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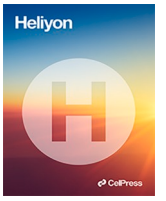

Research article

Optimizing heat inactivation for SARS-CoV-2 at 95 °C and its implications: A standardized approach

Gannon C.K. Mak *, Stephen S.Y. Lau, Kitty K.Y. Wong, Eunice K.Y. Than, Anita Y.Y. Ng, Derek L.L. Hung

All from Microbiology Division, Public Health Laboratory Services Branch, Centre for Health Protection, Department of Health, Hong Kong Special Administrative Region

A B S T R A C T

Background: Standardized and validated heat inactivation procedure for Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are not available. For heat inactivation, various protocols were reported to prepare External Quality Assessment Programme (EQAP) samples without direct comparison between different durations.
Objective: To assess the heat inactivation procedures against SARS-CoV-2. The efficacy of the optimized condition was reflected by the results from laboratories testing the EQAP samples.
Study design: The SARS-CoV-2 strain was exposed to 95 °C in a water bath for three different time intervals, 5 min, 10 min and 15 min, respectively. The efficacy of inactivation was confirmed by the absence of cytopathic effects and decreasing viral load in 3 successive cell line passages. The viral stock inactivated by the optimal time interval was dispatched to EQAP participants and the result returned were analyzed.
Results: All of the three conditions were capable of inactivating the SARS-CoV-2 of viral load at around cycle threshold value of 10. When the 95 °C 10 min condition was chosen to prepare SARS-CoV-2 EQAP samples, they showed sufficient homogeneity and stability. High degree of consensus was observed among EQAP participants in all samples dispatched.
Conclusions: The conditions evaluated in the present study could be helpful for laboratories in preparing SARS-CoV-2 EQAP samples.

1. Introduction

Currently, the gold standard to detect Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is nucleic acid tests. Real-time reverse transcription polymerase chain reaction (RT-PCR) is widely available in clinical laboratories to cope with high diagnostic demands. The quality of the test results can be assured by testing a standardized panel of samples with known amount through inter-laboratory comparison, i.e. External Quality Assessment Programme (EQAP). Inactivation of SARS-CoV-2 is necessary to facilitate different laboratories to perform the assessment under the safe conditions.

Different physical and chemical inactivation methods such as heat inactivation, UV irradiation, ozone and detergents are proven to be effective to inactivate SARS-CoV-2. The choice of specific method depends on the compatibility of the downstream experiments. Inactivation with UV irradiation and detergents can preserve the proteins which are suitable for assessment of immunological assays. However, the UV energy emitted should be even and consistent which requires a separate instrument to be installed in laboratory [1–4]. Although ozone is effective to inactivate SARS-CoV-2, optimum ozone concentration and exposure time have to be obtained [5,6]. On the other hand, detergent has to be removed after inactivation since it acts as PCR inhibitor [7,8]. Although heat inactivation

* Corresponding author. 9/F, Public Health Laboratory Centre 382 Nam Cheong Street Shek Kip Mei Kowloon, Hong Kong Special Administrative Region .
E-mail address: so_phls10@dh.gov.hk (G.C.K. Mak).

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could denature proteins, nucleic acid amplification tests would not be affected. For the ease of use without additional dedicated equipment, heat inactivation is widely adopted [9].

The efficiency of heat inactivation has been studied extensively. It depends on numerous factors such as sample volume, container material, viral load of the samples, heating temperature, and heating time [10]. Three different temperatures, 56 °C, 65 °C and 95 °C, were used and shown to be effective to inactivate SARS-CoV-2 [11,12]. However, other studies showed equivocal infectivity at lower temperature ranges, and higher temperature should be used to obtain complete inactivation [13,14]. At high temperatures, previous studies showed that heat inactivation may decrease the amount of viral nucleic acid [15,16]. Although a variety of conditions were reported, the most efficacious and cost-effective procedures were not known.

