

United States  
Department of  
Agriculture

Animal and Plant  
Health  
Inspection  
Service

Veterinary  
Services

Science,  
Technology, and  
Analysis Services

Center for  
Epidemiology  
and Animal  
Health

September 2017



# Potential Uses of a rRT-PCR Assay for FMD in Bulk-Tank Milk in the United States



## Table of Contents

Executive Summary.....	3
Purpose .....	5
Background .....	5
Disease Characteristics and Shedding in Milk.....	7
Test Characteristics.....	8
Test and Testing Considerations .....	9
Information Gaps .....	10
Evaluation Approach.....	11
Surveillance Design .....	12
Potentially Valuable Uses of the rRT-PCR Assay for FMD in BTM .....	13
Potentially valuable uses of the rRT-PCR assay in BTM when FMD is not known to be present, or at first detection in the United States.....	13
Potentially valuable uses of the rRT-PCR assay in BTM during an FMD outbreak.....	13
Not Valuable Uses of the rRT-PCR Assay for FMD in BTM.....	17
Not valuable uses of the rRT-PCR assay in BTM when FMD is not known to be present in the United States.....	18
Not valuable uses of the rRT-PCR assay in BTM when FMD is known to be present in the United States.....	19
Appendix A. Sampling Requirements for Control and Surveillance Area Scenarios.....	22
Appendix B. Surveillance Sampling for FMD from the 2014 USDA-APHIS Response Plan .....	25
References .....	26

This report was prepared by USDA-APHIS-VS-STAS-CEAH

**The authors of this report were:**

Dr. Jason Lombard – Monitoring and Modeling Unit [jason.e.lombard@aphis.usda.gov](mailto:jason.e.lombard@aphis.usda.gov)

Dr. Marta Remmenga – Surveillance, Design and Analysis Unit [marta.d.remmenga@aphis.usda.gov](mailto:marta.d.remmenga@aphis.usda.gov)

Dr. Karla Moreno-Torres – Postdoctoral Scientist/ ORISE Fellow

**The report was reviewed and/or significant contributions were made by staff from:**

Science, Technology and Analysis Services

- Center for Epidemiology and Animal Health
- National Veterinary Services Laboratories
  - Foreign Animal Disease Diagnostic Laboratories
  - National Animal Health Laboratory Network

Surveillance, Preparedness and Response Services

- Cattle Health Center
- National Preparedness and Incident Coordination Center

University of California – Davis (Dr. Pamela Hullinger)

## Executive Summary

Foot-and-mouth disease (FMD) is one of the most contagious viral diseases of cattle and other cloven-hooved animals. If FMD were to occur in the United States, it would be imperative for response agencies to identify infected animals and restrict movement to prevent further spread of the disease. Currently, detection of FMD virus (FMDV) in the field relies primarily on detection of overt clinical signs (e.g., vesicles), followed by presumptive laboratory-based diagnostic testing within the National Animal Health Laboratory Network (NAHLN) and simultaneous definitive diagnostic testing by USDA's National Veterinary Services Laboratories (NVSL) Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center. Diagnostic techniques for earlier detection during the subclinical phase are essential for optimal outbreak control and limiting spread.

The literature describes shedding of FMDV in milk from 1 to 15 days prior to the onset of clinical signs, but most studies suggest 2 to 4 days is more typical. Even when clinical signs are present, it may take additional hours or days for observation and detection of infected animals by owners or animal health officials. One potential tool for identification of infected premises during this subclinical phase or early at the onset of a disease outbreak in dairy cattle is a bulk-tank milk (BTM) test for FMD.

NVSL has validated a real-time reverse transcriptase-polymerase chain reaction assay (*simply referred to as rRT-PCR assay in BTM throughout this document*) for the detection of FMDV in BTM samples. Diagnostic sensitivity and specificity of the assay in BTM samples was reported to be 86.4 and 100.0 percent, respectively; however, the reagents for the initial assay have been discontinued and additional validation is underway using reagents currently used for other sample types.

The rRT-PCR assay detects viral RNA and does not differentiate viable versus nonviable virus. The rRT-PCR assay in BTM can be performed in about 2 hours; however, the additional time required to obtain the sample, deliver it to the lab, and report the results must be factored into the timeline. If the routine milk sample collection process is used, the additional time could range from 8 to 18 hours from milk pickup to reporting of results. Using the rRT-PCR assay in BTM allows for screening a large number of lactating dairy cattle with a single test, using fewer resources than testing individual animals. Similarly, bias is reduced because the rRT-PCR assay in BTM does not rely on producer reporting. However, the possibility of overwhelming the laboratory capacity still exists, if a large number of premises are scheduled for testing.

BTM samples from every dairy premises in the United States are routinely collected by the transport personnel immediately before milk is transported for processing and samples are tested per the regulations of the Pasteurized Milk Ordinance. On most dairy premises, the processor tests either a single BTM sample or multiple samples on a daily basis. Some small herds may only be sampled and tested every other day. This routine testing process illustrates that a system is already in place that allows easy access to BTM samples for potential use in an FMD testing program of dairy premises, but the timing of sample collection does not allow for testing prior to movement of

milk. Further evaluation could determine the coordination necessary for timely transfer of BTM samples from processors to NAHLN laboratories.

This document describes an evaluation of potential uses of the rRT-PCR assay for FMD virus in BTM collected from U.S. dairy premises. Dairy premises are premises with lactating dairy cows. A team of analysts from VS-Science, Technology, and Analytical Services (STAS) Center for Epidemiology and Animal Health (CEAH); NVSL including NAHLN and FADDL, in consultation with academic institutions, analyzed various uses of the rRT-PCR assay in BTM sample matrix before, during, and after an outbreak. The evaluation includes recommendations for uses that may be valuable as well as those considered not to be valuable. The three criteria used for determining the value of the rRT-PCR assay in BTM were:

1. Sufficiently robust for the proposed purpose
2. Augments decision-making
3. Provides an advantage over current FMD testing protocols or surveillance activities

If the proposed purpose met all of the criteria above, it was categorized as potentially valuable and a quantitative analysis, if necessary, was performed. If any one of the criteria was not met, the use was categorized as not valuable for that purpose.

This evaluation considered the following rRT-PCR assay in BTM uses to be **potentially valuable** for managing FMD in the United States based on current knowledge. All of these are for use of the rRT-PCR assay in BTM as a screening test, except the third bullet:

- Targeted/risk-based surveillance of dairy premises at first detection in the United States or if other North American countries were infected
- Testing of dairy premises not declared as infected within a control area
- Confirmation of an infected, unvaccinated dairy premises in a control area when clinical signs are also present
- Informing the classification of premises (e.g., at-risk or monitored premises) in a control area during an outbreak
- Testing of milk to be fed back to susceptible animals on premises in the control area
- Surveillance of dairy premises within surveillance zone
- Screening of infected dairy premises after the post-infection period prior to performing individual animal serology testing for quarantine release and/or disease freedom

The following rRT-PCR assay in BTM uses were considered **not valuable** for use in managing FMD in the United States based on current knowledge:

- Nationwide surveillance for early detection of an FMD outbreak. There is no reason in the current FMD-free situation for using resources (e.g., lab capacity and cost) to test in this high-volume, frequent testing scenario.
- Maintaining disease freedom status after establishment of disease freedom. Testing with the rRT-PCR assay in BTM does not provide additional evidence of disease freedom over the current passive surveillance system.

