Multiplex Detection of Nucleic Acids Using Recombinase Polymerase Amplification and a Molecular Colorimetric 7-Segment Display

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1. INTRODUCTION

Nucleic acid testing is a critical tool in diagnostics, particularly for enhancing microbial detection using sensitive, specific, and rapid methodologies.1 The advent of isothermal nucleic acid amplification methods has enabled testing highly amenable for point-of-care (POC) applications. In particular, recombinase polymerase amplification (RPA) is considered as one of the most promising candidates for POC applications, as it uses low incubation temperatures (37−42 °C), to detect as little as a single copy of nucleic acid, in less than 10 min of reaction time.2 The combination of RPA with lateral flow detection offers a rapid and simple solution for field-amenable low-resource nucleic acid testing. Expanding POC nucleic acid tests for the detection of multiple analytes is vital to improve diagnostic efficiency because increased multiplexing capacity enables higher information density combined with reduced assay time and costs. Here, we investigate expanding RPA POC detection by identifying a generic multiplex RPA format that can be combined with a generic multiplex lateral flow device (LFD) to enable binary and molecular encoding for the compaction of diagnostic data. This new technology relies on the incorporation of molecular labels to differentiate nucleic acid species spatially on a lateral flow membrane. In particular, we identified additional five molecular labels that can be incorporated during the RPA reaction for subsequent coupling with LFD detection. Combined with two previously demonstrated successful labels, we demonstrate potential to enable hepta-plex detection of RPA reactions coupled to multiplex LFD detection. When this hepta-plex detection is combined with binary and molecular encoding, an intuitive 7-segment output display can be produced. We note that in all experiments, we used an identical DNA template, except for the 5′ label on the forward primer, to eliminate any effects of nucleic acid sequence amplification bias. Our proof-of-concept technology demonstration is highly relevant for developing information-compact POC diagnostics where space and time are premium commodities.

1.1 Limitations of traditional LFDs for POC use are the restriction to single-plex detection. Expanding to multiplex POC detection is vital for (i) efficient infectious disease diagnosis, given the increasing number of biomarkers discovered; (ii) reducing false positive or negative reporting in cases where a single biomarker may be indicative of more than one disease;3 and (iii) reducing diagnostic time and costs compared to performing multiple single tests. Previously reported multiplex LFDs operate by increasing the number of lines or dots incorporated within one device, with each one representing the detection of a specific analyte.4 Indeed, this strategy was recently combined with RPA for the detection of up to three bacterial parasites.5 However, with any multiplex LFD, there is a physical limit to detection capacity expansion because the flow rate decreases with distance from the conjugate pad and interpretation becomes difficult as the number of lines or dots accumulates.

To circumvent multiplex LFD expansion limitations, we recently reported a novel LFD technology that combines binary and molecular encoding to increase multiplex detection capacity without expanding device dimensions. The binary
Figure 1. Lateral flow sandwich assay of single-plex RPA amplicons. (a) During the RPA reaction, (i) the 5′-labeled primer, reverse primer, and the 5′ FAM-labeled TwistAmp LF probe bind to DNA and (ii) the 3′ block (also known as blocker, e.g., C3-spacer) on the probe is removed by the enzyme Nfo, allowing (iii) extension by the Bst polymerase to create (iv) a dual-labeled double-stranded amplicon. Adapted from Li and Macdonald.24 

Previously, Crannell and colleagues reported a failure of RPA in combination with binary LFD detection. We used RPA to couple with multiplex LFD detection strategy and demonstrate that primers containing up to seven different 5′ molecular labels can be successfully incorporated during a single-tube RPA reaction (with a 5′ labeled probe). We verify single-plex RPA reactions using these seven different molecular tags, followed by both single-plex and multiplex LFD detection. We then demonstrate that all seven molecular labels can be combined in a multiplex RPA reaction coupled with multiplex LFD detection. Finally, we describe the coupling of this multiplex RPA reaction with our compact multiplex 7-segment display LFD, indicating that the combination of RPA with binary and molecular encoding can enable an intuitive LFD result display highly amenable for POC operation.

