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Sensitive and Label-free Detection of Bacteria in Osteomyelitis through Exo III-Assisted Cascade Signal Amplification

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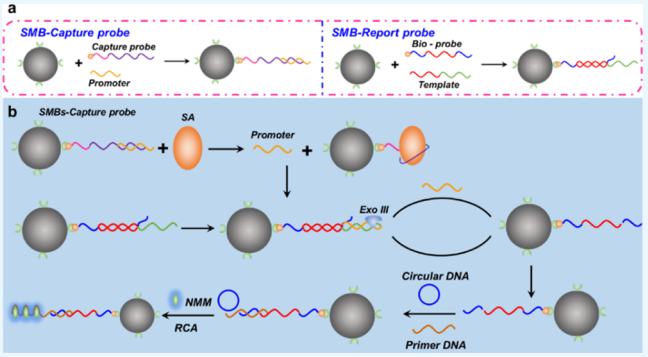
ABSTRACT: Rapid and sensitive pathogenic bacterial identification and isolation from complicated clinical specimens are of great importance for the early diagnosis and prevention of osteomyelitis. Herein, we proposed a novel methicillin-resistant *Staphylococcus aureus* (MRSA) detection strategy through two specially designed streptavidin magnetic bead-based probes, including a capture probe and a report probe. In detail, the capture probe takes the responsibility to specially bind with the surface protein of MRSA and leads to the liberation of the promoter which could subsequently initiate report probe-based signal amplification. Afterward, the hybridization of the promoter probe with the report probe could then transform the protruding 3′ terminus of template DNA in the report probe into a blunt end. With the assistance of Exo III, the template could be digested to liberate the promoter to form a recycle and to liberate the biprobe to induce the following rolling circle amplification (RCA)-based signal amplification. Through the integration of the Exo III-assisted recycle and RCA-based signal amplification, the proposed method exhibited a favorable detection performance.



INTRODUCTION

Osteomyelitis is a kind of the inflammatory state of bone mostly caused by pathogenic bacterial infection.[1] Osteomyelitis causes substantial morbidity and mortality and accounts a major challenge in a frequent complication of trauma or surgery.[1,2] Infection from open fractures is one of the most devastating complications in trauma and could lead to fracture nonunions. Among all the former reported microorganisms that could bind to the surface of host tissue or evade the host defense system, *Staphylococcus aureus* is the leading cause of osteomyelitis. Therefore, early diagnosis of infection would contribute to the prevention of osteomyelitis.[3,4] Among all the bacteria that cause osteomyelitis, methicillin-resistant *Staphylococcus aureus* (MRSA) has attracted abundant attention due to its high risk from the drug-resistant characteristics.[5,6] Therefore, rapid and sensitive detection of MRSA from clinical samples plays a pivotal role in the early diagnosis and therapy of MRSA-related osteomyelitis.

It is in high demand to establish a sensitive and label-free method for bacterial detection due to the broad potential applications in early diagnosis and the prevention of osteomyelitis. Among all the reported bacterial detection methods,[7−10] fluorescent-based assay integrated with the attached signal amplification process is a popular bacterial sensing method because of the stable signal generation and robust detection sensitivity. In these fluorescence-based methods, the aptamer is often utilized for converting amounts of bacteria into signals of nucleic acids.[11−13] Thus, with the assistance of specially designed aptamer-based probes, the signals of surface protein could be amplified from trace amounts to levels that could be monitored and improve the sensitivity. In recent years, a variety of fluorescent amplification approaches have been established and demonstrated to be successfully applied for sensitive bacterial detection,[14−16] including DNAzyme-based recycle assay, nanoparticle-based amplification, enzyme-assisted signal enhancements, and so on. Among all the abovementioned techniques, the enzyme-assisted signal enhancement strategy attracts abundant attention due to its favorable signal amplification efficiency.[17,18] For example, Xu et al.[13] proposed the sensitive and accurate MRSA detection method for the dual-functional aptamer for specifically recognizing target bacteria and CRISPR-Cas12a-assisted rolling circle amplification for signal amplification. In their methods, the dual-functional aptamer was designed based on the selected PBP2a-specific aptamer

