analytical chemistry

Article

pubs.acs.org/ac

Multiplexed Recombinase Polymerase Amplification Assay To Detect Intestinal Protozoa

Zachary Crannell

1980 West Getaway Trail, Flagstaff, Arizona 86005, United States

Alejandro Castellanos-Gonzalez

Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555.0435, United States

Gayatri Nair

Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch, 1709 Dryden Road, No. 1700, Houston, Texas 77030, United States

Rojelio Mejia

Department of Pediatrics, Section of Tropical Medicine; Department of Medicine, Section of Infectious Diseases; and National School of Tropical Medicine, Baylor College of Medicine 1709 Dryden Road, No. 1700, Houston, Texas 77030, United States

A. Clinton White

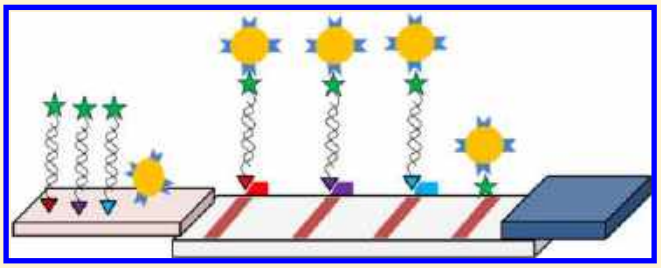
Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch, Mary Moody Northen, Room 4.230, 301 University Boulevard, Galveston, Texas 77555-0435, United States

Rebecca Richards-Kortum*

Department of Bioengineering, Rice University, Houston, Texas 77030, United States

Supporting Information

ABSTRACT: This work describes a proof-of-concept multiplex recombinase polymerase amplification (RPA) assay with lateral flow readout that is capable of simultaneously detecting and differentiating DNA from any of the diarrhea-causing protozoa *Giardia*, *Cryptosporidium*, and *Entamoeba*. Together, these parasites contribute significantly to the global burden of diarrheal illness. Differential diagnosis of these parasites is traditionally accomplished via stool microscopy. However, microscopy is insensitive and can miss up to half of all cases. DNA-based diagnostics such as polymerase chain reaction (PCR) are far more sensitive; however, they rely on expensive thermal cycling equipment, limiting their availability to centralized reference laboratories. Isothermal DNA amplification platforms, such as the RPA platform used in this study, alleviate the need for thermal cycling equipment and have the potential to broaden access to more sensitive diagnostics. Until now, multiplex RPA assays have not been developed that are capable of simultaneously detecting and differentiating infections caused by different pathogens. We developed a multiplex RPA assay to detect the presence of DNA from *Giardia*, *Cryptosporidium*, and *Entamoeba*. The multiplex assay was characterized using synthetic DNA, where the limits-of-detection were calculated to be 403, 425, and 368 gene copies per reaction of the synthetic *Giardia*, *Cryptosporidium*, and *Entamoeba* targets, respectively (roughly 1.5 orders of magnitude higher than for the same targets in a singleplex RPA assay). The multiplex assay was also characterized using DNA extracted from live parasites spiked into stool samples where the limits-of-detection were calculated to be 444, 6, and 9 parasites per reaction for *Giardia*, *Cryptosporidium*, and *Entamoeba* parasites, respectively. This proof-of-concept assay may be reconfigured to detect a wide variety of targets by re-designing the primer and probe sequences.



This work describes a multiplex recombinase polymerase amplification (RPA) assay with lateral flow readout designed to simultaneously detect and differentiate between DNA from up

Received: August 25, 2015
Accepted: December 16, 2015
Published: December 16, 2015

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

to three different diarrhea-causing protozoa. The multiplex assay builds on work we previously published describing three separate RPA assays to diagnose *Cryptosporidium* species, *Giardia lamblia* (*G. lamblia*), and *Entamoeba histolytica* (*E. histolytica*).[1−3]

RPA and other DNA diagnostics such as polymerase chain reaction (PCR) can amplify detect trace levels of pathogen DNA to levels that are easily detected. Because very low concentrations of pathogen can be detected, DNA tests are typically significantly more sensitive than traditional diagnostic methods such as microscopy or antibody-based tests.[4−7]

While PCR is considered to be the gold standard for diagnosing many infectious diseases, it requires the use of heat-labile reagents, technical expertise to avoid cross-contamination, and specialized thermal cycling equipment, which often limits its availability to centralized reference laboratories. A number of nucleic acid amplification methods have been developed recently to bypass the requirement for a thermal cycler.[8−11] These platforms use a fixed temperature heater instead of a thermal cycler, which costs an order of magnitude less, potentially allowing broader access to diagnostics based on isothermal amplification.[12]

One isothermal amplification platform, RPA, offers significant advantages. RPA enzymes are provided in an easy-to-transport format that does not require refrigeration. Moreover, RPA is tolerant to sample impurities that inhibit other nucleic acid (NA) amplification platforms and provides test results that can be easily visualized in the field using lateral flow strips.[13,14]

Detection and differentiation of multiple DNA targets allows clinicians to run syndromic panels to simultaneously test for several diseases that present similarly in the clinic but require different treatments.[17] Steps toward a multiplexed RPA assay have been reported, though these approaches require the use of a fluorescence reader.[18−20] Even basic fluorescence detectors may cost thousands of dollars and are thus not appropriate for low-resource settings.[21] The ability to detect multiple targets using a low-cost platform such as a lateral flow strip that could be interpreted visually or with a simple reader could facilitate the implementation of DNA-based diagnostics for syndromic panels.

