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Original Research

Rapidly quantification of intact infectious H1N1 virus using ICA-qPCR and PMA-qPCR

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ABSTRACT

The increase in emerging and reemerging infectious diseases has underscored the need for the prompt monitoring of intact infectious viruses and the quick assessment of their infectivity. However, molecular techniques cannot distinguish between intact infectious and noninfectious viruses. Here, two distinct methodologies have been developed for the expeditious and dependable quantification of intact infectious H1N1 virus, and several experiments have been conducted to substantiate their efficacy. One is an integrated cell absorption quantitative polymerase chain reaction (qPCR) method (ICA-qPCR), and the other is a combined propidium monoazide qPCR method (PMA-qPCR). The quantification limit is 100 cell culture infective dose 50 % ($CCID_{50}$)/mL in ICA-qPCR following a 1.5-hour cell absorption or 126 $CCID_{50}$/mL after a 15-minute incubation. For PMA-qPCR, the limit was 2,512 $CCID_{50}$/mL. The number of genome copies quantified by the ICA-qPCR and PMA-qPCR methods was strongly correlated with the infectious titer determined by the $CCID_{50}$ assay, thereby enabling the estimation of virus infectivity. The ICA-qPCR and PMA-qPCR methods are both suitable for the identification and quantification of intact infectious H1N1 virus in inactivated samples, wastewater, and biological materials. In conclusion, the ICA-qPCR and PMA-qPCR methods have distinct advantages and disadvantages, and can be used to quantify intact infectious viruses rapidly. These methodologies can facilitate the identification of the presence of intact infectious viruses in wastewater or on pathogen-related physical surfaces in high-level biosafety laboratories and medical facilities. Furthermore, these methodologies can also be utilized to detect other highly pathogenic pathogens.

1. Introduction

Human health may be at risk from infectious and pathogenic microorganisms, such as viruses and bacteria, in the environment. The incidence of emerging and reemerging infectious viruses has increased in recent years, suggesting that pathogens may spill over and spread. It is therefore necessary to monitor intact infectious viruses.

Several molecular technologies based on the amplification of virus nucleic acid and immunoassays have been used for rapid pathogen detection [1–3]; however, their certainty is limited because these molecular techniques cannot distinguish between intact infectious viruses and noninfectious viruses. It is possible that the detection of viral nucleic acid was due to the deposition of noninfectious viral material and did not represent a true infection, which would require further confirmation. For example, the environmental contamination associated with two asymptomatic cases among poultry workers in Spain did not result in a genuine infection incident [4]. Nevertheless, the situation caused social unrest prior to the announcement of the test results. It is critical to update technology to monitor intact infectious viruses. Moreover, biosafety laboratories still rely on traditional pathogen culture for biosafety assurance and sample inactivation validation, which increases operating expenses and time requirements. Thus, there is a growing demand for quick and precise methods to measure intact infectious viruses.

Some researchers have made noteworthy discoveries by combining cell absorption or cell culture processes and molecular assays to detect

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HIGHLIGHTS

Scientific question

The increase in emerging and reemerging infectious diseases has underscored the necessity for prompt monitoring of intact infectious viruses and assessment of their infectivity. There is an urgent demand to determine whether rapid quantification methods can accurately indicate intact viral infectivity in a shorter time for practical applications.

Evidence before this study

Prior research has effectively used combined cell absorption or cell culture processes and molecular assays to detect infectious viruses before the cytopathic effect (CPE) is evident. The use of propidium monoazide (PMA) has been effectively demonstrated for distinguishing between active and inactive bacteria and other species.

New findings

Two optimized methodologies were developed: an integrated cell absorption (ICA) quantitative polymerase chain reaction (qPCR) method (ICA-qPCR) and a combined PMA qPCR method (PMA-qPCR). Both methods demonstrated to be useful and adaptable in the rapid quantification of intact infectious virus, achieving quantification limits of 126 cell culture infective dose 50 % ($CCID_{50}$) /mL in ICA-qPCR after 15 minutes of absorption and 2,512 $CCID_{50}$/mL in PMA-qPCR. These methods enable estimation of viral infectivity, applicable in inactivation validation, environmental monitoring, and detection of infectious pathogens.

Significance of the study

ICA-qPCR and PMA-qPCR techniques have the potential to replace traditional culture-based methods for quantifying intact infectious viruses. They can aid in identifying intact infectious viruses in wastewater or on pathogen-related physical surfaces in high-level biosafety laboratories and medical facilities. Additionally, these methodologies can be used to detect other highly pathogenic pathogens.

infectious viruses before the cytopathic effect (CPE) is evident, providing an alternative means of measuring viral infectivity [5–8]. However, in these studies, researchers have opted to utilize cell culture for an extended period to increase the sensitivity of detection. In the event of a higher viral content in a sample, the virus is more likely to be detected through short-term cell absorption [5]. Reduction times are important for the development of techniques to detect intact infectious viruses; this is a prerequisite for the wider application of such techniques. In addition, viability polymerase chain reaction (v-PCR) has demonstrated the effective use of propidium monoazide (PMA) in distinguishing between intact and inactive viruses, including enteric viruses, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), hepatitis A virus, and norovirus [9–12], which was previously used for identifying active bacteria or other species [13]. As a high-affinity photoreactive DNA / RNA-binding dye, PMA can penetrate disrupted virus membranes, bind to nucleic acids, and consequently inhibit template amplification by polymerases [14]. Nevertheless, further investigation is needed to ascertain whether this method can accurately indicate true viral infectivity.

In this study, two methods were developed for the rapid identification and precise titration of intact infectious H1N1 virus: an integrated cell absorption quantitative polymerase chain reaction (qPCR) method (ICA-qPCR) and a combined PMA qPCR method (PMA-qPCR). Moreover, a comparison was conducted between PMA-qPCR and ICA-qPCR to evaluate their performance in practical applications, including in inactivated, wastewater, and biological samples.

2. Methods and materials

2.1. Cell line and virus

The Madin-Darby canine kidney (NBL-2) (MDCK) cell line was cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (BasalMedia, China) supplemented with 10 % fetal bovine serum (FBS) (ExCell Bio, China), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO, USA) at 37 °C with 5 % CO_2.

