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This project was conducted under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19;

14. ABSTRACT

A Plasmodium genus TaqMan assay (16S rRNA gene target) wet assay was successfully developed and tested. The wet assay showed promise as a highly sensitive and specific malaria causative agent detection test however in the freeze-dried formulation chronic cross-reactivity occurred. The freeze-dried (FD) assay was deployed for use in an established vector surveillance program at an AFRIMS field site (Mae Sot, Thailand). During field-evaluation the FD assay consistently reported cross-reactivity against an established negative control panel as well as non-infected mosquito extracts. Fluorescence above background was periodically observed for negative template control (NTC) reactions. Subsequent to field study, FD assays stored at 4oC were tested using the negative control panel, non-infected mosquito extracts, and NTC. No fluorescence above background was observed using 4oC stored FD assays. The conclusion was probable TaqMan probe degradation due to thermal instability. By the conclusion of the study the issue was not resolved. Under follow-on studies, a highly sensitive and specific Plasmodium genus TaqMan assay was developed.

15. SUBJECT TERMS

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Clinical Research Division
59th Medical Wing, Lackland AFB, Texas

And

Armed Forces Research Institute for the Medical Sciences (AFRIMS),
Bangkok, Thailand

Final Report

AFMSA O&M FY09 Project (FWH20090036H):

Plasmodium Genus Assay Transition to the Joint Biological Agent Identification and Diagnostic System (JBAIDS)

July 12, 2012

Reporting Period: October 1, 2008 to September 30, 2009

Principal Investigator: Col James A. Swaby
59th Medical Wing, Lackland, AFB Texas

Report Prepared by:

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Designated PI 18 June 2012
Clinical Research Division/59th Medical Wing

Project Objectives

1. Complete RAPID-based test and evaluation data to support AFPMB approval of the RAPID-based *Plasmodium* genus assay for use in vector/vector-borne disease surveillance on the RAPID.

Objective 1 Results: *Plasmodium* genus RAPID/JBAIDS assay development activities conducted during the FY09 study did not resolve cross-reactivity issues.

Plasmodium genus TaqMan assay development was successfully completed under the AFMSA O&M FY10 'Plasmodium' project described in DTIC submission;

"Final Report on AFMSA O&M FY10 Project (FWH20090194E): Test and Evaluation of Field-Deployable Infectious Disease Diagnostic Assays in Support of the Joint Biological Agent Identification and Diagnosis System (JBAIDS): Malaria (Plasmodium/JBAIDS)."

Follow-on RDT&E efforts conducted by Army collaborators exceeded assay development objectives proposed under this study. *Plasmodium falciparum* and *Plasmodium vivax* TaqMan assays have been developed by WRAIR/AFRIMS/USAMRU-K collaborators. These assays are formulated and optimized for use with RAPID/JBAIDS platforms and are readily transferable to diverse real-time PCR instrumentation. These assays are used regularly for vector surveillance and are the primary candidates for transfer to microarray-based analytic systems. A Material Transfer Agreement (MTA) with Idaho Technology, Inc. for transfer to microarray instrumentation (FilmArray) is under assessment.

Assay oligonucleotide sequences and formulations are available for use in future joint projects. A data sharing agreement will be formalized through the WRAIR & 59th MDW MOA (pending amendment and signature).

2. Complete JBAIDS-based test and evaluation data to support JBAIDS program manager approval of the JBAIDS-based *Plasmodium* genus assay for use in environmental (non-human) surveillance on the JBAIDS. Objective 2 Results: Results from the FY09 study do not support Molecular Assay Transition Package submission to the JBAIDS program manager.

3. Assays qualified by the JBAIDS program manager as a qualified candidate assays for future research targeted at getting FDA-clearance for human diagnostics on the JBAIDS under a separate research proposal. Objective 3 Results: Results from the FY09 study do not support submission for JBAIDS program manager qualification of this assay for FDA clearance.

4. Provide scholarly and challenging Graduate Medical Education opportunities for residents, fellows and staff. Objective 4 Results: During this study GME was not accomplished.

Summary

Develop of *Plasmodium* real-time PCR detection capability has been challenging. During 2006, the Division of Entomology, WRAIR designed and developed a *Plasmodium* genus TaqMan assay (16S rRNA gene target). The chemistry applied was minor groove binder (MGB). During 2007, the assay was redesigned and reformulated using standard TaqMan chemistry to meet JPO/JBAIDS requirements. During FY07 - FY08 *Plasmodium* genus assay optimization, freeze-dried reagent production, and preliminary sensitivity and specificity testing were successfully completed. However, during field evaluation the assay failed to meet acceptance test criteria due to probable thermal-stability issues. Testing conducted during showed cross-reactivity. Resolution of cross-reactivity (potential thermal stability issues) was not resolved by the completion of this AFMSA FY09 study.

Project funding was provided by the Air Force Medical Support Agency (AFMSA), Research, Development and Innovations Directorate (SG9), Office of the Surgeon General (AF/SGR) Falls Church, Virginia and the Military Infectious Diseases Research Program (MIDRP), USAMRC, Fort Detrick, Frederick, Maryland. Project execution was conducted by the Armed Forces Research Institute for the Medical Sciences (AFRIMS) and Clinical Research Division (CRD)/59th MDW. This project was jointly funded and executed under memorandum of agreement (MOA) between Walter Reed Army Institute of Research (WRAIR), Silver Spring, Maryland & 59th Medical Wing (MDW) Lackland AFB, Texas (MOA 2007 - 2012. Agreement No.: DODI 4000.19; AFI 25-201).

Products Completed

Development activities conducted during this study were unable to resolve cross-reactivity. Follow-on RDT&E efforts conducted by Army collaborators exceeded assay development objectives proposed under this study. A data sharing arrangement provides for use of, TaqMan assay oligonucleotide sequences and formulations, in future joint projects pending WRAIR & 59th MDW amendment and signature.

Use by the Government

This study addresses DoD priorities in protecting the health of soldiers. Malaria is ranked first among the top 40 diseases in the DoD global risk-severity index and recognized as a military significant disease.

