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

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GenAphora

- GenAphora
- *Ehrlichia canis*
- *Babesia canis*
- Singleplex Experiments
- Multiplex Experiments

GenAphora

- 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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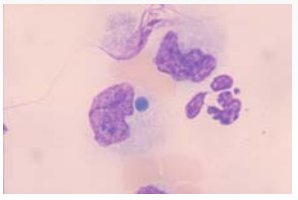
Ehrlichia canis

- 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



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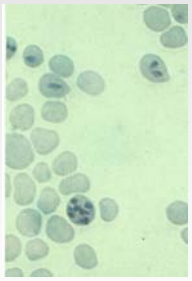
Babesia species

- *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



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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

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Aim

Construction of a multiplex qPCR for

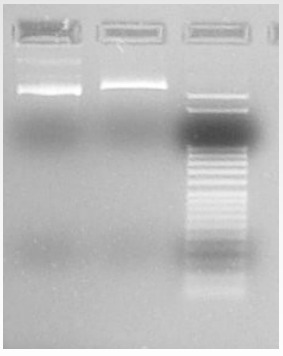
E. canis & *B. canis vogeli*

(ACTB as a reference gene)

M & M

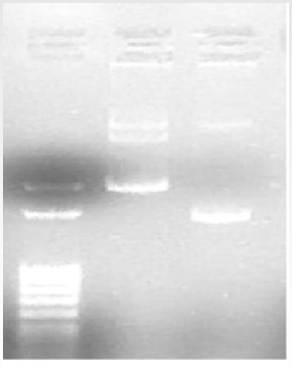
Plasmid Construction

Pst I Cut



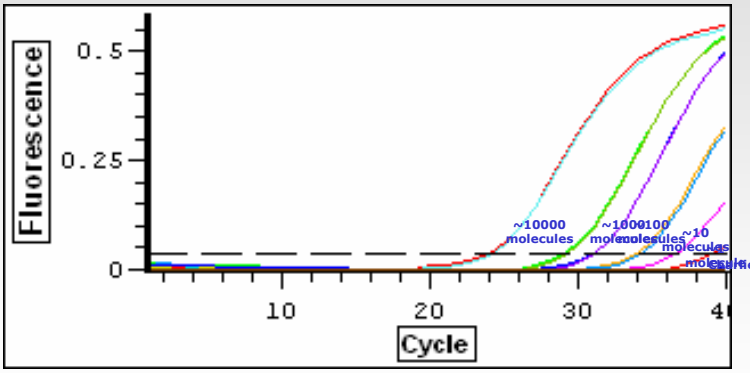
pGMT pGMT-16S Ladder

Circular



Ladder pGMT-16S pGMT

E. canis Assay Sensitivity (dual labled probe)



•Same performed with *Babesia canis vogeli*

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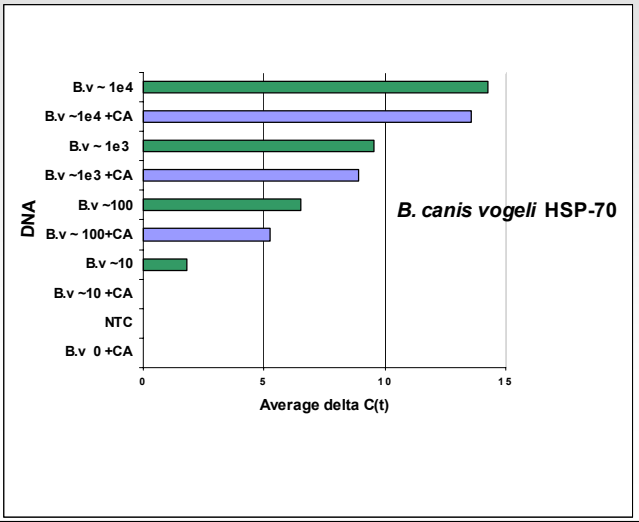
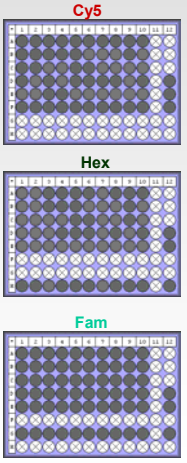
Duplex: *Babesia canis vogeli* hsp70 (Hex) with and without 10^4/ml canine β actin (Cy5)





