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Collaborative Study to Establish the First WHO International Standard for Detection of Hepatitis C Virus Core Antigen

Sigrid Nick, Peter Volkers, Stefan Ross*, Heinrich Scheiblauer
and the Collaborative Study Group**

Prüflabor für In-vitro Diagnostic Medical Devices (IVD) and Division of Biostatistics, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany

**Institute of Virology, National Reference Centre for Hepatitis C, Essen University Hospital, University of Duisburg Essen, Hufelandstr. 55, D-45147 Essen, Germany*

***See Appendix 1*

Study Coordinator: Tel +49-6103-777008, Fax +49-6103-771234
e-mail: Sigrid.Nick@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 **4 October 2014** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Department of Essential Medicines and Health Products (EMP). Comments may also be submitted electronically to the Responsible Officer: **Dr David Wood** at email: *woodd@who.int* .

Summary

A Collaborative Study was carried out to develop an international standard for Hepatitis C virus (HCV) core antigen for use with HCV core antigen detecting assays. The candidate standard is a lyophilized plasma preparation obtained from a donor infected with HCV genotype 1a. Twelve laboratories from nine countries world-wide participated in the collaborative study to evaluate and characterize the candidate material (sample A) using the assays in routine use in their laboratory alongside with the corresponding liquid-frozen bulk material (sample B) and four liquid frozen neat and diluted HCV core antigen positive plasma specimen (samples C to F). Six assays were used including two quantitative and one qualitative HCV Ag assays as well as three qualitative HCV Ag/Ab combination assays. The assays´ analytical sensitivities were determined to assess the potency and compared to the corresponding liquid bulk material. The results showed considerable differences in analytical sensitivity between the various assays yielding an endpoint titer range from 1:2 for the least sensitive assay to 1:3200 for the most sensitive assay. The same differences in sensitivity between the assays as with the candidate standard A were reflected by samples C to F. Intra-laboratory and inter-laboratory variability for sample A and the bulk material sample B were in an expected range for immunoassays and it can therefore be assumed that the candidate material was still homogenous after the lyophilization step.

To support commutability of the candidate standard, a complementary study was performed with a selected number of representative assays using low, medium and high HCV core antigen positive clinical samples from early HCV infection (seroconversion). This additional study demonstrated that seroconversion sensitivity is correlated to analytical sensitivity as determined with the candidate standard sample A.

Overall, the study results show that there was good comparability of sensitivity for HCV core antigen between the various assays and laboratories by the candidate standard sample A. The potency of the candidate standard material is however dependent on the sensitivity of the test kits. It was therefore decided to determine the units based on highest sensitivity

Accelerated and on-going real-time stability studies of the proposed 1st WHO International Standard for HCV core antigen indicate that the preparation is stable and suitable for long-term use when stored as recommended.

It is therefore proposed that the candidate material (PEI code 129096/12) is established as the 1st WHO International Standard for HCV core antigen for use with HCV core antigen assays with an assigned potency of 3,200 International Units per mL (IU/mL) when reconstituted in 0.5 mL of distilled water.

Part 1:

1. Collaborative study report

1.1. Introduction

Hepatitis C virus (HCV) infections represent an enormous global health care burden. The severity of disease may vary from a mild illness lasting a few weeks only to a serious, lifelong condition that can finally lead to liver cirrhosis or hepatocellular carcinoma (HCC). Hepatitis C virus is primarily transmitted by blood transfusion, hemodialysis, intravenous drug use and tattooing. About 150 million individuals world-wide are chronically infected with hepatitis C virus, and more than 350 000 people die every year from hepatitis C-related liver damage [1]. The incidence of liver cirrhosis and subsequently HCC ranges from 15% to 35% 25–30 years after HCV infection and is highest among recipients of HCV-contaminated blood products and hemophiliacs and lowest among women who became infected by a contaminated anti-D Immunoglobulin prophylaxis.

Hepatitis C virus is a small enveloped 55-65 nm virus with a single-stranded 9.6 kb positive sense RNA as genome that belongs to the *Flaviviridae* family. Until 1989 all pathogens causing liver disease which could not be identified as either hepatitis A virus or hepatitis B virus were classified as non-A, non-B hepatitis viruses. Hepatitis C virus was finally discovered in 1989 [2] and a first target antigen (c-100) for HCV antibody detection was soon identified thereafter [3]. HCV is a very variable virus for which 7 major genotypes and a series of 67 subtypes have been described until now [4]. The genotypes differ in 30-35% of nucleotide sites over the complete genome. The difference in genomic composition of subtypes of genotypes is estimated to be 20-25% [5]. Viral proteins are cleaved from a 3000 amino acid polyprotein precursor into at least 10 different proteins. The structural proteins are the 22 kDa core protein (C), the envelope proteins E1 and E2 and p7 and the non-structural proteins are NS2, NS3, NS4A, NS4B, NS5A and NS5B.

The HCV core protein forms the internal viral capsid. It contains three domains: a basic and hydrophilic region (domain I; residues 1 to 118), a C terminal hydrophobic domain (domain II; residues 119 to 173), and a hydrophobic signal sequence (domain III; residues 174 to 191) [6, 7]. The protein is highly conserved and constitutes an efficient target for HCV specific antibodies.

Diagnosis of HCV infection includes detection of anti-HCV antibodies and in blood screening also detection of HCV RNA. Recently, sensitive qualitative and quantitative HCV core-detecting assays as well as several HCV Ag/Ab combination assays have become available. HCV Ag/Ab assays provide a useful improvement for earlier detection of HCV infection by serological methods [8, 9]. HCV core antigen assays show a performance comparable to that of commercially available viral load assays and thus appear to be a highly suitable alternative for monitoring of the therapeutic efficacy of antiviral treatment. Due to the high sensitivity, these tests seem also appropriate for the screening of blood donations and thus may contribute to the improvement of health and blood safety [10, 11].

Hepatitis C can be treated successfully and continuous therapeutic progress has been achieved with new drugs becoming available recently [12]. For determining the efficiency of HCV therapy and its end-point, viral load assays by nucleic acid amplification techniques (NAT) have been used widely. Recently qualitative and quantitative determination of HCV core antigen in serum and plasma has gained increasing importance for the diagnosis of HCV infection and monitoring of therapeutic success [11, 13, 14, 15]. It has been acknowledged

that HCV core antigen and HCV Ag/Ab combination assays may constitute a reasonable alternative to HCV RNA detection when NAT technologies are not implemented or are too costly.

Since these assays gain more and more importance, there appears to be a rising need for a standardization. Standardization may ensure comparability of results and also contribute to an enhancement of sensitivity.

To satisfy the need for a suitable HCV core preparation and the need for panels with region-specific genotypes or polymorphisms, a national reference panel has been established already for the evaluation of Hepatitis C Virus RNA and quantitative HCV core antigen detecting assays in Japan [16].

Currently an internationally recognized standard preparation is not yet available. The proposal by the Paul-Ehrlich-Institut (PEI) to develop the 1st WHO International Standard for HCV core antigen for use with and standardization of HCV core antigen detecting assays was endorsed by the Expert Committee on Biological Standardization (ECBS) in October 2009. The HCV material being developed as the standard was characterized as genotype 1a which is one of the most frequent HCV genotypes globally.

The aims of the collaborative study were (1) to demonstrate the suitability of the candidate standard for its use in a range of typical assays performed in different laboratories, (2) to evaluate its potency, (3) to assign an internationally agreed unitage and (4) to assess commutability of the candidate material to serve as a standard for different samples being assayed.

1.2. Materials and Methods

1.2.1. Selection and characterization of candidate materials

For the selection of suitable materials specimens were collected and screened for high levels of HCV core Ag with the Architect HCV core Ag test kit. In addition, appropriate materials needed to be negative for HCV antibodies. Seven samples (W122040 to W122045) were kindly provided by the Institute of Haematology and Transfusion Medicine, Warsaw, Poland, two samples were collected by the blood donation centre in Duisburg, Germany and one sample (W126181) was obtained from ZeptoMetrix Corporation, New York, US. These nine samples were pre-tested in a feasibility study using three diagnostic test kits: ARCHITECT HCV Ag (Abbott). Murex HCV Ag/Ab combination (DiaSorin S.A.) and Monolisa HCV Ag-Ab Ultra (Bio-RAD). Results are summarized in **Table 1**. It turned out that the sensitivities of available HCV core antigen and HCV Ag/Ab combination tests varied substantially. Results from the HCV Ag/Ab with lowest sensitivity showed that there was only limited capacity for diluting the tested materials. Five samples exhibited high antigen levels > 8000 fmol/L in the Architect HCV core antigen test. Out of these, only four were of HCV genotype 1 and out of these four only one sample (W126181) was available in a large volume > 500 ml. Therefore, it was decided to use sample W126181 as starting material for the preparation of the candidate material for the 1st International HCV core antigen standard.

1.2.2. Sample A: Candidate HCV core antigen material, code 129096/12

The candidate standard coded sample A was prepared from the plasma W126181 that was collected from a US blood donor in 1996 and obtained from ZeptoMetrix via Helvetica HealthCare in 2011. The HCV genotype is 1a as determined by ABBOTT RealTime HCV Genotype II Assay and confirmed by direct sequencing of a fragment from the NS5B region.

The material was negative for anti-HCV by the Ortho HCV 3.0 Enhanced SAVe and the ADVIA Centaur HCV (Siemens Healthcare) assays. In addition, the plasma was negative for markers of HBV (HBsAg and anti-HBc) and HIV infection (HIV-1 p24 anti-HIV-1/2). The material was diluted twofold with normal human plasma negative for markers of HCV, HIV and HBV infection, mixed thoroughly and filtered through a Sartopure PP8 8 µm capsule to remove particles. The material was neither inactivated nor were stabilizers or preservatives added. Freeze-drying was performed by Greiner Diagnostic AG, 4900 Langenthal, Switzerland. The resulting preparation has residual moisture of 0.8% as determined at PEI using an accredited method according to the European Pharmacopoeia and a filling variation (CV) of 0.8% [17]. In total 1847 ampoules were obtained. The lyophilized preparation has the PEI code no. 129096/12.

1.2.3. Sample B, code 126181/11

Sample B was a frozen aliquot of sample A collected after the filtration step. The material was provided in 0.5 ml aliquots to the participants. It was neither inactivated nor were preservatives or stabilizers added. Sample B had been stored at -70°C until shipping.

1.2.4. Sample C, code 131347/12

Sample C was collected from a US blood donor in 1997 and was obtained via Helvetica Health Care. Participants were provided each with a 1.0 ml frozen undiluted aliquot of the sample and with an 1:64 dilution. The material has been found negative for markers of HBV (HBsAg. anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material was positive for HCV. The material was neither inactivated nor were preservatives or stabilizers added. HCV RNA was indicated by 37.000.000 copies /mL by the vendor. The sample is of genotype 1. HCV RNA quantification using the Roche CAP/COBAS TaqMan HCV Test quant v2.0 yielded 8.540 IU/mL. Abbott RealTime HCV yielded 9.331 IU/mL.

1.2.5. Sample D, code 131348/12

Sample D was collected from a US blood donor in 1997 and was obtained from ZeptoMetrix via Helvetica Health Care. Participants were provided each with a 1.0 ml frozen undiluted aliquot of the sample and with an 1:64 dilution. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material was positive for HCV. The material was neither inactivated nor were preservatives or stabilizers added. HCV RNA was indicated as 88.000.000 copies /mL by the vendor. The sample is of genotype 1 and HCV RNA quantification using the Roche CAP/COBAS TaqMan HCV Test quant v2.0 yielded 260.000 IU/mL.

1.2.6. Sample E, code 131349/12

Sample E was collected from a US blood donor in 1997 and was obtained via Helvetica Health Care. Participants were provided each with a 1.0 ml frozen undiluted aliquot of the sample and with an 1:64 dilution. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material was neither inactivated nor were preservatives or stabilizers added. HCV RNA was indicated as 94.000.000 copies /mL by the vendor. The sample is of genotype 1. HCV RNA quantification using the Roche CAP/COBAS TaqMan HCV Test quant v2.0 yielded 559.000 IU/mL.

1.2.7. Sample F, code 117031/09

Sample F originated from a German blood donor and was collected in 2009. The sample was provided neat and diluted 1:4 each containing 1.0 ml frozen human plasma. The material was negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV antigen and HCV RNA (13.200 IU/mL). The sample is of HCV genotype 3 and was neither inactivated nor were preservatives or stabilizers added.

1.2.8. Quantification of HCV RNA and determination of the HCV genotype

Quantitative determination of HCV RNA amount of the samples was performed using either the ROCHE COBAS AmpliPrep/ COBAS Taqman HCV Test or the ROCHE CAP/COBAS TaqMan HCV Test quant. v2 assay. HCV genotyping was performed with the ABBOTT RealTime HCV Genotype II Assay or the Roche Linear Array HCV Genotyping Test and for sample A also by direct sequencing of a fragment from the NS5B region.