With no licensed vaccine available, accurate diagnostics and real-time disease surveillance capability are essential in efficacious treatment and mitigating outbreaks. The assays facilitate timely assessments of transmission risk and time-critical implementation of personal protection and focused transmission control measures. The assays are used in US Military mosquito-borne disease agent surveillance programs.
Under a separate protocol, JBAIDS validated assay primer and probe sequences will be classified as Government Furnished Material (GFM) and Government Furnished Information (GFI) respectively and serve as deliverable to the Joint Program (JPEO) for integration in Critical Reagent Program (CRP) and potential Federal Drug Administration (FDA) clearance using the

DoD Joint Biological Agent Identification and Diagnostic System (JBAIDS). The oligonucleotides are candidates for transfer to molecular-based "Next Generation Diagnostic System" platforms. The ultimate objective of the DoD is commercialization of the assays for disease surveillance and human diagnostics.

Commercial Application

Malaria is one of the most widespread mosquito-borne diseases in the world. Commercializing the assays will allow public health organizations globally to enhance malaria surveillance programs and potentially provide an aid in diagnosis.

Purpose

To test and evaluate critical Force Health Protection disease surveillance assays in support of the JBAIDS program. Assays evaluated included mosquito-borne causative agents for malaria. This work was undertaken to address the critical need for field expedient assays to evaluate the complete epidemiology of these diseases to support "real-time" preventative and control measures. The ability of medical personnel to accurately survey and recognize infectious disease threats in an operational environment is a high priority. The rapid identification of an infectious agent will allow for prompt, appropriate treatment, thereby minimizing morbidity and mortality. Additionally, knowledge about a specific infectious disease threat will allow for the implementation of prevention and control efforts to protect the fighting force.

Problem

Naturally occurring infectious diseases pose a significant threat to military operations and can account for up to 80% of all casualties. Over 25% of marines deployed to Liberia in 2003 were infected with the potentially fatal falciparum malaria. Between 2003 and 2005, over 2,000 military personnel deployed to Iraq were infected with leishmaniasis. A variety of other infectious diseases have impacted every military operation conducted in the past century.

The Joint Biological Agent Identification and Diagnosis System (JBAIDS) is an $80 million acquisition program that is currently fielding approximately 425 JBAIDS platforms. Idaho Technology, Inc. (ITI) is the prime contractor for the JBAIDS program, responsible for delivering both the platform and associated assays. The JBAIDS program is currently funding the development of the 10 "first tier" assays contained in the JBAIDS Operational Requirements Document (ORD). All 10 of these agents are Biological Warfare Agents. The JBAIDS ORD also requires the development of an additional 10 "second tier" assays for naturally-occurring infectious disease agents. Funding has not been provided for the development, testing and evaluation of these "second tier" assays.

Background

This project was conducted through WRAIR & 59th MDW MOA FY07 - FY12. During 2006, the Division of Entomology, WRAIR designed and developed a *Plasmodium* genus TaqMan assay (16S rRNA gene target). The chemistry applied was minor groove binder (MGB). TaqMan MGB probes are typically used for allelic discrimination assays to provide for more accurate allelic discrimination. The WRAIR used MGB chemistry because it also provides more flexibility in probe design. This chemistry increases the melting temperature (Tm) thus allowing the design of shorter probes where target sequence is limited such as the *Plasmodium* genus 16S rRNA target sequences. The results of the assay were promising (Fig. 1).

However, JBAIDS TaqMan chemistry requirements prohibit the use of MGB therefore the assay was re-designed using conventional TaqMan chemistry, a probe with TAMRA dye as the quencher dye. The assay was optimized using wet JBAIDS reagents, LoD established, and preliminary specificity testing conducted. Wet assay formulation was used to prepare freeze-dried (FD) assays (Idaho Technology, Inc.). The FD assay was transitioned to the RAPID/JBAIDS and platforms and the performance compared to an existing TaqMan format (Cepheid SmartCycler). Laboratory testing using the RAPID/JBAIDS was conducted at the Entomology Department, AFRIMS and field validation testing subsequently conducted at AFRIMS Malaria field site at Mae Sot.

During FY07 - FY08 *Plasmodium* genus assay optimization, FD reagent production, and preliminary sensitivity and specificity testing were successfully completed (Fig. 2-4). However, under field test conditions the assay failed to meet acceptance test criteria presumably due to probable thermal-stability issues (results are shown in figures 5-7). Testing conducted during FY09 showed cross-reactivity that remained unresolved at the completion of the study (representative results are shown in figures 7).

During the FY07 – FY09 studies, *Plasmodium* genus assay NALOD was established with wet reagents on the RAPID, and the preliminary lower limit of detection for this assay was found to be roughly 7.5 fg/ul using *Plasmodium falciparum* template strain 3D7G1. This concentration of Plasmodium DNA corresponds to approximately 3 genome copies of *Plasmodium falciparum* DNA. Freeze-dried reagents for the *Plasmodium* genus assay were prepared by ITI using the optimized conditions of the wet reagents, and the NALOD was confirmed on the JBAIDS instrument using the standard JBAIDS thermocycling conditions for DNA target. The assessment performed by ITI showed robust amplification of the genomic DNA target (Appendix A).

Plasmodium genus FD assays were hand-carried to the Entomology Department, AFRIMS to support laboratory validation testing and field evaluation at the AFRIMS Malaria field site at Mae Sot, Thailand. Just prior to field testing, an abbreviated validation study of the freeze-dried assays confirmed LoD, sensitivity and specificity performance (Fig. 5).

Figure 1. Amplification plots of *Plasmodium* genus 16S RNA target using MGB chemistry.

