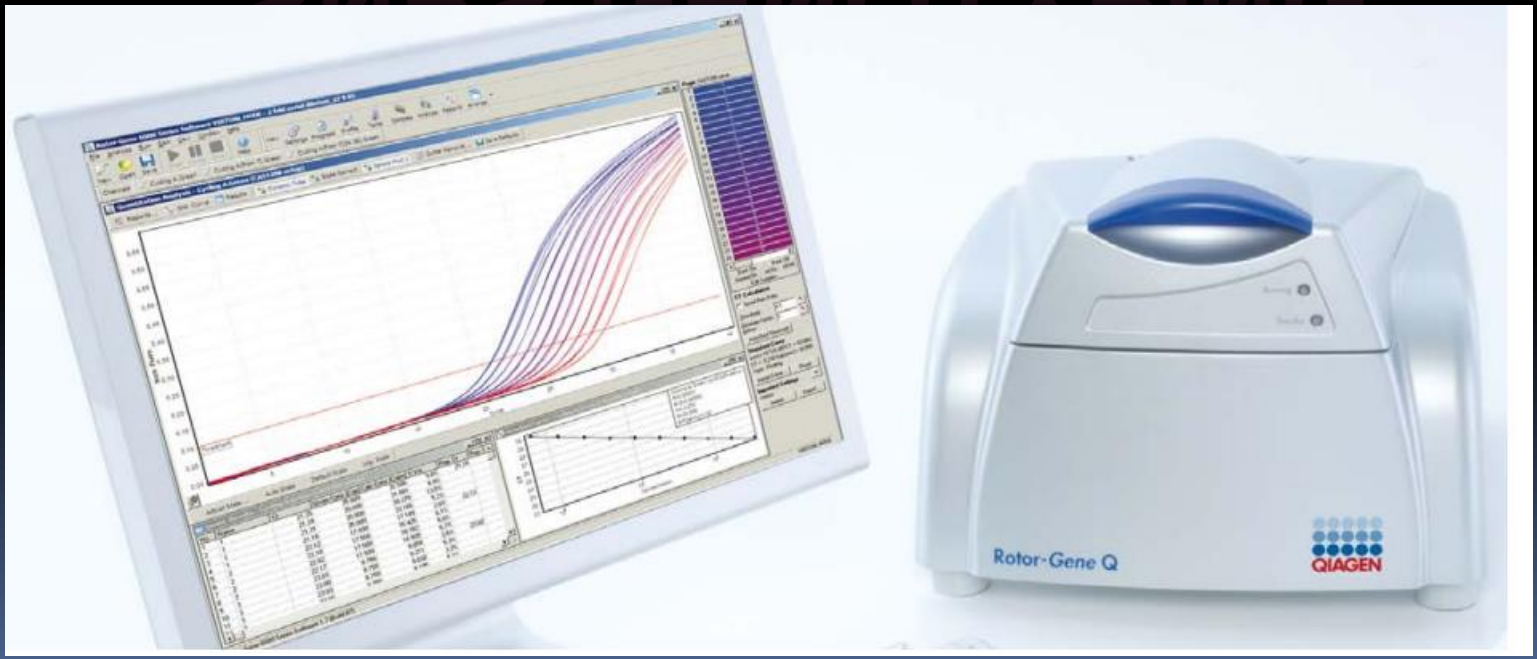
(HRMA)
HIGH RESOLUTION MELTING ANALYSIS FOR SNPS GENOTYPING



CONTANE

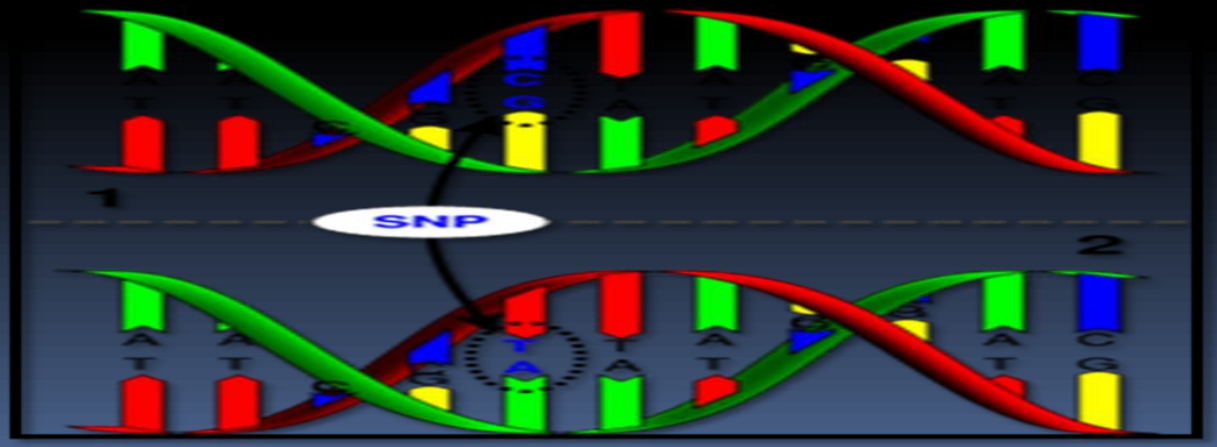
- SNPs---definition, application, analysis
- HRM ---definition, application, advantages

- SNPs genotype by HRM:-
- --Preparing samples
- --Primer design
- --HRM real time PCR RUNS
- --Software setup
- --Analysis

SNP

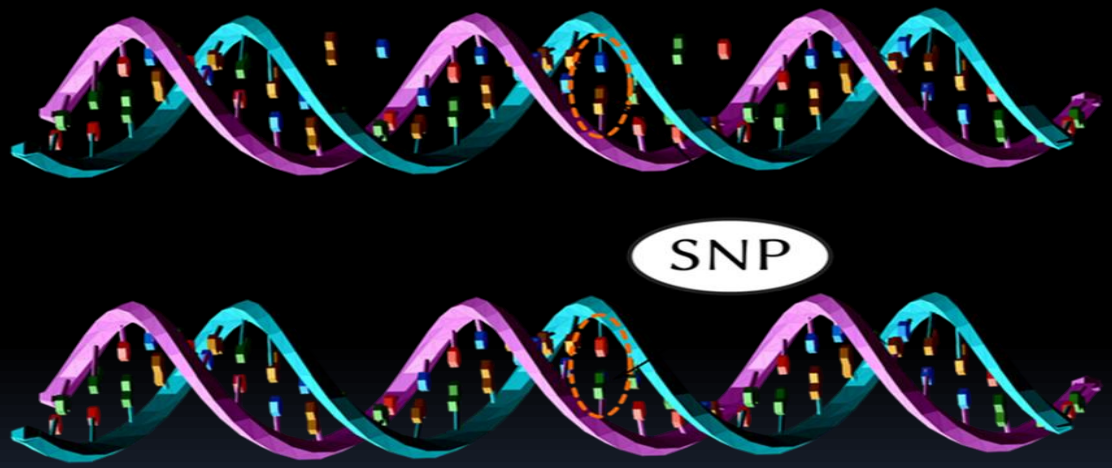
- Single nucleotide polymorphisms called SNPs. It is a DNA sequence variation occurring within a population and it's the most common and simplest type of genetic variation among people

- The difference between SNP and mutation is that a SNP represents a single nucleotide difference in DNA while mutation represents any change of DNA including single to many nucleotide differences (deletion , insertion…)



- For example, at a specific base position in the human genome, the G nucleotide may appear in most individuals, but in a some individuals, the position is occupied by an A.

- This means that there is a SNP at this position, and the two possible nucleotide variations – C or A – are said to be alleles for this position .



- The upper DNA molecule differs from the lower DNA molecule at a single base-pair location (G/A polymorphism)

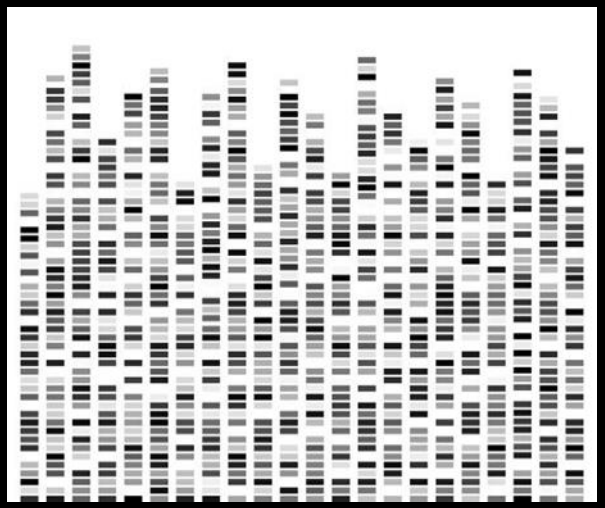
Applications of SNPs

- 1- Diagnosis of disease
- 2- Genetic similarity between parents and son
- 3- Forensics
- 4- individual's response to certain drugs

SNP analysis

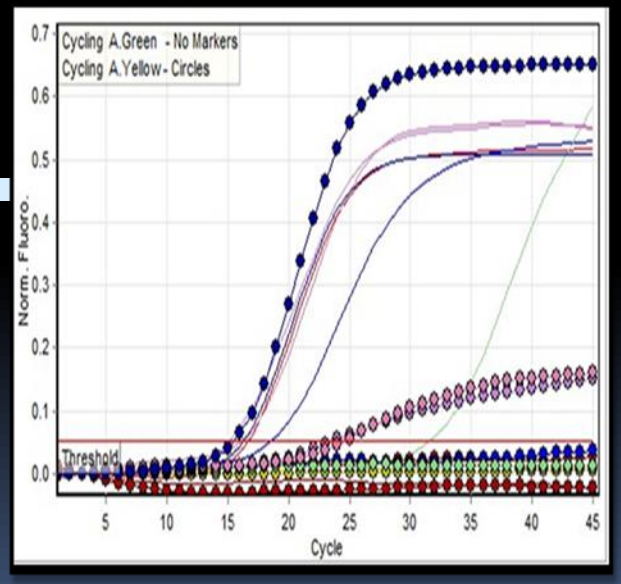
- SNPs can be easily assayed due to only containing two possible alleles and three possible genotypes involving the two alleles:
- -wild AA , hetero AB , homo BB

- leading to many possible techniques for analysis :-
- - DNA sequencing
- - Capillary electrophoresis
- - Electrochemical analysis
- - allele-specific PCR (AS-PCR)
- - TaqMan allelic discrimination assay (TaqMan)
- - High-resolution melting Analysis (HRMA)

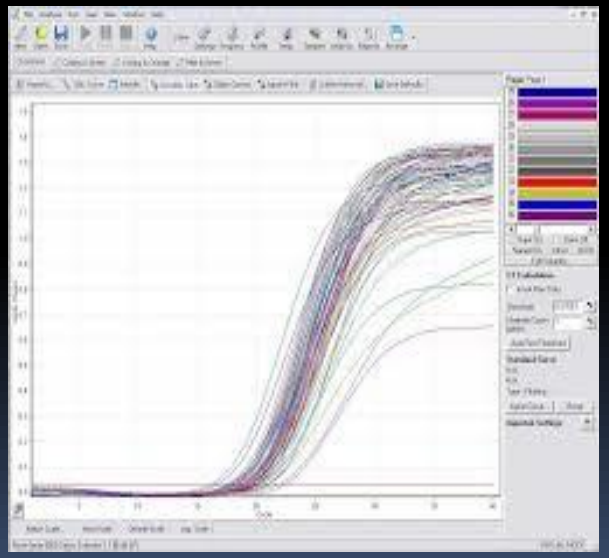


Black and white Sequencing

Coloured Sequencing



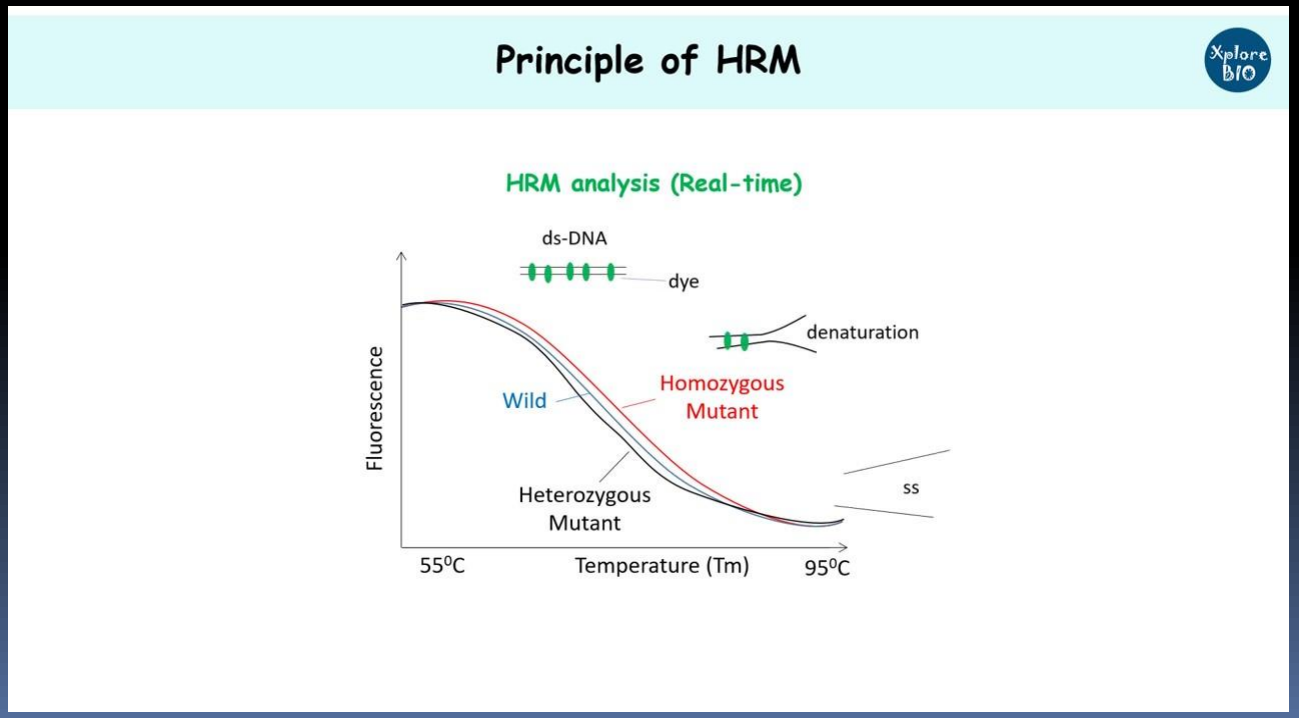
TaqMan Analysis



HRM Analysis

High-Resolution Melting Analysis (HRMA)

- It is a DNA dissociation or “melting” analyses.
- It is used to characterize DNA samples according to their dissociation behavior as they transition from double stranded DNA (dsDNA) to single stranded DNA (ssDNA) with increasing temperature by collecting fluorescent signals.

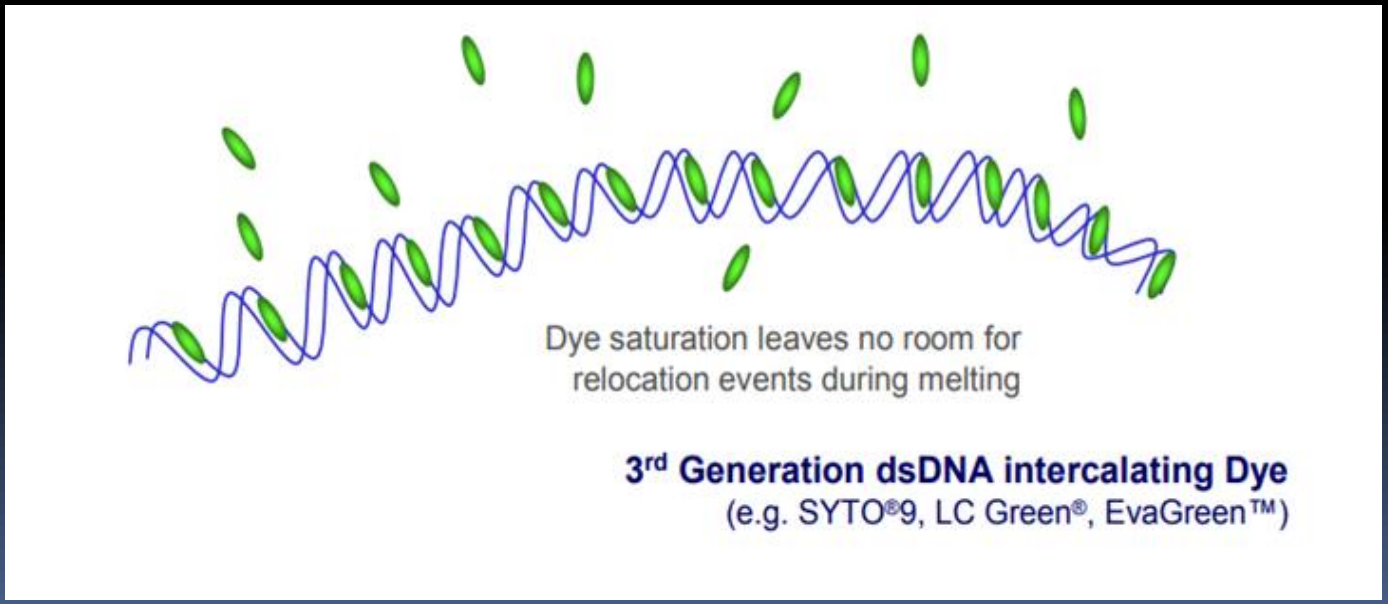


Type of machine used in HRMA

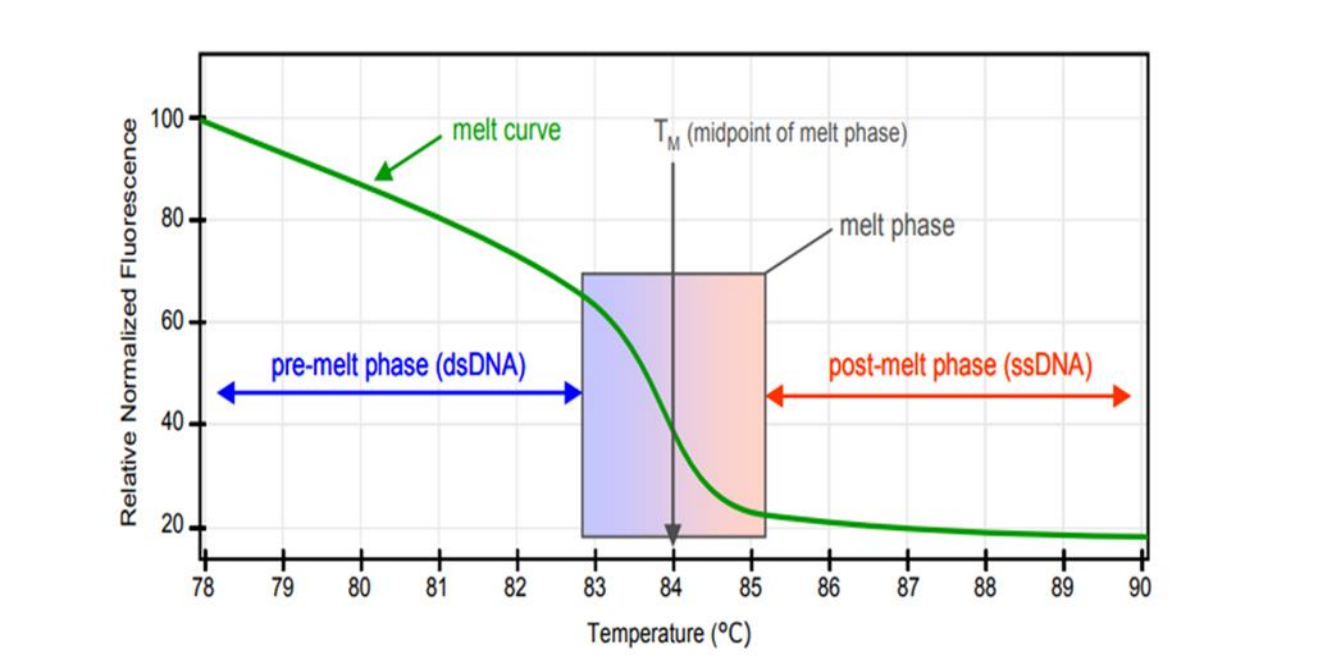
- Rotor gene -6000
- Rotor gene Q
- Qiagen PCR machine
- QIAGEN Rotor gene Q /Germany

