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A Blood Drying Process for DNA Amplification

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The presence of numerous inhibitors in blood makes their use in nucleic acid amplification techniques difficult. Current methods for extracting and purifying pathogenic DNA from blood involve removal of inhibitors, resulting in low and inconsistent DNA recovery rates. To address this issue, a biphasic method is developed that simultaneously achieves inhibitor inactivation and DNA amplification without the need for a purification step. Inhibitors are physically trapped in the solid-phase dried blood matrix by blood drying, while amplification reagents can move into the solid nano-porous dried blood and initiate the amplification. It is demonstrated that the biphasic method has significant improvement in detection limits for bacteria such as *Escherichia coli, Methicillin-resistant Staphylococcus aureus, Methicillin-Sensitive Staphylococcus aureus* using loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). Several factors, such as drying time, sample volume, and material properties are characterized to increase sensitivity and expand the application of the biphasic assay to blood diagnostics. With further automation, this biphasic technique has the potential to be used as a diagnostic platform for the detection of pathogens eliminating lengthy culture steps.

1. Introduction

In the context of human health, blood serves a critical function and provides valuable information regarding the physiological state of an individual. Blood analysis is a useful tool for detecting a range of ailments, including genetic disorders, microbial and viral infections, as well as cancer.[1–4] The standard method for nucleic acid detection from blood involves extraction and purification of nucleic acid followed by amplification techniques such as polymerase chain reaction (PCR). Amplification is initiated by the binding of primers to the template, and polymerase extends the template length by adding the nucleotides, resulting in numerous amplicons (**Figure 1**A). Fluorescent dye intercalating the double-strand DNA can be used to detect the presence of generated amplicons. However, as the target nucleic acid is present in low concentration and is surrounded by numerous inhibitor components in blood that may bind to the target, primers, or polymerase, the quality of the nucleic acid extraction and purification process has critical impact on the success of downstream nucleic amplification techniques (Figure 1B). Thus, appropriate pre-treatment and nucleic acid extraction and treatment procedures are essential for obtaining accurate and reliable results.

Despite the importance of nucleic acid extraction and purification in the blood analysis process, the current methods

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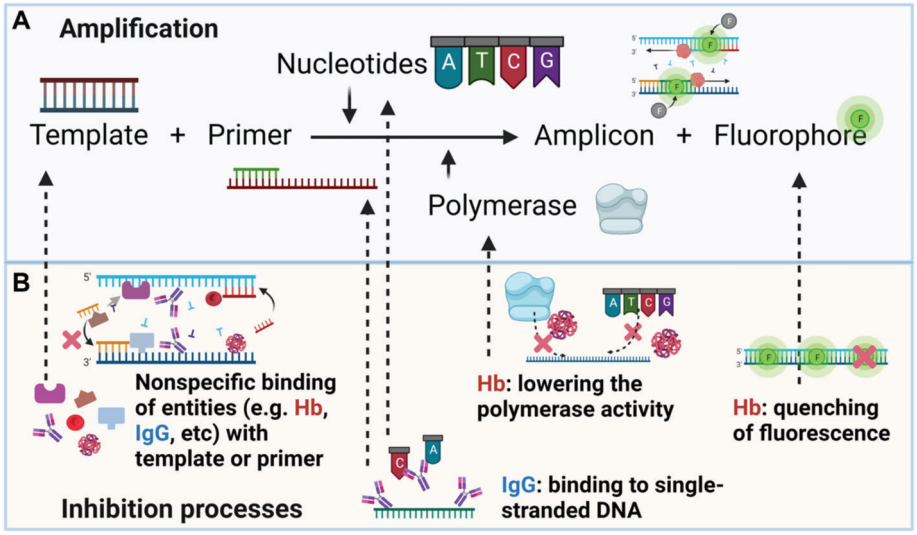

Figure 1. Nucleic acid amplification processes and inhibition mechanisms. A) Nucleic acid amplification and B) Inhibition mechanism by nonspecific binding of various inhibitor components found in blood.

have several drawbacks. First, they are expensive, time-consuming, and labor-intensive.[5,6] Moreover, there is a risk of cross-contamination during the multiple steps involved in sample processing.[7] Very importantly, removal of PCR inhibitors is challenging due to the high number of precise manual steps required.[1] The most significant issue is the loss of target nucleic acid itself during the current purification and extraction processes. This is due to inherent loss mechanisms, and the inefficiency of binding and unbinding of the nucleic acid to negatively charged silica surfaces, hence limiting the efficiency of capturing and retaining a few copies of target pathogenic DNA against millions of copies of human genomic DNA. Consequently, most purification and extraction processes are carried out with substantial amounts of DNA, exceeding 1 µg of total input DNA.[8] This poses a significant challenge for the detection of microbial or viral infections in blood.

Considering the challenges associated with traditional blood treatment methods, there is a need to develop a new blood treatment module that simplifies pre-treatment steps prior to nucleic acid amplification testing (NAAT) and bypasses extraction and purification. The two primary obstacles to overcome are the reduction of inhibitor levels in blood and the retention of target DNA molecules without loss. Blood contains numerous inhibitors that bind nonspecifically to key components of amplification reactions, reducing the efficiency of the reaction. Hemoglobin and immunoglobulin G (IgG) are two of the main inhibitors found in blood, and can lower the activity of polymerase or even inactivate it, bind with cofactors, or degrade the target or primers (Figure 1B).[6,9,10] Additionally, hemoglobin can interfere with fluorescence readout and make amplification detection difficult by the absorption and scattering of the light.[9]

Recently, our group demonstrated highly sensitive detection (limit of detection ≈1 CFU mL^{-1}) of bloodstream infections caused by bacteria, including *Methicillin-Sensitive Staphylococcus aureus* (*MSSA*), *Methicillin-resistant Staphylococcus aureus* (*MRSA*), and *Escherichia coli* (*E. coli*), without blood culture, and extraction and purification.[11] We utilized blood to create a structure within the PCR tubes by heating the blood to result in a dried blood matrix. Additionally, the use of loop-mediated isothermal amplification (LAMP) with a robust Bst polymerase results in amplification that is not affected by inhibitors.[12] The approach is termed as "biphasic reaction" since it consists of two phases: a solid phase of the dried blood matrix and a liquid phase of supernatant also allowing clear fluorescence detection.[13] The developed assay was shown to be highly sensitive and robust to inhibitors, but its mechanism has not yet been thoroughly investigated.

In this paper, the mechanism of this heating induced biphasic reaction was investigated, with a focus on understanding the inhibitory processes and ways to improve assay sensitivity of the reaction. Inhibitor levels were characterized in the liquid phase before and after biphasic thermal treatment using colorimetric or enzyme-linked immunoassay (ELISA) measurements. To determine the existence of inhibitors within the dried blood matrix (solid phase), liquid extraction surface analysis (LESA) coupled mass spectrometry (MS) was utilized. Additionally, various parameters, including drying time, diffusion distance, and material properties, were investigated to establish the optimal conditions for improving assay sensitivity when using the biphasic protocol. Lastly, we demonstrated a new application of the biphasic reaction in recombinase polymerase amplification (RPA), expanding the potential use of the technique in general nucleic acid amplification techniques.

