The Application of Digital PCR as a Reference Measurement Procedure to Support the Accuracy of Quality Assurance for Infectious Disease Molecular Diagnostic Testing

Samreen Falak [a,†] Denise M. O'Sullivan [b,†] Megan H. Cleveland [c,†] Simon Cowen [b] Eloise J. Busby [b] Alison S. Devonshire [b] Esmeralda Valiente [a] Gerwyn M. Jones [b] Martin Kammel [d,e] Mojca Milavec [f] Laura Vierbaum [d] Ingo Schellenberg [d] Heinz Zeichhardt [d,g] Andreas Kummrow [a] Peter M. Vallone [c] Rainer Macdonald [a] and Jim F. Huggett [b,h,*]

BACKGROUND: Nucleic acid amplification tests (NAATs) assist in the diagnosis of numerous infectious diseases. They are typically sensitive and specific and can be quickly developed and adapted. Far more challenging is the development of standards to ensure NAATs are performing within specification; reference materials take time to develop and suitable reference measurement procedures (RMPs) have not been available. This study investigated digital PCR (dPCR) RMP delivery of traceability for NAAT external quality assessment (EQA).

METHODS: Three National Metrology Institutes (NMIs) applied reverse transcription (RT)-dPCR as a candidate RMP to estimate the RNA quantity in 32 independent severe acute respiratory syndrome coronavirus 2 materials. The results were combined to value assign the respective materials: 21 materials were used in 6 rounds of EQA over 17 months for 61 laboratories for COVID-19 testing results compared with reference values.

RESULTS: The agreement between the 3 NMIs showed <2-fold difference between laboratories. EQA laboratory reverse transcription quantitative PCR (RT-qPCR) values estimation of viral RNA quantity showed good median agreement with RT-dPCR reference value; however, RT-qPCR differences were generally between 10- and 50-fold between laboratories.

CONCLUSION: This work demonstrates how RT-dPCR can provide reference values for whole virus materials for NAAT quality assurance. RT-dPCR values guided EQA control material selection and provided EQA participants with traceability to RNA copy number delivered through the RMP. This approach can be used to support routine reference material use as well as to standardize quality assurance for NAATs where established reference materials are not available, such as in disease outbreaks.

Introduction

Nucleic acid amplification technologies/tests (NAATs) are often the procedure of choice for diagnosing and tracking outbreaks such as influenza, Mpox, and COVID-19. Molecular diagnostic tests deploying NAATs such as the polymerase chain reaction (PCR), offer a versatile solution for detecting pathogen nucleic acids in clinical specimens. This versatility is enabled by the fact that a PCR assay, comprising oligonucleotide primer pairs and, typically, a labeled oligonucleotide

[a]Department 8.3, Biomedical Optics, Physikalisch-Technische Bundesanstalt, Berlin, Germany; [b]Biological Metrology, National Measurement Laboratory (NML), LGC, London, United Kingdom; [c]Biomolecular Measurement Division, National Institute of Standards and Technology, Gaithersburg, MD, United States; [d]Gesellschaft zur Foerderung der Qualitaetssicherung in Medizinischen Laboratorien e. V. (INSTAND), North Rhine-Westphalia, Düsseldorf, Germany; [e]GBD Gesellschaft fuer Biotechnologische Diagnostik mbH, Berlin, Germany; [f]Department of Biotechnology and Systems Biology, National Institute of Biology Ljubljana, Slovenia; [g]IQVD GmbH, Institut fuer Qualitaetssicherung in der Virusdiagnostik, Berlin, Germany; [h]School of Biosciences & Medicine, University of Surrey, Guildford, United Kingdom.

*Address correspondence to this author at: National Measurement Laboratory (NML), LGC, Queens Rd, Teddington TW11 0LY, United Kingdom. E-mail jim.huggett@lgcgroup.com.
[†]Contributed equally.

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probe, can be quickly designed by examining the sequence of interest. Consequently, a diagnostic solution to an infectious outbreak can be rapidly developed, i.e., in a matter of weeks. This undoubtedly meant that NAATs enabled a swift diagnostic response to the COVID-19 pandemic.

