World Health Organization

WHO/BS/2017.2330
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 20 October 2017

Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Chikungunya Virus for Nucleic Acid Amplification Technique (NAT)-Based Assays

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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 **12 October 2017** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr C M Nübling** (nueblingc@who.int)

Summary

This report describes the World Health Organization (WHO) project to develop an international standard (IS) for Chikungunya virus (CHIKV) RNA for use with nucleic acid amplification technique (NAT)-based assays. An international collaborative study was conducted to determine the potency of the candidate standard using a range of NAT-based assays for CHIKV, and to evaluate the suitability of the candidate for the calibration of secondary reference materials and the standardization of CHIKV viral load measurements.

The candidate standard consisted of a heat inactivated CHIKV strain of the East/South/Central African genotype (ESCA), also known as the Indian Ocean Lineage, isolated from a patient returning from India to the United States in 2006[1], diluted in human negative plasma. The lyophilized candidate preparation (Sample 1), the corresponding liquid-frozen bulk material (Sample 2) and three different clinical samples (Sample 3, Sample 4 and Sample 5) were included in the collaborative study. Twenty-five laboratories representing 14 countries participated in the study to evaluate the material using their routine CHIKV NAT assays. Twenty-four laboratories returned 31 data sets from 17 commercial assays and 14 in-house methods. Of these 31 methods, 11 were quantitative and 20 were qualitative.

The results of the study indicate the suitability of the candidate material of the CHIKV strain of ESCA genotype (Sample 1) as the proposed 1st WHO IS for CHIKV. It is therefore proposed that the candidate material (PEI code 11785/16) is established as the 1st WHO IS for CHIKV RNA for NAT-based assays with an assigned potency of 2,500,000 International Units (IU)/mL when reconstituted in 0.5 mL of nuclease-free water.

On-going studies for real-time and accelerated stability of the proposed IS indicate that the preparation is stable and suitable for long-term use under the proposed storage conditions.

Introduction

Chikungunya virus (CHIKV) is an enveloped, positive-sense single stranded RNA alphavirus in the family Togaviridae that causes a fever-rash-arthralgia syndrome in humans, known as chikungunya fever. The virus is most commonly transmitted in the urban cycle by the mosquitoes *Aedes aegypti* and *Aedes albopictus*, the same vectors that transmit dengue and Zika virus.[2]

CHIKV was first recognized in epidemic form in East Africa (Tanzania) in 1952–1953.[3, 4] Following the discovery of CHIKV, numerous subsequent outbreaks have been documented both in Africa and Asia. Since 2000, the incidence of large outbreaks has increased with spread of the virus to previously non-endemic regions. The current epidemic, ongoing since 2004, involves many tropical and sub-tropical areas of Africa, Asia, Europe, the Indian Ocean, the Caribbean and the Americas. Autochthonous transmissions have been reported in the Caribbean, the Americas and Europe.[5] In the years 2016 and 2017 current outbreaks have been reported in India and Pakistan.[6] Just recently, in August/September 2017, French and Italian authorities reported numerous clustered cases of autochthonous CHIKV infection in France and Italy.[7, 8]

Genetic analysis of strains have identified three geographically distinct CHIKV lineages: the West African lineage, the East/Central/South African (ECSA) lineage, and the Asian lineage derived from the ECSA virus.[9]

About 15% of individuals infected with CHIKV are asymptomatic, but most infections cause a febrile illness characterized by high fever and severe joint pain. Other symptoms include muscle

pain, headache, nausea, fatigue and rash. Joint pain is often debilitating and can last months to years, but death from CHIKV infection appears to be extremely rare.[10]

Owing to the lack of licensed vaccines and antiviral therapeutics, the primary response to CHIKV outbreaks is vector-control. However, *A. aegypti* and *A. albopictus* populations continue to expand in some regions due to the rapid development of insecticide resistance, the lack of education and poor infrastructure.[11]

CHIKV, like other arboviruses, has the potential to be transmitted by transfusion of blood and blood products. Measures to prevent possible CHIKV transfusion transmission include deferral of symptomatic donors, discontinuing blood collections in affected areas, and CHIKV nucleic acid amplification technique (NAT) screening of donations.[12]

NAT-based assays are considered the most sensitive detection method for laboratory diagnosis and blood screening of acute CHIKV infections. Currently, there is no standardization of NAT-based assays for the detection of CHIKV RNA.

The proposal for the establishment of an International Standard (IS) for CHIKV RNA for NAT-based assays was endorsed by the Expert Committee on Biological Standardization (ECBS) of the World Health Organization (WHO) in 2010. The Paul-Ehrlich-Institut (PEI), a WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, has developed a candidate IS for CHIKV RNA in collaboration with the Centre for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA). The International Standard was prepared in accordance with published WHO recommendations.[13]

Study materials

Candidate International Standard (11785/16)

For the preparation of the candidate IS a CHIKV isolate imported in 2006 from India to the United States[1] was selected. The CHIKV strain R91064 was kindly provided by Maria Rios from CBER/FDA. The virus has been propagated in cell culture (Vero cells), inactivated by heat-treatment and characterized within the context of a collaborative study at CBER/FDA as described previously by Añez et al..[14] The complete coding region and partial non-coding region sequences of the CHIKV strain R91064 have been determined and made available under the GenBank accession number KJ941050.[15]

For the preparation of the candidate WHO standard bulk, 35 mL of the CHIKV strain R91064 were mixed with 1965 mL of human plasma (~1:57 dilution). The bulk preparation was cooled (4-8°C) until processing (~18 hours later). The preparation was diluted using pooled citrated human plasma which was centrifuged and filtered before use. The plasma diluent tested negative for anti-CHIKV IgG and IgM, CHIKV RNA, dengue virus (DENV) RNA, Zika virus (ZIKV) RNA, human immunodeficiency virus type 1/2 (HIV-1/2) RNA, hepatitis A virus (HAV) RNA, hepatitis B virus (HBV) DNA, hepatitis C virus (HCV) RNA, hepatitis E virus (HEV) RNA and Parvo B19 virus DNA. Testing was performed at PEI by using CE marked and in-house validated test methods. In addition, the plasma was negative for hepatitis B surface antigen (HBsAg), anti-HCV and anti-HIV-1/2.

The filling and lyophilisation was performed by an EN ISO 9001- and EN ISO 13485-certified company in Switzerland. Processing took place between the 8th and 11th November 2016. For processing, 0.5 mL volumes were dispensed into 4 mL screw-cap glass vials. After completion of

the freeze-drying procedure using a CHRIST Epsilon 2-25 D freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany), the vacuum was broken by the introduction of nitrogen gas and the vials sealed with rubber seals. The vials were further secured with screw caps prior to storage at -20°C.

For the candidate WHO standard, 3524 vials were lyophilized. The mean fill weight was 508.09 mg and the coefficient of variation (% CV) of the fill volume was 0.58% with a standard deviation (SD) of ± 2.937 mg (n=24). Residual moisture was determined by Karl Fischer analysis and was 0.525% (n=12). The freeze-drying process did not significantly affect the CHIKV RNA titre of the lyophilized samples when compared to aliquots of the respective bulk preparations which were stored at -80°C (data not shown).

Because the candidate IS was to be assigned a unitage with respect to the CHIKV RNA content, homogeneity of the filling/freeze-drying was assessed using real-time PCR. Extraction of RNA was performed using 500 µL of the sample using the QIAamp DSP Virus Kit (Qiagen GmbH, Hilden, Germany). Elution of viral nucleic acid was performed using 60 µL of elution buffer, and 10 µL of the eluate was used for the RT-PCR. Amplification reactions and detection were performed using the RealStar® Chikungunya RT-PCR Kit 2.0 (altona Diagnostics, Hamburg, Germany) on the LightCycler 480 II instrument (Roche Molecular Diagnostics, Mannheim, Germany) in accordance with the manufacturer's instructions. A standard curve was prepared using an in vitro CHIKV RNA transcript kindly provided by altona Diagnostics, Hamburg, Germany. The crossing point-PCR-cycle or cycle threshold (C_T) values were determined for 12 vials of the candidate IS. For each vial 1:10 and 1:100 dilutions were tested. The mean C_T values were 24.94 (1:10) and 28.17 (1:100), respectively, with a CV of 0.44% (1:10) and 0.32% (1:100), indicating that the filling was of acceptable homogeneity.

For the international collaborative study both, the lyophilized candidate standard 11785/16 (coded as "**Sample 1**") and the liquid/frozen bulk material (coded as "**Sample 2**") were provided to the participants for parallel testing.

Vials of the candidate WHO IS are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, 63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.

All manufacturing records are held by PEI and are available on request by the WHO Expert Committee on Biological Standardization.

Clinical materials

Sample 3

This viraemic plasma sample originated from an ill traveller returning to Europe from the Indian Ocean region (Mauritius) and was kindly provided by Bernd Kupfer (University of Bonn, Institute of Virology, Germany). Plasma has been stored as liquid/frozen material at ≤ -70°C after ~1:500 dilution in pooled negative human plasma which tested negative for different viral nucleic acids including CHIKV RNA at the PEI (see preparation of candidate IS).

Sample 4

This viraemic plasma came from a Brazilian patient infected with CHIKV and was kindly provided by Lia Laura Lewis Ximenez de Souza Rodrigues (Instituto Oswaldo Cruz/Fiocruz, Brazil). Plasma

has been stored as liquid/frozen material at ≤ -70°C after ~1:200 dilution in pooled negative human plasma (see preparation of candidate IS).

Sample 5

This viraemic plasma came from a Brazilian patient infected with CHIKV and was kindly provided by Patrícia Alvarez Baptista (Bio-Manguinhos/Fiocruz, Brazil). Plasma has been stored as liquid/frozen material at ≤ -70°C after ~1:100 dilution in pooled negative human plasma (see preparation of candidate IS).

Collaborative Study

A total of 34 laboratories from 19 countries located in 4 continents were invited to participate in the study. The potential participants were selected because of their recognized expertise in the CHIKV diagnostics field and geographic distribution. They represented IVD manufacturers, regulatory authorities, as well as clinical, reference and research laboratories. Twenty-five laboratories from 14 different countries (Americas: 7, Asia: 4, Europe: 13, Australia: 1) agreed to participate in the study and received the study samples. One laboratory was unable to return data. The collaborative participants are listed in Appendix 1. For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1. Qualitative and quantitative results from the same laboratory were identified in the figures with the same laboratory code and a letter "A" and "B" to differentiate between qualitative and quantitative results, respectively.

All collaborative study materials were shipped to participating laboratories on dry ice and participants were requested to store the materials at or below -20°C (Sample 1) and at or below -70°C (Samples 2 to 5) until use. Samples represented a mixture of inactivated reference materials as well as a small number of infectious clinical samples. Participants received samples representing the ESCA CHIKV lineage (Samples 1 and 2) and unknown CHIKV lineages (Samples 3 to 5; sequencing analysis is ongoing).

The samples included in the panel (Samples 1 to 5) are described above. Sufficient materials were provided for four separate assay runs. Additional vials were provided for laboratories using larger extraction volumes. Laboratory 18 only received the inactivated Samples 1 and 2.

Participants were asked to test the panel using their routine assay for CHIKV RNA, testing the panel of samples in four separate assay runs, using fresh vials of each sample for each run.

Where laboratories performed quantitative assays, participants were requested to test Sample 1, Sample 2, Sample 3, Sample 4 and Sample 5 in the linear range of the test by a series of three one $\log_{10}$ dilution steps in all four assay runs. Suggested dilutions to test for each sample were proposed in the study protocol (Appendix 3). Results should be reported in copies/mL.

In the case of qualitative assays, participants were requested to assay Sample 1, Sample 2 and Sample 3 by a series of seven one $\log_{10}$ dilution steps to obtain an initial estimate of an end-point. For the three subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point determined in their first assay. If, in the second assay, all dilutions were positive, or all negative, then the dilution series were to be adjusted accordingly for the final assay run. The participating laboratories were asked to test Sample 4 and Sample 5 at three one $\log_{10}$ dilution steps in all four assay runs. Results were reported as either positive i.e. CHIKV RNA detected or negative.