The study employed the influenza A virus (IAV) H1N1 strain (A/Puerto Rico/8/1934). Additionally, other viral samples were employed in the experiments, including avian H1N1 (A/Wild bird/Heilongjiang/x35YG/2020, A/Wild bird/Hubei/b126/2019, A/Anatidae/Anhui/a516/2020, A/Wild bird/Anhui/a631/2020), H1N2 (A/-duck/Hubei/1654/2016), H1N8 (A/Wild bird/Anhui/a651/2020), and H1N9 (A/Wild bird/Hubei/B386/2017). These samples were kindly provided by Prof. Chai Hongliang from Northeast Forestry University, China.

2.2. Virus sequences and conservative sites analysis

A total of 345 hemagglutinin (HA) sequences of homo sapiens H1N1 viruses and 189 of avian H1N1 viruses were randomly selected from the National Center for Biotechnology Information (NCBI) virus database (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/) (Table S1). After sequence alignment (Lasergene), the conservative sites within the HA were subjected to analysis using advanced bioinformatics tools accessible via https://meme-suite.org/meme/doc/meme.html. Subsequently, specific primers were meticulously designed to target the conserved regions of the H1N1 HA. The forward primer of 5′-GCATATGTTTTTGTGGGG-3′, and the reverse primer of 5′-TGTCCAGTAATAGTTCATC-3′ (also seen in Table S2), were subsequently used in the following study.

2.3. Generation of DNA standards

The target gene sequences of viruses, including H1N1, H3N2, H5N1, H7N9, influenza B virus (IBV), human parainfluenza virus type 1 (HPIV1), and measles virus, were synthesized and inserted into the pUC57 vector (Ampicillin resistance, constructed by Ruibiotech, China). The detail information of target genes and specific primers can be seen in Table S2. The DNA plasmids were extracted using the QIAfilter Plasmid Midi Kit (Qiagen, Germany) and their concentrations were determined using the NanoPhotometer spectrophotometer (IMPLEN, Germany). The external DNA standards were created by progressively diluting the samples in tenfold from 10^{10} to 10^{1} genome copies (GCs)/μL, respectively.

2.4. Standard curve construction and the specificity, sensitivity, and repeatability assays

The standard curve was derived through the analysis of 10-fold series dilutions of DNA standards. The R squared (R^2) of linear regression and the efficiency (E) were evaluated using the following formula [15]:

$$E = \left(10^{-1/slope} - 1\right) \times 100\ \%$$

The specificity assay was performed using the specific HA primers and other DNA templates mentioned in the preceding section. To verify the reproducibility, the assay was repeated in triplicate or more within the same experiment and across different experiments, with the average (AVG), standard deviation (SD), standard error (SE), and relative standard deviation (RSD, also known as the coefficient of variation, CV) calculated. The RSD % (or CV %) of the test was calculated using the following formula [15]:

$$RSD\ \%\ (or\ CV\ \%) = \frac{SD}{AVG} \times 100\ \%$$

2.5. Virus RNA extraction and RT-qPCR

The extraction of viral RNA was carried out using TRIzol (Invitrogen, USA) by the Ultrapure RNA Kit (CWBIO, China), and were inverted to complementary DNA (cDNA) immediately or stored at a frigid −80 °C. The reverse transcription (RT) process was executed using the HiFiScript genomic DNA (gDNA) Removal cDNA Synthesis Kit (CWBIO, China) in accordance with the manufacturer's instructions, within the ProFlex™ PCR system (Applied Biosystems, USA) to facilitate the synthesis of cDNA.

All RT-qPCR reaction were carried out in QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA), coupled with the MagicSYBR Mixture (CWBIO, China). The reaction mixture, composed of 2 × MagicSYBR Mixture, the forward and reverse primers [1 μmol/L (μM) each], cDNA template, and RNase-free water, was assembled to achieve a total volume of 20 μL. The cycling protocol involved pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, annealing/extension at 60 °C for 30 s for 40 cycles, and subsequent melting curve analysis, culminating in an infinite 4 °C hold. The threshold cycle values (Ct, also known as Cq), melting curve, and amplification curve data were obtained using the QuantStudio Design & Analysis software.

2.6. Viral stock preparation and $CCID_{50}$ assay

The MDCK cells, cultivated to achieve a 90 % confluency, were exposed to the IAV at a multiplicity of infection (MOI) of 0.01. Following a two-hour incubation period, the cultured medium was supplemented with 2 % FBS and 2 μg/mL TPCK-treated trypsin (Sigma-Aldrich, Germany). The extensive CPE were observed over a period of 72 h. The resulting cultured virus supernatant was collected. The infectious titer of the virus within the stock was quantified by cell culture infective dose 50 ($CCID_{50}$) assay that was calculated according to the Reed-Muench's method [16].

2.7. ICA-qPCR assay

Approximately 2 × 10^5 MDCK cells were introduced to a pre-infected H1N1 virus in 24-well cell culture plates. The cultured supernatant and infected cells were gathered at various time points following virus infection. The entire monolayer of infected cells was washed three times with phosphate buffered saline (PBS) to remove any lingering virus particles. Viral RNA was extracted and diluted in 30 μL RNase free water, then 500 ng of RNA was immediately inverted into cDNA or stored at −80 °C. The procedures were performed following the protocols outlined in the preceding sections. The relative and absolute nucleoid acid expression of the virus within the cells was calculated respectively. The endogenous control RPL32 was employed, and the viral GCs was quantified using a standard equation developed for this study. Negative controls (NTC) consisted of MDCK cells that were not exposed to the virus. All experiments described above were run in triplicate to assess data variability.

2.8. PMA-qPCR assay

In the pursuit of optimizing the PMA-qPCR assay, the viruses were exposed to varying doses of PMA (Biotium Inc., USA) (25, 50, 100 μM). The incubation period in darkness was spanned 10, 20, and 30 min at 37 °C, and the exposure time under light (80 W) was conducted using intervals of 10, 20, and 30 min, respectively. Each experimental variable (in triplicate) listed above was subjected to RNA extraction and in dilution of 30 μL. The maximum volume (8.5 μL) of RNA was used for RT-qPCR analysis according to the instructions. The viral GCs were calculated based on a standard equation developed for this study. Virus samples lacking PMA treatment were used to establish baselines for the treatment efficacy. In experiments requiring parallel comparison, the same viral infection dose was used for both ICA-qPCR and PMA-qPCR methods.

