Calibration of native and inactivated HBV, HCV and HIV-1 standards

Classification	Confidential
Version 1.0 valid from	30 September 2016
Version 2.0 valid from	19 December 2017
Version 3.0 valid from	25 March 2020
Version 4.0 valid from	22 October 2021
Version 5.0 valid from	06 May 2022

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1. OBJECTIVE

This validation report describes the calibration of the native VQC-Sanquin and inactivated BioQ standards (and standard dilutions in ViraQ run controls) for HBV-DNA, HCV-RNA and HIV-1-RNA in:

Nucleic acid copies (SI units or mol/L)
International Units (IUs)

and the traceability chain to:

Purified nucleic acid calibrator molecules in branched DNA (bDNA) assay
WHO International Standards.
Chimpanzee infectivity plasmas

For this purpose we summarize the calibration studies for quantification of:

Native Eurohep and VQC-Sanquin liquid frozen plasma standards
Lyophilized WHO International Standards (IS)
Japanese chimpanzee plasmas of known infectivity titer
Inactivated BioQ standards used for preparation of ViraQ run controls

The standards above have been calibrated against each other in WHO collaborative studies, in VQC proficiency studies and in calibration experiments in the branched (b) DNA assay and other NAT methods performed by VQC-Sanquin and later in studies coordinated by BQC. In the calibration studies summarized in this report we estimated the amount of nucleic acid copies per IU and – in approximation – per 50% chimpanzee minimum infectious dose (CID_{50}).

2. INTRODUCTION AND METHODS

Before interpreting the calibration data in this report it is important to give some background on the history in standardization of nucleic acid amplification technology (NAT) for qualitative and quantitative detection of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV). The next chapters are intended to give more insight in our contribution to NAT standardization, the methodology used and the relevance of calibrating standards in absolute virion numbers (or nucleic acid copies) for understanding blood safety.

History of standardization of viral NAT in nucleic acid copies

Since the early 1990s the initiators of Viral Quality Control (VQC) unit in Sanquin (now BioQControl) have tried to calibrate viral standards in absolute virion numbers corresponding to nucleic acid copies or genome equivalents (qeq) per mL. The use of copies/mL or geq/mL for standardization of NAT for blood born viruses was first proposed by Mickey Urdea (Chiron Corporation, CA, USA, later Bayer) who had developed the branched DNA assays for quantification HBV-DNA, HCV-RNA and HIV-1 RNA. The in vitro HCV and HIV-1 RNA and HBV-DNA plasmid standards used for calibration of the bDNA assay have been thoroughly quantified in molecules (mol/L) by three different physico-chemical methods based on isotopic tracer, phosphate analysis against the NIST standard and extinction at 260 and 280 nm[1]. Because of the potential traceability to SI units (mol/L) the bDNA assay has been proposed to be used as an international reference measurement method following the metrological principles described in ISO17511, but after discussion with experts in a number of WHO consultation meetings it was decided that this concept was not feasible for biological WHO standards[2]. Nevertheless, the bDNA 3.0 assay was chosen by BioQControl to quantify the original Eurohep and Sanquin-VQC standards in RNA and DNA copy numbers (or genome equivalents).The liquid frozen Eurohep and VQC-Sanquin standards were kept at -80 ºC to ensure stability (VR4058) and used as standards in the first international (Eurohep and VQC) proficiency studies organized by University of Giessen, Sanquin and DDL from 1992 until 2004[3,4,5,6,7]. Since these standards have proved to be stable for more than two decades when stored at below 65 ºC they are still in use for validation of NAT methods[8].

VQC-Sanquin candidate standards in WHO collaborative studies

Large batches of gravimetrically recorded dilutions of the VQC-Sanquin standards (that fell well in the dynamic range of viral load assays) were proposed as candidate international standards and were included in the first WHO collaborative studies conducted in the late 1990s[9,10,11]. It was decided by the National Institute for Biological Standards and Control (NIBSC) and the WHO Expert Committee for Biological Standardization (ECBS) that lyophilized materials prepared by NIBSC (and not the liquid frozen VQC-Sanquin standards) became the WHO International Standards (IS). Instead of quantitative values close to viral genome copy numbers in the bDNA assay an arbitrary unitage in International Units (IU) was assigned to the International Standard. Therefore conversion factors from IU to genome copies are different for HBV-DNA, HCV-RNA, and HIV-1 RNA as is shown in the calibration studies summarized in this report.

Use of WHO standards for calibration of secondary standards

The WHO standards are intended to be used as primary reference materials for calibration of secondary standards in IUs. However, calibration of secondary standards against the WHO standard is not a trivial exercise. Well calibrated secondary standards are not widely available and if provided often not trusted by the scientific community. Therefore most laboratories use the WHO standards directly for validation of NAT methods. As a consequence the current batch size of WHO standards for viral NAT methods (1000 to 2000 vials) is exhausted more rapidly than those of other analytes. Two decades after the establishment of the first batches of lyophilised WHO standards NIBSC (Pottersbar, UK) is now distributing the 5th WHO standard for HCV, the 3rd for HBV and the 4th for HIV-1[12,13,14]. There is a serious concern that the continuity of the IU for HCV cannot longer be guaranteed because of stability and calibration issues with the 3rd and 4th HCV 06/100 and 06/102 International Standards[15,16]. The WHO does not keep a traceability chain towards the assigned value of the first International Standard, but calibrates the new standard against the preceding one in a collaborative study. The uncertainty in calibration is not reported and denied when the new average potency value is adopted. Recently the correct calibration of the International replacement standard for Parvo-B19-DNA has been subject of debate since it may have been affected by sequence variation between the standards[17]. Hence a drift in IU values assigned to subsequent WHO replacement standards cannot be excluded. The liquid frozen VQC-Sanquin standards calibrated against the first batches of WHO standard (data summarized in this report) are kept below -65 ^{o}C and are known to be stable during more than two decades (VR4058). Therefore these could serve as secondary standards for validation of IVDs. So far, the status of the WHO, and legal requirements have forced the use of the International Standards for IVD product evaluation and registration. As a consequence IVD product specifications such as lower limits of detection (LODs) may vary depending on which versions of the WHO standard have been used for the claims in the package inserts.

Value of calibration in nucleic acid copy numbers in blood safety research

Apart from the uncertainty in the continuity of the IU with WHO replacement standards there is a more fundamental reason why calibration in genome copies is necessary: one genome copy is equivalent to one potentially infectious HBV and HCV particle and two RNA copies are present in one potentially infectious HIV virion. The risk of virus transmission by blood donations in the early window period ramp-up phase starts when one infectious virion is present in the plasma of a transfused blood component. This risk is determined by the absolute number of virions in the blood component and the probability that one virion will cause infection (which is related to the 50% minimum infectious dose (MID50)). The transfusion-transmission risk in NAT screened blood is further dependent on the probability that the virus concentration in the blood donation would not be detected by the NAT screening assay employed. Weusten et al[18] used Poisson distribution statistics to develop a mathematical model for calculating the transmission risk of a NAT screened window period donation to cause infection in a recipient. This is expressed in risk day equivalents and determined by:

- the amount of plasma in the transfused component
- the estimated infectivity of the virus (expressed as MID50 or alternatively as the probability that one virion can cause infection)
- the 95% and 50% LOD of the NAT assay and the minipool size used,
- the doubling time of the virus in the window period ramp-up phase,

For this risk model it is obvious that both the 95% and 50% LODs of the NAT system and the MID50 are expressed in the same RNA or DNA copy (or virion) numbers. For this purpose HBV and HCV chimpanzee plasmas of known infectivity titres[19,20], kindly provided by Prof Yoshizawa and Tanaka (Hiroshima University, Japan) were calibrated against the Sanquin-VQC standards using multiple replicate bDNA assays. The use of the VQC-Sanquin and Japanese infectivity standards in validation studies of NAT blood screening assays has been fundamental for understanding the residual viral transmission risk and efficacy of NAT in reducing this risk[21-26] .

Inactivated viral standards in run controls

Finally this report summarizes the calibration experiments of the inactivated BioQ standards used for manufacturing of the ViraQ Check and Trend Controls of 125, 75 and 25 copies/mL that are in use for external quality control of the Ultrio, Ultrio Plus and Ultrio Elite assays as well as the TaqScreen 2.0 and cobas MPX 6800/8800 assays. The preparation process and safety of these inactivated standards used in the ViraQ run controls is described elsewhere (CE4006) as well as the particle integrity and stability of the viruses in the untreated and inactivated standards (VR4058). The traceability chain and uncertainty of the nucleic acid copy numbers assigned to the BioQ standards and those to the VQC-Sanquin standard using multiple replicate bDNA testing as the reference measurement method is described in this report.

Verification of calibration of HBV, HCV and HIV standards in copies/mL in analytical sensitivity studies

At the end of this report the correct cross calibration of viral standards in copies/mL and IU/mL by multiple replicate bDNA tests and by WHO collaborative studies[27,28] is checked in analytical sensitivity studies[8,29-33] that have been conducted since 2005 in different versions of the NAT blood screening assays, i.e the Ultrio, Ultrio Plus and Ultrio Elite assays manufactured by Hologic and distributed by Grifols Diagnostic Solutions and the TaqScreen 1.0, 2.0 and cobas MPX 6800/8800 assays made available by Roche Molecular Systems. In these studies dilution series of the WHO-standards as well as native VQC-Sanquin- and inactivated BQC-standards were tested. For cross-calibration the proportion of reactive NAT results on the standard dilutions that were found by different laboratories in one assay version were combined and the relative potencies (and 95% confidence intervals (CI)) between different standards were calculated. If the confidence limits of the potency of a secondary standard relative to the primary standard overlaps the value 1.0 , there is not a significant difference in the copy numbers, assigned to the two standards. With the interpretation of the probit analysis data one need to bear in mind that there is a certain contribution of laboratory variation to the potency results (such as the use of different NAT reagent batches over time) and the parallel line probit analysis method itself usually generates wide confidence limits.

In addition we checked the calibration of viral standards in copies/mL by limiting dilution analysis. According to Poisson distribution statistics the NAT system detects one viral nucleic acid copy per assay at the 63% lower limit of detection (LOD). Since the Ultrio TMA versions use 0.5 mL plasma as input volume and the TaqScreen PCR assays 1.0 mL (of which an estimated 850 ul seems to be used in the amplification reaction) we expect a 63% LOD at 2 copies/mL for the first and down to 1 copy/mL for the latter method if 100% efficiency of NAT reactivity would be reached. However, the NAT efficiency is expected to be less than 100% because nucleic acid extraction from the viral particles in plasma is likely to be incomplete. In this report we compared the theoretical NAT detection efficiencies (as a percentage (and 95% confidence interval (CI)) for HBV, HCV and HIV for different NAT methods in order to check the calibration of our viral standards in nucleic acid copies. Of course we cannot claim that the copy numbers assigned to the VQC-Sanquin and BQC standards are equivalent to the true amount of (either defective or potentially infectious) virions. However, when interpreting the data obtained over two decades presented in this report we believe the nucleic acid copy numbers assigned to our standards are close to the true amount of virions and therefore have been rightfully used in several publications to estimate residual transfusion-transmission risk of NAT screened blood donations[18,21-26]

Metrologic Traceability chain, ISO 17511

The IVD Directive refers to the ISO 17511:2003 standard for traceability of standards for IVDs and provides guidance on metrological levels (table 1)34.

Table 1. Metrological levels illustrated with some examples

Level	Traceable to SI unit	Int. reference measurement method	Calibrator material	example
1	Yes	yes	yes	Na
2	No	yes	yes	HbA1c
3	No	yes	no	Factor IX
4	No	no	yes	WHO IS
5	No	no	no	enterovirus

The WHO Expert Committee for Biological Standardization (ECBS), which enforces the International Standards for NAT systems, did not accept the bDNA 3.0 assay as the international reference measurement method, nor the bDNA calibrator molecules as primary reference standards traceable to SI units. WHO international standards by definition are situated at the fourth metrological level. There are limitations for accurate and precise measurement of the viral nucleic acid concentration in mol/L (SI units) in blood samples by quantitative NAT methods, mainly because the nucleic acid extraction efficiency is not controlled. Nevertheless the initiators of BQC decided to use multiple replicate bDNA test results (obtained in experiments performed over several years) to assign a value in copies/mL to the VQC-Sanquin standards. If the bDNA detection efficiency of the purified calibrator molecules and viral nucleic acid in plasma is known a calibration system at the first metrological level would become feasible. In our studies the accurate calibration of BQC standards in copies/mL is verified by limiting dilution testing in the most sensitive NAT blood screening methods available (Hologic, Grifols Ultrio Plus and Ultrio Elite and Roche cobas MPX assays). Similarly, the accurate quantification of the VQC-Sanquin and BioQ inactivated standards in copy numbers could be checked by digital PCR, which may be validated as a future international reference measurement method for quantification of viral standards in copies/mL, but also in this method the uncertainty in the nucleic acid extraction efficiency remains present.

We aim to reach metrological level 2 (table 1) for our native standards by adopting the bDNA assay as the reference method and using a variety of NAT methods and calculations to confirm the true value of nucleic acid copies in the VQC-Sanquin and BQC standards.
Again, it must be emphasized that there are limitations to apply ISO guide 17511 to WHO replacement standards and secondary NAT standards . Since the WHO International Laboratories do not accept any NAT method as international reference measurement method the metrological principles described in ISO guide 17511 are not followed for WHO biological standards. An arbitrary unitage is assigned to the International Standard and one International Unit is defined as a fraction of lyophilised material in the standard vials. The replacement international standards are calibrated against the previous IS in multi-method WHO collaborative studies and the calibration results are dependent on the NAT methods applied by the participating laboratories. With the replacement standards a certain drift in IU values is therefore inevitable. In the WHO collaborative studies no uncertainty is calculated for the IU values assigned to the replacement standard. As a consequence the IU assigned to the previous International Standard ceases to exist and is replaced by the IU value assigned to the new standard. Since the native VQC-Sanquin standards have been calibrated against the first and 2nd established WHO standards it cannot be guaranteed that the measured concentrations in IU/mL are still valid for the currently distributed 3rd, 4th or 5th WHO standards. However, this is not important because the native VQC-Sanquin and inactivated BioQ standards are calibrated independently from the WHO standards in nucleic acid copies/mL (next chapters in this report) and these values have been successfully used in several publications for validation of NAT methods and for estimating residual transfusion-transmission risk[8,18,21-26,29-33].

Metrologic traceability chain for viral standards and ViraQ™ run controls

In this report the VQC-Sanquin standards for HBV-DNA genotype A, HCV-RNA genotype 1, HIV-1 RNA subtype B are positioned as primary reference materials for calibration of the secondary inactivated BioQControl standards in copies/mL. Likewise the first versions of WHO International Standards were used as the primary reference standards for calibration of the secondary VQC-Sanquin standards in IU/mL (table 2). Because it cannot be excluded that there has been a drift in IU values with the WHO replacement standards over the last two decades we cannot guarantee that the calibration results shown in this report are reproducible with the currently distributed 3rd to 5th version of the WHO international standards.
Since the inactivated BioQ standards were not directly calibrated against the WHO International Standards they could be considered tertiary standards in the traceability chain to the first versions of WHO standards (as are the third batches of WHO standards). However for the calibration in copies/mL in the bDNA 3.0 assay the inactivated BioQ standards could be considered as secondary standards. The ViraQ run controls are gravimetrically recorded dilutions of the inactivated BioQ standards and thus can also be considered secondary standards when calibrated in copies/mL and tertiary standards when calibrated in IU/mL.

Table 2. Definition of primary and secondary standards in calibration studies in this report

primary reference standard	secondary standard	Reference method	Unit
NIST standards	Purified calibrator molecules used for calibration of bDNA assay	Phosphate analysis, isotopic tracer, E260 and E280 extinction	SI (mol/L)
bDNA calibrator molecules	Liquid deep frozen native VQC-Sanquin plasma standards	Multiple replicate bDNA assays performed over several years	Average values in copies/mL of multiple bDNA 3.0 assays
VQC-Sanquin standards	Inactivated BQC standards (and ViraQ run controls)	Multiple parallel replicate bDNA assays performed on both standards in the same test runs	copies/mL assigned to VQC-Sanquin standard
First established lyophilized WHO standard batches	VQC-Sanquin standards	Multiple parallel replicate bDNA assays performed on both standards in the same test runs or reported values in WHO collaborative studies	IU/mL values assigned to first lots of WHO standards
VQC-Sanquin standards	Chimpanzee challenge plasmas	Multiple parallel replicate bDNA assays performed on both plasmas in the same test runs	copies/mL assigned to VQC-Sanquin standard
Chimpanzee challenge plasmas		Infectivity by inoculation of 10-fold dilutions in chimpanzees and human liver chimera mice	CID_{50}/mL or MID_{50}/mL

Figure 1a, 1b and 1c illustrate the traceability chain of the HBV, HCV and HIV-1 standards described in this report. It depends on the assigned unitage whether they can be considered primary or secondary standards (table 2). For convenience the figures also present the key data of the cross calibration studies that are presented in the next chapters of this report.

