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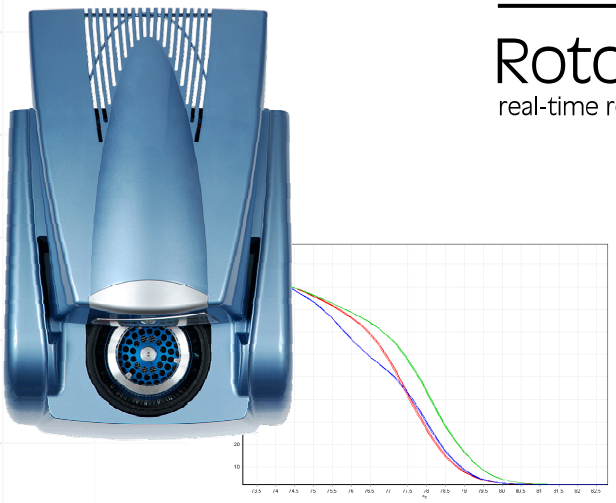
HRM™

High Resolution Melt Assay Design and Analysis



Rotor-Gene™ 6000
real-time rotary analyzer

CorProtocol™ 6000-1-July06

Contents

Introduction

High resolution melt (HRM) analysis is an extension of previous 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 (Figure 1). A HRM instrument collects fluorescent signals with much greater optical and thermal precision than previous methods to create new application possibilities.

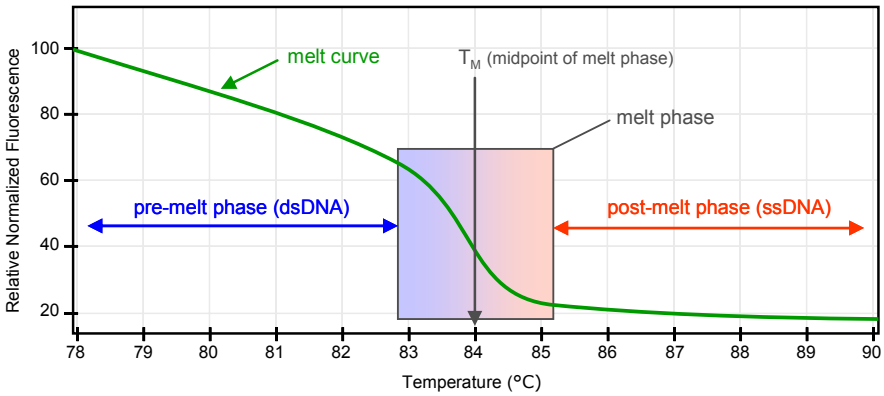


Figure 1: Fundamentals of a typical HRM (high resolution melt) plot. *The melt curve (green) plots the transition from high fluorescence of the initial pre-melt phase through the sharp fluorescence decrease of the melt phase to basal fluorescence at the post-melt phase. Fluorescence decreases as DNA intercalating dye is released from double-stranded DNA as it dissociates (melts) into single strands. The midpoint of the melt phase, at which the rate of change in fluorescence is greatest, defines the temperature of melting (T_M) of the particular DNA fragment under analysis.*

Prior to performing a HRM analysis, a target sequence must first be purified to high copy number. This is normally done using a DNA amplification reaction such as the PCR in the presence of a dsDNA intercalating fluorescent dye. The dye does not interact with ssDNA but actively intercalates with dsDNA and fluoresces brightly in this state. 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. 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. The observed "melting" behavior is characteristic of a particular DNA sample.

Using HRM, a Rotor-Gene 6000 instrument can characterize samples based on sequence length, GC content and DNA sequence complimentarity. For example, HRM can be used to detect single base sequence variations such as SNPs (single nucleotide polymorhphisms) or to discover unknown genetic mutations. It can also be used to quantitatively detect a small proportion of variant DNA in a background of wild-type sequence at sensitivities approaching 5%. This can be used, for example, to study somatically acquired mutations or changes in the methylation state of CpG islands.

HRM on the Rotor-Gene 6000 is being developed for multiple applications, including:

- Identification of candidate predisposition genes
- Association studies (comparing cases and controls, genotype to phenotype)
- Determination of allele prevalence within a population or sub group
- Screening for loss of heterozygosity
- DNA fingerprinting
- Characterization of Haplotype blocks
- DNA methylation analysis
- DNA mapping
- Species identification
- Mutation discovery
- Determining the ratio of somatic acquired mutations
- HLA typing

HRM is a simpler and more cost effective way to characterize samples than probe-based genotyping assays and, unlike conventional methods, it is a closed assay system requiring no post PCR processing. Results are comparable to more time consuming and expensive conventional methods such as SSCP, DHPLC, RFLP and DNA sequencing (White and Potts, 2006).

