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Bioluminescence DNA Hybridization Assay for *Plasmodium falciparum* Based on the Photoprotein Aequorin

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A bioluminescence DNA hybridization assay for the detection of *Plasmodium falciparum*, the most deadly species of malaria, using the photoprotein aequorin as a bioluminescent label has been developed. The current gold standard for the detection of malaria is light microscopy, which can detect down to ∼50 parasites/μL of blood, but has low-throughput, high costs, and requires high skill, which limit the applicability of the method, especially in the developing regions where malaria detection is mostly needed. The utilization of aequorin as a bioluminescence label offers the advantages of high signal-to-noise ratio and reliable detection down to attomole levels, allowing for the development of highly sensitive and miniaturized high-throughput bioluminescence assays. Herein, we developed a DNA hybridization assay for the detection of *P. falciparum* based on the competition between the target DNA and the signal generating DNA streptavidin−aequorin for hybridization with the probe DNA. This bioluminescence hybridization assay demonstrated a detection limit of 3 pg/μL and was employed for the detection of target DNA in standard and spiked human serum samples. The DNA hybridization assay was developed in a microplate format without the need for sample PCR amplification, showing the potential suitability of this method in the parallel analysis of samples by low-trained personnel, such as that typically encountered in developing regions.

Plasmodium falciparum is the most prevalent and deadly species of malaria, causing almost 90% of malaria-related deaths.[1] There are between 300 and 500 million cases of malaria each year, killing up to 3 million people annually, and 90% of these deaths occur in Africa.[2] The current gold standard for the detection of malaria is light microscopy, which has been used since the identification of the sporozoite *P. falciparum* in 1880.[3] This is a powerful method that is able to identify the number and species of the parasite present in red blood cells, but has its own limitations as it requires high technical skill, which comes with high costs and considerable health infrastructure.[3,4] Additionally, the throughput of the method is very low, as a skilled pathologist requires 1 h or more in order to determine the infection per patient.[2,3]

Several diagnostic techniques have started to emerge as promising alternatives to light microscopy. One such example includes rapid diagnostic devices, i.e., dipsticks, based on immunochromatographic assays to detect the sporozoite.[5] These devices are currently the state-of-the-art in the detection of malaria, as they are rapid and can detect levels of >100−500 parasites/μL of blood.[6,7] Nevertheless, they are not capable of giving quantitative information on the number of parasites present in the sample, which is a parameter required in the follow up of patients and for the success of their treatment.[3] Hybridization assays for the detection of *P. falciparum* have previously been developed, but these methods can be costly and time-consuming as they involve sample amplification by PCR followed by other analytical steps.[8] A self-contained electrochemical enzyme-linked hybridization assay method was recently developed for the detection of *P. falciparum*.[9] This method presented a detection limit of 1.4 ng/mL or 46 pM, which may be useful for clinical applications[3] but does not offer a high-throughput; i.e., it took 6 h to analyze 64 samples, in addition for the need of skilled personnel and special instrumentation. Laser desorption mass spectrometry has also been utilized for the diagnosis of malaria.[3,10] This method is useful for the rapid screening of blood samples, but does not have the ability to provide parasite speciation; therefore, it has to be followed by microscopy or dipstick testing in order to obtain this information.[3] Thus, there is still a need for other assays suitable for the fast and high-throughput detection of *P. falciparum* in clinical diagnostics, applicable in areas with limited resources.

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Additionally, such methods could also prove useful for the fast and high-throughput evaluation of drugs and vaccines by following the parasite behavior in the presence of these drugs.[3] To that end, our research focused on the development of a rapid and high-throughput bioluminescence DNA hybridization assay for the detection of *P. falciparum* based on the use of the photoprotein aequorin as the label.

