

Real-Time Reverse Transcription PCR Detection of Foot-and-Mouth-Disease Virus Using the R.A.P.I.D.[®] System

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Summary

This study evaluates the ability of two real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) assays to independently detect the presence of Foot-and-Mouth-Disease Virus (FMDV) RNA. Assays to amplify the internal ribosomal entry site (IRES) and RNA polymerase regions of the FMDV genome recognize all seven existing serotypes. Both assays can effectively evaluate the presence of FMDV in various bovine samples, including blood, serum, saliva, nasal swabs, and epithelial tissue.

Abstract

Foot-and-Mouth-Disease (FMD) is a severe, highly communicable disease of cattle, swine and other cloven-hoofed animals. The grave economic impact of a FMD outbreak in the United States could be in the billions of dollars in just the first year (USDA/APHIS Animal Disease Factsheet). In the event of an outbreak, be it an accidental introduction or an act of bio-terrorism, it would be critical to have a fast and reliable method to quickly identify infected animals. Real-time RT-PCR using the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) System is an ideal platform for fast, sensitive, and specific identification of FMDV. Primers and hybridization probes specific to the RNA polymerase gene and IRES were designed and optimized using synthetic DNA targets. The sensitive nature of FMDV dictates that in the U.S. all research has to take place at the USDA Plum Island Animal Disease Center on Plum Island, New York. In collaboration with the USDA, the RNA polymerase and IRES assays were tested on RNA isolates collected worldwide. Both assays recognized all seven FMDV serotypes and have limits of detection of approximately 150 (RNApol) and 1.5 (IRES) pfu/PCR reaction. Both assays positively identified FMDV RNA prepared from several bovine matrices at various days post infection. Availability of assays to detect FMDV allows rapid identification of infected animals so that appropriate actions can be taken to contain the spread of the disease.

Background

FMD is a highly contagious disease among cattle, swine, sheep, and other cloven-hoofed animals. FMDV is a small, positive-sense strand RNA virus belonging to the *Aphovirus* genus in the family *Picornaviridae*. The pathogenesis of the virus is similar in cattle and swine, resulting in vesicular lesions in the mouth and foot epithelial tissues, while other susceptible animals such as sheep often do not show obvious clinical signs. FMDV is initially inhaled into the pharynx, passes through the lymph nodes, and enters the blood stream. Initial viremia leads to infection of epithelial cells, resulting in amplification of the virus within the epithelial tissue. It takes approximately 72-120 hours for an exposed animal to become symptomatic, however, it can be as short as 24 hours. This cycle continues until vesicular lesions form or the disease is controlled by the host's immune response (1).

Presence of FMDV is typically detected by inoculating cell monolayers such as primary bovine thyroid (BTY) and primary lamb kidney cells with tissue suspensions and observing for cytopathic effects (CPE) (3). The specificity of the CPE is confirmed by detecting viral antigens using ELISA (2), complement fixation antigen test, or end-point polymerase chain reaction (PCR). This series of methods to identify FMDV takes six hours to several days depending on the final viral load in the sample being tested. A rapid alternative to cell culture inoculation and ELISA analysis is real-time RT-PCR.

In order to develop specific and sensitive RT-PCR assays to detect FMDV, a region conserved among all known FMDV serotypes must be evaluated. Regions of the IRES and RNA-dependent RNA polymerase are highly conserved among all seven known serotypes of FMDV, while having little to no identity with any viruses or other organisms. Two real-time RT-PCR assays targeting the IRES and RNA polymerase regions were developed and optimized on the R.A.P.I.D. System. This system incorporates the use of fluorescently labeled hybridization probes to detect amplification of a cDNA target in real time using fluorescence resonance energy transfer (FRET) (4).