1.3. Design of the collaborative study

The study protocol is attached in **Appendix 2**. Dilutions should be prepared from samples coded A and B and be tested in triplicates on three days apart in the assays in use in the participants´ laboratories. A fresh ampoule of sample A and sample B should be used for each of the 3 independent tests. The dilutions should cover the linear measuring ranges of the test and reach the endpoint titer (intercept with the cut-off of the assays). Normal human serum (NHS) or plasma negative for markers of HCV infection (anti-HCV and RNA) was recommended as dilution matrix. If normal HCV-negative serum or plasma were not available, a dilution matrix normally taken in the participant´s laboratory could be used as well. The diluent in use in each laboratory should be tested in triplicate as a control.

Samples C to E should be tested undiluted and in the dilutions provided in triplicates on one day only. The dilution matrix for samples C to E was provided to be included as a control.

Sample F should be used for HCV core antigen tests only. This sample was known to be low titer and not to be suitable for HCV Ag/Ab combination tests. Sample F should be tested undiluted and in a 1:4 dilution in triplicates only once (on one day only).

1.3.1. Participants

Twenty laboratories world-wide were contacted for participation in the Collaborative Study. Thirteen laboratories were willing to participate and received the samples. Out of these eleven returned results. The participants were from France (2), Japan (2), Korea (1), Russia (1), Spain (1), South Africa (1), the UK (1), the US (1) and Germany (2). Details on participants and laboratories are given in **Appendix 1**.

1.3.2. Assay methods

Six different assays were used. Three assays were HCV core antigen tests and 3 tests were combined HCV antigen/antibody (Ag/Ab) tests. Two HCV core antigen tests were instrument bound chemiluminescence immunoassays for the quantitative determination of the HCV core antigen. One HCV core antigen test was a qualitative enzyme immunoassay. All three HCV Ag/Ab combination tests were qualitative enzyme immunoassays. Assays and features are specified in **Table 2**.

1.3.3. Statistical methods

Statistical analysis was performed at PEI based on the raw data provided by the participants. The titers with the diluted sample A and sample B respectively were calculated by linear interpolation at the intersection of the dilution series (titers) with the assay's cut-off. Geometric mean titers (GMT) including their 95% confidence intervals (CI) were used to describe each assay. The geometric coefficient of variation (GCV = sqrt(exp(SD*SD)-1), where SD is the standard deviation of the ln-transformed data) was used to describe the intra-assay and inter-laboratory (where appropriate) variability. For comparison, titers were also determined by linear regression analysis using up to two values above and below the cut-off.

Spearman's rank correlation coefficient was used to assess the correlation between titers obtained with sample A and HCV core antigen recovery in samples C, D, E and F and the respective dilutions [18]. In addition, it was applied to assess the correlation between HCV core antigen and HCV RNA content in sample A.

Results and Discussion

1.3.4. Data received

Most participants adhered to the study plan. One laboratory did not perform triple determinations of the dilution series. One laboratory did not return results for sample F and one laboratory did not test the matrix provided with samples C, D and E. In addition, the results for samples C to E of that laboratory differed completely from results from the other laboratories using the same test and thus were omitted.

1.3.5. Candidate material sample A (PEI code 129096/12) and sample B (PEI code 126181/11) – Titers at cut-off

Endpoint titers of each assay performed in each laboratory were calculated for the candidate material sample A and for sample B by linear interpolation from maximum two measuring values above and below the cut-off. The geometric mean titers per assay and per laboratory (GMT) are presented in **Table 3**. Geometric mean titers of all laboratories that used the same assay are shown in **Table 4**. Titers obtained with the six HCV core antigen and HCV Ag/Ab combination tests were heterogeneous. Results for sample A ranged between a maximum total mean endpoint titer of 3209 (95%-CI 2898 - 3552) for the quantitative HCV core antigen assay no. 4 and a minimum total mean endpoint titer of 2 for the two qualitative HCV Ag/Ab tests (assays no. 1 and 6). The quantitative HCV core Ag assay no. 2 yielded a total mean titer of 314 (95%-CI 211 - 466). The total mean titer of assay no. 3 was 60 (95%-CI 23-151) and the total mean titer of assay no. 5 was 6 (95%-CI 2-16). Although the mean total results of the different assays used were quite different and spanned a wide range from borderline reactivity to high titers, the determined endpoint titers were very similar for the laboratories that used the same assay for testing. In addition, the dilution curves that for comparative purposes are depicted from the natural logarithm of the results (ln(dose)) demonstrated that the results of the laboratories that used the same assay were comparable (**Figure 1**).

For sample B which was the material used for the preparation of the candidate material sample A overall higher mean titers were observed suggesting that there was a certain loss of detectable HCV core antigen caused by the transport of sample A to Switzerland, the lyophilisation procedure and the transport back to PEI **(Table 4)**. The maximum mean endpoint titer of sample B was 5009 (95%-CI 3195 – 7853) for the quantitative HCV core Ag assay no. 4 and the minimum titer was 3 (95%-CI 2 - 3) for the qualitative HCV Ag/Ab

combination assay no. 1. This apparent loss of detectable HCV core antigen was 29.4 % for assay 3, 35.9 % for assay 4, 40.0 % for assay 5 and 42.5 % for assay 2.

Overall, the mean titers of sample A obtained with the different assays in the different laboratories were on average 36.6 % (range 29.4 to 42.5 %) lower than the titers of sample B (**Table 4** and **Figure 2a**). For the assays 1 and 6 this calculation could not be performed, as the measuring values (optical densities, OD) and thus the S/CO ratios and titers) were too low and partly below the cut-offs of both assays. The reason for this apparent loss of potency is most likely the liquid transport of sample A.

Calculation of endpoint titers of sample A were also performed by the geometric mean value of the single values of each assay or by linear regression analysis of a maximum 2 values above and 2 values below the intercept with the cut-off. A comparison to the geometric mean values obtained linear interpolation from maximum two measuring values above and below the cut-off showed that the endpoint titers were comparable and thus not biased by the statistical approach chosen (**Figure 2b**).

Generally, the dilution ranges chosen for sample A and for the starting material sample B were in the linear measuring ranges of the two quantitative HCV core antigen assays 2 and 4. The dilution curves were parallel and had similar slopes as determined by regression analysis (**Figure 3**). Dilutions of sample A and sample B were in the dynamic ranges of both assays. Correlation was significant with the coefficient of determination R^2 being 0.9945 for assay 2 and 0.9896 for assay 4 when calculation was based on single measuring values of the individual test runs in the laboratories.

For the four qualitative assays linearity and parallelism of the dilution curves for sample A to that of assay 2 or 4 could not be demonstrated: Three assays (no. 1, 5 and 6) exhibited only limited dilution capacity with sample A and hence achieved low endpoint titers. Out of the qualitative assays, only assay 3 reached a considerably higher endpoint titer. Due to the qualitative assay design, the dilution curves of assay 3 were however not parallel to those of the two quantitative assays 2 or 4 and slopes were different.

1.3.6. Intra-laboratory and inter-laboratory variability

Intra-laboratory (within-assay laboratory) and inter-laboratory variability (variability between laboratories using the same assay) for results of samples A and B at the cut-off were assessed by means of GCV. For assays 2, 3, 4 and 5 intra-laboratory variability for the three test runs performed on the same assay were between 1.6% and 82.2 % variation coefficient (CV) for sample A and 2.9 and 49.5 % CV for sample B (**Table 3**). For sample A intra-laboratory variability was lowest for assay 4 with a CV range of 1.4 % to 16.2 %. Intra-laboratory variability of sample A was highest for assay 3 with a range of 9.6 % to 82.2 %. For sample B intra-laboratory variability of assay 4 was 6.4 % to 20.0 %and thus also low. Again, assay 3 showed highest intra-laboratory variability. Intra-laboratory variability of assay 2 was very similar for samples A and B (%?). The intra-assay variation was low, except for test kit 3 in one laboratory. The reason was high variability of results in one laboratory.

Inter-laboratory variability was between 2.8 % and 64 % GCV (geometric mean coefficient of variation) for sample A and 13.0 to 46.1 % GCV for sample B (**Table 4**). The GCVs of sample A were low for both quantitative assays 2 (4.4 % GCV) and 4 (8.2 % GCV). Assays 2 and 4 yielded GCV values of 13.0 % and 28.8 %, respectively, for sample B. Assay 3 showed highest inter-laboratory variability for sample A (64 % GCV). This was due to high variability of results in one laboratory.

Inter-laboratory variability could not calculated for assays 1 and 6 as titers were low and results were partly below cut-off.

Generally, it appeared that samples A and B had comparable intra-laboratory and inter-laboratory variability and it can therefore be assumed that the candidate material was still homogenous after the lyophilization step. Overall variabilities were in an expected range for immunoassays and suggested that the features of sample A were preserved.

1.3.7. Recovery of supplemental samples C, D, E and F and correlation to titers obtained with sample A

Additional samples were included in the collaborative study to investigate whether the candidate material A performed similar to further HCV core antigen positive samples. Three supplemental samples (C, D, E) were selected. These samples were tested neat and in a 1:64 dilution (C 1:64, D 1:64, E 1:64) to cover a range from a high to a low HCV core antigen level. Participants were asked to test samples C, D, E and the respective 1:64 dilutions by all HCV core antigen and HCV Ag/Ab combination tests. In addition, as recommended during the WHO Collaborating Centers meeting in 2013, the HCV core antigen low positive sample F and the respective 1:4 dilution (F 1:4) were included in the study for use with HCV core antigen tests only.

Due to the qualitative design of most assays of the study, analysis was performed by normalizing results by calculating S/CO ratios. The results of the study are presented in **Table 5**. Geometric mean S/CO ratios per sample are presented in **Table 6** and assays are ranked according to the geometric mean titers obtained with sample A in an ascending manner starting with assay 6 that exhibited the lowest titer (titer 2) to assay 4 that exhibited the highest titer (titer 3209). The undiluted samples C to E were detected positive by all HCV core Ag assays and also by all HCV Ag/Ab combination assays. The 1:64 diluted samples C to E were detected positive only by the two quantitative HCV core antigen assays that showed the highest titers with sample A (assay 2 with a titer of 314 and assay 4 with a titer of 3209). None of the qualitative assays 1, 3, 5 and 6 (mean titers for A: 2 to 60) detected any of the 1:64 dilutions as positive. Overall, calculated Spearman´s rank correlation coefficients show that there is a significant correlation between the assays´ titers for sample A and the S/CO ratios for samples C to E and for the respective dilutions (**Figure 4**).

Sample F and the respective 1:4 dilution were investigated using the three HCV core antigen assays only (assays 2, 4 and 5). This was a sample that was known to contain only low amounts of HCV core antigen. It was detected positive only by assay 4 in all laboratories. The 1:4 dilution was borderline reactive by assay 4 with two results above and three results below cut-off. Sample F and the dilution were analyzed with assay 2 solitary by laboratory 2. The undiluted sample was borderline reactive yielding a S/CO ratio of 0.9. The 1:4 dilution was clearly negative. Assay 5 did not detect sample F at all. The results are summarized in **Tables 5** and **6** and **Figure 5**. Supplemental samples C to E were of HCV genotype 1 as was the candidate standard sample A while sample F was of genotype 3. However, it is assumed that the differences in HCV genotype are not the reason for the overall weak reactivity of sample F, as HCV-RNA levels were also low. In addition, it has been shown that HCV genotype 3 samples show similar analytical sensitivity as genotype 1 samples [15].

Generally, the results on the additional samples C to F indicate that within the same assay the candidate standard sample A behaves similar to HCV core antigen positive clinical samples. When the assays´ results in **Table 6** are ranked according to the titers of sample A, S/CO ratios of samples C to E and of the dilutions show similar sorting, demonstrating that the

supplemental samples C to E were detected with the same sensitivity as with candidate standard sample A. Spearman´s rank correlation coefficients show that there is a significant correlation between the assays´ titers for sample A and the S/CO ratios for samples C to E and the respective dilutions **(Figure 4)**.

To further support commutability of the candidate standard, a complementary study was performed and showed comparability of the results of the candidate HCV core antigen standard A to results from representative patient samples. A range of seven commercial HCV seroconversion panels **(Table 9)** was investigated. The panels were well-characterized and included negative as well as low and high titer HCV core antigen positive samples. All panels were from the HCV antibody negative phase. These panels were investigated with assays 1, 3 and 4 that span the whole titer range obtained with sample A **(Table 3)**. The study outcome is included in **Part 2** of the report.

1.4. Additional studies for characterization of sample A

1.4.1. Correlation of HCV core antigen level and HCV RNA level

For the two quantitative HCV core assays 2 and 4 it was further investigated whether the determination of HCV core antigen in sample A indicated in fmol/L correlated to the determination of HCV RNA in IU/mL. Therefore, the HCV RNA content in IU/mL was determined in the undiluted sample A and in two-fold dilutions and compared with the HCV core antigen content of the undiluted sample A and the respective dilutions. Results of this investigation showed a high correlation between HCV core antigen and RNA determination with linear curves when the RNA content was plotted against the HCV antigen content (**Figure 6**). Determination of the Spearman's rank correlation coefficient showed that the relationship between HCV RNA and HCV antigen determination in sample A was significant ($p < 0.001$) for both, assay 2 ($r=0.903$) and assay 4 ($r=0.927$). In summary, the correlation between HCV RNA and HCV core antigen content further suggests that sample A is an appropriate material for the standardization of quantitative and qualitative HCV core antigen detecting assays.