- The fluorescence dye does not interact with ssDNA but actively intercalates with dsDNA and fluoresces brightly in this state.

- Initially, fluorescence is high in a melt analysis because the sample starts as dsDNA, but fluorescence diminishes as the temperature is raised and DNA dissociates into single strands.



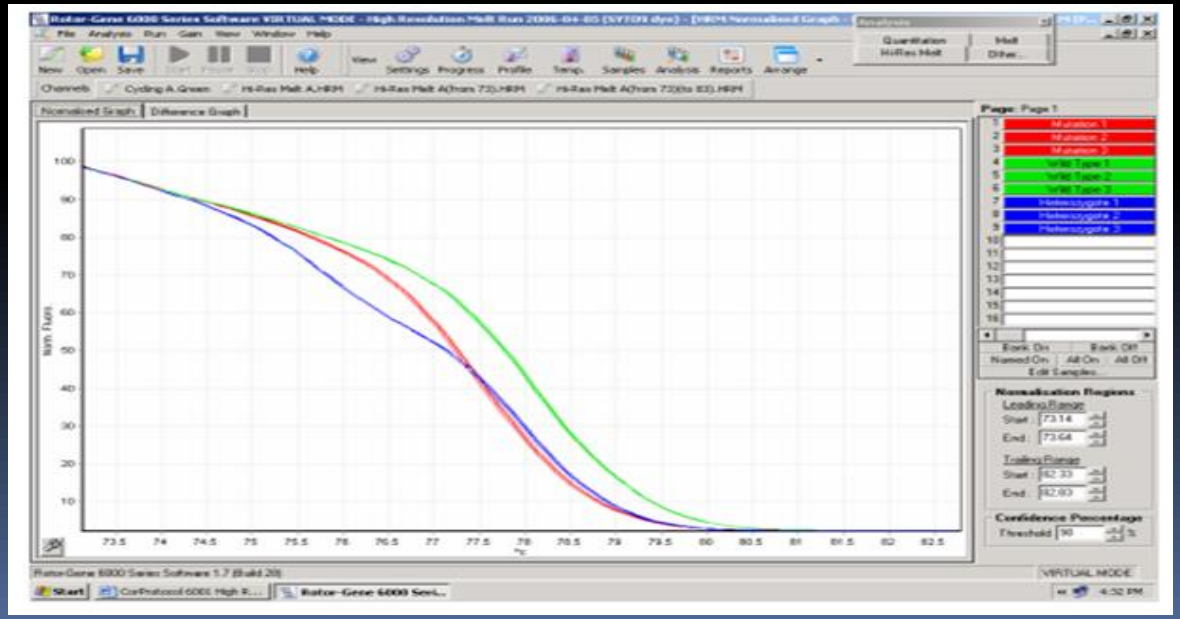
- This shift in fluorescence can be used firstly to measure the increase in DNA concentration during a pre-HRM amplification reaction and then to directly measure thermally-induced DNA dissociation by HRM.



- HRM curves are formed based on increasing temperature and decreasing amount of fluorescent dye in real-time PCR process.

- The shapes of them are unique for each species due to the sequence, length, and GC content of species' DNA (guanine – cytosin content).

- In the literature, the classification of HRM curves is usually conducted through visual inspection and a limited number of data mining methods have been used to classify these curves.

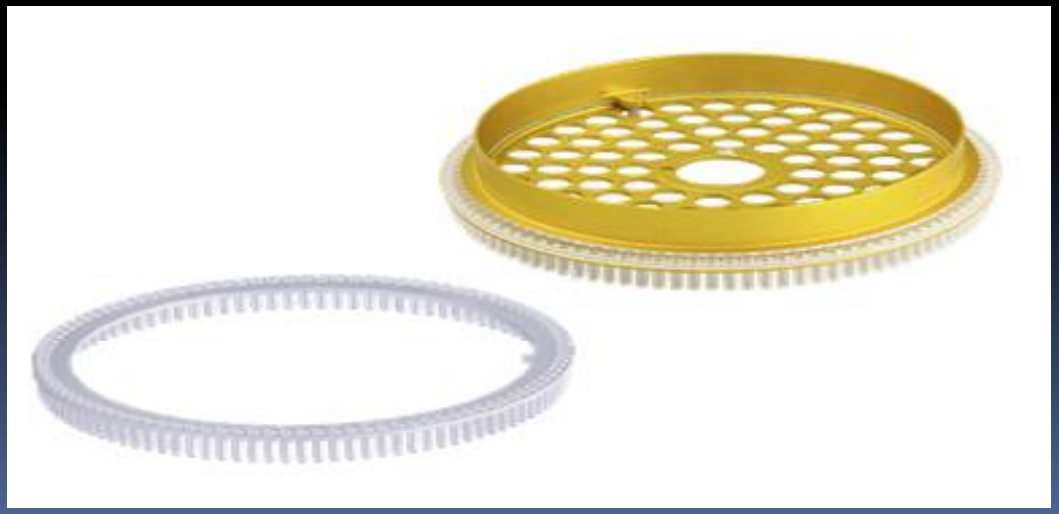


HRM Applications

- Mutation discovery
- DNA fingerprinting
- SNP genotyping
- DNA methylation analysis
- DNA mapping
- Species identification
- Somatic acquired mutation ratios
- HLA compatibility testing
- Association (case/control) studies
- Alleleic prevalence in a population
- Identification of candidate predisposition genes
- Detection of unknown (or new) mutations
- Detection of mutations and epigenetic differences in double stranded DNA samples.

Advantages

- To identify easily the SNPs genotypes ,by evaluating melting curve shape and melting temperature (Tm).
- It is massively cost effective vs. other genotyping technologies such as sequencing and Taqman SNP typing. This makes it ideal for large scale genotyping projects.
- It is fast and powerful thus able to accurately genotype huge numbers of samples in rapid time (96 sample)
- It is simple, with a good quality HRM assay powerful genotyping can be performed by non-geneticists in any laboratory with access to an HRM capable real-time PCR machine.



SNP Genotyping by HRMA

- HRM can be used to analyze virtually all types of DNA sequence variants, including single base changes, insertions, deletions and base pair substitutions.
- Representative of the smallest genetic change, the detection and genotyping of SNPs underlines the sensitivity of HRM analysis.
- In general, the more base changes in the DNA the easier they are to detect by HRM.

A
Design

1. Identify target sequence
(use an online database)

2. Amplicon design
(e.g. Primer 3, DINAMelt software)

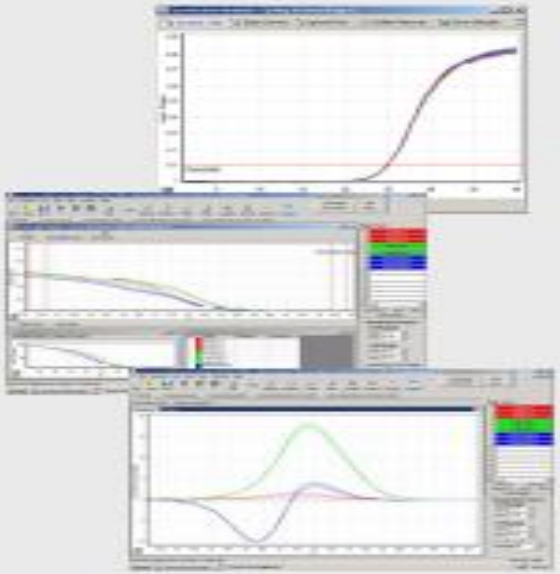
3. Check specificity
(BLAST primer sequences)

B
Run

4. Set up reactions
in desired tube format

5. Run PCR *and* HRM

C
Analyze



6. Analyze results
(real-time and HRM)

7. Auto-call genotypes
(up to 100 at a time)

- **Preparing sample**

1-Collecting blood

- About two milliliters of venous blood were taken from each subject and collected into tubes containing ethylene diamine tetra acetic acid (EDTA) to be used for molecular analysis .

- All sample was refrigerated in deep freeze .

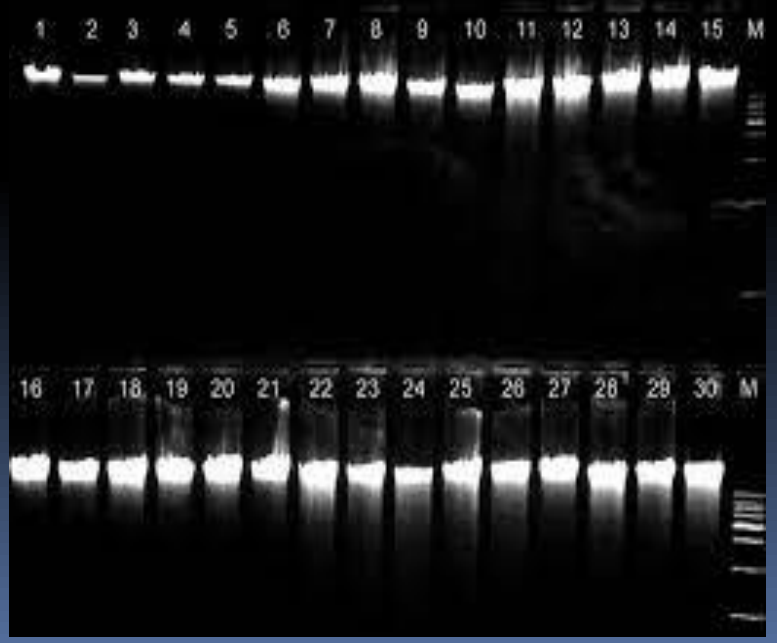
2-DNA EXTRACTION

- Genomic DNA was extracted by using EasyPure® Genomic DNA Kit (Transgene biotech).
- The manufacturer protocol was followed for extraction of the DNA from frozen blood samples .

3-DNA ELECTROPHORESIS

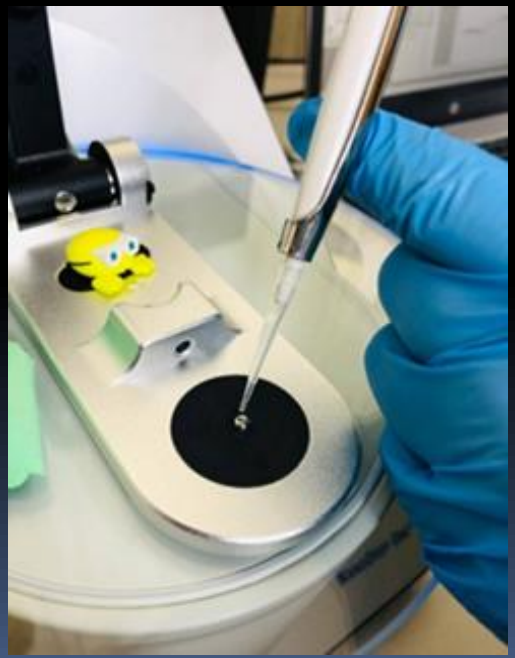
- Agarose gel electrophoresis was used to confirm the existence and quality of isolated gDNA and PCR products.

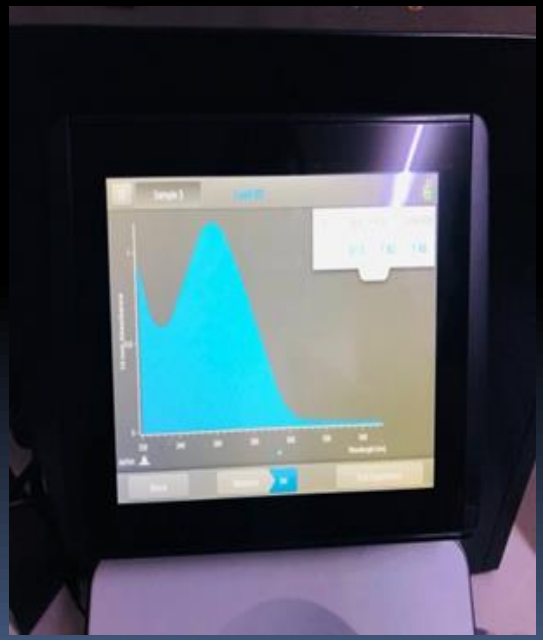
- 1 X TBE buffer, loading dye, DNA ladder, and Gel stain (Ethidium Bromide) are the main requirements for DNA electrophoresis.



4-Spectrophotometer (Nanodrop)

- The DNA concentration and purity of samples was estimated using a spectrophotometer (Nanodrop).

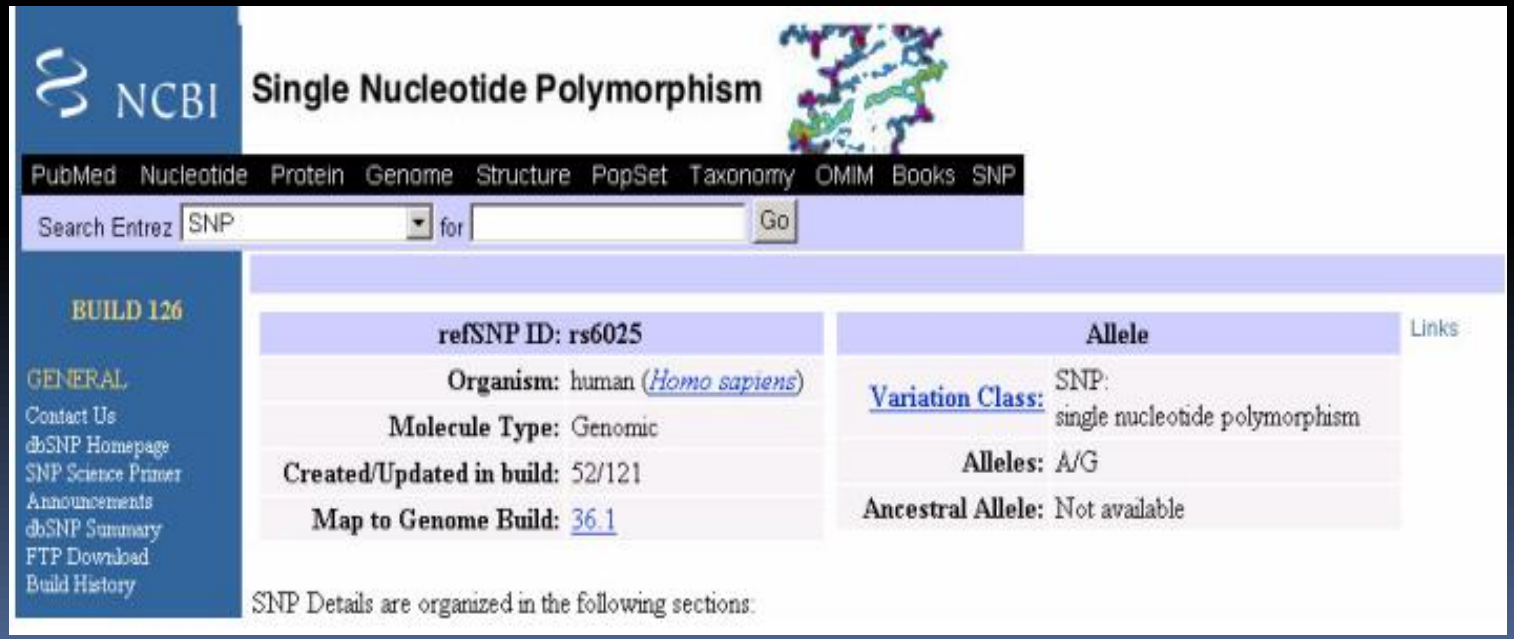




PRIMER DESIGN

1-SNPs SELECTION

- Potential functional SNPs were selected from published association studies and from the dbSNP ,database of the National Center for Biotechnology Information (NCBI). In order to capture the greatest degree of genetic variation in the gene.