2. Results and Discussion

2.1. Biphasic Reaction

Figure 2 introduces the current state of the art in extraction and purification method in comparison to the new biphasic assay. During each step, the presence of the two primary components of interests, the inhibitors and target DNA, are depicted in Figure 2A,C, while their relative level during the process is

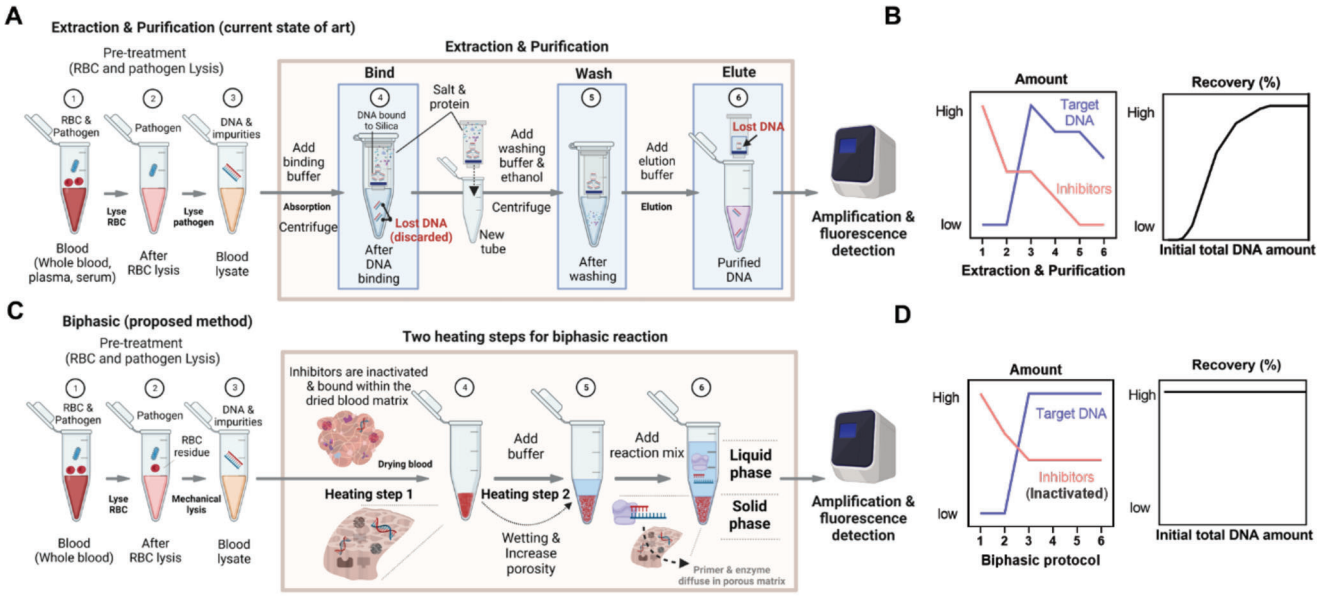

Figure 2. Biphasic reaction for highly sensitive nucleic acid amplification. A) Current state-of-art techniques involve extraction and purification with polymerase chain reaction. Target DNA is lost due to inherent loss mechanism during the binding and elution of the DNA. B) General trend of recovery rate versus extraction and purification steps in A. C) Proposed biphasic method uses two heating steps to inactivate inhibitors while retaining the target DNA within the dried blood matrix. D) There is no loss of target DNA during the process because relatively high inhibitor level does not affect the amplification since they exist in an inactivated form. As a result, recovery rate remains consistently high regardless of initial total DNA amount.

shown in Figure 2B,D. Nucleic acid extraction and purification starts with the pre-treatment steps such as red blood cell (RBC) lysis and pathogen lysis (Figure 1A, step 1–3). The starting material could be either whole blood or plasma and serum if the samples went through centrifugation (step 1). Ammonium-Chloride-Potassium (ACK) lysis buffer is employed for selective RBC lysis, using a volume 10–20 times greater than that of the blood sample to ensure complete removal of the RBCs (step 2). Chemical lysis buffers are commonly utilized for pathogen lysis (step 3), in which detergents and enzymes disrupt the cell wall of the pathogens, leading to the release of targeted nucleic acid from within the pathogen. Following the lysis of the pathogen, the sample comprises elevated levels of targeted nucleic acid alongside impurities (Figure 2B). During the centrifugation, there is absorption of the DNA onto the surface of purification materials, such as negatively charged silica (step 4). A fraction of unbound DNA may be lost during the centrifugation step, as the bottom tube is discarded. Furthermore, during the purification process, various other impurities, including proteins, polysaccharides, and salts, may adhere to the purification materials and need to be eliminated through the washing step (step 5). Finally, an elution step is performed to generate the ultimate purified DNA sample, which can subsequently be utilized for amplification and fluorescence detection. Lost DNA during the process is highlighted in red (step 4 and 6). Figure 2B conceptually illustrates the variations in inhibitor and target DNA levels that could occur during the extraction and purification process. These changes suggest that the method results in the reduction of inhibitors, albeit at the expense of inevitable DNA loss. This limitation may affect the efficiency of the extraction and purification process, particularly when starting with low DNA concentrations, as it may result in insufficient purified DNA at the end of the process. Consequently, the recovery rate of DNA is dependent on the initial total DNA amount especially at low DNA copies.

Conversely, the biphasic method utilizes a simple process of drying the blood sample (heating step 1) which results in inactivation of inhibitors, followed by a heating step 2 to enhance the sensitivity of the assay (Figure 2C). This method commences with the use of whole blood as the specimen (from step 1 to step 4 directly) or, alternatively, can start with the blood lysate by RBC lysis (step 2) and mechanical lysis of bacteria (step 3) to obtain DNA readily available for downstream analysis. In the biphasic method, the RBC lysis process involves the use of a 1:1 or 1:2 ratio of whole blood to ACK lysis buffer to partially remove RBC rather than a ratio of 1:10 or 1:20 whole blood to ACK lysis buffer.[11] This approach is employed to maintain the stability of the dried blood matrix by leaving a portion of RBC in the sample, which helps to facilitate the formation of a solid structure during the drying process. It should be noted that complete removal of RBC can lead to an unstable matrix structure, as shown in **Figure 3**A for the "100x Dilution". After completing the pre-treatment process (steps 1–3), the sample is ready to undergo the biphasic protocol. The optimal temperature and duration of heat for drying (heating step 1) the blood sample may vary based on the sample volume used. In our study, we utilized a volume of 30 µL and dried it at 95 °C for 10 min. During the blood drying (step 4), we postulate that the amplification inhibitors are inactivated and become bound within the dried blood matrix. Meanwhile, the concentration of DNA in the blood is maintained, as no additional binding and elution steps are required, unlike in state of the art extraction and purification processes. The resultant dried blood matrix could be effective in neutralizing inhibitors; however, it is suboptimal for nucleic acid amplification and detection, primarily due to its thick and nonporous structure that arises from the

