

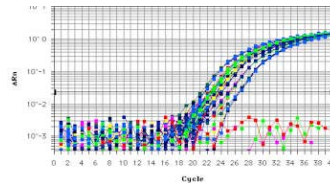


Using Melt Curve Analyses for Experimental Inquiry

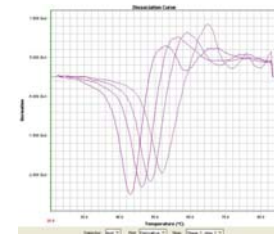
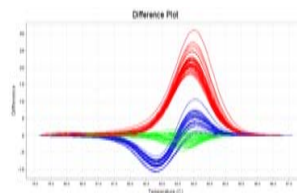
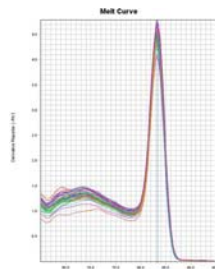
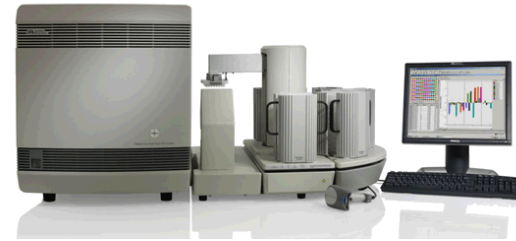
Madeline O'Donoghue
Senior Applications Specialist
– Real Time PCR Systems

Real-Time PCR and DNA Melt Curves

- 1996 – ABI 7700 System



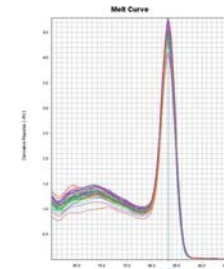
- Present – StepOne™, StepOnePlus™, 7300, 7500, 7500 Fast, 7900 HT systems



Three different Melt Curve Analyses

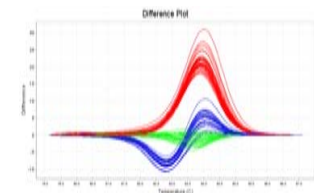
● Standard Melt Curve

- Using non-specific binding dsDNA Fluorescent Dye
- Using basic melt curve program of Real Time PCR Instrument
- Using basic data analysis within standard Real Time PCR Instrument Software



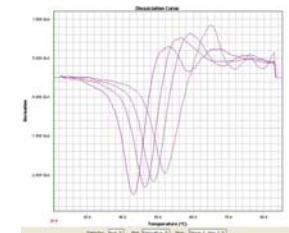
● High Resolution Melting Analysis

- Using higher concentrations of non-specific binding dsDNA Fluorescent Dye
- Collecting more data points (usually 10-30 data points per degree) during the melt curve program of Real - Time PCR Instrument
- Using advanced normalising algorithms and new plot displays for analysis

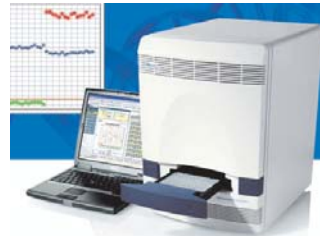
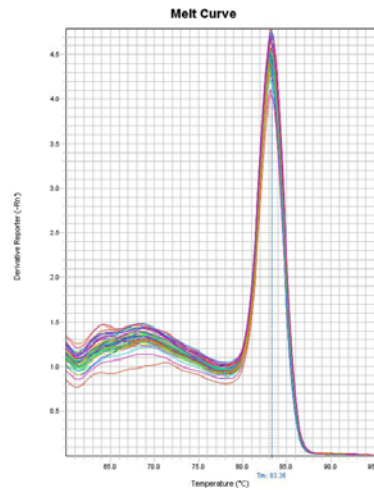


● Protein Thermal Shift Assay

- Using non-specific acting dye sensitive to hydrophobic regions of all proteins
- Using basic or advanced melt curve programs of Real - Time PCR Instrument
- Data visualised in software or exported for more complex analysis

