Methods xxx (xxxx) xxx

Contents lists available at ScienceDirect

Methods

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

M−CDC: Magnetic pull-down-assisted colorimetric method based on the CRISPR/Cas12a system

Menglu Hu [a], Debin Zhu [b,*], Xiaoming Zhou [a,*]

[a] School of Life Sciences, South China Normal University, Guangzhou 510631, PR China
[b] Guangzhou Key Laboratory of Analytical Chemistry for Biomedicine, School of Chemistry, South China Normal University, Guangzhou 510006, PR China

ARTICLE INFO

Keywords:
CRISPR
Nanoparticle
COVID-19
Detection
Colorimetric

ABSTRACT

The construction of a rapid, simple, and specific nucleic acid detection platform is of great significance to the control of the large-scale spread of infectious diseases. We have recently established a magnetic pull-down-assisted colorimetric method based on the CRISPR/Cas12a system (termed M−CDC), which effectively integrates the advantages of CRISPR/Cas12a, magnetic beads-based separation, and AuNP bioprobe to provide a simple and specific biosensing platform for nucleic acid assay. The M−CDC method is compatible with point-of-care testing and enables the detection of nucleic acid samples in less than an hour without relying on expensive and complex instruments. In this paper, step-by-step instructions for M−CDC assay, including recombinase polymerase amplification (RPA)/reverse transcription-polymerase chain reaction (RT-RPA) of DNA or RNA, Cas12a-mediated target recognition and cleavage, and subsequent magnetic beads-mediated colorimetric readouts are provided. In addition, the protocol for the expression and purification of *Lachnospiraceae bacterium*-Cas12a (LbCas12a) protein, the design and synthesis of high-efficient crRNA, and the preparation of AuNP bioprobe are also offered.

1. Introduction

There is an urgent need to develop simple, cost-effective, sensitive, and specific diagnostic tests to deal with the outbreaks of infectious diseases [1]. Recently, some new biosensing strategies for nucleic acids analysis have been reported [2–5], but the gold standard for nucleic acid diagnosis of infectious diseases is still quantitative PCR (qPCR), which only requires small amounts of samples and provides a real-time, highly sensitive, and specific readout for analysis [6]. However, due to its dependence on expensive bulky equipment and professional experimenter, qPCR is thus not conducive to the point-of-care (POC) tests. In recent decades, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) [7,8], recombinase polymerase amplification (RPA) [9], and exponential amplification reaction (EXPAR) [10] have been developed as attractive alternatives to conventional PCR method because of their simplicity and rapidity. However, most of those methods rely on a non-specific detection mode, which makes it challenging to apply them to develop accurate and reliable POC diagnoses technology [11,12].

Clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system is an adaptive immune system found in prokaryotes, which has been widely used in genome engineering, such as gene editing, gene regulation, gene imaging [13]. CRISPR-Cas system can be classified into Class 1 and Class 2 according to the organizational structure of the effector proteins [14]. Especially, the Class 2 CRISPR-Cas system, which only needs a single-protein effector and guide RNA to perform genome engineering, is popular for downstream applications. Class 2 CRISPR-Cas system can be further subdivided into three types (type II, V, VI) [15], and CRISPR-Cas12a belongs to the Class 2-type V system. CRISPR-Cas12a is a crRNA-guided DNA endonuclease that efficiently recognizes the dsDNA using a T nucleotide-rich protospacer-adjacent motif (PAM) and generates a dsDNA break with 4–5 nt protruding sticky terminal [16].

The recent discovery of the *trans*-cleavage mechanism of CRISPR-Cas12a extends its applications range from genome engineering to molecular diagnosis [16–18]. The *trans*-cleavage activity of the CRISPR-Cas12a system, activated from the target DNA recognition (ds or ssDNA), allows indiscriminately cleave surrounding nontarget ssDNA in the reaction system with thousand turnovers [17–20]. Based on this property, a series of sensitive and rapid diagnostic techniques [17,18,21–23], such as DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR) [17], one-HOur Low-cost Multipurpose highly

* Corresponding authors.
E-mail addresses: zhudb@scnu.edu.cn (D. Zhu), zhouxm@scnu.edu.cn (X. Zhou).

https://doi.org/10.1016/j.ymeth.2021.11.009
Received 30 August 2021; Received in revised form 28 October 2021; Accepted 15 November 2021
Available online 20 November 2021
1046-2023/© 2021 Elsevier Inc. All rights reserved.

Efficient System (HOLMES) [18], have been proposed. Among these methods, the fluorescence output mode is the most commonly employed. However, the fluorescence detection equipment is usually expensive and not suitable for large-scale promotion in resource-poor areas. To reduce dependence on testing instruments, the diagnostic technology based on the CRISPR-Cas12a system with lateral flow analysis (LFA) as the signal readout was also developed [24,25]. In these methods, a 5′-FAM fluorophore and 3′-Biotin dual-labeled nucleic acid reporter is usually employed as the *trans*-cleavage substrate of the CRISPR/Cas12a system. In the actual test situation, the dual-labeled nucleic acid reporter can be intact (in absence of target DNA), or completely degraded (high target DNA concentration), or partially degraded (low target DNA concentration). After the CRISPR reaction, the reaction products were subjected to lateral flow analysis. Ideally, the intact nucleic acid reporters will intercept all anti-FAM antibody-coated AuNPs probes at the streptavidin line so only one test line is rendered. And when the nucleic acid reporters are partially degraded or completely degraded, anti-FAM antibody-coated AuNPs probes will be escaped from the streptavidin line and be enriched at the second line through antibody capture. However, in the absence of target DNA, it is difficult to accurately control the complete capture of the anti-FAM antibody-coated AuNPs probes by streptavidin line. Once the anti-FAM antibody-coated AuNPs probes escape from the streptavidin line, a false-positive line signal will be generated [20,26].

To overcome these shortcomings, we here designed the streptavidin-embedded magnetic beads (SA-MBs) as the captured carrier of the AuNP bioprobe and transferred the reaction into the EP tube to develop a simple and rapid nucleic acid detection method, named the magnetic pull-down-assisted colorimetric method based on the CRISPR/Cas12a system (M−CDC) [27,28]. In this method, a Biotin-P1 probe was designed to serve as a *trans*-cleavage substrate for CRISPR/Cas12 and also as a linker-DNA for capturing AuNP bioprobes on SA-MBs. In the presence of target DNA, the *trans*-cleavage activity of CRISPR/Cas12 was activated, and the Biotin-P1 was cut off. The AuNP bioprobe could not be enriched by SA-MBs, and after a magnetic separation step, the supernatant showed red color. In the absence of target DNA, the *trans*-cleavage activity of CRISPR/Cas12 was not activated, and Biotin-P1 remained intact. The AuNP bioprobe was enriched by SA-MBs, and the supernatant was thus colorless (Fig. 1a). As shown in Fig. 1b, only a thermostatic metal bath, a rotating mixer, and a magnetic rack were required during the whole experiment process. In resource-limited areas, these two instruments (thermostatic metal bath and rotating mixer) can even be replaced by a water bath and manual mixing.

