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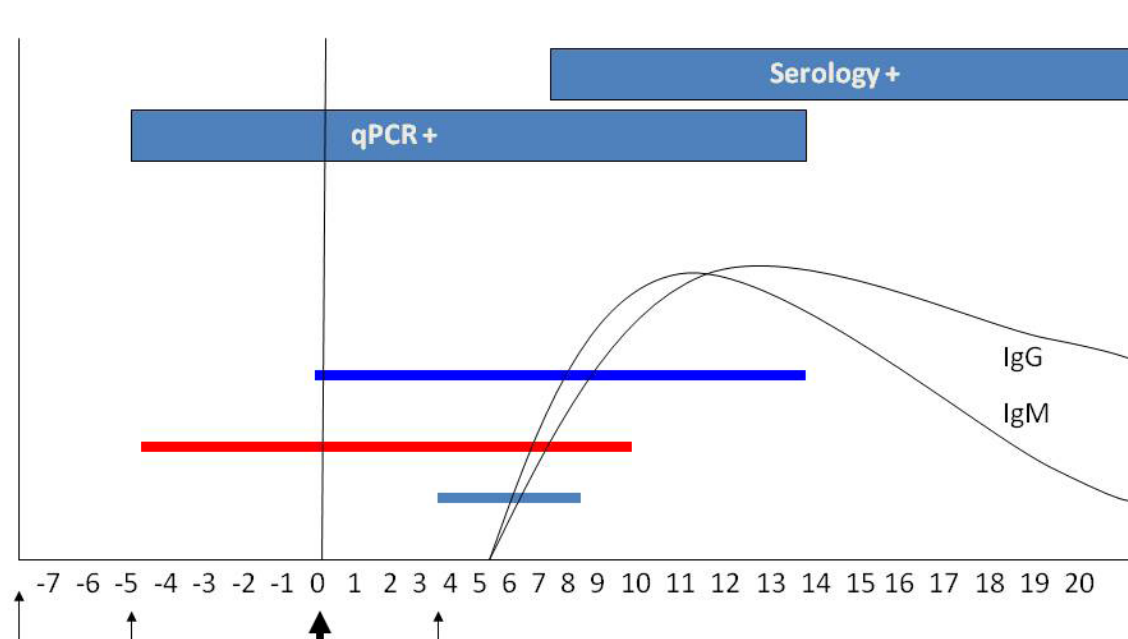
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INTRODUCTOIN

The Joint Biological Agent Identification and Diagnostic System (JBAIDS) is the Department of Defense's (DoD) Program of Record for the detection and diagnostic testing of biological warfare agents and infectious diseases of operational concern. The JPEO-CBD (Chemical Biological Medical Systems/Biosurveillance) has contracted with Idaho Technology, Inc. (ITI), the developer of the JBAIDS platform, to develop an *in vitro* diagnostic (IVD) kit for typhus. Included in this kit are two real-time PCR assays for the detection of Rickettsial organisms; one for the detection of *Orientia tsutsugamushi* (a Rickettsial organism that is the causative agent of scrub typhus), and one for the detection of all *Rickettsia* species (causative agents of both epidemic and murine typhus as well as spotted fevers).

Though easily treatable, *Rickettsia* and *O. tsutsugamushi* infections are very difficult to diagnose because symptoms of early disease are identical to other diseases of similar epidemiology. Current diagnosis methods are based on serological methods which compare acute and convalescent phase antibody titers. As such, they require sufficient time for seroconversion (1-2 weeks of illness); thus they are not always reliable to detect disease in the early stages. A delayed diagnosis (and treatment) leads to a higher mortality rate. In contrast, the real-time PCR assay in the JBAIDS Typhus detection kit is intended to provide a rapid diagnosis during the acute phase of disease leading to earlier treatment and recovery.

