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Article

Photo-Cross-Linked Probe-Modified Magnetic Particles for the Selective and Reliable Recovery of Nucleic Acids

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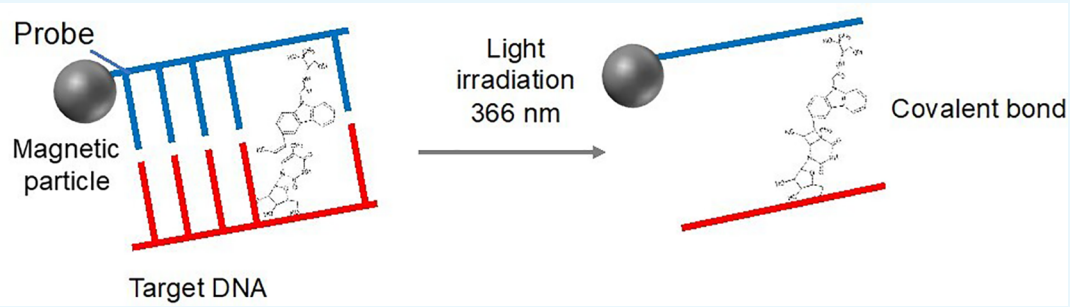

ABSTRACT: Polymerase chain reaction (PCR) assays are used to diagnose various infectious diseases such as Coronavirus disease 2019 by detecting the nucleic acids of the pathogen. However, in practice, the yield of the extraction process and the inhibition of the reverse transcription reaction and PCR by foreign substances reduce the sensitivity and may yield false negative results. The sensitivity of the PCR test can be improved by using technologies that can reliably capture the target nucleic acid and remove foreign substances. In this study, we developed photo-cross-linkable probe-modified magnetic particles (PPMPs) for the sequence-specific recovery of target nucleic acids using photo-cross-linkable artificial nucleic acid probes and magnetic particles. Nucleic acid probes modified with photo-cross-linkable artificial nucleic acids can hybridize with the target nucleic acids in a sequence-specific manner and then securely capture the target nucleic acids by UV irradiation-mediated covalent bonding. Then the target nucleic acid is detected by trapping the target-bound probe on the surface of the magnetic particles and subjecting these collected magnetic particles to PCR. Recovery of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N gene pseudo-DNA (120 bp) was performed using PPMPs. We confirmed that the PPMPs captured the target consistently even after washes were done with denaturing agents and surfactants. Even in the presence of foreign DNA fragments, PPMPs were able to specifically recover the target DNA. This method allows for a more accurate detection by recovering only the target DNA for PCR. Hence, PPMPs can be successfully used for PCR-mediated detection of SARS-CoV-2 and other pathogens whose nucleic acid sequences are known.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a viral infection that causes severe respiratory distress.[1] In December 2019, a series of patients were reported to show pneumonia of an unknown cause in Wuhan, Hubei Province, China.[2] A novel coronavirus isolated in 2019 (2019-nCOV) was obtained from the bronchoalveolar lavage fluid of one patient.[3] Subsequently, it was renamed as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses.[4] On the basis of the global spread of COVID-19 due to the rapid increase in human-to-human transmission, the World Health Organization classified the COVID-19 outbreak as a pandemic on March 12, 2020. Since SARS-CoV-2 is known to spread via infected individuals showing mild or no symptoms, the early diagnosis, control of infected individuals, and establishment of advanced virus-testing techniques are necessary to control the outbreak.[5]

Currently, COVID-19 is diagnosed via reverse transcription-polymerase chain reaction (RT-PCR) assays owing to their specificity and sensitivity.[6] PCR is used to examine the presence of viral genes in patient samples. The main limitation of the PCR test involves the possibility of developing false negative results because the PCR amplification may not occur due to degradation of viral RNA by RNase, which is also present in human saliva, sweat, and dust in the air.

