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

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Summary
The aim of the collaborative study was to evaluate candidate standards for hepatitis E virus (HEV) RNA for use in nucleic acid amplification technology (NAT)-based assays. The candidate standards consisted of lyophilized preparations of genotype 3a and genotype 3b HEV strains, obtained from blood donors, diluted in human plasma. The genotype 3a HEV strain has been developed as the candidate World Health Organization International Standard and the genotype 3b strain has been developed as the candidate Japanese National Standard. Coded duplicate samples of the two virus strains were distributed to the participating laboratories; genotype 3a HEV (Sample 1 and Sample 2) and genotype 3b HEV (Sample 3 and Sample 4). Each laboratory assayed the samples on 4 separate occasions and the data were collated and analyzed at the Paul-Ehrlich-Institut. Twenty-four laboratories from 10 countries participated in the study. Data were returned by twenty-three laboratories using both qualitative and quantitative assays. All assays were able to detect both candidate standards. It is proposed that the genotype 3a strain be established as the 1st International Standard for HEV RNA with a unitage of 250,000International Units per ml. On-going real-time and accelerated stability studies of the proposed International Standard are in progress.

Introduction
Hepatitis E virus (HEV) is a non-enveloped single stranded RNA virus belonging to the *Hepeviridae* family (Purcell and Emerson, 2008; Meng, 2010). In developing countries HEV is a major cause of acute hepatitis, transmitted by the faecal-oral route and associated with contamination of drinking water. In industrialized countries, HEV infection is being more frequently reported and whilst some cases are imported after travel to endemic areas, autochthonous cases are also increasing and infection with HEV appears more prevalent than originally believed (Ijaz *et al*., 2009). Prospects for control of HEV infection are encouraged by recent efforts in vaccine development (Shrestha *et al*., 2007; Zhu *et al*., 2010). Four main genotypes, representing a single serotype, of HEV infect humans. Genotype 1 viruses are found mainly in Africa and Asia and genotype 2 in Africa and Central America. Genotype 3 and 4 viruses are generally less pathogenic, although some exceptions have been reported, particularly for genotype 4; these genotypes infect not only humans, but also animals including swine, wild boar and deer. While genotype 4 strains are restricted to parts of Asia, genotype 3 viruses are found throughout the world. Zoonotic transmission of HEV occurs, either by consumption of contaminated meat and meat products, or by contact with infected animals (Purcell and Emerson, 2010). An alternative route of transmission is by transfusion of blood components with reports from several different countries including, for example, the UK, France and Japan (Boxall *et al*., 2006; Colson *et al*., 2007; Matsubayashi *et al*, 2004; Matsubayashi *et al*., 2008). Studies in Japan and China have identified acute HEV infections in blood donors confirmed by the detection of HEV RNA (Guo *et al*., 2010; Sakata *et al*., 2008).
It is now recognized that, in some countries at least, HEV infection is underreported, and where other causes of acute hepatitis have been excluded, HEV infection should be considered (Waar *et al*., 2005). The diagnosis of HEV infection is based upon the detection of specific antibodies (IgM and IgG), however there are issues concerning the sensitivity and specificity of these assays (Bendall *et al*., 2010; Drobeniuc *et al*., 2010). Analysis of HEV RNA using nucleic acid amplification techniques (NAT) is also used for diagnosis and can identify active infection and help to confirm serological results (Huang *et al*., 2010).
Infection with HEV may be particularly severe during pregnancy and in individuals with existing liver disease. Chronic HEV infection is an emerging problem amongst solid organ transplant recipients (Kamar *et al*., 2008; Legrand-Abravanel *et al*., 2010). In chronically infected patients, viral loads are monitored to investigate the efficacy of antiviral treatment (Haagsma *et al*., 2010;

Kamar *et al*, 2010a; Kamar *et al*., 2010b) and effects of reduction of immunosuppressive therapy (Kamar *et al*., 2010c).
Several NAT assays have been reported for the detection of HEV RNA in serum and plasma or faecal samples, including conventional reverse transcriptase polymerase chain reaction (RT-PCR) as well as real-time RT-PCR, and reverse transcription-loop-mediated isothermal amplification (Lan *et al*., 2009). The NAT tests include generic assays developed for the detection of HEV genotypes 1-4 (Jothikumar *et al*., 2006; Gyarmati *et al*., 2007). A recent study organized by the Paul-Ehrlich-Institut (PEI) on behalf of the World Health Organization (WHO), investigated the performance of HEV NAT assays in an international study (Baylis *et al*., 2011). Dilution panels of different HEV strains were blinded and tested by laboratories with experience in detection of HEV RNA. The results of the study demonstrated wide variations in assay sensitivity (100-1000 fold, for the majority of assays). The proposal by the PEI to prepare a standard for HEV RNA for use in NAT-based assays was endorsed by the WHO Expert Committee on Biological standardization (ECBS) in 2009 (WHO/BS/09.2126) and following the initial study, two virus strains were selected for further development as a candidate International Standard for the WHO and a candidate Japanese National Standard in collaboration with the National Institute of Infectious Diseases (NIID) in Japan. The viral strains being developed as standards are genotype 3a and 3b HEV strains, which were equally well detected in the initial study and belong to genotype 3 which is widely distributed. The strains are both derived from blood donors with sufficient titres of HEV RNA to prepare standards of good potency. The aim of the present study is to establish the respective standards and demonstrate their suitability for use, evaluate the potency and assign an internationally agreed unitage.

Preparation of bulk materials

After the initial proficiency/strain evaluation study (Baylis *et al*., 2011), two HEV strains were selected for the preparation of the candidate WHO International Standard and the candidate Japanese National Standard. The samples were kindly provided by Keiji Matsubayashi from the Japanese Red Cross Hokkaido Blood Center. The genotype 3a HEV strain HRC-HE104 was used to prepare the candidate WHO standard. The genotype 3b HEV strain JRC-HE3 was used to prepare the candidate Japanese National Standard. Characterization of the virus strains is shown in Table 1. The target concentration for the two bulk preparations was approximately 5.5 $\log_{10}$ HEV RNA copies/ml based upon the concentrations reported in the initial study (Baylis *et al*., 2011) and the concentrations determined by the Japanese Red Cross Hokkaido Blood Centre. The two virus strains tested negative for HIV-1/2 RNA, HBV DNA and HCV RNA using the Cobas TaqScreen MPX test (Roche Molecular Systems Inc., Branchburg, USA).
For the preparation of the candidate WHO standard bulk, 131 ml of the HEV strain HRC-HE104 were mixed with 2015 ml of plasma. For the preparation of the candidate Japanese National Standard bulk, 30 ml of the HEV strain JRC-HE3 were mixed with 1070 ml of plasma. The bulk preparations were cooled (4-8°C) until processing (~18 hours later). The respective preparations were diluted using pooled citrated plasma which had been used in the initial HEV collaborative study (Baylis *et al*., 2011). The plasma was centrifuged and filtered twice before use. The plasma diluent tested negative for anti-HEV IgG and IgM (Ulrich Mohn, Mikrogen GmbH, Neuried, Germany, personal communication) and tested negative for HEV RNA (data not shown) and HIV-1/2 RNA, HBV DNA and HCV RNA, testing was performed as described above. In addition, the plasma was negative for HBsAg, anti-HCV, anti-HBc and anti-HIV-1/2.
The filling and lyophilization was performed by an ISO 13485:2003 accredited Swiss company. For processing, 0.5 ml volumes were dispensed into 4 ml screw-cap glass vials. Rubber seals were then placed on top of the filled vials before loading into the freeze drier (CHRIST Epsilon 2-25 D) for lyophilization. After freeze-drying the vials were sealed with screw caps and vials stored at -20°C.

