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High Resolution Melt Curve Analysis- An Innovative Approach for Molecular Diagnosis

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Introduction

High Resolution Melting (HRM) is a homogeneous, extremely powerful technique for SNP genotyping, mutation scanning and sequence scanning in DNA samples [1]. Enabled by the recent availability of improved double-stranded DNA (dsDNA)–binding dyes and next-generation real-time PCR instrumentation, High Resolution Melting Analysis is based on PCR melting (dissociation) curve techniques. For several years, various researchers and instrument makers have independently investigated the utility of high-resolution DNA dissociation analysis. The team at Idaho Technology has done an admirable job of vigorously promoting their research through traditional journal publications. Conversely, Corbett Life Science does not pursue publication, but instead relies on the publications of customers to promote the technology. Regardless, both companies have independently advanced the field of high resolution dissociation analysis and successfully introduced what has now become known as high resolution melt (HRM) analysis. Idaho Technology was first to market with an instrument made specifically to do dissociation analysis; the HR-1. The HR-1 was a showpiece for the technology with the singular aim of producing the most detailed melt curve possible [2]. As such, it opened the eyes of many to the potential of HRM and remains the performance benchmark for the acquisition of an individual melt curve. However the HR-1 is not capable of thermal cycling and can only analyze a single sample from within a glass capillary per run making data analysis time consuming. The HRM technology characterizes nucleic acid samples based on their disassociation behavior and detects small sequence differences in PCR amplified sequences, just by direct melting. Samples are also discriminated according to their sequence length, GC content and strand complementarily. With the use of specific DNA dyes, high-end instrumentation and sophisticated analysis software, these differences are detected.

Working Principle

High Resolution Melting Analysis (HRM) is a post PCR method. During processing, special saturation dyes known as Intercalating dyes, are added to the reaction, that fluoresce only in the presence of double stranded DNA[3]. The region of interest within the DNA sequence is first amplified using the polymerase chain reaction. As the amplicon (amplified product) concentration in the reaction tube increases the fluorescence exhibited by the double stranded amplified product also increases. After the PCR process the HRM analysis begins. In this process the amplicon DNA is heated gradually from around 50°C up to around 95°C. As the temperature increases, at a point the melting temperature of the amplicon is reached and the sample DNA denatures and the double stranded DNA melts apart. Due to this the fluorescence fades away. This is because in the absence of double stranded DNA the intercalating dyes have nothing to bind to and they only fluoresce at a low level. This observation is plotted showing the level of fluorescence vs the temperature, generating a Melting Curve. Even a single base change in the sample DNA sequence causes differences in the HRM curve. Since different genetic sequences melt at slightly different rates, they can be viewed, compared, and detected using these curves [3, 4]. Melt curves generated after High Resolution Melting analysis is normally plotted with fluorescence on the Y axis and temperature on the X axis. These are similar to real-time PCR amplification plots but with the substitution of temperature for cycle number.

Intercalating Dyes for HRM Analysis.

Saturating fluorescent dyes are used to monitor the denaturation of sample DNA amplicons, in order to perform HRM Analysis. Some of the commercially available dyes include;

1.SYTO Dye is a cell -permeant nucleic acid stains showing a large fluorescence enhancement once they bind to double stranded DNA. These dyes are commercially available as blue-, green-, orange- or red fluorescent dyes.
2. Chromofy is a monomeric asymmetric cyanine dye. When chromofy binds to a double stranded DNA it shows a very strong increase in fluorescence. Chromofy can be used for HRM analysis for SNP

Genotyping and methylation analysis.

3. LC Green are specifically designed for High Resolution Melting analysis to detect DNA sequence variants. The addition of these dyes increases the melting temperature of DNA by 1-3°C and may require adjustment of cycling parameters.

4. EvaGreen is a green fluorescent nucleic acid dye widely used for High Resolution Melting Analysis. The dye is non-fluorescent by itself, but becomes highly fluorescent upon binding to double stranded DNA. It is non-mutagenic and non-cytotoxic and is completely impermeable to cell membranes [5].

Innovations from Instrument manufacturers-Applications of HRMA.

