Biosensors and Bioelectronics 209 (2022) 114256

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Ligation-free isothermal nucleic acid amplification

Jeong Moon [a,b], Jayeon Song [a], Hyowon Jang [a], Hyunju Kang [a,c], Yong-Min Huh [d,e,f,g], Hye Young Son [d,g], Hyun Wook Rho [d], Mirae Park [d], Chandana S. Talwar [h,i], Kwang-Hyun Park [h], Euijeon Woo [h,i], Jaewoo Lim [a,j], Eun-Kyung Lim [a,j], Juyeon Jung [a], Yongwon Jung [c], Hyun Gyu Park [b,**], Taejoon Kang [a,*]

[a] Bionanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[b] Department of Chemical and Biomolecular Engineering (BK21+ Program), Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[c] Department of Chemistry, KAIST, 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[d] Department of Radiology, College of Medicine, Yonsei University, 50-1 Yonsei-ro, Seodaemun-gu, Seoul, 03722, Republic of Korea
[e] YUHS-KRIBB Medical Convergence Research Institute, 50-1 Yonsei-ro, Seodaemun-gu, Seoul, 03722, Republic of Korea
[f] Department of Biochemistry & Molecular Biology, College of Medicine, Yonsei University, 50-1 Yonsei-ro, Seodaemun-gu, Seoul, 03722, Republic of Korea
[g] Severance Biomedical Science Institute, College of Medicine, Yonsei University, 50-1 Yonsei-ro, Seodaemun-gu, Seoul, 03722, Republic of Korea
[h] Disease Target Structure Research Center, KRIBB, 125 Gwahak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea
[i] Department of Biomolecular Science, KRIBB School of Bioscience, University of Science and Technology (UST), 217 Gajeong-ro, Yuseong-gu, Daejeon, 34113, Republic of Korea
[j] Department of Nanobiotechnology, KRIBB School of Biotechnology, UST, 217 Gajeong-ro, Yuseong-gu, Daejeon, 34113, Republic of Korea

ARTICLE INFO

Keywords:
Nucleic acid
Isothermal amplification
Ligation-free
Phosphorothioate probe
Cancer diagnosis

ABSTRACT

In this study, we uncover a ligation-free DNA extension method in two adjacent fragmented probes, which are hybridized to target RNA, for developing a ligation-free nucleic acid amplification reaction. In this reaction, DNA elongation occurs from a forward probe to a phosphorothioated-hairpin probe in the presence of target RNA regardless of ligation. The second DNA elongation then occurs simultaneously at the nick site of the phosphorothioated probe and the self-priming region. Therefore, the binding site of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) 12a is repeatedly amplified, inducing a fluorescence signal in the presence of CRISPR-Cas12a. This ligation-free isothermal gene amplification method enables the detection of target RNA with 49.2 fM sensitivity. Moreover, two types of mRNA detection are feasible, thus, demonstrating the potential of this method for cancer companion diagnostics. Notably, the proposed method also demonstrates efficacy when applied for the detection of mRNA extracted from human cells and tumor-bearing mouse tissue and urine samples. Hence, this newly developed ligation-free isothermal nucleic acid amplification system is expected to be widely used in a variety of gene detection platforms.

1. Introduction

As the messenger of genetic information, RNA plays important roles in biology, from regulating cell development and disease progression to mediating viruses infecting hosts (Cooper et al., 2009; Poltronieri et al., 2015). Therefore, using precise RNA detection methods can facilitate the investigation of gene expression and the roles of different genetic elements, while also being applied for the diagnosis of infections (Kurzawski et al., 2012; Cella et al., 2013). Reverse transcription-polymerase chain reaction (RT-PCR) has been the most common technique for RNA amplification and detection, although it requires multiple processes, a thermal cycler, and trained personnel (Nolan et al., 2006). As alternatives, several isothermal amplification methods have been developed, including nucleic acid sequence-based amplification (NASBA) (Kievits et al., 1991), nicking and extension chain reaction system-based isothermal amplification (NESBA) (Ju et al., 2021), rolling circle amplification (RCA) (Goo and

* Corresponding author.
** Corresponding author.
E-mail addresses: hgpark1@kaist.ac.kr (H.G. Park), kangtaejoon@kribb.re.kr (T. Kang).

https://doi.org/10.1016/j.bios.2022.114256
Received 7 February 2022; Received in revised form 29 March 2022; Accepted 4 April 2022
Available online 11 April 2022

Kim, 2016), strand displacement amplification (SDA) (Walker et al., 1992), and loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000). While these methods are more rapid and efficient in practical applications than PCR, they require additional components for reverse transcription.

Ligation-based nucleic acid amplification methods have been developed to detect RNA since the discovery of RNA-templated DNA ligase (Lohman et al., 2014). Ligation-mediated amplification methods show high specificity for differentiating the mismatched sequences from target RNA as the probes can only be ligated in the presence of a perfectly matched target (Gibriel and Adel, 2017). In addition, these methods offer sensitivity comparable to RT-PCR owing to their high amplification efficiency (Nouri et al., 2014; Jin et al., 2016). Ligation-mediated amplification begins with the ligation of two adjacent oligonucleotide probes that recognize target-specific sequences, in particular, the ends of each probe are modified to facilitate the subsequent nucleic acid amplification and signal generation processes. (Woo et al., 2020; Gibriel and Adel, 2017). Therefore, most ligation-based detection methods have the disadvantage that they require temperature conversion and/or multi-step procedures.

Herein, we report that *Bst* DNA polymerase enables ligation-free DNA extension of two adjacent probes. Two DNA probes were hybridized to target RNA, facilitating extension regardless of the ligation reaction. To understand the principle of ligation-free isothermal nucleic acid amplification, we performed fluorescence and gel electrophoresis analyses under various experimental conditions. The results confirm the ligation-free DNA extension and subsequent isothermal nucleic acid amplification. Thus, a novel target RNA detection method was developed by combining the continuous ligation-free DNA extension of phosphorothioated (PS)-terminal hairpin probes and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)12a-based signal amplification. Briefly, DNA was elongated from a forward (FW) probe to the PS probe in the presence of target RNA without ligation, after which continuous DNA elongation occurred at the nick site of the PS probe and the self-priming region. Consequently, the binding region of CRISPR-Cas12 was amplified, producing a fluorescence signal by the collateral activity of the CRISPR-Cas12a system. The ligation-free gene amplification method facilitated detection of the ERBB2 gene at a low concentration of 49.2 fM with high specificity. In addition, ERBB2 and GRB7 genes were selectively detected using the new method. Finally, we successfully detected target mRNA extracted from the human cell and breast cancer model mouse tissue and urine samples, thus, demonstrating the feasibility of the method for cancer diagnosis. These findings enable us to develop a ligation-free nucleic acid amplification method for the first time. Considering that the ligation-free approach exhibits comparable RNA sensing performance to ligation-dependent methods, various types of ligation-free nucleic acid amplification and detection approaches are expected to be developed in the future.

