Multiplex quantitative PCR for detection of *Ehrlichia canis*, *Babesia canis* and canine ACTB gene

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¹GenAphora, ²School of Veterinary Medicine, Faculty of Agriculture, Hebrew University of Jerusalem
• GenAphora was founded on the basis of computational biology

• The Singleplexer of GenAphora is PERL software that searching for the most suitable and complex primers & probes for qPCR

• The Multiplexer of GenAphora is a PERL software that searching for the best primers & probe combinations for multiples qPCR

• Currently, construction of singleplex and multiplex assays for pathogens detection in veterinary medicine

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**Ehrlichia canis**

• Small gram-negative bacteria
• Infecting monocytes
• Clusters called morulae – hard to visualize
• Disease prevalent worldwide
• The rickettsia can persist in infected dogs for months & years
• Variable clinical signs
• May be fatal
**Babesia species**

- *Babesia* spp. are protozoa infecting erythrocytes, causing hemolysis > resulting in anemia

- *B. canis canis*, *B. canis vogeli* *B. canis rossi* and *B. gibsoni* are commonly known organisms that infect dogs

**Common Vector**

- Both *E. canis* & *B. canis vogeli* are transmitted by *R. sanguineus*

- Ticks found throughout Asia, Africa, Europe, the Middle East, and North America

*Rhipicephalus sanguineus*  
The brown dog-tick
Aim

Construction of a multiplex qPCR for *E. canis* & *B. canis vogeli*  
(ACTB as a reference gene)

M & M

Plasmid Construction

![Pst I Cut](image1)

![Circular](image2)
E. canis Assay Sensitivity (dual labeled probe)

Same performed with Babesia canis vogeli

Duplex: Babesia canis vogeli hsp70 (Hex) with and without 10^4/ml canine β actin (Cy5)
Triplex: $\sim 10^4$/ml *B. canis vogeli* hsp70 gene (Hex) in elevated concentrations of *E. canis* 16s RNA gene (Fam) and $10^4$ copies/ml canine $\beta$ actin gene (Cy5) with or without *B. canis vogeli* hsp70 gene & *E. canis* 16s RNA gene.

\[ C(t) \text{ value} = \sim 26 \]

Elevated concentrations of canine $\beta$ actin gene (Cy5) with or without *B. canis vogeli* hsp70 gene & *E. canis* 16s RNA gene

With $10^4$ Babesia hsp 70 and $10^4$ E. canis 16s.
Triplex: $\sim 10^2$ copies/µl *B. canis vogeli* hsp70 gene in elevated concentrations of *E. canis* 16s RNA gene and $10^4$/ml canine β actin gene

dNTPs titration
Singleplex using triplex primer-probe sets

B. canis vogeli hsp70 stem loop primers
**B. canis vogeli** hsp70 stem loop primers melting curve

- Amplicon
- NTC Stem loop primers
- NTC primers set

**Primers sequencing**

![Diagram of primers sequencing](Image)
Hot start enzymes?

• These primer-dimers cannot be formed in 61°C

• The order of introducing ingredients into the reaction mix tube affects the C(t) value of the NTC

• Therefore, the enzyme is probably not 100% hot start.

• Improving tm calculation in the primer design script can reduce primer-dimers.

Children, I say plainly, watch out of taking the hot start feature for granted!
Improved primer design
primer dimmers are not inevitable

ACTB -10^4 copies/ul

NTC Blank

summary

- Triplex qPCR assay was successfully constructed
- Triplex reduces the assay sensitivity by about one order of magnitude
- The cause for this reduction is not system resources overuse
- Primer-dimers is probably the major cause
- We manage to overcome primer-dimers by improving primer design
- Improved multiplex is underway
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