The purpose of this evaluation is to assess the diagnostic use of the heat inactivation procedures at 95 °C for preparing EQAP. The aim of the first part of the study is to assess the impact on SARS-CoV-2 viability by using 95 °C for various periods of time. The second part is to use EQAP data to assess the usefulness of the optimized heat inactivation condition.

2. Methods

2.1. EQAP provider

Our laboratory, the Public Health Laboratory Services Branch (PHLSB) has been the EQAP provider for more than ten years. In 2010, PHLSB was accredited as a Proficiency Testing Provider in accordance with the ISO 17043 standard [17]. This standard focuses on management, planning and design and assuring quality. PHLSB currently organizes different EQAP programmes and distributes samples to participating laboratories for assessing their performance including the World Health Organization (WHO) EQAP for molecular detection of influenza [18]. The PHLSB has been designated as WHO COVID-19 reference laboratory since April 2020 [19] and is now EQAP provider of SARS-CoV-2 for international laboratories.

2.2. SARS-CoV-2 virus

The SARS-CoV-2 virus used in the present study was propagated from a respiratory specimen, a combined throat and nasal swab, collected from a COVID-19 patient on August 24, 2022. Whole genome sequencing results showed that it belonged to BA.5.2 lineage. It was designated as: strain hCoV-19/Hong Kong/VC22007564/2022 (GISAID Accession number: EPI_ISL_16717129). Viral culture was first conducted by inoculating the specimen onto Vero cells as described previously [19]. In order to obtain enough culture supernatants for subsequent experiments, the SARS-CoV-2 stock was then grown on Vero cells of six culture tubes while each culture tube consisted of 1 mL medium. The culture tubes were harvested upon observation of '4+' cytopathic effect (CPE) (i.e. 100% cells were affected) on day 5 after inoculation. The culture supernatants were pooled together which consisted of around 6 mL volume. It was used as the virus stock for subsequent downstream experiments.

2.3. Heat inactivation procedures

The virus stock solution was divided into six portions. Each portion consisted of 1 mL quantity which was aliquoted into a 1.5 mL screw-cap vial before heat treatment. The six vials were divided into three groups and were exposed to 95 °C in a water bath for three different time intervals, 5 min, 10 min and 15 min and then cooled on ice for 5 min. The cell debris were removed by centrifugation at 13,000 g for 1 min, while the supernatants were transferred to new 1.5 mL screw-cap vials. The samples were either kept frozen at −70 °C or directly proceeded to subsequent downstream experiments.

2.4. Analysis for viable virus post-heat inactivation

To assess the integrity of SARS-CoV-2 RNA after heat inactivation, samples with and without heat inactivation were performed for a SARS-CoV-2 real-time RT-PCR assay to compare their viral loads.

Evidence of inactivation was determined by the absence of CPE in Vero cells. Vero cells were inoculated with 50 μL of heated samples. From day 3 after inoculation onwards, the cells were observed daily for CPE. Viral isolation was considered negative when CPE was not observed by day 7. Two more passages were performed. Before each passage, freeze-thaw cycle was performed to ensure any intracellular virions in the cell line are released [20]. The amount of viral materials after each culture was determined by RT-PCR. Successful virus inactivation was determined by negative CPE and decreasing RNA loads with successive passage.

2.5. SARS-CoV-2 real-time RT-PCR assay

The viral nucleic acids were extracted with EZ1 instrument with EZ1 Virus Mini Kit v2.0 (Qiagen, Germany). The input and elution volumes were 200 μL and 90uL respectively. A 10 μL volume of the internal extraction control BVDV was added.