- Use in foreign animal disease (FAD) investigations in a non-FMD outbreak situation. The rRT-PCR assay in BTM does not have any advantage over testing normally collected FAD samples from individual animals.
- Permitting of daily movement of raw milk off-farm or rerouting of milk tankers in an FMD control area. The rRT-PCR assay in BTM results would not normally be available prior to the need to move milk to processing. Protocols for milk movement have been developed under the assumption that infected milk may be moving.
- Permitting movement of milk after pasteurization at a processing plant or movement of products made from pasteurized milk during an FMD outbreak. The rRT-PCR assay in BTM detects RNA of the virus and does not determine whether it is infectious or not.
- Permitting movement of live infected cattle to designated slaughter facilities during an FMD outbreak. The premises is already designated as infected and protocols should already be in place.
- Permitting movement of contaminated materials off infected premises for appropriate disposal during an FMD outbreak. The premises is already designated as infected and protocols should already be in place.

BTM samples provide a valuable matrix for FMD testing. Using BTM samples would conserve resources by reducing sampling of individual animals and number of tests performed. This document outlines the potential uses, given our current knowledge of the disease and test performance. The value of the uses described in this evaluation may change based on conditions and/or test performance during an outbreak. This is not an exhaustive list of potential uses and additional ones are likely in the future.

## **Purpose**

The purpose of this document is to evaluate and provide recommendations for the use of a real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) assay for foot-and-mouth disease virus (FMDV) in bulk tank milk (BTM) from U.S. dairy premises. The evaluation analyzed potential uses of the BTM samples with the FMD rRT-PCR assay during and after an outbreak.

## **Background**

FMD is one of the most contagious viral diseases of cattle and other cloven-hooved animals. The causative agent of FMD is a single-stranded RNA virus belonging to the family *Picornaviridae*. Seven serotypes (A, O, C, Asia 1, and SAT 1, 2, and 3) are recognized and many strains or topotypes have been described (Merck 2017). These different serotypes can be differentiated with serological testing, with little cross reactivity, although there is some cross reactivity among topotypes within a serotype. FMDV is primarily spread by direct or indirect contact with infected animals or fomites. Although adult cattle can survive the disease, their health and productivity are significantly compromised. Mortality can be significant in calves. If FMD were to occur in the United States, it would be imperative for response agencies, in cooperation with animal industries, to identify infected animals among many different animal livestock species and restrict movement of animals and animal products to prevent further spread of the disease.

Currently, detection of FMDV in the field relies primarily on the detection of overt clinical signs (e.g., vesicles). However, this approach could delay the detection of infected individuals and premises at a given point in time. The incubation period in cattle ranges from 2-14 days, during which time the animals may be infectious but asymptomatic and remain undetected. This delay in detection of infected individuals or premises may result in failure of control strategies. Therefore, diagnostic techniques for early detection during this subclinical phase are essential for controlling the outbreak and limiting spread.

FMDV has been detected in secretions, excretions, and blood before, during, and after clinical signs appear. For example, FMDV has been shown to be shed in milk within a few hours to a few days before overt clinical signs develop. One potential tool for use during this subclinical phase is the bulk-tank milk (BTM) test for FMD. BTM samples are easily obtained and this non-invasive sampling method can be used for early herd-level detection of FMDV. An rRT-PCR assay for the detection of FMDV in BTM samples has recently been validated (Armson et. al., unpublished). The rRT-PCR assay does not rely on farmer reporting, hence detection is less subjective and can more easily follow standardized collection and testing protocols. While the BTM test could be an important diagnostic tool, a better understanding of this diagnostic approach is needed to determine its potential use in detecting and controlling FMD.

To address the issue of business continuity in the United States, a secure food supply framework is being developed for a number of commodities, including milk, through the Secure Milk Supply (SMS) plan (<http://securemilksupply.org/>). One objective of the current plan is to allow the risk-based permitted movement of milk, a perishable product, from herds within a control area to commercial processing. One goal of the SMS is to reduce the risk of moving raw milk from infected but undetected premises and from infected premises to commercial processing during an FMD outbreak. State, Federal, and industry partners have developed biosecurity guidelines with the intent of helping producers meet guidelines for continuing to move their milk to processing. According to the current SMS plan for the movement of milk during an FMD outbreak, all premises located in a control area that are not designated as infected, suspect, or contact premises and meet the criteria required in their State may continue to move raw milk to processing plants within or outside of the control area unless notified otherwise.

Carrying out an FMD milk test surveillance program using this rRT-PCR assay in BTM requires BTM samples. BTM samples from every dairy premises in the United States are routinely collected by the transport personnel immediately before milk is transported for processing and samples are tested per the regulations of the 2015 Pasteurized Milk Ordinance (<https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/milk/ucm513508.pdf>).

Milk from smaller dairy herds (50-75 cows) might be picked up every other day, while for large herds (~1,000 cows), multiple loads may be picked up daily. The milk is sampled to evaluate quality (somatic cell count) and milk components to determine the price producers are paid. In addition to the sample collected at the dairy, a sample of the tanker, representing a single premises or multiple

premises based on size, is taken at the processing plant and tested for antibiotics. Individual dairy premises and tanker load samples would be available for FMD testing if an outbreak occurred. During an outbreak, the appropriate regulatory agency could request that an additional sample be collected from each dairy premises for FMD testing. The fact that a system is already in place highlights the potential for easy access to BTM samples for use in an FMD testing program of dairy premises.

### **Disease Characteristics and Shedding in Milk**

There are seven serotypes (A, O, C, Asia 1, and SAT 1, 2, and 3) and multiple topotypes within serotypes of FMDV. Disease progression in cattle varies and is dependent upon a number of factors including, but not restricted to, the serotype/topotype, age, route of exposure, viral dose, and immune status (Arzt et al., 2011).

FMD pathogenesis is commonly discussed in terms of stages based on the presence of viremia. There are three stages of FMD pathogenesis in cattle:

1. Pre-viremic
2. Viremic, and
3. Post-viremic

The first stage of FMDV pathogenesis in cattle is the pre-viremic stage, beginning with the onset of infection and lasting from 16-72 hours depending on the strain and dose. During this stage, FMDV replicates in the nasopharynx area if exposed intranasally. The presence of virus in nasopharyngeal fluid during this phase makes testing of a probang sample a good tool for early detection of the virus. FMDV is not detectable in blood during the pre-viremic stage. This stage of infection sometimes involves a temporary viremia that might distribute the virus to the mammary gland earlier, prior to the prolonged viremic stage.

Viremia is the second stage of infection. Experimental infection in cattle has shown that viremia precedes pyrexia and clinical signs by 1 to 2 days or more. During the viremic stage, there is widespread distribution of the virus to various organs and tissues, including the mammary gland. Studies suggest that FMDV may replicate in the mammary gland (Reid et al., 2004). All excretions and secretions can contain virus during this stage. The duration of viremia is variable, but cattle usually aren't infectious after about 5 days from the onset of clinical signs. However, they continue to shed detectable virus at amounts below the threshold of infection for other animals.

The ability to detect FMD prior to onset of clinical signs would have a positive impact on controlling the disease. The literature describes shedding of FMDV in milk from 1 to 15 days prior to the onset of clinical signs but most studies suggest 2 to 4 days is more typical (Bates, Thurmond et al. 2003, Thurmond and Perez 2006). The onset of clinical signs in an animal or herd typically occurs hours or sometimes days before those signs are detected. Moreover, FMDV shed in milk was detectable at an average of 14 days (and maximum of 28 days) after the onset of clinical signs. Presumably during this time, additional animals in a herd would become infected, increasing the likelihood of detection either by observance of clinical signs or by surveillance testing of BTM.