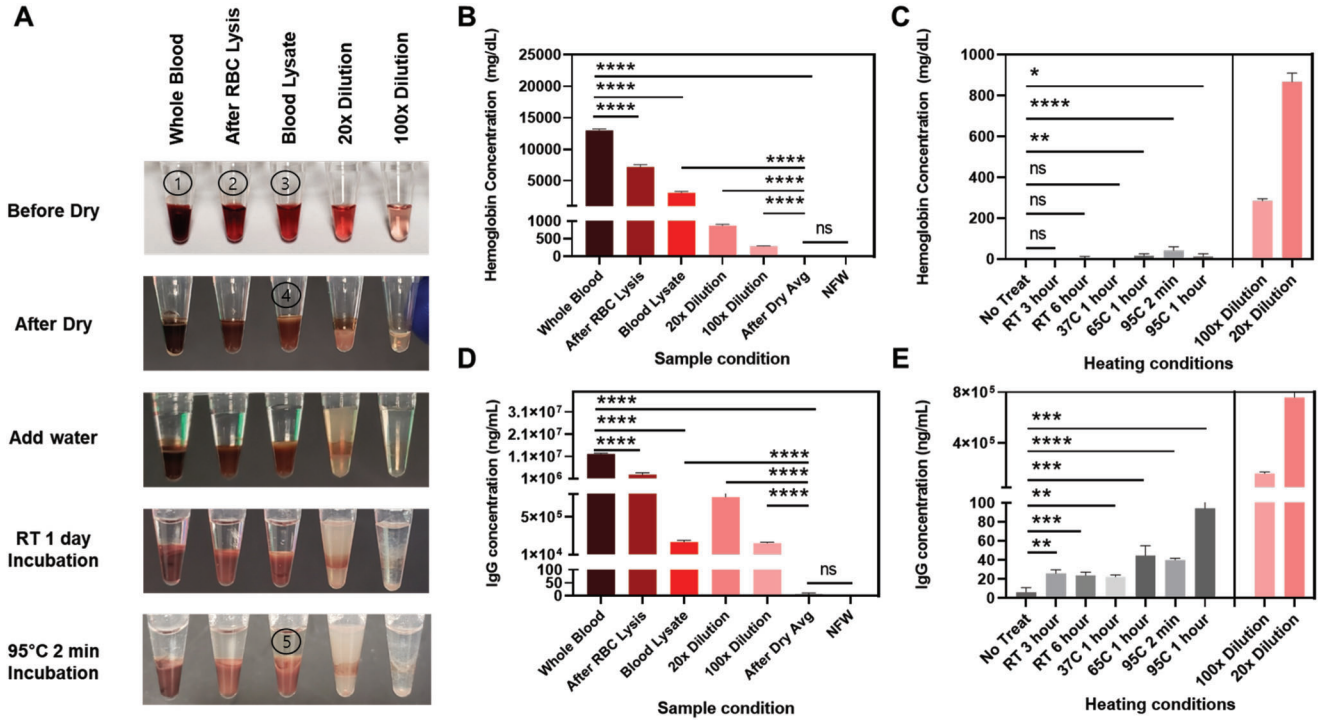

Figure 3. Inhibitor characterization using colorimetric (for hemoglobin) and ELISA (for IgG) measurement. A) Images of blood samples with various conditions along with blood drying process and after further incubation. Inhibitor concentration for B) hemoglobin and D) IgG, before and after drying. Further characterization of inhibitor level for C) hemoglobin and E) IgG, using various treatment to see if physically locked inhibitors are released out to the supernatant after incubation.

compaction of the blood during coagulation. To overcome this limitation, we introduced a buffer or water and conducted the second important step in the biphasic reaction protocol, namely a heating step 2 (95 °C for 2 min) (step 5), to increase the porosity and wetting of the dried blood matrix. Before the heating step 2, we refrained from adding primers and polymerase to the buffer solution, as the polymerase, especially could be degraded at the high temperature conditions used in heating step 2. Following heating step 2 (step 5), a reaction mix comprising of primers and polymerase is added (step 6). The tube now contains two phases, namely a liquid and a solid phase (Figure 2C, step 6). The solid phase contains the inactivated inhibitors and target DNA bound within the dried blood matrix. On the other hand, the liquid phase now contains polymerase and primers, which can diffuse into the porous dried blood matrix, and initiate amplification upon binding to the target DNA. After amplification, the resulting amplicons diffuse out from the solid phase into the liquid phase. The fluorescent intercalating dye provides a signal from the clear supernatant measurements without interference from the red components in blood. Thus, by physically separating the two phases and detecting only the liquid phase for amplification and fluorescence measurement, we can increase the signal to noise. Figure 2D demonstrates the effect of the biphasic process on the target DNA levels. The target DNA level remains preserved during the biphasic process (steps 3–6), while inhibitor levels remain constant at a steady state. However, the inhibitors exist in an inactivated form and do not participate in the amplification reaction, resulting in a high recovery rate and amplification of the target DNA, irrespective of the initial total DNA amount. This is crucial for detecting low-concentration pathogens, such as those involved in sepsis diagnosis.

2.2. Characterization of the Inhibitor Levels in Liquid Phase

To assess whether inhibitors are truly inactivated following blood drying and if they are not subsequently solubilized into the liquid phase, we undertook a characterization of two significant amplification inhibitors: hemoglobin and immunoglobulin G (IgG). **Figure 3**A illustrates the analysis of the blood sample and its derivatives associated with the biphasic protocol. The figure is organized into five groups of samples presented in columns, which include whole blood, blood after RBC lysis, blood lysate after mechanical lysis, and two control groups: blood that has been diluted with water by 20 times and by 100 times. Each group is further divided into rows that represent the process of sample preparation, including drying, addition of water, and incubation either at room temperature for one day or at 95 °C for 2 min (heating step 2 condition). The numbers (1–5) in the image correspond to the individual steps outlined in the biphasic protocol illustrated in Figure 2C. To determine if the samples can generate a stable dried blood matrix after undergoing the blood drying process, a color change was observed after adding water (row 3 in Figure 3A). It was evident that samples 1–3 produced a stable structure that prevented the solid phase from being resuspended into the liquid phase, in contrast to the 20x and 100x diluted blood samples. Furthermore, in the case of the 20x diluted blood sample, once incubated either at room temperature or at 95 °C, the solid phase

is solubilized into the liquid phase, which ultimately results in the interruption of fluorescence readings.

To obtain a quantitative assessment of inhibitor levels in accordance with the biphasic protocol, a colorimetric kit for hemoglobin and an enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G (IgG) were employed and are presented in Figure 3B–E. After allowing 10 min of incubation to enable the solubilization of inhibitors into the supernatant, measurements were conducted on the supernatant of each sample based on the vendor's protocol. Figure 3B displays the level of hemoglobin prior to and after blood drying. Distinctive reductions in hemoglobin levels were observed during each pre-treatment step (steps 1–3, as shown in Figure 2C) as the samples were processed from the initial "Whole Blood" state to "after RBC Lysis", and finally to "Blood Lysate". The concentration of hemoglobin in the blood lysate was still greater than that of the 20x diluted blood sample, indicating that the pre-treatment procedure did not completely remove hemoglobin. The phrase "After Dry Avg" in the x-axis refers to the hemoglobin level of three samples (Whole Blood, After RBC lysis, Blood Lysate) following blood drying. All three samples displayed hemoglobin levels that were close to zero after drying, with no significant variation in comparison to nuclease-free water (NFW). Subsequently, water was added to the samples (sample 1–3), which were then incubated under varying conditions to measure the hemoglobin level and to verify whether hemoglobin physically trapped within the dried blood matrix was indeed inactivated (Figure 3C). Direct samples were used for measurement for 20x and 100x diluted blood samples. Incubation at room temperature and 37 °C did not show any significant difference in comparison to the negative control sample (i.e., no treatment). Although incubation at higher temperatures utilized for loop-mediated isothermal amplification (LAMP) at 65 °C and heating step 2 at 95 °C showed a notable variation in comparison to the negative control sample, their hemoglobin levels remained significantly lower when compared to the 100x diluted blood sample. Similar to the hemoglobin analysis, characterizations were conducted on IgG and are displayed in Figure 3D,E. A considerably larger proportion of IgG was removed for the blood lysate (i.e., comparable to a 100x dilution), but similar patterns of inactivation following drying were observed (i.e., the "After Dry Avg" was near zero). In contrast, incubations steps showed some dissolve of IgG into liquid phase compared to the negative control (no treatment). While the initial IgG concentration (10^7 ng mL^{-1}) decreased down to less than 100 ng mL^{-1} (5 orders of magnitude), the hemoglobin concentration dropped from 10,000 to 0 mg dL^{-1}. Also, this level of IgG dissolved in liquid phase was substantially lower than that of 100x diluted blood (≈10^5 ng mL^{-1}). A prior investigation of the inhibitory mechanism of blood components on real-time PCR demonstrated that the presence of 2.5% blood in the reaction resulted in a 45% quenching of fluorescence intensity, and an increase in hemoglobin concentration was found to have a linear correlation with a decrease in the number of positive reactions, starting at a concentration of 39 μM.[9] Moreover, an IgG concentration of 190 μM (2.79×10^7 ng mL^{-1}) led to a delay in amplification time from 26 to 32 min.[9] It is noteworthy that the concentration of inhibitors in the supernatant of the biphasic reaction was significantly lower compared to the range that causes inhibition of amplification.

