RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2024.077

ScienceAsia 50 (4): 2024: ID 2024077: **1–10**

Effect of azo dye treatment on the detection of human (OC43) coronavirus surrogate and SARS-CoV-2 viability based on an in-house photoactivator device

Nattamon Niyomdecha[a,*], **Pirom Noisumdaeng**[b,c], **Chompunuch Boonarkart**[d], **Prasert Auewarakul**[d]

[a] Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Rangsit Campus, Pathum Thani 12120 Thailand
[b] Faculty of Public Health, Thammasat University, Rangsit Campus, Pathum Thani 12120 Thailand
[c] Thammasat University Research Unit in Modern Microbiology and Public Health Genomics, Thammasat University, Pathum Thani 12120 Thailand
[d] Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700 Thailand

[*]Corresponding author, e-mail: nattamon@tu.ac.th

Received 29 Nov 2023, Accepted 21 Jul 2024
Available online 19 Sep 2024

ABSTRACT: Innovative detection methods for discriminating between infectious and non-infectious viruses have been developed to determine the risk of transmission, quarantine policy, and medical treatment. This study evaluated the efficacy of viability quantitative reverse transcription-polymerase chain reaction (vRT-qPCR) to indicate human coronavirus surrogate strain OC43 (HCoV-OC43) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viability based on azo dye treatment combined with our in-house photoactivator device. The factors affecting azo dye efficacy were optimized in tests with free viral RNA and virus suspension. A propidium monoazide (PMAxx) had a better performance than ethidium monoazide bromide (EMA) in the exclusion of false positive PCR signals, and its activity correlated with an increasing dye concentration. Although 50 µM PMAxx could completely quench amplification of free viral RNA at a concentration of 10^8 copies/µl, the highest dose of 2 mM PMAxx was only sufficient for a complete suppression of inactivated virus suspension at 10^2 pfu/100 µl. Additionally, various techniques of dye combinations, surfactant cotreatment, and double light exposure were tested to improve the result. Only the combination of 1 mM PMAxx and 10 µM EMA showed a comparable result to 2 mM PMAxx. The other evaluated conditions had a deleterious effect on the active virus. Despite an incomplete amplification inhibition, an observed quantification cycle (Cq) value difference larger than 8 between untreated virus and inactivated virus with intact RNA and compromised capsid might suggest viral infectivity. Therefore, this research provides vRT-qPCR data in discerning the infectious status of enveloped SARS-CoV-2 and its surrogate virus.

KEYWORDS: viability qRT-PCR, azo dye, propidium monoazide, ethidium monoazide bromide, SARS-CoV-2

INTRODUCTION

Virus detection based on polymerase chain reaction (PCR) is faster and has higher sensitivity and specificity than a gold standard viral culture [1] but is unable to distinguish between infectious and inactivated non-infectious viruses. In case of coronavirus disease 2019 (COVID-19), a common and important problem of diagnosis is the prolonged persistence of viral SARS-CoV-2 RNA shedding in various clinical sample types after recovery. Therefore, viral infectivity is unclear, leading to the risk of virus transmission and an epidemiological control challenge [2, 3]. Since SARS-CoV-2 is classified into the biological risk group 3, it is impractical to use a culture-based technique for viability determination in a real-life scenario. Thus, the development of a culture-free method which can discriminate between infectious and non-infectious viruses has been attempted.

A technique known as viability PCR (vPCR) has been recently developed, which combines traditional PCR or quantitative PCR (qPCR) with photoactivatable nucleic acid-intercalating dyes. This technique has the potential to reduce the PCR drawback of undifferentiating the genome obtained between infectious and dead states [4]. Based on the principle of viral capsid integrity, azo dyes can penetrate damaged virus particles and bind with nucleic acids under dark incubation. EMA and PMAxx are 2 major azo dyes that have been used for this purpose. Exposure to a specific light source or photoactivation will cause covalent binding of the dye to nucleic acids which will result in inhibition of viral genome amplification by PCR and thereby prevent false-positive signals from damaged virus particles [4]. Consequently, the PCR amplification of the viral genome derived from intact virus particles will only produce a true-positive signal following the application of azo dye. In previous studies, vPCR has been mostly applied for the detection of foodborne and waterborne naked pathogenic viruses [5]. In contrast, the number of vPCR applications in enveloped viruses is limited. The potential infectivity of certain enveloped viruses such as avian influenza virus, influenza A virus, porcine epidemic diarrhea virus (PEDV), murine hepatitis virus (MHV),

and SARS-CoV-2 has been recently reported [4]. However, various factors that may affect or improve the vPCR results need to be more thoroughly examined.

In this study, we aimed to evaluate the effect of azo dye treatment in a vPCR assay under different treatment conditions for discriminating between live and inactivated states of HCoV-OC43 and SARS-CoV-2 viruses based on our economical in-house photoactivator device. The results provide informative data on the application of vPCR for SARS-CoV-2 detection.

MATERIALS AND METHODS

Virus titration

Virus stock of SARS-CoV-2/01/human/Jan2020/Thailand and HCoV-OC43 ATCC strains was titrated by plaque assay. Briefly, susceptible cell lines, including African green monkey kidney cells (Vero E6) for SARS-CoV-2 and human ileocecal adenocarcinoma cells (HCT-8) or human rhabdosarcoma cells (RD) for HCoV-OC43, were cultured in 24-well plates at a density of 2.3×10^5 cells/well and maintained in MEM/DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and antibiotics. After overnight incubation in a humidified 37 °C incubator with 5% CO_2, the old culture medium was removed. The cells were inoculated with 100 µl of 10-fold serial dilutions of virus stock for 1 h at 37 °C with 5% CO_2, and the virus inoculums were discarded. The cells were then overlaid with 1.56% microcrystalline cellulose (Avicel RC-591, FMC Biopolymer, Ireland) in 2% FBS-MEM/DMEM and incubated for 3 days. After that, the overlaid medium was removed, and the cells were fixed with 10% (v/v) formalin in phosphate-buffered saline (PBS) for 2 h. The fixed cells were washed with tap water and stained with 1% (w/v) crystal violet in 20% (v/v) ethanol for 15 min. Excess dyes were discarded by washes in tap water. The viral titers were evaluated by counting plaque number and calculated in plaque-forming units per ml (pfu/ml).

