ORIGINAL ARTICLE

A Lateral Flow Assay for Nucleic Acid Detection Based on Rolling Circle Amplification Using Capture Ligand-Modified Oligonucleotides

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Abstract

We introduce a lateral flow assay (LFA) integrated with a modified isothermal nucleic acid amplification procedure for rapid and simple genetic testing. Padlock probes specific for the target DNA were designed for ligation, followed by rolling circle amplification (RCA) using capture ligand-modified oligonucleotides as primers. After hybridization with detection linker probes, the amplified target DNA is flowed through an LFA membrane strip for binding of gold nanoparticles as the substrate for colorimetric detection. We established and validated the "RCA-LFA" method for detection of mecA, the antibiotic resistance gene for methicillin-resistant *Staphylococcus aureus* (MRSA). The assay was optimized using various concentrations of primers and probes for RCA and LFA, respectively. The sensitivity was determined by performing RCA-LFA using various amounts of mecA target DNA, showing a detection limit of ~ 1.3 fmol. The specificity of the assay was examined using target DNAs for other resistance genes as the controls, which demonstrated positive detection signals only for mecA DNA, when added either individually or in combinations with the control targets. Furthermore, applying the RCA-LFA method using specifically designed probes for RNA-dependent RNA polymerase (RdRp) and receptor binding domain (RBD) gene for SARS-CoV-2, which demonstrated feasibility of the method for viral gene targets. The current method suggests a useful platform which can be universally applied for various nucleic acid targets, allowing rapid and sensitive diagnosis at point-of-care.

Keywords Lateral flow assay · Rolling circle amplification · Rapid diagnosis · Genetic testing · Point-of-care

1 Introduction

The development of a rapid and simple detection method for nucleic acid analytes provides great advantages such as enabling early diagnosis, treatment monitoring, and point-of-care testing [1–3]. Genetic testing methods based on nucleic acid biomarkers allow highly sensitive and specific detection, due to the involvement of amplification procedures and sequence specificity. Nucleic acid amplification techniques have been widely utilized for laboratory and diagnostic purposes, to identify even trace amounts of genetic targets possibly in a multiplexed and high-throughput process [4, 5].

Quantitative real-time polymerase chain reaction (qPCR) has been extensively used in the clinic for diagnosis of diseases such as cancer and infections; however, the requirement of a thermal cycling process and real-time fluorescence monitoring system has limited its application as a sensing platform for diagnostics in resource-limited settings. As an alternative method, isothermal amplification procedures such as nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), and loop-mediated amplification (LAMP) have been investigated and attempted to be integrated into biosensing platforms [5–8].

Among the nucleic acid amplification methods, RCA is a unidirectional isothermal process used to synthesize periodic long single-stranded DNA which is hundreds of thousands of nucleotides long [9–11]. Incorporation of RCA into diagnostic assays can provide high sensitivity by the amplification of the target nucleic acid up to 1000-fold [9]. The amplified products must be detected by colorimetric, fluorescence, optical, or electrochemical means, and usually involves the addition of organic substrates or hybridization

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probes to induce specific target signals [12–16]. Advanced methods such as detection of RCA products based on hydrogel formation or suppression of coffee ring patterns have been reported as novel approaches to apply genetic testing as a simple and affordable assay while achieving high sensitivity [17–19]. However, these strategies require further development as a platform to be available to customers for practical use as a self-diagnostic kit for on-site or home settings.

The importance of point-of-care diagnostics has been tremendously increasing, due to the recent pandemic and the concern regarding the possible outbreaks in the future [20]. The spread of antibacterial resistance has been recognized as a major problem worldwide, with estimations of more than 10 million deaths per year by the year 2050. Multidrug-resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant Enterobacteriaceae, carbapenem-resistant *Acinetobacter baumannii*, and multidrug-resistant *Pseudomonas aeruginosa* have been reported as the major microbes that cause hospital-acquired infections. Particularly, MRSA infections have been the most prevalent among the Gram-positive infections, which can involve serious conditions such as bacteremia possibly as complications with other diseases, requiring rapid diagnosis at the bedside. As an affordable and easy-to-use platform, lateral flow assays (LFA) based on colorimetric detection have been widely investigated and developed as diagnostic kits [20–23]. LFA is generally performed by applying the analyte solution on a paper-based membrane strip, which flows by capillary force, and binds to the substrate to appear as a visible signal on the test line. AuNPs are widely used as probes in LFAs for colorimetric pathogen detection, as well as AgNPs and other metal nanoparticles such as

cerium oxide [24, 25]. However, although LFAs have great advantages and is the most feasible platform for point-of-care testing, the assay principle has been limited to antigen–antibody based detection [26–28]. Recently, many studies have reported developments on the integration of nucleic acid testing into LFAs and other membrane-based assays [29–33]. However, their poor sensitivity and selectivity limit their practical application for rapid diagnostics and clinical decision making.