Participants were asked to note if replicate extractions and replicate amplification/detection steps were performed. Electronic data sheets and a method reporting form were provided so that all

relevant information (e.g. C_T values for the respective dilutions where real-time PCR methods were used) could be reported.

For the preparation of dilutions, participants were requested to use their usual diluent representing the matrix of the normal test specimens e.g. CHIKV negative plasma. It was recommended to dilute the samples in plasma, or other types of sample matrix that might be used for CHIKV diagnostic testing.

The lyophilized preparation evaluated in the study was reconstituted before use by participants using 0.5 mL molecular grade, nuclease-free water. After addition of water, it was recommended that the samples be left for a minimum of 20 minutes with occasional agitation before use. All other samples were provided as liquid/frozen materials.

Statistical Methods

Quantitative Assays

Evaluation of quantitative results includes the complete dilution range used by the participants, as with correction of the dilution factor the data show comparable results between dilutions. Estimates (expressed in $\log_{10}$ NAT-detectable units/mL) for each laboratory, sample and assay were derived by the geometric mean from up to 6 replicates.

Qualitative Assays

For qualitative data analysis, results from all assays were pooled to give the number of positives out of the total number tested at each dilution. If it is assumed that a single 'detectable unit' will give a positive result, and that the probability of a positive result follows a Poisson distribution, the EC63 (the dilution at which 63% of the samples are expected to be positive) was chosen as the end-point. For each laboratory and sample, these end-points were estimated by means of a probit analysis. For assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps for all samples, the Spearman-Kaerber method was applied for EC63 estimation. The calculated end-point was used to give estimates expressed in $\log_{10}$ NAT-detectable units/mL after correcting for the equivalent volume of the test sample.

According to the study protocol for qualitative assays, participants were requested to test dilutions of Sample 1, Sample 2 and Sample 3 around the end-point determined in the first assay run. In contrast, Sample 4 and Sample 5 were to be tested in three ten-fold dilutions (without end-point dilution analysis). However, evaluable data for Sample 4 and Sample 5 were included in the analysis whenever the end-point was detectable.

Combination of quantitative and qualitative data

A mean estimate for quantitative data for each sample and laboratory was estimated by means of mixed linear model (random factor assay run). These mean estimates were then combined with the qualitative data for each sample and laboratory. Distribution of participants, assay types, and diluent matrices were graphically presented in histogram form.

For the candidate reference potency, data from 30 assays were combined. Data from 23 out of 24 laboratories was included with 7 participants providing data for more than one assay. Most assays were qualitative (20 assays, 10 quantitative assays). One assay had to be excluded due to different

assay units (plaque-forming units (pfu)/mL) reported, which could not be converted to copies or genome equivalents/mL.

Relative potencies

Potencies of all samples, for the quantitative assays, were estimated relative to the candidate IS 11785/16 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to the reference candidate IS 11785/16 potency) using a parallel line model for each laboratory combined for all evaluable (i.e. valid) assay runs. Relative potencies from C_T values were shown in histogram form to allow for a comparison of participants and assay types.

Parallel line model and probit analysis were performed according to methods described in chapter 5.3, "Statistical analysis of results of biological assays and tests", of the Ph. Eur..[16] The statistical analysis was performed with SAS®/STAT software, version 9.4[17], and CombiStats software, version 5.0.[18]

Inter- and intra-laboratory variation

For C_T values (relative potencies of both qualitative and quantitative data), variation between laboratories (inter-laboratory) as well as variation within laboratories and between assays (intra-laboratory) was estimated as geometric variation coefficients (%GCV) based on a mixed linear model including all individual estimates (random factors *labcode* and *assay type(labcode)*). For quantitative data, the variability (as combination of inter- and intra-laboratory variation) was estimated with a mixed linear model including all individual estimates (with random factors *labcode* and *assay run*), whereas for qualitative data the variation was estimated simply by means of the ratio of the mean estimate divided by the standard deviation (due to only one estimate per laboratory available).

Stability Studies

Stability of the candidate IS is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate WHO standard have been stored at -20°C (the recommended storage temperature) and at -80°C (to provide a baseline if there is any suggestion of instability at higher temperatures). For the accelerated thermal degradation, vials have been incubated at +4°C, +20°C and +37°C for up to 6 months. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 mL of nuclease free water and analysed by real-time PCR as described above. Aliquots of the corresponding frozen liquid bulk materials storage at -80°C were analysed in parallel.

Results

Data received

Data were received from a total of 24 of the 25 participating laboratories. In total, 31 sets of data were returned; 11 from quantitative assays and 20 from qualitative assays. Some laboratories reported results for more than one type of assay. For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing

in Appendix 1. Where a laboratory performed more than one assay method, the results from the different methods were analysed independently, as if from separate laboratories, and coded, for example, laboratory 3A and laboratory 3B.

For quantitative data, participants returned values as copies/mL or genome equivalents/mL which were assumed to be equivalent to copies/mL. Qualitative data was expressed as reactive or non-reactive. Participants who performed real-time RT-PCR methods reported C_T values in addition (qualitative and quantitative assays).

The types of methods used by the participants are listed in Table 1. Assays included in the study targeted several different regions of the non-structural and structural protein genes of the CHIKV genome. Participants used in-house developed assays based on scientific publications as well as commercial assays (including assays under development). The vast majority of assays were based on real-time RT-PCR. A range of manual and automated extraction methods as well as different types of matrix for dilution of the study samples were used (Table 1).

Exceptions were as follows:

Laboratory 9 reported quantitative results as plaque-forming units (pfu)/mL without further adjustment on the number of copies or genome equivalents quantifiable by NAT per pfu. Due to non-comparability this data set had to be omitted from the evaluation.

Laboratory 18 did not receive the infectious study material Sample 3, Sample 4 and Sample 5 for testing.

Quantitative Assay Results – Mean Estimates

A total of 11 (35%) data sets for quantitative assays were provided by the participating laboratories. Quantitative assays for each study sample were performed over a range of three ten-fold dilutions expected to fall in the linear range of the majority of assays. Participants returned values as copies/mL or genome equivalents/mL (accounted for dilution). The individual laboratory means are given in Table 2. Mean estimates are also shown in histograms in Figures 1-5. Each box represents the mean estimate from an individual laboratory and is labelled with the laboratory code number. The mean estimates for each study sample in each laboratory were expressed in $\log_{10}$ NAT-detectable units/mL. Relative variation of the individual laboratory estimates for quantitative assays was evaluated with regard to the intra-laboratory standard deviation of $\log_{10}$ NAT-detectable units/mL and %GCV (Table 8).

Qualitative Assay Results – Mean Estimates

A total of 20 (65%) data sets for qualitative assays were provided by the participating laboratories. The individual laboratory means are given in Table 3. The NAT-detectable units/mL ($\log_{10}$) for the qualitative assays is shown in histograms in Figures 1-5. Each box represents the mean estimate from an individual laboratory and is labelled with the laboratory code number. Mean qualitative results were considerably lower than quantitative results. From Figures 1-5, it can be seen that the qualitative assays are more variable than the quantitative assays, reflecting the different sensitivities of the various assays. This observation is not unexpected and is in line with other studies in this field.

Determination of Overall Laboratory Means – Combined Qualitative and Quantitative Results

The overall mean values and the precision of estimates between laboratories (95% confidence interval) for the candidate IS and the other study samples for the qualitative and quantitative assays are shown in Table 4. The combined overall mean values for both the qualitative and quantitative tests are shown in Table 5 together with the standard deviations and the range of estimates.

Qualitative assays gave consistently lower mean estimates for all study samples than for the quantitative assays, probably as a result of lower sensitivity of qualitative assays. When comparing results from Sample 1 and Sample 2 a slight potency loss of 0.27 and 0.37 $\log_{10}$ NAT-detectable units/mL for the candidate IS was observed for the mean estimates of both, quantitative and qualitative tests, respectively. In conclusion, the lyophilisation of the candidate IS had no significant impact on the CHIKV RNA integrity.

Relative Potencies

On the basis of the combined data from both qualitative and quantitative assays, the candidate WHO IS 11785/16 (Sample 1) was determined to have a potency of 6.39 $\log_{10}$ units/mL (95% CI 6.00 – 6.79). The potencies of the panel of study samples were calculated relative to Sample 1 – the candidate IS, taking the value of Sample 1 as 6.39 $\log_{10}$ units/mL. The relative potencies for quantitative and qualitative assays are shown in Table 6 and in histograms in Figures 6-9. Overall mean estimates and inter-laboratory variation for potency relative to Sample 1 $\log_{10}$ IU/mL are summarized in Table 7 for quantitative and qualitative assays as well as for combined data. For Sample 2, the liquid bulk material, as well as for the clinical samples, Sample 3, Sample 4 and Sample 5, it can be observed that in each case there is a marked reduction in assay variability when data are expressed against the common standard. These data provide some evidence for commutability of the candidate standard for evaluation of CHIKV from infected individuals and its potential to harmonize CHIKV NAT-based assays. Please recognize the scales differences between different figures.

Relative potencies C_T values

For the different laboratories and assays where C_T values have been reported, data were evaluated for both qualitative and quantitative methods relative to the candidate IS (Sample 1) potency of 6.39 $\log_{10}$ units/mL. The relative potencies from C_T values for quantitative and qualitative assays are shown in Table 9 and in histograms in Figures 10-13. Overall potency estimates from C_T values for quantitative and qualitative assays as well as for combined data are summarized in Table 10. The data show that potencies estimated by the alternative statistical method based on C_T values are comparable to those obtained using the conventional method described above. Please recognize the scales differences between different figures.

Table 11 shows the variability of relative potencies (from up to 4 assays per laboratory) based on C_T values from qualitative and quantitative assays. As already observed in the figures above (Figures 10-13), for Sample 4 and Sample 5, the between laboratory variability is distinctly higher than for Sample 2 and Sample 3.

Evaluation of C_T values

In order to evaluate the commutability of the candidate IS, comparative testing of three clinical samples (Sample 3, Sample 4 and Sample 5) was performed within the international collaborative study and C_T values reported for qualitative and quantitative real-time PCR assays have been analysed in this regard. The analysis included 30 data sets, 11 from quantitative and 19 from qualitative real-time PCR assays used in the collaborative study. Data demonstrated that the candidate IS and the clinical samples were detected with comparable efficiency (data not shown). Furthermore, the mean C_T values of the liquid bulk material (Sample 2) and the clinical samples (Sample 3, Sample 4 and Sample 5) were compared with the corresponding mean C_T values of the candidate IS (Sample 1). The relationship of mean C_T values between the Sample 1 and Sample 2, Sample 3, Sample 4 and Sample 5 (based on data of 1:10 dilution) is shown in Figure 15. The relative concentration of Sample 2 (liquid-frozen candidate IS material) from most assays is slightly higher compared to the relative concentration of Sample 1 (up to 1 C_T value), which demonstrated that the lyophilisation had no significant effect on the integrity of CHIKV RNA. Only results from assays 23A/B provided a C_T values difference of about 2. Almost all laboratories included in the evaluation detected the clinical samples (Sample 3, Sample 4 and Sample 5) with a lower concentration when compared to the candidate IS (as expected). However, for Sample 4 and Sample 5 the between laboratory variability is distinctly higher than for Sample 3.

Results of Stability Studies

Vials of the candidate WHO standard were incubated at +4°C, +20°C and +37°C for up to 6 months and tested by real-time PCR for CHIKV RNA. The heat-treated vials were assayed concurrently with control vials that had been stored at -20°C and at -80°C, in duplicate. Summarized results of stability studies are shown in Table 12 and Figure 14, respectively.