For the candidate WHO standard, 4256 vials were lyophilized; the coefficient of variation of the fill volume was 1.1%. In the case of the candidate Japanese National Standard, 2154 vials were lyophilized; the coefficient of variation of the fill volume was 1.0%. In both cases, measurements were made for a total of 26 vials. For analysis of residual moisture, vials filled with 0.5 ml volumes of plasma diluent were distributed throughout the freeze-drier. Residual moisture was 0.73%, as determined by testing of 12 vials (Karl Fischer analysis). The freeze-drying process did not affect the HEV RNA titre of the lyophilized samples when compared to aliquots of the respective bulk preparations which were stored at -80°C (data not shown).
Vials of the candidate WHO standard are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.
All manufacturing records are held by PEI and are available on request by the ECBS.

Collaborative study

The collaborative study comprised 24 laboratories from 10 countries. The participants in the collaborative study who returned data are listed in Appendix 1.
The samples analysed in the study were labelled as Sample 1, Sample 2, Sample 3 and Sample 4. Sample 1 and Sample 2 were replicates of the candidate WHO standard; and Sample 3 and Sample 4 were replicates of the candidate Japanese National Standard. The collaborative study materials were shipped to participants at ambient temperature.
Participants were asked to test the panel using their routine assay for HEV RNA, testing the samples in four separate assay runs, using fresh vials of each sample for each run. Where laboratories performed quantitative tests, they were requested to report results in copies/ml, testing samples in the linear range of the assay. In the case of qualitative assays, participants were requested to assay each sample by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For the three subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point estimated in their first assay. Participants reported diluting the materials using plasma, water or phosphate buffered saline. Data sheets and a method form were provided so that all relevant information could be recorded.

Statistical Methods

Quantitative Assays

Evaluation of quantitative assays was restricted to dilutions in the range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where the assays of most participants seem to produce comparable data. 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.

Qualitative Assays

The data from all assays were pooled to give series of number positive out of number tested at each dilution. For each participant, these pooled results were evaluated by means of probit analysis to estimate the EC50 i.e. the concentration at which 50% of the samples tested were positive (for assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps , the Spearman-Kaerber method was applied for EC50 estimation). The calculated end-point was used to give estimates expressed in $\log_{10}$ NAT-detectable units/ml after correcting for the equivalent volume of the test sample.

Relative potencies

Potencies of Samples 2, 3 and 4, for the quantitative assays, were estimated relative to Sample 1 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

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

Stability studies

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 -80°C (to provide a baseline if there is any suggestion of instability at higher temperatures). For the accelerated thermal degradation, vials have been incubated at +4°C, +20°C, +37°C and +45°C for up to 4 months. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 ml of nuclease free water and analysed by real-time PCR (Jothikumar *et al*., 2006).

Data Received

Data were received from a total of 23 participating laboratories; one laboratory failed to complete the study within the specified time frame. Data from 20 qualitative and 14 quantitative assays were reported. The types of assays used by participants are listed in Table 2; all assays were developed in-house. The assays used by participants were mainly based upon real-time PCR, although some conventional PCR methods were also used.
For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1. Where a laboratory performed more than one assay method, the results from the different methods were analyzed independently, as if from separate laboratories, and coded, for example, laboratory 16a and laboratory 16b. In the case of 9 assays, quantitative values were reported covering the linear range of the respective assays; in addition, further dilutions have been performed allowing end-point determination. These data have been analysed separately and the number of estimates therefore exceeds the number of assay sets returned by the participants.

Results

Quantitative Assay Results

Initially evaluation of quantitative assays was performed without removing any outlying data; subsequently the data was restricted to a range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where reproducible results were obtained across dilutions. The laboratory mean estimates in copies/ml ($\log_{10}$) are shown in histogram form in Figure 1. Each box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. The individual laboratory means are given in Table 3. The relative variation of the individual laboratory estimates is illustrated by the box-and-whisker plots in Figure 2.

Qualitative Assay Results

The NAT-detectable units/ml ($\log_{10}$) for the qualitative assays are shown in histogram form in Figure 3. Each box represents the mean estimate from an individual laboratory and is labelled with the laboratory code number. The individual laboratory means are given in Table 4. From Figure 3, it can be seen that the estimates of NAT detectable units/ml ($\log_{10}$) from the qualitative

assays are more variable than the quantitative assays, reflecting the different sensitivities of the various assays. This observation is not unexpected and is in line with other studies.

Determination of Overall Laboratory Means

The overall means for the laboratories performing quantitative assays are shown in Table 5a. The means for both Sample 1 and Sample 2, replicates for the candidate WHO standard, are 5.58 $\log_{10}$ and 5.60 $\log_{10}$ copies/ml HEV RNA respectively, which demonstrates excellent agreement between the replicate samples. The candidate Japanese National Standard showed identical mean results of 5.66 $\log_{10}$ copies/ml HEV RNA for replicate Samples 3 and 4. The combined mean values for the replicate samples are shown in Table 5b.
The overall means for the qualitative assays are shown in Table 6a; there is good agreement between the duplicate samples as seen previously for the quantitative assays. The combined mean values for the replicate samples are shown in Table 6b. The qualitative assays show 0.3 $\log_{10}$ lower mean estimates than the quantitative assays.

