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CRISPR/Cas13a-assisted AMP generation for SARS-CoV-2 RNA detection using a personal glucose meter

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ABSTRACT

Herein, we described a washing- and label-free clustered regularly interspaced short palindromic repeats (CRISPR)/LwoCas13a-based RNA detection method utilizing a personal glucose meter (PGM), which relies on the trans-cleavage activity of CRISPR/Cas13a and kinase reactions. In principle, the presence of target RNA activates the trans-cleavage of CRISPR/Cas13a, generating 2′,3′-cyclic phosphate adenosine, which is converted to adenosine monophosphate (AMP) by the T4 polynucleotide kinase. Subsequently, the AMP is converted to adenosine diphosphate (ADP) through phosphorylation by a myokinase; ADP is then used as a substrate in the cascade enzymatic reaction promoted by pyruvate kinase and hexokinase. The overall reaction leads to the continuous conversion of glucose to glucose-6-phosphate, resulting in a reduction of glucose concentration proportional to the level of target RNA, which can therefore be indirectly measured with a PGM. By employing this novel strategy, severe acute respiratory syndrome coronavirus-2 RNA can be successfully detected with excellent specificity. In addition, we were able to overcome non-specific responses of CRISPR/Cas13a and distinguish single nucleotide polymorphisms by introducing a single-base mismatch in the complementary RNA.

1. Introduction

Growing international trade and travel are considered major contributors to the frequent occurrence and rapid spread of communicable diseases worldwide (Patchsung et al., 2020; Woo et al., 2020; Y. Wang et al., 2021). Since its discovery in December 2019, the novel coronavirus disease 2019 (COVID-19) has spread at an alarming rate, ultimately causing the World Health Organization (WHO) to declare a global pandemic in March 2020. By August 2022, more than 600 million people had contracted the disease worldwide, of which more than six million died. Moreover, the pandemic claimed tremendous social and economic costs (Nouri et al., 2021; Woo et al., 2020). Future pandemics are expected to occur more frequently and at a greater scale and intensity, thus necessitating preparedness in terms of clinical control measures. To effectively control the spread of highly transmissible diseases, such as COVID-19, prompt, sensitive, and affordable diagnostic technology is essential (Kaminski et al., 2020; W.S. Zhang et al., 2021; Xu et al., 2020).

Although reverse transcription polymerase chain reaction (RT-PCR)—the gold standard diagnostic test for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)—has shown excellent diagnostic performance (Cai et al., 2021), the technology is bulky, costly, temperature sensitive, and can only be performed by trained personnel (T. Zhang et al., 2021; W. S. Zhang et al., 2021). As such, tests cannot be performed immediately at the point of care. Instead, samples must be sent to an equipped laboratory, resulting in variable turnaround times of 1 h to several days, depending on the distance to the laboratory (Sheng et al., 2021; Wang et al., 2021b). In contrast, rapid immunodiagnostic kits are user-friendly and generate results within minutes (Han et al., 2021; van Dongen et al., 2020). However, these tests have low specificity for early diagnosis because at least five days are required for viral
antigens and antibodies (IgM and IgG) to reach detectable levels after infection (Jacofsky et al., 2020; Muruato et al., 2020). In addition, the visual analysis of color signals can lead to human errors (Yuan et al., 2020).

Personal glucose meters (PGMs) are used to self-monitor blood glucose levels. Owing to their user-friendliness, affordability, and rapid processing time, recent studies have investigated techniques for analyzing non-glucose target substances using PGMs. Xiang and Lu (2011) developed a PGM-based bio-sensing system using DNA probes to detect various biomolecular targets, such as nucleic acids, proteins, and small molecules (Kim et al., 2020; Xiang and Lu, 2011). However, this technology requires enzyme-linked DNA or nanomaterial complexes and a separation process, which hinders its implementation in point-of-care testing (POCT) (Han et al., 2021; Kim et al., 2020b; Park et al., 2022). Ahn et al. (2018, 2019) developed an alternative washing- and label-free detection technology based on the changes in glucose concentration induced through cascade enzymatic reactions (CERs) and is subsequently analyzed using a PGM.

