



## Highlights

- *Mpara-teQ* assay detects the presence of *Mycobacterium avium subsp. paratuberculosis* (MAP), the causative organism of Johne's disease, in fecal or environmental samples.
- Quantitative assay is based on molecular detection of MAP IS900 DNA. Values correlate with the MAP shedding status in individual animals or level of environmental contamination.
- AntelBio achieved USDA certification to perform *Mpara-teQ* for 2009.
- Superior sensitivity to detect low bacteria counts which may occur in samples from cows in early stages of infection, in pooled fecal samples or in environmental samples.

## Introduction

A primary goal in any Johne's control program is the identification and elimination of infected animals shedding MAP organism via their feces into the immediate environment, the predominant mode of transmission for Johne's disease. The AntelBio *Mpara-teQ* fecal test improves the ability of veterinarians and producers to identify individual animals that are shedding MAP organism in vaccinated and non-vaccinated herds. The *Mpara-teQ* fecal test, based on real-time polymerase chain reaction (PCR) format, detects MAP IS900 DNA in fecal samples and is more sensitive and reliable than traditional culture methods. The new procedure is also quantitative, allowing the assessment of relative fecal MAP content or shedding status of individual animals. Infected animals can subsequently be stratified according to risk of Johne's transmission and confidently subjected to more stringent management protocols. Because of the enhanced sensitivity the *Mpara-teQ* test can readily be used to reduce costs of fecal testing through strategies such as fecal pooling or environmental testing.

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.

AntelBio is certified by the USDA for *Mpara-teQ*, having passed the National Veterinary Services Laboratory's 2009 Johne's Proficiency Test for real-time, PCR-based quantitative assays.

## Test Description

The AntelBio *Mpara-teQ* test directly detects the presence of MAP organism DNA in fecal and environmental samples. Following unique sample 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 fluorescent amplification signal of a test sample crosses a threshold value is called the Cycle Threshold (Ct). Since the amount of DNA present increases in predictable ways after each PCR cycle, the resulting numeric Ct value is quantitative with roughly a 3 unit change in Ct value equivalent to a 10-fold difference in relative DNA content. Ct values are inversely proportional to the amount of target DNA in the sample; the lower the Ct value the higher the original content of MAP

DNA in sample. In the current assay, a Ct value equal to 36 units or less is considered "Positive" and indicates that MAP DNA was detected in the fecal sample. Samples with Ct values of 36 units or more are considered "Negative" as detection did not take place in a range consistent with MAP DNA in feces. Samples that showed no evidence of MAP DNA within the analytical range (maximum 45 Ct) are not reported with a specific Ct value, but rather are labeled "Undetected."

For quality assurance, every test panel includes two 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 added to every sample to identify PCR inhibition or reaction failure, thereby reducing the occurrence of false negatives.

## Assay Validity

For validation, fecal samples from infected animals were serially diluted 10 fold with negative fecal samples and tested by traditional culture in Herrold's Egg Yolk media to produce a standard range of fecal samples (A-F). The average number of colony forming units (cfu) per tube of MAP organism for these standard samples is recorded in Table 1.

**Table 1: Culture and Real-time PCR Analysis of Diluted Fecal Samples from MAP Infected Cows**

	Dilution	cfu	AvgCt	Result
A	10 <sup>-0</sup>	>300	27.4±0.26	Positive
B	10 <sup>-1</sup>	27	30.5±0.25	Positive
C	10 <sup>-2</sup>	3	34.1±0.20	Positive
D	10 <sup>-3</sup>	1 <sup>†</sup>	40*	Negative
E	10 <sup>-4</sup>	0	Undetected	Negative
F	10 <sup>-5</sup>	0	Undetected	Negative

cfu: Average number (replicates = 3) of colony forming units of MAP organism per culture tube as determined by standard culture. <sup>†</sup> denotes a single replicate that showed one cfu. AvgCt: Average Ct ± standard deviation from two replicates. \* denotes that a single replicate recorded a Ct value.

