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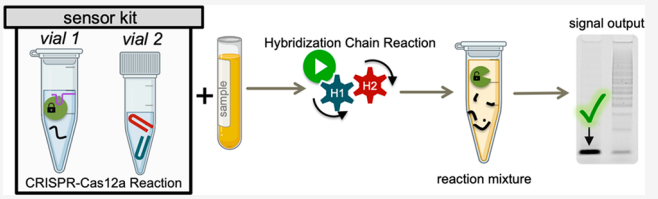
Reprogrammable Gel Electrophoresis Detection Assay Using CRISPR-Cas12a and Hybridization Chain Reaction

Mahera J. Kachwala, Christopher W. Smith, Nidhi Nandu, and Mehmet V. Yigit*

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ABSTRACT: Hybridization chain reaction (HCR) is a DNA-based target-induced cascade reaction. Due to its unique enzyme-free amplification feature, HCR is often employed for sensing applications. Much like DNA nanostructures that have been designed to respond to a specific stimulus, HCR employs nucleic acids that reconfigure and assemble in the presence of a specific trigger. Despite its standalone capabilities, HCR is highly modular; therefore, it can be advanced and repurposed when coupled with latest discoveries. To this effect, we have developed a gel electrophoresis-based detection approach which combines the signal amplification feature of HCR with the programmability and sensitivity of the CRISPR-Cas12a system. By incorporating CRISPR-Cas12a, we have achieved greater sensitivity and reversed the signal output from TURN OFF to TURN ON. CRISPR-Cas12a also enabled us to rapidly reprogram the assay for the detection of both ssDNA and dsDNA target sequences by replacing a single reaction component in the detection kit. Detection of conserved, both ssDNA and dsDNA, regions of tobacco curly shoot virus (TCSV) and hepatitis B virus (HepBV) genomes is demonstrated with this methodology. This low-cost gel electrophoresis assay can detect as little as 1.5 fmol of the target without any additional target amplification steps and is about 100-fold more sensitive than HCR-alone approach.

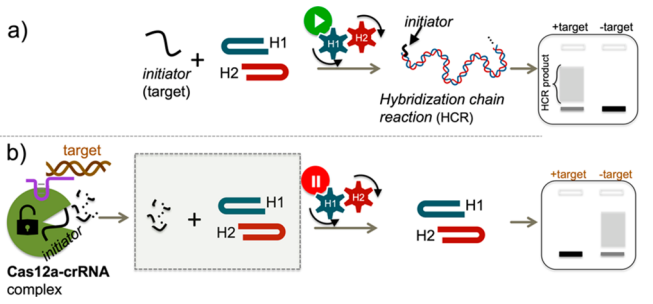


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DNA has been an invaluable material for development of advanced functional systems owing to its programmable recognition, assembly, and reconfiguration features. Several types of DNA-based technologies have been developed and evaluated for a number of scientific aims.[1,2] Hybridization chain reaction (HCR) is one such technology that has been instrumental for oligonucleotide-based detection with an exceptional enzyme-free signal amplification feature.[3,4] In HCR, two metastable hairpins (H1 and H2, <50 bp each) coexist in solution without communicating with each other. However, when a short ssDNA, termed *initiator* (target), is introduced into the reaction mixture, interaction between the hairpins is initiated, which results in the assembly of a long unbranched dsDNA polymer (≤1000 bp) (Scheme 1a). Briefly, in HCR, the initiator first hybridizes with H1, which then becomes available to bind to H2. After this step, the activated H2 opens and binds to another H1 in the environment, causing a cascade reaction termed *hybridization chain reaction* (HCR). The HCR product, which is an indication of target detection, can be measured by the appearance of a broad DNA band (assembled H1 and H2) in gel electrophoresis, (Scheme 1a).