What was limited during the initial diagnostic response to COVID-19, however, were pathways to standardize the performance of the NAATs during their development and wider application. Initially, in vitro diagnostic manufacturers used a variety of approaches to assess analytical performance with a wide variation in results (1). The development of external quality assessment (EQA) panels (2) and, early in 2021, the World Health Organization (WHO) international standard (3) provided conventional routes to support test evaluation. However, these "material standard" approaches, while important in supporting test quality, could not be developed or distributed as quickly as the NAAT tests they were intended to support. In a new outbreak this may result in a considerable period where the tests are performed with limited standardization. If, as was the case for COVID-19, the testing in question is seen to be instrumental to the pandemic response (4, 5) this reality could represent a serious hindrance in pandemic management. This is because, without standardization, such a situation could result in an unknown number of positive cases not being identified due to false-negative results. The worst-case scenario would be a poorly performing diagnostic test being directly responsible for further outbreak spread as infectious individuals continue to circulate with the false confidence that they do not carry the pathogen. This situation of test escape has been observed to facilitate the spread of other infectious diseases (6).

While material standards have provided the main route for standardization in many molecular diagnostic areas, digital PCR (dPCR) has recently been proposed as a methodological standard, termed reference measurement procedure (RMP) (7), for testing spanning applications in precision medicine (8, 9) to infectious disease (10, 11). Recently, dPCR has also been applied as a potential RMP to outbreaks such as COVID-19 (2) and Mpox (12) offering a potential methodological route for standardization, which has the advantage of being dynamic and able to be deployed at a similar pace as the diagnostic tests that need to be standardized. Such methodological standards can be used to complement material standards allowing for a route for traceability when physical sharing of material standards may be delayed or even prevented, such as during a pandemic outbreak where borders are closed.

While there have been several reports of dPCR as an RMP, broader evidence of methodological reproducibility between laboratories over time, along with examples of how this may be applied by the wider community are needed. To address this, we explored the role of dPCR as an RMP in support of routine EQA using severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) molecular diagnosis as an example. Three National Metrology Institutes (NMIs) used RT-dPCR to quantify the RNA content of whole viral materials (32 SARS-CoV-2 control materials), which were subsequently used to guide the design and evaluation in 6 rounds of EQA for the genome detection of SARS-CoV-2 over a 17-month period. Crucially, the quantification by the NMIs was performed without the need for an external material standard calibrator allowing the methodological procedure to provide the route for standardization. This work illustrates how dPCR can act as an RMP for value assignment of material standards and support test accuracy for routing infectious disease molecular diagnosis as well as pandemic diagnostic response.

Materials and Methods

QUALITY ASSURANCE PANEL PREPARATION AND DISTRIBUTIONS

In total, 6 panels for the SARS-CoV-2 molecular EQA scheme were prepared and distributed by the Society for Promoting Quality Assurance in Medical Laboratories e.V. (INSTAND e.V.) for evaluation of SARS-CoV-2 molecular diagnostic performance between June 2020 and November 2021, see Supplemental Table 1.

Prior to each of the EQA rounds, 3 national metrology laboratories received 5 tubes/units of each EQA sample and each additional sample as a lyophilized cell culture supernatant. EQA samples were labeled from 340066 to 409020. Additional samples (not used for the EQA) are labeled with "G" followed by a number (see Supplemental Table 2). For this report, the respective samples were given an additional sample number from 1–32 (see Supplemental Tables 1 and 2).

Detailed EQA reports can be found at: https://rv-online.instandev.de/index.shtml (registration required). Each EQA scheme participant was offered the opportunity to submit qualitative and/or quantitative results. When submitting quantitative results based on RT-qPCR, the target gene region was requested in addition to the name of the test used. The calibrator and detailed protocols for quantification were not requested.

