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Collaborative Study to Evaluate the Proposed 1st WHO International Standard for Human Cytomegalovirus (HCMV) for Nucleic Acid Amplification (NAT)-Based Assays

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** See Appendix 1*

Summary
This report describes the development and worldwide collaborative study evaluation of the candidate 1st WHO International Standard for human cytomegalovirus (HCMV) for use in the standardisation of nucleic acid amplification techniques (NAT). Proposals for the formulation of the candidate standard were discussed at the Standardisation of Genome Amplification Techniques (SoGAT) Clinical Diagnostics meeting at NIBSC in June 2008. The candidate is a whole virus preparation of the HCMV Merlin strain, formulated in a universal buffer comprising Tris-HCl and human serum albumin, and freeze-dried for long-term stability. Thirty-two laboratories from 14 countries participated in a collaborative study to evaluate the fitness for purpose and potency of the candidate standard using their routine NAT-based assays for HCMV. The freeze-dried candidate standard (Sample 1) was evaluated alongside the liquid bulk of the candidate preparation (Sample 2), a whole virus HCMV AD169 preparation (Sample 3) and purified Merlin DNA cloned into a bacterial artificial chromosome (Sample 4). The majority of data sets returned were from laboratory-developed quantitative assays based on real-time PCR technology. However, a wide range of extraction and amplification methodologies were used. The overall mean potency estimate for the candidate standard sample 1, across the different laboratory assays, was 5×10^6 (6.7 $\log_{10}$) 'copies/mL'. The variability in individual mean estimates for whole virus samples 1-3 was 2 $\log_{10}$ (100-fold), however, the variability for the purified DNA sample 4 was higher (>3 $\log_{10}$). The agreement between laboratories was markedly improved when the potencies of the virus samples 2 and 3 were expressed relative to the candidate standard (sample 1). In contrast, the agreement between laboratories for the purified DNA sample 4 was not improved. This suggests that purified DNA that is not extracted alongside the clinical samples is not suitable for standardising these types of assays. The overall data returned from each laboratory indicates that there was no significant loss in potency upon freeze-drying. In addition, the results obtained from accelerated thermal degradation studies at four and eight months indicate that the candidate is extremely stable and suitable for long-term use.
The results of the study indicate the suitability of the candidate HCMV Merlin standard as the proposed 1st WHO International Standard for HCMV. It is therefore proposed that the candidate standard (NIBSC code 09/162) be established as the 1st WHO International Standard for HCMV with an assigned potency of 5×10^6 International Units (IU) when reconstituted in 1 mL of nuclease-free water.

Introduction
HCMV is a ubiquitous herpesvirus with a high seroprevalence worldwide. It causes disease in the immunologically-naïve, such as newborns and infants, and immunosuppressed individuals, particularly transplant recipients and AIDS patients. Severe and life-threatening HCMV infections in immunocompromised individuals are managed through the administration of anti-herpetic agents, however, all are associated with toxicity with prolonged use.

The clinical utility of viral load measurements in the diagnosis and antiviral management of HCMV in transplant recipients has been well documented [1,2]. Two therapeutic approaches have evolved; prophylaxis, whereby antiviral drugs are administered for a fixed period from the time of transplant, and pre-emptive treatment, which is administered in response to an increased risk of CMV disease. The pre-emptive approach requires diagnosis of HCMV replication, and initiation of antiviral therapy when a predetermined level of virus in peripheral blood is reached, prior to the appearance of clinical symptoms. Subsequently, the levels of virus are frequently measured in order to monitor the response to and determine the duration of treatment. Although there is no consensus on the optimal sample type or frequency of testing, both plasma and whole blood provide prognostic information.

Consensus guidelines for the management of HCMV infection and disease in transplant recipients have been published [1,3]. These recommend the use of NAT-based approaches in order to determine viral load measurements in pre-emptive programmes for disease prevention. These NAT assays measure the quantity of HCMV DNA present in a clinical sample, following extraction of viral nucleic acid. The application and range of NAT assays used in the diagnosis and management of HCMV varies significantly. Currently, many sites use laboratory-developed assays based on real-time PCR technology, many of which have been described in the literature. A range of commercial assays are also available, and comprise either analyte-specific reagents (ASR) or assay kits specific for different amplification platforms. Each laboratory-developed or commercial assay differs in the specimen type and nucleic acid extraction method used, as well as in the reagents (including primers and probes) and instrumentation used for the amplification and detection of HCMV DNA. In addition, each assay uses proprietary quantification controls to determine the concentration of viral DNA present. These may comprise either a plasmid clone of the PCR target, or quantified viral DNA or virus particles, and may or may not be included in the extraction step.

Given the heterogeneity of these NAT-based assay systems, and the lack of traceability to a standardised reference system, it is difficult to compare viral load measurements between different laboratories and to develop uniform treatment strategies. Indeed, variability in the performance of different assays for HCMV has been documented [4,5]. These studies have highlighted the need for an internationally-accepted reference standard for HCMV. In 2004, the International Herpes Management Forum called for; 'an international quantification standard… to compare studies using different PCR-based systems and to facilitate patient management at multiple care centres' [1]. In the absence of such a standard, current clinical guidelines recommend that individual laboratories establish their own viral load thresholds for HCMV management, which are specific to their laboratory assay [1,3]. It is also recommended that the specimen type is not changed when monitoring patients.

The World Health Organisation's Expert Committee on Biological Standardisation establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards are recognised as the highest order of reference for biological substances, 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 method used [6].

Proposals for the development of the 1st WHO International Standard for HCMV were discussed at the SoGAT Clinical Diagnostics meeting held at NIBSC in June 2008 [7]. Options for source materials and formulation of the candidate standard were discussed [8]. It was agreed that the candidate standard would comprise a whole virus preparation of the prototype clinical HCMV strain Merlin, and would be formulated in a universal buffer for further dilution in the sample matrix appropriate to each assay. The use of whole virus would standardise the entire assay including both extraction and DNA amplification steps. It was also agreed that the final concentration would be in the order of 1×10^7 'copies/mL', and would be expressed in IU when established. The proposal was adopted into the WHO biological standardisation programme in October 2008.

The proposed standard is intended to be used in the *in vitro* diagnostics field and it relates to ISO 17511:2003 Section 5.5.

Aims of study

The aim of this collaborative study is to determine the potency of the candidate standard using a range of NAT-based assays for HCMV, and to evaluate the suitability of the candidate for the calibration of secondary reference materials and the standardisation of HCMV viral load measurements.

Materials

Candidate standard

The proposed candidate standard comprises a cell-free live virus preparation of the prototype clinical HCMV strain Merlin [10]. This low passage strain represents a well characterised, near complete HCMV genome compared with other laboratory strains, and has been fully sequenced (GenBank Accession number AY446894). The Merlin strain is classified as a genotype 1 virus, based on the glycoprotein B gene *UL55*. Given the wide range of samples routinely tested for HCMV, the candidate standard is formulated in a universal buffer, comprising 10 mM Tris-HCl and human serum albumin, for further dilution in the appropriate sample matrix used in each laboratory assay. This preparation has then been freeze-dried to ensure long-term stability.

Preparation of bulk material

A tissue culture supernatant sample of HCMV Merlin strain (passage 4) was propagated in MRC-5 cells, infecting at a multiplicity of infection of 0.1. Tissue culture fluid (passage 6) was harvested once a cytopathic effect (CPE) was observed, and repeated until all the cells showed CPE. The culture fluid was clarified by low speed centrifugation and virus pelleted by ultracentrifugation. Viral pellets were pooled to make a stock of virus in 200 mL 10 mM Tris-HCl buffer (pH 7.4), containing 0.5% human serum albumin (Tris-HSA buffer). The human serum albumin used in the production of the candidate standard and other study samples was derived from licensed products, and was screened and tested negative for anti-HIV-1, HBsAg, and HCV RNA.

The concentration of the HCMV Merlin stock was determined at NIBSC, using a laboratory-developed real-time PCR assay. Briefly, 400 µL of sample was extracted using the QIAamp® MinElute® Virus Spin Kit (QIAGEN, Hilden, Germany), on the QIAcube® instrument. Five microlitres of purified nucleic acid was then amplified by real-time PCR using the LightCycler® 480 Instrument (Roche Applied Science, Mannheim, Germany) [11]. The target was quantified against serial dilutions of a plasmid clone of the PCR target. The HCMV DNA concentration was also assessed at NIBSC using two commercial HCMV assays (Roche LightCycler® CMV Quant Kit and Nanogen Q-CMV Real Time Complete Kit), and in five clinical laboratories in the UK using a range of laboratory-developed and commercial assays. The stock was diluted 1/8000 in Tris-HSA buffer and dispensed in 0.5 mL volumes prior to evaluation. The remainder of the stock was stored at -80 °C until preparation of the final bulk. The geometric mean virus concentration from all assays, in 'copies/mL', was used to determine a consensus HCMV concentration for the stock.

The bulk preparation was formulated to contain approximately 1×10^7 HCMV 'copies/mL' in a final volume of 6.4 L Tris-HSA buffer, and mixed for a total of 30 minutes using a magnetic stirrer. Approximately 250 mL of the liquid bulk was dispensed in 1 mL aliquots into 2 mL Sarstedt screw cap tubes and stored at -80 °C. The remaining bulk volume was immediately processed for lyophilisation in order to prepare the final product, NIBSC code 09/162.

Filling and lyophilisation of candidate standard

The filling and lyophilisation of the bulk material was performed at NIBSC, and the production summary is detailed in Table 1. 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, Arguenteil, France) through a 'pizza door' arrangement to maintain integrity of the operation. The bulk material was kept at 4 °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 volumes, 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, and vials outside the defined specification were discarded. Filled vials were partially stoppered with halobutyl 14mm diameter cruciform closures and lyophilised in a CS150 freeze dryer. Vials were loaded onto the shelves at -50 °C and held at this temperature for 4 hrs. A vacuum was applied to 270 µb over 1 hr, followed by ramping to 30 µb over 1 hr. The temperature was then raised to -40 °C, and the vacuum maintained at this temperature for 42.5 hrs. The shelves were ramped to 25 °C over 15 hrs before releasing the vacuum and back-filling the vials with nitrogen. The vials were then stoppered in the dryer, removed and capped in the isolator, and the isolator 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 (NIBSC to act as custodian and worldwide distributor).

Post-fill testing

Assessments of residual moisture and oxygen content, as an indicator of vial integrity after sealing, were determined for twelve vials of freeze-dried product. Residual moisture was determined by non-invasive near-infrared (NIR) spectroscopy (MCT 600P, Process Sensors, Corby, UK). NIR results were then correlated to Karl Fischer (using calibration samples of the same excipient, measured using both NIR and Karl Fischer methods) to give % w/w moisture readings. Oxygen content was measured using a Lighthouse Infra-Red Analyser (FMS-750, Lighthouse Instruments, Charlottesville, USA).

Samples of the liquid bulk (n=18) and freeze-dried product (n=18) were tested by HCMV real-time PCR as described earlier, in order to determine the homogeneity of the product prior to dispatch for collaborative study.

Stability of the freeze-dried candidate

Accelerated degradation studies are underway at NIBSC in order to predict the stability of 09/162 when stored at the recommended temperature of -20 °C. Vials of freeze-dried product are being held at -70 °C, -20 °C, +4 °C, +20 °C, +37 °C, +45 °C. At specified time points during the life of the product, three vials will be removed from storage at each temperature and HCMV DNA quantified by real-time PCR (as previously described). In addition, a limited assessment of the stability of reconstituted product was performed. Reconstituted product was stored at +4 °C, +20 °C, and +37 °C, and HCMV DNA quantified by real-time PCR after 24 and 48 hrs.

Study samples

The freeze-dried candidate HCMV Merlin preparation was evaluated alongside the unprocessed liquid bulk (used to prepare the freeze-dried candidate), a live virus preparation of the HCMV strain AD169 [12], and a sample of purified HCMV Merlin DNA cloned into a bacterial artificial chromosome (BAC) [13].

The AD169 virus was propagated in MRC-5 cells as described earlier. The culture fluid was harvested once a CPE was observed, clarified at low speed centrifugation and virus pelleted by

ultracentrifugation. Virus was then diluted to approximately 1×10^7 HCMV 'copies/mL' in Tris-HSA buffer. As the prototype laboratory strain of HCMV, AD169 DNA is frequently used as a calibrator in NAT-based assays. It has been classified as a genotype 2 virus, based on the glycoprotein B gene.

The Merlin BAC had been prepared from the complete HCMV Merlin genome [10,13]. BAC DNA was purified using a Nucleobond BAC100 kit (Machery-Nagel GmbH, Düren, Germany) according to manufacturer's instructions. The concentration of purified BAC DNA was determined by absorbance at 260 nm, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and diluted to 1×10^5 HCMV 'copies/µL' in nuclease-free water. The purpose of including this purified HCMV DNA sample was to investigate the effect of the extraction step on the variability in HCMV quantification.