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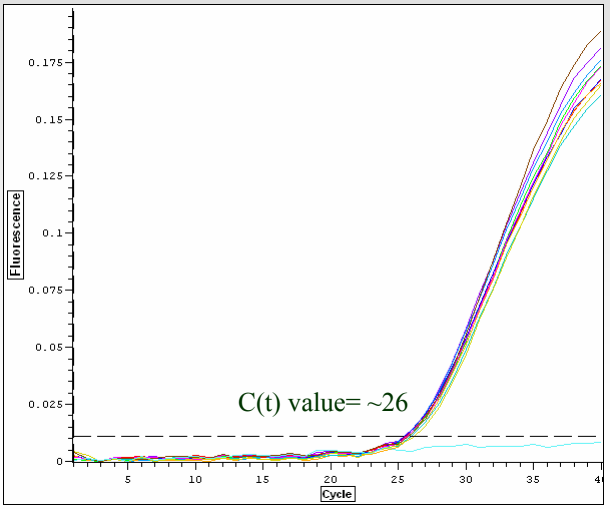
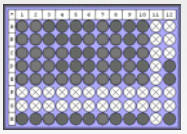
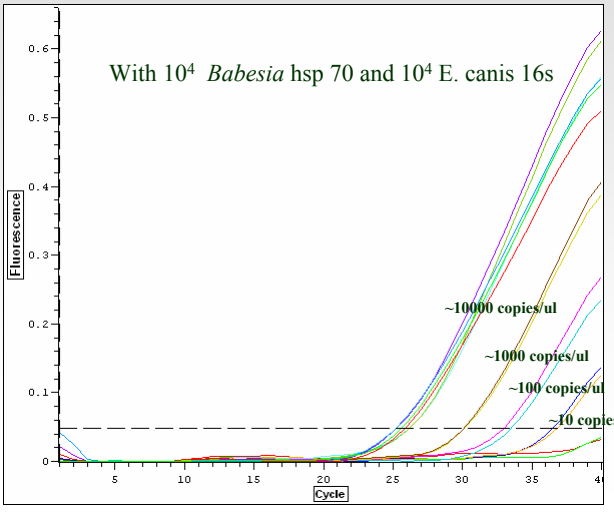
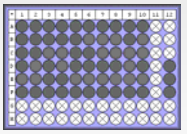
Triplex: ~10^4/ml *B. canis vogeli* hsp70 gene (Hex) in elevated concentrations of *E. canis* 16s RNA gene (Fam) and 10^4 copies/ml canine β actin gene (Cy5)



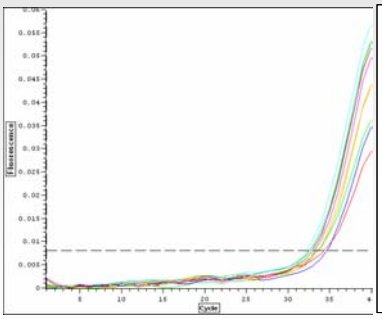


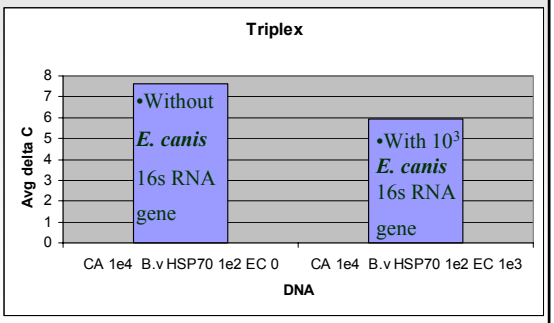
Elevated concentrations of canine β actin gene (Cy5) with or without *B. canis vogeli* hsp70 gene & *E. canis* 16s RNA gene



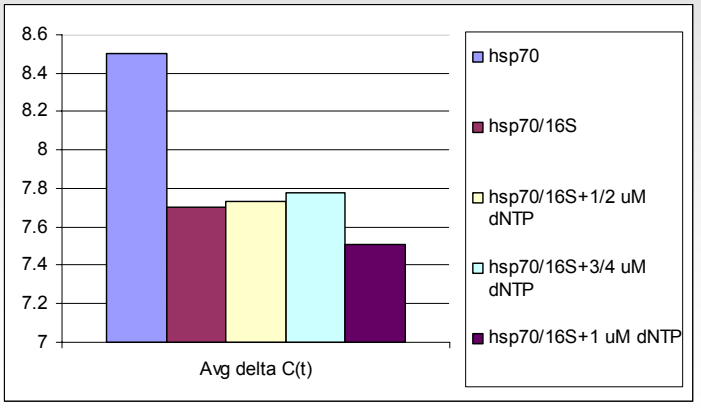


Triplex: **~10^2 copies**/μl *B. canis vogeli* hsp70 gene in elevated concentrations of *E. canis* 16s RNA gene and 10^4/ml canine β actin gene

