Facile, ultrasensitive, and highly specific diagnosis of goose astrovirus via reverse transcription-enzymatic recombinase amplification coupled with a CRISPR-Cas12a system detection

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ABSTRACT Fatal gout in geese caused by goose astrovirus (**GAstV**) has been spreading rapidly in China since 2018, causing serious economic losses in the goose breeding industry. To achieve simple, convenient and sensitive detection of GAstV, a novel diagnostic test was developed by combining reverse transcription-enzymatic recombinase amplification (**RT-ERA**) and CRISPR-Cas12a technologies. RT-ERA primers were designed to pre-amplify the conserved region of the ORF2 gene of GAstV and the predefined target sequence detected using the Cas12a/crRNA complex at 37℃ for 30 min. Specific detection of GAstV was achieved with no cross-reaction with non-GAstV templates and a sensitivity detection limit of 2 copies. The experimental procedure could be completed within 1 h, including RNA extraction (15 min), RT-ERA reaction (20 min), CRISPR-Cas12a/crRNA detection (5 min) and result readout (within 2 min) steps. In conclusion, the combination of RT-ETA and CRISPR-Cas12a provides a rapid and specific method that should be effective for the control and surveillance of GAstV infections in farms from remote locations.

Key words: goose astrovirus, reverse transcription-enzymatic recombinase, CRISPR-Cas12a, detection

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INTRODUCTION

Since 2017, outbreaks of an emerging infectious disease characterized by severe articular gout, hemorrhage and kidney swelling have been reported in 4- to 16-day-old goslings from several Chinese provinces. The incidence of the disease is up to 80% and the mortality rate is ~50%, resulting in significant economic losses in the goose industry in China. Following cell isolation and identification, as well as animal regression experiments, a novel astrovirus was identified as the main causative agent of gosling gout symptoms in 2018 (Zhang et al., 2018a,b; He et al., 2020; Wu et al., 2021). The virus was additionally detected in ducklings with similar clinical symptoms. Moreover, chickens experimentally infected with isolated GAstV displayed similar clinical characteristics as infected goslings (Chen et al., 2020; Wei et al., 2020; Li et al., 2021). These findings support the possibility of cross-species transmission of novel GAstV from infected geese to ducks and even chickens.

The ability to make immediate, specific and sensitive diagnoses at the early stages of viral infection is critical for effective control of infectious disease progression. Recently, RNA-guided CRISPR/Cas nuclease-based point-of-care testing molecular diagnostic approaches have emerged as attractive alternatives to traditional methods (Ma et al., 2020). The assay relies on the non-specific endonuclease activity of Cas13 or Cas12 upon binding of the programmable guide RNA to specific target RNA or DNA (Ding et al., 2020; Ma et al., 2020; Wang et al., 2020). The CRISPR/Cas system has been widely used to detect a variety of pathogens, including Canine parvovirus type 2 (**CPV-2**), Porcine reproductive and respiratory syndrome virus (**PRRSV**), Zika virus (**ZIKV**), Dengue virus (**DENV**), and African swine fever virus (**ASFV**) (Gootenberg et al., 2018; Khan et al., 2019; Chang et al., 2020; Lu et al., 2020; Tao et al., 2020).

Enzymatic recombinase amplification (**ERA**) is a modified version of recombinase polymerase amplification

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(**RPA**), an isothermal amplification technique for nucleic acids that can be performed at a constant temperature without the requirement for thermal cycling (Xia and Chen, 2020). In this study, a simple, rapid, specific and device-free visual assay was successfully established by combining RT-ERA isothermal amplification technology and the CRISPR-Cas12a system targeting conserved regions of the ORF2 gene of GAstV.