2. Methods

As shown in Fig. 2a, the whole M−CDC assay protocol can be divided into two stages: experimental material preparation and M−CDC assay procedure. The experimental material preparation includes the expression and purification of the LbCas12a protein, *in vitro* transcription of the crRNA, and construction of the AuNP bioprobe. M−CDC assay procedure includes the detection of nucleic acid samples and the analysis of results. The results can be analyzed by the naked-eye colorimetric method or absorption analysis. The detailed experimental procedures are shown below.

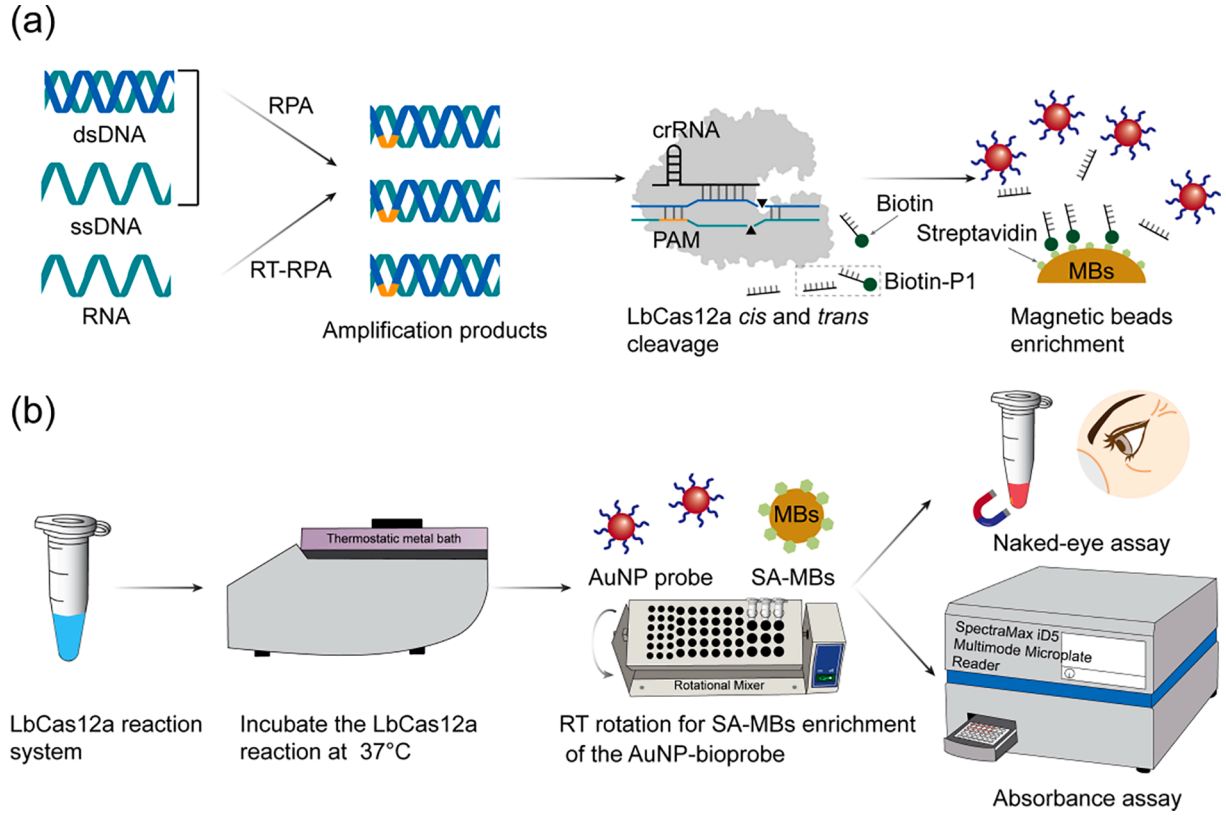


Fig. 1. Schematic diagram for nucleic acid detection using M−CDC. (a) The schematic diagram of the M−CDC method. RPA was used for the amplification of double-stranded (ds) DNA and single-stranded (ss) DNA, and reverse transcription-polymerase chain reaction (RT-RPA) was employed for the amplification of RNA. After the amplified products were added into the LbCas12a reaction assay, the target DNA can be recognized by crRNA guided CRISPR recognition. The *cis*- and *trans*-cleavage activities of LbCas12a were activated, leading to the cleavage of Biotin-P1 contained in the reaction system. The AuNP bioprobes could not be enriched by the SA-MBs, resulting in a red supernatant. (b) The operation flow diagram of the M−CDC method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

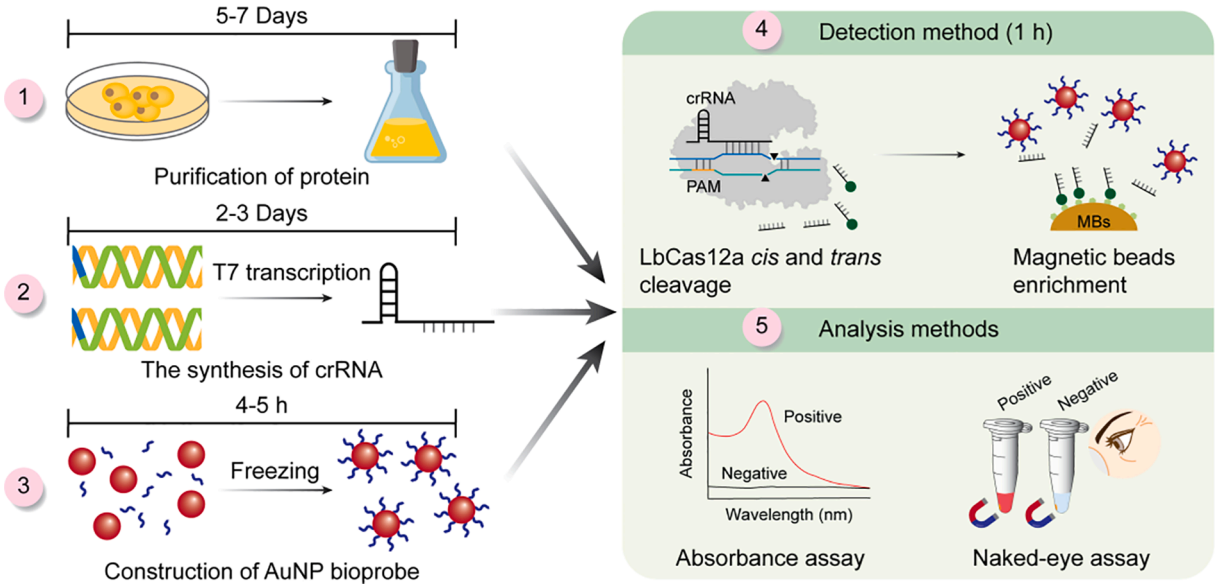


Fig. 2. The workflow of nucleic acid detection using the M−CDC method.