2.9. Virus inactivation and tested samples preparation

Three distinct means were employed to inactivate viruses in this study. These included autoclaving (AV), which the viruses were subjected to high pressure exceeding 0.1 MPa and a temperature of 121 °C for 30 min, ultraviolet sterilization (UV) at a 254 nm wavelength, 400 mW/m^2, for 30 min, and thermal inactivation (either 56 °C or 70 °C) for 30 min using a digital dry bath.

The mixed samples were contained intact and inactive H1N1 virus, with a volume ratio of 3:7. Incomplete inactivated virus samples were prepared over a range of times, from 0 to 30 min. The viral samples derived from wastewater were prepared, and the recycled samples were subjected to testing after the precipitation process of magnesium- and aluminum-based absorption [17]. The biological viral samples were also tested, the details of which have been previously outlined. All samples were subjected to testing and analysis through the utilization of conventional qPCR, ICA-qPCR, PMA-qPCR and $CCID_{50}$ assays.

2.10. Statistical analysis

The statistical analyses were conducted using GraphPad Prism (version 9) software. ANOVA analysis was used to determine statistical significance, with significance levels denoted as * ($P < 0.1$), ** ($P < 0.01$), *** ($P < 0.005$), and **** ($P < 0.0001$). Results with P values <0.05 were considered significant. The correlation coefficient was assessed using Pearson's r to evaluate the goodness of fit in simple linear regression analysis. Nonlinear regression analysis was also used to assess correlation using R-squared (R^2) and standard deviation of residuals (S, written as Sy.x in the software). The model fits the data better when the value of r or R^2 is closer to 1, while a value of Sy.x is closer to 0 [18]. The 95 % confidence intervals and prediction bands were also calculated. Bland-Altman analysis and paired t-tests were performed to compare the agreement of methods [19]. The mean bias and 95 % limit of agreement (LoA) were evaluated, and the plots should fall within the range of the LoA. A paired t-test with $P > 0.05$ indicates no significant difference between the methods.

3. Results

3.1. RT-qPCR assay demonstrates respectable levels of specificity, sensitivity, and repeatability for H1N1 HA

To quantify the viral loads of the virus accurately, a standard curve for H1N1 was initially developed utilizing DNA plasmid standards and HA-specific primers using RT-qPCR (Fig. 1A-E). A total of 345 HA sequences of homo H1N1 IAV were randomly selected from the NCBI virus database for analysis of their conserved sites. Specific primer

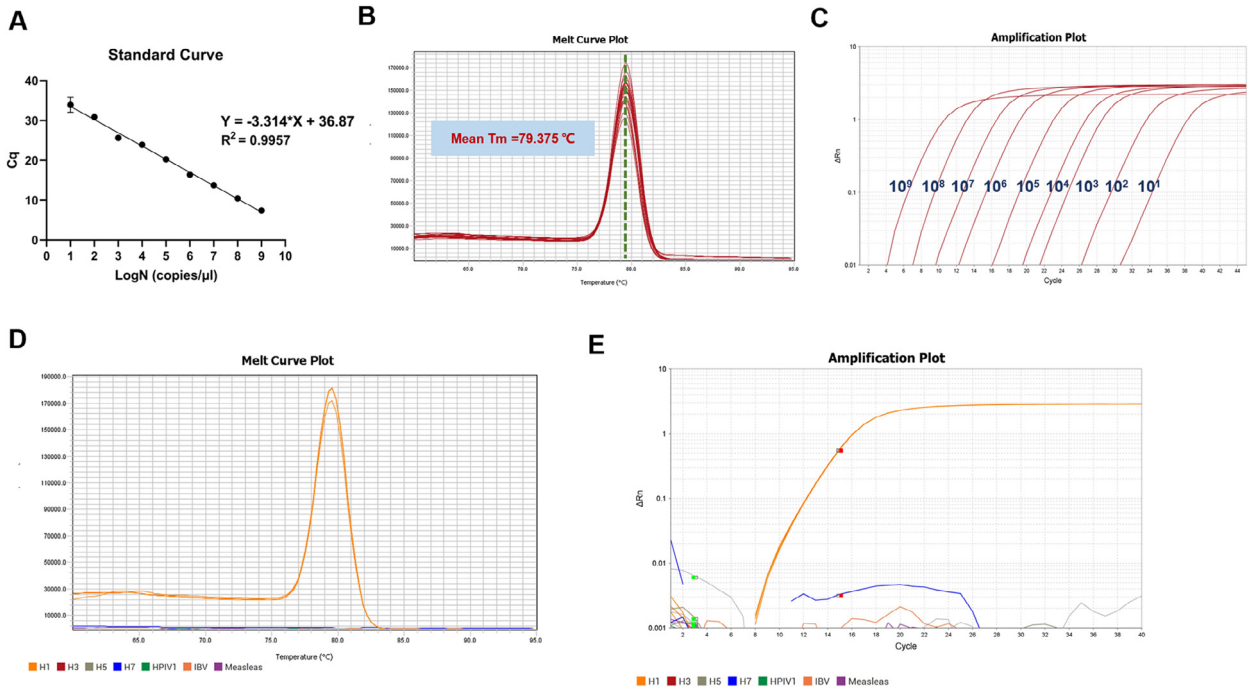

Fig. 1. Specificity and sensitivity assays for HA in RT-qPCR. A) The HA standard curve derived from 10-fold series-diluted DNA standards. The linear equation and R^2 were shown in the graph. B) The melting curves of HA obtained using DNA standards. C) The amplification curves of HA generated using a series of diluted standards. D) The melting curves for the specificity assay utilizing the specific HA primers and other viral DNA templates. E) The amplification curves for the specificity assay. Abbreviations: HA, hemagglutinin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; DNA, deoxyribonucleic acid.

