Research Article

Development of Real-Time Fluorescence CRISPR/Cas12a-Based Detection as a Portable Diagnostic System Using Integrated Circuits

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Abstract
A solution for achieving high-performance measurements in a space-constrained experimental setup was developed as a portable incubating instrument for real-time fluorescence detection of *AvrPi9* gene in rice blast fungus by using a calibrated spectrometer in CRISPR-Cas12a detection. The system demonstrates accurate temperature control with low energy consumption and low deviation of ±0.16 °C from the setpoint temperatures, with high sensitivity and accurate detection within 10 min. The CRISPR-Cas12a detection reaction was demonstrated using *AvrPi9* PCR product, crRNAs, LbCas12a and fluorescence-quencher reporter incubating at 37 °C for 10 min. Calibrated C12666MA spectrometer with 480 nm and 520 nm LEDs vs HR4000 reference exhibits low RMS of 0.54 and 1.30 and drift of 6.4 nm and 4.84 nm, respectively indicating high accuracy and reliability in fluorescence detection. Fluorescence signals were observed under an LED transilluminator, while real-time analysis was conducted through spectrometric measurements upon excitation by a 480 nm high-intensity blue LED source. Accuracy of detection between positive, non-template and non-target control was reported with no incidence of false positives observed. The instrument exhibits reliable quantitative detection capabilities with a limit of detection of 3.8 ng of DNA targets that are comparable to when running the same reaction on a commercial real-time PCR, with a detection limit of 1 ng. This study demonstrates that the CRISPR-Cas12a detection method represents a significant breakthrough in molecular diagnostics due to its advantages of rapidity, high sensitivity, and convenience allowing for the development of a compact, and energy-efficient platform that can facilitate real-time on-site diagnostics with accurate temperature control.

Keywords: Fluorescence, CRISPR-Cas12a, *AvrPi9*, Calibration, Diagnostic

1 Introduction

Conventional polymerase chain reaction (PCR) and quantitative PCR, gold standards for molecular detection, were demonstrated as reliable diagnostic tools for detecting plant pathogens in specific genome regions. However, the specialized laboratory equipment, need for skilled personnel, complicated protocols, prolonged waiting times for results, and high cost per test may limit their utility in resource-limited settings of developing countries [1]. Loop-mediated isothermal amplification (LAMP) offers a promising alternative that amplifies target genes at constant temperature at 63–65 °C and yields detectable fluorescent DNA

products. However, the complexity of LAMP primers and the potential for false positives should be carefully considered [2]–[4].

The recent advancements in clustered regularly interspaced short palindromic repeat (CRISPR)-Cas technology represent a new strategy for nucleic acid detection, utilizing Cas effector proteins (e.g., Cas9, Cas12, and Cas13) as effective identifiers, exhibiting cleavage activity upon crRNA-guided recognition of specific target sequences leading to collateral cleavage of single-stranded DNA (ssDNA) molecules. This approach employs a reporter-quencher system, in which the separation of the fluorescent dye and quencher upon ssDNA cleavage results in increased fluorescence, enabling rapid and on-site analysis for various diagnostic applications [5], [6]. The CRISPR-Cas12a detection is demonstrated at approximately 37 °C, eliminating the need for additional instruments. In DNA detection, the LbCas12a/crRNA recognizes the target DNA and cleaves the fluorescence-quencher labeled oligonucleotides (FQ-reporter), subsequently releasing fluorophores, which emit fluorescence for analysis. Due to its simplicity, efficiency, and cost-effectiveness, the CRISPR-Cas12a DNA testing method holds great potential for straightforward application in field settings, particularly for diagnosing crop diseases [2]. In recent works, using 30–40 min detection, real-time CRISPR-Cas12a-based detection has a detection limit of $10 – 10^4$ copy number/μL with high specificity in clinical studies found in SARS-CoV-2 patients, which traditional RT qPCR detection methods may not be sensitive enough [7]–[9].

As a sample to detect, rice blast disease, caused by *Magnaporthe oryzae*, is the single most catastrophic plant disease that threatens strength of rice production worldwide and affects rice yields and grain quality, resulting in massive economic losses yearly [10], [11]. The avirulence genes in this fungus share a gene-for-gene relationship with the resistance genes in its host rice [12]. *AvrPi9* is a gene that encodes an avirulence (AVR) effector protein that is produced by the rice blast fungus *Magnaporthe oryzae*. *Avr* proteins are molecules that are recognized by host resistance (R) proteins, which trigger a defense response that prevents the fungus from infecting the plant. The *Pi9* gene encodes a broad-spectrum resistance protein that recognizes *AvrPi9* and triggers resistance to a wide range of rice blast isolates [13], [14]. Understanding this gene is beneficial to develop effective strategies for controlling rice blast disease and ensuring food security [15].

Traditional techniques, such as PCR and LAMP assays, are not practical due to their substantial equipment requirements and intricate operational procedures. Recombinase polymerase amplification (RPA) can be an alternative technique for nucleic acid amplification for its rapid, on-site testing and constant temperature, which we will use in further research and development. Currently, CRISPR-Cas-based detection systems offer a simplified approach to nucleic acid identification. This paradigm shift enables the integration of on-site diagnostic, circumventing the need for complex methodologies while maintaining accuracy, sensitivity, and rapid turnaround times. CRISPR-Cas can be performed at a constant temperature in less than 20 min enabling target-specific cleavage detection through a fluorescent readout [16], [17].

In scientific terms, instrument calibration is crucial as it enables accurate validation against established standards, thus minimizing errors. Calibration involves comparing the instrument to primary, secondary, or known input sources [18]. For precise real-time fluorescence CRISPR-Cas12a detection, spectrophotometry and temperature calibration are important to ensure accuracy by comparing to a known standard (520 and 480 nm LED).