There was no evidence of instability of the samples stored at -20°C when compared to samples stored at -80°C. Thus, the results from the testing suggest that the lyophilized preparation is stable when stored at the recommended storage temperature at -20°C or below. Real-time stability studies have also indicated that the candidate IS is stable after 6 months incubation at +20°C. As there was no significant reduction in CHIKV RNA this would support shipment at ambient temperature. The most significant loss of titre (~ 1.3 $\log_{10}$) was observed for the candidate material when stored for 6 months at +37°C. Thus, the lyophilized candidate IS should be protected from extreme temperatures when transported.

The potency of the reconstituted material, after freezing and thawing, has not been investigated. Accelerated and real-time stability studies are on-going and will be communicated to the WHO.

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

Conclusions

In this collaborative study, various quantitative and qualitative assays were employed to determine the potency of the candidate IS for CHIKV RNA and to evaluate its suitability for use in NAT-based assays. Collaborators used a wide range of methods, both in-house developed and commercially available, for extraction of nucleic acids and for amplification/detection. Approximately half of the data sets received were from laboratory-developed assays. The other half of the data sets was

received from assays available commercially or under development by kit manufacturers. The different samples included in the study were well detected by the participating laboratories, with some differences in efficiency of detection of some of the samples. Results of the study provide evidence that the lyophilisation of the candidate IS had no great impact on the integrity of the viral nucleic acid.

Real-time stability studies have indicated that the candidate IS is stable under normal conditions of storage, i.e. at -20°C or below for 6 months and therefore suitable for long term use as well as at elevated temperatures, i.e. after 6 months incubation at +20°C there was no significant reduction in CHIKV RNA which would support shipment at ambient temperature. Initial accelerated thermal degradation analysis indicates a reduction in the levels of CHIKV RNA at higher incubation temperatures (e.g. +37°C). Shipment at extreme temperatures should therefore be avoided. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.

The draft "Instructions for Use" for the candidate IS includes details for storage and reconstitution of the material (Appendix 2). Each vial contains the lyophilized residue of 0.5 mL of inactivated CHIKV in human plasma to be reconstituted in 0.5 mL of nuclease-free water. Based upon the qualitative and quantitative results of the collaborative study, the candidate WHO IS was estimated to have a potency of 6.39 $\log_{10}$ units/mL.

The standard will be of value for comparison of results between laboratories, determination of assay sensitivities and for validation. It is anticipated that the standard will find application in clinical, reference, and research laboratories as well as blood transfusion services, regulatory authorities and manufacturers of in vitro diagnostic kits.

Recommendations

Based on the results of the international collaborative study, it is proposed that the heat-inactivated, lyophilized CHIKV strain (Sample 1 in this study), should be established as the 1st International Standard for CHIKV RNA with a unitage of 2,500,000 IU/mL. The standard has been given the code number 11785/16. 3200 vials are available to the WHO and the custodian laboratory is the Paul-Ehrlich-Institut.*

Responses from participants

After circulation of the draft report for comment, nine out of twenty-five participants responded to the report. There were no disagreements with the suitability of the candidate IS (PEI code 11785/16) to serve as the 1st WHO International Standard for CHIKV RNA for NAT-based assays. The majority of the comments were editorial in nature and the report has been amended accordingly where appropriate.

Acknowledgements

We are grateful to Maria Rios (Centre for Biologics Evaluation and Research, U.S. Food and Drug Administration) for kindly providing the candidate virus strain. We thank the collaborators who

* In previous discussion CBER/FDA has indicated that they could store part of the material so that they are kept at two sites.

kindly provided the clinical samples (Samples 3, 4 and 5). We gratefully acknowledge the important contributions of the collaborative study participants. We thank Ines Amberg, Sigrid Hanitsch and Christine Hanker-Dusel for technical assistance.

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Table 1. Assay protocols used by participants.

Laboratory code	Assay type (qualitative / quantitative)	Extraction method	NAT method	Assay target	Quantification standard	Diluent	Reference
1	qualitative	Exiprep™16 Dx Viral RNA kit on Exiprep™16 Dx (Bioneer Corp.)	Real-time RT-PCR (AccuPower ZIKV, DENV, CHIKV Multiplex Real-Time RT-PCR Kit, Bioneer Corp.)	Not stated	n/a	Plasma	
2	quantitative	ZR Viral RNA Kit™ (Zymo Research)	Real-time RT-PCR	Structural polyprotein gene (E1 envelope)	Synthetic E1 region RNA	Not stated	Lim CK et al., 2009
3A	quantitative	MagNA Pure LC Total Nucleic Acid Isolation Kit on MagNA Pure LC instrument (Roche)	Real-time RT-PCR (TaqMan; LightMix Modular Chikungunya virus duplex PCR, TIB Molbiol)	Structural polyprotein gene (envelope)	in vitro transcribed RNA	Human plasma	
3B	quantitative	MagNA Pure LC Total Nucleic Acid Isolation Kit on MagNA Pure LC instrument (Roche)	Real-time RT-PCR (TaqMan; LightMix Modular Chikungunya virus in multiplex PCR (CHIKV, DENV, ZIKAV, ECT), TIB Molbiol)	Structural polyprotein gene (envelope)	in vitro transcribed RNA	Human plasma	
4	qualitative	Sentosa SX Virus Total Nucleic Acid Kit v2.0 on Sentosa SX101 (Vela Research Singapore Pte Ltd)	Real-time RT-PCR	Structural polyprotein gene (E1 envelope)	n/a	EDTA plasma	
5	qualitative	Magnetic based sample processing on Panther System (Grifols Inc.)	Real-time transcription mediated amplification	Structural polyprotein gene	n/a	Not stated	
6A	qualitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan; FTD-36 Tropical fever core assay)	Structural polyprotein gene (E1 envelope)	n/a	Plasma	
6B	qualitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan; FTD-43 Dengue/Chik assay)	Structural polyprotein gene (E1 envelope)	n/a	Plasma	
7	qualitative	cobas® 6800/8800 (Roche)	Real-time RT-PCR (cobas® CHIKV/DENV (in development), Roche)	Not stated	n/a	Human plasma	
8	quantitative	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics)	Not stated	in vitro transcribed RNA	EDTA plasma	
9	quantitative	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR	Non-structural poly-protein gene (nsP1)	RNA from viral stocks	DMEM	
10	quantitative	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics)	Not stated	in vitro transcribed RNA	Human serum	
11	qualitative	MagCore® Viral Nucleic Acid Extraction Kit on MagCore® Automatic Nucleic Acid Extractor (RBC Bioscience)	Real-time RT-PCR	Nonstructural poly-protein gene (nsP1)	n/a	Nuclease-free water	
12	qualitative	QIAamp MinElute Virus Spin Kit (Qiagen)	Real-time RT-PCR (TaqMan)	Structural polyprotein gene (E1 envelope)	n/a	Supplied with the extraction kit	Pongsiri P et al., 2012
13	qualitative	Exiprep™16 Dx Viral RNA kit on Exiprep™16 Dx (Bioneer Corp.)	Real-time RT-PCR (AccuPower ZIKV, DENV, CHIKV Multiplex Real-Time RT-PCR Kit, Bioneer Corp.)	Non-structural poly-protein gene	n/a	Human plasma	

14A	qualitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan)	Non-structural poly-protein gene (nsP1)	n/a	Basematrix (Seracare)	Lanciotti RS et al., 2007 (modified)
14B	quantitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan)	Non-structural poly-protein gene (nsP1)	in vitro transcribed RNA	Basematrix (Seracare)	Lanciotti RS et al., 2007 (modified)
15	qualitative	Silica columns on BioRobot® MDx instrument (Qiagen)	Real-time RT-PCR (TaqMan; Kit Molecular ZDC Bio-Manghuinhos)	Non-structural poly-protein gene (nsP1)	n/a	Elution buffer	
16A	qualitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan)	Non-structural poly-protein gene (nsP2)	n/a	Not stated	Lanciotti RS et al., 2007
16B	qualitative	NucliSENS® easy MAG (bioMérieux)	Real-time RT-PCR (TaqMan)	Structural polyprotein gene (envelope)	n/a	Not stated	Pongsiri P et al., 2012
17	qualitative	MagNA Pure LC Total Nucleic Acid Isolation Kit on MagNA Pure LC instrument (Roche)	Real-time RT-PCR (TaqMan)	Non-structural poly-protein gene (nsP1)	n/a	Not stated	Panning M et al., 2008
18	qualitative	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR (Chikungunya Virus Real Time RT-PCR Kit, Liferiver)	Not stated	n/a	Human plasma	
19A	qualitative	QIAamp Viral RNA Mini Kit on QIACube instrument (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics)	Not stated	in vitro transcribed RNA	Foetal bovine serum	
19B	quantitative	QIAamp Viral RNA Mini Kit on QIACube instrument (Qiagen)	Real-time RT-PCR	Non-structural poly-protein gene (nsP1)	n/a	Foetal bovine serum	Panning M et al., 2008
20	qualitative	QIAamp Viral RNA Mini Kit on QIACube instrument (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit, altona Diagnostics)	Not stated	n/a	Fresh-frozen plasma	
21	quantitative	DNA and Viral NA Small Volume Kit on MagNA Pure 96 instrument (Roche)	Real-time RT-PCR	Non-structural poly-protein gene (nsP1)	in vitro transcribed RNA	Molecular Biology Water	Panning M et al., 2008
22	qualitative	QIAamp Viral RNA Mini Kit (Qiagen)	Real-time RT-PCR	Non-structural poly-protein gene	n/a	Nuclease-free water	Powers AM et al., 2010
23A	qualitative	QIAamp Viral RNA Mini Kit on QIACube instrument (Qiagen)	Real-time RT-PCR (TaqMan)	Structural polyprotein gene (E1 envelope)	n/a	Basematrix	Edwards CJ et al., 2007
23B	quantitative	QIAamp Viral RNA Mini Kit on QIACube instrument (Qiagen)	Real-time RT-PCR (TaqMan)	Structural polyprotein gene (E1 envelope)	CHIKV positive tissue culture supernatant	Basematrix	Edwards CJ et al., 2007
24A	qualitative	QIAamp DSP Virus Kit (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics)	Not stated	n/a	Human plasma	
24B	quantitative	QIAamp DSP Virus Kit (Qiagen)	Real-time RT-PCR (RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics)	Not stated	in vitro transcribed RNA	Human plasma	

n/a = not applicable

Table 2. Laboratories mean estimates from quantitative assays (log_{10} NAT-detectable units/mL).

Laboratory code	Sample 1		Sample 2		Sample 3	
	Mean	95% CI[1)]	Mean	95% CI[1)]	Mean	95% CI[1)]
2	8.59	8.34 – 8.85	8.86	8.74 – 8.98	8.43	8.11 – 8.76
3A	7.36	7.04 – 7.68	7.67	7.35 – 7.99	7.09	6.77 – 7.41
3B	7.56	7.25 – 7.87	7.86	7.74 – 7.98	7.28	6.96 – 7.60
8	7.33	7.20 – 7.46	7.62	7.50 – 7.74	7.07	6.99 – 7.14
9	[2)]	–	[2)]	–	[2)]	–
10	7.42	7.07 – 7.77	7.85	7.50 – 8.19	7.05	6.66 – 7.44
14B	6.88	6.69 – 7.07	7.13	6.91 – 7.35	6.71	6.55 – 6.86
19B	7.67	7.14 – 8.21	7.94	7.44 – 8.44	7.37	6.81 – 7.94
21	6.89	6.71 – 7.07	7.05	6.90 – 7.19	6.73	6.61 – 6.85
23B	5.23	4.72 – 5.74	5.51	5.20 – 5.82	5.01	4.76 – 5.26
24B	7.45	7.40 – 7.51	7.64	7.46 – 7.81	7.27	7.15 – 7.40

Laboratory code	Sample 4		Sample 5	
	Mean	95% CI[1)]	Mean	95% CI[1)]
2	6.94	6.80 – 7.08	6.91	6.68 – 7.13
3A	6.11	5.75 – 6.46	6.00	5.59 – 6.41
3B	6.45	6.19 – 6.71	6.43	5.97 – 6.88
8	5.70	5.59 – 5.81	5.69	5.54 – 5.84
9	[2)]	–	[2)]	–
10	5.77	5.32 – 6.22	5.94	5.58 – 6.31
14B	4.95	4.89 – 5.02	5.00	4.93 – 5.07
19B	6.06	5.50 – 6.62	6.10	5.55 – 6.66
21	6.04	5.78 – 6.30	6.02	5.85 – 6.18
23B	3.62	3.11 – 4.14	3.80	3.26 – 4.34
24B	6.28	6.08 – 6.49	6.28	6.07 – 6.49

[1)] 95% confidence interval
[2)] Data were omitted from the evaluation due to non-comparable units (pfu/mL) reported

Table 3. Laboratories mean estimates from qualitative assays (log_{10} NAT-detectable units/mL).