Aequorin is a bioluminescent protein native to the jellyfish *Aequorea victoria*, which is composed of the apoprotein and the chromophore coelenterazine.[11] In the presence of Ca^{2+}, aequorin undergoes a conformational change that results in an excited state of coelenteramide followed by the emission of light at 469 nm with a quantum yield of Φ of 0.2.[12,13] Given that bioluminescence is a rare phenomenon in nature, aequorin demonstrates a very low, virtually zero, bioluminescence background in many environments, such as that of biological fluids, thus increasing the sensitivity of the method.[14] Compared to other chemiluminescent reactions, aequorin has the highest signal-to-noise ratio and can be detected down to attomole levels, which makes it amenable for miniaturization and small-volume analysis.[15] The bioluminescent signal of aequorin is generated within 3 s after the addition of Ca^{2+}, that being a lower incubation time than most enzyme reactions.[16] Additionally, there is no need for an excitation light source. Due to these advantages, aequorin has been previously employed in the development of DNA hybridization assays, which relied on the PCR amplification of the sample.[8,17,18]

In this work, we employed the photoprotein aequorin as a highly sensitive label in the development of a bioluminescence DNA hybridization assay for *P. falciparum* that does not require PCR amplification of the samples. To accomplish this, we employed a genetically engineered mutant of the photoprotein aequorin that incorporates a unique cysteine at position five,[19] which was site-directly conjugated to the target DNA and was used as a label to report the amount of DNA hybridized on the platform. The bioluminescence DNA hybridization assay developed for *P. falciparum* showed a detection limit of 3 pg/μL and was used for the detection of target DNA in spiked human serum samples. The assay was developed in a microplate with no need of PCR amplification of the samples. This assay is potentially suitable for multiplex analysis, even in laboratories that lack sophisticated equipment.

EXPERIMENTAL SECTION

Chemicals and Materials. Tris(hydroxymethyl)aminomethane (Tris) free base and ethylenediaminetetraacetic acid (EDTA) sodium salt were purchased from Fisher Scientific. Sodium chloride was obtained from EMD Chemicals Inc. (Islandia, NY). Calcium chloride, Tween 20, bovine serum albumin (BSA), and human serum from clotted human male whole blood were purchased from Sigma-Aldrich (St. Louis, MO). Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL). Streptavidin was obtained from Chemicon. Tris(2-carboxyethyl)phosphine (TCEP), and Reacti-bind neutravidin-coated microplates were purchased from Invitrogen (Carlsbad, CA). Coelenterazine was obtained from Biosynth International (Naperville, IL). The Bradford protein assay kits were from Bio-Rad Laboratories (Hercules, CA), while the dialysis tubing with MW 50 000 was purchased from Spectrum Laboratories, Inc. All solutions were prepared by using deionized (Milli-Q water purification system, Millipore, Bedford, MA) distilled water.

DNA Probes. The DNA probes that were utilized were specific to *P. falciparum*.[20] In more detail, the oligo sequence used was specific for the small-subunit ribosomal RNA (Invitrogen) with a sequence 5′-biotin-TGTAGCATTTCTTAGGGAATGTTGATTT-TATAT-3′ (probe b-DNA, MW 10 629.23). The oligo sequences for the target DNA probes used were 5′-biotin-ATATAAAATCAA-CATTCCCTAAGAAATGCTACA-3′ (b-DNA, MW 10 501.16) and 5′-amine-ATATAAAATCAACATTCCCTAAGAAATGCTACA-3′ (target DNA, MW 10 244.84).

Conjugation of Streptavidin−Aequorin. The conjugation of streptavidin to aequorin was performed as previously described.[21] The mutant aequorin-5 that incorporated a unique cysteine at the five position[19] was expressed in *Escherichia coli* cells and purified using perfusion anion chromatography. A Bradford assay was used to determine the concentration of the purified aequorin solution. An aliquot of 2.66 mL of aequorin-5 with a concentration of 2.44×10^{-5} M was charged overnight with 130 μL of a 100 μg/mL native coelenterazine solution at 4 °C. An aliquot of 500 μL of a 5 mg/mL streptavidin stock solution was incubated with 20 μL of a 4 mg/mL solution of the linker sulfo-SMCC for 45 min at room temperature. The charged aequorin mutant was reacted with 54 μL of a 1 mg/mL TCEP stock solution for 45 min to reduce any disulfide groups. The aequorin mutant was then added to the streptavidin−SMCC solution and left to react at 4 °C overnight. The streptavidin−aequorin conjugate was then dialyzed using dialysis membranes with MW 50 000 three times for 12 h at 4 °C using a buffer solution of 30 mM Tris, 2 mM EDTA, pH 7.2 to remove the unreacted monomers.

