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# Collaborative Study to Evaluate the Proposed 1st WHO International Standard for Epstein-Barr Virus (EBV) for Nucleic Acid Amplification Technology (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 Epstein-Barr virus (EBV) for use in the standardization of nucleic acid amplification techniques (NAT). Proposals for the formulation of the candidate standard were discussed at the Standardization of Genome Amplification Techniques (SoGAT) Clinical Diagnostics meeting at NIBSC in June 2008. The candidate is a whole virus preparation of the EBV B95-8 strain, formulated in a universal buffer comprising Tris-HCl, human serum albumin, 0.1% trehalose, and freeze-dried for long-term stability. Twenty-eight laboratories from 16 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 EBV. The freeze-dried candidate standard (Sample 1) was evaluated alongside the liquid bulk of the candidate preparation (Sample 2), and preparations of Namalwa (Sample 3) and Raji (Sample 4) cells. A wide range of extraction and amplification methodologies were used. The majority of data sets returned were from commercial quantitative assays and all were based on real-time PCR technology. The overall mean potency estimate for the candidate standard sample 1, across the different laboratory assays, was $5\times10^6$ (6.7 $\log_{10}$) 'copies/mL'. The variability between individual laboratory mean estimates for samples 1-4 was 2.5 $\log_{10}$. The agreement between laboratories was markedly improved when the potency of the virus sample 2 was expressed relative to the candidate standard (sample 1). The agreement for samples 3 and 4 was also improved when the potency of these samples was expressed relative to the candidate standard, however, the improvement was less marked than for sample 2. This suggests that the whole virus reference might be most effective for standardizing assay results of clinical samples comprising cell-free material. The utility of the candidate to standardize EBV measurements in whole blood samples would benefit from further investigation. The overall data returned from each laboratory indicated that there was minimal loss in potency upon freeze-drying. In addition, the results obtained from accelerated thermal degradation studies at 4, 8 and 12 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 EBV B95-8 standard as the proposed 1st WHO International Standard for EBV. It is therefore proposed that the candidate standard (NIBSC code 09/260) be established as the 1st WHO International Standard for EBV for NAT with an assigned potency of $5\times10^6$ International Units (IU) when reconstituted in 1 mL of nuclease-free water.

## Introduction
EBV is a ubiquitous herpesvirus with a high worldwide seroprevalence. Primary infection is generally asymptomatic but occasionally leads to infectious mononucleosis (IM) in adolescents and young adults. Following primary infection virus establishes life-long persistence in B cells. EBV is associated with a range of malignancies, such as; Burkitt's lymphoma, Hodgkin and non-Hodgkin lymphoma, gastric and nasopharyngeal carcinoma (NPC), in immunocompetent individuals. Immunocompromised individuals such as transplant recipients, AIDS patients, and those with congenital immunodeficiencies, are at an increased risk of developing EBV-associated lymphoproliferative diseases, associated with primary infection or reactivation. Post-transplant lymphoproliferative disorder (PTLD) is a major complication among haematopoietic stem cell (HSCT) and solid organ (SOT) transplant recipients, with primary EBV infection being a major risk factor [1,2].

The clinical utility of viral load measurements using NAT-based assays in the diagnosis and management of EBV-associated infections has been described [1,2]. The monitoring of EBV DNA in peripheral blood or plasma is widely applied in high-risk PTLD and NPC patients and is critical for early intervention to prevent the development of disease and in monitoring the

response to therapy. However, there is no clear consensus on the timing or frequency of testing or the best blood component to monitor. In the case of NPC, virus is principally measured in plasma since EBV DNA is derived from the tumor cells and exists mainly as naked DNA. Meanwhile in PTLD, EBV is both cell-associated and is released into the cell-free fraction of blood. Although peripheral blood mononuclear cells, plasma and whole blood have all been used successfully to detect EBV in transplant recipients, some studies suggest that whole blood is the most appropriate sample to monitor as it captures both cell-free and cell-associated components (reviewed in [1-3]).

The range of NAT assays used in the diagnosis and management of EBV-associated diseases varies significantly. Laboratories use a range of DNA extraction and amplification methodologies including commercial kits, or analyte-specific reagents (ASR), and laboratory-developed assays which differ in the reagents (including primers and probes) and instrumentation used. In addition, the quantification controls used to determine the concentration of viral DNA present vary. These may comprise either a plasmid clone of the PCR target, quantified viral DNA or virus particles, or cells containing specific copy numbers of the EBV genome, and may or may not be included in the extraction step. Consequently, results may be reported as EBV copies/mL, copies/µg, or copies/number of cells, depending on which control is used. Given the heterogeneity of these NAT-based assay systems, and the lack of traceability to a standardized reference system, it is difficult to compare viral load measurements between different laboratories and to develop uniform therapeutic strategies. Indeed, variability in the performance of different assays for EBV has been documented [4,5]. These studies have highlighted the need for an internationally-accepted reference standard for EBV. In the absence of such a standard, individual centres apply their own management algorithms and these vary between centres.

The WHO's 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 are recognized 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 EBV 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 laboratory EBV strain B95-8 [9], 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 standardize the entire assay including both extraction and DNA amplification steps. It was also agreed that the final concentration would be in the order of $1\times10^7$ 'copies/mL', and would be expressed in IU when established. It was suggested that the candidate could be evaluated alongside preparations of Namalwa [10] and Raji [11] cells, which contain 2 and 50-60 copies of the EBV genome respectively, and which are present as episomes. These cells are sometimes used as secondary references for the routine control of EBV NAT assays. The proposal was endorsed by the WHO ECBS 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 EBV, and to evaluate the suitability of the candidate for the calibration of secondary reference materials and the standardization of EBV viral load measurements.

# Materials

## Candidate standard

The proposed candidate standard comprises a cell-free live virus preparation of the prototype laboratory EBV strain B95-8 [9]. This strain represents a well characterized EBV genome, and has been fully sequenced (NCBI Reference Sequence: NC_007605.1 [12]). The B95-8 strain is classified as a Type 1 virus, based on the sequence of EBNA-2 and EBNA-3 genes. The B95-8 cells were sourced from the European Collection of Cell Cultures (ECACC). Given the wide range of samples routinely tested for EBV, the candidate standard is formulated as EBV virions in a universal buffer, comprising 10 mM Tris-HCl, human serum albumin (HSA) and 0.1% trehalose (Tris-HSA-trehalose buffer), 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 cell culture supernatant sample of EBV B95-8 strain was prepared by propagation of B95-8 cells incubated with 30 mg/mL 12-O-tetradecanoylphorbol-13-acetate (TPA) (adapted from JC Lin [13]). The culture fluid was clarified by low speed centrifugation and virus pelleted by ultracentrifugation. Virus pellets were resuspended to make a stock of virus in 200 mL 10 mM Tris-HCl buffer (pH 7.4), containing 0.5% HSA (Tris-HSA buffer). The HSA used in the production of the candidate standard and other study samples was derived from licensed products. The plasma pools from which these products were derived were screened and tested negative for anti-HIV-1, HBsAg, and HCV RNA.

The concentration of the EBV B95-8 stock was determined at NIBSC, using a laboratory-developed real-time PCR assay (adapted from Kimura et al [14]). Briefly, 400 µL of sample (1/1000 dilution of stock) 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). The target was quantified against serial dilutions of a plasmid clone of the PCR target. The EBV DNA concentration was also assessed at NIBSC using three commercial EBV assays (Roche LightCycler® EBV Quant Kit, Nanogen EBV Q-PCR Alert Kit and Argene EBV R-gene™ Quantification Kit), and in four 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 EBV concentration for the stock.

The bulk preparation was formulated to contain approximately $1\times10^7$ EBV 'copies/mL' in a final volume of 6.4 L Tris-HSA-trehalose 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 lyophilization in order to prepare the final product, NIBSC code 09/260.

### *Filling and lyophilization of candidate standard*

The filling and lyophilization 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 12 $m^2$, 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 14 mm diameter cruciform closures and lyophilized 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 the 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 Analyzer (FMS-750, Lighthouse Instruments, Charlottesville, USA).

Samples of the liquid bulk (n=24) and freeze-dried product (n=24) were tested by EBV real-time PCR as described in the preparation of bulk material, 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 09/260 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 EBV DNA quantified by real-time PCR (as described above).

## Study samples

The freeze-dried candidate EBV B95-8 preparation was evaluated alongside the unprocessed liquid bulk (used to prepare the freeze-dried candidate), and preparations of Namalwa and Raji cells (both Type 1 strains). Namalwa and Raji cells were also sourced from the European Collection of Cell Cultures (ECACC). Namalwa and Raji cells contain 2 and 50-60 episomal copies of the EBV genome, respectively.

The Namalwa and Raji cells were propagated in RPMI-1640 medium containing 10% FCS, supplemented with 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. The

cells were harvested from the culture medium and resuspended in PBS at a concentration of $1\times10^6$ cells/mL. Cells were aliquotted in 1 mL volumes into 2 mL Sarstedt screw cap tubes and stored at -80 °C. Aliquots of Namalwa (n=18) and Raji cells (n=18) were tested by EBV real-time PCR (as described above), 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 -80°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 (S1) - Lyophilized preparation 09/260 in a 5 mL screw cap glass vial.
- Sample 2 (S2) - 1 mL frozen liquid preparation of the EBV B95-8 bulk (used to prepare freeze-dried candidate) in a 2 mL Sarstedt screw cap tube.
- Sample 3 (S3) - 1 mL frozen liquid preparation of Namalwa cells in a 2 mL Sarstedt screw cap tube.
- Sample 4 (S4) - 1 mL frozen liquid preparation of Raji cells in a 2 mL Sarstedt screw cap tube.

# Study design

The aim of the collaborative study was to evaluate the suitability and potency of the candidate EBV International Standard in a range of NAT based assays. Four vials each of study samples 1-4 were sent 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 EBV 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 deionized, nuclease-free molecular-grade water and left for a minimum of 20 minutes with occasional agitation before use. Meanwhile, study samples 2-4 were to be thawed and vortexed briefly before use.

Participants were requested to dilute samples 1-4 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. 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 28 participants representing 16 countries (Appendix 1). Participants were selected for their experience in EBV NAT and geographic distribution. They represented mainly clinical laboratories, but also included manufacturers of *in vitro* diagnostic devices (IVDs), reference and research 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 analyzed separately, as if from different laboratories, and are referred to as, for example, laboratory 1A, 1B 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 [15]. 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, with individual laboratories using different methods and quantification controls to determine their copy number. 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) [16] 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 for that assay is 7.2 $\log_{10}$ IU/mL. Potencies were also calculated relative to sample 3, in the same manner.

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. The significance of the inter-laboratory variation relative to the intra-laboratory variation was assessed by an analysis of variance. A component of variance analysis was used to assess the relative magnitude of the inter-laboratory and intra-laboratory variability.

# 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 of each study sample (n=24 each for study samples 1 and 2, n=18 each for study samples 3 and 4) at NIBSC prior to dispatch indicated that the homogeneity of EBV content was similar for all study samples (2SD less than 0.2 $\log_{10}$ 'copies/mL' for each sample).

Samples of the candidate standard 09/260 were stored at elevated temperatures, and assayed at NIBSC concurrently with samples stored at -20 °C and -70 °C, after 4, 8 and 12 months of storage, by EBV real-time PCR (as described in the preparation of bulk material). 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 ±0.10 $\log_{10}$ based on a pooled estimate of the standard deviation between individual vial test results. Only the results at +45 °C for 4 months and 12 months are outside these confidence limits and are statistically

significantly different from zero. There does appear to be a pattern of apparent increase in potency with increasing temperature although there is no observable change in potency across the course of the 12 month stability study at any temperature. The reason for this apparent increase in potency at the higher temperatures is not clear, but it was also observed in the stability studies for the recently established International Standard for human cytomegalovirus (HCMV) [17]. 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 °C increase in temperature (personal communication: Dr. P K Philips), and noting that there is no detectable drop in potency after 12 months at +20 °C, then there should be no detectable difference after 96 months (8 years) at -20 °C. A similar argument applied to the +37 °C data would imply no detectable loss after 30 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 degradation at any temperature after storage for 12 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 18 months, then at 2, 3, 4, and 5 years.

The stability of the material when reconstituted has not been specifically determined. Therefore, it is recommended that, when established, the standard is for single use only.

## Data received

Data were received from all 28 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 36 quantitative assays, and 2 qualitative assays. Apart from the cases noted below, there were no exclusions of data.

*Qualitative Assays:*
Laboratory 17 used 1.0 $\log_{10}$ dilutions for all assays.
Laboratory 20 had positive results for sample S1 for nearly all dilutions.
These factors will result in reduced precision for the relevant estimates. No data were excluded however.

