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An assessment of the reproducibility of reverse transcription digital PCR quantification of HIV-1

Samreen Falak [a,*,1], Rainer Macdonald [a], Eloise J. Busby [b], Denise M. O'Sullivan [b], Mojca Milavec [c], Annabell Plauth [a], Martin Kammel [d,e], Heinz Zeichhardt [d,e], Hans-Peter Grunert [f], Andreas Kummrow [a,1], Jim F. Huggett [b,g,*,1]

[a] Physikalisch Technische Bundesanstalt, Abbestr. 2-12, 10587 Berlin, Germany
[b] National Measurement Laboratory, LGC, Queens Road, Teddington, Middlesex TW11 0LY, United Kingdom
[c] Department of Biotechnology and Systems Biology, National Institute of Biology, Ljubljana, Slovenia
[d] INSTAND, Gesellschaft zur Foerderung der Qualitaetssicherung in medizinischen Laboratorien e.V., Ubierstr.20, D-40223 Düsseldorf, Germany
[e] IQVD GmbH, Institut fuer Qualitaetssicherung in der Virusdiagnostik, Potsdamer Chaussee 80, 14129 Berlin, Germany
[f] GBD Gesellschaft fuer Biotechnologische Diagnostik mbH, Berlin, Potsdamer Chaussee 80, 14129 Berlin, Germany
[g] School of Biosciences & Medicine, Faculty of Health & Medical Science, University of Surrey, Guildford GU2 7XH, United Kingdom

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ABSTRACT

Viral load monitoring in human immunodeficiency virus type 1 (HIV-1) infection is often performed using reverse transcription quantitative PCR (RT-qPCR) to observe response to treatment and identify the development of resistance. Traceability is achieved using a calibration hierarchy traceable to the International Unit (IU). IU values are determined using consensus agreement derived from estimations by different laboratories. Such a consensus approach is necessary due to the fact that there are currently no reference measurement procedures available that can independently assign a reference value to viral reference materials for molecular in vitro diagnostic tests. Digital PCR (dPCR) is a technique that has the potential to be used for this purpose. In this paper, we investigate the ability of reverse transcriptase dPCR (RT-dPCR) to quantify HIV-1 genomic RNA without calibration. Criteria investigated included the performance of HIV-1 RNA extraction steps, choice of reverse transcription approach and selection of target gene with assays performed in both single and duplex format. We developed a protocol which was subsequently applied by two independent laboratories as part of an external quality assurance (EQA) scheme for HIV-1 genome detection. Our findings suggest that RT-dPCR could be used as reference measurement procedure to aid the value assignment of HIV-1 reference materials to support routine calibration of HIV-1 viral load testing by RT-qPCR.

1. Introduction

Antiretroviral therapy (ART) has rendered infection by HIV, which initially had high mortality, a manageable chronic condition. Approximately 38.0 million people are currently living with HIV, and globally 68% of adults and 53% of children living with HIV receive lifelong antiretroviral therapy [1]. Effective ART can enable sustained suppression of viral load in the plasma (to below 50 copies per millilitre (cp/mL)) [2,3]. To ensure ART is being effective, the patient's viral load is monitored by measuring the quantity of the RNA genome in response to treatment [4,5]. Reverse transcription quantitative PCR (RT-qPCR) is the routine method for measuring HIV RNA [6] which relies on calibration for quantitative measurement [7,8]. For these measurements to be reproducible the calibration must be traceable to support

Abbreviations: ART, antiretroviral therapy; BV, blank value; CV, coefficient of variation; cp/mL, copies per millilitre; EQA, external quality assessment; HIV-1, human immunodeficiency virus type 1; IU, international unit; LOD, limit of detection; LOQ, limit of quantification; NTC, negative template control; RMs, reference materials; RNA, ribonucleic acid; RSD, relative standard deviation; RT-dPCR, reverse transcription digital PCR; RT-qPCR, reverse transcription quantitative PCR; WHO, World Health Organization.

* Corresponding authors at: Physikalisch Technische Bundesanstalt, Abbestr. 2-12, 10587 Berlin, Germany (S. Falak). National Measurement Laboratory, LGC, Queens Road, Teddington, Middlesex TW11 0LY, United Kingdom (J.F. Huggett).
E-mail addresses: samreen.falak@ptb.de (S. Falak), jim.huggett@lgcgroup.com (J.F. Huggett).
[1] Contributed equally.

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standardization.

Traceability of HIV-1 load measurements is supported by the World Health Organization (WHO) via the development and distribution of reference materials (RMs), called WHO International Standards [9,10]. The WHO RMs have enabled global comparisons of the viral load of HIV-1, and other viruses like hepatitis viruses, to be made with traceability to the international unit (IU), the value of which is assigned by consensus. These reference materials enable harmonization of the associated quantitative measurements, allow performance assessment during the development and routine application of tests, and comparison of the diagnostic services offered at national and international levels [11].

