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Collaborative Study to Establish the 1st World Health Organization International Reference Panel for Hepatitis E Virus RNA Genotypes for Nucleic Acid Amplification Technique (NAT)-Based Assays

Sally A. Baylis[1], Eriko Terao[2], Kay-Martin O. Hanschmann[1]

[1]*Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D 63225 Langen, Germany*
[2]*European Directorate for the Quality of Medicines & HealthCare, Council of Europe, 7 allée Kastner, CS 30026, F-67081 Strasbourg, France*
Principal contact: Sally.Baylis@pei.de

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 **14 September 2015** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr M Nübling** (nueblingc@who.int)

Summary

The aim of the collaborative study was to evaluate a panel of plasma samples containing different genotypes of hepatitis E virus (HEV) for use in nucleic acid amplification technique (NAT)-based assays. The panel of samples comprises eleven different members including genotype 1a (2 strains), 1e, 2a, 3b, 3c, 3e, 3f, 4c, 4g as well as a human isolate related to rabbit HEV. Each laboratory assayed the panel members directly against the 1st World Health Organization (WHO) International Standard (IS) for HEV RNA (6329/10). Also included in the study was a candidate Biological Reference Preparation (BRP) for HEV RNA prepared from an HEV 3f virus strain on behalf of the European Directorate for the Quality of Medicines and HealthCare (EDQM). The samples for evaluation were distributed to 24 laboratories from 14 different countries. The samples were assayed on three separate days and the data were collated and analysed at the Paul-Ehrlich-Institut (PEI). Data were retuned by 23 of the participating laboratories, in total 32 sets of data were returned; 17 from quantitative assays and 15 from qualitative assays. The assays used consisted of a mixture of in-house developed and commercially available assays. The results showed that all samples were detected consistently by the majority of participants.
It is proposed that the panel, consisting of 11 members, be established as the "1st International Reference Panel (IRP) for all HEV genotypes for NAT-based assays" (code number 8578/13) with no unitage being assigned to the individual panel members. In the case of the candidate BRP, a unitage of 40,850 IU/ml (4.6112 $\log_{10}$ IU/ml) is assigned to the material after calibration against the WHO IS. Real-time stability studies have indicated that the panel of HEV samples and the candidate BRP is very stable under normal conditions of storage, i.e., at -20ºC or below, and are therefore suitable for long term use. On-going real-time stability studies of the panel members and the BRP are in progress.

Introduction

The 1st WHO IS for HEV RNA for nucleic acid amplification technique (NAT)-based assays (code number 6329/10) was established in October 2011 by the WHO Expert Committee on Biological Standardization (ECBS). The WHO IS represents an HEV sub-genotype 3a strain from a Japanese blood donor diluted in negative plasma. Whilst HEV is represented by a single serotype, the virus can be classified into at least four main genotypes. Genotypes 1 and 2 can be found in humans, whilst genotypes 3 and 4 are found in both humans as well as a range of animal species, particularly pigs. The geographical distribution of HEV genotypes is complex. Genotype 1 consists of strains circulating in Africa and Asia. Genotype 2 has been found in Mexico and albeit rarely, in some African countries. Genotype 3 is widely distributed, mainly being reported in the USA, Europe and Japan. Genotype 4 tends to be restricted to India and East Asia. However, genotype 1 viruses, and more recently genotype 4 viruses, are also found in patients in Europe, North America and elsewhere after travelling to endemic areas and represent imported cases. Epidemics and sporadic cases of hepatitis E occur in areas of endemicity (genotypes 1, 2 and 4); more isolated clinical cases are diagnosed among a sizeable group of mostly asymptomatic seropositive residents in developed countries (mainly genotype 3). Chronic infection, almost exclusively with genotype 3 HEV, occurs in immunocompromised transplant patients and those with HIV infection. Given the genetic diversity of HEV and the importance of the epidemic strains in terms of the global disease burden of

hepatitis E, the PEI proposed the preparation of a genotype panel for HEV RNA; this proposal was endorsed by the WHO ECBS in October 2011 (WHO/BS/2011.2179).
In the current collaborative study, a candidate WHO HEV genotype panel, representing all four genotypes and a range of sub-genotypes, has been evaluated alongside the WHO IS (code number 6329/10). A further aim of the study was to calibrate, in IU/ml, a candidate BRP for HEV RNA for NAT testing on behalf of the Biological Standardisation Programme (BSP) of the EDQM, Council of Europe and the EU Commission. The calibration of the candidate BRP coincides with the introduction of NAT testing for HEV RNA for solvent/detergent (S/D)-treated plasma in the European Pharmacopoeia (Ph. Eur. monograph 1646).

Preparation of bulk materials

The HEV strains selected for the preparation of the candidate WHO panel and the candidate BRP are shown in Table 1. The strains used to prepare the panel were derived from a mixture of plasma and stool samples positive for HEV RNA. The samples were derived from blood/plasma donations or clinical samples – either plasma or stool samples. Stool samples were included to supplement the genotype coverage; they were of generally higher viral load than the HEV positive plasma samples. All samples were tested at PEI by NAT to determine viral loads and sequencing was performed in HEV ORF1 and ORF2 regions to confirm the (sub-)genotype; sub-genotyping was performed according to Lu *et al*., 2006. In the case of the HEV RNA-positive plasma sample stocks, the anti-HEV status (IgM and IgG) was determined by enzyme immunoassays (Wantai, Beijing, China). Phylogenetic analysis of the panel strains, the BRP and the WHO IS are shown in Figure 1.
For the preparation of the candidate materials, the HEV RNA-positive samples were diluted using pooled citrated human plasma which tested negative for HBV, HCV and HIV-1/2 using the Cobas TaqScreen MPX test (Roche Molecular Systems Inc.) and was negative for HEV RNA (testing described below) and anti-HEV IgM and anti-HEV IgG by enzyme immunoassays (Wantai, Beijing, China). All HEV RNA-positive plasma samples used in the preparation of the candidate panel and BRP were tested and found negative for the presence of HBV, HCV and HIV-1/2 using the Cobas TaqScreen MPX test. In the case of 8567/13 and 8569/13, the samples were diluted 1:500 prior to testing.

Stool samples were diluted in Dulbecco's Modified Eagle's Medium, filtered through 1 µM and then 0.2 µM filters and mixed with an equal volume of negative plasma (described above) and frozen at -80°C prior to processing. The filtered samples were tested to ensure the absence of aerobic and anaerobic microorganisms. The stool samples were diluted in plasma, and in order to stabilize the respective HEV strains, trehalose and magnesium chloride were added at final concentrations of 5% and 150 mM, respectively. The formulation of the final matrix for the stool samples was evaluated using a range of PCR and transcription-based NAT assays in order to determine if there was any interference with the downstream amplification/detection of the HEV RNA; no evidence of inhibition was observed (data not shown).

The candidate panel samples were lyophilized in batches between the 27th August 2013 and 25th of August 2014. Vials of the candidate BRP were lyophilized in December 2013. 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. The conditions for the lyophilization of the samples were as previously described (Baylis *et al*., 2011a). After completion of the freeze-

drying procedure, the vacuum was broken by the introduction of nitrogen gas and the vials sealed. The vials were further secured with screw caps prior to storage at -20°C.

The number of vials lyophilized per sample, coefficients of variation (% CV) of the respective fill volumes and residual moisture content are shown in Table 2. Measurements for the % CV of the filling process were made for 1% of the vials prepared for the panel. In the case of the BRP, measurements were made from 30 vials across different trays/shelves. For analysis of residual moisture, vials filled with 0.5 ml volumes of plasma diluent were distributed throughout the freeze-drier. Residual moisture was determined by Karl Fischer analysis for 8 vials per lyophilisation run for the panel samples and 10 in the case of the BRP.

Testing of the samples post-lyophilization revealed that there was an approximate drop in HEV RNA titre of ~0.0-0.3 $\log_{10}$ IU/ml for the different panel members when compared to aliquots of the respective bulk preparations which were stored at -80°C (data not shown). The titre of HEV RNA in the liquid/frozen bulk for the BRP was ~4.8 $\log_{10}$ IU/ml; after freeze-drying the mean titre was ~4.51 $\log_{10}$ IU/ml, indicating some loss in titre during processing. Because of the candidate BRP was to be assigned a unitage with respect to the HEV RNA content, homogeneity of the filling/freeze-drying was assessed using HEV RT-PCR. The crossing threshold or cycle threshold (C_T) values were determined for 25 vials of the BRP; the mean C_T value was 28.9, with a CV of 0.79%, indicating that the filling was of acceptable homogeneity.

Vials of the candidate WHO genotype panel 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. Vials of the candidate BRP are held at the EDQM in Strasbourg, France under the same conditions.

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

Collaborative Study

The collaborative participants are listed in Appendix 1; 24 laboratories from 14 different countries agreed to participate in the study. 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.

All collaborative study materials were shipped to participating laboratories on dry ice and participants requested to store the materials at or below -20°C until use. Participants received samples representing different HEV genotypes and sub-genotypes (including one strain prepared in two different types of matrix), the candidate BRP together with the WHO IS (Table 1). The approximate titres of the lyophilized materials were provided for guidance (Table 3). Sufficient materials were provided for three separate assay runs. Additional vials were provided for laboratories using larger extraction volumes.

Participants were asked to test the panel of samples using their routine assay for HEV RNA using fresh vials of each sample for each of the three assay runs. For each assay run, a new set of samples were reconstituted in 0.5 ml deionised, nuclease-free, molecular biology grade water and left for a minimum of 20 minutes with occasional agitation before use.

For quantitative tests, it was proposed that participants use the WHO IS 6329/10 to create a standard curve (testing the IS neat and by three ten-fold dilutions i.e. 250,000 IU/ml (neat) to 250 IU/ml) and samples reported directly in IU/ml. Participants were requested to test the candidate WHO panel samples without dilution. In the case of the candidate BRP, it was recommended to test this neat, and in three further half-$\log_{10}$ dilutions.

For laboratories performing qualitative assays, participants were requested to assay each sample alongside the IS, 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. It was suggested to dilute the materials as follows in the first assay run:

→ The WHO IS (6329/10) and 8577/13s: by five ten-fold dilutions (10^{-2} to 10^{-6}). The WHO IS has a concentration of 250,000 IU/ml.

→ The candidate BRP and 8570/13 should be tested by five ten-fold dilutions (10^{-1} to 10^{-5}).

→ All other samples tested neat and by four ten-fold dilutions (neat to 10^{-4}), with the exception of sample 8567/13 which should be tested neat and by three ten-fold dilutions (neat to 10^{-3}).

If, in the second assay, all dilutions were positive, or all negative, then the dilution series were to be adjusted accordingly for the final assay run. Participants were asked to note if replicate extractions and replicate amplification/detection steps were performed. Results were reported as either positive i.e. HEV RNA detected or negative.

For the preparation of dilutions, participants were requested to use their usual diluent representing the matrix of the normal test specimens e.g. HEV negative plasma. Participants reported diluting the materials using either water (Laboratory 3) or plasma (all other participants). Electronic data sheets and a method reporting form were provided so that all relevant information (e.g. C_T values for the respective dilutions where real time PCR methods were used or signal to cut-off (S/Co) values - e.g. for transcription-mediated assays) could be reported.

Statistical Methods

Quantitative assays

Potency estimates determined for each sample relative to the WHO IS (measured in $\log_{10}$ IU/ml) based on quantitative data were derived by means of a mixed linear model with random factors *laboratory* and *assay run*. Mean potency for the candidate BRP (relative to the WHO IS) was estimated using a mixed linear model with random factors *laboratory*, *assay run* and *test dilution*.

Qualitative assays

For qualitative data analysis, results from all assays were pooled to give the of number positives out of the total number tested at each dilution. If it is assumed that a single 'detectable unit' will give a positive result, and that the probability of a positive result follows a Poisson distribution, the EC63 (the dilution at which 63% of the samples are expected to be positive) was chosen as the end-point.

For each dilution series, this end-point was estimated by means of a probit analysis. Within the same evaluation, relative potencies were also estimated.
For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to WHO IS; quantitative data for BRP only) using a parallel line model for each laboratory and assay run, as well as combined for all evaluable (i.e. valid) assay runs.

Qualitative sample cut-off values from lab 5 and 7 were evaluated by means of a sigmoid dose-response model. Samples with less than 3 dose values and/ or non-linear or non-parallel behavior were excluded from the analysis.

Parallel line and sigmoid evaluation model as well as the combination of assays were performed according to methods as described in chapter 5.3, "Statistical analysis of results of biological assays and tests", of the Ph. Eur. The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows, and CombiStats, version 5.0, EDQM, Council of Europe.

Data received

Data were received from a total of 23 of the 24 participating laboratories. In total, 32 sets of data were returned; 17 from quantitative assays and 15 from qualitative assays. Some laboratories reported results for more than one type of assay. The types of methods used by the participants are listed in Table 4. The majority of assays designs are based on HEV ORF2/3.
Laboratory 3 tested the candidate BRP once at a single dilution, and once by half $\log_{10}$ dilutions. The candidate WHO panel was tested singly in the first assay run, in duplicate in the second assay run; no third assay run was performed.

Laboratory 6A tested the candidate BRP at a single (ten-fold) dilution in assay run 1; other assays were performed according to the protocol.

Laboratory 6B tested the candidate BRP at a single (ten-fold) dilution in all three assay runs.
Laboratory 8, sample 8571/13 gave an invalid test result in the second assay run.
Laboratory 23A only reported data for a single run; not all samples were detected.

Results

The different HEV genotypes and sub-genotypes were detected by all participants, with a single exception – Laboratory 12 was unable to detect sample 8567/13 (HEV genotype 1a) in any of the three assay runs. In some cases, differences in the efficiency of detection were observed for some of the candidate panel members. Some of the other samples were inconsistently detected by Laboratory 12. Laboratory 6 reported inhibition of some of the undiluted samples, including some of the stool-derived materials; however this was not consistent across assay runs. Laboratory 6 used the Nuclisens easyMag (Biomerieux) extraction platform and other laboratories using this system did not report inhibition of any of the samples and it is possible that inhibition may have been due to the high proportion of extracted nucleic acid used in the amplification/detection reaction.

Quantitative Assay Results

The individual laboratory mean estimates ($\log_{10}$ IU/ml) for the candidate panel samples as well as the candidate BRP assayed directly against the WHO IS 6329/10 are shown in Tables 5a and 5b. The relative variation of the individual laboratory estimates is illustrated by the box-and-whisker plots in Figure 2. Greater variation was observed with the genotypes 1, 2 and 4, whereas better agreement was observed for the genotype 3 strains, including the candidate BRP – several genotype 3 strains had been evaluated in previous studies (Baylis *et al*., 2011b; Baylis *et al*., 2013), these include the 3b strain that was established as the first HEV RNA National Standard in Japan as well as the RKI 3f strain which is the basis for the candidate BRP. In the current study, the HEV 3b strain included in the panel (8570/13) has been formulated at a different concentration compared to the previously established Japanese National Standard.