MATERIALS AND METHODS

Viruses and Clinical Samples

The GAstV strain AH/2018 (GenBank: MN099162) was obtained from Heifei city (Anhui province, China). The GPV strain WX3 (GenBank: MK333463), duck Tembusu virus strain AH-F10 (GenBank: KM102539.1), goose circovirus strain AH/2019 (GenBank: MN756799), duck circovirus strain AH2019 (GenBank: MN822911), and fowl adenovirus serotype 4 strain AH-F18 (GenBank: MN781665) were maintained in Anhui Province Engineering Laboratory for Animal Food Quality and Bio-safety (Anhui Agricultural University, China). The Kidney samples from goslings characterized as gout due to potential GAstV infection were collected from different areas of Anhui Province during 2020−2021. Clinical tissue samples were added to phosphate-buffered saline (**PBS**) at a ratio of 1:3, centrifuged at 12,000×g for 3 min, the supernatant was ground, and viral nucleic acids were extracted using the TIANamp Virus DNA/RNA kit according to the manufacturer's instructions (TIANGEN, Beijing, China). The GAstV RNA genome was reverse-transcribed with the aid of the SPARKscript Ⅱ One Step RT-PCR kit (SparkJade, Nanji, China), in keeping with the supplier's protocol. Viral genomic cDNA/DNA was stored at −80°C until use.

Reverse Transcription-enzymatic Recombinase primer Design and crRNA Preparation

The nucleotide sequences of 20 GAstV ORF2 genes uploaded at

NCBI were compared using DNASTAR for selection of conserved regions containing PAM (TTT) sequences. crRNA was designed using the online software CRISPR-DT (http://bioinfolab.miamioh.edu/CRISPR-DT/interface/Cpf1_main.php). The T7 promoter sequence was appended to the 5′ end of the forward and reverse primers of crRNA. RT-ERA primers designed for the conserved regions were synthesized by General Biological system (Anhui) Co., Ltd. Double-stranded DNA was purified via gel extraction after annealing of the primers, subsequently transcribed to crRNA using the T7 high-yield RNA transcription kit (Vazyme, Nanjing, China) and de-purified using the HiPure RNA Clean Pure Kit (Magen, China). Finally, the crRNA was stored at −80°C until use.

Reverse Transcription-Enzymatic Recombinase Amplification

Standard RT-ERA reactions were performed in a total volume of 50 μL using the basic RT-ERA Nucleic Acid Amplification Kit (GenDx Biotechnology, Suzhou, China). The reaction consisted of 20 μL of lysis agent, 2.5 μL of forward primer (10 μM), 2.5 μL of reverse primer (10 μM), 6 μL of template RNA, and 17 μL of ddH$_2$O. A 48 μL volume of premix was transferred to each tube of the RT-Basic Amplification Reagent, shaken and mixed until resuspension, and centrifuged at 6,000×g for 1 min. Subsequently, 2 μL of ERA activator was added to the lid of the reaction tube, the lid was closed tightly and shaken briefly to mix. Finally, tubes were incubated for 20 min at 40 to 42°C. RT-ERA amplification products were analyzed via agarose gel electrophoresis.

Cas12a-mediated Cleavage Assay

The Cas12a-mediated nucleic acid detection assay contained 2 μL NEB buffer 2.1, 250 nM EnGenLba Cas12a (Cpf1; New England Biolabs, Ipswich, MA), 500 nM crRNA, 1 μM single-stranded DNA fluorophore quencher-labeled probe sensor, and 4 U RNase inhibitor with 10 ng GAstV plasmid pMD-19T-ORF2 in a total reaction volume of 20 μL. The reaction tubes were incubated at 37°C on a constant temperature water bath for 30 min and photographed immediately using a Bio-Rad Molecular Imager Gel Doc TMXR+ imaging system with a built-in UV channel.

Comparison of Visual Reverse Transcription-enzymatic Recombinase-Cas12a and Fluorescent Quantitative Polymerase Chain Reaction Assays for Clinical Sample Detection

Thirty clinical samples were numbered and tested using the previously established goose astrovirus qPCR primers and reaction system (Yang et al., 2020), and their positivity rates were counted. Simultaneously, clinical samples were subjected to the RT-ERA-Cas12a assay to compare the detection compliance of the two methods.

RESULTS

Visual RT-ERA-Cas12a System Build Up

Principle of the CRISPR/Cas12a-based fluorescence platform for the sensitive and specific detection of GAstV is shown in Figure 1. To improve detection sensitivity, we designed 5 pairs of crRNA primers for screening the ORF2 gene. For evaluation of their efficiency, individual crRNAs were mixed with Cas12a, GAstV plasmid pMD-19T-ORF2 and DNA fluorophore quencher (ssDNA) reporters, respectively, and reacted at 37°C. crRNA with a 23-nucleotide spacer that was completely complementary with specific double-