2.3. Characterization of the Inhibitor Levels in Solid Phase

To further investigate the existence of inhibitors within the dried blood matrix, liquid extraction surface analysis coupled mass spectrometry (LESA-MS) was used and results are presented in **Figure 4**. Protocol was adapted from previously described methods with modification.[14,15] Figure 4A illustrates the two sample preparation protocols: one for the dried blood sample and the other for the supernatant. For the dried blood sample, after the dried blood matrix was suspended by vortexing, 5 μL of the mixture was utilized for measurement following overnight desiccation. The supernatant sample was utilized in a similar manner as in the inhibitor level measurement shown in Figure 3. Figure 4B displays the relative abundance of heme B, α-globin and β-globin, which are subunits of hemoglobin, within the dried blood matrix under various conditions. Peaks were annotated based on a previous publication.[14] As a result, a notably higher concentration of heme B, α-globin and β-globin peaks were observed in the dried blood matrix in comparison to the supernatant, highlighting that the majority of inhibitors actually exist within the dried blood matrix and were not present in the liquid phase.

2.4. Highly Sensitive Biphasic LAMP without Purification

For detection of DNA with high sensitivity, it is crucial for primers and polymerase to locate the target DNA easily and initiate the amplification faster. To identify such conditions, we performed the characterization on the biphasic protocol using three parameters: drying time, volume of the blood, and material properties. **Figure 5**A depicts a decrease in sample weight over time as the sample dries, followed by an increase in the probability of amplification simply due to increasing concentration. We utilized a starting material volume of 30 μL, dried the sample at 95 °C, and measured its weight at different time points. The liquid components of blood evaporated during drying, and the percentage of weight loss was recorded, with a starting weight of 100% and 40% remaining after 10 min of drying. After 20 min of drying, the weight remained constant and saturated at 20%. This weight loss caused by the loss of liquid can contribute to the increased concentration per unit mass or per unit volume, as the target DNA concentration remains the same during the drying process. This highlights the potential improvement in the theoretical limit of detection (LOD) using this engineering design. For instance, we simulated the probably of amplification of 10 copies/30 μL concentration sample using Poisson statistics.[16,17] The chance of detecting at least one genome copy increased from 28% when blood was not dried to 54% when blood was dried for 10 min, as the weight decreased by 44% and the DNA concentration increased proportionally to the evaporation rate. When the drying time exceeded 20 min, it caused the sample to contract and lose contact with the tube (Figure S1C, Supporting Information). This resulted in the entire dried blood matrix floating instead of remaining fixed at the bottom of the tube when buffer and reaction mix were added, causing the solid phase to intrude into the liquid phase and interfere with fluorescence reading. Thus, excessive drying time can adversely affect detection probability.

The sample volume related to the diffusion distance is the second major component for achieving better sensitivity. We used

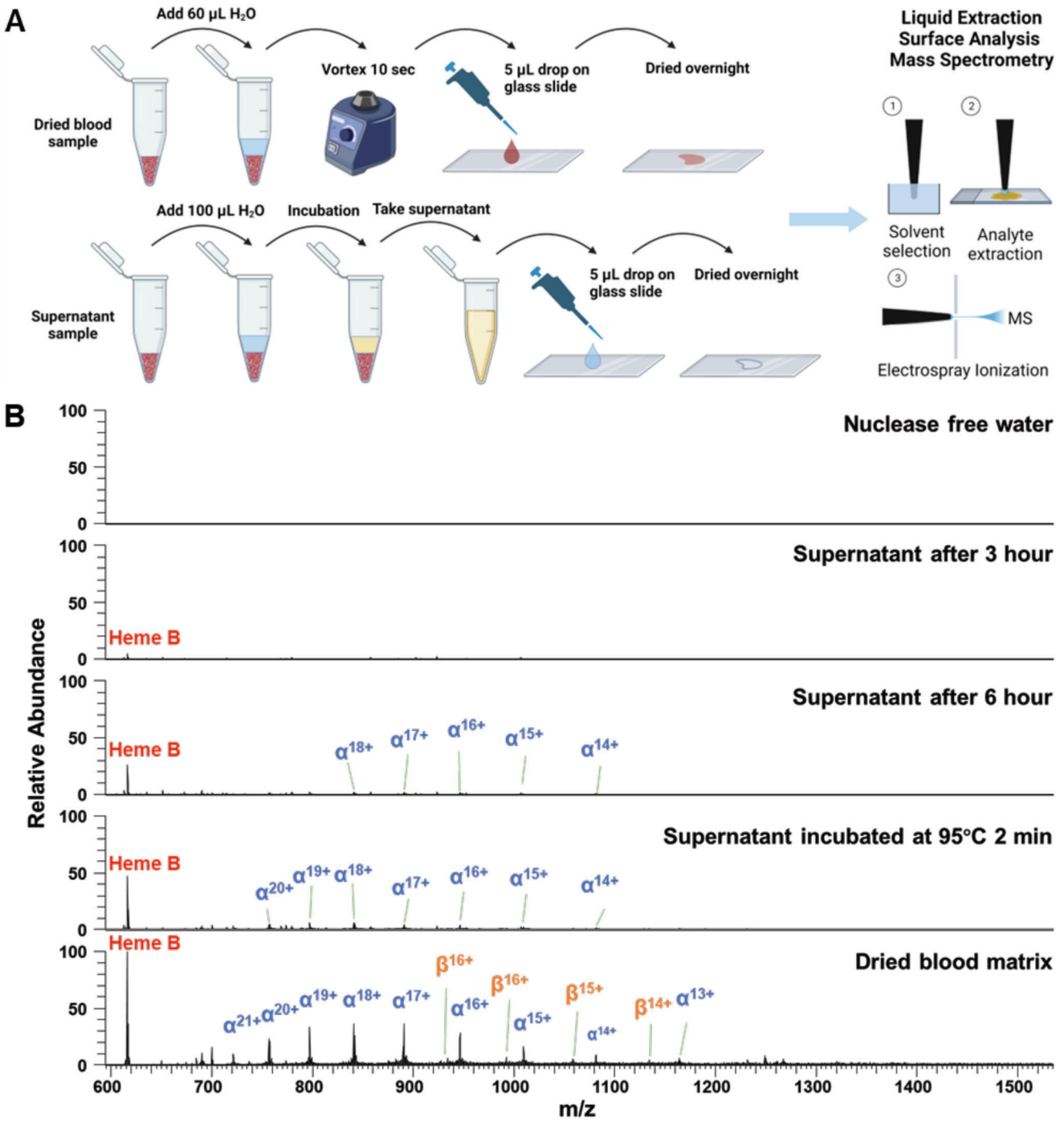

Figure 4. LESA-MS workflow and results for the analysis of inhibitor levels either inside of or released from the dried blood matrix. Protocols of the pre-processing for different types of samples: A) dried blood matrix and supernatant incubated with different conditions. B) Inhibitor levels for heme B, α-globin and β-globin measured by LESA-MS with various conditions: nuclease free water, supernatant, and dried blood matrix. Major peaks corresponding to heme B, α-globin and β-globin in different charge states were labeled. Peak abundance was normalized to heme B intensity in dried blood matrix.

