Title: METHOD, COMPOSITION AND KIT FOR HIGH THROUGHPUT DETECTION OF GENUS PLASMODIUM

Abstract: The present invention discloses a method for detecting genus Plasmodium in a sample. Furthermore, the present invention provides a composition and a kit for the detection of genus Plasmodium in a sample.
METHOD, COMPOSITION AND KIT FOR HIGH THROUGHPUT DETECTION OF GENUS
PLASMODIUM

Technical field of the invention

The present invention relates to a method for high throughput and sensitive detection of
Genus Plasmodium. The present invention also relates to the composition and kit
thereof.

Background of the invention

Malaria remains as one of the leading infectious diseases in the world. In recent years,
however, its decline of transmission in many countries makes it possible to consider
elimination of the disease (WHO. World malaria report; 2009). As countries approach
malaria elimination, disease monitoring, evaluation, and surveillance activities will need
to shift from measuring morbidity and mortality to detecting infections and measuring
transmission. This calls for new diagnostic tools with higher sensitivity for detecting
asymptomatic infections and higher throughput for mass screening and surveillance (A
research agenda for malaria eradication: diagnoses and diagnostics. PLoS Med
2011;8:e1000396). For example, with the increasing spread of the disease to
non-endemic areas as a result of global traveling and migrations, there is an urgent need
for large scale, active malaria screening in at-risk populations that may or may not show
clinical symptoms, as subclinically-infected individuals can be significant sources of
infection (Harris I, Sharrock WW, Bain LM, et al. A large proportion of asymptomatic
Plasmodium infections with low and sub-microscopic parasite densities in the low
transmission setting of Temotu Province, Solomon Islands: challenges for malaria
malaria are Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium
malariae and Plasmodium knowlesi. The most serious and fatal type of malaria is caused
by P. falciparum, while P. vivax-caused malaria is more prone to relapse (Galinski MR,
falciparum and P. vivax are the most prevalent parasites for malaria, and countries with
low, unstable transmission are encouraged to proceed to malaria elimination by focusing
on both falciparum elimination and vivax elimination (WHO. World malaria report;
2010).

Current diagnostic methods lack the sensitivity or throughput needed for monitoring and
controlling malaria transmission. The most-widely used microscopic examination
cannot detect Plasmodia infection lower than 50 parasites/µl (Moody A. Rapid diagnostic
tests for malaria parasites. Clinical Microbiology Reviews 2002;15:66), and can miss a
substantial proportion of infections in surveys of endemic populations, especially in areas
with low transmission of infection (Okell LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic
review and meta-analysis. J Infect Dis 2009; 200:1509-17). Recently developed
molecular methods, such as PCR (Snounou G, Viriyakosol S, Jarra W, Thaithong S,
Brown KN. Identification of the four human malaria parasite species in field samples by
the polymerase chain reaction and detection of a high prevalence of mixed infections.
Mol Biochem Parasitol 1993; 58:283-92), quantitative-PCR (Rougemon M, Van Saanen
M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. J Clin Microbiol 2004;42:5636-43, Nucleic Acid Sequence-Based Amplification (NASBA) (Mens PF, Sohooone GJ, Kager PA, Schallig HD. Detection and identification of human *Plasmodium* species with real-time quantitative nucleic acid sequence-based amplification. Malar J 2006;5:80) and Loop Mediated Isothermal Amplification (LAMP) (Lucchi NW, Demas A, Narayanan J, et al. Real-Time Fluorescence Loop Mediated Isothermal Amplification for the Diagnosis of Malaria. PLoS One 2010;5) are more sensitive and call for less malaria expertise, yet their dependence on DNA/RNA extraction can strongly influence the performance of the diagnosis. Furthermore, such dependence also hindered their use in high throughout patient screening. The protein-based Rapid Diagnostic Tests (RDTs) are easy, sensitive and useful for malaria screening, but they are more reliable for detecting *P. falciparum* infection and some cannot differentiate between active infection and past malaria experience, reducing their effectiveness as alternatives to microscopic diagnosis.