The in-house developed RT-PCR was used to detect the presence of SARS-CoV-2 virus nucleic acid in all samples. It was conducted using the NxtScript Enzyme and Master Mix (Roche Diagnostics GmbH, Germany) as previous [21]. Each 10 μL reaction mixture contained 5 μL RNA samples, 2 μL Reaction Mix (5X), 0.06 μL Adpta Taq DNA polymerase (50U/μL), 0.05 μL NxtScript RT Enzyme (85U/μL). Three primer/probe sets were included, two sets (Urbani-aaa and NCOV-2) were used to detect two different RdRp regions of SARS-CoV-2 while the remaining one was used to detect BVDV. The working primer/probe mixes were added as 0.9 μL, 0.9 μL and

Table 1
The sequences of primers and probes used.

Name	Sequence	Virus/Target	Final concentration (μM)	Reference
Urbani-Faa	CTCACCTTATGGGTTGGGATTA	SARS-CoV-2/RdRp	0.3	Own designed
Urbani-Raa	GTTTGCGAGCAAGAACAAGTG	SARS-CoV-2/RdRp	0.3	Own designed
Urbani-Paa	FAM-TGATAGAGCCATGCCTAACATGCT–BHQ1	SARS-CoV-2/RdRp	0.3	Own designed
NCOV–F2	AGCTTGTCACACCGTTTCTAT	SARS-CoV-2/RdRp	0.3	Own designed
NCOV-R2	GATGAGGTTCCACCTGGTTT	SARS-CoV-2/RdRp	0.3	Own designed
NCOV–P2	FAM-TGGTCATGTGTGGCGGTTCACTAT-BHQ1	SARS-CoV-2/RdRp	0.15	Own designed
BVDV-F	CCAGGATTATATAGGGCCAGTCTAC	BVDV/NPRO	0.1	[22]
BVDV-R	TATATGTGGTACAACTTGCCATCAC	BVDV/NPRO	0.1	[22]
BVDV-P-Q670	Quasar 670-CAGTCACTCTCCCTATCCGTTTGGTCGTTT-BHQ2	BVDV/NPRO	0.05	[22]

0.3 μL respectively. The working primer/probe mixes were prepared by: (1) Urbani-aaa: mixing equal volume of forward primer, Urbani-Faa (10 μM); reverse primer, Urbani-Raa (10 μM) and probe, Urbani-Paa (10 μM), (2) NCOV-2: mixing equal volume of forward primer, NCOV–F2 (10 μM); reverse primer, NCOV-R2 (10 μM) and probe, NCOV–P2 (5 μM), (3) BVDV: mixing equal volume of forward primer, BVDV-F (10 μM); reverse primer, BVDV-R (10 μM) and probe, BVDV-P-Q670 (5 μM). The sequences of the primers and probes and the final concentrations in a 10 μL reaction mixture were shown in Table 1. The reverse transcription, amplification was performed in the LC480 System (Roche Diagnostics GmbH, Germany) according to the following program: 1 cycle of 50 °C for 10 min, 1 cycle of 95 °C for 30 s, 40 cycles of 95 °C for 10 s and 56 °C for 30 s; and holding at 4 °C.

Virus concentrations in samples were estimated from cycle threshold (Ct) value. The difference of Ct values for the two concentrations were converted as $\log_{10}$ as previously described [23,24]. In brief, the Ct value difference of 3.3 is approximately equal to 1 $\log_{10}$ (i.e. 10 fold concentration difference), 6.6 is 2 $\log_{10}$, 9.9 is 3 $\log_{10}$.

2.6. SARS-CoV-2 EQAP samples

A paynel of four coded samples were prepared for laboratories to perform SARS-CoV-2 molecular testing. Three samples were SARS-CoV-2 positive while the remaining one was negative control containing virus transport medium (VTM) only. The three SARS-CoV-2 samples were prepared from the inactivated SARS-CoV-2 virus stock. The virus stock was diluted with VTM to three different target concentrations and was confirmed by RT-PCR. After dispensing to 1 mL quantity to each vial, the samples were frozen at −70 °C. According to our previous experiences, the expected number of participants would not exceed 90. We prepared a total of 180 samples for this panel which was 2 times required for the maximum number of participants. The formula, $0.4\sqrt{N}$ (where N = total number of prepared samples) [25] was used to calculate the number of prepared samples to be tested. For the 180 samples prepared, 5 samples were required for testing. The sample quality was assessed by selecting five samples each set at random to test for homogeneity and stability. For stability testing, samples were incubated at 4 °C for 1 day and then stored at −70 °C freezer to mimic the transportation condition.

