# Multiplexed, Real-time PCR Detection of *Anaplasma phagocytophillum* and *Ehrlichia* spp. in Whole Blood Specimens Using the Luminex ARIES® Instrument

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## Introduction And Objective

Human monocytic ehrlichiosis and human granulocytic anaplasmosis are tick borne bacterial diseases, found in several states in the U.S. We developed and evaluated a multiplexed, real-time PCR assay to detect *A. phagocytophilum* (ANA) and several *Ehrlichia* species [*E. chaffeensis (E. chaf)*, *E. ewingii* and *E. muris,* or Ehr spp.] in whole blood specimens using the Luminex ARIES® instrument. Selective primers, labeled with several different fluorescent dyes, facilitate the detection of ANA, Ehr spp. and an internal control in whole blood specimens in a single-step, multiplexed, PCR assay. This allows for more rapid diagnosis of illness with increased sensitivity in less time.

## Methods & Materials

Specimens: Analytical sensitivity, accuracy, precision, stability and specificity studies were assessed by using whole blood (WB), collected from separate volunteers and patients.

Analytical sensitivity: The limit of detection (LOD) was determined for two targets, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis (E. chaf).*

Accuracy: We used incoming patient specimens to run an in-house PCR and used the remaining specimen to process on the ARIES instrument. Fourteen (14) consecutive WB specimens for PCR testing were received in our reference laboratory from patients for whom a tick-borne agent (*E. chaf* positive) was detected using the in-house PCR. In addition, DNA from nine (9) positive patients were spiked into ReadyMix® tubes and thirty seven (37) negative WB patient samples were tested. A panel of twenty two (22) patient specimens, previously PCR-positive for ANA, provided by Dr. Hayley Webber (NorDx, Portland, ME), were tested with the ARIES® assay system as well.

Precision: Repeatability studies to determine precision were performed using samples with known concentrations of each target. One positive control for each target and negative control were run duplicate each day for 20 consecutive days, with different operators.

Analytical Stability: Specimen stability studies were conducted by incubation of spiked samples at 4°C, then assayed daily for 8 days.

Analytical Specificity: We tested the ability of the assay to detect only the intended target, and that the quantification of the target was not affected by cross- reactivity. We spiked 12 different organisms into negative WB and tested the specificity of the multiplex primers.

Reagents: Primer pairs, obtained from IDT, Inc., were designed to include a fluorescent reporter-labeled primer with an isoC on the 5'end and an unlabeled primer. The primer sequences for ANA were: forward 5'-CAG TCG TGA ATG TAG AGG GAA AAA C-3'; reverse 5'-GGA ATC CCC CTT CAG GAA CTT G-3' and for Pan Ehrl were: forward 5'- AAT GCT TCT ACT GCT ACT GT-3'; reverse 5'-GCT CCA CCA TGA GCT GG-3'. Each primer was used at a final concentration of 200 nM. MHV2 internal control primers were used to amplify the sample processing control in the cassette. ReadyMix® and cassettes were purchased from Luminex. Each cassette contained all reagents needed to run PCR on the Luminex ARIES® instrument. All steps, including extraction, purification, amplification, and detection, plus the sample processing control, were contained in the cassette.

## Incidence

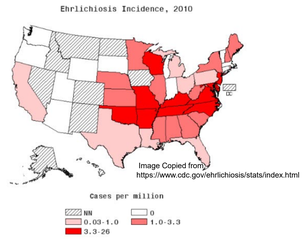



**Figure 1.** Annual reported incidence (per million population) for Ehrlichiosis in the United States for 2010. (NN= Not notifiable)

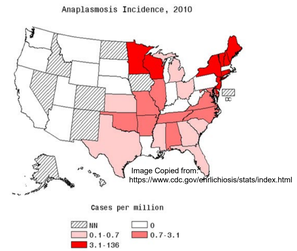



**Figure 2.** Annual reported incidence (per million population) for Anaplasmosis in the United States for 2010. (NN= Not notifiable)

## Transmission Cycles

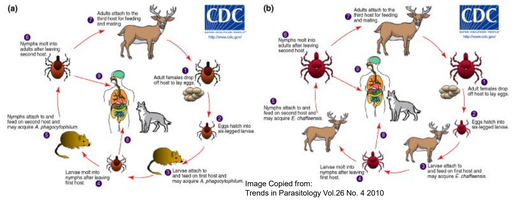



**Figure 3.** Transmission cycles responsible for maintaining rickettsial pathogens in tick populations and allowing infection of people and dogs. Transmission cycle for maintenance of *Anaplasma phagocytophilum* (a). The pathogen is acquired from sigmodontine rodents or other reservoir hosts during feeding by larval or nymphal *Ixodes* spp. ticks and then transmitted in subsequent feedings of nymphal or adult ticks. Transmission cycle for maintenance of *Ehrlichia chaffeensis* (b).

## Results

**Analytical Sensitivity:**

- Limit of detection for *Anaplasma* on ARIES® is 200 CFU/mL of blood.
- Limit of detection for *Ehrlichia* spp. on ARIES® is 200 CFU/mL of blood.

