Biomarkers for Infectious Disease Diagnostics in the Developing World:

Rapid, Home-based Diagnosis of Malaria in Symptomatic Individuals and Screening of Asymptomatic Pregnant Women

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Malaria

In this review of diagnostic biomarkers for malaria, Section 1 provides an introduction to the diagnostic needs for malaria. Sections 2 and 3 discuss the current status of biomarkers and technologies that have been used for malaria diagnosis, and their performance for the two intervention points that have been selected, which are 1) in-home rapid diagnosis for symptomatic individuals, and 2) diagnosis of asymptomatic pregnant women. Sections 4 through 8 outline the steps that need to be taken to improve existing biomarkers, or to identify and develop new biomarkers for these intervention points. Finally, in Section 9, the recommended courses of action are presented.

1. Introduction to the Diagnostic Needs for Malaria in Resource-Limited Settings

Malaria is a disease caused by infection with protozoan parasites of the genus *Plasmodium*. The parasites are transmitted from human to human via an intermediary vector (host), which are mosquitoes of the genus *Anopheles*. Four species of Plasmodia parasites cause malaria in humans, *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *P. falciparum* and *P. vivax* cause the most infections world-wide, with *P. falciparum* predominating in Africa and *P. vivax* predominating in Latin America and Asia. *P. falciparum* is also the cause of the most severe illnesses and deaths.[1] Because the accurate diagnosis of *P. malariae* and *P. ovale* is difficult using the most widely available technique (microscopy), their prevalence may be underestimated in some areas.[2]

There are several elements to the parasites' life cycle that are relevant to the utility of biomarkers for infection. Transmission of the parasite occurs when a female *Anopheles* mosquito feeds on a person who is already infected, and ingests blood containing the parasite gametocytes. During the next one to two weeks, gametocytes inside the mosquito reproduce sexually and develop into infective sporozoites. When the mosquito feeds again on another human, it inoculates sporozoites, which rapidly infect the hepatocytes of the new human host. Though hepatocyte infection does not produce illness, schizogony (asexual development of a single trophozoite into numerous merozoites) occurs within infected hepatocytes; and one to two weeks later the hepatocytes rupture and release the merozoites, which in turn invade the hosts' red blood cells (RBCs) and transform into trophozoites. The young trophozoites, which appear as rings in Geimsa-stained RBCs, grow and develop into merozoites (also called schizonts), which rupture the RBC. Merozoites released into the plasma rapidly invade new RBCs. Repeated cycles of invasion and rupture of RBCs are responsible for the clinical symptoms of malaria. A separate cycle of development occurs simultaneously, and results in the production of gametocytes along with the trophozoites in the RBCs. The gametocytes do not cause clinical symptoms, but can be ingested by anopheline mosquitoes and therefore maintain the parasite's life cycle. Schizonts in the liver may persist for two to three years in infections with *P. vivax* and *P. ovale*, but not with *P. falciparum* or *P. malariae*. This long-lived dormant stage (hypnozoites) serves as a reservoir for relapses and complicate chemotherapy because they are not killed by drugs used to treat clinical disease.[1,3]

The parasites lifecycle, which is slightly different for different species, is generally found to be synchronized within a host, and this presents challenges for diagnostic approaches such as microscopy that rely on the visualization of a particular stage in development. Studies that have looked at the daily dynamics of parasite

levels have found quite variable levels of parasites with a defined periodicity. Therefore, when a single sample is obtained from a patient, the level of parasites may be at a low point in the cycle, and therefore may not reflect the parasite levels that occur over a several day period, and an infection can even be missed.[4]

P. falciparum is unique among the human-infecting *Plasmodia* species in its hallmark behavior of late pigment-containing stage parasites (trophozoites and schizonts) which cause infected erythrocytes to temporarily withdraw from the circulation (sequester) to the capillary beds of the deep tissues, particularly liver, spleen, and bone marrow.[5,6,7] This occurs once every asexual life cycle (generally 48 hours for *P. falciparum*) and in synchrony (due to the natural synchrony of parasite developmental stages *in vivo)* when the older parasites predominate and express adhesion molecules that are trafficked to the RBC membrane. They also adhere to the vascular endothelium via a number of specific ligand-host-cell-receptor interactions.[5] This phenomenon explains the general absence of the pigment-containing stages of *P. falciparum* in the peripheral blood, which contains primarily the earlier, unpigmented ring-stage parasites. Sequestration is thought to be crucial to parasite survival because it prevents destruction of the infected erythrocytes in the spleen.[5] Because of sequestration, *P. falciparum* parasites are not found in as high numbers (compared to the other parasite species) in the circulating peripheral blood, and therefore a *P. falciparum* infection may easily be missed because there are insufficient numbers of parasites for detection.[7] The impact of sequestration on the ability to diagnose asymptomatic pregnant women in particular will be discussed at greater length in Section 3.

Malaria involves a wide variety of symptoms, ranging from no or very mild symptoms to severe disease. Malaria diagnosis, particularly in resource-limited settings without access to laboratory diagnostics, frequently relies on the clinical symptoms. The first symptoms of malaria, which often include fever, chills, sweats, headaches, muscle pains, nausea, vomiting, anorexia, and worsening malaise, are not very specific for malaria, and therefore the use of clinical signs results in a high rate of false positive diagnoses. Other infections with an overlapping set of symptoms include pneumonia, tuberculosis, and gastrointestinal infections. Symptoms of infection with *P. falciparum* can progress from mild to severe and life threatening in a matter of hours, and therefore it is recommended that a laboratory diagnosis be available within a maximum of two hours of the patient presentation.[8] In addition, in highly endemic areas, a large proportion of the population can be infected but yet have no symptoms, and therefore a significant number of individuals may never be evaluated for infection, but remain as a reservoir of the parasites and indirect infection to others. Severe disease can include coma, metabolic acidosis, severe anemia, hypoglycemia, and in adults, acute renal failure or acute pulmonary edema. Anemia and jaundice are caused by a variety of factors including the intravascular hemolysis of infected RBCs during release of merozoites, the phagocytosis of infected and uninfected RBCs in the spleen, the shortened survival of infected and uninfected RBCs, and the resulting ineffective hematopoiesis. These consequences may be much more serious if the patient is malnurished.

The morbidity and mortality posed by malaria remains significant in the inter-tropical areas of the world. Recent estimates suggest that one to three million deaths, and between 500 million and five billion episodes of clinical illness, are caused by malaria, along with an enormous morbidity burden that is more challenging to calculate.[9,10] Despite many advances in understanding malaria, and the development of interventions, more than 50% of the world's population, or about 3 billion people, are exposed to malaria.[10] Poor

populations are at the greatest risk, with close to 60% of cases occurring in the poorest 20% of the world's population, who receive the worst health care and suffer catastrophic economic consequences as a result of the disease. Several studies in Africa, which bears the vast majority of the burden of malaria, have suggested that in some regions, for every case of febrile illness that is seen in a health care facility, another four or five remain untreated in outlying communities. Other studies have shown that in some malarious areas, only ~ 50% of the children with febrile illness are being treated.[9]

Traditional treatment of *P. falciparum* included chloroquine or sulphadoxine-pyrimethamine. The emergence of resistant *P. falciparum* strains has driven a shift to a new class of anti-malaria compounds related to artemisinin. Artemisinin derivatives in combination with traditional anti-malarial compounds (artemisinin combination therapy or ACT) are currently considered the best treatment options.[11] Because *P. vivax* and *P. ovale* infection have a dormant stage parasite that can result in relapses, it is recommended that primaquine be administered in addition to the treatments mentioned above, to prevent relapses.

The first clinical decision point that has been selected for evaluation in this document is the rapid diagnosis of malaria in symptomatic individuals in settings with no laboratory resources (e.g. in the home). Because rapid and accurate diagnostic methods are not accessible, even in health outpost settings, to most of the populations at risk for malaria, healthcare organizations such as WHO and UNICEF recommend treating all febrile patients for malaria. The poor accuracy of this approach highlights the critical need for a simple, inexpensive, rapid, and reliable diagnostic product for malaria that can be performed in the homes of patients in remote areas. Such a product would improve the care of patients, conserve valuable drugs, and help prevent the emergence of resistant parasites.[9] A discussion of the currently available biomarkers for this intervention point is presented in Section 2.

The second clinical decision point that has been selected for evaluation in this document is the diagnosis of malaria in asymptomatic pregnant women in resource-limited settings, such as at a health outpost. The unique biology and diagnostic challenges for this intervention point, along with the status of the currently available biomarkers for diagnosis, are presented in Section 3.

2. Rapid Diagnostic for Patients with Symptoms in Settings with No Laboratory Resources: Status of Currently Available Biomarkers

In resource-limited settings, the decision to use anti-malarial drugs is often based on symptoms alone, and because the symptoms are poorly specific, drug is wasted in patients who do not require it. The emergence of parasites that are resistant to the traditional treatments has lead to the development of newer, more effective treatments which are more costly, and therefore there is an even greater need for accurate diagnostic methods.

Despite the development of several modern, high technology methods for diagnosing malaria, the vast majority of diagnoses rely on a combination of the clinical presentation and the century-old approach of reviewing stained blood smears by light microscopy. Light microscopy is considered the current gold standard, and if performed by highly trained personnel, can provide good sensitivity and specificity, as well as information on parasite density, stage, and species information. Blood from pricking a finger or ear lobe is considered ideal, because the density of the developed trophozoites or shizonts is greater in blood from

these capillary-rich areas. "Thick" blood smears or films are prepared from a slightly larger drop of blood that is spread circularly over an area of approximately 150 mM^2 of a microscope slide, so that blood cells are layered on top of each other. This concentrates the layers of RBCs on a small surface by a factor of 20 to 30. Thick films are stained, without fixing, using Field's stain or diluted Wright's or Giemsa stain. This provides enhanced sensitivity for the detection of low levels of parasitemia. For "thin" blood film smears, the drop of blood is spread over a wider area, fixed with methanol, and then stained with diluted Giemsa or Wright's stain. This procedure emphasizes the parasite inclusions in the RBCs, and makes the morphological identification of the species of parasite much easier, providing greater specificity.[7] Theoretically, thick film microscopy can detect approximately 50 to 100 parasites per μL of blood, though many labs routinely achieve sensitivities that are much worse, on the order of 500 to 1,000 parasites/μL.[7] However, because high quality microscopy diagnosis is labor-intensive, requires significant training, and can only be performed where electricity is available, it cannot be easily maintained in remote areas,[11] and will never be feasible for in-home diagnosis.

Nucleic acid amplification methods have been developed to detect *Plasmodia* DNA in the circulation, and it is generally agreed that these methods are more sensitive than expert light microscopy.[11,12] Polymerase chain reaction (PCR)-based methods are thought to detect as little as 5 parasites/ μL.[7] However, the nucleic acid based methods are still impractical to implement in the settings with no laboratory resources.

The need for a rapid test that is feasible to use at home has driven the development of point-of-care (POC) rapid diagnostic tests (RDTs) that use an immunodiagnostic format, which are currently the only method, other than clinical symptoms, which can be used in an in-home setting. Ideally the test would provide high sensitivity for at least the two most common *Plasmodia* species (though distinguishing all four species would be added benefit), and have sufficient specificity to distinguish a current infection from past infections, which commonly occur in endemic areas. In addition, a test that is semi-quantitative (i.e., provides some information on parasite level) would be useful, although this capability is not absolutely necessary. The performance of commercially available RDTs is summarized below.

2A. Immunodiagnostic Lateral Flow Devices

Malaria rapid diagnostic tests (RDTs) that have been developed to date utilize a lateral-flow immunochromatographic format to detect parasite-derived antigens in the host blood. In theory, this approach might provide better sensitivity than the visualization of parasites, because the levels of antigen in the blood may be reflective of many generations of parasites, not just those present in the circulation at the time a sample is taken. This is an important issue for an infectious agent that is best detected by microscopy at only one stage of its lifecycle, and that goes through wide fluctuations in the numbers of any one stage over the course of a few days.