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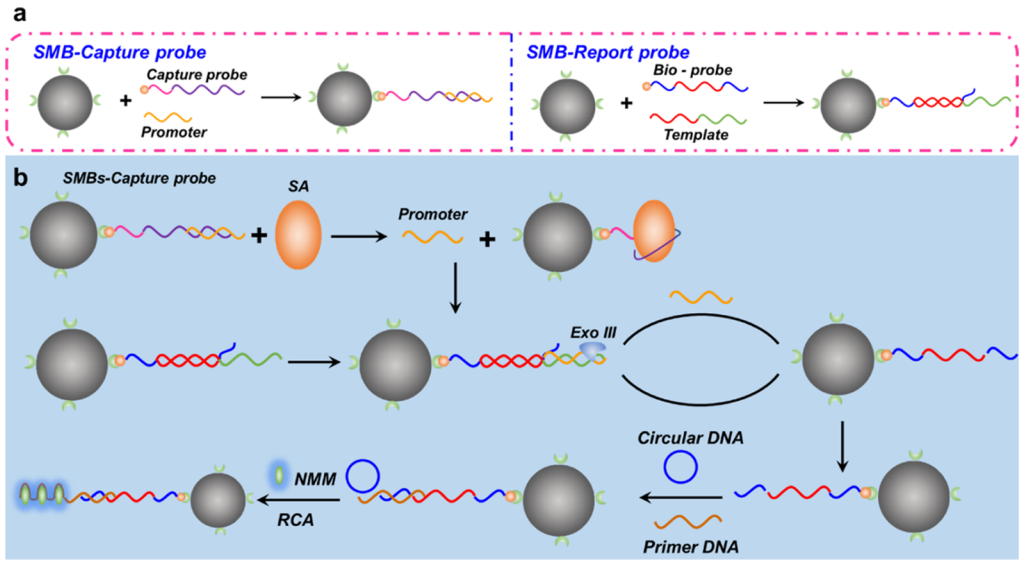
Scheme 1. Working Principle of the Proposed Method for Sensitive Bacterial Detection[a]



[a](a) The details of the design of the SMB-Capture probe and SMB-Report probe. (b) Working process of the proposed method for bacterial sensing.

