



Rapid design and fielding of four diagnostic technologies in Sierra Leone, Thailand, Peru, and Australia: Successes and challenges faced introducing these biosensors

Shawn P. Mulvaney^{a,*}, Lisa A. Fitzgerald^a, Leila J. Hamdan^a, Bradley R. Ringeisen^a, Emily R. Petersen^b, Jaimee R. Compton^b, Nina L. McAuliff^b, Tomasz A. Leski^c, Chris R. Taitt^c, David A. Stenger^c, Christopher A. Myers^d, Erin Hansen^{d,e}, Michelle Ricketts^{d,e}, Chelsea Hoegberg^{d,e}, Kamonthip Homdayjanakul^{d,e}, Rashid Ansumana^f, Joseph M. Lamin^f, Umaru Bangura^f, Joseph Lahai^f, Victoria Baio^f, Direk Limmathurotsakul^{g,h,i}, Gumphol Wongsuvan^g, Viriya Hantrakun^g, Supaporn Wacharapluesadee^j, Anek Mungaomklang^k, Opass Putcharoen^k, Pratoomtong Yatoon^l, Kriengsak Kruthakool^m, Robert D. Hontzⁿ, Christopher Moresⁿ, Crystyan Siles^o, Amy Morrison^o, Mark Mayo^p, Bart J. Currie^{p,q}, Kathryn H. Jacobsen^r, Kathleen Quinn^s, Jerold Blutman^s, Flavia Amariei^s, John Hannan^s

^a Chemistry Division, US Naval Research Laboratory, Washington, DC, USA

^b Nova Research, Inc., Alexandria, VA 22308, USA

^c Center for Biomolecular Science and Engineering, US Naval Research Laboratory, Washington, DC, USA

^d Operational Infectious Diseases Directorate, Naval Health Research Center, San Diego, CA, USA

^e Henry M. Jackson Foundation for the Advancement of Military Medicine, Bethesda, MD, USA

^f Mercy Hospital Research Laboratory, Bo, Sierra Leone

^g Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Thailand

^h Center for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, Churchill Hospital, University of Oxford, United Kingdom

ⁱ Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Thailand

^j King Chulalongkorn Memorial Hospital, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

^k Debaratana Nakhon Ratchasima Hospital, Nakhon Ratchasima, Thailand

^l Pak Thong Chai Hospital, Nakhon Ratchasima, Thailand

^m Chokchai Hospital, Nakhon Ratchasima, Thailand

ⁿ Naval Medical Research Unit 6, Lima, Peru

^o Naval Medical Research Unit 6, Iquitos, Peru

^p Global and Tropical Health Division, Menzies School of Health Research, Darwin, Northern Territory, Australia

^q Department of Infectious Diseases, Royal Darwin Hospital, Darwin, Northern Territory, Australia

^r George Mason University, Fairfax, VA, USA

^s Defense Threat Reduction Agency, Washington, DC, USA

ARTICLE INFO

Keywords:

Diagnostic device
Lateral flow immunoassay
Nested PCR
Field testing
Malaria
Dengue fever

ABSTRACT

Febrile illnesses are among the most common reasons for visits to hospitals and clinics worldwide. Since fevers can arise from a wide range of diseases, identifying the causative pathogen is essential not only for effective personal treatment but also for early detection of outbreaks. The Defense Threat Reduction Agency (DTRA) tasked a coalition of commercial, academic, and government researchers with moving diagnostic technology concepts from ideation to field use as rapidly as possible using scientifically sound evaluations. DTRA's 24 Month Challenge program examined > 30 technologies before fielding four technologies on four continents. > 10,000

Abbreviations: ADMD, Active Dengue Melioidosis Detect; AFRIMS, The Armed Forces Research Institute of Medical Sciences; Bp, *Burkholderia pseudomallei*; BSL2, Biosafety level two; BSL3, Biosafety level three; BT, Biothreat pouch; Chula, Chulalongkorn University (Thailand); CPS, Capsular polysaccharide; DoD, United States Department of Defense; DPP, Dual Path Platform; DTRA, Defense Threat Reduction Agency (USA); F1, Fraction one antigen from *Y. pestis*; HRPII, Histidine rich protein two; LFI, Lateral flow immunoassay; LOD, Limit of detection; Menzies, The Menzies School of Health Research (Australia); MHRL, Mercy Hospital Research Laboratory (Sierra Leone); MORU, Mahidol-Oxford tropical Medicine Research Unit (Thailand); NAMRU-6, Naval Medical Research Unit 6 (Peru); NHRC, Naval Health Research Center (USA); NMRC, Navy Medical Research Center (USA); NRL, Naval Research Laboratory (USA); NS1, Nonstructural protein 1; Pf, *Plasmodium falciparum*; pLDH, Plasmodium lactate dehydrogenase; SASFI, Severe acute systemic febrile illness pouch; USAMRIID, United States Army Medical Research Institute of Infectious Diseases (USA); WHO, World Health Organization; WRAIR, Walter Reed Army Institute of Research (USA); Yp, *Yersinia pestis*

* Corresponding author.

E-mail address: shawn.mulvaney@nrl.navy.mil (S.P. Mulvaney).

<https://doi.org/10.1016/j.sbsr.2018.06.003>

Received 24 April 2018; Accepted 20 June 2018

2214-1804/ © 2018 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

in field test results were recorded. Here we discuss our tiered evaluation system to assess candidate technologies developed by commercial partners and the process of field testing those technologies at various front-line clinics in Sierra Leone, Thailand, Peru, and Australia. We discuss successes and challenges for introducing two multiplexed lateral flow immunoassay (LFI) tests that detect malaria, dengue fever, melioidosis, and the plague. Additionally we discuss the use of a LFI reader that assisted the interpretation of the assay, communicated results to a data cloud, and greatly facilitated reach-back support. Lastly, we discuss the concurrent field testing of a multiplexed PCR assay on the FilmArray platform, which had an assay pouch specially designed for the 24 Month Challenge. Either standard-of-care or gold-standard testing were run alongside our fielded technologies to benchmark their performance.

1. Introduction

Presentation with febrile illness is ubiquitous at health clinics across the globe [1–3]. Correctly diagnosing these illnesses is essential for ensuring favorable patient outcomes. Some infectious diseases can be managed with rest and rehydration, but others can be fatal if not rapidly treated with appropriate antimicrobial agents. Differential diagnosis may be especially valuable in low-income countries where numerous life-threatening febrile infectious diseases are endemic, such as malaria, typhoid, dengue fever, Lassa fever, and the plague [1–3]. Conducting routine laboratory tests is difficult in places where there is no reliable source of electrical power, few facilities for cold-chain storage, frequent interruptions in supply chains, and insufficient trained personnel. New diagnostic technologies are being developed to address these challenges, and to be effective they must be able to perform well in harsh environments, have adequate sensitivity and specificity for the prevalent disease targets, and be sufficiently simple that they can be properly used and correctly interpreted by people with minimal training.

The US Department of Defense (DoD) has personnel stationed across the globe in locations where endemic disease profiles can vary greatly. Getting the needed diagnostic tools to the correct location can be a difficult task, especially when appropriate diagnostics are not commercially available and disease-specific tests must be developed to suit each new area of deployment. The DoD also faces limitations in terms of supply chain, warfighter payload, personnel training, and assay cost, each of which can limit the number and variety of diagnostic tools that can be successfully introduced at a given location. To overcome some of these barriers, the Defense Threat Reduction Agency (DTRA) launched a 24 Month Challenge Program that supported the development, validation, and field deployment of diagnostic technologies for laboratories and clinical facilities in low- and middle-income countries. Specifically, the diagnostic technologies would comprise both simple, rugged devices suitable for use by non-medically trained personnel or self-use under potentially austere conditions and laboratory-based tests for use in minimally equipped clinical laboratories. A key requirement was the ability for the diagnostic results to be transferred to a centralized cloud database for remote analysis. The overall goal was to demonstrate that custom diagnostic tools fitting DoD-mandated requirements could be smartly developed and delivered to specific global positions in a timely manner.

The 24 Month Challenge was a public-private effort that included commercial, academic, and government researchers. Commercial partners built custom diagnostic technologies, which were then independently validated by government laboratories, and lastly were fielded at clinics and laboratories across four continents. Importantly, the program was an exploration of the development lifecycle concept, not specifically focused on achieving regulatory clearance of a given prototype. The U.S. Naval Research Laboratory (NRL) was one of three government laboratories that identified commercial partners to develop a custom technology and then independently validated the resulting diagnostic. For NRL, the process began with review of proposal submissions, followed by empirical evaluation of candidate technologies with an existing assay. Next, the most promising technologies were

selected for development of custom multiplex assays for several causes of febrile illness. The custom diagnostic technologies were then independently evaluated for analytical performance and environmental suitability. Validated technologies were then supplied to laboratories and clinics on four continents. The cycle was completed rapidly: it took approximately one year to go from initial proposal to a validated technology deemed suitable for field testing. The commercial partners met very tight schedules that allotted only six months for custom test development and optimization. Naval Health Research Center (NHRC) oversaw the fielding of technologies and the test protocols performed. NHRC worked with individual clinics and laboratories to administer the program and provide clinical oversight and monitoring. Reach-back support was provided by NHRC, NRL, and at times the technology developers themselves. Through this process > 10,000 diagnostic tests were performed across all locations.

2. Materials and methods

2.1. Materials

2.1.1. Human whole blood

Control blood was purchased from Valley Biomedical (Winchester, VA, USA) with either Na Heparin or Na Citrate for anticoagulant and delivered in the blood bag used for collection. For sensitivity and specificity testing, control blood was spiked with the target antigens (or challenge samples) and diluted serially to the final desired concentration. All blood was stored at 4 °C and used within two weeks of receipt to avoid any potential issues with aging blood sample matrices.