Figure 1. Traceability chains of native VQC-Sanquin and inactivated BQC standards in ViraQ run controls

Figure 1a. Traceability chain HBV-DNA standards and calibration data

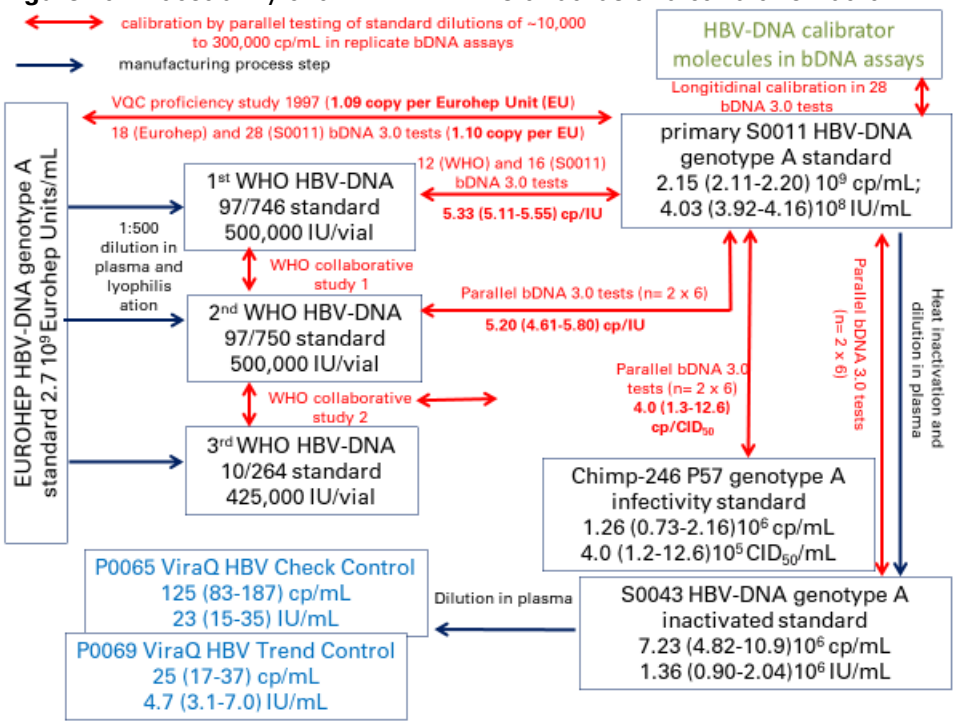


Figure 1b Traceability chain HCV-RNA standards and calibration data

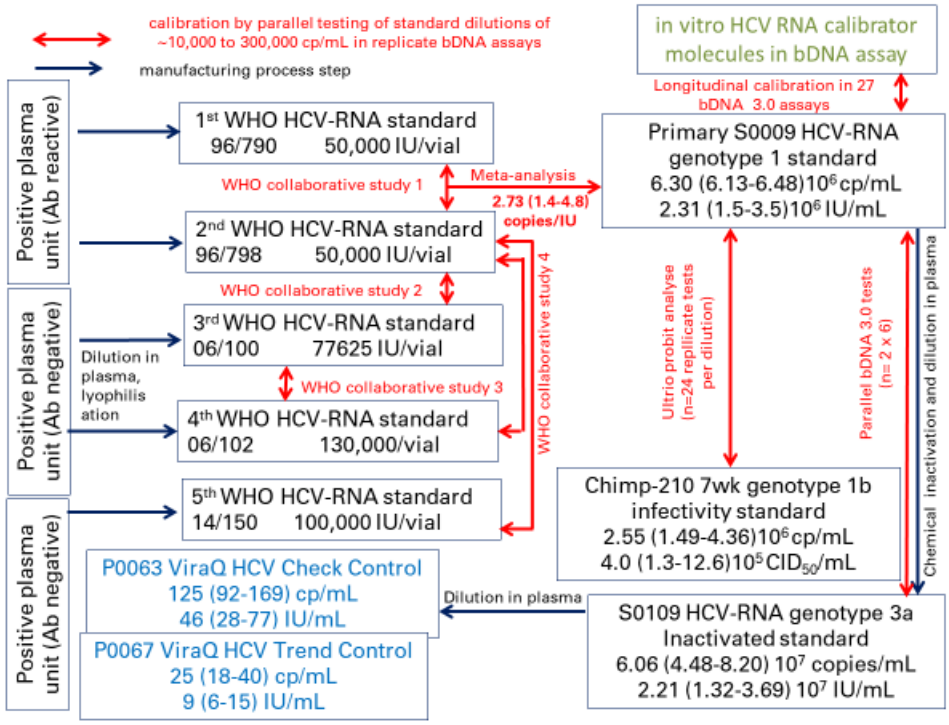
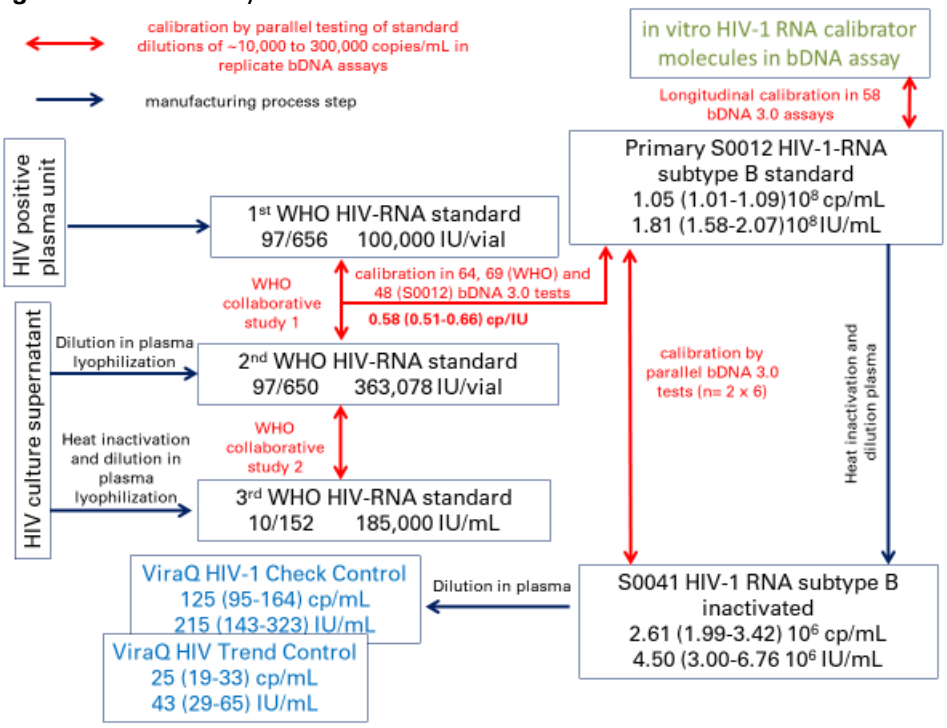


Figure 1c Traceability chain HIV-1 RNA standards and calibration data



3. QUANTIFICATION OF VQC-SANQUIN STANDARDS IN NUCLEIC ACID COPIES

Gravimetrically recorded dilutions of VQC-Sanquin HBV-DNA, HCV-RNA and HIV-1 RNA standards have been tested in different viral load assays in the Eurohep and VQC proficiency studies from 1994 to 2004[3-7], in WHO collaborative (calibration) studies[9,10,11,27,28] and by replicate testing organized by VQC-Sanquin and BioQControl (unpublished data). Table 3 summarizes the geometric quantitative values (and 95% confidence intervals) obtained in quantitative NAT methods from different manufacturers back calculated from the viral load measured on dilutions that fell well in the dynamic range of the assays. There was generally good consensus in the quantitative values in copies/mL reported by the different assays, although in some cases values were (more than) two-fold higher, such as in the Organon Teknika NASBA (NucliSens) HIV-1 assay. These assays were calibrated against the VQA HIV-RNA standard that is calibrated in VQA copies/mL[35]. As described above we have chosen the values measured by the Siemens bDNA3.0 assay as the reference method and the concentrations in bold text in table 3 have been assigned to the primary VQC-Sanquin standards.

Table 3. Quantification of primary S0011 HBV-DNA genotype A, S0009 HCV genotype 1 and S0012 HIV-1 subtype B standards (VQC-Sanquin, Amsterdam, the Netherlands) in copies/mL using different viral load assays from 1996 until 2008

HBV-DNA genotype A		**copies/mL**	**95% CI**	
Assay	N	Geomean	lower	upper
Chiron bDNA 1.0	17	3.22E+09	3.13E+09	3.32E+09
Siemens bDNA 3.0	**28**	**2.15E+09**	**2.11E+09**	**2.20E+09**
Roche Amplicor. Monitor	198	2.11E+09	2.05E+09	2.17E+09
Affigene VL	8	1.17E+09	1.96E+08	7.04E+09
Roche Taqman	8	2.38E+09	1.01E+09	5.61E+09
Artus RealArt HBV PCR Kit	9	2.17E+08	3.67E+07	1.29E+09
Digene HCS	42	1.63E+09	1.57E+09	1.69E+09
Digene HCS Ultra	4	1.30E+09	5.92E+08	2.85E+09

HCV-RNA genotype 1		**copies/mL**	**95% CI**	
Assay	N	Geomean	lower	upper
Roche Amplicor 1.0	90	4.04E+06	3.72E+06	4.39E+06
Roche COBAS Amplicor 2.0	73	4.38E+06	4.12E+06	4.65E+06
Roche MWP Amplicor 2.0	35	3.97E+06	3.51E+06	4.48E+06
Bayer bDNA 2.0	43	9.83E+06	9.41E+06	1.03E+07
Siemens bDNA 3.0	**27**	**6.30E+06**	**6.13E+06**	**6.48E+06**

HIV-RNA Group M subtype B		**copies/mL**	**95% CI**	
Assay	N	Geomean	lower	upper
Abbott LCx	18	1.91E+08	1.84E+08	1.99E+08
Chiron bDNA 1.0	13	4.71E+07	1.97E+07	1.12E+08
Bayer bDNA 2.0	57	1.09E+08	1.05E+08	1.14E+08
Siemens bDNA 3.0	**58**	**1.05E+08**	**1.01E+08**	**1.09E+08**
Organon Teknika NucliSens	119	2.41E+08	2.28E+08	2.54E+08
Organon Teknika QT-NASBA	366	3.32E+08	3.21E+08	3.43E+08
Roche Amplicor. Monitor mixed primers	63	1.53E+08	1.46E+08	1.59E+08
Roche Amplicor Monitor V1.0	437	2.25E+08	2.20E+08	2.29E+08
Roche Amplicor Monitor V1.5	316	1.36E+08	1.30E+08	1.42E+08
Roche Amplicor Monitor Ultra	142	1.24E+08	1.18E+08	1.29E+08

The assigned values in copies/mL were obtained by replicate bDNA 3.0 testing performed in studies from 2001 to 2008 and the results were comparable to the values obtained with the previous bDNA assay versions used between 1996 and 2000 (Table 4a,b and c). As can be seen in Table 4a, b and c there is some variation in average bDNA 3.0 test values over the years which is the result of variation between assay results within one run (intra-assay variation), between test runs (inter-assay variation) and bDNA reagent or calibrator lots (reagent batch variation). To reduce the impact of these variables we have chosen the geometric mean value (and 95% CI) of all longitudinally obtained bDNA 3.0 test results as the final concentration in copies/mL that has been assigned to the VQC-Sanquin standards (values in bold text in table 3 and in table 4a, b and c).

Table 4a Longitudinal calibration of the VQC-Sanquin S0011 HBV-DNA genotype A standard in bDNA assay versions

			Copies/mL	95% CI	
Test	year	n	Average	Lower	Upper
bDNA 1.0	1996	9	3.73E+09		
	1997	2	2.38E+09		
	1998	4	2.94E+09	1.46E+09	5.92E+09
	1999	2	2.69E+09		
	geomean	17	3.22E+09	3.20E+09	3.24E+09
bDNA 3.0	2001	16	2.15E+09	1.77E+09	2.62E+09
	2006	3	2.07E+09	1.45E+09	2.96E+09
	2006	3	1.40E+09	5.63E+08	3.47E+09
	2008	6	2.71E+09	2.06E+09	3.57E+09
	geomean	**28**	**2.15E+09**	**2.10E+09**	**2.20E+09**

Table 4b. Longitudinal calibration of VQC-Sanquin S0009 HCV-RNA genotype 1 standard in bDNA assay versions.

			Copies/mL	95% CI	
Test	year	n	Average	lower	upper
bDNA 1.0	1996	4	8.76E+06	raw data not available	
bDNA 2.0	1997	3	1.17E+07	3.50E+05	3.89E+08
	1998	4	6.31E+06	5.45E+06	7.30E+06
	1999	1	7.01E+06		
	2000	5	9.59E+06	3.49E+06	2.63E+07
	2000	30	1.06E+07	7.10E+06	1.58E+07
	geomean	43	9.83E+06	9.41E+06	1.03E+07
bDNA 3.0	2001	8	6.21E+06	3.97E+06	9.71E+06
	2003	6	6.43E+06	4.68E+06	8.84E+06
	2003	3	6.77E+06	1.35E+06	3.40E+07
	2004	4	8.45E+06	3.65E+06	1.96E+07
	2008	6	5.00E+06	3.83E+06	6.52E+06
	geomean	**27**	**6.30E+06**	**6.13E+06**	**6.48E+06**

Table 4c. Longitudinal calibration of VQC-Sanquin S0012 HIV-RNA subtype B standard in bDNA assay versions

			Copies/mL	95% CI	
Test	year	n	Average	Lower CI	Upper CI
bDNA 1.0	1997	13	4.71E+07		
bDNA 2.0	1999	18	1.14E+08	5.79E+07	2.25E+08
	1999	31	1.06E+08	6.95E+07	1.61E+08
	2000	8	1.13E+08	5.06E+07	2.53E+08
	geomean	57	1.09E+08	1.05E+08	1.14E+08
bDNA 3.0	2001	3	8.75E+07	3.55E+07	2.16E+08
	2002	6	1.63E+08	1.18E+08	2.25E+08
	2002	33	9.66E+07	5.71E+07	1.64E+08
	2002	2	1.66E+08		
	2003	6	8.99E+07	6.73E+07	1.20E+08
	2004	2	4.18E+07		
	2008	6	1.56E+08	1.14E+08	2.14E+08
	geomean	**58**	**1.05E+08**	**1.01E+08**	**1.09E+08**

4. CALIBRATION OF VQC-SANQUIN STANDARDS IN INTERNATIONAL UNITS

Calibration S0011 HBV-DNA standard against WHO 97/746 and 97/750 HBV standard

Cuijpers et al. (Sanquin, Amsterdam, the Netherlands) tested dilutions of 1:100, 1:1000, 1:10,000 and 1:30,000 of the S0011 VQC-Sanquin HBV genotype A standard in 4 replicates against dilutions of 1:10 and 1:100 of the 1st WHO 97/746 standard in 6 replicates in the same bDNA 3.0 test run and found HBV-DNA concentrations (95% CI) of 2.15 (1.77-2.62) x 10^9 and 5.33 (5.11-5.55) x 10^6 copies/mL respectively, which translates to a conversion factor (95%CI) of 5.33 (5.11-5.55) copies per IU (Table 5). [Note that these were not the values in copies/mL reported by the bDNA 3.0 assay but expressed to the corrected values assigned to the VQC-Sanquin standards in table 3 and 4]. More recently in 2008 we tested a 1:66667 dilution of the VQC standard (S1220) against a 1:543.2 dilution of the 2nd WHO 97/750 standard (S1231) in 6 replicates in the same bDNA 3.0 test run and found a conversion factor of 5.20 (4.61-5.80) copies per IU (table 5). A 1:10,000 dilution of the S0011 HBV genotype A standard was also tested in the WHO collaborative study reported by Saldanha et al[11] and a conversion factor of 4.12 was calculated (with wide not reported confidence limits since 1.25 logs differences in potencies between laboratories were found). [Note that for this analysis in the WHO collaborative study NAT detectable units were used as copy numbers but this can only been done if NAT efficiency would be 100%]. For further communications we adopted the conversion factor of 5.33 (5.11-5.55) copies per IU as found in the bDNA 3.0 assay on the 1st WHO 96/746 standard. Hence the undiluted S0011 VQC-Sanquin HBV-DNA genotype A standard contains 4.03 (3.92-4.16).10^8 IU/mL

Table 5. Calibration of S0011 Sanquin-VQC HBV-DNA genotype A standard (quantified in bDNA 3.0 copies/mL) against the 1st and 2nd WHO genotype A (97/746 and 97/750) standards

HBV-DNA Assays	Principal Investigator (Study)	Reference standard	n WHO	n VQC	copies (95 %CI) per IU
Siemens Versant bDNA 3.0	Cuijpers HT (Sanquin, Amsterdam, the Netherlands, unpublished)	WHO 97/746	12	16	5.33 (5.11-5.55)
Siemens Versant bDNA 3.0	Van Drimmelen H. (BQC, the Netherlands, unpublished)	WHO 97/750	6	6	5.20 (4.61-5.80)
Multi-method WHO collaborative study	Saldanha J et al. (Vox Sang 2001;80:63-71)[11]	WHO 97/746	46	23	4.12 (not reported)

Calibration S0009 VQC-Sanquin HCV-RNA standard against WHO (97/790, 97/978 and 06/100) HCV standards.

The S0009 VQC-Sanquin HCV-RNA standard containing 6.30 (6.13-6.48).10^6 copies/mL has been calibrated against the WHO 97/790, 97/978 and 06/100 standards quantified at 50.000, 50.000 and 77.625 IUs per ampoule respectively by Saldanha et al[9] and Baylis et al[15]. For HCV we have not performed a calibration study using the bDNA assay as reference method, but compared the conversion factor found in several studies (table 6). When the geometric mean conversion factor of nine calibration experiments was calculated we estimated that one IU is equivalent to 2.76 (1.4-4.8) copies, close to the value of 2.73 that was established in a WHO calibration study of secondary standards reported by Saldanha et al[28]. We therefore decided to use the value 2.73 as conversion factor from IUs to copies

Table 6. Calibration of the Sanquin-VQC HCV-RNA genotype 1 standard against the WHO genotype 1 (96/790, 96/978 and 06/100) standards.

