# Malaria Diagnostics Technology Landscape:

## Enzyme Linked Immunosorbent Assays (ELISA) for Histidine-Rich Protein 2 (HRP 2)

## Project DIAMETER (Diagnostics for Malaria Elimination Toward Eradication)

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# Enzyme Linked Immunosorbent Assays (ELISA) for Histidine-Rich Protein 2 (HRP 2)

## Introduction

Recent emphasis on elimination and eradication (E&E) goals for malaria programs is changing the way experts evaluate malaria diagnostic tools and tactics. As malaria prevalence is driven to lower levels, the focus pivots toward low-density, subclinical infections and geographically and demographically concentrated reservoirs. The proportions of species and subspecies found in these reservoirs may also change in favor of higher fitness phenotypes that are more difficult to diagnose and treat. These changes present both threats and opportunities to existing diagnostic technologies and those in the development pipeline. Furthermore, the evolution of new tactics to combat malaria in low-prevalence areas necessitates a review of technologies in the product development pipeline to encourage development of appropriate tools for new diagnostics use-scenarios. In light of shifting goals, epidemiology, and tactics, this report focuses on enzyme linked immunosorbent assays (ELISA) for histidine-rich protein 2 (HRP 2) for *Plasmodium falciparum (P. falciparum)* detection.

## The need

In the elimination context, low-parasite-density *P. falciparum* infections present two distinct challenges for a diagnostic test used for active infection detection (ID).[i] The first challenge is the obvious low total parasite biomass in the infected individual.[1,2] With fewer parasites, the limit of detection of the assay must be suitably low, and a larger volume of specimen may be required to avoid Poisson effects leading to false negatives from the sampling.

The second effect of low levels of parasitemia arises because there is a greater probability that the scarce parasites are all part of a single cohort; i.e., developing synchronously.[3] Parasites in a cohort can all sequester out of the peripheral circulation at the same time, resulting in durations when no detectable parasites are present in blood. The challenge posed by synchrony can be overcome through the collection of multiple specimens in a defined time period, but this strategy is incompatible with “single encounter” diagnosis and treatment strategies typically used in case management, mass screening and treatment (MSAT), focal screening and treatment (FSAT), reactive case detection (RACD), and other elimination diagnostic use-scenarios. Thus, consistent detection of synchronized parasitemia may require a diagnostic test that detects analytes that persist in the peripheral circulation even when the parasites are sequestered.

[i] Active case detection refers to the detection of malaria (clinical and sub-clinical) infections by health workers at community and household levels in population groups that are considered to be at high risk. Active infection detection can be conducted with a fever screening followed by parasitological examination of all febrile patients or as parasitological examination of the target population without prior fever screening.

## Histidine-rich protein 2

Histidine-rich proteins were among the first plasmodial proteins to be studied in detail. As a result, much is known about their biological structure and function.[ii] The concentration of HRP 2 in the peripheral circulation of an infected individual is directly related to the total viable parasite biomass[6] and the stage of development,[7] and has been used extensively as the analyte in *in vitro* diagnostic tests. HRP 2 can be detected in erythrocytes, serum, plasma, cerebrospinal fluid, and urine.[8]

One of the most attractive features of HRP 2 for use in active ID is the clearance time compared to other parasite biomarkers. It generally takes from two weeks to one month after successful malaria treatment for HRP 2 tests to score negative.[9,10] HRP 2 persistence is often viewed as a negative attribute in control programs, where the levels of parasitemia and frequency of re-infection are high enough that HRP 2 levels do not correlate well with time since infection and severity of disease.[11] However, in an elimination context, particularly for active ID, HRP 2 persistence would seem to mitigate the effects of the short-term temporal changes in parasitemia that result from synchronized sequestration. [iii,3]

Most HRP 2 tests rely on the detection of the HRP 2 antigen in whole blood with a rapid dipstick antigen-capture assay, lateral flow strip, or ELISA.[12,13] Rapid diagnostic tests (RDTs) that use HRP 2 as the sole or as one of several (often two) target analytes have had a major impact in many low- and middle-income countries (LMIC). Such RDTs have enabled access to diagnostic testing at lower levels of the health care system, preventing overuse of antimalarials and under-treatment of other sources of fever.[14] A large number of trials have proven the high sensitivity and specificity of these tests, relative to smear microscopy and polymerase chain reaction (PCR) testing, in endemic regions as well as in non-immune travelers.[11,12,15-21]

