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Collaborative Study for the Establishment of a 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 3 December 2020** 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.

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Summary

In support of the global response to the 2019 emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and subsequent pandemic in 2020, the World Health Organization (WHO) has highlighted the importance of the availability of an International Standard (IS) for SARS-CoV-2 RNA. The intended use of the IS is for the calibration and control of nucleic acid amplification techniques (NAT), which are considered the gold standard method for accurate diagnosis of infection. This report describes the results of a multi-centre collaborative study to evaluate two candidate materials for their ability to harmonise the potencies of a panel of SARS-CoV-2 RNA samples between different laboratories. The candidates included an inactivated virus isolate (Candidate 1; NIBSC code 20/146) and a synthetic SARS-CoV-2 RNA preparation (Candidate 2; 20/138). Seventeen laboratories returned 32 datasets from 21 methods, covering in-house and commercial assays based on digital PCR, real-time PCR and Transcription Mediated Amplification technology.

A comparable reduction in the inter-laboratory variation was achieved when expressing results as relative to each of the candidates. Unexpectedly, the synthetic SARS-CoV-2 RNA preparation, Candidate 2, gave a greater overall level of harmonisation. However, it did not perform well within one assay and it is considered that a wider evaluation of the performance of the synthetic candidate is required. As such, it is proposed that the inactivated virus isolate, Candidate 1 (20/146) is established as the WHO International Standard for SARS-CoV-2 RNA with an assigned unitage of 7.40 Log_{10} IU/ampoule. To preserve the use of the International Standard, we propose that Candidate 2 (20/138) is established as a WHO Working Reagent, calibrated to the International Standard as part of this study with a potency of 6.73 Log_{10} IU/mL (95% confidence limits: 6.58 to 6.88 Log_{10} IU/mL).

Introduction

On 31 December 2019 the World Health Organization (WHO) was notified of an outbreak of pneumonia of unknown aetiology in Wuhan, People's Republic of China. The causative agent was rapidly identified as belonging to the *Betacoronavirus* genus (subgenus *Sarbecovirus*) of the *Coronaviridae* and was subsequently named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). In a matter of days of the complete genome sequence of the RNA virus being made available in mid-January [1], the WHO had published a list of in-house PCR assay protocols [2] developed by partner laboratories to help facilitate rapid implementation of global diagnostic testing capability. By 30th January 2020, the WHO declared the outbreak a Public Health Emergency of International Concern (PHEIC) and on 11th March 2020, it was characterised as a pandemic, with cases having been identified in 114 countries at that time [3]. As of 4th November 2020, the number of countries affected has reached 215, with confirmed cases surpassing 41 million.

Primary clinical diagnostic testing to confirm coronavirus disease (COVID-19) remains via the detection of SARS-CoV-2 specific RNA by nucleic acid amplification techniques (NAT). In line with the rapid global expansion of cases, there has been an unprecedented effort by independent laboratories and diagnostic test manufacturers to meet demands for testing capability. As of 4th November 2020, over 350 NAT-based products have been released onto the market [4]. The availability of an International Standard (IS) with an assigned potency in International Units (IU) will allow for assay calibration, thus greater comparability and control of platforms being used to detect SARS-CoV-2 RNA. Harmonisation of data reporting, using a reference material, will make it possible to better define parameters such as the analytical sensitivity/limits of detection of NAT assays.

This report describes the preparation and multi-centre collaborative study evaluation of two candidate materials to select the most appropriate to serve as the WHO IS for SARS-CoV-2 NAT assays. The candidate materials evaluated include a synthetic SARS-CoV-2 RNA preparation and an inactivated virus isolate. While ISs for other RNA viruses are invariably whole virus isolates, the synthetic approach offers flexibility and speed, with production able to commence as soon as the viral sequence is published and when access to live virus or the sequence in its entirety is restricted. Indeed, this approach was used to expedite the delivery and establishment of a WHO Reference Reagent for Ebola RNA [5]. This is the first study to evaluate both the traditional and synthetic candidate, offering an important opportunity for performance comparability. To meet the needs of the global response to this PHEIC, the study was carried out in 9 months following an accelerated timeframe: typically the process can take 2-3 years [6].

The aims of this collaborative study are to:

- Assess the suitability of different SARS-CoV-2 preparations to serve as the International Standard with an assigned unitage per ampoule for use in the harmonisation of COVID-19 NAT assays
- Characterise the standard preparations in terms of reactivity/specificity in different assay systems

- Assess each preparation's potency i.e. readout in a range of typical assays performed in different laboratories
- Assess commutability i.e. to establish the extent to which each preparation is suitable to serve as a standard for the variety of different samples and assay types
- Recommend to the WHO ECBS, the standard preparation(s) found to be suitable to serve as the International Standard and propose an assigned unit

Materials and Methods

Candidate Materials

Candidate 1 - Chimeric Lentiviral Particles (LVPs) containing SARS-CoV-2 RNA covering full genome (NIBSC code 20/138)

This material comprises chimeric lentiviral particles (LVPs) in which the Human Immunodeficiency Virus (HIV-1) genes have been substituted with those of the Wuhan-1 isolate of SARS-CoV-2 (GenBank: MN908947.3). The material was produced following a similar approach to that described previously [7]. Briefly, the SARS-CoV-2 genome was divided into four overlapping fragments (Appendix 2) and inserted within the long terminal repeats of a lentiviral plasmid. Single nucleotide mutations were randomly inserted within structural genes to prevent protein expression and the promoter of the U3-defective lentiviral plasmid removed as an additional safety precaution, with the sequences of the four plasmids deposited to GenBank (MT299802, MT299803, MT299804, MT299805). The four SARS-CoV-2 lentiviral plasmids were then each individually transfected into a HEK 293T/17 cell line together with a HIV-1 packaging plasmid (p8.91) kindly donated by Prof. D. Trono [8]. The lack of an envelope protein renders the particles produced non-infectious. The chimeric LVPs contained within the culture supernatant of the transfected cells were treated with DNaseI to remove residual plasmid DNA and purified by ultracentrifugation over a 20% sucrose cushion. The chimeric LVPs were then resuspended in universal buffer (10 mM Tris-HCl (pH 7.4), 0.5% human serum albumin and 1% D-(+)- Trehalose dehydrate) containing a background of $1x10^5$ copies/mL of human genomic DNA. The human serum albumin used in the preparation of the universal buffer was tested and found negative for HBsAg, anti-HIV and HCV RNA. The amount of LVPs for each preparation was quantified by real-time RT-PCR targeting the HIV-U5 region, common to each of the four particles. An equimolar mix of the four chimeric LVPs containing SARS-CoV-2 RNA containing $5x10^7$ genomes per mL was prepared in universal buffer.

Candidate 2 - Inactivated SARS-CoV-2, England/02/2020 Isolate (NIBSC code 20/146)

This material was prepared using the England/02/2020 isolate of SARS-CoV-2 (GISAID: EPI_ISL_407073) which was kindly donated by Public Health England (PHE, UK). The viral stock was received at passage 1 and amplified at NIBSC one further passage in Vero-E6 cells (ATCC® CRL-1586) with a MOI of 0.001. The culture supernatant was collected 72 hours post-infection. The infectious titre of the stock was measured as $1.5x10^6$ infectious units/mL by plaque assay. Inactivation of the virus was performed by acid-heat treatment, with the protocol

provided in full within Appendix 3. Briefly, the virus culture was incubated for 15minutes with 3% v/v acetic acid and neutralised by the addition of sodium hydroxide before subsequent incubation for 1hour at 60°C. The inactivated virus culture was then purified by using Amicon® Ultra 10K centrifugal filter columns.

Inactivation of the virus culture was validated by serial blind passage of 10% of the inactivated stock across 3 replicates on permissive Vero-E6 target cells for a period of 3 weeks, alongside a positive and negative control. Cells were monitored for signs of cytopathic effect and culture media tested for any quantifiable increase in viral RNA by real-time RT-PCR. No viable virus was detected. The inactivation procedure was approved by the NIBSC Biological Safety committee.

To prepare the bulk material, quantification of the SARS-CoV-2 genome copies within the inactivated material was determined relative to a plasmid standard curve by in-house real-time RT-PCR using primer/probe targeting the E-gene [9]. The material was prepared to contain $1x10^8$ genomes per mL and as with the chimeric LVP, was formulated in universal buffer containing a background of $1x10^5$ copies/mL of human genomic DNA.

Filling and Lyophilisation of Candidate Materials

The filling and lyophilisation of each candidate material, 20/138 and 20/146, was performed by the Standards Processing Division at NIBSC under ISO9001 between 12-15th and 25-28th June 2020, respectively. 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 lyophilised 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 a 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 a FMS-760 Oxygen Headspace Analyzer (Lighthouse Instruments, Charlottesville, VA, USA).

Next Generation Sequencing (NGS) of Candidate Materials

NGS was performed to verify sequences contained within each of the candidate materials, 20/138 and 20/146. In each case RNA was extracted using the QIAmp viral RNA Mini Spin Kit (Qiagen) according to the method of the manufacturer, except for the omission of carrier RNA.

For 20/138, four amplicons, each spanning the SARS-CoV-2 insert were amplified from a single cDNA preparation. For 20/146, four overlapping fragments of approximately 8 kb were prepared, spanning almost the entire genome. cDNA was prepared using Maxima H Minus RT (Thermo) and PCR performed using Platinum SuperFi II DNA Polymerase (Thermo). The amplicons were purified, and a sequencing library generated using the DNA Flex Kit (Illumina), with sequencing performed on the Illumina MiSeq with data imported and analysed in Geneious R10.2.3 (Biomatters). Detailed methods are provided in Appendix 4.

A consensus sequence for each of the four LVPs comprising 20/138 has been deposited under GenBank accession MW059032, MW059033, MW059034, MW059035. Analysis showed a lower region of sequencing coverage at the 5' end of ORF1ab gene within the first construct (spanning initial 2,700 nt) which has been annotated on the GenBank accession (MW059032). However, quantitative analysis has indicated a low frequency of this variant within the population (Appendix 4).

The consensus sequence for 20/146 was generated by alignment to the England/02/2020 isolate and is available with GenBank accession MW059036. Analysis found that approximately 70% of the population contains a 24-nucleotide deletion within the furin cleavage site of the S gene. This has been widely reported in the literature, with a high propensity to occur during passaging of the England/02/2020 isolate and is regarded as the "Bristol deletion" after the initial study in which it was documented [10].

Additional Samples included in the Collaborative Study

Unless stated otherwise, additional study samples were formulated in universal buffer containing a background of $1x10^5$ copies/mL of human genomic DNA as per the candidate material and included in the study as 0.5mL liquid frozen samples.

Individual Chimeric Lentiviral Particles (LVPs) containing partial SARS-CoV-2 RNA

The four chimeric LVPs used to prepare candidate 20/138 were included individually in the study, with each construct containing only partial SARS-CoV-2 RNA. Full sequences are deposited to GenBank and summarised below:

- Construct 1 – partial ORF1a (GenBank MW059032)
- Construct 2 – partial ORF1ab (GenBank MW059033)
- Construct 3 – partial ORF1ab/partial S (GenBank MW059034)
- Construct 4 – partial S/E/M/N (GenBank MW059035)

CE-Marked SARS-CoV-2 RNA NAT Run Control (NIBSC code 20/110)

This material is available within the NIBSC catalogue and was produced to act as a PCR run control for diagnostic assays detecting SARS-CoV-2. It was prepared using an equimolar mix of four chimeric LVPs as described for 20/138 and formulated at an approximate 50-fold lower dilution in phosphate buffered saline (PBS) as a 0.5mL liquid frozen sample.

Low Titre Inactivated SARS-CoV-2, England/02/2020 Isolate

The inactivated SARS-CoV-2, candidate 20/146, was diluted 1000-fold in universal buffer to provide a low potency sample.

Low Titre Inactivated SARS-CoV-2, Australia/VIC01/2020 Isolate
A different isolate of SARS-CoV-2 (Australia/VIC01/2020 isolate) was kindly donated to NIBSC by Victoria Infectious Diseases Reference Laboratory, Royal Melbourne Hospital (Australia). The virus was received at NIBSC at passage 3, amplified in Vero cells (passage 4) and acid-heat inactivated following the protocol and validation procedure described for candidate 20/146 (Appendix 3). The material was quantified by RT-PCR targeting the E gene alongside the candidate material 20/146 and formulated at an approximate 1000-fold lower concentration to serve as a low potency sample.

Stability of the Lyophilised Candidates
Accelerated degradation studies are underway for the lyophilised candidates 20/138 and 20/146 in order to determine the duration of stability of the material. Fifteen ampoules of each candidate were stored at -20, +4, +20, +37 and +45°C. Three ampoules for each temperature were retrieved at 2 weeks, 1 month and 3 months for both candidates and held at -20°C until testing. Retrieved samples were reconstituted and analysed by in-house SARS-CoV-2 real-time RT-PCR and compared to the designated -20ºC baseline. Loss of potency will be assessed by increase in the threshold cycle from the baseline and analysed to predict stability of the product, using the Arrhenius model for accelerated degradation studies if appropriate [11]. Final ampoules for completion of the assessment are due to be retrieved after 6 months and 1 year in December 2020 and June 2021 respectively.

Coded Study Samples
Table 1 lists the collaborative study samples provided to the participants, coded and blinded, as either 0.5mL aliquots or freeze-dried preparations. Laboratories received at least 4 sets of study samples, allowing for 3 independent assays plus 1 spare per method. Where more than one method was performed or the method required an input volume greater than 0.5mL, additional sample sets were provided. The study samples were stored at -20ºC and shipped on dry ice to participants under NIBSC dispatch reference CS679.

Participants
Twenty-seven laboratories accepted to participate in the study. Due to delays in obtaining import permits for 9 laboratories and 1 laboratory not returning data, 17 laboratories completed the study within the expedited timeframe, and they are listed in Appendix 1. The participants were from 9 countries: Canada (1), France (1), Italy (1), Japan (1), Luxemburg (1), South Korea (1), Taiwan (1), United Kingdom (5) and U.S.A. (5). All laboratories are referred to by a code number randomly allocated and not reflected in the order presented in Appendix 1. Participating organisations included national control/reference laboratories, diagnostic kit manufacturers and research laboratories.

Study Design

The study protocol (Appendix 2) requested participants to extract and test each sample in their established SARS-CoV-2 NAT based assay/s. Participants were asked to perform 3 independent assays, and for each assay, to prepare at least two independent dilution series of the samples, within a matrix specific to their assay, which would optimally cover 5 to 6 dilution points, with at least one point beyond the endpoint dilution. It was advised that the dilution series could be adjusted following initial testing. A result reporting sheet was provided for participants to return all essential information and the raw data readouts (e.g. Ct, copies, +/-) from their assay, as well as providing the result as per the analysis within their laboratory. Given the public health emergency, the study was conducted to an expedited timeline, between July and October 2020, with participants asked to return results within 4 weeks of receiving materials.

Assay Methods

Assays used by the participants are summarised in Table 2. Where laboratories performed multiple assay methods, or a method provided a readout against multiple targets, laboratory codes are followed by a letter indicating the different methods/targets e.g. 1a, 1b. The methods used cover both in-house and commercial kits based on digital PCR (dPCR), real-time PCR and Transcription Mediated Amplification (TMA) technology.

Statistical Methods

Potency estimates for each of the study samples 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, the geometric mean of within-assay replicates was taken, correcting for sample input, dilution factor and linearity of dilutions where appropriate. A potency estimate was reported as Log_{10} Copies/mL calculated from the geometric mean across the independent assays. Qualitative data, reported as '+/-' or Ct without conversion to copies, was 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 in the R software program [13]. Relative potencies were calculated by two methods. Where raw data for sample dilutions was provided either in copies or Ct values, relative potency estimates were obtained by parallel line analysis (PLA) with Log_{10}-transformed response. All calculations were performed using the R software program [13]. Linearity was assessed by visual assessment and by calculating an r^2 value. Samples with a value below 0.90 were excluded from further analysis. Test samples with response ranges that did not overlap with the range for the sample selected as reference standard were also excluded from the calculation of relative potency. Non-parallelism was assessed by calculating the ratio of fitted slopes for the test and candidate reference sample under consideration. Samples were concluded to be non-parallel when the slope ratio was outside of the range 0.80 – 1.25 and the resulting relative potency estimate was considered not valid. Where

sample dilutions had not been performed or raw data was provided scored as '+/-', relative potencies were calculated as a ratio between the estimated potency of the test and candidate reference sample. All potencies are presented in Log_{10} units.

Overall analysis was based on the Log_{10} estimates of copies/mL, 'Log_{10} NAT detectable units/mL' or relative potency, as required. 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 (s) of the Log_{10} potency estimates, the % Geometric Coefficient of Variation (%GCV = $\{10^{s}-1\}\times100\%$) and the Interquartile Range (IQR) of the Log_{10} estimates. Further analysis of variability was undertaken by calculating the proportion of laboratory mean potency estimates within 0.5 Log_{10} (3-fold) of the sample median.

Results and Data Analysis

Collaborative Study Data Received

Overall, 17 laboratories returned data covering 21 methods, with 10 methods providing data for two or more target genes, thus a total of 32 datasets have been evaluated within this study (Table 2). This includes 17 quantitative datasets and 15 qualitative datasets with a good distribution across the targets genes ORF1ab(RdRp)/S/N/E. For the quantitative datasets, 10 were obtained via dPCR with Lab 2 using a commercial kit while the other laboratories used in-house methods. Lab 10 and 14a used a 2-step method, performing the cDNA synthesis by reverse transcriptase reaction separately from the amplification step. The other laboratories used a 1-step RT-dPCR. Three laboratories performing real-time RT-PCR (Lab 1, 8 and 14b) reported quantitative results, in copies, calculated from the standard curve of an internal reference. For the qualitative methods, two laboratories reported data scored as positive/negative, with Lab 12 reporting 5 datasets obtained from 3 commercial real-time RT-PCR kits and Lab 5 reporting data using a commercial TMA based kit. Remaining qualitative data were reported as Ct values with all methods based on 1-step real-time RT-PCR which comprised 3 commercial kits and 4 in-house methods. Five of the in-house methods have reported using primer-probe targets from the 2019-nCoV CDC qPCR Probe Assay [2]. All laboratories returned results from three independent assays, with data reported from a dilution series of the samples from all laboratories except 3 performing dPCR which tested a single dilution of the samples (Lab 2, 3 and 16).
Two commercial methods performed by Lab 2 and 9 include targets against the housekeeping genes RPP30 and GAPDH respectively. In each instance uniformity of detection was reported for all study samples except sample P, which is the only study sample not formulated within a background of human genomic DNA.

Laboratory Reported Potency Estimates of Study Samples

Individual laboratory mean potency estimates of the study samples are provided in Table 3. These are presented in histogram form in Figures 2A-7A for all samples except the negative sample N, Q (LVP 2) and T (LVP 1) which comprise too few datapoints for detailed analysis.