Unlike conventional nucleic acid amplification techniques,[5] HCR is a nonenzymatic reaction that magnifies the probe signal instead of the target and can be performed in ambient conditions. While its simplicity and effectiveness render HCR highly favorable, its sensitivity can be improved for clinical diagnostics or agricultural screening.[6,7] Furthermore, although

Scheme 1. Schematic Illustration of Hybridization Chain Reaction and Gel Electrophoresis Result (a) with and (b) without CRISPR-Cas12a Preamplification Reaction



HCR in combination with aptamer technology can be used for detecting other biomolecules,[8,9] its nucleic acid detection is generally limited to single-stranded DNAs or RNAs.[10−15] Thus, increasing its target pool from a ssDNA strand (initiator) to a broad spectrum of ssDNA and dsDNA targets could advance the HCR-based detection technologies. The

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CRISPR-Cas12a system is highly valuable to accomplish these advancements.

The RNA-mediated defense mechanism called clustered regularly interspaced short palindromic repeats (CRISPR), and its associated endonuclease proteins (Cas proteins), form a formidable and adaptive immune response.[16−18] CRISPR-Cas technology has led to numerous inventions in molecular biology and disease diagnostics since its discovery in 2012.[19] CRISPR-Cas type II (Cas9), V (Cas12), and VI (Cas13) are the three main systems that have been employed for these diagnostic purposes.[20−24] Cas12a is an RNA-guided endonuclease which binds to a specific ssDNA or dsDNA through a rationally designed CRISPR RNA (crRNA), containing an ortholog specific hairpin scaffold sequence and a nucleic acid-specific recognition sequence.[25] Upon recognition of the target, Cas12a gets activated and indiscriminately cleaves all DNAs in its proximity.[26] The CRISPR-Cas12a system offers improved programmability and sensitivity properties to any detection approach.[27] In this study, by integrating CRISPR-Cas12a into HCR, greater sensitivity and target diversity is aimed, simultaneously.

In this CRISPR-HCR combination approach, H1 and H2, the initiator strand, crRNA, and Cas12a are the principal elements of the assay. The crRNA is the only component that is changed to program the assay for a target of interest. The rest of the elements in the assay are not changed. Upon recognition of a target, Cas12a is activated and cleaves the initiator strand (Scheme 1b) which, in its absence, is unable to initiate HCR. In principle, if the target is present, the initiator would be cleaved which cannot react with H1 and H2 and cannot initiate HCR (Scheme 1b). However, if the target is absent, the initiator would not be cleaved, which would be available to react with H1 and H2 hairpins resulting in HCR. The results can be monitored and quantified by the gel electrophoresis assay.

Out of the multitude of signal readout techniques used for HCR detection, optical methods are the most widely used.[3,28] Gel electrophoresis-based detection approaches, on the other hand, have been overlooked for a long time despite being cost effective and readily available in almost every biochemistry lab. In recent years, however, gel electrophoresis has been evaluated for biodetection studies by measuring the reconfiguration of large DNA nanostructures termed DNA nanoswitches.[29−32] Halvorsen et al. has utilized gel electrophoresis to measure the target-induced reconfiguration of DNA nanoswitches to detect miRNA,[33] Zika virus,[34] and more.[35] These reports, along with others, emphasize that gel electrophoresis is highly competitive for development of diagnostic assays. Here, we have demonstrated a gel electrophoresis-based CRISPR-HCR assay for the detection of two model systems—conserved regions of tobacco curly shoot virus (TCSV) and hepatitis B virus (HepBV) genomes—and achieved superior sensitivity and target diversity over the HCR-alone approach. Although, researchers have previously coupled HCR with CRISPR-Cas systems, the strength of our assay is its homogeneous nature, which makes our no-wash procedure simple, convenient, and less error prone.[36,37]

First, a gel electrophoresis assay was performed for the detection of a target strand (1 pmol, initiator in this case) using the HCR-alone approach. The gel electrophoresis data showed that the intensity of band 1 (unassembled H1 and H2 pair) decreased, whereas a broad band, band 2 (assembled H1 and H2 pair), appeared upon target recognition (Figure 1a).

