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An ultrasensitive, rapid and portable method for screening oseltamivir-resistant virus based on CRISPR/Cas12a combined with immunochromatographic strips

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Abstract
Influenza is a significant public health challenge because of the emergence of antigenically shifted or highly virulent strains. The neuraminidase inhibitor oseltamivir is used as an antiviral drug in clinical treatment. However, its therapeutic effects can be greatly compromised by the emergence of drug-resistant mutant viruses. Thus, there is an urgent need to distinguish drug-resistant strains with a simple method. To address this, in the present study, we develop a rapid, sensitive and convenient molecular diagnosis method based on CRISPR/Cas12a technology and lateral flow detection (LFD). By targeting mutant sequences amplified by recombinase polymerase amplification (RPA) reaction, crRNA is designed to develop the CRISPR/Cas12a assay, and 2000 copies can be directly observed by the naked eye under blue light-emitting diode (LED) light. Combined with LFD, the limit of detection of RPA-CRISPR/Cas12a-LFD is about 20 copies of target sequence per reaction. Collectively, RPA-CRISPR/Cas12a-LFD method provides a novel alternative for the sensitive, specific and portable detection to diagnose oseltamivir-resistant mutant strains.

Key words influenza virus, CRISPR/Cas12a, sensitivity, lateral flow detection

Introduction

Influenza is a highly infectious disease characterized by recurrent annual epidemics and unpredictable major pandemics worldwide. It is mainly caused by the influenza A virus (IAV), an enveloped virus with eight negative-sense single-stranded RNA molecules and among the most common causes of human respiratory infections with high morbidity and mortality. Millions of people died from Spanish pandemic influenza in 1918 and 1919 [1,2] and 0.3 million people died from Swine Flu from 2009 to 2010 [3,4]. Furthermore, up to 50% of the world population can be infected in a single pandemic year [5]. Thus, it is important to prevent and treat the suspected influenza during the outbreak of epidemics.

Vaccines, as an effective, economical and safe way to treat the influenza pandemic, play a crucial role in protecting humans and animals against IAV [6,7]. However, it is difficult to produce large quantities of vaccines in a short period of time, resulting in protection not being given timely [8]. The vaccines cannot protect against mutant virus infection due to the continuous antigenic drift and sporadic antigenic shifts in the viral surface glycoproteins [9]. Thus, stockpiling IAV inhibitors in the earlier pandemic has been proposed to defend against pandemic strains. Over the past few years, inhibitors of the viral enzyme neuraminidase have been used to treat influenza in clinical settings, *i.e.*, neuraminidase (NA) inhibitors can prevent the efficient release of newly synthesized viral particles from infected cells by binding to the NA protein and blocking its enzymatic activity [10].

Due to the low-fidelity viral RNA polymerase lacking exonuclease proofreading capability, IAVs exist as populations of quasispecies [11]. In addition, random mutations can be introduced quickly. Then the particular mutants are selected, depending upon the evolutionary pressures, such as host environment, immune pressure leading to viral amino acid alterations and antigenic drift,

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or antiviral inhibitor pressure leading to drug-resistance [12–16]. As an antiviral compound, the therapeutic importance of neuraminidase inhibitors can be compromised by the emergence of drug-resistant mutants. In the 2009 pandemic influenza A/H1N1 virus background, H275Y, a histidine is replaced by a tyrosine in the neuraminidase at 275th residue, becoming the most frequently reported mutation, which renders oseltamivir-resistance [17]. Thus, for efficient clinical treatment or to avoid missing the best time to administer medication, it is crucial to obtain the genotype of the IAVs. Reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence polarization methods have been applied to distinguish whether it is the oseltamivir-resistant mutant or the sensitive strain [18]. However, highly specialized personnel and expensive equipment are required for the implementation of these methods. Against such a backdrop, any development toward ultrasensitive, economical, and portable diagnostic method is highly valuable.

As a new biotechnological technique, CRISPR/Cas has been recently harnessed for the *in vitro* detection of nucleic acids, thereby emerging as a powerful and precise tool for molecular diagnosis [19,20]. The detection relies on the target-activated non-specific endonuclease activity of Cas12 or Cas13 after binding to a specific target DNA or RNA via programmable guide RNAs [19–21]. It induces indiscriminate single-stranded DNA (ssDNA) collateral cleavage after target recognition. CRISPR/Cas-based diagnostic technology has been applied to identify different pathogenic microorganisms, such as Zika virus, Dengue virus, and SARS-CoV-2 [22,23]. In addition, owing to the sensitivity and specificity of the CRISPR/Cas system, it can also be applied to detect a single base mutation. Based on the Cas13-detection system, HIV DRMs (drug resistance mutations) and low-frequency cancer mutations were successfully detected in cell-free DNA (cfDNA) fragments [19,24]. Furthermore, drug resistance mutations against inhibitors of NAMPT and KIF11 were detected utilizing enAsCas12a [24].