2.1.2. *Yersinia pestis* F1 antigen

The samples used in NRL's biosafety level two (BSL2) testing are inactivated *Y. pestis* strain Colorado 92 from the Critical Reagents Program. Live agent *Y. pestis* samples were also obtained from the Critical Reagents Program by Naval Medical Research Center (NMRC) for BSL3 level testing. Three strains, Colorado 92, Java 9, and Kim 5 were used for limit of detection (LOD) and inclusivity testing. All samples were spiked in house by NMRC personnel using fresh whole blood obtained from Valley Biomedical.

2.1.3. *Burkholderia pseudomallei* CPS antigen

Circulation polysaccharide (CPS) antigen was provided by Dr. David AuCoin at the University of Nevada, Reno. Highly purified antigen was provided frozen and at a concentration of 2 mg/ml. Dilutions were made to the given target concentration tested. Live agent detection of nearly 30 different *Burkholderia* species was performed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) under BSL-3 conditions with organisms grown in house.

2.1.4. Dengue NS1 antigen

Nonstructural protein 1 (NS1) antigens were purchased from The Native Antigen Company (Upper Heyford, Oxfordshire, UK) as a recombinant NS1 protein preparation produced in a human cell line expression system. All four dengue serotypes were used for testing of the LFIs.

2.1.5. *Plasmodium falciparum* (Pf)

Pf strain D6 and C235 samples (2000 parasites/ μ l in whole blood) were prepared at the Walter Reed Army Institute of Research (WRAIR), frozen at -80°C and transferred to the testing site. Pf strains 3D7 and 7G8 were prepared at NMRC and were provided at a concentration of 1.13×10^4 and 9.5×10^3 parasites/ μ l, respectively. Clinical strains from Malawi and Thailand were prepared at the University of Maryland, Baltimore, in the laboratory of Prof. Christopher Plowe. Each of the strains were frozen at -80°C prior to transfer. Testing samples were prepared by diluting the thawed Pf stock samples in fresh whole blood to the stated concentration.

2.1.6. Exclusivity panel

BSL2 level reagents were tested as an exclusivity panel at $200 \times$ the LOD for the LFI assay at NRL. Panel members included *Leishmania donovani* acquired from ATCC, chikungunya and yellow fever viruses obtained from NMRC, influenza A (H3N2) virus obtained from NHRC, and hepatitis B and C viruses from Thermo Fisher Scientific. For BSL3 level exclusivity testing, live agent *Burkholderia species* were grown in house at USAMRIID.

2.2. Methods

2.2.1. Assay protocols for technologies developed for the 24 Month Challenge program

For this field demonstration, all experimental LFI assay cassettes were labelled generically instead of indicating any specific disease detection. This blinding was designed to prevent research-use only results being used for patient management. Control lines, to indicate valid assay performance, were labelled with the letter C.

2.2.1.1. Active dengue melioidosis detect (ADMD) protocol. The ADMD LFI assay was designed and built by Inbios International, Inc. (Seattle, WA) in collaboration with Prof. David AuCoin at the University of Nevada, Reno. The ADMD is a traditional, linear-style lateral flow immunoassay (LFI) (Fig. 1). The assay is performed by adding 20 μ l of whole blood to the reservoir and immediately adding two drops of running buffer. The LFI is read visually by the user and optoelectronically by the Deki Reader after 30 min.

2.2.1.2. Dual path platform (DPP) protocol. The DPP assay was designed and built by Chembio Diagnostic Systems, Inc. (Medford, NY). The DPP is an LFI with two nitrocellulose strips perpendicular to each other in a 'T' shape, one strip for the sample, and one strip for the antibody conjugate and capture lines (Fig. 1). To perform the test, 50 μ l of whole blood is mixed with 50 μ l of running buffer and placed in the sample

port. The sample migrates up the sample strip and transfers onto the perpendicular strip where it flow across the capture line. After 10 min, four drops of chase buffer are added to the buffer port at the end of the antibody conjugate strip. This carries the gold conjugate labels along the strip, past where the sample had merged, and across where the capture lines are printed. The DPP LFI is read after 20 min visually by the user and optoelectronically by Deki Reader. This design addresses some potential issues with reagent compatibility, striving to avoid cross-reactivity that would negatively impact test performance.

2.2.1.3. Deki reader operation. The Deki Reader was designed, built, and programmed by Fio Corporation (Toronto, OR). The Deki Reader can be used in conjunction with a large number of LFI tests and has been specially programmed to guide operation and determine results for both the ADMD and DPP assays designed for the 24 Month Challenge (Fig. 1). After starting the Deki Reader software, the user is provided the opportunity to enter metadata for the test participant, including any information about the test participant (age, gender, and a test ID), date and time the test was conducted and the GPS coordinates of where the test was performed. The software then guided the user step-by-step through completing an LFI test, including timing all steps of the protocol. At the conclusion of the assay, the Deki Reader asked the user to input their result as interpreted visually. Independent of the user's interpretation, the Deki Reader made its own determination of the LFI test result and stored in memory that information alongside an image of the completed LFI and the user's input. Finally, the software confirmed with the user that the assay has been completed and transmitted the assay results and all captured meta-data to a Health Level-7 compliant data cloud, where it could be accessed by authorized personnel. For this field demonstration, individual test results were not revealed to the test participant or user because the devices were for research purposes only.

2.2.1.4. FilmArray operation. The FilmArray instrument was designed and built by BioFire Diagnostics (Salt Lake City, UT) (Fig. 1). The BioThreat (BT) pouch, and the Severe Acute Systemic Febrile Illness (SASFI) pouch were designed and built by BioFire Defense (Salt Lake City, UT). The SASFI pouch used elements of the previously developed BioThreat pouch while adding seven new assays that detected the four serotypes of dengue virus and the various species of Plasmodium that cause malaria. The SASFI pouch used a 200 μ l whole blood sample to detect up to 20 different targets in a two-stage, nested PCR assay. The sample-to-answer time was about 80 min and could be successfully performed by minimally trained personnel. The FilmArray was reliant on an attached laptop, which contained the device software and allowed the user to enter meta-data associated with the sample. As

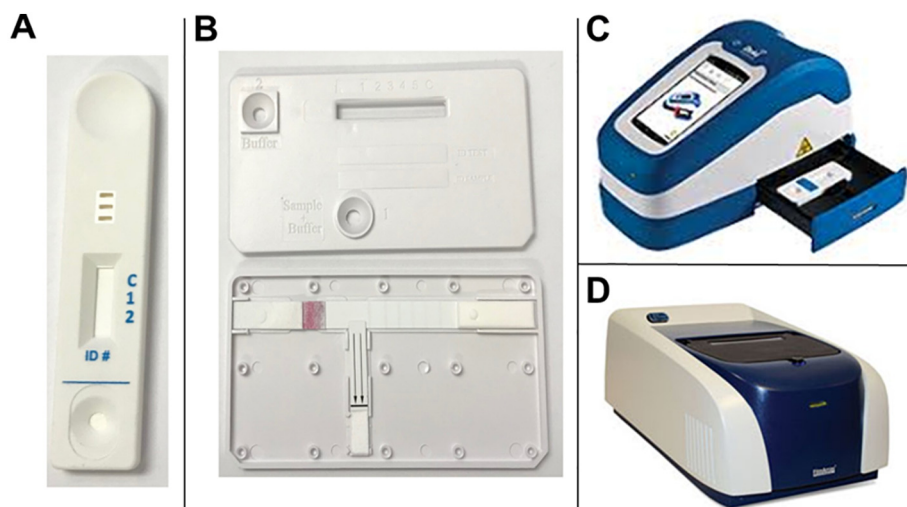


Fig. 1. Technologies developed for the 24 Month Challenge Program. (A) Image of the Active Dengue Melioidosis Detect (ADMD) lateral flow immunoassay. (B) Image of the Dual Path Platform (DPP) lateral flow immunoassay. The top image shows the cassette top with location of buffer port, sample port, and area to view capture lines. The bottom image show the interior of the DPP with perpendicular nitrocellulose strips. (C) Image of the Deki Reader, an instrument that assists with execution and evaluation of lateral flow immunoassays. (D) Image of the FilmArray instrument which is used to perform the SASFI molecular diagnostic panel developed for the 24 Month Challenge Program.

with the LFI devices, the FilmArray results were blinded from the user to prevent its use in patient management. Data from film array runs was securely transmitted over a standard internet connection or wirelessly through the associated laptop to the secure data cloud.

2.2.2. Standard-of-care complementary assays

Complementary assays used to confirm results at the test sites were based on standard-of-care testing pathways established at the test site laboratories. Commercial LFIs for dengue and malaria were used at the Sierra Leone site (Dengue Duo IgG/IgM/NS1 and Malaria Ag Pf/Pan, SD Bioline, Gyeonggi-do, Republic of Korea; Paracheck™, Orchid Biomedical Systems, Goa, India). At sites in Peru and Thailand, 24 Month technologies were run alongside gold-standard tests including blood culture, ELISA, PCR identification, and thick blood smear analysis.

2.2.2.1. For *Burkholderia melioidosis* detection. The Menzies School of Health Research in Australia (Menzies) and Chula and MORU in Thailand used locally established blood culture and PCR methodologies. All other sites used a validated PCR assay [4].