2. Results

2.1. Principle of ligation-free isothermal nucleic acid amplification

Recently, several DNA extension strategies based on self-priming and fold-back systems have been studied and applied to molecular diagnostics, as they can reduce the number of primers and provide self-contained reactions (Kato et al., 2012; Jung and Ellington, 2016; Jung et al., 2021). In particular, phosphorothioate-modified sequences have been widely adopted to reduce the reaction temperature and improve the self-folding efficiency (Cai et al., 2018; Song et al., 2021). Based on these advantages, we originally designed a ligation-dependent phosphorothioated-terminal hairpin formation and self-priming extension (PS-THSP) reaction to detect target RNA (Fig. S1). In this reaction, the repetitive DNA extension through PS-THSP is expected to occur only after the ligation of probes. However, we discovered that DNA amplification can be initiated from the 3′ end of a FW probe to a PS probe under the condition that target RNA exists, even if the two types of probes are not ligated. Once the two probes are hybridized to target RNA regardless of the ligation reaction, the continuous DNA extension through PS-THSP extension is feasible. The overall strategy of ligation-free gene amplification is illustrated in Fig. 1.

In the ligation-free isothermal nucleic acid amplification method, two types of single-stranded DNA (ssDNA) probes, including FW and PS probes, were designed to detect target RNA. The FW probe has 25 complementary sequences targeting RNA, 20-base hairpin sequences at the 3′ end, and a phosphate group at the 5′ end. The PS probe has 20-base hairpin sequences modified with phosphorothioate at its 5′ end and 23 complementary nucleotides to target RNA at its 3′ end. For the proof-of-concept and validation of each reaction step, the messenger RNA (mRNA) containing ERBB2 gene sequence is employed as a target RNA. As shown in Fig. 1, FW and PS probes can be placed close together in the presence of target RNA (The first step in Fig. 1). Subsequently, the DNA extension begins from the FW probe to the PS probe, even though the probes are not ligated (The second step in Fig. 1). This is the key step of ligation-free nucleic acid amplification. Once the DNA is extended from the FW probe to the PS probe, DNA extension can occur from the nick site at the 3′ end of the PS probe (The third step in Fig. 1), unfolding the hairpin structure of the FW probe (The fourth step in Fig. 1). Concurrently, the sequences extended to the 5′ end of the PS probe can fold themselves to initiate priming as phosphorothioates can reduce the stability of duplexes. Sequentially, the self-folded 3′ end of the complementary sequences to the PS probe can function as a primer and be extended using DNA polymerase. Therefore, the extended structure from the 3′ end of the FW probe is separated, creating a strand with the Cas12a binding region (The fifth step in Fig. 1). The other released strand forms a terminal hairpin by self-folding, and DNA extension is repeated (The sixth step in Fig. 1). Finally, the repeated extension produces long DNA structures containing the guide RNA (gRNA)-specific sequences in succession. After the production of long DNA structures, Cas12a/gRNA complexes recognize the specific sequences and the trans-cleavage activity is activated, cleaving the fluorescent-quencher probes and generating fluorescence signals (The seventh step in Fig. 1). The gRNA of CRISPR-Cas12a was designed to detect the sequences comprising each half of the complementary sequence of FW and PS probes. The protospacer adjacent motif (PAM) sequences (5′–TTTV–3′) were designed to be included in the PS probe. Thus, the fluorescence signals can only be generated when the final amplification products contain the Cas12a binding sequences.

2.2. Evaluation of ligation-free isothermal nucleic acid amplification

To evaluate ligation-free isothermal gene amplification, we sequentially checked the ligation and polymerization reactions under different component conditions. First, FW and PS probes were mixed with or without target RNA and incubated at 37 °C for 20 min under the condition with or without SplintR ligase. Next, the reactants were mixed with or without *Bst* DNA polymerase and incubated at 60 °C for 40 min. The real-time fluorescence signals were measured after the final products were mixed with Cas12a/gRNA complexes and fluorescence-quencher probes (Fig. 2A). When FW and PS probes were mixed with target RNA without polymerase, no signals were detected (1 and 2 in Fig. 2A). However, when *Bst* DNA polymerase was mixed with FW and PS probes and target RNA, the fluorescence signals increased without the ligation step (3 in Fig. 2A). This indicates that the repetitive DNA extension, including the target RNA sequence, could occur without ligation, as depicted in Fig. 1. When the ligation and polymerization steps were sequentially reacted with probes and target RNA, increased fluorescence signals were measured (4 in Fig. 2A). Note that the fluorescence signals are similarly enhanced regardless of the ligation step. This suggests that continuous DNA extension through the PS-THSP is feasible after the two probes are hybridized to target RNA, regardless of

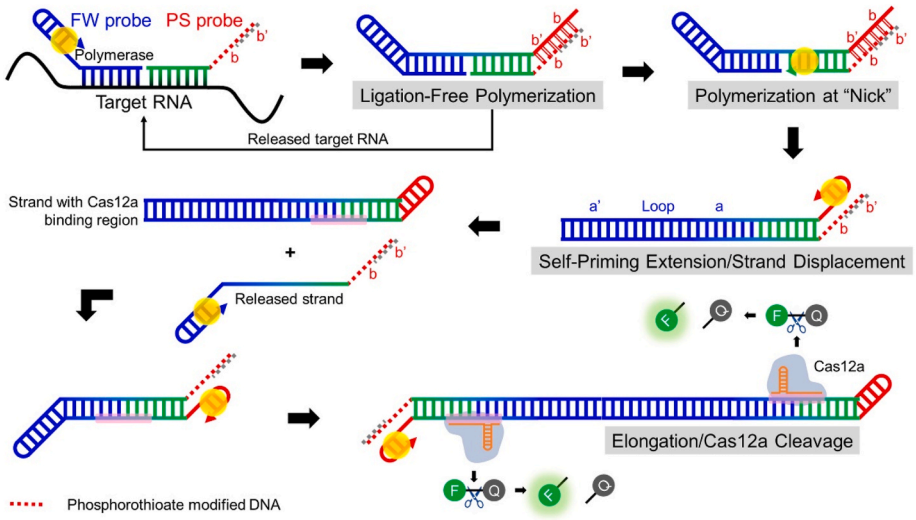

Fig. 1. Schematic illustration of ligation-free nucleic acid amplification.