HCV-RNA Assays	Principal Investigator (Study)	Reference standard	n WHO	n VQC	copy per IU
Multi-method WHO collaborative study	Saldanha J. et al (Vox Sang 2000;78:217-24)28	WHO 96/790	19	19	2.73
Multi-method WHO collaborative study	Saldanha J. et al (Vox Sang 1999;76:149-158)9	WHO 96/790	33	33	1.95
Multi-method WHO collaborative study	Saldanha J. et al (Vox Sang 1999;76:149-158)9	WHO 96/978	33	33	2.09
Ultrio probit analysis	Koppelman M et al (Transfusion 2005;45:1288-1266)29	WHO 96/790	63	48	3.28
Ultrio probit analysis	Assal A. et al (Transfusion 2009;49:289-300)30	WHO 96/978	24	12	3.52
TaqScreen probit analysis	Assal A. et al (Transfusion 2009;49:289-300)30	WHO 96/978	24	12	2.17
Ultrio Plus probit analysis	Goubran H et al (National Blood Transfusion Center, Cairo, unpublished)	WHO 06/100	24	24	2.45
Ultrio probit analysis	Grabarczyk et al (Transfusion 2013;53:2512-24)31	WHO 06/100	24	12	5.25
Ultrio Plus probit analysis	Grabarczyk et al (Transfusion 2013;53:2512-24)31	WHO 06/100	36	12	2.57

Calibration S0012 VQC-Sanquin HIV-1 RNA standard against WHO (97/656 97/650) HIV-1 RNA standards

Dr. H. Holmes (NIBSC, Potterbar, UK) kindly shared the raw data of the laboratories that participated in the first WHO collaborative study with us[10]. The individual viral load measurements in log copies/mL with five methods on sample XX, YY and ZZ representing the 1st WHO HIV subtype B, 2nd WHO HIV subtype B and the Sanquin-VQC HIV subtype B standard respectively were used to calculate the geometric mean concentration (and 95% CI) in copies/mL with the different assays. The mean concentration in sample ZZ was then corrected for the original established concentration of 1.05 (1.01-1.09) x 10^8 copies/mL assigned to the S0012 HIV-1 RNA subtype B standard. For example the bDNA 3.0 assay measured on average 11,600 copies/mL in sample ZZ which according to the original quantification contains 10,500 copies/mL (since sample ZZ was a 1:10,000 dilution of the S0012 Sanquin-VQC standard). Therefore the mean value (and 95% confidence intervals) in copies/mL in the WHO standards were corrected by a factor 0.904 and divided by 100,000 and 363,078 IU/mL concentrations assigned to the 1st and 2nd WHO standard respectively to obtain the copy/IU values. As can be seen in table 7 replacement of the first by the second WHO standard caused a significant drift in the amount of copies per IU for the bDNA 3.0 and in the NucliSens assay since the 95% confidence intervals do not overlap. One reason that could have contributed to the significant drifts in IU from the 1st to the 2nd WHO standard was the fact that there were mismatches in oligonucleotides incorporated in the NucliSens assay. This divergence was established by Dr. Holmes and colleagues after alignment against the sequence of the 2nd WHO standard, but not against the sequence in the 1st WHO standard. Unfortunately for the quantification in IU/mL the results found using the NucliSens assay in the WHO collaborative study were not rejected. More recently a third heat inactivated WHO HIV standard (10/152) has been established that contains 185.000 IU per ampoule[14]. Apparently, the calibration of the HIV-1 replacement standard by multiple methods in the WHO collaborative study could not guarantee the continuity of the IU in different assays. The copy to IU conversion factor of the VQC-Sanquin standard changed significantly ($p<0.05$) from 0.39 (0.34-0.44) to 0.58 (0.51-0.66) by replacement of the WHO 97/656 by the WHO 97/650 standard (table 7). Since both the first and the Sanquin-VQC standard were not included in the WHO collaborative study to establish the third heat-inactivated International Standard the continuity of the conversion factor from IU to bDNA copies on this latter standard could not be evaluated.

Table 7. Calibration of VQC-Sanquin HIV-RNA subtype B standard on the first (97/656) and second (97/650) WHO HIV-1 RNA subtype B standards (containing 100,000 and 363,078 IU per ampoule respectively) as calculated from individual quantitative assays on standard dilutions with five methods as reported by the laboratories participating in the first WHO collaborative study[10]

	n assays			VQC copies/IU on 1st WHO (97/656) standard		VQC copies/IU on 2nd WHO (97/650) standard	
	1st WHO	2nd WHO	VQC	mean	(95%CI)	mean	(95%CI)
Abbott LCx	14	15	14	0.76	(0.60-0.96)	0.69	(0.56-0.86)
Roche Amplicor Monitor	125	134	112	0.70	(0.60-0.81)	0.93	(0.80-1.08)
Siemens bDNA 3.0	64	69	48	0.39	(0.34-0.44)	0.58	(0.51-0.66)
Organon Teknika NucliSens	46	51	36	0.80	(0.69-0.92)	0.43	(0.36-0.50)
Roche Amplicor Monitor UltraSens	16	15	11	0.51	(0.27-0.95)	0.86	(0.49-1.51)

Calibration of the VQC-Sanquin standards against the Eurohep standards for HBV-DNA and HCV-RNA

Calibration S0011 VQC-Sanquin HBV-DNA standard against Eurohep genotype A standard

The WHO HBV-DNA standards are a 1:500 dilution of the Eurohep genotype A standard, which was then lyophilized. Heerman et al[3] estimated the HBV-DNA concentration in the undiluted Eurohep standard at 2.7 x 10^9 copies or Eurohep Units/mL. In one experiment in 2008 a 1:90,000 dilution of the Eurohep standard (S1230) was tested in parallel to 1:66,667 dilution of the VQC-Sanquin standard (S1220) in 6 replicate bDNA 3.0 tests per standard. When calibrated against the VQC-Sanquin standard the Eurohep standard contained 2.97 (1.78-4.95) x 10^9 copies/mL (table 8a and 8b). Hence, one copy assigned to the Eurohep standard by Heerman et al[3] or one Eurohep Unit contains 1.10 copy assigned to the Sanquin-VQC standard. This conversion factor was confirmed by comparing the potencies of the two standards in different NAT methods used in the VQC proficiency study of 1997 (table 8b). When comparing the replicate bDNA 3.0 test results on the S1230 and S1231 dilutions of the Eurohep and WHO 97/750 standards in the bDNA assay a conversion factor of 4.73 Eurohep Units per IU was calculated. A 1:500 dilution of the Eurohep standard should contain 5.40 x 10^6 Eurohep Units/mL. Hence the recovery of HBV-DNA after lyophilisation of the Eurohep standard in the WHO standard is estimated at 88%. Table 8a also shows the calibration data of the Eurohep genotype D standard.

Table 8a. Calibration of the Eurohep standards against the VQC-Sanquin HBV genotype A standard

HBV-DNA standard	bDNA 3.0 runs			cp/mL	95% CI (cp/mL)		95% CI (%)	
	N assays	N exp	df	weighted geomean	lower	upper	lower	upper
VQC gt A	6	1	4	2.15E+09	2.11E+09	2.20E+09	98%	102%
Eurohep gt A	6	1	4	2.97E+09	1.78E+09	4.95E+09	60%	167%
Eurohep gt D	12	2	8	2.53E+09	2.03E+09	3.17E+09	80%	125%

Table 8b. Calibration of Sanquin-VQC HBV-DNA genotype A standard (quantified in bDNA 3.0 copies/mL) against the Eurohep genotype A standard quantified at 2.7x109 Eurohep Units/mL by Heerman et al3

Principal Investigator (Study)	HBV-DNA Assays	n EH	n VQC	VQC copies (CI) per Eurohep Unit
Van Drimmelen H. (BQC, Rijswijk, the Netherlands, unpublished)	Siemens Versant bDNA 3.0	6	6	1.10
Van Drimmelen H (VQC international proficiency study 1997, unpublished)	Chiron bDNA 1.0	6	6	1.07
	Roche Ampl Monitor	5	5	0.94
	Digene HCS	9	9	1.09
	Probit qualitative PCR	54	54	1.09

Calibration of S0011 VQC-Sanquin HCV-RNA standard against the previous Eurohep HCV standard

The VQC-Sanquin HCV-RNA standard has originally been calibrated against the Eurohep HCV genotype 1 standard quantified in geq/mL by multiple bDNA 1.0 assays. These calibration data have been described in the Eurohep proficiency studies that have been performed and published in the 1990s[4,5].

Calibration of chimp infectivity plasmas against VQC-Sanquin standards

Calibration Chimp genotype A and C plasmas against S0011 VQC-Sanquin HBV-DNA standard

Table 9a and 9b summarize the results of a calibration experiment in which two Japanese chimpanzee plasmas with a known HBV infectivity titer were calibrated against the VQC-Sanquin HBV genotype A standard. According to interpretation of the chimpanzee infectivity data reported by Komiya et al[19] the 50% chimpanzee minimum infectious dose or CID_{50} (and 0% to 100% infectivity range) is 8.2 (2.6-26) copies for a genotype A plasma and 9.5 (3.0-30) for a genotype C plasma. These estimates were based on quantification in the Roche TaqMan assay. Recalibration of a 1:100 dilution of these ramp up phase plasma samples (P57 and P29 of chimpanzees C-446 and C-272) in 6 replicate bDNA assays against the Sanquin-VQC standard showed conversion factors of 2.06 and 1.62 from bDNA to TaqMan copies for genotype A and C respectively. As a consequence one CID_{50} (0% to 100% infectivity range) of the HBV genotype A and C strain contain 4.0 (1.3-12.6) copies and 5.9 (1.8-18.5) copies respectively when calibrated against the Sanquin-VQC standard. Based on these data it has been decided to use a worst case estimate of 3.16 HBV virions or copies in the VQC-Sanquin standard (half log between 1 and 10 virions) as the 50% minimum infectious dose (MID_{50}) in infectivity based risk analysis of transfusion transmission of HBV by a window period donation[21].

Table 9a. Calibration of Japanese chimpanzee plasmas with a known infectivity titer against the VQC-Sanquin HBV genotype A standard

HBV-DNA standard	bDNA 3.0 runs			copies/mL	95% CI (copies/mL)		95% CI (%)	
	N assays	N exp	df	weighted geomean	lower	upper	lower	upper
VQC gt A	6	1	4	2.15E+09	2.11E+09	2.20E+09	98%	102%
Chimp gt A	6	1	4	1.26E+06	7.30E+05	2.16E+06	58%	172%
Chimp gt C	6	1	4	1.85E+06	1.28E+06	2.67E+06	69%	144%

Table 9b. Estimation of MID_{50} in HBV-DNA copies or virion numbers by calibration of Japanese chimpanzee infectivity plasmas against the S0011 VQC-Sanquin HBV genotype A standards in multiple replicate bDNA assays

HBV-DNA Assays	Principal Investigator (Study)	Reference standard	n chimp	n VQC	copy per CID_{50} (range)
Siemens Versant bDNA 3.0	Van Drimmelen H. (BioQControl, the Netherlands, unpublished)	C-246 P-57 gt A	6	6	4.0 (1.3-12.6)
Roche TaqMan	Komiya K et al. (Transfusion 2008;48:286-9)[19]		1	1	8.2 (2.6-26)
Siemens Versant bDNA 3.0	Van Drimmelen H. (BioQControl, the Netherlands, unpublished)	C-272 P-29 gt C	6	6	5.9 (1.8-18.5)
Roche TaqMan	Komiya K et al. (Transfusion 2008;48:286-9)[19]		1	1	9.5 (3.0-30)

Calibration Chimp-210 HCV genotype 1b infectivity plasma against S0009 VQC-Sanquin HCV-RNA standard

Katayama et al[20] determined the infectivity titer of a chimpanzee HCV RNA genotype 1b window period sample taken 7 weeks after inoculation. This plasma was inoculated in two chimpanzees per 10-fold dilution. The 50% chimpanzee minimum infectious dose or CID_{50} (and 0% to 100% infectivity range) was established at 7.0 (2.2-22) copies based on quantification in the Roche TaqMan assay. The HCV-RNA concentration of a 1:100 dilution of the chimpanzee C-210 window phase plasma was below the quantification limit of the bDNA 3.0 assay. Therefore dilution panels prepared from this plasma and the Sanquin-VQC standard (see table 10a and 10b) were tested in 24 replicates in the Ultrio assay and probit analysis in parallel line model showed a conversion

factor of 1.16 from bDNA to TaqMan copies. As a consequence one CID_{50} (0% to 100% infectivity range) of the HCV genotype 1b infectivity standard contains 8.1 (2.6-25.6) copies when calibrated against the Sanquin-VQC standard. Based on these data it has been decided by Kleinman et al[21] to use a worst case estimate of 3.16 HCV virions or copies (half log between 1 and 10 virions) in the VQC-Sanquin standard as the 50% minimum infectious dose (MID_{50}) in risk analysis of transfusion of an infectious window period donation.

Table 10a. Calibration of Japanese chimpanzee HCV genotype 1b infectivity plasma against the S0009 VQC-Sanquin HCV genotype 1 standard.

HCV-RNA standard	bDNA 3.0 runs			copies/mL	95% CI (copies/mL)		95% CI (%)	
	n assays	m exp	Df	weighed geomean	lower	upper	lower	upper
VQC gt 1	27	5	17	6.30E+06	6.13E+06	6.48E+06	97%	103%
Chimp gt 1b	Ultrio probit analysis			2.55E+05	1.49E+05	4,36E+05	59%	171%

Table 10b Estimation of MID_{50} in HCV-RNA copies or virion numbers by calibration of Japanese chimpanzee infectivity plasma against the S0009 VQC-Sanquin HCV genotype 1 standard

HCV-RNA Assays	Principal Investigator (Study)	Chimp standard	n Chimp	n VQC	copy per CID50 (range)
Siemens Versant bDNA 3.0	Van Drimmelen H. (BioQControl, the Netherlands, unpublished)	C210-7wk	6	6	8.1 (2.6-25.6)
Roche Taqman	Katayama et al (Intervirology. 2004;4:57-64)[20]		1		7.0 (2.2-22)

5. CALIBRATION OF INACTIVATED BIOQ STANDARDS AGAINST NATIVE VQC-SANQUIN STANDARDS

In document CE4006 the preparation of the inactivated BioQ standards is described including the percentage recovery after the inactivation processes as measured in replicate bDNA assays. The chapters below summarize the calibration of the inactivated BioQ standards against the native VQC-Sanquin standards (table 11).

Calibration S0043 heat-inactivated HBV-DNA standard against S0011 native VQC-Sanquin standard

The HBV-DNA concentration in the pasteurized S0043 BioQ HBV-DNA genotype A plasma standard was compared with the native S0011 Sanquin-VQC HBV-DNA genotype A standard by parallel testing of 1:66 667 and 1:235 dilutions of the standards respectively in 6 replicate bDNA 3.0 assays. The geometric mean HBV-DNA concentrations in the test samples were 40 625 and 38 777 cps/mL respectively. These values became 32 250 and 30 783 copies/mL respectively when the HBV-DNA concentration in the native VQC standard was adjusted for the geometric mean concentration measured in a total of 28 bDNA 3.0 assays (table 3). After this 1.26 fold adjustment of the measured bDNA test results the HBV-DNA concentration in the pasteurized BQC HBV-DNA standard was established at 7.23 (4.82-10.9) x 10^6 copies/mL as compared to 2.15 x 10^9 copies/mL in the native Sanquin-VQC standard (table 11).

Calibration S0041 heat-inactivated HIV-RNA standard against S0012 native VQC-Sanquin standard

The HIV-RNA concentration in the S0041 pasteurized HIV subtype B standard was established at 2.61 (1.99-3.42) x 10^6 copies/mL by parallel testing of 1:8333 and 1:10^5 dilutions of the native and heat inactivated plasma standards respectively in 6 replicate bDNA 3.0 assays. A similar concentration of 2.28 x 10^6 copies/mL was measured at time point T=0 in a stability study (data not shown). However the concentration of 2.61 (1.99-3.42) x 10^6 copies/mL has been assigned to S0041 heat inactivated HIV-RNA standard (table 11).

Calibration S0109 inactivated HCV-RNA standard against S0009 native VQC-Sanquin HCV standard

In the first calibration experiment a 1:295.6 dilution of S0009 Sanquin-VQC HCV genotype 1 standard and a 1:1000 dilution of S0109 betapropiolactone inactivated BioQ HCV genotype 3a standard were tested in 6 replicate bDNA assays. The geometric mean concentrations in the undiluted standards were measured at 4.99 x 10^6 and 4.80 x 10^7 copies/mL respectively. After 1.26 fold adjustment to the Sanquin-VQC standard concentration of 6.30 x 10^6 copies/mL we established the concentration in the betapropiolactone treated standard to be 6.06 (4.48-8.20) x 10^7 copies/mL. This value has been assigned to S0109 inactivated standard (table 11).

Later in a calibration experiment for preparation of panels for an evaluation of HCV Ag/Ab combo assays[33] the geometric mean concentrations in the undiluted plasmas were determined again in triplicate tests by the bDNA assay at 3.83 x 10^7 copies/mL and 5.49 x 10^6 copies/mL in the inactivated genotype 3a and genotype 1 standards respectively. Since the concentration in the Sanquin-VQC standard has been established at 6.30 x 10^6 copies/mL the HCV-RNA concentration in the HCV genotype 3a plasma was adjusted 1.15 fold to 4.39 x 10^7 copies/mL. We have no explanation why this value is 1.47 fold lower than in the previous calibration experiment (table 11). For the moment we have not changed the value of 6.06 (4.48-8.20) x 10^7 copies/mL that was previously assigned to the inactivated S0109 standard (table 11).