Plasmodia parasites produce several proteins that have been used as biomarkers of infection. One of these molecules, histidine rich protein II (HRPII or HRP2), is unusual in its high content of histidine, alanine, and aspartic acid, which are arranged in contiguous repeats of AHHAHHAAD. The function of this protein is still unclear, but it may be involved in cytoskeletal remodeling of the RBC, or heme polymerization. HRP2 is produced in the early states of RBC infection, is found in the cytoplasm and at the cell membrane of RBCs, and is also secreted by RBCs into the circulation.[13] Merozoite surface protein 1 (msp-1) is a protein that exists as part of a non-covalently associated multimeric protein complex on the surface of merozoites.

The complex is thought to be the most abundant constituent of the merozoite surface, and it is proteolytically cleaved from the surface and shed upon invasion of an RBC.[14] The function of merozoite surface protein-2 (msp-2) is unknown, but it shows a very high level of diversity among different malaria strains.[15]

Despite the fact that more than a decade has passed since the first introduction of RDTs for malaria, only a few parasite antigens have been targeted, and these are listed in Table 1.

Table 1. Parasite Antigen Biomarkers Currently Targeted by Rapid Diagnostic Tests

Organism	Antigen Target	Abbrev.	Parasite Life Cycle Stage
Plasmodia falciparum	histidine-rich protein	HRP2	Water-soluble protein produced by asexual stages and young gametocytes of *P. falciparum*[16]
	parasite lactate dehydrogenase	pLDH	Enzyme in the glycolytic pathway produced by all blood-stage parasites[17,7]
	aldolase		Enzyme of the glycolytic pathway[7]
Plasmodia vivax	antigen not identified by manufacturer		Not specified. Antigen unique to *P. vivax*[18]
	parasite lactate dehydrogenase	pLDH	Enzyme in the glycolytic pathway produced by all blood-state parasites[17,7]
	aldolase		Enzyme of the glycolytic pathway[7]
Plasmodia malariae	parasite lactate dehydrogenase	pLDH	Enzyme in the glycolytic pathway produced by all blood-state parasites[17,7]
	aldolase		Enzyme of the glycolytic pathway[7]
Plasmodia ovale	parasite lactate dehydrogenase	pLDH	Enzyme in the glycolytic pathway produced by all blood-state parasites[17,7]
	aldolase		Enzyme of the glycolytic pathway[7]

The performance of RDT tests has been reviewed relatively recently by Moody.[7] A number of studies have reported that RDTs are capable of detecting *P. falciparum* with sensitivities of > 90% and with specificity approaching 100% at parasite densities greater than 100 to 500 parasites/μL of blood, though the sensitivity declines rapidly at lower parasite densities. However, a wide range of sensitivities, which are often much worse, are reported in other studies conducted in endemic areas.[11] In addition, sensitivity is lower and generally inadequate for the non-*P. falciparum* species in some tests,[19,20,21,22] and a decline from the maximum sensitivity generally occurs at higher parasite densities for the non-*P. falciparum* species.[11]

Selected studies that evaluated RDT performance in malaria endemic areas, using Geimsa thick- and thin-smear microscopy as reference standards, are summarized in Table 2.

Table 2. Performance of Rapid Diagnostic Tests for Malaria in Endemic Regions

Study	Products	N (% malaria)	Location	*P. falciparum*		*P. vivax*	
				sens (%)	spec (%)	sens (%)	spec (%)
Mboera et al 2006[23]	Paracheck-Pf	1655 (23.3%)	Tanzania	90[c]	96.6[c]	NA	NA
Soto Tarazona 2004[24]	OptiMAL	72 (54%)	Peru	NA	NA	92.3[b]	100[b]
Wongsric hanalai et al. 2003[25]	NOW ICT Malaria P.f./P.v.	246	Thailand	100	96.2	87.3	97.7
Coleman et al 2002[26]	ICT Pf/Pv	1137	Thailand	35.4	99.7	2[a]	99.9[a]
Guthmann et al. 2002[27]	Paracheck Pf	742 (57%)	District hospital, Uganda	97	88	NA	NA
	ParaHIT f			95	95	NA	NA
	Malaria Rapid			98	75	NA	NA
	BIO P.F.			90	93	NA	NA
Ferro et al 2002[28]	OptiMAL		Colombia	90.6	98.6	96.5[b]	97.6[b]
Huong et al 2002[29]	OptiMAL,	412/(61%)	Vietnam	49.7	100	73.7[b]	100[b]
	ICT Pf/Pv			82.6	100	20[a]	100[a]
	Paracheck-Pf			95.8	100	NA	NA
Mason et al 2002[20]	OptiMAL	229/133	Myanmar	42	97	27[b]	96.9[b]
	ICT Pf/Pv			86	77	3[a]	100[a]

a: ICT Pf/Pv kit detects an antigen produced by *P. vivax*, *P. malariae*, and *P. ovale*
b: OptiMAL detects an epitope on pLDH that is common to *P. vivax*, *P. malariae*, and *P. ovale*
c: Numbers represent overall performance in the study, which looked at performance in 5 districts. Assay performance was relatively poor in districts where the test had been stored for 12 months at room temperature (23.5 -/+3.5°C)

As demonstrated by the data in the table, the performance of even one test varies greatly from study to study, and there is no test that consistently performs well enough in endemic regions to recommend its deployment.

A major difference among the available tests is whether they detect only *P. falciparum* (e.g. Paracheck-Pf), or can also detect the other non-*falciparum* species (e.g. ICT Pf/Pv), and whether they can distinguish mixed infections. No commercially available RDT can distinguish among the non-*falciparum* species. Though not listed in Table 2, at least one report indicates that that the monoclonal antibodies that detect the parasite lactate dehydrogenase enzyme in the OptiMAL test have a lower affinity for the variant of the enzyme

produced by *P. ovale* and *P. malariae*. This fact, combined with the lower parasite densities that are generally found for these species, suggest that sensitivity for these species may be much lower than for *P. falciparum* or *P. vivax*.[30] A similar problem may occur with the reportedly pan-species antibodies used to detect parasite aldolase enzyme in the ICT Pf/Pv test, which in at least one study did not detect *P. malariae* infections.[31]

A number of factors are thought to contribute to the performance deficiencies of RDTs that are observed in resource-limited settings. Some of these pertain to the particular biomarkers that have been selected, and other factors are related to technological or study-design issues. The poorer performance in malaria-endemic regions may be a result of the gold standard method that was used, and the level/quality at which that gold standard is performed. For instance, at least one report suggests that the use of microscopy as a gold standard can affect the measured sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of an RDT. Rates of false positives can appear high (especially when using RDTs that detect HRP2) when microscopy is used as the gold standard technique because individuals who have levels of parasitemia that are lower than the detection limit by microscopy can still test positive for HRP2. Highly skilled microscopists are capable of detecting very low parasite densities in some specialized testing centers (10 to 50 parasites/μL), but the sensitivity limits of microscopy in many malaria-endemic are generally higher at 100 to 500 parasites/μL. Estimates of the sensitivity of the RDTs that detect HRP2 range from 60 to 100 parasites/μL for the more sensitive tests.[32,33] Published estimates of reproducible PCR sensitivity for a single copy gene range from 10 to 100 ring-stage parasites/μL when detecting laboratory strains, but up to 400 parasites/μL in some studies on field samples. In the report by Bell et al., 44 of 48 samples that were negative by microscopy but positive by RDT were also positive by PCR,[32] suggesting that microscopy may not be the best gold standard to use. The persistence (stability) of the different antigens used in RDTs may be either an advantage or disadvantage, depending on the characteristics of the population concerned. In areas where drug treatment is rare, HRP2 persistence may aid diagnostic accuracy, but in populations where recent drug treatment is likely, an RDT that detects a less persistent antigen, such as pLDH, might be an advantage.[32]

The inconsistency in performance that is illustrated by the data in Table 2 is likely to arise from a variety of sources, including product instability, lot-to-lot manufacturing variability, and perhaps other sources of variation. It is generally believed that malaria RDTs are degraded by heat and moisture, and gradually deteriorate, even under ideal conditions. Unfortunately, the conditions under which the devices are transported and stored in resource-limited settings are difficult to control, and often greatly exceed the manufacturers recommended condition of less than 30°C.[34] The product's overall stability will be determined by the stability of the antibodies that bind to the parasite antigens, the test-line antibodies (which must remain adhered to the nitrocellulose and maintain their ability to bind to the antigens), and the conjugates of dyes and antibodies. Though malaria RDTs no longer require cold for transport or storage, manufacturers recommend storage between 2°C and 30°C.[11] The stability issue has been exacerbated by the lack of a way to assess the condition of products after they have been transported and stored in the field. One commercially available, parasite-antigen-based positive control sample is available (pLDH Diamed OptiMAL Positive Control Well). This small plastic well is coated with a small amount of recombinant pLDH that produces a weak but clear positive reaction when using the OptiMAL IT kit. However, this positive control sample requires cool temperatures for shipment and storage as well, as it has been

demonstrated that its function deteriorated after cumulative exposure to temperatures likely to be encountered over a few months in a malaria-endemic area.[35] Therefore this commercially-available positive control sample can identify batches of devices that are likely to have deteriorated, but it does not begin to address the problem of how to prevent deterioration. The degradation of RDTs due to conditions in malaria-endemic areas is thought to be the most significant factor causing the poorer and inconsistent performance of such tests in resource limited settings, compared to their laboratory evaluations in the developed world.

Diversity in the *P. falciparum* HRP2 (PfHRP2) protein may also contribute to poor and inconsistent performance of the RDTs that target this antigen. Despite reports suggesting that PfHRP2 is antigenically invariant,[36] Baker et al. (2005) report that the naturally occurring diversity in this protein, which is extraordinarily high, affects the ability of RDTs to detect certain strains of *P. falciparum*, particularly at lower parasite densities, and that perhaps only 84% of *P. falciparum* parasites in the Asia Pacific region are likely to be detected at densities of less than 250 parasites/μL.[37] This study reports that one of 75 *P. falciparum* isolates from 19 counties has a deletion in its DNA that encompasses (at least) exon 2 of the HRP2 gene, and the ability of the RDTs to detect this strain at all (which was possible at parasite densities over 1000 parasites/μL) probably resulted from cross reactivity of the antibodies in the test strips to the related protein PfHRP3. If conservation of an antigen across isolates plays a role in test performance, it is hard to imagine a worse selection of antigen to be detected by a test. However, the repetitive nature of the protein may improve test sensitivity (for the strains that have it) if the antibodies that detect the protein are specific for an epitope that appears many times in the protein. There appears to be no published information on the specific epitopes that are recognized by either the commercially available tests or the six published monoclonal antibodies directed against PfHRP2, and because the limited supply of monoclonal antibodies, it is likely that all of the commercially available tests use one of the two commercially available antibodies.[37]

Another factor that is reported in the literature to affect the performance of RDTs is the ability of rheumatoid factor to cause false positives with malaria RDTs.[11] This artifact occurs with the Parasight F test (16.5% false positive rate[38]), which is no longer sold.

Lot-to-lot variability has been reported for some newer tests,[26,20] and a high frequency of aberrant results (which may or may not be related) is reported in some studies. For instance, Coleman et al. 2002 report that 34% of test strips produced aberrant results, where a positive *P. falciparum*-specific line appeared, but the pan-malarial line did not appear. A high rate of false positive results have been reported in a number of studies, though this may be an artifact that is due, at least in part, to cases in which the parasite level is too low to be detected via microscopy.[32]

Most of the studies that evaluate the performance of rapid malaria diagnostic devices, particularly in the developing world, have been performed in a hospital setting, rather than a setting with no laboratory resources. The goal is to have a test that performs well in the home, administered by a family member or local health care worker. In general, evaluations in these settings have not been conducted. One published report on performance of RDTs when testing was done outside a health care setting found that 9 to 32% of tourists from the developed world were unable to correctly administer a malaria self-test.[39,40] It is likely that the performance of the available RDTs will be different when it is measured in an at-home testing environment.

In summary, some studies have shown adequate performance of *Plasmodia* antigens biomarkers detected by rapid immunochromatographic devices, particularly using newer products. This approach has the advantages of being relatively easy to perform, and requiring little infrastructure or training. Evaluations under in-home conditions in resource-limited settings remain to be performed. The erratic performance reports are thought to be due to a variety of factors, such as product stability under field conditions, manufacturing inconsistencies, and the ability to detect all malaria species with roughly equal sensitivity. A clear deficiency of this approach is the ability to detect mixed infections. Overall, it seems probable that if all of these technical issues were remedied, that *Plasmodia* antigen detection using an RDT format could provide adequate performance. While the cost of RDTs is low by diagnostic standards (approximately $0.50 each through bulk procurement), this is probably still too costly to be practical in many resource-limited settings.

