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Portable, single nucleotide polymorphism-specific duplex assay for virus surveillance in wastewater

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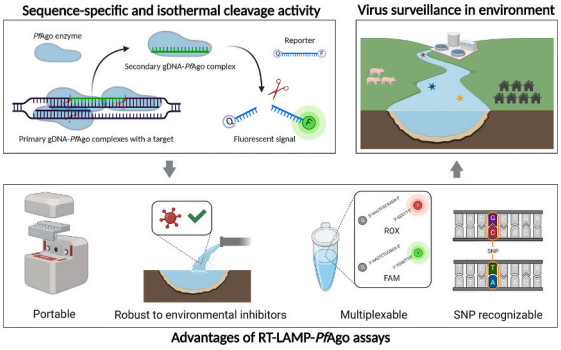
HIGHLIGHTS

- RT-LAMP-*Pf*Ago assays were evaluated for virus detection in wastewater.
- The novel assays are multiplexable and single nucleotide polymorphism-specific.
- No dilution is required for the novel assays to avoid environmental inhibitors.
- The assays are compatible with a portable device, allowing on-site applications.

GRAPHICAL ABSTRACT



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ABSTRACT

The Argonaute protein from the archaeon *Pyrococcus furiosus* (*Pf*Ago) is a DNA-guided nuclease that targets DNA with any sequence. We designed a virus detection assay in which the *Pf*Ago enzyme cleaves the reporter probe, thus generating fluorescent signals when amplicons from a reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay contain target sequences. We confirmed that the RT-LAMP-*Pf*Ago assay for the SARS-CoV-2 Delta variant produced significantly higher fluorescent signals ($p < 0.001$) when a single nucleotide polymorphism (SNP), exclusive to the Delta variant, was present, compared to the samples without the SNP. Additionally, the duplex assay for Pepper mild mottle virus (PMMOV) and SARS-CoV-2 detection produced specific fluorescent signals (FAM or ROX) only when the corresponding sequences were present. Furthermore, the RT-LAMP-*Pf*Ago assay does not require dilution to reduce the impact of environmental inhibitors. The limit of detection of the PMMOV assay, determined with 30 wastewater samples, was 28 gc/μL, with a 95 % confidence interval of [11,103]. Finally, using a point-of-use device, the RT-LAMP-*Pf*Ago assay successfully detected

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PMMOV in wastewater samples. Based on our findings, we conclude that the RT-LAMP-*Pf*Ago assay can be used as a portable, SNP-specific duplex assay, which will significantly improve virus surveillance in wastewater.

1. Introduction

Viral pathogens are shed by infected individuals and can be transported via sewage systems, eventually merging at the wastewater treatment plants (Simmons and Xagoraraki, 2011). The effluent discharged from these treatment plants and viral pathogens from non-point sources flow directly into natural water environments such as rivers, groundwaters, lakes, and oceans (Fong et al., 2010; Xagoraraki et al., 2007). Monitoring these viruses in the water environment is essential as it provides valuable information from two public health perspectives. First, it can be used to understand the disease prevalence in various community settings, such as cities, neighborhoods, universities, and nursing homes (Davó et al., 2021; Gibas et al., 2021; Oh et al., 2022c). Wastewater-based epidemiology (WBE), which involves monitoring viruses in wastewater using targeted viral genes, has been used to conduct disease surveillance for various viruses, such as SARS-CoV-2, Mpox, Influenza, and Respiratory Syncytial Virus (RSV) (Boehm et al., 2023). Second, monitoring viruses in the environment allows us to assess the risk of viral infection to their host species, including humans. Viral pathogens in water are crucial in assessing the risks of human exposure, especially when people inadvertently come into contact with pathogens in the water. For instance, the risk of viral infection through exposure to recreational and irrigation water has been determined to impact public health significantly (Fuzawa et al., 2020; Kundu et al., 2013).

One of the challenges in virus monitoring is the reliance on PCR-based assays, which utilize a thermal cycling process to amplify and detect organism-specific sequences. These assays typically require real-time, digital, or droplet digital PCR systems, which are among the most expensive instruments used in virus monitoring. As a result, only advanced laboratory facilities can afford to equip themselves with these systems. Consequently, the transportation of samples from monitoring sites to centralized laboratories can incur high costs and result in longer turnaround times (Daigle et al., 2022; Mackul'ak et al., 2021; Yang et al., 2017). Moreover, monitoring viruses in resource-limited regions, such as rural areas or low-income countries, has not been widely implemented (Medina et al., 2022). Therefore, there is a need for alternative assays that can operate in laboratories with limited resources or even on-site to expand virus monitoring capacity and ultimately enhance public health.

Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) assays have been adopted as alternatives to PCR-based assays. This is because these alternative assays utilize strand-displacing polymerase enzymes and amplify target genomic materials at a constant temperature without the need for thermocyclers. These alternative assays have been used to monitor viruses in the environment. For example, Yang et al. (2014) developed a LAMP assay to detect human astrovirus in reclaimed water, and Rames and Macdonald (2019) designed an RPA assay to detect human adenovirus in wastewater. However, both LAMP and RPA assays suffer from nonspecific and nontemplate amplification under isothermal conditions, leading to false positive results (Becherer et al., 2020; Da Silva et al., 2019; Kim et al., 2023; Patchsung et al., 2020; Wang et al., 2021). The low specificity significantly limits the application of these assays in viral pathogen surveillance (Kang et al., 2022; Schneider et al., 2019; Wang et al., 2017).

The clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated (Cas) system belong to a bacterial immune system that cleaves specific sequences of viral genomes upon recognition of viral invasion (Broughton et al., 2020; Gootenberg et al., 2017; Kaminski et al., 2021). Due to the sequence-specific properties, a CRISPR-Cas system has been employed in isothermal amplification assays, such as LAMP and RPA, to reduce false positive rates of virus detection (Cao et al., 2022; Liu et al., 2022). Although CRISPR improves specificity and is a simple assay, the CRISPR-Cas system has unique features to consider when designing a virus detection assay. First, the recognition of the CRISPR/Cas12a complex to the target genome is PAM-dependent, which limits the possible target sequences. SNPs detection is necessary for variant-specific virus detection. Oh et al. (2022b, 2023) found that specific SNPs should be targeted for specific variant detection, further narrowing down the candidate SNPs. Second, Cas12a's collateral cleavage activity is a non-sequence-specific reaction. In other words, Cas12a cleaves any single-stranded DNA (ssDNA), and Cas13a cleaves any single-stranded RNA (ssRNA) nearby regardless of sequences upon recognizing the on-target site, so it is difficult to perform an one-enzyme-based multiplex assay and thus hard to design a multiplexable one-pot assay. Virus surveillance in the environment requires multiple target monitoring for quality assurance. For example, pepper mild mottle virus (PMMOV) and phages have been measured together with SARS-CoV-2 as an internal control. These features could significantly limit the application of CRISPR assays to monitor viruses in the environment.

The Argonaute protein from the archaeon *Pyrococcus furiosus* (*Pf*Ago) is another enzyme that potentially defends the host from invasion. *Pf*Ago is a nuclease that utilizes 5′-phosphorylated ssDNA guides (gDNA) to cleave complementary ssDNA targets (Swarts et al., 2015). *Pf*Ago has unique features that differentiate it from the CRISPR systems. First, gDNA-*Pf*Ago complexes can bind to any target sequences based on their gDNA sequences, allowing them to target SNPs of virus mutation. Second, *Pf*Ago stepwise cleavage activity is sequence-specific, allowing a specific cleavage on probes when a corresponding unique sequence is present. This feature allows the assay to include multiple reporters with different dyes, which is necessary for multiplexable assay design. These features have been utilized to design an assay to detect viruses and proved multiplexability and SNP detection with clinical samples (Xun et al., 2021; Ye et al., 2022). Although *Pf*Ago has unique features that make it useful for environmental surveillance systems, it has not been applied to environmental samples.