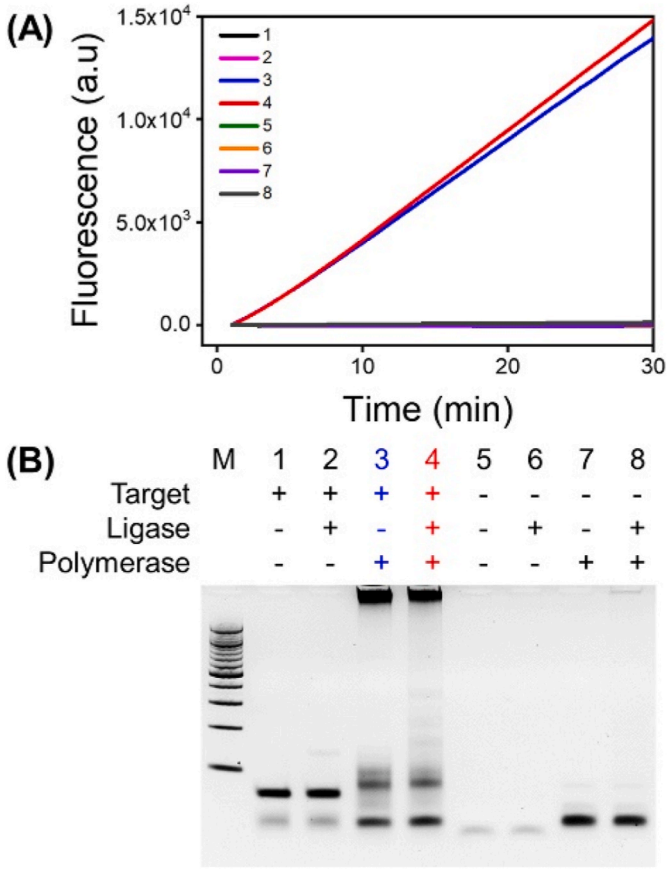

Fig. 2. Evaluation of ligation-free nucleic acid amplification reaction. (A) Real-time fluorescence responses and (B) agarose gel analyses under various component conditions. [Target RNA] = (A) 100 pM, (B) 100 nM, [FW and PS probes] = (A) 10 nM, (B) 100 nM, [*Bst* DNA polymerase] = 20 U, and [SplintR ligase] = 25 U.

the ligation reaction. In contrast. no fluorescence increase was identified in the absence of target RNA (5–8 in Fig. 2A). This result suggests that the polymerization of two probes can occur after hybridization with target RNA. The nucleic acid amplification reactions were further confirmed using agarose gel analyses (Fig. 2B). Bands indicating the long extension of the DNA structure were only observed when the probes and target RNA were incubated with *Bst* DNA polymerase (3 and 4 in Fig. 2B), which was consistent with the fluorescence results. In addition, we assessed the ligation-free nucleic acid amplification under various target and probe conditions (Fig. S2). Only in the presence of target RNA and FW and PS probes did the fluorescence signal increase (4 in Fig. S2), indicating that the proposed method can be useful for the detection of RNA.

To confirm the reaction intermediate, we conducted the ligation and polymerization reactions at low temperature. First, FW and PS probes were mixed with or without target RNA and the ligation reaction was performed at 37 °C for 20 min under the different enzyme conditions. Next, the polymerization reaction proceeded at 45 °C for 40 min with or without polymerase. At the reaction temperature of 45 °C, polymerization can occur, whereas the PS-THSP reaction cannot proceed as the low temperature cannot destabilize and dissociate the duplex of the extended PS probe (Jung and Ellington, 2016). Fig. S3 illustrates the agarose gel results after each reaction. When the probes were mixed with target RNA, a hybridized structure was observed (1 in Fig. S3). Without target RNA, only the band for the probes was observed (5 in Fig. S3). After ligation, the hybridized structure was still identified in the presence of target RNA (2 in Fig. S3) however, not without target RNA (6 in Fig. S3). When polymerase was added, the extended DNA structure was identified regardless of ligation in the presence of target RNA (3 and 4 in Fig. S3). The presumed reaction intermediates are displayed and marked as arrows. The extended structures were not observed without target RNA in the presence of polymerase and/or ligase (7 and 8 in Fig. S3). This result demonstrates the production of the reaction intermediate, as shown in Fig. 1.

To further verify the ligation-free nucleic acid amplification reaction, we added one, two, or three gap sequences to target RNA. As shown in Fig. 3A, the target RNA perfectly matched to FW and PS probes provided highly increased signals, whereas the mismatched target RNAs provided weak signals. This result suggests that polymerase can use the fragmented DNA probes as a template to extend the DNA sequences if there is no gap between two adjacent probes. Moreover, we evaluated the amplification reaction using FW probes containing one, two, or three mismatched sequences to target RNA (Fig. 3B). When the FW probe was perfectly matched to target RNA, the fluorescence signal significantly increased. When a mismatched region was present in the FW probe, the signal decreased. Thus, we concluded that the ligation-free nucleic acid amplification reaction can be operated in the presence of perfectly matched target RNA.

As shown in Fig. 1, we hypothesized that once DNA is extended from

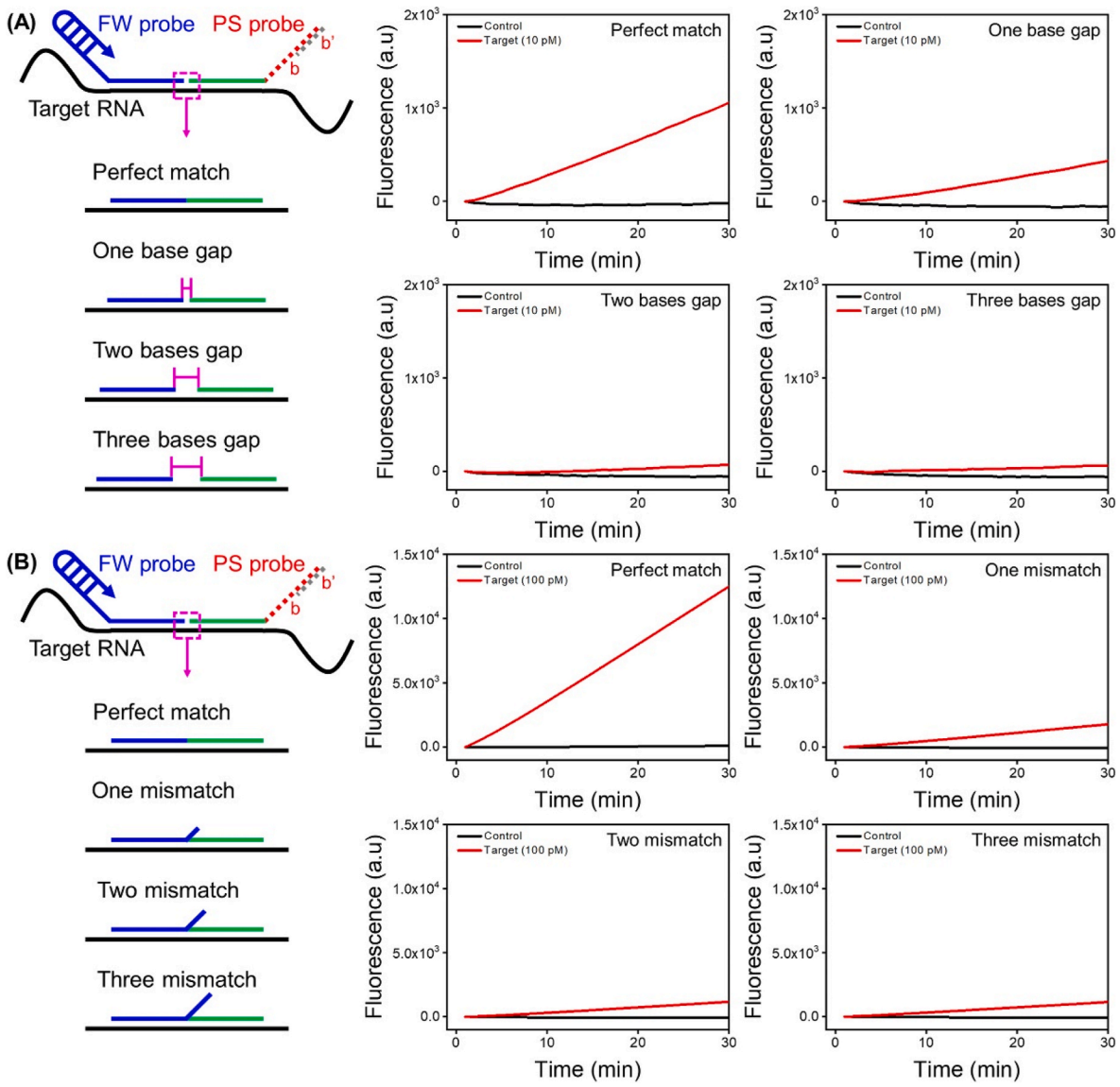

Fig. 3. Comparison of ligation-free nucleic acid amplification reaction (A) depending on the distance between FW and PS probes and (B) the number of mismatched bases in FW probe. [FW probe] = 10 nM, [PS probe] = 10 nM, and [*Bst* DNA polymerase] = 20 U.