3. Diagnosis of Malaria in Asymptomatic Pregnant Women: Status of Currently Available Biomarkers

The two populations at greatest risk for morbidity and mortality caused by malaria are children under the age of five, and pregnant women. Approximately 50 million women become pregnant each year in areas where malaria is endemic. Pregnancy-associated malaria (PAM) is thought to account for a third of the preventable low birth weight babies in sub-Saharan Africa, and 100,000 to 200,000 infant deaths annually.[41]

The biology of infection in PAM differs from that seen in *P. falciparum* infection in non-pregnant individuals (sometimes called peripheral malaria). In PAM, *P. falciparum*-infected RBCs sequester in the intervillous space of the placenta by expressing parasite-encoded surface antigens that differ from those expressed by parasites in non-pregnant individuals, which mediate their adhesion to specific molecules such as chondroitin sulfate A (CSA) in the placenta. PAM therefore appears to be a special instance of the adhesion of infected erythrocytes to molecules in the host vasculature (sequestration) that is the hallmark of *P. falciparum*-infected erythrocytes.[41] The other *Plasmodia* species do not appear to sequester to any significant degree either in the placenta or in other tissues. The sequestration of infected RBC in the placenta reduces the number of circulating ring-stage parasites in the peripheral blood, which is the stage of the parasite that can be identified in traditional Giemsa stain-based microscopy.[42]

A rapidly growing body of evidence suggests that the parasite-encoded variant surface antigens (VSA), which show genetic variation in different strains, are expressed on the surface of infected erythrocytes, and mediate the sequestration of *P. falciparum* to the host vasculature by binding to specific host proteins. There is evidence suggesting that the parasites that cause PAM express VSAs that are fundamentally different from all other VSAs, which is reviewed by Hviid 2004.[41] Some pregnant women do have peripherally detectable parasites. Several studies have also shown, using genotyping methods, that the parasite strains found in the peripheral circulation are distinct from, or are a different subset of, the parasite strains found when the placenta is examined after delivery.[43,44,45,46] Initial evidence suggested that in areas of stable transmission, women acquire antibodies against the PAM-specific VSAs (which allow adhesion to molecules in the placenta) over successive pregnancies, and this has been hypothesized to explain the high prevalence of malaria symptoms during the first pregnancy, and the decreasing susceptibility to symptoms with increasing parity.[41,47] More recent evidence suggests that increasing gravidity correlates with decreasing susceptibility to symptoms, a reduction in poor outcomes, and a reduction in microscopically detectable levels of parasites,

but does not correlate with a decrease in sub-microscopically detectable infections. It may therefore be that the acquisition of gravidity-associated immunity does not eliminate the infection, but may serve to maintain parasite levels sufficiently low that they are less often detectable by microscopy.[2] These biological phenomena make the detection of placental infection particularly challenging, when the available specimen type is peripheral blood.

The high numbers of parasites that accumulate in the intervillous spaces of the placenta induce an inflammatory response which results in an intense infiltration of macrophages. The macrophages in turn secrete many substances, including tumor necrosis factor alpha, gamma interferon, transforming growth factor beta, and inerleukin-2, which alter the function of the cells of the placenta. Severe maternal anemia (which predisposes to maternal mortality) and low birth weight babies are frequent consequences. Low birth weight, which predisposes to infant mortality, is caused by either prematurity or intrauterine growth retardation. In many studies, placental parasite density has the strongest association with poor outcomes. Unfortunately, placental parasite levels do not correlate with peripheral parasite levels, and placental parasitemia can only be diagnosed at birth, which does not provide an opportunity to intervene during the pregnancy.[48]

The HIV pandemic has resulted in an increasing percentage of the world's population being co-infected with both HIV and *Plasmodia* species. In sub-Saharan Africa, the prevalence of maternal malaria is as high as 65%, and HIV affects up to 40% of pregnant women in some areas.[49] The effects of malaria are more severe in HIV infected individuals, due to their deficient immune status. Higher parasite densities and higher rates of clinical episodes of malaria have been associated with falling CD4+ T-cell counts. HIV infection likely disrupts the development of the gravidity-dependent acquired immunity to PAM. HIV-malaria co-infected pregnant women are at increased risk for maternal anemia, low-birth-weight babies, and possibly a greater risk of transmitting HIV to their babies, though this is more controversial.[49,41]

If PAM is identified prior to delivery it can be treated with anti-malarial drugs, and it is standard practice in endemic areas to put women on Fansidar in the second trimester of pregnancy. However, the emergence of drug resistant *P. falciparum* strains has eroded the usefulness of the few drugs known to be safe for the woman and her fetus.[2,47] Anti-malarial treatment may decrease the incidence of the complications of PAM, but it does not abolish them.[48]

Because of the sequestration of parasites in the placenta, diagnostic approaches that examine peripheral blood do not perform as well in women who are pregnant. Therefore there is a specific need for a diagnostic product that performs well in asymptomatic pregnant women, and that can be performed in a resource-limited setting such as a health outpost clinic.

3A. Visualization of Parasites via Light Microscopy as a Biomarker for PAM

Because of the sequestration of parasites in the placenta, levels of parasites in the peripheral blood are more frequently below the level of detection using light microscopy than in non-pregnant individuals. Studies reporting the performance of microscopy on peripheral blood smears for the identification of PAM are summarized in Table 3.

Table 3. Performance of Parasite Visualization by Light Microscopy as a Biomarker of PAM

Study	No.	Location	Performance	
			sens (%)	spec (%)
Walker-Abbey et al. 2005[2]	278	Cameroon (three hospitals)	27[d]	not reported
Mockenhaupt et al. 2002[50]	596	Ghana	42[a]	97[a]
			27[b]	100[b]
Mankhambo et al. 2002[48]	114	Malawi (tertiary hospital setting)	52[a]	93[a]
Leke et al. 1999[51]	1077	Cameroon	79[c]	97[b]

a: Compared to placental blood smear microscopy
b: Compared to PCR-confirmed placental parasitemia
c: Compared to combined placental and peripheral blood smear microscopy
d: Compared to combined PCR-confirmed placental and peripheral parasitemia

Researchers have found that the visualization of parasites using microscopy is the least sensitive method for detecting infection during pregnancy, and may underestimate the number of infected women significantly. For instance, Walker-Abbey found that in an area on intense transmission, more than 80% of women who were asymptomatic for malaria at delivery were infected, but only 28% of the women were positive by peripheral blood smear.[2] Regardless of this poor performance, high quality microscopy diagnosis is labor-intensive, requires significant training, can only be performed where electricity is available, and is difficult to maintain in remote areas,[11] and is therefore not practical for resource-limited settings.

3B. Plasmodia Antigens Detected Using Rapid Immunodiagnostic Devices as Biomarkers of PAM

In theory, this approach might provide better sensitivity than the visualization of parasites, because the levels of antigen in the blood may be reflective of many generations of parasites, not just those present in the circulation at the time a sample is taken. This is an important issue for detecting *P. falciparum* infections which are often sequestered in the placenta. Malaria RDTs have demonstrated mixed performance in the detection of PAM. Their performance may also be affected by the same product stability and manufacturing inconsistencies that were outlined in Section 2A. The results of studies that have evaluated the performance of RDTs for detecting malaria in asymptomatic pregnant women are presented in Table 4. The data reported is for tests performed on peripheral blood samples.

The differences observed in the performance of these tests are likely to be due to a variety of factors, including differences in the products used, the gold standard methods used, as well as other product stability or manufacturing consistency issues that are harder to demonstrate. The table demonstrates that the performance of RDTs has been inconsistent, and though it is generally better than microscopy performed on peripheral blood specimens, it appears very insensitive compared to nucleic acid based approaches (see next section).

Table 4. Performance of *Plasmodia* Antigens Detected Using RDTs in Asymptomatic Pregnant Women

Study	Products	Antigen	No.	Location	Performance	
					sens (%)	spec (%)
Leke et al 1999	ICT Malaria	HRP2	181	Cameroon (University Biotechnology Center, but site where RDT performed not specified)	89[a]	95[a]
Mockenhaupt et al. 2002	ICT Malaria P.f/P.v	HRP2	596	Ghana (hospital and university setting, but site where RDT performed not specified)	80[b]	90[b]
					56[c]	97[c]
Mankhambo et al. 2002	OptiMAL	LDH	171	Malawi (tertiary hospital setting, but site where RDT was per-formed not specified)	38[b]	91[b]
Singer et al. 2004	Malaria Rapid Test (MAKROmed)	HRP2	853	Burkina Faso (2 district hospitals)	95[b]	61[b]
					92[c]	59[c]

a: Compared to combined placental and peripheral blood smear microscopy
b: Compared to microscopically confirmed placental parasitemia
c: Compared to PCR-based detection of parasitemia in placental blood

Mankhambo et al. speculate that the low sensitivity observed in their study could be due to the unusually low parasite densities (both placental and peripheral) that were observed in their study group. Their results, like those of many studies, show that the sensitivity of the RDT dropped at lower parasite densities, which in their study began at about 1,500 parasites/υL.[48] Singer et al. speculate that the low specificities observed in their study could be due to a number of reasons, such as the detection of some infections that were missed by microscopy, false positive results in individuals with treated (cleared) infections in which residual protein remained in the circulation, as well as product degradation under field conditions.[52]

In summary, the small amount of data and variation in results make it hard to decisively conclude whether *P. falciparum* antigens as biomarkers in peripheral blood can provide adequate performance in an RDT format for the detection of malaria infection during pregnancy. Their generally poor sensitivity compared to the detection of DNA sequences using PCR suggests that this approach will not be one that maximizes sensitivity.

3C. DNA Biomarkers for Diagnosing PAM

Approaches that detect *Plasmodia* DNA in peripheral blood are well documented to be more sensitive than approaches that use visualization of parasites via microscopy, or detection of antigens via immunodiagnostic methods. There are no commercially available kits or systems for detecting *Plasmodia* infection using nucleic acid amplification methods, so researchers have developed their own assays. Studies that have evaluated the performance of PCR-based approaches to detect *Plasmodia* DNA in peripheral blood samples from asymptomatic pregnant women in malaria-endemic areas are presented in Table 5.

Table 5. Performance of *Plasmodia* DNA Sequences as Biomarkers in Asymptomatic Pregnant Women

Study	Methods	Genes	No.	Location	Performance	
					sens (%)	spec (%)
Mockenhaupt et al. 2002[50]	Species-specific, nested PCR, visualized on EtBr stained gels[53]	18S rRNA	596	Ghana (hospital and university setting, but site where PCR was performed not specified)	97[a]	75[a]
					84[b]	97[b]
Mankhambo et al. 2002	Species-specific, nested PCR, visualized on EtBr stained gels[53]	18S rRNA	171	Malawi (tertiary hospital setting, but site where PCR was performed not specified)	72[a]	67[a]
Singer et al. 2004[52]	Species-specific, nested PCR, visualized on EtBr stained gels[53]	18S rRNA	853	Burkina Faso (blood spots sent to U.S. testing site)	98[c]	NA[e]
					100[d]	NA[e]

a: Compared to microscopically-confirmed placental parasitemia
b: Compared to PCR-based detection of parasitemia in placental blood
c: Compared to cases where placental parasitemia was >= 500 parasites/µL
d: Compared to cases where placental parasitemia was 100 - 499 parasites/µL
e. Study not designed to measure specificity

There is relatively little data on the performance of DNA-based biomarkers in peripheral blood for the detection of malaria infection during pregnancy. Three of the four studies shown in the table appear to utilize the same PCR approach for detecting the 18S rRNA gene. The differences in performance that are evident could arise from differences in the level of parasitemia in the populations that were studied, differences in sample preparation methods, or differences in the ability of each laboratory to conduct the analysis. Mankhambo et al. suggest that the low specificity they observed for PCR was a function of the low sensitivity of microscopy, which was the gold standard they used for comparison. While PCR was the most sensitive method in that study, it still missed 16 of 32 of cases with microscopically identified placental infections that also had with microscopically negative peripheral blood smears. They also observed 20 cases that were positive by PCR, but negative by both peripheral and placental microscopic analysis. There were no low birth weight babies associated with these 20 cases. These authors suggest that the higher sensitivity provided by PCR does not assist in the identification of cases at risk for poor outcome (with regard to infant birth weight at least), which is a topic that will be discussed further at the end of Section 3.

