DNA Molecular Detection of Mycoplasmas: Introducing Real-Time PCR

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Polymerase Chain Reaction (PCR)

- **1986 – First description of the polymerase chain reaction**
  - **Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction**
    Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H.
    Cold Spring Harb Smp Quant Biol 1986; 51 Pt 1:263-73

- **1988 – First description of a thermostable DNA polymerase**
  - **Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase**
    Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA.
    Science 1988 Jan 29; 239(4839): 487-91

- **1993 – First description of real-time polymerase chain reaction**
  - **Kinetic PCR analysis: real-time monitoring of DNA amplification reactions**
    Higuchi R, Fockler C, Dollinger G, Watson R
    Biotechnology (NY) 1993 Sep; 11(9): 1026-30

- **1993 – Nobel Prize in Chemistry**
  - **Kary B. Mullis – Polymerase Chain Reaction**
  - **Michael Smith – Site-directed mutagenesis**

- **1996 – First commercial real-time PCR instruments available**

Pathogenic Avian Mycoplasmas: Background

- **Pathogenic avian mycoplasmas**
  - ***Mycoplasma gallisepticum*: chickens and turkeys**
  - ***Mycoplasma synoviae*: chickens and turkeys**
  - ***Mycoplasma meleagridis*: turkeys**
  - ***Mycoplasma iowae*: turkeys**
- **Disease symptoms**
  - **Airsacculitis, sinusitis**
  - **Synovitis, arthritis**
  - **Skeletal deformities**
  - **Embryo mortality**
  - **Poor performance**
- **Diagnosis**
  - **Culture: gold standard**
  - **Antibody detection: Serum Plate Agglutination (SPA), Hemagglutination Inhibition (HI), ELISA**
  - **Molecular detection: PCR**

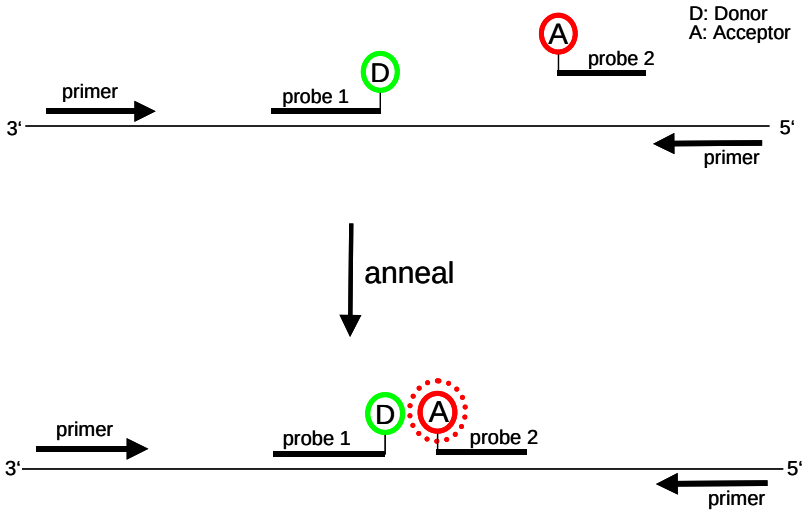
M. gallisepticum, *M. synoviae*, *M. meleagridis* Real Time PCR Assay Overview

- **Hybridization probes detection chemistry**
  - **Crossing point and T_m data values**
- **Surveillance (Screening) Testing**
  - **Crossing point value for yes/no determination**
- **Confirmatory Testing**
  - **Crossing point value**
  - **T_m value for additional data confirmation of correct target detection**
- **Sequences for primers and probes based on amplicon sequences from S. Kleven**
  - **Avian Diseases 53:103-107, 2009. Ziv Raviv and Stanley H. Kleven**

  "The Development of Diagnostic Real-Time TaqMan PCRs for the Four Pathogenic Avian Mycoplasmas"

Hybridization Probes Detection Format

- **Donor probe (D) provides energy to Acceptor probe (A) to emit signal when both probes are bound to target sequence (Fluorescence Resonance Energy Transfer)**
- **Probes are recycled throughout amplification program**
- **Signals are collected by the instrument and used to calculate the crossing point values**
- **Melting curves and T_m values may be generated with hybridization probes**



Cycling Program and Instrumentation
M. gallisepticum, *M. synoviae*, *M. meleagridis* Real-Time PCR

- **Instrumentation: LightCycler 480 with 96-well block**

- **Cycling Program: used for Mg, Ms and Mm real-time PCR assays**
  - **Activation: 1 cycle**
    95°C 10 minutes
  - **Amplification: 45 cycles**
    95°C 20 seconds
    60°C 20 seconds with a single fluorescence data acquisition
    72°C 15 seconds
  - **Melting Curve: 1 cycle**
    95°C 1 minute
    45°C 1 minute
    80°C continuous 0.14°C/second
    with collection of 4 fluorescence data acquisitions per second
  - **Cooling: 1 cycle**
    40°C 30 seconds

Sample DNA Extraction Protocol used to generate data
Tracheal Swabs and *Mycoplasma* Collection Broth

- **High Pure PCR Template Preparation Kit (Roche Applied Science)**
  -Spin column based format

- **Trachael swabs:**
  - **Swabs were rinsed in 0.5 ml PBS**
  - **400 ul PBS were used for the DNA extraction**

