Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Biologicals 79 (2022) 31–37

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

A collaborative study to establish the national standard for SARS-CoV-2 RNA nucleic acid amplification techniques (NAAT) in Taiwan

Po-Lin Lin, Ming-Sian Wu, Po-Chi Wu, Hsin-Mei Chen, Yi-Hsuan Peng, Jia-Chuan Hsu, Der-Yuan Wang *

Division of Research and Analysis, Taiwan Food and Drug Administration, Ministry of Health and Welfare, No.161-2, Kunyang St, Nangang District, Taipei City, 11561, Taiwan, ROC

ARTICLE INFO

Keywords:
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)
Nucleic acid amplification techniques (NAAT)
National standard (NS)
International standard (IS)

ABSTRACT

The conventional PCR remains a valuable method to detect the newly emergent coronavirus rapidly and accurately. Our investigation aimed to establish the standard materials of SARS-CoV-2 for NAAT detection. We provided formalin-inactivated SARS-CoV-2 and confirmed RNA copy numbers. In addition, the virus genome was confirmed with whole-genome sequencing and identified as Wuhan/WI04/2019. Seven laboratories were invited for this collaborative study, according to the reporting data, we determined the SARS-CoV-2 with the unit of 6.35 Log_{10} copies/mL as the national standard. The availability of the national standard (NS) of SARS-CoV-2 will facilitate the standardization and harmonization of SARS-CoV-2 NAAT assays.

1. Introduction

At the end of 2019, a novel coronavirus called severe acute respiratory syndrome coronavirus 2 emerged and designated coronavirus disease 2019 (COVID-19) in Wuhan, China [1]. The number of confirmed cases has already surpassed one hundred million, and it is still rising. The consistent spread of COVID-19 has already posed an unprecedented threat to global public health [2].

A wide variety of testing methods were developed to confirm SARS-CoV-2 RNA. The diagnosis of COVID-19 could be made using viral load measurement, computed tomography scan (CT scan) [3], various laboratory-developed tests (LDTs) based on NAAT-based assays, serological immunoassays, or the CRISPR-based assay [4,5]. Until now, the detection of SARS-CoV-2 specific RNA using NAAT-based assays remains the primary clinical diagnostic test. One or more viral target genes are generally applied in the assay, such as open reading frame 1a/b (ORF1a/b), RNA-dependent RNA polymerase (RdRp), envelope (E), spike (S), or nucleocapsid (N) genes [6,7]. With the detection of more than one region of SARS-CoV-2 virus genomes, higher accuracy, including risks of rapidly mutating viruses, could be achieved [8].

Numerous *in vitro* diagnostic (IVD) devices based on NAAT technology have been developed for SARS-CoV-2 qualitative or quantitative tests to fulfill the urgent local demands. As of May 31, 2021, the Taiwan Food and Drug Administration (TFDA) had already granted an emergency use authorization (EUA) for 16 NAAT-based IVD products (three products are manufactured domestically, and 13 are imported). To ensure the quality of these NAAT-based IVD devices, the availability of reference materials with the assigned potency values is of critical importance.

The availability of a calibrated national reference standard for SARS-CoV-2 RNA is urgently needed for control of the epidemic at the local level, including assurance of relevant IVD performance evaluations regardless of the assays used. Therefore, the object of this study was to establish the optimal candidate as the first NS for SARS-CoV-2 RNA NAAT assays and evaluate the SARS-CoV-2 RNA copy number concentration through a collaborative international effort. Typically, it takes two to three years to develop the biological standards. However, to satisfy local demand, this study was conducted within 10 months.

Abbreviations: NAAT, nucleic acid amplification techniques; NS, national standard; LDT, laboratory-developed test; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; E, envelope; S, spike; N, nucleocapsid; TFDA, Taiwan Food and Drug Administration; EUA, emergency use authorization; CPE, cytopathic effect; SD, Standard deviation; CV, coefficient of variation; ECBS, expert committee on biological standardization; NIBSC, National Institute for Biological Standards and Control; IS, international standard.

* Corresponding author.
E-mail address: dywang@fda.gov.tw (D.-Y. Wang).

https://doi.org/10.1016/j.biologicals.2022.08.002
Received 30 June 2021; Received in revised form 14 June 2022; Accepted 21 August 2022
Available online 31 August 2022
1045-1056/© 2022 International Alliance for Biological Standardization. Published by Elsevier Ltd. All rights reserved.

2. Materials and methods

2.1. Preparation of the national candidate standards

The candidate materials comprised whole virus preparations of CGMH-CGU-01/2020, which was kindly provided by the Chang Gung Memorial Hospital, Taoyuan, Taiwan. The virus was propagated in Vero E6 cells, and virus culture fluid was harvested once a cytopathic effect (CPE) was observed. The culture supernatant was clarified using low-speed centrifugation at 2000×*g* for 20 min. The virus supernatant was incubated with formalin (final concentration of 0.01%) at 4 °C for 10 days. Most of the frozen SARS-CoV-2 standard material was diluted to contain approximately 3.0×10^6 copies/mL in a final volume of 200 mL PBS buffer. Standard candidate material was dispensed in 0.5 mL aliquots and stored at −80 °C. The operation and storage of this material complied with the Centers for Disease Control (CDC) biosafety regulations. In the collaborative study, the SARS-CoV-2 RNA concentration was determined using the digital PCR LDT described below.