Table 11. Calibration of the inactivated BQC HBV, HCV and HIV standards against the respective native VQC-Sanquin standards in six replicate tests performed in parallel in one bDNA 3.0 assay run.

standard	bDNA 3.0 runs			copies/mL	95% CI (cps/ml)		95% CI (%)	
	N assays	N exp	df	weighted geomean	lower	upper	lower	upper
S0011 HBV gt A native	6	1	4	2.15E+09	2.11E+09	2.20E+09	98%	102%
S0043 HBV gt A inact.	6	1	4	7.23E+06	4.82E+06	10.9E+06	67%	151%
S0009 HCV gt 1 native	6	2	4	6.30E+06	6.13E+06	6.48E+06	97%	103%
S0109 HCV gt 3a inact.	6	2	4	6.06E+07	4.48E+07	8.20E+07	74%	135%
S0012 HIV gt B native	6	1	4	1.05E+08	1.01E+08	1.09E+08	96 %	104%
S0041 HIV gt B inact.	6	1	4	2.61E+06	1.99E+06	3.42E+06	76%	131%

6. VERIFICATION OF CROSS CALIBRATION OF STANDARDS

Verification of cross calibration of inactivated and native viral standards by Ct analysis in TaqScreen MPX 2.0 assay

For a stability study the native VQC-Sanquin standards and inactivated BQC standards (calibrated against each other in six bDNA3.0 assays as shown in table 11) were tested at a concentration of 2000 copies/mL in a specially prepared P0238 mixed standard panel. Each 2000 copies/mL sample was tested in 4 real time PCR tests (Roche TaqScreen 2.0 assay) by Dr. Marco Koppelman (Sanquin, Amsterdam, the Netherlands). Since the same samples in the P0238 mixed standard panel were tested at different time points and storage temperatures there were nine suitable sets of quadriplicate Ct values available for potency analysis (37°C storage data were excluded). For each time point and temperature the average Ct value (given with one decimal point) was calculated and shown in table 12 (with two decimal points).

Table 12. Potency of inactivated BQC standards as compared to native VQC-Sanquin standards tested at a concentration of 2000 copies/mL in 36 parallel TaqScreen 2.0 tests performed for a stability study

Storage		Mean Ct# HBV-DNA			Mean Ct# HCV-RNA			Mean Ct# HIV-RNA		
temp	time	S0011 native	S0043 inact	potency inact/ native	S0009 native	S0109 inact	potency inact/ native	S0012 native	S0041 inact	potency inact/ native
-70°C	8	27.98	28.20	0.86	30.88	32.00	0.46	29.05	28.95	1.07
	24	28.05	28.23	0.89	30.90	31.83	0.53	29.15	28.90	1.19
	48	28.18	28.48	0.81	30.93	31.75	0.56	28.75	28.78	0.98
2-8°C	8	28.03	28.18	0.90	30.88	32.20	0.40	29.05	28.93	1.09
	24	28.00	28.08	0.95	30.88	32.25	0.39	29.05	28.98	1.05
	48	28.00	28.25	0.84	31.23	32.35	0.46	29.25	29.30	0.97
+20°C	8	27.78	27.98	0.87	31.00	32.38	0.39	28.85	28.68	1.13
	24	27.88	27.90	0,98	30.93	32.63	0.31	29.00	28.90	1.07
	48	28.03	28.25	0.86	31.13	32.13	0.50	29.15	29.20	0.97
		Average potency (95%CI)§		0.88 (0.78-0.99)	Average potency (95%CI)§		0.44 (0.28-0.60)	Average potency (95%CI)§		1.06 (0.91-1.21)

each mean Ct value is the average of 4 measurements
§ average potency of 9 x 4 = 36 Ct values, T-value for 95% CI 2.0

The potency of the inactivated standard relative to the native material (tested in parallel in the same test runs in quadruplicate) was calculated by the formula: $2^{(Avg(Ct_{native})-Avg(Ct_{inact}))}$.

The inactivated HBV and HIV standards have been prepared from the same native plasma standards and here we did not find a significant difference in potency in the materials before and after inactivation (table 12). Table 12 shows a mean potency (95% CI) of 0.88 (0.78-0.99) for the inactivated relative to the native HBV-DNA standard, a borderline significant difference (p=0.05). However for HCV the difference in concentration was significant (p<0.05). According to the TaqScreen 2.0 Ct analysis the HCV-RNA concentration of the inactivated genotype 3a material was 44 (28-60)% of that in the native genotype 1 standard. Even if the calibration of the inactivated HCV standard was based on the second bDNA experiment with 1.47 fold lower HCV-RNA content the potency in the real time PCR would have been 0.64 (0.41-0.88), still significantly lower than the native standard. This difference in reactivity of the 2000 copies/mL samples in the TaqScreen 2.0 assay may also be caused by the genotype, which was genotype 1 for the native and genotype 3a for the inactivated standard. It is possible that HCV genotype 3 is detected with lower efficiency than genotype 1 in this real time PCR method. To investigate this further we also included the native HCV genotype 3a plasma before inactivation in the P0238 mixed standard panel. This material was also calibrated against the VQC-Sanquin HCV genotype 1 standard (in triplicate bDNA assays). When the potency of the inactivated HCV genotype 3a standard was compared with the same native HCV genotype 3a plasma before inactivation in the TaqScreen Ct analysis the potency was 0.61 (0.38-0.85). If the inactivated standard would be adjusted 1.47 fold (according to the second bDNA calibration experiment) the potency would have been 0.90 (0.56-1.24) as compared to the native genotype 3a material (which is logical since it was calibrated in the same second bDNA calibration experiment). For the moment we suspect that the lower potency of the inactivated HCV genotype 3a standard observed by Ct analysis in the TaqScreen 2.0 assay may be the result of a genuinely (approximately 1.5 fold) lower concentration but that it can also be caused by lower detection efficiency of TaqScreen for HCV genotype 3 than the bDNA 3.0 assay. In a head to head comparison study the TaqScreen 1.0 assay[30] had a 1.4 (0.7-3.4) fold lower sensitivity than Ultrio (hence not significantly different). However in a proficiency study organized by EDQM the TaqScreen assay was significantly less sensitive than Ultrio in detecting an HCV genotype 3a sample while the opposite was found for a genotype 2b sample (Marie-Laure Hecquet, EDQM, personal communication).
In another experiment Dr Marco Koppelman (Sanquin, the Netherlands) tested a two-fold HBV/ HCV/HIV multi-marker dilution panel (table 13) in multiple replicate TaqScreen 2.0 assays. The relative sensitivity of HBV and HIV were comparable, but that of HCV was significantly lower (both according to Ct analysis and probit

analysis). This would indicate that the HCV concentration in a multi-marker run control for TaqScreen 2.0 may need to be 2-fold higher than that of HBV and HIV when based on the current calibration.

Table 13. Average Ct values of TaqScreen 2.0 assay on a mixed standard dilution panel and relative sensitivities in detecting inactivated BQC HBV, HCV and HIV standards

copies/mL	reps	mean Ct value			relative sensitivity Ct analysis	
		HIV	HBV	HCV	HBV to HIV	HCV to HIV
500	4	30.63	30.78	33.80	0.76	0.11
250	4	31.53	31.47	35.03	1.00	0.09
125	12	32.61	32.96	35.75	1.00	0.11
62.5	4	33.60	33.85	37.45	0.54	0.07
31.2	12	34.72	34.93	38.65	0.57	0.07
All (95%CI)	36	Individual Ct values			0.90 (0.31-1.50)	0.12 (0.03-0.44)
		LODs probit analysis			relative sensitivity probit analysis	
	n	HIV cps/mL	HBV cps/mL	HCV cps/mL	HBV to HIV	HCV to HIV
50% LOD (CI)	12	2.0 (1.3-2.8)	2.8 (1.5-4.3)	5.2 (3.3-7.8)	0.71	0.38
63% LOD (CI)		2.6 (1.6-3.9)	4.3 (2.7-7.2)	7.6 (5.1-12.5)		
95% LOD (CI)		7.6 (4.9-21)	23.8(12.3-101)	35.2 (19.3-116)		
efficiency		38% (25-62)%	23% (14-37)%	13% (8-19)%		

Verfication of quantification of viral standards in copies/mL by limiting dilution analysis in Ultrio and Taqscreen assay versions

The internationally used standards calibrated in copies/mL as described above (VQC-Sanquin, Eurohep, BioQ, and WHO standards) were used in several validation studies of Ultrio (Plus and Elite) versions, some of which have been published before[8,25,29-33]. Knowing that there is some variation in analytical sensitivity of NAT reagent batches and in individual NAT test runs by the laboratories we combined all available data in the Ultrio versions for a parallel line probit analysis in order to compare the potency of the different HBV, HCV and HIV standards. Table 14a,b and c give the 50% and 95% LODs that were calculated from the combined analytical sensitivity studies performed with each of the Ultrio versions, but also present the 63% LODs. According to Poisson distribution the 63% LOD corresponds with one detectable viral nucleic acid copy per assay. Since the Ultrio, Ultrio Plus and Ultrio Elite assays use 0.5 mL plasma input one expects a concentration of 2 copies/mL at the 63% LOD when all DNA or RNA molecules in the viral particles would be extracted and detected with 100% efficiency (which is likely to be less). By dividing the value of 2 copies by the observed 63% LOD value the theoretical NAT efficiency (and 95%CI) can be calculated (table 15). Since the Ultrio assay was deficient in detecting double stranded HBV-DNA[36] we excluded this assay in calculating the theoretical detection efficiency of all Ultrio versions combined (last column in table 15) .

Table 14a. LODs estimated by parallel line probit analysis on HBV standard dilution series for each of the Ultrio versions and all Ultrio versions combined

standard dilution panel	LOD	n	Ultrio cps/mL (CI)	n	Ultrio Plus cps/mL (CI)	n	Ultrio Elite cps/mL (CI)	n	Ultrio Plus and Elite combined cps/mL (CI)
P0023 WHO IS 97/750	50%	32	11.1 (6.1-20.4)	303	4.3 (3.4-5.5)	252	4.4 (3.8-5.1)	555	4.3 (3.6-5.2)
	63%		18.0 (9.8-33.8)		6.7 (5.1-8.8)		6.6 (5.6-7.8)		6.6 (5.4-8.2)
	95%		120 (63.0-250)		36.4 (62.2-54)		33.6 (27-43)		35.9 (28-49)
P0007 HBV-DNA genotype A2	50%	24	15.7 (8.0-31.1)	48	4.8 (2.6-8.9)	50	2.8 (2.0-3.8)	97	3.7 (2.4-5.7)
	63%		25.4 (13-51)		7.4 (4.0-14)		4.2 (3.0-5.9)		5.6 (3.6-8.8)
	95%		170 (84-374)		40.6 (22-82)		21.5 (15-32)		30.3 (19-51)
P0031 HBV-DNA genotype A inact.	50%	58	56.1 (36-87)	24	6.6 (2.8-15)	25	5.7 (3.6-8.9)	49	6.1 (3.3-113)
	63%		90.7 (58-146)		10.1 (4.3-24)		8.6 (5.5-14)		9.4 (5.0-18)
	95%		605 (364-1097)		55.0 (23.4-140)		43.8 (27.3-73)		50.6 (26.8-100)
P0001 Eurohep HBV-DNA genotype A2	50%	48	9.4 (5.7-15.4)	96	3.6 (2.0-6.9)	24	7.9 (4.9-13)	120	4.1(2.8-6.1)
	63%		15.2 (9.2-26)		5.7 (3.1-11)		11.9 (7.4-19)		6.3 (4.2-9.5)
	95%		102 (58.9-191)		30.0 (19-52)		60.5 (37.2-102)		34.3 (22-55)
P0005 HBV-DNA Chimp genotype A	50%	48	8.4 5.1-14)	48	3.7 (2.0-6.9)			48	3.7 (2.0-7.0)
	63%		13.6 (8.2-23)		5.7 (3.1-11)				5.7 (3.0-11)
	95%		91.0 (53-171)		31.4 (17-63)				31.0 (16-62)
P0006 HBV-DNA Chimp genotype C	50%	24	32.7 (17-65)	48	4.9 (2.7-9.1)			48	4.9 (2.6-9.3)
	63%		52.9 (27-107)		7.6 (4.1-14)				7.6 (4.0-14)
	95%		353 (173-788)		41.4 (22-83)				40.9 (21-82)
P0002 Eurohep HBV-DNA genotype D	50%	48	3.3 (1.9-5.5)	48	1.9 (1.0-3.4)			48	1.9 (1.0-3.5)
	63%		5.3 (3.1-9.1)		2.8 (1.5-5.4)				2.8 (1.5-5.4)
	95%		35.4 (20-67)		15.6 (8.2-32)				15.3 (8.0-31)

Table 14b. LODs estimated by parallel line probit analysis on HCV standard dilution series for each of the Ultrio versions and all Ultrio versions combined

Standard dilution panel	LOD	n	Ultrio cps/mL(CI)	n	Ultrio Plus cps/mL(CI)	n	Ultrio Elite cps/mL(CI)	n	All combined cps/mL(CI)
P0024 WHO HCV-RNA IS 06/100	50%	32	2.5 (1.8-3.5)	291	2.9 (2.3-3.6)	250	3.4 (2.6-4.5)	573	3.1 (2.7-3.6)
	63%		3.9 (2.8-5.4)		4.3 (3.4-5.6)		5.1 (3.8-6.9)		4.6 (4.0-5.4)
	95%		21.2 (15-32)		21.4 (16-32)		24.3 (17-39		23.4 (19-30)
P0019 HCV-RNA genotype 1	50%	36	2.9 (2.1-3.9)	48	1.7 (1.1-2.9)	88	1.7 (1.1-2.5)	172	1.9 (1.5-2.4)
	63%		4.4 (3.3-6.1)		2.6 (1.6-4.4)		2.5 (1.6-3.9)		2.9 (2.2-3.7)
	95%		24.4 (17-36)		12.9 (7.4-25)		11.8 (7.2 -22)		14.5 (11-20)
P0018 HCV-RNA Chimp genotype 1b	50%	24	2.4 (1.6-3.5)					24	2.4 (1.2-4.6)
	63%		3.6 (2.5-5.4)						3.6 (1.8-7.0)
	95%		20.0 (13-32)						17.9 (9.1-37)
P0020 HCV-RNA genotype 3 inact.	50%	51	3.6 (2.8-4.7)					51	3.6 (2.3-5.7)
	63%		5.6 (4.3-7.4)						5.5 (3.5-8.7)
	95%		30.8 (23-44)						27.6 (17-46)
P10 HCV-RNA genotype 3a	50%	12	2.8 (1.6-4.6)					12	2.7 (1.1-6.8)
	63%		4.2 (2.5-7.2)						4.1 (1.6-10)
	95%		23.2 (14-42)						20.7 (8.3-54)

Table 14c LODs estimated by parallel line probit analysis on HIV-1 standard dilution series for each of the Ultrio versions and all Ultrio versions combined

Standard dilution panel	LOD	n	Ultrio cps/mL(CI)	n	Ultrio Plus cps/mL (CI)	n	Ultrio Elite cps/mL (CI)	n	All combined cps/mL (CI)
P0022 WHO HIV-RNA IS 97/650	50%	40	2.6 (1.8-3.8)	291	2.4 (2.1-2.7)	229	2.2 (1.7-2.8)	557	2.3 (2.1-2.6)
	63%		3.8 (2.6-5.6)		3.4 (3.0-4.0)		3.3 (2.6-4.3)		3.4 (3.0-3.9)
	95%		16.9 (11-287)		14.6 (12-19)		16.3 (12-25)		15.4 (13-19)
P0025 HIV-RNA subtype B	50%	60	1.5 (1.1-2.0)	48	1.7 (1.3-2.3)	24	2.0 (1.1-3.8)	132	1.6 (1.3-2.1)
	63%		2.1 (1.6-2.9)		2.5 (1.8-3.4)		3.0 (1.6-5.9)		2.4 (1.9-3.1)
	95%		9.5 (6.6-15)		10.4 (7.4-15)		15.0 (7.8-33)		10.8 (8.2-15)
P0026 HIV-RNA subtype B inact.	50%	52	3.1 (2.2-4.2)					52	3.1 (2.1-4.4)
	63%		4.4 (3.2-6.3)						4.5 (3.1-6.6)
	95%		19.7 (14-31)						20.1 (14-31)

Table 15. Percent detection efficiency based on NAT detectable copies/mL (63% LOD of 2 copies/mL is 100%)

Standard dilution panel	n	Ultrio efficiency (CI)	n	Ultrio Plus efficiency (CI)	n	Ultrio Elite efficiency (CI)	n	Ultrio versions combined# efficiency (CI)
P0023 WHO HBV-DNA IS 97/750	32	11 (6-20)%	303	30 (23-39)%	252	30 (26-36)%	555	30 (24-37)%
P0007 HBV-DNA genotype A	24	8 (4-16)%	48	27 (14-50)%	50	47 (34-65)%	97	36 (23-55)%
P0031 HBV-DNA genotype A inact.	58	2 (1-3)%	24	20 (8-46)%	25	23 (15-37)%	49	21 (11-40)%
P0001 Eurohep HBV-DNA genotype A	48	13 (8-22)%	96	36 (56-23)%	24	17 (10-27)%	120	32 (21-47)%
P0005 HBV-DNA Chimp genotype A	48	15 (9-24)%	48	35 (19-64)%			48	35 (18-66)%
P0006 HBV-DNA Chimp genotype C	24	4 (2-8)%	48	26 (14-49)%			48	26 (14-50)%
P0002 Eurohep HBV-DNA genotype D	48	38 (22-64)%	48	70 (37-131)%			48	70 (37-134)%
P0024 WHO HCV-RNA IS 06/100	32	52 (37-72%)	291	46 (36-58)%	250	39 (29-52)%	573	43 (37-50)%
P0019 HCV-RNA genotype 1	36	45 (33-61)%	48	76 (45-27)%	88	80 (51-25)%	172	70 (54-90)%
P0018 HCV-RNA Chimp genotype 1b	24	55 (37-81)%					24	56 (28-10)%
P0020 HCV-RNA genotype 3 inact.	51	36 (27-46%)					51	36 (23-57)%
P10 HCV-RNA genotype 3a native	12	47 (28-79%)					12	49 (19-21%)
P0022 WHO HIV-RNA IS 97/650	40	52 (36-76)%	291	58 (50-68)%	229	60 (47-78)%	557	58 (51-67)%
P0025 HIV-RNA subtype B	60	94 (68-127)%	48	81 (59-11)%	24	66 (34-25)%	132	83 (65-06)%
P0026 HIV-RNA subtype B inact.	52	45 (32-62)%					52	45 (30-65)%

#for HBV Ultrio Plus and Ultrio Elite combined

Overall the native VQC-Sanquin standards for HIV and HCV were detected with higher efficiency by the Ultrio versions [70 (54-90)% and 83 (65-106)%] than the one for HBV [36 (23-55)%]. We believe HBV is detected with lower efficiency than HCV and HIV by the Ultrio versions because the opposite was observed for the TaqScreen 1.0 assay (table 16). Table 16 shows the NAT efficiencies calculated from the 63% LODs as deduced from analytical sensitivity studies on the same VQC-Sanquin standard dilution panel that was tested by three laboratories (EFS in France, ARCBS in Australia and Gen-Probe/Hologic in Ca, US). For the TaqScreen assay the efficiencies (calculated for 1.0 mL plasma input per assay) were 27(15-45)% for HBV as compared to 18 (11-28)% and 19 (11-32)% for HCV and HIV-1 respectively. Hence HBV-DNA seemed to be detected with higher sensitivity by the TaqScreen 1.0 assay than HCV-RNA and HIV-RNA.

