World Health Organization

WHO/BS/2023.2459
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 16 to 19 October 2023

Collaborative Study for the Establishment of the Second WHO International Standard for SARS-CoV-2 RNA

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** Listed in Appendix 1*

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 **2 October 2023** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

The First WHO International Standard (IS) for SARS-CoV-2 RNA (20/146) was established by the WHO ECBS in December 2020, under an expedited timeframe, to support the development of diagnostic nucleic acid amplification technique (NAT)- based assays in response to the COVID-19 pandemic. Given the large number of commercial NAT-based platforms on the market, there has been a high demand for the First IS and stocks are nearing depletion. The current regulatory landscape and continued global public health importance of diagnosing infection, means that demand for the IS will continue and there is a need to provide continuity in both the unitage and availability. This report describes the results of a multi-centre collaborative study to evaluate the suitability of a candidate Second WHO IS for SARS-CoV-2 RNA (22/252) and propose an International Unit assignment. The candidate Second WHO IS was prepared to be a like-for-like replacement, comprising an inactivated pre-variant of concern (VOC) isolate of SARS-CoV-2. Performance was assessed alongside the First WHO IS and a panel of samples representing five VOC. Twenty-two laboratories took part in the study and returned 47 datasets from 34 methods, with 22 of these based on commercial platforms. The methods used cover real-time PCR, digital PCR, TMA, LAMP and qSTAR technology. Evaluation of the reduction in inter-laboratory variability when expressing the data relative to the Standards showed an ability to harmonise across the range of molecular technologies, with no discernible difference between the VOC. The First and candidate Second WHO IS were found to have a similar performance. The study shows that the agreement between data reported from qualitative and quantitative methods is improved when reported relative to a Standard, with only a 0.01 Log_{10} IU/mL difference in the mean potency for the candidate Second WHO IS relative to the First WHO IS. It is therefore proposed that 22/252 is established as the Second WHO International Standard for SARS-CoV-2 RNA for NAT assays with an assigned potency of 7.50 Log_{10} IU/ampoule based on the combined method mean potency.

Introduction

Since the first reported cases of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in December 2019 and the WHO announcement of a Public Health Emergency of International Concern (PHEIC) in January 2020 and global COVID-19 pandemic in March 2020, the demand and implementation of molecular diagnostic testing has been unprecedented. This was initially facilitated by the early availability of the complete genome sequence of the virus [1] and the WHO publishing in-house PCR assay protocols [2] to rapidly facilitate global diagnostic testing capability by nucleic acid amplification techniques (NAT). To support these efforts, the First WHO International Standard (IS) for SARS-CoV-2 RNA (20/146) was established by the WHO ECBS in December 2020, having been developed under an accelerated timeline [3]. Standardisation of molecular diagnostics through calibration to the International Unit (IU) provides a vital means by which to compare and control platforms being used to detect SARS-CoV-2 RNA. The harmonisation of data reporting to the IU makes it possible to better define parameters such as analytical sensitivity/limits of detection. At the time of its establishment, over 350 NAT-based products had been released onto the market, with the number now standing at >700 [4], and there has consequently been a high demand for the First WHO IS with more than 1,800 vials distributed to over 400 end-users/companies globally at the time of writing and stocks are nearing depletion.

The COVID-19 diagnostic landscape remains very active and the demand for the WHO IS will likely continue for the foreseeable future. With the WHO declaring the PHEIC over on 5th May 2023 [5], this has set in motion a period of transition from emergency use licensing to the traditional pre-qualification assessment pathway [6]. Globally, regulators will likely be entering into periods of transition or adapting COVID-19 molecular device legislation. Clinical diagnosis to confirm COVID-19 will remain via the detection of SARS-CoV-2 specific RNA by NAT-based methods. Further, active surveillance and assessment of circulating variants of concern (VOC) will continue. As such, continuity in the unitage and availability of the IS with a timely transition to the Second WHO IS will be needed to support the continued assessment and regulatory review of new and updated platforms. The proposal to replace the First WHO IS for SARS-CoV-2 RNA was endorsed by the WHO ECBS in October 2022.

This report describes the preparation and multi-centre collaborative study to evaluate the candidate Second WHO IS for SARS-CoV-2 RNA for NAT assays. As with the First WHO IS, the candidate Second IS is based on an inactivated pre-VOC (Wuhan-like) isolate. Although the dominant circulating variants have fallen under the Omicron lineage since early 2022 [7], the importance of maintaining continuity in the IU and the level of genetic diversity within circulating strains, together with the requirement for molecular diagnostics to detect all sequences, does not support transitioning to a more recent strain. There was, however, a requirement for this study to adapt to the change in molecular landscape since establishment of the First WHO IS. This included the inclusion of a panel of inactivated SARS-CoV-2 VOC, to demonstrate harmonisation across variants, and the recruitment of a broader range of participants (20-30) than is typically required for a replacement study to capture the wide range of molecular technologies now commercially available.

The aims of this collaborative study are to:

- Evaluate the candidate's potency/readout, in parallel to the First WHO International Standard, in a range of typical assays performed in different laboratories.
- Characterise the candidate's reactivity/specificity in different assay systems.
- Assess commutability, i.e. establish the extent to which the candidate is suitable to serve as a standard for a variety of different samples/strains.
- Propose a unitage for the Second WHO International Standard that will ensure continuity in the use of the International Unit.

Materials and Methods

Candidate Standard

The candidate replacement Standard (NIBSC code 22/252) comprises of a lyophilized preparation of an inactivated SARS-CoV-2, pre-VOC, isolate. The viral bulk was prepared using the BetaCoV/Australia/VIC01/2020 isolate (Genbank accession number MT007544.1), kindly donated to MHRA by the Victoria Infectious Diseases Reference Laboratory, Royal Melbourne Hospital (Australia). The virus was received at MHRA at passage 3, and amplified one further passage in Vero/hSLAM (ECACC #04091501) with an MOI of 0.001 and harvested 72 hours post-inoculation. The infectious titre of the viral bulk was measured as $2.15x10^7$ $TCID_{50}$/mL via endpoint titration on Vero-E6 cells (ATCC® CRL-1586). Next generation sequencing (NGS) was performed using a protocol derived from the method described in Moore *et al.* [8] to confirm the genetic integrity of the propagated batch of virus. Following extraction of the viral RNA using the QIAamp Viral RNA mini kit (Qiagen #52904), cDNA was generated using the Superscript IV first-strand cDNA synthesis kit (ThermoFisher #18091050) and PCR performed using the Q5 high fidelity 2x master mix (NEB #M0492S). The amplicons were purified using AMPure XP beads (Beckman Coulter #A63880), and a sequencing library generated using the Illumina DNA Nextera Flex. The libraries were sequenced on the Illumina MiSeq and variants were called against the Wuhan-1 reference sequence (Genbank accession number NC_045512.2) using ivar and lofreq variant callers as part of a bioinformatic pipeline developed in-house. A table of variations is provided in Appendix 2.

Inactivation of the virus was performed using a validated acid-heat treatment protocol (described in full within Appendix 3). Briefly, the viral culture was incubated for 15 minutes with 3% v/v acetic acid and neutralized by the addition of sodium hydroxide before subsequent incubation for 1 hour at 60°C. Inactivation of the viral stock was verified by passaging 10% of the stock 3 times in the highly susceptible Vero-E6/TMPRSS2 cell line (NIBSC #100978) over a period of 3 weeks, alongside positive and negative controls. Cells were monitored for signs of cytopathic

effect and culture media tested for any quantifiable increase in viral RNA by RT-qPCR. No viable virus was detected. The inactivation procedure was approved by the MHRA Biological Safety committee.

To formulate the bulk of 22/252 in preparation for filling, the inactivated viral culture was purified using Amicon® Ultra 50kDa centrifugal filter columns and resuspended within universal buffer (10 mM Tris-HCl (pH 7.4), 0.5% human serum albumin and 1% D-(+)-Trehalose dehydrate). Quantification of the SARS-CoV-2 genome copies within the stock was determined relative to the First WHO International Standard, 20/146 ($1x10^{7.7}$ IU/mL) by in-house RT-qPCR using primer/probe targeting the E-gene [9]. The stock was diluted 1:100 in universal buffer which included a background of ~$1x10^5$ copies/mL of human genomic DNA (referred herein as UB^+), to give a final bulk volume of 2.8 L with a potency in the order of ~$1x10^{7.8}$ IU/mL.

Filling and Lyophilization of the Candidate

The filling and lyophilization of 22/252 was performed by the Manufacturing team at MHRA under ISO9001 between 24-28th November 2022. Material was dispensed in 0.5mL volumes into 2.5mL glass DIN ampoules at 4°C on an AVF5090 filling line (Bausch & Stroebel, Ilshofen, Germany). The homogeneity of the fill was maintained by on-line check-weighing of a proportion of the filled ampoules. Filled ampoules were partially stoppered with halobutyl 13mm diameter igloo closures and lyophilized in the CS100 freeze dryer. Ampoules were loaded onto the shelves at 4°C and primary freezing was performed to -50°C over 1.5 hours. Primary drying was performed at -30°C for 40 hours at 30µb vacuum, then raising the shelf temperature to 25°C and holding vacuum in secondary drying for at least 15 hours, before releasing the vacuum and back-filling the vials with nitrogen. Ampoules were flame sealed on the same filling line. The sealed vials are stored at -20°C under continuous temperature monitoring for the lifetime of the product.

Assessments of residual moisture and oxygen content, as indicators of freeze-drying completion and vial integrity after sealing, were determined for twelve vials of freeze-dried product. Residual moisture was measured destructively using colorimetric Karl Fischer (CA-200, Mitsubishi Instruments obtained through A1-Environsciences Ltd, Blyth, UK) operated within a dry box and checking performance before analysis using an Aquamicron Check P water standard (A1 Environsciences) to give % w/w moisture readings. Oxygen content was measured non-invasively by frequency modulated infra-red spectroscopy using an FMS-760 Oxygen Headspace Analyzer (Lighthouse Instruments, Charlottesville, VA, USA).

Stability of the Lyophilized Candidate

Accelerated degradation studies commenced in January 2023 for the lyophilized candidate Standard 22/252 to predict the stability of the material. Fifteen ampoules of the candidate were stored at -20, +4, +20, +37 and +45°C. Three ampoules for each temperature were retrieved at 2 weeks, 1 month, 3 months and 6 months and held at -20°C until testing. Retrieved samples were

reconstituted and analyzed in-house by SARS-CoV-2 RT-qPCR and potencies calculated relative to the designated -20^{o}C baseline. Degradation was assessed by a drift in potency relative to the baseline. The Arrhenius model was used to predict long-term stability when stored at a range of temperatures, with the equation relating degradation rate to absolute temperature assuming first-order decay [10]. Additional ampoules will be retrieved after 1 year in January 2024 and stored in case further assessment of stability is required.

Additional Study Samples

The candidate 22/252 was evaluated alongside the current First WHO International Standard for SARS-CoV-2 RNA (20/146) [3] and a panel of 8 other study samples. This included:

First WHO International Standard for SARS-CoV-2 Antigen (21/368)

Established by the WHO ECBS in December 2022[11]. It comprises a 0.25mL lyophilized preparation of an Omicron BA.1 variant of SARS-CoV-2 (hCoV19/England/PHEP-YYNGSX6/2021), which was inactivated via 12 days incubation at 37°C with 0.01% formaldehyde and neutralized with a 1:8 solution of sodium bisulfate. The final bulk was prepared to contain 50ng/mL of nucleocapsid protein and formulated in COPAN UTM® Universal Transport Medium™ (UTM).

Liquid Dilution of the Candidate Standard

A portion of the liquid bulk used to prepare 22/252 was diluted 100-fold in UB^{+} to provide a lower potency sample, provided as a 0.5mL liquid frozen sample.

SARS-CoV-2 Variants of Concern (VOCs)

Five inactivated SARS-CoV-2 VOC samples were prepared following the same acid-heat treatment and subsequent inactivation verification as described for the candidate Standard. They were all formulated within UB^{+} to a target potency approximately within the same order of magnitude (10^{6} IU/mL) based on in-house RT-qPCR and provided as 0.5mL liquid frozen samples. The isolates were kindly donated by the UK Health Security Agency (UKHSA) and provided through the NIBSC Research Reagent repository, with stocks propagated on Vero/hSLAM (ECACC #04091501) to a passage no higher than 4, with sequences confirmed via NGS:

- Alpha (hCoV-19/England/204820464/2020, lineage B.1.1.7; NIBSC #101027)
- Delta (hCoV-19/England/PHEC-30C951/2021, lineage B.1.617.2; NIBSC #101030)
- Omicron (hCoV-19/England/PHEP-YYNGSX6/2021, sublineage BA.1; NIBSC #101047)
- Omicron (sublineage BA.2; NIBSC #101055)
- Omicron (hCoV-19/England/PHEP-YYR69B6/2022, sublineage BA.5.2.1; NIBSC #101061)

An additional inactivated SARS-CoV-2 Omicron (SARS-CoV-2/human/USA/COR-22-063113/2022, sublineage BA.5.5) sample was included in the study panel, which was produced by BEI Resources, Manassas, USA. The isolate was inactivated via heat treatment at 65°C for 30 minutes and inactivation verified by inoculating 10% of the viral stock onto Vero E6-TMPRSS2-T2A-ACE2 cells (NR-54970) followed by 14 days incubation. Cells were monitored for cytopathic effect and the cell lysate and supernatant were then blind passaged for a further 14 days, with samples from all timepoints also tested for the presence of viral RNA by RT-qPCR. No viable virus was detected. The material was provided formulated with universal buffer, as a 0.5mL liquid frozen sample.