In the present study, we sought to develop a molecular diagnosis method based on CRISPR/Cas12a to distinguish the sequence of the oseltamivir-resistant mutant (H275Y). Then, we attempted to make the result readout more sensitive and easier using the lateral flow detection method. As such, pathogens surveillance and diagnosis would greatly benefit from this approach which can simplify subsequent identification of the relevant drug-resistant mutant virus.

Materials and Methods

Target sequence and lentivirus (harboring target sequence) preparation

The wild-type (WT) and H275Y mutant gene fragment were synthesized and inserted into vector plasmid pJET1.2 provided with the CloneJET PCR Cloning kit (Thermo Fisher Scientific, Waltham, USA). Target sequences cleaved by Cas12a-RNP were generated by polymerase chain reaction (PCR) using specific primers (forward primer: 5′-tccaatggaaccattaaaga-3′ and reverse primer: 5′-ggatatgctgctcccgctag-3′). The lentiviral vector plasmid pSIN-NA (WT or H275Y) was generated from pSIN-GFP [25]. Lentivirus were generated by transfecting 293T cells with lentivirus vector plasmids and packaging plasmids.

cDNA preparation from lentivirus

RNA was extracted from lentivirus (harboring target sequence) using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. RNA was used to synthesize cDNA using GoScript Reverse Transcription System (Promega, Wisconsin, USA). Then, the RT-RPA pruducts were subject to genotyping analysis by CRISPR/Cas12a assay or CRISPR/Cas12a-LFD assay.

Protein expression and purification

The Cas12a (FnCas12a, also known as "FnCpf1") protein was expressed and purified as described previously [20]. The purified Cas12a protein was concentrated into storage buffer [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, and 2 mM DTT], quantitated using the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China), and frozen at −80°C until use.

In vitro synthesis of crRNA

The template for transcription of crRNA was generated by PCR using specific primers listed in Table 1, then *in vitro* synthesis of crRNA was performed using purified PCR products as the template using the MEGA shortscript T7 Transcription kit (Thermo Fisher Scientific). crRNAs were purified with Trizol method. RNA was aliquoted and stored at −80°C until use.

Isothermal amplification

The isothermal amplification of the H275Y and WT sequences were performed with a commercial RPA kit according to the manufacturer's instructions of Twist Amp Exo (TwistDx) kits (Cambridge, UK). The RPA primers were designed according to the instructions (forward primer: 5′-taatgaccgatggaccaagtgatggacaggcctca-3′ and reverse primer: 5′-gcacacacatgtgatttcactagaatcagg-3′) and all the primers and probes were synthesized by Sangon Biotech (Shanghai, China). Then, the RPA reaction was transferred to the CRISPR/Cas12a detection assay.

CRISPR/Cas12a detection reaction

Detection assays were performed with 200 ng purified Cas12a, 20 pM ssDNA FQ probe sensor, 1 μM crRNA, and 1 μL sample in 50 μL of reaction volume at 37°C for 120 min. A TECAN NanoQuant reader (Beijing, China) was used for fluorescence detection. Fluorescence kinetics were monitored using a monochromator with excitation at 485 nm and emission at 525 nm.

CRISPR/Cas12a-LFD detection reaction

CRISPR/Cas12a-LFD assays were performed with 100 ng purified Cas12a, 2 pM ssDNA BFQ probe sensor, 1 μM crRNA, and 1 μL sample in 50 μL of reaction volume at 37°C for 90 min. Then, reaction products were added to the strip for lateral flow detection. After 10 min, LED blue light and immunochromatographic strip readers was used for fluorescence band intensity detection.

