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RAPID DIAGNOSTIC TESTS FOR RESOURCE-POOR AREAS

Teppo Salminen

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To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (**I-IV**).

I Salminen T, Juntunen E, Khanna N, Pettersson K, Talha SM. Anti-HCV immunoassays based on a multiepitope antigen and fluorescent lanthanide chelate reporters. *J Virol Methods.* **228**:67-73. (2016)

II Salminen T, Knuutila A, Barkoff AM, Mertsola J, He Q. A rapid lateral flow immunoassay for serological diagnosis of pertussis. *Vaccine* **36**:1429-1434. (2018)

III Salminen T, Juntunen E, Talha SM, Pettersson K. High-sensitivity lateral flow immunoassay with a fluorescent lanthanide nanoparticle label. *J Immunol Methods*. **465:**39-44. (2019)

IV Salminen T, Rohila D, Kumar M, Talha SM, Pettersson K, Khanna N, Batra G. Up-converting nanophosphor based ultra-sensitive point-of-care immunoassay for the detection Plasmodium falciparum malaria [manuscript]

ABBREVIATIONS

2DPN	2-dimensional paper network
ACT	Artemisin-based combination therapy
AI	Avidity index
ASSURED	Affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, delivered
ATPS	Aqueous two-phase system
DAA	Directly acting antiviral
DALY	Disability-adjusted life year
DHFR-TS	Dihydrofolate reductase-thymidylate synthase
DTP3	Diphteria-tetanus-pertussis vaccine
EDC	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
EIA	Enzyme immunoassay
EIR	Entomological inoculation rate
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous hemagglutinin
FIND	The Foundation For Innovative New Diagnostics
fPSA	Free prostate specific antigen
G6PD	Glucose 6-phosphate dehydrogenase
GLURP	Glutamate rich protein
HCV	Hepatitis C virus
HCVcAg	Hepatitis C virus core antigen
HDP	Heme detoxification protein
HIV	Human Immunodeficiency virus
HRP2	P. falciparum histidine rich protein 2
HRP3	P. falciparum histidine rich protein 3
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ITP	Isotachophoresis
LDH	Lactate dehydrogenase
LF	Lateral flow
LFIA	Lateral flow immunoassay
M+2-NTA	Metal ion nitrilotriacetic acid
MDA	Mass drug administration
MEP	Multiepitope protein
MSAT	Mass-screen and treat

$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NAT	Nucleic acid test
NHS	N-hydroxysuccinimide
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pLDH	*Plasmodium* lactate dehydrogenase
PSA	Prostate specific antigen
PT	Pertussis toxin
RDT	Rapid diagnostic test
RNA	Ribonucleic acid
UCNP	Up-converting nanophosphor
UN	United Nations
UV	Ultraviolet
WHO	World Health Organization

ABSTRACT

Rapid diagnostic tests, such as lateral flow immunoassays may enable diagnostics in resource poor areas, thus improving health outcomes by helping to control and eliminate infectious diseases. This thesis focuses on the development of sensitive tools for the rapid diagnosis of three infectious diseases prevalent in resource poor areas: hepatitis C, pertussis and malaria. For malaria and hepatitis C, rapid diagnostic tests with improved sensitivity could help in ongoing efforts to eliminate the diseases. For pertussis (whooping cough), a field-usable rapid serodiagnostic test would help in disease surveillance and control.

New methods to enhance the sensitivity of lateral flow immunoassays include pre-concentrating the sample before the immunoassay takes place, controlling the flow of reagents in the lateral flow strip to allow more complex assays and using high-sensitivity luminescent instrument-read labels. All the above methods involve trade-offs between the sensitivity, complexity, and affordability of the test.

In publication **I** of this thesis, immunoassays for anti-HCV antibodies were developed using a single multiepitope protein antigen and a luminescent europium-chelate label. In **II** and **III**, quantitative lateral flow immunoassays utilizing luminescent europium nanoparticle labels were developed and assessed. The results obtained with patient samples using the lateral flow immunoassay for anti-pertussis antibodies (**II**) correlated well with a traditional enzyme immunoassay. In **IV**, an higly sensitive lateral flow immunoassay utilizing up-converting nanophosphor labels was developed for *Plasmodium falciparum* infection i.e. malaria, and the test performance was evaluated with *P. falciparum* culture samples. The analytical sensitivity of *P. falciparum* detection was improved up to 250-fold as compared to a standard lateral flow test.

The results of the publications included in this thesis show that the use of intrument-read luminescent labels in rapid lateral flow immunoassays allows the development of highly sensitive and quantitative point-of-care tests, which could be used in-resource poor areas. Particularly, an ultrasensitive test for the detection of *P. falciparum* could detect asymptomatic carriers of the malaria parasite and thus support malaria elimination efforts.

TIIVISTELMÄ

Pikadiagnostiikka-testit, kuten lateraalivirtaus-immunomääritykset mahdollistavat diagnostiikan käytön köyhillä ja eristyneillä alueilla, mikä auttaa kontrolloimaan ja hävittämään infektiotauteja. Tämä väitöskirja käsittelee kolmen infektiotaudin pikadiagnostiikkaa: hepatiitti C , hinkuyskä ja malaria. Herkemmät pikadiagnostiikka-testit erityisesti malarialoiselle ja hepatiitti C –infektioille voisivat auttaa yrityksissä hävittää nämä taudit. Lisäksi vieritestaukseen soveltuva pikatesti hinkuyskälle auttaisi taudin valvonnassa ja helpottaisi taudin leviämisen estämistä.

Lateraalivirtaus-immunomääritysten herkkyyden parantamiseksi on kehitetty useita menetelmiä. Näihin kuuluvat näytteen konsentrointi ennen testin suorittamista, määrityksen komponenttien virtauksen kontrollointi ja luminoivien mittalaitteilla luettavien leimojen käyttö. Kaikki nämä metodit edellyttävät kompromisseja kehitetyn testin herkkyyden, helppokäyttöisyyden ja edullisuuden välillä.

Väitöskirjatyön ensimmäisessä julkaisussa kehitettiin immunomäärityksiä HCV vasta-aineille käyttäen multiepitooppiproteiini-antigeenia ja luminoivaa europium-kelaattileimaa. Toisessa ja kolmannessa julkaisussa kehitettiin luminoivia europium-nanopartikkeleita käyttäviä kvantitatiivisia lateraalivirtaus-immunomäärityksiä. Toisessa julkaisussa kehitetty hinkuyskän vasta-aineita tunnistavaa lateraalivirtaus-immunomääritys korreloi hyvin potilasnäytteillä perinteisen entsyymi-immuno-määrityksen kanssa. Neljännessä julkaisussa kehitettiin herkkä käänteisviritteisiä nanopartikkelileimoja käyttävä lateraalivirtaus-immunomääritys *Plasmodium falciparum* malarialoisen havaitsemiseksi verestä. Kehitetty testi oli noin 250 kertaa herkempi havaitsemaan malarialoisia kuin aikaisemmin käytetyt lateraalivirtaustestit.

Tämän väitöskirjatyön tulokset osoittavat, että käyttämällä mittalaitteilla luettavia leimoja lateraalivirtaus-immunomäärityksissä voidaan kehittää erittäin herkkiä kvantitatiivisia pikatestejä resurssiköyhille alueille. Erittäin herkkää malaria-pikatestiä voitaisiin erityisesti käyttää havaitsemaan myös malarialoisen oireettomat kantajat ja täten tukemaan malarian hävittämisohjelmia.

1 INTRODUCTION

Infectious diseases are a significant burden to the health care systems, especially in the poorest countries. In vitro diagnostics can help lower the burden by guiding correct treatment for individual patients and by helping to map and control the spread of infections. However, in resource-poor areas access to reliable diagnostic testing is very limited and lack of access to diagnostic tests impedes health improvement.[1]

From the diagnostics point of view, resource-poor areas or settings can be defined as areas with little to no access to healthcare resources, laboratory diagnostics in particular. Resource-poor settings exist in many countries, for example in remote areas or disaster zones where the normal healthcare system cannot function. However, the most extensive resource-poor settings are found in countries that do not have a developed healthcare system with comprehensive access to central clinical chemistry laboratories due to country-wide lack of resources[2]. The World Bank divides countries into low, lower-middle, upper-middle and high income groups, based on gross national income[3]. Most often the countries with significant resource-poor settings are in the low or lower-middle income groups. I will use these income groups, instead of the ill-defined term developing countries, throughout this thesis.

The lateral flow (LF) immunoassay (LFIA) can provide simple and accurate infectious disease diagnostics of at the point-of-care with low cost[4]. The structure and function of a typical LFIA is presented in **Figure 1**. The first LFIAs for infectious disease detection in low-income countries were developed for HIV, tuberculosis and hepatitis B[5]. Later, tests important for low-income countries have been developed for infectious diseases such as influenza[6], meningitis[7], chlamydia[8], ebola[9], syphilis[10], hepatitis C[11], and perhaps most importantly, malaria[12].

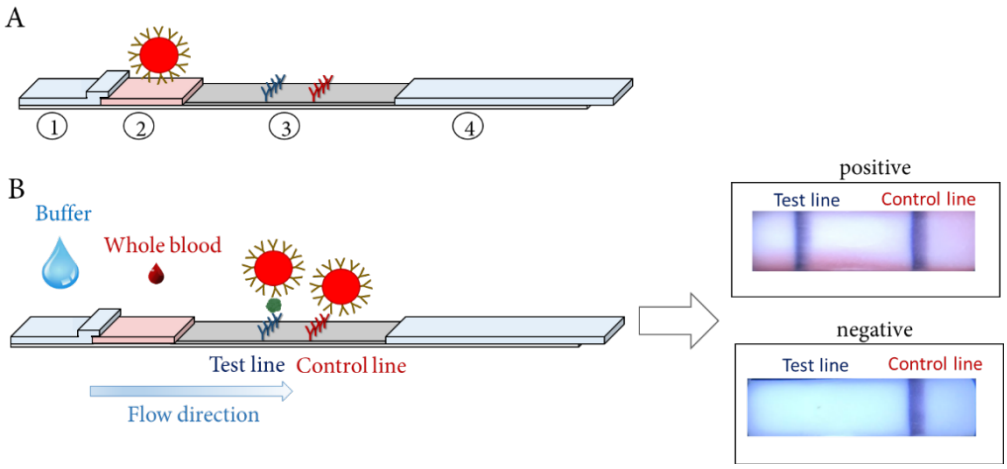

Figure 1. The structure and function of a typical lateral flow immunoassay. (A) The structure of a lateral flow immunoassay strip. The setup presented here is used for malaria assays and consists of (1) buffer pad for applying running buffer, (2) conjugate/sample pad where the label conjugate is dried and the whole blood sample is added, (3) analytical membrane with the capture antibody immobilized on the test line and a secondary antibody immobilized on the control line, (4) absorbent pad. (B) When the assay is run, the blood sample is added to the sample pad and buffer to the buffer pad. The liquids then flow with the reconstituted label conjugate by capillary action through the porous nitrocellulose analytical membrane. A sandwich of label conjugate, analyte and capture antibody forms on the test line and unbound label conjugate is bound on the control line, forming visible lines. A visible signal on the test line means the test is positive, while all valid tests should have a visible signal from the control line. The absorbent pad absorbs excess liquids, which maintains the capillary flow.

While the term LFIA is well defined, the term rapid diagnostic test (RDT) is more ambiguous. The EU defines RDTs as being in vitro diagnostic devices, which involve non-automated procedures and give a fast result[13]. In malaria diagnostics, RDT refers to diagnostic tests that are inexpensive, require minimal instrumentation, and are easy and quick to perform with minimal training. Typically, malaria RDTs use the LFIA format and indeed the terms LF test, LFIA and RDT are used interchangeably. In other contexts, the term RDT is used for tests with a short turnaround time but requiring laboratory equipment, as opposed to true point-of-care tests[14]. In this thesis, the term RDT is used to refer to tests that are LFIAs or are similar to LFIAs, where the heterogenous sandwich immunoassay takes place in a membrane with minimal liquid handling by the operator and the test is aimed to be usable in resource-poor locations.

This thesis discusses the development of improved rapid tests for three of the infectious diseases: hepatitis C, pertussis and *P. falciparum* malaria. The sensitivity of these RDTs

is improved, particularly through the use of instrument-read luminescent labels. In the literature review I will discuss other methods, which can be used to improve the sensitivity of RDTs, and the current state of rapid diagnostics for the three diseases.

2 REVIEW OF THE LITERATURE

2.1 Malaria diagnostics

According to the latest World Health Organization (WHO) world malaria report, in 2016 there were 216 million cases of malaria and 445 000 deaths globally[15]. After years of decreasing morbidity and mortality, the progress in malaria elimination seems to have stalled. Improvements in malaria diagnostic technologies would help combat malaria, both by improving the outcomes of individual patients as well as by supporting efforts to eradicate malaria.

The malaria parasite, genus *Plasmodium*, is transmitted by mosquitoes of the genus *Anopheles*. The sexual reproduction of the parasite takes place in the gut of the mosquito. An infected mosquito transmits sporozoite forms of the parasite to the bloodstream during blood meal (**Figure 2**). The sporozoites invade hepatocytes starting a liver stage, during which merozoites are produced. Some *P. vivax* and *P. ovale* parasites remain dormant and undetectable in the liver as hypnozoites, causing delayed relapse of malaria[16, 17]. The merozoites then infect red blood cells, reproduce asexually, induce cell rupture and infect more red blood cells (blood stage), and in parallel a portion of parasites develop into gametocytes that can infect a new mosquito. Symptoms of malaria start when the merozoites reach roughly 50 parasites / µl of blood, which corresponds to approximately 100 million parasites in blood circulation[18].

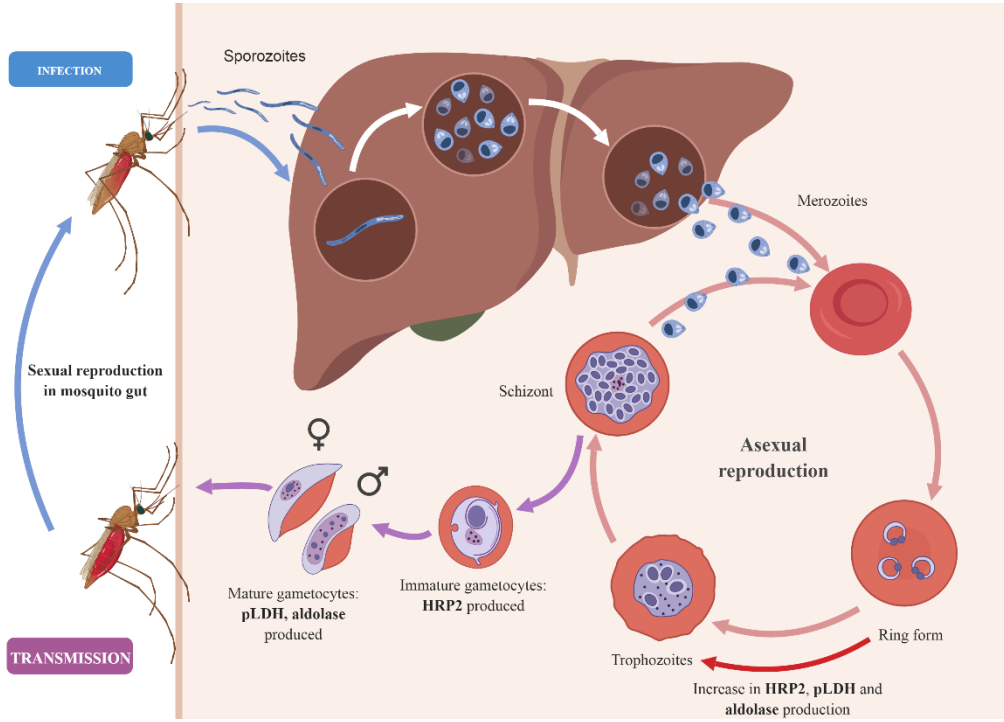

Figure 2. Life-cycle of the malaria parasite. The life cycle includes transmission between *Anopheles* mosquitoes and humans as well as life cycles inside both hosts. The life cycle in the human host starts with the injection of sporozoites into the dermis by a feeding mosquite. The sporozoites migrate to the liver and infect hepatocytes. The infected hepatocytes release merozoites into the blood circulation, where they infect red blood cells and start the asexual reproduction cycle. Some infected red blood cells produce gametocytes, which can be ingested by a feeding mosquito. During the asexual repdoction cycle, the production of the main antigen markers of *P. falciparum*, histidine rich protein 2 (HRP2), *Plasmodium* lactate dehydrogenase (pLDH) and aldolase increase as the ring form matures into a trophozoite. Immature *P. falciparum* gametocytes also contain HRP2, while pLDH and aldolase is found in mature gametocytes[19]. (Figure created with Biorender.com)

In total, five species of the genus *Plasmodium* are known to infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Of these *P. falciparum* and *P. vivax* are the most common causes of malaria. Also, monkey-infecting *P. knowlesi* present in Southeast Asia occasionally infects humans[20].

P. falciparum causes the most severe form of malaria and accounts for almost all deaths from malaria. It is most prevalent in Africa and is limited to tropical regions[21]. *P. vivax* is found in parts of Africa, Asia and Latin America and is not as climatically constrained[22]. In fact, *P. vivax* was probably endemic even in parts of Finland up to the beginning of the twentieth century[23]. In Asia and Latin America, *P. falciparum* and *P.*

vivax have roughly equal prevalence, while the total malaria prevalence is low compared with sub-Saharan Africa[22].