**Summary of the invention**

The present invention provides an easy, sensitive and reliable method suitable for large-scale detection of Genus *Plasmodium*. We adopted our previously developed sandwich RNA hybridization assay (Zheng Z, Luo Y, McMaster GK. Sensitive and quantitative measurement of gene expression directly from a small amount of whole blood. Clin Chem 2006;52:1294-302; and US patent application US2007/016015) to detect the presence of genus *Plasmodium* in a sample. All of the contents of the references are incorporated herein.

In detail, the present provides a method for detection of genus *Plasmodium* in a sample, comprising:

(a) providing a test sample suspected of containing target genus *Plasmodium*;

(b) optionally, releasing the polynucleic acids from the test samples of (a);

(c) contacting the said test sample or the released polynucleic acids with probe mixture containing one or more capture extender (CE) probes and one or more label extender (LE) probes;

wherein the CE probe consists of nucleotides hybridizing to a region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently or non-covalently attached to a solid support;

and wherein the LE probes consists of nucleotides hybridizing to a region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule;

for a time and under conditions sufficient for hybridization to take place; and

(d) contacting the said test sample with a detectable molecule after the said step (c), for a time and under conditions sufficient for hybridization to take place;

(e) detecting any hybridization which has taken place in step (c); wherein the presence or absence of hybridization is indicative of the presence or absence of said genus *Plasmodium* in said test sample.
In another aspect of the invention, the present provides a method for detection of genus *Plasmodium* in a sample, comprising:

(a) providing a test sample suspected of containing target genus *Plasmodium*;

(b) optionally, releasing the polynucleic acids from the test samples of (a);
(c) contacting the said test sample or the released polynucleic acids with probe mixture containing one or more CE probes, one or more LE probes and at least one blocking probe;

wherein the CE probe consists of nucleotides hybridizing to the region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently or non-covalently attached to a solid support;

and wherein the LE probes consists of nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule;

and wherein the blocking probe consists of nucleotides hybridizing to 18S ribosomal RNA of genus Plasmodium to reduce or eliminate non-specific hybridization of other probes to the sequence, and to enhance the hybridization of adjacent probes by stacking effect.

for a time and under conditions sufficient for hybridization to take place; and

(d) contacting the said test sample with a detectable molecule after the said step (c), for a time and under conditions sufficient for hybridization to take place;

(e) detecting any hybridization which has taken place in step (c); wherein the presence or absence of hybridization is indicative of the presence or absence of said genus *Plasmodium* in said test sample.

In a preferable embodiment, the amount of CE probe is from 1 wt% to 99 wt% by total weight of the composition; the amount of LE probe is from 1 wt% to 99 wt% total weight of the composition; the amount of blocking probe is from 1 wt% to 99 wt% total weight of the composition.

In a preferable embodiment, the said blocking probe has a sequence as shown in SEQ ID NO:22.

In a preferable embodiment, the said genus *Plasmodium* is selected from the group consisting of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*.

In another embodiment, the test sample is a blood sample including plasma, serum or blood clot, cultured erythrocytes, or blood sample dried on a filter paper.

In a preferable embodiment, the test sample is treated with proteinase K. Preferably, the test sample is treated with proteinase K at 50°C to 60°C for 1 hour. More preferably, the test sample is treated with proteinase K at 56°C for 1 hour.

In a preferable embodiment, the said one or more CE probes are bound to a solid support. More preferably, the solid support is a planar solid support or a bead. Most preferably, the support is a 96-well plate.
In a preferable embodiment, the detectable molecule is labeled with an enzyme or a fluorescent label.

In a preferable embodiment, the nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* have at least 80% identity, more preferably, at least 85%, more preferably 90%, most preferably 95% identity to the nucleotides as shown in SEQ ID NOs 23 to 43.

In other embodiments, the at least one or more CE probes are selected from the group consisting of SEQ ID NOs 1 to 7; and the at least one or more LE probe is selected from the group consisting of SEQ ID NOs 8 to 21.

In another aspect, the present invention provides a method for detection of genus *Plasmodium* in a sample, comprising:

(a) providing a test sample suspected of containing target genus *Plasmodium*;

(b) optionally, releasing the polynucleic acids from the test samples of (a);

(c) contacting the said test sample or the released polynucleic acids with probes mixture containing all of the probes represented as SEQ ID NOs 1 to 22; for a time and under conditions sufficient for hybridization to take place; and

(d) contacting the said test sample with a detectable molecule after the said step (c), for a time and under conditions sufficient for hybridization to take place;

(e) detecting any hybridization which has taken place in step (c); wherein the presence or absence of hybridization is indicative of the presence or absence of said genus *Plasmodium* in said test sample.