Sensitivity detection of target sequence with real-time PCR

To compare the sensitivity of different detection methods, serial

Table 1. Sequence of primers used in the synthesis of crRNA

Primer	Sequence (5′→3′)
F0	cttggagcaggttccattca
R0	atctacaatagtagaaattccctatagtgagtcgtatta
R1	caggagcattcctcatagtaaatctacaatagtagaaattc
R2	gcccctaattattactatgagatctacaatagtagaaattc
R3	taacaggagcattcctcatagatctacaatagtagaaattc

dilution of the standard pJET recombinant plasmids (H275Y) as the target sequences were detected by RPA-CRISPR/Cas12a, RPA-CRISPR/Cas12a-LFD and real-time PCR assays, respectively. The real-time PCR detection were performed with forward primer (5′-ATGTGCATGTGTAAATGGTTCTTGCTTTAC-3′), reverse primer (5′-ACACATGTGATTTCACTAGAATCAGG-3′) and TaqMan probe (5′-FAM-TCCTCATAGTAATAATT-MGB-3′). The PCR conditions are as follows: 95°C for 3 min and 40 cycles of 95°C for 10 s and 60°C for 35 s. A cycle threshold value (Ct) < 35 was regarded as H275Y positive.

Statistics analysis

All data generated from at least three independent experiments are presented as the mean ± SD. Statistical analyses and graphing were carried out using GraphPad Prism 7.0 and SPSS. $P < 0.05$ was considered to be statistically significant.

Results

CRISPR/Cas12a-based oseltamivir-resistant mutant detection

Scheme of Cas12a-mediated oseltamivir-resistant mutant detection system with Cas12a protein, specific CRISPR RNAs (crRNAs), and a single-stranded DNA (ssDNA) reporter was generated as illustrated in Figure 1A. The ssDNA reporters labeled with a quenched fluorescent molecule were used for fluorescence detection. Specifically, the ssDNA-FQ reporter was a 5 nucleotide (nt) single-stranded DNA (5′-TTATT-3′) labeled by 5′ 6-FAM fluorophore and 3′ TAMRA quencher. After incubation at 37°C for 60–120 min, Cas12a nuclease would be triggered by the binding of crRNA to target sites, and activated Cas12a engages in "collateral" cleavage of nearby non-targeted DNAs. This crRNA-programmed collateral-cleavage activity allows Cas12a to detect the presence of a specific DNA *in vitro* by non-specific degradation of the labeled ssDNA-FQ reporter. As a result, a super-bright fluorescence signal can be directly visualized under a blue LED illuminator, and the fluorescence signal value correlates with the amount of target sequence.

As CRISPR/Cas12a recognizes a T (thymine) nucleotide-rich protospacer-adjacent motif (PAM), three crRNAs with a 5′-TTN-3′ PAM targeting H275Y sequence were designed (Figure 1B). The Cas12a-RNP targeting the H275Y mutant sequence and WT sequence were tested *in vitro* to evaluate the cleavage activity and specificity of crRNA. PCR products harboring the target sequences were used as the template for the cleavage. The results indicated that each crRNA of crRNA1, crRNA2, and crRNA3 plus Cas12a protein would trigger robust cleavage with target sequences (Figure 1C). However, with the WT sequence no cleavage was observed by agarose gel electrophoresis. Collectively, these results revealed that CRISPR/Cas12a plus crRNA can distinguish variants with missense mutations.

Optimization of crRNA for CRISPR/Cas12a assay

We then compared three crRNAs based on the CRISPR/Cas12a assay to screen the best one for the detection assay. As shown in Figure 2A–C, CRISPR/Cas12a assay with crRNAs (crRNA1, crRNA2, and crRNA3) shows a similar fluorescence curve respectively when PCR products (harboring WT or H275Y mutant sequence) were used as the target template. The fluorescence signal of crRNA2 was much more obvious than that of the other two, as observed with naked eyes. In addition, fluorescence signal intensity of crRNA2 was higher than that of crRNA1 or crRNA3 (Figure 2D). Thus, we selected crRNA2 for the further assay. Surprisingly, all three crRNAs plus CRISPR/Cas12a can be activated in the presence of the WT

