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Collaborative Study to Evaluate the Proposed 4th WHO International Standard for Hepatitis C Virus (HCV) for Nucleic Acid Amplification Technology (NAT)-Based Assays

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*** See Appendix 1***

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Summary
This report describes the evaluation of the replacement 4th WHO International Standard for hepatitis C virus (HCV) for use in the standardization of nucleic acid amplification technology (NAT). A replacement freeze-dried preparation (NIBSC code 06/102) was prepared from the same bulk as the current International Standard (NIBSC code 06/100), in 2006, but was filled and freeze-dried in a separate processing run. Both preparations were characterized in a worldwide collaborative study in 2007. In this present study, seven laboratories from five countries participated in the evaluation of the potency and real-time stability of 06/102, by comparison with the current and previous HCV International Standards (NIBSC codes 06/100 and 96/798 respectively), using their routine NAT-based assays for HCV. Six different HCV NAT assays were used in the evaluation, the majority of which were commercial quantitative assays based on real-time PCR technology. The overall mean potency estimates for sample 1 (06/102), sample 2 (06/100) and sample 3 (96/798) from quantitative assays were 5.24, 5.08, and 5.05 log_{10} IU/mL respectively. The range in individual laboratory mean estimates for these samples from quantitative assays was approximately 0.39-0.44 log_{10} IU/mL, suggesting that inter-laboratory agreement was good.

For sample 1 (06/102) and sample 2 (06/100) there was a drop in potency of approximately 0.2 log_{10} when compared to the results obtained in the original collaborative study evaluation performed in 2007. Meanwhile, there was no corresponding drop in potency for sample 3 (96/798). The results of in-house testing and a subsequent additional collaborative study suggest that the drop in potency for preparations 06/100 and 06/102 is likely to be due to instability of these freeze-dried preparations when stored at elevated temperatures (for example, during shipping), rather than a deterioration of the product during storage at -20 °C.

Overall, the results of this study indicate the suitability of the candidate as the replacement 4th WHO International Standard for HCV, when maintained at or below -20 °C, and that the concentration determined in the original 2007 collaborative study should be applied. It is therefore proposed that the candidate sample 1 (NIBSC code 06/102) is established as the 4th WHO International Standard for HCV for NAT with an assigned potency of 260,000 IU/mL (~5.41 log_{10} IU/mL) when reconstituted in 0.5 mL of nuclease-free water. It is also proposed that when established, the standard should be shipped to customers on dry ice, with specific instructions for storage of the material at -20 °C.

Introduction
Stocks of the 3rd WHO International Standard for HCV RNA are diminishing and need to be replaced. The HCV International Standard is used by IVD manufacturers, blood transfusion centres, control authorities, and clinical laboratories, to calibrate secondary reference materials and in the validation of HCV NAT assays.

The need to standardize NAT-based assays for HCV and maintain the availability of the WHO International Standard for HCV for NAT is ongoing. HCV remains a major public health problem worldwide. Approximately 130-170 million people (2.2-3% of the world's population) are infected with HCV [1]. Chronic infection can lead to liver cirrhosis, liver failure and hepatocellular carcinoma [2]. The introduction of routine screening for HCV antibody from the late 1980's greatly reduced the risk of transmission via infected blood and blood products, however, there remained a major risk of transfusion-transmitted infection due to window-period donations [3]. As a consequence, NAT was introduced for the detection of HCV RNA in human plasma, used in the safety testing of blood and blood products, and it is now mandated in many parts of the

world [4,5]. HCV NAT is also widely used in the clinical management of HCV, particularly in the diagnosis of infection and in monitoring the response to antiviral therapies [6]. A range of both commercial and laboratory-developed NAT-based assays are currently in use.

International Standards are prepared in accordance with published WHO recommendations [7]. The 1st and 2nd WHO International Standards for HCV RNA were prepared by dilution of a high-titre HCV-positive donation (HCV antibody-positive), genotype 1 virus, in HCV-negative pooled human plasma. Both materials were prepared from the same bulk, but filled and freeze-dried on two separate occasions. The preparations were evaluated in parallel in a worldwide collaborative study using a range of NAT-based assays for HCV [8,9]. The first candidate (NIBSC code 96/790) was established as the 1st WHO International Standard for HCV RNA in 1997, with an assigned potency of 100,000 International Units (IU)/mL when reconstituted in 0.5 mL nuclease-free water. In 2003 the WHO Expert Committee on Biological Standardization (ECBS) established the second candidate (NIBSC code 96/798) as the replacement 2nd WHO International Standard for HCV RNA (applying the same unitage) following a smaller collaborative study [10,11]. In 2006, two replacement batches (NIBSC codes 06/100 and 06/102) were prepared from genotype 1a, HCV antibody-negative, HCV RNA positive plasma, diluted in human plasma, and were evaluated in a worldwide collaborative study in parallel with 96/798 [12,13]. Mean relative potencies for 06/100 and 06/102 were 5.19 and 5.41 $\log_{10}$ IU/mL respectively, when compared against the 2nd WHO International Standard for HCV RNA (96/798). The first candidate (06/100) was established as the 3rd WHO International Standard for HCV RNA in October 2007 with a unitage of 154,881 IU/mL.

This report now describes the collaborative study evaluation of the second candidate 06/102 as the replacement 4th WHO International Standard for HCV for NAT. The proposal to replace the 3rd WHO International Standard for HCV RNA was endorsed by the WHO ECBS in October 2010. The proposal and an update were also presented at the XXII Scientific Working Group on the Standardization of Genome Amplification Techniques for the Safety Testing of Blood, Tissues and Organs for Blood Borne Pathogens in Rome in April 2011. The proposed standard is intended to be used in the *in vitro* diagnostics field and relates to ISO 17511:2003 Section 5.5.

Aims of study

The aim of this collaborative study was to examine the potency and real-time stability of 06/102 by comparison with the 3rd WHO International Standard for HCV RNA (NIBSC code 06/100) and previous International Standard (NIBSC code 96/798), using a range of NAT-based assays.

Materials

Candidate standard

The candidate preparation 06/102 comprises freeze-dried human plasma and HCV (genotype 1a) sourced from three window-period donations [12]. The virus stock was tested and found negative for HIV-1 RNA, HBV DNA, HAV RNA and parvovirus B19 DNA. The pooled human plasma diluent was sourced from blood donations and had been tested and found negative for HIV antibody, HCV antibody, HBsAg, syphilis antibody, HTLV antibody, as well as HIV and HCV RNA.

The filling and freeze-drying of both 06/100 and 06/102 was performed in two separate processing runs at BioReliance Ltd., Glasgow, Scotland, in April and May 2006 and has been described previously [12]. The bulks were dispensed in 0.5 mL volumes into 3 mL crimp-cap glass vials (Adelphi Tubes, Haywards Heath, UK). The homogeneity of each fill was determined by

performing check-weighing of approximately every thirtieth vial, with vials outside the defined specification being discarded. Filled vials were partially stoppered, lyophilized and then fully stoppered in the freeze dryer. A total of 2257 vials were prepared for 06/102. The percentage coefficient of variation (%CV) of the fill was 1.5%. The sealed vials were returned to NIBSC for storage at -20 °C under continuous temperature monitoring for the lifetime of the product.

Assessments of residual oxygen and moisture were not performed at the time of production because of the infectious nature of these preparations and the lack of non-invasive methods available at NIBSC. Instead, data from stability assessments were taken to give an indication of residual moisture levels which can impact on the stability of freeze-dried preparations. Subsequently, assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, have been determined at NIBSC (February 2011) for 6 vials of the 06/102 product. Residual moisture was determined by non-invasive near-infrared (NIR) spectroscopy (MCT 600P, Process Sensors, Corby, UK). NIR results were then correlated to Karl Fischer (using calibration samples comprising freeze-dried human plasma, measured using both NIR and Karl Fischer methods) to give % w/w moisture readings. Oxygen content was measured using a Lighthouse Infra-Red Analyzer (FMS-750, Lighthouse Instruments, Charlottesville, USA).

Stability of the freeze-dried candidate

Ongoing accelerated degradation studies have been underway at NIBSC since 2006 in order to predict the stability of 06/100 and 06/102 when stored at the recommended temperature of -20 °C. Vials of freeze-dried product have been held at -70 °C, -20 °C, +4 °C, +20 °C, +37 °C and +45 °C. Accelerated thermal degradation studies were initiated on 29th June 2006. Preliminary stability assessments were performed in 2007 on vials stored at +4 °C for 10.5 months and at +20 °C for 4 weeks, and compared with samples stored at -20 °C [12]. It was noted that it was not possible to reconstitute samples stored at +20 °C for 8 months or samples stored at +37 °C or +45 °C.

As part of ongoing stability assessments, three vials each of 06/100 and 06/102 were examined on three separate occasions after storage for 4.5 years. The HCV RNA concentration was determined by NAT using the COBAS® AmpliPrep/ COBAS TaqMan® HCV Test (Roche Diagnostics GmbH, Mannheim, Germany).

The stability of 06/102 during simulated shipping at ambient temperature was also investigated. Vials stored at +4 °C and +20 °C for 1 week were compared with vials stored continually at -20 °C. The HCV RNA concentration was determined by NAT as above.

Study samples

The freeze-dried candidate 06/102 was evaluated alongside the 3rd WHO International Standard for HCV RNA (NIBSC code 06/100) and previous International Standard (NIBSC code 96/798). Study samples were stored at -20 °C prior to shipping to participants.

Collaborative study (NIBSC code CS458) samples were shipped to participants at ambient temperature coded as samples 1-3 and were as follows:

- Sample 1 (S1) - Lyophilized preparation 06/102 in a 3 mL crimp cap glass vial.
- Sample 2 (S2) - Lyophilized preparation 06/100 in a 3 mL crimp cap glass vial.
- Sample 3 (S3) - Lyophilized preparation 96/798 in a 3 mL crimp cap glass vial.

Study design

The aim of this collaborative study was to examine the potency and real-time stability of 06/102 by comparison with the 3rd WHO International Standard for HCV RNA (NIBSC code 06/100)

and the 2nd International Standard (NIBSC code 96/798) using a range of NAT-based assays. Three vials each of study samples 1-3 were sent to participating laboratories by courier at ambient temperature, with specific instructions for storage and reconstitution.

Study protocol

Participants were requested to test dilutions of each sample using their routine HCV NAT-based assay on three separate occasions, using a fresh vial of each sample in each independent assay. In accordance with the study protocol (Appendix 2), the lyophilized samples were to be reconstituted with 0.5 mL of deionized, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use.

Participants were requested to dilute samples 1-3 to within the quantitative range of the assay, using the sample matrix specific to their individual assay, and to extract each dilution prior to amplification. For quantitative assays, participants were requested to test a minimum of two serial ten-fold dilutions within the linear range of the assay. For qualitative assays, participants were requested to test half-$\log_{10}$ serial dilutions of each sample, around the assay end-point (in order to determine the actual assay end-point). For subsequent assays, participants were asked to test the dilution at the predetermined end-point, and a minimum of two half-$\log_{10}$ serial dilutions either side of the end-point (i.e., at least five dilutions in total).

Participants were requested to report the concentration of each sample in IU/mL (positive/negative for qualitative assays) for each dilution of each sample and return results, including details of methodology used, to NIBSC for analysis.

Participants

Study samples were sent to seven participants representing five countries (Appendix 1). Participants were selected for their experience in HCV NAT and geographic distribution. They represented manufacturers of *in vitro* diagnostic devices (IVDs), control, reference and contract testing laboratories, and blood transfusion centres. All participating laboratories are referred to by a code number, allocated at random, and not representing the order of listing in Appendix 1. Where a laboratory returned data using different assay methods, the results were analyzed separately, as if from different laboratories, and are referred to as, for example, laboratory 3A, 3B etc.

Statistical methods

Qualitative and quantitative assay results were evaluated separately. In the case of qualitative assays, for each laboratory and assay method, data from all assays were pooled to give a number positive out of number tested at each dilution step. A single 'end-point' for each dilution series was calculated, to give an estimate of 'NAT detectable units/mL', as described previously [8]. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome copy number/mL. In the case of quantitative assays, analysis was based on the results supplied by the participants. Results were reported as IU/mL. For each assay run, a single estimate of $\log_{10}$ IU/mL was obtained for each sample, by taking the mean of the $\log_{10}$ estimates of IU/mL across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of IU/mL across assay runs.

All analysis was based on the $\log_{10}$ estimates of IU/mL or 'NAT detectable units/mL'. Overall mean estimates were calculated as the means of all individual laboratories. Variation between laboratories (inter-laboratory) was expressed as standard deviations (SD) of the $\log_{10}$ estimates and % geometric coefficient of variation (%GCV) [14] of the actual estimates. Potencies relative to

sample 3, the 2nd WHO International Standard for HCV RNA (96/798), were calculated as the difference in estimated $\log_{10}$ 'units per mL' (test sample – standard) plus the value in $\log_{10}$ IU/mL for the International Standard. So for example, if in an individual assay, the test sample is 0.5 $\log_{10}$ higher than the International Standard, assigned 5.0 $\log_{10}$ IU/mL, the relative potency of the test sample is 5.5 $\log_{10}$ IU/mL.