Table 16a. Analytical sensitivity of Ultrio, Ultrio Plus and TaqScreen 1.0 estimated by probit analysis on the proportions of reactive results on the same PeliCheck dilution panels of the VQC-Sanquin HBV, HCV and HIV standards tested by EFS, Gen Probe and ARCBS (unpublished data).

VQC-Sanquin Standard	50% LOD (CI) in copies/mL			95% LOD (CI) in copies/mL		
	Ultrio (n=24)	Ultrio Plus (n=12)	TaqScreen (n=12)	Ultrio (n=24)	Ultrio Plus (n=12)	TaqScreen (n=12)
HBV-DNA genotype A	18.6 (13-27)	5.7 (3.4-9.7)	2.8 (1.7-4.8)	160 (99-302)	49.4 (27-103)	24.5 (14-519)
HCV-RNA genotype 1	3.6 (2.6-4.9)	2.8 (1.8-4.3)	4.9 (3.2-7.5)	15.9 (10.8-27)	12.2 (7.5-23)	21.5 (13-40)
HIV-1 RNA subtype B	1.4 (1.0-1.9)	2.6 (1.6-4.1)	3.2 (2.0-5.0)	6.9 (4.6-12)	12.8 (7.7-25)	16 (9.5-30)

Table 16b. Detection efficiencies of Ultrio, Ultrio Plus and TaqScreen 1.0 in an analytical sensititivity study using proportions of reactive results data on a VQC-Sanquin PeliCheck standard dilution panel tested by EFS, Gen Probe and ARCBS (unpublished data).

VQC-Sanquin Standard	63% LOD (CI) in copies/mL			% NAT efficiency (CI)		
	Ultrio (n=24)	Ultrio Plus (n=12)	TaqScreen (n=12)	Ultrio (n=24)	Ultrio Plus (n=12)	TaqScreen (n=12)
HBV-DNA genotype A	28.7 (19-44)	8.9 (5.2-15.6)	4.4 (2.6-7.7)	7 % (5-10)%	23% (13-39)%	27% (15-45)%
HCV-RNA genotype 1	4.9 (3.5-7.0)	3.7 (2.4-6.0)	6.6 (4.2-10.5)	41% (29-57)%	54% (33-83)%	18% (11-28)%
HIV-1 RNA subtype B	4.0 (2.8-6.0)	4.3 (2.6-7.5)	6.1 (3.6-10.5)	50% (33-72)%	46% (27-77)%	19% (11-32)%

Table 17a, 17b and 17c compare the LODs found on different standard dilutions in TaqScreen 1.0 and 2.0 with those more recently found in the cobas 6800/8800 TaqScreen 3.0 version (data provided by Marco Koppelman, Sanquin, the Netherlands). The oligonucleotide design of the TaqScreen 3.0 assay has been modified to detect all genetic variants of HCV and HIV-1 with optimal sensitivity. For this purpose additional HCV oligo's and a second set of primers and probe has been added for dual region HIV detection. It is not known to us whether the oligonucleotide design for HBV has also been changed or whether other modifications (e.g. in extraction method or amplification cycles) have occurred. For HBV the VQC-Sanquin standard seems to be detected with slightly higher sensitivity in the cobas MPX 6800 assay than in TaqScreen 1.0. The same may hold for the inactivated HBV standard, although the difference in LODs did not reach significance. For HCV both the native VQC-Sanquin genotype 1 standard and the inactivated BioQ HCV genotype 3 standard were detected with significantly higher sensitivity by the latest cobas 6800 TaqScreen assay version. For HIV it seems that sensitivity of TaqScreen 2.0 has been improved as compared to version 1.0 although not the same standard dilution panels were tested. The sensitivity of the cobas MPX 6800 assay for HIV-1 RNA has definitely been improved as can be deduced from the low LODs on the inactivated HIV subtype B standard (data provided by Dr Marco Koppelman, Sanquin, the Netherlands) and the native HIV subtype C standard (data provided by Dr Marion Vermeulen, SANBS, South Africa).

Table 17a. LODs estimated by parallel line probit analysis on HBV standard dilution series for each of the TaqScreen versions

standard dilution panel	LOD	n	TaqScreen 1.0 cps/mL (CI)	n	TaqScreen 2.0 cps/mL (CI)	n	TaqScreen 3.0 cps/mL (CI)
EFS WHO HBV-DNA 97/746	50%	24	3.6 (2.6-4.9)				
	63%		5.6 (4.0-7.6)				
	95%		25.1(17.2-41.8)				
S2384 PeliCheck HBV genotype A2	50%	12	2.8 (1.7-4.6)				
	63%		4.3 (2.7-7.1)				
	95%		19.6 (11.4-40.7)				
P0001 Eurohep HBV-DNA genotype A2	50%	12	2.2 (1.3-3.7)				
	63%		3.5 (2.1-5.8)				
	95%		15.6 (8.8-32.4)				
Multi-marker HBV DNA genotype A2 inact.	50%			12	2.8 (1.5-4.3)		
	63%				4.2 (2.4-6.6)		
	95%				23.8 (12.4-99.3)		
P0023 WHO HBV IS 97/750	50%					12	1.6 (0.9-2.8)
	63%						2.4 (1.4-4.3)
	95%						11.0 (6.2-22.4)
P0007 HBV-DNA genotype A2	50%					12	1.9 (1.3-2.6)
	63%						2.7 (1.9-4.1)
	95%						12.6 (8.0-23.4)
P0031 HBV-DNA genotype A2 inact.	50%					12	2.4 (1.5-4.0)
	63%						3.6 (2.1-6.1)
	95%						16.3 (9.3-33.8)

Table 17b. LODs estimated by parallel line probit analysis on HCV standard dilution series tor each of the TaqScreen versions

standard dilution panel	LOD	n	TaqScreen 1.0 cps/mL (CI)	n	TaqScreen 2.0 cps/mL (CI)	n	TaqScreen 3.0 cps/mL (CI)
EFS WHO HCV 96/798	50%	24	6.2 (4.7-8.1)				
	63%		9.5 (7.3-12.5)				
	95%		39.2 (26.3-68.1)				
S2391 PeliCheck HCV genotype 1	50%	12	4.8 (2.9-7.9)				
	63%		7.4 (4.5-12.2)				
	95%		30.7 (17.7-61.6)				
Multi-marker HCV-RNA genotype 3 inact.	50%			12	5.2 (3.3-7.8)		
	63%				8.0 (5.1-12.0)		
	95%				35.2 (19.3-114)		
P0019 HCV-RNA genotype 1	50%					36	2.9 (2.2-3.9)
	63%						4.5 (3.3-5.9)
	95%						20.2 (13.4-35.8)
P0020 HCV-RNA genotype 3 inact.	50%					12	2.4 (1.4-4.3)
	63%						3.8 (2.1-6.6)
	95%						17.1 (9.3-36.6)

Table 17c. LODs estimated by parallel line probit analysis on HIV-1 standard dilution series tor each of the TaqScreen versions

Standard dilution panel	LOD	n	Ultrio cps/mL(CI)	n	Ultrio Plus cps/mL (CI)	n	Ultrio Elite cps/mL (CI)
EFS WHO HIV subtype B 97/650	50%	24	4.4 (3.4-5.7)				
	63%		6.8 (5.3-8.8)				
	95%		23.1 (16.0-38.4)				
S2398 PeliCheck HIV subtype B	50%	12	4.2 (2.6-6.6)				
	63%		6.4 (4.0-10.2)				
	95%		21.6 (13.0-41.3)				
Multi-marker HIV-RNA subtype B inact.	50%			12	2.0 (1.3-2.8)		
	63%				3.1 (2.0-4.4)		
	95%				7.6 (4.9-21.4)		
P0022 WHO HIV-RNA IS 97/650	50%					12	2.7 (1.7-4.3)
	63%						4.1 (2.6-6.6)
	95%						12.5 (7.5-24.0)
P0026 HIV-RNA subtype B inact.	50%					12	1.0 (0.6-1.5)
	63%						1.5 (1.0-2.3)
	95%						4.4 (2.7-8.3)
P0027 HIV-RNA subtype C	50%					24	0.7 (0.5-0.9)
	63%						1.0 (0.8-1.4)
	95%						3.1 (2.1-5.4)

Table 18. Percent detection efficiency based on NAT detectable copies/mL (63% LOD of 1 copy/mL is 100%)

standard dilution panel	n	TaqScreen MPX 1.0 % efficiency (CI)	n	TaqScreen MPX 2.0 % efficiency (CI)	n	Cobas MPX % efficiency (CI)
EFS WHO HBV-DNA 97/746	24	18 (13- 25) %				
S2384 PeliCheck HBV genotype A	12	23 (14-37) %				
P0001 Eurohep HBV-DNA genotype A2	12	29 (17-48) %				
Multi-marker HBV DNA gt A inact.			12	24 (15-42) %		
P0023 WHO HBV IS 97/750					12	42% (23-74) %
P0007 HBV-DNA genotype A2					24	37 (24-53) %
P0031 HBV-DNA genotype A inact.					12	28 (16-47) %
EFS WHO HCV 96/798	24	11 (8-14) %				
S2391 PeliCheck HCV genotype 1	12	13 (8-22) %				
Multi-marker HCV-RNA gt 3 inact.			12	13 (8-20) %		
P0019 HCV-RNA genotype 1					36	22 (17-30) %
P0020 HCV-RNA genotype 3 inact.					12	26 (15-46)%
EFS WHO HIV subtype B 97/650	24	16 (10-25) %				
S2398 PeliCheck HIV subtype B	12	15 (11-19) %				
Multi-marker HIV-RNA subt B inact.			12	32 (23-50) %		
P0022 WHO HIV-RNA IS 97/650					12	24 (15-39) %
P0026 HIV-RNA subtype B inact.					12	68 (44-105) %
P0027 HIV-RNA subtype C					24	97 (71-133) %

The inactivated BioQ HBV, HCV and HIV standards were detected with 21 (11-40)%, 36 (23-57)% and 45 (30-65)% by the Ultrio versions (table 15) and with 24 (15-42)%, 13 (8-20)% and 32 (23-50)% by the TaqScreen 2.0 assay (table 13). However, in the cobas 6800 TaqScreen 3.0 version the detection efficiency improved to 28 (16-47)%, 26 (15-46)% and 68 (44-105) % for HBV, HCV and HIV respectively. Interestingly the detection efficiency of the native HCV genotype 1 and the inactivated HCV genotype 3 standard has become comparable in the cobas 6800 TaqScreen assay, which may indicate that the lower potency of the inactivated genotype 3 standard in the TaqScreen 2.0 assay (table 12) was caused by a difference in genotype detection efficiency rather than a 1.5 fold underestimation in the original bDNA 3.0 calibration experiment. Interestingly the detection efficiency of the inactivated HIV subtype B standard in the cobas 6800 assay has become higher than that found in the Ultrio assay.

Of course one should be careful with comparing the theoretical detection efficiencies of the inactivated BioQ standards since they are influenced by the uncertainty of the calibration against the native standards by replicate bDNA assays. Also it may be possible that the extraction of nucleic acid from inactivated standards is less efficient, for example because of formation of aggregates by heat treatment. There is reason to assume that the HBV particle configuration has been changed after pasteurization. This becomes clear when comparing the detection efficiencies of the HBV standards before and after heat treatment by the Ultrio versions. The efficiency of Ultrio HBV detection reduced significantly from 8 (4-16)% to 2 (1-3)% after pasteurization of the VQC-Sanquin standard, whereas the efficiency in the Ultrio Plus and Elite assay only reduced from 36 (23-55)% to 21(11-40)%. It may be that the target enhancer reagent (Lithium hydroxide) in the Ultrio Plus and Elite versions not only denatures double stranded HBV-DNA in the target region but also dissociates aggregates of HBV particles and plasma proteins (since pasteurization reduced detection efficiency in Ultrio 3.5 fold and in Ultrio Plus and Elite 1.7-fold). Hence, it may be that the effect of the alkalic shock on the HBV-DNA detection efficiency in the newer Ultrio versions is still incomplete. There was no clear change in HBV detection efficiency between the TaqScreen 1.0 and 2.0 versions (table 13 and 16). Despite an approximately two-fold higher sensitivity of the TaqScreen 2.0 and 3.0 assays for HBV than Ultrio Plus when testing the same VQC-Sanquin standard dilution panel, the detection efficiency of the two methods was similar

[27(15-45)% versus 23 (13-39)% respectively]. The reason for this is that the plasma input in the TaqScreen assay is two-fold higher (1 mL) than that in the Ultrio versions (0.5 mL). Recently the same inactivated standard dilution panels were tested in the new Roche cobas MPX 6800 assay by Dr Marco Koppelman (Sanquin, the Netherlands) and this assay version the LODs for HBV and HCV were comparable, while HIV-1 RNA was detected with approximately two-fold higher sensitivity (compare table 13 and 17). It may be that the HCV genotype 3a standard and HIV subtype B standards are more efficiently detected by the new (cobas MPX 6800 assay because of the addition of additional oligonucleotides (HCV) or the change from single to dual region detection (HIV-1).

When interpreting the limiting dilution data over the different Ultrio and TaqScreen versions (tables 14, 15, 16, 17 and 18) we conclude that our original quantification of the VQC-Sanquin standards in copies/mL by the bDNA assay is likely close to true absolute virion numbers (or at least within a factor 2, which is good enough for the use of LODs of NAT methods in estimating the lengths of window periods and residual viral transmission risk).

Verification of cross calibration of viral standards by parallel line probit analysis

Table 19 shows the potencies (and 95%CI) of the different standards as compared to the primary VQC-Sanquin standard as the reference standard which was estimated by parallel line probit analysis in each of the Ultrio versions and for all Ultrio versions combined. Hence, if the calibration in copies/mL by the bDNA assay would be confirmed by the Ultrio versions a potency value is expected that differs not significantly from 1.00. Since not all standards have been tested in parallel in the same experiments the differences in potency may also be caused by batch variation of Ultrio reagents or BioQ standard dilution panels as well as by laboratory variation.

Table 19. Relative potency of HBV, HCV and HIV-1 standards calibrated in copies/mL against the VQC-Sanquin standards in Ultrio versions

Standard dilution panel	n	Ultrio potency (CI)	n	Ultrio Plus potency (CI)	n	Ultrio Elite potency (CI)	n	Ultrio versions combined# Potency (CI)
P0007 HBV-DNA genotype A	24	1.00 reference	48	1.00 reference	50	1.00 reference	97	1.00 reference
P0023 WHO HBV-DNA IS 97/750	32	1.42 (0.59-3.48)	303	1.12 (0.58-2.15)	252	0.64 (0.44-0.91)	555	0.84 (0.52-1.36)
P0031 HBV-DNA genotype A inact.	58	0.28 (0.12-0.63)	24	0.74 (0.26-2.08)	25	0.49 (0.28-0.85)	49	0.60 (0.28-1.27)
P0001 Eurohep HBV-DNA genotype A	48	1.66 (0.74-3.83)	96	1.35 (0.65-2.86)	24	0.34 (0.19-0.63)	120	0.88 (0.49-1.59)
P0005 HBV-DNA Chimp genotype A	48	1.85 (0.82-4.31)	48	1.29 (0.55-3.08)			48	0.98 (0.45-2.11)
P0006 HBV-DNA Chimp genotype C	24	0.48 (0.18-1.21)	48	0.98 (0.41-2.31)			48	0.74 (0.34-1.59)
P0002 Eurohep HBV-DNA genotype D	48	4.74 (2.0-12.3)	48	2.61 (1.09-6.59)			48	1.98 (0.91-4.41)
P0019 HCV-RNA genotype 1	36	1.00 reference	48	1.00 reference	88	1.00 reference	172	1.00 reference
P0024 WHO HCV-RNA IS 06/100	32	1.15 (0.74-1.79)	291	0.60 (0.33-1.03)	250	0.48 (0.26-0.82)	573	0.62 (0.45-0.83)
P0018 HCV-RNA Chimp genotype 1b	24	1.22 (0.75-1.99)					24	0.81 (0.39-1.64)
P0020 HCV-RNA genotype 3 inact.	51	0.79 (0.53-1.17)					51	0.52 (0.31-0.87)
P10 HCV-RNA genotype 3a native	12	1.05 (0.58-1.91)					12	0.70 (0.27-1.79)
P0025 HIV-RNA subtype B	60	1.00 reference	48	1.00 reference	24	1.00 reference	132	1.00 reference
P0022 WHO HIV-RNA IS 97/650	40	0.56 (0.33-0.90)	291	0.72 (0.50-1.00)	229	0.92 (0.46-1.79)	557	0.70 (0.53-0.92)
P0026 HIV-RNA subtype B inact.	52	0.48 (0.28-0.75)					52	0.54 (0.34-0.84)

#for HBV Ultrio Plus and Ultrio Elite combined

Relative potencies of HBV standards in Ultrio versions

The combined Ultrio Plus and Ultrio Elite confirm the calibration of the VQC-Sanquin, WHO 97/750. Eurohep and Chimp genotype A standards since the confidence limits of the relative potencies overlap the value 1.0. Hence the conversion factor of 5.33 copies per IU in the bDNA assay is confirmed. However the heat-inactivated HBV standard seems to be detected with somewhat lower efficiency, although the potency of 0.60 (0.28-1.27) was not significantly different from the native standard in contrast to the Ultrio assay that detected HBV in the heat inactivated standard with significantly lower efficiency (potency 0.28 (0.12-0.63)).