Virus RNA extraction and concentration measurement

RNA was extracted using a QIAamp Viral RNA Mini Kit (Cat no. 52904, QIAGEN, Germany) according to manufacturer's instructions. RNA was eluted in 20–50 µl of RNase-free water and stored at −80 °C. The concentration of the purified RNA from virus stock was measured as ng/µl and converted to copies/µl based on using the following formula:

$$\text{No. of copies} = \frac{\text{A} \times 6.022 \times 10^{23}}{\text{M.W.} \times 10^9}$$

where A = amount of sample (ng), M.W. = molecular weight = (nucleotide length × 320.5) + 159.0 Daltons or g/mol. Nucleotide length of HCoV-OC43 = 30,746 nucleotides; nucleotide length of SAR-CoV-2 = 29,903 nucleotides; 6.022×10^{23} = Avogadro's constant; 10^9 = conversion factor.

Inactivation of viruses

Dilutions of 10^2–10^4 pfu/100 µl were prepared in UDW/PBS or other matrices depending on the experimental purpose. Aliquots of 100 µl of virus suspension were inactivated by the following methods: heating at 65 °C for 20 min, 99 °C for 5 min, UV-C radiation for 15 min, disinfection with either 70% ethanol or 1% sodium hypochlorite (NaOCl) at a ratio of 1:1 for 30 s. Viral culture or blind subculture was employed to verify that the inactivated virus was completely non-infectious. Briefly, 100 µl of inactivated virus suspension, either derived from physical inactivation methods or a tenfold dilution from chemical methods, was added to susceptible cell lines specific for each virus and incubated at 37 °C with 5% CO_2 for 3 days. A continuous blind subculture was conducted twice on the supernatant of the virus that had been treated with disinfectant agents after 3 days. The viral cytopathic effect (CPE) was daily observed and compared to mock-uninfected cells and virus-infected cells under a light microscope. The inactivated virus must not produce CPE throughout the incubation time.

Azo dye preparation

EMA (Biotium, USA) and PMA derivative or PMAxx (Biotium) were used as azo dye or photoreactive viral genome-binding dye for vRT-qPCR in this study. Purchased PMAxx was ready to use at a concentration of 20 mM in H_2O, but EMA was in powdered form and 5 mg powder were dissolved in 1 ml of absolute ethanol or dimethylsulfoxide (DMSO) to prepare a 12 mM stock concentration. Stock dye solutions were stored light protected at −20 °C.

The azo dyes were diluted to working concentrations of 50 and 100 µM in different matrices representing various real-world samples, including ultrapure distilled water (UDW), PBS, DMEM cell culture medium, 0.3% bovine serum albumin (BSA), and cell culture medium including 0.3% BSA depending on the purpose of each experiment.

Construction and optimization of in-house photoactivator device

The in-house photoactivator device was assembled from 3 major parts: a 1.5 ml microtube sample holder, blue light emitting diodes (LEDs) as the light source, and a temperature probe detector. The device was created in different heights, i.e., 1, 2, 4, 7, and 10 cm. The height was the distance between tube bottom and light source for optimization purpose.

To determine the optimal condition for azo dye photolysis reaction, the working concentrations of PMAxx and EMA prepared in the different matrices were tested at various time intervals of light exposure

at each device height, and the color changes from dye structural modification and accumulated temperature of the samples were recorded. The optimal condition was accepted when the apparent color of azo dye changed from pale yellow/yellow into pale orange in clear with/without turbid matrices or from fuchsia pink into reddish in colored solution, and the accumulated temperature in all tested exposure times must not exceed 30 °C.

Azo dye treatments of free viral RNA

Working concentrations of SARS-CoV-2 and HCoV-OC43 RNA were prepared in a range of 10^4–10^9 copies/µl. To evaluate the optimal concentration and dye type, RNA was incubated with EMA and PMAxx at the final concentrations of 5, 15, 50, and 100 µM in the dark for 15 min. The RNA-dye samples were then placed into the sample holder of the in-house photoactivator device and exposed to blue LED light for 15 min to activate a photolysis reaction for covalent crosslinking with the viral RNA. Treated RNA and untreated RNA control samples were used as the template for RT-qPCR, and the Cq or cycle threshold (Ct) values were compared. Additionally, to determine the remaining activity of the activated azo dye after being light exposed, it was exposed to blue LED light for 15 min prior to using the same protocol for testing.

The protocol was modified to evaluate the effect of sample matrix (diluting solution) and time for light exposure on the azo dye treatment efficacy. Briefly, RNA samples were prepared in 3 different matrices: cell culture medium, 0.3% BSA, and cell culture medium and 0.3% BSA. The RNA samples were incubated with the most effective dye at the optimal concentration for 15 min in the dark. Then, the samples were exposed to light for 15 and 30 min and analyzed by RT-qPCR.

Azo dye treatment of infectious and inactivated viruses

Infectious and inactivated viruses were treated with PMAxx, combined PMAxx and EMA, or combined PMAxx and Triton X-100 or Tween 20 surfactant, and light exposure was done once or twice as specified in Tables 4 and 5. One-hundred µl of infectious or inactivated virus suspension was incubated with the indicated reagent for 15 min in the dark and then exposed to blue LED light for 30 min. Dark incubation and light exposure were repeated in double light exposure experiments. RNA was extracted from infectious and inactivated virus samples and used for RT-qPCR.

RT-qPCR assay for SARS-CoV-2 and HCoV-OC43 detection

Specific primers to detect the envelope (E) gene of SARS-CoV-2 were E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT and E_Sarbeco_R: ATATTGCAGCAGTACGCACACA [6], and the primers for the nucleocapsid (N) gene of HCoV-OC43 were GC-HCoV-OC43-F: ACGTGCGCGATGTCAATACCCCGGCTGAC and GC-HCoV-OC43-R: CCAGGCGGTGGCTCTACTACGCGATCCTG [7].

The same amplification protocol was employed by using both primer sets. The reaction was conducted with a Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, USA) to detect the amplified products via a SYBR Green reporter system. The final reaction mixture included 1X one-step reaction mix, 1X Warm-Start RT enzyme mix, 200 nM of each primer, equal volume of the RNA template, and nuclease-free water for volume adjustment to 20 µl. A positive control of viral RNA and a negative control of nuclease-free water were included in each run. The amplification process was done using a CFX-96 real-time PCR detection system (Bio-Rad, USA). The conditions were reverse transcription for 10 min at 55 °C, initial denaturation for 1 min at 95 °C, 40 cycles of denaturation for 10 s at 95 °C, and extension for 30 s at 60 °C, followed by melt curve analysis to confirm the presence of target gene.

Statistical analysis

Data were obtained from duplicate experiments and are presented herein as mean ± SD. Statistical analysis was performed by using paired and unpaired t-tests for comparing the differences within group and between groups. Significance was accepted at $p < 0.05$.