For the quantitative assays, variation within laboratories, and between assays, (intra-laboratory) was expressed as standard deviations of the individual assay mean $\log_{10}$ estimates. These estimates were calculated for the different samples individually, and also pooled across all samples. The significance of the inter-laboratory variation relative to the intra-laboratory variation was assessed by an analysis of variance.

Additional collaborative study investigation of stability during shipping – 'transportation study'

An additional collaborative study (NIBSC code CS474) was performed to investigate whether the observed drop in potency between the 2007 and 2011 studies could have resulted from shipping the samples at ambient temperature rather than an actual loss in potency upon storage at -20 °C. A subset of the laboratories that had participated in the 2011 study (CS458) were sent samples of 06/102, 06/100 and 96/798 that had been stored at +20 °C for one week, along with samples that had been stored continually at -20 °C. Participants have been allocated the same code number as for CS458. All samples were shipped on dry ice. Participants were requested to test all samples concurrently using quantitative assay methods, in three independent assays as for CS458. This study is referred to as the 'transportation study' in this report, to distinguish it from the '2011 study'.

Collaborative study (NIBSC code CS474) samples shipped to participants were coded as samples 1-6 and were as follows:

- Sample 1 (S1) - Lyophilized preparation 06/102 in a 3 mL crimp cap glass vial.
- Sample 2 (S2) - Lyophilized preparation 06/100 in a 3 mL crimp cap glass vial.
- Sample 3 (S3) - Lyophilized preparation 96/798 in a 3 mL crimp cap glass vial.
- Sample 4 (S4) - Lyophilized preparation 06/102 in a 3 mL crimp cap glass vial. Stored at +20 °C for 1 week.
- Sample 5 (S5) - Lyophilized preparation 06/100 in a 3 mL crimp cap glass vial. Stored at +20 °C for 1 week.
- Sample 6 (S6) - Lyophilized preparation 96/798 in a 3 mL crimp cap glass vial. Stored at +20 °C for 1 week.

Results and data analysis

Validation of study samples and stability assessment

Production data for the candidate standard sample 1 (06/102) showed that the CV of the fill mass was within acceptable limits for a WHO International Standard [7]. The non-invasive moisture measurements were out of range of the calibration curve, therefore, an estimate was made by extrapolating the calibration curve beyond the last calibration point. The residual moisture and oxygen contents were 4.68% and 18.2% respectively. These were higher than expected and may have been due to ingress of atmospheric air during storage of the product at -20 °C.

Samples of the candidate standards 06/100 and 06/102 were stored at elevated temperatures, and assayed at NIBSC in parallel with samples stored at -70 °C by HCV NAT (as described above). Three vials of each sample were evaluated on three separate occasions after storage at each temperature for approximately 4.5 years. The mean estimated $\log_{10}$ IU/ml and differences ($\log_{10}$

IU/ml) from the -70 °C baseline sample are shown in Table 1. A negative value indicates a drop in potency relative to the -70 °C baseline. Samples stored at +20 °C and above could not be reconstituted at all. In addition, samples stored at +4 °C could not be fully reconstituted. Therefore, it was only possible to compare samples stored at -20 °C and -70 °C. The results indicate that there has been a drop in potency for 06/102 of 0.13 log_{10} IU/mL. This observed drop is close to the borderline of statistical significance ($p=0.052$), based on the three assay means. For 06/100, there was no observed drop at -20 °C compared to the -70 °C baseline, although the assay variability was large.

Stability of 06/102 during simulated shipping at ambient temperature was also investigated in-house. Vials stored at +4 °C and +20 °C for 1 week were compared with vials stored at -20 °C. The results, in Table 2, show a drop in potency upon storage at both +4 °C (-0.04 log_{10}) and +20 °C (-0.24 log_{10}) for 1 week. Although only three vials (total of 6 replicates) stored at each temperature were examined in one assay run, the observed drop in potency after storage at +20 °C for 1 week was highly significant ($p<0.0001$), when assessed relative to the intra-run variability for the assay.

The stability of 06/102 when reconstituted has not been specifically determined. Therefore, it is recommended that the reconstituted material is for single use only.

Data received

Data were received from seven laboratories, performing a variety of assay methods. Laboratory 3 returned data from three different assay methods (coded 3A, 3B and 3C), while laboratories 4 and 7 each returned data from two different methods (coded 4A, 4B, 7A and 7B). Laboratory 1 performed qualitative assays, while all other laboratories performed quantitative assays. All quantitative assays gave results that were calibrated in IU. In total, 11 data sets were received from 10 quantitative assays, and 1 qualitative assay.

Assay methodologies for qualitative and quantitative assays are summarized in Table 3. The majority of participants prepared dilutions of study samples 1-3 using negative human plasma, however, Siemens VERSANT Sample Preparation 1.0 Reagent Kit Elution Buffer and AcroMetrix NAT Dilution Matrix were also used. The range of the dilutions performed varied slightly between each laboratory.

Estimated IU/mL or 'NAT detectable units/mL'

The qualitative results from laboratory 1 were analyzed to give estimates of 'NAT detectable units/mL' for all three study samples. For sample 1 (06/102) all dilutions tested were positive, and so it was not possible to obtain an estimate of units/mL for this sample.

The laboratory means of the estimates provided by the various assay methods are shown in Table 4 for the three study samples. The laboratory mean estimates for the quantitative assays are also shown in histogram form in Figure 1. Each box represents the mean estimate from one laboratory, and the boxes are labeled with the laboratory and assay code. The overall mean estimates and inter-laboratory variation (standard deviation of log_{10} estimates, %GCV of absolute estimates, minimum and maximum) for the quantitative assays are shown in Table 5. The qualitative assay gives estimates of 'NAT detectable units/mL', which do not allow for extraction efficiency and are not calibrated in International Units. Therefore, these qualitative results are not combined with the results for the quantitative assays for the overall means.

From the tables and figures it can be seen that there is very good agreement between laboratories, with SD's between 0.11 and 0.16 $\log_{10}$, and %GCV's from 28% to 46%.

Potencies relative to the 2nd WHO International Standard for HCV (Sample 3)

The potencies of samples 1 and 2 (06/102 – candidate; 06/100 – current 3rd WHO International Standard for HCV RNA) were expressed relative to the concurrently tested sample 3 (96/798 – 2nd WHO International Standard for HCV RNA). The laboratory means are shown in Table 6, with the overall mean and inter-laboratory variation for the quantitative assays shown in Table 7. The mean estimates are also shown in histogram form in Figure 2. Again, there is good agreement between laboratories, with SD's of 0.16 and 0.19 $\log_{10}$, and %GCV's of 43% and 54% for 06/102 and 06/100 respectively.

The inter-laboratory variation is slightly higher after expressing results relative to 96/798. Expressing results relative to a concurrently tested reference is usually expected to improve the inter-laboratory agreement. However, in situations where there is already very good inter-laboratory agreement, as is the case with the established HCV assays, the use of relative potencies may increase the inter-laboratory variability. This is because the relative potency is based on results for two samples rather than just one, with a subsequent increase in the potential for assay variability. It is recognised that the use of relative potency is most effective for removing large differences between laboratories when different assay methods do not give consistent results.

The overall mean for the current 3rd WHO International Standard for HCV RNA (06/100) relative to the 2nd International Standard (96/798) is 5.03 $\log_{10}$ IU/mL, lower than the assigned value of 5.19 $\log_{10}$ IU/mL (but within the SD). This assigned value was derived from the results of the collaborative study conducted in 2007 to establish the 3rd International Standard [12].

Intra-laboratory variation

The intra-laboratory (between-assay) variability is shown in Table 8 as the SD of the $\log_{10}$ estimates. The intra-assay variability for 96/798 was generally good, with a pooled SD across laboratories of 0.08 $\log_{10}$. The variability was much greater for 06/102 and 06/100, with SDs of 0.22 and 0.17 $\log_{10}$ respectively. The inter-laboratory variability was significantly greater than the intra-laboratory variability for 96/798 ($p<0.0001$). However, for 06/102 and 06/100 it was not ($p=0.657$ and $p=0.050$ respectively), as a result of the much higher intra-assay variability for these samples.

Comparison of 2011 (CS458) and 2007 study results

The three CS458 study samples were all included in the 2007 collaborative study carried out to establish the 3rd WHO International Standard for HCV RNA [12]. The overall mean estimates and inter-laboratory variation for both studies are shown in Table 9, for the quantitative assays. The results are based on the direct kit estimates of IU/mL. The individual laboratory mean estimates are shown in Figure 3, with the 2011 results shaded in grey, and the 2007 results in white. From Table 9 it can be seen that the overall means from the 2011 study for 06/102 and 06/100 are lower than those from the original 2007 study, by 0.27 and 0.24 $\log_{10}$ respectively. These differences were statistically significant ($p<0.0001$ and $p=0.0060$ respectively). For 96/798 there is little difference, with the 2011 study mean being 0.05 $\log_{10}$ lower than the 2007 study mean. This was not significant ($p=0.4706$).

This apparent shift for 06/102 and 06/100 can be seen in Figures 3a and 3b, where the 2011 results (shaded grey) are generally in the lower half of the overall distribution of laboratory

results, although still within the overall distribution of results. For 96/798 the 2011 results are more central to the overall distribution (Figure 3c). From Table 9 it can also be seen that for all three study samples the agreement between laboratories for the quantitative assays has improved for the 2011 study, with lower SD's and %GCV's than for the 2007 study.

The overall mean estimates and inter-laboratory variation for both studies for the potencies of 06/102 and 06/100 relative to 96/798 are shown in Table 10. The results from the quantitative and qualitative assays from the 2007 study are shown separately. The distributions of results are shown in Figure 4, for the quantitative assays. The overall means from the 2011 study for both samples are lower than for the 2007 study, by 0.23 and 0.19 log_{10} for 06/102 and 06/100 respectively, for the quantitative assays. Again, these differences were statistically significant ($p=0.0006$ and $p=0.0042$ respectively).

The overall means from the quantitative and qualitative assays in the 2007 study are in reasonably close agreement but the inter-laboratory variation is much higher for the qualitative assays, with the %GCV's 2-fold higher or more. This is expected as the determination of an estimated 'NAT detectable unit' from a dilution series with limited replication is inherently imprecise. The distributions of results relative to 96/798 including qualitative and quantitative assays are shown in Figure 5. Qualitative assay methods are labeled with an asterisk following the assay code. The inclusion of the qualitative assays leads to a greater spread of results, but the overall pattern of a shift in values for the 2011 study is still clear.

Comparison of studies by assay method

To investigate whether the observed differences for 2007 and 2011 studies could be due to the different representation of assay methods between studies, the results from the two most frequently used assays (Abbott RealTime HCV assay and Roche COBAS® AmpliPrep/ COBAS® TaqMan® HCV Test) were grouped and analyzed separately. Table 11 shows the mean of all laboratories using either method in both studies, based on direct kit estimates, while Table 12 gives the equivalent means based on potencies expressed relative to the concurrently tested 96/798.

From Table 11, based on the direct kit estimates of IU, for 06/102 the mean difference between studies was 0.31 log_{10} with the Abbott RealTime HCV assay, and 0.33 log_{10} with the Roche COBAS® AmpliPrep/ COBAS® TaqMan® HCV Test. For 06/100 the differences were 0.22 and 0.30 log_{10} respectively. The differences for 06/102 were statistically significant ($p=0.0030$ and $p=0.0233$ respectively). The differences for 06/100 were significant with the Abbott RealTime HCV assay ($p=0.0296$) but not with the Roche COBAS® AmplPrep/COBAS® TaqMan® Test ($p=0.0969$). The actual observed differences with each assay are of a similar order to each other and to the difference in overall study means. This indicates that the observed overall differences between studies is consistent across these two assay methods and suggests that it is not assay dependent. Differences in the level of significance are likely to be the result of the reduced sample sizes when restricting analysis to a single assay method.

From Table 12, based on the estimates relative to 96/798, for 06/102 the mean difference between studies was 0.15 log_{10} with the Abbott RealTime HCV assay, and 0.21 log_{10} with the Roche COBAS® AmpliPrep/COBAS® TaqMan® Test. For 06/100 the differences were 0.05 and 0.17 log_{10} respectively. None of these were statistically significant. As noted above, the use of relative potencies may increase the inter-laboratory variability in situations where there is already very good inter-laboratory agreement, as is the case with the established HCV assays.

This may explain the lack of statistical significance compared to the results from the direct kit estimates of IU.

Results of additional collaborative study investigation (CS474) of stability during shipping – 'transportation study'

An additional collaborative study was performed to investigate the stability of samples 06/102, 06/100 and 96/798 during shipping at ambient temperature. The results of residual moisture and oxygen testing for these samples, described above, suggested possible ingress of air into the vials since manufacture. It was hypothesized that this may affect stability of the freeze-dried preparations when exposed to elevated temperatures, for example, during shipping. Therefore, a subset of the laboratories that had participated in the 2011 study (CS458) were sent samples of 06/102, 06/100 and 96/798 that had been stored at +20 °C for one week, along with samples that had been stored continually at -20 °C. All samples were shipped to participants on dry ice.