In this study, we introduce a simple diagnostic method by combining a modified RCA procedure with LFA (RCA-LFA) as a versatile platform to rapidly detect nucleic acid targets at point-of-care (Fig. 1). RCA of the target nucleic acids with capture ligand-modified oligonucleotides as primers (biotin-primer) produces functionalized, long single-strand DNA. The amplified biotin-modified target DNA is sequentially labeled by hybridization with fluorescein amidite (FAM)-modified oligonucleotides (FAM-probe), which are complementary to the RCA-amplified target DNA, as detection probes. Upon flow through the LFA strip, the biotin-modified target DNA is captured by streptavidin immobilized on the test line. The control line is immobilized with anti-rabbit antibody, which binds to the residual anti-FAM antibody on the gold nanoparticles, after passing through the test line. The target DNA hybridized with the probes binds with anti-FAM antibody coated on gold nanoparticles (AuNP-aFAM), resulting in a colorimetric 'red signal' on the test line. We demonstrate that the current RCA-LFA method can be used to detect mecA, which is known as the antibiotic resistance gene for methicillin-resistant *Staphylococcus aureus* (MRSA) [34]. We anticipate that the current assay, with advantages in enabling amplification of nucleic
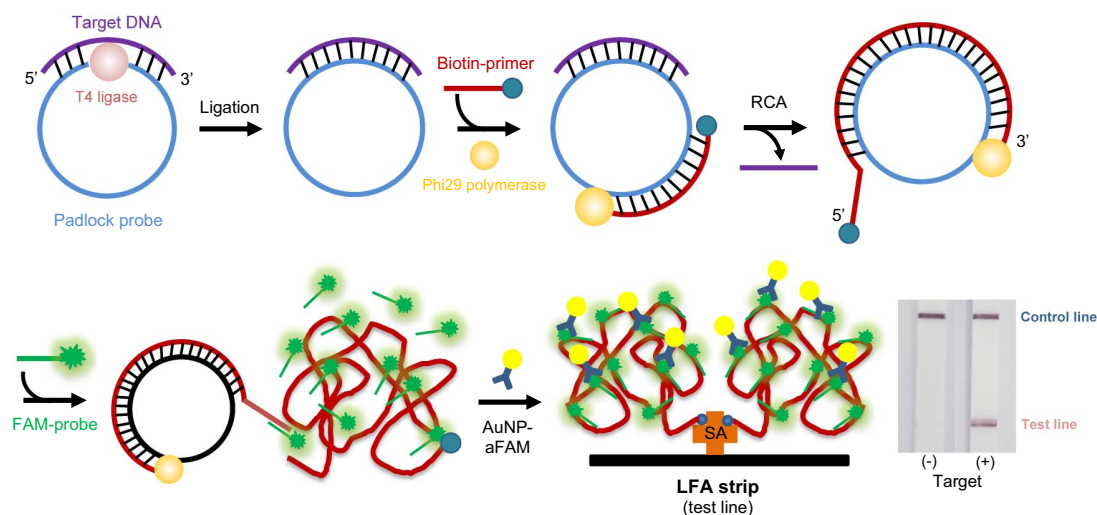

Fig. 1 Schematic illustration of the RCA-LFA assay: a padlock probe specific to the target DNA is used to induce target-dependent ligation followed by RCA using biotin-primer, hybridized with FAM-probe, and bound with AuNP-aFAM during flow through on an LFA strip for detection. The control line is immobilized with anti-rabbit antibody, which binds to the residual AuNP-aFAM

acid targets at ambient temperature and visualization without the requirement of any instrumentation, can be applied as a versatile diagnostic platform for detecting bacteria or viral pathogens which require rapid and early detection at resource-limited settings.

2 Experimental Section

2.1 Materials

Synthetic oligonucleotides for padlock probes, biotin-primers, FAM-probes, and target DNAs were custom-synthesized and provided by Bioneer, Inc (Republic of Korea). T4 ligase was purchased from Enzynomics (Republic of Korea). NxGen®phi29 polymerase was provided by Lucigen. dNTPs and dithiothreitol (DTT) were purchased from Promega. Exonuclease 1 (Exo1) and pyrophosphatase were purchased from New England Biolabs. Millenia Genline Hybridetect for LFA test was purchased from Milenia Biotec GmbH (Germany).

2.2 Padlock Probe Design and Ligation

Ligation and RCA was performed according to a previous report with modification in the materials and procedures [19]. The sequences for padlock probes, biotin-primers, FAM-probes, and target DNAs are listed in Table 1. Specific regions in mecA, New Delhi metallo-beta-lactamase 1 (NDM-1), *Klebsiella pneumoniae* carbapenemase (KPC), RdRp, and RBD were selected by obtaining cDNA

sequences from the National Center for Biotechnology Information (NCBI) database. Padlock probes for mecA, RdRp, and RBD were designed based on the selected target regions using the NUPACK thermodynamic analysis software. For target-dependent ligation, 0–100 pmol of synthetic target DNA was added to 50 pmol of padlock templates in ligation buffer containing 800 U of T4 ligase, followed by incubation at 37 °C for 30 min. For characterization, the reacted ligation solution was treated with 10 U Exo1 and incubated at 37° C for 30 min. Gel electrophoresis was performed at 12% polyacrylamide in TBE buffer and visualized with a gel imaging system (LSG1000, Optinity).

2.3 RCA and Probe Hybridization

For the RCA reaction, the solution including ligated product was added to 2–50 pmol of biotin-primer, 0.2 mM dNTP mix, 20 U NxGen®phi29 DNA polymerase, 0.05 U pyrophosphatase, and 0.8 mM DTT in phi29 reaction buffer to a final volume of 50 μl and incubated at 37 °C for 30 min. The RCA product was characterized by polyacrylamide gel electrophoresis. The solution including RCA product was purified using Illustra Microspin G-25 columns (Cytiva), to remove the enzyme, DTT, and buffer components. The purified product was then mixed with 1–100 pmol of FAM-probe to a final volume of 10 μl and heated to 95 ℃ for 10 min, followed by cooling to 25 ℃ and incubation for 20 min for hybridization of the FAM-probe to the amplified target DNA produced by RCA.