In another aspect, the present invention provides a composition for detection of genus *Plasmodium* in a sample, comprising:

(a) one or more CE probes; wherein the said CE probe consists of nucleotides hybridizing to a region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a solid support; and

(b) one or more LE probes; and wherein the LE probe consists of nucleotides hybridizing to a region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule.

In another aspect, the present invention provides a composition for detection of genus *Plasmodium* in a sample, comprising:

(a) one or more CE probes; wherein the said CE probe consists of nucleotides hybridizing to the region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a solid support;

(b) one or more LE probes; and wherein the LE probe consists of nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule; and
(c) at least one blocking consisting of nucleotides hybridizing to 18S ribosomal RNA of genus *Plasmodium* to reduce or eliminate non-specific hybridization of other probes to the sequence, and to enhance the hybridization of adjacent probes by stacking effect.

In a preferable embodiment, the nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* have at least 80% identity, more preferably, at least 85%, more preferably 90%, most preferably 95% identity to the nucleotides as shown in SEQ ID NOs 23 to 43.

In a preferable embodiment, the said probe is selected from the group consisting of SEQ ID NOs 1 to 7; and the LE probe is selected from the group consisting of SEQ ID NOs 8-21.

In a preferable embodiment, the said blocking probe has a sequence as shown in SEQ ID NO:22.

In another respect, the present invention provides a composition for detection of genus *Plasmodium* in a sample, comprising all of the probes as shown in SEQ ID NOs 1 to 23.

In another respect, the present invention provides a kit for detection of genus *Plasmodium* in sample, comprising

(a) a composition comprising:

(i) one or more CE probes; wherein the said CE probe consists of nucleotides hybridizing to a region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a solid support; and

(ii) one or more LE probes; and wherein the LE probe consists of nucleotides hybridizing to a region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule;

(b) a solid support;

(c) a detectable molecule.

In another respect, the present invention provides a kit for detection of genus *Plasmodium* in sample, comprising

(a) a composition comprising:

(i) one or more CE probes; wherein the said CE probe consists of nucleotides hybridizing to a region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a solid support; and

(ii) one or more LE probes; and wherein the LE probe consists of nucleotides hybridizing to a region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule;

(iii) at least one blocking consisting of nucleotides hybridizing to 18S ribosomal RNA of genus *Plasmodium* to reduce or eliminate non-specific hybridization of other probes to the sequence, and to enhance the hybridization of adjacent probes by stacking effect.
(b) a solid support;
(c) a detectable molecule.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. The following definitions supplement those in the art and are directed to the current application and are not to be imputed to any related or unrelated case, e.g., to any commonly owned patent or application. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. Accordingly, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a molecule" includes a plurality of such molecules, and the like.

The terms “polynucleic acid”, “polynucleotide”, “nucleotide” and “nucleic acid” are interchangeably used herein and encompass any physical string of monomer units that can be corresponded to a string of nucleotides, including a polymer of nucleotides (e.g., a typical DNA or RNA polymer), peptide nucleic acids (PNAs), modified oligonucleotides (e.g., oligonucleotides comprising nucleotides that are not typical to biological RNA or DNA, such as 2′-O-methylated oligonucleotides), and the like.

The term “capture extender probe” or “CE probe” is a polynucleotide that is capable of hybridizing to 18S ribosomal RNA of genus Plasmodium and is capable of hybridizing to nucleic acids covalently or non-covalently attached to a solid support. The capture extender probe (CE probe) typically has a first polynucleotide portion, which is complementary to 18S ribosomal RNA of genus Plasmodium, and a second polynucleotide portion, which is complementary to a polynucleotide covalently or non-covalently attached to a solid support. The first and the second polynucleotide are typically not complementary to each other. The CE probe is preferably single-stranded.

The term “label extender probe” or “LE probe” is a polynucleotide that is capable of hybridizing to 18S ribosomal RNA of genus Plasmodium and is capable of hybridizing to a detectable molecule. The label extender probe (LE probe) typically has a first polynucleotide portion, which is complementary to 18S ribosomal RNA of genus Plasmodium, and a second polynucleotide portion, which is complementary to a detectable molecule. The first and the second polynucleotide are typically not complementary to each other. The LE probe is preferably single-stranded.