*Quantitative Assays:*
Laboratory 1 returned no data for S4 in assay 4, reporting an 'empty vial'.
Laboratory 3 noted for S3 and S4 'after defrosting sample contained small particles'.
Laboratory 4 (A&B) had results, after correction for dilution factors, that were high at higher dilutions ($10^{-4}$ & $10^{-5}$) compared to other dilutions. Also the results at neat were high compared to those from the $10^{-1}$ to $10^{-3}$ dilutions. Only the results from the $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions were used for subsequent analysis.
Laboratory 6 had no data for S4 from assay 4. They reported some estimates of S3 as 0 at the $10^{-3}$ dilution. All data for S3 at $10^{-3}$ were excluded.
Laboratory 7 reported one estimate for S3 from assay 2 at the $10^{-2}$ dilution as outside the range of the assay. All estimates for S3 at this dilution for this assay were excluded.
Laboratory 9 continued their dilution range until they obtained 'undetectable' for their quantitative assays. Only estimates > 100 copies/mL were used in subsequent calculations.
Laboratory 10 had variable estimates with the results for assay 1 for S1, S2 and S4 being approximately 1.0 log lower than for subsequent assays. No data were excluded however.
Laboratory 14 had results that were 'undetected' or below the linear range of the assay at higher dilutions. Only results from the first two dilution steps were used in subsequent analysis.

Laboratory 16 had very low estimates at higher dilutions. Only estimates > 50 copies/mL were used in subsequent calculations.
Laboratory 18 had results for S3 at higher dilutions that were low relative to the neat sample. They were also the lowest estimates for all samples. Only the estimates from the neat sample for S3 were used, in case the higher dilutions were giving estimates outside the range of the assay.
Laboratory 20 had some results at $10^{-3.5}$ and $10^{-4}$ that appeared inconsistent with other dilutions. Only the results from the $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions were used for subsequent analysis.
Laboratory 21 had 'positive but non-quantifiable' results for some replicates at the $10^{-3}$ dilution. All data at the $10^{-3}$ dilution were excluded.

## *Summary of assay methodologies*

The majority of participants prepared dilutions of study samples 1-4 using either plasma or whole blood, however, other diluents (PBS, DMEM, 10 mM Tris, 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; QIAGEN's QIAsymphony SP and RG Q, BioRobot, QIAcube, and EZ1 Advanced, bioMérieux NucliSENS® easyMag®, Roche MagNA Pure LC and MagNA Pure Compact. Manual extraction protocols included; Roche High Pure Viral Nucleic Acid Kit, Nanogen EXTRAgen®, EXTRAcell®, and EXTRAblood®, QIAGEN QIAamp (DNA Blood and DNA) Mini Kits, QIAGEN QIAamp DSP Virus Kit, Cepheid affigene® DNA Extraction Kit, bioMérieux NucliSENS® miniMAG®, and heat treatment (60 °C for 2 hrs then 95 °C for 10 mins) in 'Cracking buffer'.
All data sets reported the use of real-time PCR technology. Fourteen participants used commercial assays and reagents (20 data sets), while 12 participants used laboratory-developed assays (14 data sets). Two participants used both commercial and laboratory-developed assays (4 data sets). Commercial assays and reagents included; Nanogen EBV Q-PCR Alert, Argene EBV R-gene™, QIAGEN *artus*® EBV (LC and RG) PCR Kits, Roche LightCycler® EBV Quant Kit, Cepheid's affigene® EBV trender and SmartEBV™, ELITech/Epoch EBV ASR, and Quantification of HHV4 PrimerDesign™ Ltd. The range of EBV PCR targets included; EBNA-1, EBNA-2, BNRF1 p143, BXLF1, EBER1, BALF5 and BamHI-W. Amplification platforms included; Roche LightCycler® 1.5, 2.0 and 480 systems, 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 Cepheid SmartCycler™ II. Given the range of assay combinations and variables, and the fact that no two assays were alike (apart from two laboratories using the Roche MagNA Pure LC instrument in combination with the QIAGEN *artus*® EBV LC PCR Kit), it was not possible to group methods and perform analysis according to the method used.

## *Estimated potencies of study samples*

The laboratory mean estimates of 'copies/mL' ($\log_{10}$) from the quantitative assays and 'NAT detectable units/mL' ($\log_{10}$) from the qualitative assays are shown in Tables 3 and 4 respectively. The individual laboratory mean estimates are also shown in histogram form in Figures 1a-1d. Each box represents the mean estimate from one laboratory, and the boxes are labeled with the laboratory code number. The results from the qualitative assays are labeled Q.
From the figures it is clear that there is considerable variation between laboratories, for all samples, with estimates differing by up to 2.5 $\log_{10}$. The estimates of 'NAT detectable units/mL' from the qualitative assay from laboratory 17 are low for all samples. The estimates from the qualitative assay from laboratory 20 are closer to the estimates from the majority of quantitative assays. However, the results from qualitative and quantitative assays are not directly comparable, as 'NAT detectable units/mL' obtained from dilution series of qualitative assays are not

necessarily equivalent to the estimates of 'copies/mL' from quantitative assays. The method of determining an estimate of 'NAT-detectable units/mL' is based on an end-point dilution series (diluting until negative), makes no allowance for extraction efficiency, and is not calibrated against any measure of genuine copy numbers.

The estimates of 'copies/mL' from laboratory 10 are lower than other laboratories for all samples. The results from laboratory 16 are also generally low for S1, S2 and S4. The quantitative results from laboratory 17 are low for S3 and S4 (cellular material) but not for S1 and S2. It is also notable that laboratory 12 has estimates that are central or slightly lower compared to the majority of other laboratories for samples S1 and S2, but estimates that are high compared to other laboratories for the cellular materials S3 and S4. Individual laboratory results in Figures 1a-d are colour coded according to the diluent used in the assay (i.e. dark grey, whole blood; light grey, plasma; white, other) and show no apparent relationship between matrix and estimate of 'copies/mL'.

Table 5 shows the overall mean estimates of $\log_{10}$ 'copies/mL' from the quantitative assays, and $\log_{10}$ 'NAT-detectable units/mL' from the qualitative assays, along with the standard deviation (of $\log_{10}$ estimates) and the %GCV (of actual estimates). Standard deviations and %GCVs were not calculated for the qualitative assays, as there were only two laboratories performing qualitative assays. The quantitative assays have SD's around 0.6 $\log_{10}$, and %GCVs around 270 - 330%. These figures are consistent with the observed ranges of estimates of over 2.5 $\log_{10}$.

Comparison of overall mean estimates for the freeze-dried candidate sample 1 and liquid bulk sample 2 indicates that there was a small decrease of 0.04 $\log_{10}$ in potency upon freeze-drying (Table 5), which was marginally statistically significant (p=0.029).

Table 6 shows the overall mean estimates from all the quantitative assays again, along with equivalent figures obtained by excluding the results from laboratories 10, 12, 16 and 17. These laboratories were noted above as having either low estimates, or an apparent differential efficiency for cellular material compared to the cell-free virus preparations (S3 & S4 compared to S1 & S2), and did not share similarities in assay methodology. After excluding these laboratories, the SD's are around 0.5 $\log_{10}$, with %GCVs around 200%, which is similar to the variability observed between laboratories in the recent collaborative study to establish an International Standard for HCMV [17]. This comparison illustrates that the large variability observed between results is not simply due to a few individual laboratories. There is no justification for excluding these laboratories simply because of the results achieved, and the comparisons are presented for illustration only.

## *Potencies relative to sample 1*

The expression of potency of samples 2-4 relative to sample 1 allows an assessment of the suitability of the candidate standard for the standardization of EBV NAT assays.

The estimated concentrations of samples 2-4 were expressed as relative potencies in IU, by comparison to the candidate standard sample 1, using a hypothetical unitage of $5x10^6$ IU/mL (6.7 $\log_{10}$ IU/mL) for sample 1, as described in the statistical methods section (in this case, the hypothetical unitage is based on the overall mean laboratory estimate for sample 1). The laboratory mean estimates are shown in Tables 7 and 8 for the quantitative and qualitative assays and respectively. Units are candidate $\log_{10}$ IU/mL in both cases. The results are also shown in histogram form in Figures 2a-2c.

From Figure 2a, it is clear that for sample 2 there is a big improvement in agreement between laboratories, with the vast majority of laboratories obtaining estimates within 0.5 $\log_{10}$. For samples 3 and 4 (Figures 2b and 2c), there is some improvement but there are still ranges of estimates of up to 2.0 $\log_{10}$.

The overall mean estimates of samples S2, S3 and S4 relative to S1, in candidate IU/mL, are shown in Table 9 for the qualitative and quantitative assays, along with the standard deviation

(of $\log_{10}$ estimates) and the %GCV (of actual estimates). For S2, the SD is reduced to 0.09 $\log_{10}$ from 0.57 $\log_{10}$, while the %GCV is reduced to 23% from 270%. For S3 and S4 there is a reduction from an SD of around 0.6 $\log_{10}$ to 0.46 $\log_{10}$, and a reduction in %GCV from around 300% to 187%. The improvement from expressing results in IU relative to the candidate standard S1 is therefore very marked for S2, the same unprocessed liquid bulk material used in the preparation of S1, but less marked for the cellular materials S3 and S4.
Table 10 shows the overall mean estimates from all the quantitative assays again, along with equivalent figures obtained by excluding the results from laboratories 10, 12, 16 and 17. Exclusion of data from these four individual laboratories does improve the agreement between the remaining laboratories for S3 and S4 expressed relative to S1, reducing the %GCV from 187% to 84% for S3 and 130% for S4. These figures are still much higher than the 23% for S2. Looking at Figures 2b and 2c, it is clear that laboratory 17 (quantitative) produced very low estimates for S3 & S4 relative to S1, while laboratory 12 produced high estimates relative to S1. This is a result of the apparent differential sensitivity of the assays from these laboratories for the cell preparations of S3 & S4, compared to their results with S1 & S2 (cell-free virus preparations). Laboratory 17 produced lower results for cellular material compared to the cell-free virus, while laboratory 12 is the other way round. In contrast, laboratory 10, which has low results compared to other laboratories for all samples, has estimates for S2, S3 and S4 relative to S1 that are consistent with the other laboratories.

### *Potencies relative to sample 3*

The estimated concentrations of samples S1, S2 and S4 were also expressed relative to sample S3, using a hypothetical unitage of $10^6$ IU/mL (6.0 $\log_{10}$ IU/mL) for sample 3 (in this case, the hypothetical unitage is based on the overall mean laboratory estimate for sample 3). The laboratory mean estimates are shown in Tables 11 and 12 for the quantitative and qualitative assays respectively. Units are candidate $\log_{10}$ IU/mL in both cases. The results are also shown in histogram form in Figures 3a-3c.
The overall mean estimates of samples S1, S2 and S4 relative to S3, in candidate IU/mL are shown in Table 13 for the qualitative and quantitative assays, along with the standard deviation (of $\log_{10}$ estimates) and the %GCV (of actual estimates).
From the figures and Table 13, there is some reduction in between-laboratory variability, with the SD for quantitative assays for S1 and S2 reducing from 0.58/0.57 to 0.46/0.47, and %GCVs reducing from 277%/270% to 187%/197%. The improvement is greatest for S4 relative to S3, with SD reducing from 0.63 to 0.32, and %GCV reducing from 327% to 107%.
Table 14 shows the overall mean estimates from all the quantitative assays again, along with equivalent figures obtained by excluding the results from laboratories 10, 12, 16 and 17. Exclusion of data from these four individual laboratories does improve the agreement between the remaining laboratories for S1, S2 and S4 expressed relative to S3, reducing the %GCV from 187% to 84% for S1, from 197% to 86% for S2, and from 107% to 76% for S4. Looking at Figures 3a and 3b, laboratories 12 and 17 produced results for S1 and S2 relative to S3 that are outside the range of other laboratories. Again, this is a result of the differential sensitivity of the assays from these laboratories for the cell preparations (S3 & S4), compared to their results with cell-free virus preparations (S1 & S2), noted above.

### *Inter and intra-laboratory variation*

Table 15 shows the intra-laboratory (between assay) standard deviations and %GCVs for the quantitative assays for each laboratory. There are differences between the repeatability of laboratory estimates across assays for all samples. The average standard deviation is 0.14/0.15

$\log_{10}$ for samples S1 and S2 (a %GCV of around 40%). For samples S3 and S4 the average SD is higher at 0.26 $\log_{10}$ (a %GCV of 81%). This indicates that in general assays of the cell preparations were less repeatable than for the cell-free virus samples. Some individual laboratories have poor repeatability, for example laboratory 10 for all samples, or laboratory 7A for samples S3 and S4 (7A used a separate cell-specific extraction method for S3 and S4).
Table 16 shows the results of a 'Components of Variance' analysis for the quantitative assays. Briefly, this analysis breaks down the observed variability in results to a 'between-laboratory' component, and a 'between-assay' component, allowing for the fact that the observed laboratory means are based on a mean of four assays in most cases. The analysis assumes that the between-assay component is the same across laboratories. The results in Table 15 suggest that this is not the case, so the results are very much an "average" component.
In all cases, the between-laboratory variation was statistically significant ($p<0.0001$), i.e. the observed differences between laboratories was not accounted for by between-assay variability alone. This was true for the results expressed relative to either sample S1 or sample S3 as well as for the absolute estimates of 'copies/mL'. Table 16 shows the variance components as both standard deviations (of $\log_{10}$ estimates) and %GCVs. The equivalent results excluding laboratories 10, 12, 16 & 17 are also shown in Table 17.
Looking at the %GCVs in Table 16, the components for estimates of absolute 'copies/mL' confirm that the within laboratory (between-assay) variability for S3 and S4 is double that for S1 and S2. The figures are equivalent to the pooled estimates in Table 15, with slight rounding differences based on the methods of calculation. The between-laboratory component is much greater at around 300%, compared to 40% between-assay for S1/S2 and 80% for S3/S4. The between-laboratory component is reduced to around 200% if laboratories 10, 12, 16 and 17 are excluded (Table 17), while the within-laboratory components are reduced to 30 or 70%. The reduction in average within-laboratory (between-assay) variability when excluding these four individual laboratories is because laboratory 10 in particular has a very high between-assay variability (see Table 15).
When expressing results relative to sample S1, the between-assay components are similar for S3 and S4, and lower for S2. The between-laboratory components are reduced from around 300%, greatly for S2 to 19%, and less so to a little below 200% for S3 and S4. When expressing results relative to sample S3, the between-assay components are around 90% for all samples. The between-laboratory components are around 200% for S1 & S2, and around 100% for S4.
In conclusion, the use of a relative potency can reduce the between-laboratory variation. However, this is most effective when using a standard of a similar nature to the test sample. S1 is the most effective for reducing variability in estimates for S2, while S3 is most effective for reducing variability in estimates for S4. Expressing S2 relative to S1 gives extremely good agreement between laboratories. However, since S1 and S2 are essentially the same material, this is to be expected. The within-laboratory (between-assay) variability is greater for S3 & S4 than S1 & S2. For all samples, there is significant between-laboratory variation in addition to the between-assay variation.