2. RESULTS AND DISCUSSION

2.1. Single-Plex RPA Combined with Single-Plex Lateral Flow Detection. In this study, we aimed to demonstrate that RPA could be combined with multiplexed LFD detection via incorporation of multiple 5′-labeled primers during the RPA reaction, followed by sandwich assay LFD detection. We used RPA to combine a varying 5′ molecular label on the forward primer, with a 5′ FAM-labeled probe, to
obtain dual-labeled double-stranded amplicons (Figure 1a). The S’-labeled primer, reverse primer, and the S’ FAM-labeled probe (TwistAmp LF probe; the 3’ blocker prevents DNA extension) bind specifically to complementary sequences in a DNA template. Once the probe binds, the enzyme (Nfo) detects the abasic residue (dSpacer) in the probe. Subsequently, Nfo chops off the remaining segment behind it, thereby removing the 3’ blocker and simultaneously producing a 3’ hydroxide group (OH) that can be elongated. The DNA polymerase elongates from 3’ ends of the primers and probe and replaces the downstream sequences. After several rounds of reactions, dual-labeled double-stranded amplicons are generated for subsequent LFD detection.25 LFD sandwich detection was achieved by depositing anti-label detection antibodies in the test zone of the nitrocellulose membrane and by using gold nanoparticles (AuNPs) conjugated to an anti-FAM capture antibody to transform the sandwich complex (capture antibody/AuNPs/nucleic acid/detection antibody) into a colorimetric signal (Figure 1b). We chose to incorporate the S’ labels into the forward primer based on our previous successful demonstration of multiplex LFD detection using synthetic dual-labeled single-stranded DNA.7 The analytical sensitivity and specificity of the multiplex LFD had previously been reported in the absence of nucleic acid amplification; this study thus assessed the ability of the system to be combined with a prior nucleic acid amplification step. We targeted the Rift Valley fever virus S (RVFV S) gene as a proof-of-concept DNA template for RPA amplification and LFD detection because it had previously been demonstrated effective for real-time RPA detection.26 Importantly, we kept the RVFV S DNA analyte sequence identical in all tests (apart from the incorporated S’ forward primer molecular labels) to eliminate any differences in behavior because of subtle changes in nucleotide sequences.

We first tested RPA incorporation of each S’ molecular label in separate RPA reactions, followed by single-plex LFD detection. Each RPA reaction contained a different S’-labeled forward primer (either biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, DNP-X C6-NH, or Dansyl-X C6-NH). Incorporation of the labeled primer was demonstrated, as observed by the appearance of a colored test dot when the DNA template was supplied into the RPA reaction (Figure 1c). No background amplification was observed in the absence of a DNA template, indicating that the reactions were DNA template-specific (Figure 1c).

Next, we hypothesized that the varying S’ molecular labels may affect the analytical sensitivity [also known as limit of detection (LOD)] of the RPA-LFD assay. To address this hypothesis, we measured the LOD for all seven S’ molecular labels using 10-fold serial dilutions of the RVFV S gene target DNA and performed digital analysis (Figure 1d, digoxigenin; Figure S1, Dansyl, DNP, TAMRA, Cascade Blue, Texas Red, biotin). Incorporating digoxigenin, Dansyl, and TAMRA as S’ molecular labels resulted in a LOD of S1 synthetic gene copies per reaction, whereas biotin showed a LOD of S11 synthetic gene copies per reaction. LOD for these four labels is similar to previously reported LOD using digoxigenin, Alexa-Fluor 488, fluorescein, and biotin.6 Notably, LODs from primers labeled with Cascade Blue (5.11 × 10^10 copies/μL), DNP (5.11 × 10^10 copies/μL), and Texas Red (5.11 × 10^10 copies/μL) were 10–1000× higher, suggesting that LODs for different S’ molecular labels significantly vary. It is likely that the detection antibodies have a significant impact on the LOD, suggesting that further optimization is required to diminish the impact of different antigen (S’ molecular labels) and detection antibody combinations within an assay.