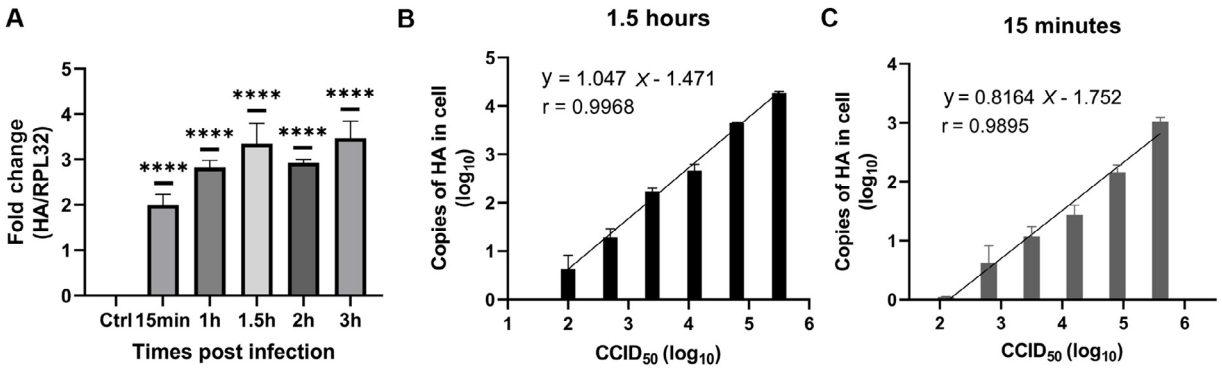

Fig. 2. Intact infectious H1N1 virus can be quantified by ICA-qPCR. A) The relative nucleic acid levels of the H1N1 virus in cells at varying absorption times. Significance levels were denoted as *** ($P < 0.005$) and **** ($P < 0.0001$) when compared to the control group. B) – C) The correlation between infectious titers determined by $CCID_{50}$ assay and genome copies calculated by ICA-qPCR following 1.5 h and 15 minutes of cell incubation. The linear equation and correlation coefficient (Pearson's r) value were presented in the graphs. Abbreviations: $CCID_{50}$, cell culture infective dose 50 %; HA, hemagglutinin; ICA-qPCR, integrated cell absorption quantitative polymerase chain reaction; Ctrl, control.

pairs were subsequently designed based on these sites for use in further studies. The standard curve was determined using the following equation: $y = -3.314x + 36.87$, with $R^2 > 0.99$ (Fig. 1A–C), and the E value was determined to be 100.33 %. The range of quantification was between 10^1 and 10^9 GCs, with a detection limit of 2.51 GCs, which demonstrates excellent detection sensitivity. A specificity assay was conducted, which successfully distinguished the virus from other viruses, including the IAV of the H3, H5, and H7 subtypes, influenza B virus (IBV), human parainfluenza virus type 1 (HPIV1), and measles virus (Fig. 1D, E, and Fig. S1). All of these viruses have genomes that encode functional HA or HA-like proteins. Moreover, a repeatability assay was performed to assess both within-group and intergroup assessment reproducibility by calculating the RSD. The statistical results demonstrated excellent repeatability, with an RSD of less than 5 % (Table S3).

3.2. ICA-qPCR is able to quantify intact infectious H1N1 virus titers in a shorter time

To identify and quantify the infectious viral titer in a shorter time frame, we developed an integrated cell absorption and qPCR method, which we term ICA-qPCR for the H1N1 virus. To determined the optimal time for the collection of intact infectious viruses and explore the relationship with the actual infectious titer, the nucleic acid level of the virus in the infected cells was detected. The results revealed a significant linear correlation between the GCs evaluated using ICA-

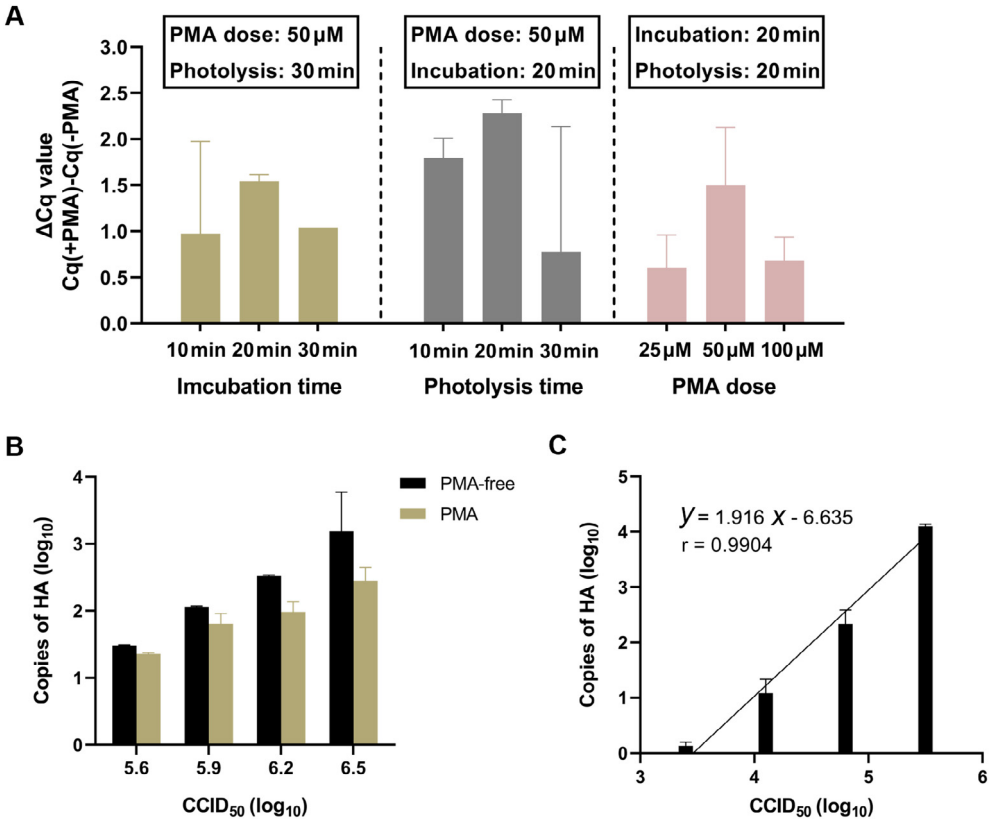

Fig. 3. PMA-qPCR represents a rapid and effective method for quantifying intact infectious H1N1 virus A) The optimal conditions for PMA dosage, incubation time and photoactivation duration in PMA-qPCR assay. B) The comparation of GCs detected by conventional RT-qPCR and PMA-qPCR at various viral titers. C) The correlation between infectious titers determined by $CCID_{50}$ assay and GCs estimated by PMA-qPCR. The linear equation and r value were presented in the graph. Abbreviations: $CCID_{50}$, cell culture infective dose 50 %; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; GCs, genome copies; μM, μmol/L.

qPCR after 1.5 h of inoculation and the infectious titer measured using the $CCID_{50}$ assay, with an equation of $y = 1.047x - 1.471$, $r > 0.99$, $P < 0.05$ (Fig. 2A and B). The limit of detection for ICA-qPCR was determined to be 100 $CCID_{50}$/mL after 1.5 h of cell absorption. Furthermore, we attempted to optimize the ICA-qPCR method by shortening the collection time. Our experiment revealed that intact active H1N1 virus could be detected within 15 min of infection (Fig. 2A and C). The titer evaluated using ICA-qPCR also showed a significant linear correlation with that measured by $CCID_{50}$, although it was weaker than that quantified at 1.5 h. The equation was $y = 0.8164x - 1.752$, with $r > 0.98$ and $P < 0.05$ (Fig. 2C), and the quantification limit reached 126 $CCID_{50}$/mL. Consequently, we have developed the ICA-qPCR method, which is capable of accurately quantifying intact infectious H1N1 virus within a total of three hours, a significantly shorter timeframe than that required by the traditional method.

