in phase two; our assay detected the samples with nearly identical Ct values between phase 1 and phase 2. However our transcript seemed to lose its potency over the time between phase 1 and phase 2, resulting in the lower quantitation of the samples in phase 2. We have rectified this problem which was highlighted by the discrepancy in this evaluation.*

Lab 12: *On the figure 3 (page 21) related to the section "Results of the quantitative procedure for S1, S2 and S3" (page 7) each point of our curve should be elevated 5 cycles up because we have 5 blind cycles in the program. This correction doesn't change the slope of the curve.*

Acknowledgements

We gratefully acknowledge the important contributions of all participants in the collaborative study.

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Table 1. HDV markers of the clinical plasma specimens

Sample	HDV RNA (log_{10} copies/mL)			Anti-HDV total[3]
	NAT Assay 1[1]	NAT Assay[2]	Δ	S/Co
N6356	5,58	6,78	1,20	15,24
N6357	7,38	8,72	1,34	15,75
N6358	6,40	7,33	0,93	15,75
N6359	5,14	6,21	1,07	15,75
N6360	6,24	7,70	1,46	14,77
N6361	6,82	7,60	0,78	14,77
N6362	5,95	6,33	0,38	14,77

[1] In-house TaqMan PCR, Laboratory Ankara; [2] RoboGene HDV RNA Quantification Kit (aj Roboscreen), Langen; [3] Murex Anti-Delta, Laboratory Langen.

Table 2. Characterization of the HDV positive plasma samples

Sample	HBV DNA (IU/mL)[1]	HBV DNA (IU/mL)[2]	HBsAg (IU/mL)[3]	Anti-HBc total[4]	HBeAg[5]	Anti-HBe[6]	Anti-HCV[7]	Anti-HIV 1/2[8]
N6356	228	203	5.570	pos	neg	pos	neg	neg
N6357	<120	20	15.625	pos	neg	pos	neg	neg
N6358	<120	102	16.190	pos	pos	pos	neg	neg
N6359	9.140	4.250	1.600	pos	neg	pos	neg	neg
N6360	1.470	1.323	14.545	pos	neg	pos	neg	neg
N6361	<120	10	18.945	pos	neg	pos	neg	neg
N6362	<120	10	22.730	pos	neg	pos	neg	neg

[1] Cobas AmpliPrep/Cobas TaqMan HBV Test, v2.0, Roche Diagnostics GmbH; [2] Abbott RealTime HBV assay, Abbott; [3] Architect HBsAg, Abbott; [4] Architect Anti-HBc, Abbott; [5] Elecsys HBeAg, Roche Diagnostics GmbH; [6] Elecsys Anti-HBe, Roche Diagnostics GmbH; [7] HCV Ag/Ab Combo, Murex; [8] Axsym Ag/Ab Combo, Abbott. pos, positive; neg, negative.

Table 3. List of HDV real-time NAT assays used in the collaborative study

Lab code	Sample preparation	Assay type	Target region	Quantification standard	Sample equivalent (µL)	Dilution factor
1	m2000sp	quant	NTR upstream HD gene	cDNA	35.71	28
2	QIAamp Viral RNA	quant	Ribozyme region	RNA transcript	35	28.6
3	easyMAG	quant	HD gene	RNA transcript	11.67	86
4	Manual GuSCN	quant	HD gene	RNA transcript	40	25
5	Cobas AmpliPrep	quant	HD gene	plasmid	26.67	37.5
6	m2000sp	quant	Ribozyme region	Synthetic DNA	27.8	36
7	EZ1 Advanced	quant	HD gene	cDNA	20	50
8	Manual Kit	quant[1]	HD gene	RNA transcript	16,67	60
9	MagnaPure	quant	HD gene	RNA transcript	20	50
10	QIAamp MiniElute	qual	HD gene	—	70	14.3
11	MagnaPure	qual	HD gene	—	40	25
12	Manual RIBO-prep	Quant[2]	HD gene	plasmid	50	20
13	QiaSymphony	qual	HD gene	—	83.33	12
14	QIAamp Viral RNA	quant	between autocatalytic cleavage sites	cDNA	11.67	86
		qual		—.		
15	HPS Viral RNA	quant	HD gene	Armored RNA	20	50

[1]Commercial research-use-only kit: Instant Virus RNA Kit in combination with RoboGene HDV RNA Quantification Kit (aj Roboscreen, Leipzig, Germany); [2]Commercial research-use-only kit: AmpliSens HDV-Monitor-FL PCR kit (Central Research Institute of Epidemiology, Moscow, Russia); [3] Sample volume which is used for the amplification/detection.