Data Received

Data were returned from five laboratories, two of which used two different assay methods, resulting in seven data sets from quantitative assays. All laboratories returned data from three independent assays, except for laboratory 3, which performed two assays with each of two methods (coded 3A and 3B). All laboratories tested all samples at two dilutions.

For laboratories 3A (+20 °C-heated samples) and 4A (all samples) the estimates obtained at the higher dilution (less concentrated) were around 0.15 $\log_{10}$ higher after correcting for dilution factor than at the more concentrated dilution. All estimates were included in the combined laboratory mean however.

For each laboratory and assay, a single assay mean was calculated as the mean across replicates and dilutions, after correcting for dilution factor, of the estimates of $\log_{10}$ IU/mL. A single laboratory mean was calculated as the mean of the assay means. Intra-laboratory variation was calculated as described in the statistical methods section.

Results

The laboratory mean estimates (based on direct assay kit results) for the heated (+20 °C for one week) and unheated (-20 °C) samples are shown in Tables 13 to 15 for 06/102, 06/100 and 96/798 respectively. The drop ($\log_{10}$) in potency for the +20 °C samples relative to the -20 °C samples and the results obtained by the individual laboratories in the earlier 2011 study (using the same assay methods) are also shown. The mean drops in potency found after one week at +20 °C are 0.34, 0.25 and 0.08 $\log_{10}$ for 06/102, 06/100 and 96/798 respectively. These were all statistically significant ($p<0.001$, $p=0.001$ and $p=0.006$ respectively). However, for 96/798 the drop of 0.08 $\log_{10}$ is small relative to the assay precision. Statistical significance is not a measure of the scientific relevance of any difference.

From the tables, the results for laboratory 3, particularly 3A, are lower than for other laboratories, and for all samples laboratory 3A finds the largest drop in potency for the +20 °C sample. These results contrast with those of laboratory 4B, which uses an identical assay. For the -20 °C samples of 06/102 and 06/100 the differences between the results of laboratories 3A and 4B are around 0.5 – 0.6 $\log_{10}$, using the same assay. The reasons for this are unknown.

For both 06/102 and 06/100 the results for the -20 °C samples shipped on dry ice are higher than the results obtained by the same laboratories with the same assays in the earlier 2011 study

CS458). This suggests that there was a loss of potency during shipping of samples in the 2011 study.

The intra-laboratory (between-assay) variability is shown in Table 16 as the SD of the $\log_{10}$ estimates. The intra-laboratory variability was generally good for all samples stored continuously at -20 °C and shipped on dry ice. There were two instances where the SD was just over 0.2 $\log_{10}$, based on the three assays. However, the pooled figures across laboratories are around 0.1 $\log_{10}$ for 06/102 and 06/100, and 0.05 for 96/798. For the samples stored for one week at +20 °C, the intra-laboratory variability is generally good for 96/798, but for 06/102 it is poor, and worse than for 06/100. This pattern of intra-laboratory variation was also found for the same samples shipped at ambient temperature in the 2011 (CS458) study. This suggests that the loss of potency that occurs for samples 06/100 and particularly 06/102 at elevated temperatures is variable across vials. Overall in this additional transportation study, the inter-laboratory variability was significantly greater than the intra-laboratory variability for all samples ($p<0.0001$, or $p=0.003$ for 06/102 stored at +20 °C for one week).

A summary of the overall mean estimates of $\log_{10}$ IU/mL for 06/102, 06/100 and 96/798 across studies is shown in Table 17, for direct assay kit estimates. Table 18 shows the same summary for estimates relative to 96/798 for sample 06/102 and 06/100. The rows labeled ‘2011’ and ‘2011 subset’ give the overall means of all quantitative assays in the 2011 study, and the means of the subset of laboratories that took part in the latest transportation study (as listed in Tables 13-15). The ‘Transport -20’ row gives the results from the latest round of study for the samples stored at -20 °C and shipped on dry ice.

The results for the ‘2011 subset’ and ‘2011’ studies are very close, indicating that the subset of laboratories who took part in the latest round of study is representative of the results of the full 2011 study.

Results for 96/798, the 2nd WHO International Standard for HCV RNA, are reasonably consistent across all studies, and there was only a small drop of 0.08 $\log_{10}$ on heating at +20 °C for one week. The results from the 2011 and the latest transportation studies are also very close to the assigned value of 5.0 $\log_{10}$ IU/ml for the 2nd International Standard, 96/798.

When results are expressed relative to 96/798, the estimates for the -20 °C samples of 06/102 shipped on dry ice are the same as for the original 2007 study. For 06/100, the results are also much closer to the 2007 study results than the lower potencies seen in the 2011 study. These results indicate that the most likely cause of the apparent loss of potency between the 2007 and 2011 studies is due to the shipping of materials at ambient temperature, rather than an actual loss during long-term storage at -20 °C.

Discussion and conclusions

In this study, a range of NAT-based assays for HCV have been used to evaluate the real-time stability and suitability of the candidate standard as the replacement 4th WHO International Standard for HCV for NAT-based assays. The candidate was prepared from the same bulk as the current 3rd WHO International Standard for HCV in 2006, but was filled and freeze-dried in a separate processing run. The production data suggests that the batch is homogeneous.

In the collaborative study, the candidate 4th International Standard was evaluated alongside the 3rd WHO International Standard for HCV RNA (sample 2, 06/100) and the previous 2nd International Standard (sample 3, 96/798). The overall mean estimate for the 2nd WHO

International Standard for HCV determined by quantitative assays was 5.05 $\log_{10}$ IU/mL. This reflects the long-term stability of this preparation over a period of approximately 15 years. The overall mean estimates for the candidate standard (sample 1) and the current International standard (sample 2), as determined by quantitative assays, were 5.24 and 5.08 $\log_{10}$ IU/mL respectively, based on the calibration of quantitative assay kits in IU/mL. When the results were expressed in IU/mL directly relative to the concurrently tested 2nd WHO International Standard for HCV RNA, the overall mean estimates from the quantitative assays were 5.19 and 5.03 $\log_{10}$ IU/mL respectively. There was good agreement between laboratories (overall range in laboratory mean estimates for study samples 1, 2 and 3 was 0.39, 0.41 and 0.44 $\log_{10}$ IU/mL respectively, for quantitative assays), as expected with established IU-calibrated assays. However, overall mean results for samples 1 and 2 (06/102 and 06/100) were significantly lower than equivalent values determined in the original collaborative study in 2007. Meanwhile, the overall mean estimates for sample 3 (96/798) were not.

The results obtained from ongoing accelerated thermal degradation studies performed in-house after approximately 4.5 years did indicate a possible loss in potency at -20 °C for 06/102, with means of 5.48 and 5.35 $\log_{10}$ IU/mL for the -70 °C and -20 °C samples respectively. However, the loss in potency was on the borderline of statistical significance (p=0.052) based on the three assays alone. The results of accelerated thermal degradation studies reported in the original collaborative study in 2007 suggested that there was some loss in potency for both 06/100 and 06/102 after storage at +4 °C for 10.5 months (0.22 and 0.34 $\log_{10}$ respectively) and at +20 °C for 4 weeks (0.2 and 0.24 $\log_{10}$ respectively) [12]. However, the report concluded that it was not possible to relate these results to predicted stability at -20 °C. The report noted the stability the 2nd WHO International Standard for HCV RNA (96/798) manufactured in 1996, over a period of 9.5 years at -20 °C. Indeed, in this present study, the ongoing stability of this preparation is demonstrated after 15 years at -20 °C. Overall, given the intrinsic variability of results for the NAT assays in use, there is insufficient evidence to demonstrate a loss in potency for 06/102 when stored at -20 °C. The stability of 06/102 during simulated shipping was also investigated in-house, by assaying vials at +4 °C and +20 °C for 1 week with those stored continually at -20 °C. The in-house results showed that the drop in potency upon storage was significant and suggested that the material should be shipped on dry ice.

These results were presented at the XXII Scientific Working Group on the Standardization of Genome Amplification Techniques for the Safety Testing of Blood, Tissues and Organs for Blood Borne Pathogens in Rome in April 2011. The consensus was that an additional collaborative study to further investigate stability during shipping should be performed in order to determine whether the drop in potency determined for 06/102 and 06/100 in the 2011 collaborative study was due to shipment of samples at ambient temperature rather than an actual loss in potency during prolonged storage at -20 °C. The results observed for 06/102 for samples stored continually at -20 °C and shipped on dry ice suggest that the primary cause for the discrepancies between the original 2007 collaborative study and the 2011 study are a loss during shipping for the 2011 study that did not occur during shipping for the 2007 study. Although transport conditions were the same (not shipped on dry ice), the oxygen and moisture levels in both 06/102 and 06/100 may have deteriorated in the intervening time period. The results suggest that 06/102 and 06/100 are not stable at +20 °C, losing between 0.25 and 0.35 $\log_{10}$ after one week. This is similar to the investigation of simulated shipping performed at NIBSC. These results indicate that the loss of potency during transportation is of sufficient magnitude to explain the differences seen between the original 2007 study and the more recent 2011 study. It is therefore proposed that the unitage assigned to 06/102 should be the same as that determined in the original 2007 collaborative study [12].

The matter of commutability [15] of the candidate standard for HCV-positive samples has not been specifically assessed in this study. However, the material is derived from the same source as the 3rd WHO International Standard for HCV and is expected to perform in a similar manner.

This study demonstrates the stability and suitability of the candidate for the replacement 4th WHO International Standard for HCV, when shipped at -20 °C or below. There are ongoing plans to replace this preparation as soon as appropriate stocks of virus can be sourced.

Proposal

It is proposed that the candidate standard, NIBSC code 06/102, is established as the 4th WHO International Standard for HCV for use in NAT-based assays, with an assigned potency of 260,000 IU/mL (~5.41 $\log_{10}$ IU/mL) when reconstituted in 0.5 mL of nuclease-free water. The uncertainty can be derived from the variance of the fill and is 1.5 %. It is also proposed that when established, the standard should be shipped to customers on dry ice, with specific instructions for storage of the material at -20 °C. After collaborative study evaluation and stability assessments 1737 vials are available to the WHO. The proposed standard is intended to be used by IVD manufacturers, blood transfusion centres, control authorities, and clinical laboratories, to calibrate secondary reference materials and in the validation of HCV NAT assays. Proposed Instructions for Use (IFU) for the product are included in Appendix 3.

Comments from participants

Four of seven participants responded to the report. There were no disagreements with the suitability of the candidate standard (NIBSC code 06/102) to serve as the 4th WHO International Standard for HCV for NAT-based assays. Some comments suggested minor editorial changes and these have been implemented.

Acknowledgements

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

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Table 1. Ongoing in-house stability testing of 06/100 and 06/102 after approximately 4.5 years.

Temperature (°C)	Mean log_{10} IU/mL		Difference in log_{10} IU/mL from -70 °C baseline sample	
	06/100	06/102	06/100	06/102
-70	5.22	5.48	-	-
-20	5.28	5.35	0.06	-0.13

Table 2. In-house stability testing of 06/102 after storage at +4 °C and +20 °C for 1 week.

Temperature (°C)	Mean log_{10} IU/mL	Difference in log_{10} IU/mL from -20 °C baseline sample
	06/102	06/102
-20	5.50	-
+4	5.46	-0.04
+20	5.26	-0.24

Table 3. Collaborative study (CS458) assay methods and codes.

Quantitative Assays		
Assay Code	Assay	No. of data sets
AB	Abbott RealTime HCV	3
CTM	COBAS® AmpliPrep/COBAS® TaqMan® HCV Test	3
CTMH	COBAS® TaqMan® HCV Test For Use With The High Pure System	2
bDNA	VERSANT® HCV RNA 3.0 Assay (bDNA)	1
kPCR	VERSANT® HCV RNA 1.0 Assay (kPCR)	1
Qualitative Assays		
Assay Code	Assay	No. of data sets
ULT	PROCLEIX® ULTRIO® Assay	1

Table 4. Laboratory mean absolute (direct kit) estimates. Log_{10} IU/mL for quantitative assays or log_{10} 'NAT detectable units/mL' for qualitative assays. -, not determined.

Lab	Assay type	Assay	Sample		
			S1 06/102	S2 06/100	S3 96/798
1	Qualitative	ULT	-	5.04	5.04
2	Quantitative	CTM	5.39	5.20	5.11
3A		CTM	5.17	5.00	5.13
3B		CTMH	5.00	4.84	5.08
3C		AB	5.19	4.97	4.93
4A		CTM	5.32	5.22	5.15
4B		CTMH	5.19	4.84	5.02
5		AB	5.28	5.25	4.96
6		AB	5.28	5.23	4.93
7A		bDNA	5.24	5.02	4.86
7B		kPCR	5.32	5.22	5.30
Overall Mean (Quantitative)			5.24	5.08	5.05

Table 5. Overall means and inter-laboratory variation – absolute (direct kit) estimates for quantitative assays only (log_{10} IU/mL).

