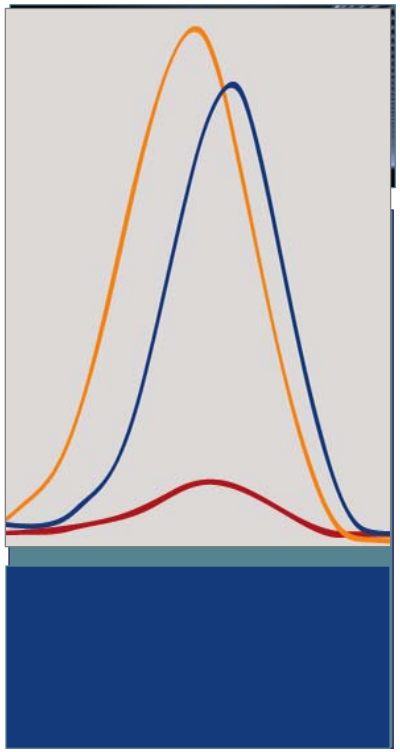
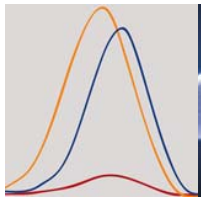
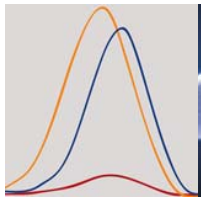


HRM Powered by Precision Melt Analysis™ Software



Overview

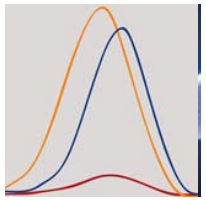
- Introduction to High Resolution Melt (HRM)
- Applications
- HRM versus Melt Curve
- Assay Design and Optimization
- Precision Melt Analysis software

High Resolution Melt

- Post-PCR melt analysis method
- Discriminates dsDNA based on sequence length, GC content or strand complementarity
- Detects a single base difference
- Rapid, inexpensive sequence screening method
  - Mutation sequence can be unknown
  - Samples are further processed to identify mutation sequence

Sample → PCR → HRM

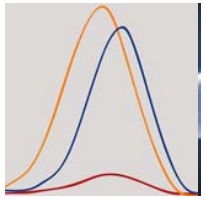


HRM Applications

- Mutation discovery/gene scanning
- SNP genotyping
- DNA methylation analysis

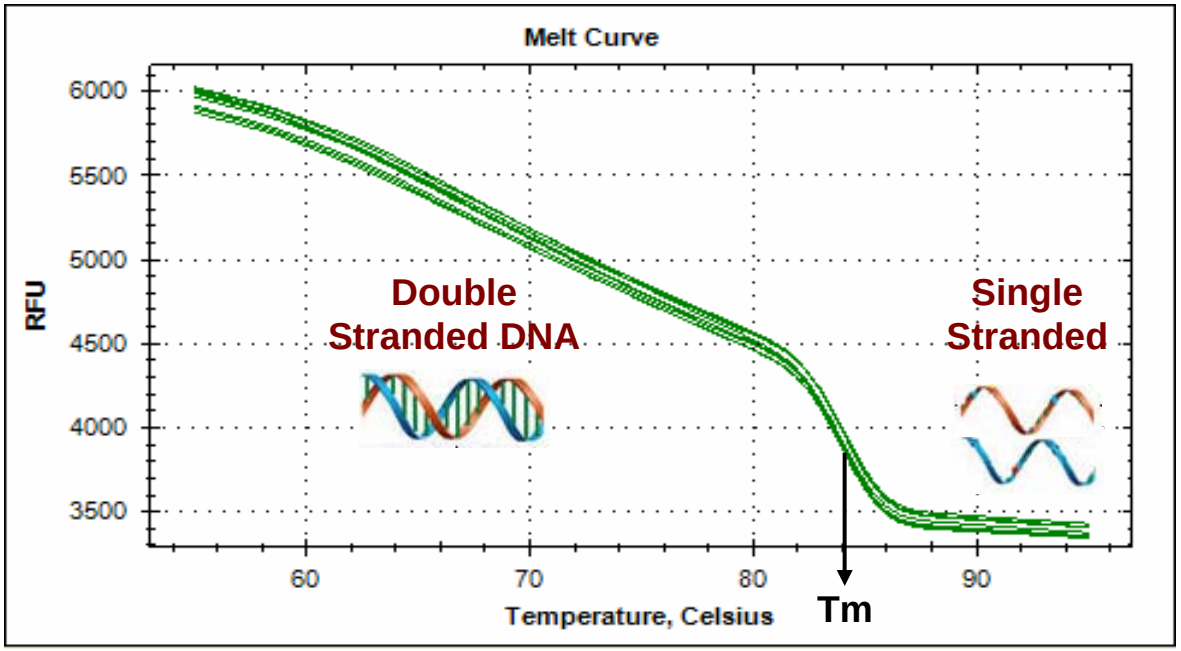
95% of all applications

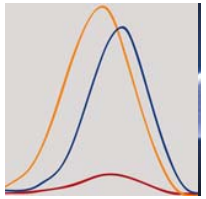
- Species identification
- DNA fingerprinting
- Screening for loss of heterozygosity
- Allelic prevalence in a population
- Characterization of haplotype blocks
- HLA compatibility testing
- Identification of candidate predisposition genes

Melt Curve Analysis

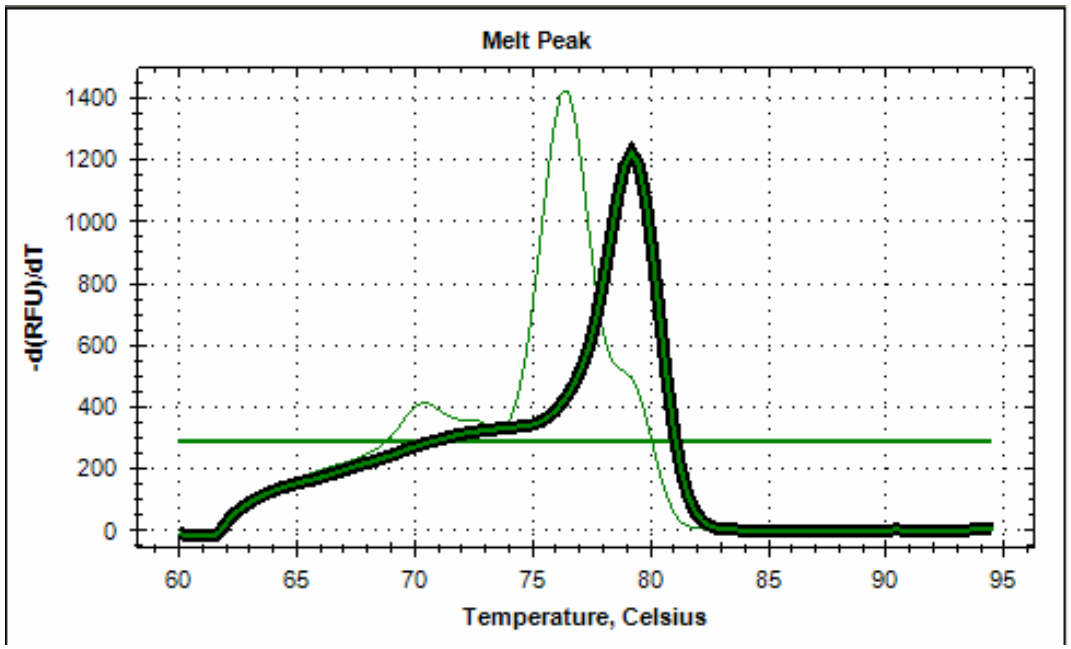
- After real-time PCR amplification, a melt curve is performed in presence of a DNA binding “saturation dye”
- Melting temperature (Tm)
  - DNA is half double and half single-stranded
  - Depends on nucleotide content and length



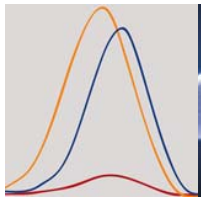


Melt Curve Analysis

- Distinguish products based on their Tms
  - Plot negative rate of change of fluorescence vs. temp (-dI/dT) for easy discrimination of Tms