Table 4. End-point dilution results of S1, S2, and S3 in Phase 1 of the WHO collaborative study

Lab code	Assay Type	S1	S2	S3	Study protocol Phase 2
1	quant	1,E-05	1,E-05	1,E-05	ED
2	quant	1,E-04	1,E-04	1,E-04	ED
3	quant	1,E-02	1,E-02	1,E-02	quant
4	quant	1,E-05	1,E-05	1,E-05	ED
5	quant	1,E-02	1,E-03	1,E-02	quant
6	quant	1,E-03	1,E-03	1,E-03	quant +ED
7	quant	1,E-05	1,E-05	1,E-05	ED
8	quant	1,E-04	1,E-04	1,E-04	ED
9	quant	1,E-03	1,E-03	1,E-04	quant
10	qual	1,E-04	1,E-05	1,E-05	ED
11	qual	1,E-05	1,E-03	1,E-05	ED
12	quant	1,E-05	1,E-06	1,E-05	ED
13	qual	1,E-03	1,E-03	1,E-03	ED
14	quant	1,E-01	1,E-02	1,E-02	quant
	qual	1,E-02	1,E-03	1,E-03	–
15	quant	1,E-04	1,E-04	1,E-04	ED

ED, end-point dilution; quant, quantitative; qual, qualitative.

Table 5. Mean estimates of S1-S3 from assays followed the end-point dilution protocol (log_{10} NAT detectable units/mL)

	S1			S2			S3		
Lab code	Estimate	95%-Confidence interval		Estimate	95%-Confidence interval		Estimate	95%-Confidence interval	
1	6.52	6.23	6.78	6.61	6.32	6.87	6.37	6.08	6.63
2	5.81	5.54	6.07	5.39	5.15	5.64	5.52	5.30	5.73
4	6.57	6.33	6.79	6.73	6.48	6.95	6.51	6.26	6.73
6A	5.13	4.92	5.36	5.24	4.97	5.43	5.06	4.85	5.34
7	7.04	6.82	7.24	7.07	6.86	7.27	7.33	7.10	7.58
8	5.53	5.24	5.78	5.59	5.31	5.84	5.90	5.61	6.16
10	5.39	4.78	5.96	5.40	4.80	5.97	4.62	4.02	5.24
11	5.24	4.73	5.68	4.97	4.40	5.43	4.81	4.20	5.27
12	6.26	5.99	6.50	6.36	6.09	6.60	6.39	6.12	6.63
13	4.39	4.26	4.50	4.51	4.37	4.65	4.46	4.32	4.59
15	6.20	6.05	6.35	6.12	6.12	6.12	6.12	6.12	6.12

Table 6. Mean estimates S1-S3 from assays followed the quantitative protocol (log_{10} copies/mL)

	S1			S2			S3		
Lab code	Estimate	95%-Confidence interval		Estimate	95%-Confidence interval		Estimate	95%-Confidence interval	
3	5.51	4.95	6.06	5.51	5.07	5.95	5.23	4.56	5.89
5	4.22	3.80	4.63	4.23	4.01	4.45	4.20	3.89	4.51
6B	5.70	4.83	6.57	5.61	3.31	7.91	5.71	4.75	6.68
9	6.23	5.35	7.10	6.42	5.73	7.10	6.48	5.75	7.21
14	5.72	5.15	6.29	5.83	5.48	6.18	5.70	5.35	6.06

Table 7. Mean estimates of S4 from quantitative assays (log_{10} copies/mL)

	S4		
Lab code	Estimate	95%-Confidence interval	
1	5.66	4.09	7.23
2	6.72	5.51	7.92
3	4.75	3.51	5.99
4	6.57	5.76	7.38
5	4.39	4.22	4.55
6B	6.04	5.89	6.18
7	5.45	4.40	6.49
8*	5.10	3.49	6.70
9	5.88	4.11	7.66
12	5.74	5.40	6.08
14	4.82	2.79	6.84
15	5.65	4.71	6.59

*Quantitation procedure modified, see chapter 'Data Received'.

Table 8a. Overall mean estimates from assays followed the end-point dilution protocol (log_{10} NAT detectable units/mL)

Sample	N	Mean	SD[1]	95% Confidence interval		Min	Median	Max
S1	11	5.82	0.78	5.30	6.35	4.39	5.81	7.04
S2	11	5.82	0.81	5.27	6.36	4.51	5.59	7.07
S3	11	5.73	0.92	5.12	6.35	4.46	5.90	7.33

N, number of dilutions analyzed; SD, standard deviation.

Table 8b. Overall mean estimates from assays followed the quantitative protocol (log_{10} copies/mL)

Sample	N	Mean	SD[1]	95% Confidence interval		Min	Median	Max
S1	64	5.49	0.80	4.36	6.62	4.00	5.54	6.88
S2	66	5.49	0.80	4.23	6.75	4.07	5.54	6.88
S3	65	5.44	0.82	4.18	6.70	4.02	5.51	6.88
S4	224	5.56	0.64	4.68	6.45	4.12	5.60	6.89

N, number of dilutions analysed; SD, standard deviation.