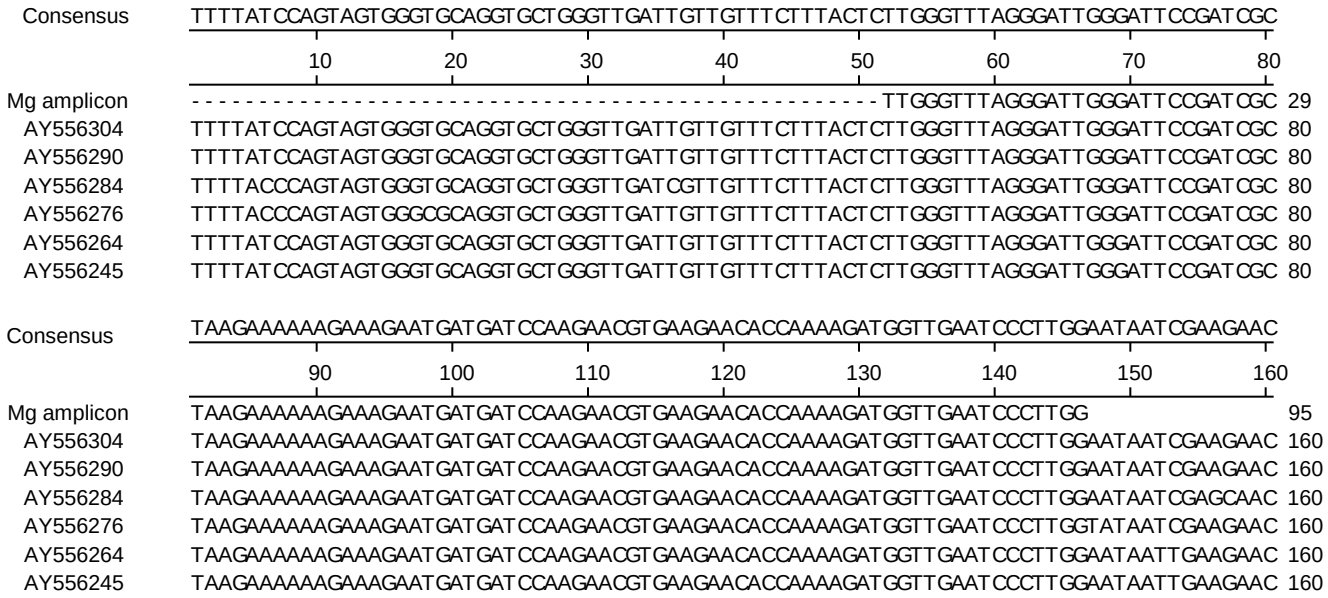
- ***Mycoplasma* Collection Broth:**
  - **1 ml broth was centrifuged at 1000xg for 3 minutes**
  - **Pellet was resuspended in 200 ul PBS**

- **All DNA was stored at -80°C**

- **5 µl Sample DNA was used per PCR assay reaction (total volume was 20 µl)**

M. gallisepticum Amplicon Sequence

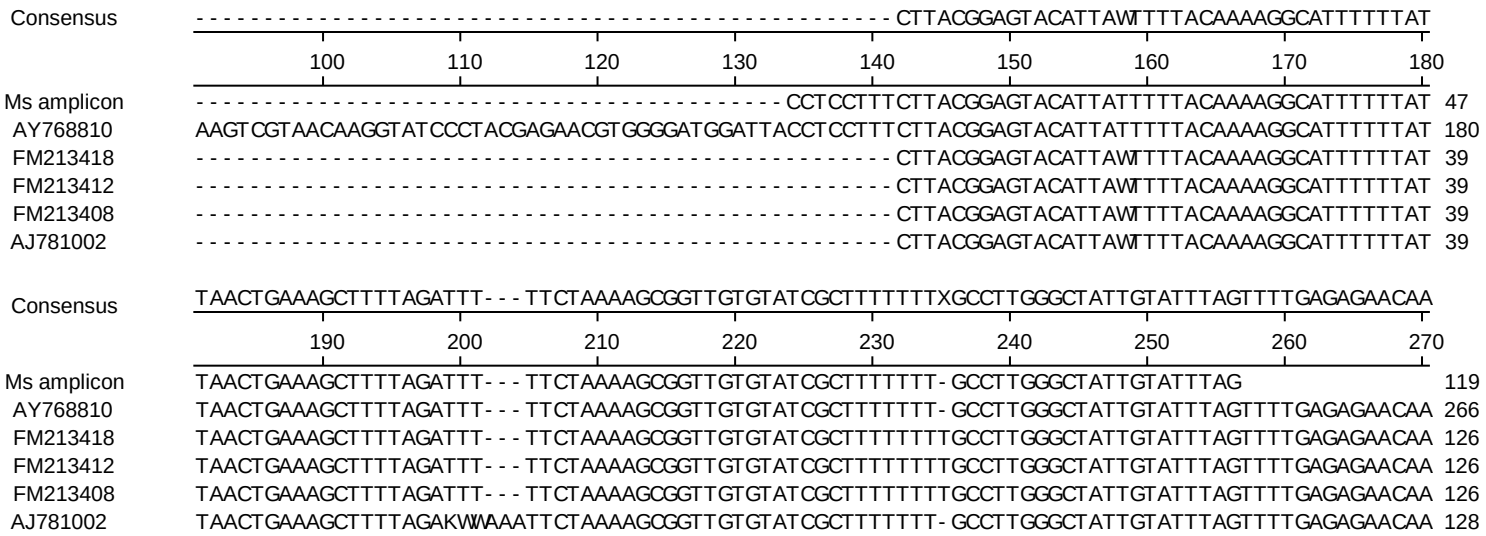
Alignment with Wild Type *mgc2* Sequences