Malaria endemic areas can be categorized by transmission intensity and stability of transmission. In high transmission-intensity areas, sub-Saharan Africa and parts of Oceania, the entomological inoculation rate (EIR, the number of infectious mosquito bites per person per year[24]) can reach 1000/year[25]. In these areas, malaria-associated deaths occur most often in childhood, whereas infections in adults are commonly asymptomatic due to immunity[26]. In low transmission intensity areas, the EIR is typically below one. Stable transmission means constant year-round infections, while unstable transmission areas have intense transmission only during the rainy season. Low transmission intensity and unstable transmission areas can suffer from malaria epidemics when conditions change, and in such occasions symptomatic and severe malaria occurs in all age classes[18]. The transmission characteristics in different areas are important considerations when planning the use of diagnostics, either for routine diagnosis or supporting an eradication campaign.

Historically, direct observation of stained parasites from blood by microscopy served as the "gold standard" for diagnostic testing of malaria. Microscopy does not require electricity and the cost per test is very low. However, testing is time-consuming and accurate diagnosis requires extensive training of the microscopist. In laboratory conditions with a trained microscopist the detection limit is 4 – 20 parasites / µl, while in field conditions the detection limit is often 50 – 100 parasites / µl[27].

RDTs, in practice synonymous with LFIAs, have been used for malaria diagnosis since the 1990s[28]. The shift to the more expensive artemisin-based combination therapies has sharply increased the use of RDTs with to minimize the wasteful use of medication[29]. Increasingly, RDTs and other point-of-care diagnostic methods are also helping with malaria eradication efforts[30].

2.1.1 RDTs for malaria HRP2 antigen

The widely used RDTs for malaria diagnosis share the same basic technology of LFIA with visual labels[12]. The most significant differences between the RDTs are in the target antigens. By far the most important target antigens are *P. falciparum* histidine rich protein 2 (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH).

HRP2 is only produced by *P. falciparum* and as the name suggests, the amino acid composition is 35 % histidine with multiple repeating AHHAAD, AHH and AHHAA

epitopes. These allow multiple binding of the same paratope to the same antigen, for example when using an IgM binder. This in turn increases the avidity of the binding in an immunoassay, thus increasing the sensitivity[31, 32]. HRP2 is produced inside erythrocytes, but is released to the bloodstream upon cell rupture. Since HRP2 is very stable in blood, with an approximate half-life of 3 – 4 days, its concentration is not affected by the life-cycle of the parasite. This enables detection of HRP2 even when the parasites are sequestered into organs and non-detectable by microscopy[33]. On the other hand, the long half-life of HRP2 makes it useless for evaluating the response to anti-malaria therapy. In fact, HRP2 RDTs can lead to false positive results from patients that have recently cleared the parasite[34]. The persistence of HRP2 in blood leads to false-positive results with standard HRP2 RDTs for up to 4 – 5 weeks after microscopy-confirmed parasite clearance[35, 36]. Interestingly, HRP2 has even been detected from 5,200 year-old Egyptian mummies[37].

The use of HRP2 as a diagnostic antigen is further challenged by the fact that *P. falciparum* strains lacking the *pfhrp2* circulate in Africa, South America and Asia[38]. These strains cause false negative results with tests that rely solely on HRP2 as the diagnostic antigen[39]. There might even be positive selection favoring deletions in regions where diagnosis and treatment rely solely on HRP2 RDTs[40]. *P. falciparum* also produces HRP3, which a high sequence homology with HRP2 and is recognized by some antibodies used in HRP2 RDTs[41]. However, there are strains with concurrent loss of both HRP2 and HRP3[42]. All in all, to minimize the risk of false negatives due to mutated strains, the RDTs should comprise an additional antigen to HRP2, especially in the areas where strains with HRP2 deletions circulate.

Some studies have also suggested that the different number of epitope repeats of different HRP2 types may influence RDT performance[43]. For example, Jimenez et al. found that four out of five tested RDTs recognized the cultured PH1 strain (type C) at lower concentrations than either the Benin I (type A) or Santa Lucia (type B) strains[44] However, Baker et al. did not find a correlation between HRP2 type and RDT performance in an extensive study of highly polymorphic *P. falciparum* samples with the parasite concentrations equalized to 200 parasites / µl. Altogether, the effect of HRP2 types on RDT performance is currently considered to be insignificant[12, 41].

Quantitative detection of HRP2

Typically, malaria RDTs with visual labels are designed to give qualitative results of the presence or absence of infection. Is there a need for quantitative HRP2 RDTs? Generally, a quantitative immunoassay for an infectious disease can be useful for monitoring the

response to treatment, assessing disease severity or improving specificity in high-prevalence areas. The persistence of HRP2 in blood makes treatment response monitoring impractical using this antigen, but potentially useful for the other two quantitative applications. Even though there is only a limited correlation between parasitemia and HRP2 level in blood[45], there seems to be a stronger correlation with the severity of infection and disease progression to severe malaria[46]. In fact, it seems that plasma HRP2 concentrations distinguish patients with severe malaria from patients with another severe febrile illness better than quantifying parasitemia by microscopy[47]. A possible explanation is that the number of sequestered parasites, invisible to microscopy yet producing HRP2, are important in severe malaria[48].

In general, quantitative assessment of HRP2 levels is done with immunoassays in central laboratories, and a quantitative RDT could bring the benefits to the point-of-care. Sinha et al. showed that even the traditional RDTs can be used in a semi-quantitative fashion by testing several dilutions of the same sample, and either quantifying the absorbance with a scanner or estimating the line intensity visually[49]. However, this approach has limitations: running multiple dilutions from the sample complicates the test and the dynamic range is narrow. Therefore, a simple to perform, yet truly quantitative HRP2 RDT, would be useful in areas where severe malaria is common.

Clinical and analytical sensitivities of HRP2 RDTs

The latest product testing programme by WHO and FIND for malaria RDTs evaluated the clinical sensitivity, specificity and other characteristics of a total of 202 RDTs in the market. Of the evaluated tests, 32 % detected only *P. falciparum* and 76 % both *P. falciparum* and non-*P. falciparum* malaria, with one test detecting only *P. vivax*. The sensitivity evaluation was done with a panel of malaria samples diluted to densities of 200 and 2000 parasites/µl. The sensitivity of both *P. falciparum* and *P. vivax* detecting test lines is presented in **Figure 3** for 200 parasites/µl dilutions of the wild type samples. It must be noted that many RDTs detect *P. falciparum* also with an anti-pLDH test line, so in the case of RDTs with combined HRP2 and pLDH detection, the reported clinical sensitivity is a combination of HRP2 and pLDH detection.[50]

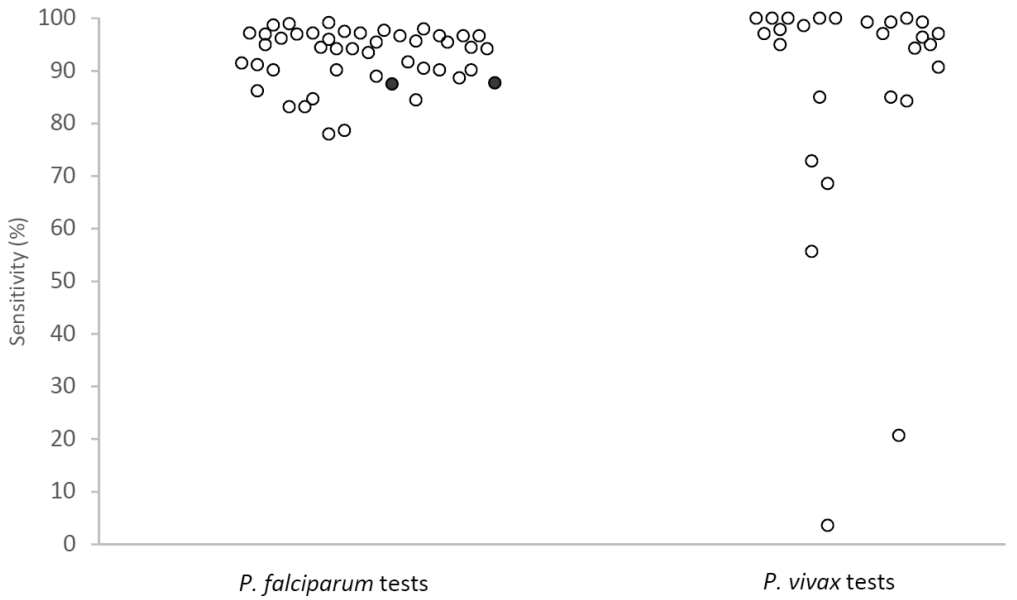

Figure 3. Clinical sensitivity of P. falciparum and P. vivax detecting RDTs from the WHO product testing programme phase 2, calculated from the wild type samples[50]. In practice, many tests contain both *P. falciparum* and *P.vivax* or pan-malaria test lines. Although the tests can detect *P. falciparum* also with an anti-pLDH test line, usually the *P. falciparum* result is from a test line detecting HRP2. The two RDTs that detected *P. falciparum* solely by pLDH are shown as filled circles. In total 400 *P. falciparum* positive samples and 140 *P. vivax* positive samples were tested.

A meta-analysis of *P. falciparum* RDT evaluation studies finds the average HRP2 RDT sensitivity 95.0 % and specificity 95.2 %[51]. The evaluations were typically done against microscopy. It is important to note that the clinical sensitivities found in the studies of malaria RDTs are affected by the choice of the reference. Evaluations against microscopy generally result in higher RDT sensitivities than evaluations done against nucleic acid test (NAT) methods, since a NAT can detect low parasitemia asymptomatic samples[52]. Overall, the sensitivities of the best HRP2 RDTs currently available seem to be sufficient for detecting symptomatic patients with a parasitemia of >100 parasites/µl with a high probability.

Similarly to the clinical sensitivities, also the analytical sensitivities of HRP2 RDTs vary widely. The analytical sensitivity can be evaluated based on two values: the density of parasites or the actual HRP2 concentration in the sample. Evaluating the analytical sensitivity in terms of parasite densities has the benefit of easy comparison to other diagnosis methods such as microscopy and NAT. The drawback is the limited correlation between parasitemia and HRP2 concentration in patient samples as well as

in cultured sample strains, depending on the strain as well as the duration and phase of the infection[45]. The discrepancy between parasitemia and HRP2 concentration has led to some differences in the reported analytical sensitivities of HRP2 RDTs. Marquart et al. developed a mathematical model to estimate the production of HRP2 in different parasitemias and stages of infection[34]. After finding HRP2 detection limits ranging from 6900 to 27800 pg/ml for four RDTs, they concluded that according to the model, the best performing RDTs should be able to detect parasitemias of 50 – 100 parasites/µl and chronic infections with a minimum parasitemia in the range of 8 to 17 parasites/µl.

The FIND target product profile for point-of-care diagnosis of submicroscopic *P. falciparum* sets the optimal detection limit requirement at 10 parasites/µl, which according to FIND would correspond to 6000 pg/ml[53]. However, a study of five unidentified WHO evaluated, best-in-class, HRP2 RDTs demonstrated analytical sensitivities of 400 – 1600 pg/ml for HRP2 with both cultured and recombinant HRP2[44]. These detection limits are an order of a magnitude lower than the detection limits evaluated by Marquart et al. and would suggest that current tests are already meeting the FIND target product profile, even though the estimated parasitemia detection limits of the tests are at 50 – 100 parasites/µl[34]. To further complicate the issue, the HRP2 detection limit of a single RDT varies widely when tested in different locations with actual patient samples[54]. One possible cause of the observed discrepancies may be that the methods used in the evaluations have not been standardized, e.g. HRP2 enzyme-linked immunosorbent assays (ELISAs), for quantifying HRP2 in the tested samples. In order to standardize the evaluations of the analytical sensitivities of *P. falciparum* RDTs, WHO and FIND have recently developed an international standard for *P. falciparum* antigens[55].

An RDT for HRP2 detection with a tenfold improvement in analytical sensitivity as compared to the traditional RDTs was recently developed. The limits of detection with cultured samples and recombinant HRP2 were 40 – 80 pg/ml and 40 – 125 pg/ml, respectively. The corresponding parasitemic detection limit of a cultured strain was 3 parasites/µl. The test has not yet been evaluated with the new international standard, and thus a direct comparison to the analytical sensitivities of the traditional RDTs is difficult. Nevertheless, the reported sensitivity is very promising and this test may be useful in malaria elimination campaigns, as discussed below.[56]

2.1.2 Rapid diagnostic tests for malaria pLDH antigen

pLDH is an enzyme in the glycolytic pathway of the parasite, which is an important metabolic pathway in all *Plasmodium* parasite species[57]. The pLDH protein has only a

25 – 26 % amino acid sequence homology to human LDHs, which makes it a specific marker for malaria parasites. In contrast, the sequence homology of pLDH between different *Plasmodium* species is over 90 % and there are common epitopes between the species[58]. This enables design of RDTs using pan-species monoclonal antibodies reacting with the five human-infecting malaria species. On the other hand, careful design of an RDT with species-specific monoclonal antibodies also enables the specific identification of an infecting species. It is particularly important to differentiate between the two most common species, *P. falciparum* and *P. vivax,* since the treatments applied are significantly different[59]. Apart from *P. malariae*, the pLDH amino acid sequence does not seem to vary between different strains of a single species, rendering the risk of false negative results due to mutated strains small[60].

Taken together, there are RDTs with multiple combinations of different pLDH binder test lines as well as HRP2 lines for either the pan-specific detection of all *Plasmodium* species or for the specific determination of an infecting species. **Figure 4** shows the combinations of test lines used in RDTs. The only malaria species that cannot currently be specifically identified by RDTs is *P. knowlesi*, which can be mistakenly identified as *P. falciparum* or *P. vivax*, depending on the RDT[61].

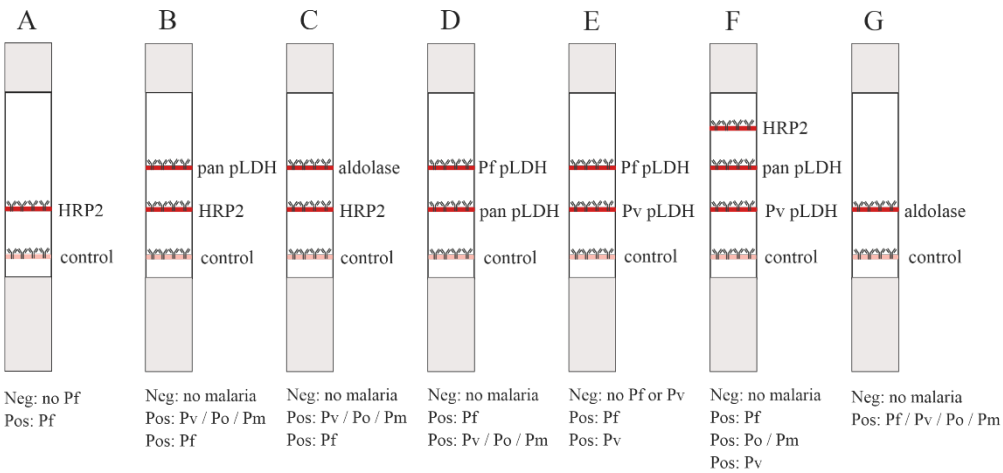

Figure 4. Combinations of test lines detecting different malaria species in RDTs. Strip A can only detect *P. falciparum* (Pf) malaria. Strips B, C and D can differentiate between Pf and non-Pf malaria (Pv = *P. vivax*, Po = *P. ovale*, Pm = *P. malariae*). Strip E can differentiate between Pf and Pv, but cannot detect non-Pf or Pv malaria. Strip F with three test lines can differentiate between the major species and strip G can detect all species, but not identify the species. Data from[19].

Clinical and analytical sensitivities of pLDH RDTs

The WHO and FIND product testing programme has also evaluated the sensitivity of RDTs for *P. vivax* detection with a protocol similar to the *P. falciparum* RDT evaluation[50]. The RDTs for *P. vivax* predominantly detect pLDH, and the sensitivity of these tests is shown in **Figure 3** together with the *P. falciparum* tests. Also, two tests for *P. falciparum* exclusively detected pLDH and are also shown.

A meta-analysis of *P. vivax* and non-*P. falciparum* detecting RDTs found that the RDTs reached clinical sensitivities above 90 % only when the parasitemia of the patient samples was above 500 to 1000 parasites/µl[62]. In contrast, the specificities of the tests in all the studies were consistently above 98 %.

The lower clinical sensitivity of pLDH tests as compared to HRP2 tests has several potential explanations. Firstly, *P. falciparum* parasitemia is typically higher than *P. vivax*, and the fact that HRP2 RDTs are only used for *P. falciparum* biases the comparison of sensitivities between species[63]. Yet, the sensitivities of the pLDH RDTs with *P. falciparum* are lower than with most HRP2 RDTs. A meta-analysis of studies that directly compared HRP2 and pLDH RDTs for *P. falciparum*, showed the HRP2 test to have a pooled sensitivity of 96.3 %, while the pooled sensitivity of the pLDH RDTs reached only 82.6 % (**Table 1**)[52]. pLDH is produced in smaller quantities than HRP2 by *P. falciparum*, which leads to poorer pLDH RDT analytical sensitivities especially with low parasitemia samples[64, 62].

The limited clinical sensitivity of the pLDH RDTs can also be partly explained by their lack of analytical sensitivity as compared to the HRP2 RDTs. To evaluate the analytical sensitivities of different types of pLDH RDTs, Jimenez et al. tested nine pLDH-based RDTs with *P. vivax* patient isolates, *P. falciparum* culture samples as well as *P. falciparum* and *P. vivax* recombinant proteins[44]. The RDTs comprised three tests detecting both *P. vivax* and *P. falciparum* pLDH (pan-pLDH), three detecting *P. vivax* exclusively, two detecting *P. falciparum*, and one detecting *P. vivax*, *P. ovale* and *P. malariae*. Overall, the analytical sensitivities for the *P. vivax* samples ranged from 12.5 to 25 ng/ml for most of the RDTs tested. The *P. falciparum* specific RDTs had analytical sensitivities ranging from 0.5 to 5 ng/ml for the *P. falciparum* culture samples. In particular, the study pointed out a clear difference in the analytical sensitivities of tests for *P. vivax* pLDH as compared to the analytical sensitivities of the HRP2-based RDTs. Similar analytical sensitivities were also reported in another study for pLDH RDTs evaluated with diluted *P. vivax* samples[65]. One reason for the difference in the analytical sensitivities may be the differing avidities of the pLDH and HRP2 binders. The

difference in avidity is due to the multiple repeating epitopes of HRP2, which allows for very high avidity binding both on the solid-phase and on the particle label[31, 32]. Secondly, the parasites may be produce a lower amount of pLDH as compared to HRP2.