2.2.2.2. For malaria detection. Samples collected at NAMRU-6, MORU and Chula used local microscopic thick smear examination as established in each individual laboratory. In Sierra Leone, the Malaria Ag Pf/Pan (SD Bioline, Gyeonggi-do, Republic of Korea) was used. Samples from Menzies were not tested for malaria.

2.2.2.3. For dengue virus detection. A validated PCR assay that detects all strains was used by NAMRU-6 in Iquitos and by Chula [5]. The Armed Forces Research Institute of Medical Sciences (AFRIMS) performed dengue PCR for samples collected by MORU. In Sierra Leone the Dengue Duo IgG/IgM/NS1 LFI (SD Bioline, Gyeonggi-do, Republic of Korea) was used. Samples from Menzies were not tested for dengue virus.

2.2.2.4. For *Yersinia pestis* detection. Samples from both Chula and MORU were tested at Chulalongkorn University with two PCRs distributed through the US DoD's Critical Reagent Program (Catalog numbers: PCR-YPT-1FB-K and PCR-YPT-2FB-K). As these are DOD-controlled assays, testing was performed by personnel from the Navy Medical Research Center, or under their direct supervision. Samples from NAMRU-6 Iquitos were tested by PCR at the NAMRU-6 reference laboratory in Lima. Samples from Menzies were not tested for *Yersinia pestis*.

2.3. Participant recruitment and eligibility

The testing sites were selected due to the presence of established research and surveillance programs, the high incidence of at least one pathogen of interest, and the existing infrastructure necessary to carry out the study procedures. The study protocol was either nested in an ongoing surveillance program or was a standalone project. Testing sites were Mercy Hospital Research Laboratory (MHRL) in Sierra Leone, Chulalongkorn University (Chula) and Mahidol-Oxford Tropical Medicine Research Unit (MORU) in Thailand, The Menzies School of Health Research in Australia, and the Naval Medical Research Unit 6 (NAMRU-6) in Peru.

At NAMRU-6, the study was carried out in Iquitos, Peru where ongoing community research and surveillance programs are well-established. Iquitos is an urban community located in the Amazon Basin and functions as a socio-epidemiological island due to access being limited to air and river travel. Study enrollment in Iquitos, Peru occurred at six hospitals: Army Hospital Santa Rosa, Hospital Apoyo, Tupac Amaru, Air Force Hospital, Clinica Naval de Iquitos and Bellavista Nanay. In addition, ongoing cohort studies were utilized for enrollments during routine home visits by community health workers. All participants were Peruvian citizens and residents of Iquitos.

In Thailand, Chula enrolled subjects at a total of four study enrollment hospitals, three located in the northeastern Thai province of Nakhon Ratchasima and one in Bangkok. Elsewhere in Thailand, MORU's collection occurred at a public hospital in the northeastern province of Ubon Rathchathani. All of the MORU participants enrolled were inpatient Thai nationals that had infection as the primary cause of illness.

At Menzies, the study was carried out at Darwin Royal Hospital as part of an ongoing *Burkholderia melioidosis* study, with a significant portion of the patient population being indigenous Australian.

In Peru, Sierra Leone, and Thailand, patients presenting with febrile illnesses (self-reported fever or measured temperature of $\geq 38^\circ\text{C}$ within onset with the previous seven days) who were ≥ 5 years of age and who provided informed consent were enrolled into the study, barring any exclusion criteria; parental consent was obtained for minor children participating in the study. Patients with febrile symptoms of ≥ 10 days, an identifiable focus of infection that could be treated by surgical means (such as debridement or an incision and drainage procedure), non-consenting individuals, and patients who had previously enrolled in the study within the past 10 days were excluded from enrollment. At all sites, participants were enrolled by trained study personnel. There was no exclusion criteria related to gender, ethnicity, or race. Children < 5 years of age were excluded due to the inherent difficulties in obtaining blood from these participants. Pregnant women were allowed to participate because they represent a group at high risk for febrile illness and the study procedures did not represent an additional risk. For this demonstration, results from LFIs – whether interrogated visually or optoelectronically using the Deki readers – were blinded from both the participant and user because the test was conducted for research purposes only and the result had not been approved as a tool for diagnostic decision making. Diagnostic criteria were based on previously approved, local standard-of-care tests.

2.4. Ethics approval

2.4.1. Institutional review board (IRB) – Sierra Leone

The human participant research protocol was approved by the IRBs of Njala University, George Mason University (protocol: 7909), the U.S. Naval Research Laboratory (protocol: NRL.2012.0007), and the Sierra Leone Ethics and Scientific Review Committee.

2.4.2. Institutional review board (IRB) – Thailand

The human participant research protocol for the Chula site was approved by the IRB of Chulalongkorn University (protocol: 009/57). The human participant research protocol for the MORU site was approved by the Human Research Ethics Committee of Mahidol University (protocol: TMEC 12-017).

2.4.3. Institutional review board (IRB) – Peru

The human participant research protocol for the NAMRU-6 site was approved by the IRB of Naval Medical Research Unit No. 6 (protocol: NAMRU6.2014.0003).

2.4.4. Institutional review board (IRB) – Australia

The human participant research protocol for the Menzies site was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (protocol: 04/09).

2.4.5. Institutional review board (IRB) – naval health research center

The non-research role determination was approved by the IRB of the Naval Health Research Center (NHRC). NHRC's role is project management, monitoring, and oversight. (Protocol: NHRC.2015.0035).

3. Results

3.1. Technology validation studies

Two multiplexed LFIs were built for the 24 Month Challenge program. The Dual Path Platform (DPP) LFI was a 4-plex assay for the detection of NS1 produced by humans in response to the dengue virus, CPS that is shed by *B. pseudomallei*, F1 antigen present in *Y. pestis*, and both HRP2 and pLDH produced by *Plasmodium* species. The Active Dengue Melioidosis Detect (ADMD) LFI was a 2-plex assay for the detection of NS1 and CPS. Both assays were designed to work with finger-prick volumes of whole blood and their procedures could be completed in < 30 min. Details for each are given in the Materials and Methods section.

Prior to deployment to field sites, both LFIs were extensively tested for analytical performance and robustness at NRL under BSL2 conditions using inactivated *Y. pestis*, purified CPS, NS1 antigens, and blood samples containing *Pf* D6 and C235 strains. The analytical sensitivity of each test was determined by successively narrowing down the detection range and then confirming that concentration with 20 replicates at the suspected limit of detection. Multiple replicate assays for each test concentration were performed by two individuals, and no statistically significant user-to-user variability was observed. Table 1 lists the various sensitivities measured for each target. The limits of detection recorded here compare favorably or exceed those of commercially available LFIs [6–14]. Additionally, an exclusivity panel was performed with at least 10 different pathogens and no false positives were observed for either the DPP or ADMD assays (Table 2).

Complementing the BSL2 testing, both LFIs were tested against live samples of *Y. pestis* and *B. pseudomallei*. Trained personnel in BSL3 laboratories completed the inclusivity (Table 3) and exclusivity testing (Table 4) at NMRC and USAMRIID, respectively. For *Y. pestis*, the DPP LFI was two orders of magnitude more sensitive for live strain Colorado 92 than for inactivated antigen, possibly indicating that the inactivation process affected the antigenic character of the F1 protein. Despite showing greater sensitivity for Colorado 92, the DPP failed to detect live Kim 5 or Java 9 strains at the same concentration in all 20 assays performed. Detection of higher concentrations of *Y. pestis* Kim 5 or Java 9 was possible, but not confirmed due to the limited number of tests available for live agent work. Both the DPP and ADMD assays showed broad inclusivity for *B. pseudomallei*, recognizing all 18 strains tested. As would be expected for both LFIs, two strains (BURK135 and BURK149) that do not produce CPS were not detected. In live agent exclusivity studies, the DPP showed no response to six near neighbors of *Y. pestis* and neither LFI gave a response to a 15-member exclusivity panel for non-pseudomallei *Burkholderia* species. However four non-pseudomallei *Burkholderia* strains (BURK009, BURK010, BURK113, and BURK186) known to display a polysaccharide returned the expected

Table 2

Exclusivity testing for ADMD and DPP LFIs in BSL2 conditions.

Exclusivity panel					
Pathogen	CONC.	ADMD		DPP	
		Positive	Negative	Positive	Negative
<i>Leishmania donovani</i>	Lot 610-207-07 ^a	0%	100%	0%	100%
Chickungunya	3 × 10 ⁸ pfu/ml	0%	100%	0%	100%
Flu A H3N2	3 × 10 ⁴ TCID50	0%	100%	0%	100%
Yellow Fever	2 × 10 ⁶ pfu/ml	0%	100%	0%	100%
Hepatitis B	2 × 10 ⁷ IU/ml	0%	100%	0%	100%
Hepatitis C	2 × 10 ⁷ IU/ml	0%	100%	0%	100%
Dengue virus - Type 1	1 × 10 ⁶ GE/μl	0%	100%	0%	100%
Dengue virus - Type 2	1 × 10 ⁶ GE/μl	0%	100%	0%	100%
Dengue virus - Type 3	1 × 10 ⁶ GE/μl	0%	100%	0%	100%
Dengue virus - Type 4	1 × 10 ⁶ GE/μl	0%	100%	0%	100%
<i>Yp</i> Colorado 92 (inactivated)	1 × 10 ⁷ cfu/ml	0%	100%		
<i>P. falciparum</i> - D6	200 parasites/μl	0%	100%		
<i>P. falciparum</i> - C235	200 parasites/μl	0%	100%		

^a Exact concentration of *Leishmania donovani* not determined.

positive response. All exclusivity testing was done at 10-times the limit of detection or higher concentration.