The laboratory mean absolute estimates of IU/ml ($\log_{10}$) are shown in histogram form in Figure 3. For the quantitative assay results, each white box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. From Figure 3, it can be seen that there is, in general, good agreement between the laboratories performing quantitative assays. For the laboratories performing quantitative assays for the calibration of the BRP, the results across the range of dilutions tested were in the main parallel; non-parallel data was excluded. Table 6 shows the laboratory mean estimates ($\log_{10}$ IU/ml) for quantitative assays (based on C_T values) for the candidate BRP relative to WHO IS 6329/10. In the histograms (Figure 3 – candidate BRP), results based on analysis of C_T values from quantitative methods are shown in the light grey boxes. The overall means from all laboratories for the quantitative assays are shown in Table 7.

Qualitative Assay Results

The individual laboratory relative potency estimates ($\log_{10}$ IU/ml), for the candidate panel samples as well as the candidate BRP, assayed in parallel against the WHO IS 6329/10 are shown in Tables 8a and 8b; the data are based upon end-point dilution analysis. The overall means from all laboratories for the qualitative end-point dilution assays are shown in Table 9. In addition, mean estimates were also evaluated by analysis of C_T and S/Co values for the qualitative assays (Tables 10a and 10b). The relative variation of the individual laboratory estimates for the qualitative assays is illustrated by the box-and-whisker plots in Figure 4. The overall means from all laboratories for the analysis of C_T and S/Co values for the qualitative assays are shown in Table 11.

The qualitative assays are much more variable than the quantitative assays, reflecting the different sensitivities of the different assays. This observation is not unexpected and is in keeping with previous studies. The potencies of the candidate panel samples and the candidate BRP for the qualitative assay results are shown in Figure 3, each dark grey box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number – these data represent the end-point dilution analysis; values determined by analysis of the C_T values are shown in the black boxes. In general, the results from the qualitative assay are in good agreement with those of the quantitative assays, although they are more variable, as expected (Figure 3).

Determination of Overall Laboratory Means – Combined Qualitative and Quantitative Results

The overall mean values for the candidate panel samples and the candidate BRP are shown in Table 12. The respective overall means are a combination of the quantitative data (IU/ml), the qualitative data (based on end-point dilution); potencies determined by analysis of reported C_T values or S/Co

values from the qualitative assays for the candidate panel samples and BRP relative to the WHO IS; and, for the candidate BRP potencies determined by analysis of reported C_T values relative to the WHO IS for the quantitative assays.
In general, the overall means are in line with the expected results (Table 3). There will be no unitage assigned to the candidate panel samples; nevertheless, the data will be informative for users of the panel in the future. The candidate BRP is assigned a potency of 40,850 IU/ml (4.6112 $\log_{10}$ IU/ml).

Stability Studies

Stability of the candidate panel samples is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate panel samples 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 were incubated at +4°C, +20°C, +37°C and +45°C for up to 6 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 as previously described (Baylis *et al*., 2013).

Results of Stability Studies

Preliminary studies demonstrated that in general the plasma-derived viruses showed good stability comparable to that of the WHO IS. However, it was noticed that dilution of the genotype 2 HEV stool-derived virus strain into pooled plasma and then lyophilized resulted in a very unstable preparation and after overnight incubation at +37°C there was a 2-3 $\log_{10}$ drop in titres and after 1 week of storage at +37°C or higher, HEV RNA was no longer detectable. No loss of signal was observed under normal storage conditions i.e. -20°C or below (data not shown).

Despite containing higher levels of residual moisture, the plasma matrix prepared with the stabilizing agents, gave not only improved levels of stability of HEV RNA for all three HEV stool samples, but also resulted in improved solubility of the cake after incubation at +45°C for 6 months compared to the diluted HEV-positive plasma samples diluted in the same pooled plasma matrix.
In line with the study to establish the WHO IS (Baylis *et al*., 2011a), it is expected that the panel of samples will show good long term stability. After 3 years and 1 month storage at -20°C, +4°C, +20°C, the titre of the WHO IS was 5.44, 5.50 and 5.13 $\log_{10}$ IU/ml, respectively; the assigned concentration of the WHO IS is 5.40 $\log_{10}$. The samples were compared to baseline samples of the IS stored at -80°C. In the case of the candidate BRP, there was a 0.3 $\log_{10}$ IU/ml drop in titre after storage at 20°C for 6 months, in the case of the individual panel members there was a drop of between 0.0-0.3 $\log_{10}$ IU/ml (Tables 11a and 11b); it is difficult to compare stability at higher temperatures with the WHO IS because of problems encountered in reconstituting vials of the IS after prolonged incubation similar to other IS preparations formulated in plasma.

Conclusions

In this study, a wide range of quantitative and qualitative assay formats were used to evaluate the candidate HEV RNA genotype panel of samples as well as the candidate BRP in parallel with the WHO IS. Approximately half of the assays were developed in-house with the rest being

commercially available, this is in contrast to the initial study to evaluate laboratory performance where a single commercial assay was included (Baylis et al., 2011b) and the study to establish the WHO IS where all assays were developed in-house (Baylis et al., 2011a; Baylis et al., 2013). With a single exception, all participants were able to detect the different HEV genotypes and sub-genotypes included in the panel as well as the candidate BRP. One in-house assay, used by a single laboratory, failed to detect 8567/13, genotype 1a HEV representing the sample with the lowest titre in the panel, and may be due to a lack of assay sensitivity or possibly due to primer design. There were noticeable differences in the potencies for some of the samples – particularly genotype 2 for which there are very few reported sequences, with only a single full length sequence determined. In contrast the potencies reported for the genotype 3 strains were less variable and reflect the availability of a large number of sequences and evaluation of several of these strains in previous studies (Baylis *et al*., 2011b; Baylis *et al*., 2013).

The sample 8577/13 (HEV genotype 2a) was included as a matrix control. Because of the instability of 8577/13 at ambient temperature, the formulation of the sample was revised to include stabilizers (8577/13s). There was no evidence of inhibition of this revised matrix, either in testing prior to the collaborative study or during the collaborative study itself. In the final panel composition, sample 8577/13 will be excluded due to its instability at elevated temperatures.

In accordance with WHO policy, no unitage will be assigned to the panel members, although the potencies and range, determined in the collaborative study will be included in the Instructions for Use (Appendix 3) in order to inform users of the panel performance. It is anticipated that the panel will be used by laboratories to ensure that the detection of different HEV genotypes and sub-genotypes is adequate.

The other aim of the study was to establish a potency of the candidate BRP in IU/ml, combining all the data (quantitative and qualitative (end-point dilution, analysis of C_T values as well as S/Co values) the overall mean potency was 40,850 (4.6112 $\log_{10}$ IU/ml). The characterization of the candidate BRP will be reviewed separately by the EDQM BSP Steering Committee.

Real-time stability studies have indicated that the panel of HEV samples are very stable under normal conditions of storage, i.e., at -20ºC or below for 6 months and are therefore suitable for long term use. The assessment of the stability of the HEV genotype panel is on-going. Similarly, the stability analysis of the BRP has not indicated loss of titre after storage at -20ºC or below. Each vial of the panel contains 0.5 ml of lyophilized material. The draft "Instructions for Use" for the panel, include details for storage and reconstitution of the individual panel members (Appendix 3); each sample vial contains the dried residue of 0.5 ml of HEV-containing plasma and should be reconstituted in 0.5 ml of water.

Recommendations

Based upon the results of the collaborative study, it is proposed that the panel should be established as the 1st International Reference Panel for Hepatitis E Virus RNA Genotypes (code number 8578/13). The custodian laboratory is the Paul-Ehrlich-Institut. The panel is not intended to replace the WHO IS for HEV RNA. No unitage is assigned to the individual panel members, however, potencies and the minimum and maximum ranges for each panel member are to be provided, for information only.

Responses from participants

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

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Table 1 Details of HEV strains evaluated in the study

Code	Genotype	Origin	Sample Type	Anti-HEV IgM/IgG
8567/13	1a	India	Plasma	n.d.
8568/13s	1a	India	Stool	n.a.
8569/13	1e	Sudan	Plasma	+/-
8570/13*	3b	Japan	Plasma	-/-
8571/13*	3c	Sweden	Plasma	-/-
8572/13*	3e	Germany	Plasma	-/-
8573/13*	3f	Sweden	Plasma	-/-
8574/13s	3 (rabbit)	France	Stool	n.a.
8575/13*	4c	Japan	Plasma	-/-
8576/13*	4g	Japan	Plasma	-/-
8577/13	2	Mexico	Stool	n.a.
8577/13s	2	Mexico	Stool	n.a.
Candidate BRP*	3f	Germany	Plasma	+/+

n.d. not determined
n.a. not applicable
*Samples obtained from blood/plasma donors

Table 2 Characterization of the filled and lyophilized materials

Code	Number of vials filled	% CV	Residual moisture %
8567/13	1013	1.1	1.1
8568/13s	1048	1.1	2.8
8569/13	1020	1.1	1.1
8570/13	1012	1.1	1.1
8571/13	1025	1.1	1.1
8572/13	995	1.0	1.6
8573/13	1025	1.0	1.6
8574/13s	1050	1.1	2.8
8575/13	1020	1.0	1.6
8576/13	1023	1.0	1.6
8577/13	1059	0.7	0.6
8577/13s	1050	1.1	2.8
Candidate BRP	6061	1.0	0.6

Table 3 Collaborative study materials – approximate expected concentrations

Code number/ Name of preparation	Nominal concentration (log_{10} IU/ml)	Status
6329/10*	5.4	WHO IS
BRP	~4.5	Candidate BRP
8567/13	~2.5-3.0	Candidate WHO panel and control sample
8568/13s	~4.0	
8569/13	~3.0-3.5	
8570/13	~4.0	
8571/13	~3.0-3.5	
8572/13	~3.5	
8573/13	~3.5	
8574/13s	~4.5-5.0	
8575/13	~4.0	
8576/13	~3.5	
8577/13s	~5.0-5.5	
8577/13	~3.5-4.0	

*250,000 IU/ml upon reconstitution in 0.5 ml water

Table 4 Assay protocols used by participants

Laboratory code	Assay type*	Extraction method	NAT method	Reference
1	Quant.	High Pure Viral Nucleic Acid Kit Large Volume (Roche)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
2	Quant.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
3A/3B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (HepatitisE@ceeramTools™)	
4	Quant.	EZ1 Virus Mini Kit v2.0 (Qiagen)	Real-time RT-PCR (TaqMan)	Wenzel *et al*., 2011
5	Qual.	Automated proprietary magnetic target capture method – Procleix Panther System	Transcription mediated amplification and hybridization protection assay (Procleix HEV Assay, Hologic Inc.)	
6A/B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
6C/D	Quant./Qual.	chemagic Viral DNA/RNA Kit (PerkinElmer)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
6E	Qual.	NucliSENS® easyMag® (bioMérieux)	*ampli*Cube HEV 2.0 (Mikrogen GmbH)	
6F	Qual.	chemagic Viral DNA/RNA Kit (PerkinElmer)	*ampli*Cube HEV 2.0 (Mikrogen GmbH)	
7	Qual.	Automated proprietary magnetic target capture method – Procleix Panther System	Transcription mediated amplification and hybridization protection assay (Procleix HEV Assay; Hologic Inc.)	
8	Qual.	QIAamp MinElute Virus Spin Kit/QIAcube (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
9	Quant.	QIAsymphony DSP Virus/Pathogen Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	
10	Quant.	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Matsubayashi *et al*., 2008
11	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
12	Quant.	SMI-TEST EX-R&D (Medical Biological Laboratories Co., Ltd.)	Real-time RT-PCR (TaqMan)	Tanaka *et al*. 2007
13	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (TaqMan)	
14	Qual.	Roche cobas® 6800/8800	Real-time RT-PCR (cobas® HEV, Roche Molecular Systems)	
15	Quant.	QIAamp MinElute Virus Spin Kit/QIAcube (Qiagen)	Real-time RT-PCR (TaqMan)	

16	Quant.	MagNA Pure 96 Viral NA Large Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Abravanel *et al*., 2012
17	Quant.	MagNA Pure LC (Roche)	Real-time RT-PCR (SYBR Green)	Jothikumar *et al*. 2006
18	Quant.	MagNA Pure 96 Viral NA Large Volume Kit (Roche)	Real-time RT-PCR (TaqMan)	Jothikumar *et al*. 2006; Garson et al., 2012
19A/B	Quant./Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (TaqMan)	Zhao *et al*., 2007
20	Qual.	NucliSENS® easyMag® (bioMérieux)	Real-time RT-PCR (TaqMan)	
21A/B	Quant./Qual.	GenMag Viral DNA/RNA Kit (GenMagBio)	Real-time RT-PCR (TaqMan)	Jothikumar *et al*. 2006
22	Qual.	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (Diagnostic kit hepatitis E virus RNA, Beijing Kinghawk Pharmaceutical Co., Ltd.)	Zhao *et al*., 2007
23A	Quant.	High Pure Viral Nucleic Acid Kit (Roche)	Real-time RT-PCR (RealStar® HEV RT-PCR Kit 1.0, altona Diagnostics)	
23C	Quant.	High Pure Viral Nucleic Acid Kit (Roche)	Real-time RT-PCR (TaqMan)	Jothikumar *et al*. 2006

*Assay type:Qualitative (Qual.) and quantitative (Quant.) assays

Table 5a Laboratory mean estimates (log_{10} IU/ml) for quantitative assays relative to WHO IS 6329/10; mean for different assay runs and dilutions for samples 8567/13, 8568/13s, 8569/13, 8570/13, 8571/13, 8572/13

Laboratory	Sample					
	8567/13	8568/13s	8569/13	8570/13	8571/13	8572/13
1	2.67	4.74	3.21	4.32	3.26	3.60
2	1.97	4.27	3.19	4.12	3.29	3.42
3A	1.96	4.22	3.00	4.18	3.40	3.50
4	2.54	4.10	2.93	4.11	3.52	3.12
6A	2.68	4.66	3.50	4.38	3.63	3.54
6C	2.60	4.25	3.07	4.21	3.44	3.60
9	2.23	3.67	2.82	4.21	3.80	3.40
10	1.82	3.62	2.64	3.99	3.49	3.16
12		4.29	3.10	4.39	3.60	3.62
15	2.71	4.40	3.36	4.33	3.32	3.54
16	2.79	4.57	3.57	4.17	3.47	3.55
17	2.51	4.04	2.61	3.95	3.00	3.30
18	2.69	4.23	3.02	4.16	3.49	3.38
19A	2.72	4.73	3.43	4.21	3.22	3.77
21A	3.87	5.37	4.59	4.28	3.34	3.77
23A						
23C	3.91	4.81	3.97	4.22	3.27	3.81

It was not possible to calculate mean values for some of the samples for Laboratories12 and 23A

Table 5b Laboratory mean estimates (log_{10} IU/ml) for quantitative assays relative to WHO IS 6329/10; mean for different assay runs and dilutions for samples 8573/13, 8574/13s, 8575/13, 8576/13, 8577/13s and the candidate BRP (cBRP)

