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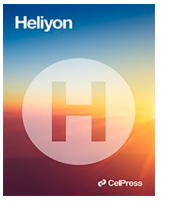

Research article

Establishment of national standards of SARS-CoV-2 variants in Taiwan

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ABSTRACT

Objectives: In response to the pandemic, the Taiwan Food and Drug Administration (TFDA) established an initial SARS-CoV-2 RNA national standard based on the original Wuhan strain. However, with the depletion of the first national standard and continued mutation of the virus, the establishment of new national standards was imminent.

Methods: Hence, new candidate national standards were established by heat-inactivation for 30 min for six representative strains of SARS-CoV-2, comprising the original strain and five variants with anticipated concentrations of 7.70 Log_{10} international units (IU)/mL each. To enhance the credibility of these national standards, the TFDA extended invitations to both domestic and international institutions to participate in a collaborative study. A total of eight participants contributed eleven datasets, incorporating two methods and targeting four distinct genes.

Results: Based on these collective findings, the quantified viral RNA concentrations for each SARS-CoV-2 national standard strain are 7.69, 7.70, 7.69, 7.44, 7.52, and 7.29 Log_{10} IU/mL with Wuhan, alpha, beta, gamma, delta, and omicron strain, respectively.

Conclusions: These newly established national standards will continue to be made available to the industry, serving as a fundamental reference for the development and quality control of nucleic acid *in vitro* diagnostic (IVD) reagents in Taiwan.

1. Introduction

The COVID-19 pandemic, resulting from the widespread transmission of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has presented a profound global health crisis since its emergence in December 2019 [1]. This ongoing pandemic has, to date, documented over 775 million confirmed cases and more than 6 million fatalities, prompting transformative adaptations in society and healthcare practices [2,3].

In response to this crisis, numerous research institutions and diagnostic reagent manufacturers embarked on developing *in vitro* diagnostic (IVD) reagents to satisfy the escalating need for reliable SARS-CoV-2 detection [4]. Nucleic acid amplification techniques (NAAT) became pivotal in this field, with reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and reverse transcription digital polymerase chain reaction (RT-dPCR) serving as primary diagnostic modalities [5]. The necessity for standardized rapid diagnostic procedures for SARS-CoV-2 prompted the Taiwan Food and Drug Administration (TFDA) to define a national

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SARS-CoV2 RNA standard (also referred to as the first-generation national standard) at an early stage in the pandemic [6]. This national standard, derived from the Wuhan-Hu-1 viral strain, was disseminated to institutions and facilities beginning in July 2020. The national standard was primarily aimed at facilitating the consistent calibration of analytical data across distinct testing platforms, particularly across secondary developers conducting "in-house" assays, thereby enhancing the comparability of SARS-CoV-2 RNA detection results and reducing inter-laboratory variability [7].

Throughout the pandemic, the virus underwent rapid genomic mutations, resulting in the emergence of diverse viral variants across the globe [8]. Following the World Health Organization's (WHO) classification, SARS-CoV-2 strains were categorized as variants of interest (VOIs), variants of concern (VOCs), or variants under monitoring (VUMs) [9]. VOIs included highly transmissible strains, strains that evaded the immune system, and strains that were resistant to treatment [10]. VOCs exhibit distinctive characteristics of global public health significance, including increased transmissibility, alterations in epidemiological patterns, stronger virulence or modified clinical presentations, and decreased susceptibility to public health measures [11]. The WHO has officially designated five VOCs, identified as alpha, beta, gamma, delta, and omicron. These VOCs have posed considerable challenges in terms of accurate viral validation [12].

To address these challenges, the TFDA collected the major strains, encompassing the original viral strain and the aforementioned five VOCs to develop new candidate national standards (also referred to as candidate second-generation national standards). Diverging from the initial SARS-CoV-2 national standard prepared using a formalin-inactivated virus, the second-generation national standards were developed as heat-inactivated viruses [13].