The term “detectable molecule” comprises one or more polynucleotides that collectively comprise a label and a polynucleotide sequence M-1, which is capable of hybridizing to
at least one LE probe. The label provides a signal, directly or indirectly. Polynucleotide sequence M-1 is typically complementary to the second polynucleotide in the LE probe.

The terms “hybridization” and “hybridizing” are used herein to refer to denote the paring of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Defining appropriate hybridization conditions is within the skill of the art.

Brief Description of the Drawings

Figure 1 indicates the correlation of assay signal with cultured P. falciparum in human erythrocytes. P. falciparum in human erythrocyte culture was determined as described in Method for detection of Genus Plasmodium. The limit of detection was determined as the minimal amount of cultured Plasmodia added to the erythrocytes that gave a net signal above 3 times the SD of the background erythrocyte control. Each dilution is prepared using culture medium. Triplicate samples were used in the assay and the data is representative of three independent runs. Averaged limit of detection was 0.04 parasite/μl blood. RLU: relative light unit

Figure 2 indicates the correlation between assay signals and parasitemia of poorly stored blood samples. Stored blood samples from 59 patients, which underwent multiple freeze-and-thaw cycles after collection due to limiting resources, were assayed (20μl each) and the net signals were plotted against parasitemia determined microscopically at admission. Each sample was assayed in duplicate and the averaged signal was used. Signal intensities beyond 1x10^7 RLU were considered approaching detection saturation of the photon detector.

Detailed Descriptions of the Invention

Examples

Sample collection

Veinous heparin blood samples were collected from 202 patients with fever of unknown origin in Kachin, Myanmar, and in Yunnan, China. Among them, 59 were collected in 2008, and 143 was collected in 2011. Control blood samples (n=13) used for analytical evaluation were taken from healthy subjects in Beijing, China. All samples were frozen and stored at -80 °C before use. Samples were collected with written informed consent. Ethical approval was granted by the Institutional Review Board (IRB) of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Due to limited resources, the 59 blood samples have suffered several freeze-and-thaw cycles before the test.

Method for detection of Genus Plasmodium

1. Microscopy

At time of admission, 2 slides, each containing a thick and a thin blood film, were
collected from each patient and dyed with 2% Giemsa Stain solution for 30 minutes. Slides were then examined independently by two professional microscopists for plasmodium parasites, and were pronounced negative only when a minimum of 500 fields had been carefully examined by each microscopist for the absence of parasites. The parasite density was counted if the slide was positive.

2. RDT

RDT tests in this study were done with CARESTART™ (Accessbio, Monmouth Junction, NJ) according to the manufacturer’s protocol. The kit is among the list of RDT procurement recommendations issued by WHO.

3. Real time qPCR

DNA was extracted from 200 µl of thawed blood with QIAamp DNA Blood Mini Kit (QIAGEN), according to the manufacturer’s instructions. The genus Plasmodium 18s screening primer and probe sequences and real time qPCR condition were adopted from previously published work. If the fluorescent signal did not increase within 40 cycles (Ct ≤ 40), the sample was considered negative. At least 1 positive control and 1 negative control were included to each experiment. Each sample was tested in duplicate.

4. Sandwich RNA hybridization assay

22 oligonucleotide probes (having the sequences indicated in SEQ ID Nos: 1 to 22) targeting at several highly conserved regions in 18S ribosomal RNA of the genus Plasmodium, including P. falciparum (GeneBank accession number M19172.1), P. vivax (U03079.1), P. malariae (AF488000.1), P. ovale (L48987.1) and P. knowlesi(L07560.1) was used for the assay. The sequences of the probes are indicated as follows:

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>SEQ ID NO:</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE1</td>
<td>SEQ ID NO:1</td>
<td>atcaaaatagtctagtccacaaattcaTTTTTcatctggagaagaagt</td>
</tr>
<tr>
<td>CE2</td>
<td>SEQ ID NO:2</td>
<td>ccaaggtaggctaccaacccatTTTTTcatctggagaagaagt</td>
</tr>
<tr>
<td>CE3</td>
<td>SEQ ID NO:3</td>
<td>gcttcagaacctcctctttaaatTTTTTctctggagaagaagt</td>
</tr>
<tr>
<td>CE4</td>
<td>SEQ ID NO:4</td>
<td>aattggcctgggttgtgttattTTTTTcatctggagaagaagt</td>
</tr>
<tr>
<td>CE5</td>
<td>SEQ ID NO:5</td>
<td>actecctaatcttccttcgtctgtgatTTTTTctctggagaagaagt</td>
</tr>
<tr>
<td>CE6</td>
<td>SEQ ID NO:6</td>
<td>ccaagtgggctatagtaattgtgatTTTTTctctggagaagaagt</td>
</tr>
<tr>
<td>CE7</td>
<td>SEQ ID NO:7</td>
<td>gctctgggaggaTTTTTctctggagaagaagt</td>
</tr>
<tr>
<td>LE1</td>
<td>SEQ ID NO:8</td>
<td>ttatttagcgtgattcctcctttaaatTTTTTgaagttacgcttt</td>
</tr>
<tr>
<td>LE2</td>
<td>SEQ ID NO:9</td>
<td>tgcgttgatacacactaaataaatTTTTTctgatgctacaagcact</td>
</tr>
<tr>
<td>LE3</td>
<td>SEQ ID NO:10</td>
<td>accatccacaaataaaccacaaTTTTTgaagttacgcrttt</td>
</tr>
<tr>
<td>LE4</td>
<td>SEQ ID NO:11</td>
<td>tctgggaaggtttttatatcTTTTTctgatgctacaagcact</td>
</tr>
<tr>
<td>LE5</td>
<td>SEQ ID NO:12</td>
<td>gttacaccaacagtaaatctttactTTTTTgaagttacgcrttt</td>
</tr>
<tr>
<td>LE6</td>
<td>SEQ ID NO:13</td>
<td>tgcgttttgcattcagatatTTTTTctgatgctacaagcact</td>
</tr>
<tr>
<td>LE7</td>
<td>SEQ ID NO:14</td>
<td>etctatattcgcactaatgacctTTTTTgaagttacgcrttt</td>
</tr>
<tr>
<td>LE8</td>
<td>SEQ ID NO:15</td>
<td>gattcggaggtttttatatcTTTTTctgatgctacaagcact</td>
</tr>
<tr>
<td>LE9</td>
<td>SEQ ID NO:16</td>
<td>actaagattacctctgacactcTTTTTgaagttacgcrttt</td>
</tr>
<tr>
<td>LE10</td>
<td>SEQ ID NO:17</td>
<td>cgccagtttgtcgcactTTTTTctgatgctacaagcact</td>
</tr>
<tr>
<td>LE11</td>
<td>SEQ ID NO:18</td>
<td>tccccatatatattgcataagttacccatTTTTTgaagttacgcrttt</td>
</tr>
<tr>
<td>LE12</td>
<td>SEQ ID NO:19</td>
<td>ttaataattgcaaatatactatcgcagTTTTTttgagtcagaatcat</td>
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<tr>
<td>------</td>
<td>-------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>LE13</td>
<td>SEQ ID NO:20</td>
<td>taagtgcattctctgtcaagaTTTTTgaagttacgtttt</td>
</tr>
<tr>
<td>LE14</td>
<td>SEQ ID NO:21</td>
<td>agcacaatctgatgaatcatcatgctTTTTTtctgagctaaagcat</td>
</tr>
<tr>
<td>Blocking probe</td>
<td>SEQ ID NO:22</td>
<td>tgaaggtatctgtacgtaccc</td>
</tr>
</tbody>
</table>