The most common specimen used for PCR analysis is a nasopharyngeal swab.[6] The collected specimens can get

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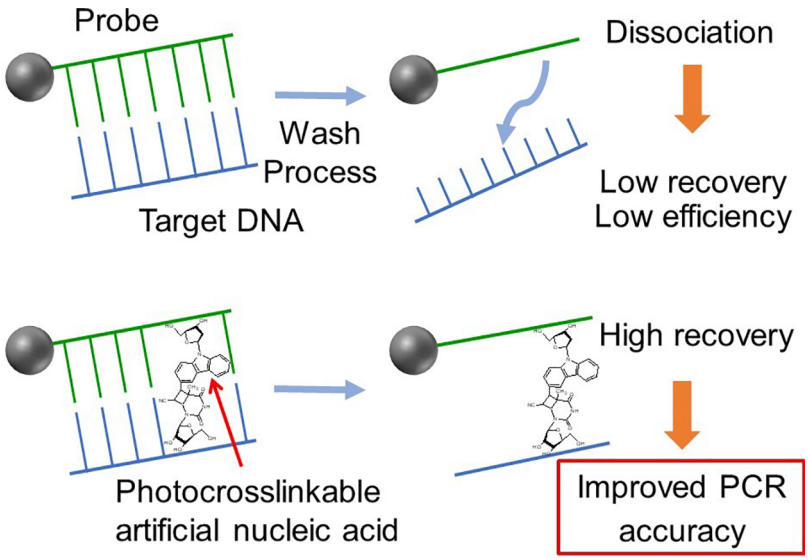

Figure 1. Schematic illustration of the recovery of target DNA using photo-cross-linkable artificial nucleic acid probes.

contaminated with substances other than DNA and RNA. In the commonly used spin column extraction, the concentration of target nucleic acid in the purified product is relatively low, since the total RNA in the specimen is recovered. False negatives occur due to a decrease in the concentration of target nucleic acids and inhibition of the reaction by foreign substances. Therefore, to improve the accuracy of the PCR assay, a method to specifically capture the target nucleic acid in the extraction process and to increase nucleic acid concentration is required.

To specifically capture target RNA, a method using magnetic particles (MPs) modified using nucleic acid probes has been developed.[7−12] Nucleic acid probes containing specific sequences for target RNA capture target RNA via hydrogen bonding. Since MPs can be collected using a magnet while the target RNA is bound, the cleaning and collection processes do not require specialized equipment and can be performed anywhere. However, while using this method, the target nucleic acid may potentially adsorb nonspecifically to particle surfaces or the probe via electrostatic or hydrophilic interactions.[12] Although washing with denaturing agents or surfactants can effectively remove these nonspecifically adsorbed substances, the target nucleic acids trapped via hydrogen bonding with the nucleic acid probe are simultaneously desorbed. To ensure the recovery of the target nucleic acid by the nucleic acid probe, there is a need to develop technologies for consistently capturing the target nucleic acid even under conditions in which hydrogen bonds dissociate.

To address this issue, we focused on photo-cross-linkable artificial nucleic acids, 3-cyanovinylcarbazole nucleotide (CNV-K). The strands of the nucleic acids are held together by hydrogen-bonding interactions. CNV-K form covalent bonds with pyrimidine bases between nucleic acid duplexes upon light irradiation. Cross-linking of nucleic acids by UV light does not require any other reagents and can be controlled spatially and temporally by simply selecting the appropriate irradiation method.[13−15] If a probe modified using a CNV-K is cross-linked with a target nucleic acid, the probe can maintain its binding state with the target nucleic acid even after the dissociation of hydrogen bonds.[16] This technology enables the use of washing procedures using denaturing agents and surfactants to remove nonspecific adsorbents. It also prevents the loss of target nucleic acids due to dissociation from the probe during magnetic separation and processes other than washing.

We designed a photo-cross-linkable probe by introducing a CNV-K into a DNA probe that recognizes an SARS-CoV-2 sequence specifically. The photo-cross-linkable probe-modified magnetic particles (PPMPs) were prepared by adding biotin to the end of the photo-cross-linkable probe following affinity binding with streptavidin-coated magnetic beads. The prepared particles consistently captured the target SARS-CoV-2-derived RNA even after a vigorous washing and in the presence of foreign substances (Figure 1).