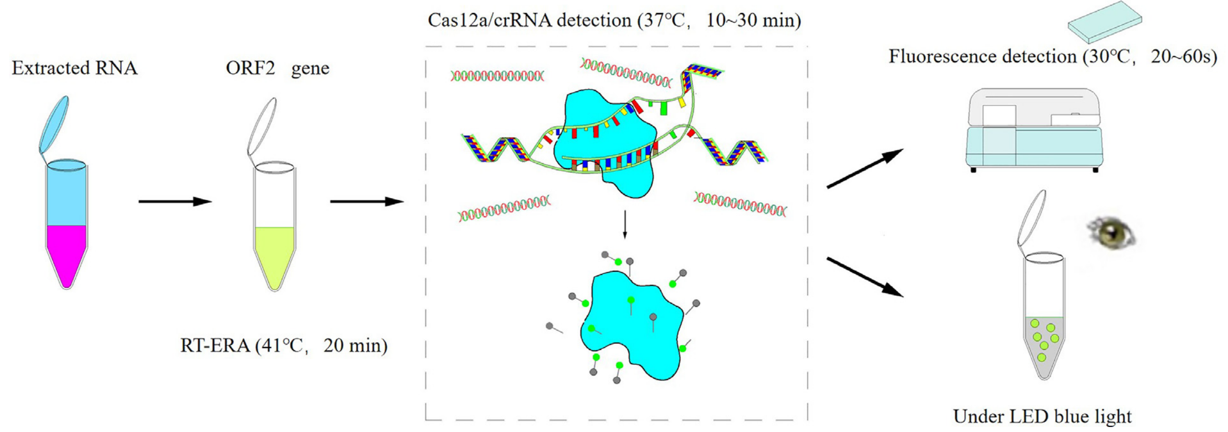

Figure 1. Schematic diagram of CRISPR-Cas12a coupled with a reverse transcription-enzymatic recombinase amplification assay for enhancement of fluorescence and visualization.

stranded DNA sequence (Figure 2A). Three of the 5 crRNAs displayed fluorescence intensity, among which crRNA-3 produced the strongest signal (Figures 2B,C). The ssDNA-FQ reporter could only be efficiently cleaved (based on the released fluorescence signals) in the presence of GAstV and the Cas12a/crRNA complex (Figure 2C). Thus, crRNA-3 was selected for GAstV detection in subsequent experiments.

Optimization of the Cas12a-mediated Cleavage Assay

Thereafter, we optimized the reaction time for the Cas12a-mediated cleavage assay. Nucleic acid samples containing 500 copies RNA templates were prepared, and after amplification via RT-ERA, products were subjected to Cas12a/crRNA detection. RNase-free ddH_2O was used as a negative control. The results of Cas12a/crRNA reads were analyzed at 0, 1, 5, 10, 15, 20, 25, 30, 35, 40 min using a real-time fluorescence instrument and LED blue light. As shown in Figure 3, fluorescence intensity was observed after 1 min of incubation. Fluorescence values gradually increased with time. After the reaction had proceeded for 30 min, fluorescence showed a plateau with no significant changes. Accordingly, the optimal cleavage time for the Cas12a/crRNA assay was taken as 30 min.

Sensitivity and Specificity of the Cas12a-mediated Cleavage Assay

We used 10-fold serial dilutions of standard plasmid pMD-19T-ORF2 as the template to examine the