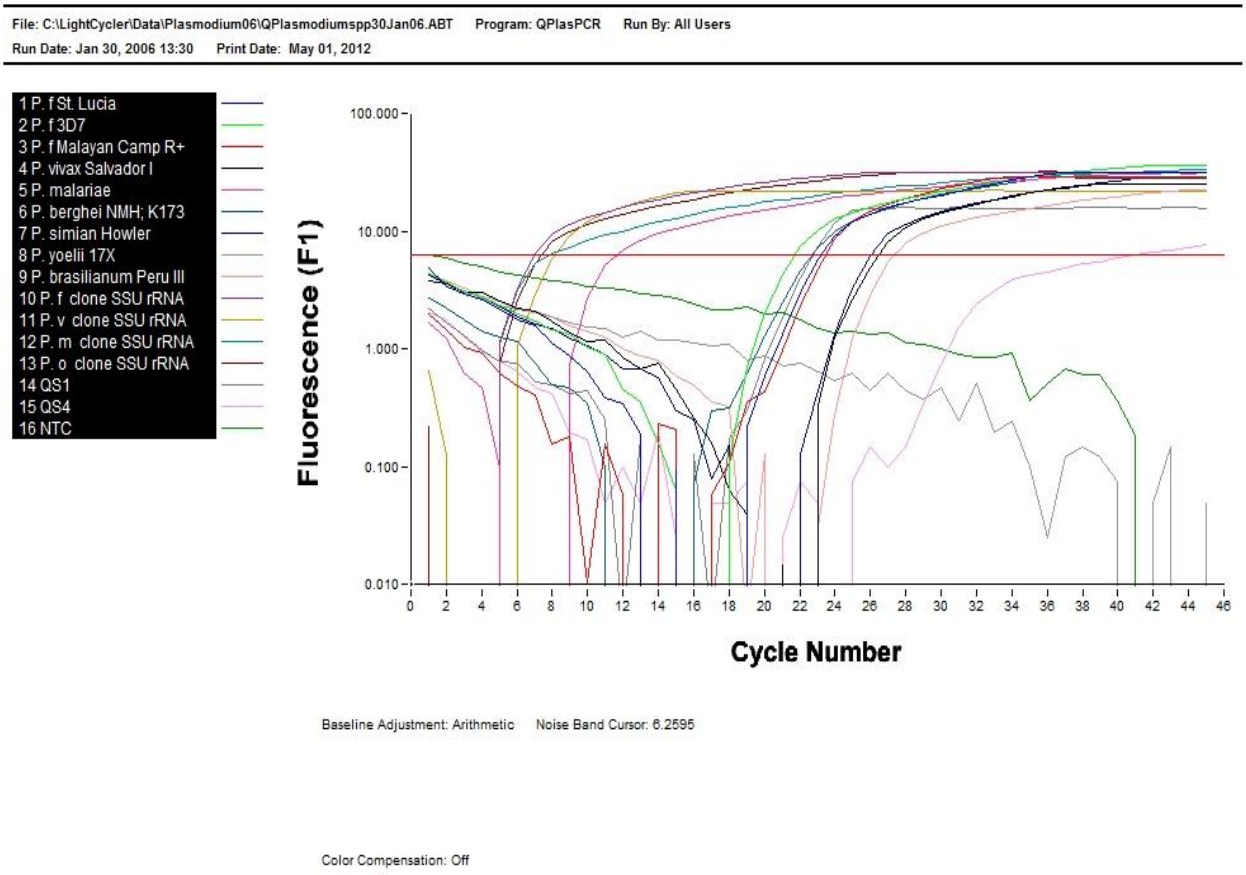


Figure 2. Amplification plots of *Plasmodium* genus MgCl2 optimization using TaqMan chemistry.

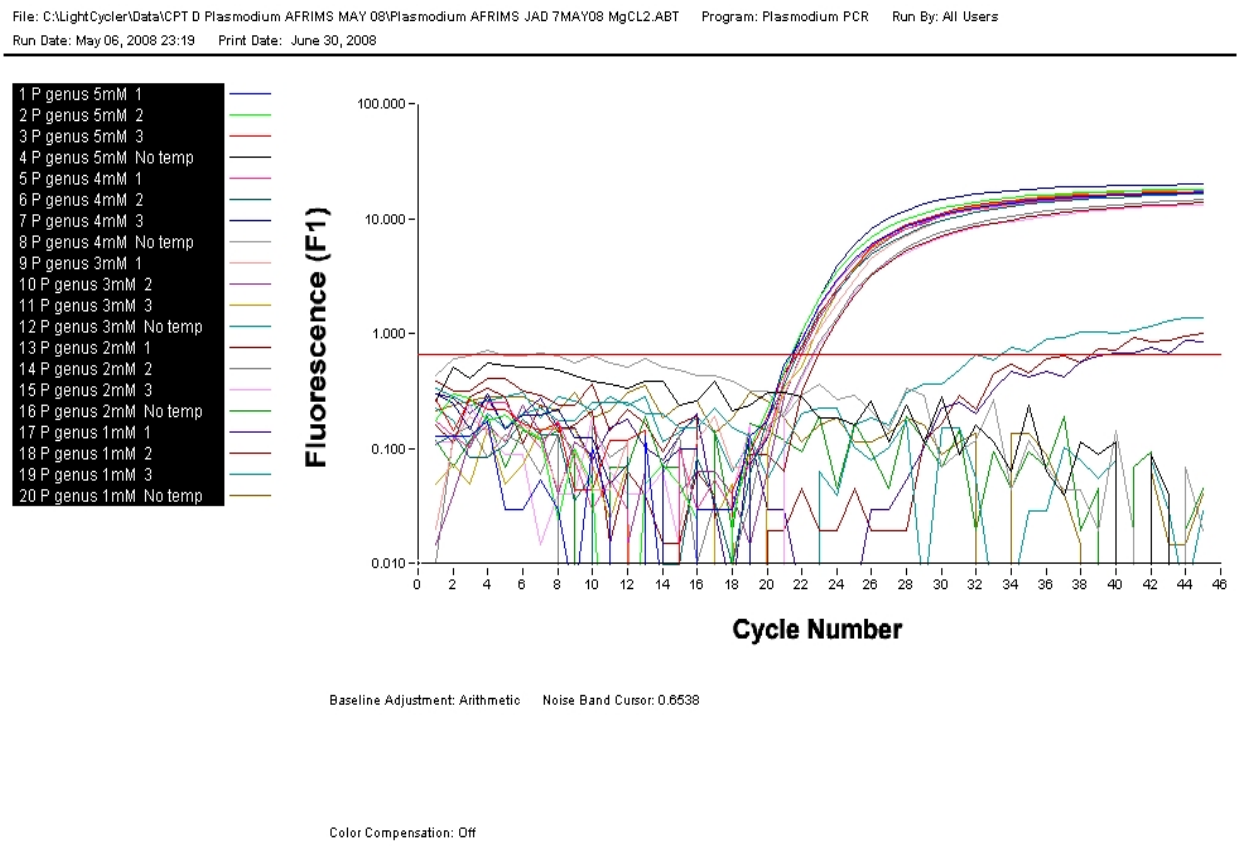


Figure 3. Amplification plots of *Plasmodium* genus primer titration using TaqMan chemistry.