Table 8c. Combined mean estimates of samples S1-S3 (log_{10} NAT detectable units/mL log_{10} copies/mL)

	S1			S2			S3		
Assay	Estimate	95% Confidence interval			95% Confidence interval			95% Confidence interval	
ED	5.82	5.30	6.35	5.82	5.27	6.36	5.73	5.12	6.35
Quant	5.49	4.36	6.62	5.49	4.23	6.75	5.44	4.18	6.70
All	5.75	5.32	6.18	5.75	5.30	6.20	5.68	5.19	6.17
S1 and S2 combined	5.76	5.32	6.19						

Table 9a. Potency relative to S1 for assays followed the end-point dilution protocol (log_{10} NAT detectable units/mL)

	S2			S3		
Lab code	Relative potency	95%-Confidence interval		Relative potency	95%-Confidence interval	
1	5.85	5.47	6.23	5.61	5.23	5.99
2	5.34	4.98	5.71	5.47	5.13	5.81
4	5.91	5.58	6.24	5.70	5.37	6.02
6A	5.88	5.49	6.14	5.69	5.39	6.04
7	5.79	5.50	6.09	6.05	5.74	6.39
8	5.82	5.45	6.20	6.13	5.75	6.51
10	5.77	4.94	6.60	4.99	4.17	5.87
11	5.49	4.79	6.15	5.33	4.61	5.98
12	5.86	5.50	6.22	5.89	5.54	6.25
13	5.89	5.71	6.07	5.83	5.66	6.01
15	5.68	5.53	5.83	5.68	5.53	5.83
Mean[1]	5.77	5.68	5.85	5.67	5.45	5.89

[1]Combined potency

Table 9b. Potency of samples S2-S4 relative to S1 for assays followed the quantitative protocol (log_{10} copies/mL)

	S2			S3			S4			
Lab code	Relative potency	95%-Confidence interval		Relative potency	95%-Confidence interval		Relative potency	95%-Confidence interval		Measured
3	5.78	5.49	6.07	5.49	5.19	5.78	4.99	4.69	5.29	copies
3	5.77	5.48	6.07	5.51	5.20	5.80	5.01	4.71	5.32	Ct values
5	5.77	5.66	5.88	5.71	5.60	5.82	5.91	5.80	6.02	copies
5	5.82	5.66	5.97	5.86	5.71	6.01	6.02	5.86	6.18	Ct values
6B	5.67	5.52	5.82	5.77	5.62	5.92	6.02	5.88	6.15	copies
6B	5.68	5.54	5.83	5.77	5.63	5.92	6.02	5.89	6.15	Ct values
9	5.88	5.75	6.00	5.93	5.80	6.06	5.23	5.08	5.38	copies
9	5.80	5.65	5.94	5.80	5.66	5.94	4.84	4.68	5.01	Ct values
14	5.83	5.53	6.12	5.68	5.38	5.98	4.90	4.59	5.20	copies
14	5.84	5.54	6.15	5.71	5.40	6.01	4.93	4.61	5.24	Ct values
Mean[1]	5.78	5.73	5.83	5.72	5.62	5.82	5.39	5.01	5.77	