One advantage of pLDH- over HRP2-based RDTs is the fact that the pLDH-based tests do not seem to suffer from the prozone effect (also known as the hook effect). In the prozone effect the label and capture antibodies are saturated by a sample from a hyperparasitemic patient having a high concentration of the antigen[66]. With *P. vivax* samples, this can be explained by the typically lower parasitemias compared to *P. falciparum*, but with *P. falciparum* samples the cause could be the lower production of pLDH as compared to HRP2.

In field studies, the clinical specificity of the pLDH RDTs is typically higher than that of the HRP2 RDTs[51, 52, 62]. The best demonstration of the differences in specificities is the aforementioned meta-analysis of head-to-head comparison studies of *P. falciparum* HRP2 and pLDH RDTs, the pooled specificity of HRP2 RDTs was only 86.1 % as compared to 95.9 % for pLDH RDTs (**Table 1**)[52]. The observed difference could be due to the longer persistence of the HRP2 antigen in the bloodstream, which would lead to false positive results in patients with resolved infections[34]. On the other hand, the shorter half-life of pLDH can affect the clinical sensitivity of the RDTs, since pLDH concentration in blood is more prone to variation depending on the life-stage of the parasite[67, 68, 69].

Table 1. Pooled clinical sensitivity and specificity of HRP2 and pLDH RDTs from a meta-analysis of evaluations of RDTs for P. falciparum[52].

Test type	Sensitivity % (95% CI)	Specificity (95% CI)
HRP2	96.3 (95.8–96.7)	86.1 (85.3–86.8)
LDH	82.6 (81.7–83.5)	95.9 (95.4–96.3)

2.1.3 Other malaria target antigens

Aldolase is another glycolytic enzyme of the parasite, with a 60 – 70 % sequence homology to human aldolases[70]. It is also highly conserved between *P. falciparum* and *P. vivax*[71]. However, in contrast to pLDH, the clinical sensitivities of RDTs targeting aldolases have been unsatisfactory[72]. One aldolase-targeting RDT for *P. vivax* was evaluated by WHO, and had a clinical sensitivity of 85 % (**Figure 3**). Typically, aldolase has been included in a two-line test format together with HRP2 to enable *P. vivax* detection. The reason for low sensitivity is likely due to the fact that the amount of

aldolase produced by the parasite is too low to meet the analytical detection limits of the traditional RDTs.

High-affinity antibodies have also been generated against *P.falciparum* and *P. vivax* glutamate rich protein (GLURP), dihydrofolate reductase-thymidylate synthase (DHFR-TS) and heme detoxification protein (HDP), but apparently they have not been applied in RDTs[73].

2.1.4 RDTs for Glucose 6-phosphate dehydrogenase (G6PD) deficiency

Tests for the detecting human G6PD deficiency are also critical for malaria treatment and elimination. The genetic G6PD deficiency increases the haemolysis of aged erythrocytes, which affords a degree of protection against the malaria parasites. Indeed, polymorphic G6DP deficiencies are most common in populations of malaria-endemic areas, with 8 % of the population in malaria-endemic countries affected, a total of 400 million people[74, 75, 76]. The deficiency is X-linked and deficient homozygote and heterozygous individuals may have variable levels of G6PD activity depending on the exact genotype[77].

Primaquine is a drug of the 8-aminoquinoline type and it is the only drug licensed to clear hypnozoites and prevent the relapse of *P. vivax* infection[77]. It is thus critical in the treatment and elimination of *P. vivax.* Unfortunately, intensive primaquine treatment to clear the dormant parasites can lead to acute hemolysis[78]. The risk of serious side effects in a sizable fraction of the patient population in the absence of practical G6PD deficiency testing has led to under-prescription of primaquine, thus hampering the efforts to control and eliminate *P. vivax*[77]. Primaquine can also be used to reduce the transmission of *P. falciparum*, since it kills gametocytes[79]. For the best treatment outcomes and for the malaria elimination efforts to succeed optimally, at least the *P. vivax* diagnostic RDTs should be complemented by rapid G6PD deficiency testing[80] Point-of-care G6PD deficiency tests are based on measuring the activity of the enzyme, which catalyzes the following reaction:

$$\text{Glucose 6-phosphate} + NADP^+ \rightleftharpoons \text{6-Phosphogluconate} + NADPH + H^+$$

Currently, the most widely used G6PD deficiency test is the fluorescence spot test, in which fluorescent reaction product NADPH is detected using UV-light[81]. It is a relatively easy test to perform, but requires laboratory equipment and training. RDTs with a membrane containing the substrates and a compound that forms a colored substance when reduced by the H^+ product have been recently developed[82]. These tests

are as easy to perform as LFIAs, but they only provide qualitative results and often fail to detect heterozygous females who have 30 – 40 % of the normal G6PD activity[83]. Altogether, a quantitative G6PD test incorporated into a malaria RDT could be particularly useful for malaria elimination campaigns[84].

2.1.5 Role of RDTs in malaria elimination

WHO has set an ambitious goal of 90 % reduction in malaria incidence rate, and elimination of malaria from 35 countries by the year 2030[85]. Malaria elimination refers to the removal of malaria transmission from a certain area, while eradication means the complete interruption of transmission of human-infecting forms of malaria. Most of the efforts are aimed towards the elimination of *P. falciparum* and *P.vivax*.[86] A major challenge in this effort is the long duration of infectivity of the human hosts. In the case of patients with functional acquired immunity, a low-density asymptomatic infection can persist for over a year.[87, 88]. These patients can still infect mosquitoes and are an important reservoir for the parasite in low- and medium-endemicity areas[89]. In fact, in many populations most infections are submicroscopic and asymptomatic[90]. Since these individuals can account for 20 to 50 % of malaria transmissions in the pre-elimination and elimination settings in regions of low prevalence, identifying these infections through active case detection using RDTs, and treating them, would help in elimination by interrupting the parasite life-cycle[91, 92, 84].

There are two strategies for eliminating the asymptomatic reservoir: 1) mass drug administration (MDA), and 2) mass-screen and treat (MSAT) only the carriers found with the screening test. In MDA the entire population is administered medication, an artemisin-based combination therapy (ACT) in the case of a *P. falciparum* infection. The key to a successful MSAT campaign is in detecting a sufficient proportion of the carriers. Usually, RDTs are used for this purpose.[93] However, the analytical sensitivity of the current *P. falciparum* or *P.vivax* RDTs is not sufficient to detect asymptomatic or submicroscopic infections with parasite densities below 100 parasites/µl of blood[94]. The studies of MSAT programmes have indicated that the sensitivity of the current RDTs is not sufficient for reducing *P. falciparum* malaria incidence rate, possibly because they detect only 10 – 30 % of the NAT-positive carriers [95, 96, 97].

The key factors in estimating the probability of MSAT success are: the infectiousness of the patients with varying parasitemias, the detection limit of the RDT used, the EIR, and the seasonality of malaria transmission in the region[98]. Parasitemia is correlated with the density of gametocytes in patient blood, which positively correlates with the probability of infecting a feeding mosquito[99]. For example, according to mathematical models, an

RDT with a sensitivity of 2 parasites/µl used in MSAT campaigns could interrupt malaria transmission in an area where the EIR is 4 and transmission is highly seasonal[100].

Lately, a new ultrasensitive, RDT (uRDT) for *P. falciparum* HRP2 with a detection limit close to 2 parasites/µl, RDT (uRDT) for has been developed to improve MSAT elimination strategies[56]. This is a 10-fold improvement to the detection limit of the traditional RDTs, and in theory the increased sensitivity should expand the applicability of MSAT into areas with EIR ranging from 1 to 4. When the uRDT was compared with a standard RDT in several cross-sectional studies of populations from varying endemic regions, the uRDT detected approximately twice the number of infections[101, 102, 103]. Despite the improvement, the uRDT still only detects approximately 50 % of the patients with gametocytes, which can transmit malaria[103]. Unfortunately, improving the detection limit of the RDTs does not significantly improve the diagnosis of malaria from febrile patients, since most of the symptomatic patients have values of 100 parasites/µl when they seek medical treatment[104]. Taken together, another 10-fold improvement in the detection limit of RDTs would be necessary to increase the efficacy of MSAT campaigns.

While the control and elimination of the more severe and more common *P. falciparum* are the most urgent issues, the elimination of *P. vivax* is also necessary. In fact, in elimination settings the prevalence of *P. vivax* decreases less rapidly than *P. falciparum.* This indicates that *P. vivax* could ultimately become the main barrier to the elimination of malaria. Generally, the strategies used for *P. falciparum* elimination would also be applicable to *P. vivax*. However, three characteristics of *P. vivax* complicate the diagnosis and elimination of this species.[63]

First, *P. vivax* parasitemia is typically lower than that of *P. falciparum,* since *P. vivax* parasites predominantly invade immature red blood cells. This combined with the lower production of the pLDH antigen as compared to *P. falciparum* specific HRP2 leads to a requirement of very high analytical sensitivity for *P. vivax* RDTs used in MSAT programmes.[63]

Secondly, *P. vivax* produces gametocytes at an early phase of the infection, even before the symptoms appear[105]. This increases the need for a highly sensitive RDTs capable of identifying these patients before they can infect mosquitoes. Thirdly, the *P. vivax* hypnozoites sequestered in the liver cannot be detected by the current diagnostic tests, and thus relapsing carriers can restart the parasite life-cyle after completion of the MSAT[63].

2.2 Pertussis diagnostics

Pertussis, or whooping cough, is a disease caused by a bacteria, *Bordetella pertussis*. It is highly contagious, and despite widespread vaccinations it continues to be a significant source of morbidity. The highest morbidity and mortality rates are seen in infants too young for vaccination[106].

In vitro diagnostics of pertussis poses some unique challenges when compared with the malaria or hepatitis C diagnostics. While malaria and hepatitis C are blood borne infections and their antigens are found in the bloodstream, *Bordetella pertussis* colonizes the respiratory tract[106]. An additional challenge in pertussis diagnostics are the vaccinated individuals, since the protective immunity wanes after several years thus enabling infections in vaccinated individuals[107].

Pertussis diagnostics can be divided into methods suitable for early and late stage diagnosis or surveillance. Early stage diagnosis is important for diagnosing life-threatening infections in infants and toddlers in the acute phase, since antibiotic treatment is only effective if started within one week after the onset of symptoms. Early stage diagnosis is commonly based on bacterial culture or PCR from nasopharyngeal swabs or aspirates.[108]

For older children and adults, the symptoms are typically mild and unspecified such as a prolonged cough. In these cases, the diagnostic testing typically takes place only after a long period of coughing. The sensitivity of pertussis PCR tests decrease around two weeks after the onset of symptoms, at the time when antibodies become detectable, and therefore serological tests are recommended for late stage diagnosis. The serodiagnostic testing can be done using either single or paired sample serology. In paired samples serology the first sample is taken as soon as possible and the second sample weeks later at the convalescence phase. The paired sample serology provides a more accurate diagnosis, since the change in the immune response between the samples enables differentiation between vaccine- and infection-induced immunity[109, 110]. Single sample serology is nevertheless far more convenient for diagnosis as well as surveillance and thus more widely used.[107]

2.2.1 Immunoassays for chronic pertussis and surveillance

The most common immunological diagnostic test for pertussis is an enzyme-linked immunosorbent assay (ELISA) for anti-pertussis toxin (PT) antibodies. *B. pertussis* displays several antigens that can be used in immunoassays, the antigens include

filamentous hemagglutinin (FHA), fimbria and pertactin. While these antigens are produced by several *Bordetella* species, PT is produced solely by *B. pertussis* and is recommended as the only specific antigen for *B. pertussis* testing.[111]

PT is included in all acellular or whole cell vaccines against pertussis[112]. Most adults and adolescents in the high-income countries have detectable levels of anti-PT antibodies due to the high vaccine coverage and the circulation of pertussis in the adult population[111]. Therefore anti-PT immunoassays need to be quantitative and equipped with a cutoff to separate recent natural infections from vaccination-induced or past infection immunity. The need for quantitative testing in turn has thus far precluded the development of a LFIA for anti-PT testing.

The WHO 06/140 and 06/142 standards for anti-PT antibody detection have been used to set diagnostic cutoffs ranging from 40 to 125 IU/ml[113]. Laboratories typically use in-house anti-PT ELISAs and the WHO standards have helped in standardizing the results of different laboratories. For normal testing the cutoff is usually 100 to 125 IU/ml, while lower cutoffs are used in outbreak situations[111]. If the anti-PT test is used for an epidemiological study, a value higher than 100 IU/ml indicates the patient has had an infection within a year, and 50 to 100 IU/ml indicates an infection in the past few years. A result below 50 IU/ml can stem from exposure to pertussis several years ago, which may lead to an overestimation of the pertussis prevalence in epidemiological studies. Since the assessment of the diagnosis solely by anti-PT is never clear-cut, the anti-PT result should always be assessed in combination with the patient's vaccination history and possible exposure information.[114]

Pertussis burden is the highest in low-income countries, particularly in Africa. In a ranking of leading causes for years of potential life lost, pertussis ranked on average 32nd in sub-Saharan Africa, 67th globally, and 95th in Western Europe[115]. Furthermore, there are large areas in Africa with lower than 50 % pertussis vaccine coverage[116]. While the morbidity and mortality burden of pertussis can be estimated from hospitalized cases, current prevalence estimates for pertussis in Africa are very uncertain[117]. According to WHO pertussis surveillance objectives, laboratory confirmation of suspected cases would help in pertussis surveillance, both for better estimates of prevalence and for the development of vaccination strategies[117, 118].

To improve the surveillance, an RDT for anti-PT antibodies could be used to complement ELISAs in resource-poor areas with limited access to hospital laboratories. Line immunoblot tests with antigens immobilized on a nitrocellulose strip are used as confirmatory tests for anti-pertussis ELISA and are a step towards portable testing. Line

immunoblots can separate between IgG, IgA and IgM responses, and can include several antigens in addition to PT. They can also provide a semi-quantitative assessment of anti-PT levels. [119] However, the use additional antigens or detection of IgA and IgM responses do not benefit the sensitivity or specificity, and the interpretation of the semi-quantitative results of line immunoblots is difficult[120]. Due to the above the use of line immunoblots is not recommended as the sole test for pertussis serodiagnosis[121].

LFIAs, on the other hand, could be used also for anti-PT serodiagnostics. Semi-quantitative visual LFIAs would face the same problem of interpretation, but instrument-read tests can provide rapid quantitative results[122]. There are thus far no published anti-PT lateral flow immunoassays, but qualitative serological LF tests for viral infections are widely used[123].

2.2.2 Immunoassays for acute pertussis diagnosis

The main diagnostic methods for acute phase pertussis infections, PCR and bacterial culture, require centralized laboratories[124]. Therefore, rapid immunoassays would improve the acute phase diagnostics in resource-poor areas. Direct detection of the bacteria or the secreted PT with immunoassays from the same nasopharyngeal aspirate used for PCR and culture could provide a simpler method for acute pertussis diagnostics[125].

Already in 1989, Friedman et al. published an interesting dot blot immunoassay for PT and demonstrated that PT can be detected from nasopharyngeal aspirates[125]. The analytical sensitivity of the dot blot test was 10 ng of PT per dot and the test recognized all six positive nasopharyngeal aspirate samples. However, the study was limited to a small number of positive samples, and showed a 12 % false positive rate with presumed healthy control samples. No further studies related to this test have been published. Capture ELISAs for PT have also been developed, but these are solely used for screening cultured *B. pertussis* isolates for strains that lack this important vaccine immunogen[126,127]. Therefore, their sensitivity with nasopharyngeal aspirates has not been studied. Although the evidence thus far is quite limited, the results of the dot blot immunoassay study suggest that a more sensitive LFIA for PT could reach sufficient clinical sensitivity to be useful in rapid diagnosis of acute pertussis.

2.3 Rapid diagnostic tests for hepatitis C virus (HCV)

HCV is a single-strand positive-sense RNA virus of the family *Flaviviridae*[128]. HCV infection causes the disease hepatitis C, which develops into a chronic disease in 85 % of cases. Acute hepatitis C is asymptomatic in most cases, but the chronic disease leads over decades to liver disease, cirrhosis and even liver cancer.[129] There are an estimated 70 million people living with chronic hepatitis C, resulting in 700,000 deaths annually. Low- and middle-income countries account for 80 % of this global burden.[130, 131] Due to the vagueness of symptoms, globally fewer than 20 % of those with chronic hepatitis C are aware of the infection, and <1 % in low- and middle-income countries[132, 133].

Hepatitis C is a blood-borne infection and it spreads through contaminated blood products, medical procedures and via needle-sharing of intravenous drug users. From the Second World War to the 1980s HCV spread widely through transfusions of contaminated blood. Currently, in high-income countries transfusion-transmitted infections have been practically eliminated by the testing of donated blood, but in low-income countries iatrogenic infections remain a problem.[134] Starting from 2014, increased knowledge of HCV molecular biology has led to the revolutionary development of directly acting antiviral drugs (DAAs), which provide complete viral clearance in nearly 100 % of patients with a single 12 week orally administered treatment regimen[129]. Development of the DAAs has opened the door to the possibility of eradicating HCV. In fact, one of the goals of the WHO Global Hepatitis Health Sector Strategy is that 90 % of HCV infections would be found and treated by the year 2030[135].