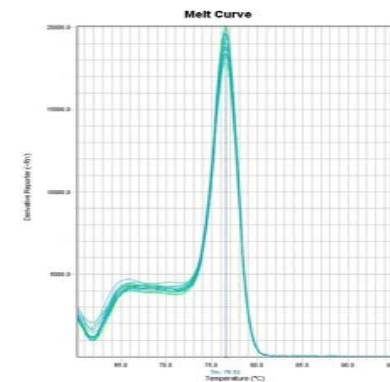
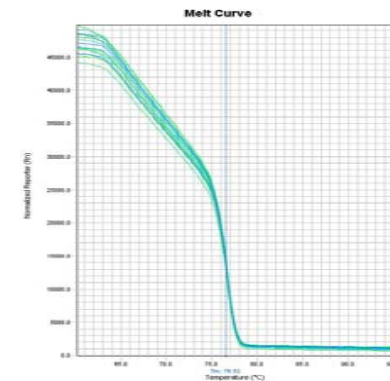


Standard Melt Curve Analysis

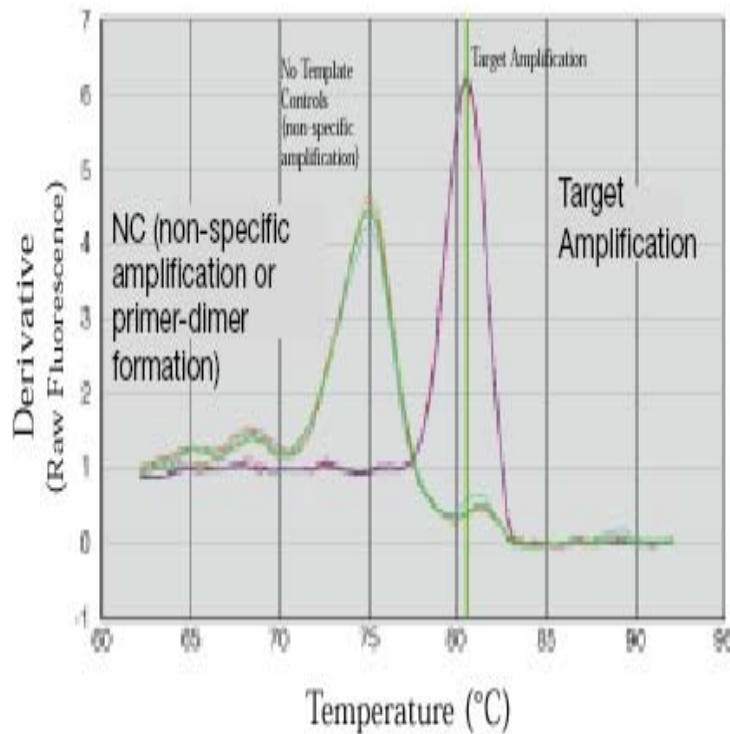


Standard Melt Curve Analyses

- Generated by slowly denaturing (melting) the DNA sample through a range of temperatures in the presence of a dsDNA binding dye
- When the dsDNA melts into its fully denatured form, a sharp decrease in fluorescence is detected
- The T_m is defined as the point in the melting curve where 50% of the DNA is double-stranded, and 50% is single-stranded (melted)
- To visualize the T_m more clearly, the negative first derivatives are often plotted, making the T_m s of the products appear as peaks