Table 1 Sequences of oligonucleotide probes and targets

Type of oligonucleotide	Length (base)	Sequences
Padlock probe for mecA	115	5'-ACCTGAGCCATATTGCCAATTCGAGTTCGAATTGGACCCTACTTACCCATTCTTACCCTA CACCTTCACCCTCACCCTCATAACTTAACCCGGAACCGGACTC GTGGATAGCAGT-3'
Padlock probe for RdRp	115	5'-CAAGTGAGGCTATTGCCAATTCGAGTTCGAATTGGACCCTACTTACCCATTCTTACCCTA CACCTTCACCCTCACCCTCATAACTTAACCCGGAACCGGACTC TGCGAGCAAGAA-3'
Padlock probe for RBD	115	5'-TCCAAGCATATATTGCCAATTCGAGTTCGAATTGGACCCTACTTACCCATTCTTACCCTA CACCTTCACCCTCACCCTCATAACTTAACCCGGAACCGGACTC TTCTCTTTCGGT-3'
Biotin-primer	20	5'-GGGTAAGAATGGGTAAGTAG-3'
FAM-probe	20	5'-TCACCCTCACCCTCATAACT-3'
Target DNA for mecA	65	5'-TGGCTCAGGTACTGCTATCCACCCTCAAACAGGTGAATTATTAGCACTTGTAAGCACACC TTCAT-3'
Target DNA for NDM-1	65	5'-CAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGG TATGGACG-3'
Target DNA for KPC	65	5'-CAGTCGGAGACAAAACCGGAACCTGCGGAGTGTATGGCACGGCAAATGACTATGCCG TCGTCTGG-3'
Target DNA for RdRp	68	5'-GCCTCACTTGTTCTTGCTCGCAAACATACAACGTGTTGTAGCTTGTCACACCGTTTCTAT AGATTAGC-3'
Target DNA for RBD	65	5'-TATGCTTGGAACCGAAAGAGAATCTCCAATTGCGTAGCTGATTACTCCGTTCTCTATAAC AGTGC-3'

2.4 LFA Detection and Analysis

Ten microliters of the solution containing the hybridized, amplified target DNA/FAM-probe complex were mixed with 90 µl LFA buffer (Millenia Genline Hybridetect) including AuNP-aFAM in a 96-well microplate. The LFA strip (Millenia Genline Hybridetect) was then immersed in the solution in the microwell for 30 min, to induce binding of the reagents and flow through the membrane. The final results which appeared as colorimetric signals on test line and control line were directly visualized. Images were also acquired using a smartphone camera (Samsung GalaxyNote 8) and the detection signals were quantified using ImageJ.

2.5 Statistical Analyses

All data were obtained by performing experiments in duplicates. Quantitative data are expressed as mean $\pm$ standard deviation (stdev). Detection limit was determined using the formula: [signal > background + 3*stdev]. At least three independent sets of experiments were performed for each condition to confirm reproducibility of the assay.

3 Results and Discussion

3.1 Probe Design and Establishment of RCA-LFA

The RCA reaction includes a ligation step of a padlock probe specific for the target DNA, followed by isothermal amplification with phi29 DNA polymerase [11]. The padlock probe was designed to complementarily bind to the target region within the antibiotic resistance gene mecA gene as shown in Fig. 2a. By binding to the target DNA, the 5′ end of the padlock probe is placed in close proximity to the 3′ end, inducing ligation by T4 ligase into a circularized form. Ligation of the padlock probe for mecA was validated by treating the nuclease Exo1 and gel electrophoresis. Exo1 removes the nucleotides of linear single-stranded DNA in the 3'–5' direction, resulting in degradation of the target DNA and padlock probe which has not gone through ligation. As shown in Fig. 2b, the band for the ligated padlock probe is observed only when added with the target DNA for mecA, whereas the bands do not appear when target DNA is absence or when added with control target DNAs for other resistance genes NDM-1 and KPC [35, 36]. Exo1 treatment of the padlock probe ligated by mecA target DNA resulted in a shift of the band to the (−) electrode, due to the decrease in size by removal of single-strand target DNA which used to be bound to the padlock probe [19]. This result demonstrates the specificity of the padlock probe, which is able to ligate only upon complementary binding of mecA target DNA.

Amplification was then performed with the ligated padlock probe by adding phi29 DNA polymerase and RCA primer under isothermal conditions (37 ℃). Amplification of the ligated padlock results in long, single-strand DNA including multiple repeats of the amplicon as the RCA product, which can continuously take place as long as the dNTPs are sufficiently supplied. Biotin-modified oligonucleotides were used as the RCA primer, for the following procedures in performing LFA. Figure 2c shows the presence of a band for an extremely long as the RCA product located on top of the gel, which is unable to migrate to the (+) electrode due to their large size. The RCA product was observed only when the padlock probe was ligated in the presence of mecA target DNA, while the addition of control target DNAs did not produce any amplified product. This result confirms the successful amplification of the padlock probe, which is induced by target-dependent ligation, and subsequent amplification by RCA. The resultant RCA product, which is functionalized with biotin, can be further applied for capture and detection on the LFA strip.