In this study, we developed a portable incubating instrument for detecting *Avr* gene/allele of the rice blast fungus, using *AvrPi9* DNA target as a model. The target region was amplified by PCR, followed by the detection of PCR product, and the test result is analyzed in fluorescence, FAM signal within the 517 nm emission range upon excitation with a 480 nm high intensity blue LED source, was measured by a real-time UV-visible spectrometer, processed by a microcontroller inside the detection device and displayed on the connected USB laptop in real-time. These improvements in real-time CRISPR-Cas12a detection demonstrate a higher efficient method in the detection of rice blast, making it suitable for the on-site diagnostics of other diseases in the future.

2 Materials and Methods

2.1 *Preparation AvrPi9 gene PCR product*

Genomic DNA of two *M. oryzae* isolates, 10100 and

NYK56003, was extracted from mycelium using CTAB method. 342 bp region of *AvrPi9* gene was amplified using *AvrPi9* specific primers (forward: 5'-CAGCCCAACATGCAGTTCTC-3' and reverse: 5'-CTACCAGTGCGTCTTTTCGAC-3') [14]. The amplification was executed in a 50 µL reaction mixture, consisting of 5 µL of 10X PCR buffer, 2 µL of 50 mM MgCl2, 1 µL of 10 mM dNTP mixture, 2 µL of 5 µM of each primer [14], 0.5 µL of Taq DNA polymerase (Vivantis Technologies, Malaysia), and 50 ng/µL of *M. oryzae* DNA, with the process carried out in an Eppendorf Mastercycler® nexus gradient thermal cycler (Eppendorf Co, USA) under a specific cycling protocol: initial denaturation at 95 °C for 5 min, followed by 35 cycles comprising 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing), and 72 °C for 1 min (extension), and a final extension step at 72 °C for 5 min.

The PCR product was analyzed by 1% agarose gel electrophoresis and purified using a PCR purification kit (BIO-HELIX, Taiwan). The quality of purified PCR product was determined via 1% agarose gel electrophoresis, quantified using NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.2 *CRISPR-Cas12a fluorescence-based detection*

The CRISPR-Cas12a detection reactions targeting *AvrPi9* gene/allele were carried out in 20 µL volume, comprising 2 µL of 10X NEBuffer r2.1 Reaction Buffer, 1 µL of 1 µM EnGen® Lba Cas12a (Cpf1) (New England BioLabs, USA), 1 µL of 1 µM crRNA: 5'UAAUUUCUACUAAGUGUAGAUCCCGUCU UGUGGCAGUUGGGG-3' [15], 1 µL of 10 µM FQ reporter: 5'- /56-FAM/ TTATT /3BHQ_1/ -3' (Integrated DNA Technologies, USA) and 50 ng PCR product of isolate 10100, isolate NYK56003 and ddH20 were used as *AvrPi9* target DNA, non-target DNA and non-template controls (NTC), respectively. To prevent enzyme degradation, reaction mixes were maintained on ice. The reactions were carried out in 0.1 mL PCR microtubes (PakGent Bioscience, China) and incubated at 37 °C for a 10 min duration and included the PCR reaction time of 80 min.

During a reaction, LbCas12a/crRNA will recognize target DNA leading to the cleavage of FQ-reporter. As a result, the FAM is released from the FQ-reporter and generates a fluorescence signal. The increase in fluorescence serves as a readout for the presence of the target DNA in the sample. This signal can be visually observed using an LED transilluminator and quantitatively measured via real-time detection systems employing a spectrometer. PCR method can be used to amplify target DNA copies to the amount that can be detected as a selective in-lab method. For on-site diagnostic, RPA coupled with CRISPR-Cas12a detection can be more efficient, rapid, and no need for complicated equipment. And the total operation time including reaction preparation, DNA amplification and CRISPR-Cas12a detection can be reduced from about 2 h to 1 h. Figure 1 shows the CRISPR-Cas12a detection protocol.

2.3 *CRISPR-Cas12a detection instrument*

2.3.1 Temperature control

An aluminum incubator had a hole (8 mm deep, 5 mm diameter) to accommodate a 0.1 ml PCR microtube in each device, with a through hole to conform to the microtube's taper shape and allow the bottom 2.5 mm diameter, 3 mm deep to protrude. A DS18B20 one-wire thermometer sensor with 0.5 °C accuracy was inserted into another hole (6.2 mm diameter) to monitor the reaction temperature, and record temperature data every 1 s. DS18B20 was employed a calibration process to assess the accuracy and precision of measurement, where the sensor was subjected to compare with higher accuracy equipment to determine its measurement error by measuring in the temperature-controlled incubator in a range of 30–40 °C in ten replicates. A thermoelectric heater tube (5 mm diameter) supplied by an isolated 12V 3A power source was controlled by a pulse-width modulation (PWM) signal from a PWM module, maintaining a constant temperature of 37 °C for 10 min during the CRISPR-Cas12a detection. The PWM duty cycle was updated every second by employing a modified proportional-integral control algorithm, which adjusted the system based on the deviation between the observed temperature and the desired set point [19]. Temperature value and PWM signal were operated by Arduino IDE software regulated by an Arduino Uno microcontroller.