2-PRIMER DESIGNE

- Primer is a short single-stranded DNA fragment used in certain laboratory techniques, such as the polymerase chain reaction (PCR) to detect the position of mutant allele.

- The primers were designed using the Primer 3plus, primer Blast, and double checked by the University Code of Student Conduct (UCSC) programs, and with their reference sequences in the National Center for Biotechnology Information (NCBI) database.

An official website of the United States government Here's how you know

National Library of Medicine
National Center for Biotechnology Information

Log in

Primer-BLAST

A tool for finding specific primers

Finding primers specific to your PCR template (using Primer3 and BLAST).

Primers for target on one template | Primers common for a group of sequences

Retrieve recent results | Publication | Tips for finding specific primers

Save search parameters | Reset page

PCR Template

Enter accession, gi, or FASTA sequence (A refseq record is preferred) Clear

Range Clear

	From	To
Forward primer		
Reverse primer		

Or, upload FASTA file Choose File No file chosen

Primer Parameters

Use my own forward primer (5'->3' on plus strand) Clear

Use my own reverse primer (5'->3' on minus strand) Clear

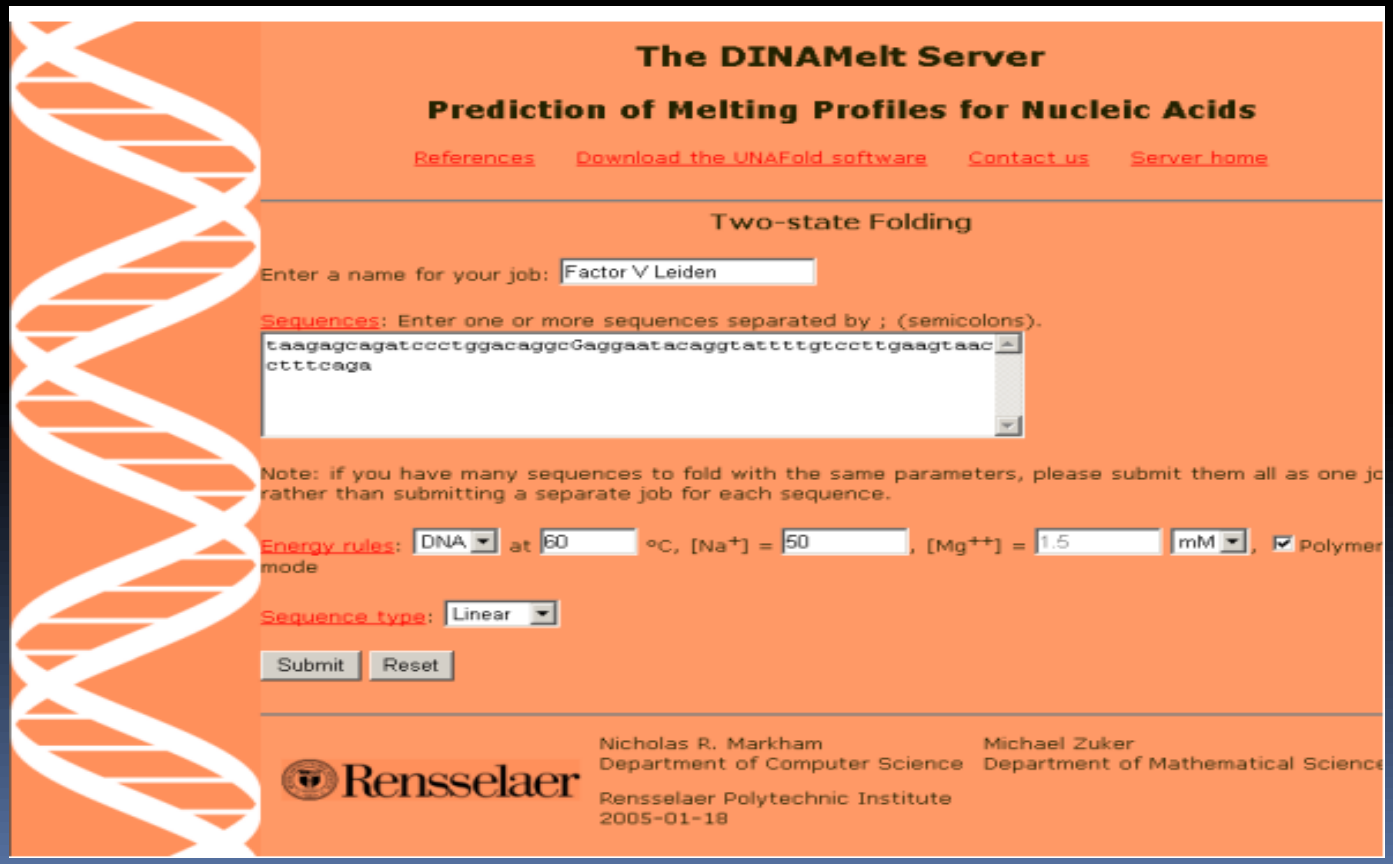
	Min	Max
PCR product size	70	1000

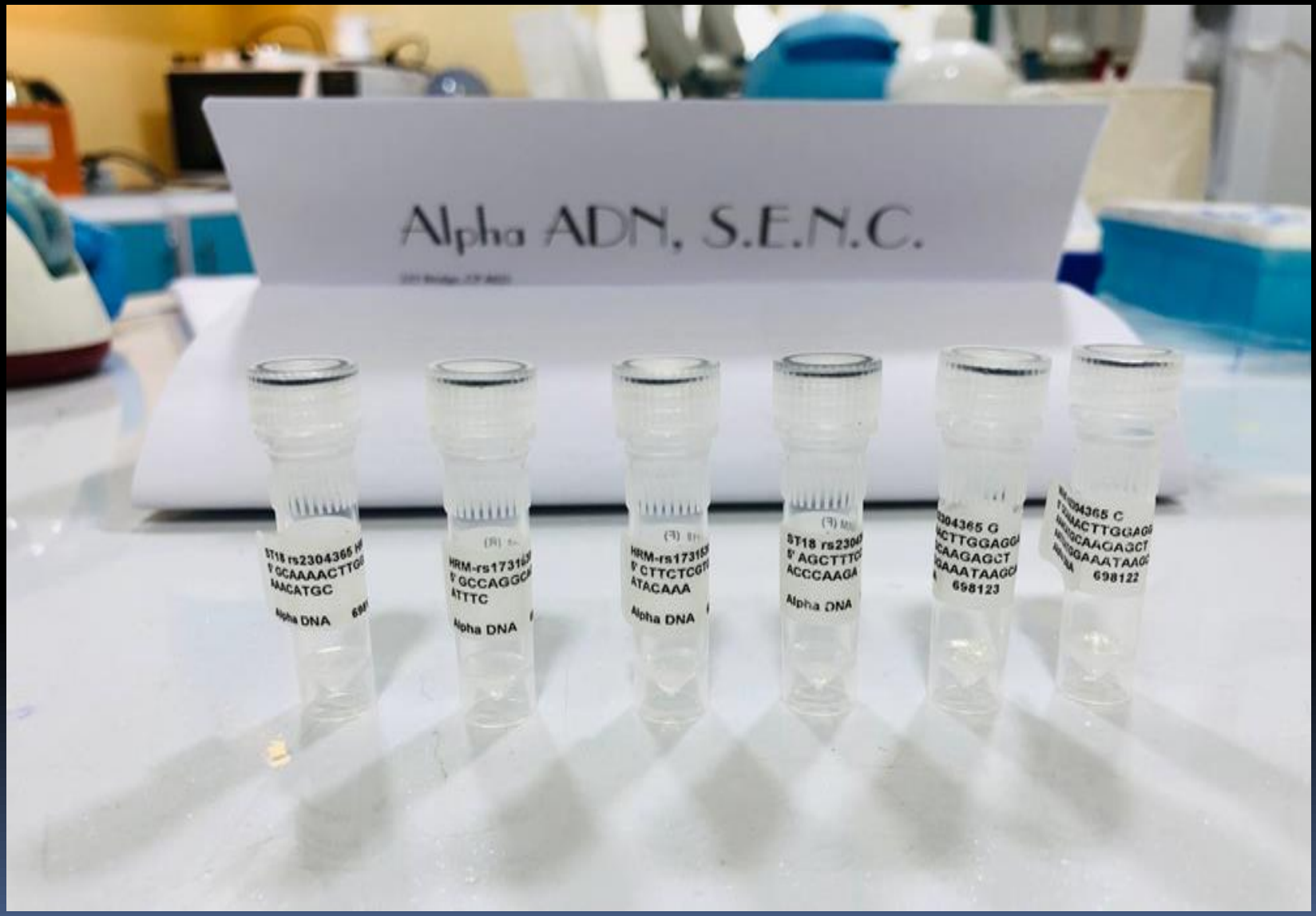
- They were synthesized by specialized genetic companies like Alpha DNA Ltd (Canada).

Primer	Sequence (5'→3' direction)	primer size bp
ST18 (RS2304365)		
Forward	5'AGCTTTCCAAATTCAACCCAAGA-3'	23
Reverse	5'-GCAAAACTTGGAGGAAAACATGC-3'	23
ST18(Rs17315309)		
Forward	CTTCTCGTGATTAGCATACAAA-3'	22
Reverse	5'-GCCAGGCAGCAATTCATTTC-3'	20

3-Choosing appropriate temperature for each primer

- The DINAMelt Servers from the Rensselaer Polytechnic Institute are an appropriate software solution as corrections are made for both salt and magnesium concentration

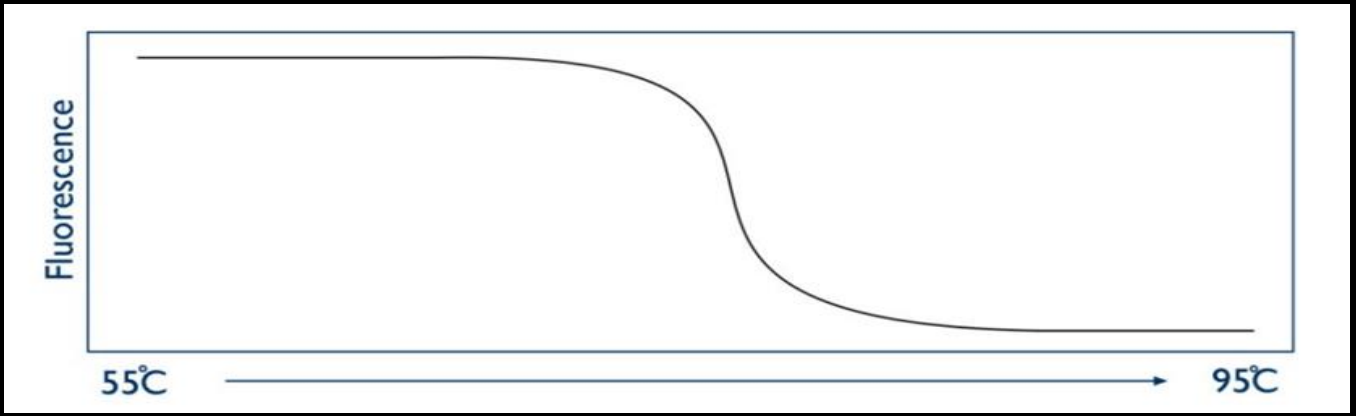



Alpha ADN, S.E.N.C.
Alpha DNA
Alpha DNA
Alpha DNA
Alpha DNA
698123
698122

Fluorescent Dye

- The secret of HRM is to monitor this process happening in real-time. This is achieved by using a fluorescent dye (SYTO®9, LC Green®, LC Green™Plus+, EvaGreen™)
- This called intercalating dyes and have a unique property; they 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.
- So 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.

- But as the sample is heated up and the two strands of the DNA melt apart there is no longer any double stranded DNA present and thus fluorescence is reduced.



- The temperature that the amplicon melts and the two DNA strands come apart is an entirely predictable process. It is dependent on the sequence of the DNA bases.

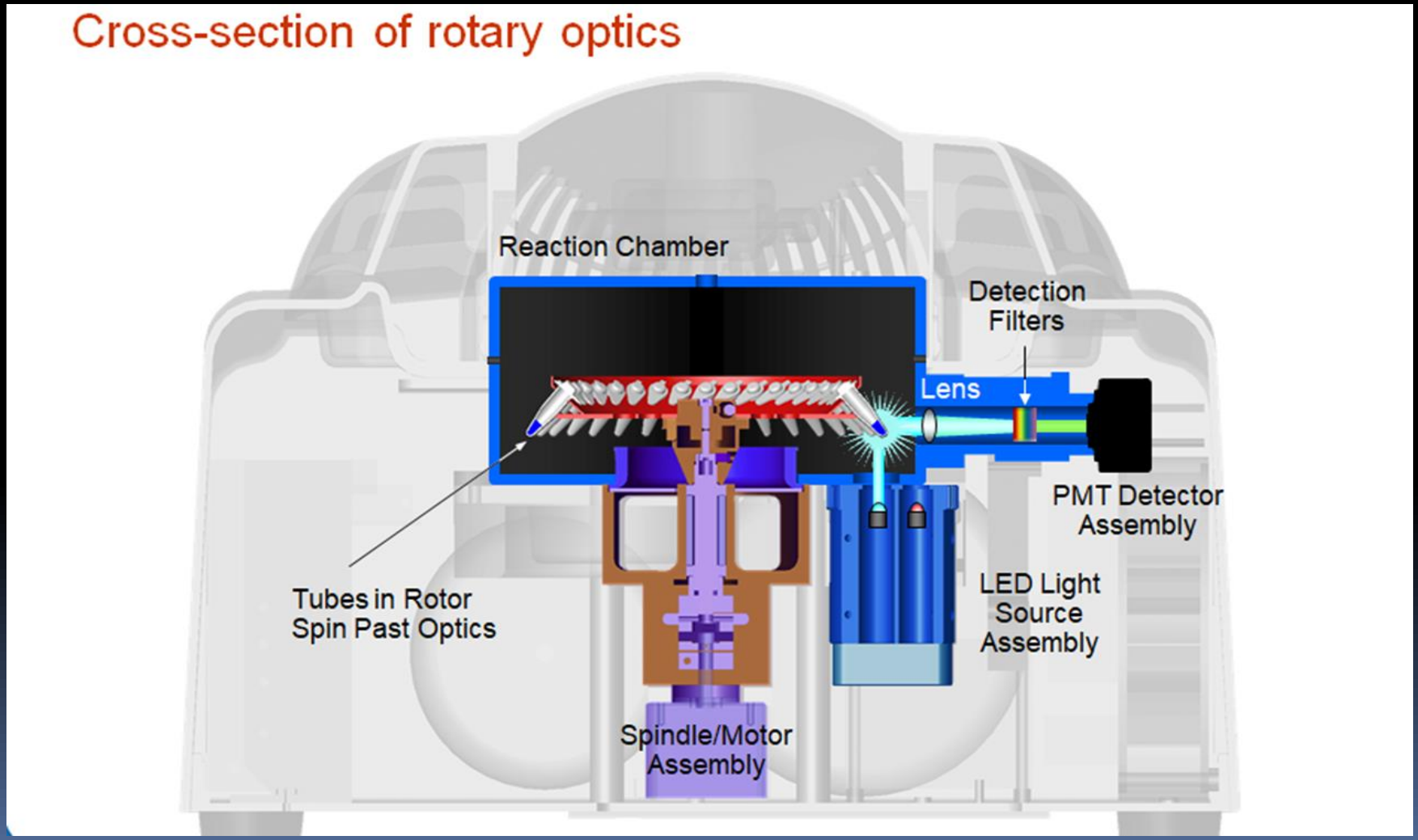
- If comparing two samples from two different people they should give exactly the same shaped melt curve.