1.4.2. Preliminary stability study

Ampoules of sample A were incubated each at -20°C, 4°C, 20°C and 37°C. At the end of the respective temperature treatments, ampoules were reconstituted and 1:10, 1:50, 1:250, 1:1250 dilutions were prepared and then frozen immediately at -70°C until analysis. The dilutions were tested in two replicates using assay 4. As a baseline, the activity of a freshly reconstituted ampoule was used. This ampoule had been continuously stored at -70°C until measurement. The results of the study are presented in **Table 7** and **Figure 7**. Results from the "accelerated" study suggest that the candidate material may be sufficiently stable when stored at -20°C or below and when shipped refrigerated on dry-ice. **Table 8** summarizes in-use studies mimicking conditions at the users´ sites where ampoules are reconstituted, frozen again at -20°C, re-opened and frozen again. So far, these studies suggest that the material may be stable when stored at the recommended temperature or below. In addition, the reconstituted material seems stable for three freeze-thaw cycles so far. The reconstituted material was however not stable when stored at 4°C for 7 days. Further real time and in-use stability studies are ongoing.

1.4.3. Expanded Anti-HCV testing

As suggested by participants a further study was carried out on HCV antibody content of sample A (PEI code 129096/12). Results are summarized in **Table 11.** The bulk material from which sample A was prepared had been screened HCV antibody negative upon collection using Ortho HCV 3.0 Enhanced SAVe and Siemens ADVIA Centaur Anti-HCV. This was repeated and the initial findings could be confirmed. Both assays do not detect HCV antibodies in the bulk and sample A. Furthermore, Abbott ARCHITECT Anti-HCV, Murex Anti-HCV 4.0 (DiaSorin S.A.) and DS-EIA-Anti-HCV (RPC Diagnostic Systems) were also used for the investigation. Also, Abbott Architect was negative with the bulk and sample A and the respective dilutions. It is however noted that the S/CO ratio is elevated with the bulk. Murex Anti-HCV 4.0 detected HCV-specific antibodies up to a dilution of 1:2 in both materials and DS-EIA-Anti-HCV was positive with the bulk (1:16) and sample A up to 1:8. Two supplemental assays (Mikrogen *recom*Line HCV IgG) and MP Biomedicals Asia Pacific Pte Ltd. HCV Blot 3.0) were performed. Both immunoblots showed reactivity of the core bands with overall indeterminate results. In summary, the sample is considered from early HCV seroconversion where HCV antibody tests react differently with some assays being negative and some more evolved assays being reactive.

Specific antibodies may especially influence the results of HCV Ag/Ab combination tests even though the level is still low. A comparison of the HCV antibody results for assay 3 to its antibody counterpart shows that there is still a considerable difference between the geometric mean titers of sample A and the dilution in which antibodies can be found (**Table 4**). So the titer of 60 is mainly attributed to the HCV core antigen. This is also supported by the complementary study in Part 2 which was performed to address commutability of the candidate material.

1.5. Conclusions

Sample A which is the candidate material for the 1st WHO International Standard for HCV core antigen was investigated in a collaborative study by twelve participating laboratories that used 6 different HCV core antigen detecting assays. These six assays exhibited diverse features: three assays detected HCV core antigen only, while 3 assays were qualitative HCV Ag/Ab combination assays. Out of the HCV core antigen detecting assays one was qualitative and two were quantitative assays.

Overall, sensitivities with sample A between the assays differed significantly by several magnitudes depending on the performance of the respective assay. It was therefore decided to define the potency of the candidate 1st WHO International Standard for HCV core antigen based on the results of assay 4 which showed highest sensitivity. In addition, the results showed high correlation of the sensitivity for sample A with that for the supplemental samples C to E, for samples from early HCV infection and with HCV-RNA detection.

The candidate material was prepared from a HCV core antigen positive plasma sample belonging to genotype 1a which in a feasibility study showed a high HCV antigen content, lack of HCV specific antibodies and was available in sufficient amounts for the preparation of a candidate material. The candidate material was freeze-dried to ensure long term stability and to allow for a global shipment. The material had not undergone special manufacturing steps, inactivation procedures, heat treatment or any stabilization measures that could lead to denaturation and thus affect its antigenic properties.

The proposed international standard may contribute to the development of new sensitive HCV core antigen and later to an improvement of the sensitivity and general performance of HCV core antigen assays. Such assays are especially useful in settings where the introduction of

NAT is difficult and are performed on the same equipment as anti-HCV tests. Since the use of more advanced HCV antibody assays lateron revealed that there are low level antibodies detectable by some assays, its value for HCV Ag/Ab combination tests needs to be further evaluated.

The outcome of the collaborative study demonstrated that the candidate material representing HCV genotype 1a is suitable as a reference preparation to standardize and harmonize HCV core antigen detecting assays. The results from the accelerated and real-time stability studies indicate that the preparation is stable at the recommended storage temperature and can be shipped globally.

1.6. Proposal

It is proposed that the candidate material with the PEI code 129096/12 be established as the 1st WHO International Standard for HCV core antigen for use with HCV core antigen assays with an assigned potency of 3,200 IU/mL when reconstituted in 0.5 ml distilled water. The proposed unitage does not carry an uncertainty associated with its calibration. The only uncertainty is therefore derived from the filling variability which had a coefficient of variation of 0.8%. The proposed standard is intended for the calibration of HCV core antigen assays, the determination of analytical sensitivity and for quality control. The proposed Instructions for Use (IFU) for the reference preparation are included in **Appendix 3**.

1.7. Comments from participants

All participants responded to the request for comments on the report. Seven participants agreed to the report, the proposal and the suitability of the material without further comments.

One participant recommended that 3 different commercially available anti-HCV antibody-only assays be used and that one or two commercially available immunoblot assays be performed (even if the screening anti-HCV assay is negative). Another participant communicated the detection of anti-HCV antibodies in the candidate material and in dilutions that may form complexes with the HCV core antigen. The comments of these two participants were addressed and a further investigation of HCV antibodies was carried out, although the starting material of the candidate standard had been tested negative for anti-HCV when choosing the material. These results were added to the report. As a consequence the proposal was changed and the use of the material was limited to HCV core antigen assays that detect the antigen in the presence of antibodies and a note was included in the draft Instructions for Use that is attached in **Appendix 3**.

One participant requested an explanation of how results were normalized to S/CO ratios which was added to the respective Table.

One participant claimed that assay 6 has better performance than assay 1 especially with the antigen part and was surprised about sample A results and that, on the contrary, results on samples C, D and E confirm a better Ag sensitivity with assay 6 than assay 1. In addition, the participant questioned the selection of the seroconversion panels of the complementary study of **Part 2** of the report performed to address commutability and suggested seroconversion results on all evaluated assays to really conclude that analytical sensitivity determined with the candidate standard sample A is correlated with clinical sensitivity. As it is not possible to conduct a study to address commutability on all assays, an explanatory note was added to the complementary study.

None of the participants disagreed with the proposal that 129096/12 (Sample A) be established as the 1st WHO International Standard for HCV core antigen with a potency of 3,200 IU/mL for use with HCV core antigen assays that detect the antigen in the presence of antibodies.

Part 2. Complementary characterization of the proposed HCV core antigen standard

2. Complementary study to demonstrate commutability

The outcome of the Collaborative Study and the results of the four additional HCV core positive clinical samples showed that the candidate 1st HCV core antigen International standard (IS) showed performance similar to clinical samples. To further address commutability of the candidate 1st International HCV core antigen standard, an additional study was performed at the Paul-Ehrlich-Institut. One potential use of the candidate 1st HCV core antigen IS will be determination of the analytical sensitivity of HCV core antigen detecting assays. Therefore, sensitivities of representative HCV core antigen-detecting assays were determined by measuring HCV seroconversion panels and compared to analytical sensitivities determined using the candidate 1st International HCV core antigen standard.

2.1. Materials and methods

2.1.1. Assays

One HCV core antigen (assay 4) and two HCV Ag/Ab combination tests (assays 1 and 3) were used for this additional investigation. The three assays were representative of the sensitivity range of HCV core antigen detecting assays. The HCV core antigen test is a chemiluminescence test exhibiting a quantitative assay format and the two HCV Ag/Ab combination tests were qualitative ELISA tests. Test features are described in **Table 1** of the Collaborative Study report.

2.1.2. HCV seroconversion panels

Seven commercial HCV seroconversion (SC) panels were obtained from ZeptoMetrix Corporation (872 Main Street Buffalo, NY 14202, USA). SC panels comprise sequential plasma samples from one individual drawn during a certain time frame. Samples were generally collected in plasmapheresis centers in short intervals and represent early HCV infection. The panels are well-characterized for all markers of HCV infection including RNA and antibodies (**Table 9**). The members of the seven HBV SC panels varied from 3 to 19 samples. In total, 82 samples were investigated comparatively by all three tests.

2.2. Results and conclusions of the complementary study

In the complementary study, sensitivities of three representative HCV core antigen detecting tests were investigated using samples from early stages of HCV infection that contained different quantities of HCV core antigen (**Table 10**). Results were compared to analytical sensitivity relative to the candidate IS. Assay 4 showed the highest analytical sensitivity relative to the candidate IS and also the highest sensitivity using the clinical samples. Assay 1 showed the lowest analytical sensitivity and also the lowest sensitivity when testing samples

from early HCV infection. Assay 3 which exhibited a medium analytical sensitivity also showed medium sensitivity in seroconversion. When ranking the test kits according to their titer or their clinical sensitivity, they clustered exactly at the same position (Assays 4 > 3 > 1). The higher the titer with sample A, the more samples from early infection were detected as positive (**Figure 8**). Thus correlation between the determination of analytical sensitivity using the proposed HCV core antigen standard and determination of sensitivity using clinical samples was very high (**Figure 8**) suggesting that the proposed 1st International HCV core antigen standard is suitable for its purpose and may serve as a standard for the variety of different samples being assayed.

Please note: The complementary study was designed and performed with selected HCV core detecting assays covering the reaction range to address commutability. The results do not allow an overall picture of the performance of the two HCV Ag/Ab combination tests as the antibody part was not addressed with these panels.

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Table 1. Pre-tests for selection of a suitable candidate material