Laboratory	Sample						
	8573/13	8574/13s	8575/13	8576/13	8577/13s	8577/13	cBRP
1	3.96	5.29	4.07	3.61	5.58	3.93	4.51
2	3.63	4.67	3.79	3.43	5.22	3.54	4.44
3A	3.50	4.96	3.71	3.48	5.43	3.49	4.65
4	3.60	5.15	3.93	3.47	5.23	3.26	4.68
6A	3.72	4.97	4.17	3.86	5.57	3.91	4.85
6C	3.77	4.72	4.00	3.75	5.43	3.71	4.58
9	3.73	4.49	3.88	3.68	4.60	3.46	5.03
10	3.55	4.74	3.68	3.37	4.58	3.00	4.49
12	3.84	5.26	4.09	3.81	5.31	3.40	4.85
15	3.72	5.09	4.01	3.65	5.57	4.04	4.46
16	3.80	4.96	4.03	3.68	5.69	3.57	4.61
17	3.81	6.10	3.93	4.04	5.51	2.66	4.46
18	3.61	5.05	3.98	3.57	5.31	3.36	4.53
19A	4.14	4.93	4.34	3.72	5.78	3.36	4.49
21A	5.03	4.60	5.27	5.02	5.83	5.23	4.71
23A	2.40	4.35	3.03	4.18	3.27	3.59	3.47
23C	4.48	4.71	4.60	4.23	5.81	3.27	4.33

*Candidate BRP potency estimates, accounted for dilution effect

Table 6 Laboratory mean estimates (log_{10} IU/ml) for quantitative assays (based on C_T values) for the candidate BRP relative to WHO IS 6329/10

Laboratory	Mean (log_{10} IU/ml)	95% Confidence Interval	
1	4.44	4.32	4.56
2	4.49	4.42	4.55
3A	4.70	4.63	4.76
4	4.58	4.52	4.63
6A	5.07	4.68	5.46
6C	4.55	4.43	4.67
9	5.03	4.96	5.10
10	4.40	4.38	4.42
12	4.81	4.75	4.87
15	4.49	4.42	4.55
16	4.61	4.58	4.64
17	4.38	4.25	4.51
18	4.57	4.46	4.69
19A	4.76	4.65	4.87
21A	4.68	4.58	4.78
23C	4.38	4.19	4.57

Data have been evaluated in IU/ml for quantitative assays (Table 5b).

Table 7 Overall mean estimates from quantitative assays (log_{10} IU/ml)

Sample	Estimate*	95% CI**		Median	Std	Min	Max	Range
#8567/13	2.64	2.22	3.06	2.64	0.60	1.44	4.10	2.66
#8568/13s	4.25	3.87	4.63	4.33	0.43	3.55	5.46	1.91
#8569/13	3.25	2.91	3.59	3.25	0.51	2.40	4.81	2.41
#8570/13	4.20	4.12	4.28	4.19	0.18	3.80	4.57	0.78
#8571/13	3.40	3.27	3.53	3.43	0.22	2.80	3.88	1.08
#8572/13	3.50	3.36	3.64	3.50	0.22	2.99	4.02	1.04
#8573/13	3.84	3.60	4.07	3.76	0.41	2.40	5.14	2.74
#8574/13s	4.98	4.73	5.24	4.98	0.38	4.35	6.88	2.52
#8575/13	4.07	3.82	4.31	3.97	0.38	3.03	5.37	2.34
#8576/13	3.77	3.49	4.04	3.70	0.38	3.13	5.05	1.92
#8577/13s	5.42	5.10	5.74	5.47	0.49	3.27	6.10	2.83
#8577/13	3.57	3.18	3.95	3.53	0.55	2.40	5.29	2.89
cBRP	4.61	4.44	4.78	4.53	0.33	3.47	5.03	1.55

*Estimated mean accounting for laboratory and number of performed assays
**95% Confidence interval
Std Standard deviation

Table 8a Laboratory relative potency estimates (log_{10} IU/ml) for qualitative assays (end-point) relative to WHO IS 6329/10 for samples 8567/13, 8568/13s, 8569/13, 8570/13, 8571/13, 8572/13

Laboratory	Sample					
	8567/13	8568/13s	8569/13	8570/13	8571/13	8572/13
3B	2.57	4.43	3.29	4.41	3.18	3.73
5	2.19	4.14	2.93	4.41	3.34	3.41
6B	2.53	4.18	2.96	4.17	3.35	3.96
6D	1.47	3.64	3.25	3.47	2.83	3.04
6E	1.8	3.36	2.58	3.36	2.36	3.21
6F	2.16	4.13	3.13	4.16	3.33	3.54
7	2.14	4.88	3.27	5.05	2.77	3.79
8	2.97	3.23	3.39	3.97	3.68	3.22
11	2	3.79	2.4	4.4	3.4	3.05
13	2.1	4.6	3.31	4.1	3.29	3.38
14	1.97	3.39	2.64	4.15	3.18	3.56
19B	2.67	4.4	3.47	4.47	3.4	3.9
20	3.19	4.17	2.98	4.79	4.17	3.19
21B	3.56	4.9	4.23	4.06	3.4	3.56
22	2.15	4.65	2.81	4.65	3.15	3.65

Table 8b Laboratory relative potency estimates (log_{10} IU/ml) for qualitative assays (end-point) relative to WHO IS 6329/10 for samples 8573/13, 8574/13s, 8575/13, 8576/13, 8577/13s and the candidate BRP (cBRP)

Laboratory	Sample						
	8573/13	8574/13s	8575/13	8576/13	8577/13s	8577/13	cBRP
3B	3.76	5.09	4.25	3.91	6.2	3.27	5.07
5	3.47	4.43	3.62	3.38	5.39	3.69	4.72
6B	2.96	4.79	3.76	3.96	4.64	3.17	4.4
6D	3.22	4.25	3.43	3.25	4.25	2.83	4.25
6E	2.58	4.37	3	2.37	4.17	2.37	3.8
6F	2.93	4.49	3.49	3.27	4.94	3.37	4.36
7	3.11	4.18	3.92	3.33	4.94	3.23	5.27
8	3.18	5.02	3.33	3.18	5.23	3.81	4.39
11	3.4	4.41	3.71	3.4	3.92	2.14	4.9
13	3.28	4.8	3.6	3.21	5.29	3.31	4.49
14	3.9	2.4	3.38	3.4	4.34	2	4.97
19B	3.9	5.4	4.06	3.9	5.64	3.32	4.71
20	4.17	5.17	4.19	3.79	5.40	3.79	5.17
21B	5.06	4.4	5.06	4.56	5.73	4.56	4.56
22	3.81	4.65	3.98	3.65	5.15	3.65	5.15

Table 9 Overall mean estimates from qualitative (end-point) assays (log_{10} IU/ml)

Sample	Estimate*	95% CI**		Median	Std	Min.	Max.	Range
#8567/13	2.37	2.06	2.67	2.16	0.55	1.47	3.56	2.10
#8568/13s	4.12	3.82	4.42	4.17	0.54	3.23	4.90	1.67
#8569/13	3.11	2.86	3.36	3.13	0.44	2.40	4.23	1.83
#8570/13	4.24	4.00	4.49	4.17	0.44	3.36	5.05	1.69
#8571/13	3.25	3.03	3.48	3.33	0.41	2.36	4.17	1.81
#8572/13	3.48	3.32	3.65	3.54	0.30	3.04	3.96	0.93
#8573/13	3.52	3.18	3.85	3.40	0.61	2.58	5.06	2.48
#8574/13s	4.52	4.14	4.91	4.49	0.69	2.40	5.40	3.00
#8575/13	3.79	3.51	4.06	3.71	0.49	3.00	5.06	2.07
#8576/13	3.50	3.23	3.78	3.40	0.49	2.37	4.56	2.19
#8577/13s	5.01	4.66	5.37	5.15	0.65	3.92	6.20	2.28
#8577/13	3.24	2.86	3.61	3.31	0.68	2.00	4.56	2.56
Candidate BRP	4.68	4.45	4.91	4.71	0.41	3.80	5.27	1.47

*Estimated mean accounting for laboratory and number of performed assays
**95% Confidence interval
Std Standard deviation

Table 10a Laboratory mean estimates (log_{10} IU/ml) for qualitative assays relative to WHO IS 6329/10 – data based upon analysis of C_T and S/Co values; mean for different assay runs and dilutions for samples 8567/13, 8568/13s, 8569/13, 8570/13, 8571/13, 8572/13

Laboratory	Sample					
	8567/13	8568/13s	8569/13	8570/13	8571/13	8572/13
3B	2.61	4.04	3.08	4.21	3.38	3.63
5		5.56	4.14	4.14	3.36	3.3
6B	2.77	4.51	3.64	4.34	3.6	3.55
6D	2.88	4.15	3.57	3.71	3.25	3.71
6E	2.26	3.62	2.48	4.01	3.44	3.44
6F	2.67	3.98	3.69	4.47	3.48	3.54
7	2.86	4.82	4.31	5.49	2.57	2.91
8	3.71	3.46	3.94	4.57	3.86	3.02
11	2.06	3.95	2.65	4.16	2.96	3.19
13		4.38	3.14	4.26	3.56	3.41
14	2.4	3.49	2.45	4.14	2.75	3.78
19B	3.03	4.7	3.65	4.31	3.54	3.59
20	1.93	3.73	2.46	4.13	2.92	3
21B	3.82	5.45	4.66	4.37	3.2	3.67
22	2.49	4.54	3.11	4.37	3.45	3.74

It was not possible to determine the end-point data from sample 8567/13 (Laboratories 5 and 13)

Table 10b Laboratory mean estimates (log_{10} IU/ml) for qualitative assays relative to WHO IS 6329/10 – data based upon analysis of C_T and S/Co values; mean for different assay runs and dilutions for samples 8573/13, 8574/13s, 8575/13, 8576/13, 8577/13s and the candidate BRP (cBRP)

Laboratory	Sample						
	8573/13	8574/13s	8575/13	8576/13	8577/13s	8577/13	cBRP
3B	3.56	4.99	3.9	3.34	5.63	3.72	4.72
5	4.21	4.8	4.14	3.83	6.69	4.69	4.71
6B	4.05	4.79	4.23	4.12	5.34	3.97	4.65
6D	3.51	4.36	4.08	3.88	5.37	3.48	4.67
6E	3.36	4.24	3.58	3.07	4.99	3.06	4.32
6F	3.47	4.61	3.86		5.11	3.77	4.37
7	3.42	3.91	3.71		4.5	5.9	4.98
8	3.7	4.75	4.2	3.74	5.66	3.79	4.19
11	3.6	4.63	3.66	3.29	3.25	1.02	4.49
13	3.41	4.79	3.43	3.29	5.14	3.78	4.69
14	3.73	2.8	4.07	3.72	4.77	3.09	5.26
19B	4.28	4.91	4.55	3.94	6.5		4.68
20	3.36	4.55	3.68	3.27	4.35	2.5	4.43
21B	5.14	4.56	5.34	5.06	6.11	5.14	4.65
22	3.94	4.86	4.02	3.62	5.33	3.8	5.09

It was not possible to determine the end-point data from sample 8576/13 (Laboratories 6F and 7) and for sample 8577/13 (Laboratory 19B). S/Co values - Laboratories 5 and 7, all other means based on C_T values

Table 11 Overall mean estimates from qualitative assays analysed by C_T and S/Co values (log_{10} IU/ml)

Sample	Estimate*	95% CI**		Median	Std	Min.	Max.	Range
#8567/13	2.78	2.51	3.04	2.66	0.59	1.93	3.91	1.99
#8568/13s	4.35	4.11	4.60	4.33	0.67	2.75	5.56	2.81
#8569/13	3.40	3.09	3.70	3.30	0.79	2.24	5.39	3.15
#8570/13	4.30	4.14	4.47	4.28	0.47	2.76	5.65	2.89
#8571/13	3.31	3.15	3.46	3.40	0.43	2.29	4.12	1.84
#8572/13	3.48	3.37	3.60	3.51	0.32	2.69	4.07	1.38
#8573/13	3.86	3.65	4.07	3.81	0.59	2.51	5.31	2.80
#8574/13s	4.51	4.28	4.73	4.68	0.66	2.73	5.54	2.81
#8575/13	4.09	3.88	4.29	4.04	0.56	3.12	5.51	2.39
#8576/13	3.74	3.53	3.95	3.69	0.55	2.86	5.07	2.22
#8577/13s	3.76	3.39	4.14	3.84	0.92	1.02	5.24	4.22
#8577/13	5.47	5.16	5.77	5.37	0.80	3.25	6.87	3.62
Candidate BRP	4.72	4.60	4.85	4.64	0.35	4.23	5.70	1.46

*Estimated mean accounting for laboratory and number of performed assays
**95% Confidence interval
Std Standard deviation

Table12 Overall laboratory means – combined qualitative and quantitative results (log_{10} IU/ml)

Sample	Mean*	95% CI**		Median	Std	Min.	Max.	Range
#8567/13	2.57	2.37	2.78	2.57	0.58	1.47	3.91	2.45
#8568/13s	4.28	4.09	4.48	4.24	0.55	3.23	5.56	2.34
#8569/13	3.25	3.05	3.45	3.16	0.56	2.40	4.66	2.26
#8570/13	4.24	4.13	4.36	4.21	0.34	3.36	5.49	2.13
#8571/13	3.32	3.22	3.43	3.36	0.33	2.36	4.17	1.81
#8572/13	3.47	3.39	3.56	3.54	0.26	2.91	3.96	1.06
#8573/13	3.69	3.50	3.88	3.67	0.54	2.40	5.14	2.74
#8574/13s	4.73	4.52	4.94	4.73	0.57	2.40	6.10	3.70
#8575/13	3.96	3.80	4.13	3.93	0.47	3.00	5.34	2.34
#8576/13	3.68	3.51	3.85	3.65	0.48	2.37	5.06	2.69
#8577/13s	5.22	4.98	5.47	5.33	0.71	3.25	6.69	3.45
#8577/13	3.48	3.22	3.74	3.48	0.75	1.02	5.23	4.21
Candidate BRP	4.61	4.50	4.72	4.61	0.31	3.47	5.27	1.79

*Estimated mean accounting for laboratory and number of performed assays
**95% Confidence Interval
Std Standard deviation

Table 13a Stability analysis of panel samples 8567/13, 8568/13s, 8569/13, 8570/13, 8571/13, 8572/13 – titres $\log_{10}$ IU/ml

Temp.	Time	8567/13	8568/13s	8569/13	8570/13	8571/13	8572/13
-20°C	3 mo.	2.74	4.19	3.39	4.06	3.22	3.48
	6 mo.	2.56	4.19	3.21	4.15	3.39	3.41
+4°C	3 mo.	2.67	4.12	3.20	4.16	3.26	3.31
	6 mo.	2.57	4.20	3.10	4.26	3.21	3.41
+20°C	3 mo.	2.04	3.89	2.90	3.96	2.88	3.24
	6 mo.	2.27	4.09	2.82	4.03	3.03	3.28
+37°C	3 mo.	2.00	3.82	2.16	3.29	2.43	2.18
	6 mo.	0.00	4.06	1.95	2.67	2.07	1.41
+45°C	3 mo.	-	3.82	-	2.68	-	-
	6 mo.	-	3.95	-	-	-	-

