

Use of the HRM Analysis in Pathogen Detection

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Introduction

The high resolution melt (HRM) analysis can be used as a cost-effective post-PCR method enabling the specific discrimination among tested samples. For this purpose, special fluorescent incorporating dyes are used. When the temperature rises within the melting phase, the double-strands of the amplicon separate gradually. This is connected with the drop of the emitted fluorescence which is, in turn, plotted as a peak representing the specific melting temperature of the amplicon. Compared to the conventional melting analysis, the differentiation among samples is enabled thanks to the specific shape of the melting curve of the particular amplicons even so they have the same melting temperature.

Results

The usefulness of the HRM assay is demonstrated on the examples of the tick-borne encephalitis virus (TBEV) and the bacteria *Brucella abortus* and *B. melitensis*.

TBEV is a flavivirus transmitted to humans through ticks and is a cause of a serious illness affecting the central nervous system. TBEV comprises the three subtypes – European (Eu), Far Eastern (FE) and Siberian (Sib). Conserved regions for particular subtypes were found in the envelope protein used as a target. The HRM approach was chosen to enable the detection and distinguishing of all the subtypes in the same reaction.

Brucellosis represents one of the main zoonoses worldwide. A part of *Brucella* differentiation assay is presented as an efficient tool for the distinguishing the closely related agents according to the only particular region of variability in the nucleotide sequence. The following graphs are focused on the differentiation between *B. abortus* and *B. melitensis* as the causes of illness in humans and the possible misuse as biological weapons, as well.