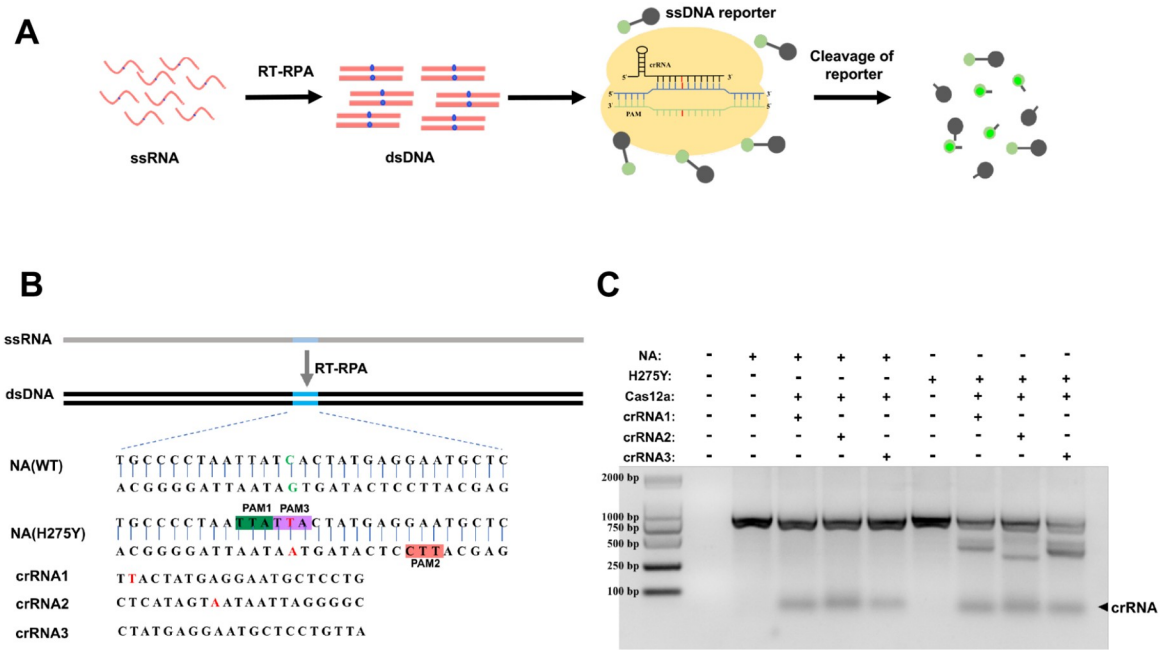


Figure 1. Molecular diagnosis of drug-resistant influenza virus based on CRISPR/Cas12a assay (A) Schematic of H275Y mutant discrimination using CRISPR/Cas12a. Specific crRNAs targeting the H275Y mutant were designed. Under the guidance of the specific crRNA, the Cas12a proteins introduce double-stranded DNA (dsDNA), and then trigger cleavage of nearby non-targeted single-stranded DNA (ssDNA). The cleavage of the reporter separates the quencher from the fluorophore, resulting in the generation of fluorescence. (B) The target sequence with the detailed mutant information of the designed crRNA. The mutant target nucleotide is highlighted in red. (C) The cleavage of WT or H275Ymutant PCR products with CRISPR/Cas12a.