Aliquots of AD169 (n=18) and Merlin BAC (n=18) were tested by HCMV real-time PCR (as previously described), in order to determine the homogeneity of the samples prior to dispatch for collaborative study. Study samples were stored at -20 °C (sample 1) and -70°C (samples 2-4) prior to shipment to participants.

Study samples shipped to participants were coded as samples 1-4 and were as follows:
- Sample 1 - Lyophilised preparation 09/162 in a 5 mL screw cap glass vial.
- Sample 2 - 1 mL frozen liquid preparation of the HCMV Merlin bulk (used to prepare freeze-dried candidate) in a 2 mL Sarstedt screw cap tube.
- Sample 3 - 1 mL frozen liquid whole virus preparation of HCMV AD169 in a 2 mL Sarstedt screw cap tube.
- Sample 4 - 50 µL frozen liquid preparation of purified BAC-cloned Merlin DNA in a 0.5 mL Sarstedt screw cap tube.

Study design

The aim of the collaborative study was to evaluate the suitability and potency of the candidate HCMV International Standard in a range of NAT based assays. Four vials each of study samples 1-4 were delivered to participating laboratories by courier on dry ice, with specific instructions for storage and reconstitution.

Study protocol

Participants were requested to test dilutions of each sample using their routine NAT-based assay for HCMV on four separate occasions, using a fresh vial of each sample in each independent assay. In accordance with the study protocol (Appendix 2), the lyophilized sample 1 was to be reconstituted with 1 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use. Meanwhile, study samples 1-3 were to be thawed and vortexed briefly before use.

Participants were requested to dilute samples 1-3 to within the quantitative range of the assay, using the sample matrix specific to their individual assay, and to extract each dilution prior to amplification. Meanwhile, participants were requested to dilute sample 4 in nuclease-free water, and add an aliquot of each dilution directly to the amplification reaction. For quantitative assays, participants were requested to test a minimum of two serial ten-fold dilutions within the linear range of the assay. For qualitative assays, participants were requested to test ten-fold serial dilutions of each sample to determine the assay end-point, and then a minimum of two half-log serial dilutions either side of the predetermined end-point, for subsequent assays.

Participants were requested to report the viral load in 'copies/mL' (positive/negative for qualitative assays) for each dilution of each sample and return results including details of methodology used to NIBSC for analysis.

Participants

Study samples were sent to 32 participants representing 14 countries (Appendix 1). Participants were selected for their experience in CMV NAT and geographic distribution. They represented mainly clinical laboratories, but also included a range of manufacturers of *in vitro* diagnostic devices (IVDs), as well as reference, research and quality assurance laboratories. All participating laboratories are referred to by a code number, allocated at random, and not representing the order of listing in Appendix 1. Where a laboratory returned data using different assay methods, the results were analysed separately, as if from different laboratories, and are referred to as, for example, laboratory 9A, 9B etc.

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 'NAT detectable units/mL', as described previously [14]. It should be noted that these estimates are not necessarily directly equivalent to a genuine genome equivalent number/mL. In the case of quantitative assays, analysis was based on the results supplied by the participants. Results were reported as 'copies/mL' although the relationship to genuine genome equivalence numbers is unknown. For each assay run, a single estimate of $\log_{10}$ 'copies/mL' was obtained for each sample, by taking the mean of the $\log_{10}$ estimates of 'copies/mL' across replicates, after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of 'copies/mL' across assay runs.

Overall analysis was based on the $\log_{10}$ estimates of 'copies/mL' or 'NAT detectable units/mL'. 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) [15] of the actual estimates. Potencies relative to sample 1, the candidate International Standard, were calculated as the difference in estimated $\log_{10}$ 'units per mL' (test sample – candidate standard) plus a candidate assigned value in International Units/ml (IU/mL) for the candidate standard. So for example, if in an individual assay, the test sample is 0.5 $\log_{10}$ higher than the candidate standard, and the candidate standard is assigned 6.7 $\log_{10}$ IU/mL, the relative potency of the test sample is 7.2 $\log_{10}$ IU/mL. The same approach was used to calculate the potencies relative to sample 4, in order the evaluate the utility of purified DNA to standardise HCMV assays.

Variation within laboratories and between assays (intra-laboratory), was expressed as standard deviations of the $\log_{10}$ estimates and %GCVs of the individual assay mean estimates. These estimates were pooled across samples 1 to 3, but calculated separately for sample 4. The significance of the inter-laboratory variation relative to the intra-laboratory variation was assessed by an analysis of variance.

Results and data analysis

Validation of study samples and stability assessment

Production data for the candidate standard sample 1 showed that the CV of the fill mass and mean residual moisture were within acceptable limits for a WHO International Standard (Table

1). Residual oxygen content was within the NIBSC working limit of 1.1%. Evaluation of multiple aliquots (n=18) of each study sample at NIBSC prior to dispatch indicated that the homogeneity of HCMV content was similar for all study samples (2SD less than 0.3 $\log_{10}$ 'copies/mL' for each sample).

Samples of the candidate standard 09/162 were stored at elevated temperatures, and assayed at NIBSC concurrently with samples stored at -20 °C and -70 °C, after 4 months or 8 months storage, by HCMV real-time PCR (as described earlier). At each time point, three vials of samples stored at each temperature were extracted and amplified in triplicate. The mean estimated $\log_{10}$ 'copies/ml' and differences ($\log_{10}$ 'copies/ml') from the -70 °C baseline sample are shown in Table 2. A negative value indicates a drop in potency relative to the -70 °C baseline. 95% confidence intervals for the differences are ±0.16 $\log_{10}$ based on a pooled estimate of the standard deviation between individual vial test results. Considered individually, only the difference of +0.204 for the 45 °C samples stored for 8 months is therefore statistically significant. However, there does appear to be a pattern of apparent increase in potency with increasing temperature and length of storage. The reason for this is not clear. As there is no observed drop in potency it is not possible to fit the usual Arrhenius model for accelerated degradation studies, or obtain any predictions for the expected loss per year with long term storage at -20 °C. However, using the 'rule of thumb' that the decay rate will approximately double with every 10 °K increase in temperature (personal communication: Dr P K Philips), and noting that there is no detectable drop in potency after 8 months at +20 °C, then there should be no detectable difference after 64 months at -20 °C. A similar argument applied to the +37 °C data would imply no detectable loss after 256 months (over 20 years) at -20 °C. However, with the unexplained trend for an apparent increase in potency at the higher temperatures, extrapolations based on the +37 °C data may not be reliable. In summary, there is no evidence of any degradation at any temperature after storage for 8 months. It is not possible to obtain precise estimates of any degradation rates for long term storage at -20 °C. All available data indicates adequate stability. Subsequent testing will take place at 12 and 18 months, then at 2, 3, 4, and 5 years.

The limited assessment of the stability of reconstituted product stored at +4 °C, +20 °C, and +37 °C for 24 and 48 hrs showed that there was no marked decrease in HCMV DNA concentration in vials stored at +20 °C and +37 °C compared with those stored at 4 °C, as determined by real-time PCR (data not shown).

Data received

Data were received from all 32 participating laboratories. Participants performed a variety of different assay methods, with some laboratories performing more than one assay method. In total, data sets were received from 53 quantitative assays, and 5 qualitative assays. Apart from the cases noted below, there were no exclusions of data.

Qualitative Assays:
Laboratories 24 and 25 used 1-log dilution steps for all 4 assays. For laboratory 24, the majority of the results for sample 4 were positive. Estimates for this laboratory will therefore be less precise than from those using half-log dilution steps.
Laboratory 31 had anomalous results for sample 1 in assay 4 (negative at $10^{-4.5}$ to 10^{-6} but positive at $10^{-6.5}$ dilutions). These results were excluded for this assay.

Quantitative Assays:

Laboratories 2B, 4, 19B, 19C and 25 did not return results for sample 4. This was principally because it was not possible to determine viral load without extracting the sample.
Laboratory 12A reported problems with their second assay for most replicates of samples 1, 2 & 3. This assay was excluded from further analysis.
Laboratory 16 only provided data from 2 assays. The second assay was on freeze/thawed extractions and was excluded. The first assay did not have valid results for sample 3 (noted by participant as possible technical error).
Laboratory 20A reported that "Samples were frozen between dilution/extraction and PCR assay".
Laboratory 22B returned data from 4 assays, but the last 2 were after freeze-thaw cycles and were excluded from further analysis.
For some laboratories and assays, results from individual dilutions were excluded when they were noted as being outside the linear range of the assays.

Summary of assay methodologies

The majority of participants prepared dilutions of study samples 1-3 using either plasma or whole blood, however, urine, PBS, and nuclease-free water were also used. The extent of the dilutions performed varied slightly between each laboratory. Extractions were predominantly automated, and employed a range of instruments including; Abbott *m*2000sp, QIAGEN's QIAsymphony SP and RG Q, BioRobot, MDx, and EZ1, bioMérieux NucliSENS® easyMag®, Roche MagNA Pure LC and COBAS® AmpliPrep, and Siemens VERSANT® kPCR. Manual extraction protocols included Roche High Pure Viral Nucleic Acid Kit, Nanogen EXTRAgen®, QIAGEN QIAamp (Blood DNA, DNA and Viral RNA) Mini Kits, QIAGEN QIAamp DSP Virus Kit, Cepheid affigene® DNA Extraction Kit, and phenol-chloroform extraction.
The majority of datasets reported the use of real-time PCR technology. Seventeen participants used commercial assays and reagents (37 data sets), while 13 participants used laboratory-developed assays (17 data sets). Two participants used both commercial and laboratory-developed assays (4 data sets). Commercial assays and reagents included; Roche COBAS® AMPLICOR CMV MONITOR Test, Nanogen Q-CMV Real Time Complete Kit, Argene CMV R-gene™ and CMV HHV6,7,8 R-gene™, QIAGEN *artus* CMV (LC and RG) PCR Kits, Roche COBAS® TaqMan® CMV Test, Cepheid's affigene® CMV trender and SmartCMV™, Abbott RealTime CMV (in development), 'ELITech/Epoch CMV 3.0', and Quantification of CMV PrimerDesign™ Ltd. The range of HCMV genes targeted included; *UL122*/*UL123* (MIE/IE19), *UL54* (DNA polymerase), *UL83* (pp65), *UL55* (glycoprotein B), *US8*, HXFL4, and *UL34* and *UL80.5*. Amplification platforms included; Roche LightCycler® 1.5, 2.0 and 480 systems, COBAS® TaqMan® and COBAS® AMPLICOR Analyzer, Applied Biosystems™ 7300, 7500, 7500 Fast, and 7900 HT Fast Real-Time PCR Systems, Agilent Mx3000P® qPCR System, QIAGEN Rotor-Gene Q, Rotor-Gene 3000 and 6000 instruments, Cepheid SmartCycler™ II and Bio-Rad MyCycler™. Given the range of assay combinations and variables, and the fact that no two assays were alike (apart from two laboratories using the Roche COBAS® AMPLICOR CMV MONITOR Test), it was not possible to group methods and perform analysis according to the method used.

Estimated potencies of study samples

The laboratory mean estimates for each study sample for quantitative assays (in $\log_{10}$ 'copies/mL') and qualitative assays (in $\log_{10}$ 'NAT detectable units/mL') are shown in Tables 3 and 4 respectively. The individual laboratory mean estimates for each assay and study sample are also shown in histogram form in Figures 1a-d. Each box represents the mean estimate from one laboratory, and the boxes are labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

Results for samples 1-3 show considerable variation in viral load reported between different assays, with estimates differing by up to 2 $\log_{10}$ (100-fold) (Table 5). The estimates from the qualitative assays were typically lower than those for quantitative assays. Meanwhile, the variability for sample 4 was greater than that of samples 1-3, although this was principally due to outlying results from five different assays (Figure 1d). Evaluation of the spread of results based on individual assay parameters, such as the dilution matrix, showed that there was no observed relationship between these factors and the HCMV concentration for each sample (data not shown).
Table 5 shows the overall mean estimates for each study sample, for quantitative and qualitative assays, along with the standard deviation (of $\log_{10}$ estimates) and the %GCV (of actual estimates). For samples 1 – 3, the standards deviation for quantitative assays is approximately 0.5 log, and %GCV is approximately 200%. These figures are consistent with the observed 2-log range of estimates. The spread for the qualitative assays is similar. The SD and %GCV for sample 4, are higher than those for samples 1-3, again most likely due to the outlying results.

Comparison of overall mean estimates for freeze-dried candidate sample 1 and liquid bulk sample 2 indicates that there was no significant loss in potency upon freeze-drying (Table 5). In addition, comparison of overall mean estimates for Merlin sample 2 and AD169 sample 3 indicates the suitability of all assays to equally quantify these two strains.

