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(54) Title: METHODS FOR EXTRACTING NUCLEIC ACIDS FROM DRIED BLOOD AND USES THEREOF

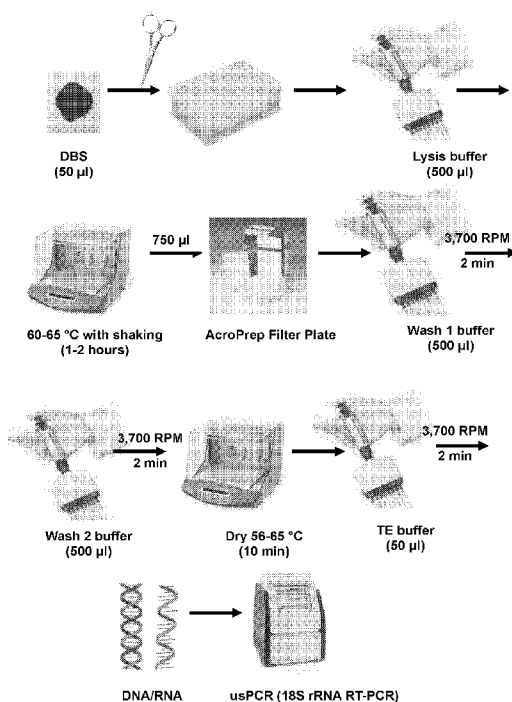


FIG. 1

(57) Abstract: The present invention provides a method for extracting nucleic acids from a dried blood sample that obviates the need for drawing venous blood or the need for preserving blood in a cold-chain. The present invention provides a method for screening a dried blood sample for an infectious agent, such as a parasite, a virus or a bacterium, that utilizes the steps for extracting the nucleic acids. In addition a method for monitoring the level of parasitemia in a population by concurrently screening blood samples from a plurality of subjects in the absence of cross-contamination and in the absence of interference from fungal contamination.



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METHODS FOR EXTRACTING NUCLEIC ACIDS FROM DRIED BLOOD AND USES THEREOF

Cross-Reference to Related Application

This international application claims benefit of priority under 35 U.S.C. §119(e) of provisional application U.S. Serial No. 62/244,867, filed October 22, 2015, the entirety of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention generally relates to the fields of medicine and molecular biology and to the detection of blood borne parasites. In particular, the invention relates to an ultrasensitive method for efficient and rapid extraction of nucleic acids, which then can be used, for example, in the detection of ultra low parasitemias.

Description of the Related Art

The emergence of drug resistance is a serious setback to the management of blood-borne communicable diseases. This is further complicated by presence of a significant number of asymptomatic individuals who serve as transmission reservoirs, unwittingly spreading the disease to vulnerable individuals. In many cases, these silent carriers outnumber the number of clinical cases at least ten fold (1, 2). There is therefore the need to develop improved strategies for diagnosing early stage and asymptomatic disease, so that these individuals may be treated in a timely manner. Traditional diagnostic tools used for detection of blood-borne diseases including, rapid diagnostic tests (RDTs) and microscopy are disadvantageous due to poor sensitivity in detecting low parasitemias associated with subclinical infections (3, 4). As a result, this large asymptomatic reservoir has only recently been identified with the development of very sensitive PCR methods (2, 5-7). For example, by using PCR for malaria screening, a vast majority of the infections were found to reside in asymptomatic individuals who were never identified by microscopy or RDT based assays (1, 2, 8).

While PCR techniques are sensitive, there still remain difficulties in implementing this

test in the field. Oftentimes this requires laborious sample collection procedures - usually a venous blood draw, coupled with centrifugation to remove the buffy coat, which is time consuming. Moreover, blood samples have to be stored at a low temperature during transport and processing (constant cold-chain), further adding to equipment and transportation costs. There is hence the need for developing new, highly sensitive methods for detection of subclinical levels of parasites in the blood, that have the added benefits of long term sample stability, reduced labor, and reduced handling/processing costs.

Overall, there is a deficiency in the art for optimal preparation and handling of blood-derived nucleic acid samples due to limitations imposed by low sensitive detection methods, absence of a simple, user-friendly interface and poor cost-effectiveness. Thus, there is a recognized need for improved methods to screen subclinical parasitemia. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

The present invention is directed to a method for extracting nucleic acids from a dried blood sample. The method comprises obtaining a blood sample from a subject and drying the blood sample on a filter paper. Cells in the dried blood sample are lysed to obtain a lysate and nucleic acids are extracted from the lysate. The present invention is directed to a related method further comprising storing the dried blood sample prior to the lysing step.

The present invention also is directed to a method for screening a dried blood sample for an infectious agent. The method comprises obtaining a blood sample from a subject and drying the blood sample on a filter paper. Cells in the dried blood sample are lysed to obtain a lysate and nucleic acids are extracted from the lysate. A PCR is performed to determine a presence or an absence of a target gene specific to the infectious agent where the presence of a target amplicon indicates the presence of the infectious agent in the blood sample. The present invention is directed to a related method further comprising determining a copy number for the target gene and comparing the copy number to a standard curve that correlates copy number of the of the target gene with a density in infectious agent/ml to determine a density of the infectious in the dried blood sample. The present invention is directed to another related method further comprising storing the dried blood sample prior to the lysing step.

The present invention is directed to a high throughput method for monitoring a level of parasitemia in a population. The method comprises obtaining a blood sample from each of a plurality of subjects in the population and drying the blood samples on filter paper. Cells in each blood sample are lysed to obtain a plurality of lysates and nucleic acids are

extracted from each of the lysates. A PCR is performed concurrently on each of the extracted nucleic acids to determine a copy number of a target gene specific to the parasite; and comparing each copy number to a standard curve that correlates copy number of the target gene with a density in parasites/ml where the parasite density is indicative of the level of parasitemia in the population. The present invention is directed to a related method further comprising repeating the method steps one or more times. The present invention is directed to another related method further comprising storing the dried blood sample prior to the lysing step.

Other and further aspects, features, benefits, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a work-flow of the steps involved in extraction of nucleic acids from dried blood spot (DBS) for ultrasensitive PCR (usPCR) detection of parasites.

FIG. 2 shows the dried blood spot collection strategy. **FIG. 2A** shows Whatman 3 MM filter paper pre-cut to yield four 2 X 0.5 cm strips. **FIG. 2B** shows that each strip, which corresponds to a 50 µl blood spot can be fitted into a standard 96 well plate.

FIG. 3 shows plate covers used to prevent cross-contamination. **FIG. 3A** shows a typical plastic plate cover that is used during nucleic acid extraction and PCR setup. This plate cover allows multichannel pipetting of an entire column (8 wells) but covers the remainder of the 96-well plate. **FIG. 3B** shows a plate cover for a single well of a 96-well plate and is used during cutting to isolate the well receiving the cut DBS.

FIG. 4 shows a comparison of limits of detection of parasites using PCR, Rapid diagnostic test (RDT) and PCR following the ultrasensitive extraction method (us PCR) claimed here.

FIGS. 5A-5D show methods to control for contamination and false positives. **FIG. 5A** shows wiping scissors and forceps three times with a Kimwipe sprayed with 70% ethanol is sufficient to prevent contamination when cutting from high (600,000 parasites/ml) to low (0 parasites/ml) parasitemic samples. **FIG. 5B** The amplification curves for all of the 0 parasites/ml samples are shown indicating lack of amplification. **FIG. 5C** Representative plates for analysis of *P. falciparum* levels in a field survey from Myanmar showing that even in high malaria burden areas, negative control samples consisting of blank filter papers (indicated by *) remain negative, confirming lack of cross-contamination. **FIG. 5D** Representative plates for analysis of *P. vivax* levels in a field survey from Myanmar showing

that even in high malaria burden areas, negative control samples consisting of blank filter papers (indicated by *) remain negative, confirming lack of cross-contamination.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

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For convenience, before further description of the present invention, certain terms employed in the specification, examples and appended claims are collected herein. These definitions should be read in light of the remainder of the disclosure and understood as by a person of skill in the art. Unless defined otherwise, all technical and scientific terms used
10 herein have the same meaning as commonly understood by a person of ordinary skill in the art.

The articles "a" and "an" when used in conjunction with the term "comprising" in the claims and/or the specification, may refer to "one", but it is also consistent with the meaning of "one or more", "at least one", and "one or more than one". Some embodiments of the
15 invention may consist of or consist essentially of one or more elements, components, method steps, and/or methods of the invention. It is contemplated that any composition, component or method described herein can be implemented with respect to any other composition, component or method described herein.

The term "or" in the claims refers to "and/or" unless explicitly indicated to refer to
20 alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or".

The terms "comprise" and "comprising" are used in the inclusive, open sense, meaning that additional elements may be included.

The term "including" is used herein to mean "including, but not limited to". "Including"
25 and "including but not limited to" are used interchangeably.

The term "about" is used herein to mean a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term "about" generally refers to a range of numerical values (e.g., +/- 5-10% of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g.,
30 having the same function or result). In some instances, the term "about" may include numerical values that are rounded to the nearest significant figure.

In one embodiment of the present invention there is provided a method for method for extracting nucleic acids from a dried blood sample comprising the steps of obtaining a blood sample from a subject; drying the blood sample on a filter paper; lysing cells in the
35 dried blood sample to obtain a lysate; and extracting nucleic acids from the lysate. Further to this embodiment the method comprises storing the dried blood sample prior to the lysing

step.

In one aspect of both embodiments the lysing step may comprise incubating with a lysis buffer that has a pH of about 6.3 to about 6.5 and comprises 3 M guanidine thiocyanate, 2% v/v TRITON-X100, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol, 6 mM hydrochloric acid and 0.5 % v/v 2-mercaptoethanol. In this aspect the dried blood sample may be incubated in the lysis buffer for 30 min at 70 °C, for 60 min at 60 °C, or for 120 min at 56 °C.