Coded Study Panel

Table 1 lists the collaborative study samples provided, coded and blinded to the participants. For each method in use in their laboratory, participants received 4 sample panels to allow for a preliminary assay to determine optimal dilution range, followed by 3 independent tests per method. Where more than one method was performed additional sample panels were provided. Sample SV-10 was not provided to participants performing qualitative methods with a +/- results readout, based on limiting usage of stocks of the WHO IS for SARS-CoV-2 antigen, 21/368. The study samples were stored at either -80°C (SV-01 to 07) or -20°C (SV-08 to 10) and shipped on dry ice to participants under NIBSC dispatch reference CS714.

Participants

Twenty-six laboratories accepted to participate in the study; however, one participant did not receive the study panel in time due to delays in obtaining an import permit and three participants withdrew from the study due to resource constraints preventing timely evaluation of the samples. Overall, the 22 laboratories returning results were from 11 countries: China (2), Germany (3), Italy (1), Japan (1), Luxemburg (1), Sweden (1), Switzerland (1), South Korea (2), Taiwan (1), United Kingdom (2) and U.S.A. (7) and are listed in Appendix 1. All laboratories' data are referred to by a code number randomly allocated and not reflected in the order presented in Appendix 1. Participating organizations included diagnostic kit manufacturers, national control/reference laboratories and research laboratories.

Study Design

The study protocol (Appendix 4) requested participants to test the sample panel in their established SARS-CoV-2 NAT based assay/s. Participants were asked to perform 3 independent tests, and for each test, to prepare at least two independent dilution series of the samples, within a matrix specific to their assay. For quantitative methods, which were defined as those reporting a numerical readout in copies, International Units or Cq values with good linearity, participants were asked to test the panel across a minimum of 4 serial 10-fold dilutions. For qualitative methods, which were defined as those reporting a +/- readout or numerical values which do not provide good linearity, participants were asked to test the panel across dilutions spanning the

endpoint of the assay, including at least 2 positive and 2 negative points. Participants performing qualitative assays were requested not to test SV-10. In both cases, a sample panel was provided for a preliminary assay to determine the potency range/endpoint within the assay method. A results-reporting sheet was provided for participants to return all essential information and the raw data readouts (e.g. Cq, copies, +/-) from their assay, as well as providing the result as per the analysis within their laboratory. Participants were asked to return results by the end of April 2023.

Assay Methods

Assays used by the participants are summarised in Table 2. Where laboratories performed multiple assay methods, laboratory codes are followed by a letter indicating the different methods (e.g. Lab 1a, 1b). In cases where a single method provides separate data points for multiple assay targets, these are differentiated as Lab 1^{T1}, Lab 1^{T2} etc., with T1/2 indicating the target number. The methods used cover in-house and commercial kits based on reverse transcription digital PCR (RT-dPCR), real-time RT-PCR (RT-qPCR), isothermal amplification (Transcription mediated (TMA) & Loop-mediated (LAMP)) and non-isothermal amplification (quantitative Selective Temperature (qSTAR)) technology.

Statistical Methods

Mean potency estimates for each of the study samples were calculated as laboratory reported estimates (i.e. not relative to a common standard) and also as estimates relative to the First IS, 20/146 or candidate Second IS, 22/252.

Laboratory reported estimates were calculated from raw data returned by participants. A different approach was applied dependent on whether the data was quantitative or qualitative. For quantitative data, reported as copies or IU, the geometric mean of all replicates was taken, correcting for sample input and dilution factor where required. A potency estimate was reported as Log_{10} Copies or IU/mL calculated from the geometric mean across the independent assays. Qualitative data, reported as Cq or pos/neg, were evaluated to provide a potency estimate in Log_{10} NAT detectable units/mL (NDU/mL) which is corrected for the equivalent volume of sample amplified. This was calculated by pooling sample data across independent assays within a laboratory to provide a number positive out of number tested at each dilution step. A single endpoint for each sample dilution series was calculated using the method of maximum likelihood [12]. The model assumes that the probability of a positive result at a given dilution follows a Poisson distribution and that a single ‘copy’ will provide a positive result. The estimated endpoint is equivalent to the dilution at which there is an average of a single copy per sample tested, or the dilution at which 63% of samples tested are positive. Calculations were performed using R software [13].

Relative potencies were calculated by two methods. Where raw data for sample dilutions was provided either in copies or Cq values, relative potency estimates were obtained by parallel line analysis (PLA) with Log_{10}-transformed response. All calculations were performed using CombiStats [14]. Linearity was assessed by visual assessment and by calculating Pearson correlation r value. Assays with a value below 0.99 (quantitative assays) or 0.95 (qualitative

assays where PLA of Cq values was possible) were excluded from further analysis. Non-parallelism was assessed by calculating the ratio of fitted slopes for the test and reference sample under consideration. Samples were concluded to be non-parallel when the slope ratio was outside the range 0.80 – 1.25 and a relative potency estimate was not calculated. Where qualitative assay data did not allow PLA to be performed, differences in Log_{10} NDU estimates (calculated as described above) were used to estimate relative potencies.

Overall mean estimates were calculated as the arithmetic mean across all laboratories, combined, as well as segregated into quantitative and qualitative methods. Statistical analysis was performed on combined data to evaluate inter-laboratory variation. This was expressed as the standard deviation (SD) of the Log_{10} estimates and the % Geometric Coefficient of Variation (%GCV = $\{10^{s}-1\}\times100\%$). Laboratory mean estimates further than 1.5 times the interquartile range from the upper and lower quartiles of the distribution of estimates were identified as 'outliers' and overall summary statistics were calculated with and without exclusion of these values.

Results and Data Analysis

Collaborative Study Data Received

Overall, 22 laboratories returned data covering 34 methods, with 12 of these providing readouts against two or more target genes which have been analyzed individually; thus, a total of 47 datasets have been evaluated in this study (Table 2). This includes 16 datasets from methods providing a quantitative result (copies or IU) and 31 datasets from qualitative methods. Most assays had a target within the ORF1ab (RdRp) gene (20), N gene (13) or E gene (11), with a further 5 assays targeting the S (3), M (1) or ORF8 (1). Seven assays provide a single readout against a dual target, for 5 assays both targets fall within in the ORF1ab region (Lab 7a-d & 13b), with 2 targeting the ORF1ab and N (Lab 2 & 13c). As would be expected, for the quantitative assays there is a higher distribution targeting structural proteins at the 3'-end of the SARS-CoV-2 genome (N=8, E=4, ORF1ab =4).

Of the quantitative datasets, 9 were obtained via RT-dPCR with 7 based on in-house 1-step methods operating on the same Bio-Rad QX200 ddPCR system (Lab 4a-c, 5, 9b, 19a-b) and 2 with a commercial kit (Lab 12). The remaining 7 datasets were obtained via RT-qPCR, with 5 based on in-house methods reporting copies (Lab 3a-c & 17) or International Units (Lab 9a), and 2 based on either a commercial kit, reporting copies (Lab 8), or a commercial fully automated closed system reporting International Units (Lab 13b). All laboratories returned results from three independent assays, with data reported from a dilution series of the samples from all laboratories except 2 performing RT-dPCR which tested a single dilution of the samples (Lab 4a-c & 19a-b).

The qualitative datasets included methods based on RT-qPCR (26), TMA (2), RT-LAMP (1) and qSTAR (2) technology, all are commercial kits/systems. For those based on RT-qPCR, this included 2 datasets from a single direct method with a Cq readout (Lab 1), 5 datasets from 3 cartridge-based closed systems with 2 providing pos/neg readouts (Lab 10, 13c) and one providing a Cq readout (Lab 20), 8 datasets from 5 fully automated closed systems with 2 providing Cq readouts (Lab 7c-d) and the rest pos/neg (Lab 13a, 14, 18) and the remaining 11 datasets from 6 methods using off-board extraction all reporting Cq readouts (Lab 2, 11, 12b, 15, 16, 22). Only one method is represented twice (Lab 14 and 18). For the TMA methods, one is based on chemiluminescent technology providing end-point readouts in kRLU (Lab 7a) and the second on fluorophore-probes to provide real-time readouts as Ttime (Lab 7b). The RT-LAMP method reported data as pos/neg (Lab 6) and the 2 datasets based on qSTAR provide a Cq readout (Lab 21). All laboratories returned results from three independent assays, with data reported against a dilution series of the samples.

Reported Potency Estimates of Study Samples

Based on the reported raw data provided by the participating laboratories, mean potency estimates were calculated for each of the study samples and are provided in Table 3. These mean values are also presented by a box and whisker plot in Figure 1. For the quantitative datasets these are reported as Log_{10} copies/mL or Log_{10} IU/mL and for qualitative datasets Log_{10} NAT detectable units (NDU)/mL as described in the statistical methods. As the sample data reported for Labs 1, 18 and 22 did not reach a dilution endpoint it was not possible to calculate NDU/mL, however this was due to the study protocol not specifying an endpoint should be met for labs following the quantitative method (when Cq values provided good linearity). For Labs 20 and 21, calculations are based on data from a single experiment due to either a high level of negativity in the dilutions selected for testing in the subsequent experiments or a high level of variability in the dilutions; it was only possible to calculate a potency estimate for target 1 (T1) data for Lab 21. As per the study protocol, only labs following the quantitative method included sample SV-10 in their testing.

Both labs (9a and 13b) reporting data in IU/mL estimated the potency of the First IS, 20/146 (SV-08) to be within 0.04 Log_{10} IU/mL of the assigned potency (7.70 Log_{10} IU/mL). Across the remaining quantitative methods reporting copies/mL, the potency estimate for 20/146 ranged 27-fold from 7.40 – 8.83 Log_{10} copies/mL (Table 4). This is reduced to 3-fold when considering only the RT-dPCR methods where the data ranges from 7.66 – 8.14 Log_{10} copies/mL (Table 4, Figure 2). For the qualitative methods, the potency estimates were lower and ranged 73-fold from 6.06 – 7.92 Log_{10} NDU/mL, with no clear bias based on method. When considering the combined methods (quantitative & qualitative) the inter-laboratory variation, measured as the geometric coefficient of variation (GCV), for 20/146 is 335% which is in line with the 2020 collaborative study for its establishment which reported a GCV of 375% [3]. The performance of the candidate Second IS, 22/252 (SV-09) is similar to 20/146 in terms of both the %GCV and standard deviation (SD) of the reported potency estimates (Table 4).

As for the other study samples, the panel of VOC samples (SV-01 to -05 & -07) have a similar level of inter-laboratory variation when considering the quantitative methods (146 – 174 %GCV), with potency estimates across the samples falling within a 31- to 47-fold range (Table 4). Similar to 20/146, SV-08, this is reduced to 4-fold across the samples when considering the RT-dPCR datasets alone (Figure 2). For the qualitative methods the inter-laboratory variability for the VOC samples falls between 135 – 311 %GCV and a 20- to 386-fold range, with the VOC-Delta sample, SV-02, giving the highest level of variability (Table 4). The combined average potency estimates across samples SV-01 to -05 fall within 4-fold of each other (5.80 – 6.44 Log_{10} 'various units'/mL), as expected based on their formulation to be a similar target potency. Similarly, SV-06, the low potency liquid 1:100 dilution of 22/252 is approximately 78-fold less potent than SV-09 based on the combined study estimates. While there is no discernable difference in the performance of SV-06 to the other study samples, the WHO IS for antigen, 21/368 (SV-10) has the highest %GCV across the study samples, particularly in the case of the quantitative methods (291%). No biases could be identified across the samples based on method or assay target to account for differences in performance.

Potencies Relative to the First International Standard for SARS-CoV-2 RNA (20/146)

The potencies of the study samples were expressed in IU/mL, relative to the current IS (sample SV-08) which has an assigned unitage of 7.70 Log_{10} IU/mL (Table 5), by either parallel line analysis or as a ratio with quality criteria applied as described in the statistical methods. This is additionally presented by box and whisker plots in Figure 3. Samples SV-05 and SV-06 were found to be non-parallel for Lab 1 and the data provided by Lab 22 was found to be both non-linear and non-parallel. Outliers were identified and are excluded from the overall estimates and inter-laboratory variation presented in Table 6. A high proportion of outliers for Lab 20 may be accounted by the data analyzed representing a single experiment.

The overall mean of the candidate Second IS, 22/252 (SV-09) is 7.78 Log_{10} IU/mL, with only a 0.01 Log_{10} IU/mL difference between quantitative and qualitative methods. The level of inter-laboratory variability is lowest for the quantitative methods in comparison to the qualitative, with a GCV of 16% *vs* 35% and an SD of 0.07 *vs* 0.13 (Table 6).