This study aimed to apply the *Pf*Ago enzyme to design a novel assay for virus detection in the environment. We designed a reverse transcriptase (RT)-LAMP-*Pf*Ago assay where *Pf*Ago assay generates fluorescent signals upon from the target DNA was amplified by RT-LAMP assay. We chose the RT-LAMP assay because it generates the highest yield of amplicons through an isothermal reaction, potentially improving the limit of detection (LOD) for the *Pf*Ago assay (Oliveira et al., 2021). This assay has been systematically evaluated with synthetic controls of PMMOV, generic SARS-CoV-2, and the Delta variant of SARS-CoV-2 and 30 wastewater samples. We demonstrated that this RT-LAMP-*Pf*Ago assay can be a duplex assay that is variant-specific and robust to inhibitors in the environment. In addition, we proved that the assay is performed in a single tube and is compatible with a lab-made portable device, which can make it a point-of-use assay that can be operated in a decent laboratory or even in the field. These portable and SNP-specific duplex assays will improve environmental surveillance capacity, especially for viruses spreading through the environment.

2. Materials and methods

2.1. Wastewater sample collection and process

We collected 30 wastewater samples from five locations: four manholes receiving neighborhood-scale sewage (about 1000 residents) and one from the meat-packing industry from January 2021 to December

2021 (Table S1). All samples were obtained using an autosampler (Teledyne ISCO, USA), programmed to collect a 1 to 2 L composite sample comprised of samples pumped for about four days. The composite samples were transferred to sterile sampling bags (14–955-001, Fisher Scientific, USA), and 20 mL of 2.5 M $MgCl_2$ was added to the samples (i.e., final $MgCl_2$ concentrations were from 25 to 50 mM) to coagulate solids including virus particles (Ahmed et al., 2020; Oh et al., 2022a). The samples were transported on ice to a laboratory at the University of Illinois Urbana-Champaign within three hours. Upon arrival at the laboratory, the composite samples stayed on a bench for about 30 min to settle wastewater solids. Next, the supernatant from each composite sample was discarded. The resultant 35 mL of solid suspension was transferred to a 50 mL tube (12–565-271, Fisher Scientific, USA). The sewage samples were centrifuged at 10,000*g* for 30 min (Sorvall™ RC 6 Plus, Thermo Scientific, USA). Note that virus settlement under these centrifugation conditions is negligible (Oh et al., 2020). Supernatants were discarded again, and a portion of the concentrated sludge (100 μL) was transferred to a sterile 1.5 mL tube (1415–2600, USA Scientific, USA). Viral nucleic acid is known to be enriched in wastewater solids compared to liquid wastewater (Wolfe et al., 2021), and as a result, the sludge produced in this step provides concentrated viral nucleic acids. Nucleic acids were extracted from the sludge with QIAamp Viral RNA mini kits (Qiagen, Germany) following the manufacturer's procedure. This extraction kit includes two washing steps to reduce the impact of potential inhibitors on downstream molecular analysis. Sewage collection and processing were conducted on the same day, and the aliquots of RNA samples were stored in separate cryogenic tubes at −80 °C for a maximum of two years. We analyzed the RNA extracts for PMMOV RNA after two different storage periods: one aliquot within a week and the other after approximately two years. Our results indicate that there was no significant difference in PMMOV RNA concentrations between these two analyses ($p > 0.05$ from a paired *t*-test), suggesting that RNA in the samples did not undergo significant degradation during the two-year storage period at −80 °C (Fig. S1).

2.2. Taqman reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay to determine PMMOV RNA concentrations of the wastewater samples

The Taqman-based RT-qPCR started with mixing 5 μL of RNA sample, 5 μL of Taqman Fast Virus 1-step Master Mix (Applied Biosystems, USA), 1 μL of primers/probe mixture (i.e., final concentrations of 400 nM for primers and 200 nM for probes), and 9 μL of nuclease-free water. The RNA samples from the wastewater were diluted 10-fold before the analysis to avoid the impact of inhibitors on quantification (Oh et al., 2022c). The information on primers and probes for PMMOV analysis is summarized in Table S2. The PCR cocktail was placed in 96-well plates (Applied Biosystems, USA) and analyzed by QuantStudio 3 (Thermofisher, USA) with a thermal cycle of 5 min at 50 °C, 20 s at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The cycles of quantification (Cq) were determined by QuantStudio Design & Analysis Software (v1.5.1). For every RT-qPCR assay, at least three replicates were analyzed for serial dilution of synthetic DNA (for a standard curve and a positive control), nuclease-free water (as a negative control), and samples. All positive samples (synthetic DNA or RNA controls) were amplified and negative samples (nuclease-free water) were not amplified in all RT-qPCR analyses. The linear dynamic range for the serial dilutions of synthetic DNA was between 10^0 and 10^5 gene copies (gc)/μL. The PCR efficiencies for RT-qPCR were higher than 85 % ($R^2 > 0.99$). For these RT-qPCR assays, the concentration at which the positive rate was 0.95, which was defined as the limit of detection (LOD), was 1.6 gc/μL, and the lowest concentration with a coefficient of variation (CV) of <35 %, which was defined as the limit of quantification (LOQ) was 12.6 gc/μL (Oh et al., 2022c). The details for RT-qPCR assays are summarized in Table S3 following MIQE guidelines (Bustin et al., 2009).

2.3. PfAgo expression and purification

*Pf*Ago expression and purification were performed using a previously mentioned protocol (Enghiad and Zhao, 2017). The *Pf*Ago gene was cloned into the pET28a plasmid, which was transformed into *Escherichia coli* KRX (Promega, USA). This *E. coli* strain was cultivated overnight at 37 °C in LB medium supplemented with 0.4 % (*w/v*) glucose and 50 μg/mL kanamycin. Following overnight incubation, this culture was centrifuged at 3220 *g* for 5 min, and the supernatant was removed. The cell pellets were resuspended in Terrific Broth containing 50 μg/mL kanamycin and incubated at 37 °C until the OD600 reached 1.0–1.5. The culture was then cold-shocked by incubation on ice for 15 min, protein expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) and L-rhamnose to final concentrations of 1 mM and 0.1 % (w/v), respectively. The mixture was further incubated at 30 °C at 300 rpm for 20 h. The mixture was centrifuged at 5000 rpm for 10 min (4 °C), and the supernatant was discarded. The cells at the bottom were resuspended with 25 mL of 20 mM Tris-HCl buffer (pH 8.0 and 1 M NaCl) and sonicated (5 s pulses at 60 % power with 10 s pause between pulses) to denature cell membranes. After the sonication step, the cells were pelleted at 20,000 rpm for 30 min at 4 °C, and the supernatant was purified using the strep-tag system (Swarts et al., 2015). The purified protein was concentrated using Amicon Ultra centrifuge filters (<30 kDa). Finally, the purified protein sample was run on SDS-PAGE, and the resolved bands were shown in our previous work (Xun et al., 2021). The purified protein was stored in a storage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 40 % (*v*/v) glycerol), and the aliquots were stored at −80 °C before use.