- **AY556304 and AY556290: Chicken isolates**
- **AY556284 and Ay556276: Turkey isolates**
- **AY556264 and AY 556245: House Finch isolates**
- **All sequences from PDRC (N. Ferguson and S. Kleven)**

M. synoviae Amplicon Sequence

Alignment with Intergenic Spacer Region Sequences



- **AY768810: USDA**
- **FM213418: University of Liverpool (J. Bradbury)**
- **FM213412: University of Liverpool (J. Bradbury)**
- **FM213408: University of Liverpool (J. Bradbury)**
- **AJ781002: University of Liverpool (J. Bradbury)**

M. meleagridis Amplicon Sequence

Alignment with Intergenic Spacer Region Sequences

```
Consensus    ---------AACAAGGTATCCCTACGAGAACGTGGGGATGGATTACCTCCTTTCTACGGAGTACATTAGTTAATTTATTT
                      90        100       110       120       130       140       150       160
Mm amplicon  ---------AACAAGGTATCCCTACGAGAACGTGGGGATGGATTACCTCCTTTCTACGGAGTACATTAGTTAATTTATTT   71
AY762641     TTAAGTCGTAACAAGGTATCCCTACGAGAACGTGGGGATGGATTACCTCCTTTCTACGGAGTACATTAGTTAATTTATTT   160
AJ780999     -----------------------------------------------------CTACGGAGTACATTAGTTAATTTATTT   27

Consensus    AATCATTGGGACTAAAATTAACCTTTAAGCCTGTTATTTTAATATTGTCATGACTTGGTTTAAGGCTCTGAGTXATATAT
                      170       180       190       200       210       220       230       240
Mm amplicon  AATCATTGGGACTAAAATTAACCTTTAAGCCTGTTATTTTAATATTGTCATGACTTGGTTTAAGGCTCTGAG           143
AY762641     AATCATTGGGACTAAAATTAACCTTTAAGCCTGTTATTTTAATATTGTCATGACTTGGTTTAAGGCTCTGAGTGATATAT   240
AJ780999     AATCATTGGGACTAAAATTAACCTTTAAGCCTGTTATTTTAATATTGTCATGACTTGGTTTAAGGCTCTGAGTTATATAT   107
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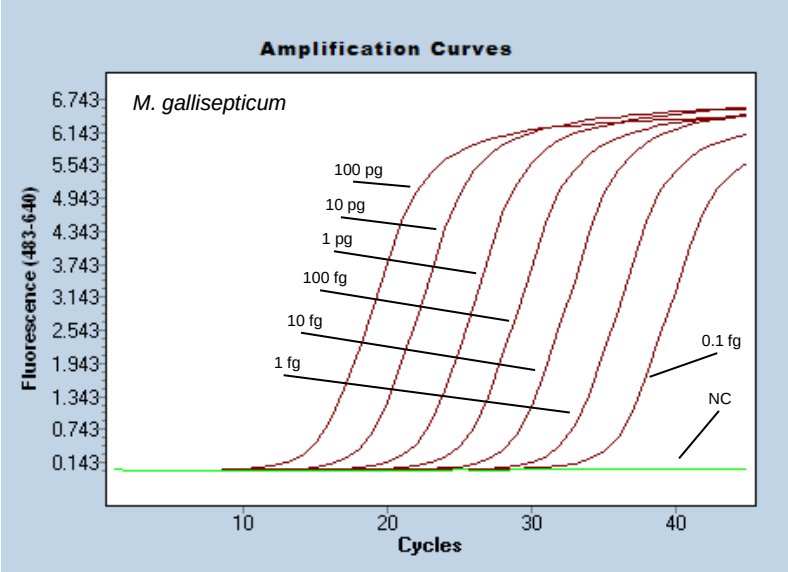
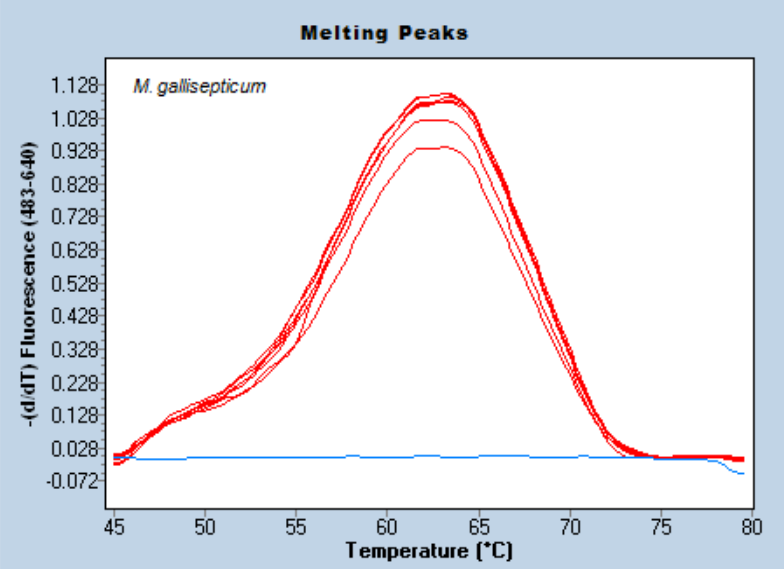
- AY762641: USDA
- AJ780999: University of Liverpool (J. Bradbury)

Analytical Sensitivity and Specificity

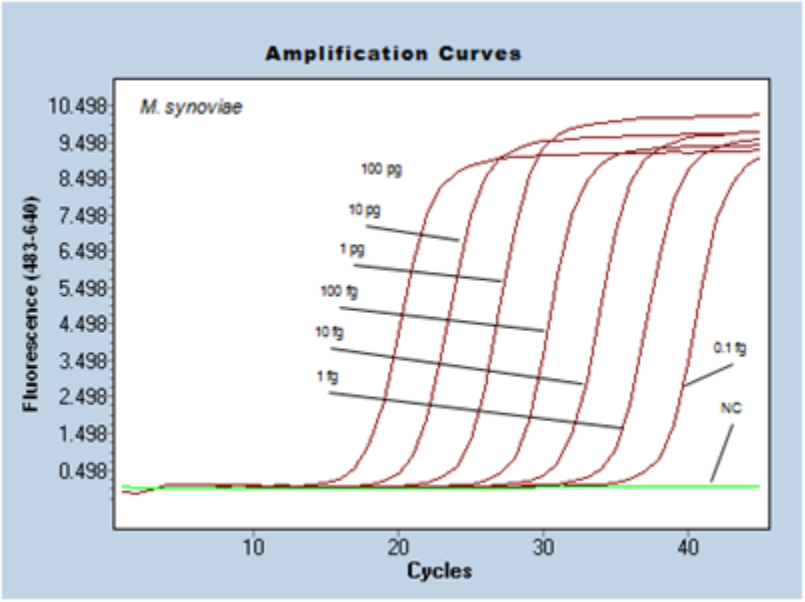
M. gallisepticum Real-Time PCR
Analytical Sensitivity

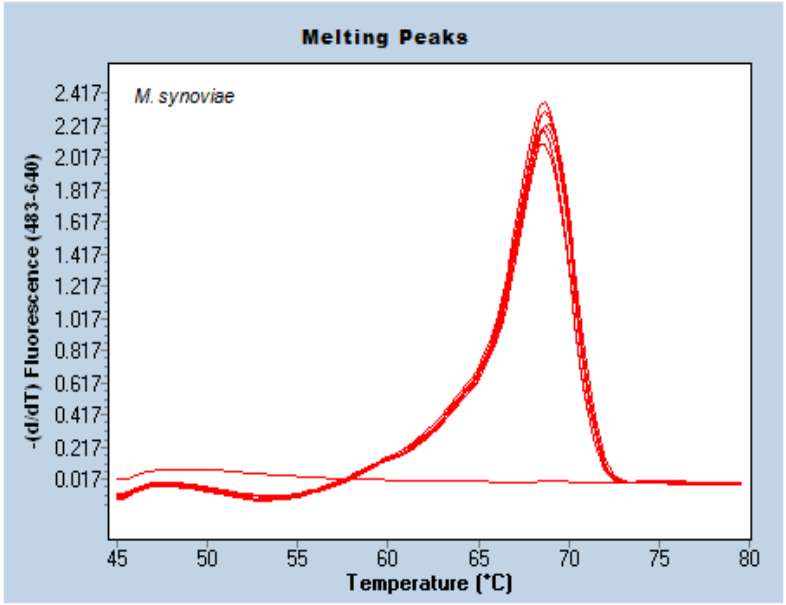




Dilution	CP Value	T_m value °C
10^7 copies	15.29	62.55
10^6 copies	18.73	62.55
10^5 copies	22.44	62.55
10^4 copies	25.60	62.55
10^3 copies	28.90	62.55
10^2 copies	32.53	62.55
10^1 copies	35.91	62.55
nc	negative	negative

M. synoviae Real-Time PCR
Analytical Sensitivity





Sample	CP Value	T_m value
10^7 copies	**17.08**	**68.91**
10^6 copies	**20.55**	**68.81**
10^5 copies	**24.02**	**68.73**
10^4 copies	**27.51**	**68.67**
10^3 copies	**30.87**	**68.71**
10^2 copies	**34.23**	**68.67**
10^1 copies	**37.41**	**68.66**
nc	**negative**	**negative**

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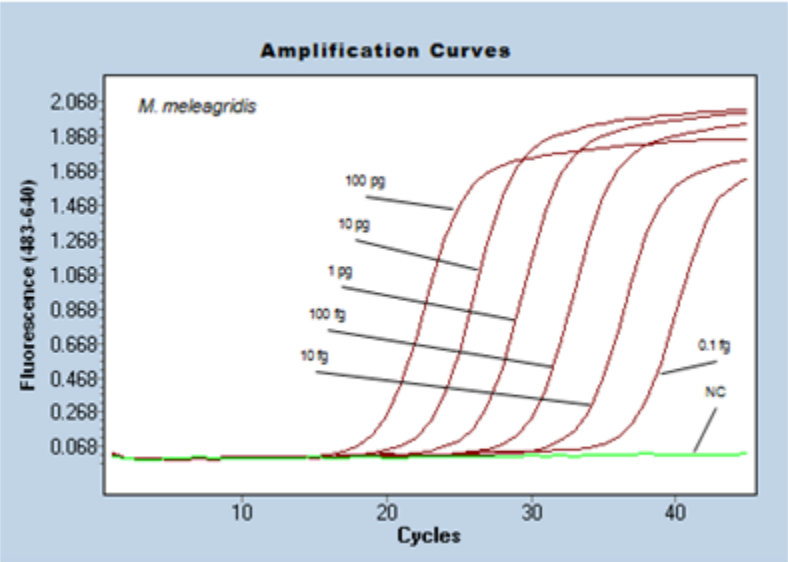
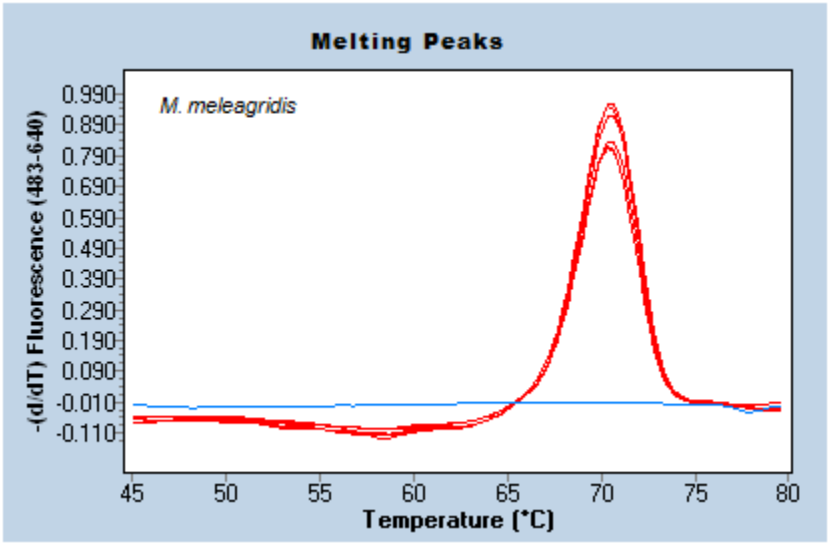
M. meleagridis Real-Time PCR
Analytical Sensitivity





Sample	CP Value	T_m value
10^6 copies	**19.29**	**70.90**
10^5 copies	**22.86**	**70.90**
10^4 copies	**26.16**	**70.89**
10^3 copies	**29.69**	**70.88**
10^2 copies	**33.22**	**70.77**
10^1 copies	**36.77**	**70.90**
nc	**negative**	**negative**

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Analytical Specificity