Mutation Detection, SNP typing, Methylation, Alternative for Gel Electrophoresis, Multi-well instruments with greater practical utility were utilized for all the mentioned applications. The first multi-well HRM instruments were the Rotor-Gene 6000 (Corbett Life Science). These two instruments were introduced at about the same time but employed fundamentally different technical innovations to achieve HRM. The Light Scanner uses a modified block-based design available in 96-well or 384-well versions. Despite advanced engineering, it still suffers from measurable sample-to-sample thermal and optical variation and is unable to match the performance benchmark set by the original HR-1 instrument. Like the HR-1, the light scanner is not capable of thermal cycling. The Rotor-Gene 6000, was the first of the multi-well instruments capable of both thermal cycling and HRM. This dual capability enables samples to be fully processed in the one instrument (i.e. pre-amplification and HRM done consecutively in the one run). A major advantage of this is that amplification plots can be used to help interpret HRM results since aberrant amplification plots (i.e. those that amplified differently to what was expected) also produce aberrant HRM data. In this way compromised samples can be easily identified and removed from downstream HRM analysis. The main advantage of the Rotor-Gene for HRM stems from its rotary design, in which samples spin under centrifugal force past a common optical detector. This is seemingly ideal for HRM as thermal or optical variation between samples is insignificant. The result is that the Rotor-Gene HRM performance closely matches the HR-1 benchmark with the compromise that samples are not arranged in a conventional array format (as they are in block-based instruments) but are instead arranged around the perimeter of a spinning rotor. The more recently introduced Light Cycler 480 (Roche Molecular Systems) is capable of HRM and thermal cycling. The Light Cycler 480 is a block-based instrument design and it has better thermal uniformity than other block-based instruments, it nevertheless does exhibit measurable thermal and optical non-uniformity [6]. Other instrument providers are now rushing to introduce HRM capability and some are planning to release software upgrades to support HRM analysis. The danger here is that instruments not specifically engineered for HRM will deviate so much from the HR-1 performance benchmark that careful investigation will need be done before accepting those instruments as HRM capable. High Resolution Melt (HRM) analysis is a powerful technique in molecular biology for the detection of mutations, polymorphisms and epigenetic differences in double-stranded DNA samples. It was discovered and developed by Idaho Technology and the University of Utah. Idaho Technology was first to market with an instrument made specifically to do dissociation analysis; the HR-1. The HR-1 was a showpiece for the technology with the singular aim of producing the most detailed melt curve possible. As such, it opened the eyes of many to the potential of HRM and remains the performance benchmark for the acquisition of an individual melt curve. However the HR-1 is not capable of thermal cycling and can only analyze a single sample from within a glass capillary per run making data analysis time consuming. High Resolution Melting Analysis (HRM or HRMA) is a recently developed technique for fast, high-throughput post-PCR analysis of genetic mutations or variance in nucleic acid sequences. It enables researchers to rapidly detect and categorize genetic mutations (e.g. single nucleotide polymorphisms (SNPs) identify new genetic variants without sequencing (gene scanning) or determine the genetic variation in a population (e.g. viral diversity) prior to sequencing. The first step of the HRM protocol is the amplification of the region of interest, using standard PCR techniques, in the presence of a specialized double-stranded DNA (dsDNA) binding dye. This specialized dye is highly fluorescent when bound to dsDNA and poorly fluorescent in the unbound state. This change allows the user to monitor the DNA amplification during PCR (as in quantitative PCR). After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile; this is termed melting analysis. The amplified target denatures gradually, releasing the dye, which results in a drop in

fluorescence. When set up correctly, HRM is sensitive enough to allow the detection of a single base change between otherwise identical nucleotide sequences. HRM uses low-cost dyes and requires less optimization than similar systems based on TaqMan and fluorescence resonance energy transfer (FRET) probes. Compared to other technologies for mutation scanning such as sequencing, denaturing HPLC (dHPLC), and denaturing gradient gel electrophoresis (DGGE), HRM offers a far easier, less time-consuming procedure that consumes significantly less reagent It is more reproducible than either dHPLC or DGGE, requiring less optimization and interpretation by the investigator. In some cases, it also provides better accuracy and sensitivity. In addition, HRM analysis is a closed-tube procedure, greatly reducing the risk of contamination from earlier PCR products. Because HRM analysis does not destroy samples, it is particularly well-suited for genetic variant scanning. Compared to these methods HRM is a simpler and more cost-effective way to characterize multiple samples.

The Applied Biosystems HRM Software provides an easy and intuitive workflow that:

1. Shortens analysis time by auto-calling genotypes and automatically omitting the no template controls.
2. Minimizes subjective analysis by automatically grouping unknown variant clusters.
3. Allows easy data review with customizable plot views, expandable windows, and one-click color assignment to highlight curves of interest.
4. Ability to analyze multiple targets (assays) on one plate.
5. A variant of RT PCR includes 7500 Fast Real-Time PCR System and 7900HT Fast Real-Time PCR System.

Advantages of High Resolution Melt Curve Analysis.

Cost Effectiveness is one of the most significant advantages when the technology is applied to routine molecular diagnosis. High Resolution DNA Melt Analysis is cost effective than other genotyping technologies such as sequencing and TaqMan SNP typing [7]. This makes it ideal for large scale genotyping projects. Easy and Fast Work Flow after performing PCR amplification of the target sequence, no additional instrumentation is required for HRM Analysis. Fast and powerful HRM analysis is fast and powerful, due to which it is able to accurately genotype huge number of samples in a short time, with a high level of accuracy. Low reagent consumption High Resolution Melting Analysis requires only PCR reaction volume for analysis of each sample, eliminating the need for HPLC solvents or Denaturing Gradient Gel Electrophoresis (DGGE) gels [8].

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Illustrations

Illustration 1

Figure1: DNA Melt Curve plot generated by High Resolution Melting Analysis Technique

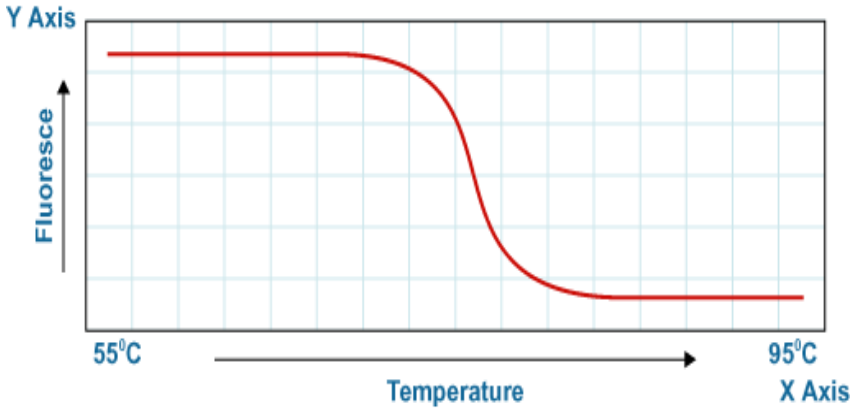

Figure1. DNA Melt Curve plot generated by High Resolution Melting Analysis Technique

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