PEI Sample No. W	122040			122041			122042			122043			122045			W117029	W117031	W126181	W126181
Anti-HCV		negative			negative			negative			negative			negative		negative	negative	negative	negative
RNA HCV [IU/ml]		4.3E+06			6.7E+06			6.9E+06			7.8E+06			9.2E+06		n.a.	n.a.	3.4E+07	3.4E+07
Genotype		1b			1b			1b			1b			1b / 4a		n.a.	n.a.	1	1a
Test	ARCHI-TECT HCV Ag	Monolisa HCV Ag/Ab Ultra	Murex HCV Ag/Ab Combination	ARCHI-TECT HCV Ag	Monolisa HCV Ag/Ab Ultra	Murex HCV Ag/Ab Combination	ARCHI-TECT HCV Ag	Monolisa HCV Ag/Ab Ultra	Murex HCV Ag/Ab Combination	ARCHI-TECT HCV Ag	Monolisa HCV Ag/Ab Ultra	Murex HCV Ag/Ab Combination	ARCHI-TECT HCV Ag	Monolisa HCV Ag/Ab Ultra	Murex HCV Ag/Ab Combination	ARCHITECT HCV Ag Reagent Kit	ARCHITECT HCV Ag Reagent Kit	ARCHI-TECT HCV Ag	Murex HCV Ag/Ab Combination
Manufacturer	Abbott	Bio-Rad	DiaSorin (Murex)	Abbott	Bio-Rad	DiaSorin (Murex)	Abbott	Bio-Rad	DiaSorin (Murex)	Abbott	Bio-Rad	DiaSorin (Murex)	Abbott	Bio-Rad	DiaSorin (Murex)	Abbott	Abbott	Abbott	DiaSorin (Murex)
ID-No.	LN6L47-27	72556	4J24-04	LN6L47-27	72556	4J24-04	LN6L47-27	72556	4J24-04	LN6L47-27	72556	4J24-04	LN6L47-27	72556	J24-04	6L47-27	6L47-27	6L47-27	4J24-04
Sample Dilution (1:)	fmol/l	S/CO	S/CO	fmol/l	S/CO	S/CO	fmol/l	S/CO	S/CO	fmol/l	S/CO	S/CO	fmol/l	S/CO	S/CO	fmol/l	fmol/L	fmol/L	S/CO
1	n.d.	3.88	9.42	n.d.	3.32	8.82	n.d.	3.59	9.01	n.d.	3.05	7.55	n.d.	4.02	8.44	3.51	8.86	n.d.	n.d.
2	n.d.	2.38	7.66	n.d.	2.59	6.22	n.d.	2.54	6.47	n.d.	2.01	5.23	n.d.	2.52	6.41	n.d.	4.42	n.d.	n.d.
4	n.d.	1.31	4.84	n.d.	1.65	3.98	n.d.	1.54	4.22	n.d.	1.17	3.02	n.d.	1.46	4.10	n.d.	1.87	n.d.	3.76
8	n.d.	0.85	4.44	n.d.	0.90	2.83	n.d.	0.96	2.34	n.d.	0.70	1.84	n.d.	0.94	2.41	n.d.	0.28	n.d.	2.24
16	n.d.	0.64	5.77	n.d.	0.59	2.33	n.d.	0.63	2.24	n.d.	0.48	1.20	n.d.	0.62	1.36	n.d.	n.d.	n.d.	1.20
32	1724.75	0.41	2.39	1057.91	0.46	1.91	1710.48	0.46	0.79	1296.87	0.39	0.82	1435.59	0.44	0.92	n.d.	n.d.	783.56	0.85
64	795.13	0.35	0.63	518.70	0.35	1.47	835.63	0.34	0.61	676.18	0.35	0.56	729.56	0.37	0.62	n.d.	n.d.	421.28	0.70
128	384.13	n.d.	n.a.	248.55	0.31	0.51	408.66	0.30	0.53	314.01	0.32	0.49	373.28	n.d.	n.d.	n.d.	n.d.	230.28	0.56
256	201.08	n.d.	n.a.	133.15	0.28	0.45	201.40	0.29	0.45	154.42	n.d.	n.d.	189.02	n.d.	n.d.	n.d.	n.d.	128.22	n.d.
512	88.39	n.d.	n.a.	61.00	0.28	0.42	98.60	0.29	0.45	74.52	n.d.	n.d.	91.82	n.d.	n.d.	n.d.	n.d.	61.01	n.d.
1024	49.03	n.d.	n.d.	27.14	n.d.	n.d.	47.81	n.d.	n.d.	34.54	n.d.	n.d.	46.30	n.d.	n.d.	n.d.	n.d.	34.78	n.d.
2048	23.52	n.d.	n.d.	13.32	n.d.	n.d.	21.55	n.d.	n.d.	16.26	n.d.	n.d.	22.87	n.d.	n.d.	n.d.	n.d.	18.74	n.d.
4096	10.14	n.d.	n.d.	6.75	n.d.	n.d.	11.72	n.d.	n.d.	7.99	n.d.	n.d.	11.24	n.d.	n.d.	n.d.	n.d.	8.94	n.d.
8192	4.82	n.d.	n.d.	2.72	n.d.	n.d.	6.79	n.d.	n.d.	3.71	n.d.	n.d.	5.29	n.d.	n.d.	n.d.	n.d.	3.99	n.d.
16384	2.17	n.d.	n.d.	1.40	n.d.	n.d.	2.15	n.d.	n.d.	1.52	n.d.	n.d.	2.86	n.d.	n.d.	n.d.	n.d.	2.72	n.d.
Titer	12476.3	6.0	52.9	7659.8	7.1	84.7	13847.3	7..5	28.0	9776.8	4.9	21.7	15491.5	7.2	27.1	< 2	2.8	13424.3	22.4

Reactive results are indicated by a green colour.
S/CO sample to cut-off ratio

Table 2. HCV core antigen and HCV antigen/ antibody (Ag/Ab) combination assays used in the collaborative study

Assay Code	Product Name	Cat. No.	Manufacturer	Procedure / Instrument	Detection of	Antibody to HCV core	Test format	Technology
1	Monolisa HCV Ag-Ab ULTRA	72556	Bio-RAD	manual	Ag/Ab	monoclonal antibodies	qualitative	EIA
2	Lumipulse Ortho HCV Ag	603006	Fujirebio Inc.	Lumipulse	Ag	not known	quantitative	CLEIA
3	Murex HCV Ag/Ab combination	4J24-03	DiaSorin S.A.	manual	Ag/Ab	monoclonal	qualitative	EIA
4	ARCHITECT HCV Ag	6L4727	Produced by Denka Seiken Co. LTD for Abbott Laboratories	ARCHITECT	Ag	monoclonal	quantitative	CMIA
5	DS-EIA-HCV-AG	C-1962	Diagnostic Systems	manual	Ag	monoclonal antibodies	qualitative	EIA
6	Monolisa HCV Ag-Ab ULTRA V2	72561	Bio-RAD	manual	Ag/Ab	monoclonal	qualitative	EIA

CMIA Chemiluminescent Microparticle Immunoassay
CLEIA Chemiluminescent enzyme immunoassay
EIA Enzyme Immunoassay
Ag Antigen

Table 3. Mean endpoint titers of sample A and sample B

		Sample A							Sample B						
Assay Code	Laboratory Code	1	Run 2	3	Geom. mean	95%	- CI	CV %	1	Run 2	3	Geom. mean	95%	- CI	CV %
1	8	< 2	< 2	< 2					3	3	3	3	2	- 3	6.5
	11	3	3	2	3	2	- 3	8	4	4	4	4	4	- 4	2.9
2	2	306	350	262	304	212	- 437	14.7	675	530	n/a	598	129	- 2781	17.2
	6	325	395	263	324	195	- 535	20.5	466	584	454	498	352	- 703	14.0
3	3	27	18	73	33	6	- 197	82.2	63	41	59	53	30	- 95	23.7
	7	52	77	83	69	38	- 128	24.9	89	170	117	121	54	- 271	33.3
	11	114	138	128	126	99	- 160	9.6	79	187	138	127	43	- 376	46.0
	12	50	27	60	43	16	- 121	43.2	75	38	94	65	20	- 207	49.5
4	4	3125	3182	3081	3129	3006	- 3258	1.6	5615	6378	6064	6011	5125	- 7050	6.4
	8	3534	3089	4272	3599	2402	- 5394	16.4	5420	5721	7153	6053	4200	- 8724	14.8
	9	2881	n/a	n/a	2881				3321	n/a	n/a	3321			
	10	3186	3107	3181	3158	3049	- 3270	1.4	>8192	>8192	>8192	>8192			
	12	3018	3033	3999	3320	2225	- 4954	16.2	4300	5149	6390	5211	3184	- 8528	20.0
5	5	9	5	4	6	2	- 16	42.5	12	9	9	10	7	- 15	16.0
6	1	2	3	2	3	2	- 3	12.1	4	5	6	5	3	- 8	17.3
	11	< 2	2	3	2	0	- 21	24.5	< 4	< 4	<4				

Geom. mean	geometric mean value
< co	measuring values below cut-off
CI	Confidence Interval

Table 4. Overall geometric mean titers at cut-off and variation coefficients of sample A and sample B per assay

			Sample A					Sample B					Difference in detectable HCV core antigen [%]
Assay Code	Detection of	Laboratory Codes	Geom. mean titer	95%	-	CI	GCV %	Geom. mean titer	95%	-	CI	GCV %	
1	Ag/Ab	8 (for sample A excluded[1]), 11	3	2	-	3	n.a.	3	0	-	33	n.a.	n.a.
2	Ag	2, 6	314	211	-	466	4.4	546	170		1752	13.0	42.5
3	Ag/Ab	3, 7, 11, 12	60	23	-	151	64	85	42	-	171	46.1	29.4
4	Ag	4, 8, 9, 10 (sample B excluded[2]). 12	3209	2898	-	3552	8.2	5009	3195	-	7853	28.8	35.9
5	Ag	5	6	2	-	16	42.5	10	7	-	15	16.0	40.0
6	Ag/Ab	1, 11 (sample B excluded[3])	2	2	-	3	n.a.	5	3	-	8	n.a.	n.a.

Reasons for exclusion of results from the calculation of the geometric mean value:
1) Results of sample A below cut-off for Assay 1 in laboratory 8
2) Results of sample B beyond the dilution range cut-off for Assay 4 in laboratory 10
3) Results of sample B below cut-off for Assay 6 in laboratory 11
GCV geometric mean coefficient of variation
n.a. not applicable due to low measuring values close to the cut-off and hence low titers

Table 5. Results of supplemental samples C, D, E and F and of respective dilutions

Assay	Laboratory	C S/CO*	C 1:64 S/CO	D S/CO	D 1:64 S/CO	E S/CO	E 1:64 S/CO	F S/CO	F 1:4 S/CO
1	8	1.220	0.271	1.689	0.286	5.327	0.354	n.d.	n.d.
1	11	1.408	0.381	1.843	0.376	5.199	0.433	n.d.	n.d.
2	2	376.796	5.954	575.586	10.001	1344.661	18.258	0.895	0.359
2	6	264.309	4.793	478.407	8.660	984.685	16.815	n.d.	n.d.
3	7	3.558	0.472	4.683	0.506	7.727	0.550	n.d.	n.d.
3	11	2.273	0.530	3.385	0.496	5.418	0.591	n.d.	n.d.
3	12	2.975	0.441	5.385	0.406	8.248	0.477	n.d.	n.d.
4	4	2895.806	40.369	4893.070	82.210	> 6666.667	139.227	4.766	1.374
4	8	3532.577	54.960	6129.148	99.051	> 6666.667	184.896	5.957	2.187
4	9	3373.467	55.275	5951.544	92.389	> 6666.667	178.354	5.729	0.959
4	10	2559.768	39.174	5377.455	79.671	> 6666.667	135.661	3.855	0.984
4	12	2632.672	41.574	5536.253	81.410	> 6666.667	138.865	3.681	0.588
5	5	4.721	0.252	2.098	0.196	9.117	0.213	0.350	0.150
6	1	2.683	0.317	2.333	0.280	8.546	0.397	n.d.	n.d.
6	11	2.256	0.273	2.519	0.296	6.843	0.373	n.d.	n.d.

*For comparability all results were normalized to S/CO ratios by dividing the measuring values through the assays´ cut-offs

Table 6. Geometric mean values of supplemental samples C, D, E and F and of respective dilutions and comparison to titer of sample A

Assay	C S/CO	C 1:64 S/CO	D S/CO	D 1:64 S/CO	E S/CO	E 1:64 S/CO	F S/CO	F 1:4 S/CO	Mean Titer of Sample A
6	2.460	0.294	2.424	0.288	7.647	0.385	n.d.	n.d.	2
1	1.311	0.321	1.764	0.328	5.263	0.392	n.d.	n.d.	3
5	4.721	0.252	2.098	0.196	9.117	0.213	0.350	0.150	6
3	2.887	0.480	4.403	0.467	7.016	0.537	n.d.	n.d.	60
2	315.580	5.342	524.752	9.306	1150.681	17.522	n.d.	n.d.	314
4	2973.767	45.718	5560.077	86.632	> 6666.667	153.956	4.706	1.108	3209

Table 7: Preliminary stability study on sample A (candidate standard) using Assay 4

Temperature	Sample A dilution	Day 0 fmol/L	Day 7 fmol/L	Day 14 fmol/L	Day 21 fmol/L	Day 28 fmol/L
-20°C	1:10	1424.77	1382.07	1193.62	1210.99	1481.83
	1:50	304.35	384.13	340.75	285.34	312.00
	1:250	78.085	92.73	87.71	52.08	62.67
	1:1250	15.16	21.02	21.97	17.95	10.73
	Diluent control	0.00	0.00	0.00	0.00	0.00
+4°C	1:10	1424.77	1150.84	1186.09	1257.72	1206.08
	1:50	304.35	317.65	337.65	282.10	271.39
	1:250	78.085	77.36	92.98	58.39	49.38
	1:1250	15.16	21.59	24.42	10.06	7.89
	Diluent control	0.00	0.00	0.00	0.00	0.00
+25°C	1:10	1424.77	1272.46	1198.95	1211.56	1142.84
	1:50	304.35	338.99	307.76	268.40	222.62
	1:250	78.085	80.11	84.19	55.85	49.22
	1:1250	15.16	19.18	20.91	9.78	6.99
	Diluent control	0.00	0.00	0.00	0.00	0.00
+37°C	1:10	1424.77	1250.00	1146.68	1178.19	1045.77
	1:50	304.35	331.84	268.57	233.78	238.91
	1:250	78.085	85.35	77.25	49.59	49.37
	1:1250	15.16	18.35	15.99	7.15	7.79
	Diluent control	0.00	0.00	0.00	0.00	0.00

Table 8: Results of in-use stability study

Temperature	Sample A dilution	Day 0 fmol/L	Day 3 fmol/L	Day 5 fmol/L	Day 7 fmol/L	Day 14 fmol/L	Day 28 fmol/L
-20°C	1:10	1424.77	n.d.	n.d.	1307.22	1285.49	1375.05
	1:50	304.35	n.d.	n.d.	258.59	346.45	332.13
	1:250	78.09	n.d.	n.d.	82.84	87.76	94.1
	1:1250	15.16	n.d.	n.d.	24.16	23.37	23.18
	Diluent control	0.00	n.d.	n.d.	0.00	0.00	0.00
2-8°C	1:10	1424.77	870.18	1040.21	935.36	898.29	n.a.
	1:50	304.35	188.22	203.83	309.51	281.58	n.a.
	1:250	78.09	40.39	38.75	78.23	80.07	n.a.
	1:1250	15.16	9.40	6.68	21.28	23.03	n.a.
	Diluent control	0.00	0.00	0.00	0.00	0.00	n.a.