Laboratory code	Sample 1		Sample 2		Sample 3	
	Mean	95% CI[1)]	Mean	95% CI[1)]	Mean	95% CI[1)]
1	5.12	4.88 – 5.31	5.27	5.04 – 5.46	4.90	4.65 – 5.09
4	4.69	4.54 – 4.83	5.11	4.96 – 5.25	4.99	4.85 – 5.13
5	6.24	6.09 – 6.38	6.40	6.30 – 6.51	6.40	6.30 – 6.51
6A	7.56	6.95 – 8.55	8.36	7.49 – 9.78	7.77	7.10 – 8.87
6B	6.11	5.89 – 6.31	6.29	6.07 – 6.49	6.32	6.10 – 6.52
7	6.74	6.52 – 6.93	6.93	6.71 – 7.13	6.71	6.49 – 6.90
11	4.41	4.17 – 4.64	4.66	4.42 – 4.90	4.21	3.92 – 4.51
12	4.44	4.06 – 4.80	4.87	4.47 – 5.28	4.82	4.45 – 5.17
13	5.59	5.42 – 5.76	5.72	5.54 – 5.88	5.24	5.07 – 5.40
14A	6.09	5.87 – 6.31	6.44	6.22 – 6.67	6.07	5.86 – 6.29
15	6.10	5.69 – 6.55	6.41	5.96 – 6.92	6.34	5.90 – 6.83
16A	5.87	5.73 – 6.01	6.08	6.00 – 6.15	5.87	5.73 – 6.01
16B	5.99	5.83 – 6.15	6.19	6.01 – 6.35	5.99	5.83 – 6.15
17	6.58	6.33 – 6.82	7.28	7.04 – 7.51	6.60	6.36 – 6.83
18	5.28	5.02 – 5.55	5.62	5.62 – 5.62	[2)]	–
19A	6.95	6.82 – 7.08	6.87	6.73 – 7.01	[3)]	–
20	7.12	6.50 – 7.62	7.61	6.99 – 8.11	7.56	6.97 – 8.06
22	5.47	5.15 – 5.75	6.58	6.27 – 6.86	5.87	5.57 – 6.15
23A	6.09	5.81 – 6.35	6.58	6.30 – 6.84	6.31	6.03 – 6.57
24A	7.02	6.66 – 7.34	7.49	7.13 – 7.85	6.86	6.50 – 7.18

Laboratory code	Sample 4		Sample 5	
	Mean	95% CI[1)]	Mean	95% CI[1)]
1	3.47	3.25 – 3.74	3.40	3.17 – 3.62
4	3.92	3.27 – 4.56	3.92	3.27 – 4.56
5	[4)]	–	[4)]	–
6A	[4)]	–	[4)]	–
6B	[4)]	–	[4)]	–
7	[4)]	–	[4)]	–
11	3.05	2.74 – 3.35	3.37	3.06 – 3.67
12	3.91	3.40 – 4.40	3.47	2.97 – 3.93
13	3.37	3.18 – 3.55	3.27	3.06 – 3.44
14A	4.07	3.79 – 4.33	4.07	3.79 – 4.33
15	4.34	3.89 – 4.89	4.94	4.26 – 5.84
16A	4.97	4.70 – 5.24	4.80	4.52 – 5.09
16B	4.91	4.73 – 5.08	4.96	4.78 – 5.14
17	[4)]	–	[4)]	–
18	[2)]	–	[2)]	–
19A	[4)]	–	[4)]	–
20	5.46	4.56 – 6.26	5.46	4.56 – 6.26
22	4.52	4.12 – 4.93	4.52	4.12 – 4.93
23A	4.14	3.79 – 4.48	4.15	3.79 – 4.49
24A	[4)]	–	[4)]	–

[1)] 95% confidence interval
[2)] Laboratory 18 did not receive Sample 3, Sample 4 and Sample 5 for testing
[3)] Invalid (curve not parallel)
[4)] All dilutions positive, no cut-off detectable

Table 4. Overall mean estimates and inter-laboratory variation from quantitative and qualitative assays (log_{10} NAT-detectable units/mL).

Sample	Assay type	N[1)]	Mean[2)]	95% CI[2)]	SD[2)]	%GCV[2)]	Min	Max
1	quantitative	10	7.24	6.36 – 8.12	0.91	13	5.23	8.59
	qualitative	20	5.97	5.56 – 6.39	0.89	15	4.41	7.56
2	quantitative	10	7.51	6.64 – 8.38	0.90	12	5.51	8.86
	qualitative	20	6.34	5.89 – 6.78	0.95	15	4.66	8.36
3	quantitative	10	7.00	6.14 – 7.86	0.91	13	5.01	8.43
	qualitative	18	6.05	5.58 – 6.51	0.94	16	4.21	7.77
4	quantitative	10	5.79	4.87 – 6.72	0.99	17	3.62	6.94
	qualitative	12[3)]	4.18	3.73 – 4.63	0.71	17	3.05	5.46
5	quantitative	10	5.82	4.94 – 6.70	0.95	16	3.80	6.91
	qualitative	12[3)]	4.19	3.72 – 4.66	0.74	18	3.27	5.46

[1)] Number of data sets
[2)] Estimates, standard deviation (SD), geometric coefficient of variation (%GCV), and 95% confidence interval (95% CI) based on mixed linear model including random factors participant and assay for quantitative data; for qualitative data based mean of participant's cut-offs
[3)] Evaluable data from 10-fold dilution series

Table 5. Overall mean estimates and inter-laboratory variation – combined data from quantitative and qualitative assays (log_{10} NAT-detectable units/mL).

Sample	N[1)]	Mean[2)]	95% CI[3)]	SD[4)]	%GCV[5)]	Min	Median	Max
1	30	6.39	6.00 – 6.79	1.05	16	4.41	6.41	8.59
2	30	6.73	6.33 – 7.13	1.07	16	4.66	6.73	8.86
3	28	6.39	6.00 – 6.78	1.01	16	4.21	6.50	8.43
4	22	4.91	4.41 – 5.42	1.14	23	3.05	4.93	6.94
	10[6)]	5.79	4.87 – 6.72	0.92	16	3.62	6.05	6.94
5	22	4.93	4.43 – 5.43	1.14	23	3.27	4.95	6.91
	10[6)]	5.82	4.94 – 6.70	0.86	15	3.80	6.01	6.91

[1)] Number of data sets from qualitative and quantitative data
[2)] Overall mean estimate (log_{10} NAT-detectable units/mL) based on mean estimates of each data set
[3)] 95% confidence interval
[4)] Standard deviation
[5)] Geometric coefficient of variation
[6)] Excluding data sets from qualitative assays, as not tested according to the end-point dilution protocol

Table 6. Laboratory estimates of potency relative to Sample 1 $\log_{10}$ IU/mL from quantitative and qualitative assays (taking the candidate WHO IS as 6.39 $\log_{10}$ IU/mL).

Laboratory code	Assay type	Sample 2	Sample 3	Sample 4	Sample 5
1	qualitative	6.54	6.17	4.74	4.66
2	quantitative	6.66	6.23	4.74	4.71
3A	quantitative	6.71	6.13	5.14	5.03
3B	quantitative	6.70	6.11	5.28	5.26
4	qualitative	6.82	6.70	5.63	5.63
5	qualitative	6.56	6.56	[1]	[1]
6A	qualitative	7.20	6.61	[1]	[1]
6B	qualitative	6.57	6.60	[1]	[1]
7	qualitative	6.58	6.36	[1]	[1]
8	quantitative	6.68	6.13	4.77	4.76
10	quantitative	6.82	6.02	4.75	4.92
11	qualitative	6.65	6.19	5.04	5.36
12	qualitative	6.82	6.78	5.86	5.42
13	qualitative	6.52	6.04	4.17	4.07
14A	qualitative	6.75	6.38	4.37	4.37
14B	quantitative	6.65	6.22	4.47	4.51
15	qualitative	6.70	6.63	4.64	5.23
16A	qualitative	6.60	6.39	[1]	[1]
16B	qualitative	6.59	6.39	5.31	5.36
17	qualitative	7.10	6.42	[1]	[1]
18	qualitative	6.73	[2]	[2]	[2]
19A	qualitative	6.31	[1]	[1]	[1]
19B	quantitative	6.66	6.10	4.78	4.83
20	qualitative	6.89	6.83	4.73	4.73
21	quantitative	6.55	6.23	5.54	5.52
22	qualitative	7.51	6.79	5.45	5.45
23A	qualitative	6.89	6.61	4.45	4.46
23B	quantitative	6.68	6.18	4.79	4.96
24A	qualitative	6.87	6.24	[1]	[1]
24B	quantitative	6.58	6.21	5.22	5.22

[1] Data not estimable
[2] Laboratory 18 did not receive Sample 3, Sample 4 and Sample 5 for testing.

Table 7. Overall mean estimates and inter-laboratory variation for potencies relative to Sample 1 log_{10} IU/mL (taking the candidate WHO IS as 6.39 log_{10} IU/mL) – quantitative and qualitative assays as well as combined data.

Sample	Assay type	N[1)]	Mean[2)]	95% CI[3)]	SD[4)]	%GCV[5)]	Min	Max
2	quantitative	10	6.67	6.62 – 6.72	0.07	1	6.55	6.82
	qualitative	20	6.76	6.63 – 6.89	0.27	4	6.31	7.51
	combined	30	6.73	6.64 – 6.81	0.23	3	6.31	7.51
3	quantitative	10	6.16	6.11 – 6.21	0.07	1	6.02	6.23
	qualitative	18	6.48	6.37 – 6.60	0.23	4	6.04	6.83
	combined	28	6.37	6.27 – 6.46	0.25	4	6.02	6.83
4	quantitative	10	4.95	4.71 – 5.19	0.33	7	4.47	5.54
	qualitative	11[6)]	4.95	4.57 – 5.32	0.55	11	4.17	5.86
	combined	21	4.95	4.74 – 5.15	0.45	9	4.17	5.86
5	quantitative	10	4.97	4.76 – 5.19	0.30	6	4.51	5.52
	qualitative	11[6)]	4.98	4.62 – 5.33	0.53	11	4.07	5.63
	combined	21	4.97	4.78 – 5.17	0.43	9	4.07	5.63

[1)] Number of evaluable data sets
[2)] Mean estimate
[3)] 95% confidence interval
[4)] Standard deviation
[5)] Geometric coefficient of variation
[6)] Evaluable data from 10-fold dilution series

Table 8. Intra-laboratory standard deviation of log_{10} NAT-detectable units/mL and %GCV for quantitative assays.

