# VYUŽITÍ HRM ANALÝZY PŘI IDENTIFIKACI PATOGENŮ

# USE OF THE HRM ANALYSIS IN PATHOGEN IDENTIFICATION

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## Abstrakt

Analýza HRM – analýza křivek tání s vysokým rozlišením – představuje post-PCR metodu umožňující odlišení podle sekvence amplikonu. Amplikon je tedy analyzován hned po dokončení real-time PCR v tom samém přístroji bez nutnosti dalších manipulací se vzorky. V reakci jsou používána interkalační barviva tzv. třetí generace a speciální software nezbytný pro analýzu hrubých dat. Pro předvedení užitečnosti analýzy HRM byly vybrány vzorky viru klíšťové encefalitidy (TBEV) a bakterií *Brucella sp.* jakožto zástupci patogenních biologických agens. TBEV zahrnuje tři různé subtypy spojené s rozmanitým průběhem onemocnění, mezi *Brucella sp.* je řazeno velké množství geneticky blízce příbuzných druhů, z nichž některé představují pro člověka velké riziko. Proto je analýza HRM v tomto případě zaměřena na odlišení *B. abortus, B. melitensis* a *B. suis* od ostatních brucel.

***Klíčová slova:*** *analýza HRM, patogen, odlišení, TBEV, Brucella*

## Abstract

The HRM – high resolution melting – analysis represents a post-PCR method enabling the differentiation according to the sequence of the amplicon. Thus, the amplicon is analyzed directly after the real-time PCR in the same machine without additional manipulation with samples. The so-called third generation intercalating dyes are used in the reaction, and special software for the analysis of raw data is essential. To demonstrate the usefulness of the HRM analysis, tick-borne encephalitis virus (TBEV) and *Brucella sp.* were chosen as examples of pathogenic biological agents. The former involves three distinct subtypes connected with a varied course of the disease, the latter comprises a high number of genetically closely related species, although some of them possess a high risk for humans. Accordingly, the HRM analysis was selected to distinguish *B. abortus, B. melitensis* and *B. suis* from the rest of *Brucella sp.*

***Keywords:*** *HRM analysis, pathogen, differentiation, TBEV, Brucella*

## 1. INTRODUCTION

The HRM analysis comprises an efficient tool for the characterization of a pre-amplified nucleic acid target. The basic melting analysis is often used to confirm the specificity of the amplified product when no specific probes are used. The HRM analysis involves further discrimination of amplicons on a more detailed level.


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The proper choice of chemistry for the HRM analysis is necessary, though. For this purpose, SYTO 9, the fluorescent nucleic acid incorporating dye, was chosen. It is the third generation of incorporating dyes with a very low level of fluorescence when unbound and strongly fluorescent after binding to a double-stranded target nucleic acid.

Ideally, target sequences used for the HRM analysis should involve only one region of variability like SNP (*Single Nucleotide Polymorphism*), but it is proved here that it is also possible to distinguish agents with a high portion of the variability in the amplicon. Another recommendation applies to the length of the amplicon which should not exceed 250 bp. Too long amplicons negatively affect the precision of melting curves as well as the discrimination of differences in target sequences etc.

Prior to the HRM analysis, a target sequence has to be amplified by the PCR using appropriate chemistry. The proper choice of chemistry for the HRM analysis is necessary and involves fluorescent intercalating dyes which are incorporated into the newly arising double strand of the amplified target nucleic acid during each extension step of the PCR. When the binding on the double strand nucleic acid occurs, then the emitted fluorescence is detected on the device.

Afterward, the HRM itself consists of a gradual increase in the temperature connected with the monitoring of the emitted fluorescence. During the increase of the temperature, double strands are dissociated and the emitted fluorescence decreases which can be monitored as a change in the slope of the melting curve characterized by a melting temperature (Tm). Tm is the temperature at which half the amplicons is in the form of double strands and the half is single-stranded. Finally, the detected curve specific to the particular amplicon is obtained. Subsequently, the analysis using the special software is needed. The measured fluorescence and temperature are then plotted in normalization and difference graphs.

To summarize, HRM analysis serves not only as a proof of the specificity of the amplified product but also as a useful discrimination tool for differences in particular sequences.