Although the WHO International Standards for HIV-1 are assigned to IU, diagnostic reporting is frequently still based on copies/mL taking into account a conversion factor [9]. This is due to the introduction and application of quantitative PCR systems for HIV-1 viral load testing before the development of the first WHO International Standard for HIV-1. Although International Standards have revolutionized global viral measurement, the reliance of measurement traceability on a reference material brings challenges associated with its supply and continuity between different batches. In clinical chemistry this challenge is often resolved by using units that are traceable to either a reference measurement procedure or, preferably, the International System of Units [11,12]. Reference measurement procedures provide an accurate characterization of reference material to a high metrological order and provide stability in the reference measurement system. To date, it is not clear whether such an approach could assist in improving the standardization of global viral load measurements as suitable reference measurement procedures have not existed.

Digital PCR (dPCR) is a method that can be performed as an SI-traceable reference measurement procedure when measuring DNA in buffered solution [13] and can perform with high reproducibility when incorporating extraction protocol to measure DNA from whole bacteria [14] and viruses [15]. In combination with reverse transcription (RT), dPCR was proposed as method to provide accurate and robust quantification of HIV RNA in plasma samples in the clinically relevant low concentration range [16]. Previous dPCR studies reported to date have quantified HIV-1 DNA as well as cell associated and synthetic RNA [7]. In several studies, dPCR has been used in the measurement of HIV DNA from patients and was found to be more robust to mismatches between primers and probes and target sequence of HIV [17–21].

Despite these promising studies, the measurement of RNA by RT-dPCR has not been investigated to the same extent as for DNA. Such assessments are required if the method is to support reference material production as a reference measurement procedure for HIV-1, hepatitis C or coronavirus. In this study, we developed a procedure incorporating extraction and RT-dPCR to reproducibly quantify HIV-1 RNA from whole virus samples and evaluated it on EQA samples and the WHO 4th HIV-1 International Standard.

2. Materials and methods

The “Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020” (dMIQE2020) checklist [22] is given in (Supplementary Table S1).

2.1. Sample collection

HIV-1 positive samples (group M, subtype F) derived from External Quality Assessment (EQA) schemes performed by INSTAND e.V. (https://www.instand-ev.de/en). The sample sets corresponded to panels of the INSTAND EQA schemes No. 360 and No. 382, distributed in June 2017, March 2018 and March 2019 (https://www.instand-ev.de/no_cache/en/eqas-online/service-for-eqa-tests/#rvp//360/-2021/). The samples were prepared by the manufacturer spiking HIV stock material (heat inactivated) into human plasma at different dilution levels. Viral loads are available as consensus value from the EQA schemes. In addition, the WHO 4th HIV-1 International Standard of HIV-1 subtype B virus was included in the study (WHO-IS NIBSC code: 16/194, NIBSC Hertfordshire UK). The nominal concentration for the WHO Standard is 5.10 $\log_{10}$ IU/mL [9].

2.2. Comparison of HIV-1 RNA extraction kits

Three different RNA extraction kits were assessed for HIV-1 RNA extraction from plasma samples: i) QIAamp viral RNA mini kit (#52904, Qiagen Hilden, Germany), ii) High Pure Viral RNA kit (#11858882001, Roche Diagnostics Manheim, Germany) and iii) Nucleo-Spin RNA virus (#740956.50 Macherey-Nagel, Germany). All kits used silica gel membrane columns for extractions and centrifugation. Six replicate extractions were performed on three different days, for each of the three methods. For evaluation of these RNA extraction methods, the EQA sample 360126 (term March 2018) was used. For each extraction, 200 µL of reconstituted plasma sample was processed following the manufacturer instructions for the respective kit. A negative control for the extraction method was included consisting of 200 µL deionized water instead of plasma sample. DNase digestion was performed on-column using RNase-Free DNase Set (50), (#79254 Qiagen Hilden, Germany) according to manufacturer instructions. The RNA was eluted applying 60 µL elution buffer. Following extraction, the RNA concentration was measured when eluted in water using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and used immediately for dPCR.

2.3. HIV-1 primers and probes

Previously published primers and probe sequences targeting the HIV-1 *gag* (p24 sequence of *gag*) and *pol* (exon/intron boundary of *pol* and *vif* genes) were chosen [23,24]. Sequence of primers and probes are listed in Supplementary Table S2. The assays were selected to target specific sequences from various HIV-1 groups M, N, O with major subtypes.