Sample	Assay type	No. of data sets	Mean	SD	%GCV	Min	Max	Range
S1 06/102	Quantitative	10	5.24	0.11	28	5.00	5.39	0.39
S2 06/100	Quantitative	10	5.08	0.16	46	4.84	5.25	0.41
S3 96/798	Quantitative	10	5.05	0.13	36	4.86	5.30	0.44

Table 6. Laboratory mean estimates of potency relative to 96/798 (log_{10} IU/mL). -, not determined.

Lab	Assay type	Assay	Sample S1 06/102	Sample S2 06/100
1	Qualitative	ULT	-	-
2	Quantitative	CTM	5.28	5.09
3A		CTM	5.03	4.87
3B		CTMH	4.92	4.76
3C		AB	5.26	5.03
4A		CTM	5.17	5.07
4B		CTMH	5.17	4.82
5		AB	5.32	5.30
6		AB	5.35	5.29
7A		bDNA	5.38	5.16
7B		kPCR	5.01	4.91
Overall Mean (Quantitative)			5.19	5.03

Table 7. Overall means and inter-laboratory variation – potency relative to 96/798 (log_{10} IU/mL).

Sample	Assay type	No. of data sets	Mean	SD	%GCV	Min	Max
S1 06/102	Quantitative	10	5.19	0.16	43	4.92	5.38
S2 06/100	Quantitative	10	5.03	0.19	54	4.76	5.30

Table 8. Intra-laboratory standard deviation of the individual mean log_{10} IU/mL estimates for quantitative assays for the 2011 study (CS458).

Lab	S1 06/102	S2 06/100	S3 96/798	Overall
2	0.23	0.15	0.02	0.16
3A	0.21	0.02	0.02	0.12
3B	0.25	0.04	0.05	0.15
3C	0.28	0.26	0.10	0.23
4A	0.30	0.01	0.01	0.17
4B	0.31	0.17	0.02	0.20
5	0.11	0.36	0.05	0.22
6	0.12	0.14	0.08	0.12
7A	0.12	0.10	0.16	0.13
7B	0.10	0.15	0.15	0.14
Overall	0.22	0.17	0.08	0.17

Table 9. Comparison of 2007 and 2011 studies: quantitative assays. Overall means and inter-laboratory variability - absolute (direct kit) estimates (log_{10} IU/mL).

Sample	Study and assay type	No. of data sets	Mean	SD	%GCV	Min	Max
S1 06/102	2011 Study - Quantitative	10	5.24	0.11	28	5.00	5.39
	2007 Study - Quantitative	25	5.51	0.17	48	5.18	5.92
S2 06/100	2011 Study - Quantitative	10	5.08	0.16	46	4.84	5.25
	2007 Study - Quantitative	25	5.32	0.24	74	4.62	5.73
S3 96/798	2011 Study - Quantitative	10	5.05	0.13	36	4.86	5.30
	2007 Study - Quantitative	25	5.10	0.19	55	4.74	5.50

Table 10. Comparison of 2007 and 2011 studies: quantitative assays. Overall means and inter-laboratory variability - potency relative to 96/798 (log_{10} IU/mL).

Sample	Study and assay type	No. of data sets	Mean	SD	%GCV	Min	Max
S1 06/102	2011 Study - Quantitative	10	5.19	0.16	43	4.92	5.38
	2007 Study - Quantitative	25	5.42	0.16	44	5.01	5.64
	2007 Study - Qualitative	14	5.40	0.28	90	4.86	5.99
S2 06/100	2011 Study - Quantitative	10	5.03	0.19	54	4.76	5.30
	2007 Study - Quantitative	25	5.22	0.16	45	4.81	5.48
	2007 Study - Qualitative	13	5.12	0.34	119	4.54	5.73

Table 11. Comparison of 2007 and 2011 studies by assay kit. Absolute (direct kit) estimates (quantitative log_{10} IU/mL).

Sample	Study	Abbott RealTime assay		Roche COBAS® AmpliPrep/ COBAS® TaqMan ® HCV Test	
		No.	Mean	No.	Mean
S1 06/102	2011	3	5.25	3	5.29
	2007	7	5.56	6	5.62
S2 06/100	2011	3	5.15	3	5.14
	2007	7	5.37	6	5.44

Table 12. Comparison of 2007 and 2011 studies by assay kit. Potencies relative to 96/798 (log_{10} IU/mL).

Sample	Study	Abbott RealTime assay		Roche COBAS® AmpliPrep/ COBAS® TaqMan ® HCV Test	
		No.	Mean	No.	Mean
S1 06/102	2011	3	5.31	3	5.16
	2007	7	5.46	6	5.37
S2 06/100	2011	3	5.21	3	5.01
	2007	7	5.26	6	5.18

Table 13. Comparison of -20 °C and +20 °C (1 week) 06/102 samples shipped on dry ice.
*Only two independent assays were performed for 3A and 3B during the transportation study.

Lab	Assay	Study 2011	Transportation		
			-20 °C	+20 °C	Drop
2	CTM	5.39	5.48	5.25	0.23
3A	CTMH	5.00	5.08*	4.52*	0.57
3B	AB	5.19	5.38*	5.04*	0.35
4A	CTM	5.32	5.64	5.41	0.24
4B	CTMH	5.19	5.56	5.20	0.35
5	AB	5.28	5.44	5.14	0.29
7	bDNA	5.24	5.47	5.10	0.38
Mean		5.23	5.44	5.09	0.34

Table 14: Comparison of -20 °C and +20 °C (1 week) 06/100 samples shipped on dry ice.
*Only two independent assays were performed for 3A and 3B during the transportation study.

Lab	Assay	Study 2011	Transportation		
			-20 °C	+20 °C	Drop
2	CTM	5.20	5.33	5.13	0.20
3A	CTMH	4.84	4.69*	4.34*	0.35
3B	AB	4.97	5.10*	4.86*	0.25
4A	CTM	5.22	5.44	5.21	0.23
4B	CTMH	4.84	5.28	4.94	0.33
5	AB	5.25	5.26	4.96	0.30
7	bDNA	5.02	5.13	5.07	0.06
Mean		5.05	5.17	4.93	0.25

Table 15: Comparison of -20 °C and +20 °C (1 week) 96/798 samples shipped on dry ice.
*Only two independent assays were performed for 3A and 3B during the transportation study.

Lab	Assay	Study 2011	Transportation		
			-20 °C	+20 °C	Drop
2	CTM	5.11	5.19	5.10	0.09
3A	CTMH	5.08	4.83*	4.66*	0.17
3B	AB	4.93	4.98*	4.90*	0.08
4A	CTM	5.15	5.20	5.18	0.02
4B	CTMH	5.02	5.06	5.00	0.07
5	AB	4.96	4.94	4.86	0.08
7	bDNA	4.86	4.96	4.92	0.04
Mean		5.02	5.02	4.94	0.08

Table 16: Intra-laboratory standard deviation of the individual mean $\log_{10}$ IU/mL estimates for quantitative assays for the transportation study (CS474). *Only two independent assays were performed for 3A and 3B.

Lab	Stored at -20 °C			1 week at +20 °C			Overall
	S1 06/102	S2 06/100	S3 96/798	S4 06/102	S5 06/100	S6 96/798	
2	0.23	0.12	0.07	0.24	0.08	0.09	0.16
3A*	0.01	0.07	0.07	0.51	0.03	0.02	0.21
3B*	0.01	0.11	0.05	0.30	0.07	0.03	0.14
4A	0.05	0.02	0.02	0.31	0.08	0.04	0.13
4B	0.01	0.02	0.03	0.19	0.10	0.05	0.09
5	0.07	0.04	0.02	0.11	0.07	0.01	0.06
7	0.05	0.21	0.08	0.20	0.27	0.16	0.18
Overall	0.10	0.11	0.05	0.29	0.12	0.07	

Table 17: Summary of quantitative results; absolute assay estimates.

Study	No. of data sets	S1 06/102	S2 06/100	S3 96/798
2007	25	5.51	5.32	5.10
2011	10	5.24	5.08	5.05
2011 subset	7	5.23	5.05	5.02
Transport -20 °C	7	5.44	5.17	5.02

Table 18: Summary of quantitative results; potencies relative to S3, 96/798.

Study	No. of data sets	S1 06/102	S2 06/100
2007	25	5.42	5.22
2011	10	5.19	5.03
2011 subset	7	5.21	5.03
Transport -20 °C	7	5.41	5.15

Figure legends

Figure 1. Individual laboratory mean estimates for study samples 1 (06/102) (a), 2 (06/100) (b) and 3 96/798 (c) from quantitative NAT assays. Units are $\log_{10}$ IU/mL. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code.

Figure 2. Individual laboratory mean relative potency estimates for study samples 1 (06/102) (a) and 2 (06/100) (b), relative to sample 3 (96/798), from quantitative NAT assays. Units are $\log_{10}$ IU/mL. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code.

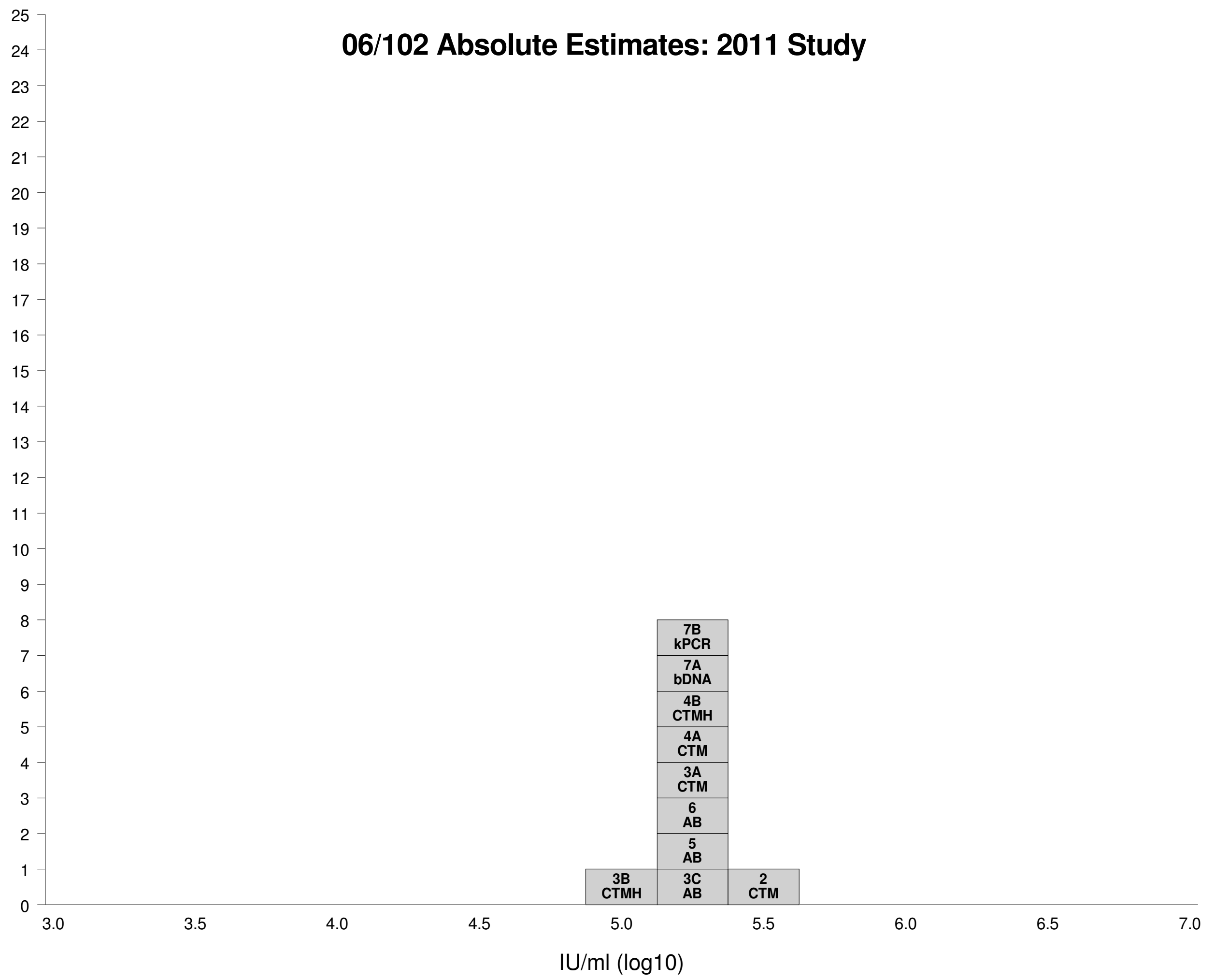
Figure 3. Individual laboratory mean estimates for study samples 06/102 (a), 06/100 (b) and 96/798 (c) from quantitative NAT assays from both 2007 and 2011 collaborative studies. Units are $\log_{10}$ IU/mL. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code. Results from the 2011 study are shaded in grey and results from the 2007 study are shaded white.