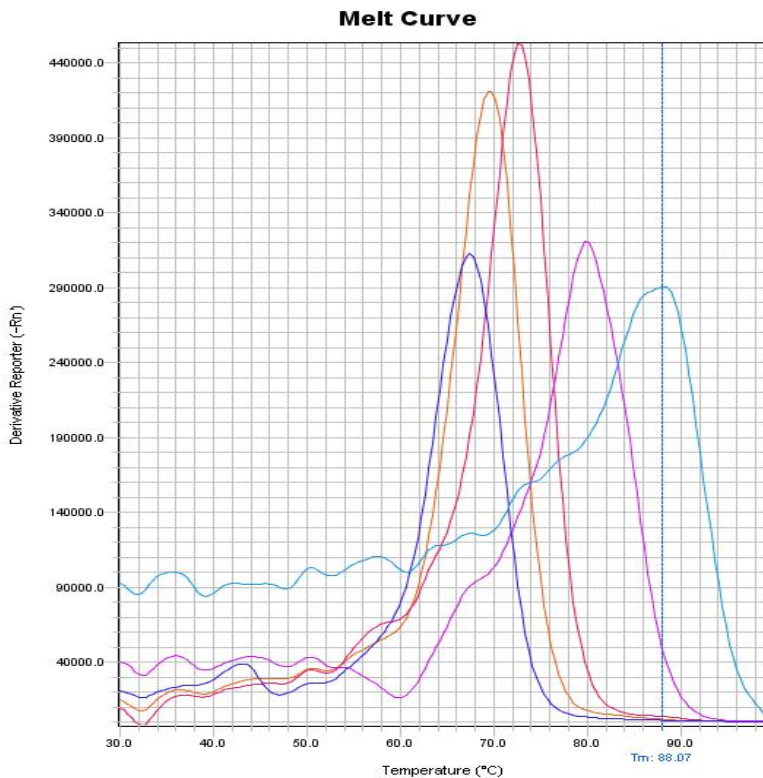


Standard Melt Curve as Quality Control



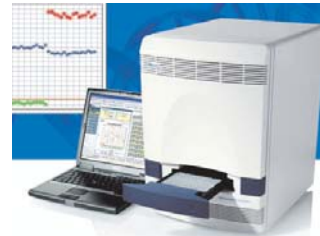
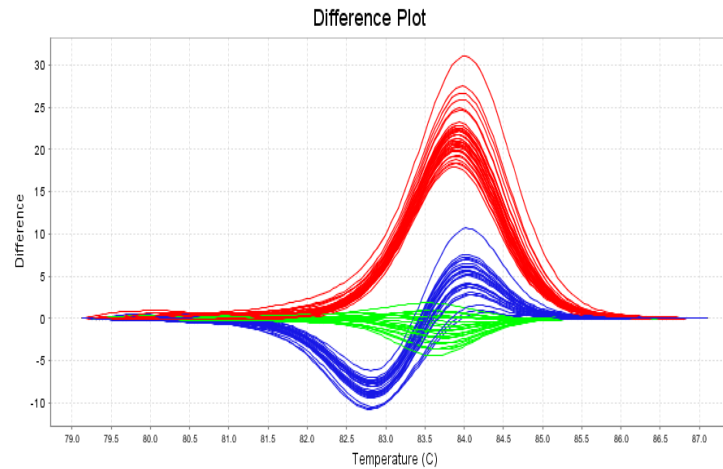
- Primer dimers are the products generated when two primers anneal to each other instead of the target of interest
 - Amplicon size is significantly shorter than product of interest
 - T_m values are significantly lower than product of interest
- Non-specific products generated when primers anneal to an area different from the region of interest
 - Amplicon size may not vary from expected amplicon size
 - T_m values may not differ significantly from product of interest

More Complex Analysis with Standard Melt Curves



- Tm values are used to aid in identification of different products from mixtures of unknown samples
- Modifying amplicon length or tagging primers can allow for multiplexing
 - Ensuring a 2-3°C Tm difference between expected amplicons

High Resolution Melting Analysis



High Resolution Melting

HRM is different from a regular SYBR[®]Green dye melt curve in three ways:

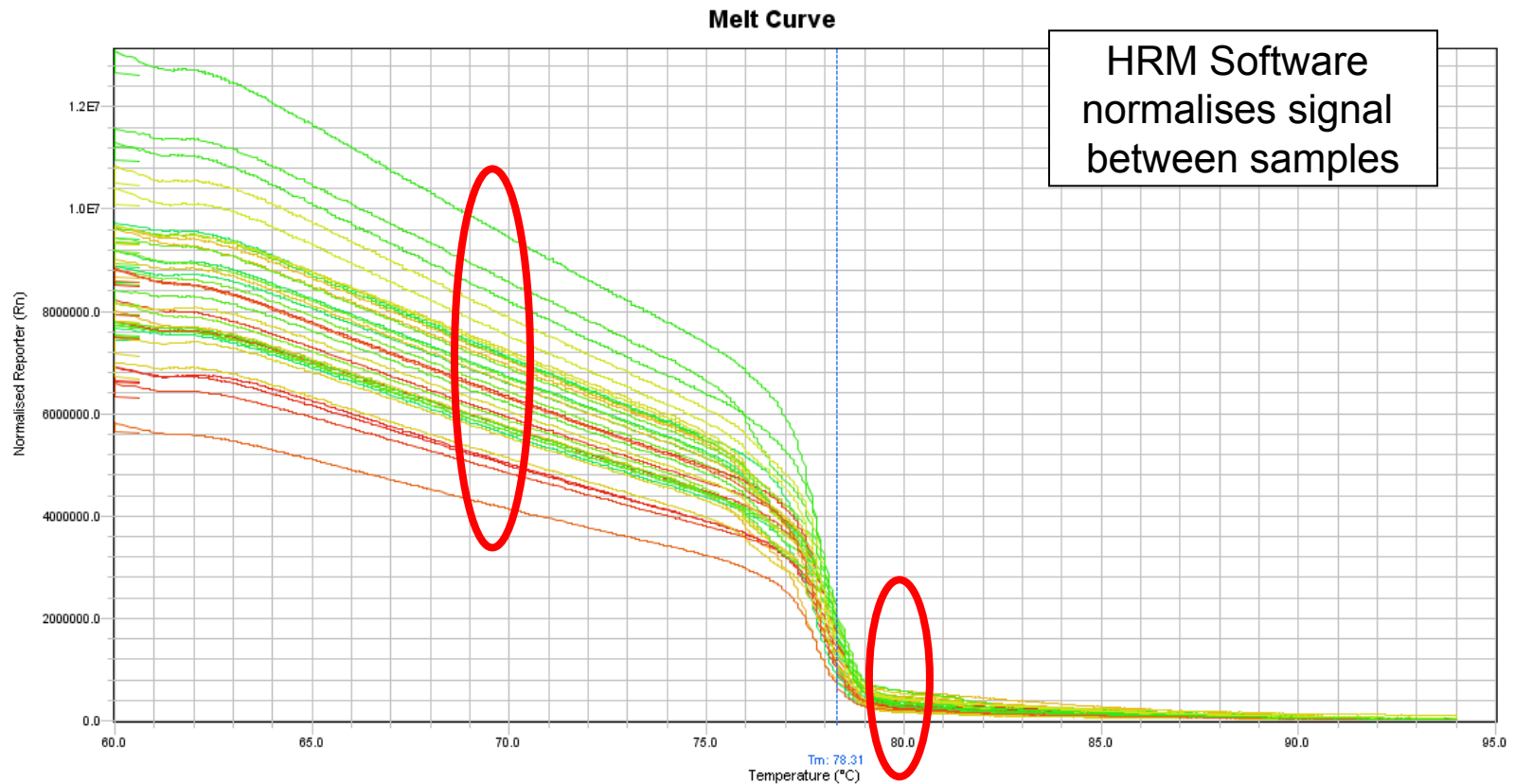
1. Chemistry: Uses brighter dsDNA binding dyes that do not inhibit PCR at high concentrations
2. Instrument: More data points are collected than a standard melt curve
3. Software: New fluorescence normalization algorithms and plots

It enables:

- Determination of a more exact melting point of a DNA fragment
- Significantly improved discrimination power to visualise differences between DNA fragment melting behaviour