Potencies relative to sample 1

The expression of potency of samples 2-4 relative to sample 1 (as described in the statistical methods section), allows an assessment of the suitability of the candidate standard for the standardisation of CMV NAT assays. The relative potencies of samples 2-4 against sample 1, for each quantitative and qualitative assay, are shown in Tables 6 and 7 respectively. Units are expressed as candidate $\log_{10}$ IU/ml in both cases. The relative potencies are also shown in histogram form in Figures 2a-c. Figures 2a and 2b show that when the mean estimates of samples 2 and 3 are expressed relative to sample 1, there is a marked improvement in agreement between laboratories, compared with Figures 1b and 1c. While the results from the qualitative assays remain more variable, they are now centred around the overall mean. However, when the mean estimates of sample 4 are expressed relative to sample 1 (Figure 2c), there is no significant improvement in agreement between laboratories, compared with Figure 1d.
Table 8 shows the overall mean relative potency estimates (in 'candidate $\log_{10}$ IU/mL') for samples 2-4, for quantitative and qualitative assays, along with the standard deviation (of $\log_{10}$ estimates) and the %GCV (of actual estimates). For the quantitative assays, the SD has reduced from approximately 0.5 $\log_{10}$ to 0.12 and 0.19 $\log_{10}$ for samples 2 and 3 respectively. This demonstrates that the use of a sample 1 as a standard would lead to significant reductions in inter-laboratory variability in the estimation of CMV concentrations for clinical samples similar to virus samples 2 and 3. Meanwhile for sample 4, the there is no reduction in the SD for quantitative assays. As sample 1 requires extraction, and sample 4 does not, differences in extraction efficiency between laboratories and methods will still contribute to the observed variation between laboratories for sample 4.

Potencies relative to sample 4

The estimated concentrations of samples 1-3 were also expressed in 'candidate IU', relative to sample 4, using a hypothetical unitage of 10^7 IU/ml for sample 4. The relative potencies of samples 1-3 against sample 4, for each quantitative and qualitative assay, are shown in Tables 9 and 10 respectively. The results are also shown in histogram form in Figures 3a-c. These results show that when the purified DNA sample 4 is used as a standard there is no improvement in agreement between laboratories, as compared with Figure 1a-c. From Table 11, it can be seen

that the standard deviation between laboratories has in fact increased from around 0.5 $\log_{10}$ to 0.64 $\log_{10}$, while the %GCVs have increased to over 300%. These results show that as the purified DNA sample 4 is not extracted alongside clinical samples, it cannot control for differences in extraction methods or efficiency between laboratories.

Intra-laboratory variation

Table 12 shows the intra-laboratory standard deviations and %GCVs for each laboratory, calculated by pooling estimates for samples 1-3, but separately for sample 4. For all samples, the inter-laboratory variation was greater than the intra-laboratory variation ($p<0.0001$). For samples 1-3 there were differences between the repeatability of laboratory estimates across assays, with the average standard deviation being 0.11 $\log_{10}$ or a %GCV of 30%. For sample 4, there was a greater range of values between laboratories, with the average standard deviation being 0.21 $\log_{10}$ or a %GCV of 63%.

Discussion and conclusions

In this study, a range of NAT-based assays for HCMV have been used to evaluate the suitability and potency of the candidate standard preparation as the 1st WHO International Standard for HCMV.

The candidate standard comprises a whole virus preparation of the prototype clinical HCMV strain Merlin. This strain was chosen as it is well characterised and more likely to represent a clinical virus than other laboratory-adapted strains. The use of a whole virus preparation, allows the candidate standard to be extracted alongside clinical samples thereby standardising the entire HCMV assay. In addition, because of the range of patient samples routinely tested for HCMV, the candidate has been formulated in a universal buffer for further dilution in the sample matrix appropriate to each HCMV test. This allows the matrix of the candidate standard to be as similar as possible to that of the test analyte.

In the collaborative study, the freeze-dried candidate standard was evaluated alongside whole virus preparations of Merlin and AD169 strains. In the analysis of results, these samples represent the samples to be calibrated by the candidate standard and enable the study to evaluate the suitability of the candidate standard to harmonise assay results for these samples. In addition, a purified DNA sample comprising the entire Merlin sequence cloned into a BAC was included in the study. The purpose of including this sample was to evaluate the effect of the extraction step on the variability of HCMV viral load measurements. It also enabled the investigation of the utility of a purified DNA sample to standardise assays of whole virus samples.

The study results showed that all assays detected both Merlin and AD169 strains, demonstrating the suitability of the Merlin strain for use as the candidate International Standard, and confirming its ability to calibrate secondary references comprising the AD169 strain.

The overall mean estimate for the candidate standard sample 1 was 5×10^6 (6.7 $\log_{10}$) 'copies/mL'. Individual laboratory mean estimates ranged from 5.4 to 7.5 $\log_{10}$ ‘copies/mL’. The target concentration for the candidate standard was 1×10^7 ‘copies/mL’, based on preliminary testing of the Merlin stock at NIBSC and in a selection of UK clinical laboratories. The overall mean estimate for the liquid bulk sample 2 was similar to that of the freeze-dried sample 1, indicating that there was no significant loss in potency upon freeze-drying. The small difference between the overall mean estimate and the target concentration for the candidate standard is likely to be a result of the selection of a small subset of laboratories for preliminary testing, and the large inter-laboratory variation observed in assay results.

The overall range in laboratory mean estimates for the whole virus study samples 1-3 was 2 $\log_{10}$. This variability reflects the range and differences in diagnostic testing procedures between laboratories and is similar to levels previously reported for HCMV NAT assays [4,5]. The overall range in laboratory mean estimates for the purified DNA sample 4 was higher. This was unexpected since the purified DNA sample 4 was not extracted, however, the spread was principally due to outlying results from five assays (there was no observed relationship between these five assays). Inter-laboratory variability was significantly greater than intra-laboratory variability. This was also reported by Pang *et al*., [4].

The agreement between laboratories for virus samples 2 and 3 was markedly improved when the potencies of these study samples was expressed relative to the candidate standard (sample 1), demonstrating the suitability of the candidate to standardise assays of whole virus samples. However, when the purified DNA sample 4 was used as the standard, it did not lead to any improvement in agreement between laboratories, and such a preparation would not be suitable for the standardisation of assays of whole virus samples.

The matter of commutability of the candidate standard for clinical HCMV samples has not been specifically assessed in this study. Commutability is affected by a range of factors including matrix and molecular variants of the analyte (in this case HCMV DNA). The idea behind preparing the candidate in a universal matrix for subsequent dilution in the sample matrix appropriate to each assay was to control for matrix effects. In this study, the marked improvement of all assay results for samples 2 and 3 when expressed relative to sample 1, independent of the sample matrix used to dilute the samples, might suggest that this approach does control for different sample matrices. However, it is difficult to control for differences in HCMV forms that are present in different clinical samples. In this study, the candidate standard is derived from a crude cell-free preparation of HCMV from cell culture, which comprises both whole virus and naked HCMV DNA (as determined by DNase digestion experiments – data not shown). However, patient samples derived from peripheral blood are likely to comprise a range of HCMV forms including whole and disrupted virions, and fragmented genomic DNA, with different forms predominating in different blood compartments. Plasma and serum samples from renal transplant recipients have been reported to contain highly fragmented HCMV DNA [16]. Meanwhile, whole blood samples from the same patients comprised a mixture of highly fragmented and large DNA forms, some of which may have been derived from whole virus. Therefore, further studies are needed to assess the commutability of this material with respect to HCMV DNA forms present in different clinical samples.

The results obtained from accelerated thermal degradation studies at four and eight months indicate that the candidate is stable and suitable for long-term use.

The results of this study have demonstrated that the candidate standard, NIBSC code 09/162, has been shown to be suitable for use as a standard in a range of NAT-based assays for the quantification of HCMV DNA. As this is proposed as the 1st WHO International Standard for HCMV the assignment of an International Unit is arbitrary. In the case of this study a value of 5×10^6 International Units has been chosen as this represents the consensus estimate for the candidate across all laboratory assays. The uncertainty can be derived from the variance of the fill and is 0.23%.

Proposal

It is proposed that the candidate standard (NIBSC code 09/162) is established as the International Standard for HCMV with an assigned potency of 5×10^6 International Units when reconstituted in 1 mL of nuclease-free water. The proposed standard is intended to be used by clinical laboratories and IVD manufacturers to calibrate secondary references used in routine NAT-based assays for HCMV. Proposed Instructions for Use (IFU) for the product are included in Appendix 3.

Comments from participants

Fifteen of thirty-two participants responded to the report. There were no disagreements with the suitability of the candidate standard (NIBSC code 09/162) to serve as the 1st WHO International Standard for HCMV. The majority of comments suggested editorial changes and these have been implemented where appropriate. Two participants commented on the proposal to assign the candidate a concentration of 6.7 $\log_{10}$ IU/mL, despite the initial proposal to assign a concentration of 7 $\log_{10}$ IU/mL.

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Table 1. Production summary for the candidate standard (sample 1).

NIBSC code	09/162
Product name	Human Cytomegalovirus
Dates of processing	Filling; 25 September 2009 Lyophilisation; 25-29 September 2009 Sealing; 29 September 2009
Presentation	Freeze-dried preparation in 5mL screw-cap glass vial
Appearance	Robust opaque white cake
No. of vials filled	6029
Mean fill weight (g)	1.00 (n=126)
CV of fill weight (%)	0.23
Mean residual moisture (%)	0.6 Karl Fischer, 0.41 NIR units (n=12)
CV of residual moisture (%)	7.2
Mean oxygen content (%)	0.22 (n=12)
CV of oxygen content (%)	40.6
No. of vials available to WHO	5100

Table 2. Stability of 09/162 at 4 and 8 months.

Temperature (°C)	Mean $\log_{10}$ 'copies/mL'		Difference in $\log_{10}$ 'copies/mL' from -70°C baseline sample	
	4 months	8 months	4 months	8 months
-70	6.92	6.77	-	-
-20	6.92	6.78	0.008	0.015
+4	6.86	6.72	-0.054	-0.048
+20	6.96	6.84	0.044	0.071
+37	7.02	6.91	0.103	0.141
+45	7.07	6.97	0.155	0.204

Table 3. Laboratory Mean Estimates from Quantitative Assays (log_{10} 'copies/mL').

Participant	Sample			
	S1	S2	S3	S4
01	6.91	6.92	6.93	7.26
02A	6.05	6.14	5.96	7.81
02B	6.93	7.00	6.99	.
03	6.25	6.30	6.12	7.20
04	7.24	7.14	7.09	.
05	5.71	5.89	5.99	7.21
06	6.31	6.24	6.16	6.80
07	6.03	5.96	6.00	6.56
08	6.74	6.90	6.84	8.81
09A	6.78	6.85	6.68	7.24
09B	7.24	7.20	7.00	7.34
09C	7.04	6.99	6.80	7.21
09D	6.66	6.59	6.43	6.80
09E	7.21	7.29	7.21	7.23
09F	7.32	7.46	7.39	7.15
09G	7.30	7.39	7.35	7.21
09H	7.13	7.03	7.10	6.87
09I	6.67	6.78	6.82	7.21
09J	6.84	6.89	6.85	6.73
09K	7.06	7.03	7.08	7.23
09L	7.29	7.35	7.34	7.15
09M	7.04	7.11	7.11	7.21
09N	6.92	6.74	6.83	6.87
11	7.04	7.11	7.06	7.18
12A	6.31	6.38	6.05	7.43
12B	6.36	6.35	6.57	6.55
13	5.65	5.77	5.73	5.06
14	6.76	6.78	6.76	7.34
15	6.95	6.87	6.82	6.67
16	5.97	5.58	.	6.91
17	6.66	6.75	6.73	7.64
18	6.15	5.99	5.97	5.81
19A	6.65	6.53	6.47	7.37
19B	6.49	6.42	6.36	.
19C	6.76	6.57	6.49	.
20A	6.57	6.58	7.32	7.19
20B	6.18	6.22	6.66	6.88
21	7.46	7.53	7.36	7.14
22A	6.02	5.84	6.12	6.86
22B	6.11	6.09	6.36	8.39
23	6.94	7.02	7.15	7.08
24	6.09	6.13	6.06	7.10
25	7.05	6.91	6.87	.
26A	6.82	6.75	6.65	6.41
26B	7.03	6.97	6.95	7.91
27	7.23	7.30	7.30	7.69
28A	6.85	6.65	6.66	6.55
28B	6.52	6.72	6.24	7.03
29	7.08	7.13	7.10	7.79
30A	6.67	6.82	6.72	6.09
30B	7.05	7.24	7.23	8.02
31	6.24	6.13	6.03	6.69
32	7.43	7.41	7.39	7.49

Table 4. Laboratory Mean Estimates from Qualitative Assays (log_{10} 'NAT detectable units/mL').