Figure 4. Individual laboratory mean relative potency estimates for samples 06/102 (a) and 06/100 (b), relative to 96/798, from quantitative and qualitative NAT assays from both 2007 and 2011 collaborative studies. Units are $\log_{10}$ IU/mL. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code. Results from the 2011 study are shaded in grey and results from the 2007 study are shaded white. Qualitative assay methods are labeled with an asterisk after the assay code.

Figure 5. Individual laboratory mean relative potency estimates for samples 06/102 (a) and 06/100 (b) relative to 96/798 from quantitative and qualitative NAT assays from both 2007 and 2011 collaborative studies. Units are $\log_{10}$ IU/mL. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory and assay code. Results from the 2011 study are shaded in grey and results from the 2007 study are shaded white. Qualitative assay methods are labeled with an asterisk after the assay code.

Figure 1

a



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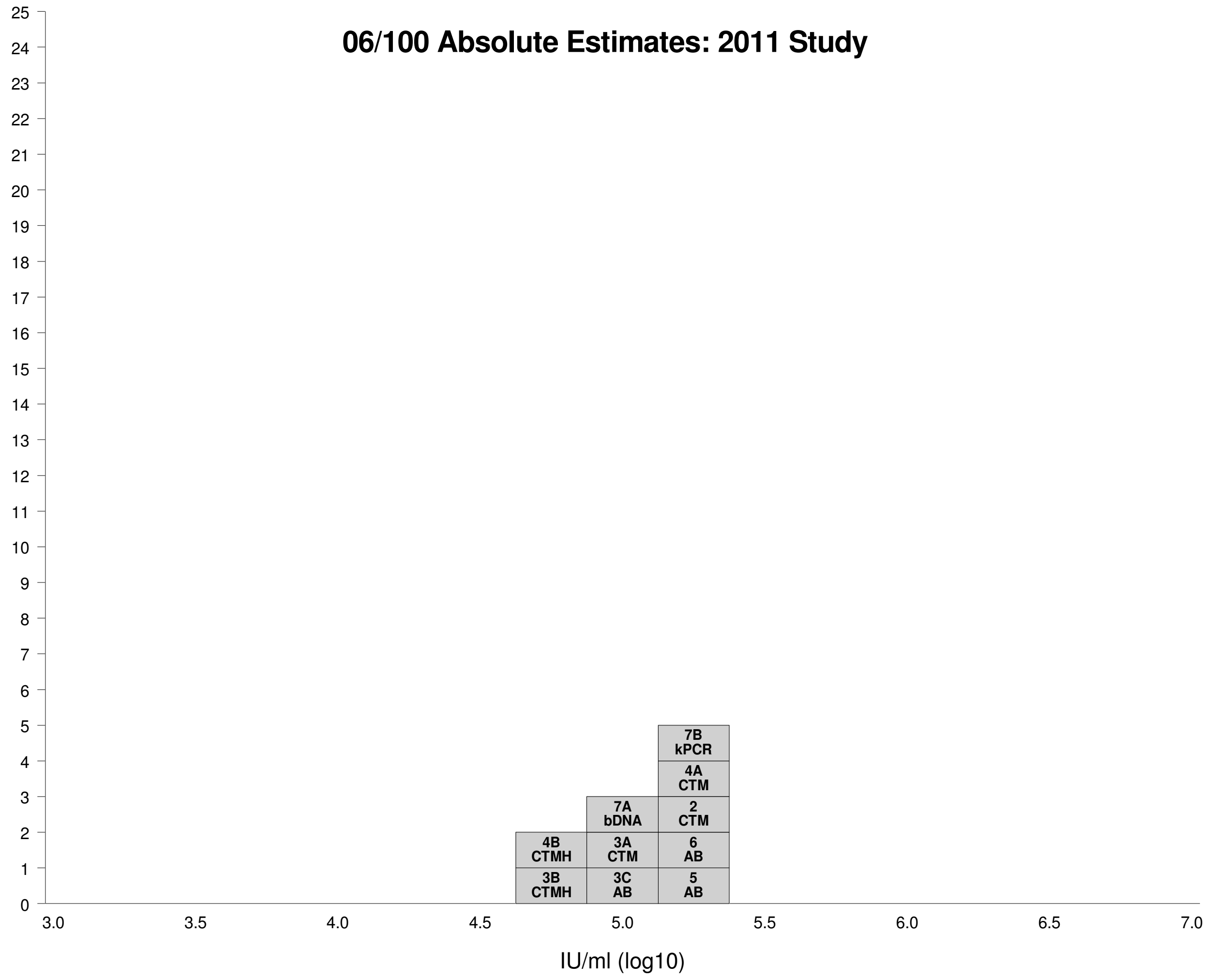

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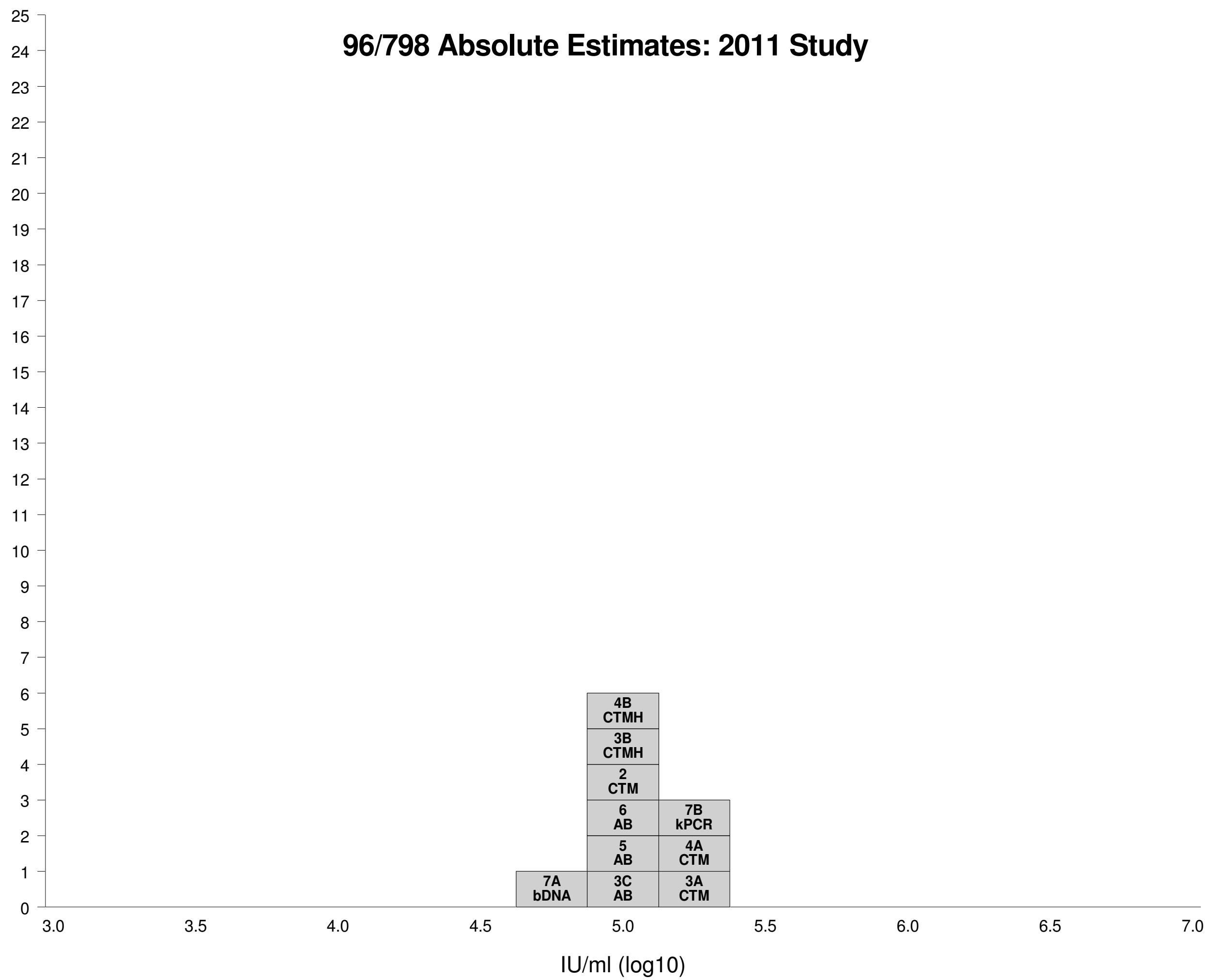

Figure 2

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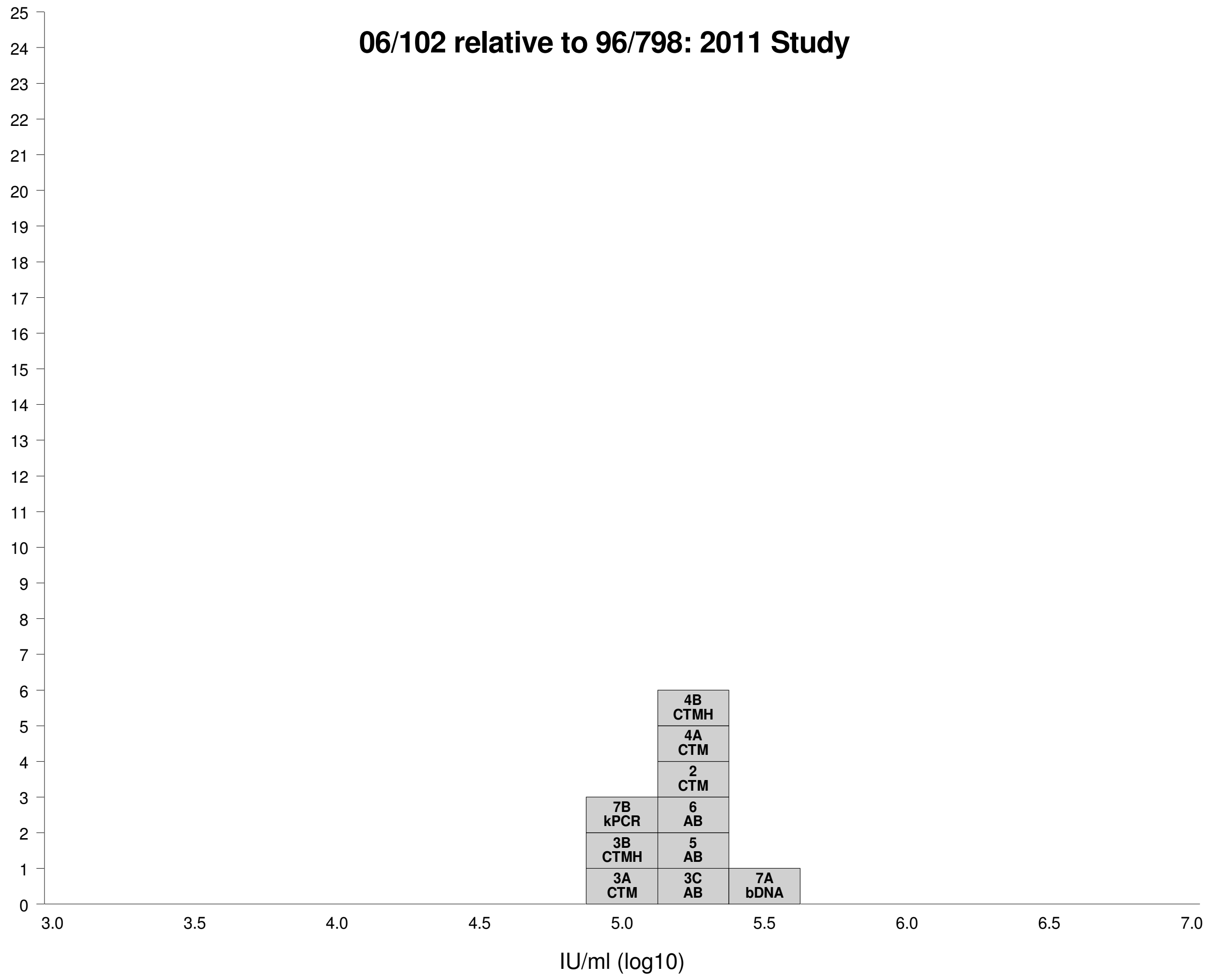

