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A collaborative study to evaluate the proposed 1st WHO International Standard for Varicella Zoster Virus (VZV) for nucleic acid amplification techniques detection (NAT) assays

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **17 September 2021** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technical Standards and Specifications (TSS). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

Summary

An international collaborative study was conducted to establish the 1st WHO International Standard (IS) for use in the standardisation of Varicella Zoster Virus (VZV) nucleic acid amplification technology (NAT) detection assays. A candidate freeze-dried VZV preparation, propagated in cell culture from a primary clinical isolate, was formulated in 10mM Tris-HCl pH 7.4, 0.5% Human serum albumin (HSA), 2.0% D-(+)-Trehalose dehydrate, and evaluated by 12 laboratories from 9 countries, each using their routine VZV NAT-detection assays.

The candidate standard was evaluated alongside two proprietary VZV comparator materials (Ellen and v-Oka strains) and 4 clinical samples and 2 spiked samples for a preliminary commutability assessment. A difference of up to 2.10 $\log_{10}$ copies/ mL in the mean potency estimates were reported across the laboratories for both qualitative and quantitative assays for the study samples. The agreement between the laboratories was significantly improved when the potencies were expressed relative to the candidate preparation, resulting in the reduction of interlaboratory variation.

Results from the accelerated thermal degradation stability studies performed at 12 months and 18 months post lyophilisation have demonstrated that the candidate material is stable and suitable for long-term use. Further stability assessments will be performed to follow the long-term stability of the candidate IS. Next-generation sequencing data indicates single nucleotide polymorphism (SNP) patterns consistent with a phylogenetic VZV classification into Clade 3 and gave no indication of major genome re-arrangements.

Based upon the returned dataset, the results of this study corroborate the suitability of the candidate as a calibrator for VZV NAT assays. Therefore, it is proposed that the candidate IS (NIBSC code 19/164) be established as the 1st WHO International Standard for VZV nucleic acid amplification technique (NAT)-based assays with an assigned potency of 7.0 $\log_{10}$ IU/vial.

Introduction

Varicella zoster virus (VZV) belongs to the Herpesvirus family of DNA viruses, categorised into the alpha subfamily along with Herpes simplex virus (HSV) 1 and 2 (Lui *et al*., 2007). VZV is a common and highly contagious pathogen, widely distributed in the human population causing significant public health burden.

It is the etiological agent of the primary infection varicella (chickenpox), typically occurring in early childhood in temperate regions and in late childhood in tropical regions (Gershon *et al*., 2015). VZV is characterised by a disseminated vesicular rash with mild to moderate illness in immunocompetent patients. Whilst the disease course is usually benign, 2-4% of children are hospitalised for complications caused by secondary bacterial infection, pneumonia and encephalitis (Heininger *et al*., 2006). The incidence of varicella hospitalisation in Europe is reported to range from 1.3 to 4.5 per 100,000 population/year with complications and hospitalisations occurring predominantly in immunologically healthy children (Bonanni *et al*., 2009).

VZV persists in the body asymptomatically after primary infection establishing latency in the trigeminal and dorsal root ganglia. Here it can reactivate from dormancy to cause Herpes Zoster (HZ) in adulthood. The symptoms of HZ normally resolve within 2–4 weeks, however patients can develop postherpetic neuralgia (PHN) a sequel of HZ with incidence increasing

particularly in older individuals, that causes chronic persistent pain, significantly impacting quality of life (Nalamachu *et al*., 2012).
Congenital varicella syndrome occurs in ~2% of the offspring of women who develop varicella between the 8th and 26th week of pregnancy. Sequela including severe damage to the central and automimic nervous system, eye and limb abnormalities can occur in affected infants, furthermore infants born with congenital varicella often do not survive infancy (Gershon and Gershon., 2013).
Transplant patients are at increased risk for developing HZ because of their immunocompromised status. The type of transplantation, immunosuppression, and antiviral prophylaxis may influence HZ incidence. Based on trials and retrospective studies the incidence of HZ is reported to vary between up to 9% in kidney, up to 7.2% in liver, up to 14.3% in lung, and around 16% in heart transplant recipients (Wang *et al*., 2018).
Disseminated VZV infection is potentially life-threatening in patients after organ transplantation, where some patients develop severe disseminated VZV infection with visceral involvement including pneumonitis, hepatitis, meningoencephalitis, glomerulonephritis or haemorrhagic complications, that can result in multiple organ failure (Yamada *et al*., 2015).

VZV diagnostics is primarily achieved using molecular PCR-based detection methods for rapid and sensitive VZV detection in a number of sample types (serum, plasma, whole blood, vesicle fluid, vesicle swab, and cerebrospinal fluid (CSF)). Commercial assays can include PCR detection/ differentiation tests using combined rapid diagnostics due to similarities in the clinical presentation of VZV and HSV1 and HSV2 infection. Quantitative viral load assays and anti-viral (acyclovir) resistance testing for transplantation patients and vaccine vs wildtype differentiation tests are also performed in the clinical setting. Clinical guidelines for VZV transplantation, recommend laboratory confirmation using PCR as the method of choice for detection of VZV in vesicle fluid, serum/blood, spinal fluid, and other tissues (Pergham *et al*., 2019).

Five VZV clades have been categorised, distinguishable by single nucleotide polymorphisms. Geographically Clades 1 and 3 are found in European countries and areas of the world predominantly settled by Europeans, whilst Clade 2 is predominantly present in Japan and surrounding countries. Clades, 4 and 5, are most prevalent in Asian and African countries (Breuer *et al*., 2010).

In 1998, the World Health Organization (WHO) recommended routine childhood varicella vaccination in countries particularly where a vaccination program is affordable, and where sustained vaccine coverage (85-90%) can be achieved. Whilst varicella vaccine is used in childhood immunisation programmes in many countries, including Australia, Canada, Germany, Japan, Saudi Arabia, and USA, in other countries such as the UK, vaccination is limited to susceptible or high-risk groups (Bonanni *et al*., 2009). The live attenuated varicella vaccine called VZV-Oka (v-Oka) was developed in the 1970s from the parental strain p-Oka from Clade 2 (Takahashi *et al*., 1974), which is administered globally for childhood vaccinations and has also been subsequently reformulated for the prevention of HZ in older adults (Peters *et al*., 2012).

The World Health Organization's (WHO) Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards (IS) are recognized as

the highest order of reference for biological substances. They are developed through the processes of biological standardization and are arbitrarily assigned a potency in International Units (IU). Their primary purpose is to calibrate secondary references used in routine laboratory assays, in terms of the IU, thereby providing a uniform result reporting system, and traceability of measurements, independent of the methodology used. The following documents the development and the evaluation of a proposed candidate WHO IS for use in the standardisation of Varicella Zoster Virus (VZV) molecular detection assays.

Aim of the study

The purpose of this study is to establish the 1st WHO International Standard for varicella Zoster Virus (VZV) for use as a calibrant in NAT detection assays for clinical diagnostics. The candidate was evaluated by expert laboratories in a multicentre collaborative study to determine a potency estimation based on data from both quantitative and qualitative molecular assays. The returned data has been used to determine the suitability of the candidate material for use as a primary biological reference material by its ability to improve agreement between the represented NAT-detection assays. Furthermore the evaluation conducted aimed to provide a preliminary indication of the commutability of the candidate formulation.

Bulk Material and Processing

Candidate Standard and Comparators

The candidate material was prepared from a primary clinical isolate donated from a paediatric VZV infection. The vesicle swab was maintained in viral transport medium at -80ºC and subsequently used for propagation in cell culture using MRC-5 human foetal lung fibroblast cell line. Two further VZV isolates were obtained for inclusion in the study and were also propagated using the same method. Varicella vaccine (live) BRP batch 1 (Product code Y0001198), a freeze-dried preparation of live varicella vaccine (Oka strain) was purchased from EDQM (European Directorate for the Quality of Medicines & HealthCare) and approved for use as a comparator strain for the purpose of the collaborative study by GSK (GlaxoSmithKline plc). Varicella Zoster virus (Strain Ellen Product code 1367) was purchased from ATCC (American Type Culture Collection) and was also approved for use as a comparator strain for the purpose of the collaborative study.

Since the detection of VZV is performed in multiple analytes, a universal buffer formulation was selected, to permit subsequent dilution by the end-user, into a clinical matrix pertinent to the analyte. The candidate preparation of VZV comprises of cell-free, live virus from productively infected cell culture supernatant diluted into a universal buffer formulation comprising 10mM Tris-HCl pH 7.4, 0.5% Human serum albumin (HSA), 2.0% D-(+)-Trehalose dehydrate, that underwent a freeze-drying procedure to ensure long term stability of the product and permit shipment at ambient temperatures. This formulation was selected based on previously established DNA virus WHO IS preparations that have demonstrated a consistently stable potency profile when assessed in accelerated thermal degradation studies.

Viral propagation of bulk material

The propagation of VZV isolates was achieved in MRC-5 cell line. Initial studies included MeWO human melanoma as an alternative permissive cell line (Ng *et al*., 2001), however

due to the excessive lytic nature of the infection in MeWO cells, propagation was limited to MRC-5 cells to reduce the presence of human cellular DNA in the final preparation.
MRC-5 cells (Passage 21) stored in liquid nitrogen were thawed in a 37ºC water bath and resuspended in 10mL of growth medium; Minimum Essential Medium Eagle (Sigma) supplemented with 10% Foetal bovine serum (Pan-Biotech GmbH P30-3306 Origin South America), 1.5% HEPES (Sigma) , 2% L-Glutamine Solution (Sigma) and 1% Penicillin-Streptomycin (Sigma) and briefly centrifuged at 1250 RPM for 5 minutes to recover the cellular pellet which was resuspended in fresh media and maintained at 37^0C with 5% CO2.
For infection a 1:1 dilution of the viral stock was used to inoculate 90% confluent of MRC-5 cells in a T175 flask. The inoculum was diluted into PBS buffer containing 5% sucrose and 5mM L-Glutamic acid potassium salt monohydrate (Sigma). The media was removed from the flask and an inoculum volume of ~0.50mL/ flask was added and gently distributed across the cell monolayer. Flasks were incubated for ~1 hour, then 30ml of media containing 3% FBS was added to the flasks and maintained at 35^0C with 5% CO_2. Periodic testing of the supernatant using quantitative PCR was performed to track the increase in viral load.
Cells were harvested 14 days post infection. The total cell culture lysate was stored at -80ºC until required for bulk preparation.
For v-Oka the freeze-dried pellet was resuspended in 0.5mL water as described in the IFU, then diluted as described above. An estimate of 4.37log10 pfu/vial was provided by the manufacturer, equating to approximately 0.437 log10 pfu/ flask used for infection. Plaques were observed around 3 days post-infection. By 7 days 50% of the flask was infected. Flasks were harvested at 10 days post-infection. For the Ellen strain the course of plaque formation was similar to the v-Oka strain but not to the same extent. Culture supernatant was harvested at 17 days post-infection with around 25% plaque formation. The monolayer was largely intact compared with v-Oka infection.

Pre-fill testing

Viral DNA quantification

The concentration of the VZV viral stocks were determined using the CE marked RealStar® VZV PCR Kit 1.0 *in vitro* diagnostic (IVD) assay that quantifies VZV DNA using a probe-based qPCR detection (Altona Diagnostics, Germany). Briefly nucleic acid extractions were performed using 200 µL of test sample using the QIAamp DNA mini Kit (QIAGEN, Germany). Extractions were performed using the QIAcube, an automated extraction platform (QIAGEN). Extractions were performed with the inclusion of an internal control (IC) which is included in the RealStar® VZV PCR Kit, intended as an extraction and PCR inhibition control. During the course of the study, extractions were subsequently performed using the MagNA Pure 24 System, a magnetic bead-based automated extraction platform (Roche Diagnostics). Purified nucleic acid samples were amplified by qPCR on the Mx3005P Real-time PCR instrument (Mx3005P QPCR System, Agilent Genomics) enabling VZV detection (FAM fluorophore) and IC (JOE fluorophore) with ROX as a passive reference. Viral quantification was achieved with the inclusion of 4 kit plasmid quantification standards, to generate standard curve with a dynamic range of 10^1-10^4 quantifiable copies/µL (Altona Diagnostics, Germany). Reactions were set up according to manufacturer's instruction using 10µl of test DNA sample added to 20µl of the combined master mixes A and B. Reactions conditions were performed according to the manufacturers instruction: denaturation at 95ºC for 10 minutes followed by 45 cycles of two-step denaturation and

annealing at 95°C for 15 seconds and 58°C for 1 minute respectively. Data were analysed using the Mx3005P software v4.0. According to the manufacturers specification the quantification results are valid if the generated standard curve reaches the control parameter values of $R^2 > 0.98$.

Preparation of bulk material

A universal buffer formulation: 10mM Tris-Cl pH 7.4, 0.5% Human serum albumin (HSA), 2.0% D-(+)-Trehalose dehydrate, 0.2µm filtered was prepared at NIBSC. The HSA used in the production of the candidate standards was derived from a licensed product (Zenalb® 20, a 200 g/L of human albumin solution 20% Solution) screened at NIBSC to be negative for anti-HIV-1, HIV-2, HBsAg, and HCV. Frozen aliquots of the viral supernatant were thawed using a 30^{0}C water bath. Prior to dilution into universal buffer, the viral supernatants were cleared of cellular debris by centrifugation for 5 minutes at 3500 rpm and a 1:50 fold dilution was made in order that the bulk preparation should contain approximately ~1 x 10e7 copies/ml, in a final volume of 6.0L of universal buffer. Approximately 200mL of the liquid bulk was divided into aliquots of 0.25mL, 0.55ml, and 1.0ml in 2ml screw cap Starstedt tubes and stored at -80°C for viral copy determination as well as for inclusion in the collaborative study panel. 10mL of the liquid bulk was also reserved for sterility testing for independent microbiology analysis within NIBSC. The remaining bulk volume was processed for lyophilisation and designated NIBSC product code 19/164 for the VZV candidate.

Filling and lyophilisation of candidate standard

The filling and lyophilisation of the bulk material was performed at NIBSC Standards Processing Division under ISO9001. The filling was performed in a Metall and Plastic GmbH (Radolfzell, Germany) negative pressure isolator that contains the entire filling line and is interfaced with the freeze dryer (CS150 12m2, Serail, Argenteuil, France) through a 'pizza door' arrangement to maintain containment of the operation. The bulk material was kept at 18°C throughout the filling process and stirred constantly using a magnetic stirrer. The bulk was dispensed into 5 mL screw cap glass vials in 1 ml aliquots, using a Bausch & Strobel (Ilfshofen, Germany) filling machine FVF5060. The homogeneity of the fill was determined by on-line check-weighing of the wet weight against the dry weight, and vials outside the defined specification were discarded. Filled vials were partially stoppered with halobutyl 14mm diameter cruciform closures and lyophilized in a CS150 freeze dryer. Vials were loaded onto the shelves at +4°C, then cooled to -50^{0}C and held at this temperature for 2 hours. A vacuum was applied to 200 µb over 1 hour, followed by ramping to 100 µb over 1 hour. The temperature was then raised to -15°C, and the vacuum maintained at this temperature for 31 hours. The vacuum was lowered to 30 µb and the shelves were ramped to 25°C, over 10 hours and this was then held for 20 hours before releasing the vacuum and back-filling the vials with nitrogen, produced by evaporation of liquid nitrogen with an analysis of 99.999% purity. The vials were then stoppered in the dryer, removed and capped in the isolator. The isolator was decontaminated with formaldehyde before removal of the product. The sealed vials are stored at -20°C at NIBSC under continuous temperature monitoring for the lifetime of the product.

Post-fill testing

Stability assessment of candidate product

To predict the stability of the freeze-dried material, vials of the candidate VZV IS were subject to accelerated thermal degradation. This entails the storage of multiple vials of the candidate postproduction at -70^{0}C, -20^{0}C, +4^{0}C, +20^{0}C, +37^{0}C and +45^{0}C for up to 10 years. Periodically 3 vials are removed from each temperature and tested for viral potency using the real-time PCR method described above, to provide an indication of stability at each of the storage temperatures relative to -20^{0}C. Typically 2-3 tests are performed in the first year pre-establishment then and annually thereafter post-establishment.

The assessment of residual moisture and oxygen content are critical parameters when considering the stability and shelf life of lyophilized products. Non-invasive moisture and oxygen determinations were made as follows. Vials of excipient only formulation for the proposed standard were prepared to be used to compare between destructive and non-invasive moisture analysis by near Infra-Red reflectance (NIR, Process Sensors MT 600P, Corby, UK). Results obtained from the non-infectious samples by NIR would then be correlated to coulometric Karl Fischer (KF, Mitsubishi CA-100, A1 Environsciences, Cramlington, UK) to give % w/w moisture readings. Moisture determinations were compared against values from a standard curve, which was made using 12 vial replicates of non-infectious excipient only samples, which were subjected to varying exposure times (0, 5, 10, 15, 30, 45, 60 and 90 minutes) to atmospheric air, by removing screw caps and raising the stopper to the filling position for the designated period of time before the stoppers were fully re-inserted and the caps re-sealed. Subsequently, several vials of each time point were tested by coulometric Karl Fischer and the standard curve generated. Moisture values were assigned based on the calibration curve generated from the data from the non-infectious excipient vials. A CV% is therefore not applicable. Oxygen headspace content is an indicator of the success of the nitrogen back-filling process in the dryer and subsequent integrity of the seal on the vials. Oxygen headspace was measured using non-invasive headspace gas analysis (FMS-760 Lighthouse, Charlottesville, VA). This correlates the NIR absorbance at 760nm (for oxygen) based on a NIR laser source and is calibrated against equivalent vials, sealed with traceable oxygen gas standards. Calibration of the unit was achieved using 5ml screw capped vials containing oxygen standards of 0% and 20%.

Homogeneity and genome integrity of candidate IS

The freeze-dried candidate (19/164) was tested to determine the homogeneity of the viral contents of the lyophilised material post-production. Lyophilised samples were reconstituted in 1 mL of nuclease-free water (QIAGEN, Germany), mixed gently on a vortex and left for 20 minutes. The reconstituted sample was serially diluted or tested neat after nucleic acid extraction using either the QIAamp Mini DNA kit on the QIAcube or on the MagNA Pure 24 extraction platform using the Total NA Isolation Kit with the Pathogen 200hp 1.0 protocol (Roche). Extracted DNA was used for amplification using the Altona Diagnostics RealStar® VZV PCR Kit 1.0 assay as described above.

The candidate standard as well as the comparator samples underwent full Next-Generation Sequencing (NGS) analysis by our NIBSC NGS core facility to verify the sequence integrity of the viral genomes. Briefly before NGS evaluation, 20 mL of viral supernatant was processed for DNA extraction. Total cell culture lysates stored at -80ºC were thawed in a

30ºC water bath and centrifuged at 1500 rpm for 5 minutes to remove cellular debris. The resultant supernatant was ultra-centrifuged at 18,000 rpm for 2 hrs in a Beckman swing out rotor SWTi28. The supernatant was discarded, and the pellet was gently resuspended in the remaining culture medium. The extract was treated with 10% SDS and Proteinase K overnight at 37ºC in water bath (sealed within a 50mL Falcon tube). 200 µL of the sample was processed using the Genomic DNA clean & concentrator kit (Zymo Research) and 140 µL was processed manually using the QIAamp Viral kit (QIAGEN), according to manufacturer's instructions. The concentration and purity were confirmed using a nanodrop spectrophotometer.