For quantitative datasets providing results in copies, this is reported as Log_{10} copies/mL and for qualitative datasets reporting data as '+/-' or Ct values, the calculated Log_{10} NAT detectable units (NDU)/mL is reported. The two units are not directly comparable since NDU/mL determined from end-point dilutions is not necessarily equivalent to an absolute copy number. Qualitative data reported for sample L (LVP 4), O (Candidate 1, inactivated virus) and S (Candidate 2, LVP) by Lab 9 had not reached a dilution endpoint, thus a potency estimate could not be calculated and the result is reported as undetermined. Where values are missing the sample was reported by the participant as negative or below the limit of quantification of the assay.
The collaborative study samples provided to participants (Table 1) included one negative sample (N) and 9 positive samples. However only 1 out of the 4 positive study samples K, L, Q and T should have been detected by an assay with a single target gene owing to them each comprising an individual portion of the full-length SARS-CoV-2 RNA, as LVPs 1-4. Where a value is reported which is not consistent with the sample or target of the assay, this is noted. There are a high number of inconsistent results reported for sample K, which comprises chimeric LVP 3 containing SARS-CoV-2 RNA partial ORF1b/partial-S. It is not clear whether this is sample or assay dependent. A single inconsistent value is reported for sample L which comprises LVP 4 by Lab 2b; this is excluded from overall estimates due to heavily skewing the data.

All assays were able to detect the three inactivated SARS-CoV-2 preparations included in the study panel; the inactivated England/02/2020 isolate as the freeze dried Candidate 1 (sample O) the 1000-fold lower potency liquid preparation (sample M), and the same low potency liquid preparation of the inactivated VIC01/2020 isolate (sample R). The combined average potency estimates for sample M, inactivated England low was similar to that of sample R, inactivated VIC01 low (4.73 Log_{10} and 4.85 Log_{10}) which were formulated at similar potencies, suggesting that there is no discernible difference in detection between the two isolates (Table 3). The study samples containing the full SARS-CoV-2 genome within chimeric LVPs, the low potency liquid preparation (sample P) and freeze dried Candidate 2 (sample S), were correctly scored as positive in all the assays except for Lab 12c and 12e (E gene target) and Lab8d (RdRp gene target) which could also not detect the low potency sample P. Of note, Lab 12c and 12e could detect sample L, LVP 4, containing the E gene target, although provided a low potency estimate compared to the overall mean. Since the assay could detect the inactivated virus candidates, this signifies sub-optimal performance of Candidate 2. Overall, the data do not display any notable bias amongst the samples based on assay technology or target.

Expression of Potencies Relative to Candidates

To assess the suitability of each of the Candidates to serve as an International Standard and harmonise data reporting, potency estimates were expressed relative to the inactivated virus sample O (Candidate 1) and chimeric LVP sample S (Candidate 2). Where data were returned testing dilutions of samples associated with a numerical value (copies or Ct values), the relative potencies were calculated by parallel line analysis with quality criteria applied as described within the statistical methods. Little difference is observed in the performance of the two candidates as a reference when observing slope ratios, with Candidate 2 offering a slightly

reduced interquartile range yet a higher proportion of outlying points (Figure 1). Remaining data were analysed as a ratio of the sample relative to the candidate.

Candidate 1 – Inactivated virus (Sample O)

Potency estimates of the study samples relative to sample O were calculated based on an assigned arbitrary unitage of 7.7 Log_{10} units/mL and are presented in Table 4 and Figures 2-7. Sample K (LVP 3), which showed a high number of inconsistent results clustering as lower potencies, show only a small reduction in the spread of data reported when relative to Candidate 1 (5-fold, Figure 2A-B). The other single LVP 4, sample L, demonstrated an overall 8-fold reduction in the total spread of data (Figure 3A-B), although this is skewed by data from Lab 12c and 12e which provide distant relative potency estimates. For the chimeric LVP containing the full SARS-CoV-2 RNA sequence, the higher potency sample S (Candidate 2) had a reduction in the total spread of data from 630-fold to within an 85-fold range (Figure 7A-B) and for the lowest potency study sample P, this was reduced from 1995-fold to 250-fold (Figure 5A-B). The highest levels of reduction in the inter-laboratory variation were observed for sample M, inactivated England low (10-fold, Figure 3A-B) and sample R, inactivated VIC01 low, 14-fold from 630-fold to 45-fold (Figure 6A-B).

Candidate 2 – Chimeric LVP (Sample S)

Potency estimates of the study samples relative to S were calculated based on an assigned arbitrary unitage of 6.7 Log_{10} units/mL and are presented in Table 5 and Figures 2-7. Lab 12c and 12e were excluded from the analysis since they were not able to detect sample S. Expressing the potency of sample K, LVP3 relative to Candidate 2 was able to reduce the inter-laboratory variation slightly more than Candidate 1, although the data remains spread over a 33,000-fold range (Figure 2A-C). Sample L (LVP 4) shows a much greater degree of harmonisation when expressed relative to Candidate 2 than 1, with a 2,000-fold reduction in the difference between laboratories in comparison to 8-fold achieved by Candidate 1 (Figure 3A-C). This is in part due to the absence of outlying data points, with Lab 12c and e excluded from this analysis. While it might be expected that similarity in the formulation of the chimeric LVP preparations would lead to a greater degree of harmonisation, the low potency LVP sample P is harmonised to a similar extent as observed with Candidate 1 (Figure 5A-C). Interestingly, the inactivated VIC01 isolate, sample R, is harmonised to a lower extent than with Candidate 1 (6-fold, Figure 6A-C), however the similar potency England isolate, sample M, shows a greater 18-fold reduction to give a 30-fold range when relative to Candidate 2 (Figure 3A-B). As for the higher potency England isolate, sample O (Candidate 1), a slightly lower reduction was seen but the total spread remains below 85-fold (Figure 4A-B).

Evaluation of Inter-Laboratory Variation

Measures of the inter-laboratory variation for each of the samples based on the combined potency estimates are summarised in Table 6. Samples N, Q and T should not be considered in the analysis due to the small sample size, and sample K is excluded based on a high number of inconsistent results. The inter-laboratory variation measured as the Geometric Coefficient of Variation (%GCV) is reduced when sample potencies are expressed relative to each of the candidates, but similar to the overall spread of data, a greater reduction is seen in all but one case

with Candidate 2 (sample S). Candidate 2 gives a greater reduction for sample L LVP 4 (77% Candidate 2 *vs* 562% Candidate 1), M inactivated England (103% *vs* 122%) and P low potency LVP (328% *vs* 401%). However, as with the overall spread of data, for sample R inactivated VIC01 Candidate 1 offers better improvement, reducing the GCV to 137% compared to 169% with Candidate 2. When considering the proportion of laboratory potencies falling within 0.5 Log_{10} of the overall sample median (% within Med±0.5), and the interquartile range (IQR) which are less sensitive to outliers, a similar pattern is observed. When using Candidate 1 there is only a slight improvement in the Med±0.5 for sample P low potency LVP, yet a greater reduction to the IQR (16.4 to 5.7), whereas with Candidate 2 both show a good level of improvement (Med±0.5 at 80% and IQR of 4.4). However, for the low potency inactivated VIC01, sample R, the improvement is similar but favorable to Candidate 1, providing Med±0.5 of 79% compared to 76% with Candidate 2. Interestingly, the IQR is slightly better with Candidate 2 at 2.3 *vs* 2.1 with Candidate 1. Lastly, in both cases there is a greater overall reduction in the inter-laboratory variation for the low potency inactivated England, sample M with the IQR reducing from 5.2 to 1.9 and 1.7 for Candidate 1 and 2 respectively.

Production of the Candidate Material and Stability Assessment

The two candidate reference materials were freeze dried at NIBSC in June 2020 and given the production codes 20/138 (Candidate 2, chimeric LVP – sample S) and 20/146 (Candidate 1, inactivated virus - sample O). A product summary is provided in Table 7. The mean residual moisture content of 20/138 is slightly higher than the ideal 1%, however, provided stability of the product can be demonstrated, this is within acceptable limits for a WHO reference material [14]. The residual oxygen content falls within the NIBSC quality requirement limit of <1%. There are approximately 2200 vials of 20/138 and 2500 vials of 20/146 available for distribution. Accelerated degradation studies are ongoing. Candidate materials have currently been retrieved and tested for samples at 2 weeks, 1 month and 3 months from storage temperatures of -20, +4, +20, +37 and +45°C. Data have been analysed for the difference in Ct value from the baseline stored at -20°C (Table 8). Very little increase in Ct, which signifies a reduction in potency, is detected in all cases with 3month samples at +4 and +20°C not varying by more than ±0.1 from the -20°C baseline. The data is not suitable for fitting to the Arrhenius model to predict loss of potency. Current data suggests a good level of stability and further evaluation will be performed at the remaining timepoints of 6 months and 1 year.

Discussion

Within this study two candidate materials have been evaluated for their ability to harmonise results from NAT based assays and serve as an International Standard for SARS-CoV-2 RNA. The candidate material consisted of an inactivated whole virus isolate (Candidate 1) and a synthetic material comprising of 4 chimeric LVPs covering the full SARS-CoV-2 genome (Candidate 2). While the former is a traditional approach having been applied to other viruses requiring level 3 (BSL-3) laboratory containment, the synthetic material has only been evaluated on one other occasion where it was not possible to compare performance with a whole virus isolate [5]. Following evaluation by 17 laboratories across a total of 21 methods, covering 3

platform technologies (dPCR / real-time PCR /TMA), it has been shown that both candidates are able to harmonise data and reduce the inter-laboratory variation. Surprisingly, the synthetic Candidate 2 achieved an overall greater degree of harmonisation than the inactivated virus isolate Candidate 1 (Table 6).

It has been suggested in other reports for the establishment of International Standards that a better agreement between laboratories is obtained when sample potencies are expressed relative to a candidate most like itself. Yet, within this study the synthetic Candidate 2 offered a lower level of inter-laboratory variation for the low potency England isolate of inactivated virus (a dilution of Candidate 1), giving the greatest reduction in the spread of data to within a 30-fold range. Although the data are encouraging as to the potential of a synthetic approach, it should be noted that assays from one participant (Lab 12c & e) targeting the E-gene did not detect Candidate 2, yet the assays could efficiently detect the inactivated virus isolate Candidate 1. We speculate that the introduction of single nucleotide mutations within the synthetic sequence, to prevent protein expression, could be responsible. Two mutations, not naturally occurring, have been introduced within the E-gene. Although performing well with other assays targeting this gene, it highlights that variability in target regions may mean the synthetic material is not compatible with all assay set-ups.

We suggest that continued evaluations into the performance of the synthetic material across further platform technologies are required before this type of material can be considered as an International Standard. While this study does provide performance data on the 3 predominant platforms, other technologies such as loop-mediated isothermal amplification (LAMP) have received EUA status [4] and are being evaluated for large-scale testing. In this case it is uncertain how the segregation of the genomic RNA in the synthetic material, as opposed to a full transcript, will impact the technology which typically includes multiple primer sets. With the huge number of different NAT based assays on the market, it is considered more appropriate to propose that the inactivated virus isolate Candidate 1 serves as the highest order International Standard to which assays should be calibrated. The synthetic Candidate 2 will be made available as a Working Reagent, providing a routine laboratory control with feedback on performance requested to facilitate an evaluation of performance across other platforms.

The lowest potency sample included in the study, sample P LVP full, did not show as good a degree of harmonisation, particularly when data were reported relative to Candidate 1. This sample has been made available as a run control for PCR-based diagnostic assays detecting SARS-CoV-2 (NIBSC code 20/110). It is formulated to be close to the limit of detection of majority of assays, with the lowest raw potency estimate between all the collaborative study samples (3.86 Log_{10} units/mL); as such, the intended use of the sample is to be tested as provided, with no further dilutions. However, the study design required serial dilutions for all the samples to calculate relative potencies by parallel line analysis. Sample P could not be reliably detected across enough dilution points and therefore several datasets did not meet quality criteria for assigning a relative potency by parallel line analysis. The reduced level of harmonisation is thus likely representative of the higher variability in assay sensitivity at the lower limits of detection.

It has not been possible to fully evaluate commutability, which measures how closely a reference material behaves to a clinical sample, of the candidates within this study. This is determined by factors such as genetic variability of most recent clinical isolates, effect of the inactivation procedure and impact of the sample matrix. Restrictions on both the ability to source clinical samples and distribution of a hazard group 3 infectious pathogen meant that it was not feasible to include such samples in the expedited timeframe of the study. However, inclusion of two SARS-CoV-2 isolates; England/2/20 and VIC01/2020, showed no variation in their quantification, and a comparable reduction in the inter-laboratory variation of potencies relative to Candidate 1. Further, an aspect of commutability has been addressed from the range of methods used by participants, which included using in-house diluents and mostly manual but also some automated or closed system RNA extraction steps, with no notable performance issues.

Accelerated degradation studies to evaluate stability of the candidates are ongoing. So far, evaluation at 3 months indicates the candidates are sufficiently stable for storage at -20°C and shipment at ambient temperature within temperate climates. Until completion of the study, it is recommended that the candidates are shipped on ice packs or dry ice to hot climates.

Proposal

It is proposed that Candidate 1, the inactivated SARS-CoV-2 England isolate, NIBSC code 20/146 is established as the WHO International Standard for NAT based assays detecting SARS-CoV-2 RNA with an assigned potency of 7.40 Log_{10} IU/ampoule (7.70 Log_{10} IU/mL following reconstitution). Proposed Instructions for Use (IFU) for the product are included in Appendix 5.

There are approximately 2500 ampoules (0.5 mL/ ampoule) available for distribution. It is recommended that the International Standard is stored at -20˚C.

Furthermore, it is proposed that Candidate 2, the chimeric LVP containing SARS-CoV-2 RNA, NIBSC code 20/138 is made available as a Working Reagent calibrated to the International Standard for SARS-CoV-2 RNA within this study. The potency of the reagent is 6.73 Log_{10} IU/mL, with 95% confidence limits of 6.58 to 6.88 (n = 30). Proposed Instructions for Use (IFU) for the product are included in Appendix 6.

There are approximately 2000 ampoules (0.5 mL/ ampoule) available for distribution. It is recommended that the International Standard is stored at -20˚C.

Comments from Participants

Nine participants returned comments following circulation of the draft report. These included minor text changes and corrections to two participant's datasets due to misinterpretation of results returned. Three main comments were reported:

1) One participant suggested it be made clear the recommended matrix which should be used to reconstitute and dilute the International Standard, which has now been incorporated within the proposed IFU.

2) It was also suggested to highlight in the IFU for the Working Reagent (chimeric LVP, candidate 2) that the inclusion of single nucleotide mutations in the construct used to generate a non-infectious RNA, could adversely impact detection if occurring within the assay target region. Information on the design of the Working Reagent has been incorporated within the proposed IFU, which includes availability of full sequences from GenBank to allow users to check primer compatibility.
3) A concern was raised that by providing names of the commercial assays used it was not possible to maintain anonymity of participant's data and as such these have been removed.

Overall, no disagreements were received on the suitability of 20/146 to serve as the candidate WHO International Standard for SARS-CoV-2 RNA.

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Tables

Table 1. Collaborative Study Samples

Sample Code	Sample Description	Abbreviation	Formulation, volume (mL)
K	Chimeric LVP containing SARS-CoV-2 RNA partial ORF1b/partial-S (Construct 3)	LVP 3 (ORF1ab/S)	Frozen liquid, 0.5
L	Chimeric LVP containing SARS-CoV-2 RNA partial S(partial)/E/M/N (Construct 4)	LVP 4 (S/E/M/N)	Frozen liquid, 0.5
M	Inactivated SARS-CoV-2, England/20/2 isolate, low titre	Inactivated England (Low)	Frozen liquid, 0.5
N	Buffer only	Negative	Frozen liquid, 0.5
O	**Candidate 1** Inactivated SARS-CoV-2, England/02/2020 isolate	Candidate 1 (Inactivated England)	Lyophilised, 0.5
P	Chimeric LVP containing SARS-CoV-2 RNA covering full genome, low titre (NIBSC 20/110)	LVP full (Low)	Frozen liquid, 0.5
Q	Chimeric LVP containing SARS-CoV-2 RNA partial ORF1ab (Construct 2)	LVP 2 (ORF1ab)	Frozen liquid, 0.5
R	Inactivated SARS-CoV-2, Australia/VIC01/2020 isolate, low titre	Inactivated VIC (Low)	Frozen liquid, 0.5
S	**Candidate 2** Chimeric LVP containing SARS-CoV-2 RNA covering full genome	Candidate 2 (LVP full)	Lyophilised, 0.5
T	Chimeric LVP containing SARS-CoV-2 RNA partial ORF1a (Construct 1)	LVP 1 (ORF1a)	Frozen liquid, 0.5

LVP: lentiviral particles

Table 2. Laboratory Codes and Assay Methods
Quantitative assays reporting copies are indicated by red shading, with Lab 1, 8 and 14b performing real-time RT-PCR and all others dPCR. Unshaded labs have all performed qualitative assays, reporting Ct or '+/-' by real-time RT-PCR, except Lab 5 which performed Transcription Mediated Amplification (TMA) and is shaded blue.

Lab	NAT Method	Extraction Method	Assay Target	Use	Reported Readout
1	Real-time RT-PCR	QIAGEN QIAamp Viral RNA mini kit	N	In-house	Ct; Copies
2a	Commercial RT-dPCR Kit	Beaver Beads Viral RNA/DNA kit	N	RUO	Copies
2b	Commercial RT-dPCR Kit	Beaver Beads Viral RNA/DNA kit	ORF1ab	RUO	Copies
3a	RT-ddPCR	QIAGEN QIAamp Viral RNA mini kit	CDC N2*	In-house	Copies
3b	RT-ddPCR	QIAGEN QIAamp Viral RNA mini kit	ORF1ab (China CDC)	In-house	Copies
4	Real-time RT-PCR; 2019-nCoV CDC qPCR Probe Assay	Homemade kit based on Sridhar *et al.* 2020 [15]	CDC N1*	In-house	Ct
5	Commercial TMA Assay	Procleix Panther System (Grifols)	Not stated	CE	+/-
6a	Commercial real-time RT-PCR Kit	N/A	S	CE; EUA	Ct; +/-
6b	Commercial real-time RT-PCR Kit	N/A	ORF1ab	CE; EUA	Ct; +/-
7	Commercial real-time RT-PCR Kit	Biomerieux NucliSENS easyMAG	N/ORF1ab	CE; EUA	Ct; +/-
8a	Real-time RT-PCR; 2019-nCoV CDC qPCR Probe Assay	QIAGEN QIAamp Viral RNA mini kit	CDC N1*	In-house	Ct; Copies
8b	Real-time RT-PCR; 2019-nCoV CDC qPCR Probe Assay	QIAGEN QIAamp Viral RNA mini kit	CDC N2*	In-house	Ct; Copies

8c	Real-time RT-PCR	QIAGEN QIAamp Viral RNA mini kit	E (pan-Sarbeco)	In-house	Ct; Copies
8d	Real-time RT-PCR	QIAGEN QIAamp Viral RNA mini kit	RdRp	In-house	Ct; Copies
8e	Real-time RT-PCR	QIAGEN QIAamp Viral RNA mini kit	ORF1ab	In-house	Ct; Copies
9a	Commercial real-time RT-PCR Kit	QIAGEN QIAamp Viral RNA mini kit	N	RUO	Ct; +/-
9b	Commercial real-time RT-PCR Kit	QIAGEN QIAamp Viral RNA mini kit	S	RUO	Ct; +/-
10	2-Step RT-ddPCR	TRIzol extraction (Phenol/Chloroform)	E	In-house	Copies
11	Real-time RT-PCR	Macherey-Nagel RNA Isolation NucleoSPin RNA Plus	N	In-house	Ct; +/-
12a	Commercial real-time RT-PCR Kit	N/A	RdRp/N	CE; EUA	+/-
12b	Commercial real-time RT-PCR Kit	N/A	ORF1ab	CE; EUA	+/-
12c	Commercial real-time RT-PCR Kit	N/A	E (pan-Sarbeco)	CE; EUA	+/-
12d	Commercial real-time RT-PCR Kit	N/A	ORF1ab	CE; EUA	+/-
12e	Commercial real-time RT-PCR Kit	N/A	E (pan-Sarbeco)	CE; EUA	+/-
13	RT-ddPCR	QIAGEN QIAamp Viral RNA mini kit	CDC N1*	In-house	Copies
14a	2-Step RT-dPCR	TANBead Nucleic Acid Extraction Kit	E	In-house	Copies

14b	2-Step Real-time RT-PCR	TANBead Nucleic Acid Extraction Kit	E	In-house	Ct; Copies
15a	Real-time RT-PCR; modified 2019-nCoV CDC qPCR Probe Assay	QIAGEN QIAamp Viral RNA mini kit	CDC N1*	In-house	Ct
15b	Real-time RT-PCR; 2019-nCoV CDC qPCR Probe Assay	QIAGEN QIAamp Viral RNA mini kit	CDC N2*	In-house	Ct
16a	RT-dPCR	QIAGEN QIAamp Viral RNA mini kit	CDC N2*	In-house	Copies
16b	RT-dPCR	QIAGEN QIAamp Viral RNA mini kit	E (pan-Sarbeco)	In-house	Copies
17	RT-ddPCR	Roche MagNA Pure 24 Total NA Isolation Kit	CDC N2*	In-house	Copies

CE = CE-marked i*n vitro* diagnostic; EUA = emergency use authorization; RUO = research use only; N/A = not applicable due to use of closed system; * = primer sequences published by the CDC [16]

Table 3. Laboratory Mean Potency Estimates of SARS-CoV-2 RNA Study Samples
Quantitative data is reported as Log_{10} Copies/mL and indicated by red shading, with Lab 1, 8 and 14b performing real-time RT-PCR and all others dPCR. Qualitative data is reported as NAT-detectable units/mL, with all labs performing real-time RT-PCR except Lab 5 which performed TMA and is shaded blue.