In this study, we present a technique to detect the target RNA of SARS-CoV-2 with a PGM through CER without needing gene amplification. This technology is based on the clustered regularly interspaced short palindromic repeats (CRISPR)/LwaCas13a system (Cox et al., 2017; Gootenberg et al., 2017; Shi et al., 2021). The CRISPR/Cas13a system was discovered in bacteria and archaea as an RNA-guided adaptive immunity system to cleavage foreign genetic material from invading viruses and phages. Furthermore, the revelation that CRISPR-Cas12, 13, 14 demonstrate the nonspecific breakdown of non-target nucleic acids (trans-cleavage) after specific identification of nucleic acids suggests that CRISPR/Cas biology has the potential of speedy, accurate, and portable diagnostic tools. Based on these methods, recently discovered diagnostic procedures for infectious and noninfectious disorders have pushed next-generation diagnostics with CRISPR (Shan et al., 2019; Sheng et al., 2021). Once activated, the CRISPR-R/Cas13a system randomly cleaves ssRNAs, and one activated Cas13a/complementary RNA (crRNA) complex performs multiple trans-cleavages (Myhrvold et al., 2018; Tian et al., 2021; Wang et al., 2021). In our system, the trans-cleavage response by the CRISPR/Cas13a system is activated by the presence of target RNA, resulting in the production of adenosine monophosphate (AMP) from specially designed probes (Gootenberg et al., 2018; Zhou et al., 2020, 2021). The AMP serves as a substrate for the kinases, which induce changes in glucose concentrations, ultimately enabling the quantitative detection of target RNA by measuring the glucose level with a PGM (Kim et al., 2020a).

2. Materials and methods

2.1. Materials

All synthetic sequences (Table S1) were purchased from Integrated DNA Technologies Inc. (Coralville, IA, USA). D-glucose, pyruvate kinase from rabbit muscle, hexokinase from Saccharomyces cerevisiae, phospho (enol)pyruvic acid monophosphate salt (PEP), adenosine 5′-monophosphate disodium salt hydrate (AMP), cytidine 5′-monophosphate disodium salt (CMP), uridine 5′-monophosphate disodium salt (UMP), guanosine 5′-monophosphate disodium salt (GMP), adenosine 5′-diphosphate disodium salt hydrate (ADP), acetonitrile (ACN), 3-hydroxy-2-picolinic acid (3-HPA), myokinase from rabbit muscle, sodium triphosphate penabasic, and the DL-dithiothreitol (DTT) solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). LwaCas13a protein was purchased from MCLAB (San Francisco, CA, USA). T4 polynucleotide kinase (T4 PNK) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). CutSmart® buffer was purchased from New England Biolabs Inc. (Ipswich, MA, USA). Diethyl pyrocarbonate distilled water (DEPC-DW) was purchased from Bioneer (Daejeon, Korea). The PGM was purchased from Accu-Chek Active (Roche, Basel, Switzerland).

2.2. RNA virus detection with the PGM

A 20 μL mixture of Cas13a (20 nM) protein and T4 PNK (50 U) with target RNA (10 nM), crRNA (10 nM), DTT (2 mM), and AMP probe (450 μM) was incubated in 1 × CutSmart buffer at 37 °C for 90 min. The reaction solutions were then treated with a 30 μL mixture of myokinase and CER system containing myokinase (5 U), D-glucose (5 mM), triphosphate penabasic (1 μM), pyruvate kinase (5 U), hexokinase (5 U), and PEP (2 mM) and incubated in the 1 × CutSmart buffer at 35 °C for 30 min. Then, using 5 μL of the reaction solution, the glucose concentration was measured with the PGM.

2.3. Fluorescence spectra analysis

The fluorescence quenching (FQ) AMP probe (5 μM) and target RNA (25 nM) were added to a reaction solution consisting of Cas13a (50 nM) and crRNA (25 nM) and incubated in 1 × CutSmart buffer at 37 °C for 60 min. The total reaction volume was 30 μL. The fluorescence of the mixture was measured from 500 nm to 550 nm at a fixed value of λex (470 nm) using an Infinite 200 Pro Multi-Mode Microplate Reader (Tecan, Männedorf, Switzerland) in the fluorescence intensity scan mode.