For each assay, 20 µl of fresh or thawed blood or erythrocyte culture of P. falciparum was lysed with 50 µl of Lysis Mixture (Pomics/Affymetrix), 28 µl of water and 2 µl of 50 µg/ml proteinase K at 60 °C for 1 h with vigorous shaking. The lysate were mixed with probes and hybridized to the probe set comprising the above 22 oligonucleotide probes, wherein the amounts of CE, LE and BE probes are 150, 600 and 300 fmol, respectively. The mixture was incubated at 58 °C overnight without shaking. After the unbound probes were washed off, captured targets were sequentially hybridized, with washing in between, with Preamplicfer, Amplifier, Label Probe and Substrate as described in the Quantigene Assay Kit (Pomics/Affymetrix). The resulting chemiluminescence was quantified in a Modulus plate reader (Turner Biosciences) as previously described Zheng Z et al., 2006 (Zheng Z, Luo Y, McMaster GK. Sensitive and quantitative measurement of gene expression directly performed from a small amount of whole blood. Clin Chem 2006;52:1294-302) For malaria diagnosis, the background signal from blank control was subtracted from sample signal to obtain the net signal, and samples with net signal above the detection threshold (3xSD of blank control) was diagnosed as positive, while below as negative. Each sample was tested in duplicate.

Example 1

We investigated the detection limit and the quantification capability of the RNA hybridization assay we developed. We tested a 3-fold serial dilution of fresh human erythrocyte-cultured P. falciparum. The assay gave a detection limit of about 0.04 parasite/µl, with signals above the threshold proportional to parasite numbers (R²=0.999) (Figure 1), indicating that our assay is highly sensitive, quantitative and able to detect low parasitemia in intact samples. All samples collected from health volunteers (n=13) are negative, showing that the method is highly specific.

Example 2

We tested our assay with clinical blood samples from 202 febrile patients with undetermined cause. Among these samples, 66 were determined by microscopy as positive (P. falciparum (n=27) and P. vivax (n=39)), with parasitemia ranging from 320 parasites/µl to 6×10³ parasites/ µl, while the remaining 136 samples were microscopy-negative. Our assay identified all 66 microscopy-positive samples. There was no apparent difference between detecting P.falciparum infection and P.vivax infection. Although an overnight incubation was required, the overall hands-on time of the assay was less than 2 hours as the samples were assayed in parallel in 96-well plates. The blood lysates for this assay can be stored at -20° C with stability for at least 6 months (data not shown). These results suggest the assay is well sufficient for simultaneous qualitative detection of malaria infection in a large number of samples.

Despite broad agreement between microscopy and our assay (66 positive and 131
negative), there were, as may be expected, 5 microscopy negative samples which showed positive in our assay. We decided to further investigate microscopic-negative samples with RDT and real-time qPCR, and compared the results (Table 1 and Table 2). We tested all 136 microscopy-negative and 7 microscopic positive samples with RDT. The two methods have good agreement for 134 negative samples and 4 positive samples. However, 3 microscopy positive and 2 microscopy negative samples have opposite result in RDT. All these 5 samples were later proved to be positive both by our assay and by real time qPCR.

Table 1 Results of microscopy, real time qPCR, RDT and RNA hybridization assay

<table>
<thead>
<tr>
<th>Microscopy (n=202)</th>
<th>RNA hybridization assay (n=202)</th>
<th>Positive (n=71)</th>
<th>Negative (n=131)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>66(27P.f+39 P.v*)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>5</td>
<td>131</td>
</tr>
<tr>
<td>Real time qPCR</td>
<td>Positive</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>(n=44)</td>
<td>Negative</td>
<td>1</td>
<td>23</td>
</tr>
<tr>
<td>RDT (n=143)</td>
<td>Positive</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6</td>
<td>131</td>
</tr>
</tbody>
</table>

*P.f=plasmodium falciparum, P.v=plasmodium vivax

Table 2 Results of the microscopy false-negative samples

<table>
<thead>
<tr>
<th>Patients</th>
<th>Microscopy</th>
<th>RDT</th>
<th>Real qPCR (Ct)</th>
<th>time</th>
<th>RNA hybridization assay (net RLU)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative</td>
<td>Negative</td>
<td>41</td>
<td></td>
<td>9.89E+03</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>Negative</td>
<td>34</td>
<td></td>
<td>4.33E+06</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>Negative</td>
<td>39</td>
<td></td>
<td>6.87E+03</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>Positive</td>
<td>32</td>
<td></td>
<td>7.89E+05</td>
</tr>
<tr>
<td>5</td>
<td>Negative</td>
<td>Positive</td>
<td>38</td>
<td></td>
<td>6.06E+03</td>
</tr>
</tbody>
</table>

*RLU detection threshold for RNA hybridization assay is 5029.