In another aspect of both embodiments the extracting step may comprise immobilizing the nucleic acids in the lysate to a silica substrate; washing the silica substrate with a first wash buffer; washing the silica substrate with a second wash buffer; drying the silica substrate for 10 min at 56-65 °C; and eluting the nucleic acids from the silica substrate with an elution buffer. Examples of the silica substrate may be a borosilicate glass fiber filter plate, a glass filter plate, a silica resin, or silica beads. Also in this aspect the first wash buffer may have a pH of about 6.3 to about 6.5 and may comprises 3 M guanidine thiocyanate, 2% v/v Triton, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol and 6 mM hydrochloric acid. In addition, the second wash buffer may have a pH of about 7.4 and may comprise 25% v/v ethanol, 25% v/v isopropanol, 100 mM sodium chloride and 10 mM TRIZMA hydrochloride. Furthermore the elution buffer may have a pH of about 8.0 and comprises 10 mM Tris and 1 mM EDTA.

In another embodiment of the of the present invention there is provided a method for screening a dried blood sample for an infectious agent, comprising the steps of obtaining a blood sample from a subject; drying the blood sample on a filter paper; lysing cells in the dried blood sample to obtain a lysate; extracting nucleic acids from the lysate; and performing a PCR to determine a presence or an absence of a target gene specific to the infectious agent; wherein the presence of a target amplicon indicates the presence of the infectious agent in the blood sample.

Further to this embodiment the method comprises determining a copy number for the target gene; and comparing the copy number to a standard curve that correlates copy number of the target gene with a density in infectious agent/ml to determine a density of the infectious agent in the dried blood sample. In another further embodiment the method comprises storing the dried blood sample prior to the lysing step.

In aspects of these embodiments the lysing step, the extracting step and the eluting step may be as described *supra*. Particularly, the dried blood sample may be incubated in the described lysis buffer for 30 min at 70 °C, for 60 min at 60 °C, or for 120 min at 56 °C. Also, in the extracting step, the nucleic acids immobilized on a silica substrate may be washed with the first and second wash buffers and eluted with the elution buffer as

described *supra*. Examples of the silica substrate may be a borosilicate glass fiber filter plate, a glass filter plate, a silica resin, or silica beads.

In all embodiments and aspects thereof the subject may be a human or a non-human mammal. Also, the infectious agent may be a parasite, a virus or a bacterium or a combination thereof. Examples of the parasite are a *Plasmodium* species, a *Leishmania* species, a *Trypanosoma* species, a *Toxoplasma* species, or a *Babesia* species. Examples of the virus are an RNA virus, a DNA virus or a DNA-RT virus. Examples of the bacterium a *Rickettsia* species or a *Borrelia* species. In addition the PCR is a nested PCR, a reverse transcription-PCR (RT-PCR) or a nested RT-PCR. Furthermore, the target gene may be 18S ribosomal RNA.

In another embodiment of the of the present invention there is provided a high throughput method for monitoring a level of parasitemia in a population, comprising the steps of a) obtaining a blood sample from each of a plurality of subjects in the population; b) drying each of the blood samples on a filter paper; c) lysing cells in each blood sample to obtain a plurality of lysates; d) extracting nucleic acids from each of the lysates; e) performing a PCR concurrently on each of the extracted nucleic acids to determine a copy number of a target gene specific to the parasite; and f) comparing each copy number to a standard curve that correlates copy number of the parasite with a density in parasites/ml wherein the parasite density is indicative of the level of parasitemia in the population. Further to this embodiment the method may comprise repeating steps a) to f) one or more times. In another further embodiment the method comprises storing the dried blood sample prior to the lysing step. In all embodiments and aspects thereof the subject may be a human or a non-human mammal.

In aspects of these embodiments the lysing step, the extracting step and the eluting step and the lysis buffer, the first and second wash buffers and the elution buffer may be as described *supra*. In all embodiments and aspects thereof the silica substrate may be as described *supra*. Also the subject may be a human or a non-human mammal. In addition the parasite may be those species or those viruses or a combination thereof as described *supra*. In addition the PCR may be a nested PCR, a reverse transcription-PCR (RT-PCR) or a nested RT-PCR and the target gene may be 18S ribosomal RNA.

Provided herein is a method for extracting nucleic acids from a dried blood sample. The subject from which the blood sample is obtained may be a mammal, preferably a human. Alternatively, the subject may be a non-human mammal including, but not limited to, mice, rabbits, cat, dogs, birds, horses, and farm animals.

The blood sample may be obtained using various methods that are well known in the art, such as a venous blood draw or using a lancet whereby one can draw small amounts of

blood, typically 10 μ l -100 μ l, from body extremities. The volume of collected blood that is dried on the filter paper may be from about 50 μ l to about 200 μ l. Filter papers used for this purpose may include Whatman filter paper. Specifically a Whatman 3 MM filter paper may be used. Alternatively, a Whatman 903 Protein Saver filter paper, or any other filter paper that may be cut into the desired shapes and sizes also may be used. The filter paper may be in the form of a circular dot, a 2 x 0.5 cm rectangle, or any other suitable shape and size that allows one to dry and process the dried blood sample immediately or to dry the sample for long term storage under ambient field conditions.

Once dried, the nucleic acids may be extracted immediately or the filter paper containing the blood sample may be stored at an ambient temperature or below ambient temperature prior to carrying out the lysing step at an in-field or off-field facility. The method does not require drawing venous blood or the need for preserving blood in a cold-chain. The dried blood spots have a shelf life of several months.

The method of extracting the nucleic acids requires lysing the cells in the blood sample to obtain a lysate. The lysing step is performed by placing the filter paper containing the dried blood sample in a well of a multi-well plate. Different multi-well plate formats may be used depending on the size of the filter paper and the number of samples being processed. Examples of multi-well formats include 12-well, 24-well, 48-well, 96-well, and 384-well. An increase in well number increases throughput.

Lysis buffers having a pH of about 6.3 to about 6.5 may be used for releasing the nucleic acids from the cells. Lysis buffer may comprise a chaotropic agent for denaturing the macromolecules in the blood sample, a non-ionic detergent for solubilizing and stabilizing the lysate, a metal ion chelator to deactivate nucleases, an alcohol compound as a dehydration agent, a thiol compound as a reducing agent and a weak base/inorganic acid buffering system. A non-limiting example of a lysis buffer comprises the chaotropic agent guanidine thiocyanate at a concentration of about 3 M, the non-ionic detergent Triton-X100 having a final concentration of about 1% v/v to about 2% v/v, the metal ion chelator EDTA from about 5 mM to about 10 mM, the alcohol isopropanol at about 10-20% v/v, the thiol compound 2-mercaptoethanol at a concentration from about 0.5% v/v to about 1% v/v in a 5 mM to 10 mM TRIZMA/hydrochloride buffer. Particularly, the lysis buffer contains isopropanol at a preferred concentration of 16.7% v/v. The inclusion of isopropanol in the lysis buffer significantly and substantially improves sensitivity.

Incubations with lysis buffer may be optimized by varying the incubation time, incubation temperature and/or by using a speed controlled rotary shaker. For example, incubations may be performed for about 30 min to about 120 min at temperatures of about 70 °C to about 56 °C. Preferably, incubations are performed for 30 min at 70 °C, for 60 min

at 60 °C, or for 120 min at 56 °C in the presence or absence of shaking. One of ordinary skill in this art can easily vary each of these conditions one at a time, and evaluate its effect on extraction efficiency and quality of the extracted nucleic acids by performing a quantitative PCR or RT-PCR for a suitable target gene, such as an actin gene, a glyceraldehyde 3-phosphate dehydrogenase gene, or any other known gene or genes specific to the parasite.

Extracting nucleic acids requires immobilizing the nucleic acids in the lysate on a silica substrate. The silica substrate may be in the form of a glass, a fiberglass, a glass powder, silica particles or borosilicate fibers. The silica substrate may be immobilized with or without a binder to a solid phase support, such as beads, a filter plate or any other suitable support. The filter plates, preferably a borosilicate fiber filter plate, have a format that allows insertion into a 96-, 12-, 24-, 48- or 384- multiwell plate to enable one to process lysates from multiple dried blood samples in a high-throughput process. The method of extracting the nucleic acids requires washing the filter plates containing the immobilized nucleic acids to remove non-nucleic acid contaminants that may be non-selectively adsorbed to the silica substrate.

This encompasses a two-step sequential washing procedure that employs two wash buffers having different compositions. The first wash buffer has a pH of about 6.3 to about 6.5 and comprises the components of the lysis buffer described herein, without the 2-mercaptoethanol. The second wash buffer has a pH of about 7.4, and comprises, 25% v/v ethanol, 25% v/v isopropanol, 100 mM sodium chloride and 10 mM TRIZMA/hydrochloride buffer. Each of the washing steps may be performed one or more times depending on the amount of non-nucleic acid contaminants in the lysate. The wash volumes as well as the number of washes can be easily adjusted and adapted by one having ordinary skill in this art.

Extracting the nucleic acids further comprises a drying step to remove trace amounts of the alcohol carryover from the washing steps, prior to eluting the nucleic acids. Drying may be performed by placing the silica substrate to which the nucleic acids are adsorbed for about 10 min in an oven maintained from about 56 °C to about 65 °C. The dried silica substrate/immobilized nucleic acids is placed in a new, uncontaminated multi-well plate and the immobilized nucleic acids eluted using an elution buffer having a pH of about 8.0 and comprising about 10 mM Tris and about 1 mM EDTA. The drying conditions and the volume of the elution buffer can easily be optimized for maximizing nucleic acid quality and yields, by one of ordinary skill in this art. Each of the steps encompassed in extracting nucleic acids as described herein requires centrifuging the multi-well plate at 37,000 rpm for at least 1 min to allow the lysis, wash and elution buffers to pass through into the well of the multi-well plate.

Also provided is a method for screening a dried blood sample for an infectious agent. The method utilizes the nucleic acid extraction method described in detail herein. PCR run on the extracted nucleic acids determines the presence or the absence of a target gene specific to the parasite. The presence of a target gene amplicon indicates the presence of the infectious agent in the blood sample. Non-limiting examples of blood-borne parasites that may be screened by this method include, a *Plasmodium* species, a *Leishmania* species, a *Trypanosoma* species, a *Toxoplasma* species, a *Babesia* species, a *Rickettsia* species, a *Borrelia* species. Viruses, either blood-borne or parasitic, such as an RNA virus, a DNA virus or a DNA-RT virus, also may be detected with these methods. The method described herein may be used to screen for a single infectious agent or a combination of infectious agents.