When considering the other study samples, a substantial and similar level of harmonization in the potency estimates can be seen through the reduction in the measures of inter-laboratory variation (%GCV and SD) for the VOC samples, SV-01 to -05 & -07, and the low potency sample, SV-06. Combined estimates have a GCV between 51-76% and an SD of 0.15-0.25. The lowest level of variability is achieved across the samples when considering the quantitative methods in isolation. The data does therefore not highlight any concerns with the ability of the Standard to harmonize data based on VOCs. In contrast, the antigen Standard, 21/368 (SV-10) does not demonstrate a reduction in inter-laboratory variation when data is expressed relative to the First IS, 20/146. As there is no clear method or assay target bias, it is considered the formaldehyde inactivation method may be responsible for the performance of this sample.

Potencies Relative to the Second International Standard for SARS-CoV-2 RNA (22/252)

As described for 20/146, the potencies of the study samples were expressed relative to the candidate Second IS (SV-09) with an assigned potency of 7.80 Log_{10} IU/mL (Table 7, Figure 4). The same quality criteria to exclude non-linear/parallel data and the identification of outliers were applied, with a similar number identified as to when using 20/146 as the Standard (22 vs 26). Considering the measures of inter-laboratory variation (Table 8), the candidate Second IS achieves a greater level of harmonization across all study samples SV-01 to -07, with combined estimates of GCV between 24-57% and an SD of 0.09-0.20. As with 20/146, the candidate Second IS demonstrates the ability to harmonize across VOC.

Production of the Candidate Second IS and Stability Assessment

The candidate Second IS, NIBSC code 22/252, was freeze-dried at NIBSC in November 2022. A product summary is provided in Table 9. The mean residual moisture and oxygen content falls within the recommended limit of <1% for a WHO reference material [15]. All microbiological tests for bacteria and mold/yeast colony count returned a negative result. There are approximately 5000 vials of 22/252 available for distribution.

Accelerated degradation studies were performed to determine the long-term stability of the candidate Standard 22/252. Samples were retrieved at 2 weeks, 1 month, 3 months and 6 months from storage temperatures of -20, +4, +20, +37 and +45°C. Two vials of 22/252 from each temperature were evaluated and the mean estimated potencies relative to the -20°C baseline are shown in Table 10. There is no loss of potency observed, indicated by negative values compared to the -20°C baseline, up to 1 month stored at 37°C demonstrating adequate stability to be shipped at ambient temperature. The Arrhenius model was applied to the relative potencies measured at 3 and 6 months to predict loss per year with long term storage. The 6-month potencies at 4°C and 45°C were omitted from the analysis due to causing a significantly poor model fit, which also provided lower estimates of predicted loss. The model predicts the loss in potency of 22/252 when stored at -20°C to be 0.07% per year (Table 11).

Discussion

Within this study, the suitability of a candidate replacement Second WHO IS for SARS-CoV-2 RNA (22/252) has been evaluated in a range of NAT-based assays. The candidate was prepared in the same manner as the First WHO IS (20/146), through acid-heat inactivation of whole virus and formulated within universal buffer with a background of human genomic DNA [3]. A different pre-VOC isolate (VIC01/2020; GenBank: MT007544.1) was used, since the First IS was found to have a 24-nucleotide deletion in the furin cleavage site (England/02/2020; GenBank: MW059036). The two isolates have >99% sequence homology. The study included a larger number of samples and participants than is typical for the evaluation and potency assignment of a replacement Standard. This was to account for the evolution of VOC, with only pre-VOC isolates included in the previous study, and to reflect the large number of commercial molecular assays that have undergone development, covering various platform technologies, that were not previously captured.

The panel of 10 study samples were evaluated by 22 laboratories across a total of 34 methods, covering 5 platform technologies (qPCR, dPCR, TMA, LAMP, qSTAR). This compares to 21 methods covering only the first three platform technologies listed in the previous 2020 study. Most notably, there is a higher proportion of methods using commercial kits in this study (22 *vs* 8), with 5 commercial methods common between the two. Of note, the studies include an equivalent number of quantitative datasets (16 *vs* 17) which are in both cases based on a similar distribution of qPCR and dPCR. This is reflective of the fact most commercial assays provide qualitative results. In addition to the inclusion of methods using LAMP and qSTAR technology, this study also captured data from qPCR technologies using a direct and cartridge-based set-up, which were not previously reflected. Although the evaluation is limited in number of datasets falling within these new categories, no method-based outliers were detected and as such the study demonstrates the ability of the Standards to harmonize data reported, to comparable levels, across the range of molecular technologies.

A key component in evaluating the performance of a Standard is to assess how closely it behaves to a clinical sample. This is determined by factors such as genetic variability of clinical isolates, effect of inactivation procedure and the impact of sample matrix. As the study to establish the First IS in 2020 only included two pre-VOC isolates relevant at the time, this study has addressed a need to extend this assessment by including seven samples representing Alpha, Delta, Omicron BA.1, BA.2 and BA.5 VOC. This included Omicron BA.5 samples inactivated by acid-heat (SV-05) and heat alone (SV-07) and Omicron BA.1 samples inactivated by acid-heat (SV-03) and formaldehyde inactivated (SV-10). No discernible difference in the ability of the Standards to harmonize across the VOC could be detected, with near equivalent %GCV values for the relative potencies when comparing across variants inactivated by the same method or using heat alone in the case of Omicron BA.5 (SV-05 *vs* SV-07). In contrast, data reported for the formaldehyde inactivated Omicron BA.1 sample (SV-10) failed to be harmonized when expressed relative to the Standards, with a worsening %GCV. This sample is the First WHO IS for SARS-CoV-2 Antigen [11] and was included within this study to determine if a unitage relative to the WHO IS for SARS-CoV-2 RNA could be assigned. Whilst formaldehyde inactivation has been shown to preserve antigenicity, the cross-linking of nucleic acids and protein can impact amplification of viral RNA in a method dependent manner [16, 17] and is likely attributable to the variability within this study. This highlights the importance of insuring inactivation methods are appropriate for the intended use of a sample.

Overall, the data presented within this report shows the candidate Standard has a comparable performance to the First WHO IS. The %GCV of the laboratory reported potency estimates of the First WHO IS is similar to that reported in the 2020 study for its establishment. For laboratories reporting a readout in IU, the estimates for the First WHO IS are within 0.04 Log_{10} IU/mL of the assigned potency (7.70 Log_{10} IU/mL). Of the quantitative methods, it was found that dPCR, which were largely based on one method, had a lower spread (3-fold) in the reported potency estimates than the qPCR methods (27-fold). However, when reporting data relative to either the First or candidate Second IS the relative potencies of the combined quantitative methods fall within a 3-fold range, demonstrating the value of calibrating to the IU. It was also

found that reporting data relative to the Standard improved the agreement between the quantitative and qualitative methods. The potency of the candidate Second IS relative to the First IS was found to be 7.78 Log_{10} IU/mL, with only a 0.01 Log_{10} IU/mL difference between quantitative and qualitative methods. Outliers were removed from the relative analysis to prevent the impact of anomalous results on the potency assignment, which could contribute to a drift in the value of the IU. The distribution of outliers did not highlight any sample specific issues. Finally, it was found that an overall higher level of agreement between the laboratories and methods is achieved when reporting data relative to the candidate Second WHO IS.

Proposal

It is proposed that, the inactivated SARS-CoV-2, NIBSC code 22/252 is established as the Second WHO International Standard for NAT based assays detecting SARS-CoV-2 RNA with an assigned potency of 7.50 Log_{10} IU/ampoule. Proposed Instructions for Use (IFU) for the product are included in Appendix 5.

There are approximately 5000 ampoules (0.5 mL/ ampoule) available for distribution. It is recommended that the Second International Standard is stored at -20˚C.

Comments from Participants

Fifteen participants returned comments following circulation of the draft report. All were complimentary of the study and supportive of the proposal. Three participants commented that the explanation of the analysis applied to qualitative data in the results section could be improved and updates were made to the text, with reference to the statistical methods section. Following the suggestion of one participant, the report terminology for reporting of the fractional PCR cycle used for quantification was updated from threshold cycle (Ct) to quantification cycle (Cq) in line with MIQE guidelines [18]. Remaining comments included minor text or affiliation changes which have been addressed.

Acknowledgements

We gratefully acknowledge the important contributions of the collaborative study participants, particularly in meeting the tight timeframes of this study. We express our thanks to Victoria Infectious Diseases Reference Laboratory, Royal Melbourne Hospital (Australia) for the provision of the BetaCoV/Australia/VIC01/2020 isolate as well as UKHSA and BEI resources for provision of the VOC isolates of SARS-CoV-2. We also thank MHRA Manufacturing and Logistics teams for formulation of the candidate and distribution of the study material. We would like to acknowledge Ute Ströher (*In Vitro* Diagnostics Assessment Prequalification Unit, WHO), Uwe Scherf and Mayra Garcia (Office of *In Vitro* Diagnostics and Radiological Health, the Food and Drug Administration), Megan Cleveland and Peter Vallone (U.S. National Institute of Standards and Technology) and the UKHSA-MHRA VOC assurance group for constructive discussions on the isolate selection for the candidate replacement and collaborative study panel composition.

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Tables

Table 1. Collaborative Study Panel
Samples were shipped under study reference CS714

Sample Code	Sample Description (Inactivation method, Buffer)	Variant	Abbreviation	Appearance, volume (mL)
SV-01	VOC Sample (A/H, UB$^+$)	Alpha, B.1.1.7	VOC - Alpha	Frozen liquid, 0.5
SV-02	VOC Sample (A/H, UB$^+$)	Delta, B.1.617.2	VOC - Delta	Frozen liquid, 0.5
SV-03	VOC Sample (A/H, UB$^+$)	Omicron, BA.1	VOC - BA.1	Frozen liquid, 0.5
SV-04	VOC Sample (A/H, UB$^+$)	Omicron, BA.2	VOC - BA.2	Frozen liquid, 0.5
SV-05	VOC Sample (A/H, UB$^+$)	Omicron, BA.5.2.1	VOC - BA.5.2.1	Frozen liquid, 0.5
SV-06	Dilution of SV-09	Pre-VOC	Low - 22/252	Frozen liquid, 0.5
SV-07	VOC Sample (H, UB)	Omicron, BA.5.5	VOC – BA.5.5	Frozen liquid, 0.5
SV-08	20/146 - First WHO International Standard for SARS-CoV-2 RNA (A/H, UB$^+$)	Pre-VOC	20/146	Lyophilised, 0.5
SV-09	22/252 - Candidate Second WHO International Standard for SARS-CoV-2 RNA (A/H, UB$^+$)	Pre-VOC	22/252	Lyophilised, 0.5
SV-10	21/368 - First WHO IS for SARS-CoV-2 Antigen (Formaldehyde, UTM)	Omicron, BA.1	21/368 (Ag)	Lyophilised, 0.25

VOC: variant of concern; A/H: acid-heat; UB: universal buffer; UB$^+$: UB with human genomic DNA; H: heat; UTM: COPAN UTM® Universal Transport Medium™

Table 2. Laboratory Codes and Assay Methods

Quantitative assays reporting results in copies or International Units (IU) are shaded blue, with all other labs performing qualitative methods.