To evaluate the PPMPs, recovery and detection experiments were performed using single-stranded DNA (ssDNA) mimicking the SARS-CoV-2 N gene sequence as a pseudotarget. In the recovery experiment, after hybridization of the PPMPs with the pseudotarget, the particles were irradiated with ultraviolet light and subjected to various washing procedures. PCR was performed using the particles recovered via magnetic separation, and the target recovery performance of the PPMPs was evaluated by detecting and quantitatively evaluating the pseudotargets.

RESULTS

Optimization of Photo-Cross-Linking Reaction Conditions. After the hydrogenation of the photo-cross-linkable probe with the target nucleic acid, the C−C double bond of the cyanovinyl group of CNV-K and the C5═C6 double bond of the corresponding thymine of the target nucleic acid cyclize [2 + 2] to form a covalent bond via light irradiation at 365 nm.[13] The photo-cross-linking efficiency between the photo-cross-linkable probe and the target nucleic acid is affected by the amount of energy of the irradiated light.[14] Turbidity is generated in the reaction solution when PPMPs are used, which may reduce the photo-cross-linking reaction rate.

The photo-cross-linking reaction between the probe sequence and the target nucleic acid introduced with a CNV-K was evaluated via high-performance liquid chromatography (HPLC). In this evaluation, a 9-mer probe sequence (TGCCXGCGT: X = CNV-K) and target nucleic acid

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ACGGGCGCA were prepared to evaluate the cross-linking reaction.

In the solution containing a mixture of the target DNA and probe (a), the peaks attributed to the target DNA and probe were observed at retention times of 12 and 18 min, respectively (Figure 2). In the case of DNA after light irradiation (b), the

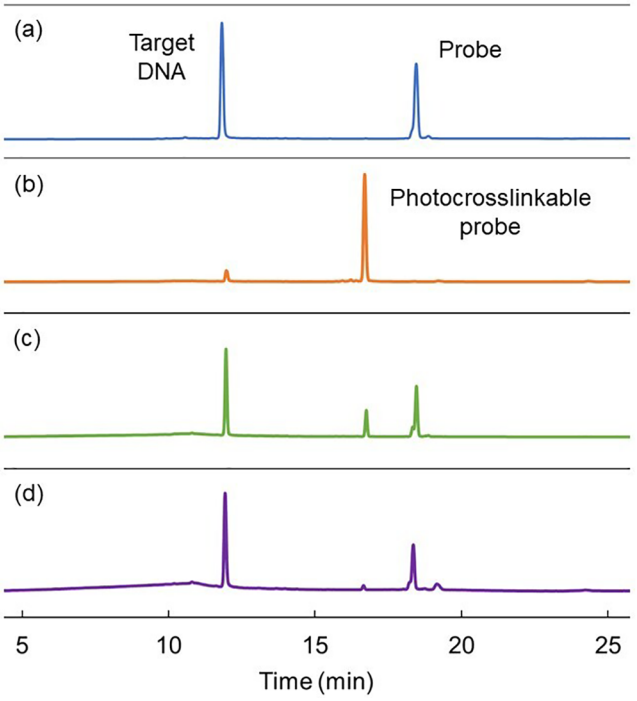

Figure 2. HPLC analysis for confirmation of cross-linking between 9-mer model target nucleic acid and photo-cross-linkable probe. (a) Before light irradiation. (b) After light irradiation without MPs. (c) 1400 mW irradiation without MPs. (d) 150 mW irradiation with MPs.

peaks associated with the target DNA and probe were weakened or absent, and a complex peak due to photo-cross-linking was observed at ∼17 min. The photo-cross-linking ratio was calculated as the ratio of the areas of the cross-linked peaks under each condition, with the peak area of the cross-linked complex in (b) being 100% (Table 1). Since the duration of