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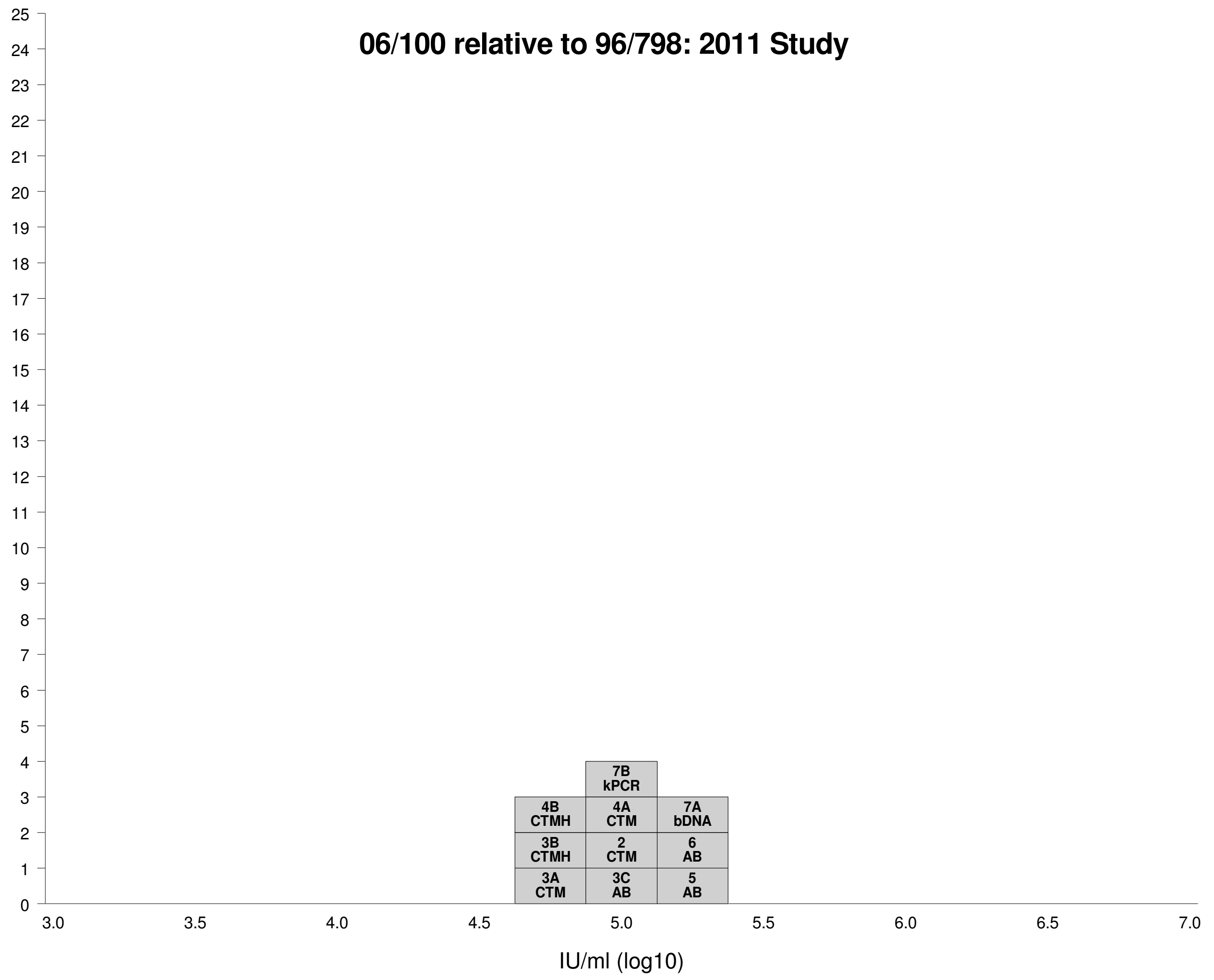

Figure 3

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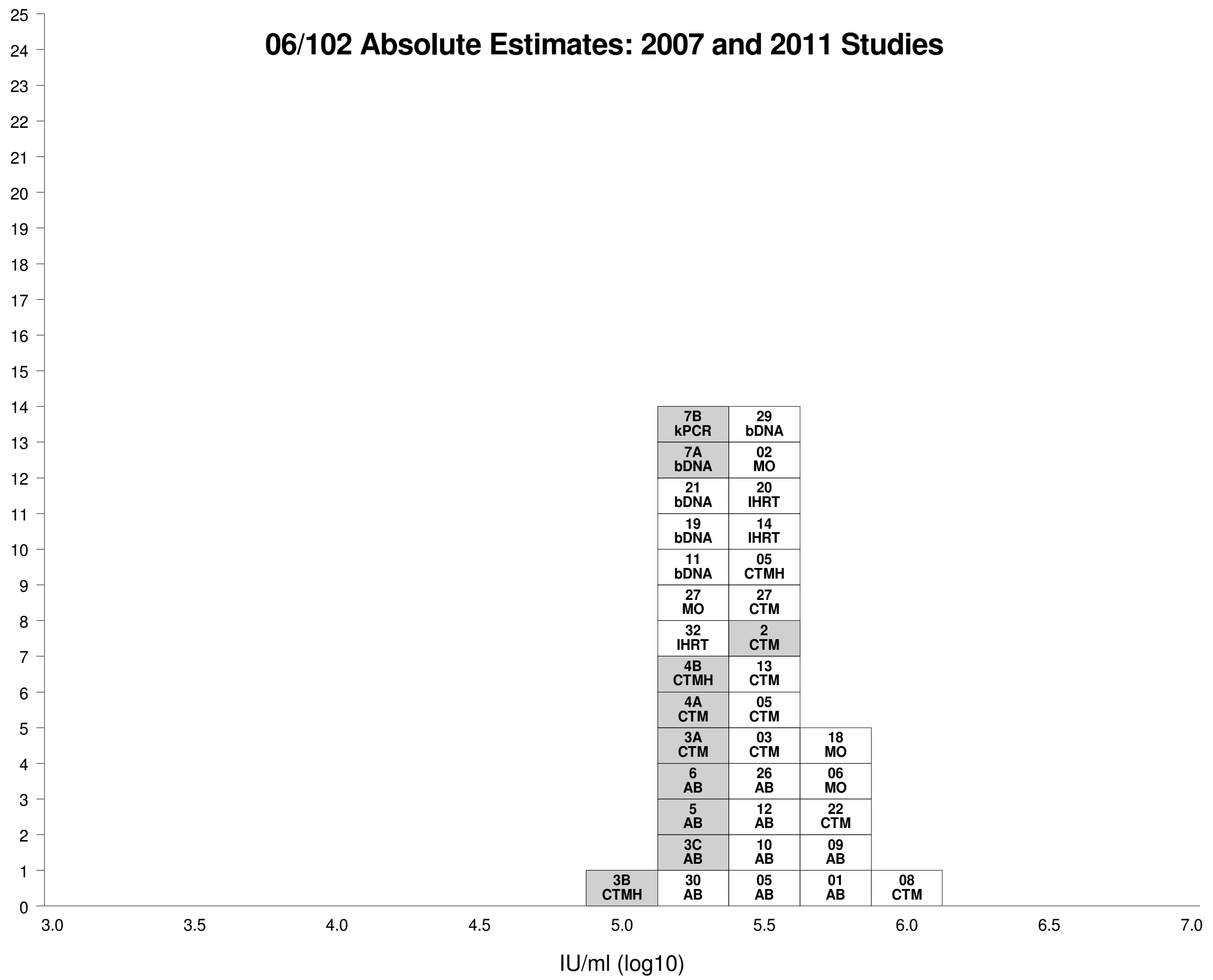

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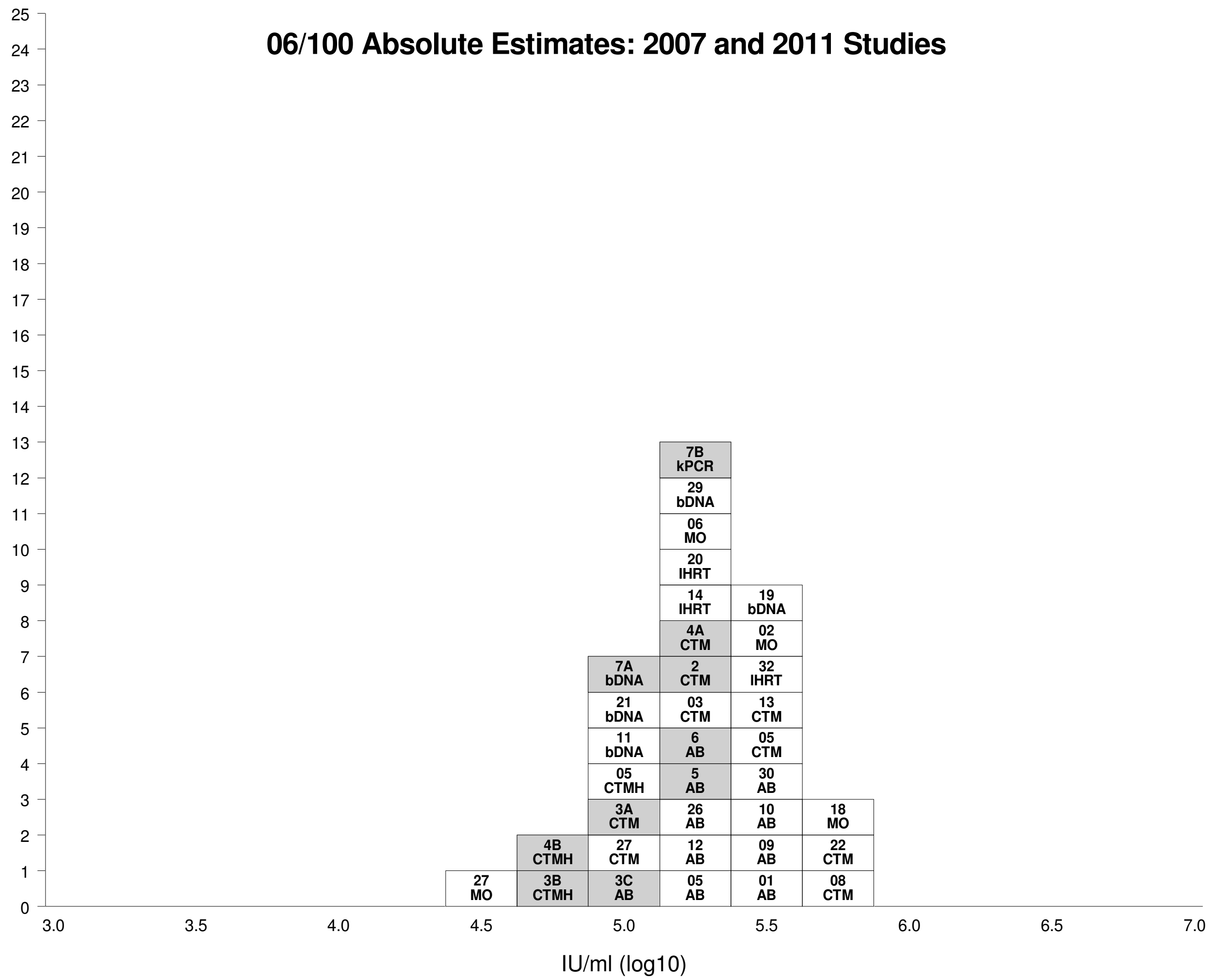

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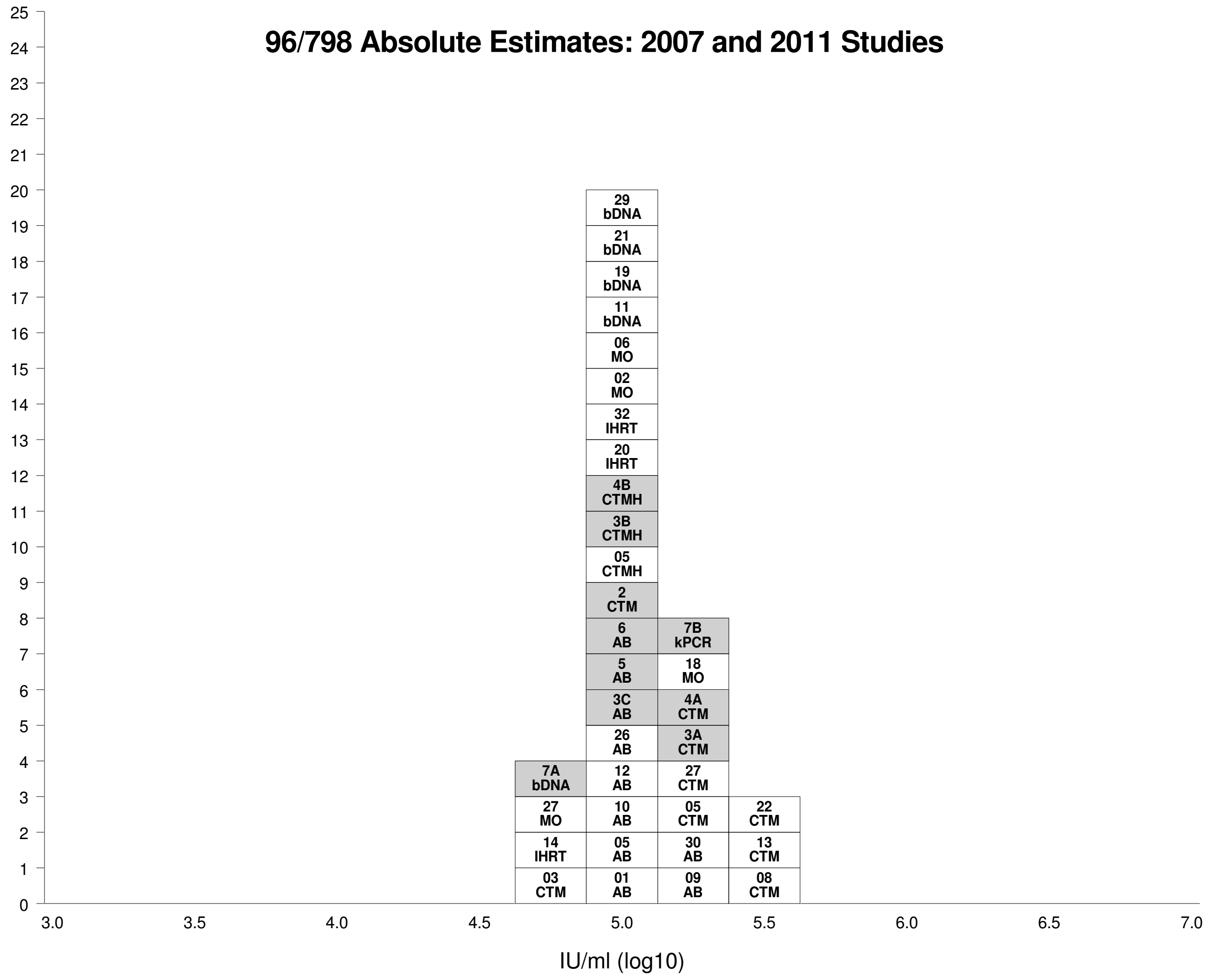

