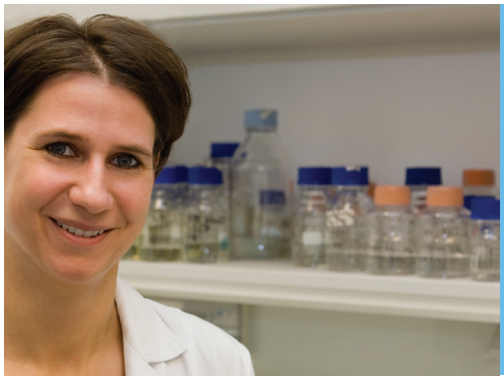
Identifying Variants by High Resolution Melting (HRM) in Difficult Samples

Applied Biosystems 7500 Fast Real-Time PCR System, HRM Software v2.0, 3730 DNA Analyzer



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Applications

SNP Genotyping

Technologies

7500 Fast Real-Time PCR System, HRM Software v2.0, 3730 DNA Analyzer

What is High Resolution Melting (HRM)?

HRM is a DNA melting analysis used to characterize nucleic acid samples based on DNA strand dissociation or melting curve analysis. Amplicon length, GC content, and potential mismatches (heterozygosity) are some of the factors that can influence the melting profile of a PCR product. HRM analysis is used to identify these differences and requires special instrumentation capable of capturing enough data points per change in temperature in combination with software that can interpret this type of data. HRM also requires a nonspecific DNA-binding dye such as SYTO® 9, which will not inhibit the PCR when binding at high density.

Dr. Birgit Ebner, along with coworkers from the Department Core Facility Molecular Biology at the Center for Medical Research in Graz, Austria, used Applied Biosystems HRM technology to perform single nucleotide polymorphism (SNP) genotyping on formalin-fixed, paraffin-embedded (FFPE) tissues. The HRM Software v2.0 was used along with the 7500 Fast Real-Time PCR System for genotyping six DNA samples isolated from FFPE tissues. Results showed that the Applied Biosystems HRM workflow can be an effective tool to screen for unknown SNPs across genes of interest in these difficult-to-use samples.

ISOLATING DNA FROM FFPE-TREATED TISSUES

Although the degree of fragmentation of DNA that has already occurred in FFPE tissues cannot be reversed, Ambion's RecoverAll™ Total Nucleic Acid Isolation Kit yields sufficient DNA for successful SNP genotyping in many FFPE samples. FFPE tissue sections are first deparaffinized using a series of xylene and ethanol washes. The sections are then subjected to rigorous protease digestion with an incubation time tailored for recovery of RNA or DNA. The nucleic acid is purified using a rapid glass filter methodology that includes an on-filter nuclease treatment. Finally, recovered DNA is eluted into either water or the low-salt buffer provided.

The Challenges of Working with FFPE Samples

Formalin fixation and paraffin embedding is a process that was developed to preserve tissue morphology. It was not designed as a nucleic acid preservation method. As a result, nucleic acids in

FFPE tissues are often degraded. This is influenced by several factors:

- Formaldehyde is a promiscuous cross-linker. It reacts primarily with proteins, creating a tightly locked, three-dimensional network of proteins, which is also linked to other macromolecules.
- Standard embedding protocols require heating the formaldehyde-soaked samples. The temperatures used for embedding in paraffin cause further molecular reactions, some irreversible.
- The nucleic acid in tissue blocks can degrade further during standard storage.

HRM analysis is a high-resolution method designed to detect small differences between two DNA sequences in a double-stranded DNA molecule. Sample degradation, including introduction of a nucleic acid modification, can result in a heterogeneous pool of amplicons after PCR, causing an artificial spread of the population, and as a consequence, an increasing variation during HRM analysis.

Experimental Design

1. **DNA isolation**—DNA was isolated from six FFPE samples using a commercially available kit, after deparaffination by xylene and ethanol washes.

2. **DNA amplification**—Triplicate samples of 20 ng DNA were amplified by PCR using the universal thermal cycling protocol on a 7500 Fast Real-Time PCR System for 40 cycles. Each PCR contained SYTO® 9 dye and primers for the PTEN SNP rs2248293 (T/C) class 1 SNP (customer-designed), which have a melting temperature of 59–60˚C and produce a 212 bp amplicon.