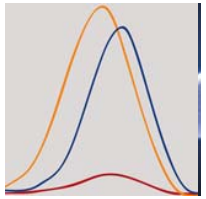


BIO-RAD

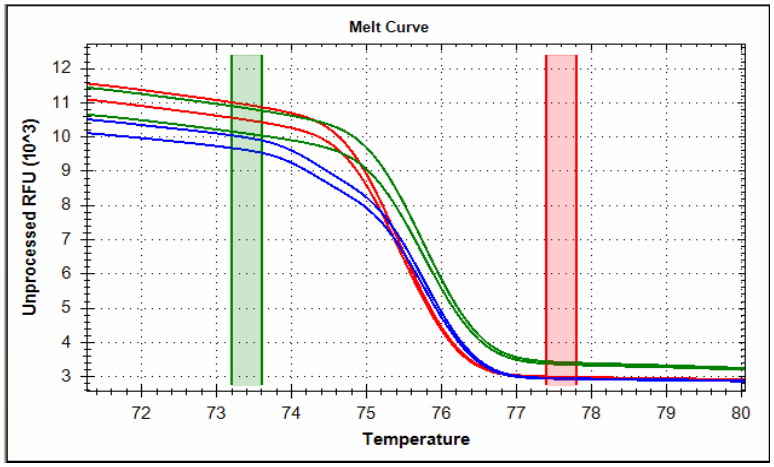
HRM Versus Melt Curve

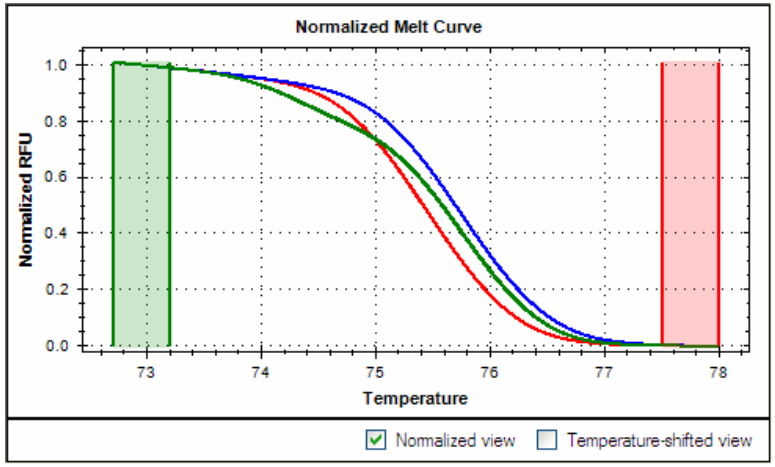
- HRM is an extended analysis of a melt curve
- Requires additional analysis software
  - Normalize melt curves
  - Apply an optional temperature shift
  - Plot curves in a difference graph for easy visualization
  - Clusters curves into groups representing different genotypes/sequences

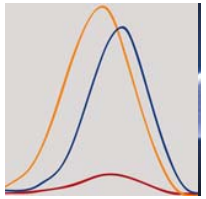



Normalization

- Pre-melt (initial) and post-melt (final) fluorescence signals of all samples are normalized to relative values of 100% and 0%
- Eliminates differences in background fluorescence between curves

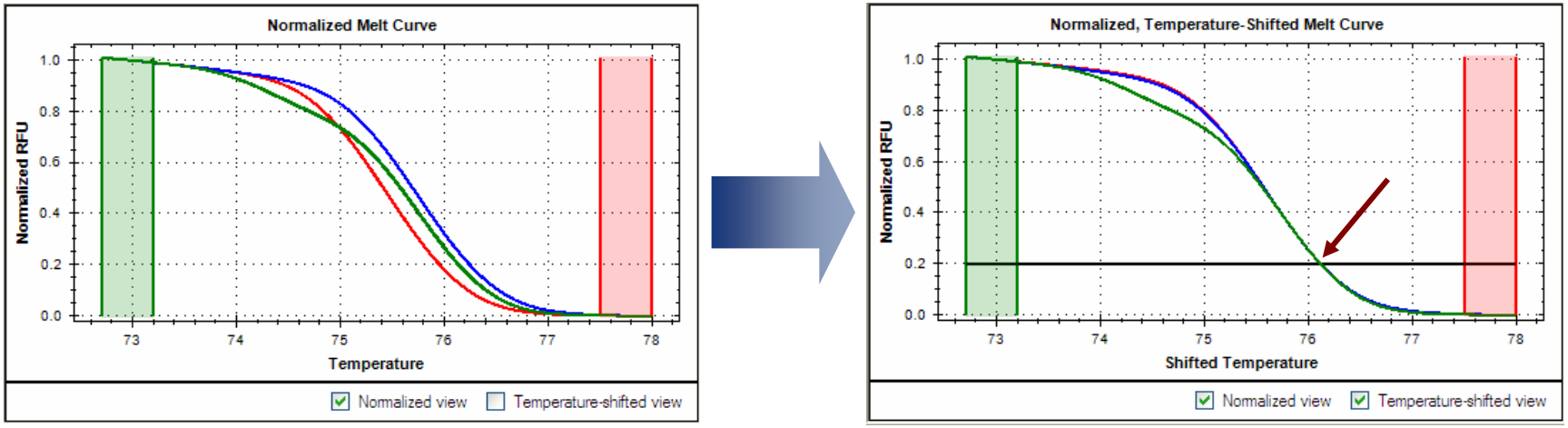


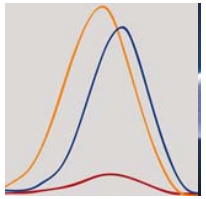


Optional Temperature Shift

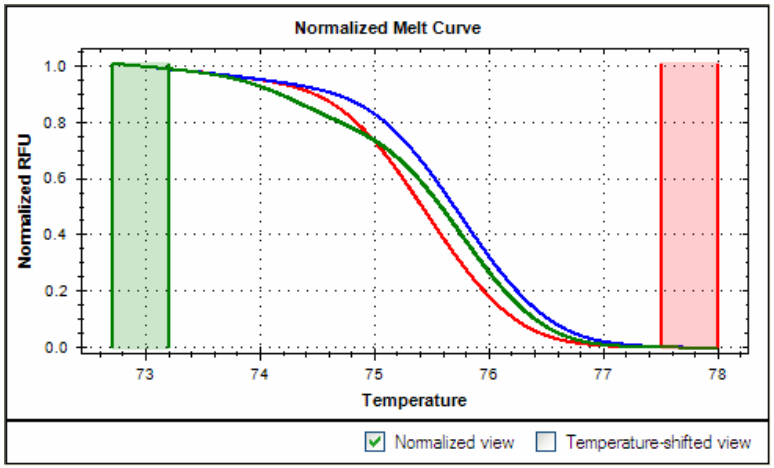
- Shift curves along the temperature axis so they meet at a specific temperature at which the DNA is denatured
- Easier to distinguish heterozygous samples from the now superimposed wild type homozygous samples

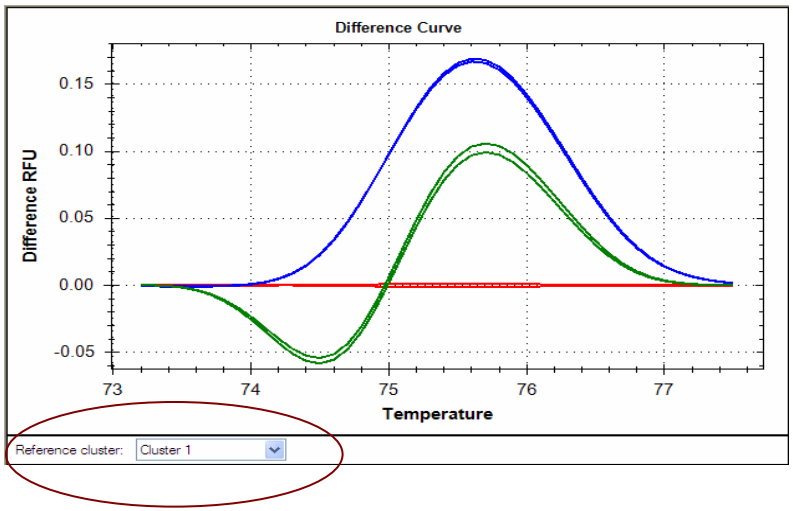


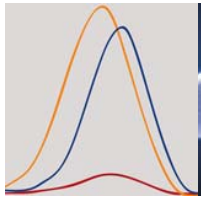


Difference Plot

- Magnify curve differences by subtracting each curve from the most abundant type or from a user-defined reference
- Sets a baseline, so small differences become visible

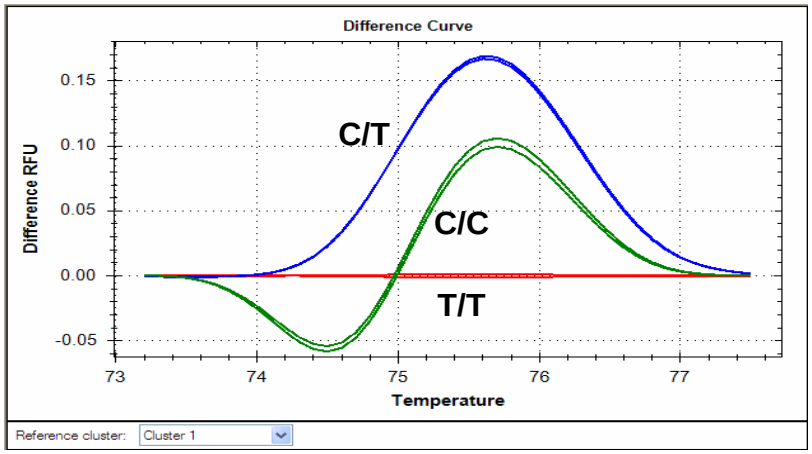




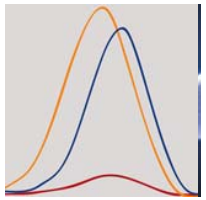



Cluster Analysis

- Software clusters similar curve shapes automatically into groups representing different genotypes (sequences)
- Software then auto-calls samples to a genotype depending on where their curve shape clusters