Table 1. Cross-Linking Rate of Samples Based on High-Performance Liquid Chromatography Analysis[a]

sample	(a)	(b)	(c)	(d)
light irradiation	−	1400 mW	150 mW	150 mW
magnetic particle	−	−	−	+
cross-linking rate (%)	0	100	17	≤10

[a]−: without, +: with.

irradiation was the same, the photo-cross-linking rate is considered to differ according to the irradiation intensity (c). Under condition (d), where MPs were present in the solution, the cross-linking rate was lower than that in conditions (b) and (c), where MPs were absent in the buffer solution. It was found that the presence of MPs in the solution scattered the irradiated light and inhibited the photo-cross-linking reaction. Therefore, a high-intensity light source should be used for completing the photo-cross-linking reaction in the absence of MPs in the solution. However, when irradiated with 1400 mW, the solution was heated, which caused the solution to evaporate. Therefore, in this paper, we decided to irradiate the solution with 150 mW for the photo-cross-linking reaction and then collect the hybridization products by avidin-modified magnetic particles.

Detection of SARS-CoV-2 pseudo DNA. To evaluate the PPMPs, recovery and detection experiments were performed using ssDNA mimicking the SARS-CoV-2 N gene sequence as a pseudotarget. In the recovery experiment, after hybridization of the PPMPs with the pseudotarget, the particles were irradiated with light and subjected to various washing procedures. The particles recovered via magnetic separation were analyzed using PCR to check for the presence of target nucleic acids.

The results of agarose gel electrophoresis of the PCR-amplified products under each condition are shown in Figure 3. The (a) positive control and (b) PPMPs showed a band of

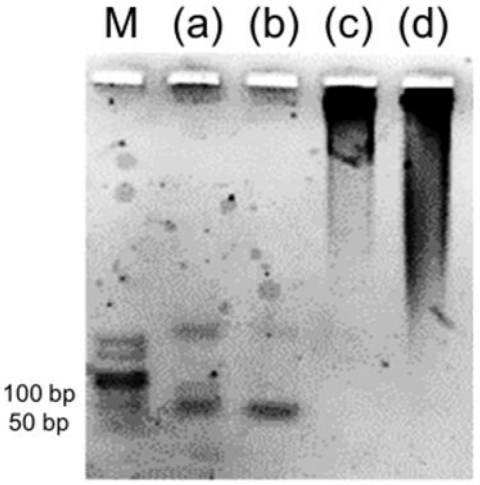

Figure 3. Separation of samples via agarose gel electrophoresis after PCR amplification. (a) Target nucleic acids. (b) Photo-cross-linked probe-modified MPs. (c) MPs. (d) Photo-cross-linked probe-modified MPs without light irradiation.

∼80 bp in size. In contrast, no bands were observed for (c) MPs and (d) PPMPs without light irradiation. The samples comprising (c) MPs and (d) PPMPs without light irradiation showed no bands, suggesting that the target DNA was not captured and recovered because the probe was not bound to the MPs. The possibility of nonspecific adsorption on the surface of the MPs exists; however, it was prevented by washing with a surfactant, and it is considered that the particles were not nonspecifically adsorbed onto the surface. In the case of (d), covalent bonds were not formed via photoirradiation; therefore, the target DNA could not be captured and recovered, since the hydrogen bonds dissociated and the target DNA was desorbed when washed with a denaturant and surfactant.

Detection of Target DNA in the Presence of Contaminant Foreign Substances. During the actual extraction of nucleic acids, the presence of proteins and other viral nucleic acids may interfere with the specific binding of target nucleic acids to the probe. Therefore, we added substances other than the target DNA to the experimental system to mimic conditions similar to those of the actual system. Since a short DNA sequence was used as the target in this study, fragmented methicillin-resistant *Staphylococcus*

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aureus (MRSA) genomic DNA fragments were used as the foreign material.

