

Note: This fictitious example of validation study for a real time PCR assay of a non-cultivable or not easily cultivable bacterium is a guideline and includes minimum needed information. This example does not necessarily apply to all situations, additional information may be requested: please contact CLEP if you need assistance.

Validation study for the detection of *Anaplasma phagocytophilum* using real-time PCR (Polymerase Chain Reaction) in whole blood

Validation study overview:

The purpose of this study is to evaluate the utility of a real-time PCR assay as a diagnostic tool for detection of *A. phagocytophilum*. Known gene copy numbers (high, medium and low amounts) of *A. phagocytophilum* cells are seeded into PCR-negative whole blood samples. The gene copy number of the seed is determined by extracting genomic DNA and then measuring the quantity of DNA and calculating gene copy number based on gene copy (GC) per picogram of DNA for the organism. The samples are processed to extract DNA from the sample and real-time PCR is performed as in the standard operating procedure manual in a blinded fashion. The results are compared to the known seeded data. Typically the low seeded samples should have resulting Ct's of 34-39, medium samples have Ct's of 29-34, and high samples have Ct's <29.

Sensitivity of the *Anaplasma phagocytophilum* assay

A cell suspension of *Anaplasma phagocytophilum* was diluted 10-fold out to 10^{-8} , and 50 μ L of the 10^{-2} to 10^{-8} dilutions were added to 150 μ L of PCR buffer for analytical sensitivity and blood for matrix sensitivity and extracted according to the protocol. These results reflect that this assay is sensitive to 0.2 gene copies per reaction at 40 PCR cycles (Table 1 and 2). Results are as follows:

Table 1- <i>Anaplasma phagocytophilum</i> cells diluted in buffer				
Dilution	Calculated Gene copy number	target gene detection		
		Ct1	Ct2	Ave. Ct
10^{-2}	24,550	20.98	20.82	20.90
10^{-3}	2,455	23.08	22.72	22.90
10^{-4}	245	25.26	25.94	25.60
10^{-5}	24	28.30	27.90	28.10
10^{-6}	2	32.47	32.08	32.27
10^{-7}	0.2	36.12	35.90	36.01
10^{-8}	0.02	undet	undet	undet

Table 2- <i>Anaplasma phagocytophilum</i> cells diluted in blood				
Dilution	Calculated Gene copy number	target gene detection		
		Ct1	Ct2	Ave. Ct
10^{-2}	24,550	21.98	21.82	21.90
10^{-3}	2,455	24.08	23.72	23.90
10^{-4}	245	26.26	26.94	26.60
10^{-5}	24	29.30	29.50	29.40
10^{-6}	2	33.47	33.08	33.27
10^{-7}	0.2	38.10	39.30	38.70
10^{-8}	0.02	undet	undet	undet

Specificity of the *Anaplasma phagocytophila* assay

Table 2 displays the organisms that were tested for specificity by the real-time PCR assay. Approximately 1×10^6 genome copies of each organism was used for specificity testing. All were negative except for the *Anaplasma phagocytophilum*. In addition to PCR testing the DNA sequence of the target was matched against the sequences of the specific genome projects. *Anaplasma marginale* and *Bartonella quintana* are not easily available so these organisms were exclusively checked by comparison to the genome sequence.

Table 2- Specificity Table

Specificity panel	Source	PCR Result	target Genome Match
<i>Anaplasma phagocytophilum</i>	NYSDOH	positive	yes
<i>Babesia microti</i>	NYSDOH	negative	N/A
<i>Bartonella henselae</i>	NYSDOH	negative	No
<i>Borrelia burgdorferi B31</i>	NYSDOH	negative	No
<i>Coxiella burnetii</i>	NYSDOH	negative	No
<i>Escherichia coli</i>	ATCC 25922	negative	No
<i>Ehrlichia chaffeensis</i>	NYSDOH	negative	No
<i>Enterococcus faecalis</i>	ATCC 51299	negative	N/A
<i>Staphylococcus aureus</i>	ATCC 25923	negative	No
<i>Streptococcus pneumoniae</i>	ATCC 49619	negative	No
<i>Treponema pallidum</i>	NYSDOH	negative	No
<i>Treponema denticola</i>	ATCC 35405	negative	No
<i>Anaplasma marginale</i>	St. maries	ND	No
<i>Bartonella quintana</i>	Toulouse	ND	No

Assay Verification

We spiked 30 whole blood specimens with 50 ul of 3 different dilutions of *A. phagocytophilum* cells representing high, medium, and low amounts (Table 3). An additional 10 blood samples had 50 ul of 1X PCR buffer added to simulate a negative sample. These samples were blinded and randomized by one member of the laboratory. DNA was extracted from each specimen and was tested in duplicate in the real-time PCR assay by another laboratorian. The results reflecting a 100% correlation are shown in Tables 4. All seeded specimens are PCR positive and those that were not seeded remain PCR negative. The raw data for these is shown in Table 5.