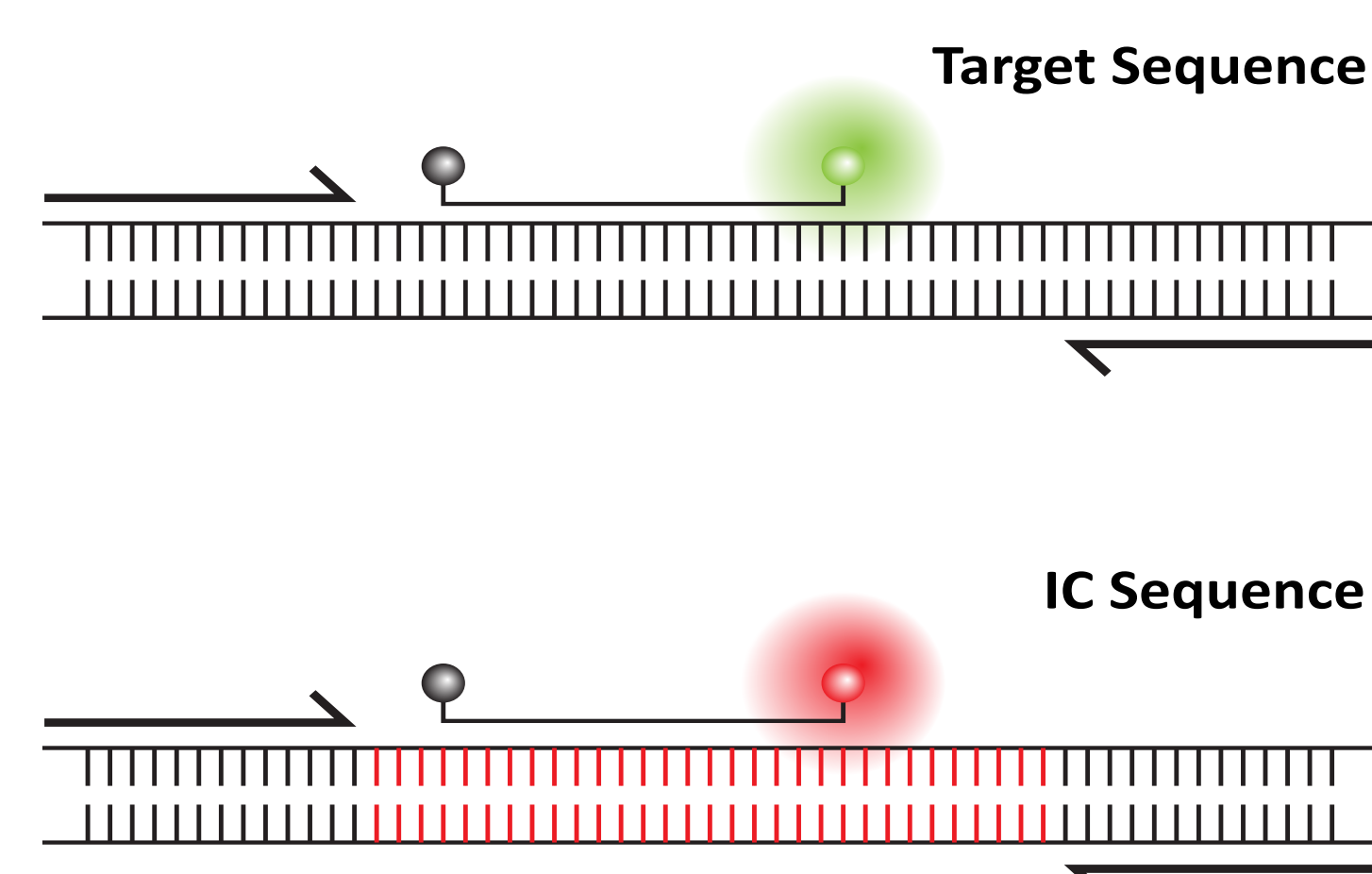
Rickettsial Infection & Host Response



The JBAIDS system is an FDA-cleared device for *in vitro* diagnostic testing and consists of the following components:

- JBAIDS instrument with thermocycler and real-time fluorimeter
- Room temperature-stable reagents that are freeze-dried and specific for the target organism
- Ruggedized laptop loaded with specific, user-friendly software
- Specific sample purification kits and protocols.

The JBAIDS Typhus Detection Kit contains two freeze-fried real-time PCR assays: one for the specific detection of *Orientia tsutsugamushi* and another for the detection of *Rickettsia* spp. Both assays have an internal inhibition control that consists of a synthetic DNA construct containing the primer binding sequences of the target assay and a heterologous intervening sequence to which a novel probe binds. The target assay is read in Channel 1 (530 nm); the IC is read in Channel 3 (705 nm).



RESULTS

The pan-*Rickettsia* Assay Detects Multiple *Rickettsia* species

The pan-*Rickettsia* primers and probes were designed against a target that was selected from a consensus sequence of 21 *Rickettsia* species. The assay was tested using genomic DNA from 14 different *Rickettsia* species and strains as well as two non-*Rickettsia* species. The pan-*Rickettsia* assay successfully detected all *Rickettsia* organisms and no non-*Rickettsia* (Table 1). Sensitivities for the ancestral species, *R. bellii* and *R. canadensis*, were low. However, these species are not thought to be clinically relevant.

Table 1: pan-*Rickettsia* assay inclusivity and exclusivity

Strain	Detected	Strain	Detected
<i>R. amblyommii</i> 85-1084	+++	<i>R. prowazekii</i> Breinl	+++
<i>R. bellii</i> G2D	+	<i>R. prowazekii</i> Madrid E	+++
<i>R. bellii</i> OT-2006	+	<i>R. rickettsii</i> VR891 R	+++
<i>R. canada</i> * MCK-29(29)	+	<i>R. sibirica</i> 246 CWPP	+++
<i>R. canadensis</i> CA410	+	<i>R. slovakia</i> Arm25	+++
<i>R. conorii</i> VR597 ITT	+++	<i>R. typhi</i> Wilmington	+++
<i>R. felis</i> Cal2	+++	<i>O. tsutsugamushi</i> Kato	-
<i>R. montanensis</i> OSU 85-930	+++	<i>C. burnetti</i> NMQ	-

The *O. tsutsugamushi* assay primers and probes were designed to a target using the consensus sequence of Karp, Kato, Gilliam and Boryong strains. In previous tests, this primer pair successfully detected 19 different strains of *O. tsutsugamushi* but no non-*Orientia* (not shown; Jiang et al. *Am. J. Trop. Med. Hyg.* 70(4), 2004, pp. 351-356).