Figure 1 illustrates real-time PCR amplification curves of the serially diluted fecal samples after processing. The amplification curves for fecal sample A crosses the threshold (green bar) at an average Ct value of 27.4, whereas the last sample in the dilution series (F – not shown on graph) did not produce any amplification signal. The fecal samples from A-C are considered positive for the presence of MAP DNA as the Ct value of the replicate samples is below 36.

## Assay Controls

Real-time PCR assay controls provide quality assurance in the interpretation and analysis of test results. Internal control DNA is added to each real-time PCR reaction to monitor DNA amplification efficiency. Figure 1B and 1C show the amplification of internal control DNA (light blue lines) present in the same reactions as the serially diluted fecal samples (Table 1). If there is a failure in the performance of real-time PCR due to the presence of PCR inhibitors or sub-optimal reac-

-tion conditions, the internal 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 addition of internal control DNA greatly reduces the possibility of false negatives and serves as a measure of DNA amplification consistency across each reaction.

**Figure 1: Real-time PCR Output for Fecal Samples**

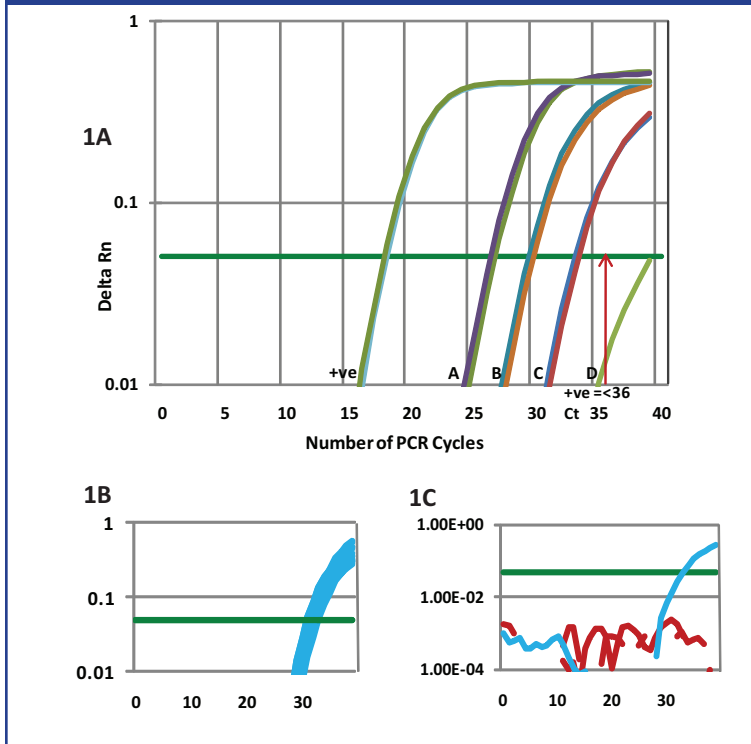


Figure 1A also shows the results of the positive control sample included in each test panel (+ve, green amplification curve) and Figure 1C shows results of the negative control sample (red line) in addition to the positive control signals (light blue lines) shown in 1B and 1C. The positive control sample is designed to show that conditions are appropriate for amplification of IS900 DNA, the specific molecular target for MAP. The negative control is used to monitor for cross-contamination during sample processing and analysis, and should yield no detectable target sequence amplification.

Test results are reported only when real-time PCR conditions have been systematically validated, 1) no cross contamination as shown by the absence of IS900 signal in the negative control sample, 2) appropriate reaction conditions for amplification of available target sequence as shown by IS900 DNA signal in the positive control sample, and 3) absence of reaction inhibitors as shown by internal control DNA signals in every sample (Ct<30).

