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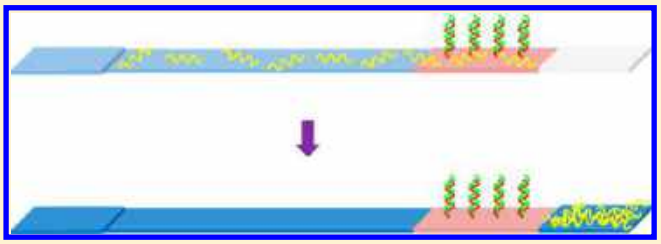
Inhibition of Recombinase Polymerase Amplification by Background DNA: A Lateral Flow-Based Method for Enriching Target DNA

Brittany Rohrman*,† and Rebecca Richards-Kortum†

†Department of Bioengineering, Rice University, 6100 Main St MS-142, Houston, Texas 77005, United States

Supporting Information

ABSTRACT: Recombinase polymerase amplification (RPA) may be used to detect a variety of pathogens, often after minimal sample preparation. However, previous work has shown that whole blood inhibits RPA. In this paper, we show that the concentrations of background DNA found in whole blood prevent the amplification of target DNA by RPA. First, using an HIV-1 RPA assay with known concentrations of nonspecific background DNA, we show that RPA tolerates more background DNA when higher HIV-1 target concentrations are present. Then, using three additional assays, we demonstrate that the maximum amount of background DNA that may be tolerated in RPA reactions depends on the DNA sequences used in the assay. We also show that changing the RPA reaction conditions, such as incubation time and primer concentration, has little effect on the ability of RPA to function when high concentrations of background DNA are present. Finally, we develop and characterize a lateral flow-based method for enriching the target DNA concentration relative to the background DNA concentration. This sample processing method enables RPA of 10^4 copies of HIV-1 DNA in a background of 0−14 μg of background DNA. Without lateral flow sample enrichment, the maximum amount of background DNA tolerated is 2 μg when 10^6 copies of HIV-1 DNA are present. This method requires no heating or other external equipment, may be integrated with upstream DNA extraction and purification processes, is compatible with the components of lysed blood, and has the potential to detect HIV-1 DNA in infant whole blood with high proviral loads.



Nucleic acid tests (NATs) are the gold standard for diagnosis of many infectious diseases. For example, because infants may harbor maternal antibodies against HIV for more than a year after birth, early infant diagnosis requires detection of proviral DNA or viral RNA.[1] NATs usually require three main steps: sample preparation, amplification, and detection. Because these steps typically require trained operators and expensive, electrically powered equipment, NATs are often unavailable in low resource settings where the infectious disease burden is high.[2]

To increase accessibility to NATs, much research has focused on developing methods for sample preparation, amplification, and detection that are appropriate for resource-limited settings. In this paper, we present a sample preparation method that enables recombinase polymerase amplification (RPA) of DNA from whole blood samples. RPA is an isothermal technique that may serve as an alternative to polymerase chain reaction (PCR) for amplifying nucleic acids.[3] While PCR requires a thermal cycler and several hours to produce a result, RPA may produce a result in less than 30 min using only a fixed-temperature heater or heat from the human body.[4] In addition, RPA products may be detected rapidly using lateral flow strips.[5−8] A variety of RPA assays have been developed to detect pathogens in food and clinical samples.[5−10] These assays have the potential to serve as point-of-care diagnostics in low-resource settings.

Although some sample types only require minimal processing before RPA, whole blood and nucleic acids purified from whole blood are incompatible with RPA. Previous work has shown that RPA remains active in the presence of many known PCR inhibitors, including undiluted serum, heparin (0.5 U), hemoglobin (50 g/L), and ethanol (4% v/v).[5] Two commercially available RPA tests from TwistDx only require lysis of cells before performing amplification.[11,12] Other work has shown that pathogens may be detected directly in urine with no sample preparation.[13] However, whole blood completely inhibits RPA,[5] and RPA assays that target nucleic acids purified from whole blood or samples containing blood suffer from low sensitivity.[10,14] The incompatibility of RPA and blood is a significant drawback because blood is the preferred sample matrix for many diagnostic tests.