Screening encompasses identifying one or more target genes specific for the infectious agent being screened, which are well known in the art, and may be performed by a PCR, such as a nested PCR, an RT-PCR or a nested RT-PCR. The target gene may be any known infectious agent gene or a species-specific gene. An example of one such gene is the gene coding for 18S ribosomal RNA. Generic or species specific primers for these genes may be easily designed from gene sequences which are available from open access databases. Methods for performing a PCR, or RT-PCR or a nested PCR using extracted DNA or RNA as templates are well known in the art, and one having ordinary skill can easily adapt these methods to arrive at the copy number of the infectious agent in the dried blood sample. Copy numbers of target genes amplified by PCR may be compared with a Copy Number versus Parasite Density standard curve. The standard curve may be generated using a healthy blood sample free of the infectious agent that is doped with parasites to give a range of standard blood samples having known parasite densities. Methods for making standards and arriving at a suitable standard curve are well known in the art.

In addition a high throughput method to monitor a level of parasitemia in a population is provided. The high throughput method utilizes the nucleic acid extraction method and concurrent PCR amplification of one or more target genes of interest in a plurality of samples as described in detail herein. If present, the copy number of the target gene determined from the amplicon is compared with a standard curve to arrive at the parasite density. The cut-off values for sub-clinical and clinical parasitemia expressed as the number of parasites per volume of blood sample are readily available in the art. The skilled person can therefore compare the parasite densities obtained from members of the population with established cut-off values and correlate to an absence of parasitemia, the presence of sub-clinical parasitemia or the presence of clinical parasitemia in individual members or in the population as a whole.

The method may be repeated as required or necessary to track and monitor an increase or decrease in parasitemia within the population. This information is useful to determine the efficacy of a treatment program or to track potential or current outbreaks of disease. Moreover, the methods described herein enable large numbers of samples to be collected in the field and stored for later processing.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

10 Materials

Pall AcroPrep 96 Filter Plate (BIORESCO cat# VWR-89135-690-PK), 96-well multi-well plates, multichannel pipettes, centrifuge, cold chain Whatman 3 MM filter paper.

Buffer composition

15 Lysis buffer:

3 M Guanidine Thiocyanate

2% v/v TRITON-X100

10 mM EDTA

5 mM Trizma hydrochloride

20 16.7% v/v isopropanol

6 mM HCl

0.5% v/v 2-mercaptoethanol- added before use.

pH between 6.3 and 6.5

25 First wash buffer:

3 M Guanidine Thiocyanate

2% v/v TRITON-X100

10 mM EDTA

5 mM Trizma hydrochloride

30 16.7% v/v isopropanol

6 mM HCl

pH between 6.3 and 6.5

Second wash buffer:

35 25% v/v ethanol

25% v/v isopropanol

100 mM sodium chloride
10 mM Trizma hydrochloride
pH 7.4

5 Elution buffer:

10 mM Tris
1 mM EDTA
pH 8.0

10 **EXAMPLE 2**

Optimization of nucleic acid extraction from dried blood spots

The extraction procedure from dried blood spots (DBS) was systematically optimized by varying one condition at a time, such as constitution of lysis and wash buffers, length of incubation time and temperature with lysis buffer, drying time, and elution volume, and
15 evaluating the effect quantitatively by qPCR for *P. falciparum* 18S rRNA. This allowed for identification of conditions that incrementally improved extraction of 18s rRNA. Once an optimized method was found, effort was taken to simplify the protocol by reducing the number of steps without compromising sensitivity. As a result, the final protocol was optimized both for sensitivity and simplicity (FIG. 1).

20

Reproducibility considerations

Solutions were made in large batches to maximize efficiency and minimize variability. Lysis and first wash buffer were made 20 liters at a time and second wash buffer was made 10 liters at a time, which was enough for at least 10,000 extractions, and stored in 500 ml
25 bottles at room temperature in the dark or at 4°C long-term. After every batch, a bridge study was performed with previously created lab samples to ensure the quality of solutions. 2-mercaptoethanol was added fresh to the lysis buffer at 0.5% final concentration before use.

q-Reverse transcriptase polymerase chain reaction (q-RTPCR)

30 Ultrasensitive reverse-transcriptase PCR for *P. falciparum* and *P. vivax* 18s rRNA/DNA, and human actin RNA/DNA as an internal control, was performed with the primer or probe sequences with conditions as shown in Table 1 (9, 6). Cycling conditions are: 50 °C for 20 min (RT), 95 °C for 15 min and 94 °C for 45 seconds (repeat 44 times) and 60 °C for 75 seconds (repeat 44 times).

35

Table 1: q-RTPCR for *P. falciparum* and *P. vivax* 18 S rRNA

Species	Primer or probe ^a	Sequence (5'-3') ^b	SEQ ID NO
<i>P. falciparum</i>	Falciparum F	CCGACTAGGTGTTGGATGAAAGTGTTAA	1
	Plasmodium R	AACCCAAAGACTTTGATTTCTCATAA	2
	Falciparum P	Cy5-AGCAATCTAAAAGTCACCTCGAAAGATG ACT-BHQ2	3
<i>P. vivax</i>	Vivax R	CCGACTAGGCTTTGGATGAAAGATTTTA	4
	Vivax P	FAM-GCAATCTAAGAATAAACTCCGAAGAGAA AATTCT-BHQ2	5
Human	Actin F	ACCGAGCGCGGCTACAG	6
	Actin R	CTTAATGTCACGCACGATTTC	7
	Actin P	VIC-TTCACCACCACGGCCGAGC-MGB	8

^aProbe sequence as previously published (9) with modified fluorphores (6).

^bCy5, cyanine; BHQ2, black hole quencher 2; FAM, carboxyfluorescein; VIC (Life Technologies, Carlsbad CA); MGB, minor-groove binder

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Testing of commercial buffers

The following commercially available lysis buffers were tested and compared to the lysis buffer described *supra* in this invention: AL, ATL, RLT, and RLT-PLUS (Qiagen); PURELINK Viral/RNA Lysis Buffer (ThermoFisher); GENELUTE Lysis Buffer (SIGMA); 6 M

10

Guanidine Hydrochloride solution (VWR); and 6 M Guanidine Thiocyanate solution (SIGMA). The following commercially available first wash buffers were tested: AW1, RW1, RLT, and RLT-PLUS (Qiagen); WASH BUFFER 1 (SIGMA); PURELINK Viral/RNA Wash 1 Buffer (ThermoFisher).

The following commercially available second wash buffers were tested: AW2, RPE, and RWT (Qiagen); WASH BUFFER 2 (SIGMA); PURELINK Viral/RNA Wash 2 buffers (ThermoFisher); 70% ethanol; 70% ethanol with 30% PBS.

15

Commercial lysis, first wash, and second wash buffers that gave similar results as the buffers described *supra* in this invention were chosen for further testing in combination with each other.

20

Limit of detection for *Plasmodium falciparum*

Experiments were performed as previously reported with the exception that dried blood spots were both dried and stored in simulated field conditions (28 °C with 80% relative humidity) for two weeks before use in experiments (2). Three independent analyses were

performed to obtain the limit of detection (LoD), which was determined by a probit analysis using the software, Statistical Analysis System 9.2 (SAS Institute, Cary NC).

EXAMPLE 3

5 Development of a new extraction method for ultrasensitive detection of malaria using dried blood spots

The 18S rRNA of the malarial parasite exists in thousands of copies per parasite, leading to a dramatic improvement in sensitivity compared to traditional DNA based assays (6-7). To adapt the ultrasensitive PCR protocol to dried blood spots (DBS) Qiagen-based
10 methods were applied to compare existing sensitivities with a new method that could be applied to DBS. A new protocol based on traditional guanidine/silica technology was developed that was exquisitely optimized for purification of parasite 18S rRNA, and yet could be applied in a simple and high throughput fashion. After testing a multitude of conditions, a few key variables that markedly improved sensitivity was determined. These included, using
15 a higher volume of DBS (50 µl); inclusion of isopropanol in the lysis buffer (at a final concentration of 16.7%); incubating the DBS with lysis buffer at a temperature between 60 and 65°C on a shaker; and heat-drying the filter plate containing bound DNA prior to eluting.

The procedure was then simplified by reducing the number of steps without compromising sensitivity. Consequently, the final protocol (FIG. 1) used the buffers
20 described in Example 1 to optimize both sensitivity and simplicity. This method when tested on standards that were dried and stored in simulated field conditions (28°C, 80% relative humidity), yielded a limit of detection (LoD) for *P. falciparum* at 20 parasites/mL for Whatman 3 MM filter paper. In comparison, techniques using venous blood or preserved capillary blood gave a LoD of 22 parasites/ml and ≤16 parasites/ml, respectively.

25

Cost effectiveness.

Substituting Whatman 3 MM filter paper with the more expensive, Whatman 903 PROTEIN SAVER cards, which are coated with a proprietary preservative, did not result in further improvement of sensitivity (LoD = 23 parasites/ml). However, Whatman 903 Protein
30 Saver completely prevented the appearance of fungal contamination when compared with Whatman 3 MM filter paper.

To obviate the encumbrance from having to use homemade extraction buffers, which is problematic in the field, this technique was adapted by substituting commercially available buffers for the home-made solutions. After extensive testing, Qiagen RLT-PLUS,
35 supplemented with 16.7% isopropanol and 0.5% 2-mercaptoethanol achieved an identical sensitivity as the homemade buffers. This convenience however increased the cost per

sample by approximately 50% when compared with homemade solutions. Nonetheless, both the homemade new extraction method and commercial new extraction method were still substantially less expensive than traditional Qiagen kit-based methods. Based on list prices, the approximate cost per sample when using the homemade new extraction method and commercial new extraction method, including all consumables, was \$1.77 and \$2.66 respectively. In contrast, the cost per sample when using Qiagen QIAamp was \$7.87.