In pursuit of ensuring the integrity and credibility of these standard materials, an international collaborative study was conducted in parallel with the establishment of the SARS-CoV-2 RNA national standards. Each SARS-CoV-2 strain was quantified in international units (IU), with the expected concentration of the national standards set at 7.70 Log_{10} IU/mL. This study enlisted the participation of seven Taiwanese institutions and one institution from the United Kingdom, as outlined in Supplementary Table 1. The collaborative study united ten quantitative datasets and one qualitative dataset from the eight participating entities. This research elucidates the critical role of standardized national standards in the context of the global response to the COVID-19 pandemic, encompassing the challenges posed by variant strains and the imperative for international collaboration in calibration and validation efforts.

2. Materials and methods

2.1. Virus preparation of the national candidate standards

The international standard for SARS-CoV-2 was purchased from the National Institute for Biological Standards and Control (NIBSC, UK) (code 20/146) and supplied as 7.7 $\log_{10}$ IU/mL. The international standard was reconstituted with 0.5 mL of phosphate-buffered saline following the manufacturer's instructions and stored at −20 °C. The six virus strains were propagated in Vero E6 cells (Cat. No. CRL-1586, American Type Culture Collection, Manassas, VA, USA) namely the original strain (BetaCoV/Taiwan/4/2020, Taiwan Centers for Disease Control [TCDC], Taipei, Taiwan), alpha strain (NR-54011, BEI Resources, Manassas, VA, USA), beta strain (NR-54009, BEI Resources), gamma strain (NR-54982, BEI Resources), delta strain (hCoV-19/Taiwan/1144/2021, TCDC), and omicron strain (hCOV-19/Taiwan/16804/2021, TCDC). All virus strains mentioned above followed the biosafety regulations from TCDC [14]. Virus infectivity was verified through the observation of cytopathic effects (CPE).

Once significant CPE was observed, the virus supernatant was collected and clarified using low-speed centrifugation at 2000×*g* for 20 min. Subsequently, a preliminary analysis of viral RNA concentrations was performed. For virus inactivation, the aforementioned viral culture was incubated at 65 °C for 30 min in a water bath. Cell CPE analysis and real-time reverse transcription polymerase chain reaction (RT-PCR) RNA monitoring were conducted to confirm virus inactivation. Finally, a quantitative assessment of the RNA content of the inactivated virus was performed and was dispensed in 0.5 mL aliquots and stored at −80 °C by the TCDC biosafety regulations. The digital PCR test developed using the collected virus samples is described in Sections 2.2 and 2.3.

2.2. Viral genomic sequencing

Dual-index libraries were generated from viral RNA using the Stranded Total RNA Prep Ligation with Ribo-Zero Plus kit (Illumina, San Diego, CA, USA). Viral quality and yield were assessed using a Qubit 2.0 fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and a Qsep1 Bio-Fragment Analyzer (BiOptic Inc, New Taipei City, Taiwan). Genomic sequencing was performed using a MiSeq Instrument (Illumina) with two 151-cycle reads. The files were processed using fastp (v.0.21.0) to remove low-quality reads and filter out duplicate reads. The cleaned reads were aligned to the SARS-CoV-2 reference genome 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 (version 2.1.5) was used to call variants to generate a consensus genome (Supplementary Fig. 1) [15].

2.3. RT-dPCR and RT-qPCR

The RNA concentration of the viruses was determined using reverse transcription digital polymerase chain reaction (RT-dPCR) and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The detected conserved four regions are the *RdRp*, *E*, *N,* and *ORF1ab* genes. The steps were followed as previously described [6].

2.4. Study participants

This collaborative study involved eight laboratories at Academia Sinica (Taipei, Taiwan), Chang Gung University (Taoyuan, Taiwan), National Cheng Kung University (Tainan, Taiwan), the National Health Research Institutes (Zhunan, Taiwan), National Taiwan University (Taipei, Taiwan), the TCDC (Taipei, Taiwan), the TFDA (Taipei, Taiwan), and the Medicines and Healthcare products Regulatory Agency (London, UK; Supplementary Table 1). All participating laboratories were referred to by a code number, allocated at random, and not represented by order of the list in Supplementary Table 1. The test results were analyzed independently, as if from different laboratories (e.g., laboratory 1a or 1b).

2.5. Stability study of the national candidate standards

Numerous vials of virus from different strains were maintained at −80 °C, −20 °C, 4 °C, and 24 °C until predetermined time points during the product's shelf-life period. Three vials were retrieved from each temperature, and the RNA content was quantified using RT-qPCR described above.