Biosafety approval

All procedures involving biological risk group viruses were approved by the Thammasat University Institutional Biosafety Committee (064/2564) and the Mahidol University Biosafety Committee (MU 2021-013). Biosafety lab level and practice guidelines were applied in accordance with the biological risk group of the analyzed viruses.

RESULTS

Optimization of experimental conditions for using an in-house photoactivator device on azo dye activity

This study did not use a sophisticated commercial photoactivator device but a simple in-house photoactivator device using blue LEDs as the light source for azo dye photolysis activation (Fig. 1). Two parameters, firstly, distance between light source and dye tube and, secondly, exposure time, were varied to evaluate whether this tool could activate azo dye structure modification and to determine the optimal conditions for photoactivation. PMAxx and EMA azo dyes were prepared for testing different matrices of clear, turbid, and color reagents. The optimal condition was selected based on the apparent color change of azo dye from pale yellow/yellow into pale orange in clear with/without turbid matrices or from fuchsia pink into reddish in

Table 1 Optimization for azo dye activation in different matrices by using an in-house photoactivator device.

Matrix of Azo dye[a]	Result of color change and accumulated temperature (°C)[b]					
	Exposure time (min)	Light source distance (cm)				
		1	2	4	7	10
UDW 1X PBS 0.85% NSS Cell culture media 0.3% BSA Cell culture media + 0.3% BSA	5	+ 29.5	+ 29.5	+ 29.2	+ 29.2	+ 29.2
	10	+ 30.1	+ 30.1	+ 29.5	+ 29.5	+ 29.5
	15	+ 32.3	+ 32.3	+ 29.7	+ 29.6	+ 29.6
	30	+ 32.5	+ 32.5	+ 29.7	+ 29.7	+ 29.7

[a] PMAxx and EMA azo dyes were prepared at 50 μM and 100 μM concentrations in different matrices.
[b] Color changes of the tested azo dyes were observed by naked eye. A positive reaction caused azo dye color change from pale yellow/yellow into pale orange in clear with/without turbid matrices or from fuchsia pink into reddish in colored solution.
Abbreviations and symbols: UDW, ultra distilled water; PBS, phosphate buffer saline; NSS, normal saline; BSA, bovine serum albumin; min, minute; cm, centrimeter; °C, degree Celsius; and +, positive reaction of azo dye activation.

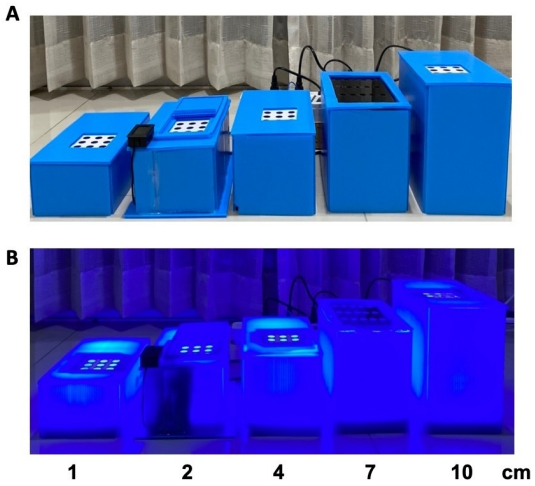

Fig. 1 Illustration of in-house photoactivator devices. (A) Five in-house photoactivator devices built with 1, 2, 4, 7, and 10 cm distance of the blue LED light source to the test tube. (B) Working in-house photoactivator devices to determine color change from azo dye activation and accumulated temperature (°C) by probe detector.

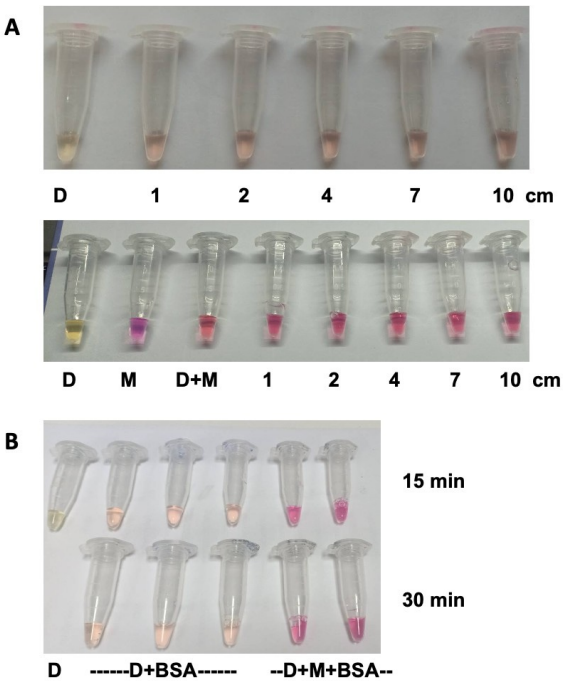

Fig. 2 Illustration of azo dye color before and after blue LED light activation. (A) Azo dyes in UDW (PMAxx/EMA, D), cell culture medium (M), and dyes with cell culture medium (D+M) before and after activation at different light source distances. (B) Azo dyes in 0.3% bovine serum albumin (D+BSA) and in cell culture medium with BSA (D+M+BSA) at 4 cm light source distance and 15 min and 30 min exposure time.

colored solution, and the accumulated temperature in all tested exposure times had to be at $\leqslant 30$ °C to preserve sample quality.

As shown in Table 1 and Fig. 2, all tested light source distances and exposure times could activate and modify azo dye structure in all matrices and confirmed the function of the device. However, considering the accumulated temperature, only light source distances $\geqslant 4$ cm were found acceptable. The parameters selected for further experiments were a distance of 4 cm and an exposure time of 15 or 30 min.

Evaluation of the efficacy of azo dye treatment on amplification inhibition of free viral RNA

Amplification inhibition in dead cells in vPCR mainly depends on azo dye type and concentration, sample matrix, and dye treatment. We evaluated these factors with treated/untreated free viral RNA using the optimal light source distance and exposure time for the photoactivator device. The efficacy of amplification inhibition in vRT-qPCR was determined by comparison of the Cq value of treated and untreated RNA.

Initially, SARS-CoV-2 RNA at 108 copies/µl was used to evaluate the optimal dye type and concentration (Table 2 section A). The Cq value was found to increase with increasing dye concentration, i.e., inhibition of amplification increased along increasing dye concentration. Between the 2 dyes, PMAxx showed higher inhibition of amplification than EMA at all tested concentrations. Indeed, PMAxx at 50 µM completely inhibited the amplification of SARS-CoV-2 RNA and RNA of HCoV-OC43 surrogate virus (data not shown). This dye concentration was further tested at lower and higher concentrations of virus RNA for both dyes. Amplification was completely inhibited at all lower RNA concentrations (10^4–10^7 copies/µl) by 50 µM PMAxx, while a weak positive signal was observed at 10^9 copies/µl. PMAxx at a concentration of 100 µM completely inhibited amplification at all RNA concentrations.