The robustness of each custom LFI was examined by challenging each with elevated temperature and humidity during both storage and operation. Each individual LFI was sealed in a foil pouch prior to use, and no test pouch evidenced signs of being compromised following storage. LFIs were stored for 10 or 30 days at 37 °C and 95% humidity and then tested at the same elevated environmental conditions using targets at the determined limits of detection, 3-fold, and 9-fold higher concentrations; both NS1 and CPS were tested on the ADMD platform while a wider variety of NS1 antigens were tested on the DPP platform. The data are summarized in Table 5. Using NS1 test results to assess performance, both LFIs demonstrated operability under these stressed environmental conditions. There was some loss in sensitivity near the limit of detection for both LFIs, with the ADMD faring better than the DPP in that regard. Details of these environmental studies are given in the Supporting Information Topics 3 and 4.

A core benefit of LFIs is that they can be read visually, making them particularly well-suited for use in limited-resource settings. During BSL2 validation testing at NRL, staff made a visual interpretation of every test line's intensity. We used a zero (0) to five point scale, with '0' indicating there was no line observed (i.e., a negative) and scores from '1–5' indicating the observation of a progressively darker, or more intense, line

Table 1

Sensitivity of ADMD, DPP, and SASFI custom assays.

Pathogen	Target/strain	Limit of detection		Target/strain	LOD
		ADMD	DPP		SASFI
Dengue	NS1 Type 1 (recombinant)	17.5 ng/ml	30 ng/ml	Dengue Virus Type 1 (West Pac 74)	1 × 10 ⁴ GE/ml
	NS1 Type 2 (recombinant)	8 ng/ml	15 ng/ml	Dengue Virus Type 2 (S16803)	1 × 10 ⁴ GE/ml
	NS1 Type 3 (recombinant)	25 ng/ml	70 ng/ml	Dengue Virus Type 3 (CH5381)	1 × 10 ⁴ GE/ml
	NS1 Type 4 (recombinant)	40 ng/ml	70 ng/ml	Dengue Virus Type 4 (341750)	1 × 10 ³ GE/ml
<i>B. pseudomallei</i>	CPS	300 pg/ml	500 pg/ml	<i>B. pseudomallei</i> (1026b)	1 × 10 ² cfu/ml
<i>Y. pestis</i>	F1 from Colorado 92 (inactivated)		1 × 10 ⁵ cfu/ml	Colorado 92	1 × 10 ² cfu/ml
<i>Plasmodium falciparum</i>	D6		8 parasites/μl	D6	66 parasites/ml
	C235		4 parasites/μl	C235	13 parasites/ml
	3D7		20 parasites/μl	3D7	100 parasites/ml
	7G8		20 parasites/μl	7G8	100 parasites/ml
	Malawi		50 parasites/μl	Malawi	
	Thailand		40 parasites/μl	Thailand	10 parasites/ml

Table 3
Inclusivity testing of live agents for ADMD and DPP LFIs and the SASFI pouch.

Live agent inclusivity panel							
Pathogen	CONC.	ADMD		DPP		SASFI	
		Positive	Negative	Positive	Negative	Pos/neg	
	<i>Yp - Colorado 92</i>			97%	3%		
	<i>Yp - Kim 5</i>			0%	100%		
	<i>Yp - Java 9</i>			0%	100%		
BURK003	<i>B. pseudomallei - 1026B</i>	1 × 10 ³ cfu/ml		100%	0%	Positive	
BURK017	<i>B. pseudomallei - 229</i>	1 × 10 ³ cfu/ml	100%	100%	0%	Negative	
BURK018	<i>B. pseudomallei - 295a</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK089	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK094	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK095	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK096	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK097	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Negative	
BURK098	<i>B. pseudomallei</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK119	<i>B. pseudomallei - K96243</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK132	<i>B. pseudomallei - 406E</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Negative	
BURK133	<i>B. pseudomallei - 112</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK142	<i>B. pseudomallei - 1106a</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK147	<i>B. pseudomallei - MSHR668</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Negative	
BURK152	<i>B. pseudomallei -MSHR2543</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK155	<i>B. pseudomallei - JCU-NCTC13179</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK181	<i>B. pseudomallei -HBPU10134a</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Positive	
BURK182	<i>B. pseudomallei - HBPU10303a</i>	1 × 10 ⁵ cfu/ml	100%	100%	0%	Negative	
BURK135 ^a	<i>B. pseudomallei - Pasteur 52,237</i>	1 × 10 ⁵ cfu/ml	0%	0%	100%	Positive	
BURK149 ^a	<i>B. pseudomallei - MSHR1655</i>	1 × 10 ⁵ cfu/ml	0%	0%	100%	Positive	

^a Burk 135 and Burk 149 do not produce CPS, expected LFI result is negative.

(see Supporting information Topic 1 for scale). A score of '5' was a very intense line and a line score of '1' was a positive line near the LFI's limit of detection. There is documented bias for interpretation of LFI tests near their limit of detection in that only ~50% of users will call a faint line positive [15, 16]. NRL confirmed this bias with the performance of 48 LFI tests for detection of *P. falciparum* (commercial CareStart™ Malaria

HRP2/pLDH (Pf/Pv) Combo test; data shown in Supporting Information Topic 2). High, medium, low, and zero samples were analyzed in triplicate by four users, and all lines were read by five individuals. While there was 87.5% concordance between the five individuals' overall interpretations of the results, there was only a 50% concordance for the interpretation of 'low' samples where lines were the least intense.

Table 4
Exclusivity testing of live agents for ADMD and DPP LFIs and the SASFI pouch.

Live agent exclusivity panel							
Pathogen	CONC.	ADMD		DPP		SASFI	
		Positive	Negative	Positive	Negative	Pos/neg	
	<i>Y. pseudo TB</i>			0%	100%		
	<i>Y. entero</i>			0%	100%		
	<i>Y. frederiksenii</i>			0%	100%		
	<i>C. freundii</i>			0%	100%		
	<i>P. aeruginosa</i>			0%	100%		
	<i>S. flexneri</i>			0%	100%		
ACIN001	<i>Acinetobacter baumannii</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BACI008	<i>Bacillus anthracis</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK101	<i>Burkholderia multivorans</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK102	<i>Burkholderia cepacia</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK103	<i>Burkholderia cenocepacia</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK104	<i>Burkholderia multivorans</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK105	<i>Burkholderia cepacia</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK106	<i>Burkholderia cepacia</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK126	<i>Burkholderia vietnamiensis</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK162	<i>Burkholderia ubonensis</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK191	<i>Burkholderia multivorans</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
ESCH004	<i>Escherichia coli 0157:H7</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
SALM003	<i>Salmonella typhimurium</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
SHIG001	<i>Shigella flexneri</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
STAP002	<i>Staphylococcus aureus</i>	~10 ⁸ cfu/ml	0%	0%	100%	Negative	
BURK009 ^a	<i>Burkholderia mallei</i>	~10 ⁸ cfu/ml	100%	100%	0%	Positive	
BURK010 ^a	<i>Burkholderia mallei</i>	~10 ⁸ cfu/ml	100%	100%	0%	Positive	
BURK113 ^a	<i>Burkholderia thailandensis</i>	~10 ⁸ cfu/ml	100%	100%	0%	Negative	
BURK186 ^a	<i>Burkholderia stabilis</i>	~10 ⁸ cfu/ml	100%	100%	0%	Negative	

^a BURK009, BURK010, BURK113, and BURK186 all produce a polysaccharide, expected result is positive.

Table 5
Performance of ADMD and DPP LFIs in Austere Environmental Conditions.

Assays stored & performed at 37 °C/95% humidity							
Pathogen	CONC.	ADMD		PATHOGEN	CONC.	DPP	
		Positive	Negative			Positive	Negative
NS1 - Type 2	24 ng/ml	11	0	NS1 - Type 1	90 ng/ml	6	0
NS1 - Type 2	16 ng/ml	9	1	NS1 - Type 1	60 ng/ml	6	0
NS1 - Type 2*	8 ng/ml	10	0	NS1 - Type 1*	30 ng/ml	4	2
CPS	900 pg/ml	9	0	NS1 - Type 2	45 ng/ml	6	0
CPS	600 pg/ml	11	0	NS1 - Type 2	30 ng/ml	6	0
CPS*	300 pg/ml	5	3	NS1 - Type 2*	15 ng/ml	3	3
blank	0	1	21	NS1 - Type 3	210 ng/ml	6	0
*Limit of detection determined in analytical testing				NS1 - Type 3	140 ng/ml	4	2
				NS1 - Type 3*	70 ng/ml	2	4
				NS1 - Type 4	210 ng/ml	3	3
				NS1 - Type 4	140 ng/ml	4	2
				NS1 - Type 4*	70 ng/ml	3	3
				Blank	0	11	13

This known user bias near the cutoff highlighted the need for an unbiased instrument to read LFIs. Several LFI readers were evaluated, and the Deki Reader was selected for the program because it quantitatively analyzes custom LFIs by optoelectronic readings, guides the user through execution of the LFI protocol, communicates the LFI results to a data cloud using cellular or WiFi capabilities, and it demonstrates the necessary robustness for field deployment. We note that our observations agree well with three literature reports for the in-field use of the Deki Reader [16–18]. Initial reliability tests of the Deki Reader utilized a reference LFI cassette that used red dye to simulate the line positions. First, the reference cassette was placed in the Deki Reader and read eight consecutive times. Second, eight consecutive readings were made with the reference cassette being removed from and then replaced in the Deki Reader between readings. Finally, the same two experiments were conducted on a second Deki Reader instrument. Both the intra- and inter-instrument reliability demonstrated better than 97% reproducibility (Supporting Information Topic 6).