Binder Dilution Study of the Probe b-DNA. Neutravidin-coated microplates were incubated overnight at 4 °C with 100 μL of blocking buffer (BB) containing 30 mM Tris, 2 mM EDTA, and 0.1% w/w BSA. Serial dilutions of the probe b-DNA were made in the standard buffer (SB), which contained 30 mM Tris, 2 mM EDTA, 0.1 M NaCl, and 1% w/w BSA, pH 7.2, in order to prepare the probe b-DNA solutions with concentrations varying from 0.1 to 100 nmol/L. A volume of 100 μL of each probe b-DNA solution was added to neutravidin-coated microplates in triplicate and was incubated with shaking for 1 h at room temperature. The excess probe b-DNA was then removed by washing three times with wash buffer (WB), which contained 30 mM Tris, 2 mM EDTA, 0.1% Tween 20, 1 M NaCl, and 1% w/w BSA, pH 7.2. An aliquot of

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100 μL of the 10 nmol/L b-DNA solution in SB was then added to each well, and the resultant mixture was incubated under shaking for 1 h at room temperature. Unbound DNA was then removed by washing three times with the WB. Then, 100 μL of a 2.44×10^{-4} M streptavidin−aequorin conjugate was added to each well, and the resultant mixture was incubated with shaking for 1 h at room temperature. Excess streptavidin−aequorin was removed by washing three times with the WB. Control solutions were also prepared using b-DNA and streptavidin−aequorin and tested to ensure that the luminescence signal obtained was not from nonspecific binding. The bioluminescence signal was triggered by the addition of an aliquot of 100 μL of 100 mM Tris-HCl, 100 mM $CaCl_2$, pH 7.5, in each well, and it was measured over a 5-s interval using a POLARstar Optima microplate luminometer from BMG labtech (Offenburg, Germany).

Binder Dilution Study of the b-DNA. The neutravidin-coated microplates were incubated at 4 °C overnight with BB to prevent any nonspecific binding. Then, an aliquot of 100 μL of a 10 nmol/L probe b-DNA solution in SB was added to each well of the neutravidin-coated microplates, and the resultant mixture was incubated with shaking for 1 h at room temperature. Excess DNA was then removed by washing three times with WB. Serial dilutions of the b-DNA stock solution were made using SB, and 100 μL of each b-DNA solution was added to each well, followed by incubation under shaking for 1 h at room temperature. The unbound b-DNA was then removed by washing three times with the WB. Then, 100 μL of a 2.44×10^{-4} M streptavidin−aequorin conjugate solution was added to each well, and the resultant mixture was incubated with shaking for 1 h at room temperature. The unreacted streptavidin−aequorin conjugate was removed by washing three times with the WB. Controls were also prepared using the probe b-DNA and streptavidin−aequorin to ensure that the bioluminescence signal measured was not a result of nonspecific binding on the wells of the microplate. The bioluminescence signal was triggered by the addition of 100 μL of 100 mM Tris-HCl, 100 mM $CaCl_2$, pH 7.5 in each well, and it was measured over a 5-s interval using the POLARstar Optima microplate luminometer

Dose−Response Curve in the Standard Buffer. The neutravidin-coated microplates were coated overnight with the BB in order to prevent nonspecific binding. An aliquot of 100 μL of a 10 nmol/L probe b-DNA solution was added to each well of the neutravidin-coated microplates, and the resultant mixture was incubated on a shaker for 1 h at room temperature. The wells were washed three times with the WB. Aliquots of 100 μL of target DNA solutions with different concentrations in SB were added to each well, were left to incubate for 1 h under shaking at room temperature, and then were washed three times with the WB. A volume of 100 μL of 10 nmol/L b-DNA in SB was added to each well and, after 1 h incubation, was washed three times with the WB. An aliquot of 100 μL of a 2.44×10^{-4} M streptavidin−aequorin conjugate was added to each well and incubated with shaking for 1 h at room temperature. The unbound streptavidin−aequorin was removed from each well by washing three times with the WB. The bioluminescence signal was triggered by 100 μL of 100 mM Tris-HCl, 100 mM $CaCl_2$, pH 7.5, and it was measured over a 5-s interval using the POLARstar Optima microplate luminometer.