Instrumentation and analysis software

Herrmann *et al* (2006) published a cross-platform comparison of instruments and dyes for high-resolution DNA melt analysis. While this comparison was made prior to the availability of the Rotor-Gene 6000, it nevertheless clearly highlighted the limitations of conventional systems for HRM use. HRM requires demanding real-time and thermo-optical analysis capabilities, specifically;

- High intensity illumination
- High sensitivity optical detection
- A fast data acquisition rate
- The ability to exquisitely control sample temperature
- An absolute minimum of sample-to-sample thermal and optical variation

In addition, the methods used to normalize and display HRM data for intuitive manual analysis or automated genotyping are specific and continue to improve (see data analysis section).

Chemistry

HRM is made possible not only by specialized instrumentation and software, but also by the introduction of third generation fluorescent dsDNA dyes. Third generation intercalating dyes such as SYTO 9 (Invitrogen Corp., Carlsbad, CA), LC Green (Idaho Technologies, Salt Lake City, UT) and Eva Green (Biotium Inc, Hayward, CA) have been successfully used for HRM analysis on the Rotor-Gene 6000 (Reja and Bassam, in preparation). These dyes have low toxicity in an amplification reaction and can therefore be used at higher concentrations for greater saturation of the dsDNA sample (Figure 2). Greater dye saturation means measured fluorescent signals have higher fidelity, apparently because there is less dynamic dye redistribution to non-denatured regions of the nucleic strand during melting and because dyes do not favor higher melting temperature products (Wittwer *et al* 2003). The combination of these characteristics provides greater melt sensitivity and higher resolution melt profiles.

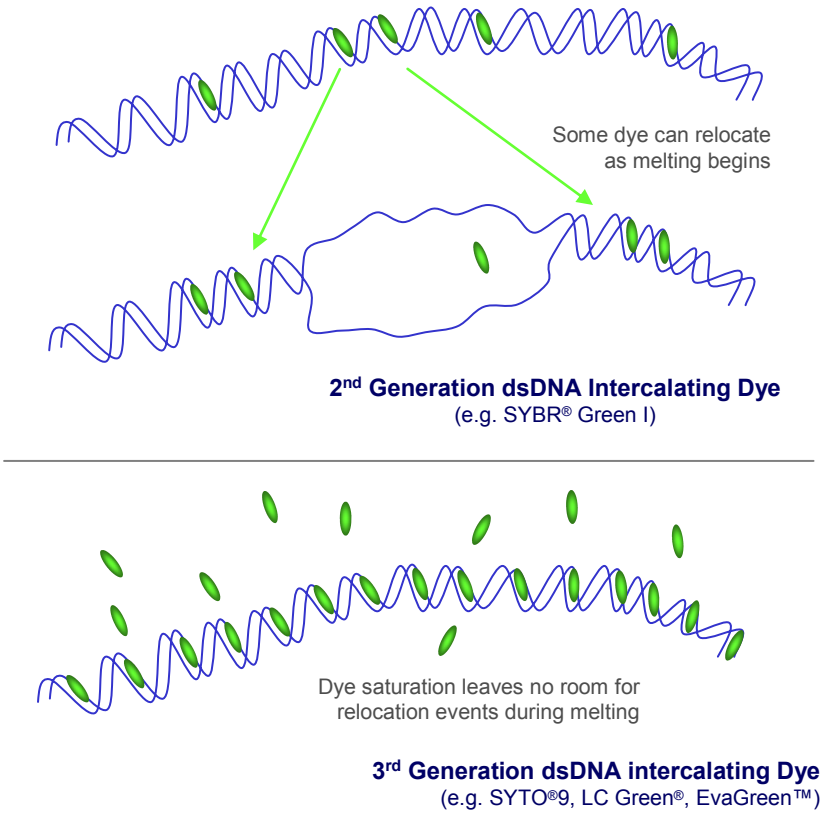


Figure 2: Redistribution of DNA intercalation dyes during DNA dissociation.
The reduced reaction toxicity of 3rd generation dyes means that a higher concentration of dye can be used. Higher dye concentration increases the level to which the DNA becomes saturated with dye molecules. Saturation is believed to reduce dye redistribution effects during DNA dissociation (as illustrated) which increases the resolution of melt analysis.