The gel electrophoresis results for the final PCR-amplified products are shown in Figure 4. Similar to the experiment in which foreign DNA was absent (Figure 3), a band (~80 bp) was observed for (a) target nucleic acids and (b) PPMPs. Therefore, it was confirmed that (b) PPMPs consistently captured the target nucleic acids even after washes with denaturing agents and surfactants and that it could capture the target nucleic acids regardless of the presence of foreign substances.

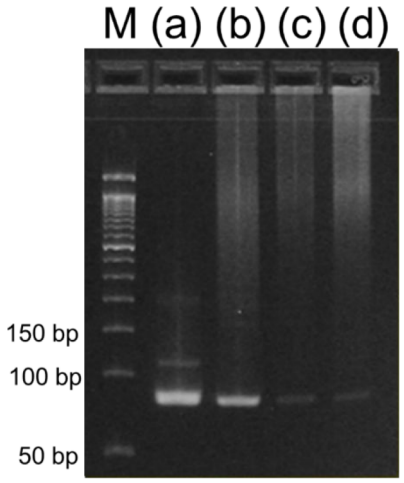

Figure 4. Separation of samples via agarose gel electrophoresis after PCR amplification of crude samples. (a) Target nucleic acids. (b) Photo-cross-linked probe-modified MPs. (c) magnetic particle. (d) Photo-cross-linked probe-modified MPs without light irradiation.

Furthermore, quantitative measurements were performed using real-time PCR. When (b) the expression of PPMPs was compared to that of (d) PPMPs without light irradiation, a difference in threshold cycle values was obtained. The target DNA recovered by (b) PPMPs was higher than that of (d) PPMPs without light irradiation. It was confirmed that the amount of recovery increased with successful photo-cross-linking.

DISCUSSION

The low yield of nucleic acid extraction processes and the inhibition of PCR by foreign substances reduce the sensitivity of PCR assays and may yield false negative results; therefore, methods to reliably capture nucleic acids are required to increase the sensitivity and specificity of PCR assays.

The purpose of this study was to establish a highly accurate detection method by using PPMPs to achieve a reliable capture of target DNA while simultaneously eliminating the foreign substances. We developed the PPMPs for sequence-specific retrieval of target DNA. Furthermore, we devised a method to detect the target by subjecting the target-captured PPMPs to PCR. (Figure 5).

The probe containing CNV-K is covalently bound to the complementary sequence of the target DNA by photo-cross-linking reaction via irradiation with 366 nm light. We observed that the cross-linking rate differs depending on the power of the UV light source. Furthermore, it was confirmed that the cross-linking rate was affected by the presence and absence of MPs in the solution during light irradiation. One of the limitations of this method is the inhibition of the photo-cross-linking reaction due to the scattering of light by MP. Hence, to circumvent this drawback and maximize the cross-linking efficiency, we invented a method in which the binding of the probe to the target (hybridization and photo-cross-linking reactions) is carried out first, and then the resulting nucleic acid complexes are trapped by the MPs.

The double-stranded nucleic acid, cross-linked by the photo-cross-linking reaction, does not dissociate even when heated or exposed to a highly concentrated formamide solution. The avidin−biotin affinity between the probe and MP is remarkably