There are in total 8 disagreements out of 202 samples between the results of microscopy, RDT and our assay. We tested all these 8 samples, along with other 36 randomly chosen samples, by real time qPCR. For these 8 samples with discrepancy, both qPCR (including DNA extraction) and our assay were repeated twice for conformation. For 43 samples out of 44, the result is consistent with diagnosis of our assay (20 positive, 23 negative), the
remaining one sample is positive in our assay but is negative in real time qPCR and other two methods. For this one, our assay gave rather weak signal, but above detection threshold, indicating the rather low parasite load; the Ct value of the qPCR assay for this sample is 41, close to the threshold cycle number of 40 (Table 2). Doubling the DNA input in the qPCR assay did not improve the Ct value of this sample.

Using microscopy as the gold standard, our assay has a sensitivity of 100% (66/66); using real time qPCR as the gold standard, our assay has a sensitivity of 100% (20/20). There was no sample that was negative by our assay but was positive by other assays tested.

Example 3

To test for the assay’s robustness and to determine if the assay is capable of diagnosing samples of sub-optimal quality, we examined the assay results of the 59 malaria samples that were poorly stored and likely partially degraded. These samples were microscopy-positive, and included both P. falciparum samples and P. vivax samples that underwent repeated freeze-thaw during collection and storage. Signals of all 59 patient samples were above detection threshold, and a majority of them approached detection saturation (Figure 2), despite the fact that the samples may have suffered from RNA degradation due to several freeze-and-thaw cycles and prolonged storage. The assay had a mean intra-assay CV of 5%.

Reference
1. WHO. World malaria report; 2009.
5. WHO. World malaria report; 2010.
What is claimed is:

1. A method for detection of genus Plasmodium in a sample, comprising the steps of
   (a) providing a test sample suspected of containing target genus Plasmodium;
   (b) optionally, releasing the polynucleic acids from the test samples of the step
   (a);
   (c) contacting the said test sample or the released polynucleic acids with probe
   mixture containing one or more CE probes and one or more LE probes for a time and
   under conditions sufficient for hybridization to take place, wherein the CE probe consists
   of nucleotides hybridizing to a region of the 18S ribosomal RNA of genus Plasmodium
   and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a
   solid support; and the LE probes consists of nucleotides hybridizing to a region of 18S
   ribosomal RNA of genus Plasmodium and the nucleotides hybridizing to a detectable
   molecule,
   (d) contacting the said test sample with a detectable molecule after the said step
   (c), for a time and under conditions sufficient for hybridization to take place; and
   (e) detecting any hybridization which has taken place in the step (c); wherein the
   presence or absence of hybridization is indicative of the presence or absence of said
   genus Plasmodium in said test sample.

2. The method according to claim 1, wherein the method further comprises the steps of
   contacting the said test sample with at least one blocking probe consisting of nucleotides
   hybridizing to 18S ribosomal RNA of genus Plasmodium to reduce or eliminate
   non-specific hybridization of other probes to the sequence, and to enhance the
   hybridization of adjacent probes by stacking effect.

3. The method according to claim 2, wherein the said blocking probe has a sequence as
   shown in SEQ ID NO:22.

4. The method according to any one of claims 1 to 3, wherein the said genus Plasmodium
   is selected from the group consisting of P. falciparum, P. vivax, P. malariae, P. ovale and P.
   knowlesi.

5. The method according to any one of claims 1 to 4, wherein the said test sample is a
   blood sample, cultured erythrocytes, or blood sample dried on a filter paper.

6. The method according to any one of claims 1 to 5, wherein the said one or more CE
   probes are bound to the said solid support.

7. The method according to claim 6, wherein the said support is a planar solid support or
   a bead.

8. The method according to claim 7, wherein the said support is a 96-well plate.

9. The method according to any one of claims 1 to 8, wherein the said detectable
   molecule is labeled with an enzyme or a fluorescent label.
10. The method according to any one of claims 1 to 9, wherein the nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* are selected from the nucleotides having at least 80% identity to the nucleotides as shown in SEQ ID NOs 23 to 43.

11. The method according to any one of claims 1 to 10, wherein the said CE probe is selected from the group consisting of SEQ ID NOs 1 to 7; and the said LE probe is selected from the group consisting of SEQ ID NOs 8 to 21.