Laboratory code	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	SD[1)]	%GCV[2)]	SD[1)]	%GCV[2)]	SD[1)]	%GCV[2)]	SD[1)]	%GCV[2)]	SD[1)]	%GCV[2)]
2	0.17	2.0	0.19	2.1	0.19	2.3	0.15	2.2	0.18	2.5
3A	0.21	2.9	0.20	2.6	0.22	3.1	0.32	5.3	0.42	6.9
3B	0.24	3.2	0.14	1.8	0.24	3.3	0.31	4.8	0.37	5.8
8	0.09	1.2	0.09	1.1	0.09	1.3	0.17	2.9	0.26	4.6
10	0.54	7.3	0.53	6.8	0.42	5.9	0.53	9.1	0.56	9.4
14B	0.12	1.7	0.13	1.9	0.10	1.5	0.07	1.4	0.05	0.9
19B	0.36	4.6	0.35	4.4	0.43	5.8	0.42	7.0	0.46	7.5
21	0.20	2.9	0.16	2.3	0.13	2.0	0.23	3.8	0.16	2.7
23B	0.42	8.1	0.31	5.6	0.48	9.6	0.55	15.0	0.57	14.9
24B	0.09	1.2	0.12	1.6	0.13	1.8	0.32	5.1	0.32	5.1

[1)] Standard deviation
[2)] Geometric coefficient of variation

Table 9. Laboratory estimates of potency relative to Sample 1 $\log_{10}$ IU/mL from quantitative and qualitative assays based on C_T values (taking the candidate WHO IS as 6.39 $\log_{10}$ IU/mL).

Laboratory code	Assay type	Sample 2	Sample 3	Sample 4	Sample 5
1	qualitative	6.59	6.23	4.76	4.61
2	quantitative	6.67	6.22	4.68	4.62
3A	quantitative	6.74	6.14	5.21	5.22
3B	quantitative	6.73	6.07	5.22	5.17
4	qualitative	6.76	6.48	5.56	5.45
5	qualitative	[1]	[1]	[1]	[1]
6A	qualitative	6.67	6.51	5.60	5.55
6B	qualitative	6.56	6.49	5.57	5.55
7	qualitative	6.58	6.16	5.24	5.19
8	quantitative	6.69	6.15	4.76	4.75
9	quantitative	6.87	6.42	5.74	5.68
10	quantitative	6.59	5.31	3.62	3.94
11	qualitative	6.63	6.11	5.98	5.97
12	qualitative	6.71	6.24	5.24	4.81
13	qualitative	6.66	6.09	4.11	4.09
14A	qualitative	6.62	6.12	5.53	4.41
14B	quantitative	6.69	6.18	4.59	[2]
15	qualitative	6.65	6.39	4.92	5.05
16A	qualitative	6.62	6.23	5.37	5.40
16B	qualitative	6.64	6.33	5.19	5.25
17	qualitative	6.77	6.08	5.26	5.21
18	qualitative	6.41	[3]	[3]	[3]
19A	qualitative	6.53	7.47	5.26	4.56
19B	quantitative	6.72	5.92	[2]	4.03
20	qualitative	7.17	6.14	[2]	[2]
21	quantitative	6.54	6.19	5.46	5.36
22	qualitative	7.22	6.64	5.40	5.48
23A	qualitative	6.58	6.17	4.63	4.77
23B	quantitative	6.75	5.96	4.83	4.89
24A	qualitative	6.44	6.44	5.16	5.09
24B	quantitative	6.59	6.19	[2]	[2]

[1] Assay is not based on real-time PCR (no C_T values available)
[2] Data not estimable
[3] Laboratory 18 did not receive Sample 3, Sample 4 and Sample 5 for testing.

Table 10. Overall mean estimates for potencies relative to Sample 1 $\log_{10}$ IU/mL based on C_T values (taking the candidate WHO IS as 6.39 $\log_{10}$ IU/mL) – quantitative and qualitative assays as well as combined data.

Sample	Assay type	N[1)]	Mean[2)]	95% CI[3)]
2	quantitative	11	6.69	6.63 – 6.75
	qualitative	19	6.67	6.58 – 6.77
	combined	30	6.68	6.28 – 7.07
3	quantitative	11	6.07	5.88 – 6.26
	qualitative	18	6.35	6.19 – 6.51
	combined	29	6.21	4.41 – 8.01
4	quantitative	9	4.90	4.43 – 5.37
	qualitative	17	5.16	4.93 – 5.40
	combined	26	5.06	3.43 – 6.69
5	quantitative	9	4.85	4.40 – 5.30
	qualitative	17	5.08	4.83 – 5.33
	combined	26	5.00	3.57 – 6.43

[1)] Number of evaluable data sets
[2)] Mean estimate
[3)] 95% confidence interval

Table 11. Variability of relative potencies based on C_T values.

Source of Variation[1)]	Sample 2	Sample 3	Sample 4	Sample 5
Laboratory[2)]	14%	46%	143%	166%
Assay Type[3)]	11%	34%	47%	35%
Residual[4)]	41%	83%	57%	36%
Total (UM)[5)]	46%	113%	198%	194%

[1)] Geometric coefficient of variation (GCV) for variability of relative potencies
[2)] Inter-laboratory variability
[3)] Inter-assay variability (qualitative/quantitative)
[4)] Intra-assay variability (between up to 4 assays per lab)
[5)] Total variability (UM = uncertainty of measurement)

Table 12. Stability and accelerated degradation studies after 1, 2 and 3 weeks and after 1, 2, 3 and 6 months – difference to baseline samples stored at -20°C and -80°C is shown.

Temperature	**Mean log_{10} copies/mL***						
	1 week	2 weeks	3 weeks	1 month	2 months	3 months	6 months
-80°C	7.39	7.30	7.32	7.35	7.27	7.42	7.33
-20°C	7.34	7.30	7.34	7.34	7.30	7.34	7.31
+4°C	7.32	7.36	7.31	7.30	7.32	7.39	7.33
+20°C	7.30	7.31	7.25	7.35	7.30	7.32	7.15
+37°C	7.23	7.20	7.06	7.17	6.80	6.66	5.96
Temperature	**Difference in log_{10} copies/mL from -20°C baseline sample**						
+4°C	0.02	-0.06	0.03	0.04	-0.02	-0.05	-0.02
+20°C	0.04	-0.01	0.09	-0.01	0.00	0.02	0.16
+37°C	0.11	0.10	0.28	0.17	0.50	0.68	1.35
Temperature	**Difference in log_{10} copies/mL from -80°C baseline sample**						
+4°C	0.07	-0.06	0.01	0.05	-0.05	0.03	0.00
+20°C	0.09	-0.01	0.07	0.00	-0.03	0.10	0.18
+37°C	0.16	0.10	0.26	0.18	0.47	0.76	1.37

* RealStar® Chikungunya RT-PCR Kit 2.0, altona Diagnostics

Figure 1. Laboratories mean estimates for the candidate WHO standard 11785/16 (Sample 1). Estimates of $\log_{10}$ NAT-detectable units/mL are indicated on the x-axis. White squares represent quantitative assays and grey squares represent qualitative assays.

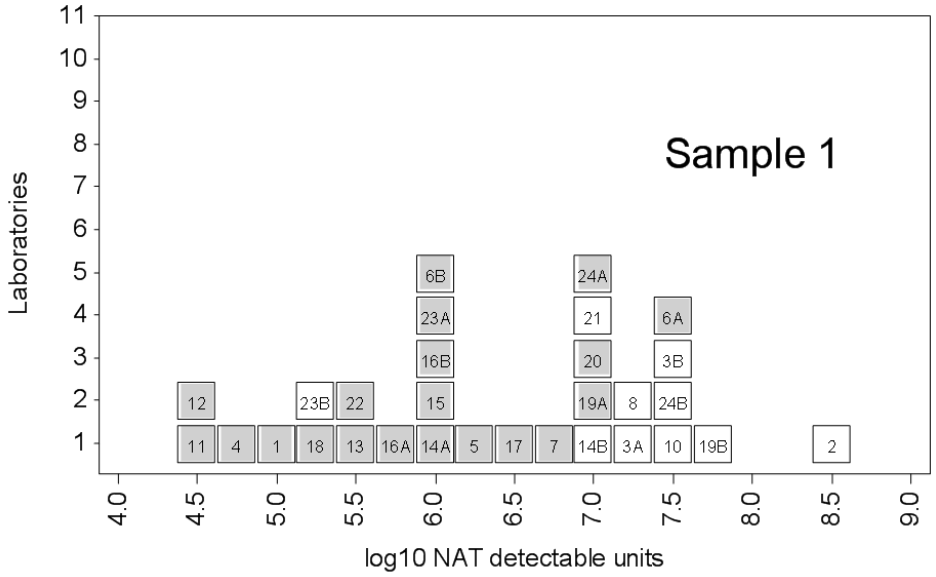


Figure 2. Laboratories mean estimates for the liquid/frozen bulk material (Sample 2). Estimates of $\log_{10}$ NAT-detectable units/mL are indicated on the x-axis. White squares represent quantitative assays and grey squares represent qualitative assays.

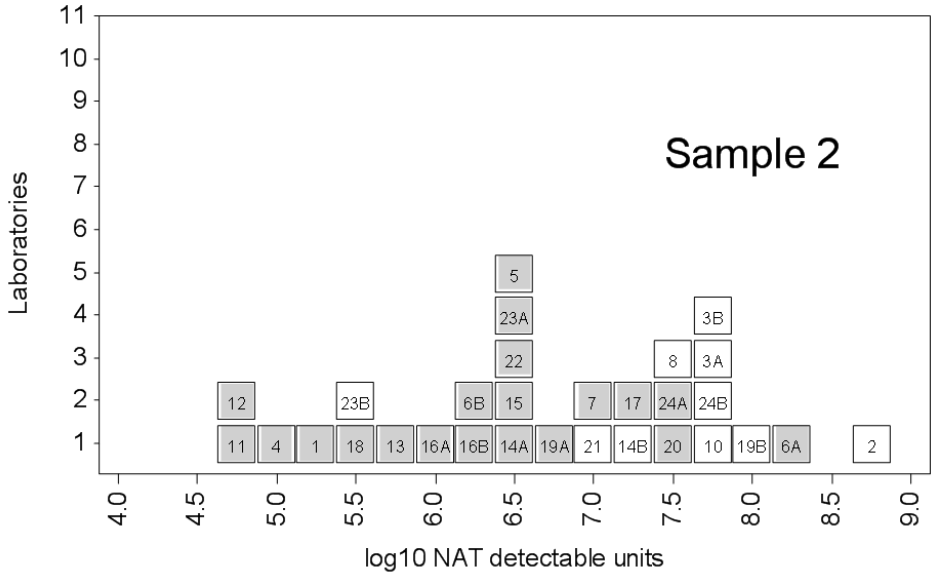


Figure 3. Laboratories mean estimates for Sample 3 (clinical sample). Estimates of $\log_{10}$ NAT-detectable units/mL are indicated on the x-axis. White squares represent quantitative assays and grey squares represent qualitative assays.

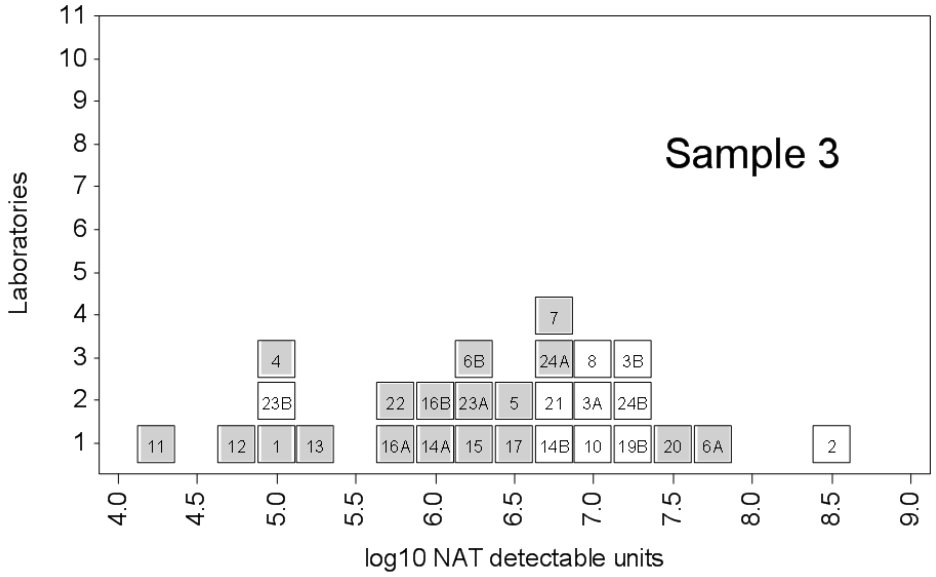


Figure 4. Laboratories mean estimates for Sample 4 (clinical sample). Estimates of $\log_{10}$ NAT-detectable units/mL are indicated on the x-axis. White squares represent quantitative assays and grey squares represent qualitative assays.