n.d. not determined; n.a. not applicable

Table 9: HCV seroconversion panels of the complementary study

Panel catalogue no.	Donor no.	HCV Genotype	Panel members
6225	62999	unknown	19
9055	66732	3a	11
10000	69089	1a	10
10003	69158	3	13
10040	66154	unknown	3
10051	67787	1b	14
10052	67861	3a	12

Table 10: Results of HCV seroconversion panels

Panel (Donor no.)	Panel member	Days after 1st bleed	Zeptometrix NAT	Anti-HCV	Assay 1 s/co	Assay 3 s/co	Assay 4 fmol/L
6225 (62999)	11*	39	670	neg	0.20	0.18	0.00
6225 (62999)	12	45	3.90E+05	neg	0.32	2.42	3114.97
6225 (62999)	13	47	1.30E+06	neg	1.43	9.96	10346.07
6225 (62999)	14	52	1.30E+06	neg	1.21	10.96	16973.67
6225 (62999)	15	56	8.10E+05	neg	0.98	8.42	6433.87
6225 (62999)	16	60	2.40E+06	neg	2.62	13.09	20000.00
6225 (62999)	17	73	5.10E+05	pos (2/17)**	0.76	2.14	3205.41
6225 (62999)	18	78	4.60E+05	pos (12/17)	3.44	1.62	1794.54
6225 (62999)	19	80	2.30E+05	pos (13/17)	4.11	1.35	1203.78
9055 (66732)	5	27	neg	neg	0.14	0.23	0.00
9055 (66732)	6	31	pos	neg	0.18	0.35	329.53
9055 (66732)	7	34	pos	neg	1.79	9.14	19763.40
9055 (66732)	8	38	pos	neg	2.39	9.52	20000.00
9055 (66732)	9	43	pos	neg	1.09	6.26	13380.18
9055 (66732)	10	46	pos	neg	1.70	5.60	11489.04
9055 (66732)	11	65	pos	pos (8/14)	2.54	3.29	13496.42
10000 (69089)	3	13	neg	neg	0.20	0.32	0.00
10000 (69089)	4	31	pos	neg	0.25	0.55	216.73
10000 (69089)	5	36	pos	neg	0.29	0.68	524.51
10000 (69089)	6	39	pos	neg	0.23	1.40	968.81
10000 (69089)	7	43	pos	neg	0.28	1.14	943.03
10000 (69089)	8	46	pos	neg	0.18	0.60	227.46
10000 (69089)	9	50	pos	neg	0.31	1.24	979.38
10000 (69089)	10	53	pos	neg (4/4)***	0.39	0.54	214.98
10003 (69158)	5	23	neg	neg	0.20	0.20	0.00
10003 (69158)	6	26	pos	neg	0.21	0.24	12.96
10003 (69158)	7	30	pos	neg	0.19	5.68	4870.09
10003 (69158)	8	35	pos	neg	0.25	4.14	2518.89
10003 (69158)	9	37	pos	neg	0.22	3.45	2534.60
10003 (69158)	10	42	pos	neg	0.19	2.21	899.06
10003 (69158)	11	44	pos	neg	0.18	1.54	1322.66
10003 (69158)	12	49	pos	neg	0.15	1.54	1456.52
10003 (69158)	13	51	pos	neg (7/7)	0.16	2.22	1899.99
10040 (66154)	1	0	pos	neg	0.87	1.86	17426.76
10040 (66154)	2	3	pos	neg	1.32	2.89	19123.69
10040 (66154)	3	7	pos	neg (6/6)	1.00	2.72	17596.85
10051 (67787)	8	29	neg	neg	0.17	0.33	1.01
10051 (67787)	9	31	pos	neg	0.16	0.36	19.85

10051 (67787)	10	36	pos	neg	0.18	0.43	96.95
10051 (67787)	11	38	pos	neg	0.43	0.88	3056.99
10051 (67787)	12	43	pos	neg	0.31	1.19	1620.50
10051 (67787)	13	46	pos	neg	0.93	2.36	5061.42
10051 (67787)	14	50	pos	neg (5/5)	0.75	2.21	4286.70
10052 (67861)	8	45	neg	neg	0.13	0.34	0.00
10052 (67861)	9	74	pos	neg	0.20	0.82	870.02
10052 (67861)	10	76	pos	neg	0.17	0.35	72.54
10052 (67861)	11	91	pos	neg	0.17	0.34	63.14
10052 (67861)	12	93	pos	neg (5/5)	0.14	0.61	694.36

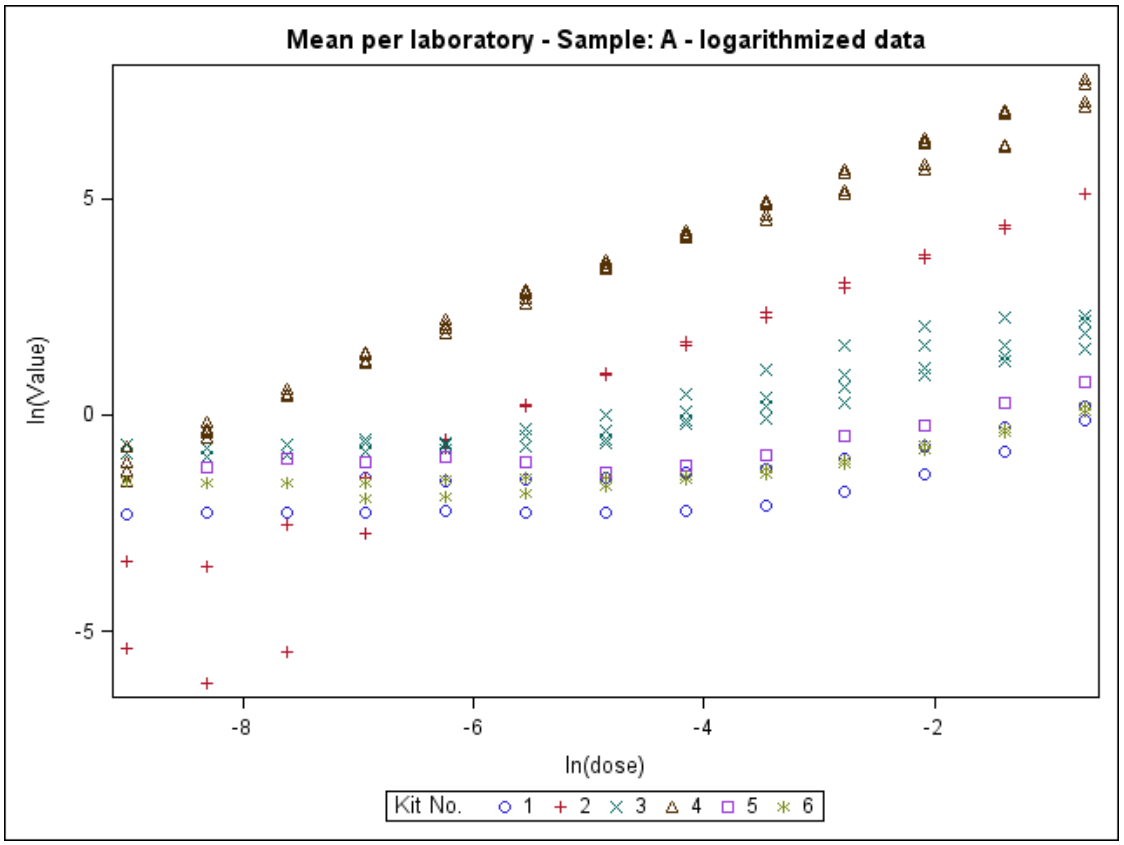
*Results are displayed starting with the last HCV Antigen negative panel member.
S/CO ≥1.0 and fmol/L ≥ 3 are considered to be reactive. Reactive results are indicated in green colour.
Neg: negative; pos: positive; ** indicates numbers of reactive (pos) or negative (neg) tests out of the total tests performed. *** One assay is borderline reactive throughout the whole panel and hence considered unspecific which is also supported by the vendor´s data.

Table 11. HCV-specific antibodies of sample A

		Assay	ADVIA Centaur HCV	Ortho HCV 3.0 ELISA with Enhanced Save	ARCHITECT Anti-HCV	MUREX anti-HCV 4.0	DS-EIA-Anti-HCV
		Manufacturer	Siemens Healthcare	Ortho-Clinical Diagnostics	Abbott	DiaSorinS.A.	RPC Diagnostic Systems
Sample	Code	Dilution 1:	Index	S/CO	S/CO	S/CO	S/CO
Starting material (bulk)	126181	1	0.29	0.56	0.84	n.d.	n.d.
	126181	2	0.17	0.19	0.47	1.97	8.23
	126181	4	0.10	0.09	0.27	0.93	5.61
	126181	8	0.09	0.05	0.17	0.54	3.45
	126181	16	0.07	0.04	0.12	0.33	1.88
	126181	32	0.07	0.03	0.09	0.22	0.89
	126181	64	0.07	0.03	0.09	0.18	0.47
Sample A	129096/12	1	0.23	0.24	0.48	n.d.	n.d.
	129096/12	2	0.14	0.08	0.30	1.32	5.37
	129096/12	4	0.10	0.04	0.17	0.69	3.25
	129096/12	8	0.08	0.03	0.14	0.42	1.66
	129096/12	16	0.08	0.02	0.09	0.27	0.79
Dilution matrix	114126	n.a.	0.06	0.02	0.06	0.16	0.09

n.a.: not applicable; n.d. not determined.
Reactive results are indicated in green colour.

Figure 1. Mean results of dilution series of samples A and B per assay and per laboratory.



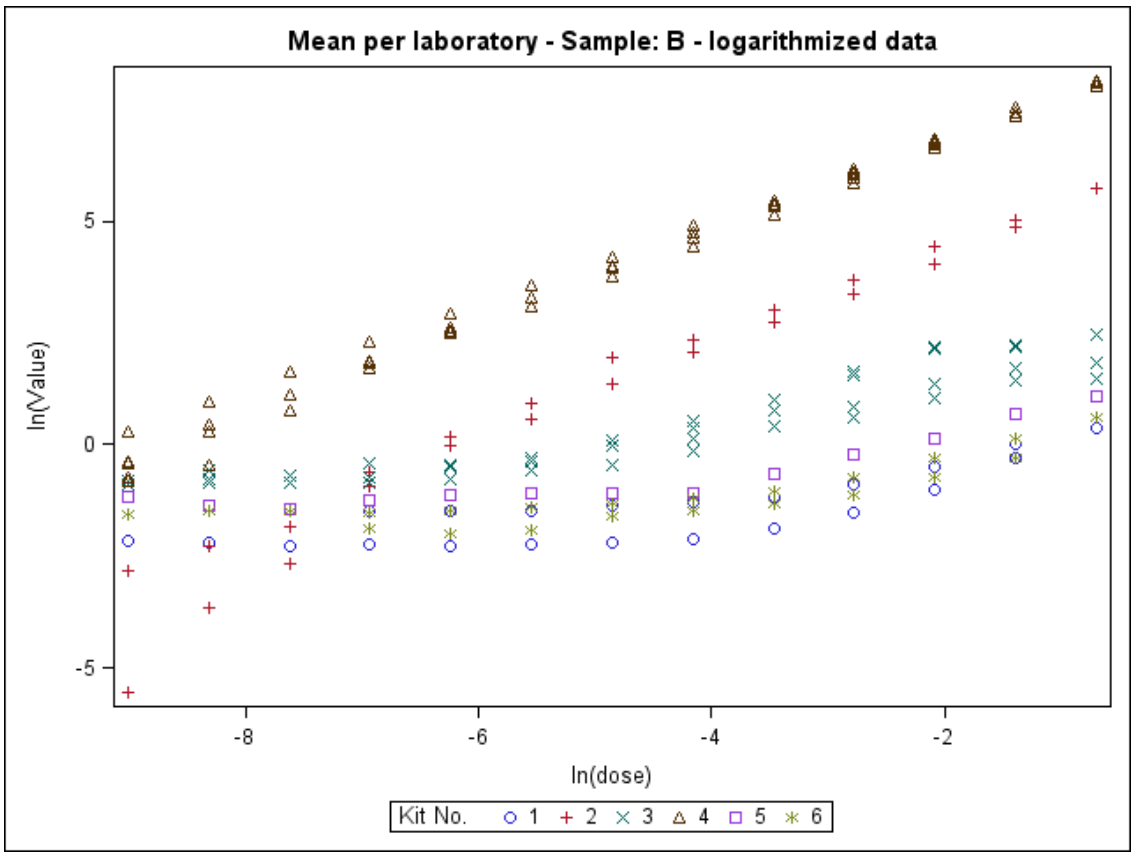


Figure 2a. Mean endpoint titers of sample A and sample B per assay and per laboratory.

