High Resolution Melt: species identification in theory and practice

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"In theory, theory and practice are the same. In practice, they are not." This quotation from Albert Einstein describes well the theoretical expectations and the practical reality of High Resolution Melt (HRM) method, especially from the aspect of species identification in two-case-studies described here. HRM is a post PCR technique based on real-time detection of dissociation (melt) of dsDNA molecules by raising the temperature of reaction mixture in presence of dsDNA interchelating fluorescence dye. The dissociation profile of dsRNA molecules is based on sequence, length, GC-content, and strand complementary. These differences can be utilized, in addition to species identification, also in single nucleotide polymorphism (SNP), DNA fingerprinting, DNA methylation, heterozygote, and in many other analysis applications.

The first generation interchelating fluorescence dyes (e.g. Sybr Green I) are cost effective, but they inhibit PCR-reaction itself and can be used only in nonsaturating concentrations if used as PCR additive. Second generation dyes, in general, inhibit less PCR reaction, have better thermal stability and can be nonmutagenic or noncytotoxic (e.g. Eva Green). Other 2\textsuperscript{nd} generation dyes are LC Green, Chromofy, BEBO, and SYTO9 each with different characteristics.

HRM is most conveniently performed as closed tube experiment after the PCR. However, originally this method was developed for a separate HRM-machine manufactured by Idaho Technology which is incapable to perform PCR amplification. Nowadays many block-based quantitative PCR machines are equipped with HRM feature. These machines however suffer from low thermal uniformity within the metal-block (±0.2°C). This problem can be solved in a rotary design with "kind of" mono-block used in Rotor-Gene 6000 (Rotor-Gene Q). Rotary design can reach extremely high sample to sample uniformity (±0.01°C) and enables fixed light path length without complex optics.

In case study I Picea abies trees were infected with a mixture of five different mycorrhiza fungi (Laccaria, Piloderma, Amphinema, Thelephora, Paxillus, and "black fungi"). After infection root tips were isolated and Phire plant direct PCR-kit was applied (ITS1f & ITS4 primers). HRM-technique was applied by adding 0.5x Sybr Green I (1 µM) to the PCR-mixture. When clean template DNA from pure cultures was used each of the fungi species could be identified without any problems. However, when using DNA from the root tips unreliable melting patterns were produced due to nonuniform template composition and heteroduplex formation (multiple infections in single root tip). Original plan to use restriction fragment length polymorphism and agarose gel electrophoresis was applied.

In case study II a method to detect Melampsoridium alni, M. betulinum and M. hiratsukanum infecting leafs of alder was developed. Purified (commercial kit and polyethylene glycol precipitation) DNA was amplified with Solis Biodyne EvaGreen HRM mix –kit as described by the manufacturer. A five primer mixture was applied to target ITS2 region. In HRM analysis M. betulinum had the lowest melting temperature and the M. alni highest one. Melting temperatures differed by 1.31°C (betulinum - hiratsukanum) and 0.3°C (hiratsukanum – alni). Results were reproducible.

The HRM has many benefits. It is a closed-tube method (easy handling, practically free of contamination problems) and cost effective compared to other identification methods (sequencing, DGGE, T-RFLP etc.). In addition, HRM is fast and simple to perform. However, HRM has also limitations. Resolution is low. DNA melting is dependent on many factors (e.g. DNA / salt concentration, template quality). In silico DINAMelt
program could be applied to design suitable amplicons for HRM analysis. In practice this is not the case and manual optimization is often required.

In Practice when applying HRM you need:
- Well amplifying amplicons
- Good template quality
- Limited number of alleles per tube (this may vary from study to study)

For more information about HRM. Please visit, http://hrm.gene-quantification.info