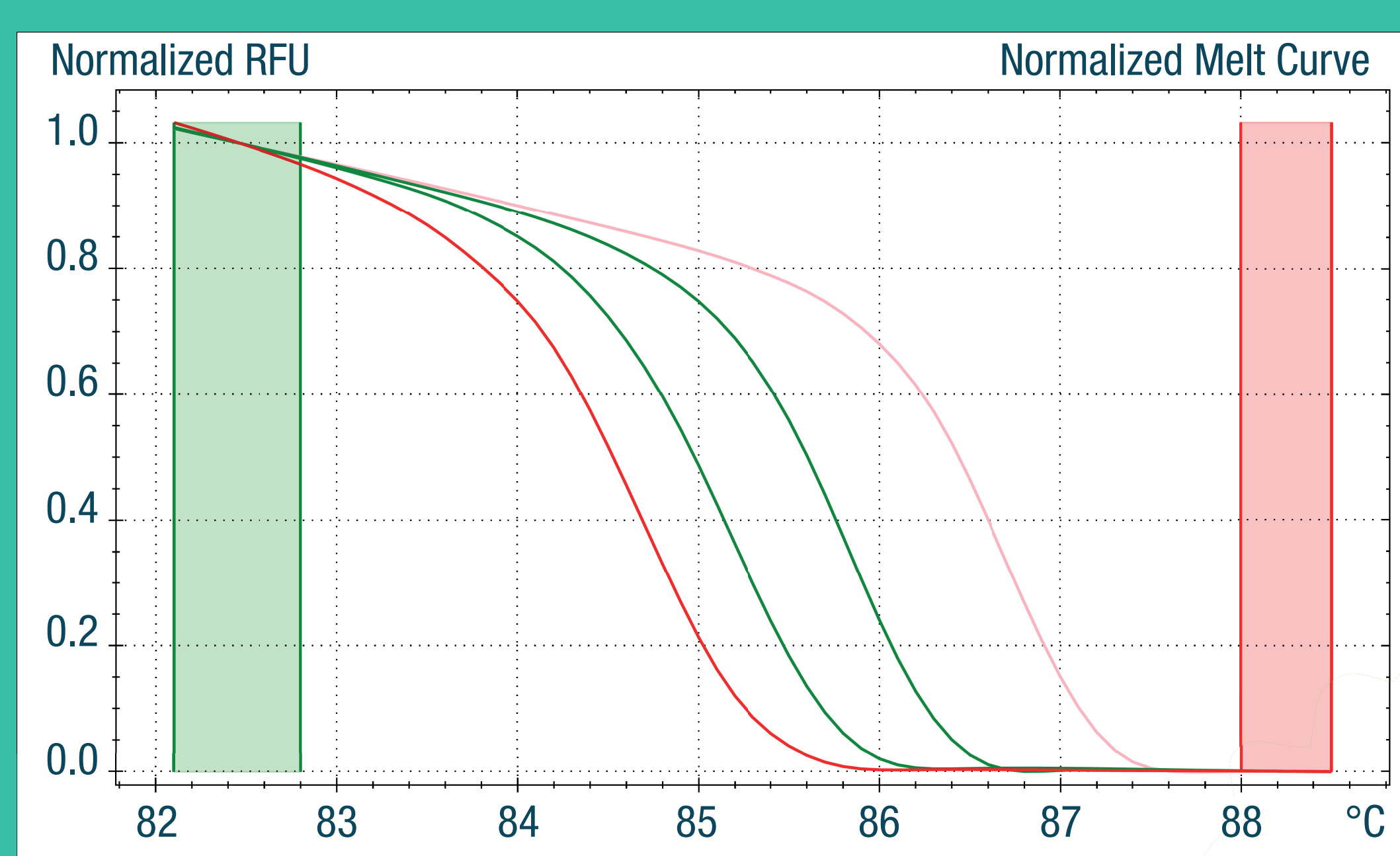


Figure 1: The Normalized Melt Curve Graph of TBEV

In the first stage of the analysis, the melt curves have to be normalized with each other. The normalisation regions in green and red have to be adjusted properly.

Conclusion

Here, two points of view are showed to present the advantages of the HRM assay: firstly, a discrimination model with a portion of the variability in the amplified sequence and the same length of the amplicon; secondly, the differentiation according to the one specific site of variability in the amplicon – a SNP or an insertion/deletion. Moreover, there is no demand for further manipulation with the samples after the PCR and so the cross-contamination is prevented.