[1]combined potency

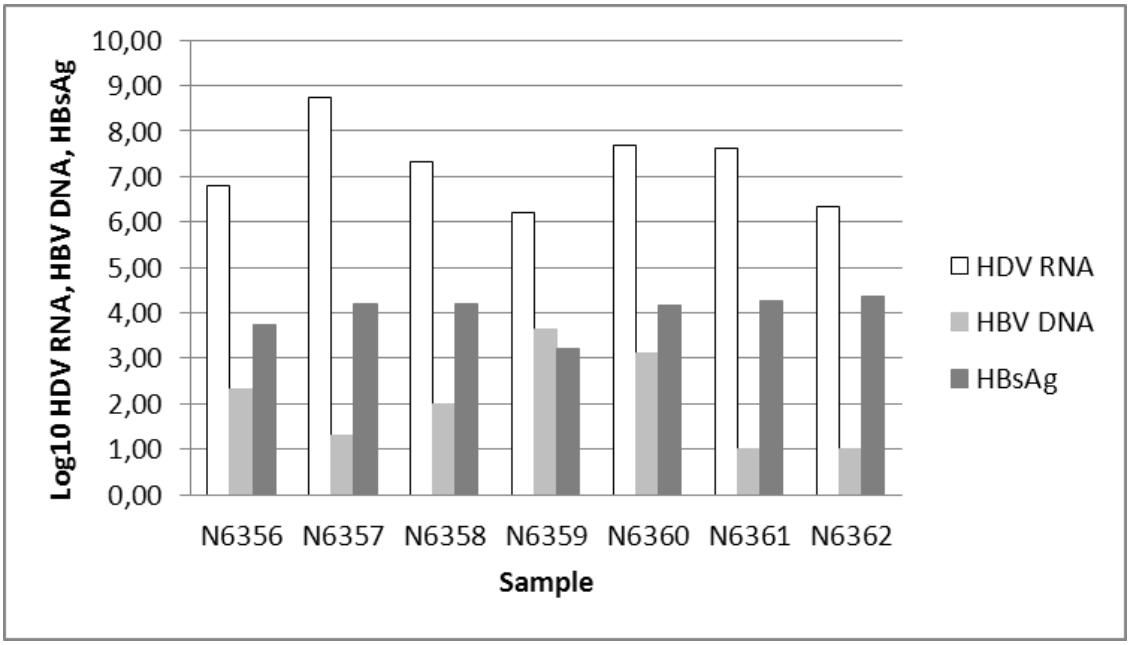

Figure 1. Comparison of direct HDV and HBV markers of seven plasma samples from chronic HDV infected patients. HDV RNA (copies/mL by RoboGene assay); HBV DNA, HBsAg (IU/mL).

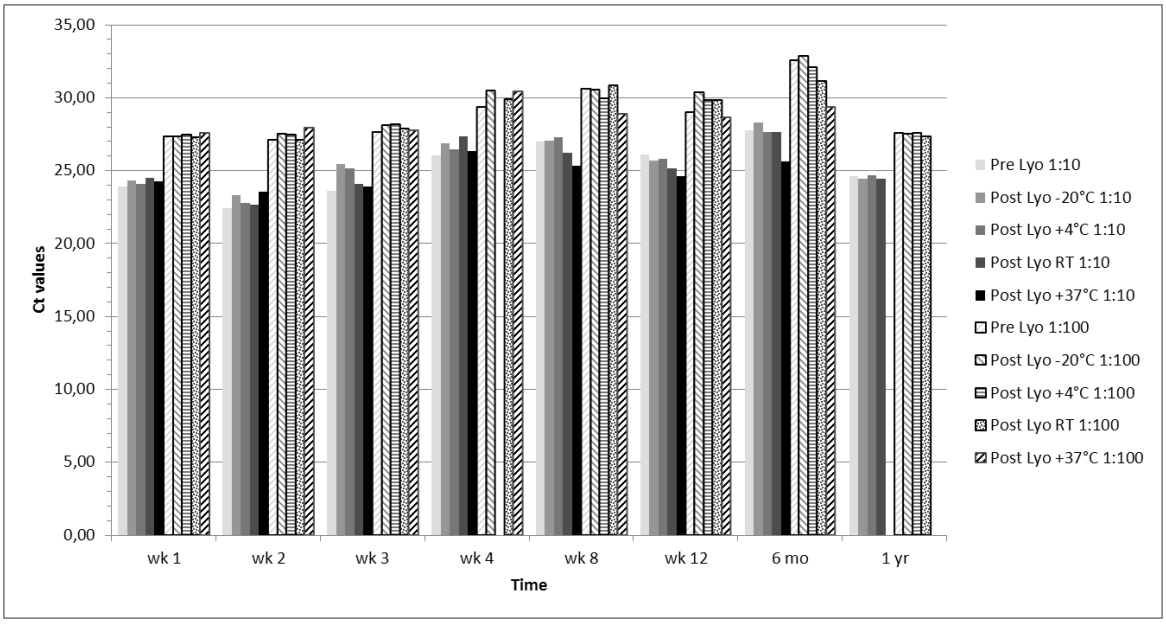

Figure 2. Results from stability testing of the candidate WHO standard 7657/12. Liquid bulk material (Pre Lyo) was stored in aliquots at -80°C.