Results

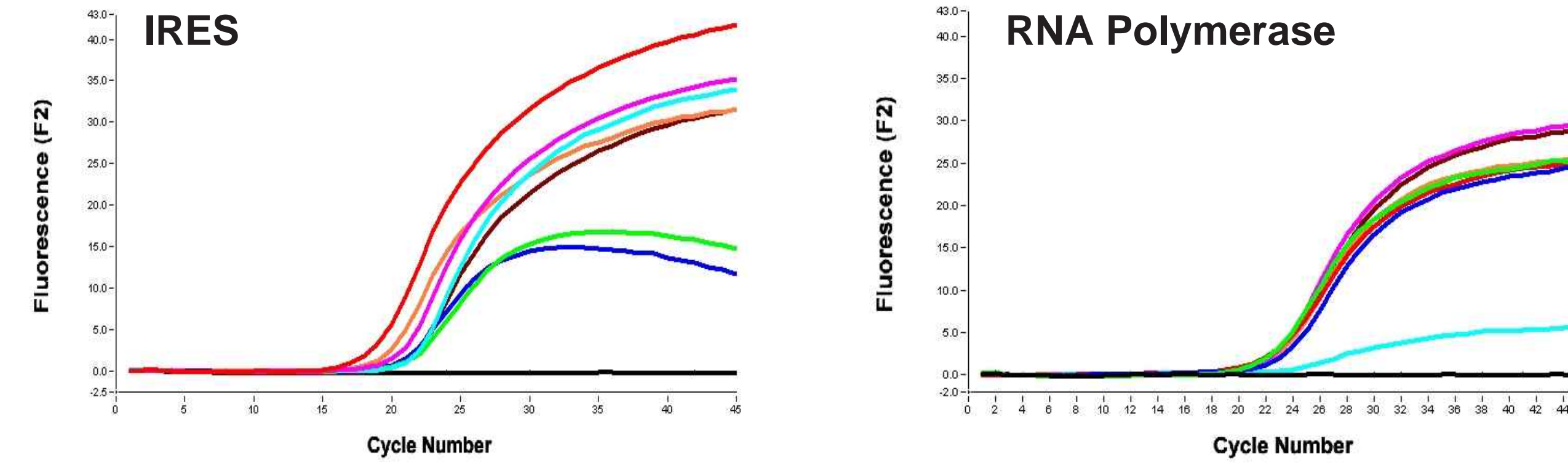


Figure 1. All seven known FMDV serotypes are recognized by both the IRES and RNA Polymerase assays.

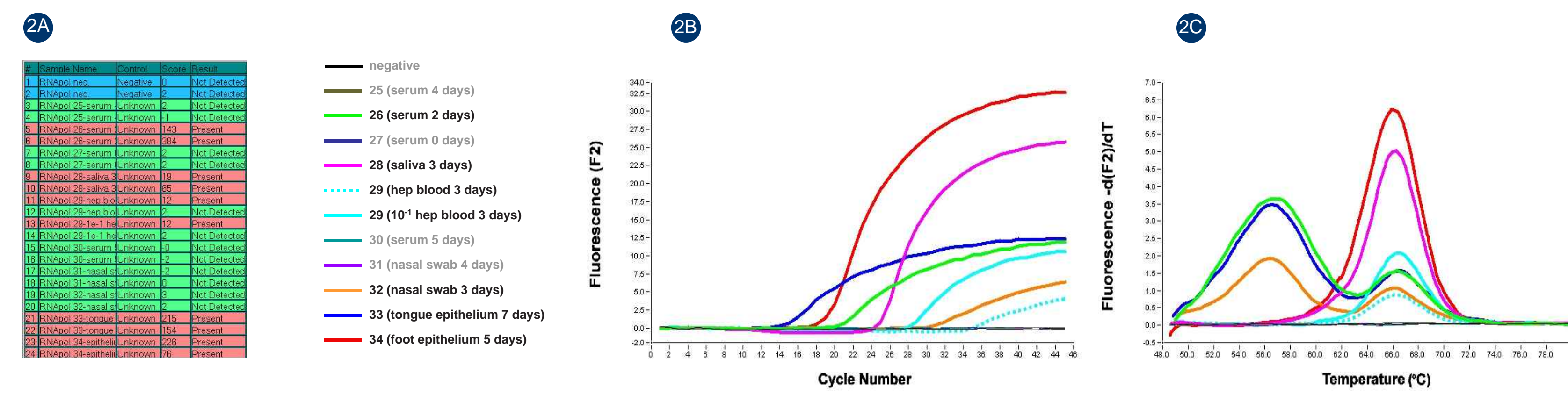


Figure 2. IRES Assay. 2A) R.A.P.I.D. Detector identification of the presence of FMDV in bovine samples. 2B) R.A.P.I.D. LCDA quantitative analysis of amplification of FMDV from bovine samples. 2C) Melt curve analysis of bovine samples positive for the presence of FMDV.