In this paper, we show that the concentrations of background DNA found in whole blood prevent the amplification of target DNA by RPA. For blood sample volumes typically used for diagnostic tests (50−1000 μL), the concentration of genomic DNA ranges from 20 to 100 ng/μL, corresponding to a total DNA content of 1−100 μg.[15−18] Using an HIV-1 RPA assay, we first demonstrate RPA inhibition in the presence of several target DNA concentrations with concentrations of background DNA found in whole blood. We then characterize the inhibition of three other RPA assays by background DNA.

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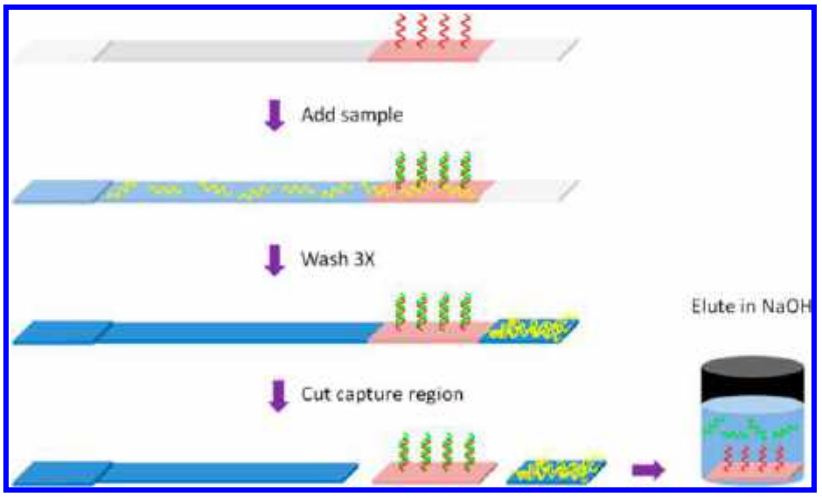

Figure 1. Lateral flow-based enrichment of target DNA. Capture oligonucleotides are shown in red, target DNA is shown in green, and background DNA is shown in yellow. Target DNA binds to the capture oligonucleotides, background DNA is washed away, the capture region is excised, and target DNA is eluted.

Next, we show that RPA fails to function in the presence of background DNA even after modifying the RPA reaction conditions. Finally, we develop and characterize a lateral flow-based method for enriching the target DNA concentration relative to the background DNA concentration.

EXPERIMENTAL SECTION

Inhibition of an HIV-1 RPA Assay by Background DNA. The inhibition of RPA by background DNA was first characterized using an assay to detect HIV-1 DNA. Reactions were assembled as recommended by the manufacturer (TwistAmp nfo kit, TwistDx, Cambridge, U.K.) using primer and probe oligonucleotides purchased from Integrated DNA Technologies (Novato, CA, U.S.A.). Primer and probe sequences were published previously.[6] Each 50 μL reaction contained 29.5 μL of rehydration buffer, 2.1 μL of biotin-labeled forward primer [10 μM], 2.1 μL of reverse primer [10 μM], 0.6 μL of FAM-labeled probe (FAM = 6-carboxyfluorescein) [10 μM], 3.2 μL of water, one supplied enzyme pellet, 2.5 μL of magnesium acetate [280 mM], and 10 μL of sample. Samples contained 0, 0.1, 0.2, 0.5, 1, 2, or 5 μg of sheared salmon sperm DNA (AM2980, Ambion, Foster City, CA, U.S.A.) as background DNA and various concentrations of the plasmid pHIV-IRES-eYFPΔEnvΔVifΔVpr, a generous gift from R. Sutton, as target DNA.[19] For preliminary experiments, 50, 10^3, and 10^6 copies of HIV-1 DNA were used as target DNA in order to cover the entire range of HIV-1 proviral DNA concentrations found in clinical samples. Reactions were incubated in a heat block for 20 min at 37 °C.