2.4. RT-dPCR methods

For two-step RT-dPCR, RNA was reverse transcribed to cDNA using the SuperScript IV First-Strand Synthesis System (#18091050, Applied Biosystems, Life Technologies, Darmstadt, Germany) details shown in (Supplementary Table S3). Two-step RT-dPCR reactions (total volume 20 µL) contained 10 µL ddPCR Supermix for probes (No dUTP) (BioRad, USA), primers and probe mix (900 nM / 250 nM probe), 5 µL of cDNA and nuclease-free water.

One-step RT-dPCR reactions was carried out with One-Step RT-ddPCR Advanced kit for probes (#1864021, BioRad, USA). The final reaction volume 20 µL contained 5 µL Supermix (BioRad, USA), 2 µL reverse transcriptase (20 U/µL; BioRad, USA), 1 µL 300 mM dithiothreitol (BioRad, USA), primers and probes (900 nM/250 nM), nuclease-free water, and 8 µL RNA sample. The samples were analysed as at least three technical replicates with each run including a non-template control and an RT-negative control.

Singleplex and duplex assay was performed using *gag* and *pol* gene with primers and probe concentrations of (900 nM and 250 nM, respectively), and were optimised by varying annealing temperature. In the duplex assay, the primers and probes were added in the same concentration (900 nM and 250 nM) of each target as in the singleplex assay.

Droplets were generated in DG8 cartridges using QX200 Droplet Digital System manual droplet generator (QX200, BioRad, USA). The generated water-in-oil emulsions were transferred to a 96-well PCR plate and the PCR reactions carried out using a thermal cycler (C1000 BioRad, USA) under the following conditions, 60 min reverse transcription at 50 °C and 10 min enzyme activation at 95 °C followed; by 45 cycles using a two-step thermal profile, of 30 s denaturation at 95 °C and 60 s annealing and extension at 55 °C; followed by 10 min at 98 °C and then cooled at 4 °C. The thermal cycling conditions for two step dPCR

were exactly the same as for one step dPCR, except exclusion of the reverse transcription step (60 min at 50 °C) prior to amplification. Following thermal cycling, PCR plates were transferred into a droplet reader (QX200 BioRad, USA) and data were collected and analysed using the dPCR provided software package (QuantaSoft 1.7.4.0917; BioRad, USA). The duplex assays temperature optimization is shown in Supplementary Fig. S1.

2.5. Intermediate precision

To test the intermediate precision, five extracts were prepared from WHO 4th HIV-1 International Standard material (WHO-IS NIBSC code: 16/194, NIBSC UK) on different days. RT-dPCR was performed with a duplex assay of *gag* and *pol* primers and probes (See Supplementary Table S4).

2.6. Limit of detection and quantification method

Eleven dilution series of extracted RNA were produced from the WHO 4th HIV-1 International Standard material using nuclease free water. Eleven dilution steps 2×, 4×, 8×, 16×, 32×, 64×, 128×, 256×, 512×, 1024×, 2048× and a negative template control (NTC) consisting of nuclease free water were tested. Sixteen replicates of each dilution were measured by RT-dPCR in four separate runs in consecutive days, containing four technical replicates except for dilutions series 2×, 1024× and 2048× which were measured once, and each contained four technical replicates. The verified values from RT-dPCR were used to calculate the assigned copy number of the targets for the dilution series.

2.7. EQA participation

In EQA scheme March 2019, 140 laboratories have participated in the Virus Genome Detection HIV-1 (RNA) Program 1 (INSTAND ring trial number also denominated as (360)) and 44 laboratories in the Virus Genome Detection-HIV-1 (RNA) additional Training Program 2 also denominated (382). Each program covers samples with four different dilution levels. The target value for each EQA sample is determined as consensus value from all quantitative results for the respective sample (based on the robust average according to algorithm A/DIN ISO 13528/ Annex C).

2.8. Data acquisition and analysis

The dPCR data acquisition was performed with QuantaSoft™ version 1.7.4.0917 (BioRad, US) and data processing used QuantaSoft™ Analysis Pro 1.0.596 (BioRad, US). Software counts the number of valid droplets and records the associated fluorescence signals of positive droplets (amplified products) and negative droplets without the amplification product as described previously [25]. The threshold was applied automatically by the software or set manually (if required) for both channels FAM and HEX. The data generated by the QX200 droplet reader were excluded from subsequent analysis if the number of accepted droplets were below 10.000 per well. Exported data were further analysed using Microsoft Excel spreadsheets and CV was calculated as relative standard deviation and expressed as percentage value. Grubbs outlier test was performed using Origin 2019 software. Respective examples for positive and negative samples are shown in Supplementary Fig. S2.