However, with a limit of detection around 100p/ul, RDTs lack the performance required for active ID tactics.[iv] ELISA occupies an interesting "middle ground" between RDTs and more resource-intensive techniques like quantitative polymerase chain reaction (qPCR). In an ELISA format, it may be possible to use a larger initial sample volume (especially with some pre-processing) than with an RDT, thereby allowing for a lower limit of detection. ELISA is also a higher throughput method, making it more suitable for some elimination use-scenarios such as epidemiological surveys. ELISA is generally performed in a "batch" fashion, with multiple assays (and the required standards and controls) set up and run on a multi-well plate. When ELISA is used in a setting where throughput enables use of a full plate per run, the cost can be lower than RDTs, but if turnaround constraints dictate that partial plates are run, the cost can be higher than RDTs (break-even thresholds vary by individual assay). While ELISA is more complex than RDTs, ELISA is a technique that is widely used in LMIC in labs that are at a level of the health care system where some elimination surveillance will be done. ELISA, however, is significantly easier to perform, requires fewer

[ii] There is evidence that HRP-2 is involved in detoxifying free heme by its polymerization to inactive hemozoin[4], mediated by direct binding of heme to a hexapeptide repeat sequence that appears 33 times in HRP-2. The exact mechanism of polymerization is unclear, but it has been proposed that HRP-2 may facilitate the transport of hemoglobin to the food vacuole and catalyze the polymerization. Absorbance spectroscopy studies suggest that chloroquine—one of the most effective malaria treatments— is capable of extracting heme bound to HRP-2 by disrupting the heme–HRP-2 complex and replacing it with a complex that is toxic to the parasite.[5]

[iii] This hypothesis has not been definitively proven.

[iv] Quality of HRP-2 RDTs varies by manufacturer, but the best RDTs have sensitivities approaching 100% versus field microscopy,[12] ≥90% versus expert reference microscopy,[17,19,21] and ~90% versus PCR[16] (although the number of studies that make this comparison are low). HRP-2 RDT sensitivity declines rapidly as levels of parasitemia go down,[12] a direct result of the limit of detection of these tests—around 100 parasites/μL[11] — compared to typical microscopy and PCR at 10–100 parasites/μL[22] and 0.5–10 parasites/μL,[18,23] respectively. Specificity for HRP-2 RDTs is generally >95%, although there is some variation with study populations.[12,16,17]

capital resources, and is less expensive than qPCR. A properly designed qPCR should be a more accurate test than ELISA, but will still have limitations deriving from sample volume and pre-processing.

The aim of the remainder of this report is to assess the utility and opportunities for inclusion of HRP 2 ELISA to increase the efficiency of active ID tactics in malaria elimination.

## Landscape of technology solutions

The HRP 2 ELISA landscape has recently been dominated by Cellabs Malaria antigen (Ag) CELISA™, a sandwich assay based on commercially available monoclonal antibodies (MAbs) from Immunology Consulting Labs (ICL), and faster (Noedl) modification of the Malaria Ag CELISA.

### CELISA

The main commercialized assay, Malaria Ag CELISA (Cellabs, Sydney, New South Wales, Australia), has been extensively tested in the field by the Centers for Disease Control and Prevention (CDC), US Army Medical Component of the Armed Forces Research Institute of the Medical Sciences (USAMC-AFRIMS), and Harald Noedl of the University of Vienna.[24,25] Cellabs appears to be privately held since 1985. Their ELISA assay has received CE certification (a symbol of free marketability in Europe), and has been shown to have sensitivity and specificity comparable to qPCR in symptomatic outpatients.[25] Despite its excellent performance attributes, the Cellabs assay has been limited in its uptake due to the perception by some researchers that it is too expensive.[26] If the Cellabs CELISA is to have maximum impact, it must be available at a price point that is acceptable to most end-users. If additional research proves the price is actually too high, some form of a subsidy, some other incentive for Cellabs to reduce their margins, and/or another way to reduce costs through economies of scale could be required for widespread adoption. The primary advantage of working with Cellabs is that they have already commercialized their product.

### Noedl assay

In response to the perceived high cost of the CELISA assay, Harald Noedl and colleagues (funded by the Department of Defense Global Emerging Infections Surveillance and Response System (GEIS), the Austrian Science Fund Project No. 15754-B02, and the Medicines for Malaria Venture) developed a sandwich ELISA using commercially available monoclonal antibodies from Immunology Consultants Laboratories, Inc. (Newport, Oregon, USA). This assay, which is purported to cost only 20% of the Malaria Ag CELISA kit, has been shown to be similar in performance to the CELISA assay[26] and has subsequently been shown to be useful in several published studies in Cambodia[27,28] and in a larger network of surveillance labs (personal communication with Harald Noedl, July 2013). The work done by Noedl and colleagues is focused on drug susceptibility testing (DST). The protocol for such testing includes a 72-hour pre-culture of samples in a plate with several candidate drug treatments to enable the differentiation of relative susceptibility. While evidence suggests that this approach may be useful for active ID tactics,[v] the time-limiting pre-culture step seems to be a critical factor in that it undoubtedly improves the lower limit of detection but may not be practical for elimination use-scenarios where throughput and turn-around time are priorities. Also, the

[v] The no-drug control wells enable inferences about the assay's performance outside of the DST format.

criterion for judging the performance of these tests was DST-based[vi] and, therefore, is not the best measure of a possible elimination application.