Lab	NAT Method		Extraction Method	Assay Target(s)	Readout
	Technology	Description			
1[T1]	RT-qPCR (Direct)	Simplexa Covid-19 Direct	N/A	S	Cq
1[T2]				ORF1ab	Cq
2	RT-qPCR	FTD™ SARS-CoV-2 Assay	VERSANT® kPCR Molecular System	ORF1ab, N	Cq
3a	RT-qPCR	In-house, TaqMan™ RNA-to-Ct One-Step kit; Applied Biosystems	Qiagen QIAamp Viral RNA Mini Kit	N1	Copies
3b				N2	Copies
3c				RdRp	Copies
4a	RT-dPCR	In-house, 1-step, Bio-Rad QX200 Droplet Digital PCR System	Qiagen QIAamp Viral RNA mini kit	N2	Copies
4b				E	Copies
4c				ORF	Copies
5	RT-dPCR	In-house, 1-step, Bio-rad QX200 Droplet Digital PCR System	Qiagen QIAamp Viral RNA mini kit	N	Copies
6	RT-LAMP	RT-LAMP, SARS-Q Kit	N/A	N	pos/neg
7a	TMA	Aptima® SARS-CoV-2 Assay	N/A	ORF1ab (2 targets)	kRLU
7b	Real-time TMA	Aptima® SARS-CoV-2/Flu Assay	N/A	ORF1ab (2 targets)	Ttime
7c	RT-qPCR	Panther Fusion® SARS-CoV-2 Assay	Closed system	ORF1ab (2 targets)	Cq
7d	RT-qPCR	Panther Fusion® SARS-CoV-2/Flu A/B/RSV Assay	Closed system	ORF1ab (2 targets)	Cq
8	RT-qPCR	GoTaq® Enviro Wastewater SARS-CoV-2 System-N1	Maxwell® Purefood GMO and Authentication Kit	N1	Copies
9a	RT-qPCR	In-house, LightCycler® Multiplex RNA Virus Master Kit	Qiagen EZ1 virus mini kit v2.0	E	IU
9b	RT-dPCR	In-house, 1-step, Bio-rad QX200 Droplet Digital PCR System	Qiagen EZ1 virus mini kit v2.0	E	Copies
10[T1]	RT-qPCR	BioFire® Respiratory Panel 2.1 plus	Closed system - cartridge based	S	pos/neg
10[T2]				M	pos/neg
11[T1]	RT-qPCR		Qiagen QIAamp Viral RNA mini Kit	ORF1ab	Cq
11[T2]				N	Cq

11[T3]		Novel Coronavirus (SARS-CoV-2) Real-Time Multiplex RT-PCR Kit		E	Cq
12a[T1]	RT-dPCR	TargetingOne 2019-nCoV Nucleic Acid Detection Kit	Qiagen QIAamp Viral RNA mini Kit	ORF1ab	Copies
12a[T2]				N	Copies
12b[T1]	RT-qPCR	Sansure Biotech Inc. 2019-nCoV Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing)	Magnetic bead extraction	ORF1ab	Cq
12b[T2]				N	Cq
13a[T1]	RT-qPCR	cobas® SARS-CoV-2 & Influenza A/B for cobas® 5800/6800/8800	Closed system	ORF1ab	pos/neg
13a[T2]				E	pos/neg
13b	RT-qPCR	cobas® SARS-CoV-2 Duo for cobas® 5800/6800/8800	Closed system	ORF1a, ORF1ab	IU
13c	RT-qPCR	cobas® Liat® SARS-CoV-2	Closed system - cartridge based	ORF1ab, N	pos/neg
14[T1]	RT-qPCR	cobas® SARS-CoV-2 Qualitative Test for cobas® 5800/6800/8800	Closed system	ORF1ab	pos/neg
14[T2]				E	pos/neg
15[T1]	RT-qPCR	AltoStar® SARS-CoV-2 RT-PCR Kit 1.5	AltoStar® Purification Kit 1.5	E	Cq
15[T2]				S	Cq
16[T1]	RT-qPCR	PowerChek™ SARS-CoV-2 Real-time PCR Kit	Automated purification system using paramagnetic particles	ORF1ab	Cq
16[T2]				E	Cq
17	RT-qPCR	In-house, AgPath-ID™ One-Step RT-PCR Reagents; Applied Biosystems	Qiagen QIAamp Viral RNA mini Kit	N	Copies
18[T1]	RT-qPCR	cobas® SARS-CoV-2 Qualitative Test for cobas® 5800/6800/8800	Closed system	ORF1ab	pos/neg
18[T2]				E	pos/neg
19a	RT-dPCR	In-house, 1-step, Bio-Rad QX200 Droplet Digital PCR System	Qiagen QIAamp Viral RNA mini kit	N2	Copies
19b				E	Copies
20[T1]	RT-qPCR	Xpert® Xpress SARS-CoV-2	Closed system - cartridge based	E	Cq
20[T2]				N2	Cq
21[T1]	qSTAR	LumiraDx™ Dual-Target SARS-CoV-2 STAR Complete	N/A	nsp1 or ORF8	Cq
21[T2]				nsp1 or ORF8	Cq
22	RT-qPCR	GenomEra® SARS-CoV-2, Flu A/B + RSV 2.0 Assay Kit	GenomEra® Extraction Columns	RdRp	Cq

Table 3. Laboratory Reported Mean Potency Estimates of Study Samples

Quantitative data (blue) is reported as Log_{10} copies or IU/mL and qualitative data as Log_{10} NDU/mL, datasets which were not suitable to perform this calculation are indicated (-). Results are derived from 3 experiments (n=3), unless indicated (*: n=1)

Lab	Technology	Target	SV-01	SV-02	SV-03	SV-04	SV-05	SV-06	SV-07	SV-08	SV-09	SV-10
			VOC - Alpha	VOC - Delta	VOC - BA.1	VOC - BA.2	VOC - BA.5.2.1	Low - 22/252	VOV – BA.5.5	20/146	22/252	21/368 (Ag)
1^{T1}	RT-qPCR (Direct)	S	-	-	-	-	-	-	-	-	-	-
1^{T2}		Orf1ab	-	-	-	-	-	-	-	-	-	-
2	RT-qPCR	Orf1ab, N	6.65	6.58	5.94	6.11	6.43	6.05	6.78	7.83	7.56	nt
3a	RT-qPCR	N1	7.53	7.56	7.02	7.40	7.56	6.78	8.21	8.60	8.77	7.31
3b		N2	7.31	7.69	7.30	7.59	7.79	6.85	8.44	8.83	8.92	7.50
3c		RdRp	7.27	6.36	6.63	6.99	7.41	6.73	7.77	8.17	8.28	7.00
4a	RT-dPCR	N2	6.63	6.80	6.35	6.51	6.83	5.79	7.44	7.75	7.77	6.37
4b		E	6.53	6.69	6.22	6.43	6.74	5.69	7.25	7.76	7.75	6.22
4c		Orf	6.45	6.53	6.05	6.34	6.66	5.69	7.17	7.69	7.69	5.65
5	RT-dPCR	N	7.01	7.13	6.66	6.93	7.26	6.16	7.90	8.14	8.23	6.75
6	RT-LAMP	N	4.85	5.46	5.30	5.20	5.54	4.62	6.14	6.55	6.46	nt
7a	TMA	ORF1ab (2 targets)	5.81	6.18	5.10	5.48	5.72	4.89	6.48	6.81	6.91	nt
7b	Real-time TMA	ORF1ab (2 targets)	6.37	6.29	5.78	6.18	6.34	5.52	7.07	7.60	7.59	nt
7c	RT-qPCR	ORF1ab (2 targets)	6.26	6.51	6.18	6.29	6.48	5.59	7.18	7.48	7.76	7.38
7d	RT-qPCR	ORF1ab (2 targets)	6.15	6.65	5.65	5.98	6.52	6.07	6.98	7.59	8.15	7.41
8	RT-qPCR	N1	6.03	6.17	5.52	5.92	6.12	5.16	7.04	7.40	7.61	7.61
9a	RT-qPCR	E	6.51	6.61	6.12	6.35	6.65	5.73	7.17	7.68	7.76	7.51
9b	RT-dPCR	E	6.40	6.59	6.09	6.28	6.63	5.65	7.14	7.66	7.73	7.47
10^{T1}	RT-qPCR	S	5.53	5.69	5.21	5.54	5.27	4.75	6.21	6.68	6.62	nt
10^{T2}		M	5.12	5.35	4.87	4.97	5.37	4.84	6.09	6.57	6.54	nt
11^{T1}	RT-qPCR	ORF1ab	6.30	6.30	5.90	6.30	6.42	5.56	6.90	7.31	7.31	6.30
11^{T2}		N	6.30	6.30	6.08	6.30	6.30	5.30	6.30	7.31	7.31	6.08

11[T3]		E	5.90	5.90	5.30	5.56	6.30	5.42	6.30	7.08	7.31	6.08
12a[T1]	RT-dPCR	ORF1ab	6.60	6.77	6.29	6.49	6.93	5.95	7.33	7.94	7.97	6.45
12a[T2]		N	6.72	6.90	6.43	6.58	6.99	5.98	7.44	7.92	7.96	6.44
12b[T1]	RT-qPCR	ORF1ab	5.91	5.77	4.95	5.64	6.06	5.27	6.62	6.60	7.14	6.12
12b[T2]		ORF1ab	5.83	5.72	5.00	5.54	6.12	5.23	6.49	6.57	6.85	6.10
13a[T1]	RT-qPCR	ORF1ab	5.97	6.41	5.52	5.84	6.33	5.57	6.80	7.52	7.52	nt
13a[T2]		E	6.30	6.10	5.77	5.73	6.10	5.22	6.45	7.40	7.47	nt
13b	RT-qPCR	ORF1a, ORF1ab	6.45	6.45	5.99	6.21	6.59	5.68	6.98	7.74	7.82	7.38
13c	RT-qPCR	ORF1ab, N	6.03	5.73	5.29	5.99	6.18	5.11	6.75	6.75	7.44	nt
14[T1]	RT-qPCR	ORF1ab	6.30	5.98	5.61	5.91	6.29	5.40	6.57	7.47	7.58	nt
14[T2]		E	6.03	5.97	5.79	5.76	6.09	5.27	6.62	7.23	7.27	nt
15[T1]	RT-qPCR	E	6.42	6.87	5.71	6.04	6.57	5.42	6.87	7.23	7.87	nt
15[T2]		S	6.15	6.10	5.87	6.04	6.32	5.83	6.71	7.05	7.57	nt
16[T1]	RT-qPCR	ORF1ab	6.01	6.35	5.78	6.30	6.35	5.78	6.53	6.18	6.18	nt
16[T2]		E	6.46	6.49	5.78	6.15	6.53	5.90	6.18	6.03	6.03	nt
17	RT-qPCR	N	7.00	7.12	6.64	6.85	7.18	6.17	7.90	8.09	8.18	6.52
18[T1]	RT-qPCR	ORF1ab	-	-	-	-	-	-	-	-	-	-
18[T2]		E	-	-	-	-	-	-	-	-	-	-
19a	RT-dPCR	N2	6.69	6.86	6.38	6.57	6.91	5.87	7.49	7.80	7.97	6.52
19b		E	6.59	6.73	6.23	6.44	6.81	5.80	7.28	7.76	7.89	6.30
*20[T1]	RT-qPCR	E	6.19	4.60	4.29	5.19	5.86	5.29	7.34	7.34	7.34	5.29
*20[T2]		N2	5.29	4.29	4.86	5.60	5.86	5.86	6.88	7.34	6.88	5.86
*21[T1]	qSTAR	nsp1 or ORF8	5.25	5.25	4.52	5.85	5.52	4.47	5.76	6.06	6.52	nt
21[T2]		nsp1 or ORF8	-	-	-	-	-	-	-	-	-	nt
22	RT-qPCR	RdRp	-	-	-	-	-	-	-	-	-	-

nt: not tested

Table 4. Summary of Laboratory Reported Mean Estimates and Inter-Laboratory Variation

	SV-01	SV-02	SV-03	SV-04	SV-05	SV-06	SV-07	SV-08	SV-09	SV-10
	VOC - Alpha	VOC - Delta	VOC - BA.1	VOC - BA.2	VOC - BA.5.2.1	Low - 22/252	VOC – BA.5.5	20/146	22/252	21/368 (Ag)
Quantitative										
Mean	6.73	6.81	6.37	6.62	6.94	5.98	7.50	7.93	8.02	6.81
SD	0.39	0.41	0.42	0.44	0.42	0.46	0.43	0.37	0.38	0.59
Min	6.03	6.17	5.52	5.92	6.12	5.16	6.98	7.40	7.61	5.65
Max	7.53	7.69	7.30	7.59	7.79	6.85	8.44	8.83	8.92	7.61
N	16	16	16	16	16	16	16	16	16	16
%GCV	146%	156%	165%	174%	162%	190%	168%	132%	139%	291%
Qualitative										
Mean	5.98	5.95	5.44	5.82	6.11	5.37	6.60	7.02	7.17	6.29
SD	0.45	0.61	0.49	0.37	0.38	0.43	0.37	0.51	0.54	0.69
Min	4.85	4.29	4.29	4.97	5.27	4.47	5.76	6.03	6.03	5.29
Max	6.65	6.87	6.18	6.30	6.57	6.07	7.34	7.83	8.15	7.41
N	25	25	25	25	25	25	25	25	25	9
%GCV	183%	311%	211%	135%	138%	168%	136%	224%	248%	387%
Combined										
Mean	6.27	6.29	5.80	6.13	6.44	5.61	6.95	7.38	7.50	6.62
SD	0.57	0.68	0.65	0.56	0.56	0.53	0.59	0.64	0.64	0.67
Min	4.85	4.29	4.29	4.97	5.27	4.47	5.76	6.03	6.03	5.29
Max	7.53	7.69	7.30	7.59	7.79	6.85	8.44	8.83	8.92	7.61
N	41	41	41	41	41	41	41	41	41	25
%GCV	268%	383%	347%	260%	266%	239%	289%	335%	334%	362%

SD: Standard deviation; N: number of laboratories; %GCV: percentage geometric coefficient of variation

Table 5. Potency Estimates of Study Samples Relative to the First WHO IS, 20/146 (SV-08)
Potency estimates determined based on the assigned potency of 7.70 Log_{10} IU/mL. Outliers are indicated by red highlight and underlined, datasets which were not suitable to perform this analysis are indicated (-), results are derived from 3 experiments (n=3), unless indicated (*: n=1)