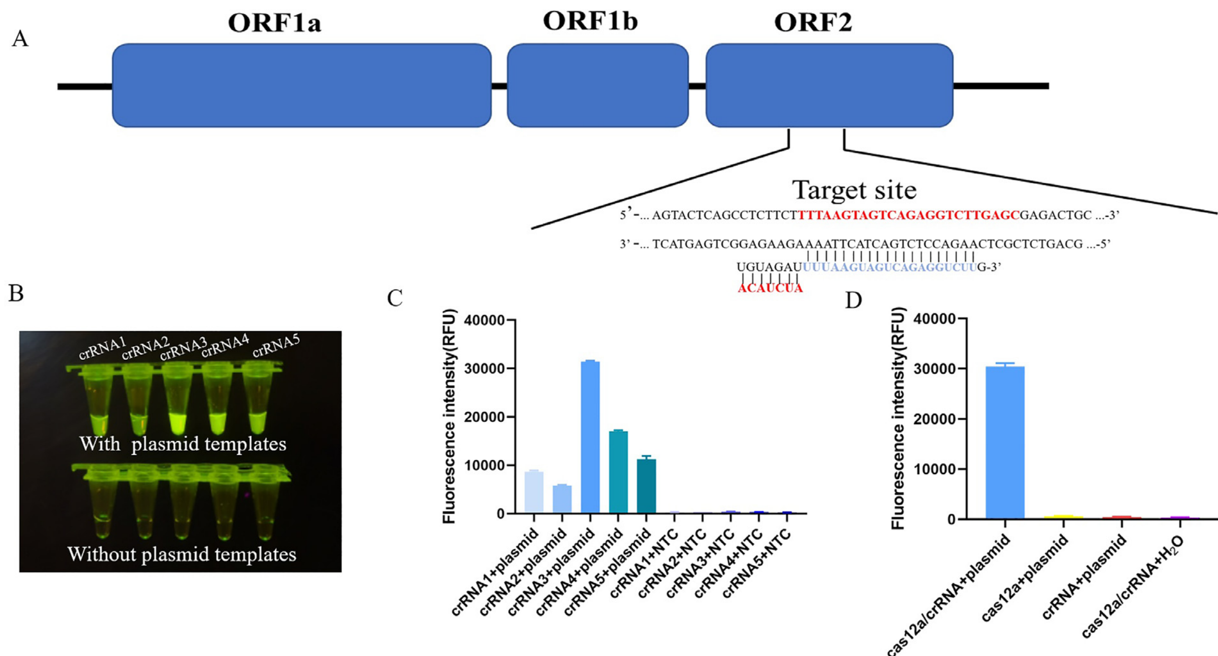

Figure 2. Feasibility analysis of the Cas12a platform for plasmid detection, Specific validation and screening of five GAstV crRNAs designed for the ORF2 gene. (A) Design of the crRNA sequence based on the ORF2 gene of GAstV. (B,C) ORF2 gene was recognized by corresponding five pairs of crRNAs. The fluorescent images and intensities at 30 min of the reaction were shown. (D) Fluorescence analysis of the feasibility of the Cas12a platform for nucleic acids detection.

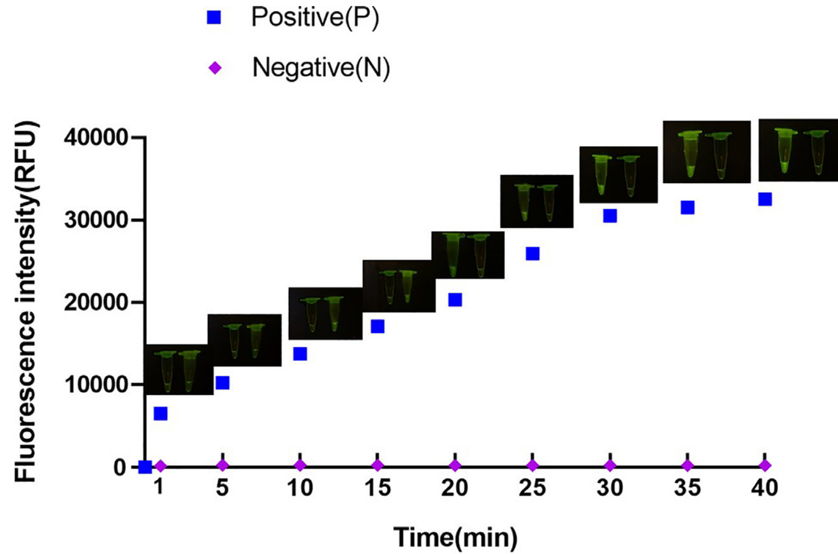

Figure 3. RT-ERA coupled with Cas12a at optimization of timing of the Cas12/crRNA reaction.

sensitivity of Cas12a-mediated cleavage under LED blue light or a UV transilluminator. Repeat three times for each dilution with good repeatability and stability. The assay could successfully detect plasmids containing the ORF2 gene down to 2 copy numbers, both under blue light and fluorescence detection (Figure 4 A, B), which was more sensitive than our previously established SYBR Green I-based method. In addition, we evaluated the specificity of RT-ERA-Cas12a for GAstV detection. GAstV was specifically detected with no cross-reactivity to other viruses using the one-step Cas12a assay (Figure 4C, D).