The following steps were performed within the NIBSC NGS core facility: Samples were mixed with 1 volume (100 µl) of AMPure beads (Beckman Coulter, A63881) and purified / concentrated according to the manufacturer's instructions. DNA was eluted in 12 µl of sterile DNase-free water and the concentrations were measured using the Qubit fluorometer using the Qubit dsDNA HS Kit (Thermo Fisher, Q32854). Sequencing libraries were constructed using the Nextera DNA Flex kit (Illumina, 20018705) with 5 µl of each concentrated DNA sample according to manufacturer's instructions. Samples were dual-indexed using Nextera XT Index kits (Illumina, FC-131-2002). Pooled libraries were sequenced on a MiSeq v2 500 sequencing kit (Illumina, MS-102-2003) with 250 bp paired-end sequencing reads. For the bioinformatic analysis, a custom pipeline was used, including: Read trimming using Trimgalore (v0.5.0), De-Duplication using Picard (v2.18.23), Alignments using BWA (v0.7.17), variant Calling using LoFreq (v2.1.3) and plot generation using custom R (v3.5.3) scripts.

Collaborative Study

Study samples

A total of 10 study samples coded A- J, were prepared for evaluation in this study (Table 1). Participating laboratories were sent a questionnaire (Appendix II) prior to sample dispatch, to ascertain the types of clinical samples routinely assayed in their laboratory and to determine the types and quantity of sample required for their extraction procedure. Sample sets were thereby customised to each participant based on the responses received. All participants received the candidate 19/164 materials in both the liquid and lyophilised form (Samples B and D) and the comparator strains Ellen and v-Oka, Samples A and C respectively. In addition, four clinically relevant adult (Sample E and F) and paediatric (Samples I and J) swab samples were also included in the panel for testing alongside the candidate material, kindly donated by the St Georges Hospital (received as anonymised residual samples that were diluted in UTM as required). Two further samples were generated using the candidate 19/164 spiked into VZV negative plasma and CSF respectively (Samples G and H). Plasma was obtained from the National Blood Service and CSF was sourced from Raighmore Hospital, Scotland.

Diluents were tested prior to dilution to confirm the absence of VZV DNA and the samples were further tested post dilution with the positive clinical samples to confirm the viral load of the study samples before shipment. Once diluted the VZV were aliquoted into volumes proportionate to the volumes required by participants to perform duplicate nucleic acid extractions in 3 separate runs as part of the collaborative study protocol. All liquid samples were stored at -80^{0}C until required for dispatch. All samples were shipped on dry ice from NIBSC care of our dedicated dispatch team.

Design of the study protocol

Participants typically received 6 vials of each of the samples for duplicate analysis of each sample and were instructed to use a fresh vial for each data point across 3 independent runs. For the qualitative assay an extra vial was included so that an initial test could be conducted to ascertain the potency of the samples for subsequent repeat analysis. Sufficient volumes were sent to enable duplicate analysis of each sample for each data point across 3 independent runs. In some instances, participants were also supplied with UTM to perform their dilutions for the study.

Upon receipt, participants were directed to store the study samples either at -80^{0}C (liquid samples A-C and E-J) or at -20^{0}C (lyophilised sample D). Participants were directed to reconstitute the lyophilised Sample D in 1mL of nuclease-free molecular grade water for a minimum of 20 minutes with occasional gentle agitation before use.

Participants performing quantitative analysis, were directed to test samples A to D at a minimum of 3-4 serial ten-fold dilutions in a single sample matrix commonly used in their laboratory (e.g. CSF, UTM plasma etc.). For example, dilutions of 1/10, 1/100 etc. were suggested such that at least 2 of these dilutions should fall within the linear range of quantification in their assay.

Participants performing qualitative analysis were requested to test samples A-D to a minimum of 5-7 serial 1:10 fold serial dilutions in a single sample matrix commonly used in their laboratory, to determine the end point of detectable VZV viral DNA. Participants were requested to ensure their data included at least 2 dilution points at which a positive VZV signal was no longer detectable. For the 2 remaining qualitative assays, participants were requested to re-test the dilutions around the assay end point as determined in the first assay, and where practicable to include a minimum of two half-log serial dilutions either side of the determined end point dilution.

A result reporting form was provided to each participant. This included a table to provide details of extraction and amplification procedure in the assays performed. Separate sheets were provided to submit data values from each assay performed. Study protocols for both qualitative and quantitative assays are included in Appendix II.

Participants

27 participants were initially recruited however due to the COVID-19 pandemic the number was reduced to 18 participants who received their samples between September-December 2020. A randomly assigned laboratory code was given to each participant to assure laboratory anonymity. Due to the second COVID-19 wave in some countries a further 6 laboratories were unable to commit to performing their analysis. Therefore 12 study participants from 9 countries (listed in Appendix 1) representing clinical laboratories, manufacturers of VZV NAT *in-vitro* diagnostic (IVD) kits and an EQA provider performed the analysis.

Statistical Methods

Qualitative and quantitative assay results were evaluated separately. In the case of qualitative assays, for each laboratory and assay method, data from all assays were pooled to give a number positive out of number tested at each dilution step. A single ‘end-point’ for each

dilution series was calculated, to give an estimate of '$\log_{10}$ NAT detectable units/mL. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome equivalent number/mL (Saldanha *et al*., 1999).
In the case of quantitative assays, analysis was based on the results supplied by the participants, reported in copies/mL. To calculate laboratory reported potency estimates, first the mean $\log_{10}$ value at each dilution was calculated in each sample and assay run. Then a single estimate of $\log_{10}$ copies/mL was obtained for each sample within an assay run by correcting for the corresponding dilution factors and taking the arithmetic mean value across the results. A single estimate for each sample within the laboratory and assay method was then calculated as the arithmetic mean of the $\log_{10}$ estimates of copies/mL across assay runs.
Relative potencies where data was available across a range of doses for both reference and test sample were estimated using a parallel line model with $\log_{10}$-transformed copies/mL values as responses (Finney *et al*., 1978). Calculations were performed using the R software package (R Core Team 2021). Estimates were calculated from this analysis, plus an assigned value in proposed $\log_{10}$ International Units/mL (IU/mL) for the candidate standard where appropriate. Estimates from all valid assays were combined to generate an arithmetic mean in for each laboratory and assay type.
Relative potencies for samples with only a single dilution were obtained by fitting a linear model to $\log_{10}$-transformed copies against $\log_{10}$-dose and interpolating relative potency values for test samples from the model. Calculations were performed using the R software package (R Core Team 2021). Potency estimates were calculated from this analysis, plus an assigned value in proposed $\log_{10}$ International Units/mL (IU/mL) for the candidate standard where appropriate. Estimates from all valid assays were combined to generate an arithmetic mean in for each laboratory and assay type. One exception to this was for laboratory 15, that reported data for some samples that were not matched in the same assay runs due to the way the machine functions. In this case, the relative potency of a sample to the proposed candidate was taken as the ratio of the laboratory mean reported value of that sample to the laboratory mean reported value of the candidate (e.g. potency of Sample A divided by potency of Sample D), rather than calculations done on an assay by assay basis. Due to this difference in calculation, these values are not included in the calculations for overall sample potencies and variability measures.
Overall analysis was based on the laboratory $\log_{10}$ mean estimate values. Overall mean estimates were calculated as the means of all individual laboratories. Variation between laboratories (inter-laboratory) was expressed as standard deviations (SD) of the $\log_{10}$ estimates and % geometric coefficient of variation (GCV = $\{10^{s}\text{-}1\}\times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates) of the actual estimates. Variation within laboratories and between assays (intra-laboratory) was expressed as standard deviations of the $\log_{10}$ estimates of the individual assay mean estimates.

Validity Criteria

For reported estimate calculations, dilutional linearity was considered acceptable if the slope of the fitted regression line for $\log_{10}$-transformed copies against $\log_{10}$-dose was within the range of 0.80 to 1.25; results were excluded from further calculations if this was not the case. For relative potency analyses model fit was assessed visually and by calculating an r^2 value from the fitted model. Samples with an r^2 value below 0.95 were excluded from further analysis (marked NL in tables). Where relevant, non-parallelism was assessed by calculation of the ratio of fitted slopes for the test and reference samples under consideration. The samples were concluded to be non-parallel when the slope ratio was outside of the range 0.80

– 1.25 and no estimates are reported in these cases (marked NP in tables). If the range of response values for a test sample did not fall within the range of response values for a reference, then no estimates is reported (marked RR in tables).
As all reported potency estimates across all samples for laboratory 2 are around 2 $\log_{10}$ units lower (100-fold difference) than for all other laboratories (an outlier according to Grubbs' test), they have been excluded from further summary calculations.

Matrix Effects

Apart from one laboratory assay that used water, three clinically relevant matrices were used in this study: universal transport media (n=7), plasma (n=5) and CSF (n=1). One laboratory used both plasma and CSF in their analysis of the candidate and comparator samples. All other laboratories used only a single matrix exclusively.
Due to the low sample sizes, CSF and water matrices were not included in analysis to compare matrices. Using the laboratory mean relative potency estimates (in $\log_{10}$ IU/mL), calculated relative to sample D, a mixed-effects model was fit using potency as response, sample as a random factor and matrix as a fixed factor. This showed no significant difference in results based on matrix used (p=0.289).

Results and analysis

Validation of candidate IS

Postproduction data analysis was performed on the lyophilised candidate material Sample D (19/164) confirming the quality assurance of the freeze-drying and filling process. For a 1.0g product fill, a CV of <0.25% was met for the fill mass. The mean residual moisture and oxygen content were also determined, 0.15 % and 1.27% respectively and deemed to be within the acceptance criteria for the preparation of WHO biological reference materials (Table 2).

The mean VZV viral nucleic acid content from 8 randomly selected vials of the candidate IS preparation were tested in duplicate to ensure the potency homogeneity across vials. Aliquots of the undiluted and 1:10 diluted candidate were extracted using two different automated extraction platforms using either plasma or TRIS buffer. An average of each duplicate was then used to determine the viral copy number using a quantitative CE marked IVD kit (Table 3). Average potency estimates between the platforms ranged between 6.77-6.84 $\log_{10}$ copies/mL with comparable standard deviations and GCV% values.

Two thermal accelerated degradation assessments were performed at 12 and 18 months post-production on the candidate standard by assessing the change in potency if any, of detectable VZV viral nucleic acid across the range of storage temperatures. One vial was tested at each of the storage temperatures in 3 independent assays. Each vial was tested at three 10-fold dilutions (10, 100 and 1000 fold dilution). The averaged values obtained at 12 and 18 months for 19/164 show no observable drop in potency at temperatures up to $+45^0$C relative to the storage temperature potency (Table 4a). Relative potencies to the -20°C baseline and 95% confidence limits were also calculated by pooling all results for each temperature at each timepoint (Table 4b). As no loss of activity was observed at the elevated temperatures, it was

not possible to predict the rate of degradation during long-term storage at -20°C using an Arrhenius equation.

Sequence alignment of the Illumina sequence reads obtained for candidate 19/164 showed that 97.246 reads mapped to the reference genome strain Dumas (GenBank accession no. NC_001348) from Clade 1. A second reference genome for Clade 3 GenBank Accession AJ871403.1 strain HJ0 returned 98.212 aligned reads whereas DQ479957.1 strain 03-500 also from Clade 3 retuned a slightly lower number of 96.791. To elucidate the Clade designation further, the candidate VZV strain was typed using the SNP method based on the genotyping scheme described by Breuer *et al* (Breuer *et al*., 2010). Based on the pattern of a minimum complement of 27 SNPs positions across 15 ORFs to identify a putative clade designation, the candidate was identified as being most closely aligned to Clade 3 (Table 5).

The VZV genome includes two main coding regions, unique long (UL) and unique short (US) and flanking inverted repeats, termed terminal and internal repeats long (TRL, IRL) and short (TRS, IRS) In addition to these repeats, there are five genomic regions with tandem reiterations, designated R1 to R5 (Davidson *et al*., 2000; Zell et al., 2012). Since long repeat regions are not easily resolvable with short-read sequencing methods such as Illumina sequencing-by-synthesis, these areas of low sequence coverage have been identified and primers have been selected to PCR amplify the remaining gaps in the Illumina genome alignment. Sanger sequencing of PCR derived amplicons will be used to elucidate the repeat regions in the candidate IS genome. Additionally, long-read sequencing technology could also be applied for full genome sequencing.

Collaborative Study Data

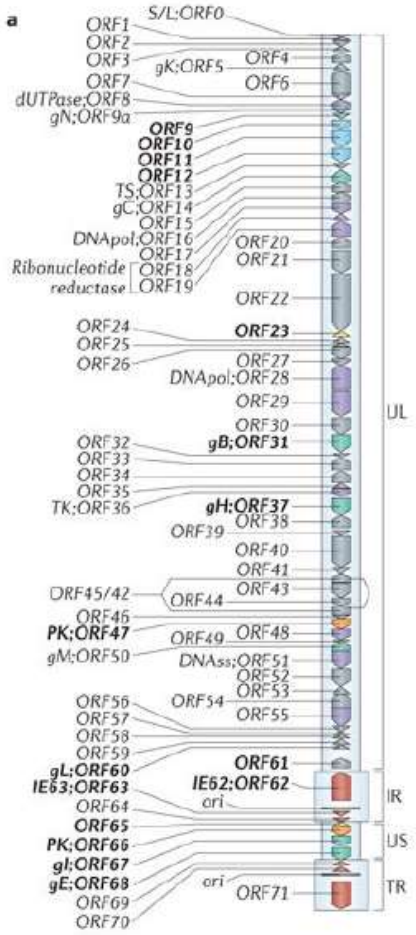
Data were received from 12 laboratories from 9 different countries. From the 12 laboratories 14 datasets were returned for the candidate materials and comparator Samples A-D, eleven quantitative and three qualitative datasets. For quantitative data all assays were performed using conventional real-time qPCR assays from which participants returned values as copies/mL. Qualitative data evaluations were returned as either positive or negative detection.

In general, participants performed their experimental runs using one assay method with the use of one matrix type for the dilution of Sample A-D. With the exception of Participant 1 who performed their analysis using both plasma and CSF. Most participants used UTM for their dilutions (n=7), the remainder used either plasma (n=4), or CSF (n=1) and water (n=1). The distribution of participants included IVD manufacturers (n=6), clinical laboratories (n=5) and an EQA provider. Expectedly the number of assays using commercial vs laboratory developed assays (LDT) followed this distribution, with commercial assays representing n=7 and LDT representing n=5 of the dataset (Table 6).

Summary of assay methodologies

Almost all participants used commercially available automated nucleic acid extraction platforms, with the exception of 1 laboratory that performed extraction manually using QIAamp DNA Blood mini Kit. Eight different extraction platforms were represented including: AltoStar Automation System, Antolia Geneworks/ Magrev NA ExtrMag , BioMerieux EasyMAG, MAX KingFisher systems Thermofisher, NeuMoDx Molecular System and QIAcube, QIAsymphony, ZJ Bio-Tech Auto NA Extra (Table 6).

Five datasets were obtained using laboratory developed tests (LDT), compared with 7 datasets obtained using commercial amplification assays, which included; AltoStar® VZV PCR Kit 1.5 (altona Diagnostics GmbH); Anchor VZV PCR Kit (CE); Bosphore VZV (HHV-3) Detection Kit v1 (Anatolia Geneworks®); HSV1&2 VZV R-GENE® (ref.: 69-014B – bioMérieux); NeuMoDx VZV Quant Assay (QIAGEN In development) and Varicella Zoster Virus (VZV) Real Time PCR Kit. (Shanghai ZJ Bio-Tech Co., Ltd) (Table 6).
A range of amplification platforms are also represented which included
ABI Prism 7500 Thermofisher/Applied Biosystems, CFX96 Bio-Rad, LC480 Lightcycler 480 (Roche), m2000 RealTime System (Abbott), Montania 4896 Real-time PCR Instrument (Anatolia Geneworks), MxPro3000P (Agilent), NeuMoDx Molecular System, QuantStudio 3 (Applied Biosystems), Rotorgene Q (QIAGEN) and Viia 7 (Thermofisher) (Table 6).



Zerboni et al; Nat Rev Microbiol. 2014;12(3):197-210

The VZV genome is a linear double-stranded DNA molecule of ~125,000 bp that encodes at least 71 unique ORFs. The genome consists of a unique long region (UL) of ~105,000 bp, a unique short region (US) of ~5,232 bp, and internal repeat (IR) and terminal repeat (TR) regions (Zerboni *et al*., 2014). Where disclosed several VZV genes were used as amplification targets, including gp19/ORF 17, polymerase gene ORF28, gB ORF 31, ORF 38, ORF 11/29 and ORF 62. Only two of the participants used the same target region ORF 38. Five of the 12 datasets did not disclose the amplification region (Table 6). It is reported that the genes that encode ORF62 and ORF70, ORF63 and ORF71, and ORF64 and ORF69 are duplicated (Zerboni *et al*., 2014).

Reported estimated potencies of study samples

Inter-laboratory variation

The individual laboratory potency estimates for the study samples from 11 quantitative and 3 qualitative datasets are presented in Table 7. The overall mean potency estimates are also provided in Table 7 with the averaged potency estimates for each sample across the qualitative and quantitative assays provided along with the corresponding standard deviations and GCV percentage values. Quantitative potency estimates are provided in $\log_{10}$ copies/mL and qualitative potency estimates as NAT detectable units/ mL Samples A and D were assayed by all laboratories. As samples E-J were not received for testing by all laboratories (based on responses to the Participants Questionnaire in Appendix II regarding routinely tested samples) an asterisk denotes "not received". For qualitative assays potency estimates were derived from end-point dilution series as described in the statistical methods.
The quantitative potency estimates for samples A-D are based on combined corrected mean estimates across dilutions as described in the statistical methods. For quantitative assays all clinical samples were tested undiluted and potency estimates were therefore derived from the mean of the neat reported values. For qualitative assays any clinical samples tested were serially diluted to end-point detection to derive a potency estimate. All the individual estimates returned across the replicate assays performed by each participant are included in the Appendix. (Appendix I: Table 1).

The overall mean potency estimates are further summarised in Table 8 with the averaged potency estimates for each sample across the combined assays including the range of the estimates, along with the corresponding standard deviations and GCV percentage values. Based on the exclusion criteria described in the statistical methods, data from laboratory 2 have been omitted, and results are discussed accordingly.
Up to a 2.10 $\log_{10}$ difference (1.19-2.10) in reported viral potency estimates is evident across the 13 assays for Samples A-D. The $\log_{10}$ copies/mL range for the liquid candidate IS Sample B combining both the qualitative and quantitative estimates is 6.39-7.82 showing a 1.43 $\log_{10}$ spread of the data and for the candidate IS sample D (lyophilised) the minimum and maximum values are 6.44-7.79 showing a 1.35 $\log_{10}$ spread of the data. Correspondingly the overall mean potency estimates are 6.94 $\log_{10}$ copies/mL for both of the samples. The reported potency estimates for Sample A, the comparator Ellen strain gave a range of 6.66-7.85 showing a 1.19 $\log_{10}$ spread of the data. Whereas for the v-Oka vaccine strain, Sample C the minimum and maximum values are 7.38-9.50 showing the highest spread at 2.10 $\log_{10}$ of reported estimates (Table 8).

For the clinical samples E-J fewer datasets were returned and differences across the estimates ranged from 0.81-1.71 $\log_{10}$. For the adult and paediatric swab samples F and J, seven datasets were evaluated showing minimum and maximum values of 6.20-7.85 and 5.41-6.89 respectively. For Samples E and I where only 5 quantitative estimates were evaluated, similar potency estimate differences of 1.71 $\log_{10}$ and 1.46 $\log_{10}$ respectively were seen. Similarly, for Sample G the candidate spiked plasma sample, the estimates ranged 3.79- 4.60 $\log_{10}$ copies/mL, a difference of 0.81 $\log_{10}$ across 8 laboratories. Finally, for Sample H estimates from five quantitative assays ranged from 4.02-4.96 $\log_{10}$ copies/mL, a variation of 0.94 $\log_{10}$ (Table 8). Summary calculations are also provided with the inclusion of laboratory 2 dataset, for comparison (Table 7 and 8).