M. gallisepticum, *M. synoviae*, *M. meleagridis*

Sample DNA	*M. gallisepticum*		*M. synoviae*		*M. meleagridis*	
	Crossing Point	T_m	Crossing Point	T_m	Crossing Point	T_m
M. iowae	negative	negative	negative	negative	negative	negative
M. lipofaciens	negative	negative	negative	negative	negative	negative
M. cloacale	negative	negative	negative	negative	negative	negative
M. imitans	negative	negative	negative	negative	negative	negative
M. gallisnarum	negative	negative	negative	negative	negative	negative
M. gallinaceum	negative	negative	negative	negative	negative	negative
M. glycophilum	negative	negative	negative	negative	negative	negative
M. pullorum	negative	negative	negative	negative	negative	negative
M. gallopavonis	negative	negative	negative	negative	negative	negative
10 fg	28.58	63.99	28.92	68.48	28.71	71.48
1 fg	31.70	63.64	32.22	67.92	32.84	71.42
nc	negative	negative	negative	negative	negative	negative
nc	negative	negative	negative	negative	negative	negative

Non-pathogenic *Mycoplasma* DNA received from PDRC, University of Georgia
All DNA tested negative with the three assays
Assays did not recognize the non-pathogenic avian *Mycoplasma* DNA

Reproducibility

M. gallisepticum Real-Time PCR
Inter-assay and Intra-assay Performance

Dilution	Assay 1			Assay 2			Assay 3			Standard Deviation
	Crossing Point Replicate 1	Crossing Point Replicate 2	Crossing Point Replicate 3	Crossing Point Replicate 1	Crossing Point Replicate 2	Crossing Point Replicate 3	Crossing Point Replicate 1	Crossing Point Replicate 2	Crossing Point Replicate 3	
10 pg	18.59	18.62	18.60	18.64	18.69	18.65	18.57	18.55	18.49	0.059
1 pg	21.94	22.08	21.93	22.09	22.09	21.96	21.87	21.81	21.75	0.124
100 fg	25.17	25.47	25.30	25.33	25.25	25.23	25.20	25.13	25.03	0.126
10 fg	28.68	28.75	28.73	28.74	28.70	28.68	28.63	28.64	28.05	0.219
1 fg	31.95	31.84	31.95	31.72	31.87	31.88	31.85	32.06	31.85	0.094
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	

Dilution	Assay 1			Assay 2			Assay 3			Standard Deviation
	T_m Replicate 1	T_m Replicate 2	T_m Replicate 3	T_m Replicate 1	T_m Replicate 2	T_m Replicate 3	T_m Replicate 1	T_m Replicate 2	T_m Replicate 3	
10 pg	64.52	64.42	64.14	64.11	64.01	64.02	63.97	63.99	63.94	0.207
1 pg	64.07	64.07	64.00	63.97	63.92	63.89	63.90	63.94	63.88	0.073
100 fg	64.10	63.95	63.84	63.80	63.75	63.70	63.90	63.91	63.85	0.118
10 fg	63.75	63.71	63.70	63.12	63.12	63.06	63.75	63.81	63.71	0.321
1 fg	63.11	63.09	62.96	63.18	63.06	63.08	63.49	63.56	63.57	0.238
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative

Standard Deviation is $\leq$ 3% indicating acceptable inter-assay performance
Target was *M. gallisepticum* plasmid ten-fold dilution series

M. synoviae Real-Time PCR
Inter-assay and Intra-assay Performance

Dilution	Assay 1 Crossing Point Replicate 1	Assay 1 Crossing Point Replicate 2	Assay 1 Crossing Point Replicate 3	Assay 2 Crossing Point Replicate 1	Assay 2 Crossing Point Replicate 2	Assay 2 Crossing Point Replicate 3	Assay 3 Crossing Point Replicate 1	Assay 3 Crossing Point Replicate 2	Assay 3 Crossing Point Replicate 3	Standard Deviation