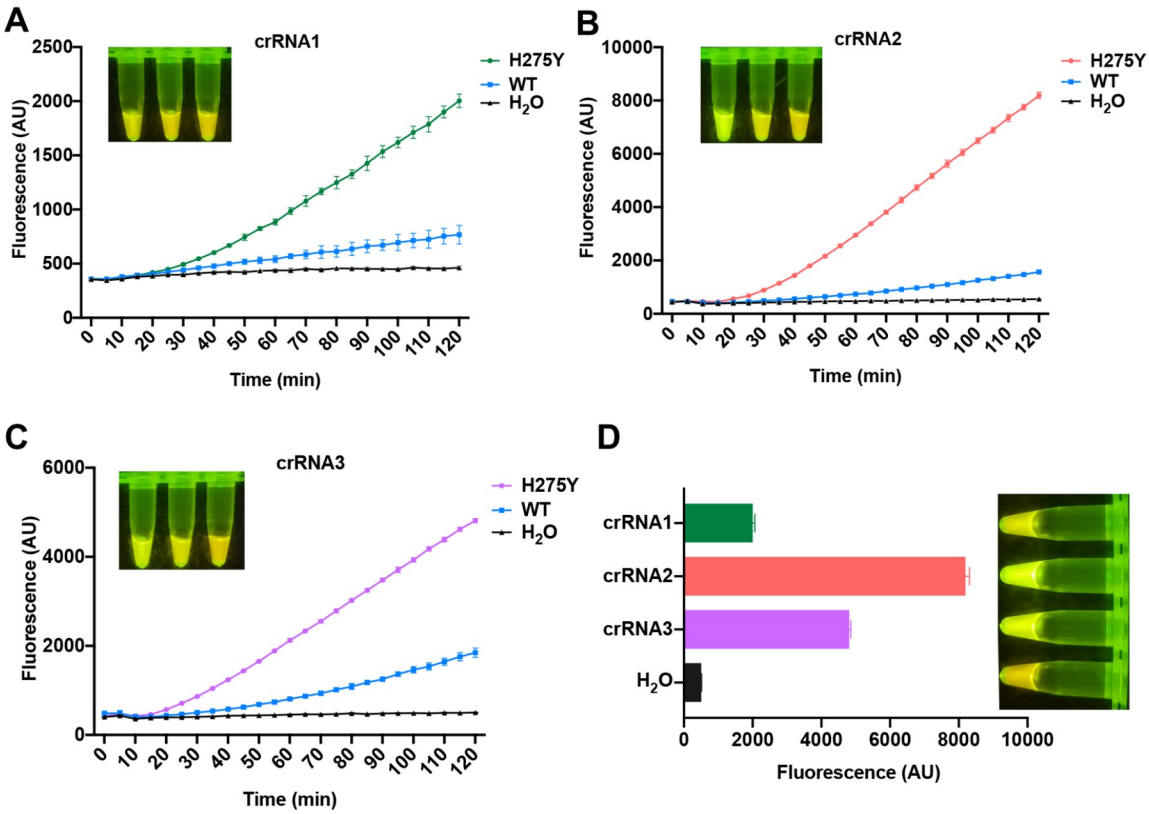


Figure 2. Optimization of crRNA for CRISPR/Cas12a assay Specificity and cleavage efficiency of (A) crRNA1, (B) crRNA2, and (C) crRNA3 with CRISPR/Cas12a fluorescent assay at different time points. (D) Optimization of crRNA for detection by comparing the fluorescence readout of three crRNA in 2 h. Data points represent independent crRNA from at least three independent experiments, and data are shown as the mean ± SD ($n = 3$).

sequence. However, compared with the mutant sequence (H275Y), the fluorescence signal from the WT sequence was substantially lower (2.6:1, 5.2:1, and 2.6:1, respectively).

CRISPR/Cas12a assay for the detection of H275Y mutant sequence

Plasmids of pJET-NA (H275Y) and pJET-NA (WT) were generated as the DNA standard to assess the sensitivity of the CRISPR/Cas12a assay. Serially diluted concentrations of 2×10^{10} copies to 2×10^{0} copies of the plasmids were evaluated, and the results indicated that the detection limit of CRISPR/Cas12a direct fluorescence readout was 2×10^{9} copies per reaction (Figure 3A). To improve the detection sensitivity, we took advantage of isothermal recombinase polymerase amplification (RPA), and then 2×10^{3} copies can be detected. Notably, the fluorescence signal can be directly visualized under a blue LED illuminator clearly (Figure 3B), which is six orders of magnitude higher than the recombinant plasmid without RPA. In addition, with the increase of dsDNA in the appropriate concentration range, the ssDNA reporter cleavage activity of RPA-CRISPR-Cas12a also increased in a linear manner (Supplementary Figure S1). Next, we detected the lentivirus genome (harboring WT or H275Y) with diluted concentrations of 2×10^{4} copies to 2×10^{1} copies by RT-RPA-CRISPR/Cas12a assay (Figure 3C,D), and found that the results are consistent with the pJET recombinant plasmids results.

Detection of H275Y mutant sequence with the CRISPR/Cas12a-LFD assay

As the sensitivity and rapid readout of CRISPR/Cas12a assay is crucial for clinical diagnosis, we introduced an ssDNA reporter labeled with a fluorophore-biotin and quencher into an *in vitro* reaction, and cleavage event signals may be detected with a lateral flow test strip reader. Firstly, we tested the sensitivity of the CRISPR/Cas12a assay with pJET recombinant plasmids (WT and H275Y) diluted concentrations of 2×10^{10} copies to 2×10^{0} copies. After the reporter concentration was reduced from 10 μM to 1 μM, the RPA-CRISPR/Cas12a assay showed a direct fluorescence readout detection limit of 2×10^{4} copies per reaction (Figure 4A,B). LFD is sensitive, which can amplify the signal, with the advantages of simplicity, rapid analysis, low cost, and ease of use without the requirement of sophisticated instrumentation [26]. Based on the CRISPR/Cas12a-LFD assay (Figure 4C), we evaluated the sensitivity of it with diluted concentrations of 2×10^{7} copies to 2×10^{0} copies. The results showed that the detection limit of CRISPR/Cas12a-LFD assay can be improved to approximately 20 copies with RPA (Figure 4D), demonstrating that RPA-CRISPR/Cas12a-LFD assay with strip reader can be rendered portable without loss in sensitivity compared to the RPA-CRISPR/Cas12a assay. In addition, with the increase of dsDNA in the appropriate concentration range, the ssDNA reporter cleavage activity of RPA-CRISPR-Cas12a-LFD also increased in a linear manner (Supplementary Figure S2). The recombinant lentivirus harboring H275Y NA sequence can be distinguished with this method (Figure 4E).