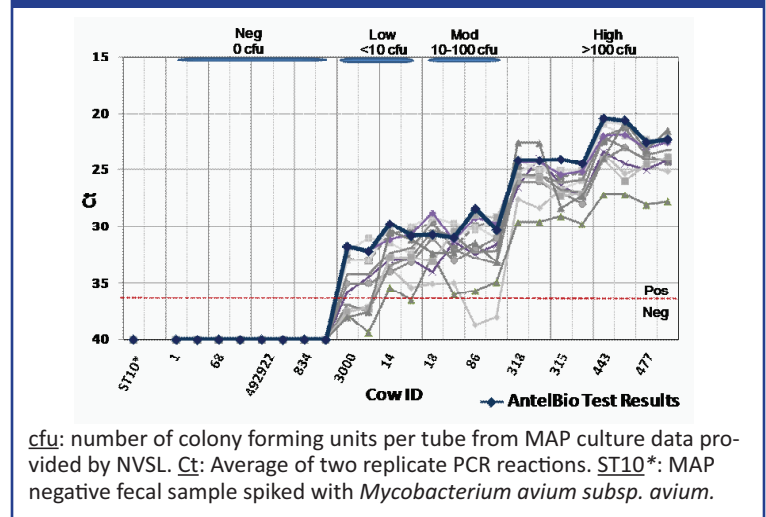
### Assay Sensitivity

The Ct values for the fecal dilution series are listed in Table 1. Average Ct values of 27.4, 30.4 and 34.1 were obtained from fecal samples with traditional culture values of 300, 27, and 3 respectively. The relationship between culture value (10-fold dilutions) and Ct value (increments of 3) demonstrates the quantitative ability of *Mpara-teQ*. The sensitivity of *Mpara-teQ* fecal readily detects the traditional culture equivalent of 3 cfu of MAP organism at a Ct value of 34.1. With a cutoff of 36 Ct the test is theoretically capable of detecting MAP levels below the lower detection limit of 1 cfu in traditional culture. The MAP dilution series was tested alongside AntelBio's existing gel-based PCR assay (Johne's Rapid Fecal Test) and *Mpara-teQ* fecal showed a ten-fold higher sensitivity in detecting the presence of MAP organism (data not shown).

## National Veterinary Services Laboratory (NVSL) 2009 Johne's Proficiency Test

The AntelBio *Mpara-teQ* fecal test passed the NVSL 2009 Johne's Proficiency Test panel for direct PCR with 100% accuracy. Figure 2 illustrates the *Mpara-teQ* proficiency test results (samples 1-26) where Ct value for each test sample is graphed. The colony counts (cfu) were determined by NVSL and are averages of three cultures for each fecal sample. The Ct value for each sample correlated with the shedding status of individual animals; Ct values below 26 corresponded to high shedders (>100 cfu per tube of sample), Ct values between 26 and 30 corresponded to moderate shedders (100-10 cfu per tube), and Ct values between 31 and 36 corresponded to low shedders (<10 cfu). All negative samples were correctly identified indicating 100% specificity. Additionally, the *Mpara-teQ* fecal test did not show any undesired cross-reactivity to *Mycobacterium avium subsp. avium* present in one negative sample from NVSL.

**Figure 2: 2009 NVSL Johne's Proficiency Test**



cfu: number of colony forming units per tube from MAP culture data provided by NVSL. Ct: Average of two replicate PCR reactions. ST10\*: MAP negative fecal sample spiked with *Mycobacterium avium subsp. avium*.

### Conclusions

The AntelBio *Mpara-teQ* test is a superior method for detection of MAP in fecal samples from individual animals or the environment. Greater sensitivity allows veterinarians and producers to identify infected animals or environmental areas with even minimal MAP content. The inclusion of process controls yield results that allow implementation of management procedures with greater confidence whether testing individual animal or pooled samples, ultimately facilitating more cost effective testing programs. The quantitative information obtained from the test provides knowledge of MAP content in each sample, and faster turnaround times, compared to fecal culture, allow for more rapid implementation of management decisions and better control of Johne's disease.



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