10 pg	18.72	18.70	18.68	18.65	18.63	18.63	18.71	18.63	18.58	0.046
1 pg	22.19	22.24	22.17	22.10	22.11	22.06	22.08	22.05	22.00	0.076
100 fg	25.73	25.66	25.78	25.78	25.71	25.70	25.75	25.70	25.70	0.040
10 fg	29.14	29.20	29.12	29.13	29.05	29.09	29.13	29.00	29.02	0.064
1 fg	32.68	32.44	32.62	32.58	32.61	32.60	32.59	32.61	32.45	0.079
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	

Dilution	Assay 1 T_m Replicate 1	Assay 1 T_m Replicate 2	Assay 1 T_m Replicate 3	Assay 2 T_m Replicate 1	Assay 2 T_m Replicate 2	Assay 2 T_m Replicate 3	Assay 3 T_m Replicate 1	Assay 3 T_m Replicate 2	Assay 3 T_m Replicate 3	Standard Deviation
10 pg	69.10	69.06	69.05	69.34	69.21	69.14	69.32	69.21	69.14	0.105
1 pg	69.08	68.91	68.89	69.23	69.04	68.96	69.25	69.07	68.99	0.128
100 fg	69.08	68.91	68.90	69.17	68.97	68.94	69.24	69.05	69.00	0.118
10 fg	69.16	69.07	69.07	69.18	69.06	69.05	69.34	69.19	69.19	0.094
1 fg	68.27	69.03	68.15	69.06	68.04	68.04	68.33	68.19	68.19	0.396
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	

Standard Deviation is $\leq$ 3% indicating acceptable inter-assay performance
Target was *M. synoviae* plasmid ten-fold dilution series

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M. meleagridis Real-Time PCR
Inter-assay and Intra-assay Performance

Dilution	Assay 1 Crossing Point Replicate 1	Assay 1 Crossing Point Replicate 2	Assay 1 Crossing Point Replicate 3	Assay 2 Crossing Point Replicate 1	Assay 2 Crossing Point Replicate 2	Assay 2 Crossing Point Replicate 3	Assay 3 Crossing Point Replicate 1	Assay 3 Crossing Point Replicate 2	Assay 3 Crossing Point Replicate 3	Standard Deviation
10 pg	19.43	19.42	19.24	19.47	19.49	19.48	19.45	19.47	19.45	0.076
1 pg	22.92	22.81	22.79	23.00	22.96	22.88	22.81	22.99	22.91	0.080
100 fg	26.22	26.42	26.15	26.49	26.32	26.34	26.18	26.29	26.18	0.117
10 fg	29.76	29.71	29.70	29.89	29.76	29.75	29.66	29.69	29.69	0.068
1 fg	33.16	33.20	33.09	33.31	33.52	33.28	32.85	32.95	32.90	0.217
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	

Dilution	Assay 1 T_m Replicate 1	Assay 1 T_m Replicate 2	Assay 1 T_m Replicate 3	Assay 2 T_m Replicate 1	Assay 2 T_m Replicate 2	Assay 2 T_m Replicate 3	Assay 3 T_m Replicate 1	Assay 3 T_m Replicate 2	Assay 3 T_m Replicate 3	Standard Deviation
10 pg	70.23	70.09	70.00	70.27	70.15	70.04	71.14	71.21	71.31	0.553
1 pg	70.20	70.07	70.03	70.23	70.10	70.05	71.00	71.08	71.21	0.499
100 fg	70.27	70.17	70.14	70.30	70.21	70.16	70.96	70.01	71.18	0.405
10 fg	70.26	70.17	70.11	70.27	70.19	70.15	71.07	71.09	71.18	0.464
1 fg	70.25	70.09	70.01	70.28	70.12	70.05	71.04	71.01	71.12	0.470
nc	negative	negative	negative	negative	negative	negative	negative	negative	negative	

Standard Deviation is $\leq$ 3% indicating acceptable inter-assay performance
Target was *M. meleagridis* plasmid ten-fold dilution series

Performance

M. gallisepticum Real-Time PCR

Sample	Crossing Point	T_m
Mg-1	31.24	64.26