Sensitivity comparison of CRISPR/Cas12a assay or CRISPR/Cas12a-LFD assay with real-time PCR assay

To compare the sensitivity of CRISPR/Cas12a assay, CRISPR/

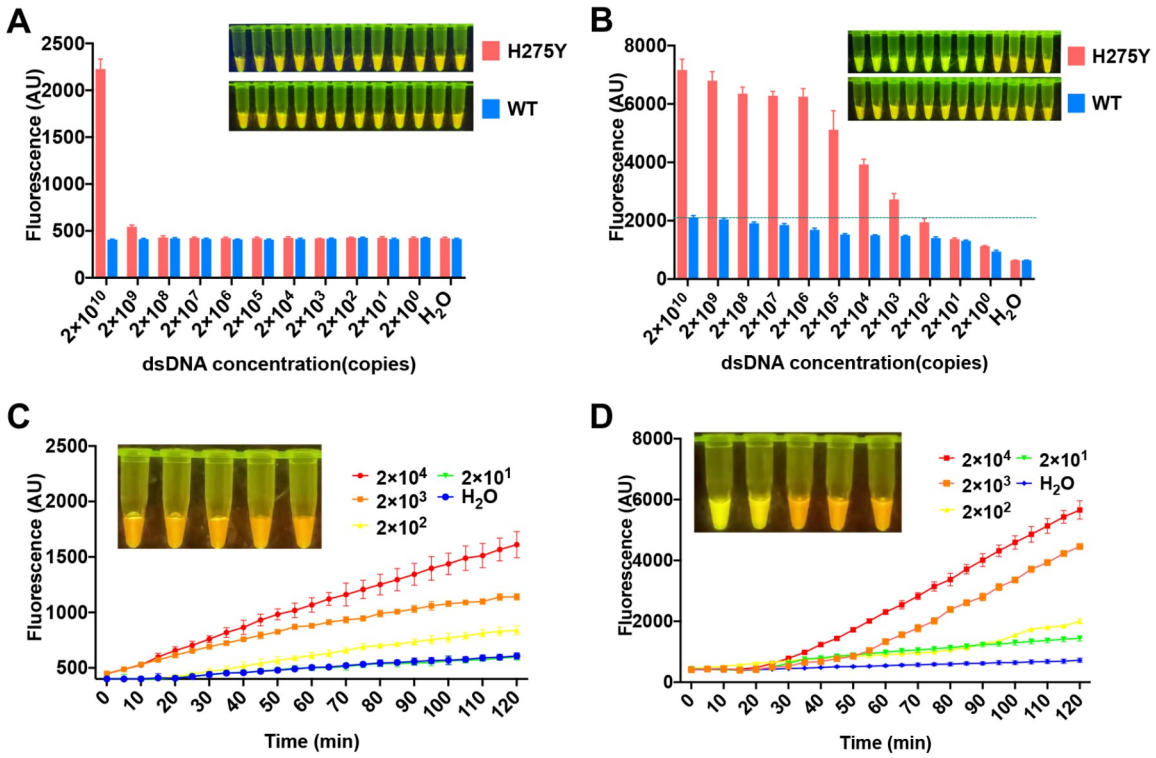


Figure 3. The sensitivity of target sequence detection with CRISPR/Cas12a assay The CRISPR/Cas12a assay detecting the serial dilution of standard plasmids without RPA (A) or with RPA (B) as the target sequence. The fluorescence signal was detected after incubation for 2 hours. The dynamic fluorescent signal of lentivirus harboring WT (C) or H275Y (D) gene fragments of influenza virus can be detected with CRISPR/Cas12a assay. The RNA of lentivirus was isolated and then amplified with RT-RPA. The RT-RPA products were used to distinguish the WT and H275Y mutant sequence with CRISPR/Cas12a assay. Data are shown as the mean ± SD ($n = 3$).