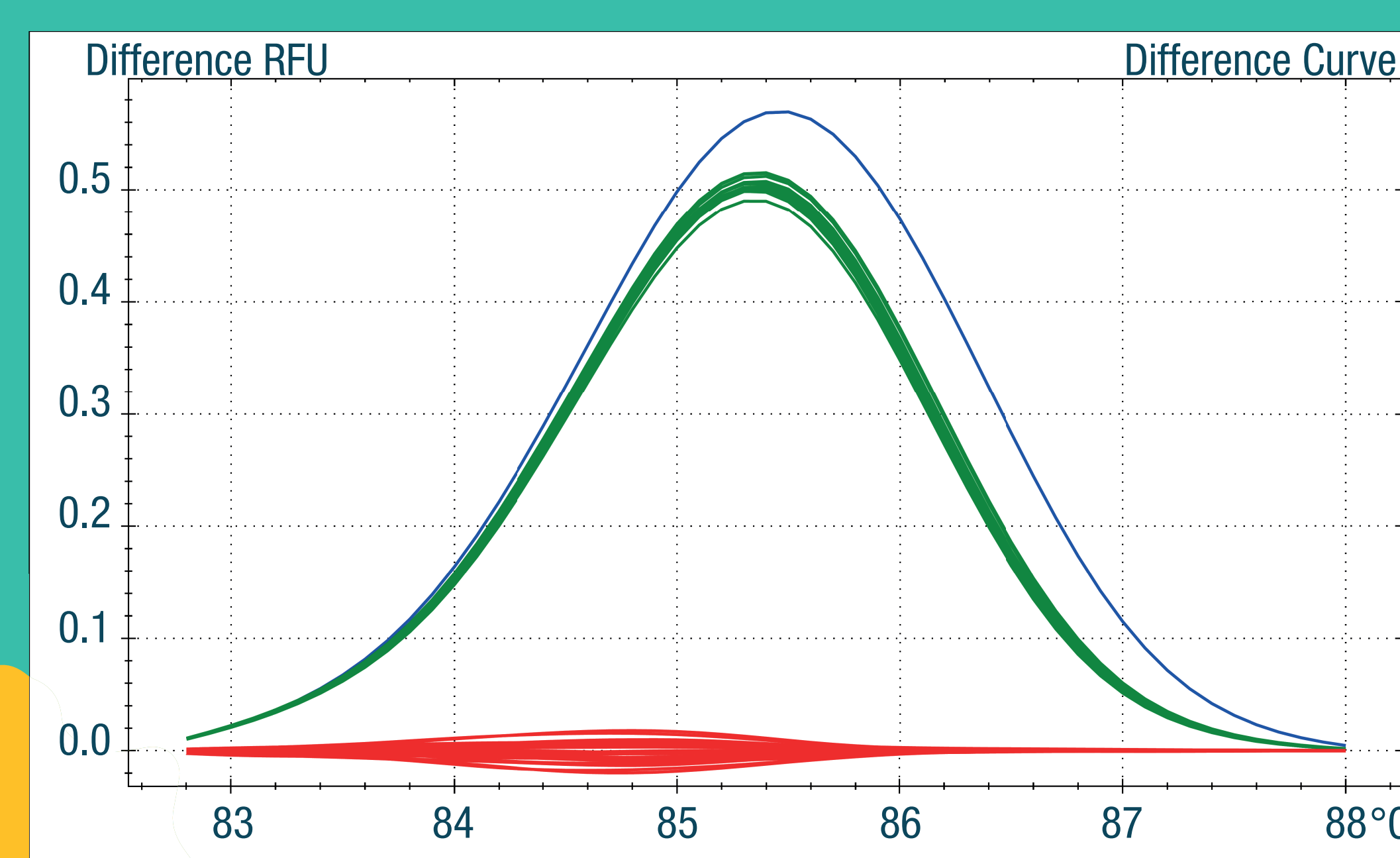


Figure 3: The HRM Differentiation of the *B. abortus* + *B. melitensis* Group from Other *Brucella* sp.

According to the 7 bp deletion, the target *Brucellae* can be discriminated. *B. abortus* and *B. melitensis* in red; other *Brucella* sp. in green and blue.