Intra-laboratory variation

Table 9 presents the intra-laboratory variation of the $\log_{10}$ copies/ mL potency estimates for each of the samples assayed using quantitative analysis. Each laboratory is represented showing the SD values across the samples assayed. For laboratory 1 each of their component assays are represented for 1a and 1b datasets. A large majority of the SD values are extremely low (< 0.1) across the individual laboratories reflecting excellent single assay agreement and reproducibility. The remaining standard deviation values were 0.20-0.31. Using >0.50 as a cut-off SD value, only one of the laboratories datasets returned a higher SD value for Sample C only. A standard deviation 0.79 was seen due to the divergence in estimates in one of the three assay replicates they performed. This was the exception to otherwise excellent intra-laboratory SD values for this laboratory. There was no observable difference in SD variability between the sample types.

Comparison of laboratory reported estimates and relative potencies

Figures 1-4 present histogram plots of the mean estimates returned by the study participants for the candidate and comparator samples (A-D) and Figures 5-10 present histogram plots the clinical samples (E -J). For each of the samples quantitative assay estimates are represented by purple boxes and the qualitative datasets are represented by the green boxes. Each dataset is shown by the individually assigned laboratory number inside each box. Where laboratories provided more than one dataset a further designation of “a” or “b” alongside the laboratory number is provided. The mean estimates of each sample are plotted on the x axis either as $\log_{10}$ copies/mL for the quantitative assays or NAT detectable units/ mL for the qualitative assays against the frequency of the estimated mean values on the Y axis. Each histogram provides a representation of where each laboratory lies (including laboratory 2) in the distribution of the total dataset positioned in relation to the calculated mean potency estimate.

For Sample A, B and D there is a consistent distribution of the reported mean potency estimates across the laboratories as depicted in the histogram plots (Figure 1a, 2a and 4 respectively). Laboratory 2, one of the three qualitative assays lies to the extreme left of the histogram plots representing the lowest mean potency estimates reported for each of the samples. Interestingly the other qualitative assays report estimates that are consistent with or close to the majority of quantitative assays. From the quantitative assays, laboratory 10 and 17 consistently report the highest potency estimates (Figure 1a, 2a and). Overall, the quantitative assays are in very good agreement, with around half or more of the estimates falling within a small range; Sample A (6.81-7.06) 0.25 $\log_{10}$ difference, for Sample B (6.79-7.07) 0.28 $\log_{10}$ difference and for Sample D (6.87-7.06) 0.19 $\log_{10}$ difference.
For Sample C (Figure 3a) the distribution is comparatively broader but again, Laboratory 2 lies to the extreme left of the histogram plot representing the lowest mean potency estimate reported and laboratory 17 reports the highest potency estimate. Qualitative potency estimates returned by Laboratory 7 and 11 again are consistent with the majority of quantitative estimates (Figure 3a).

Using Sample D as a common reference, to apply a relative potency assessment we observe a considerable improvement in agreement across the reported mean estimate values (Table 10). These values are obtained by taking the difference between the laboratory derived estimates

for candidate sample D from the laboratory derived estimates of the test sample and then adding the nominative assigned potency of 7.0 $\log_{10}$ IU/vial.
The impact of the relative potency is best demonstrated in the histogram plots (Figures 1-10b). For Sample B the outlier estimates from laboratory 2, and 17 now sit within a narrow consensus across all the laboratories (Figure 2b). For sample B the SD and GCV% values drop from 0.69 and 386% respectively to SD 0.13 and GCV 35% (Table 11 including laboratory 2). The relative potency estimation brings the interlaboratory variation down from 3.06 $\log_{10}$ to 0.50 $\log_{10}$ with the inclusion of laboratory 2. Excluding laboratory 2 the interlaboratory variation declines from 1.43 $\log_{10}$ to 0.50 $\log_{10}$.
Similarly applying a relative potency assessment using Sample D as a common reference, to the comparator Samples A and C we observe an improvement in agreement across the reported mean estimates (Figure 1b and 3b). For Sample A, the outlier estimates from laboratory 2, and 17 now sit within the consensus range across all the laboratories. The SD and GCV% values drop from 0.69 and 391% to SD 0.18 and GCV 53% respectively (Table 11 including laboratory 2). The relative potency estimation brings the interlaboratory variation of Sample A down from 3.10 $\log_{10}$ to 0.58 $\log_{10}$ (including laboratory 2). Excluding laboratory 2 the interlaboratory variation declines from 1.19 $\log_{10}$ to 0.58 $\log_{10}$. For Sample C the SD and GCV% values drop from 0.82 (0.54 excluding laboratory 2) and 553% (248% excluding laboratory 2) respectively to SD 0.37 (0.38 excluding laboratory 2) and GCV 132% (141% excluding laboratory 2) (Table 11). Whilst the relative potency estimation brings the interlaboratory variation for Sample C down from 3.57 $\log_{10}$ to 1.11 $\log_{10}$ (2.10 $\log_{10}$ to 1.11 $\log_{10}$ excluding laboratory 2) this is less pronounced compared with the change in inter-laboratory variation for Sample A and B.

For the clinical samples the histogram plots of the potency estimates are shown in Figures 5a-10a and the corresponding potency estimates relative to Sample D are shown in histogram plots 5b-10b. For Sample E only five quantitative datapoints were received with four the estimates in close agreement. Laboratory 17 potency estimate however shows a difference of 1.24 $\log_{10}$ from the highest of the other laboratory estimates (Laboratory 18) and is positioned to the right of the other estimates (Figure 5a). Using Sample D to apply a relative potency assessment, we observe an improvement in the agreement of laboratory 17 with the other laboratory estimates (Figure 5b). However assays from laboratories 5, 8, 12b, 17 and 18 had response values outside of the range of the data for sample D, the relative potency values shown are read from the model for D, which is outside of the validity criteria quantitative range for D in each assay, therefore the relative potency estimates squares have been shaded to depict this.
For Sample F, eight datapoints were received, three of which were qualitative. Whilst the majority are in close agreement laboratory 2 and 17 potency estimates differ, with laboratory 2 showing a difference of 1.44 $\log_{10}$ from the lowest of the other laboratory estimates and laboratory 17 showing a 1.2 $\log_{10}$ difference from the highest of the other estimates (Figure 6a). Using Sample D to apply a relative potency assessment, we observe an improvement in the agreement of laboratory 2 and 17 with the other laboratory estimates (Figure 6b).

For the spiked plasma Sample G, eight quantitative estimates were returned. These potency estimates are in good agreement with each other (Figure 7a). Laboratory 17 estimate is in agreement with the other laboratories in this instance. A modest improvement is observed when the estimates are shown as a relative potency of the candidate (Figure 7b). For the spiked CSF Sample H, five quantitative estimates and one qualitative estimate were returned. Whilst the quantitative potency estimates were in reasonable agreement with each other,

laboratory 2 was significantly different, showing a ~2.0 log_{10} difference from the lowest (12b) of the other laboratory estimates (Figure 8a). An improvement is observed when the estimates are shown as a relative potency of the candidate (Figure 8b) with laboratory 2 in agreement with remaining potency estimates. Using Sample D to apply a relative potency assessment, we observe an improvement in the agreement of laboratory 2 with the other laboratory estimates, which are also in closer agreement. Interestingly laboratory 17 potency estimates for Sample H is not as divergent from the other laboratories as seen with Sample G above.

The observations for the paediatric swab samples I and J, were similar to the adult swab samples as the estimates were returned by the same laboratories. For Sample I, five quantitative datapoints were received with the majority already in close agreement, with the exception of laboratory 17 (Figure 9a). However, agreement of laboratory 17 with the other laboratory estimates was achieved when estimates were expressed relative to Sample D as a common reference (Figure 9b). For Sample J, of the eight datapoints three of were qualitative. Whilst the majority are in close agreement, discrepant estimates were observed with laboratory 2 under reporting and 17 over reporting. Laboratory 2 shows a difference of 1.72 log_{10} from the lowest of the other laboratory estimates and laboratory 17 shows a 0.92 log_{10} difference from the highest of the other estimates (Figure 10a). Using Sample D as a common reference, to apply a relative potency assessment, the outlier laboratories are now in closer agreement reducing the variability across the laboratories (Figure 10b).

Assessment of diluent effects

Since the proposed IS preparation is intended for use in multiple diluents we wanted to see if there was any difference in the potency estimates depending on the diluent used. Most participants used UTM for their dilutions (n=7), the remainder were plasma (n=4), CSF (n=1) and water (n=1). Figure 11 shows the laboratory mean estimates for the candidate IS Sample D coded for the diluents used. Each diluent used is represented by a colour. There is no clear trend across the potency estimates. Mean estimates obtained using either UTM or plasma are interspersed suggesting little influence based on the diluent used. A mixed-effects model was applied but returned no significance as described in the statistical methods. Since laboratory 1 used both CSF and plasma for their evaluation of the candidate IS, their data was subdivided into 4 different methods per analyte for comparison. A *t* test was performed but no significant difference between the estimates was observed.

Comparison of candidate IS relative potencies with comparator samples

We also looked at the performance of the comparator samples (A and C) to assess their ability to reduce variability across the laboratory reported estimates compared with the proposed candidate IS. Table 12 shows the change in the GCV percentage values relative to either Sample D, A or C, compared to the GCV% values for the reported estimates of each study sample. The results are discussed excluding laboratory 2 from the combined dataset, based on the validity criteria described in the statistical methods. Summary calculations are provided in Table 12, including and excluding laboratory 2 for comparison.
For Sample B the overall extent of variability in relation to the mean of the estimates was 139% combining both qualitative and quantitative estimates. This was reduced to 37% when the potency estimates were expressed relative to the candidate IS. Sample A was able to

reduce variation similarly, to GCV of 52%, whereas for Sample C there was no reduction in the GCV 138% for Sample B.
For the clinical samples that included both qualitative and quantitative estimates, the GCV% for Sample F was reduced from 246% to 26% when the potency estimates were expressed relative to Sample D, the candidate IS. Sample A was able to reduce the variability to 65%, whereas Sample C saw a modest reduction in the GCV% to 168%. Interestingly for Sample J the change in the GCV% was similar for both the candidate IS and Sample C, with the reported GCV of 205% reducing to 101% and 105% relative to D and C respectively. Sample A resulted in a greatest reduction in variability with the reported GCV reduced to 86%.
For the clinical samples where only, quantitative estimates were returned; for Sample G the candidate IS and Sample A performed similarly reducing variability, however Sample C resulted in an increase in variability (104% to 453%). For Sample H, the comparator Sample A outperformed the candidate reducing variability from 167% GCV down to 22% GCV, compared with a reduction in GCV% to 79% by the candidate Sample D. Sample C had a limited impact, with the GCV % almost unchanged at 130%. Finally, for Sample I a similar pattern was observed with Sample A outperforming the candidate Sample D, and Sample C having no effect. The calculated relative potency estimates of the study samples calculated relative to Sample A and C are provided in Appendix I: Table 3 and 4. A nominal potency value was not been given to either of the comparator samples, therefore the values remain as $\log_{10}$ relative values.

Discussion

The intended purpose of this study was evaluate the suitability of the proposed candidate WHO International Standard preparation for use as a primary reference material, for the standardisation of VZV NAT-detection assays, and thereby assign a potency to the proposed candidate based on the collective dataset from a broad range of diagnostic assays.

The proposed candidate standard (19/164) comprises of a whole virus preparation of VZV Clade 3, propagated from a clinical isolate and formulated in a TRIS-based universal buffer. The formulation will enable the dilution of the preparation into a suitable clinical matrix for the preparation of a multi-point standard curve for calibration purposes. The use of a whole virus preparation permits processing of the reference preparation in a manner comparable to routine clinical specimens, thereby standardising the entire assay procedure to include the extraction process as well as the DNA amplification steps. This is of particular importance as interlaboratory variability has been widely assigned to variation in extraction procedures (Hayden *et al*., 2012). Here despite the small number of participants, eight different extraction platforms are represented. A further consideration to the selection of a whole virus preparation is the variability in the amplification targets across assays. Here of the targets that were disclosed, 7 different gene targets are represented spanning a region covering ~75% of the entire 125kB VZV genome (ORF 11-62).

This preparation was propagated directly from a primary isolate and has not undergone successive rounds of cell culture propagation. Typically, WHO standards of this type have been generated from well characterised laboratory strains. The inclusion of the Ellen strain was intended to serve as a comparator strain. This strain, which was first described in 1964, originally isolated from a child with varicella in Atlanta, GA and is described to have become

highly attenuated after at least 90 passages in cultured cells (Grose *et al*., 2012). This VZV strain is widely used as a laboratory reference strain, featuring in VZV EQA panels and commercial reference preparations for assay validation. The performance of the proposed candidate IS in the laboratory assays was very similar to the Ellen strain, with the distribution of the individual laboratory mean potency estimates being almost identical. A comparison of reduction in SD and GCV% following the relative potency assessment using either the candidate or Ellen strain, also demonstrates the close performance of the two materials in improving the agreement across the assays for each of the study samples. This contrasted with the v-Oka strain, the live attenuated varicella vaccine, that overall did not improve agreement across laboratories and in one instance increased inter-laboratory variability. These findings support the suitability and use of the candidate VZV isolate as a biological reference preparation, that goes some way to meeting the criteria of a clinically commutable reference preparation. Although its use in global clinical settings will test this assumption.

Our NGS analysis of the candidate IS demonstrated ~98% sequence homology to Clade 3 reference strain HJ0 (Gene accession # AJ871403.1). Despite the small dataset, the candidate was able to successfully improve agreement of the clinical isolates (E, F, I and J). These clinical isolates have yet to be characterised for their Clade designation. As they were donated from within the United Kingdom, they may belong to either Clade 1 or 3, however this remains to be determined. Whilst an attempt was made to acquire clinical isolates from a wider geographical reach, we were unsuccessful. Generally, VZV genomes are thought to be highly conserved. In 2008 Breuer *et al* reported that isolates within a clade display >99.9% identity to one another, while members of one clade compared to a second clade show 99.8% identity to one another (Breuer *et al*., 2010). It remains to be seen if there could be an impact on the standardisation of VZV genotypes in Clades 2, 4 and 5, endemic to Japan and neighbouring regions, Africa, and Southeast Asia respectively.

The production data analysis of the residual oxygen and moisture content of the lyophilised formulation is within the acceptable limits (<1.14% and <1.0% respectively) indicative of long-term stability. Furthermore, the fill CV and the potency estimates are indicative of a homogeneous product. The viral copy values obtained for the VZV candidate post-production shows good homogeneity across the vial contents. The accelerated thermal degradation analyses performed at 12 and 18 months post-production, shows no loss in potency relative to the recommended storage temperature of -20 ºC. Any observed variation in potency is within the expected standard deviation for sample replicates within assay performance. The Arrhenius model depends on a scale of observed loss in potency with increasing temperature and time. With little to no loss observed the accelerated degradation data failed to meet the model criteria. Therefore, a prediction on the long-term stability of the candidate could not be made with certainty. Further accelerated degradation evaluations are scheduled for future time points which will provide additional data for analysis and an indication of continued stability and suitability of the candidate for long-term use.

Unfortunately, as a consequence of the pandemic many participants that were recruited in Q4 of 2019 were unable to commit to the collaborative study and further participants were forced to drop out due to ongoing commitments to the COVID-19 response in their respective countries. Nevertheless, the collaborative study group represents a variety of end-users of VZV NAT assays across a reasonable geographical distribution.
The details supplied by participants on the assay methodologies highlight the heterogeneity of the assay methodologies in use, for both the extraction of samples and amplification of VZV, where no two methods of the returned datasets were identical. This heterogeneity illustrates

the potential difficulty in comparing viral load threshold limits across institutions for patient treatment.

The overall reported mean estimate of the lyophilised candidate sample D is 6.94 $\log_{10}$ copies/ mL (SD 0.33), this is equivalent to the mean of the candidate liquid bulk (Sample B) 6.94 $\log_{10}$ copies/ mL SD 0.38. This demonstrates the stability of the preparation post-lyophilisation and is also indicative of the consistency in the execution and performance of the collaborative study assays. Interestingly the reported potency estimates of the candidate standard (Sample B and D) and the comparator Ellen strain (Sample A) across a proportion of the laboratories was in very good agreement. These estimates were all derived using IVD kits with <0.28 $\log_{10}$ difference between the reported estimates. The remaining assays were almost exclusively represented by laboratory developed tests, which showed greater interlaboratory variability.

Reported estimates from Laboratory 2 made a significant contribution to the interlaboratory variation, and the exclusion of their data significantly reduces the observed variation across Samples A-D, F, H and J from (2.91-3.57 $\log_{10}$) to (0.94-2.10). The reported potency estimates across the samples reported by laboratory 2, are ~2 $\log_{10}$ units lower (100-fold difference) compared with other laboratories. Based on previous collaborative studies of this nature, the observed difference is not unexpected, where a proportion of qualitative assay potency estimates are typically lower than quantitative assay estimates. Of the recruited laboratories that were unable to return data, five represented qualitative assays which could have provided a more balanced representation. For VZV genome detection proficiency panels provided by External Quality assessment scheme providers such as INSTAND and QCMD, data is predominantly submitted from qualitative assays (INSTAND November 2018: 125 qualitative and 51 quantitative). For QMCD the VZV proficiency scheme is reported as a qualitative assessment, despite accepting quantitative data. Therefore, the dataset presented here may not be entirely representative. Nevertheless, for the study reported herein, the decision was made to classify laboratory as an outlier, justified based on the Grubbs statistical test.

The inclusion of clinical samples in the collaborative study provides a preliminary assessment of the ability of the candidate to harmonise agreement of viral potency estimates of true clinical samples, an indication of commutability. Commutability describes the extent to which a reference material behaves in a like manner to patient samples (Hayden and Caliendo 2020). However, commutability is defined as the equivalence of the mathematical relationships between the results of different measurement procedures for a reference material and for representative samples from healthy and diseased individuals. The small number of samples included in this study do not meet this criterion and any meaningful conclusion would require the analysis of a greater sample size conducted in a separate bespoke analysis using linear regression analysis or principle component analysis (Hayden and Caliendo 2020).

PCR detection is currently the most widely employed and recommended method for rapid and sensitive VZV detection in many clinical laboratories worldwide. The findings of the collaborative study data support the use of a common primary biological reference material to standardise and harmonise both qualitative and quantitative detection methods. The observed variation across the laboratories for all the samples was markedly improved when the potencies were expressed relative to the candidate material, demonstrating the suitability of

the candidate to standardise discordant results. Implementation and traceability to a common reference preparation could ensure better comparability between laboratories and direct therapeutic management decisions where applicable. The results of the study demonstrate that the proposed candidate standard NIBSC code 19/164, would be suitable for use as a standard of VZV DNA detection assays.

Whilst the international unit is in its conception an arbitrary unit, based on the collaborative study data and the decision to exclude the outlier laboratory 2 (significant according to Grubb's test) we propose, the potency assignment could be based on the average 6.94 $\log_{10}$ of the remaining dataset (range 6.44-7.79). For the purposes of the relative potency analysis presented in this draft report a provisional value of 7.0 $\log_{10}$ IU/vial was selected which may be more practical in use. Therefore, considering the findings of this collaborative study we would recommend a value of 6.94 $\log_{10}$ rounded up to 7.0 $\log_{10}$ International Units/ vial be assigned to the 1st WHO International Standard for VZV NAT detection assays.

Proposal

It is proposed that the candidate standard (NIBSC code 19/164) is established as the 1st WHO International standard for VZV DNA for nucleic acid amplification technique (NAT) detection assays with an assigned potency of 7.0 $\log_{10}$ International Units when reconstituted in 1 mL of nuclease-free water (7.0 $\log_{10}$ IU/vial). The proposed standard is intended for use by IVD manufactures for kit calibration and for use by clinical, reference and research laboratories for the calibration of secondary reference reagents used in routine NAT-assays for VZV detection.