Evaluation of Clinical Samples Using Reverse Transcription-enzymatic Recombinase-Cas12

Thirty tissue samples from goslings with gout symptoms from Anhui Province (China) were collected and tested for GAstV infection using both RT-ERA-Cas12 and real-time PCR. The positive detection rate of RT-ERA-Cas12 was the same as that of real-time PCR, with 10 of 30 samples showing positive results, a detection rate of 33.33% (10/30) (Figure 5). These findings clearly indicate that our novel method involving RT-

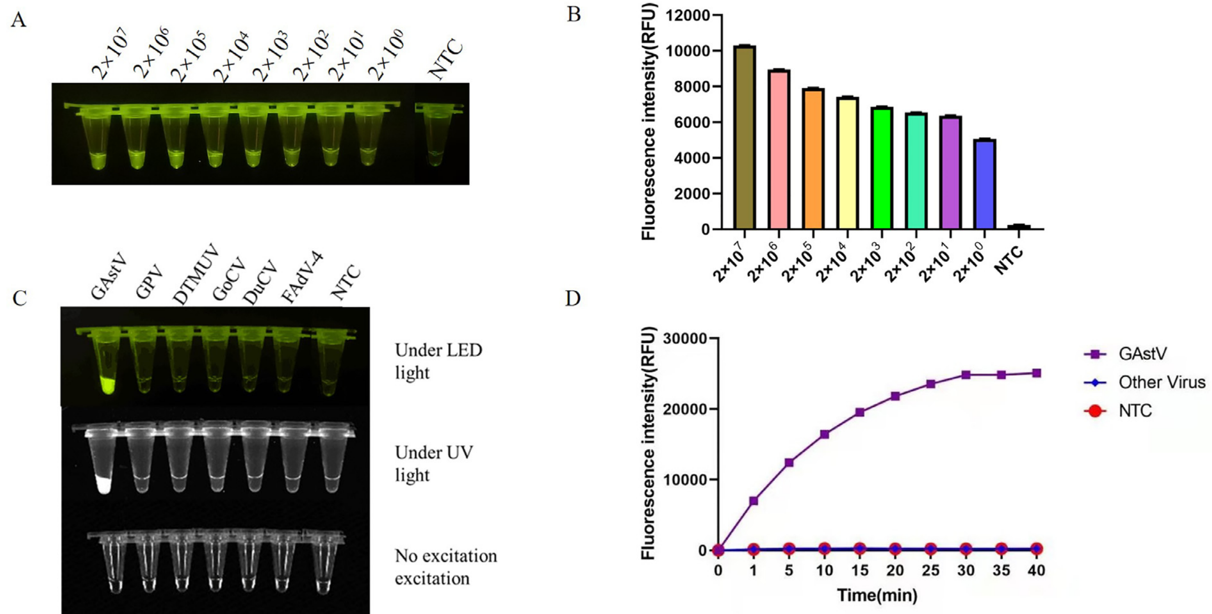

Figure 4. Validation of the specificity and sensitivity of Cas12a/crRNA for the detection of GAstV. (A) Validation of crRNA sensitivity reacts with the target gene. Sensitivity assay using crRNA to detect gradient ORF2 gene plasmid from 2×10^7 to 2×10^0 copies. (B) The fluorescence intensity of different dilution copy numbers at 30 min was shown. (C) Validation of crRNA specifically reacts with the target gene. (D) GAstV and other virus were detected Fluorescence intensity at every 5 min.

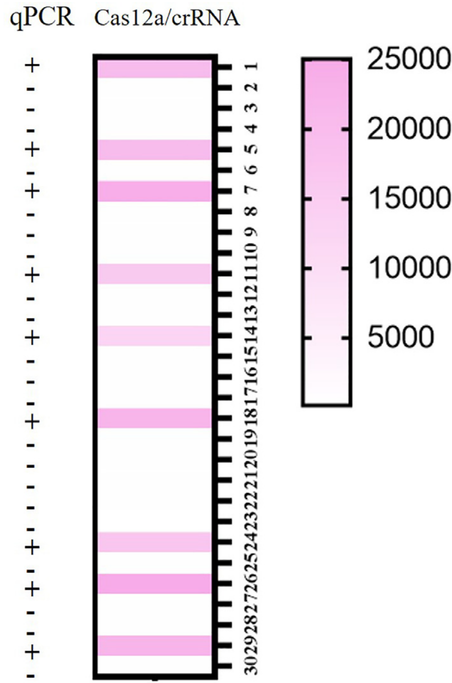

Figure 5. Detection of GAstV in 30 clinical samples by the conventional culture method (qPCR) and Cas12a/crRNA assay. The RT-ERA-based Cas12a heatmap represents normalized mean fluorescence values. Lanes 1−30: the clinical sample number.