Dose−Response Curve in Human Serum. To test the sensitivity of the DNA hybridization assay in biological samples, a dose−response curve was obtained as outlined above using aliquots of 100 μL of a human serum sample, which was spiked with varying amounts of target DNA (either 0.001, 0.01, 0.1, 1, 10, or 100 nmol/L final target DNA concentration). The standard addition and the analysis of each of these six spiked human serum samples was performed in five replicates, and the data presented for each sample were based on the average response of these five replicates ± the standard deviation. The detection limit of the aequorin-based DNA hybridization assay was calculated by the S/N of 3, by fitting of the curve to the resulted signal utilizing the software GraphPad Prism 4.0 for Windows (GraphPad Software, San Diego, CA).

Time Optimization. Time studies were performed in order to optimize the assay detection time. For this reason, each step of the dose−response curve was carried out as outlined above except for its duration, which lasted for either 15 or 30 min.

RESULTS AND DISCUSSION

In this work, we describe the design and development of a bioluminescence DNA hybridization assay that does not require PCR amplification of the sample for detection of the most deadly species of malaria, namely, *P. falciparum*. For that, we have employed the photoprotein aequorin, a highly sensitive bioluminescent label suitable for the development of miniaturized and ultrasensitive assays.[15] Specifically, a genetically engineered mutant of aequorin that contains a unique cysteine at position 5 (aequorin-5) has been utilized. The mutation at position 5 of the protein was found to result in a stable aequorin mutant that presented a greater bioluminescence activity than that of the wild-type aequorin. Moreover, this mutant aequorin retained its biological activity upon site-directed conjugation through the unique cysteine.[19] Aequorin-5 was conjugated to a single streptavidin molecule using the heterobifunctional cross-linker sulfhydryl- and amine-reactive sulfo-SMCC. The conjugate streptavidin−aequorin produced was then used as the bioluminescent label for the development of the assay.

The DNA hybridization assay was preformed by first immobilizing the probe b-DNA on neutravidin microplates through the strong interaction between biotin (b) and neutravidin (Figure 1). An aliquot of the target DNA at different concentrations was then incubated in the microplate, to promote the interactions with the immobilized probe DNA. Finally, the b-DNA (b-DNA) was added in the microplate in order to hybridize with the probe DNA that was left unbound by the target DNA. The bioluminescent label streptavidin−aequorin was then employed for interaction with the biotin of the b-DNA and the quantitation of the amount of b-DNA bound on the microplate (Figure 1). In other words, in this assay the target DNA, i.e., the analyte, competes with the b-DNA for hybridization on the microplate, which is detected by the signal of the streptavidin−aequorin. Therefore, this assay format yields an emission of bioluminescence by aequorin that is related to the amount of the target *P. falciparum* DNA and can thus give information about the status of a malarial infection of an individual.

A binder dilution study was first performed for the optimization of the concentration of the probe b-DNA used. More specifically, an aliquot of 100 μL of 10 nmol/L b-DNA was incubated with a

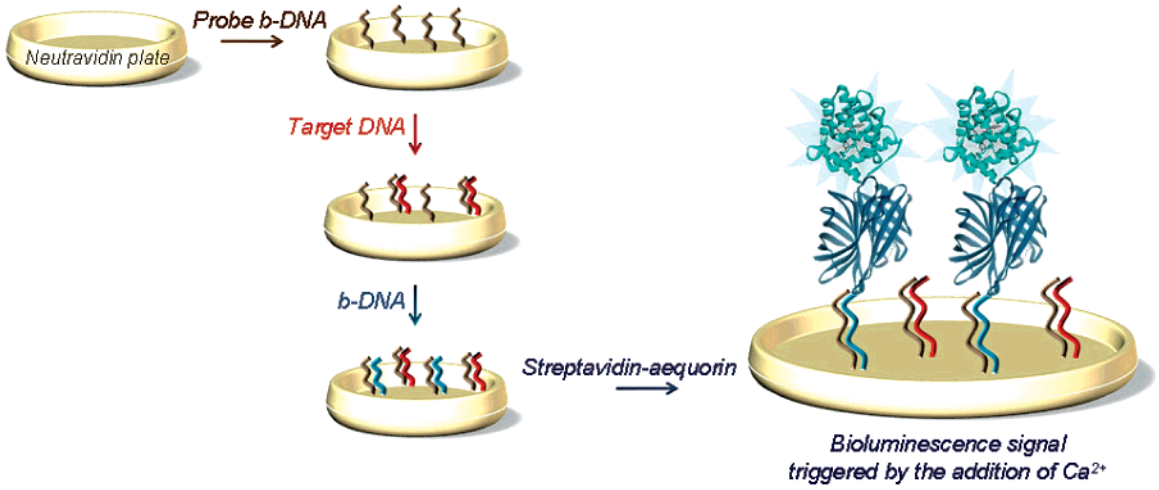

Figure 1. Schematic representation of the bioluminescence DNA hybridization assay developed for the detection of *P. falciparum*.