## Discussion and conclusions

In this study, a range of NAT-based assays for EBV have been used to determine the potency and evaluate the suitability of the candidate standard preparation as the 1st WHO International Standard for EBV. The candidate comprises a whole virus preparation of the prototype laboratory EBV strain B95-8. This strain was chosen because it is well characterized and widely used to design NAT-based assays for EBV. It is also used as a secondary reference for routine control of assays. The use of a whole virus preparation, allows the candidate to be extracted alongside clinical samples thereby standardizing the entire EBV assay. In addition, because of the range of patient samples routinely tested for EBV, the candidate has been formulated in a

universal buffer for further dilution in the sample matrix appropriate to each EBV assay. This allows the matrix of the candidate standard to be as similar as possible to that of the test analyte. The material was freeze-dried to ensure long-term stability, and the production data suggests that the batch is homogeneous.

In the collaborative study, the freeze-dried candidate standard was evaluated alongside the liquid bulk of the B95-8 strain and preparations of Namalwa and Raji cells. These cell preparations were chosen as they are also sometimes used as secondary references for the routine control of EBV NAT assays. The overall mean estimate for the candidate standard (S1) was $5\times10^6$ (6.7 $\log_{10}$) 'copies/mL'. Individual laboratory mean estimates ranged from 5.03 to 7.56 $\log_{10}$ 'copies/mL'. The target concentration for the candidate standard was $1\times10^7$ 'copies/mL', based on preliminary testing of the B95-8 stock at NIBSC and in a selection of UK clinical laboratories. 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 all study samples was 2.5 $\log_{10}$. This variability reflects the range and differences in diagnostic testing procedures between laboratories and is similar to levels previously reported for EBV NAT assays [4]. The agreement between laboratories for virus sample 2 was markedly improved when the potency was expressed relative to the candidate standard (S1), demonstrating the suitability of the candidate to standardize assays of whole virus samples. The agreement between laboratories for cell samples 3 and 4 was also improved when the potencies were expressed relative to the candidate standard (S1), however, the improvement was less marked than for sample 2. This may be due to greater variability in the extraction efficiencies for cellular samples or possible heterogeneity of the cell samples S3 and S4.

Inter-laboratory variability was significantly higher than intra-laboratory variability, as determined for other studies [4,5]. Intra-laboratory variability was higher for cell-based samples S3 and S4 compared to virus samples S1 and S2. This finding was also reported by Preiksaitis *et al* [4], illustrating the possible differences in and the need to validate extraction efficiencies between cell-free and cell-based samples. The overall data returned from each laboratory indicates that there was minimal loss in potency upon freeze-drying. The results obtained from accelerated thermal degradation studies at 4, 8 and 12 months indicate that the candidate is stable and suitable for long-term use.

It was noted that a PCR target based on the BamHI-W repeat fragment was used by two participants (laboratories 10 and 15). This fragment is repeated approximately 6-20 times in different EBV isolates, which can lead to over-quantification of EBV DNA in certain sample types (e.g. plasma), especially when these contain fragmented DNA. Therefore, the use of this sequence in EBV viral load measurement is generally not advised (personal communication: Prof. J Middeldorp). However, in this evaluation laboratories 10 and 15 did not report higher EBV concentrations for these study samples compared to other participants.

The matter of commutability of the candidate standard for clinical EBV samples has not been specifically assessed in this study. Commutability is determined by a range of factors including the matrix and molecular variants of the analyte (i.e. intracellular vs. extracellular or encapsidated vs. naked nature of the DNA, and EBV strain variants), both of which can also be affected by processing steps.

The differing pathology of EBV infections in the various disease states is such that a range of types of patient samples (described in the introduction), particularly different blood components, are routinely tested for EBV 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 some matrix effects. Study results suggested that there was no apparent relationship between matrix used as the diluent and the estimate of 'copies/mL'. However, it is evident that cell-free virus diluted in whole blood does not represent an EBV-positive whole blood from an immunosuppressed individual, where virus is cell-associated at least in part. Indeed, the results suggest that there was a difference in the ability of the candidate to reduce the variability of viral load measurements of cell-free vs. cell-associated EBV preparations. This may again be due to the different efficiencies in extracting different sample types, possible heterogeneity of the cell samples S3 and S4, or to other matrix effects.

In addition, the nature of the EBV DNA present within each sample type differs depending on the disease state and stage of infection. EBV DNA in the plasma of NPC patients is principally naked and fragmented [18]. Meanwhile in PTLD, it is cell-associated although smaller amounts of naked DNA and encapsidated virus (in primary infection) may also be present in plasma. In this study, the candidate standard is derived from a crude cell-free preparation of EBV from cell culture, which comprises both whole virus and naked EBV DNA (as determined by DNase digestion experiments – data not shown). All assays used in the collaborative study evaluation were able to detect the three EBV Type I strains represented in the study, suggesting that the candidate International Standard would be suitable for the calibration of secondary references comprising these viruses. It was not feasible to examine further EBV strains (such as Type II strains) within the context of this collaborative study because of the work load required by the participants. However, the majority of EBV NAT assays do not distinguish between EBV types.

The complexity of EBV pathology, in terms of the sample types and nature of virus examined in the management of EBV infections, makes it difficult to produce a universal reference material to be used as an International Standard. A further commutability study will be performed to assess the commutability of this material with respect to EBV DNA forms present in different clinical samples, and to determine whether a cell-associated EBV reference might be more suitable for the standardization of assays used to monitor EBV in whole blood from transplant recipients. It is likely that the preparation of such a freeze-dried reference would be difficult because of the need to maintain cell structure during the freeze-drying process. Despite this, the implementation and use of the candidate preparation evaluated in this study as an International Standard for EBV will facilitate the characterization and standardization of the factors that contribute to assay variability, and assist in the development of uniform management strategies for EBV-associated diseases.

# Proposal

It is proposed that the candidate standard (NIBSC code 09/260) is established as the 1st WHO International Standard for EBV for use in NAT-based assays, with an assigned potency of $5\times10^6$ International Units when reconstituted in 1 mL of nuclease-free water. This value is arbitrary but represents the consensus estimate for the candidate across the wide range of qualitative and quantitative laboratory assays used in this collaborative study. The uncertainty can be derived from the variance of the fill and is 0.23%. 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 EBV. Proposed Instructions for Use (IFU) for the product are included in Appendix 3.

## Comments from participants

Ten of twenty-eight participants responded to the report. There were no disagreements with the suitability of the candidate standard (NIBSC code 09/260) to serve as the 1st WHO International Standard for EBV for NAT. The majority of comments suggested editorial changes and these have been implemented where appropriate.

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

| | |
|---|---|
| NIBSC code | 09/260 |
| Product name | Epstein-Barr virus |
| Dates of processing | Filling; 25 January 2010<br>Lyophilization; 25-29 January 2010<br>Sealing; 29 January 2010 |
| Presentation | Freeze-dried preparation in 5 mL screw-cap glass vial |
| Appearance | Robust opaque white cake |
| No. of vials filled | 6084 |
| Mean fill weight (g) | 0.9997 (n=60) |
| CV of fill weight (%) | 0.23 |
| Mean residual moisture (%) | 0.7 Karl Fischer, 0.48 NIR units (n=11) |
| CV of residual moisture (%) | 9.5 |
| Mean oxygen content (%) | 0.18 (n=12) |
| CV of oxygen content (%) | 26.6 |
| No. of vials available to WHO | 5677 |

**Table 2.** Stability of 09/260 at 4, 8 and 12 months.

| Temperature (°C) | Mean $\log_{10}$ 'copies/mL' | | | Difference in $\log_{10}$ 'copies/mL' from -70 °C baseline sample | | |
|---|---|---|---|---|---|---|
| | 4 months | 8 months | 12 months | 4 months | 8 months | 12 months |
| -70 | 6.865 | 6.992 | 7.041 | - | - | - |
| -20 | 6.882 | 6.971 | 7.048 | 0.017 | -0.021 | 0.007 |
| +4 | 6.909 | 6.999 | 7.088 | 0.044 | 0.007 | 0.047 |
| +20 | 6.959 | 7.02 | 7.092 | 0.094 | 0.028 | 0.051 |
| +37 | 6.956 | 6.935 | 7.126 | 0.091 | -0.057 | 0.085 |
| +45 | 6.994 | 7.061 | 7.151 | 0.129 | 0.069 | 0.110 |

**Table 3.** Laboratory mean estimates from quantitative assays ($log_{10}$ 'copies/mL').

| Laboratory | Sample | | | |
|---|---|---|---|---|
| | S1 | S2 | S3 | S4 |
| 01 | 6.25 | 6.42 | 5.65 | 7.39 |
| 02A | 7.03 | 7.13 | 6.25 | 8.07 |
| 02B | 6.06 | 6.03 | 5.31 | 6.79 |
| 03 | 6.42 | 6.63 | 5.61 | 7.31 |
| 04A | 6.95 | 6.97 | 6.01 | 7.24 |
| 04B | 6.59 | 6.65 | 5.62 | 7.07 |
| 05 | 6.11 | 6.15 | 5.53 | 6.97 |
| 06 | 6.52 | 6.58 | 6.05 | 7.66 |
| 07A | 6.35 | 6.56 | 5.37 | 6.97 |
| 07B | 6.24 | 6.10 | 5.47 | 7.37 |
| 08A | 6.73 | 6.82 | 5.86 | 7.36 |
| 08B | 6.83 | 6.92 | 5.89 | 7.42 |
| 09 | 6.43 | 6.62 | 6.30 | 7.85 |
| 10 | 5.03 | 5.02 | 4.52 | 5.96 |
| 11 | 6.75 | 6.77 | 5.88 | 7.52 |
| 12 | 6.39 | 6.35 | 6.75 | 8.17 |
| 13A | 7.32 | 7.33 | 6.33 | 8.23 |
| 13B | 7.50 | 7.53 | 6.51 | 8.41 |
| 14 | 7.26 | 7.19 | 6.12 | 7.51 |
| 15 | 6.55 | 6.53 | 5.87 | 7.24 |
| 16 | 5.67 | 5.62 | 5.71 | 6.03 |
| 17 | 6.96 | 7.01 | 4.41 | 6.29 |
| 18 | 6.77 | 6.76 | 6.02 | 7.16 |
| 19 | 7.28 | 7.24 | 6.37 | 7.99 |
| 20 | 7.08 | 7.09 | 6.26 | 7.21 |
| 21 | 7.56 | 7.54 | 6.78 | 8.29 |
| 22A | 7.40 | 7.51 | 6.82 | 8.56 |
| 22B | 5.61 | 5.74 | 4.87 | 7.00 |
| 23 | 7.38 | 7.29 | 6.62 | 7.85 |
| 24 | 6.55 | 6.71 | 4.97 | 6.67 |
| 25 | 7.35 | 7.41 | 5.97 | 7.33 |
| 26A | 7.33 | 7.30 | 6.53 | 8.08 |
| 26B | 6.97 | 6.89 | 6.17 | 7.74 |
| 27 | 6.56 | 6.72 | 6.17 | 7.64 |
| 28A | 6.70 | 6.73 | 5.88 | 7.74 |
| 28B | 7.22 | 7.28 | 6.57 | 8.22 |

**Table 4.** Laboratory mean estimates from qualitative assays ($log_{10}$ 'NAT detectable units/mL').

| Laboratory | Sample | | | |
|---|---|---|---|---|
| | S1 | S2 | S3 | S4 |
| 17 | 5.49 | 5.74 | 3.83 | 5.96 |
| 20 | 6.59 | 6.20 | 5.46 | 6.45 |

**Table 5.** Overall mean estimates and inter-laboratory variation ($log_{10}$ 'copies/mL' for quantitative or 'NAT-detectable units/mL' for qualitative assays). - = not determined.