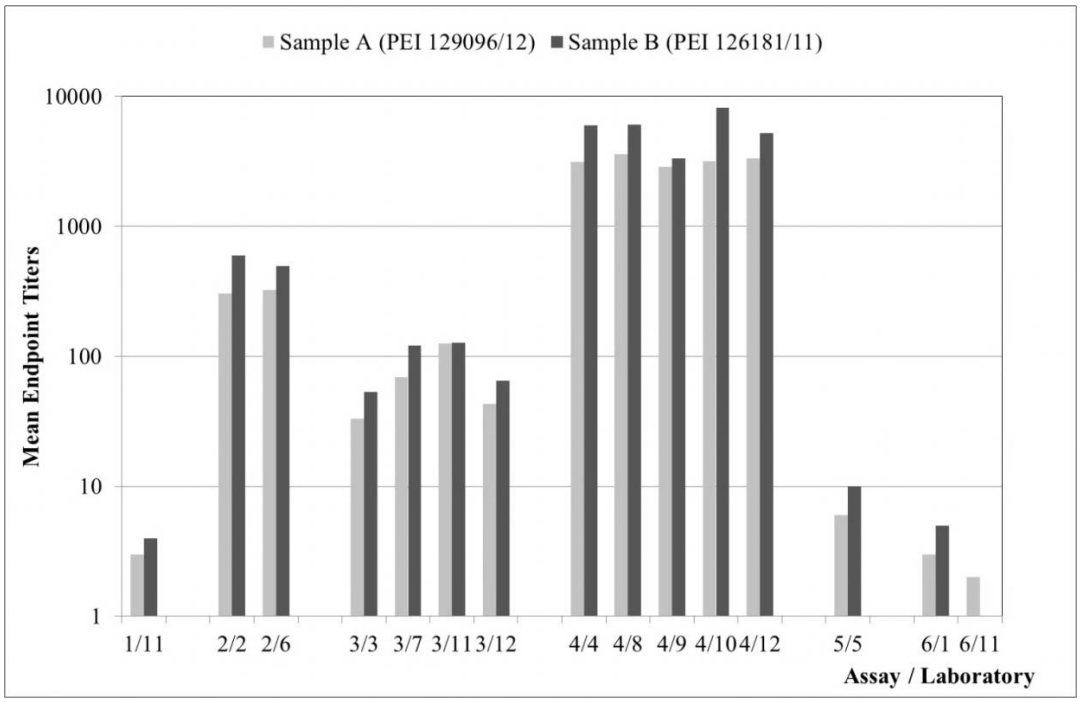


Figure 2b. Comparison of mean endpoint titers of sample A per assay as determined by different statistical methods.

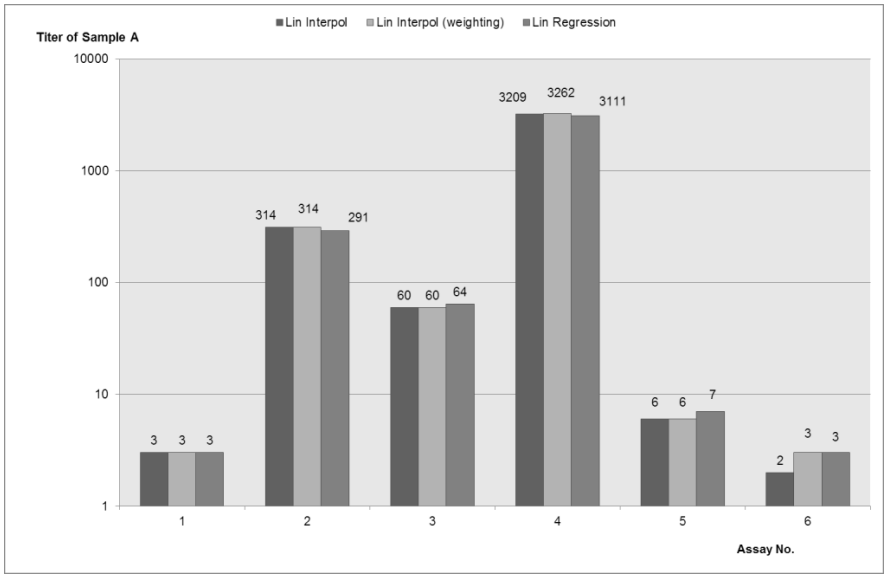


Figure 3. Parallelism of results of samples A and B by the quantitative assays 2 and 4.

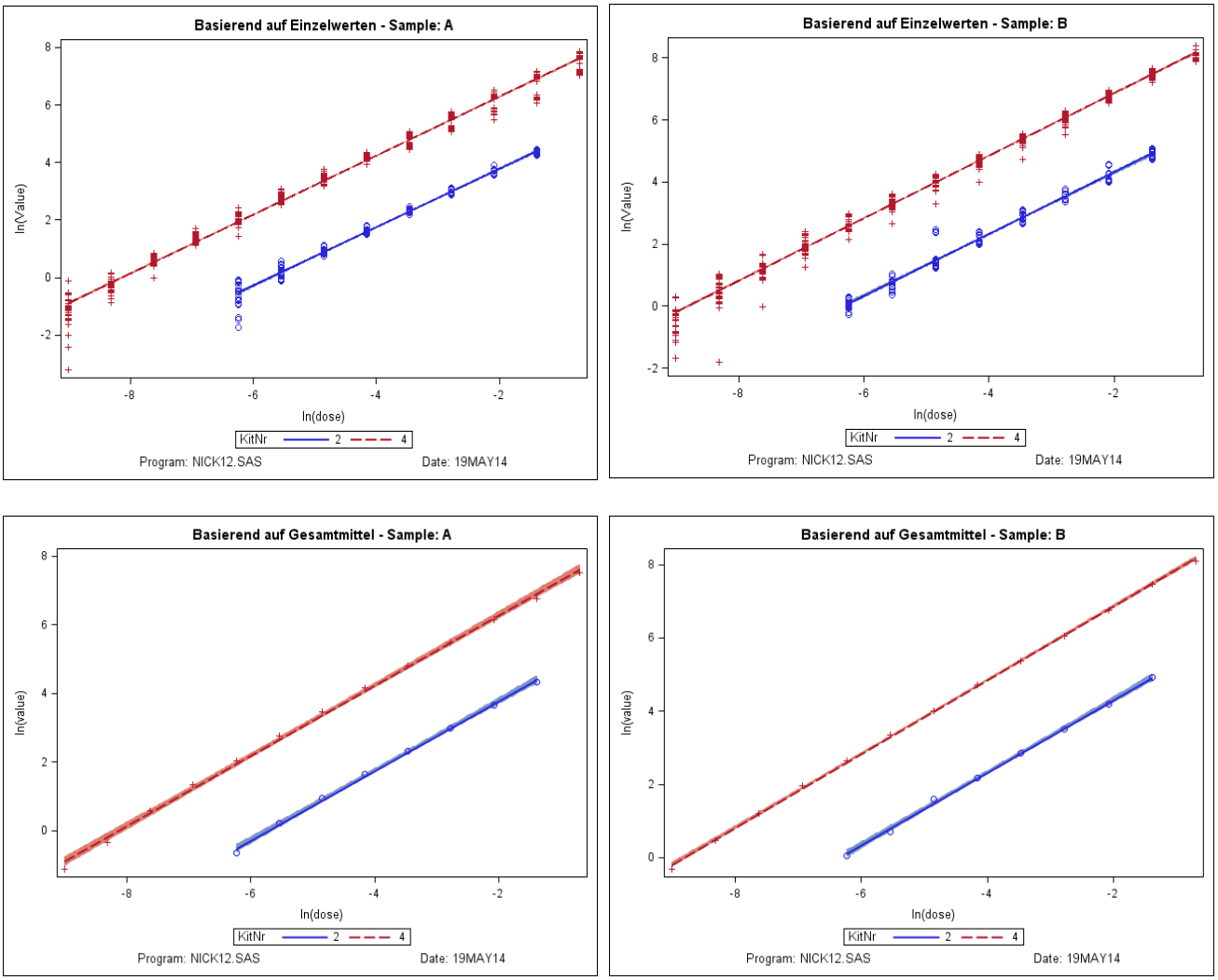
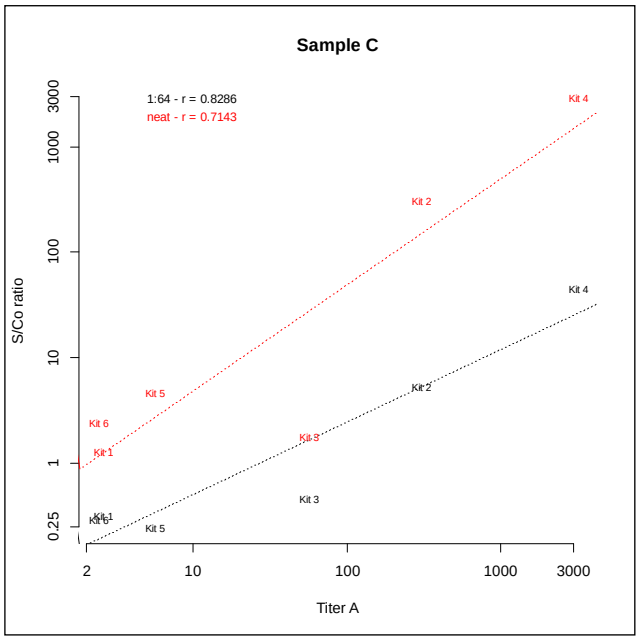
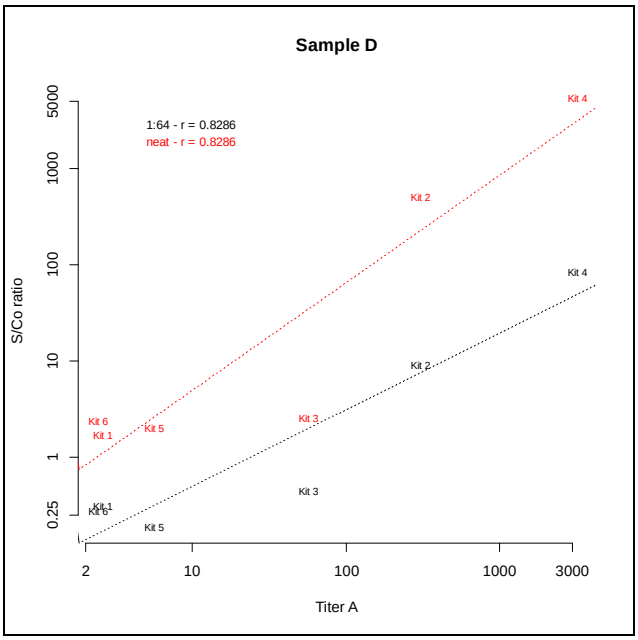


Figure 4. Comparison of titers of sample A and mean S/CO ratios of samples C, C 1:64, D, D 1:64, E and E 1:64 of all assays and laboratories.





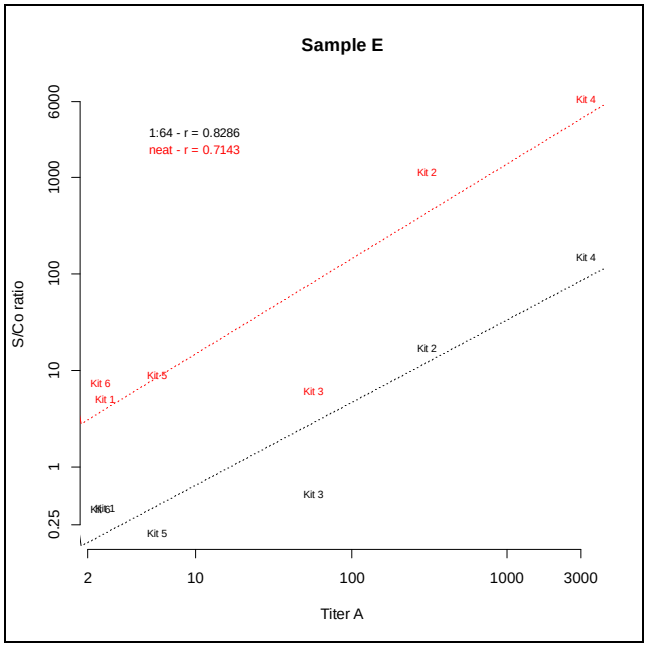


Figure 5. Comparison of titers of sample A and mean S/CO ratios of samples F and F 1:4 of all HCV core antigen assays and the respective laboratories.