In this work we detail the development and optimization of a multiplex RPA reaction capable of amplifying DNA from three different diarrheal-causing protozoa in a single tube. The results can be read with easy-to-make multiplex lateral flow strips containing three different detection zones and a positive control zone. The multiplex RPA assay amplifies different DNA targets from the *G. lamblia*, *E. histolytica*, and *Cryptosporidium* species genomes. These three parasites were selected for this proof-of-concept assay because they are significant contributors to diarrheal disease and resulting malnutrition around the world, all have similar clinical presentations, but require different treatment regimens to be managed effectively.[22−24]

The multiplex assay was characterized with three synthetic DNA targets, and its performance was compared to singleplex assays using the same targets. The singleplex assays had limits-of-detection of 10−40 gene copies, roughly an order of magnitude more sensitive than the limits-of-detection for the multiplex assay for each of the respective targets. The limits-of-detection for the multiplex assay using stool spiked with live parasites was around 10 parasites per reaction for *Entamoeba* and *Cryptosporidium* and 400 parasites per reaction for *Giardia*.

This is the first lateral-flow-based multiplex RPA assay that is capable of simultaneously amplifying three different genetic targets. This work contributes to a better understanding of how to integrate RPA assays into syndromic panels and therefore expand the use of syndromic RPA panels.

METHODS

Singleplex RPA Assays. Singleplex RPA reactions were assembled according to the manufacturer's recommended protocol, but with slight modifications to probe design described in later text.[14] Each reaction contained an RPA TwistAmp nfo enzyme pellet that was rehydrated with 45.5 μL of a master-mix consisting of 29.5 μL of supplied rehydration buffer, 11.2 μL of nuclease free water, 2.1 μL of 10 μM forward primer, 2.1 μL of 10 μM 5′-biotinylated reverse primer, and 0.6 μL of 10 μM 5′-labeled probe. The probe was designed according to the manufacturer's recommendations with a 5′ label, an internal spacer replacing a base, and a 3′ carbon blocker. The 5′ labels for the *Giardia*, *Entamoeba*, and *Cryptosporidium* probes were Alexa Fluor488, fluorescein, and digoxigenin, respectively. See Table 1 for a complete description of primers and probes for each assay.

After rehydrating the enzyme pellet with 45.5 μL of the master-mix, 2 μL of target (synthetic DNA standard or DNA extracted from stool) were added to each reaction. Next, 2.5 μL of the supplied magnesium acetate was added to the lid of each tube containing a reaction. All reactions were simultaneously initiated by centrifuging the magnesium acetate into the reaction mixture and transferring the tubes to a 37 °C heat block for 35 min.

After incubation in the heat block, 5 μL of RPA amplicons were removed and diluted in a tube containing 95 μL of tris-buffered saline solution (25 mM tris, 150 mM NaCl, 0.05% Tween 20). The conjugate pad end of one of the multiplex lateral flow strips was then placed vertically inside the tube. After 10 min of flow, the strip was removed and immediately scanned using a flatbed scanner (V500, Epson, Long Beach, CA, USA).

Agarose gel electrophoresis was used to visualize reaction products. In the presence of target DNA, RPA reactions generated two RPA products: a long product from the forward and reverse primer and a shorter, dual-labeled product from the probe and the reverse primer. Primers and probe with standard fluorescein modifications were initially screened via gel electrophoresis to ensure visualization of desired products. Alternative probe modifications were similarly screened using standard probe modifications as a control.

The dual-labeled DNA amplicons (labeled with biotin and either Alexa Fluor488, fluorescein, or digoxigenin depending on which target and probe(s) were in the assay) were detected via lateral flow strips as shown schematically in Figure 1. Addition of the reaction product to the lateral flow strips rehydrates the dried gold nanoparticles and initiates flow. If present, biotin-labeled DNA product binds to the streptavidin-coated gold nanoparticles and is captured at one of the three detection zones in a traditional sandwich assay, causing a color change that is easily visualized with the naked eye (Figure 3). Any unbound streptavidin-coated gold is captured at the positive control line by a biotinylated IgG.