| Sample | Assay | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S1 | Qualitative | 2 | 6.04 | - | - | 5.49 | 6.59 |
| | Quantitative | 36 | 6.71 | 0.58 | 277 | 5.03 | 7.56 |
| | | | | | | | |
| S2 | Qualitative | 2 | 5.97 | - | - | 6.20 | 5.41 |
| | Quantitative | 36 | 6.75 | 0.57 | 270 | 5.02 | 7.54 |
| | | | | | | | |
| S3 | Qualitative | 2 | 4.64 | - | - | 3.83 | 5.46 |
| | Quantitative | 36 | 5.92 | 0.60 | 294 | 4.41 | 6.82 |
| | | | | | | | |
| S4 | Qualitative | 2 | 6.20 | - | - | 5.96 | 6.45 |
| | Quantitative | 36 | 7.45 | 0.63 | 327 | 5.96 | 8.56 |

**Table 6.** Overall mean estimates and inter-laboratory variation ($log_{10}$ 'copies/mL') for quantitative assays.

| Sample | | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S1 | All Labs | 36 | 6.71 | 0.58 | 277 | 5.03 | 7.56 |
| | Excluding 10, 12, 16 & 17 | 32 | 6.80 | 0.48 | 205 | 5.61 | 7.56 |
| | | | | | | | |
| S2 | All Labs | 36 | 6.75 | 0.57 | 270 | 5.02 | 7.54 |
| | Excluding 10, 12, 16 & 17 | 32 | 6.85 | 0.46 | 188 | 5.74 | 6.70 |
| | | | | | | | |
| S3 | All Labs | 36 | 5.92 | 0.60 | 294 | 4.41 | 6.82 |
| | Excluding 10, 12, 16 & 17 | 32 | 5.99 | 0.48 | 205 | 4.87 | 6.82 |
| | | | | | | | |
| S4 | All Labs | 36 | 7.45 | 0.63 | 327 | 5.96 | 8.56 |
| | Excluding 10, 12, 16 & 17 | 32 | 7.56 | 0.49 | 209 | 6.67 | 8.56 |

**Table 7.** Laboratory estimates of potency relative to S1 from quantitative assays $\log_{10}$ IU/mL taking sample 1 as $5\times10^6$ (6.7 $\log_{10}$ ) IU/mL.

| Laboratory | Sample | | |
|---|---|---|---|
| | S2 | S3 | S4 |
| 01 | 6.87 | 6.10 | 7.84 |
| 02A | 6.80 | 5.92 | 7.73 |
| 02B | 6.67 | 5.95 | 7.43 |
| 03 | 6.90 | 5.89 | 7.59 |
| 04A | 6.72 | 5.76 | 6.99 |
| 04B | 6.77 | 5.74 | 7.18 |
| 05 | 6.74 | 6.12 | 7.56 |
| 06 | 6.75 | 6.23 | 7.84 |
| 07A | 6.91 | 5.72 | 7.32 |
| 07B | 6.56 | 5.93 | 7.83 |
| 08A | 6.80 | 5.83 | 7.33 |
| 08B | 6.80 | 5.76 | 7.29 |
| 09 | 6.89 | 6.57 | 8.12 |
| 10 | 6.69 | 6.19 | 7.63 |
| 11 | 6.73 | 5.83 | 7.47 |
| 12 | 6.66 | 7.05 | 8.48 |
| 13A | 6.72 | 5.71 | 7.61 |
| 13B | 6.72 | 5.71 | 7.60 |
| 14 | 6.63 | 5.56 | 6.95 |
| 15 | 6.69 | 6.02 | 7.39 |
| 16 | 6.65 | 6.74 | 7.06 |
| 17 | 6.75 | 4.15 | 6.03 |
| 18 | 6.68 | 5.94 | 7.09 |
| 19 | 6.67 | 5.79 | 7.41 |
| 20 | 6.71 | 5.88 | 6.83 |
| 21 | 6.67 | 5.92 | 7.43 |
| 22A | 6.81 | 6.13 | 7.86 |
| 22B | 6.83 | 5.96 | 8.09 |
| 23 | 6.61 | 5.94 | 7.16 |
| 24 | 6.86 | 5.13 | 6.83 |
| 25 | 6.76 | 5.32 | 6.68 |
| 26A | 6.67 | 5.89 | 7.45 |
| 26B | 6.62 | 5.91 | 7.47 |
| 27 | 6.86 | 6.32 | 7.78 |
| 28A | 6.73 | 5.88 | 7.74 |
| 28B | 6.76 | 6.06 | 7.70 |

**Table 8.** Laboratory estimates of potency relative to S1 from qualitative assays $\log_{10}$ IU/mL taking sample 1 as $5\times10^6$ (6.7 $\log_{10}$ ) IU/mL.

| Laboratory | Sample | | |
|---|---|---|---|
| | S2 | S3 | S4 |
| 17 | 6.69 | 5.04 | 7.17 |
| 20 | 6.31 | 5.57 | 6.56 |

**Table 9.** Overall mean estimates and inter-laboratory variation for potency relative to sample 1 $\log_{10}$ IU/mL taking sample 1 as $5\times10^6$ (6.7 $\log_{10}$ ) IU/mL. - = not determined.

| Sample | Assay | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S2 | Qualitative | 2 | 6.63 | - | - | 6.31 | 6.96 |
| | Quantitative | 36 | 6.74 | 0.09 | 23 | 6.56 | 6.91 |
| | | | | | | | |
| S3 | Qualitative | 2 | 5.30 | - | - | 5.04 | 5.57 |
| | quantitative | 36 | 5.90 | 0.46 | 187 | 4.15 | 7.05 |
| | | | | | | | |
| S4 | Qualitative | 2 | 6.86 | - | - | 6.56 | 7.17 |
| | Quantitative | 36 | 7.43 | 0.46 | 187 | 6.03 | 8.48 |

**Table 10.** Overall mean estimates and inter-laboratory variation for potency relative to sample 1 quantitative assays $\log_{10}$ IU/mL taking sample 1 as $5x10^6$ (6.7 $\log_{10}$ ) IU/mL.

| Sample | | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S2 | All Labs | 36 | 6.74 | 0.09 | 23 | 6.56 | 6.91 |
| | Excluding 10, 12, 16 & 17 | 32 | 6.75 | 0.09 | 23 | 6.56 | 6.91 |
| | | | | | | | |
| S3 | All Labs | 36 | 5.90 | 0.46 | 187 | 4.45 | 7.05 |
| | Excluding 10, 12, 16 & 17 | 32 | 5.89 | 0.26 | 84 | 5.13 | 6.57 |
| | | | | | | | |
| S4 | All Labs | 36 | 7.43 | 0.46 | 187 | 6.03 | 8.48 |
| | Excluding 10, 12, 16 & 17 | 32 | 7.46 | 0.36 | 130 | 6.68 | 8.12 |

**Table 11.** Laboratory estimates of potency relative to S3 from quantitative assays $\log_{10}$ IU/mL taking sample 3 as $10^6$ (6.0 $\log_{10}$ ) IU/mL.

| Laboratory | Sample | | |
|---|---|---|---|
| | S1 | S2 | S4 |
| 01 | 6.60 | 6.77 | 7.74 |
| 02A | 6.78 | 6.88 | 7.81 |
| 02B | 6.75 | 6.72 | 7.48 |
| 03 | 6.81 | 7.01 | 7.70 |
| 04A | 6.94 | 6.96 | 7.23 |
| 04B | 6.96 | 7.03 | 7.44 |
| 05 | 6.58 | 6.62 | 7.44 |
| 06 | 6.47 | 6.53 | 7.61 |
| 07A | 6.98 | 7.20 | 7.60 |
| 07B | 6.77 | 6.63 | 7.90 |
| 08A | 6.87 | 6.97 | 7.50 |
| 08B | 6.94 | 7.03 | 7.53 |
| 09 | 6.13 | 6.32 | 7.55 |
| 10 | 6.51 | 6.51 | 7.44 |
| 11 | 6.87 | 6.90 | 7.64 |
| 12 | 5.65 | 5.61 | 7.42 |
| 13A | 6.99 | 7.00 | 7.90 |
| 13B | 6.99 | 7.02 | 7.90 |
| 14 | 7.14 | 7.07 | 7.39 |
| 15 | 6.68 | 6.66 | 7.37 |
| 16 | 5.96 | 5.91 | 6.32 |
| 17 | 8.55 | 8.60 | 7.88 |
| 18 | 6.76 | 6.74 | 7.14 |
| 19 | 6.91 | 6.88 | 7.62 |
| 20 | 6.82 | 6.83 | 6.95 |
| 21 | 6.78 | 6.75 | 7.51 |
| 22A | 6.57 | 6.69 | 7.73 |
| 22B | 6.74 | 6.87 | 8.13 |
| 23 | 6.76 | 6.67 | 7.23 |
| 24 | 7.57 | 7.73 | 7.70 |
| 25 | 7.38 | 7.44 | 7.36 |
| 26A | 6.81 | 6.77 | 7.56 |
| 26B | 6.79 | 6.71 | 7.56 |
| 27 | 6.38 | 6.54 | 7.46 |
| 28A | 6.82 | 6.85 | 7.86 |
| 28B | 6.64 | 6.70 | 7.64 |

**Table 12.** Laboratory estimates of potency relative to S3 from qualitative assays $\log_{10}$ IU/mL taking sample 1 as $10^6$ (6.0 $\log_{10}$ ) IU/mL.

| Laboratory | Sample | | |
|---|---|---|---|
| | S1 | S2 | S4 |
| 17 | 7.66 | 7.92 | 8.13 |
| 20 | 7.13 | 6.74 | 6.99 |

**Table 13.** Overall mean estimates and inter-laboratory variation for potency relative to sample 3 $\log_{10}$ IU/mL taking sample 1 as $10^6$ (6.0 $\log_{10}$ ) IU/mL. - = not determined.

| Sample | Assay | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S1 | Qualitative | 2 | 7.40 | - | - | 7.13 | 7.66 |
| | Quantitative | 36 | 6.80 | 0.46 | 187 | 5.65 | 8.55 |
| | | | | | | | |
| S2 | Qualitative | 2 | 7.33 | - | - | 6.74 | 7.92 |
| | Quantitative | 36 | 6.84 | 0.47 | 197 | 5.61 | 8.60 |
| | | | | | | | |
| S4 | Qualitative | 2 | 7.56 | - | - | 6.99 | 8.13 |
| | Quantitative | 36 | 7.53 | 0.32 | 107 | 6.32 | 8.13 |

**Table 14.** Overall mean estimates and inter-laboratory variation for potency relative to sample 3 $\log_{10}$ IU/mL taking sample 1 as $10^6$ (6.0 $\log_{10}$ ) IU/mL.

| Sample | | No. of data sets | Mean | SD | %GCV | Min | Max |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| S1 | All Labs | 36 | 6.80 | 0.46 | 187 | 5.65 | 8.55 |
| | Excluding 10, 12, 16 & 17 | 32 | 6.81 | 0.26 | 84 | 6.13 | 7.57 |
| | | | | | | | |
| S2 | All Labs | 36 | 6.84 | 0.47 | 197 | 5.61 | 8.60 |
| | Excluding 10, 12, 16 & 17 | 32 | 6.86 | 0.27 | 86 | 6.32 | 7.73 |
| | | | | | | | |
| S4 | All Labs | 36 | 7.53 | 0.32 | 107 | 6.32 | 8.13 |
| | Excluding 10, 12, 16 & 17 | 32 | 7.57 | 0.25 | 76 | 6.95 | 8.13 |