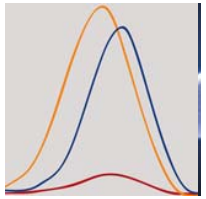


Well	Content	Sample	Cluster
C06	Unkn-10	A t/t	Cluster 1
C07	Unkn-10	A t/t	Cluster 1
B06	Unkn-09	C c/c	Cluster 2
B07	Unkn-09	C c/c	Cluster 2
A06	Unkn-08	H c/t	Cluster 3
A07	Unkn-08	H c/t	Cluster 3

Experiment Considerations

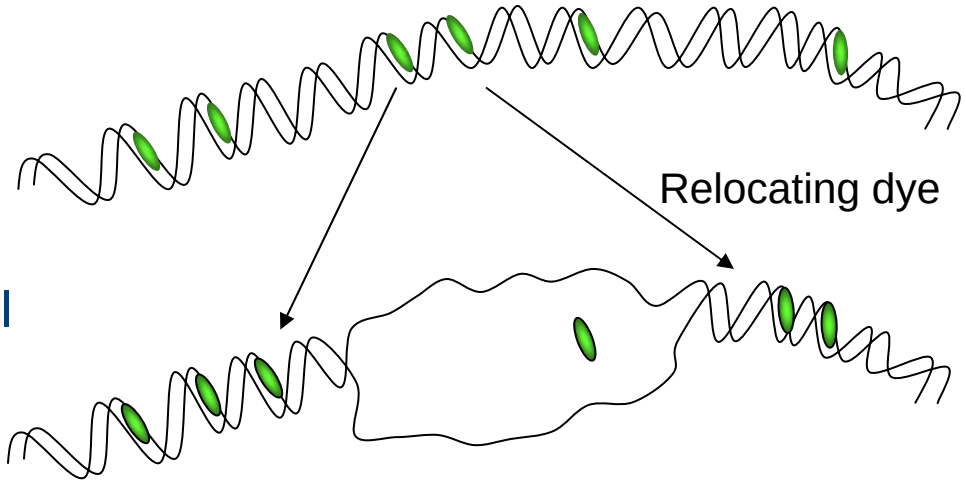
- Amplify a single product at high efficiency
  - No primer-dimers or non-specific products
- Generate sufficient PCR product (C(t)s ≤ 30)
- Samples need equal PCR efficiencies and plateau fluorescence for comparison
- Analyze short PCR products, the smaller the better
- Uniform reaction mix/sample concentrations
- Capture data over at least a 10 °C melt curve range

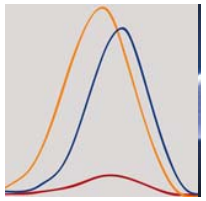


Why Special Reagents?

- SYBR Green inhibits PCR at high concentrations
- As DNA melts, freed SYBR relocates and rebinds dsDNA
- Consequently, the change in fluorescence may not accurately reflect DNA sequence composition

SYBR® Green I



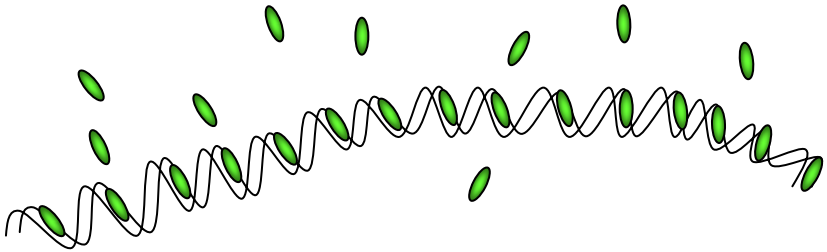



Why Special Reagents?

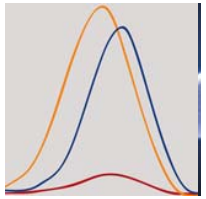
- “Saturation” dyes are less toxic to PCR than SYBR
- At higher concentrations, the frequency of dye relocation events is reduced, improves results

Saturation Dyes

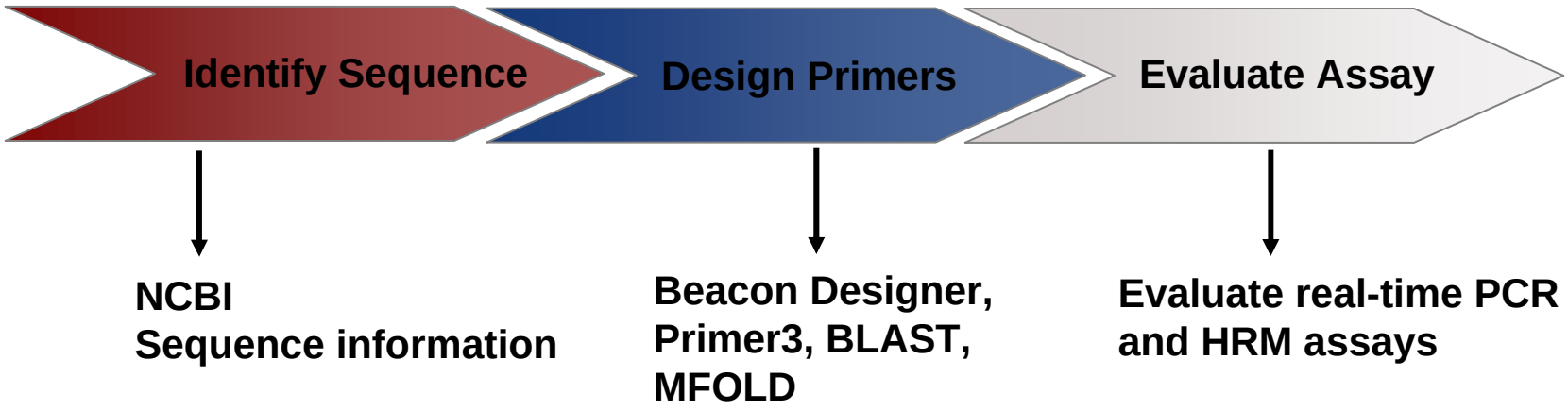
LC Green (Plus)	Idaho Technologies
SYBR GreenER	Life Technologies
Syto9	Life Technologies
Resolight	Roche
Bebo	TATAA Biocenter

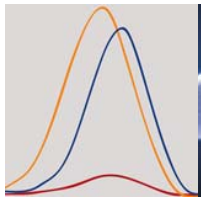


Dye saturation leaves no room for relocation events during melting



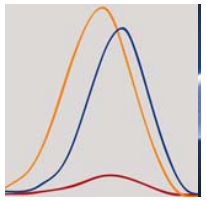
Assay Design Workflow



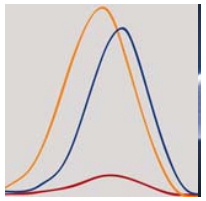
Target Sequence

- SNP analysis
  - Identify the right sequence
  - Search for SNPs based on gene, location or function
  - Find variation sites (avoid variations that impact melt curves)
- Methylation Assays
  - CpG sites in primers and within the sequence
  - Fragment length
- NCBI SNP Databases
  - http://www.ncbi.nlm.nih.gov/SNP

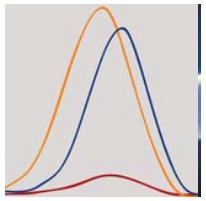
Primer Design

- Primer guidelines
  - BLAST primer sequences
  - 18-24 bases
  - 40-60% G/C
  - Balanced distribution of G/C and A/T bases
  - Annealing between at 55-65°C
  - No internal secondary structures (hair-pins)
- Primer pairs
  - Similar Tms, within 2-3°C
  - No significant complementarity (> 2-3bp), especially in 3’ ends
- Primer binding sites
  - Avoid targets with secondary structure
  - Avoid pseudogenes

Amplicon Design

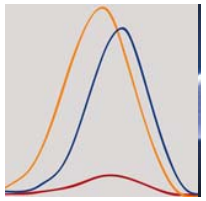
- Use similar criteria for SYBR Green assay
- Short amplicons maximize differences in melting behavior between similar sequences
  - 70-150bp is desired (50-250bp acceptable)
  - Longer amplicons yield more complex profiles, with multiple melting domains, consider melt domain complexity (M-fold)
  - Local sequence context can influence mutation detection
  - Overall GC content and position of the mutation in the fragment does not have a significant effect on mutation detection
- Determine Tm of amplicon variations in case of SNP determination