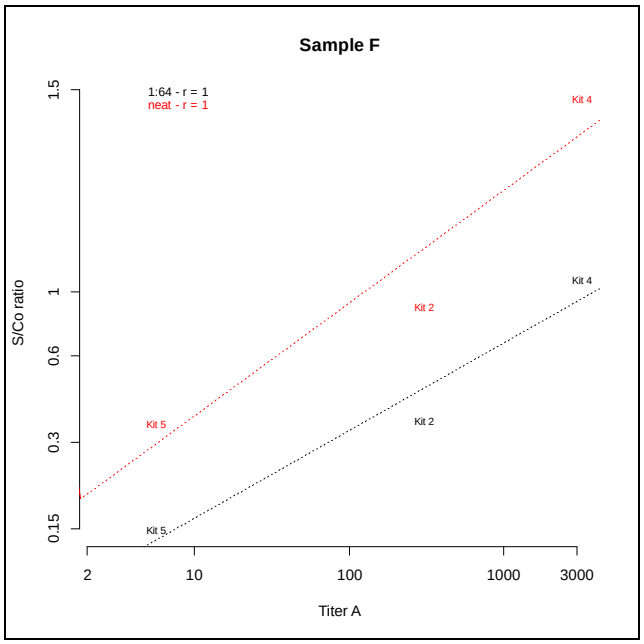
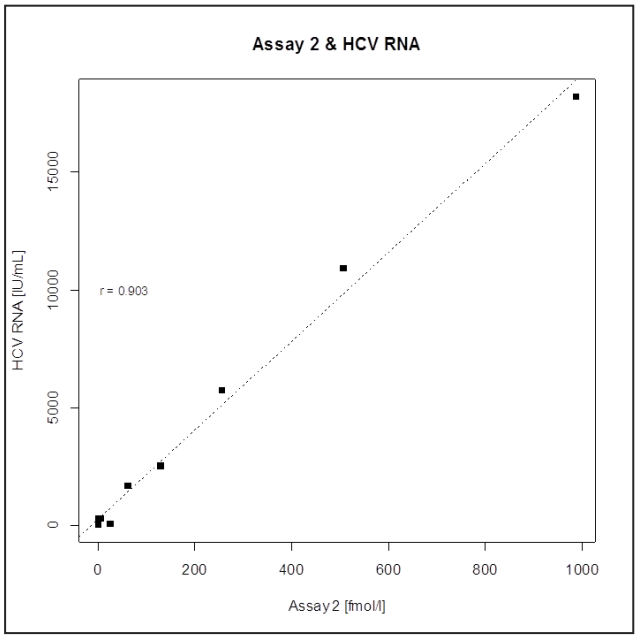


Figure 6. Correlation of HCV core antigen and HCV RNA content of Sample A as determined using the quantitative assays 2 and 4.



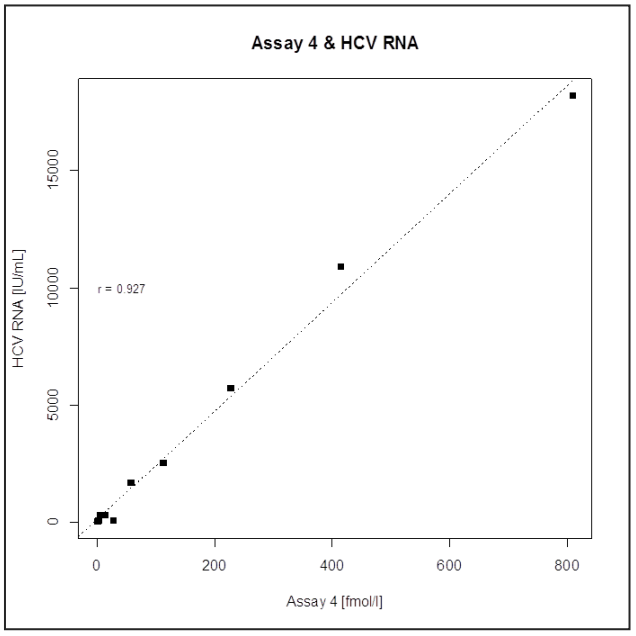


Figure 7. Storage of the candidate material sample A at recommended temperature.

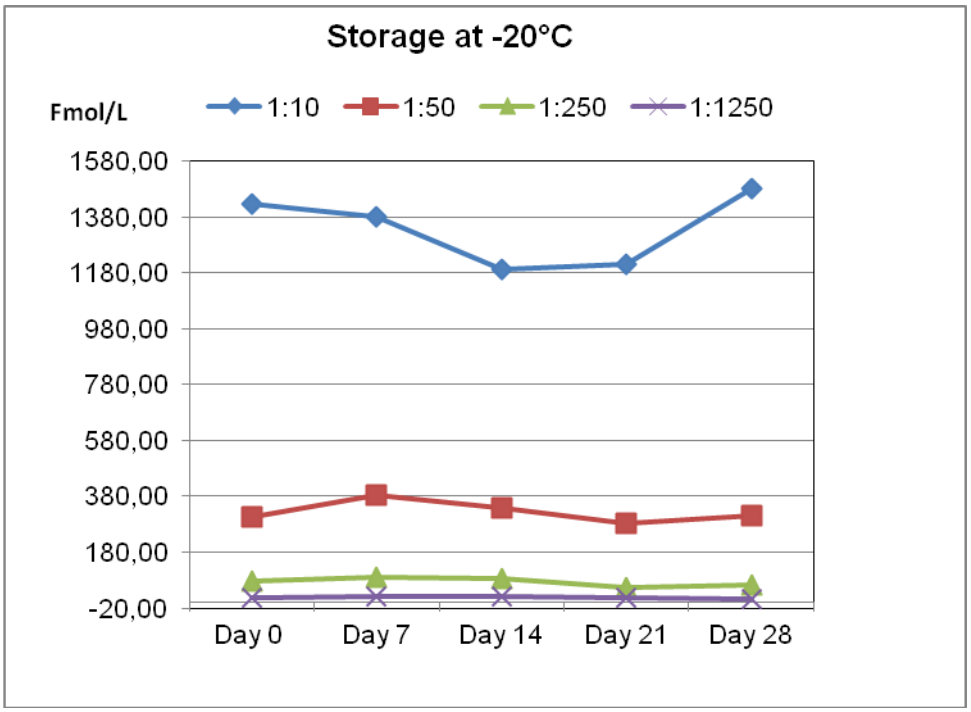


Figure 8. Detection of samples from early HCV infection and correlation to the titer obtained relative to sample A.

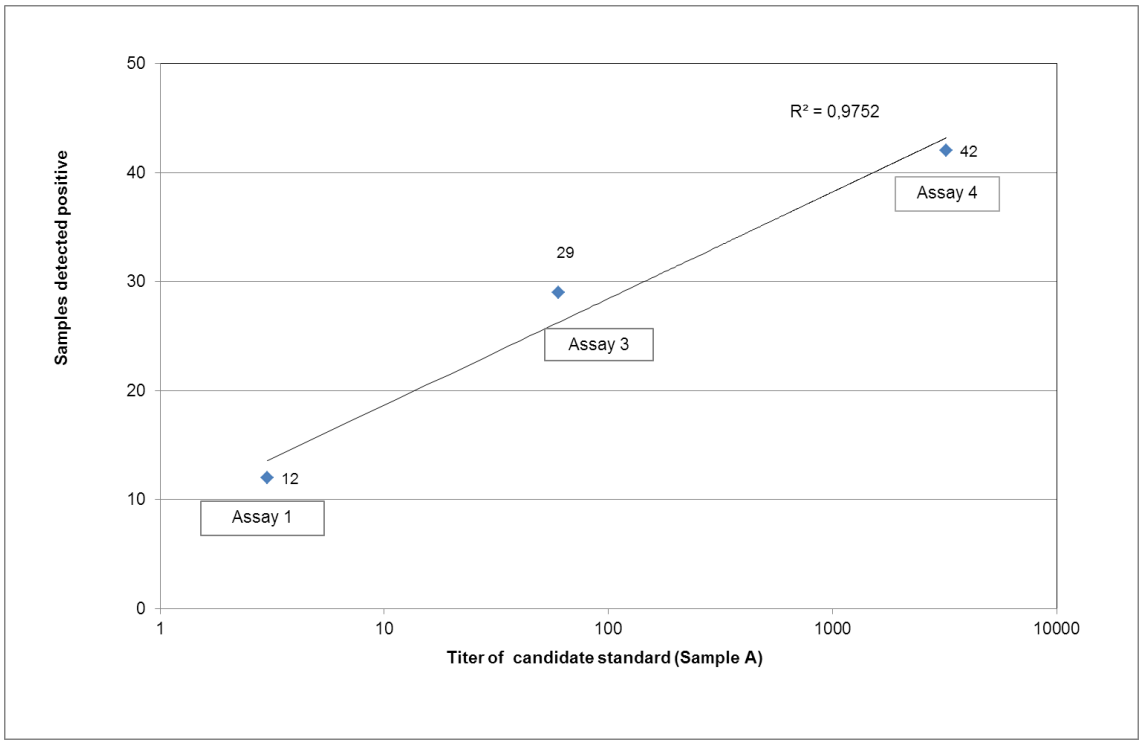


Figure legends

Figure 1. Mean results of dilution series of samples A and B per assay and laboratory. The dilution curves for samples A and B are derived from the natural logarithm of the results (ln(dose)).

Figure 2a. Mean endpoint titers of Sample A and Sample B per assay and per laboratory. Endpoint titers of each assay performed in each laboratory were calculated for the candidate material sample A and for sample B by linear interpolation from maximum two measuring values above and below the cut-off. Logarithmized titers are plotted for each assay and each laboratory.

Figure 2b. The titers were calculated as the (1) geometric mean of all geometric mean values per laboratory (lin interpol), (2) geometric mean value of the single values of each assay (lin interpol weighting) or (3) the individual titer was determined by linear regression analysis of a maximum 2 values above and 2 values below the intercept with the cut-off. The overall titer is the calculated from the geometric mean value per laboratory (lin regression).

Figure 3. Parallelism of results of samples A and B by the quantitative assays 2 and 4. Parallelism and slopes of the curves were determined by regression analysis.

Figure 4. Comparison of titers of sample A and mean S/CO ratios of samples C, C 1:64, D, D 1:64, E and E 1:64 of all assays and laboratories. Geometric mean titers for sample A are plotted against the geometric mean S/CO ratios. Correlation between the assays´ titers for sample A and the S/CO ratios for samples C to E and for the respective dilutions was established by calculation of Spearman´s rank correlation coefficients (r).

Figure 5. Comparison of titers of sample A and mean S/CO ratios of samples F and F 1:4 of all HCV core antigen assays and the respective laboratories. Geometric mean titers for sample A are plotted against the geometric mean S/CO ratios for sample F and for the respective dilutions. Correlation between the assays´ titers for sample A and the S/CO ratios for samples F was established by calculation of Spearman´s rank correlation coefficients (r).

Figure 6. Correlation of HCV core antigen and HCV RNA content of sample A as determined using the quantitative assays 2 and 4. HCV RNA levels (in IU/mL) were plotted against HCV core antigen levels (in fmol/L) as determined with assays 2 and 4. Correlation was established by calculation of Spearman´s rank correlation coefficients (r).

Figure 7. Storage of the candidate material sample A at recommended temperature. Sample A was stored at -20°C. At the indicated time points each, one ampoule was thawed and 1:10, 1:50, 1:250 as well as 1:1250 dilutions were prepared and measured using the ARCHITECT HCV core Ag assay.

Figure 8. Detection of samples from early HCV infection and correlation to the titers obtained relative to sample A. Assays 1, 3 and 4 were used to measure all bleeds of 7 HCV seroconversion panels. The number of samples detected reactive by each assay was plotted against the titers achieved with that assay. Correlation between the assays´ titers for sample A and the number of seroconversion samples detected reactive was established by calculation of Spearman´s rank correlation coefficients (r).

Appendix 1

Participants of the WHO HCV Core Antigen Collaborative Study

Contact	Address
Dr. Sangja Ban Dr. Sangjin Park	Biologics Research Division National Institute of Food and Drug Safety Evaluation (NIFDS), Ministry of Food and Drug Safety(MFDS) Osong Health Technology Administration Complex. 187, Osongsaengyeong2(i)-ro, Osong-eup, Heungdoek-gu, Cheongju-si, Chungcheongbuk-do, Korea, 363-951
Dr Pascal Chesne	Bio-Rad Laboratories, 3 blvd Raymond Poincaré, 92430 Marnes la Coquette, France
Dr David Padley, Dr Neil Almond	Blood and Tissue Pathogens. Adventitious Agents and Diagnostics, Division of Virology, NIBSC. POTTERS BAR, Hertfordshire, EN6 3QG, UK
Dr Mary C. Kuhns	Dept. 9AA, Bldg. AP20, 100 Abbott Park Rd., Abbott Park, IL 60064, USA
Dr Syria Laperche, Dr Annabelle Servant-Delmas	Institut National de la Transfusion Sanguine, 6 rue Alexandre CABANEL, 75739 PARIS cedex, France
Dr Ana Avellón Calvo	Jefe Unidad Hepatitis, Área de Virología, Centro Nacional de Microbiología Instituto de Salud Carlos III., Majadahonda 28220 MADRID, Spain
Prof Dr med. Stefan Ross	Laboratorium für virologische Diagnostik Nationales Referenzzentrum für Hepatitis C Universitätsklinikum Essen Institut für Virologie, Robert-Koch-Haus, Virchowstr. 179 45147 Essen, Germany
Dr Takanobu Kato	Laboratory of Tumor Viruses, Department of Virology II, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan
Lyndon Oldfield, Etienne Du Toit	Operations Executive DiaSorin South Africa (Proprietary) Ltd. 22 Kyalami Blvd, Kyalami Business Park Kyalami, 1684 South Africa
Dr KM Aoyagi, Dr Kazuya Takahashi	Product Lifecycle Management Department. Fujirebio Inc. 51 Komiya-cho, Hachiouji-shi, Tokyo, 192-0031, Japan
Dr Sigrid Nick	Prüflabor für In-Vitro-Diagnostika, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, 63225 Langen, Germany
Dr A.P. Obriadina, Dr Tatjana Ulanova	RPC Diagnostic Systems, Yablonevaya, 22 MS69 603093 Nizhniy Novgorod, Russia

Appendix 2

PROTOCOL FOR THE HEPATITIS C VIRUS CORE ANTIGEN (HCV CORE AG) WHO COLLABORATIVE STUDY TO CHARACTERISE A CANDIDATE INTERNATIONAL STANDARD

Background

Hepatitis C Virus (HCV) is distributed world-wide and a major cause of acute and chronic liver disease, including cirrhosis and liver cancer. Globally, an estimated 170 million persons are chronically infected with HCV and 3 to 4 million persons are newly infected each year. A relatively high number of these people do not have access to appropriate HCV testing. Highly sensitive quantitative HCV core-detecting assays as well as several HCV Ag/Ab combination assays have become available recently. Especially HCV core antigen assays show a performance comparable to that of commercially available viral load assays and thus appear to be a suitable alternative for screening of blood donations and for monitoring of the therapeutic efficacy of antiviral treatment. Consequently, HCV core antigen assays may represent a reasonable alternative to HCV-RNA detection and quantification and thus may contribute to the improvement of health and blood safety.