Multiplex RPA Assay. The three, separate singleplex assays were then integrated into one multiplex assay. Multiplex RPA reactions capable of amplifying up to three different targets were assembled by rehydrating an RPA nfo enzyme pellet with 41.5−45.5 μL of a master-mix (depending on the volume of target in each reaction). The master-mix consisted of 29.5 μL of rehydration buffer, 7−11 μL of nuclease free water, 0.74 μL each of *Giardia* forward and reverse primers, 0.28 μL of *Giardia* probe, 0.81 μL of *Entamoeba* forward and reverse primers, 0.38 μL of *Entamoeba* probe, 0.53 μL of *Cryptosporidium* forward and

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

Table 1. DNA Sequences of All Primers and Probes

target		sequence
Giardia lamblia	forward primer	5′-TAC GCT CAC CCA GAC GAT GGA CAA GCC CG-3′
	reverse primer	5′-biotin-TGT GCG ATG GCG TCC TTG ATC ATC TTC ACG C-3′
	probe	5′-alexa fluor488-TAC GCT CAC CCA GAC GAT GGA CAA GCC CG/internal spacer/CGA CCT CAC CCG CAG-carbon spacer-3′
Entamoeba histolytica	forward primer	5′-GTA CAA AAT GGC CAA TTC ATT CAA TG-3′
	reverse primer	5′-biotin-ACT ACC AAC TGA TTG ATA GAT CAG 3′
	probe	5′-fluorescein-GTA CAA AAT GGC CAA TTC ATT CAA TG/internal spacer/ATT GAG AAA TGA CAT-carbon spacer-3′
Cryptosporidium spp.	forward primer	5′-GTG GCA ATG ACG GGT AAC GGG GAA TTA GGG-3′
	reverse primer	5′-biotin-AAT TGA TAC TTG TAA AGG GGT TTA TAC TTA ACT C-3′
	probe	5′-digoxigenin-GTG GCA ATG ACG GGT AAC GGG GAA TTA GGG/internal spacer/TCG ATT CCG GAG AGG-carbon spacer-3′

reverse primers, and 0.2 μL of *Cryptosporidium* probe (all primers and probes stored at 10 μM concentration). After rehydrating the enzyme pellet, 2−6 μL of target was added to each reaction. Limit-of-detection (LOD) experiments were conducted using 2 μL of 1 target. Experiments when the multiplex RPA assay was used to amplify two or three targets simultaneously utilized a total of 4 or 6 μL (2 μL for each of up to three different targets). Otherwise, multiplex RPA assays were conducted exactly as singleplex RPA assays were.

Multitarget Amplification. The multiplex RPA assay was used to simultaneously amplify various combinations and concentrations of *Giardia*, *Entamoeba*, and *Cryptosporidium* synthetic targets. All combinations contained DNA from two or three targets using either a low target DNA concentration (10^3 gene copies per reaction) or a high target DNA concentration (10^5 gene copies per reaction) for each target. Each of the 20 combinations was tested three different times.

Objective Determination of Test Results. Objective determination of positive and negative test results was accomplished using a custom MATLAB script that has been previously described.[1,15] Briefly, a signal-to-background ratio (SBR) at each detection zone was calculated and compared to a threshold SBR for that zone. The threshold SBR was set at the average plus three standard deviations of the SBR from nine negative control reactions. If the signal intensity of a given detection zone was greater than the threshold for that zone, the test was considered positive for that target. In this study, as with previous studies, visual determination almost always corresponded with the objective determination.

Assembly of Lateral Flow Strips. Dual-labeled RPA products were detected with lateral flow strips as described in Methods on the "singleplex RPA assay". Lateral flow strips were assembled as follows and stored for up to 1 month prior to use. Streptavidin-coated gold colloid for lateral flow strips was made according to a protocol adapted from Toubanaki et al.[25] Briefly, the pH of 1 mL of 50 nm gold colloid (15707-1, Ted Pella, Redding, CA, USA) was adjusted to 9.5 using 1 μL of 200 mM sodium borate (S248, Fisher, Pittsburgh, PA, USA). In a separate microcentrifuge tube, 2 μL of 2 mg/mL streptavidin (S000-01, Rockland, Limerick, PA, USA) was mixed with 398 μL of 2 mM sodium borate. The diluted streptavidin was added to the pH-adjusted gold in 50 μL increments, vortexing after each addition. The gold and streptavidin were incubated for 45 min at room temperature. They were then mixed with 155.6 μL of 10% bovine serum albumin (A3059-50g, Sigma-Aldrich, St. Louis, MO, USA) in 2 mM sodium borate and incubated at room temperature for 15 min. The gold was then washed 3 times by centrifuging at 5,000 rcf for 8 min, removing the supernatant, and resuspending the gold in 1 mL of 1% BSA in 2 mM sodium borate. Finally, the gold was centrifuged at 5,000 relative centrifugal force (rcf) for 8 min again, the supernatant removed, and resuspended in 250 μL of a buffer containing 5% bovine serum albumin, 137 mM sodium chloride (S671, Fisher), and 0.025% Tween 20 (P9416, Sigma-Aldrich).

Sixteen separate tubes of streptavidin-coated gold were prepared in parallel according to the protocol described previously and then mixed to obtain a final volume of 4 mL of streptavidin-coated gold. A 1 mL aliquot of gold was then pipetted onto four separate 7 mm × 300 mm laser cut glass fiber pads (GFCP203000, Millipore, Billerica, MA, USA), spread evenly, and dried on the benchtop overnight.