EXAMPLE 4

Field validation of the new extraction method for molecular surveillance of asymptomatic malaria

Two cross-sectional surveys were performed in Myanmar, one during the dry season in the Tanintharyi Region and one during the wet season in Buthitaung, Ann, and Ingapu. All studies were approved by the Ethics Review Board of the Department of Medical Research of the Myanmar Ministry of Health and University of Maryland School of Medicine. Two types of samples were taken from each volunteer. Using a BD MICRO-FINE Contact-Activated Lancet (Fisher Scientific), 0.3 ml of capillary blood from each volunteer was mixed with 0.75 ml of DNA/RNA Shield (Zymo Research). This method has previously been shown to stabilize the nucleic acids for up to two weeks in field conditions and have a limit of detection of ≤ 16 parasites/mL (2). From the same volunteer a 50 μ l dried blood spot (DBS) was obtained with Whatman 3 MM filter paper corresponding to a 2 cm x .5 cm area (FIG. 2A-2B), and Whatman 903 Protein Saver (one circle, when applicable) Samples were dried for two hours in ambient conditions during the dry season and up to overnight during the wet season. After drying, samples were placed in a sealed plastic pouch with desiccant and stored at ambient conditions until analyzed, usually several months later. Upon return to the laboratory, DBS were inspected for the presence of fungus. Samples that had fungus were rated on a three-point scale (slight, moderate, or severe) and separated from the rest of the group for independent testing, along with the corresponding Whatman 903 PROTEIN SAVER and preserved capillary blood.

To minimize the chance of carry-over cross-contamination, plate covers were used during cutting, extraction and PCR procedures (FIG 3A-3B). As an internal control, every eighth sample consisted of a blank filter paper sample that was cut, extracted and used for ultrasensitive PCR alongside actual field samples. This allowed for identification of any contamination issues resulting from any step in the process that would otherwise yield false positives.

Results

In a cross-sectional survey performed during the rainy season consisting of 595 individuals, DBS gave nearly identical prevalence rates as preserved capillary blood (PCB) (Table 2). A second survey performed during the dry season consisting of 1,750 individuals found near identical *P. falciparum* prevalence and slightly lower (but statistically insignificant) *P. vivax* prevalence of 5.9% versus 5.3% PCB versus DBS (Table 3). These data confirm that dried blood spots can be used in molecular surveillance studies of asymptomatic malaria, even in Southeast Asia where parasitemias are quite low. FIG. 4 shows the limit of detection of parasites using PCR, RDT and, PCR performed after the ultrasensitive extraction method.

Table 2: Survey performed during the rainy season (n = 595)

Sample: Extraction method: Detection method:	Preserved Capillary Blood Qiagen Ultra-sensitive PCR	Dried Blood Spot New extraction method Ultra-sensitive PCR
<i>P. falciparum</i> mono-infections (%)	6.4	6.7
<i>P. vivax</i> mono-infections (%)	4.9	4.9
<i>P. falciparum</i> + <i>P. vivax</i> (%)	1.2	1.2

Table 3: Survey performed during the dry season (n = 1,750)

Sample: Extraction method: Detection method:	Preserved Capillary Blood Qiagen Ultra-sensitive PCR	Dried Blood Spot New extraction method Ultra-sensitive PCR
<i>P. falciparum</i> mono-infections (%)	1.1	1.2
<i>P. vivax</i> mono-infections (%)	5.9	5.3
<i>P. falciparum</i> + <i>P. vivax</i> (%)	0.3	0.1

EXAMPLE 5

Controlling for False Positives and Fungal Contamination.

Increased sensitivity is oftentimes accompanied by decreased specificity. The use of plate covers (FIG. 3A-3B) help preventing cross-contamination during sample extraction and ultra-sensitive PCR. Use of dried blood spots require additional considerations such as, cross-contamination when cutting the filter paper. Previous work has attempted to circumvent this by use of laser-cutting, which although effective is not readily adaptable in the field (10). Hence the minimal safeguards needed to reliably prevent contamination from cutting were systematically analyzed. Three wipes of the scissor blades and forceps with a damp paper towel (or KIMWIPE) sprayed with 70% ethanol was determined to be sufficient to consistently prevent contamination when cutting from a high (600,000 parasites/ml) to a low (0 parasites/ ml) parasitemic sample (FIG. 5A-5B). The benefits of these techniques

became apparent in our field surveys where use of plate covers and adaptation of the above decontamination method was sufficient to prevent the appearance of false-positives in blank filter paper (negative controls), even in high malaria burden areas (FIG. 5C-5D).

Finally, the collection of dried blood spots during the rainy season has the potential for fungal contamination on account of the high humidity. Since the quality of these samples is unknown and so they are frequently discarded. To overcome this, the ability fungus to affects detection of malaria in field samples were systematically analyzed using the decontamination technique described herein. Preserved capillary blood (PCB) was used as the positive control, and compared the results with Dried Blood Spot (DBS) on Whatman 3 MM and Whatman 903 Protein Saver filter paper. One immediate observation was that, unlike 3 MM Whatman, 903 Protein Saver completely prevented the appearance of fungal contamination (and thus served as an additional control). As seen in Table 4, in 14/15 cases the presence of fungus had no qualitative impact on the detection of *P. falciparum* and *P. vivax*, even in the three cases where fungal contamination was severe. There was no quantitative loss in Ct value in malaria positive samples that had fungus compared to those that were free of fungus. Therefore DBS is a robust sampling strategy, even under non-ideal field conditions, and is therefore a reliable method for obtaining field surveillance data.

Table 4: Controlling for False Positives and Fungal Contamination

	Capillary blood	903 DBS		3 MM DBS	
Sample #	Malaria status	Malaria status	Fungus status	Malaria status	Fungus status
1	PF+	PF+	None	PF+	Slight
2	PF+	PF+	None	PF+	Moderate
3	PV+	PV+	None	PV+	Slight
4	PV+,PF+	PV+, PF+	None	PV+, PF+	Severe
5	PV+	PV+	None	PV+	Slight
6	PV+	PV+	None	PV+	Severe
7	PV+	PV+	None		Slight
8	PF+	PF+	None	PF+	Slight
9	PV+	PV+	None	PV+	Slight
10	PV+,PF+	PV+, PF+	None	PV+, PF+	Slight
11	PV+	PV+	None	PV+	Moderate
12	PF+	PF+	None	PF+	Moderate
13	PV+	PV+	None	PV+	Moderate
14	PV+,PF+	PV+, PF+	None	PV+, PF+	Moderate
15	PV+	PV+	None	PV+	Severe

The following references are cited herein.

1. Imwong M. *et al. Malar J.* 14:381, 2015.

2. Adams M. *et al. Malar J.* 14:520, 2015..
3. Okell LC *et al. J Infect Dis.* 200:1509-17, 2009.
4. Cheng Q. *et al. PLoS Negl Trop Dis.* 9:e3413, 2015.
5. Imwong M. *et al. J Clin Microbiol.* 52:3303-9, 2014.
6. Kamau *et al. J Clin Microbiol.* 49:2946-53, 2011.
7. Murphy *et al. Am J Trop Med Hyg.* 86:383-94, 2012.
8. Imwong *et al. J Infect Dis.* 2016. 213:1322-9, 2016.
9. Seder *et al. Science*, 341(6152):1359-65, 2013.
10. Murphy *et al. J Clin Microbiol.* 2012. 50:4128-30, 2012.

10 The present invention is well adapted to attain the ends and advantages mentioned as well as those that are inherent therein. The particular embodiments disclosed above are illustrative only, as the present invention may be modified and practiced in different but equivalent manners apparent to those skilled in the art having the benefit of the teachings herein. Furthermore, no limitations are intended to the details of construction or design
15 herein shown, other than as described in the claims below. It is therefore evident that the particular illustrative embodiments disclosed above may be altered or modified and all such variations are considered within the scope and spirit of the present invention. The terms in the claims have their plain, ordinary meaning unless otherwise explicitly and clearly defined by the patentee.

WHAT IS CLAIMED IS:

1. A method for extracting nucleic acids from a dried blood sample comprising the steps of:

obtaining a blood sample from a subject;
drying the blood sample on a filter paper;
lysing cells in the dried blood sample to obtain a lysate; and
extracting nucleic acids from the lysate.

2. The method of claim 1, further comprising:
storing the dried blood sample prior to the lysing step.

3. The method of claim 1 wherein the subject is a human or a non-human mammal.

4. The method of claim 1, wherein the lysing step comprises:
incubating with a lysis buffer that has a pH of about 6.3 to about 6.5 and comprises 3 M guanidine thiocyanate, 2% v/v TRITON-X100, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol, 6 mM hydrochloric acid and 0.5 % v/v 2-mercaptoethanol.

5. The method of claim 4, wherein the dried blood sample is incubated in the lysis buffer for 30 min at 70 °C, for 60 min at 60 °C, or for 120 min at 56 °C.

6. The method of claim 1 wherein the extracting step comprises;
immobilizing the nucleic acids in the lysate to a silicate substrate;
washing the silicate substrate with a first wash buffer;
washing the silicate substrate with a second wash buffer;
drying the silicate substrate for 10 min at 56-65 °C; and
eluting the nucleic acids from the silicate substrate with an elution buffer.

7. The method of claim 6, wherein the silica substrate is a borosilicate glass fiber filter plate, a glass filter plate, a silica resin, or silica beads.

8. The method of claim 6, wherein the first wash buffer has a pH of about 6.3 to about 6.5 and comprises 3 M guanidine thiocyanate, 2% v/v Triton, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol and 6 mM hydrochloric acid.

9. The method of claim 6, wherein the second wash buffer has a pH of about 7.4 and comprises 25% v/v ethanol, 25% v/v isopropanol, 100 mM sodium chloride and 10 mM TRIZMA hydrochloride.

5 10. The method of claim 6, wherein the elution buffer has a pH of about 8.0 and comprises 10 mM Tris and 1 mM EDTA.