2.4. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

We placed 1 μL of MALDI matrix (50 mg/mL 3-hydroxy-2-picolinic acid (3-HPA) in 1:1 DW/acetoniitrate (ACN)) on a MALDI sample plate to air-dry. We added the sample resuspended in 1 μL of 1:1 CH3CN/DW to the dried matrix crystals and allowed it to air dry. MALDI-TOF-MS analysis was done on a Bruker Daltonics Autoflex III mass spectrometer (Billerica, MA, USA) using a nitrogen laser at 355 nm with an initial accelerating voltage of 20 kV and a delay time of 100 ns. The instrument was run in reflector mode using negative ion detection with external instrument calibration.

2.5. Real-sample test of SARS-CoV-2 detection system

Various concentrations of SARS-CoV-2 RNA were spiked into each 5% human serum, 5% human plasma, and 100% human saliva to mimic real samples. To calculate the amount of spiked SARS-CoV-2 RNA, a calibration curve was provided using a standard set of known concentrations of SARS-CoV-2 RNA in the real samples. The unknown amount of SARS-CoV-2 RNA was then calculated based on this curve.

3. Results and discussion

3.1. Detection of SARS-CoV-2 RNA using the PGM

Fig. 1 illustrates the overall workflow for the detection of SARS-CoV-2 RNA using the CRISPR/Cas13a system and four kinase reactions. First, in the presence of target RNA, crRNA binds to the target RNA and activates the CRISPR/Cas13a system (Fig. S1). Next, the activated CRISPR/Cas13a system cleaves the junction between the adenine ribonucleotide (rA) and uracil ribonucleotide (rU) of the AMP probe near it, generating two fragments of the AMP probe. T4 PNK acts on the 3′-end and repairs the 2′,3′-cyclic phosphate to produce a complete AMP. We designed the AMP probe as an RNA/DNA chimera structure containing only two ribonucleotides (rA and rU) at the 5′-end. Because the trans-cleavage activity of the CRISPR/Cas13a system does not affect DNA strands, cleavage occurs only at the junction between rA and rU in the sequences of the AMP probe. The resulting AMP is converted to ADP through phosphorylation by a myokinase, and ADP is used as a substrate in the CER with pyruvate kinase and hexokinase. This cascade reaction continuously converts glucose into glucose-6-phosphate (G6P). Finally, the target RNA can be determined by simply measuring the reduced glucose concentration.
3.2. Validity of PGM-based SARS-CoV-2 detection

To verify target RNA detection by the PGM, we prepared five different reaction mixtures to compare PGM signals (Fig. 2). First, we measured PGM signals in the presence of pyruvate kinase and hexokinase with AMP or ADP. In the presence of ADP, a reduced PGM signal was observed, reflecting the use of ADP as a substrate in the CER. The addition of myokinase reduced the PGM signals to a level comparable to those generated in the presence of ADP (bars 2, 3, and 4; Fig. 2). This indicates that while AMP does not serve as a substrate in CER, its conversion to ADP promoted by myokinase can accelerate CER.

Next, PGM signals were measured in the presence or absence of a target SARS-CoV-2 RNA with all components incorporated (CRISPR/Cas13a system, myokinase, pyruvate kinase, hexokinase, and T4 PNK). The initial PGM signal decreased considerably in the presence of the target RNA (bar 5, Fig. 2). However, we observed a nearly identical PGM signal between reaction systems without T4 PNK or target RNA (bars 6 and 7, Fig. 2). This is consistent with our previous findings that the 2′,3′-cyclic phosphate structure at the 3′-end of the AMP probe is converted to a hydroxyl group (-OH) by T4 PNK (Zhou et al., 2021). These results indicate that fully formed AMPs are generated only in the presence of both an activated CRISPR/Cas13a system and T4 PNK. Furthermore, our results confirm that myokinase, pyruvate kinase, and hexokinase activities cause a reduction in glucose concentration by acting on AMP.

3.3. Design of AMP probe for AMP generation

We examined whether Cas13a/crRNA specifically cleaves the rArU junction in the 5′-end of the AMP probe using MALDI-TOF-MS. As shown in Fig. 3A, the peak corresponding to the AMP probe (7-mer) was observed in the absence of an RNA target. However, this peak disappeared, and another peak corresponding to one less base (~340 m/z) was observed. Certainly, this is because one base was cleaved off during the CER. We performed the same analysis using probes involving rUrU and rArA instead of rArU at the 5′-end. The peak corresponding to the one-base-lower position was found only when the probe with rUrU was used, suggesting that trans-cleavage occurs only at the rUrU junction. These results correspond with the previously reported features of the sequence-specific trans-cleavage activity of CRISPR/Cas13a (Gootenberg et al., 2018; Patchtsung et al., 2020).