The next day lateral flow strips were prepared by striping capture antibodies onto a plastic-backed nitrocellulose card

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

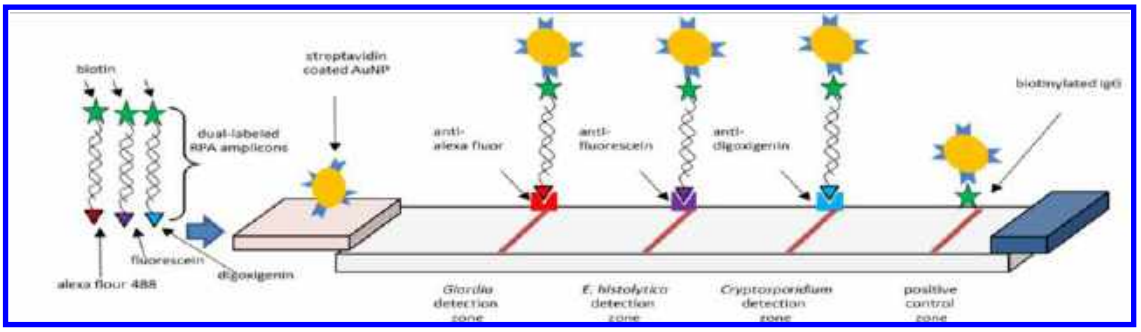


Figure 1. Schematic of simultaneous lateral flow detection of multiple targets. The biotin end of dual-labeled amplicons binds to streptavidin-coated gold and wicks down the lateral flow strips. The other end of the dual-labeled amplicons is captured at one of the three detection zones.

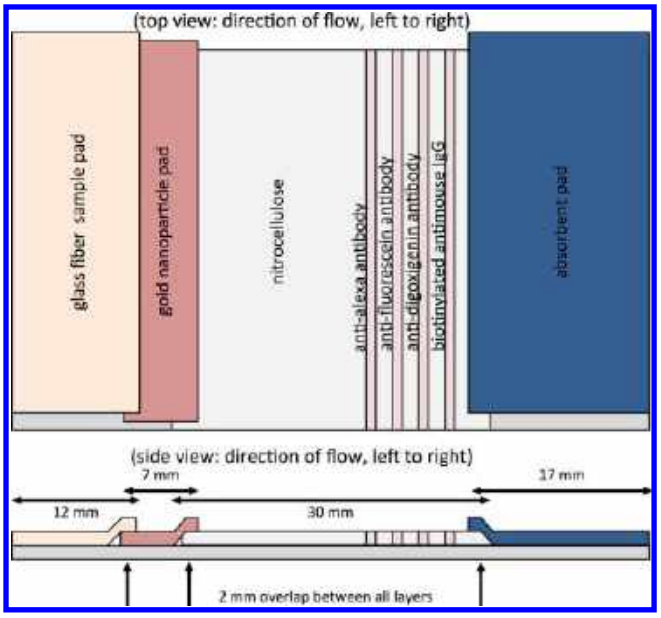


Figure 2. Schematic of lateral flow card assembly. Cards were made by striping antibodies onto plastic-backed nitrocellulose and then layering a glass fiber pad containing dried gold nanoparticles, and a sample pad, on the upstream ends, and an absorbent pad on the downstream end.

(HF135MC100, Millipore). Anti-Alexa antibodies (A619224, Life Technologies, Carlsbad, CA, USA) were diluted to a final concentration of 0.5 mg/mL using the antibody buffer containing 5% methanol (494437 Sigma-Aldrich) and 2% sucrose (IB37160, IBI Scientific, Peosta, IA, USA) in 100 mM sodium bicarbonate (S233 Fisher). Similarly, anti-FITC (ab19224, abcam), antidigoxigenin (ab64509, abcam, Cambridge, MA, USA), and biotinylated antimouse IgG (B7401, Sigma-Aldrich) antibodies were diluted to final concentrations of 0.5 mg/mL, 2.5 mg/mL, and 1 mg/mL respectively. All antibodies were striped onto the nitrocellulose cards using a lateral flow reagent dispenser (LFRD, Claremont BioSolutions, Upland, CA, USA) set to a head speed of 2 cm/s and a syringe pump (F200, Chemyx, Stafford, TX, USA) set to a flow rate of 0.1 mL/min. After the four antibodies were striped on the card, the card was dried at 37 °C for 1 h.

The cards were then assembled by first placing a 17 mm × 300 mm absorbent pad (CFSP223000, Millipore) on the downstream side of the plastic-backed nitrocellulose card, overlapping with the nitrocellulose by 2 mm (Figure 2). Next, the 7 mm × 300 mm glass fiber pad containing the dried gold nanoparticles was placed on the upstream side of the plastic-backed card, overlapping the nitrocellulose by 2 mm. Finally, a 12 mm × 300 mm GF sample pad was placed overlapping the upstream edge of the gold nanoparticle pad by 2 mm. After assembly, the cards were immediately cut into 3 mm wide strips using an A-Point Digital guillotine cutter (Arista Biologicals, Allentown, PA, USA) and stored with desiccant for up to 1 month. The total cost per strip was under $1, significantly less than the cost of commercially available strips that can only detect one or two targets (MGHD 1 and MGHD2 1, Milenia, Germany).