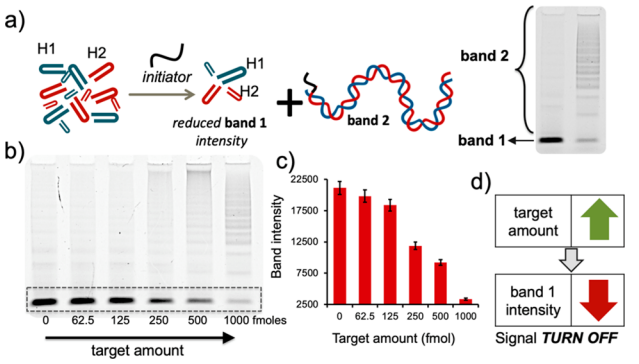


Figure 1. (a) Schematic illustration of reduced band 1 intensity and increased band 2 intensity caused due to HCR. (b) Gel showing decreasing band 1 intensity with increasing target amount. (c) Graphical representation of band intensities against the increasing target amounts. Data are represented as mean ± SD.

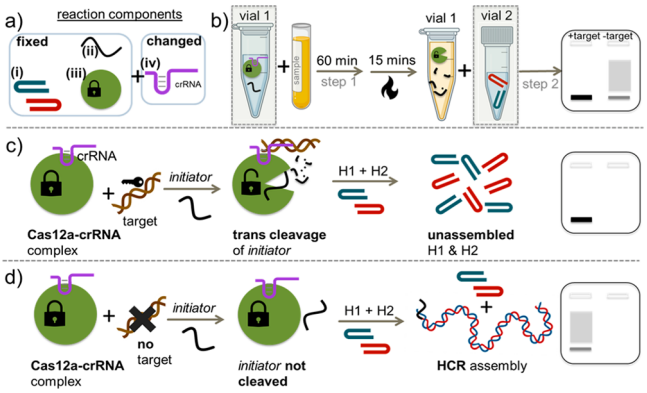
The high background interference for a broad band as compared to a sharp band (band 1) also makes it relatively difficult to quantify band 2. Further, because band 1 specifically represents unassembled H1 and H2, and the reduction of its intensity is proportional to the assembly of H1 and H2, the detection of this band is easier and more reliable. The decrease in band 1 is an indication of the H1 and H2 consumption upon target recognition. Thus, we have used band 1 to evaluate the detection performance of HCR using gel electrophoresis.

Different amounts (0, 62.5, 125, 250, 500, and 1000 fmol) of the target strand (initiator) were used to evaluate the detection performance by measuring band 1 intensity. The studies suggest that as the target amount increases, the band 1 intensity decreases (TURN OFF) in a concentration-dependent manner (Figure 1b−d).

After demonstrating that gel electrophoresis could be instrumental for the detection of a specific target strand using HCR, we have investigated to see whether CRISPR-Cas12a can be utilized in the detection scheme to (i) improve the sensitivity and (ii) diversify the target type, without making any changes in the H1 and H2 pair and initiator composition of the assay.

In the CRISPR-HCR combination detection approach, H1 and H2, the initiator strand, and Cas12a are the key unchanged components of the detection kit (Scheme 2a, b). crRNA is the

Scheme 2. Depiction of (a) Reaction Components and (b) Reaction Protocol of CRISPR-Cas12a Coupled HCR Assay and Schematic Illustration of Detection System in (c) Presence of Target and (d) Absence of Target



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only component that is redesigned and changed to program the detection kit for the target of interest. The reaction is carried out by first incubating the sample with vial 1 containing the Cas12a−crRNA complex and the initiator. If the target is present in the sample, the initiator is degraded by the Cas12a−crRNA complex (Scheme 2c); however, in its absence, the initiator will remain intact (Scheme 2d). After 60 min of incubation, the reaction is heated to 65 °C to inactivate Cas12a and stop it from degrading hairpins in the next step (Scheme 1b). The resulting mixture is mixed with vial 2, which contains H1 and H2 hairpins (Scheme 2b). At the end of this period, gel electrophoresis is performed to monitor the change in the band 1 intensity. If during step 2 the initiator was shredded, the H1 and H2 hairpins will not react, and no change in band 1 intensity could occur (Scheme 2c). Conversely, if the initiator was not shredded, it would initiate HCR, and H1 and H2 hairpins would react to form the HCR assembly. As a result of this, band 1 intensity would decrease (Scheme 2d)