whole blood sample into two groups: one with 30 µL in one tube and the other with three tubes, each containing 10 µL. The 30 µL sample was dried at 95 °C for 10 min, resulting in a 57% weight loss, while the three tubes of 10 µL samples were dried at 95 °C for 5 min, resulting in a 61% weight loss (Figure S1E, Supporting Information). LAMP biphasic reaction was conducted for both samples, and the results were compared. We considered the 30 µL sample in one tube to be the same as the three tubes of 10 µL samples. This division doesn't change the fact that combining the amplification results from all three tubes yields a single result. Given this equivalence, their expected amplification values are identical. In other words, when we divide the sample (1 copy

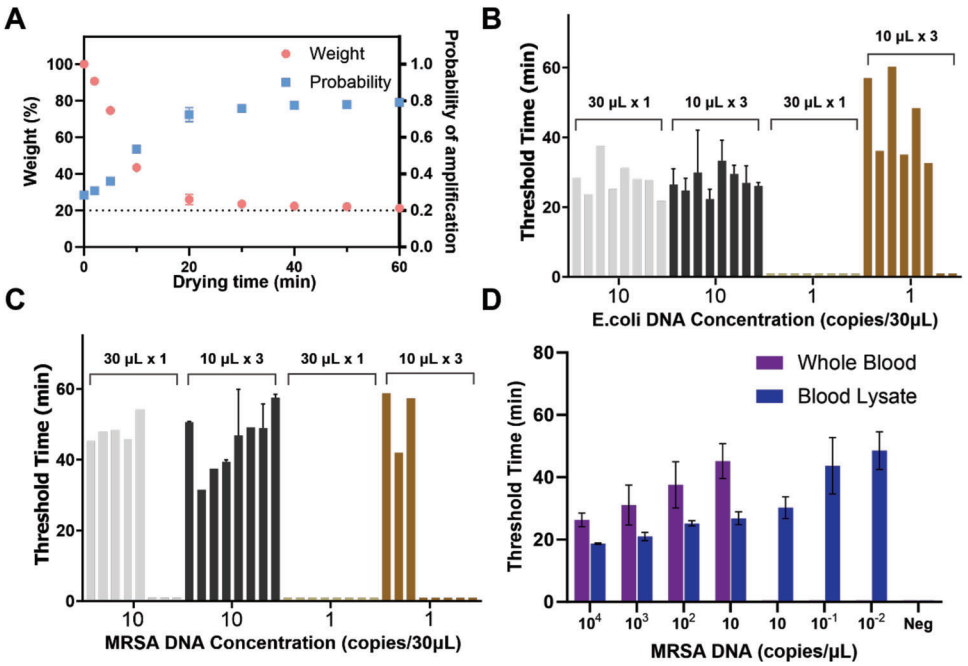

Figure 5. Characterization of biphasic assay sensitivity with various conditions (drying time, sample volume and material properties). A) Longer drying time results in increased target concentration (target amount/unit volume) and increased calculated theoretical probability of amplification. B,C) Sample volume affects the diffusion distance and hence the probability of amplification. LAMP characterization with the comparison between two sample groups: 30 µL in a one tube versus three tubes with 10 µL each using B) *E. coli* DNA and C) *MRSA* DNA spiked in whole blood. D) Effects of material properties on the detection sensitivity using whole blood (WB) and blood lysate (BL).

of *E. coli* DNA in a 30 µL volume of whole blood) into 3 tubes, any one of the three tubes could potentially contain the 1 copy of *E. coli* DNA within a 10 µL volume of whole blood. The threshold time was characterized using whole blood spiked with either *E. coli* DNA (Figure 5B) or MRSA DNA (Figure 5C) at concentrations of 10 copies and 1 copy/30 µL. In the case of 10 copies/30 µL *E. coli* DNA spiked in whole blood (Figure 5B), both sample types (30 µL x 1 and 10 µL x 3) showed all 8 replicates being amplified. However, when 1 copy/30 µL *E. coli* DNA was spiked in whole blood, only the 10 µL x 3 sample group showed amplification (6 out of 8 replicates). Similar observations were made when using MRSA DNA (Figure 5C). The 10 copies/30 µL MRSA DNA spiked in whole blood showed more amplification replicates when using the 10 µL x 3 sample, while the 1 copy/30 µL MRSA DNA demonstrated amplification only for the 10 µL x 3 sample (3 out of 8 replicates). The observed phenomenon can be explained due to changes in diffusion distance.[18] Since the target DNA is retained during blood drying, the key factor is whether the amplification agents can reach the target DNA, which is mainly driven by diffusion within the dried blood matrix. In the case of the 30 µL sample in one tube, the diffusion length is longer. Consequently, primer and polymerase may not be able to reach the target DNA due to the dead-end microfluidic structure within the dried blood matrix. By dividing the sample into multiple tubes, the physical diffusion distance can be reduced. This distance change could also affect the diffusion distance of amplicons diffusing out to the supernatant for fluorescence detection once amplification is complete. Further explanation based on the estimation and simulation data is shown in Figure S2 (Supporting Information). Additionally, using a smaller volume per well with much larger number of wells can reduce the time required for drying and potentially lowering the drying temperature. Application of this approach to larger volume used in clinical applications (≈5 mL) using automated division of sample, drying, distribution of amplification reagents, and fluorescence reading provide exciting opportunities for next steps. Furthermore, the ability of the primers to initiate and amplify the target DNA at a sufficient speed is another crucial parameter for a successful biphasic assay. The difference in the threshold time and the number of amplified replicates between *E. coli* and MRSA primer highlights the importance of the primer's ability for the amplification process.

Finally, the material properties can significantly impact the sensitivity of the assay, as demonstrated in Figure 5D. We compared the characteristics of whole blood and blood lysate, which are two primary starting materials for blood diagnostics. We first prepared the whole blood and blood lysate, spiked the MRSA DNA into each material, and tested them with biphasic LAMP reaction. Surprisingly, there was a three-magnitude order difference in LOD (10 copies μL^{-1} for whole blood and 0.01 copy μL^{-1} for blood lysate using a volume of 30 µL, Figure 5D). In case of blood lysate, it could successfully detect all 0.1 copy μL^{-1} 6 out of 6 replicates and 4 out of 6 replicates for 0.01 copy μL^{-1}. To begin with, we compared the physical properties, such as the shape of the dried blood matrix, between whole blood and blood lysate. Once we dried the two materials, we cut the bottom of the tube and examined the surface (Figure S3, Supporting Information). In the case of whole blood, it did not create a hollow space in the center, whereas blood lysate formed a large hole in the middle of the structure. This can be explained by the coffee ring effect, where drying occurs faster at the edges and leaves