Comments from participants

A previous version of this report was circulated to all participants for comment. Participants were asked directly regarding the decision on value assignment. One participant commented: To my understanding, a WHO international standard is devoted to assessing quantitative results and as such, assignment should take into account quantitative kits results and not extrapolation of qualitative results. An error in a participant's data analysis was also queried which has been rectified, minor editorial changes were also addressed. The report was re-written based on the recommendation of our statistician, to exclude qualitative data from one participant. This report has been recirculated to all participants and any further comments will be requested to be submitted within the consultation period. During the consultation period we received a response from one participant, querying the exclusion of some of their data from the analysis. The exclusion was due to samples not being tested together across successive runs. The participant explained that for their random continuous access system, tests are not run as badges, and the time when the runs are performed should not matter as with traditional batch-based systems. Where their findings were initially excluded their data has been re-calculated (as described in statistical methods) and now included in the relevant tables and figures.

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Table 1: Study sample details

CS652 sample	Study sample ID	VZV Sample ID
A	Comparator	Ellen strain (ATCC)
B	Liquid candidate	19/164 Liquid
C	Comparator	v-Oka strain (EDQM)
D	Candidate standard	19/164 Lyophilised
E	Clinical sample	Shingle vesicle swab
F	Clinical sample	Shingle vesicle swab
G	Candidate spiked clinical matrix	Spiked plasma
H	Candidate spiked clinical matrix	Spiked CSF
I	Clinical sample	Paediatric vesicle swab
J	Clinical sample	Paediatric vesicle swab

Table 2: Production summary for the candidate standard (Sample D)

NIBSC code	19/164
Product name	Varicella Zoster Virus
Dates of processing	Filling: 23/9/19
	Lyophilisation: 23/9/19
	Sealing: 26/9/21
Presentation	Freeze-dried preparation in 5ml screw-cap glass vial
Appearance	Well-formed robust cake
No of vials available to WHO	5679
Mean fill weight (g)	1.0064
CV of fill weight (%)	0.2260 (n=192)
Mean residual moisture (%)	0.15%
CV of residual moisture (%)	n/a (n=12)
Mean of Oxygen content (%)	1.27
CV of Oxygen content (%)	8.09 (n=12)
Microbial testing outcome (cfu/ml)	Pre-fill/Post-fill/Post-freeze dried: Negative

Table 3: Homogeneity testing of lyophilised candidate material 19/164 (Sample D)

Lyophilised Candidate	Average Log_{10} copies/ mL		
	***MagNA 24 Plasma**	**QIAcube Plasma**	**QIAcube TRIS buffer**
Vial 1	6.70	6.92	6.76
Vial 2	6.75	6.92	6.79
Vial 3	6.77	7.00	6.81
Vial 4	6.80	6.97	6.75
Vial 5	6.82	6.98	6.82
Vial 6	6.77	6.84	6.90
Vial 7	6.80	6.91	6.94
Vial 8	6.76	-	6.91
Overall Av Log_{10} copies/ mL	6.77	6.93	6.84
SD	0.04	0.05	0.07
GCV (%)	10	12	17

*MagNA 24 extraction platform is incompatible with TRIS buffer samples (data excluded)

Table 4a: Accelerated Degradation assessment- Stability at 12 and 18 months

Temperature (°C)	Mean log_{10} copies/mL at **12** months	Difference in log_{10} copies/mL from -20°C baseline sample	Mean log_{10} copies/mL at **18** months	Difference in log_{10} copies/mL from -20°C baseline sample
-70	6.91		6.84	
-20	6.86		6.81	
+4	6.96	0.1	6.82	0.01
+20	6.84	-0.02	6.83	0.02
+37	6.84	-0.02	6.81	-
+45	6.85	-0.01	6.79	-0.02

Table 4b: Accelerated Degradation assessment- Relative potencies to the -20°C baseline

Storage Temperature	Time point	Relative potency	95% confidence interval
+4°C	12 months	1.01	0.86 – 1.19
+20°C		0.96	0.82 – 1.13
+37°C		0.95	0.79 – 1.14
+45°C		1.00	0.87 – 1.14
+4°C	18 months	1.09	0.91 – 1.32
+20°C		1.04	0.92 – 1.17
+37°C		1.02	0.90 – 1.14
+45°C		0.96	0.86 – 1.07

Collated data from 6 vials at each temperature/ timepoint, each analysed at three 10 fold dilutions (10, 100 and 100). Extractions performed on MagNA 24 using serum as a diluent.

Table 5: Minimum complement of SNPs required to identify a putative candidate IS clade (adapted from Breuer *et al*., 2010)

		NIBSC isolate		
ORF	SNP	VZV244	Clade 1	Clade 3
1	508	C	C	C
1	685	G	G	G
1	790	T	T	T
6	5827	A	C	A
6	6850	G	G	G
12	17834	C	C	C
12	18082	C	C	C
16	23294	G	A	G
17	24533	A	A	A
17	24578	G	A	G
21	33725	C	T	C
22	37902	A	A	A
22	38019	G	G	G
22	38055	T	T	T
22	38081	A	A	A
22	38177	G	G	G
22	39394	A	G	A
35	64703	G	G	G
37	68101	C	T	C
37	68172	A	G	A
37	68254	T	C	T
50	87841	T	C	T
54	95241	T	T	T
55	98437	T	T	T
56	98825	T	T	T
60	101464	C	C	C
66	113243	C	A	C

Table 6: Laboratory assay methods

Lab	Diluent	Extraction Platform	Amplification Assay	Target	Amplification Platform
2	UTM	BioMerieux EasyMAG	LDT	Poly gene/ORF28	Rotorgene Q
7	UTM	ZJ Bio-TechAuto NA Extra	VZV Rt-PCR Kit ZJ Bio-Tech	Proprietary	ABI 7500
11	UTM	QIAsymphony	LDT	ORF 38	Rotorgene Q
1a	CSF	BioMerieux EasyMAG	BioMerieiux HSV/VZV R0Gene Kit	gp19/ORF17	CFX96/RotorgeneQ/ ABI7500/Lightcycler 480
1b	Plasma	BioMerieux EasyMAG	BioMerieiux HSV/VZV R0Gene Kit	gp19/ORF17	CFX96/RotorgeneQ/ ABI7500/Lightcycler 480
5	water	BioMerieux EasyMAG	LDT	Proprietary	ABI 7500
6	Plasma	AltoStar Automation System	AltoStar VZV PCR kit 1,5	Proprietary	CFX96
8	Plasma	QIAamp DNA Blood Mini kit (manual)	LDT	gB/ORF 31	Quantstudio 3
10	UTM	Antolia Geneworks/ Magrev NA Extr	Bosphore VZV Quantification kit	ORF 38	Montania 4896
12a	UTM	EZ1 DSP QIAGEN	Anchor VZV PCR kit	Proprietary	Rotorgene Q
12b	UTM	MagMAX KingFisher systemsThermofisher	Anchor VZV PCR kit	Proprietary	Rotorgene Q
15	Plasma	NeuMoDx Molecular System	NeuMoDx VZV Quant Assay	ORF 11 and 29	NeuMoDx Molecular System
17	UTM	DSP Midi QIAsymphony	LDT	ORF 62	ABI 7500/Viia 7
18	UTM	QIAamp DNA Blood Mini QIAcube Connect	Altona Real Star kit 1.0	Proprietary	Abbott m2000rt

Table 7. Laboratory reported potency estimates of samples (in log_{10} copies/mL and in log_{10} NAT-detectable units for qualitative assays)

Lab	Method	A	B	C	D	E	F	G	H	I	J
1a	Quant	7.06	7.03	8.61	6.99	*	*	+	4.37	*	*
1b	Quant	7.02	7.00	8.58	6.98	*	*	4.25	+	*	*
2	Qual	4.76	4.76	5.93	4.69	*	4.76	*	2.05	*	3.69
5	Quant	7.29	6.81	7.38	6.73	6.93	6.65	*	*	5.22	5.61
6	Quant	7.02	7.07	8.20	7.06	*	*	4.51	*	*	*
7	Qual	6.85	6.79	8.08	6.66	*	6.58	*	*	*	5.97
8	Quant	6.66	6.39	7.40	6.44	6.60	6.20	3.87	*	4.70	5.41
10	Quant	7.52	7.50	8.61	7.18	*	*	4.31	4.91	*	*
11	Qual	6.97	6.53	8.06	6.64	*	6.41	*	*	*	5.80
12a	Quant	6.85	6.86	8.35	6.87	**	**	**	**	**	**
12b	Quant	6.65	6.72	8.37	6.99	6.96	6.49	3.90	4.02	5.04	5.75
15	Quant	6.81	6.79	8.38	6.93	*	*	3.79	*	*	*
17	Quant	7.85	7.82	9.50	7.79	8.31	7.85	4.60	4.96	6.16	6.89
18	Quant	6.85	6.89	8.14	6.89	7.07	6.54	3.98	4.18	4.85	5.62
Average	Qual	6.19	6.02	7.36	6.00		5.91		2.05		5.16
	Quant	7.05	6.99	8.32	6.99	7.18	6.75	4.15	4.49	5.19	5.86
Std Dev	Qual	1.25	1.10	1.23	1.13		1.01				1.27
	Quant	0.37	0.39	0.58	0.33	0.66	0.64	0.31	0.43	0.57	0.59
GCV	Qual	1661%	1172%	1607%	1255%		913%				1770%
	Quant	134%	144%	284%	114%	356%	336%	104%	167%	274%	288%

*sample not included in study panel for this laboratory; ** samples not tested using method 12a; +sample tested according to matrix used

Excluding Laboratory 2

Average	Qual	6.91	6.66	8.07	6.65		6.49				5.89
	Quant	7.05	6.99	8.32	6.99	7.18	6.75	4.17	4.49	5.19	5.86
Std Dev	Qual										
	Quant	0.37	0.39	0.58	0.33	0.66	0.64	0.33	0.43	0.57	0.59
GCV	Qual										
	Quant	134%	144%	284%	114%	356%	336%	112%	167%	274%	288%

Table 8. Overall mean reported potency estimates and inter-laboratory variation (in log_{10} copies/mL and log_{10} NAT detectable units/mL)

Quantitative and Qualitative assays combined								
CS652 sample	VZV Sample ID	No. of datasets	Av Potency Estimate	SD	GCV	Min log_{10}	Max log_{10}	Range log_{10}
A	Ellen strain (ATCC)	14	6.87	0.69	391%	4.76	7.85	3.09
B	19/164 Liquid	14	6.78	0.69	386%	4.76	7.82	3.06
C	v-Oka strain (EDQM)	14	8.11	0.82	553%	5.93	9.50	3.57
D	19/164 Lyophilised	14	6.77	0.68	375%	4.69	7.79	3.10
E	Shingle vesicle swab	5	7.18	0.66	356%	6.60	8.31	1.71
F	Shingle vesicle swab	8	6.43	0.84	595%	4.76	7.85	3.10
G	Spiked plasma	8	4.15	0.31	104%	3.79	4.60	0.81
H	Spiked CSF	6	4.08	1.07	1067%	2.05	4.96	2.91
I	Paediatric vesicle swab	5	5.19	0.57	274%	4.70	6.16	1.46
J	Paediatric vesicle swab	8	5.59	0.89	676%	3.69	6.89	3.20

Excluding laboratory 2

CS652 sample	VZV Sample ID	No. of datasets	Av Potency Estimate	SD	GCV	Min log_{10}	Max log_{10}	Range log_{10}
A	Ellen strain (ATCC)	13	7.03	0.34	120%	6.66	7.85	1.19
B	19/164 Liquid	13	6.94	0.38	139%	6.39	7.82	1.43
C	v-Oka strain (EDQM)	13	8.28	0.54	248%	7.38	9.5	2.10
D	19/164 Lyophilised	13	6.94	0.33	112%	6.44	7.79	1.35
E	Shingle vesicle swab	5	7.18	0.66	356%	6.60	8.31	1.71
F	Shingle vesicle swab	7	6.67	0.54	246%	6.20	7.85	1.65
G	Spiked plasma	8	4.17	0.33	112%	3.79	4.60	0.81
H	Spiked CSF	7	4.49	0.43	167%	4.02	4.96	0.94
I	Paediatric vesicle swab	5	5.19	0.57	274%	4.70	6.16	1.46
J	Paediatric vesicle swab	7	5.87	0.48	205%	5.41	6.89	1.48

Table 9. Standard deviation of reported potency estimates within quantitative assays

Lab	A	B	C	D	E	F	G	H	I	J
1a1	0.06	0.05	0.07	0.02				0.01		
1a2	0.09	0.03	0.05	0.01				0.02		
1a3	0.07	0.01	0.06	0.02				0.05		
1a4	0.08	0.06	0.08	0.01				0.03		
1b1	0.05	0.04	0.07	0.03			0.03			
1b2	0.06	0.07	0.07	0.02			0.03			
1b3	0.05	0.03	0.07	0.01			0.04			
1b4	0.05	0.09	0.10	0.02			0.00			
5	0.03	0.10	0.04	0.10	0.15	0.17			0.02	0.09
6	0.05	0.05	0.10	0.05			0.14			
8	0.20	0.27		0.22	0.29	0.31	0.17		0.18	0.25
10	0.05	0.04	0.16	0.14			0.05	0.02		
12a	0.06	0.13	0.01	0.13						
12b	n< 3	n< 3	n< 3	n< 3	0.10	0.14	0.12	0.13	0.10	0.11
15	0.04	0.06	0.05	0.05			0.03			
17	0.09	0.17	0.79	0.11	0.06	0.08	0.25	0.11	0.17	0.14
18	0.08	0.24	0.02	0.03	0.13	0.07	0.05	0.11	0.12	0.10

n<3 insufficient replicates performed

Table 10: Potency estimates of samples (log_{10} IU/mL) calculated relative to Sample D with assumed potency of 7.00 IU/vial

Lab	Method	A	B	C	E	F	G	H	I	J
1a	Quant	7.05	7.04	8.66				4.37		
1b	Quant	7.01	7.03	8.69			4.27			
2	Qual	7.07	7.07	8.24		7.07		4.36		6.00
5	Quant	7.53	7.07	7.58	RR				5.39	5.75
6	Quant	6.95	7.01	8.25			4.46			
7	Qual	7.19	7.13	8.41		6.91				6.31
8	Quant	7.21	6.95	7.69	RR		4.46		5.24	5.46
10	Quant	7.34	7.33	8.51			4.13	4.74		
11	Qual	7.33	6.88	8.42		6.77				6.16
12a	Quant	6.97	6.89	8.59	RR					
15	Quant	6.88*	6.86*	8.46*			3.85			
17	Quant	7.06	7.00	8.68	RR	6.96	3.90	4.24	5.37	6.05
18	Quant	6.96	6.83	8.26	RR		3.99	4.17	4.93	5.91
Average	Qual	7.20	7.03	8.36		6.92		4.36		6.16
	Quant	7.12	7.02	8.32		6.96	4.15	4.38	5.23	5.79
	All	7.14	7.02	8.33		6.93	4.15	4.38	5.23	5.95
Std Dev	Qual	0.13	0.13	0.10		0.15				0.16
	Quant	0.20	0.14	0.42			0.25	0.25	0.21	0.25
	All	0.18	0.13	0.37		0.12	0.25	0.22	0.21	0.28
GCV	Qual	36%	34%	26%		41%				43%
	Quant	59%	38%	166%			78%	79%	62%	78%
	All	53%	35%	132%		33%	78%	66%	62%	90%
Excluding Laboratory 2										
Average	Qual	7.26	7.01	8.42		6.84				6.24
	Quant	7.12	7.02	8.32		6.96	4.17	4.38	5.23	5.79
	All	7.15	7.02	8.34		6.88	4.17	4.38	5.23	5.94
Std Dev	Qual									
	Quant	0.20	0.14	0.42			0.26	0.25	0.21	0.25
	All	0.19	0.14	0.38		0.10	0.26	0.25	0.21	0.30
GCV	Qual									
	Quant	59%	38%	166%			81%	79%	62%	78%
	All	55%	37%	141%		26%	81%	79%	62%	101%

*****Calculated via a different method, not included in summary calculations**. RR** Out of range of response values for reference

Table 11: Summary of inter-laboratory variation for reported estimates and relative estimates to Sample D using assumed potency of 7.00 log_{10} IU/vial

Sample	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Reported Average cp/mL	6.87	6.78	8.11	6.77	7.18	6.43	4.15	4.08	5.19	5.59
Reported StdDev	0.69	0.69	0.82	0.68	0.66	0.84	0.31	1.07	0.57	0.89
Reported GCV%	391%	386%	553%	375%	356%	595%	104%	1067%	274%	676%
Relative Average IU/mL	7.14	7.02	8.33			6.93	4.15	4.38	5.23	5.95
Relative StdDev	0.18	0.13	0.37			0.12	0.25	0.22	0.21	0.28
Relative GCV%	53%	35%	132%			33%	78%	66%	62%	90%

Excluding laboratory 2

Sample	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Reported Average cp/mL	7.03	6.94	8.23	6.94	7.18	6.67	4.15	4.49	5.19	5.87
Reported StdDev	0.34	0.38	0.54	0.33	0.66	0.54	0.31	0.43	0.57	0.48
Reported GCV%	120%	139%	248%	112%	356%	246%	104%	167%	274%	205%
Relative Average IU/mL	7.15	7.02	8.34			6.88	4.15	4.38	5.23	5.95
Relative StdDev	0.19	0.14	0.38			0.10	0.25	0.25	0.21	0.30
Relative GCV%	55%	37%	141%			26%	78%	79%	62%	101%

Sample E Out of range of response values for reference

Table 12. Summary of GCV values of reported and relative estimates

		A	B	C	D	E	F	G	H	I	J
Reported	Qual	1661%	1172%	1607%	3%		913%				1770%
	Quant	134%	144%	284%	114%	356%	336%	104%	167%	274%	288%
	All	391%	386%	553%	112%	356%	595%	104%	1067%	274%	676%
Relative to D	Qual	36%	34%	26%			41%				43%
	Quant	59%	38%	166%				78%	79%	62%	78%
	All	53%	35%	132%			33%	78%	66%	62%	90%
Relative to A	Qual		74%	17%	36%		91%				40%
	Quant		45%	242%	59%			87%	22%	45%	85%
	All		50%	191%	53%		74%	87%	19%	45%	79%
Relative to C	Qual	17%	53%		26%		75%				22%
	Quant	242%	157%		166%	216%	297%	453%	130%	273%	150%
	All	191%	131%		132%	216%	164%	453%	103%	273%	93%

Excluding Laboratory 2

		A	B	C	D	E	F	G	H	I	J
Reported	Qual										
	Quant	134%	144%	284%	114%	356%	336%	112%	167%	274%	288%
	All	120%	139%	248%	112%	356%	246%	112%	167%	274%	205%
Relative to D	Qual										
	Quant	59%	38%	166%				81%	79%	62%	78%
	All	55%	37%	141%			26%	81%	79%	62%	101%
Relative to A	Qual										
	Quant		45%	242%	59%			71%	22%	45%	85%
	All		52%	205%	55%		65%	71%	22%	45%	86%
Relative to C	Qual										
	Quant	242%	157%		166%	216%	297%	453%	130%	273%	150%
	All	205%	138%		141%	216%	168%	453%	130%	273%	105%

Sample E Out of range of response values for reference D and A

Figure legends

Figure 1a: Graph of Sample A potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples A (Comparator Ellen Strain) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 1b: Graph of Sample A potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample A (Comparator Ellen strain) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 2a: Graph of Sample B potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples B (Liquid candidate) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 2b: Graph of Sample B potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample B (liquid candidate IS) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 3a: Graph of Sample C potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples C (Comparator v-Oka Strain) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 3b: Graph of Sample C potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample C (Comparator v-Oka Strain) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 4: Graph of Sample D potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples D (lyophilised candidate IS) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 5a: Graph of Sample E potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples E (adult swab) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 5b: Graph of Sample E potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample E (adult swab) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code. Where the response values for the test sample did not fall within the range of response values these boxes are shaded (RR).

Figure 6a: Graph of Sample F potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples F (adult swab) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 6b: Graph of Sample F potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample F (adult swab) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code. Where the response values for the test sample did not fall within the range of response values these boxes are shaded (RR).

