High Resolution Melt (HRM) Analysis Information

Supplementary HRM material for year 10 genetics module

This supplementary material covers the background of HRM, plus prompts educators & students to consider its applications.

Activities & tasks: This information can feed into various options for activities & tasks (see end of section).

High Resolution Melt (HRM)

High Resolution Melt (HRM) is a relatively new, powerful technique used for detecting differences in genetic material, often conducted after PCR. These genetic differences include mutations, polymorphisms and epigenetic differences in double-stranded DNA samples.

Terminology check: at the start of this HRM section, it may be useful to have a brief term-check & refresher of the following definitions (as a class or small groups):

- DNA – term, role, structure, components, including nucleotide bases (types (A,T,G,C).
- Mutation – change in the genetic sequence of an organism (can be in DNA or RNA material). mutation – example, see short video & genetics overview visual below on this page from the Genetic Science Learning Centre (US)
- Polymorphism - the presence of two or more variant forms of a specific DNA sequence that can occur among different individuals or populations. The most common type of polymorphism involves variation at a single nucleotide base (also called a single-nucleotide polymorphism, or 'SNP').
- Epigenetics – the process where the environment & behaviours can cause changes that affect the way that genes operate. Unlike genetic changes, epigenetic changes are reversible and do not change the DNA sequence, but they can change how an organism reads a DNA sequence.

HRM has advantages over other technologies that can determine the genotype (genotyping technology), including:

- HRM is fast and powerful, and therefore able to accurately genotype many samples rapidly.
- The process is relatively straightforward. With a good quality HRM assay, powerful genotyping can be performed by non-geneticists in any laboratory with access to the equipment – i.e., a HRM capable 'real-time' PCR machine.
- HRM is cost effective compared to other genotyping technologies such as full genetic sequencing and Taq SNP (single-nucleotide polymorphism) typing. This makes HRM ideal for large scale genotyping tasks & projects. Robot technology can assist with the preparation of many samples post-PCR, ready for the HRM step. In the case shown below, robotic technology can be used to prepare genetic samples for HRM analysis, with around 100 samples rapidly prepared. This occurs in an enclosed glass frame, with the robot scanning the area & guiding robotic arms that rapidly move between solutions of chemicals and samples, waste containers and changing tips of the pipettes between solutions. An ultraviolet (UV) light source also automatically cleans the equipment prior to preparing the samples.
    - Question: what benefits might robot technology (such as that shown in the following photo) have for these genetic analysis tasks such as HRM? *(Think: speed; reduction of manual error by humans - especially due to very small volumes of liquids; cleanliness of sample preparation – reducing contamination risk).*
    - Question: Why would contamination by humans, bacteria etc. be an issue with this genetic analysis? What sorts of steps may a researcher take to reduce the likelihood of this

happening? (This also applies to HRM, PCR, gel electrophoresis etc. techniques already covered). *(Think: contamination from other genetic material sources can lead to misleading results – e.g., genetic material from bacteria may appear in the pea genetic sample).* This includes using sterile (clean) equipment such as tubes, pipettes for measuring and transferring samples and liquid chemicals. Wearing personal protective equipment (PPE) is also important– clean gloves, laboratory coat, safety glasses, not touching your face/hair etc.).

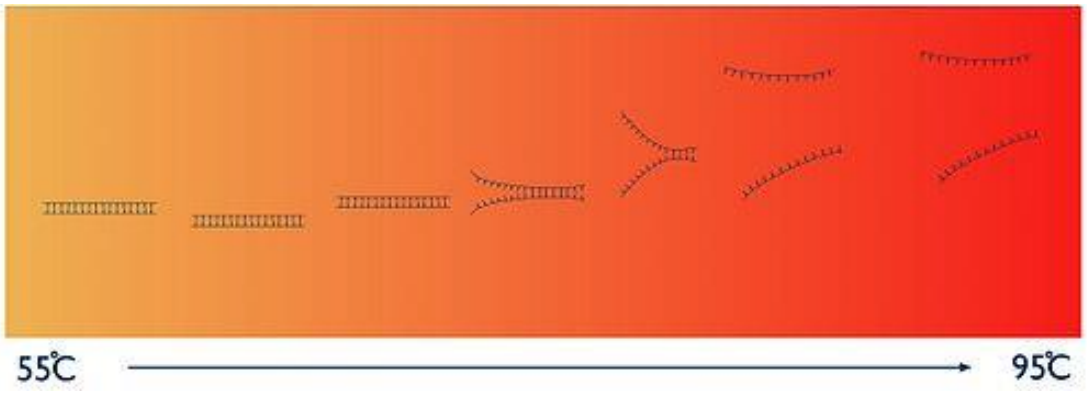
Below: Robotic technology used for the preparation of many genetic samples for HRM analysis. In this photo, the machine's glass case is open so we can view the components. This will close once the robot commences its process, therefore reducing contamination issues & mechanical hazards. The large ring is a row of tubes that will receive genetic samples for the next step, HRM analysis.