DETERMINATION OF SARS-COV-2 CONCENTRATION VALUES BY METROLOGY INSTITUTES

From 2020 to 2021, prior to the EQA round, the materials were analyzed by 3 NMIs [the National Measurement Laboratory (NML at LGC, UK), the

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National Institute of Standards and Technology (NIST, USA) and the National Metrology Institute of Germany, Physikalisch-Technische Bundesanstalt (PTB, Germany)] to determine an estimated SARS-CoV-2 RNA concentration within the respective panels using reverse transcription dPCR (RT-dPCR).

For all analyses, 3 tubes of each sample were analyzed on 3 different days applying the same validated extraction method and 1-step RT-dPCR method for the SARS-CoV-2 nucleocapsid (*N*) protein gene specific assays (see next).

RNA EXTRACTION AND RT-DPCR BY NMIS

After receipt, the samples were stored between 2 and 8°C prior to reconstitution in 1.1 mL nuclease free water (ddH_2O) according to the instructions. RNA was extracted from 140 to 200 µL aliquots from each sample unit using QIAamp Viral RNA mini kit (Qiagen) and eluted according to the manufacturer's instructions (the protocol used by NML, NIST, and PTB is described in (11)). The extracted RNA was either analyzed immediately or stored at −80°C for further use. Negative extraction controls were included in each experiment (details can be found in the online Supplement).

RT-DPCR

RT-dPCR experiments were performed using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad). The detailed protocol and thermal cycling conditions used by each NMI are described in the online Supplement. Negative template controls, without template, were included in each experiment and all reactions were performed in triplicate.

dPCR was performed using the QX200 Droplet Digital PCR System (Bio-Rad). 20 µL pre-reaction was pipetted into the sample-well of a DG8 cartridge, and droplets were generated as previously described (10). Thermocycling conditions varied by NMI (see online Supplement). The temperature ramp rate for each step was 2°C/s. After thermocycling was completed, plates were read on a QX200 Droplet Reader (Bio-Rad) and the data analyzed using QuantaSoft version 1.7.4.0917. Only the reactions with >10 000 accepted droplets were used for further analysis. Details of the molecular methods are outlined below and in PCR primer sequences can be found in the online Supplement (Supplemental Table 3). Examples of dPCR amplification plots and method linearity can be found in Supplemental Figs. 1 and 2 respectively. Information is also available in the online Supplement on analytical precision (Supplemental Table 4), limit of detection (Supplemental Table 5), and measurement uncertainty (Supplemental Table 6). The Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020 (13), dMIQE 2020 checklist providing additional methodological information can be found in Supplemental Table 7.

DATA ANALYSIS

All RT-dPCR EQA SARS-CoV-2 genome detection datasets (INSTAND code 340 and code 409) from 2020 to 2021 conducted by the NMIs were submitted to NML for analysis. RT-dPCR data from the 3 NMIs on the 6 rounds of the INSTAND SARS-CoV-2 molecular EQA scheme were aggregated to form a single dataset with the following variables:

1. Laboratories: NIST, NML, and PTB used different assays targeting the Nucleocapsid (N) protein gene.
2. Materials: 32 distinct SARS-CoV-2 containing materials covering a concentration range of between approximately 10^3 and 10^7 copies/mL (Supplemental Tables 1 and 2). Three of these were measured in >1 round; Sample 8 was measured in both November 2020 and June 2021, Sample 9 in November 2020, March 2021, and June 2021; and EQA Sample 5 in November 2020 and March 2021. This provided some information on the consistency of the overall mean between rounds.
3. Replicates: every sample was measured by each laboratory over 3 or more days, with repeated measurements on each day (mostly 3 replicates per day but varying between laboratories and samples).
4. Time: June 2020, November 2020, March 2021, June 2021, September 2021, and November 2021. Eleven samples (340068, 340070, 340072, 340074, 340076, 340078, 409004, 409007, 409010, 409017, 409019) were control samples and did not contain SARS-CoV-2 and were removed from the dataset. In addition to these samples, sample 340073 was not included in the analysis as its mean positive count was only 16, resulting in the expected higher relative standard deviation. The remaining dataset contained a total of 1278 observations.
5. Sample 340071 was not included in the data analysis because it was at a concentration level that resulted in an average count of roughly 2 positive partitions per reaction (213 ± 77 copies/mL), which was below the limit of detection. However, these samples were used for EQA analysis (Supplemental Table 1).