3. **SNP analysis**—Melting curve data were collected using the Expert Mode function within the 7500 Fast SDS v1.4 Software, to allow data collection in a single filter. Melting curves were then analyzed using the HRM Software v2.0.

4. **Sequencing**—SNP analysis results were confirmed by sequencing using the Applied Biosystems 3730 DNA Analyzer, BigDye® Terminator v1.1 Cycle Sequencing Kit, and SeqScape® software v2.5, according to recommended protocols.

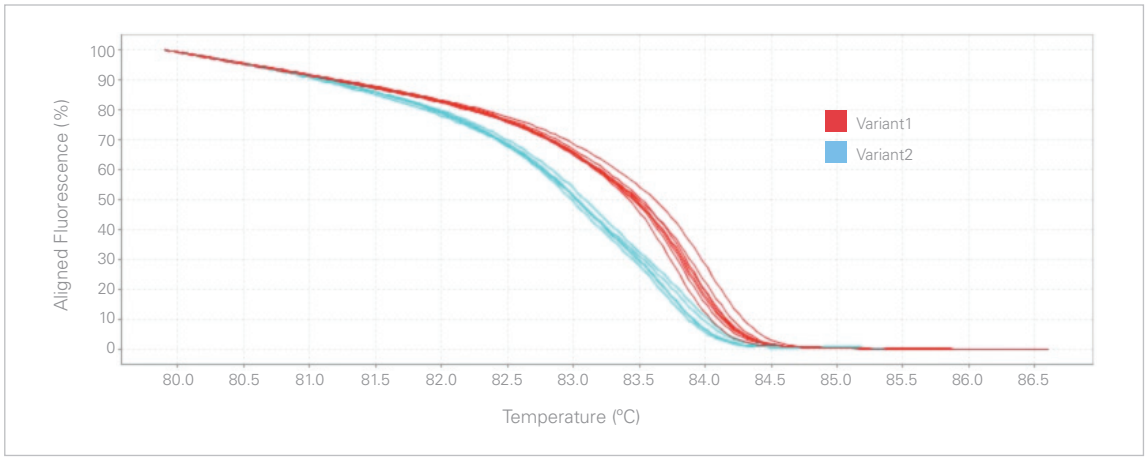

Figure 1. Aligned Melting Curve Data Generated from HRM v2.0 Default Analysis Settings. Distinct differentiation of heterozygous (variant 2) and homozygous populations (wild type and homozygous-variant samples) (variant 1) is shown.

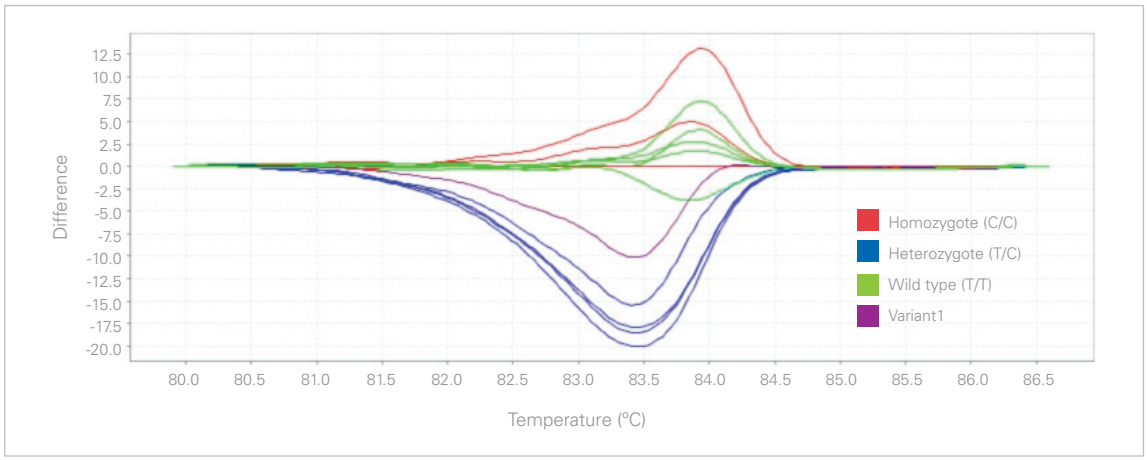

Figure 2. Difference Plot Delineating Sample Groupings. The grouping of wild type, homozygous variant, and heterozygous samples after defining controls for each population.