Mg-2	25.46	64.28
Mg-3	negative	negative
Mg-4	24.73	63.90
Mg-5	negative	negative
Mg-6	31.83	62.71
10^3 copies	28.64	63.83
10^2 copies	31.74	63.39
NC	negative	negative

DNA samples from chickens
***M. gallisepticum* DNA positive**

M. gallisepticum Real-Time PCR

Sample	Crossing Point	T_m	Sample	Crossing Point	T_m
Sample 1	negative	negative	Sample 17	negative	negative
Sample 2	negative	negative	Sample 18	negative	negative
Sample 3	negative	negative	Sample 19	negative	negative
Sample 4	negative	negative	Sample 20	negative	negative
Sample 5	negative	negative	Sample 21	negative	negative
Sample 6	negative	negative	Sample 22	negative	negative
Sample 7	negative	negative	Sample 23	negative	negative
Sample 8	negative	negative	Sample 24	negative	negative
Sample 9	negative	negative	Sample 25	negative	negative
Sample 10	negative	negative	Sample 26	negative	negative
Sample 11	negative	negative	Sample 27	negative	negative
Sample 12	negative	negative	Sample 28	negative	negative
Sample 13	negative	negative	Sample 29	negative	negative
Sample 14	negative	negative	Sample 30	negative	negative
Sample 15	negative	negative	Sample 31	negative	negative
Sample 16	negative	negative	Sample 32	negative	negative
10 fg	28.60	63.83	nc	negative	negative
1 fg	31.82	63.39	nc	negative	negative

DNA Samples from chickens
***M. gallisepticum* negative**

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M. gallisepticum Real-Time PCR

DNA samples from turkeys
***M. gallisepticum* DNA positive**

Sample	Crossing Point	T_m
TK-1	24.20	63.02
TK-2	29.01	62.79
TK-3	31.53	62.64
TK-4	31.02	62.73
TK-5	31.71	62.67
TK-6	25.23	62.79
TK-7	26.26	62.81
TK-8	26.77	62.85
TK-9	30.99	62.73
TK-10	23.66	62.92
10 fg	28.65	63.60
1 fg	31.98	63.39
nc	negative	negative
nc	negative	negative

DNA samples from turkeys
***M. gallisepticum* DNA negative**

Sample	Crossing Point	T_m
Tk-11	negative	negative
Tk-12	negative	negative
Tk-13	negative	negative
Tk-14	negative	negative
Tk-15	negative	negative
Tk-16	negative	negative
Tk-17	negative	negative
Tk-18	negative	negative
Tk-19	negative	negative
Tk-20	negative	negative
Tk-21	negative	negative
Tk-22	negative	negative
Tk-23	negative	negative
Tk-24	negative	negative
Tk-25	negative	negative
10 fg	28.70	63.69
1 fg	31.84	63.16
nc	negative	negative
nc	negative	negative

M. synoviae Real-Time PCR

DNA samples from chickens
***M. synoviae* DNA positive**

Sample	Crossing Point	T_m	Sample	Crossing Point	T_m	Sample	Crossing Point	T_m
MS CH 1	32.30	68.46	MS CH 26	28.54	67.46	MS CH 51	26.29	67.23
MS CH 2	29.01	67.61	MS CH 27	34.54	68.31	MS CH 52	31.91	68.04
MS CH 3	33.61	68.32	MS CH 28	29.93	67.58	MS CH 53	27.86	67.38
MS CH 4	27.45	67.62	MS CH 29	30.05	67.55	MS CH 54	24.57	67.29
MS CH 5	32.05	67.59	MS CH 30	28.58	67.47	MS CH 55	29.69	67.25
MS CH 6	31.86	67.57	MS CH 31	31.48	68.15	MS CH 56	26.50	67.39
MS CH 7	negative	negative	MS CH 32	29.09	67.45	MS CH 57	26.75	67.46
MS CH 8	negative	negative	MS CH 33	25.16	67.54	MS CH 58	26.86	67.40
MS CH 9	32.92	67.63	MS CH 34	33.82	68.06	MS CH 59	24.54	67.30