Lab	Technology	Target	SV-01 VOC - Alpha	SV-02 VOC - Delta	SV-03 VOC - BA.1	SV-04 VOC - BA.2	SV-05 VOC - BA.5.2.1	SV-06 Low - 22/252	SV-07 VOC – BA.5.5	SV-09 22/252	SV-10 21/368 (Ag)
1^{T1}	RT-qPCR (Direct)	S	6.30	6.62	5.90	6.20	NP	NP	7.26	7.75	7.73
1^{T2}		Orf1ab	6.20	6.48	5.94	5.99	NP	NP	7.02	7.50	7.14
2	RT-qPCR	Orf1ab, N	6.61	6.73	6.25	6.41	6.70	5.74	7.25	7.87	nt
3a	RT-qPCR	N1	6.57	6.60	6.02	6.43	6.60	5.79	7.27	7.89	6.33
3b		N2	*6.04*	6.46	6.11	6.35	6.57	5.56	7.27	7.80	6.26
3c		RdRp	6.51	*5.83*	6.13	6.53	6.87	*5.92*	7.35	7.87	6.50
4a	RT-dPCR	N2	6.58	6.75	6.30	6.46	6.78	5.74	7.39	7.72	6.32
4b		E	6.48	6.63	6.16	6.38	6.68	5.63	7.19	7.69	6.16
4c		Orf	6.46	6.54	6.06	6.35	6.67	5.70	7.18	7.71	5.66
5	RT-dPCR	N	6.57	6.69	6.22	6.49	6.82	5.72	7.45	7.79	6.31
6	RT-LAMP	N	5.99	6.60	6.44	6.34	6.69	5.76	7.29	7.61	nt
7a	TMA	ORF1ab (2 targets)	6.70	7.07	6.00	6.38	6.61	5.78	7.38	7.80	nt
7b	Real-time TMA	ORF1ab (2 targets)	6.48	6.39	5.89	6.28	6.45	5.63	7.18	7.70	nt
7c	RT-qPCR	ORF1ab (2 targets)	6.42	6.62	6.10	6.44	6.62	5.77	7.31	7.90	7.60
7d	RT-qPCR	ORF1ab (2 targets)	6.46	6.56	6.13	6.38	6.68	5.88	7.19	7.88	7.51
8	RT-qPCR	N1	6.33	6.47	*5.82*	6.21	6.42	*5.45*	7.34	7.91	7.90
9a	RT-qPCR	E	6.53	6.63	6.14	6.37	6.67	5.75	7.19	7.78	7.53
9b	RT-dPCR	E	6.45	6.63	6.14	6.32	6.68	5.70	7.17	7.77	7.51
10^{T1}	RT-qPCR	S	6.55	6.70	6.23	6.55	6.28	5.77	7.23	7.63	nt
10^{T2}		M	6.25	6.48	6.00	6.10	6.50	5.97	7.22	7.67	nt
11^{T1}	RT-qPCR	ORF1ab	6.47	6.58	6.00	6.32	6.82	5.69	7.23	7.90	6.02

11[T2]		N	6.51	6.72	6.22	6.22	6.79	5.63	7.28	7.89	6.39
11[T3]		E	6.47	6.67	6.12	6.39	6.87	5.84	7.18	7.87	6.29
12a[T1]	RT-dPCR	ORF1ab	6.38	6.54	6.07	6.27	6.71	5.73	7.11	7.74	6.23
12a[T2]		N	6.49	6.67	6.21	6.35	6.76	5.75	7.22	7.74	6.21
12b[T1]	RT-qPCR	ORF1ab	*7.01*	6.87	6.05	6.74	7.16	*6.37*	*7.72*	*8.24*	7.22
12b[T2]		ORF1ab	6.96	6.85	6.13	6.67	7.25	*6.36*	7.62	7.98	7.23
13a[T1]	RT-qPCR	ORF1ab	6.15	6.59	5.70	6.02	6.51	5.75	6.98	7.70	nt
13a[T2]		E	6.60	6.40	6.07	6.03	6.40	5.52	6.76	7.77	nt
13b	RT-qPCR	ORF1a, ORF1ab	6.42	6.42	5.98	6.19	6.57	5.66	6.95	7.78	7.35
13c	RT-qPCR	ORF1ab, N	6.98	6.68	6.24	*6.94*	7.13	6.06	*7.70*	*8.39*	nt
14[T1]	RT-qPCR	ORF1ab	6.53	6.21	5.84	6.14	6.51	5.63	6.80	7.81	nt
14[T2]		E	6.50	6.44	6.26	6.23	6.56	5.74	7.09	7.74	nt
15[T1]	RT-qPCR	E	6.46	6.52	6.02	6.26	6.63	5.56	7.08	7.78	nt
15[T2]		S	6.40	6.51	6.02	6.27	6.61	5.72	7.11	7.78	nt
16[T1]	RT-qPCR	ORF1ab	6.33	6.36	5.65	6.02	6.40	5.77	7.17	7.70	nt
16[T2]		E	5.78	6.13	5.55	6.08	6.13	5.55	7.34	7.70	nt
17	RT-qPCR	N	6.63	6.71	6.27	6.42	6.79	5.75	7.51	7.78	6.16
18[T1]	RT-qPCR	ORF1ab	6.31	6.35	5.90	6.04	6.43	5.60	6.99	7.74	7.33
18[T2]		E	6.34	6.44	5.98	6.08	6.45	5.59	6.96	7.75	7.49
19a	RT-dPCR	N2	6.58	6.76	6.28	6.46	6.81	5.76	7.39	7.87	6.41
19b		E	6.53	6.68	6.17	6.38	6.76	5.74	7.22	7.84	6.25
*20[T1]	RT-qPCR	E	6.55	*4.96*	*4.64*	*5.55*	6.22	5.64	*7.70*	7.70	5.64
*20[T2]		N2	*5.64*	*4.64*	*5.22*	5.96	6.22	6.22	7.23	*7.23*	6.22
*21[T1]	qSTAR	nsp1 or ORF8	6.90	6.90	6.16	*7.50*	7.16	6.12	7.40	8.16	nt
21[T2]		nsp1 or ORF8	-	-	-	-	-	-	-	-	nt
22	RT-qPCR	RdRp	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP

nt: not tested; NP: non-parallel; NL: non-linear

Table 6. Summary of Mean Estimates and Inter-Laboratory Variation Relative to the First WHO IS, 20/146 (SV-08)

	SV-01	SV-02	SV-03	SV-04	SV-05	SV-06	SV-07	SV-09	SV-10
	VOC - Alpha	VOC - Delta	VOC - BA.1	VOC - BA.2	VOC - BA.5.2.1	Low - 22/252	VOC – BA.5.5	22/252	21/368 (Ag)
Quantitative									
Mean	6.50	6.61	6.15	6.37	6.70	5.71	7.26	7.79	6.57
SD	0.08	0.11	0.09	0.09	0.12	0.06	0.14	0.07	0.63
Min	6.33	6.42	5.98	6.19	6.42	5.56	6.95	7.69	5.66
Max	6.63	6.76	6.30	6.53	6.87	5.79	7.51	7.91	7.90
N	15	15	15	16	16	14	16	16	16
%GCV	21%	27%	24%	24%	31%	15%	38%	16%	330%
Qualitative									
Mean	6.45	6.57	6.03	6.25	6.62	5.76	7.17	7.78	6.91
SD	0.26	0.21	0.20	0.21	0.30	0.18	0.19	0.13	0.70
Min	5.78	6.13	5.55	5.96	6.13	5.52	6.76	7.50	5.64
Max	6.98	7.07	6.44	6.74	7.25	6.22	7.62	8.16	7.73
N	27	27	27	26	27	25	26	26	13
%GCV	83%	62%	58%	62%	98%	51%	54%	35%	397%
Combined									
Mean	6.47	6.59	6.07	6.30	6.65	5.74	7.21	7.78	6.72
SD	0.22	0.18	0.18	0.18	0.25	0.15	0.17	0.11	0.67
Min	5.78	6.13	5.55	5.96	6.13	5.52	6.76	7.50	5.64
Max	6.98	7.07	6.44	6.74	7.25	6.22	7.62	8.16	7.90
N	42	42	42	42	43	39	42	42	29
%GCV	65%	51%	51%	52%	76%	40%	49%	29%	370%

SD: Standard deviation; N: number of laboratories; %GCV: percentage geometric coefficient of variation

Table 7. Potency Estimates of Study Samples Relative to the Candidate Second WHO IS, 22/252 (SV-09)
Potency estimates determined based on an assigned potency of 7.80 Log_{10} IU/mL. Outliers are indicated by red highlight and underlined, datasets which were not suitable to perform this analysis are indicated (-), results are derived from 3 experiments (n=3), unless indicated (*: n=1)

Lab	Technology	Target	SV-01	SV-02	SV-03	SV-04	SV-05	SV-06	SV-07	SV-08	SV-10
			VOC - Alpha	VOC - Delta	VOC - BA.1	VOC - BA.2	VOC - BA.5.2.1	Low - 22/252	VOC – BA.5.5	20/146	21/368 (Ag)
1[T1]	RT-qPCR (Direct)	S	6.35	6.67	5.96	6.26	NP	NP	7.31	7.75	7.78
1[T2]		Orf1ab	6.50	6.77	6.24	6.28	NP	NP	7.32	8.00	7.44
2	RT-qPCR	Orf1ab, N	6.54	6.66	6.19	6.34	6.63	5.67	7.18	7.63	nt
3a	RT-qPCR	N1	6.48	6.51	5.94	6.34	6.52	5.70	7.18	7.61	6.25
3b		N2	6.04	6.46	6.11	6.34	6.57	5.56	7.27	7.70	6.25
3c		RdRp	6.44	5.76	6.06	6.46	6.80	5.85	7.28	7.63	6.43
4a	RT-dPCR	N2	6.66	6.83	6.38	6.54	6.86	5.82	7.47	7.78	6.40
4b		E	6.58	6.74	6.27	6.48	6.78	5.74	7.29	7.81	6.27
4c		Orf	6.56	6.63	6.15	6.44	6.76	5.79	7.27	7.79	5.75
5	RT-dPCR	N	6.58	6.70	6.23	6.50	6.83	5.73	7.47	7.71	6.32
6	RT-LAMP	N	6.18	6.80	6.64	6.54	6.88	5.96	7.48	7.89	nt
7a	TMA	ORF1ab (2 targets)	6.70	7.07	5.99	6.37	6.61	5.78	7.37	7.70	nt
7b	Real-time TMA	ORF1ab (2 targets)	6.58	6.49	5.99	6.38	6.55	5.73	7.28	7.80	nt
7c	RT-qPCR	ORF1ab (2 targets)	6.32	6.52	6.00	6.35	6.52	5.67	7.21	7.60	7.51
7d	RT-qPCR	ORF1ab (2 targets)	6.38	6.48	6.05	6.30	6.60	5.80	7.11	7.62	7.43
8	RT-qPCR	N1	6.22	6.36	5.71	6.11	6.31	5.35	7.23	7.59	7.80
9a	RT-qPCR	E	6.55	6.65	6.16	6.39	6.69	5.77	7.21	7.72	7.55
9b	RT-dPCR	E	6.48	6.66	6.17	6.35	6.71	5.73	7.20	7.73	7.54
10[T1]	RT-qPCR	S	6.71	6.87	6.39	6.72	6.45	5.93	7.39	7.87	nt
10[T2]		M	6.39	6.61	6.13	6.23	6.63	6.10	7.36	7.83	nt
11[T1]	RT-qPCR	ORF1ab	6.36	6.48	5.90	6.21	6.72	5.58	7.12	7.60	5.92
11[T2]		N	6.42	6.63	6.13	6.13	6.70	5.55	7.19	7.61	6.30