Relative potencies of HIV standards in Ultrio versions

Based on a conversion factor of 0.58 copies/IU we found a significant difference in potency between WHO 97/650 and VQC-Sanquin standard in the Ultrio assay, but not in the Ultrio Plus and Elite assays. Overall there was a borderline significantly lower potency of 0.70 (0.53-0.92) observed. We suspect that this result may be influenced by the fact that not always the same TMA reagent batches were used for testing the two standards. The same may also be true for the heat-inactivated standard that was tested with a lower potency of 0.48 (0.28-0.74) by Ultrio in Denmark and Ireland, while the VQC-Sanquin standard was tested in Poland and South Africa.

Relative potencies of HCV standards in Ultrio versions

The conversion factor of 2.73 copies per IU (established mainly in studies with the first and second (96/790 and 97/798) WHO standards) was confirmed by a potency of 1.15 (0.74-1.79) in the Ultrio assay. However, in later experiments with the same standard dilution panels the potency reduced to 0.60 (0.33-1.03) and 0.48 (0.26-0.82) in Ultrio Plus and Ultrio Elite respectively. We suspect that this may be caused by instability of the WHO 06/100 standard (personal communication Phil Minor, NIBSC, data presented at SoGAT meetings). The potency of the betapropiolactone inactivated genotype 3a standard was estimated at 0.79 (0.53-1.17) in Ultrio, not significantly different from the VQC-Sanquin genotype 1 standard. If however the VQC-Sanquin standard dilution data in Ultrio Plus and Elite were added to the data base the difference became significant (potency 0.52 (0.31-0.87)).

Relative potencies of HBV, HCV and HIV-1 standards in TaqScreen versions

Table 20 summarizes the relative potencies of the standard dilution panels calculated by parallel line probit analysis against the native VQC-Sanquin standards calibrated in copies/mL. Both in TaqScreen 1.0 and 2.0 there was not a significant difference in potency between the native VQC-Sanquin and the first established WHO standards, which confirm the original calibration and conversion factors from IU to copies of 5.33, 2.73 and 0.58 for HBV, HCV and HIV-1 respectively. When comparing the potency of the inactivated BQC standards against the native VQC-Sanquin standards there was no significant difference either for HBV and HCV, again confirming the calibration in the bDNA assay. Unfortunately no data were available for the native VQC-Sanquin HIV-1 standard. When compared to the assumed copy number of the WHO 97/650 standard the potency of the inactivated HIV-1 subtype B standard was significantly higher in the cobas MPX 6800 assay. We have no explanation for this result. However when compared to the native VQC-Sanquin HIV subtype C standard (calibrated against the VQC-Sanquin HIV subtype B standard) the potency was not significantly different.

Table 20. Relative potency of HBV, HCV and HIV-1 standards calibrated in copies/mL against the VQC-Sanquin standards in TaqScreen versions

standard dilution panel	n	TaqScreen 1.0 potency (CI)	n	cobas MPX 6800 potency (CI)
S2384 PeliCheck HBV genotype A	12	1.00 (reference)		
EFS WHO HBV-DNA 97/746	24	0.79 (0.41-1.39)		
P0001 Eurohep HBV-DNA genotype A2	12	1.27 (0.64-2.61)		
P0007 HBV-DNA genotype A2			24	1.00 (reference)
P0023 WHO HBV IS 97/750			12	1.14 (0.59-2.17)
P0031 HBV-DNA genotype A inact.			12	0.77 (0.41-1.39)
S2391 PeliCheck HCV genotype 1	12	1.00 reference)		
EFS WHO HCV 96/798	24	0.78 (0.43-1.36)		
P0019 HCV-RNA genotype 1			36	1.00 (reference)
P0020 HCV-RNA genotype 3 inact.			12	1.18 (0.63-2.24)
S2398 PeliCheck HIV subtype B	12	1.00 (reference)		
EFS WHO HIV subtype B 97/650	24	0.94 (0.55-1.56)		
P0025 HIV-RNAsubtype B		n.d.		n.d
P0022 WHO HIV-RNA IS 97/650			12	1.00 (reference)#
P0026 HIV-RNA subtype B inact.			12	2.82 (1.11-6.92)
P0027 HIV-RNA subtype C			24	4.04 (1.96-11.0)

P0025 HIV-RNA subtype B VQC-Sanquin standard not tested

Verification of calibration of inactivated BQC standards against WHO standards by Ct analysis in cobas MPX version

Marco Koppelman (Sanquin, the Netherlands) kindly provided the Ct values on the WHO and inactivated standard panels found with the cobas MPX assay. When using the following formula one obtains a value for each of the replicate tests on the standard dilutions which is constant for all standard dilutions.

2Log(copies/mL in standard dilution) + Ct value on standard dilution

When comparing the individual values for two standards using dosages above the 95% LOD by Student t-test one can calculate the potency (and 95% CI).
Unfortunately no Ct values were available on the native VQC-Sanquin standard dilutions. Therefore the potency (and 95%CI) of the inactivated BQC standards were calculated against the WHO standards as the reference (except for HCV the VQC-Sanquin standard was used since the frozen WHO 06/100 standard dilution panel was found to be not stable). Table 21 compares the relative potencies found by Ct analysis with those obtained by PLA probit analysis. Surprisingly for HCV the potency values for the Ct analysis and probit analysis did not overlap indicating that in this particular experiment the Ct values were not predictive for the analytical sensitivity.

Table 21. Comparison of potency calculated by Ct analysis and PLA probit analysis

standard dilution panel	n series	n values	Ct analysis potency (CI)	n series	PLA probit analysis potency (CI)
P0007 HBV-DNA genotype A2			n.d.	24	1.00 (reference)
P0023 WHO HBV IS 97/750	12	58	1.00 (reference)	12	1.14 (0.59-2.17)
P0031 HBV-DNA genotype A inact.	12	41	1.29 (1.03-1.62)	12	0.77 (0.41-1.39)
P0019 HCV-RNA genotype 1	12	60	1.00 (reference)	36	1.00 (reference)
P0020 HCV-RNA genotype 3 inact.	12	20	0.41 (0.33-0.51)	12	1.18 (0.63-2.24)
P0025 HIV-RNA subtype B			n.d.		n.d.
P0022 WHO HIV-RNA IS 97/650	12	36	1.00 (reference)	12	1.00 (reference)#
P0026 HIV-RNA subtype B inact.	12	36	2.00 (1.66-2.42)	12	2.82 (1.11-6.92)
P0027 HIV-RNA subtype C				24	4.04 (1.96-11.0)

Verification of calibration of WHO replacement standards by parallel line probit analysis in TMA assay versions

Over the years Chiron Corporation and Novartis Diagnostics have supported customer validation studies by providing the same batches of WHO standard dilution series to different laboratories. The available TMA data on the dilution panels allowed for comparison of the potency of the first and second (and third) WHO standards. As can be seen in table 22 there was no significant difference in the virus concentration per IU in the first and second WHO standards for HBV-DNA, HCV-RNA and HIV-RNA according to probit analysis in the TMA assay versions. This is not surprising for HBV and HCV since these were different freeze-dry runs starting from the same batch of liquid material. However the potency of the 3rd HCV WHO standard (06/100) was significantly lower, indicating that the amount of HCV per IU has become lower. This may be due to stability problems that have been observed with the 3rd and 4th WHO HCV standards (personal communication Phil Minor, NIBSC, SOGAT meetings Rome, Ljubljana, London).

Table 22. Potency of WHO HBV, HCV and HIV replacement standards against the first established International Standards estimated by probit analysis on data from validation studies of users of Procleix assays of similar sensitivity

Virus	Assay	WHO standard	n	Potency (95% CI)
HBV	Ultrio	97/746	733	1.00 (reference)
	Ultrio	97/750	88	1.14 (0.57-2.35)
HCV	Duplex Ultrio	96/798	72 522	
	All Procleix		594	1.00 (reference)
	Duplex Ultrio	96/790	24 24	
	All Procleix		48	1.04 (0.71-1.50)
	Ultrio Ultrio Elite Ultrio Plus	06/100	86 245 419	
	All Procleix		750	0.78 (0.67-0.89)
HIV	Ultrio Ultrio Elite Ultrio Plus	97/650	94 231 393	
	All Procleix		718	1.00 (reference)
	dHIV Duplex	97/656	48 83	
	All Procleix		131	1.00 (0.7-1.30)

Summary of potency data of inactivated BQC and native VQC-Sanquin standards in different methods

Table 23 summarizes the potency results that were found in the experiments described above. When comparing the potency values one has to bear in mind that the data of the Ultrio versions were not obtained in the same test runs and can be influenced by laboratory and reagent batch variation. However the Ct analyses in TaqScreen 2.0 and 3.0 versions were performed in parallel assays. When interpreting all data we suspect that the copy numbers assigned to the inactivated HCV standard may be slightly overestimated. It also may be that Ultrio versions are less efficient in detecting viral nucleic acid in the inactivated standards.

Table 23. Summary of potency values of viral nucleic acid copies of inactivated BioQ standards relative to those of native VQC-Sanquin standards in different experiments/methods.

Method	n	Potency of inactivated relative to native standard (95%CI)		
		HBV	HCV	HIV
Original calibration in bDNA assay (table 11)#	6	1.00 (reference)	1.00 (reference)	1.00 (reference)
Second bDNA experiment	3, 22		0.68 (not calculated)	0.87 (not calculated)
Ultrio versions combined (table 17)	49-172	0.60 (0.28-1.27)	0.52 (0.31-0.87)	0.54 (0.34-0.84)
TaqScreen 2.0 Ct analysis (table 12)	36	0.88 (0.78-0.99)	0.44 (0.28-0.60)	1.06 (0.91-1.21)
TaqScreen 3.0 Ct analysis (table 21)	20-60	nd	0.41 (0.33-0.51)	nd
TaqScreen 3.0 PLA probit analysis (table 21)	12	nd	1.18 (0.63-2.24)	nd
Geometric mean value all methods		0.81	0.65	0.84

Table 24. Summary of analytical sensitivity studies of NAT blood screening assays on native VQC-Sanquin and inactivated BQC standard dilutions

Standard dilutions	Assay	LOD	LODs (95%CI) in copies/mL_		
			HBV	HCV	HIV
native VQC-Sanquin*	Ultrio Plus (table 16a)	50%	5.7 (3.4-9.7)	2.8 (1.8-4.3)	2.6 (1.6-4.1)
		95%	49.4 (27-103)	12.2 (7.5-23)	12.8 (7.7-25)
	TaqScreen 1.0 (table 16a)	50%	2.8 (1.7-4.8)	4.9 (3.2-7.5)	3.2 (2.0-5.0)
		95%	24.5 (14-51)	21.5 (13-40)	15.7 (10-30)
native VQC-Sanquin#	Ultrio versions combined (table 14)	50%	3.7 (2.4-5.7)	1.9 (1.5-2.4)	1.6 (1.3-2.1)
		95%	30.3 (19-51)	14.5 (11-20)	10.8 (8.2-15)
	Cobas MPX 6800 (table 17)	50%	1.9 (1.3-2.7)	3.2 (2.2-4.6)	nt
		95%	13.0 (7.7-29.7)	22.6 (13.4-51.7)	nt
Inactivated BioQ#	Ultrio versions (table 14)	50%	6.1 (3.3-11.3)	3.6 (2.3-5.7)	3.1 (2.1-4.4)
		95%	50.6 (27-100)	27.6 (17-46)	20.1 (14-31)
	TaqScreen 2.0 (table 13)	50%	2.8 (1.5-4.3)	5.2 (3.3-7.8)	2.0 (1.3-2.8)
		95%	23.8 (12-101)	35.2 (19-116)	7.6 (4.9-21)
	Cobas MPX 6800 (table 17)	50%	2.4 (1.4-4.2)	2.5 (1.3-4.4)	1.0 (0.6-1.6)
		95%	18.6 (9.1-75.9)	15.6 (7.6-77.7)	5.8 (3.0-23.2)
Inactivated ViraQ (Trend Controls)§	Ultrio versions hit rate at 25 copies/mL		179/193 (92.7%)	2497/2702 (92.4%)	2583/2690 (96.0%)
	Ultrio versions hit rate at 50 copies/mL		2381/2411 (98.8%)		

*panels manufactured by VQC-Sanquin #panels manufactured by BioQControl

When comparing LODs of the native and inactivated standards (table 24), the latter were indeed significantly higher in the Ultrio versions but not in the TaqScreen versions. This indicates that the differences in LODs in the Ultrio versions are to a large extent caused by laboratory variation.
When comparing the 95% LODs in the Ultrio versions with response rates on the 25 copies/mL concentrations in the ViraQ run controls (table 24) the data were in line with the 95% LODs on the inactivated standard dilution panels for HIV and HCV when taking the calibration data of this report into account. It may be that with approximately 1.5 fold correction of the HCV concentration in the HCV standard the response rate on 25 copies/mL Trend Controls would become comparable to that of HIV. For HBV the reactivity on 50 copies/mL concentrations in the ViraQ run control were somewhat higher than expected and for that reason we decided to introduce a 25 copies/mL Trend Control that had comparable reactivity to the HCV control. Probably a concentration of 37.5 copies/mL of heat inactivated HBV would give a response rate closer to 95%. The available data on the inactivated standard dilutions indicate that a 25 copies/mL Trend control would give response rates above 95% for HBV and HCV and probably near 100% for HIV in the cobas MPX assay.

Summary of limiting dilution data on native VQC-Sanquin and inactivated BioQ standards in different methods

Table 25. Theoretical efficiency of NAT methods as deduced from 63%LODs in analytical sensitivity studies

Standard dilutions	Assay	Percent NAT efficiency (95%CI)		
		HBV	HCV	HIV
native VQC-Sanquin*	Ultrio Plus (table 16b)	23% (13-39)%	54% (33-83)%	46% (27-77)%
	TaqScreen 1.0 (table 16b)	27% (15-45)%	18% (11-28)%	19% (11-32)%
native VQC-Sanquin#	cobas MPX 6800 (table 17)	36% (23-53)%	21% (14-31)%	n.d.
	Ultrio versions combined (table 15)	36% (23-55)%	70% (54-90)%	83% (65-106)%
Inactivated BioQ#	Ultrio versions combined (table 15)	21% (11-40)%	36% (23-57)%	45% (30-65)%
	TaqScreen 2.0 (table 13)	23% (14-37)%	13% (8-19)%	38% (25-62)%
	cobas MPX 6800 (table 17)	27% (13-48)%	28% (13-52)%	73% (36-125)%

*panels manufactured by VQC-Sanquin #panels manufactured by BioQControl

From the data in Table 25 it seems that the Ultrio versions are less efficient in detecting HBV, HCV and HIV in the inactivated standards than in the native standards. By contrast detection efficiencies on native and inactivated standards were comparable in the cobas MPX 6800 assay. The data in the Ultrio versions may indicate that that the HCV concentration in the inactivated standard is genuinely lower than in the native standard as can be deduced from the potency results. However this is not confirmed when the data are compared in the cobas MPX 6800 assay. One has to bear in mind that the variation in detection efficiency can also be caused by reagent batch variation. There seems to be a significant increase in the detection efficiency of TaqScreen 3.0 for HIV.

Although the detection efficiency of HBV is significantly lower than that of HCV and HIV in the Ultrio versions we believe that the quantification in copy numbers by the bDNA assay was correct. This conclusion can be drawn from comparison of the detection efficiencies in the TaqScreen versions.

When the calibration in copies/mL by the bDNA assay on the native VQC-Sanquin standards is correct the Ultrio versions reach the highest NAT efficiencies for HCV and HIV and may fail to detect 10-20% of virions in the samples. Obviously the combination of target capture and RNA amplification by TMA is highly efficient for HCV and HIV. Probably the double stranded fractions of HBV-DNA and the tightly attached polymerase protein makes it more difficult to capture single stranded HBV-DNA in the Ultrio versions and this is probably the reason for the lower efficiency for this marker[36]. It may be that the DNA extraction technology of the Roche MPX system in combination with the heating cycles and availability of two strands for amplification in PCR makes the TaqScreen assay relatively more efficient for HBV detection. If a higher efficiency of HBV detection as observed in TaqScreen versions would also be possible for HBV by modification of the Ultrio assays the HBV detection efficiency would likely become more comparable with that of HIV and HCV in TMA. This reasoning and the recently observed higher detection efficiencies on the inactivated BioQ HIV standard tested in the dual region cobas 6800 TaqScreen assay further supports that the calibration in copies/mL in the VQC-Sanquin standards has been close to true (defective or infectious) virion concentrations.

Summary of potency data of who standards against VQC-Sanquin standards

In our analysis we assigned copy numbers to the first established WHO standards based on bDNA 3.0 experiments using the VQC-Sanquin standard as the reference (Figure 1). As described above the conversion

factors determined by these calibration experiments were 5.33, 2.73 and 0.58 copies/IU for HBV, HCV and HIV respectively. Table 26a and 26b compare the potency values of WHO standards against the VQC-Sanquin standards as the reference using the data of analytical sensitivity studies with the different Ultrio and TaqScreen assay versions. The combined probit analysis data of multiple experiments per assay version generally confirm the original calibration (and thus the copy number per IU). In some cases the fiducial limits did not overlap the potency value 1.00. This can be explained by laboratory and reagent variation or stability issues with the WHO HCV 06/100 standard.