The final stage of infection is the post-viremic stage. Cattle in this stage are usually no longer infectious, although reportedly up to 50 percent of cattle recovered from infection may be classified as persistently infected if viral shedding continues for more than 28 days post-infection. Persistent infection may occur regardless of vaccination status. Using the BTM test at this stage might not be as useful, because virus may no longer be secreted in milk at detectable levels but could remain in the naso-pharyngeal areas of cattle recovered from clinical disease.

## Test Characteristics

### *Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) Background*

PCR is a technique used to amplify nucleic acid. The unique aspect of the PCR is that small areas of targeted nucleic acid are amplified to a billion or more copies that can then be readily detected. As the genome of FMD consists of RNA rather than DNA, the rRT-PCR used for FMDV detection and quantification in bulk-tank milk is termed real-time reverse transcriptase-polymerase chain reaction because reverse transcriptase is needed to make DNA copies of the RNA genome. Use of a fluorescent reporter molecule further allows detection of amplified products in real-time (Elsevier, 2007). This test is able to detect all seven serotypes of FMD. The assay is not able to differentiate viable from nonviable virus.

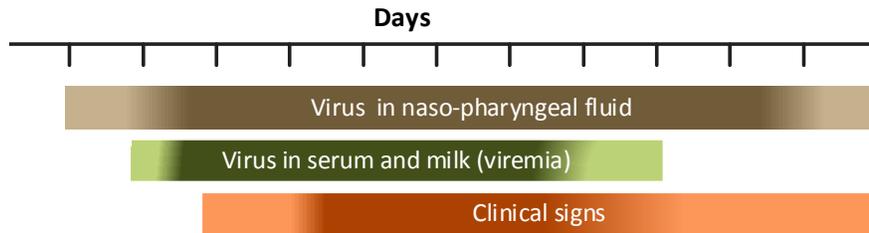
The rRT-PCR was evaluated in BTM for both analytical and diagnostic sensitivity and specificity<sup>1</sup> (Armson et. al., unpublished). *Diagnostic sensitivity and specificity will be referred to as simply sensitivity and specificity throughout this document.* The sensitivity of the rRT-PCR assay in BTM using TaqMan EZ (discontinued reagents) and PathID multiplex kits (current reagents) was 86.4 percent (5 percent error) and 100 percent (5 percent error), respectively. The specificity was 100 percent (95 percent CI: 0.99, 1.00). Reproducibility and robustness were demonstrated using a blinded test evaluation. To assess the effects of dilution on the ability of the assay to detect FMDV, milk samples from contact FMDV-infected lactating cows containing various viral titer levels were used, resulting in FMDV detection from a 250-fold dilution to an approximately 30,000-fold dilution depending on starting titer. This approach supports the contention that the test can reasonably detect FMDV from a BTM sample in which a single infected dairy cow out of a herd size of 100-1,000 is shedding virus. This assumption is based on sampling during the onset of infection, which is when viral shedding in milk is the greatest and the average milk production decreases only about 7.9 percent and no greater than 50 percent on any one day.

rRT-PCR assays have detected viral RNA in the mouth and nose of infected cattle 24 to 96 hours before the onset of clinical disease (Callahan, Brown et al. 2002) [Figure 1]. Detection of FMDV in milk was early, coincident with clinical signs, and 1-2 days before the onset of characteristic clinical signs such as foot lesions. A modeling study for early detection of FMDV in BTM found that the virus

---

<sup>1</sup>*Analytical sensitivity refers to the smallest concentration of a substance that can be reliably measured by the analytical method while diagnostic sensitivity is the conditional probability that an animal with disease will be correctly identified by a clinical test.*

could be detected in a dairy herd 5-6 days earlier than the observance of clinical signs (Thurmond and Perez, 2006).



**Figure 1. An approximate timeline of virus distribution and clinical disease in cattle.**

In general, the breed of cow (Jersey, Holstein, and mixed) from which the milk was obtained had little effect on the recovery and detection of FMDV by the assay in bulk-tank milk. Mean cycle threshold (Ct) values were slightly lower for the Holstein milk as compared to Jersey and mixed bulk-tank milk, which were similar. This is an important result, since fat content may affect the performance of the test. Jersey milk contains 45 percent more fat than Holstein milk (4.57 and 3.15 percent, respectively). Feasibility studies showed that potential milk inhibitor problems were negligible for the rRT-PCR assay. In addition, other studies found that sample matrices such as the cream layer and skim milk fraction were difficult to extract DNA or RNA from, and not practical for high-throughput testing (USDA-APHIS-VS, 2014).

In addition, other studies found that samples such as the cream layer and skim milk fraction were difficult to extract DNA or RNA from these sample matrices and not practical for high-throughput testing (USDA-APHIS-VS, 2014).

Milk tested during an outbreak would most likely be chilled, raw milk that had never been frozen; however, studies demonstrated no significant difference in results obtained using fresh or frozen milk. In its study, FADDL spiked fresh and frozen whole milk samples with FMD virus and compared two extraction methods. Results showed that these two extraction methods using either fresh or frozen milk resulted in an amplification efficiency of greater than 90 percent. Amplification efficiency is calculated based on the assumption that the number of RNA copies will double in each cycle. An efficiency of greater than 90 percent means that rRT-PCR inhibitors in fresh or frozen whole milk samples were not a problem using either extraction method.

### **Test and Testing Considerations**

- Sensitivity of the rRT-PCR assay in BTM is very important because of the likelihood of dilution of viral load in milk at the premises level. Milk from a small number of infected cows is diluted by the milk from uninfected cows in the BTM sample, but it is further diluted by the decreased amount of milk that is produced by infected cows.
- In some cases, more than a single tanker truck may need to be sampled to include milk from all lactating cows on a premises, depending on the number of lactating cows and the amount of milk produced. Premises with about 1,000 cows producing 70 lbs of milk per cow per day will fill an 8,000 gallon (68,800 lbs) tanker (Appendix A). A single milk sample

collected from a premises milking 2,000 cows producing 70 lbs of milk per cow per day would still represent at least one milking for all cows.

- The probability of detection from a single BTM sample is, at best, the sensitivity of the test. To ensure at least 0.95 probability of detection at the premises level, at least three samples must be collected over time (see Appendix A.) These sampling requirements assume, if infected, the prevalence of FMD among lactating animals represented by any single tank sample is at least 1 percent. For premises with more than 2,500 cows where multiple tanks are required to represent all milking cows, testing all of the tanks provides 0.95 probability of detection, assuming the infected cows are distributed throughout the milking herd and the prevalence is at least 1 percent. To be conservative, we employed a sampling scheme of collecting BTM samples three consecutive days from all premises to ensure at least 0.95 probability of detection.
- The rRT-PCR assay is used on a pooled milk sample, representing multiple dairy cows in a single sample.
- This rRT-PCR assay detects viral RNA and does not determine viability of the virus. For example, a positive outcome of a rRT-PCR assay in BTM does not provide evidence to determine if the milk in the bulk tank is infectious.
- The rRT-PCR assay can be performed in about 2 hours. Additional time is required to obtain the sample, deliver it to the lab, and report the results. Time estimates range between 8 and 18 hours from milk pickup to reporting of results.
- A consistent State-by-State coordination plan is needed to ensure an efficient transfer of BTM samples from processors to NAHLN laboratories.
- A proficiency test program is already in place for the FMD rRT-PCR assay used in foreign animal disease investigations. Additional approval and/or proficiency testing for the rRT-PCR assay in BTM may be required.
- Preserved milk samples contain rRT-PCR inhibitors and should not be tested with the rRT-PCR assay.
- BTM only represents lactating cows producing salable milk. Young cattle, non-lactating cows, cows that have recently calved, and sick cows would not be represented in BTM samples.