Figure 7a: Graph of Sample G potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples G (Candidate spike in Plasma) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 7b: Graph of Sample G potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample G (Candidate spike in Plasma) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 8a: Graph of Sample H potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples H (Candidate spike in CSF) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 8b: Graph of Sample H potency estimates, calculated relative to Sample D
Laboratory mean estimates of Sample H (Candidate spike in CSF) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 9a: Graph of Sample I potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples I (paediatric swab) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log_{10} copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 9b: Graph of Sample I potency estimates calculated relative to Sample D
Laboratory mean estimates of Sample I (paediatric swab) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 10a: Graph of Sample J potency estimates from laboratory reported results
Laboratory reported mean estimates of Samples J (paediatric swab) from quantitative (purple boxes) and qualitative assays (green boxes) reported as Log10 copies/mL for quantitative assays and Log_{10} NAT-detectable units for qualitative assays. Each box is labelled with a laboratory code.

Figure 10b: Graph of Sample J potency estimates calculated relative to Sample D
Laboratory mean estimates of Sample J (paediatric swab) calculated relative to the Sample D VZV candidate IS (19/164) from quantitative (purple) and qualitative (green) assays. Units are expressed as Log_{10} IU/mL. Each box is labelled with a laboratory code.

Figure 11: Graph of Sample D reported potency estimates with matrix references

Sample D VZV candidate IS (19/164) laboratory reported mean estimates from quantitative and qualitative assays depicting the diluent used for analysis. CSF (yellow), Plasma (red), Universal transport medium (UTM) (green) and Molecular grade water (Blue).

Figure 1a. Graph of Sample A potency estimates from laboratory reported results

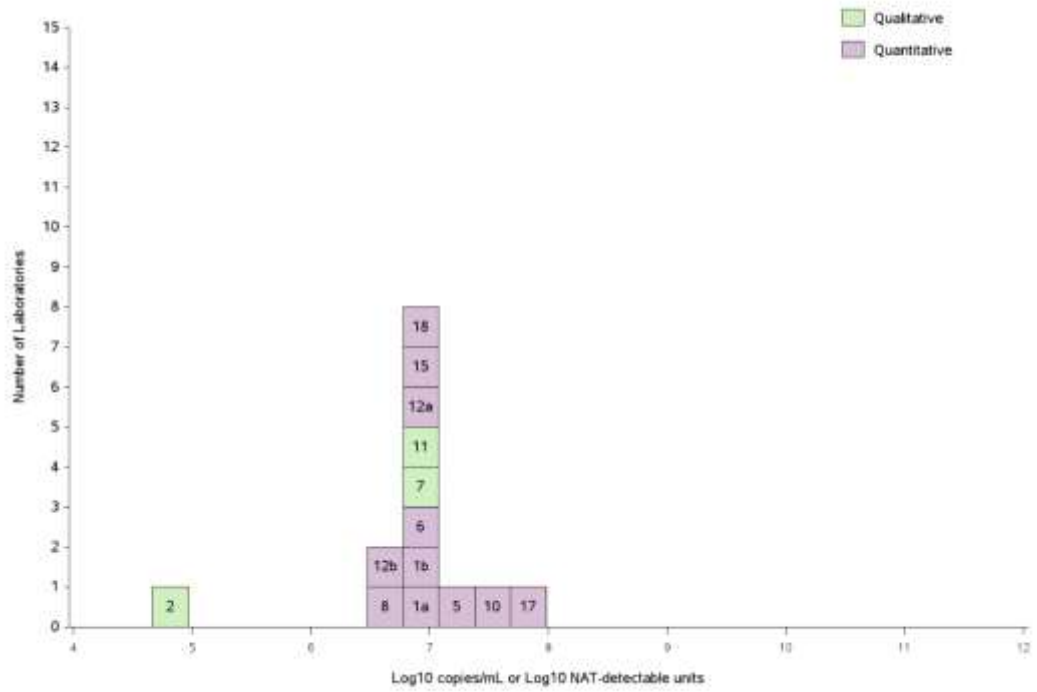


Figure 1b. Graph of Sample A potency estimates, calculated relative to Sample D

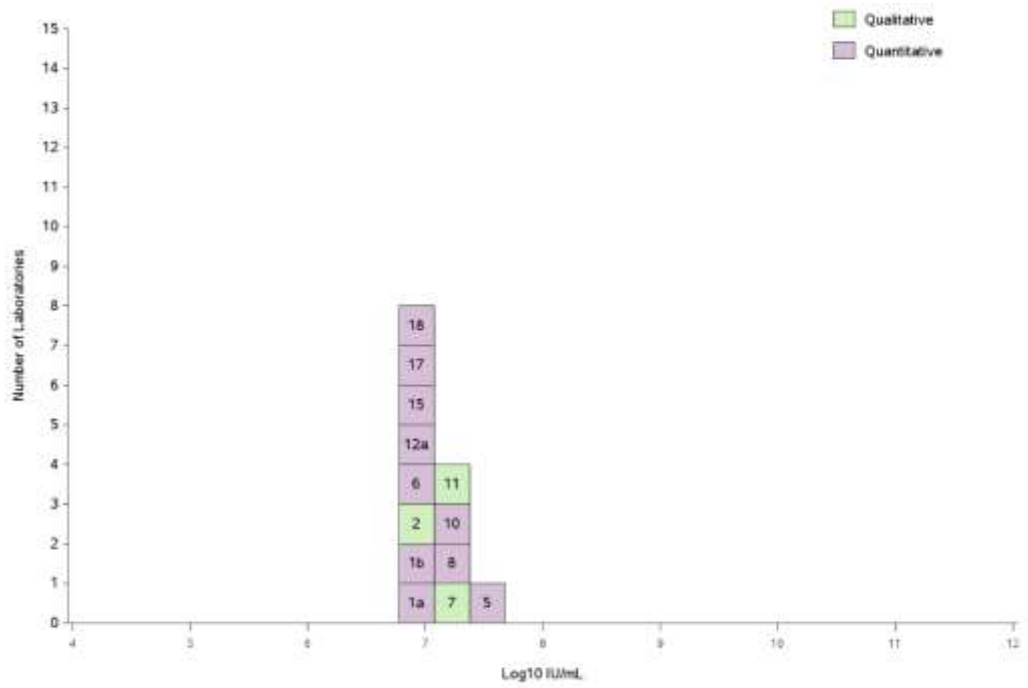

Qualitative
Quantitative
Number of Laboratories
Log10 IU/mL
18
17
15
12a
6
11
2
10
1b
8
1a
7
5