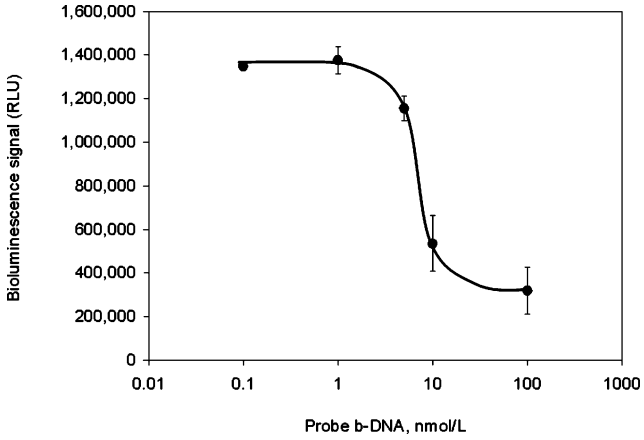

Figure 2. Optimization of the concentration of the probe b-DNA used for the development of the DNA hybridization assay for *P. falciparum*. The data are presented on logarithmic scale and are based on an average response of three samples ± the standard deviation (the standard deviations for some experimental points are so small they cannot be seen).

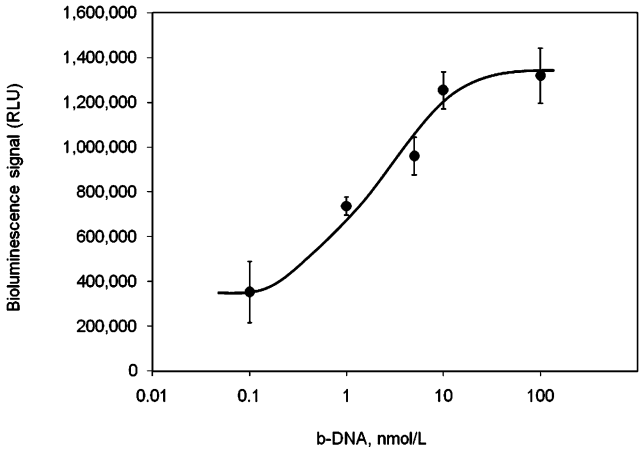

Figure 3. Optimization of the concentration of the b-DNA utilized for the development of the DNA hybridization assay for *P. falciparum*. The concentration of b-DNA is shown on logarithmic scale, while the error bars reflect the average response of three samples ± the standard deviation.

volume of 100 μL of different concentrations of probe b-DNA in the neutravidin microplates (Figure 2). During this study, an excess of the bioluminescent label streptavidin−aequorin was employed to ensure complete labeling and, therefore, the accurate detection of the b-DNA molecules immobilized on the microplate. From the binder dilution curve obtained, the concentration of 10 nmol/L probe b-DNA was found to be the minimum concentration of probe b-DNA able to provide an ample bioluminescence signal. Therefore, this concentration was chosen as optimum and was employed in further studies for the optimization of the DNA hybridization assay.

Another parameter that was evaluated in the development of the DNA hybridization assay for *P. falciparum* was the amount of b-DNA that needed to be employed. For that, a second binder dilution study was performed using an aliquot of 100 μL of 10 nmol/L probe b-DNA along with a solution of 100=μL fixed volume containing different concentrations of b-DNA (Figure 3). From this study, it was concluded that a concentration of 1 nmol/L b-DNA was the minimum concentration of b-DNA that provided a significant amount of triggered bioluminescence signal. Therefore, this concentration was the one used to perform the rest of the experiments needed for the development of the DNA hybridization assay for *P. falciparum*.