Component	Final Volume reaction
Template of DNA	4 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
TransStart® Tip Green qPCR Super Mix	10 µl
Nuclease-free Water	6 µl
Total volume	20 µl

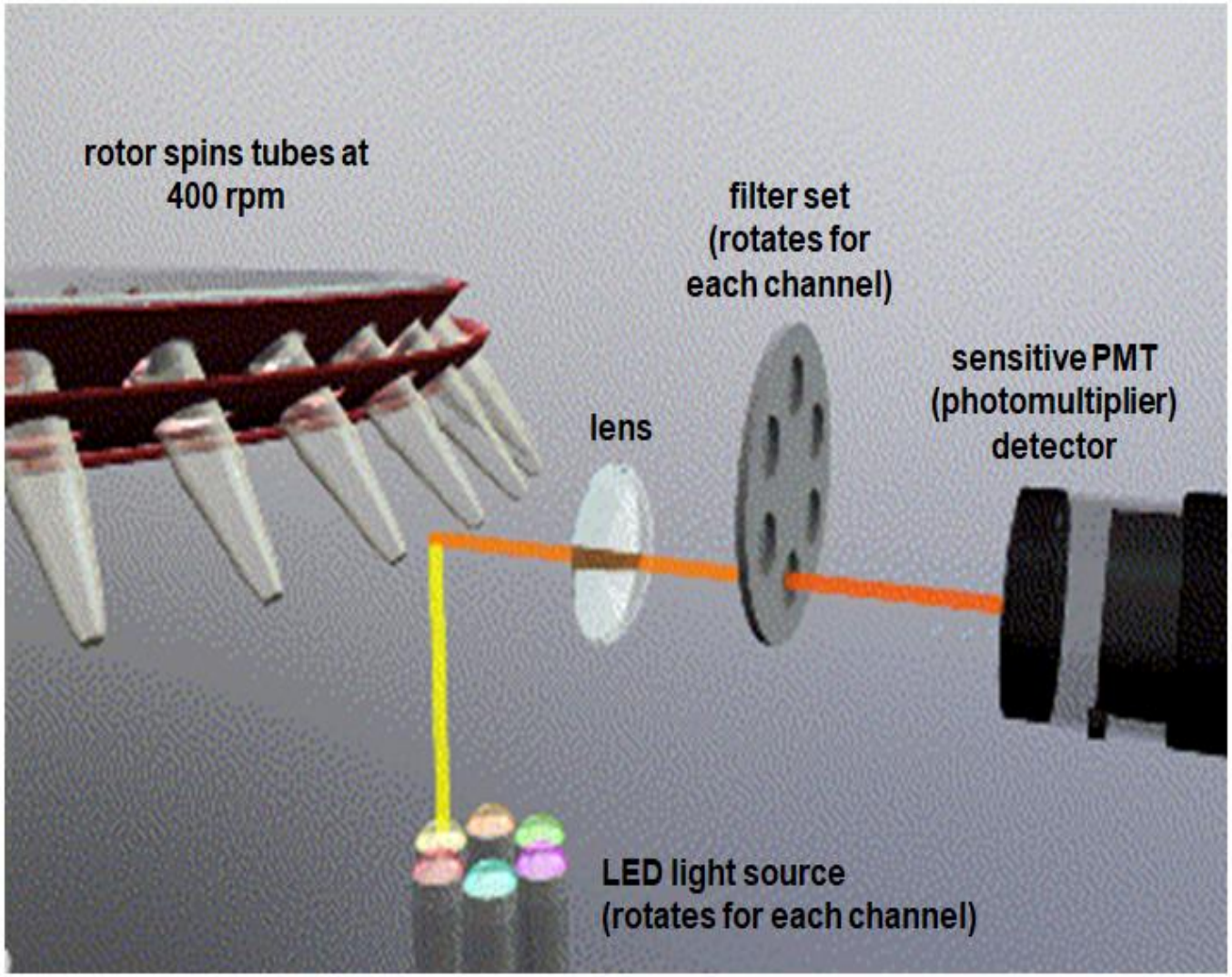

Place the capped tubes into the blue Rotor-Disc 72 rotor.
Always make sure to fill the rotor completely, placing blank tubes in any empty positions.


Attach the corresponding locking ring onto the filled rotor.

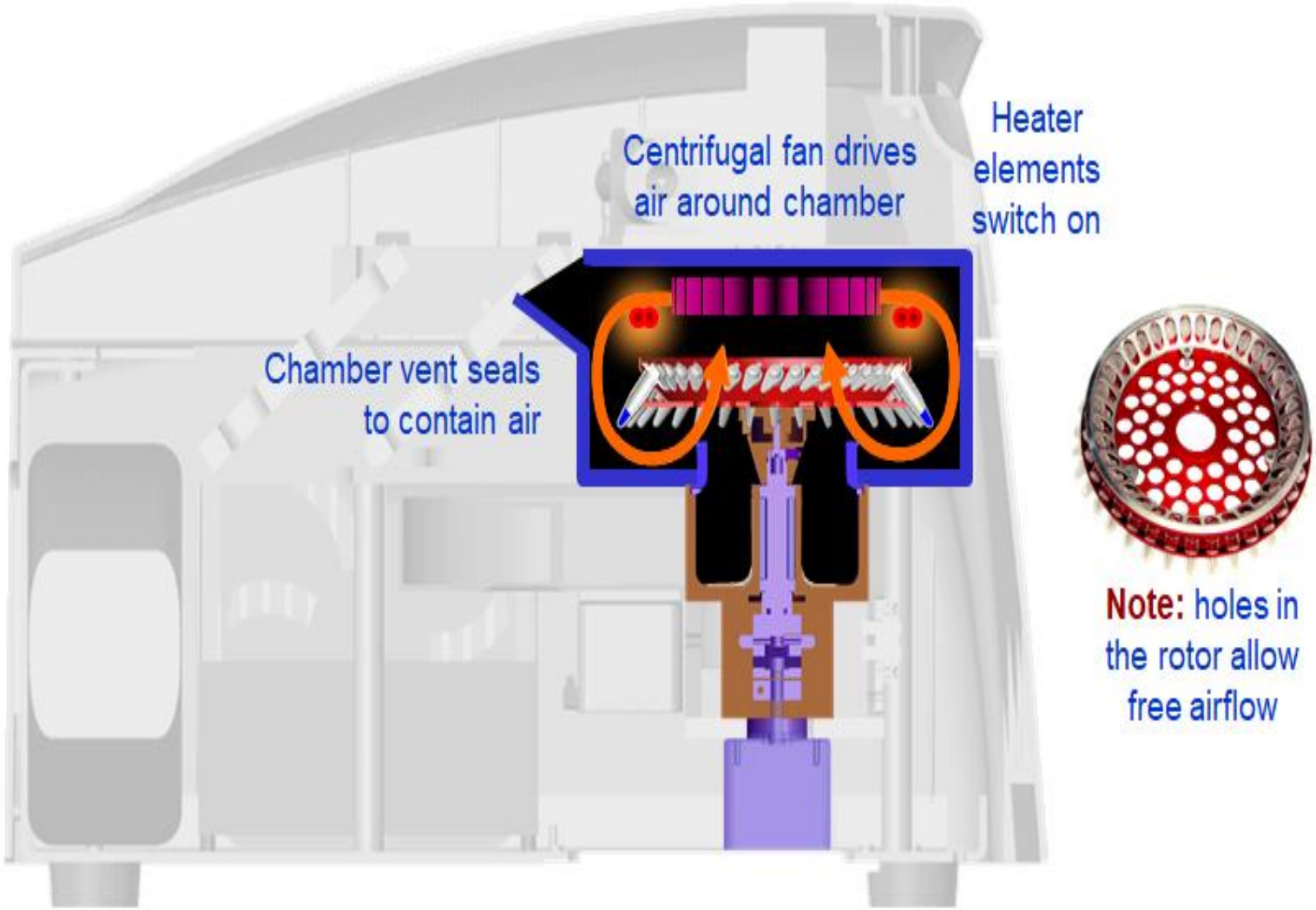
- The HRM machine has a camera that watches this process by measuring the fluorescence.



Rotary optics 3D animation



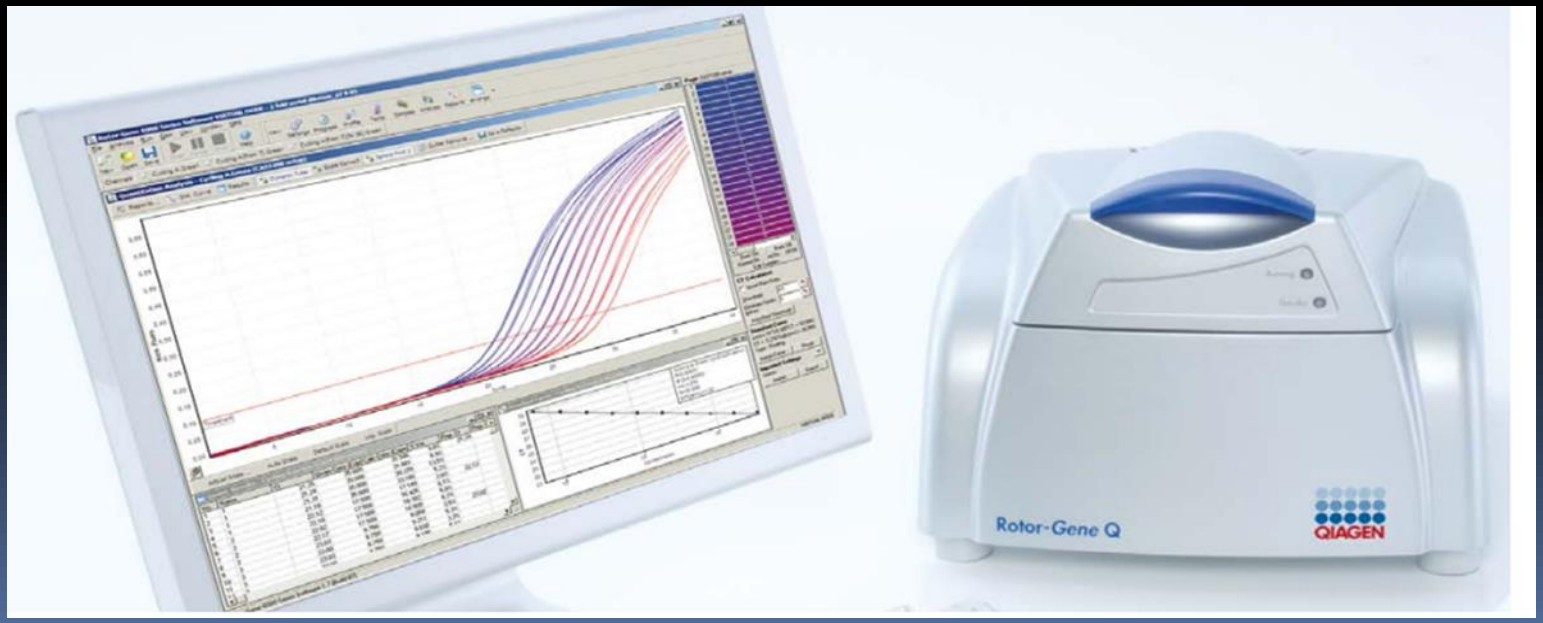
Heating mechanism



- HRM REAL TIME PCR RUNS

- Rotor gene Q ,Real-time PCR System (QIAGEN /Germany) was used to perform qPCR-HRM

- Followed by an HRM analysis with from 55 - 95 °C. 2xTransStart® Tip Green qPCR Super Mix Synthetic SNP sequences were evaluated using duplicates.

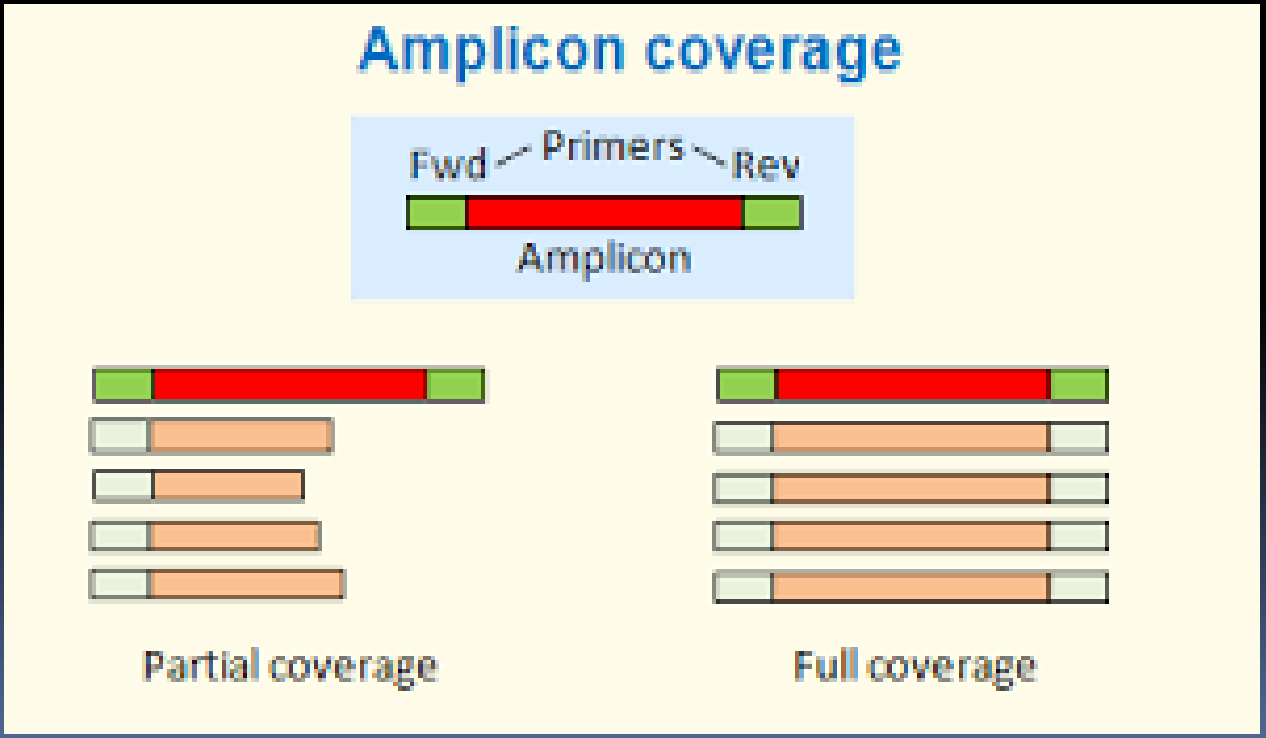


How does it work?

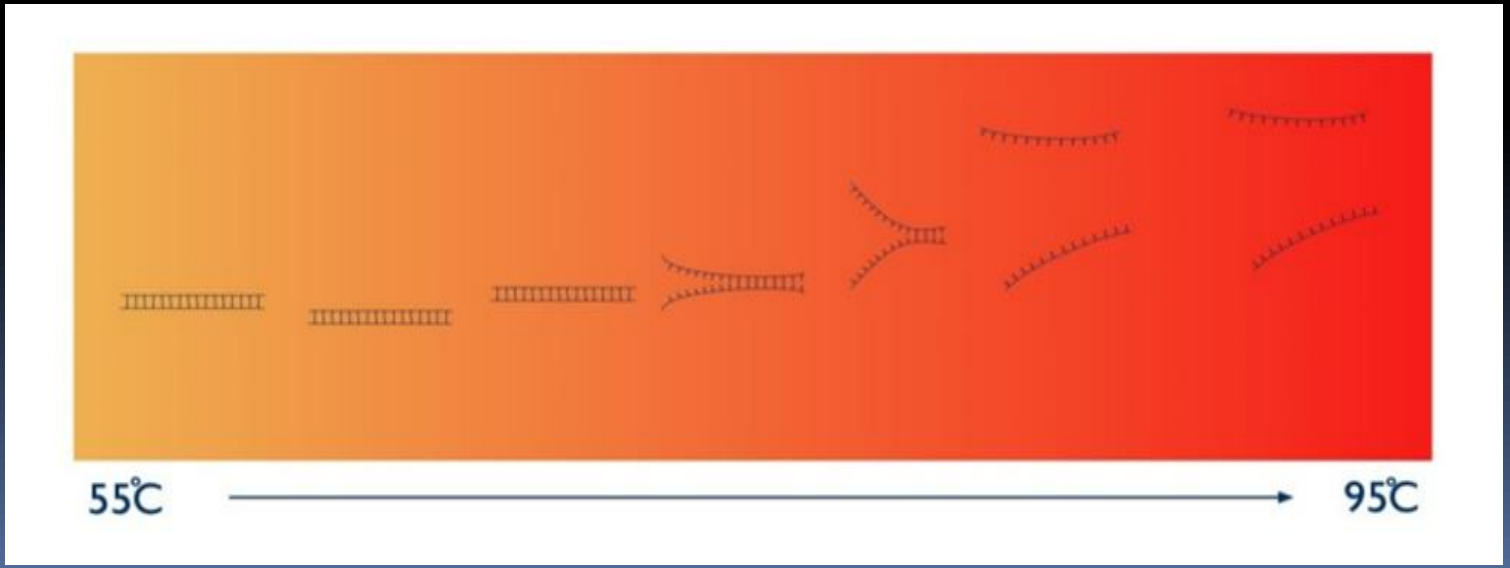
- Typically the user will use the real-time polymerase chain reaction prior to HRM analysis to amplify the DNA region in which their mutation of interest lies.

- Essentially the real-time PCR process turns a tiny amount of DNA of interest in to a large amount so there is enough to be worth analyzing.

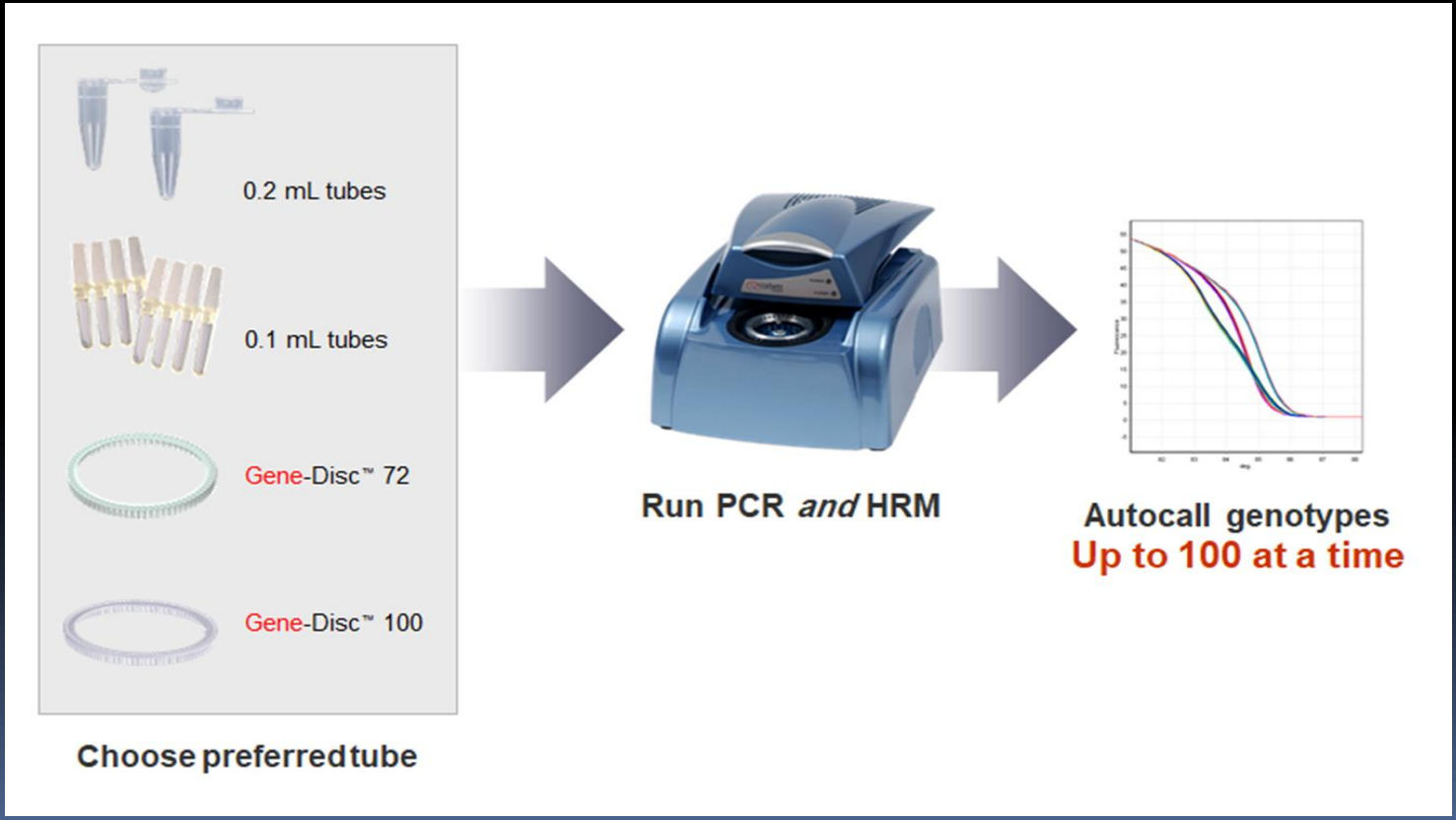
- This region that is amplified is known as the amplicon.