Example Application: SNP Genotyping

HRM on the Rotor-Gene 6000 can be used to analyze virtually all types of DNA sequence variants, including single base changes, insertions, deletions and base pair substitutions (White and Potts 2006). Representative of the smallest genetic change, the detection and genotyping of SNPs underlines the sensitivity of HRM analysis. By way of example, this protocol outlines the principles used to design and analyze a typical SNP assay suitable for routine automated genotyping by HRM.

The assay design and analysis principles outlined in this protocol are valid for other HRM applications, such as the detection of unknown (or new) mutations. Unknown mutations are often a single nucleotide change, but they may also comprise multiple base changes, insertions and/or deletions. In general, the more base changes in the DNA the easier they are to detect by HRM.

SNPs have been divided into four classes as summarized in Table 1. The most difficult to genotype are the class 4 (A/ T conversions). However, the near-perfect thermal and optical uniformity of the Rotor-Gene can allow even class 4 SNPs to be resolved (Figure 3).

SNP Class	Base Change	Typical T_M Melt Curve Shift	Rarity (in the 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%

Table 1: SNP classes as defined by Venter et al. (2001).

For SNP analysis, homozygous allelic variants are characterized by a temperature (x-axis) shift in a HRM melt curve whereas heterozygotes are characterized by a change in melt curve shape (Figure 3). The change in curve shape is a result of destabilized heteroduplex annealing between some of the wild type and variant strands. The heterozygote melting curve is thus a composite of both heteroduplex and homoduplex components, and, because it dissociates more readily, shifts left to lower temperature.

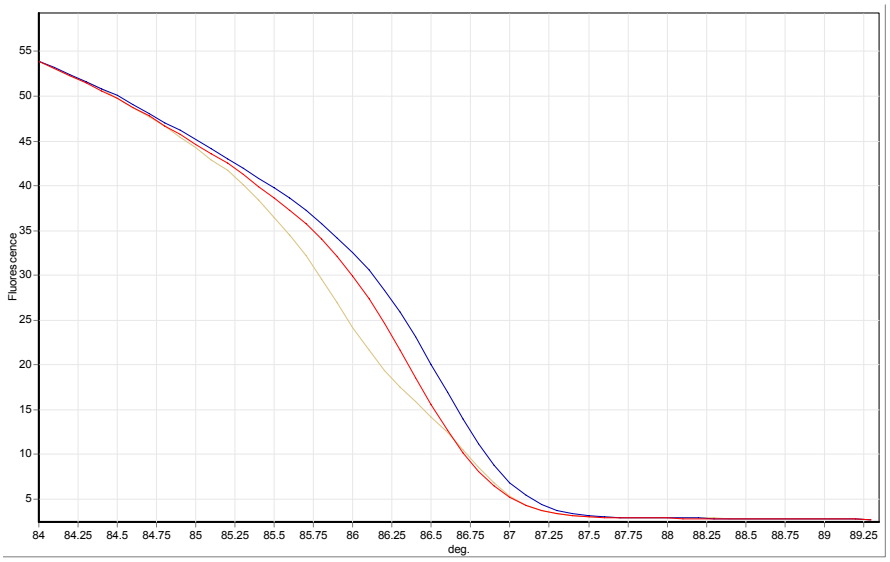


Figure 3. Example of Class 4 SNP genotyping by HRM on the Rotor-Gene 6000; discrimination of monocarboxylate transporter 1(MCT1; A1470T) alleles. *MCT1 is a representative class 4 SNP (A to T conversion)—the rarest and most difficult SNP type to discriminate. HRM analysis identified a characteristically subtle change in T_M of only about 0.2°C between homozygous samples. The melt profiles shown are; AA homozygote, blue (right); TT homozygote, red (middle) and the AT heterozygote, yellow (left) . Heteroduplexes are discriminated by a change in the shape of the melt curve. Homoduplexes usually have the same curve shape, as seen here, and are differentiated primarily by a shift in the curve on the temperature axis (T_M shift). The length of the MCT1 amplicon analyzed here was 66 bp, and was amplified and melted using LCGreen I dye.*

Guidelines for successful HRM assays

While usually highly effective, the success of HRM analysis depends largely on the particular sequence under investigation. Certain sequence motifs, such as hairpin loops or other secondary structures, localized regions of unusually high or low GC content, or repeat sequences can all affect the outcome in unpredictable ways. However, here are some simple guidelines to help ensure success:

1. **Analyze small DNA fragments**
   Analyze fragments no greater than about 250 bp. Larger products can be analyzed successfully but usually with lower resolution. This is simply because a single base variation affects the melting behavior of a 100 bp amplicon more than a 500 bp amplicon, for example.