The performance of the Deki Reader with the two custom LFIs was evaluated next. When the Deki Reader analyzes a LFI cassette, it assigns

a numerical value for the intensity or optical density of the line observed. Table 6 compares the results of assays run at NRL as measured by user eye and quantified by the Deki Reader. For users' visual readings of '0', or blank line positions, the Deki Reader reported an average optical density of 0.75 on the ADMD and 0.95 on the DPP cassettes. User readings of '1–5' evidenced increasingly higher optical densities for both custom LFIs. Interestingly, the difference between the user observation of a line (score of '1') and no line (score of '0') was greater for the ADMD than the DPP, which correlates well with the overall observation that the ADMD had a better performance against false positive readings. This limitation is likely a function of two constraints. First, the short timeframe allotted for development and production of both LFIs (~6 months) greatly limited the amount of assay optimization that could be accomplished while remaining on schedule. Second, the DPP is a four-plex assay and the ADMD is a duplex assay. We examined the DPP results more closely to determine average line intensity for each target (Tables 7 and 8). The data are divided into categories where the target was present and should have scored '1' by user reading and where the target was not present. The differential scores suggest that

Table 6
Comparison of LFI interpretation by user eye and Deki Reader evaluation.

User reading by eye	Deki reading of DPP											
	All lines		YP line		Burk line		Dengue line		HRP2 line		pLDH line	
	Avg	St Dev	Avg	St Dev	Avg	St Dev	Avg	St Dev	Avg	St Dev	Avg	St Dev
0	0.95	0.39	0.68	0.43	1.00	0.32	1.09	0.31	1.04	0.34	1.03	0.36
1	1.18	0.52	0.85	0.55	1.21	0.51	1.17	0.47	1.21	0.53	1.35	0.59
2	3.50	1.77	1.69	0.83	2.17	0.75	2.80	1.01	5.32	1.47	2.97	0.46
3	6.66	2.94	6.17	2.30	7.87	3.04	4.72	1.71	8.45	3.22	–	–
4	13.59	7.71	12.76	6.85	15.30	8.82	8.59	1.61	–	–	–	–
5	45.16	25.40	63.15	25.57	30.76	13.86	–	–	–	–	–	–
Pos Ctrl	33.88	15.92										

User reading by eye	Deki reading of ADMD	
	All lines	
	Avg	St Dev
0	0.75	0.36
1	1.55	0.49
2	2.97	1.11
3	7.01	3.17
4	17.69	8.99
5	66.65	16.97
Pos Ctrl	51.70	6.17

Table 7

Comparison of DPP interpretation near cutoff by user eye and Deki Reader evaluation: number of results at each test line.

DPP LFI						
Cohort of DPP tests performed at NRL with all lines read visually by user						
	F1	CPS	NS1	HRP2	pLDH	
Total number of tests with by eye user reading ≥ 1	129	200	309	216	87	
Total number of tests with a line observed at a visual intensity of ≥ 1 , but the corresponding antigen is not present in sample	59	138	178	110	34	
Percentage of tests with a false positive as determined by visual reading	12%	29%	38%	23%	7%	

background clearance is an area of potential improvement and that in particular the NS1, CPS, and HRP2 lines would benefit from further optimization on the custom DPP LFI.

Synchronous with the development of the custom LFIs and compatible Deki Reader, a PCR system was further developed for the 24 Month Challenge Program. The FilmArray instrument by Biofire Diagnostics is a nested-PCR system with multiplexed assays for a wide variety of diseases and can include detection for up to 20 targets in any one assay. For the 24 Month Challenge, two assay panels were made available, the BioThreat (BT) panel and a custom panel developed specifically for the 24 Month Program, the Severe Acute Systemic Febrile Illness (SASFI) panel. Additionally, the FilmArray was hardened for use in the field. The SASFI pouch was designed to work with a 200 μ l whole blood sample and sample preparation was highly automated. This simplified sample preparation method meant the FilmArray could be operated by minimally trained personnel. Analytical performance for detection of *Plasmodium* spp., dengue, *Y. pestis*, and *B. pseudomallei* are shown in Table 1. As would be expected for a PCR device, the limits of detection were well below those observed for LFIs. Multiple targets and strains were detected spanning the four diseases the 24 Month Challenge focused upon. Additional inclusivity and exclusivity testing demonstrated that the SASFI pouch was ready for in-field studies (Tables 3 and 4).

3.2. In-field testing of technologies

Following validation testing in the United States, the four candidate technologies were introduced in multiple laboratories and clinics across the globe, including in Sierra Leone, Thailand, Peru, and Australia. The technologies were tested with samples collected from febrile participants who consented to be enrolled in the program. All specimens and resulting data were collected and handled with strict regard to the approved IRB protocols at each test location. The introduced tests were for research use only, and the results were not used for clinical diagnosis and care. The four technologies implemented by the 24 Month Challenge were performed in parallel with standard-of-care tests specific to each test location. For MHRL in Bo, Sierra Leone, the standard-

of-care tests included commercial LFI tests for malaria and dengue. At sites in Peru, Australia and Thailand, 24 Month technologies were analyzed alongside gold-standard tests including blood culture, ELISA, PCR identification, and thick blood smear analysis.

In total, 3029 DPP, 4830 ADMD, and 3395 SASFI assays were performed across all testing sites (Table 9). Over 17,000 separate standard-of-care complementary tests were performed in parallel with the implemented 24 Month Challenge technologies. The original intent of the project was for each site to field-test identical numbers of samples using each platform. However, delays caused by IRB review and approval, issues with shipping and customs, production delivery schedules, and in Sierra Leone, the 2014–2016 Ebola outbreak, resulted in an uneven distribution of the tests at the various sites.

In Sierra Leone, 1570 DPP, 1109 ADMD, and 513 SASFI assays were performed from January 2014 to September 2016. Although the study ran during the 2014–2016 Ebola outbreak in West Africa, MHRL remained operational during most months. However, safety considerations, roadblocks, travel bans, and quarantines restricted the MHRL's ability to resupply on a set schedule and to transport samples for confirmatory analysis. Because of these constraints, it was not possible for every participant to be tested with all three 24 Month Challenge assays. Malaria was by far the most frequently detected disease at this site. For any patient who had more than one test performed, the concordance statistics are presented in Table 10. A more granular analysis of concordance is provided in the Supporting Information Topic 9. Malaria was positively detected in ~20% of samples tested using LFIs, independent of whether custom or commercial standard-of-care tests were used. However, concordance between the DPP test and the standard-of-care LFIs was only about 70%. SASFI returned about 60% malaria positive samples, but only had a concordance with DPP of about 30% for overlapping tests. However, the lack of concordance between SASFI (PCR-based) and all LFIs is consistent as all malaria standard-of-care tests showed concordance at 40% or lower at MHRL.

In Peru and Thailand, the study ran from May 2015 until January 2017. At the MORU site in Thailand, 601 DPP, 1820 ADMD, and 940 SASFI tests were performed (Table 9). All four disease targets were identified within the collected samples, with dengue being the most commonly identified pathogen. ADMD, SASFI, and gold-standard testing identified dengue in 11% of the samples, with 90% or better concordance (Table 10), but the DPP assay only identified 2% of samples as dengue positive. At the Chula site in Thailand, 552 DPP, 1542, ADMD, and 1008 SASFI tests were performed. Again the predominant positive result was for dengue and likewise the ADMD identified many more incidences than the DPP. Comparisons with gold-standard results were similar to those observed in MORU.

In Peru, the four technologies were used in six clinics across Iquitos (Peru data consolidated into Tables 9 & 10). A smaller total enrollment was achieved, but 171 DPP, 224 ADMD, and 467 SASFI tests were performed. Dengue and malaria were the two most commonly observed diseases. Overall prevalence as determined by gold-standard tests was in looser agreement with the fielded technologies.

At the Menzies School of Health Research, the study ran from October of 2014 until May of 2017. A total of 135 samples were run on both the

Table 8

Comparison of DPP interpretation near cutoff by user eye and Deki Reader evaluation: average value at each test line determined by the Deki Reader.

	User's Visual Score	Average Deki Reading Value for DPP LFIs									
		F1 line		CPS line		NS1 line		HRP2 line		pLDH line	
		Avg	St Dev	Avg	St Dev	Avg	St Dev	Avg	St Dev	Avg	St Dev
Target antigen present in sample	1	1.68	0.75	1.42	0.54	1.37	0.51	1.38	0.77	1.48	0.55
	0	0.69	0.39	0.81	0.12	1.13	0.31	0.91	0.33	1.04	0.35
Target antigen not present in sample	1	0.82	0.46	1.24	0.45	1.14	0.32	1.40	0.94	1.17	0.37
	0	0.69	0.39	1.02	0.31	1.07	0.29	1.04	0.33	1.04	0.33

Table 9
In-field performance of deployed technologies.