11. A method for screening a dried blood sample for an infectious agent, comprising the steps of:

10 obtaining a blood sample from a subject;
drying the blood sample on a filter paper;
lysing cells in the dried blood sample to obtain a lysate;
extracting nucleic acids from the lysate; and
performing a PCR to determine a presence or an absence of a target gene specific to
15 the infectious agent; wherein the presence of a target amplicon indicates the presence of the infectious agent in the blood sample.

12. The method of claim 11, further comprising:
determining a copy number for the target gene; and
20 comparing the copy number to a standard curve that correlates copy number of the target gene with a density of infectious agent/ml to determine a density of the infectious agent in the dried blood sample.

13. The method of claim 11, further comprising:
25 storing the dried blood sample prior to the lysing step.

14. The method of claim 11 wherein the subject is a human or a non-human mammal.

30 15. The method of claim 11, wherein the lysing step comprises:
incubating with a lysis buffer that has a pH of about 6.3 to about 6.5 and comprises 3 M guanidine thiocyanate, 2% v/v TRITON-X100, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol, 6 mM hydrochloric acid and 0.5 % v/v 2-mercaptoethanol.

35 16. The method of claim 15, wherein the dried blood sample is incubated in the lysis buffer for 30 min at 70 °C, for 60 min at 60 °C, or for 120 min at 56 °C.

17. The method of claim 11 wherein the extracting step comprises:
immobilizing the nucleic acids in the lysate on a silicate substrate;
washing the silicate substrate with a first wash buffer;
washing the silicate substrate with a second wash buffer;
5 drying the silicate substrate for 10 min at 56-65 °C; and
eluting the nucleic acids from the silicate substrate with an elution buffer.

18. The method of claim 17, wherein the silica substrate is a borosilicate glass
fiber filter plate, a glass filter plate, a silica resin, or silica beads.

19. The method of claim 16 wherein the first wash buffer has a pH about 6.3 to
about 6.5 and comprises, 3 M guanidine thiocyanate, 2% v/v Triton, 10 mM EDTA, 5 mM
TRIZMA hydrochloride, 16.7% v/v isopropanol and 6 mM hydrochloric acid.

20. The method of claim 17, wherein the second wash buffer has a pH of about
7.4 and comprises, 25% v/v ethanol, 25% v/v isopropanol, 100 mM sodium chloride and 10
mM TRIZMA hydrochloride.

21. The method of claim 17, wherein the elution buffer has a pH of about 8.0 and
comprises, 10 mM Tris and 1 mM EDTA.

22. The method of claim 11, wherein the infectious agent is a parasite, a virus or
a bacterium or a combination thereof.

23. The parasite of claim 22, wherein the parasite is a *Plasmodium* species, a
Leishmania species, a *Trypanosoma* species, a *Toxoplasma* species, or a *Babesia* species.

24. The method of claim 22, wherein the virus is an RNA virus, a DNA virus or a
DNA-RT virus.

25. The method of claim 22, wherein the bacterium is a *Rickettsia* species, or a
Borrelia species.

26. The method of claim 11, wherein the PCR is a nested PCR, a reverse
transcription-PCR (RT-PCR) or a nested RT-PCR.

27. The method of claim 11, wherein the target gene is 18S ribosomal RNA.

28. A high throughput method for monitoring a level of parasitemia in a population, comprising the steps of:

- 5 a) obtaining a blood sample from each of a plurality of subjects in the population;
 b) drying the blood samples on filter papers;
 c) lysing cells in each blood sample to obtain a plurality of lysates;
 d) extracting nucleic acids from each of the lysates;
 e) performing a PCR concurrently on each of the extracted nucleic acids to
10 determine a copy number of a target gene specific to the parasite; and
 f) comparing each copy number to a standard curve that correlates copy
number of the target gene with a density in parasites/ml, wherein the parasite density is
indicative of the level of parasitemia in the population.

15 29. The method of claim 28 further comprising repeating steps a) to f) one or
more times.

 30. The method of claim 28, further comprising:
storing the dried blood sample prior to the lysing step.

20

 31. The method of claim 28, wherein the parasite is a *Plasmodium* species, a
Leishmania species, a *Trypanosoma* species, a *Toxoplasma* species, or a *Babesia* species.

 32. The method of claim 28, wherein the population comprises humans or non-
25 human mammals.

 33. The method of claim 28, wherein the lysing step comprises:
incubating with a lysis buffer that has a pH of about 6.3 to about 6.5 and comprises 3
M guanidine thiocyanate, 2% v/v TRITON-X100, 10 mM EDTA, 5 mM TRIZMA hydrochloride,
30 16.7% v/v isopropanol, 6 mM hydrochloric acid and 0.5 % v/v 2-mercaptoethanol.

 34. The method of claim 33, wherein the dried blood sample is incubated in the
lysis buffer for 30 min at 70 °C, for 60 min at 60 °C, or for 120 min at 56 °C.

35 35. The method of claim 28, wherein the extracting step comprises:
immobilizing the nucleic acids extracted from each lysate to a silica substrate;

washing the silica substrate with a first wash buffer;
washing the silica substrate with a second wash buffer;
drying the silica substrate for 10 min at 56-65 °C; and
eluting the nucleic acids from the silica substrate with an elution buffer.

5

36. The method of claim 35, wherein the silica substrate is a borosilicate glass fiber filter plate, a glass filter plate, a silica resin, or silica beads.

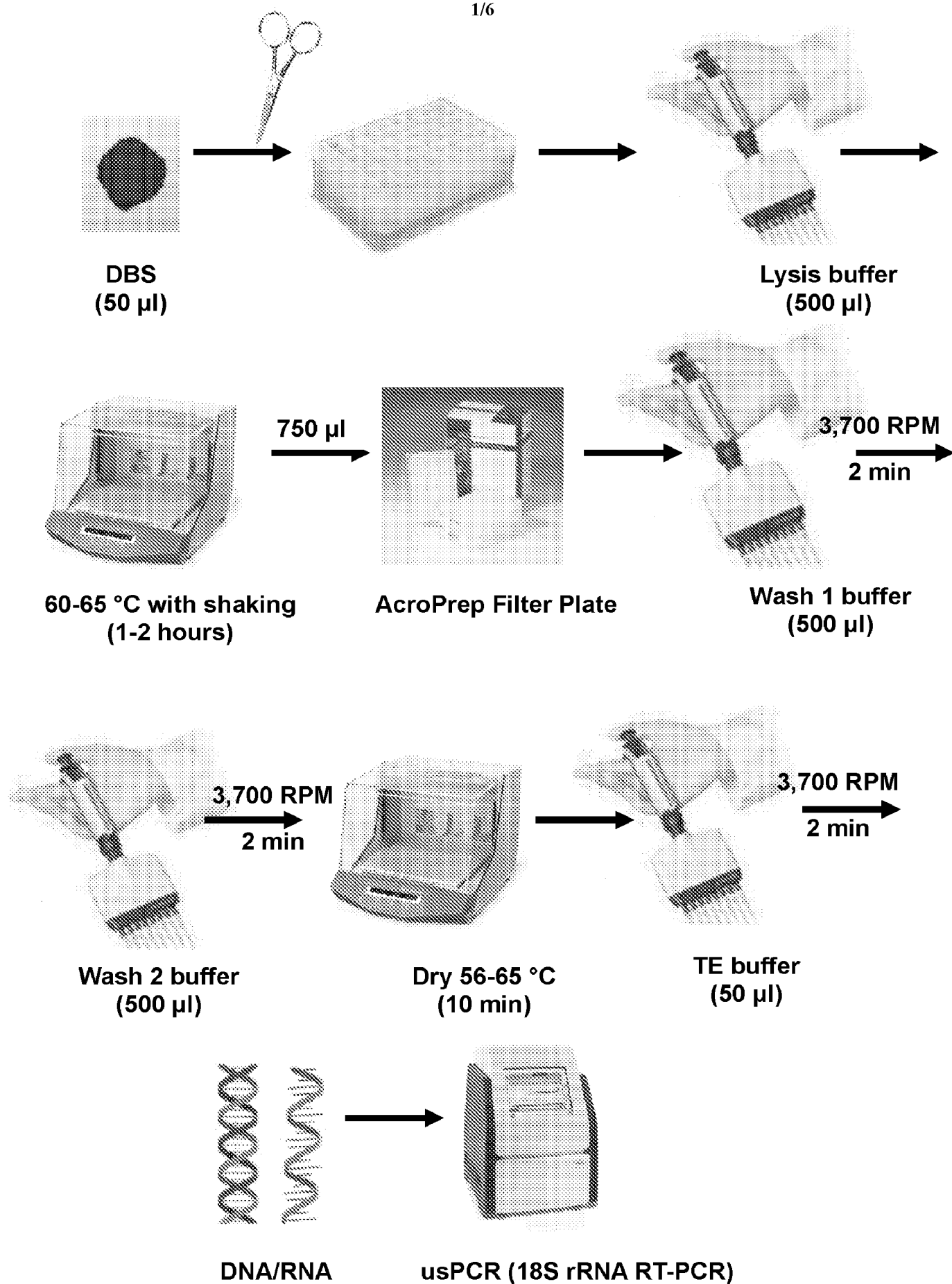
37. The method of claim 35 wherein the first wash buffer has a pH about 6.3 to about 6.5 and comprises 3 M guanidine thiocyanate, 2% v/v Triton, 10 mM EDTA, 5 mM TRIZMA hydrochloride, 16.7% v/v isopropanol and 6 mM hydrochloric acid.

38. The method of claim 35, wherein the second wash buffer has a pH of about 7.4 and comprises, 25% v/v ethanol, 25% v/v isopropanol, 100 mM sodium chloride and 10 mM TRIZMA hydrochloride.