In some experiments, RPA products were analyzed via electrophoresis on a 2% agarose gel after purification (QIAquick PCR Purification Kit, Qiagen, Valencia, CA, U.S.A.). In other experiments, RPA products were detected using commercially available lateral flow strips (MGHD 1, TwistDx). For detection on lateral flow strips, 2 μL of RPA products were diluted into 98 μL of the supplied running buffer. Ten microliters were dispensed on the sample pad of the strip, and the strip was placed into 100 μL of running buffer in a 96-well plate. After 3 min of incubation, strips were scanned using a flatbed scanner. The signal-to-background ratio (SBR) of each strip was calculated in MATLAB as described previously.[20] The SBR threshold for a positive strip was defined as three standard deviations above the average SBR of 28 no-target-control strips.

Inhibition of Other RPA Assays by Background DNA. The inhibition of RPA by background DNA was then characterized for three additional assays, which were designed to detect *Plasmodium falciparum*, *Entamoeba histolytica*, and *Giardia duodenalis* DNA. The sequences of the primers and probe for these assays have been published previously.[5,21,22] A 1388 bp double-stranded synthetic DNA segment of the *18S rRNA* gene (accession number M19173) served as the target for the *P. falciparum* assay. A 135 bp PCR product amplified from the *E. histolytica SSU rRNA* gene served as the target for the *E. histolytica* assay. A 250 bp double-stranded synthetic DNA segment of the *G. duodenalis* assemblage A *beta giardin* gene served as the target for the *G. duodenalis* assay. For all assays tested, samples contained 1000 copies of target DNA and 0, 0.1, 0.2, 0.5, 1, 2, or 5 μg of sheared salmon sperm DNA. RPA samples were incubated for 30 min, and lateral flow detection was performed as described in the previous section.

Attempts to Restore RPA Activity in the Presence of Background DNA. The reaction conditions for RPA were modified with the goal of restoring RPA activity in the presence of background DNA. The original HIV-1 assay was performed with an incubation time of 20 min, a magnesium acetate concentration of 14 mM, primer concentrations of 420 nM, and a probe concentration of 120 nM. To test the effects of these parameters, the following reaction conditions were compared in independent experiments using 1000 copies of HIV-1 DNA and 0, 0.2, 0.5, 1, or 2 μg of sheared salmon sperm DNA: incubation times of 20, 40, and 60 min; magnesium acetate concentrations of 14, 22.4, and 33.6 mM; primer concentrations of 200, 420, and 600 nM; and probe concentrations of 50, 100, 150, 200, and 250 nM. RPA products were detected on lateral flow strips as described earlier.

Lateral Flow-Based Method for Sequence-Specific Enrichment of Target DNA. A sequence-specific lateral flow capture strategy was developed to enrich the concentration of HIV-1 target DNA relative to the concentration of background DNA (Figure 1). The lateral flow strips were designed to bind target DNA in the sample using capture oligonucleotides impregnated in the strip. After the target DNA is immobilized, three wash steps reduce the concentration of

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background DNA. Then the capture region of the strip is excised and placed in a 96-well plate. The DNA is denatured for 10 min at room temperature and then added to RPA reactions.