2. **Analyze a single pure product**
   Samples contaminated with post-amplification artifacts such as primer-dimer or non-specific products can make HRM results difficult to interpret.

3. **Use sufficient pre-amplification template**
   The capture and analysis of real-time amplification data can be extremely useful when troubleshooting HRM analyses. Amplification plots should have a C_T (threshold cycle) of no more than 30 cycles. Products that amplify later than this (due to too little starting template amount or template degradation effects) typically produce variable HRM results due to amplification artifacts.

4. **Check for aberrant amplification plots**
   Prior to running HRM, examine real-time plot data carefully for abnormal amplification curve shape. Plots having a log-linear phase that is not steep, is jagged, or that reaches a low signal plateau compared to other reactions can indicate poor amplification or a fluorescence signal that is simply too low. Poor reactions can be caused by reaction inhibitors, too little dye, incorrect reaction set-up, etc. HRM data from such samples can be inconclusive or of lower resolution.

5. **Keep post-amplification sample concentrations similar**
   The concentration of a DNA fragment affects its temperature of melting (T_M). For this reason sample DNA concentrations must be kept as similar as possible. When analyzing amplification products, ensure every reaction has amplified to the plateau phase. At plateau, all reactions will have amplified to a similar extent irrespective of their starting amount. Note however that "poor" reactions might not reach plateau with the same amplified quantity due, for example, to inconsistent assay set-up (e.g. the primer concentration was too low).

6. **Ensure sample-to-sample uniformity**
   All samples must be of equal volume and should contain the same concentration of dye. DNA melting behavior is affected by salts in the reaction mix, so it is important that the concentration of buffer, Mg and other salts are as uniform as possible in all samples. Similarly, use only identical reaction tubes from the same manufacturer to avoid variations due to plastic thickness and auto-fluorescence properties.

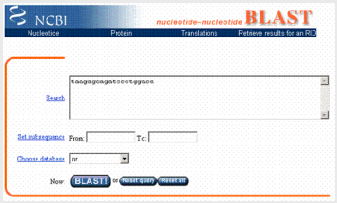
7. **Allow sufficient data collection for pre-and post-melt phases**
   Capture HRM data points over about a 10°C (or greater) window, centered around the observed T_M (see Figure 1). This provides enough baseline data points for effective curve normalization and will result in tighter replicates and easier data interpretation.

Protocol Overview

A
Design

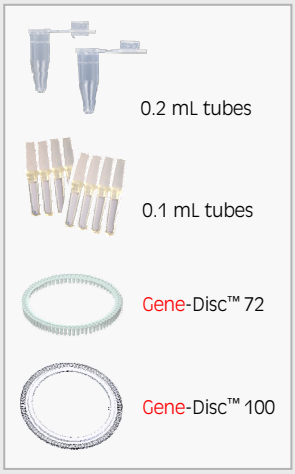
1. Identify target sequence
(use an online database)

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

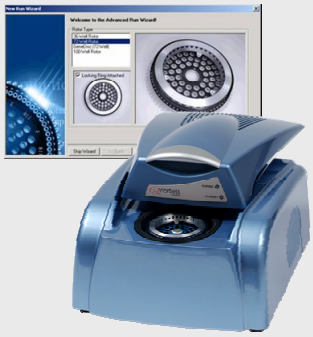


3. Check specificity
(BLAST primer sequences)

B
Run

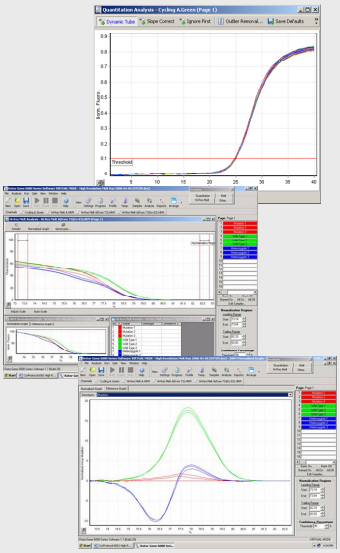


4. Set up reactions
in desired tube format



5. Run PCR *and* HRM

C
Analyze



6. Analyze results
(real-time and HRM)

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)

Protocol Validation

This protocol has been functionally developed on the Rotor-Gene 6000 Real-Time Thermal Analyzer using standard Corbett Life Science consumables and standard commercial reagents.