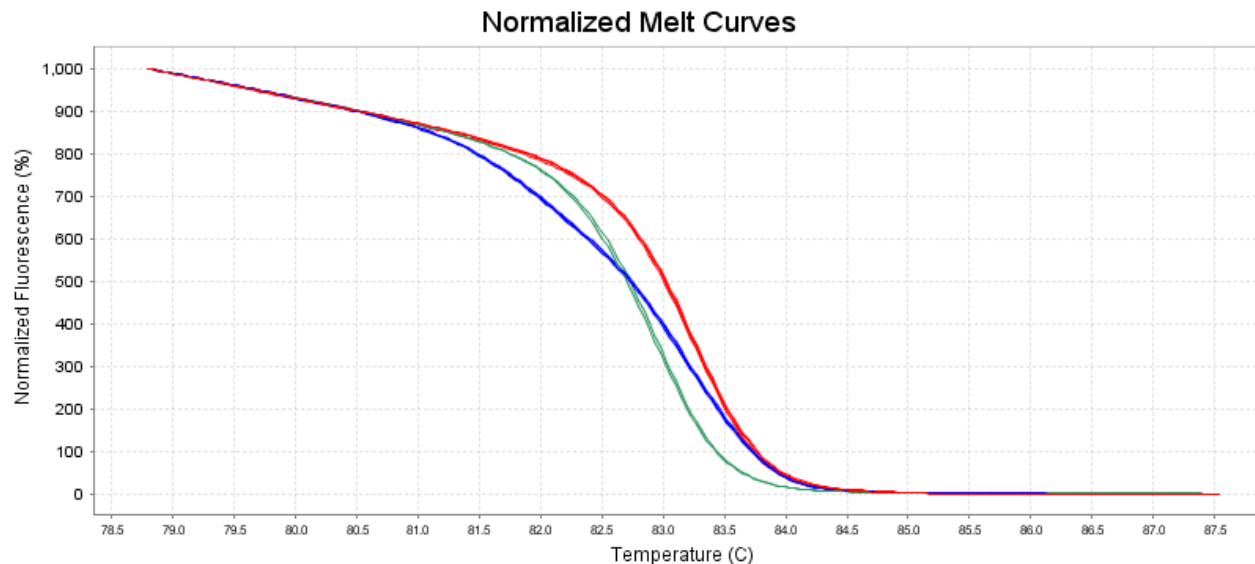
HRM New Software Algorithms

- High resolution data, collecting a minimum of 10 data points per degree
 - on average 20-30 data points/degree



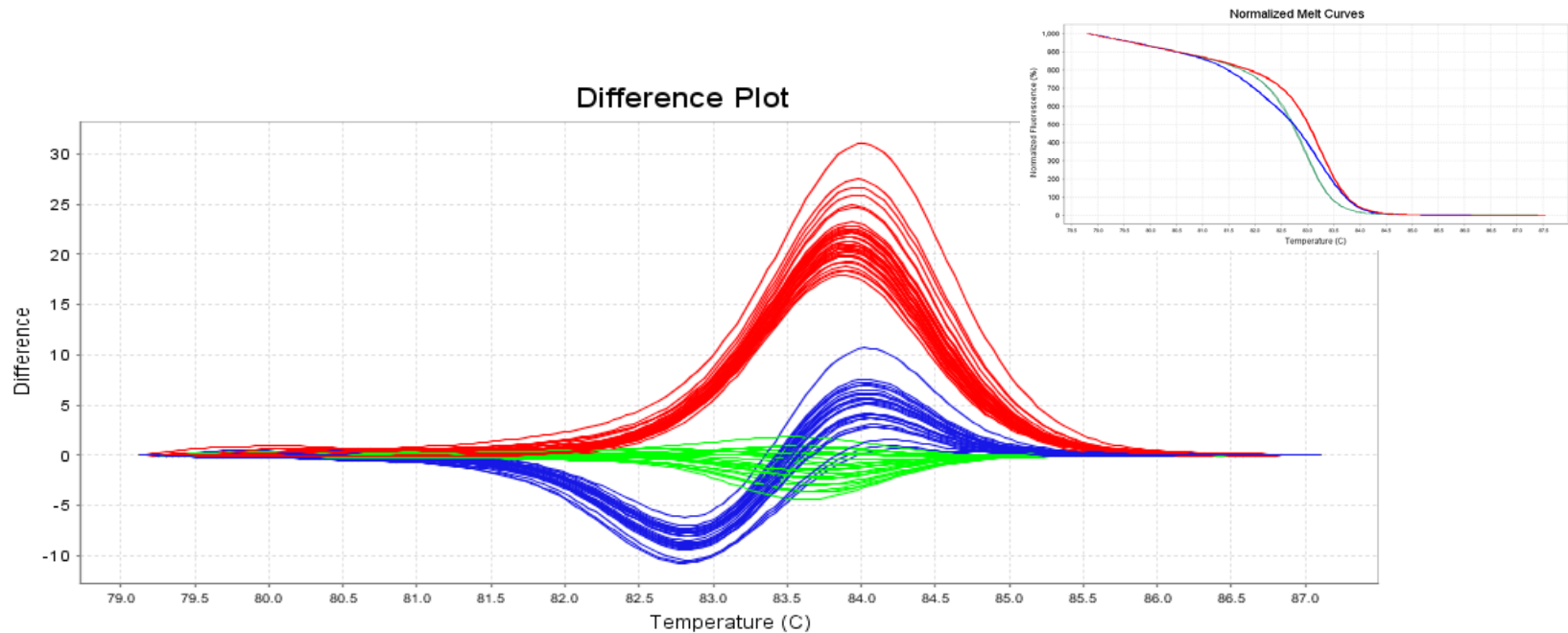
Reading a High Resolution Melt Curve

- The analysis is based on the difference in curve shape as well as T_m .
 - Heterozygotes (blue) have a different curve shape compared to the wild type homozygote (green) and the variant homozygote (red).
 - The two homozygotes are distinguished from each other based on the difference in T_m .
 - Note the T_m difference between homozygous curves is less than 0.5°C