In Figure 2, the temperature control diagram of the system, an Arduino microcontroller acquires data from DS18B20 temperature sensor within the

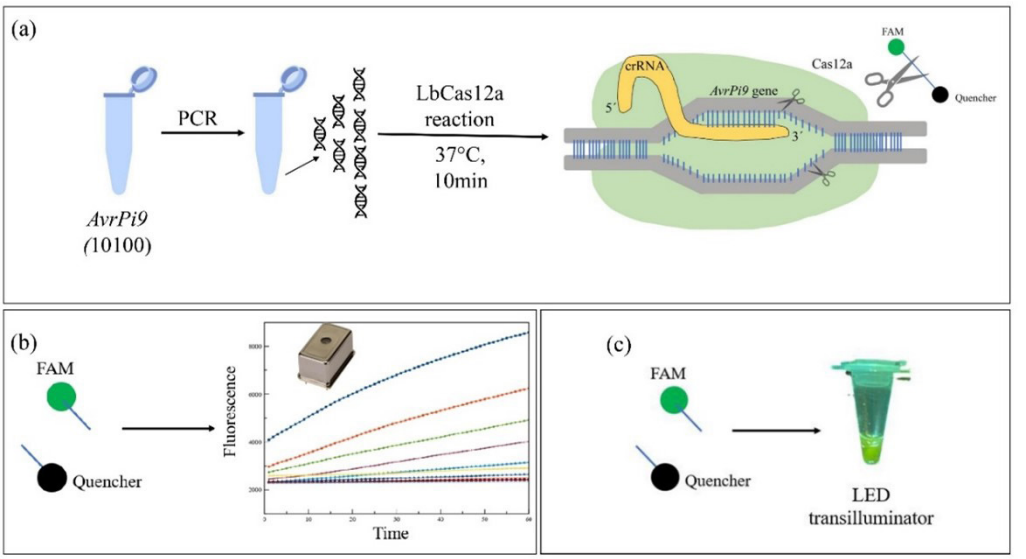

Figure 1: Assay workflow of the CRISPR-Cas12a detection of *AvrPi9*: (a) Target DNA is amplified by PCR amplification, followed by the detection, LbCas12a/crRNA recognizes target DNA leading to the cleavage of FQ-reporter, (b) Measurement of fluorescence signal from FQ-reporter by spectrometer in CRISPR-Cas12a detection, (c) Fluorescence from cleavage of the reporter is visualized under LED transilluminator.

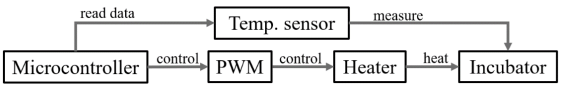

Figure 2: Temperature control diagram of the device for CRISPR-Cas12a detection.

incubator, regulating PWM module. The PWM signal is manipulated to minimize the deviation between the actual temperature and the desired setpoint of 37 °C. When the system's temperature falls below 37 °C, the PWM module transmits a signal to activate the heating mechanism, elevating the temperature; conversely, when the temperature exceeds 37 °C, the PWM module transmits a signal to the heater to reduce the temperature. The adjustment of the PWM cycle refines the alignment of the actual temperature with the desired setpoint according to the implemented algorithm.

2.3.2 Real-time fluorescence measurement

In detection, fluorescent measurements were performed using a UV-visible C12666MA spectrometer (Hamamatsu, Japan) with a spectral response range of 340–780 nm, a resolution of 15 nm, and a 256-pixel detector. The excitation source was a high-intensity blue 480 nm LED (Cree, Inc.) positioned at the bottom of the setup with an angle of 90° directed through an aperture in the incubator, enabling real-time analysis of PCR microtubes as depicted in Figure 3. The spectrometer was interfaced with an Arduino microcontroller to control and display spectral values in real-time. C12666MA spectrometer was programmed to analyze the 520 nm channel only every 1 s, focusing on the FQ reporter cleavage range of 517 nm FAM emission over a 10 min reaction duration.

As a reference method for quantitative analysis, the real-time PCR platform [Figure 3(d)] employed in this study, namely the CFX Connect system offers a precise temperature control range of 0 to 100 °C with an accuracy of ±0.2 °C, ensuring optimal thermal cycling conditions for nucleic acid amplification. Additionally, the system provides a heated lid up to 105 °C, preventing sample evaporation during the reaction process. This platform is equipped with a broad spectrum of excitation/emission wavelengths ranging from 450–580 nm, enabling the detection and differentiation of multiple fluorophores. Furthermore, the CFX connect system demonstrates exceptional sensitivity, with the capacity to detect as low as a single copy of the target

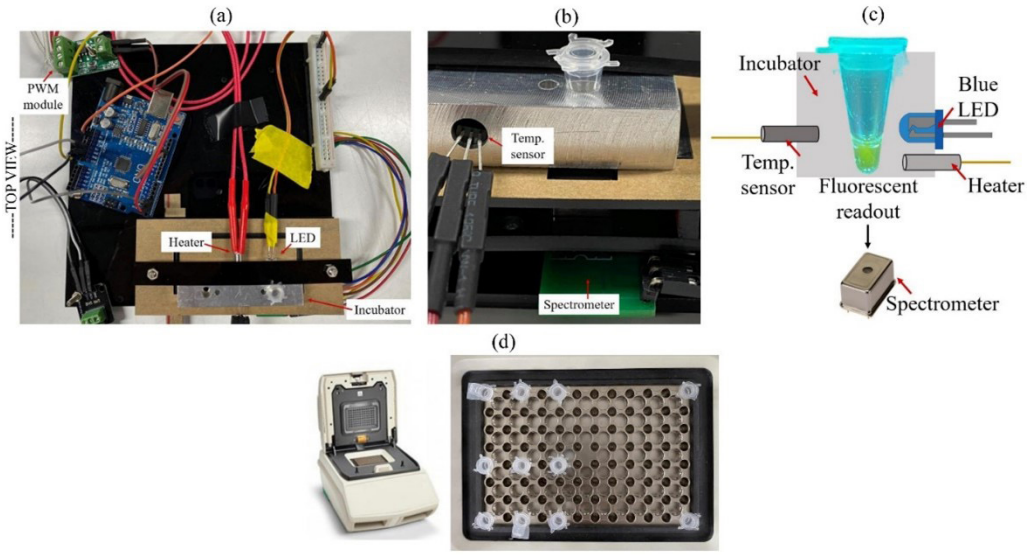

Figure 3: Real-time system (a) Schematic of the real-time fluorescence detection system, (b) The front view of the fluorescence detector, (c) Diagram of the real-time fluorescence detection system, (d) The real-time PCR, CFX Connect system (Bio-Rad).

nucleic acid sequence, ensuring accurate quantification of target molecules in the samples [20].