2.7. Evaluating the results

Participants were laboratories in Hong Kong performing SARS-CoV-2 tests. They were requested to collect samples at our laboratory with icebox/cooler and to test all samples according to their laboratory testing strategies as far as practicable. Participants were asked to submit the final interpreted qualitative test results for each sample (i.e. positive or negative) with the standard result form. As more than one method can be used for a laboratory to diagnose a sample, this result form was used to collect details on the detection method and target genes used, together with their respective Ct values. We did not score incorrect results for each target gene, but only the final interpreted result.

The final interpreted result for a sample can comprise of up to three target genes for a detection method used. The test results generated by the target genes may not be concordant, the laboratory could interpret the final results according to its own testing strategies. A maximum of three detection methods and three target genes per method could be submitted per laboratory. The Ct values from a target gene constitute a dataset. The design of the result form was capable of analyzing results in terms of 'dataset' level in addition to 'laboratory' level.

3. Results

3.1. Inactivation of SARS-CoV-2, integrity of SARS-CoV-2 and inactivation efficiency

All three inactivation protocols, (1) 95 °C, 5 min; (2) 95 °C, 10 min; (3) 95 °C, 15 min showed RNA loss when inactivating the virus stock of Ct 9.34. The RNA loss was higher for longer inactivation time as demonstrated by higher increasing of Ct values. The RNA loss ranged from an increase of Ct value by 1.25 to 3.26 (Table 2).

All three inactivation protocols, (1) 95 °C, 5 min; (2) 95 °C, 10 min; (3) 95 °C, 15 min were able to inactivate the virus at concentration of Ct 9.34. No CPE was observed in all cell cultures vials by 7 days for the three passages. In addition, a continuous decrease in viral load was also shown through 3 passages for all the duration of heat inactivation due to the dilution effect of the inoculum and

Table 2
Heat inactivation and impact on the RNA detection.

Heating condition	Ct value				
	Before inactivation	After inactivation			Difference (fold change)
		replicate 1	replicate 2	mean	
95 °C, 5 min	9.34	10.50	10.67	10.59	1.25 (<1 $\log_{10}$)
95 °C, 10 min		11.53	11.26	11.40	2.06 (<1 $\log_{10}$)
95 °C, 15 min		12.44	12.76	12.60	3.26 (<1 $\log_{10}$)

the culture medium. RT-PCR results for all three passages are shown in Table 3. A combination of these two findings demonstrated the three inactivation protocols could abolish the viability of the SARS-CoV-2 virus completely. Balancing the ease of operations, the RNA loss and margin of sample variability, eventually we decided the condition of 95 °C 10 min was optimum for this EQAP. One of the two culture supernatant tubes after heating for 95 °C 10 min was chosen as the inactivated SARS-CoV-2 virus stock to prepare the EQAP samples.

3.2. EQAP results

A total of 66 laboratories received the EQAP samples and returned results for analysis in January 2023. These laboratories included 38 testing agencies/private laboratories, 25 public/private hospitals, 2 universities and 1 government laboratory. The EQAP samples demonstrated sufficient homogeneity and stability prior to dispatch to participants. All of the 66 laboratories reported correct results for all samples according to the final interpreted qualitative test results (Table 4).