The dose−response curve obtained for the DNA hybridization assay developed for *P. falciparum* is shown in Figure 4. This curve showed that the bioluminescence signal of streptavidin−aequorin obtained was dependent on the concentration of b-DNA and inversely proportional to the concentration of the target DNA present in the sample. In addition, the DNA hybridization assay developed demonstrated a detection limit of 0.03 nM, or 3 pg/μL of DNA based on a S/N of 3, demonstrating the potential suitability of the method for the detection of *P. falciparum* without PCR amplification.[3] The potential usefulness of the method for the detection of malaria in real samples was demonstrated by analyzing human serum samples spiked with different amounts of target DNA. The results obtained (shown in Figure 4) demonstrate the suitability of this method for the detection of *P. falciparum* in human serum samples without PCR amplification. Additionally, it should be noted that there was no significant change in the bioluminescence signal obtained from the spiked human serum samples as compared to the signal recorded from the standard samples, demonstrating the feasibility of employing this DNA hybridization assay in biological fluids.

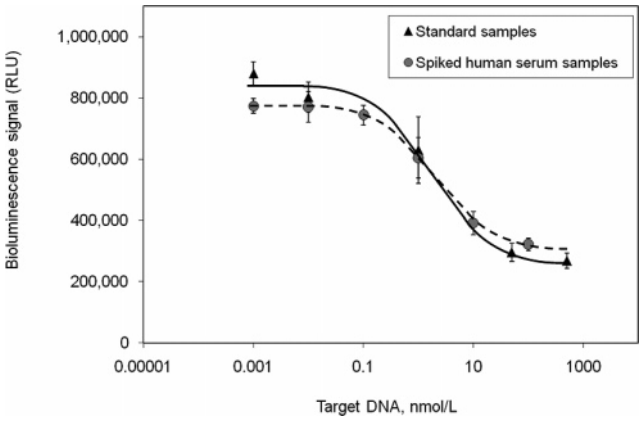

Figure 4. Dose−response curves for *P. falciparum* generated by incubating a fixed amount of probe b-DNA and b-DNA in 100 μL of varying concentrations of target DNA in standard or spiked human serum samples. The data are presented on logarithmic scale and are based on an average response of five samples ± the standard deviation.

After the development of the DNA hybridization assay, studies were performed in order to optimize the analysis time of the method. So far, the incubation time of each step of the assay was performed in the time frame of 1 h. In order to improve the analysis time of the method, shorter incubation times were also tested in the order of 15 and 30 min for each step performed (Table 1). During these tests, it was seen that the assay was still able to run successfully in a microplate format, demonstrating a detection limit of 100 pg/μL, allowing the parallel analysis of multiple samples within a time frame of 2 h.

CONCLUSIONS

A bioluminescence DNA hybridization assay was developed for the detection of *P. falciparum*, the most deadly species of malaria. The assay employed the highly sensitive bioluminescence label streptavidin−aequorin to report the hybridization of the target DNA on a 96-well microplate format, without the need of PCR amplification of the sample. The DNA hybridization assay developed presented a detection limit of 3 pg/μL DNA and was utilized for the detection of target DNA in spiked human serum samples, demonstrating the suitability of the method for use in biological fluids. This method is able to offer the parallel analysis of multiple samples on microplate formats of 96 wells or even more, i.e., 384 wells, and is suitable for use in clinical diagnostics or for the evaluation of drugs and vaccines for *P. falciparum*. The ability of storage of the assay reagents in a lyophilized form permits the preservation and transferring of the assay, making it suitable for utilization in the developing regions. Additionally, this method can be integrated on microfluidic platforms,[22,23] like the compact disc (CD) microfluidic platform.[24,25] In this case, the sample preparation steps needed prior to performing the DNA hybridization assay, such as the cell lysis, the release, and the denaturation of the DNA from the parasite bound to erythrocytes, could potentially be all implemented on a single CD.[26,27] Such CD devices offer the reduction of labor, risk of sample contamination, and analysis time as they provide faster and more efficient analytical processes.[28,29] Furthermore, such platforms provide the ability for automated multitask analysis, requiring limited resources and use by minimally trained personnel, therefore, being ideal for the development of automated diagnostic systems that can be used in developing regions, even in the field, where they will be mostly beneficial.[30]

Table 1. Characteristics of the *P. falciparum* DNA Hybridization Assay Performed under Various Incubation Times

incubation time (min)e	detection limit (pg/μL)	sensitivity (RLU/nM)
15	100	1.1×10^3
30	36	1.8×10^3
60	3	2.4×10^3

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