Data from the EQA participants who submitted RT-qPCR copy number values were evaluated to determine median and data distribution.

The INSTAND data were analyzed by fitting mixed effects models with restricted maximum likelihood estimation (14). EQA round, sample nested within round and assay were treated as fixed effects, and the random effects consisted of the following: laboratory, day nested

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within laboratory, and the interaction between round and laboratory. The last of these was included because of the observed differences in the between-laboratory variation across EQA rounds (see Supplemental Fig. 3).

Results

QUANTIFICATION OF INSTAND EQA SARS-COV-2 MATERIAL AND VALUE ASSIGNMENT BY THREE METROLOGY LABORATORIES

All SARS-CoV-2 negative samples (MERS CoV, human coronaviruses such as 229E, NL63, OC43, and CoV negative MRC-5-cell lysates) were consistent with a negative result (examples of negative results are included in Supplemental Fig. 1). The EQA material properties and the NMI consensus values obtained by the participating laboratories for the 6 EQA rounds are presented in Supplemental Table 1.

During the period examined, the overall reproducibility of the methods was consistent when looking at different EQA rounds between the 3 NMIs over a wide dynamic range of >4 orders of magnitude (roughly 10^3 to 10^7 copies/mL). Differences within the results derived from the different SARS-CoV-2 containing materials were further explored. The consistency of the between-laboratory variation was assessed using interaction plots. Supplemental Fig. 3 demonstrated that the mean value of a given sample generally varied in a similar manner between the 3 NMIs. However, the relative laboratory means were not the same in different rounds. This suggests that the 2 variables (round and laboratory) were not independent of each other, or that an additional source of variation was present. To account for this, an additional random interaction term was included in the model.

SAMPLES MEASURED MORE THAN ONCE

Although the 32 materials were almost completely nested within NMI analysis rounds, 3 samples were analyzed by the NMIs more than once in different rounds (Fig. 1, Table 1 and Supplemental Fig. 4). This was not considered in the model, with sets of repeat results being distinguished by using unique sample identifiers, and was considered justifiable as the departure from complete nesting was small.