Laboratory	Sample			
	S1	S2	S3	S4
05	5.39	5.53	5.53	6.92
10	6.55	6.38	6.62	6.68
24	5.96	5.83	6.02	7.16
25	5.98	6.43	5.98	6.86
31	6.15	5.47	5.18	6.51

Table 5. Overall Mean Estimates and Inter-Laboratory Variation (log_{10} 'copies/mL' for quantitative or 'NAT-detectable units/mL' for qualitative assays).

Sample	Assay	No. data sets	Mean	SD	%GCV	Min	Max
S1	qualitative	5	6.01	0.42	161	5.39	6.55
	quantitative	53	6.71	0.46	188	5.65	7.46
S2	qualitative	5	5.93	0.46	185	5.47	6.43
	quantitative	53	6.71	0.49	207	5.58	7.53
S3	qualitative	5	5.86	0.54	249	5.18	6.62
	quantitative	52	6.72	0.46	190	5.73	7.39
S4	qualitative	5	6.82	0.25	77	6.51	7.16
	quantitative	48	7.11	0.61	307	5.06	8.81

Table 6. Laboratory Estimates of Potency Relative to S1 from Quantitative Assays $\log_{10}$ IU/mL taking Sample 1 as 5×10^6 (6.7 $\log_{10}$) IU/mL.

Participant	Sample		
	S2	S3	S4
01	6.70	6.71	7.05
02A	6.79	6.61	8.45
02B	6.77	6.76	.
03	6.75	6.57	7.64
04	6.60	6.55	.
05	6.88	6.98	8.20
06	6.63	6.56	7.20
07	6.63	6.67	7.23
08	6.87	6.81	8.77
09A	6.77	6.61	7.16
09B	6.66	6.46	6.81
09C	6.64	6.46	6.87
09D	6.63	6.47	6.83
09E	6.78	6.70	6.72
09F	6.84	6.78	6.54
09G	6.80	6.75	6.61
09H	6.60	6.67	6.44
09I	6.81	6.86	7.24
09J	6.75	6.70	6.59
09K	6.67	6.72	6.87
09L	6.76	6.75	6.57
09M	6.77	6.77	6.87
09N	6.51	6.61	6.65
11	6.77	6.72	6.84
12A	6.77	6.44	7.82
12B	6.69	6.91	6.89
13	6.82	6.79	6.11
14	6.72	6.70	7.29
15	6.62	6.57	6.42
16	6.31	.	7.64
17	6.79	6.77	7.68
18	6.54	6.52	6.36
19A	6.59	6.52	7.43
19B	6.63	6.56	.
19C	6.51	6.43	.
20A	6.71	7.46	7.32
20B	6.74	7.18	7.41
21	6.77	6.60	6.38
22A	6.52	6.80	7.55
22B	6.69	6.95	8.99
23	6.78	6.90	6.83
24	6.74	6.66	7.70
25	6.57	6.52	.
26A	6.63	6.53	6.29
26B	6.65	6.62	7.58
27	6.77	6.77	7.16
28A	6.50	6.50	6.39
28B	6.89	6.42	7.20
29	6.75	6.72	7.40
30A	6.86	6.75	6.13
30B	6.90	6.88	7.67
31	6.59	6.49	7.15
32	6.68	6.66	6.76

Table 7. Laboratory Estimates of Potency Relative to S1 from Qualitative Assays $\log_{10}$ IU/mL taking Sample 1 as 5×10^6 (6.7 $\log_{10}$) IU/mL.

Laboratory	Sample		
	S2	S3	S4
05	6.84	6.84	8.23
10	6.52	6.77	6.83
24	6.57	6.76	7.90
25	7.16	6.70	7.58
31	6.02	5.73	7.06

Table 8. Overall Mean Estimates and Inter-Laboratory Variation for Potency Relative to Sample 1 $\log_{10}$ IU/mL taking Sample 1 as 5×10^6 (6.7 $\log_{10}$) IU/mL.

Sample	Assay	No. data sets	Mean	SD	%GCV	Min	Max
S2	qualitative	5	6.62	0.42	163	6.02	7.16
	quantitative	53	6.70	0.12	31	6.31	6.90
	combined	58	6.69	0.16	44	6.02	7.16
S3	qualitative	5	6.56	0.47	192	5.73	6.84
	quantitative	52	6.69	0.19	56	6.42	7.46
	combined	57	6.68	0.23	68	5.73	7.46
S4	qualitative	5	7.52	0.58	280	6.83	8.23
	quantitative	48	7.12	0.64	341	6.11	8.99
	combined	53	7.16	0.64	340	6.11	8.99

Table 9. Laboratory Estimates of Potency Relative to S4 from Quantitative Assays $\log_{10}$ IU/mL taking Sample 4 as 10^7 (7.0 $\log_{10}$) IU/mL.

Participant	Sample		
	S1	S2	S3
01	6.65	6.66	6.67
02A	5.25	5.34	5.15
02B			
03	6.06	6.10	5.92
04			
05	5.50	5.68	5.79
06	6.50	6.43	6.36
07	6.47	6.39	6.44
08	4.93	5.10	5.04
09A	6.54	6.61	6.45
09B	6.89	6.85	6.66
09C	6.83	6.78	6.59
09D	6.87	6.79	6.64
09E	6.98	7.06	6.98
09F	7.16	7.30	7.24
09G	7.09	7.18	7.14
09H	7.26	7.16	7.23
09I	6.46	6.57	6.61
09J	7.11	7.16	7.11
09K	6.83	6.80	6.85
09L	7.13	7.20	7.19
09M	6.83	6.90	6.90
09N	7.05	6.87	6.96
11	6.86	6.93	6.88
12A	5.88	5.95	5.62
12B	6.81	6.80	7.02
13	7.59	7.71	7.68
14	6.41	6.43	6.41
15	7.28	7.20	7.15
16	6.06	5.68	.
17	6.02	6.11	6.08
18	7.34	7.18	7.16
19A	6.27	6.16	6.10
19B			
19C			
20A	6.38	6.39	7.13
20B	6.29	6.34	6.77
21	7.32	7.39	7.22
22A	6.15	5.98	6.25
22B	4.71	4.70	4.97
23	6.87	6.94	7.07
24	6.00	6.04	5.96
25			
26A	7.41	7.34	7.24
26B	6.12	6.07	6.04
27	6.54	6.61	6.60
28A	7.31	7.11	7.11
28B	6.50	6.69	6.21
29	6.30	6.35	6.31
30A	7.57	7.73	7.62
30B	6.03	6.23	6.21
31	6.55	6.45	6.34
32	6.94	6.92	6.90

Table 10. Laboratory Estimates of Potency Relative to S4 from Qualitative Assays $\log_{10}$ IU/mL taking Sample 4 as 10^7 (7.0 $\log_{10}$) IU/mL.

Laboratory	Sample		
	S1	S2	S3
05	5.47	5.61	5.61
10	6.87	6.70	6.94
24	5.80	5.67	5.86
25	6.12	6.58	6.12
31	6.64	5.97	5.68

Table 11. Overall Mean Estimates and Inter-Laboratory Variation for Potency Relative to Sample 4 $\log_{10}$ IU/mL taking Sample 4 as 10^7 (7.0 $\log_{10}$) IU/mL.

Sample	Assay	No. data sets	Mean	SD	%GCV	Min	Max
S1	qualitative	5	6.18	0.58	280	5.47	6.87
	quantitative	48	6.58	0.64	341	4.71	7.59
	combined	53	6.54	0.64	340	4.71	7.59
S2	qualitative	5	6.10	0.51	221	5.61	6.70
	quantitative	48	6.59	0.64	333	4.70	7.73
	combined	53	6.54	0.64	334	4.70	7.73
S3	qualitative	5	6.04	0.54	246	5.61	6.94
	quantitative	47	6.60	0.63	325	4.97	7.68
	combined	52	6.54	0.64	334	4.97	7.68

Table 12. Intra-Laboratory standard deviation of $\log_{10}$ 'copies/mL' and %GCV for quantitative assays.

Participant	Samples 1-3		Sample 4	
	SD	%GCV	SD	%GCV
01	0.12	33	0.13	35
02A	0.10	27	0.07	17
02B	0.09	23	-	-
03	0.10	24	0.13	35
04	0.10	25	-	-
05	0.13	34	0.04	10
06	0.08	21	0.31	103
07	0.12	32	0.11	30
08	0.10	27	0.09	23
09A	0.10	27	0.12	32
09B	0.06	14	0.14	38
09C	0.09	23	0.07	18
09D	0.11	30	0.10	27
09E	0.06	16	0.12	32
09F	0.09	23	0.16	44
09G	0.05	12	0.07	18
09H	0.08	19	0.11	28
09I	0.10	25	0.07	18
09J	0.13	35	0.23	68
09K	0.08	20	0.12	32
09L	0.09	22	0.16	44
09M	0.07	18	0.07	18
09N	0.12	31	0.11	28
11	0.01	3	0.09	24
12A	0.22	66	0.22	65
12B	0.03	6	0.16	44
13	0.11	28	0.32	108
14	0.03	6	0.13	35
15	0.05	12	0.05	13
16	-	-	-	-
17	0.17	48	0.08	20
18	0.05	12	0.09	24
19A	0.08	20	0.07	18
19B	0.15	42	-	-
19C	0.06	15	-	-
20A	0.16	45	0.30	97
20B	0.12	33	0.07	18
21	0.09	24	0.37	134
22A	0.36	132	0.06	14
22B	0.16	45	0.05	12
23	0.06	14	0.13	35
24	0.08	21	0.29	96
25	0.05	11	-	-
26A	0.07	16	0.50	215
26B	0.04	9	0.72	427
27	0.10	25	0.14	38
28A	0.05	13	0.02	5
28B	0.10	25	0.10	26
29	0.07	17	0.10	25
30A	0.24	72	0.17	47
30B	0.08	21	0.09	22
31	0.14	37	0.34	117
32	0.06	14	0.47	194
Mean	0.11	30	0.21	63

Figure legends

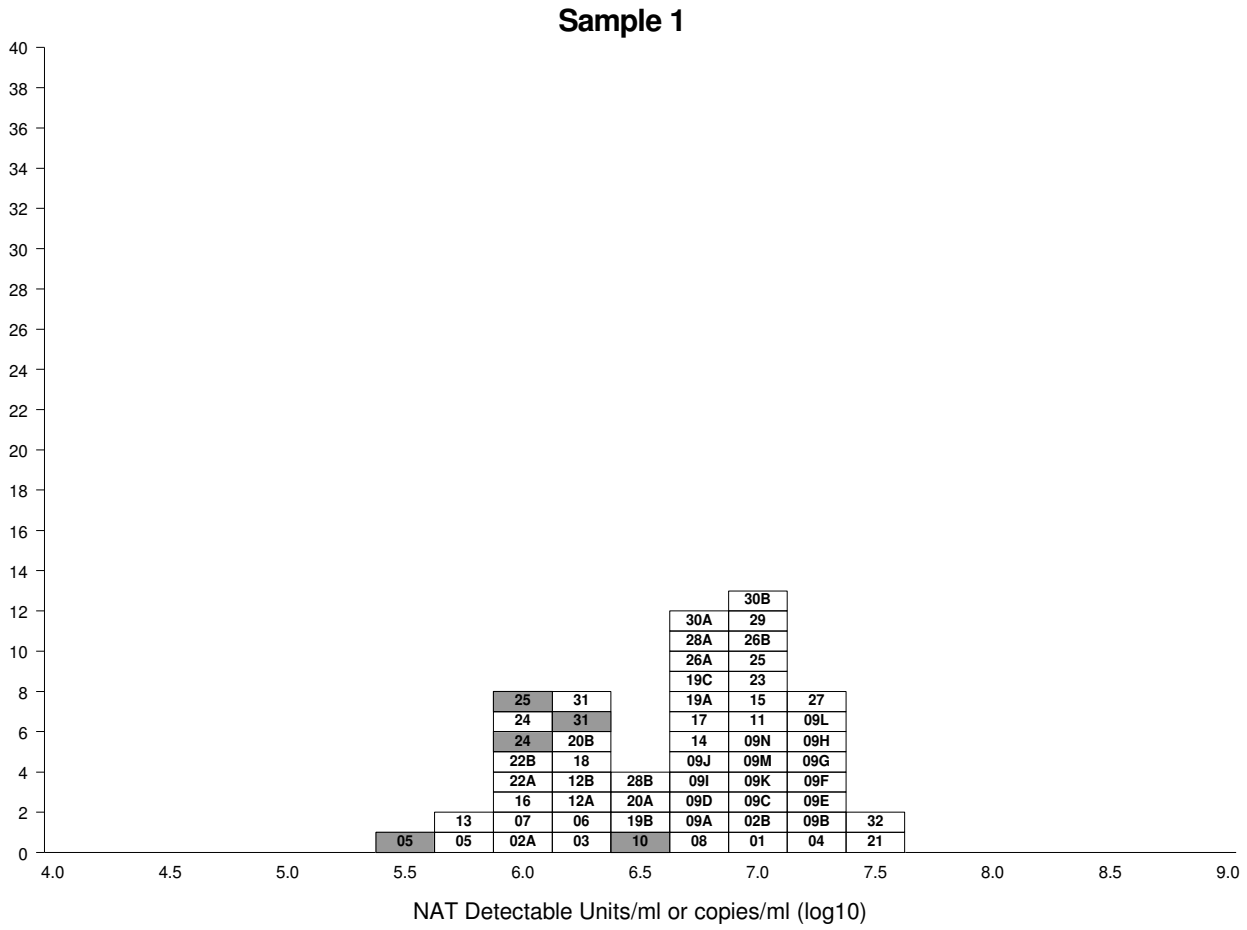
Figure 1. Individual laboratory mean estimates for study samples 1-4 obtained using qualitative or quantitative NAT assays. Each box represents the mean estimate from each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

Figure 2. Relative potencies of samples 2-4 against sample 1, for each quantitative and qualitative assay. Units are expressed as candidate $\log_{10}$ IU/mL in both cases. Each box represents the relative potency for each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

Figure 3. Estimated concentrations of samples 1-3 expressed in IU, relative to sample 4, using a hypothetical unitage of 10^7 IU/mL for sample 4. Each box represents the relative potency for each laboratory assay and is labelled with the laboratory code number. The results from the qualitative assays are shaded in grey.