Figure 2a. Graph of Sample B potency estimates from laboratory reported results

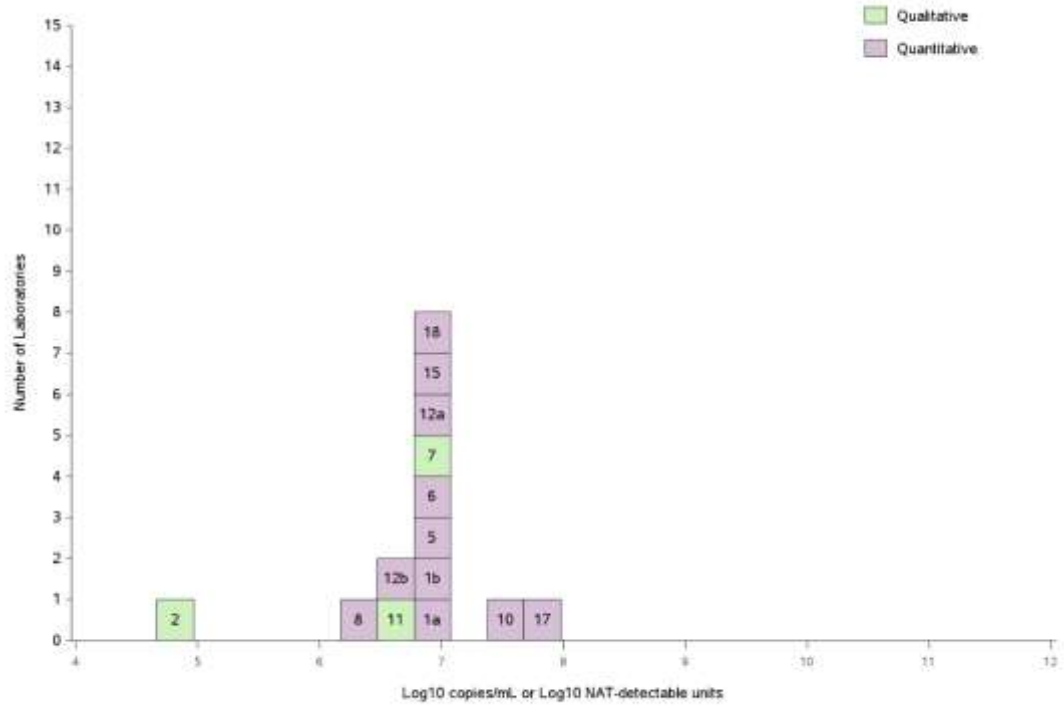


Figure 2b. Graph of Sample B potency estimates, calculated relative to Sample D

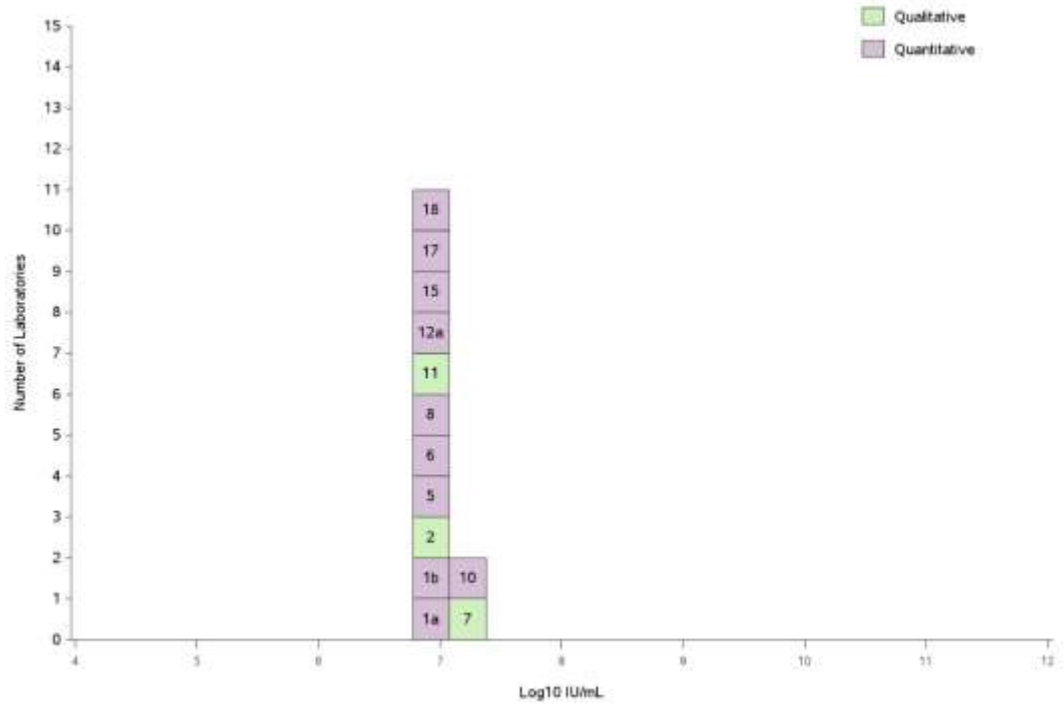


Figure 3a. Graph of Sample C potency estimates from laboratory reported results

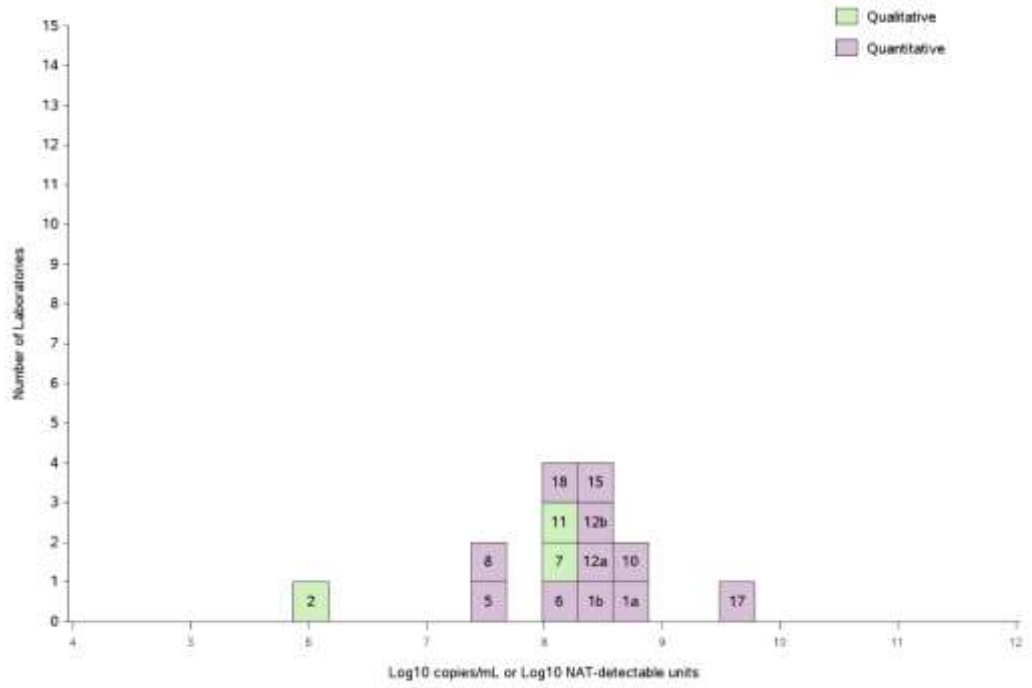


Figure 3b. Graph of Sample C potency estimates, calculated relative to Sample D

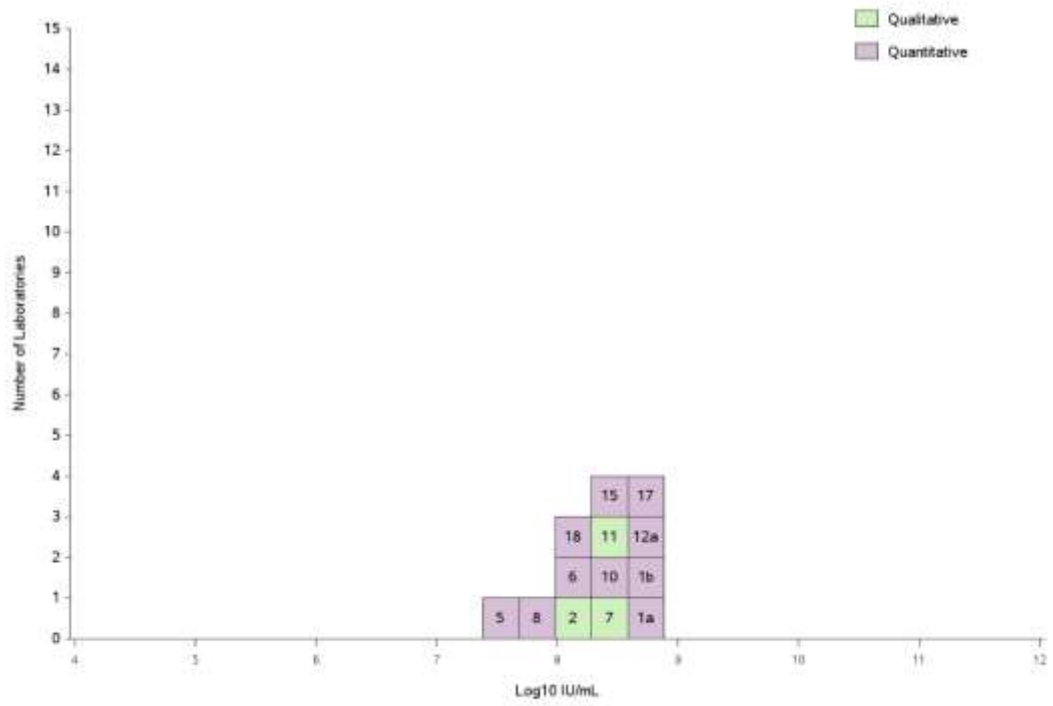


Figure 4. Graph of Sample D potency estimates from laboratory reported results

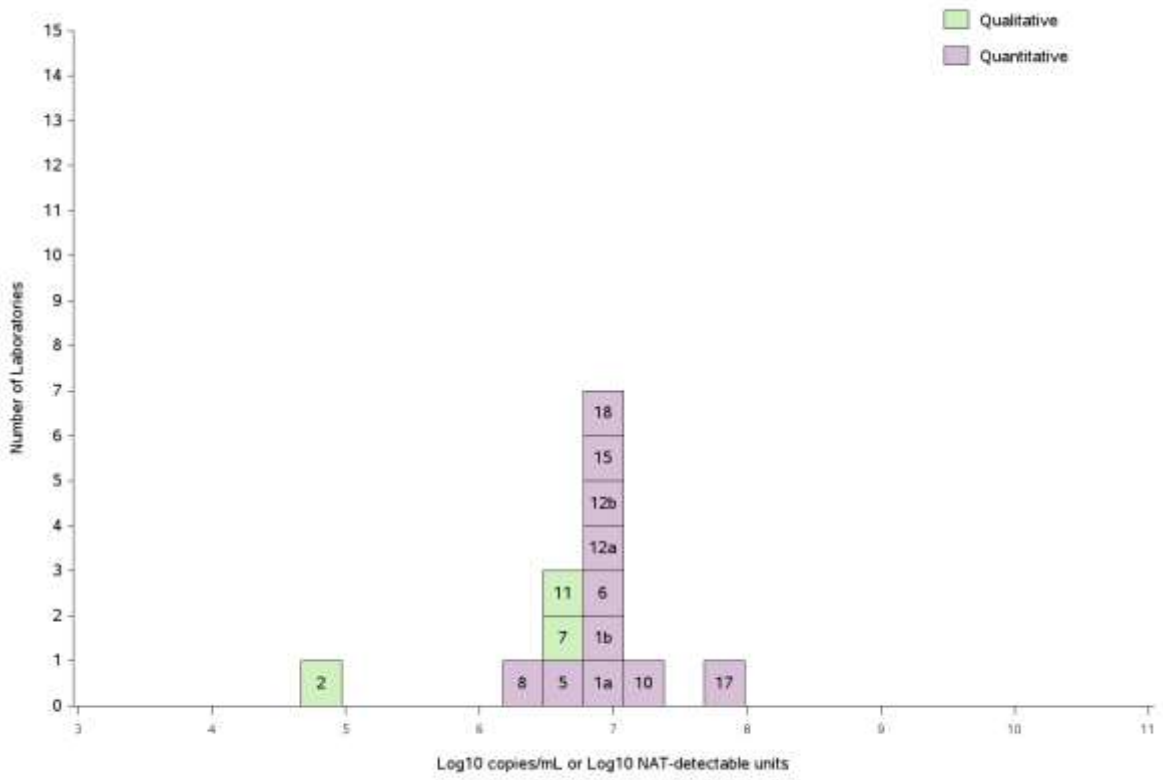


Figure 5. Graph of Sample E potency estimates from laboratory reported results

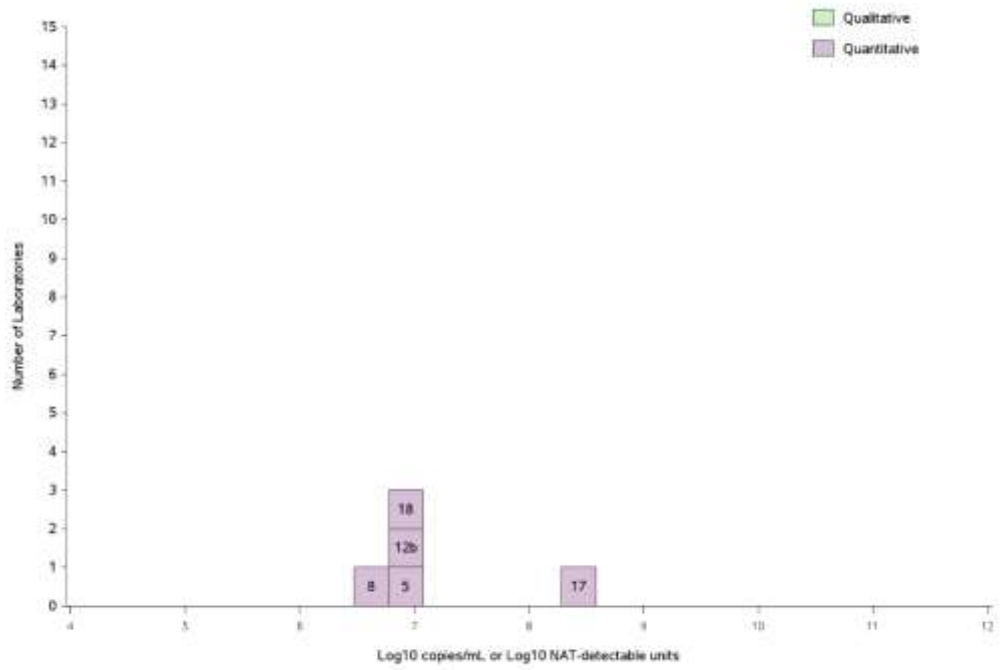


Figure 5b. Graph of Sample E potency estimates, calculated relative to Sample D

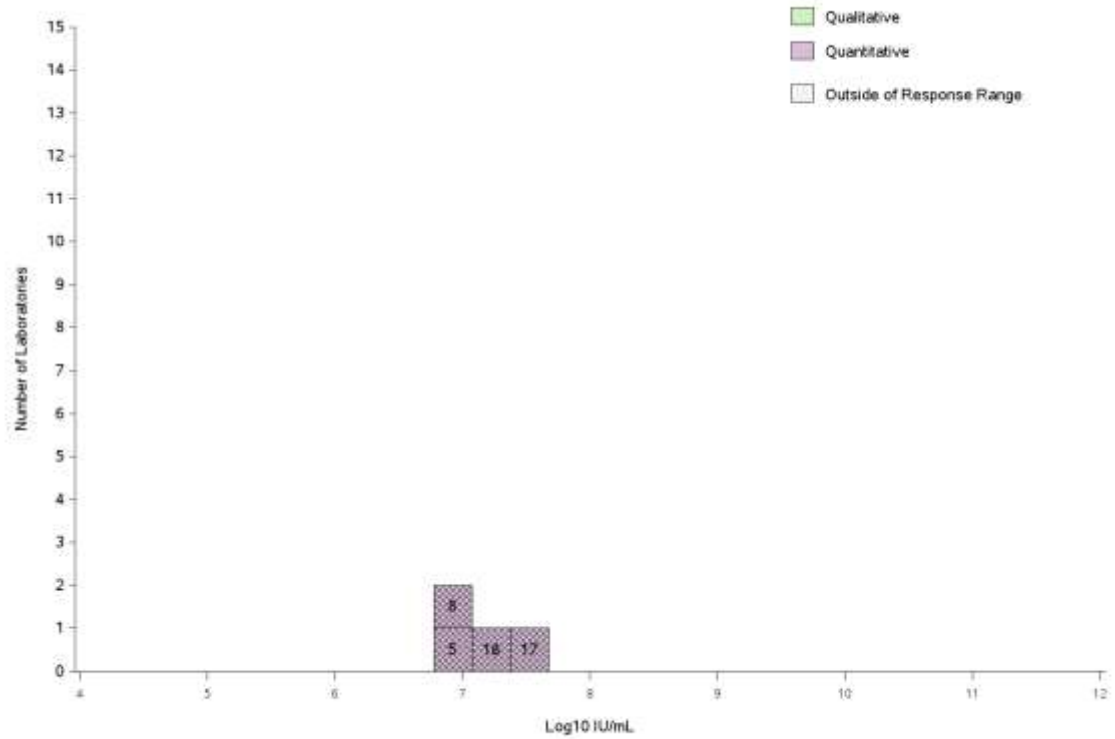


Figure 6a. Graph of Sample F potency estimates from laboratory reported results

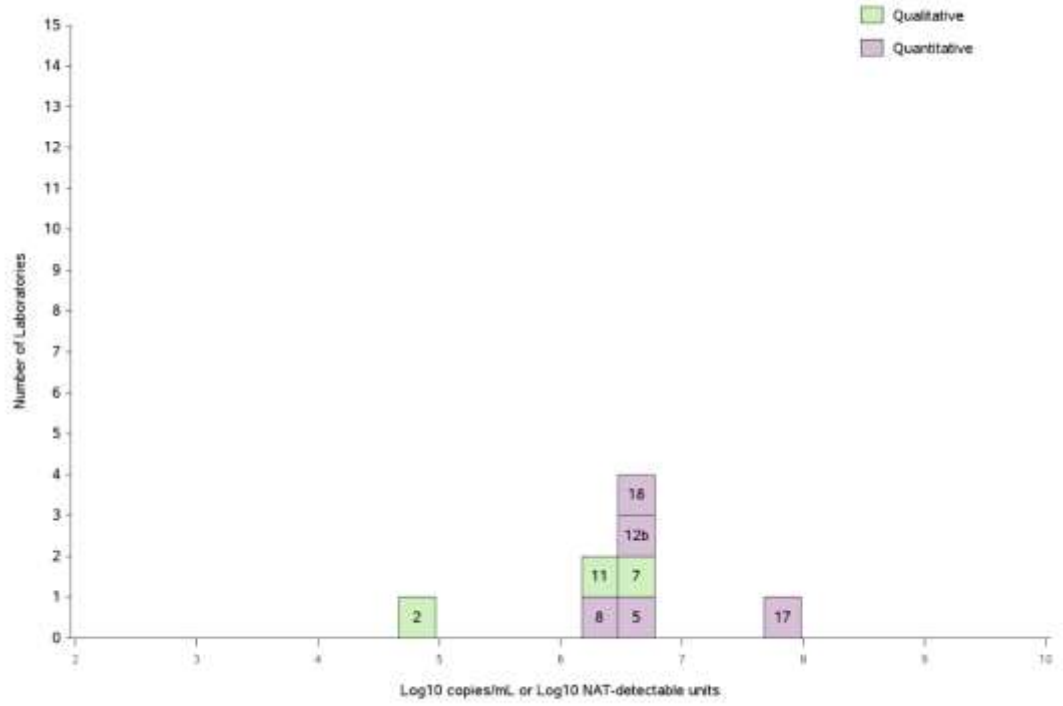


Figure 6b. Graph of Sample F potency estimates, calculated relative to Sample D

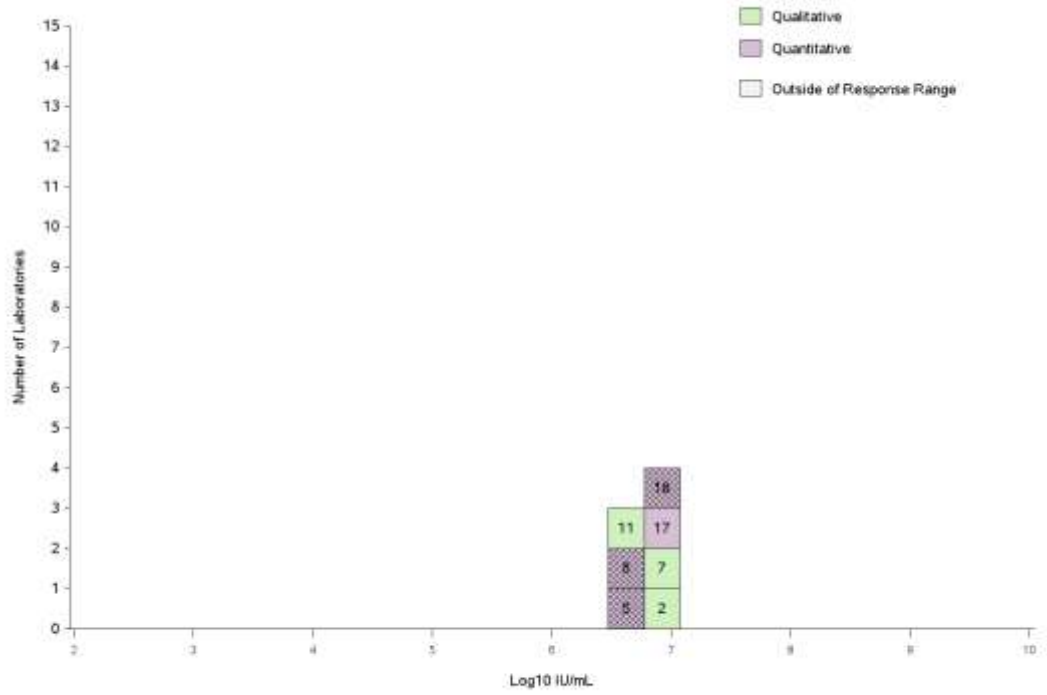


Figure 7a. Graph of Sample G potency estimates from laboratory reported results

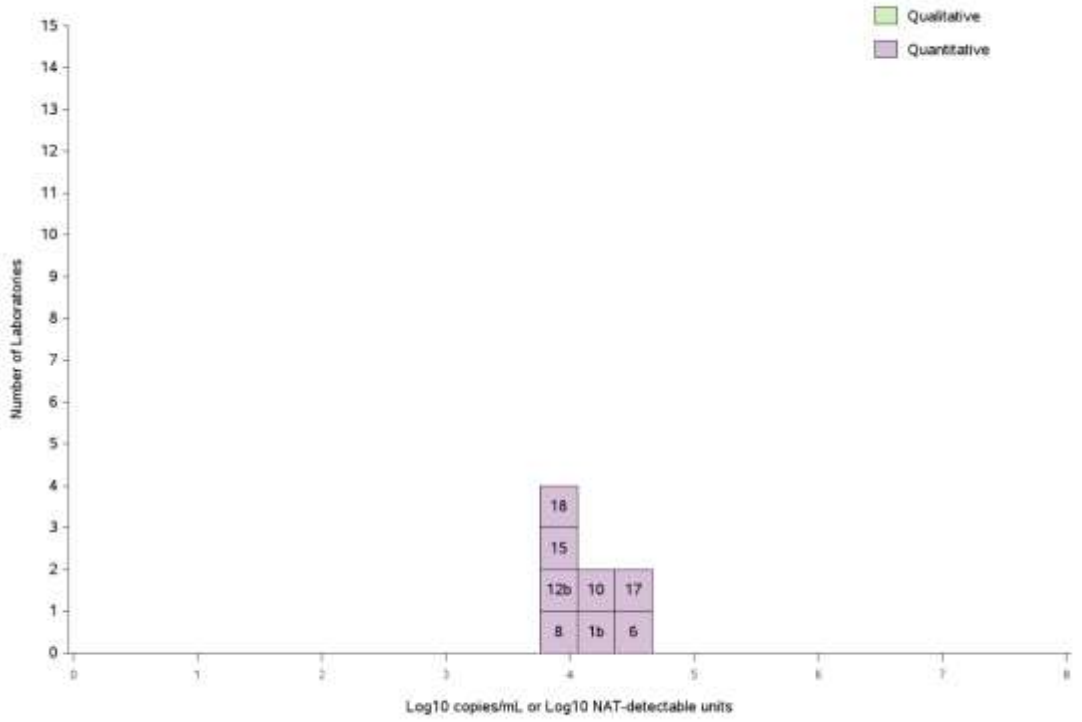


Figure 7b. Graph of Sample G potency estimates, calculated relative to Sample D

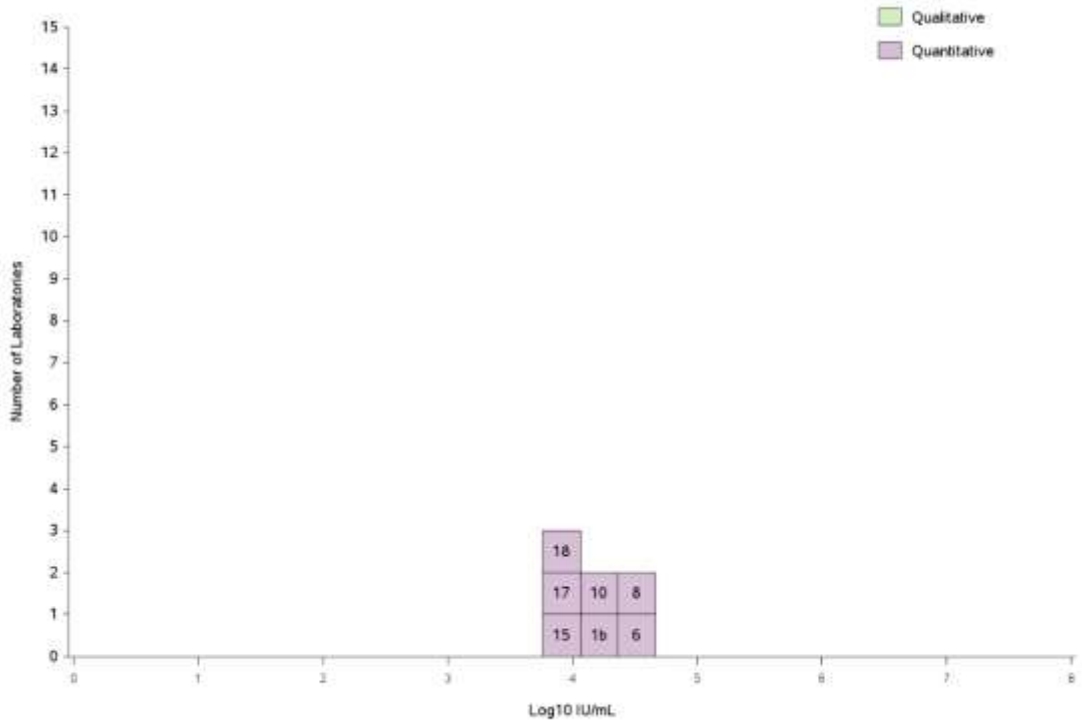


Figure 8a. Graph of Sample H potency estimates from laboratory reported results

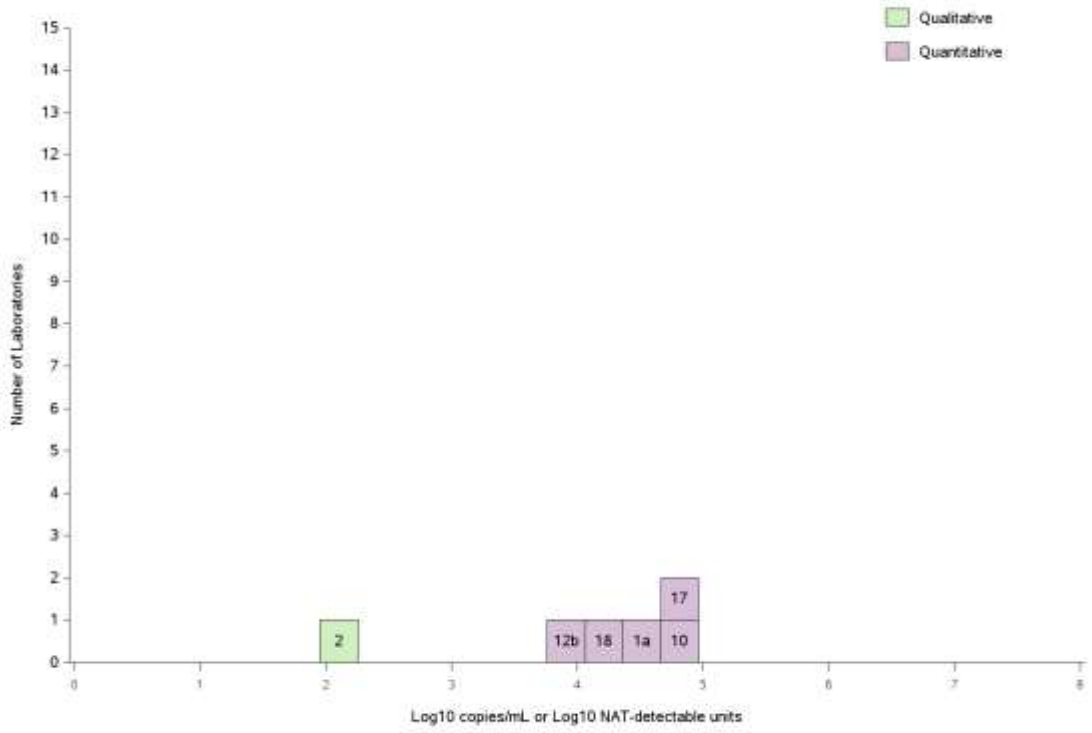


Figure 8b. Graph of Sample H potency estimates, calculated relative to Sample D

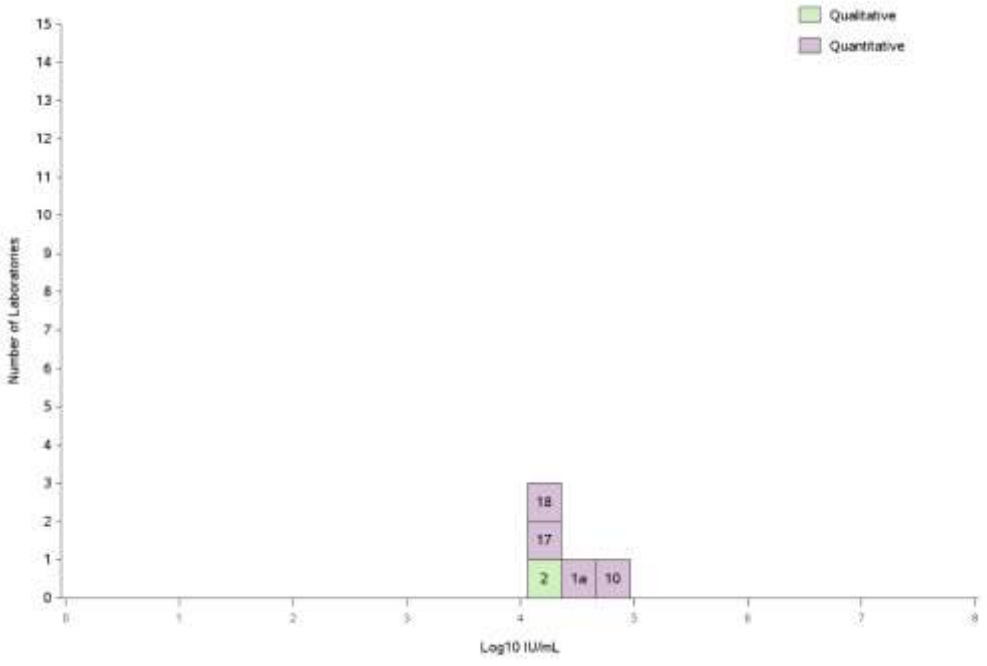


Figure 9a. Graph of Sample I potency estimates from laboratory reported results

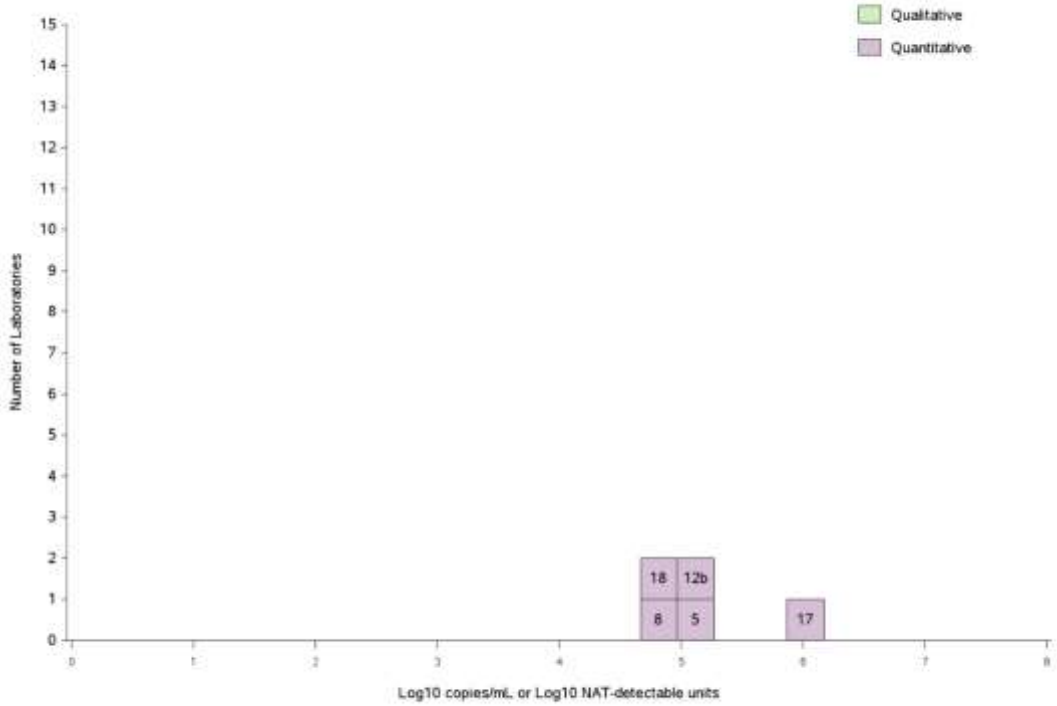


Figure 9b. Graph of Sample I potency estimates, calculated relative to Sample D

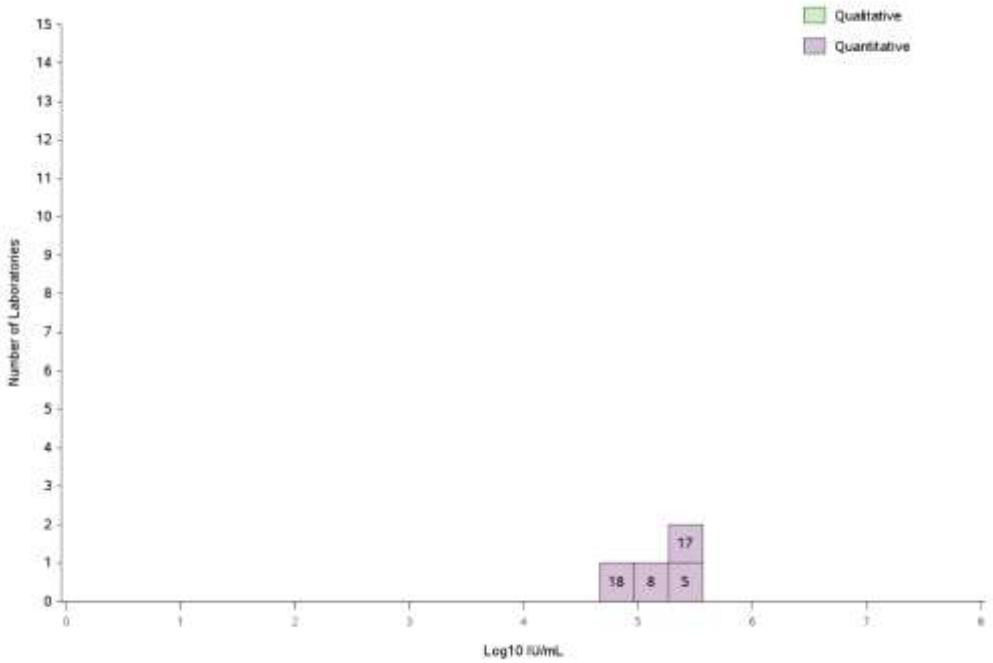


Figure 10a. Graph of Sample J potency estimates from laboratory reported results

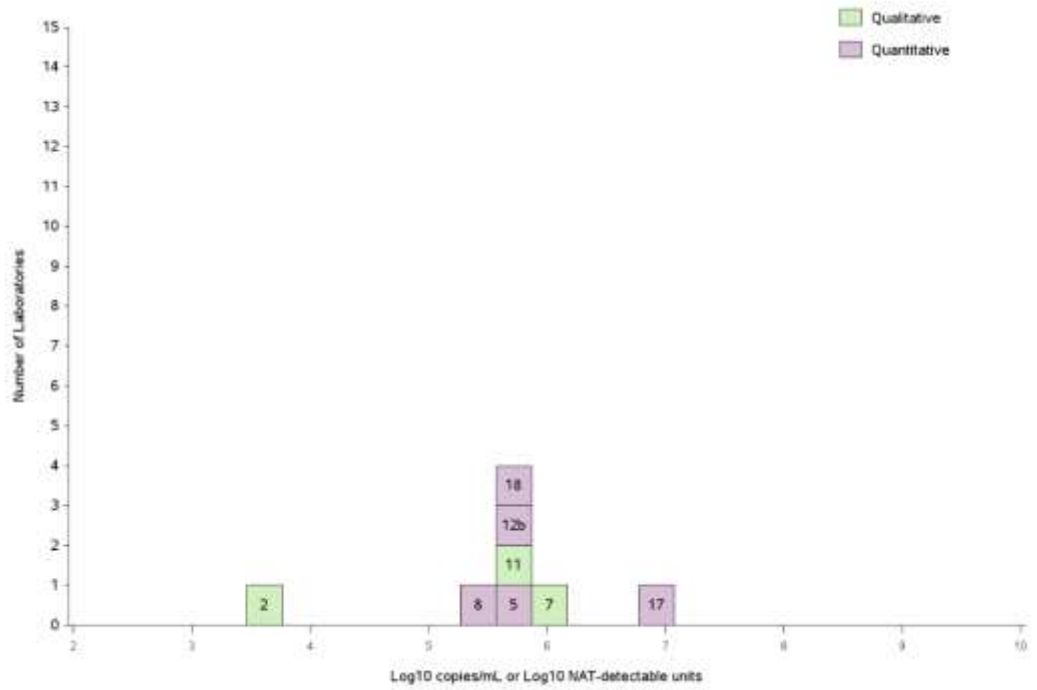


Figure 10b. Graph of Sample J potency estimates, calculated relative to Sample D

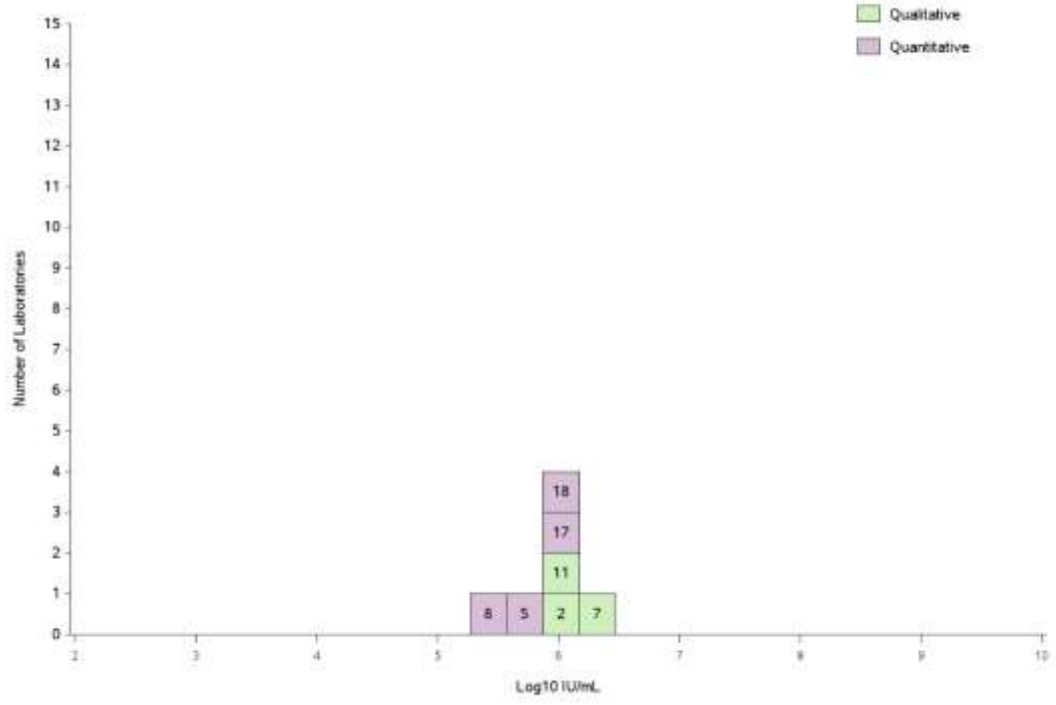
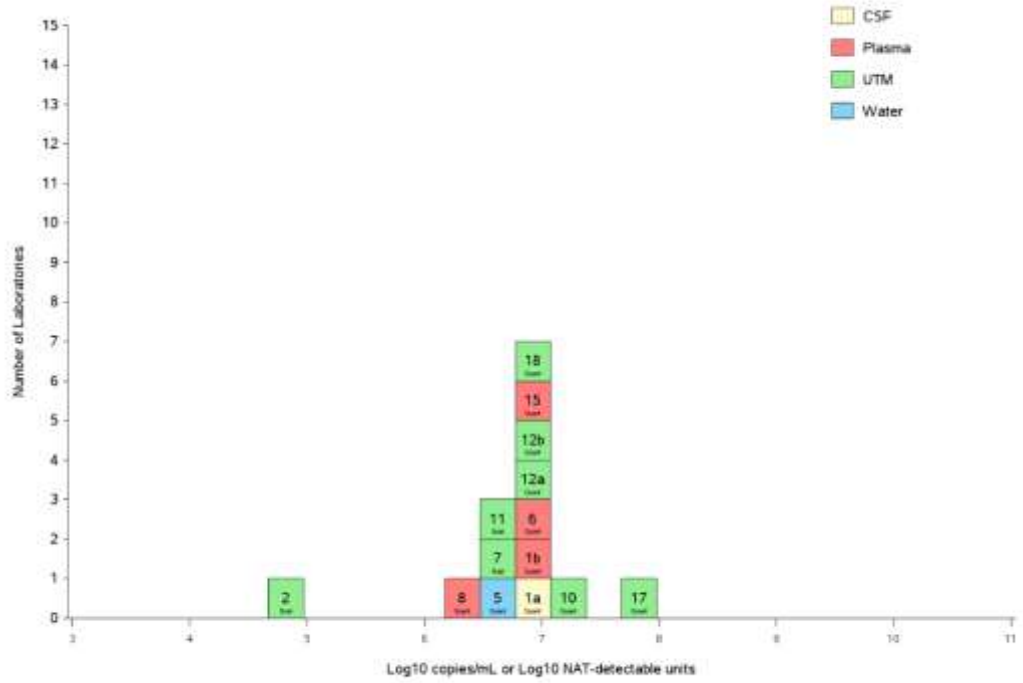


Figure 11. Graph of Sample D reported potency estimates with matrix references



Appendix I.

Table 1: Individual reported potency estimates in quantitative assays (in log_{10} copies/mL).

Lab	Assay	A	B	C	D	E	F	G	H	I	J
1a1	1	7.19	7.15	8.71	7.14				4.50		
1a1	2	7.18	7.18	8.64	7.13				4.49		
1a1	3	7.07	7.08	8.57	7.09				4.50		
1a2	1	7.11	7.00	8.67	6.94				4.31		
1a2	2	7.00	7.00	8.60	6.96				4.29		
1a2	3	6.94	6.94	8.56	6.94				4.33		
1a3	1	7.12	7.04	8.63	6.97				4.39		
1a3	2	7.03	7.03	8.59	6.94				4.32		
1a3	3	6.97	7.01	8.51	6.97				4.41		
1a4	1	7.09	7.02	8.66	6.95				4.32		
1a4	2	7.02	7.01	8.62	6.96				4.27		
1a4	3	6.94	6.90	8.50	6.95				4.33		
1b1	1	7.09	7.11	8.64	7.06			4.38			
1b1	2	7.16	7.15	8.64	7.11			4.35			
1b1	3	7.18	7.08	8.53	7.07			4.32			
1b2	1	6.90	6.94	8.63	6.94			4.23			
1b2	2	6.97	6.99	8.60	6.95			4.18			
1b2	3	7.02	6.86	8.50	6.90			4.18			
1b3	1	6.95	6.99	8.60	6.97			4.28			
1b3	2	7.00	6.99	8.61	6.98			4.21			
1b3	3	7.04	6.94	8.49	6.96			4.26			
1b4	1	6.91	7.02	8.59	6.92			4.21			
1b4	2	6.98	7.04	8.65	6.96			4.21			
1b4	3	7.00	6.88	8.46	6.94			4.20			
5	1	7.32	6.92	7.41	6.84	7.08	6.84			5.20	5.64
5	2	7.29	6.73	7.37	6.64	6.79	6.51			5.22	5.69
5	3	7.25	6.77	7.34	6.72	6.94	6.61			5.24	5.51
6	1	7.06	7.11	8.20	7.10			4.44			
6	2	7.03	7.08	8.30	7.08			4.66			
6	3	6.96	7.02	8.11	7.00			4.42			
8	1	6.88	6.68	8.11	6.68	6.27	5.88	4.07		4.57	5.14
8	2	6.62	6.16	6.69	6.24	6.70	6.21	3.74		4.64	5.46
8	3	6.49	6.32	6.68*	6.40	6.83	6.51	3.79		4.90	5.64
10	1	7.58	7.54	8.51	7.14			4.36	4.91		
10	2	7.47	7.46	8.54	7.06			4.30	4.89		
10	3	7.50	7.51	8.79	7.33			4.28	4.93		

Appendix I

Table 1 continued: Individual reported potency estimates in quantitative assays (in log_{10} copies/mL).

Lab	Assay	A	B	C	D	E	F	G	H	I	J
12a	1	6.79	7.01	8.34	6.79						
12a	2	6.90	6.82	8.34	7.02						
12a	3	6.86	6.76	8.36	6.81						
12b	1	6.65	6.72	8.37	6.99	6.86	6.33	3.80	3.90	4.94	5.64
12b	2					7.06	6.57	4.03	4.16	5.13	5.74
12b	3					6.96	6.59	3.89	3.99	5.06	5.87
15	1				6.87			3.75			
15	2				6.96			3.82			