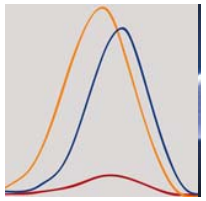
PCR Evaluation

- Poor PCR optimization = Poor HRM resolution
- No primer-dimers or non-specific products
  - Thermal gradient optimization of annealing temperature
  - Increase annealing temperature/decrease $MgCl_2$ concentration to increase specificity
  - Run No Template Controls (NTC)
  - Optimize primer concentrations if needed (500nM recommended to start)
- Efficient PCR amplification, want similar plateau fluorescence
- Generate sufficient product, C(t) values below 30
  - Degraded material or too little material, increase concentration

Always check amplification curves prior to HRM analysis



Precision Melt Analysis software

Workflow

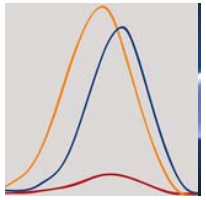
1.) Set up reactions using HRM compatible reagents

SsoFAST Eva Green Supermix

2.) Run amp + high resolution melt protocol on CFX96 or CFX384

98 ℃ for 2 min
98 ℃ for 2-5s
55-60 ℃ for 2-10sec (plate read)
Go to step 2, 39 more times
98 ℃ for 1 min
70 ℃ for 1 min
Melt curve 75 ℃ to 95 ℃ increment
***0.2 ℃ 10 sec hold** (plate read)*

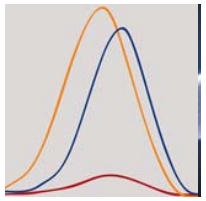
3.) In Precision Melt Analysis software, import the data file (.pcrd) to create a melt file (.melt)



HASP Key

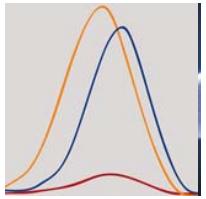
- The software will work only with a licensed HASP key. The HASP key presence will be checked at:

- 1. Startup of software
- 2. Importing pcrd file
- 3. Opening melt file
- 4. Opening melt study file



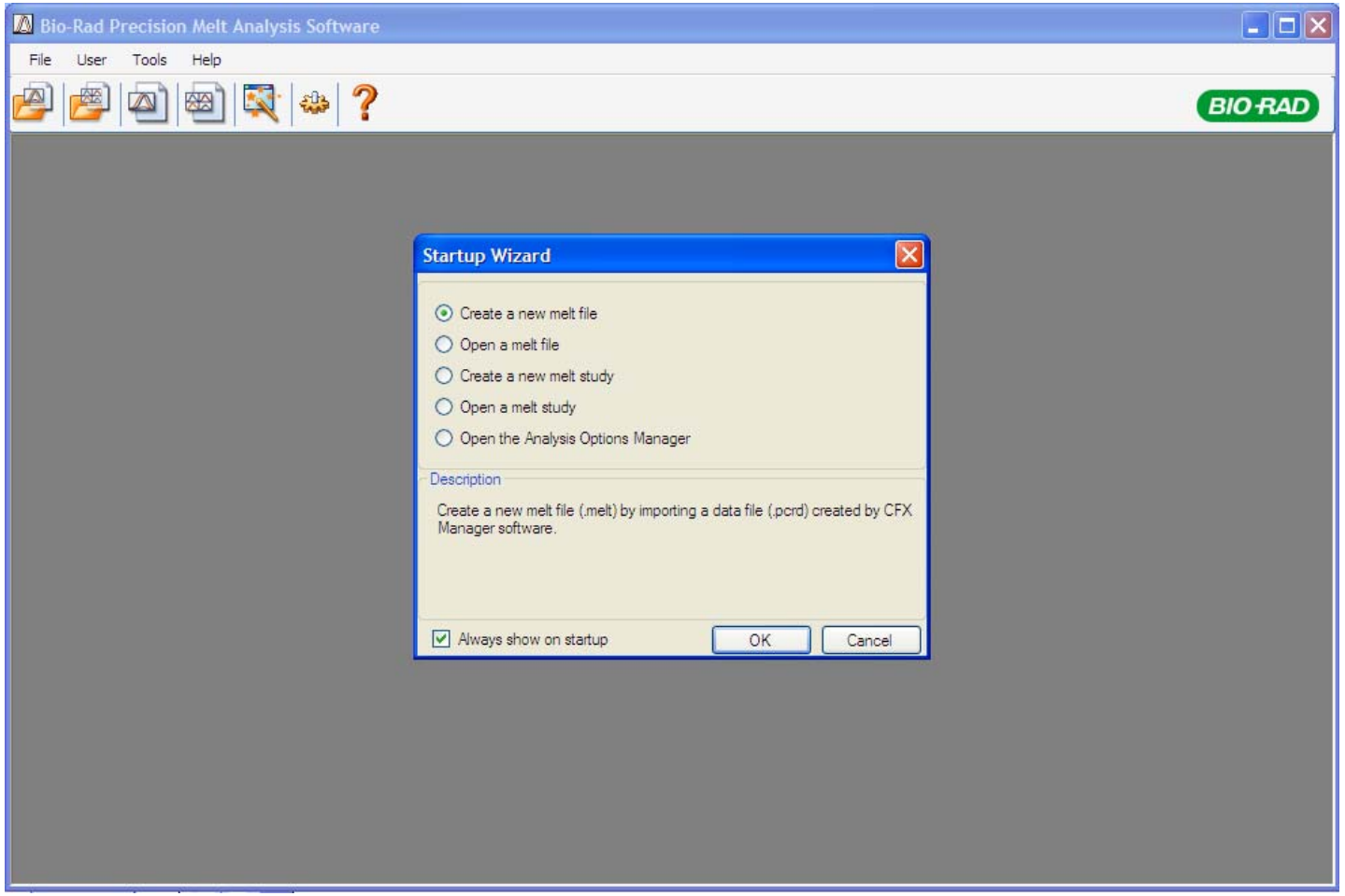
Precision Melt Analysis Software
Unique Features

- Compare data between multiple experiments by combining experiments into a single Melt Study
- View data in a plate view, for easy identification of sample results
- Share analysis settings between melt experiments using the Analysis Options Manager tool
- Analyze multiple experiments from a single plate using the Well Group feature
- View all charts in a single view



Startup Wizard

- Same intuitive navigation as CFX Manager software



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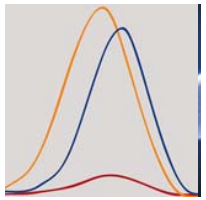
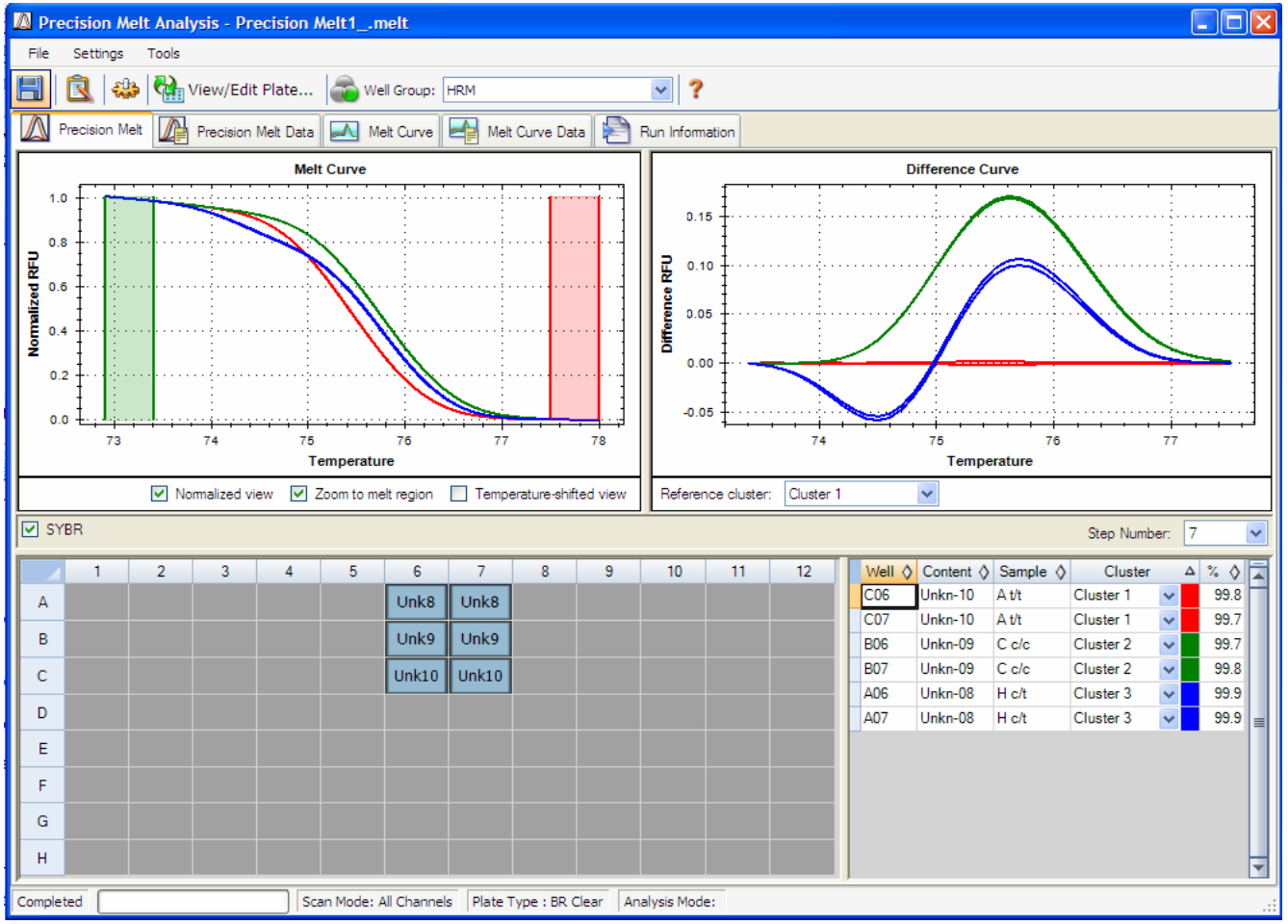