In the field of metrology, a calibration of the C12666MA spectrometer was carried out using two reference LEDs with distinct wavelengths of 480 and 520 nm (Farnell), respectively, specifically targeting the fluorescence resonance energy transfer process involving the FQ-reporter with a cleavage range corresponding to a 517 nm FAM emission wavelength. This calibration process was conducted in parallel with an HR4000 spectrometer (Ocean Insight), which served as a reference standard for comparative purposes. The primary objective of this study was to assess the repeatability and stability of the C12666MA spectrometer, as well as its suitability for fluorescent measurements in the context of this research. A calibration of fluorescent measurement by comparison between an HR4000 UV-NIR spectrometer and a C12666MA UV-visible spectrometer with ten replicate measurements of two LEDs was performed under ambient conditions. These replicate measurements were provided to minimize the impact of random errors and fluctuations that could influence the results and ensure the reliability and consistency of the data. The root mean square (RMS), as defined by Equation (1), was calculated to quantify the deviations for the set of ten measurements of a spectrometer. It proves to be a useful way to characterize the reliability of the measurements [21]. Thus, the drift in wavelength measurement of a spectrometer was estimated by comparison of the spectral curves of two reference LEDs measured by the HR4000.

$$RMS = \sqrt{\frac{\Sigma(x_i - \overline{x})^2}{N}} \quad (1)$$

where x_i = measured value, $\overline{x}$ = averaged value of measurements, N = number of measurements

2.4 *Accuracy of detection*

To evaluate the accuracy of CRISPR-Cas12a detection of fluorescence in real-time, the reaction was prepared with the total volume of 20 µL using 50 ng PCR product with *AvrPi9* (10100) as a positive reaction, one without *AvrPi9* (NYK56003) and non-template control (NTC) as a negative control. The samples were incubated at 37 °C for 10 min. To ensure the reliability of the results and enable comparative analysis, each reaction was performed in triplicate and the mean values of the fluorescence signals were calculated. The obtained fluorescence curves were then analyzed.

The same reactions were demonstrated in the real-time PCR in triplicate as a reference method to compare the detection accuracy between the CRISPR-Cas12a system and the reference real-time PCR approach.

2.5 *Limit of detection (LoD)*

The sensitivity of CRISPR-Cas12a detection by real-time fluorescence measurement was evaluated by using a spectrometer. Assay sensitivity, defined as the lowest concentrations that yielded positive reaction in triplicates, was determined by performing 2-fold serial dilutions of DNA targets. The reaction was prepared with a total volume of 20 µL containing 2 µL of 10X NEBuffer r2.1 Reaction Buffer, 1 µL of 1 µM EnGen® Lba Cas12a (Cpf1), 1 µL of 1 µM crRNA (New England BioLabs, USA), 1 µL of 10 µM FQ reporter, and 1 µL of diluted PCR product and incubated at 37 °C for 10 min. The fluorescence curve from the FQ reporter was analyzed and compared to real-time PCR as a reference method.

2.6 *Statistical analysis*

Curve fitting analysis was employed to model the relationship between fluorescence values and DNA concentrations. The resulting standard curve was then analyzed using statistical methods to determine the detection limit of DNA in the detection system and real-time PCR. The linear model $y = mx + c$ was demonstrated, where y is the fluorescence values and x is DNA concentration, m is slope and c is intercept.

3 Results and Discussion

3.1 *Thermal performance of the system*

The DS18B20 temperature sensor (±0.5 °C accuracy) was calibrated by comparison unit under test into stabilized calibration bath with a Standard Platinum resistance probe as a reference for assessing the performance of the DS18B20 sensor. The root mean square error (RMSE) was determined to be 0.3 °C to a reference. To ensure consistent and precise temperature control, the sensor was integrated into a microcontroller-based temperature control system that enabled accurate tracking of the 37 °C reaction setpoint as shown in Figure 4, with a 10 min short rise time and an RMSE from a setpoint of ±0.16 °C. The sensor was selected for its high precision and low error rate to ensure accurate monitoring of the temperature throughout the duration of the experiment. This low deviation value was the efficiency and effectiveness of the system in maintaining accurate temperature control and ensuring the reliability of the data generated under these controlled conditions. In the steady-state phase, for a duration of 1 h, the temperature control system successfully sustained the target temperature of 37 °C. This level of precision is crucial in ensuring the reliability and reproducibility of the experimental results, as it minimizes the influence of temperature fluctuations on the reaction outcomes. The high accuracy of the sensor and the microcontroller-based temperature control system's performance contributed to the robustness of the experimental setup of real-time fluorescent CRISPR-Cas12a detection.