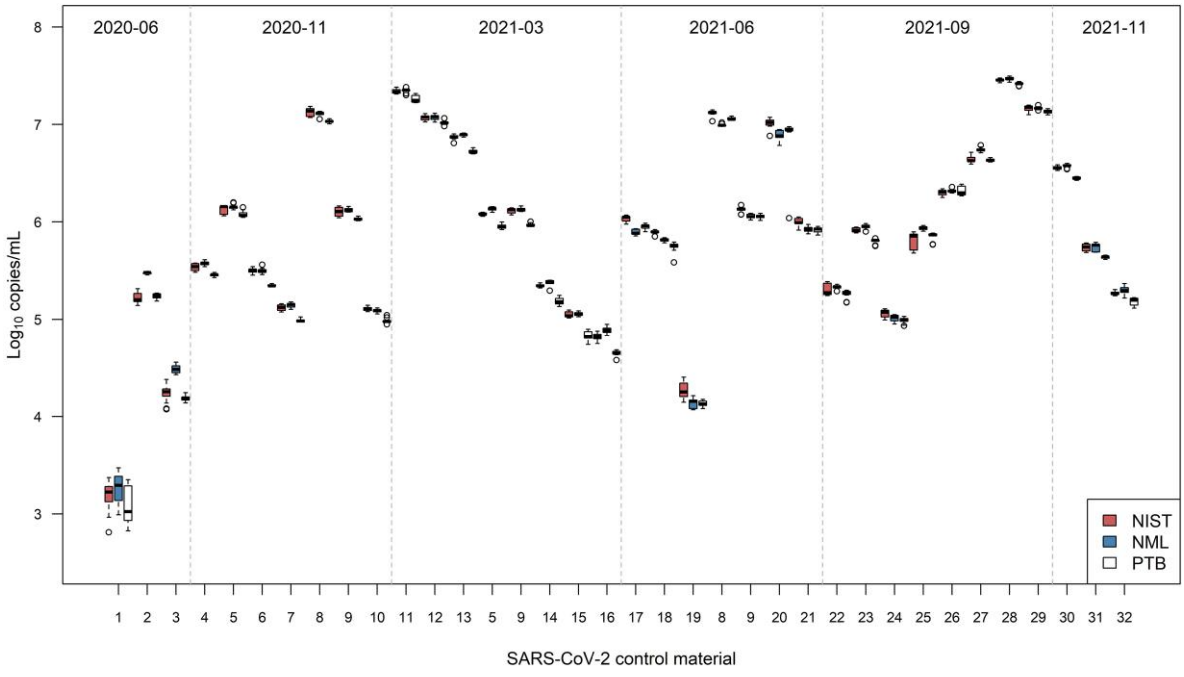

Fig. 1. Box plot showing results in $\log_{10}$ copies/mL (*N* gene RT-dPCR) in chronological order of the 32 SARS-CoV-2 control materials analyzed between June 2020 and November 2021. Each sample has 3 box and whisker results (median, interquartile, and range comprising 9 data sets), one per NMI. Color figure available at https://academic.oup.com/clinchem.

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VALUE ASSIGNMENT

The combined NMI RT-dPCR results were used to value assign RNA quantities within the materials prior to the EQA round providing an indicator of the quantity of SARS-CoV-2 RNA within the respective tubes (see Supplemental Table 1). Further investigation of the uncertainty illustrated that NMI-to-NMI RT-dPCR variation was the predominant contributor to the uncertainty on the value assignment and generally differed by <2-fold (Fig. 2).

Table 1. Estimates and standard errors for sample G21125 measured by the 3 NMIs in 3 separate rounds showing good agreement.

Round	Estimate (copies/mL)	Standard error (K = 1) (copies/mL)
November 2020	1 223 570	97 040
March 2021	1 194 730	97 240
June 2021	1 206 400	97 250

EQA USE AND WIDER APPLICATION OF VALUES ASSIGNMENT INFORMATION WITHIN THE EQA MATERIALS

In April 2020 INSTAND conducted the first SARS-CoV-2 EQA scheme to support the rapid deployment of molecular diagnostic tests for COVID-19 (15). Owing to the urgent need for the confidence afforded by the EQA, INSTAND opted to release interim results of 3 samples 1½ weeks before the deadline; a process that was both unorthodox and complicated. To support this early data release, the NMI laboratories began measuring the respective materials before the EQA round began. For the June 2020 EQA, the values were provided for one of the materials (sample 1, dPCR assigned concentration 1570 ± 360 copies/mL) on receipt of the materials. This allowed the participants to assess their diagnostic method against the reference values during the ongoing EQA scheme.

As the EQA rounds progressed, prior NMI value assignment continued to occur for 6 rounds in total over 17 months. The values were used by the EQA provider to guide EQA design and to inform participants of values including to support the determination of clinical