39. The method of claim 35, wherein the elution buffer has a pH of about 8.0 and comprises, 10 mM Tris and 1 mM EDTA.

40. The method of claim 28, wherein the PCR is a nested PCR, a reverse transcription-PCR (RT-PCR) or a nested RT-PCR.

41. The method of claim 28, wherein the target gene is 18S ribosomal RNA.

**FIG. 1**

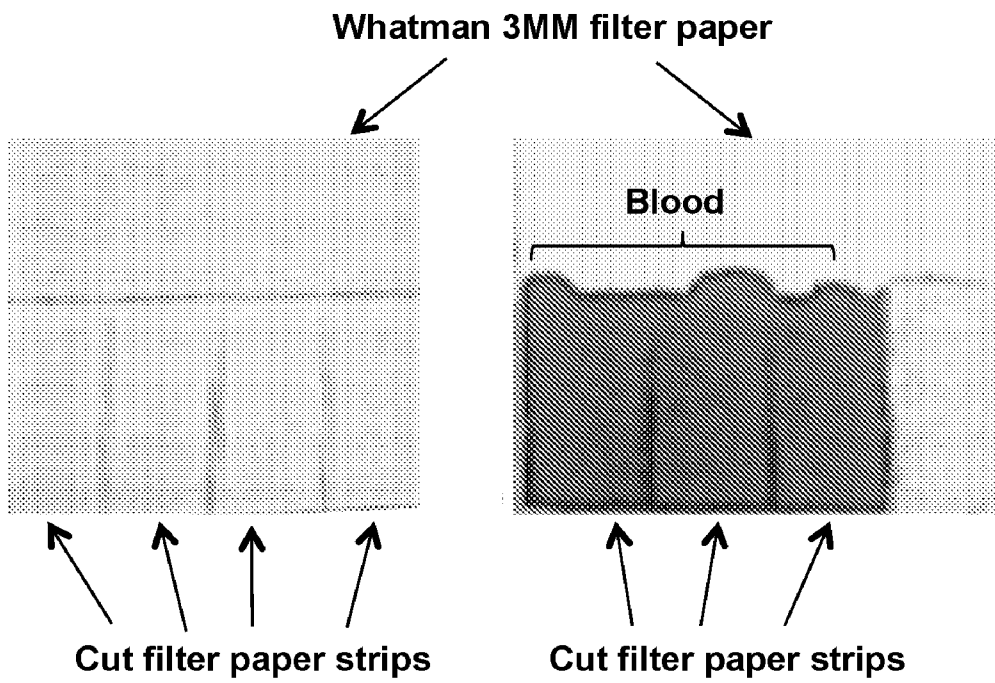


FIG. 2A

FIG. 2B