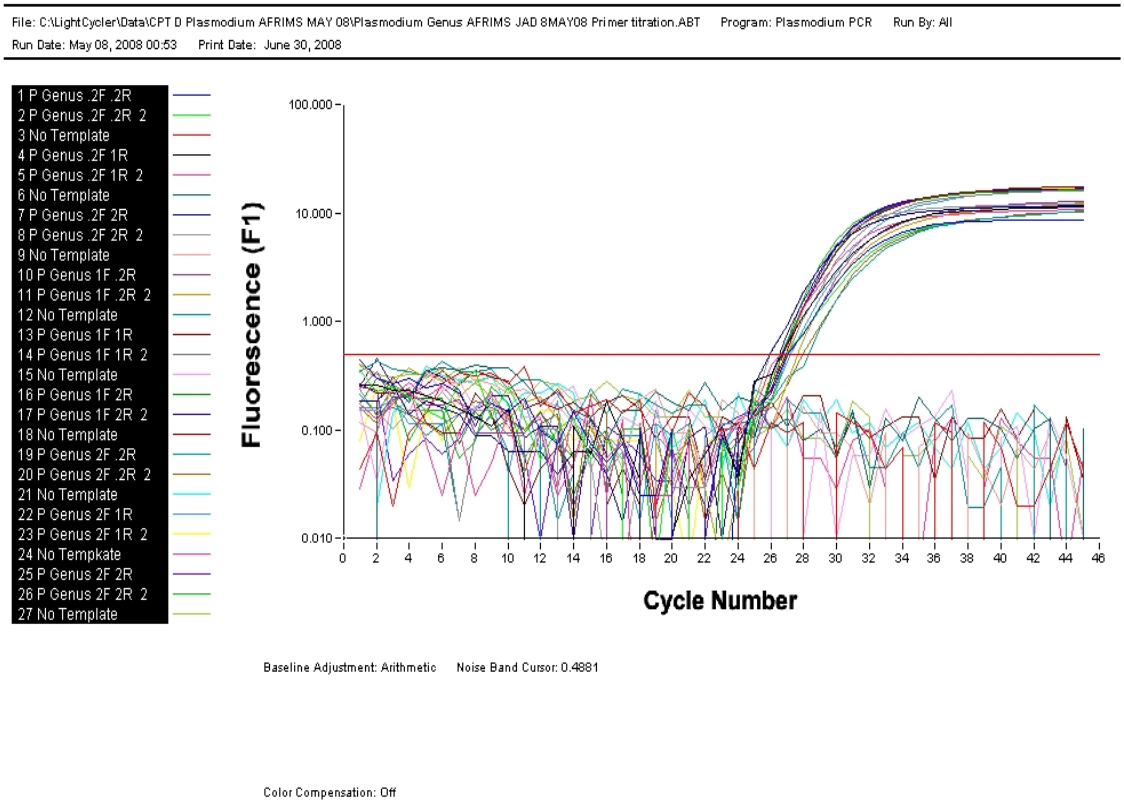


Figure 4. Amplification plots of *Plasmodium* genus TaqMan probe titration.