2.2. Genomic sequencing of the national candidate standards

Viral RNA was used to generate dual-index libraries using the Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit. The quality and yield of the prepared libraries were assessed using a Qubit 2.0 fluorometer (ThermoFisher Scientific, USA) and a Qsep1 Bio-Fragment Analyzer (BiOptic Inc., Taiwan). Sequencing was conducted with a MiSeq Instrument (Illumina, USA) with two 151-cycle reads (2 x 151). All protocols were carried out following the manufacturer's instructions. The obtained files were treated using fastp (v.0.21.0) to remove low-quality reads by trimming low-quality residues and filtering out duplicate reads. The modified (cleaned) reads were aligned to the SARS-CoV-2 reference genome (GeneBank accession: NC_045512) using the Burrows-Wheeler Aligner MEM algorithm (BWA-MEM, v0.7.17). SAMtools (v.1.11) was used to sort BAM files and generate alignment statistics. LoFreq (v2.1.5) was performed to call variants for generating the consensus genome.

2.3. Verification of the inactivated virus

To verify the consequence of virus inactivation, both positive and negative control and testing samples were added to a confluent monolayer of Vero E6 cells; cell pathological effects were subjected to phase-contrast microscopic observation. The inactivated virus culture medium was examined to establish the initial inoculum level of viral RNA. This was accomplished using RT-qPCR described below, which detected the E and RdRp gene of SARS-CoV-2. The flasks were kept in culture for seven days, observing for the presence of a CPE. The SARS-CoV-2 infection of Vero E6 resulted in a visible CPE by day three in culture. On day seven, a sample of the culture supernatant was again collected for quantitative analysis and 1 mL was transferred to a fresh flask of Vero E6 cells. The cell culture supernatant for all samples continued to be passaged two times, incubating for seven days to allow the CPE to become visible. Samples were collected for quantitative analysis at the beginning and end of each passage.

2.4. Digital PCR and RT-qPCR analyses

The RNA concentration of the SARS-CoV-2 virus stocks was determined using digital PCR and RT-qPCR. The assay amplifies and detects conserved regions of the RdRp, E, and N genes. The viral RNA extraction was conducted according to the manufacturer's instructions. In that process, nucleic acid is purified using capture oligonucleotides in a magnetic field and eluted in a 60 μL elution buffer. For digital PCR, 10 μL of extracted RNA was subjected to reverse transcription using the SuperScript®IV Reverse Transcriptase (Thermo Fisher, USA). 1 μL of RT product was then amplified by digital PCR in 15 μL of total reaction volume, using the QuantStudio™ 3D Digital PCR (Thermo Fisher, USA) and all samples were amplified in duplicate. For RT-qPCR, LightCycler® Multiplex RNA Virus Master (Roche, Germany) was used in RT-qPCR. 5 μL of extracted RNA was then transcribed and amplified by RT-qPCR in 20 μL of total reaction volume, using the LightCycler® 480 Instrument (Roche, Germany). All samples were amplified in triplicate. The primer sequences used in this study were listed in Supplementary Table 1.

2.5. Study participants

Seven laboratories from five countries participated in the collaborative study and are listed in Table 1. All participating laboratories were referred to by a code number, allocated at random, and not representing the order of the list in Table 1. When a laboratory returned data using different assay methods, the results were analyzed separately, as if from different laboratories, for example, laboratory 1A, 1B, etc.

2.6. Design of the international collaborative study

Four vials of the SARS-CoV-2 candidate NS were delivered to participating laboratories by courier on dry ice with specific instructions for storage. If the participating laboratories used more than one method, an additional four vials of candidate standards were provided for each method. Participants were requested to test each sample using their routine SARS-CoV-2 NAAT assay on three separate assay runs, using a fresh vial in each assay. Study samples were thawed and vortexed briefly before use. For quantitative assays, participants were asked to test a minimum of two serial ten-fold dilutions within the assay's linear range. For qualitative assays, participants were requested to test ten-fold serial dilutions of each sample in the first assay to determine the assay end-point. The next step was to fine-tune the end-point by testing a minimum of three $\log_{10}$ serial dilutions on either side of the predetermined end-point for subsequent assays. Participants were asked to dilute samples using the sample matrix specific to their individual assay and extract each dilution before amplification. Participants were requested to report the viral load in 'copies/mL' (positive/negative for qualitative assays) for each dilution of each sample and return the results, including details of the methodology used, to the TFDA for analysis.

2.7. Stability study of the national candidate standard

The stability of the frozen SARS-CoV-2 preparation is being assessed in an ongoing accelerated thermal degradation study to predict the stability of the product when stored at the recommended temperature. The numerous vials of the SARS-CoV-2 preparation are being maintained at −80 °C, −20 °C, +4 °C, and +24 °C. At specified time points during the product's shelf period (Fig. 4a), three vials are retrieved from each temperature and the SARS-CoV-2 RNA are quantified using the RT-qPCR described above.

Table 1
Collaborative study participants.

Organisation	Country
Center for Biologics Evaluation and Research (CBER), US FDA	United States
National Centre for the Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome (CNCF)	Italy
National Institute for Biological Standards and Control (NIBSC)	United Kingdom
Paul-Ehrlich-Institute (PEI)	Germany
Taiwan Centers for Disease Control (TCDC)	Taiwan
Section of Biologics, Division of Research and Analysis, Taiwan Food and Drug Administration (TFDA)	Taiwan
Section of Food Biology, Division of Research and Analysis, Taiwan Food and Drug Administration (TFDA)	Taiwan

2.8. Statistical methods

The NAAT qualitative and quantitative assay results were evaluated separately. For the qualitative assays, data obtained from individual laboratory and assay method were pooled to give a positive number out of a total number tested at each dilution step.