Lab (Target)	**K** **LVP 3 (ORF1ab/S)**	**L** **LVP 4 (S/E/M/N)**	**M** **Inactivated England (Low)**	**N** **Negative**	**O** **Candidate 1 (Inactivated England)**	**P** **LVP full (Low)**	**Q** **LVP 2 (ORF1ab)**	**R** **Inactivated VIC (Low)**	**S** **Candidate 2 (LVP full)**	**T** **LVP 1 (ORF1a)**
1 (N)	3.08*	7.06	5.05		7.86	4.14		4.99	7.01	
2a (N)	3.01*	6.81	4.88	2.29*	7.75	4.57	3.28*	5.06	6.77	2.97*
2b (ORF)	6.53	2.46*†	4.81	2.26*	7.76	4.42	3.16	4.96	6.66	2.88
3a (N2)	3.26*	6.77	4.99		7.88	4.17		5.08	6.82	
3b (ORF)			4.95		7.76	4.18	6.53	4.94	6.70	
4 (N1)	2.85*	7.11	3.94		7.11	3.56		3.96	6.95	
5	2.66	6.66	4.26		7.68	3.26		4.26	6.26	
6a (S)	3.15*	7.16	4.70		7.66	2.94		4.70	6.93	
6b (ORF)	6.54		4.97		7.78	2.65		5.03	6.97	
7 (N/ORF)	2.29	5.99	4.40		7.66	4.48	6.05	4.89	6.82	
8a (N1)		7.22	5.54		8.21	5.09		5.52	7.29	
8b (N2)		7.52	5.75		8.68	5.31		5.97	7.66	
8c (E)		7.69	5.64		8.66	4.60		5.88	7.91	
8d (RdRp)	7.14		5.34		8.04			5.25	7.40	
8e (ORF)			6.00		8.97	5.22	7.75	6.29	7.90	
9a (N)	1.71*	*Undt.*	3.99		*Undt.*	3.37		4.22	*Undt.*	
9b (S)		*Undt.*	4.05		*Undt.*	3.36		3.77	*Undt.*	
10 (E)		6.83	5.94		7.65	4.32		5.64	6.74	
11 (N)		5.41	3.71		6.71	3.71		3.71	5.57	
12a (RdRp/N)	6.27	5.92	3.81		6.91	2.64		4.46	6.03	
12b (ORF)	6.82		4.30		7.14	2.03		4.39	6.15	
12c (E)		3.15	4.46		7.16			4.84		
12d (ORF)	6.30		4.46		7.16	2.54		4.42	6.81	
12e (E)		3.96	4.61		7.41			5.51		

13 (N1)		6.39	4.65		7.35	4.07		4.80	6.49	
14a (E)		5.12	3.28		5.93	2.90		3.55	5.07	
14b (E)		5.16	3.43		5.98	2.47		3.49	5.06	
15a (N1)	2.56*	6.26	4.90		7.56	4.56		4.56	6.11	
15b (N2)	2.90*	7.56	4.90		7.81	3.52		4.90	6.97	
16a (N2)	3.23*	6.87	4.99		7.89	4.22		5.11	6.98	
16b (E)		6.91	4.97		7.84	4.47		5.06	7.02	
17 (N2)		7.36	5.77		8.28	5.17		5.92	7.53	
Average										
Qualitative	4.00	5.92	4.36		7.37	3.28	6.05	4.51	6.51	
Quantitative	4.38	6.75	5.05	2.28	7.79	4.33	5.18	5.15	6.88	2.93
Combined	4.13	6.39	4.73	2.28	7.61	3.86	5.35	4.85	6.74	2.93
N										
Qualitative	11	10	15	0	13	13	1	15	11	0
Quantitative	6	13	17	2	17	16	4	17	17	2
Combined	17	23	32	2	30	29	5	32	28	2

* = inconsistent result; † = value excluded from average estimates; *Undt.* = value undetermined due to assay dilutions not reaching an endpoint; N = number of datasets

Table 4. Laboratory Mean Potency Estimates of SARS-CoV-2 RNA Study Samples Relative to Candidate 1 (Sample O) with an assigned arbitrary value of 7.7 Log_{10} units/mL

Relative potency estimates determined by either parallel line analysis (PLA) based on reported copies (cp) or Ct values where sample dilutions have been performed or as a ratio of the sample relative to the candidate

Lab (Target)	**K** **LVP 3 (ORF1ab/S)**	**L** **LVP 4 (S/E/M/N)**	**M** **Inactivated England (Low)**	**N** **Negative**	**P** **LVP full (Low)**	**Q** **LVP 2 (ORF1ab)**	**R** **Inactivated VIC (Low)**	**S** **Candidate 2 (LVP full)**	**T** **LVP 1 (ORF1a)**	**Analysis**
1 (N)	3.89*	7.29	5.69		4.99		5.87	7.10		PLA(Ct)
2a (N)	2.89*	6.67	4.81	2.42*	4.63	3.49*	4.98	6.76	3.11*	Ratio
2b (ORF)	6.49	2.44*†	4.76	2.52*	4.49	3.48	4.95	6.64	3.26	Ratio
3a (N2)	3.09*	6.60	4.81		4.00	-	4.90	6.64		Ratio
3b (ORF)			4.88		4.12	6.47	4.88	6.63		Ratio
4 (N1)	2.82*	7.25	-		-		-	7.60		PLA(Ct)
5	2.68	6.68	4.28		3.28		4.28	6.28		Ratio
6a (S)	-	7.31	-		-		4.98	7.14		PLA(Ct)
6b (ORF)	6.76		4.95		4.61		4.98	6.77		PLA(Ct)
7 (N/ORF)	-	6.34	5.05		4.63	6.38	5.10	6.81		PLA(Ct)
8a (N1)		6.60	4.68		4.58		4.83	6.71		PLA(Ct)
8b (N2)		6.45	4.73		-		4.87	6.62		PLA(Ct)
8c (E)		6.62	4.54		3.88		4.85	6.78		PLA(Ct)
8d (RdRp)	6.81		5.09				4.69	7.02		PLA(Ct)
8e (ORF)			4.67		4.14	6.41	4.94	6.58		PLA(Ct)
9a (N)	-	6.83	4.80		-		-	6.85		PLA(Ct)
9b (S)		6.78	4.96		-		-	6.85		PLA(Ct)
10 (E)		6.90	6.00		4.88		5.93	6.71		PLA(cp)
11 (N)		6.08	-				5.06	5.67		PLA(Ct)
12a (RdRp/N)	7.06	6.71	4.60		3.42		5.25	6.82		Ratio
12b (ORF)	7.38		4.86		2.59		4.95	6.71		Ratio
12c (E)		3.70	5.00				5.38			Ratio

12d (ORF)	6.84		5.00		3.08		4.97	7.35		Ratio
12e (E)		4.26	4.90				5.81			Ratio
13 (N1)		6.79	5.17		4.64		5.26	6.86		PLA(cp)
14a (E)		6.95	5.14		-		5.40	6.86		PLA(cp)
14b (E)		6.87	4.98		4.94		5.24	6.74		PLA(Ct)
15a (N1)	3.05*	6.38	4.58		-		4.88	5.69		PLA(Ct)
15b (N2)	-	6.31	4.34		3.06		4.41	6.12		PLA(Ct)
16a (N2)	3.05*	6.68	4.81		4.03		4.93	6.79		Ratio
16b (E)		6.76	4.83		4.32		4.91	6.88		Ratio
17 (N2)		6.91	5.22		4.87		5.47	6.88		PLA(cp)
Average										
Qualitative	5.23	6.22	4.78		3.53	6.38	5.00	6.67		
Quantitative	4.37	6.78	4.99	2.47	4.46	4.96	5.11	6.78	3.19	
Combined	4.83	6.51	4.90	2.47	4.15	5.25	5.07	6.73	3.19	
N										
Qualitative	7	12	12	0	7	1	12	13	0	
Quantitative	6	13	17	2	14	4	17	17	2	
Combined	13	25	29	2	21	5	29	30	2	

* = inconsistent result; † = value excluded from average estimates; - = data excluded as outside quality parameters; N = number of datasets analysed

Table 5. Laboratory Mean Potency Estimates of SARS-CoV-2 RNA Study Samples Relative to Candidate 2 (Sample S) with an assigned arbitrary value of 6.7 Log_{10} units/mL

Relative potency estimates determined by either parallel line analysis (PLA) based on reported copies (cp) or Ct values where sample dilutions have been performed or as a ratio of the sample relative to the candidate

Lab (Target)	**K** **LVP 3 (ORF1ab/S)**	**L** **LVP 4 (S/E/M/N)**	**M** **Inactivated England (Low)**	**N** **Negative**	**O** **Candidate 1 (Inactivated England)**	**P** **LVP full (Low)**	**Q** **LVP 2 (ORF1ab)**	**R** **Inactivated VIC (Low)**	**T** **LVP 1 (ORF1a)**	**Analysis**
1 (N)	3.23*	6.88	5.15		7.30	3.82		4.94		PLA(Ct)
2a (N)	2.93*	6.74	4.80	2.21*	7.64	4.49	3.20	4.99	2.90*	Ratio
2b (ORF)	6.57	2.50*†	4.85	2.30*	7.76	4.45	3.20	5.00	2.92	Ratio
3a (N2)	3.15*	6.66	4.87		7.76	4.06		4.96		Ratio
3b (ORF)			4.95		7.77	4.18	6.53	4.94		Ratio
4 (N1)	2.84*	6.36	4.49		6.80	-		3.96		PLA(Ct)
5	3.10	7.10	4.70		8.12	3.70		4.70		Ratio
6a (S)	-	6.90	-		7.26	-		4.79		PLA(Ct)
6b (ORF)	6.69		-		7.63	4.49		-		PLA(Ct)
7 (N/ORF)	-	6.20	4.91		7.59	4.47	6.26	4.96		PLA(Ct)
8a (N1)		6.59	4.72		7.69	4.37		4.93		PLA(Ct)
8b (N2)		6.54	4.69		7.78	-		4.89		PLA(Ct)
8c (E)		6.54	4.56		7.62	3.82		4.83		PLA(Ct)
8d (RdRp)	6.50		4.76		7.38			4.33		PLA(Ct)
8e (ORF)			4.81		7.82	4.13	6.53	5.08		PLA(Ct)
9a (N)	-	6.55	4.64		7.55	-		4.75		PLA(Ct)
9b (S)		6.61	4.65		7.55	-		4.73		PLA(Ct)
10 (E)		6.83	5.83		7.69	4.42		5.86		PLA(cp)
11 (N)		6.90	-		8.73			5.56		PLA(Ct)
12a (RdRp/N)	6.94	6.59	4.48		7.58	3.31		5.13		Ratio
12b (ORF)	7.36		4.84		7.69	2.57		4.94		Ratio
12c (E)										Ratio

12d (ORF)	6.19		4.35		7.05	2.43		4.31		Ratio
12e (E)										Ratio
13 (N1)		6.63	4.96		7.54	4.50		5.10		PLA(cp)
14a (E)		6.75	4.83		7.54	-		5.11		PLA(cp)
14b (E)		6.82	4.86		7.66	4.88		5.17		PLA(Ct)
15a (N1)	4.07*	6.67	5.22		8.71	-		5.98		PLA(Ct)
15b (N2)	-	7.43	5.46		8.28	-		5.80		PLA(Ct)
16a (N2)	2.95*	6.59	4.71		7.61	3.94		4.84		Ratio
16b (E)		6.59	4.65		7.52	4.15		4.74		Ratio
17 (N2)		6.55	4.58		7.39	4.35		5.11		PLA(cp)
Average										
Qualitative	5.31	6.73	4.77		7.73	3.49	6.26	4.97		
Quantitative	4.22	6.67	4.86	2.26	7.61	4.25	4.87	4.99	2.91	
Combined	4.81	6.70	4.83	2.26	7.67	4.03	5.15	4.98	2.91	
N										
Qualitative	7	10	10	0	13	6	1	12	0	
Quantitative	6	13	17	2	17	14	4	17	2	
Combined	13	23	27	2	30	20	5	29	2	

* = inconsistent result; † = value excluded from average estimates; (-) = data excluded as outside of quality parameters; N = number of datasets analysed

Table 6. Overall Mean Estimates and Inter-Laboratory Variation in the Study Samples

The percentage geometric coefficient of variation (%GCV), the proportion of potencies within 0.5 Log_{10} of the median (%within Med±0.5) and the interquartile range (IQR) are calculated from the raw (Table 3), relative to Candidate 1 (Table 4) and relative to Candidate 2 (Table 5) potency estimates. Data from laboratory 2b for sample L is excluded. Colored shading is graduated from light-dark representing the degree of improvement in inter-laboratory variation, excluding samples K, N, Q and T due to inconsistent results and small sample size.

Potency Estimates	K LVP 3 (ORF1ab/S)	L LVP 4 (S/E/M/N)	M Inactivated England (Low)	N Negative	O Candidate 1 (Inactivated England)	P LVP full (Low)	Q LVP 2 (ORF1ab)	R Inactivated VIC (Low)	S Candidate 2 (LVP full)	T LVP 1 (ORF1a)
Raw										
Mean	4.13	6.39	4.73	2.28	7.61	3.86	5.35	4.85	6.74	2.93
GCV (%)	8239%	1339%	407%	4%	375%	717%	10994%	415%	421%	17%
Median	3.15	6.81	4.84	2.28	7.72	4.14	6.05	4.92	6.82	2.93
%within Med±0.5	47%	48%	53%	100%	60%	45%	40%	53%	57%	100%
Antilog(IQR)	2818.1	15.2	5.2	1.0	4.7	16.4	1789.7	5.4	3.8	1.1
N	17	23	32	2	30	29	5	32	28	2
Relative to Candidate 1 (O)										
Mean	4.83	6.51	4.90	2.47		4.15	5.25	5.07	6.73	3.19
GCV (%)	10184%	562%	122%	19%		401%	3964%	137%	148%	23%
Median	3.89	6.68	4.86	2.47		4.32	6.38	4.96	6.78	3.19
%within Med±0.5	8%	76%	86%	100%		57%	60%	79%	83%	100%
Antilog(IQR)	5850.8	2.6	1.9	1.1		5.7	843.6	2.3	1.7	1.2
N	13	25	29	2		21	5	29	30	2
Relative to Candidate 2 (S)										
Mean	4.81	6.70	4.83	2.26	7.67	4.03	5.15	4.98		2.91
GCV (%)	7358%	77%	103%	16%	149%	328%	5914%	169%		3%
Median	4.07	6.63	4.80	2.26	7.62	4.17	6.26	4.94		2.91
%within Med±0.5	8%	96%	93%	100%	83%	80%	60%	76%		100%
Antilog(IQR)	1836.4	1.8	1.7	1.1	1.7	4.4	2127.8	2.1		1.0
N	13	23	27	2	30	20	5	29		2

GCV = geometric coefficient of variation; Med = Median; IQR = Interquartile Range; N = number of datasets analysed

Table 7. Production Summary of the Candidate Reference Material
Microbiological tests for bacterial and mould/yeast colony count returned negative

NIBSC Code	20/138	20/146
Product Description	Chimeric LVP containing SARS-CoV-2 RNA (Candidate 2)	Inactivated SARS-CoV-2, England/02/2020 (Candidate 1)
Dates of processing	Filling; 12Jun20 Lyophilisation; 12-15Jun20 Sealing; 15Jun20	Filling; 25Jun20 Lyophilisation; 25-28Jun20 Sealing; 28Jun20
Presentation	Freeze-dried preparation in 2.5mL DIN Ampoule	Freeze-dried preparation in 2.5mL DIN Ampoule
No. vials filled	2371	2590
Mean fill weight (g)	0.52 (n = 108)	0.52 (n = 208)
CV of fill mass (%)	0.39	1.04
Mean residual moisture (%)	1.72 (n = 12)	0.89 (n = 12)
CV of residual moisture (%)	57.87	59.54
Mean of oxygen content (%)	0.27 (n = 12)	0.28 (n = 12)
CV of oxygen content (%)	44.41	48.72

n = number of samples tested

Table 8. Accelerated Stability Assessment of Candidate Material
Thermal stability of 20/138 (Candidate 2, chimeric LVP – sample S) and 20/146 (Candidate 1, inactivated virus - sample O) at 2 weeks, 1 month and 3 months expressed as the difference in threshold cycle (Ct) from the -20°C baseline sample, quantified in duplicate by real-time RT-PCR. A 3.3 Ct difference indicates an approximate 10-fold change in detection

Time point	Storage Temperature (°C)	Difference from -20°C Baseline (Ct)	
		20/138	20/146
2 weeks	**4**	0.00	0.15
	20	0.00	-0.30
	37	0.15	-0.40
	45	0.25	-0.35
1 month	**4**	0.00	-0.15
	20	0.15	-0.15
	37	0.65	-0.45
	45	0.55	0.00
3 months	**4**	0.05	-0.05
	20	-0.05	-0.10
	37	0.35	-0.25
	45	0.40	-0.10