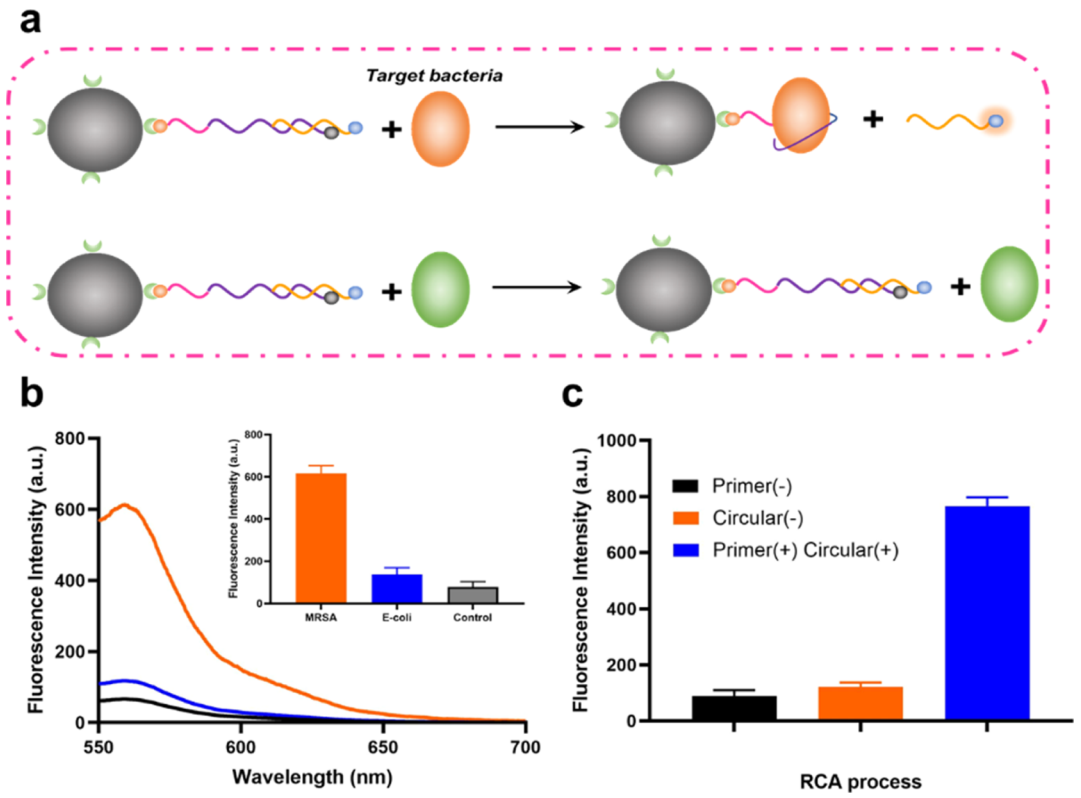


Figure 1. Investigation of the feasibility of the two designed probes. (a) Illustration of the fluorescence assay to study the target bacterial recognition specificity of the SMB-Capture probe. (b) Fluorescence spectrum of the SMB-Capture probe when incubated with target bacteria or not. The inset is a histogram of the calculated fluorescence intensity. (c) Fluorescence intensity generated in the RCA process.

and protein A-specific aptamer from ssDNA library and could rapid recognize trace MRSA from clinical samples. Consequently, the proposed method was proved to be with an improved sensitivity and selectivity toward MRSA detection. However, the label of the fluorescence moiety on the designed fluorescence probes in the abovementioned method could be easily interfered by reaction conditions (e.g., temperature), thus inevitably decreasing the experimental stability and increasing risks of false-positive results. Therefore, development of a label-free method with a favorable detection sensitivity and specificity is in urgent demand.

Herein, we propose here a novel bacterial detection method in a label-free manner through two specially designed streptavidin magnetic bead (SMB)-probe complexes. In detail,

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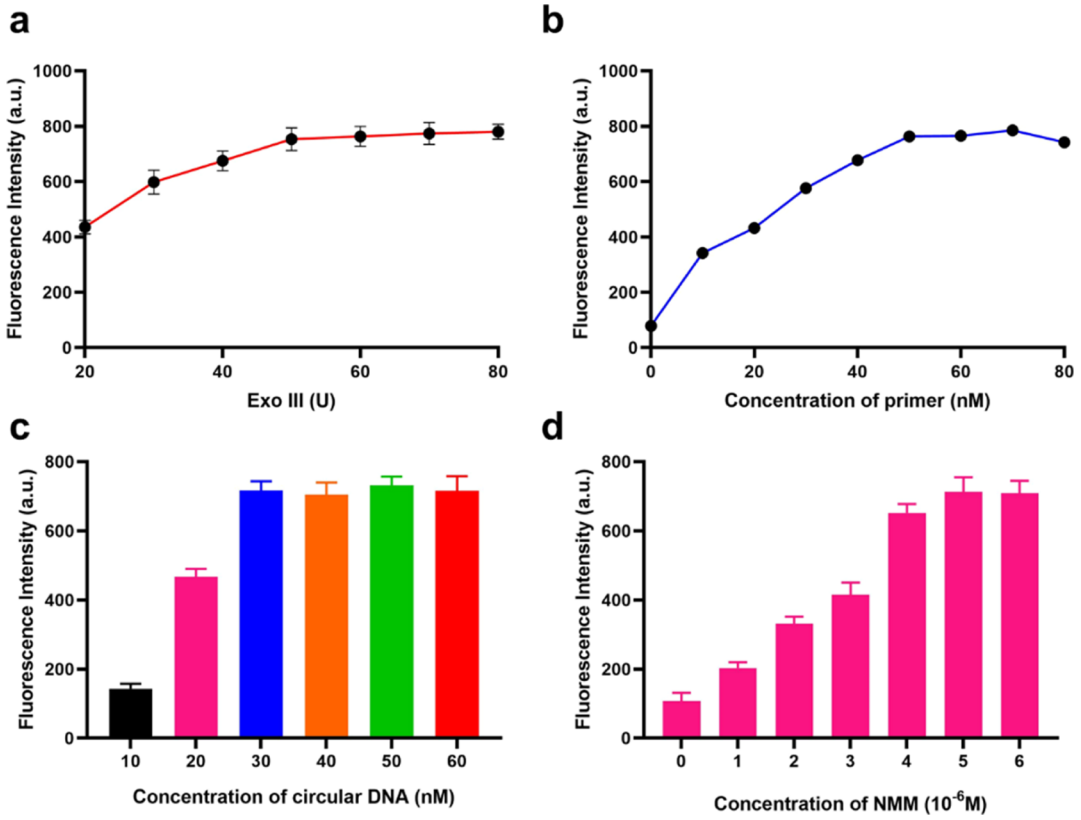