Cas12a-LFD assay and real-time PCR assay, the limit of detection (LoD) of these assays were assessed. The results showed the limit of detection of RPA-CRISPR/Cas12a was about 2000 copies, while that of RPA-CRISPR/Cas12a-LFD and real-time PCR were both approximately 20 copies (Table 2 and Supplementary Figure S3). These results indicate that the sensitivity of real-time PCR assay is similar to that of RPA-CRISPR/Cas12a-LFD assay, but higher than that of the RPA-CRISPR/Cas12a assay. However, compared with the real-time PCR assay, CRISPR/Cas12a assay and CRISPR/Cas12a-LFD assay are more convenient.

Discussion

Influenza is a contagious, acute febrile respiratory infection caused by the influenza virus that can evolve rapidly and cause pandemics and epidemics of acute respiratory disease in domestic poultry, lower mammals and humans [9]. The development of fast and efficient methods for detecting viral mutant sequences directly in clinical specimens would improve the evaluation of heterogeneity and temporal changes in drug-resistant virus populations during antiviral therapy. In addition, it can allow better management of patients. Thus, developing an ultrasensitive, economical, and portable diagnostic detection method for patients is highly valuable.

CRISPR/Cas assay was recently harnessed for rapid on-site detection [27]. Based on the CRISPR/Cas12a assay, the detection limit for coronavirus sequences was estimated to be up to 10 copies [28]. The exquisite specificity of Cas13 also enables CRISPR/Cas13 assay to identify clinically relevant viral mutations in the multiplex, such as those that confer drug resistance [19]. In this study, we sought to develop a novel detection method that combines CRISPR/Cas12a with lateral flow readout to identify the mutant virus strain. We designed three crRNA (crRNA1, crRNA2, and crRNA3) and found that crRNA2 performs better than the other two crRNAs in distinguishing a C-T single base mutation. In the cleavage of PCR products assay by agarose gel electrophoresis, we found only mutant sequence (H275Y) could be recognized with CRISPR/Cas12a plus one crRNA (crRNA1, crRNA2, or crRNA3). While, with WT sequence as target, we can also detect the fluorescence signal in the CRISPR/Cas12a assay. We speculated that this fluorescence signal may be due to the leakage from the activated Cas12a with the WT sequence and crRNA. As only one nucleotide between the WT and mutant sequence and the low fidelity of Cas12a, all three crRNAs with the WT sequence may still trigger the cleavage of ssDNA-FQ reporter with low efficiency. Encouragingly, based on the CRISPR/Cas12a assay and CRISPR/Cas12a-LFD assay (Figures 3 and 4), we found that the detection assay can clearly discriminate between the mutant (H275Y) and WT sequences. Kean *et al.* [29] has demonstrated that the fidelity of LbCas12a is very sensitive to imperfect base pairing between the gRNA and the target sequences, as any mismatch along the spacer reduces the fluorescence output to near-background level, while AsCas12a and enAsCas12a can tolerate some of the mismatches. In our study, the reason why we choose FnCas12a is that LbCas12a requires a 5′-TTTN-3′ PAM sequence which may not be present in some sequence of single base mutations. Compared with LbCas12a, FnCas12a only requires 5′-KYTV-3′ as a PAM and presents a greater choice of the target sequence [30]. We aim to design a rapid, portable and broad