Figure 1. Comparison of Slope Ratios when Analyzing Samples Relative to Candidate 1 (Sample O) and Candidate 2 (Sample S)

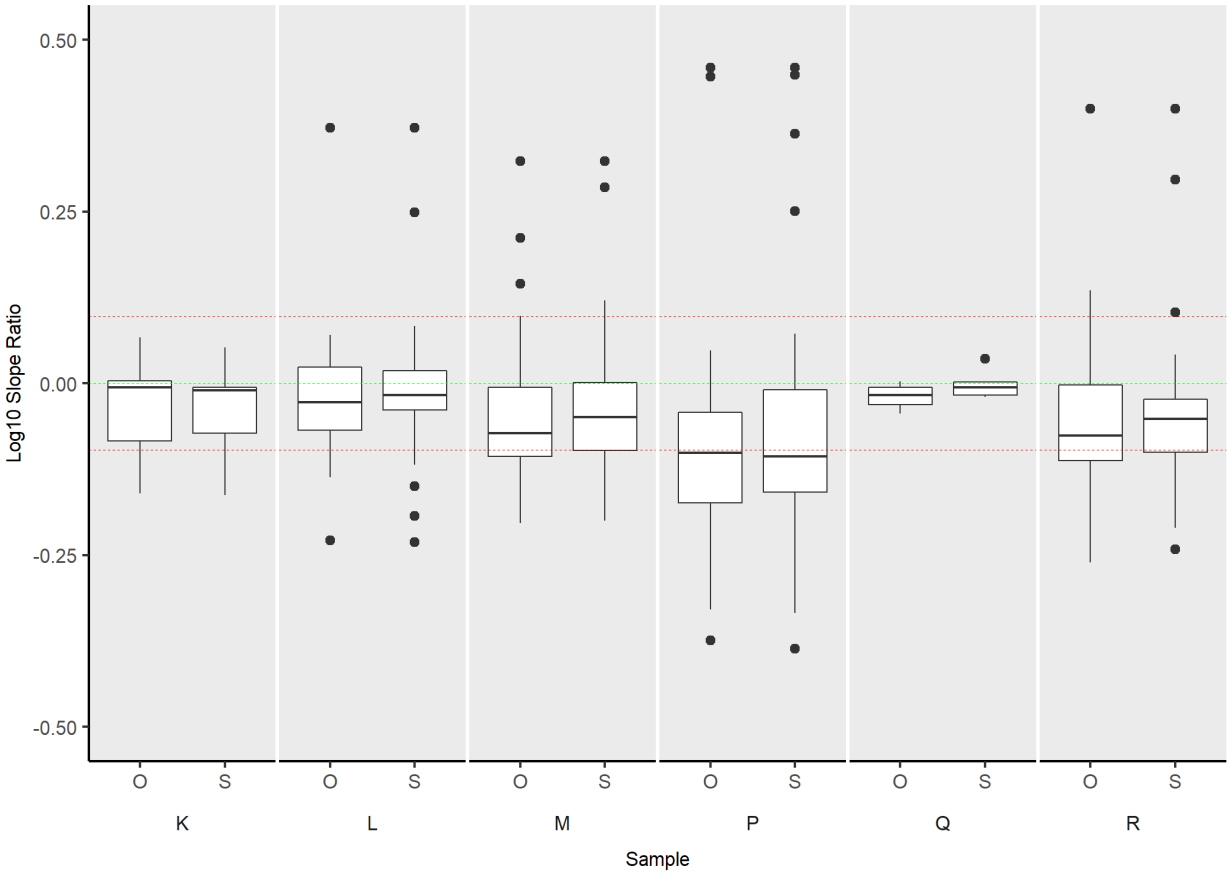


Figure 2A. Potency Estimates for Sample K (LVP 3)

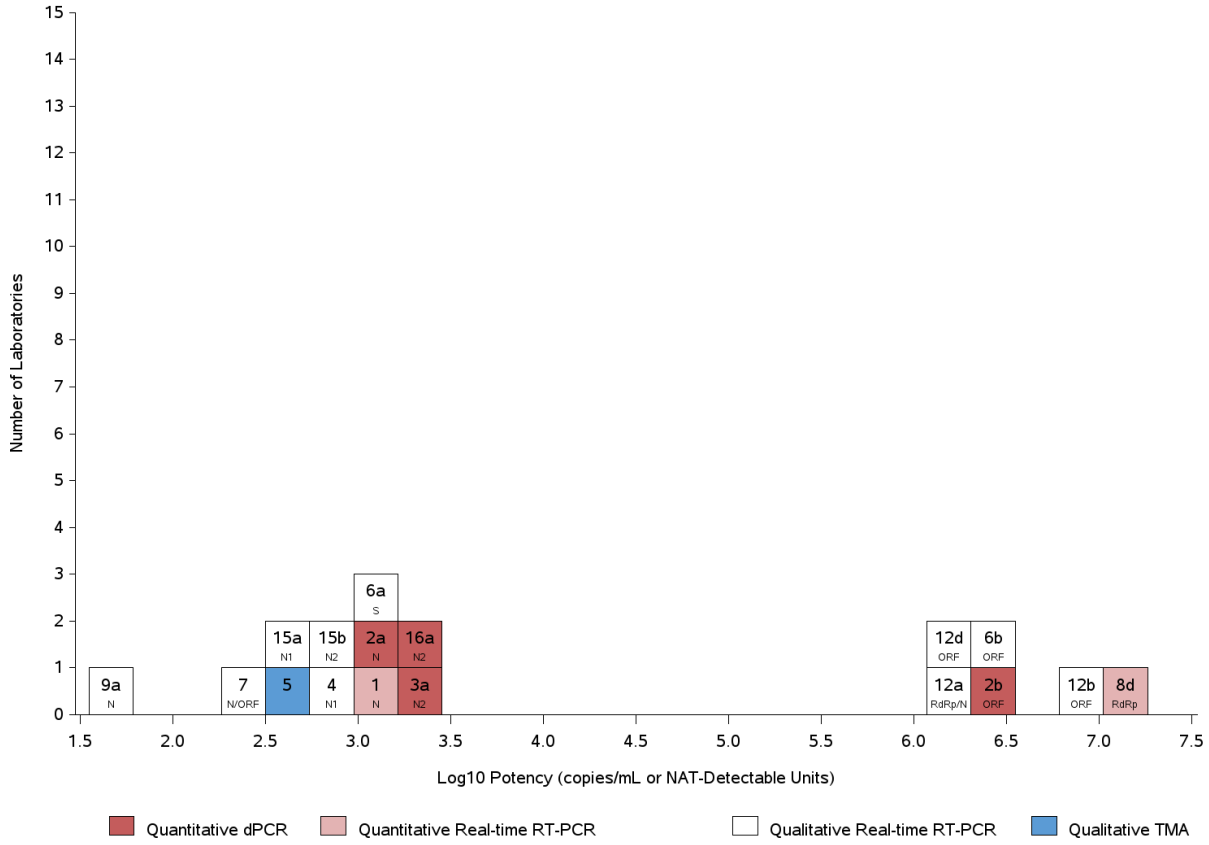


Figure 2B. Potency Estimates for Sample K (LVP 3) Relative to Candidate 1

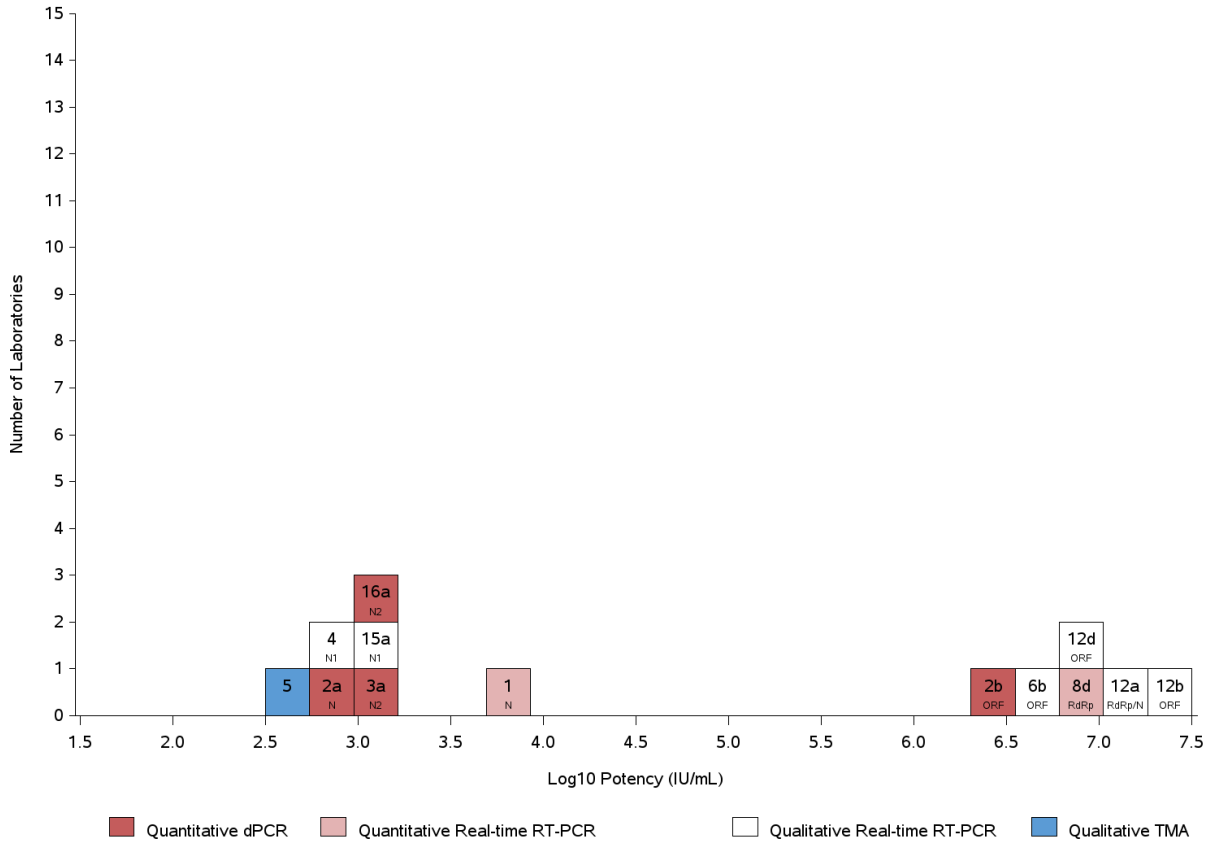


Figure 2C. Potency Estimates for Sample K (LVP 3) Relative to Candidate 2

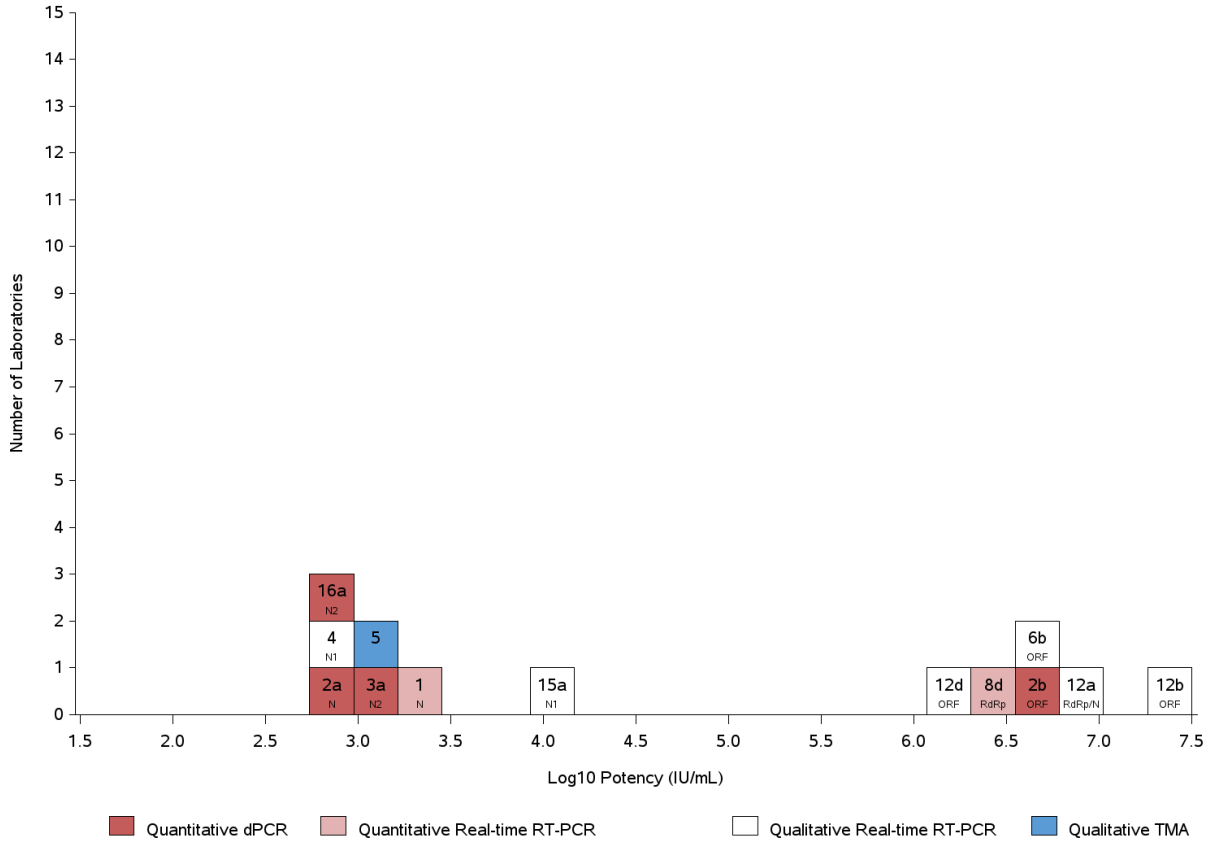


Figure 3A. Potency Estimates for Sample L (LVP 4)