A single end-point for each dilution series was calculated to estimate NAAT detectable units/mL based on method LOD. In the case of quantitative assays, analysis was based on the results reported by the participants in copies/mL. For each assay run, a single estimate of $\log_{10}$ 'copies/mL' was obtained for each sample by taking the mean of the $\log_{10}$ estimates of 'copies/mL' across replicates after correcting for any dilution factor. A single estimate for the laboratory and assay method was then calculated as the mean of the $\log_{10}$ estimates of 'copies/mL' across assay runs. Overall analysis was based on the $\log_{10}$ estimates of 'copies/mL' or 'NAT detectable units/mL.' General mean estimates were calculated as the means for all individual laboratories. The variation between laboratories (inter-laboratory) was expressed as the SD of the $\log_{10}$ estimates and the percentage coefficient of variation (%CV) of the actual estimates.

3. Results

3.1. Verification of the cadidate national standard

As the previous study concerns the rapid increases of genetic variants in the SARS-CoV-2 genome when cultured in Vero E6 cells, especially in the furin cleavage site (FCS, aa: 680–685). Their data demonstrated that 90% of the virus in the second passage (P2) contained a 24-nucleotide deletion in the spike region and resulted in the frameshift changes in FCS [9]. Thus, the virus genome was sequenced after viral propagation and analyzed by Illumina Miseq relative to the reference Wuhan virus genome. Eight base variants could be observed in our viral materials cultivated under normal culture conditions (Fig. 1) [10,11] and less than 2% of the P2 virus has changed in the FCS (data not shown). In addition, the primer nucleotides were aligned with original stock and P2 virus target genes and no nucleotide changes were found in the primer detection region in both two target genes. (Supplementary Fig. 1). Next, the candidate national standards were prepared by dispensing 500 μL of the formalin-treated SARS-CoV-2 homogenate into each vial. The approach for SARS-CoV-2 cultivation and subsequent inactivation was described previously.

To validate the SARS-CoV-2 inactivation, the supernatant of the SARS-CoV-2 virus or inactivated virus was harvested and further inoculated with the freshly-prepared cells for three cycles. To rule out the cytotoxicity of formalin, an MTT assay was performed to examine the maximum tolerance concentration of formalin (data not shown). There was no observable CPE at the formalin-treated SARS-CoV-2 after three cycles (Fig. 2a). The expression of target genes of formalin-treated SARS-CoV-2 harvests did not show any amplification tendency from the real-time analysis (Fig. 2b). Furthermore, the viral proteins of spike and nucleoprotein were undetected in formalin-treated samples (Fig. 2c). These results showed that no infectious virus had been detected in the proposed NS.

3.2. Laboratory codes and assay methods

A total of 17 datasets were collected from all the participating laboratories and evaluated within this collaborative study (Table 2). Participants used either the laboratory-derived methods or commercial kits, with some laboratories performing more than one assay. The assay methodologies covered three platform technologies, including digital PCR, RT-qPCR, and insulated isothermal PCR. All laboratories returned the results with three independent assays, including 12 quantitative datasets and five qualitative datasets. The datasets had the distribution to cover the target genes, including ORF1ab, RdRp, N, and E genes. For the quantitative results, 10 were obtained from the digital PCR analysis. Two (Lab 1d, Lab 2) reported the quantitative results by performing RT-qPCR analysis using the comparative calculation from the standard curve of the internal references. Lab 2 reported the results in IU because the proposed World Health Organization (WHO) IS was included in the test. For the qualitative results, two datasets (Lab 5d, 7) reported positive/negative, obtaining the results from the insulated isothermal PCR platform. The remaining qualitative results (Lab 6a, 6b, and 6c) were reported as cycle threshold (Ct) values with the in-house RT-PCR method.

3.3. Estimated value of the candidate national standard

The mean estimates from the individual laboratories for the candidate standard by quantitative assays (in $\log_{10}$ "copies/mL"), along with the SD and the CV are listed in Table 3, and the qualitative assays (in $\log_{10}$ "NAAT detectable copies/mL") are listed in Table 4. Results for qualitative assays show considerable variation in the reported viral load between different assays, with estimates differing by up to 3000-fold copies/mL. Due to the value of qualitative assays were only determined by the LOD, qualitative assays were excluded from the candidate standard estimates. However, the estimate results from most of the

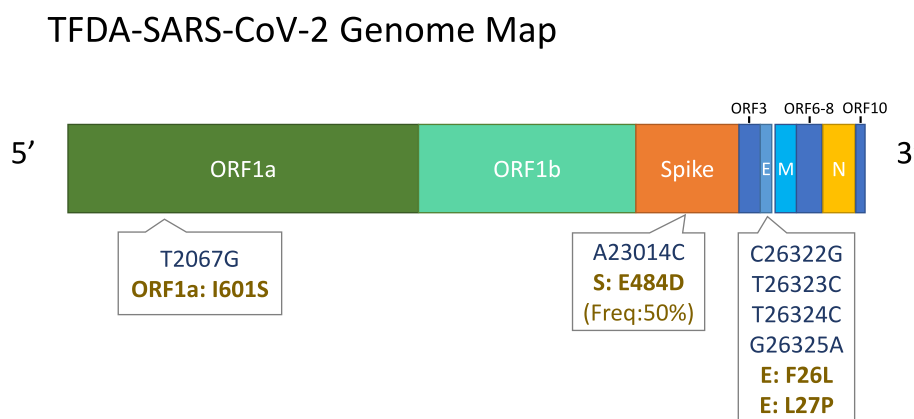

Fig. 1. The entire genome sequencing of SARS-CoV-2. The entire virus genome was sequenced, analyzed using Illumina Miseq., and compared with the reference genome (NC_045512.2 Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome). Eight nucleotides and two amino acid variants can be observed. Abbreviations: ORF = open reading frames; E = envelope glycoprotein gene; M = membrane glycoprotein gene; N = nucleocapsid gene.

