

Microsphere Hybridization-Based Molecular Assay Development: Transitioning PCR Assays for the U.S. Army Public Health Command

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Abstract

There are many human pathogens transmitted by ticks, including *Borrelia burgdorferi* (Lyme disease agent) and *Rickettsia rickettsii* (Rocky Mountain spotted fever agent). The U.S. Army Public Health Command (USAPHC) monitors these and other pathogens by analysis of ticks collected from the environment and those found on military and civilian personnel. This analysis is commonly performed with conventional or quantitative PCR (qPCR). However, the process is time-consuming since qPCR instruments are often limited to a triplex, reducing the number of pathogens and targets that can be simultaneously detected.

USAPHC has a need for greater sample throughput via multiplex analysis methods which allow for additional targets to be detected using fewer assays. The Luminex MAGPIX detects nucleic acid targets by coupling multiplex PCR with microsphere hybridization, allowing detection of 50 targets per sample in a 96 well plate. To leverage this technology, the BioSciences Division at the U.S. Army Edgewood Chemical Biological Center is partnering with USAPHC to transition tick-borne pathogen qPCR assays to the MAGPIX. We have developed two focused multiplex PCR panels. The Rickettsia panel is a 4-plex that includes a genus-specific assay, as well as species-specific assays for *R. rickettsii*, *Rickettsia parkeri* and *Rickettsia montanensis* for analysis of *Dermacentor* and *Amblyomma* ticks. The Black-legged tick (*Ixodes*) panel is a 7-plex, and includes two assays for *Babesia microti*, two assays for *Anaplasma phagocytophilum*, one assay that identifies both *B. burgdorferi* and *Borrelia miyamotoi*, as well as a species-specific assay for each.

All panels include four controls: positive PCR, DNA extraction, positive hybridization and negative hybridization. We have analyzed 22 ticks previously tested by USAPHC with the Rickettsia panel, and all were identified correctly. Fifty ticks were analyzed by the *Ixodes* panel, and were called correctly 86% of the time. Limit of detection testing suggests that most of the included assays can accurately identify targets as low as 100 copies. The panels will be further validated by analysis of a larger number of ticks previously tested by USAPHC to determine assay performance. The results of these efforts can have an immediate impact within USAPHC by increasing throughput capabilities.

There are MAGPIX instruments at USAPHC as well as other federal laboratories, placed by the Global Biosurveillance Technology Initiative (GBTI), the Joint United States Forces Korea Portal and Integrated Threat Recognition (JUPITR) Advanced Technology Demonstration (ATD).



Introduction

Ticks require a blood meal as larvae, nymphs and as adults, and can be infected by a human pathogen at any of these stages. Most of the human pathogens are not transmitted transovarially; the *Rickettsiae* are a notable exception. An infected tick may in turn infect a human during their next blood meal, which can take up to 72 hours to complete. The chance of infection increases the longer the tick feeds on the host. There are many different pathogens that can infect ticks, however, there is some species-specificity. For example, *B. burgdorferi* (agent of Lyme disease) is not thought to be transmitted by *Amblyomma americanum* ticks due to a borreliacidal agent in their saliva (1). In addition, their range (southeastern US), host preferences and habits preclude these types of infections. There are similar reasons for pathogen specificity to other tick species as well.

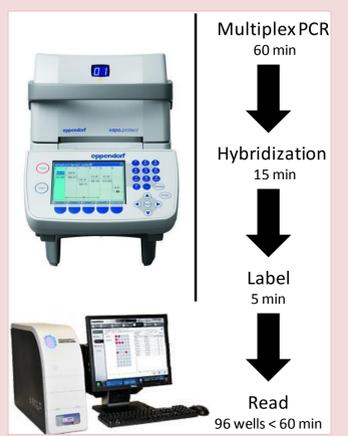
USAPHC tests ticks for human pathogens. To reduce workload while maintaining specificity, USAPHC uses PCR primer assays specific to each tick species. For example, *A. americanum* ticks are not tested for *B. burgdorferi*, although the tick species *Ixodes scapularis* is tested. These robust assays have been tested in USAPHC hands with thousands of ticks. Therefore, we will transfer these experimentally well-defined PCR assays to the Luminex MAGPIX instead of developing new PCR assays.

MAGPIX protocol:

Multiplex PCR: MAGPIX assays are set up like conventional PCR, with primers (one biotinylated primer per amplicon), master mix, and template for 35-40 cycles.

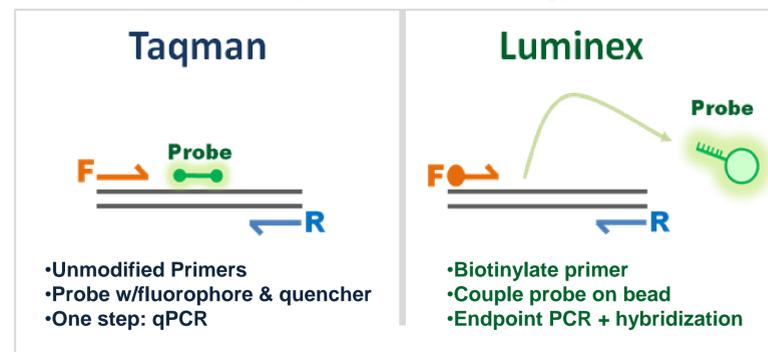
Hybridization: The biotinylated PCR amplicons are mixed with microspheres coupled to the sequence-specific probes of interest.