Currently, the Noedl assay is a lab-developed "home-brew" assay that requires a significant investment in quality assurance/quality control (QA/QC) by the user to ensure its long-term performance. If the Noedl assay were produced as a commercial kit, the user variability inherent in the "home-brew" approach could be eliminated. Funding for commercialization could be made contingent on keeping margins to a sustainable minimum, ensuring access to performance data. This investment would possibly be larger and the timeline longer, however. It is also not certain that the end result would be any less costly to the user than CELISA. It is likely that the lower limits of detection (LOD) for this assay could be reduced even further for an elimination scenario, given that Noedl and colleagues actually traded off LOD to give the quantitation required of a DST (personal communication with Harald Noedl, July 2013).

**Walter Reed Army Institute of Research (WRAIR) assay**

A research group at WRAIR and the Kenya Medical Research Institute (KEMRI) (funded by the Malaria Vaccine Development Program, USAID, and University of Edinburgh) has chosen to use the Cellabs assay, but has modified the protocol to make it faster.[29,30] This approach has been shown to give a lower LOD and prevalence estimates similar to qPCR and superior to an lactate dehydrogenase (LDH) ELISA and microscopy.[vii,31] The work on the modified CELISA protocol at WRAIR was done to evaluate the utility of the assay for a vaccine monitoring program. In this case, the criterion of judgment was more appropriate for an elimination use-scenario, but the specimens evaluated were not.[viii] Nevertheless, the lower LOD inferred may be appropriate for some elimination use cases.[31]

**Standard Diagnostics (SD) assay**

The SD Malaria Pf Ag ELISA (Standard Diagnostics, Korea) is a commercially available HRP 2 assay, made using SD's proprietary antibodies (personal communication with Dr. Steve D. La, September 2013). It has not been as extensively characterized on clinical samples as the Cellabs CELISA. Standard Diagnostics was established with a Korean Ministry of Science and Technology grant in 1999, attracted Korean venture capital in 2000, and received project-based funding from a variety of Korean government agencies and private sources until it was acquired by Alere in 2010. Public statements suggest that SD/Alere is committed to supporting products for the global health market and malaria elimination (http://www.standardia.com/en/home/about.html).

There are a few additional ELISA technologies that could be applicable. There are also at least 12 companies offering antibodies (Abs) for research ELISA use (see Table 1). Depending on the properties of the Abs, one of these alternative Ab sources could represent a superior assay for an elimination setting—although this hypothesis is untested.

[vi] Acceptable performance was judged to be comparability of $IC_{50}$ to other reference DSTs.
[vii] Prevalence estimates: HRP-2 ELISA, 36.9% (CI 34.4–45.5%); qPCR, 39.9% (CI 31.4–42.4%); pLDH ELISA, 16.8% (CI 12.5–21.0%); microscopy, 13.4% (CI 9.6–17.3%). Estimates for qPCR and HRP-2 were not significantly different (P = 0.80), while both qPCR and HRP-2 ELISA estimates were significantly different than pLDH and microscopy (P<0.0001).
[viii] Performance claims were based mainly on contrived samples prepared by diluting recombinant antigen, cultured parasites, and a few clinical samples from symptomatic patients.

It is important to note that all the assays described here have neither been evaluated against standards nor with samples from populations with low-density, asymptomatic infections, based on our literature searches. Thus, we are uncertain how these tests will perform in an elimination setting. This lack of understanding will be discussed in greater detail in the gap analysis section. A summary of the ELISA assay principle, workflow, and optional formats is given in Appendix A.