- Resuspension of samples was not possible

Table 13b Stability analysis of panel samples 8573/13, 8574/13s, 8575/13, 8576/13, 8577/13s and the candidate BRP (cBRP) – titres $\log_{10}$ IU/ml

Temp.	Time	8573/13	8574/13s	8575/13	8576/13	8577/13s	cBRP
-20°C	3 mo.	3.68	4.43	3.86	3.56	5.11	4.55
	6 mo.	3.60	4.49	3.71	3.56	5.32	4.52
+4°C	3 mo.	3.61	4.74	3.71	3.41	5.35	4.53
	6 mo.	3.65	4.62	3.92	3.46	5.33	4.43
+20°C	3 mo.	3.46	4.13	3.74	3.42	4.92	4.43
	6 mo.	3.56	4.55	3.66	3.34	5.02	4.19
+37°C	3 mo.	2.52	4.38	3.02	2.89	4.83	3.91
	6 mo.	1.66	4.81	2.76	2.01	5.06	3.50
+45°C	3 mo.	-	3.34	2.51	2.61	4.96	3.73
	6 mo.	-	4.67	-	-	4.98	2.74

- Resuspension of samples was not possible

Figure 1. Phylogenetic analysis

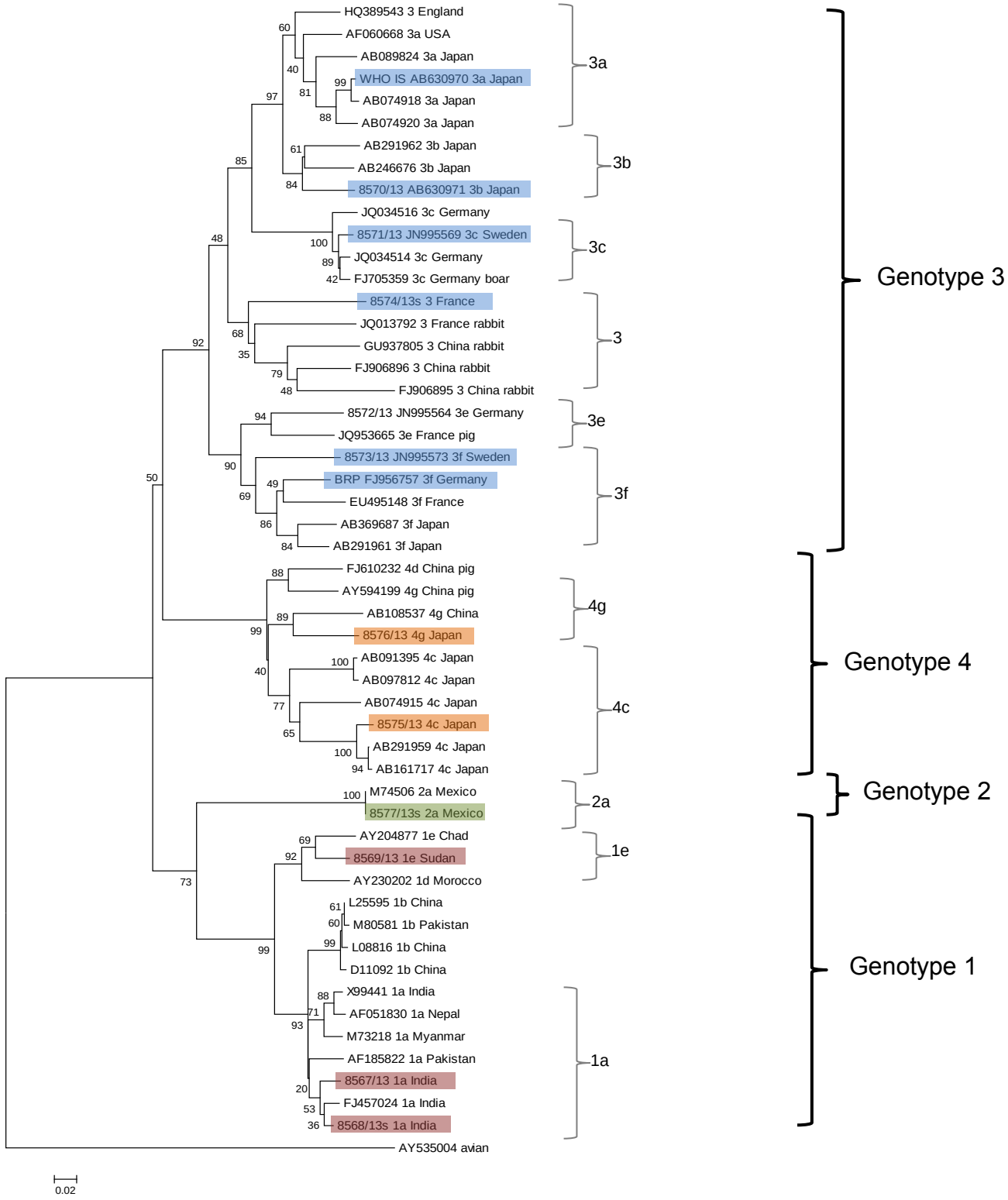
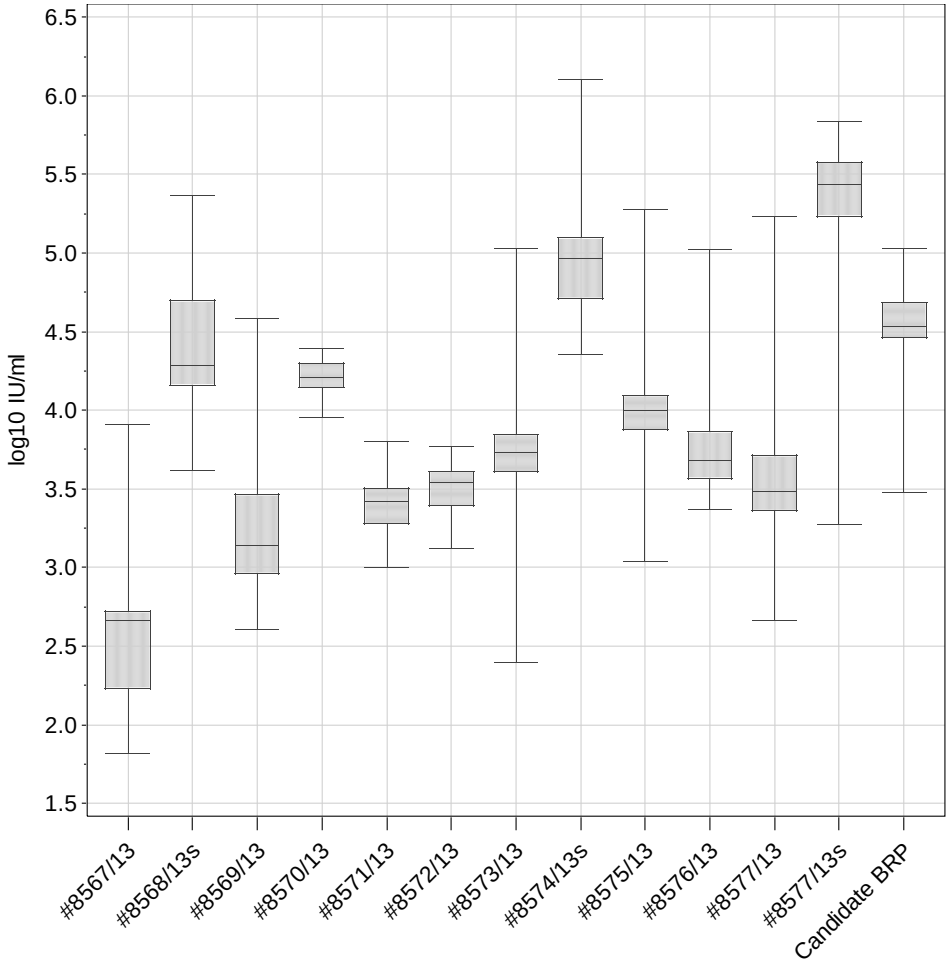


Figure 1. Phylogenetic analysis of HEV strains evaluated in the study. The code numbers of the candidate WHO panel are indicated; the sequences from the WHO IS and candidate BRP as also included (shown boxed). All strains represent human isolates unless otherwise indicated. The sequences are based upon analysis of part of the RdRp region of the HEV genome. Phylogeny was conducted using MEGA5 (www.megasoftware.net) using the neighbour-joining algorithm with bootstrap re-sampling (1000 replicates). Scale bar indicates genetic distance.
N.B. Full length sequence data is available for the WHO IS genotype 3a strain (AB630970), genotype 3b strain (AB630971) as well as the 3f strain representing the candidate BRP (FJ956757). Accession numbers are shown for partial sequences for ORF2 for some of the remaining panel members. Partial sequences from all panel members for the HEV RpRd RNA polymerase are shown in Appendix 2. Sequencing was performed according to Drexler *et al*., 2012. Sequencing in ORF2 confirmed the genotyping analysis as well as the sub-genotyping analysis for the genotype 3 strains (data not shown).