This is the first report of successful RPA incorporation of primers containing TAMRA, Texas Red, Cascade Blue, DNP, and Dansyl (biotin and digoxigenin have previously been described3), with TAMRA and Dansyl demonstrating particularly high LODs when coupled with LFD. The successful incorporation of seven labels during RPA-LFD detection suggests that RPA generally tolerates S’ label incorporation, despite a previous report that five different S’ labels (Cy5, Cy3, bromodeoxyuridine, tetrachlorofluorescein, and hexachloro-fluorescein)26 were not successfully detected using a RPA-LFD detection. We previously demonstrated, using dual-labeled single-stranded DNA, that Cy5 is not well tolerated for LFD detection because of low sensitivity and thus similarly excluded it from our present study. Additionally, while a different S’ molecular label, Alexa-Fluor 488, has been shown to be successfully incorporated during RPA-LFD,6 we previously demonstrated that the Alexa-488 molecular label cross-reacts with an anti-Texas Red antibody,7 so we also excluded this S’ molecular label from the present study.

2.2. Specificity Test of the Seven Dual-Labeled RPA Amplicons on a Multiplexed Lateral Flow Detection. In order to achieve multiplexed lateral flow detection using these RPA dual-labeled DNAs, we performed a specificity test by running each dual-labeled RPA amplicon on a LFD that was predesigned with all seven corresponding antibodies. The results showed that all the seven RPA dual-labeled amplicons only produced a colored test dot at the position corresponding to the antibody specific to their S’ forward primer molecular label (Figure 2a,b), indicating specific detection in each case.

This is consistent with our previous report that single-stranded dual-labeled DNA containing these same labels was specific only to their corresponding antibodies.

2.3. Seven Single-Plex RPA Reactions Combined with Multiplexed Lateral Flow Detection. Because all the seven RPA dual-labeled amplicons were specific only to their
corresponding anti-label antibodies, we combined these amplicons as a mixture (with running buffer) and confirmed the capacity of the multiplex LFD to display all seven test dots in a single assay. Results indicated that all the seven RPA dual-labeled amplicons can be detected simultaneously on a single LFD (Figure 3a, right). However, we found that extra volumes of S′ FAM/S′ biotin and S′ FAM/S′ Dansyl-X C6−NH (3.0 μL instead of 2.0 μL for the other RPA dual-labeled amplicons) in the sample mixture were required to achieve approximately equal test dot intensities for all seven antigen−antibody detections with multiplexed LFD detection (Figure S2b,c). It is likely that the binding affinities of biotin to anti-biotin and Dansyl-X C6−NH to anti-dansyl were weaker in comparison to the other antigen−antibody combinations, as suggested by the intensity of the test dot during the single-plex LFD detection results (Figure 1c).

Next, we combined the RPA dual-labeled amplicons as mixtures to display numbers on our previously described 7-segment display LFDs. Numbers 0−9 were clearly visible when the defined RPA dual-labeled amplicon mixture was applied (Figure 3e). Alphanumeric solution-phase displays detecting synthetic oligonucleotides have previously been reported as proof-of-concept demonstrations for identifying filoviruses and lyssaviruses and also Mycobacterium tuberculosis drug susceptibility. Here, we demonstrate that a solid-phase alphanumerical display can be linked to a simple isothermal amplification method, indicating potential for the amplification of pathogen nucleic acid signatures to improve detection on such displays, in a more portable format suitable for use in low-resource situations. Interestingly, the number display represents an unbiased test of different label combinations toward a specific end goal (the display of a numeral). Our seven different labels have the potential to be uniquely combined to produce 127 (27 − 1) different display patterns using defined combination of RPA dual-labeled amplicons. However, testing each combination would be an exhaustive and costly exercise. By applying these combinations to produce a 7-segment display, we sample and demonstrate success for a smaller test set of ten different combinations. Such sampling testing strategies are common in systems with complex decision trees such as integrated circuits, and formal sampling strategies will need to be adopted for LFD detection as system complexity increases.

Multiple parallel RPA reactions coupled with multiplexed detection have been demonstrated previously on a solid phase in a microarray format. The highest previous multiplexing was sixteen parallel RPA reactions (as four analytes in quadruplex replicates) on a single solid phase, followed by detection using specific equipment (e.g., microarray scanner). In comparison to these, our research demonstrates the potential for hepta-plex detection using an amplicon mixture that contained up to seven different parallel RPA reactions on a self-operated device with direct result interpretation by the end user. Notably, although we used the same DNA template for all the parallel RPA reactions, our detection technology is generic or DNA template independent, which means that it can be applied for amplifying seven different DNA templates. Importantly, using the same DNA template for all RPA reactions, followed by multiplex LFD, combined with our previous study on antigen−antibody affinities for the same template, allowed us to investigate the impact of hybridization rates of chosen antigen−antibody pairs, which critically influence the multiplex LOD. Our results indicate that future multiplex assay design for seven different DNA templates must take not only variances in primer RPA incorporation into account but also hybridization rates during incorporation into multiplex LFD.