**Table 1.** Comparison of technologies

| Name | Stage of product development (discovery, development, validation, commercialization, scale-up) | Quality of evidence | Sensitivity (%) | Specificity (%) | Limit of detection | Point-of-care (POC) compatible? | Throughput | Required training | Target analyte | Species specificity | Comments |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cellabs CELISA** | Commercial | 3.5 | 98.8% (95% CI: 93.6–100%)* | 100% (95% CI: 99.5–100%)* | No claim or test; advertised as 5–50 p/ul in brochure | No | ~ 40/3-hour Batch | Moderate | Histidine-rich protein 2 (HRP 2) | Plasmodium *Falciparum* (P*f*) | * vs. smear + polymerase chain reaction (PCR) |
| **Noedl Assay ICL MPFM- 45A (Commercial antibody (Ab)), MPFG-45P (R-Ab)** | Commercial (Ab), home-brew assay | 3.5 | Similar to CELISA | Similar to CELISA | 0.002% parasi-temia* | No | ~ 40/3-hour batch | High (ELISA set-up) | HRP 2 | P*f* | * With Drug susceptibility testing (DST) pre-culture |
| **Walter Reed Army Institute of Research (WRAIR)/ Waitumbe** | CELISA assay Standard operating procedure (SOP) modifications | 2 | No data | No data | ~4 ng/mL (serum) 5.8 infected red blood cells (iRBC)/µL = 6–200 ng/ml (whole blood) | No | 38/2-hour batch | Moderate | HRP 2 | P*f* | Mostly a combination of RPfHRP2 spikes and cultured samples |
| **Standard Diagnostics Malaria P*f* antigen (Ag) ELISA** | Commercial | 2.5 | 98% | 100% | No claim or test | No | 91 singletons*/ 4-hour batch | Moderate | HRP 2 | P*f* | Little detail in insert about 300 clinical samples tested *Insert does not specify number of sample replicates |
| **MyBiosource** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Monoclonal, raised against |

| | | | | | | | | | | | native Ag |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Name** | **Stage of product development (discovery, development, validation, commercialization, scale-up)** | **Quality of evidence** | **Sensitivity (%)** | **Specificity (%)** | **Limit of detection** | **POC compatible?** | **Throughput** | **Required training** | **Target analyte** | **Species specificity** | **Comments** |
| **GeneTex** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Monoclonal |
| **LSBio** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Monoclonal, raised against native Ag |
| **Creative Diagnostics** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Monoclonal, raised against native Ag |
| **Thermo-Fisher** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **Protein Tech Group** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **antibodies-online.com** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **Acris Antibodies** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **Aviva Systems Biology** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **Abbiotec** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **ProSci** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |
| **Santa Cruz Biotechnology** | Raw material research Abs | 0 | No data | No data | No data | No | No data | No data | No data | No data | Polyclonal |

## Gap analysis

The biggest limitation in the current ELISAs is a lack of data or understanding on how they will perform in elimination use-scenarios. Two domains of data require better characterization: (1) the lower LOD of these assays with real clinical samples, and (2) how the biological variability of the HRP 2 protein in different sample types and populations will affect the performance of the individual tests. Note that many of the gaps in understanding expanded on below apply to antigen (Ag)-detection RDTs as well as ELISAs, since they also rely on antibodies to HRP 2 for capture and detection.

LOD is a key indicator of the potential utility of an assay in an elimination use-scenario. Levels of *P. falciparum* parasitemia should produce correspondingly lower concentrations of circulating HRP 2 (although definitive research on the correlation between HRP 2 expression and low parasitemia has not been done). Given the current uses of these ELISAs (DST and vaccine efficacy testing), their lower LOD have not been well characterized or, at least, these data have not been publicized. Where LOD data is available, it has been generated with spiked recombinant protein or cultured reference clones[27,29] that do not adequately approximate wild-type variations.[ix] In addition, the impact of the biological variation in clinical samples on the performance of the individual antibodies used in different assays can have a large effect on true LOD, since it is the variation in the response of the negative and the sample near the LOD that determine this value.

**Variation across sample types**

Variation across sample types is primarily variability in detectable HRP 2 concentration. As noted by Kifude et al., "In a clinical sample, HRP 2 may either be (1) "free"; (2) bound by antibody in plasma (soluble or through large complexes); (3) inside infected red blood cells (iRBCs); (4) bound to uninfected RBCs as part of immune complexes via complement receptors on the surfaces of iRBCs; or (5) bound to other cells, such as leukocytes. Each of these HRP 2 blood compartments may have separate complex kinetics, which also further prevents reconciliation of the HRP 2 concentrations with the numbers of parasites in patients..."[29] This intrinsic variability can be further magnified by differences in the affinity and avidity of the particular antibodies used for different assays to Ag in different compartments, making standardization across assays challenging and a currently unmet need. For an elimination use-scenario, lysed whole blood may give the lowest LOD, due to the presence of all the compartments outlined by Kifude et al., but may also come with a trade-off in background response and variability that should be characterized.[29] There is also temporal variation in sample concentrations through synchronous sequestration and schizont rupture[3] that contribute to the components of variation, and must be better understood to inform any sampling instructions in a package insert or surveillance protocol. Given the approximate four-day half-life of HRP 2, this may not be a major issue, but explicit data should be