Relative Potencies

Based upon the data from both qualitative and quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml (95% confidence limits 5.15 – 5.63). This value was estimated with a combined end-point evaluation of qualitative and quantitative (restricted to dilutions in the range of 0.0 $\log_{10}$ to - 2.5 $\log_{10}$) data by means of a mixed linear model.
The potencies of Samples 2, 3 and 4 were calculated relative to Sample 1, taking the value of Sample 1 as 5.39 $\log_{10}$ units/ml. The relative potencies are shown in Tables 7 and 8 for the quantitative and qualitative assays, respectively. For the quantitative data from laboratory 9, no potency was estimable since there was only one dilution tested for each sample. The data is plotted in histogram form (Figures 4-6). The data demonstrate that expressing the results as potencies relative to Sample 1, as a standard with an assumed unitage of 5.39 $\log_{10}$ units/ml gives a marked improvement in the agreement between the majority of methods and laboratories. These data provide some evidence for commutability of the candidate standard for evaluation of HEV from infected individuals, since Samples 1 and 2 represent a different strain of HEV compared to Samples 3 and 4.

Results of Stability Studies

Vials of the candidate WHO standard were incubated at +4°C, +20°C, +37°C and +45°C for up to four months and tested by real-time PCR for HEV RNA. The heat-treated vials were assayed concurrently with vials that had been stored at -20°C and at -80°C. All samples were tested in duplicate and were compared to a standard curve prepared using vials of the candidate WHO standard stored at -80°C.
There was no evidence of instability of the samples stored at -20°C when compared to samples stored at -80°C. After 4 months incubation at +20°C a small loss of titre was observed.The observed drop in titre at higher temperatures (+37°C and +45°C) may be related to problems with reconstitution of the samples rather that actual degradation and has previously been observed for some other preparations, particularly for RNA viruses formulated in pooled plasma. The potency of the reconstituted material, after freezing and thawing, has not been investigated. Further stability studies (both real-time and accelerated) are on-going and will be communicated to the WHO.

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

Conclusions

In this study, a wide range of quantitative and qualitative assays were used to determine the suitability and evaluate the HEV RNA content of the candidate standards. Although the methods used by the study participants were all developed in-house, the majority of assays were able to detect the two HEV strains consistently. Based upon the data from the qualitative and the quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml. Since the unitage assigned to the 1st WHO standard of a preparation is essentially arbitrary, for practical purposes, the candidate International Standard has been assigned a unitage of 250,000 International Units/ml. Since there was only a negligible difference in the overall means for the candidate Japanese National Standard compared to the WHO preparation, the two materials have therefore been assigned the same value i.e. 250,000 International Units/ml. In the case of the quantitative assays, laboratories reported values in HEV RNA copies/ml. The participants used plasmid DNA containing HEV sequences, synthetic oligonucleotides and *in vitro* transcribed HEV RNA to control for copy number. In some cases laboratories used HEV-containing plasma which had been calibrated against *in vitro* transcribed HEV RNA. Another laboratory prepared standard using stool-derived virus, the titre of which was determined by end-point dilution and analysis by Poisson distribution. No standard method or common quantitation standard material was used, and this is reflected in the variation observed for the quantitative results, with a variation in the order of 2 $\log_{10}$, which were improved by expressing the results against Sample 1 as a common standard. In the case of the qualitative assays, the variation in NAT-detectable units was at least 3 $\log_{10}$, and again expressing potencies relative to Sample 1 improved the agreement between the different laboratories and methods.

The collaborative study materials have been dispatched at ambient temperature, replicating the intended shipping conditions. Initial accelerated thermal degradation analysis indicates a reduction in the levels of HEV RNA at higher incubation temperatures. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.

The standard will be of value for comparison of results between laboratories, determination of assay sensitivities and for validation. It is anticipated that the standard will find application in clinical laboratories, particularly hepatitis reference laboratories performing diagnosis and monitoring HEV viral loads in chronically infected transplant patients, research laboratories, blood and plasma centres which implement HEV NAT screening, regulatory agencies and organizations developing HEV vaccines as well as manufacturers of diagnostic kits.

Each vial of the HEV RNA standard contains the lyophilized residue of 0.5 ml of HEV RNA positive plasma. Predictions of stability indicate that the standard is stable and suitable for long-term use when stored as directed in the accompanying proposed “Instructions For Use” data sheets for the panel (Appendix 2).

Recommendations

Based upon the results of the collaborative study, it is proposed that the genotype 3a HEV strain (Samples 1 and 2, in this study) should be established as the 1st International Standard for hepatitis E virus RNA and be assigned a unitage of 250,000 International Units/ml. The standard has been given the code number 6329/10; 3800 vials are available to the WHO and custodian laboratory is the Paul-Ehrlich-Institut.

Comments from participants

After circulation of the draft report for comment, replies were received from all participants. The majority of the comments were editorial in nature and the report has been amended accordingly. All participants were in agreement with the conclusions of the report.

One participant commented on the possible incorrect estimation of the viral load by the participants who used DNA standards (synthetic oligonucleotides or plasmid DNA) due to lack of control for reverse transcription of virus RNA into cDNA. This might be better controlled using *in vitro* transcribed RNA or a virion-based preparation.

Another participant remarked that many laboratories have used the same method, showing quite different sensitivities, possibly due to differences in extraction and amplification/detection reagents and instrumentation and its set up.