3.3. PMA-qPCR can be used for the rapid quantification of intact infectious H1N1 virus without cell absorption

Although we established that ICA-qPCR could be utilized for the rapid quantification of intact infectious H1N1 virus titers, the process was contingent upon the availability of sensitive cells that exhibited a high degree of susceptibility to a specific viral strain, as well as the requisite time for cell inoculation. In our subsequent study, we developed and verified an alternative cell-free approach using PMA dye for the accurate quantification of intact infectious H1N1 virus. The optimal reaction system and conditions were determined to be a 50 μM dose of PMA, 20 min of incubation in darkness, and 20 min of photolysis in this study (Fig. 3A). Compared with the untreated H1N1 virus, the intact infectious H1N1 virus treated with PMA could be detected (Fig. 3B). The GCs measured using PMA-qPCR were strongly correlated with the infectious titer measured using the $CCID_{50}$ assay, with an equation of $y = 1.916x - 6.635$, $r > 0.99$, $P < 0.05$ (Fig. 3C). The quantification limit was 2,512 $CCID_{50}$/mL. In conclusion, PMA-qPCR was demonstrated to be an effective method for accurately quantifying intact infectious H1N1 virus, negating the necessity for cell culture and achieving results within a time frame of 2.5 h.

3.4. ICA-qPCR and PMA-qPCR show moderate correlations in the quantification of intact infectious viruses

To further substantiate the efficacy of ICA-qPCR and PMA-qPCR in quantifying intact infectious H1N1 virus titers, reproducibility experiments were conducted. The results of the repeat experiments revealed a consistent and reliable correlation between the GCs quantified using ICA-qPCR or PMA-qPCR and the infectious titers measured by $CCID_{50}$ assays. The coefficient of variation was weak over $\log_{10}$ 4.1 $CCID_{50}$/mL in the ICA-qPCR assays between groups (Fig. 4A and Table 1), and over $\log_{10}$ 4.8 $CCID_{50}$/mL in the PMA-qPCR assays between groups (Fig. 4B and Table 1). Moreover, the GCs obtained through ICA-qPCR and PMA-qPCR exhibited variability when 30 distinct viral samples with different infectious titers were tested. A moderate correlation was observed between the two methods, with $R^2 = 0.7837$ and Sy. x = 0.6163 (Fig. 4C). The mean and median ΔGC values were 1.534 and 1.683, respectively, with a slight variation of 39.86 % (Fig. S2A). Although the ICA-qPCR and PMA-qPCR assays exhibited weak agreement in the Bland-Altman analysis (Fig. 4D), they both demonstrated a satisfactory linear relationship with the infectious titers measured by the $CCID_{50}$ assays (Fig. 2C, Fig. 3C, Fig. 4A, and

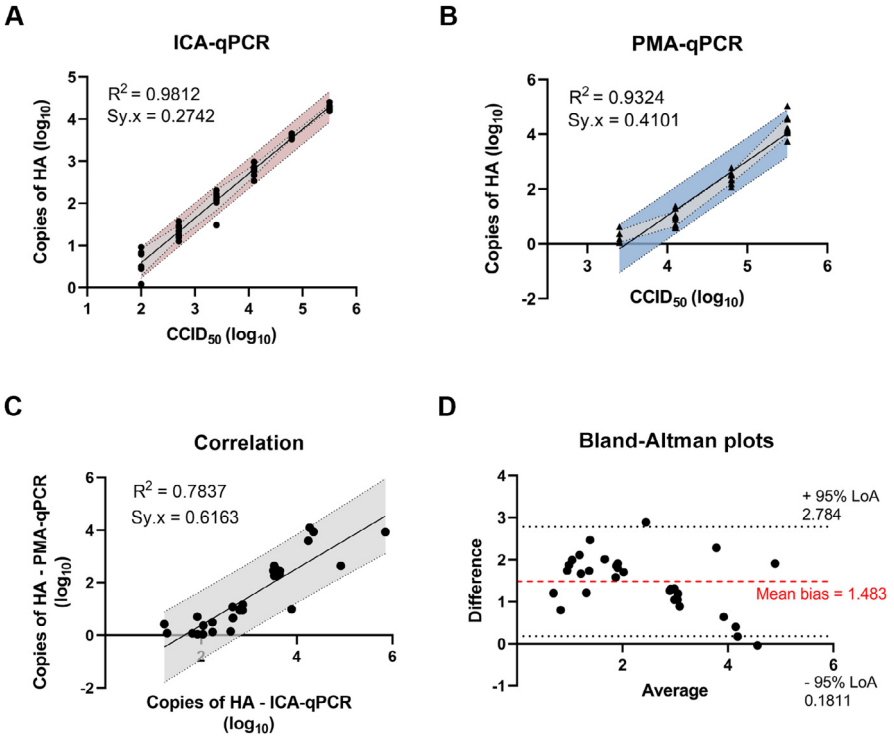

Fig. 4. The moderate correlation was observed between the ICA-qPCR and PMA-qPCR methods. A) – B) Repeatability assay for the correlation between infectious titers determined by the $CCID_{50}$ assay and the estimated GCs determined by the ICA-qPCR and PMA-qPCR methods. The r values were presented in the graphs. C) The correlation between values quantified by ICA-qPCR and PMA-qPCR techniques by measuring 30 samples. The graph displayed the r value and standard deviation of residuals (Sy.x). The 95 % confidence intervals and prediction bands were illustrated in the graphs. D) Bland-Altman plots for quantifying intact infectious H1N1 virus using ICA-qPCR and PMA-qPCR. The red line represented the average of the measurements, while the black line represented the 95 % limits of agreement. Abbreviations: $CCID_{50}$, cell culture infective dose 50 %; ICA, integrated cell absorption; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction; GCs, genome copies; LoA, limits of agreement.