Current hepatitis C diagnostic tests are based on detecting anti-HCV antibodies in blood with fourth generation anti-HCV enzyme-immunoassays (EIA)[11]. Since the HCV genome is heterogeneous with six major genotypes, the fourth generation EIAs use as test antigens a mixture of recombinant proteins and synthetic peptides recognizing all six HCV genotypes[136]. An anti-HCV positive screening sample is usually confirmed with a NAT to ascertain that the infection is active, since anti-HCV antibodies remain for life even after virus clearance[11]. NAT can also be used to identify window period infections, meaning early acute phase infections in which viremia is present but the antibodies are not yet detectable (**Figure 5**). Due to the risk of window period donations, testing of donated blood with NAT is also routine in blood banks of high-income countries[134]. The risk of anti-HCV negative but NAT positive infections to the blood supply is considered to be minimal in countries with effective pre-donation screening of blood donors[134]. Immunoassays for HCV core antigen (HCVcAg) are a possible alternative for NAT, since they can also cut up to six weeks from the window period and works as a confirmatory test[137].

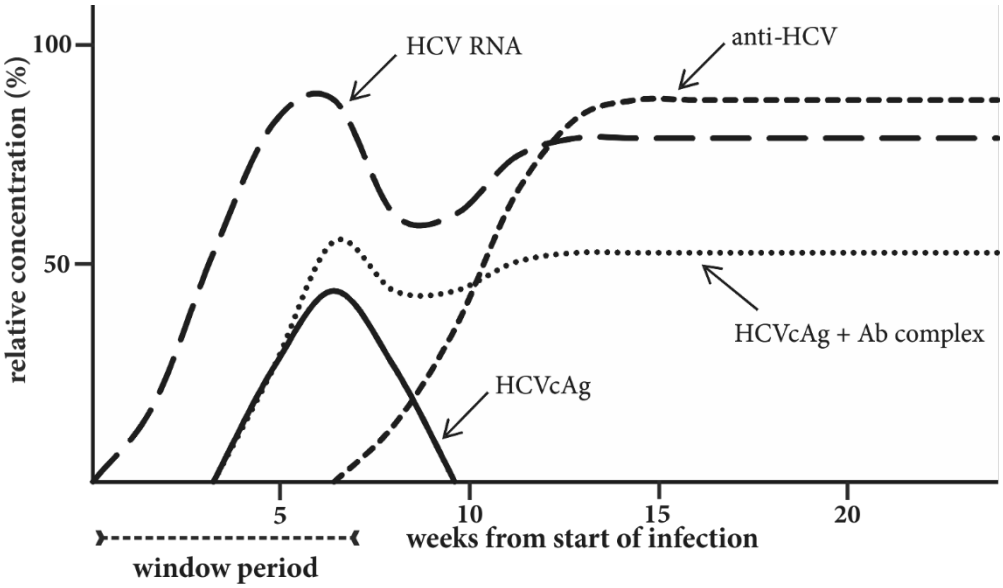

Figure 5. Kinetics of HCV markers detectable from patient's serum. HCV-RNA is detectable at the earliest phase through NAT methods. HCVcAg can be detected after HCV-RNA, both in free form and later bound with antibodies. The anti-HCV immune response can be detected after a window period, which can vary between 4 to 8 weeks[137].

RDTs for anti-HCV antibodies using the LFIA platform are widely used in low-income countries, with two of the tests already prequalified by WHO (**Table 2**)[138]. WHO prequalification of a diagnostic test means it has sufficient quality, safety and performance so that WHO can recommend it to be purchased by health services and non-governmental organizations and it is also eligible for procurement by UN agencies[135].

Table 2. Comparison of some key characteristics of the two WHO prequalified anti-HCV RDTs[139, 140]. Both of the prequalified tests perform well compared with standard EIAs.

Charateristic	SD Bioline HCV	OraQuick HCV Rapid Antibody Test Kit
Year prequalified	2016	2017
Clinical sensitivity %[a] (95% CI)	100 (97.8–100)	100 (97.8–100)
Clinical specificity %[a] (95% CI)	100 (98.9–100)	99.7 (98.3–100)
Seroconversion sensitivity index[b]	+2.0 days	+ 2.75 days
Mixed titer panel positivity	15/15	20/25
Time to result	5 min	20 min

[a]Sensitivity and specificity were tested against an agreed reference standard

[b]Average sensitivity on four seroconversion panels in comparison with standard EIA

Rapid testing for HCV can have a significant impact on the fight against HCV on two fronts. First, the availability of rapid tests can improve donated blood safety in low and lower-middle income countries. Second, a rapid test capable of detecting an active HCV infection without a confirmatory test would allow a one-step detect and treat approach, considerably improving HCV eradication efforts.

2.3.1 Rapid HCV tests for transfused blood

The challenges for donated blood safety in low-income countries, above all in Africa, are severe. Particularly, three problems present in low-income countries all compound the risk to the blood supply. First, the blood supply is mainly from family or paid donors with a higher infection risk than regular voluntary donors[141]. Secondly, the prevalence of HCV is higher in low- and middle-income countries, further increasing the risk[142, 143]. Thirdly, screening of donated blood may be insufficient, and in the case of HCV is often done with anti-HCV RDTs, which therefore have great responsibility to screen out the infectious donors **(Table 3)**[141].

Table 3. The distibution of HCV blood screening tests used in blood banks by WHO region[141]. The majority of countries in Africa rely solely on anti-HCV testing for screening of donated blood and many of these tests are RDTs.

	Number of countries by HCV screening tests used		
Region	**anti-HCV**	**anti-HCV + HCVcAg**	**NAT**
Africa	31	13	2
Americas	21	6	4
Eastern Mediterranean	10	4	5
Europe	15	5	23
South-East Asia	8	1	2
Western Pacific	16	1	7

Is the performance of the current anti-HCV RDTs sufficient for the task of blood screening? WHO recommends a minimum clinical sensitivity and specificity of 99.5 % for any test for donated blood screening[144]. Studies comparing the sensitivity of RDTs with EIAs or NAT show somewhat contradictory results. In a meta-analysis of 52 studies with patient samples, Tang et al. found an overall sensitivity of 99 % for different RDTs when compared with EIAs and 97 % when compared with NATs[145]. The overall specificity was 99 %. The analysis by Tang et al. also contained studies that were analyzed in an earlier meta-analysis that found similar sensitivities, supporting the conclusions

of the analysis and showing that there have not been any significant changes in available HCV RDTs the last few years[14]. While there was high variability in the sensitivities of RDTs from different manufacturers, the now WHO prequalified OraQuick HCV RDT and SD Bioline HCV RDT showed consistently good sensitivities and specificities across studies[14, 145].

However, two studies of RDTs used in real conditions in blood centers around Africa found a much lower than expected sensitivity with a standardized panel of HCV-positive samples including low antibody titer samples[146, 147]. The sensitivity of HCV RDTs varied from 47 % to 100 %, with the now prequalified SD Bioline HCV RDT having an average sensitivity of only 72 %. The studies had two weaknesses: There is a large variation between the sensitivities of the same RDT between different blood centers, pointing to operator errors as a source of poor sensitivity. Also, the sample panels used in the studies were not representative of the local HCV population and contained diluted samples. Nevertheless, the studies show that EIA-based blood screening does have better sensitivity compared to RDTs in African blood centers and HCV RDTs have problems detecting possible low antibody titer samples.

Anti-HCV RDTs, particularly the WHO prequalified tests, provide a validated cost-effective alternative to donated blood screening in low-income countries[139 140]. Anti-HCV RDTs naturally cannot detect the early-phase window-period infections currently detected by NAT or HCVcAg immunoassays. The current HCVcAg immunoassays have been shown to be a cost-effective alternative to NAT in window-period testing although with a lower sensitivity[148].

It is hypothesized that reliable detection of window-period infections would be more relevant in the high-prevalence donor pool of many low and lower-middle income countries than in the high-income countries, where the probability of a window-period donation is very small[149]. However, a comparison study of nearly 2000 real blood donations in high-prevalence Cameroon tested both with a RDT and an Ag/Ab combination immunoassay did not find any window-period donations, and the sensitivity improvement of the Ag/Ab immunoassay was simply caused by higher antibody-detection sensitivity of the Ag/Ab test[150]. Nevertheless, a RDT capable of screening both for anti-HCV antibodies and HCVcAg could provide improved blood safety in low and lower-middle income countries. There are currently no RDTs for HCVcAg detection, and the major limiting factor in their development is the very high analytical sensitivity required[123]. The current EIAs for HCVcAg reach analytical sensitivities in the low femtomolar range, which is almost impossible to achieve with

traditional RDT technologies[151]. Rapid HCVcAg assays should therefore provide an ideal test case for the technologies used to improve the analytical sensitivity of RDTs.

2.3.2 Rapid HCV tests for single-test and treat

Since the arrival of the new DAA medications has simplified the treatment of hepatitis C, it has become possible to envision a protocol where a single diagnosis of active infection is followed by a treatment regimen expected to clear the virus completely from the patient[152]. The simplification of treatment could allow realistic efforts to eradicate hepatitis C[153]. However, in order to have a chance of success, any eradication campaign requires a diagnostic test that can detect an active HCV infection rapidly and without a confirmatory test[154]. Since the current HCV RDTs detect anti-HCV antibodies they cannot be used as a single-test and treat type test to find active infections requiring treatment. There is currently a push to automate and miniaturize HCV NAT technologies to provide a rapid test, but a true point-of-care NAT for HCV with results from finger-prick whole blood has not been realized[153].

A sufficiently sensitive RDT for HCVcAg could also fulfill the criteria for this type of single test, since the core antigen indicates the presence of an active infection and core antigen levels correlate with HCV RNA levels[155]. Current EIAs for HCVcAg have been found to be sufficient for clinical decision-making[156]. The FIND target product profile for a point-of-care NAT or HCVcAg test sets the optimal analytical sensitivity to 15 IU/ml and minimal analytical sensitivity to 1000 IU/ml[157]. The minimal analytical sensitivity criterion corresponds to approximately 3 fM concentration of HCVcAg, which is also the detection limit of the best current HCVcAg EIAs. The lower optimal limit is especially critical in detecting early pre-seroconversion infections, but a test with the acceptable minimal analytical sensitivity would still be able to detect 95 % of patients with an active infection[158]. For screening and treatment purposes, this is considered acceptable. Even so, the few published proof-of-concept LF tests for HCVcAg are still far from reaching this sensitivity[159, 160].

2.4 Techniques to improve rapid diagnostic test sensitivity

The greatest limitation for many RDTs lies in the analytical sensitivity. The most popular methods for improving RDT analytical sensitivity are optimizing the affinity of the binders used the tests and switching from visually read colloidal gold nanoparticle (Au-np) labels to instrument-read labels. The different alternatives to the visually read Au-np labels have recently been expertly reviewed by Juntunen[161]. However, other

methods related to sample pre-concentration, visual label enhancement and control of the liquid flow on the tests can also improve sensitivity of RDTs. These different types of sensitivity enhancement methods are not mutually exclusive and could be used in combination to further improve test sensitivity. The parameters influencing the formation of the sandwich immunoassay in a lateral flow test format is quite complex to model[162], but in its simplest form can be considered in the terms of the binding constants of the binders:

$$K_{eq} = \frac{[A][B]}{[AB]}$$

The concentration of the binder *B* is limited by the background signal caused by excess label or the binding capacity of nitrocellulose on the test line. The affinity constant K_{eq} can be improved by optimizing the affinity of the antibodies used. The concentration of the free ligand *A*, the analyte, can be increased with sample pre-concentration methods. The signal generated from the bound complex *AB* can be improved by label enhancement methods without needing to affect the concentration of *AB*. Thus,

cumulative improvements on sensitivity could be achieved by developing these different facets of the LF test.

2.4.1 Sample pre-concentration

In sample pre-concentration methods, the goal is either to increase the usable sample volume on a single lateral flow strip, increase the effective concentration of the analyte during the binding reactions, or a combination of these. They can be divided into methods to enrich the sample before it starts to flow in the actual LF test and methods where the analyte concentration takes place during the flow in the test strip.

Magnetic particles

Magnetic particles are a popular tool for enriching the analyte from a larger sample volume to a volume suitable for a LF assay. The problem is that binding the analyte to magnetic iron oxide particles for separation precludes binding the analyte to the Au-np label particles for the LF test. This problem has been circumvented with a mix of Au-np and magnetic particles forming a co-aggregate, without the analyte binding directly to the magnetic particles. Both the Au-np with bound analyte as well as the iron oxide magnetic nanoparticles were coated with a linear coblock polymer. When the Au-np conjugated with the tracer antibody and magnetic nanoparticles are mixed with the sample containing *Pf*HRP2, the normal Au-np tracer + analyte complex is formed.

Then, when free polymer is added and the mixture is heated to 40 °C, the Au-np coaggregates with the magnetic nanoparticles. This aggregate can be captured by applying a magnet and the supernatant can be removed. The aggregates are re-dissolved in buffer and the resulting enriched Au-np + analyte complex can be added to a standard LF test format.[163, 164]

Nash et al. applied this technique to a HRP2 LF test and achieved a 25-fold enrichment of the analyte and a 2.5-fold improvement in the detection limit. A significant advantage of this method is that it can be easily applied to different analytes with a streptavidin-coated Au-np and a biotinylated binder. However, the enrichment phase has many pipetting steps , a heating step, and takes 30 minutes to complete.[164]

The *P. falciparum* HRP2 antigen is especially suitable for pre-enrichment protocols, since its amino acid composition is 35 % histidine and a chelated divalent metal ion can reversibly bind the imidazole functional group of the histidine through metal chelation chemistry[165]. Accordingly, the divalent metal ion chelate (M^{+2}-NTA) can be bound to solid phase, the sample exposed to the solid phase, unbound sample washed away and the bound and concentrated HRP2 can then be eluted with free imidazole. This immobilized metal affinity chromatography principle has been used for histidine-rich or histidine-tagged protein purification since 1975[166].

Subsequently, magnetic particles with M^{+2}-NTA surface have been used to develop a series of HRP2 enrichment methods[165, 167]. In the simplest format, the M^{+2}-NTA magnetic particles are mixed with the sample for 5 to 30 min, after which they are directly transferred to the sample pad of a normal malaria HRP2 RDT, and eluted with a test running buffer with added imidazole[168]. This enrichment achieved a 50-fold concentration of the analyte with 70 – 80 % of HRP2 captured from the sample, resulting in a 21-fold improvement in sensitivity. This is a relatively simple HRP2 enrichment format, which was estimated to increase the cost of a test for only 1 USD on top of the 0.55 – 1.5 USD per test of normal malaria RDTs.

An alternative method eliminating the need for magnetic particles is a modified pipette tip with M^{+2}-NTA resin. HRP2 capture can be achieved from a larger sample by pipetting the sample back and forth through the resin, and then eluted by pipetting in imidazole elution buffer and ejecting the eluent to the LF strip. This pre-concentration method achieved only a 5-fold improvement in sensitivity, but the pipetting steps take only 5 minutes and require nothing more than a modified pipette tip and a standard pipette.[169]

Integrated sample pre-concentration

There are three appealing methods for concentrating the analyte in the LF strip itself. The method simplest to implement is an integrated dialysis pad on the LF test strip. The sample along with a dialysis buffer rich in polyethylene glycol (PEG) can be added onto a semi-permeable membrane in front of the conjugate pad, where the sample is concentrated through dialysis for 10 minutes before running the LF test[170]. This resulted in a modest 2-fold improvement in the detection limit.

A more complex analyte concentration method uses an aqueous two-phase system (ATPS) to separate biomolecules and the label into two phases based on their hydrophilicity and size. In ATPS, a mixture of a polymer and a salt is separated into a polymer-rich and salt-rich phase and larger as well as more hydrophilic biomolecules and other larger particles are concentrated in the salt-rich phase[171]. When an ATPS mixture is mixed with a sample and Au-np labels before loading onto an LF strip, the phase separation is further enhanced since the PEG-rich phase is held back at the beginning of the nitrocellulose, while the PEG-poor phase with the analyte and large Au-nps moves through the sample pad and nitrocellulose[172]. The separation can be further enhanced by a thick 3D sample pad, on which phase separation can take place before flowing into the nitrocellulose. Au-np tracers binding the analyte can thus be concentrated on the leading front of the flow in the LF strip, in practice concentrating the analyte from a high volume of sample to smaller volume that flows through the LF strip. This ATPS-based concentration method has been used in a malaria pLDH LF test to concentrate the sample 10-fold with corresponding 10-fold improvement in the detection limit, with a 5-minute pre-incubation and a total assay time of 25 min[173]

More recently, ionic liquids have been used in an ATPS concentration system to improve the phase separation speed outside the LF membranes. This allows a 5-minute sample concentration step before adding the concentrated phase to a normal LF test. Thus, the method could be used to improve the sensitivity of current tests without costly modifications to the LF strip manufacturing process. Overall, ATPS concentration methods still need to be optimized according to the analyte and the sample matrix used. Particularly, some antibody-antigen interactions may suffer from the high ionic strength of the salt phase where the sample is concentrated.[174]

Isotachophoresis

The most elaborate concentration method, yet also most promising from the sensitivity improvement point of view, is the use of isotachophoresis (ITP) to concentrate proteins

on a LF strip. ITP is a type of electrophoresis where charged analytes are separated and concentrated based on their ionic mobility in a medium.[175]

When ITP is used in LF, the sample is introduced into a nitrocellulose with a solution of slow moving trailing electrolyte ions. Fast moving leading electrolyte ions are introduced to the other end of the nitrocellulose and when an electric current is applied the analyte moves through the nitrocellulose and accumulates at the boundary of leading and trailing electrolyte ions. The analyte is concentrated from a large volume into a narrow plug approximately 100 μm wide.[175] When this narrow plug moves over the test line capture zone, the effective concentration of the analyte is up to 1000-fold higher than in the original sample [176]. The electric current can even be decreased when the plug is migrating over the test line to increase the efficient reaction time.