Results will vary depending upon DNA template quality and the particular sequence analyzed (see Guidelines for Successful HRM Assays, above). DNA quality will influence amplification efficiency and specificity. Sequences with strong secondary structures or rich in GC content may hinder the detection of difficult to detect base substitutions (e.g. A>T). Consistency in reaction setup and reagent use is paramount.

Inconsistencies in melt behavior can occur due to variations in the following:

- $MgCl_2$ concentration
- Buffer salts
- Taq storage buffer additives
- Intercalating dye type and concentration
- Reaction volume
- Melt ramp rate (=rate of temperature change)
- Reaction vessel

The protocol outlined here describes the design, reaction setup and genotype analysis of the Factor V Leiden (G313A) Class 1 polymorphism. However, this protocol has also been used to develop a wide variety of successful SNP assays, including:

- Human Monocarboxylate transporter 1 (A1470T)
- Human Glutathione S-transferase P1 (A313G)
- Human Manganese superoxide dismutase (T175C)
- Human Methylene tetrahydrofolate reductase (C677T)
- Human Tumor necrosis factor alpha (G-308A)
- Human Collagen type 1 alpha 1 (G-Sp1T)
- Human Beta-2 adrenergic receptor (Gln27Glu)
- Human Endothelial nitric oxide synthase (T-786C)

Reagents and Consumables Required

The following reagents and consumables are required by this protocol:

Item	Requirement for a Full 72-Well Rotor	Part No.	Supplier
Consumables			
72-Well High Profile Rotor	1	6001-001	Corbett Life Science
72-Well Rotor Locking Ring	1	6001-002	Corbett Life Science
0.1 mL Strip Tubes and Caps	18 strips of tubes & caps	3001-002	Corbett Life Science
Reagents			
5 U/µL Platinum® Taq DNA polymerase	18 µL	10966-083	Invitrogen
10X PCR Buffer (no $MgCl_2$)	180 µL	53286	Invitrogen
50 mM $MgCl_2$	54 µL	52723	Invitrogen
100 mM dATP Solution	144 µL[A]	55082	Invitrogen
100 mM dCTP Solution	144 µL[A]	55083	Invitrogen
100 mM dGTP Solution	144 µL[A]	55084	Invitrogen
100 mM dTTP solution	144 µL[A]	55085	Invitrogen
SYTO® 9 green fluorescent nucleic acid stain	54 µL[B]	S34854	Invitrogen

[A] *At a concentration of 2.5 mM*
[B] *At a concentration of 50 µM*

Reagent Handling and Storage

Upon receipt of reagents, unpack and store the individual reagents as follows:

Reagent	Pack Size	Storage Temp.	Storage State
5 U/µL Platinum® Taq DNA polymerase	500 rxn	–20°C	Not Critical
10X PCR Buffer (no $MgCl_2$)	50 mL	–20°C	Not Critical
50 mM $MgCl_2$	10 mL	18–24°C	Not Critical
100 mM dATP Solution	250 µL	–20°C	Not Critical
100 mM dCTP Solution	250 µL	–20°C	Not Critical
100 mM dGTP Solution	250 µL	–20°C	Not Critical
100 mM dTTP solution	250 µL	–20°C	Not Critical
SYTO® 9 green fluorescent nucleic acid stain	100 µL	–20°C	Dark

Reagent Preparation

dNTPs
Prepare the dNTPs by aliquoting 20 µL of each to 720 µL of molecular biology grade water to achieve an 800 µL working stock concentration of 2.5 mM. Store the prepared working stock at –20°C until required.

SYTO® 9 Dye
Dilute 5 µL of the 5 mM stock concentration of SYTO 9 green fluorescent nucleic acid stain into 495 µL of molecular biology grade water to obtain a working stock of 50 µM. Cover the tube with aluminium foil to avoid light and store the prepared working dye stock at –20°C until required (up to 6 months). Prepared working stock solution can be kept at 4°C for 2–3 weeks.

Sample Preparation

Samples must be isolated and stored in a manner that will prevent degradation. Avoid excessive amounts of inhibitors such as ethanol carry-over. To improve HRM results it is recommend that the amount of template used be consistent between samples. The use of spectral analysis for determining DNA concentration and purity is recommended.