Acknowledgements

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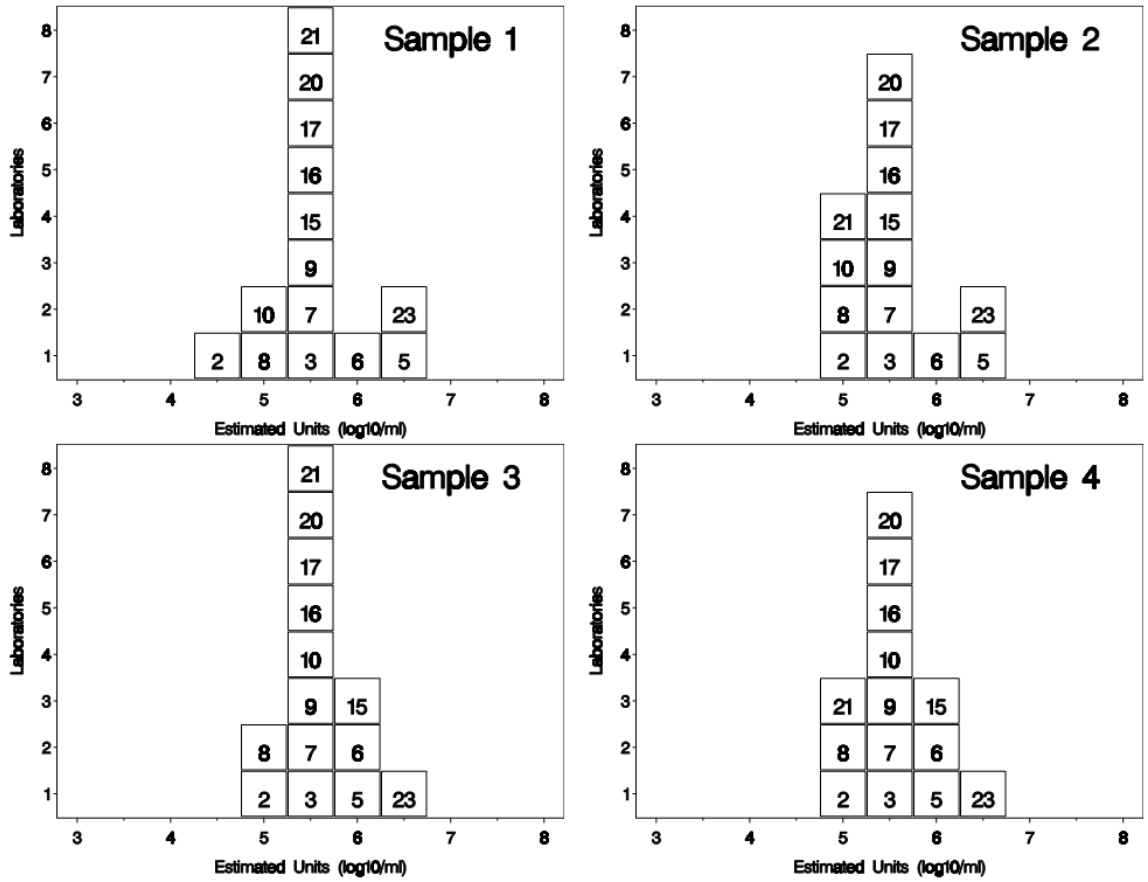
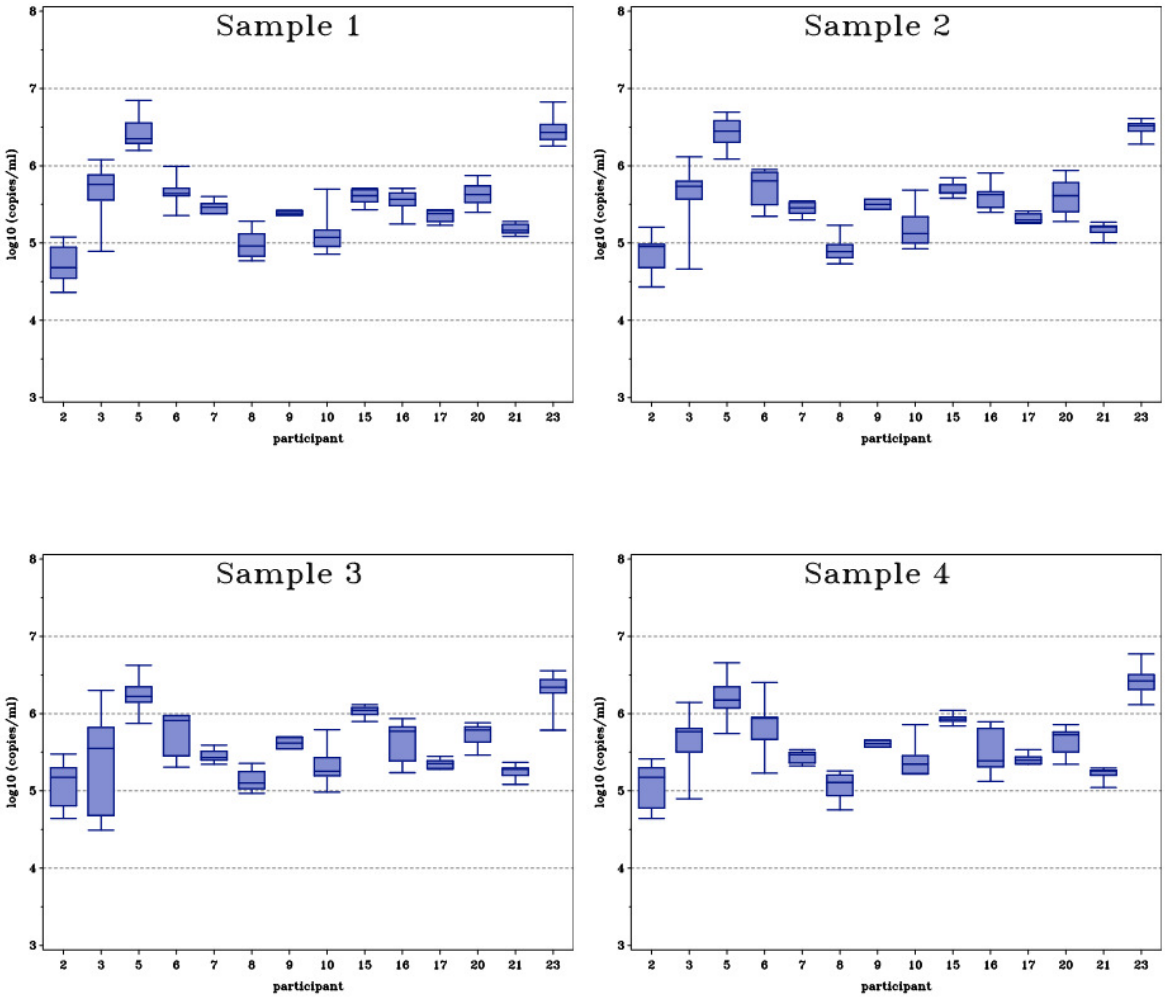
Figure 1 Estimates for quantitative assays



Histograms of the quantitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ copies/ml are indicated on the x-axis. Data are shown for laboratory 16a.