2.4. Multiplex RPA Reactions Combined with Multiplexed Lateral Flow Detection. Successful operation of the single-plex RPA combined with multiplex LFD encouraged us to trial multiplex RPA detection by combining forward primers labeled with different tags in a single tube (with reverse primer, probe, and other RPA reagents) (Figure 4a−c). This multiplexing RPA is notably different from that of Crannell and colleagues, who performed triplex detection of bacterial parasites using three different DNA templates. In our proof-of-concept multiplex RPA demonstration, we continued to use an identical DNA template while adding different combinations of labeled primers. This allowed us to eliminate any binding bias toward the DNA template among the labeled primers during the RPA reaction to fully determine the specific competitive effect of the labels themselves and assess the impact of...
Multiplexing RPA could theoretically detect 127 (27 combinations in the RPA reaction (a form of barcoding), our display), providing exceptional con-

detect the dual-labeled amplicons. We note that the antibody deposition (of the test dots) in our research was performed by hand pipetting, and thus, the quality of the test dots was not smooth and evenly distributed, which led to the appearance of comet tails for some of the test dots. To reduce these artifacts, we applied ethanol precipitation prior to applying amplicons to the LFD (although RPA amplicons can be directly applied to LFD), which removes proteins and crowding agents that affect wicking performance and alleviate “ghost band” effects.21 Although these issues limit portability and usability, they can be solved by the application of advanced deposition techniques, such as inkjet printing.16 Inkjet printing sprays anti-label antibodies in a series of microdots rather than depositing a single large drop by hand (e.g., the Symbolics technology7a~7e), so that the anti-label antibodies distribute evenly in an optimized manner on the matrix. In this case, the microdots prevent perturbation of the flow of liquid and conjugate as it traverses the device, enabling clarity of text and improving performance and portability (after judicious optimization of parameters such as reagent concentration, reagent on-rates, drop size, drop spacing, surface tension, and viscosity16).

The successful results also demonstrated that the forward primers labeled with different tags (except for the biotin tag) showed approximately equal incorporation efficiencies into the DNA template (Figure 4d). However, we note that a double concentration of the S’ biotin-labeled forward primer was required in comparison to the other labeled forward primers in order to form dual-labeled amplicons that could be visualized on the multiplexed lateral flow detection. Any further increases in the concentration of the S’ biotin-labeled primer interfered with the binding efficiency of the S’ Dansyl-X C6-NH-labeled primer to the DNA template and reduced test dot intensity of Dansyl label pair on the multiplex display of number 8 (Figure S3b). In addition, the position of the antibodies itself on the multiplex strip impacts the test dot intensity when detecting differently labeled RPA amplicons. Note that antigen (S’ molecular label) and detection antibody combinations with higher LOD (Figures 2c and S1) were placed closer to the conjugate/sample pads to enhance the AuNP conjugate/capture antibody efficiency. We ensured that the multiplex RPA assay can detect the RVFV S gene target DNA at predetermined concentrations by performing digital analysis (Figure S4b).