Figure 2. Optimization of the whole sensing system. (a) Obtained fluorescence intensity of the sensing system when incubated with Exo III with different concentrations. (b) Fluorescence intensity of the sensing system with different concentrations of the primer. (c) Fluorescence intensity of the sensing system with different concentrations of circular DNA. (d) Fluorescence intensity of the sensing system with different concentrations of NMM.

we first designed a capture probe to specially recognize target bacteria and thus liberate single-stranded DNA sequences to initiate the following signal amplification process under the assistance of Exo III and result readout. Through a series of experiments, we have demonstrated its feasibility for trace amounts of target bacterial detection after optimizing the experimental conditions. Therefore, we believe that the method could provide a new tool for early diagnosis and prognosis of osteomyelitis.

RESULTS AND DISCUSSIONS

The Working Principle of the Proposed Method for Bacterial Detection. The working mechanism of the proposed method is illustrated in Scheme 1. In the established method, we have first designed two SMB probes: SMB-Capture probe and SMB-Report probe (Scheme 1a). In the SMB-Capture probe, we choose the PBP2a specific aptamer for specific capture of target bacteria. The SMB-Capture probe is eventually designed with the PBP2a-specific aptamer sequence and a biotin at the 3′ end to tether on the streptavidin-coated magnetic bead (MB). Furthermore, we have also added a blocker sequence that is partially complementary with the aptamer on the 5′ terminal. In the presence of target bacteria, the capture probe in the SMB-Capture probe could specially bind to the PBP2a on the surface of target bacteria and thus lead to the liberation of the blocker. In the SMB-Report probe, the bioprobe which could partially be hybridized with template DNA was labeled on the surface of SMBs through the interaction between affidavin and streptomavidin.

There are two steps in the whole sensing system: (i) SMB-Capture probe-based target bacterial recognition and blocker release; (ii) SMB-Report probe-based signal amplification and signal generation. In the first step, the recognition of target bacteria by the SMB-Capture probe could lead to the release of the blocker to participant in the next step. In the second step, the template DNA was originally designed with a protruding 30-terminal in which state it could not be digested by Exo III. Upon the existence of the released blocker, the hybridization of the template and promoter formed a blunt 30-terminus in template DNA which could be stepwise hydrolyzed by Exo III from its 30-terminus. Notably, there are still 6-nt single-stranded sequences at the 3′-terminus of the template even though the released blocker could totally hybridize with it with the aim to avoid degradation by Exo III under the assistance of Exo III to remove mononucleotides from the template, and the blocker is then released to initiate the next recycle and to liberate more SMB-Report probes. Afterward, primer DNA was added to bind with the template and then anneal circular DNA. Under the assistance of ligase, the circular DNA was cyclized. Afterward, with the primer DNA as a primer and cyclized DNA as a template, the RCA process was initiated under the assistance of phi29 DNA polymerase and a long-repeated G-quadruplex ssDNA sequence was produced. Eventually, the added *N*-methyl mesoporphyrin IX (NMM) could strongly interact with the G-quadruplex product, bringing a great fluorescence enhancement.

Investigation of the Feasibility of the Two Designed Probes. The SMB-Capture probe is responsible for specific target bacterial recognition and release of the blocker to initiate