### Information Gaps

- Pathogenesis
  - Literature suggests significant variation in the pathogenesis and clinical presentations of FMD-infected animals depending on species, age, serotype/topotype, dose, exposure route, and host immunity. This variation makes it difficult to predict the timing of FMDV in milk.
  - The carrier state (persistent infection) of the disease is still not completely understood; therefore, any discussion around this topic pertains to current knowledge. For example, detection of carriers via testing of BTM samples is not currently supported due to a lack of knowledge about carriers and viral shedding in milk.
  - A list of high priority knowledge gaps related to the pathogenesis is listed in Table 1 of Arzt et al., 2011.

- Testing
  - We are not aware of any studies that have estimated the sensitivity of an rRT-PCR assay in BTM in infected herds. All sensitivity studies have been done on individual animals. Cattle infected with FMDV typically develop oral lesions, resulting in reduced feed consumption and decreased milk production. Small numbers of clinical cows may go unnoticed in large herds. So, not only is milk from infected cows diluted by the milk from uninfected cows in the BTM, but it is further diluted by the decreased amount of milk that is produced by infected cows.
  - Reagents for this test have changed since the initial test validation was conducted (Armson et. al., unpublished). More recent study results suggest the sensitivity of the rRT-PCR assay in BTM has improved with the currently available reagents, but the results have not yet been published (United States Department of Agriculture Updated January 2016). Field trials are being conducted by Pirbright Institute ([www.pirbright.ac.uk](http://www.pirbright.ac.uk)) using the methods that NVSL and NAHLN use for the detection of FMDV in other sample matrices to confirm the sensitivity and specificity. In this regard, a field trial using an updated version of the USDA BTM test involving in-line and bulk tank milk samples tested over time from rural and industrial dairy farms in Kenya and the Kingdom of Saudi Arabia is underway this year. ***For this paper, we assumed that the sensitivity and specificity of the rRT-PCR assay using the new reagents are the same as the old reagents. We also assumed that the sensitivity of the test in field settings will be the same as in the experimental setting.***
  - It is not clear from the literature how many Holstein cows, which represent about 90 percent of cows in the United States, were used in the validation study by Armson et.al. The Pirbright Institute is currently conducting a study to determine the diagnostic sensitivity of the rRT-PCR assay in BTM among breeds, including Holstein (Lyons and Armson 2017).

## Evaluation Approach

In this document, we evaluate whether milk is an appropriate sample matrix for each proposed purpose during and after an outbreak situation. First, we qualitatively assessed whether the test is sufficiently robust for the proposed purpose, if use of the test could augment current decision making, and whether it provides an advantage over current FMD protocols or surveillance activities. If these criteria were met, a quantitative evaluation, if necessary, was conducted. If any one of the criteria were not met, the use was categorized as not valuable for that purpose.

The evaluation used the following assumptions about pathogenesis and test characteristics:

- All cattle on an infected premises are assumed to be infected.
- When applied in a field setting, the test sensitivity for detecting virus in a BTM sample is 90 percent on the upper end and 80 percent on the lower end when it contains milk from at least one viremic cow per 100 cows milked, or at least 1 percent prevalence. Some anecdotal information suggests this is a very conservative threshold of detection based on this within-herd prevalence.

- FMDV may be detected in milk 2 to 4 days prior to the onset of clinical signs. Observation of clinical signs usually occurs hours or days after onset of infection. The additional time between onset of infection and detection of clinical signs makes testing BTM samples a potential early detection method for FMD in the lactating herd.
- This evaluation should be updated if studies provide new information about the pathogenesis of FMD, particularly as it relates to the presence of virus in milk that differs from these assumptions.

## Surveillance Design

An effective surveillance design should achieve a minimum probability of 0.95 of detecting at least one infected unit (animal or premises depending on the scale of the design) within the design period. The surveillance designs explored here focus either on the detection of infection on an individual premises by increasing sampling at the individual premises level or detection within a zone or area by increasing the number of premises sampled.

Surveillance designs adjust for imperfect test performance by increasing the number of tests required to meet a desired level of detection. The lower the sensitivity of the test, the greater the sample size required to be tested from a premises to achieve 0.95 probability of detection. The sensitivity of the test when used in a field setting depends on the timing of sampling relative to viral shedding in the milk, the amount of virus shed in milk by individual cows, the number of infected and uninfected cows contributing milk to the bulk tank, the sensitivity of the test on a known infected sample of milk, and the potential presence of other contaminants in the milk sample.

The sample size for an effective design is also determined by the design prevalence. The lower the prevalence required for detection in the surveillance design, the larger the sample size required to achieve 0.95 probability of detection. The design prevalence and the sampling time frame are defined by each specific surveillance goal and by the disease characteristics.

BTM samples are aggregated samples. Each BTM sample represents the milk from all the lactating cows that is collected in that tank. When all of the cows on a premises are milked into a single tank, there is no way to increase the sample size to compensate for imperfect test performance until the animals are milked again. The probability of detection from a single BTM sample is, at best, the sensitivity of the test. Similarly, on a premises where multiple bulk tanks are needed to hold the milk for all lactating cows, sampling all the tanks has a probability of detection equal to the sensitivity of the test if only one bulk tank contains infected milk. To ensure at least 0.95 probability of detection at the premises level given test characteristics, at least three samples must be collected over time (see Appendix A). These sampling requirements assume, if infected, the prevalence of FMD among lactating animals represented by any single tank sample is at least 1 percent. For premises with more than 2,500 cows where multiple tanks are required to represent all milking cows, testing all of the tanks provides 0.95 probability of detection, assuming the infected cows are distributed throughout the milking herd and the prevalence is at least 1 percent. To be conservative, we employ the sampling scheme of collecting BTM samples 3 consecutive days from all premises to ensure at least 0.95 probability of detection.

For surveillance within an area, two-stage sampling is required. The number of premises required to achieve 0.95 probability of detecting at least one infected premises in the area depends on the within-herd probability of detection, as well as the herd-level design prevalence. Sampling all tanks on a premises for three consecutive days to ensure the 0.95 probability of detection of at least 1 percent within-herd prevalence and sampling at the 5 percent herd-level design prevalence for area surveillance were used as example surveillance designs in the following evaluation. If the BTM test is approved for any such use, the design prevalence values could be adjusted to create the most appropriate surveillance design for an actual outbreak.

### **Potentially Valuable Uses of the rRT-PCR Assay for FMD in BTM**

A variety of potential uses for the milk testing have been proposed. For this document, we evaluated uses of this sample matrix before, during, and after a disease outbreak and categorized them as potentially valuable or not valuable based on current performance and knowledge.

#### **Potentially valuable uses of the rRT-PCR assay in BTM when FMD is not known to be present, or at first detection in the United States**

##### **1. Targeted/risk-based surveillance of dairy premises at first detection in the United States or if other North American countries were infected**

If FMD was detected in the United States, all premises in a specific region of the State, the entire State, or a region of the country could be sampled and tested. The rRT-PCR assay in BTM could be used as a screening test. Contact premises not located in the targeted surveillance area could also be sampled. Similarly, if FMD was detected in Canada or Mexico, the BTM test could be used in specific areas most likely to be at risk for infection. The surveillance design would be similar to that for a surveillance zone mentioned above.