Aim of the Collaborative Study

The WHO Expert Committee of Biological Standardisation in Geneva on October 2009 proposed to develop an International Hepatitis C Virus (HCV) core antigen standard. The standard may be used to estimate the analytical sensitivity of HCV core antigen-detecting test devices as well as that of HCV Ag/Ab combination tests. Such a standard may be used for the calibration of quantitative HCV core tests that are used for the monitoring of HCV infected patients undergoing antiviral therapy. In addition, it may contribute to better standardising such devices and serve for quality control purposes. It will also be useful for manufacturers to foster product development and for the calibration of the devices. This collaborative study will assess the suitability of a candidate HCV core antigen standard for use in a range of HCV core antigen as well as HCV Ag/Ab combination tests for a wide range of different countries and laboratories. Further available HCV core antigen-containing plasma samples are also included in the WHO Collaborative Study to compare the results and to address commutability of the preparation.

Materials

All study materials will be distributed coded, i.e. Samples A, B, C, D, E and F.

1. Sample A (129096/12): contains freeze-dried human plasma. Ampoules should be reconstituted with 0.5 ml distilled water. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.
2. Sample B (126181/11): contains 0.5 ml frozen plasma. Ampoules should be stored at -70°C. Short term storage at -20°C is possible. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.
3. Sample C (131347/12): is provided neat and diluted 1:64 each containing 1.0 ml frozen human plasma. Ampoules should be stored at -70°C. Short term storage at -20°C is possible. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV

infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.

4. Sample D (131348/12): is provided neat and diluted 1:64 each containing 1.0 ml frozen human plasma. Ampoules should be stored at -70°C. Short term storage at -20°C is possible. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.
5. Sample E (131349/12): is provided neat and diluted 1:64 each containing 1.0 ml frozen human plasma. Ampoules should be stored at -70°C. Short term storage at -20°C is possible. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.
6. Sample F (117031/09): is provided neat and diluted 1:4 each containing 1.0 ml frozen human plasma. Ampoules should be stored at -70°C. Short term storage at -20°C is possible. The material has been found negative for markers of HBV (HBsAg, anti-HBc) and HIV infection (HIV-1 p24, anti-HIV-1/2). The material is positive for HCV. It is not inactivated.
7. The diluent used for the preparation of the samples A-F is included as a control.

SAFETY ADVICE

Samples A – F have <u>not</u> undergone viral inactivation and should be considered infectious and handled as such. The laboratories should follow their own safety procedures. Care should be taken when opening the vials, in particular glass ampoules, to avoid cuts and injuries.

Assay Methods

The assay methods as filled out before in the questionnaire by the participating laboratories should be used according to the instructions for use of the respective manufacturer. If there is any deviation from the instructions in the laboratory, please justify and describe.

Design of the Study

1. From the Samples coded A and B a series of dilutions should be prepared and tested as shown in the results sheets on three days apart.

   Diluent: Normal human serum (NHS) or plasma negative for markers of HCV infection (anti-HCV and RNA) would be favoured. If normal HCV-negative serum or plasma is not available, a dilution matrix normally taken in the participant´s laboratory may well be used.
2. From a feasibility study it is known that the dilution ranges for samples A and B given in the results sheet below cover the detection range of known HCV core Ag and HCV Ag/Ab combination assays. Ideally the dilutions should cover the linear range and reach the endpoint titre (intercept with the cut-off of the assays).
3. The diluent used in your laboratory should be tested in triplicate as a control.
4. Dilutions of samples A and B are requested to be tested in HCV core antigen assays or HCV Ag/Ab combination assays in triplicates independently on 3 three different days. A fresh ampoule of sample A and sample B should be used for each of the 3 independent tests.
5. Samples C to E should be tested neat and in a dilution provided in triplicates only once (on one day only). The dilution matrix for samples C to E is provided to be included as a control.

6. Sample F should be used for HCV Core tests only. This sample is in general not suitable for HCV Ag/Ab combination tests. Sample F should be tested neat and in a dilution provided in triplicates only once (on one day only).
7. All samples (A-F) should be tested concurrently in each assay.
8. In order to be able to assess stability of the various materials (A-F) participants are asked to store any material left over according to the storage instructions below. This will also facilitate retesting when needed.

Results

The results should be reported in the results sheets prepared below. The participants will get the same result´s sheets as electronic file by e-mail.

Please return the filled in embedded excel Results Data Sheet (see below) to Dr Sigrid Nick, Testing Laboratory for IVD, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, 63225 Langen/ Germany, by e-mail: Sigrid.Nick@pei.de (cc pei-ivd@pei.de) or by fax: +49 6103 77-1267.

Storage

Liquid materials left over after the study should be stored at -20°C or -70°C. Avoid multiple freeze/thaw cycles.

Results Sheet Layout

The use of these sheets facilitates the analysis and interpretation of results. The results data sheet is embedded below as an Excel file so that it may be filled out electronically.

Laboratory:	
Assay kit: (Kit name/ version/ catalogue no./ manufacturer.)	
Date of reconstitution / opening of vial:	
Date of test:	
Diluent used in your laboratory:	
Cut-off formula:	
Calculated cut-off value:	
Negative Control:	
Positive Control:	

Sample A:

Perform each HCV core antigen or each HCV Ag/Ab combination assay in triplicates independently on 3 three different days using a freshly reconstituted ampoule for each day. Please be aware of the different dilution series recommended for HCV core Ag tests and HCV Ag/Ab combination tests in the Table below. A separate data sheet should be completed for each experiment and each assay used.

HCV core Ag/ HCV Ag/Ab combination tests		Assay response: e.g. optical density or RLU etc.		
Sample A	Dilution*	Replicate 1	Replicate 2	Replicate 3
129096/12	1:2			
	1:4			
	1:8			
	1:16			
	1:32			
	1:64			
	1:128			
	1:256			
	1:512			
	1:1024			
	1:2048			
	1:4096			
	1:8192			
Dilution matrix	Neat			
Cut-off value		N/A	N/A	N/A

*Dilutions > 1:128 may not be necessary for HCV Ag/Ab combination tests; N/A = not applicable

Sample B:
Please perform each HCV core Ag or each HCV Ag/Ab combination assay in triplicates independently on 3 three different days using a freshly opened ampoule for each day. Please be aware of the different dilution series recommended for HCV core Ag tests and HCV Ag/Ab combination tests in the Table below. A separate data sheet should be completed for each experiment and each method used.

HCV core Ag/ HCV Ag/Ab combination tests		Assay response, e.g. optical density or RLU etc.		
Sample B	Dilution*	Replicate 1	Replicate 2	Replicate 3
126181/11	1:2			
	1:4			
	1:8			
	1:16			
	1:32			
	1:64			
	1:128			
	1:256			
	1:512			
	1:1024			
	1:2048			
	1:4096			
	1:8192			
Dilution matrix	Neat			
Cut-off value		N/A	N/A	N/A

*Dilutions > 1:128 may not be necessary for HCV Ag/Ab combination tests; N/A = not applicable

Samples C, D and E:

Please perform each HCV core Ag or HCV Ag/Ab combination assay in triplicates on one day.

HCV core Ag/ HCV Ag/Ab combination tests		Assay response, e.g. optical density or RLU etc.		
	Dilution*	Replicate 1	Replicate 2	Replicate 3
Sample C (131347/12)	neat			
	1:64			
Sample D (131348/12)	neat			
	1:64			
Sample E (131349/12)	Neat			
	1:64			
Dilution matrix (128928/12)	Neat			

*Dilutions are already provided. N/A = not applicable

Sample F:

Please perform each HCV Core Antigen assay in triplicates on one day only. Sample F is not for evaluation in HCV Ag/Ab combination tests.

HCV core Ag tests		Assay response, e.g. optical density or RLU etc.		
Sample F	Dilution	Replicate 1	Replicate 2	Replicate 3
117031/09	neat			
	1:4			

Results Data Sheet

The results should be filled in an Excel sheet as shown above. The use of this sheet facilitates data analysis and interpretation of results. The sheet provided here as an embedded file may be used:

Data-sheet-Coll-stud
y-HCV core Ag.xlsx

Appendix 3

Proposed instructions for use

See next page.

1st World Health Organization International Standard for Hepatitis C Virus (HCV) core antigen

PEI code 129096/12

(Version 1.0, dated)

1. INTENDED USE

The 1st World Health Organization (WHO) International Standard for Hepatitis C Virus (HCV) core antigen is intended to be used in the standardization and calibration of quantitative and/or qualitative diagnostic HCV core antigen assays, for the determination of analytical sensitivity and for quality control purposes. The establishment of an international standard is an urgent need in the standardization, harmonization and quality control of HCV core antigen assays that detect HCV in the presence of specific antibodies.

The standard represents a lyophilized plasma preparation of a originating from a HCV genotype 1a infected blood donor. 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 12 laboratories performing six different HCV core antigen-detecting assays. Further details of the collaborative study are available in the report WHO/BS/2014.xxxx.

Please note that the material contains low level antibodies that may interfere with HCV Ag/Ab combination tests.

2. UNITAGE

This reagent has been assigned a unitage of 3,200 International Units/ml.

3. CONTENTS

Each vial contains 0.5 ml of a lyophilized plasma preparation containing infectious HCV.

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

The preparation contains material of human origin, and infectious HCV. It was characterized as follows: high positive for HCV RNA, and HCV core antigen. The material tested negative for markers of HIV and HBV infection. This preparation should be regarded as hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures probably will include the wearing of protective gloves and avoiding the generation of aerosols. Care should be taken in opening ampoules or vials, to avoid cuts.

5. USE OF MATERIAL

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

The material is supplied lyophilized and should be stored at or below -20ºC. Each vial should be **reconstituted in 0.5 ml sterile ultrapure water**. The product should be reconstituted just prior to use, once reconstituted, multiple freeze thawing of the product is not recommended. If not all the material is used immediately, laboratories may aliquot the remaining material into suitable volumes which should be stored at or below -20ºC. Do not store the reconstituted material in the refrigerator. It may not be stable.

6. STABILITY

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

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

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

7. REFERENCES

Nick S., Volkers P., Ross S., Scheiblauer H., and the Collaborative Study Group: Collaborative Study to Establish a World Health Organization International Standard for Hepatitis C Virus core antigen.
WHO Report, WHO/BS/2014.xxxx

8. ACKNOWLEDGEMENTS

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

9. FURTHER INFORMATION

Further information for this material can be obtained as follows: pei-ivd@pei.de or whoccivd@pei.de
WHO Biological Reference Preparations:
http://www.who.int/biologicals/en/

10. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to pei-ivd@pei.de or 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, when stored as recommended	Corrosive: No
Hygroscopic:	No	Oxidising: No
Flammable:	No	Irritant: No
Other (specify): CONTAINS HUMAN PLASMA & INFECTIOUS HEPATITIS C VIRUS (HCV)		
Handling:	See caution, section 4	
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 - *contains infectious HCV*		
Ingestion: Seek medical advice - *contains infectious HCV*		
Contact with eyes: Wash thoroughly with water. Seek medical advice – *contains infectious HCV*		
Contact with skin: Wash thoroughly with water. Seek medical advice – *contains infectious HCV*		
Action on Spillage and Method of Disposal		
Spillage of vial contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.		

13. LIABILITY AND LOSS

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

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

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

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

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

The total liability of the Paul-Ehrlich-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 Paul-Ehrlich-Institut 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.