Method

HRM analysis is performed on double stranded DNA samples. Typically the user will do a polymerase chain reaction (PCR) prior to HRM analysis to amplify the DNA region in which the genetic mutation of interest is located.

After the PCR process the HRM analysis begins. The process is simply a precise warming of the DNA from around 55˚C up to around 95˚C. At some point during this process, the melting temperature of the DNA is reached and the two strands separate or "melt" apart.

Diagram: The diagram below illustrates the effect of raising the temperature on the double-stranded DNA, resulting in this 'melting' or separating into two strands.



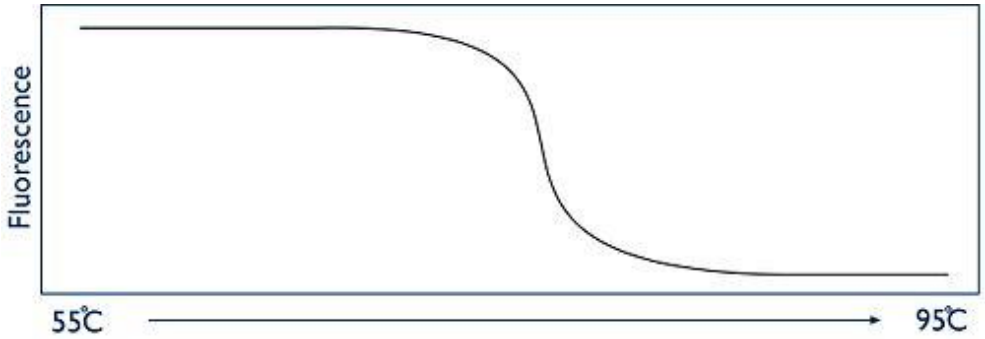
Real-time monitoring & dyes

The secret of HRM is to monitor this process happening in real-time (monitored constantly, for slight changes). This is achieved by using a fluorescent dye. The dyes that are used for HRM are known as intercalating dyes and have a unique property:

- The dye bind specifically to double-stranded DNA and when they are bound they fluoresce brightly.
- In the absence of double stranded DNA they have nothing to bind to and they only fluoresce at a low level.

Illustrating this relationship of dye fluorescence & temperature in a HRM

1. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions of copies of the amplicon.
2. However, as the sample is heated and the two strands of the DNA melt apart, presence of double stranded DNA decreases and therefore the fluorescence is also reduced.
3. The HRM machine measures the levels of fluorescence as this process happens. The machine then simply plots this data as a graph known as a melt curve, showing the level of fluorescence vs the temperature - see an example 'melt curve' below:



Spot the difference

The melting temperature at the point at which two DNA strands separate is **entirely predictable**. This is because the **melt temperature depends on the sequence of the DNA bases**, and the chemical bonds that they have between their pairs.

The nucleic acid base pairs (A-T & G-C) have numbers of chemical bonds, specifically **hydrogen bonds** connecting them across the double-strand of DNA.

- **Adenine–thymine (A-T)** base pair has **two** hydrogen bonds
- **Guanine–cytosine (G-C)** base pair has **three** hydrogen bonds