**Table 15.** Intra-laboratory standard deviation of $\log_{10}$ 'copies/mL' and %GCV for quantitative assays.

| Laboratory | Sample | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | S1 | | S2 | | S3 | | S4 | |
| | SD | %GCV | SD | %GCV | SD | %GCV | SD | %GCV |
| 01 | 0.11 | 29 | 0.11 | 29 | 0.12 | 32 | 0.28 | 90 |
| 02A | 0.04 | 9 | 0.03 | 7 | 0.16 | 46 | 0.21 | 63 |
| 02B | 0.15 | 41 | 0.14 | 38 | 0.14 | 39 | 0.21 | 64 |
| 03 | 0.08 | 20 | 0.08 | 19 | 0.41 | 157 | 0.29 | 96 |
| 04A | 0.06 | 15 | 0.24 | 74 | 0.10 | 27 | 0.17 | 47 |
| 04B | 0.08 | 21 | 0.21 | 61 | 0.16 | 44 | 0.12 | 33 |
| 05 | 0.05 | 13 | 0.04 | 9 | 0.21 | 64 | 0.29 | 96 |
| 06 | 0.08 | 20 | 0.09 | 22 | 0.13 | 36 | 0.23 | 71 |
| 07A | 0.06 | 14 | 0.01 | 3 | 0.76 | 473 | 0.54 | 245 |
| 07B | 0.17 | 47 | 0.18 | 51 | 0.28 | 92 | 0.23 | 69 |
| 08A | 0.02 | 4 | 0.02 | 6 | 0.17 | 48 | 0.13 | 33 |
| 08B | 0.04 | 10 | 0.04 | 9 | 0.14 | 38 | 0.09 | 24 |
| 09 | 0.11 | 29 | 0.12 | 32 | 0.19 | 55 | 0.18 | 53 |
| 10 | 0.49 | 206 | 0.54 | 251 | 0.29 | 94 | 0.49 | 210 |
| 11 | 0.04 | 10 | 0.05 | 13 | 0.06 | 15 | 0.19 | 54 |
| 12 | 0.20 | 58 | 0.10 | 27 | 0.54 | 245 | 0.38 | 141 |
| 13A | 0.07 | 18 | 0.10 | 26 | 0.15 | 41 | 0.10 | 25 |
| 13B | 0.07 | 18 | 0.06 | 15 | 0.06 | 15 | 0.07 | 19 |
| 14 | 0.17 | 48 | 0.13 | 35 | 0.05 | 13 | 0.05 | 11 |
| 15 | 0.15 | 41 | 0.14 | 39 | 0.23 | 69 | 0.27 | 88 |
| 16 | 0.04 | 9 | 0.03 | 6 | 0.19 | 53 | 0.34 | 121 |
| 17 | 0.09 | 23 | 0.08 | 21 | 0.52 | 230 | 0.33 | 114 |
| 18 | 0.10 | 26 | 0.05 | 12 | 0.20 | 58 | 0.13 | 34 |
| 19 | 0.24 | 73 | 0.31 | 106 | 0.12 | 33 | 0.23 | 71 |
| 20 | 0.10 | 26 | 0.16 | 43 | 0.45 | 182 | 0.19 | 56 |
| 21 | 0.04 | 11 | 0.06 | 14 | 0.19 | 54 | 0.37 | 134 |
| 22A | 0.11 | 29 | 0.04 | 10 | 0.28 | 92 | 0.41 | 158 |
| 22B | 0.23 | 70 | 0.12 | 32 | 0.14 | 37 | 0.16 | 43 |
| 23 | 0.22 | 64 | 0.20 | 60 | 0.18 | 51 | 0.37 | 133 |
| 24 | 0.08 | 19 | 0.08 | 19 | 0.04 | 11 | 0.02 | 5 |
| 25 | 0.06 | 14 | 0.06 | 14 | 0.12 | 32 | 0.07 | 19 |
| 26A | 0.07 | 17 | 0.05 | 13 | 0.05 | 13 | 0.14 | 38 |
| 26B | 0.17 | 47 | 0.23 | 68 | 0.12 | 33 | 0.09 | 24 |
| 27 | 0.13 | 34 | 0.13 | 35 | 0.06 | 16 | 0.34 | 116 |
| 28A | 0.09 | 24 | 0.04 | 11 | 0.26 | 83 | 0.26 | 84 |
| 28B | 0.07 | 18 | 0.05 | 11 | 0.18 | 51 | 0.21 | 63 |
| Mean | 0.14 | 38 | 0.15 | 42 | 0.26 | 81 | 0.26 | 81 |

**Table 16.** Components of variation, inter vs. intra contributions for all quantitative assays (36 data sets). CPM = copies per mL.

| Component | | SD | | %GCV | |
|---|---|---|---|---|---|
| | | Lab | Assay | Lab | Assay |
| Absolute CPM | S1 | 0.57 | 0.14 | 273 | 38 |
| | S2 | 0.56 | 0.15 | 267 | 41 |
| | S3 | 0.60 | 0.26 | 293 | 81 |
| | S4 | 0.62 | 0.26 | 320 | 82 |
| | | | | | |
| Potencies Relative to S1 | S1 | - | - | - | - |
| | S2 | 0.07 | 0.10 | 19 | 24 |
| | S3 | 0.46 | 0.28 | 185 | 90 |
| | S4 | 0.44 | 0.25 | 177 | 79 |
| | | | | | |
| Potencies Relative to S3 | S1 | 0.46 | 0.28 | 185 | 90 |
| | S2 | 0.47 | 0.27 | 196 | 87 |
| | S3 | - | - | - | - |
| | S4 | 0.30 | 0.28 | 98 | 88 |

**Table 17.** Components of variation, inter vs. intra contributions for quantitative assays, excluding laboratories 10, 12, 16 & 17 (32 data sets). CPM = copies per mL.

| Component | | SD | | %GCV | |
|---|---|---|---|---|---|
| | | Lab | Assay | Lab | Assay |
| Absolute CPM | S1 | 0.48 | 0.11 | 203 | 30 |
| | S2 | 0.46 | 0.12 | 185 | 33 |
| | S3 | 0.48 | 0.23 | 200 | 71 |
| | S4 | 0.48 | 0.24 | 202 | 73 |
| | | | | | |
| Potencies Relative to S1 | S1 | - | - | - | - |
| | S2 | 0.08 | 0.09 | 19 | 24 |
| | S3 | 0.24 | 0.24 | 75 | 75 |
| | S4 | 0.34 | 0.24 | 120 | 72 |
| | | | | | |
| Potencies Relative to S3 | S1 | 0.24 | 0.24 | 75 | 75 |
| | S2 | 0.25 | 0.24 | 78 | 75 |
| | S3 | - | - | - | - |
| | S4 | 0.21 | 0.27 | 63 | 84 |

## Figure legends

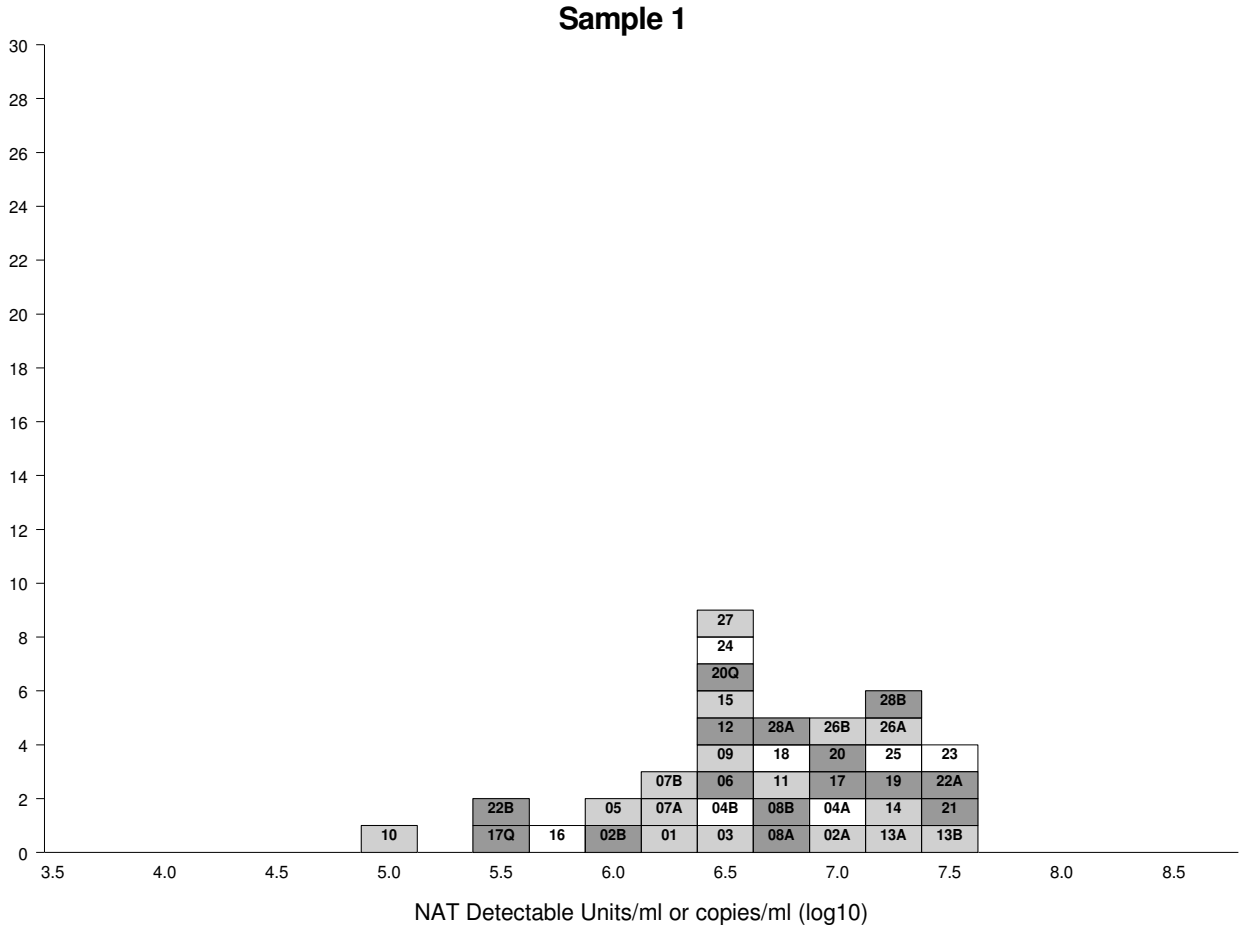
**Figure 1.** Individual laboratory mean estimates for study samples 1-4 obtained using qualitative or quantitative NAT assays. Each box represents the mean estimate from each laboratory assay and is labeled with the laboratory code number. The results from the qualitative assays are labeled Q. The results are colour coded according to the diluent used in the assay (dark grey, whole blood; light grey, plasma; white, other).

**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 labeled with the laboratory code number. The results from the qualitative assays are labeled Q. The results are colour coded according to the diluent used in the assay (dark grey, whole blood; light grey, plasma; white, other).

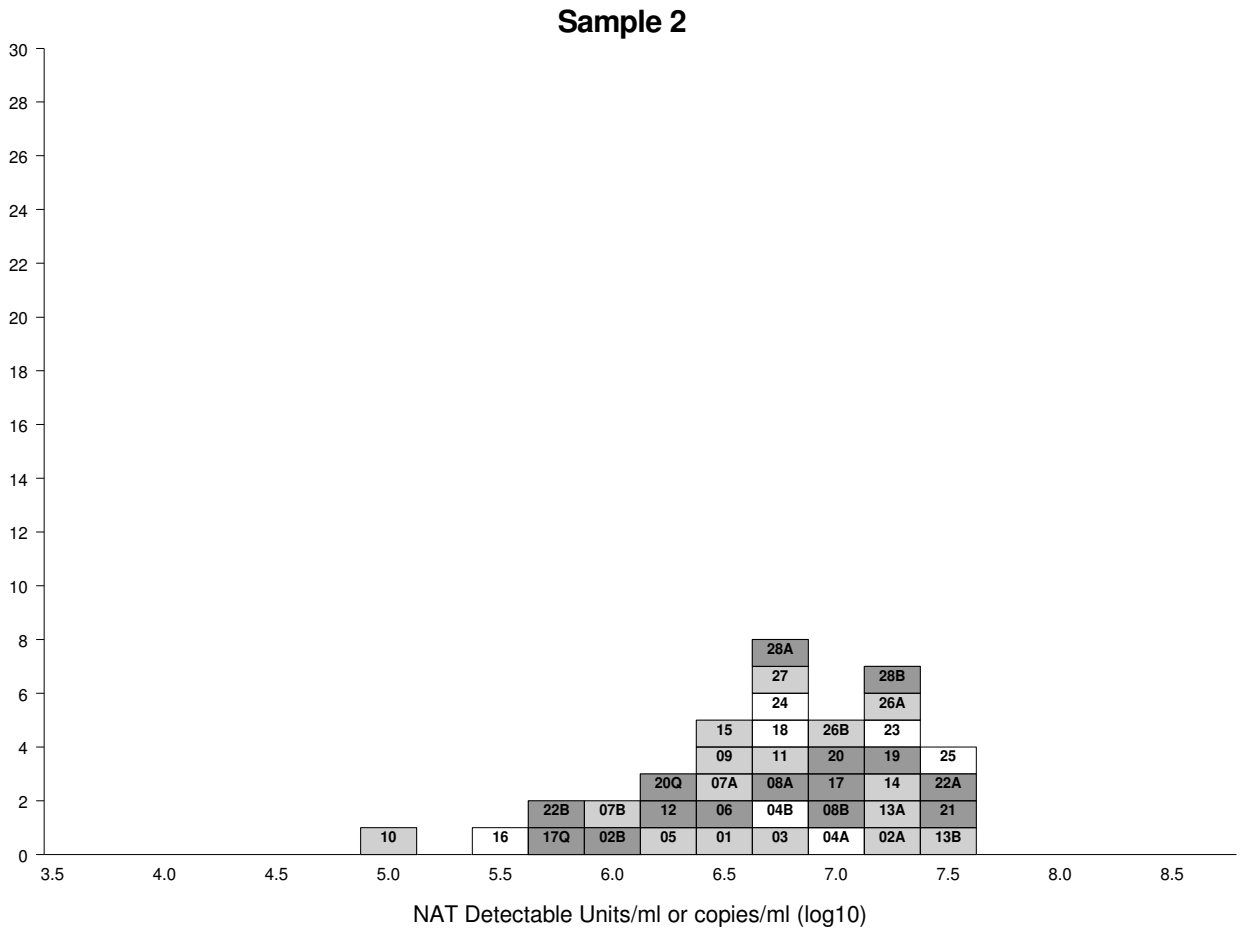
**Figure 3.** Estimated concentrations of samples 1, 2 and 4 expressed in IU, relative to sample 3, using a hypothetical unitage of $10^6$ IU/mL for sample 3. Each box represents the relative potency for each laboratory assay and is labeled with the laboratory code number. The results from the qualitative assays are labeled Q. The results are colour coded according to the diluent used in the assay (dark grey, whole blood; light grey, plasma; white, other).