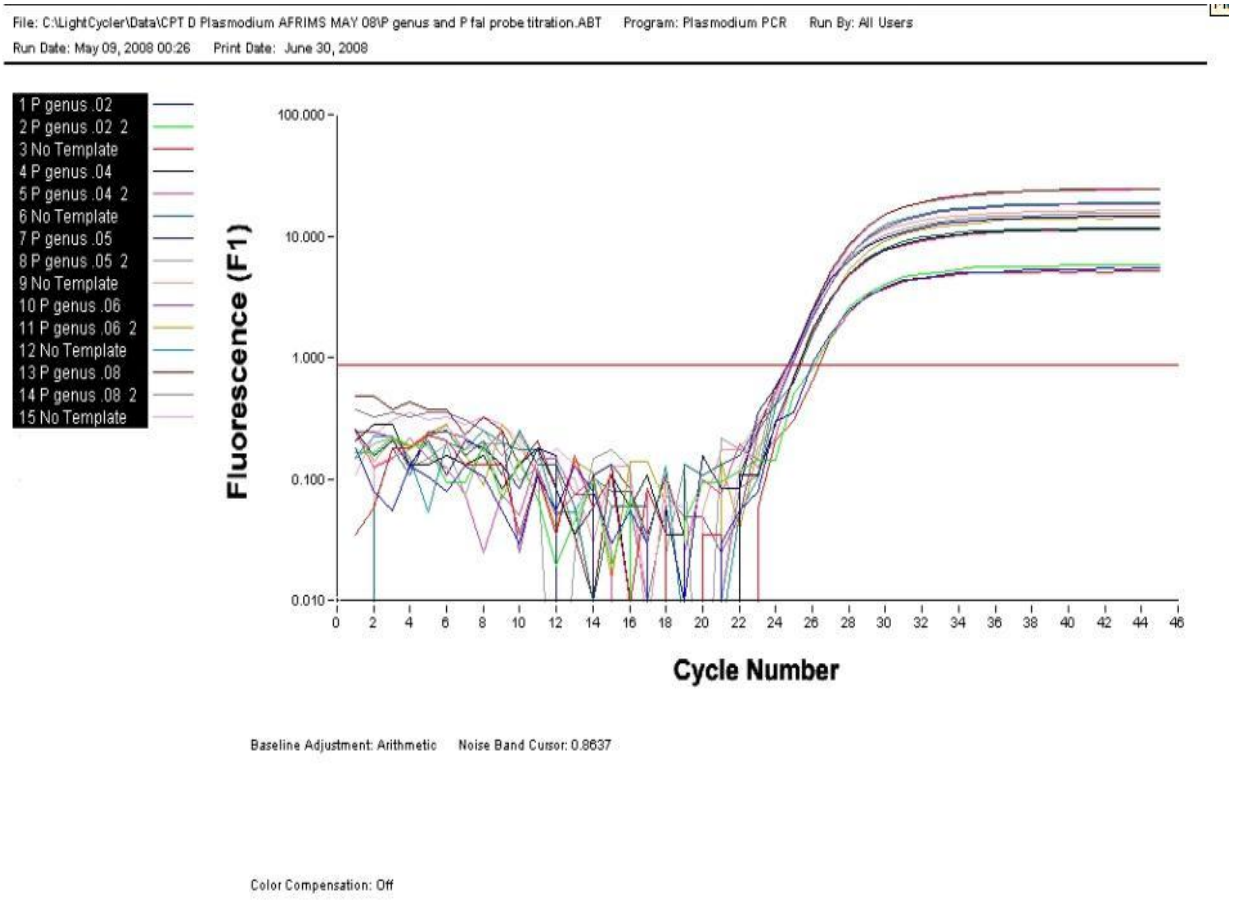
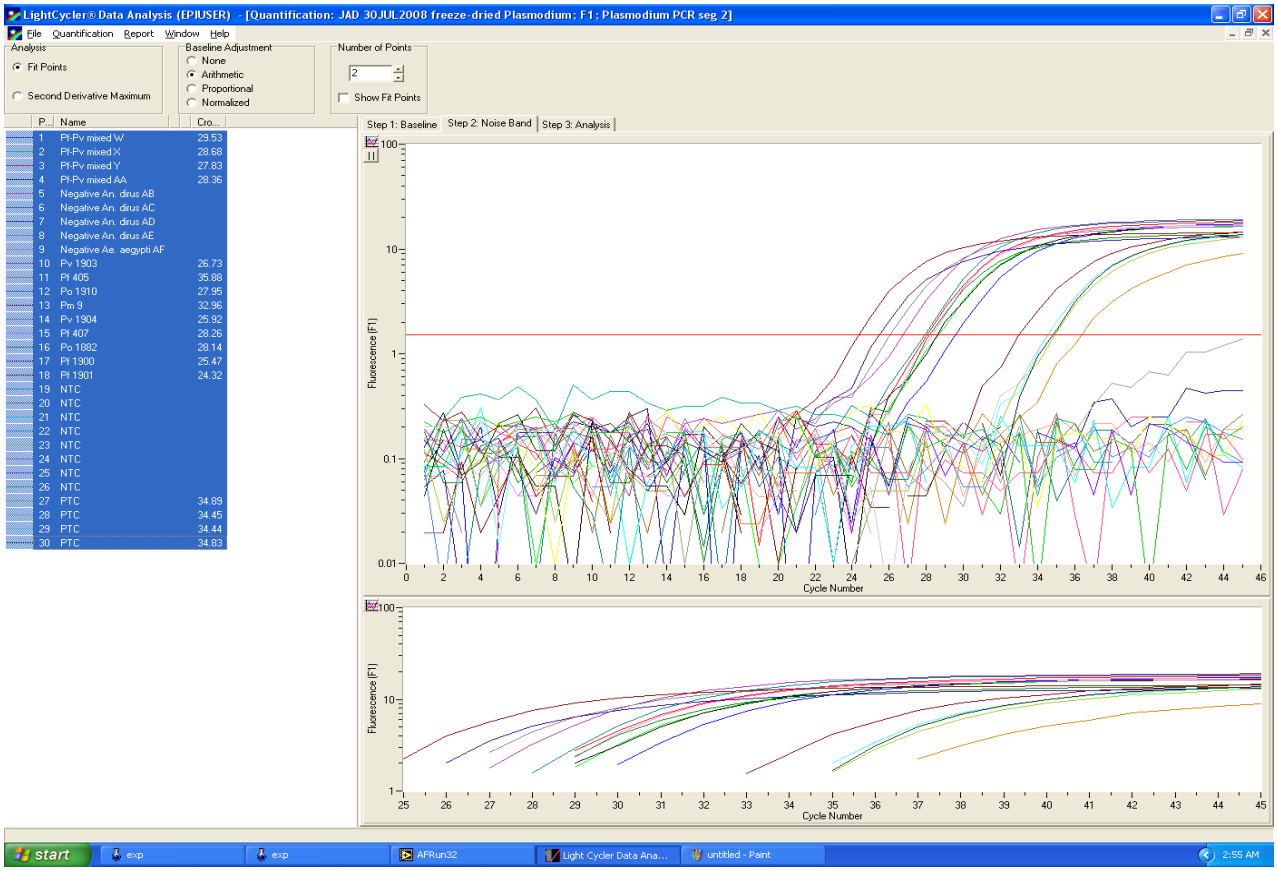


Figure 5. *Plasmodium* genus TaqMan RAPID assay sensitivity and specificity test results.



This validation work was followed by vector surveillance studies at the Mae Sot field site in Thailand during the week of 14 July, 2008. The PI, co-investigators, and GME student traveled to the Mae Sot province for initial field-based testing. Collections and processing of genomic DNA from female anopheles mosquitoes proceeded the testing of the assay on the JBAIDS and RAPID. Once the first set of mosquitoes was collected and processed, an initial reagent QC run was performed. Immediately, the presence of false-positives was discovered (Fig. 6). The false positives initially were attributed to the presence of contamination, so the entire PCR working section of the field laboratory was decontaminated, and the samples were re-run. Again, false positives were noted. The following run (performed at the same location) was a complete rotor run of negative controls and positive controls (28 negatives and 4 positives). 30% of the negative controls produced false positives.