General multiplex nucleic acid amplification coupled with multiplexed LFD detection has been demonstrated previously; however, the majority employ the nonsothermal amplification method – polymerase chain reaction (PCR).3a~3c,18 only a few employ the isothermal amplification methods [e.g., RPA3a,10,15g~i and loop-mediated isothermal amplification (LAMP)].19 The highest multiplexing in these systems was hepta-plex detection of six human papillomavirus and a human β-globin gene using PCR amplicons.23 However, a post-probe detection of multiple DNA templates would require judicial bioinformatics selection and experimental testing of primer and probe combinations because interactions between primers, probe, and multiple DNA targets could lead to nonspecific amplification products. Additionally, variation in amplification efficiency must be considered when different DNA templates are amplified with their corresponding primers and probe. The optimization of primers and probe ratios will be crucial to design a highly sensitive multiplex RPA-LFD. Further, special care must be taken to identify a suitable high-affinity antibody to
hybridization was required for the subsequent LFD detection, and a fluorescent reader is required for signal measurement.\textsuperscript{20} Our detection technology employs RPA and shows feasibility of DNA labeling using 5’-labeled primers and probe, which is much faster (35 min in comparison to 1 h PCR) and negates the need for a 30 min probe hybridization before the LFD detection. Also, our signals are colorimetric (not fluorescent) and can be inspected by eye without external readers. Moreover, in comparison to PCR and LAMP, using RPA to combine with LFD for POC diagnostics has several advantages: (1) no requirement to predenature the DNA template (LAMP can be performed with the non-denatured DNA template; however, the reaction efficiency decreases and reaction time increases\textsuperscript{20}); (2) low reaction temperature (PCR requires rising temperature to 95 °C; LAMP performs between 60 and 65 °C); (3) highest amplification efficiency (10\textsuperscript{6}-fold in 10 min);\textsuperscript{24} PCR amplifies to 10\textsuperscript{9}-fold in about 2 h; LAMP achieves 10\textsuperscript{9}-fold amplification in approximately 1 h\textsuperscript{21}; (4) commercially available lyophilized reaction pellets\textsuperscript{22} with a shelf-life of several months stored at −room temperature for days without loss of activity) and (5) much faster (35 min in comparison to 1 h PCR) and negates the need for predenaturation of DNA template, which was used to rehydrate the lyophilized reaction pellets. The DNA template was added to the resulting mixture, which was used to rehydrate the lyophilized reaction pellets with a shelf-life of several months stored at −20 °C (can tolerate room temperature for days without loss of activity) and (5) commercially available portable devices for field use.

3. CONCLUSIONS

We have developed a potentially field-deployable nucleic acid assay by combining an RPA reaction with multiplexed lateral flow detection. Our research demonstrated potential for the highest multiplexing of an RPA reaction (hepta-plexing) to date. In addition, we demonstrated the first intuitive LFD result display to be coupled with any nucleic acid amplification reactions. Our results showed successful incorporation of seven 5’-labeled primers (containing either biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, DNP-X C6-NH, or Dansyl-X C6-NH, together with a 5’ FAM-labeled probe) to the DNA template during RPA, followed by LFD detection. In particular, the tags TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, DNP-X C6-NH, and Dansyl-X C6-NH have not been used in RPA DNA labeling before. The combination of RPA with lateral flow detection can be performed in less than an hour, which allowed a low quantity of DNA to be amplified and labeled and produced a visual signal on the LFD based on a sandwich assay. Our intuitive result display is highly relevant for POC applications because it provides easy result interpretation compared to detecting multiple lines or dots and does not require special equipment for signal visualization.

4. EXPERIMENTAL SECTION

4.1. Oligonucleotides and Antibodies. DNA template, RPA primers (5’-labeled with biotin, digoxigenin (NHS ester), TAMRA (NHS ester), or Texas Red-X (NHS ester)), and probe were synthesized and high-performance liquid chromatography (HPLC) purified by Integrated DNA Technologies, Inc (IDT, Coralville, USA). The RPA primer 5’-labeled with Cascade Blue C6-NH was synthesized and HPLC purified by Invitrogen (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia). The RPA primers 5’-labeled with dinitrophenyl (DNP)-X C6-NH or Dansyl-X C6-NH were synthesized by TriLink BioTechnologies (TriLink BioTechnologies, San Diego, USA) and PAGE purified.

A monoclonal anti-fluorescein antibody (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia) was used for AuNP conjugation. Antibodies corresponding to the 5’-labeled DNA were as follows: (i) anti-biotin (mouse) monoclonal antibody (Rockland Immunochemicals Inc., Limerick, PA, USA); (ii) polyclonal anti-digoxigenin antibody (Rock Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia); (iii) monoclonal anti-TAMRA antibody (Thermo Fisher Scientific, Scoresby, VIC, Australia); (iv) monoclonal anti-Texas Red antibody (Invitrogen Corporation, Carlsbad, CA, USA); (v) polyclonal anti-Alexa Fluor 405/Cascade Blue antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); (vi) polyclonal anti-dinitrophenyl-KLH antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia); and (vii) polyclonal anti-Dansyl antibody (Life Technologies Australia Pty Ltd., Mulgrave, VIC, Australia). These antibodies were deposited at the test zone of the LFD. In addition, a polyclonal rabbit anti-mouse antibody (Sapphire Bioscience Pty. Ltd., Waterloo, NSW, Australia) was deposited at the control zone.