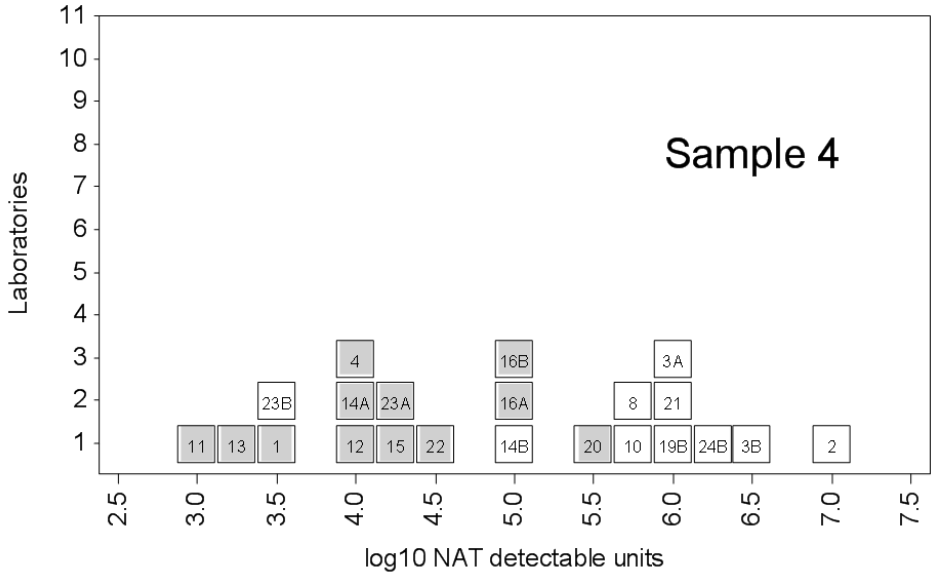


Figure 5. Laboratories mean estimates for Sample 5 (clinical sample). Estimates of $\log_{10}$ NAT-detectable units/mL are indicated on the x-axis. White squares represent quantitative assays and grey squares represent qualitative assays.

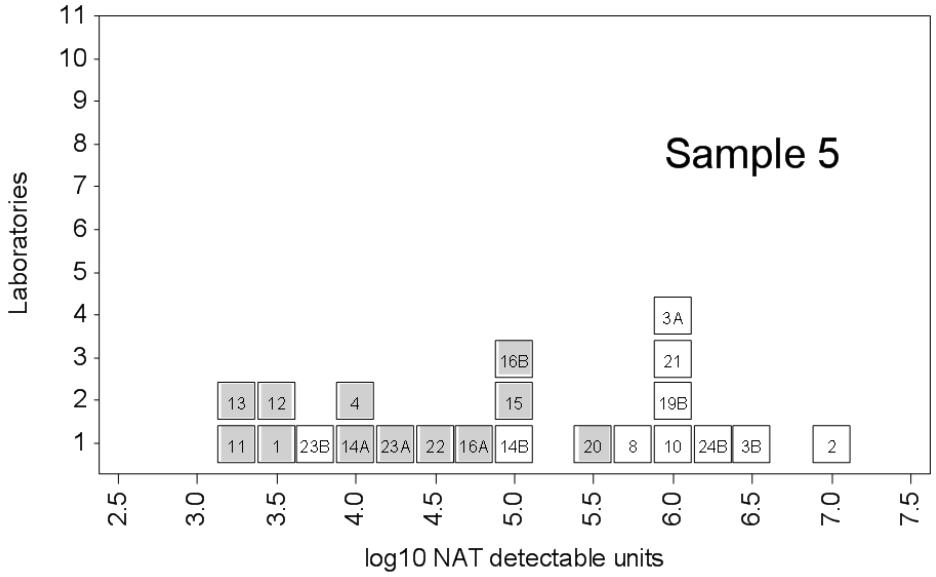


Figure 6. Potency of Sample 2 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

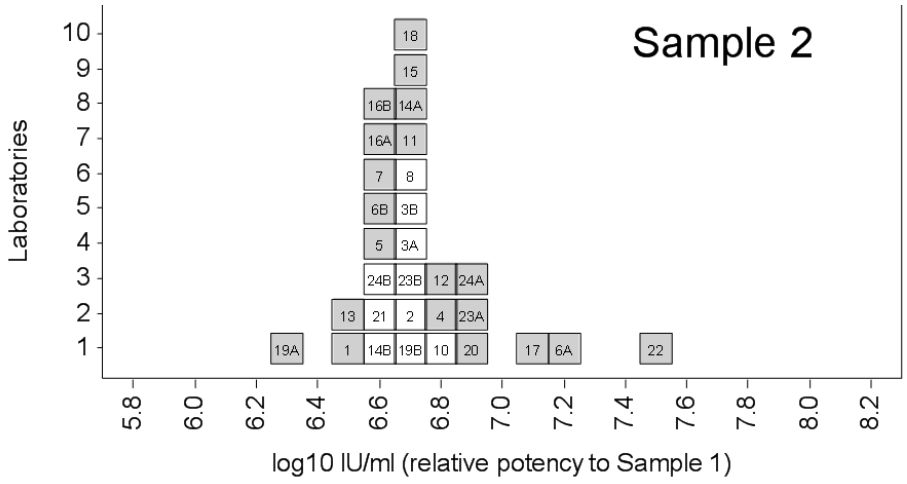


Figure 7. Potency of Sample 3 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

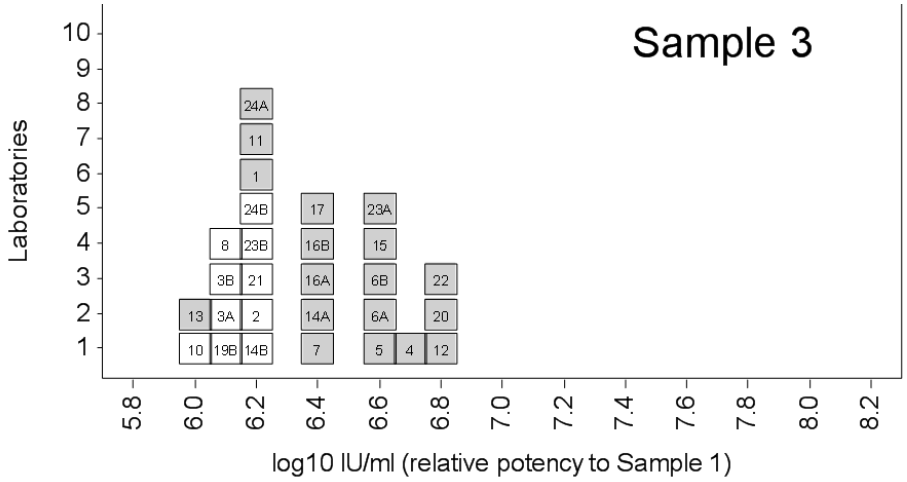


Figure 8. Potency of Sample 4 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

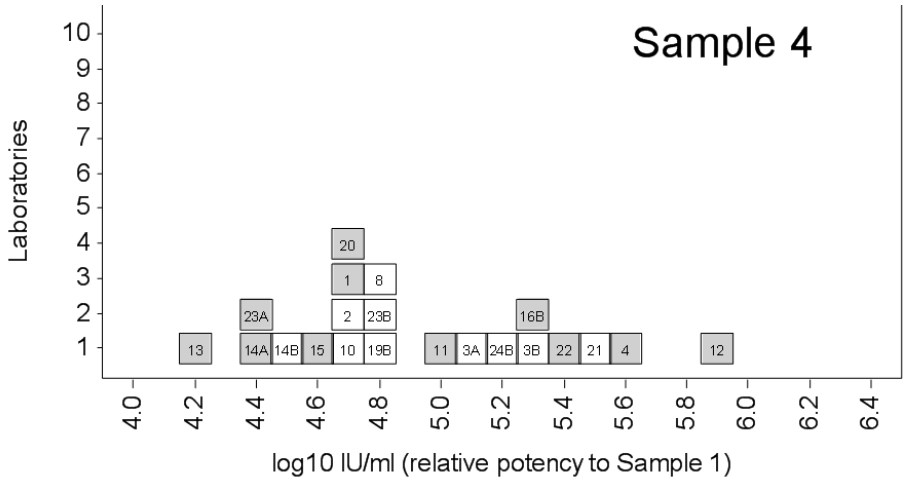


Figure 9. Potency of Sample 5 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

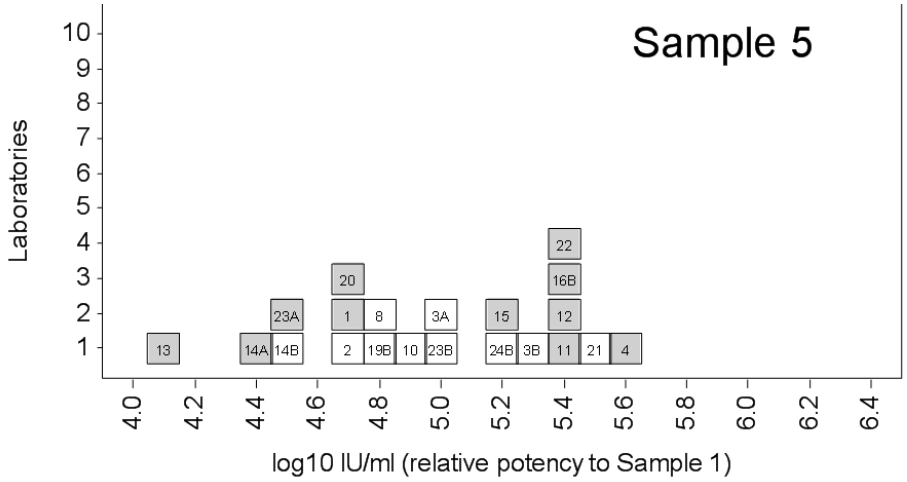


Figure 10. C_T value-based potency of Sample 2 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

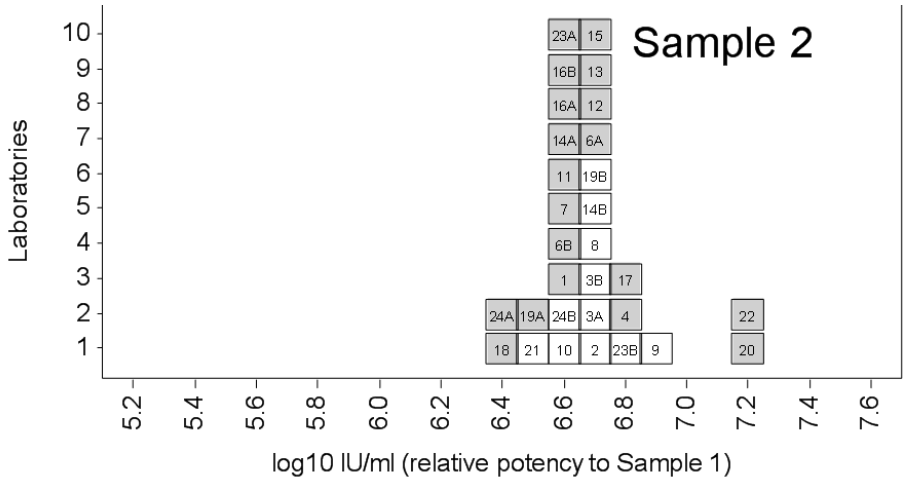


Figure 11. C_T value-based potency of Sample 3 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