The work was subsequently moved near the anopheles collection site at Mae Sot, and the process of running samples of extracted anopheles gDNA resumed; however, false-positives were observed (Fig. 7 - 8). Additional rotor runs of negative controls were run; however, approximately 30% of the negative controls were again coming up positive. At this point, we began to consider the possibility that there may be a problem with the thermostability of the freeze-dried chemistry or specific lots of the reconstitution buffer. The following day (Tuesday, July 17, 2008), we tested all available lots of the reconstitution buffer in an attempt to rule out

the possibility of a problem with a specific lot of buffer. The results from this experiment indicated no link of the false-positive problem to a particular lot of reconstitution buffer. The team returned to AFRIMS (Entomology Division) on Friday, July 18, 2008, and conducted repeat testing using a specific portion of freeze-dried reagents that were maintained at 4 degrees Celsius for the duration of the TDY. Results from these experiments deviated from the false-negative results we had encountered in previous experiments with freeze-dried reagents maintained at ambient temperatures.

Laboratory versus field test results indicated probe degradation when stored at ambient temperatures. Examples of assay performance when tested under cold chain and ambient temperatures are shown below (Fig. 9 and 10). The probable cause of probe degradation was assay thermal instability resulting from an uncoupling of probe re-design with reformulation either at the research or production level.

Figure 6. *Plasmodium* genus RAPID TaqMan assay QC field 15 Jul test results.

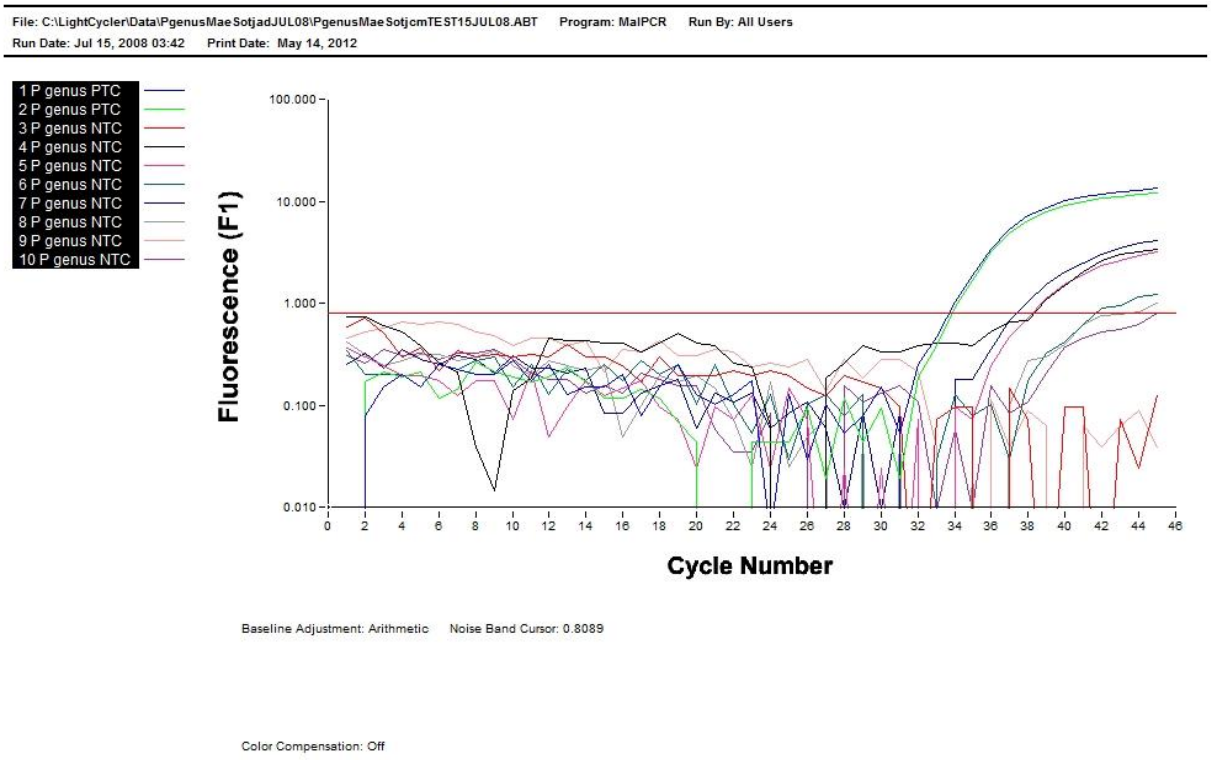


Figure 7. *Plasmodium* genus TaqMan RAPID assay field test 17 JUL results.