Exposure of azo dyes to light causes dye activation and structural modification and, thereby, triggers covalent binding to nucleic acids. However, at 100 µM concentration, the light-activated azo dye still caused significant inhibition of viral RNA amplification (Table 2 section B). Considering these results, PMAxx at 50 µM concentration was selected for further experiments.

Next, the effect of different matrices on azo dye treatment was tested. Viral RNA derived from SARS-CoV-2 and HCoV-OC43 was prepared in cell culture media, 0.3% BSA, and cell culture media with 0.3% BSA as representative of color clear, turbid, color and turbid matrices, respectively. The samples were incubated with PMAxx at 50 µM concentration, and the dye was then activated by exposure to blue LED light for 15 min and 30 min. Amplification inhibition in all matrices was very high after 15 min and complete at 30 min exposure time (Table 3). In conclusion, the optimal conditions for vRT-qPCR were using PMAxx at a concentration of 50 µM and a light exposure time of 30 min.

Efficacy of discrimination between infectious and inactivated viruses after azo dye treatment

HCoV-OC43 is one of the endemic strains of low-risk coronaviruses that has attracted attention as a valuable research alternative for a high-risk SARS-CoV-2 study [8]. HCoV-OC43 was employed as a surrogate virus for SARS-CoV-2 in the initial experiments due to biosafety facility limitations that require biosafety level 3 (BSl-3) to cooperate with SARS-CoV-2. Therefore, using this surrogate virus facilitated the study under a variety of test parameters. Viability RT-qPCR was performed using the optimal conditions as described in the previous section. Success of vRT-qPCR was defined as no effect on the amplification signal of infectious virus and a significant decrease in false-positive signal in inactivated virus.

A suspension of infectious HCoV-OC43 at a concentration of 10^4 pfu/100 µl was used to prepare samples of infectious and inactivated viruses (Table 4). Inactivation was achieved by heating at 65 °C and

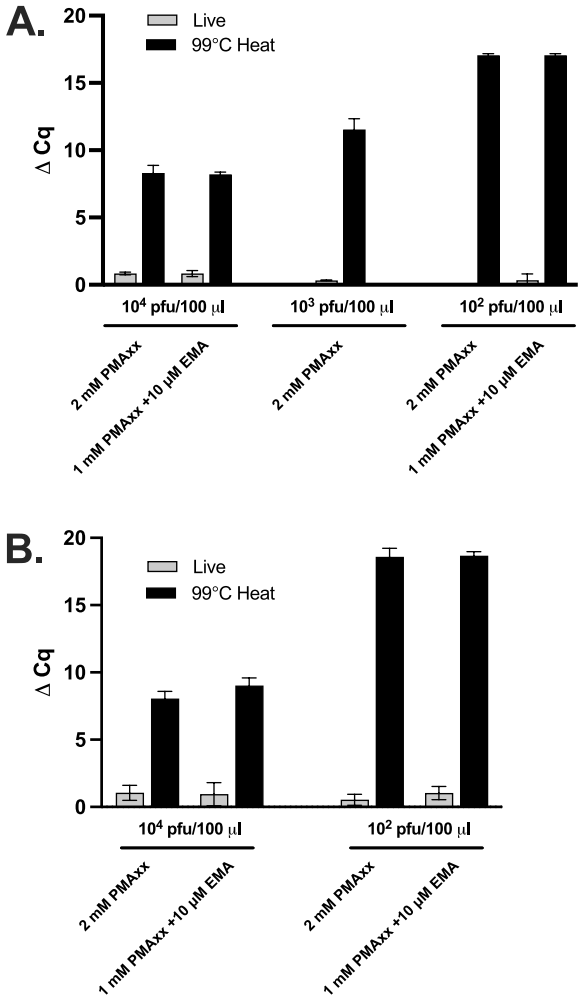

Fig. 3 Cq value difference (ΔCq) between azo dye-treated and untreated virus samples. Infectious (gray bars) and 99 °C heat-inactivated viruses (black bars) of (A) HCoV-OC43 and (B) SARS-CoV-2 at the indicated viral titers treated at the indicated optimal azo dye conditions and after vRT-qPCR. The Cq value difference (ΔCq) to untreated viruses was determined. Data are shown as mean ± SD from duplicate experiments.

Table 2 Efficacy of treatment of SARS-CoV-2 RNA with PMAxx and EMA at different concentrations.

A. Cq value[a] of treatment of SARS-CoV-2 RNA with non-activated azo dye[b]									
Copies/µl	RNA	5 µM		15 µM		50 µM		100 µM	
	control	EMA	PMAxx	EMA	PMAxx	EMA	PMAxx	EMA	PMAxx
10^9	5.04 ± 0.06	–	–	–	–	–	33.22 ± 0.06	30.68 ± 0.13	> 40.00[c]
10^8	10.91 ± 0.24	12.62 ± 0.15	18.20 ± 0.23	13.28 ± 0.24	21.99 ± 0.08	19.46 ± 0.28	> 40.00	36.43 ± 0.13	> 40.00
10^7	14.59 ± 0.38	–	–	–	–	20.59 ± 0.32	> 40.00	–	–
10^6	18.87 ± 0.20	–	–	–	–	25.39 ± 0.24	> 40.00	–	–
10^5	24.47 ± 0.40	–	–	–	–	31.48 ± 0.40	> 40.00	–	–
10^4	28.53 ± 0.34	–	–	–	–	32.78 ± 0.24	> 40.00	–	–
B. Cq value of treatment of SARS-CoV-2 RNA with activated azo dye[b]									
10^8	10.91 ± 0.24	10.86 ± 0.16	10.32 ± 0.13	10.49 ± 0.64	11.46 ± 0.18	12.06 ± 0.11	12.76 ± 0.04	17.69 ± 0.08	32.43 ± 0.23

[a] Data are the mean ± SD from duplicate experiments. Statistical significance ($p < 0.05$) of data is indicated by blue and red when EMA and PMAxx were compared to non-treated RNA (control), respectively.
[b] Classification of non-activated or activated azo dye is based on whether it was photoactivated or not before incubation with viral RNA.
[c] Cq > 40.00 means undetectable.
Abbreviations and symbols: Cq, quantification cycle or cycle threshold; PMAxx, propidium monoazide derivative; EMA, ethidium monoazide bromide; and –, not done.