15	3				6.95			3.80			
15	4	6.76	6.73	8.34							
15	5	6.85	6.85	8.37							
15	6	6.81	6.77	8.43							
17	1	7.95	8.01	9.44	7.92	8.36	7.95	4.88	5.08	6.34	7.04
17	2	7.78	7.74	9.45	7.74	8.25	7.80	4.39	4.89	6.01	6.80
17	3	7.84	7.71	9.61	7.73	8.33	7.81	4.53	4.89	6.12	6.82
18	1	6.95	6.68	8.17	6.92	7.19	6.62	3.95	4.10	4.84	5.55
18	2	6.79	6.84	8.13	6.89	7.10	6.49	3.95	4.13	4.73	5.74
18	3	6.81	7.15	8.13	6.87	6.93	6.50	4.05	4.31	4.98	5.58

* Not included in further calculations

Appendix I

Table 2: Potency estimates in quantitative assays, calculated relative to sample D (in log_{10} copies/vial).

Lab	Assay	A	B	C	E	F	G	H	I	J
1a1	1	7.05	7.03	8.70				4.38		
1a1	2	7.06	7.06	8.64				4.35		
1a1	3	6.98	6.99	8.56				4.40		
1a2	1	7.11	7.07	8.72				4.36		
1a2	2	7.05	7.04	8.66				4.33		
1a2	3	7.00	7.00	8.59				4.40		
1a3	1	7.09	7.06	8.73				4.42		
1a3	2	7.09	7.09	8.69				4.37		
1a3	3	7.01	7.05	8.60				4.45		
1a4	1	7.06	7.04	8.72				4.34		
1a4	2	7.06	7.05	8.70				4.30		
1a4	3	6.99	6.96	8.58				4.38		
1b1	1	7.03	7.06	8.75			4.30			
1b1	2	7.06	7.05	8.73			4.21			
1b1	3	7.03	6.99	8.61			4.26			
1b2	1	6.96	7.00	8.73			4.29			
1b2	2	7.02	7.04	8.68			4.23			
1b2	3	7.01	6.99	8.60			4.27			
1b3	1	6.98	7.03	8.72			4.30			
1b3	2	7.02	7.01	8.74			4.21			
1b3	3	6.99	7.00	8.63			4.33			
1b4	1	6.99	7.10	8.73			4.28			
1b4	2	7.02	7.08	8.72			4.25			
1b4	3	6.99	7.00	8.61			4.31			
5	1	7.48	7.09	7.56	7.28^{RR}	7.03^{RR}			5.37	5.81
5	2	7.61	7.08	7.63	6.72^{RR}	6.49^{RR}			5.40	5.80
5	3	7.50	7.05	7.55	6.90^{RR}	6.61^{RR}			5.40	5.64
6	1	6.96	7.01	8.20			4.37			
6	2	6.94	6.99	8.35			4.59			
6	3	6.96	7.02	8.20			4.41			
8	1	7.20	7.01	8.41	6.61^{RR}	6.21^{RR}	4.39		4.90	5.46
8	2	7.34	6.93	7.41	7.21^{RR}	6.77^{RR}	4.56		5.36	6.09^{RR}
8	3	7.08	6.92	7.24	7.30^{RR}	7.00^{RR}	4.42		5.47	6.17^{RR}
10	1	7.44	7.41	8.42			4.21	4.77		
10	2	7.42	7.41	8.65			4.22	4.84		
10	3	7.17	7.18	8.46			3.96	4.60		

Appendix I

Table 2 continued. Potency estimates in quantitative assays, calculated relative to sample D (in log_{10} copies/vial).

Lab	Assay	A	B	C	E	F	G	H	I	J
12a	1	6.98	6.96	8.58						
12a	2	6.87	6.77	8.35^{NP}						
12a	3	7.05	6.95	8.59						
12b	1	6.63	6.73	8.55	7.18^{RR}	6.58^{RR}	3.72	3.84	5.01	5.80
15	1	6.88^{LM}	6.86^{LM}	8.46^{LM}			3.87			
15	2						3.84			
15	3						3.83			
17	1	7.14	7.08	8.66	7.34^{RR}	6.96	4.13	4.32	5.48	6.12
17	2	6.97	6.98	8.65	7.37^{RR}	6.95	3.71	4.18	5.24	6.00
17	3	7.08	6.95	8.74	7.45^{RR}	6.97	3.87	4.22	5.38	6.03
18	1	7.03	6.73	8.29	7.64^{RR}	7.00^{RR}	3.97	4.14	4.98	5.79

RR = Outside of the response range of the reference
NL = Non-linear
NP = Non-parallel
LM = Overall laboratory mean only

Appendix I

Table 3. Relative potency estimates of samples calculated relative to Sample A (log_{10} relative values)

Lab	Method	B	C	D	E	F	G	H	I	J
1a	Quant	-0.01	1.62	-0.05				-2.68		
1b	Quant	0.02	1.67	-0.01			-2.74			
2	Qual	0.00	1.18	-0.07		0.00		-2.71		-1.07
5	Quant	-0.47	0.08	-0.53					-2.07	-1.66
6	Quant	0.05	1.29	0.05			-2.50			
7	Qual	-0.07	1.22	-0.19		-0.28				-0.88
8	Quant	-0.25	0.49	-0.21			-2.73		-1.98	-1.33
10	Quant	-0.01	1.15	-0.34			-3.21	-2.61		
11	Qual	-0.45	1.09	-0.33		-0.56				-1.17
12a	Quant	-0.07	1.51	0.03						
15	Quant	-0.02	1.58	*0.12**			*-3.02**			
17	Quant	-0.06	1.61	-0.06		-0.14	-3.14	-2.81	-1.70	-1.03
18	Quant	0.04	1.26	0.04			-2.87	-2.67	-2.00	-1.22
	Qual	-0.17	1.16	-0.20		-0.28		-2.71		-1.04
Average	Quant	-0.08	1.23	-0.12		-0.14	-2.87	-2.69	-1.94	-1.31
	All	-0.10	1.21	-0.14		-0.24	-2.87	-2.70	-1.94	-1.19
	Qual	0.24	0.07	0.13		0.28				0.14
Std Dev	Quant	0.16	0.53	0.20			0.27	0.09	0.16	0.27
	All	0.18	0.46	0.18		0.24	0.27	0.07	0.16	0.25
	Qual	74%	17%	36%		91%				40%
GCV	Quant	45%	242%	59%			87%	22%	45%	85%
	All	50%	191%	53%		74%	87%	19%	45%	79%
					Excluding Laboratory 2					
	Qual	-0.26	1.16	-0.26		-0.42				-1.02
Average	Quant	-0.08	1.23	-0.12		-0.14	-2.84	-2.69	-1.94	-1.31
	All	-0.11	1.21	-0.15		-0.33	-2.84	-2.69	-1.94	-1.22
	Qual									
Std Dev	Quant	0.16	0.53	0.20			0.23	0.09	0.16	0.27
	All	0.18	0.48	0.19		0.22	0.23	0.09	0.16	0.27
	Qual									
GCV	Quant	45%	242%	59%	0%	0%	71%	22%	45%	85%
	All	52%	205%	55%	0%	65%	71%	22%	45%	86%

* Calculated via a different method, not included in summary calculations.