# Figure 1

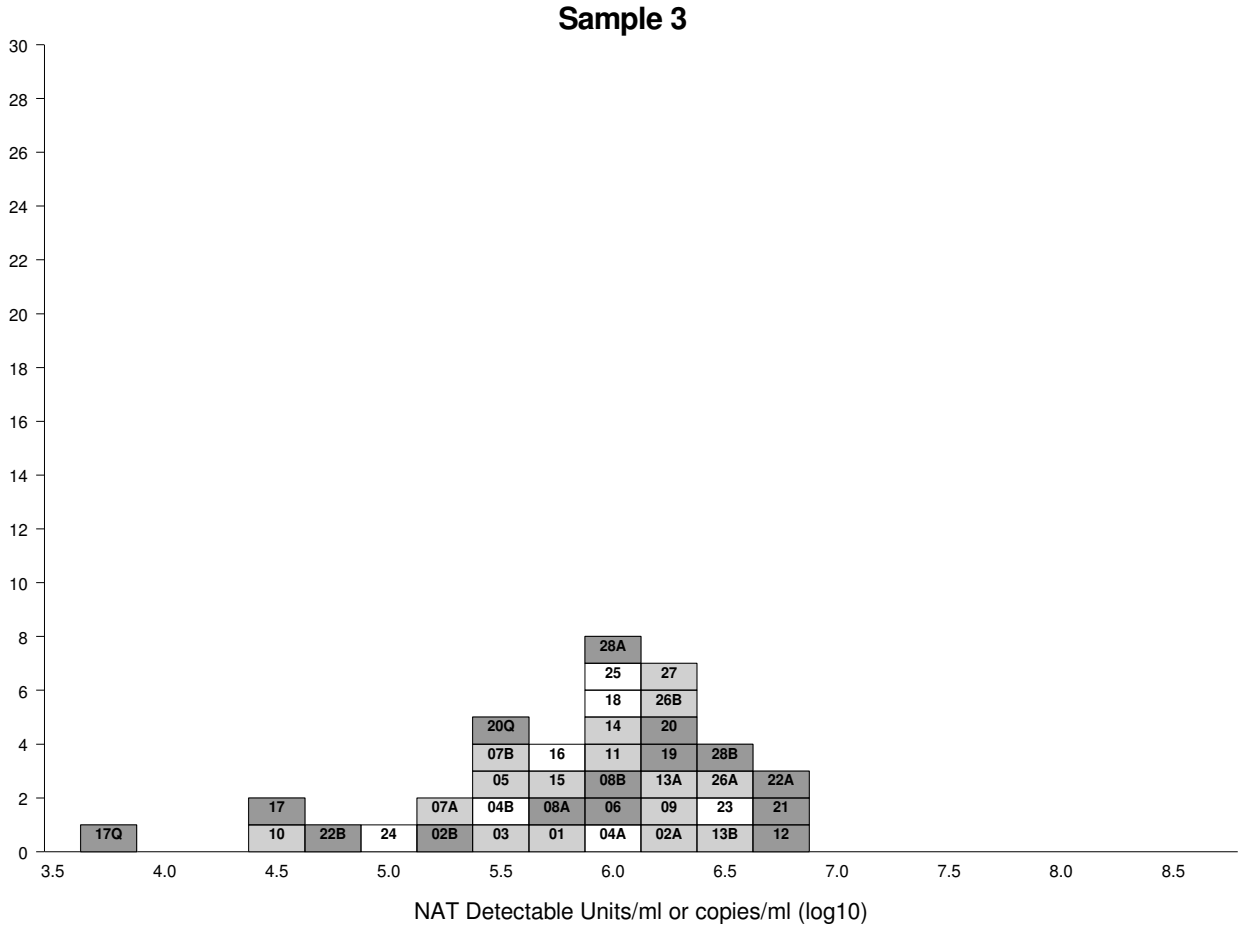
**a**


Sample 1
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
NAT Detectable Units/ml or copies/ml (log10)
10
22B
17Q
16
05
02B
07B
07A
01
27
24
20Q
15
12
09
06
04B
03
28A
18
11
08B
08A
26B
20
04A
02A
28B
26A
25
19
14
13A
23
22A
21
13B

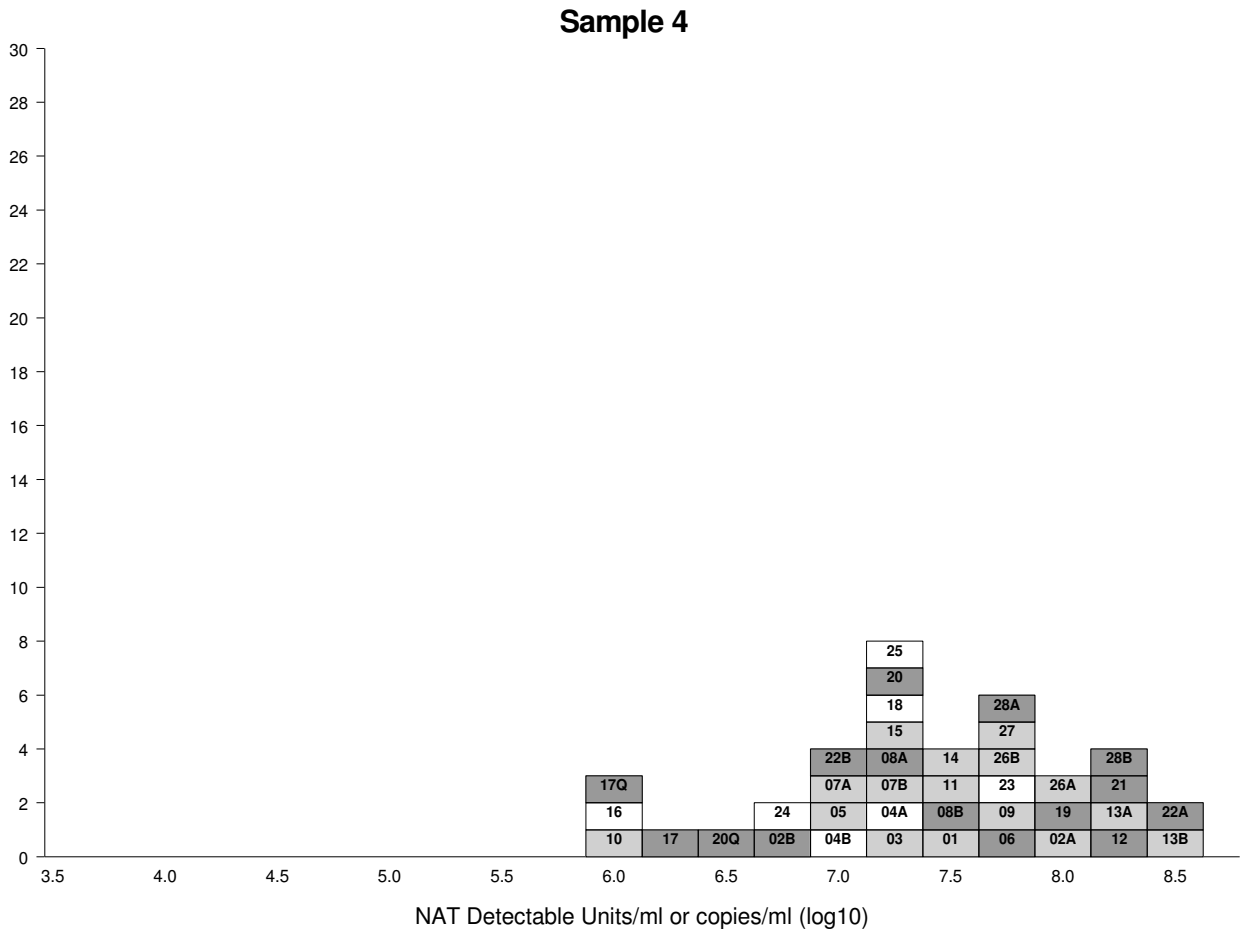
**b**


Sample 2
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
NAT Detectable Units/ml or copies/ml (log10)
10
16
22B
17Q
07B
02B
20Q
12
05
15
09
07A
06
01
28A
27
24
18
11
08A
04B
03
26B
20
17
08B
04A
28B
26A
23
19
14
13A
02A
25
22A
21
13B

**c**

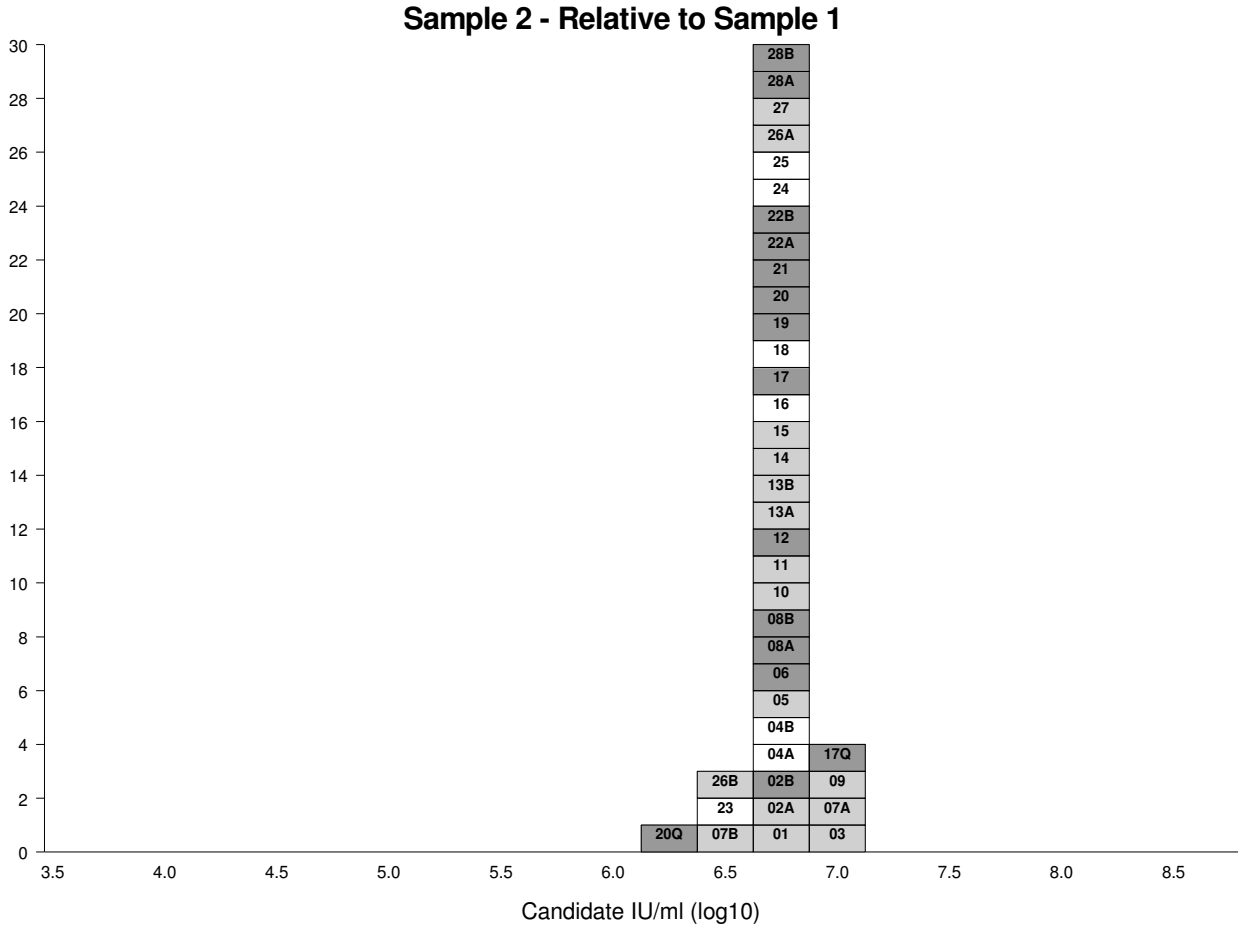

Sample 3
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
17Q
17
10
22B
24
07A
02B
20Q
07B
05
04B
03
16
15
08A
01
28A
25
18
14
11
08B
06
04A
27
26B
20
19
13A
09
02A
28B
26A
23
13B
22A
21
12
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
NAT Detectable Units/ml or copies/ml (log10)