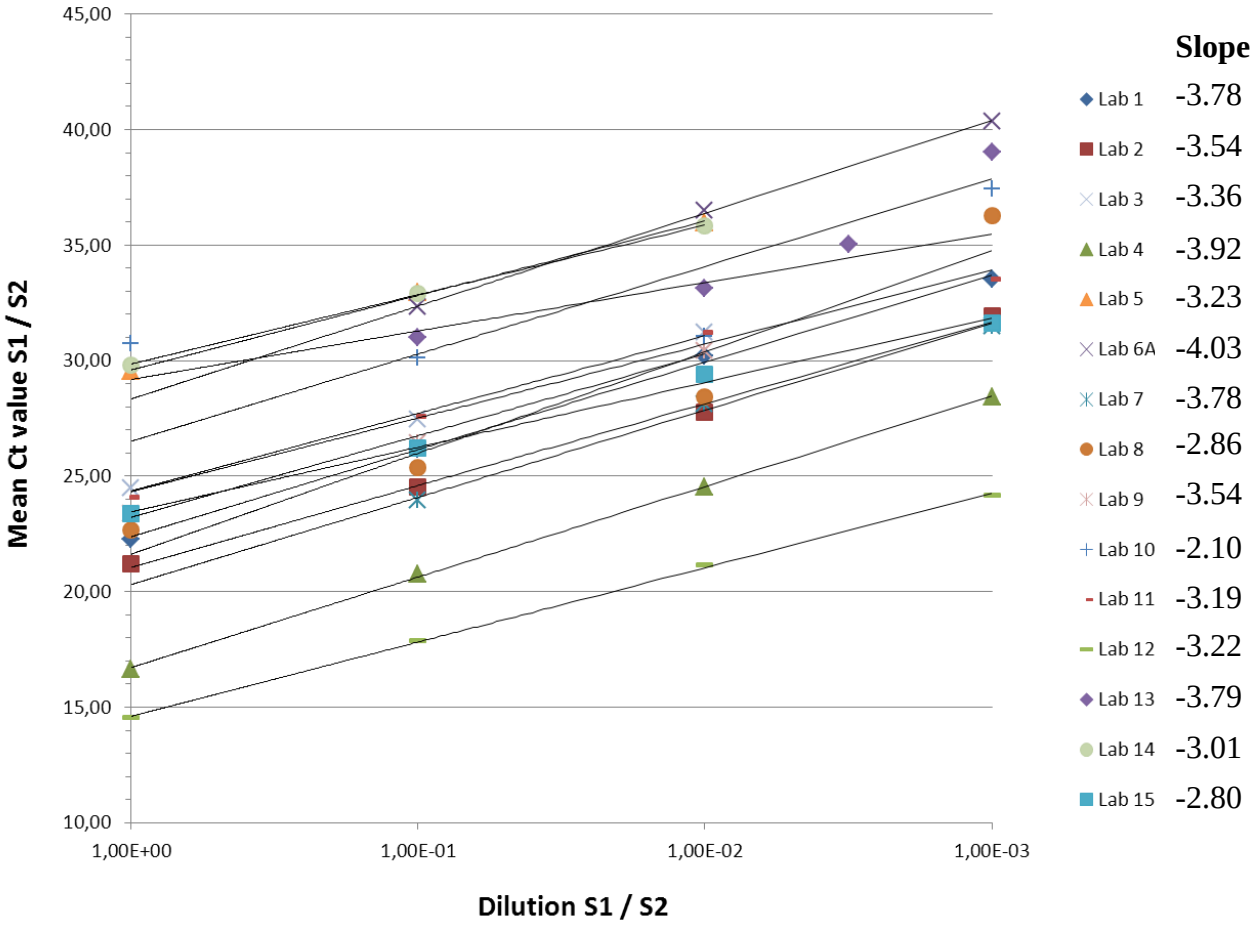

Figure 3. Linear relationship between $\log_{10}$ dilution and mean Ct values of S1 and S2.

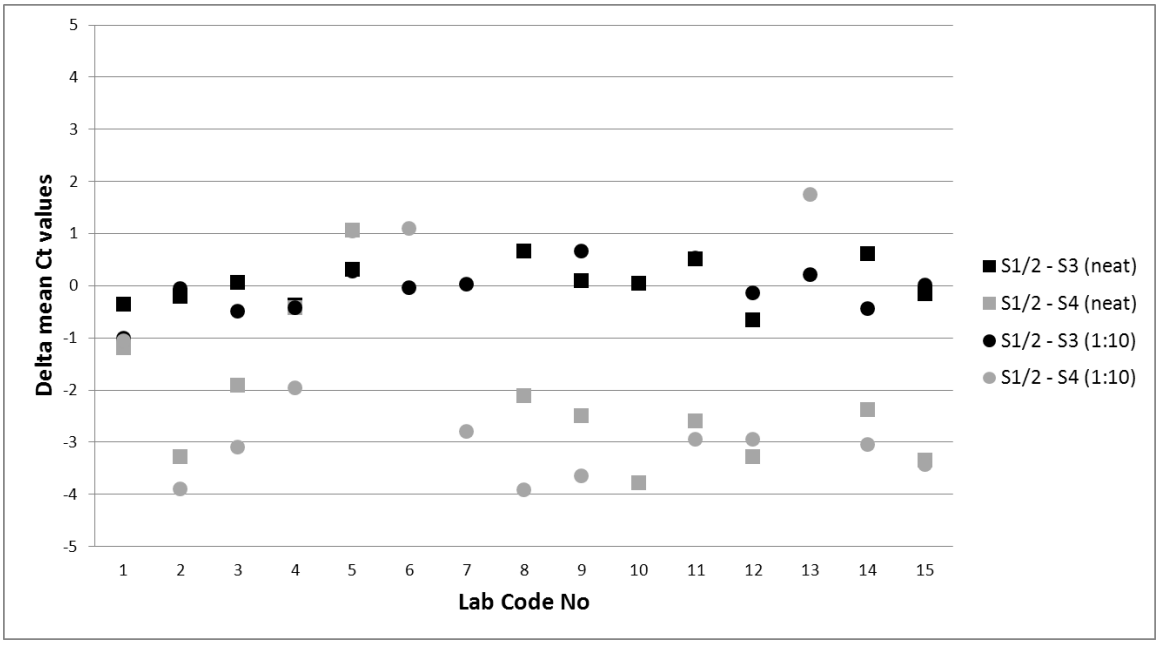

Figure 4. Relationship of mean Ct values between S1/S2 and S3 and between S1/S2 and S4. Laboratories 6, 7 and 13 tested S1/S2 not undiluted.