Table 3 Efficacy of treatment of viral RNA with PMAxx in different matrices.

Matrix	Virus RNA[a]	Cq value[b]		
		RNA control	Light exposure time of PMAxx 50 µM treatment	
			15 min	30 min
Cell culture medium (M)	SARS-CoV-2	10.97 ± 0.45	39.77 ± 0.08	> 40.00[c]
	HCOV-OC43	13.04 ± 0.13	39.94 ± 0.06	> 40.00
0.3% BSA	SARS-CoV-2	11.56 ± 0.13	39.14 ± 0.09	> 40.00
	HCOV-OC43	13.17 ± 0.13	39.50 ± 0.09	> 40.00
M + 0.3% BSA	SARS-CoV-2	11.28 ± 0.12	39.48 ± 0.18	> 40.00
	HCOV-OC43	13.78 ± 0.20	39.31 ± 0.04	> 40.00

[a] Virus RNA concentration derived from SARS-CoV-2 and HCOV-OC43 was 10^8 copies/µl.
[b] Data are the mean ± SD from duplicate experiments.
[c] Cq > 40.00 means undetectable.
Abbreviations: Cq, quantification cycle or cycle threshold; PMAxx, propidium monoazide derivative; SARS-CoV-2; severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2); HCOV-OC43; human coronavirus strain OC43; and BSA, bovine serum albumin.

99 °C, radiation with UV-C, 70% alcohol, and 1% NaOCl, and it was confirmed by cell-based viral culture that CPE was not observed (Fig. S1). As expected, at 0 µM PMAxx (untreated condition), the qPCR results were similar for infectious virus and heat or alcohol inactivated virus whereas inactivation by UV-C and especially NaOCl caused reduction in amplification.

Unfortunately, the previously determined optimal conditions for vRT-qPCR with free viral RNA (50 µM PMAxx and 30 min light exposure) failed to discriminate between infectious and heat-inactivated viruses. Thus, higher PMAxx concentrations (100 µM to 2 mM) were tested at 30 min light exposure. These concentrations did not show negative effects on infectious virus detection (Table 4). The results were dose-dependent, and significant differences between infectious and heat-inactivated viruses were observed at 1 and 2 mM PMAxx. Likewise, a significant reduction of false positive signals was observed at 2 mM PMAxx for 70% alcohol and 1% NaOCl-inactivated viruses. It is important to note that PMAxx is only capable of entering viruses that have been damaged, and the inactivation of viruses by 65 °C heating or UV-C appears to have caused less damage. Tests of 1 and 2 mM PMAxx at lower HCoV-OC-43 concentrations (10^2 and 10^3 pfu/100 µl) showed better suppression of false-positive amplification of heat-treated virus, and 2 mM PMAxx caused complete amplification inhibition of 10^2 pfu/100 µl inactivated virus. The detection results of virus suspension prepared in UDW and 0.3% BSA treated by PMAxx were not different (data not shown).

Several alternative PMAxx treatment protocols were examined including combination of PMAxx and EMA, combination of PMAxx and surfactant (Triton X-

Table 4 Evaluation of optimal azo dye treatment conditions in discrimination of infectious and inactivated human coronavirus OC43 strain by vRT-qPCR.

Treatment condition	Cq value[a] of infectious and inactivated virus detection from each condition by vRT-qPCR									
	10^4 pfu/100 µl						10^3 pfu/100 µl		10^2 pfu/100 µl	
	Infectious	H-65 °C	H-99 °C	UV-C	70% Alc	NaOCl	Infectious	H-99 °C	Infectious	H-99 °C
Light exposure: S										
A) PMAxx										
0 µM	15.13±0.05	15.64±0.13	16.53±0.25	18.43±0.17	15.31±0.27	34.99±0.42	18.17±0.15	19.25±0.39	21.78±0.28	22.95±0.11
50 µM	15.60±0.45	15.91±0.16	17.65±0.08	–	–	–	–	–	–	–
100 µM	15.80±0.19	16.32±0.04	18.34±0.33	–	–	–	–	–	–	–
200 µM	15.67±0.28	16.78±0.11	18.56±0.27	–	–	–	–	–	–	–
500 µM	15.76±0.08	18.08±0.18	20.00±0.25	–	–	–	–	–	–	–
1 mM	16.06±0.06	18.31±0.06	22.89±0.08	–	–	–	18.52±0.10	29.97±0.11	21.51±0.40	34.89±0.57
2 mM	15.97±0.15	18.41±0.05	24.83±0.33	20.26±0.74	21.97±0.15	> 40.00[b]	18.50±0.18	30.79±0.41	21.57±0.12	> 40.00
B) PMAxx + EMA										
100 µM+100 µM	23.49±0.45	23.60±0.13	29.01±0.30	–	–	–	–	–	–	–
1 mM+10 µM	15.97±0.18	18.61±0.16	24.74±0.08	–	–	–	–	–	21.87±0.40	> 40.00
1 mM+100 µM	23.03±0.08	25.22±0.76	28.08±0.61	–	–	–	–	–	–	–
C) PMAxx + surfactant										
50 µM+1% Tx	30.37±0.40	–	35.86±0.36	–	–	–	–	–	–	–
50 µM+2% Tx	29.82±0.85	–	36.44±0.54	–	–	–	–	–	–	–
50 µM+3% Tx	31.24±0.19	–	37.51±0.04	–	–	–	–	–	–	–
50 µM+1% Tw	28.66±0.55	–	33.43±0.67	–	–	–	–	–	–	–
50 µM+2% Tw	30.53±0.49	–	35.31±0.08	–	–	–	–	–	–	–
50 µM+3% Tw	31.36±0.15	–	36.03±0.30	–	–	–	–	–	–	–
Light exposure: Db										
D) PMAxx										
1 mM	17.76±0.18	18.96±0.30	24.85±0.74	–	–	–	–	–	–	–
E) PMAxx + EMA										
100 µM+100 µM	24.53±0.30	25.04±0.10	28.09±0.88	–	–	–	–	–	–	–
1 mM+10 µM	17.76±0.12	18.05±0.14	24.95±0.13	–	–	–	–	–	–	–
1 mM+100 µM	23.63±0.19	24.61±0.42	28.90±0.35	–	–	–	–	–	–	–

[a] Data are the mean ± SD from duplicate experiments. Statistical significance ($p < 0.05$) of data is indicated by blue and red when compared to untreated infectious virus and its untreated inactivation condition, respectively.
[b] Cq > 40.00 means undetectable.
Abbreviations and symbols: Cq value, quantification cycle or cycle threshold; vRT-qPCR, viability reverse transcription-quantitative polymerase chain reaction; pfu, plaque forming unit; Infectious, infectious virus; H, heat-inactivated virus; Alc, ethanol alcohol, NaOCl, 1% sodium hypochlorite; S, single time; Db, double time; PMAxx, propidium monoazide derivative; EMA, ethidium monoazide bromide; Tx, Triton X-100; Tw, Tween 20; and –, not done.