The method has been shown to enable both more sensitive as well as faster LF tests, with a 160-fold improvement in the detection limit after 5 minutes of running compared with the same assay in a normal LF setup. Even so, the method has not been tested with an actual sandwich immunoassay or a biological sample matrix and the electric current requires a small battery-powered system. Also, the speed of ITP concentration is limited by the heating and evaporation of the sample when using higher electric currents. There is nonetheless great potential in this concentration methods and it could be possible to combine ITP with an instrument-read LF label, where a simple portable instrument could perform both ITP and label detection.[175]

2.4.2 Flow control

Many of the presented methods for improving the sensitivity of RDTs introduce extra steps to the LF assay protocol. These steps can be automated or simplified by adding manual or automatic flow controls to the LF strip architecture. In a LF strip the flow speed of the liquid front is powered by capillary action formed in the porous nitrocellulose membrane, described by the Lucas-Washburn equation (1)[177, 178]:

$$L = \sqrt{\frac{\gamma r t \cos(\theta)}{2\eta}} \quad (1)$$

Where L is the distance the liquid has penetrated the capillary, γ is the surface energy, r is the radius of the pores, θ is the contact angle between the liquid and the pore wall and η is the viscosity of the liquid. Similarly, the flow rate of the fully wetted capillary membrane is described by the Darcy equation (2) [179, 178]:

$$Q = -\frac{\kappa wh}{\eta L}\Delta P \quad (2)$$

Where Q is the volumetric flow rate, κ is the permeability coefficient that is dependent on the porous structure of the membrane, wh is the membrane area, η is the viscosity, L the length of the membrane and ΔP the drop in pressure occurring over the length of the membrane.

In a normal LF nitrocellulose membrane it can usually be assumed that the average radius of the pores and the nitrocellulose material stays constant, thus r, θ and κ can be assumed to remain constant over the whole length of the LF strip. If all the used liquids in a single test are similar, γ, η and μ can be assumed to stay constant. In this typical scenario for a LF test, the flow and timing of the different assay liquids can be manipulated simply by the length of the membrane they have to flow through. In its simplest form this means that the reaction time of the label and the analyte and the flow speed of this complex over the test line can be optimized by situating the test line closer or further from the start of the nitrocellulose[4].

The predictable flow speeds of liquids on the porous membrane can also be used to automate the sequential steps of a multi-step immunoassay process on the LF strip. For example, separate rinse and signal amplification steps can be automatically timed with a 2-dimensional paper network (2DPN) so that the end user can only add sample and buffer to different wells of the LF strip and start the test (**Figure 6A**)[180]. The 2DPN format has been used to improve the sensitivity of malaria HRP2 detection 4-fold with an Au-np enhancement step without significantly increasing the complexity of the test for the end user[181, 182]. This type of automated timing of assay steps can be also used with other signal amplification methods such as silver enhancement of Au-np and enzymatic labels that have been used to improve LF sensitivity[183, 184, 185]. In a further improvement to HRP2 2DPN LF tests, a horseradish-peroxidase enzyme label together with streptavidin – biotin chemistry was used in the paper network LF format, achieving a respectable detection limit of 0.1 ng/ml of HRP2 and 90 min running time[186].

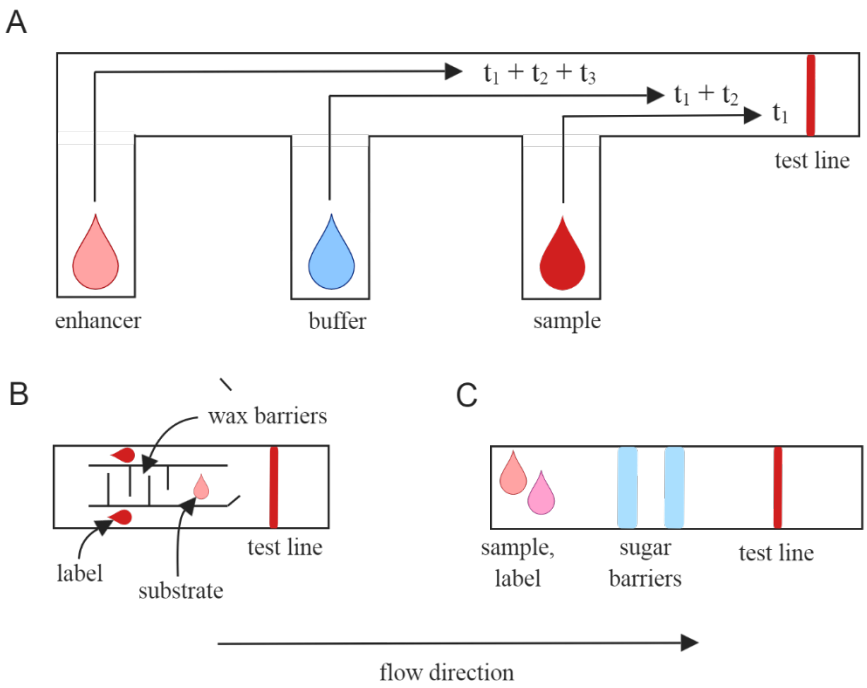

Figure 6. Flow control methods for LF strips. (A) 2-dimensional paper networks can automate the application of different liquids to the LF strip at different time-points. When the three liquids are added to the 2PDN strip at the same time, the sample and label will arrive at the test line at time t_1, the washing buffer at time $t_1 + t_2$, and the label enhancer at time $t_1 + t_2 + t_3$. (B) Wax barriers are relatively simple to print to LF strips to slow the flow speed of the liquids at desired points. The barriers slow the flow of liquid to the substrate compartment, so that the substrate of the label enzyme arrives to the test line after the label enzyme itself. (C) Dissolvable sugar barriers can stop the flow on the strip for a pre-defined time, allowing the sample and label to react on the strip.

One drawback of the 2DPN method is that the manufacturing of the large and relatively complicated 2DPN strips may be difficult for the normal LF production lines. Printing of wax pillar barriers on the nitrocellulose of a standard LF strip is a simpler to manufacture method for increasing the effective distance the liquid has to flow. This method has been used to develop an enzymatically enhanced LF immunoassay where the flow of the substrate for the enzyme-labeled tracer is automatically delayed by 11 seconds (**Figure 6B**).[187]

The flow timing of liquids in the membrane can also be manipulated by temporarily increasing the viscosity (η) of the liquid with a dissolvable sugar barrier. A barrier formed by printing and drying sucrose in the membrane temporarily increases the viscosity of the liquid flow, and thus printed sucrose lines of different saturations can be used on 2PDN strips to delay the flow of different components for up to 50 minutes[188].

This allows more flexible automated multi-step 2PDN strip assays without lengthening the side-pads too much (**Figure 6C**).

A further issue that can be seen from equations 1 and 2 is that the speed of liquid flow in the LF membrane may be affected by variation in sample surface energy and viscosity. The viscosity of human blood and urine varies between samples, which can introduce variation in the test results[189]. Flow speed differences caused by sample viscosity variation can be circumvented by combining the traditional LF strip with a stronger upstream capillary pump in a sealed system[190]. While this method has not yet been tested with real assays, it can equalize the flow of a wide variety of sample types, perhaps opening the door to future simple tests capable of incorporating multiple sample types.

All in all, the key compromise in all these sensitivity improvement methods is the proportional decrease of the detection limit against added complexity and added time. Typically, more time is required if the volume of sample or the proportion of analyte that is separated or captured is increased. Another important open question in many of the concentration and flow control methods is how well they would function with actual sample matrices such as blood or urine.

2.5 The impact of RDTs

2.5.1 Disease burdens

The practical impact of clinical diagnostics depends on the severity and prevalence of the disease the test detects. Regarding the diagnostics discussed in this thesis, the burden of the three diseases in disability-adjusted life years (DALY) are presented in **Figure 7**, together with the burden of HIV/AIDS for comparison. DALY as a metric combines the life years lost to the disease with the possible reduction in the quality of life due to the disability caused by the disease. Malaria, particularly *P. falciparum* malaria, causes by far the heaviest burden and thus improved diagnostics for malaria have the potential for the greatest impact[133]. Particularly, very sensitive malaria RDTs could support malaria elimination efforts. Also the advent of DAA medications for HCV has opened the possibility of eradicating hepatitis C, and rapid diagnostics can be invaluable in helping with this task. Thus far, WHO has prequalified RDTs for the diagnosis of malaria, HIV, Hepatitis B, HCV and syphilis (**Figure 8**).

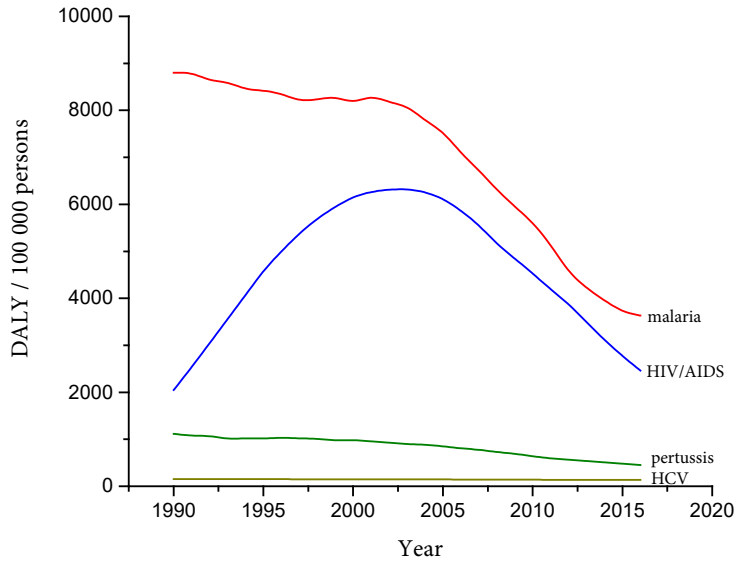

Figure 7. Burden of diseases in low socio-demographic index countries, measured in disability-adjusted life years per 100 000 persons. Included are malaria, pertussis (whooping cough), combined HCV-related diseases (hepatitis C, liver cirrhosis and liver cancer caused by HCV) and HIV/AIDS as comparison. Despite a significant decrease, malaria burden is still the highest[191].

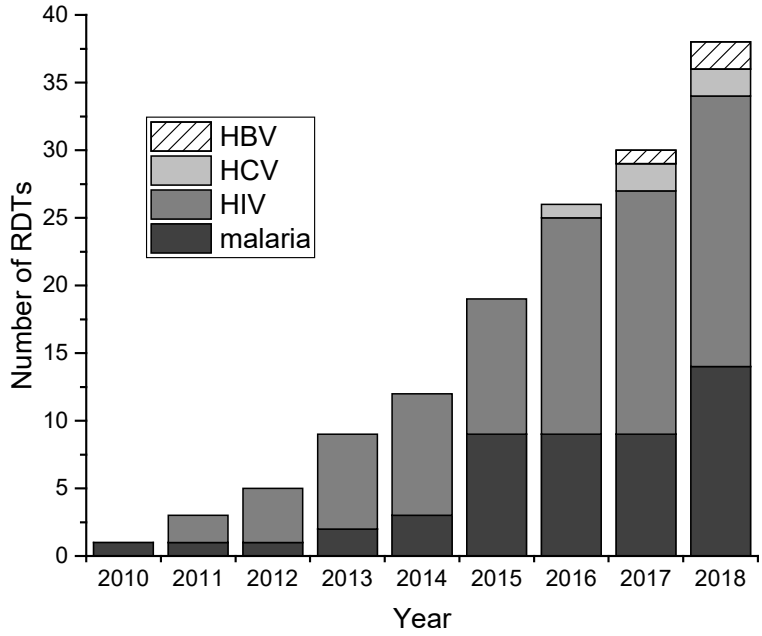

Figure 8. Cumulative number of WHO prequalified RDTs detecting different diseases. The first RDT for malaria was prequalified in 2010. By 2018, there were 14 prequalified RDTs for malaria, 20 for HIV, 2 for HCV and 2 for HBV. One HIV RDT prequalified in 2015 included also a test for syphilis[138].

2.5.2 The ASSURED criteria

The impact of a developed RDT depends not only on the sensitivity and performance of the test, but also on the availability of the test in the places where it is needed the most. In order to comprehensively evaluate the relevant qualities of a RDT, WHO has developed the ASSURED criteria for the ideal diagnostic test for resource-poor areas[192]:

Affordable by those at risk of infection

Sensitive (few false-negatives)

Specific (few false-positives)

User-friendly (simple to perform and requiring minimal training)

Rapid (to enable treatment at first visit) and Robust (does not require refrigerated storage)

Equipment-free

Delivered to those who need it

How do the current diagnostic tests for malaria, pertussis and HCV fit the ASSURED criteria? For *P. falciparum* as well as *P. vivax* malaria, many of the currently available RDTs have sufficient sensitivity and specificity for case-detection and are affordable, rapid, easy to use, equipment free and widely used in resource-poor areas, fulfilling the criteria well[50]. The criteria of sensitivity, specificity, cost and the resulting health outcomes have also been modeled for different malaria RDTs to further evaluate their suitability for different settings[193]. When considering tests useful for supporting MSAT efforts, the only currently available tests with sufficient sensitivity to detect individuals with very low parasitemia are NATs, which are not yet usable for point-of-care.

In principle, there are RDTs for the screening of anti-HCV antibodies from donated blood that fulfill the ASSURED criteria[138]. However, the discrepancy between sensitivities achieved in laboratory and field evaluations of HCV RDTs would suggest that even the RDTs that should be simple to use have problems with user-friendliness and robustness[146, 147]. Current RDTs for HCVcAg are far from meeting the sensitivities required for accurate diagnosis[159, 160]. For pertussis, the field of RDTs is in its infancy, and tests with any relevance for ASSURED criteria are still in the future.

The methods for improving the sensitivity of LF tests have different drawbacks when evaluated with the ASSURED criteria. Sample pre-concentration methods have the potential to improve the sensitivity of RDTs irrespective of the analyte. In particular, efforts have been made to improve the sensitivity of malaria HRP2 RDTs. Although most of these methods would not increase the price of the test prohibitively, they typically lengthen the running time of the RDT and increase the number of handling steps needed.

Flow control methods such as 2PDN and flow barriers can improve the sensitivity without making the RDT more complex for the end-user. However, the concern is that these types of modifications require changes in design and manufacturing, making the tests less affordable. Also, the multi-step or delayed flow methods almost by definition increase the running time of the test.

3 AIMS OF THE STUDY

The overall aim of this thesis was to develop rapid point-of-care *in vitro* –diagnostic tests for use in resource-poor areas, using luminescent lanthanide-based labels to achieve highly sensitive and quantitative tests in the LFIA format.

More specifically, the aims were:

I To develop a simple yet sensitive immunoassay for anti-HCV antibodies, based on a multiepitope protein (MEP) containing HCV epitopes, and on luminescent europium chelate labels.

II To develop a quantitative serological LFIA with luminescent Eu-nanoparticle labels for the diagnosis of pertussis, which is easy to use and suitable for POC environments, and to evaluate this assay against a standardized ELISA with patient samples.

III To develop an ultrasensitive LFIA with luminescent Eu-nanoparticle labels and explore the critical parameters of a quantitative LFIA that affect the dynamic range, assay duration, simplicity and sensitivity of the test.

IV To develop a simple to use and ultransensitive RDT for *P. falciparum* malaria HRP2 antigen using the LF platform and luminescent upconverting nanophosphor labels, and to evaluate the sensitivity of this test against a traditional RDT using visual labels.

4 SUMMARY OF MATERIALS AND METHODS

A brief summary of the materials and methods with some additional information is presented here. More complete descriptions of the materials and methods can be found in the original publications (**I – IV**).

4.1 Photoluminescent reporters

4.1.1 Europium-chelate labels

The europium chelate label (**I**), was an isothiocyanate-modified intrinsically fluorescent nonadentate europium chelate. The synthesis and conjugation of the label to tracer proteins was done as described by von Lode et al.[194]

4.1.2 Europium nanoparticle labels

The carboxyl modified polystyrene nanoparticle labels containing europium chelates were from Thermo Fisher Scientific (USA) (**II**) and Seradyn (USA) (**III**). The particles used had diameters of 99 nm (**II**) and 107 nm (**III**). The goat anti-human IgG (**II**) and mouse monoclonal anti-PSA (prostate specific antigen) 5A10 (**III**) antibodies were covalently conjugated to the active carboxyl groups by NHS-EDC chemistry based on a method described by Valanne et al.[195].

4.1.3 Upconverting nanophosphor labels

Up-converting nanophosphor (UCNP) reporter particles (RD Upcon®540-L-C1-COOH, product number 46-05RD, Kaivogen, Finland) were used as reporters in publication **IV**. The particles had a hydrophilic coating with an average thickness of 10 nm (SD 0.8 nm) and a total diameter of 68 nm (SD 2.7 nm) (**Figure 9**). The particles were covalently conjugated with the anti-HRP2 monoclonal antibody by NHS-EDC chemistry, and the conjugated particles were resuspended into storage buffer.