the FW to the PS probe, DNA extension begins from the nick at the 3′ end of the PS probe. Concurrently, the extended structure from the 3′ end of the PS probe is dissociated by sequential extension initiated from the self-folded 3′ end of the complementary sequence to the PS probe. Thus, the strand with the Cas12a binding region and the self-folded strand, including PS sequences, are produced. The self-folded strand with PS sequences leads to the PS-THSP reaction, generating long DNA structures that contain several Cas12a binding regions. To confirm polymerization at the nick of the PS probe, we blocked the 3′ end of the PS probe with biotin and proceeded with the reaction under various target RNA and probe conditions. Fig. 4 shows that the fluorescence signal significantly decreased when the 3′ blocked PS probe was employed instead of the normal PS probe. This result indicates that DNA extension can simultaneously start from the nick at the 3′ end of the PS probe and the self-folded complementary sequences to the PS probe. Overall, the proposed ligation-free nucleic acid amplification reaction was proven feasible and thus can detect RNA biomarkers.

2.3. Optimization, sensitivity, and selectivity

The PS-THSP reaction is highly affected by the length of PS sequences (Jung and Ellington, 2016). Therefore, we investigated the ligation-free DNA amplification efficiency based on the length of PS sequences. First, PS probes were prepared to contain the different lengths of PS sequences (12, 20, and 25 bases). Moreover, a PS probe that contains the phosphodiester sequences (20 bases) was prepared. Each PS and FW probe was mixed with or without target RNA and amplified for 45 min at 60 °C. As shown in Fig. S4, the highest fluorescence signal was observed when the PS probe containing 20 bases of PS sequences was used. Based on this result, we designed the PS probe to have 20 bases of PS sequences. Next, the ligation-free PS-THSP reaction was conducted under different temperatures ranging from 50 to 65 °C. Below 50 °C, the amplification efficiency of the PS-THSP reaction significantly decreased because the extended DNA strands were hybridized with PS sequences rather than by self-folding (Jung and Ellington, 2016). Above 55 °C, the PS-THSP reaction occurred, and the optimum temperature was selected as 60 °C (Fig. S5). In addition, as a result of the ligation-free gene amplification reaction over time, it was confirmed that the fluorescence signal was saturated after 40 min of reaction time (Fig. S6).

To compare the reaction efficiencies for different enzymes, four types of enzymes (*Bst* DNA polymerase large fragment, *Bst* 2.0 WarmStart

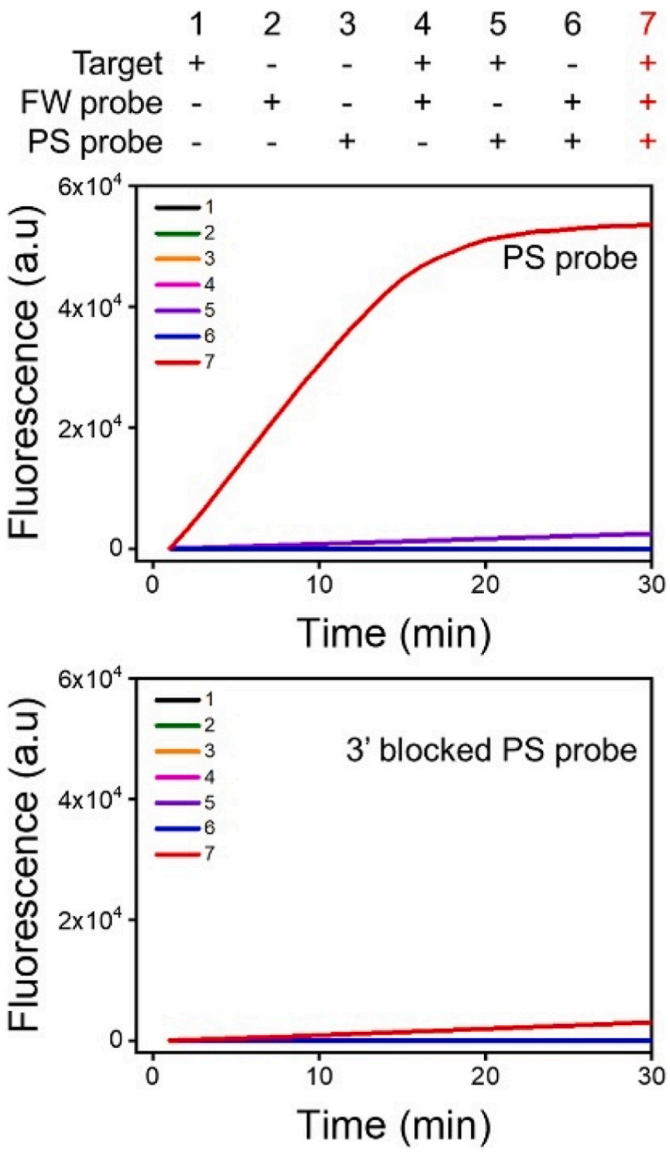

Fig. 4. Comparison of ligation-free nucleic acid amplification reaction using normal PS probe and 3′ blocked PS probe. [Target RNA] = 100 pM, [FW probe] = 10 nM, [PS probe] = 10 nM, and [*Bst* DNA polymerase] = 20 U.

DNA polymerase, *Bst* 3.0 DNA polymerase, and Vent (exo-) DNA polymerase) were examined. *Bst* DNA polymerase large fragment showed fast fluorescence signal increase during the CRISPR/Cas12-cleavage reaction, and Vent (exo-) DNA polymerase showed no signal (Fig. S7A). The agarose gel results correspond to the fluorescence results (Fig. S7B). Enzyme concentration, probe concentration, and reaction buffer condition were also optimized (Figs. S8, S9, S10, and S11). Based on these results, 20 U of *Bst* DNA polymerase large fragment, 10 nM probes, and SplintR ligase buffer containing 2 mM $MgSO_4$ were used in this strategy.