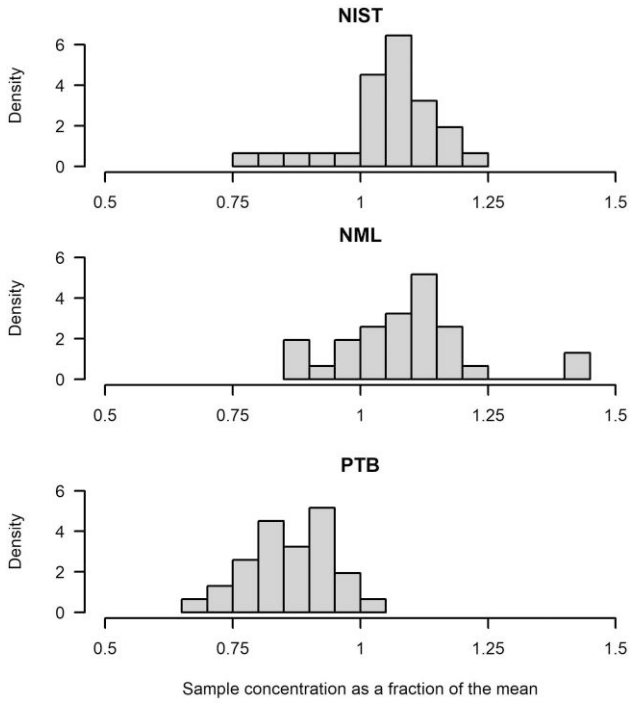

Fig. 2. Histogram showing the multiple of the consensus value for each experiment (fold difference) deviation of individual national metrology institute RT-dPCR result from the estimated sample concentration for 32 SARS-CoV-2 samples (concentration in copies/mL). A systematic difference from 1 indicates a relative measurement bias showing the difference relative to the overall mean for each sample, i.e., the relative difference between the laboratory result and the mean of the 3 laboratory results. The approximate range is around ±40%.

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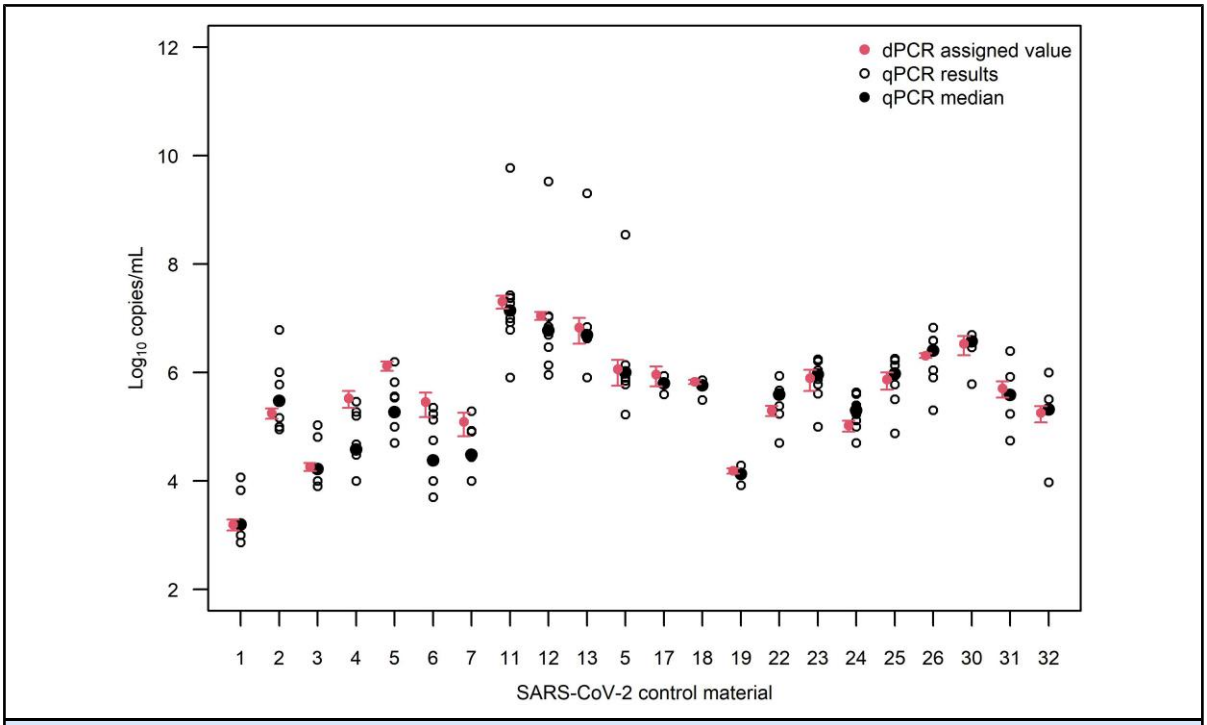

Fig. 3. Results obtained from 22 SARS-CoV-2 control materials used for EQA from Fig. 1 in $\log_{10}$ *N* gene copies/mL shown in chronological order and used for EQA between June 2020 and November 2021. Red points (with error bars) show national metrology institute consensus RT-dPCR values with the error bars indicating the expanded uncertainties (Supplemental Table 1). Black points (without error bars) show the median RT-qPCR copy number estimates from the EQA participants and clear points show the actual RT-qPCR results contributing to this median value. Color figure available at https://academic.oup.com/clinchem.