[ix] Characterization of an assay with consistent and standardized material is a very important part of manufacturing quality control in a commercial product, but it is not a substitute for similar data generated with clinical samples. Since there is an accepted gold-standard assay for the presence of parasites with suitably low LOD for comparison (qPCR), a panel of very low parasitemia samples should be evaluated to verify the LOD claims derived from synthetic material, following a consensus guideline; e.g., Clinical Laboratory Standards Institute protocol EP-17-A. (CLSI, *Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline*, CLSi document EP-17-A [ISBN 1-56238-551-8]. CLSI, 940 West Valley Road, Wayne, Pennsylvania 19087-1898, USA 2004). This step is especially important if the assay is re-optimized to achieve a lower LOD. Care must be taken in assembling this panel to insure that qPCR false negatives resulting from synchronous sequestration do not confound the results.

generated to test this hypothesis; i.e., the 1997 study by Farnert et al.[3] should be repeated with an HRP 2 ELISA performed along with the microscopic evaluation and nested PCR.

**Variation across populations**

Variation across population comes primarily from variations in the structure of the HRP 2 proteins associated with different clones of the parasite. Despite many recent investigations on that variability, however, it is still not well understood how that variation affects binding of the particular antibodies used in different tests. As a result, it is not clear which MAbs are most sensitive for active infections in populations where this variation is displayed. It has been shown that polymorphism in the repeated sequence motifs of HRP 2 can result in variation in the number of antibodies bound to the protein[32,33] but how that affects the final performance of an assay depends on how that assay is formatted and optimized. Individual incidences of discordance between two antibody assays supposedly both sensitive and specific to HRP 2[25] is circumstantial evidence that there are significant differences in HRP 2 recognition by different antibody clones in some clinical samples; however, no systematic correlation has been found between polymorphisms and performance of RDTs by the World Health Organization (WHO) criteria.[34] If differences in HRP 2 recognition do prove to be important, they may become magnified and more problematic as the parasite clonal diversity in individual infections is reduced in the lower transmission populations that indicate an approach to elimination. Certain populations of *P. falciparum* may not be identifiable by HRP 2 immunoassays that have limited avidity for the HRP 2 produced by certain clones. In a "diagnose and treat" scenario that relies on such diagnostic tests, the less-detectable phenotypes may be selected for.[35] Although this hypothesis rests on several as-yet-untested assumptions about the origins of the HRP 2 polymorphism and the selective strength of the different phenotypes, it is advisable to consider the possibility.

Even more problematic are populations that display deletions of the HRP 2 gene. The frequency of this deletion varies widely with region and population. It may be reactively infrequent, like the 2.4% prevalence observed in Dakar, Senegal,[36] to very frequent, like the 25.7%[37] to 41% in deletion rates observed in Peru.[38] Where the HRP 2 gene is absent, it can greatly reduce the efficacy of HRP 2 based diagnostic tests; e.g., in one study, in Mali, approximately 50% of RDT false negatives could be attributed to HRP 2 gene deletions.[39] In populations where this deletion is common, tests that are specific to HRP 2 will be inaccurate in proportion with the frequency of the deletion, and no amount of binding optimization will alleviate that if the analyte is not universally present.

The HRP 2 protein has considerable biological variation across populations and sample types that cannot be avoided solely through assay design; this variation will affect the outcome of an ELISA (or Ag detection RDT). Acknowledging that there is a paucity of information available on important assay features such as the binding affinity and avidity of MAbs to their target epitopes, and their durability to heat, Lee et al. recently[40] mapped the epitopes recognized by eight HRP 2-specific MAbs commonly used in RDTs. Parasites from geographically diverse regions were assessed, and MAb stability was also evaluated. The results suggest that certain antibodies have defined characteristics that make them more desirable for immune-assay detection schemes. While none of the MAbs tested mapped well to the "ideal" amino acid motifs identified, the C1-13 MAb demonstrated the highest all-around versatility, followed closely by 2G12 and PTL-3.