Figure 2 Box and whisker plots of the quantitative data (log_{10} copies/ml)



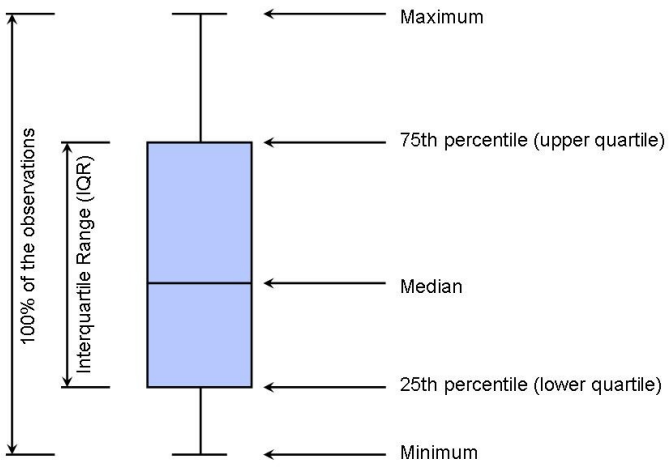
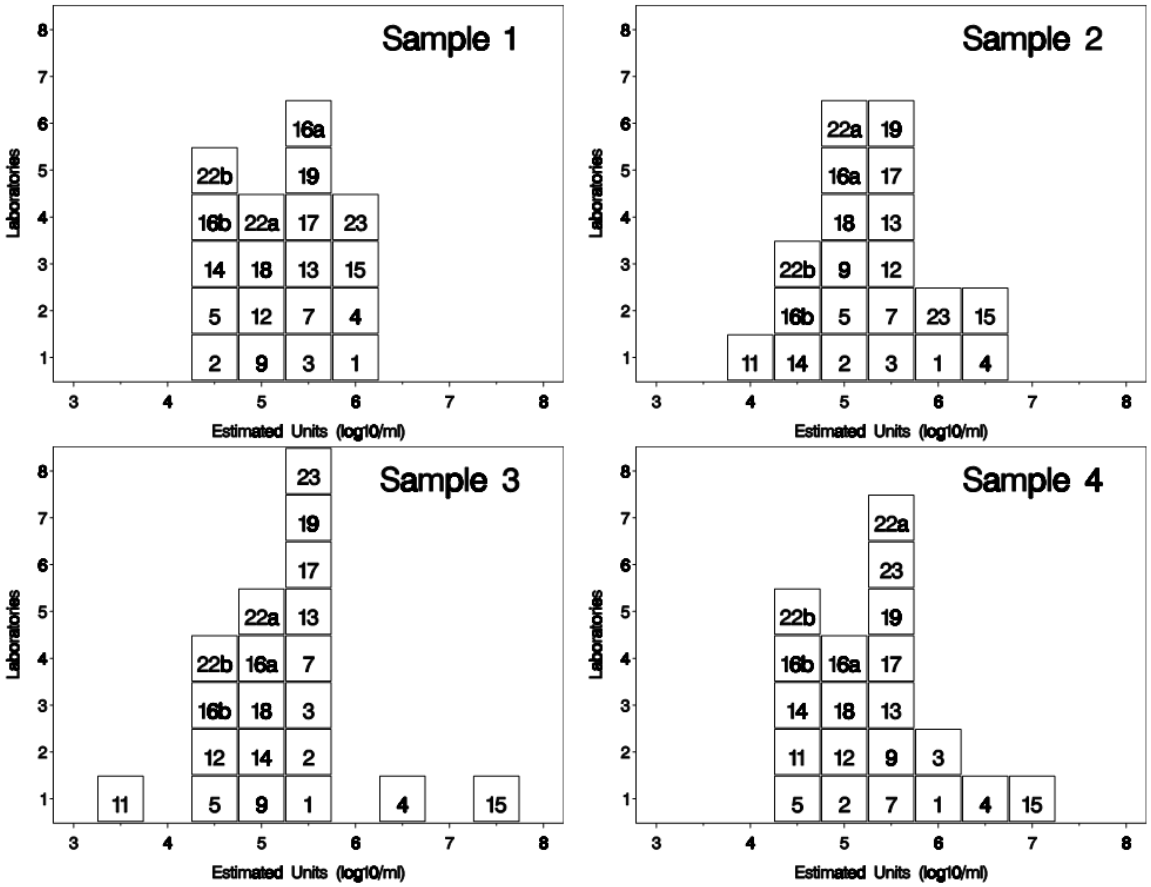
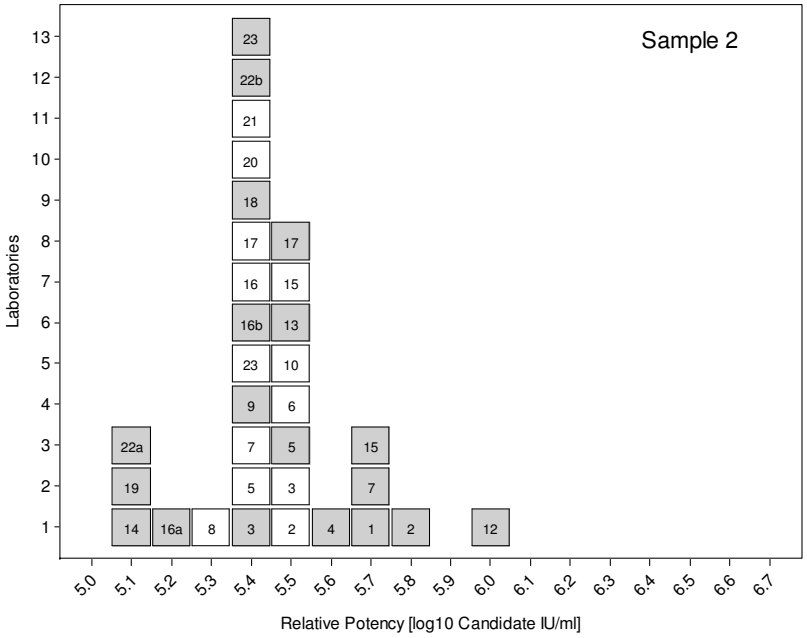


Figure 3 Estimates for qualitative assays



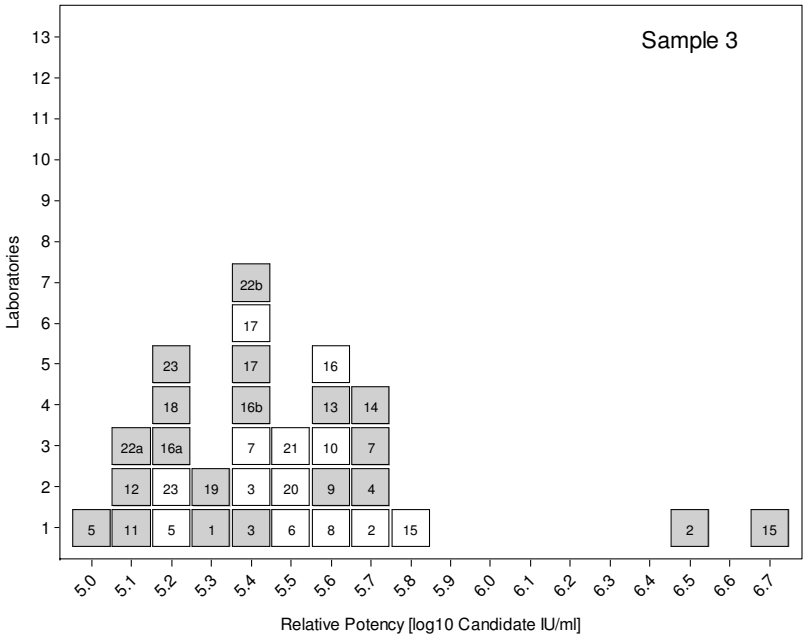
Histograms of the qualitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ NAT-detectable units/ml are indicated on the x-axis. In the case of laboratory 11, data for Sample 1 have been omitted due to a 2 $\log_{10}$ higher cut-off.