#### **Potentially valuable uses of the rRT-PCR assay in BTM during an FMD outbreak**

##### **2. Testing of dairy premises not declared as infected within a control area**

Testing of apparently uninfected premises in the control area or testing dangerous contact premises using the rRT-PCR assay in BTM as a screening test during an outbreak could provide early detection of new premises and may also reduce testing costs. The FMD Response Plan (USDA, 2014) states that approximately 50–75 percent of the herd might be infected before morbidity is likely to appear abnormally high. BTM testing has the potential to detect infected premises 2 to 4 days prior to the onset of clinical signs. This earlier detection of infected premises should result in less disease spread.

Appendix F in the FMD Response Plan recommends individual animal sampling and testing all premises in the control area every 5 days for 28 days and sampling animals according to a sample size calculator using herd-level design prevalence values ranging from 1 to 10 percent (Appendix B, Table B.1 [Table F-2 in the FMD Response Plan]). The number of animals tested

varies from all animals on premises with less than 100 animals to more than 300 animals on premises with 2,000 or more animals.

Using BTM, all dairy premises in a control area could be sampled daily and all BTM on each premises could be tested. This approach would result in one to four tests per premises each day in most cases, although very large dairy herds (>30,000 cows) may require up to 11 tests each day (Appendix A, Table 1).

To demonstrate, we looked at four counties with moderate to high density of dairy premises: Tulare County, CA; Jerome County, ID; Yakima County, WA; and Clark County, WI (see Appendix A). Table 1 lists a conservative estimate of number of tests per premises to achieve 0.95 probability of detection when the prevalence among the milking animals is 1 percent, by sampling all tanks for 3 consecutive days.

**Table 1. Estimated average number of individual animal and BTM samples/tests required per premises to achieve 0.95 probability of detection when within-herd prevalence is at least 1 percent in the lactating cows.**

County, State	Average Herd Size	Estimated average number of individual animal tests per premises <sup>1</sup>	Estimated average number of BTM samples/tests per premises per day required to represent all milking cows <sup>2</sup>	Estimated number of BTM samples/tests per premises <sup>3</sup>
Tulare, CA	1,912	292	2	6
Jerome, ID	1,748	292	2	6
Yakima, WA	1,026	292	1	3
Clark, WI	76	76	1	3

<sup>1</sup>Sample sizes selected from Table F-2 of the FMD Response Plan (also shown in Appendix B)

<sup>2</sup>See Appendix A

<sup>3</sup>Three consecutive days of testing to achieve at least 0.95 probability of detection. See Appendix A

The FMD Response Plan recommends that surveillance (by testing individual animals) be conducted every 5 days for 28 days. We presume that zone-based surveillance would continue 28 days beyond the last detected case and that the premises might be quarantined for more than 1 month. Matching the frequency of testing in the FMD Response Plan, testing each premises every 5 days would translate to collecting BTM samples 3 days in a row and skipping 2 days. Daily herd testing using the rRT-PCR assay in BTM would be more straightforward and would improve the probability of early detection for newly infected herds in the zone.

Table 2 provides estimates for the number of tests required in a control area (10 km radius) for each of the four example counties. Estimates include the number of tests required when testing each premises every 5 days for 28 days using individual animal testing at a 1 percent design prevalence, when sampling every premises every 5 days for 28 days using BTM testing

of all tanks for three consecutive days, and when using daily BTM testing on every premises for 28 days. These estimates meet the standards outlined in the USDA Red Book, but the total amount of testing in the control area would depend on the length of the quarantine period and the timing of detection of the infected herds. These numbers are for lactating dairy cattle only and do not include testing of other cattle or susceptible species in the zone.

Based on the estimated sample numbers from Table 2, the use of BTM samples reduces the amount of testing by 93 to 98 percent as compared with testing individual animals. In addition, the BTM samples are already collected as part of the dairy testing program while individual samples would require additional resources to collect.

Regardless of whether the premises testing is conducted on individual animals or using the BTM sample, milk is a perishable product. If milk continues to move off-farm, by the time test results are available, the milk would typically already be in or through processing.

**Table 2. Estimated total number of tests required in the Control Area under three sampling schemes: sampling each premises to achieve 0.95 probability of detection at the 1 percent design within-herd prevalence level every 5 days for 28 days using 1) individual animal testing and 2) testing BTM, or 3) conducting daily testing of BTM testing on every premises.**

County, State	75 <sup>th</sup> percentile of number of premises in the Control Area <sup>1</sup>	Estimated total number of tests <sup>2</sup> when sampling premises every 5 days for 28 days		Estimated total number of tests <sup>2</sup> when testing daily for 28 days
		Individual animal testing <sup>3</sup>	BTM testing	BTM testing
Tulare, CA	20	35,400	720	1,120
Jerome, ID	6	5,256	216	336
Yakima, WA	14	24,528	252	392
Clark, WI	95	43,320	1,710	2,660

<sup>1</sup>Table A.2 provides numbers of premises from simulations of the control area (10 km radius) plus the surveillance zone (10 km beyond the control area) in these four counties. The numbers used in this table for the control area are ¼ of those in Table A-3 because the control area comprises approximately ¼ of the area of the control area plus surveillance zone.

<sup>2</sup>Test numbers reflect an upper bound because they are based on the 75<sup>th</sup> percentile of premises estimated to be in potential control areas in these counties.

<sup>3</sup>Sample sizes selected from Table F-2 of the FMD Response Plan (Table B-1 in Appendix B)

### 3. Confirmation of an infected, unvaccinated dairy premises in a control area

The rRT-PCR assay in BTM could be used as the confirmatory test for unvaccinated herds in the control area. The authors suggest confirmation be based on both a positive rRT-PCR assay in BTM and the presence of clinical signs. The current FMD Response Plan states that an unvaccinated herd on a premises in the control area can be declared as infected based on a positive rRT-PCR assay conducted at any NAHLN laboratory (FMD Response Plan Figure 5-3, USDA 2014). The plan states that NVSL would still have to confirm FMD in vaccinated herds in a control area. This approach would result in reduced costs and labor.

**4. Informing the classification of premises (e.g., at-risk or monitored premises) in a control area during an outbreak**

Consecutive negative rRT-PCR assays in BTM could be used to classify premises (e.g., at-risk or monitored premises). This classification, in combination with Secure Milk Supply planning, could guide the requirements for moving raw milk for processing, live cattle, manure, etc., off an at-risk or monitored premises.

**5. Testing of milk to be fed back to susceptible animals on premises in the control area**

Milk fed to calves may be able to be held/stored until assay results are known. Milk that tests negative via rRT-PCR could be fed to calves or other animals if pasteurized. Pasteurization is highly recommended because the minimum infective dose of FMDV is not known relative to the limit of detection of the rRT-PCR assay and pasteurization would reduce viable virus in the milk if present.

**6. Surveillance of dairy premises within the surveillance zones**

Surveillance of dairy premises outside the control zone could result in earlier identification of infected premises than detection via clinical signs alone. The rRT-PCR assay in BTM could be used as a screening test and would be less resource-intensive than individual animal testing or observational surveillance performed by regulatory officials.

Once a control area has been established, surveillance of premises in the surveillance zone and possibly other parts of the free area (perhaps by county or State) should be initiated to demonstrate that set boundaries are appropriate. Because premises in the free area wouldn't be under movement restrictions, these premises are important to monitor. If FMD spreads outside of the control area, newly infected premises need to be identified quickly. A positive rRT-PCR assay in BTM from a premises outside of the control area could lead to revised boundaries of the control area and other zones to include the test-positive premises. To conduct surveillance in the free area, a sample of premises could be tested unless the number of premises is small and all can be tested. This type of surveillance plan involves two phases in that a sample of premises is selected and then BTM samples are collected only from those premises.