In summary, there are relatively few studies that report the performance of approaches that use DNA biomarkers in peripheral blood to detect *Plasmodia* infection of the placenta in asymptomatic pregnant women, and there is still uncertainty about the clinical relationship between biomarkers that can be detected in the peripheral blood and an infection in the placenta. DNA biomarkers have the advantage of being the most sensitive method available (reports range from 72 to 100% for sensitivity). Their poor specificity is likely to be a reflection on the relatively insensitive method (microscopy) to which they are compared. The advantages that nucleic-acid based methods have in performance are accompanied by a number of disadvantages, including the lack of commercially available products, the requirement for a high level of

resources, and their relatively high cost. In addition, they have the potential for cross-contamination of specimens.

3D. Detection of Hemozoin as a Biomarker for PAM

Plasmodia parasites produce a substance called hemozoin, which is a crystallized form of heme that the parasite makes as part of hemoglobin catabolism. Originally thought to be produced as a method of detoxifying the heme that is naturally found in RBCs, recent evidence suggests that hemozoin inhibits dendritic cell activity, and therefore plays a role in immunosuppression.[54]

Inside the red blood cell, the parasite digests up to 0.4 femptomoles of hemoglobin. Trophozoites can be seen to contain hemozoin crystals by light microscopy using Geimsa stains. Though hemozoin is not visible under light microscopy in ring-stage parasites, electron micrographs demonstrate the presence of hemozoin crystals during this stage as well.[42] Hemozoin is released into the circulation when infected RBCs are ruptured, and therefore the detection of free hemozoin in the circulation detects the molecular remnants of previous generations of parasite, not just the ring-stage parasites that are found in circulating erythrocytes.[42] In addition to the free hemozoin in the circulation, phagocytic monocytes and neutrophils ingest hemozoin.

The clinical utility of two approaches for the detection of the pigment hemozoin, flow cytometry and mass spectrometry, have been explored. Flow cytometry approaches utilize full blood count analysis instruments, such as the Cell-Dyn line of instruments (Abbott Diagnostics, Santa Clara, USA) which use a multi-angle polarized scatter separation (MAPSS) technology. When samples from patients with malaria are analyzed, the plots are abnormal, and this is thought to be because of the presence of birefingent, depolarizing hemozoin in some monocytes and neutrophils, which normally do not have any depolarizing properties.[55] Initial studies in non-pregnant populations appear promising, with sensitivities and specificities reported in the 80 to 90% range.[55,56] Unfortunately, there is no data on the performance of this approach in asymptomatic pregnant women. One challenge to the approach are apparent false positive results in individuals who have been treated, and the infection cleared, because the pigment-containing leukocytes appear to be observed up to two to three weeks later in the circulation. Hanscheid et al. also point out that neither the sample preparation techniques, nor the analytical algorithms on the off-the-shelf Cell-Dyn instruments, were designed to detect the patterns generated by malaria infection, and that the sensitivity could be improved by modifying both of these aspects of the analysis.[55] In any case, these instruments are not portable, costly ($ 20,000 refurbished[57]), require a blood sample drawn via phlebotomy and a laboratory environment, and are therefore not at all designed for use in a health-outpost setting.

The second approach for the detection of hemozoin utilizes mass spectrometry. When analyzed by laser desorption mass spectrometry (LDMS), hemozoin crystals efficiently absorb UV photons from the laser, which liberates intact iron protoporphyrin IX (heme) molecules and heme ions from the blood sample. Therefore a photoabsorbing matrix is not required to assist in the ionization. The fragmentation that occurs during the desorption process produces a characteristic spectral signature, which is generated primarily from the heme ions, because little else is ionized from the sample when a matrix is not used. Heme bound to hemoglobin in uninfected RBCs is not detected by LDMS.[42] A very small amount of whole blood (1 – 2 uL) is simply diluted and then spotted in multiple locations on to a small metal plate, which is then allowed to dry. The plates cost about $ 5 and can be washed and re-used.

The study by Nyunt et al. investigated the ability of hemozoin, detected using LDMS, to diagnose malaria infection in asymptomatic pregnant women in Zambia. The study included 45 women who were all microscopy-negative for malaria infection. PCR amplification of five genes (MSP2, chloroquine-resistance (CDRT), quinoline-resistance modulation (MDR1), pyrithehamine-resistance (DHFR), and sulfadoxine-resistance (DHPS) genes) detected infection in 29 of the women. To perform the LDMS analysis, 2.0 μL of whole blood was diluted with water, and 0.2 μL aliquots of the mixture were spotted onto a metal slide, which was air-dried. The slides were then shipped back to the U.S., and each slide was analyzed using a commercial time-of-flight mass spectrometer (Kratos Discovery, Shimadzu Analytical, Chestnut Ridge, NY). Two thousand laser shot spectra were acquired for each sample. The number of "shots" from which a heme spectrum is produced ranges from one to several hundred per 2000 shots, and provides a rough correlation with parasite density. The LDMS detection method, using 0.1 μL of blood, identified malaria infection in 15 of the 29 women who were PCR-positive, and identified infection in two women who were PCR-negative, for a sensitivity of 52% and a specificity of 92%, when compared to PCR analysis.[42]

This analytical approach could in theory be modified to achieve a much higher sensitivity by analyzing a larger effective sample size. The effective sample size currently analyzed is 0.1 μL of whole blood, in comparison to 10 to 40 μL that might be analyzed via microscopy or PCR. Efforts are underway to develop an instrument that is optimized to detect malaria, which would include modifications such as a wider beam, which would increase the effective sample size.[58] The authors also speculate that because the later, trophozoite stage parasites contain more hemozoin, this approach should be better at detecting the trophozoite stage than the ring stage parasites. This suggests the method could be more sensitive for the non-*falciparum* species, because the later stage parasites of these species circulate in the blood, rather than sequester in internal organs. Preliminary studies have verified that all four species can be detected by the method, but thus far there is no evidence that the species can be distinguished, or that mixed infections can be identified. It is possible that this LDMS approach could be combined with a MALDI-MS approach that would allow species identification, perhaps by distinguishing the species-specific variants of lactate dehydrogenase or aldolase.[59]

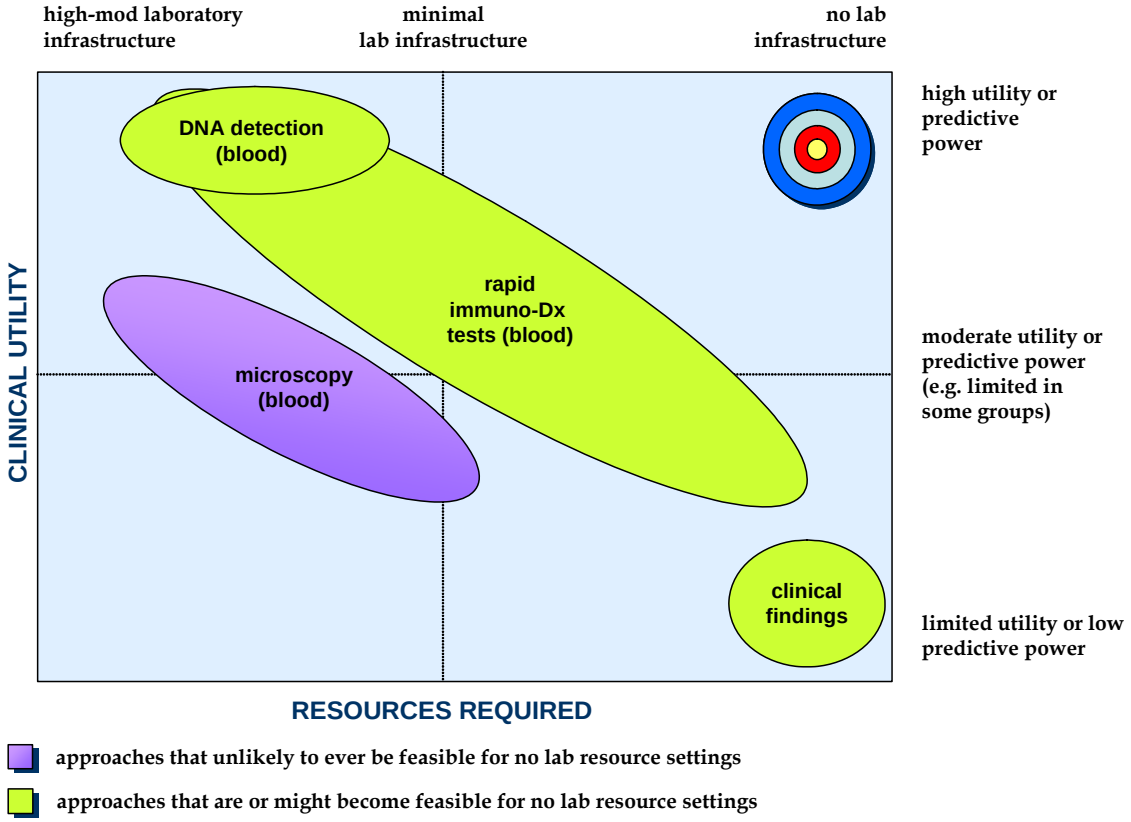
In summary, one published study reported that the detection of hemozoin by LDMS was more sensitive than microscopy, but less sensitive than PCR. Additional studies remain to be performed to replicate this data, to compare the performance of LDMS to the performance to immuno-chromatographic detection of antigens, as well as the ability of hemozoin detection to identify the women and babies who are at risk for poor outcomes. The LDMS approach has the advantages that a small sample (e.g., from a finger prick) can be used, very little sample preparation is required, no reagents are required other than water, the consumable required (a metal plate) is relatively inexpensive and re-usable. and a prototype for a field-usable instrument apparently exists (see Section 5C). The disadvantages are a sensitivity that may be lower than PCR (though this remains to be verified), a high instrument cost, potential issues regarding the power source required for the instrument, and the current requirement for training to perform the test and interpret the output of the instrument.

Summary of the Status of Biomarkers

The relative merits of the biomarkers and diagnostic approaches discussed in Section 2 and Section 3 are presented diagrammatically in Figures 1 and 2. In these figures, the ideal approach would be in the upper

right quadrant, where the target is shown, because of its high predictive power (clinical utility in resource limited settings), and the low level of resources required for successful implementation. The figures identify the approaches that are easy to perform, but are limited by the biology of *Plasmodia* or its hosts. Such approaches appear in the lower right quadrants. In addition, approaches with high predictive power, but that currently require significant resources, are seen in the upper left quadrants.