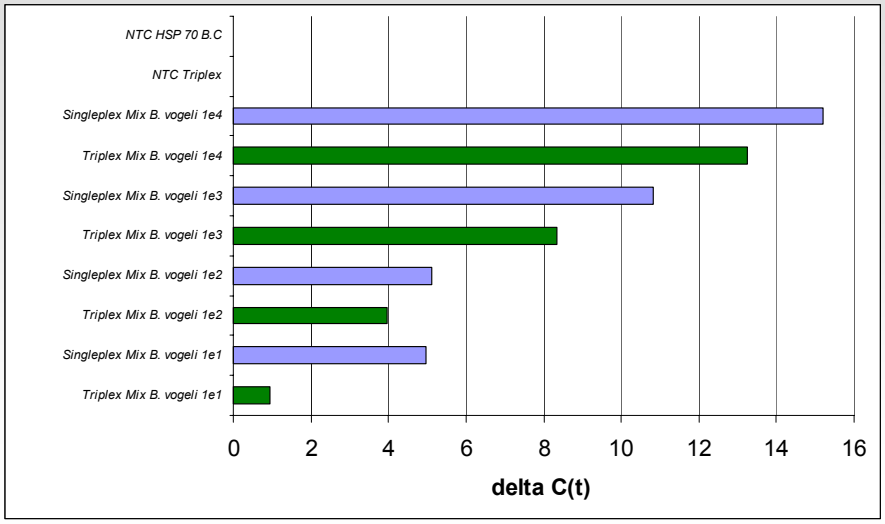




dNTPs titration



Singleplex using triplex primer-probe sets



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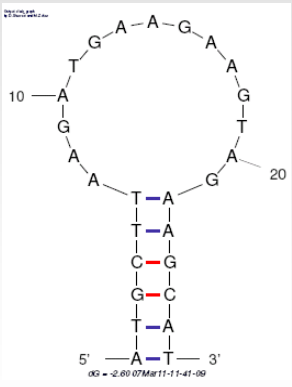
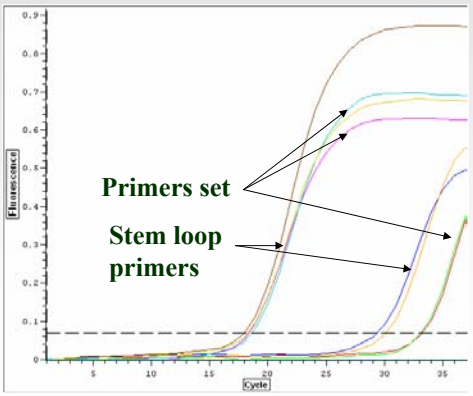
B. canis vogeli hsp70 stem loop primers





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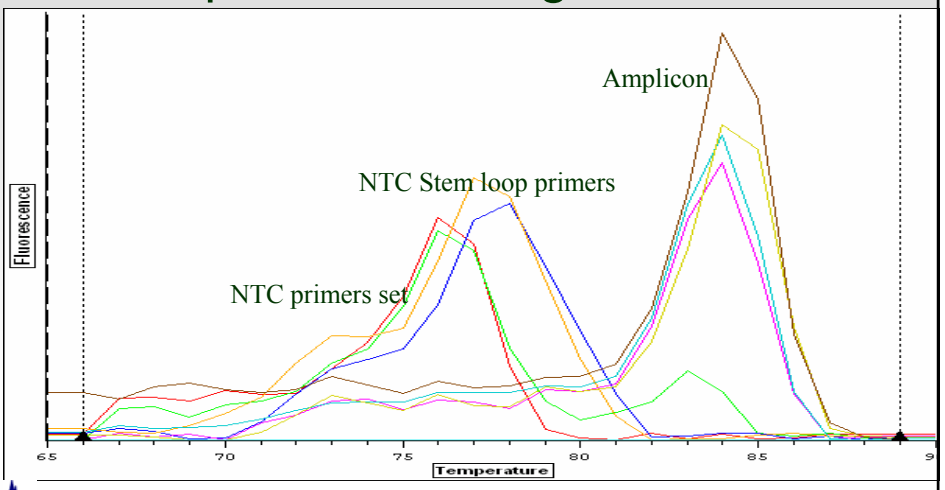
B. canis vogeli hsp70 stem loop primers melting curve



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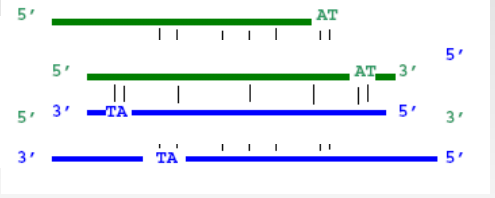
Primers sequencing



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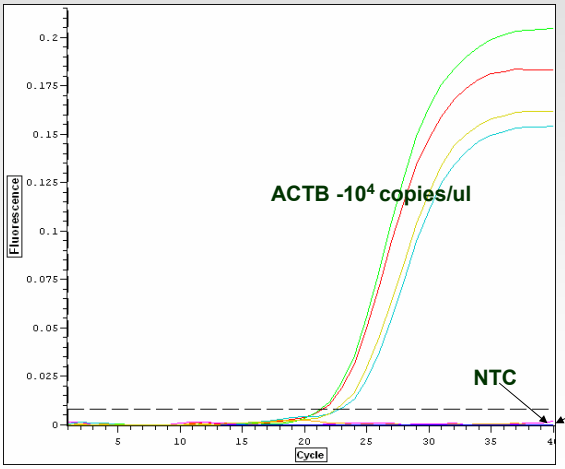
Hot start enzymes?

- These primer-dimers cannot be formed in 61C
- The order of introducing ingredients into the reaction mix tube effects 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



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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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