2.1. *Expression and purification of LbCas12a protein*

All buffers used for protein expression and purification are listed in Appendix B. The workflow for protein expression and purification was presented in Fig. 3a. 6His-MBP-TEV-huLbCpf1 was a gift from Feng Zhang (Addgene plasmid # 90096; http://n2t.net/addgene:90096; RRID: Addgene_90096). The purchased LbCas12a (LbCpf1) plasmid was transformed into Escherichia coli Rosetta2 (DE3) cells, and the competent cells were coated on the surface of LB Agar Plate (containing 100 mg/mL Ampicillin and 50 mg/mL Chloramphenicol) to obtain single colonies. After incubation for 16–18 h at 37°C, single colonies were selected and inoculated in 2 mL LB liquid medium (containing 100 mg/mL Ampicillin and 50 mg/mL Chloramphenicol) as a starter culture. The liquid medium was placed on a shaker at 37°C with 200 rpm for overnight culture. And then the starter culture was added into 400 mL TB liquid medium (containing 100 mg/mL Ampicillin and 50 mg/mL chloramphenicol) for expanded culture at 37°C with 270 rpm for 3–4 h. When the OD_{600} of the culture was reached 0.5, isopropyl β-D-

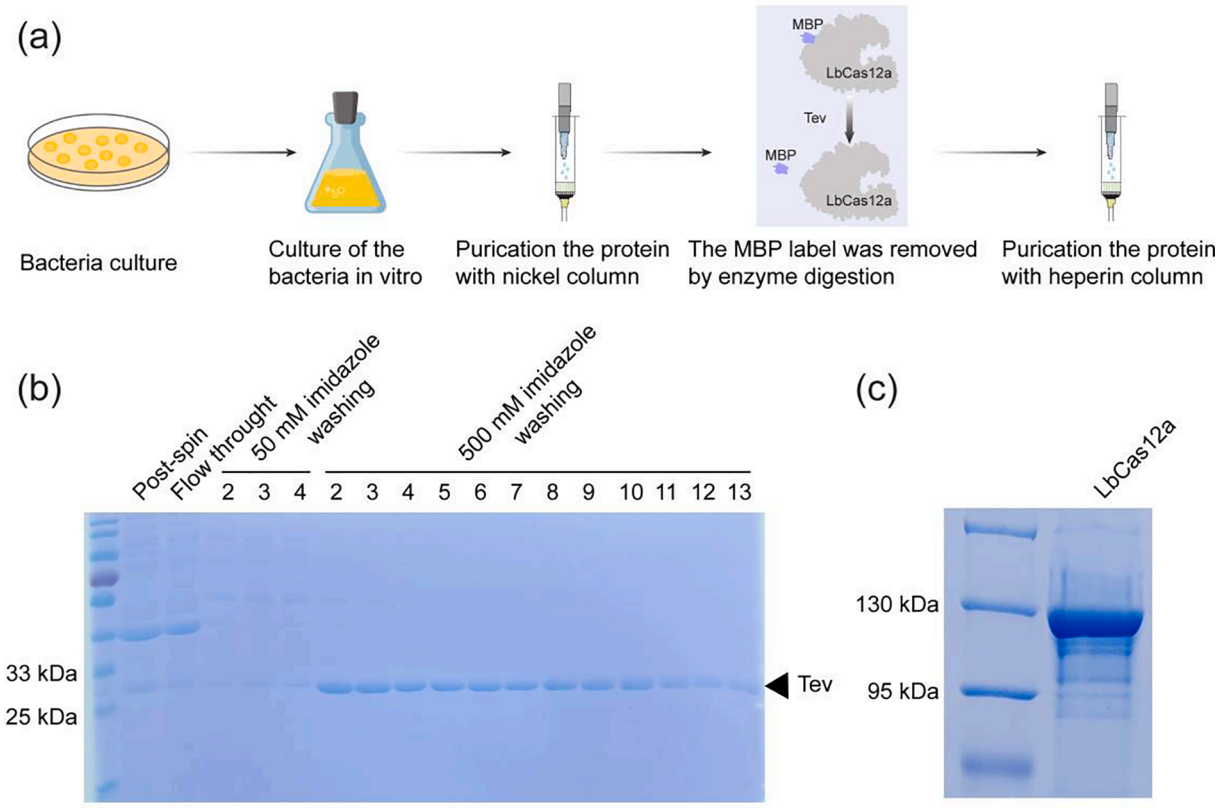


Fig. 3. The expression and purification of LbCas12a protein. (a) The workflow of LbCas12a protein expression and purification. (b) The SDS-PAGE analysis of the eluate from the nickel column of TEV protein. (c) The SDS-PAGE analysis of the LbCas12a protein.

thiogalactoside (IPTG, the final concentration was 1 mM) was added into the medium to induce the expression of the LbCas12a protein at 20℃ for another 18 h. Subsequently, cells were harvested by centrifugation (8000 rpm, 4℃, 10 min) and then resuspended in Lysis buffer, followed by disrupted using an ultrasonic cell crushing apparatus. The sonicated solution was clarified by centrifugation at 8000 rpm for 10 min. The supernatant was loaded onto a nickel column, washed with Eluent buffer 1, and eluted by Eluent buffer 2, and analyzed by SDS-PAGE. The TEV protein (Fig. 3b) was added to the collected purified products containing LbCas12a protein, and then the mixture was placed in a refrigerator at 4℃ overnight for enzymatic hydrolysis to remove the 6His-MBP label. The mixture was then loaded onto the heparin column, washed with Eluent buffer 3, and eluted by Lysis buffer, and analyzed by SDS-PAGE. After the protein solution was collected and placed at 4℃ overnight for dialysis. Glycerin was added to the protein solution to a final concentration of 50%. And the solution was stored in a refrigerator at −80℃ for later use (Fig. 3c).

2.2. The preparation of the crRNA

The mature crRNA contains two regions, namely repetitive sequence region in 5′ end and spacer sequence region in 3′ end. The repetitive sequence region was universal (the detail sequence was referred to the paper published by the Doudna group in 2018 [17]), while the spacer region needs to be designed according to the target sequence. LbCas12a protein recognizes a T nucleotide-rich PAM and prefers the TTT(N)-PAM site. Accordingly, we first need to found the region containing this PAM site in the target sequence and select the 20 bp base sequence downstream of the site as the spacer sequence. The presence of unnecessary secondary structures in the crRNA sequence will affect the activity of the CRISPR-Cas12 system. In addition, it is especially important to maintain the integrity of the secondary structure in the repetitive sequence region [29]. Based on this characteristic, some secondary structure prediction software, such as Integrated DNA Technologies (IDT) software (https://sg.idtdna.com/calc/analyzer), should be used to analyze and compare the secondary structure of the designed crRNA to find the optimal crRNA sequence. Then an efficient chemical synthetic strategy, such as the phosphoramidite method, was used for the synthesis of the crRNA.

In addition, the crRNA can also be synthesized by *in vitro* transcription. All the sequences are list in Table 1. The detailed procedures are as follows: First, import the crRNA sequence with the T7 promoter sequence tag at the 5′ end into the NovoPro software to obtain the reverse complementary DNA sequence (crDNA). Then the T7 promoter sequence tag and the crDNA were synthesized by chemical methods. The T7 promoter sequence and crDNA were annealed. The annealing system was prepared as shown in Table 2. The reaction system was incubated at 95℃ for 5 min, then transferred to room temperature for natural cooling. The annealed product was used as a template for the T7 transcription reaction. And the preparation of the related reaction system was shown in Table 3. After reaction at 37℃ for 4 h, RNA purification was performed by the RNA Clean & Concentrator Kits (RCC). Finally, the crRNA solution was collected and analyzed by polyacrylamide gel electrophoresis (PAGE) to assess RNA integrity (Fig. 4a). Furthermore, as shown in Fig. 4b, the activity of the crRNA was evaluated by the *trans*-cleavage method of LbCas12a. And then stored it at −80℃ for later use.