11[T3]		E	6.40	6.60	6.05	6.32	6.80	5.77	7.12	7.63	6.22
12a[T1]	RT-dPCR	ORF1ab	6.44	6.60	6.13	6.32	6.77	5.79	7.17	7.76	6.29
12a[T2]		N	6.55	6.74	6.27	6.42	6.83	5.81	7.28	7.76	6.28
12b[T1]	RT-qPCR	ORF1ab	6.57	6.42	5.60	6.30	6.71	5.93	7.28	*7.26*	6.78
12b[T2]		ORF1ab	6.78	6.67	5.95	6.49	*7.07*	6.18	7.44	7.52	7.05
13a[T1]	RT-qPCR	ORF1ab	6.25	6.69	5.80	6.12	6.61	5.85	7.08	7.80	nt
13a[T2]		E	6.64	6.43	6.10	6.06	6.43	5.56	6.79	7.73	nt
13b	RT-qPCR	ORF1a, ORF1ab	6.44	6.44	6.00	6.21	6.59	5.68	*6.98*	7.72	7.37
13c	RT-qPCR	ORF1ab, N	6.39	6.09	5.65	6.35	6.54	5.47	*7.11*	7.11	nt
14[T1]	RT-qPCR	ORF1ab	6.52	6.20	5.83	6.13	6.50	5.62	6.79	7.69	nt
14[T2]		E	6.56	6.50	6.32	6.28	6.62	5.80	7.15	7.76	nt
15[T1]	RT-qPCR	E	6.48	6.54	6.04	6.28	6.65	5.58	7.10	7.72	nt
15[T2]		S	6.41	6.53	6.04	6.28	6.63	5.74	7.13	7.72	nt
16[T1]	RT-qPCR	ORF1ab	6.43	6.46	5.75	6.12	6.50	5.87	7.27	7.80	nt
16[T2]		E	*5.88*	6.23	5.65	6.18	6.23	5.65	7.44	7.80	nt
17	RT-qPCR	N	6.64	6.73	6.29	6.43	6.81	5.77	*7.52*	7.72	6.18
18[T1]	RT-qPCR	ORF1ab	6.37	6.41	5.97	6.11	6.50	5.67	7.05	7.76	7.40
18[T2]		E	6.39	6.48	6.03	6.13	6.50	5.64	7.00	7.75	7.54
19a	RT-dPCR	N2	6.51	6.69	6.21	6.40	6.74	5.69	7.32	7.63	6.34
19b		E	6.49	6.64	6.14	6.35	6.72	5.71	7.19	7.66	6.21
*20[T1]	RT-qPCR	E	6.65	*5.06*	*4.74*	*5.65*	6.32	5.74	*7.80*	7.80	5.74
*20[T2]		N2	6.21	*5.21*	5.78	6.53	6.78	*6.78*	*7.80*	*8.27*	6.78
*21[T1]	qSTAR	nsp1 or ORF8	6.53	6.53	5.80	*7.13*	6.80	5.75	7.04	*7.34*	nt
21[T2]		nsp1 or ORF8	-	-	-	-	-	-	-	-	nt
22	RT-qPCR	RdRp	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP	NL/NP

nt: not tested; NP: non-parallel; NL: non-linear

Table 8. Summary of Mean Estimates and Inter-Laboratory Variation Relative to Candidate Second WHO IS, 22/252 (SV-09)

	SV-01	SV-02	SV-03	SV-04	SV-05	SV-06	SV-07	SV-08	SV-10
	VOC - Alpha	VOC - Delta	VOC - BA.1	VOC - BA.2	VOC - BA.5.2.1	Low - 22/252	VOC – BA.5.5	20/146	21/368 (Ag)
Quantitative									
Mean	6.53	6.63	6.17	6.40	6.73	5.74	7.27	7.71	6.58
SD	0.07	0.13	0.12	0.08	0.10	0.07	0.09	0.07	0.61
Min	6.44	6.36	5.94	6.21	6.52	5.56	7.17	7.59	5.75
Max	6.66	6.83	6.38	6.54	6.86	5.85	7.47	7.81	7.80
N	14	15	15	15	15	15	14	16	16
%GCV	18%	35%	30%	21%	26%	18%	24%	16%	309%
Qualitative									
Mean	6.47	6.53	5.98	6.27	6.59	5.75	7.19	7.74	6.91
SD	0.15	0.18	0.20	0.13	0.15	0.17	0.18	0.11	0.68
Min	6.18	6.09	5.60	6.06	6.23	5.47	6.79	7.52	5.74
Max	6.78	6.87	6.39	6.54	6.88	6.18	7.48	8.00	7.78
N	28	26	27	26	26	26	27	25	13
%GCV	41%	51%	58%	35%	41%	48%	50%	28%	379%
Combined									
Mean	6.49	6.56	6.04	6.32	6.64	5.75	7.22	7.72	6.73
SD	0.13	0.17	0.20	0.13	0.15	0.14	0.16	0.09	0.65
Min	6.18	6.09	5.60	6.06	6.23	5.47	6.79	7.52	5.74
Max	6.78	6.87	6.39	6.54	6.88	6.18	7.48	8.00	7.80
N	42	41	42	41	41	41	41	41	29
%GCV	35%	47%	57%	35%	41%	38%	44%	24%	351%

SD: Standard deviation; N: number of laboratories; %GCV: percentage geometric coefficient of variation

Table 9. Production Summary of the candidate Second IS, 22/252
Microbiological tests for bacterial and mould/yeast colony count returned negative

NIBSC Code	22/252
Product Description	Inactivated SARS-CoV-2 candidate Second WHO IS
Dates of processing	Filling; 24Nov22 Lyophilisation; 24-27Nov22 Sealing; 27Nov22
Presentation	Freeze-dried preparation in 2.5mL DIN Ampoule
No. vials filled	5169
Mean fill weight (g)	0.52 (n = 183)
CV of fill mass (%)	0.35
Mean residual moisture (%)	0.61 (n = 12)
CV of residual moisture (%)	51.30
Mean of oxygen content (%)	0.40 (n = 12)
CV of oxygen content (%)	30.49

n = number of samples tested

Table 10. Thermal Stability Assessment of candidate Second WHO IS, 22/252
Potency estimates of 22/252 retrieved from storage at increasing time intervals and temperatures relative to the baseline sample stored at -20°C (assigned a potency of 7.8 Log_{10} IU/mL), quantified by in-house SARS-CoV-2 RT-qPCR with measures taken from two vials and experiments performed in duplicate. All values are expressed in Log_{10} IU/mL.

Timepoint	Temperature (°C)	Relative potency			Difference from -20°C baseline
		95% LCL	Mean	95% UCL	
2 weeks	4	7.83	7.87	7.92	0.07
	20	7.81	7.86	7.90	0.06
	37	7.78	7.82	7.87	0.02
	45	7.75	7.80	7.85	0.00
1 month	4	7.69	7.82	7.94	0.02
	20	7.62	7.82	8.02	0.02
	37	7.74	7.80	7.85	0.00
	45	7.71	7.77	7.82	-0.03
3 months	4	7.60	7.79	7.99	-0.01
	20	7.65	7.80	7.96	0.00
	37	7.75	7.78	7.81	-0.02
	45	7.68	7.76	7.84	-0.04
6 months	4	7.65	7.69	7.74	-0.11
	20	7.74	7.79	7.83	-0.01
	37	7.70	7.76	7.82	-0.04
	45	7.59	7.63	7.68	-0.17

LCL: lower confidence level; UCL: upper confidence level

Table 11. Predicted Long-Term Stability of candidate Second WHO IS, 22/252
Calculations made using the Arrhenius model [10], based on potency estimates from samples stored at -20, 4, 20, 37 and 45°C for 3 months and at -20, 20 and 37°C for 6 months.

Temp (°C)	K	S.E. (K)	% loss per year	95% UCL
-20	0.0007	0.00493	0.070	2.503
4	0.00948	0.03601	0.944	17.265
20	0.04232	0.08256	4.144	36.563
37	0.17524	0.08328	16.074	44.658

K: degradation rate constant; S.E.: standard error of K; UCL: upper confidence level

Figure 1. Box and Whisker Plots of the Laboratory Reported Potency Estimates of Study Samples

Plots represent the overall mean and variation in data reported (Table 3), separated by qualitative (Qual) and quantitative (Quan) methods. Individual data points are outliers, further than 1.5 times the interquartile range from the lower and upper quartiles

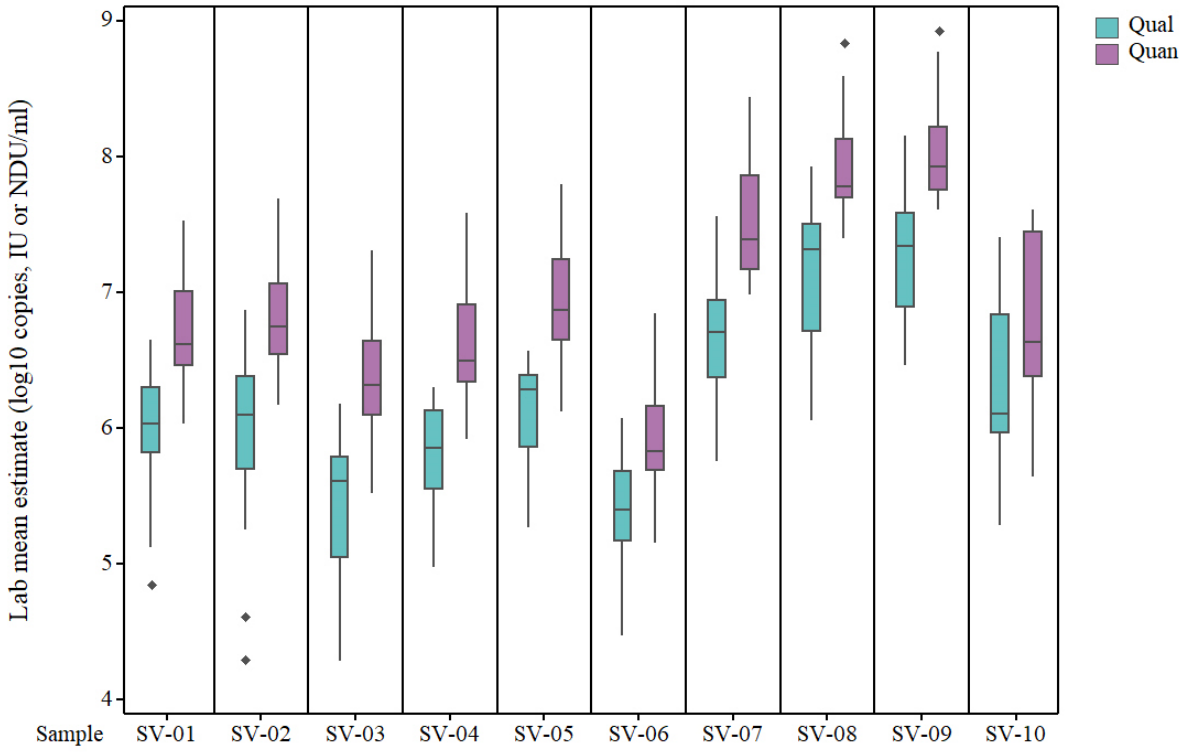


Figure 2. Reported Potency Estimates for Quantitative Methods Separated by PCR Technology

Bars represent the spread of data (min to max), with lines at the mean, of the reported potency estimates across the study samples based on real time PCR (n = 7) or digital PCR (n = 9) technology (data taken from Table 3)

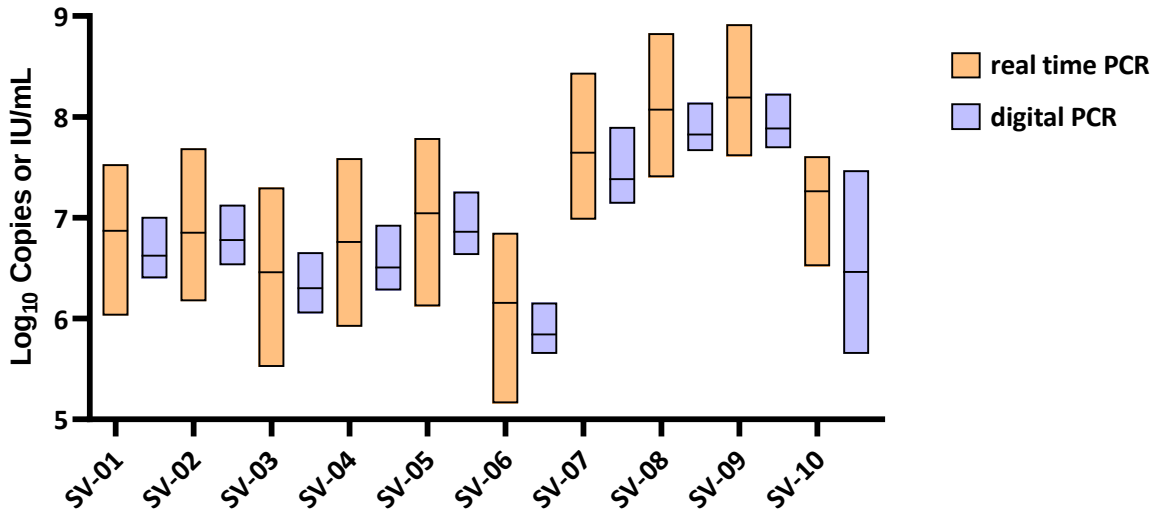


Figure 3. Box and Whisker Plots of the Potency Estimates Relative to the First WHO IS, 20/146 (SV-08)

Plots represent the overall mean and variation in data reported (Table 5) relative to the First IS, 20/146 (7.70 Log10 IU/mL), separated by qualitative (Qual) and quantitative (Quan) methods. Individual data points are outliers, further than 1.5 times the interquartile range from the lower and upper quartiles.