12. A method for detection of genus *Plasmodium* in a sample, comprising the steps of
(a) providing a test sample suspected of containing target genus *Plasmodium*;
(b) optionally, releasing the polynucleic acids from the test samples of the step (a);
(c) contacting the said test sample or the released polynucleic acids with probes mixture containing at least one probe selected from the group consisting of the probes represented as SEQ ID NOs 1 to 22 for a time and under conditions sufficient for hybridization to take place;
(d) contacting the said test sample with a detectable molecule after the step (c), for a time and under conditions sufficient for hybridization to take place; and
(e) detecting any hybridization which has taken place in the step (c); wherein the presence or absence of hybridization is indicative of the presence or absence of said genus *Plasmodium* in said test sample.

13. A composition for detection of genus *Plasmodium* in a sample, comprising:
(a) one or more CE probes; wherein the said CE probe consists of nucleotides hybridizing to the region of the 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to nucleic acids covalently non-covalently attached to a solid support; and
(b) one or more LE probes; and wherein the LE probe consists of nucleotides hybridizing to the region of 18S ribosomal RNA of genus *Plasmodium* and the nucleotides hybridizing to a detectable molecule;
wherein the said CE probes and LE probes are in effective amounts sufficiently for hybridization to take place.

14. The composition according to claim 13, further optionally comprising a blocking probe in effective amounts sufficiently for hybridizing to 18S ribosomal RNA of genus *Plasmodium* to reduce or eliminate non-specific hybridization of other probes to the sequence, and to enhance the hybridization of adjacent probes by stacking effect.

15. The composition of claim 14, wherein the amount of CE probe is from 1 wt% to 99 wt% by total weight of the composition; the amount of LE probe is from 1 wt% to 99 wt% total weight of the composition; the amount of blocking probe is from 1 wt% to 99 wt% total weight of the composition.

16. The composition according to claim 13 or 14, wherein the nucleotides hybridizing
to the region of 18S ribosomal RNA of genus *Plasmodium* are selected from the nucleotides having at least 80% identity to the nucleotides as shown in SEQ ID NOs 23 to 43.

17. The composition according to any one of claims 13 to 15, wherein the said CE probe is selected from the group consisting of SEQ ID NOs 1 to 7; and the said LE probe is selected from the group consisting of SEQ ID NOs 8-21.

18. The composition according to any one of claims 14 to 16, wherein the said blocking probe has a sequence as shown in SEQ ID NO:22.

19. A composition for detection of genus *Plasmodium* in a sample, comprising all of the probes as shown in SEQ ID NOs 1 to 22.

20. A kit for detection of genus *Plasmodium* in sample, comprising a) a composition according to any one of claims 13 to 18; b) a solid support; c) a detectable molecule and d) an instruction.
Figure 1

Figure 2
# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

See the extra sheet

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: C12Q; C07H

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

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

VEN, CNKI, CNABS, CPSABS, CNTXT, EPTXT, USTXT, WOTXT, JPTXT, ISI web of Knowledge, EMBASE, NCBI: plasmodium, detect, 18s, RNA, probe, hybridize+, sequences search on DB ID NOs: 1-43

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>CN101541978 A (HARRIS N) 23 Sep.2009 (23.09.2009) see claim 1, description page 17, line 23-page 20, line 8.</td>
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<td>CN10545168 A (ASTRA AB) 18 Sep.1991 (18.09.1991) see the whole document.</td>
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☐ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
  - “A” document defining the general state of the art which is not considered to be of particular relevance
  - “E” earlier application or patent but published on or after the international filing date
  - “L” document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - “O” document referring to an oral disclosure, use, exhibition or other means
  - “P” document published prior to the international filing date but later than the priority date claimed
  - “T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  - “X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  - “Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  - “&” document member of the same patent family

Date of the actual completion of the international search
15 Jan.2013 (15.01.2013)

Date of mailing of the international search report
07 Feb. 2013 (07.02.2013)

Name and mailing address of the ISA/CN
The State Intellectual Property Office, the P.R.China
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Facsimile No. 86-10-62019451

Form PCT/ISA/210 (second sheet) (July 2009)

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Continuation of CLASSIFICATION OF SUBJECT MATTER:
C12Q 1/68 (2006.01)i
C07H 21/04 (2006.01)i