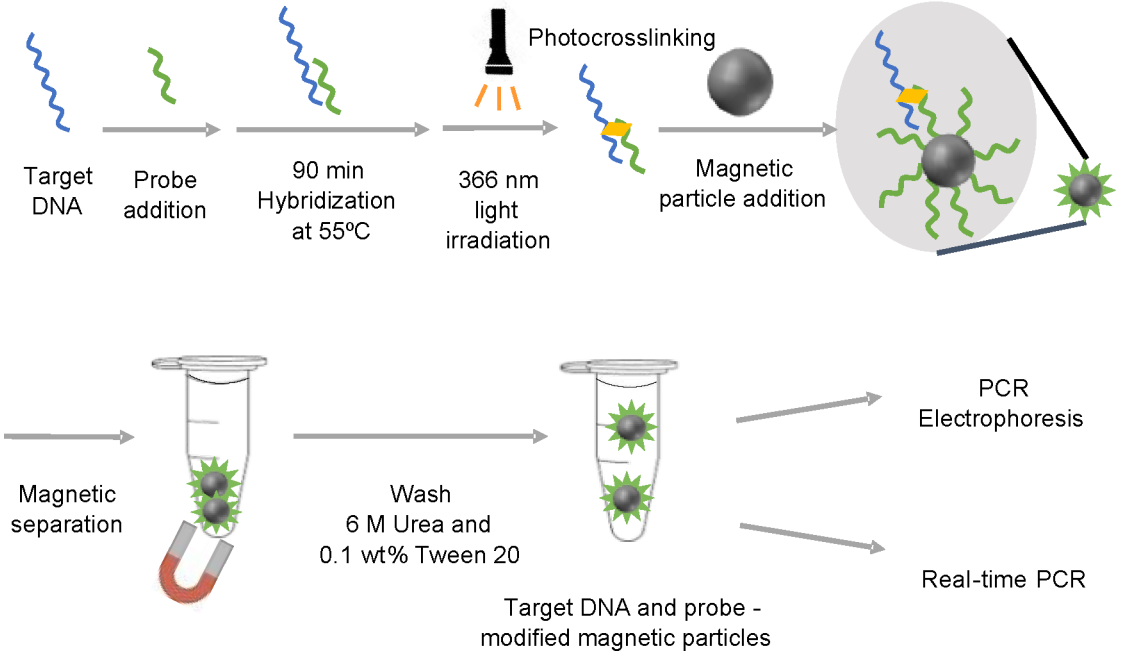

Figure 5. Recovery of target nucleic acids using photo-cross-linkable artificial nucleic acid probes.

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strong, and the formed complex is resistant to extreme pH, temperature, organic solvents, and denaturing agents.[17] Photo-cross-linking enhanced the stability of the binding of the target to the probe, so much so that, even when the target-captured PPMP was washed with denaturant and surfactant solution, the target could still be detected in subsequent PCR. On the contrary, MPs immobilized with probes that were captured and bound to the target by hydrogen bonding alone were subsequently not detected by PCR due to the possible dissociation of the target from probe during washing. Hence, in this technique, the target nucleic acid maintained a very strong bond with PPMPs. In addition, we were able to detect the target using PPMPs even in the presence of foreign DNA, confirming that the probe containing CNV-K selectively captured the target. Therefore, the target recovery by PPMP is sequence-specific.

Additionally, PPMP can also be used for a quantitative evaluation of targets by real-time PCR. Although the enzymatic reaction and detection of the target using fluorescent probes on the surface of PPMPs was slightly inhibited compared to normal PCR, the difference in cycle threshold (Ct) values showed that the target recovery was significantly improved by UV irradiation.

In this study, we developed a target recovery method using an optically linkable probe and magnetic particles for more efficient detection of target nucleic acids in liquids. Also, we confirmed that PPMPs can be subjected to PCR, which is extensively useful for diagnostic purposes and detection of infectious diseases. If a suitable detection method for PPMPs can be designed, this recovery method is expected to provide a more accurate diagnosis of COVID-19 and other viral infections.

MATERIALS AND METHODS

Synthetic DNA. The photolinkable probe (5-biotin-GTTCTGGACCACTGCCGAXAG-3′X = CNV-K) was designed from the N gene sequence of SARS-CoV-2. Synthesized ssDNA (5′-CTTTCGGCAGACGTGGTCCAGAACC-AACCCAAGGAAATTTTGGGGACCAGGAACT-AATCAGACAAGGACAATGTCCGCCATTGGT-TGACCTACACAGGTGCCATCA-3′) was used as a target for pseudo-SARS-CoV-2. The sequences from the National Institute of Infectious Diseases N2 set (NIID_2019-nCOV_N_F2:5′-AAATTTTGGGGACCAGGAAC-3′, NIID_2019-nCOV_N_R2:5′-TGGCAGCTGTGTAG-GTCAAC-3′) were used as primers for PCR and real-time RT-PCR.