4.2. RPA Amplification. RPA amplification was performed using synthetic double-stranded gBlock DNA encoding a segment of the RVFV S gene (s segment) (Genbank accession number NC_014395.1, nucleotides 1428–1535),\textsuperscript{9} a pair of forward [5’-labeled with either biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, DNP-X C6-NH, or Dansyl-X C6-NH] and reverse primers, and a Nfo probe [5’-labeled with 6-carboxyfluorescein (FAM), containing an internal abasic residue known as dSpacer replacing a base, and a 3’ C3-Spacer carbon blocker]. The following sequences were used: RVFV S forward, 5’-X-CAT TTT CAT CAT CAT CCT CCK GGG TTG RTT G-3’ (X varying 5’-molecular label: biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, DNP-X C6-NH, or Dansyl-X C6-NH); RVFV S reverse, 5’-GAR CTC YTA AAG CAG TAT GGT GGG GCT GAC T-3’; RVFV-S Nfo probe, 5’-FAM-GGG AGA AGG ATG CCA AGA AAA TGA TGG TT (dSpacer) TGG CTC TRA CTC GTG (C3-Spacer)-3’; RVFV-DNA, 5’-GCT TTG CCT TCT TGC ATT TTO ATC ATC ATC ATC ACT CTC CTT GGG TTG TTG CGG CCA CGT AGA GCC AGA ACA ATC ATT TTT TTG GCA TCC TTA TCC CAG TCA GGC CCA CCA TAC TGC TTT TAT AGT TCG ATC ACT GTA CGG GCA TCA AAC CC-3’.

4.2.1. Single-Plex RPA Amplification. The RPA reaction was conducted at 39 °C for 35 min using the TwistAmp nfo kit (TwistDx Ltd., UK). Primers (420 nM), probe (120 nM), rehydration buffer (29.5 μL), magnesium acetate (14 mM), and DNA template (2.5 μL, 0.5 mM) were combined in a 50 μL reaction volume. All the reagents, except for the DNA template and magnesium acetate, were prepared in a master mix, which was used to rehydrate the lyophilized reaction pellets. The DNA template was added to the resulting mixture, and magnesium acetate was pipetted into the cap of each tube and centrifuged down to initiate amplification. Subsequent RPA amplicons were purified by the addition of 100% ice-cold ethanol and incubation for 20 min on ice before centrifugation of precipitated nucleic acids (13 500g, 8 min), followed by a 70% ice-cold ethanol wash and recentrifugation (13 500g, 8 min). Pellets were resuspended in TE buffer (Tris 10 mM, EDTA 0.1 mM, pH 8.0) to the original RPA volume of 50 μL.

4.2.2. Multiplex RPA Amplification. The RPA reaction was conducted at 39 °C for 35 min using the TwistAmp nfo kit (TwistDx Ltd., UK). For numerical displays (Table 1), the 50 μL reaction volume contained either (i) for numbers “0”, “2”,...
**Table 1. Forward Primer 5’ Molecular Labels for Each Multiplex RPA Numerical Display**

| number | tags labeled at 5’ of forward primers |
|--------|---------------------------------------|
| 0      | biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), dinitrophenyl-X C6-NH, and Dansyl-X C6-NH |
| 1      | biotin and TAMRA (NHS ester) |
| 2      | digoxigenin (NHS ester), TAMRA (NHS ester), Cascade Blue C6-NH, dinitrophenyl-X C6-NH, and Dansyl-X C6-NH |
| 3      | biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Cascade Blue C6-NH, and Dansyl-X C6-NH |
| 4      | biotin, TAMRA (NHS ester), Texas Red-X (NHS ester), and Cascade Blue C6-NH |
| 5      | biotin, TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, and Dansyl-X C6-NH |
| 6      | biotin, digoxigenin (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, dinitrophenyl-X C6-NH, and Dansyl-X C6-NH |
| 7      | biotin, digoxigenin (NHS ester), TAMRA (NHS ester) |
| 8      | biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, dinitrophenyl-X C6-NH, and Dansyl-X C6-NH |
| 9      | biotin, digoxigenin (NHS ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, and Dansyl-X C6-NH |

**4.4. Preparation of Single-Plex Lateral Flow and Multiplex Lateral Flow Devices.** Conjugate and sample pads (Millipore, Billerica, MA, USA) were blocked with blocking solution (1% polyvinyl alcohol, 20 mM Tris base, pH 7.4) for 30 min and dried at room temperature for 2 h. The two pads were soaked in borate running buffer for 30 min before drying at 25 °C for 6 h.