After optimizing the amplification conditions, the sensitivity of the ligation-free isothermal nucleic acid amplification reaction was evaluated using synthetic ERBB2 target RNA ranging from 1 fM to 100 pM. Target RNA was mixed with FW and PS probes (10 nM each) and *Bst* DNA polymerase large fragment (20 U) and incubated at 60 °C for 40 min. Next, the fluorescence signal was measured in real-time after mixing it with the Cas12a/gRNA complex. As the amount of target RNA increased, the rate of the fluorescence response increased, with greater observed intensity (Fig. 5A and B). Moreover, the fluorescence intensity obtained after the CRISPR reaction for 30 min depended linearly on the logarithm of the target RNA concentration ($y = 0.6112x + 10.3239$, $R^2 = 0.9622$), indicating that the concentration of target RNA can be quantified with the developed ligation-free gene amplification method (Fig. 5C). The limit of detection (LOD) was estimated as 49.2 fM according to the formula LOD = 3 × standard deviation of linear regression/slope, which is comparable to prior research based on THSP (Jung and Ellington, 2016; Abdullah Al-Maskri et al., 2020).

To evaluate the selectivity, three types of RNAs (GAPDH, GRB7, and PPP1R1B sequence) and non-target ssDNAs were employed instead of target ERBB2 RNA. As shown in Fig. 5D, the fluorescence signal could only be observed in the presence of target ERBB2 RNA, which indicates that the ligation-free gene amplification could specifically detect the target RNA. Moreover, three types of RNAs containing a single-mismatch region in different sites were also tested. As a result, the fluorescence signal was highly increased in the presence of perfectly matched target RNA, whereas the fluorescence signal was reduced when a single-mismatched region existed in the target RNA sequence. It represents this system can be applied for a single-nucleotide polymorphism detection assay (Fig. S12).

2.4. Practical utility

Amplification and overexpression of the human epidermal growth factor receptor 2 (HER2; ERBB2) gene are observed in 15–30% of invasive breast cancer cases (Sahlberg et al., 2013). In addition, certain genes, such as the growth factor receptor bound protein 7 (GRB7), the star related lipid transfer domain containing 7 (STARD7), and the protein phosphatase 1 regulatory inhibitor subunit 1B (PPP1R1B), are co-amplified with the ERBB2 gene to accelerate breast cancer cell growth and cause further drug resistance (Kauraniemi and Kallioniemi, 2006). Therefore, detecting multiple genes in a single assay is highly important. Therefore, we attempted to detect ERBB2 and GRB7 simultaneously using the ligation-free gene amplification method.

To investigate the simultaneous detection of RNAs, different RNA samples (control, ERBB2, GRB7, mixture of ERBB2 and GRB7) were prepared. The samples were mixed with all four probe pairs (FW and PS probe of ERBB2, and FW and PS probe of GRB7) and reacted in the buffer at 60 °C for 50 min. Next, the final reactant was mixed with Cas12a/gRNA recognizing ERBB2 and GRB7, respectively. As shown in Fig. S13, when the reactants were mixed with Cas12a/gRNA for ERBB2, the fluorescence signal selectively increased in samples containing the ERBB2 sequence (ERBB2 and mixture); when the reactants were mixed with Cas12a/gRNA for GRB7, the fluorescence signal increased in samples containing the GRB7 sequence (GRB7 and mixture). This result shows that the developed assay effectively detected the target RNAs in a single tube and maintained the specificity of each probe pair.

Finally, we examined the ligation-free nucleic acid amplification method for practical RNA analysis. First, total RNA samples extracted from human cell lines of HCC1954 and HCC1143, respectively, were prepared as HCC1954 cells are HER2-positive, whereas HCC1143 cells are HER2-negative (Grigoriadis et al., 2012). ERBB2 and GRB7 have been reported as overexpressed in HER2-positive breast cancer cell lines (Dean-Colomb and Esteva, 2008). Therefore, we compared the ERBB2 and GRB7 mRNA expression levels between HCC1954 and HCC1143 cell lines using the proposed RNA detection method. The extracted human mRNA reacted with four probe pairs and the fluorescence signal was measured after mixing with Cas12a/gRNA targeting ERBB2 and GRB7, respectively (Fig. S14A). As shown in Fig. S14B, the fluorescence response of the HCC1954 sample was faster than HCC1143, indicating that ERBB2 and GRB7 are overexpressed in HCC1954 cells. The identical cellular RNA samples were analyzed using quantitative RT-PCR (qRT-PCR), and the results were correlated with the ligation-free gene amplification results (Fig. S14C).

We further assessed the ligation-free gene amplification reaction for the RNA analysis of tumor-bearing mouse tissue and urine samples (Fig. 6A). A tumor-bearing mouse model was prepared by injecting

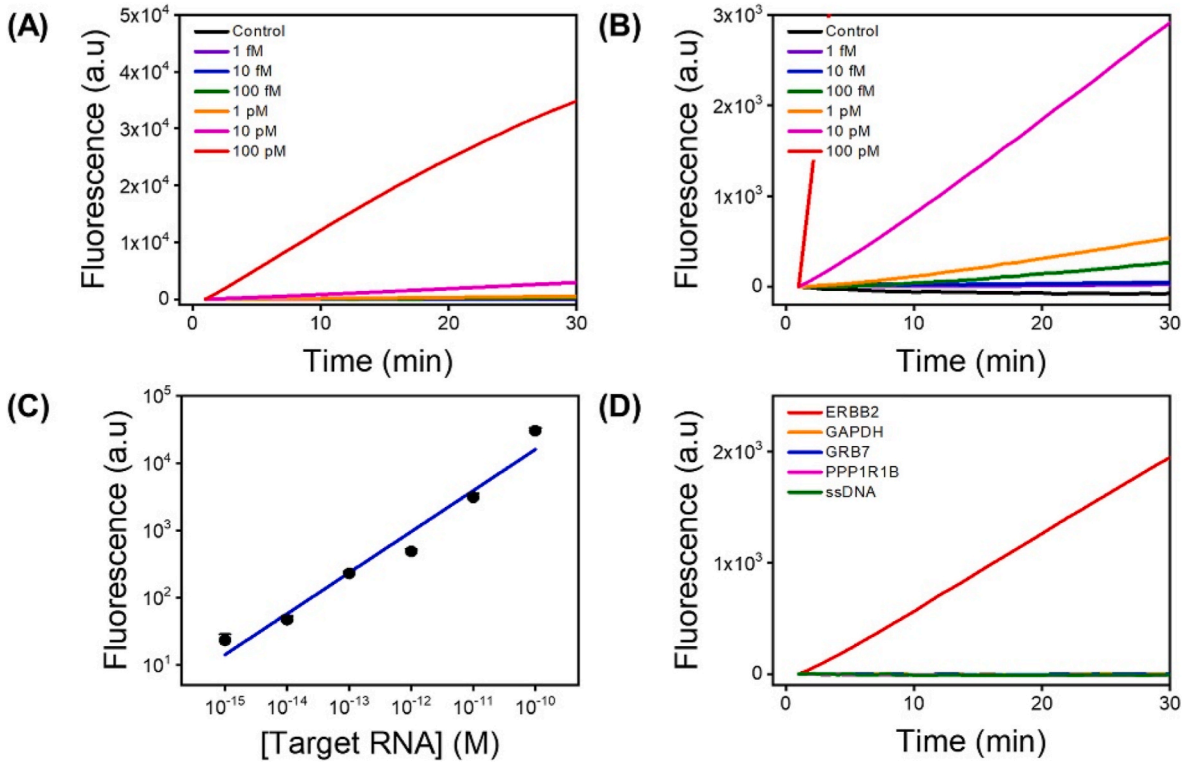

Fig. 5. Sensitivity and selectivity of ligation-free nucleic acid amplification reaction. (A, B) Real-time fluorescence responses at various target RNA concentrations. (C) Fluorescence intensity as a function of target RNA concentration (n = 3, error bar = standard deviation). (D) Real-time fluorescence responses with various RNAs (ERBB2, GAPDH, GRB7, and PPP1R1B) and non-target ssDNA. Concentration of RNAs and ssDNA was 10 pM.