NOTE

At 260 nm one absorbance unit is equal to 50 µg/mL of DNA

Pure DNA will provide a 260 nm to 280 nm ratio of 2

Assay Design Criteria

The following criteria should be used when designing HRM assays:

- First obtain a clear understanding of the sequence targeted. Where possible, determine all the variations present within the sequence of interest. Check for species homology, intron-exon boundaries, splice sites, known SNPs, etc.
- Design primers with anneal temperatures of 60°C that will amplify short products (100–250 bp). Longer products can be used, however, using products above 250 bp can reduce sensitivity due to the increased potential for multiple melt domains with complicated melt curves.
- Determine the folding characteristics of the product and primers at the annealing temperature (e.g. use DINAMelt, see below) and test for specificity (BLAST search).
- (OPTIONAL) Determine the theoretical T_M difference between the wild type and variant amplification products. While predicted T_{Ms} may vary compared with experimental results (due to the limitations of the software algorithm as well as salt and concentration variables) they can nevertheless be a useful guide.

Amplicon Design Protocol

Step 1: Identify the correct flanking sequence
Find the sequence containing the polymorphic site using an appropriate sequence database. Here we use the NCBI SNP web search engine (http://www.ncbi.nlm.nih.gov/SNP). Using a search for Coagulation Factor V Leiden on the NCBI Nucleotide page we find the sequence and position of the SNP along with other valuable information such as the Accession number of the gene. Example screenshots for this process from the NCBI web site are shown below.

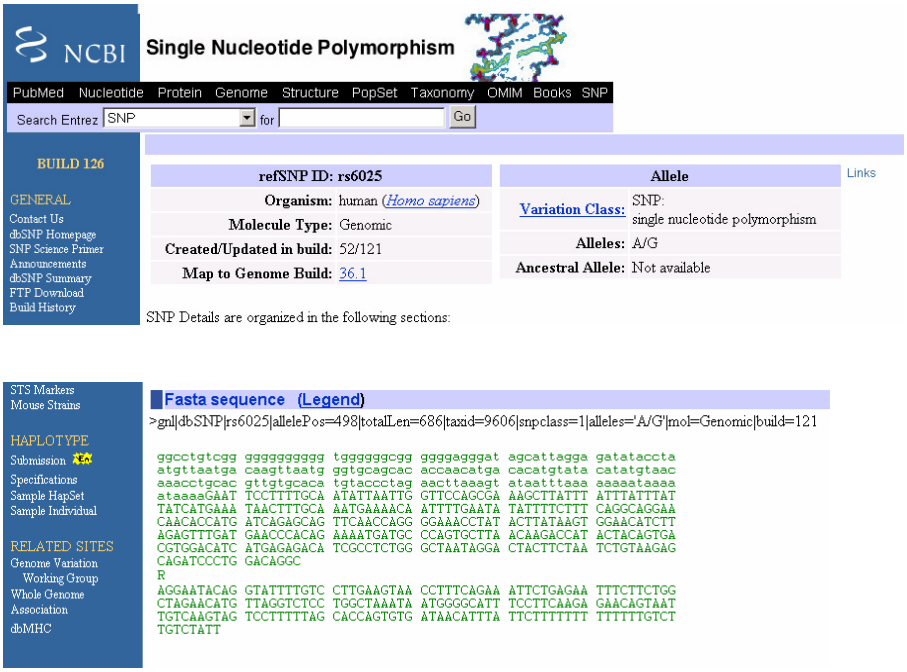


***Figure 4*: NCBI Single Nucleotide Polymorphism search result for Factor V Leiden.** *Fasta sequence shows the position of the SNP in relation to the complete genomic sequence. A portion of this sequence should be used to find the whole genomic sequence using BLAST.*

Step 2: Identify other sequence features

BLAST a portion of the Fasta sequence to locate the SNP in the whole genomic sequence. Part of the sequence page will contain information regarding the location of various features and qualifiers such as variation sites and intron–exon boundaries. Using this information we can find other possible variations present near our region of interest. These regions are highlighted in blue while our variation is highlighted in yellow (shown below). It is important to avoid other variations that can cause a change in the resulting melt curves.

```
     variation       38224
                     /gene="F5"
                     /frequency="0.47"
                     /replace="t"
     variation       38337
                     /gene="F5"
                     /frequency="0.01"
                     /replace="c"
     variation       38529
                     /gene="F5"
                     /frequency="0.17"
                     /replace="a"
     variation       38592
                     /gene="F5"
                     /frequency="0.01"
                     /replace="a"
     misc_feature    38767..41751
                     /gene="F5"
                     /note="Region not scanned for variation"
```

***Figure 5**: **Sequence features and qualifiers reported by the NCBI data base**. Reported features include known variations along with the position in the genomic sequence. Variations other than those being investigated should be avoided as they will influence HRM results.*