**Elimination populations**

If the HRP 2 ELISA is to become a useful tool for malaria elimination, a significant investment should be made in understanding how the different candidate ELISAs perform head-to-head in a wide variety of populations displaying the biological variability discussed above. The CELISA and Noedl home-brew ELISA assays have been reasonably well characterized in a few populations in Southeast Asia and Kenya, but not in South America, where HRP 2 variation appears to be highest, or in any other populations. The SD assay and the commercially available raw materials have not been comprehensively characterized with clinical samples at all. A head-to-head comparison should focus on samples that have low levels of parasitemia to challenge the lower limits of detection of the candidates, even if dilution of the samples is required. Challenging the candidates near the LOD will determine early on if the ELISA can be used immediately on samples after they have been collected, or if a pre-culture step (as is done with drug susceptibility testing) is required to get adequate sensitivity in a low-parasitemia, elimination setting. To assess analytical specificity, the study should be done with a population that contains typical comorbid infections.

The number of candidates to include in the head-to-head is a trade-off between a desire to find viable assays for all populations and the additional costs of including less-well-characterized candidates. At a minimum, the CELISA, the Noedl assay, and the SD assay should be considered. Each sample should also be tested by the consensus gold standard, currently nested or qPCR, to enable calculation of sensitivity, specificity, positive predictive value, and negative predictive value. Given that qPCR is capable of testing only for the presence of parasite in blood while HRP 2 ELISA should be capable of detecting sequestered parasites, false positives by the index test that are actually false negatives by the reference test will be expected. For the most meaningful results, a composite reference method that better represents true diagnostic accuracy may be required—although the appropriate composite reference has yet to be defined.

Although there has been considerable work done already on characterizing the global distribution of HRP 2 polymorphisms and deletions, and how these effect RDTs,[32,34,36,38,40-42] one of the challenges of qualifying an HRP 2 ELISA for elimination will be in properly defining the test population. We are faced with a "chicken and egg" dilemma—in order to understand the antibodies, you need a well-characterized population, but in order to understand the antigen distribution in the population, you need a well-characterized set of immunoassays. An orthogonal technique, like qPCR, can help resolve some of the immune response related issues, but is limited by sequestration and sample size, as discussed above. Genetic analysis of the parasites (sequencing, fingerprint mapping) may give you richer information on the parasite genotypes involved but are not useful for evaluating an ELISA unless results can be mapped back to the immunoassay performance.

## Investment opportunity

The suggested investments can be categorized into the typical sequence of product development activities:

- Discovery: The commercially available assays have already passed this phase. If novel antibodies are considered as candidates, they will require some degree of assay design (determining

concentrations, buffers, assay protocols, etc.). Any activities directed at better understanding the test populations and global distribution of HRP 2 polymorphisms may also fall into this phase.

- Develop/validate: A head-to-head comparison with clinical samples from populations spanning the spectrum of global diversity will validate which assays can be used for which populations and generate the kind of data that supports downstream activities. Some classical product development may be needed for the less-characterized materials or assays to adapt them to the target product profiles (TPPs) of an elimination scenario (e.g., if culture is required to reach the required LOD). It is also advisable that a small panel of standards be formulated for this work to insure that all candidate assays are evaluated against the same material in every run, and not against a manufacturer's control that may not be representative of real specimens. Ideally, a neutral third party (such as National Institute of Standards and Technology [NIST], WHO, Foundation for Innovative New Diagnostics [FiND], or PATH) would prepare and certify these materials. Validation should be performed with an eye to regulatory requirements.
- Regulatory/policy: Once a candidate is validated for a particular population, advocacy and appropriate regulatory filings may be undertaken to insure that the product is accepted by the target market. Additional studies may be required at this stage to meet requirements in particular settings. If a candidate assay has not been commercialized, a technology transfer to a commercial manufacturer will be required. Some validation activities will need to be repeated after technology transfer to a commercial manufacturer.
- Introduction: It will be important at this stage (ideally, long before this stage) to differentiate the use-scenarios where this technology can have the most benefit to elimination programs. Working with early adopters of new technology, demonstration projects will be critical here to build the evidence base.
- Scale up: In order to achieve scale, it will be important that the manufacturers of the candidates understand the market size and supply chain and are prepared to supply the assays to the end users without stockouts. This may require capacity-building for some of the smaller manufacturers currently making these tests.

Commercialization and scaling can be very expensive endeavors. A single commercialized product may be adequate and thus investment might be focused on that ELISA and kept to a minimum. However, it may be the case that multiple products are required to account for the regional genetic diversity of *P. falciparum*, in which case, investment would be larger. However, if the market is well defined and sufficiently large, manufacturers may be willing to assume a significant fraction of the cost. It should be noted that SD is a major supplier of malaria RDTs and that some degree of market forecasting would be appropriate to estimate whether an ELISA test would be a benefit to their business interests or would dilute the RDT market.