Table 5

Treatment condition under single light exposure	Cq value[a] of infectious and inactivated virus detection from each condition by vRT-qPCR			
	10^4 pfu/100 µl		10^2 pfu/100 µl	
	Infectious	H-99 °C	Infectious	H-99 °C
A) PMAxx				
0 µM	14.82 ± 0.49	16.69 ± 0.08	19.94 ± 0.10	21.26 ± 0.40
2 mM	15.87 ± 0.08	24.74 ± 0.46	20.48 ± 0.30	> 39.84
B) PMAxx + EMA				
1 mM + 10 µM	15.79 ± 0.35	25.71 ± 0.66	20.98 ± 0.39	> 39.93

[a] Data are the mean ± SD from duplicate experiments. Statistical significance ($p < 0.05$) of data is indicated by red highlight when compared to its non-treated inactivation condition.
Abbreviations and symbols: Cq value, quantification cycle or cycle threshold; vRT-qPCR, viability reverse transcription-quantitative polymerase chain reaction; pfu, plaque forming unit; Infectious, infectious virus; H, heat-inactivated virus; PMAxx, propidium monoazide derivative; and EMA, ethidium monoazide bromide.

100, Tween 20), and repeated light exposure. Combinations that included 100 µM EMA or surfactant had a significant negative effect on infectious virus detection. The same negative effect was observed with repeated light exposure. On the other hand, the combination of 1 mM PMAxx and 10 µM EMA showed a comparable result with using only 2 mM PMAxx.

Finally, vRT-qPCR was tested with infectious and

99 °C heat-inactivated SARS-CoV-2. The virus was used at concentrations of 10^2 and 10^4 pfu/100 µl with 2 mM PMAxx and a combination of 1 mM PMAxx and 10 µM EMA. In line with the HCoV-OC43 results, the amplification signal was significantly reduced in heat-inactivated SARS-CoV-2. The Cq value difference between infectious and heat-inactivated viruses was larger than 8 as shown in Fig. 3.

DISCUSSION

Amid the ongoing global transmission of COVID-19, powerful genomic detection methods with high sensitivity and specificity are key to identification and discrimination of infected and uninfected individuals. A major limitation of PCR, which has been widely used during the pandemic era, is its inability to identify the true infection status or the true amount of infectious virus particles in the tested samples. SARS-CoV-2 RNA can be detected in symptomatic infected individuals, asymptomatic infected individuals, and convalescent patients. Virus detection in the latter 2 groups contributes to infectivity quandary, i.e., a problem of justification in quarantine ending, determination of reinfection/recurrent, or medical treatment [9, 10]. In general, infectivity seems to correlate with the Cq value obtained by qPCR, but a recent study found evidence of viral replication at high Cq values of 36–39 [11]. This suggests that the Cq value is insufficient for definitive indication of the infection status and that diagnosis by standard qPCR must be improved to find the true infection status.

Recently, a novel PCR technique termed capsid integrity PCR (ciPCR) or more general vPCR has been used to reduce the amplification of nucleic acids from non-infectious viruses. It has been mostly applied to non-enveloped enteric viruses in environmental surveillance and food safety [12], including human norovirus (NoV), murine norovirus (MNV), enterovirus (EV), rotavirus (RV), hepatitis A virus (HAV), hepatitis E virus (HEV), and Adenovirus (AdV) [4, 12]. Most studies demonstrated a consistent reduction in false positive signals following azo dye treatment [12]. Nevertheless, the assessment of the PMA azo dye treatment with MNV [13] and bacteriophage T4 [14] naked viruses indicated a failure. Compared to the investigation of naked viruses, the number of azo dye applications in enveloped viruses is limited. Some enveloped viruses, including avian influenza virus, influenza A virus, PEDV, MHV, and SARS-CoV-2, have been recently evaluated [4]. Similarly, almost all enveloped virus studies illustrated a positive trend in the reduction of false positive signals from damaged viruses [15–17], except for the application of vPCR to the avian influenza virus [18]. Depending on the viability reagents employed, the degree of false positive signal reduction varied. However, most studies used laboratory samples like free viral nucleic acids and infectious and inactivated virus suspensions as controls to evaluate the efficacy of their vPCR protocols rather than testing with real-world samples, resulting in an inability to determine the diagnostic performance statistics of a vPCR test by comparing it with a gold standard virus culture and a traditional qPCR [4]. Previous studies used a small sample size of the natural water samples to primarily validate the developed vPCR protocol in discriminating between infectious and non-infectious enteric viruses [15] and SARS-CoV-2 [17]. These studies have demonstrated a decrease in the false positive detection rate when compared to the qPCR assay.

To study the vPCR based on azo dye treatment, an expensive commercial photoactivator device is required in the light activation process by exposing the blue light LED on the azo-treated samples at an optimized time. Light exposure is a critical step for activating the covalent bond of bound intercalating azo dye with free nucleic acids or within damaged capsid particles and for inactivating the residual unbound dye to prevent its binding with nucleic acids after the extraction process [4]. In this study, an economical in-house photoactivator device that generates blue light LEDs has been created instead of using an expensive device and evaluated to determine the optimal conditions for vRT-qPCR with PMAxx and EMA azo dyes in the infectivity determination of SARS-CoV-2 and HCoV-OC43 (SARS-CoV-2 surrogate virus).

In line with previous reviews [5], incubation with PMAxx or PMA derivative resulted in less false positive signal amplification than EMA at all tested concentrations. PMAxx or PMA showed superior results to EMA when testing with PEDV and SARS-CoV-2, 2 coronaviruses [16, 17]. The strength of false positive signal reduction depended on the dose of the dye. Not only the kind of dye and its concentration but also the matrix type of the sample might affect the result because suspended solids and color of complex matrices could interfere with photoactivation of the dye. In this study, an extension of light exposure from 15 min to 30 min compensated matrix effects on dye activity that could improve the vPCR result as previously described [19]. It is necessary to optimize the concentration of azo dye treatment when it is applied to various viruses, as it is likely to be dependent on the target viruses [4]. Although our preliminary data showed that the amplification of free viral RNA at a concentration of 108 genome copies/µl was successfully inhibited by 50 µM PMAxx, this dye concentration failed to suppress RNA amplification of heat-inactivated viruses as reported in recent studies [20, 21]. This might be due to the viral envelope/capsid that impeded dye penetration at low concentration. However, when the highest documented value of 2 mM PMAxx was used in this study [4], it was significantly more effective treatment at the lowest virus titers tested (10^2 pfu/100 µl) compared

to that at the higher virus titers (10^3–10^4 pfu/100 µl). The residual false positive signal level at high viral titers after azo dye treatment depended on the virus inactivation method. Especially UV-C and heating at 65 °C seem to have caused less damage to virus envelope/capsid and, therefore, less dye penetration to reduce false positive signal.