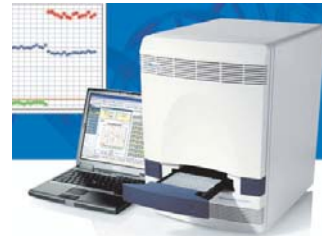
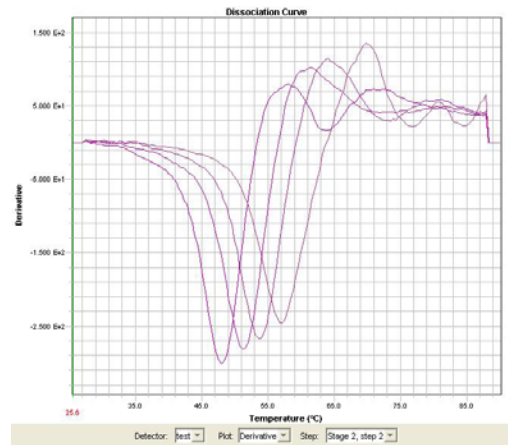


Reading High Resolution Melt Curves

- An alternative display is by the difference plot.
 - This applies a sample as the 'normal' baseline and displays the others as a function of similarity to 'normal'



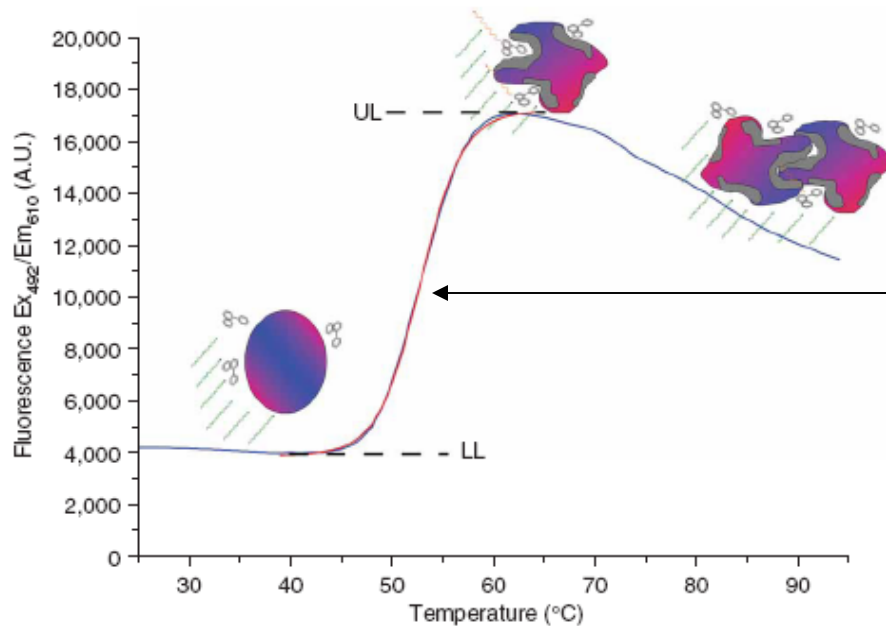
Protein Melt Curve Analysis



What is Protein Melting?

- a.k.a. Protein Thermal Shift Assay or Protein Stability Assay
- Uses a dissociation curve and ‘protein-binding’ dyes
 - e.g. SYPRO[®] Orange
- During heating, the protein is unfolded and the hydrophobic region of the protein is exposed
- SYPRO[®] Orange and other similar dyes, are sensitive to this change in the environment and this leads to an increase in fluorescence during the unfolding process

Protein Stability Assay



Calculate the inflection point of the curve

$$y = LL + \frac{(UL - LL)}{1 + \exp\left(\frac{T_m - x}{a}\right)}$$

The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability

Frank H Niesen, Helena Berglund & Masoud Vedadi

Nature Protocols Published online 13 September 2007; doi:10.1038/nprot.2007.321

Boltzmann Equation: Schellman, J.A. Temperature, stability, and the hydrophobic interaction. Biophys. J. 73, 2960–2964 (1997).