Table 3. Number of GC's seeded into negative **whole blood** for blinded validation

Cell concentration	Average number of GC/5 μ L DNA tested
High	2,455
Medium	245
Low	24
Negative	0

Table 4*geneX* PCR

Seeded whole blood		POS	NEG
		POS	30
NEG		0	10

Table 5. Extraction of spiked whole blood samples – Validation raw data. (con't on next page)

Sample Number	Concentration of <i>A. phagocytophilum</i>	Ct1	Ct2	Average Ct
1	Medium	33.18	33.33	33.255
2	Low	34.00	33.10	33.55
3	Low	36.15	35.19	35.67
4	High	23.37	22.73	23.05
5	High	22.88	23.12	23.00
6	High	24.49	24.4	24.45
7	High	23.04	22.33	22.69
8	Medium	30.09	29.62	29.86
9	High	24.92	25.6	25.26
10	High	25.29	26.29	25.79
11	Low	36.05	37.59	36.82
12	Negative	undet	undet	
13	Negative	undet	undet	
14	Negative	undet	undet	
15	Negative	undet	undet	
16	Low	38.52	38.02	38.27
17	Medium	31.17	31.82	31.50
18	Low	37.54	39.27	38.41
19	Low	34.84	35.06	34.95
20	Low	35.00	35.20	35.10
21	Medium	32.37	32.25	32.31
22	Medium	32.38	31.85	32.11
23	Medium	31.69	31.67	31.68
24	Low	33.73	34.27	34.00
25	Low	33.84	34.34	34.09
26	Medium	30.36	30.22	30.29
27	Low	38.77	38.33	38.55
28	High	27.32	27.51	27.42
29	Medium	27.31	27.52	27.42
30	High	27.19	27.2	27.20
31	High	26.38	26.41	26.40

32	Medium	28.63	29.25	28.94
33	High	23.24	22.98	23.11
34	Negative	undet	undet	
35	Negative	undet	undet	
36	Negative	undet	undet	
37	Negative	undet	undet	
38	Negative	undet	undet	
39	Negative	undet	undet	
40	Medium	31.37	31.34	31.40

Inhibition testing: To test for the presence of inhibitors in each acceptable specimen type for this assay, an aliquot of each sample was spiked with a low concentration of sample target DNA. In addition, a comparative sample was prepared as an inhibition control that contained only a low concentration of sample target DNA. Table 6 displays the raw data of the negative samples that were spiked with the inhibition control and the inhibition control alone for comparison. The negative spiked samples and the inhibition control had average Ct values that were within 3 Ct values. Table 6 contains data from the negative samples only. Every sample tested using this assay was also tested for inhibition. Samples found to be positive in this assay that were spiked with additional target DNA produced Ct values that were less than the inhibition control alone since they contained both the spiked target DNA as well as varying amounts of whole organism (data not shown). Every sample must be tested to demonstrate the absence of inhibitors.

Table 6- Inhibition Testing

Sample Number	Type of Specimen (Negative Sample)	Negative sample replicate spiked with low concentration of target DNA Ct	Inhibition Control (Low concentration of target DNA) Ct
1	Blood	34.68	34.93
2	Blood	35.99	34.93
3	Blood	34.98	34.93
4	Blood	35.95	34.93
5	Blood	33.26	34.93
6	Blood	34.00	34.93
7	Blood	35.46	34.93
8	Blood	34.65	34.93
9	Blood	35.04	34.93
10	Blood	36.89	34.93

Interassay reproducibility: The Ct value of the positive extraction/lysis control which should be at a low but readily detectable concentration was analyzed over a period of 15 days with various technicians performing the assay. The results in Table 7 show consistent reproducibility over this period of time on the same sample.

Table 7- InterAssay Reproducibility

Date of Assay	geneX target Ct
6/12/06	34.47
6/13/06	34.82
6/14/06	36.04
6/15/06	36.52
6/16/06	35.96
6/17/06	34.56
6/18/06	35.55
6/19/06	36.64
6/20/06	35.17
6/21/06	35.95
6/22/06	36.06
6/23/06	35.34
6/24/06	36.58
6/25/06	36.49
6/26/06	36.84
4/26/06	37.43
average Ct	35.90
Std Deviation	0.86
Coefficient of Variation-%CV	2.40%

Intra-assay reproducibility: Ten samples of varying low, medium and high concentrations were tested in triplicate on the same plate. The results below in Table 8 show excellent reproducibility.

Table 8- Intra-assay Reproducibility

Sample	Ct value 1	Ct value 2	Ct value 3	Average Ct	Std Dev Ct	% CV
1-Low	38.62	36.84	37.43	37.63	0.91	2.42%
2-Low	34.18	34.05	34.55	34.26	0.26	0.76%
3-High	22.3	22.21	22.06	22.19	0.12	0.54%
4-High	22.37	22.63	22.51	22.50	0.13	0.58%
5-Med	25.01	25.29	25.47	25.25	0.23	0.91%
6-Med	25.88	25.64	26.34	25.95	0.36	1.39%
7-Med	25.15	25.76	26.17	25.69	0.51	1.99%
8-High	26.88	26.67	26.93	26.83	0.14	0.52%
9-High	26.12	27.25	27.04	26.80	0.60	2.24%
10-Low	36.58	36.49	36.56	36.54	0.05	0.14%

Conclusion: Our conclusion is that this real-time PCR assay can be used as a diagnostic tool for the detection of *Anaplasma phagocytophilum* in whole blood samples. This assay is 100% specific, sensitive to 0.2 gene copies, reproducible, robust and stable. The blinded panel demonstrated that low amounts of HGA can be detected in mock samples using this assay.