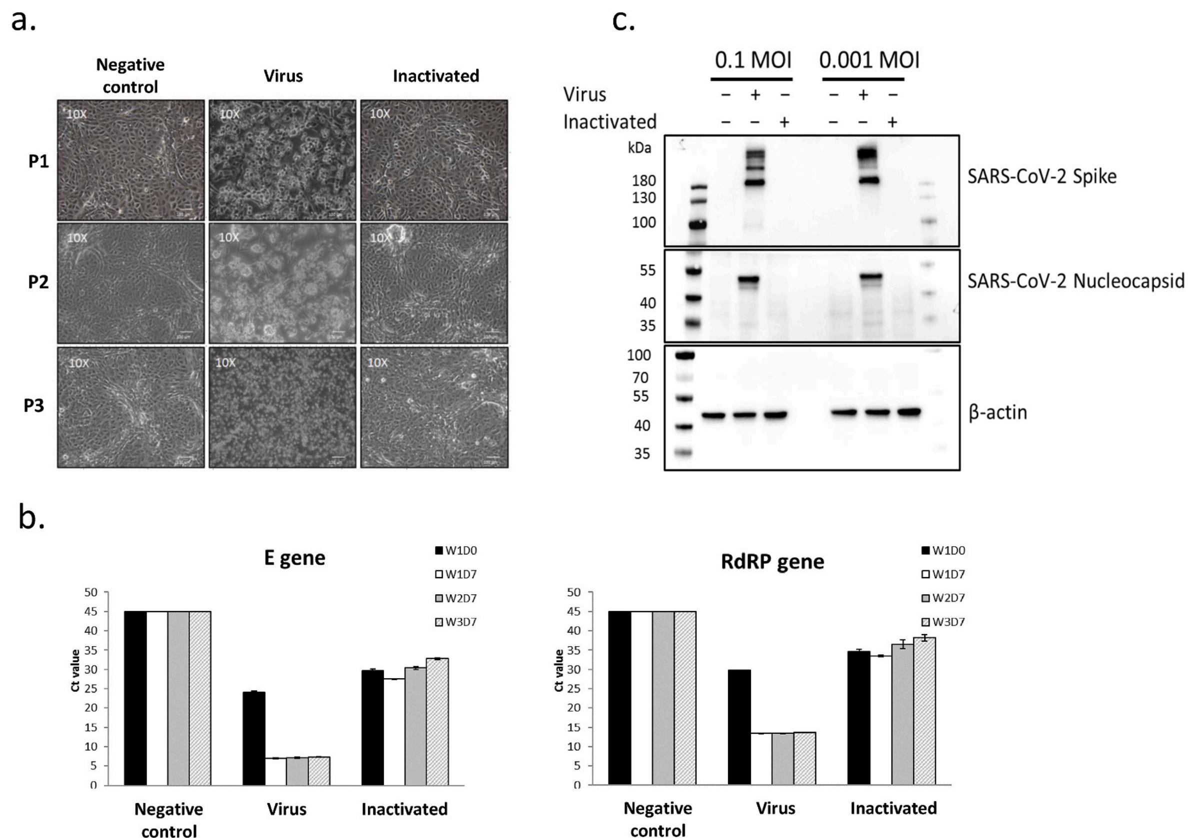

Fig. 2. The preparation of inactivated SARS-CoV-2 national candidate standard was verified using three methods
a) Vero E6 cells were inoculated with/without SARS-CoV-2 and formalin-inactivated virus. Significant CPE was observed in cells infected with the virus but not in the other two groups.
b) Ct values quantitated by RT-qPCR with primer-probe targeting E and RdRp genes. After seven days of incubation, the supernatant was harvested and used to infect new prepared cells for another seven days for a total of three weeks. Error bars represent the standard deviation of duplicate reactions. Undetermined values were assigned a Ct value of 45.
c) A total of 20 μg of protein were used to detect spike and nucleocapsid proteins. Protein lysate of 0.1 M.O.I was harvested on third day, while lysate of 0.001 MOI was harvested seven days after infection.

qualitative assays were close to those of the quantitative assays. The quantitative values of SARS-CoV-2 RNA estimates from each laboratory are shown in histogram form (Fig. 3), and each data is also within the range of the overall average ± 2SD. All of the values, show the distributions within the intervals of 5.5–7.2 log_{10} copies/mL. Supplementary Table 2 shows the intra-laboratory SDs and CVs. The intra-laboratory SDs is approximately 0.02–0.14 log_{10} copies/mL, the mean SD is approximately 0.07 log_{10} copies/mL, the intra-laboratory CVs is approximately 0.08–1.96%, the mean CV is approximately 1.07%. For intra-laboratory study, both digital PCR and qRT-PCR method demonstrated consistent data with different gene in every individual participant. The inter-laboratory variation was slightly higher than the intra-laboratory variation, the inter-laboratory SD is approximately 0.50 log_{10} copies/mL and the CVs is approximately 7.80%. The overall mean of the candidate standard from the qualitative assays is 6.35 log_{10} copies/mL (2.24×10^6 copies/mL), and the 95% confidence intervals are 5.35 log_{10} copies/mL (2.24×10^5 copies/mL) to 7.34 log_{10} copies/mL (2.19×10^7 copies/mL).