## Conclusion

HRP 2 is a marker specific to *P. falciparum,* so by definition HRP 2 detection technologies cannot address the speciation component of a differential diagnosis. ELISA is also not very amenable to point-of-care application nor rapid turn-around. Therefore, technologies reviewed in this report may represent a niche market within the overall elimination diagnostics market. Nonetheless, this analyte and format have some unique advantages, particularly with regard to identification of infection despite low or sequestered parasitemia, that may make it impactful in elimination use-scenarios.

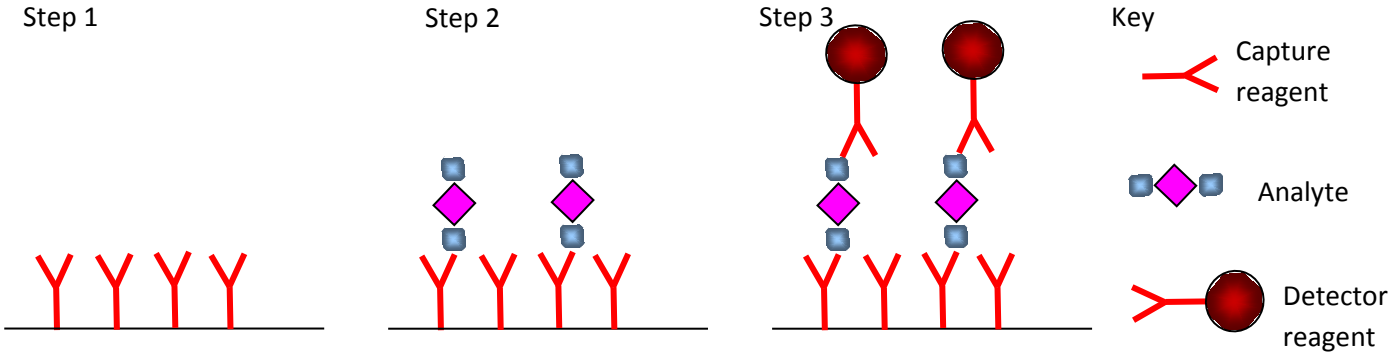
Before advancing HRP 2 ELISA as a useful tool for elimination use, however, any investment in HRP 2 ELISA technologies should include closing of the gaps discussed above. Specifically, the LOD must be better characterized on representative populations, performance of the ELISA should be mapped to the genetic diversity of *Pf* epitopes, and the ability of HRP 2 ELISA to identify sequestered parasites should be verified.

As elimination is approached regionally, the HRP 2 ELISA may be a useful tool in active ID use-scenarios such as MSAT, FSAT, and reactive ID. These use-scenarios favor higher throughput and a lower LOD than is usually realized by RDTs, and aim to identify all infections, including those that are subclinical, subpatent, and/or sequestered.

# Appendix A: Antigen detection ELISA

These assay formats focus on the detection of the antigen (HRP 2) in a sample by using its specific cognate antibody. There are three different formats that could potentially be used (sandwich, competitive, and inhibition); however, the extant assays discussed in the text are all sandwich assays. The sample is usually serum or plasma (or in the case of the drug susceptibility assays, culture media), and is introduced to the solid phase (functionalized 96-well plate, or similar) in a mobile phase that retains the native protein conformation. Typically, there are wash steps in between the steps shown below to eliminate possible interfering substances. Result readout depends on the signal produced by the detector (color, fluorescence, etc.) but requires a reader instrument for precise quantitation.

**Figure 1.** Sandwich assay.[43]

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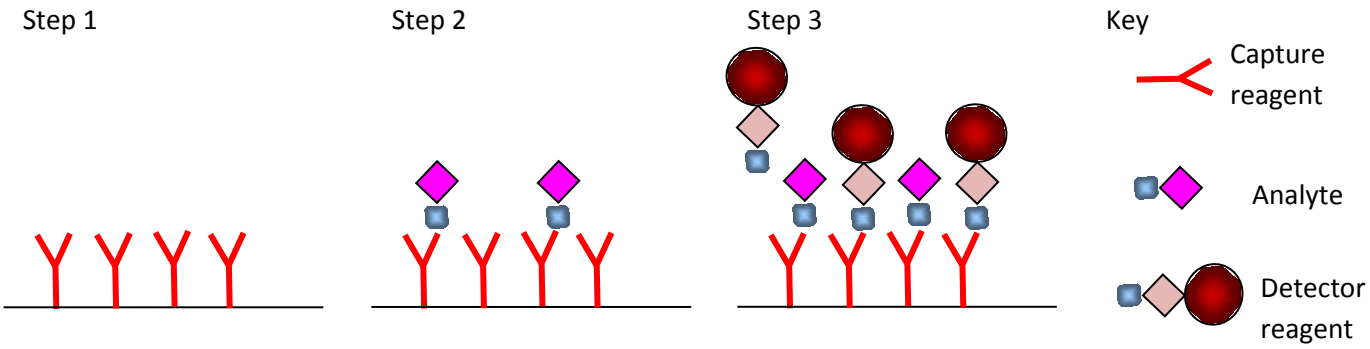
Step 1: A capture reagent specific to the analyte is immobilized on the solid phase. Step 2: The sample is introduced through the mobile phase. If the analyte is present in the sample, it will bind to the capture reagent on the solid phase through its cognate binding site. Step 3: The detector reagent that is specific to the analyte is introduced through the mobile phase. If the analyte is present in the sample, the detector reagent will specifically bind to it, forming a sandwich and resulting in a signal. If no analyte is present, the detector reagent has no place to bind and is washed away. This format works well when the analyte has at least two distinct epitopes or multiple repeats of the same epitope, like HRP 2.