Figure 4 Potency of Sample 2 relative to Sample 1



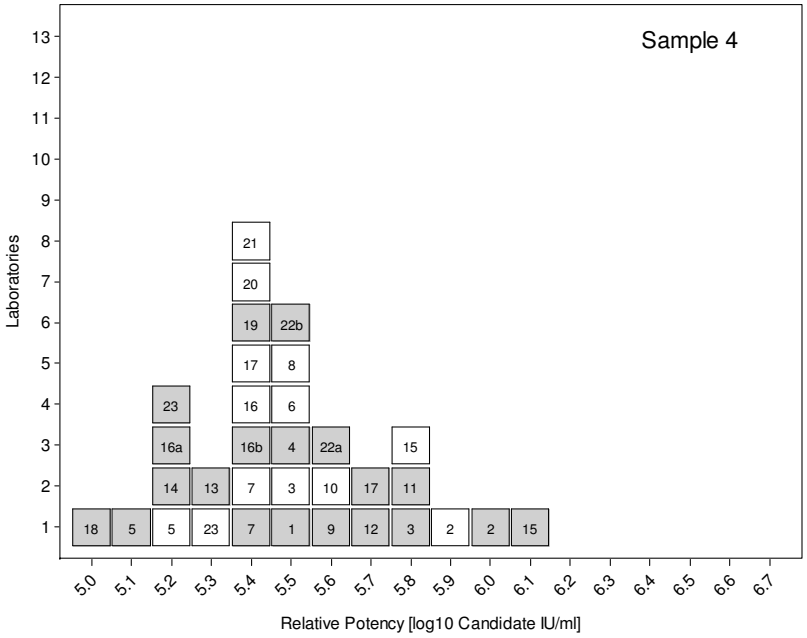
Histogram of the potency of Sample 2 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). No relative potency is shown for laboratory 11 for sample 2, since no value had been determined for Sample 1 (i.e. the data were outlying and did not perform as the replicate i.e. Sample 2).

Figure 5 Potency of Sample 3 relative to Sample 1



Histogram of the potency of Sample 3 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Figure 6 Potency of Sample 4 relative to Sample 1



Histogram of the potency of Sample 4 relative to Sample 1 (=5.39 $\log_{10}$ units/ml); qualitative data (grey boxes) and quantitative data (white boxes). In the case of Laboratory 11, the data have been calculated relative to Sample 2.

Table 1 Details of HEV strains lyophilized as candidate standards

Virus strain	HEV RNA (copies/ml)*	Genotype	Accession No.**	Anti-HEV IgM/IgG	ALT (IU/L)
HRC-HE104	1.6×10^7	3a	AB630970	-/-	36
JRC-HE3	2.5×10^7	3b	AB630971	+/-	398

*Concentrations determined by the Japanese Red Cross Hokkaido Blood Center
**Full length sequence

Table 2 Assay protocols used by participants

Laboratory code	Assay type (qualitative or quantitative)	Extraction method	NAT method	Assay target	Reference
1	Qual.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
2	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2	Adlhoch *et al*. 2009
3	Qual./Quant.	High Pure Viral Nucleic Acid kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
4	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
5	Qual./Quant.	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
6	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
7	Qual./Quant.	QIAamp MinElute Virus Spin kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al*. 2008
8	Quant.	SMI-TEST EX-R&D (Medical Biological Laboratories Co., Ltd.)	Real-time RT-PCR (TaqMan)	ORF2/3	Tanaka *et al*. 2007
9	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
10	Quant.	COBAS AmpliPrep Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
11	Qual.	COBAS AmpliScreen Multiprep Specimen Preparation and Control kit (Roche)	Conventional one step RT-PCR; analysis by agarose gel electrophoresis	ORF1	
12	Qual.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
13	Qual.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
14	Qual.	Viral DNA/RNA Isolation kit (GenMag Biotechnology)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	
15	Qual./Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006 (modified)
16a	Qual./Quant.	MagNA Pure LC (Roche)	Real-time PCR (SYBR Green)	ORF2/3	Jothikumar *et al*. 2006

					(modified)
16b	Qual.	MagNA Pure LC (Roche)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Meng *et al*. 2001
17	Qual./Quant.	QIAamp Virus BioRobot MDx kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Matsubayashi *et al*. 2008
18	Qual.	MagNA Pure LC Total Nucleic Acid Isolation kit (Roche)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
19	Qual.	easyMag (bioMérieux)	Real-time RT-PCR (TaqMan)	ORF2	
20	Quant.	QIAamp Viral RNA Mini kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	
21	Quant.	BioRobot Universal (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
22a	Qual.	QIAamp RNA Mini kit (Qiagen)	Nested RT-PCR; analysis by agarose gel electrophoresis	ORF2	Gyarmati *et al*. 2007
22b	Qual.	QIAamp RNA Mini kit	Real-time RT-PCR (TaqMan)	ORF2/3	Jothikumar *et al*. 2006
23	Qual./Quant**.**	QIAamp DNA Mini Blood kit (Qiagen)	Real-time RT-PCR (TaqMan)	ORF2/3	Wenzel *et al*., in press

Qualitative (Qual.) and quantitative (Quant.) assays

Table 3 Mean estimates from quantitative assays (log_{10} copies/ml)

Laboratory code	Sample			
	1	2	3	4
2	4.69	4.82	5.09	5.08
3	5.69	5.62	5.43	5.65
5	6.51	6.48	6.24	6.20
6	5.75	5.80	5.77	5.83
7	5.50	5.46	5.45	5.44
8	5.07	4.97	5.14	5.06
9	5.43	5.52	5.62	5.61
10	5.18	5.22	5.30	5.39
15	5.66	5.73	6.02	5.93
16a	5.59	5.62	5.64	5.51
17	5.40	5.34	5.35	5.41
20	5.70	5.65	5.74	5.65
21	5.25	5.23	5.25	5.23
23	6.54	6.53	6.31	6.41

Table 4 Mean estimates from qualitative assays (log_{10} NAT detectable units/ml)