a stain around the perimeter.[19] However, in the case of whole blood, this effect is reduced due to compaction toward the inside caused by coagulation factors such as platelets (Figure S3B,D, Supporting Information). Additionally, whole blood is a multicomponent agent, minimizing the coffee ring effect.[20] Due to the absence of a hole in the center, the dried blood matrix created from whole blood requires a longer diffusion distance, significantly limiting sensitivity. Conversely, biphasic LAMP in the dried blood matrix from blood lysate can be easily achieved because the reagents can reach the target DNA without significant diffusion distance. This highlights that material properties can have a substantial impact on the limit of detection (LOD) and optimizing the starting material could be a solution for current blood diagnostics. As a result, faster amplification (i.e., reduced threshold times) were achieved when using blood lysate as a starting material for blood drying (10^4-10 copies μL^{-1} concentration in Figure 5G5D), as well as 1000 times higher sensitivity (from 10 to 0.01 copies μL^{-1}). This suggests that using less thick, homogeneous, and platelet-free blood can lead to highly sensitive detection. Interestingly, our previous research verified that for dried blood lysate, sensitivity of the biphasic assay was not compromised under the absence of the heating step 2 process. To be specific, dried blood matrix with (66.2% porosity) and without (11.3% porosity) heating step 2 has shown the same LOD of ≈1 CFU mL^{-1} for *E. coli* and MRSA, meaning that in case of blood lysate, there is no need for heating step 2 and high porosity for diffusion of reagents. This suggests that material properties, rather than porosity, can have a more significant impact on assay sensitivity. However, it is important to note that this sample processing can result in target molecule loss, and therefore, optimization of the biphasic protocol should be based on the specific target molecule and assay purpose. In addition to the three properties mentioned, there are other characteristics that can affect sensitivity. For example, the difference in density between whole blood (985 g μL^{-1}) and blood lysate (946 g μL^{-1}) can impact the evaporation rate (Figure S1F,G, Supporting Information), with blood lysate evaporating at a higher rate due to its lower density (63% evaporation compared to 54% for whole blood). This evaporation difference can result in a higher concentration of target molecules in blood lysate, increasing the chances of successful amplification. Another study has reported that a lower hematocrit (HCT) value and a lower ratio of red blood cells (RBCs) in whole blood may result in increased cracking and subsequently higher surface area when the blood is dried.[21] This finding suggests that blood lysate samples may exhibit greater sensitivity due to the increased surface area.

The biphasic method offers versatility and potential as a diagnostic tool for detecting viruses such as HBV, HCV, and HIV. For example, viruses with a thinner envelop covering genetic material compared to bacteria, such as HBV, may be detected directly using simple blood drying. This is because the process of drying at high temperature can have the additional effect of viral lysis and release of DNA, resulting in both DNA extraction and inhibitor inactivation (purification). This provides advantages over current HBV diagnostic methods, which typically require processing whole blood through centrifugation to obtain plasma or serum samples since centrifugation can result in the loss of a significant amount of HBV due to its low density.[22] However, processing RNA, which is highly sensitive to heat treatment, presents a significant challenge. The use of high temperatures, such as 95 °C, during blood drying intended to expedite the drying process could damage genetic materials of interest. Reduction in drying time for minimizing the damage could leave the blood undried due to inadequate drying time and cause the dried blood matrix to resuspend into the liquid phase, decreasing amplification efficiency due to inhibitors and interfering with fluorescence readings. As the volume of the sample increases, drying time is also prolonged, making high temperatures necessary to speed up the process. To detect RNA-based viruses, it is necessary to use RNA-preserving agents and perform drying at low temperatures and new approaches would have to developed to address this important need.

2.5. Application of Biphasic Reaction to RPA

Next, we demonstrated that recombinase polymerase amplification (RPA) is compatible our biphasic protocol. RPA is also an isothermal amplification process operating at 37 °C and can be used at point the of care or in low resource setting a with simple heater.[23] We utilized the commercial RPA Exo kit from TwistDX company and adapted it for biphasic reaction. We performed heating step 2 only with nuclease-free water and did not include any buffer component from the RPA kit (**Figure 6**A). The reaction master mix from the RPA Exo kit, comprising forward and reverse primers, Exo probe, and rehydration buffer, was added to the sample prepared through heating step 2. The RPA reaction with fluorescence detection was carried out at 37 °C for 60 min. RPA employs a recombinase-primer complex to target the homologous sequence, similar to the mechanism used by PCR.[24] However, RPA requires an Exo probe for real-time fluorescence detection, as conventional Taq-Man probes cannot be used due to Taq polymerase's exonuclease activity.[25] This activity progressively digests the displaced strand during the strand displacement process, inhibiting DNA amplification. The Exo probe contains an abasic residue that serves as a substrate for exonuclease, which can cleave tetrahydrofuran (THF) only after binding to the target sequence. This cleavage separates the fluorophore (F) from its quencher (Q), allowing for fluorescence detection (Figure 6B). Utilizing the biphasic protocol with the RPA method, the amplification threshold time of Methicillin-sensitive Staphylococcus aureus (MSSA) DNA spiked in whole blood was determined and depicted in Figure 6C.[16] The influence of heating step 2 on the biphasic RPA assay was evaluated by testing MSSA DNA spiked in whole blood with and without heating step 2. The detection sensitivity increased to a single copy when heating step 2 was employed, compared to 100 copies μL^{-1} without heating step 2. The advantage of utilizing the biphasic method over the direct RPA without blood drying was demonstrated in Figure 6D, where two concentrations of MSSA DNA (1000 and 100 copies μL^{-1}) were used, and their fluorescence levels were compared. The direct RPA assay using whole blood exhibited minimal fluorescence increment even after amplification due to the interference of red blood cells (RBC) with the fluorescence reading. Conversely, a fluorescence difference ten times higher was shown in the biphasic RPA assay because the the fluorescence reading was performed in the clear liquid phase. Finally, the biphasic RPA assay successfully amplified the MSSA pathogen spiked