In a routine scenario, a concentration of 2 mM PMAxx might not be cost-effective. Based on the literature, a combination of PMAxx/EMA, PMAxx/surfactant cotreatment, and double light exposure were tested to find better assay conditions. Notably, most of these conditions seemed to have a negative effect on the integrity of infectious virus particles and caused substantial amplification inhibition. The effect of surfactant-assisted vPCR might depend on virus type and surfactant type and concentration. Hong et al [20] reported that 0.005% SDS with 50 µM PMA caused a Cq value difference larger than 9 between infectious and heat-inactivated SARS-CoV-2, while our study used a higher surfactant concentration of 1–3% of Tween 20 or Triton X-100 that was not appropriate for intact virus particles causing the Cq shift to the signal of damaged particles. In this study, a combination of 1 mM PMAxx and 10 µM EMA caused results comparable to those of 2 mM PMAxx with a Cq value difference in the same range as previously reported [20].

To the best of our knowledge, application of viability PCR based on azo dyes to coronaviruses has faced the challenge of incomplete signal suppression from capsid-compromised virions [21, 22]. Recently, platinum compounds such as platinum chloride (IV) ($PtCl_4$) and CDDP have been used as alternative viability reagent for vPCR [4] and shown superior performance when compared to azo dyes in SARS-CoV-2 studies [17, 21, 22]. Conversely, Chen et al([23] reported that PMAxx had better activity than PtCl4 in discriminating infectious and inactivated viruses. Thus, further studies are required to compare the efficacy of these compounds in vPCR assay.

CONCLUSION

In summary, this study revealed the application of an affordable in-house photoactivator device combined with azo dyes for culture-free vRT-qPCR to discriminate infectious and inactive SARS-CoV-2 and HCoV-OC43 under various treatment conditions. Although the optimized conditions were not sufficient for complete amplification inhibition of capsid-damaged viruses at high concentration of azo dye, the observed different Cq values for intact and damaged virions might be sufficient to define cut-off criteria for a valid viability interpretation. Furthermore, future research should compare the efficacy of vPCR between using azo dyes and platinum compounds in laboratory and real-world samples such as clinical or environmental samples. This will help to establish diagnostic performance statistics that could support the plausibility of vPCR application especially in the newly emerging virus diseases to determine the risk of transmission, quarantine policy, and medical treatment.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2024.077.

Acknowledgements: The RNA extraction reagent was provided by Thammasat University Research Unit in Modern Microbiology and Public Health Genomics. This study was supported by Thammasat University Research Fund, Contract No. TUFT 17/2565.

REFERENCES

1. Capraro GA (2022) Replacement of culture with molecular testing for diagnosis infectious diseases. *Clin Lab Med* **42**, 547–555.
2. Carmo A, Pereira-Vaz J, Mota V, Mendes A, Morais C, da Silva AC, Camilo E, Pinto CS, et al (2020) Clearance and persistence of SARS-CoV-2 RNA in patients with COVID-19. *J Med Virol* **92**, 2227–2231.
3. Herrero Hernando C, Álvarez Serra JA, Elizari Saco MJ, Martínez-Nadal S, Vila Cerén C (2020) PCR test for SARS-CoV-2 persistently positive. Virus detection is not always COVID-19. *An Pediatr (Engl Ed)* **93**, 264–265.
4. Canh VD, Liu M, Sangsanont J, Katayama H (2022) Capsid integrity detection of pathogenic viruses in waters: Recent progress and potential future applications. *Sci Total Environ* **827**, 154258.
5. Leifels M, Cheng D, Sozzi E, Shoults DC, Wuertz S, Mongkolsuk S, Sirikanchana K (2021) Capsid integrity quantitative PCR to determine virus infectivity in environmental and food applications: A systematic review. *Water Res X* **11**, 100080.
6. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brünink S, et al (2020) Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* **25**, 2000045.
7. Wan Z, Zhang Y, He Z, Liu J, Lan K, Hu Y, Zhang C (2016) A melting curve-based multiplex RT-qPCR assay for simultaneous detection of four human coronaviruses. *Int J Mol Sci* **17**, 1880.
8. Kim MI, Lee C (2023) Human coronavirus OC43 as a low-risk model to study COVID-19. *Viruses* **15**, 578.
9. DeBiasi RL, Delaney M (2021) Symptomatic and asymptomatic viral shedding in pediatric patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2): Under the surface. *JAMA Pediatr* **175**, 16–18.
10. Wei L, Lin J, Duan X, Huang W, Lu X, Zhou J, Zong Z (2020) Asymptomatic COVID-19 patients can contaminate their surroundings: an environment sampling study. *mSphere* **5**, e00442-20.
11. Romero-Gómez MP, Gómez-Sebastian S, Cendejas-Bueno E, Montero-Vega MD, Mingorance J, García-Rodríguez J, et al (2021) Ct value is not enough to discriminate patients harbouring infective virus. *J Infect* **82**, e35–e37.
12. Elizaquível P, Aznar R, Sánchez G (2014) Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. *J Appl Microbiol* **116**, 1–13.