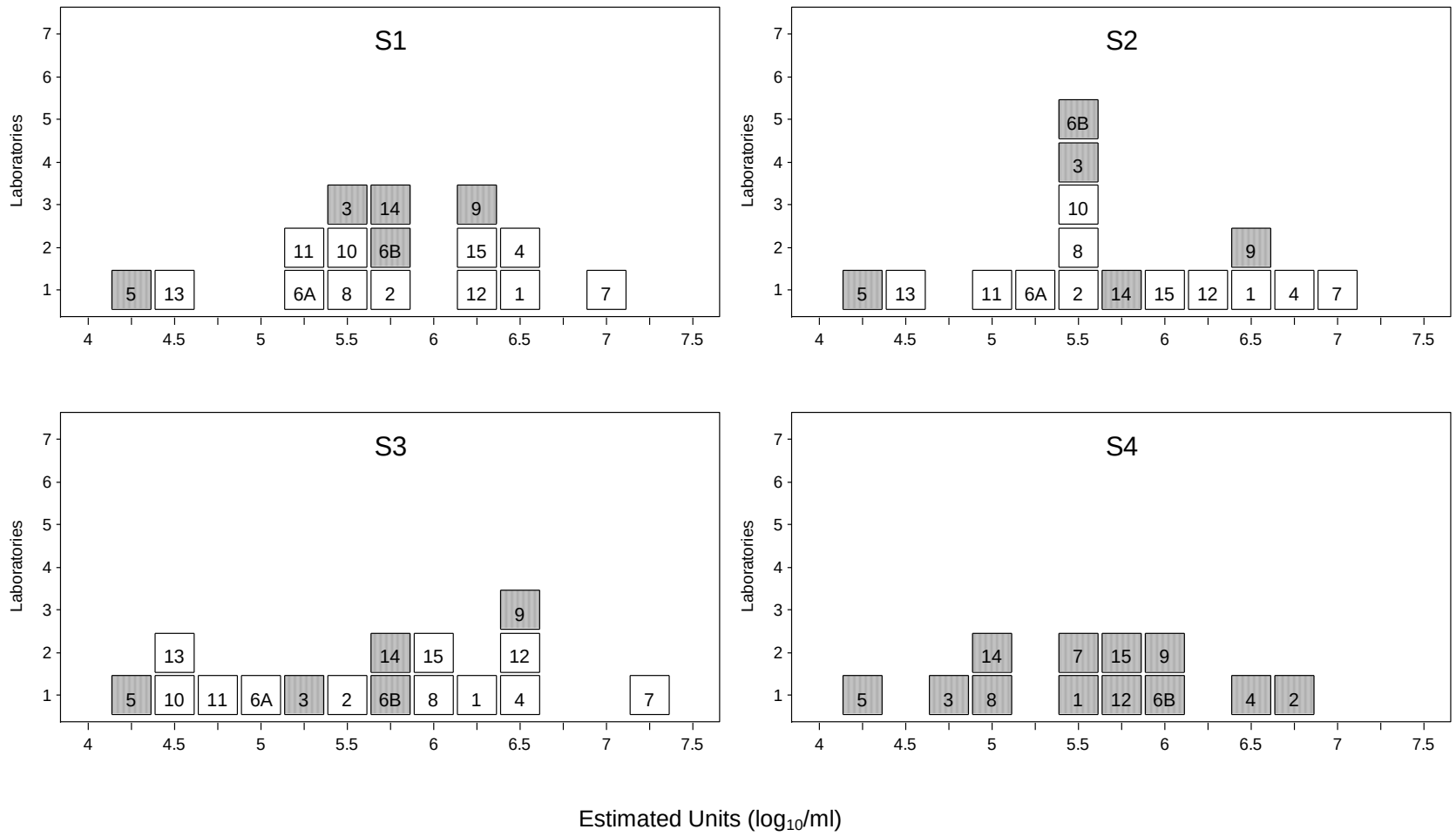

Figure 5. Histograms of the results for participating laboratories followed either the end-point dilution protocol or the quantitative protocol for samples S1-S4. Estimated concentrations indicated on the x-axis are expressed as $\log_{10}$ NAT-detectable units/mL (white boxes) or $\log_{10}$ copies/mL (grey boxes), respectively.