2.4. RT-LAMP-PfAgo assay

An RT-LAMP-*Pf*Ago assay is a combination of two assays: an RT-LAMP assay amplifying a target genome to improve assay sensitivity and a *Pf*Ago assay generating fluorescent signals to specifically detect target sequences. The RT-LAMP-*Pf*Ago assay was conducted either in a one-step or two-step manner. For the two-step RT-LAMP-*Pf*Ago assay, the RT-LAMP and *Pf*Ago reactions occurred sequentially. First, the RT-LAMP reagent mixture includes 12.5 μL of WarmStart® LAMP Kit (E1700S, New England Biolabs, USA), 2.5 μL of a 10× primer mixture prepared according to the manufacturer's recommendation (16 μM for FIP and BIP, 2 μM for F3 and B3, and 4 μM for LF and LB), 5 μL of a sample, and nuclease-free water (W4502, Millipore Sigma, USA) to make up the solution volume to 25 μL in a 200 μL PCR tube. The samples include a synthetic DNA control for PMMOV (Table S2) and synthetic RNA controls for wildtype SARS-CoV-2 and the Delta variant (TWIST Bioscience, USA; part numbers 102,024 and 104,533, respectively). The reaction was incubated at 65 °C for 75 min. A set of six LAMP primers were designed using the NEB LAMP Primer Design Tool. The length between the F1 and B1c primers exceeded 60 bp to ensure the target sequence was present in all shapes of LAMP amplicons (Table S4). Second, the *Pf*Ago reagent was added to the product from the RT-LAMP assay. The *Pf*Ago assay reagent contains 0.625 μL of 100 μM gDNA1, gDNA2, and gDNA3 each, 1.563 μL of 10 μM probe, 2.11 μL of 33 μM *Pf*Ago enzyme, 2 μL of 100 mM $MgSO_4$, and nuclease-free water (W4502, Millipore Sigma, USA) to fill up the reaction to 25 μL. The mixture was incubated at 95 °C for 15 min for the stepwise cleavage activity of *Pf*Ago enzymes to produce fluorescence signals from the target sequences. The fluorescent signals were measured by QuantStudio 3 (Thermofisher, USA). Information about gDNA and reporters for *Pf*Ago assays and primers for RT-LAMP assays is presented in Table S4.

The one-step RT-LAMP-*Pf*Ago assay consists of three layers of reagents in a 200 μL PCR tube arranged in the following order from bottom to top: 25 μL of *Pf*Ago reagent mixture, 30 μL of paraffin wax, and 25 μL of RT-LAMP reagent mixture. The constituents of RT-LAMP and *Pf*Ago reagents were the same as what was used for the two-step assays. These three layers of reagents in a PCR tube were incubated at 65 °C for 75

min, followed by 95 °C for 15 min. At the first incubation at 65 °C, the RT-LAMP assay synthetizes dsDNA from the target genome. During the initial incubation at 65 °C, the RT-LAMP reagent amplifies the target nucleic acids, while the solidified wax physically separates the RT-LAMP reagent and *Pf*Ago reagent. This separation was designed based on the finding that the RT-LAMP reaction is inhibited by the *Pf*Ago enzyme (Xun et al., 2021). When the temperature increases to 95 °C, the wax melts, and the *Pf*Ago reagent can cleave the target nucleic acid if the LAMP successfully amplifies the target genome. This one-pot assay was used to detect PMMOV in sewage or groundwater using the RT-LAMP-*Pf*Ago assay in a portable and simple manner. We developed a portable device comprising 3D-printed structural parts, a machined copper heat block, optical filters for FAM and ROX, and electrical components for the operation of the RT-LAMP-*Pf*Ago assay (Xun et al., 2021). We determined the coefficient of variation (CV) of the lab-made portable device using the measurements from this study, which were 8.9 ± 8.4 %. The CV of the lab-made portable device was not significantly different from that of the qPCR instrument (6.8 ± 4.7 %), showing $p = 0.52$ by the two-sample *t*-test. Thus, we concluded that the reproducibility of the lab-made portable device is acceptable for RT-LAMP-*Pf*Ago assays. In this study, the fluorescence was measured by the portable device, which was modified slightly to accommodate a 200 μL PCR tube, a commercial

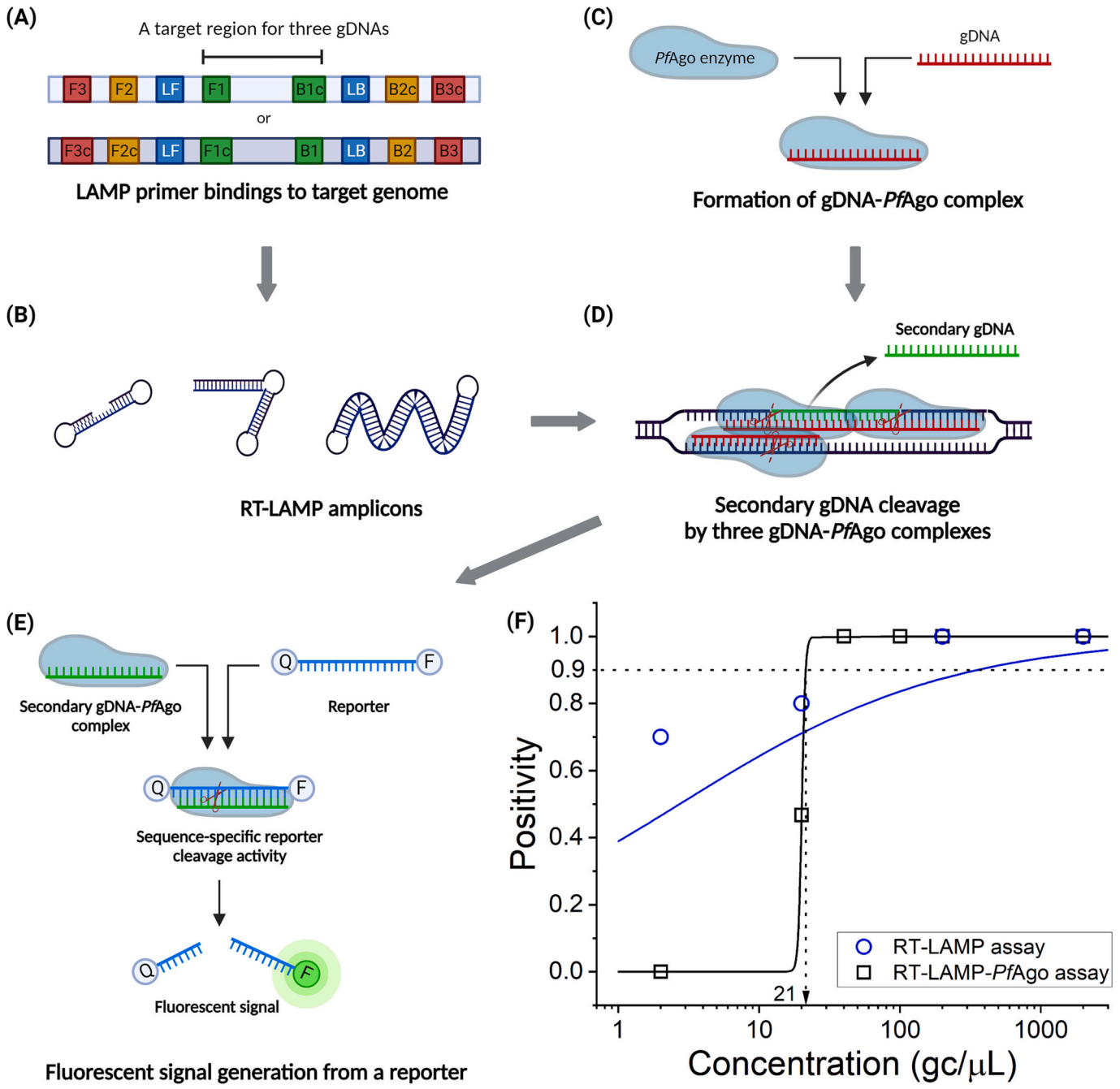

Fig. 1. Schematic diagram showing sequence-specific fluorescent signal production using an RT-LAMP-*Pf*Ago assay (A-E). (F) Positivity for PMMOV determined by RT-LAMP and RT-LAMP-*Pf*Ago assays. The x-axis shows the concentration of synthetic PMMOV DNA, and the y-axis presents positivity determined by the qPCR system. Ten or twenty replicates were analyzed for concentrations between 10 and 100 gc/μL samples, while five replicates were for 2 and 2000 gc/μL samples. The two-step RT-LAMP-*Pf*Ago assay for PMMOV showed 0.9 positivity at 21 gc/μL of synthetic DNA concentrations, which is defined as the limit of detection in this study.

qPCR instrument (Quantstudio 3, Thermo Fisher Scientific, USA), and a Gel Doc EZ System (Bio-Rad, USA). Because of different optical systems, such as a light source and detector, each instrument should have its fluorescence intensity threshold to make either positive or negative results. We empirically set 10,000 and 70,000 as the threshold for the lab-made portable device and the qPCR instrument.