**Figure 2.** Competitive assay. [43]

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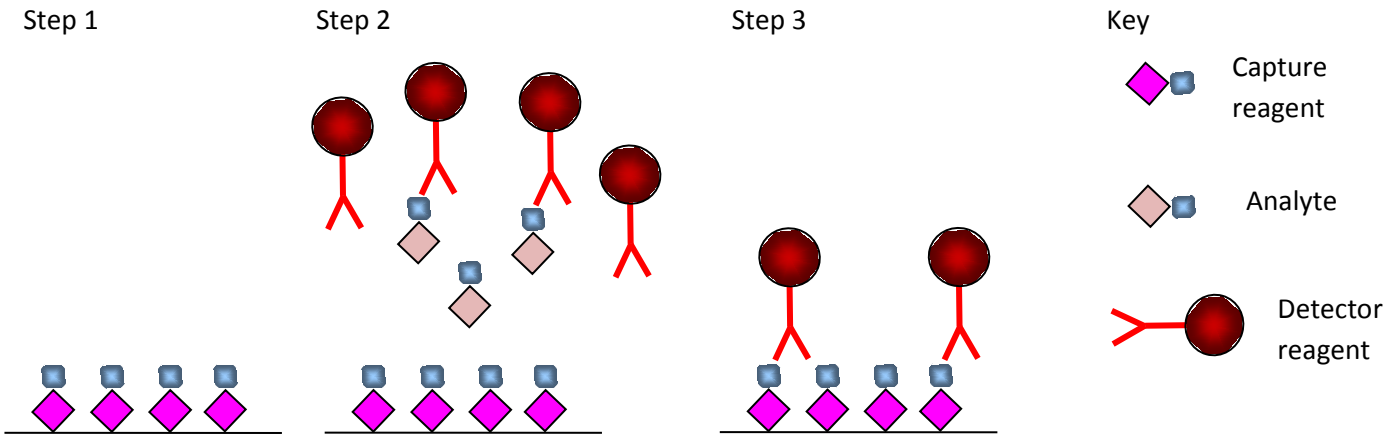
Step 1: A capture reagent specific to the analyte is immobilized on the solid phase. Step 2: The sample is introduced through the mobile phase. If the analyte is present in the sample, it will bind to the capture reagent on the solid phase through its cognate binding site. Step 3: The detector reagent is introduced through the mobile phase. In this case, the detector reagent presents an epitope analogous to that of the analyte. If the analyte is present in the sample it will compete with the detector reagent and no signal (or a reduced signal) will be present. If no analyte is present, the capture reagent is available to bind the detector reagent, resulting in a maximal signal. Interpretation of this format is somewhat counterintuitive since the analytical signal is greatest when the analyte is not present in the sample. This format is well suited for analytes with only one epitope, such as a small protein or xenobiotic.

**Figure 3.** Inhibition assay.[43]

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Step 1: A capture reagent analogous to that of the analyte is immobilized on the solid phase. Step 2: The detector reagent is specific to the analyte. The sample is premixed with the detector reagent prior to introduction to the solid phase. If the analyte is present in the sample, it will bind to the capture reagent on the solid phase through its cognate binding site. Step 3: If the sample contains the analyte, it will bind to the detector reagent and inhibit its ability to be captured on the solid phase, resulting in no signal or a reduced signal. If no analyte is present in the sample, the detector reagent is available to bind to the capture reagent on the solid phase resulting in a maximal signal. Similar to the competitive assay, interpretation of this format is somewhat counterintuitive since the analytical signal is greatest when the analyte is not present in the sample. This format is well suited for analytes with only one epitope, such as a small protein or xenobiotic.

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