Figure 2 Quantitative results (log_{10} IU/ml) shown as as Box-and-Whisker-Plot



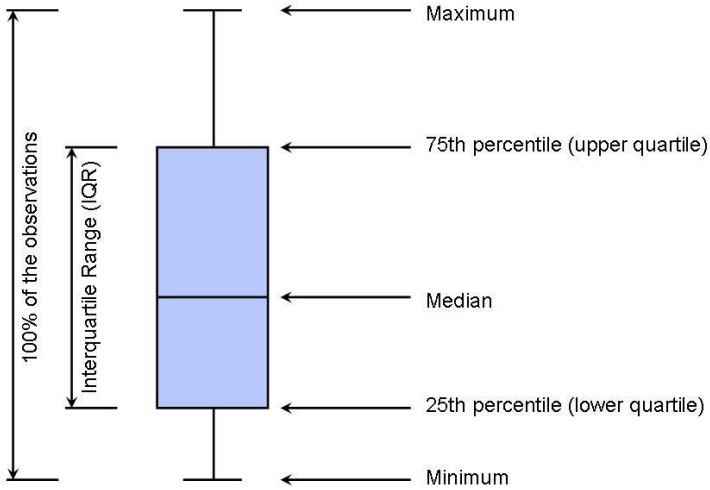
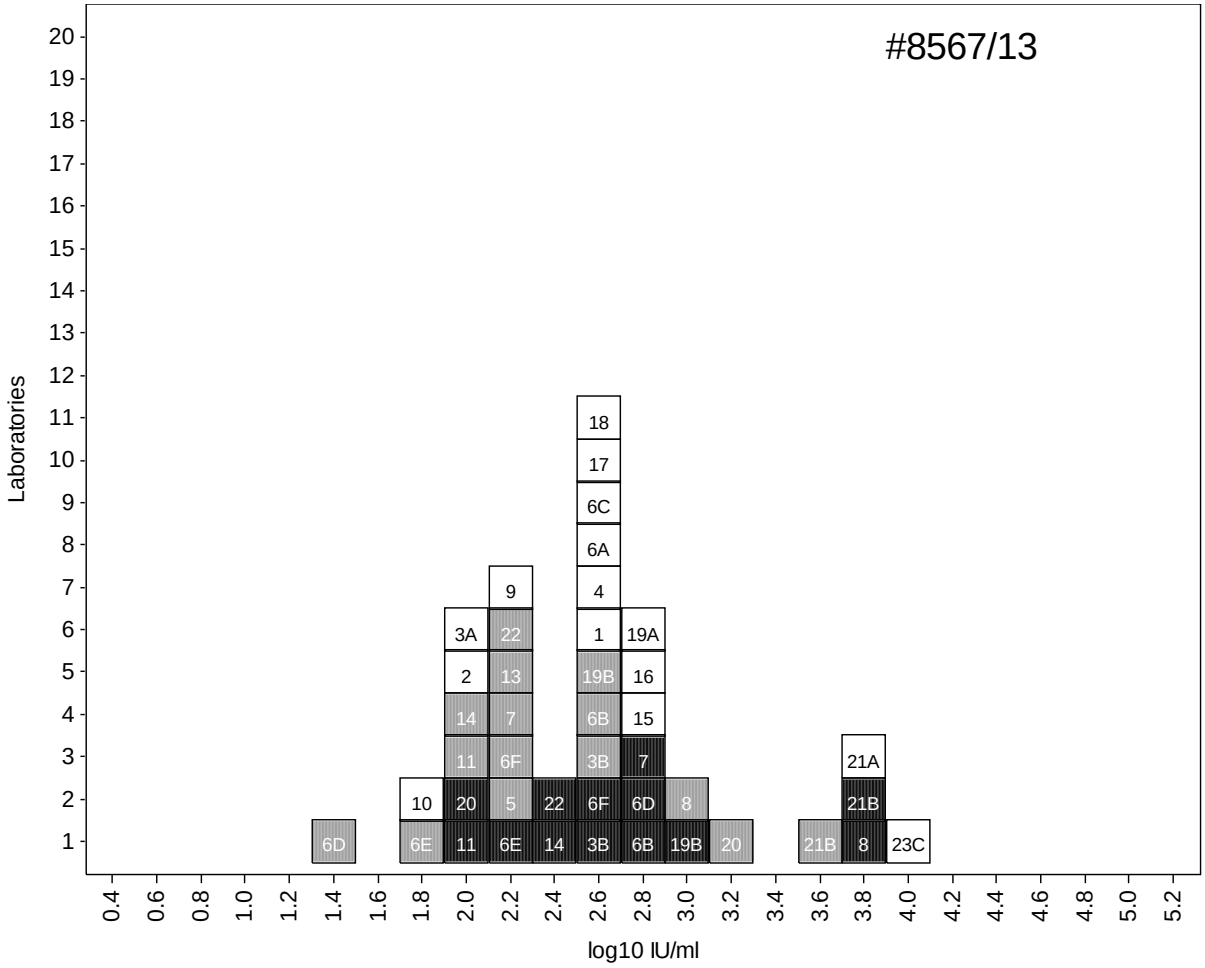
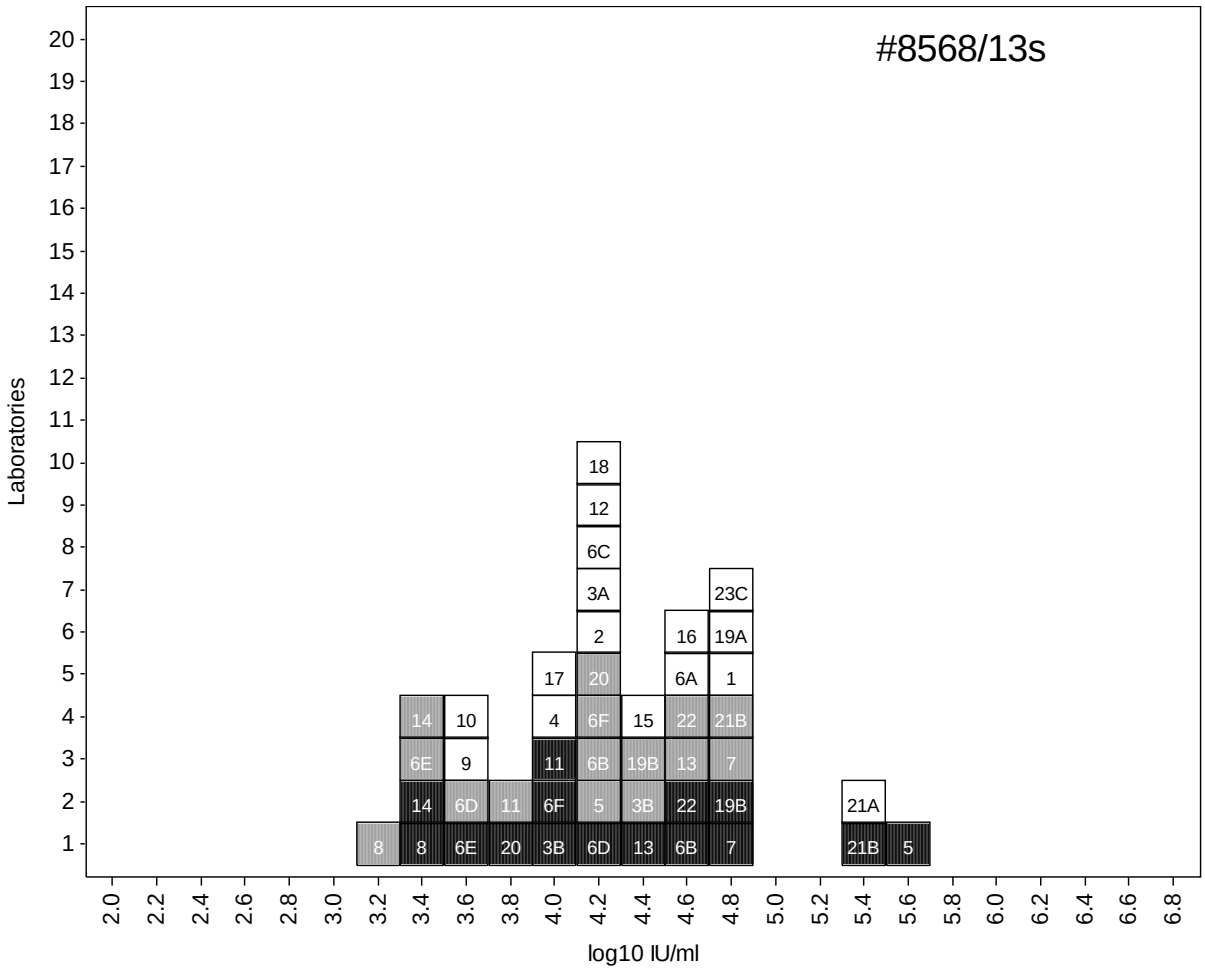
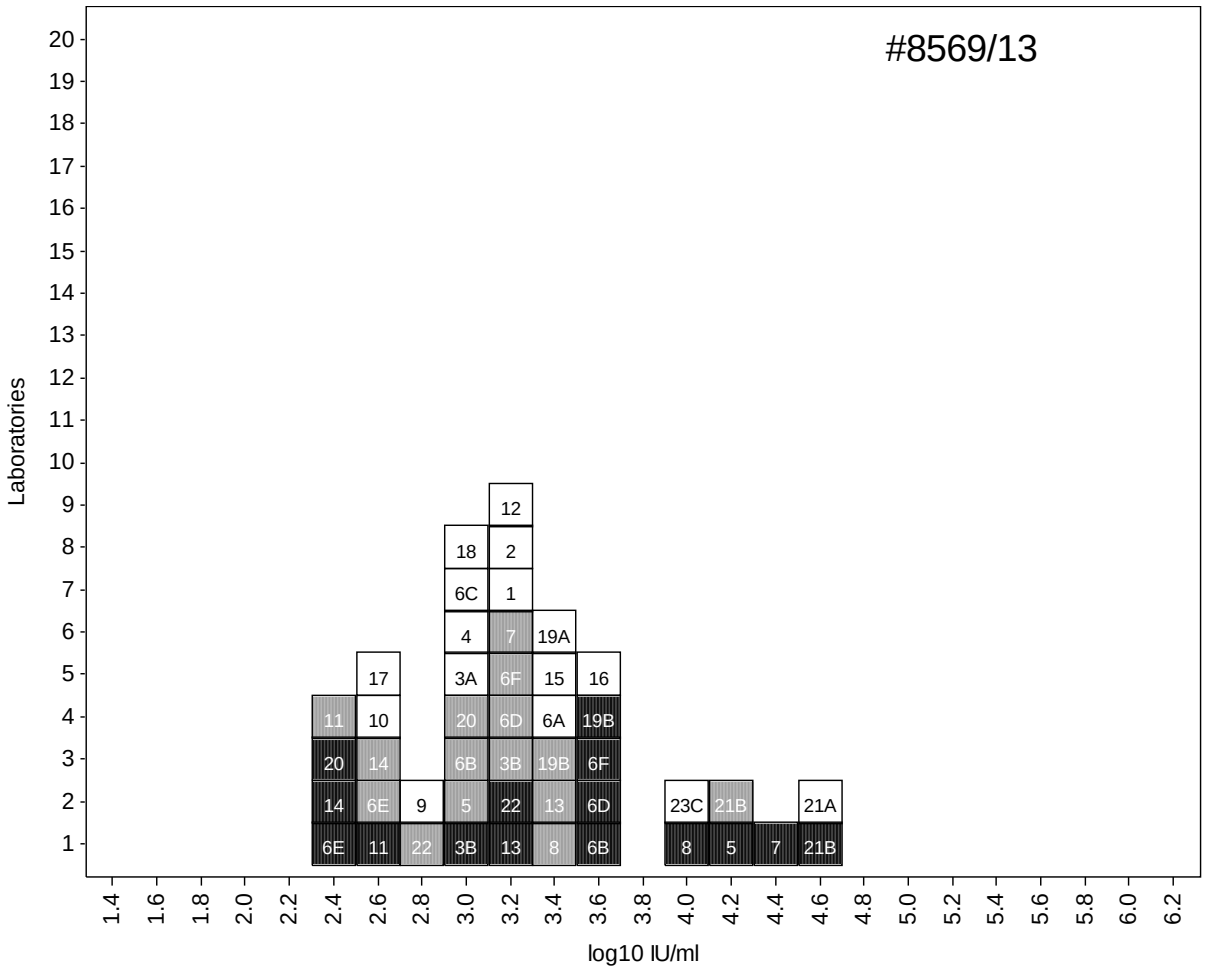


Figure 3 Potency Comparison for all samples by participant and assay type




#8568/13s
Laboratories
log10 IU/ml
8
14 8
6E 9 6E
14 6D 11 6F
14 10 4 6F 15 22 21B
6E 9 11 6B 19B 13 7
17 20 6A 1
18 2 16 19A
12 6C 3A 23C
21A
21B 5
20 3B 6D 13 6B 7
5 3B 22 19B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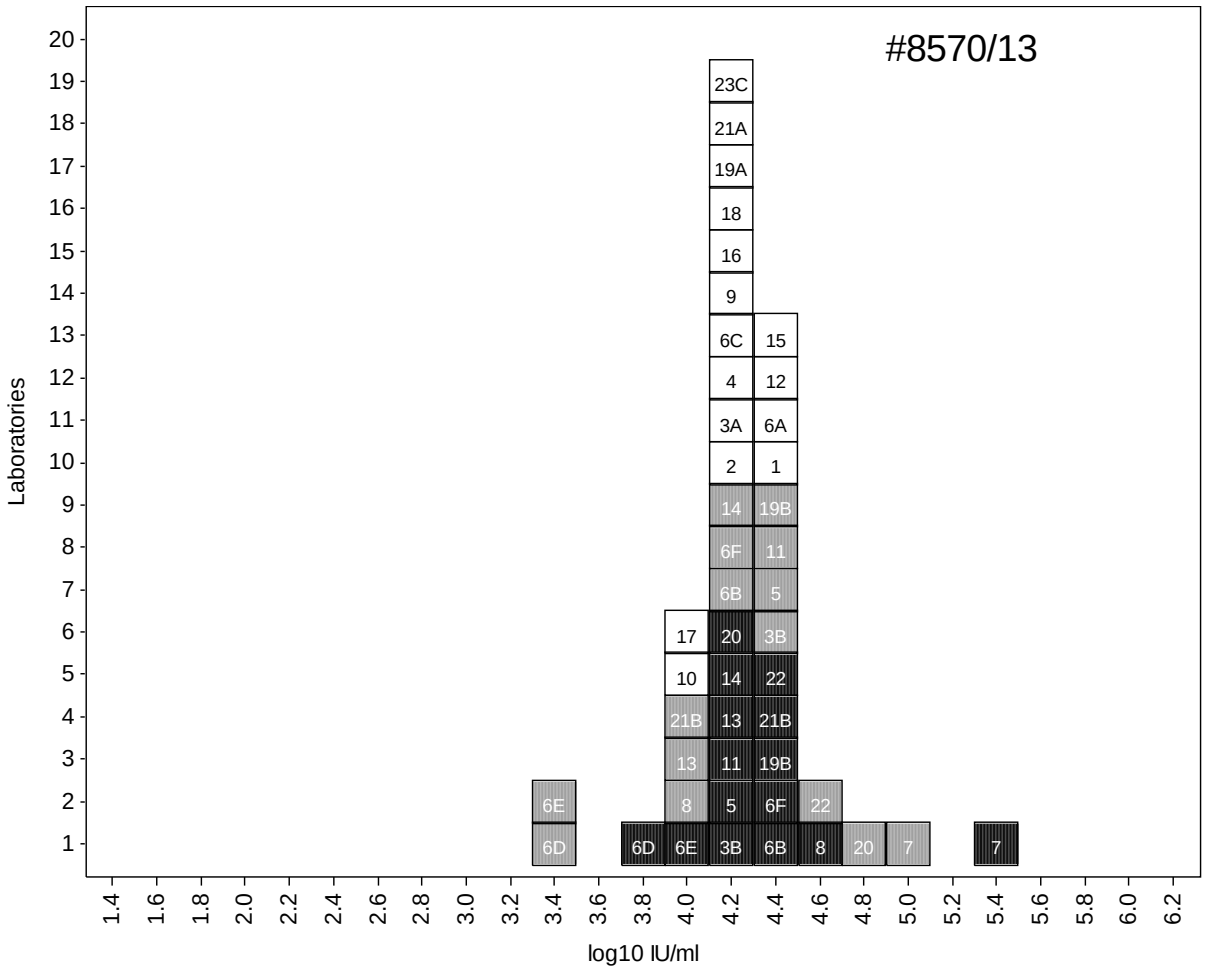
6E 11 22 3B 13 8 6B
14 6E 9 5 22 13 6D 23C 21B 21A
20 14 6B 3B 19B 6F 8 5 7 21B
11 10 20 6D 6A 19B
17 3A 6F 15 16
4 7 19A
6C 1
18 2
12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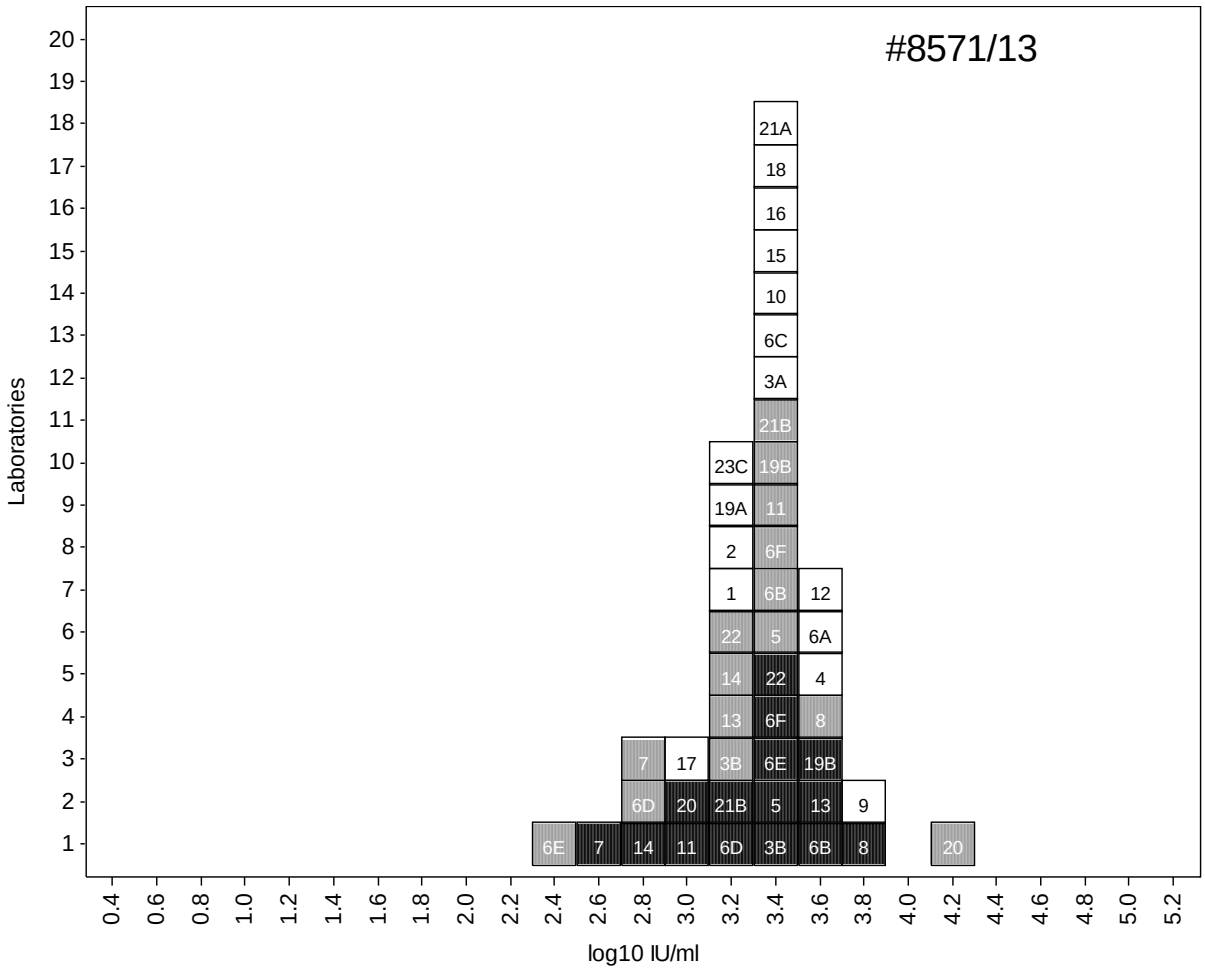
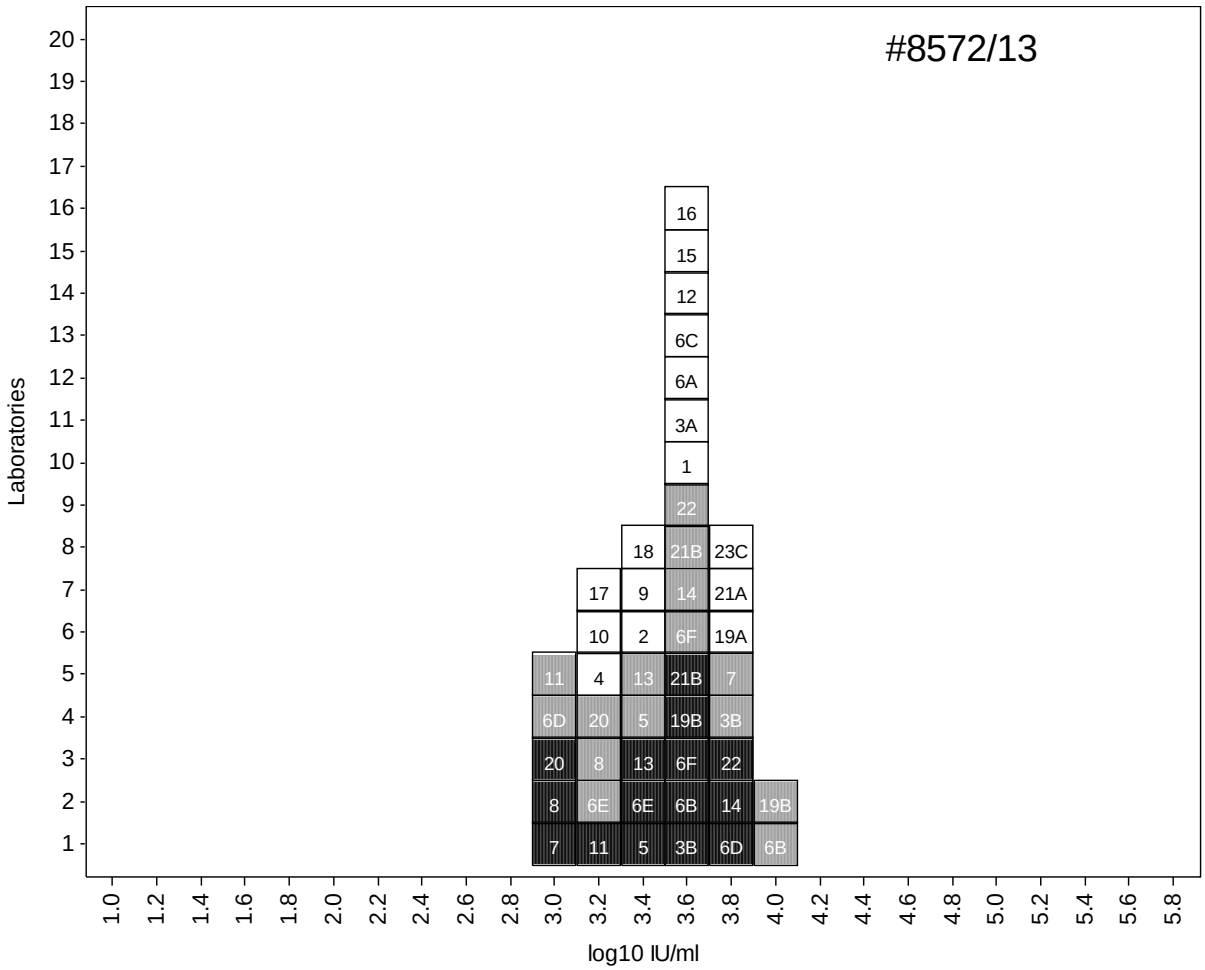
6D 6E 6D 6E 3B 6B 8 20 7 7
6E 8 5 6F 22
13 11 19B
21B 13 21B
10 14 22
17 20 3B
6B 5
6F 11
14 19B
2 1
3A 6A
4 12
6C 15
9
16
18
19A
21A
23C