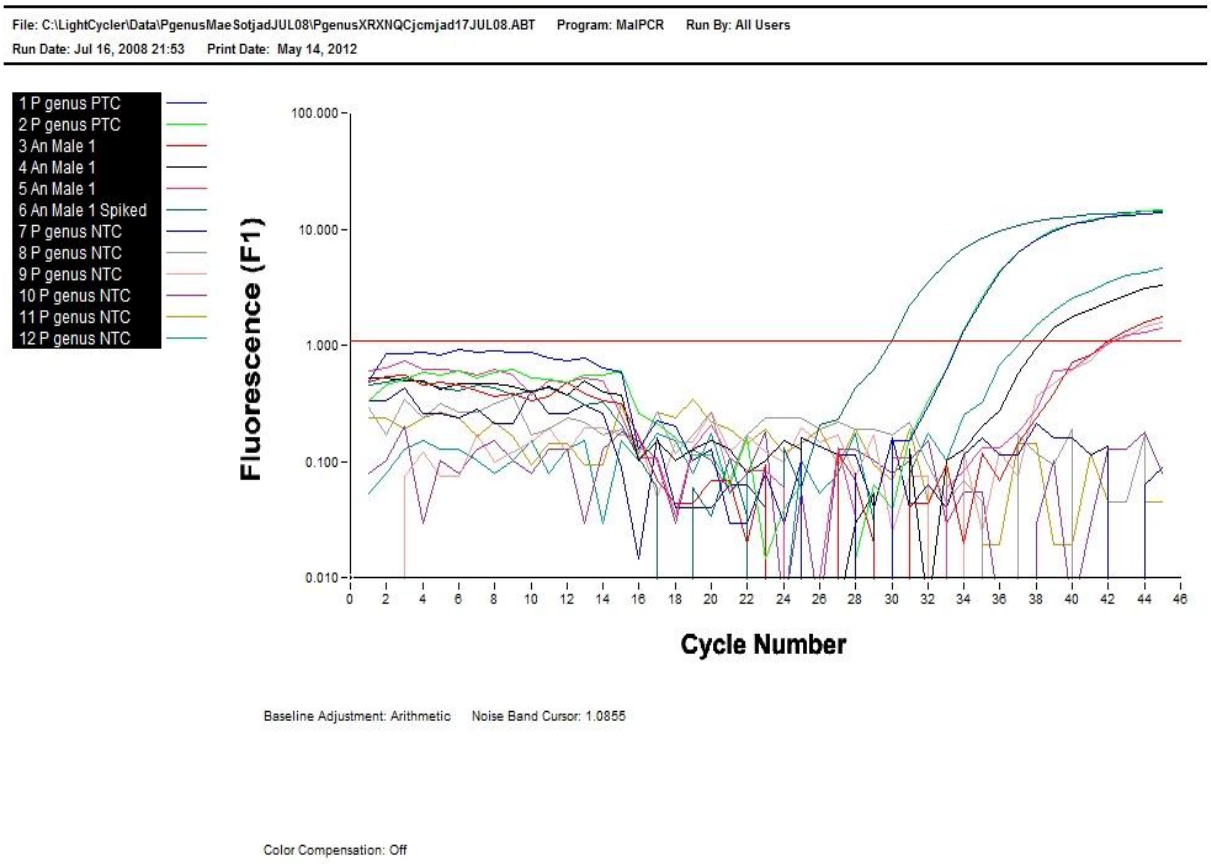


Figure 8. *Plasmodium* genus TaqMan assay field 17 JUL test results.

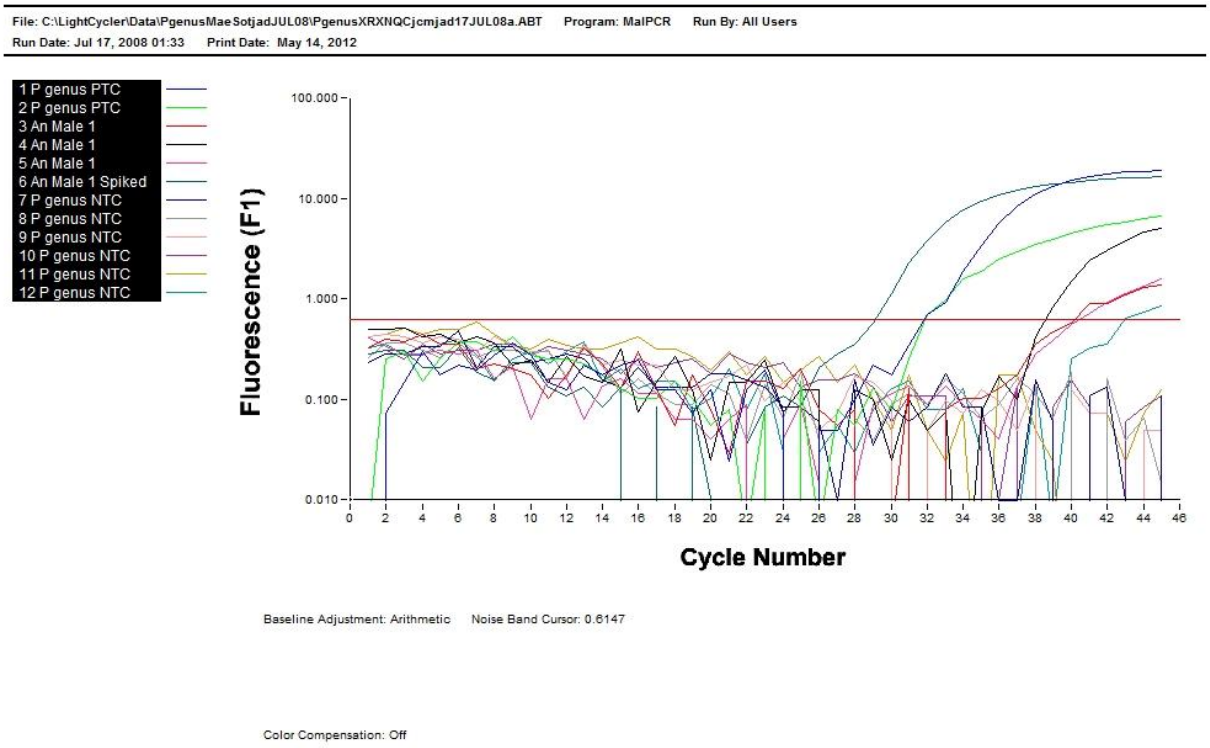


Figure 9. Post field test evaluation: performance of the *Plasmodium* genus TaqMan RAPID assay when stored at 4º centigrade