Step 3: Copy the target sequence

Copy a section of the gene that contains the variation (highlighted in yellow, SNP highlighted in red) avoiding regions highlighted in blue that contain variations that may influence the melt.

```
38461 tgatgaaccc acagaaaatg atgcccagtg cttaacaaga ccatactaca gtgacgtgga
38521 catcatgaga gacatcgcct ctgggctaat aggactactt ctaatctgta agagcagatc
38581 cctggacagg cgaggaatac aggtattttg tccttgaagt aacctttcag aaattctgag
38641 aatttcttct ggctagaaca tgttaggtct cctggctaaa taatggggca tttccttcaa
38701 gagaacagta attgtcaagt agtccttttt agcaccagtg tgataacatt tattcttttt
```

Step 4: Paste target sequence into the amplicon design software

Paste the copied segment of the sequence into Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Indicate to the Primer3 software the SNP position using square brackets [ ] so that the primers must flank the SNP. To help you find the SNP in Primer3, capitalize the base prior to importing.

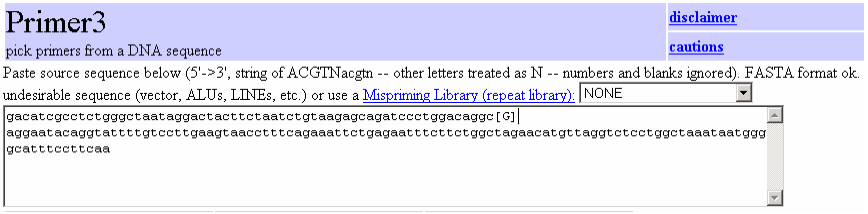
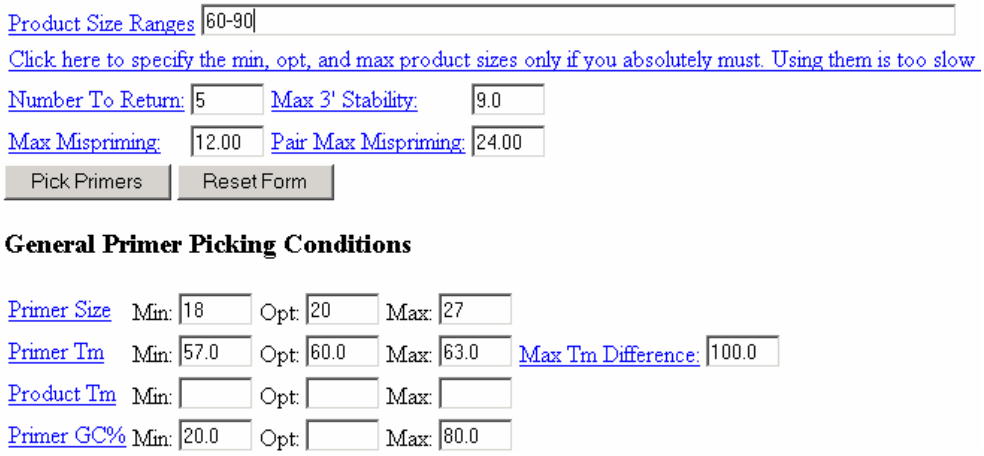


***Figure 6**: **Primer3 sequence input**. To help identify the SNP the letter was capitalized. Square brackets were used to ensure that the primers flanked the SNP.*

Step 5: Establish design parameters

Select the product size to between, for example 60–90 bases (up to 250 bp). Primers size is set to be between 18 and 27 bases long, have T_M values between 57°C and 63°C and a GC content of 20 to 80% (as shown below). Leave the other parameters such as Max 3' stability and Max mis-priming as defaulted.



Step 6: Choose primers

Primer3 will generate primer sequences based on the criteria specified. The primer sequence, length, T_M, GC%, and amplicon length are reported. The position of the primers in relation to the sequence submitted and the SNP are also shown. Alternative primer sequences are also reported below and we recommend selecting 2–3 forward and reverse primers. Remember, these designs are virtual and you may need to empirically test a combination of forward/reverse primers for the best possible assay.

```
OLIGO          start  len      tm     gc%   any   3' seq
LEFT PRIMER       39   20   58.39   50.00  4.00  2.00 taagagcagatccctggaca
RIGHT PRIMER     101   24   59.34   37.50  5.00  1.00 tctgaaaggttacttcaaggacaa
SEQUENCE SIZE: 170
INCLUDED REGION SIZE: 170

PRODUCT SIZE: 63, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00
TARGETS (start, len)*: 62,1

    1 gacatcgcctctgggctaataggactacttctaatctgtaagagcagatccctggacagg
                                              >>>>>>>>>>>>>>>>>>>

   61 cGaggaatacaggtattttgtccttgaagtaacctttcagaaattctgagaatttcttct
       *              <<<<<<<<<<<<<<<<<<<<<<<<

  121 ggctagaacatgttaggtctcctggctaaataatggggcatttccttcaa


KEYS (in order of precedence):
****** target
>>>>>> left primer
<<<<<< right primer
```