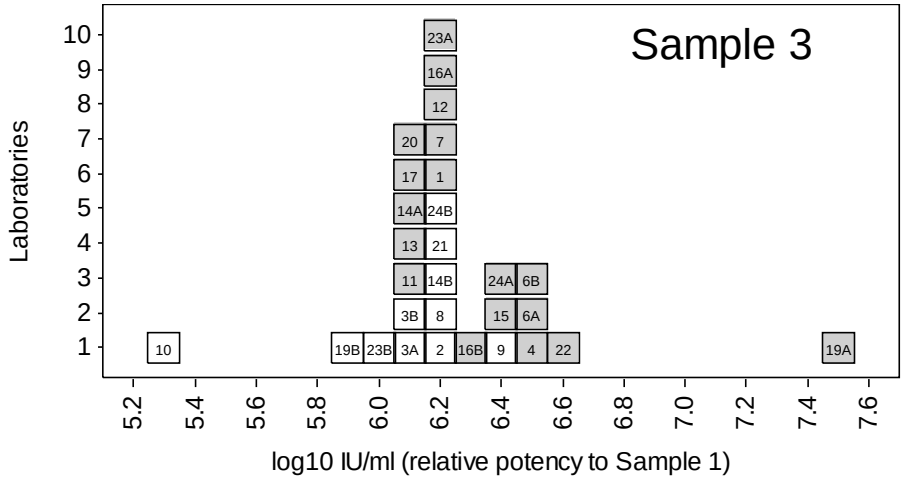


Figure 12. C_T value-based potency of Sample 4 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

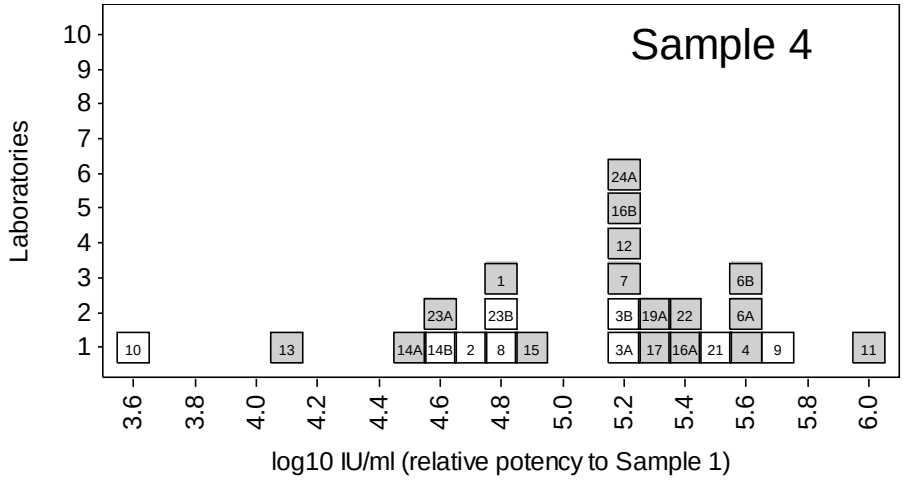


Figure 13. C_T value-based potency of Sample 5 relative to the candidate WHO standard 11785/16 (Sample 1; 6.39 $\log_{10}$ IU/mL). Units are expressed as candidate $\log_{10}$ IU/mL. White squares represent quantitative assays and grey squares represent qualitative assays.

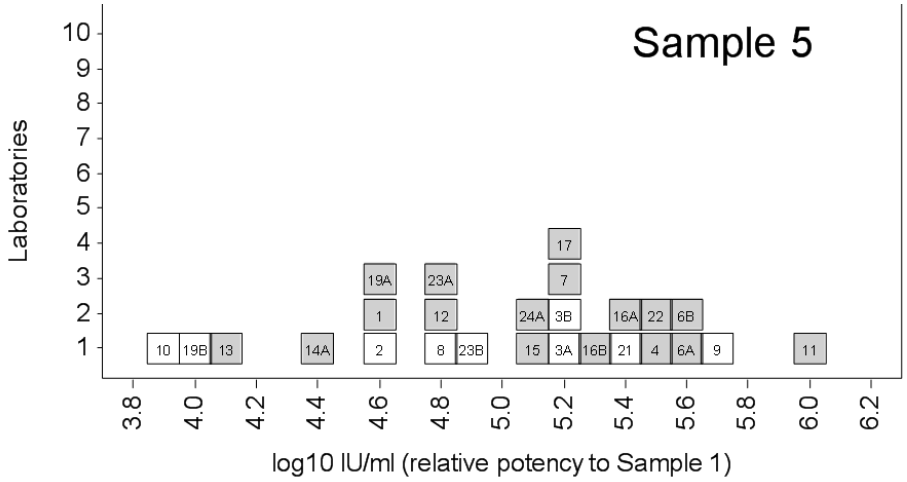


Figure 14. Results from stability testing of the candidate WHO standard 11785/16 (Sample 1).

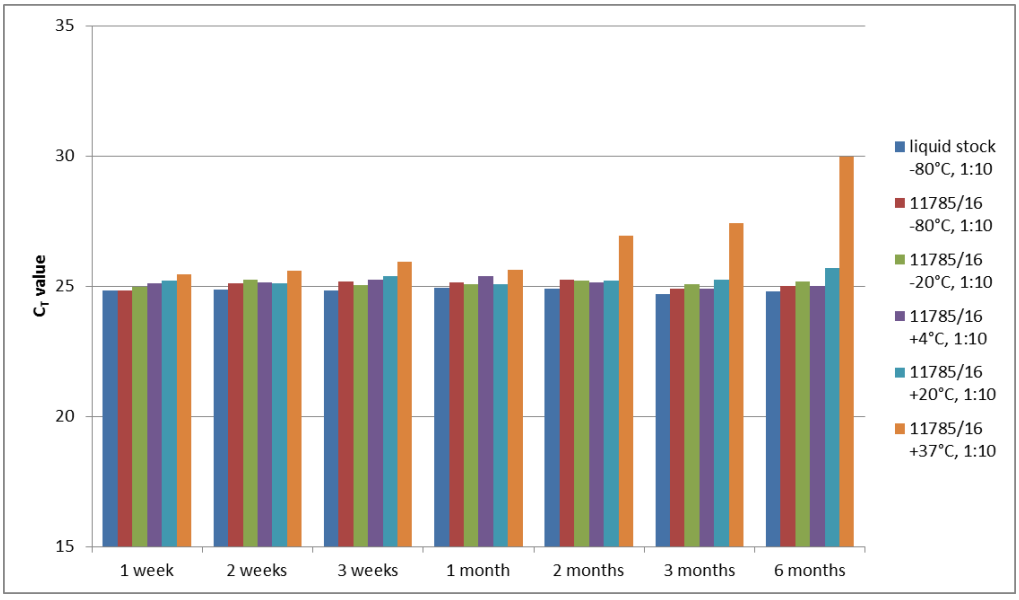
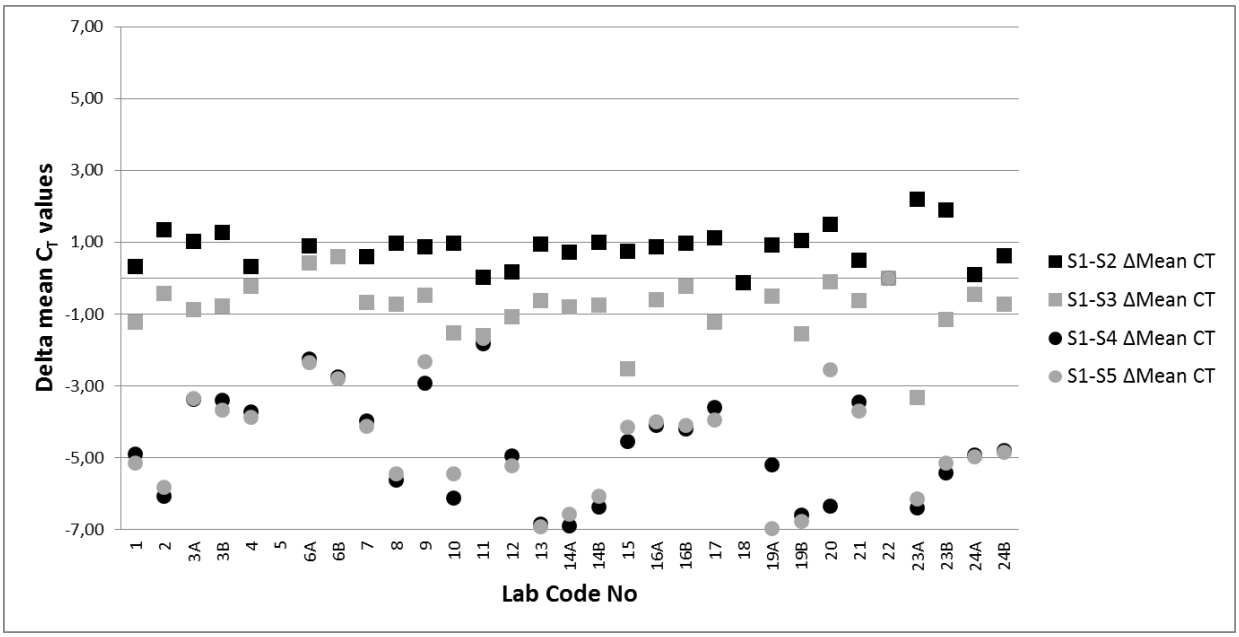


Figure 15. Relationship of mean C_T values between the candidate IS (Sample 1, S1) and Sample 2 (S2), Sample 3 (S3), Sample 4 (S4) and Sample 5 (S5) (based on data of 1:10 dilution).



Appendix 1. List of participants, alphabetically according to organization.

Participant(s)	Affiliation
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Suzan Pas, Chantal Reusken	Erasmus MC Rotterdam, The Netherlands
Madeleine Heckmann, Nicolas Emilien	Fast-track Diagnostics Luxembourg S.à.r.l. Esch-sur-Alzette, Luxembourg
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Jeffrey Linnen, Kui Gao	Grifols Diagnostic Solutions Inc. San Diego, United States of America
Giulio Pisani, Matteo Simeoni, Sara Fabi	Istituto Superiore di Sanità, National Centre for the Control and Evaluation of Medicines (CNCF) Rome, Italy
Moi Meng Ling	Nagasaki University, Institute of Tropical Medicine Nagasaki, Japan
Maria João Alves, Líbia Zé-Zé	National Institute of Health Dr. Ricardo Jorge, Center for Vectors and Infectious Diseases Research Águas de Moura, Portugal
Amy B. Dean	New York State Department of Health, Wadsworth Centre Albany, United States of America
Constanze Yue, Sally Baylis	Paul-Ehrlich-Institut Langen, Germany
Julia Kreß	Paul-Ehrlich-Institut Langen, Germany
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John Aaskov	Queensland University of Technology, Institute of Health and Biomedical Innovation Brisbane, Australia
Cristina Domingo, Andreas Nitsche	Robert Koch Institute Berlin, Germany
Patrick Albrecht, Sabine Locher, Marina Hegner	Roche Diagnostics International AG Rotkreuz, Switzerland
Walter Zhang, Lei Xiong, Yong Li	Shanghai ZJ Bio-Tech Co., Ltd. (Liferiver) Shanghai, China
Olfert Landt, Pranav Patel	TIB Molbiol Syntheselabor GmbH Berlin, Germany
Jan Felix Drexler, Carlo Fischer	University of Bonn Medical Centre, Institute of Virology, German Centre for Infection Research (DZIF) Bonn, Germany
Marcus Panning	University of Freiburg Medical Centre, Centre for Microbiology and Hygiene, Institute of Virology Freiburg, Germany
Scott C. Weaver, Dawn I. Auguste	University of Texas Medical Branch Galveston, United States of America
Tan Si Kee, Charlie Lee	Vela Research Singapore Pte Ltd Singapore, Singapore

Appendix 2. Instructions for Use for 11785/16.

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

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

1 st World Health Organization International Standard for Chikungunya virus RNA for Nucleic Acid Amplification Techniques (NAT)-Based Assays

PEI code 11785/16

(Version 2.0, Dated 23/10/2017)

1. INTENDED USE

The World Health Organization (WHO) International Standard for Chikungunya virus (CHIKV) RNA is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for CHIKV. The standard has been prepared using an East/Central/South African (ESCA) strain of CHIKV (R91064), isolated from a patient returning from India to the U.S. in 2006 (1). 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 25 laboratories performing a wide range of CHIKV NAT assays. Further details of the collaborative study are available in the report (2).