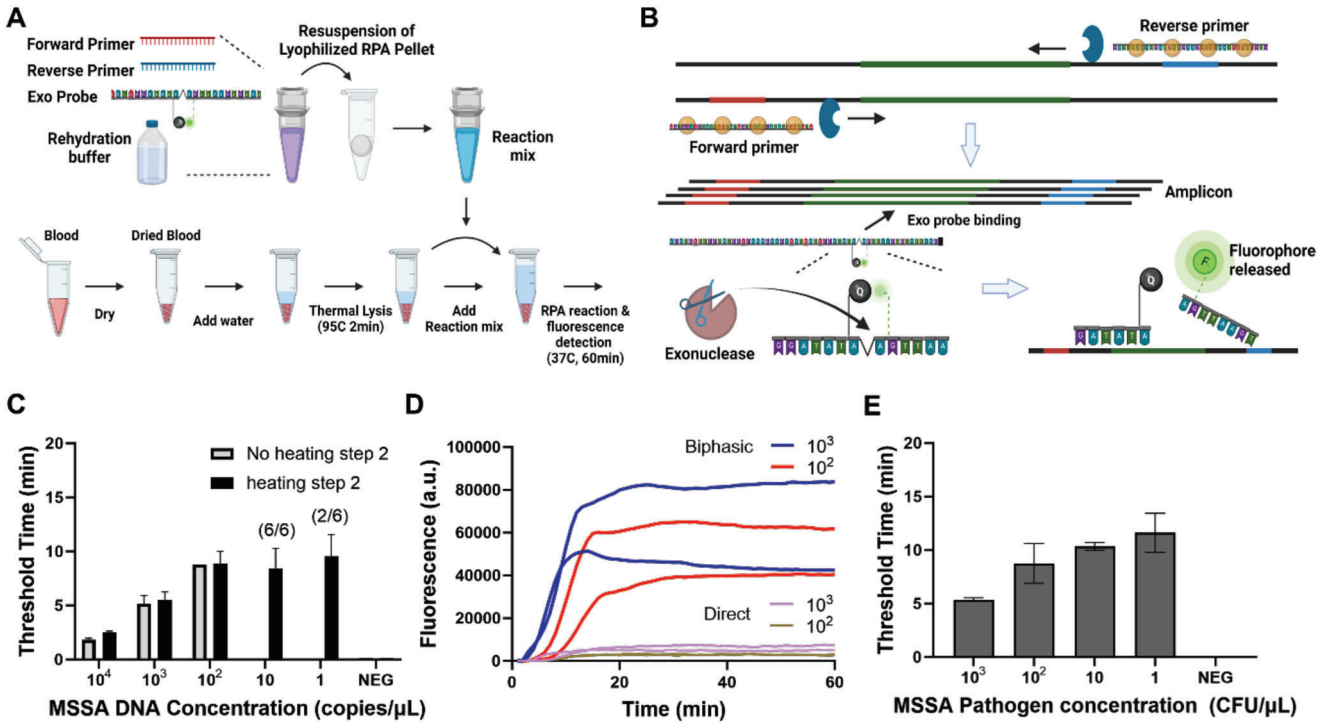

Figure 6. Application of blood drying and biphasic reaction using recombinase polymerase amplification (RPA). A) Schematic workflow of RPA for biphasic reaction. B) Fluorescence detection method using the RPA Exo probe. C–E) Limit of detection characterization using different DNA concentration and pathogen concentration. C) Effects of the heating step 2 for achieving a higher sensitivity. Two replicates were used for 10^4 –10^2 copies µL^{-1} concentration and 6 replicates were used for 10 and 1 copy experiments D) Fluorescence level comparison between biphasic and direct reaction using 10^3 and 10^2 copies µL^{-1}. 10 times higher signal to noise ratio was obtained. E) Assay characterization using pathogen spiked in whole blood.

in whole blood at a concentration of 1 colony-forming unit per µL without any nonspecific amplification for the negative control (Figure 6E).

The LAMP method is advantageous for use in the biphasic reaction due to its optimal conditions. First, it is an isothermal amplification technique that does not damage the dried blood matrix and does not release the inactivated inhibitors from the structure (Figure 3C,E). Additionally, it uses a robust Bst polymerase that can function at higher concentrations of inhibitors compared to other techniques such as PCR or RPA.[26] The tolerance of the Bst polymerase is not limited to blood inhibitors and can process crude samples such as nasal swabs and saliva.[27,28] Therefore, the biphasic approach can be extended to other specimens that form a solid-phase structure for the diffusion of amplification reagents.[13] RPA, on the other hand, has some drawbacks due to its assay format and the use of lyophilized products and viscous reagents, limiting their diffusion within the dried blood matrix. Moreover, additional steps such as adding Magnesium Acetate with centrifugation may make the automation of the assay difficult. In this paper, instead of focusing on optimizing the RPA assay for such large volumes (≈1 mL), we aimed to provide proof of concept by demonstrating the possibility of detecting both DNA and the pathogen directly in whole blood. Achieving effective detection in large volumes would require additional optimization of the RPA assay, and our goal was to showcase the versatility of the biphasic method by applying it to different isothermal amplification techniques. Regardless, we show the compatibility of RPA with our biphasic protocol.

3. Conclusion

In summary, we investigated the biphasic reaction mechanism with a focus on understanding the inhibitor inactivation to improve assay sensitivity. Our findings showed that most inhibitors exist in the solid phase of the dried blood matrix, as measured using LESA-MS. We also observed that the biphasic treatment efficiently purifies the liquid phase, resulting in low inhibitor levels even when compared to highly diluted blood samples. We optimized various parameters, such as drying time, diffusion distance, and material properties, to enhance assay sensitivity using the biphasic protocol. Also, the study demonstrated a novel application of the biphasic reaction in recombinase polymerase amplification (RPA), expanding its potential use in general nucleic acid amplification techniques. An added benefit of the biphasic method that bypasses the extraction and purification is that the entire sample processing steps only require a simple heating instrument, reducing both cost and instrumentation complexity. We believe that application of this solution to existing methods will have widespread use in the detection of various blood-borne diseases, including bacteria and viruses, and could usher in a new era of blood diagnostics.

4. Experimental Section

Blood Preparation: Whole venous blood samples were purchased from the vendor BIOIVT using the HUMANWBK2-0101184, Human Whole Blood K2EDTA Gender Unspecified. After collection, the blood

samples were kept in a sample rotisserie at 4 °C until used for experiments. Tenfold serial dilutions of DNA or bacterial stocks were performed to achieve the appropriate concentration.

Biphasic Protocol: Pre-Treatment, Heating step 1 (Drying), and Heating step 2: The pre-treatment of whole blood to obtain blood lysate was performed following the protocol developed by Ganguli et al. RBC lysis was achieved using ACK lysis buffer (ThermoFisher Scientific), while pathogen mechanical lysis was carried out using 100 µm glass disruptor beads (Scientific Industries, Inc.). In brief, 800 µL (or 1 mL) of blood was mixed with 40 µL (90 mg) of glass disruptor beads and 600 µL of ACK lysis buffer in a 1.5 mL tube. The blood and lysis buffer were mixed by manual pipetting and left to incubate at room temperature for 5 min. After centrifugation at 6000 g for 10 min, the lysed blood supernatant was removed, and 200 µL of TE buffer was added to the tube for bead lysis. The tubes were vortexed at 3000 rpm for 10 min, followed by a brief 10 s centrifugation. The resulting material, i.e., blood lysate, was stored at 4 °C until further use.

For drying, either whole blood or blood lysate (30 µL) was heated at 95 °C for 10 min in PCR tubes, followed by heating step 2 after adding water at 95 °C for 2 min. Inhibitor characterization was performed using mainly water, but buffer mix components without polymerase and primers were used for heating step 2 in the LAMP reactions.

Hemoglobin Assay: The detection of hemoglobin was carried out using the Hemoglobin Assay Kit (ab234046, abcam), which utilizes colorimetric analysis based on the manufacturer's protocol. First, the Hemoglobin Standard provided in the kit was used to generate a standard curve. Subsequently, 20 µL of each sample was added to individual wells in a 96-well plate, followed by the addition of 180 µL of Hemoglobin Detector to form a color complex. The absorbance was measured at 575 nm using the Synergy HT (BioTek) Microplate Reader, and the hemoglobin concentration was calculated using the standard curve. For dried blood samples, 96 µL of water was added and incubated for 10 min before measuring 20 µL of the resulting solution. For the 20x and 100x diluted samples, 20 µL of the sample was directly added to the 96-well plate.

Immunoglobulin G (IgG) Assay: The detection of IgG was performed using the Human IgG ELISA Kit (ab195215, abcam) according to the manufacturer's protocol. First, a standard curve was generated using the serially diluted standards provided in the kit. Next, 50 µL of each sample was added to individual wells in a 96-well plate, followed by the addition of 50 µL of Antibody Cocktail to each well. The plate was then incubated at room temperature on a plate shaker set to 400 rpm for 40 min. Following three washes with 1X Wash Buffer PT, 100 µL of TMB Development Solution was added, and the plate was incubated for 5 min in the dark on a plate shaker set to 400 rpm. Subsequently, 100 µL of Stop Solution was added to each well, and the plate was shaken for 1 min. The absorbance at 450 nm was measured using the Synergy HT (BioTek)Microplate Reader. For dried blood samples, 96 µL of water was added and incubated for 10 min before measuring 50 µL of the resulting solution. For the 20x and 100x diluted samples, 50 µL of the sample was directly added to the 96-well plate.