Table 2
Components and concentrations of Annealing mix.

	1 reaction	1.1 N reaction
crDNA (100 μM)	2 μL	2 × 1.1 N μL
T7 sequence (100 μM)	2 μL	2 × 1.1 N μL
10 × T7 buffer	2 μL	2 × 1.1 N μL
RNase-free Water	14 μL	2 × 1.1 N μL
Total	20 μL	22 N μL

Table 3
Components and concentrations of crRNA transcription mix.

	1 reaction	1.1 N reaction
T7 RNA polymerase (50 U/μL)	1 μL	1 × 1.1 N μL
Recombinant RNase inhibitor (40 U/μL)	0.5 μL	0.5 × 1.1 N μL
NTP Mixture (10 mM)	10 μL	10 × 1.1 N μL
Template	2 μL	2 × 1.1 N μL
10 × T7 buffer	5 μL	5 × 1.1 N μL
RNase-free Water	31.5 μL	31.5 × 1.1 N μL
Total	50 μL	55 N μL

2.3. Synthesis of the AuNP

13 nm AuNP was prepared by sodium citrate reduction method according to the published paper [30]. To avoid the influence of the residual impurities in the experimental materials, a conical flask, brown bottle, and magnetic stir bar should be soaked in aqua regia for 30 min. And then all the experimental materials were desiccated after being rinsed with deionized water. Add 96 mL ultrapure water, 4 mL 24.3 mM $HAuCl_4$ solution, and magnetic stir bar to 250 mL conical flask respectively. Cover the mouth of the conical flask with tin foil (do not seal). Place it on a Hotplate & Magnetic Stirrer, and set the temperature to 150℃. After the solution was boiled evenly, the speed of the Hotplate & Magnetic Stirrer was turned to 1200 rpm/min, and then 10 mL of 38.8 mM trisodium citrate solution was added quickly. When the color of the solution turns deep-red, allow it to heat for another 15–20 min. Turn off the heating, but keep stirring until the solution has cooled to room temperature. Then transfer it to a clean brown bottle and allow it to stand at room temperature for 12 h. Store in a refrigerator at 4℃ and keep away from light.

2.4. Preparation of AuNP bioprobe

All the buffers used for this experiment were listed in Appendix B. AuNP bioprobe was prepared according to the published paper by our group [28]. DNA/AuNP mixture was prepared according to the ratio of adding 5 μL 100 μM P1-DNA to each 100 μL AuNP solution. After vortexing and mixing, it was placed in a refrigerator at −20℃ until completely frozen. Remove the mixture from the refrigerator and thaw it

Table 1
DNA and RNA sequence used in the preparation of the crRNA.

Name	Sequence (5′-3′)
T7 promoter	GAAATTAATACGACTCACTATAGGG
crDNA	**GAACGCTGAAGCGCTGGGGG**ATCTACACTTAGTAGAAATTA**CCCTATAGTGAGTCGTATTAATTTC*
crRNA	[#]**UAAUUUCUACUAAGUGUAGAU**CCCCCAGCGCUUCAGCGUUC

*The sequence in italics represents the reverse complementary of the spacer sequence region; The sequence highlighted in bold represents the reverse complementary of the repetitive sequence region; The unmarked sequence represents the reverse complementary of the T7 promoter sequence tag.

[#] The sequence highlighted in bold represents the repetitive sequence region; The unmarked sequence represents the spacer sequence region.

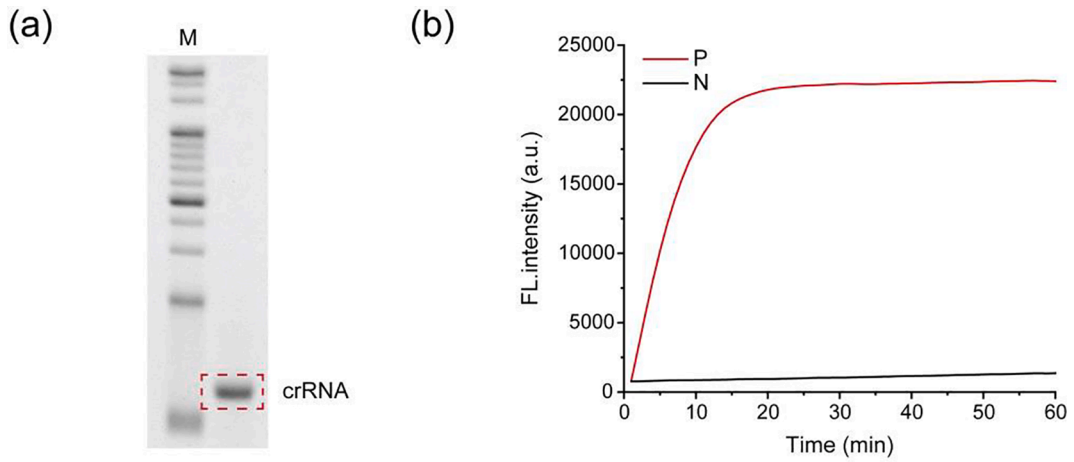


Fig. 4. Characterization of crRNA. (a) The crRNA synthesized by *in vitro* transcription was analyzed by PAGE. (b) The activity of crRNA was assessed by *trans*-cleavage assay of LbCas12a. P and N represents positive and negative samples.

at room temperature. And then the mixture was transferred to a centrifuge which was precooled at 4℃, centrifuged at 12000 rpm/min for 20 min to remove the supernatant. The AuNP bioprobe deposited on the bottom of the tube was resuspended with an equal volume of Wash buffer and centrifuged again. The cleaning process would be repeated three times. And the resulting precipitate from the final centrifugation would be resuspended with a Resuspension buffer of equal volume. Store the P1-AuNP bioprobe at 4℃ and keep it away from light. In the AuNP bioprobe preparation process, the colorimetric results of each stage were shown in Fig. 5a. And the absorption spectrum of AuNP solution and AuNP bioprobe were shown in Fig. 5b.

Moreover, there are several methods available for preparing the AuNP bioprobe, for example, salt-aging [31], low-pH [32,33], freezing [34], and instant dehydration butanol (INDEBT) [35]. Those methods have different characteristics, which can be selected according to experimental requirements.