Data Analysis Window

- Multiple views of data, with easy interpretation of results



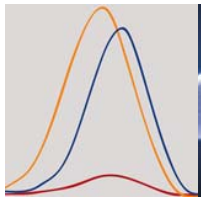
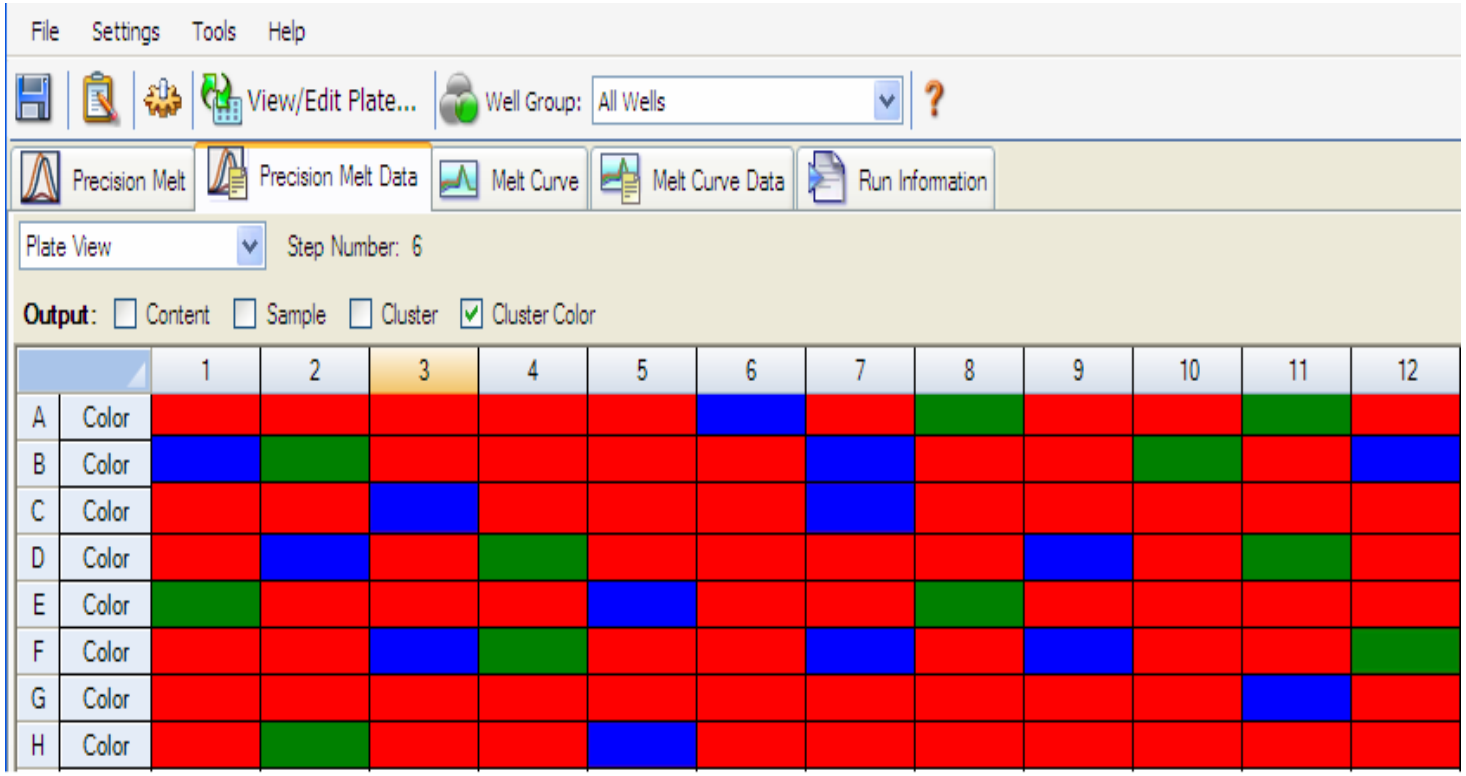
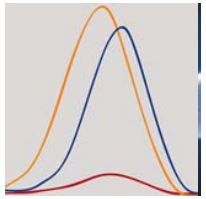


Plate View of Results

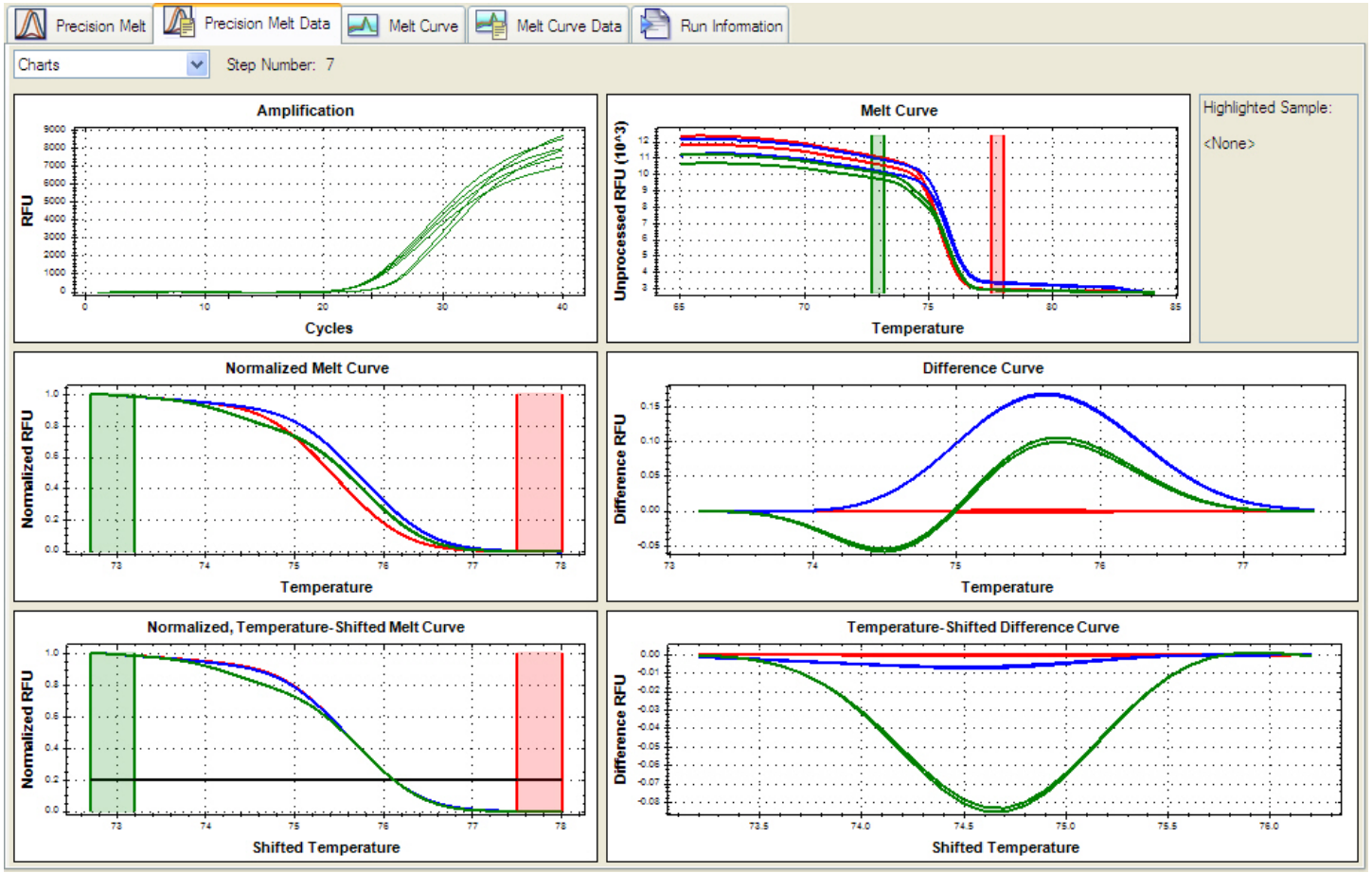
- View sample genotype results in a plate grid