Table F-2 in the FMD Response Plan (see Appendix B) recommends testing a randomly selected number of premises from the surveillance zone every 3 weeks until the quarantine is lifted. Premises could be sampled using a herd-level design prevalence greater than 1 percent, but we have chosen to make comparisons keeping the design prevalence at 1 percent because of the test characteristics of the rRT-PCR assay in BTM. Table 3 lists the estimated number of samples required to conduct surveillance in the surveillance zone using individual animal testing and testing of BTM. Although the sampling would continue every 3 weeks until the end of the quarantine, in Table 3 we provide estimates of one round of sampling in the zone.

**Table 3. Estimated total number of dairy premises to be tested in the surveillance zone to achieve 0.95 probability of detecting at least one infected premises at the 5 percent herd-level design prevalence when within-herd prevalence is 1 percent.**

County, State	75 <sup>th</sup> percentile of number of dairy premises in the Surveillance Zone <sup>1</sup>	Number of premises to sample <sup>2</sup>	Estimated total number of tests <sup>2</sup>	
			Individual animal testing	BTM testing
Tulare, CA	60	47	13,724	564
Jerome, ID	18	18	5,256	216
Yakima, WA	42	37	108,804	222
Clark, WI	285	56	4,256	336

<sup>1</sup>Table A.2 provides numbers of premises from simulations of the control area (10 km radius) plus the surveillance zone (10 km beyond the control area) in these four counties. The numbers used in this table for the surveillance zone are ¾ of those in Table A-3 because the surveillance zone comprises approximately ¾ of the area of the control area plus surveillance zone.

<sup>2</sup>Sample sizes selected from Table F-2 of the FMD Response Plan (Appendix B). Numbers reflect an upper bound because they are based on the 75th percentile of premises estimated to be in potential surveillance zone in these counties.

Premises outside of the control area require confirmatory testing conducted by NVSL, and a positive rRT-PCR assay in BTM could lead to designation as a suspect premises with the appropriate Federal-State-Tribal-industry response; containment measures will be initiated during FMD investigations (USDA 2014).

**7. Screening of infected dairy premises after the post-infection period prior to performing individual animal serology testing for quarantine release/disease freedom.**

Assuming all infected premises are not depopulated, quarantine release of infected premises will require negative individual animal serology results to meet OIE requirements for disease freedom. Testing BTM could be used to determine if individual animal serology is likely to be negative. If the rRT-PCR assay in BTM is positive, then individual animal testing could be delayed until the test is negative. This may reduce resources required to test individual animals in a herd that are likely to test positive via serology.

**Not Valuable Uses of the rRT-PCR Assay for FMD in BTM**

The following uses of the rRT-PCR assay in BTM were considered **not valuable** for use in managing FMD in the U.S. based on current knowledge and regulatory application. For the uses below, either the test was insufficiently robust for the proposed purpose, or the use of the test did not augment current decision making or provide an advantage over current FMD protocols.

## **Not valuable uses of the rRT-PCR assay in BTM when FMD is not known to be present in the United States**

### **1. Nationwide surveillance for early detection of an FMD outbreak**

Early detection using any test requires frequent sampling of a large number of premises. When testing BTM, high-volume, frequent testing is possible because of current, routine daily testing of each load of milk shipped from dairies across the United States. However, there is currently no reason for the dairy industry or regulatory agencies to incur these testing costs. If the risk of introduction to the United States were to increase, then there may be an incentive to add FMD testing to the current milk testing program. However, before including FMD testing in routine milk testing, a clear procedure for ruling out false-positives would be needed because of the large number of tests performed. Even with a specificity of 99.99 (0.0001 probability of a false-positive), there would be approximately five false-positive tests each day if 50,000 premises were tested. The necessary regulatory reaction to positive tests, regardless of being true positive or false-positive, may overwhelm FADDL's ability to keep up with the routine confirmatory testing done for all suspect-positives identified prior to an outbreak.

### **2. Maintaining disease freedom status after establishment of disease freedom**

Currently, the United States has an established FMD-free status because of historical absence of disease. Disease freedom status is maintained, at least in part, through a passive surveillance approach in which any cases with compatible clinical signs are tested at approved laboratories via an FAD investigation (USDA FADPreP Manual, 2015). This approach relies on accredited veterinarians to report suspicious lesions/illness in cattle that may be associated with a foreign animal disease. Although BTM samples are already collected for other testing, their use for active surveillance to provide additional evidence of disease freedom is not currently required by any trade partner and would therefore not provide additional benefit over current passive surveillance activities to maintain disease freedom status. The number of BTM samples to test for disease freedom would be high and the cost of testing would be very expensive unless integrated into the current milk testing program at a nominal cost.

### **3. Using in FAD investigations in a non-FMD outbreak**

A protocol currently in place specifies which samples are taken and tested during an FAD investigation, which generally involves sampling individual animals. Testing BTM samples doesn't have any advantage over testing normally collected FAD samples from individual animals and may have the disadvantage of reduced sensitivity.

## **Not valuable uses of the rRT-PCR assay in BTM when FMD is known to be present in the United States**

### **4. Permitting of daily movement of raw milk off-farm or re-routing of milk tankers in an FMD control area**

Permitting of raw milk movement off-farm is not practical given the timing of the test results and the rapid movement of milk to processing. To be a useful tool for permitting daily milk movement or to re-route milk transportation vehicles, the testing with results could be accomplished in less than 30 minutes. Without immediate test results, milk movement would remain governed according to the premises designation and additional permitting requirements.

### **5. Permitting movement of milk after pasteurization at a processing plant or products made from pasteurized milk**

Because the rRT-PCR assay detects RNA and doesn't differentiate viable versus nonviable virus, the test in BTM is not useful in terms of determining whether pasteurized milk poses a risk in terms of movement. Pasteurized milk also falls under the jurisdiction of the FDA in the U.S. Department of Health and Human Services, not USDA. FMDV is not a threat to public health.

### **6. Permitting movement of live animals from an infected premises to designated slaughter facilities**

Current policy is for no movement of animals, especially infected animals, from a known infected premises although the secure food supply plans are constructing a framework for permitted animal movements from a control area during an outbreak. These types of movements will likely be regulated by premises status and/or the zone or area in which the premises is located.

### **7. Permitting movement of contaminated materials (carcasses and waste products: manure, bedding, etc.) off infected premises for appropriate disposal**

Current policy is for no movement of materials from a known infected premises, although the secure food supply plans are constructing a framework for permitted animal movements from a control area during an outbreak. These types of movements will likely be regulated by premises status and/or the zone or area in which the premises is located.