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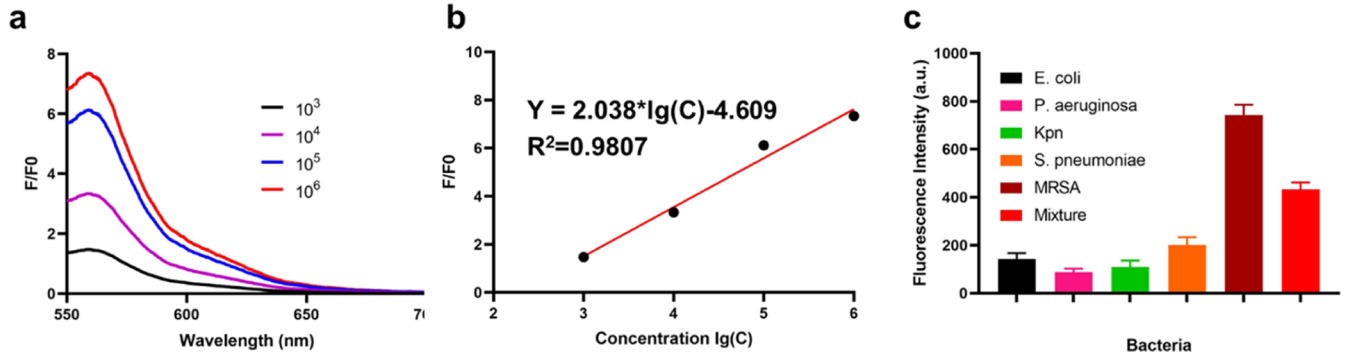

Figure 3. Sensitivity of the cascade signal amplification strategy. (a) Obtained fluorescence spectrum of the sensing system when incubated with different concentrations of target MRSA. (b) Correlation equation between the obtained fluorescence intensity of the sensing system and concentrations of bacteria. (c) Fluorescence intensity of the sensing system for different bacterial detection.

the following recycle and signal amplification processes. Therefore, we first investigated the recognition efficiency of the designed SMB-Capture probe through a fluorescence assay (Figure 1a). From the result in Figure 1b, we have obtained a significantly enhanced fluorescence intensity when the SMB-Capture probe was incubated with target bacteria, while no significant increase was observed when the target bacteria were absent, indicating that the existence of target bacteria could be recognized by the SMB-Capture probe and thus lead to the release of the blocker. Furthermore, we have also observed no significant fluorescence intensity increase when the SMB-Capture probe was incubated with other bacteria, suggesting the specificity recognition between the SMB-Capture probe and target bacteria. Afterward, we studied the RCA process through detection of generated fluorescence signals. From the result in Figure 1c, the RCA product was successfully generated and an obvious signal enhancement was observed when the SMB-Report probe was used to capture primer DNA and circular DNA, while no significant fluorescence signal enhancements were observed when the primer and circular DNA were absent.

Optimization of Experimental Conditions. With the aim to obtain a favorable detection performance, we have first evaluated the experimental parameters that affect Exo III-based recycling and RCA-based signal amplification, including Exo III concentrations, the amounts of circular DNA and primer DNA, and dosage of NMM. Notably, the ratio between F and F0 was used to minimize the interference from background signals (F and F_0 refer to the fluorescence signals when target bacteria existed or were absent, respectively). Considering the important role of Exo III in the signal amplification process, we have first investigated the dosage of Exo III. We have observed an obvious influence on the obtained fluorescence signals by Exo III (Figure 2a). The obtained fluorescence signals increased with the addition of more Exo III, and the fluorescence intensity reached a plateau with 50 U of Exo III. Thus, 50 U of Exo III was applied for the following experiments. We then optimized the concentrations of circular DNA and primer DNA during the RCA process. From the result in Figure 2b, we have observed a gradually increased fluorescence intensity with the increased concentrations of primer sequences and no more increase was observed when the concentration of primer sequences goes more than 50 nM. Thus, we believe that 50 nM primer DNA could provide a favorable detection performance. We have obtained a similar optimization result upon the concentration of circular DNA (Figure 2c), which produced significantly enhanced signals at 2×10^{-7} M. Therefore, 2×10^{-7} M of circular DNA was chosen for the following experiments. We at last studied the amounts of NMM used for signal generation. From the result in Figure 2d, the fluorescence signals peaked with 3×10^{-6} M NMM in the presence of target bacteria and 3×10^{-6} M was chosen as the optimal concentration of NMM.