We measured PGM signals in cases wherein the AMP probe contained rArU, rUrU, or rArA at the 5′-end. As shown in Fig. 3B, the peak corresponding to the AMP probe (7-mer) was observed in the absence of an RNA target. However, this peak disappeared, and another peak corresponding to one less base (~340 m/z) was observed. Certainly, this is because one base was cleaved off during the CER. We performed the same analysis using probes involving rUrU and rArA instead of rArU at the 5′-end. The peak corresponding to the one-base-lower position was found only when the probe with rUrU was used, suggesting that trans-cleavage occurs only at the rUrU junction. These results correspond with the previously reported features of the sequence-specific trans-cleavage activity of CRISPR/Cas13a (Gootenberg et al., 2018; Patchtsung et al., 2020).

We performed the same analysis using a probe containing rArU without phosphate modification at the 5′-end but found...
no changes in PGM signals (Fig. 3B). This implies that since the product of the trans-cleavage activity of the CRISPR/Cas13a system does not contain phosphate at the 5′-end, glucose is not consumed.

3.4. Optimization of SARS-CoV-2 detection system

We conducted several optimization experiments to ensure the highest efficiency of target RNA detection. First, we examined the change in PGM signal (ΔP; defined as P0 – P, where P0 and P indicate the PGM signal from the samples before and after CER involving myokinase, respectively) according to the initial glucose concentration. We found that PGM signals were comparable regardless of the initial glucose concentration (Fig. S2). We selected 5 mM as the optimal glucose concentration based on the measurable range of the PGM, and we found the PGM signal to change with or without a target RNA. Furthermore, we compared detection performance in the absence and presence of DTT because enzymatic activity, particularly that of T4 PNK, is dependent on DTT (Fig. S3) (Feng et al., 2018). As shown in Fig. S3, the addition of DTT was effective for detecting target RNA at a low concentration (2.5 nM), while it was not helpful at a high concentration (10 nM). Our reaction temperature optimization for CER involving myokinase revealed that the enzymes were most active at 35–45 °C, but activity was lost beyond 50 °C (Fig. S4A). 35 °C was selected as the optimal temperature for CER involving myokinase. Finally, we optimized the reaction time to 90 min for the CRISPR/Cas13a system and 30 min for CER involving myokinase (Fig. S4B, S4C). Further experiments were conducted under these conditions.

3.5. Evaluation of SARS-CoV-2 detection system performance

To determine the sensitivity of the proposed system, SARS-CoV-2 RNA at varying concentrations (0–10 nM) was subjected to the assay. The PGM signal (ΔP) increased with increasing concentrations of SARS-CoV-2 RNA (C_{SARS-CoV-2, RNA} Fig. 4A). We observed a strong positive correlation between PGM signal and C_{SARS-CoV-2, RNA} over the range of 0–1.28 nM RNA (ΔP = 0.6123 C_{SARS-CoV-2, RNA} [nM] + 0.6054, R^2 = 0.9943). This confirms that our technique can quantitatively and reliably determine SARS-CoV-2 RNA levels. Based on 3σ/slope (σ is the standard deviation of a blank sample), the limit of detection (LOD) was estimated at 27 pM. Next, we verified the detection selectivity of our technology by measuring ΔP for a combination of SARS-CoV-2, Zika virus (ZIKV), and dengue virus (DENV) and their crRNA (Fig. 4B). The PGM signal changes (ΔP) were observed only when the crRNA and the target RNA matched. This confirms that this detection method is highly selective for the target RNA.