#8571/13
Laboratories
log10 IU/ml
21A
18
16
15
10
6C
3A
21B
23C 19B
19A 11
2 6F
1 6B 12
22 5 6A
14 22 4
13 6F 8
7 17 3B 6E 19B
6D 20 21B 5 13 9
6E 7 14 11 6D 3B 6B 8 20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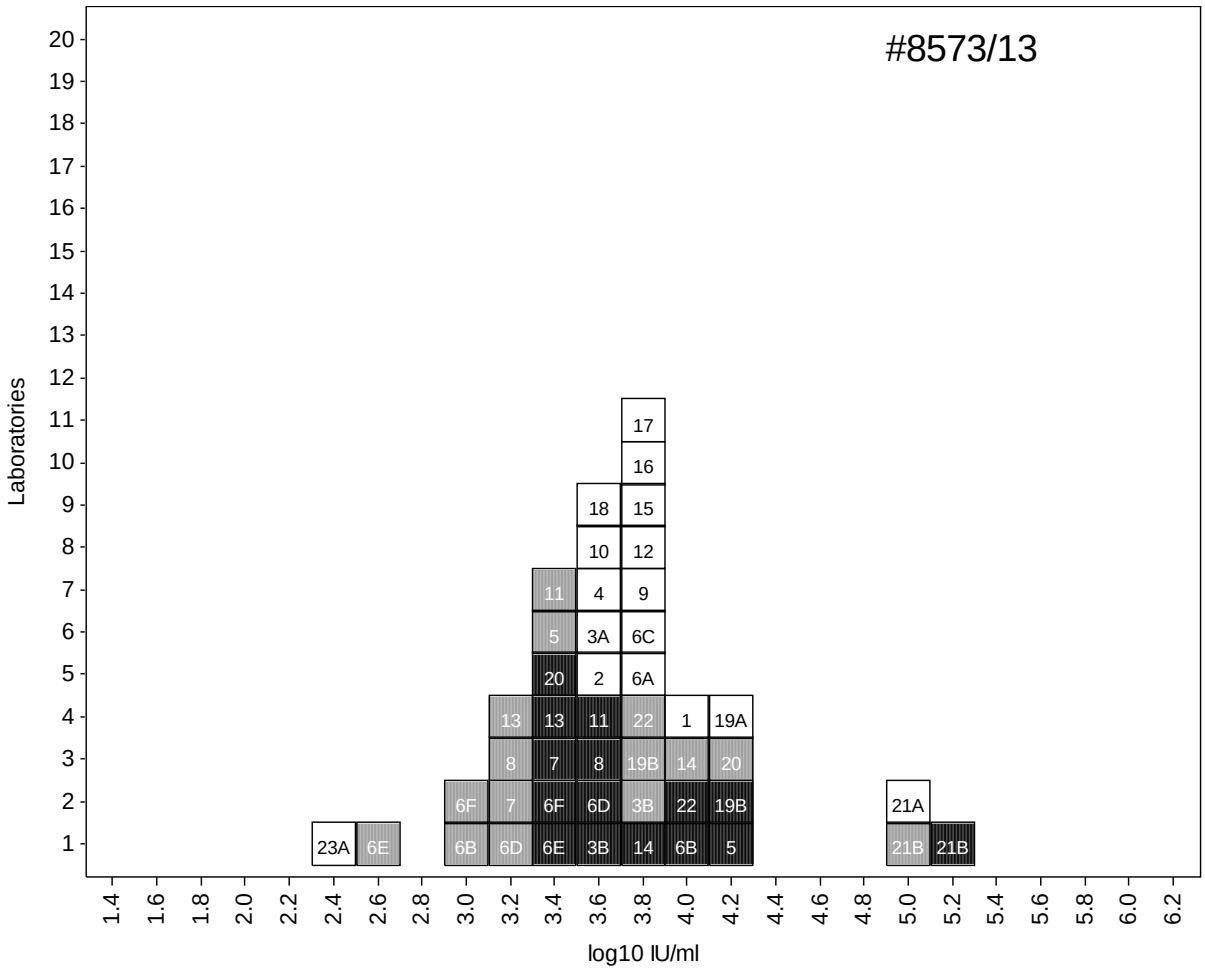
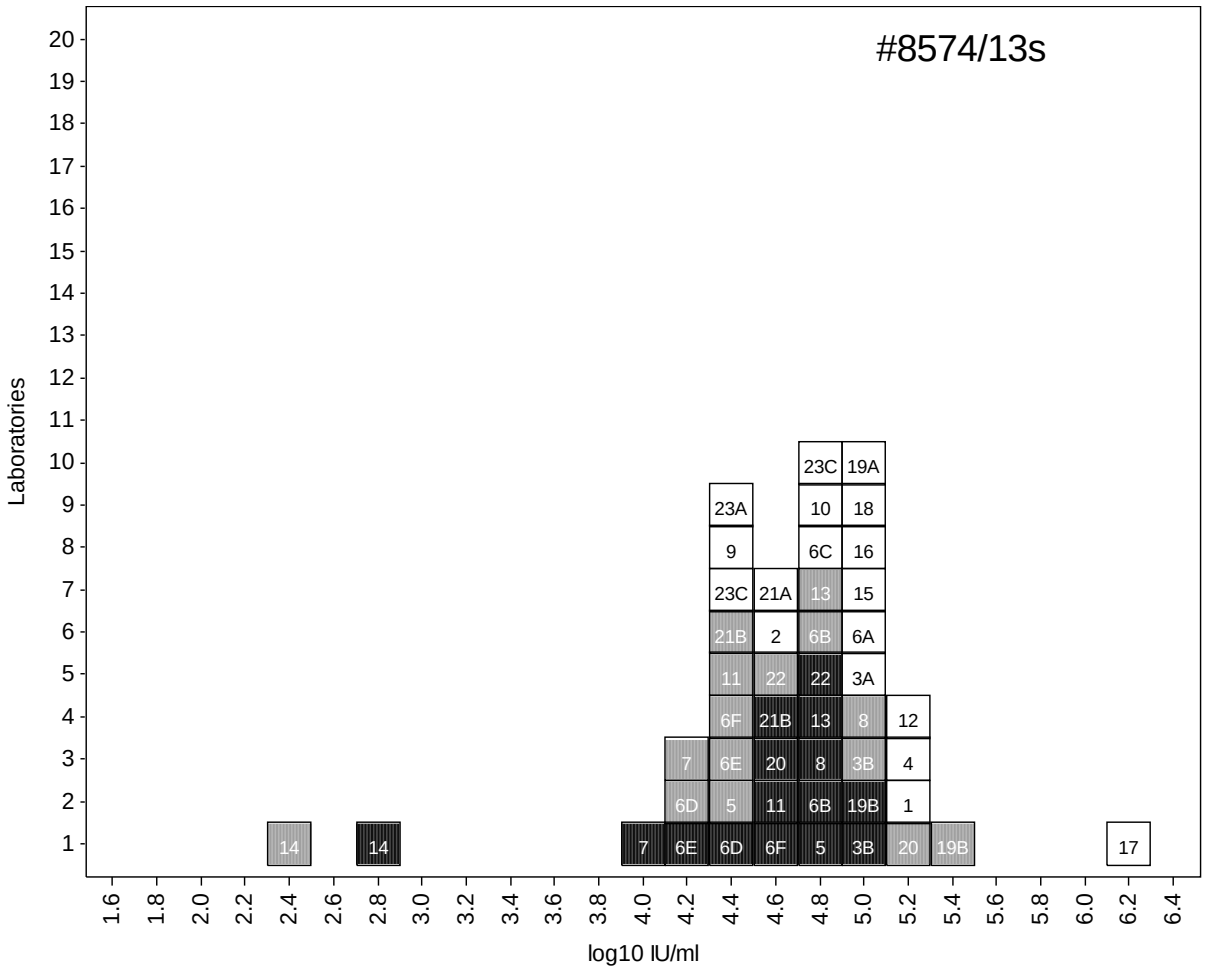
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
7 11 5 3B 6D 6B
8 6E 6E 6B 14 19B
20 8 13 6F 22
6D 20 5 19B 3B
11 4 13 21B 7
10 2 6F 19A
17 9 14 21A
18 21B 23C
22
1
3A
6A
6C
12
15
16