Results

Despite the challenges posed for genotypic analysis of FFPE tissues, the HRM Software v2.0 was able to distinguish heterozygous from homozygous samples (Figure 1) when default analysis settings in the HRM Software v2.0 with no controls entered were used. These data were also confirmed by sequence analysis (data not shown). Once controls were added for each population, the software separated and annotated samples into homozygous variant, homozygous wild type, or heterozygote groups (Figure 2). Two replicates from one sample and one replicate from another sample failed to amplify successfully. These samples were omitted from subsequent analyses.

Conclusion

Dr. Ebner found that the 7500 Fast Real-Time System and the HRM Software v2.0 provide a user-friendly and easy-to-interpret system for SNP genotyping, when performing HRM analyses on FFPE samples. Furthermore they demonstrated that results were consistent with other methods.

"The user-friendly HRM software enables simple discovery and detection of SNPs and provides accurate results, even from FFPE materials. We use HRM analysis successfully as a cost-effective method for the detection of several mutations e.g., B-Raf and K-Ras mutations in various oncological questions. " —Dr. Ebner

Scientific Advisors

Thomas Halama, Madeline O'Donoghue, Simone Günther, Applied Biosystems

Acknowledgement

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TIPS FOR GREAT SNP GENOTYPING USING HRM WITH FFPE TISSUES

1. PCR primer design
   a. Primers should have a high level of specificity as HRM dyes bind generically —Use SYBR® Green primer design principles
   b. Amplicon length should be kept short to maximize different melting behaviors of similar products (100–200 bp is ideal)
   c. Best primer concentration is approximately 300 nM
   d. Test different primer pairs to determine which is optimal

2. Template concentration and quality
   a. If possible, use the same starting concentration for all samples
   b. Use high-quality DNA whenever possible—when using FFPE tissues, consider using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues

3. PCR reaction components
   a. Starting $MgCl_2$ concentrations should be between 1.5 and 2.0 mM—concentration can be increased for difficult assays
   b. Use AmpliTaq Gold® DNA Polymerase or AmpliTaq Gold® 360 Master Mix at a concentration of 1.25–1.5 U/µL
   c. Use a suitable dsDNA binding dye, such as SYTO® 9 (1.5 µM concentration) or EvaGreen® (1X working concentration)

4. Optimize PCR
   a. C_T values should be between 20 and 30
   b. Samples should amplify similarly
   c. Exponential phases should be strong and steep
   d. Allow reactions to reach plateau

5. Include proper controls for PCR and variants; include a proper number of replicates

6. Use good laboratory practices, including:
   a. Accurate pipetting and use of reaction volumes that are large enough to minimize pipetting errors
   b. Prepare fresh master mix as needed
   c. Run samples within two hours after preparation
   d. Perform melt curve analyses immediately following amplification

ORDERING INFORMATION

Description	Size	P/N
7500 Real-Time PCR System with Dell™ Notebook	1 instrument	4351104
7500 Fast System Upgrade Kit	1 instrument	4362143
7500 Real-Time PCR System with Dell™ Tower	1 instrument	4351105
High Resolution Melting (HRM) Software v2.0	1 software	4397808
3730 DNA Analyzer	1 instrument	3730S
BigDye® Terminator v1.1 Cycle Sequencing Kit	100 reactions	4337450
BigDye® Terminator v1.1 Cycle Sequencing Kit	1,000 reactions	4337451
BigDye® Terminator v1.1 Cycle Sequencing Kit	5,000 reactions	4337452
BigDye® Terminator v1.1 Cycle Sequencing Kit	24 reactions	4337449
SeqScape® Software v2.6 upgrade from v2.1.x and earlier	1 CD	4332045
SeqScape® Software v2.5, 45-Day Demo	5 licenses	4327099
SeqScape® Software v2.6, Initial License	1 license	4327091

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