2.6. Design of the collaborative study

Three vials of candidate RNA national standards of each strain are provided for each method used by each participant and should be stored at −70 °C or below while the first WHO international standard (IS) for SARS-CoV-2 RNA (NIBSC code: 20/146) was provided for each method used by each participant and stored at −20 °C. Three independent tests using the SARS-CoV-2 WHO IS and the 6 candidate RNA national standards for SARS-CoV-2 will be conducted with the laboratory's routine SARS-CoV-2 NAAT methods. Fresh samples will be used for each assay. For quantitative tests, the samples were diluted in at least three serial 10-fold dilutions within the linear range of the method (e.g., 10^0, 10^{-1}, and 10^{-2}). The diluted samples will be subjected to NAAT analysis after RNA extraction using the appropriate method. At least two replicates will be performed for each dilution in the same assay. For qualitative tests, the samples were diluted at least five half-log dilutions on both sides of the predetermined endpoint dilution (e.g., 10^{-3}, $10^{-3.5}$, 10^{-4}, $10^{-4.5}$, 10^{-5}) and were subjected to NAAT analysis with at least two replicates.

2.7. Statistical methods

The SARS-CoV-2 WHO IS dilution series will be used as a quantitative standard curve for quantitative tests. The curve should have the coefficient of the determination (R^2) of ≥97 % and a slope between −3.1 and −3.6 (corresponding to 90 %–110 % PCR efficiency) to confirm its suitability. The RNA content of the 6 candidate RNA national standards will be calculated using the geometric mean. For qualitative tests, the RNA content will be calculated using the Spearman-Karber method based on the SARS-CoV-2 WHO IS as the reference. The average of the logarithmically transformed quantitative and qualitative results, excluding outliers (values outside the mean ± 2 standard deviations [SDs]), will be considered the consensus value expressed as Log_{10} IU/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. The RNA concentrations are expressed in

Table 1
Laboratory codes and assay methods.

Lab Code	Extraction method	NAT method	Assay type	Target	Reported Readout
1a	MagPurix Viral Nucleic Acid Extraction Kit	*In-house* Real-time RT-PCR	Quantitative	N	IU/mL
1b	MagPurix Viral Nucleic Acid Extraction Kit	*In-house* Real-time RT-PCR	Quantitative	ORF1ab	IU/mL
1c	MagPurix Viral Nucleic Acid Extraction Kit	*In-house* Real-time RT-PCR	Quantitative	E	IU/mL
2	QIAzol Lysis Reagent	*In-house* Real-time RT-PCR	Quantitative	E	IU/mL
3	Alinity m System for RNA extraction	Abbott Alinity m SARS-CoV-2 AMP Kit. Real-time RT-PCR	Quantitative	RdRp/N	IU/mL
4	QIAamp Viral RNA Mini Kit	*In-house* Real-time RT-PCR	Quantitative	E	IU/mL
5	QIAamp Viral RNA Mini Kit	*In-house* Real-time RT-PCR	Quantitative	E	IU/mL
6	COBAS SARS-CoV-2	COBAS SARS-CoV-2 Real-time RT-PCR	Qualitative	ORF1ab/E	+/−
7a	Qiagen EZ1 Virus Mini Kit v2.0	*In-house* Digital RT-PCR	Quantitative	E	copies/mL
7b	Qiagen EZ1 Virus Mini Kit v2.0	*In-house* Digital RT-PCR	Quantitative	RdRp	copies/mL
8	Qiagen Viral RNA mini-kit	*In-house* Real-time RT-PCR	Quantitative	E	IU/mL

mean with SD. All graphs were drawn using GraphPad Prism version 8.02 (Boston, MA, USA).

