



## Highlights

- Detects the presence of *Mycobacterium avium paratuberculosis* (MAP), the causative organism of Johne's disease, in individual animal or bulk milk samples.
- Quantitative assay is based on molecular detection of MAP IS900 DNA in fresh or preserved milk samples. Values correlate with the MAP shedding status in dairy herds.
- Provides a convenient and cost effective screening tool to detect the presence of MAP organism in dairy herds.
- Could serve as a tool to screen milk pools for traceability should the presence of MAP be used for quality assessment.

## Introduction

In response to prioritization of bulk milk analysis by the Johne's Committee of the United States Animal Health Association, Antel BioSystems developed a real-time, polymerase chain reaction (PCR) assay for the detection of *Mycobacterium avium paratuberculosis* (MAP) in milk samples. According to USDA's National Animal Health Monitoring System (NAHMS) Dairy 2007 Study, sixty-eight percent of US dairies have MAP in their immediate environment. Using the AntelBio Mpara-teQ Milk assay, the study found bulk tank samples from forty percent of these infected dairies contained MAP. These and similar studies continue to demonstrate the growing dilemma Johne's disease presents to the dairy industry.

The AntelBio Mpara-teQ Milk assay is an effective tool for routine screening of bulk tank or individual animal milk samples. The real-time PCR assay is based on MAP DNA isolation, amplification and detection making it much quicker than traditional culture methods. Additionally, the assay is quantitative, allowing interpretation of milk MAP content and/or shedding status within herds. Milk preservative does not affect the performance of the assay. The convenience of bulk tank sample collection and the rapid turnover of the assay offer an efficient mechanism to evaluate milking herds for the presence of MAP infection.

Samples submitted to AntelBio are routinely batched; results are available within two weeks of sample submission. When possible, AntelBio will expedite analytical services upon request.

## Test Description

While the Johne's Milk ELISA detects antibodies against MAP organism, the AntelBio Mpara-teQ Milk assay directly detects MAP organism DNA. Following unique processing steps, a DNA sequence in the MAP-specific IS900 element is amplified in successive cycles by real-time PCR. The increase in measurable fluorescence signal after each cycle indicates amplification of the target sequence.

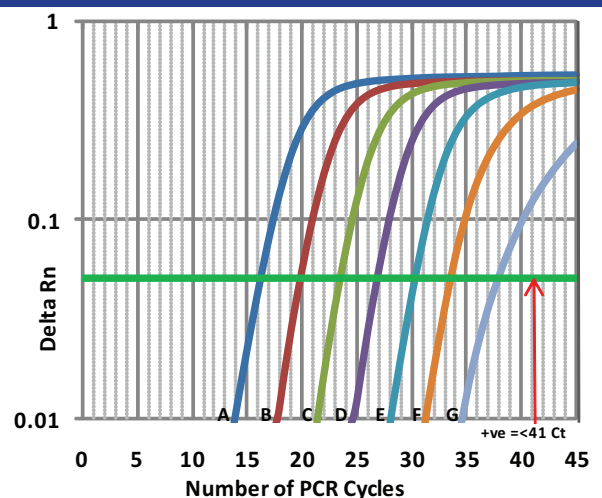
The PCR cycle at which the amplification signal of a test sample crosses the baseline or threshold value, is called the Cycle Threshold (Ct). The resulting numeric Ct value is quantitative, with Ct values inversely proportional to the amount of target DNA in the sample. The lower the Ct value, the higher the content of MAP DNA; in this assay, a Ct value less than 41 is considered "Positive" for MAP organism. All samples without detectable MAP DNA are labeled as "Undetected" and considered negative for the presence of MAP organism.

For quality assurance, every test panel includes two essential control reactions; purified MAP DNA as a positive control, and a negative sample without MAP DNA. Moreover, each real-time PCR reaction simultaneously amplifies internal control DNA to identify PCR inhibition or reaction failure, thereby reducing the occurrence of false negatives.

## Assay Validity

To determine the analytical sensitivity of the assay, milk samples (A-H) were spiked with serial dilutions of MAP organism and tested by quantitative real-time PCR. Figure 1 illustrates PCR amplification curves of these serially diluted samples. The amplification curve for milk sample A crosses the reaction threshold (green bar) at a Ct value of 16.18, whereas the last sample in the dilution series (H – not shown on graph) did not produce any amplification signal. The milk samples from A-G are considered positive for the presence of MAP DNA, as the Ct value is below 41.

Figure 1: MAP Dilution Series in Milk



DNA extracted from milk samples spiked with MAP organism was analyzed by quantitative real-time PCR (A-G). Delta reaction (Delta Rn) is the level of fluorescence intensity, which is a measure of the PCR amplification signal. The cycle at which the amplification signal of a test sample crosses the threshold (green bar) is the cycle threshold (Ct) for that sample. Ct values below 41 are considered positive for the presence of MAP DNA.