3.4. *Stability of the national candidate standard*

To evaluate the stability of our standard materials, fifteen to eighteen vials of candidate standard were selected and stored at 24 °C, +4 °C, −20 °C, and −80 °C. Three vials from each group were randomly examined for stability tests at specified time intervals as shown in Fig. 4a.

Candidate standards were validated at zero-, first-, second-, fourth- and eighth-week for 24 °C and additional twelveth-week for 4 °C. Those held at −20 °C were validated at zero, third-, sixth-, twelfth-, eighteenth- and twenty-fourth-month. For −80 °C storage condition, samples were validated at zero-, sixth-, twelfth-, eighteenth- and twenty-fourth-month (Fig. 4a). The viral RNA was extracted and subjected to quantitative analysis and the current stability results were shown in Fig. 4b, demonstrated the mean concentration (in log_{10} "copies/mL") in three independent tests for each time point at different temperatures. The results show that the candidate samples had no observable changes in potency at −20 °C and −80 °C storage after six months. However, the potency of the candidate samples fell when they were stored at +4 °C and +24 °C after four weeks. The results suggest that the proposed NS samples stored at −20 °C or −80 °C exhibited substantially more stability during long-term storage than those stored above zero degrees Celsius.

4. Discussion

This study aimed to establish a NS for nucleic acid amplification tests of SARS-CoV-2. The first WHO IS for SARS-CoV-2 RNA was established by the WHO expert committee on biological standardization (ECBS) in 2020. This IS (code number: 20/146) can be purchased from National Institute for Biological Standards and Control (NIBSC) However, the availability of the IS for SARS-CoV-2 is limited. Therefore, the NS development is critically required to validate the quality of commercial

Table 2
Laboratory codes and assay methods.

Lab Code	Extraction method	NAAT method	Assay type	Target	Reported Readout
1a	QIAamp Viral RNA Mini Kit	dPCR Bio-Rad	Quantitative	N1	copies
1b	QIAamp Viral RNA Mini Kit	dPCR Bio-Rad	Quantitative	RdRp	copies
1c	QIAamp Viral RNA Mini Kit	dPCR *in-house* method	Quantitative	E	copies
1d	QIAamp Viral RNA Mini Kit	RT-PCR	Quantitative	E	Ct; copies
2	QIAamp Viral RNA Mini Kit	RT-PCR	Quantitative	E	Ct; IU
3a	QIAamp Viral RNA Mini Kit	dPCR Bio-Rad	Quantitative	N1	copies
3b	QIAamp Viral RNA Mini Kit (DNAse)	dPCR Bio-Rad	Quantitative	N1	copies
4a	TANBead Nucleic Acid Extraction Kit	dPCR Bio-Rad	Quantitative	RdRp	copies
4b	TANBead Nucleic Acid Extraction Kit	dPCR Bio-Rad	Quantitative	E	copies
5a	TANBead Nucleic Acid Extraction Kit	dPCR QS3D	Quantitative	RdRp	copies
5b	TANBead Nucleic Acid Extraction Kit	dPCR QS3D	Quantitative	E	copies
5c	TANBead Nucleic Acid Extraction Kit	dPCR QS3D	Quantitative	N	copies
5d	POCKIT™ Central Cartridge Set	POCKIT™ Central SARS-CoV-2 (iiPCR, CE; EUA)	Qualitative	orf 1 ab	+/-
6a	TANBead Nucleic Acid Extraction Kit	RT-PCR	Qualitative	RdRp	Ct
6b	TANBead Nucleic Acid Extraction Kit	RT-PCR	Qualitative	E	Ct
6c	TANBead Nucleic Acid Extraction Kit	RT-PCR	Qualitative	N	Ct
7	Procleix Panther System (Grifols)	Procleix SARS-CoV-2 Assay (TMA, CE; EUA)	Qualitative	RdRp	+/-

SARS-CoV-2 NAAT-associated reagents and instruments. An ideal standard material is an analyte that performs well in various types of assays and can reflect the results of testing samples in an assay.

The purpose of the SARS-CoV-2 standard material was to set up the calibration and quality control of the nucleic acid amplification analysis. This investigation invited multi-center laboratories to collaboratively evaluate the candidate materials for their ability to harmonize the potencies of the SARS-CoV-2 RNA sample among the various institutions. In addition, such reference materials would help calibrate the data of NAAT-based IVD device for the pre-market products evaluation. Those evaluation results would be valuable for defining parameters such as the analytical sensitivity and limitations of NAAT detecting assays. Therefore, the TFDA is actively working to establish the primary SARS-CoV-2 NS with the formalin treatment virus, expected to be credible reference materials for further performance evaluation. In addition to the higher potency of the NS, the secondary working standards with lower copy numbers of viral RNA were also under evaluation. This evaluation would help reduce the variability of testing results from the various laboratories and compare their proficiency.

In general, reference materials should exhibit the properties of

Table 3
Quantitative method mean copies estimates of SARS-CoV-2 RNA National standard.