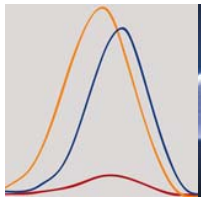




Charts View

- Easily compare amplification plots and melt curves



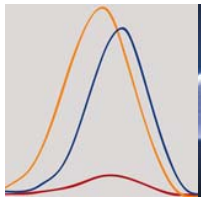



Percent Confidence

- Provides a percentage chance that a given well is correctly categorized within the assigned cluster

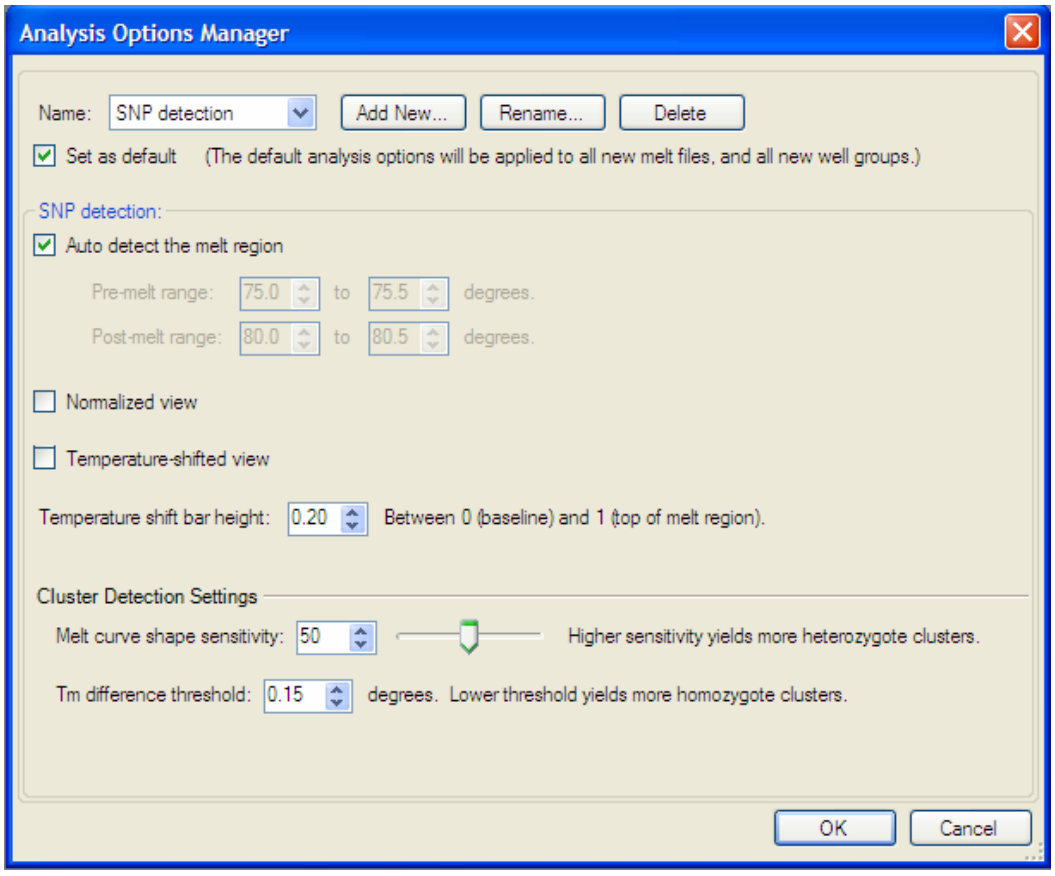
- It is based on the number of standard deviations the sample is from the mean of the cluster. This assumes that the found "cluster means and standard deviations" are accurate descriptions of the real probability distributions of the data

Well	Content	Sample	Cluster	Percent Confidence	Call Type
B02	Unkn-7	0%	Cluster 1	98.5	Auto
B09	Unkn-7	0%	Cluster 1	99.6	Auto
F02	Unkn-7	0%	Cluster 1	99.7	Auto
F09	Unkn-7	0%	Cluster 1	99.2	Auto
A05	Unkn-5	5%	Cluster 2	99.6	Auto

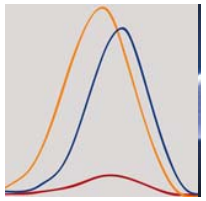


Analysis Options Manager

- Streamline data analysis using customizable analysis settings

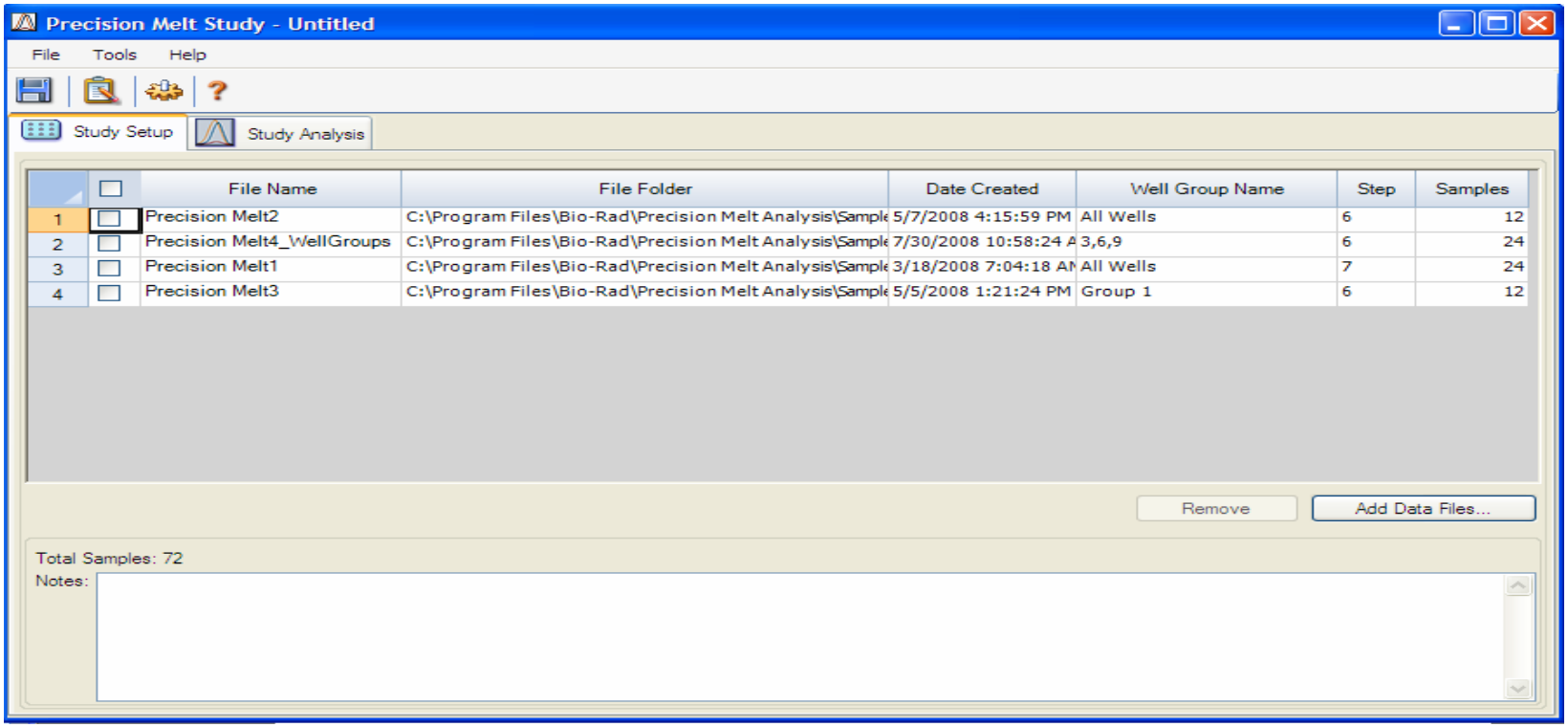


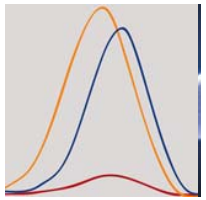
BIO-RAD



Melt Study

- Easily examine results from multiple files, without exporting data



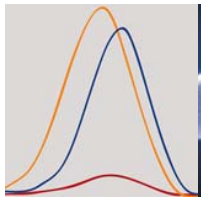



SNP Genotyping

- A single base substitution, prevalent to ≥ 1% in a population
- Use HRM analysis to identify samples containing known single nucleotide polymorphisms
- Not all SNPs are equally easy to differentiate