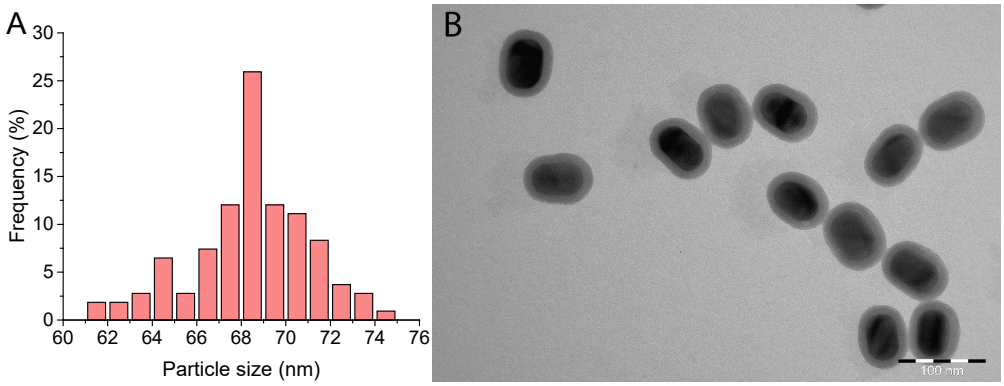

Figure 9. (A) Histogram of the particle size distribution of UCNPs used as labels in publication **IV**. (B) Transmission electron micrograph of the UCNP label particles, magnification 80 000x. The average size of the coated particles is 68 nm (SD 2.7 nm). TEM-image and size data courtesy of Kaivogen Oy.

4.2 Sample panels

The various sample panels used in publications **I**, **II** and **IV** are summarized in **Table 4**. Publication **III** used a free-PSA (fPSA) standard diluted in pooled female serum as the sample and 11 female sera as the negative samples.

Table 4. Sample panels used in the original publications. Number of samples positive for the relevant infection

Sample panel	Positive samples	Negative samples	Notes	Publication
HCV in-house panel	81	51	61 of the positive samples used for double-antigen assay	**I**
Worldwide HCV Performance Panel WWHV301[a]	18	2	samples from HCV genotypes 1 to 4	**I**
Worldwide HCV Performance Panel WWHV302[a]	14	1	samples from HCV genotypes 1 to 6	**I**
Seroconversion panel PHV901[a]	9	2	Seroconversion samples from single patient	**I**
Pertussis panel I[b]	138	0		**II**
Pertussis panel II[b]	32	28		**II**
P. falciparum WHO manufacturer's panel	5	0	Cultured parasite strains	**IV**
P. falciparum Zeptometrix culture panel	6	0	Pre-diluted cultured parasite strains	**IV**
Malaria-negative in-house panel	0	8	Assumed parasite-negative individuals	**IV**

[a] Purchased from SeraCare Life Sciences (USA), [b] Received from Department of Medical Microbiology and Immunology, University of Turku

4.3 HCV immunoassays

4.3.1 HCV multiepitope protein

A recombinant multiepitope protein (MEP) containing seven conserved and immunodominant epitopes from the non-structural and structural proteins of HCV was

designed and produced in *E. coli* (**Figure 10**). Briefly, the synthetic gene, codon optimized for E. coli expression, was custom-synthesized by Geneart (Germany). The gene was subcloned into vectors and expressed in *E. coli* BL21(DE3) strain. The insoluble fractions of the cell lysate were purified in denaturing conditions with Ni-NTA affinity chromatography and dialyzed into a solubilizing buffer.

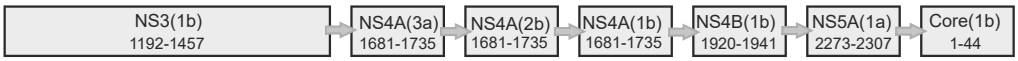

Figure 10. The HCV epitopes in the HCV-MEP. Epitopes from non-structural proteins NS3, NS4 and NS5 as well as from the core protein were used. The amino acid position of each of the epitopes in the original HCV polyprotein is shown, as well as the genotype origin in brackets.

4.3.2 Immunoassay procedures

The HCV MEP was used in both a double-antigen immunoassay and a secondary antibody immunoassay. In the both immunoassays, the capture surface consisted of streptavidin coated wells with immobilized *in vivo* biotinylated MEP. In the one-step double-antigen immunoassay, the sample and Eu-chelate labeled MEP were then added to the well (**Figure 11B**). The secondary antibody immunoassay was done in a two-step format, where the sample is added in the first step, unbound sample is washed away and Eu-chelate labeled goat antihuman-IgG was added to the well. After a wash step, the Eu-chelate luminescence of both immunoassays was then measured with a time-resolved luminescence reader.

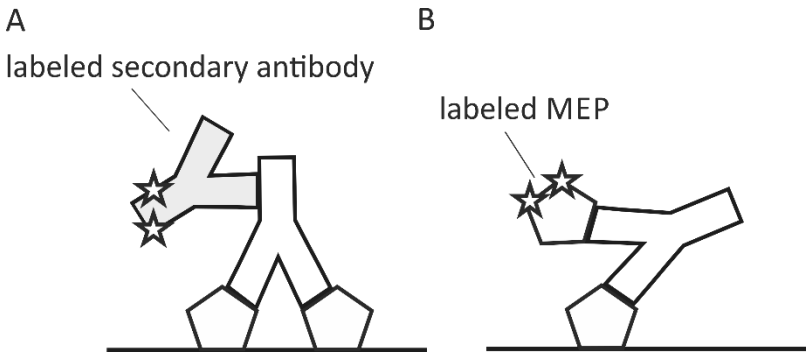

Figure 11. Formats of the HCV immunoassays. (A) In the two-step secondary antibody immunoassay, a labeled antihuman-IgG antibody was used as reporter. (B) In the one-step double-antigen immunoassay, a labeled MEP was used as reporter.

4.4 Avidity index immunoassay

An avidity index immunoassay was performed in **I** and with the pertussis samples of **II**, but the avidity index data for pertussis samples were not included in the publication. In an avidity index immunoassay, the antigen-specific antibodies of the sample are first bound to the surface capture antigen as in a normal well immunoassay. Then, a dissociation step is performed by incubating the wells containing the bound antibodies with a chaotropic agent, which dissociates weakly bound antibodies. After a wash step, the bound antibodies are detected with a labeled secondary antibody and this signal is compared with a signal from the same sample acquired without the dissociation step. The ratio between dissociation step signals and normal assay signals is the avidity index.

In the HCV avidity index immunoassay (**I**), the dissociation step was performed by adding 8 M urea, a relatively strong chaotropic agent, which can be used to detect increase of the anti-HCV IgG avidity index for up to 36 months from the start of infection[196]. With the pertussis samples, the avidity index immunoassay was performed based on the avidity index immunoassay described by Barkoff et al. in wells coated with the PT antigen[197]. The avidity indexes of 56 patient samples were determined. The dissociation step was performed with 30 mM diethylamine as the chaotropic agent and with the antihuman-IgG conjugated Eu-np as labels.

4.5 LFIAs

LFIAs were performed in **II – IV**. The assay procedure consists of the preparation of LF strips and the actual LF immunoassay procedure.

4.5.1 Preparation of LF strips

The LF test strips were assembled on an adhesive plastic support from G&L Precision Die Cutting (**II**, **III**) (USA) or from Kenosha Tapes (**IV**) (Netherlands). The parts added onto the support included a 10 mm wide buffer pad (**IV**) (Ahlstrom cotton fiber 238, Ahlstrom, Finland), and a sample/conjugate pad of 10 mm wide G041 glass fiber (Millipore, USA) (**II**, **III**) or 10 mm wide Ahlstrom 8950 glass fiber (**IV**). The nitrocellulose membranes used were Millipore HF90 (**II**, **III**), Millipore HF180 (**III**) and LFNC-C-SS22-70 (Nupore, India) (**IV**), all 25 mm wide. The test and control lines were dispensed on the nitrocellulose with the Linomat 5 non-contact printer (Camag, Switzerland).

For the anti-pertussis LF assays (**II**), the pertussis toxin (PT) (GlaxoSmithKline, Belgium) test line was printed in a 10 mM Tris-HCl buffer (pH 8.0) at a speed of 80 nL/s to a density of 200 ng/cm. A control line of rabbit anti-goat IgG (Sigma-Aldrich, USA) was printed 8 mm from the test line in the same buffer at a speed of 200 nL/s to a density of 400 ng/cm.

For the PSA LF immunoassay (**III**), the streptavidin test line was printed in 10 mM citrate-phosphate buffer (pH 5.0) at a speed of 100 nL/s to a density of 4000 ng/cm. Additionally, a biotinylated PSA capture antibody H117 (University of Turku) was printed on top of the streptavidin line in 10 mM Tris-HCl buffer (pH 8.0) at a speed of 100 nL/s to a density of 250 ng/cm. A control line of rabbit anti-mouse IgG (Dako, Denmark) was printed 6 mm from the test line in 10 mM Tris-HCl buffer (pH 8.0) at a speed of 200 nL/s to a density of 400 ng/cm.

For the HRP2 malaria LF immunoassay (**IV**), the anti-HRP2 PTL3 IgM (National Bioproducts Institute, South Africa) was printed in 10 mM Tris-HCl buffer (pH 8.0) containing 5 % ethanol and 1 % sucrose at a speed of 50 nL/s to a density of 600 ng/cm. A control line of rabbit anti-mouse IgG (Dako, Denmark) was printed 5 mm from the test line in 10 mM Tris-HCl buffer (pH 8.0) at a speed of 200 nL/s to a density of 400 ng/cm.

After the printing of test and control lines, all LF cards were dried for 2 – 3 h at +35°C, then cut into 4.8 mm wide strips and stored in room temperature, protected from humidity. For the HRP2 malaria LF strips (**IV**), 25 ng/strip of UCNP-label conjugate was dried onto the conjugate pad in 10 mM Tris-HCl buffer (pH 8) containing 0.1 % casein, 2.9 % NaCl, 1 % Triton-X-100m 1 % Tween-20, 0.2 % bovine gamma globulin, 2 mM KF, 5 % sucrose, 5 % trehalose.

4.5.2 Anti-pertussis LFIA procedure

The anti-pertussis LF immunoassay (**II**) had a four-step procedure, in which the strips were sequentially moved to microtiter wells containing the reagents (dip-stick procedure). First, 20 µl of 1:250 diluted sample serum was added to the strips, followed by a wash step. After this, the label of goat anti-human IgG conjugated Eu-np was added to the strips. Finally, a second wash step was performed as before. The total time for these steps was 30 min.

The Eu-np luminescence from dry strips was measured by scanning the test and control line areas with measurement points at 0.35 mm intervals with a Victor 3 plate reader,

using a 615 nm emission filter. The measurement was time-resolved with a 1200 µs delay time, a 400 µs counting window and a duty cycle of 1800 µs.

4.5.3 fPSA LFIA procedure

The fPSA LFIA (**III**) had a two-step dip-stick procedure. First, 10 µl of sample serum and together with the label consisting of anti-PSA antibody 5A10 conjugated Eu-np were added to the strips. Immediately after this, the strip was washed by adding 20 µl of assay buffer. The strips were measured at different time-points with a Victor X4 plate reader (PerkinElmer), using a 615 nm emission filter. The measurement was time-resolved with a 400-µs delay time, a 400-µs counting window and a duty cycle of 1000 µs.

4.5.4 Malaria HRP2 LFIA procedure

The malaria HRP2 UCNP-LFIA had a simple two-step procedure, with the LF strip in a cassette. First, 5 µl of sample whole blood was applied to the conjugate/sample pad, then 80 µl of running buffer was added to the buffer pad. After 20 min, the test and control line area of the strip was scanned at 0.2 mm intervals with an Upcon UCNP-reader device (Labrox, Finland).

The traditional visual label HRP2 RDTs (First Response Malaria Antigen test kit, Premier Medical Corporation, India) were performed according to manufacturer's instructions and the signal was read visually as well as with an image analysis reader (Inme reader, Inme, Finland) and a reflective reader (ESEQuant LR3, Qiagen, Germany).

4.6 LF signal analysis

With all the anti-pertussis and fPSA assays, the maximum reading from the test line peak was considered the signal from the sample. With the malaria HRP2 LF assay, the signal was extracted by a formula, where the background signal of the individual strip is subtracted from the maximum signal of the test. This background-subtracted signal is then divided by the signal of the control line. This ratiometric analysis is illustrated in **Figure 12A**. The equation is:

$$S = \frac{(T - \frac{A + B}{2})}{C}$$

Wherein S is the final signal from the strip, T is the maximum reading from the test line, A is the background reading from the nitrocellulose 1 mm before the test line, B is the similar background reading 1 mm after the test line and C is the maximum reading from the control line.

Subtracting the luminescence background level of the individual strip from the maximum reading from the test line improved the performance of the assay. This is because individual strips are in somewhat different stages of UCNP label flow when the strips are measured after 20 min, which causes different levels of background (**Figure 12B**).

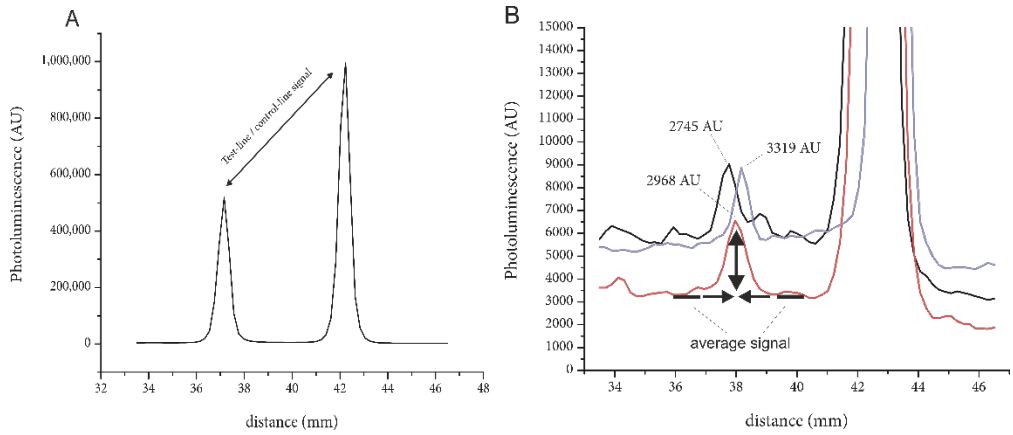

Figure 12. Illustration of LF signal analysis methods. (A) In ratiometric analysis, the ratio of the maximum signals of the test and control line is used. (B) In the peak detection and background subtraction system, the average signal level from around the test line is automatically subtracted from the test line peak signal. This decreases the variation between replicates, as illustrated. The background-subtracted peak signals for three replicates are shown in the figure.

For the anti-pertussis LF assay, the strips were analysed both according to the maximum reading from the test line and with ratiometric analysis. When test line readings were used directly, the average coefficient of variation between two replicates of a same sample was 17.6 %, while with ratiometric analysis the variation was 14.5 %.

5 SUMMARY OF RESULTS AND DISCUSSION

5.1 Immunoassay for hepatitis C antibodies

In **I**, a single recombinant multiepitope protein (MEP) was developed and evaluated as a diagnostic binder antigen in an anti-HCV antibody immunoassay. Two types of anti-HCV immunoassays were developed and evaluated: a secondary antibody immunoassay and a double-antigen immunoassay, both using fluorescent Eu-chelates as labels. The sensitivity of the secondary antibody immunoassay with the in-house and worldwide performance panels was 95.6 % (108 / 113 positive samples) and the specificity was 100 % (58 / 58 negative samples) (**Table 5**). A "hi-lo" graph showing the separation of positive and negative results of both assay types are presented in **Figure 13**. Three of the five false-negative samples of the in-house panel had been categorized as positive with both commercial reference assays, while two of the false-negative samples had been categorized as positive only with one of the reference assays. With the worldwide performance panels, the secondary antibody immunoassay detected all the 32 positive samples consisting of all the six major genotypes.

The double-antigen immunoassay was tested with the same panels as the secondary antibody immunoassay, but only 61 of the total of 81 positive in-house panel samples were tested due to the larger sample volume required for the double-antigen assay required. The sensitivity of the double-antigen immunoassay was 91.4 % (85/93 positive samples) and the specificity was 100 % (55/55 negative samples). With the worldwide performance panels, the double antigen immunoassay detected 31 out of 32 samples, the false negative result was obtained with a weak positive genotype 2a/2c sample.

The results of the secondary antibody immunoassay with a MEP capture antigen had a high sensitivity and specificity, and it recognized all the main HCV genotypes. Taken together, the results show that the epitopes chosen for the MEP were sufficiently diverse. Furthermore, a single simple-to-produce antigen including several epitopes could be used instead of multiple recombinant proteins as an affordable diagnostic intermediate in a HCV immunoassay.

The sensitivity of the MEP-based double-antigen immunoassay was lower than in the secondary antibody immunoassay. The issues of double-antigen antibody immunoassays are discussed in the avidity of antibody response in immunoassays section (Section 5.5.).

Table 5. Performance of the two MEP-based HCV assays in the in-house panel.[a]

Assay	% Sensitivity (95 % CI)	% Specificity (95 % CI)
secondary antibody assay	95.6 (90.0-98.6)	100 (93.8-100)
double-antigen assay	91.4 (83.6-96.2)	100 (93.8-100)

[a]95 % CI: 95 % confidence interval calculated according to the Exact Confidence Interval method (Clopper and Pearson, 1934)

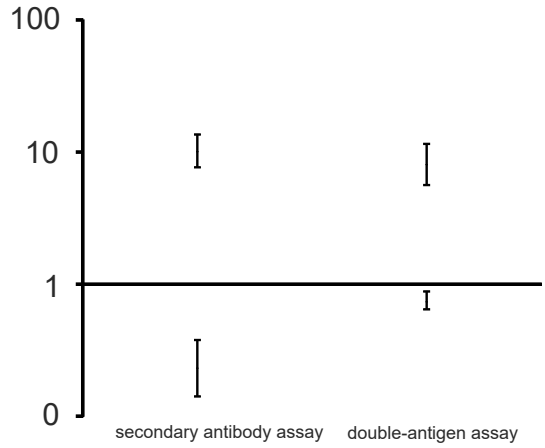

Figure 13. The 95 % confidence limits of the positive and negative populations of the anti-HCV secondary and double-antigen immunoassays presented in a Hi-Lo graph (adapted from **I**).