Table 26a Summary of potency values of WHO standards against VQC-Sanquin standards in analytical sensitivity studies.

BQC Standard dilution panel	n	Ultrio potency (CI)	n	Ultrio Plus potency (CI)	n	Ultrio Elite potency (CI)	n	TaqScreen 3.0 potency (CI)
P0007 HBV-DNA genotype A	24	1.00 (reference)	48	1.00 (reference)	50	1 (reference)	24	1 (reference)
P0023 WHO HBV-DNA IS 97/750	32	1.42 (0.59-3.48)	303	1.12 (0.58-2.15)	252	0.64 (0.44-0.91)	12	1.14 (0.59-2.17)
P0019 HCV-RNA genotype 1	36	1.00 (reference)	48	1.00 (reference)	88	1.00 (reference)		
P0024 WHO HCV-RNA IS 06/100	32	1.15 (0.74-1.79)	291	0.6 (0.33-1.03)	250	0.48 (0.26-0.82)		
P0025 HIV-RNA subtype B	60	1.00 (reference)	48	1.00 (reference)	24	1.00 (reference)		
P0022 WHO HIV-RNA IS 97/650	40	0.56 (0.33-0.90)	291	0.72 (0.50-1.00)	229	0.92 (0.46-1.79)		

Table 26b. Summary of potency values of WHO standards against VQC-Sanquin standards in analytical sensitivity studies (continued)

VQC-Sanquin standard dilution panel	n	TaqScreen 1.0 potency (CI)
S2384 PeliCheck HBV genotype A	12	1.00 (reference)
EFS WHO HBV-DNA 97/746	24	0.79 (0.41-1.39)
P0001 Eurohep HBV-DNA genotype A2	12	1.27 (0.64-2.61)
S2391 PeliCheck HCV genotype 1	12	1.00 (reference)
EFS WHO HCV 96/798	24	0.78 (0.43-1.36)
S2398 PeliCheck HIV subtype B	12	1.00 (reference)
EFS WHO HIV subtype B 97/650	24	0.94 (0.55-1.56)

7. DISCUSSION

This report summarizes 20 year data on the VQC-Sanquin standards that are calibrated in copies/mL in multiple replicate bDNA 3.0 assays. Limiting dilution data in the blood screening assays used during the last decade strongly indicate that the calibration in bDNA 3.0 tests has generated values that are close to the true amounts of viral particles in the VQC-Sanquin standards. Cross calibration of Japanese chimpanzee plasmas of known infectivity indicate that the 50% chimpanzee infectious dose would have been approximately 4 to 8 virions in the HBV and HCV acute phase challenge plasmas. This result and the maximum TMA and PCR efficiency levels reaching 70-80% for HCV and HIV further support the correct calibration in copy numbers in the VQC Sanquin standards. The maximum TMA efficiency observed for HBV (36%) was significantly lower than for the RNA viruses but since the opposite was found for PCR TaqScreen 1.0 (27% for HBV versus 18-19% for HCV and HIV on the same standard dilution panels) we believe the bDNA calibration in copies/mL must have been reliable.

We therefore use the LODs in copies/mL on the VQC-Sanquin standards in combination with worst case 50% minimum infectious doses of 3.16 (half log value between 1 and 10 virions) to estimate residual window period transmission risk by blood transfusion.[18,21]. Hence the calibration data of the BQC and VQC-Sanquin standards in copies/mL described in this report are fundamental for understanding blood safety[22-26.]

The VQC-Sanquin standards have also been extensively calibrated against the first two lots of WHO standards[9-11,27,28]. The VQC-Sanquin standards can be considered secondary standards when calibrated in IU/mL and primary standards when calibrated in copies/mL. We have calculated 95% confidence limits for both the copy numbers and the IU values in the VQC-Sanquin standards following the metrological principles of the ISO guide 17511:2003. The average conversion factors were found to be 5.33, 2.73 and 0.58 copies/IU for HBV, HCV and HIV respectively. Currently the 3rd to 5th WHO standards are in use. It must be emphasized that we cannot guarantee that historically measured IU values in the VQC-Sanquin standards will be the same when measured against the current WHO replacement standards.

The native VQC-Sanquin standards have been subjected to inactivation processes of proven virucidal efficacy (CE4006) to obtain secondary standards suitable for large scale production of external quality control reagents. The inactivated BioQ standards have been calibrated in copies/mL against the native VQC-Sanquin standards using replicate bDNA assays in the same test runs. The correct calibration against the native VQC-Sanquin standards has been checked and confirmed for HBV and HIV with different methods as shown in this report. For the inactivated HCV genotype 3 standard it may be that the original calibration in the bDNA 3.0 assay has overestimated the concentration as in a second calibration experiment the values were 1.5 fold lower. Also according to Ct value analysis in TaqScreen 2.0 and cobas MPX the relative concentration in the inactivated BioQ genotype 3 standard 2.3-2.5 fold lower than in primary VQC-Sanquin standard. However there was no difference between the two standard in limiting dilution parallel line probit analysis in the cobas MPX assay (limited data). By contrast the potency in the Ultrio assay also confirmed a 2-fold lower potency of the copy number assigned to the inactivated genotype 3 standard. Therefore in 2019 we performed another calibration study.

8. SUPPLEMENTAL CALIBRATION DATA AFTER 2018

Supplemental calibration study of native and inactivated HCV standard performed in 2019

P0344 ViraQ HCV Quant 1000 (a 1000 copies/mL dilution of the S0009 primary VQC-Sanquin HCV genotype 1 standard) was tested against 1000 copies/mL samples (S1431) of the S0109 inactivated HCV genotype 3 standard in two quantitative NAT assays. Each product was tested in 8 replicates in the Roche cobas MPX assay (by Dr Marco. Koppelman, Sanquin, Amsterdam) and in the Hologic Aptima HCV Quant assay (by Dr. David Kwa, OLVG, Amsterdam). The cobas MPX was performed according to the batch release control procedure (QCF4007-106).

Potency comparison in cobas MPX assay.
The potency of the S1431 (1000 cp/mL genotype 3 standard dilution) was 0.40 (0.24-0.68) of the P0344 1000 copies/mL genotype 1 standard, comparable to the potency of 0.41 (0.33-0.51) found before (table 21 and 23). Hence based on calibration in the cobas MPX assay the concentration of S1431 would be 400 copies/mL instead of 1000 copies/mL and the concentration in P0273 ViraQ Multi-Marker would be 30 copies/mL instead of 75 copies/mL.

Potency comparison in Grifols Ultrio assay versions and in Hologic Aprima HCV Quant assay
In the Ultrio assay versions the potency of the inactivated HCV genotype 3 as compared to the native genotype 1 standard was 0.52 (0.31-0.87). Since the Grifols Ultrio and Hologic Aptima are both based on TMA we decided to also test the same two 1000 copies/mL products (P0344 and S1431) in the Hologic Aptima viral load assay (table 27). In the first Aptima experiment the 1 mL samples were not thawed in a water bath at 37˚C and this may have introduced a bias if there would have been differences in stability between the two standards. However if two potential outlier values were removed the potency results of the two experiments were comparable and overall the same potency result of 52% was found with and without the removal of the

potential outlier values. According to this calibration experiment in the Hologic Aptima assay the S1431 sample contains 520 copies/mL, comparable to the overall potency in the Ultrio assay versions.

Table 27. Comparison of 1000 copies/mL samples of the native S0009 HCV genotype 1 with 1000 copies/mL samples of the inactivated S0109 HCV genotype 3 standard in the Hologic Altima HCV Quant assay

Test: Hologic Aptima HCV viral load (IU/ml)			
Date: 2019-11-15		Date: 2019-12-17	
P0344 B4328-001		P0344 B4328-001	
Nr.	IU/mL	Nr.	IU/mL
1	143	1	219
2	115	2	203
3	101	3	254
4	107	4	199
5	129	5	203
6	(272)	6	263
7	152	7	274
8	128	8	225
geomean	136.6	geomean	228.4
S1431 B4996-001		S1431 B4996-001	
Nr.	IU/mL	Nr.	IU/mL
1	62	1	54
2	46	2	78
3	66	3	(123)
4	65	4	61
5	56	5	94
6	52	6	66
7	89	7	75
8	81	8	92
geomean	63.3	geomean	77.9
Potency (95%CI)	46%(N.C)		57% (N.C)
potency^	51%		53%
potency overall		52%	

^without potential outlier values between brackets
(N.C) 95% CI could not be calculated

The Hologic assay is calibrated in IU/mL and the data show approximately two-fold differences in concentration between the 1000 copies/mL standards. When the last experiment was taken as the most reliable (because in this experiment samples were handled correctly the conversion factor in the primary S0009 HCV standard was 4.38 copies/IU in the Hologic assay, whereas according to historical calibration described in this report the factor was 2.73. Hence according to calibration against the 1st and 2nd WHO standard the P0344 ViraQ Quant 1000 control contains 366.3 IU/mL whereas 228.4 IU/mL was measured by the Aptima assay. This is 62% of the expected value according to historical calibration.

Potential impact of 2.5 fold recalibration of S0109 HCV standard on P0273 ViraQ run control performance in cobas MPX assay

Table 28 gives the average Ct values on the P0273 ViraQ Multimarker 75 control in one lot of the cobas MPX assay in two blood screening labs (Netherland and Belgium). Based on historical calibration in the in activated HBV, HCV and HIV standards in the bDNA 3.0 assay the product contains 75 copies/mL for all markers. As described above the Ct values in the cobas MPX are higher in the HCV than in the HBV and HIV assays but these are not predictive for the 95% and 50% LOD. The assay is designed so that HCV generates higher Ct values than HBV and HIV. If calibration based on the Ct values in the cobas MPX was used and the concentration in the run control would be increased 2.5 fold it can be estimated that the Ct values would reduce by a Ct distance of 1.32. The average Ct values would then still be 1.78-1.90 higher than for HIV and HBV (which would correspond to a dilution factor of 3.5-3.7). Hence the Ct values are not indicative for the viral concentration of the three markers in copies/mL.

Table 28. Average Ct values on P0273 ViraQ Multimarker 75 control in two labs and potential impact on Ct values if HCV concentration would be increased 2.5 fold

marker	Copies/mL based on original bDNA 3.0 calibration	Copies/mL with 2.5 fold S0109/S0009 recalibration based on Ct values in cobas MPX	n NL	n BE	Avg (Std) NL	Avg (Std) BE
HBV	75	~75	42	22	34.33 (0.42)	34.33 (0.31)
HCV	75	30	42	22	37.43 (0.55)	37.43 (0.64)
HCV 2.5 fold adjusted	*188*	*75*			*36.11 (0.55)*	*36.11 (0.55)*
HIV-1	75	~75	42	22	34.21 (0.40)	34.33 (0.31)

Recalibration of S0109 HCV standard based on multiple methods and expected impact on ViraQ HCV run control performance

Table 29 gives an overview of the reactivity rates on the native S0009 HCV genotype 1 standard dilution panels and the inactivated S0109 HCV genotype 3 standard dilution panels in the Grifols Ultrio versions and cobas MPX assay. The reactivity rates on the standard dilution panels are compared with those found on the P0067 HCV Trend 25 and P0273 Check 75 controls.

Table 30a and b present the 50% and 95% LODs in Ultrio versions based on the data in table 29. According to these data the potency of the inactivated S0109 HCV genotype 3 copies to the native S0009 HCV genotype 1 copies is 48 (36-62)% (close the 52% estimated by the Hologic Aptima assay). Increasing the concentration of the P0067 trend control from 25 to 50 copies/mL of the S0109 inactivated HCV genotype 3 would increase the reactivity rate from 93% to 98% (table 30a).

For comparison the reactivity rate on P0068 ViraQ HIV-1 Trend 25 was 96.0% in 2689 Ultrio Plus and Elite runs test runs (table 29). Table 30a gives an impression of how much the concentration should be increased in the P0067 ViraQ HCV Trend 25 control to increase the reactivity rate from the observed 93% on the HCV trend control to 96.0% as observed on the HIV trend control. To increase the reactivity rate by 3% in this range around the 95% LOD the concentration in the HCV trend control should increase 1,41 fold.

Table 29. Reactivity rates on HCV genotype 1 and inactivated HCV genotype 3 standard dilution panels as comparted to observed reactivity rates

panel	name	member	copies /mL	Ultrio r/n (%)	Ultrio Plus r/n (%)	Ultrio Elite r/n (%)	cobas MPX r/n (%)
P0019	HCV-RNA genotype 1	B4016-01	2720	30/30 (100%)	24/24 (100%)	64/64 (100%)	12/12 (100%)
P0019	HCV-RNA genotype 1	B4016-02	992	24/24 (100%)	24/24 (100%)	64/64 (100%)	12/12 (100%)
P0019	HCV-RNA genotype 1	B4016-03	272	30/30 (100%)	36/36 (100%)	88/88 (100%)	60/60 (100%)
P0019	HCV-RNA genotype 1	B4016-04	99.2	36/36 (100%)	48/48 (100%)	112/112 (100%)	60/60 (100%)
P0019	HCV-RNA genotype 1	B4016-05	27.2	36/36 (100%)	48/48 (100%)	112/112 (100%)	60/60 (100%)
P0019	HCV-RNA genotype 1	B4016-06	9.92	29/36 (81%)	45/48 (94%)	108/112 (96%)	47/60 (83%)
P0019	HCV-RNA genotype 1	B4016-07	2.72	15/36 (42%)	29/48 (60%)	69/112 (62%)	30/60 (50%)
P0019	HCV-RNA genotype 1	B4016-08	0.99	6/36 (17%)	10/48 (21%)	33/112 (29%)	12/60 (33%)
P0019	HCV-RNA genotype 1	B4016-09	0.27	3/36 (8%)	5/48 (10%)	6/88 (7%)	1/36 (0%)
P0019	HCV-RNA genotype 1	B4016-10	0.099	0/24 (0%)	2/36 (6%)	0/88 (0%)	0/36 (0%)
P0272	MM HCV-RNA genotype 1	B4263-01	50.00				48/48 (100%)
P0272	MM HCV-RNA genotype 1	B4263-02	25.00				48/48 (100%)
P0272	MM HCV-RNA genotype 1	B4263-03	12.50				44/46 (96%)
P0272	MM HCV-RNA genotype 1	B4263-04	6.25				34.48 (71%)
P0272	MM HCV-RNA genotype 1	B4263-05	3.12				21/48 (44%)
P0272	MM HCV-RNA genotype 1	B4263-06	1.56				17/48 (35%)
P0272	MM HCV-RNA genotype 1	B4263-07	0.78				4/46 (9%)
P0020	HCV-RNA genotype 3 inact.	B4031-01	3450	49/49 (100%)			
P0020	HCV-RNA genotype 3 inact.	B4031-02	1160	52/52 (100%)			
P0020	HCV-RNA genotype 3 inact.	B4031-03	345	51/51 (100%)			
P0020	HCV-RNA genotype 3 inact.	B4031-04	116	52/52 (100%)			10/10 100%)
P0020	HCV-RNA genotype 3 inact.	B4031-05	34.5	52/52 (100%)			10/10 (100%)
P0020	HCV-RNA genotype 3 inact.	B4031-06	11.6	38/51 (75%)			9/10 (90%)
P0020	HCV-RNA genotype 3 inact.	B4031-07	3.45	24/48 (50%)			7/10 (70%)
P0020	HCV-RNA genotype 3 inact.	B4031-08	1.16	8/51 (16%)			1/10 (10%
P0020	HCV-RNA genotype 3 inact.	B4031-09	0.345	3/52 (6%)			1/10 (10%
P0020	HCV-RNA genotype 3 inact.	B4031-10	0.116	0/52 (0%)			
P0067	ViraQ HCV Trend 25	B4062	25			3160/3393 (93.1%)	
P0067	ViraQ HCV Trend 25	B4062-009-013	25				70/80 (87.5%)
P0273	ViraQ Multi-Marker 75	B4254-001-005	75				80/80 (100%)
P0068	*ViraQ HIV-1 Trend 25*	*for ref*	*25*			*2582/2689 (96.0%)*	

Table 30a. LODs in parallel line probit analysis using dose response data presented in table 29

S0009 HCV genotype 1 copies/mL	Ultrio versions probit %	Potency factor to 95% LOD	S0109 HCV genotype 3 copies/mL	Ultrio versions probit %	Potency factor to 95% LOD
5,4	80%	0,37	11,2	80%	0,37
6,8	85%	0,47	14,2	85%	0,47
9,2	90%	0,64	19,3	90%	0,64
9,9	91%	0,69	20,7	91%	0,69
10,8	92%	0,74	22,4	92%	0,74
11,7	93%	0,81	24,5	93%	0,81
12,9	94%	0,89	27	94%	0,89
14,5	95%	1	30,1	95%	1
16,5	96%	1,14	34,3	96%	1,14
19,4	97%	1,34	40,3	97%	1,34
23,9	98%	1,66	49,9	98%	1,66
33,5	99%	2,32	69,8	99%	2,32

Table 30b . LODs in parallel line probit analysis using dose response data presented in table 29.

Assay	n	standard	50% LOD (CI)	95% LOD (CI)
Ultrio versions	196	S0009 HCV genotype 1	1.91 (1.68-2.17^	14.5 (12.1-17.7)
Ultrio versions	48-3393	S0109 inact HCV genotype 3	3.97 (3.42-4.54)^	30.1 (27,9-32.7)

^ 2.08 (1.62-2.77) fold difference in S0009/S0109 copy potency in Ultrio versions

Table 30c present the 50% and 95% LODs in cobas MPX on the S0009 HCV genotype 1 standard and the S0109 HCV genotype 3 standard in parallel probit analysis using the data presented in table 29. According to this experiment the potency (95% CI) of the copies in the S0109 standard is 0.68 (0.43-1.0) of those in the S0009 standard. Conversely, the copy-units assigned to the HCV genotype 3 standard are 1.47 (0.98-2.3) fold weaker than those assigned to the S0009 HCV genotype 1 standard.