Lab Code	NAAT Method	Target	mean (Log_{10} copies/mL)	SD	CV %
1a	dPCR Bio-Rad	N1	5.80	0.06	1.08
1b	dPCR Bio-Rad	RdRp	5.53	0.08	1.43
1c	dPCR *in-house* method	E	5.63	0.02	0.30
1d	RT-PCR	E	6.16	0.08	1.23
Lab 1			5.78	0.25	4.35
2	RT-PCR	E	6.33	0.07	1.14
3a	dPCR Bio-Rad	N1	6.51	0.05	0.08
3b	dPCR Bio-Rad	N1	7.11	0.07	1.00
Lab 3			6.81	0.31	4.54
4a	dPCR Bio-Rad	RdRp	6.79	0.12	1.79
4b	dPCR Bio-Rad	E	7.16	0.14	1.96
Lab 4			6.98	0.23	3.30
5a	dPCR QS3D	RdRp	6.35	0.06	1.02
5b	dPCR QS3D	E	6.74	0.02	0.27
5c	dPCR QS3D	N	6.01	0.09	1.50
Lab 5			6.35	0.31	4.83
Calculated value			**6.35**	**0.50**	**7.80**

Table 4
Qualitative method estimate approximatively copies of SARS-CoV-2 RNA National standard.

Lab Code	NAAT Method	Target	Estimate copies (Log_{10} copies/mL)
5d	POCKIT™ Central SARS-CoV-2 (iiPCR)	orf 1 ab	6.28
6a	RT-PCR	RdRp	6.12
6b	RT-PCR	E	5.82
6c	RT-PCR	N	5.73
7	Procleix SARS-CoV-2 Assay (TMA)	RdRp	8.45–9.42

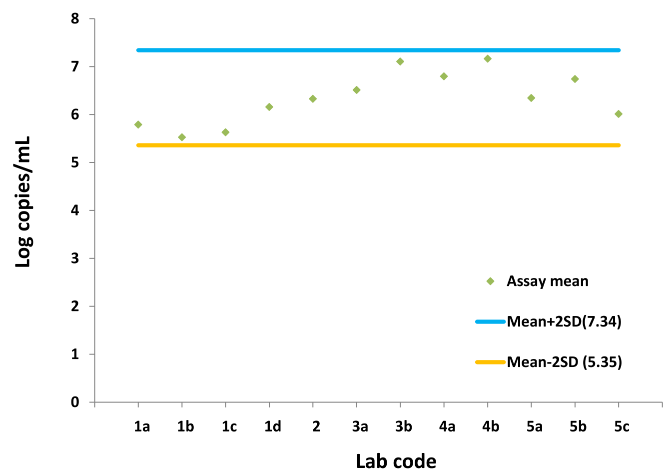

Fig. 3. Distribution chart of the quantitative results of the SARS-CoV-2 NS. The mean estimates from the individual laboratories for the SARS-CoV-2 NS were obtained using quantitative NAAT assays. The upper blue line is the mean + 2SD; the lower yellow line is the mean value -2SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