## 2. MATERIAL AND METHODS

The initial material was represented either by infected cell cultures or by bacterial suspensions of TBEV and *Brucella* sp., respectively. Nucleic acid extraction was done by using the appropriate purification kit (STRATEC Biomedical AG, Germany). In the case of TBEV, the purified RNA sample was treated with the DNase I enzyme (AMPD1, SIGMA-ALDRICH, USA) ensuring the elimination of DNA contamination. The cDNA was synthesized by the ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, UK). The primers used in both assays were designed in the Oligo 7 software (Molecular Biology Insights, Inc., USA). Primer sequence specificity using the blast web tool [1], secondary structure tests using the mfold web tool [2] or Oligo 7 software, as well as melting predictions in DINAMelt [3] or Oligo 7 software were carried out where applicable. For sequence alignments were used the ClustalX2 software [4] or the MAFFT web alignment tool [5]. Specificity of both assays was proved by laboratory tests on the panel of viruses or bacteria.


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Following primer sequences were used for the detection of TBEV:

(F) 5'-AGTGATMGGAGARCAYGCCTG-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. For the HRM analysis, qPCR Master Mix containing the SYTO 9 green fluorescent nucleic acid stain was used (Top-Bio).

Following primer sequences were used for the detection of target *Brucella*:

Br_AM1_araC_F: 5'-GCTTTCGAACGTAGCCTGC-3';

Br_AM1_araC_R: 5'-AGTCCGAGCAATATCCGCAA-3';

Br_suis_F55: 5'-AAAATGCCAATCAATTCAACA-3';

Br_suis_R110: 5'-TGAGAATTCCCGTTCCCTC-3'

Br_mel_BAB1_0255b_F: 5'-TGGAAAGGCCGAGATTGAGC-3';

Br_mel_BAB1_0255b_R: 5'-CACACAATCAGCTTGTCACCC-3'

Br_canis_F: 5'-GTTCACGCGATATTTGGCCAGA-3';

Br_canis_R2: 5'-TTTTCACTGCGCCGGCAC-3'.

qPCR cycling conditions for the *Brucella* 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 HRM analysis, qPCR Master Mix containing the SYTO 9 green fluorescent nucleic acid stain was used.

Both assays were performed on the CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, USA).

## 3. RESULTS AND DISCUSSION

Two different points of view on the use of HRM analyses are presented here. The first one shows a case when there is a variety in the amplicon sequence, the second one involves highly related agents with only minor differences in amplicon sequences as it is represented by TBEV and *Brucella* species, respectively. Final results obtained by the special analysis software are described in the following subchapters focused on a particular model species.

### 3.1 TBEV

TBEV is a part of the *Flaviviridae* family and is a causative agent of the serious disease of the central nervous system which is transmitted to humans by ticks. The virus comprises three different subtypes – European (Eu), Far-Eastern (FE) and Siberian (Sib) differing in the locations as well as the vector [6].

The selected genomic region encoding the envelope protein of the virus was chosen thanks to the fact that particular subtypes can be divided according to its sequence [7]. Due to the use of degenerated primers, as well as the variability in the inner sequence, it is difficult to use tools for the melting prediction and the suitability of amplicons has to be tested practically.



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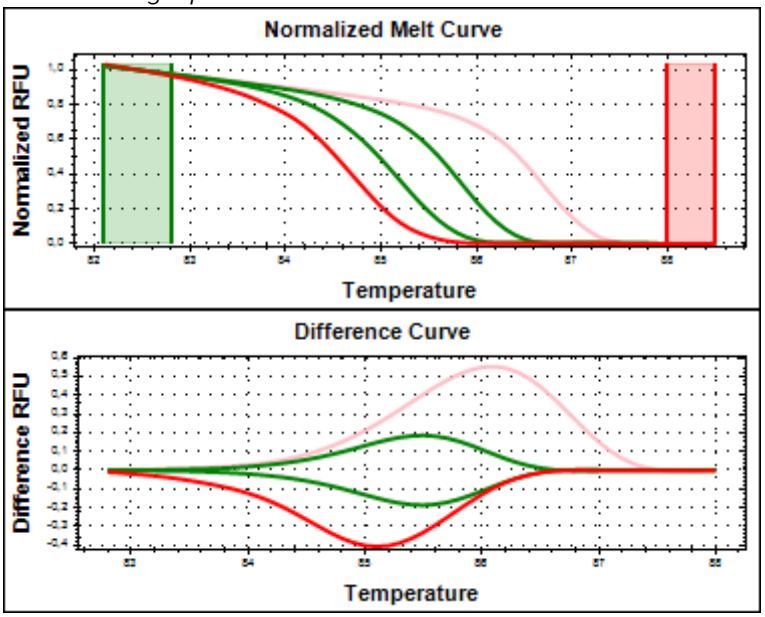
Moreover, the DINAMelt tool does not enable to make predictions with sequences longer than 100 bp. The best resolution capability of the HRM was obtained under melting conditions in the range of 60-95 °C using the step 0.1 °C per 2 s, see Figure 1.