We examined the occurrence of false positives by the RT-LAMP assay and the RT-LAMP-*Pf*Ago assay for PMMOV to investigate if the *Pf*Ago assay improves the specificity of the RT-LAMP assay. These two assays were applied to serial dilutions of synthetic DNA controls for PMMOV (Table S4). The positivity of each assay with varying PMMOV concentrations ranging from 2×10^0 to 2×10^5 gc/μL was analyzed for the comparisons. We also investigated whether an RT-LAMP-*Pf*Ago assay can identify SNPs. The S: P681R mutation of SARS-CoV-2, which was exclusive to the Delta variant in our study area (IL, USA) (Oh et al., 2022a, 2022b, 2022c), was chosen for the target SNP in this study. We obtained synthetic RNA controls for wildtype SARS-CoV-2 and the Delta variant (TWIST Bioscience, USA; part numbers 102,024 and 104,533, respectively) for the experiment. To enhance the specificity of the SNP target, we included an affinity-plus base in the *Pf*Ago assay reporter for the Delta variant (Integrated DNA Technology, USA) (Table S4).

2.5. Statistical analysis

A paired *t*-test was conducted to compare PMMOV RNA degradation with storage periods at −80 °C (Fig. S1). Two-sample *t*-tests were applied for comparisons between two fluorescent signals (Fig. 2B, Fig. 3B, and Fig. 5). Mean-squared errors (MSE) were calculated to compare the goodness-of-fit of a non-linear regression model (sigmoidal curve) (Fig. 1F). All statistical analyses were conducted using OriginPro 2023.

3. Results

3.1. A design strategy for gDNAs and a probe of PfAgo assays

We aimed to combine the *Pf*Ago assay with an RT-LAMP assay, referred to as an RT-LAMP-*Pf*Ago assay. In this combined assay, the RT-LAMP assay amplifies a target region of the genome, and the *Pf*Ago assay detects the RT-LAMP amplicons with the target sequence. Our hypothesis is that the *Pf*Ago assay provides sequence-specific detection of RT-LAMP amplicons, thereby enhancing the specificity of the RT-LAMP assay. Fig. 1 illustrates the reaction orders that occur in an RT-LAMP-*Pf*Ago assay. First, six RT-LAMP primers bind to (Fig. 1A) and amplify target genomes (Fig. 1B), producing various shapes of RT-LAMP amplicons. Next, the *Pf*Ago enzyme can form a complex with a gDNA, a 16-nucleotide long ssDNA with phosphorylated 5′ end (Fig. 1C). The gDNA-*Pf*Ago complex can bind to its complementary ssDNA sequence and perform the cleavage on the target ssDNA, specifically the cleavage occurs between the 10th and 11th nucleotides from the 5′ end of the gDNA. Three different gDNAs can be designed to form complexes with *Pf*Ago enzymes, all of which collectively cleave 16-nucleotide long ssDNA from double-stranded DNA (dsDNA). Specifically, two gDNA-*Pf*Ago complexes perform the cleavage on the same strand of the dsDNA and generate a 16-nucleotide long cleavage fragment at 95 °C. And the third gDNA-*Pf*Ago complex cleaves the complementary strand of the dsDNA, which helps releasing the cleavage fragment aforementioned (Fig. 1D). This resulting 16-nucleotide long ssDNA fragment can serve as a secondary gDNA that forms a new complex with an apo-*Pf*Ago enzyme. This secondary gDNA-*Pf*Ago complex can bind to a reporter probe that is designed to have the complement sequence of the secondary gDNA. The secondary gDNA-*Pf*Ago complex cleaves the reporter upon binding, generating a fluorescent signal (Fig. 1E). Consequently, the measurement of this fluorescent signal can be utilized to identify the presence of the sequences of three gDNAs and the secondary gDNA in the samples.

An RT-LAMP-*Pf*Ago assay requires a design of one reporter, three gDNAs, and six primers. Because a sequence of secondary gDNA is included in sequences of three gDNAs (Fig. 1D), and the three gDNA sequences are encompassed by sequences of six LAMP primers (Fig. 1E), choosing a sequence of the secondary gDNA can be the first step to design an RT-LAMP-*Pf*Ago. In this study, we chose a secondary gDNA sequence to contain conservative genes, the N gene for generic SARS-CoV-2 assay, and the replicase gene for PMMOV assay to detect generic PMMOV species usually served as an internal control for human virus detection. Once the secondary gDNA sequence is selected, the reporter sequence is determined accordingly because it is a complementary sequence of the secondary gDNA. Also, three gDNA sequences are determined to have cleavage activity happening at the right locations, as described above. The six LAMP primers were chosen to include the three gDNA sequences between F1 and B1c (or F1c and B1) primers (Fig. 1A and Fig. S1), whose sequences are synthesized on all shapes of LAMP amplicons (Fig. 1B).

3.2. PfAgo assays improve the specificity of an RT-LAMP assay

We hypothesized that the *Pf*Ago assay provides an additional sequence-specific check beyond RT-LAMP assay, thereby enhancing its specificity. In this way, potential non-specific RT-LAMP amplicons do not lead to fluorescence generation in the *Pf*Ago assay. We found that the positivity of the RT-LAMP assay was higher than the non-linear regression analysis at low DNA concentrations (Fig. 1F). Specifically, the discrepancy became bigger as the PMMOV DNA concentration decreased ($p < 0.05$ from a linear regression analysis). The level of differences between the measured and predicted positivity values was quantified by a mean squared error (MSE) of 0.08. Considering that three negative controls were also tested positive, the measurements with the low DNA concentrations were expected to include false positives. This finding suggests that the positivity determined by the RT-LAMP assay was significantly overestimated. In contrast, the positivity of the RT-LAMP-*Pf*Ago assay was well-aligned with the non-linear regression analysis, exhibiting a much lower MSE of 0.00. Furthermore, all three negative controls tested negative. These results indicate that the inclusion of the *Pf*Ago assay effectively eliminated false positives, thereby enhancing the specificity of the RT-LAMP assay. The LOD of the RT-LAMP-*Pf*Ago assay for PMMOV determined with synthetic DNA controls were 21 gc/μL.

3.3. An RT-LAMP-PfAgo assay recognizes a single nucleotide polymorphism in the Delta variant of SARS-CoV-2

We designed an RT-LAMP-*Pf*Ago assay specifically targeting S: P681R mutation to detect the Delta variant of SARS-CoV-2 (Fig. 2A). This single nucleotide polymorphism was confirmed to be exclusive to the Delta variant in our study area (IL, USA), and thus it was used for the Delta variant-specific PCR assay design (Oh et al., 2022b, 2023). The secondary gDNA sequence of the Delta variant-specific RT-LAMP-*Pf*Ago assay was selected to incorporate the S: P681R mutation at the 10th nucleotide from the 5′-end. The reporter sequence was then the complement sequence of the secondary gDNA, except for one intentional mismatch. The reporter is intentionally designed to have a mismatch at the 6th nucleotide from the 5′-end, corresponding to the 11th nucleotide from the 5′-end of the secondary gDNA. This single mismatch is not sufficient to impede the binding and cleavage activity of the secondary complex, resulting in the production of a fluorescent signal. However, if the nucleic acid being tested does not contain the target SNP, it instead forms another mismatch at the 7th nucleotide from the 5′-end of the reporter. In that case, two consecutive mismatches significantly reduce the cleavage activity of the secondary complex (Fig. 2B). The discrepancy in fluorescent signals due to the presence or absence of the target SNP was hypothesized to be significant enough to identify its presence.