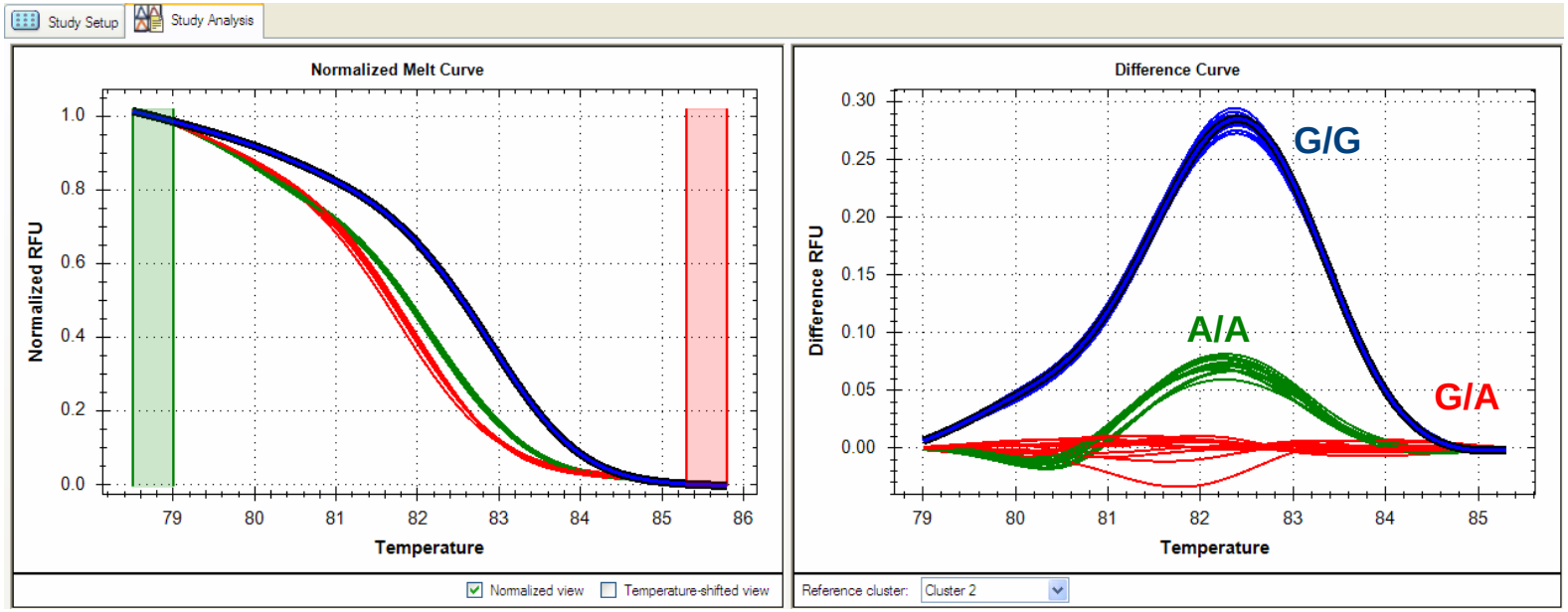
SNP Class	Base Change	Typical Tm Shift	Rarity (in human genome)
1	C/T and G/A	Large (>0.5°C)	64%
2	C/A and G/T	↓	20%
3	C/G		9%
4	A/T	Very Small (<0.2°C)	7%

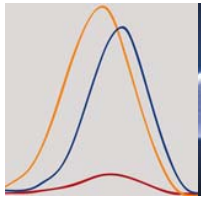
SNP classes defined by Venter et al (2001)



Class 1 SNP Mutation G>A

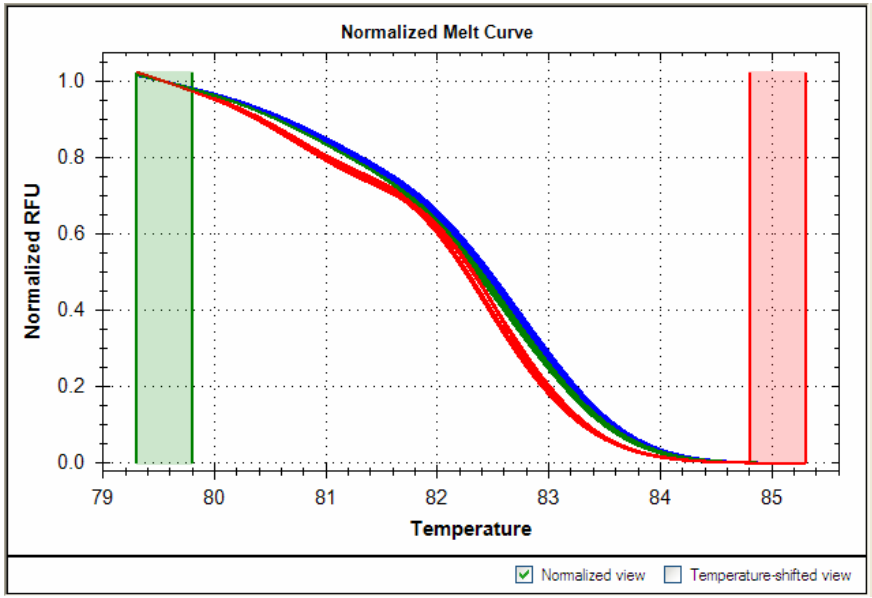
- Hemochromatosis (HFE), C282Y mutation
- 75bp amplicon
- Genomic DNA from human blood, using SsoFAST Eva Green Mix
- Melt Study results from 3 melt files (12 samples per genotype)

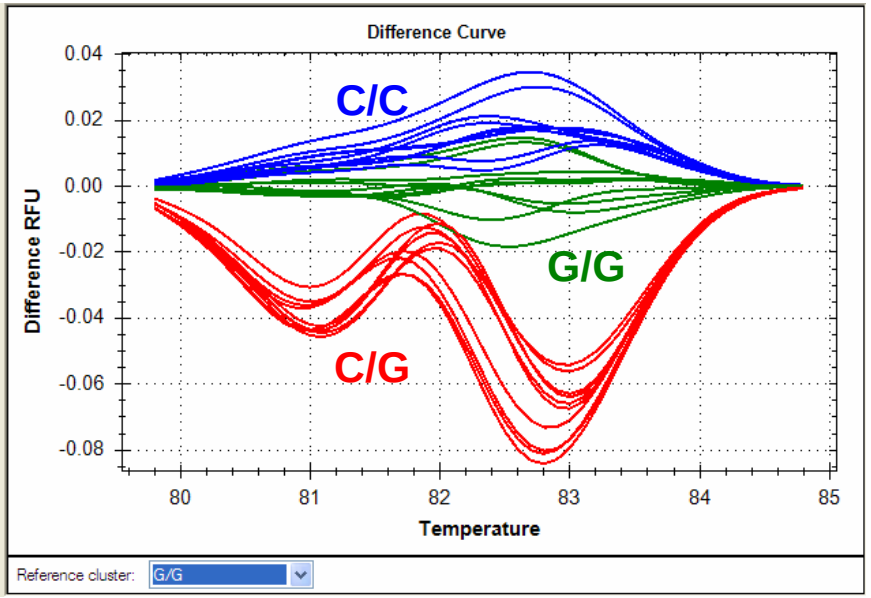


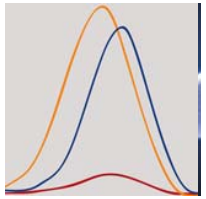



Class 3 SNP Mutation C>G

- Hemochromatosis (HFE) gene, H63D mutation
- 100bp amplicon
- Genomic DNA from human blood, using SsoFAST Eva Green Mix
- 10 samples of each genotype