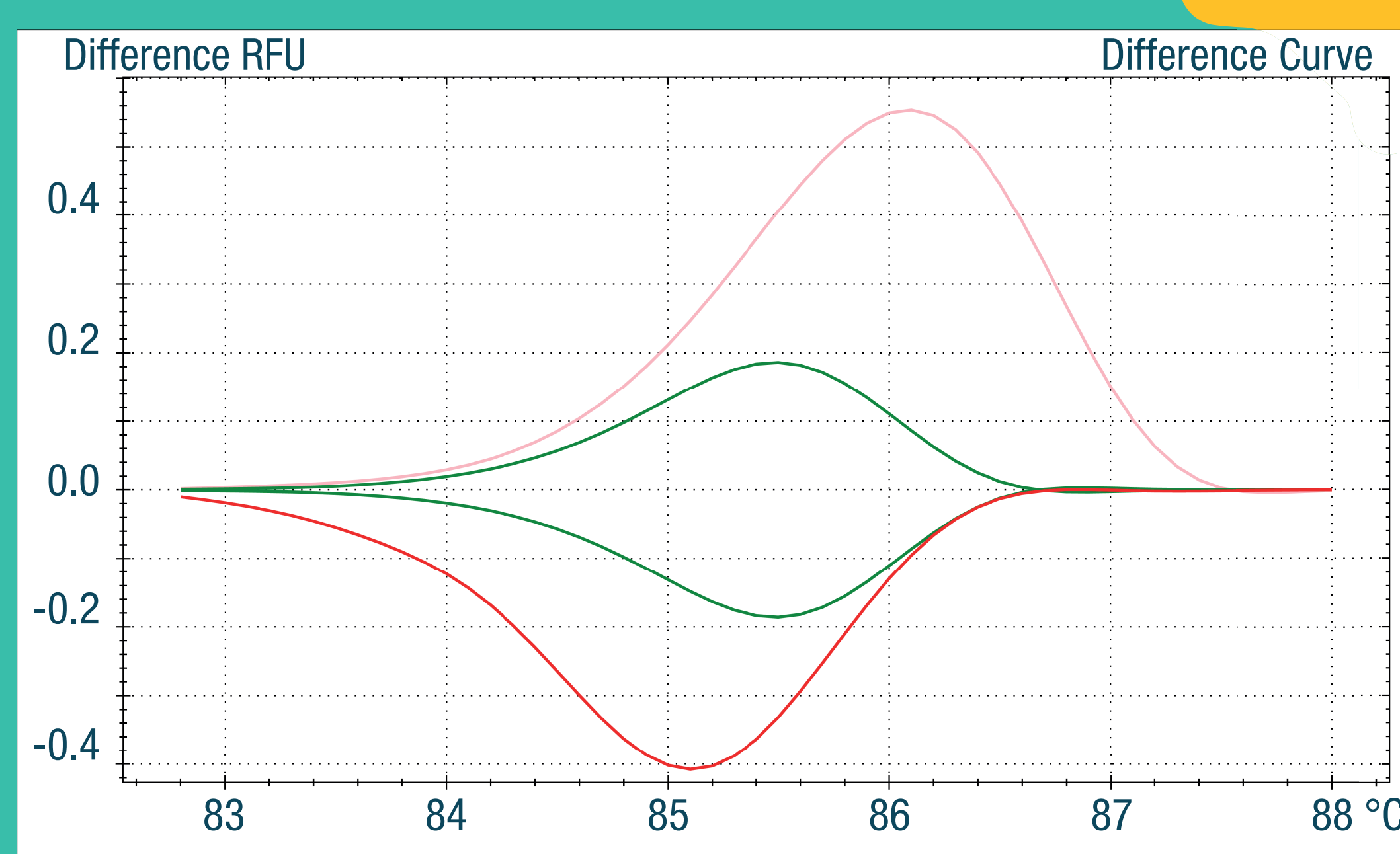


Figure 2: The HRM Differentiation among the Particular Subtypes of TBEV

The area between the two normalisation regions is plotted in the difference graph enabling the discrimination of the samples in detail. Eu subtype in red, FE in green and Sib in pink.