Synthetic DNA Standards. Bench-top characterization of each assay was performed using double-stranded synthetic DNA. Sequences corresponding to each of the three genetic targets used in previously published work on singleplex assays (Supporting Information Table S1) were purchased from IDT (gblock, Integrated DNA Technologies, Coralville, IA, USA).[1−3] The synthetic sequences were serially diluted in a buffer containing 10 mM Tris (AM9855G, Life Technologies), 0.1 mM EDTA (AM9261, Life Technologies), and 1 ng/μL carrier gDNA (AB360486, Life Technologies) to obtain standards. These standards were used in RPA reactions to determine the LOD for each of the three singleplex assays and for each target in the multiplex assay.

DNA Extraction of Spiked Stool Samples. *G. lamblia* and *Cryptosporidium parvum* (*C. parvum*) parasites were purchased from Waterborne (P101 and P102C, Waterborne, New Orleans, LA, USA). *E. histolytica* were purchased from ATCC (50525, ATCC, Manassas, VA, USA). Aliquots of 200 μL each of stool samples from normal healthy volunteers were spiked with 10^5 parasites in accordance with University of Texas Medical Branch (UTMB) approved IRB protocol 07-285. DNA was then extracted using the QuickGene Mini80 DNA extractor (Autogen, Holliston, MA, USA) and the QuickGene tissue kits (FK-DTS, Autogen). The spiked stool was incubated in 200 μL of lysis buffer at 56 °C for 1 h and passed through the DNA binding columns. The DNA binding columns were washed 2 times with 350 μL of washing buffer, and then the DNA was eluted in 200 μL of nuclease free water.

Statistical Analysis. The LOD at 95% probability for all targets and assays was determined by probit analysis using the dose−effect function of XLSTAT for Microsoft Excel (Addinsoft, New York, NY, USA) with at least six replicates per concentration.

RESULTS

Singleplex and Multiplex Limits-of-Detection. Typical scanned images from lateral flow strips are shown in Figure 3. These scanned images were used to objectively determine positive and negative test results as described in Objective Determination of Test Results.

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

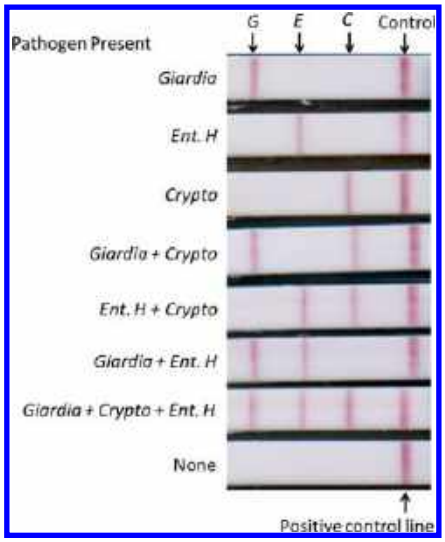


Figure 3. Multiplex lateral flow detection strips with three detection zones and a positive control zone. Strips tested positive (shown from top to bottom) for *Giardia*, *E. histolytica*, *Cryptosporidium*, *Giardia* + *Cryptosporidium*, *E. histolytica* + *Cryptosporidium*, *Giardia* + *E. histolytica*, and *Giardia* + *Cryptosporidium* + *E. histolytica*, and no pathogens.

Probe Optimization. Initial integration of the three assays using the previously published probe sequences resulted in false positives in the *Cryptosporidium* detection zone any time the *Giardia* target was present.[1−3] The LOD of the multiplex assay for the *Entamoeba* target was also 5−6 orders of magnitude higher in the multiplex assay as compared to the singleplex assay for the same target. After reviewing the sequence alignment for the targets, primers, and probes, we found there was significant sequence overlap between the three probes. The probes were then re-designed such that the functional part of the probes (the portion to the 3′ side of the cleavable spacer) overlapped with the forward primer. These regions had significantly less sequence alignment and also reduced the functional number of different DNA sequences interacting in the master-mix. Re-designing the probes eliminated the *Cryptosporidium* and *Giardia* cross-talk and improved the LOD for the *Entamoeba* target by 4 orders of magnitude.

When tested using synthetic DNA targets, the LOD of the singleplex *Giardia* assay was 39 gene copies (95% probability by probit analysis with six replicates per concentration). Similarly, the LODs of the singleplex *E. histolytica* assay and singleplex *Cryptosporidium* assay were 11 and 36 synthetic gene copies per reaction, respectively.

The LODs for each target in the multiplex assay were 403 synthetic gene copies per reaction of the *Giardia* target, 425 of the *Cryptosporidium* target, and 368 of the *Entamoeba* target (95% probability by probit analysis with six replicates per concentration). Figure 4 compares the average SBR of the singleplex assays and the same target in the multiplex assay. The markers represent the average SBR of six separate reactions using a synthetic DNA target, and the error bars represent one standard deviation of the SBR.