3. Results and discussions

3.1. Singleplex and duplex RT-dPCR

To characterize and compare the performance of a singleplex and a duplex assay, the absolute concentration of HIV-1 RNA was determined for an EQA plasma sample (360126), and the WHO 4th HIV-1 International Standard. RNA was extracted using QIAamp viral RNA mini kit. For both assays, copy number ratios of duplex to singleplex assays are presented in Table 1.

Typical CV values for the results shown in Table 1 were 10%–12%. The ratio between duplex to singleplex shows that the concentration measured by the duplex assay is on average lower by 5% compared to the singleplex approach (Table 1) which is less than the observed CV. This shows that the duplex assay does not compromise quantification when compared to the singleplex approach and the former was chosen for the remainder of the study. Duplex formats provide an additional level of confidence and are commonly used for a wide range of molecular testing applications [4,26,27].

3.2. Comparison of RNA extraction kits for the detection of HIV-1

When different extraction procedures were compared using RNA from EQA sample 360126, we observed that the choice of RNA extraction kit resulted in a clear difference in the measured viral concentrations (Fig. 1). A large difference in the viral RNA concentration was observed between High Pure Viral RNA kit and the other two kits. The QIAamp Viral RNA mini kit and NucleoSpin RNA Virus yielded comparable concentration for both assays and days. This demonstrated that selection of the extraction method is critical as it can influence copy number concentration estimates following downstream molecular analyses as it has been observed in *Mycobacterium tuberculosis* [14].

Overall, extraction analysis showed that the QIAamp viral RNA mini kit consistently yielded the highest signal for the plasma-based samples, and consequently was chosen for the subsequent HIV-1 RNA measurements performed in this study.

It is known that the extraction can contribute towards a major source of bias in dPCR based workflows [14]. Therefore, different RNA extraction kits used in this study contributed to some order of discordance in RNA extraction and led to differences being observed in the copy number concentration obtained from different extraction kits.

3.3. Reverse transcription RT-dPCR assay for HIV

A comparison of the one-step RT-dPCR supermix for probes with the two-step approach using SuperScript IV reverse transcriptase was performed (Fig. 2). All four dilution levels of EQA 2018 samples (EQA scheme no. 360) and the WHO 4th HIV-1 Standard were included in the analysis. The concentrations measured when using the one-step RT-dPCR supermix were consistently higher, despite matched input concentrations of RNA. Our results demonstrate that the supermix for one-step RT-dPCR provided greater efficiency (Fig. 2).

In one-step format, both the RNA conversion and gene specific PCR amplification occur in a single tube. In contrast, two-step needed two separate reactions for RNA conversion and amplification. In addition, gene-specific primers in one-step have revealed an efficient cDNA synthesis compared to using random hexamers and oligo dT primers in two-step particularly for samples like HIV-1 with low copy number concentrations. This observation has previously been reported for one- and two-step RT-qPCR [28].

This observation is in line with recent findings of Myerski et al. [29]. Other studies [30,31] reported that one-step RT-dPCR had higher precision and repeatability. In addition, one-step reaction is time efficient

Table 1
Performance of singleplex and duplex assays.

Sample	Target	Mean concentration (cp/µL)		Ratio
		Singleplex	Duplex	
EQA plasma sample (360126)	*gag*	13.5	12.5	0.93
	pol	10.8	10.1	0.94
WHO 4th HIV-1 Standard	*gag*	32.2	30.9	0.96
	pol	28.9	27.2	0.94