**d**

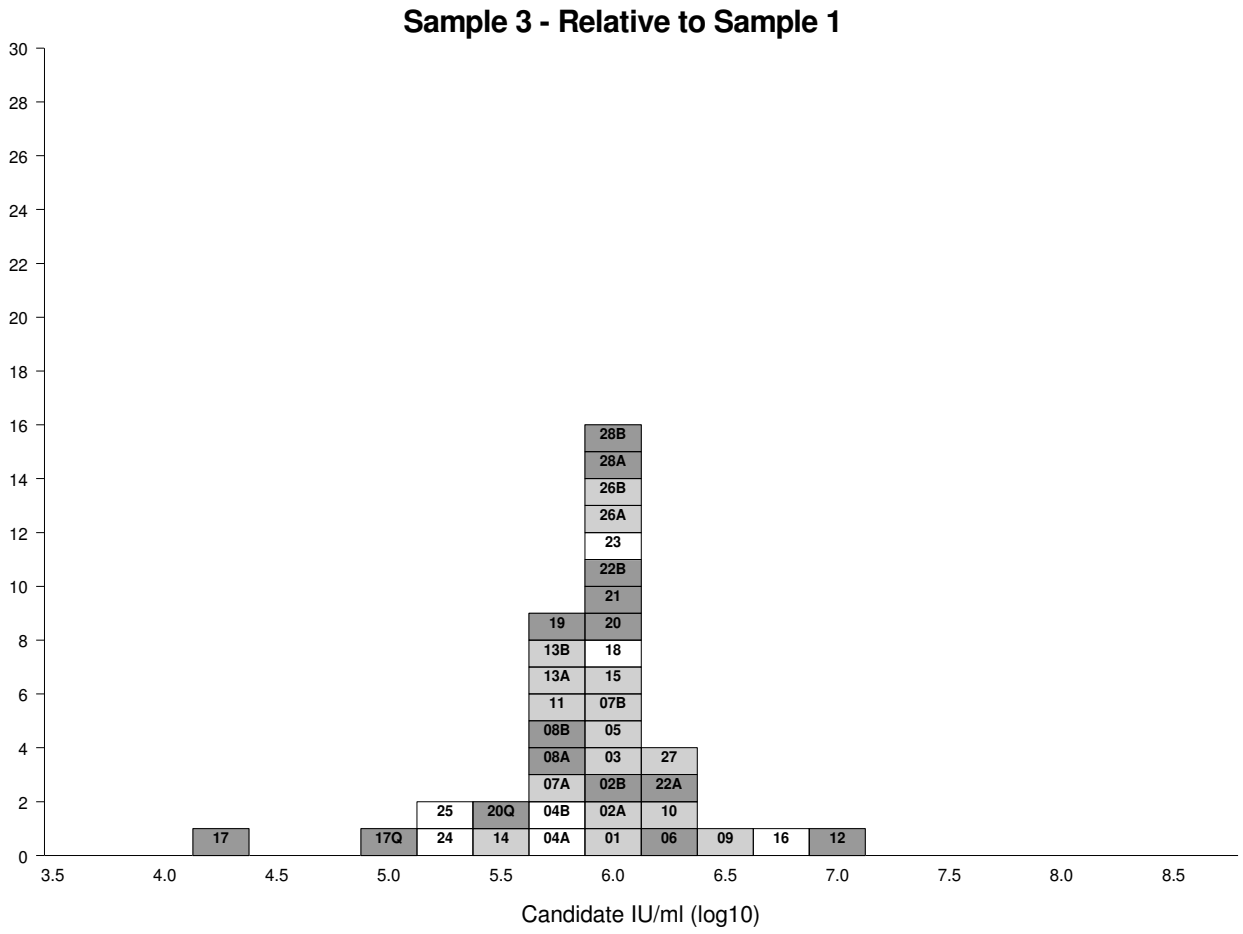

Sample 4
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
NAT Detectable Units/ml or copies/ml (log10)
17Q
16
10
17
20Q
24
02B
22B
07A
05
04B
25
20
18
15
08A
07B
04A
03
14
11
08B
01
28A
27
26B
23
09
06
26A
19
02A
28B
21
13A
12
22A
13B

# Figure 2

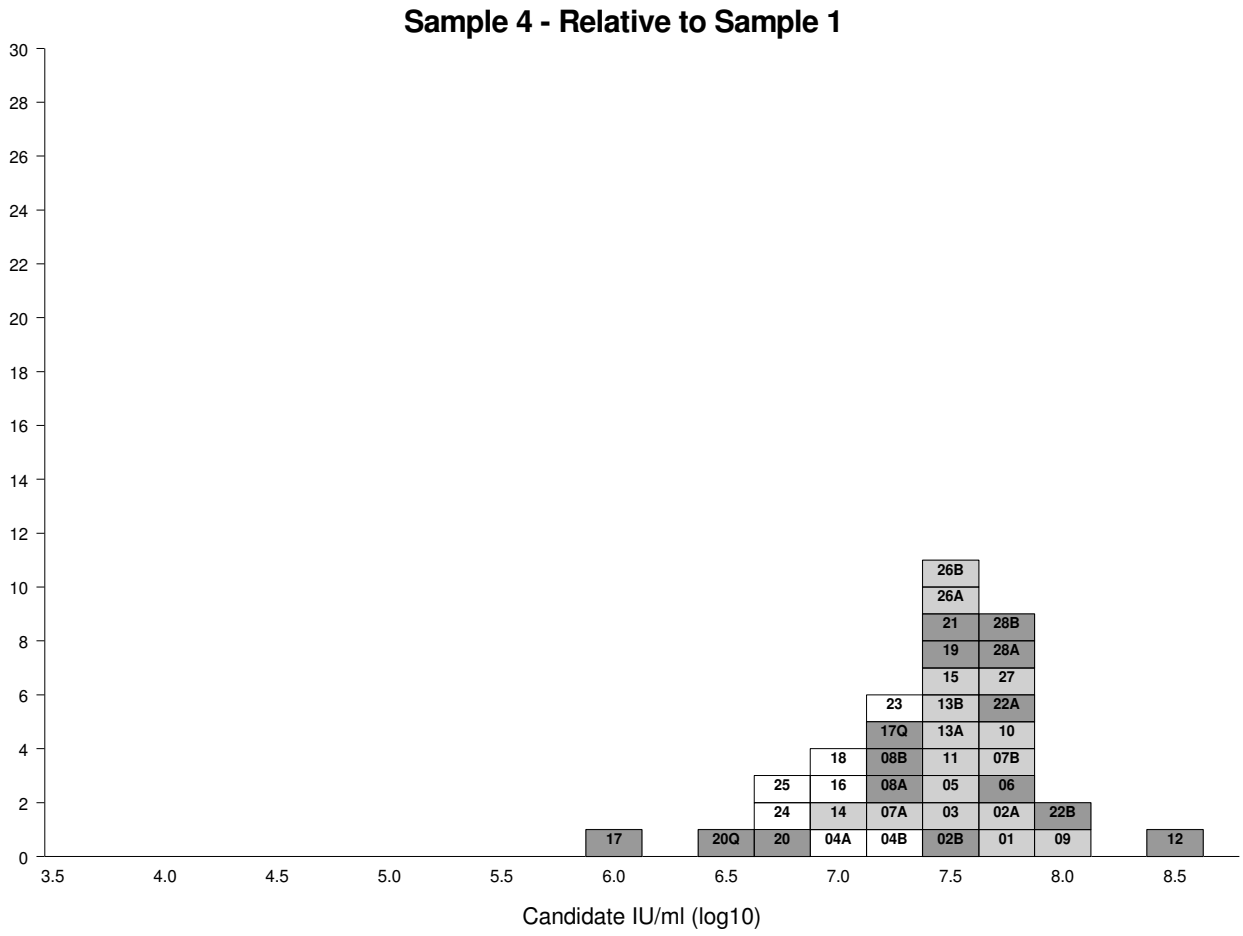
**a**


Sample 2 - Relative to Sample 1
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)
28B
28A
27
26A
25
24
22B
22A
21
20
19
18
17
16
15
14
13B
13A
12
11
10
08B
08A
06
05
04B
04A
17Q
26B
02B
09
23
02A
07A
20Q
07B
01
03

**b**

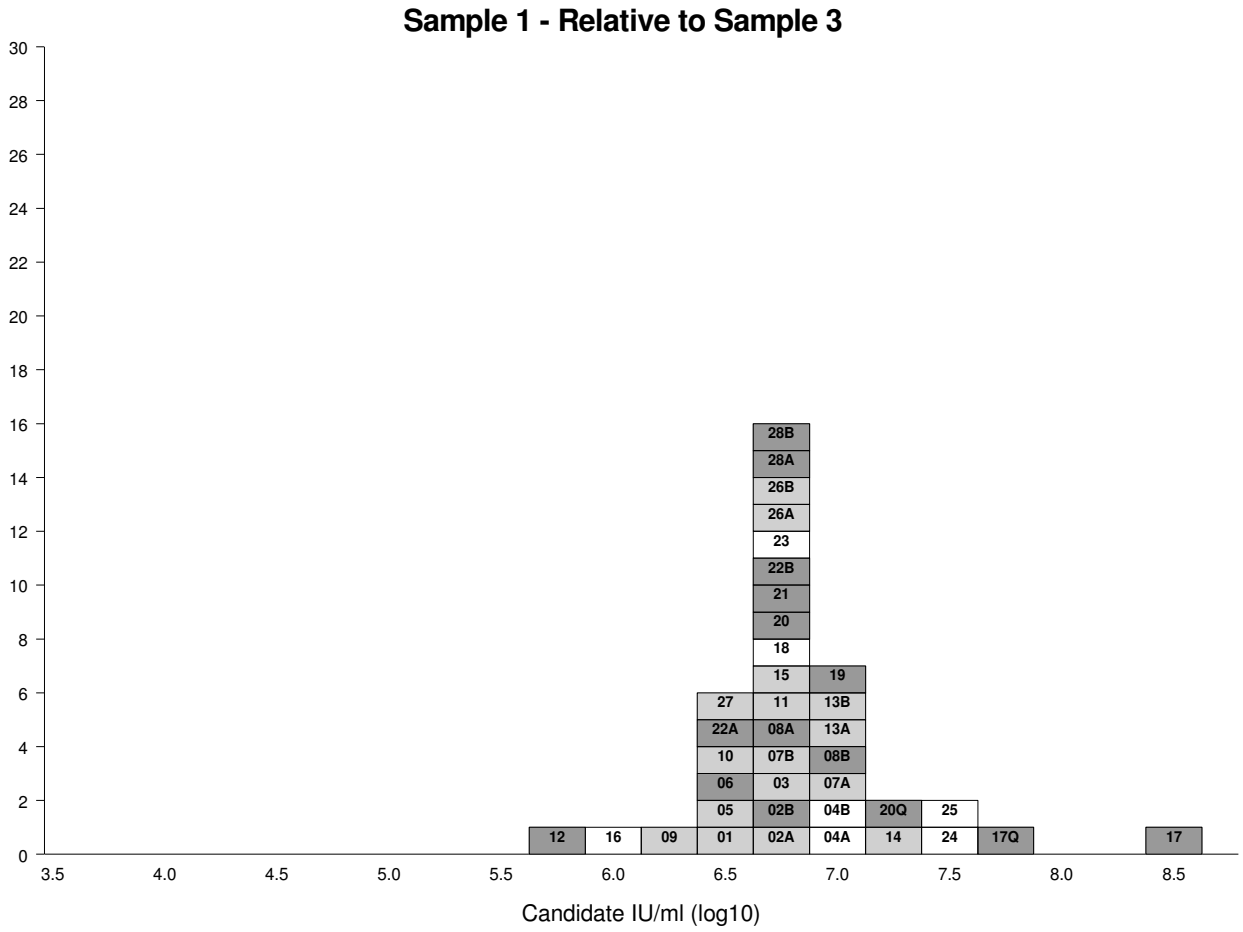

Sample 3 - Relative to Sample 1
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)
17
17Q
25
24
20Q
14
19
13B
13A
11
08B
08A
07A
04B
04A
28B
28A
26B
26A
23
22B
21
20
18
15
07B
05
03
02B
02A
01
27
22A
10
06
09
16
12

**c**

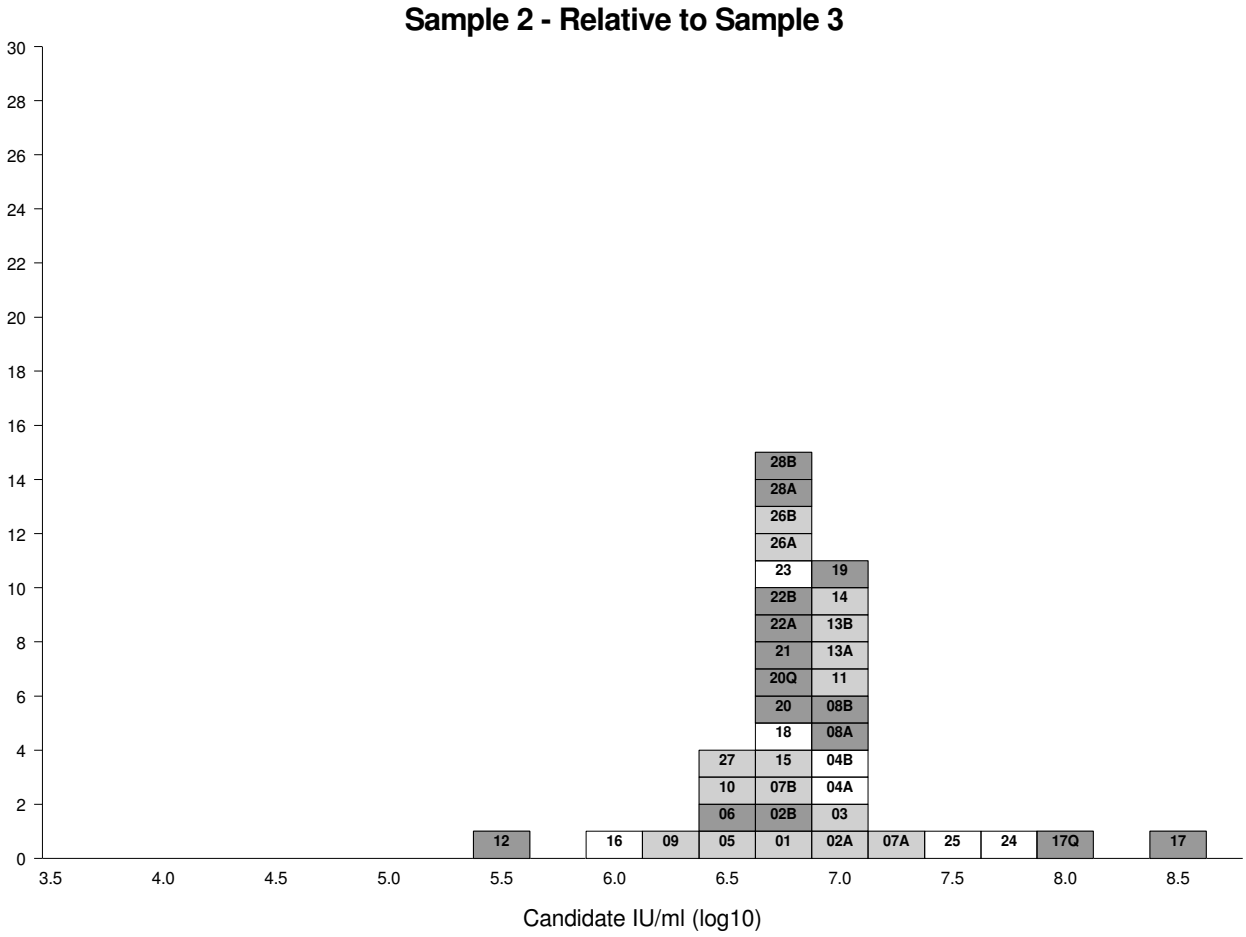

Sample 4 - Relative to Sample 1
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)
26B
26A
21
28B
19
28A
15
27
23
13B
22A
17Q
13A
10
18
08B
11
07B
25
16
08A
05
06
24
14
07A
03
02A
22B
17
20Q
20
04A
04B
02B
01
09
12