Figure 1

a


Sample 1
40
38
36
34
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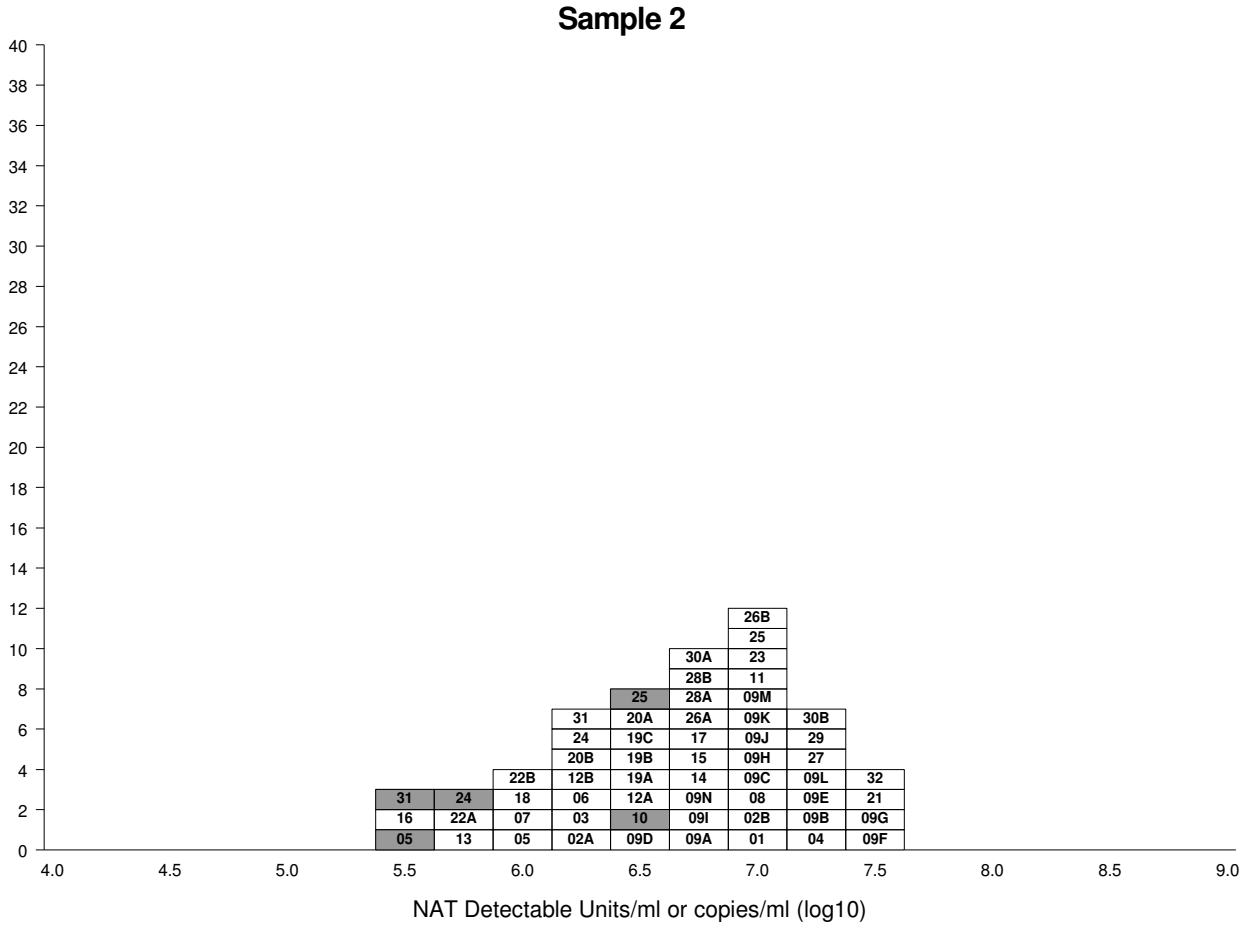
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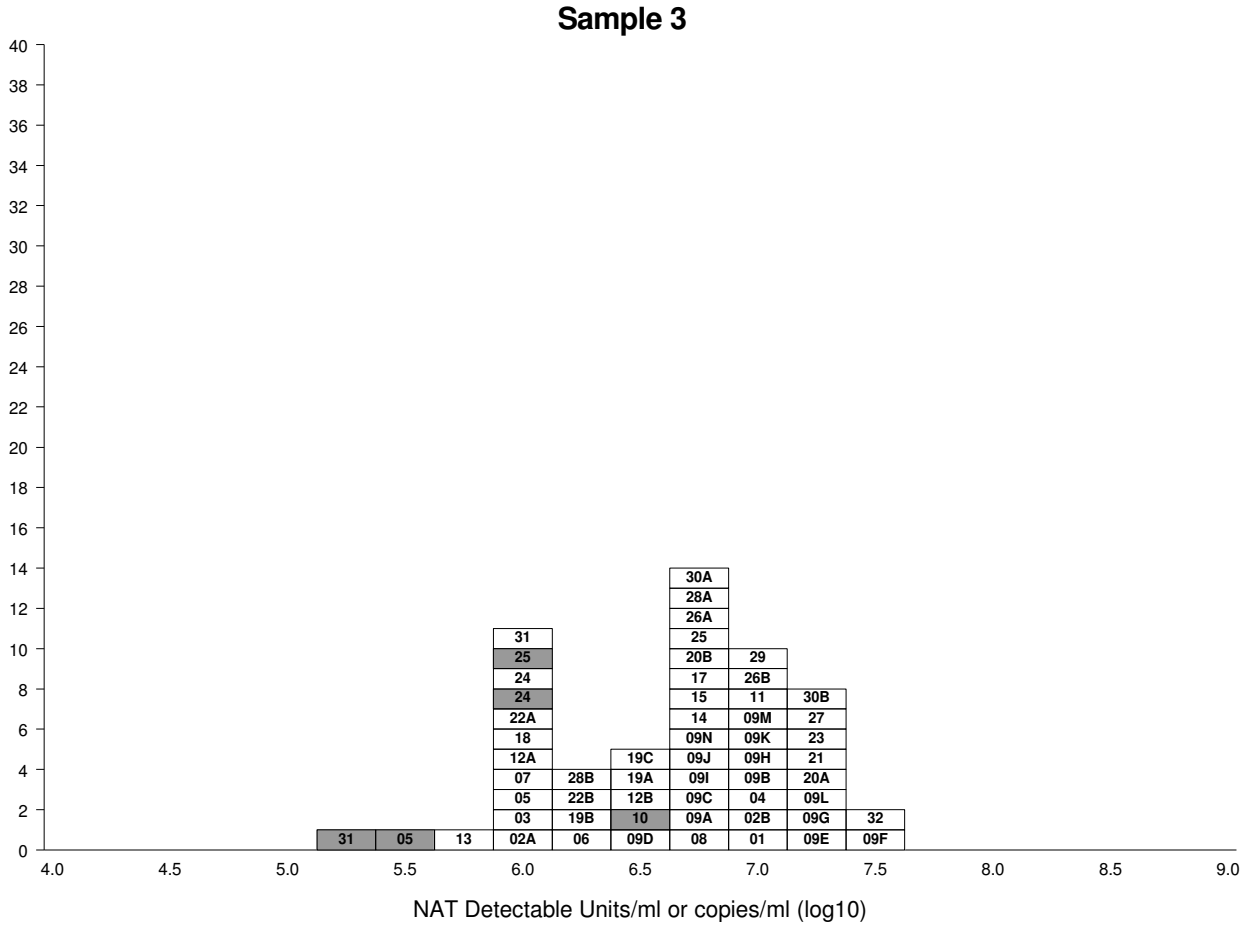
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NAT Detectable Units/ml or copies/ml (log10)

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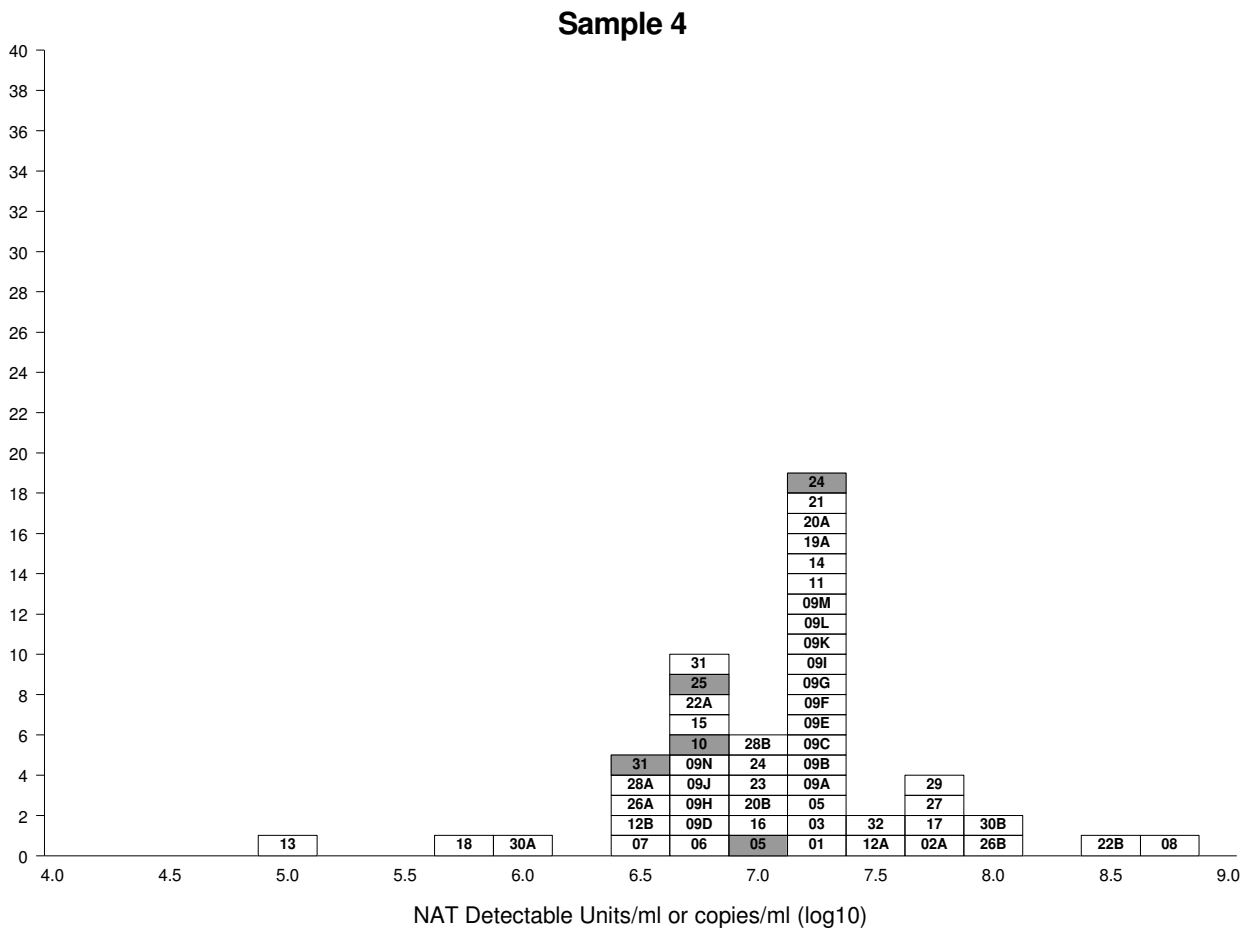
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NAT Detectable Units/ml or copies/ml (log10)
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Figure 2