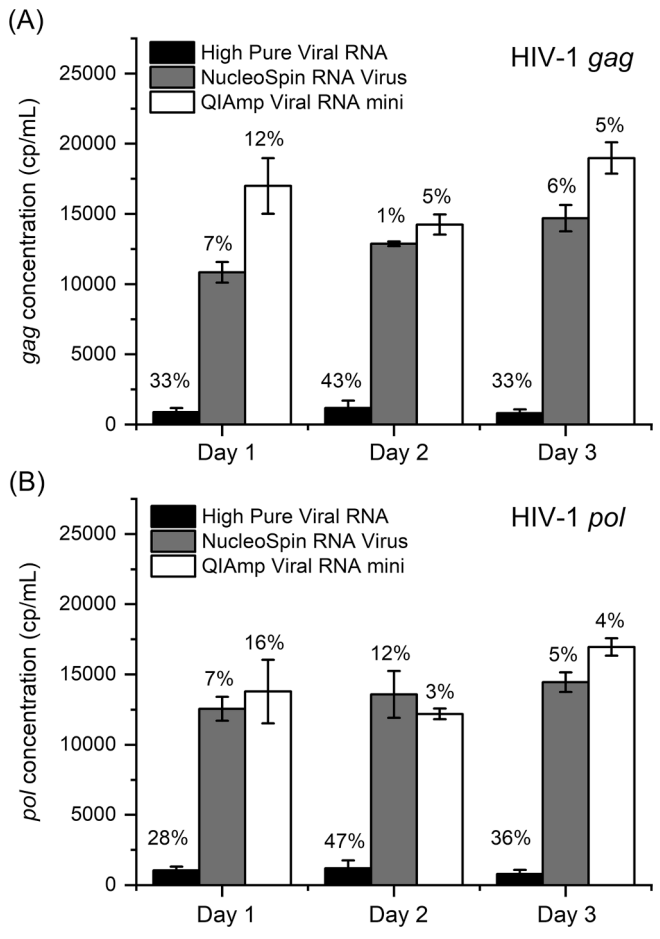

Fig. 1. Evaluation of extraction methods with commercial kits. Absolute RNA concentrations were shown in cp/mL for (A) *gag* and (B) *pol* genes separately. Error bars depict standard deviations with the numbers above bars outlining coefficient of variation. ($n = 6$).

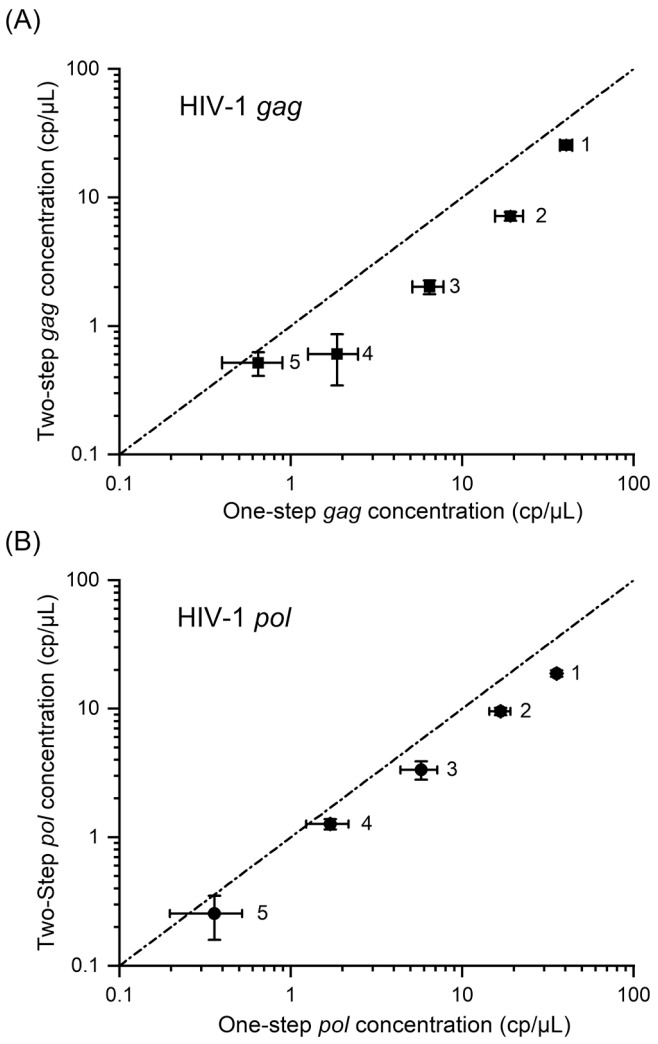

Fig. 2. Assessment of one-step dPCR on X-axis and two-step dPCR on Y-axis. A) representing *gag* and B) *pol* targets (1: WHO 4th HIV-1 International Standard; 2: INSTAND EQA (March 2018) sample 360126; 3: 360128; 4: 360125; 5: 360127).

and minimizes the risk of contamination. Based on this, one-step format was chosen for the remaining experiments in this study.

3.4. Intermediate precision of RT-dPCR

The intermediate precision is defined as the precision obtained within a single laboratory over a longer period of time (generally several months at least) and takes into account more changes than repeatability [32]. The intermediate precision of the selected RT-dPCR measurement procedure was assessed by repeated experiments including repeated extractions conducted on separate days over a period of more than three months.

The intermediate precision of the duplex assay was analysed using the WHO 4th HIV-1 Standard material at a nominal concentration of 125,900 IU/mL. The intermediate precision was examined by measuring 31 replicates in five days. Grubbs outlier testing did not indicate outliers at a significance value of 0.05 when applied to all replicates or when applied to average values from individual extracts. The intermediate precision expressed as % CV was 8.8% for *gag* and 12.3% for *pol* as shown in Table 2 (Details shown in Supplementary Table S4).

The variation of our results as reflected by the intermediate precision is negligible when compared to the variation allowed in EQA for virologic laboratories. The intermediate precision found here is clearly lower than the interlaboratory variation of 0.43 on a log10 scale reported for the same material [9]. The measurement of WHO 4th HIV-1 International Standard is summarized in Supplementary Note S1 and Table S5.