Sensitivity of the Cascade Signal Amplification Strategy for Target Bacterial Detection. Under the optimized experimental conditions, we have then assessed the sensitivity of the method through applying it for the detection of bacteria with different concentrations. Notably, the target bacteria were incubated and calculated through colony culture. The series of bacteria with different concentrations were obtained through diluting it with PBS buffer. From the result in Figure 3a, the fluorescence intensity of the sensing system gradually increased with the bacterial concentration ranging from 10^3 to 10^6, indicating a relationship between the obtained fluorescence signals and amounts of bacteria. Afterward, the correlation equation between the fluorescence intensity and amounts of bacteria was calculated as $Y = 2.038 \times \log C - 4.609$ with a correlation coefficient of 0.9807. The sensitivity of this cascade signal amplification method for the bacterial detection was superior to many existing Exo III-based signal amplification methods and comparable to other signal amplification strategies (Figure 3b). The results implied good precision and repeatability of this amplification strategy for MRSA detection. It remains a huge significant challenge for the existing bacterial sensing system to accurately identify and isolate MRSA from a diversity of bacteria. Therefore, we have then assessed the selectivity of the proposed method toward target bacteria through applying it for the detection of Kpn, S, *Escherichia coli*, pneumonia, *Pseudomonas aeruginosa*, MRSA, and their mixture. We have eventually obtained neglectable fluorescence signals for the detection of Kpn, S, *E. coli*, pneumonia, and *P. aeruginosa*, while the fluorescence signals for MRSA detection were significantly enhanced, indicating that the method could specially recognize target bacteria even from complicated clinical samples (Figure 3c).

CONCLUSIONS

We developed here a cascade signal amplification strategy that could detect bacteria in a sensitive and label-free way. In the proposed method, we have elegantly designed two SMB-based probes, including the capture probe and a report probe.

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Table 1. Brief Comparison of the Bacterial Detection Method[a]

title	sensitivity	signal amplification	signal output	label-free	ref
the method	10^2	RCA + recycle	fluorescence	yes	
CRISPR-Cas12a	10^2	RCA + trans-cleavage	fluorescence	no	13
allosteric probe	10^3	trans-cleavage	fluorescence	no	19

[a]RCA, rolling circle amplification.

Among them, the capture probe could specially identify target bacteria and thus lead to liberation of the promoter and the report probe could induce the signal amplification process. In detail, the released promoter could initiate the recycle process under the assistance of Exo III after hybridization with template DNA and the report probe could induce the RCA process for signal amplification. Through comparison with some of the traditional signal amplification methods, this strategy exhibited a comparable signal amplification efficiency in a label-free way (Table 1).

EXPERIMENTAL SECTION

Chemical Regents. All related nucleic acids used in this method were designed according to some former proposed methods[20,21] and synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). The obtained purified sequences were first diluted with DEPC water to 10 μM and stored at 4 °C for subsequent experiments. Related enzymes used in the experiment, including T4 DNA ligase, Phi29 DNA polymerase, Exo III, dNTPs, and NMM, were obtained from New England Biolabs Co. Ltd. (Shanghai, China). SMBs were purchased from Thermo Fisher Scientific Co. Ltd. (Shanghai, China). Tween-20 was purchased from Sigma (Suzhou, China). Other regents used in the experiment are in the analytical level.

Assembly of the SMB-Based Probe. We first placed the obtained SMBs on a vortex shaker for 20 s to resuspend them. Afterward, 100 μL of SMBs was taken into a new centrifuge tube which is placed on the magnetic stand to remove the supernatant after incubation for 1 min and resuspended it through adding the corresponding buffer.

Detection Process of the Method. After the assembly of the two SMB-based probes, we have first added the 5 μL capture probe (5 μM) with 5 μL of target bacteria for about 2 h at 37 °C. Afterward, the SMB capture probe that binds with target bacteria was removed under the assistance of a magnet for 10 min and resuspend with 1× PBS buffer. Furthermore, the 5 μL supernatant was mixed with the 5 μL report probe (5 μM) and incubated for 30 min. Afterward, 2 μL of the Exo III enzyme and 5 μL of 10 reaction buffer were added in the mixture and incubated at 37 °C for 2 h. After that, the RCA process was then conducted through the following procedures: We first added 10 μL of 0.5 mM primer DNA to the report probe and incubated it at room temperature for 1 h. Afterward, 0.5 μL of T4 DNA ligase was added in the mixture, and we incubated it for 1 h. After being heated at 60 °C for about 10 min, 5 μL of Phi29 DNA polymerase, 10 μL of reaction buffer, and 3 μL of dNPTs were added to the mixture and incubated at 37 °C for 2 h. After that, 4 mL of NMM was added to the solution and incubated at 37 °C for 0.5 h.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c01107.

Details of the sequences (PDF)

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Notes

The authors declare no competing financial interest.

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