**Table 4. Summary of potentially valuable uses of the rRT-PCR assay in BTM for testing dairy premises**

Use	Advantages
Targeted/risk-based surveillance of dairy premises at first detection in the U.S. or if other North American countries were infected	The rRT-PCR assay in BTM as a screening test could be used to evaluate the initial spread of FMD in dairy premises in a region/State. The test could be used for surveillance in areas adjacent to Canada or Mexico if an outbreak was occurring in those countries. Determine extent of outbreak or incursion of disease from neighboring country.
Testing of dairy premises not declared as infected within a control area	BTM samples are already collected as part of the dairy testing program. Testing of BTM samples has the potential to detect infected premises 2 to 4 days prior to onset of clinical signs. Detection of clinical signs could take additional days after onset. Earlier detection should result in less disease spread. Testing of BTM would result in greater than 93 percent reduction in test numbers compared with testing individual animals. This use of the rRT-PCR assay in BTM as a screening test would result in additional testing or active surveillance before the premises would be confirmed as infected. Negative test results show the control zone is appropriately sized and that biosecurity practices are limiting spread of disease.
Confirmation of an infected, unvaccinated dairy premises in a control area	The resources needed to test and confirm individual animals as infected would be reduced if the rRT-PCR assay in BTM was used as an approved, confirmatory test with the presence of clinical signs.
Informing the classification of premises in a control area during an FMD outbreak	Consecutive negative rRT-PCR assays in BTM could be used in determining premises classification (e.g., at-risk or monitored premises). This classification, in combination with Secure Milk Supply planning, could guide the requirements for moving raw milk for processing, live cattle, manure, etc., off an at-risk or monitored premises.
Testing of milk to be fed back to susceptible animals on premises in the control area	Milk fed to calves may be able to be held/stored until rRT-PCR assay in BTM results are known. Milk samples that test negative could be fed to calves or other animals if pasteurized. Pasteurization is highly recommended because the minimum infective dose of FMDV is not known relative to the limit of detection of the rRT-PCR assay in BTM and pasteurization would reduce viable virus in the milk if present.
Surveillance of dairy premises within surveillance zone	Surveillance of dairy premises outside the control area could result in earlier identification of infected premises than detection via clinical signs alone. The rRT-PCR assay in BTM as a screening test would be less resource-intensive than individual animal testing. Negative test results show the zones are appropriately sized and that biosecurity practices are limiting spread of disease.
Screening of infected dairy premises after the post-infection period prior to performing individual animal serology testing for quarantine release/disease freedom.	The resources required to test individual animals in a herd that are likely to test positive via serology would be reduced as only individual animals in BTM rRT-PCR-negative premises would be tested.

**Table 5. Summary of not valuable uses of the rRT-PCR assay in BTM for testing dairy premises or products**

Use	Limitations
Nationwide surveillance for early detection of an FMD outbreak	Early detection using any test requires frequent sampling of a large number of premises. The number of BTM samples to test would be very high and the cost of testing would be very expensive unless integrated into the current milk testing program at a nominal cost. There is currently no reason for the dairy industry or regulatory agencies to incur these testing costs.
Maintaining disease freedom status after establishment of disease freedom	The use of any BTM rRT-PCR testing to provide additional evidence of disease freedom is not currently required by any trade partner and would not provide additional benefit over the current passive surveillance activities. The number of BTM samples to test would be high and the cost of testing would be very expensive unless integrated into the current milk testing program at a nominal cost.
Using in FAD investigations in a non-FMD outbreak situation	Current protocol specifies that individually infected animals be tested. Testing BTM samples doesn't have an advantage over testing individual animals and may have the disadvantage of reduced sensitivity.
Permitting of daily movement of raw milk off-farm or rerouting of milk tankers in an FMD control area	Using the current milk sampling scheme, test results would not be available before milk is moved to processing. Without immediate test results, milk movement would remain governed according to the premises designation and additional permitting requirements.
Permitting movement of milk after pasteurization at a processing plant or products made from pasteurized milk during an FMD outbreak	Because the rRT-PCR assay detects RNA and doesn't differentiate viable versus nonviable virus, the test in BTM is not useful in terms of determining whether pasteurized milk poses a risk in terms of movement.
Permitting movement of live animals from an infected premises to designated slaughter facilities during an FMD outbreak	Current policy is for no movement of animals from a known infected premises although the secure food supply plans are constructing a framework for these types of movements. Off-farm movements will likely be regulated by premises designation and/or the zone or area where the premises is located.
Permitting movement of contaminated materials (carcasses and waste products; manure, bedding, etc.) off infected premises for appropriate disposal during an FMD outbreak	Current policy is for no movement of materials from a known infected premises although the secure food supply plans are constructing a framework for these types of movements. Off-farm movements will likely be regulated by premises designation and/or the zone or area where the premises is located.

## Appendix A. Sampling Requirements for Control and Surveillance Area Scenarios

### 1. Number of samples needed for herds of different sizes and production levels

The number of samples required to test all eligible lactating cows in a herd depends on the size of the herd, the number of milking shifts per day and the daily milk production per cow (Table A.1). The following equation will specify the number of samples and therefore tests needed to represent all lactating cows.

- Number of cows \* daily milk production per cow = total milk per day
- Total milk per day / number of milking shifts = milk produced per milking shift
- Milk produced per milking shift / 68,000 lbs (tanker capacity) = number of samples/tests required (round up to integer)

**Table A.1. Examples showing the number of tankers representing all lactating animals on a premises for varying premises sizes and daily milk production.**

Number of cows	Milk per cow per day	Total milk per day	Number of milking shifts	Milk produced per shift	Tanker capacity (68,000 lbs)	Number of tankers and tests required to represent all lactating animals
100	50	5,000	2	2,500	2,500/68,000	1*
1,000	60	60,000	3	20,000	20,000/68,000	1*
2,500	90	225,000	3	75,000	75,000/68,000	2
5,000	100	500,000	2	250,000	250,000/68,000	4
10,000	90	900,000	2	450,000	450,000/68,000	7
30,000	75	2,250,000	3	750,000	750,000/68,000	11

\*Could test an entire day's production with a single sample/test

Using the results of sensitivity testing, which estimated that the rRT-PCR assay could reasonably detect FMDV from a BTM sample in which a single infected dairy cow is shedding virus out of a herd size of 100-1,000, the detection limit in a tanker is 1 to 10 infected cows assuming 1,000 cows per tanker. For this evaluation, we chose the more conservative level of 10 infected cows per 1,000, or 1 percent prevalence.

Sampling serves two purposes in surveillance design: more sampling increases the probability of detecting an infected animal among those tested, and increased sampling compensates for imperfect test performance by increasing the probability of detection from multiple tests. Pooling samples greatly reduces the sampling requirements for increasing the probability of detecting an infected animal. However, testing only one or two samples does not compensate for imperfect test performance.

The BTM test is reported to have a sensitivity of 86.4 percent (margin of error, 5 percent) and a specificity of 100 percent (margin of error, 1 percent). With nearly perfect specificity, false positives will be rare. We used 80 percent for a lower bound and 90 percent for an upper bound on

sensitivity in this evaluation. A single BTM sample from a group of cows with prevalence of at least 1 percent has an 80 to 90 percent probability of a positive test result. The only way to compensate for the limits of the test performance is to conduct more tests. Samples from the same tank would be nearly perfectly correlated and would not improve the detection probability. One approach would be to collect milk from independent subsets of the milking population, but logistically this could be very difficult and does not take advantage of the convenience of the BTM samples currently collected.

The best option to make use of BTM samples while increasing the probability of detection would be to test BTM samples on consecutive days. Three BTM samples would be sufficient to increase the probability of detection to above 0.80 in most cases. Only in cases where the virus spread was slow and test results on consecutive days were highly correlated would a fourth test be necessary.

Therefore, to achieve 0.95 probability of detection, three consecutive days of sampling are required. This translates to 3 tests for premises where 1 tanker represents all lactating animals each day, but 12 tests for premises where 4 tankers represents all lactating animals each day.

The need to sample consecutive days impacts the “early detection” capability of the test. Quantifying the effect would require simulation studies because the correlation between samples collected from the same cows 1 or 2 days apart is not known, particularly when disease is spreading. However, less than 0.95 probability of detection does not translate to zero probability of detection, making testing of BTM samples more likely to detect infected premises compared with the observation of clinical signs.