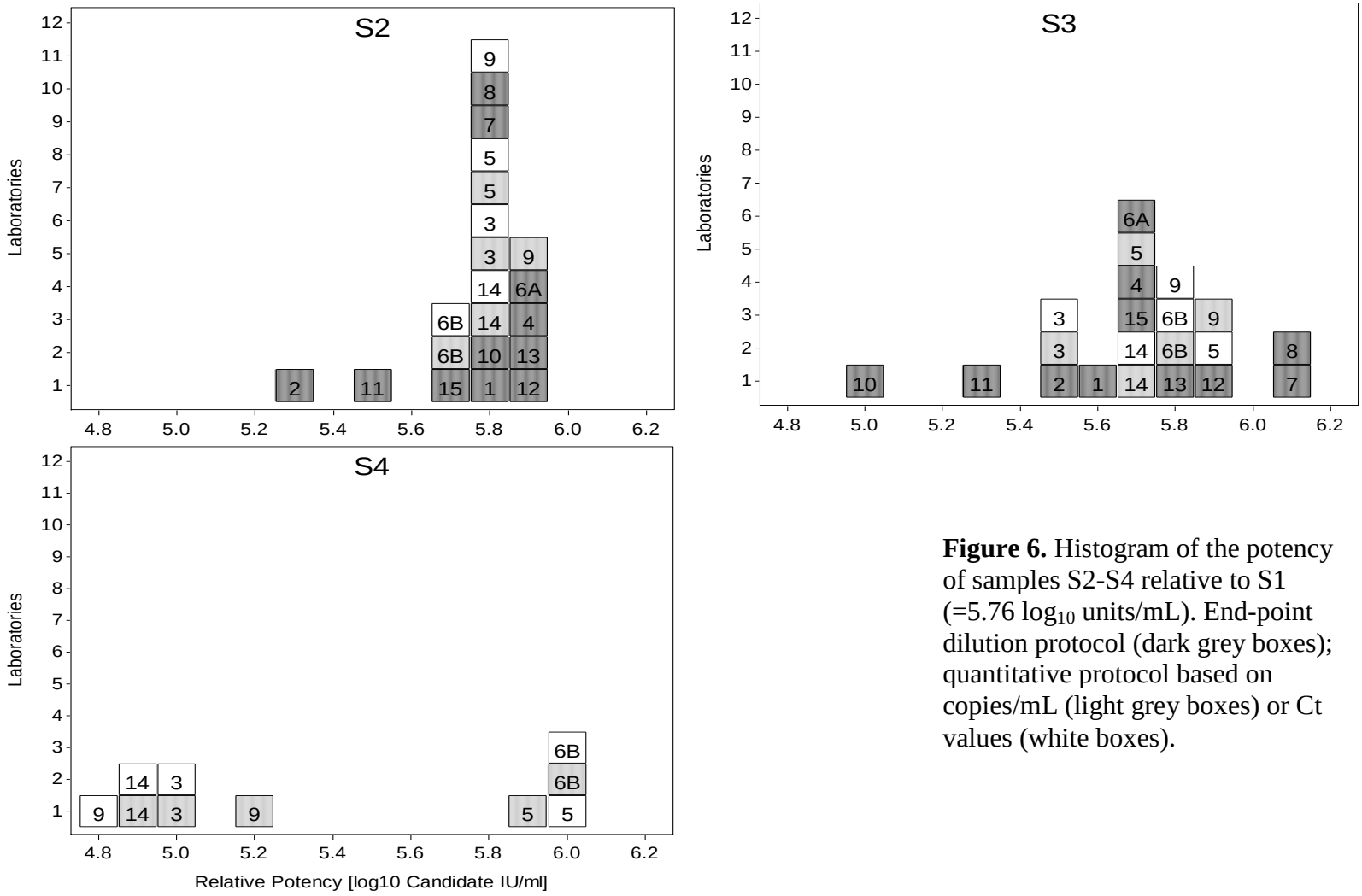

Figure 6. Histogram of the potency of samples S2-S4 relative to S1 (=5.76 $\log_{10}$ units/mL). End-point dilution protocol (dark grey boxes); quantitative protocol based on copies/mL (light grey boxes) or Ct values (white boxes).