To test this hypothesis, we applied the RT-LAMP-*Pf*Ago assay targeting the S: P681R mutation of SARS-CoV-2 to nuclease-free water,

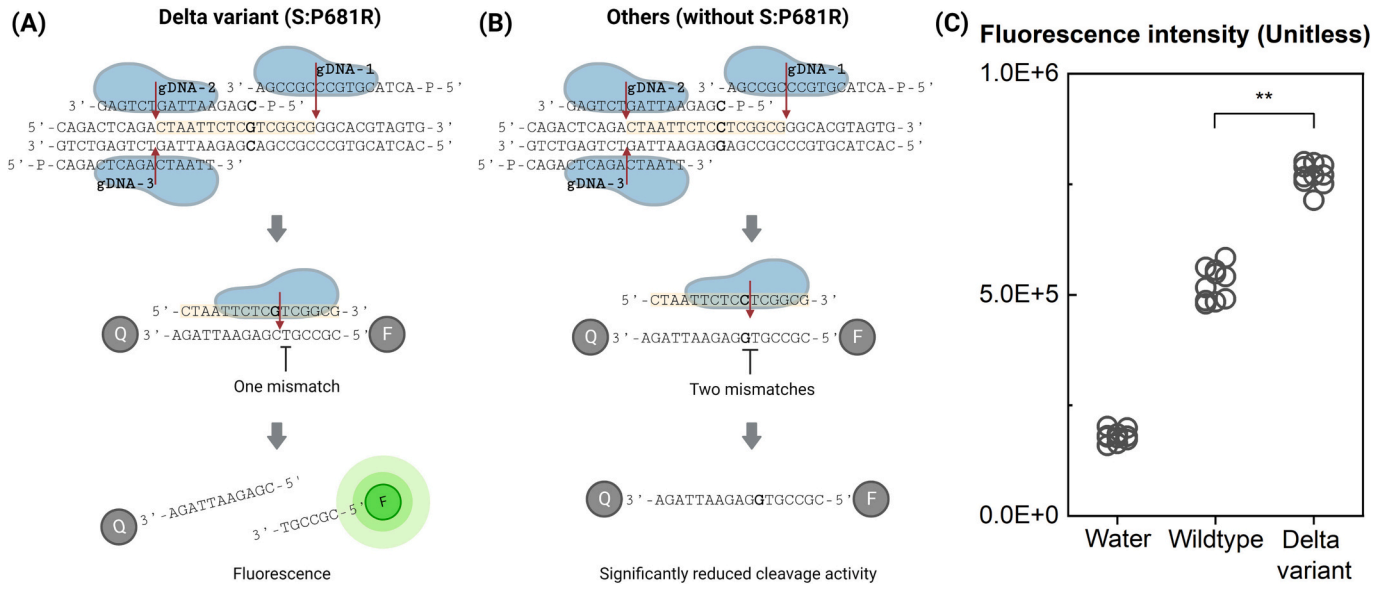

Fig. 2. Schematic illustration showing reactions between an RT-LAMP-*Pf*Ago assay targeting the S: P681R mutation and (A) synthetic RNA control for the Delta variant or (B) synthetic RNA control for the wildtype. (C) Application of the two-step RT-LAMP-*Pf*Ago assay targeting the S: P681R mutation to nuclease-free water (left column), the synthetic RNA control for the wildtype (10,000 gc/μL, middle column), and synthetic RNA control for the Delta variant (1000 gc/μL, right column). The y-axis presents fluorescence intensity determined by the qPCR system. Ten replicates were tested for each sample. The two-sample *t*-test was applied for comparisons between two data (** indicates $p < 0.001$).

synthetic RNA controls for the Delta variant, and synthetic RNA controls for the wildtype SARS-CoV-2. Note that the synthetic controls for the Delta variant and wildtype had a single nucleotide difference in the target sequences of the RT-LAMP-*Pf*Ago assay. Fig. 2C illustrates the fluorescence intensity observed in each sample. Our results proved that the fluorescent signals from both synthetic RNA controls for the Delta variant and the wildtype SARS-CoV-2 were significantly higher than that from the nuclease-free water. However, the fluorescent signals from 1000 gc/μL of synthetic RNA controls for the Delta variant were significantly higher than those from 10,000 gc/μL of the synthetic RNA controls for the wildtype SARS-CoV-2 (two-sample *t*-test; $p < 0.001$). We can differentiate between the two samples by setting an appropriate threshold of 700,000 (unitless). For example, the fluorescence signals from the Delta variant were significantly higher (one-sample t-test; $p < 0.001$), while those from the wildtype SARS-CoV-2 were significantly lower (one-sample t-test; $p < 0.001$) than the threshold. Note that we did not conduct an experiment with mixtures of the wildtype and the Delta variant at different mixing ratios. This experiment would provide conclusive evidence to determine the lowest proportion of the Delta variant that can be detected by the RT-LAMP-*Pf*Ago assay. However, we have confirmed that the fluorescence intensity from RT-LAMP-*Pf*Ago assays does not significantly reduce as target concentrations decrease from 2000 to 20 gc/μL as long as they test positive (Fig. S2). This finding suggests that the RT-LAMP-*Pf*Ago assay would identify SNP-containing variants present at low proportion.

3.4. RT-LAMP-PfAgo can be a duplex assay for virus detection

The formation of the secondary gDNA-*Pf*Ago complex is a result of the sequence-specific cleavage activity of three primary gDNA-*Pf*Ago complexes. Additionally, the cleavage of the reporter, leading to the production of a fluorescent signal, is a result of the sequence-specific cleavage activity of the secondary gDNA-*Pf*Ago complex. Therefore, the unique feature of the cleavage activity of the gDNA-*Pf*Ago complex can include a mixture of various sequences and multiple reporters in a single assay, making it a potentially multiplexable assay. We designed a duplex RT-LAMP-*Pf*Ago targeting PMMOV and SARS-CoV-2. The reporter for PMMOV is labeled with the FAM dye, while the reporter for SARS-CoV-2 is labeled with the ROX dye. To confirm the sequence-specific cleavage activity, we applied the duplex assay to nuclease-free water, synthetic controls for PMMOV or SARS-CoV-2, and a mixture of the two synthetic controls (Fig. 3A).FAM signals (for PMMOV detection) produced from PMMOV and a mixture of PMMOV and SARS-CoV-2 were significantly higher than those from water (negative control) ($p < 0.001$, two-sample t-test, Fig. 3B). Additionally, ROX signals (for SARS-CoV-2 detection) from SARS-CoV-2 and a mixture of PMMOV and SARS-CoV-2 were significantly higher than those from water (negative control) ($p < 0.001$, two-sample t-test, Fig. 3B). These results indicate that the presence of the target sequence led to the formation of a secondary gDNA-*Pf*Ago complex, resulting in the generation of a corresponding fluorescent signal (solid line in Fig. 3A). Furthermore, FAM signals (for PMMOV detection) from SARS-CoV-2 were not significantly higher than those from water samples ($p < 0.001$, two-sample *t*-tests, Fig. 3B). This finding suggests that the unwanted target (SARS-CoV-2) did not form the secondary gDNA-*Pf*Ago complex for PMMOV, and the secondary gDNA-*Pf*Ago complex for SARS-CoV-2 did not cleave the reporter for PMMOV (dashed line in Fig. 3A). Similarly, ROX signals (for SARS-CoV-2 detection) from PMMOV were not significantly higher than those from water samples ($p < 0.001$, two-sample t-tests, Fig. 3B), supporting the findings observed in the FAM signals. Based on these findings, we can conclude that the duplex assay only produces a designed fluorescent signal when the specific target sequences are present in the samples.