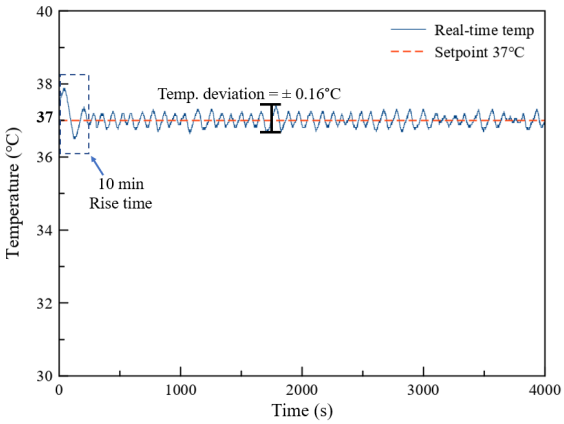

Figure 4: Temperature curve during real-time detection: real-time temperature (-----) set point temperature (- - -).

The integration of microcontroller allows for the optimization of assay parameters, such as temperature, reaction time, and reagent concentrations, to achieve the desired level of sensitivity and specificity. Furthermore, the microcontroller's computational capabilities enable real-time data processing and analysis, facilitating rapid and reliable diagnostic outcomes.

3.2 *Calibration of the spectrometer*

Ten replicate measurements were conducted using a UV-visible spectrometer C12666MA, to deliver

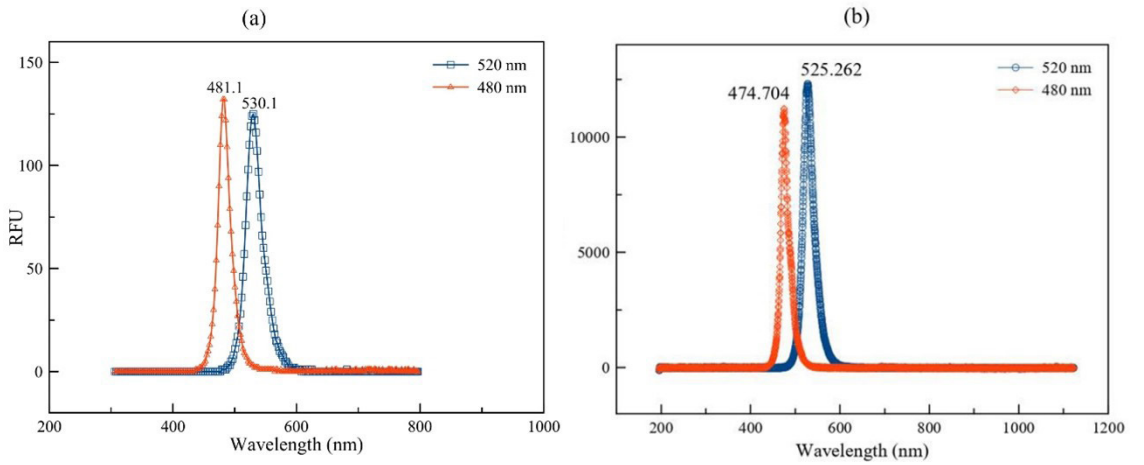

Figure 5: Spectral curves of RFU vs wavelength as a comparison of spectral curves of 480 and 520 nm LED (a) measured by a spectrometer, (b) measured by an HR4000 UV-NIR spectrometer as a reference.

optimal accuracy and reliability, and to evaluate the calibration in the 480 and 520 nm LED as a reference incorporated within the experimental configuration. This calibration process of the LED's spectral properties ascertained the precision of the results, thereby contributing to the overall robustness of the experimental methodology.

The calibration measurement of reference intensity in ten replicates was shown in Table 2 demonstrating a high level of stability and repeatability in its performance. This implies that the instrument can reliably produce the same results under identical conditions to this detection with a spectral resolution of 15 nm. The detector is less capable of differentiating between small changes in the measurement values due to the limited view, restricting its capacity to capture only a fraction of the incident light. From an analysis of the data, a mean and RMS were shown in Table 2, reflecting the accuracy of the spectrometer in this experiment. The RMS value serves as an important metric to evaluate the performance of the spectrometer, as it quantifies the difference between the measurements and mean values in the 480 and 520 nm LED measurements. As a result, a low RMS of 0.54 for the 480 nm LED and 1.30 for the 520 nm LED were determined. The relatively low RMS value suggests that the spectrometer was able to accurately measure the reference LEDs, with minimal fluctuations.

It is essential to compare various measurement techniques to ascertain the reliability and accuracy of the C12666MA spectrometer, a HR4000 UV-NIR spectrometer as a reference spectrometer with higher accuracy and resolution was employed to measure at 480 and 520 nm LEDs.

Figure 5(a) shows that the spectral curves of a spectrometer in 480 nm LED drifted from a reference spectrometer of 6.4 nm and in a 520 nm LED of 4.84 nm from a reference in Figure 5(b). These low drift values, when compared to the reference spectrometer, together with low RMS from Table 2 of spectrometer suggest that C12666MA can provide reliable and precise measurements for fluorescent applications and can deliver accurate and trustworthy results in metrology applications, particularly in those involving the analysis of fluorescent materials or light sources within the context of metrology and optical measurements.

To assess the fluorescent accuracy and sensitivity of the Cas12a-based detection method within the

Table 2: RFU of ten replicate measurements of 520 nm and 480 LED

λ (nm)	1	2	3	4	5	6	7	8	9	10	Mean	RMS
480 nm	301	302	302	302	302	302	302	302	303	303	302.10	0.54
520 nm	260	262	258	258	261	261	260	261	259	261	260.10	1.30

experimental setup, a spectrophotometric approach was employed. Spectrophotometry, a widely accepted and reliable technique, enables the quantification of light absorption and emission properties of molecules, providing essential information about their behavior and interactions. The C12666MA UV-visible mini-spectrometer, a crucial component of the detection system, was utilized as a portable diagnostic for this work. This innovative spectrometer is characterized by its rapid response time, high sensitivity, and ultra-compact design, which is comparable in size to a human fingertip. The miniaturized spectrometer head has been meticulously engineered using Micro Electro-Mechanical Systems technology and an advanced image sensor, allowing for precise measurements and enhanced portability [22].