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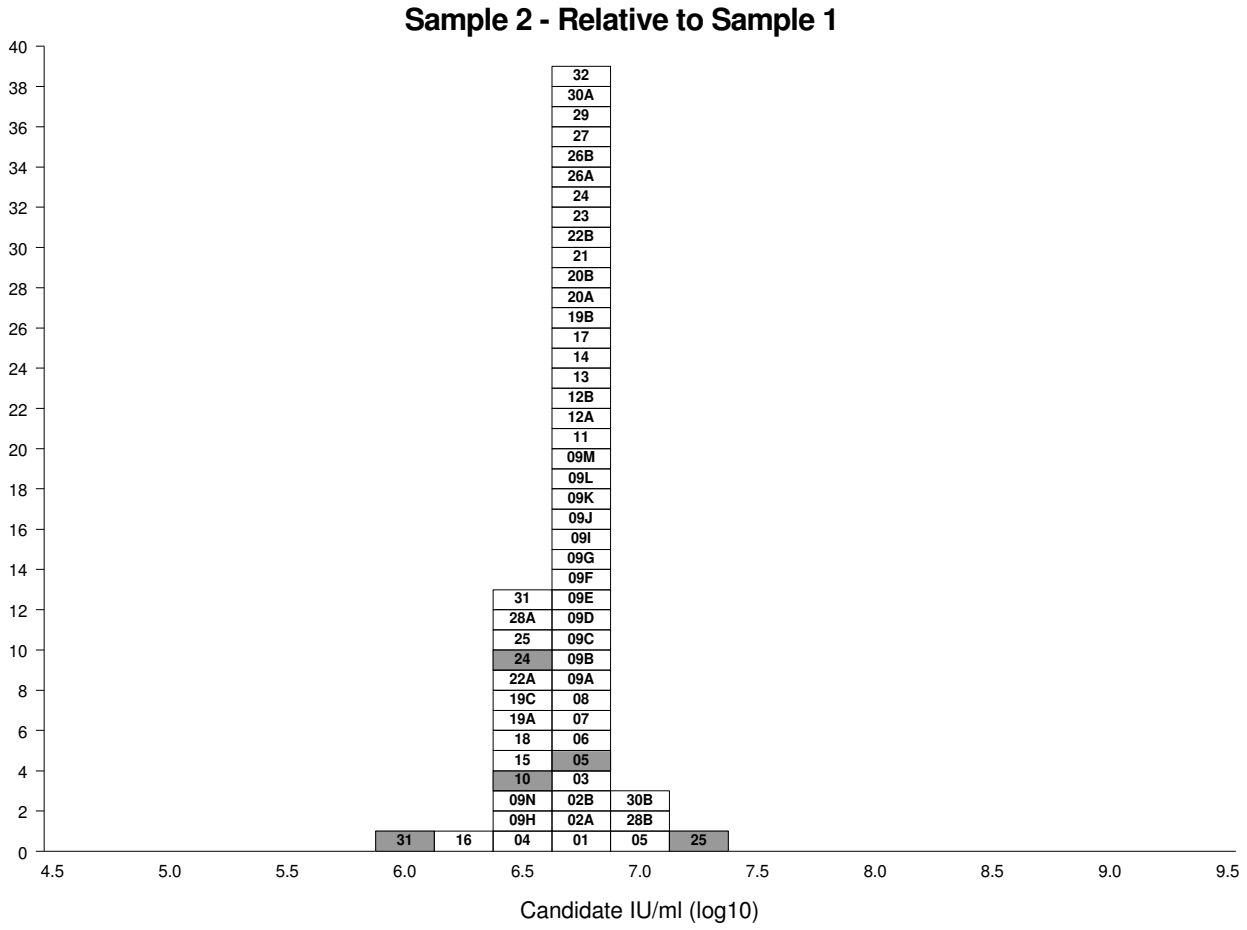
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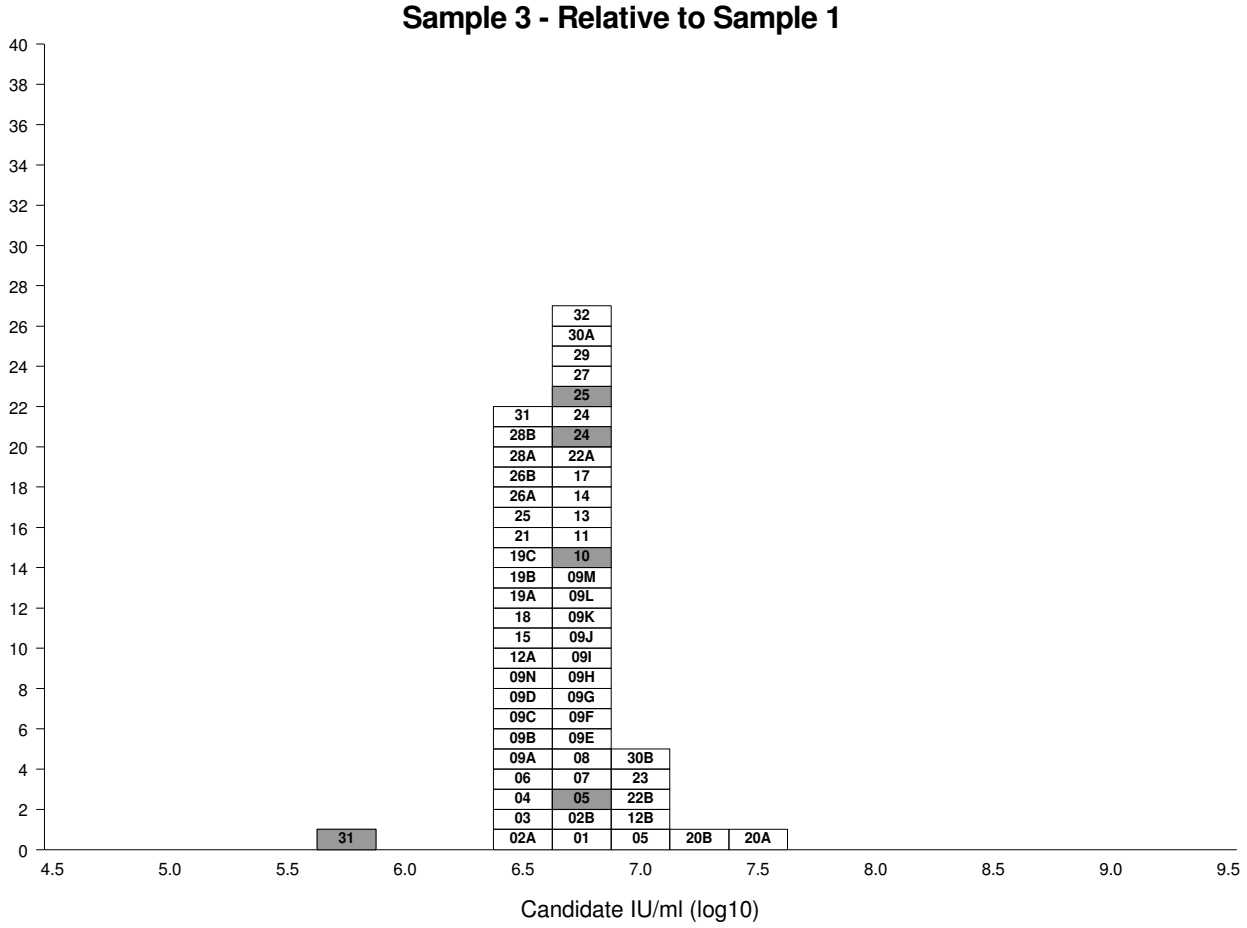
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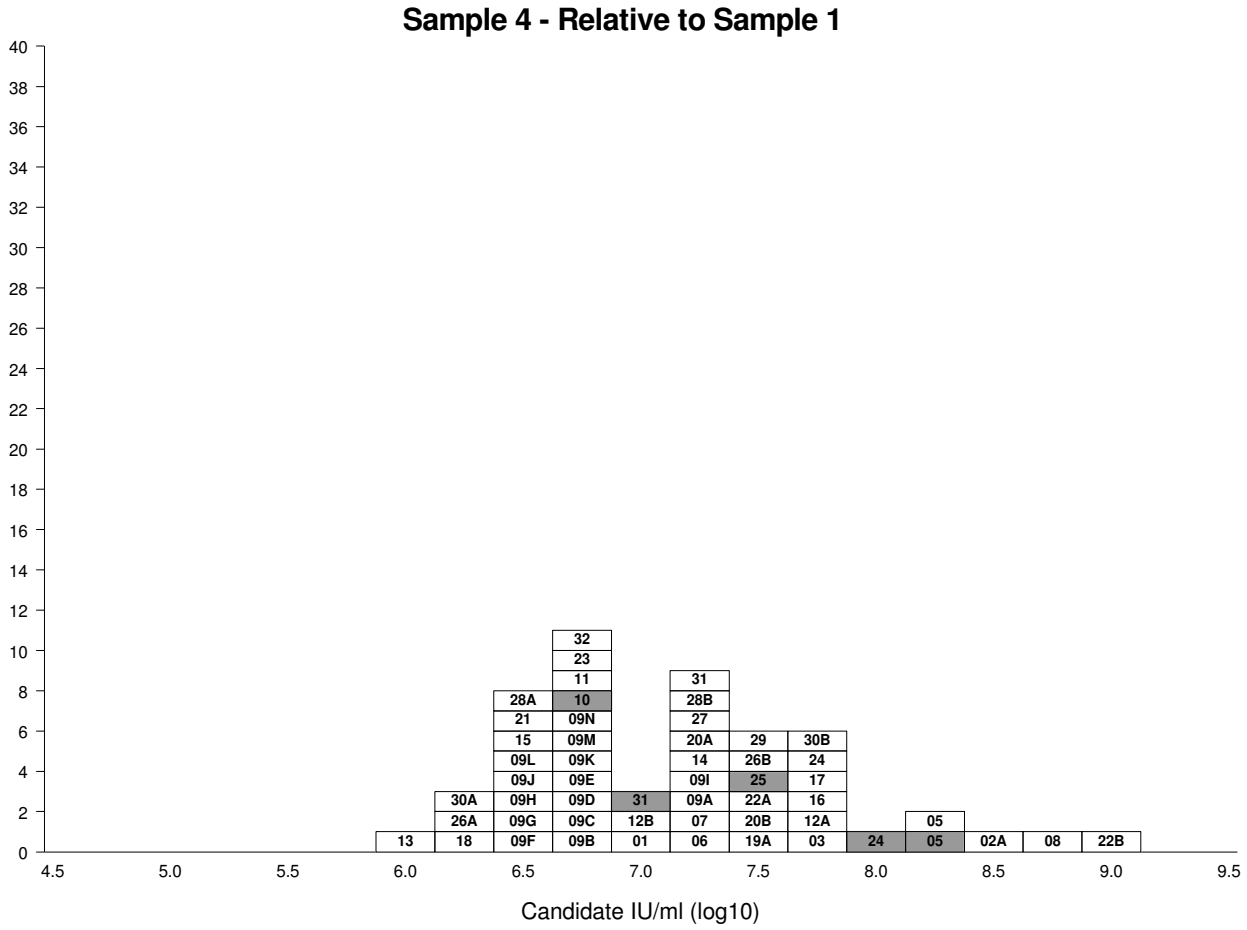
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Candidate IU/ml (log10)
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Figure 3