Site	Diagnostic	n =	<i>B. pseudomallei</i>	Dengue	<i>Y. pestis</i>	<i>Plasmodium</i> sp.	
			CPS	NS1	F1	pLDH	HRP2
MORU	DPP	601	7 (1.2%)	15 (2.5%)	17 (2.8%)	26 (4.3%)	12 (2.0%)
	ADMD	1820	14 (0.8%)	103 (5.7%)	N/A	N/A	N/A
	SASFI	940	19 (2.0%)	111 (11.8%)	0	23 (2.4%)	
	Blood culture for <i>B. pseudomallei</i>	2077	59 (2.8%)	N/A	N/A	N/A	
	PCR for dengue	2066	N/A	165 (8.0%)	N/A	N/A	
	Thick smear	1294	N/A	N/A	N/A	1 (0.001%)	
Chula	DPP	552	1 (0.2%)	5 (0.9%)	0	3 (0.5%)	1 (0.2%)
	ADMD	1542	0	136 (8.8%)	N/A	N/A	N/A
	SASFI	1008	0	193 (19.1%)	0	1 (0.1%)	
	PCR for <i>B. pseudomallei</i>	1789	0	N/A	N/A	N/A	N/A
	PCR for dengue	1827	N/A	313 (17.1%)	N/A	N/A	N/A
	Thick smear	1723	N/A	N/A	N/A	1 (0.001%)	
NAMRU-6	PCR for <i>Y. Pestis</i>	1540	N/A	N/A	0	N/A	
	DPP	171	3 (1.8%)	3 (1.8%)	2 (1.2%)	0	1 (0.6%)
	ADMD	224	0	5 (2.2%)	N/A	N/A	N/A
	SASFI	467	0	37 (7.9%)	0	62 (13.3%)	
	PCR for <i>B. pseudomallei</i>	853	1 (0.1%)	N/A	N/A	N/A	N/A
	PCR for dengue	934	N/A	79 (8.5%)	N/A	N/A	N/A
Sierra Leone	Thick smear	949	N/A	N/A	N/A	60 (6.3%)	
	PCR for <i>Y. Pestis</i>	853	N/A	N/A	0	N/A	
	DPP	1570	30 (1.9%)	41 (2.6%)	22 (1.4%)	340 (21.7%)	
	ADMD	1109	24 (2.2%)	24 (2.2%)	N/A	N/A	
	SASFI	513	0	2 (0.4%)	2 (0.4%)	305 (59.5%)	
	BioThreat PCR pouch	219	0	N/A	0	N/A	
Menzies	Parachek (Pf)	858	N/A	N/A	N/A	162 (18.9%)	
	SD Bioline (Pf/Pan)	1098	N/A	N/A	N/A	207 (18.9%)	
	Dengue Duo	396	N/A	4 (1.0%)	N/A	N/A	
	DPP	135	9 (6.6%)	1 (0.7%)	0	2 (1.5%)	2 (1.5%)
	ADMD	135	5 (3.7%)	4 (3.0%)	N/A	N/A	
	SASFI	128	7 (4.7%)	5 (3.1%)	0	2 (0.8%)	
	Blood culture for <i>B. pseudomallei</i>	105	21 (20%)	N/A	N/A	N/A	

DPP and ADMD LFIs, and 128 samples were run on the SASFI pouch. The most commonly detected pathogen was *Burkholderia* with dengue detections a close second. The total number of positive samples was relatively low ($N = 9$) making comment on performance difficult. Moreover, due to the study we leveraged at Menzies the inclusion criteria were more subjective than the other sites and so the Menzies data is not included in the concordance data comparisons presented in Table 10.

4. Discussion

There are many obstacles that must be overcome to build an effective diagnostic technology. The device must not only be sensitive and selective for the biomarker targeted, it must be compatible with the appropriate biological matrix, be it blood, plasma, urine, saliva, etc.

Taking that diagnostic technology into the field adds further layers of complication; temperature, humidity, power requirements, sample preparation, and ease of use are all critical factors in its successful introduction. The latter two considerations are particularly important since sample preparation in a field setting is made more difficult by the lack of supplies that would otherwise be readily available in a well-stocked laboratory, and by contamination challenges that could introduce inhibitory substances into the sample. The complexity of sample preparation is compounded by capability of users and training provided to those users in the field. The number and complexity of these issues typically mean that it takes years to bring technologies from ideation to field deployment.

The 24 Month Challenge program sought to accelerate this paradigm by demonstrating how partners with a track record in diagnostic

Table 10
Concordance of technologies used in the field.

Concordance with Other Diagnostics											
Site	Diagnostic	ADMD		SASFI		Standard of Care or Site's Gold Standard					
		Bp	Den	Bp	Den	Yp	Mal	Bp	Den	Mal	Yp
Chula	DPP	99%	91%	100%	75%	100%	99%	99%	83%	99%	100%
MORU		98%	92%	99%	86%	95%	96%	98%	89%	98%	N/A
Iquitos		N/A	N/A	N/A	N/A	N/A	N/A	98%	88%	98%	99%
Sierra Leone		98%	98%	98%	96%	97%	24%	N/A	97%	68%	N/A
Chula	SASFI	100%	79%					100%	93%	100%	100%
MORU		97%	86%					99%	94%	97%	N/A
Iquitos		N/A	N/A					99%	95%	98%	100%
Sierra Leone		96%	95%					N/A	N/A	32%	N/A
Chula	ADMD							100%	88%	N/A	N/A
MORU								98%	94%	N/A	N/A
Iquitos								100%	98%	N/A	N/A
Sierra Leone								N/A	99%	N/A	N/A

development could work together to quickly move new technologies toward field deployment. As a first demonstration, we did not pursue truly jumping from ideation to fielded device on a compressed schedule. Instead we began with technologies where many aspects of their field deployment had already been explored. This narrowing meant that new assays began at ideation, but the overall platforms had shorter development pathways to traverse. We also sought to challenge these devices in multiple field settings spanning from modern hospital laboratories, to point-of-care clinical testing sites with and without modern conveniences, and even in the subject's home environment.

LFI has a long history of use in field forward settings and in low- and middle-income countries. The tests require no power, can be stored, shipped, and used in ambient conditions, are relatively easy to perform, use a small sample volume, and have been adapted for a wide variety of diseases [1, 2]. LFIs have been used with great results across the globe. In 2015, the World Health Organization (WHO) completed its sixth in a series of biannual reviews of LFIs that detect malaria [14]. The WHO data, as well as additional studies, have shown that several malaria LFIs exhibit performance as good as or better than the current gold-standard, microscopy of blood smears. These LFIs are easier to complete, require less training and experience than microscopy, and provide a shorter sample-to-answer timeline. The success with malaria detection by LFI has been mirrored for other diseases, including dengue, HIV, etc., and the WHO notes that LFIs have become the standard-of-care in locations with limited resources. The main drawback to LFIs is the relative sensitivity of the technique, but commercial manufacturers are exploring ways to improve it with each passing year.

In our study two custom LFIs were developed. The DPP format is notable in that the sample is introduced down one arm of the device and first interacts with the capture lines printed on the nitrocellulose [19–22]. Next, the gold particle conjugates are introduced down an orthogonal arm and they label any captured target molecules. This design addresses some potential issues with reagent compatibility, striving to avoid cross-reactivity that would negatively impact test performance. The test is highly multiplexed, with five capture lines for the detection of antigens that indicate malaria, dengue, melioidosis, and the plague. The ADMD is a duplex assay using a traditional linear LFI architecture. The sample first passes through the gold particle conjugates, binds target to the gold and then the complex is captured at a line further down the nitrocellulose strip. The ADMD detects for melioidosis and dengue. Both custom LFIs were prepared on a very compressed schedule and do not necessarily reflect a typical development lifecycle, but the purpose of the 24 Month Challenge was to explore the pathway of an accelerated production.

The Deki Reader proved to be a powerful tool in this study for several critical reasons. First, the software provides a pre-programmed operating procedure to help the user successfully complete the LFI test protocol, addressing ease-of-use challenges. When used properly, the step-by-step instructions greatly reduces common errors such as incorrect timing of the various incubation periods. The built-in timer ensures the LFI is interrogated at the exact time and also notifies the user if the LFI was incubated too long, requiring the LFI to be discarded. These built-in system checks reduce the proportion of invalid test results provided the user starts the timer as instructed. Another benefit is that the Deki Reader records an image of each LFI and uses image analysis software to determine the presence or absence of each LFI test line. Third, the image, plus the user's interpretation of the test result, are communicated to a data cloud using the Deki Reader's cellular or WiFi capability. When the Deki Reader is not able to use either mode of communication, several thousand results can be stored on the instrument and then transmitted upon the next connection to the cloud. The resulting data upload can be used to monitor potential disease outbreaks in a given location. The uploaded data can also be monitored by the clinical laboratory team as part of quality assurance protocols. For this study, handwritten logs of test results were kept and then transposed to a spreadsheet for data analysis. For the MHR log, < 1% of

data between the transposed database and Deki uploaded results were in conflict. However, the uploaded images allowed the research team to rectify all errors associated with transposed data, even when not located on-site. Image analysis also proved useful for monitoring longitudinal LFI performance and any user-to-user variability. The images verified the observation of test lines, and were used to document instances of streaking, spots or uneven backgrounds, overloaded membranes, and seemingly dry LFI cassettes. The Deki Reader performed well at identifying problems such as streaking or overload with a given LFI, but the analysis was not foolproof. Human interpretation may be suboptimal at limit of detection positives, but human visualization still provides more accurate decisions on test validity than possible when solely implementing image analysis software. For example, a test with a streak that also has a clearly positive test line might be read as negative or invalid by the detection algorithm, but would be correctly classified as positive based on human visualization. Therefore, for diagnostic purposes visual confirmation is still recommended, but for surveillance studies, statistical models could be validated using the Deki Reader's analysis. In our hands, an electronic LFI reader proved to be an essential tool for both performing LFIs in the field and for monitoring the study from remote locations.