In the field of molecular detection research, this type of spectrometer, the C12666MA model, has been utilized in a few prior studies. Its advantages lie in the unique combination of cost-effectiveness, portability, and potential for integration within on-site diagnostic systems. These attributes render the C12666MA spectrometer able to deliver rapid and reliable results that can be used in the decision-making process for the management of a wide range of diseases. Due to the limited resolution of 15 nm exhibited by the detection system, it becomes difficult to accurately detect variations in the observed values. Consequently, this versatile analytical tool represents a promising avenue for further investigation and development within biomedical research and clinical practice.

3.3 *Accuracy of the detection system*

The accuracy of the CRISPR-Cas12a detection system was conducted through a series of experiments using 50 ng PCR product of isolate 10100 (*AvrPi9* target DNA), isolate NYK56003 (non-target DNA), and ddH2O (NTC). To guarantee the robustness and reproducibility of the findings, each sample was tested in triplicate, with each reaction mixture incubated at 37 °C for 10 min. The real-time fluorescence curve was analyzed to evaluate the sensitivity and specificity of the detection approach. The results demonstrated that the *AvrPi9* PCR product of isolate 10100 appeared high fluorescence readings increasing over time, signifying the successful detection of the target DNA sequence. In contrast, both the NYK56003 and NTC

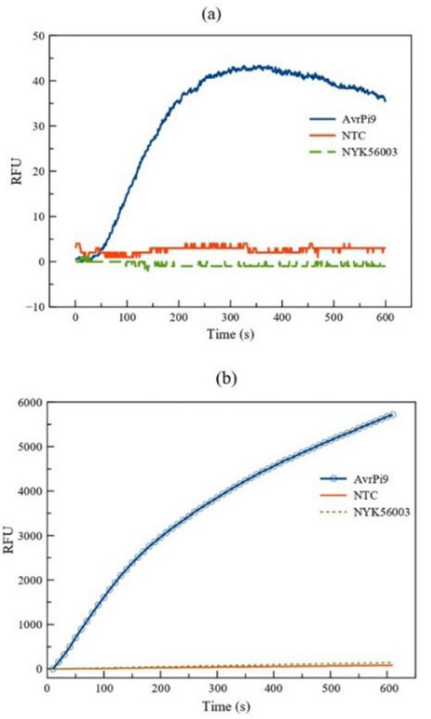

Figure 6: (a) Fluorescence accuracy curve averaged for triplicate from isolate 10100 PCR product (*AvrPi9*), isolate NYK56003 and NTC in CRISPR-Cas12a detection system and (b) in real-time PCR as a reference method.

samples showed no observable fluorescence curves. This finding is significant as it indicates the absence of false-positive results, thereby highlighting the specificity of the CRISPR-Cas12a detection system. The lack of fluorescence in these samples also effectively eliminates concerns regarding potential cross-reactivity and non-specific binding, which are crucial factors to consider when evaluating the overall efficacy and accuracy of the detection system as shown in Figure 6.

To further validate the results obtained from the detection system, a comparison was conducted using real-time PCR as a reference method [Figure 6(b)]. The relationship between the two methods supported the reliability and accuracy of the CRISPR-Cas12a detection system in targeting *AvrPi9* gene. The experimental outcomes effectively confirm that the CRISPR-Cas12a detection system eliminates potential cross-reactivity and non-specific binding, thus ensuring

the reliable detection of target DNA sequences. These findings not only showcase the system's ability to produce accurate and reliable results but also emphasize its potential as an indispensable tool in the field of genetics and molecular biology research.

3.4 *Sensitivity of CRISPR-Cas12a detection*

The sensitivity of a real-time CRISPR-Cas12a detection system was assessed using a series of 2-fold serial dilutions of *AvrPi9* PCR products at concentrations ranging from 30 ng/µL to 0.1 ng/µL, along with NTC for negative control. The experiments were conducted in triplicate to ensure the reliability and reproducibility of the results and were performed at a temperature of 37 °C for 10 min. Real-time fluorescent measurements were performed using a C12666MA spectrometer. The resulting fluorescence curves corresponding to target concentration were analyzed by comparing the signal intensities and concentrations of the different serial dilutions to determine the detection limits of the system.

The results revealed that the real-time CRISPR-Cas12a detection system had a detection limit of 3.8 ng for the *AvrPi9* PCR product. In comparison, the detection limit for a real-time PCR system was found to be 1 ng as a reference result. The sensitivity tests were proved by the fluorescence curves shown in Figure 7(a), (e) and (c), (f) which provided the correlation between the target concentration and the fluorescent intensities. And we observed the quenching of fluorescence in the microtubes under the LED transilluminator and UV by Gel Doc XR system (Bio-rad) as a reference to visualize the changes in fluorescence to determine the efficiency of detection at varying concentrations shown in Figure 7(b) and (d). These sensitivity tests systematically evaluated LoD of CRISPR-Cas12a detection using *AvrPi9* PCR product and compared to the real-time PCR system using a combination of fluorescent curves and quenching analysis under the LED transilluminator and under UV by Gel Doc XR system to provide valuable insights into the system's ability to detect trace amounts of targets accurately and reliably from the samples. This information is crucial for evaluating the diagnostic potential of the detection system in various applications, such as pathogen detection, genotyping, and gene editing analysis. Furthermore, the detection limit from standard curves in Figure 7(e) and (f) was determined by the values above the baseline critically. The graphs determine the minimum amount of DNA to be detected in the detection system and real-time PCR. The detection system has a detection limit of 3.8 ng for the *AvrPi9* PCR product and 1 ng for real-time as more sensitive. R^2 values from graphs indicate a linear relationship between fluorescence values of each concentration.