Laboratory code	Sample			
	1	2	3	4
1	5.76	6.05	5.62	5.91
2	4.42	4.85	5.49	5.02
3	5.35	5.40	5.35	5.76
4	6.20	6.37	6.47	6.33
5	4.70	4.84	4.27	4.42
7	5.34	5.62	5.62	5.34
9	5.02	5.03	5.18	5.26
11		4.00	3.72	4.42
12	4.91	5.48	4.61	5.18
13	5.51	5.66	5.71	5.44
14	4.71	4.43	5.00	4.57
15	6.11	6.36	7.42	6.87
16a	5.32	5.17	5.17	5.17
16b	4.74	4.74	4.74	4.74
17	5.39	5.52	5.42	5.67
18	5.13	5.13	4.98	4.76
19	5.68	5.42	5.56	5.71
22a	5.21	4.92	4.91	5.44
22b	4.53	4.53	4.52	4.68
23	5.76	5.76	5.60	5.60

Laboratory 11, sample 1, omitted due to 2 log_{10} higher cut-off

Table 5a Overall mean estimates from quantitative assays (log_{10} copies/ml)

Sample	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
1	123	5.58	0.29	5.32	5.85	5.46	4.36	6.85	98%
2	125	5.60	0.28	5.33	5.87	5.46	4.43	6.69	94%
3	124	5.66	0.20	5.40	5.93	5.50	4.49	6.63	77%
4	125	5.66	0.20	5.40	5.93	5.48	4.64	6.77	76%

n – number of dilutions analysed (in linear range), sd – standard deviation, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 5b Combined mean estimates from quantitative assays (log_{10} copies/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	248	5.59	0.30	5.33	5.86	5.46	4.36	6.85	99%
NIID	249	5.66	0.20	5.40	5.93	5.48	4.49	6.77	76%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)

Table 6a Overall means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Sample	n	mean	sd	Lower cl	Upper cl	median	min	max	cv_geo
1	19	5.25	0.51	5.01	5.50	5.32	4.42	6.20	150%
2	20	5.26	0.62	4.97	5.56	5.29	4.00	6.37	179%
3	20	5.27	0.79	4.90	5.64	5.27	3.72	7.42	226%
4	20	5.31	0.64	5.02	5.61	5.30	4.42	6.87	183%

n – number of tests, lowercl/uppercl – 95% confidence limits for the mean, cv_geo – geometric coefficient of variation [%]

Table 6b Combined means of estimates from qualitative assays (log_{10} NAT detectable units/ml)

Candidate	n	mean	sd	lowercl	uppercl	median	min	max	cv_geo
WHO	39	5.26	0.56	5.08	5.44	5.32	4.00	6.37	163%
NIID	40	5.29	0.71	5.07	5.52	5.30	3.72	7.42	202%

Combined data for Samples 1 and 2, replicate samples of the candidate IS (WHO); combined data for Samples 3 and 4, replicate samples of the candidate Japanese National Standard (NIID)

Table 7 Potency relative to Sample 1 (quantitative assays)

Sample	Laboratory code	Relative potency (log_{10} copies/ml)	95% Confidence Interval	
2	2	5.54	5.29	5.78
	3	5.45	5.15	5.74
	5	5.39	5.15	5.63
	6	5.45	5.20	5.71
	7	5.38	5.28	5.47
	8	5.31	5.17	5.45
	9			
	10	5.47	5.34	5.59
	15	5.53	5.46	5.60
	16a	5.40	5.22	5.59
	17	5.36	5.29	5.43
	20	5.36	5.26	5.46
	21	5.39	5.35	5.44
	23	5.41	5.29	5.53
3	2	5.74	5.50	5.97
	3	5.36	5.07	5.65
	5	5.21	4.97	5.46
	6	5.48	5.21	5.75
	7	5.38	5.29	5.47
	8	5.55	5.41	5.69
	9			
	10	5.55	5.43	5.68
	15	5.83	5.76	5.90
	16a	5.55	5.36	5.73
	17	5.39	5.31	5.46
	20	5.52	5.42	5.62
	21	5.46	5.41	5.50
	23	5.20	5.09	5.32
4	2	5.90	5.66	6.15
	3	5.45	5.17	5.74
	5	5.17	4.93	5.42
	6	5.54	5.29	5.80
	7	5.37	5.28	5.46
	8	5.46	5.32	5.60
	9			
	10	5.63	5.50	5.76
	15	5.75	5.68	5.83
	16a	5.35	5.17	5.53
	17	5.44	5.37	5.52
	20	5.43	5.33	5.52
	21	5.44	5.39	5.48
	23	5.27	5.16	5.39

It was not possible to estimate the relative potency for laboratory 9 since there were only two assay runs performed, each at a different dilution

Table 8 Potency relative to Sample 1 (qualitative assays)

Sample	Laboratory code	Relative potency (log_{10} NAT detectable units/ml)	95% Confidence Interval	
2	1	5.68	5.10	6.27
	2	5.82	5.26	6.38
	3	5.44	4.81	6.08
	4	5.56	4.90	6.22
	5	5.53	5.09	5.97
	7	5.68	5.16	6.23
	9	5.40	5.15	5.66
	12	5.96	5.35	6.51
	13	5.54	5.14	5.91
	14	5.11	4.71	5.50
	15	5.65	4.90	6.40
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.52	4.96	6.08
	18	5.39	4.88	5.90
	19	5.13	4.71	5.56
	22a	5.10	4.57	5.63
	22b	5.39	4.79	5.99
	23	5.39	4.74	6.04
3	1	5.25	4.67	5.81
	2	6.46	5.90	7.14
	3	5.39	4.76	6.02
	4	5.66	5.00	6.32
	5	4.96	4.53	5.39
	7	5.68	5.16	6.23
	9	5.55	5.30	5.80
	11	5.11	4.52	5.69
	12	5.09	4.51	5.64
	13	5.59	5.19	5.96
	14	5.67	5.27	6.08
	15	6.67	5.90	7.44
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.43	4.87	5.98
	18	5.24	4.73	5.75
	19	5.28	4.85	5.70
	22a	5.10	4.56	5.63
	22b	5.38	4.78	5.97
	23	5.24	4.59	5.89
4	1	5.54	4.96	6.12
	2	5.99	5.43	6.55
	3	5.80	5.15	6.48
	4	5.52	4.86	6.18
	5	5.11	4.70	5.51
	7	5.39	4.87	5.92
	9	5.64	5.38	5.90