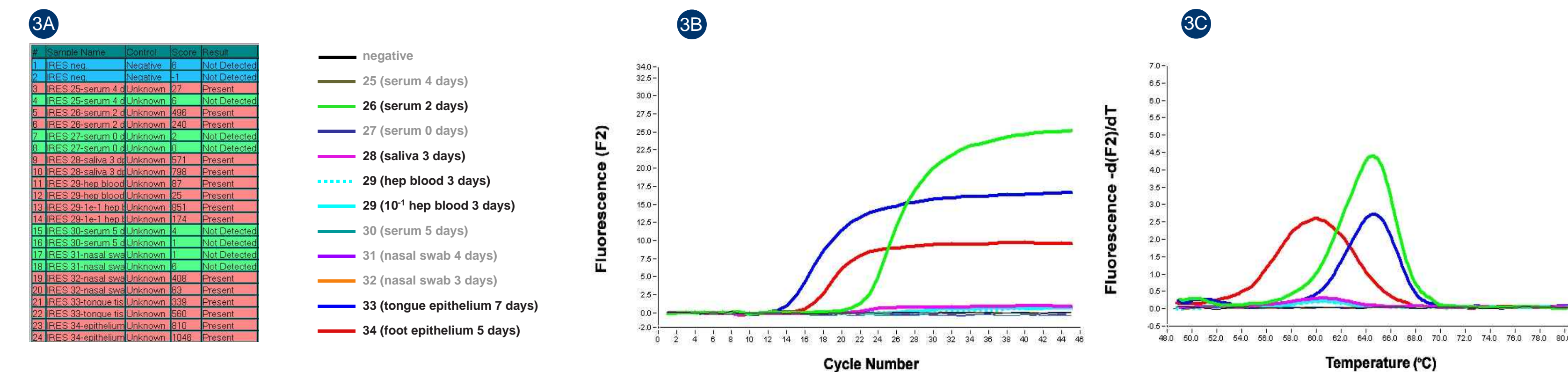


Figure 3. RNA Polymerase Assay. 3A) R.A.P.I.D. Detector identification of the presence of FMDV in bovine samples. 3B) R.A.P.I.D. LCDA quantitative analysis of amplification of FMDV from bovine samples. 3C) Melt curve analysis of bovine samples positive for the presence of FMDV.

Materials and Methods

Polymerase Chain Reaction (PCR). Primers were optimized to specifically amplify DNA from the RNA polymerase and IRES regions of the FMDV genome using synthetic DNA targets. Fluorescent probes were designed to hybridize with the amplification products, allowing real-time detection. Real-time PCR analysis was performed using the R.A.P.I.D. System. PCR reactions were performed in LightCycler[®] capillary tubes in 20 μ L volumes containing the synthetic DNA template, specific primers and hybridization probes, stabilization buffer, MgCl₂ buffer, dNTPs, KlenTaq1[™] DNA polymerase, and TaqStart[™] antibody. The primers and probes that yielded the greatest sensitivity with the synthetic DNA target were selected for testing of the actual FMDV genomic RNA by RT-PCR (see below).

RNA Isolation from Cell Culture. A volume of 250 μ L of cell culture medium was collected from monolayers of primary BTY cells. Three volumes of Triazol Reagent were added, mixed by vortexing, and incubated at room temperature for five minutes. The dissolved RNA was separated from cellular debris by extraction with chloroform:isoamyl alcohol (24:1) followed by isopropanol precipitation and a 70% ethanol wash. The resulting RNA pellet was briefly dried under vacuum and subsequently dissolved in 50 μ L of RNA-grade water and stored at -70°C.

RNA Isolation from Tissue. RNA was isolated from several bovine tissues (Figures 2 & 3). To approximately 50 mg of tissue, 500 μ L of Triazol Reagent was added. The tissue was mechanically homogenized, followed by the addition of another 500 μ L of Triazol Reagent, and continued gentle homogenization. The dissolved RNA was separated from the debris using chloroform extraction. Glycogen (1 μ L of 20 mg/mL) was added to the aqueous phase before isopropanol precipitation. The pellet was washed with 70% ethanol, dried briefly under vacuum, and subsequently dissolved in 50 μ L of RNA-grade water and stored at -70°C.