## **2. Control and Surveillance Area Scenarios**

To estimate the number of tests of BTM needed from premises in the control area and surveillance area, we chose four counties: Tulare County, CA; Jerome County, ID; Yakima County, WA; and Clark County, WI, due to their moderate to high density of dairy premises and dairy cows (Table A.2). To simulate the number of premises in the zone/area, we applied the Farm Locator and Animal Population Simulator (FLAPS) (Burdett et al., 2015). The simulator uses county-level information collected by USDA’s National Agricultural Statistics Service Census of Agriculture to distribute and assign geographic locations of dairy premises and dairy cattle in each county. For each of the four counties above, simulations were conducted where an individual dairy premises was selected and then the simulated number of premises within a 20 km radius were counted. This process was repeated until the number of sample iterations were 50 percent of the number of dairy premises in each county. Simulations at the county perimeter only included premises within the county and not adjacent counties.

The average herd size in each county suggests that most premises and all lactating cows on the premises would be able to be tested with a single BTM sample. The mean number of premises within the 20 km radius ranged from 18 in Jerome County, ID, to 301 premises in Clark County, WI. Because these premises would be located in a control area or surveillance zone, a portion of these premises would be infected premises and not necessitate daily or less frequent bulk milk testing.

The number of premises in the zone/area ranged from 24.2 to 43.9 percent of premises in the county.

**Table A.2. County-level demographic information and simulation results**

County	State	Number of Dairy Premises	Number of Dairy Cows	Average Herd Size	Simulated number of premises in a 20 km radius (Control Area and Surveillance Zone)					Percent of Premises in the county
					25 <sup>th</sup> percentile	Mean	Median	Mode	75 <sup>th</sup> percentile	
Tulare	CA	256	489,436	1,912	50	62	63	50	79	24.2
Jerome	ID	41	71,680	1,748	16	18	19	15	21	43.9
Yakima	WA	97	99,532	1,026	24	40	41	53	53	41.2
Clark	WI	948	71,641	76	240	301	304	280	377	31.8

To apply the results of the quantitative analyses to other counties, compare the demographics (number of dairy premises and the average herd size) for the county of interest to this information for the example counties. Herd size impacts the number of tankers required to represent all lactating animals each day (Table A.1) and therefore number of BTM samples collected and tested for each premises each day. The number of dairy premises and the simulated number of premises in the 20 km radius zone are used to scale the number of samples per premises to the area or zone level. The sampling estimates for these example counties can be used to provide upper and/or lower bounds for the estimated number of rRT-PCR assay in BTM tests that might be required in other locations by comparing demographics.

## Appendix B. Surveillance Sampling for FMD from the 2014 USDA-APHIS Response Plan

Table B.1. Minimum sample sizes with various design prevalence levels needed to detect FMD in apparently healthy herds/animals.<sup>2</sup>

Herd Size or Number of Premises	Minimum Number of Individual Animal Samples or Individual Premises			
	Prevalence			
	1%	2%	5%	10%
<=50	ALL	ALL	37	23
51-100	ALL	82	47	27
101-200	164	111	54	28
201-300	199	124	56	29
301-400	222	131	58	29
401-500	237	136	59	30
501-600	248	139	62	30
601-700	256	141	62	30
701-800	262	143	62	30
801-900	268	144	62	30
901-1,000	272	146	62	30
1,001-2,000	292	157	62	30
>2,000	314	157	62	30

Note: These sample sizes are based on an rRT-PCR sensitivity of 95% for detecting FMDV in appropriately collected samples from infected cattle. The sizes provide 95% confidence that the premises or area has an FMD prevalence less than the design prevalence, given that the virus is there and all animals test negative. Prevalence in this table indicates:

1. If determining the number of animals in a herd, then the within-herd prevalence is the level chosen.
2. If determining the number of herds in a zone to test, then the herd-level prevalence is the level chosen.

<sup>2</sup> Based on table F2 in USDA-APHIS FAD Preparedness and Response Plan for FMD, September 2014.

## References

Armson, B., C. Doel, S. Parida, V.L. Fowler, M. Madi, V. Mioulet, D.P. King, M. Bounpheng, A. Clavijo, K. Toohey-Kurth, F. Cigel, Md A. Hossain, K. Lemire, A. Das, D. Holder, and M.T. McIntosh. 2016. Diagnostic validation of a high-throughput real-time RT-PCR for the rapid detection of foot-and-mouth disease virus in bulk tank milk. Manuscript in preparation.

Arzt, J., N. Juleff, Z. Zhang, and LL. Rodriguez. 2011. The pathogenesis of foot-and-mouth disease I: viral pathways in cattle. *Transboundary and Emerging Diseases*, 58: 291–304. doi:10.1111/j.1865-1682.2011.01204.x

Bates, T. W., M. C. Thurmond, S. K. Hietala, K. S. Venkateswaran, T. M. Wilson, B. W. Colston, Jr., J. E. Trebes and F. P. Milanovich. 2003. Surveillance for detection of foot-and-mouth disease. *J Am Vet Med Assoc* 223(5): 609-614.

Burdett, C.L., B.R. Kraus, S.J. Garza, R.S. Miller, K.E. Bjork. 2015. Simulating the distribution of individual livestock farms and their populations in the United States: An example using domestic swine (*Sus scrofa domesticus*) farms. *PLoS ONE* 10(11): e0140338. doi:10.1371/journal.pone.0140338

Callahan, J.D., F. Brown, F.A. Osorio, J.H. Sur, E. Kramer, G.W. Long, J. Lubroth, S.J. Ellis, K.S. Shoulars, K.L. Gaffney, D.L. Rock and W.M. Nelson. 2002. Use of a portable real-time reverse transcriptase-polymerase chain reaction assay for rapid detection of foot-and-mouth disease virus. *J Am Vet Med Assoc* 220(11): 1636-1642.

Lyons, N. and B. Armson. 2017. Use of milk for FMD surveillance in endemic settings. Unpublished work, The Pirbright Institute.

Reid, S.M., S. Parida, D.P. King, G.H. Hutchings, A.E. Shaw, N.P. Ferris, Z. Zhang, J.E. Hillerton, D.J. Paton. 2014. Use of automated RT-PCR to detect FMDV in milk. Accessed March 2017 at <http://www.fao.org/ag/againfo/commissions/docs/greece04/app43.pdf>

Thurmond, M.C. and A.M. Perez. 2006. Modeled detection time for surveillance for foot-and-mouth disease virus in bulk tank milk. *American Journal of Veterinary Research* Vol. 67, No. 12, Pages 2017-2024 doi: 10.2460/ajvr.67.12.2017

USDA APHIS Foot-and-Mouth Disease (FMD) Response Plan: The Red Book. September 2014. Accessed March 2017 at [https://www.aphis.usda.gov/animal\\_health/emergency\\_management/downloads/fmd\\_responseplan.pdf](https://www.aphis.usda.gov/animal_health/emergency_management/downloads/fmd_responseplan.pdf)

USDA APHIS Foreign Animal Disease Preparedness and Response Plan. October 2015. Accessed March 2017 at

[https://www.aphis.usda.gov/animal\\_health/emergency\\_management/downloads/documents\\_manuals/fadprep\\_manual\\_2.pdf](https://www.aphis.usda.gov/animal_health/emergency_management/downloads/documents_manuals/fadprep_manual_2.pdf)

USDA-APHIS-VS. 2014 Optimization and validation of a real-time RT-PCR assay for rapid detection of foot-and-mouth disease virus in bulk tank milk. Unpublished internal analysis.

USDA Assay Performance Characteristic Summary Sheet. Updated January 2016. Internal document.