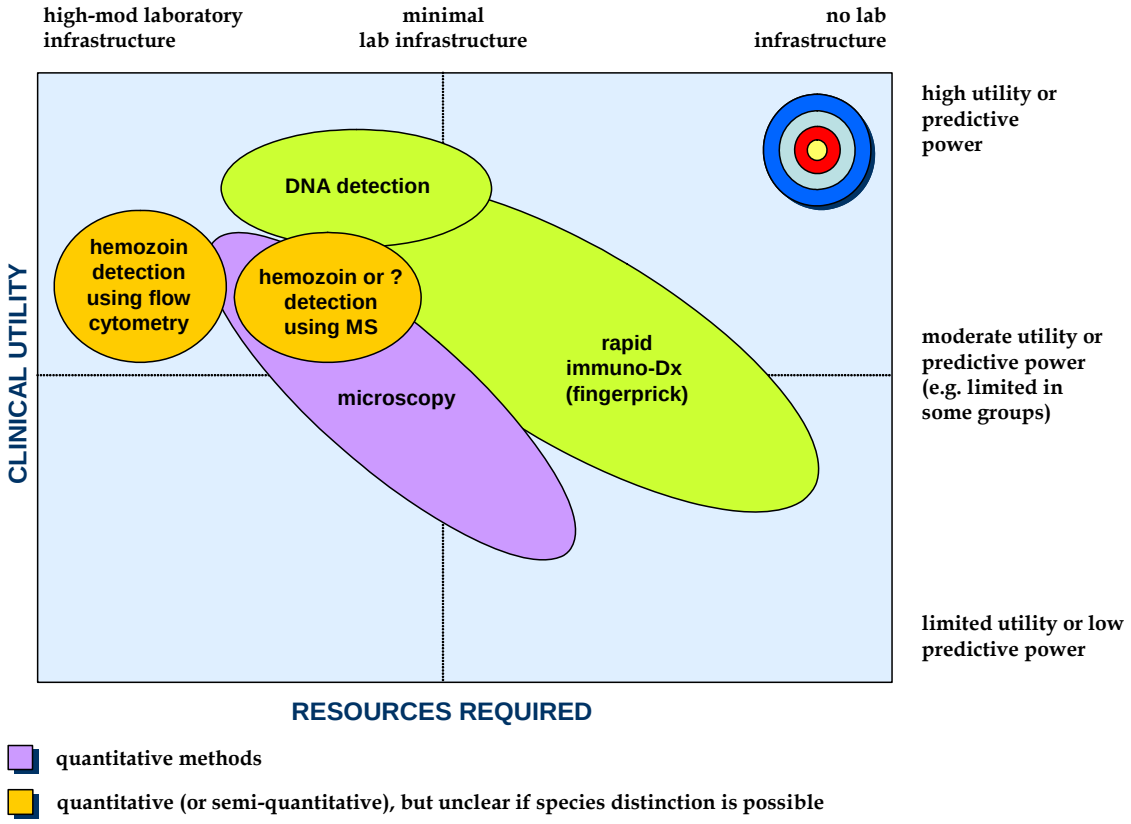
Figure 1. Map of Currently Available Diagnostic Approaches for the Diagnosis of Malaria in Symptomatic Individuals



The angle of the oval that represents microscopy indicates that in settings with greater resources (such as laboratories with highly skilled microscopists), greater clinical utility is achieved. The angle of the oval that represents the RDT immunodiagnostic devices indicates that in settings with greater resources (such as a cool chain for storage and transport of the diagnostic devices), greater clinical utility is achieved.

The only diagnostic approach that is anywhere close to being feasible for in-home use is the RDT immunodiagnostic device. Though the RDTs have demonstrated mixed performance, they can achieve better sensitivity than microscopy in resource-limited settings, and are already close to being in a field-useable format. The figure indicates that one approach, microscopy, is not really feasible in an in-home setting. This approach is included in the diagram for comparison to the other methods.

Figure 2. Map of Currently Available Approaches for Diagnosing Malaria in Asymptomatic Pregnant Women



The angle of the oval that represents microscopy indicates that in settings with greater resources (such as in laboratories with highly skilled microscopists), greater clinical utility is achieved. The angle of the oval that represents the RDT immunodiagnostic devices indicates that in settings with greater resources (such as cool temperatures for storage and transport of the diagnostic devices), greater clinical utility is achieved.

Antigen biomarkers perform better than microscopy in the detection of PAM, at least in part because they are able to diagnose sequestered *P. falciparum* parasites by detecting freely circulating antigens, such as HRP2, aldolase, or lactate dehydrogenase, that are released as the schizont-stage parasites rupture the infected RBCs in the placenta. Though the RDTs performed on peripheral blood have demonstrated mixed performance, they do appear to have better sensitivity than microscopy, and are already close to being in a field-useable format.

Another facet worth considering is how well the different biomarkers and approaches, particularly RDTs, can predict poor outcomes, rather than just diagnosing PAM infection. There are conflicting reports regarding whether infections that are not detectable by microscopy (i.e. sub-microscopic infections) do confer,[2,50,43] or do not confer,[50] an increased risk for maternal anemia. In addition, at least one study has shown that women identified by RDT (and microscopic) analysis of peripheral blood had lower birth weight babies than those who were not infected,[48] but that women with PAM that was detected ONLY by PCR (i.e., with submicroscopic infections) did not have lower birth weight babies.[48] Therefore the lower sensitivity of the RDTs for detecting a placental infection is not replicated when the RDTs are used to predict poor outcomes,

and therefore to identify women who would most benefit from interventions. Additional studies would be required to clarify the performance of RDTs in predicting poor outcomes. In addition, policy-makers would need to consider whether it is most beneficial to detect and treat all women that have a PAM infection, or to treat only those women that are detected by less-than-optimal methods (PAM that is likely to be "clinically significant"), knowing that the test will miss many submicroscopic infections, but can adequately identify the women who have the greatest risk of poor outcome. These decisions would also consider the potential impact of leaving some infected women undetected and therefore untreated, and who therefore might serve as a reservoir for the parasite.

4. Current Deficiencies in the Diagnosis of Malaria in Resource-Limited Settings

For the diagnosis of malaria in symptomatic individuals in settings with no laboratory infrastructure, there are two main deficiencies with regard to the biomarkers that are currently used in the only diagnostic approach that is feasible for in-home use (RDTs). First, there is some evidence which suggests that genetic diversity within *P. falciparum* may adversely affect the sensitivity of the RDTs that use this HRP2 antigen as a biomarker (see page 11). Second, the currently available RDTs generally do not detect all four species equally well and/or identify mixed infections. The ideal test would detect all species equally well, and provide sufficient information to allow the correct therapy to be selected, which is based in part on the infecting species.

Other deficiencies that are pertinent to an RDT designed for in-home malaria diagnosis in resource limited settings are technological, and include lack of stability of the products under conditions commonly found in malaria endemic areas, product formats are not currently designed to quantify parasite levels (or even be semi-quantitative), possible manufacturing inconsistencies, and lack of information on how well the tests perform in an in-home setting, compared to a health-outpost or regional hospital setting.

For the diagnosis of malaria in asymptomatic pregnant women, the data necessary to make definitive conclusions does not appear to be available. There is still insufficient data on the relevance between biomarkers that can be detected in peripheral circulation and the presence of placental infection. The little available data suggests that the lower-resource approaches lack sensitivity, while the approach with the best sensitivity (detection of DNA sequences using PCR amplification) currently requires a high level of resources to perform. The utility of antigen biomarkers detected using RDTs is also compromised by the same issues just described in the previous two paragraphs.

The deficiencies of the current approaches are summarized in Table 6. All of the methods listed can use whole blood from finger prick or heel stick, and can therefore use a practical specimen type. Cells that are filled in light blue are the characteristics that limit the utility of the biomarker or diagnostic approach in resource-limited settings.

Table 6. Summary of Current Deficiencies in Diagnostic Tests for Malaria

Clinical Decision	Test	TAT	Sensitivity/Specificity (Limit Of Detection)	Species Distinction	Resources
Diagnosis of symptomatic individuals in settings with no laboratory infrastructure	Clinical symptoms	Min	Poor sensitivity Poor specificity	No	Low
	Plasmodia antigens detected using rapid, point-of-care immunodiagnostic devices (RDTs)	Min	Sensitivity can be good (down to 100 parasites/μL), but can be compromised by environmental challenges & species differences. Specificity can be good, but can be compromised by environmental challenges.	Limited	Moderate-Low (cool temperatures for storage and transport, some training, cost)
Diagnosis of asymptomatic pregnant women in setting with minimal laboratory infrastructure	Parasites visualized using microscopy	Min	Misses one in five women with PAM	Yes	Moderate
	Parasite antigens detected using rapid, point-of-care immunodiagnostic devices (RDTs)	Min	Very little data, and quite variable results (38 – 95% sensitivity reported). Can be compromised by environmental challenges, species differences. Sensitivity generally better than microscopy. Specificity can be good, but may be compromised by environmental challenges	Limited	Moderate-Low (cool chain for storage and transport, some training, cost)
	Parasite DNA detected using nucleic acid amplification methods	Min - Hrs	Highest sensitivity of all the approaches, but some studies report sensitivity as low as 72%. Challenges remain in estimating specificity, because the gold standard method (microscopy) is known to be much less sensitive.	Yes	High
	Hemozoin detection using LD-MS	Min	Very little data. Sensitivity probably better than microscopy, but probably less than DNA detected using PCR	Unclear	High - Moderate

In summary, therefore, there are two major categories of deficiencies with malaria tests that are available today, which affect their usefulness and the feasibility of their implementation in resource-limited sites. These categories of deficiencies are presented in Table 7.

Table 7. Categories of Deficiencies in Diagnostic Tests for Malaria to be Used in Resource-Limited Settings

Deficiency	Biomarkers and Approaches	Specific Issues
The currently-used biomarkers are or may be inadequate to fully inform the clinical decision	Parasite antigens detected using RDTs	High degree of diversity in some antigens (HRP2), lack of biomarkers or antibodies that allow all 4 species to be detected equally well, and products not configured to identify mixed infections, or provide even semi-quantitative information on parasite densities
	Hemozoin detection	Unclear if species distinction and/or mixed infections could be identified. Validation of quantitativeness remains to be performed
The resources required to perform the test are too high	Parasite antigens detected using RDTs:	The current requirement for a cool chain for storage and delivery needs to be eliminated
	Parasite DNA sequences detected using PCR	A field-ready, easy to use, and inexpensive platform without special supply chain needs would have to be implemented
	Hemozoin detection using full blood count instruments:	Requires laboratory environment, phlebotomy, instrument costs are high
	Hemozoin detection using LDMS:	Instrument costs are high, High level training required for assay and interpretation

5. Opportunities to Improve the Clinical Performance of Existing Biomarkers

Many improvements could be made to existing test technologies that might allow the currently available biomarkers to deliver adequate performance in resource limited settings.

5A. Opportunity to Improve the Stability, Manufacturing Quality, and Sensitivity of RDTs

Existing lateral flow RDTs would be improved by selecting better antibodies, or adding additional capture antibodies that would allow all four important *Plasmodium* species to be detected equally, as well as to identify mixed infections. It is likely that additional binders would need to be added that have adequate (and perhaps unique) binding properties for the non-*falciparum* variants of the existing biomarkers.

The sensitivity of lateral flow RDTs might also be improved through a variety of mechanisms, including improved signal from detection chemistries. A number of groups have explored the use of detection chemistries, such as europium (II) chelate nanoparticles, which have provided sensitivities reported to be as low as 10 parasites/μL.[60] Because these strategies require a "reader" instrument, such a product would be much less practical for settings with no laboratory infrastructure. Nevertheless, it is worth considering the incorporation of recent technology improvements to lateral flow devices, and how their incorporation might affect cost and practicability.

As discussed in Section 2A, the inability of RDTs to withstand the shipping and storage conditions that are commonly found in areas where malaria is endemic is suspected to be a major problem. Improvements to the binders, conjugation chemistries, dyes, and other components of the RDTs would address this issue. Though studies should be specifically designed to assess the stability of each of the components, it is easy to

speculate that the antibodies used as binders are likely candidates for the most unstable part of the system. Synthetic binders, specifically selected for their ability to withstand high temperatures and other environmental challenges, might significantly improve the stability of malaria RDTs, and once developed, might reduce their manufacturing cost and complexity. A variety of methods to develop synthetic binders have been developed, but in general, it has been difficult to replicate the specificity that can be obtained using antibodies. Recombinant antibody fragments (e.g., single chain antibodies) might provide an incremental improvement in stability, though a synthetic polymer or small molecule might ultimately be required to withstand 40°C for long periods of time. It is possible that a company that has capabilities for the high-throughput use of combinatorial chemistry approaches to develop novel materials and surfaces, such as Symyx,[61] might be able attempt an entirely new approach. A review of such approaches has been presented in another manuscript.[62]

Because it is not always clear if product performance problems have arisen due to storage and transport conditions, or from manufacturing inconsistencies, it is possible that manufacturing issues will be found to have contributed to the performance problems of RDTs. If this is the case, the specific issues that are causing manufacturing inconsistencies would need to be identified and remedied.