Our experiments demonstrated that the detection system exhibited an LoD of 3.8 ng for *AvrPi9*. This performance is noteworthy when compared to existing PCR-based methods, which have been reported to possess an LoD ranging from 10 to 100 fg [23]–[25] as well as LAMP methods of 1 to 100 fg [26]–[29], respectively employed in laboratory diagnostics. Our method introduces a marked paradigm shift from laboratory-based detections to more portable, rapidity with RPA technique and CRISPR-Cas12a detection within 30–40 min, and less complicated settings as a future work of on-site diagnostic solutions. Although our current detection system demonstrates the potential for further development, it is crucial to enhance its sensitivity to make it more competitive with conventional-based methods.

4 Conclusions

As the enhancement from the previous study of CRISPR-Cas12a detection [15]. We have developed and demonstrated a portable diagnostic system using the real-time fluorescence CRISPR-Cas12a detection integrated with microcontroller technology, allowing for increased precision and quantification in the analysis process, leading to enhanced performance and reliability. An Arduino Uno-based microcontroller serves as a critical component of the system, providing efficient regulation and control over the various components of the device. In the calibration process of the system, the DS18B20 temperature sensor (±0.5 °C accuracy) exhibits an RMSE of 0.3 °C when compared to a reference standard. This level of accuracy facilitates precise monitoring of the 37 °C reaction setpoint in the temperature control system, yielding an RMSE of 0.16 °C with respect to the desired setpoint value. A C12666MA spectrometer was also calibrated using two reference LEDs with wavelengths of 480 nm and 520 nm, benchmarked against an HR4000 spectrometer. Ten replicate measurements were conducted to assess

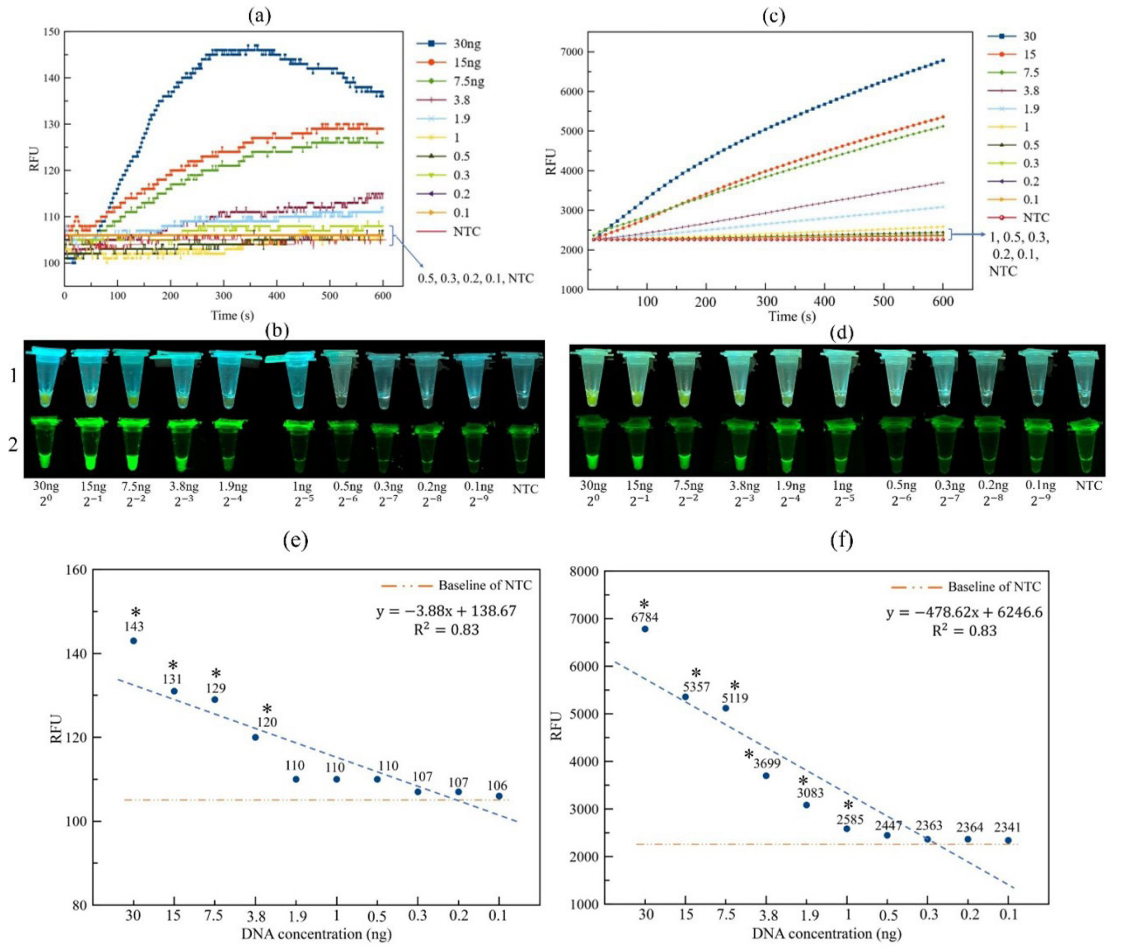

Figure 7: Sensitivity of CRISPR-Cas12a detection with 2-fold serial dilution of *AvrPi9* PCR product (a) the fluorescent curve of the detection system, and (b-1) the quenching of fluorescence under the LED transilluminator, (b-2) under UV by Gel Doc XR system in the microtubes in the detection system, (c) the fluorescence curve of real-time PCR, and (d-1) the quenching of fluorescence under the LED transilluminator, (d-2) under UV by Gel Doc XR system in microtubes in the real-time PCR system as reference, and standard curve of fluorescence values of different DNA concentration at 10 minutes of (e) the detection system and (f) real-time PCR (* designates significant difference between the values at a DNA concentration and NTC baseline).