Table 2
Intermediate precision of duplex one-step RT-dPCR assay.

Mean concentration (cp/µL) per extract						CV %
Gene	#1	#2	#3	#4	#5	
gag	36.5	37.0	32.6	40.5	33.4	8.8
pol	33.0	28.6	29.3	36.3	27.0	12.3

3.5. Limit of detection and limit of quantification

The Limit of detection (LOD) is defined as the HIV-1 RNA concentration, for which the probability of falsely claiming the absence of HIV RNA is 5% [32]. In digital dPCR, the statistical distribution in repeat measurements is not Gaussian at low sample concentration but discrete (Fig. S4). Therefore, it is not possible to derive the LOD from the standard deviation of repeat measurements. In the absence of a blank value (BV) the theoretical LOD for dPCR can be calculated from counting statistics assuming Poisson distribution. Details shown in (Supplementary Note S2 and Fig. S3). The LOD concentration for RNA in the sample material is

$$c_{LOD} = \frac{-ln0.05}{N_{tot} \cdot V_d \cdot D} \quad (1)$$

where N_{tot} is the number of accepted droplets and V_d is the droplet size. In Eq. (1) the LOD is corrected by the dilution factor D that results from concentration changes introduced by extraction and addition of reagents. For $N_{tot} = 13{,}000$, $V_d = 0.85$ nL and $D = 1.29$ this gives $c_{LOD} = 210$ cp/mL when using a single RT-dPCR replicate. In this study, a single reaction uses an approximate equivalent volume of ~26 μL of plasma. Much larger volumes of plasma are used with many diagnostic solutions which provides improved limit of detection per mL (e.g. 500 μL). For the RT-dPCR reaction, the same could be achieved by pooling results obtained by a number of wells and or adapting the pre experimental steps (this increasing N_{tot}). This approach was used in the measurements discussed in the next section, since low viral concentration is considered in the ring trial. The ultimate limit of detection was in this case calculated using the NTCs (by measuring 60 replicates) and to be 16 cp/mL.

The limit of quantification (LOQ) is defined here as the minimum concentration for which the relative standard deviation (RSD) is smaller than a predefined value R, e.g. $R = 0.2$. The ultimate limit for LOQ is given by counting statistics and can be calculated (Supplementary Note S2) from

$$c_{LOQ} = \frac{1}{R^2 N_{tot} \cdot V_d \cdot D} \quad (2)$$

For $R = 0.2$, $N_{tot} = 13{,}000$ and $V_d = 0.85$ nL Eq. (2) gives $c_{LOQ} = 1750$ cp/mL. The averaged results shown in Fig. 3A demonstrate that the LOQ can also be improved by averaging replicate measurements.

The average quantity of HIV-1 target concentration measured for WHO 4th HIV-1 Standard by duplex RT-dPCR was approximately 32,000 cp/mL in the plasma sample. The LOQ was calculated and compared on a serial dilution of WHO 4th HIV-1 International Standard (Fig. 3).

Our data demonstrate that the observed concentration determined by averaging all replicate measurements followed the expected concentration down to about 100 cp/mL for the assay used here (Fig. 3A). In Fig. 3B, the vertical dashed line indicates the theoretical LOQ determined assuming a threshold level of $R = 20\%$. Counting statistics was assumed in derivation of above formulas. The observed relative standard deviation does not exceed the threshold level of 20% significantly above the calculated LOQ expected from Eq. (2). This demonstrates that quantification is limited by counting statistics (Supplementary Note S2).

3.6. Interlaboratory comparison

In 2019, PTB and NML participated in EQA schemes organized by the German EQA provider INSTAND e.V. for HIV-1 virus genome detection. Both participants were blind to the content of the respective samples and used the protocol developed by the study. Results are shown as symbols in Fig. 4A. RT-dPCR results demonstrate good reproducibility between laboratories (target gag). The acceptance range in these EQA schemes is ±0.6 on a log 10 concentration scale in respect of the target value [33]. In this EQA scheme, the centre of the acceptance range is calculated as the robust average of the concentrations of all participating laboratories (106 clinical laboratories used RT-qPCR, two used RT-dPCR). The EQA scheme covers a wide concentration range of 42 cp/mL to 37,000 cp/mL as required for medical diagnosis [34]. Direct comparison of concentration determined in cp/mL revealed that both RT-dPCR results were below the mean value, although, all were within the acceptance range with the exception of one result (Fig. 4a). To discuss this observation, we must consider that participants of the EQA scheme who used RT-qPCR report their results in cp/mL that this is harmonised to the WHO International Standard [9,33] which uses International Units (IU) (Supplementary Note S1 and Table S5). Given that the IU is determined by consensus value and avoids an absolute measure of virus genomes we cannot expect the RT-dPCR to agree with RT-qPCR. When the RT-dPCR values are aligned to the RT -qPCR (IU supported) copies/mL (Fig. 4b) the agreement is improved between the methods.