When analyzing the results in terms of dataset level, the protocols used for detecting the EQAP samples varied among 66 laboratories. The nucleic acid extraction platforms, real-time RT-PCR reagents and instruments used by participating laboratories are listed in Tables 5–6. Altogether 143 detection methods were reported by participants with 275 datasets generated. Almost all of them were based on commercial real-time RT-PCR reagents. Majority (93.1%, 256/275) were based on N gene (41.8%, 115/275), Orf1ab gene (33.8%, 93/275), and E gene (17.5%, 48/275). Irrespective of the detection methods used, there was no significant difference of the Ct values for the N, Orf1ab and E genes used for detecting the SARS-CoV-2 positive samples, 2023–01, 2023–03, 2023–04 (Fig. 1).

4. Discussion

The SARS-CoV-2 molecular EQAP have been reported in many other countries. Different formats have been utilized including virus like particles [26], RNA control [27] and inactivated culture virus [13]. Inactivated culture virus format closely resembles the clinical specimens, requiring nucleic acid extraction step. A full validation of heat inactivation procedures is important. There are no standardized heat inactivation protocols to inactivate SARS-CoV-2 samples. The decrease in viral nucleic acid after heat inactivation has been reported with RNA loss of >5 Ct [16]. Variations in the heat inactivation procedure such as sample volume, temperature applied, duration and method of heat treatment result in inconsistent reported outcome of nucleic acid stability; and most of the methodologies are not directly compared. In the present study, the heating procedures were described in detail. The increase in Ct value shown in the present study was only 1.25, 2.03 and 3.26 for 5 min, 10 min and 15 min respectively in 95 °C. All three conditions were capable of inactivating high viral load of samples of Ct < 10 with 1 mL volume. Although SARS-CoV-2 can be successfully inactivated at 95 °C for 5 min, we chose the protocol with 10 min for a wider margin to account for possible variation in the viral stock conditions in future preparations. In case the inactivation is incomplete as demonstrated by the subsequent viral culture passages, it would incur additional time and labor cost [13]. The viral load difference between 5 min and 10 min was less than 1 Ct value, which is practically insignificant at such high viral load.

The current study added values in the field for preparing EQAP samples using infectious materials. Although there was potential RNA loss when using heat inactivation, it is insignificant using high viral load stock. According to our current experiences, 1 mL sample of Ct~10 could be used to prepare at least 2 times quantity required for the number of our participants (N = 66). We also assessed the sequence identity for the inactivated virus stock by next generation sequencing, the result was 100% identical before and after inactivation. The Ct values reported by participants were not statistically different among the three most common target genes used. In addition, although different nucleic acid extraction platforms, real-time RT-PCR reagents and real-time RT-PCR instruments were used by the 66 laboratories, the performance of laboratories was not affected by the varied protocols used. The methodology used here demonstrated a success of preparing SARS-CoV-2 EQAP samples.

There were several limitations in the current study. Firstly, the sample size included into the current study was small. Only two samples per inactivating condition were tested using one concentration. However, as our virus stock was obtained from '4+' CPE culture supernatant at Ct 9.34, it was unlikely samples containing viral load higher than Ct 9.34 would be achieved. In the present study, 95 °C 5 min was shown to be effective to inactivate SARS-CoV-2. Although it is not known whether other virus stock at other conditions could be successfully inactivated with 5 min, adding an extra 5 min (i.e. 95 °C 10 min) should be enough to cater for unforeseen variations. Also, despite only 1 sample volume (1 mL) was assessed, higher volume is likely not necessary as it is highly concentrated and one vial could be enough to prepare up to 180 samples with concentration ranging from Ct value 23 to 30. Secondly, whether the heat inactivation would be affected by other sample matrix e.g. sputum was not assessed. However, as the specimens were

Table 3
RT-PCR results for the inactivated virus stock after three passages in Vero cells.