P1-DNA: AAAAAAAAAATTTTTCACAACGAACATCAT

2.5. RPA/RT-RPA amplification reaction

2.5.1. RPA primer design

Primer Premier 5.0 software was used for primer design. The primers of the RPA amplification reaction were designed according to a relevant protocol supplied by TwistDx company. The rules were shown below: the length of the primers is 30–36 bp; the GC% content is between 20 and 70; the Tm value is between 50 and 100; the repetition of a single base in the sequence should not exceed 5 bp. Usually, we would design 4–5 sets of primers, and compare the performance of these primers through experiments to determine the optimal one. The primers for RT-RPA amplification of SARS-CoV-2 N-gene were as follows:

Forward primer:
AGACGTGGTCCAGAACAAACCCAAGGAAATT
Reverse primer:
TGTGTAGGTCAACCACGTTCCCGAAGGTGT

2.5.2. Sample treatment

Commercial nucleic acid extraction kits can be used for the pre-treatment of the nucleic acid samples. For example, the MagZol™ Reagent Kit can be used for RNA extraction and the TIANamp Bacterial DNA Kit can be used for DNA extraction. Moreover, in order to simplify the experimental operation, some methods that do not extract nucleic acid can be used for the sample treatment. For example, a protocol, named heating unextracted diagnostic samples to obliterate nucleases (HUDSON) [36], published by the Sabeti group in 2018, can be used for the bodily fluids sample treatment. The detailed procedures of this protocol are as follows: TCEP and EDTA were added to the sample at final concentrations of 100 mM and 1 mM respectively and placed the mixture in a thermostatic metal bath for 5 min at 50℃, 5 min at 64℃ for inactivation of DNase or RNase. After low-speed centrifugation, the supernatant was collected.

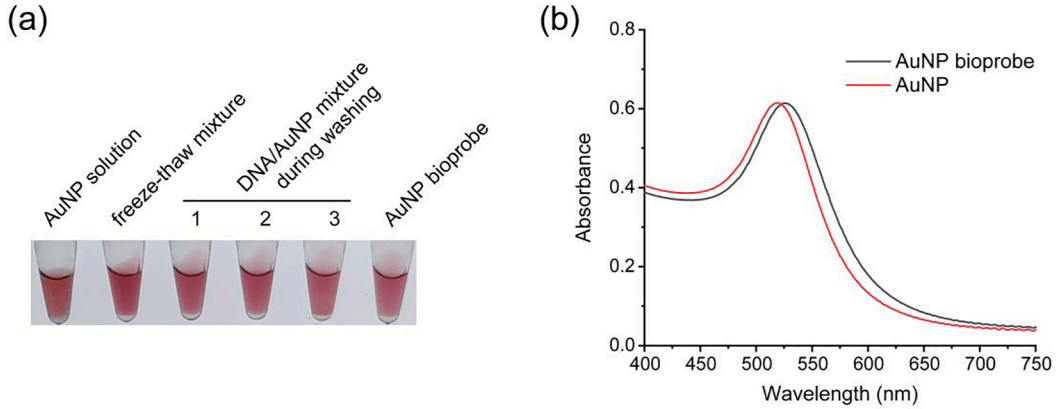


Fig. 5. Construction of the AuNP bioprobe. (a) A photograph of the labeling process for AuNP bioprobe. From left to right: the AuNP solution, the DNA/AuNP mixture after freeze–thaw, the three washing rounds of DNA/AuNP mixture in Wash buffer, the AuNP bioprobe resuspended in Resuspension buffer. (b) The absorption spectrum analysis of the AuNP solution and AuNP bioprobe solution.

2.5.3. RPA/RT-RPA reaction

The amplification reaction used in different nucleic acid samples must be selected carefully. DNA samples were amplified using RPA. The amplification reaction of RPA was prepared according to Table 4. RNA samples were amplified using RT-RPA. The amplification reaction of RT-RPA was prepared according to Table 5. Then those reaction systems were transferred to the thermostatic metal bath for 30 min at 42℃. The products were stored at −20℃ until further use.

2.6. M−CDC Reaction assay

2.6.1. LbCas12a assay

The preparation of the LbCas12a reaction was according to Table 6. Then the mixture was transferred to a thermostatic metal bath at 37℃ for 20 min. The products were stored at −20℃ until further use.

2.6.2. Colorimetric reaction

A 1.5 mL EP tube containing 40 μL 7.2 mg/mL SA-MBs was placed on a magnetic rack. After standing for 1–2 min, the supernatant was removed. 20 μL LbCas12a reaction solution from the 2.6.1 experiment and 80 μL Resuspension buffer were added into the EP tube together. After vortexing and mixing, the EP tube was placed on a rotating mixer and incubated under room temperature conditions for 5 min. Subsequently, the supernatant in the EP tube was removed again, 70 μL 5 nM P1-AuNP bioprobe was added. And the resulting solution was placed on a rotating mixer and incubated at room temperature for another 5 min. Finally, 70 μL supernatant in the EP tube was pipetted for colorimetric analysis, and its absorption value at 520 nm was determined by the SpectraMax iD5 multimode microplate reader.

2.7. Analysis

For the absorbance-based assay, a positive sample was determined on the basis that its absorption value at 520 nm was greater than the mean value of the negative sample plus three times the standard deviation. For the colorimetric-based assay, a positive sample was determined on the basis that the solution color was redder than that of the negative control. If false-negative results were found in the experiment, these reasons should be considered. (i) Failure of the extraction of the sample. (ii) Failure of the amplification of RPA/RT-RPA. (iii) Failure of the cleavage reaction of CRISPR-Cas12a. If there were false-positive results, the following reasons should be ruled out. (i) Occurrence of aerosol contamination (ii) The Biotin-P1 probe or P1-AuNP bioprobe were degraded.

3. Results

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) belongs to coronavirus, is the pathogenic bacterium that causes Coronavirus Disease 2019 (COVID-19), has the characteristics of high infectivity and high concealment. Since December 2019, the global pandemic has had a profound impact on the global political, economic, social, and other fields [25]. Therefore, the detection of SARS-CoV-2 has an important application value. To evaluate the application potential of the M−CDC method in SARS-CoV-2 detection, SARS-CoV-2 RNA sequence was analyzed. As can be seen from Fig. 6a, the RT-RPA amplification products of SARS-CoV-2, bat-SL-CoVZC45 and SARS-CoV cannot be distinguished by agarose gel electrophoresis. However, from the data in Fig. 6b, there was a significant difference between the colorimetric and absorption results of SARS-CoV-2 and other groups (bat-SL-CoVZC45 and SARS-CoV), indicating that the M−CDC method has the potential to be applied to SARS-CoV-2 detection.

Table 4
Components and concentrations of RPA amplification mix.

	1 reaction	1.1 N reaction
Primer F (10 μM)	2.4 μL	2.4 × 1.1 N μL
Primer R (10 μM)	2.4 μL	2.4 × 1.1 N μL
Primer Free Rehydration buffer	29.5 μL	29.5 × 1.1 N μL
Template	2 μL	2 × 1.1 N μL
Magnesium Acetate (280 mM)	2.5 μL	2.5 × 1.1 N μL
RNase-free Water	11.2 μL	11.2 × 1.1 N μL
Total	50 μL	55 N μL

The mixture was prepared according to the operating manual provided with the TwistAmp Basic Kit.

Table 5
Components and concentrations of RT-RPA amplification mix.

	1 reaction	1.1 N reaction
Primer F (20 μM)	0.5 μL	0.5 × 1.1 N μL
Primer R (20 μM)	0.5 μL	0.5 × 1.1 N μL
Primer Free Rehydration buffer	38 μL	38 × 1.1 N μL
SuperScript IV Reverse Transcriptase (200U/μL)	0.5 μL	0.5 × 1.1 N μL
RNase H (5 U/μL)	1 μL	1 × 1.1 N μL
Template	2 μL	2 × 1.1 N μL
Magnesium Acetate (700 mM)	1 μL	1 × 1.1 N μL
RNase-free Water	6.5 μL	6.5 × 1.1 N μL
Total	50 μL	55 N μL

The mixture was prepared according to a published paper [37].