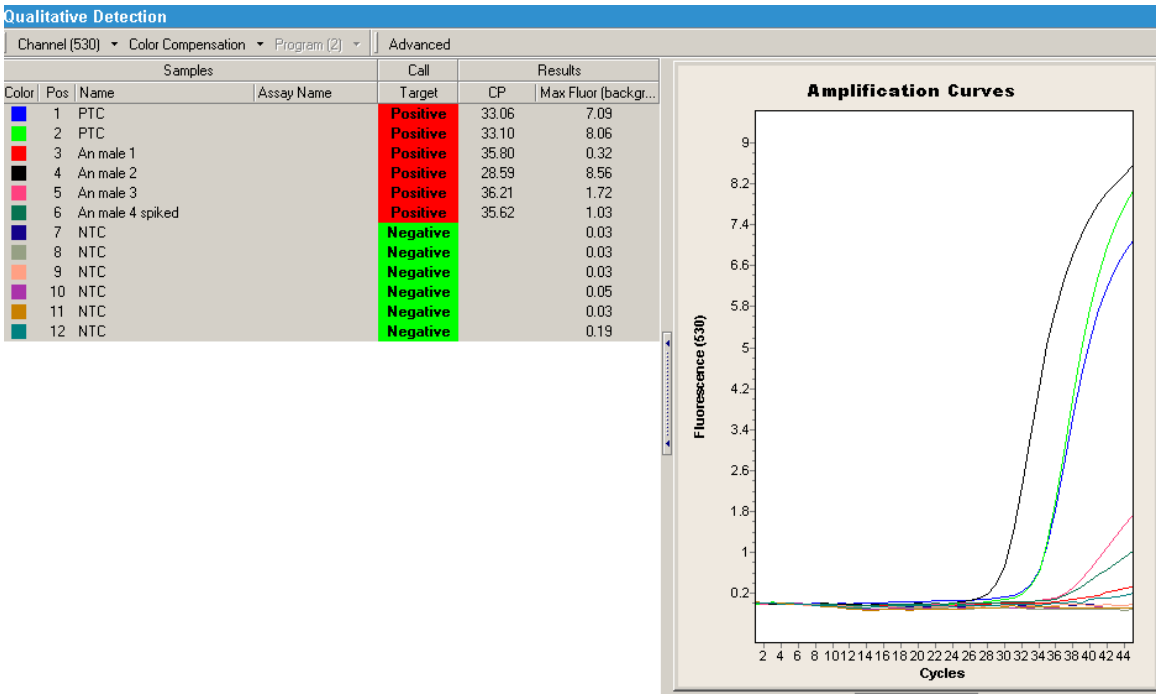
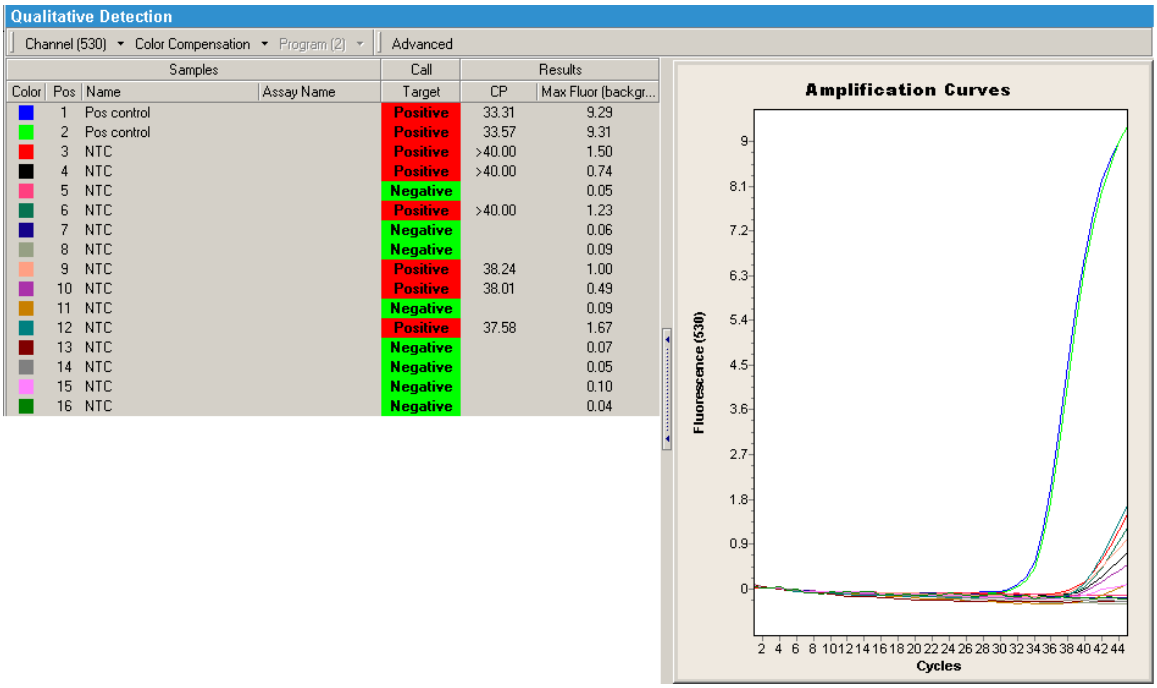


Figure 10. Post field test evaluation: performance of the *Plasmodium* genus TaqMan RAPID assay when exposed to ambient temperatures



Coordination on the resolution of potential thermal stability issues, production of assays, and planning for re-testing was attempted during FY09. The intent was to thermal-stabilize the assay and establish AFPMB and JBAIDS program manager approved RAPID/JBAIDS malaria causative agent detection capability. However, during the FY09 study FD assay QC testing conducted at WRAIR cross-reactivity was observed with uninfected mosquito homogenate and NTC. As such, primer and probe sequences required redesign.

During March through May, 2012 AFRIMS collaborators provided funding, technical staff, facility use, and supplies to complete AFMSA O&M FY10 Plasmodium Project funded deliverables. Results are described in the AFMSA O&M FY10 Final Report.

Conclusion

For this AFMSA O&M FY09 project, testing conducted during the study was not able to resolve *Plasmodium* genus assay cross-reactivity.

Follow-on RDT&E efforts conducted by Army collaborators exceeded assay development objectives proposed under this study. *Plasmodium* genus, *Plasmodium* genus, and *Plasmodium* vivax TaqMan assays have been developed by WRAIR/AFRIMS/USAMRU-K collaborators. These assays are formulated and optimized for use with RAPID/JBAIDS platforms and are readily transferable to diverse real-time PCR instrumentation. These assays are used regularly for vector surveillance and are the primary candidates for transfer to microarray-based analytic systems. A Material Transfer Agreement (MTA) with Idaho Technology, Inc. for transfer to microarray instrumentation (FilmArray) is under assessment.

Assay oligonucleotide sequences and formulations are available for use in future joint projects. A data sharing agreement will be formalized through the follow-on WRAIR & 59th MDW MOA (FY13 amendment).