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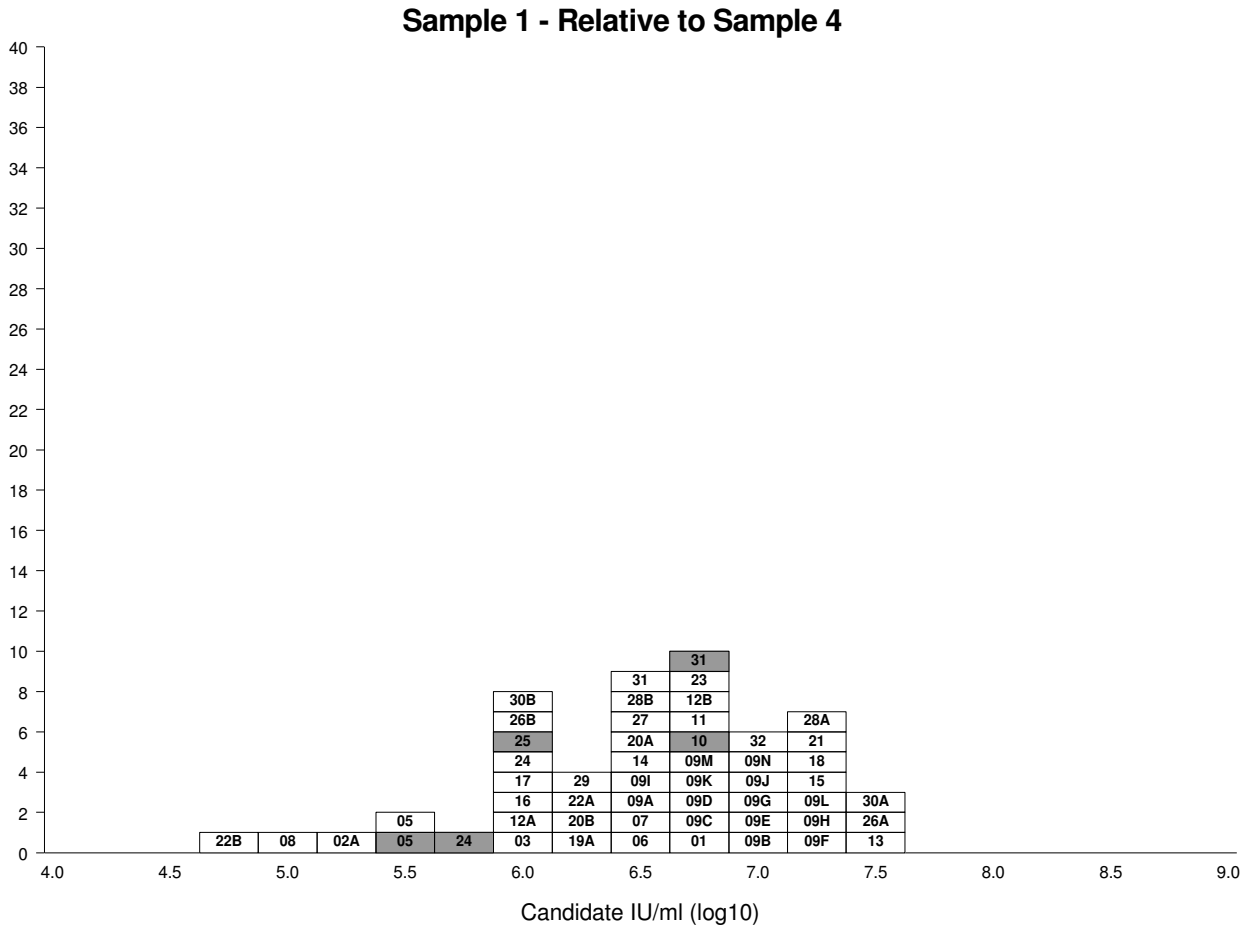
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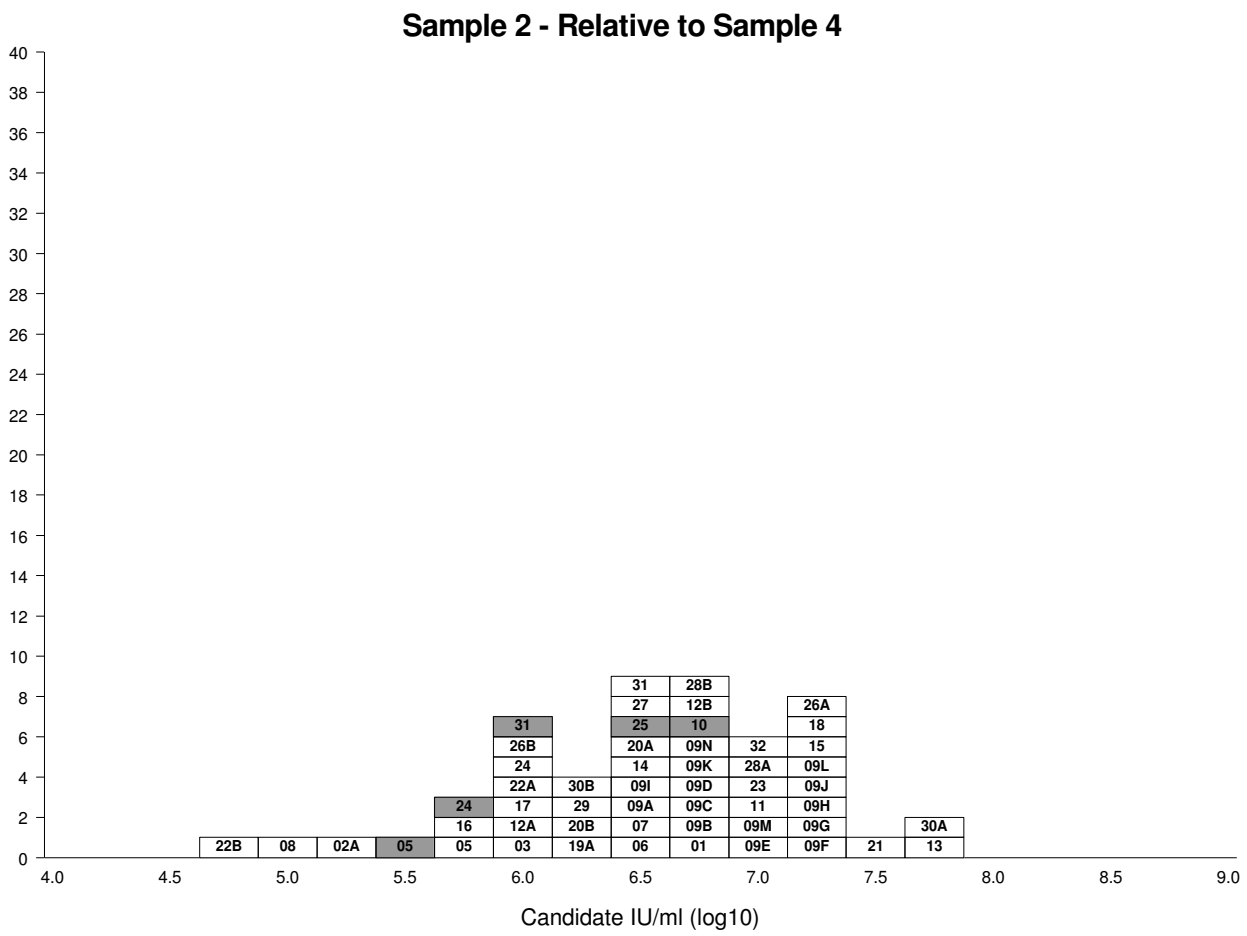
Sample 2 - Relative to Sample 4
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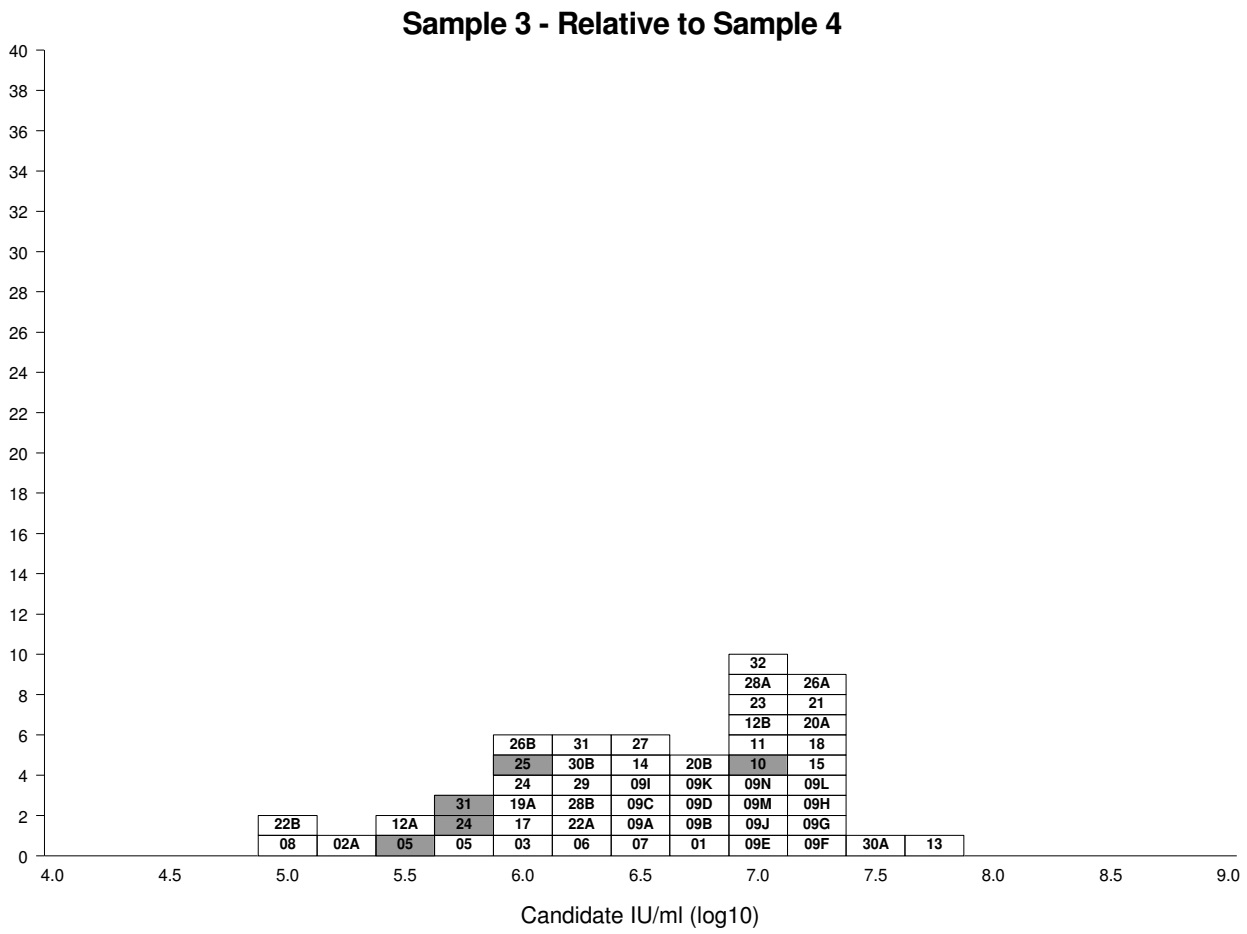
Sample 3 - Relative to Sample 4
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Appendix 1

Collaborative study participants

(In alphabetical order by country)

Name	Laboratory	Country
Prof. Dr. Harald Kessler	Mol. Diagnostics Lab / IHMEM / Medical Univ. of Graz, Graz	Austria
Prof. William Rawlinson	Virology Division, SEALS Microbiology, Randwick	Australia
Dr Guy Boivin	Centre de recherché en infectiologie-CHUQ, Québec	Canada
Dr Jutta Preiksaitis Dr Xiao-Li Pang	Provincial Laboratory for Public Health / University of Alberta, Alberta	Canada
Côme Barranger	Argene SA, Verniolle	France
Dr Sophie Alain	French National Reference Centre for Cytomegalovirus, Limoges	France
Dr Céline Bressollette-Bodin	Virology Laboratory, Nantes University Hospital, Nantes	France
Prof. Dr. Klaus Hamprecht	Institute of Medical Virology, University Hospital of Tübingen, Tübingen	Germany
Dr Thomas Grewing	QIAGEN Hamburg GmbH, Hamburg	Germany
Dr Pantelis Constantoulakis	Locus Medicus SA, Athens	Greece
Dr Valeria Ghisetti	Laboratory of Microbiology and Virology, Amedeo di Savoia Hospital, Turin	Italy
Dr Maria R Capobianchi Dr Isabella Abbate	Laboratory of Virology, National Institute for Infectious Diseases "L. Spallanzani", Rome	Italy
Dr Cristina Olivo	Nanogen Advanced Diagnostics, Buttigliera Alta	Italy
Dr Tiziana Lazzarotto	Operative Unit of Microbiology, Laboratory of Virology, Bologna	Italy
Dr Fausto Baldanti	Molecular Virology Unit, Virology and Microbiology, Fondazione IRCCS Policlinico San Matteo, Pavia	Italy
Dr Naoki Inoue	Laboratory of Herpesviruses, Department of Virology I, National Institute of Infectious Diseases, Tokyo	Japan
Prof. Fredrik Müller	Department of Microbiology, Oslo University Hospital, Rikshospitalet,	Norway
Dr Craig Corcoran	Ampath Pathology Laboratories, Pretoria	South Africa
Dr Diana Hardie	Diagnostic Virology Laboratory Groote Schuur Hospital, Cape Town	South Africa
Dr Jacqueline Prieto	Cepheid AB, Bromma	Sweden
Dr Rob Schuurman Dr Anton van Loon	University Medical Center Utrecht, Dept. Virology, Utrecht	The Netherlands
Dr Shiaolan Ho	Abbott Molecular, Inc., Des Plaines	USA
Dr David Hillyard	ARUP Laboratories, Inc. (University of Utah enterprise), Salt Lake City	USA
Dr Richard Hodinka	Clinical Virology Laboratory, Children's Hospital of Philadelphia, Philadelphia	USA
Dr Marie Louise Landry	Clinical Virology Laboratory, Yale New Haven Hospital, New Haven	USA
Dr Angela Caliendo	Emory University Hospital, Altanta	USA
Dr Nell Lurain	NIH/DAIDS/NIAID Viral Quality Assurance Laboratory, Rush University Medical Center, Chicago	USA
Dr Lee Sung	Roche Molecular Systems Inc., Pleasanton	USA
Dr Margaret Gulley	University of North Carolina, Chapel Hill	USA
Claire Atkinson	Department of Virology, Royal Free Hospital, London	UK
Dr Jon Bible	GSTS Pathology, London	UK
Dr Malcolm Guiver	HPA Laboratory NorthWest, Department of Virology, Manchester	UK

Appendix 2

Study protocol

Collaborative study to evaluate the candidate 1st WHO International Standard for human cytomegalovirus (HCMV) for NAT-based assays

Study Protocol

Objective

The World Health Organisation (WHO) Expert Committee on Biological Standardisation (ECBS) has endorsed a proposal to develop the 1st WHO International Standard for human cytomegalovirus (HCMV) for the calibration of nucleic acid amplification technology (NAT)-based assays. The aim of this collaborative study is to determine the suitability and potency of the proposed candidate standard, using a range of NAT-based assays for HCMV.