- After the PCR process the HRM analysis begins.
- The process is simply a precise warming of the amplicon DNA from around 50˚C up to around 95˚C.
- At some point during this process the melting temperature of the amplicon is reached and the two strands of DNA “melt” apart.



- The machine then simply plots this data as a graph known as a melt curve showing the level of fluorescence vs the temperature



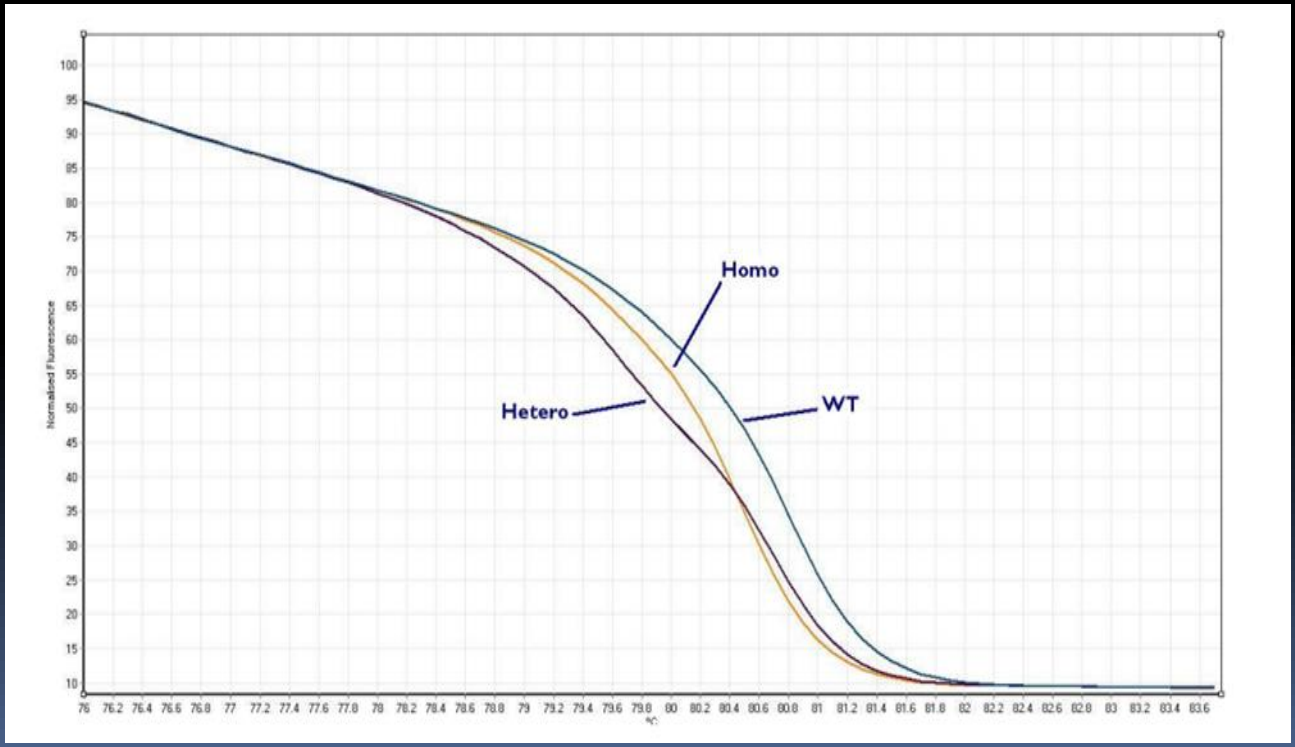
Wild type, heterozygote or homozygote?

- If a sample is taken from a patient and amplified using PCR both copies of the region of DNA (alleles) of interest are amplified.

- So if we are looking for mutation there are now three possibilities:
- 1. Neither allele contains a mutation (wild)
- 2. One or other allele contains a mutation (hetero)
- 3. Both alleles contain a mutation (homo)

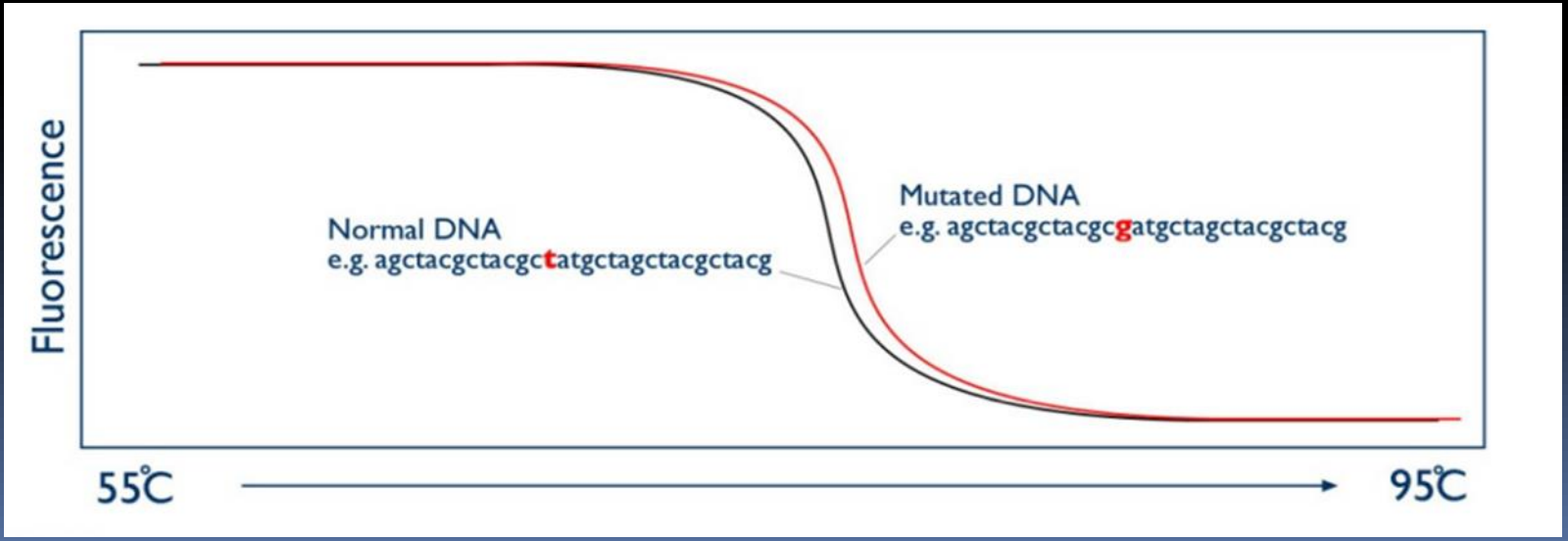
- Each gives a melt curve that is slightly different. With a high quality HRM assay it is possible to distinguish between all three of these scenarios.

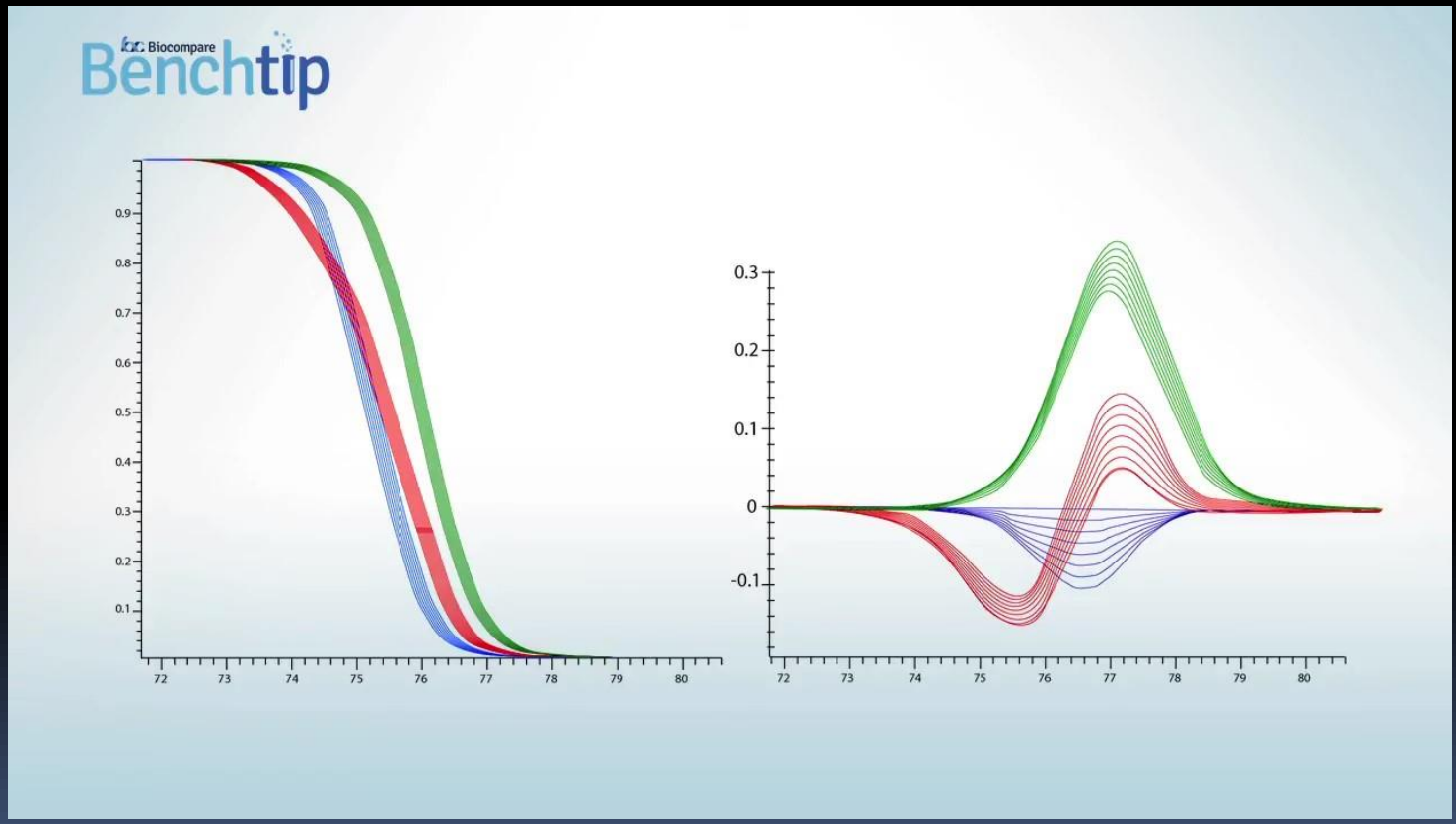
- There are three different patients here. one of the patients has the Wild type genotype, one is Homozygous (homo) and the other has the Heterozygous (hetero) genotype.



- However if one of the people has a mutation in the DNA region , the two melt curves appear different ,because mutation will alter the temperature.

- The difference may only be tiny, perhaps a fraction of a degree, but because the HRM machine has the ability to monitor this process in “high resolution” it is possible to accurately document these changes and therefore identify if a mutation is present or not.




Biocompare
Benchtip
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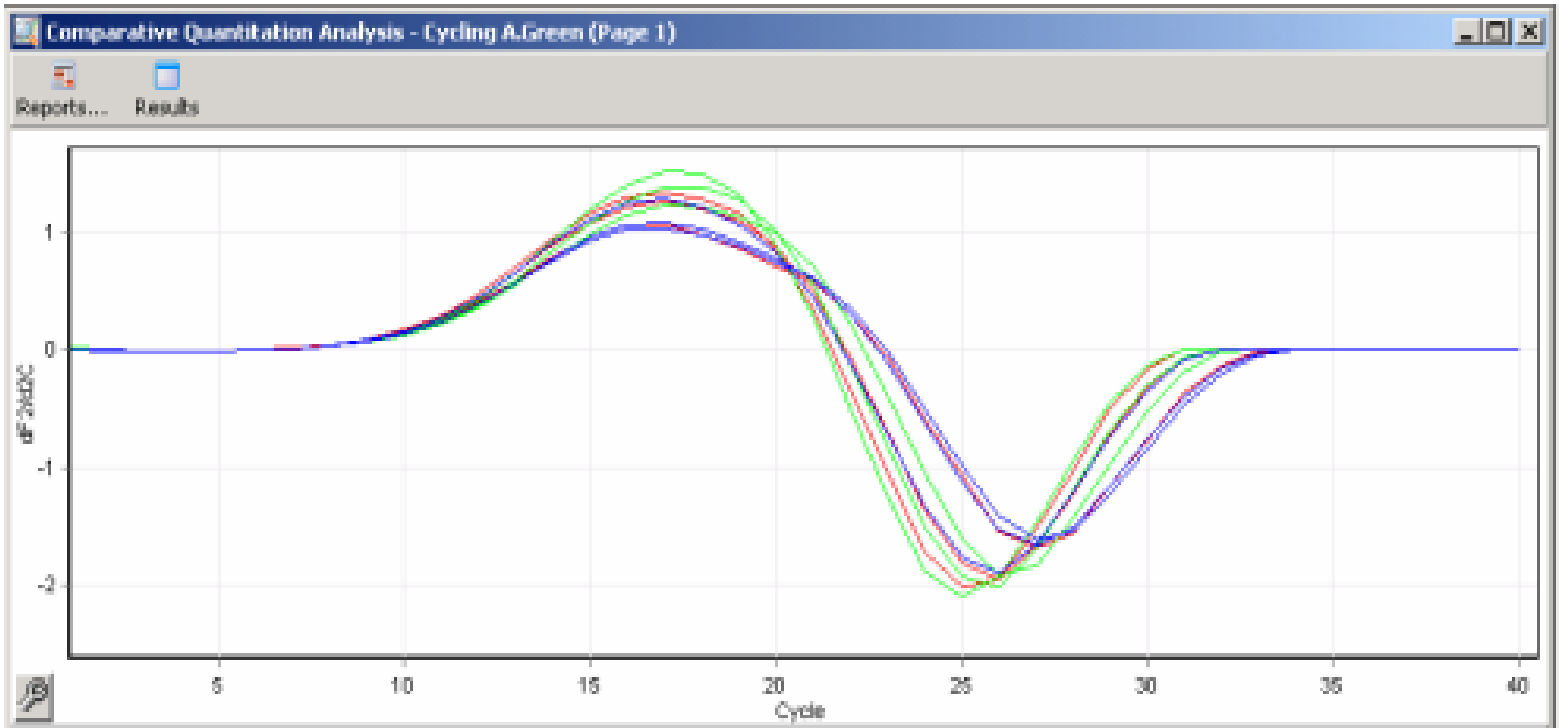


Comp. Quant Results - Cycling A.Green (Page 1)

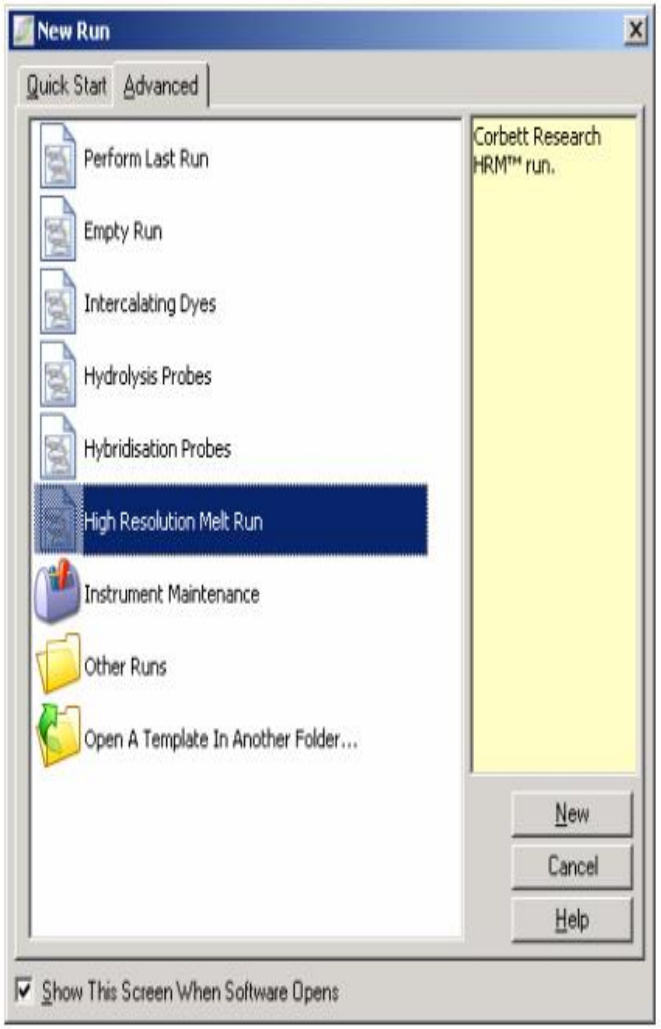
No.	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)	Rep. Amp.	Rep. Amp. (95% CI)	Rep. Conc.	Rep. C
1	mutation 1	11.2	1.66	1.00E+00	11.2		1.66		1.00E+00	Calibra
2	mutation 2	11.4	1.65	9.04E-01	11.4		1.65		9.04E-01	
3	mutation 3	11.4	1.64	9.04E-01	11.4		1.64		9.04E-01	
4	wild type 1	11.9	1.66	7.01E-01	11.9		1.66		7.01E-01	
5	wild type 2	11.8	1.66	7.38E-01	11.8		1.66		7.38E-01	
6	wild type 3	11.9	1.66	7.01E-01	11.9		1.66		7.01E-01	
7	heterozygote 1	11.4	1.67	9.04E-01	11.4		1.67		9.04E-01	
8	heterozygote 2	11.5	1.66	8.59E-01	11.5		1.66		8.59E-01	
9	heterozygote 3	11.3	1.67	9.51E-01	11.3		1.67		9.51E-01	

Software setup

Amplification and HRM

Step 1: Open a new run file

From the *File>New...* menu, select *High Resolution Melt Run* from the *Advanced* wizard.