All materials for lateral flow strips were cut using a 60-W laser cutter (Universal Laser Systems, Scottsdale, AZ, U.S.A.). Strips (3 mm wide) were cut from nitrocellulose cards (Hi-Flow 135, Millipore, Billerica, MA, U.S.A.), sample pads (0.5 cm × 1 cm) were cut from glass fiber sheets (GFCP203000, Millipore), and absorbent pads (0.75 cm × 1 cm) were cut from cellulose sheets (CFSP223000, Millipore). Two microliters of capture oligonucleotide [0.1 mM] were dispensed near the end of each strip. The 34 bp capture sequence was the same sequence as the reverse primer for RPA. After drying at room temperature for 30 min, strips were exposed to UV light (UVP HL-2000 HybriLinker) at 125 mJ/cm^2 to cross-link the oligonucleotides to the nitrocellulose. Strips were then blocked in 100 μg/mL sheared salmon sperm DNA on a rotisserie for 30 min, followed by 1 h of drying at room temperature. Strips were then washed twice in 1X SSC (0.15 M sodium chloride, 0.015 M sodium citrate) for 15 min before drying at room temperature for 1 h.

To enrich the target DNA on lateral flow strips, samples containing target HIV-1 DNA and background DNA were diluted in a 20 μL volume with a final concentration of 6X SSC (0.9 M sodium chloride, 0.09 M sodium citrate). Each 20 μL sample was dispensed onto the sample pad of a lateral flow strip. After 10 min of incubation at room temperature, 30 μL of wash buffer (1X SSC) were dispensed onto the sample pad. This step was performed a total of 3 times. After the last 10 min incubation, the region of the strip containing the capture oligonucleotides (about one-third of the strip) was excised, halved, and transferred to a well of a 96-well plate using a clean razor blade. Twenty microliters of 0.15 M sodium hydroxide were dispensed into each well containing strip fragments. After 10 min of incubation at room temperature, 10 μL of eluate from each well were added to an RPA reaction for amplification and detection on lateral flow strips as described earlier.

This enrichment method was tested using samples with and without background DNA. Samples without background DNA contained 0, 10^2, 10^3, 10^4, or 10^5 copies of HIV-1 plasmid DNA. In some samples, 10 μL of blood lysed by three freeze–thaw cycles were added to determine whether blood components interfered with the enrichment method. Samples with background DNA contained 10^3 or 10^4 copies of HIV-1 plasmid DNA and 0, 1, 2, 4, 6, 8, 10, 12, or 14 μg of sheared salmon sperm DNA.

RESULTS

When the HIV-1 assay was performed with varying amounts of target and background DNA, the maximum amount of background DNA tolerated by the reactions depended on the target DNA concentration. RPA was completely inhibited by 0.5 μg of background DNA when 50 copies of HIV-1 target DNA were present (Figure 2A). However, when 10^3 or 10^6 copies of HIV-1 target DNA were present, RPA was completely inhibited by 2 or 5 μg of background DNA, respectively. Gel electrophoresis results confirm that amplification is entirely inhibited by high concentrations of background DNA (Figure S1, Supporting Information). These results show that RPA tolerates more background DNA when higher HIV-1 target concentrations are present.

Experiments using three additional RPA assays showed that the primer, probe, and target sequences used in the assay may also affect the maximum concentration of background DNA that may be tolerated by the reaction. When 10^3 copies of target DNA were present, 2 μg of background DNA completely inhibited both the HIV-1 and *P. falciparum* assays (Figure 2). However, the *E. histolytica* and *G. duodenalis* assays were completely inhibited by 1 and 0.5 μg of background DNA, respectively, when the same amount of target DNA was present. Thus, the maximum amount of background DNA that may be tolerated by RPA reactions is assay-dependent.