Figure 4

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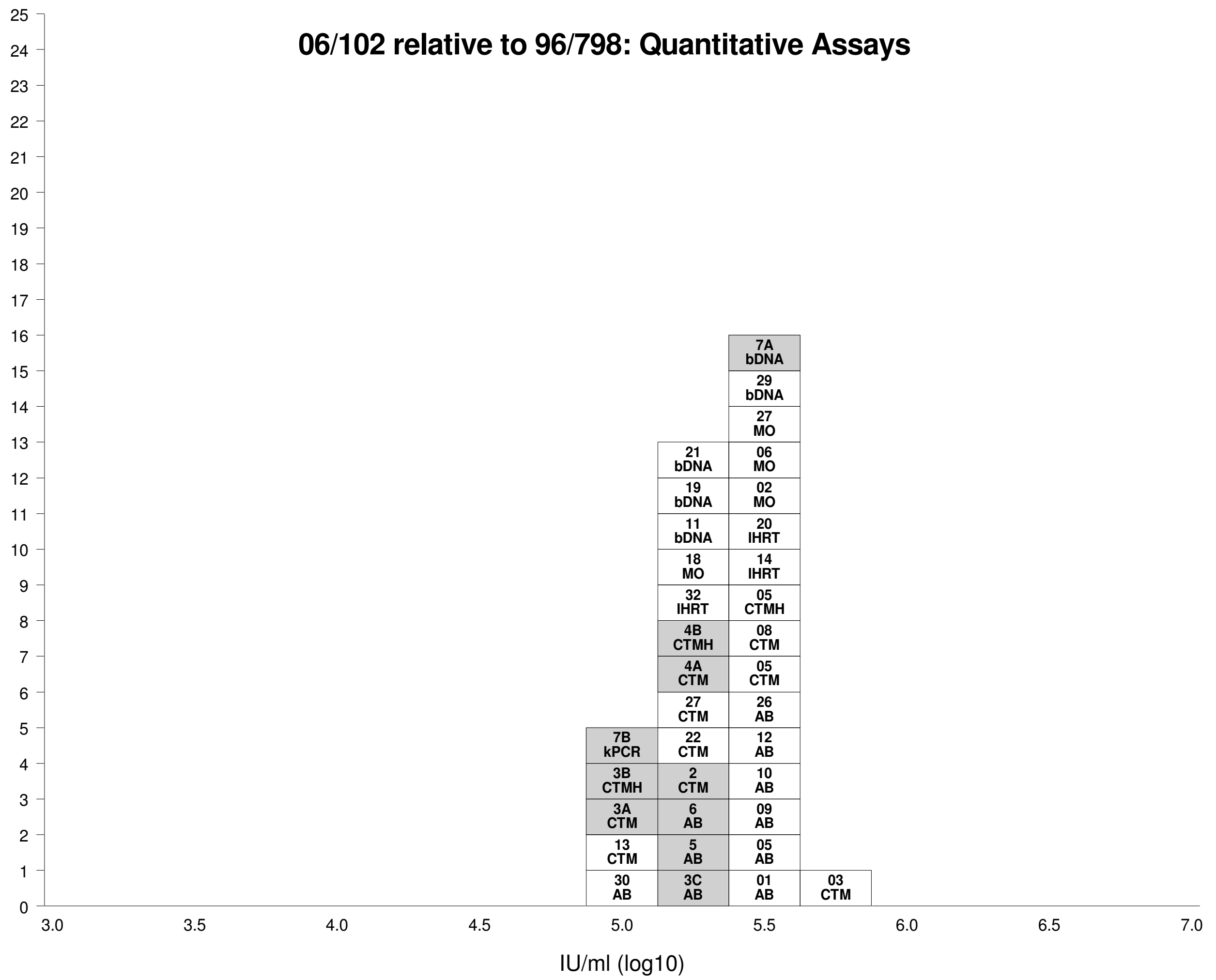


b

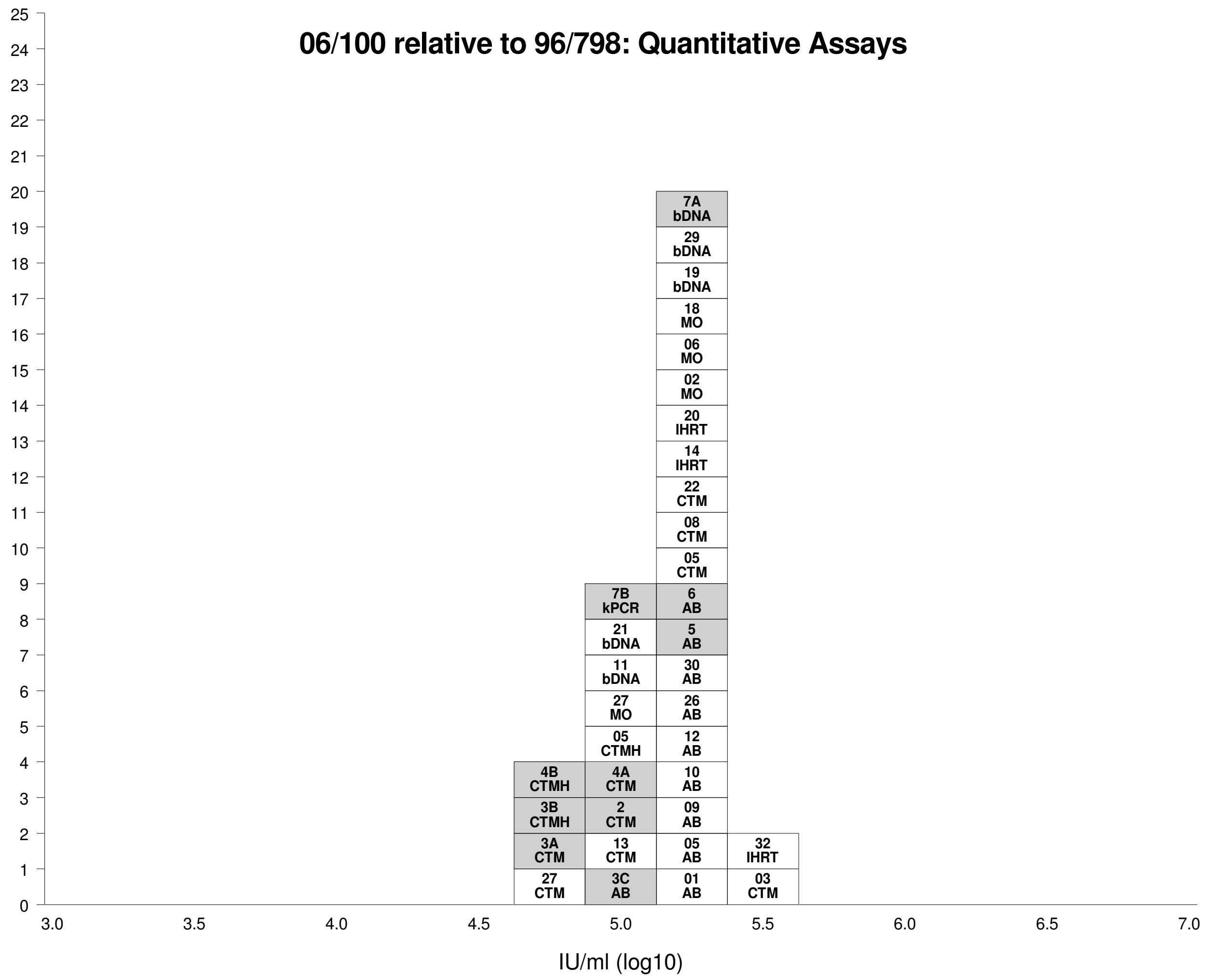
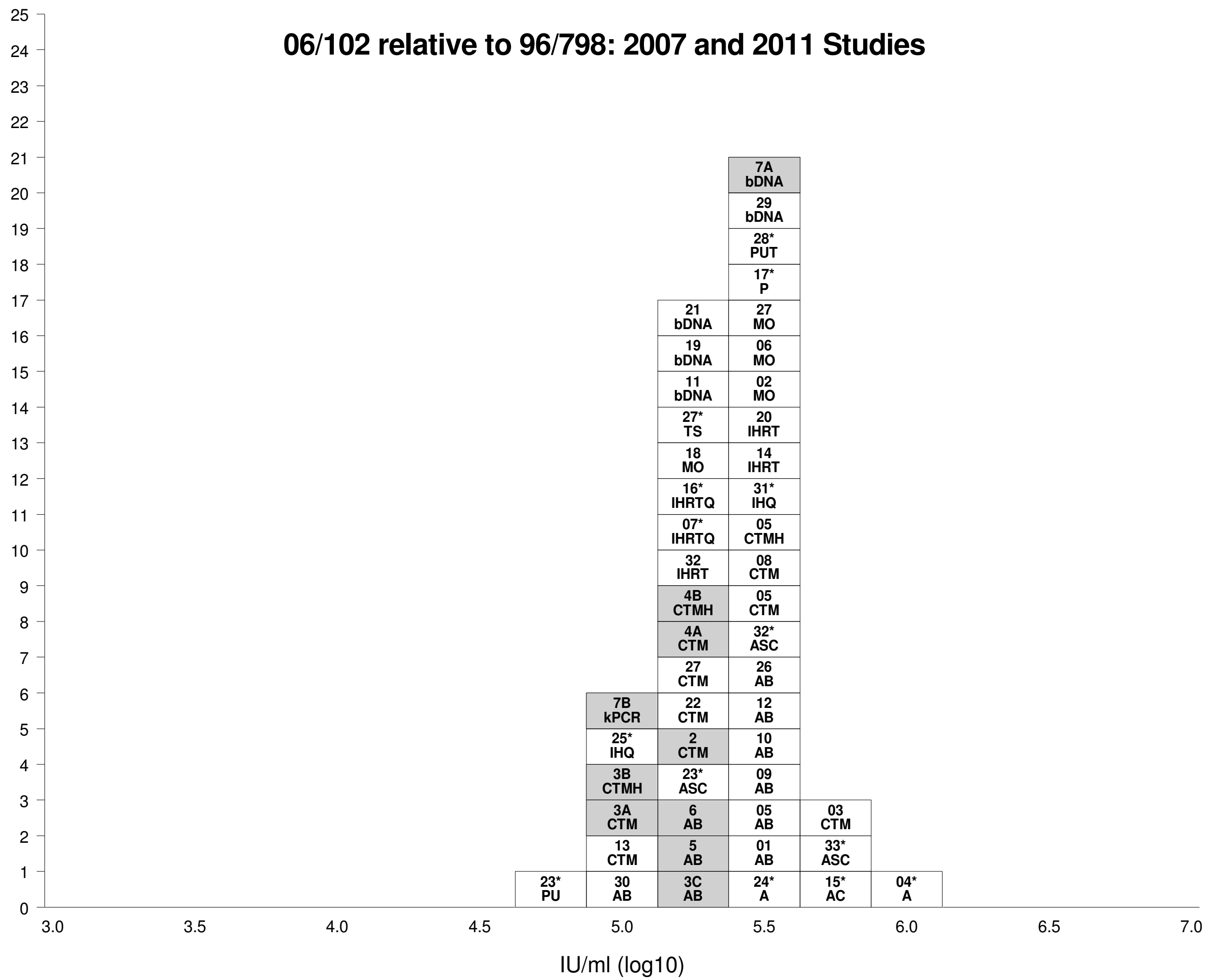
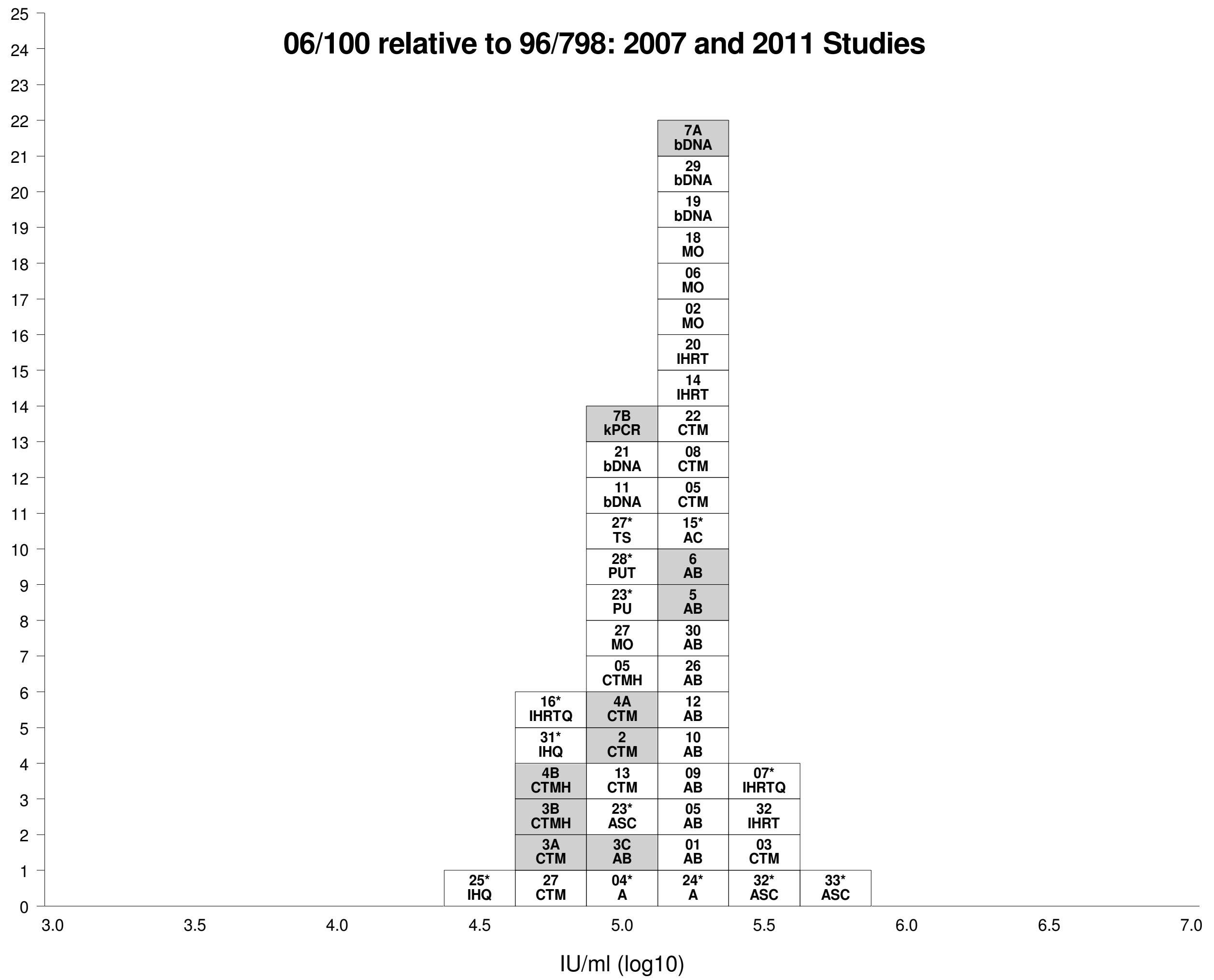