ETA coupled with CRISPR-Cas12a is comparable to real-time fluorescence PCR and suitable for point of-care testing (**POCT**) as no equipment is required and the results can be obtained within a relatively short time-period of 1 h.

DISCUSSION

GAstV outbreaks primarily causing gout in goslings have been ongoing in China since 2018. Gosling gout has been widely reported in the Chinese provinces of Hunan, Fujian, Guangdong, Henan, Liaoning, Zhejiang, Jiangsu, Shandong and Anhui, and breeding geese infected with GAstV can transmit the virus vertically to their offspring, resulting in huge economic losses in the goose breeding industry (Jin et al., 2018; Yang et al., 2018; Yang et al., 2020; Wang et al., 2020a,b; Wei et al., 2020a,b). However, the majority of farms are located in remote areas and lack sophisticated and expensive instruments and professional testing personnel. A portable and instantaneous detection method should facilitate effective prevention and control of disease transmission. The development of rapid, simple, and convenient diagnostic tools for the detection of GAstV with high sensitivity and specificity remains an urgent requirement (Table 1).

To date, multiple procedures have been developed for diagnosis of GAstV. Yuan et al. (2018) established a highly specific reverse transcription-quantitative PCR (**RT-qPCR**) method for rapid and accurate diagnosis and monitoring of GAstV in clinical samples with minimum sensitivity detection as low as 52.5 copies/μL. Ji et al. (2020) reported a sensitive and rapid method for GAstV detection based on one-step reverse transcription loop-mediated isothermal amplification (one-step RT-LAMP) using a reverse transcription-active Bst 3.0 DNA polymerase and a primer set targeting the GAstV open reading frame 1b (**ORF1b**) with a 30 min incubation in water at 61℃.

In the current study, RT-ERA and Cas12a technologies were combined with the aim of developing a simple and rapid test for GAstV detection in remote areas, without the need for expensive equipment. Compared to LAMP and TaqMan-based fluorescence quantification, our method does not require high temperatures; the reaction can be performed at room temperature and completed within 1 h. In addition, detection is more sensitive than for other methods (as low as 8 copies).

CONCLUSION

We have established a novel method that combines RT-ERA with the Cas12a-mediated cleavage assay in a single reaction for the detection of GAstV with significant advantages, including high sensitivity and specificity, easy operation, short experimental time and the requirement for less equipment. The assay detection process only requires a portable heater for the reaction and

Table 1. The crRNA, primers, and probes used in this study

Primers	Sequences (5'-3')	Size/bp
ORF2-F	ATGGCAGACAGGGCGGTGGC	2115
ORF2-R	TCACTCATGTCCGCCCTTCTC	
RT-ERA-F	CAGGTCGACGATTATGGCAGACAGGGCGGT	230
RT-ERA-R	TCGTGGTAGTCATTTTGTCATTAACGGGTG	
crRNA-1-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATACAGTTAGTGGCGCATTGCA	66
crRNA-1-R	TGCAATGCGCCACTAACTGTATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC	66
crRNA-2-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATTGATCACGAACCATCCTCCT	66
crRNA-2-R	AGGAGGATGGTTCGTGATCAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC	66
crRNA-3-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATAGTAGTCAGAGGTCTTGAGC	66
crRNA-3-R	GCTCAAGACCTCTGACTACTATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC	66
crRNA-4-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATTCAGACAGTTCCACCGAACA	66
crRNA-4-R	TGTTCGGTGGAACTGTCTGAATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC	66
crRNA-5-F	GAAATTAATACGACTCACTATAGGGTAATTTCTACTAAGTGTAGATGAGGGTTCAACCCAGGTTGA	66
crRNA-5-R	TCAACCTGGGTTGAACCCTCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC	66
Probe	FAM-N6-BHQ1	

visual blue light. However, the clinical potential of our newly developed method requires further evaluation.

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DISCLOSURES

The authors declare no conflict of interest

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102208.

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