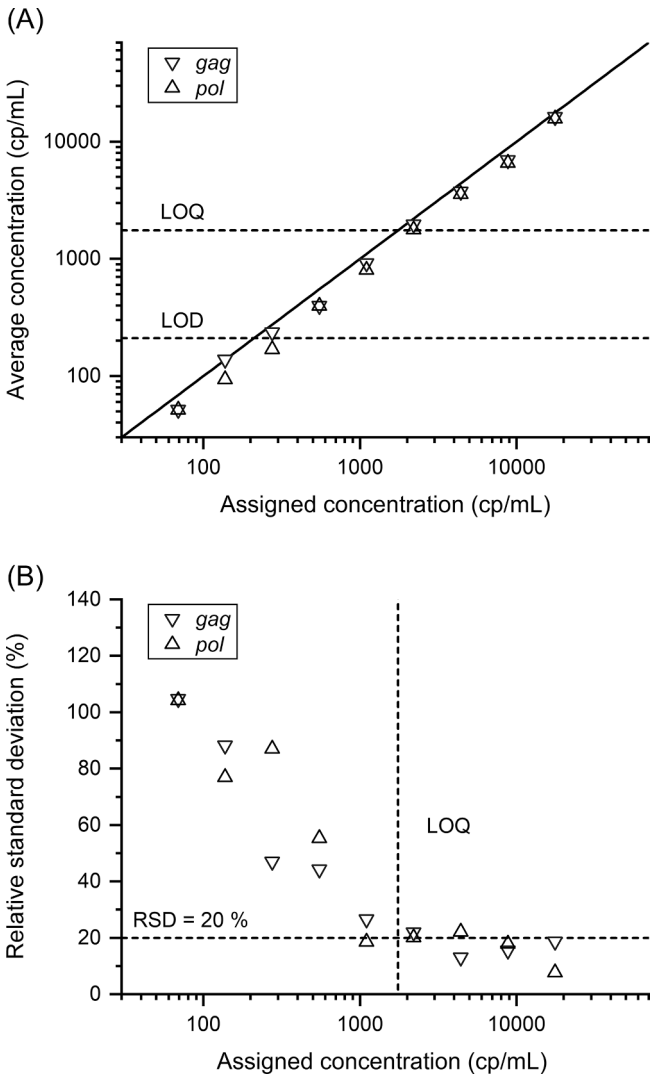

Fig. 3. A dilution series using the WHO 4th HIV-1 Standard measured by the duplex one step RT-dPCR in the plasma for *gag* and *pol* genes: A) observed average concentration (symbols) closely follow expected concentration in plasma calculated from dilution factor (solid line); B) plot of the relative standard deviation of replicate measurements to determine limit of quantification.

Overall, results obtained by two metrology laboratories using RT-dPCR with and without applying conversion factors to determine IU were in good agreement and fit well to the results obtained by conventional qPCR and met the requirements of the EQA scheme.

4. Conclusions

This paper demonstrates that RT-dPCR has the potential to be a reference measurement procedure for HIV-1 RNA measurement. Sources of bias affecting HIV-1 RT-dPCR measurements were identified including comparison of different methods for HIV RNA extraction from whole virus, comparison of different RT enzymes performed one or two-step formats. Intermediate precision (between days) and reproducibility (between two laboratories participating in clinical EQA scheme) was determined. These data demonstrate applicability and reproducibility of the developed RT-dPCR assay for HIV-1 RNA quantification in a complex genomic background. Future work is required to explore the use of the method for calibration value assignment, such as to put values on secondary reference materials aligned to the WHO International Standard, and determine if this can improve RT-qPCR harmonization. We did not