Table 6
Components and concentrations of LbCas12a reaction mix.

	1 reaction	1.1 N reaction
LbCas12a (1 μM)	2 μL	2 × 1.1 N μL
crRNA (2 μM)	2 μL	2 × 1.1 N μL
10 × NEBuffer 2.1	2 μL	2 × 1.1 N μL
*Target	2 μL	2 × 1.1 N μL
Biotin-P1 (1 μM)	3.6 μL	3.6 × 1.1 N μL
RNase-free Water	8.4 μL	8.4 × 1.1 N μL
Total	20 μL	22 N μL

Biotin-P1: Biotin-AAAAAATGATGTTCGTTGTG.
*Target represents the amplified product of RPA/RT-RPA.

4. Conclusions

In this study, we systematically described the detailed experimental procedure of the M−CDC assay. The M−CDC is a fast, visible, and simple nucleic acid detection method, which is very promising in the early prevention of infectious diseases. Compared with the other biosensing platform based on the CRISPR/Cas12 system, the M−CDC method mainly has the following advantages: (i) SA-MBs are used to control the capture and separation of AuNP bioprobe, which can improve the signal-to-noise ratio and only needs a simple magnetic separation device. (ii) The signal amplification and recognition advantages of the RPA/RT-RPA and CRISPR/Cas12 system are effectively integrated, making the M−CDC method highly sensitive and specific. (iii) M−CDC is a nucleic acid detection method that uses AuNP bioprobe as the signal output mode. It only requires a thermostatic metal bath, a rotating mixer, and a magnetic rack, making this method potentially applicable to resource-poor areas.

CRediT authorship contribution statement

Menglu Hu: conceived the concept, designed and performed all the experiments, writing original draft. **Debin Zhu:** funding acquisition. **Xiaoming Zhou:** conceived the primary idea, writing-review & editing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

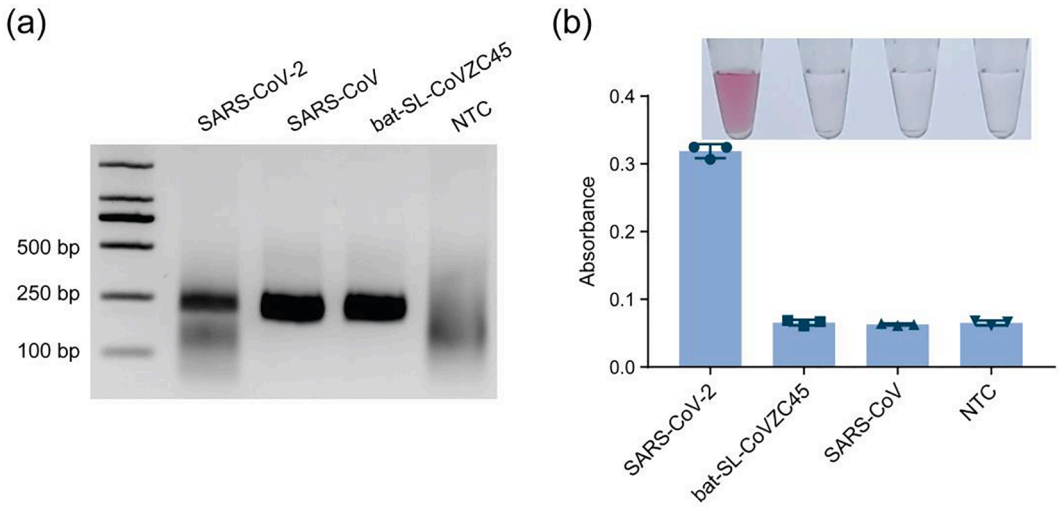


Fig. 6. Valuation of the specificity of the M−CDC method for SARS-CoV-2 RNA detection. (a) The RT-RPA amplification products of SARS-CoV-2, SARS-CoV, bat-SL-CoVZC45, and RNase-free water were analyzed by agarose gel electrophoresis. (b) The RT-RPA amplification products of SARS-CoV-2, bat-SL-CoVZC45, SARS-CoV, and RNase-free water were detected by the M−CDC method. The results were analyzed by the colorimetric method, and the UV–vis absorption values of the solution at 520 nm were measured by the SpectraMax iD5 multimode microplate reader. NTC represents no-template control.

the work reported in this paper.

Acknowledgements

This work was supported by the Special Project of Science and Technology Development of Guangdong Province, Grant/Award Number: 2017B020207011; National Natural Science Foundation of China, Grant/Award Numbers: 81772246, 21874049; and the Open Funds of the State Key Laboratory of Electroanalytical Chemistry, Grant/Award Number: SKLEAC202001.

Appendix

A. Equipment and Reagents

Equipment

1. Thermostatic metal bath (MIULAB, Hangzhou, China)
2. Rotational Mixer (Kylin-Bell, Jiangsu, China)
3. Magnetic rack (Monad, Jiangsu, China)
4. SpectraMax iD5 Multimode Microplate Reader (Molecular Devices, CA, USA)
5. Centrifuge (Eppendorf Co. Hamburg, Germany)
6. Hotplate & Magnetic Stirrer (DLAB, Beijing, China)
7. Micro flow peristaltic pump (Chuangrui, Baoding, China)

Reagents

1. 6His-MBP-TEVhuLbCpf1 (Addgene, 90096)
2. LB Agar Plate (Kanamycin) (Sangon Biotech, B530113)
3. Terrific Broth (Sangon Biotech, B540123)
4. BL21 (DE3) competent E. Coli cells (New England Biolabs, C2527H)
5. Kanamycin (Sigma-Aldrich, 25389–94–0)
6. Isopropyl β-D-thiogalactopyranoside (Sigma-Aldrich, 367–93-1)
7. Imidazole (Sangon Biotech, A500529)
8. $HAuCl_4$ (Aladdin, 16903–35-8)
9. NTP Mixture (Sangon Biotech, B600056)
10. Dynabeads™ M−270 Streptavidin (ThermoFisher Scientific, 65306)
11. T7 RNA Polymerase (TaKaRa, 2540A)
12. Recombinant RNase inhibitor (TaKaRa, 2313A)
13. SuperScript IV Reverse Transcriptase (ThermoFisher Scientific, 18090200)
14. RNase H (New England Biolabs, M0297S)
15. RNase-free Water (TaKaRa, 9012)
16. NEBuffer 2.1 (New England Biolabs, B7202S)
17. TwistAmp Basic Kit (TwistDx Inc.)
18. The RNA Clean & Concentrator Kits (RCC) (ZYMO RESEARCH, R1017)
19. The MagZol^{TM} Reagent Kit (Magen, R4801-01)

20. The TIANamp Bacterial DNA Kit (TIANGEN, DP302-02)
21. $Na_3C_6H_5O_7 \cdot 2H_2O$ (Sangon Biotech, A100101)
22. Tris-HCl (Sangon Biotech, B548124)
23. NaCl (Sangon Biotech, A100241)
24. Glycerol (Sangon Biotech, A100854)
25. NaH_2PO_4 (Sangon Biotech, A100571)
26. Na_2HPO_4 (Sangon Biotech, A501727)
27. Forward Primer (Sangon Biotech)
28. Reverse Primer (Sangon Biotech)
29. crRNA (Sangon Biotech)
30. FAM-Quench Reporter, /5′-FAM/TTTTTT/BHQ1-3′/ (Sangon Biotech)
31. P1-DNA (Sangon Biotech)
32. Biotin-P1 (Sangon Biotech)