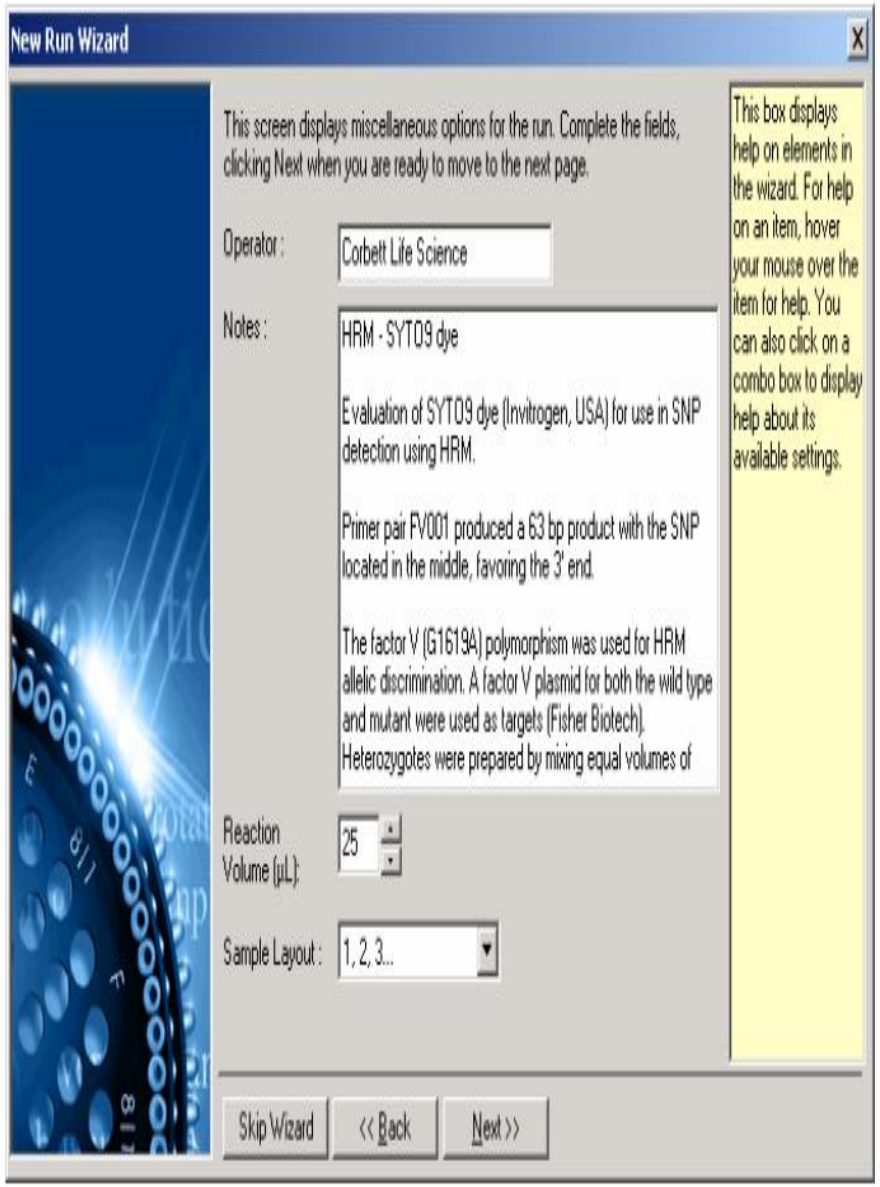


Step 2: Set the rotor type

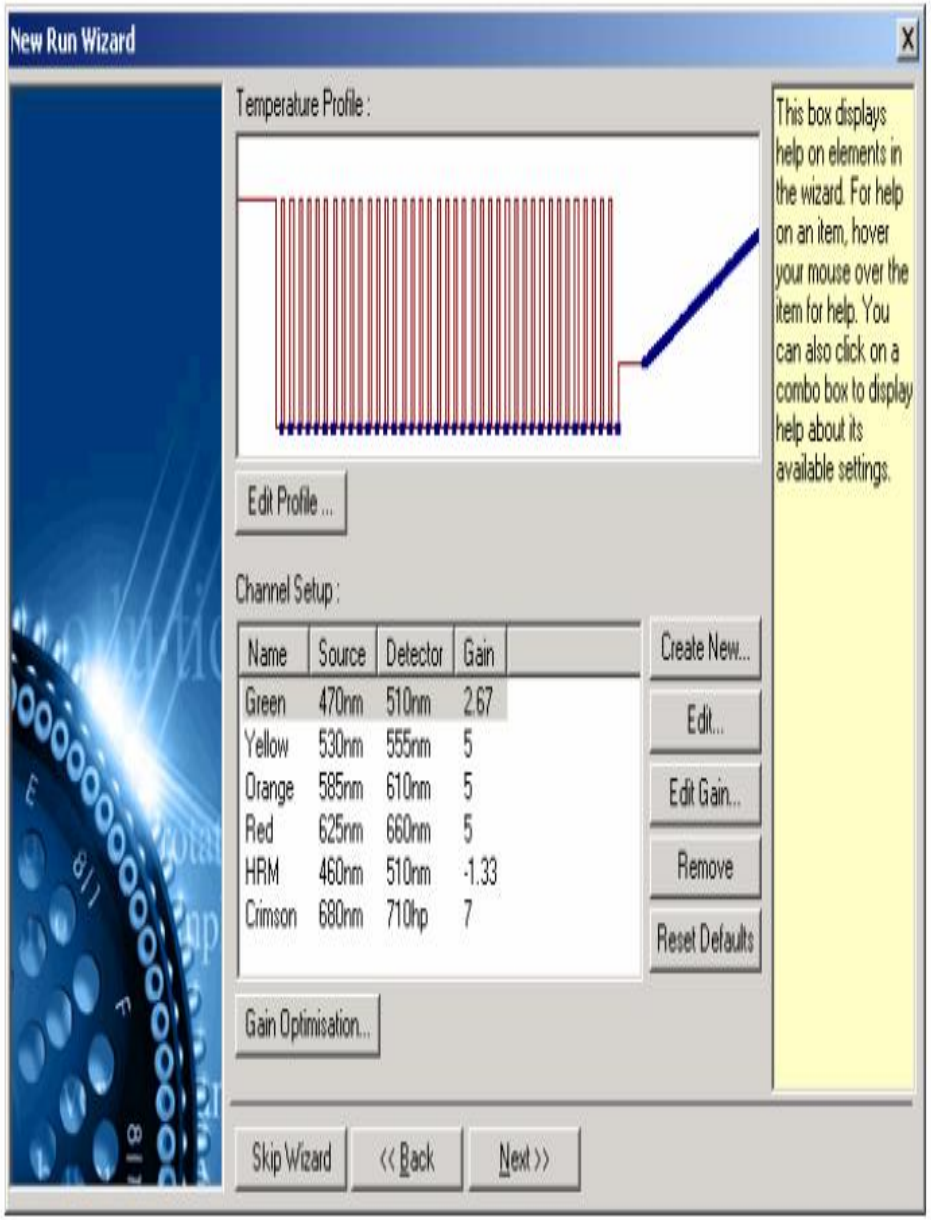
For this example the 72-Well Rotor is used. Ensure that the locking ring is in place and the *Locking Ring Attached* checkbox is checked before proceeding to the next step.

Step 3: Set run details

Type in the *Operator* name (optional) and add any *Notes* about the experiment to be recorded in the run file and incorporated into a post-run report (optional). Select the *Reaction Volume* (required) and *Sample Layout* desired (default is consecutive 1, 2, 3...)

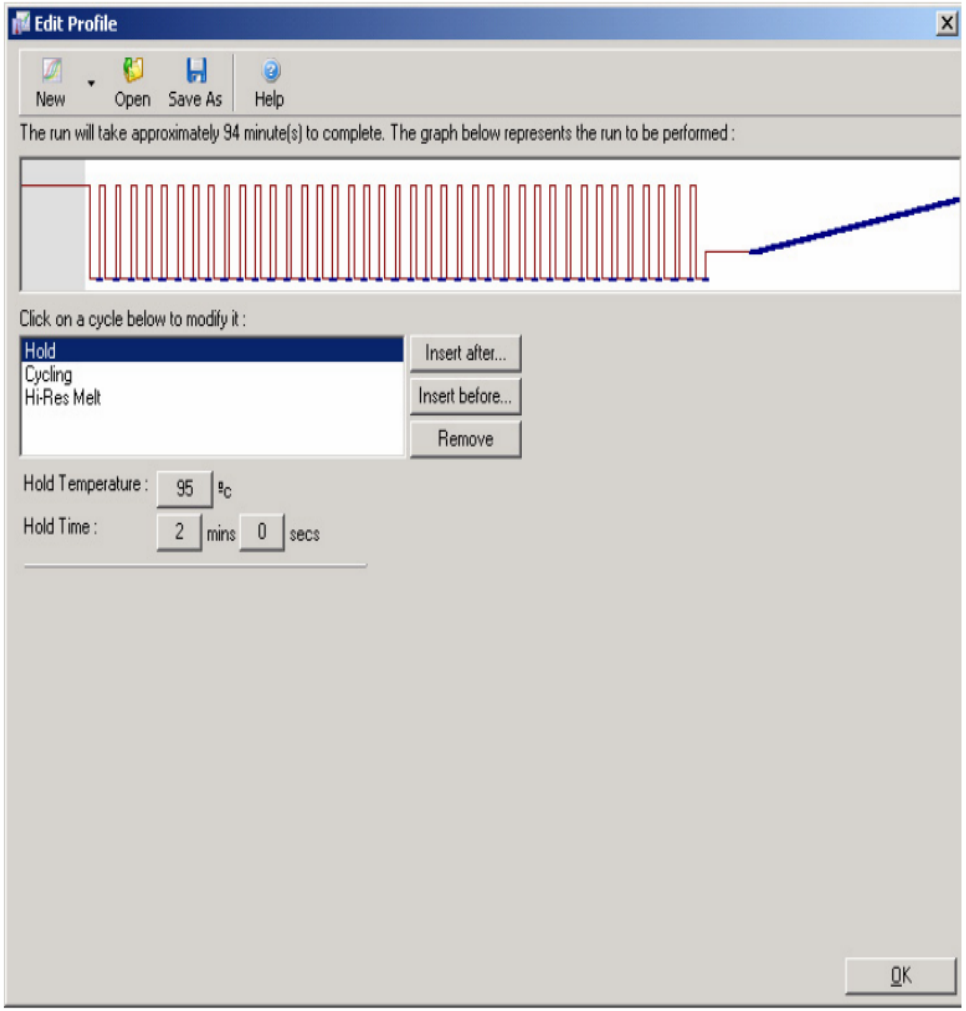


Step 4: Open *Edit Profile...* to modify the programmed times and temperatures for the reaction.



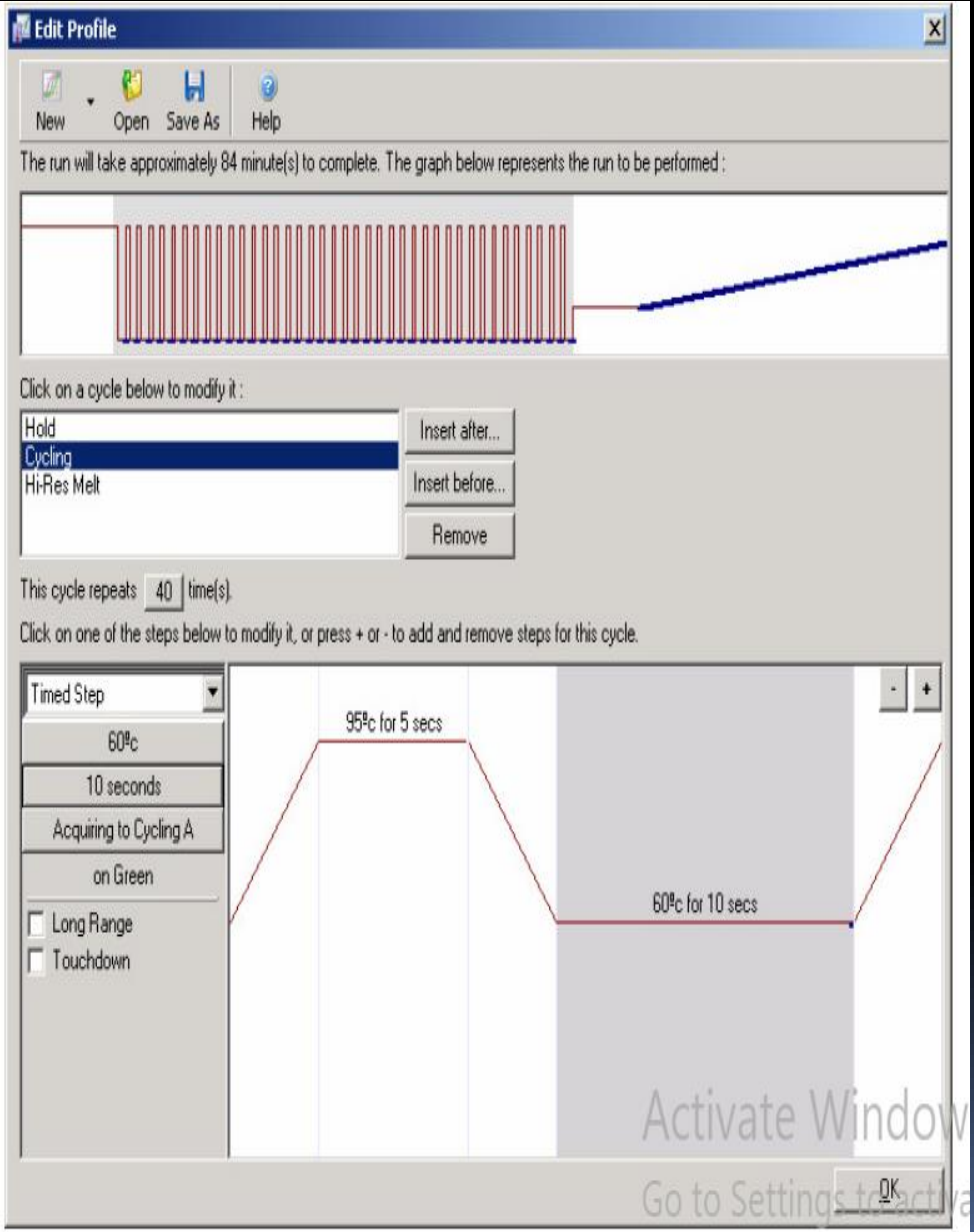
Step 5: Set an appropriate initial hold time

This time depends on the type of DNA polymerase used. For this assay we used Platinum® Taq DNA polymerase from Invitrogen Corp, which requires a 2 min activation time only. The default activation time is 10 min.



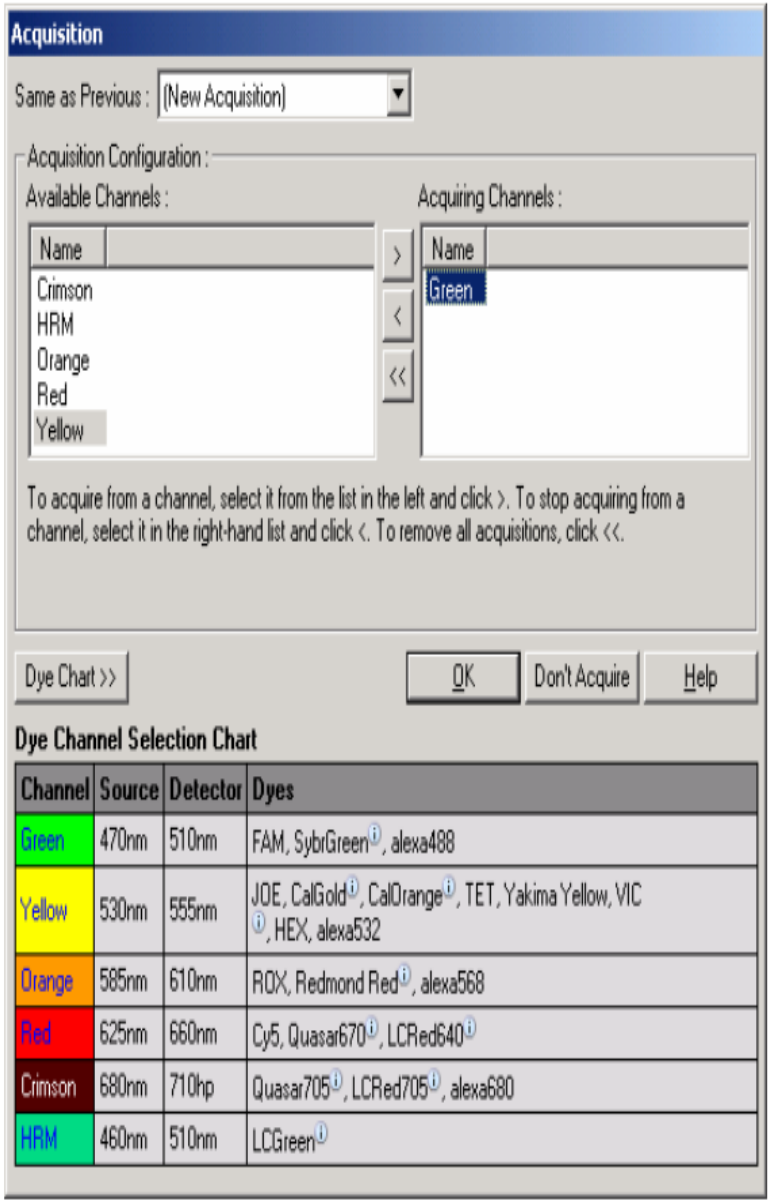
Step 6: Modify cycling to suit the amplicon

For short products use the default of 5 sec denature at 95°C and 10 sec anneal at 60°C (annealing temperatures may vary between assays).