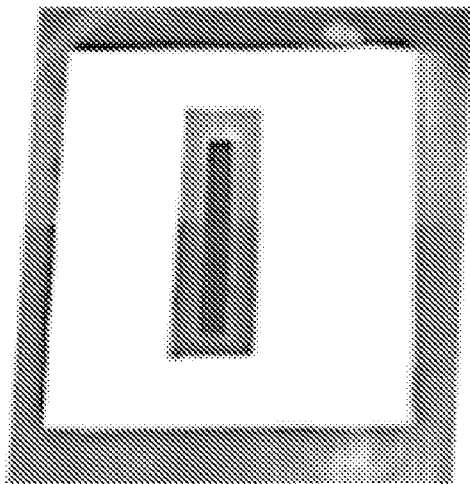


FIG. 3A

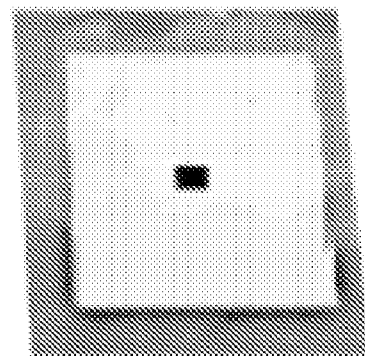


FIG. 3B

parasites/ml ~1,000

~100,000

~10

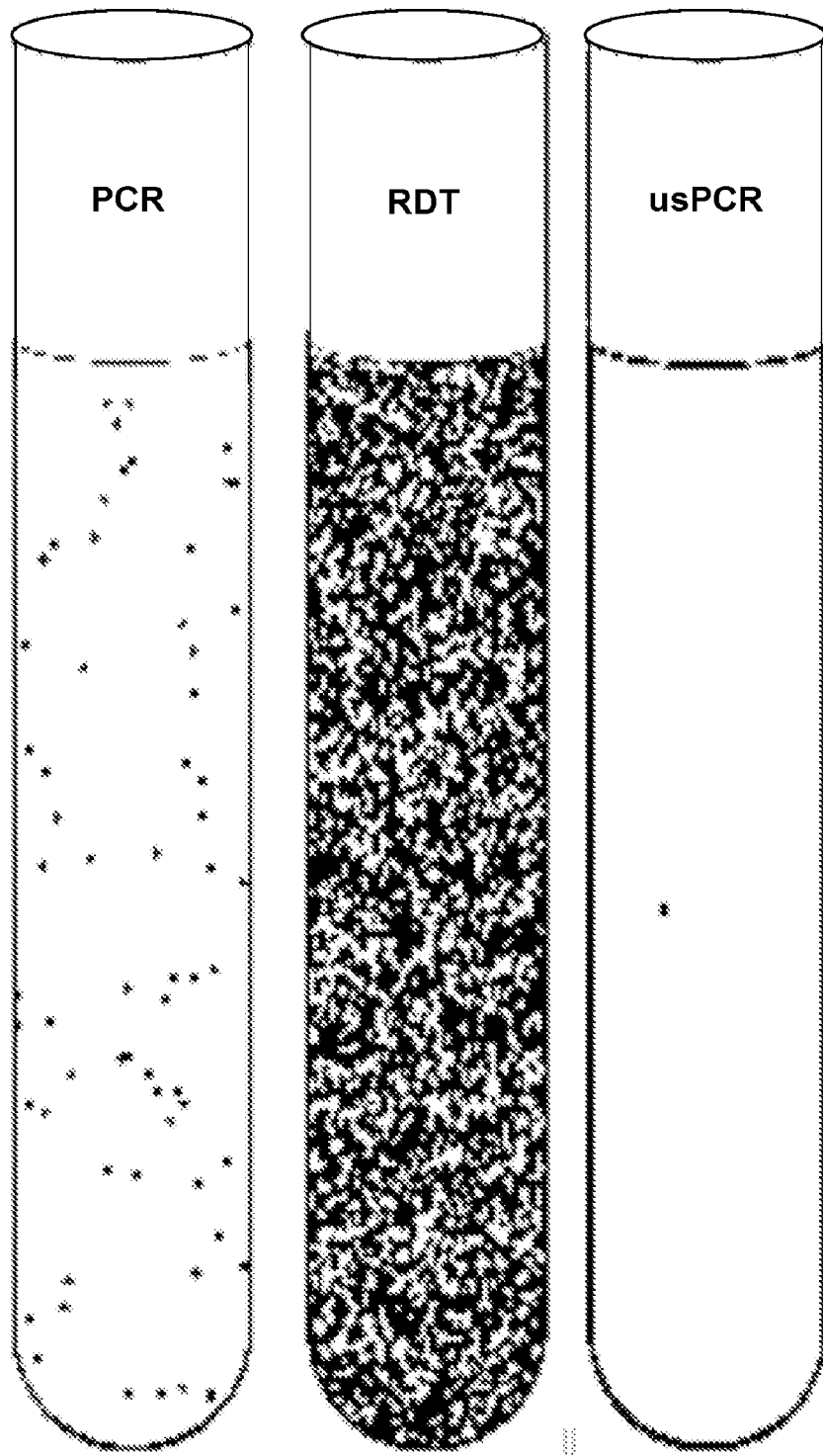


FIG. 4

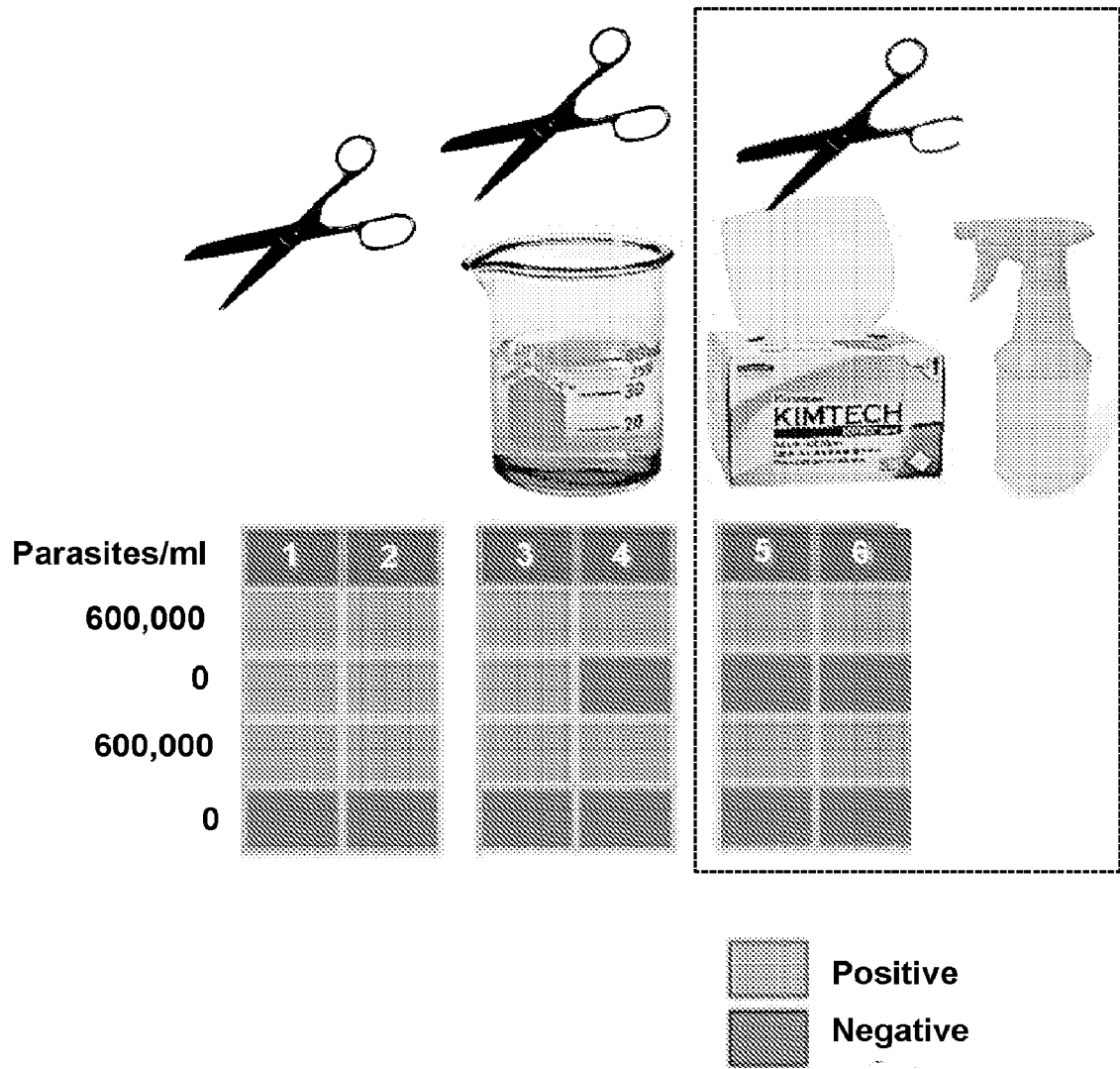


FIG. 5A

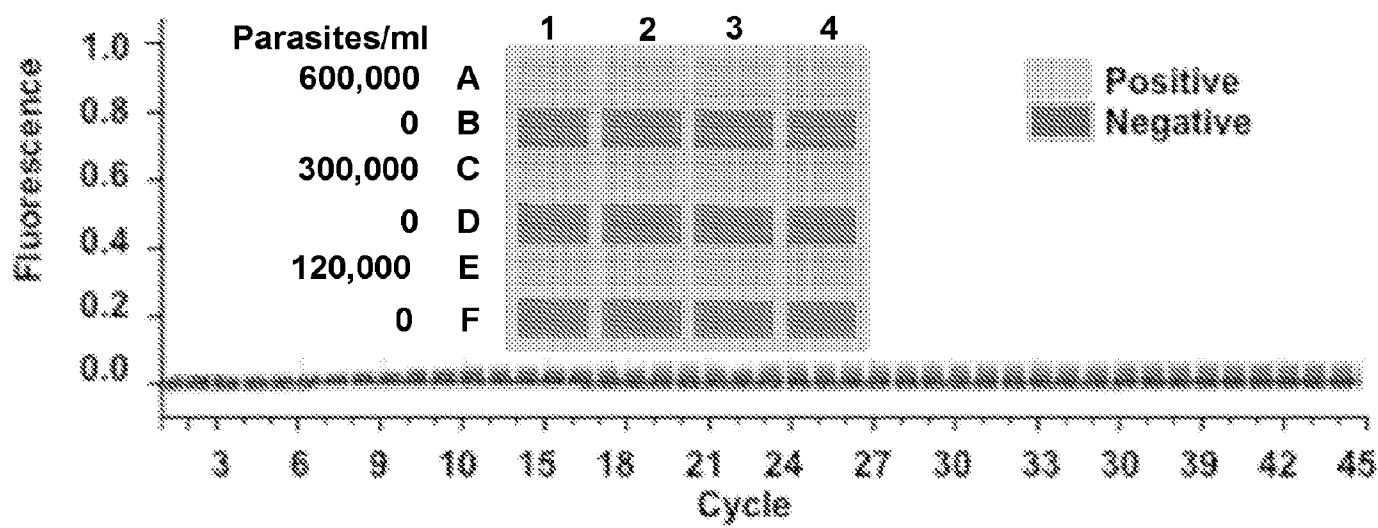


FIG. 5B

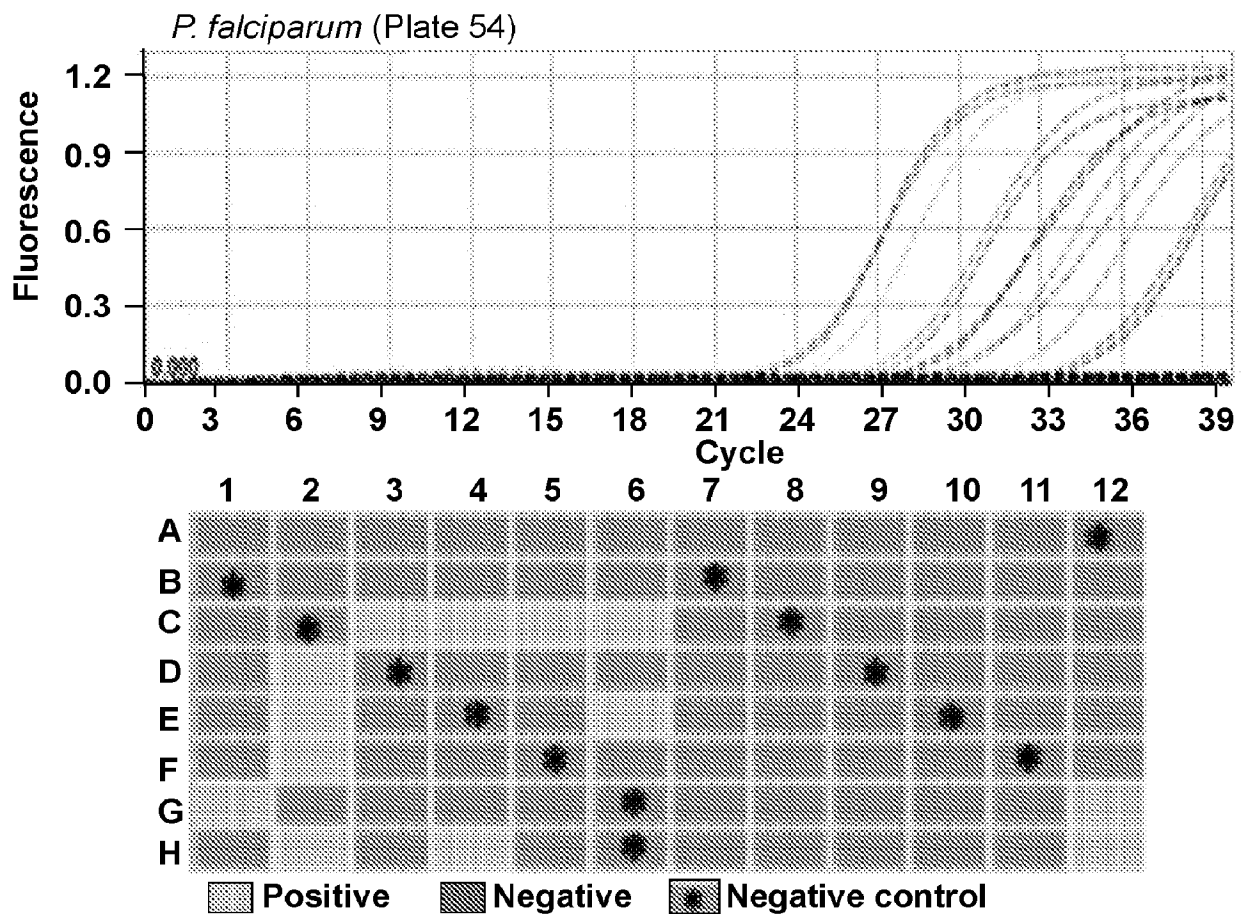
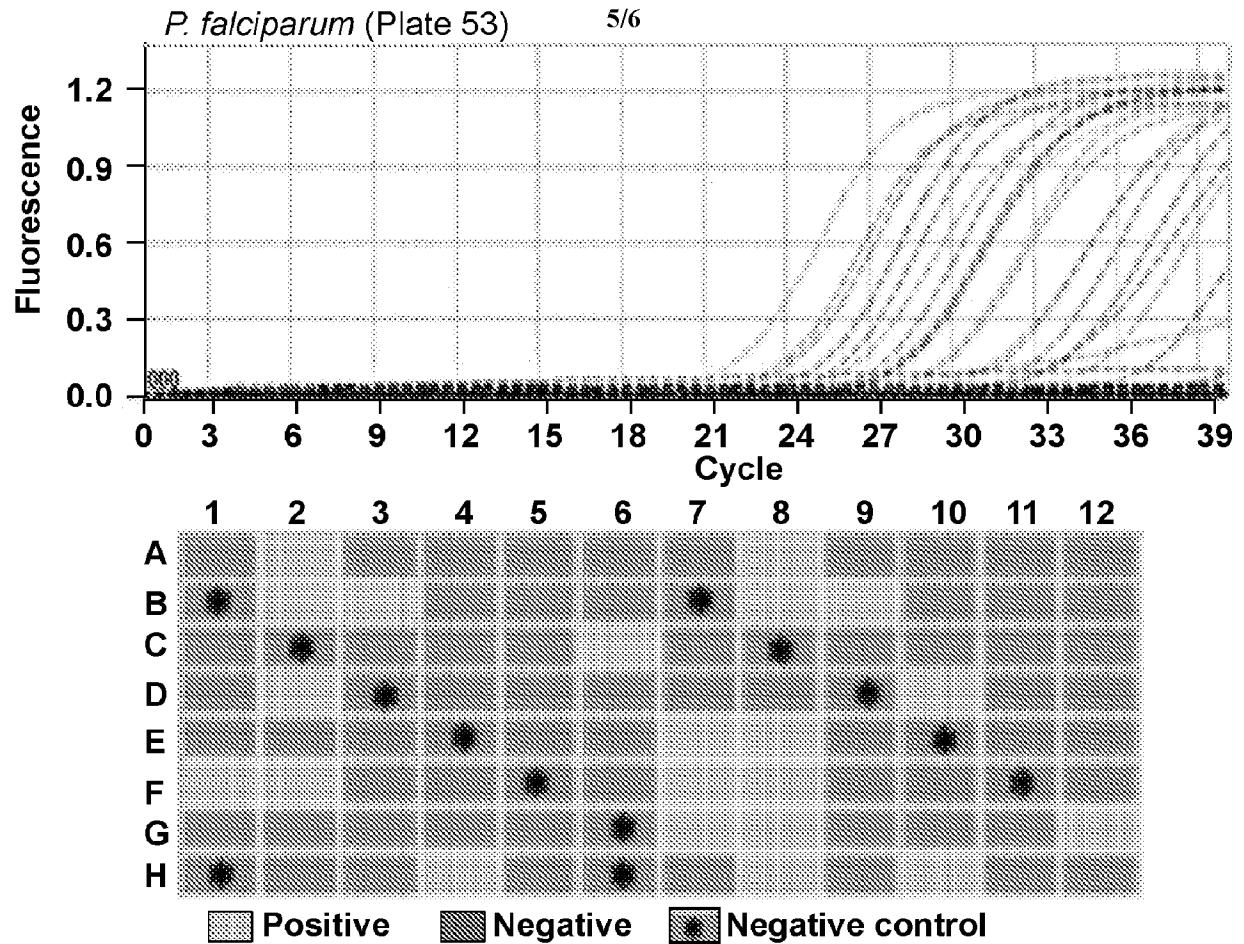