First, a model dsDNA target TCSV (a highly specific fragment from tobacco curly shoot virus) is identified, and Cas12a is programmed for the detection using a specific crRNA ($crRNA_{TCSV}$) against TCSV. When the Cas12a−crRNA complex recognizes TCSV (5 fmol), the complex is activated and cleaves initiator in the mixture. The degraded initiator is unable to trigger the HCR reaction in the next step, and consequently, a sharp band 1 (unassembled H1 and H2 band) is observed as anticipated (Figure 2a, b; lane 4). In the

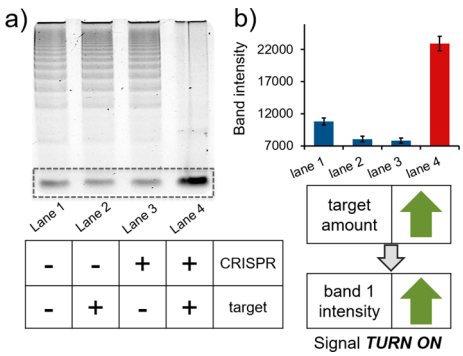


Figure 2. Depiction of CRISPR-Cas12a coupled HCR detection system when neither Cas12a or target is present, in the presence of 5 fmol of target only, in the presence of Cas12a only, and in the presence of both Cas12a and target. This is shown by (a) a gel image and (b) a graphical representation of band 1's intensity. Data are represented as mean ± SD.

absence of the target or Cas12a or both of them, the band 1 intensity is diminished, suggesting the occurrence of HCR and the consumption of the hairpins. The upper broad bands are also indicative of HCR taking place (Figure 2a, b; lanes 1−3). The data demonstrate that HCR can be programmed for detection of a specific dsDNA using the Cas12a−crRNA complex without changing any of the detection components (H1 and H2, initiator, and Cas12a) besides crRNA (Scheme 2a). Furthermore, instead of observing a TURN OFF (lighter band) signal in the HCR-alone approach as in Figure 1, now in the presence of the target, a TURN ON (intense band) is observed (Figure 2). Overall, we have demonstrated that Cas12a can be integrated in gel electrophoresis-based HCR approach and we have reversed the signal readout from TURN OFF to TURN ON upon target detection.

DNA-based detection approaches, including HCR, are typically limited to detection of single-stranded targets due Watson−Crick base pairing. However, Cas12a is able to recognize both dsDNA and ssDNA targets through the formation of an RNA−dsDNA heterotriplex or an RNA−ssDNA heteroduplex. Here, we have demonstrated that this approach is able to detect both ssDNA and dsDNA due to the strength of Cas12a−crRNA recognition. Without changing any of the detection components (H1, H2, initiator, and Cas12a) or changing the crRNA, the system was able to detect both ssDNA and dsDNA forms of the target (Figure 3).

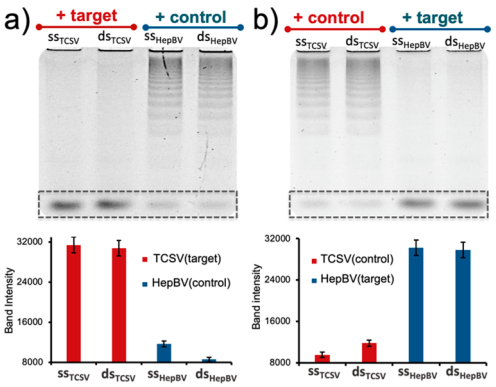


Figure 3. Programmability of CRISPR-Cas12a coupled HCR system for target (5 fmol)-specific detection using (a) $crRNA_{TCSV}$ and (b) $crRNA_{HepBV}$. Data are represented as mean ± SD.