Various technologies have been developed based on the RNA-specific trans-cleavage activity of the CRISPR/Cas13a system. However, non-specific crRNA containing single-base mismatches can also activate the CRISPR/Cas13a system (Gootenberg et al., 2017). To address this issue, Gootenberg et al. (2018) employed a mismatched site on crRNA to distinguish between the wild-type and single nucleotide polymorphism (SNP). Likewise, we investigated the ability of the system to differentiate between SNPs by intentionally adding a mismatched site on crRNA sequences. As shown in Fig. 5A and B, perfectly matched crRNA exhibited nearly identical PGM signal changes in both the wild-type and single nucleotide polymorphism (SNP). Likewise, we investigated the ability of the system to differentiate between the wild-type and single nucleotide polymorphism (SNP). Lastly, the practical applicability of our SARS-CoV-2 RNA detection system using PGM was examined by determining SARS-CoV-2 RNA present in the real sample such as human serum, human plasma, and
human saliva. As shown in Fig. S5, the patterns of PGM signal change ($\Delta P$) at various RNA concentrations in the real samples were nearly the same as that obtained in the buffer solution (Fig. 4 (A)). In addition, the PGM signal change increased with increasing concentration of SARS-CoV-2 RNA in human serum, human plasma, and human saliva, and a good linear correlation existed in the range from 0 to 2.5 nM, from 0 to 5 nM, and from 0 to 2.5 nM, respectively. Our strategy’s excellent reproducibility and precision were confirmed by a coefficient of variation (CV) of less than 8.69% and a recovery ratio between 97.4 and 104.09% (Table 1). Overall, these results confirm that the developed SARS-CoV-2 RNA detection system could be utilized to reliably determine the amount of SARS-CoV-2 RNA present in real samples.

### 4. Conclusions

In this study, we developed a washing- and label-free CRISPR/Cas13a-based target RNA detection method utilizing a PGM. This novel strategy is based on the mechanism wherein single nucleotides generated after trans-cleavage by the CRISPR/Cas13a system in response to target RNA are used as substrates of subsequent kinase reactions. Changes in the glucose concentration induced by the target RNA can then be conveniently measured with a PGM. Based on this technology, target RNA was successfully detected with a LOD of 27 pM and excellent specificity. We also validated the sensitivity of our system in discriminating between crRNA targets that differ by a single-base mismatch. Our method does not require gene amplification and thus does not require bulky and costly equipment, making it an excellent alternative to RT-PCR. We propose that our approach can be applied in POCT for RNA detection, even in resource-limited facilities and low-income populations. To the best of our knowledge, our study is the first to determine target RNA using a PGM based on the AMP substrate generated from the CRISPR/Cas13a system.

**CRediT authorship contribution statement**

**Junhyun Park:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization.

**Hyogu Han:** Validation, Resources.

**Jae Hoon Jeung:** Validation, Resources.

**Chihyun Park:** Validation, Resources.

**Jun Ki Ahn:** Writing – review & editing, Supervision, Project administration, Funding acquisition.
Table 1  
Table 1 Determination of SARS-CoV-2 RNA spiked into human serum, human plasma, and human saliva.

| Real sample | Added RNA (nM) | Measured RNA (nM) | SD | CV (%) | Recovery (%) |
|-------------|---------------|-------------------|----|--------|--------------|
| Human serum | 0.5           | 0.51              | 0.02 | 4.14   | 101.98       |
|             | 1             | 1.01              | 0.05 | 5.18   | 101.01       |
| Human plasma| 0.5           | 0.49              | 0.01 | 2.66   | 99.78        |
|             | 1             | 1.00              | 0.05 | 4.98   | 100.44       |
|             | 2             | 1.95              | 0.10 | 5.23   | 97.41        |
| Human saliva| 0.5           | 0.49              | 0.04 | 7.42   | 98.93        |
|             | 1             | 1.04              | 0.09 | 8.69   | 104.09       |
|             | 2             | 2.00              | 0.10 | 5.14   | 100.02       |

*To determine the concentration of SARS-CoV-2 RNA in real samples, a calibration curve was first generated by utilizing standards with the known concentration of SARS-CoV-2 RNA in each real sample: 5% human serum, 5% human plasma, and 100% human saliva (Fig. S5). Based on this calibration curve, the PGM signals from unknown samples were used to determine the concentration of SARS-CoV-2 RNA in real samples.

- Mean of three measurements.
- Standard deviation of three measurements.
- Coefficient of variation = SD/mean × 100.
- Measured value/added value × 100.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

Funding: This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (IPET) through the Crop Viruses and Pests Response Industry Technology Development Program funded by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) (321104-3) and the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science and ICT (MSIT) (No. 2020R1F1A10706052).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/bios.2022.100283.

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