Heating condition	Ct value											
	After 1st passage				After 2nd passage				After 3rd passage			
	replicate 1	replicate 2	mean	Difference[a] (fold change)	replicate 1	replicate 2	mean	Difference[a] (fold change)	replicate 1	replicate 2	mean	Difference[a] (fold change)
95 °C, 5 min	23.32	23.33	23.33	12.74 (>3 $\log_{10}$)	34.47	33.28	33.88	10.55 (>3 $\log_{10}$)	neg	neg	neg	NA
95 °C, 10 min	24.75	24.66	24.71	13.31 (>3 $\log_{10}$)	35.35	35.50	35.43	10.72 (>3 $\log_{10}$)	neg	neg	neg	NA
95 °C, 15 min	25.40	25.24	25.32	12.72 (>3 $\log_{10}$)	36.19	35.71	35.95	10.63 (>3 $\log_{10}$)	neg	neg	neg	NA

[a] Values were based on the mean of current passage minus to the mean of previous measurements.

Table 4
The panel composition and the overall performance for 66 laboratories.

Sample No.	Sample content	Viral load				No. of laboratories reported correct results
		Homogeneity[a]		Stability[a]		
		Mean	SD	Mean	SD	
2023–01	SARS-CoV-2	23.62	0.28	24.94	0.12	66
2023–02	Negative	Negative	Not applicable	Not applicable	Not applicable	66
2023–03	SARS-CoV-2	26.05	0.54	25.52	0.26	66
2023–04	SARS-CoV-2	30.41	0.47	30.03	0.32	66

[a] Values were obtained from the five sets of samples. The raw data for homogeneity and stability results were shown in Appendix 1.

Table 5
The detection methods used by 66 laboratories for detecting EQAP samples.

Detection method	N = 143	%
Extraction nature		
- sample-to-answer platform	55	38.5
- automated	76	53.1
- manual	9	6.3
- not specified	3	2.1
Pooling		
- no	92	64.3
- yes	39	27.3
- 2-sample pool	3[a]	7.7[a]
- 3-sample pool	20	51.3
- 4-sample pool	9	23.1
- 5-sample pool	7	17.9
- not specified	12	8.4
Detection		
- commercial	139	97.2
- in-house designed	2	1.4
- not specified	2	1.4

[a] Calculated within the specified group.

Table 6
The nucleic acid extraction platforms/kits, real-time RT-PCR reagents and real-time RT-PCR instruments for the 143 detection methods used by 66 laboratories for detecting EQAP samples.

Items[a]	N = 143	%
Nucleic acid extraction platforms/kits		
- Sample-to-answer platform	55	38.5
- Thermo Fisher Scientific	14	9.8
- TianLong	12	8.4
- Yaneng	8	5.6
- Roche	6	4.2
- Hangzhou Bioer Technology	5	3.5
- Qiagen	5	3.5
- TANBEAD	5	3.5
- AllSheng	4	2.8
- Hybribio	4	2.8
- Others/not specified	25	17.5
Real-time RT-PCR reagent		
- Sample-to-answer platform	55	38.5
- Cepheid	28[b]	50.9[b]
- Roche	11	20.0
- Hologic	5	9.1
- Qiagen	4	7.3
- Others/not specified	7	12.7
- Non Sample-to-answer platform	88	61.5
- Sansure Biotech	15[b]	17.0[b]
- DaAn Gene	11	12.5
- Roche	9	10.2
- Chaozhou Hybribio Biochemistry	6	6.8
- Seegene	6	6.8
- IDT	5	5.7

(*continued on next page*)

Table 6 (*continued*)

Items[a]	N = 143	%
- BGI	4	4.5
- Others/not specified	32	36.4
Real-time RT-PCR instrument		
- Sample-to-answer platform[c]	55	38.5
- Non Sample-to-answer platform	88	61.5
- ABI	36[b]	40.9[b]
- BioRad	14	15.9
- Roche	14	15.9
- Hangzhou Bioer Technology	4	4.5
- Suzhou Molarray	4	4.5
- Others/not specified	16	18.2

[a] Only items reported in ≥4 methods were shown; otherwise, they were grouped into the category, 'Others/not specified'.
[b] Calculated within the specified group.
[c] The sub-items were identical to 'Real-time RT-PCR reagent'.