B. Buffer Preparation for Protein Purification

Buffer	Reagents	Concentration
Lysis buffer (pH 7.5)	Tris-HCl	20 mM
	NaCl	1 M
	Glycerol	10% (v/v)
Eluent buffer 1 (pH 7.5)	Tris-HCl	20 mM
	NaCl	150 mM
	Imidazole	20 mM
	Glycerol	10% (v/v)
Eluent buffer 2 (pH 7.5)	Tris-HCl	20 mM
	NaCl	150 mM
	Imidazole	500 mM
	Glycerol	10% (v/v)
Eluent buffer 3 (pH7.5)	Tris-HCl	20 mM
	NaCl	150 mM
	Glycerol	10% (v/v)
PB buffer (pH7.0)	NaH_2PO_4	78 mM
	Na_2HPO_4	122 mM
Wash buffer (pH 7.4)	NaCl	0.1 M
	PB buffer	0.01 M
Resuspension buffer (pH 7.4)	NaCl	0.3 M
	PB buffer	0.01 M

References

[1] L. Bokelmann, O. Nickel, T. Maricic, S. Paabo, M. Meyer, S. Borte, S. Riesenberg, Point-of-care bulk testing for SARS-CoV-2 by combining hybridization capture with improved colorimetric LAMP, Nat. Commun. 12 (1) (2021) 1467, https://doi.org/10.1038/s41467-021-21627-0.

[2] S.J. Smith, C.R. Nemr, S.O. Kelley, Chemistry-Driven Approaches for Ultrasensitive Nucleic Acid Detection, J. Am. Chem. Soc. 139 (3) (2017) 1020–1028, https://doi.org/10.1021/jacs.6b10850.

[3] G. Li, S.A. Henry, H. Liu, T.S. Kang, S.C. Nao, Y. Zhao, C. Wu, J. Jin, J.T. Zhang, C. H. Leung, P. Wai Hong Chan, D.L. Ma, A robust photoluminescence screening assay identifies uracil-DNA glycosylase inhibitors against prostate cancer, Chem Sci 11 (7) (2020) 1750-1760, https://doi.org/10.1039/c9sc05623h.

[4] M. Debiais, A. Lelievre, M. Smietana, S. Muller, Splitting aptamers and nucleic acid enzymes for the development of advanced biosensors, Nucleic Acids Res. 48 (7) (2020) 3400–3422, https://doi.org/10.1093/nar/gkaa132.

[5] S. Yamazoe, Q. Liu, L.E. McQuade, A. Deiters, J.K. Chen, Sequential gene silencing using wavelength-selective caged morpholino oligonucleotides, Angew. Chem. Int. Ed. Engl. 53 (38) (2014) 10114–10118, https://doi.org/10.1002/anie.201405355.

[6] Z. Qin, R. Peng, I.K. Baravik, X. Liu, Fighting COVID-19: Integrated Micro- and Nanosystems for Viral Infection Diagnostics, Matter 3 (3) (2020) 628–651, https://doi.org/10.1016/j.matt.2020.06.015.

[7] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, T. Hase, Loop-mediated isothermal amplification of DNA, Nucleic Acids Res. 28 (12) (2000) E63, https://doi.org/10.1093/nar/28.12.e63.

[8] Y. Mori, K. Nagamine, N. Tomita, T. Notomi, Detection of Loop-Mediated Isothermal Amplification Reaction by Turbidity Derived from Magnesium Pyrophosphate Formation, Biochem. Biophys. Res. Commun. 289 (1) (2001) 150–154, https://doi.org/10.1006/bbrc.2001.5921.

[9] O. Piepenburg, C.H. Williams, D.L. Stemple, N.A. Armes, DNA Detection Using Recombination Proteins, PLOS Biology 4(7) (2006) e204, https://doi.org/10.1371/journal.pbio.0040204.

[10] J. Van Ness, L.K. Van Ness, D.J. Galas, Isothermal reactions for the amplification of oligonucleotides, Proc Natl Acad Sci U S A 100 (8) (2003) 4504–4509, https://doi.org/10.1073/pnas.0730811100.

[11] J.C. Rolando, E. Jue, J.T. Barlow, R.F. Ismagilov, Real-time kinetics and high-resolution melt curves in single-molecule digital LAMP to differentiate and study specific and non-specific amplification, Nucleic Acids Res 48(7) (2020) e42, https://doi.org/10.1093/nar/gkaa099.

[12] B. Tian, G.A.S. Minero, J. Fock, M. Dufva, M.F. Hansen, CRISPR-Cas12a based internal negative control for nonspecific products of exponential rolling circle amplification, Nucleic Acids Res 48(5) (2020) e30, https://doi.org/10.1093/nar/gkaa017.

[13] G.J. Knott, J.A. Doudna, CRISPR-Cas guides the future of genetic engineering, Science 361 (6405) (2018) 866–869, https://doi.org/10.1126/science.aat5011.

[14] S. Shmakov, A. Smargon, D. Scott, D. Cox, N. Pyzocha, W. Yan, O.O. Abudayyeh, J. S. Gootenberg, K.S. Makarova, Y.I. Wolf, K. Severinov, F. Zhang, E.V. Koonin, Diversity and evolution of class 2 CRISPR-Cas systems, Nat. Rev. Microbiol. 15 (3) (2017) 169–182, https://doi.org/10.1038/nrmicro.2016.184.

[15] K.S. Makarova, Y.I. Wolf, O.S. Alkhnbashi, F. Costa, S.A. Shah, S.J. Saunders, R. Barrangou, S.J. Brouns, E. Charpentier, D.H. Haft, P. Horvath, S. Moineau, F. J. Mojica, R.M. Terns, M.P. Terns, M.F. White, A.F. Yakunin, R.A. Garrett, J. van der Oost, R. Backofen, E.V. Koonin, An updated evolutionary classification of CRISPR-Cas systems, Nat. Rev. Microbiol. 13 (11) (2015) 722–736, https://doi.org/10.1038/nrmicro3569.

[16] B. Zetsche, J.S. Gootenberg, O.O. Abudayyeh, I.M. Slaymaker, K.S. Makarova, P. Essletzbichler, S.E. Volz, J. Joung, J. van der Oost, A. Regev, E.V. Koonin, F. Zhang, Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system, Cell 163 (3) (2015) 759–771, https://doi.org/10.1016/j.cell.2015.09.038.

[17] J.S. Chen, E. Ma, L.B. Harrington, M. Da Costa, X. Tian, J.M. Palefsky, J.A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (6387) (2018) 436–439, https://doi.org/10.1126/science.aar6245.

[18] S.Y. Li, Q.X. Cheng, J.M. Wang, X.Y. Li, Z.L. Zhang, S. Gao, R.B. Cao, G.P. Zhao, J. Wang, CRISPR-Cas12a-assisted nucleic acid detection, Cell Discov 4 (2018) 20, https://doi.org/10.1038/s41421-018-0028-z.