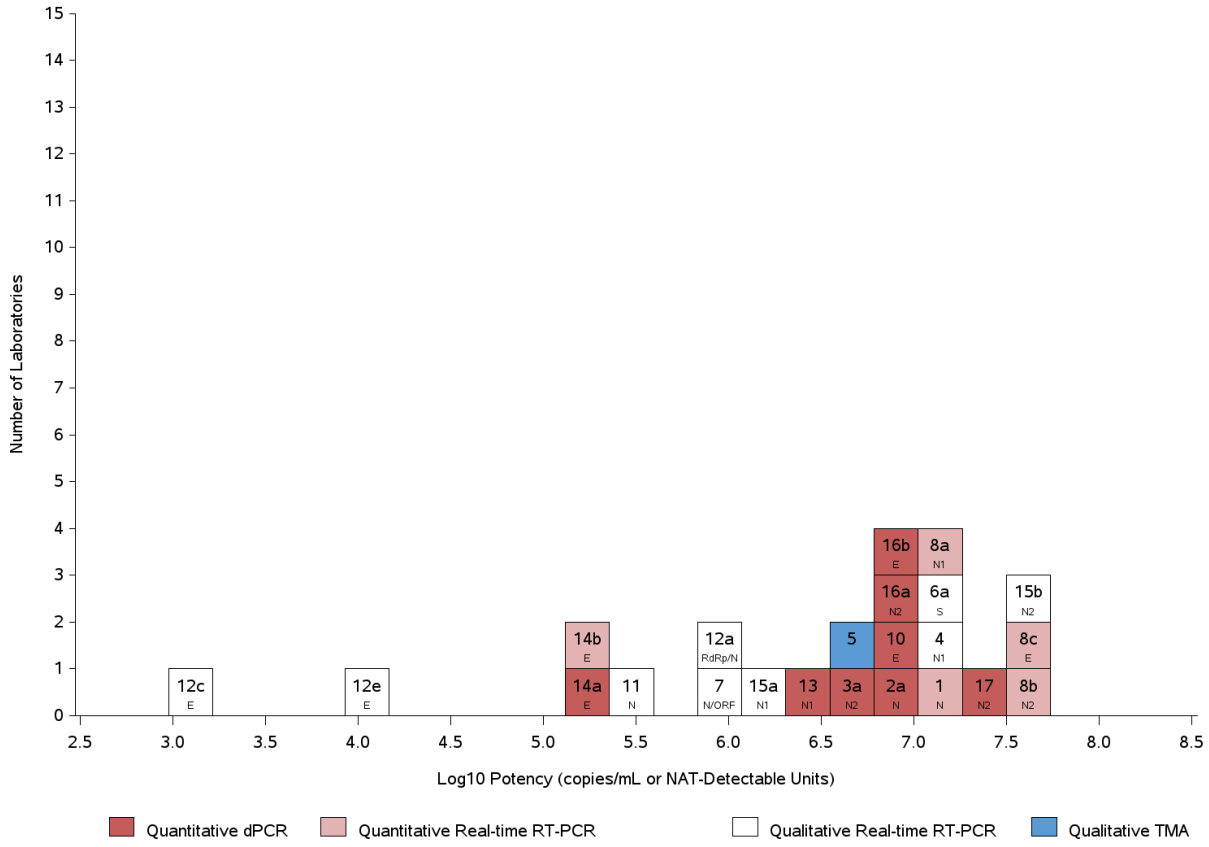


Figure 3B. Potency Estimates for Sample L (LVP 4) Relative to Candidate 1

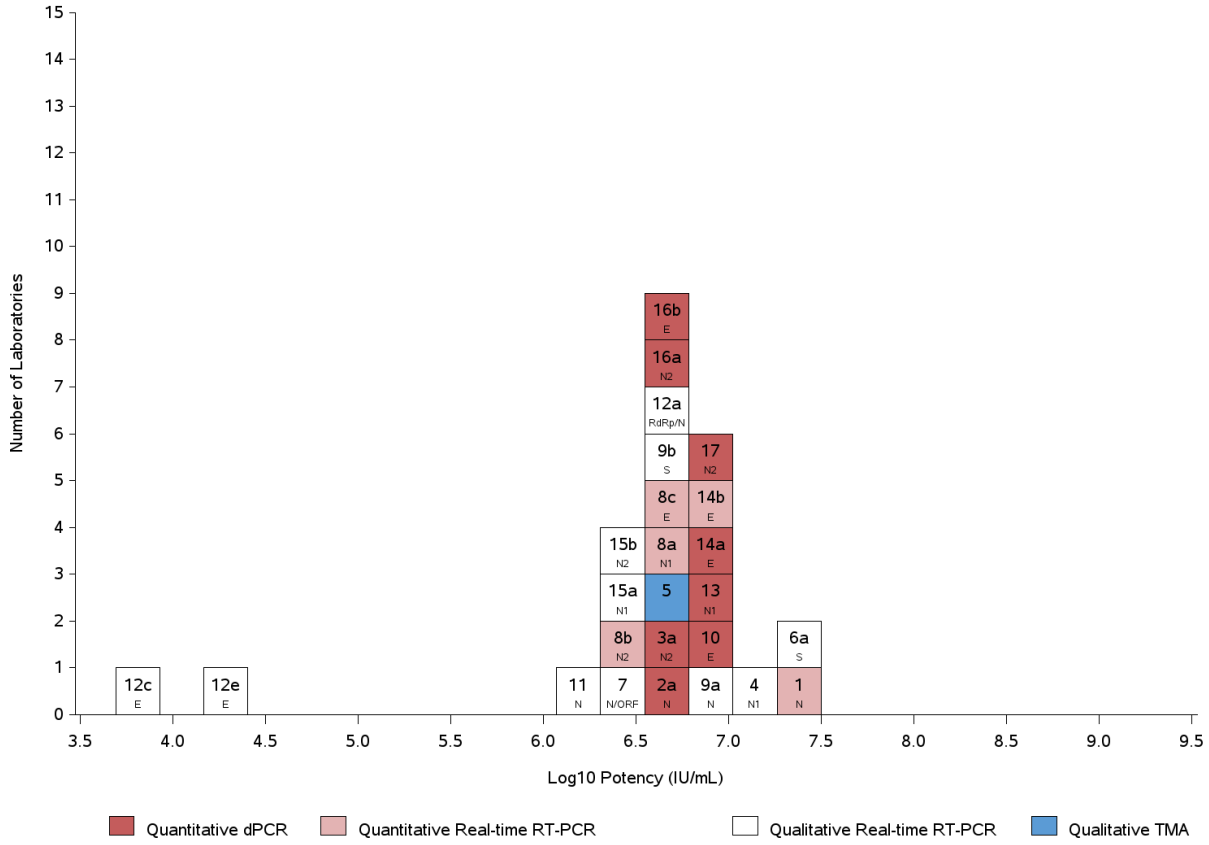


Figure 3C. Potency Estimates for Sample L (LVP 4) Relative to Candidate 2

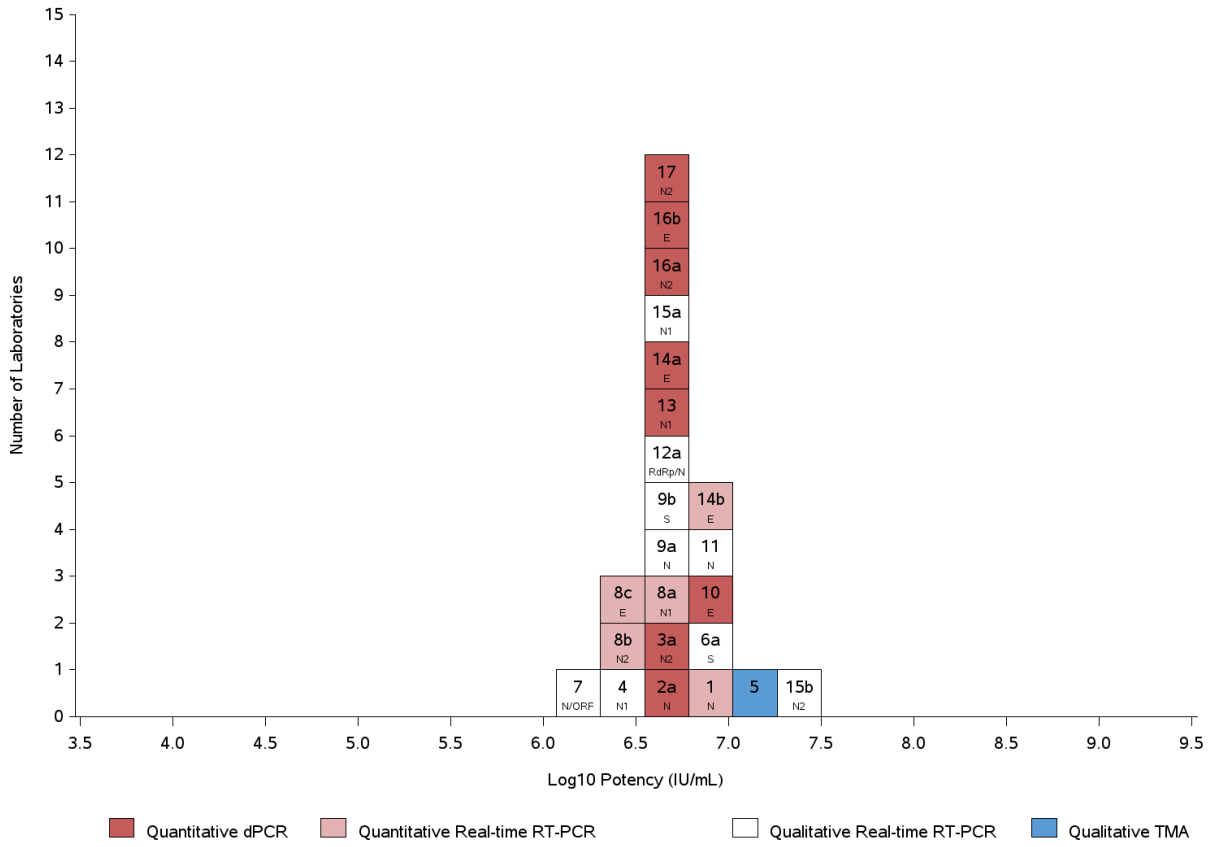


Figure 4A. Potency Estimates for Sample M (Inactivated England - Low)

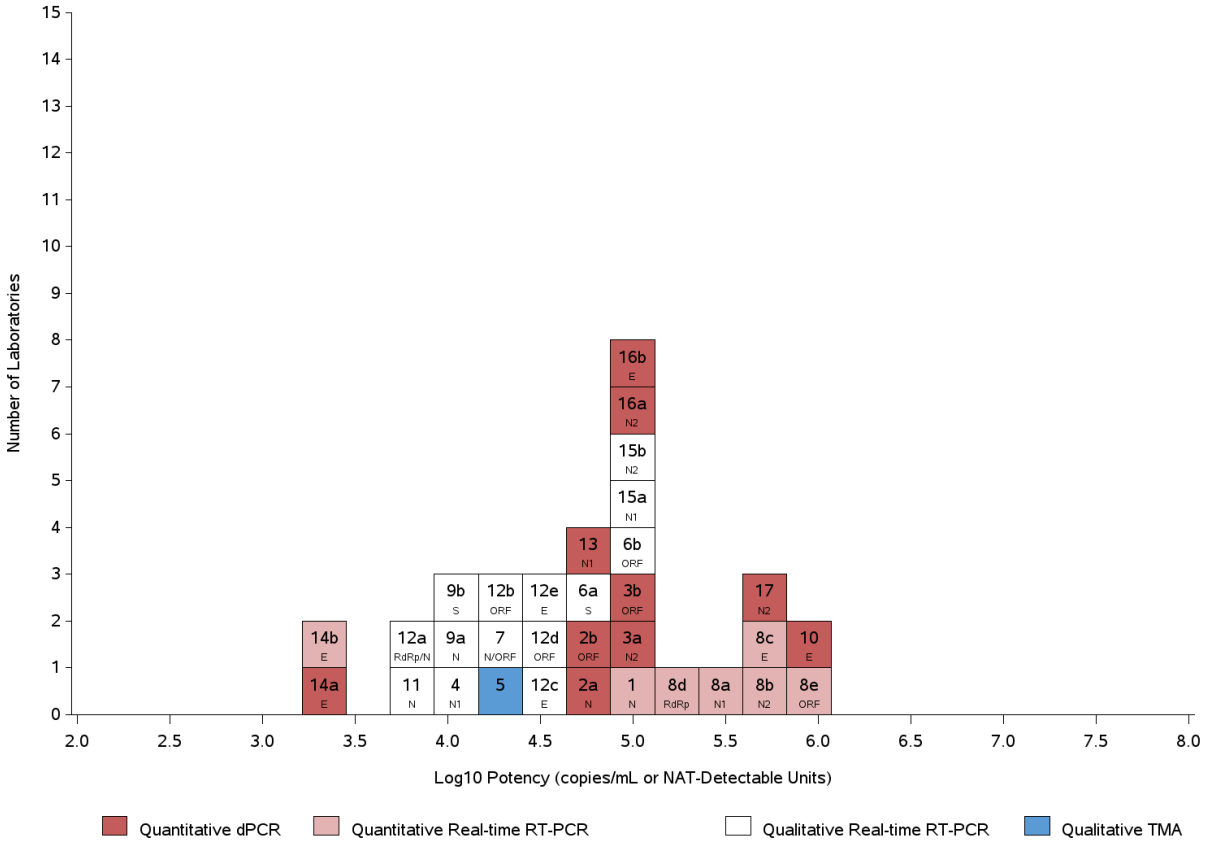


Figure 4B. Potency Estimates for Sample M (Inactivated England - Low) Relative to Candidate 1

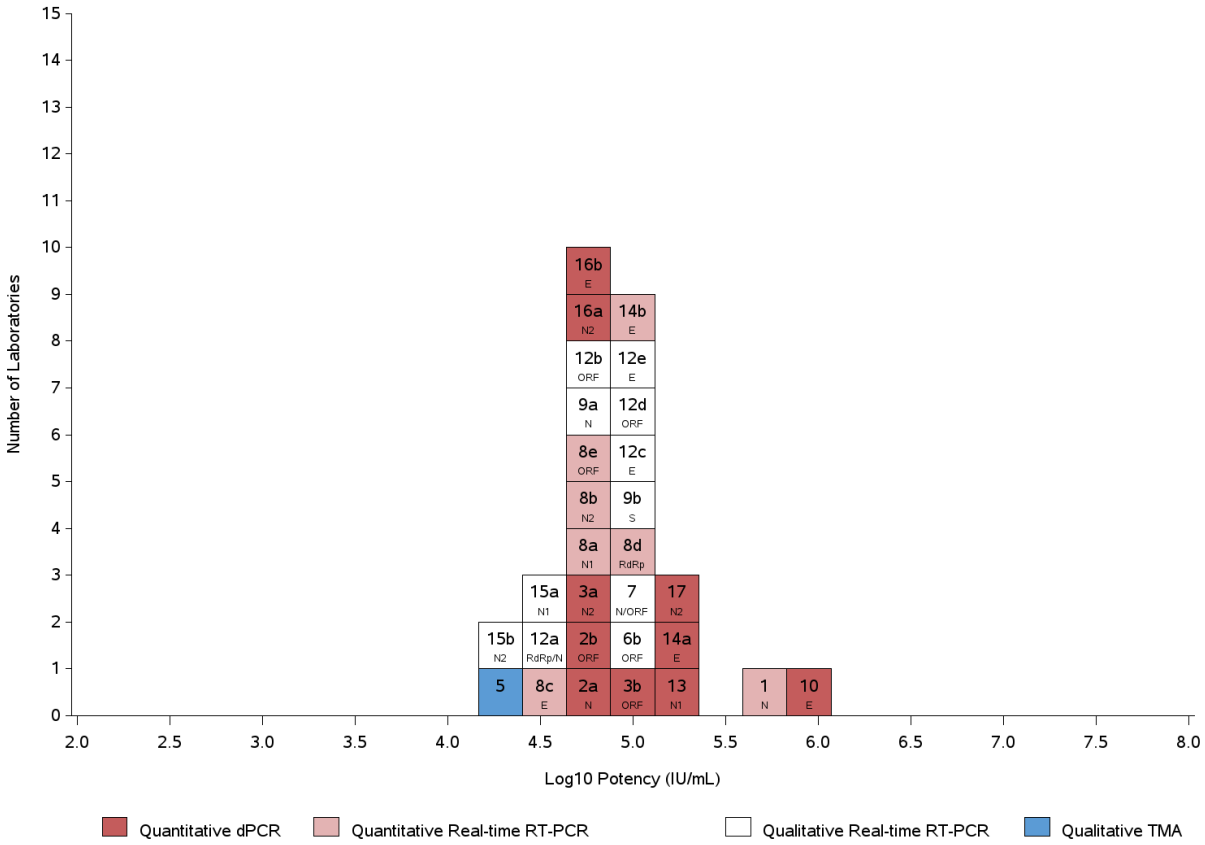


Figure 4C. Potency Estimates for Sample M (Inactivated England - Low) Relative to Candidate 2

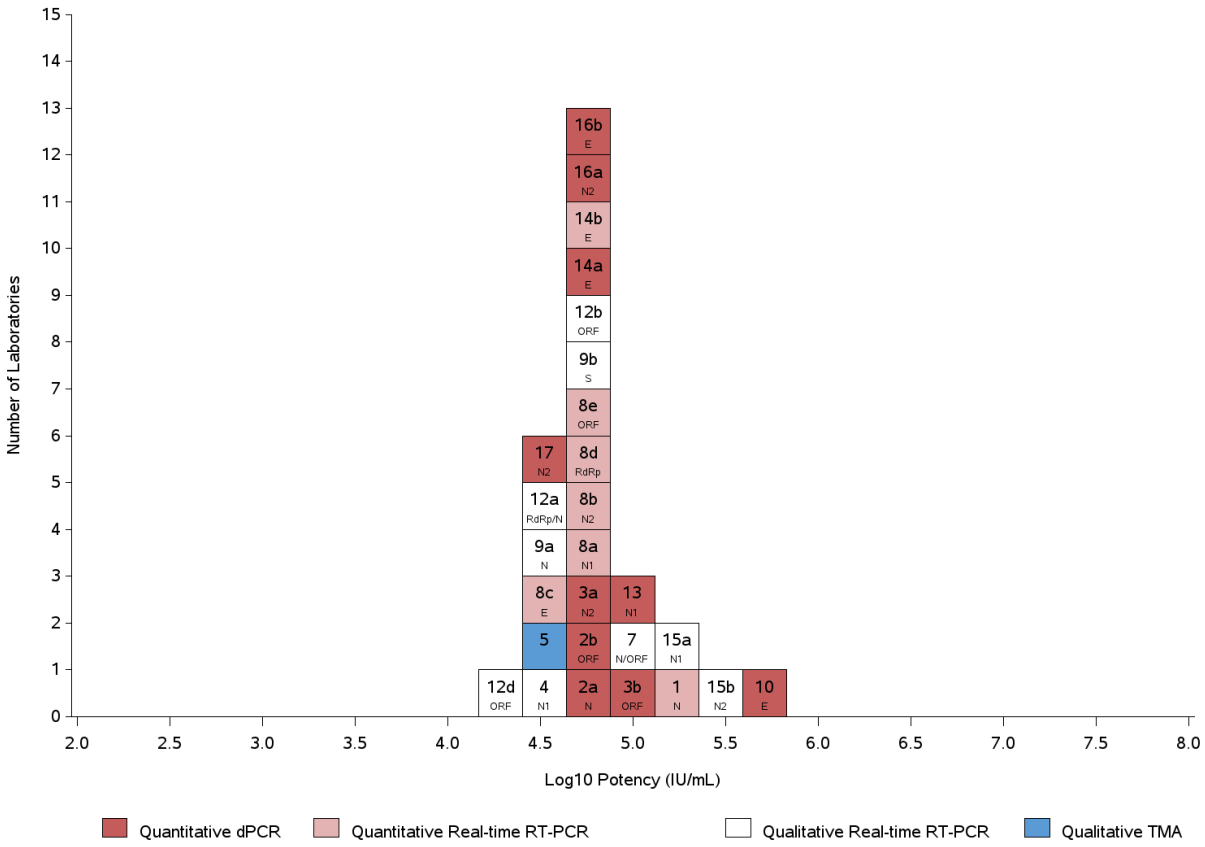


Figure 4A. Potency Estimates for Sample O (Candidate 1 – Inactivated England)

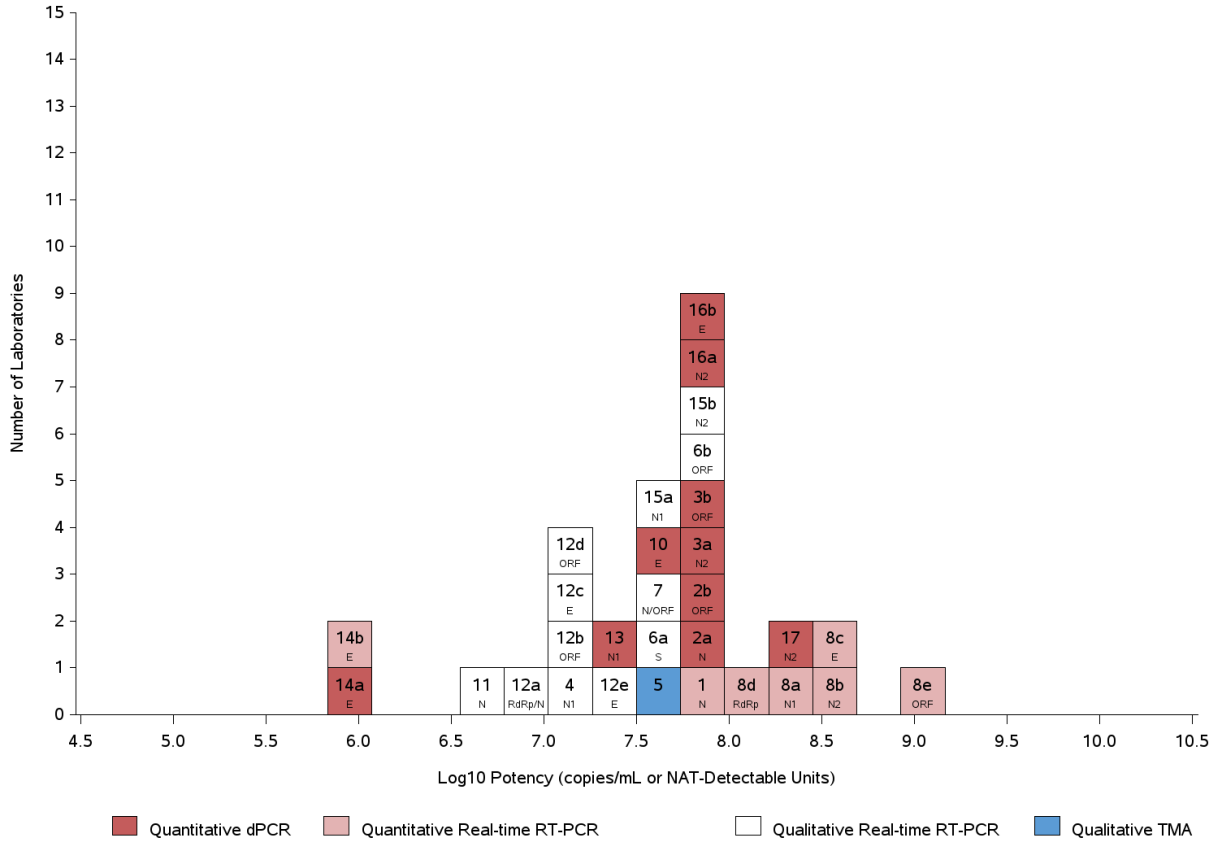


Figure 4B. Potency Estimates for Sample O (Candidate 1 – Inactivated England) Relative to Candidate 2