thresholds to guide intensive care patient management (2). The data from the EQA participant laboratories that submitted copy number values (contributing laboratory numbers varied with round and are detailed in Supplemental Table 1) was examined and the median and 2 times MADE(E) standard deviation calculated (Fig. 3); the median values generally showed good agreement with the RT-dPCR value assignment although the reproducibility was poor with 95% distributions often >2 orders of magnitude. For this assessment the dPCR values were not used to evaluate EQA participant laboratory performance; instead, the standard practice of consensus values was used.

Discussion

Digital PCR (dPCR) offers a range of potential advantages for nucleic acid analysis, one of which is the accurate quantification of nucleic acid molecules per unit volume. Unlike other molecular techniques, dPCR is capable of absolute quantification without calibration. Several publications have demonstrated that dPCR can perform with high accuracy when measuring purified DNA (9, 16) or RNA (17) in an aqueous/buffered solution. In addition, several studies also have shown high reproducibility when conducting measurements from whole microbes incorporating extraction of bacteria (10) and viruses (11, 18).

The references highlighted in the preceding paragraph demonstrate the applicability of dPCR as an RMP whereby the method offers the routes to support traceability for testing in conjunction with reference materials. This notion is further recognized by the inclusion of dPCR as a potential RMP in the recent update of the ISO standard 17511:2020 (In Vitro Diagnostic Medical Devices—Requirements For Establishing Metrological Traceability Of Values Assigned To Calibrators, Trueness Control Materials And Human Samples) (19) and the inclusion of a number of dPCR protocols on the database of the Joint Committee for Traceability in Laboratory Medicine (20).

In this study, we explored how RT-dPCR could act as an RMP to support different rounds of SARS-CoV-2 EQA schemes in support of NAAT diagnosis of

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COVID-19. The RMP (incorporating extraction and RT-dPCR) was applied by 3 NMIs to estimate the quantities of viral RNA present in 32 materials, 21 of which were used in 6 EQA rounds. The findings not only illustrated the level of high agreement between different NMI laboratories, with samples ranging in concentration by >4 orders of magnitude (Figs. 1 and 2), but also that this was reproducible over a 17-month time period, during which new batches of different genetic variants were produced (Fig. 1). We also illustrate how the RMP can be used to support EQA and aid in the assessment of results by providing a reference value on which to compare routine test findings (Fig. 3).

The quantity of analyte is an important consideration for preparing EQA materials when evaluating both quantitative and, as in this case, nonquantitative tests. Well defined materials allow more accurate assessment of the quantitative range at which a method can perform including when considering lower concentrations of material that may be used to evaluate the limit of detection. Conventional practice for estimating material concentration includes estimating viral abundance by plaque forming units or $TCID_{50}$ determined using viral culture. Such methods measure live virus quantities, but they can be variable (over time and between laboratories), and it may be unclear how such measurements are traceable to the amount of nucleic acid present. While these methods may correlate with the nucleic acid quantity (21), they do not provide a reliable and accurate estimate of the actual amount of nucleic acid within the sample. As it is the nucleic acid that is the molecular target of the NAATs, not knowing the concentration makes assessment of analytical performance specifications, such as limit of detection, challenging when using culture-based methods to estimate the reference value.

By using the dPCR estimated values, reported by the NMIs in this work, INSTAND could tailor selection of the SARS-CoV-2 EQA material concentrations with a more accurate knowledge of the amount of RNA analyte present within the EQA materials. This included when responding to changes in variants of concern to match the epidemiology of the changing pandemic.

While these findings demonstrate how dPCR can provide a reproducible estimation of the nucleic acid concentration to support molecular EQAs, additional research is needed to develop the method further and improve accuracy. The 3 NMI laboratories differed in mean estimation by a factor of up to roughly 2-fold when measuring the different materials (Fig. 2); the reason for the observed between-laboratory technical bias remains of interest. Future work focusing on procedural differences, such as instrument choice, reagent batch, or other subtleties may reduce variability, further improving accuracy.