HCC1954 cells into the breast skin of mice (Fig. 6B). After observation of tumor growth, tumor tissue was excised for RNA analysis. We also excised livers, lungs, kidneys, spleens from both normal and tumor-bearing mice. The collected organs were cut into small pieces and homogenized with lysis buffer. Next, total RNAs were extracted and the ERBB2 and GRB7 gene expression levels were compared between each organ and tumor tissue from normal and tumor-bearing mice using the ligation-free gene amplification method, respectively. The extracted RNA samples were mixed with Bst DNA polymerase and four types of probes that hybridize to ERBB2 and GRB7, followed by incubation. By measuring the fluorescence signal after mixing the reactants with Cas12a/gRNA targeting ERBB2 and GRB7, respectively, both ERBB2 and GRB7 fluorescence was confirmed faster in tumor tissue than in other organ tissues, showing that both genes were overexpressed in tumor tissues (Fig. 6C). The qRT-PCR results were also correlated with the ligation-free gene amplification results (Fig. S15).

Next, we detected the urinary exosomal RNA using the ligation-free nucleic acid amplification reaction. Exosomes, vesicles secreted into body fluids from cells, are considered promising biomarkers for liquid biopsy as the exosome is abundant in body fluids and carries many types of biomarkers, such as nucleic acids, lipids, and proteins (Kalluri and LeBleu, 2020). We detected urinary exosomal RNA by collecting urine samples from normal and tumor-bearing mice, respectively, and by isolating exosomes from urine. The extracted exosomes were analyzed by transmission electron microscopy (TEM) image and nanoparticle tracking analysis (NTA) (Fig. S16). RNA was extracted from the exosome samples and used for further experiments as in the cell and tissue experiments. The measurement of ERBB2 and GRB7 expression levels using the ligation-free gene amplification method revealed that both genes were overexpressed in the urinary exosome samples of tumor-bearing mice (Fig. 6D). The successful diagnostic results of the tumor-bearing mouse tissue and urine samples provide empirical support for the practical applications of the ligation-free nucleic acid amplification method for disease diagnosis.

3. Discussion

Various RNA detection methods have been reported for diagnosing the onset of many infectious diseases and cancers (Esbin et al., 2020). The current PCR-based technologies are highly sensitive and accurate; however, they require complex procedures, heavy instrumentation, and expertise (Nolan et al., 2006). The developments in diverse isothermal gene amplification methods that can be performed at a constant temperature using a simple device have provided benefits for point-of-care detection over PCR-based methods (Yan et al., 2014). However, these methods still entail complex primer design procedures, many types of primers and enzyme mixtures, and reverse transcription processes. Recently, ligation-dependent RNA detection methods have emerged as they have a simpler primer design and do not require reverse transcription (Gibriel and Adel, 2017; Jin et al., 2016; Woo et al., 2020). They use two types of oligo probes, which are joined by a ligase when they have hybridized adjacently to a target nucleic acid template. Nevertheless, the ligated oligo probes are amplified by PCR or isothermal gene amplification for signal processing and require additional primers and enzymes and/or temperature control (Gibriel and Adel, 2017).

In this study, we developed a new ligation-free isothermal nucleic acid amplification method. The method can amplify a target RNA sequence at a constant temperature with a DNA polymerase and two types of probes, which can hybridize adjacent to the target RNA. The two probes contained a single-stranded target binding region and hairpin-structured domain at each end of the probes. One of the hairpin-structured sequences can easily promote DNA polymerase binding and consequent polymerization without a separate primer. Another PS-hairpin sequence can reduce the melting temperature, leading to the self-priming extension. We hypothesized that continuous self-priming