3.5. RT-LAMP-PfAgo assays are applicable to wastewater samples

Environmental samples, particularly wastewater samples, are known to contain various types of inhibitors that can impact the sensitivity of virus detection assays. Even though nucleic acid extraction includes a purification step for reducing environmental inhibitors, the remaining inhibitors could still impact downstream molecular analysis depending on the wastewater samples. We assessed the impact of inhibitors present in wastewater on an RT-LAMP-*Pf*Ago assay by conducting the assay to detect PMMOV in wastewater collected from different sewersheds at various time points, ensuring a wide range of inhibitor concentrations. Viral RNA samples were extracted and purified from the concentrated wastewater sludge using a commercial extraction kit. Next, we

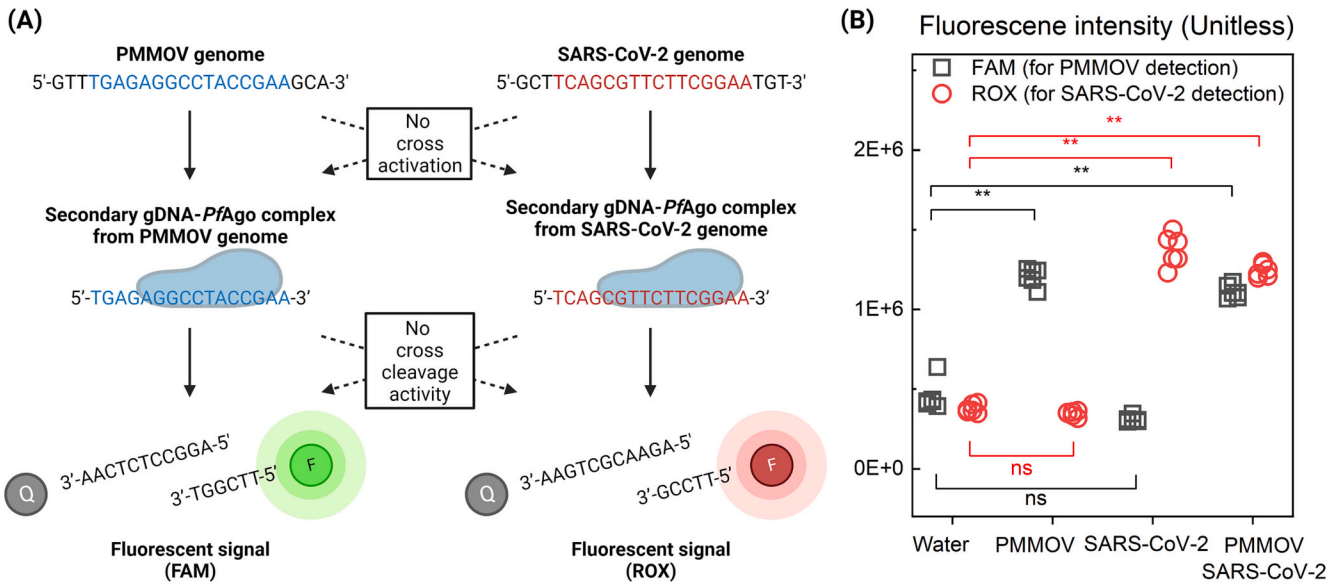

Fig. 3. (A) Cross-reactivity of the RT-LAMP-*Pf*Ago assay. (B) The two-step RT-LAMP-*Pf*Ago assays were applied for PMMOV and SARS-CoV-2 N gene detection. The x-axis shows types of samples, including molecular biology grade water, PMMOV (2.5×10^5 gc of synthetic DNA), SARS-CoV-2 N gene (2.5×10^5 gc of synthetic RNA), and a mixture of the PMMOV and the SARS-CoV-2 samples. The y-axis presents unitless fluorescence signals ($n = 6$) detected by a FAM channel and an ROX channel determined by the qPCR system.

determined the optimal dilution factor for the viral RNA samples that balances the reduction in inhibitors with the preservation of target sequences using five wastewater samples in no, 2.5-, and 5-fold dilution. Fig. 4A illustrates the fluorescent signals obtained from these samples when subjected to the RT-LAMP-*Pf*Ago assay for PMMOV detection. A sample with 0 gc/μL of PMMOV tested negative, while two samples with concentrations of 207 and 586 gc/μL tested positive across all three dilutions in the RT-LAMP-*Pf*Ago assay. However, different dilution factors resulted in varying test results for the wastewater samples containing 63 and 183 gc/μL of PMMOV. For instance, although the no-dilution samples tested positive for all three replicates of the 63 and 183 gc/μL samples, the 2.5- and 5-fold dilutions yielded partially negative results. Specifically, two out of three replicates for the 2.5-fold dilution of the 63 gc/μL sample were tested negative, while two and one replicates were tested negative for the 10-fold dilution of the 63 and 183 gc/μL samples, respectively (Fig. 4A). Based on these findings, it can be inferred that the RT-LAMP-*Pf*Ago assay does not require dilution to detect PMMOV in our wastewater samples.

Then, we applied the RT-LAMP-*Pf*Ago assay for PMMOV to 30 wastewater samples, and the positivity of PMMOV by the RT-LAMP-*Pf*Ago assay is presented in Fig. 4B. In this study, the LODs of RT-LAMP-*Pf*Ago assays are defined as the concentration at which a positivity of 0.9 is achieved. We found that the LOD of the RT-LAMP-*Pf*Ago assay for PMMOV determined by wastewater samples was 28 gc/μL. The 95 % confidence interval of the LOD, ranging from 11 to 103 gc/μL, included that of this assay determined with synthetic controls in nuclease-free water (21 gc/μL). This finding indicates that the residual inhibitor in the wastewater RNA extract did not interfere with RT-LAMP-*Pf*Ago assays. Note that these wastewater samples required 10-fold dilution to be free from the impact of inhibitors on quantitative RT-qPCR analysis (Oh et al., 2022c). In summary, the RT-LAMP-*Pf*Ago assay presented no significant impact of potential inhibitors in wastewater on the detection of nucleic acid.

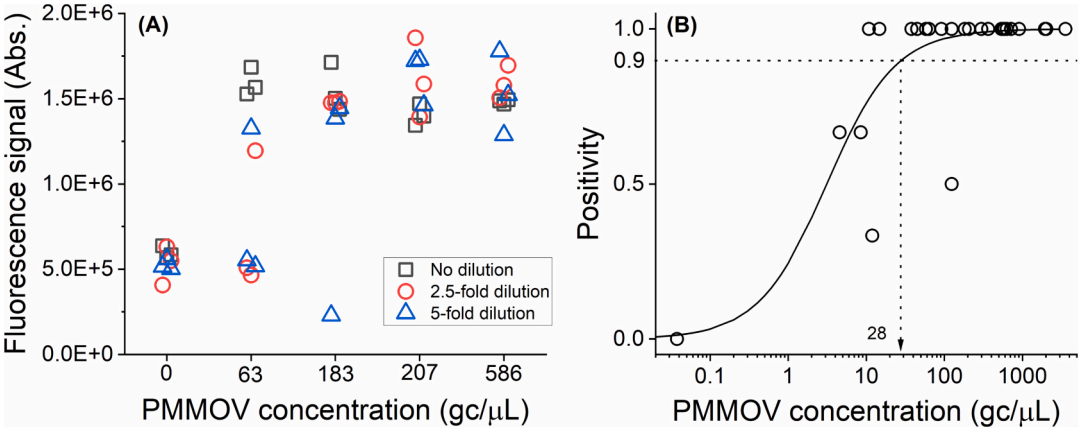

Fig. 4. (A) Application of the two-step RT-LAMP-*Pf*Ago assay for PMMOV to five wastewater samples. The x-axis shows the PMMOV concentrations of each wastewater sample determined by RT-qPCR. False positives and negatives for PMMOV were avoided through positive and negative controls. The y-axis presents fluorescence signals from wastewater samples with different dilution factors (no, 2.5-, and 5-fold dilution) quantified by the qPCR system. (B) Application of the RT-LAMP-*Pf*Ago assay for PMMOV to 30 wastewater samples without dilution. The y-axis presents the positivity of two or three replicates of each wastewater sample.