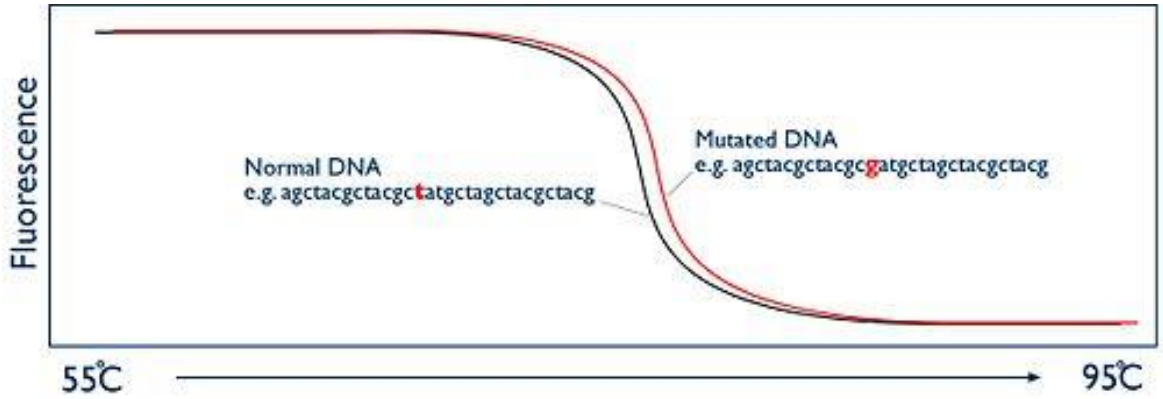
Comparing samples: If you are comparing two DNA samples from two different plants, they should show exactly the same shaped 'melt curve' (see later diagram below).

Mutation in the sequence?

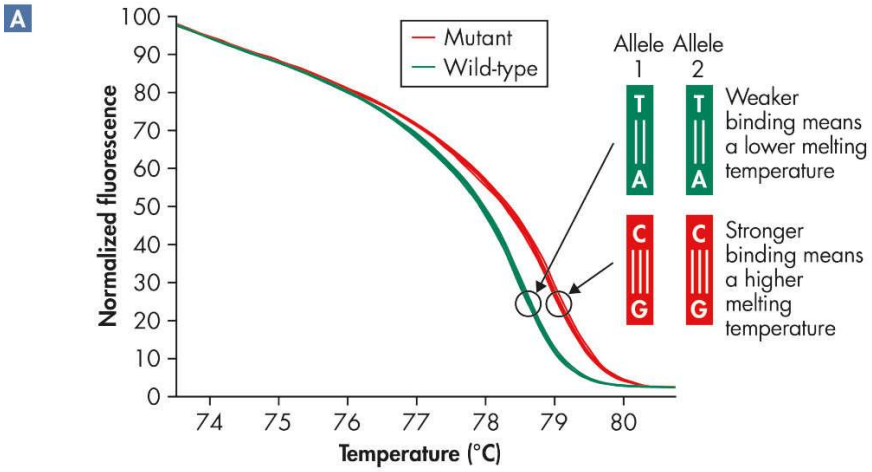
If one of those DNA samples has a **mutation in the DNA** region that you have amplified, then this will alter the temperature at which the DNA strands melt apart (i.e., separate from each other). This is because **breaking apart chemical bonds takes energy** (in this case, the energy is the temperature applied to the DNA strands). The more hydrogen bonds in the strand (i.e., 3 x hydrogen bonds in G-C pairs) will require more energy to break apart than A-T pairs (only 2 x hydrogen bonds present). **So, if there's a change in the DNA sequence of a plant, there will be a different temperature required to separate the strands and the 'melt curves' will appear different.**

The difference in the 'melt curve' may only be slight (perhaps only a fraction of a temperature degree), but because the **HRM machine can monitor this process in "high resolution" in real-time,** it is possible to accurately document these changes and therefore identify if a mutation is present or not.

Below shows a **melt curve** showing an example of a shift in the genetic sequence, and the resulting HRM curve difference. *(See description below for unpacking the graph*).



Description of melt curve: Graph axes: X-axis (temperature), Y-axis is the level of fluorescence detected. In this example, there was a **base pair change from a T (thymine) to guanine (G),** resulting in a different number of hydrogen bonds in the base pair set (resulting in a G with 3 H-bonds across the DNA strand). Due to this change, there was a higher temperature is required to separate the double DNA strands, seen in the red curve slightly shifting to the higher temperature before the DNA strands separated.



Wild type, heterozygote or homozygote?

The analysis of the HRM can become slightly more complicated because **organisms contain two (or more) copies of each gene**, known as the **two alleles**.