We next examined whether the FAM-probe can induce target-specific detection signal upon hybridization with the target DNA-derived RCA product and capture on the LFA strip. The FAM-probe was designed to include a complementary sequence to the RCA amplicon, and was compared with a control FAM-probe which was not complementary to the amplicon. As shown in Fig. 2d, we observed that a red signal appeared on the test line of the LFA strip only when the target DNA was added, and hybridized with the FAM-probe, while using the control FAM-probe did not give rise to any signal. This confirms that target induced ligation of the padlock probe by specific binding of mecA target DNA, followed by isothermal amplification with biotin-primer, and sequential binding with FAM-probe and AuNP-aFAM, leads to successful detection of the target by LFA.

3.2 Optimization of RCA-LFA

Utilizing the padlock probe and RCA characterized above, the RCA-LFA method was established and validated for target detection using synthetic DNA for mecA target. LFA performed with the amplified target DNA and probes gives rise to a signal on the membrane strip based on the following principle: the amplified target DNA, which is the single-strand RCA product modified with biotin as the capture ligand, binds to FAM-modified oligonucleotides as the linker probe (FAM-probe). Upon flow through the LFA membrane strip, the hybridized target DNA binds to anti-FAM antibodies coated on AuNPs (AuNP-aFAM). The resultant sandwich hybridization complex of biotin-modified target DNA, FAM-probe, and AuNP-aFAM is captured at
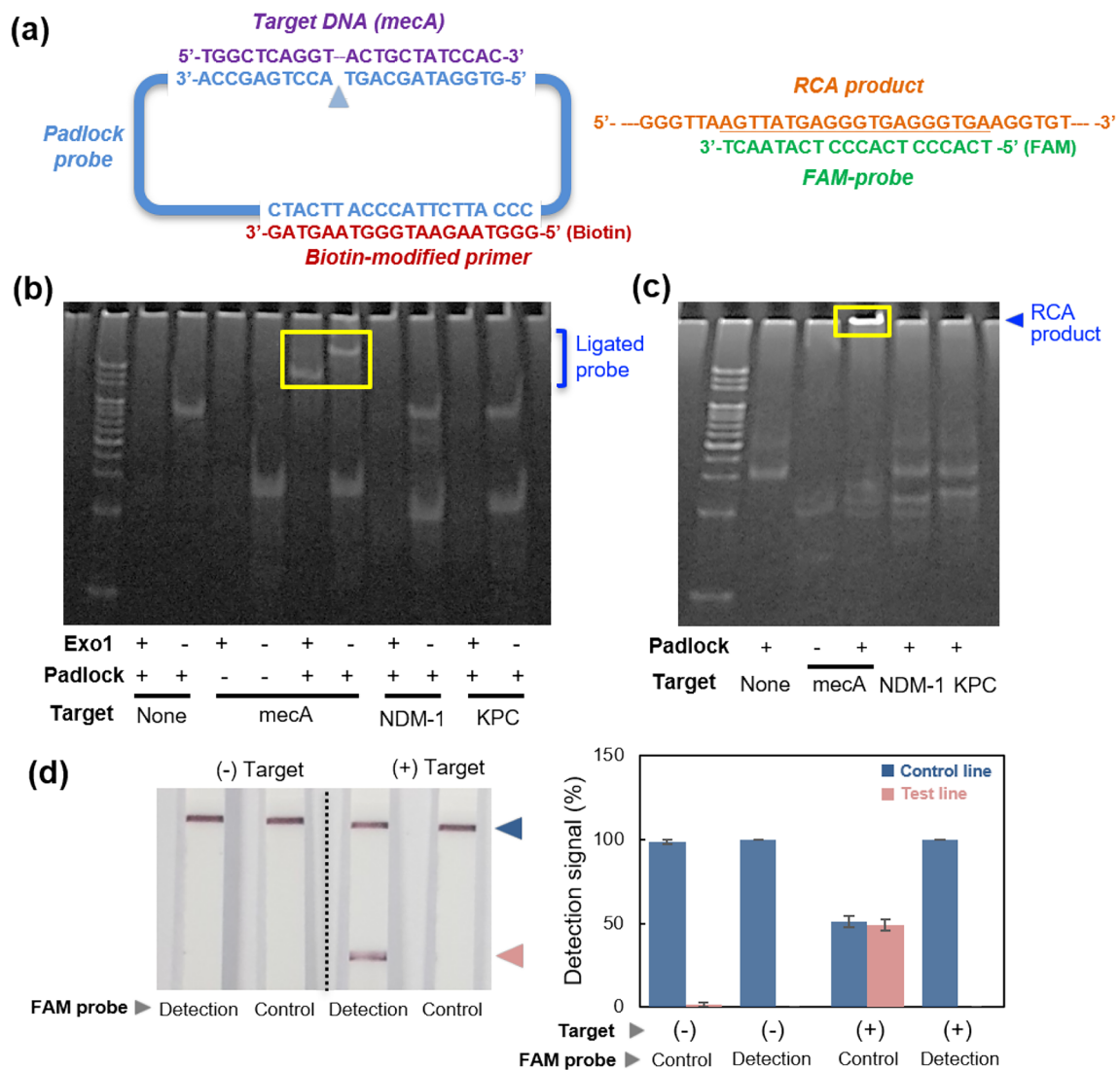
Fig. 2 Probe characterization and establishment of RCA-LFA. **a** Scheme including the aligned sequences of probes and target DNA for mecA. **b** Characterization of ligation. Padlock probe specific for mecA (16.7 μM) added with synthetic target DNA (3.33 μM) and treated with Exo1. **c** RCA product after reaction using Phi29 poly-merase and biotin-primer. **d** RCA-LFA performed by adding FAM-probe (100 nM) to RCA product (10 nM biotin-primer, 16.7 nM target DNA). Control FAM-probe used for comparison. Blue and pink arrowheads mark the control line and test line, respectively