Molecular diagnostics are highly prized for their high sensitivity and ability to specifically identify agents of disease based on genotypes, but they have limitations in terms of their sample-to-answer time and ability to be performed in field settings [23, 24]. The SASFI pouch provided our researchers with information on 20 diseases in a single run performed on-site, but the sample-to-answer time of nearly 80 min – as well as its inability to test multiple samples in parallel – did limit our overall throughput. However, a major strength of the FilmArray platform is the ease of sample preparation (Supporting Figure Topic 8), which minimizes the number of manual manipulations and mitigates potential introduction of contaminants. The sample is loaded into a syringe, which is used to load the pouch, the pouch is then placed into the instrument and then the FilmArray performs the lysis, collection, and testing of the sample. This simplified sample preparation was successfully completed at all sites by minimally trained personnel, with meaningful results collected at each site. We do note that the cost of a multiplex PCR assay is high when compared to the relative cost of an LFI, so while a rich level of information can be achieved using the FilmArray assays, it is also potentially cost prohibitive to perform routine diagnostics using these assays as the main tool. Nevertheless, it is a powerful tool that can also serve as a field confirmatory test platform. The absolute cost of a single FilmArray test is high, but when it can replace the gold-standard testing of 20 different disease targets using individual target-specific tests, the relative cost may be a bargain. It is no small feat to maintain all of the gold-standard testing equipment and training in resource-constrained settings, and in these places the FilmArray system has proven to be an excellent complement to the LFI technologies.

Across the four technologies fielded in the 24 Month Challenge, > 10,000 assays were completed on four continents, by > 30 different users. The successful completion of so many tests revealed a number of challenges, many of which are typically encountered for applications of field forward diagnostics. Effective instructions are perhaps the most important aspect for deploying any diagnostic. Instructions must be interpretable and appropriate for the user community, a task made more difficult when tests are disseminated across multiple global locations with different languages, levels of education, and technical expertise. When possible, pictorial instructions proved to be the most easily transferred from one country to the next. For example, an image of a dropper bottle and a number of drops was effective for demonstrating the application and volume of chase buffer used with an LFI assay step. Surprisingly, this image was not universally understood; some users not familiar with screw top caps did not understand how to remove the cap. In such situations, simple text instructions might supplement the pictogram, but they require translation

to the local language(s) or dialect(s). When practical to use, short videos supplemented with pictorial instructions proved to be the most effective training for new diagnostic users. The video often cleared any lingering confusion with regards to sample versus buffer port or which buffer bottle to be used, both of which are items for the custom DPP assay. Additionally, use of larger, simpler labels and color coding buffer bottles with care not to use known color-blindness combinations can assist with use of the proper reagent. In the end, the quality of the instructions directly impacts the successful execution of the diagnostic technology and the length and breadth of training needed to assure the next user group is prepared.

Sample collection was also a challenge faced in our in-field studies. Finger prick collection of blood samples was not straightforward for all participants. In particular, we found that many adult men in Sierra Leone and Peru who earn their living with manual labor would often have thick skinned and calloused fingers. Even when using large-diameter lancets and the side of the participant's finger, 50 μ l blood volumes were sometimes difficult to obtain. These and other issues with finger prick blood collection required instructions on that procedure to be appended to the LFI instructions for initial training in some locations. We note that should one have difficulty obtaining a blood sample by finger prick, both LFIs tested herein can be completed with venous puncture blood draws. Familiarity with items such as adhesive bandages was also not universal, a reminder that effective instructions need to consider whether an item is widely used or must be described for users who might not be familiar with that item.

Lastly, the storage and transport of reagents and materials across the globe without losing their efficacy must be carefully considered. The government research laboratories that validated the candidate technologies prior to field deployment used accelerated storage at elevated temperatures to measure robustness. As has been shown previously in the literature, these studies are effective, but are not absolute replacements for true aging [25, 26]. For the FilmArray pouches stored at ambient conditions, a higher rate of failure was recorded for reagents stored at $> 30^{\circ}\text{C}$ for longer than two months. Subsequent storage of new PCR pouches at temperatures below 25°C corrected the failure rate spike, suggesting that a further examination of long term storage is advisable. Regardless, this is an unavoidable condition of an accelerated technology development program such as the 24 Month Challenge. Part of the delay in bringing a diagnostic to the field is the time required for robustness testing.

5. Conclusions

The 24 Month Challenge was a successful public-private partnership that resulted in two multiplexed LFI technologies, a LFI reader for assisting with assay performance and transmitting results to a cloud database, and an orthogonal PCR technology being field tested at multiple remote sites on an accelerated schedule. Leveraging commercial knowledge and investment in conjunction with government validation and resources allowed for science-first decisions to be reached at every go/no-go gate. Down selection at every step meant that the DoD could more effectively identify those technologies most ready to be pushed toward the field and was not reliant only on data presented in a proposal to make funding decisions. Importantly, technologies selected were not necessarily commercial-ready and technologies passed over were not deemed failures, but instead at too low a technology readiness level to progress.

The rapid development cycle proved to be a serious challenge for all parties involved in the process. We consciously chose technologies that had enough maturity to meet schedule, with true ideation-to-prototype only being the final multiplexed assay delivered. Even those efforts were atypically fast for commercial assays. As discussed earlier, assay testing for robustness is a clear tradeoff made with an accelerated development schedule. These tradeoffs are not only for long term storage, but are revealed in the ultimate limit of detection and rejection of false

positive and false negative results achieved. Optimization is by nature a late stage development activity and therefore most likely to be limited with compressed schedules. Additionally we chose to explore the viability of our tiered selection method with both low complexity LFIs and higher complexity PCR technology.

In total, we successfully performed over 10,000 tests, on four continents, with > 30 unique users in a variety of assay use settings. Successful detection of four different diseases was achieved. We compared the results of the custom technologies with standard-of-care or gold-standard tests. The various challenges of working in the field, including supply, shipping, ambient environments, and the 2014–2016 Ebola outbreak in Africa, resulted in a study that could not test every participant's sample on every platform. Regardless, concordance data collected shows a $> 85\%$ agreement for all platforms. The one exception is the concordance of malaria detection, which is reflective of two crucial factors. First the performance of smear analysis is known to be technically demanding and requires significant training. Our partner laboratories began with greatly varying levels of knowledge and a challenge faced was better preparing all clinics for smear analysis. Second, the DPP assay identified both HRP2 and pLDH positive samples. Sensitivity to multiple species of *Plasmodium* further complicates both confirmatory testing and the application of concordance algorithms. Moreover, HRP2 is an excellent marker for *P. falciparum*, but its presence is not necessarily indicative of active infection as the antigen is known to linger in vivo [27, 28]. Validation of malaria detection is clearly an area of improvement required for future studies. Third, we remain mindful that the custom technologies fielded in this study were developed on a rapid schedule and are not necessarily reflective of a final prototype or product our commercial partners would put into the field.

Overall, the 24 Month Challenge demonstrated that technologies could be identified, vetted, further developed, and fielded on a rapid schedule. We faced many challenges. Some challenges were expected, well-document obstacles for fielding any technology, and our methodology was intentional to address as many of those challenges as possible. Other challenges were inherent to a rapid development process, and they highlighted the many reasons why more time is typically used to bring technologies to the field. Nevertheless, we believe the method offers several advantages including science-first down selection at each step, independent validation of technologies by third party scientists, and the use of transmitted data to provide monitoring and reach-back support throughout the field study.

These initial results point toward two promising paths for future studies, both of which our research team is currently pursuing. First, lessons learned have improved our team's preparedness and infrastructure, thus new technologies can more readily be accepted into our tiered evaluation pathway and in-field feedback can be realized on a more rapid timescale. The gathered information is of great value to both the technology developer and the sponsor. Second, past performers can apply lessons learned in taking a technology through the tiered pathway and apply them to a second round of testing. In the second round of testing, the base technology would have an established baseline and a more limited amount of testing is required to evaluate any improvements. Concurrently, the improved assay from the first round or a new multiplexed assay could be field tested in a manner to include data relevant for regulatory approval.

Acknowledgements

The authors acknowledge numerous individuals and organizations for their contribution to this work. Dr. Kathleen Santos and colleagues at Johns Hopkins University Applied Research Laboratory and Dr. George Farquar and colleagues at Lawrence Livermore National Laboratory were, along with the NRL team, the government laboratories that served as evaluation centers for the 24 Month Program. Johns Hopkins University Applied Research Laboratory worked with

BioFire on the SASFI pouch and evaluated its analytical performance. Lawrence Livermore National Laboratory was instrumental in identifying the Deki Reader as a candidate technology for the program. The authors thank Dr. David AuCoin at the University of Nevada Reno, LCDR Todd Myers and colleagues at NMRC, CPT Scott Seronello and colleagues at WRAIR, and Dr. Christopher Plowe and colleagues at the University of Maryland for their contribution of samples and/or antigens. The authors thank LT Andrea McCoy, LT Luis Estrella, Mr. Anthony Bassett, and CDR Guillermo Pimentel at NMRC and Dr. David Norwood, Dr. Mark Wolcott, and colleagues at USAMRIID for their work in BSL3 evaluation of the custom technologies. The authors also acknowledge the commercial partners who developed the technologies fielded. Drs. Syamal Raychaudhuri, James Needham, Ray Houghton and colleagues at Inbios International, Inc., and Dr. David AuCoin and colleagues at the University of Nevada Reno developed the ADMD duplex LFI. Drs. Javan Esfandiari, Angelo Gunasekera, and colleagues at ChemBio Diagnostic Systems, Inc. developed the DPP multiplexed LFI. Drs. Roman Zastawny, Ian Fine, and colleagues developed the Deki Reader and the custom software.

Funding

This work was supported by the Defense Threat Reduction Agency through multiple funding streams to the author's institutions [HDTRA1515207, HDTRA1518257, HDTRA1619699, HDTRA1722044, HDTRA1721948]. DTRA was active in both research design and project management.