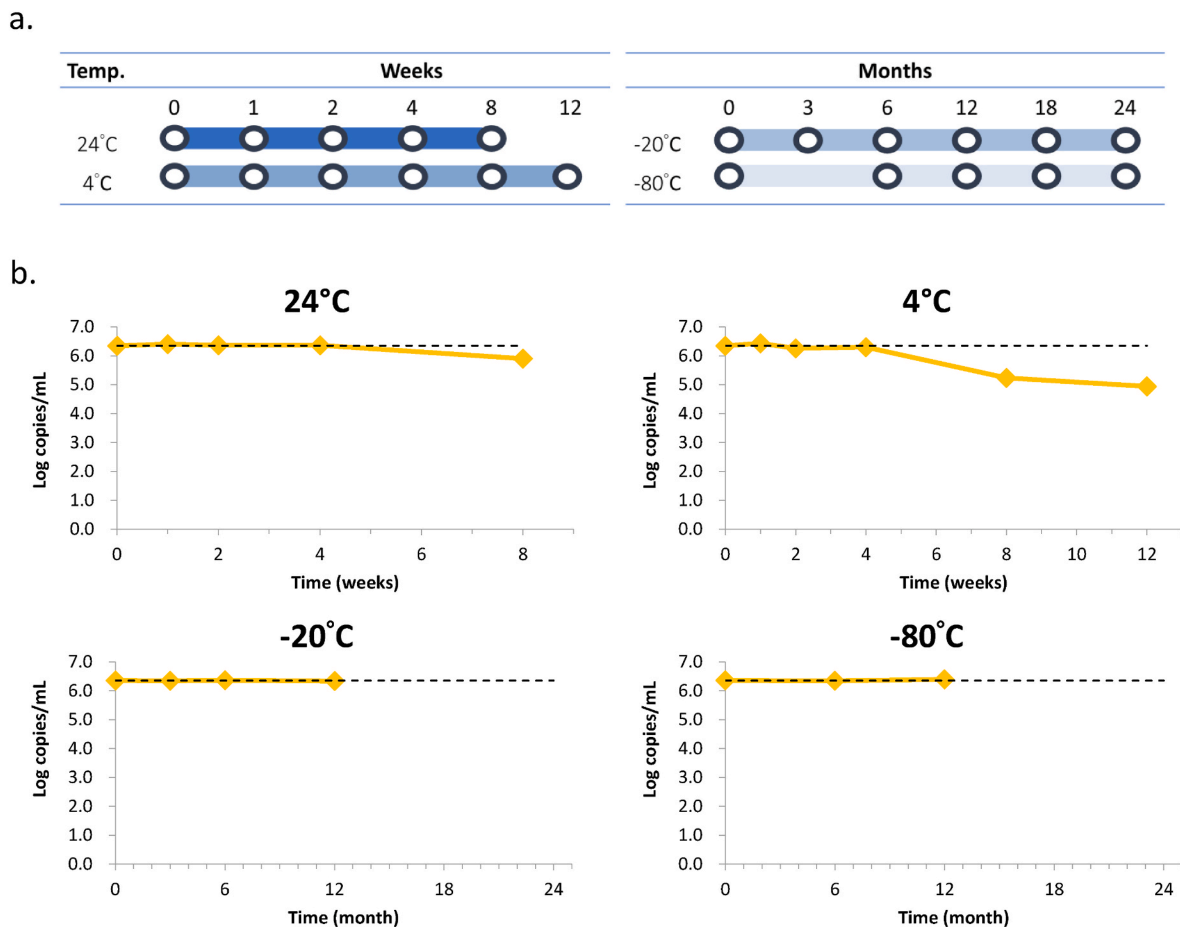

Fig. 4. The stability assessment of the SARS-CoV-2 national standard.
a) The SARS-CoV-2 national standard was stored at four temperatures: 24 °C, 4 °C, −20 °C, and −80 °C at specified time points.
b) The RNA concentration of four temperatures were evaluated at corresponding times using RT-qPCR.

commutability. There are many starting materials for preparing the standards, including the inactivated virus isolates or synthetic materials. The starting materials for our prepared candidate standards were the intact SARS-CoV-2 virus containing the entire genome. That genome would be more representative for clinical samples when presented with different matrixes. Considering that SARS-CoV-2 is a high-risk infectious agent, inactivation treatments are required to assure its biosafety and enable it to be handled in a laboratory with a lower biosafety level.

There are many strategies to inactivate the virus [12–17]. The physical inactivation can be performed using heat or exposure to UV. Heat inactivates the virus by denaturing the structure of the proteins, affecting the attachment and replication of the virus in the host cell [16]. Because of the known accidental exposure cases of SARS in the laboratory [17], SARS-CoV-2 inactivation was highly emphasized in our laboratory before transferred from BSL3 to BSL2 environment. Inactivation can be achieved by either a physical or chemical process and which approach should be considered is often related to their further application. During the initial outbreak of the novel SARS-CoV-2, the characteristics of the virus remained unclear and being the research and analytic team of TFDA, we had the responsibility to promptly provide the nucleic acid standard to validate the authenticity of IVD reagents. In this study, SARS-CoV-2 was successfully inactivated with a final formalin concentration of 0.01%, followed by incubation of 10 days at 4 °C. However, according to the previous study, inactivation with formalin would result in crosslinking of RNA to nucleocapsid proteins, causing a block of genome reading. In our study, the relative folds of two target genes (E and RdRp) decreased 10 folds after formalin treatment (data not shown). However, the final assigned values of RNA copy number of the standard is as high as 6.35 Log_{10} copies/mL, and all collaborative studies demonstrate consistent data with less than 2 stand error values between different laboratories. Thus, formalin inactivation is capable of producing a practical SARS-CoV-2 NS. With the increasing interest in COVID-19, many researchers are now applying their knowledge and expertise to different topics to address this global problem. The inactivation strategy for SARS-CoV-2 presented here can provide valuable samples for researchers who are restricted to limited facilities. Their manipulation of the live SARS-CoV-2 will support research to perform their downstream work on SARS-CoV-2.

In recent years, the digital PCR technique became an essential method for official laboratories in various countries when primary standards were established. At beginning of outbreak of the novel SARS-CoV-2, most official organizations were exhausted in coping with the virus, including virus isolation, whole-genome analysis, transmission pathway investigation, and inactivation methods examination, etc. To expedite detection of the SARS-CoV-2, RT-qPCR was considered as a standard and sensitive method for clinical detection of SARS-CoV-2, but lacking standardization of reference had hindered its usefulness. In other others, digital PCR offers highly accurate, direct quantification without a calibration curve and thus was expeditious applied during the tremendous epidemic. Currently, there are two available digital PCR systems on the market: chip-based and droplet-based. Both are included in this collaborative study. The 12 quantitative data included 10 digital PCR results, and the quantitative concentration ranged from 5.53 to 7.16 Log_{10} copies/mL, with a variation of about 42 folds. For the different extraction methods, RT efficiency, PCR platforms, and target sequences may cause deviations in quantification. Compared with the previous

collaborative studies held by the TFDA, the data were consistent, and the variations were less than 10-fold, while a unique standard was used for calibration [18]. This variation was more significant within qualitative experiments. In qualitative experiments, Proleix SARS-CoV-2 Assay was applied by one laboratory. Proleix SARS-CoV-2 Assay uses transcription-mediated nucleic acid amplification (TMA) method to qualify the viral RNA and this data was excluded from the quantitative results. According to the previous study [19], TMA method demonstrated higher sensitivity for the detection of HCV RNA in comparison with reverse transcription polymerase chain reaction method. TMA method may have highly efficient isothermic autocatalytic amplification step and more RNA can be transcribed for detection. In Procleix assay, the RNA detection limit of 5 copies was described according to manufacturing's instructions. In the collaborative study, serial dilution was necessary for the accurate quantify the viral RNA. Thus, the Proleix assay demonstrated the results of 8.45–9.42 Log_{10} copies/mL after serial dilution in the absence of reference standard control. Therefore, to use a credible standard compensate for the deviation caused by a few variables, like extraction efficiency or target sequence stability, it should also obtain comparable data, even if the digital PCR can be absolutely applied quantitative.