5.2 Anti-pertussis LFIA

In **II**, a LFIA was developed for detecting anti-pertussis toxin (anti-PT) IgG antibodies from serum. The developed assay was compared with an in-house standardized ELISA with 198 patient samples. Correlation between the pertussis LFIA and the ELISA was calculated with 170 of the samples that had measurable anti-PT in the ELISA.

The correlation between the LFIA and the ELISA as well as two typically used clinical cutoffs are shown in **Figure 14**. The 100 IU/ml cutoff is typically used in standard patient testing while a lower 50 IU/ml cutoff can be used during epidemics[121]. At the 100 IU/ml cutoff, the overall agreement between ELISA and LFIA was 157/170 (92 %). The LFIA sensitivity compared with ELISA was 63/72 (88 %; CI 77-94 %) and specificity 94/98 (96 %; CI 89 – 99 %). At the lower 50 IU/ml cutoff, the overall agreement was 150/170 (88 %). The sensitivity was 78/93 (84 %; CI 75 – 90 %) and specificity 72/77 (94%; CI 85 – 98%).

The analytical sensitivity of the LFIA was 20 IU/ml, which is below any clinically relevant cutoff. This analytical sensitivity could have been lower with the same LF setup, but the

dynamic range of the test was optimized for the clinically relevant concentration range so that the highest discrimination of samples could be achieved at the 100 IU/ml cutoff.

The correlation between the developed LFIA and the in-house ELISA was good (R^2 = 0.83, p<.001), considering that there is also variation between different commercial ELISA kits[120, 198, 199]. Naturally, ELISA and LF are two very different types of immunoassays. In this case, the assay steps of the ELISA and the developed LFIA are identical, with sequential introduction of the sample and the label into the strip with wash steps in between. Still, there are two major differences that can cause discrepancies between ELISA and LF results. First, the avidity of the antibody response against the PT antigen varies between vaccinated and naturally infected patients, as well as during affinity maturation in the course of the infection[197, 200]. The effect of antibody avidity on the results is further discussed in Section 5.5.

Secondly, in the LF strip, the sample has to pass through the porous nitrocellulose material while travelling to the capture test line, while in ELISA the liquid-to-solid capture interface is much simpler. Therefore, there may be different types of matrix interference from the serum samples in these two immunoassay types.

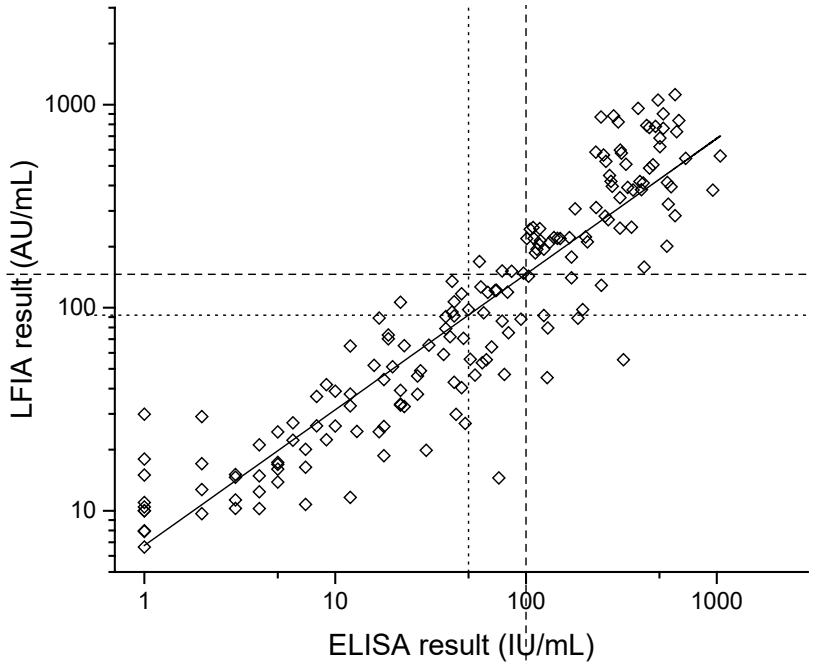

Figure 14. Correlation of LFIA and ELISA results of the positive samples (from **II**). The LFIA results are presented as arbitrary units (AU). The 100 IU/mL cutoff is shown with dashed lines and the 50 IU/mL cutoff is shown with dotted lines.

5.3 LFIA for malaria HRP2 antigen

In **IV**, a high-sensitivity LF RDT was developed for *P. falciparum* HRP2 antigen, using photoluminescent up-converting nanophosphor reporter particles. The developed LF assay with luminescent labels (UCNP-LF) was compared with a WHO prequalified commercial RDT with parasite culture panels.

The UCNP-LF and commercial RDTs were tested with two panels of culture samples. For the Zeptometrix panel, only PfHRP2 concentration information was provided while the WHO panel had both parasite density and PfHRP2 concentration information. The detection limit with UCNP-LF in PfHRP2 concentration varied from 2 to 40 pg/ml (mean 13.5 pg/ml, median 12 pg/ml) with the 11 culture samples of a total of eight different *P. falciparum* strains (**Table 6**). The detection limit of the commercial RDT varied from 400 to 820 pg/ml (mean 625 pg/ml, median 700 pg/ml) with the same samples.

For the five WHO panel samples, also the parasite density values were provided. The detection limit of the UCNP-LF in terms of parasite density varied from 0.2 to 2 parasites/µl (mean 0.8 parasites/µl, median 0.5 parasites/µl) (**Table 7**). The corresponding detection limit of the commercial RDT varied from 20 to 100 parasites/µl (mean 48 parasites/µl, median 50 parasites/µl). The sensitivities achieved with the commercial RDT corresponded well to the sensitivities of five WHO-evaluated HRP2 RDTs tested by Jimenez et al. (**Table 8**)[44]. Overall, the UCNP-LF had a 50 to 250 –fold improved sensitivity compared with the commercial RDT.

Table 6. Analytical sensitivities of UCNP-LF and First Response HRP2 RDT in pg/ml of HRP2 in Zeptometrix and WHO manufacturer's panels (from **IV**). The results for the First Response HRP2 RDT are shown from both visual inspection and the image analysis based reader.

			Test analytical sensitivity, pg/ml HRP2		
			UCNP-LF	First Response HRP2	
Sample type	**Sample**	**Type**		Visual inspection	Inme reader
Zeptometrix culture panel	FCQ79	A	12[a] (400)	400[a] (800)	400 [a] (8000)
	Benin I	A	12	800	800
	Borneo	C	12	400	400
	Santa Lucia	B	12	800	800
	W2	B	40	800	800
	PH1	C	12	800	800
WHO manufacturer's panel	US05F Benin I	A	2	490	490
	US05F Santa Lucia	B	10	420	420
	US08F Nigeria XII	B	7	700	700
	US05F FC27/A3	B	20	410	820
	US05F PH1	C	10	450	450

[a] Own dilutions, official dilutions in parentheses. The official dilutions of the Zeptometrix culture panel FCQ79 samples gave discrepant results, the same concentration were also diluted and tested from the highest concentration sample. The Zeptometrix PH1 sample was also tested with own dilutions as a comparison, there was no difference between these results.

Table 7. Analytical sensitivities of UCNP-LF and First Response HRP2 RDT in parasites/µl in WHO manufacturer's panel (from **IV**). The results for the First Response HRP2 RDT are shown from both visual inspection and the image analysis based reader.

			Test analytical sensitivity, parasites/µl		
			UCNP-LF	First Response HRP2	
Sample type	**Sample**	**Type**		Visual inspection	Inme reader
WHO manufacturer's panel	US05F Benin I	A	0.2	50	50
	US05F Santa Lucia	B	2	100	100
	US08F Nigeria XII	B	0.2	20	20
	US05F FC27/A3	B	0.5	10	20
	US05F PH1	C	1	50	50

Table 8. Comparison of the analytical sensitivities reported by Jimenez et al. for five WHO-evaluated RDTs and sensitivities reported in publication **IV** for the commercial RDT, with both panels.

		Test analytical sensitivity, pg/ml HRP2		
Sample strain	**Type**	**Jimenez et al. RDTs**	**WHO RDT**	**Zeptometrix RDT**
Benin I	A	800 - 1600	490	800
Santa Lucia	B	800 - 1600	420	800
PH1	C	400 -800	450	800

WHO recommends that malaria RDTs should remain stable even with temperature peaks up to 50 °C for a minimum of two to six months[201, 202]. The UCNP-LF tests remained stable for at least five months, when stored at room temperature, +40 °C, and at +50 °C. The prozone effect (hook-effect) was tested for the UCNP-LF with recombinant HRP2 concentrations up to 1 mg/ml without adverse effects to the test result.

To avoid subjective interpretation of the results from commercial RDT utilizing visual labels, the line intensities were observed visually and with an instrument detecting the lines with image analysis, and additionally with an instrument with reflective measurement. There were small differences between the results from visual examination and image analysis (**Tables 6 & 7**). The observed differences are due to the red coloring of the nitrocellulose from lysed red blood cells. In fact, measurement of the commercial RDT with the reflective detection instrument was not feasible, since the uneven red color on the nitrocellulose caused numerous false positive readings. Examples of strips and readings with false positive results are presented in **Figure 15**. This is a problem unique for the malaria RDTs, since lysis of red blood cells is required to release the HRP2 antigen, as opposed to being filtered at the beginning of the strip in most RDTs. While the human eye can see the test line quite clearly even with a colored nitrocellulose, the coloring problem should be taken into account when choosing the instruments for reading the visual malaria RDTs.

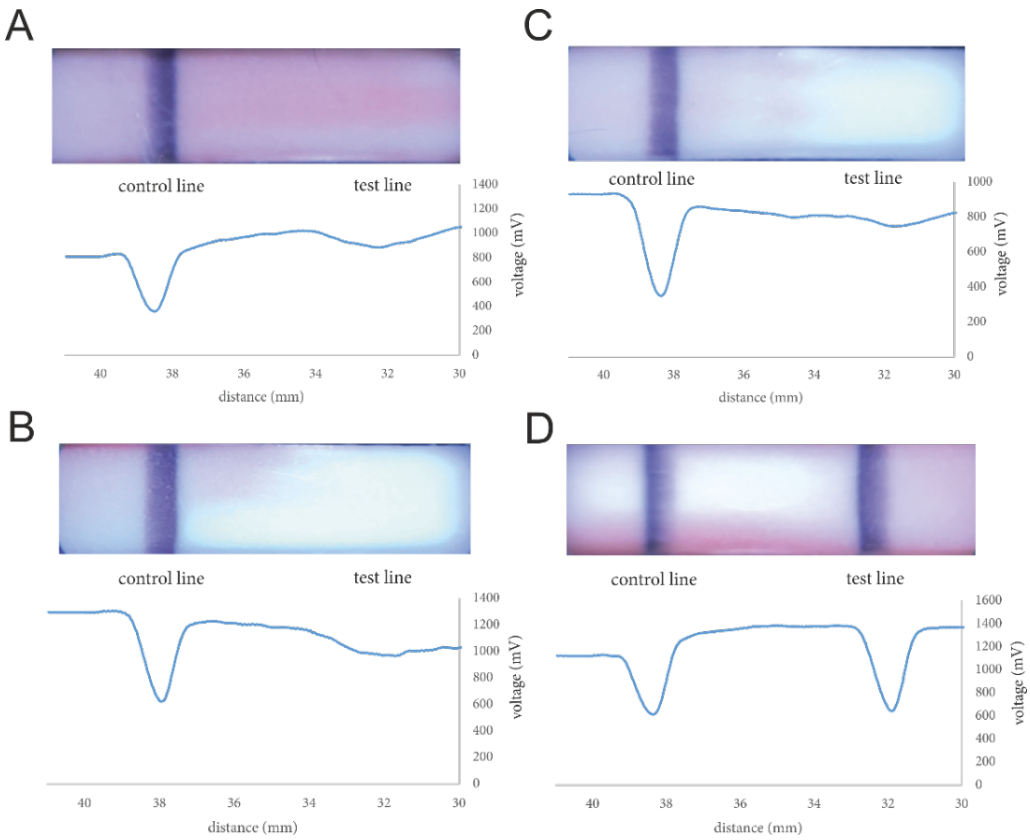

Figure 15. Examples of conventional RDT strips with red coloring due to lysed red blood cells. The image above shows the strip and the reflectance scanning result of the strip with a reflective instrument is shown below. Strips A, B and C are examples of false positive results when read by a reflective instrument. Strip D is an example of a true positive result.

The UCNP-LF assay employs a widely used commercial antibody pair (purchased from National Bioproducts Institute (South Africa)) as binders[203]. Therefore, it is likely that the dramatic improvement in the sensitivity of UCNP-LF as compared to the standard HRP2 RDT is due to the use of the luminescent UCNP-label in the place of classically used labels. The advantages and drawbacks of the luminescent instrument-read labels are discussed the Section 5.6.

Recently, an RDT with visually read labels for HRP2 with improved sensitivity was described[56]. The test had a detection limit of 3 parasites/µl with cultured parasite strains and the detection limit with recombinant and native HRP2 varied from 40 to 125 pg/ml. Population screening studies with the new test in endemic areas showed that an RDT with improved sensitivity is useful for detecting a larger proportion of the parasite carriers than the traditional RDTs[101, 102]. Nonetheless, in screening studies conducted

with samples from the endemic population both by the sensitive RDT as well as with NAT-methods show that the RDT can detect only about 50 % of the infectious parasite carriers[103]. This suggests that supporting malaria elimination efforts needs even more sensitive RDTs.

5.4 Kinetics of LFIAs

In LFIAs, the signal from the strip can theoretically be measured at any time-point after starting the assay. Naturally, a rapid test would preferably be measured at the earliest feasible time-point. However, in practice the detection time is always a compromise between the early measurement time and the development of the test line signal. The use of instrument-read luminescent labels allows real-time measurement of the development of the signal on the LF strip. In **III**, the accumulation of the label on the test line of the LF strip and the resulting increase in measured signal was followed on LF strips with a slow (small pore size) nitrocellulose and fast (large pore size) nitrocellulose membrane (**Figure 16**). The accumulation of the Eu-np label reached the maximum signal levels were after 17 minutes with the fast Millipore HF75 nitrocellulose as compared to 21 minutes with the slow Millipore HF180 nitrocellulose. However, due to the faster flow and resulting unfavorable binding kinetics, the detection limit of the assay on the faster nitrocellulose increased from 10 pg/ml to 14 pg/ml.

Overall, the effect of nitrocellulose pore size on the sensitivity and speed of the assay was quite limited, at least in this particular case: The flow rate of the fast HF75 nitrocellulose was 2.4-fold faster than the slower HF180 nitrocellulose, yet the difference in assay time decreased only 4 minutes, from 21 to 17 minutes. Also, the detection limit increased only from 10 pg/ml to 14 pg/ml. The measurement time-point for each LF test can of course be set to any required time, but there will be a compromise with the detection limit of the test. Additionally, in this case the measurement time for a quantitative assay needs to be exact, since the calibration of the assay will be done for a specific time-point and the signal will be significantly different even one minute before or after the calibrated time-point, as can be seen in **Figure 16**.

For this reason, in **II** all the pertussis antibody LF assays were measured after the strips had dried overnight and the flow of label particles had ceased. In this way, the large number of samples could be analyzed without the measurement time affecting the quantitative results. In **IV**, the measurement time-point of the malaria HRP2 LF assay was set to 20 minutes according to the FIND target product profile and the assay was

optimized for this time-point [53]. The malaria HRP2 LF assay strips can also be measured at any time after 20 minutes, so the strips can be stored and measured at a later date.

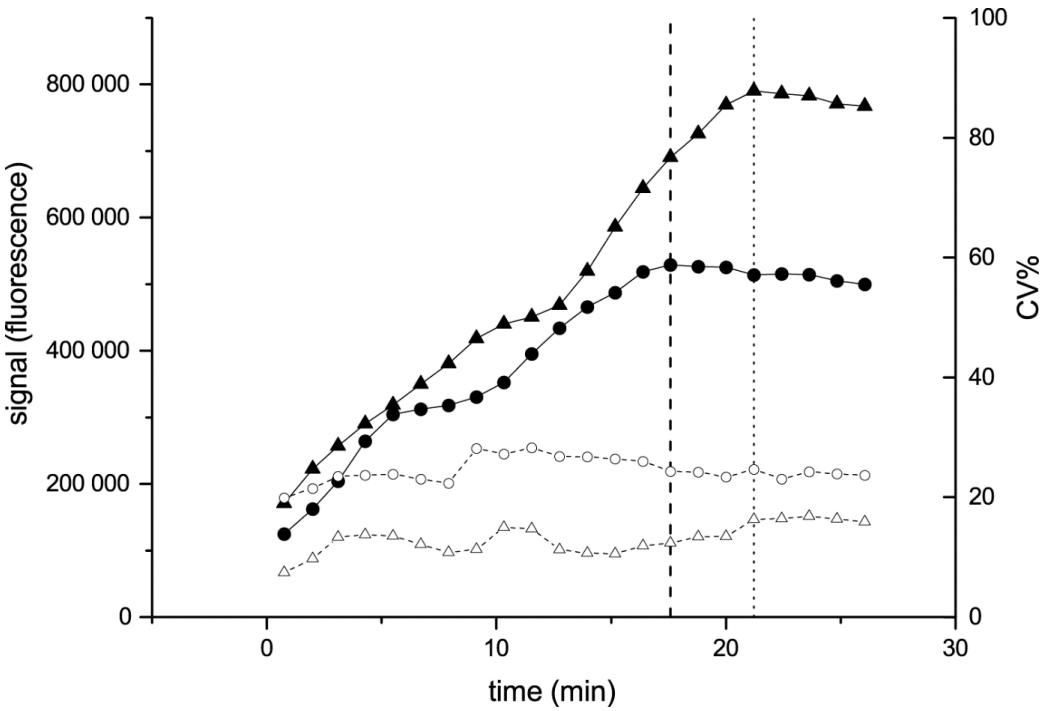

Figure 16. fPSA LF immunoassay signals with luminescent Eu-np labels at different time-points with the fast HF75 (●) and slow HF180 (▲) nitrocellulose membranes (from **III**). The assay with fast HF75 nitrocellulose reached maximum signal after 17 minutes (dashed line), while with the slow HF180 maximum signal was reached after 21 minutes (dotted line). Coefficients of variation with three replicates for both test types are shown with empty symbols.