In contrast to the roughly 2-fold laboratory difference observed with RT-dPCR applied by the 3 NMIs, the calibrated RT-qPCR estimation, from the respective diagnostic laboratories differed by 10- to 50-fold (Fig. 3). When the quantification cycle (C_q) (also termed cycle threshold, C_t) is used alone, even greater variability (>100-fold) was reported (22, 23). The results here add considerable evidence to earlier work (10, 11, 18) suggesting that (RT-) dPCR offers a paradigm shift in methodological agreement given the orders of magnitude of variability observed in quantification using qPCR. This work adds to earlier findings as it illustrates both the high reproducibility of the RMP over time and between NMI values, as well as how an EQA provider can use RT-dPCR measurements of RNA quantity within the various materials to understand the various ranges of the analyte covered by the scheme.

By using SARS-CoV-2 as a test case, we were able to evaluate dPCR as an RMP to support an EQA for an infectious disease scenario caused by an RNA virus. This procedure includes several components that contribute to the measurement error: the extraction step, reverse transcription of RNA (24) and a virus that must be detected over a wide dynamic range. The findings of this study will be readily applicable to similar scenarios, such as value assignment of other RNA and DNA viral materials, following validation of such methods using similar approaches outlined here. However, additional considerations may be needed when using dPCR as an RMP for pathogens that are more challenging to extract (due to the nature of the pathogen and/or the associated matrix) to ensure longer term reproducibility. For other diseases where molecular diagnosis is popular, such as tuberculosis (and associated drug resistance) the causative agent, *Mycobacterium tuberculosis*, is challenging to extract. Additional work is required to assess how these initial findings (10) can be applied to support the value assignment of more challenging EQA materials and the approach outlined here offers a template on how to approach such an evaluation.

The use of dPCR as an RMP offers a methodological route for metrological traceability to support test accuracy for a wide range of molecular testing applications. Such approaches may be of particular value where diagnosis is depended on to manage and prevent disease outbreaks by tracking and identifying infected patients. dPCR may also be useful as an RMP when testing for neglected and emerging infectious diseases where primary reference materials may not yet be available. Such methods could become essential in providing traceability to the in vitro diagnostic tests used during the early stages of a pandemic where vaccines and treatments are not available and nontherapeutic interventions, guided by accurate diagnostics, offer the main solution for stopping disease spread. dPCR could also be used to determine the reference ranges of the

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nucleic acid targets within the clinical specimens that could also be used in the selection and deployment of test modalities that may vary in analytical performance (25), such as PCR and lateral flow devices.

Conclusion

This work demonstrates that (RT-)dPCR can provide a reproducible estimation of the amount of nucleic acid diagnostic analyte present within the EQA panels, providing quantitative information for EQA schemes, intended to support molecular testing. This may be particularly important in challenging situations such as pandemic response. (RT-)dPCR could also be used to link the findings of different panels over time or between different EQA providers. The reproducibility demonstrated by this study also suggests that dPCR could be a valuable method for value-assigning EQA panels to align with the WHO standard, improving global harmonization. While this approach has the potential to support traceability for a wide range of molecular diagnostic applications, the fact that dPCR protocols can be developed at a similar pace to the tests they can support means they offer an ideal approach for underpinning the quality of diagnostic tests deployed at speed in response to disease outbreaks. The concepts and results presented here are not only specific for SARS-CoV-2 EQA, but transferable to other infectious disease EQA and, due to the speed with which RMPs can be developed, especially relevant to supporting the prevention of future pandemics.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: NAATs, nucleic acid amplification tests; RMPs, reference measurement procedures; dPCR, digital PCR; EQA, external quality assessment; NMIs, National Metrology Institutes; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-dPCR, reverse transcription digital PCR; RT-qPCR, reverse transcription quantitative PCR; NIST, National Institute of Standards and Technology; PTB, Physikalisch-Technische Bundesanstalt (National Metrology Institute of Germany).

Gene: *N*, nucleocapsid gene.

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