the test line immobilized with streptavidin, and appears as a red signal which can be directly visualized. To optimize the RCA condition for LFA, various concentrations of biotin-modified RCA primer (biotin-primer) was used and LFA was performed. Figure 3a shows that when RCA-LFA was performed using a higher concentration of biotin-primer, the detection signal on the test line of the LFA strip appeared to increase, while the signal on the control line decreased. That is, when the concentration of the biotin-primer was 2 nM, 10 nM, and 50 nM, the detection signals were 30%, 47%, and 58% (vs No target as control), respectively, or 42% to 139% (vs signal for Control line). It was noticed that capture of the target DNA/FAM-probe/AuNP-aFAM complex onto

the test line resulted in reduced binding of the AuNP-aFAM to the control line (71, 53, and 42% for 2 nM, 10 nM, and 50 nM, respectively), which should be due to the limited availability of AuNP-aFAM. However, increasing the biotin-primer concentration to 50 nM also resulted in a significant detection signal for the No target control. This result demonstrates that increasing the biotin-primer amount to some extent leads to improved detection sensitivity, however, an excessive amount of primer results in false-positive signals due to the non-specific binding of the amplified target DNA/FAM-probe/AuNP-aFAM complex on the control line [23]. Therefore, biotin-primer concentration of 10 nM was used as the optimal condition for further experiments.
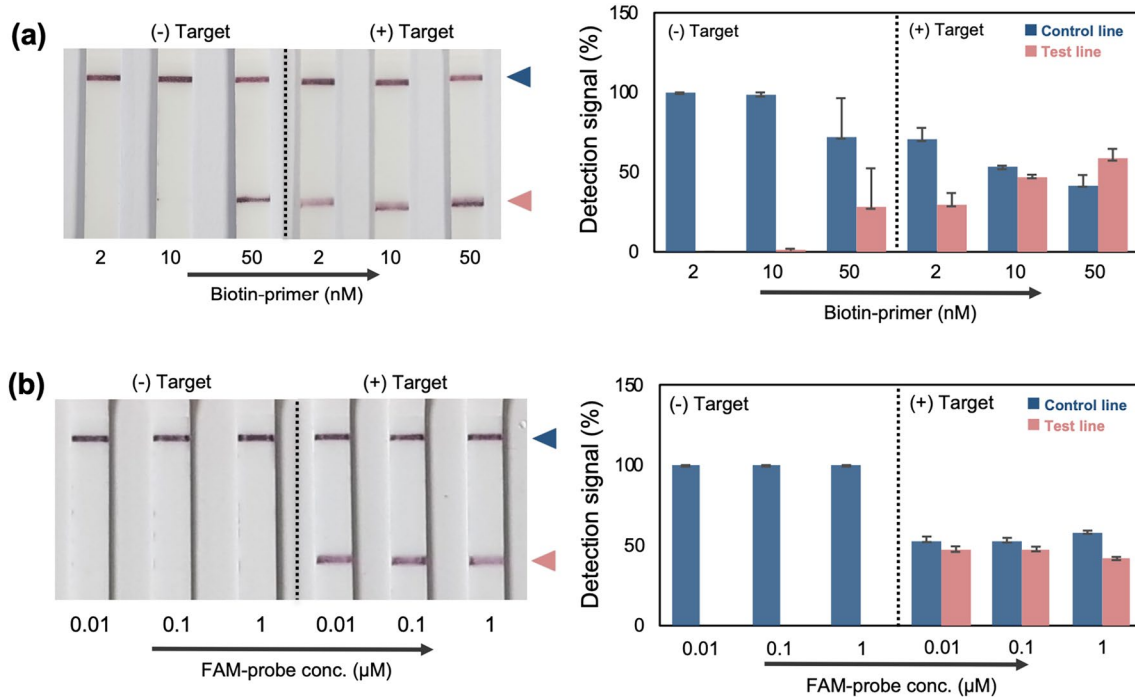
Fig. 3 Optimization of RCA-LFA. **a** Ligation and RCA was performed for mecA with various concentrations of biotin-primer, hybridized with FAM-probe (100 nM) followed by LFA. **b** Various FAM-probe concentrations used for hybridization with RCA product for mecA (10 nM biotin-primer), followed by LFA. Ligation performed with 8.33 nM padlock probe (mecA) and 16.7 nM mecA target DNA. Blue and pink arrowheads mark the control line and test line, respectively

Various concentrations of FAM-probe were used for hybridization following RCA, and LFA was attempted. When the RCA products amplified from mecA target DNA were hybridized with increasing concentrations (0.01 µM to 1 µM) of FAM-probe and flowed through the LFA strip, the detection signals on the test line appeared for all RCA products for mecA target DNA, but slightly decreased as the concentration was increased (47–42% vs (−)Target control, Fig. 3b). The signals for the control line also slightly increased at when the FAM-probe concentration was raised from 0.1 to 10 µM (53–58% vs (−)Target control). The decrease in detection signal at higher FAM-probe concentration can be due to the presence of unbound FAM-probes, which competitively binds to the AuNP-aFAM and reduces the formation of the target DNA/FAM-probe/AuNP-aFAM complex [24]. According to this result, the optimal concentration of the FAM-probe was determined to be 0.01 µM.