Step 7: Ensure fluorescence data will be acquired

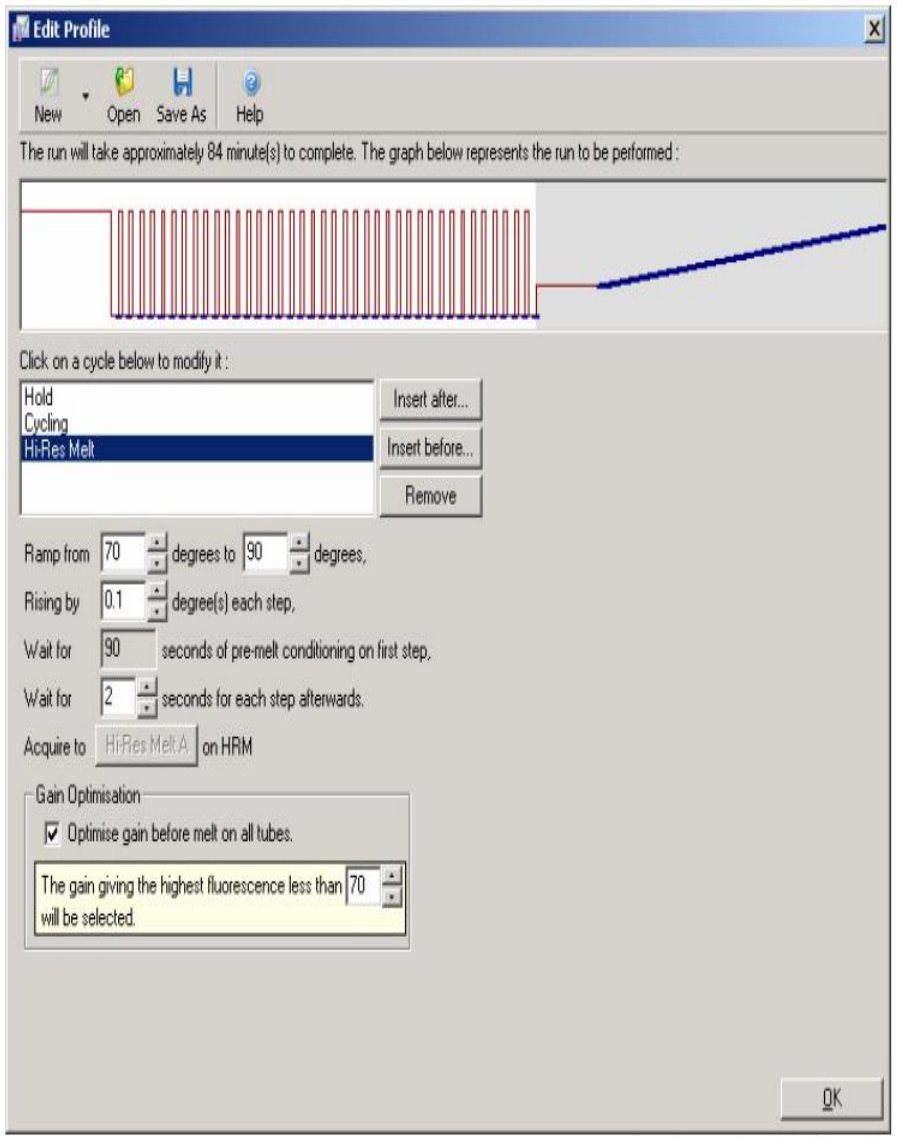
Acquire amplification data to the Green channel at the end of the anneal step.



Channel	Source	Detector	Dyes
Green	470nm	510nm	FAM, SybrGreen, alexa488
Yellow	530nm	555nm	JOE, CalGold, CalOrange, TET, Yakima Yellow, VIC, HEX, alexa532
Orange	585nm	610nm	ROX, Redmond Red, alexa568
Red	625nm	660nm	Cy5, Quasar670, LCRed640
Crimson	680nm	710hp	Quasar705, LCRed705, alexa680
HRM	460nm	510nm	LCGreen

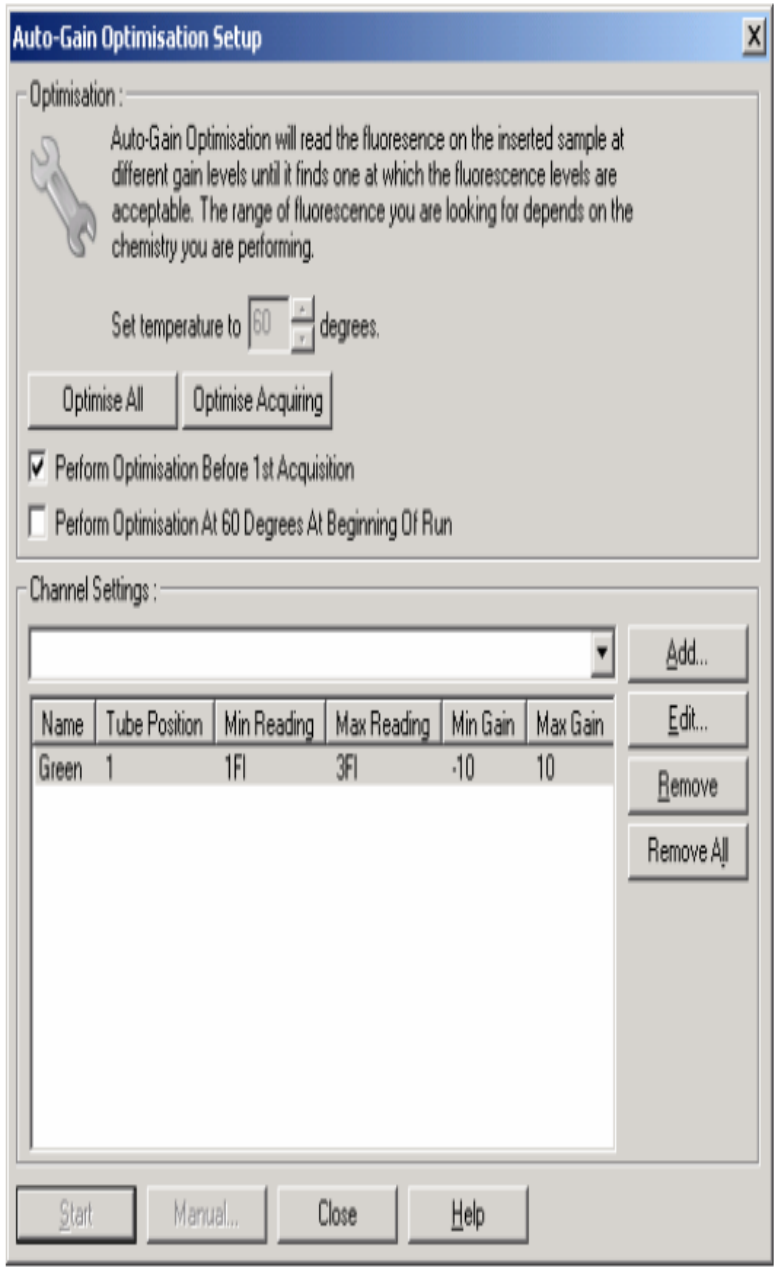
Step 8: Set HRM run conditions

Modify the HRM conditions to suit the amplicon. For the first set of experiments allow for a wide melt domain. Use the theoretical T_M to guide you to a suitable range. Once you have determined where the product will melt, reduce the melt domain to no greater than 10°C. Ensure that the start of the melt will occur 5°C prior to the first melt transition. The default ramp is set to 0.1°C with a hold of 2 sec at each step. The minimum ramp transition is 0.05°C with a second hold at each step. Data is automatically acquired to the HRM channel. Automatic gain (sensitivity) optimization will be done on all tubes by default. The software will search for the optimal gain setting so that the highest fluorescence value reported is no greater than 70 units on a scale of 100. Note this can be increased to a maximum of 100.



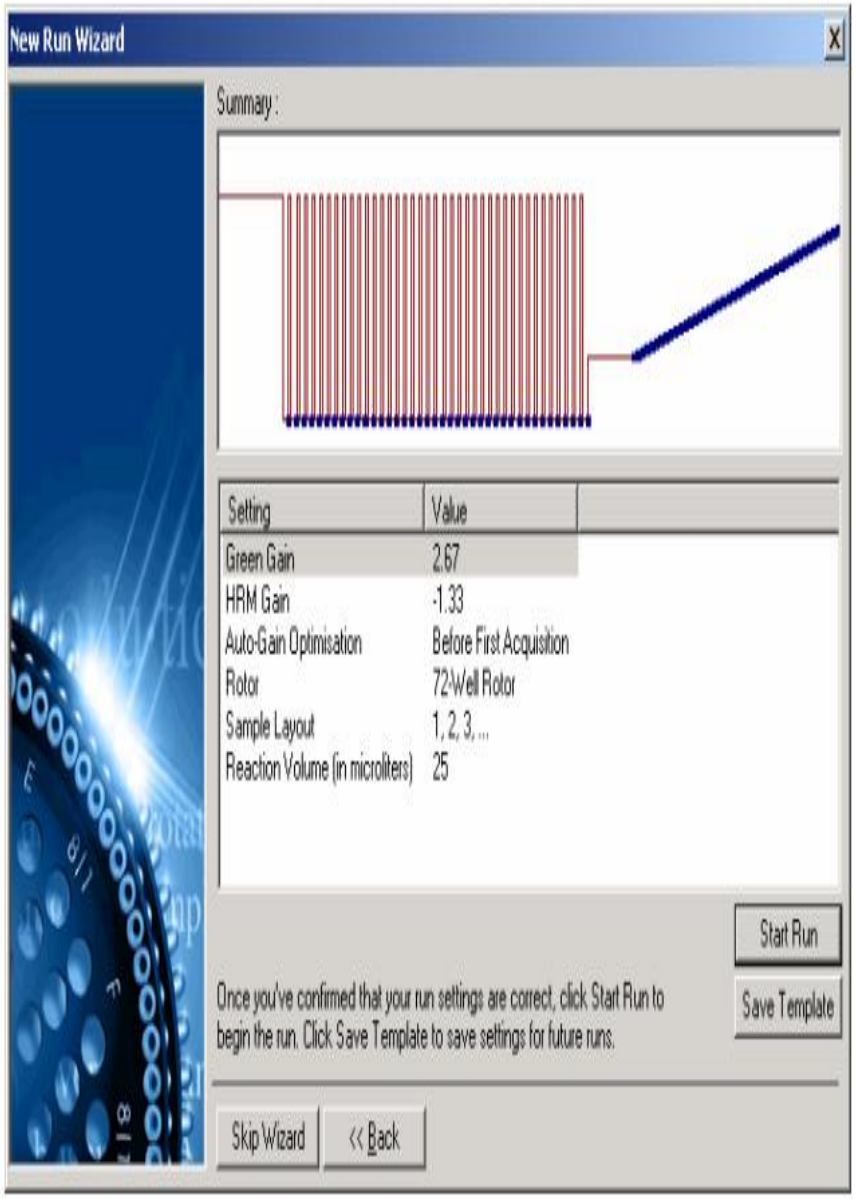
Step 9: Set Auto-Gain Optimization (Optional)

This applies to the real-time amplification step only and is set for the Green channel. Click the Optimize Acquiring button (to optimize only those channels actually used by a run). Optimization is best performed just prior to the first acquisition step, so click the Perform Optimization Before First Acquisition checkbox. The recommended background fluorescence range for intercalating dyes is between 1-3 Fluorescence units. To change this setting, click the channel name to select it in the list and then click the Edit button.



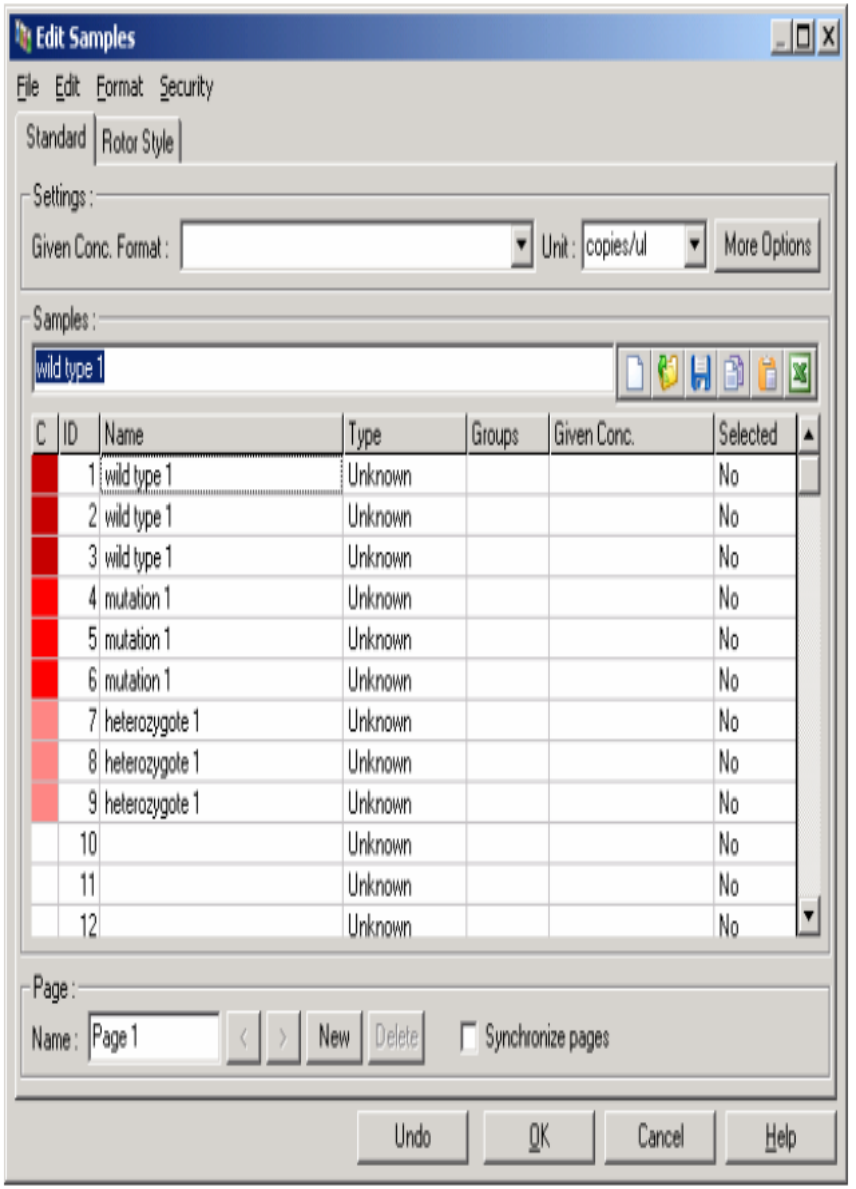
Step 10: Start the run

Click Start Run and save the run file to your computer.



Step 11: Edit Sample Names (*Optional*)

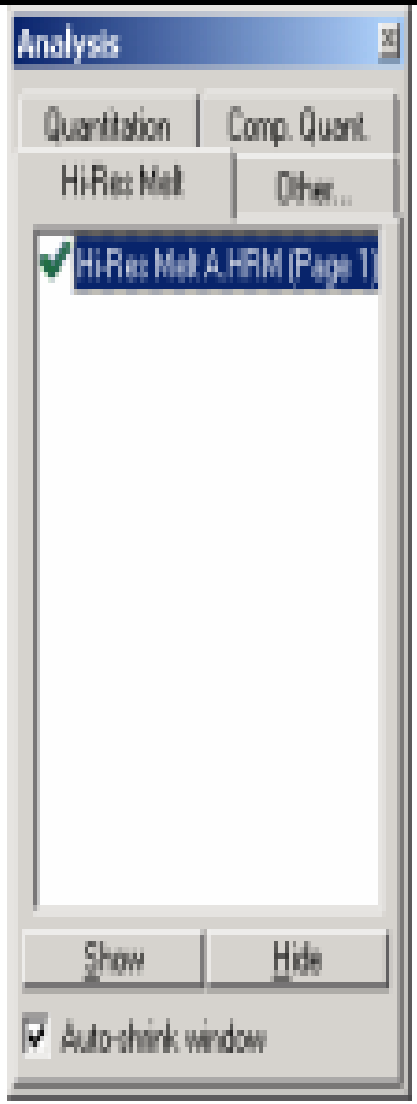
Note that sample names can be edited during or after a run, so this step can be skipped & completed later to save set-up time and expedite starting a run.