3.6. RT-LAMP-*Pf*Ago assays are compatible with a portable device

Because it consists of two isothermal reactions (RT-LAMP amplification and *Pf*Ago cleavage activity), an instrument for conducting RT-LAMP-*Pf*Ago assays can be fabricated much simpler than a thermocycler. In this study, we utilized the lab-made portable device that incubates samples at two sequential temperatures at 65 °C and 95 °C and performs end-point fluorescence measurement (Xun et al., 2021). To streamline the process and minimize pipetting errors, we combined the RT-LAMP and *Pf*Ago assay reagents into a single tube, using a wax barrier to separate the two components. Note that our previous study demonstrated that two constituents, *Pf*Ago enzyme and $MnCl_2$, of *Pf*Ago assay interfere with RT-LAMP assay (Xun et al., 2021), necessitating running an RT-LAMP assay free from the *Pf*Ago reagent. This approach makes the RT-LAMP-*Pf*Ago assay suitable as a point-of-use virus detection assay.

To validate the one-step RT-LAMP-*Pf*Ago assay for PMMOV, we applied it to nuclease-free water and four wastewater samples with varying concentrations of PMMOV from 15 to 1945 gc/μL. Our results indicate that the assay produces significantly higher fluorescent signals with wastewater samples, which contain PMMOV RNA, compared to water samples, which do not contain PMMOV RNA. Specifically, Fig. 5A illustrates the brighter fluorescent signals observed in the wastewater samples (labeled as #8, #14, #16, and #22) compared to water (labeled as Water). Furthermore, Fig. 5B presents the quantified fluorescent signals obtained using the portable device. We found that the four wastewater samples' fluorescent signals significantly differed from those of the negative controls. Note that although the previous statement is supported by a two-sample *t*-test ($p < 0.001$) and statistical power β (>0.8), a larger sample size (ideally >30) will be necessary to support the normal distribution of the data, making the statement more reliable. Using a threshold of 10,000 (unitless), all wastewater samples tested positive, while the negative control tested negative. This finding demonstrates the feasibility of operating the one-step RT-LAMP-*Pf*Ago assay on a portable device.

4. Discussion

Isothermal amplification and specific nucleic acid detection offer advantages over PCR-based assays (e.g., qPCR or digital PCR), enabling pathogen detection in resource-constrained laboratories and even on-site. Due to its rapid amplification and high amplicon concentration, RT-LAMP is the most widely used isothermal amplification assay for pathogen detection, with various approaches applied to read the amplification signals. This study investigated the utilization of the *Pf*Ago enzyme to read nucleic acid amplification by RT-LAMP, particularly from wastewater samples. Table 1 provides comparisons of various approaches used to read RT-LAMP amplification signals, emphasizing the advantages of the *Pf*Ago assay over conventional and other emerging methods, as well as its potential for further improvement.

*Pf*Ago assay provides a sequence-specific read-out of RT-LAMP amplification. The viral genome mutation rate is typically several orders of magnitude faster than that of other microorganisms, such as bacteria (Duffy et al., 2008; Peck and Lauring, 2018), resulting in genetic variation within the virus population (Sanjuán and Domingo-Calap, 2021). While sequencing provides accurate identification of viruses by generating reads that span long genome regions (>1000 bp), its analysis capacity is limited due to high costs and a slow turnaround time (>12 h). In contrast, nucleic acid-based assays, like RT-qPCR, are more commonly used for virus detection due to their affordability and scalability (Graber et al., 2021; Lee et al., 2022). However, nucleic acid-based assays only amplify a short piece of the viral genome (a few hundred base pairs), necessitating programmability to target specific sequences to accurately identify viral species. For instance, Oh et al. (2022b) found that only a few virus mutations of SARS-CoV-2 are possible targets of variant-specific PCR assay design. Furthermore, Oh et al. (2023) revealed that endemic viruses, such as human noroviruses, possess limited regions of conserved genome due to their significant genetic diversity. In this study, we confirmed that RT-LAMP-*Pf*Ago assays are programmable by demonstrating that the RT-LAMP-*Pf*Ago assay for the Delta variant specifically targeted the S: P681R mutation. The sequence-specific cleavage activity of the gDNA-*Pf*Ago complex is a unique feature of the *Pf*Ago enzyme that makes it SNPs specific assay, thereby being advantageous for virus surveillance (Table 1).

The *Pf*Ago assay allows the detection of multiple targets in a single reaction. When monitoring host-specific viral pathogens, it is important to measure internal controls that indicate the presence of host genes. Without internal controls, we might incorrectly attribute the absence of target pathogen genes in samples to low disease prevalence in the watershed population, which could lead to false negatives. For instance, the human RNase P gene has been utilized to monitor COVID-19 clinical samples, and CrAssphage or PMMOV has been employed in wastewater-based epidemiology to confirm their human origin (Greaves et al., 2020; Yüce et al., 2021). Different types and numbers of internal controls may

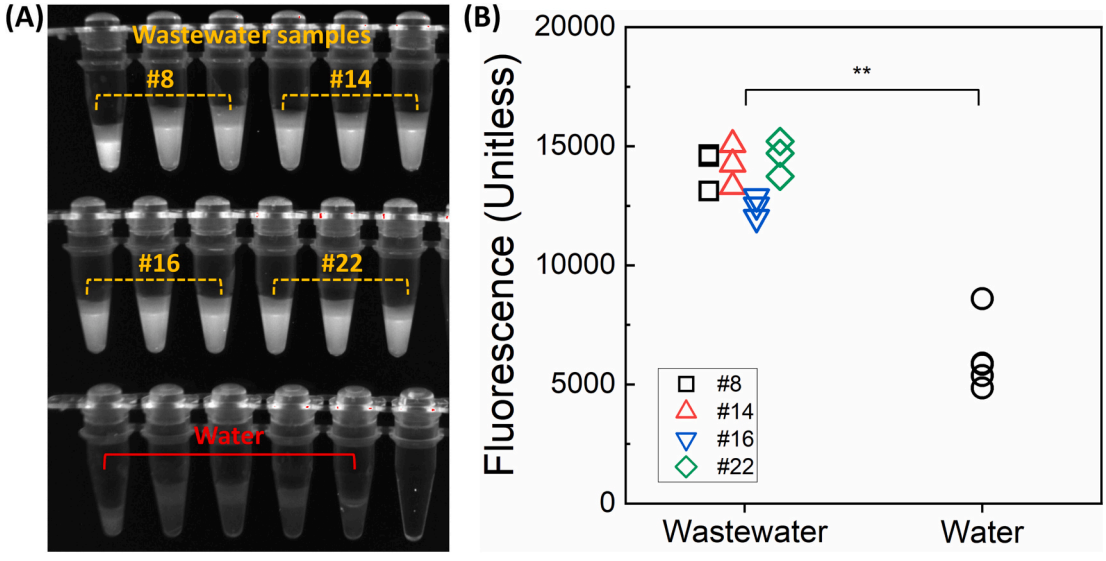

Fig. 5. Comparisons of fluorescence between four wastewater samples ($n = 3$) and nuclease-free water ($n = 5$) produced by the one-step RT-LAMP-*Pf*Ago assay for PMMOV. (A) Gel Doc image of fluorescent signals and (B) quantitative measurements of fluorescence by the lab-made portable device.