Table 1
The repeatability assay result of ICA-qPCR and PMA-qPCR.

GCs (log_{10})	ICA-qPCR		PMA-qPCR	
	GCs[a] (≥3)	RSD (%)	GCs[a] (≥3)	RSD (%)
2.0	0.60 ± 0.32	53.68	/	/
2.7	1.32 ± 0.16	11.95	/	/
3.4	2.07 ± 0.25	12.14	0.28 ± 0.22	78.35
4.1	2.78 ± 0.13	4.79	0.91 ± 0.27	29.83
4.8	3.59 ± 0.05	1.48	2.42 ± 0.20	8.31
5.5	4.28 ± 0.07	1.54	4.28 ± 0.36	8.40

Abbreviation: GCs, genome copies; SD, standard deviation; RSD, relative standard deviation; ICA, integrated cell absorption; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction.
[a] GCs average ± SD.

B). These results indicated that both techniques can be used to calculate the titers of intact infectious H1N1 virus rapidly and with reasonable efficiency.

3.5. ICA-qPCR and PMA-qPCR are both useful for identifying intact H1N1 virus in inactivation experiments

To evaluate the effectiveness of the ICA-qPCR and PMA-qPCR techniques in quantifying intact infectious viruses, several practical experiments were performed. In virus inactivation experiments, both methods exhibited the capacity to discriminate intact infectious H1N1 virus (Fig. 5A, B, C, and D). Moreover, both the ICA-qPCR and PMA-qPCR techniques demonstrated the ability to distinguish intact infectious H1N1 virus under conditions of incomplete inactivation compared with conventional qPCR detection (Fig. 5C and D). When the H1N1 virus was subjected to UV treatment at 254 nm for 30 min or thermal treatment at 70 °C for 5 min, there was no CPE production (Fig. 5C and D). Additionally, following the confirmation of the H1N1 virus's noninfectious status through $CCID_{50}$ assays, an average of log_{10} 1.37 GCs were detected in heat inactivation experiments, and log_{10} 1.16 GCs were detected in UV-irradiation inactivation experiments using ICA-qPCR methods. The results obtained using PMA-qPCR were significantly greater than those obtained using ICA-qPCR. Thus, the developed ICA-qPCR and PMA-qPCR methods were able to detect the presence of intact infectious H1N1 viruses following the completion of the inactivation treatments. ICA-qPCR demonstrated greater reliability in measuring intact infectious H1N1 viruses under conditions of incomplete inactivation.

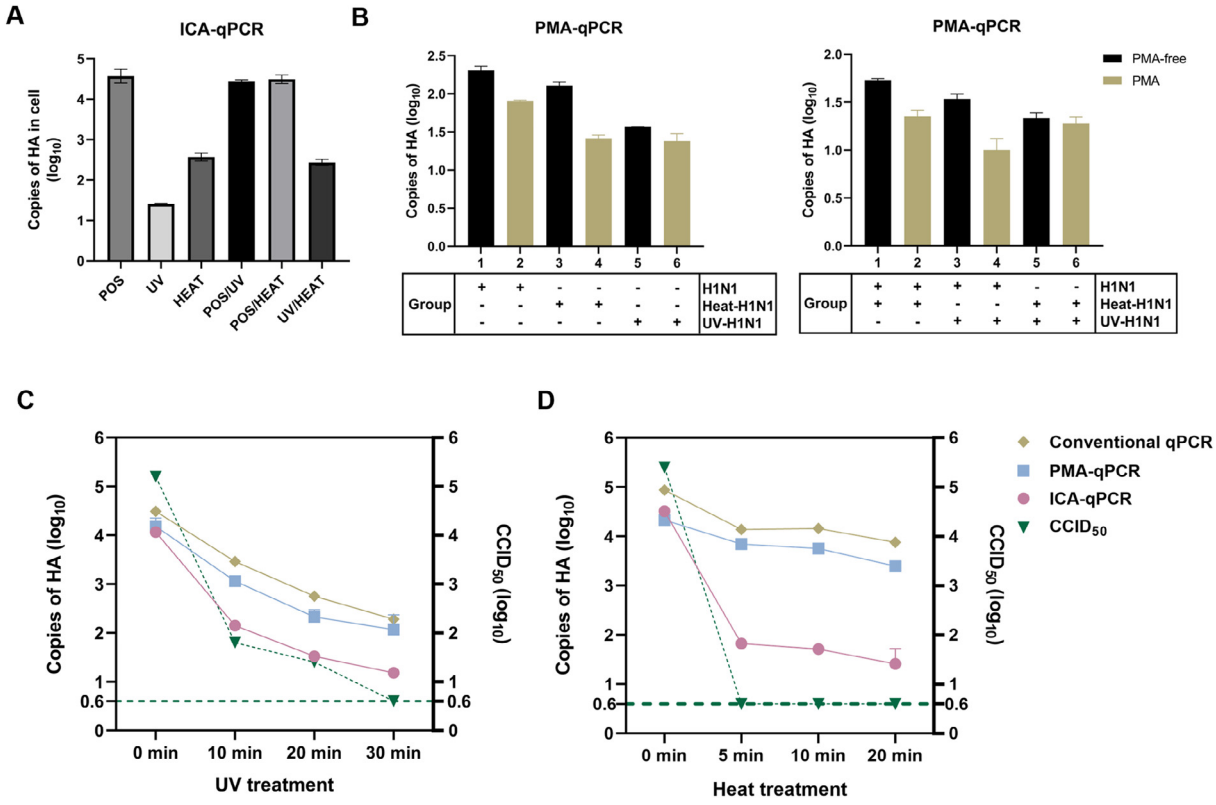

Fig. 5. ICA-qPCR and PMA-qPCR were both capable of distinguishing intact infectious H1N1 virus in inactivation experiments. A) – B) The efficacy of ICA-qPCR and PMA-qPCR in identifying and quantifying intact infectious H1N1 viruses in mixed samples. C) – D) The comparative analysis of the detection efficacy of conventional RT-qPCR, ICA-qPCR, PMA-qPCR, and $CCID_{50}$ assays in quantifying intact infectious H1N1 virus under incomplete UV and thermal inactivation experiments. Abbreviations: $CCID_{50}$, cell culture infective dose 50 %; ICA, integrated cell absorption; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction; RT, reverse transcription; HA, hemagglutinin; UV, ultraviolet sterilization.