Background

Viral load measurements using NAT-based assays are important in the diagnosis and management of CMV disease. However, the variability between the molecular methods employed, and the lack of traceability to a reference system, makes it difficult to compare assay performance and to develop uniform treatment strategies [1].

The WHO ECBS establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human disease. WHO International Standards are recognised as the highest order of reference for biological substances, 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 method used.

As a WHO-designated International Laboratory for Biological Standardisation the National Institute for Biological Standards and Control (NIBSC) prepares and coordinates collaborative studies for the development of many biological standards.

Candidate standard

Proposals for the development of the 1st WHO International Standard for HCMV were discussed at the Standardisation of Genome Amplification Techniques (SoGAT) Clinical Diagnostics meeting held at NIBSC in June 2008 (Meeting report; www.nibsc.ac.uk/spotlight/sogat/clinical_diagnostics/past_meetings.aspx).

The proposed candidate standard comprises cell-free a live virus preparation of the prototype clinical HCMV strain Merlin [2], at a concentration of approximately $1x10^7$ copies/mL, and freeze-dried to ensure long-term stability. This virus represents a well characterised, near complete HCMV genome compared with other laboratory strains. Given the wide range of samples routinely tested for HCMV, the candidate standard is formulated in a universal buffer comprising 10mM Tris-HCl and human serum albumin, for further dilution in the appropriate sample matrix used in each laboratory assay system.

Outline of the study

The potency and suitability of the candidate standard, as the proposed 1st WHO International Standard for HCMV, will be assessed in a worldwide collaborative study, involving participants performing a range of NAT-based assays. The freeze-dried candidate HCMV Merlin preparation will be evaluated alongside the unprocessed liquid bulk, a live virus preparation of the HCMV AD169 strain [3], and purified HCMV Merlin DNA cloned into a bacterial artificial chromosome (BAC).

The study samples have been prepared from material provided by a donor, and must be treated as proprietary. They MUST NOT be used for any purpose other than for the performance of this study.

Four vials of each study sample are provided and participants are asked to test dilutions of each sample, using their routine HCMV NAT assay, on four separate occasions. Where possible, we would encourage laboratories to use quantitative methods, returning viral load results to NIBSC for analysis, however, data from qualitative assays will also be acceptable.

Study samples

Four study samples are to be evaluated, these are coded; sample 1, sample 2, sample 3, and sample 4. Upon receipt, sample 1 should be stored at -20 °C or below. Sample 2, 3 and 4, should be stored at -60 °C or below.

- Sample 1 is a lyophilised preparation in a 5 mL screw cap glass vial. This sample **must be reconstituted with 1 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use.** This sample must be extracted prior to amplification.

- Samples 2 and 3 both comprise 1 mL frozen liquid preparations, and should be thawed fully and vortexed briefly before use. These samples must be extracted prior to amplification.

- Sample 4 comprises a 50 µl frozen liquid preparation of purified DNA. It should be thawed fully and vortexed briefly before use. **This sample must NOT be extracted**, but should be added directly to the amplification reaction.

CAUTION: These preparations are not for administration to humans. Study samples 1, 2 and 3, contain infectious HCMV and should be handled only in appropriate containment facilities by fully trained and competent staff in accordance with national safety guidelines. These preparations contain material of human origin, which has been tested and found negative for HBsAg, HIV antibody, and HCV RNA by PCR. Study sample 4 comprises purified non-infectious plasmid and does not contain viral or cellular constituents. See instructions for use for further details.

Study protocol

Below, are specific instructions for the dilution and testing of study samples, using either quantitative or qualitative assays. Four vials of each study sample are provided and participants are asked to test dilutions of each, using their routine HCMV NAT assay, on four separate occasions.

Samples 1, 2 and 3 should be diluted in a sample matrix appropriate to your assay (e.g. human plasma or whole blood, etc). Please use the same diluent throughout the evaluation of these samples. Meanwhile, sample 4 should be diluted in nuclease-free water.

For each independent assay, study samples 1, 2, 3 and 4 should ideally be tested within the same assay run. Independent assays should be performed on separate days, using a fresh vial of each sample.

For quantitative assays:

For the first assay, participants are requested to test each sample, at a minimum of two serial ten-fold dilutions (e.g. 10^{-1}, 10^{-2}), to ensure the results are within the linear range of the assay.

- Please dilute samples 1, 2 and 3 in the sample matrix appropriate to the assay (e.g. human plasma or whole blood, etc), and record the diluent used on the Result Reporting form. Each dilution of samples 1, 2 and 3 must be extracted prior to amplification. *[Samples 1, 2 and 3 are estimated to contain approximately $1x10^7$ HCMV copies/mL]*

- Please dilute sample 4 in nuclease-free water (this sample can also be tested neat if the result is within the linear range of the assay). Dilutions of sample 4 must NOT be extracted, but should be added directly to the amplification reaction. Please record the volume amplified on the Result Reporting form. *[Sample 4 is estimated to contain approximately $1x10^5$ HCMV copies/µL]*

For the remaining three assays, participants are requested to test a minimum of two serial ten-fold dilutions of each sample, that fall within the linear range of the assay. If the dilutions tested in assay 1 did not fall within the linear range, these must be adjusted so that each dilution is within this range for subsequent assays. A fresh vial of each sample should be used in each independent assay.

If practicable, it would be advantageous to test each dilution of each sample in duplicate.

For qualitative assays:

For the first assay, participants are requested to test ten-fold serial dilutions of each sample, in order to determine the end point (e.g., 10^{-1} to 10^{-7}).

- Please dilute samples 1, 2 and 3 in the sample matrix appropriate to the assay (e.g. human plasma or whole blood, etc), and record the diluent used on the Result Reporting form. Each dilution of samples 1, 2 and 3 must be extracted prior to amplification. *[samples 1, 2 and 3 are estimated to contain approximately $1x10^7$ HCMV copies/mL]*

- Please dilute sample 4 in nuclease-free water (this sample can be tested neat if the result is within the linear range of the assay). Dilutions of sample 4 must NOT be extracted, but should be added directly to the amplification reaction. Please record the volume amplified on the Result Reporting form. *[Sample 4 is estimated to contain approximately $1x10^5$ HCMV copies/µL]*

For the remaining three assays, participants are requested to test the dilution at the assay end point, and a minimum of two half-log serial dilutions either side of the pre-determined end point (i.e., five dilutions in total).

Reporting of results

The results of each assay (viral load expressed as copies/mL) and methodology used, should be recorded on the Result Reporting form accompanying the samples. Separate forms are provided for quantitative or qualitative assay results. Results should be returned to NIBSC before the **28th February 2010** to allow sufficient time for statistical analysis and preparation of the final report for submission to the WHO Expert Committee on Biological Standardisation by July 2010.

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.

All completed Result Reporting forms should be returned electronically to Dr J Fryer: **Jacqueline.Fryer@nibsc.hpa.org.uk**

Alternatively, results may be mailed or faxed to:

Address: Dr J. Fryer, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG.

Fax: +44 (0)1707 641366

Data analysis

All data from the study will be analysed at NIBSC. The analysis will assess the concentration of each sample, relative to each other, and the sensitivities of the different assay methods. Individual participants' data will be coded and reported "blind" to other participants during the preparation of the study report, and also in subsequent publications. Participants will receive a copy of the report of the study and proposed conclusions and recommendations for comment before it is further distributed. It is normal practice to acknowledge participants as contributors of data rather than co-authors in publications describing the establishment of the standard.

References

1. Pang XL, Fox JD, Fenton JM, Miller GG, Caliendo AM, Preiksaitis JK; American Society of Transplantation Infectious Diseases Community of Practice; Canadian Society of Transplantation. Interlaboratory comparison of cytomegalovirus viral load assays. Am J Transplant. 2009;9:258-68.

2. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, Dargan DJ, McGeoch DJ, Gatherer D, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. Genetic content of wild-type human cytomegalovirus. J Gen Virol. 2004;85:1301-12.

3. Chee MS, Bankier AT, Beck S, Bohni R, Brown CM, Cerny R, Horsnell T, Hutchison CA 3rd, Kouzarides T, Martignetti JA, et al. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr Top Microbiol Immunol. 1990;154:125-69.

Appendix 3

Proposed instructions for use

WHO International Standard
1st WHO International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Technologies
NIBSC code: 09/162
Instructions for use
(Version 2.00, Dated)

1. INTENDED USE

The 1st WHO International Standard for human cytomegalovirus (HCMV), NIBSC code 09/162, is intended to be used in the standardisation of nucleic acid amplification (NAT)-based assays for HCMV. The reference comprises a whole virus preparation of the HCMV Merlin strain [1], formulated in a universal buffer comprising Tris-HCl and human serum albumin. The material has been lyophilised in 1 mL aliquots and stored at -20 °C. The material was evaluated in a worldwide collaborative study involving 32 laboratories performing a range of NAT-based assays for HCMV [2].

2. CAUTION

This preparation is not for administration to humans.

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

This material has been assigned a concentration of $5x10^6$ International Units (IU) when reconstituted in 1 mL of nuclease-free water, based on the results of a worldwide collaborative study.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each vial contains the lyophilised equivalent of 1mL of HCMV in 10 mM Tris-HSA buffer (pH 7.4) and 0.5% human serum albumin.

5. STORAGE

Vials of lyophilised standard should be stored at -20 °C.

6. DIRECTIONS FOR OPENING

Vials have a screw cap; an internal stopper may also be present. The cap should be removed by turning anti-clockwise. Care should be taken to prevent loss of the contents. Please note: if a stopper is present on removal of the cap, the stopper should remain in the vial or be removed with the cap.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
The materials should be reconstituted with 1 mL of deionised, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use. The reconstituted material has a final concentration of $5x10^6$ IU/mL.
The International Standard should be used to calibrate whole virus secondary reference materials, for example, by determining the equivalent concentration of secondary reference reagent to be calibrated against the International Standard in parallel. The secondary reference reagent can then be assigned a concentration in terms of the IU. The International Standard should be diluted in the matrix appropriate to the material being calibrated.

8. STABILITY

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

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

9. REFERENCES

1. Dolan A, Cunningham C, Hector RD, Hassan-Walker AF, Lee L, Addison C, Dargan DJ, McGeoch DJ, Gatherer D, Emery VC, Griffiths PD, Sinzger C, McSharry BP, Wilkinson GW, Davison AJ. Genetic content of wild-type human cytomegalovirus. J Gen Virol. 2004;85:1301-12.
2. Fryer JF. Heath AB, Anderson R, Minor PD. Collaborative study to evaluate the proposed 1st WHO International Standard for human cytomegalovirus (HCMV) for nucleic acid amplification (NAT)-based assays. In preparation.

10. ACKNOWLEDGEMENTS

None

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material:
enquiries@nibsc.hpa.org.uk
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.who.int/biologicals/reference_preparations/en/
Ordering standards from NIBSC:
Http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
Http://www.nibsc.ac.uk/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.hpa.org.uk

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

Physical and Chemical properties			
Physical appearance: Lyophilized powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains infectious human cytomegalovirus and human serum albumin		
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		

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A World Health Organization Laboratory for Biological Standards

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

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

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

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

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

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

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

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: 1g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

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