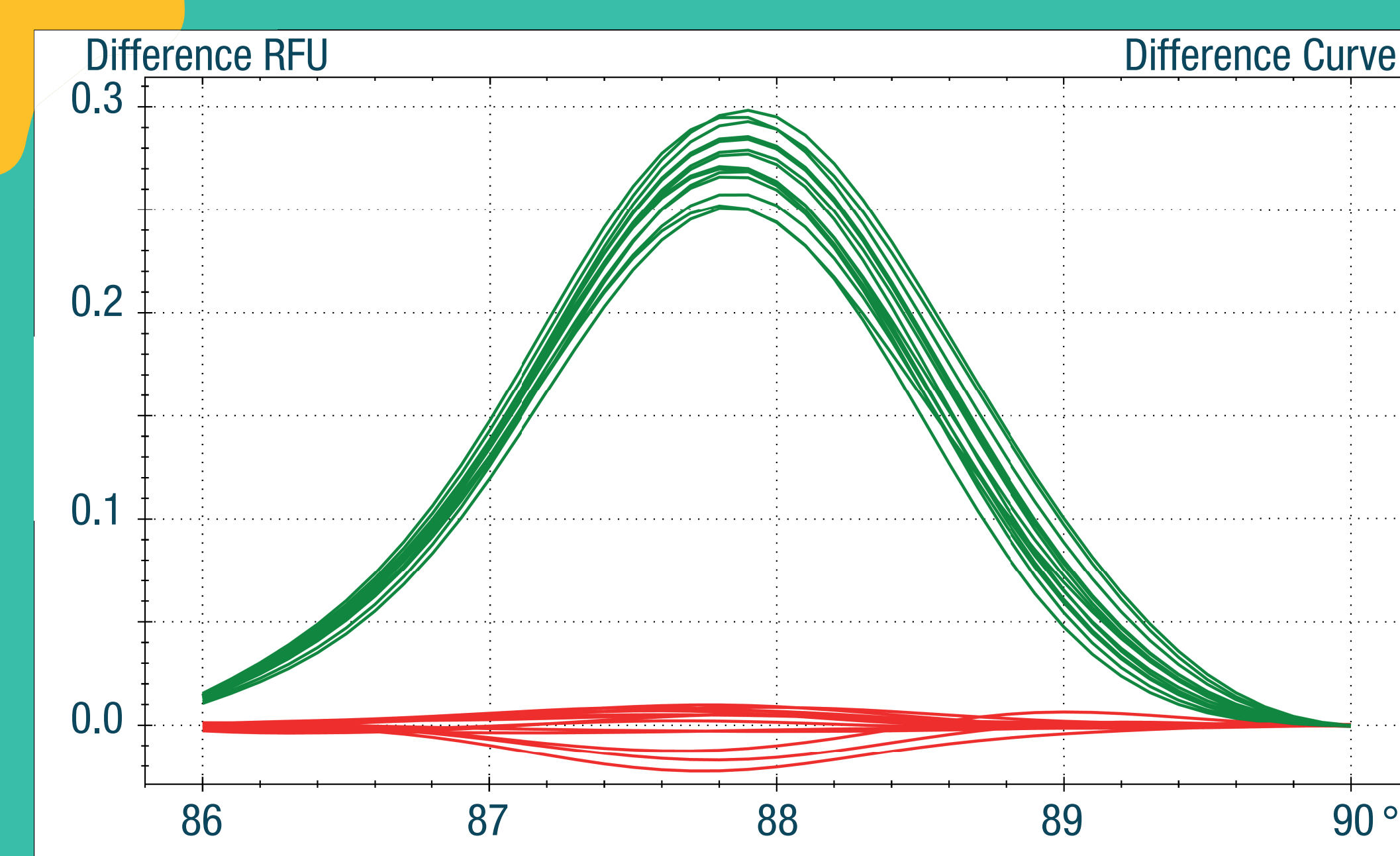


Figure 4: The Final HRM Differentiation between *B. abortus* and *B. melitensis*

The discrimination was carried out thanks to the SNP in the target sequence. *B. abortus* in green; *B. melitensis* in red.

Material and Methods

Primer sequences used for the detection of TBEV: F 5'-AGTGATMGAGARCAAGCCTG-3'; R 5'-TCATRTTYARGCCYAACCA-3'.

qPCR cycling conditions for the TBEV detection: 94 °C/1 min; 40 cycles of 94 °C/20 s, 55 °C/25 s, 72 °C/30 s; 95 °C/10 s; HRM 60-95 °C/0.1 °C/2 s.

Primers bordering the deletion in both *B. abortus* and *B. melitensis*: F 5'-GCTTTCGAACGTAGCCTGC-3'; R 5'-AGTCCGAGCAATATCCGCAA-3'.

Primers bordering the SNP enabling the differentiation between *B. abortus* and *B. melitensis*: F 5'-TGGAAAGGCCGAGATTGAGC-3'; R 5'-CACACAATCAGCTTGTACCCC-3'.

qPCR cycling conditions for the *B. abortus* and *B. melitensis* detection: 94 °C/1 min; 40 cycles of 94 °C/10 s, 60 °C/15 s, 72 °C/20 s; 95 °C/10 s; HRM 60-95 °C/0.1 °C/2 s.

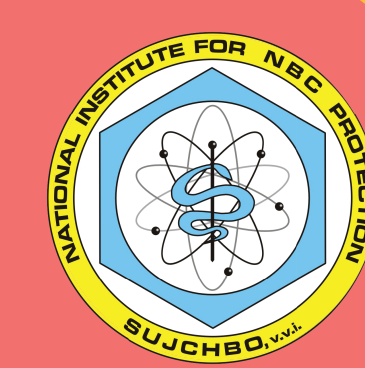
For the amplification as well as the HRM part is used the SYTO-9 intercalating dye. The results of the HRM analysis were evaluated using the Precision Melt Analysis Software (Bio-Rad). The data were verified for the Bio-Rad CFX96 machine. The specificity of PCR products was verified by both melting analysis and gel electrophoresis. Both strategies were validated in concert with the MIQE guidelines.

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