Assay Development

Single stranded oligos corresponding to the primer and probe-binding region of each assay were designed for use in assay optimization experiments. The assay optimization workflow included:

- MgCl₂ titration
- Primer titration (symmetrical and asymmetrical)
- Probe titration
- False positive assessment
- Room temperature hold
- Annealing temperature variability

Once assays were optimized, the nucleic acid limit of detection (naLoD) was determined for both the target and IC assays by testing serial dilutions of synthetic oligo template. The naLoD was found to be 25 copies for both target assays and the *O. tsutsugamushi* IC. The naLoD for the pan-*Rickettsia* IC was found to be 10 copies. naLoDs were confirmed by two independent operators on two different instruments (60 replicates total for each assay).

Following optimization, target assays were multiplexed with the appropriate IC (spiked in at 5x naLoD) and freeze-dried assay formulations were made. The naLoD was then confirmed using these freeze-dried reagents by reconstituting them with buffer and spiking with target template at the naLoD. It was found that the freeze-dried, multiplexed assays had the same naLoD as the wet singleplex assays (Table 2).

Table 2: Confirmation of naLoD using freeze-dried reagents

Target	<i>O. tsutsugamushi</i>		pan- <i>Rickettsia</i>	
	Cp (SD)	F _{max} (SD)	Cp (SD)	F _{max} (SD)
Target	33.35 (0.31)	2.94 (0.91)	33.32 (0.46)	6.15 (1.38)
IC	33.00 (0.23)	1.17 (0.13)	32.19 (0.24)	1.55 (0.28)

Detection of *Rickettsia* and *Orientia* in Whole Blood Samples

Next, we tested the assay performance on contrived samples. Intact, inactivated *R. prowazekii* Brein 1 and *O. tsutsugamushi* Kato were spiked into fresh whole blood at 1,000, 2,000 and 5,000 organisms/mL. Results are presented in Table 6. The pan-*Rickettsia* assay was capable of detecting spiked *R. prowazekii* at all spike levels. Result for *O. tsutsugamushi* show the assay is not as sensitive; this is consistent with naLoD data from genomic DNA (see Table 5).

Table 6: Detection of spiked organism in whole blood

Sample	Theoretical Organism Recovery**	<i>R. prowazekii</i>			<i>R. prowazekii</i> Target 530		<i>O. tsutsugamushi</i>			<i>O. tsutsugamushi</i> Target 530	
		P	N	I	CP (SD)	F _{max} (SD)	P	N	I	CP (SD)	F _{max} (SD)
NC	n/a	0	2	0	N/A	0.19 (0.03)	0	2	0	N/A	0.07 (0.03)
PC	n/a	2	0	0	31.37 (0.21)	15 (0.5)	2	0	0	31.77 (0.19)	5.04 (1.03)
Whole Blood	0	0	2	0	N/A	0.51 (0.1)	0	2	0	N/A	0.13 (0.2)
1,000 org/mL	40	2	0	0	30.51 (0.5)	10.49 (3.38)	1	1	0	33.96 (N/A)	0.19 (0.02)
2,000 org/mL	80	2	0	0	30.04 (0.23)	12.11 (1.0)	2	0	0	33.33 (0.83)	1.29 (0.02)
5,000 org/mL	200	2	0	0	28.97 (0.15)	15.09 (0.94)	2	0	0	33.65 (0.07)	1.66 (0.46)

*P= positive, N=Negative, I= inhibited, ** Yield from purification assumes 100% recovery from 800 µL input volume eluted in 200 µL and 10 µL added per reaction.

Nucleic acids are isolated from whole blood and serum specimens for analysis with the JBAIDS Typhus Detection Kit by purification using the IT 1-2-3 Platinum Path sample purification kit. In this simple-to-use kit, bead-beaten specimens are incubated with magnetic beads which bind nucleic acids. The beads are then moved through a series of washes using a magnetic tool and finally eluted in buffer. Purified sample is combined with reconstitution buffer to rehydrate freeze-dried PCR reagents, which are then loaded into glass capillaries and run on the JBAIDS instrument. The JBAIDS instrument is controlled by a simple-to-use Wizard-based interface. The instrument automatically calculates crossing point (Cp) values for each sample and makes a Positive, Negative or Inhibited call.



Sample Purification



Rehydrate Freeze-Dried PCR Reagents



Wizard-based software and Auto Results

ONGOING STUDIES

Several studies are planned to continue the development of the JBAIDS Typhus Detection Kit. These include:

- Development of Channel 2 chemistry to increase robustness of IC assay
- Clinical Validation
 - A multi-site prospective study involving collection and testing of serum, whole blood and buffy coat samples from patients with suspected tick-borne or "typhus-like" illness
- Trials to support a 510(k) application to the FDA for approval of the JBAIDS Typhus Detection Kit as an *in vitro* diagnostic assay
 - Analytical evaluation studies
 - Clinical trial at multiple sites world-wide, anticipated May-October 2011