5.5 Avidity of antibody response in immunoassays

While affinity refers to the direct binding interaction between two molecules, antibody avidity reflects the effective average affinity of the specific antibodies to an immunogen as a whole in an immune response[204]. The avidity of an immune response typically increases over time after exposure to the immunogen as the immune response matures[200]. The avidity index can be tested with immunoassay protocols that have a dissociation incubation phase, in which a chaotropic agent dissociates the weakly bound low affinity antibodies. Since avidity increases during affinity maturation in the acute phase of the infection, the avidity index immunoassays can be used to separate recent and established infections, for example in the case of HCV and HIV antibody

immunoassays[205, 206, 207]. The avidity of the antibody response is also important for the efficiency of the resulting protective immunity, and the avidity index immunoassay can be used for studies of immunity, as in the case of *P. falciparum* malaria[208] or pertussis[197].

The avidity of the antibody response in the patient sample panels was investigated in the anti-HCV antibody immunoassay (**I**) and the anti-pertussis antibody LF immunoassay (**II**). In the case of the anti-HCV antibody immunoassay, the goal was to compare the effect of sample antibody avidity to the two types of developed assays, the secondary antibody immunoassay and the double-antigen immunoassay. The avidity index for the in-house panel patient samples ranged from 0.05 to 0.87. The patient samples were sorted into low avidity index (<0.3) and high avidity index (>0.5) cohorts according to a classification by Hedman and Rousseau[209]. In the double-antigen immunoassay, the low avidity index cohort had a mean signal to cutoff ratio of 2.1 (95 % confidence interval: 1.2 – 3.0) and the high avidity cohort a mean signal to cutoff ratio of 42.9 (95 % CI: 24.2 – 61.6) (**Table 9**). In the secondary antibody immunoassay with the same sample cohorts, the low avidity index cohort had a mean signal to cutoff ratio of 8.0 (95 % CI: 2.8 – 13.2) and the high avidity index cohort a signal to cutoff ratio of 20.5 (95 % CI: 17.7 – 23.2).

Table 9. The different signal to cutoff ratios (S/Co) of secondary antibody immunoassay in the low avidity index (<0.3) and high avidity index (>0.5) patient sample groups. The 95 % confidence intervals are shown in brackets.

Assay	S/Co in low avidity index cohort, AI<0.3 (95% CI)	S/Co in high avidity index cohort, AI>0.5 (95% CI)
secondary antibody assay	8.0 (2.8 – 13.2)	20.5 (17.7 – 23.2)
double-antigen assay	2.1 (1.2 – 3.0)	42.9 (24.2 – 61.6)

The avidity index of the HCV seroconversion panel was also investigated and compared between the secondary antibody immunoassay and double-antigen immunoassay results (**Figure 17**). Notably, the secondary antibody immunoassay detected seroconversion already 97 days after first bleed where the sample had an avidity index of 0.18, the double-antigen immunoassay detected seroconversion only with the sample taken 159 days after first bleed, which had an avidity index of 0.53.

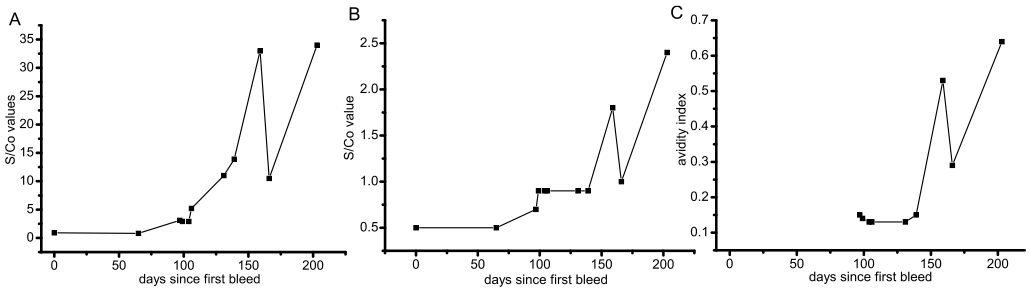

Figure 17. Signal to cutoff ratios of (A) secondary antibody immunoassay (B) double antigen immunoassay, and the avidity index of the positive seroconversion samples (adapted from **I**).

These results suggest that the double-antigen immunoassay is vulnerable to low affinity antibodies in the patient samples. This is due to the necessity of two independent binding events in the double-antigen immunoassay. The antibody needs to bind specifically to both the capture and tracer antigen to be detected. In contrast, in the secondary antibody immunoassay the antibody can bind bivalently to the capture surface, wherein it is recognized by the high-affinity labeled secondary antibody. The end result is that the practical avidity of the antibody binding increases with bivalent binding, which in turn decreases the affinity effect of any antibody on the secondary antibody immunoassays[210 211].

With the anti-pertussis antibody LFIA, the avidity indexes of the patient samples were measured to investigate the samples that had discrepant results between the LFIA and ELISA. In the LFIA, the sample antibodies have a very limited time to react with the capture PT antigen on the test line, whereas in ELISA the sample incubation time with the capture PT is typically hours[212]. Therefore, there may be a risk that lower avidity samples have anomalously low signals in the LFIA as compared to ELISA. However, while there was wide variability in the avidity indexes of the patient samples, the four discrepant samples did not show dramatically lower avidity indexes when compared to the rest of the samples (**Figure 18**). The results suggest that in this case the LFIA is not vulnerable to low AI samples.

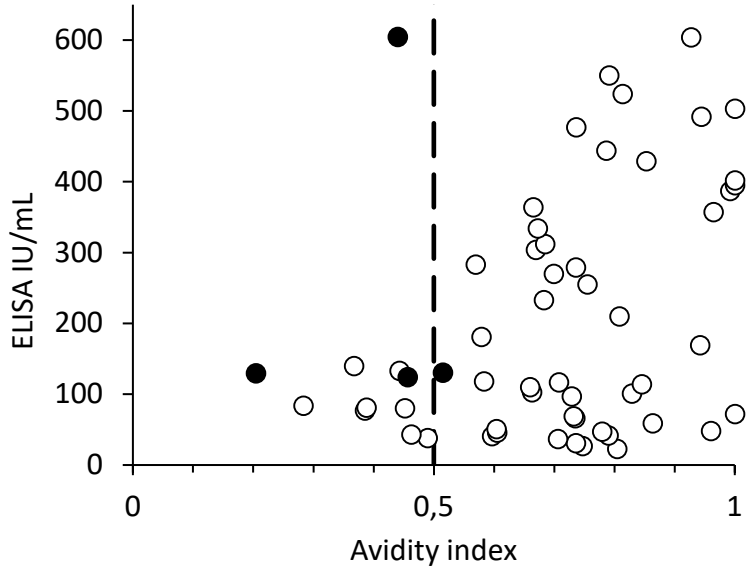

Figure 18. Avidity index of anti-PT antibodies was measured from 56 serum samples. A threshold of 50% AI (dashed line) was used to determine strong and weak binding. The four discordant samples are shown as filled circles.

In order to simplify the assay protocol for the anti-pertussis LFIA, it could be possible to develop a double-antigen LFIA with the PT antigen. In this case the differences in the sample avidities could cause larger discrepancies between the double-antigen LFIA and ELISA, as indicated by the anti-HCV immunoassay results. Also, the double-antigen LFIA would detect the anti-PT IgM and IgA responses in addition to the IgG, further weakening the correlation to ELISA.

5.6 Luminescent labels in RDTs

Three types of luminescent labels were used in the publications of this thesis. In **I** a europium chelate label was used, while in **II** and **III** europium chelate –doped nanoparticle (Eu-np) labels were used and upconverting nanophosphor (UCNP) labels were used in **IV**. In **I**, the luminescent chelate labels were used in place of the enzyme labels typically used in well-based enzyme immunoassays. In **II**, **III** and **IV**, the luminescent particles were used as labels in LFIAs, replacing the typically used visual labels.

Eu-np are polystyrene nanoparticles doped with Eu-chelate labels. They produce very high specific activity signals, since lanthanide chelate fluorescence does not suffer from

self-quenching, and can be measured with minimal background fluorescence due to their large Stokes' shift and long-lifetime luminescence[213]. Eu-np labels have been used before to develop quantitative and very sensitive LFIAs.[214-220].

UCNP labels have an inorganic crystal core containing lanthanide ions, which can up-convert long wavelength infrared radiation to shorter wavelength long-lifetime phosphorescence. The resulting anti-Stokes' shift is not generally found in nature, which helps eliminate any background photoluminescence from the LF strip materials or the sample. Also, UCNPs can withstand high intensity excitation light, which allows the use of high-energy infrared lasers as the excitation source. Together, these qualities make UCNPs an excellent label for high-sensitivity assays[221]

The research in this thesis did not attempt to directly compare the sensitivities of different types of labels. However, the sensitivity of the UCNP-LFIA (**IV**) was compared with a commercial RDT using visual labels, using the dilutions of *P. falciparum* malaria culture samples. An example of the signal-to-cutoff levels achieved by the two different assays with the WHO Benin I culture sample are presented in **Figure 19**. The signal-to-cutoff levels of the commercial First Response HRP2 RDT are measured with an instrument detecting the test line concentration with image analysis. Overall, the UCNP-LF improved the detection limit for *P. falciparum* HRP2 50- to 250-fold.

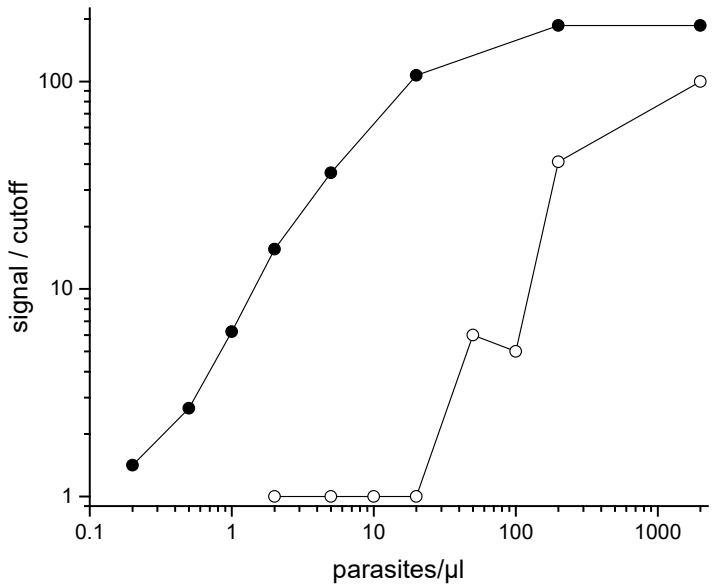

Figure 19. Comparison of the signals from WHO Benin I panel sample dilutions. UCNP-LF results shown with filled markers, traditional First Response HRP2 RDT using visual labels shown with empty markers.

It is difficult to directly compare the detection limits achieved by different LFIAs using different types of labels. However, an overview of the published detection limits converted into molarity can be used to gain a general picture of the sensitivities of different label technologies (**Figure 20**). The fPSA LFIA (**III**) with Eu-np labels achieved a detection limit of 3.5 x 10^{-13} M, and the HRP2 malaria UCNP-LF (**IV**) had detection limits ranging from 1.3 x 10^{-12} to 6.7 x 10^{-14} M depending on the culture sample.

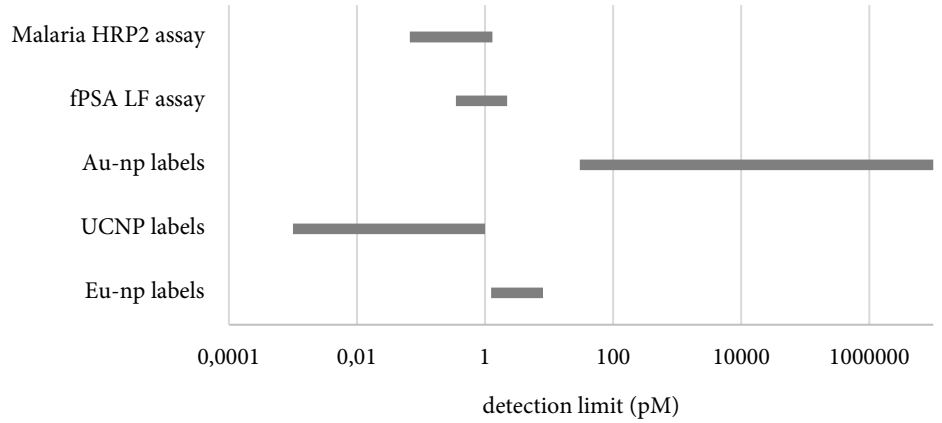

Figure 20. Comparison of detection limits achieved in published LFIAs using different label technologies with the detection limits of the fPSA LF assay using Eu-np labels and malaria HRP2 LF assay using UCNP-labels.

Previously described LFIAs utilizing Eu-np reporters for a variety of analytes had their detection limits in the 10^{-12} M range[216-219]. LFIAs using UCNP-labels have reached detection limits ranging from 10^{-12} to 10^{-15} M[222-225]. The detection limits of LFIAs based on Au-np labels vary from 10^{-5} to 3 x 10^{-11} [226].

For point-of-care applications, a portable, simple and affordable reader is required to achieve a feasible field-usable test. Portable readers capable of reading Eu-np LFIAs have been developed for both research and commercial use[216, 218-222, 227]. Similarly, several readers for UCNP-LFIAs have been described[228-232].

In addition to the significant improvement in sensitivity, the instrument-read LF assays have other advantages over visually read LF assays. An instrument can objectively interpret, archive and transmit the results, simplifying the technical aspects of the assay, by reducing the requirements for operator experience and removing sources of subjectivity and error[233]. Also, any quantitative LF assay requires an instrument for

reading the signal. Luminescent labels are very suitable for quantitative assays since they have lower background and wider dynamic range than typical visual absorbance-based labels[234].

6 CONCLUSIONS

There is a need for more sensitive simple-to-use, yet affordable RDTs, both for routine case detection as well as in support of control and eradication efforts. For malaria, the current RDTs are widely available, affordable and sufficiently sensitive for passive case detection. Nevertheless, more sensitive malaria RDTs are needed to support the elimination efforts. The case for HCV RDTs is similar: current RDTs are usable as the first screening test, but point-of-care tests, which could help in eradication efforts by confirming an active hepatitis C infection are not available. For pertussis, there is a need for both serological and direct antigen detection point-of-care immunoassays.

When considering the techniques to improve LF test sensitivity, the key question is the compromise between sensitivity on the one hand and test complexity as well as added time on the other. Test complexity includes manufacturing complexities, and increases the number of assay steps performed by the end user. The complexities of manufacturing can be further divided into more expensive manufacturing of LF strips and extra liquid handling equipment, and the need for a separate portable reader for the instrument-read labels.

The main conclusions based on the original publications are presented below.

I A single recombinant MEP antigen incorporating different epitopes that is simple to produce in *E. coli* can recognize all different HCV genotypes. A secondary antibody immunoassay using the MEP as a capture binder and a luminescent europium-chelate label showed a good clinical sensitivity and specificity when tested with patient samples. A double-antigen immunoassay with the MEP used as capture and as a labeled tracer suffers from low sensitivity with low antibody avidity patient samples.

II The developed pertussis antibody LFIA with luminescent Eu-np labels has a good correlation and high sensitivity and specificity when compared to a standard ELISA with patient samples. The developed point-of-care LF may be used in situations in which a convential ELISA for diagnosis of pertussis is not available, such as in resource-poor areas.

III It is possible to develop an ultrasensitive and quantitative LFIA for fPSA by using luminescent Eu-np labels. The LF assay can be simplified to a rapid two-step format without a significant loss of sensitivity.

IV An RDT for *P. falciparum* malaria HRP2 with instrument-read luminescent UCNP-labels was developed. An up to 250–fold improvement in thedetection limit compared with a standard RDT was shown with *P. falciparum* culture samples. The developed LF test is as rapid and simple to use as the traditional RDTs.

In conclusion, the findings of this thesis show that the use of luminescent labels in LF immunoassays allows the development of highly sensitive and quantitative rapid tests with potential for use in resource-poor areas. The findings particularly show that the analytical sensitivity of lateral flow immunoassays can be dramatically improved by using luminescent labels. The simplicity and rapidity of the IAs developed were particularly demonstrated with the malaria HRP2 LF assay, which is as rapid and easy to use as the traditional RDTs.

The main challenge for the practical usability of these tests in resource-poor areas is the need for an affordable, portable and robust reader for the luminescent labels. However, this type of a reader is especially feasible for the UCNP-labels, thanks to their robustness and unique luminescence properties. The instrument-read tests also provide the advantages of objectivity and reliable communication of results. Taken together, the tests with instrument-read labels are needed in cases where the current RDTs cannot achieve sufficient analytical sensitivity, such as in screening of non-symptomatic carriers of malaria parasites, or in cases where a quantitative rapid test is needed to replace ELISA, such as in pertussis serology.

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