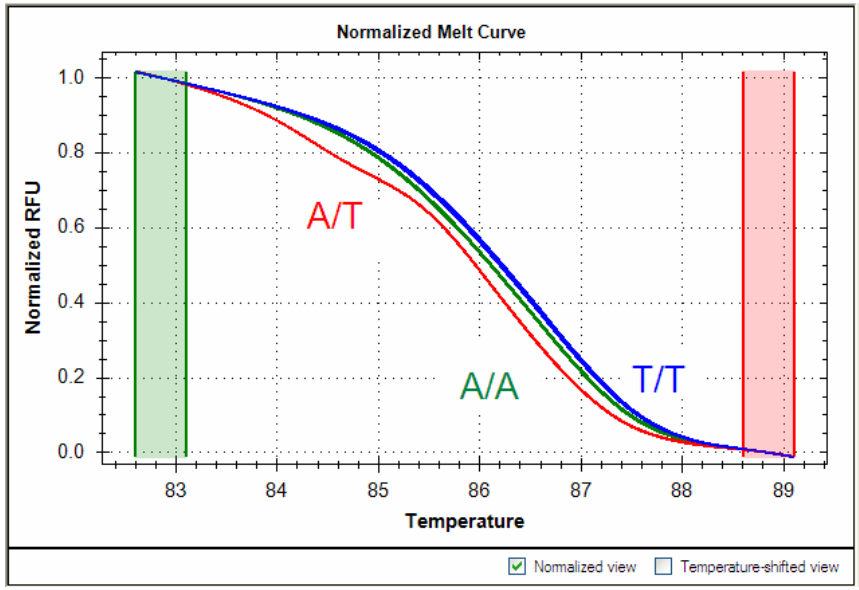


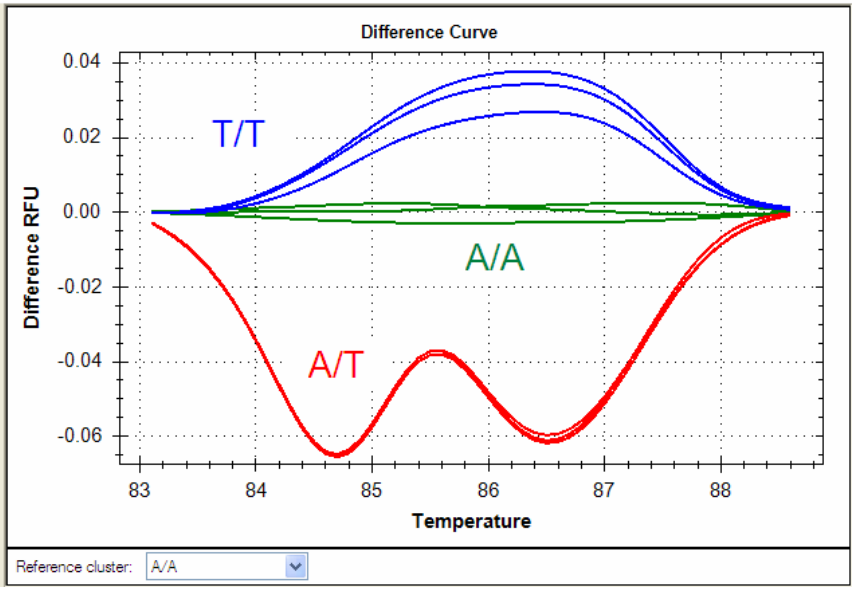


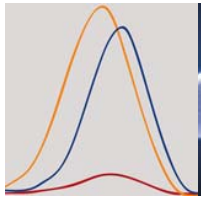



Class 4 SNP Mutation A>T

- Hemochromatosis (HFE) gene, S65C mutation
- 100bp amplicon
- Genomic DNA from human blood, using SsoFAST Eva Green Mix
- 3 samples of each genotypes

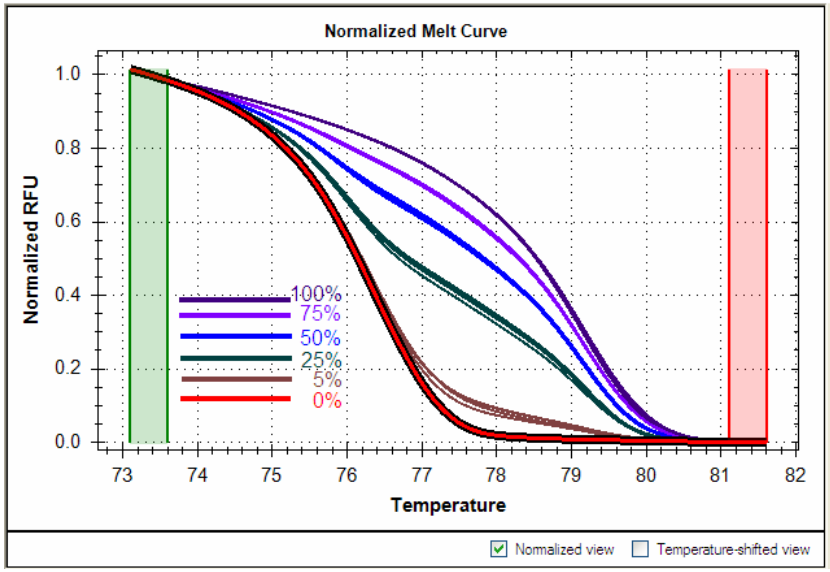


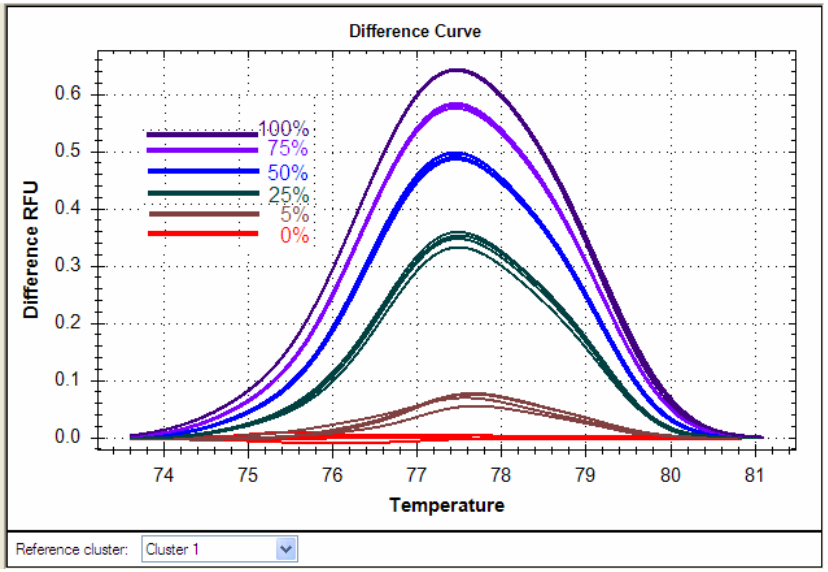


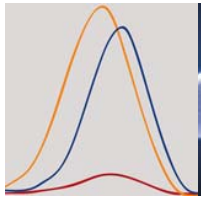


Methylation Assay

- CDH1 (Cadherin E),
- 113bp amplicon
- iQ SYBR Green Supermix
- Methylated gDNA diluted with unmethylated gDNA

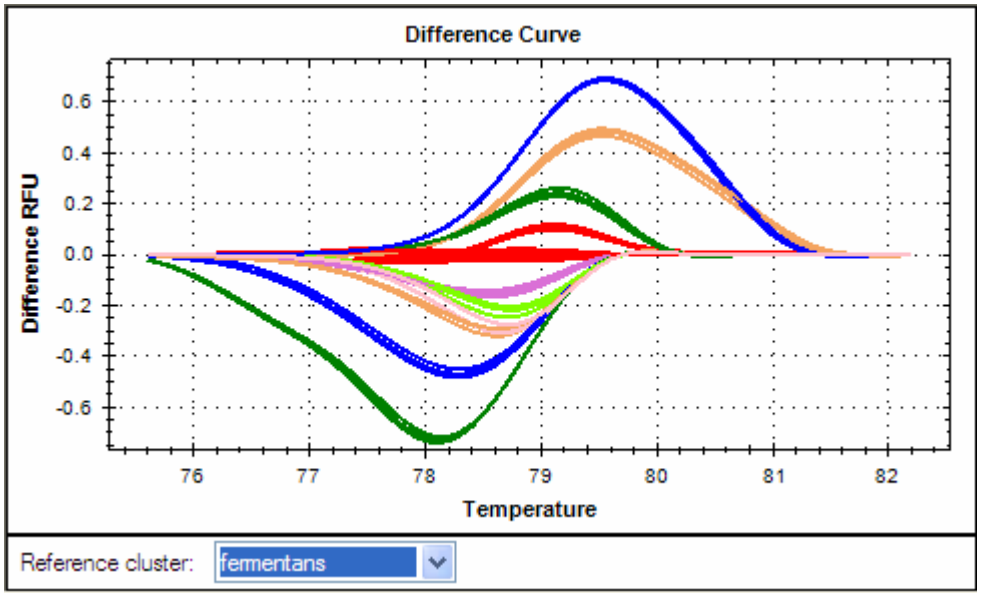


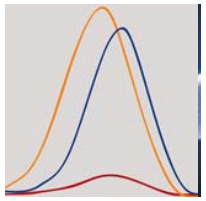


Species Identification

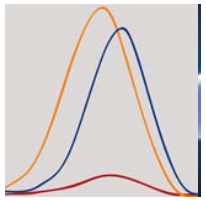
- Testing tissue culture supernates to identify the source of mycoplasma contamination
- Testing 4 samples against 10 known mycoplasma species
- One primer set to **rpoB gene**, 400-600bp amplicons



Summary

- **HRM is a powerful tool for certain applications that want to differentiate amplicons**
- **Saturation dyes help to achieve optimal results**
- **Precision Melt software is an advanced solution for HRM applications**



Questions?