3. Results

3.1. Collaborative study and assay establishment

Eight participants were invited to participate in this study to jointly calibrate the SARS-CoV-2 candidate RNA national standards prepared by the Taiwan Food and Drug Administration (TFDA) (Supplementary Table 1). These participating laboratories conducted three independent assays using their routine SARS-CoV-2 RNA quantitative/qualitative analysis methods on the candidate national standards and the WHO SARS-CoV-2 International Standard (IS). Six extraction reagents were used in the study: the MagPurix Viral Nucleic Acid Extraction Kit (Zinexts Life Science Corp, New Taipei City, Taiwan), QIAzol Lysis Reagent (Qiagen, Venlo, the Netherlands), Alinity m System for RNA extraction (Abbott Molecular Inc, Des Plaines, IL, USA), QIAamp Viral RNA Mini Kit (Qiagen), COBAS SARS-CoV-2 test (Roche Molecular Systems, Branchburg, NJ, USA), and Qiagen EZ1 Virus Mini Kit 2.0 (Qiagen). The NAAT methods employed in the study included commercially available kits and laboratory-developed in-house methods.

These assays targeted four distinct genes, namely *E*, *N*, *RdRp*, and *ORF1ab*, resulting in a total of 11 test results (Table 1). Among these, there were ten quantitative assays and one qualitative assay using either real-time RT-PCR or digital RT-PCR. Six laboratories (Lab 1, 2, 3, 4, 5, 8) reported quantitative results by conducting RT-qPCR, and one (Lab 7) used RT-dPCR analysis with comparative calculations based on the standard curve of the IS, and all datasets were expressed in IU/mL. For the qualitative results, one dataset (Lab 6) reported results as positive/negative, obtained fully (walked-away) automated sample preparation and robust PCR platform.

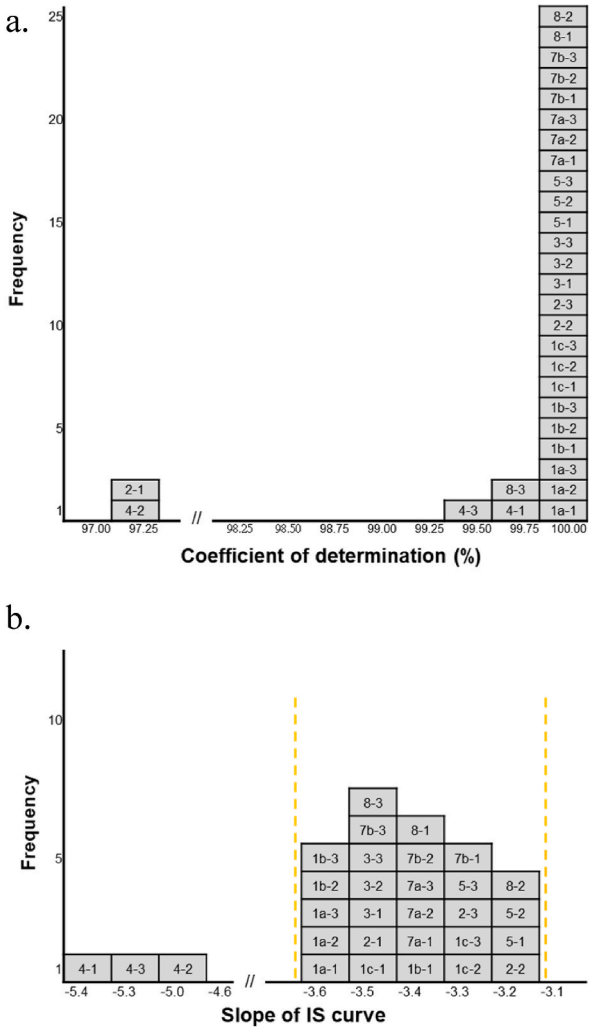

Fig. 1. The linearity of the data received from different labs to the international standard. a) the distribution of the coefficient of determination (R^2, %). b) slope of the standard curves for each quantitative assay.

3.2. Potency estimation of candidate RNA national standards

The data analysis for each assay included assessing the linearity of the IS standard curve dilution levels and the PCR efficiency. The coefficient of determination (R^2) greater than or equal to 97 %, and a slope between −3.1 and −3.6 for PCR efficiency between 90 % and 110 % were required (Fig. 1a). The coefficient of determination for all the data in the linear portion of each laboratory exceeded 97 % (Fig. 1b). From most datasets of research laboratories, the slopes fell within the range of −3.1 to −3.6 except for Lab 4 (4-1, 4-2, and 4-3).