Assembled devices (6.1 cm × 0.3 cm) for single-plex LFDS comprised treated sample pad (1.5 cm), treated conjugate pad (0.6 cm), a nitrocellulose membrane (2.5 cm; Hi-Flow Plus HF135), and an absorbent pad (1.5 cm) (Millipore, Billerica, MA, USA) all combined on an adhesive backing card (KENOSHA c.v., Schweitzerlaan, Amstelveen, Netherlands), with a 0.1 cm overlap between components. The multiplex LFDS (7.1 cm × 0.5 cm) were assembled using the same components and procedure using the treated sample pad (0.5 cm), treated conjugate pad (0.6 cm), a nitrocellulose membrane (3.5 cm), and an absorbent pad (2.5 cm).

For single-plex LFD detection, antibodies (either anti-biotin (1.0 mg/mL), anti-digoxigenin (0.75 U/μL), anti-TAMRA (1.0 mg/mL), anti-Texas Red (1.0 mg/mL), anti-Alexa Fluor 405/Cascade Blue (3.0 mg/mL), anti-Dinitrophenyl-KLH (2.0 mg/mL), or anti-Dansyl (1.0 mg/mL)) were pipetted (0.4 μL) onto the test zone of the nitrocellulose membrane. Rabbit antimouse antibody (1 mg/mL in 50% glycerol) was pipetted (0.4 μL) at the control zone. Test and control antibodies were spotted 0.5 cm apart and dried at 25 °C for 45 min. For multiplex LFD detection, each detection antibody (0.2 μL) was deposited and, as a control, rabbit anti-mouse antibody was pipetted (0.2 μL) in triplicate at the end of each array.

**4.5. Single-Plex Lateral Flow and Multiplex Lateral Flow Device Test Procedures.** Single-plex LFD detection was performed as described previously but used purified RPA amplicons, except for analytical sensitivity assays which used unpurified RPA amplicons. Briefly, anti-fluorescein/AuNP conjugate (1 μL) was pipetted onto the conjugate pad, and the LFD was dipped into a mixture containing 100 μL of running buffer and 5 μL of RPA amplicon. An additional 1 μL of anti-fluorescein/AuNP conjugate was pipetted onto the conjugate pad once the borate running buffer reached the bottom of the absorbent pad as this double-run method has been demonstrated to be effective for developing high signal intensity with reduced anti-fluorescein/AuNP consumption. The LFD was developed for 15 min. All experiments were repeated at least three times to demonstrate the consistency of results.

For the single-plex RPA combined with multiplex LFD detection, purified RPA amplicons (2.0 μL, S’ FAM/S’ X, X = digoxigenin (NHS Ester), TAMRA (NHS ester), Texas Red-X (NHS ester), Cascade Blue C6-NH, or DNP-X C6-NH) and/or purified RPA amplicons (3.0 μL, S’ FAM/S’ X, X = biotin or Dansyl-X C6-NH) were mixed with 220 μL of running buffer. Briefly, anti-fluorescein/AuNP conjugate (2.5 μL) was pipetted onto the conjugate pad, and the LFD was dipped into the RPA amplicon and running buffer mixture. An additional 2.5 μL of anti-fluorescein/AuNP conjugate was pipetted onto the conjugate pad once the running buffer reached the bottom of the absorbent pad. The LFD was developed for 25 min. All experiments were repeated at least twice to demonstrate the consistency of results.