To demonstrate the adaptability of the approach, we have programmed Cas12a for the detection of TCSV (Figure 3a) and HepBV (Figure 3b) separately. As seen in Figure 3a, when programmed to detect TCSV using $crRNA_{TCSV}$, the detection band is distinctive to both ss and ds forms of TCSV. However, in Figure 3b, the crRNA ($crRNA_{HepBV}$) is programmed for the detection of HepBV, and an intense band 1 is observed for the HepBV target but not for TCSV (control). The data suggest that by simply changing the crRNA, but nothing else, the HCR-based detection approach can be programmed for any target of interest with no observable off-target activity.

With CRISPR, not only has the target pool expanded significantly but also the sensitivity of this gel-based detection has improved remarkably. Compared to the HCR-alone approach (Figure 4a, b), where a drop (TURN OFF) in band 1 intensity is observed at 150 fmol of the target, the

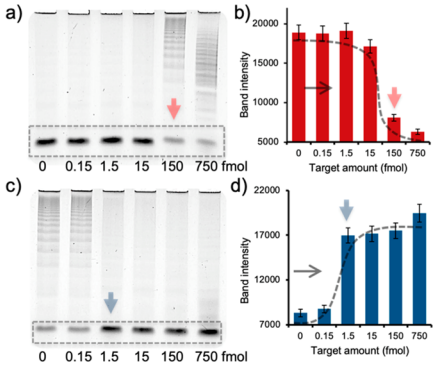


Figure 4. Target amount-dependent study (a, b) without (c, d) with CRISPR-Cas12a reaction. Data are represented as mean ± SD.

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HCR−CRISPR combination approach is ∼100 times more sensitive with an increase (TURN ON) in band 1 intensity with as little as 1.5 fmol of the target (Figure 4c, d). This improved sensitivity is seen because a single target recognition event can induce Cas12a to cleave multiple initiators of HCR, thus amplifying the signal and band 1 intensity. The data overall demonstrate that we were able to reverse the signal output from TURN OFF to TURN ON and improve the sensitivity by 2 orders of magnitude. Typical homogeneous CRISPR-Cas assays for pathogen detection employ target amplification steps to achieve higher sensitivity using flourescence-based approaches.[23,38−40] Although the sensitivity of our approach can be improved using similar target amplification steps, our current assay setup is budget friendly and an accessible alternative to current approaches.

In conclusion, we have demonstrated that a readily available, simple gel electrophoresis setup can be highly instrumental for the detection of a DNA of choice using combined hybridization chain reaction (HCR) and CRISPR-Cas12a approach. By integrating the novel CRISPR-Cas12a technology into HCR, we were able to broaden our target pool from one single ssDNA target (initiator) to any DNA molecule of choice irrespective of their ssDNA or dsDNA form. The signal readout was reversed from TURN OFF to TURN ON. The sensitivity of the assay was improved ∼100 folds, and as little as 1.5 fmol of the target was visualized on the gel. This novel assay was programmed and tested for two different viral biomarkers, TCSV and HepBV, individually. In the future, this approach can be repurposed for PCR-free clinical diagnostics or agricultural screening.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c04949.

Experimental details (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Mehmet V. Yigit − *Department of Chemistry, University at Albany, State University of New York, Albany, New York 12222, United States; The RNA Institute, University at Albany, State University of New York, Albany, New York 12222, United States;* orcid.org/0000-0002-4349-3701; Phone: 518-442-3002; Email: myigit@albany.edu

Authors

Mahera J. Kachwala − *Department of Chemistry, University at Albany, State University of New York, Albany, New York 12222, United States*

Christopher W. Smith − *Department of Chemistry, University at Albany, State University of New York, Albany, New York 12222, United States*

Nidhi Nandu − *Department of Chemistry, University at Albany, State University of New York, Albany, New York 12222, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.analchem.0c04949

Notes

The authors declare no competing financial interest.

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