FIG. 5C

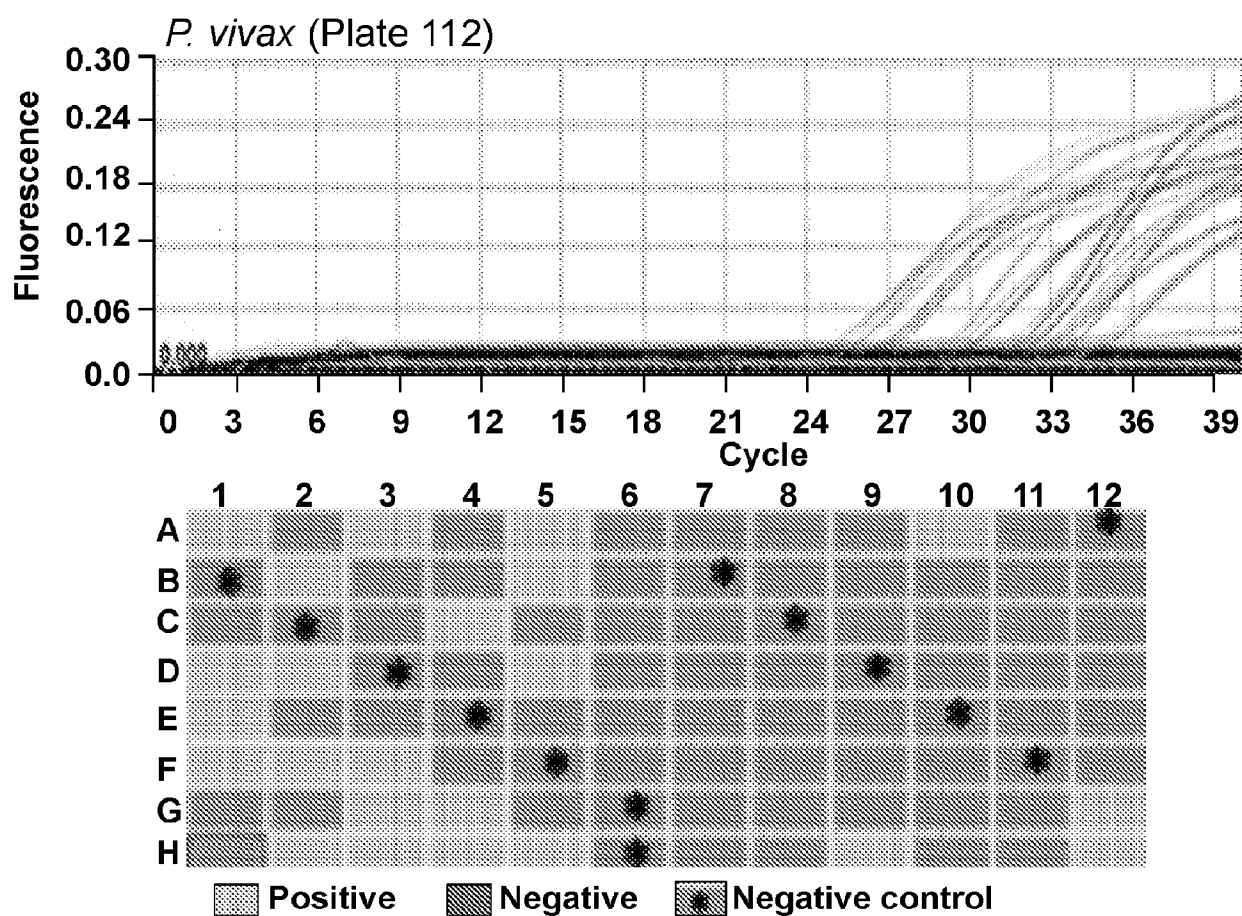
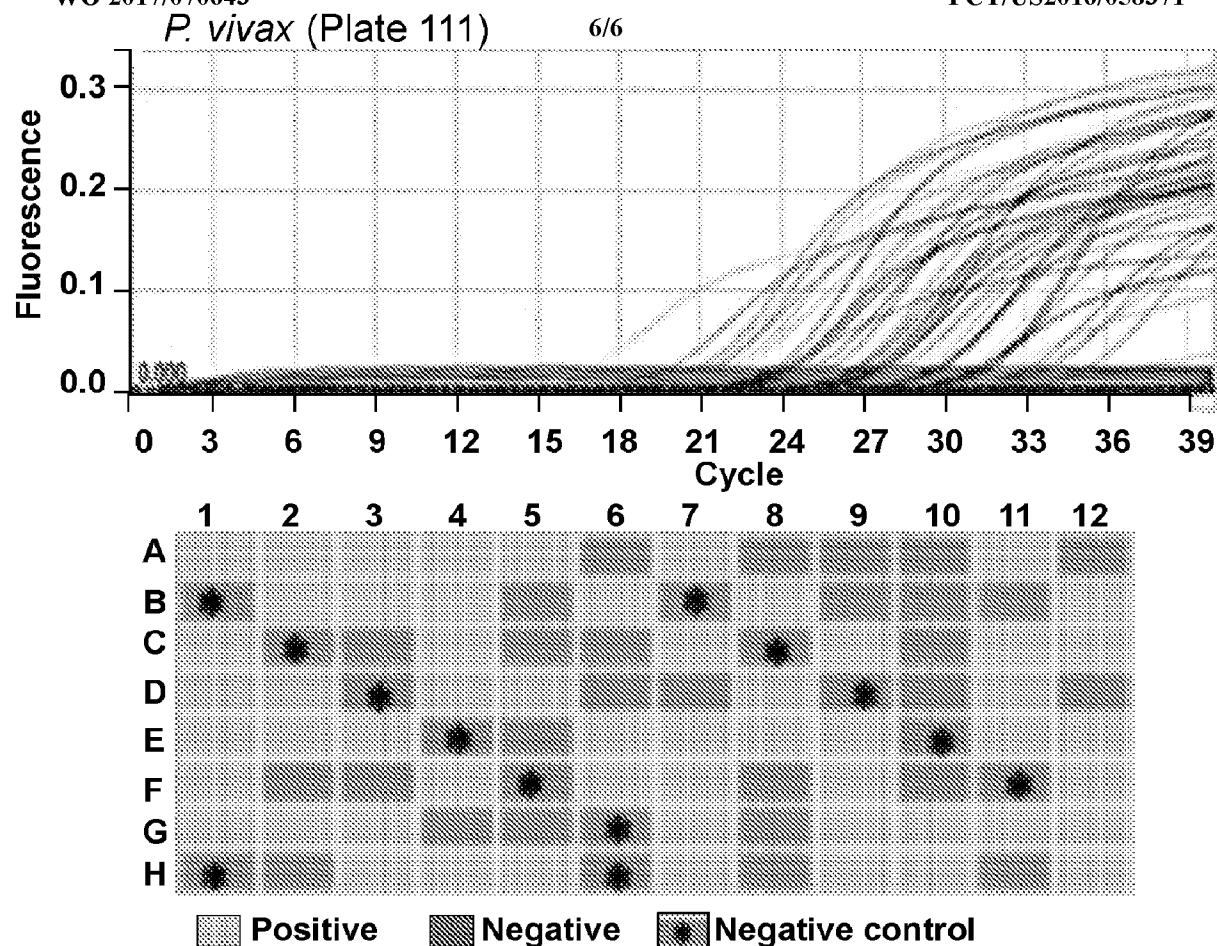


FIG. 5D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/058371

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/10; C12Q 1/68 (2016.01)

CPC - C12N 15/1003; C12Q 1/6806 (2016.11)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC - C12N 15/10; C12Q 1/68; G01N 33/49; G01N 33/50

CPC - C12N 15/1003; C12N 15/1006; C12N 15/1017; C12Q 1/6806; G01N 33/49; G01N 33/491; G01N 33/5002; G01N 33/5308

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 536/25.42; 536/25.41; 536/25.4 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar, PubMed

Search terms used: Dried blood; Extract; Lysis; Nucleic acids

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 6,410,725 B1 (SCHOLL et al) 25 June 2002 (25.06.2002) entire document	1-5 --- 6-27, 34
X --- Y	US 2015/0040688 A1 (UNIVERSITY OF WASHINGTON) 12 February 2015 (12.02.2015) entire document	28-33, 40, 41 --- 12, 23, 27, 34-39
Y	US 2010/0285578 A1 (SELDEN et al) 11 November 2010 (11.11.2010) entire document	6-27, 35-39
Y	US 2014/0018529 A1 (DU PONT et al) 16 January 2014 (16.01.2014) entire document	8, 19, 37
Y	US 6,548,256 B2 (LIENAU et al) 15 April 2003 (15.04.2003) entire document	9, 20, 38
Y	US 4,683,202 A (MULLIS) 28 July 1987 (28.07.1987) entire document	10, 21, 39
Y	US 2010/0105024 A1 (XU et al) 29 April 2010 (29.04.2010) entire document	25
A	US 2013/0338351 A1 (GENERAL ELECTRIC CO.) 19 December 2013 (19.12.2013) entire document	1-41

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

13 December 2016

Date of mailing of the international search report

03 JAN 2017

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