Figure 5

a



b



Appendix 1

Collaborative study participants

(In alphabetical order by country)

Name	Laboratory	Country
Prof. Jean-Michel Pawlotsky Dr. Stephane Chevaliez	French National Reference Center for Hepatitis B, C and Delta, Créteil	France
Dr. Micha Nübling Dr. Michael Chudy Dr. Julia Kress	Paul-Ehrlich-Institut, Langen	Germany
Dr. HTM Cuÿpers Dr. Marco Koppelman	Sanquin Diagnostic Services, Amsterdam	The Netherlands
Stephen Dicks Dr. Alan Kitchen	The National Transfusion Microbiology Reference Laboratory (NTMRL), NHS Blood and Transplant (NHSBT), London	UK
Dr. George Schneider William Caminiti	Abbott Molecular Inc., Des Plaines	USA
Dr. Zhuang Wang Dr. Sung Lee	Roche Molecular Systems (Branchburg and Pleasanton sites)	USA
Dr. Jennifer Chen Dr. Gregg Gorrin	Siemens Clinical Laboratory, Berkeley	USA

Appendix 2

Study protocol

Collaborative study to evaluate the candidate 4th WHO International Standard for hepatitis C virus (HCV) for NAT-based assays

Study Protocol

Background and outline of the study

The World Health Organisation (WHO) Expert Committee on Biological Standardisation (ECBS) has endorsed a proposal to replace the 3rd WHO International Standard for hepatitis C virus (HCV) for NAT-based assays in order to ensure continued supply of this reference material.

A replacement freeze-dried preparation (NIBSC code 06/102) was prepared from the same bulk as the current International Standard (NIBSC code 06/100), and was characterised in the original collaborative study in 2007 [1]. This small scale study now aims to examine the potency and real-time stability of 06/102 by comparison with the current and previous HCV International Standard.

Three lyophilised preparations are to be evaluated. Three vials of each study sample are provided. Participants are asked to test dilutions of each sample, using their routine HCV NAT assay, on three separate occasions. Where possible, we would encourage laboratories to use quantitative methods, however, data from qualitative assays will also be acceptable.

Study samples

Study samples comprise three lyophilised preparations in 2 mL crimp top glass vials, and are coded; sample 1, sample 2, and sample 3. Three vials of each study sample are provided for evaluation on three separate occasions. Upon receipt, all samples should be stored at -20 °C or below.

CAUTION: Study samples 1, 2 and 3, contain infectious HCV and should be handled only in appropriate containment facilities by fully trained and competent staff in accordance with national safety guidelines. These preparations contain human plasma, which has been tested and found negative for HBsAg, and HIV antibody. Care should be taken when opening vials to avoid cuts. See instructions for use for further details.

Study protocol

Participants are requested to test dilutions of each study sample, using their routine HCV NAT assay, on three separate occasions.

Prior to each assay run, samples 1-3 must be reconstituted with 0.5 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use.

Dilutions should be prepared in the diluent normally used in the assay system (e.g. anti-HCV and HCV RNA negative human plasma).

Each sample must be extracted prior to amplification.

For each independent assay, study samples 1, 2, and 3 should be tested within the same assay run. Independent assays should be performed on separate days, using a fresh vial of each sample.

Below, are specific instructions for the dilution and testing of study samples, using either quantitative or qualitative assays.

For quantitative assays:

For each of three assays, participants are requested to test each sample, at a minimum of two serial ten-fold dilutions within the linear range of the assay (e.g. 10^{-1} and 10^{-2}). If practicable, please test as many replicates as possible of each dilution of each sample within the same assay run.

For qualitative assays:

For each of three assays, participants are requested to test the dilution at the assay end-point (limit of detection), and a minimum of two half-log serial dilutions either side of the end-point (i.e., at least five dilutions in total). If practicable, please test as many replicates as possible of each dilution of each sample within the same assay run.

NB: Samples 1-3 contain approximately $1x10^5$ IU/mL HCV RNA when reconstituted in 0.5 mL nuclease-free water.

Reporting of results

The results of each assay (HCV concentration in IU/mL or qualitative result; positive / negative) and methodology used, should be recorded on the Result Reporting Form accompanying the samples. Where applicable, please also include the crossing point / threshold cycle for each result. Results should be returned to NIBSC **as soon as possible and before 25th February 2011**, to allow sufficient time for statistical analysis and preparation of the final report for submission to the WHO Expert Committee for Biological Standardisation by July 2011.

The data should not be published or cited before the formal establishment of the standard by the WHO ECBS, without the expressed permission of the NIBSC study organiser.

All completed Result Reporting forms should be returned electronically to Dr J Fryer:
Jacqueline.Fryer@nibsc.hpa.org.uk

Alternatively, results may be mailed or faxed to:

Address: Dr J. Fryer, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG.

Fax: +44 (0)1707 641366

Data analysis

All data from the study will be analysed at NIBSC. The analysis will assess the concentration of each sample, relative to each other, and the sensitivities of the different assay methods. Individual participants' data will be coded and reported "blind" to other participants during the preparation of the study report, and also in subsequent publications. Participants will receive a copy of the report of the study and proposed conclusions and recommendations for comment before it is further distributed. It is normal practice to acknowledge participants as contributors of data rather than co-authors in publications describing the establishment of the standard.

References

1. Baylis SA, Heath AB and the collaborative study group. WHO collaborative study to establish a replacement WHO International Standard for hepatitis C virus RNA nucleic acid amplification technology (NAT)-based assays. WHO ECBS Report 2007; WHO/BS/07.2055.

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A World Health Organization Laboratory for Biological Standards

World Health Organization

Appendix 3

Proposed instructions for use

WHO International Standard
4th WHO International Standard for Hepatitis C Virus for Nucleic Acid Amplification Techniques
NIBSC code: 06/102
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE
The 4th WHO International Standard for hepatitis C virus (HCV), NIBSC code 06/102, is intended to be used in the standardization of nucleic acid amplification technology (NAT)-based assays for HCV. The standard comprises genotype 1a HCV antibody-negative, HCV RNA-positive plasma, diluted in pooled human plasma. The virus stock was tested and found negative for HIV-1 RNA, HBV DNA, HAV RNA and parvovirus B19 DNA. The pooled human plasma diluent was sourced from blood donations and had been tested and found negative for HIV antibody, HCV antibody, HBsAg, syphilis antibody, HTLV antibody, as well as HIV and HCV RNA. The standard has been lyophilized in 0.5 mL aliquots and stored at -20 °C. The material has been calibrated in International Units (IU), in parallel with the 2nd and 3rd WHO International Standards for HCV [1,2].

2. CAUTION
<u>**This preparation is not for administration to humans**</u>.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg and HIV antibody. 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 should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE
This material has been assigned a unitage of 260,000 IU/mL (~5.41 log10 IU/mL) when reconstituted in 0.5 mL of nuclease-free water.

4. CONTENTS
Country of origin of biological material: United Kingdom.
Each vial contains 0.5 mL of lyophilized plasma containing infectious HCV.

5. STORAGE
Vials of lyophilized standard should be stored at -20 °C.

6. DIRECTIONS FOR OPENING
Vials have a 'flip-up' circular cap. Either on the cap or the collar of the vial, there is an indication of the point at which to lever off the cap. This exposes an area of the stopper through which reconstitution and withdrawal of the preparation can be made using a hypodermic needle and syringe. If use of a pipette is preferred, then fully remove the metal collar using, for example, forceps, taking care to avoid cuts by wearing appropriate gloves. Remove the stopper for access. Care should be taken to prevent loss of the contents.

7. USE OF MATERIAL
<u>No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution</u>
The material should be reconstituted with 0.5 mL of deionized, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use. The reconstituted material has a final concentration of 260,000 IU/mL.

The International Standard should be used to calibrate secondary reference materials, for example, by determining the equivalent concentration of secondary reference reagent being calibrated, against the International Standard, in parallel. The secondary reference reagent can then be assigned a concentration in IU. Once reconstituted, the International Standard should be diluted in the matrix appropriate to the material being calibrated, and should be extracted prior to HCV RNA measurement.

8. STABILITY
Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. The stability of 06/102 when reconstituted has not been specifically determined. Therefore, it is recommended that the standard is for single use only

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES
1. Baylis SA, Heath AB and the Collaborative Study Group. WHO collaborative study to establish a replacement WHO International Standard for Hepatitis C virus RNA nucleic acid amplification technology (NAT)-based assays. WHO ECBS Report 2007;WHO/BS/07.2055.
2. Fryer JF, Heath AB, Wilkinson DE, Minor PD and the collaborative study group. Collaborative study to evaluate the proposed 4th WHO International Standard for hepatitis C virus (HCV) for nucleic acid amplification technology (NAT)-based assays. WHO ECBS Report 2011;WHO/BS/2011.XXXX.

10. ACKNOWLEDGEMENTS
We gratefully acknowledge the important contributions of the collaborative study participants.

11. FURTHER INFORMATION
Further information can be obtained as follows;
This material:
enquiries@nibsc.hpa.org.uk
WHO Biological Standards:
Http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
Http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.who.int/biologicals/reference_preparations/en/
Ordering standards from NIBSC:
Http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
Http://www.nibsc.ac.uk/terms_and_conditions.aspx

12. 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 enquiries@nibsc.hpa.org.uk

13. CITATION
In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: Lyophilized powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No

Flammable:	No	Handling:See caution, Section 2
Other (specify):	Contains human plasma and infectious HCV	

Toxicological properties

Effects of inhalation:	Avoid, contains infectious HCV
Effects of ingestion:	Avoid, contains infectious HCV
Effects of skin absorption:	Avoid, contains infectious HCV

Suggested First Aid

Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal

Spillage of ampoule 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.

15. 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 standards or reference 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 dependant 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 contract 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.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.5 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached**: No