13. Kim SY, Ko G (2012) Using propidium monoazide to distinguish between viable and nonviable bacteria, MS2 and murine norovirus. *Lett Appl Microbiol* **55**, 182–188.
14. Fittipaldi M, Rodriguez NJ, Codony F, Adrados B, Peñuela GA, Morató J (2010) Discrimination of infectious bacteriophage T4 virus by propidium monoazide real-time PCR. *J Virol Methods* **168**, 228–232.
15. Prevost B, Goulet M, Lucas FS, Joyeux M, Moulin L, Wurtzer S (2016) Viral persistence in surface and drinking water: Suitability of PCR pre-treatment with intercalating dyes. *Water Res* **91**, 68–76.
16. Puente H, Randazzo W, Falcó I, Carvajal A, Sánchez G (2020) Rapid selective detection of potentially infectious porcine epidemic diarrhea coronavirus exposed to heat treatments using viability RT-qPCR. *Front Microbiol* **11**, 1911.
17. Canh VD, Torii S, Yasui M, Kyuwa S, Katayama H (2021) Capsid integrity RT-qPCR for the selective detection of intact SARS-CoV-2 in wastewater. *Sci Total Environ* **791**, 148342.
18. Graiver DA, Saunders SE, Topliff CL, Kelling CL, Bartelt-Hunt SL (2010) Ethidium monoazide does not inhibit RT-PCR amplification of nonviable avian influenza RNA. *J Virol Methods* **164**, 51–54.
19. Shirasaki N, Matsushita T, Matsui Y, Koriki S (2020) Suitability of pepper mild mottle virus as a human enteric virus surrogate for assessing the efficacy of thermal or free-chlorine disinfection processes by using infectivity assays and enhanced viability PCR. *Water Res* **186**, 116409.
20. Hong W, Xiong J, Nyaruaba R, Li J, Muturi E, Liu H, Yu J, Yang H, et al (2021) Rapid determination of infectious SARS-CoV-2 in PCR-positive samples by SDS-PMA assisted RT-qPCR. *Sci Total Environ* **797**, 149085.
21. Cuevas-Ferrando E, Randazzo W, Pérez-Cataluña A, Falcó I, Navarro D, Martin-Latil S, Díaz-Reolid A, Girón-Guzmán I, et al (2021) Platinum chloride-based viability RT-qPCR for SARS-CoV-2 detection in complex samples. *Sci Rep* **11**, 18120.
22. Cuevas-Ferrando E, Girón-Guzmán I, Falcó I, Pérez-Cataluña A, Díaz-Reolid A, Aznar R, Randazzo W, Sánchez G (2022) Discrimination of non-infectious SARS-CoV-2 particles from fomites by viability RT-qPCR. *Environ Res* **203**, 111831.
23. Chen J, Wu X, Sanchez G, Randazzo W (2020) Viability RT-qPCR to detect potentially infectious enteric viruses on heat-processed berries. *Food Control* **107**, 106818.

Appendix A. Supplementary data

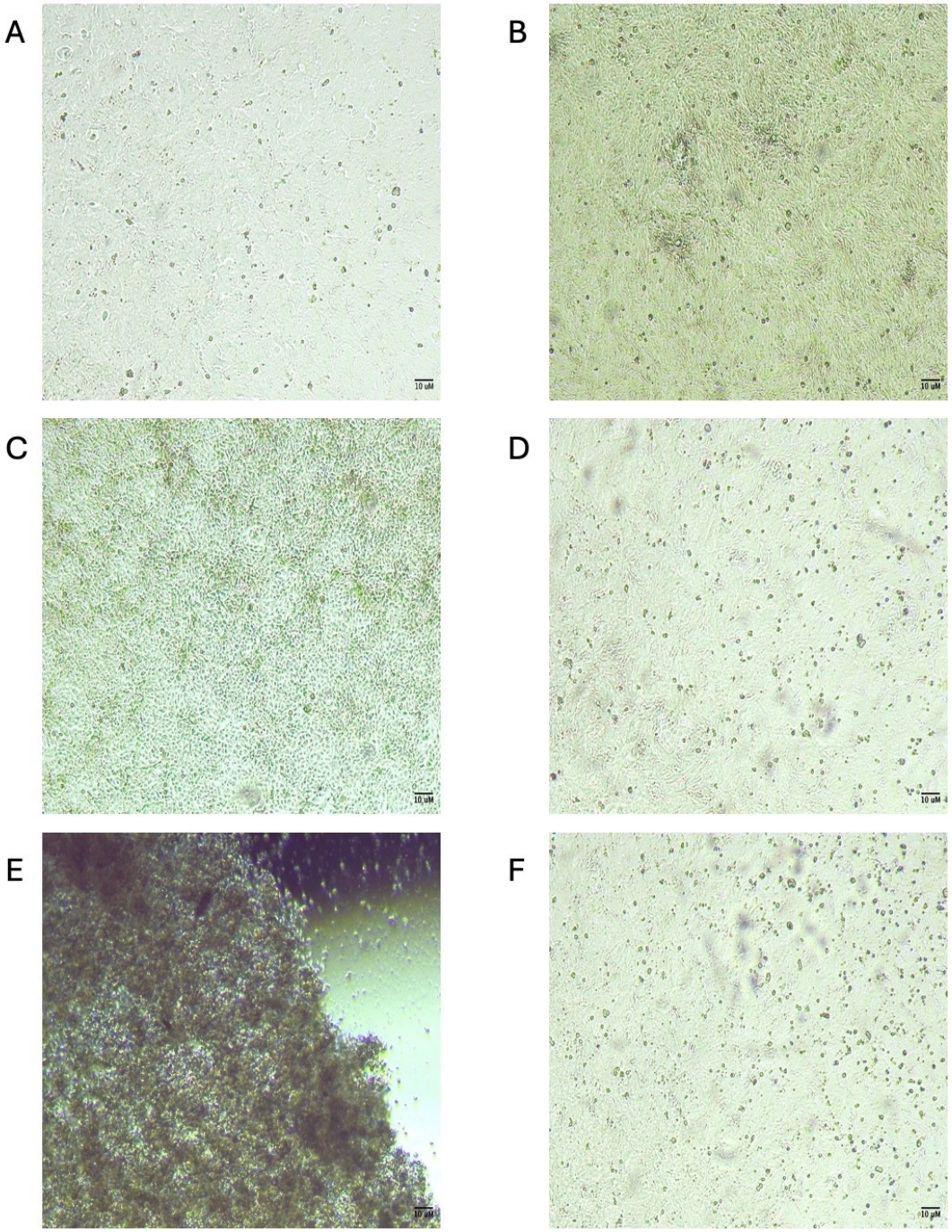

Fig. S1 A representative result of the virus inactivation method confirmation procedure. Inactivated viruses were confirmed through the virus culture. Inoculated cells were observed for the presence of CPE by comparing them with mock-infected cells and virus-infected cells at day 3 under a light microscope (10 × ; scale bar 10 µm). The upper panel showed (A) mock-infected cells and (B) HCoV-OC43 virus-infected HCT-8 cells. The middle panel showed (C) a direct suspension of 70% alcohol-treated HCoV-OC43 and (D) its ten-fold dilution. The lower panel showed (E) a direct suspension of 1% NaOCl-treated HCoV-OC43 and (F) its ten-fold dilution. Dilution was done to reduce the toxicity of chemical treatment to cell culture, and its supernatant was twice blind-subpassaged to confirm that all virus particles were destroyed completely.