In addition, in order to compare the difference in the detection signal according to the concentration of the mecA padlock probe, the initial amount of the padlock probe was set in the range of 10–100 pmol (final concentration 1.67–16.7 nM), and ligation was performed. Then, RCA and LFA were performed to compare the detection signals. As a result, when the concentration of the padlock probe was 1.67 nM, the signal was 36%, whereas it was 32% at 8.33 nM and 33% at 16.7 nM, showing no significant difference

according to the padlock concentration (Fig. S1). Meanwhile, experiments were performed to optimize the ligation time and amplification time in order to confirm a sufficient time for detection. For determining ligation time, the reaction was performed for 15, 30, and 60 min at concentrations of 8.33 nM padlock probe and 16.7 nM mecA target. Then, RCA was performed for 30 min followed by LFA to compare the results. As the result, the detection signal was 37% at 15 min and ~40% at 30–60 min (Fig. S2). Since there was no significant difference between 30 and 60 min, ligation was performed for 30 min in further experiments for rapid detection. For optimizing amplification time, ligation was performed for 30 min, followed by RCA for 15, 30, and 60 min to compare the detection signals of LFA. The detection signal according to the amplification time was 33% at 15 min and 37% at 30—60 min (Fig. S3). There was no significant difference between 30 and 60 min, and a false-positive signal appeared for a sample without target DNA at 60 min, so we decided to proceed with a reaction of 30 min for rapid detection.

3.3 Sensitivity of RCA-LFA

The feasibility of the RCA-LFA method was validated by determining assay sensitivity. Various amounts of RCA products obtained by adding mecA target DNA with padlock

probe for ligation and RCA, were added with the FAM-probe for hybridization. LFA was then performed to obtain the colorimetric signals for detection. As shown in Fig. 4a, we could observe positive signals on the test line when adding equivalent target DNA amounts of 0.03 pmol or higher for the ligation-RCA reaction, while the control samples without target DNA did not generate any signal. The detection signals on the test line gradually increased from 16 to 48%, when the target DNA amount was increased from 0.03 to 1.7 pmol. On the other hand, signals on the control line decreased from 84 to 52% upon increasing the target DNA. This result is due to the generation of larger amounts of RCA product when increasing the amount of target DNA, which leads to higher efficiencies of target DNA/FAM-probe/AuNP-aFAM complex formation to be captured on the LFA strip for detection. The inverse proportional relationship between the signals for the test line and control line show good correlation with the results in Figs. 2 and 3. However, since the amount of biotin ligand varies according the amount of RCA product applied to the LFA process, the assay conditions might be discrepant from diagnosing real samples, and not optimal to achieve maximum sensitivity.

To represent sample solutions with various target amounts, the initial amount of mecA target DNA was varied prior to addition of the padlock probe for ligation, followed by RCA and hybridization with the FAM-probe. Figure 4b shows that increasing the initial target DNA amount from 0.8 to 167 fmol results in gradually increased signals on the test line up to 43%, while target amounts above 167 fmol does not further increase, but exhibits saturation or slight decreases in the signals (33–43%). This can be caused by the limited availability of the biotin-primer. Another possible mechanism can be due to the larger sizes of RCA products formed by extremely high amounts of target DNA, which are entangled and difficult to flow through the membrane [37]. The signals for the control lines gradually decreased to 57% upon increasing target amounts to 167 fmol, and plateaued or slightly increased at higher target amounts (57–66%). By visualization, signals gradually appeared between 1.3 and 5 pmol. According to the results from quantification based on image processing, the limit of detection was also determined to be 1.3 fmol. The first-order increase in signals from 1.3 to 167 fmol demonstrate that sample solutions including target DNA at this range, which is a range of 3 orders of magnitude, can also be quantified by the RCA-LFA method with the assistance of image processing of the results. The results demonstrate the broad detection range of the assay, which can be further improved based on optimization by fine tuning of the primer and probe concentrations, and incubation time. The result also reveals the relatively high sensitivity of the current technology compared to other point-of-care methods, including LFA and other colorimetric methods which did not require any instrumentation for detection.
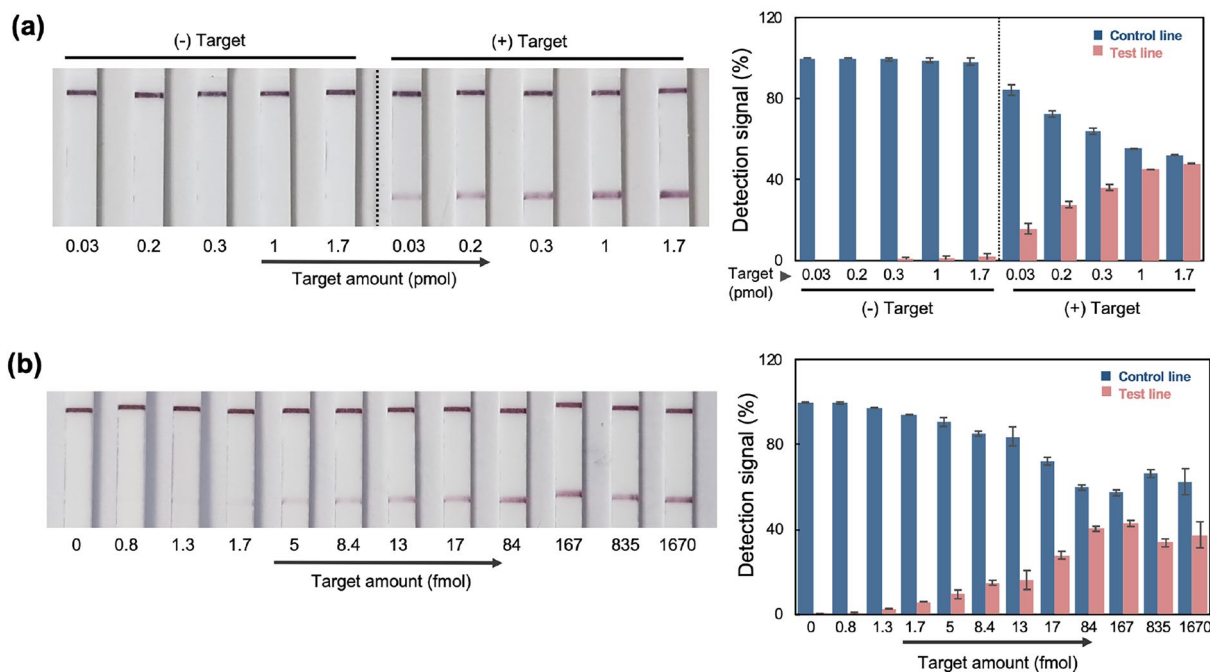