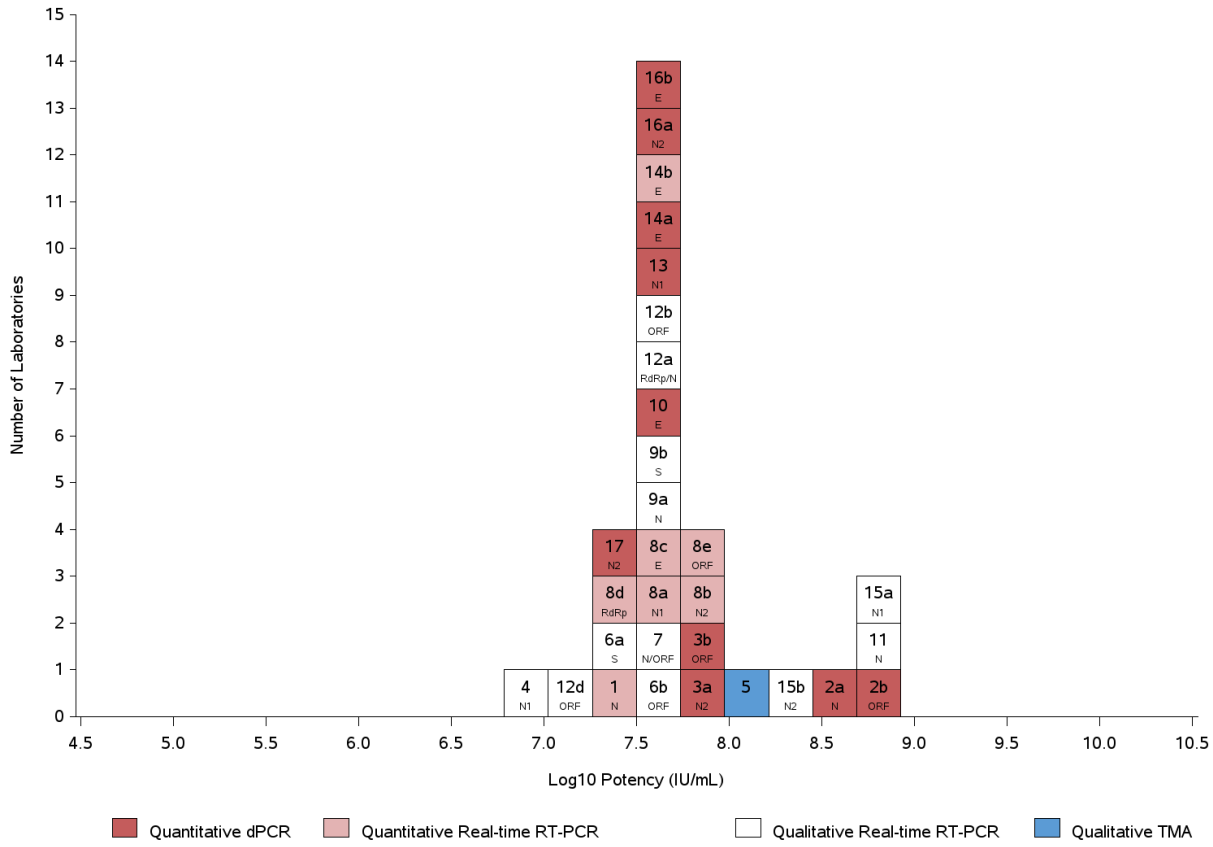


Figure 5A. Potency Estimates for Sample P (LVP full – Low)

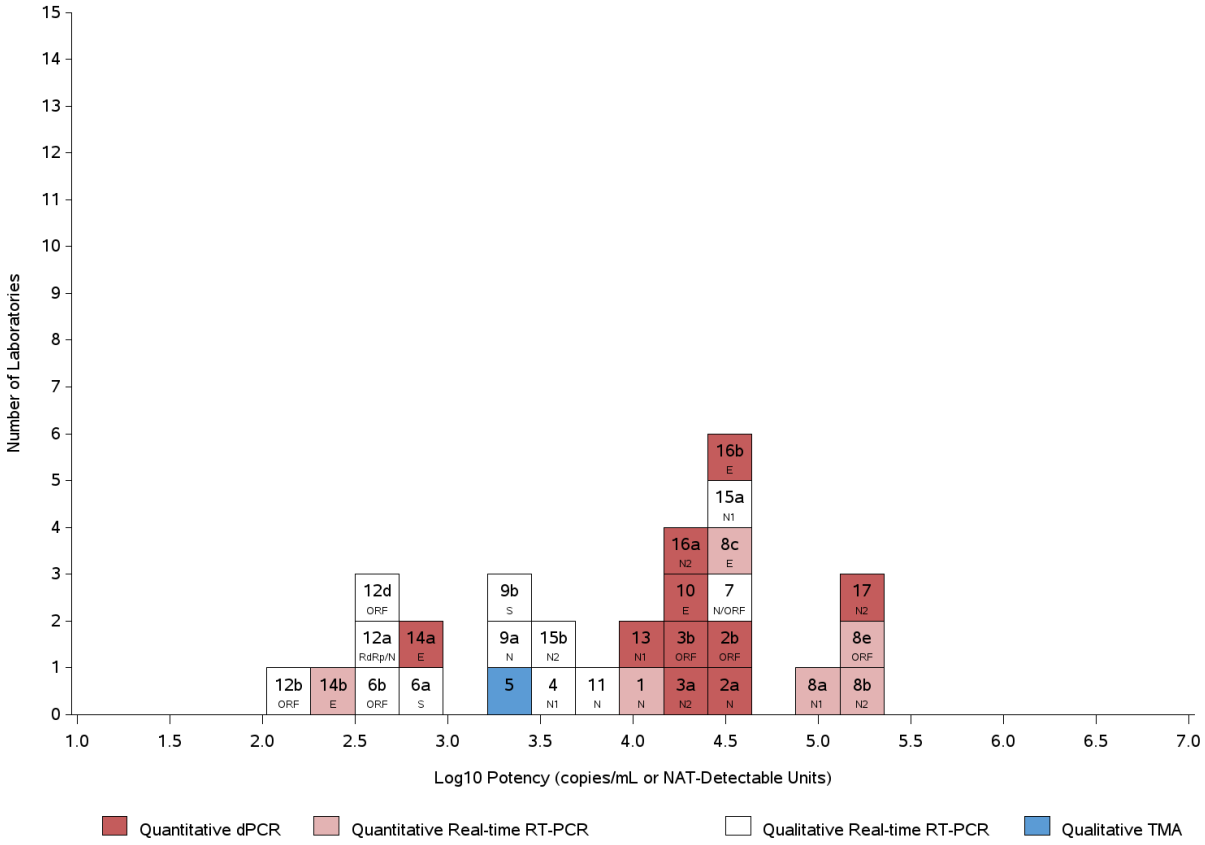


Figure 5B. Potency Estimates for Sample P (LVP full – Low) Relative to Candidate 1

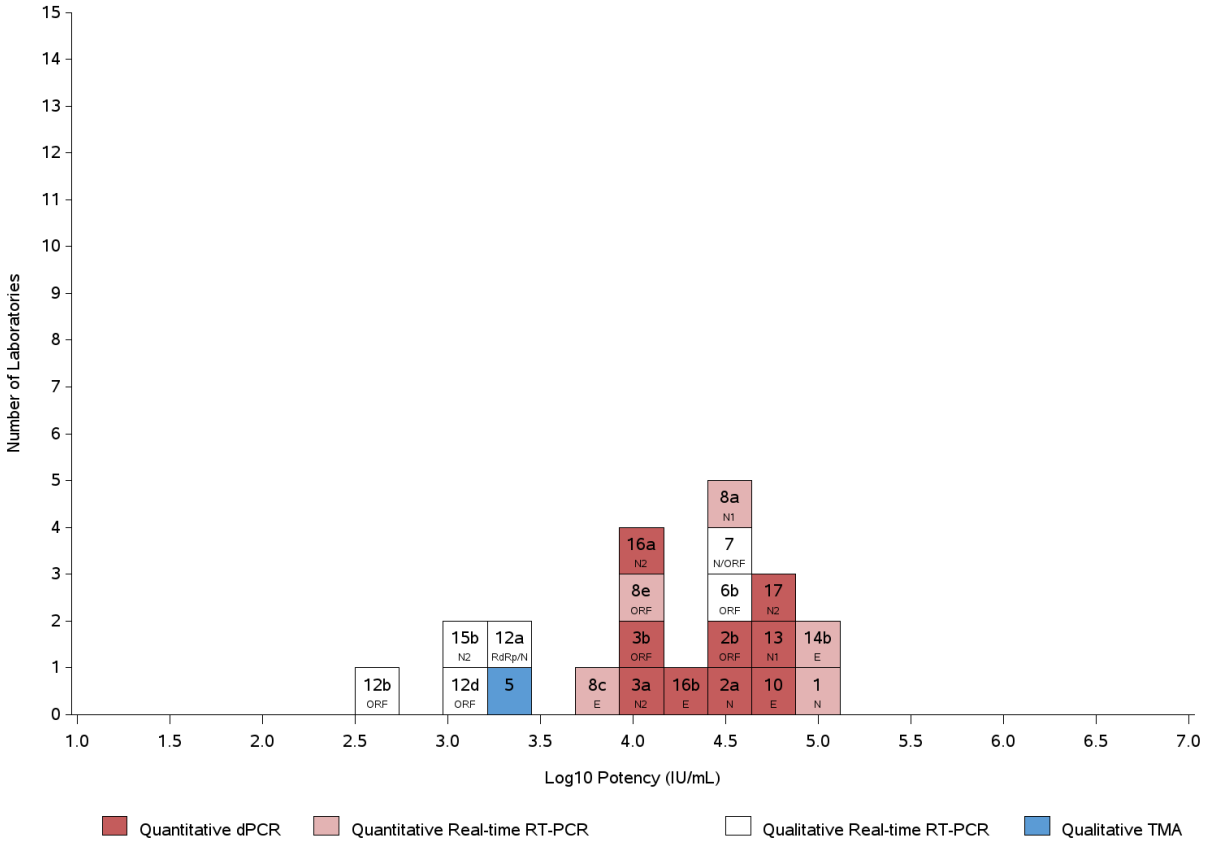


Figure 5C. Potency Estimates for Sample P (LVP full – Low) Relative to Candidate 2

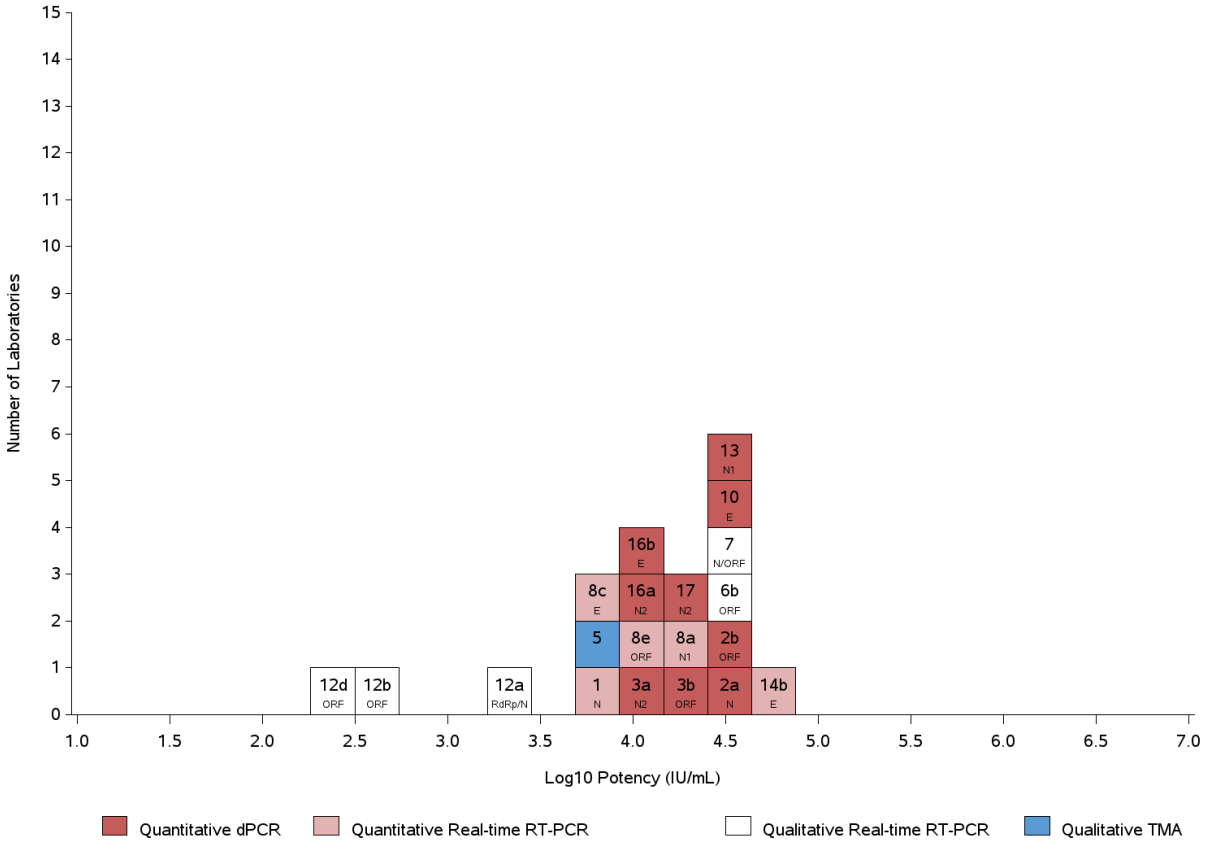


Figure 6A. Potency Estimates of Sample R (Inactivated VIC01 – Low)

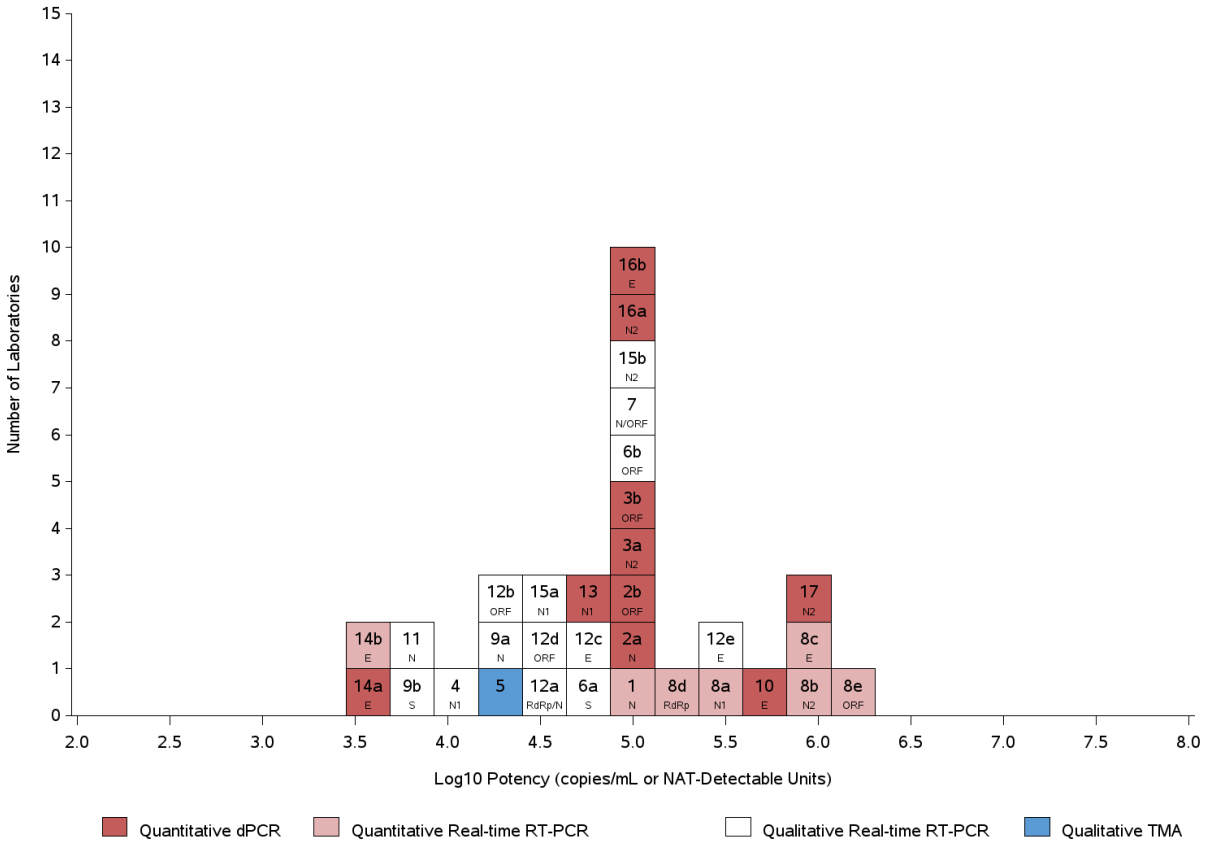


Figure 6B. Potency Estimates of Sample R (Inactivated VIC01 – Low) Relative to Candidate 1

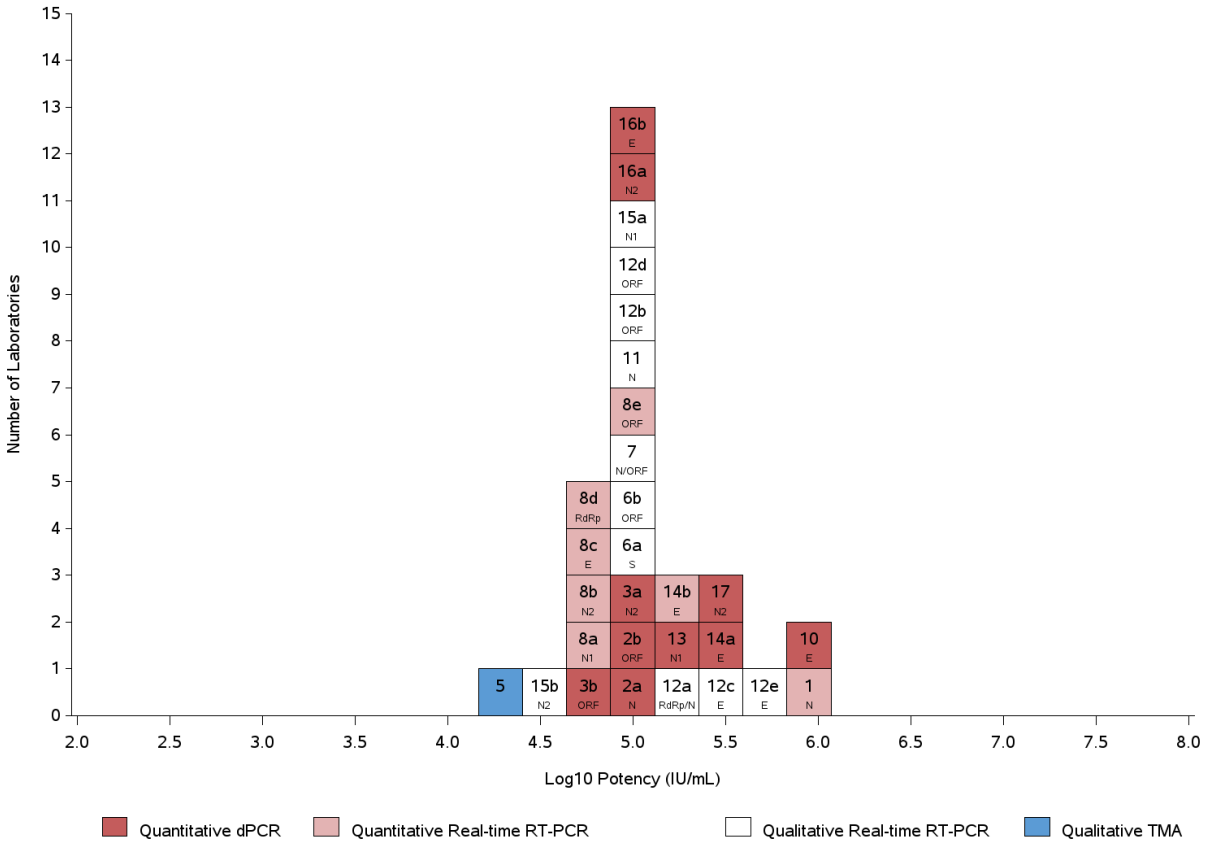


Figure 6C. Potency Estimates of Sample R (Inactivated VIC01 – Low) Relative to Candidate 2