When the multiplex assay was tested using DNA extracted from live parasites spiked into stool at various concentrations, the respective LOD at 95% probability for *Giardia*, *Entamoeba*, and *Cryptosporidium* were 444, 6, and 9 parasites per reaction.

Simultaneous Multitarget Amplification. For all three replicates of each of the 20 different combinations of targets and target concentrations tested, the multiplex assay correctly detected all positive tests. None of the 60 tests yielded false positive test results.

DISCUSSION

We previously developed and tested three separate lateral flow assays to detect *Cryptosporidium sp.*, *G. lamblia*, and *E. histolytica*.[1,2] To integrate these three individual assays into a multiplexed assay, we developed a lateral flow detection strip with distinct detection zones for each of the three different targets (Figure 1). In the previously published studies, these singleplex assays were developed according to the TwistDx recommended protocol utilizing a biotin-labeled reverse DNA primer and a fluorescein-labeled DNA probe. However, to allow for differentiation between amplicons from three different targets on the same lateral flow strip, alternative probes were designed for the *Giardia* and the *Cryptosporidium* assays utilizing Alexa Fluor488 and digoxigenin instead of fluorescein.

These alternative haptens were selected as probe labels based on their relatively low cost and the availability of complementary high-affinity antibodies capable of capturing the large hapten−DNA−gold nanoparticle complex. There were several probe labels that either inhibited amplification (as visualized by the absence of the expected probe-reverse-primer band on the gel electrophoresis) or proved difficult to match with a high-affinity antibody. These included the Integrated DNA Technologies 5′ modifications Cy3, Cy5, bromodeoxyuridine, tetrachlorofluorescein, and hexachlorofluorescein. It should be noted that it was necessary to screen several antibody vendors since products from different vendors demonstrated widely varying performance.

After the alternative probes were designed, the LODs using the alternative labels and noncommercial strips were compared to the LODs using the traditional fluorescein label and commercially available single-target strips (MGHD1, Milenia, Germany). There was no significant difference in the LODs (data not shown). The singleplex assays were also optimized by varying the primer-to-probe ratio to maximize the signal-to-background ratio (SBR) for each target (data not shown). The three singleplex assays were then integrated into the multiplex format. Lastly, the reaction master-mix was optimized by adjusting the amount of magnesium acetate, the total reaction volume, the total amount of primers, and the relative amount of primers between each target. In the end, the optimized master-mix did not vary significantly from that recommended by the manufacturer (roughly 5 μL of total primers, 2.5 μL of magnesium acetate, and 50 μL of total reaction volume).

As seen in Figure 4, even after optimization, the LOD for each target in the multiplex assay was roughly an order of magnitude higher than for the same target in the singleplex assays. Also, even for high target concentrations, the SBR of the multiplex assay was significantly lower than that for the singleplex assays. Interestingly, when multiple targets were amplified in the same multiplex RPA reaction, the SBR was greater than when only one target was present. The reduced SBR for single-target amplification in the multiplex assay was likely the result of reduced amplification efficiency. When only one target sequence is present, the primers and probes for the other targets have no high-affinity target to bind to and may interfere with the efficient binding between the primers and target that are present.

While the LODs for the three different targets in the multiplex assay were comparable using synthetic targets, the LOD using live parasites was significantly higher for *Giardia*. The lower limit-of-detection for *Cryptosporidium* and *Entamoeba* is unsurprising given that there are up to 200 copies of the targeted 18s RNA

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

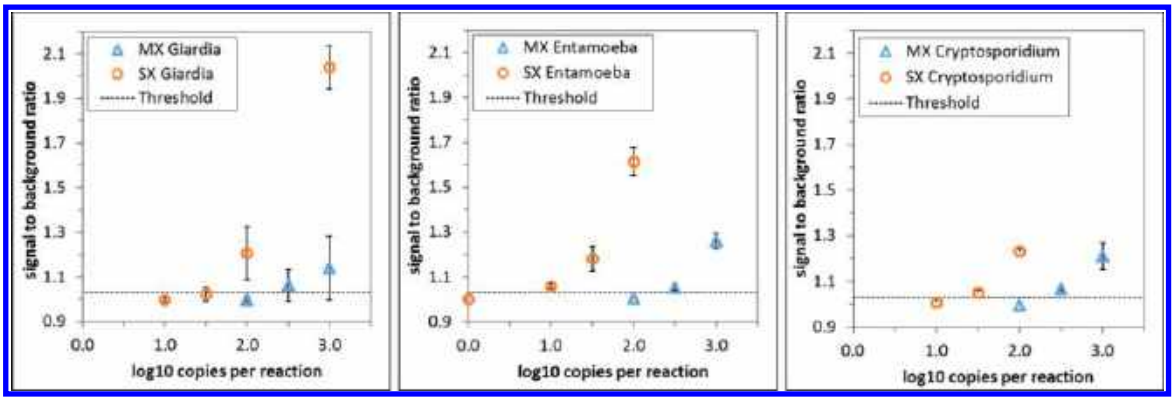


Figure 4. Signal-to-background ratios for singleplex (SX) assays and the same target in the multiplex (MX) assay. Left to right: SBR for various target concentrations for *Giardia*, *Entamoeba*, and *Cryptosporidium*. For a given target concentration, the SBR of the singleplex assay was significantly higher than that for the same target concentration in the multiplex assay.