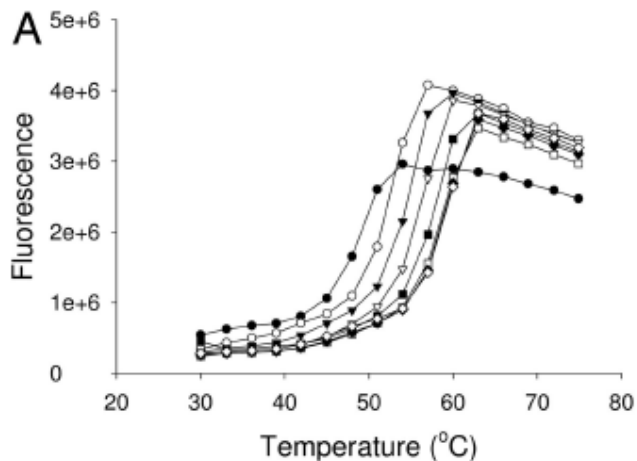
Thermal Shift Assays Using Real-Time PCR Systems

- Main use and applications:
 - Identification of unknown protein ligands
 - Protein inhibitor screening
 - Identification of inhibitors of target proteins from compound libraries
 - Protein stability assays
 - Protein-substrate interactions

- Benefits
 - Fast, universal method for screening a range of different conditions
 - Small reaction volumes requiring few μg of protein input
 - Ability to identify favourable ligand interactions with no prior knowledge of compound
 - Flexible workflow for use on any Applied Biosystems Real-Time PCR System

Thermal Shift Assay Example

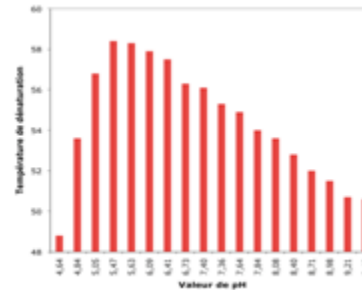
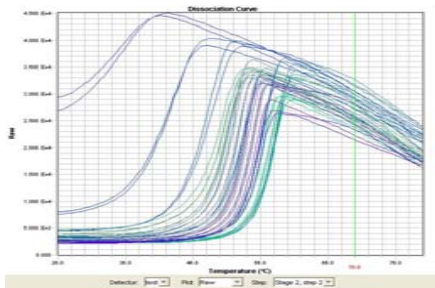
- Screening to find ligands that promote protein stability
 - Analysis of Sult1C1 and its binding to PAP (3-phosphoadenosine-5-phosphate)



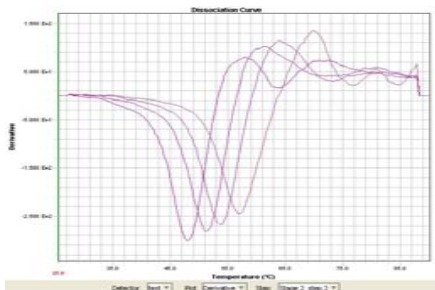
- Notice the shift of the T_m of each curve as the concentration of PAP increases

0 (●), 0.08 (○), 0.35 (▼), 1.4 (△), 5.6 (■), 11.2 (□), 22.5 (◆), and 45 (◇)
 mM PAP

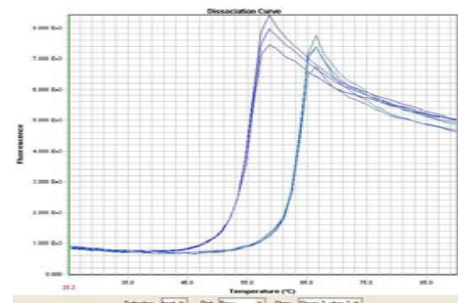
Protein Melting Example Data



- Variation in T_m as a function of pH



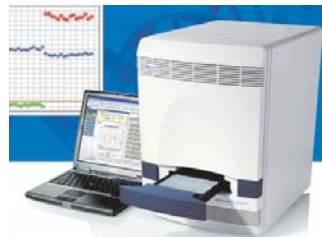
- Effect of salt concentration on T_m



- Ligand binding increases relative thermal stability of protein

Versatility of all AB Real-Time PCR Systems

- Utilise the flexibility of your Real-Time PCR Systems to the maximum potential
 - Quality control of Real-Time PCR Assays
 - Multiplexing with non-specific dyes
 - Mutation scanning with HRM
 - Genotyping with HRM
 - Protein - Ligand affinity screening (screening compound libraries)
 - Supporting protein crystallography workflows and drug discovery pathways
 - And more!



Acknowledgements

- Naima Nhiri and Eric Jacquet
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