MS CH 10	32.71	68.15	MS CH 35	24.86	67.46	MS CH 60	24.69	67.52
MS CH 11	32.74	67.43	MS CH 36	26.56	67.55	MS CH 61	29.63	67.47
MS CH 12	27.65	67.64	MS CH 37	31.62	67.46	MS CH 62	28.05	67.36
MS CH 13	27.02	67.50	MS CH 38	23.49	67.54	MS CH 63	24.13	67.33
MS CH 14	28.34	67.42	MS CH 39	25.69	67.42	MS CH 64	26.34	67.36
MS CH 15	26.66	57.41	MS CH 40	24.93	67.48	MS CH 65	30.62	67.50
MS CH 16	28.00	67.53	MS CH 41	27.05	67.45	MS CH 66	24.95	67.35
MS CH 17	31.01	67.56	MS CH 42	25.69	67.23	MS CH 67	25.46	67.31
MS CH 18	28.19	67.44	MS CH 43	24.72	67.28	MS CH 68	25.96	67.41
MS CH 19	27.47	67.42	MS CH 44	27.32	67.38	MS CH 69	30.20	67.41
MS CH 20	35.06	68.19	MS CH 45	24.55	67.36	MS CH 70	27.49	67.30
MS CH 21	31.72	67.53	MS CH 46	25.14	67.29			
MS CH 22	35.60	67.83	MS CH 47	26.01	67.29	10 fg	30.92	68.34
MS CH 23	37.76	68.76	MS CH 48	28.52	67.39	1 fg	34.79	68.32
MS CH 24	34.52	68.09	MS CH 49	26.11	67.46	nc	negative	negative
MS CH 25	29.04	67.63	MS CH 50	23.61	67.30	nc	negative	negative

M. synoviae Real-Time PCR

Sample	Crossing Point	T_m
MS 1	negative	negative
MS 2	negative	negative
MS 3	negative	negative
MS 4	negative	negative
MS 5	negative	negative
MS 6	negative	negative
MS 7	negative	negative
MS 8	negative	negative
MS 9	negative	negative
MS 10	negative	negative
MS 11	negative	negative
MS 12	negative	negative
MS 13	negative	negative
MS 14	negative	negative
MS 15	negative	negative
MS 16	negative	negative
nc	negative	negative
10 fg	30.86	67.91
1 fg	35.00	68.05

DNA Samples from chickens
***M. synoviae* negative**

M. synoviae Real-Time PCR

Sample	Crossing Point	T_m	Sample	Crossing Point	T_m
MS TK 1	negative	negative	MS TK 32	negative	negative
MS TK 2	negative	negative	MS TK 33	negative	negative
MS TK 3	negative	negative	MS TK 34	negative	negative
MS TK 4	negative	negative	MS TK 35	negative	negative
MS TK 5	negative	negative	MS TK 36	negative	negative
MS TK 6	negative	negative	MS TK 37	negative	negative
MS TK 7	negative	negative	MS TK 38	negative	negative
MS TK 8	negative	negative	MS TK 39	negative	negative
MS TK 9	negative	negative	MS TK 40	negative	negative
MS TK 10	negative	negative	MS TK 41	negative	negative
MS TK 11	negative	negative	MS TK 42	negative	negative
MS TK 12	negative	negative	MS TK 43	negative	negative
MS TK 13	negative	negative	MS TK 44	negative	negative
MS TK 14	negative	negative	MS TK 45	negative	negative
MS TK 15	negative	negative	MS TK 46	negative	negative
MS TK 16	negative	negative	MS TK 47	negative	negative
MS TK 17	negative	negative	MS TK 48	negative	negative
MS TK 18	negative	negative	MS TK 49	negative	negative
MS TK 19	negative	negative	MS TK 50	negative	negative
MS TK 20	negative	negative	MS TK 51	negative	negative
MS TK 21	negative	negative	MS TK 52	negative	negative
MS TK 22	negative	negative	MS TK 53	negative	negative
MS TK 23	negative	negative	MS TK 54	negative	negative
MS TK 24	negative	negative	MS TK 55	negative	negative
MS TK 25	negative	negative	MS TK 56	negative	negative
MS TK 26	negative	negative	MS TK 57	negative	negative