Table 1
Comparisons of various approaches for reading RT-LAMP amplification results.

Method	Purpose	Isothermal Reaction	Sequence-specific reaction	Multiplex	LOD (gc/uL)	Quantification	Target Samples	Reference
PCR platform	Conventional pathogen detection	No (thermal cycle)	Yes	Yes	<5	Yes	Diverse clinical and environmental samples	(Nyaruaba et al., 2022)
Colorimetry	Application to RT-LAMP assays	Yes	No	No	~100	No	Nasal Swab and saliva	(Aoki et al., 2021; Dao Thi et al., 2020)
Fluorescence		Yes	No	Yes	<5	No	Nasal Swab and saliva	(Ooi et al., 2022)
Lateral flow		Yes	No	Yes	<5	No	Blood and Nasal swab	(Lee et al., 2016; Zhu et al., 2020)
CRISPR-Cas12a		Yes	Limited to PAM	No	10	No	Nasal swab	(Broughton et al., 2020)
		Yes	Limited to PAM	No	<5	No	Nasal swab	(Joung et al., 2020)
*Pf*Ago		Yes	Yes, even for SNPs	Yes	28	No	Wastewater	This study
		Yes	Yes, even for SNPs	Yes	<5	No	Saliva	(Xun et al., 2021)

serve better depending on water sources. Therefore, the use of multiplexable assays can be advantageous in minimizing the volume of reagents and genome samples compared to repeating singleplex assays multiple times. In this study, we confirmed that the sequence-specific cleavage activities of an RT-LAMP-*Pf*Ago assay, specifically the formation of the secondary gDNA-*Pf*Ago complex and the cleavage of the reporter, allow for the inclusion of mixtures of various sequences and multiple reporters in a single assay. This unique feature enabled us to design a duplex RT-LAMP-*Pf*Ago assay targeting PMMOV and the SARS-CoV-2 N gene. Moreover, the number of targets in RT-LAMP-*Pf*Ago assays can be increased as there are various dyes with distinct excitation wavelengths and instruments capable of identifying these dyes. For example, Li et al. (2023) developed a pentaplex assay that simultaneously detects hepatitis B virus, hepatitis C virus, hepatitis E virus, *Treponema pallidum*, and the RNase P gene using FAM, VIC, CY5, TAMRA, and ROX dyes along with an RT-qPCR system. Note that the demonstration of a duplex assay in this study does not necessarily guarantee the functionality of the RT-LAMP-*Pf*Ago assay for three or more targets, as it may be affected by interference from overlapping fluorescence spectra. Future studies should focus on designing and demonstrating the RT-LAMP-*Pf*Ago assay for incorporating multiple targets.

Environmental surveillance has emerged as a crucial epidemiological tool, facilitating public health decision-making. For example, wastewater-based epidemiology has enabled us to estimate the prevalence of COVID-19 more accurately in communities, especially where clinical testing capacity is limited. One of the unique features of environmental samples differentiating them from the clinical samples is that different inhibitors have been identified in wastewater, such as polysaccharides, lipids, organic matter, polyphenols, and inorganic ions, which may interfere with molecular analysis (Schrader et al., 2012). In this study, we confirmed that RT-LAMP-*Pf*Ago assay can be conducted in a single tube, operated on a portable device, and is applicable to environmental samples. This study is the first application of *Pf*Ago assay to RT-LAMP assay for virus detection in environmental samples. Based on our findings, we concluded that this novel approach, a specific, portable, and single nucleotide polymorphism-specific duplex assay for virus surveillance in wastewater, will improve environmental surveillance.

While viruses such as noroviruses, adenoviruses, and enteroviruses are excreted at high concentrations by infected individuals, they become significantly diluted in the water environment through various water sources (Haramoto et al., 2018). Consequently, achieving a lower LOD value is advantageous for virus detection assays to sensitively monitor specific viruses in the environment. PCR-based assays typically present the lowest LODs among nucleic acid-based assays, with LODs mostly below 10 gc/rxn (Oh et al., 2022b, 2022c). Alternative nucleic acid-based assays, such as RT-LAMP-CRISPR-Cas12 assays, have also reported LOD values as low as 10 gc/rxn (Ali et al., 2020; Broughton et al., 2020). Although the LOD of the RT-LAMP-*Pf*Ago assay for PMMOV in wastewater was 28 gc/μL or 70 gc/rxn, which is higher than the previously reported LOD values, it is possible to achieve a lower LOD by optimizing the assay or using an advanced instrument. For instance, in our previous study, we optimized the assay for saliva sample analysis by investigating various factors that could impact the performance of the RT-LAMP-*Pf*Ago assay. These factors included the GC content of the fluorescent reporters, *Pf*Ago cleavage time, gDNA numbers, type of Ago enzymes, and LAMP reaction time. Through these optimizations, we successfully reduced the LOD to 17.5 gc/rnx for an RT-LAMP-*Pf*Ago assay targeting the N gene of SARS-CoV-2 (Xun et al., 2021). Note that the sample matrix in the current study differs from our previous research. As a result, a separate optimization process may be necessary to enhance the LOD of the RT-LAMP-*Pf*Ago assay for the analysis of wastewater samples. One approach to improve the LOD may involve designing a novel reporter for the *Pf*Ago assay. As depicted in Fig. 2C, the RT-LAMP-*Pf*Ago assay produces a background fluorescence signal that is not negligible. This background signal depends on the distance between the fluorophore and the quencher of the reporter. Intentionally forming dimers of the reporter, thereby reducing the distance between the fluorophore and quencher, could lower background noise and enhance the LOD (Johansson, 2006). Additionally, Park et al. (2021) found that using an array of microwells (0.7 nL) can lower the LOD of an RT-LAMP-CRISPR-Cas12 assay by 10-fold compared to the reaction in bulk (10 μL). Furthermore, RT-LAMP assays with various signal-readout approaches, including the *Pf*Ago assay, do not provide quantitative measurements as accurately as PCR assays. It should also be noted that the development of an RT-LAMP-*Pf*Ago assay involves the design of six LAMP primers, a reporter, and three gDNAs, which is more complex than PCR-based assays. Therefore, future research can focus on improving the LOD and providing quantitative measurements to accurately evaluate viruses in environmental samples.

5. Conclusion

This study represents the first application of the *Pf*Ago diagnostic platform for detecting viruses in wastewater. We confirmed that the sequence-specific cleavage activity of *Pf*Ago allows RT-LAMP-*Pf*Ago assays to be an SNP-specific duplex assay, providing unique advantages in virus detection. Furthermore, we demonstrated that the RT-LAMP-*Pf*Ago assays are compatible with portable devices and robust to environmental inhibitors, making them suitable for efficient environmental surveillance. Considering that virus surveillance in the environment plays a crucial role in providing essential information on community-wide disease prevalence and risk assessment for infections, this novel assay holds the potential to significantly contribute to improving public health.

Author contributions statement

Chamteut Oh and Guanhua Xun conducted experiments and wrote a draft.

Stephan Thomas Lane, Vassily Andrew Petrov, and Huimin Zhao designed experiments and revised the draft.

Thanh H. Nguyen managed the entire project.

All authors contributed to data interpretation and reviewed the final manuscript.

Declaration of competing interest

Thanh H. Nguyen reports financial support was provided by US Environmental Protection Agency.

Data availability

Data will be made available on request.

Acknowledgment

We acknowledge funding support from the EPA grant (R840487). This study has not been formally reviewed by EPA. The views expressed in this document are solely those of Professor Thanh H. Nguyen and do not necessarily reflect those of the Agency. EPA does not endorse any products or commercial services mentioned in this publication. We also thank Hankeun Lee for his advice on a portable fluorimeter.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.168701.

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