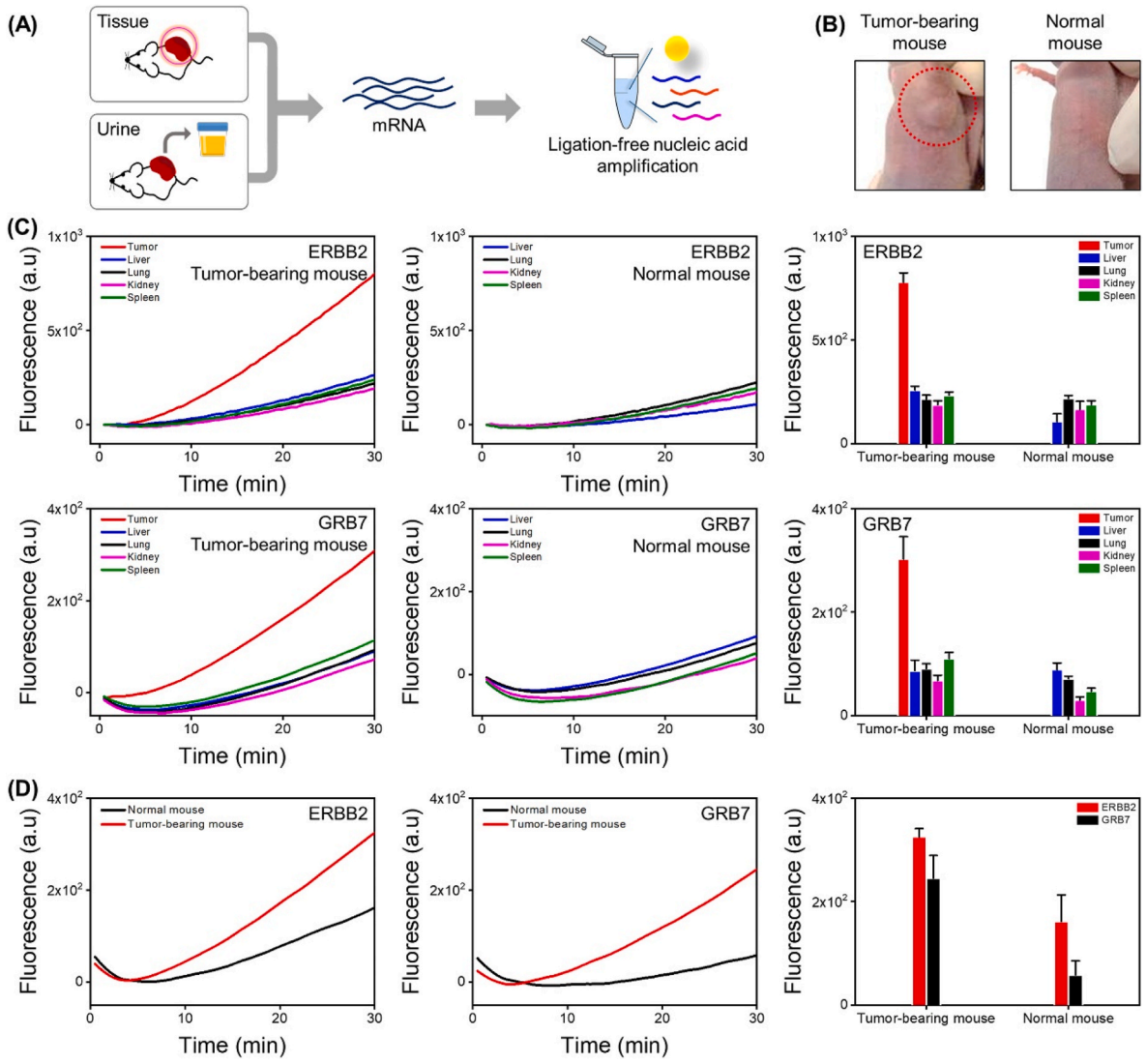

Fig. 6. (A) Schematic illustration of mRNA detection from tumor-bearing mouse tissue and urine samples using ligation-free nucleic acid amplification reaction. (B) Image of tumor-bearing mouse and normal mouse. (C, D) ERBB2 and GRB7 gene analysis results from (C) tissue and (D) urine samples using ligation-free nucleic acid amplification reaction. Left and middle panels are real-time fluorescence responses and right panels show the plots of fluorescence intensity at 30 min as a function of the mouse model (n = 3, error bar = standard deviation). [Total tissue RNA] = 10 ng, [Total exosomal RNA] = 1 ng.

extension could occur when two probes are joined by ligase and elongation initiated via ssDNA as a template (Fig. S1). We then discovered that the ligation-free gene extension (Fig. 1) was possible and thus investigated the reaction mechanism. Based on this discovery, we designed the ligation-free isothermal nucleic acid amplification method and successfully detected target RNAs in human cell lines and tumor-bearing mouse tissues and urine samples.

Another improvement in our approach combines ligation-free isothermal nucleic acid amplification with CRISPR/Cas12a-based signal amplification. Several gene amplification methods use a self-priming extension; however, these cannot readily measure real-time fluorescence signals, as fluorescence signals are highly dependent on the type of intercalating dye (Jung and Ellington, 2016; Cai et al., 2018). Moreover, non-specific signals increase when the final products are mixed with dyes to measure the fluorescence signal (Jung and Ellington, 2016; Cai et al., 2018). In this study, we adopted the CRISPR/Cas12a system for additional signal amplification while identifying the ligation-free gene amplification reaction. We selected the LbCas12a enzyme as it provides outstanding cleavage activity when comparing several types of Cas12a proteins, including LbCas12a, AsCas12a, and EeCas12a (Talwar et al., 2021). LbCas12a/gRNA can recognize the target sequence specifically and amplify the fluorescence signal by its trans-cleave activity. We designed the gRNA to include each half sequence of target binding regions within the two probes to prevent Cas12a/gRNA from being activated in the presence of two probes. Because Cas12a/gRNA facilitates very selective recognition of the target RNA sequence, the non-specific signal by the probe was barely detected. The developed method was compared with previous ligation-mediated gene amplification methods (Table S2).

Compared to the tissue biopsies that invasively extract tissue samples, liquid biopsies that diagnose diseases using body fluids are increasingly preferred (Ignatiadis et al., 2021). However, it is challenging to detect biomarkers in liquid biopsies because of their low abundance and high mutation rate (Esagian et al., 2020). Therefore, more accurate detection is required for liquid biopsies (Agrawal et al. 2020a, 2020b; Kaur et al., 2022; Zhu et al., 2020). Here, we improved the target detection specificity by introducing two adjacent probes and CRISPR/Cas12a and thus could amplify and detect two target genes simultaneously. Therefore, the mRNA expressed in cells, tissues, and urinary exosomes could be successfully analyzed, which demonstrates that our technique can be applied to not only tissue biopsy but also liquid biopsy.

Despite the advantages and novelty of our approach, we note that the amplification and signal detection processes are separated because of the different optimal temperatures for gene amplification and Cas12a/gRNA reaction. Thus, the one-step reaction can be improved through a thermophilic Cas12a/gRNA or by reducing the reaction temperature for gene amplification. Our research group is working on a one-step isothermal gene amplification technique based on ligation-free gene amplification.

4. Conclusion

In this study, we discovered a ligation-free gene amplification system using two adjacent probes and verified the principle using fluorescence and gel electrophoresis. Further, we developed a ligation-free isothermal nucleic acid amplification method based on PS-terminal hairpin mediated self-priming extension combination with CRISPR/Cas12a signal amplification to detect target RNA. Using this method, target RNA was selectively detected with a LOD of 49.2 fM and two target RNAs could be analyzed. Moreover, the assay can specifically detect target RNA in cancer cell lines, tissues of mice, and urine samples of tumor-bearing mice, demonstrating that the method can be used for tissue diagnostics and liquid biopsy. This is the first demonstration of RNA detection based on ligation-independent gene amplification and provides significant benefits for the future of molecular diagnostics. First, this method can continuously amplify target RNA sequences without the reverse transcription step. Second, this strategy can operate with two probes and an enzyme without complicated multiple primers or enzymes. Considering that the PS-hairpin sequence can be maintained and only the target sequence must be considered, the probes can be easily designed for various target sequences. Third, the trans-cleave activity of Cas12a that can specifically recognize the target sequence improves signal sensitivity and specificity compared to the previous THSP reactions (Cai et al., 2018; Abdullah Al-Maskri et al., 2020). We expect that the developed ligation-free gene amplification method could evolve into an efficient isothermal amplification method for molecular diagnoses.

Funding

This work was supported by National R&D Programs through National Research Foundation (NRF) of Korea funded by the Ministry of Science and ICT (MSIT) of Korea [NRF-2020R1A2C1010453, NRF-2021M3E5E3080844, NRF-2021M3H4A1A02051048, NRF-2021M3E5E3080379, NRF-2018M3A9E2022821, NRF-2018M3A9E2022828, NRF-2019H1A2A1073468, NRF-2021R1A2B5B03001739, and NRF-2017M3A7B4041975]; Global Frontier Program through Center for BioNano Health-Guard funded by MSIT of Korea [H-GUARD_2014M3A6B2060507 and H-GUARD_2013-M3A6B2078950]; Technology Development Program for Biological Hazards Management in Indoor Air through Korea Environment Industry & Technology Institute (KEITI) funded by Ministry of Environment (ME) of Korea [2021003370003]; Industrial Technology Alchemist Program funded by the Ministry of Trade, Industry, and Energy (MOTIE) of Korea [20012435]; Nanomedical Devices Development Program of National Nano Fab Center [CSM2105M101]; and KRIBB Research Initiative Program [1711134081].

CRediT authorship contribution statement

Jeong Moon: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Jayeon Song:** Methodology, Investigation. **Hyowon Jang:** Methodology, Investigation. **Hyunju Kang:** Investigation. **Yong-Min Huh:** Resources. **Hye Young Son:** Resources. **Hyun Wook Rho:** Resources. **Mirae Park:** Resources. **Chandana S. Talwar:** Resources. **Kwang-Hyun Park:** Resources. **Euijeon Woo:** Resources. **Jaewoo Lim:** Validation, Writing – review & editing. **Eun-Kyung Lim:** Project administration. **Juyeon Jung:** Project administration. **Yongwon Jung:** Project administration. **Hyun Gyu Park:** Writing – review & editing, Supervision, Project administration. **Taejoon Kang:** Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114256.