Appendix 1 Collaborative study participants (In alphabetical order)

Scientist	Affiliation
Scott Bowden	Victorian Infectious Diseases Reference Laboratory, Victoria, Australia
Mithat Bozdayi	Dept. of Gastroenterology, Ankara University, Turkey
Michael Chudy	Dept. of Virology, Paul-Ehrlich-Institut, Langen, Germany
Vladimir Chulanov	Reference Center for Viral Hepatitis, Moscow, Russia
Bridget Ferns/ Jeremy Garson	Clinical Microbiol. & Virology, University College London Hospitals NHS Foundation Trust, London, UK
Emmanuel Gordien	Lab. de Virologie, Hopital Avicenne, Laboratoire associé au Centre National de Référence des Hépatites B, C et delta, Université Paris, Bobigny, France
Francesca Luciani	Instituto Superiore di Sanita, Rome, Italy
Bernhard Miller	Medizinisches Versorgungszentrum Labor PD Dr. Volkmann und Kollegen GbR, Karlsruhe, Germany
Tonya Mixson-Hayden	Division of Viral Hepatitis, Centers for Disease Control and Prevention, Atlanta, GA, USA
Antonella Olivero/ Mario Rizzetto	Dept. of Medical Sciences, University of Torino, A.O. Città della Salute e della Scienza di Torino, Italy
Elizaveta Padalko	Clinical Virology, University, Ghent, Belgium
Ulrike Protzer	Institute of Virology, TU Munich, Munich, Germany
Kate Tettmar	Blood Borne Virus Unit, Health Protection Agency, London, UK
Peter Tilston	Dept. of Clinical Virology, Manchester Royal Infirmary, Manchester, UK
Dorothee von Witzendorff/ Heiner Wedemeyer	Dept. of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Appendix 2 Proposed instruction for use

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

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

1st World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays

PEI code 7657/12

(Version 1.0, October 2013)

1. INTENDED USE

The 1st World Health Organization (WHO) International Standard for hepatitis D virus (HDV) RNA is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for HDV. The establishment of an international standard is an urgent need in the standardization, harmonization and quality control of the NAT tests and patient management (1). The standard has been prepared using a genotype 1 strain of HDV, derived from the HDV positive human plasma and further diluted in human negative plasma. 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 15 laboratories performing a wide range of HDV real-time NAT assays. Further details of the collaborative study are available in the report WHO/BS/2013.xxxx (2).

2. UNITAGE

This reagent has been assigned a unitage of 575,000 International Units/ml.

3. CONTENTS

Each vial contains 0.5 ml of lyophilized plasma containing infectious HDV.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

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

As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL

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

The material is supplied lyophilized and should be stored at or below-20ºC. Each vial should be **reconstituted in 0.5 ml of sterile nuclease-free water**. The product should be reconstituted just prior to use, once reconstituted, 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 -70ºC.

6. STABILITY

As the stability studies with accelerated conditions indicate high stability of the lyophilized reference material under the recommended storage conditions (at or below-20ºC), there is no expire date assigned to the international standard. This approach complies with the recommendations for the preparation, characterization and establishment of international and other biological reference standards (3). The reference material is held at the Paul-Ehrlich-Institut (PEI) within assured, temperature-controlled storage facilities. During its life cycle the stability is monitored at regular intervals. The international standard remains valid with the assigned potency and status until withdrawn or amended.

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

(1) Wedemeyer H, Manns MP. Epidemiology, pathogenesis and management of hepatitis D: update and challenges ahead. Nat Rev Gastroenterol Hepatol 2010, 7:31-40.

(2) Chudy M, Hanschmann KM, Bozsayi M, Kreß J, Nübling CM. Collaborative Study to Establish a World Health Organization International Standard for Hepatitis D Virus RNA for Nucleic Acid Amplification Technology (NAT) –Based Assays. WHO Report 2013, WHO/BS/2013.xxxx.

(3) Recommendations for the preparation, characterization and establishment of international and other biological reference standards. W HO Expert Committee on Biological Standardization. Fifty-fifth report, 2004. (WHO Technical Report Series, No. 932).

8. ACKNOWLEDGEMENTS

We are grateful to the Department of Gastroenterology of the Ankara University, Turkey for supplying the candidate materials and for their collaboration and to all study participants.

9. FURTHER INFORMATION

Further information for this material can be obtained as follows: whoccivd@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

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Federal Institute for Vaccines and Biomedicines

A WHO Collaborating Centre
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in vitro Diagnostic Devices

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 PLASMA & INFECTIOUS HEPATITIS D VIRUS (HDV)		
Handling:	See caution, section 4	
Toxicological properties		
Effects of inhalation: *contains infectious HDV*		Avoid –
Effects of ingestion: *contains infectious HDV*		Avoid –
Effects of skin absorption: *infectious HDV*	Avoid – *contains*	
Suggested First Aid		
Inhalation	Seek medical advice - *contains infectious HDV*	
Ingestion	Seek medical advice - *contains infectious HDV*	
Contact with eyes	Wash thoroughly with water. Seek medical advice – *contains infectious HDV*	
Contact with skin	Wash thoroughly with water. Seek medical advice – *contains infectious HDV*	
Action on Spillage and Method of Disposal		
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.		

13. LIABILITY AND LOSS

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

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

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

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

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

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

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