Liquid Extraction Surface Analysis Mass Spectrometry: LESA-MS was performed by TriVersa NanoMate LESA (Advion, Inc., USA) coupled with a Thermo Scientific Q Exactive mass spectrometer. The dried water, supernatant, and dried blood matrix on slide was attached onto the LESA universal adaptor plate. The image was acquired using an Epson Perfection V370 photo scanner with 300 DPI resolution. Advion ChipSoftX software was used to select sample spots and edit sampling methods. A solution of 50% acetonitrile in H2O with 0.1% formic acid was used for extraction and ionization. All solvents and regents are LC-MS grade. For surface extraction, 7.0 µL solvent was aspirated from solvent reservoir. Then a robotic arm moved solvent above the sample spot, and dispensed 4.0 µL was onto surface at height set as −0.3 mm. The solvent stayed for 4 s there and 4.5 µL was aspirated at −0.5 mm height. The dispense-aspirate cycle was repeated once before infused into the mass spectrometer via chip-based electrospray ionization through an HD_A_384 nano ESI chip (Advion, Inc). An airgap was applied prior to engaging chip. The gas pressure was set at 1.00 psi and voltage was 1.70 kv at positive mode. For mass spectrometry, spectra were acquired in positive full scan mode at range m/z 600–4000. The capillary temperature was set at 200 °C. The resolution was set as 140000 at 200 m/z. AGC target was set as 1e6, and the maximum injection time was 100 ms. Each scan was composed by 2 microscans.

Poisson Statistics: Given a certain number of target DNA molecules (m) distributed across a dried blood matrix (n), the probability of the matrix containing k copies of the targets could be calculated using the Poisson distribution ($\Pr(X = k)$). The expected value of this distribution corresponds to the average occupancy rate (λ), which was defined as the ratio of the number of target molecules (m) to the volume of dried blood matrix (n).

$$\lambda = \frac{m}{n} \tag{1}$$

$$\Pr(X = k) = \frac{e^{-\lambda}\lambda^{k}}{k!} \tag{2}$$

To determine the probability of at least one amplification occurring in the dried blood matrix, the probability of no molecule being allocated should be subtracted from 1. This can be mathematically expressed as:

$$\Pr(\textit{at least one amplification}) = 1 - \Pr(X = 0) = 1 - e^{-\lambda} \tag{3}$$

Thus, the probability of at least one amplification occurring is a function of the average occupancy rate (λ), which can be influenced by factors such as the drying process and subsequent weight loss.

DNA and Bacteria: The genomic DNA of MRSA strain HFH-30106, NR-10320, was acquired from BEI Resources, and subsequently aliquoted for experimentation or diluted to the appropriate concentration in either whole blood or blood lysate. Similarly, the genomic DNA of *E. coli* (O157:H7), NR-4629, was obtained from BEI Resources and prepared accordingly. For experiments involving pathogenic bacteria, MSSA strain MN8 was obtained from BEI Resources, and all stocks were stored at −80 °C.

Primer Sequences: All primer sequences for the LAMP and RPA reactions were synthesized by Integrated DNA Technology. Primer sequence information is shown in Table S1 (Supporting Information). The primer sequences employed in the detection of Escherichia coli mal B gene and MRSA mec A gene via LAMP were sourced from previously published LAMP primers, as documented in references.[29,30] Additionally, the primer sequence used for the detection of MSSA vicK gene through RPA was obtained from previously published RPA primers, as documented in reference.[16]

LAMP Reactions: The LAMP assay includes several components, including a final concentration of 1x isothermal amplification buffer (New England Biolabs), 1.025 mmol L^{-1} of each deoxyribonucleoside triphosphate (dNTP), 4 mmol L^{-1} of MgSO4 (New England Biolabs), and 0.29 mol L^{-1} of Betaine (Sigma-Aldrich). Each component was stored according to the manufacturer's instructions, and a fresh mix containing all components was prepared prior to each reaction. In addition to the buffer components, the reaction also includes 0.15 µM of F3 and B3, 1.17 µM of FIP and BIP, and 0.59 µM of LoopF and LoopB primers, 0.47 U μL^{-1} of Bst 2.0 WarmStart DNA Polymerase (New England Biolabs), 1 mg mL^{-1} of BSA (New England Biolabs), and 0.74x of EvaGreen (Biotium), a double-stranded DNA intercalating dye. In the biphasic reaction, the total final reaction volume was 96 µL, consisting of 72 µL of buffer mix and 24 µL of reaction mix. After drying the blood, the buffer mix was added for wet heating step 2 at 95 °C for 2 min, followed by the addition of the reaction mix for the final LAMP biphasic reaction. LAMP reactions were performed using 0.2-mL PCR tubes in the QuantStudio 3 system (Applied Biosciences) at a constant temperature of 65 °C for 60 min. Fluorescence data were measured and recorded every minute. Normalization of the raw fluorescence data was performed, and the amplification threshold time was determined by identifying the point at which 20% of the normalized fluorescence threshold was reached. For the 10 µL x 3 tubes reactions, 1/3 of the reaction volume (32 µL, composed of 24 µL of buffer mix and 8 µL of reaction mix) was used in each tube to maintain a consistent volume ratio between the blood sample and reaction mix. The results from the three

tubes were then combined to form one set of samples and compared with the 30 µL x 1 tube sample.

RPA Reactions: A commercial RPA kit, TwistAmp exo, was purchased from TwistDx (United Kingdom), and the RPA assay was conducted as per the manufacturer's manual with some adaptations. First, in a 1.5-mL tube, 2.3 µL of forward and reverse primers (10 µM), 0.7 µL of exo probe, and 32.3 µL of rehydration buffer were added and mixed manually before transferring to the lyophilized RPA pellet for resuspension. Following the preparation of the dried blood matrix, water (instead of buffer mix) was added for heating step 2 at 95 °C for 2 min, followed by the addition of the reaction mix. For the sample, 20 µL of whole blood was used with 8 min of drying at 95 °C, followed by adding 15 µL of water for heating step 2 and 35 µL of reaction mix. Before loading the sample, 2.5 µL of 280 mM Magnesium Acetate (MgOAc) was added to the lid of the PCR tubes and centrifuged. The RPA reactions were performed using 0.2-mL PCR tubes in the QuantStudio 3 system (Applied Biosciences) at a constant temperature of 37 °C for 60 min. Fluorescence data were measured and recorded every minute. Normalization of the raw fluorescence data was carried out, and the amplification threshold time was determined by identifying the point at which 20% of the normalized fluorescence threshold was reached.

Bacterial Culture: The media and agar plates were obtained from the Cell Media Facility at the University of Illinois Urbana-Champaign (UIUC). Tryptic soy broth and agar were utilized for the culture of MSSA. Bacteria were grown in the broth at 37 °C for 16 h overnight, following which PBS stocks were prepared. The PBS stocks of pathogens were prepared as follows: 250 µL of the overnight culture was centrifuged at 5000 × g for 10 min to create a bacterial pellet, which was then washed twice with 1× PBS. Finally, the bacterial pellet was diluted in 1 mL of PBS, and the resulting solution was aliquoted and kept at room temperature. Each PBS stock was used for a maximum of 4 days after culture.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

J.L., E.V., J.S., and R.B. designed research; J.L., S.Z., J.B., and A.Y.K., performed research; J.L., S.Z., E.V., J.S., and R.B. analyzed data; J.L., S.Z., E.V., J.S., and R.B. wrote the paper.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords

blood-borne pathogens, blood drying, DNA amplification, inhibitor inactivation, extraction and purification free amplification

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