repeatability in intensity detection. The mean and RMS values were determined, revealing a low RMS of 0.54 for the 480 nm LED and 1.30 for the 520 nm LED. These values suggest a satisfactory level of stability and reliability in intensity measurement, making the system suitable for fluorescence measurements in this study. Comparison with the reference spectrometer revealed minimal drift in wavelength ranges, amounting to 6.4 nm for the 480 nm LED and 4.84 nm for the 520 nm LED. These findings suggested that the C12666MA spectrometer demonstrates a high degree of accuracy in wavelength detection, further supporting its suitability for application in fluorescent measurements. Overall, the calibration process and subsequent analysis provided valuable insights into the performance and capabilities of the C12666MA spectrometer within the metrology and optical measurements. And the C12666MA UV-Vis spectrometer model combined with the portable CRISPR-Cas12a detection system, has been utilized in a few prior studies.

Our experiments demonstrated that the sensitivity of the detection system exhibited a LoD of 3.8 ng for *AvrPi9* PCR product, signifying its potential utility in various research and diagnostic settings.

In comparison, we also investigated the detection capabilities of a real-time PCR system as the reference method and found it to have an LoD of 1.9 ng for the same target. The detection accuracy from three types of samples reveals that the positive control *AvrPi9* consistently exhibits a high fluorescence curve within a 10 min interval, signifying the identification of the target DNA sequence. Concurrently, no observable fluorescence curves were detected in both non-target (NYK56003) and negative template control (NTC) samples. This observation underscores the lack of false-positive outcomes and highlights the specificity of the detection methodology. This CRISPR-Cas12a detection has advantages over PCR and LAMP in simplicity, time efficiency, and reduced incidence of false-positive and cross-activity. The main constraint for this device is the sample preparation as it is currently capable of processing only one sample per reaction. Despite this constraint, the development of the real-time detection platform has proven to be highly specific, sensitive, accurate, and cost-effective for detecting the rice blast *Avr* gene. This device makes a significant step forward for on-site diagnostic applications, not only for rice blast but also for other plant and human diseases in the future.

Rice blast is a devastating disease that can cause significant yield losses in rice crops. The identification of *AvrPi9* that are resistant to rice blast is an important step in developing new detections that can detect rice blast [30]. The emergence of virulent strains of *M. oryzae* highlights the need for continuous research into plant-pathogen resistance. By continuing to invest in research of resistance gene, we can help to ensure that farmers have access to crops that are resistant to the latest strains of plant pathogens [31], [32]. The development of CRISPR-Cas12a detection of *AvrPi9* is an important step in protecting rice production from the devastating rice blast disease. This detection has been shown to be effective in detecting *AvrPi9* genes in accuracy and specificity. This technology has the potential to help farmers to grow more rice and to reduce the use of pesticides. However, it's important to note that this detection system would require overcoming several technical challenges, such as the development of a higher sensitive assay for the *AvrPi9* gene, as well as the ability to sample and analyze fungal populations in a cost-effective and timely manner. To ensure the efficacy of the method across both controlled laboratory conditions and field samples, the implementation of on-site testing is necessary. This on-site evaluation is critical to validate the procedure's performance and to maintain the rigor of the scientific investigation.

In the future work, we will develop new prototype devices that can execute 6–12 reactions and have multiple fluorescence channels, thereby facilitating the detection of various fluorophores. To further increase the sensitivity and specificity of the detection, advancements in spectrometer resolution and accuracy would enhance the overall quantification of measurements, making the instrument more suitable for demanding fine distinctions between closely spaced values of wavelengths. The calibration of the spectrometer will be conducted by employing multiple reference LEDs spanning the UV-Vis spectral range (λ = 340–780 nm) wavelengths to enhance accuracy and reliability in fluorescence detection.

PCR amplification is required to increase target DNA. However, for on-site applications, other amplification techniques like RPA can substitute PCR. The development using RPA combined with CRISPR-Cas12a can be more efficient, this method can amplify at a constant temperature of 37–42 °C in less than 20 min, with its high sensitivity, selectivity, and the absence of need for a thermocycler between different temperatures, paves the way for the development of more accessible, cost-effective, suitable for use in resource-limited settings and user-friendly nucleic acid detection systems [33]. These systems can have a significant impact on various areas including clinical diagnostics, field-based testing, and resource-limited settings. For further RPA coupled with the CRISPR-Cas12a detection system allows for easy readouts, often using fluorescence-based methods by precise and accurate spectrometer calibrated in this work, which can provide results once activated by the target DNA, Cas12a cleaves a reporter molecule to produce a fluorescent signal that can be detected in real-time. Overall, the combination of RPA and CRISPR-Cas12a detection offers a simpler, faster, more sensitive, and specific method for DNA detection and amplification compared

to traditional PCR and LAMP methods. This makes it a promising tool for various applications, including disease diagnosis, genetic testing, and research purposes. Combining with direct DNA extraction from infected leaves (10 minutes) [34], RPA coupled with CRISPR-Cas12a pathogen detection can be done within 40 minutes. This method has advantages, such as simple operation, requiring less instruments and equipment, high sensitivity and specificity and is expected to replace RT-qPCR in the future. As a portable fluorescence detection, it can reduce the occurrence of false positive and achieve qualitative nucleic acid detection.

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Author Contributions

C.T.: research design, data analysis, writing an original draft; P.K.: research design, reviewing and editing; S.C.: research design, reviewing and editing, project administration, funding acquisition; C.K.: research design, reviewing and editing; J.J.C.: mentoring. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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