# Figure 3

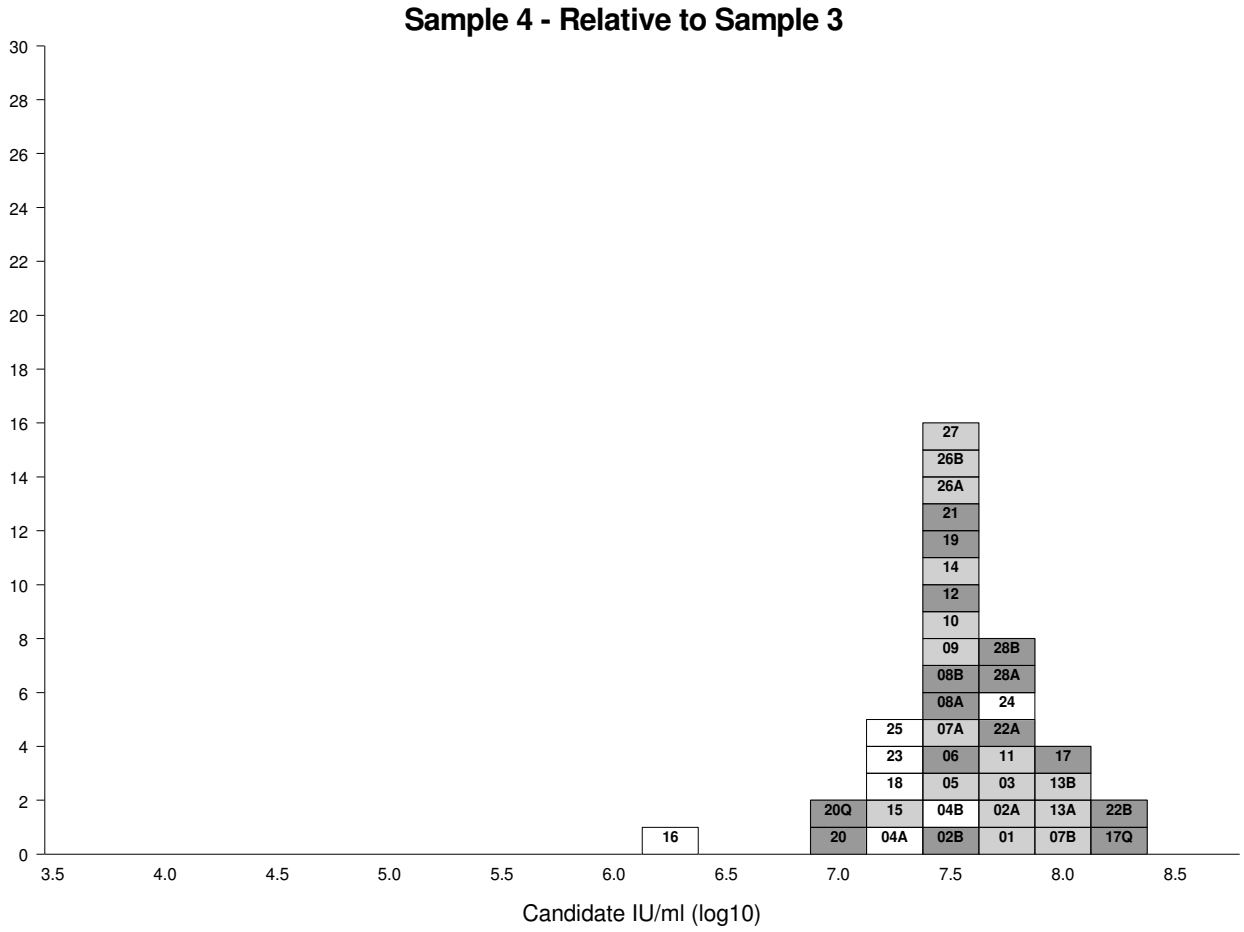
**a**


Sample 1 - Relative to Sample 3
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)
28B
28A
26B
26A
23
22B
21
20
18
15
19
27
11
13B
22A
08A
13A
10
07B
08B
06
03
07A
05
02B
04B
20Q
25
12
16
09
01
02A
04A
14
24
17Q
17

**b**


Sample 2 - Relative to Sample 3
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
28B
28A
26B
26A
23
22B
22A
21
20Q
20
18
27
10
06
19
14
13B
13A
11
08B
08A
15
07B
02B
04B
04A
03
12
16
09
05
01
02A
07A
25
24
17Q
17
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)

**c**


Sample 4 - Relative to Sample 3
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
Candidate IU/ml (log10)
16
20Q
20
25
23
18
15
04A
27
26B
26A
21
19
14
12
10
09
08B
08A
07A
06
05
04B
02B
28B
28A
24
22A
11
03
02A
01
17
13B
13A
07B
22B
17Q

# 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 |
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| Dr. Jutta Preiksaitis<br>Dr. Xiao-Li Pang | Provincial Laboratory for Public Health / University of Alberta, Alberta | Canada |
| Côme Barranger | Argene SA, Verniolle | France |
| Dr. Patrice Morand | Laboratoire de Virologie, CHU Grenoble | France |
| Dr. Céline Bressollette-Bodin | Virology Laboratory, Nantes University Hospital, Nantes | France |
| Dr. Thomas Grewing<br>Stephan Charpian | QIAGEN Hamburg GmbH, Hamburg | Germany |
| Dr. Pantelis Constantoulakis | Locus Medicus SA, Athens | Greece |
| Prof. Dennis Lo<br>Dr. Allen Chan | Department of Chemical Pathology, The Chinese University of Hong Kong | Hong Kong |
| Dr. Maria R Capobianchi<br>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. Hiroshi Kimura | Dept. of Virology, Nagoya Graduate School of Medicine, Nagoya | Japan |
| Prof. Fredrik Müller | Department of Microbiology, Oslo University Hospital, Rikshospitalet, | Norway |
| Dr. Diana Hardie | Diagnostic Virology Laboratory Groote Schuur Hospital, Cape Town | South Africa |
| Dr. Won Seog Kim | Advanced Genetic Testing Center, Samsung Medical Center, Seoul | South Korea |
| Inger Bokliden<br>Karin Bergsten | Cepheid AB, Bromma | Sweden |
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| Dr. Eithne MacMahon<br>Dr. Jon Bible | GSTS Pathology, London | UK |
| 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 |
| Jill Crouch<br>Dr. John Greg Howe | Molecular Diagnostics Laboratory, Yale New Haven Hospital, New Haven | USA |
| Dr. Angela Caliendo | Emory University Hospital, Altanta | USA |
| Dr. Margaret Gulley | University of North Carolina, Chapel Hill | USA |
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# Appendix 2

## Study protocol



**Collaborative study to evaluate the candidate 1st WHO International Standard for Epstein-Barr virus (EBV) 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 Epstein-Barr virus (EBV) 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 EBV.

### Background

Viral load measurements using NAT-based assays are important in the diagnosis and management of EBV-associated diseases. 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 EBV 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 a cell-free live virus preparation of the prototype EBV strain B95-8 [2], at a concentration of approximately $1x10^7$ copies/mL, and freeze-dried to ensure long-term stability. Given the wide range of samples routinely tested for EBV, the candidate standard is formulated in a universal buffer comprising 10mM Tris-HCl (pH7.4), human serum albumin and trehalose, 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 EBV, will be assessed in a worldwide collaborative study, involving participants performing a range of NAT-based assays. The freeze-dried candidate EBV B95-8 preparation will be evaluated alongside the unprocessed liquid bulk, and cell suspensions of Namalwa and Raji cells containing $1x10^6$ cells in 1 mL of phosphate buffered saline.

The study samples have been prepared from cell lines sourced from the European Collection of Cell Cultures (ECACC), 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 EBV 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, 3 and 4 comprise 1 mL frozen liquid preparations, and should be thawed fully and vortexed briefly before use. These samples must be extracted prior to amplification.

**CAUTION:** These preparations are not for administration to humans. Study samples 1 and 2, contain infectious EBV 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 samples 3 and 4 comprise human cells, and it is recommended that these are also handled at ACDP Category 2 containment. 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 EBV NAT assay, on four separate occasions.

Each sample 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.

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 each sample 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 1-4 must be extracted prior to amplification. *[Samples 1 and 2 and are estimated to contain approximately $1x10^7$ EBV copies/mL. Sample 3 is estimated to contain approximately $2x10^6$ EBV copies/mL. Sample 4 is estimated to contain approximately $5x10^7$ EBV copies/mL]*

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 each sample 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 1-4 must be extracted prior to amplification. *[Samples 1 and 2 and are estimated to contain approximately $1x10^7$ EBV copies/mL. Sample 3 is estimated to contain approximately $2x10^6$ EBV copies/mL. Sample 4 is estimated to contain approximately $5x10^7$ EBV copies/mL]*

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 preferably before the **28th May 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. Preiksaitis JK, Pang XL, Fox JD, Fenton JM, Caliendo AM, Miller GG; American Society of Transplantation Infectious Diseases Community of Practice. Interlaboratory comparison of epstein-barr virus viral load assays. Am J Transplant. 2009;9:269-79.

2. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, et al. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature. 1984;310:207-11.

# Appendix 3

## Proposed instructions for use

**WHO International Standard**
**1st WHO International Standard for Epstein-Barr Virus for Nucleic Acid Amplification Techniques**
**NIBSC code: 09/260**
**Instructions for use**
**(Version 1.00, Dated )**

### 1. INTENDED USE

The 1st WHO International Standard for Epstein-Barr virus (EBV), NIBSC code 09/260, is intended to be used in the standardization of nucleic acid amplification technique (NAT)-based assays for EBV. The reference comprises a whole virus preparation of the EBV B95-8 strain [1,2], formulated in a universal buffer comprising Tris-HCl, human serum albumin (HSA) and trehalose. The material has been lyophilized in 1 mL aliquots and stored at -20 °C. The material was evaluated in a worldwide collaborative study involving 28 laboratories performing a range of NAT-based assays for EBV [3].

### 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 $5x10^8$ 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 lyophilized equivalent of 1 mL of EBV in 10 mM Tris-HAS buffer (pH7.4), 0.5% HSA, and 0.1% trehalose.

### 5. STORAGE

Vials of lyophilized 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 deionized, 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 $5x10^8$ IU/mL.
The International Standard should be used to calibrate 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. Once reconstituted, the International Standard should be diluted in the matrix appropriate to the material being calibrated, and should be extracted prior to EBV DNA measurement.

### 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. The stability of 09/260 after reconstitution has not been specifically determined. Therefore, it is recommended that the standard is for single use only.

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

### 9. REFERENCES

1. Baer R, Bankier AT, Biggin MD, Deininger PL, Farrell PJ, Gibson TJ, Hatfull G, Hudson GS, Satchwell SC, Séguin C, et al. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature. 1984;310:207-11.
2. NCBI reference sequence: NC_007605.1.
http://www.ncbi.nlm.nih.gov/nuccore/NC_007605.1.
3. Fryer JF, Heath AB, Wilkinson DE, Minor PD and the collaborative study group. Collaborative study to evaluate the proposed 1st WHO International Standard for Epstein-Barr virus (EBV) for nucleic acid amplification (NAT)-based assays. WHO ECBS Report 2011; WHO/BS/11.XXXX.

### 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.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: Lyophilised powder | | Corrosive: | No |
| Stable: | Yes | Oxidising: | No |
| Hygroscopic: | No | Irritant: | No |
| Flammable: | No | Handling:See caution, Section 2 | |
| Other (specify): | Contains infectious Epstein-Barr virus and human serum albumin | | |

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A World Health Organization Laboratory for Biological Standards

| 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**

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<br>* 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 g |
| **Toxicity Statement:** Non-toxic |
| **Veterinary certificate or other statement** if applicable.<br>**Attached:** No |