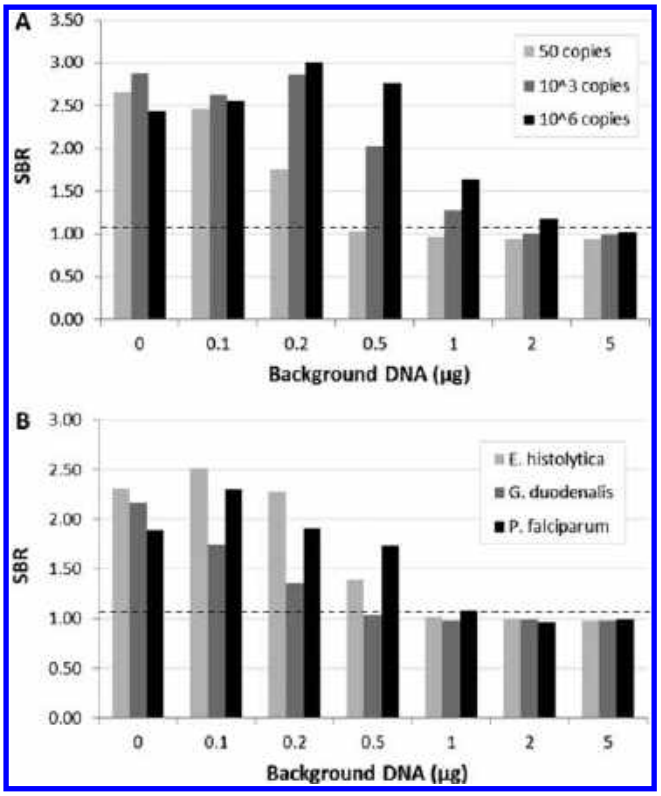

Figure 2. RPA inhibition by background DNA. (A) Results of lateral flow assay to detect RPA products in reactions with different amounts of HIV-1 target DNA and background DNA. (B) Results of lateral flow assay to detect RPA products in reactions with 1000 copies of target DNA and various amounts of background DNA for three different assays. The threshold for positive samples (1.05) is shown by the black dashed line.

Despite modifying the HIV-1 RPA assay in an attempt to restore RPA in the presence of background DNA, RPA reactions were inhibited by nearly the same concentrations of background DNA. Using the same HIV-1 target concentration, reactions with different incubation times (Figure S2A, Supporting Information), magnesium acetate concentrations (Figure S2B), and primer concentrations (Figure S2C) were all inhibited completely at 1 or 2 μg of background DNA. Reactions with probe concentrations ranging from 100 to 250 nM were also completely inhibited by 1 or 2 μg of background DNA, whereas reactions with a probe concentration of 50 nM were completely inhibited by 0.5 μg of background DNA (Figure S2D). These results show that modification of the RPA reaction conditions over this range does not rescue RPA from inhibition by background DNA.

To avoid inhibition of RPA by background DNA, a lateral flow-based method was developed to enrich the concentration of HIV-1 target DNA relative to the concentration of background DNA. When HIV-1 DNA was captured without

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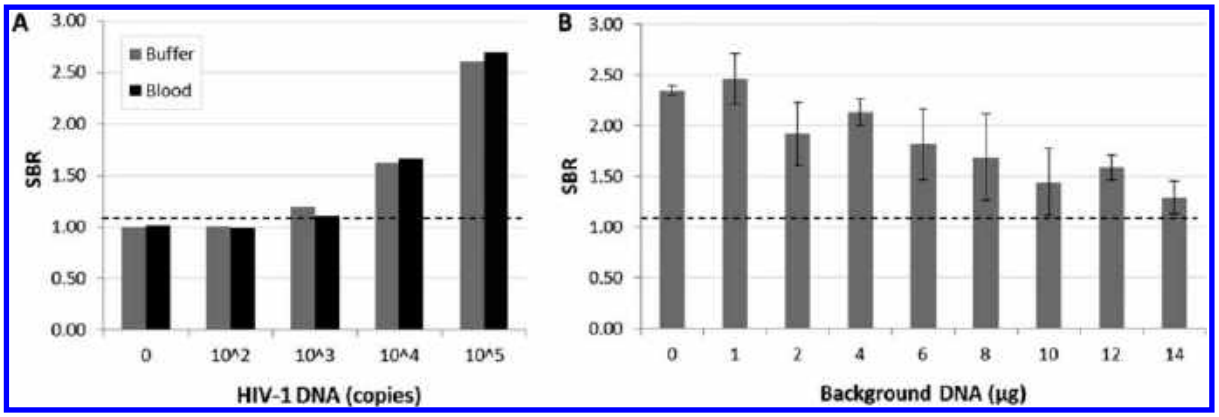