Fig. 4 Sensitivity of RCA-LFA. **a** Various equivalent amounts of target DNA (mecA) which were amplified by RCA and added with FAM-probe, followed by LFA. **b** Various initial amounts of mecA target DNA was used for ligation before RCA, followed by LFA. Ligation performed with 8.33 nM padlock probe (mecA), RCA with 10 nM biotin-primer, and hybridization with 100 nM FAM-probe

3.4 Specificity of RCA-LFA

The specificity of the RCA-LFA method to discriminate different target DNAs was evaluated. The specificity of the assay is majorly determined by the target-specific ligation of the padlock probes, which can be individually designed for any target sequence in versatile fashion. Only the padlock probe in circularized form can carry out amplification by RCA, and lead to a positive signal by LFA. In the case of adding a single target DNA, we could observe positive signals on the test line (44%) and decreased signals on the control line (56%) in the LFA membrane only when the mecA target DNA was added during the ligation reaction, while the addition of control DNAs for NDM-1 and KPC did not generate any signal test line (Fig. 5a and b). When adding combinations of two DNAs, that is the mecA target DNA with control DNAs NDM-1 or KPC, detection signals of 47% and 48%, respectively, were observed on the test line, while decreased signals to 53% and 52%, respectively, were observed on the control line of the LFA strip. The signals on the test line resembled the value for single addition of mecA target DNA, which provides supporting evidence that the signals were specifically produced only by the mecA target DNA and not by the control DNAs. A combination of all three DNAs, which are for mecA, NDM-1, and KPC, also resulted in a positive LFA signal of 46% on the test line, and reduced signal on the control line (54%), which also confirms the specificity of the assay. The ability for the RCA-LFA method to detect target DNA when mixed as combinations with control DNAs demonstrates the potential
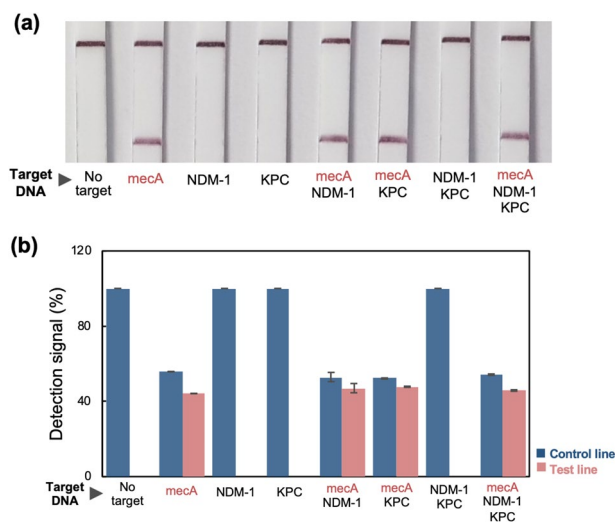
for diagnosing complex specimens which can involve polymicrobial infections or include both pathogenic and non-pathogenic bacteria [3].

3.5 Applicability of RCA-LFA for Other Targets

We also attempted to apply the RCA-LFA technique to detect nucleic acid for targets other than bacteria. We selected specific regions in the RdRp gene and RBD within the S gene, from the viral genome of SARS-CoV-2, and designed specific padlock probes for each of the targets (Fig. 6a). We carried out the ligation and RCA reactions, followed by hybridization and LFA and acquired the signals by observation as well as image processing. As shown in Fig. 6b, the addition of RdRp target DNA could generate a positive LFA signal (49%) on the test line and a decreased signal on the control line (51%). No addition of target DNA or addition of mecA target DNA as the negative controls did not induce significant signals. This result confirms the specificity of the assay for RdRp, which can be contributed to the design of the padlock probe specific for RdRp and its function on inducing target-dependent ligation. In the case of the RBD target, Fig. 6c shows that only addition of RBD target DNA to the padlock probe specific for RBD results in positive LFA signals on the test line (55%), and decrease signal on the control line (44%), while no signals were observed for the No target and mecA target DNA as the negative controls. These results demonstrate that the RCA-LFA method can be universally applied to nucleic acids from a wide range of targets, including both bacterial and viral pathogens. Since the principle of RCA-LFA is based on genetic testing determined by specificity from design of padlock probes specified by nucleotide sequence, the technique can be potentially applied for an extensive range of targets which involve genetic markers, and when the nucleic acid can be obtained from the sample.