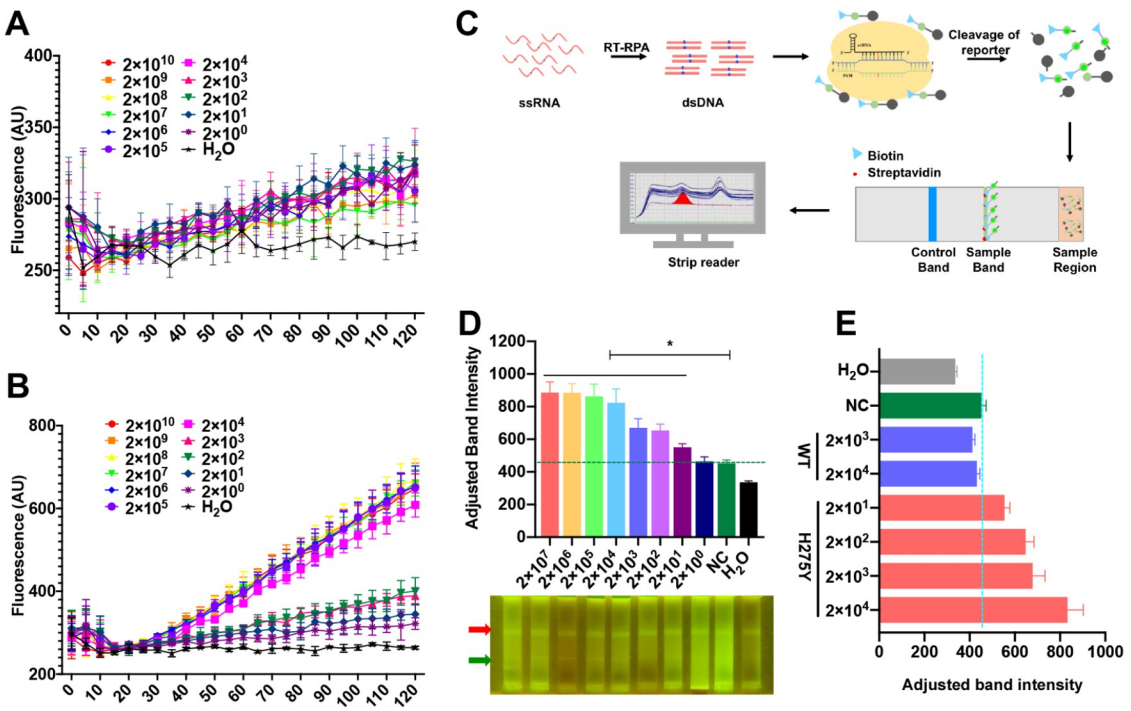


Figure 4. CRISPR/Cas12a plus lateral flow detection (CRISPR/Cas12a-LFD) for the detection of oseltamivir-resistant sequence (H275Y) Sensitivity of (A) WT and (B) H275Y detection by biotin-fluorophore-quencher (BFQ) labeled reporter assay. Diluted concentrations of 2×10^{10} copies to 2×10^{0} copies of standard plasmids (WT or H275Y mutant) with RPA were evaluated, respectively. (C) Schematic representation of target sequence detection with combination of CRISPR/Cas12a and lateral flow detection. The ssDNA reporter was labeled with biotin and fluorophore (ssDNA-BFQ-reporter) at the 5′ and 3′ termini, respectively. ssDNA-BF-reporter conjugated with streptavidin in the immunochromatographic strip was used to show the readout. The band was only shown when the BFQ-reporter was cleaved by CRISPR/Cas12a, which is activated by the target sequence. (D) The sensitivity of CRISPR/Cas12a-LFD with standard plasmids (H275Y) with diluted concentrations of 2×10^{7} copies to 2×10^{0} copies, and standard plasmids (WT) with a concentration of 2×10^{7} copies as a negative control. The green arrow indicates the test bands, while the red arrow indicates the control bands. (E) Lentivirus genome harboring oseltamivir-resistant (H275Y) or WT fragments were amplified with RT-RPA and then detected by CRISPR/Cas12a-LFD assay. Data are shown as the mean ± SD ($n = 3$). *$P < 0.05$.

Table 2. Comparison of sensitivities of different assay methods

Method	Real-time PCR	CRISPR/Cas12a	CRISPR/Cas12a-LFD
LoD (copies/μL)	20	2000	20

detection method to discriminate drug-resistant virus, it is not only applicable to H275Y, but also to additional mutations, *i.e.*, E119V, R292K or I223R [31]. So high fidelity of CRISPR/Cas12a variant, and its variant with stringent PAM recognition may be required to further improve the signal-to-noise ratio.

We showed that the detection limit of CRISPR/Cas12a-LFD can be improved to approximately 20 copies with RPA (Figure 4D), which is six orders of magnitude lower than the viral load found in the patient sample (10^7 copies/μL) [32]. With the introduction of avian myeloblastosis virus reverse transcriptase, the CRISPR/Cas12a-LFD assay may be further developed as a one-step RT-CRISPR/Cas12a-LFD assay to detect RNA targets such as SARS-CoV-2 RNA [22]. These signs of progress would greatly facilitate CRISPR/Cas12a-based RNA detection without requiring the preparation of cDNA. Here we only demonstrated mutant for oseltamivir-resistant virus (H275Y), it could be transferred to the detection of additional IAV mutations and molecular diagnose for other mutant virus strains.

In conclusion, we developed a CRISPR/Cas12a-LFD assay method to detect the oseltamivir-resistant virus (H275Y). This platform has great potential for drug-resistant virus detection, which may pave the way to allow better management of patients.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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