MS TK 27	negative	negative			
MS TK 28	negative	negative	10 fg	30.00	68.32
MS TK 29	negative	negative	1 fg	30.00	negative
MS TK 30	negative	negative	nc	negative	negative
MS TK 31	negative	negative	nc	negative	negative

DNA Samples from turkeys
***M. synoviae* negative**

Test With Confidence™

M. meleagridis Real-Time PCR

Sample	Crossing Point	T_m
Sample 1	21.80	69.74
Sample 13	19.75	69.74
Sample 25	23.20	69.27
Sample 37	21.03	69.74
Sample 49	20.45	69.74
Sample 61	22.93	69.47
Sample 73	24.88	69.43
Sample 85	18.75	69.72
1 pg	25.93	69.93
100 fg	29.85	69.94
nc	negative	negative

DNA Samples from turkeys
***M. Meleagridis* positive**

Test With Confidence™

M. meleagridis Real-Time PCR

Sample	Crossing Point	T_m	Sample	Crossing Point	T_m
MM TK 1	negative	negative	MM TK 32	negative	negative
MM TK 2	negative	negative	MM TK 33	negative	negative
MM TK 3	negative	negative	MM TK 34	negative	negative
MM TK 4	negative	negative	MM TK 35	negative	negative
MM TK 5	negative	negative	MM TK 36	negative	negative
MM TK 6	negative	negative	MM TK 37	negative	negative
MM TK 7	negative	negative	MM TK 38	negative	negative
MM TK 8	negative	negative	MM TK 39	negative	negative
MM TK 9	negative	negative	MM TK 40	negative	negative
MM TK 10	negative	negative	MM TK 41	negative	negative
MM TK 11	negative	negative	MM TK 42	negative	negative
MM TK 12	negative	negative	MM TK 43	negative	negative
MM TK 13	negative	negative	MM TK 44	negative	negative
MM TK 14	negative	negative	MM TK 45	negative	negative
MM TK 15	negative	negative	MM TK 46	negative	negative
MM TK 16	negative	negative	MM TK 47	negative	negative
MM TK 17	negative	negative	MM TK 48	negative	negative
MM TK 18	negative	negative	MM TK 49	negative	negative
MM TK 19	negative	negative	MM TK 50	negative	negative
MM TK 20	negative	negative	MM TK 51	negative	negative
MM TK 21	negative	negative	MM TK 52	negative	negative
MM TK 22	negative	negative	MM TK 53	negative	negative
MM TK 23	negative	negative	MM TK 54	negative	negative
MM TK 24	negative	negative	MM TK 55	negative	negative
MM TK 25	negative	negative	MM TK 56	negative	negative
MM TK 26	negative	negative	MM TK 57	negative	negative
MM TK 27	negative	negative			
MM TK 28	negative	negative	10 fg	29.76	70.26
MM TK 29	negative	negative	1 fg	33.20	70.09
MM TK 30	negative	negative	nc	negative	negative
MM TK 31	negative	negative	nc	negative	negative

DNA Samples from turkeys
***M. meleagridis* negative**

Test With Confidence™

M. gallisepticum, *M. synoviae*, *M. meleagridis* Real-Time PCR Summary

- **Sensitivity: Analytical sensitivity limit of detection is 10 copies of target sequence**
- **Specificity: Non-pathogenic Mycoplasma are not detected**
- **Data:**
  - **Crossing Point (CP): value indicates presence of target DNA**
  - **Tm: value confirms correct target sequence detected**
- **Clinical performance: Assays performed as expected**
  - ***Mycoplasma* negative chicken and turkey flocks were correctly identified**
  - ***Mycoplasma* positive chicken and turkey flock were correctly identified**