The stability tests showed that SARS-CoV-2 RNA NS have long-term stability when stored at −20 °C and −80 °C. We recommended that the SARS-CoV-2 RNA NS should be stored below −20 °C. Therefore, approximately 500 vials of the first NS for SARS-CoV-2 RNA NAAT assays were established in Taiwan. This NS could be used as the quality control and quantitative reference for the pre-marketing approval testing or post-marketing surveillance. Compelled by the pending emergency, the SARS-CoV-2 NS was supplied by the national producers or related research agencies to evaluate the products' performance without charging fees. The service is universally recognized, receiving positive feedback from many customers. The availability of SARS-CoV-2 RNA NS provides a higher order of references at the national level, and quality evaluation of relevant imported or domestic IVDs regardless of the assay used.

Declaration of competing interest

The authors have disclosed no conflicts of interests.

Acknowledgments

We want to thank the Chang Gung Memorial Hospital (Taoyuan, Taiwan) for providing the SARS-CoV-2 virus to prepare the candidate materials. This work was supported from the Food and Drug Administration, Ministry of Health and Welfare of Taiwan. We also gratefully acknowledge the vital contributions of the collaborative study participants:

•Dr. Arifa Khan and Dr. Sandra Fuentes, Center for Biologics Evaluation and Research (CBER), US FDA, USA.

•Dr. Giulio Pisani, Dr. Matteo Simeoni and Dr. Antonio Martina, National Centre for the Control and Evaluation of Medicines, Istituto Superiore di Sanità, Rome (CNCF), Italy.

•Dr. Giada Mattiuzzo and Dr. Emma Bentley, National Institute for Biological Standards and Control (NIBSC), UK.

•Dr. Julia Gerbeth and Dr. Sally Baylis, Paul-Ehrlich-Institute (PEI), Germany.

•Dr. Chi-Jung Yang and Chuan-Yi Kuo, Taiwan Centers for Disease Control (TCDC), Taiwan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2022.08.002.

References

[1] Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020;395:497–506.
[2] Binns C, Low WY, Kyung LM. The COVID-19 pandemic: public health and epidemiology. Asia Pac J Publ Health 2020;32:140–4.
[3] Udugama B, Kadhiresan P, Kozlowski HN, Malekjahani A, Osborne M, Li VYC, et al. Diagnosing COVID-19: the disease and tools for detection. ACS Nano 2020;14:3822–35.
[4] Alpdagtas S, Ilhan E, Uysal E, Sengor M, Ustundag CB, Gunduz O. Evaluation of current diagnostic methods for COVID-19. APL Bioeng 2020;4:041506.
[5] Broughton JP, Deng X, Yu G, Fasching CL, Servellita V, Singh J, et al. CRISPR-Cas12-based detection of SARS-CoV-2. Nat Biotechnol 2020;38:870–4.
[6] Chan JF, Yip CC, To KK, Tang TH, Wong SC, Leung KH, et al. Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/hel real-time reverse transcription-PCR assay validated in vitro and with clinical specimens. J Clin Microbiol 2020;58.
[7] Cordes AK, Heim A. Rapid random access detection of the novel SARS-coronavirus-2 (SARS-CoV-2, previously 2019-nCoV) using an open access protocol for the Panther Fusion. J Clin Virol 2020;125:104305.
[8] Penarrubia L, Ruiz M, Porco R, Rao SN, Juanola-Falgarona M, Manissero D, et al. Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak. Int J Infect Dis 2020;97:225–9.
[9] Funnell SGP, Afrough B, Baczenas JJ, Berry N, Bewley KR, Bradford R, et al. A cautionary perspective regarding the isolation and serial propagation of SARS-CoV-2 in Vero cells. NPJ Vaccines 2021;6:83.
[10] Liu Z, Zheng H, Lin H, Li M, Yuan R, Peng J, et al. Identification of common deletions in the spike protein of severe acute respiratory syndrome coronavirus 2. J Virol 2020;94.
[11] Ramirez S, Fernandez-Antunez C, Pham LV, Ryberg LA, Feng S, Pedersen MS, et al. Efficient culture of SARS-CoV-2 in human hepatoma cells enhances viability of the virus in human lung cancer cell lines permitting the screening of antiviral compounds. bioRxiv 2020.
[12] Darnell ME, Taylor DR. Evaluation of inactivation methods for severe acute respiratory syndrome coronavirus in noncellular blood products. Transfusion 2006;46:1770–7.
[13] Jureka AS, Silvas JA, Basler CF. Propagation, inactivation, and safety testing of SARS-CoV-2. Viruses 2020:12.
[14] Kim YI, Casel MAB, Kim SM, Kim SG, Park SJ, Kim EH, et al. Development of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) thermal inactivation method with preservation of diagnostic sensitivity. J Microbiol 2020;58:886–91.
[15] Rabenau HF, Cinatl J, Morgenstern B, Bauer G, Preiser W, Doerr HW. Stability and inactivation of SARS coronavirus. Med Microbiol Immunol 2005;194:1–6.
[16] Darnell ME, Subbarao K, Feinstone SM, Taylor DR. Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV. J Virol Methods 2004;121:85–91.
[17] Normile D. Infectious diseases. Mounting lab accidents raise SARS fears. Science 2004;304:659–61.
[18] Yang YC, Yang-Chih Shih D, Tsai MH, Cheng CH, Cheng HF, Lo CF, et al. A collaborative study to establish the first National Standard for HIV-1 RNA nucleic acid amplification techniques (NAT) in Taiwan. J Virol Methods 2013;191:122–7.
[19] Hofmann WP, Dries V, Herrmann E, Gartner B, Zeuzem S, Sarrazin C. Comparison of transcription mediated amplification (TMA) and reverse transcription polymerase chain reaction (RT-PCR) for detection of hepatitis C virus RNA in liver tissue. J Clin Virol 2005;32:289–93.