The analysis software of the platform used has the advantage which enables to choose a whole cluster of samples as a reference in comparison to other platforms where only one reference sample can be chosen or there is another option which involves to carry out the analysis in an alternative software. The HRM analysis allows differentiating among serotypes in order to distinguish the particular serotype in the sample. This approach can mainly be used in areas where all the subtypes occur simultaneously or in case of unknown samples which need further characterization.

*Figure 1: The HRM Analysis of TBEV*
*Each subtype is marked by a different color. Eu in red, FE in green, Sib in pink. Melting curves are normalized in the upper graph, further differentiation is obtained by plotting the curves in the bottom graph.*




## 3.2 *Brucella*

These gram-negative bacteria are found endemically in many areas of the world, like Latin America or the Middle East [8]. Especially *B. abortus, melitensis* and *suis* still present a considerable risk for humans. Pathogenicity of these bacteria together with the low infectious dose or the transmission via aerosol could possess a risk of misuse as a biological weapon [8, 9].

Unlike TBEV, there is a high sequence similarity among *Brucella* sp., so, these agents are suitable targets for the HRM analysis. Four different genomic targets were chosen for the assay design. The first target (locus dAM) is the 7 bp long deletion in the inner sequence of the 759 bp PCR product [10]. The SNP (locus MEL) was found in the gene coding for E protein in GI3 region [11]. The third one is represented by the SNP (locus SU+CAN) in the 1682 bp PCR product [12]. The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last target site encompasses the 12 bp deletion (locus CAN) in the 300 bp PCR product [13]. Completely new primers were designed in close proximity of selected targets ensuring the right size of PCR products.

When designing primers, melting predictions were carried out as well in order to find out specific differences in Tm between discriminated groups of *Brucella* sp. in particular target sites. For this purpose, predictions were made using different tools. According to Table 1, it is obvious that more accurate predictions were found out when using the Oligo 7 software than using the DINAMelt tool [3]. It is also able to compare the results gathered from predictions with real measured Tm values.

The assay consists of two duplex reactions, therefore the analysis has to be optimized accordingly. This is ensured by the definition of normalization regions of particular targets of the duplex reaction, see Table 2 and Figure 2.

*Table 1: Tm Predictions and Real Data Comparison*
*Targets designated as non- carry a different type of SNP or the insertion instead of the deletion.*

| Locus | DINAMelt | Oligo 7 | Real Data |
|---|---|---|---|
| dAM | 86.3 °C | 85.0 °C | 84.6-85.1 °C |
| non-dAM | 90.9 °C | 86.4 °C | 85.9-86.4 °C |
| MEL | 89.8 °C | 87.8 °C | 87.4-87.8 °C |
| ABO | 89.7 °C | 88.2 °C | 88.0-88.5 °C |
| SU+CAN | 83.1 °C | 76.1 °C | 75.9-76.7 °C |
| non-SU+CAN | 83.6 °C | 76.7 °C | 76.3-77.1 °C |
| SUIS | 83.8 C | 84.4 °C | 84.1-84.5 °C |
| CAN | 84.7 °C | 83.9 °C | 83.9-84.6 °C |

*Table 2: Normalisation Regions for the Evaluation of Brucella Loci*

| Locus | Pre-Melt Region | Post-Melt Region |
|---|---|---|
| dAM | 82.3-82.8 | 88.0-88.5 |
| MEL | 85.5-86.0 | 90.0-90.5 |
| SU+CAN | 73.3-73.8 | 78.5-79.0 |
| CAN | 81.8-82.3 | 86.5-87.0 |



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*Figure 2: Melt Curves in Duplex Reaction*
*The data analysis of duplex reaction requires the definition of separate normalization regions for particular targets. For the first and second part of the melting, the change in the slope of the curve is visible, compare the left and the right graph.*