The estimated values of six strains of SARS-CoV-2 RNA, relative to the IS from the WHO, were plotted in a distribution graph for the candidate standards from each laboratory (Supplementary Fig. 2). Subsequently, values that exceeded the mean ± 2 SD were indicated in orange and values within the mean ± 2 SD range in blue.

The quantitative and qualitative values of different strains of SARS-CoV-2 RNA from each laboratory are presented in scattergram form (Fig. 2). For Lab 4, the data of the original strain, the beta strain, the delta strain, and the gamma strain exceeded the mean + 2 SD. The alpha strain and omicron strain of Lab 4's values were close to the mean + 2 SD, with differences of 0.06 and 0.03, respectively. Based on the evaluation of the parameters mentioned above, the results from Lab 4 were not suitable for inclusion in the quantitative calculations. Overall, the RNA concentrations of the original, alpha, beta, gamma, delta, and omicron strains were 7.69 ± 0.19, 7.70 ± 0.23, 7.69 ± 0.16, 7.44 ± 0.20, 7.52 ± 0.23, and 7.29 ± 0.30 Log_{10} IU/mL, respectively. The average point value ranges from 7.29 to 7.7 Log_{10} IU/mL, and the CV percentage is from 2.12 % to 4.17 % (Table 2).

3.3. Temperature stability of the national standards

To assess the stability of the candidate national RNA standards, three vials from each group were randomly examined for stability assays at predetermined time intervals at four specific temperatures of −80 °C, −20 °C, 4 °C, and 24 °C (Fig. 3a).