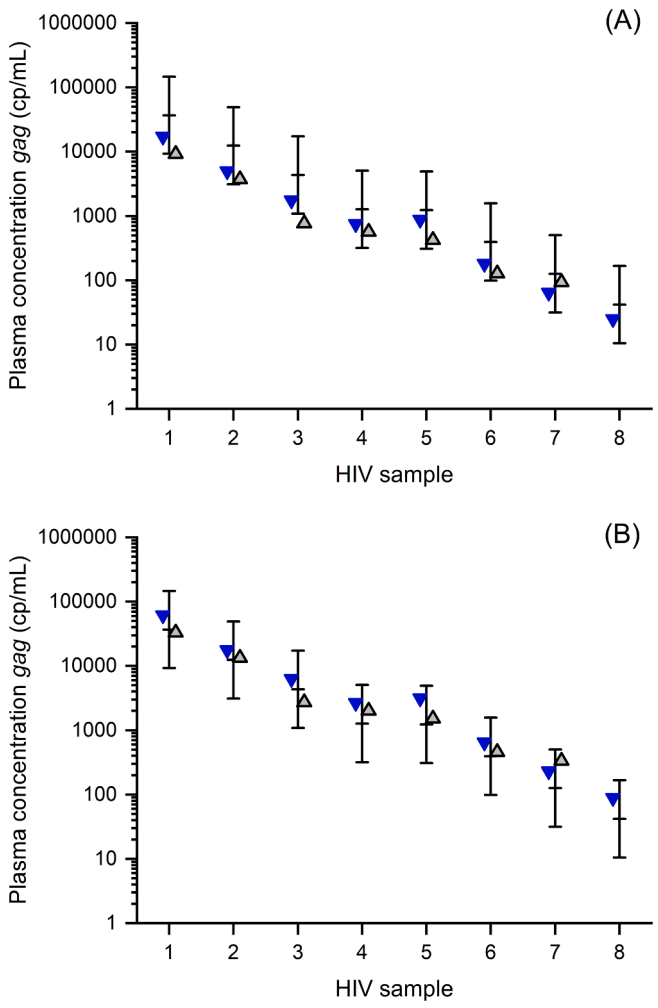

Fig. 4. Results of the EQA schemes performed in March 2019 by INSTAND: A) represents the HIV-1 EQA scheme results directly obtained by RT-dPCR; B) HIV-1 EQA scheme with RT-dPCR corrected to WHO 4th HIV-1 Standard. Bars show the acceptance range based on the consensus value from all quantitative results for the respective sample for the Virus Genome Detection HIV-1 (RNA) Program 1 (360) and the Virus Genome Detection-HIV-1 (RNA) additional Training Program 2 (382) (INSTAND) of the EQA scheme. The acceptance range is ±0.6 on a log10 concentration scale [33]. Symbols represent the results submitted by the two laboratories using duplex RT-dPCR assay demonstrating reproducibility between metrological laboratories. For sample designation see legend to Fig. S4. n.b. Samples 4 and 5 are identical.

explore the performance of these protocols on clinical samples as we did not set out to demonstrate that RT-dPCR could be used as a clinical test. However, in the short term this protocol could be used to define clinical reference ranges and calibration material commutability and this would be an important area to explore. Additional work is also required to better understand why the analytical variation during routine testing by RT-qPCR is so large (Fig. 4) and it is likely the high reproducibility of RT-dPCR demonstrated here will aid in defining sources of error to improve the reproducibility of routine testing by RT-qPCR.

To the best of our knowledge, this is the first study to apply RT-dPCR for absolute quantification of viral HIV-1 RNA in terms of copies/mL applied to plasma-based EQA samples and the WHO HIV-1 4th Standard. Our results demonstrated the applicability of simultaneous use of *gag* and *pol* primers for detecting HIV-1 RNA in a duplex RT-dPCR assay. Our findings demonstrate RT-dPCR offers good linearity, intermediate precision (within laboratory) in measurement of HIV viral load. The experiments did not require specific modifications on technical instrumentation so that measurements should also be feasible for a range of other laboratories. Very high reproducibility (in between laboratories) was possible from direct measurement, without calibration, between laboratories performing the same protocol.

We show that RT-dPCR has the potential to quantify HIV viral RNA for value assignment of quality control and reference materials. The methods described here also demonstrated to achieve sufficient consensus, sensitivity and reproducibility required for medical diagnosis as demonstrated by successful participation in the INSTAND EQA schemes 2019. This opens the possibility, as methods and instrumentation are developed, for RT-dPCR to also be considered in the near future as a diagnostic tool in its own right for HIV and other RNA viruses like influenza and SARS-CoV-2.

Author contributions

The manuscript was written through contributions of all authors.

CRediT authorship contribution statement

Samreen Falak: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Validation, Resources. **Rainer Macdonald:** Conceptualization, Formal analysis, Writing - review & editing, Visualization. **Eloise J. Busby:** Investigation, Validation, Writing - review & editing, Resources. **Denise M. O'Sullivan:** Investigation, Validation, Writing - review & editing, Resources. **Mojca Milavec:** Conceptualization, Writing - review & editing. **Annabell Plauth:** Formal analysis, Writing - review & editing. **Martin Kammel:** Conceptualization, Formal analysis, Resources. **Heinz Zeichhardt:** Formal analysis, Writing - review & editing, Resources. **Hans-Peter Grunert:** Conceptualization, Investigation, Resources. **Andreas Kummrow:** Conceptualization, Formal analysis, Methodology, Writing - original draft, Writing - review & editing, Visualization. **Jim F. Huggett:** Conceptualization, Formal analysis, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2021.03.006.

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