#8573/13
Laboratories
log10 IU/ml
23A 6E
6B 6D 6E 3B 14 6B 5
6F 7 6F 6D 3B 22 19B
8 7 8 19B 14 20
13 13 11 22 1 19A
20 2 6A
5 3A 6C
11 4 9
10 12
18 15
17
16
21A
21B 21B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Laboratories
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14 14 7 6E 6D 6F 5 3B 20 19B 17
6D 5 11 6B 19B 1
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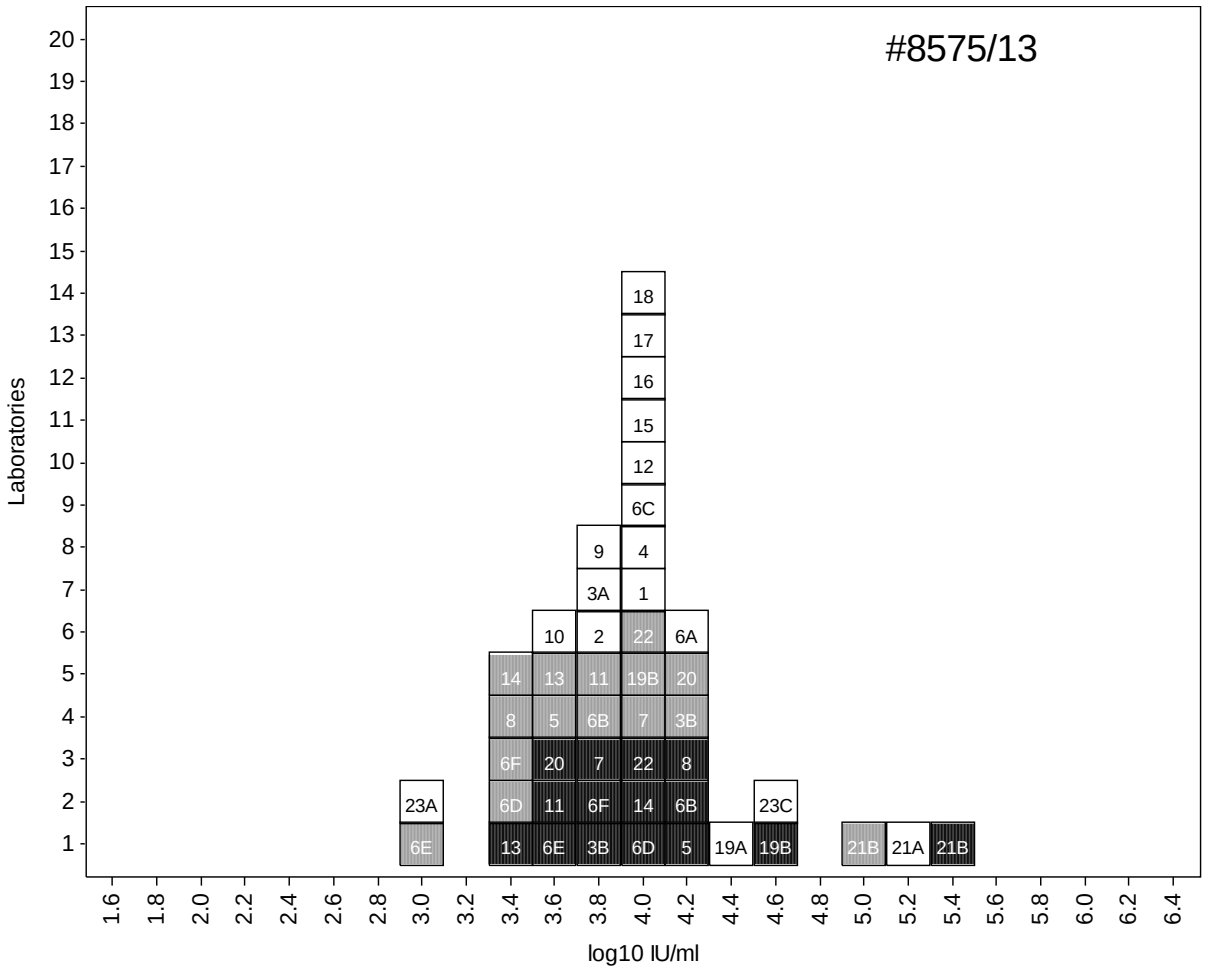
23C 21A 13 15
9 6C 16
23A 10 18
23C 19A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Laboratories
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23A 6E
6D 13 6E 11 3B 6F 6D 14 5 6B 19A 23C 19B
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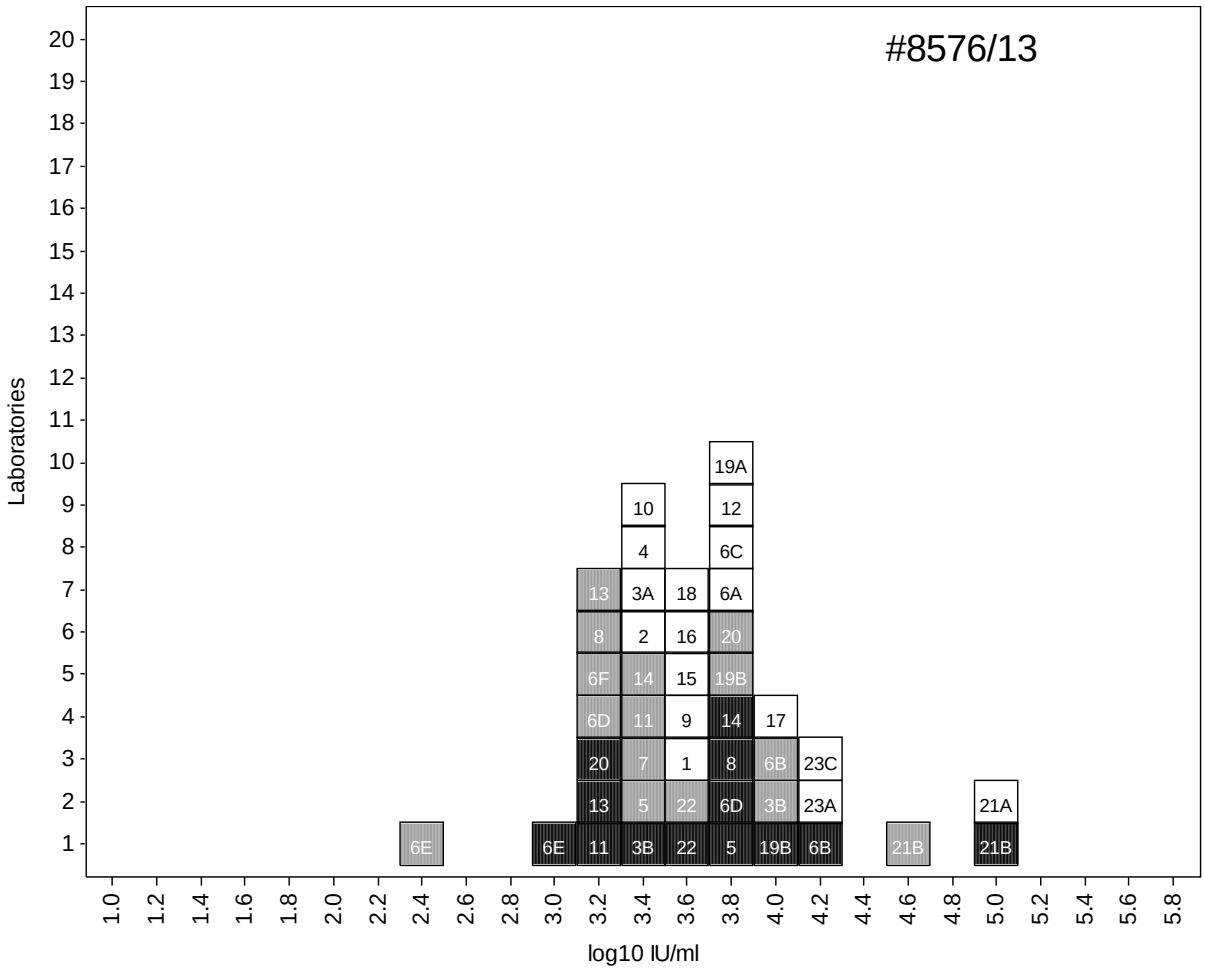
10 2 3A 9 1 4 6C 12 15 16 17 18
21B 21A 21B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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
6E
6E 11 3B 22 5 19B 6B
21B
21B
13 5 22 6D 3B 23A
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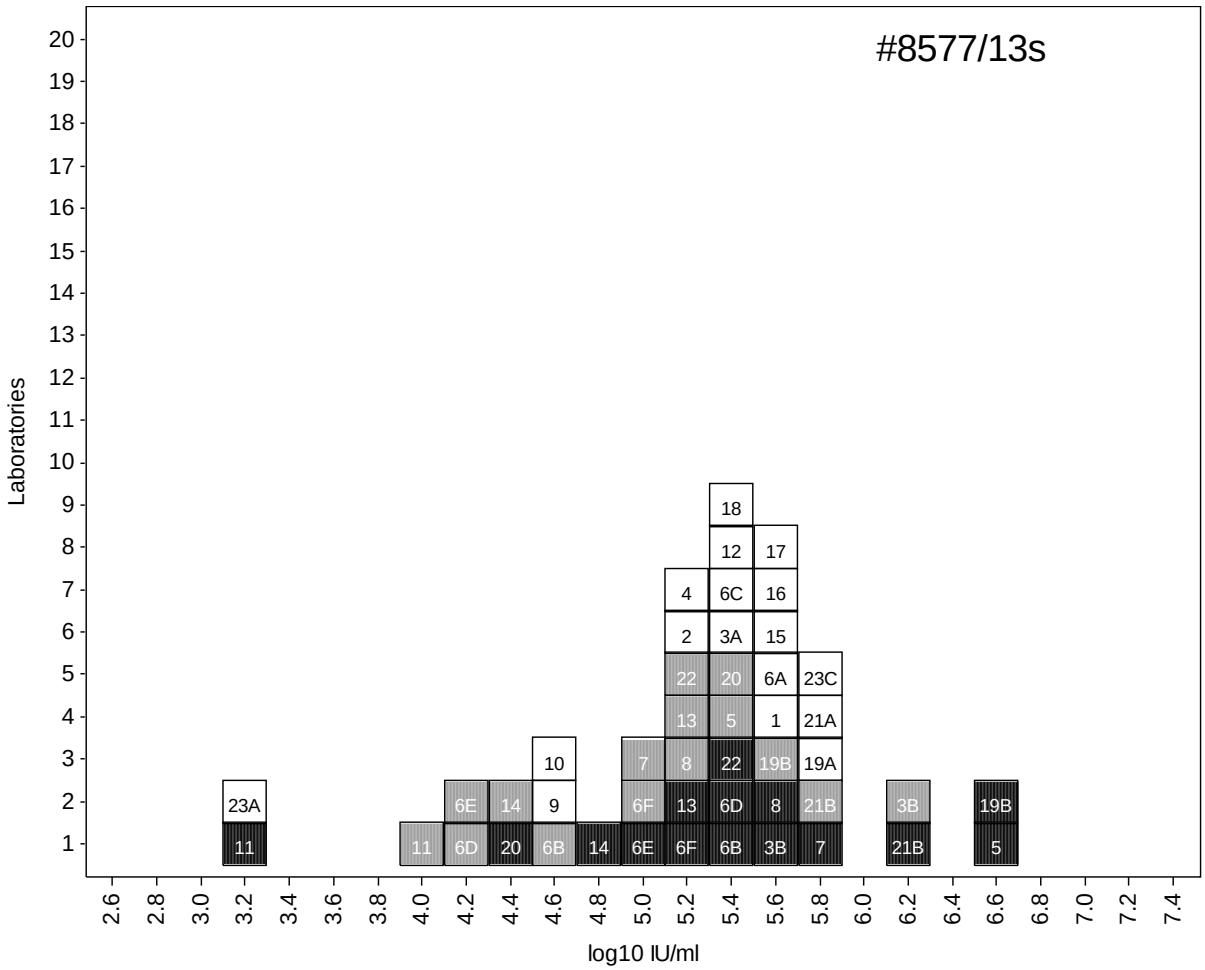
6D 11 9 14 17
6F 14 15 19B
8 2 16 20
13 3A 18 6A
4 6C
10 12
19A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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
23A 11
11
6E 6D
14 20
10 9 6B
14
7 6F 6E
8 13 6F
22 13 5 2 4
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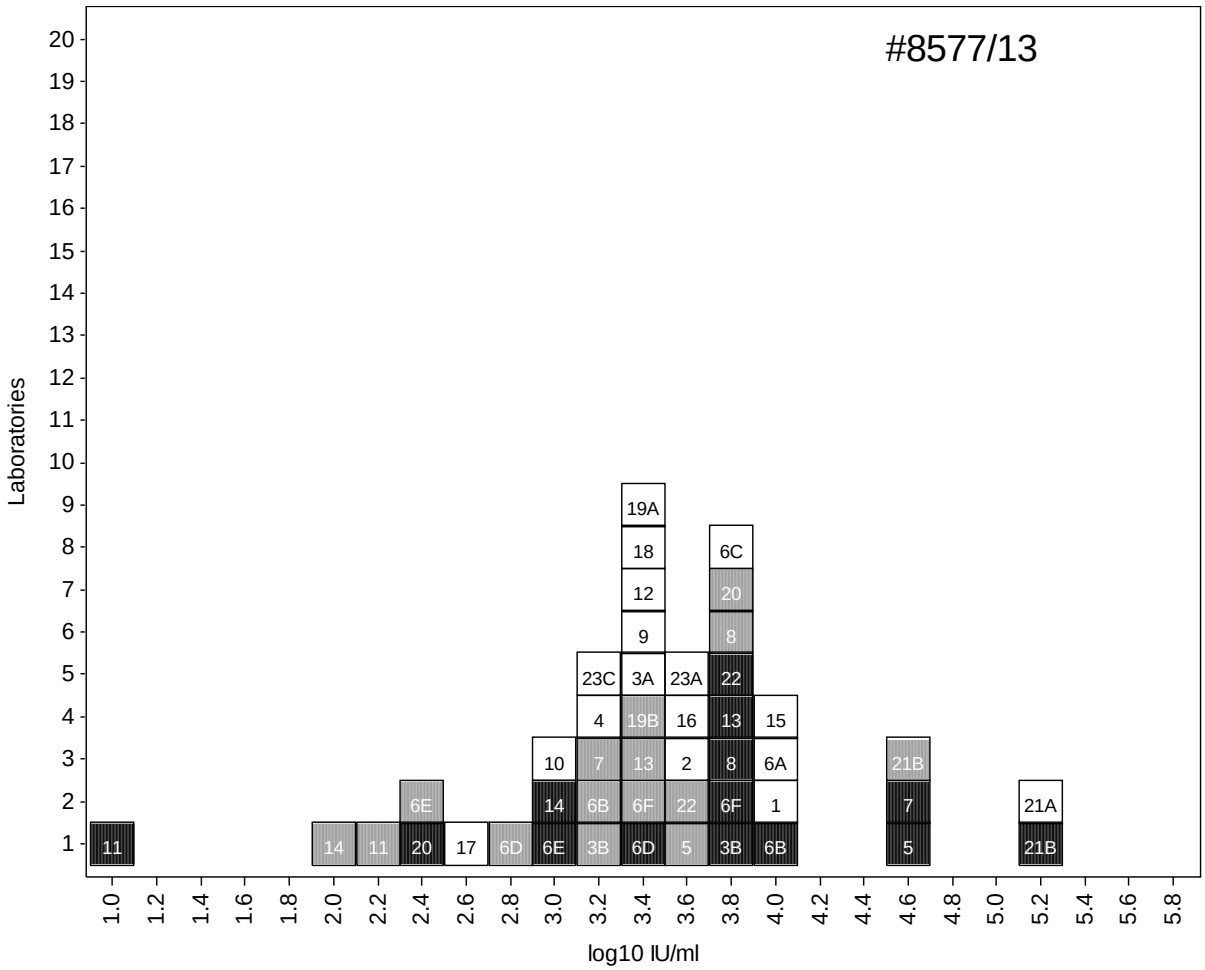
22 6D 6B
19B 8 3B
6A 1 15 16 17
23C 21A 19A 21B 7
3B 21B
19B 5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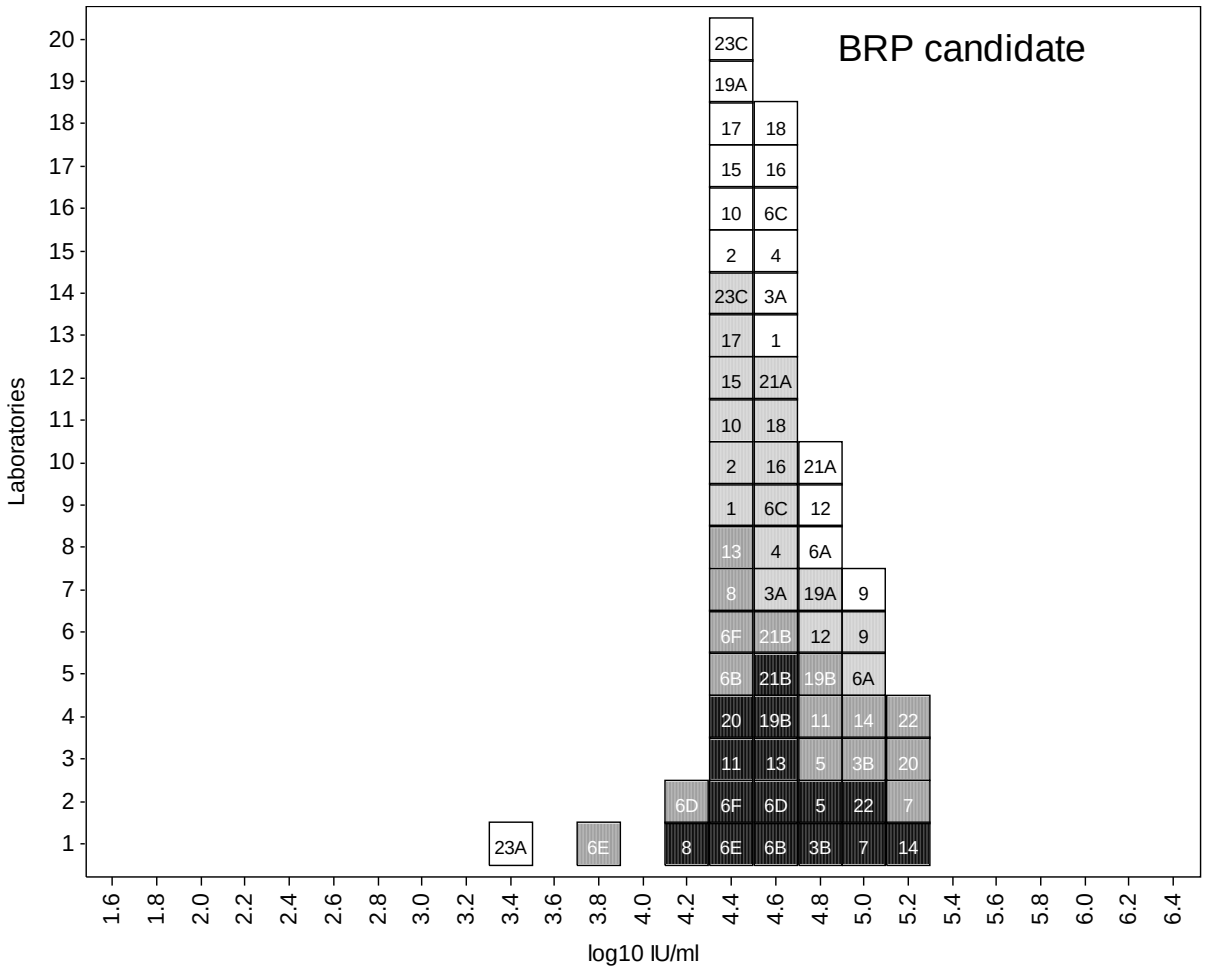
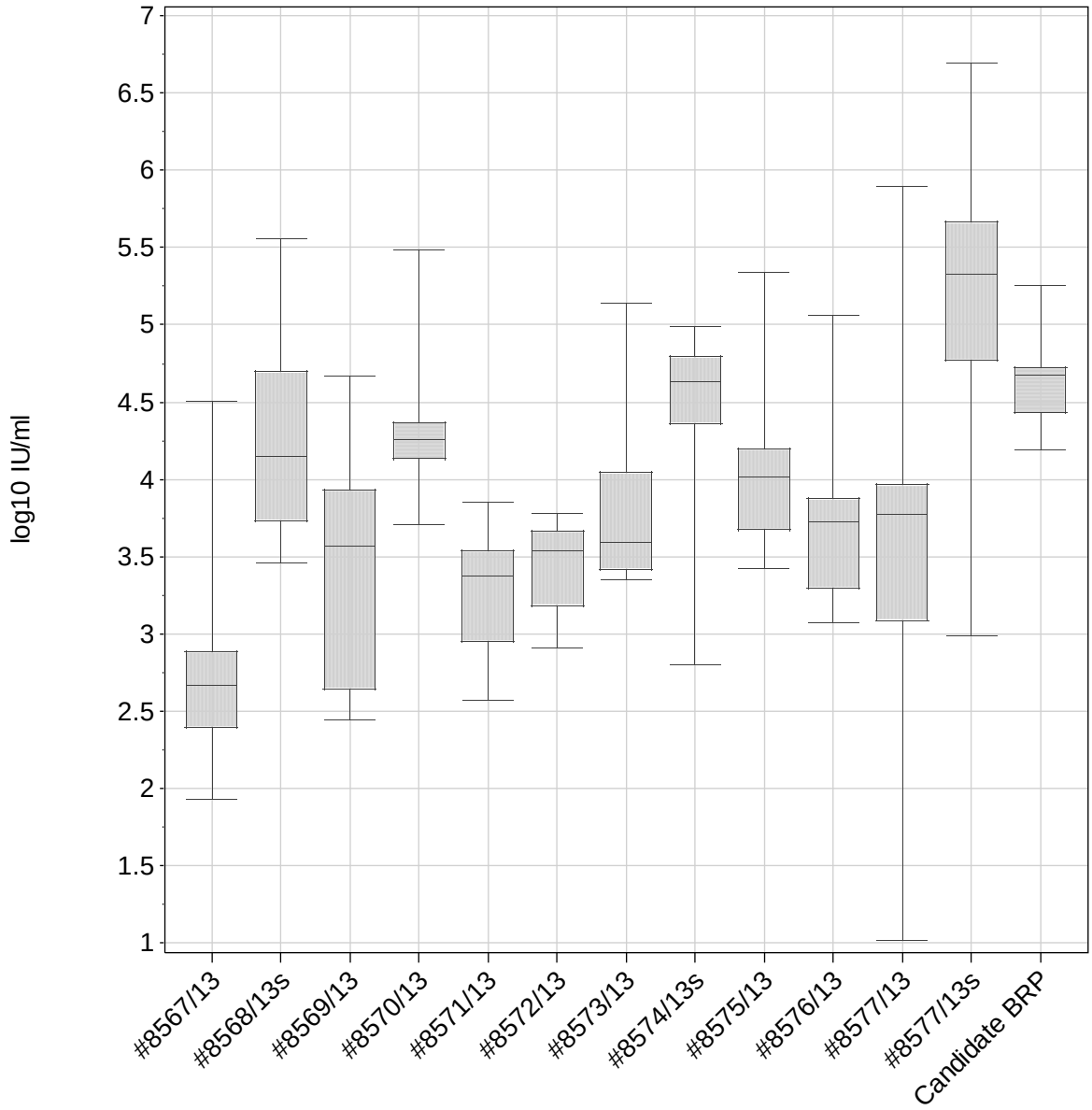
11
14 11 20 6E 17 6D 6E 14 10 3B 6B 7 4 23C 6D 6F 13 19B 9 3A 19A 12 18 5 22 2 16 23A 3B 8 13 6F 20 8 6C 6B 1 6A 15 21B 7 5 21A 21B