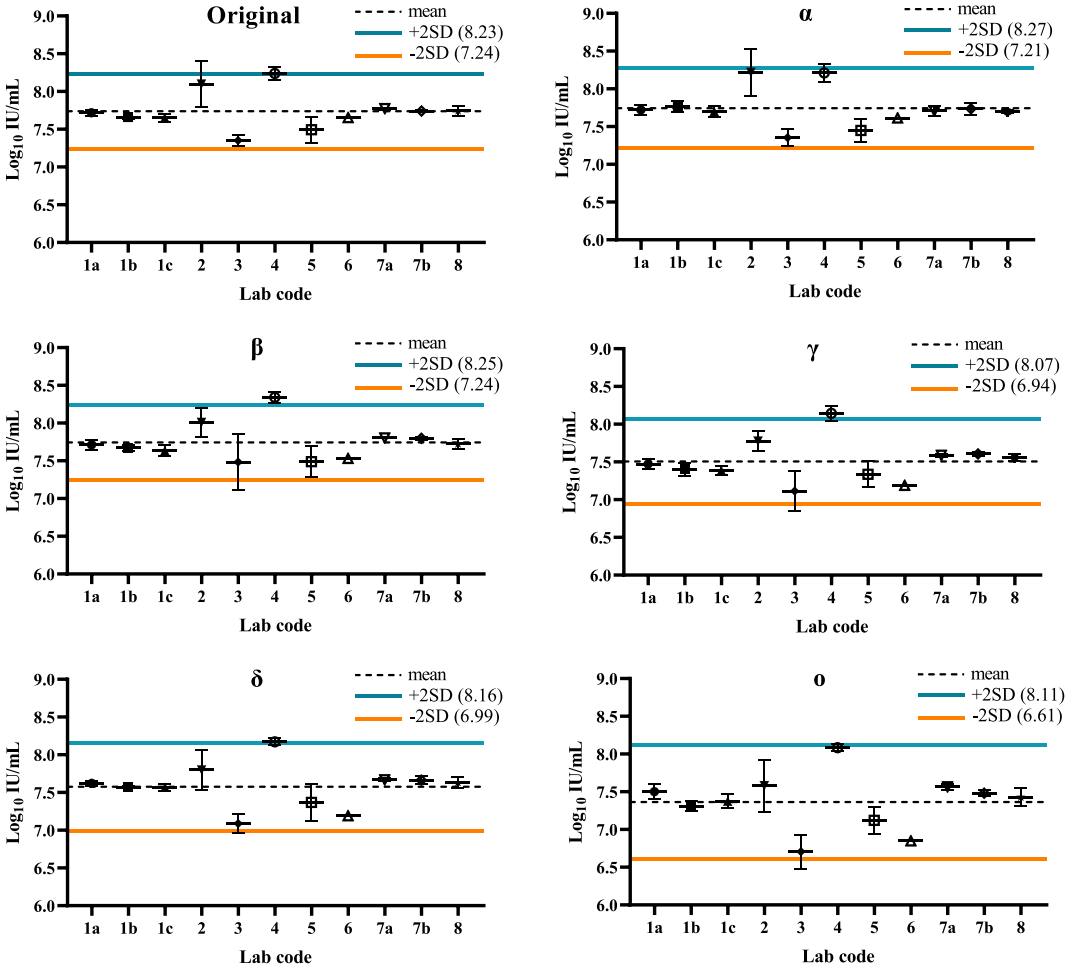

Fig. 2. The estimated values (Log 10 IU/mL) of SARS-CoV-2 RNA candidate national standards were determined by eight laboratories of six strains. Lab code symbols are designated as follows: 1a: ●; 1b: ■; 1c: ▲; 2: ▼; 3: ◆; 4: ○; 5: □; 6: △; 7:a: ▽; 7b: ◇; 8: ★.

Table 2
Mean IU estimates of SARS-CoV-2 RNA national standards.

Lab Code	Original	α	β	γ	δ	ο
	(Log_{10} IU/mL)	(Log_{10} IU/mL)	(Log_{10} IU/mL)	(Log_{10} IU/mL)	(Log_{10} IU/mL)	(Log_{10} IU/mL)
1a	7.72	7.72	7.71	7.47	7.62	7.5
1b	7.66	7.77	7.67	7.4	7.57	7.31
1c	7.65	7.7	7.63	7.39	7.57	7.37
2	8.1	8.22	8.01	7.78	7.8	7.58
3	7.35	7.35	7.48	7.11	7.09	6.71
4	8.24[a]	8.21[a]	8.34[a]	8.14[a]	8.17[a]	8.08[a]
5	7.49	7.45	7.49	7.34	7.37	7.12
6	7.65	7.61	7.53	7.19	7.19	6.85
7a	7.77	7.71	7.8	7.59	7.67	7.57
7b	7.74	7.74	7.8	7.61	7.66	7.48
8	7.74	7.7	7.72	7.56	7.63	7.43
Average	7.69 ± 0.19	7.70 ± 0.23	7.69 ± 0.16	7.44 ± 0.20	7.52 ± 0.23	7.29 ± 0.30
CV	2.52 %	2.95 %	2.12 %	2.69 %	3.02 %	4.17 %

[a] The data has been excluded due to the slope of the standard curve not conforming to the criteria.

Vials stored at 24 °C were evaluated after 0, 1, 2, 4, and 8 weeks of storage, and those stored at 4 °C were evaluated after 0, 1, 2, 4, 8 and 12 weeks of storage. Moreover, vials stored at −20 °C were evaluated after 0, 3, 6, 12, 18, and 24 months of storage. Vials stored at −80 °C were evaluated after 0, 6, 12, 18, and 24 months of storage. The viral RNA was subjected to quantitative analysis and presented in log_{10} ″IU/mL" (Fig. 3b). The results revealed no significant change in viral potency when stored at −80 °C and −20 °C. However, for the omicron strain, the sample's potency significantly decreased by 10-fold after the first week at both 4 °C and 24 °C, whereas the potency of the other strains decreased 10-fold after 4 weeks of storage.