genes in both the *Cryptosporidium* and *Entamoeba* genomes, whereas there is just a single copy of the targeted β giardin gene in the *Giardia* genome.[26−28]

For the first time, we demonstrated that it is possible to use RPA to simultaneously detect and amplify DNA from multiple parasites and detect those products using lateral flow strips. It should be noted that the multiplex format required significant further optimization of the individual component assays and still increased the LOD by roughly 1 order of magnitude compared to a singleplex assay targeting the same sequence, which may be of clinical consequence depending on the expected pathogen burden and the number of target copies per pathogen. Given the absence of any false positives after probe optimization, it was decided that the minimal benefits of a positive control were outweighed by the potential for reduced sensitivity associated with integrating a fourth set of primers. For assays prone to false positives, a positive control should be considered. Experiments involving live parasites should be conducted early in the optimization process, especially for genetic targets with low copy numbers.

A number of steps are needed to refine the multiplexed assay so that it is more amenable to use at the point-of-care, including incorporation of sample preparation. While this particular assay targets robust parasites that require extensive sample preparation, multiplexed RPA assays for less robust targets could easily integrate sample preparation at the point-of-care. Future studies should also explore methods to mitigate reduced amplification efficiency in multiplex assays, perhaps by using self-avoiding molecular recognition systems (SAMRS), which have been used in other RPA assays to enhance sensitivity.[29] For large-scale clinical studies, researchers should collaborate with the RPA enzyme manufacturers to have their final primer mix lyophilized with the enzymes to reduce the likelihood of user error when assembling reactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03267.

Sequences corresponding to each of the three genetic targets used in previously published work on singleplex assays (Table S1) purchased from IDT (gblock, Integrated DNA Technologies, Coralville, IA, USA)[1−3] (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Fax: (713) 348-5877. E-mail: rkortum@rice.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by a grant from the Bill & Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, Grant OPP1028843.

■ REFERENCES

(1) Crannell, Z. A.; Castellanos-Gonzalez, A.; Irani, A.; Rohrman, B.; White, A. C.; Richards-Kortum, R. *Anal. Chem.* **2014**, *86* (5), 2565−2571.
(2) Crannell, Z. A.; Cabada, M. M.; Castellanos-Gonzalez, A.; Irani, A.; White, A. C.; Richards-Kortum, R. *Am. J. Trop. Med. Hyg.* **2015**, *92* (3), 583−587.
(3) Nair, G.; Mauricio, R.; White, A. C. J.; Crannell, Z. A.; Richards-Kortum, R. R.; Pinilla, A. E.; Ramírez, J. D.; López, M. C.; Castellanos-Gonzalez, A. *Am. J. Trop. Med. Hyg.* **2015**, in press.
(4) Gitman, M. R.; Ferguson, D.; Landry, M. L. *J. Clin. Microbiol.* **2013**, *51* (11), 3765−3769.
(5) Wang, B.; Han, S.-S.; Cho, C.; Han, J.-H.; Cheng, Y.; Lee, S.-K.; Galappaththy, G. N. L.; Thimasarn, K.; Soe, M. T.; Oo, H. W.; Kyaw, M. P.; Han, E.-T. *J. Clin. Microbiol.* **2014**, *52* (6), 1838−1845.
(6) Patel, V. B.; Theron, G.; Lenders, L.; Matinyena, B.; Connolly, C.; Singh, R.; Coovadia, Y.; Ndung'u, T.; Dheda, K. *PLoS Med.* **2013**, *10* (10), e1001536.
(7) Liu, J.; Kabir, F.; Manneh, J.; Lertsethtakarn, P.; Begum, S.; Gratz, J.; Becker, S. M.; Operario, D. J.; Taniuchi, M.; Janaki, L.; Platts-Mills, J. a; Haverstick, D. M.; Kabir, M.; Sobuz, S. U.; Nakjarung, K.; Sakpaisal, P.; Silapong, S.; Bodhidatta, L.; Qureshi, S.; Kalam, A.; Saidi, Q.; Swai, N.; Mujaga, B.; Maro, A.; Kwambana, B.; Dione, M.; Antonio, M.; Kibiki, G.; Mason, C. J.; Haque, R.; Iqbal, N.; Zaidi, A. K. M.; Houpt, E. R. *Lancet Infect. Dis.* **2014**, *14* (8), 716−724.
(8) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. *Nucleic Acids Res.* **2000**, *28* (12), E63.
(9) Guatelli, J. C.; Whitfield, K. M.; Kwoh, D. Y.; Barringer, K. J.; Richman, D. D.; Gingeras, T. R. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (19), 7797.
(10) Piepenburg, O.; Williams, C. H.; Stemple, D. L.; Armes, N. a. *PLoS Biol.* **2006**, *4* (7), e204.
(11) Asiello, P. J.; Baeumner, A. J. *Lab Chip* **2011**, *11* (8), 1420−1430.
(12) *VWR Analog Dry Block Heaters*, VWR, Radnor, PA, USA, https://us.vwr.com/store/catalog/product.jsp?catalog_number=12621-114.
(13) Kim, T.-H.; Park, J.; Kim, C.-J.; Cho, Y.-K. *Anal. Chem.* **2014**, *86*, 3841−3848.