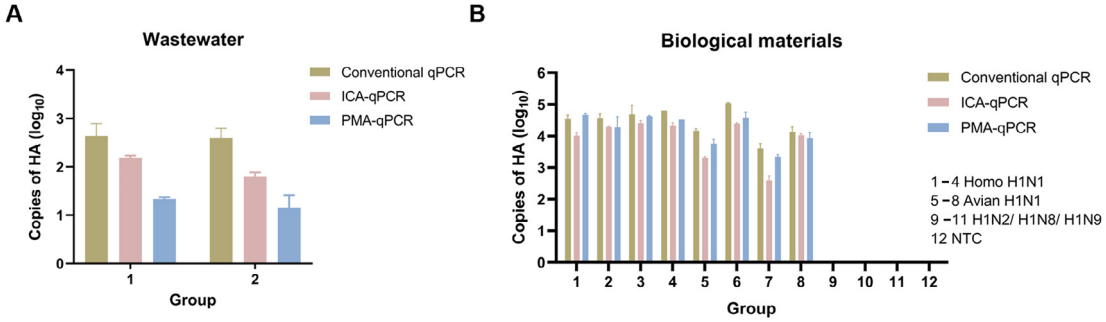

Fig. 6. ICA-qPCR and PMA-qPCR can identify intact infectious H1N1 virus in wastewater samples and biological materials. A) The GCs of intact infectious H1N1 virus in wastewater samples detected using conventional RT-qPCR, ICA-qPCR, and PMA-qPCR. B) The GCs of intact infectious H1N1 virus in 12 unidentified allantoic fluid samples using conventional RT-qPCR, ICA-qPCR, and PMA-qPCR. Samples 1-4 were identified as belonging to the homo sapiens H1N1 virus, samples 5-8 belong to the avian H1N1 virus, while samples 9-11 belong to the H1N2, H1N8, H1N9 respectively, and sample 12 was NTC. Abbreviations: ICA, integrated cell absorption; PMA, propidium monoazide; qPCR, quantitative polymerase chain reaction; GCs, genome copies; HA, hemagglutinin; NTC, negative control.

3.6. ICA-qPCR and PMA-qPCR are suitable for identifying intact infectious pathogens in wastewater and biological materials

In addition to validating the effectiveness of virus inactivation, we utilized ICA-qPCR and PMA-qPCR techniques for wastewater and biological surveillance. In a laboratory setting, pathogen-contaminated water was enriched, and both the ICA-qPCR and PMA-qPCR methods were able to successfully detect intact infectious H1N1 viruses in wastewater samples that had been recycled (Fig. 6A). Moreover, intact infectious H1N1 viruses were identified in 12 previously uncharacterized samples of allantoic fluid. The ICA-qPCR and PMA-qPCR methods exhibited 100 % sensitivity and specificity in identifying and quantifying intact H1N1 infectious viruses from these samples (Fig. 6B). Furthermore, the methodology was shown to be applicable to avian H1N1 viruses, as samples of these viruses were also successfully detected; this was confirmed by the identification of similar conserved sites in 189 HA sequences of avian H1N1 viruses (Fig. S2B). Additionally, our findings revealed that PMA treatment did not influence the capacity of H1N1 virus to infect cells (Fig. S2C), suggesting the potential integration of PMA and cell absorption for quantifying intact infectious viruses. In conclusion, both ICA-qPCR and PMA-qPCR are suitable for the rapid quantification of intact infectious viruses in prac-

Table 2
Comparison of the conventional qPCR, $CCID_{50}$, ICA-qPCR, and PMA-qPCR methods.

Methods	Advantages	Disadvantages
Conventional qPCR	a. It is time-efficient. b. It dose not require additional treatment or conditions.	It is unable to distinguish intact infectious viruses.
$CCID_{50}$	It is the gold standard for measuring the infectious viral titers.	a. Sensitive cell lines are required. b. CPE production is time-consuming, and not all CPE can be observed. c. Impurities and contamination in samples would have a severe effect on the results.
ICA-qPCR	a. It can quickly quantify intact infectious virus. b. It can be done before CPE production. c. Shorter cell culture time can help avoid cell contamination. c. It is effective in various applications, including inactivation validation, environment surveillance, and clinical practices.	a. Sensitive cell lines are required. b. Additional time for cell absorption is necessary. c. The optimal collection time for the specific virus needs to be determined.
PMA-qPCR	a. It can rapidly quantify intact infectious virus. b. It has potential applications in various fields.	a. Pre-treatment for samples is necessary. b. It might perform poorly in incomplete thermal inactivation of enveloped viruses.

Abbreviations: qPCR, quantitative polymerase chain reaction; $CCID_{50}$, cell culture infective dose 50 %; ICA, integrated cell absorption; PMA, propidium monoazide; CPE, cytopathic effect.

tical scenarios and can be further applied to wastewater or pathogen-related physical surfaces in high-level biosafety laboratories or medical facilities.

4. Discussion

One of the main challenges in public health is the prompt assessment of the risks associated with virus infectivity to effectively control viral transmission. Many researchers have focused their efforts on this issue [1–3,20–22]. Nevertheless, a positive result on a molecular test for a virus does not necessarily indicate that the virus is fully infectious. Virus infectivity is achieved when the virus has a complete structure. A transmissible infectious viral particle typically comprises nucleic acids (either DNA or RNA) in its core and proteins in its outer shell, some of which are encased in an outer membrane [23,24]. Some virus inactivation methods can disrupt a virus's protein structure and cleave its nucleic acid [25,26]. Solely relying on molecular techniques is insufficient for the precise recognition of intact infectious viruses. In this study, we successfully developed ICA-qPCR and PMA-qPCR methods for the rapid titration of intact infectious H1N1 viruses. Both the copy number and the viral infectious titer are related to the amount of virus present in the sample, yet they are distinct in terms of their meanings and methods of quantification. GCs represent the number of target genomes integrated into the genome of the cells, whereas the viral titer denotes the concentration of infectious viral particles present in the sample. In the field of gene delivery in cell and gene therapy, the quantification of the vector copy number (VCN) using qPCR and dPCR can assist in determining the concentration of infectious viral particles in a sample [27,28]. A strong correlation was observed between the number of genome copies quantified by the methodologies developed in this study and the infectious titer determined by the conventional $CCID_{50}$ assay, allowing us to estimate the infectivity of the viruses.