2. UNITAGE

This reagent has been assigned a unitage of 2,500,000 International Units/mL.

3. CONTENTS

Each vial contains 0.5 mL of lyophilized material containing inactivated CHIKV. The virus has been diluted in human negative plasma and lyophilized. The material is intended for dilution in a diluent appropriate for the assay matrix being tested (e.g. plasma).

4. CAUTION

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS.

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

5. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
The material is supplied lyophilized and should be stored at or below -20°C. Each vial should be reconstituted in 0.5 mL of sterile nuclease-free water. The product should be reconstituted just prior to use. The product should be used for the calibration of secondary reference preparations for CHIKV RNA. If not all the material is used immediately, laboratories may aliquot the remaining material into suitable single use volumes which should be stored at or below -70°C.

6. STABILITY

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

Reference materials should be stored on receipt as indicated on the label. Once, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

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

7. REFERENCES

The accession number for the CHIKV virus strain is KJ941050 (4).

1. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, Campbell GL. 2007. Chikungunya virus in U.S. travelers returning from India, 2006. Emerg. Infect. Dis. 13:764-767.

2. Kreß JA, Hanschmann KMO, Chudy M. Collaborative Study to Evaluate a Candidate World Health Organization International Standard for Chikungunya Virus for Nucleic Acid Amplification Technique (NAT)-Based Assays WHO/BS/2017.2330.

3. World Health Organization. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). WHO Technical Report Series 2006. 932, 73-131.

4. Añez G, Heisey DA, Rios M. 2014. Complete coding region sequence of a chikungunya virus strain used for formulation of CBER/FDA RNA reference reagents for nucleic acid testing. Genome Announc. 2(4):e00587-14.

8. ACKNOWLEDGEMENTS

We thank M. Rios (Center for Biologics Evaluation and Research, U.S. Food and Drug Administration) for kindly providing the CHIKV virus strain. We gratefully acknowledge the important contributions of the collaborative study participants.

9. FURTHER INFORMATION

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

10. CUSTOMER FEEDBACK

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

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

Email: whoccivd@pei.de
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Paul-Ehrlich-Institut
Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

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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
Hygroscopic: No
Flammable: No
Corrosive: No
Oxidising: No
Irritant: No
Other (specify): Contains inactivated CHIKV
Handling: See caution, section 4
Toxicological properties
Effects of inhalation: Not established - avoid
Effects of ingestion: Not established - avoid
Effects of skin absorption: Not established - avoid
Suggested First Aid
Inhalation: Seek medical advice
Ingestion: Seek medical advice
Contact with eyes: Wash thoroughly with water. Seek medical advice
Contact with skin: Wash thoroughly with water. Seek medical advice
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. CERTIFICATE OF ANALYSIS

PEI does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognized primary reference materials fully described in the instructions for use.
The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (3). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

14. LIABILITY AND LOSS

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

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

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

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

The total liability of the Institute in connection with this agreement, whether for negligence or breach of agreement or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.
If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

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Appendix 3. Study protocol, results and methods reporting forms.

Bundesinstitut für Impfstoffe und biomedizinische Arzneimittel
Federal Institute for Vaccines and Biomedicines

Paul-Ehrlich-Institut

Study Protocol

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Background

Currently, there is no standardization of nucleic acid amplification technique (NAT)-based assays for the detection of Chikungunya Virus (CHIKV) RNA. The World Health Organization (WHO) has approved a proposal for the establishment of an International Standard for CHIKV RNA for NAT-based assays.

The Paul-Ehrlich-Institut (PEI), Federal Institute for Vaccines and Biomedicines, as a WHO Collaborating Centre involved in the Biological Standardization Program, has developed a candidate International Standard for CHIKV RNA in collaboration with the Center for Biologics Evaluation and Research (CBER) of the U.S. Food and Drug Administration (FDA). The candidate material has now been prepared and lyophilized.

Objective

The objective of this study is to evaluate the proposed candidate WHO IS for CHIKV RNA (lyophilized) along with four liquid frozen CHIKV positive plasma samples. Dilutions of the study samples should be tested on four separate occasions using your routine CHIKV NAT assay (qualitative or quantitative). The potency of the candidate standard preparation will be determined using a range of NAT-based assays for CHIKV RNA with the aim of assigning an internationally agreed unitage.

Study Samples

Study samples are as follows:

Sample Name	Formulation	Volume	Storage Temperature
Sample 1	Lyophilized CHIKV, heat inactivated	0.5 mL	≤ -20°C
Sample 2	Liquid Frozen CHIKV, heat inactivated	1.0 mL	≤ -70°C
Sample 3	Liquid Frozen CHIKV	1.0 mL	≤ -70°C
Sample 4	Liquid Frozen CHIKV	1.0 mL	≤ -70°C
Sample 5	Liquid Frozen CHIKV	1.0 mL	≤ -70°C

Participants will receive differing numbers of vials, depending upon the extraction volume which has been communicated to PEI prior to the start of the study. Please let us know as soon as possible if you think that you may have insufficient samples for testing on four separate occasions.

CAUTION: Study samples 3, 4 and 5 contain infectious CHIKV and should be handled only in appropriate containment facilities by fully trained and competent staff in accordance with national safety guidelines. All study samples contain human plasma, which has previously been tested and found negative for CHIKV RNA, HAV RNA, HBV DNA, HCV RNA, HEV RNA, HIV-1/2 RNA, Parvo B19V DNA, anti-CHIKV IgG/IgM, anti-HCV anti-HIV-1/2. Care should be taken when opening vials to avoid cuts.

These materials are not for administration to humans or animals. They are not for *in vitro* diagnostic use, they are for evaluation purposes only and should not be used to determine the validity of assays for CHIKV RNA.

Shipment and Storage of Samples

The collaborative study materials will be shipped on dry ice. Upon receipt, lyophilized samples should be stored at -20°C or below. Liquid frozen samples should be stored at -70°C or below.

Participants are asked to check the incoming material. Receipt of the study samples should be confirmed and any anomaly should be reported on the "Acknowledgment of Receipt" form accompanying the study samples.

Study Protocol

Participants are requested to test dilutions of each study sample using their routine NAT-based assay for CHIKV on four separate occasions, using a fresh vial of each sample in each independent assay. The dilutions should be prepared in the normal diluent used in the laboratory (ideally this will represent the matrix of the normal test specimens).

On the day of each test run, the lyophilized sample (Sample 1) must 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.

For qualitative assays:

For the first test run, participants are requested to assay Sample 1, Sample 2 and Sample 3 by a series of seven ten-fold dilution steps (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) to obtain an initial estimate of an end-point. It is important that the dilution series spans the limit of detection for the assay.

For the three subsequent assays, participants are requested to test the dilution at the assay end-point (limit of detection) determined in the first test run, and a minimum of two half-$\log_{10}$ serial dilutions (i.e. 1:3.2 dilutions) either side of the pre-determined end-point (i.e., at least five dilutions in total). If, in the second assay, all dilutions are positive, or all negative, then the dilution series are to be adjusted accordingly for the next two assay runs.

Sample 4 and Sample 5 should be tested at three serial ten-fold dilutions (e.g. 10^{-1}, 10^{-2} and 10^{-3}) in all four assay runs.

If practicable, please test replicates (at least duplicates) of each dilution of each sample within the same assay run. Replicate extractions should be preferred over replicate amplification/detection steps.

For quantitative assays:

For each of the four assays, participants are requested to test Sample 1, Sample 2, Sample 3, Sample 4 and Sample 5 by a series of three ten-fold dilution steps within the linear range of the assay (e.g. 10^{-1}, 10^{-2} and 10^{-3}).

If practicable, please test replicates (at least duplicates) of each dilution of each sample within the same assay run. Replicate extractions should be preferred over replicate amplification/detection steps.

Reporting of Results

The results of each assay should be recorded on the appropriate "Results Reporting Form" provided with the study material. Methods used should also be reported on the "Method Reporting Form". Please use additional reporting forms as required.

For qualitative assays:

Results should be reported as reactive or non-reactive. Where real-time assays are used, please also include the crossing point / threshold cycle (C_T) for each result.

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For quantitative assays:

Results should be reported in copies/mL. For real-time methods please record the crossing point / threshold cycle (C_T) as well as the concentration of CHIKV RNA in copies/mL.

Data should be returned as soon as possible and **before** the **12th of April 2017.**

All completed forms should be returned preferably **by e-mail** to Dr. Julia Kreß: **Julia.Kress@pei.de**

Alternatively, results may be faxed or mailed to:

Dr. Julia Kreß
Paul-Ehrlich-Institut
Federal Institute for Vaccines and Biomedicines
Paul-Ehrlich-Str. 51-59
63225 Langen / Germany
Fax +49 6103 77 1285

Data Analysis

All data from the collaborative study will be analyzed at PEI. The analysis will assess the concentration of each sample, relative to each other, and the sensitivities of the different assay methods. Individual participant's data will be coded and reported "blind" to other participants during the preparation of the study report, and also subsequent publications. Participants will receive a copy of the draft 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.

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 PEI study organizer.

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Results Reporting Form 1 (Qualitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 1:

Estimation of Putative End-Point

Sample 1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Result replicate 1							
Result replicate 2							
Result replicate 3							
Sample 2	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Result replicate 1							
Result replicate 2							
Result replicate 3							
Sample 3	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Result replicate 1							
Result replicate 2							
Result replicate 3							

Sample 4	10^{-1}	10^{-2}	10^{-3}
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	10^{-1}	10^{-2}	10^{-3}
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record qualitative results as follows: R for reactive, NR for non-reactive
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 2 (Qualitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 2:

	(10^{1})	($10^{0.5}$)	(end-point)	($10^{-0.5}$)	(10^{-1})
Sample 1	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 2	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 3	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					

Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record qualitative results as follows: R for reactive, NR for non-reactive
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 3 (Qualitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 3:

	(10^{1})	($10^{0.5}$)	(end-point)	($10^{-0.5}$)	(10^{-1})
Sample 1	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 2	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 3	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					

Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record qualitative results as follows: R for reactive, NR for non-reactive
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 4 (Qualitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 4:

	(10^{1})	($10^{0.5}$)	(end-point)	($10^{-0.5}$)	(10^{-1})
Sample 1	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 2	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					
Sample 3	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**	**10^{-x}**
Result replicate 1					
Result replicate 2					
Result replicate 3					

Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record qualitative results as follows: R for reactive, NR for non-reactive
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 1 (Quantitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 1:

Sample 1	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 2	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 3	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record quantitative results in copies/mL.
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 2 (Quantitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 2:

Sample 1	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 2	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 3	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record quantitative results in copies/mL.
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 3 (Quantitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 3:

Sample 1	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 2	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 3	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record quantitative results in copies/mL.
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Results Reporting Form 4 (Quantitative)

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:
Organization name:
Date of assay run 4:

Sample 1	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 2	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 3	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 4	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			
Sample 5	**10^{-1}**	**10^{-2}**	**10^{-3}**
Result replicate 1			
Result replicate 2			
Result replicate 3			

Please record quantitative results in copies/mL.
For real-time methods please also include the crossing point / threshold cycle (C_T) for each result.
Please indicate replicate extractions and/or amplification/detection reactions.

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Method Reporting Form

Collaborative Study to Evaluate a Candidate WHO International Standard for Chikungunya Virus RNA for NAT-based Assays

Name of participant:			
Organization name:			
Extraction method:			
Instrument:			
Diluent used:			
Volume extracted:		Volume eluted:	
Amplification method:			
Instrument:			
Volume of RNA used for amplification:			
Kind of assay (e.g. real-time PCR):			
Assay reference (if available):			
CHIKV target region:			
Qualitative:		Quantitative:	
Material used for quantitation:			
Other comments:			

Please use additional sheets as necessary.