Label: Streptavidin-phycoerythrin is added to the reaction, binding to biotinylated amplicons and interrogated by the MAGPIX LED.



Methods

Assay Transfer Methodology

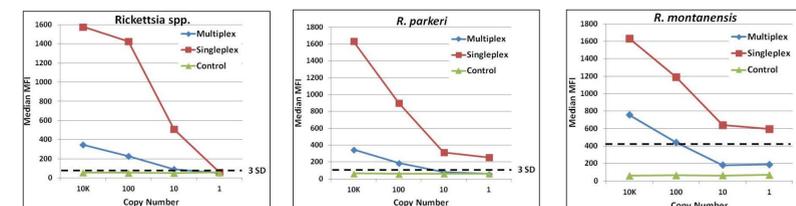


Assay Format: Two Panels

Controls		Black Legged Tick	Rickettsia
Positive Hybridization	Optimized for	<i>Ixodes</i> Ticks	<i>Amblyomma</i> Ticks
Negative Hybridization			<i>Dermacentor</i> Ticks
Positive PCR			<i>Rhipicephalus</i> Ticks
DNA Extraction	Pathogens Identified	<i>A. phagocytophilum</i>	Rickettsia spp.
		<i>B. microti</i>	<i>R. rickettsii</i>
		<i>B. burgdorferi</i>	<i>R. parkeri</i>
		<i>B. miyamotoi</i>	<i>R. montanensis</i>

Above: We developed two multiplex panels, each with four controls. The Black Legged Tick Panel includes two confirmatory assays per species, as well as a *B. miyamotoi* assay. The Rickettsia panel includes one screening assay and one confirmatory assay per species.

Limit of Detection



Above: Initial limit of detection (LOD) analysis has been performed for 12/14 assays. Examples shown above. Primer set performance was tested as a singleplex, in the final multiplex assay format, and as a singleplex with one of the other primer assays within the panel as a control. Most assays suggest a multiplex LOD of 10-100 copies.

Results

Sample	Pathogen	Rickettsia spp.	<i>R. rickettsii</i>	<i>R. parkeri</i>	<i>R. montanensis</i>	Control pass?
gDNA	<i>R. rickettsii</i>	+	+	+/-	-	Y*
gDNA	<i>R. parkeri</i>	+	-	+/-	-	Y
QC Plasmid	<i>R. montanensis</i>	-	-	-	+	Y
<i>A. maculatum</i>	<i>R. parkeri</i>	+	-	+	-	Y
<i>D. variabilis</i>	<i>R. montanensis</i>	+	-	-	+	Y
<i>A. maculatum</i>	negative	+	-	-	-	Y
<i>D. variabilis</i>	negative	-	-	-	-	Y

*Only two controls in this experiment- positive hybridization and negative hybridization.

Above: Rickettsia multiplex panel performance. Samples are genomic DNA, a synthetic plasmid for QC, or tick DNA tested for Rickettsia pathogens by USAPHC via qPCR. A positive result is at least 3 SD above background Median Fluorescent Intensity (MFI). Samples are positive if they are positive for both the genus and species-specific assay.

Sample	Pathogen	<i>A. phago</i> Msp2	<i>A. phago</i> Msp4	<i>B. microti</i> Sa1	<i>B. microti</i> 18S	<i>B. burg</i> FltD	<i>Borrelia</i> gB31	<i>B. miya</i> GIpQ	Control Pass?
gDNA	<i>A. phago</i>	+	+	-	-	-	-	-	Y*
gDNA	<i>B. burg</i>	-	-	-	-	+	+	-	Y
gDNA	<i>B. microti</i>	-	-	+	+	-	-	-	Y
gDNA	<i>B. miya</i>	-	-	-	-	-	+/-	+	Y
Tick	<i>A. phago</i> & <i>B. burg</i>	+	+	-	-	+	+	-	Y
Tick	<i>A. phago</i>	+	+	-	-	+	-	-	Y
Tick	<i>B. burg</i>	-	-	-	-	+	+	-	Y
Tick	neg	-	-	-	-	-	-	-	Y
Tick	neg	-	-	-	-	-	+/-	+	Y

*Only three controls in this experiment- positive hybridization, negative hybridization, and DNA extraction.

Above: Black legged tick multiplex panel performance. Samples are genomic DNA or tick DNA tested for pathogens by USAPHC via qPCR. A positive result is at least 3 SD above background MFI. Samples are positive if they are positive for both species-specific assays.

References

Ledin KE, Zeidner NS, Ribeiro JM, Biggerstaff BJ, Dolan MC, Dietrich G, Vredevoe L, Piesman J. 2005. Borreliacidal activity of saliva of the tick *Amblyomma americanum*. Med. Vet. Entomol. 19:90-95.

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