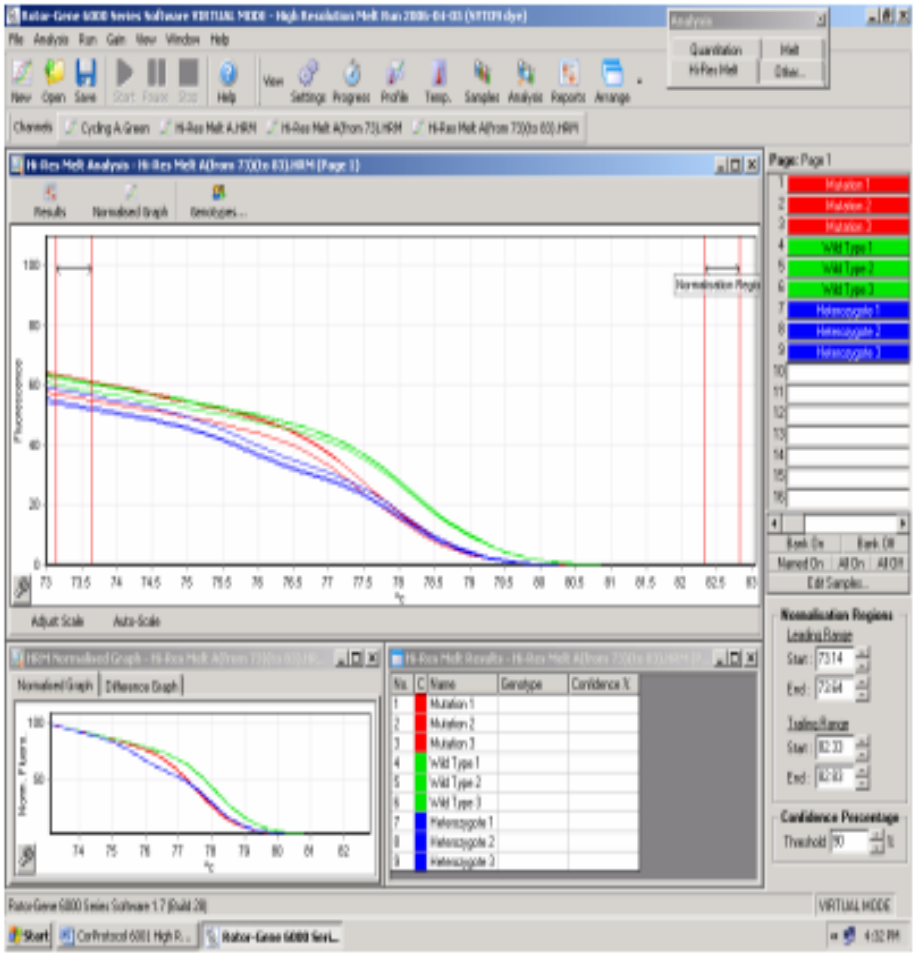
HRM Data Analysis

- HRM analysis allows for both visual- and auto-calling of genotypes.
- Results can be viewed as either a normalized melt plot or a difference plot.
- Normalized curves provide the basic representation of the different genotypes based on curve shifting (for homozygotes) and curve shape change (for heterozygotes).
- Difference plots are an aid to visual interpretation.
- They plot the difference in fluorescence of a sample to a selected control at each temperature transition.
- Difference plots provide an alternative view of the differences between melt curve transitions.

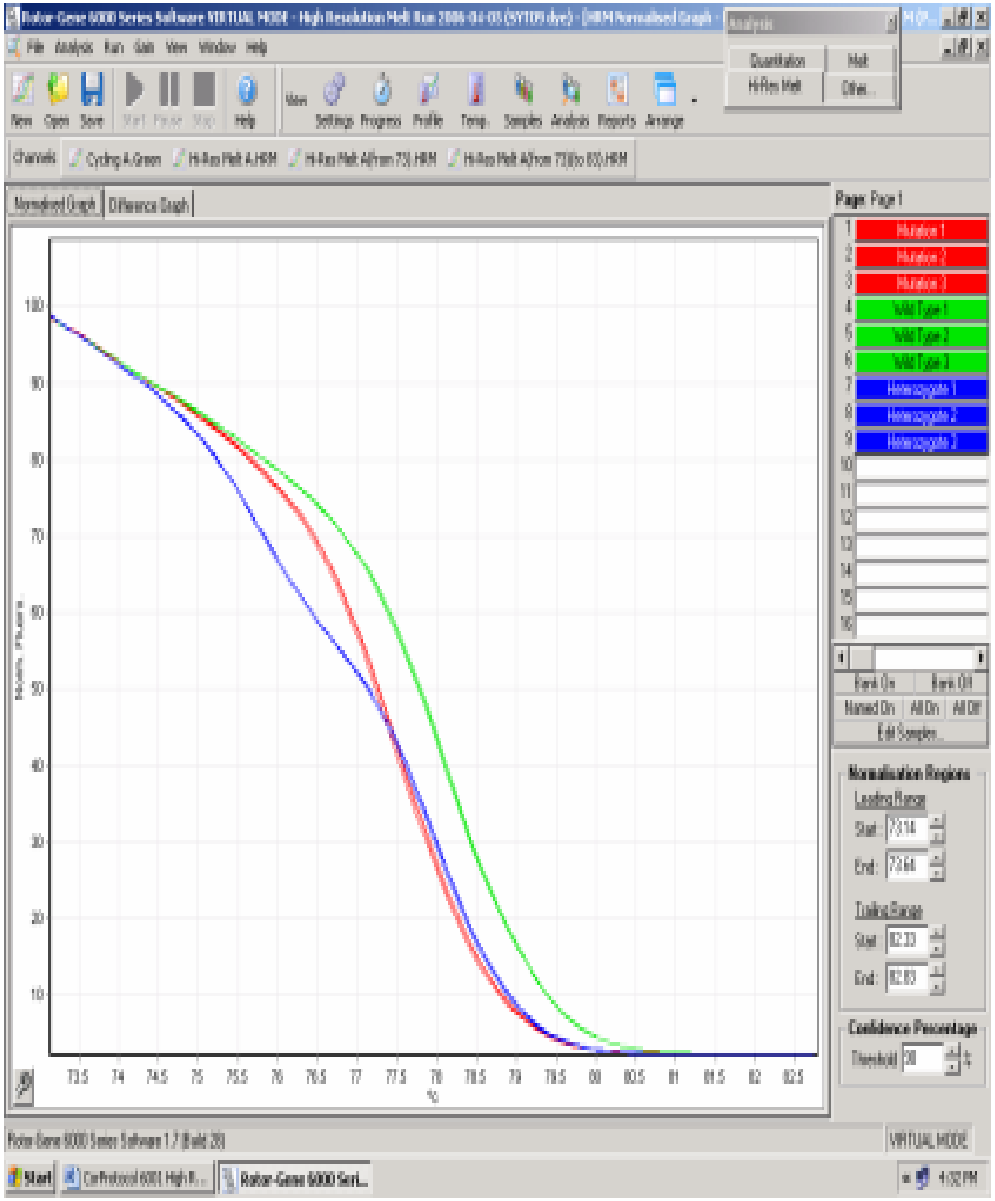
Step 1: Select the HRM analysis option from the *Analysis* palette.



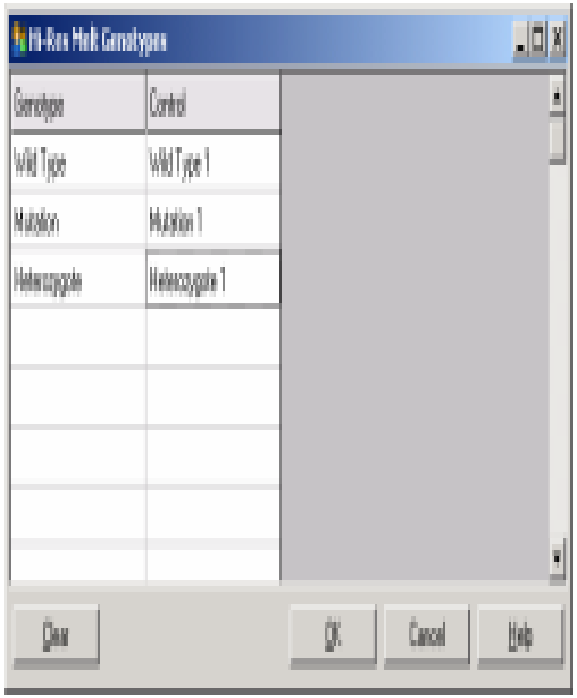
Step 2: The software will present three windows; raw data, normalized graph and results (shown opposite). The raw data window allows you to adjust the regions of normalization. Normalization allows all the curves to be compared with the same starting and ending fluorescent signal level to aid interpretation and analysis. Two cursors per region are provided, defaulted to the ends of the curve. The data points within the regions are used to normalize fluorescence (the Y axis only) for the start (Region 1) and end (Region 2) of the melt plot. Data outside the set regions is ignored. Adjust the regions to encompass representative baseline data for the pre-melt and post-melt phases. Widening the regions (by click and drag) allows the software to adjust for the slope of the baseline. To ensure curves normalize effectively, avoid widening the normalization regions into the melt phase.

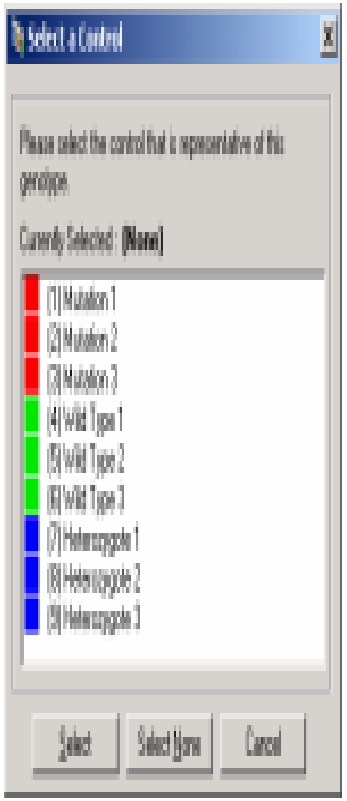


Step 3: The second window (bottom left corner) displays the normalized melt curves. Samples can also be viewed as a difference plot against one of the controls.

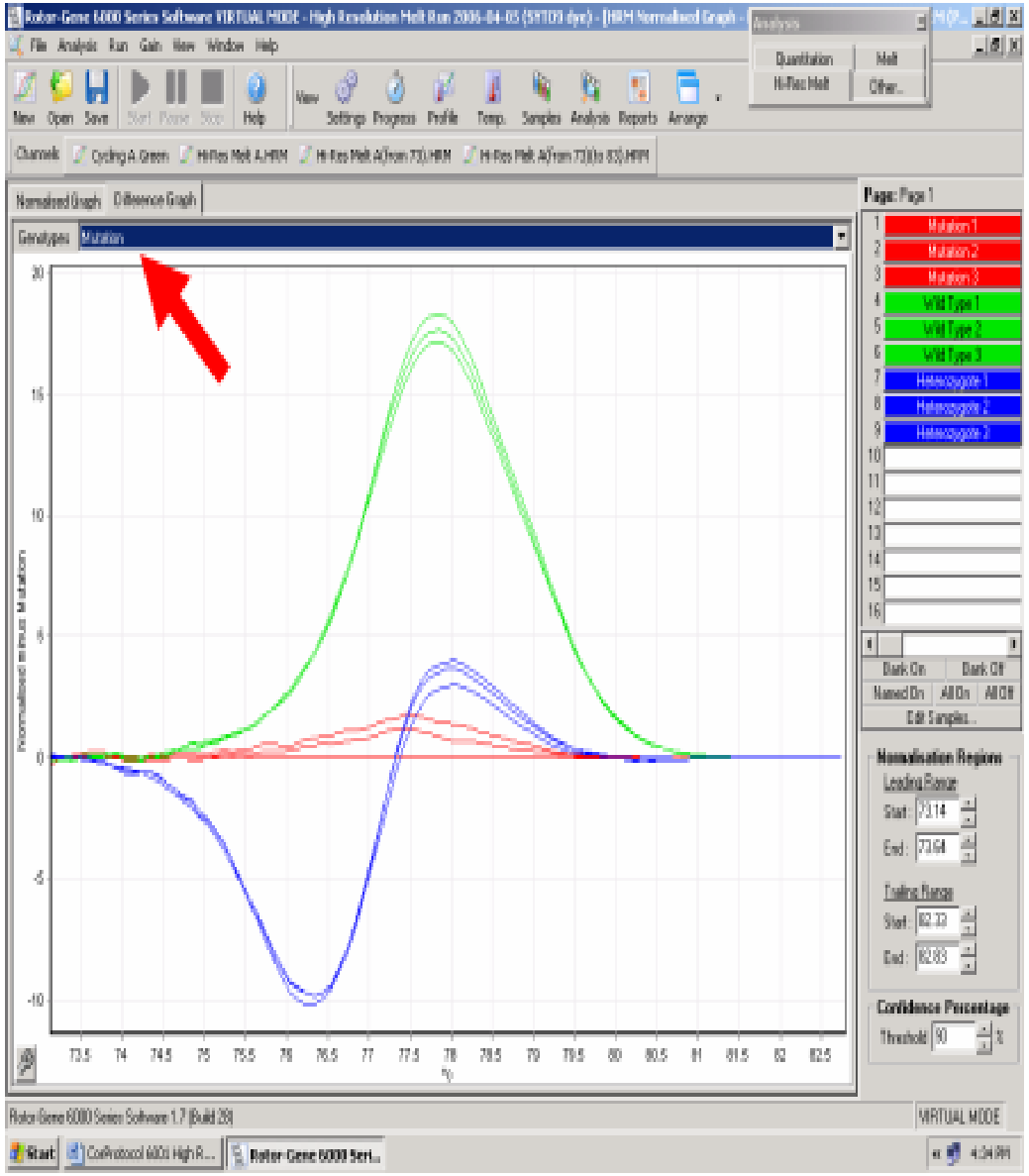


Step 4: In order to view the difference plot, representative genotypes must be defined. By clicking the *Genotypes...* button (top of the raw data window). Input each genotype category name and select a representative sample for each from the sample list, as shown in the example opposite.

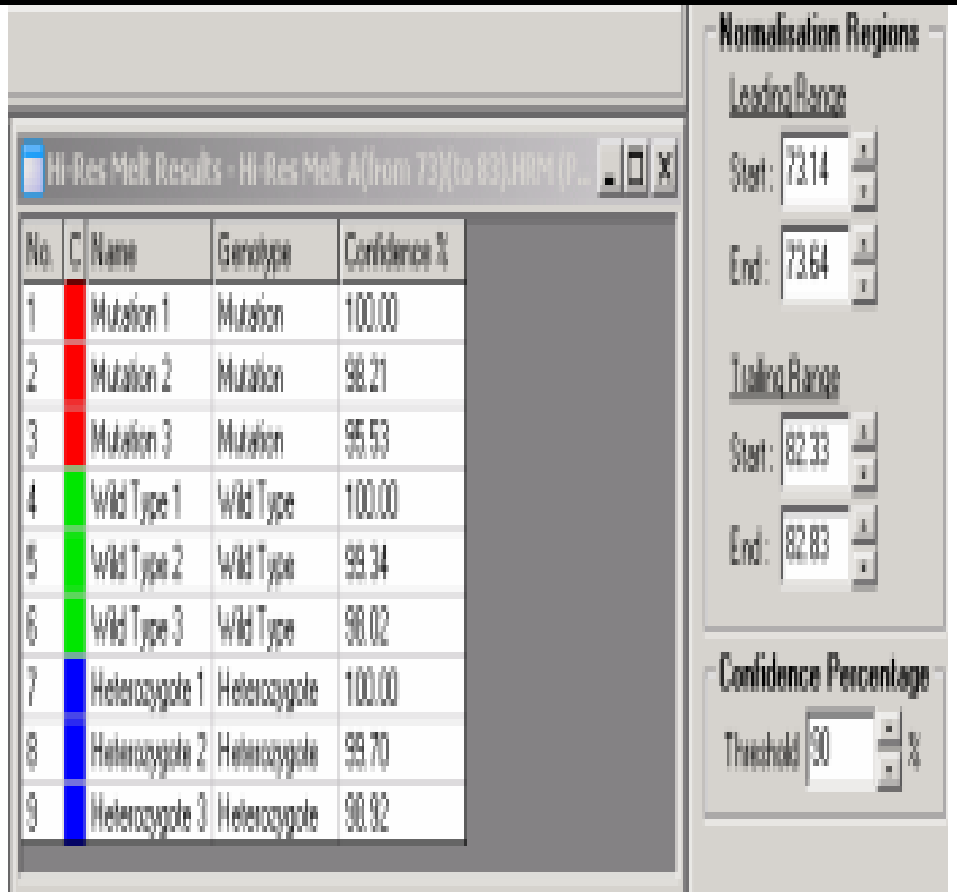




Step 5: View the difference plot by selecting the *Difference Graph* tab. Then select the *Genotype* you wish to compare all other samples against using the drop-down menu (arrowed). In the example shown, all samples are compared to the first sample "Mutation 1".



Step 6: Genotypes will be called automatically by the software in the third window. A confidence value is provided as an integrity check of auto-called results. The threshold value, above which auto-calls are made, can be edited. Samples that fall below the set threshold will be flagged as a variation for closer investigation or re-testing.



No.	C	Name	Genotype	Confidence %
1		Mutation 1	Mutation	100.00
2		Mutation 2	Mutation	98.21
3		Mutation 3	Mutation	95.53
4		Wild Type 1	Wild Type	100.00
5		Wild Type 2	Wild Type	99.34
6		Wild Type 3	Wild Type	98.02
7		Heterozygote 1	Heterozygote	100.00
8		Heterozygote 2	Heterozygote	99.70
9		Heterozygote 3	Heterozygote	98.92

7. Auto-call genotypes
(up to 100 at a time)

THANK U