One other potential modification to RDTs, which involves the use of a urine specimen instead of a blood specimen, has recently been explored. This group has adapted monoclonal antibodies to the HRP2 antigen of *P. falciparum* to a dipstick format that uses urine as the specimen type. A small validation study they conducted had sufficiently promising results to suggest that a larger study should be conducted, that would compare both the performance as well as the ease-of-use of this method to other available methods[63]

5B. Opportunity to Implement a Field-Ready, Easy-to-Use Nucleic Acid-Based Test Platform

Though a field-ready, easy-to-use, and inexpensive platform to detect nucleic acids would be very useful, this is an extremely challenging undertaking that has been the goal of many groups for many years. In order to use *Plasmodia* DNA sequence biomarkers, a multiplex assay that detects all four important species equally well would need to be adapted for a platform that could be used in resource-limited settings, which does not truly exist today. Platforms that are relatively close to meeting the requirements for a resource limited setting, such as the GeneXpert™ (Cepheid, Sunnyvale, U.S.) platform,[64] the LIAT™ (IQuum, Allston, U.S.) platform,[65] or the Handylab platform,[66] are likely to require additional engineering and manufacturing improvements, to reduce reagent and consumable costs, reduce manufacturing costs, reduce energy consumption, improve the robustness, improve the ability of local (perhaps less-specialized) technicians to make repairs in the country in which the test is being used, and to improve the ability of the reagents to withstand stability challenges throughout the supply chain. Even if all these things could be achieved, it seems more likely that such a device or instrument would be feasible for use at a health outpost by a trained health worker, rather than used in an in-home setting by a family member.

5C. Opportunity to Implement a Field-Ready, Easy-to-Use Platform for Hemozoin Detection

Because of its unique chemical structure and properties, there may be many ways to detect the hemozoin pigment produced by *Plasmodia* parasites. One method for detection of hemozoin that has been evaluated is using laser desorption mass spectrometry (MS). Interestingly, there are reports in the literature indicating

that other MS (SELDI MS) analyses performed on serum can also distinguish a range of diseases, including trypanosomiasis.[67] These analyses were performed in research laboratories in the developed world. For this strategy to be feasible for a health-outpost setting, it would be necessary to develop a field-ready, portable, easy-to-use, and low-cost instrument. Because this approach requires an instrument, it would be more appropriate for use in a health-outpost, and does not seem feasible for an in-home test. Though the method apparently requires no reagents other than water, it is possible that simplification of the procedure, and simplification of the interpretation of the output of the instrument, would need to be achieved as well.

Because MS approaches detect many molecular entities in a sample at the same time, it is possible that a signature, which includes hemozoin along with other entities unique to malaria infection, would be discovered. For this approach to be able to distinguish the four important *Plasmodium* species, it is likely that the simultaneous detection of entities other than hemozoin would be essential.

There are reports in the literature of a prototype MS instrument that at least partly satisfies the requirements for a health outpost setting. A group led by Andrew Feldman at the Johns Hopkins University Applied Physics Laboratory has developed a rugged MALDI TOF MS instrument that is the size of a suitcase (17 inches by 10 inches by 7 inches), with biodefense applications in mind. The instrument overcomes some of the issues of size, weight, and power requirements that have previously prevented deployment of MS instruments to field applications.[68] The instrument has been designed with an automated sample preparation station so that the sample, once it is in the collection vial, is then inserted into a cartridge that has all the consumables prepackaged in it, which is then put into a sample preparation station. The sample is processed and deposited on a metal plate/card, and is then ready for MALDI analysis. The sample processing and analysis apparently requires approximately 15 minutes. It is unclear whether such a device could be run using an alternative energy source, such as solar power, whether the analysis and interpretation steps can be sufficiently simplified to be feasible in a health outpost setting, and whether the cost of the consumables (sample prep cartridge and metal plate) will be sufficiently inexpensive to make this platform viable.

A suitcase-sized MALDI-TOF MS instrument[68]

6. Evaluation of Known Molecules That Have Not Yet Been Clinically Validated

There has been a tremendous amount of research into the biology of *Plasmodia* in recent years, and from this research, a variety of other options for biomarkers of infection are now known. Two of the more promising avenues are presented in the following sections.

6A. Evaluation of Known Protein Molecules as Biomarkers for RDTs

Many other *Plasmodia* proteins which could serve as biomarkers have been identified in recent years, either through efforts to identify antigens for vaccines, efforts to understand the parasite's biology, and through analyses spurred by the availability of the *P. falciparum* genome sequence, which became available in 2002.[69] The most commonly detected protein in existing RDTs is HRP2, which is found in the RBC

cytoplasm and is also secreted by RBCs into the circulation. The high degree of diversity found in PfHRP2 may affect the ability of this protein to be detected in all isolates using a single monoclonal antibody. Because of this, it is worth considering other molecules with less variation, or perhaps combining the detection of PfHRP2 with other molecules, as a way to reduce the risk that a particular isolate is not detected because of strain diversity.

The molecules that are proteolytically cleaved upon invasion, and whose cleavage products are released into the circulation, make interesting candidates because they may be present in the peripheral circulation even when parasites are sequestered. A number of merozoite surface proteins are proteolytically cleaved as the surface coat proteins are "shed" lateral to the moving junction at the point of apposition between the parasite and erythrocyte membrane.[14]

Apical membrane antigen 1 (AMA1) is such a surface protein that is found in all *Plasmodia* species. Research suggests that the N-terminal ectoplasmic region of AMA must be cleaved from the parasite surface for host-cell invasion to proceed.[70] Through an analysis of the crystal structure of the *P. vivax* AMA1, both polymorphic regions and invariant regions have been identified, and monoclonal antibodies that detect these different epitopes already exist.[70] This type of analysis might identify the best epitopes for binders to be used in an RDT. Other interesting candidates are parasite encoded proteins that function in the RBC cytosol to mediate merozoite release, such as falcipain-2 and plasmepsin-2, which might be released into the circulation upon RBC rupture.[71]

Two groups have recently reported the identification of large number of proteins predicted to be secreted from (exported from) the parasites into the host RBC. Both groups used bioinformatic approaches to identify specific amino-acid sequences that serve to target proteins that are produced by the parasite but that must be transported out of the parasitophorous vacuole (PV) and into the RBC. From this work, these groups predicted the existence of a "secretome" of 250 to 400 proteins for *P. falciparum*.[72] Yet another study used proteomic and bioinformatic approaches to identify the proteins that coat the surface of the extracellular forms of *P. falciparum*. They identified 11 proteins that represent 94% of the GPI-anchored proteins on the merozoite surface, and predicted the existence of another 19. They determined that MSP-1 and MSP-2 comprised about two thirds of the total membrane-associated surface coat proteins.[73] Though the quantity of these two proteins makes them interesting, their high level of diversity might make them risky choices when trying to develop a binder that will work in all strains.[74] The proteins identified in these types of analyses could serve as a starting point from which to select potential candidates for new antigens to be detected by RDTs. It is conceivable that a panel of antigens would need to be detected.

When choosing potential new biomarkers to replace or add to existing RDTs, it might be necessary to make a choice between a stable, long-lived protein (which might provide better sensitivity, because it detects multiple generations of parasites), and a shorter-lived protein which would indicate active infection, but which might not provide as great a sensitivity. For the intervention points under consideration in this document - the detection of infection, but not monitoring response to therapy - the longer-lived antigens are more likely to provide better performance.

6B. Evaluation of Known Molecules as Biomarkers for Malaria in Asymptomatic Pregnant Women

This manuscript has assumed that a specimen that can be easily obtained (i.e., peripheral blood) would need to be used to detect placental infection. Though a few studies have evaluated the performance of several biomarkers found in the peripheral blood, their precise relevance to placental infection has not be unequivocally determined, and therefore additional studies that definitively establish the relationship of these biomarkers to placental infection remain to be completed.

Another novel analytical approach, which remains to be validated, has already been shown to detect a pattern that is unique to malaria, but the particular molecules in the sample that generate the pattern are not known at this time. In this approach, a specific spectrum that is unique to malaria is detected using multi-wavelength ultraviolet/visible (UV-VIS) spectroscopy in a process called spectral acquisition process detection (SAPD). Garcia-Rubio et al. suggest that SAPD is a portable, quantitative, rapid, reliable method for detecting, identifying, and enumerating microorganisms in biological samples. It requires no reagents or expensive equipment. The spectrum that is generated from a sample is determined by a number of properties of the sample, including the cellular and chemical composition, as well as morphological structures within it. For the analysis, 2 to 5 μL of blood is collected, diluted with normal saline, and the spectra is generated using a Hewlett Packard 8453 spectrophotometer with an acceptance angle of less than 2 degrees. The results are downloaded to a laptop computer, where the interpretation is made. The authors do not state what particular molecules are being detected, but they claim they can detect one infected cell per million uninfected RBCs.[75] The performance of this approach in asymptomatic pregnant women remains to be evaluated.

7. Approaches for the Discovery of Novel Biomarkers for Diagnosis of Malaria

In addition to improvements that could be made to existing diagnostic test methods through technology improvements, or the incorporation of known molecules into these tests, there are also opportunities for the discovery of new biomarkers that might be better suited for deployment in resource limited settings.

It is known that malaria infection is asymptomatic in many individuals, and researchers and clinicians have been interested in identifying a biomarker that would distinguish the individuals who will develop clinical symptoms from those who will remain infected but relatively symptom-free. Parasite density observed in peripheral blood by microscopy provides a relatively loose correlation to disease severity, and to a greater degree in children, but a biomarker with greater predictive power would be useful.[76] Such a biomarker could be parasite-derived, or host-derived, or might be a combination of the two. It is possible that some of the analytical methods already discussed in this manuscript, such as LDMS, or UV-VIS spectroscopy, which identify patterns of many molecules, could be used to search for such a biomarker. The design of the validation studies which remain to be completed for these approaches might be modified to determine if there are elements of the spectra that correlate with disease severity or likelihood of developing specific symptoms.

The category of biomarkers that has not yet been explored, but which merits at least an initial evaluation, is volatile organic compounds. Though this category of biomarkers may be a long shot for diagnosis of malaria, it appears to be a very promising category of biomarkers for other febrile illnesses that are common in resource-limited settings, such as acute lower respiratory infection (ALRI) and tuberculosis (TB). In this

context, it would be extremely valuable to have a single diagnostic platform that could distinguish at least these three diseases, and the detection of volatile organics holds particular promise. The discovery of potential volatile organic biomarkers for malaria infection is discussed in the following section.

7A. Discovery of Volatile Organic Biomarkers

Volatile organic compounds (VOCs) that are indicative to the status of an individual or sample can be detected in breath, and possibly in the headspace of a whole blood sample. It does not appear particularly likely that volatile organics in the breath might be able to identify an individual with malaria, because the infection does not aggregate in the lungs. However, it is believed that VOCs found in the breath are mainly blood borne, and therefore provide a non-invasive way to monitor biological processes that can be detected in the blood.[77]

A hypothesis-free approach could be undertaken which would involve an analysis of the VOCs in the breath or in the headspace of a blood sample using either electronic nose or mass spectrometry instruments. Devices characterized as electronic noses (e-noses) utilize a variety of technologies, though perhaps the most prevalent utilizes an array of non-specific chemical sensors that bind volatile chemicals in the vapor headspace over a sample. The most common types of sensors are metal oxide sensors, conducting polymers, and piezoelectric-based sensors. The sensors typically have a partial specificity, in that they respond to certain classes of chemicals, such as alcohols and aldehydes, rather than to single compounds. The interaction of volatile compounds with the sensor surface results in changes in the physical properties of the sensor, such as its resistance, conductivity, and frequency, which are then measured. Therefore the nature and relative ratio of the molecules in the headspace determines the response pattern of the sensor array. The device is used to sample a set of "case" samples (e.g., known cases of malaria) and control samples (e.g., patients with other febrile illnesses, and healthy individuals), and pattern-matching algorithms are used to identify a pattern that segregates the cases from the controls. The device can then use the pattern to classify unknown samples. Several research groups have shown that they can discriminate between a variety of infectious organisms, either in the headspace of culture samples or in clinical breath, sputum, or serum samples.[78,79,80]

At least one commercially available e-nose model is portable and battery powered. The Cyranose 320, developed by Cyrano Sciences but now sold by Smiths Detection, is a hand-held, battery-powered device designed for field-use.[81] One drawback to the e-nose approach is that molecular identity of the molecules that make up the pattern cannot be determined, because the device only recognizes the pattern.