	11	5.81	5.23	6.40
	12	5.65	5.07	6.20
	13	5.32	4.93	5.71
	14	5.24	4.85	5.64
	15	6.13	5.39	6.88
	16a	5.24	4.85	5.64
	16b	5.39	4.77	6.01
	17	5.68	5.12	6.23
	18	5.02	4.51	5.52
	19	5.43	5.00	5.87
	22a	5.62	5.08	6.18
	22b	5.54	4.94	6.17
	23	5.24	4.59	5.89

N.B. The relative potency for laboratory 11 was estimated relative to Sample 2 (Sample 1 had a cut-off 2 $\log_{10}$ dilutions higher)

Table 9 Stability testing

Incubation time	Incubation temperature				
	-20°C	+4°C	+20°C	+37°C	+45°C
1 month	ND	ND	ND	ND	5.03
2 months	ND	ND	ND	4.98	4.55*
4 months	5.56	5.52	5.33	ND	ND

ND Not determined
*Material could not be completely reconstituted
Titres expressed as $\log_{10}$ candidate International Units/ml

Appendix 1 List of participants

Scientist	Affiliation
Akihiro Akaishi	Nihon Pharmaceuticals Co., Ltd. Chiba, Japan
Martijn Bouwknegt/Saskia Rutjes	National Institute for Public Health and the Environment Bilthoven, The Netherlands
Silvia Dorn	Mikrogen GmbH Neuried, Germany
Thomas Gärtner	Octapharma Frankfurt am Main, Germany
Samreen Ijaz/Renata Szypulska	Health Protection Agency London, UK
Jacques Izopet	Institut Fédératif de Biologie Purpan Toulouse, France
Shintaro Kamei/Katsuro Shimose	Chemo-Sero-Therapeutic Research Institute Kumamoto, Japan
Li Ma/Mei-ying Yu	Center for Biologics Evaluation and Research/Food and Drug Administration Bethesda, USA
Thomas Laue	Astra Diagnostics Hamburg, Germany
Keiji Matsubayashi/Hidekatsu Sakata	Japanese Red Cross Hokkaido Blood Center Sapporo, Japan
Birgit Meldal/Daniel Candotti	Cambridge University and NHS Blood and Transplant Cambridge, UK
Takao Minagi	Benesis Corporation Kyoto, Japan
Saeko Mizusawa/Yoshiaki Okada	National Institute of Infectious Diseases Tokyo, Japan
Elisa Moretti/Francesca Bonci	BioSC-Kedrion S.p.A. Bolognana-Lucca, Italy
Tonya Mixson/Saleem Kamili	Centers for Disease Control and Prevention Atlanta, USA
Andreas Nitsche/Marco Kaiser	Robert Koch-Institut Berlin, Germany
Mats Olsson/Anders Olofsson	Octapharma Stockholm, Sweden
Giulio Pisani/Francesco Marino	CRIVIB, Istituto Superiore di Sanità Rome, Italy
James Wai Kuo Shih	Xiamen University Fujian, China
Ko Suzuki	Central Blood Institute, Japanese Red Cross Society Tokyo, Japan
Isabelle Thomas	Scientific Institute of Public Health,Brussels, Belgium
Youchun Wang/Yansheng Geng	National Institutes for Food and Drug Control Beijing, China
Jürgen Wenzel/Wolfgang Jilg	University of Regensburg Regensburg, Germany

Appendix 2 Draft Instructions For Use for 6329/10

Paul-Ehrlich-Institut → → A WHO Collaborating Centre
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel → for Quality Assurance of Blood Products and
Federal Institute for Vaccines and Biomedicines → → in vitro Diagnostic Devices → →

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

PEI code 6329/10

(Version 1.0, 7th July 2011)

1. INTENDED USE

The 1st World Health Organization International Standard for hepatitis E virus (HEV) is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for HEV. The need to develop a standard was demonstrated in an initial study investigating performance of HEV NAT assays (Baylis *et al.*, J. Clin. Microbiol. 2011). The standard has been prepared using a genotype 3a strain of HEV, derived from the plasma of a blood donor and further diluted in human 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 23 laboratories performing a wide range of HEV NAT assays. Further details of the collaborative study are available in the report WHO/BS/11.XXXX.

2. UNITAGE

This reagent has been assigned a unitage of 250,000 International Units/ml.

3. CONTENTS

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

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

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

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 -20°C. 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.

6. STABILITY

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

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

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

7. REFERENCES

Baylis, S. A., K. M. Hanschmann, J. Blümel, and C. M. Nübling; on behalf of the HEV Collaborative Study Group. 2011. Standardization of hepatitis E virus (HEV) nucleic acid amplification technique (NAT)-based assays: an initial study to evaluate a panel of HEV strains and investigate laboratory performance. J. Clin Microbiol. 49:1234-1239.

S. A. Baylis, K. M. Hanschmann. Collaborative Study to Establish a World Health Organization International Standard for Hepatitis E Virus RNA for Nucleic Acid Amplification Technology (NAT)–Based Assays. WHO Report 2011, WHO/BS/YY.XXXX.

8. ACKNOWLEDGEMENTS

We are grateful to the Japanese Red Cross Hokkaido Blood Center for supplying the candidate materials, the National Institute of Infectious Diseases, Japan for their collaboration and to the study participants.

9. FURTHER INFORMATION

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

10. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to whoccivd@pei.de

11. CITATION

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

12. MATERIAL SAFETY SHEET

Physical properties (at room temperature)	
Physical appearance → →	Lyophilized powder
Fire hazard → → →	None
Chemical properties	
Stable → → → Yes	Corrosive: No
Hygroscopic → → No	Oxidising: No
Flammable → → No	Irritant: No
Other (specify) → CONTAINS HUMAN PLASMA & INFECTIOUS HEPATITIS E VIRUS (HEV)	
Handling: → ..... See caution, section 4	
Toxicological properties	

Paul-Ehrlich-Institut → → → Tel: +49 (0)6103 77 0
Paul-Ehrlich-Str. 51-59 → → → Fax: +49 (0)6103 77 123
63225 Langen, Germany → → → Email: whoccivd@pei.de
→ → Web: http://www.pei.de

¶

Effects of inhalation: → → → Avoid – *contains infectious HEV*	¤
Effects of ingestion: → → → Avoid – *contains infectious HEV*	¤
Effects of skin absorption: → Avoid – *contains infectious HEV*	¤
Suggested First Aid	¤
Inhalation → ..... Seek medical advice – *contains infectious HEV*	¤
Ingestion → ..... Seek medical advice – *contains infectious HEV*	¤
Contact with eyes ..... Wash thoroughly with water. Seek medical advice – *contains infectious HEV*	¤
Contact with skin ..... Wash thoroughly with water. Seek medical advice – *contains infectious HEV*	¤
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.¶