Appendix I

Table 4. Relative potency estimates of samples calculated relative to Sample C (log_{10} relative values)

Lab	Method	A	B	D	E	F	G	H	I	J
1a	Quant	-1.62	-1.62	-1.66						
1b	Quant	-1.67	-1.67	-1.69						
2	Qual	-1.18	-1.18	-1.24		-1.18		-3.89		-2.24
5	Quant	-0.08	-0.52	-0.58		-1.04			-2.19	-1.84
6	Quant	-1.29	-1.24	-1.25			-3.77			
7	Qual	-1.22	-1.29	-1.41		-1.50				-2.10
8	Quant	-0.49	-0.72	-0.69	-1.87	-2.24	-2.79		-2.46	-1.83
10	Quant	-1.15	-1.18	-1.51			^	-3.81		
11	Qual	-1.09	-1.54	-1.42		-1.65				-2.26
12a	Quant	-1.51	-1.60	-1.59						
15	Quant	-1.58	-1.57	*-1.46**			*-4.59**			
17	Quant	-1.61	-1.69	-1.68	-1.16	-1.62	-4.83	-4.48	-3.33	-2.59
18	Quant	-1.26	-1.26	-1.26			-4.14	-3.91	-3.26	-2.44
Average	Qual	-1.16	-1.33	-1.36		-1.44		-3.89		-2.20
	Quant	-1.23	-1.31	-1.32	-1.51	-1.64	-3.88	-4.07	-2.81	-2.17
	All	-1.21	-1.31	-1.33	-1.51	-1.54	-3.88	-4.02	-2.81	-2.19
Std Dev	Qual	0.07	0.18	0.10		0.24				0.09
	Quant	0.53	0.41	0.42	0.50	0.60	0.85	0.36	0.57	0.40
	All	0.46	0.36	0.37	0.50	0.42	0.85	0.31	0.57	0.29
GCV	Qual	17%	53%	26%		75%				22%
	Quant	242%	157%	166%	216%	297%	613%	130%	273%	150%
	All	191%	131%	132%	216%	164%	613%	103%	273%	93%
Excluding Laboratory 2										
Average	Qual	-1.16	-1.41	-1.42		-1.58				-2.18
	Quant	-1.23	-1.31	-1.32	-1.51	-1.64	-3.85	-4.07	-2.81	-2.17
	All	-1.21	-1.33	-1.34	-1.51	-1.61	-3.85	-4.07	-2.81	-2.18
Std Dev	Qual									
	Quant	0.53	0.41	0.42	0.50	0.60	0.74	0.36	0.57	0.40
	All	0.48	0.38	0.38	0.50	0.43	0.74	0.36	0.57	0.31
GCV	Qual									
	Quant	242%	157%	166%	216%	297%	453%	130%	273%	150%
	All	205%	138%	141%	216%	168%	453%	130%	273%	105%

^ Outside of the response range of the reference, no potency calculated
* Calculated via a different method, not included in summary calculations.

Appendix II

List of collaborative study participants (in alphabetical order by country)

Name	Laboratory	Country
Dr Marijke Reynders/ Patrick Descheemaeker	Laboratory Medicine-Molecular Microbiology AZ Sint-Jan Brugge-Oostende AV, Brugge	Belgium
Dr. Walter Zhang	Shanghai ZJ Bio-Tech Co., Ltd., Shanghai	China
Dr. Come Barranger/ Dr. Audrey Delariviere	BioMerieux SA, Verniolle	France
Dr.Thomas Grewing	Anchor Diagnostics GmbH, Hamburg	Germany
Dr. Alke Heitmann	Altona Diagnostics GmbH, Hamburg	Germany
Dr. Marcus Panning/ Dr. Sibylle Bierbaum	Institute of Virology, Medical Center-University of Freiburg, Freiburg	Germany
Prof. Yoshinori Ito	Department of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya	Japan
Dr. Jaco J. Verweij	Laboratory for Medical Microbiology and Immunology, ETZ hospital, Tilburg	The Netherlands
Dr. Elif Akyuz	Anatolia Tani ve Biyoteknoloji Urunleri Ar-Ge San. Ve Tic. A.S., Istanbul	Turkey
Dr. Shabana Kazi	Quality Control for Molecular Diagnostics, Glasgow	United Kingdom
Dr. Surendra Parmar	Addenbrookes Hospital, National Infection Services, PHE Cambridge	United Kingdom
Dr. Betty Wu	NeuMoDx Molecular, Ann Arbor, Michigan	USA

Appendix II
Participant Questionnaire

Participant Questionnaire

Please complete the following details:

1. **Laboratory information**

To which category does your laboratory belong?

- Clinical laboratory
- Manufacturer of in vitro diagnostic devices (IVDs)
- Reference laboratory
- Other laboratory (please specify):

Is the laboratory accredited to a quality management system?

2. **Sample type**

In which types of samples do you routinely test for VZV? (e.g. vesicular fluid, CSF etc.)

Please state the purpose of detection.

You will be provided with our test panel which will include anonymised frozen liquid samples as well as freeze-dried samples. Please provide the following details that you will use to analysis these samples.

3. **Dilution matrix for freeze-dried samples only**

Please state the matrix you would use for dilution.

4. **Extraction method**

Please name and give brief details of your extraction protocol.

If a laboratory developed method, please provide a protocol or article reference

If using a commercial kit, please provide kit details: Name and supplier

Manual or Automated extraction (instrument details if used):

What is the required starting volume for your extraction protocol?

Appendix II
Participant Questionnaire continued

5. Amplification methodology: Description of your VZV NAT method

Is the assay qualitative or quantitative?

Lab developed method: Please provide details, if published provide a reference:

Commercial Kit details: Kit and manufacturer

Which VZV gene does the assay target?

Instrument used for amplification

Current reference standard used in laboratory for quantification

Please return to by email: Sheila.Govind@nibsc.org

Terms of the study

I confirm that I would like to participate in the collaborative study towards the establishment of the 1st WHO International Standard for VZV DNA assays. I understand that these samples contain infectious VZV and human material. The samples will be handled only in appropriate containment facilities, by fully trained and competent staff, in accordance with national safety guidelines. I accept full responsibility for the use and disposal of the material. Any residual material shall be discarded. No material shall be distributed to third parties.

One of the samples has been acquired from ATCC, therefore you are also required to sign an ATCC MTA acknowledging the associated rights and responsibilities related to the use, handling, and storing of the ATCC Biological Materials. Your signed copy will be forwarded to ATCC for their records. The ATCC MTA will be forwarded to you separately.

Signed: **Date:**

Name:

Appendix II
Qualitative Study Protocol

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

Collaborative study to evaluate the candidate for the establishment of the 1st WHO International Standard for Varicella Zoster Virus (VZV) for DNA detection Assays

Study Protocol for Qualitative Assays

Participant #

Background and outline of the study

The World Health Organization (WHO) have endorsed the proposal to develop the 1st International Standard for VZV for the standardisation of nucleic acid amplification technology (NAT)-based laboratory assays.

The candidate material to be evaluated is a cell cultured preparation of VZV in a TRIS-based buffer provided as lyophilised and liquid samples. You are also provided with two liquid frozen comparator VZV strains in UTM (Universal transport Medium). The aim of this collaborative study is to determine the suitability of the proposed candidate standard and assign a consensus potency to the preparation, based on the range of NAT-based assays included in this study. Up to two VZV patient samples and a spiked sample are included for your evaluation depending on your assay criteria. The volumes provided have been tailored to you assay requirements.

Study samples

Sample Name	Formulation	Volume	Storage Temperature
Sample A	Liquid frozen VZV in UTM	7 vials 0.50mL	-20 °C or below
Sample B	Liquid frozen VZV in TRIS-buffer	7 vials 0.50mL	-20 °C or below
Sample C	Liquid frozen VZV in UTM	7 vials 0.50mL	-20 °C or below
Sample D	Lyophilised VZV in TRIS-buffer	7 vials to be resuspend with 1mL water	-20 °C
Sample F	Liquid frozen clinical isolate/UTM	7 vials 0.15mL	-20 °C or below
Sample H	Liquid frozen spiked CSF	7 vials 0.50mL	-20 °C or below
Sample J	Liquid frozen clinical isolate/UTM	7 vials 0.15mL	-20 °C or below

Study samples comprise of a lyophilised preparation in a screw top glass vial (Sample D), and six liquid frozen samples (Sample A-C, Sample F, H and J) in Sarstedt vials. Upon receipt, the lyophilised sample should be stored at -20 °C and liquid frozen samples should be stored at -20 °C or below. Sufficient vials of each study sample are provided for evaluation, for an initial analysis to determine the approximate end point and then for a further evaluation on three separate occasions in duplicate. Replicates are required for a robust statistical analysis.

World Health Organization

Appendix II
Qualitative Study Protocol continued

*** CAUTION:** All samples contain infectious VZV and should be handled in appropriate containment facilities by fully trained and competent staff in accordance with national safety guidelines. Care should be taken when opening vials of lyophilised material. Transit may have resulted in fragments of material coming loose. Briefly centrifuge liquid samples before opening. See instructions for use for further details.

Study protocol

Participants are requested to test each study sample, using their routine VZV NAT assay across three independent evaluations. Please ensure the same method is employed across the evaluations.

Prior to each assay run, Sample D must be reconstituted with 1.0 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use.

Dilutions of samples A-D, F, H and J should be prepared in a matrix appropriate for the extraction procedure (120mL of Universal transport medium has been included for your dilutions. Please do not use water). Each sample must be extracted prior to amplification.

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

Below, are specific instructions for the dilution and testing of study samples.

For qualitative assays:

For the first assay, participants are requested to test a single vial using ten-fold serial dilutions of each sample, in order to determine the end-point (up to 6-7 dilutions may be required depending on your assay for samples A-D and F, 4-5 dilutions for samples H and J). The results from the first assay must be reviewed to determine the dilutions for subsequent assays.

For the remaining three assays, participants are requested to test each sample in duplicate performing $\log_{10}$ serial dilutions to the assay end-point (limit of detection) determined in assay 1, and if practicable a minimum of two half-log10 dilution either side of the pre-determined end-point. **It is important that the dilution series spans the limit of detection for the assay and the data includes positive and negative results.**

Please view the Results reporting form that will guide you on the dilutions required. If further clarification on the study protocol is required, please do not hesitate to contact me.

Appendix II
Qualitative Study Protocol continued

Reporting of results

The results of each assay should be recorded as positive or negative and should be recorded on the Result Reporting Form with your participant number. Where applicable, please also include the threshold cycle for each result. Results should be returned to NIBSC within 8-10 weeks from the time of receipt of the samples. More time if required will be granted however all data should ideally be returned before the start of December 2020.

The data should not be published or cited before the formal establishment of the standard by the WHO ECBS (Oct/Nov 2021) without the expressed permission of the NIBSC study organiser.

All requests for further information and completed Result Reporting Forms should be returned electronically to **Sheila.govind@nibsc.org**

Data analysis

Data from the study will be analysed at NIBSC. Individual participant data will be coded and reported "blind" to other participants during the preparation of the study report, and in subsequent publications. Participants will receive a copy of the report of the study including proposed recommendations, for comment, before it is submitted to the ECBS Committee. Please be aware it is normal practice to acknowledge participants as contributors of data rather than co-authors in any publications describing the establishment of the standard.

Appendix II
Quantitative Study Protocol

Medicines & Healthcare products Regulatory Agency

NIBSC

Collaborative study to evaluate the candidate for the establishment of the 1st WHO International Standard for Varicella Zoster Virus (VZV) for DNA detection Assays

Study Protocol for Quantitative Assays

Participant #

Background and outline of the study

The World Health Organization (WHO) have endorsed the proposal to develop the 1st International Standard for VZV towards the standardisation of nucleic acid amplification technology (NAT)-based laboratory assays.

The candidate material to be evaluated is a cell cultured preparation of VZV in a TRIS-based buffer provided as lyophilised and liquid samples. You are also provided with two liquid frozen comparator VZV strains in UTM. The aim of this collaborative study is to determine the suitability of the proposed candidate standard and assign a potency to the preparation, based on the range of NAT-based assays included in this study. Up to four VZV patient samples and two spiked samples are also included for your evaluation depending on your assay specification. The volumes provided have been tailored to you assay requirements.

Study samples

Sample Name	Formulation	Volume	Storage Temperature
Sample A	Liquid frozen VZV in UTM	6 vials 1.0mL	-20 °C or below
Sample B	Liquid frozen VZV in TRIS buffer	6 vials 1.0mL	-20 °C or below
Sample C	Liquid frozen VZV in UTM	6 vials 1.0mL	-20 °C or below
Sample D	Lyophilised VZV in TRIS buffer	6 vials resuspend to 1ml with water	-20 °C
Sample E	Liquid frozen clinical isolate/UTM	6 vials 1.0mL	-20 °C or below
Sample F	Liquid frozen clinical isolate/UTM	6 vials 1.0mL	-20 °C or below
Sample G	Liquid frozen spiked Plasma	6 vials 1.0mL	-20 °C or below
Sample H	Liquid frozen spiked CSF	6 vials 0.50mL	-20 °C or below
Sample I	Liquid frozen clinical isolate/UTM	6 vials 1.0mL	-20 °C or below
Sample J	Liquid frozen clinical isolate/UTM	6 vials 1.0mL	-20 °C or below

Study samples comprise of a lyophilised preparation in a screw top glass vial (Sample D), and up to nine liquid frozen samples (Sample A-C and E-J) in Sarstedt vials. Upon receipt, the lyophilised sample should be stored at -20 °C and liquid frozen samples should be stored at -20 °C or below. Sufficient vials of each study sample are provided for evaluation on three separate occasions in duplicate. Replicates are required for a robust statistical analysis.

World Health Organization

Appendix II
Quantitative Study Protocol continued

*** CAUTION:** All samples contain infectious VZV and should be handled in appropriate containment facilities by fully trained and competent staff in accordance with national safety guidelines. Care should be taken when opening vials of lyophilised material. Transit may have resulted in fragments of material coming loose. Briefly centrifuge liquid samples before opening. See instructions for use for further details.

Study protocol

Participants are requested to test each study sample, using their routine VZV NAT assay across three independent evaluations. Please ensure the same method is employed across the evaluations.

Prior to each assay run, Sample D must be reconstituted with 1.0 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use.

Dilutions of samples A-D, should be prepared in a matrix appropriate for the extraction procedure. You have been provided with 130mL of Universal Transport Medium (UTM) please do not use water. Each sample must be extracted prior to amplification.

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

For quantitative assays

For the first assay participants are requested to test **Samples A-D,** at a minimum of 4-5 serial $\log_{10}$ (ten-fold 1/10, 1/100 and 1/1000 etc) dilutions in a single negative sample matrix. A proportion of your dilutions should fall within the linear range of your assay. Please see the results reporting form for clarification. **Samples E -J** need only to be tested undiluted. All samples must be extracted prior to amplification.

For the remaining assays, participants are requested to test a minimum of 3-4 serial dilutions of sample A and D that fall within the linear range of your assay. Test Samples E -J undiluted.

Reporting of results

The results of each viral quantification should be returned as copies/mL or otherwise and the methodology used should be recorded on the Result Reporting form. Results should be returned to NIBSC within 8-10 weeks from the time of receipt of the samples. More time if required will be granted however all data should ideally be returned before the start of December 2020.

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

Please do not hesitate to contact me should you require further information. Please return completed Result Reporting Forms electronically to **Sheila.govind@nibsc.org**

Data analysis

Data from the study will be analysed at NIBSC. Individual participant data will be coded and reported "blind" to other participants during the preparation of the study report, and in subsequent publications. Participants will receive a copy of the report of the study including proposed recommendations, for comment, before it is submitted to the ECBS Committee. It is normal practice to acknowledge participants as contributors of data rather than co-authors in any publications describing the establishment of the standard.

Appendix III
Interim IFU

Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

WHO International Standard
1st WHO International Standard for VZV NAT Assays
NIBSC code: 19/164
Instructions for use

1. INTENDED USE

The 1st WHO International Standard for Varicella Zoster Virus (VZV) NAT assays, NIBSC code 19/164, is intended for the standardisation of nucleic amplification technique-based assays for VZV. It should be used primarily for the calibration of secondary reference standards. The material has been evaluated in a worldwide collaborative study involving 12 laboratories using a range of VZV NAT-based assays.

2. CAUTION

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

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

3. UNITAGE

The unitage has yet to be assigned

4. CONTENTS

Country of origin of biological material: United Kingdom.
The preparation comprises of lyophilized whole Varicella Zoster virus (Clade 3), formulated in a universal buffer (10mM Tris-HCl pH 7.4, 0.5% Human serum albumin (HSA), 2.0% D-(+)-Trehalose dehydrate). Each vial contains the lyophilized equivalent of 1 mL of VZV in 10mM Tris HCl pH 7.4, 0.5% Human serum albumin (HSA), 2.0% D-(+)-Trehalose dehydrate.

5. STORAGE

Vials of lyophilized material should be stored at -20ºC. Once reconstituted, contents are for single use only. This material has not been assessed for in use stability of reconstituted material. Reconstituted material should not be stored without in house validation studies performed by the end user. **Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.**

6. DIRECTIONS FOR OPENING

Vials have a screw cap; an internal stopper is also present. The cap should be removed by turning anti-clockwise. Care should be taken to prevent loss of the contents.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
The total contents of the ampoule should be reconstituted at room temperature with 1 mL of nuclease-free molecular-grade water and left for 20 minutes with occasional gentle agitation before use. Recommended for single use only.
Once reconstituted, the International Standard should be further diluted into a matrix comparable to the clinical samples being tested. The material is designed to be used in conjunction with the extraction step of the NAT procedure. The International Standard should be used to generate a standard curve for the calibration of secondary reference materials which can then be assigned a concentration in International Units.

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.
It is the policy of WHO not to assign an expiry date to their International reference materials. Accelerated degradation studies have indicated that this lyophilized material is suitably stable, when stored at -20ºC, for the assigned values to remain valid until the material is withdrawn or replaced. This material has undergone accelerated thermal degradation studies.
Users who have data supporting any deterioration in the characteristics of this reference preparation are encouraged to contact NIBSC.

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

9. REFERENCES

Manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: Calibration to WHO International Standards.
http://www.who.int/biologicals/expert_committee/WHO_Manual_Calibration_of_secondary_standards_final_mn.pdf?ua=1

10. ACKNOWLEDGEMENTS

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

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.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: White solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains infectious VZV		

National Institute for Biological Standards and Control,
Potters Bar, Hertfordshire, EN6 3QG, T +44 (0)1707 641000, nibsc.org
WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

Appendix III
Interim IFU

Medicines & Healthcare products Regulatory Agency

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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: 1.0g
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