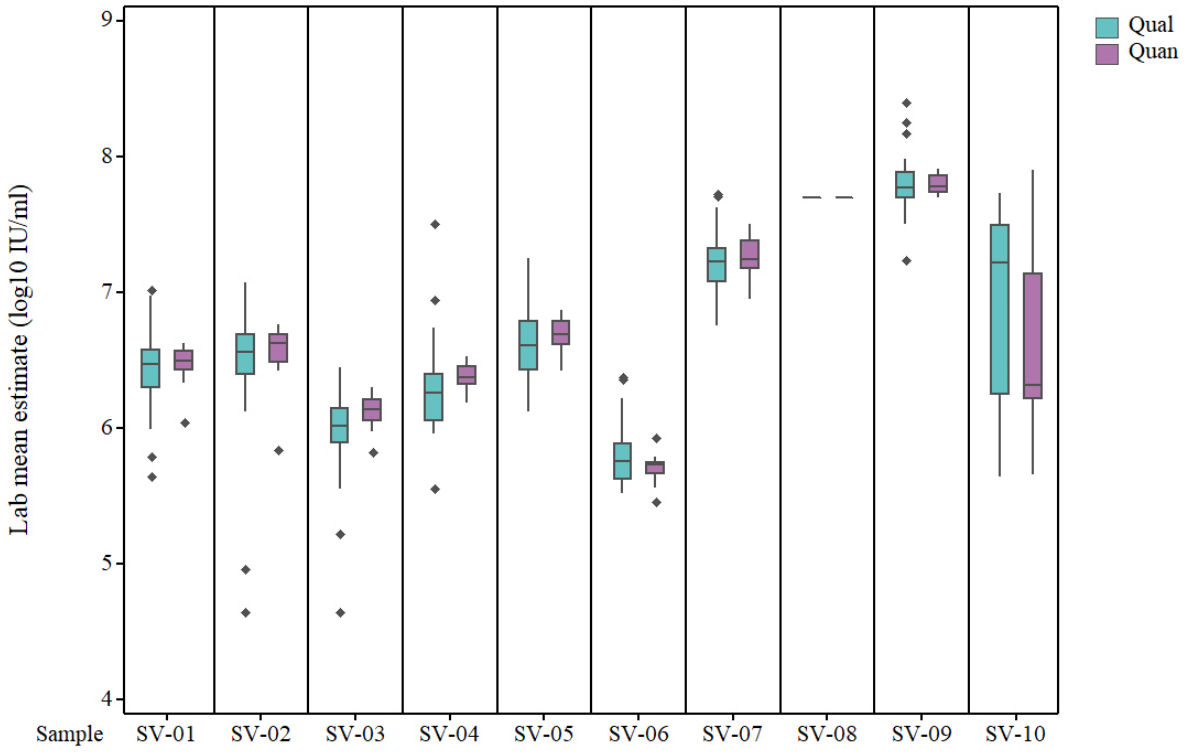
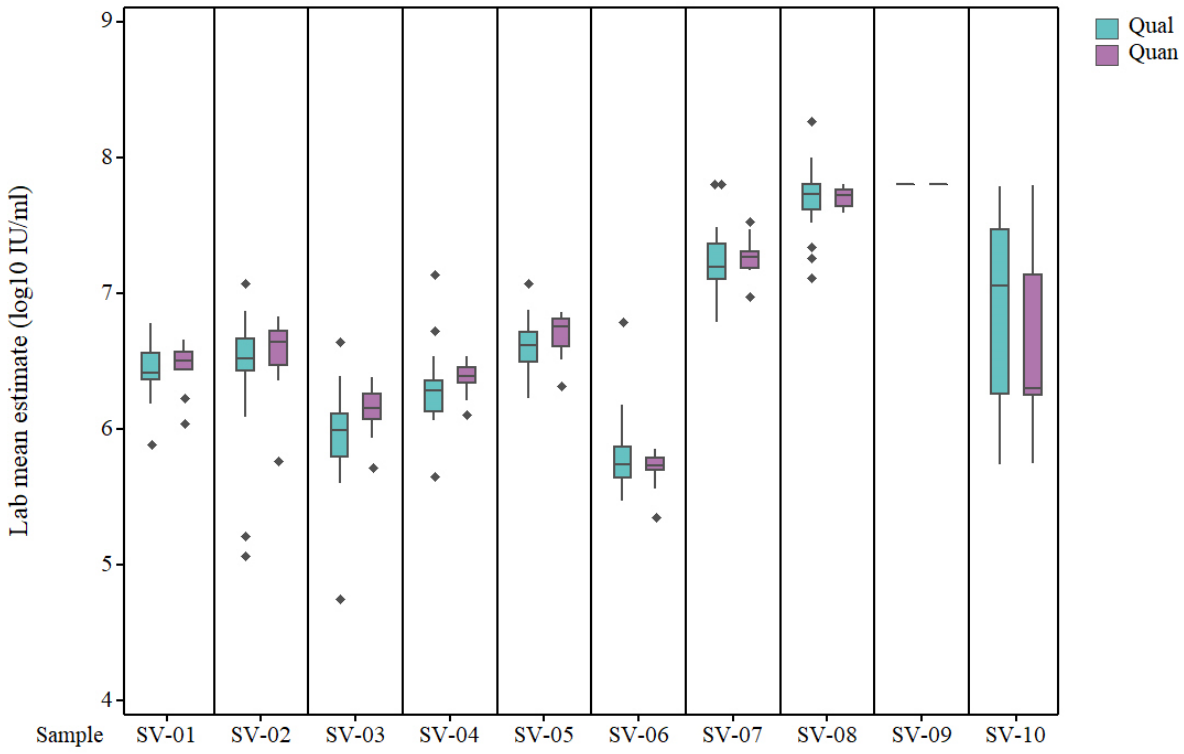


Figure 4. Box and Whisker Plots of the Potency Estimates Relative to the Candidate Second WHO IS, 22/252 (SV-09)

Plots represent the overall mean and variation in data reported (Table 5) relative to the candidate Second IS, 22/252 (assigned potency of 7.80 Log10 IU/mL), separated by qualitative (Qual) and quantitative (Quan) methods. Individual data points are outliers, further than 1.5 times the interquartile range from the lower and upper quartiles.



Appendix 1

Collaborative study participants

(in alphabetical order by organization)

Participant	Organization	Country
Angeles Jurado-Jimenez	Altona Diagnostics GmbH	Germany
Elizabeth Amiott, Chad Russell	BioMérieux (BioFire) Diagnostics	USA
David McGivern, Rafaelle Gusmao, Sakthivel Subramaniam	Center for Biologics Evaluation and Research (CBER), US FDA	USA
Nobuhiro Takemae, Doan Hai Yen	Center for Emergency Preparedness and Response, National Institute of Infectious Diseases (NIID)	Japan
Blenda Hedin, Pia Larssen, Malin Ewers, Rebecka Bergsten	Cepheid AB	Sweden
Sihong Xu, Tingting Ma	China National Institutes for Food and Drug Control (NIFDC)	China
Alessandra Gentile, Chiara Lanceni, Chiara Montrasio, Elena D'Agostini	DiaSorin Molecular LLC	Italy
Benedito Eduardo Correia, Christian Harz	Fast Track Diagnostics S.à r.l – A Siemens Healthineers Company	Luxemburg
Analee Williams, Miranda Ng, Jimmykim Pham	Hologic, Inc.	USA
Il-Hwan Kim, Seil Kim, Hee Min Yoo, Changwoo Park, Dong U Ju	Korea Research Institute of Standards and Science (KRISS)	South Korea
Verena Breuer, Daniel Hinze, Jana Liebing	LAMPseq Diagnostics GmbH	Germany
Ana Cubas Atienzar, Jacob Parkes	Liverpool School of Tropical Medicine	UK
Thuy Nguyen, Melissa Obtera, Jesse Braun	LumiraDx Ltd.	USA

Eungtae Lee, Young-Hyeon Jeon, Eung-Tae Lee, Ha-Yeon Lee, Joon-Ho Eom, Chang-Won Park	National Institute of Food and Drug Safety Evaluation (NIFDS), Ministry of Food and Drug Safety (MFDS)	South Korea
Denise O'Sullivan	National Measurement Laboratory, LGC	UK
Julia Kress, Eva Zojer-Fuchs	Paul-Ehrlich-Institut, Molecular Virology	Germany
Nathan Feirer, Subhanjan Mondal	Promega Corporation	USA
Daniel Jarem, Alison Kuchta, Sandro Sigrist	Roche Diagnostics Inc	Switzerland
Daniel Jarem, Alison Kuchta, Elissa Robbins, Diane Romo	Roche Molecular Systems	USA
Walter Zhang	Shanghai ZJ Bio-Tech Co., Ltd. ("Liferiver")	China
Po-Lin Lin, Po-Chih Wu	Taiwan Food and Drug Administration (TFDA)	Taiwan
Megan Cleveland, Peter Vallone	U.S. National Institute of Standards and Technology (NIST)	USA

Appendix 2

Summary of the variant call for the candidate Second WHO IS, 22/252

Libraries were sequenced on the Illumina MiSeq and variants were called against the Wuhan-1 reference sequence (Genbank accession number NC_045512.2) using ivar and lofreq variant callers as part of a bioinformatic pipeline developed in-house. Mutations with a proportion >5% of the population are listed.

Position (NC_045512.2)	Ref	Alt	Depth	SNP/indel	Syn/NonSyn	Proportion	Gene
19065	T	C	37464	SNP	Syn	0.9992	ORF1ab
22303	T	G	113605	SNP	Ser-->Arg	0.9995	S
26144	G	T	28965	SNP	Gly-->Val	0.999	ORF3a
29749	ACGATCGAGTG	A	17751	deletion	N/A	0.9834	ORF10-CHR_END

Appendix 3

Protocol for the Inactivation of SARS-CoV-2 by Acid-Heat Treatment

MATERIALS

- SARS-CoV-2 harvest within cell culture supernatant containing phenol-red
- 99% Acetic Acid Glacial (VWR: #20104.334)
- 2M NaOH sterile filtered
- Phosphate buffered saline (PBS)-A
- 0.45µM filter unit (Whatman: #10462100)
- Amicon® Ultra-15 50kDa centrifugal filter column (SigmaAldrich: UFC9050)
- Sterile syringe 10, 20 or 50mL
- 15 or 50mL Falcon tube
- 500 mL plastic screw-cap flask
- 60°C water bath
- 2x water bath thermometers
- Centrifuge capable of rotation at 4000 *xg*

PROCEDURE

Step 1 - Acid Inactivation:

1.1. Prepare the lab according to CoP and place within the microbiological safety cabinet (MSC) all the consumables needed

1.2. Collect the SARS-CoV-2 viral stock within a secondary container from storage

1.3. Place virus stock in MSC on industrial methylated spirit (IMS) 70%-soaked tissue to defrost and equilibrate to ambient temperature.

1.4. If required, aliquot SARS-CoV-2 viral stock(s) into a larger vessel for acid-inactivation (e.g. 500 mL screw-cap flask).

It is recommended to acid-treat the full volume of material as a single batch

1.5. Add 3% v/v Acetic Acid to the SARS-CoV-2 stock and gently mix (e.g. for 35 mL culture, add 1.05 mL acetic acid)

Addition of acetic acid will lower the pH to 3-4 which is indicated by a colour change from red to yellow of the phenol-red present within the culture media

1.6. Incubate for 15 minutes at ambient temperature.

1.7. Neutralise the acid by addition of 2M NaOH, dropwise while mixing, until the phenol indicator colour is restored to red representing pH 7.4 (e.g. to neutralise a volume of 36 mL requires addition of 8.5-9mL of 2M NaOH)

Step 2 – Heat Inactivation:

2.1. Place water bath within the MSC and add the required volume of water

It is important to record the volume of water added to the water bath, to allow the correct volume of disinfectant to be added upon completion of work

2.2. Set the water bath to 60 ± 2°C and monitor temperature with an external thermometer

2.3. Allow the temperature to stabilise for at least 30 minutes prior to use

2.4. Transfer the acid inactivated SARS-CoV-2 stock to a 50 mL falcon tube, if not already.

Do not exceed a total volume of 30 mL

The volume of stock should not exceed 30 mL to allow it to be submerged below the water level in the water bath

2.5. Prepare a temperature control 50 mL falcon tube with the equivalent volume of water/untreated media at ambient temperature, to be able to monitor the temperature within the tube

2.6. Transfer samples and the temperature control to the 60°C water bath and ensure the virus stock is submerged below the water line for even heat dispersion

2.7. Place the temperature probe within the temperature control tube and monitor until the 60°C inactivation temperature is reached (approx. 10 minutes)

2.8. Incubate for 1 hour, monitoring the temperature at 15-minute intervals

2.9. Following incubation, remove samples from the water bath and allow to equilibrate back to ambient temperature while preparing for clarification steps

Step 3 –Clarification:

It is suggested to clarify the inactivated SARS-CoV-2 culture to remove protein aggregates formed during inactivation and to limit the presence of inhibitory chemicals which may interfere with the downstream intended use (e.g. added to cells for validation of the inactivation)

3.1. Carefully decant the inactivated culture to a clean 50 mL falcon tube, avoiding the transfer of liquid vapour droplets within the lid

3.2. Transfer falcon tubes to centrifuge buckets within the MSC.

Ensure tubes are balanced by volume or weight and decontaminate with 70%IMS before removal from the MSC

3.3. Spin at 4000 rpm (3180 xg) for 20 minutes at ambient temperature

3.4. Transfer buckets back to the MSC and remove tubes

3.5. Carefully decant the sample to a clean 50 mL falcon tube, not disrupting the protein pellet

3.6. Repeat the centrifugation steps 3.2. – 3.4., spinning at 4000 rpm (3180 xg) for 20 minutes at ambient temperature

3.7. Without disrupting the pellet, proceed to transfer the inactivated culture to a sterile syringe and pass through a 0.45 μm filter

3.8. Prepare appropriate aliquots of clarified material for storage in a labelled box at 4°C (short-term) or -80°C (long term)

Step 4 –Purification:

Where required, the inactivated SARS-CoV-2 culture can be purified via a buffer exchange to formulate within a matrix appropriate for downstream use (e.g. universal buffer)

4.1. Before proceeding, it is important that clarification of the inactivated culture has been performed following steps 3.1. – 3.8.