**Accuracy:**

- ***Anaplasma* Accuracy:** Of the 22 previously PCR-positive ANA patient specimens, 21 were confirmed positive for ANA in the new PCR assay. We calculated 95.5% accuracy (21/22). The range of the resulting Ct values was 30.3 to 39.5.
- ***Ehrlichia chaffeensis* Accuracy:** Of the 23 previously PCR-positive *E. chaf* patient specimens, 21 were confirmed positive for *E. chaf* in the new PCR assay. We calculated 91.3% accuracy (21/23). The range of the resulting Ct values was 21.5 to 38.2.
- **Negative Samples:** Of 37 previously PCR negative for ANA and *E. chaf* patient specimens, all 37 specimens were confirmed negative for for ANA and *E. chaf* in the new PCR assay.

**Precision:**

- Reproducibility of the resultant Ct value did not change over the course of the different testing period by different operators.

**Analytical stability:**

- Spiked positive specimens were stored in refrigerator for 8 days. WB specimens appeared stable at 4ºC temperature for up to 8 days after collection.

**Analytical specificity:**

- 12 different organisms were spiked into negative WB and tested for specificity. There was no cross reactivity observed. These result shows that these primers are very specific to ANA and *Ehr* spp.

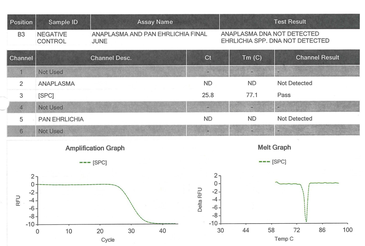
**Clinical Peformance:**

- Between January and August 2017, a total of 631 specimens were processed in our laboratory, with 16 *Ehr* spp. positive specimens, but no specimen positive for ANA. These results are consistent with disease epidemiology.

**3A. Negative Control Result**

| Position | Sample ID | Assay Name | Test Result |
|---|---|---|---|
| B3 | NEGATIVE CONTROL | ANAPLASMA AND PAN EHRLICHIA FINAL JUNE | ANAPLASMA DNA NOT DETECTED EHRLICHIA SPP. DNA NOT DETECTED |

| Channel | Channel Desc. | Ct | Tm (C) | Channel Result |
|---|---|---|---|---|
| 1 | Not Used | - | - | - |
| 2 | ANAPLASMA | ND | ND | Not Detected |
| 3 | [SPC] | 25.8 | 77.1 | Pass |
| 4 | Not Used | - | - | - |
| 5 | PAN EHRLICHIA | ND | ND | Not Detected |
| 6 | Not Used | - | - | - |




**3B. Positive Control Result**

| Position | Sample ID | Assay Name | Test Result |
|---|---|---|---|
| B2 | POSITIVE CONTROL | ANAPLASMA AND PAN EHRLICHIA FINAL JUNE | ANAPLASMA DNA DETECTED EHRLICHIA SPP. DNA DETECTED |

| Channel | Channel Desc. | Ct | Tm (C) | Channel Result |
|---|---|---|---|---|
| 1 | Not Used | - | - | - |
| 2 | ANAPLASMA | 30.1 | 82.3 | Detected |
| 3 | [SPC] | N/A | N/A | N/A |
| 4 | Not Used | - | - | - |
| 5 | PAN EHRLICHIA | 30.1 | 81.6 | Detected |
| 6 | Not Used | - | - | - |

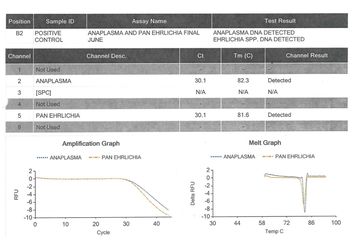



**Figure 3. Sample Data from ARIES® Run**

## Instrument

Instrument: The ARIES® is an *in vitro* diagnostic medical device for detection of nucleic acids by fluorescence based PCR (Figure 4).

Instrument Preparation: 6 µL of primer mix were added to each ReadyMix® tube, attached to the cassettes, and loaded in the ARIES®. After loading, the reaction then proceeded to completion in a hands-off manner. The ARIES® generates PCR amplification and melt curves for each target, with resulting Ct values calculated for both the sample processing control and the targets.

**Figure 4. Luminex ARIES® instrument**

## Discussion and Conclusions

Due to limited availability of *E. ewingii* and *E. muris, E. ewingii* DNA was spiked into PCR tubes, while *E. muris* and EML organisms were spiked into whole blood and tested on the ARIES®. All were detected by the new multiplexed PCR assay.

The detection technology is based on the unique MultiCode® base pair, isoC: isoG. Multicode bases form hydrogen bonds only with each other and are site-specifically incorporated during amplification.

Molecular recognition between isobases combined with a two primer system enables detection of almost any nucleic acid target. Automated extraction of nucleic acid and detection is complete in 2 hours, and involves minimal technologist time in sample preparation. ARIES® is easily adapted to additional target sequences.

The availability of sensitive and specific multiplexed PCR assay for the detection of tick-borne agents in WB specimens will improve our ability to rapidly diagnose infections caused by these agents.

## ACKNOWLEDGEMENTS

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