**Table 1: MAP Dilution Series Milk**

	Dilution	cfu	AvgCt	MAP Result
A	10 <sup>-1</sup>	3.0X10 <sup>6</sup>	16.18±0.06	Positive
B	10 <sup>-2</sup>	3.0X10 <sup>5</sup>	19.83±0.09	Positive
C	10 <sup>-3</sup>	3.0X10 <sup>4</sup>	23.48±0.07	Positive
D	10 <sup>-4</sup>	3.0X10 <sup>3</sup>	26.75±0.09	Positive
E	10 <sup>-5</sup>	3.0X10 <sup>2</sup>	30.28±0.03	Positive
F	10 <sup>-6</sup>	3.0X10 <sup>1</sup>	33.87±0.32	Positive
G	10 <sup>-7</sup>	3	38.36±1.48	Positive
H	10 <sup>-8</sup>	0	Undetected	Negative

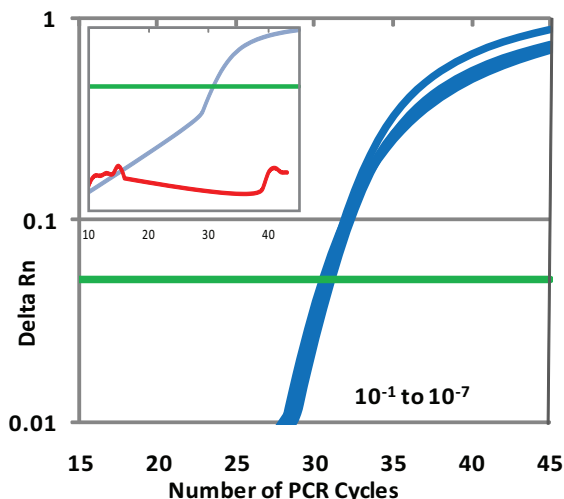
cfu: number of colony forming units of MAP organism per ml of milk, as determined by standard culture method. AvgCt: average Ct of dilution series from three replicates ± standard deviation.

The number of colony forming units (cfu) of MAP organism used to spike each milk sample (A-H) was determined by standard culture method (Table 1). The Ct values for the milk dilution series are listed in Table 1. Replicate (n=3) milk samples in the first dilution (A: 3.0X10<sup>6</sup> cfu) produced an average Ct value of 16.18±0.06, while samples in the last quantifiable dilution (G: 3 cfu) produced an average Ct value of 38.36±1.48. The milk sample (H) that did not contain any MAP organism, was negative for MAP DNA by real-time PCR. The sensitivity of the AntelBio *Mpara*-teQ Milk assay allows detection of as few as 3 cfu of MAP organism in one milliliter of milk sample.

Performance of the *Mpara*-teQ Milk assay was also assessed in the presence of milk preservative (bronopol and natamycin) and did not show any significant effect (data not shown).

## Assay Controls

*Mpara*-teQ Milk assay controls provide quality assurance in the interpretation and analysis of test results.

**Figure 2: *Mpara*-teQ Milk Assay Controls**

All PCR reactions contain internal positive control DNA that is simultaneously amplified by real-time PCR (blue lines). The inset shows positive control (blue line) and negative control (red line) PCR amplification plots.

Internal positive control DNA is added to each *Mpara*-teQ reaction to monitor DNA amplification efficiency. Figure 2 shows the amplification of internal positive control DNA (blue lines) present in the same reactions as the milk dilution series (Table 1). If there is a failure in the performance of real-time PCR, due to the presence of PCR inhibitors or sub-optimal reaction conditions, the internal positive control DNA will not be amplified. When the internal control signal is absent in any diagnostic sample, the assay is considered invalid and repeated. The internal positive control DNA greatly reduces the possibility of false negatives and serves as a measure of DNA amplification consistency across reactions.

The graph inset (Figure 2) shows the positive control (blue line) and the negative control (red line) in each assay panel. The positive control, with a Ct below 30, indicates that the test works efficiently for target gene amplification, while the negative control, with no detectable target sequence amplification, monitors cross-contamination. Test results are reported only when real-time PCR conditions have been systematically validated.

## NAHMS Dairy 2007 Study

AntelBio provided bulk milk analysis for the NAHMS Dairy 2007 Study. Altogether, 17 of the Nation's major dairy states representing 79.5 percent of U.S. dairy operations and 82.5 percent of U.S. dairy cows, participated in the study<sup>1</sup>. The AntelBio *Mpara*-teQ Milk assay detected the presence of MAP organism in forty percent of bulk tank samples from herds determined to be infected with Johne's disease. Bulk tank levels of MAP correlated with herd prevalence as estimated by environmental fecal analysis. The level of MAP in bulk tank samples can be used to screen herds for the relative severity of Johne's infection.

More information is available at: <http://www.antelbio.com/News/BulkMilkTestingforJohnesDiseaseDetectionattheFarmLevel.htm>

## Conclusions

The AntelBio *Mpara*-teQ Milk test is an effective tool for the detection of *Mycobacterium avium paratuberculosis* in individual or bulk milk samples. The test supplies quantitative information, providing knowledge of shedding status, while easy sample collection and fast turnaround time allow for improved management and tracking of Johne's disease.

<sup>1</sup>USDA-APHIS. Info Sheet #N521.0408 (2008): Johne's disease on U.S. dairies, 1991-2007; April, 2008.



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