References

Abdullah Al-Maskri, A.A., Ye, J., Talap, J., Hu, H., Sun, L., Yu, L., Cai, S., Zeng, S., 2020. Anal. Chim. Acta 1126, 1–6. https://doi.org/10.1016/j.aca.2020.06.007.
Agrawal, N., Zhang, B., Saha, C., Kumar, C., Kaushik, B.K., Kumar, S., 2020a. IEEE. Trans. Biomed. 67, 1542–1547.
Agrawal, N., Zhang, B., Saha, C., Kumar, C., Pu, X., Kumar, S., 2020b. J. Lightwave Technol. 38, 2523–2529.
Cai, S., Jung, C., Bhadra, S., Ellington, A.D., 2018. Anal. Chem. 90, 8290–8294. https://doi.org/10.1021/acs.analchem.8b02062.
Cella, L.N., Blackstock, D., Yates, M.A., Mulchandani, A., Chen, W., 2013. Crit. Rev. Eukaryot. Gene Expr. 23, 125–137. https://doi.org/10.1615/critreveukaryotgeneexpr.2013006974.
Cooper, T.A., Wan, L., Dreyfuss, G., 2009. Cell 136, 777–793. https://doi.org/10.1016/j.cell.2009.02.011.
Dean-Colomb, W., Esteva, F.J., 2008. Eur. J. Cancer 44, 2806–2812. https://doi.org/10.1016/j.ejca.2008.09.013.
Esagian, S.M., Grigoriadou, G.I., Nikas, I.P., Boikou, V., Sadow, P.M., Won, J.K., Economopoulos, K.P., 2020. J. Cancer Res. Clin. Oncol. 146, 2051–2066. https://doi.org/10.1007/s00432-020-03267-x.
Esbin, M.N., Whitney, O.N., Chong, S., Maurer, A., Darzacq, X., Tjian, R., 2020. RNA 26, 771–783. https://doi.org/10.1261/rna.076232.120.
Gibriel, A.A., Adel, O., 2017. Mutat. Res. Rev. Mutat. Res. 773, 66–90. https://doi.org/10.1016/j.mrrev.2017.05.001.
Goo, N.I., Kim, D.E., 2016. BioChip J. 10, 262–271. https://doi.org/10.1007/s13206-016-0402-6.
Grigoriadis, A., Mackay, A., Noel, E., Wu, P.J., Natrajan, R., Frankum, J., Reis-Filho, J.S., Tutt, A., 2012. BMC Genom. 13, 619. https://doi.org/10.1186/1471-2164-13-619.
Ignatiadis, M., Sledge, G.W., Jeffrey, S.S., 2021. Nat. Rev. Clin. Oncol. 18, 297–312. https://doi.org/10.1038/s41571-020-00457-x.
Jin, J., Vaud, S., Zhelkovsky, A.M., Posfai, J., McReynolds, L.A., 2016. Nucleic Acids Res. 44, e116. https://doi.org/10.1093/nar/gkw399.
Ju, Y., Kim, H.Y., Ahn, J.K., Park, H.G., 2021. Nanoscale 13, 10785–10791. https://doi.org/10.1039/d1nr00564b.
Jung, C., Ellington, A.D., 2016. Anal. Bioanal. Chem. 408, 8583–8591. https://doi.org/10.1007/s00216-016-9479-y.
Jung, Y., Song, J., Park, H.G., 2021. Sci. Rep. 11, 8399. https://doi.org/10.1038/s41598-021-87948-8.
Kalluri, R., LeBleu, V.S., 2020. Science 367. https://doi.org/10.1126/science.aau6977.
Kato, T., Liang, X., Asanuma, H., 2012. Biochemistry 51, 7846–7853. https://doi.org/10.1021/bi3010413.
Kauraniemi, P., Kallioniemi, A., 2006. Endocr. Relat. Cancer 13, 39–49. https://doi.org/10.1677/erc.1.01147.
Kaur, B., Kumar, S., Kaushik, B.K., 2022. Biosens. Bioelectron. 197, 113805.
Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., Lens, P., 1991. J. Virol. Methods 35, 273–286. https://doi.org/10.1016/0166-0934(91)90069-C.
Kurzawski, G., Dymerska, D., Serrano-Fernández, P., Trubicka, J., Masojć, B., Jakubowska, A., Scott, R.J., 2012. Hered. Cancer Clin. Pract. 10, 17. https://doi.org/10.1186/1897-4287-10-17.
Lohman, G.J., Zhang, Y., Zhelkovsky, A.M., Cantor, E.J., Evans Jr., T.C., 2014. Nucleic Acids Res. 42, 1831–1844. https://doi.org/10.1093/nar/gkt1032.
Nolan, T., Hands, R.E., Bustin, S.A., 2006. Nat. Protoc. 1, 1559–1582. https://doi.org/10.1038/nprot.2006.236.
Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Nucleic Acids Res. 28, E63. https://doi.org/10.1093/nar/28.12.e63.
Nouri, N., Fazel-Najafabadi, E., Salehi, M., Hosseinzadeh, M., Behnam, M., Ghazavi, M. R., Sedghi, M., 2014. Adv. Biomed. Res. 3, 72. https://doi.org/10.4103/2277-9175.125862.
Poltronieri, P., Sun, B., Mallardo, M., 2015. Curr. Genom. 16, 327–335. https://doi.org/10.2174/1389202916666150707160613.
Sahlberg, K.K., Hongisto, V., Edgren, H., Mäkelä, R., Hellström, K., Due, E.U., Moen Vollan, H.K.M., Sahlberg, N., Wolf, M., Børresen-Dale, A.L., Perälä, M.,

Kallioniemi, O., 2013. Mol. Oncol. 7, 392–401. https://doi.org/10.1016/j.molonc.2012.10.012.
Song, J., Kim, H.Y., Kim, S., Jung, Y., Park, H.G., 2021. Biosens. Bioelectron. 178, 113051 https://doi.org/10.1016/j.bios.2021.113051.
Talwar, C.S., Park, K.H., Ahn, W.C., Kim, Y.S., Kwon, O.S., Yong, D., Kang, T., Woo, E., 2021. Biosensors 11. https://doi.org/10.3390/bios11090301.
Walker, G.T., Fraiser, M.S., Schram, J.L., Little, M.C., Nadeau, J.G., Malinowski, D.P., 1992. Nucleic Acids Res. 20, 1691–1696. https://doi.org/10.1093/nar/20.7.1691.
Woo, C.H., Jang, S., Shin, G., Jung, G.Y., Lee, J.W., 2020. Nat. Biomed. Eng. 4, 1168–1179. https://doi.org/10.1038/s41551-020-00617-5.
Yan, L., Zhou, J., Zheng, Y., Gamson, A.S., Roembke, B.T., Nakayama, S., Sintim, H.O., 2014. Mol. Biosyst. 10, 970–1003. https://doi.org/10.1039/c3mb70304e.
Zhu, G., Agrawal, N., Singh, R., Kumar, S., Zhang, B., Saha, C., Kumar, C., 2020. Opt Laser. Technol. 127, 106156.