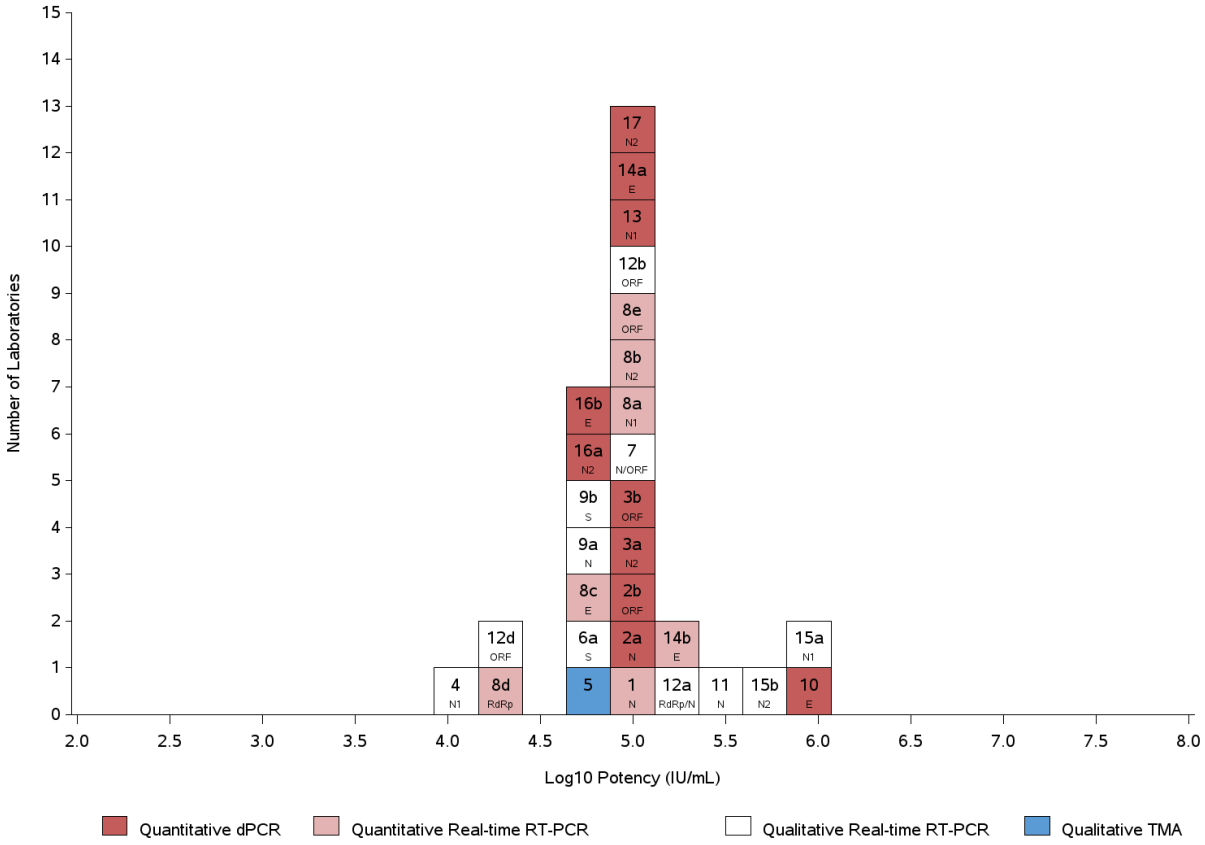


Figure 7A. Potency Estimates of Sample S (Candidate 2 – LVP full)

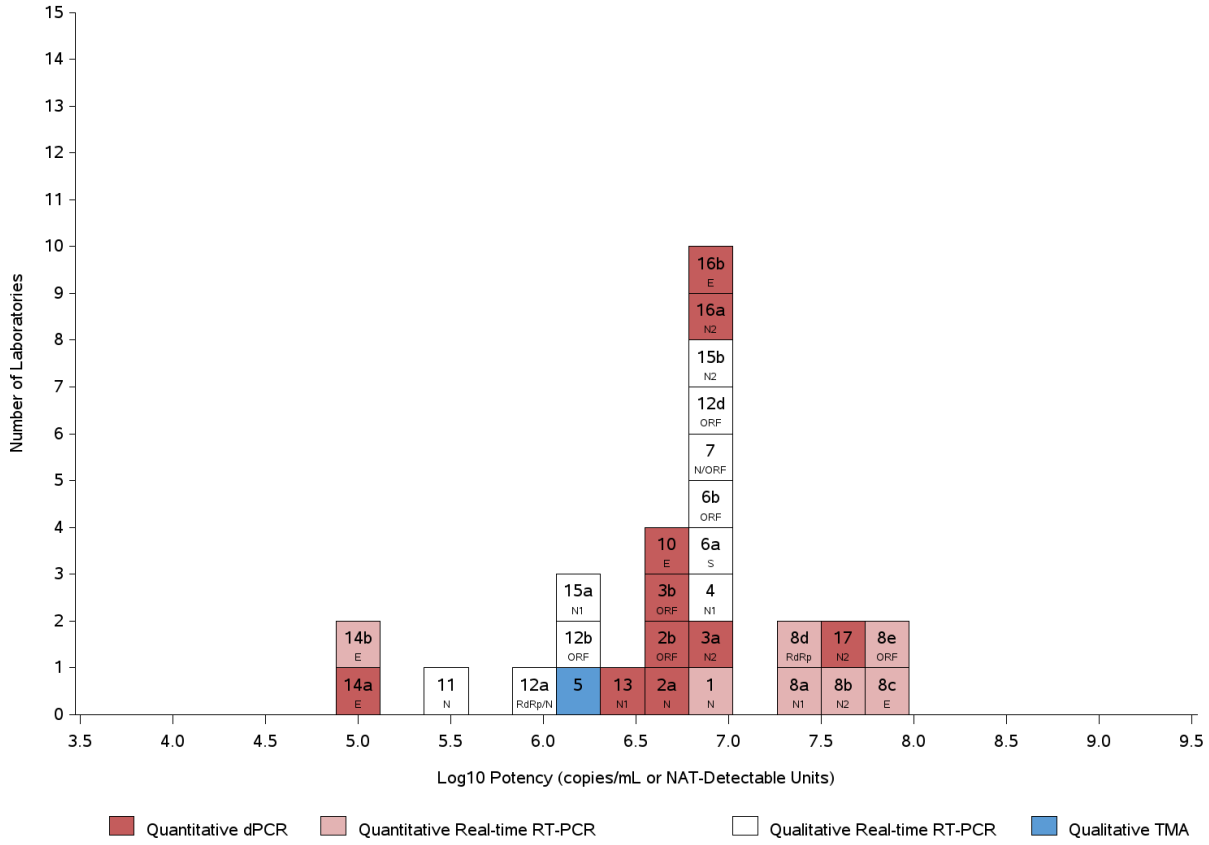
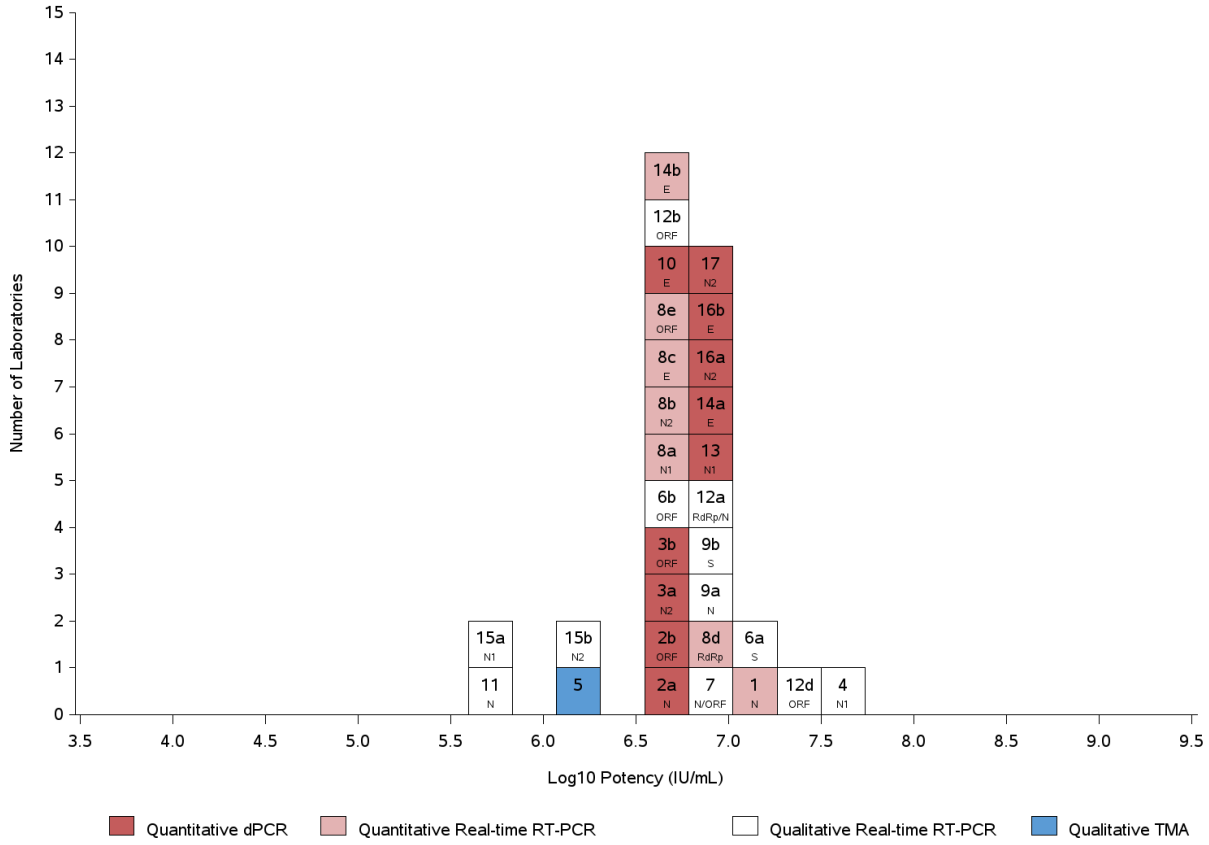


Figure 7B. Potency Estimates of Sample S (Candidate 2 – LVP full) Relative to Candidate 1



Appendix 1

Collaborative study participants

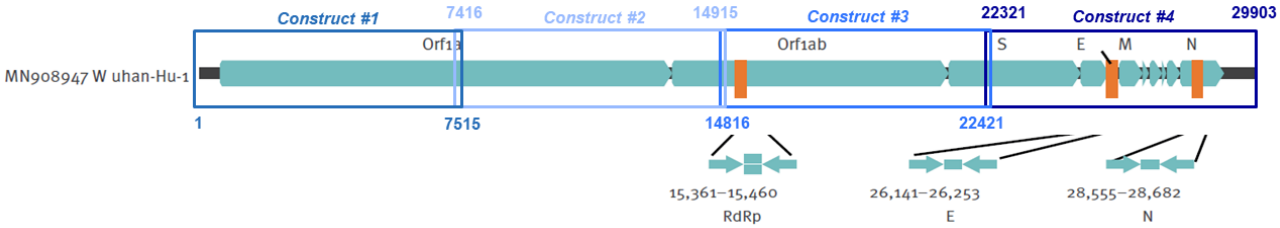
(in alphabetical order by organization)

Participant	Organisation	Country
David Edge, James Turton, Nelson Nazareth	BioGene	UK
Aaron Farnsworth, Susan Van Zanten, Louise Larocque	Biologic and Radiopharmaceutical Drugs Directorate (BRDD), Health Canada	Canada
Arifa Khan, Sandra Fuentes	Center for Biologics Evaluation and Research (CBER), US FDA	USA
David McGivern, Rafaelle Gusmao, Sakthivel Subramaniam	Center for Biologics Evaluation and Research (CBER), US FDA	USA
Paul Carlson	Center for Biologics Evaluation and Research (CBER), US FDA	USA
Shutoku Matsuyama, Kazuya Shirato	Department of Virology III, National Institute of Infectious Diseases (NIID)	Japan
Elena D'Agostini, Giulia Minnucci, Veronica Tettamanzi, Kandarp Shah	DiaSorin Molecular LLC	Italy / USA
Petra Leidinger-Kaufmann	Fast Track Diagnostics	Luxemburg
Antony Carr, Thomas Etheridge	Genome Damage and Stability Centre, University of Sussex	UK
Ji Yuon Lee, Ino Park	Korea Research Institute of Standards and Science (KRISS)	South Korea
Malcolm Hawkins, Jacqueline Fryer, Pia Sanzone	National Institute for Biological Standards and Control (NIBSC)	UK
Denise O'Sullivan, Alison Devonshire, Jim Huggett	National Measurement Laboratory, LGC	UK
Daniel Jarem	Roche Molecular Systems	USA

Carol-Anne McInally, Carol Imlach	Scottish National Blood Transfusion Service, NHS National Services Scotland	UK
Alexandra Martin, Cecile Aslanian, Rosario Tizzone	Stilla Technologies	France
Jia-Chuan Hsu, Po-Chih Wu, Po-Lin Lin	Taiwan Food and Drug Administration (TFDA)	Taiwan
Megan Cleveland, Peter Vallone	U.S. National Institute of Standards and Technology (NIST)	USA

Appendix 2

Schematic representation of the segregation of the SARS-CoV-2 RNA sequence inserted within the lentiviral plasmid used to produce chimeric lentiviral particles. Numbers denote nucleotide position.



Adapted from Corman et al., Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25(3), (2020)

Appendix 3 - CS679 Study Protocol

Protocol for the WHO collaborative study to establish the 1st International Standard for SARS-CoV-2 RNA

This multi-centre International collaborative study aims to evaluate candidate preparations to serve as 1st WHO International Standard for SARS-CoV-2 RNA organized by NIBSC in collaboration with the World Health Organization (WHO).

International Standards (IS) are recognized as the highest order of reference materials for biological substances and they are assigned potencies in International Units (IU). International Standards are used to quantify the amount of biological activity present in a sample in terms of the IU, allowing assays from different laboratories to be compared. This makes it possible to better define parameters such as the analytical sensitivity of tests. The availability of an IS for SARS-CoV-2 RNA will facilitate the standardisation and harmonisation of COVID19 nucleic acid amplification technique (NAT) assays used for detection of SARS-CoV-2 infection. The establishment of such a standard will follow published WHO guidelines and be submitted for formal endorsement by the WHO Expert Committee on Biological Standardization (ECBS) [1].

Aims

The aims of this WHO international collaborative study are to

- assess the suitability of different SARS-CoV-2 preparations to serve as the International Standard with an assigned unitage per ampoule for use in the harmonisation of COVID-19 NAT assays.
- characterise the standard preparations in terms of reactivity/specificity in different assay systems.
- assess each preparation's potency i.e. readout in a range of typical assays performed in different laboratories.
- assess commutability i.e. to establish the extent to which each preparation is suitable to serve as a standard for the variety of different samples and assay types.
- recommend to the WHO ECBS, the standard preparation(s) found to be suitable to serve as the International Standard and propose an assigned unit.

Materials

Coded study samples

The study samples should be stored at -20°C or below. The study samples shall not be administered to humans or animals in the human food chain.

All samples will be provided coded and blinded to the participants. The samples are labelled "SARS-CoV-2 RNA CS679 Sample x" where x is K, L, M, N, O, P, Q, R, S, T. The coded samples may include negative samples as well as reactive samples. Laboratories will receive at least 4 sets of study samples which should allow for 3 independent assays (plus 1 spare) by one method. Laboratories with more than one method or which require more than 0.5 mL of material per method will receive additional sample sets to allow 3 independent tests per method.

Packaged SARS-CoV-2 RNA in lentiviral particles, armoured RNA

This material is based on a lentiviral vector (LVV) plasmid in which the Human Immunodeficiency Virus (HIV-1) genes have been substituted with Wuhan-Hu-1 isolate (GenBank MN908947.3) SARS-CoV-2 using a similar approach as described in [2]. The SARS-CoV-2 genome has been divided into four overlapping fragments and inserted within the long terminal repeats of the LVV plasmid. Single nucleotide mutations have been randomly inserted in the SARS-CoV-2 sequences to prevent protein expression. The sequences of the four constructs have been deposited to GenBank: accession numbers MT299802, MT299803, MT299804, MT299805. The four LVV-SARS-CoV-2 constructs were each individually transfected into HEK293T/17 cells together with a HIV-1 packaging plasmid p8.9, kindly donated by Prof. D. Trono [3]. Particles contained in the supernatant of the transfected cells were purified by ultracentrifugation on a 20%-sucrose cushion and resuspended in universal buffer.

Study samples comprise particles containing one of the four individual constructs or an equimolar mix of the four particles.

Inactivated SARS-CoV-2

Two SARS-CoV-2 isolates are included in this study. BetaCoV/Australia/VIC/01/2020 was kindly provided by the Victorian Infectious Disease Reference Laboratory, Royal Melbourne Hospital, Australia [4]. England/2/2020 was kindly donated by Public Health England, UK. The viral stocks were amplified at NIBSC in VERO-E6 cells (one further passage from that received), the Melbourne strain was provided at passage 4 and the England strain at passage 1.
Viruses were inactivated by acid and heat treatment as indicated in Annex 1. Every batch was validated for inactivation by serial blind passage for over 3 weeks on permissive cells, monitoring for CPE and any quantifiable increase in viral RNA. The inactivation procedure was approved by NIBSC Biological Safety sub-committee.

All study samples, LVV-SARS-CoV-2 RNA and the inactivated SARS-CoV-2, are formulated in 10 mM Tris-HCl (pH 7.4), 0.5% human serum albumin and 1% D-(+)- Trehalose and human genomic DNA dehydrate. Preparations are either freeze-dried into 2.5mL ampoules (samples O and S) or liquid-frozen filled in 0.5 mL aliquots within screw cap tubes.

Lyophilised material (sample O and S) requires reconstitution in 0.5 mL of DNase and RNase-free water (see Instruction for Use).

ALL THE STUDY SAMPLES REQUIRE EXTRACTION PRIOR TO TESTING

Assay Methods

For testing the study samples, participants are requested to follow the RNA extraction procedure and use the NAT assays in routine use in their laboratory for the detection of SARS-CoV-2. Laboratories may use multiple methods to test the study materials, provided that the study design (see below) is followed for each method.