Table 30 c LODs in parallel line probit analysis using dose response data presented in table 29

Assay	n	standard	50% LOD (CI)	95% LOD (CI)
Cobas MPX	48-60	S0009 HCV genotype 1	2.9 (2.5-3.3)^	19.5 (15.4-25.9)
Cobas MPX	10-80	S0109 inact HCV genotype 3	4.2 (2.9-6.0)^	28.7 (20.5-41.0)

^ 1.47 (0.98-2.3) fold difference in S0009/S0109 copy potency in cobas MPX

Table 31 compares the calibration of the S0009 HCV genotype 1 and the S0109 HCV genotype 3 standard with 5 different methods. The overall geometric mean recalibration factor of the S0109 HCV genotype 3 standard against the HCV genotype 1 standard is **1.67-fold** less according to which factor the concentration in the undiluted S0109 sHCV genotype 3 standard would decrease from 6.06E+07 copies/mL to **3.64+07** copies/mL. If the concentration in the S0109 standard would be 1.67 fold reduced the concentration in the P0067 HCV Trend 25 Control would become 41.7 copies/mL based on the original assigned value and this would generate a reactivity rate of approximately 97%.

After discussion with the Notified Body and PEI experts we announced that we have recalibrated the S0109 HCV standard to the bold value in table 31.

Table 31. Overview of potency of inactivated S0109 HCV genotype 3a standard against native S0009 HCV genotype 1 standard

Assay	n	S0009/S0109 potency	Potency of S1431 HCV genotype 3a In copies/mL	Undiluted S0109 HCV genotype 3a inactivated copies/mL
original bDNA 3.0 tests	6	1.00	1000	60600000
bDNA 3.0 repeat tests	3	1.47	680	41224490
Probit analysis Ultrio versions	48-3393	2,08	481	29134615
Probit analysis Cobas MPX	10-80	1.47	680	41224490
Hologic Aptima	16	1.92	520	31512000
Cobas MPX Ct value	16	2.47	405	24543000
Overall geomean		**1.67**	**600**	**36385863**

The impact of recalibration of the S0109 standard (table 31) on the distance of the concentration in the ViraQ run controls to the 95% LOD HCV concentrations is presented in table 32.

Table 32. Impact of 1.67-fold recalibration of S0109 HCV standard (table 31) on the distance of the HCV concentration in ViraQ Controls of 25, 75 and 125 copies/mL to the 95% LOD

		95% LOD		Distance (factor) to 95% LOD	
		old calibration	new calibration	old calibration	new calibration
Product	Target assay	Original bDNA 3.0 experiment	Consensus multiple methods	Original bDNA3.0 experiment	Consensus multiple methods
		S0109 60,600,000 copies/mL	S0109 36,385,863 copies/mL	S0109 60,600,000 copies/mL	S0109 36,385,863 copies/mL
P0273 Multi-Marker Check 75	cobas MPX	28.7 (20.5-41.0)	17.2 (12.3-24.6)	2.61 (1.93-3.66)	4.36 (3.05-6.11)
P0067 HCV Trend 25	Ultrio Elite	30.1 (27,9-32.7)	18.0 (16.7-19.7)	0.83 (0.76-0.90)	1.39 (1.27-1.50)
P0063 HCV Check 125	Ultrio Elite	30.1 (27,9-32.7)	18.0 (16.7-19.7)	4.15 (3.80-4.48)	6.94 (6.34-7.48)

The 1.67-fold lower concentration by recalibration of the S0109 HCV standard is necessary to have sufficient distance to the 95% LOD in the P0273 ViraQ Multi-Marker Control. The distance then becomes comparable to the that of the inactivated HBV standard on which a 95% LOD of 18.6 (9.1-75.9) copies/mL was found.

Impact of recalibration of HCV standard on Cobas MPX Ct values

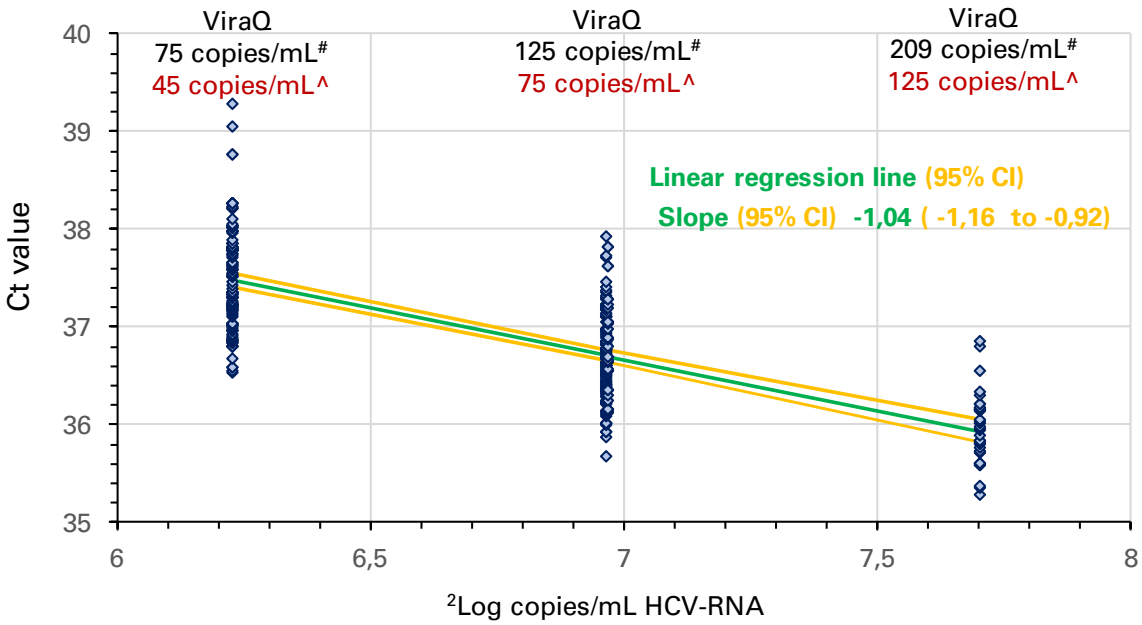
Table 33 summarizes the HCV batch release tests according to QCF4007 until May 2022. In five batch release tests either the test batch or reference batch had a 1.67-fold higher HCV-RNA concentration. Overall the average delta Ct value of 0.79 was not significantly different from the expected value of 0.74 with a slope of -1 (see Figure 2)

Table 33. Overview of cobas MPX Ct values and potencies of ViraQ test batches relative to reference batch in QCF4007 batch release testing. Yellow shaded data represent values in release tests whereby there was a 1.67-fold difference in HCV concentration between test batch and reference batch.

QCF4007	Test batch product Cat no	Ref/Test batch HCV cp/mL	n	Avg Ct Ref batch	SD Ref batch	Avg Test batch	SD Test batch	Delta Ct (ref-test)	Potency
95	P0063	125/125	8	37,05	0,41	36,46	0,29	0,59	151 (123-184)%
96	P0063	125/125	8	36,31	0,49	36,22	0,24	0,09	107 (85-134)%
107	P0063	125/125	8	36,82	0,43	36,92	0,49	-0,10	93 (72-121)%
128	P0063	125/125	8	36,61	0,30	36,81	0,32	-0,20	87 (73-104)%
157	P0063	125/125	8	36,58	0,45	36,63	0,45	-0,05	96 (74-124)%
170	P0063	125/125	8	36,82	0,41	36,82	0,39	0,01	101 (79-128)%
All			48	36,70		36,64		0,06	104 (..- ..)%
165	P0063	125/209	8	36,73	0,32	36,03	0,28	0,70	163 (133-199)%
171	P0063	209/209	8	35,93	0,28	36,42	0,47	-0,48	71 (55-93)%
177	P0063	209/209	8	35,69	0,29	35,89	0,27	-0,20	87 (74-103)%
All			16	35,81		36,15		-0,34	79 (..-..)%
91	P0067	25/25	8	39,02	0,97	39,01	0,48	0,01	101 (62-163)%
91	P0067	25/25	8	39,21	0,48	38,44	0,51	0,77	171 (120-243)%
113	P0067	25/25	8	38,92	0,62	38,87	1,00	0,05	104 (56-191)%
136	P0067	25/25	8	38,95	0,64	39,66	1,62	-0,71	61 (27-139)
All				39,03		39,00		0,03	102 (..-..)%
183	P0067	42/42	8	38,56	0,71	39,15	0,91	-0,59	66 (40-111)%
89	P0273	75/75	8	37,24	0,37	37,36	0,81	-0,12	92 (64-132)
89	P0273	75/75	8	37,31	0,56	37,45	0,49	-0,13	91 (68-123)%
127	P0273	75/75	8	37,39	0,56	37,33	0,39	0,07	105 (79-138)%
138	P0273	75/75	8	37,57	0,38	37,37	0,33	0,20	115 (94-141)%
143	P0273	75/75	8	37,71	0,51	37,60	0,71	0,12	108 (76-154)%
160	P0273	75/75	8	37,57	0,38	37,37	0,33	0,20	115 (89-147)%
160	P0273	75/75	8	37,71	0,51	37,60	0,71	0,12	108 (70-168)%
All			56	37,50		37,44		0,06	105 (..-..)%
163	P0273	75/125	8	37,53	0,46	36,94	0,60	0,60	151 (104-220)%
132	P0273	125/75	8	36,80	0,36	37,61	0,72	-0,82	57 (40-80)%
132	P0273	125/75	8	36,56	0,24	37,57	0,38	-1,01	50 (42-60)%
139	P0273	125/75	8	36,56	0,24	37,37	0,33	-0,80	57 (49-67)%
All			24	36,64		37,52		-0,88	
164	P0273	125/125	8	36,65	0,44	36,94	0,60	-0,28	82 (57-119)%
169	P0273	125/125	8	36,82	0,40	36,55	0,30	0,27	121 (94-155)%
All			16	36,74		36,74		0,00	
Overall Delta Ct for 1.67 higher S0109 concentration = 0,786 (n=40)									

When the individual cobas MPX Ct values (n=382) on Check Controls of 75, 125 and 209 copies/mL were plotted against the ^{2}Log HCV concentration in copies/mL we found a slope of -1.04 (95% CI: -1.16 to -0.92) by linear regression analysis (Figure 2). Since the slope does not differ significantly form -1 this justified our assumption that the Ct values of the post-market performance follow up (PMFU) data obtained by two national blood centers can be reduced with ^{2}Log 1.67 = 0.74 to predict the expected Ct values on the P0273 run controls with 1.67-fold higher HCV concentration. Figure 3 shows the predicted Ct values in the KI4268 package insert version 7.1 that were obtained by adjusting the Ct values with delta = -0.74. In Table 34 we compared the predicted Ct values from the PMFU data with the observed data in bath release testing on batches with 1.67-fold higher concentration (~125 copies/mL) and as expected the 95% CI's completely overlap.

Figure 2. Linear regression analysis on Ct values versus ^{2}Log copies/mL in three HCV concentrations of ViraQ Controls evaluated in batch release testing (table 33).



old calibration inactivated S0109 HCV standard
^ new calibration inactivated S0109 HCV standard

Figure 3. Post-market follow up (PMFU) data for cobas MPX Ct values obtained by two national blood centers testing the P0273 ViraQ Check 75 run control in 884 runs whereby individual Ct values were reduced by delta Ct = 0.74 to account for 1.67-fold higher HCV concentration in future P0273 batches.

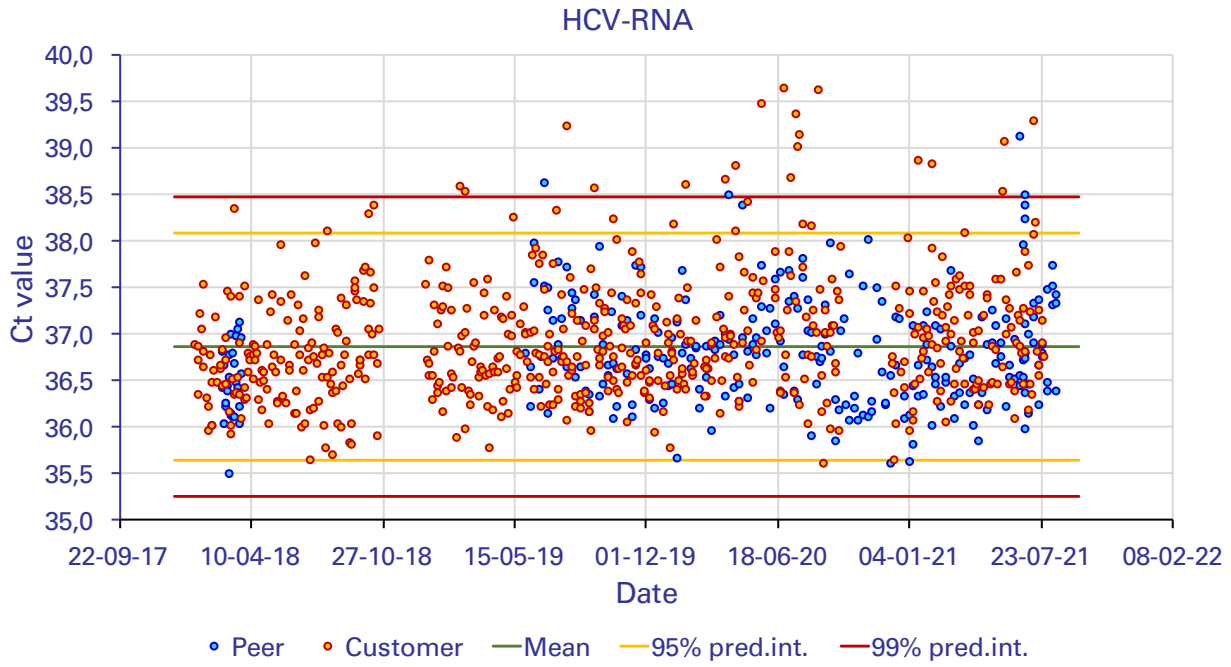


Table 34. Verification of predicted cobas MPX Ct values on P0273 ViraQ Multi-Marker Control and observed Ct values on run control with 1.67-fold higher HCV concentration.

study	Explanation	n	average Ct HCV	(95 % CI) Ct HCV
Predicted from PMFU data	Ct values on 75 copies/mL adjusted#	884	36.9	(35.6-38.1)
Observed in batch release testing	Ct values on 125 copies/mL	166	36.7	(35.8-37.6)

observed Ct values minus 0.74 to predict Ct value on 1.67-fold higher concentration of 125 copies/mL

The verification study in Table 34 proves that it is justified to have adjusted the observed Ct values of PMFU studies presented in package insert KI4268 with delta 0.74 to predict the Ct values on future batches of P0273 Multi-Marker Control with a 1.67-fold higher HCV concentration.

Supplemental calibration study of native and inactivated HIV-1 standard in different quantitative methods performed in 2018

For this study we refer to the manuscript of Lelie and Van Drimmelen published in JMV[37].

Conclusion supplemental calibration experiments

The calibration results of the inactivated HCV genotype 3 standard against the native genotype 1 standard is assay dependent and varies up to 2.5 fold between different methods and calibration experiments. Both the difference in genotype (nucleic acid sequence) and chemical inactivation as well as experimental variation may contribute to these differences.

Similar up to 2.5 fold differences in calibration of HIV-1 viral load assays in copies/mL have been observed on the native S0012 HIV-1 subtype B standard, whereas calibration of the heat-inactivated HIV-1 subtype B standard on the native standard also turned out to be assay dependent with up to 2.3 fold differences in relative quantification between methods (Lelie and Van Drimmelen, JMV)[37].

In the light of the established differences in assay calibration for HIV-1 RNA[37] and assay dependence on standard calibration for HCV-RNA (this chapter) there is no scientific reason to assign other copy numbers to the native and inactivated HBV, HCV and HIV standards than those based on historical calibration in the bDNA 3.0 assay as the reference measurement method. However since a second bDNA 3.0 calibration experiment also gave 1.47 fold higher values than the first bDNA 3.0 experiment we decided that it is better to use the overall consensus value of multiple methods to recalibrate the S0109 HCV standard 1.67-fold to **3.64E+07 copies/mL.** With this recalibration factor of the inactivated S0109 HCV genotype 3 standard the P0273 Multi-Marker Check 75 Control will contain 4.5 times the 95% LOD, comparable to the distance for HBV.

With the 1.67-fold decrease of the copy number assigned to the S0109 HCV genotype 3a standard it is expected that the P0067 ViraQ HCV Trend 25 Control generates a reactivity rate of approximately 97% in the Ultrio Elite assay. The 95% LOD in the Ultrio assay versions would then reduce from to 28.7 (20.5-41.0) copies/mL to 17.2 (12.3-24.6) copies/mL. The new P0063 ViraQ HCV Check 125 Control would then contain 7 times the 95% LOD instead of 4 times the 95% LOD. We expect that this change in the concentration of the P0063 HCV HCV Check 125 will make the run control more robust in case the new Ultrioplex E assay would be somewhat less sensitive than the Ultrio Elite assay.

The impact of 1.67-fold higher HCV concentration in the P0063 HCV Check 125, P0067 HCV Trend 25 and P0273 Multi-Marker Check 75 controls on cobas MPX Ct values was verified in the batch release tests according to QCF4007, whereby reference batches with and without the 1.67-fold shift in HCV concentration were compared. Moreover when adjusting the Ct values in the PMFU study to account for the 1.67-fold higher HCV concentration in future ViraQ Check Controls the predicted values were comparable to those observed on batches with a 1.67-fold higher HCV concentration.

These changes in the calibration of the S0109 HCV standard and in the design of the P0063, P0067 and P0273 ViraQ Controls have been announced to the Notified Body and PEI in order improve the performance of the ViraQ Check and Trend Controls and narrow the confidence bounds for HCV in PMFU studies and batch release testing of the P0273 Multi-Marker Control in the cobas MPX assay (VR4095).

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