Potency in $\log_{10}$ IU/ml based on quantitative methods (white boxes); potency based on analysis of C_T values from qualitative methods (black boxes); potency based on qualitative, end-point dilution analysis (dark grey boxes); potency based on analysis of C_T values from quantitative methods (light grey boxes – candidate BRP only).
Values determined by Laboratory 23A were based on a single assay run, the samples were inconsistently detected.

Figure 4 Qualitative results (log_{10} IU/ml) shown as as Box-and-Whisker-Plot; data are based upon end-point dilution analysis



Appendix 1
List of participants

Scientist	Affiliation
Elisabet Ekvärn	Octapharma Stockholm, Sweden
Thomas Gärtner/Maike Schönborn/Christiane Beckort	Octapharma Frankfurt am Main, Germany
Tonya Hayden/Saleem Kamili	Centers for Disease Control and Prevention Atlanta, USA
Markus Hess/Manuela Tillack/Daniel Brischke	altona Diagnostics Hamburg, Germany
Boris Hogema	Sanquin Blood Supply Foundation Amsterdam, the Netherlands
Samreen Ijaz/Steve Dicks/Becky Haywood	Public Health England London, UK
Jacques Izopet/Sébastien Lhomme/Martine Dubois	Institut Fédératif de Biologie Purpan Toulouse, France
Lia Laura Lewis Ximenez de Souza Rodrigues/Marcelo Alves Pinto	Fundação Oswaldo Cruz Rio de Janeiro, Brazil
Jeffrey Linnen/Edgar Ong/Robin Cory	Hologic Inc. San Diego, USA
Keiji Matsubayashi	Japanese Red Cross Hokkaido Block Blood Center Sapporo, Japan
Saeko Mizusawa	National Institute of Infectious Diseases Tokyo, Japan
Joan O'Riordan/Jessica Murphy/Fiona Boland	Irish Blood Transfusion Service Dublin, Ireland
Barbara Pacini	Kedrion Biopharmaceuticals Bolognana, Lucca, Italy
Giulio Pisani/Matteo Simeoni/Sara Fabi	Istituto Superiore di Sanità Rome, Italy
Dieter Pullirsch/Brigitte Hottowy	AGES PharmMed Vienna, Austria
Anne-Marie Roque-Afonso	Hôpital Paul Brousse Villejuif, France
Oliver Schär	Roche Diagnostics International Ltd. Rotkreuz, Switzerland
Tim Shuurman/Hubert Niesters	University Medical Center Groningen, Groningen, the Netherlands
Isabelle Thomas/Magali Wautier	Scientific Institute of Public Health, Brussels, Belgium
Shigeharu Uchida	Central Blood Institute, Japanese Red Cross Society Tokyo, Japan
Tanja Vollmer/Jens Dreier	Institut für Laboratoriums- und Transfusionsmedizin, Herz- und Diabeteszentrum Nordrhein-Westfalen,

	Bad Oeynhausen, Germany
Youchun Wang/Weijin Huang/Chenyan Zhao	National Institutes for Food and Drug Control Beijing, China
Jürgen Wenzel/Jasmin Klein	University of Regensburg Regensburg, Germany
Zizheng Zheng/James Wai Kuo Shih/Zi-Min Tang/Wen-Fang Ji	Xiamen University Fujian, China

Appendix 2
Sequences obtained for the RpRD region of HEV ORF1

>WHO_IS_AB630970_3a_Japan
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GGAAC

>BRP_FJ956757_3f_Germany
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>8572/13_JN995564_3e_Germany
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TCCCTAGGTCTTGAGTGCGCCATTATGGAAGAGTGTGGTATGCCCCAGTGGCTTGTCAGGTTGTACCATGCCGTCCGGTC
GGCGTGGATCCTGCAGGCCCCAAAAGAGTCTTTGAGAGGGTTCTGGAAGAAGCATTCTGGTGAGCCGGGCACGTTGCTC

Appendix 3
Draft Instructions for Use for the 1st IRP for HEV Genotypes for NAT-based Assays

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 Reference Panel for Hepatitis E Virus (HEV) Genotypes for Nucleic Acid Amplification Technique (NAT)-Based Assays

PEI code 8578/13

(Version 1.0, July 2015)

1. INTENDED USE

The current WHO International Standard (IS) material for hepatitis E virus (HEV) is genotype 3a (1). In order to reflect HEV genetic diversity and the global disease burden of acute hepatitis E, a genotype panel has been prepared which contains representative strains of all four HEV genotypes and important sub-genotypes.
The reference panel consists of 11 HEV positive samples (plasma and stool-derived) further diluted in pooled human plasma. The samples, their code numbers, as well are their HEV (sub-)genotype are listed in the appended Table. The panel has been evaluated in an international collaborative study where the samples were tested concurrently with the 1st WHO IS (6329/10).
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 24 laboratories performing a wide range of HEV NAT assays. Further details of the collaborative study are available in the report WHO/BS/2015.2264.

2. UNITAGE

No unitage has been assigned to the individual panel members. The IU for HEV RNA is defined by the current WHO International Standard, which is a genotype 3a virus. The panel is *not* intended to replace the IS for HEV RNA.
However, in the international collaborative study (based upon data returned by 17 laboratories from quantitative assays), the *mean titres* of panel members were determined and are provided below, for information only.
The values were obtained by comparison to the genotype 3a International Standard using current assays represented in the collaborative study. Further details of the collaborative study are available in the report WHO/BS/15.2264.

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 HBV DNA, HCV RNA, HEV RNA, HIV-1/2 RNA, HBsAg, anti-HEV, 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 reference panel. 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.
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) Baylis SA, Blümel J, Mizusawa S, Matsubayashi K, Sakata H, Okada Y, Nübling CM, Hanschmann KM; HEV Collaborative Study Group. World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA. Emerg Infect Dis. 2013;19:729-35.
(2) Baylis SA, Terao E, Hanschmann KM. Collaborative Study to Establish the 1st World Health Organization International Reference Panel for Hepatitis E Virus RNA Genotypes for Nucleic Acid Amplification Technology (NAT)-Based Assays. WHO Report 2015, WHO/BS/2015.2264.
(3) Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Expert Committee on Biological Standardization, Fifty-fifth report, 2004. (WHO Technical Report Series, No. 932).

8. ACKNOWLEDGEMENTS

We are grateful to all study participants and the providers of the materials used in the preparation of the panel (2).

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

Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

Email: whoccivd@pei.de
Web: http://www.pei.de

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 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 E VIRUS (HEV)		
Handling:	See caution, section 4	
Toxicological properties		
Effects of inhalation: *contains infectious HEV*	Avoid –	
Effects of ingestion: *contains infectious HEV*	Avoid –	
Effects of skin absorption: *infectious HEV*	Avoid – *contains*	
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.

Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

Email: whoccivd@pei.de
Web: http://www.pei.de

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

Results from the Collaborative Study to Evaluate a Hepatitis E Virus genotype Panel

Panel Member	Genotype	Overall mean (log_{10} IU/ml)	95% Confidence Intervals (log_{10} IU/ml)		Standard Deviation	Min	Max	Range
8567/13	1a	2.64	2.22	3.06	0.60	1.44	4.10	2.66
8568/13s	1a	4.25	3.87	4.63	0.43	3.55	5.46	1.91
8569/13	1e	3.25	2.91	3.59	0.51	2.40	4.81	2.41
8570/13	3b	4.20	4.12	4.28	0.18	3.80	4.57	0.78
8571/13	3c	3.40	3.27	3.53	0.22	2.80	3.88	1.08
8572/13	3e	3.50	3.36	3.64	0.22	2.99	4.02	1.04
8573/13	3f	3.84	3.60	4.07	0.41	2.40	5.14	2.74
8574/13s	3 (rabbit-like)	4.98	4.73	5.24	0.38	4.35	6.88	2.52
8575/13	4c	4.07	3.82	4.31	0.38	3.03	5.37	2.34
8576/13	4g	3.77	3.49	4.04	0.38	3.13	5.05	1.92
8577/13s	2a	5.42	5.10	5.74	0.49	3.27	6.10	2.83

Overall mean estimates (log_{10} IU/ml) relative to the concurrently tested 1st International Standard for HEV RNA (6329/10); the data are based upon results obtained from 17 laboratories performing a range of quantitative assays. Further details of the collaborative study are available in the report WHO/BS/15.2264

Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
63225 Langen, Germany

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