Real-Time RT-PCR. Real-time RT-PCR analysis was performed using the R.A.P.I.D. System. RT-PCR reactions were performed in LightCycler capillary tubes in 20 μ L volumes containing the RNA template, specific primers and hybridization probes, stabilization buffer, MgCl₂ buffer, dNTPs, DTT, RNaseOUT[™], M-MuLV-RT (USB), KlenTaq1 DNA polymerase, and TaqStart antibody. Assays were also performed using freeze-dried IT BioReagents[™]. Each vial was enough for two 20 μ L RT-PCR reactions and contained the same ingredients listed above for RT-PCR. For one vial, 2 μ L of extracted RNA sample was added to 38 μ L of PCR-grade water plus hydration buffer. Reagent rehydrated with the sample was transferred to two LightCycler capillary tubes and pulse-centrifuged. The capillary tubes were loaded into the R.A.P.I.D. instrument for amplification and detection.

Dilution	IRES C _t	RNApol C _t	Avg. pfu/rxn
1.00E+00	18.71	19.90	15000
1.00E-01	22.29	22.48	1500
1.00E-02	29.48	24.98	150
1.00E-03	30.98	-	15
1.00E-04	31.54	-	1.5

Table 1. Limits-of-detection of both the IRES and RNA polymerase assays using serotype O1 Manissa RNA prepared from cell culture.

Discussion

This study evaluates the ability of two RT-PCR assays to independently detect the presence of FMDV RNA in real-time. Assays to amplify the IRES and RNA polymerase regions of the FMDV genome recognize all seven known serotypes of FMDV (Figure 1) using either wet or freeze-dried chemistry. Both assays can also effectively evaluate the presence of FMDV in various bovine samples including blood, serum, saliva, nasal swabs, tongue epithelium, and foot epithelium (Figures 2 & 3). The presence of FMDV in various bovine tissue samples collected at different times correlates with the model for pathogenesis. FMDV is detected in serum and blood samples early on in infection, while it is not detected in epithelial tissues until a later time post-infection.

Both assays detect RNA from saliva (#28) and heparinized blood (#29), but the RNA polymerase assay generated a low signal. This signal was adequate for the R.A.P.I.D. LightCycler Data Analysis and Detector software programs to verify the presence of FMDV RNA in these samples and was confirmed by melt analysis (Figure 3C). RNA prepared from a nasal swab sample (#32) was only detected by the IRES assay and it is not clear why the RNA polymerase assay repeatedly did not detect FMDV in this sample. Detection of FMDV RNA in samples 28, 29, and 32 emphasizes the value of two independent assays to provide confirmation through redundancy.

When the IRES assay was used to detect FMDV RNA from serum (#26), nasal swab (#32), and tongue epithelium (#33), the overall fluorescence was lower than the other positives and the melt analysis revealed two peaks instead of the expected single peak (Figure 2). Typically, a double melt peak is indicative of single or multiple nucleotide polymorphisms in a portion of the template to which the probes hybridize. This results in the probes melting from the region of divergent sequence at a lower temperature than the fully matched sequence, thus resulting in two melt peaks. Samples with two melt peaks often have decreased overall fluorescence in the quantification analysis because the probes are interacting with two populations and thus the signal is reduced.

It is interesting to note that a ten-fold dilution of the heparinized blood sample (#29) resulted in a cycle threshold significantly earlier than undiluted. Heparin is an anticoagulant and a known PCR inhibitor. Even though the RNA in this sample was separated from the rest of the sample, some of the heparin remained with the RNA and a ten-fold dilution reduced the concentration of heparin present, thus improving the efficiency of PCR.

Conclusions

- Both the IRES and the RNA polymerase assays recognize all seven serotypes of FMDV RNA using real-time RT-PCR.
- Both assays positively identify bovine samples that contain detectable amounts of FMDV.
- Freeze-dried IRES and RNA polymerase IT BioReagents used with the R.A.P.I.D. System are a fast and reliable means to identify FMDV RNA prepared from various cell culture and tissue samples.

References

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