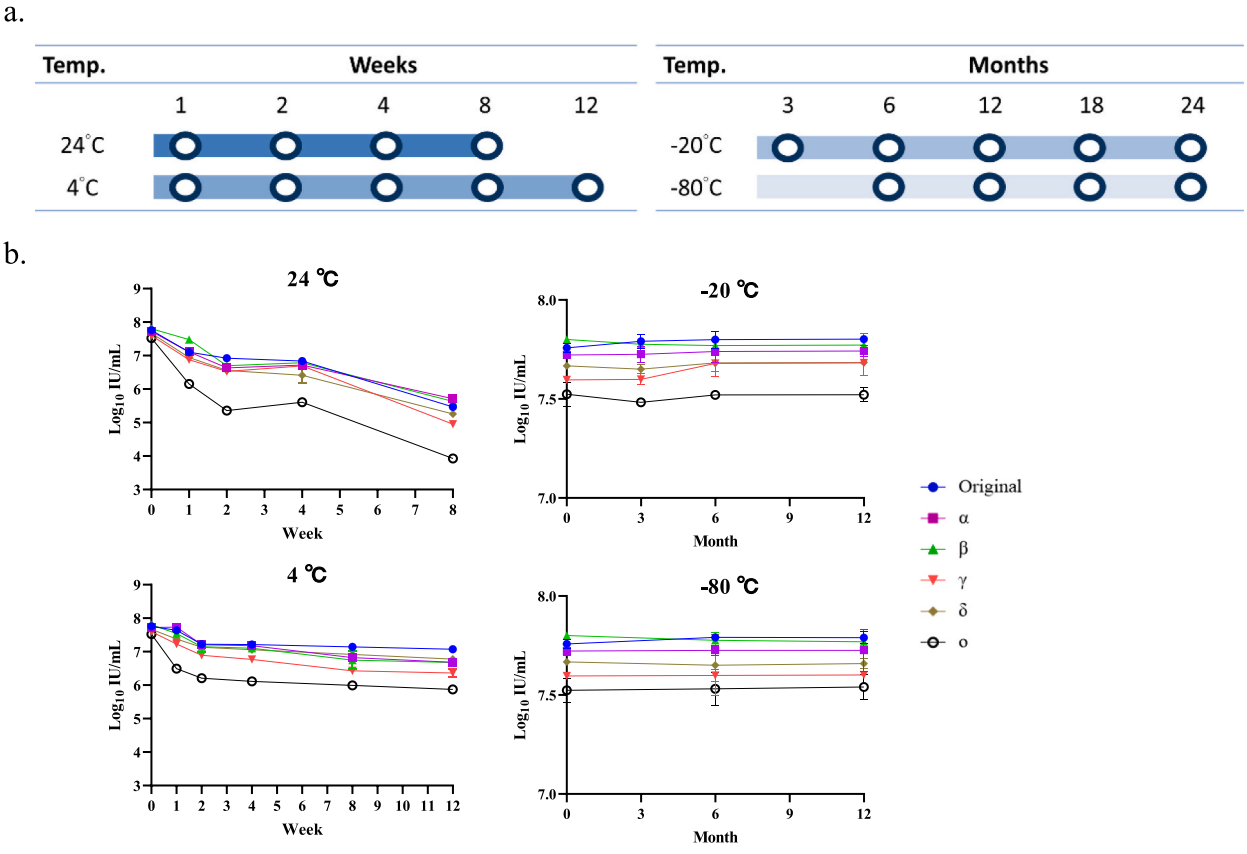

Fig. 3. The stability analysis of the SARS-CoV-2 candidate standards. a) the standards are kept at 24 °C, 4 °C, −20 °C, and −80 °C. b) The RNA concentration was measured using RT-qPCR.

4. Discussion

In light of the dynamic landscape of the SARS-CoV-2 virus, characterized by the emergence of VOIs, VOCs, and VUMs, it becomes imperative to establish formal national standards to address these evolving viral strains. Notably, the previous development of the first national standard (i.e., first-generation standard) for the SARS-CoV-2 strain (denoted as the original strain), accomplished within a span of ten months period and was driven by ty the urgent need for robust standards for evaluating the performance of IVD tools [6].

Among these variants, VOCs received significant attention due to their potential for heightened fatality rates and the possibility of eluding prevailing detection modalities [16]. The fundamental aim of establishing these standards is to facilitate precise calibration and stringent quality control of nucleic acid amplification analysis. Simultaneously, these standards underpin the development of nucleic acid amplification test (NAAT)-based IVD devices, thus expediting their pre-market evaluations [17].