Another hypothesis-free approach to discovering biomarkers in the breath utilizes mass spectrometry detection methods, which can often identify the particular volatile substances that are unique to a disease etiology. Ruzsanyi et al. reported the use of multi-capillary chromatography columns coupled to an ion mobility spectrometer to analyze breath samples from individuals with a variety of respiratory diseases, and their results indicated the presence of specific peaks that were unique to particular pathogens, compared to emphysema or general inflammation.[82] Other work recently published by Phillips et. al. (2006) identified 12 principle components in the VOCs in the breath of TB patients, such as 1-methyl-naphthalene and 1,4-dimethyl-cyclohexane that served as a fingerprint for *Mycobacteria tuberculosis* infection. This pattern identified sputum-positive patients with 83% sensitivity and 100% specificity.[83]

The Cyranose 320[81]

An experimental program would therefore need to be undertaken to determine if VOCs that are unique to *Plasmodium* infection could be detected in the breath of patients with malaria. This initial program could utilize either MS or an e-nose detection approach. It is possible that if the important principle components of the VOCs in the breath are identified via MS approaches, then an e-nose instrument (of which at least one is already field-deployable) could be specifically designed to detect and discriminate the important molecules. If it is not possible to identify malaria infections based on the VOCs found in the breath or in the headspace of a blood sample, then perhaps the blood sample could be treated or processed in some way to induce the production or release of volatile organics from the sample. One possibility would be to develop a substrate that can be acted upon by an enzyme that is unique to *Plasmodia*, such as lactate dehydrogenase (pLDH), that when acted upon, releases a volatile organic substance that can be detected.

8. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

There do not appear to be any organized sample banking efforts for malaria, though individual researchers may have some sample types stored in their "private collections." In the absence of banked samples, the preliminary studies for many diagnostic development programs (e.g., for new RDTs or hemozoin detection) could be expedited by the use of normal whole blood that is spiked with cultured parasites, or samples obtained from individuals with malaria who live near the research and development laboratories, which might be cases of "imported" malaria. Ideally these would be whole blood samples that have associated parasite density and species determined via microscopy, and possibly even strain variation information determined by PCR-based approaches. These preliminary studies might examine a small number of samples (perhaps dozens) to make an initial estimation of a new methods sensitivity and specificity, as well as determine whether the method can detect all four *Plasmodium* species and identify mixed infections, even at lower parasite densities.

In addition, the studies that would evaluate the causes of RDT product instability, and the initial studies to evaluate the success of potential remedies to these issues, could be expedited by the use of normal whole blood spiked with cultured parasites, or from individuals with imported malaria. However, once a new prototype RDT for in-home use is developed, a large part of its evaluation must occur in malaria endemic areas, and incorporate an in-home evaluation of performance. Such studies would need to evaluate hundreds of samples, compare performance to at least microscopy (but possibly also to PCR-based approaches), and be conducted in a variety of geographical settings with different prevalence of the *Plasmodia* species and endemicities. This suggests that an entire diagnostic unit, capable of performing microscopy, the method under evaluation (e.g., a new prototype RDT, MS, or spectroscopy-based approach), and possibly PCR-based methods, would need to be set up and maintained in a variety of remote locations for the duration of the study.

The study design issues are more complex for a test to diagnose asymptomatic pregnant women. Microscopic and PCR analysis of the placenta should be performed after the birth of the baby, to serve as the gold-standard measures against which the performance of any new tests on peripheral blood are measured. Ideally, a study would examine peripheral blood specimens at different points during the pregnancy, not just at birth, and also collect outcome data on the maternal health as well as the baby's health. This outcome data can be problematic, due to the inaccuracies of ascertaining the causes of morbidity and mortality in resource-limited areas with many potential concurrent conditions.[76] Therefore, these sample collections, if they must be performed prospectively on hundreds of women, could take a relatively long period of time and require a great deal of resources.

Because of the lack of sample banks, breath or other specimen types from which volatile organics might be measured will need to be collected prospectively. These initial discovery studies could be conducted in a smaller number of individuals (perhaps dozens) with imported malaria, provided that sufficient species diversity could be obtained, and that the researchers also had access to at least a small number of individuals who have infections that are to be distinguished, such as TB and ALRI. If this approach looks promising, additional evaluation and validation studies would need to be conducted, perhaps in hospitals that already have functioning malaria diagnostic laboratories near malaria endemic areas. If this round of evaluations continues to show promise, then yet another round of validations would need to be performed in a health-outpost setting. This again suggests that an entire diagnostic unit, capable of performing microscopy, the method under evaluation (e.g., measurement of volatile organic compounds), and possibly PCR-based methods, would need to be set up and maintained in a number of health outpost settings in geographically diverse regions.

9. Discussion and Recommendations for the Improvement of Diagnostics for Malaria

The following recommendations are presented for consideration, based upon the deficiencies of current diagnostic test methods for deployment in resource-limited settings, the opportunities for improving the deployment of existing biomarkers, and the approaches that might be used for discovering novel biomarkers that are more appropriate for use in resource-limited settings.

9A. What clinical information and user specifications are required for the design and development of the diagnostic products needed for the intervention points selected for malaria?

For identifying which febrile patients have malaria infection using a diagnostic test that can be performed in the home, and therefore identifying which patients should be treated with appropriate anti-malaria therapeutics, an assay is needed that can provide good sensitivity and specificity for at least *P. falciparum* and *P. vivax*, can withstand the rigors of the transport and storage chain that are likely to be encountered, and is simple enough to be performed by a local health care worker. The assay should offer high specificity to reduce the instances in which antibiotics are used inappropriately. Ideally, the test could distinguish and provide good sensitivity for the four important malaria-causing *Plasmodium* species, detect mixed infections, and provide semi-quantitative information on parasite density. The test should use whole blood from a finger prick, or some other similarly simple/accessible specimen type (e.g., urine, saliva).

For identifying which asymptomatic pregnant women have pregnancy-associated malaria, using a diagnostic test that can be performed in a health-outpost setting, and therefore identifying which women should be treated with appropriate anti-malaria therapeutics and/or other interventions, an assay is needed that can sensitively detect *P. falciparum* infection that is sequestered in the placenta using an easily obtainable specimen type such as whole blood from a finger prick specimen. The test supplies would need to be able to withstand the rigors of the transport and storage chain that is likely to be encountered, and is simple enough to be performed by health care worker. Ideally, the test might be able to also identify and distinguish the other important malaria-causing *Plasmodium* species which may be in the blood, and provide some semi-quantitative information on parasite density in the placenta.

The tests capabilities that are necessary and those that are desirable-- but perhaps not absolutely necessary-- are listed in Table 8.

Table 8. Required and Optional Capabilities for Malaria Diagnostic Tests for Use in Resource-Limited Settings

Intervention Point	Required Capabilities	Desirable Capabilities
Diagnosis of malaria in symptomatic patients in settings with no laboratory resources	• High sensitivity and specificity for *P. falciparum* and *P. vivax* • Withstand the rigors of the transport and storage chain that are likely to be encountered • Simple enough to be performed in the home, perhaps by a family member • Uses a practical specimen type, such as a finger-prick or heel-stick	• Ability to distinguish disease from parasitemia • High sensitivity and specificity for all four species that infect humans • Ability to distinguish mixed infections • Provide semi-quantitative information regarding levels of parasitemia
Diagnosis of malaria in asymptomatic pregnant women	• High sensitivity and specificity for *P. falciparum* infections sequestered in the placenta • Withstand the rigors of the transport and storage chain that are likely to be encountered • Simple enough to be performed in a health-outpost setting • Uses a practical specimen type, such as peripheral blood from a finger-prick	• High sensitivity and specificity for all four species that infect humans • Ability to distinguish mixed infections • Provide semi-quantitative information regarding levels of parasitemia

9B. What biomarkers, sample types, and technologies are most appropriate for the intervention points selected for malaria?

For a test to detect malaria in settings with no resources, the available evidence suggests that protein biomarkers are likely to be able to provide adequate performance in an rapid immunodiagnostic format using a practical sample type (whole blood from a heel stick or finger prick) if modifications were made to the immuno-diagnostic device that provided the capability to detect all species, to improve the product stability, and possibly to distinguish disease from parasitemia. DNA sequences as biomarkers provide better performance in resource-rich settings, but the technical challenges of implementing the necessary platform, and implementing it at a very low cost per test, are significantly greater than those faced in improving RDTs.

It is theoretically possible to obtain the information required to make the clinical decisions using antigen-based tests. Therefore, until it is shown that the technical challenges facing RDTs CANNOT be overcome, it does not make sense to attempt to migrate to a DNA-based approach to be feasible as a simple "no laboratory resources" test.

For a test to identify malaria in asymptomatic pregnant women greater sensitivity is required, and it is less clear whether RDTs will be able to provide sufficient performance. A small amount of data suggests that the tests can provide adequate sensitivity to detect women with "clinically significant" malaria and who are at a greater risk of poor outcomes; but, a policy decision would need to be made that prioritizes the detection of women at greater risk for poor outcomes, rather than detecting all the women who are infected. If the chosen path forward were RDTs, then all the improvements indicated above would need to be implemented. In addition, other changes to the format that might improve the sensitivity should be considered, if they can be implemented in a way that does not significantly impact the feasibility of the test for a health outpost setting.

The approaches that detect hemozoin, such as mass spectrometry, and/or other chemical entities in the sample, such as UV-VIS spectroscopy (e.g., SAPD), require further evaluation and validation. It is unclear if these methods could distinguish *Plasmodia* specie, or if they will be sufficiently quantitative. DNA sequences as biomarkers provide the greatest sensitivity based upon available lab methods (e.g. PCR), but there are still significant technical challenges remaining to implementing a reliable platform at a very low cost per test in sites with minimal laboratory resources.

Exploring the use of volatile organics in either the breath or in the headspace of a blood sample is quite risky due to the lack of data supporting the feasibility of this approach. Even though there is no data that directly suggests the diagnosis of malaria is feasible using this approach, or whether any species distinction might be possible, it is intriguing as an approach because it does not require a liquid specimen, field-deployable instruments are already available, and it might be possible to simultaneously diagnose several of the major febrile illnesses that are common in the developed world. It is also possible that a VOC biomarker could distinguish individuals with disease from those who have only parasitemia.

The potential diagnostic approaches that are reviewed in this section are illustrated in Figures 3 and 4. In these figures, the current approaches are shown light gray spheres and the potential future approaches are shown as colored spheres. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource-limited setting (y-axis). In these figures, therefore, an ideal biomarker and test method will be in the upper right quadrant, with a high predictive power and low resource requirements. Figure 3 shows the most promising opportunities for an in-home test to identify malaria in symptomatic individuals, while Figure 4 depicts the most promising opportunities to detect infection in asymptomatic pregnant women.

Figure 3. Future Approaches for Diagnosing Malaria in Settings with No Laboratory Infrastructure