Design of Study

Participants are requested to:

- Perform 3 independent tests on different days for detection of SARS-CoV-2 nucleic acid
- Reconstitute freeze-dried samples according to the Instructions for Use (IFU) supplied with the sample shipment. Use a freshly thawed/reconstituted sample for each independent test
- For the liquid frozen samples, use a freshly thawed aliquot for each independent test.
- For each independent test, prepare a series of dilutions from each coded sample, using the sample matrix specific to the individual assay(s) (e.g. PBS, buffer, others). **The optimal dilution range should cover at least 5 to 6 points including one point beyond the endpoint dilution.** Adjust dilutions accordingly for subsequent assays if needed. Record in the Excel reporting sheet any changes to the dilutions tested.
- Use the Excel reporting sheet to record for each dilution the raw assay readout (e.g. Ct, copy number, etc.). Additionally, provide the result as per analysis in your laboratory. Our statistician will use the raw data readouts to perform statistical analysis.
- If feasible, include all study samples in each assay so that the quantification of the SARS-CoV-2 nucleic acid relative to one another may be calculated. Please note in the reporting sheet, if it is not practical to test all samples concurrently and indicate which samples were tested concurrently.
- Record in the Excel reporting sheet any deviations from the assay protocol.

Results and Data Analysis

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

The confidentiality of each laboratory is assured with each participant being anonymous to the other laboratories. Analysis of the study will assess the potencies of each material relative to each other, and the sensitivity of 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 most appropriate preparation to serve as the 1st WHO IS for SARS-CoV-2 RNA. Participants' comments will be included in the report prior to submission to the WHO ECBS. Study participants will be notified of the outcome of the study after the WHO ECBS meeting.

Participation in the feasibility 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;
- 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 NIBSC study organiser.

NIBSC, 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, NIBSC 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.

Deadline for return of completed results reporting sheet is 4 weeks from receipt of study materials. If it is not practical to return results within 4 weeks, please inform Giada Mattiuzzo.

All completed results spreadsheets should be returned electronically to:

Dr Giada Mattiuzzo
Senior Scientist
Emerging Viruses Group
Division of Virology
National Institute for Biological Standards and Control
Blanche Lane
South Mimms
Hertfordshire
EN6 3QG
UK
Tel. +44(0)1707 641283
Giada.Mattiuzzo@nibsc.org

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.

[2] Mattiuzzo et al., Development of lentivirus-based reference materials for Ebolavirus nucleic acid amplification technology-based assays. PLoS One, 2015 10(11): e0142751.

[3] Zufferey et al., Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo.Nat Biotechnol 15: 871–875

[4] Caly et al. Isolation and rapid sharing of the 2019 novel coronavirus (SARS-CoV-2) from the first patient diagnosed with COVID-19 in Australia. Med J Aust 2020; 212 (10): 459-462

Annex 1

Division of Virology

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

Author: Emma Bentley
Authoriser: Giada Mattiuzzo
Date: 21st June 2020

SUMMARY

The purpose of the protocol is to inactivate SARS-CoV-2, preserving the integrity of the RNA. The inactivated virus can be used for standardisation of nucleic acid amplification techniques. A similar protocol was used by Public Health England for inactivation of Ebola virus. The principle of the inactivation procedure is a combination treatment of low pH (addition of acetic acid) followed by heat-treatment. This protocol has been validated for SARS-CoV-2 stock at 1.5 x 10^6 infectious units/mL.

MATERIALS

- SARS-CoV-2 harvest within cell culture supernatant containing phenol red, amplified as described in SOP "VIR/FCSCL3/Amplification of Coronaviruses Stocks" (w/b no. *tbc*)

- 99% Acetic Acid Glacial (VWR: #20104.334)
- 2M NaOH sterile filtered (Media)
- Gibco DMEM (1X) Phenol Free (ThermoFisher: # 31053-028)
- PBS-A (Media)
- Amicon® Ultra-4 or -15 10K Centrifugal Filter (Merck: #UFC8010 or #UFC9003)
- 15 or 50mL Falcon tube
- Sealable, zip lock, bag
- 60°C water bath
- 2x water bath thermometers
- Centrifuge capable of rotation at 4000*xg*

PROCEDURE

All the documents associated with this SOP must been read and understood

Step 1 - Acid Inactivation:

1. Before entering the CL3 lab locate the viral stock on Item tracker.
2. Update storage records on ItemTracker
3. Turn on MSC and ensure appropriate checks are performed on MSC and recorded on log sheet prior to use

All work with virus must be performed within the MSC

4. Prepare the lab according to the CoP and insert in the MSC all the consumables needed
5. Collect the viral stock from storage

Wear thermal gloves when handling material at low temperature (e.g. -80°C and dry ice)

6. Place virus stock in MSC on IMS70%-soaked tissue to defrost.
7. Add 3% v/v Acetic Acid to the SAR-CoV-2 culture and mix by inversion (e.g. for 35mL culture, add 1.05mL acetic acid)

   NOTE: 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
8. Incubate for 15 minutes at ambient temperature
9. Neutralise the acid by addition of 2M NaOH, dropwise while mixing, until the phenol red indicator colour is restored to red representing pH 7.4 (e.g. to neutralise a volume of 36mL requires addition of 8.5-9mL of 2M NaOH)

Step 2 – Heat Inactivation:

1. Set a water bath to 60°C ± 2 and monitor temperature with two external thermometers. Allow to stabilise for at least 30 minutes prior to use
2. Transfer acid inactivated SARS-CoV-2 culture to a 15 or 50mL falcon tube, if not already, and then seal within a zip lock bag making sure to remove air

3. Spray the bag with IMS70% and let dry before removing from MSC
4. Transfer to 60°C water bath and ensure the virus culture is submerged below the water line for even heat dispersion
5. Incubate for 1 hour, checking the temperature at least twice
6. Following incubation, allow virus culture to equilibrate to ambient temperature
7. The virus is now inactivated and can be removed from CL3 lab

Step 3 – Purification, if required:

It is only suggested to purify the inactivated SARS-CoV-2 culture, removing inhibitory chemicals, where they may interfere with the downstream intended use (e.g. adding to cells for validation of the inactivation)

1. Apply the acid-heat inactivated SARS-CoV-2 culture to an Amicon® Ultra-4 or -15 10K Centrifugal Filter
2. Spin at 4000*xg* for 25 minutes
3. Check residual volume has reached approximately 250µL or 500µL if using an Ultra-4 or -15 filter respectively. If not, continue to spin at 4000*xg* for 20-minute intervals
4. Once complete, apply equal volume of either phenol free DMEM or PBS-A to the column as added at Step 1
5. Repeat centrifugation at 4000*xg* until residual volume has reached 250µL or 500µL
6. Once complete, reconstitute and remove from column into desired matrix

Appendix 4

Full Methods for RT-PCR and Sequencing of 20/138 and 20/146

cDNA preparation using Maxima H Minus RT (Thermo)
For 20/138, 10 µl RNA was incubated with 10 pmol Oligo-dT, 10 nmol dNTP and 0.4 µl DMSO in a volume of 15 µl. For 20/146, 10 µl RNA was incubated with 1.25 pmol specific primer (each in a separate reaction), 10 nmol dNTP and 0.4 µl DMSO in a volume of 15 µl. Reactions were incubated at 65°C for 5 minutes followed by addition of 4 µl 5 × RT Buffer and 1 µl Maxima RT. Reverse transcription was performed at 60°C for 60 minutes followed by inactivation at 85°C for 5 minutes. 0.4 U RNaseH were added and the reaction incubated for 20 minutes at 37°C. cDNA was diluted 1:2.5 in water prior to PCR.

PCR using Platinum SuperFi II DNA Polymerase (Thermo)
2.5 µl diluted cDNA was incubated with 200µM dNTPs, 0.35 µM each primer, 1 % (v/v) DMSO and 0.5 µl Platinum SuperFi II in a final volume of 25 µl 1 × reaction buffer. Following an initial denaturation at 98°C for 60s, 35 cycles of amplification were performed with denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 72°C for 6 minutes. Final extension was performed at 72°C for 10 minutes. Products were electrophoresed on 0. 7 % agarose-TBE alongside GeneRuler Plus 1 kb ladder (Thermo) and visualised with SybrSafe (Thermo).

Preparation of Sequencing Library and Data Analysis
Amplicons were purified using AmpureXP beads (Beckman Coulter), eluted in 10 mM Tris-Cl and quantified using the Qubit High Sensitivity dsDNA Kit (Thermo). An equimolar pool of the four overlapping amplicons was prepared and sequencing library generated using the DNA Flex Kit (Illumina), according to the manufacturer's recommendations. Sequencing was performed on Illumina MiSeq, with 2 × 251 PE reads. Data were imported into Geneious v10.2.3 (Biomatters) and trimmed for residual sequencing adapters and low quality (< Q30) bases. Reads of $\geqslant$ 50 bases were mapped to the lentiviral plasmid (MT299802-5) or hCoV-19/England/02/2020|EPI_ISL_407073 reference with a minimum overlap of 50 bases at 90 % identity and maximum 10 % mismatches per read. Strict consensus sequences were generated and compared to the lentiviral plasmid or England/02/2020 reference sequence (GISAID accession: EPI_ISL_407073).

Table 1: PCR primers for 20/138

Amplicon (length (bp))	Primer name	Sequence (5' – 3')
1 (7507)	CoV2_6_ F	AGGTTTATACCTTCCCAGGTAAC
1	CoV2_Con_1_10610_R	GCAGGCTATTACGTTTGTAACACA
2 (7703)	CoV2_2_3094_F	TCGACACATCTTCTTTGCATCA
2	pSF-lenti_10742_R	TGGCTAAGATCTACAGCTGCC
3 (7575)	CoV2_Con_3_3095_F	CGACATCTACCAACAATGTGTGA

3	CoV2_Con3_10669_R	AAAGTCCTAGGTTGAAGATAACCC
4 (7558)	Construct_4_3095_F	CGACTCTTCTTCAGGTTGGAC
4	CoV2_Con4_10652_R	TTTTTGTCATTCTCCTAAGAAGCTATT

Table 2: Reverse transcription and PCR primers for 20/146

Amplicon (length (bp))	Primer name	Sequence (5' – 3')
1	CoV2_7520_R	TTGCTCTATTACGTTTGTAACACA
2	CoV2_Con_2_10585_R	ACAGCCACCATCGTAACAATCA
3	CoV2_Con_3 10669_R	AAAGTCCTAGGTTGAAGATAACCC
4	CoV2_Con_4 10652_R	TTTTTGTCATTCTCCTAAGAAGCTATT
1 (7515)	CoV2_6_ F	AGGTTTATACCTTCCCAGGTAAC
1	CoV2_7520_R	TTGCTCTATTACGTTTGTAACACA
2 (7491)	CoV2_7411_ F	AATGTACATCTTCTTTGCATCA
2	CoV2_Con2_10585_R	ACAGCCACCATCGTAACAATCA
3 (7575)	CoV2_14812_F	TATAATCTACCAACAATGTGTGA
3	CoV2_Con3_10669_R	AAAGTCCTAGGTTGAAGATAACCC
4 (7577)	CoV2_22299_F	GAAGTTATTTGACTCCTGGTGATTC
4	CoV2_Con4_10652_R	TTTTTGTCATTCTCCTAAGAAGCTATT

Quantitative Analysis of Low Frequency Variant within 20/138 – Construct 1

Analysis of NGS data for construct 1 of 20/138 revealed a region of low coverage at the 5’ end of the ORF1ab gene spanning approximately 2,700 nucleotides (Figure 1). This data cannot be considered quantitative as PCR amplification during sample preparation can lead to an exaggerated dominance due to competition between the fragments amplified. To evaluate the relative proportion of constructs containing this probable deletion, Real-time RT-PCR was performed using 20/146 as a reference with three primer sets spanning the construct as indicated in Figure 1. Evaluation of the data revealed an approximate 0.5 Ct discrepancy between Primer set 1 targeting the region of low coverage in comparison to Primer sets 2 and 3 (Figure 2).

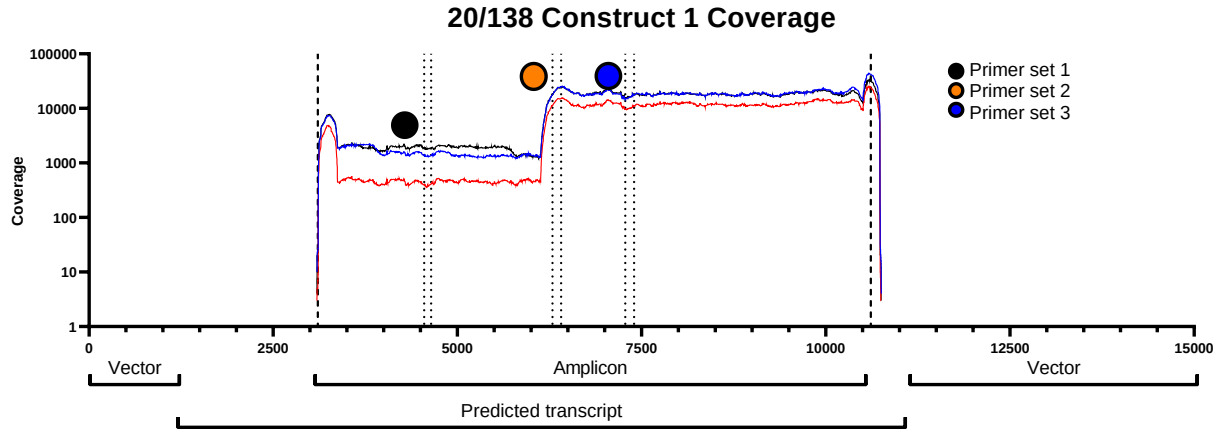


Figure 1. NGS Coverage of 20/138 Construct 1 RNA transcript. Plots indicate coverage across amplicon region in three independent replicates. Dashed lines indicate locations of primers used to generate the amplicon. Expected transcript and vector regions are marked. The locations of Primer set 1-3 used for Real-time RT-PCR are annotated.

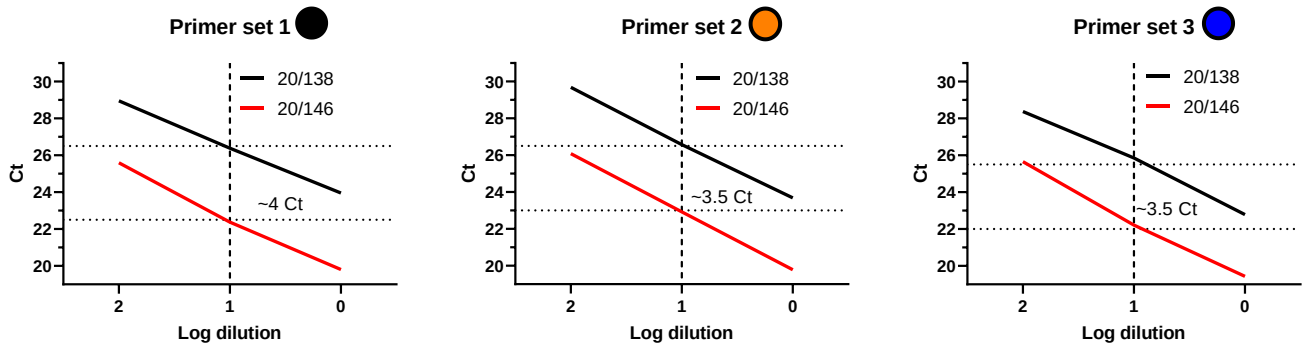


Figure 2. Relative potency of 20/138 compared to 20/146 by Real-time RT-PCR quantification using three primer sets. There is an approximate 0.5 Ct shift between the standard curves using primer set 1 which targets the region of lower coverage, in comparison to primer sets targeting the junction (primer set 2) and region of higher coverage (primer set 3).

Appendix 5 - Proposed IFU for candidate International Standard

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO International Standard
WHO International Standard for SARS-CoV-2 RNA
NIBSC code: 20/146
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The WHO International Standard for anti-SARS-CoV-2 RNA consists of acid-heat inactivated England/02/2020 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 of nucleic acid amplification technique (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 material is not of human or bovine origin. 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 is 7.40 Log10 IU/ampoule. After reconstitution in 0.5mL of molecular grade water or PBS, the final concentration of the preparation is 7.70 Log10 IU/mL. .

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial of 20/146 contains 0.5mL of lyophilised, non-infectious, England/02/2020 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. The full consensus sequence of the RNA is deposited under GenBank accession MW059036.

5. STORAGE

The International Standard 20/146 should be stored at -20°C or below upon receipt.
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 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, 20/146 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. Reference Materials should be stored on receipt as indicated on the label.

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

9. REFERENCES

(1) Bentley et al., Collaborative Study for the Establishment of a WHO International Standard for SARS-CoV-2 RNA, 2020, WHO Expert Committee on Biological Standardization. WHO/BS/2020.xxxx

10. ACKNOWLEDGEMENTS

We express our thanks to Public Health England (PHE) for provision of the England/02/2020 isolate of SARS-CoV-2. We gratefully acknowledge the important contributions of the collaborative study participants, particularly in meeting the tight timeframes of this studyWe would also like to thank NIBSC Standards Production and Development staff for the formulation and distribution of materials.

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):	material of human origin.		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

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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: 0.25 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

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.

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Appendix 6 - Proposed IFU for candidate Working Reagent

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO Reference Reagent
Working Reagent for SARS-CoV-2 RNA
NIBSC code: 20/138
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The WHO Working Reagent for SARS-CoV-2 RNA comprises chimeric lentiviral particles (LVPs) in which the Human Immunodeficiency Virus (HIV-1) genes have been substituted with those of the Wuhan-1 isolate of SARS-CoV-2. The preparation has been evaluated in parallel to the WHO International Standard for SARS-CoV-2 RNA in a WHO International Collaborative study (1). The intended use of the working reagent is as secondary standard or positve control in nucleic acid amplification technique (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.

This product is a genetically modified material; It is the responsibility of the end user to seek local biosafety approval for the storage and handling of the material in their workplace. 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 Working Reagent 20/138 was calibrated as part of the collaborative study for the establishment of a WHO International Standard for SARS-CoV-2 RNA (NIBSC cat. no. 20/146) and was found to have a unitage of 6.73 Log10 IU/mL, with 95% confidence limits of 6.58 to 6.88 when reconstituted within 0.5mL of molecular grade water or PBS.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial of 20/138 contains 0.5mL of lyophilised, non-infectious, synthetic SARS-CoV-2 RNA packaged within HIV-1 particles. Single nucleotide mutations have been randomly inserted into the SARS-CoV-2 RNA sequences to prevent protein expression and the material was prepared by dividing the SARS-CoV-2 genome into four overlapping fragments [1]. The chimeric LVPs are formulated equimolar 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. The full consensus sequence of the four chimeric LVPs containing SARS-CoV-2 RNA are deposited under GenBank accessions MW059032, MW059033, MW059034, MW059035

5. STORAGE

The Working Reagent 20/138 should be stored at -20°C or below upon receipt.
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, 20/138 should be diluted in the matrix appropriate to the material/assay being used.

8. STABILITY

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

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

9. REFERENCES

(1) Bentley et al., Collaborative Study for the Establishment of a WHO International Standard for SARS-CoV-2 RNA. 2020, WHO Expert Committee on Biological Standardization. WHO/BS/2020.xxxx

10. ACKNOWLEDGEMENTS

We gratefully acknowledge the important contributions of the collaborative study participants, particularly in meeting the tight timeframes of this studyWe would also like to thank NIBSC Standards Production and Development staff for the formulation and distribution of materials.

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

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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	

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

World Health Organization

Medicines & Healthcare products Regulatory Agency

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Other (specify):	material of human origin.
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: 0.25 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

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.