Calculation of Photo-Cross-Linking Reaction Rate. Two types of oligo DNA (100 μM; oligo DNA containing CNV-K and its complementary strand) were mixed in a buffer solution and photo-cross-linked via ultraviolet light irradiation from 15 mm above with OmniCure LX505 (SUNSTAR) and ABL-5L (Alfa Mirage Co, Ltd.) for 5 min on ice. Each sample was analyzed via HPLC using the following column: COSMOSIL 2.5C18-MS-II (3.0 I.D. × 100 mm), with elution in 2−20% acetonitrile/50 mM triethylammonium acetate buffer trifluoroacetic, in a linear gradient for over 30 min.

Target DNA Recovery. Prior to the recovery procedure, 10 mg/mL Dynabeads M-270 streptavidin solution (Thermo Fisher Scientific) was washed and adjusted to 5 mg/mL according to the manufacturer's protocol. Target DNA solution (10 μL, 1 μM in a Tris/ethylenediaminetetraacetic acid (Tris/EDTA, TE) buffer (pH 8.0)) was heated at 90 °C for 5 min, mixed with the probe solution (10 μL, 3 μM in TE buffer (pH 8.0)). The mixture was incubated at 55 °C for 90 min for the hybridization of the target DNA and the probe. After irradiation of the hybridization mixture with UV (366 nm, 150 mW) for 5 min on an ice bath, 10 μL of 5 mg/mL Dynabeads M-270 streptavidin solution was added. The mixture was mixed by rotation for 15 min at room temperature (25−28 °C). Thus, hybridization products were immobilized on the surfaces of the MPs. Subsequently, a magnetic separation was performed using a magnetic separation rack, and the supernatant was removed. The collected MPs were washed with 10 μL of 6 M urea (×1), 200 μL of TE buffer (×3), and 10 μL of 0.1 wt % Tween 20 (×1) to remove the nonspecifically bound DNA. Finally, the MPs were washed with 200 μL of TE buffer (×3) and subsequently suspended in 10 μL of TE buffer.

PCR. PCR was performed using KOD DNA Polymerase (Toyobo). Collected MPs or PPMPs solution (5 mg/μL) was used as a template of the amplification reaction. DNA in the reaction mixture was amplified using the Thermal Cycler WAKO RECT-0240 (Fujifilm). Amplification was performed in 25 cycles of initial denaturation at 98 °C for 2 min, denaturation at 94 °C for 15 s, annealing at 60 °C for 2 s, and extension at 74 °C for 4 s.

Real-Time PCR. Real-time PCR was performed using TB Green Premix Ex *Taq*II (Tli RNaseH Plus) (Takara Bio). Collected MPs or PPMPs solution (5 mg/μL) was used as the template for the amplification reaction. DNA in the reaction mixture was amplified using the Thermal Cycler Dice Real Time System II (Takara Bio). The initial denaturation was performed at 95 °C, and amplification was performed in 45 cycles with repetitive denaturation at 95 °C for 15 s and extension at 60 °C for 30 s.

Preparation of Contaminant DNA. Genomic DNA of MRSA ATCC-700698 was obtained from the American Type Culture Collection (ATCC), and the bacteria were grown overnight on Luria−Bertani (LB) culture medium at 30 °C. NucleoBond AXG column (Macherey-Nagel) was used to extract genomic DNA from cultured MRSA.[18] The purified genomic DNA of MRSA was fragmented via sonication at 25 kHz for 1 h and used as the foreign DNA. The foreign DNA was added at 1 μM concentration, which was the same as that of the target DNA, and recovery experiments using MPs, PCR, and real-time PCR were performed.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MP, magnetic particle; PPMP, photo-cross-linked probe-modified magnetic particle; CNK-V, photo-cross-linkable artificial nucleic acid; MRSA, methicillin-resistant *Staphylococcus aureus*

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