"3", "5", "6", "9", and "8": each forward primer (140 nM; except for biotin at 280 nM), reverse primer (840 nM), probe (240 nM), rehydration buffer (29.5 μL), magnesium acetate (14 mM), and DNA template (2.5 μL, 0.5 nM) or (ii) for numbers "1", "4", and "7": each forward primer (140 nM; except for biotin at 280 nM), reverse primer (420 nM), probe (120 nM), rehydration buffer (29.5 μL), magnesium acetate (14 mM), and DNA template (2.5 μL, 0.5 nM). Amplicons were again purified by ethanol precipitation as described for the single-plex reactions.

**4.2.3. Analytical Sensitivity of RPA Amplification.** The above-described RVFV S DNA template was quantitated using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Mulgrave, VIC, Australia). To evaluate the sensitivity of the RPA amplification assay, 10-fold serial dilutions of the RVFV S template DNA in nuclease free water were tested. The RPA reaction was conducted as described above at 39 °C for 35 min using the TwistAmp nfo kit (TwistDx Ltd., UK).

**4.3. Preparation of AuNP Conjugates.** Anti-fluorescein antibody was coupled to AuNPs, which served as the signaling molecule (red in color) to allow the visualization of the immuno-sandwich complex by eye observation. Coupling to AuNPs (40 nm, 20 OD/vial, which is equivalent to 9 × 10^10 particles per vial in 50 μL) was performed using the InnovaCoat GOLD 10x Multi Explorer labelling kit (BioNovus Life Sciences, Cherrybrook, NSW, Australia). Briefly, reagents were thawed to 25 °C, and 12 μL of antibody (diluted to 0.1 mg/mL using the diluent provided) was mixed with 42 μL of reaction buffer. The mixture (45 μL) was used to resuspend a vial of InnovaCoat GOLD nanoparticles, which was incubated for 10 min before the addition of 5 μL quencher, resulting in a final 20 OD solution (50 μL) of anti-fluorescein/AuNP. The conjugates were washed twice with the borate running buffer (100 mM H3BO3, 100 mM Na2B4O7, 1% bovine serum albumin, 0.05% Tween 20, pH 8.8) by centrifuging at 14 500g for 6 min before resuspension to the original (50 μL) volume. Scaled-up preparations to obtain 500 μL AuNP conjugates were achieved using the InnovaCoat GOLD Midi kit (BioNovus Life Sciences, Cherrybrook, NSW, Australia). Conjugates were stored at 4 °C.
For the multiplex RPA in combination with multiplex lateral flow detection, anti-fluorescein/AuNP conjugate (2.5 μL) was pipetted onto the conjugate pad, and the LFD was dipped into a mixture containing 220 μL of running buffer and 5 μL of purified RPA amplicon (corresponding to display numbers 0–9). An additional 2.5 μL of anti-fluorescein/AuNP conjugate was pipetted onto the conjugate pad once the running buffer reached the bottom of the absorbent pad. The LFD was developed for 25 min. All experiments were repeated at least twice to demonstrate the consistency of results.

4.6. Image and Statistical Data Analysis. Reacted LFDs were dried, imaged using the MultiDoc-ItTM Digital Imaging System (Upland, CA, USA), and analyzed using ImageJ software (National Institutes of Health, MD, USA). Image brightness/contrast and color balance were autoadjusted. To determine the analytical sensitivity, grayscale-converted images were used to determine dot intensity by measuring the mean gray value (limit to threshold), using a fixed area measurement, and subtracting from the maximum mean gray value (255). For each test dot, the average of two neighboring relative white spaces was subtracted from the band intensity to normalize the each test dot intensity 3 times higher than the standard deviation of the two neighboring white space values.

ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b01097.

Results of single-plex RPA analytical sensitivity assays, optimization data of single-plex RPA with multiplex LFDs, and optimization data of multiplex RPA with multiplex LFDs (PDF)

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Notes

The authors declare the following competing financial interest(s): Joanne Macdonald is the founder of and shareholder in diagnostics company BioCifer Pty. Ltd, which was not involved in the study.

ACKNOWLEDGMENTS

This work was supported by the Queensland Government, Department of Science, Information Technology, Innovation and the Arts (DSITIA, Australia), an internal Higher Degree by Research (HDR) grant (University of the Sunshine Coast, Australia) and in part through matching funding provided by the CSIRO Synthetic Biology Future Science Platform and the University of the Sunshine Coast, Australia. We thank John Bartlett, David McMillan, and Fabrice Rossignol for support and advice.

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