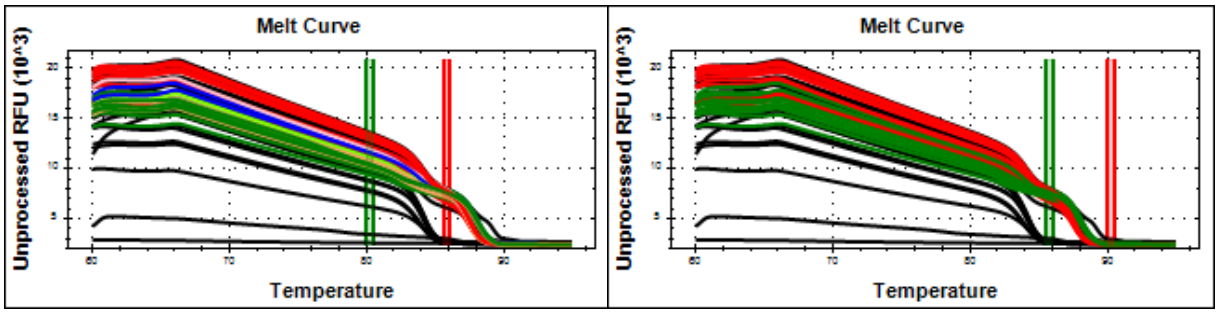
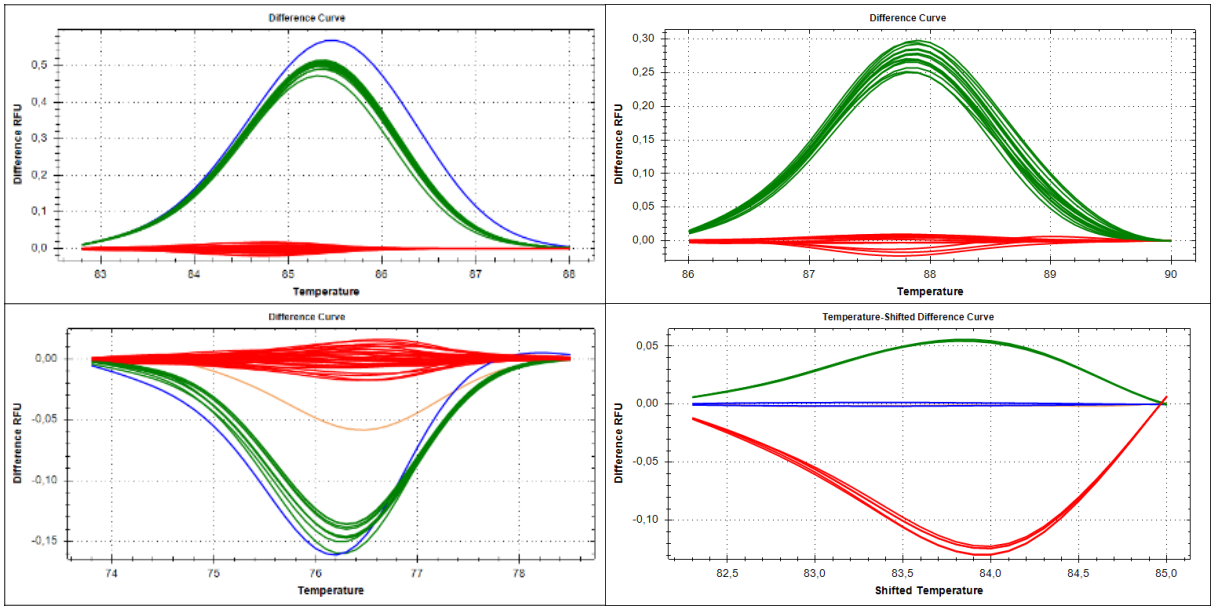



*Figure 3: The HRM Analysis of Brucella*
*The left upper graph shows the discrimination between B. abortus and B. melitensis in red and other Brucella sp. in green and blue according to the locus dAM. The right upper graph shows the subsequent discrimination between B. abortus in green and B. melitensis in red according to the locus MEL. The left bottom graph shows the discrimination between B. suis and B. canis in blue, green and orange and other Brucella sp. in red on the basis of the locus SU+CAN. The right bottom picture shows consequent discrimination between B. suis bv. 1, 3, 4 in green, B. suis bv. 2 in red and B. canis in blue on the basis of the locus CAN.*




There is complete discrimination among chosen *Brucella* sp. in Figure 3. The analysis comprises a two-step process to categorize target *Brucella* sp. In graphs on the left, there is the first step of the analysis belonging to the first duplex reaction. The second step of the analysis involves the evaluation of the second duplex reaction as it is shown in graphs on the right side of Figure 3. In the right bottom graph of Figure 3, the temperature-shifted view was used for better resolution between *B. suis* and *B. canis* samples. This feature enables a shift of the normalized curve along the x-axis.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Even though the sequences of *Brucella* sp. possess a high degree of similarity, it is able to clearly set three chosen *Brucellae* apart thanks to the HRM analysis carried out in a new two-step discrimination model.

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