Purification is challenging without prior removal of protein aggregates, which block the centrifugal column

4.2. Apply the inactivated culture to an Amicon® Ultra-15 50kDa centrifugal filter tube

4.3. Transfer tubes to appropriate centrifuge buckets

4.4. Following manufacturer’s instructions, spin at 4000 xg for 20 minutes at ambient temperature

4.5. Remove tubes and check residual volume has reached approximately 200μL

4.6. If not, continue to spin at 4000 xg for 10-minute intervals

4.7. Once complete, wash the column by applying an equal volume of PBS-A to the column as added at Step 4.1.

4.8. Repeat centrifugation steps 4.3. – 4.5.

4.9. Repeat wash steps 4.6. – 4.7.

4.10. Once complete, reconstitute and remove from the column into desired matrix (e.g. universal buffer)

Appendix 4

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STUDY PROTOCOL

Collaborative Study CS714
Second WHO International Standard for SARS-CoV-2 RNA

Study Background and Aims

During the October 2022 meeting of the WHO Expert Committee on Biological Standardization (ECBS), a proposal to replace the First WHO International Standard for SARS-CoV-2 RNA (NIBSC product code 20/146) was endorsed. The Standard has supported the development and calibration of molecular assays detecting SARS-CoV-2 RNA to the International Unit and there is a continuing high demand with rapidly depleting stocks. The timely establishment of a replacement Standard will provide continuity in availability.

This multi-centre International collaborative study will evaluate a candidate replacement preparation to serve as the Second WHO International Standard for SARS-CoV-2 RNA. The study will follow published WHO guidelines [1] and be submitted for formal establishment by the WHO ECBS.

The aims of this study are to:

- Evaluate the candidate replacement's potency/readout, in parallel to the First WHO International Standard, in a range of typical assays performed in different laboratories.
- Characterise the candidate's reactivity/specificity in different assay systems.
- Assess commutability, i.e. establish the extent to which the candidate is suitable to serve as a standard for a variety of different samples/strains.
- Propose a unitage for the Second WHO International Standard that will ensure continuity in the use of the International Unit.

Study Samples

All study samples are provided coded and blinded to participants (Table 1.). Each participant is provided with 4 study sample panels to allow for a preliminary assay to determine optimal dilution range, followed by 3 independent tests per method.

The samples comprise inactivated SARS-CoV-2 isolates, which includes pre-Variant of Concern (VOC), Alpha, Delta and Omicron variants. Inactivation was undertaken via validated procedures of either: treatment with 3% acetic acid followed by heat treatment at 60°C for 60 minutes, heat treatment at 65°C for 30 minutes, or incubation with 0.01% formaldehyde at 37°C for 12 days. In all cases, approximately 10% of the batch was tested *in vitro* to verify that no infectious virus could be detected.

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Table 1. CS714 Study Sample Panel.

Except for samples SV-07 and SV-10, all study samples are formulated within a background of human genomic DNA.

Sample Code	Formulation	Volume (mL)	Vial	Storage Temperature
SV-01	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-02	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-03	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-04	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-05	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-06	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-07	Liquid frozen (UB)	0.5	Screw Cap Vial	-80°C
SV-08	Freeze-dried (UB)	0.5	DIN Ampoule	-20°C
SV-09	Freeze-dried (UB)	0.5	DIN Ampoule	-20°C
SV-10*	Freeze-dried (UTM)	0.25	DIN Ampoule	-20°C

UB: Universal Buffer (10 mM Tris-HCl (pH 7.4), 0.5% human serum albumin and 1% D-(+)- Trehalose); UTM: Copan UTM® Universal Transport Medium™

***Not provided for testing to labs performing qualitative assays with a +/- results readout**

CAUTION: These preparations are not for administration to humans or animals in the human food chain. As with all materials of biological origin, the material should be regarded as potentially hazardous to health.

Study Protocol

As per the Instructions for Use (IFU), freeze-dried samples SV-08 and SV-09 require reconstitution in 0.5mL of sterile PBS and sample SV-10 should be reconstituted in 0.25mL of ultra-pure H_2O. Allow 20 minutes for complete dissolution and mix gently. All other samples should be thawed at room temperature prior to assay.

ALL THE STUDY SAMPLES REQUIRE EXTRACTION PRIOR TO TESTING

Participants are requested to:

- Perform 3 independent tests on different days or with different operators.
- Use a fresh set of samples for each independent test, ideally testing all samples (SV-01 to SV-10) within the same assay run.
- Test samples at least in duplicate.
- Prepare dilutions of the samples in PBS, or a matrix routinely used for the assay system/extraction platform within your laboratory, according to the guidelines for quantitative or qualitative methods provided below.

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Quantitative Methods

For the purpose of this study quantitative methods includes assays reporting numerical readouts in copies or International Units as well as those reporting Ct values with good linearity.

- Samples SV-01 to SV-10 should be tested at a minimum of 4 serial 10-fold dilutions and it is suggested to start testing from a 1/10 dilution (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). At least two of the points should fall within the linear range of the assay.
- It is recommended to test all samples from the same starting dilution. Testing of samples SV-01 to SV-07 and SV-10 should include a minimum of 2 points which fall within the standard curve (potency range) of samples SV-08 and SV-09.
- A set of samples is provided which can be used for a preliminary assay to determine the potency range of samples within your method.

Qualitative Methods

This includes assays reporting a +/- readout or numerical values (Ct or alternative units) which do not provide good linearity. Sample SV-10 should be omitted from testing for methods falling within this category.

- Samples SV-01 to SV-09 should be tested at dilutions which span the endpoint of the assay, including at least 2 positive and 2 negative points. If practical, it is recommended to test 3-fold dilutions of the samples.
- A set of samples is provided for a preliminary assay to determine the endpoint within your method. For this purpose, it is suggested to test up to five 10-fold dilutions starting from 1/1000 (e.g. 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}).

Results Reporting

An Excel reporting sheet is provided so that all essential information can be recorded, including details of test method and the raw data obtained from each assay. The use of the reporting sheet facilitates the analysis and interpretation of results.

- Use the Excel reporting sheet to record the raw assay readout for each dilution tested (e.g. Ct, copy number, +/- etc.). Ensure that the dilutions tested are correctly recorded. Our statistician will use the raw data readouts to perform statistical analysis.
- Provide the 'Result' as per analysis in your laboratory (e.g. copies/mL , +/- etc.).
- Where multiple methods have been used or an assay provides a readout against multiple targets, complete one reporting sheet per method/target.
- Record in the Excel reporting sheet any deviations from the study protocol and complete all fields requesting additional method details.

Deadline for return of results is by the end of April 2023

All completed results spreadsheets should be returned electronically to: emma.bentley@nibsc.org

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Data Analysis

The confidentiality of each laboratory is assured, with each dataset being blind coded. Analysis of the study will assess the potencies of each material relative to each other, and their performance within the different assay methods.

A draft study report will be sent to participants for comment. The report will include data analysis, proposed conclusions and recommendations on the selection, use and unitage of the candidate Second WHO IS for SARS-CoV-2 RNA. Participants' comments will be included in the report prior to submission to the WHO ECBS in July 2023. Study participants will be notified of the outcome of the study after the WHO ECBS meeting due to be held in October 2023.

References

[1] WHO, Recommendations for the preparation, characterization and establishment of international and other biological reference standards. WHO Technical Report Series, No. 932., in Expert Committee on Biological Standardization. 2006.

Participation in the collaborative study is conducted under the following conditions

- The study samples have been prepared from materials provided by donors and therefore must be treated as proprietary. The materials must not be used for any other purpose other than for this study without prior consent of the MHRA study organiser;
- The materials provided must not be shared with anyone outside of the study;
- The materials must not be used for application in human subjects or animals in the human food chain in any manner or form;
- There must be no attempt to reverse engineer, ascertain the chemical structure of, sequence or modify, or make derivatives of any of the materials;
- Participants accept responsibility for safe handling and disposal of the materials provided in accordance with the local regulations in their organization/country.
- Data obtained through testing of the materials must not be published or cited before the formal establishment of the standard by the World Health Organization and without the express permission of the MHRA study organiser.

MHRA, as the Collaborative Study coordinator, notes that:

- It is normal practice to acknowledge all participants as contributors of data rather than co-authors in publications;
- Data published from participating labs will be anonymised;
- Participation of this study is at the participant's discretion and does not include remuneration costs;
- Prior to the establishment of the standard, MHRA reserves the right to disclose specific information about the use of the material(s), without acknowledgement of the study participants;
- Participants will receive a copy of the report of the study with proposed conclusions and recommendations for comment before it is further distributed.

Appendix 5

Proposed Instructions For Use

WHO International Standard
Second WHO International Standard for SARS-CoV-2 RNA
NIBSC code: 22/252
Instructions for use

1. INTENDED USE

The Second WHO International Standard for SARS-CoV-2 RNA for Nucleic acid Amplification Technique (NAT)-based assays consists of acid-heat inactivated BetaCoV/Australia/VIC01/2020 isolate of SARS-CoV-2. The preparation has been evaluated in a WHO International Collaborative study [1]. The intended use of the International Standard is for the calibration and harmonisation of NAT-based assays for the detection of SARS-CoV-2 RNA.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The assigned potency of the WHO International Standard for SARS-CoV-2 RNA for NAT-based assays is 7.50 Log10 IU/ampoule. After reconstitution in 0.5mL of molecular grade water or PBS, the final concentration of the preparation is 7.80 Log10 IU/mL.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial of 22/252 contains 0.5 mL of lyophilised, non-infectious, BetaCoV/Australia/VIC01/2020 isolate of SARS-CoV-2. The virus has been inactivated by treatment with acetic acid, followed by 1 hour incubation at 60oC and validated for inactivation by serial blind passage on permissive cells, with full details provided within the study report [1]. The material is formulated in universal buffer comprising 10 mM Tris-HCl (pH 7.4), 0.5% human serum albumin and 1% D-(+)- Trehalose dehydrate and contains a background of 1x10^5 copies/mL of human genomic DNA. Variants were called against

the Wuhan-1 reference sequence (Genbank accession number NC_045512.2) using ivar and lofreq variant callers as part of a bioinformatic pipeline developed in-house [1]. Mutations with a proportion >5% of the population are listed:

Position (NC_045512.2)	Ref	Alt	Depth	SNP/indel	Syn/NonSyn	Proportion	Gene
19065	T	C	37464	SNP	Syn	0.9992	ORF1ab
22303	T	G	113605	SNP	Ser-->Arg	0.9995	S
26144	G	T	28965	SNP	Gly-->Val	0.999	ORF3a
29749	ACGATCGAGTG	A	17751	deletion	N/A	0.9834	ORF10-CHR_END

5. STORAGE

The ampoules should be stored at -20°C or below until use.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Various types of ampoule breaker are available commercially. To open the ampoule, tap the ampoule gently to collect material at the bottom (labelled) end and follow manufactures instructions provided with the ampoule breaker.

7. USE OF MATERIAL

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

The material should be reconstituted in 0.5 mL of molecular grade water or PBS. Following addition, the ampoule should be left at ambient temperature for 20 minutes and then mixed thoroughly, avoiding generation of excess foam. Once reconstituted, 22/252 should be diluted in the matrix appropriate to the material/assay being calibrated.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities and they should be stored on receipt as indicated on the label. 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.

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

9. REFERENCES

[1] Bentley et al., Collaborative Study for the Establishment of the Second WHO International Standard for SARS-CoV-2 RNA. 2023, WHO Expert Committee on Biological Standardization. WHO/BS/2023.xxxx

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants, particularly in meeting the tight timeframes of this study. We express our thanks to Victoria Infectious Diseases Reference Laboratory, Royal Melbourne Hospital (Australia) for the provision of the BetaCoV/Australia/VIC01/2020 isolate as well as UKHSA and BEI resources for provision of the VOC isolates of SARS-CoV-2. We also thank MHRA Manufacturing and Logistics teams for formulation of the candidate and distribution of the study material. We would like to acknowledge Ute Ströher (In Vitro Diagnostics Assessment Prequalification Unit, WHO), Uwe Scherf and Mayra Garcia (Office of In Vitro Diagnostics and Radiological Health, the Food and Drug Administration), Megan Cleveland and Peter Vallone (U.S. National Institute of Standards and Technology) and the UKHSA-MHRA VOC assurance group for constructive discussions on the isolate selection for the candidate replacement and collaborative study panel composition.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

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

13. CITATION

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

14. MATERIAL SAFETY SHEET

Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties	
Physical appearance: Freeze dried	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.5 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached: Not Applicable**

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised 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 http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). 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.