The TFDA collected the major strains of the original SARS-CoV-2 and the five VOCs to develop new candidate national standards (also referred to as candidate second-generation national standards). The primary difference between the first-generation national RNA standard and the candidate second-generation RNA national standards lies in the viral inactivation method used for sample preparation (Supplementary Fig. 3). The first-generation national standard was established using formalin at a final concentration of 0.01 % for viral inactivation [6]. Although formalin-based inactivation is acknowledged for its safety and efficacy, it exacts a 10-fold reduction in total RNA content following inactivation, through reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis as previously described [6]. Numerous strategies for viral inactivation, including chemical agents, ultraviolet (UV) irradiation, and thermal treatment, have been explored [18–20]. Chemical agents, such as Triton X-100 and TRIzol LS reagent (Invitrogen), function as surfactants which result in viral envelope destabilization, but this may interfere with downstream NAAT-based analyses [21]. Meanwhile, UV inactivation induces crosslinks between pyrimidines and nucleic acids, introducing the possibility of undesirable chemical alterations [22]. In the present study, viral heat-inactivation at 65 °C for 30 min was adopted, in consideration of the World Health Organization's (WHO) recommendation of a 65 °C temperature threshold for 10 min [13]. However, it is noteworthy that elevated temperatures may induce conformational changes in the viral envelope proteins and also cause RNA degradation [23,24]. To assess the impact of heat inactivation, a comprehensive evaluation of viral RNA quantity was undertaken, revealing no substantial deviations in RNA potency before and after the heat treatment (Supplementary Fig. 4), demonstrating the suitability of this method for subsequent NAAT applications.

The research endeavors to be an international collaborative study, encompassing eight participants from the United Kingdom to Taiwan. The primary objective of this research was to calibrate national standards for the original strain and its variant strains in relation to the international standard of SARS-CoV-2 set at 7.7 Log_{10} IU/mL by the World Health Organization (WHO) [7]. It was essential to subject the data generated by these research centers in calibration against this international standard, ensuring the coefficient of determination exceeding 97 % and a slope value within the range of −3.1 to −3.6. However, it should be noted that one of the participating laboratories, referred to as Lab 4, exhibited a slope value falling outside this specified range. This outlier status was further noticed by the distribution analysis, as depicted in Fig. 2, indicating that Lab 4's data deviated from the mean ± 2 standard deviations (SD) range for the original, beta, delta, and gamma strains. Consequently, the data generated by Lab 4 was considered as an outlier and subsequently excluded from the dataset. The remaining values were normalized and were given a concentration marked as the second generation of the national standards of SARS-CoV-2 RNA.

Notably, this study evaluated the long-term stability of the six strains of SARS-CoV-2 RNA when stored at temperatures of −80, −20, 4, and 24 °C. RNA instability was observed with a decline in concentration when stored at 4 °C and 24 °C over weeks of preservation, indicating the need to maintain RNA at frozen status for long-term storage. A recommended storage temperature below −20 °C is essential to preserve the RNA standards over an extended duration which is the same as previously described [6].

A total of 324 vials of resulting standards, each containing 0.5 mL of a viral strain, are poised to serve as national benchmarks for pre-market approval testing of NAAT-based IVDs, as well as for the evaluation of post-marketing surveillance of commercial products, whether of domestic or international origin. Despite the removal of the alpha (α), beta (β), gamma (γ), delta (δ), and omicron (ο) strains from the WHO's list of VOCs, the availability of SARS-CoV-2 original and variant strains continue to provide a higher-order reference at national-level support in the development of IVDs and quality assessments across diverse diagnostic assays.

In summary, the establishment of robust national standards for diverse SARS-CoV-2 strains plays an indispensable role in ensuring the precision and reliability of diagnostic tests, especially in the context of evolving viral variants. Ongoing updates and refinements of these standards are essential to align with the ever-evolving understanding of this virus.

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Ethical approval

Not required.

Ethics statement

Not applicable.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

CRediT authorship contribution statement

Ming-Sian Wu: Methodology, Formal analysis, Data curation, Conceptualization. **Pu-Chieh Chang:** Writing – review & editing, Writing – original draft, Formal analysis. **Po-Lin Lin:** Supervision, Resources, Project administration, Formal analysis. **Chun-Hsi Tso:** Software, Resources, Project administration, Methodology, Investigation. **Hsin-Mei Chen:** Visualization, Validation, Investigation, Data curation. **Yi-Hsuan Peng:** Project administration, Methodology, Data curation. **Po-Chih Wu:** Supervision, Project administration. **Jia-Chuan Hsu:** Writing – review & editing, Visualization, Validation. **Der-Yuan Wang:** Writing – review & editing, Validation, Supervision, Funding acquisition.

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

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