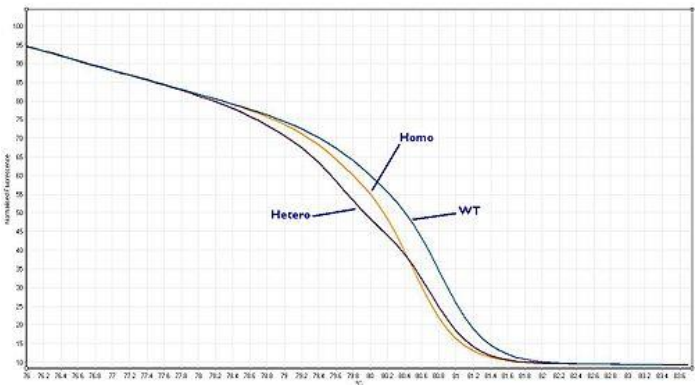
So, if a DNA sample is taken from an individual organism and amplified using PCR, both copies of the region of DNA (alleles) of interest are amplified.

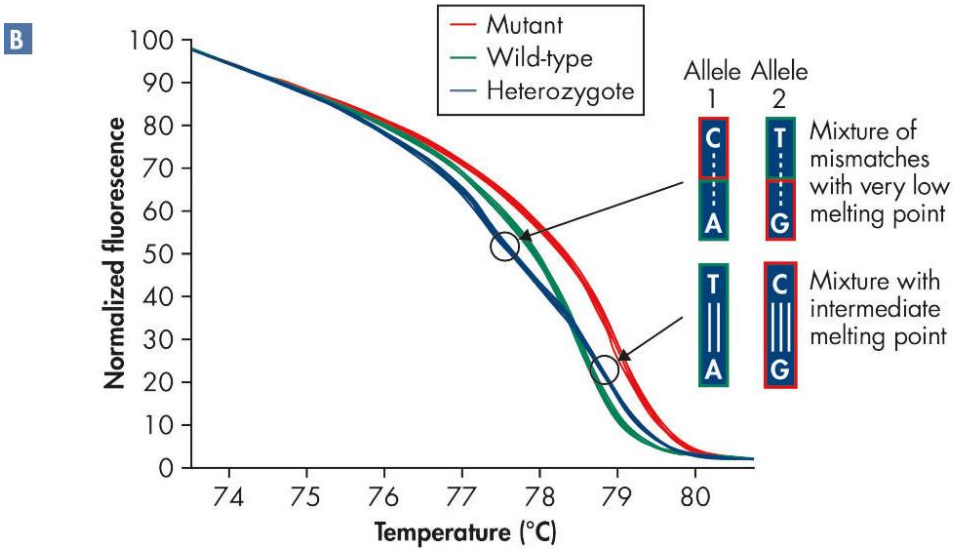
Therefore, if we are looking for a mutation in the DNA there are **now three possibilities:**

1. **Neither allele** contains a mutation ("wild-type")
2. **One or other allele** contains a mutation ("heterozygote")
3. **Both alleles** contain a mutation ("homozygote")

These three scenarios are known as **"Wild –type", "Heterozygote" or "Homozygote"** respectively. (Could link to punnet square learning & terminology of allele crosses). Each gives a melt curve that is slightly different. With a high quality HRM assay it is possible to distinguish between all three of these scenarios.

An example HRM curve graph shows these three options below: wild-type, heterozygote, and homozygote.





Bringing it all together: The above melt curve combines multiple learning concepts covered in this module: the relationship of the differences in melting temperatures & the genetic sequences, as well as allele combinations & the resulting HRM melt curves.

Suggested Activities & Tasks

This technology highlight focussed on HRM could feed into **various student activities & engagement opportunities,** including:

- **Discussions:** whole-class or small group. **Facilitate the connecting of key genetics** terms and scientific technologies discussed prior in genetics module. Bring it all together.
- **Group projects** – bringing together multiple genetics concepts, small group projects could highlight key terms of genetics, showcase a technology & its application to solving a particular challenge or issue (e.g., development of improved food crops).
- **Personal inquiry tasks**– e.g., focussing upon a specific application of this technology to improving our understanding of genetics & broader applications (why is it important?) Could use various sources including peer-reviewed research or other sources to inform this.
- **Science communication poster or presentations** – communicating the key elements of this technique in a digestible manner for peers to understand. The what, how & why.
- **Guest feature in-class** – e.g., a scientist who uses this genetics technology in their research field – e.g., geneticist/ molecular biologist (focus & aims of their research, career pathways etc). This could also be a local science communicator – search for them in your region & connect.