Infectious viruses can enter sensitive cells through the process of absorption. The integrated cell absorption and qPCR assay method represents an effective approach for rapidly measuring viral titers prior to the onset of CPE or in instances where CPE is not observed. This technique has been utilized to validate virus inactivation, and to detect pathogens in water, including pseudorabies virus, enteric virus, etc [5–8,29]. However, the aforementioned research also required several hours for cell absorption and culture. This reduction is highly beneficial for the development of techniques to detect intact infectious viruses, which is a prerequisite for the wider application of such techniques. In our study, a 15-minute cell absorption period was sufficient to estimate the titers of the H1N1 virus, due to the strong correlation with infectious titers measured by the $CCID_{50}$ assay. However, the detection efficiency of ICA-qPCR is markedly diminished in the absence of a suitable cell line for the target virus. Accordingly, a cell-free methodology is essential for the development of an infectious virus quantification method. One such method commonly used for quantifying viable bacteria is v-PCR [30–33], which uses photoactivatable PMA dye that can penetrate compromised membranes and bind to nucleic acid with high affinity. The combination of molecular techniques with PMA dye also has the potential to quantitatively detect intact infectious viruses [10,11,34], including those that are challenging to culture in cells, such as norovirus [35]. However, A. Okada et al. [36] reported that the detection rates of the PMA-qPCR and culture methods were not always consistent, indicating that there is still significant room for improvement in the PMA-qPCR technique. The discrepancy between the Ct values obtained by qPCR and those obtained by PMA-qPCR is an important parameter for discerning membrane integrity initially [37,38]. These differences can be used to investigate the efficacy of PMA treatment. However, the high slope value observed between the GCs quantified by PMA-qPCR and the infectious titer measured by $CCID_{50}$ assay may be attributed to the presence of a greater number of noninfectious viruses that are not discernible through PMA treatment. This also provides an explanation for the correlation observed between GCs measured by ICA-qPCR and PMA-qPCR, respectively. Further investigation is necessary to determine whether the PMA-qPCR method can accurately indicate true viral infectivity. Our study demonstrated that both ICA-qPCR and PMA-qPCR represent effective alternatives to traditional methods for quantifying intact infectious H1N1 viruses and estimating their infectivity. Although there was no strong correlation or agreement between the ICA-qPCR and PMA-qPCR methods, they both demonstrated a satisfactory linear relationship with the infectious titers determined by the $CCID_{50}$ assay.

In practical applications, both ICA-qPCR and PMA-qPCR can differentiate between intact infectious H1N1 virus and inactive virus in mixed samples. The efficient inactivation of viruses is of critical importance in many fields, including environmental monitoring, food hygiene, and laboratory biosafety. IAV is an enveloped virus with a segmented, negative-sense RNA genome [39]. Previous studies have indicated that elevated temperatures can cause light damage to the nucleic acid and envelope of the virus, and severe disruption to the capsid [25]. Complete thermal inactivation of the H1N1 virus is reported to occur at temperatures exceeding 70 °C for 5 min [40]. In instances of incomplete thermal inactivation, the outer membrane of the virus is only partially damaged [40], rendering PMA unable to enter and bind to the nucleic acid. The less severe destruction of the envelope may explain why ICA-qPCR is more effective than PMA-qPCR in measuring intact infectious viruses under incomplete thermal inactivation, as observed in our study. Additionally, the presence of impurities in the samples may have directly inhibited the PCR, and culturing cells, until CPE production occurred, was challenging due to contamination by other pathogens in the water and biological samples. For the detection of wastewater and biological materials, ICA-qPCR

and PMA-qPCR have emerged as valuable and expedient tool for the identification and quantification of intact infectious viruses.

Consequently, we have assembled a comprehensive overview of the relative advantages and disadvantages associated with the conventional qPCR, $CCID_{50}$, ICA-qPCR, and PMA-qPCR techniques (Table 2); this suggests that optimal rapid quantitative assays for intact infectious viruses should be selected based on the specific research objectives and the inherent limitations of the assay. Furthermore, the ICA-qPCR and PMA-qPCR techniques can be utilized to detect additional pathogens, not just H1N1 virus. To further substantiate the efficacy and suitability of these two techniques for quantifying intact infectious viruses, additional research involving more subjects is needed. Moreover, additional research is necessary to confirm the wider range of applications and develop a platform for quickly identifying intact infectious viruses.

5. Conclusion

In summary, our study validated two rapid and precise techniques, namely, ICA-qPCR and PMA-qPCR, for the titration of intact infectious H1N1 virus. The two approaches were demonstrated to be both useful and adaptable. The quantification limit was 100 $CCID_{50}$/mL in ICA-qPCR following a 1.5-hour cell absorption or 126 $CCID_{50}$/mL after a 15-minute incubation. For PMA-qPCR, the limit was 2,512 $CCID_{50}$/mL. The number of genome copies quantified by the ICA-qPCR and PMA-qPCR methods was strongly correlated with the infectious titer determined by the $CCID_{50}$ assay, thereby enabling the estimation of virus infectivity. PMA-qPCR does not require the use of sensitive cell lines, whereas ICA-qPCR is more sensitive. These methods can be effectively utilized in a variety of fields, including the validation of inactivation procedures, environmental monitoring, and the detection of infectious pathogens. Rapid techniques for the quantification of intact infectious viruses have the potential to supplant traditional culture-based methods. These techniques can rapidly identify intact infectious viruses in critical locations, such as high-level biosafety laboratories and medical facilities. This provides compelling and substantial technical validation for the improvement and dissemination of intact virus identification and quantification techniques, thereby enhancing global biosafety surveillance and detection efforts. Furthermore, these methodologies can be utilized to detect additional pathogens, thereby facilitating the monitoring of potential pathogens with high pathogenicity.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

Chudan Liang: Writing – original draft, Investigation, Formal analysis. **Zequn Wang:** Software, Resources, Investigation. **Linjin Fan:** Resources, Investigation. **Yulong Wang:** Resources, Investigation. **Yuandong Zhou:** Investigation. **Xiaofeng Yang:** Investigation. **Jingyan Lin:** Resources. **Pengfei Ye:** Resources. **Wendi Shi:** Resources. **Hongxin Huang:** Resources. **Huijun Yan:** Resources. **Linna Liu:** Writing – review & editing, Supervision, Conceptualization. **Jun Qian:** Writing – review & editing, Supervision, Conceptualization.

Supplementary data

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