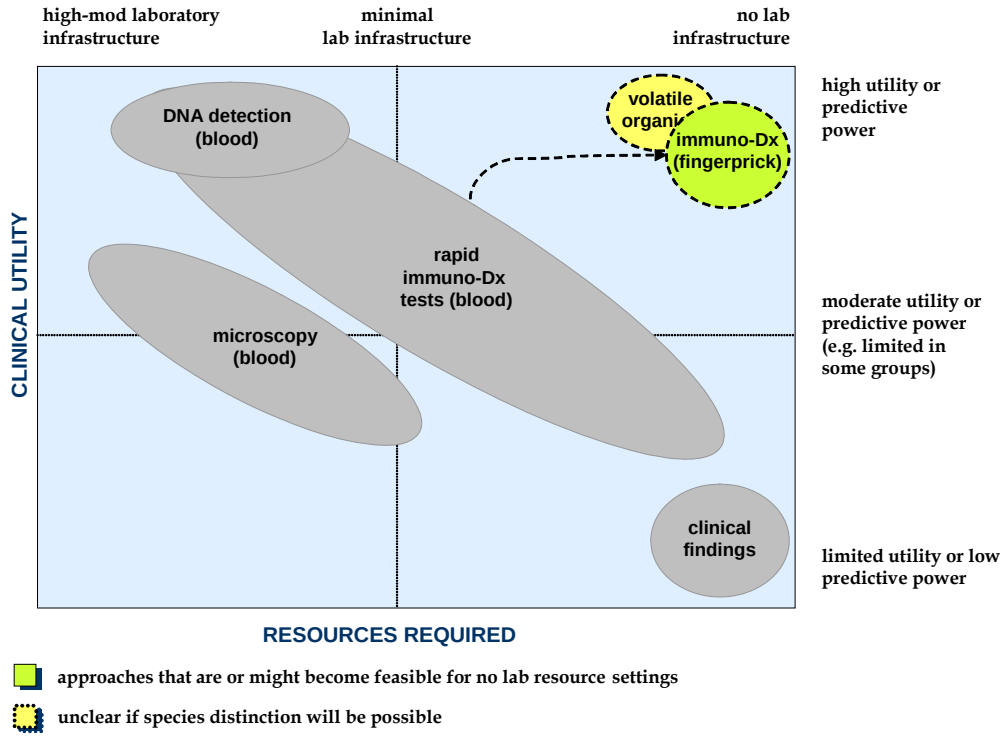
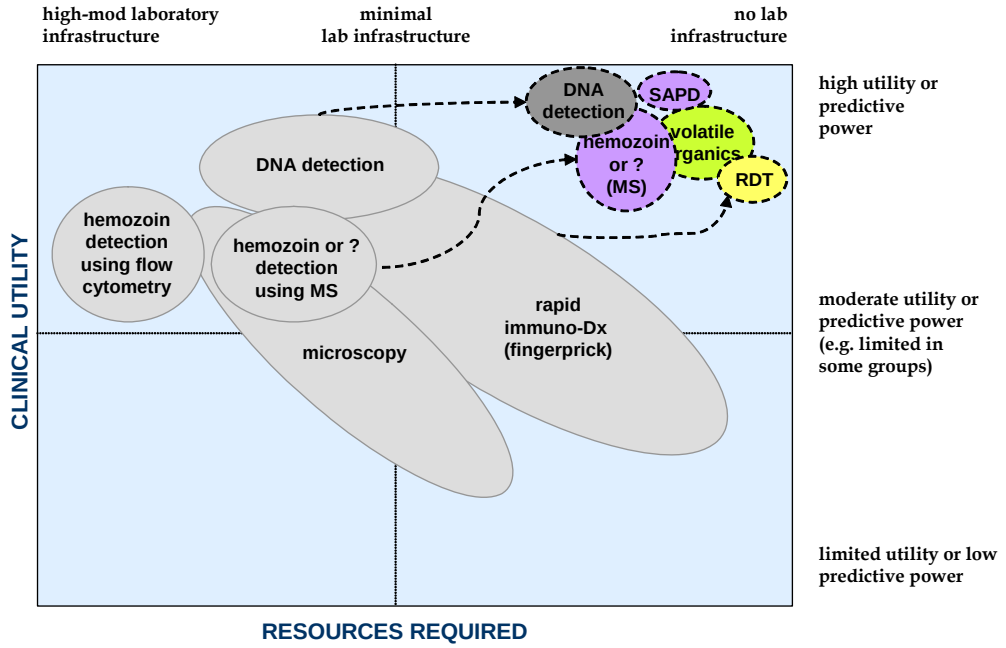


Figure 4. Future Approaches for Diagnosing Malaria in Asymptomatic Pregnant Women



The vertical placement of methods that detect hemozoin or volatile organic compounds is purely speculative, as there is little or no data on the clinical utility of these methods for the diagnosis of asymptomatic pregnant women.

9C. Recommended Course of Action and Resources Required

There are five potential paths forward which are discussed in the following sub-sections.

Potential Path Forward 1: Improvements to RDTs

In order to improve the performance of malaria RDTs, a variety of changes to the existing tests should be undertaken by a group that is very experienced in the development of point of care immunodiagnostic tests. Additional biomarkers would need to be selected to adequately detect all four species, identify mixed infections, and to distinguish disease from parasitemia. The stability of the components of existing tests, and their stability in the face of other environmental challenges, should be systematically evaluated, and a plan should be devised to develop alternative components for those that are not sufficiently stable. If the test were to be used to detect malaria in asymptomatic pregnant women, additional improvements to sensitivity would make the test more useful. Such improvements might include changes to the detection chemistry, and possibly the addition of a hand held detection instrument.

The initial evaluations during the new test's development could be expedited by the use of banked samples and/or the use of samples from individuals with "travelers malaria" which might be more readily accessible to the test development laboratories. The ultimate validation of the new test would need to be performed in diverse regions of the world where malaria is endemic, and include in-home testing. Because of the challenges in obtaining blood from asymptomatic pregnant women with demonstrated placental infection, the evaluations of a new test for diagnosing PAM would be greatly expedited by the use of banked samples, if they exist. Otherwise, prospective testing of women in endemic areas will be required.

This path forward has a low degree of biological risk and a moderate degree of technical risk. The biological risk is low because useful antigens are already known for *P. falciparum* and *P. vivax* detection that can provide relatively good performance, and the large body of information that has emerged in the literature in recent years regarding the biology of *Plasmodia* suggests that additional antigens could be fairly easily selected that would provide the additional diagnostic information that is required. The technology risk is moderate because high level of multiplex would probably be required (5 or more targets) and the immunochromatographic device would need to be re-designed to lower the resources required. Stability of test components in high humidity and temperature environments will also be a significant issue.

Potential Path Forward 2: Detection of Hemozoin Using Mass Spectrometry Approaches

Because a sophisticated instrument is required, it is assumed that the use of a mass spectrometry approach would be more feasible in a minimal laboratory resource setting compared to a no laboratory resource setting .Additional exploratory research is still required to determine if the detection of hemozoin in peripheral blood can provide sufficient sensitivity for detecting asymptomatic pregnant women with placental infection and to determine if the four important *Plasmodia* species can be differentiated. It is likely that a signature, which combines the detection of hemozoin with other entities, would need to be identified in order to be able to differentiate parasite species. The initial evaluations would be expedited by the use of banked samples which might be more readily accessible to the test development laboratories. If a small initial exploratory study suggests that this approach holds promise, larger studies conducted in regional hospitals in diverse parts of the world would need to be conducted, perhaps using the field-deployable prototype LD-MS

instrument that already exists. If it were demonstrated that this approach can provide sufficient performance, then an engineering program could need to be initiated to improve the affordability and robustness of the analytical instrument.

This path forward has a moderate degree of biological risk and a moderate degree of technical risk. The biological risk is moderate because there is little data suggesting that this approach can provide sufficient sensitivity using peripheral blood to detect placental infection or whether the approach will be able to provide species discrimination, though theoretically it should be able to. The technology risk is moderate because a prototype for a field-usable instrument already exists, and no reagents other than water are required, but it is unclear if the instrument can be made sufficiently robust to function in a health-outpost setting, and whether the instrument and the consumables required can be produced at a cost that make the test practical for a resource-limited setting.

Potential Path Forward 3: Detection of Malaria-Specific Spectra Using UV-VIS Spectroscopy

Because a sophisticated instrument is required, it is assumed that the use of UV-VIS spectrometry approach would be more feasible in settings with minimal laboratory infrastructure compared to sites with no laboratory infrastructure. Additional exploratory research is still required to determine if the spectra identified by the Garcia-Rubio group would provide sufficient sensitivity when using peripheral blood to detect asymptomatic pregnant women with placental infection and to determine if the four important *Plasmodia* species can be detected. The initial evaluations would be expedited by the use of banked samples which might be more readily accessible to the test development laboratories. If it were demonstrated that this approach can provide sufficient performance, then an engineering program would need to be initiated, which would improve the affordability and robustness of the analytical instrument.

This path forward has a moderate degree of biological risk and a moderate degree of technical risk. The biological risk is moderate because there is no data suggesting that this approach can provide sufficient sensitivity using peripheral blood to detect placental infection, or whether the approach will be able to provide species discrimination, though theoretically it should be able to. The technology risk is moderate because there is relatively little published on this approach, though instruments that are relatively close to being field-usable already exist, and no reagents other than water are required. In addition it is unclear if the instrument can be made sufficiently robust to function in settings with minimal laboratory resources, and whether the instrument and the consumables required can be produced at a cost that make the test practical for a resource-limited setting.

Potential Path Forward 4: Volatile Organic Biomarkers Detected Using Electronic Noses

The discovery and validation of volatile organic biomarkers for the diagnosis of malaria is a long shot. It is worth evaluating in the context of the potential benefits that would be provided by a single diagnostic platform that could simultaneously distinguish several of the major infective organisms that commonly cause febrile illness in the developed world. It is also possible that disease could be distinguished from parasitemia using this approach. Given that the analytical technology already exists for discovery (e.g., an e-nose, or multi-capillary-column-ion-mobility-mass spectrometry) it should be possible to complete an initial evaluation in the U.S. or Europe fairly rapidly in a lab that already has the analytical approach working. An

important question to be addressed would be whether it is possible to provide species discrimination using this approach. Any biomarkers that are discovered would then need to be validated in populations that are served by resource-limited settings, which would almost certainly require prospectively-collected samples to be analyzed in the field. If the biomarkers were validated, then it is likely that an engineering program would need to be initiated, to improve the affordability and robustness of the analytical instrument. This would require additional time, and the cost-benefits of waiting for this more robust and affordable instrument could be weighed against the option of distributing an existing, more costly, and less-robust model immediately.

This experimental program has a high degree of biological risk and a moderate degree of technical risk. The biological risk is high because a there is no evidence indicating that malaria infection could be identified by volatile organic compounds in breath samples or in the headspace of a blood sample. The technology risk is moderate because it is still unclear that the existing e-nose instruments have an adequate set of sensors that would be required for the discrimination necessary, though there are already commercially available, portable MCC-IMS and e-nose instruments that could be used in the evaluation studies, and which might be migrated to a truly field-deployable commercialization platform. If useful biomarkers were identified using mass spectrometry approaches, then perhaps the knowledge of the specific molecules to be detected could be used to fine-tune the array of sensors in an electronic nose, which might result in a better field-usable instrument.

Potential Path Forward 5: Detection of DNA Sequences

The performance demonstrated by diagnostic approaches that detect parasite DNA sequences make this an attractive approach for diagnosing malaria infection in asymptomatic pregnant women, but the challenges involved in implementing this type of platform in resource-limited settings are significant. It is also unclear that DNA biomarkers could ever distinguish disease from parasitemia. With existing technical approaches sample purification and complex reagents and devices would be required, it seems reasonable to give priority to other options that do not have these constraints. This is especially true if some of the other approaches have the potential to simultaneously diagnose other illnesses or infections using the same sample. If these other options (outlined in Paths 2, 3 and 4) are incapable of providing the necessary performance, then DNA biomarkers would be the next option.

This path forward has a low degree of biological risk and a high degree of technical risk. The biological risk is low, because DNA sequences as biomarkers have already been shown to provide the necessary performance in well-resourced testing environments. The technology risk is high because a significant decrease in the resources required would need to be achieved in even the existing platforms (e.g., GeneXpert or LIAT), including the platform robustness, power required, ease-of-use, and cost. It is also possible that improvement to the reagents would be required to enhance their stability throughout the supply and storage chain.

Summary of Recommendations

For the diagnosis of malaria infection in settings with no laboratory resources, improvements to RDTs are a clear path forward that seems logical to pursue, given the very low resources required in this approach and the potential for antigen biomarkers to provide adequate performance. It seems likely that new biomarkers

or better antibodies to existing markers will be needed to improve the detection of all species, and to distinguish disease from parasitemia. Improved stability for reliable field deployment is also essential. Exploration of the potential of VOC biomarkers might allow the development of a single platform or assay to detect a variety of diseases associated with fever, and could be pursued for malaria if it appears to be feasible for at least one other disease.

For the diagnosis of malaria in asymptomatic pregnant women, additional feasibility work remains to be conducted for four approaches, including 1) improvements to RDTs, 2) detection of hemozoin using mass spectrometry, 3) detection of a malaria-specific spectra using UV-VIS spectroscopy, and 4) the detection of volatile organic compounds either in the breath or in the headspace of a blood sample. Because it is difficult to predict which of these will provide the best performance and be the easiest to implement, the initial feasibility experiments for these four approaches could pursued in parallel. Ideally, the proof of concept would be demonstrated or eliminated for these pathways in a relatively short period of time (hopefully without requiring prospective sample collection in the developing world), at which time the most promising pathway could be prioritized. These initial feasibility experiments would help determine whether any of these approaches might be feasible as an in-home test as well. If none of these first four pathways appear feasible, then the decision could be made to prioritize the fifth path forward, the detection of DNA sequences using a nucleic acid testing platform, which is likely to be the most technically challenging.

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