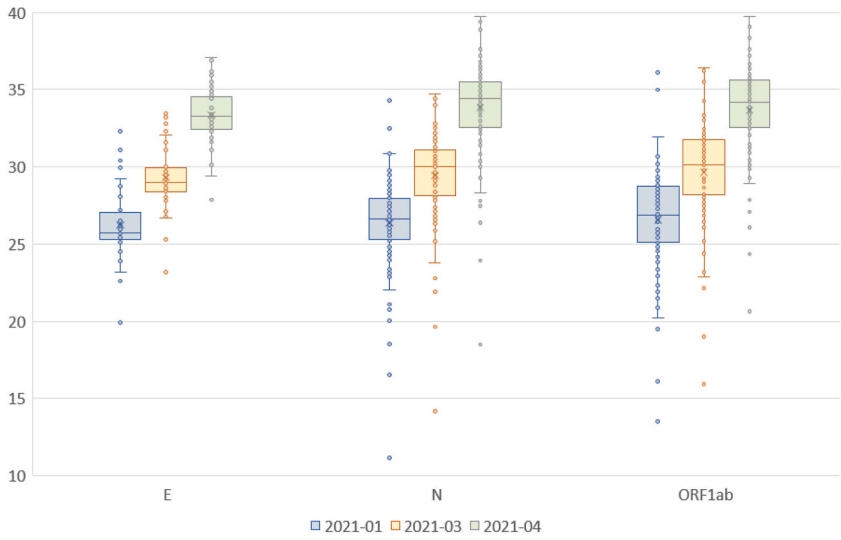

Fig. 1. The Box-whisker-plot for the Ct values generated by the 256 datasets, E gene (N = 48), N gene (115), Orf1ab gene (93).

first propagated in viral culture for EQAP preparation, this issue was not a major concern for this purpose. Thirdly, the concentrations of the samples were not accurately quantified as copies/μL unit. As the aim of our current EQAP focused on qualitative results, as long as the methods to assess the viral load were standardized, homogeneous and stable, we had no concerns of using Ct values as a surrogate marker of the viral load of the samples.

In conclusion, with limited RNA loss of <1 $\log_{10}$ using 95 °C on 1 mL high viral load samples, the heat inactivation conditions evaluated in the present study were useful for preparing EQAP samples. The samples were homogeneous and stable, and participants returned results with high level of consensus. At the time of preparing this report, the heat inactivation conditions have been shown successful to inactivate both SARS-CoV-2 and Respiratory Syncytical Virus which are incorporated in the 2023 dispatch of the WHO EQAP samples. This validated procedure provides a solid evidential basis to successfully prepare EQAP samples for other emerging pathogens in future.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Gannon C.K. Mak: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stephen S.Y. Lau:** Investigation. **Kitty K.Y. Wong:** Investigation. **Eunice K.Y. Than:** Resources. **Anita Y.Y. Ng:** Resources. **Derek L.L. Hung:** Writing – review & editing, Writing – original draft.

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 1. The raw data of the homogeneity and stability results

Sample No.	Sample content	Viral load		
		Replicate	Homogeneity	Stability
2023–01	SARS-CoV-2	Set 1	23.65	24.93
		Set 2	23.17	24.97
		Set 3	23.81	25.09
		Set 4	23.88	24.75
		Set 5	23.60	24.95
2023–02	Negative	Set 1	Negative	Not applicable
		Set 2	Negative	Not applicable
		Set 3	Negative	Not applicable
		Set 4	Negative	Not applicable
		Set 5	Negative	Not applicable
2023–03	SARS-CoV-2	Set 1	25.86	25.83
		Set 2	25.97	25.76
		Set 3	26.64	25.23
		Set 4	25.28	25.36
		Set 5	26.49	25.44
2023–04	SARS-CoV-2	Set 1	30.31	30.42
		Set 2	29.70	30.35
		Set 3	30.68	29.86
		Set 4	30.39	29.81
		Set 5	30.97	29.73

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