4 Conclusions

Nucleic acid detection has been widely utilized for disease diagnosis due to the versatility for different targets and high accuracy. For diagnosis at point-of-care or home settings, a simple assay platform is needed, which can be miniaturized and requires minimal technical components. Herein, we developed the RCA-LFA method based on a modified RCA procedure using capture oligonucleotides (biotin-primer), followed by LFA. Specific padlock probes are designed which undergoes target-specific ligation and allows isothermal amplification of the target to produce biotin-modified long, single-strand DNA. After hybridization of detection oligonucleotides (FAM-probe), the

Fig. 5 Selectivity of RCA-LFA. Assay performed with padlock probe (mecA) and samples including different synthetic target DNAs. NDM-1 and KPC were used as the negative control (16.7 nM target DNA, 8.33 nM padlock probe, 10 nM biotin-primer, 100 nM FAM-probe)

Fig. 6 Applicability of the RCA-LFA for viral targets (SARS-CoV-2). Assay performed with synthetic target DNA (16.7 nM) for **a** RdRp, and **b** RBD (8.33 nM padlock probe for RdRp or RBD, 10 nM biotin-primer, 100 nM FAM-probe)
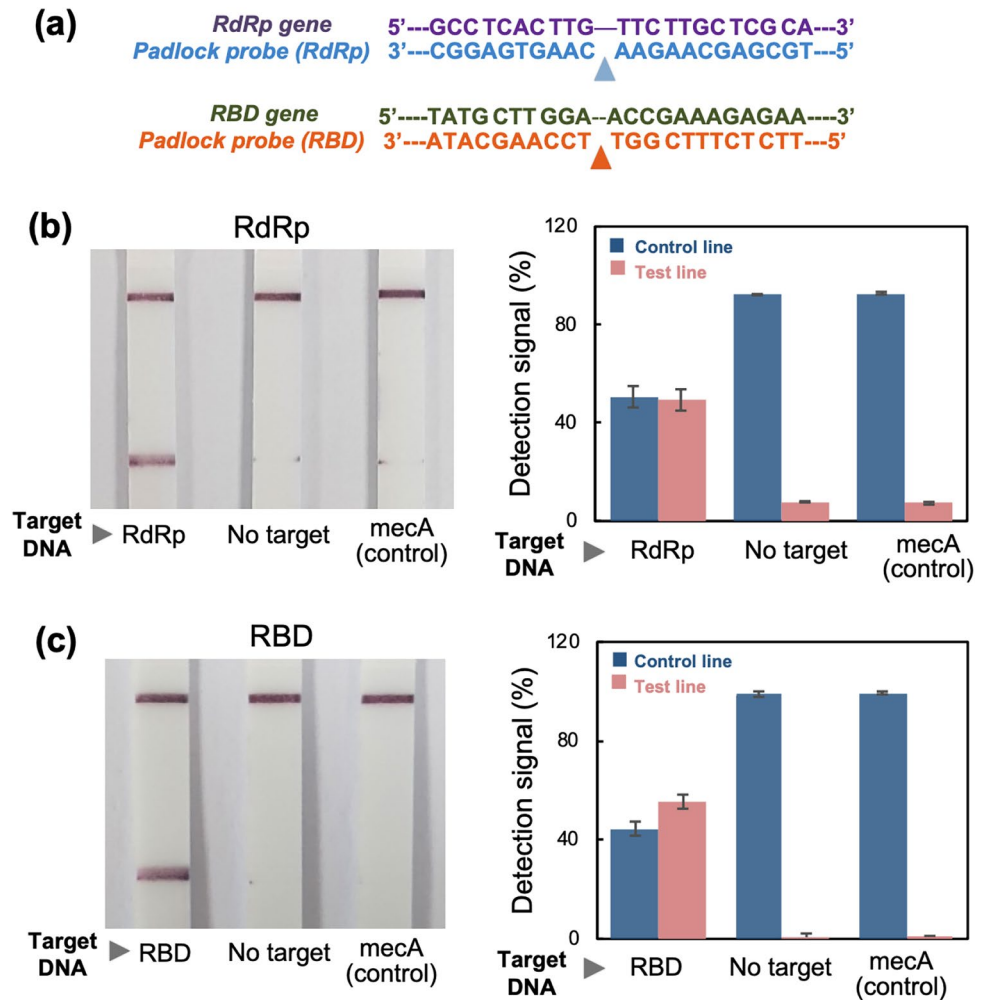
amplified target DNA is captured on the LFA membrane while it binds to AuNP-aFAM, producing a colorimetric signal. The assay is able to specifically detect target DNA for the mecA gene, with high sensitivity showing a detection limit of 1.3 fmol. The versatility of the RCA-LFA method is demonstrated by examining feasibility of the assay for viral target genes from SARS-CoV-2. The integration of isothermal nucleic acid amplification with LFA brings out the possibility of applying genetic testing to affordable, point-of-care systems, and can overcome the current limitations of conventional methods, such as the high instrumental cost of qPCR or low accuracy of immunoassay-based diagnostic kits.

Declarations

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