[19] D.C. Swarts, M. Jinek, Mechanistic Insights into the cis- and trans-Acting DNase Activities of Cas12a, Mol Cell 73(3) 73 (3) (2019) 589–600.e4, https://doi.org/10.1016/j.molcel.2018.11.021.

[20] J. Joung, A. Ladha, M. Saito, N.G. Kim, A.E. Woolley, M. Segel, R.P.J. Barretto, A. Ranu, R.K. Macrae, G. Faure, E.I. Ioannidi, R.N. Krajeski, R. Bruneau, M. W. Huang, X.G. Yu, J.Z. Li, B.D. Walker, D.T. Hung, A.L. Greninger, K.R. Jerome, J. S. Gootenberg, O.O. Abudayyeh, F. Zhang, Detection of SARS-CoV-2 with

SHERLOCK One-Pot Testing, N. Engl. J. Med. 383 (15) (2020) 1492–1494, https://doi.org/10.1056/NEJMc2026172.
[21] L. Li, S. Li, N.a. Wu, J. Wu, G. Wang, G. Zhao, J. Wang, HOLMESv2: A CRISPR-Cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation, ACS Synth. Biol. 8 (10) (2019) 2228–2237, https://doi.org/10.1021/acssynbio.9b0020910.1021/acssynbio.9b00209.s001.
[22] L.T. Nguyen, B.M. Smith, P.K. Jain, Enhancement of trans-cleavage activity of Cas12a with engineered crRNA enables amplified nucleic acid detection, Nat. Commun. 11 (1) (2020) 4906, https://doi.org/10.1038/s41467-020-18615-1.
[23] L.T. Nguyen, J. Gurijala, S.R. Rananaware, B.L.M. Pizzano, B.T. Stone, P.K. Jain, CRISPR-ENHANCE: An enhanced nucleic acid detection platform using Cas12a, Methods (2021), https://doi.org/10.1016/j.ymeth.2021.02.001.
[24] J.S. Gootenberg, O.O. Abudayyeh, M.J. Kellner, J. Joung, J.J. Collins, F. Zhang, Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6, Science 360 (6387) (2018) 439–444, https://doi.org/10.1126/science.aaq0179.
[25] J.P. Broughton, X. Deng, G. Yu, C.L. Fasching, V. Servellita, J. Singh, X. Miao, J. A. Streithorst, A. Granados, A. Sotomayor-Gonzalez, K. Zorn, A. Gopez, E. Hsu, W. Gu, S. Miller, C.Y. Pan, H. Guevara, D.A. Wadford, J.S. Chen, C.Y. Chiu, CRISPR-Cas12-based detection of SARS-CoV-2, Nat. Biotechnol. 38 (7) (2020) 870–874, https://doi.org/10.1038/s41587-020-0513-4.
[26] M.J. Kellner, J.G. Koob, J.S. Gootenberg, O.O. Abudayyeh, F. Zhang, SHERLOCK: nucleic acid detection with CRISPR nucleases, Nat. Protoc. 14 (10) (2019) 2986–3012, https://doi.org/10.1038/s41596-019-0210-2.
[27] Y. Jiang, M. Hu, A.-A. Liu, Y.i. Lin, L. Liu, B.o. Yu, X. Zhou, D.-W. Pang, Detection of SARS-CoV-2 by CRISPR/Cas12a-enhanced colorimetry, ACS Sens 6 (3) (2021) 1086–1093, https://doi.org/10.1021/acssensors.0c0236510.1021/acssensors.0c02365.s001.
[28] M. Hu, C. Yuan, T. Tian, X. Wang, J. Sun, E. Xiong, X. Zhou, Single-step, salt-aging-free, and thiol-free freezing construction of aunp-based bioprobes for advancing CRISPR-based diagnostics, J. Am. Chem. Soc. 142 (16) (2020) 7506–7513, https://doi.org/10.1021/jacs.0c0021710.1021/jacs.0c00217.s001.
[29] S.C.A. Creutzburg, W.Y. Wu, P. Mohanraju, T. Swartjes, F. Alkan, J. Gorodkin, R.H. J. Staals, J. van der Oost, Good guide, bad guide: spacer sequence-dependent cleavage efficiency of Cas12a, Nucleic Acids Res. 48 (6) (2020) 3228–3243, https://doi.org/10.1093/nar/gkz1240.
[30] J. Liu, Y. Lu, Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes, Nat. Protoc. 1 (1) (2006) 246–252, https://doi.org/10.1038/nprot.2006.38.
[31] C.A. Mirkin, R.L. Letsinger, R.C. Mucic, J.J. Storhoff, A DNA-based method for rationally assembling nanoparticles into macroscopic materials, Nature 382(6592) (1996) 607, https://doi.org/https://doi.org/10.1038/382607a0.
[32] X. Zhang, B. Liu, N. Dave, M.R. Servos, J. Liu, Instantaneous attachment of an ultrahigh density of nonthiolated DNA to gold nanoparticles and its applications, Langmuir 28 (49) (2012) 17053–17060, https://doi.org/10.1021/la3035424.
[33] X. Zhang, M.R. Servos, J. Liu, Instantaneous and quantitative functionalization of gold nanoparticles with thiolated DNA using a pH-assisted and surfactant-free route, J. Am. Chem. Soc. 134 (17) (2012) 7266–7269, https://doi.org/10.1021/ja3014055.
[34] H. Pei, F. Li, Y. Wan, M. Wei, H. Liu, Y. Su, N. Chen, Q. Huang, C. Fan, Designed diblock oligonucleotide for the synthesis of spatially isolated and highly hybridizable functionalization of DNA-gold nanoparticle nanoconjugates, J. Am. Chem. Soc. 134 (29) (2012) 11876–11879, https://doi.org/10.1021/ja304118z.
[35] Y. Hao, Y. Li, L. Song, Z. Deng, Flash synthesis of spherical nucleic acids with record DNA density, J. Am. Chem. Soc. 143 (8) (2021) 3065–3069, https://doi.org/10.1021/jacs.1c0056810.1021/jacs.1c00568.s00110.1021/jacs.1c00568.s002.
[36] C. Myhrvold, C.A. Freije, J.S. Gootenberg, O.O. Abudayyeh, H.C. Metsky, A. F. Durbin, M.J. Kellner, A.L. Tan, L.M. Paul, L.A. Parham, K.F. Garcia, K.G. Barnes, B. Chak, A. Mondini, M.L. Nogueira, S. Isern, S.F. Michael, I. Lorenzana, N. L. Yozwiak, B.L. MacInnis, I. Bosch, L. Gehrke, F. Zhang, P.C. Sabeti, Field-deployable viral diagnostics using CRISPR-Cas13, Science 360 (6387) (2018) 444–448, https://doi.org/10.1126/science.aas8836.
[37] J. Qian, S.A. Boswell, C. Chidley, Z.X. Lu, M.E. Pettit, B.L. Gaudio, J.M. Fajnzylber, R.T. Ingram, R.H. Ward, J.Z. Li, M. Springer, An enhanced isothermal amplification assay for viral detection, Nat. Commun. 11 (1) (2020) 5920, https://doi.org/10.1038/s41467-020-19258-y.