Figure 3. Lateral flow capture of DNA. (A) Results of lateral flow assay to detect RPA products in reactions following lateral flow capture of HIV-1 target DNA in buffer or lysed blood. (B) Detection of 10^4 HIV-1 DNA copies after lateral flow-based enrichment. All samples were positive. As background DNA increases, the SBR decreases but remains above the threshold for positive samples (1.05), shown by the black dashed line. Error bars represent the standard deviation for three experiments.

any background DNA present, the limit of detection was 10^3 copies (Figure 3A). The addition of 10 μL of lysed blood to the samples had no effect on the limit of detection, demonstrating that DNA may be captured on lateral flow strips even in the presence of blood components. When the enrichment method was performed with samples containing both HIV-1 DNA and background DNA, all samples containing 10^3 copies were negative while all samples containing 10^4 copies were positive (data not shown). The signal-to-background (SBR) of samples with 10^4 copies HIV-1 DNA decreased with increasing background DNA concentration (Figure 3B), but all SBRs were significantly greater than the threshold for positive samples, even for 14 μg of background DNA ($p < 0.05$). Without lateral flow sample enrichment, the maximum amount of background DNA tolerated was 2 μg when 10^6 copies of HIV-1 DNA were present. These results suggest that the enrichment method described here may enable RPA-based detection of DNA samples with high concentrations of background DNA.

DISCUSSION

The clinical range of proviral HIV-1 DNA concentrations found in infant whole blood varies from ~20−400 000 copies/mL and depends on age, treatment status of the infant, and treatment status of the mother.[23−26] A 0.1 mL blood sample from an infant with HIV-1 would have 2−10 μg of background DNA and 2−40 000 HIV-1 proviral DNA copies. The sample processing method introduced in this paper enables RPA of 10^4 copies of HIV-1 DNA in 0−14 μg of background DNA. Therefore, this method may enable detection of HIV-1 DNA in infant samples with high proviral loads, although further improvements are necessary for detecting lower proviral loads. In addition to detecting clinically meaningful DNA concentrations, this method may be suitable for use at the point of care. The method requires no heating or other external equipment, may be integrated with upstream DNA extraction and purification processes, and tolerates sample impurities found in blood. Because of these advantages, this method is promising for enriching target DNA from blood samples for RPA assays.

However, this method suffers from several disadvantages that must be addressed in future work. The main problem is that the assay cannot detect the entire clinical range of proviral loads found in infant blood samples. The lack of sensitivity is likely due to the fact that capturing double-stranded DNA with a single-stranded capture oligonucleotide is energetically unfavorable. The limit of detection may be improved in future versions of the assay by using capture oligonucleotides composed of RNA or LNA (locked nucleic acid), which have a greater affinity for DNA. Another issue is that the target enrichment process increases assay time by 50 min and requires five user steps. This drawback may be mitigated by incorporating the assay into a two-dimensional paper network (2DPN).[27] Once these shortcomings are addressed, this assay may serve as a clinically useful tool for sample preparation before RPA.

CONCLUSION

Here, we demonstrated that RPA is inhibited by concentrations of DNA found in blood volumes commonly used for diagnostic tests. Thus, background DNA is one component of whole blood that inhibits RPA. While inhibition depends on target DNA concentration, background DNA concentration, and the sequences used in the assay, inhibition is frequently observed at lower background concentrations than are typically found in whole blood. These factors should be considered when developing RPA assays designed for use with samples with high background DNA concentrations. The lateral flow-based method for enriching target DNA presented in this paper offers one approach for avoiding inhibition of RPA by background DNA.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: b.a.rohrman@gmail.com. Fax: (713) 348-5877.

Notes

The authors declare no competing financial interest.

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the *Plasmodium falciparum*, *Entamoeba histolytica*, and *Giardia duodenalis* assays.

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