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616

(14) *Recombinase Polymerase Amplification*, a breakthrough alternative to PCR, TwistDx, Cambridge, U.K., http://www.twistdx.co.uk/our_technology/.
(15) Crannell, Z. A.; Rohrman, B.; Richards-Kortum, R. *PLoS One* **2014**, *9* (11), e112146.
(16) Rohrman, B. a; Richards-Kortum, R. R. *Lab Chip* **2012**, *12* (17), 3082−3088.
(17) Lafleur, L.; Stevens, D.; McKenzie, K.; Ramachandran, S.; Spicar-Mihalic, P.; Singhal, M.; Arjyal, A.; Osborn, J.; Kauffman, P.; Yager, P.; Lutz, B. *Lab Chip* **2012**, *12* (6), 1119−1127.
(18) Kersting, S.; Rausch, V.; Bier, F. F.; von Nickisch-Rosenegk, M. *Microchim. Acta* **2014**, *181* (13−14), 1715−1723.
(19) Crannell, Z. A.; Rohrman, B. A.; Richards-Kortum, R. *Anal. Chem.* **2014**, *86*, 5615−5619.
(20) Hill-Cawthorne, G. a.; Hudson, L. O.; El Ghany, M. F. A.; Piepenburg, O.; Nair, M.; Dodgson, A.; Forrest, M. S.; Clark, T. G.; Pain, A. *PLoS One* **2014**, *9* (6), e101419.
(21) *Twista portable real-time fluorometer*, TwistDx, Cambridge, U.K., http://www.twistdx.co.uk/products/devices_and_accessories/twista/.
(22) Kotloff, K. L.; Nataro, J. P.; Blackwelder, W. C.; Nasrin, D.; Farag, T. H.; Panchalingam, S.; Wu, Y.; Sow, S. O.; Sur, D.; Breiman, R. F.; Faruque, A. S.; Zaidi, A. K.; Saha, D.; Alonso, P. L.; Tamboura, B.; Sanogo, D.; Onwuchekwa, U.; Manna, B.; Ramamurthy, T.; Kanungo, S.; Ochieng, J. B.; Omore, R.; Oundo, J. O.; Hossain, A.; Das, S. K.; Ahmed, S.; Qureshi, S.; Quadri, F.; Adegbola, R. a; Antonio, M.; Hossain, M. J.; Akinsola, A.; Mandomando, I.; Nhampossa, T.; Acácio, S.; Biswas, K.; O'Reilly, C. E.; Mintz, E. D.; Berkeley, L. Y.; Muhsen, K.; Sommerfelt, H.; Robins-Browne, R. M.; Levine, M. M. *Lancet* **2013**, *382* (9888), 209−222.
(23) Acosta, A. M.; et al. *Clin. Infect. Dis.* **2014**, *59* (Suppl. 4), S193−S206.
(24) Guerrant, R., Walker, D., Weller, P., Eds. *Tropical Infectious Diseases*, 3rd ed.; Saunders: Philadelphia, PA, USA, 1996.
(25) Toubanaki, D. K.; Christopoulos, T. K.; Ioannou, P. C.; Flordellis, C. S. *Anal. Chem.* **2009**, *81* (1), 218−224.
(26) Holberton, D. V.; Marshall, J. *Nucleic Acids Res.* **1995**, *23* (15), 2945−2953.
(27) Torres-Machorro, A. L.; Hernández, R.; Cevallos, A. M.; López-Villaseñor, I. *FEMS Microbiol. Rev.* **2010**, *34* (1), 59−86.
(28) Abrahamsen, M. S.; Templeton, T. J.; Enomoto, S.; Abrahante, J. E.; Zhu, G.; Lancto, C. A.; Deng, M.; Liu, C.; Widmer, G.; Tzipori, S.; Buck, G. a; Xu, P.; Bankier, A. T.; Dear, P. H.; Konfortov, B. a; Spriggs, H. F.; Iyer, L.; Anantharaman, V.; Aravind, L.; Kapur, V. *Science* **2004**, *304* (5669), 441−445.
(29) Sharma, N.; Hoshika, S.; Hutter, D.; Bradley, K. M.; Benner, S. A. *ChemBioChem* **2014**, *15* (15), 2268−2274.

DOI: 10.1021/acs.analchem.5b03267
Anal. Chem. 2016, 88, 1610−1616