The authors declare no conflict of interest with the work reported in the manuscript. We do note that author S. Mulvaney is an editorial board member of Sensing and Bio-sensing Research.

Declaration of conflict of interest

The authors declare no conflict of interest with the work reported in the manuscript. We do note that author S. Mulvaney is an editorial board member of Sensing and Bio-Sensing Research.

Appendix A. Supporting data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sbsr.2018.06.003>.

References

- [1] J.A. Crump, M.D. Kirk, Estimating the burden of febrile illnesses, *PLOS Neglected Trop. Dis.* 9 (2015), <http://dx.doi.org/10.1371/journal.pntd.0004040>.
- [2] N. Prasad, D.R. Murdoch, H. Reyburn, J.A. Crump, Etiology of severe febrile illness in low- and middle-income countries: a systematic review, *PLoS One* 10 (2015), <http://dx.doi.org/10.1371/journal.pone.0127962>.
- [3] P. Teparrukkul, V. Hantrakun, N.P.J. Day, T.E. West, D. Limmathursakul, Management and outcomes of severe dengue patients presenting with sepsis in a tropical country, *PLoS One* 12 (2017), <http://dx.doi.org/10.1371/journal.pone.0176233>.
- [4] M. Kaestli, L.J. Richardson, R.E. Colman, A. Tuanyok, E.P. Price, et al., Comparison of TaqMan PCR assays for detection of the Melioidosis agent *Burkholderia pseudomallei* in clinical specimens, *J. Clin. Microbiol.* 50 (2012) 2059–2062, <http://dx.doi.org/10.1128/jcm.06737-11>.
- [5] G.A. Santiago, E. Vergne, Y. Quiles, J. Cosme, J. Vazquez, et al., Analytical and clinical performance of the CDC real time RT-PCR assay for detection and typing of dengue virus, *PLOS Neglected Trop. Dis.* 7 (2013), <http://dx.doi.org/10.1371/journal.pntd.0002311>.
- [6] S. Githinji, A.M. Noor, J. Malinga, P.M. Macharia, R. Kiptui, et al., A national health facility survey of malaria infection among febrile patients in Kenya, 2014, *Malar. J.* 15 (2016), <http://dx.doi.org/10.1186/s12936-016-1638-2>.
- [7] E.W. Wanja, N. Kuya, C. Moranga, M. Hickman, J.D. Johnson, et al., Field evaluation of diagnostic performance of malaria rapid diagnostic tests in western Kenya, *Malar. J.* 15 (2016), <http://dx.doi.org/10.1186/s12936-016-1508-y>.
- [8] E.M. Linares, L.T. Kubota, J. Michaelis, S. Thalhammer, Enhancement of the detection limit for lateral flow immunoassays: evaluation and comparison of bio-conjugates, *J. Immunol. Methods* 375 (2012) 264–270, <http://dx.doi.org/10.1016/j.jim.2011.11.003>.
- [9] P.D. Sinawang, V. Rai, R.E. Ionescu, R.S. Marks, Electrochemical lateral flow immunoassay for detection and quantification of dengue NS1 protein, *Biosens. Bioelectron.* 77 (2016) 400–408, <http://dx.doi.org/10.1016/j.bios.2015.09.048>.
- [10] P. Zhang, X. Liu, C. Wang, Y. Zhao, F. Hua, et al., Evaluation of up-converting phosphor technology-based lateral flow strips for rapid detection of *Bacillus anthracis* spore, *Brucella* spp., and *Yersinia pestis*, *PLoS One* 9 (2014), <http://dx.doi.org/10.1371/journal.pone.0105305>.
- [11] Z. Yan, L. Zhou, Y. Zhao, J. Wang, L. Huang, et al., Rapid quantitative detection of *Yersinia pestis* by lateral-flow immunoassay and up-converting phosphor technology-based biosensor, *Sensors Actuators B Chem.* 119 (2006) 656–663, <http://dx.doi.org/10.1016/j.snb.2006.01.029>.
- [12] G. Robertson, A. Sorenson, B. Govan, N. Ketheesam, R. Houghton, et al., Rapid diagnostics for melioidosis: a comparative study of a novel lateral flow antigen detection assay, *J. Med. Microbiol.* 64 (2015) 845–848, <http://dx.doi.org/10.1099/jmm.0.000098>.
- [13] R.L. Houghton, D.E. Reed, M.A. Hubbard, M.J. Dillon, H. Chen, et al., Development of a prototype lateral flow immunoassay (LFI) for the rapid diagnosis of melioidosis, *PLoS Neglected Trop. Dis.* 8 (2014), <http://dx.doi.org/10.1371/journal.pntd.0002727>.
- [14] Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs, Round 6 (2014–2015) (1–154 pp.).
- [15] S.A. Harvey, L. Jennings, M. Chinyama, F. Masaninga, K. Mulholland, et al., Improving community health worker use of malaria rapid diagnostic tests in Zambia: package instructions, job aid and job aid-plus-training, *Malar. J.* 7 (2008), <http://dx.doi.org/10.1186/1475-2875-7-160>.
- [16] S. Shekalaghe, M. Cancino, C. Mavere, O. Juma, A. Mohammed, et al., Clinical performance of an automated reader in interpreting malaria rapid diagnostic tests in Tanzania, *Malar. J.* 12 (2013), <http://dx.doi.org/10.1186/1475-2875-12-141>.
- [17] D.O. Soti, S.N. Kinoti, A.H. Omar, J. Logedi, T.K. Mwendwa, et al., Feasibility of an innovative electronic mobile system to assist health workers to collect accurate, complete and timely data in a malaria control programme in a remote setting in Kenya, *Malar. J.* 14 (2015) 8, <http://dx.doi.org/10.1186/s12936-015-0965-z>.
- [18] S. Herrera, A.F. Vallejo, J.P. Quintero, M. Arevalo-Herrera, M. Cancino, et al., Field evaluation of an automated RDT reader and data management device for *Plasmodium falciparum*/*Plasmodium vivax* malaria in endemic areas of Colombia, *Malar. J.* 13 (2014), <http://dx.doi.org/10.1186/1475-2875-13-87>.
- [19] M. Gunasekera, M. Narine, M. Ashton, J. Esfandiari, Development of a Dual Path Platform (DPP®) immunoassay for rapid detection of *Candida albicans* in human whole blood and serum, *J. Immunol. Methods* 424 (2015) 7–13, <http://dx.doi.org/10.1016/j.jim.2015.04.014>.
- [20] S.A. Nabity, G.S. Ribeiro, C.L. Aquino, D. Takahashi, A.O. Damiao, et al., Accuracy of a dual path platform (DPP) assay for the rapid point-of-care diagnosis of human leptospirosis, *PLOS Neglected Trop. Dis.* 6 (2012), <http://dx.doi.org/10.1371/journal.pntd.0001878>.
- [21] K.C. Iregbu, J. Esfandiari, J. Nnorom, S.A. Sonibare, S.N. Uwaezuoke, et al., Dual Path Platform HIV 1/2 assay: evaluation of a novel rapid test using oral fluids for HIV screening at the National Hospital in Abuja, Nigeria, *Diagn. Microbiol. Infect. Dis.* 69 (2011) 405–409, <http://dx.doi.org/10.1016/j.diagmicrobio.2010.10.011>.
- [22] R. Greenwald, O. Lyashchenko, J. Esfandiari, M. Miller, S. Mikota, et al., Highly accurate antibody assays for early and rapid detection of tuberculosis in African and Asian elephants, *Clin. Vaccine Immunol.* 16 (2009) 605–612, <http://dx.doi.org/10.1128/cvi.00038-09>.
- [23] P. Ruggiero, T. Mcmillen, Y.W. Tang, N.E. Babady, Evaluation of the BioFire FilmArray respiratory panel and the GenMark eSensor respiratory viral panel on lower respiratory tract specimens, *J. Clin. Microbiol.* 52 (2014) 288–290, <http://dx.doi.org/10.1128/jcm.02787-13>.
- [24] K. Messacar, G. Breazeale, C.C. Robinson, S.R. Dominguez, Potential clinical impact of the film array meningitis encephalitis panel in children with suspected central nervous system infections, *Diagn. Microbiol. Infect. Dis.* 86 (2016) 118–120, <http://dx.doi.org/10.1016/j.diagmicrobio.2016.05.020>.
- [25] S.L. Snyder, K.B. McAuley, P.J. McLellan, E.B. Brouwer, T. McCaw, Modeling the thermal stability of enzyme-based in vitro diagnostics biosensors, *Sensors Actuators B Chem.* 156 (2011) 621–630, <http://dx.doi.org/10.1016/j.snb.2011.02.008>.
- [26] N.A. Chaniotakis, Enzyme stabilization strategies based on electrolytes and poly-electrolytes for biosensor applications, *Anal. Bioanal. Chem.* 378 (2004) 89–95, <http://dx.doi.org/10.1007/s00216-003-2188-3>.
- [27] J.C. Mouatcho, J.P.D. Goldring, Malaria rapid diagnostic tests: challenges and prospects, *J. Med. Microbiol.* 62 (2013) 1491–1505, <http://dx.doi.org/10.1099/jmm.0.052506-0>.
- [28] M.H. Nyunt, M.P. Kyaw, K.K. Win, K.M. Myint, K.M. Nyunt, Field evaluation of HRP2 and pan pLDH-based immunochromatographic assay in therapeutic monitoring of uncomplicated falciparum malaria in Myanmar, *Malar. J.* 12 (2013), <http://dx.doi.org/10.1186/1475-2875-12-123>.