Step 7: Check for secondary structures

Determine the folding characteristic of both primers and amplicon using secondary structure profiling software. Secondary structures can affect the efficiency of the amplification reaction. Strands with high delta-G values produce less secondary structures and so are favored in the amplification reaction. Ensure that the folding temperature applied is equal to the annealing temperature that will be used for the reaction (e.g. 60°C). Submit the sequence (as shown below). The DINAMelt Servers from the Rensselaer Polytechnic Institute are an appropriate software solution as corrections are made for both salt and magnesium concentration (http://www.bioinfo.rpi.edu/applications/hybrid/twostate-fold.php).

View the DINAMelt results. Low delta-G values indicate a strong and high level of secondary structure. Delta-G values above –1 are recommended.

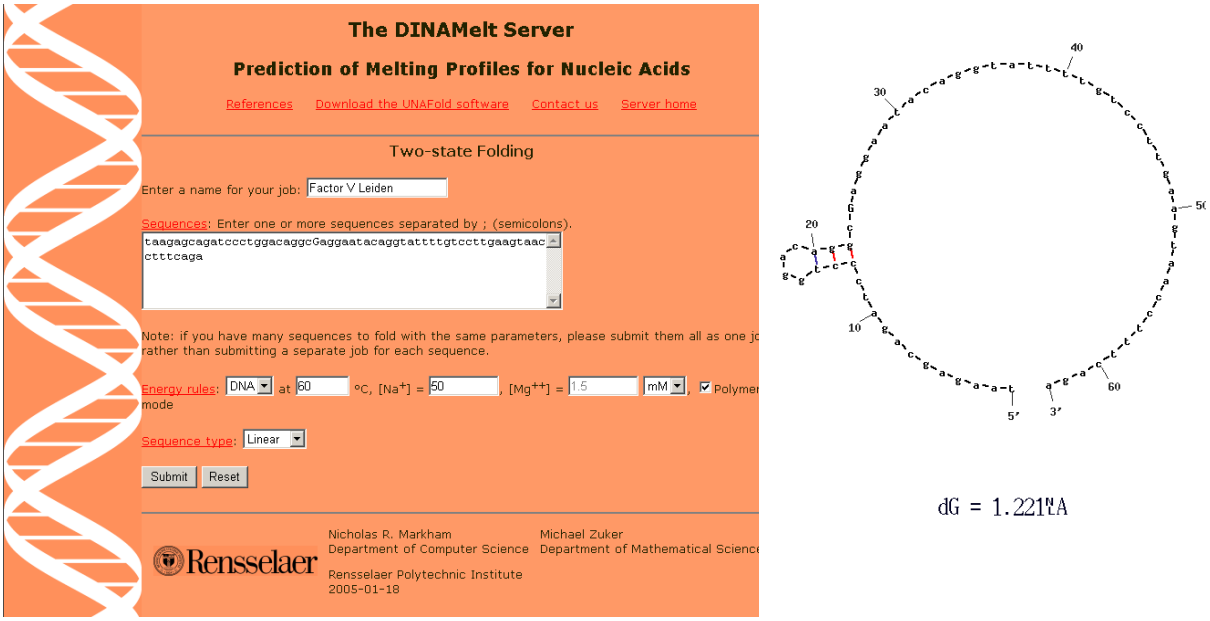


***Figure 7**: **DINAMelt web page and calculated amplicon structure**. The amplicon sequence and set-up parameters are shown in the web page (left). After clicking the* Submit *button and* View Structure *on the results page the calculated structure and delta-G value is shown (right). The calculated structure has very little secondary structure and is thus close to ideal.*

Step 8: BLAST primer sequences to ensure specificity toward the target species and gene.

Figure 8A: BLAST input screen.

gi|55588727|ref|XM_513984.1| PREDICTED: Pan troglodytes coagu...	40.1	0.028
gi|94963080|gb|BC111588.1| Homo sapiens coagulation factor V ...	40.1	0.028
gi|10518500|ref|NM_000130.2| Homo sapiens coagulation factor ...	40.1	0.028
gi|2769646|emb|Z99572.1|HS86F14 Human DNA sequence from clone...	40.1	0.028
gi|33867366|gb|AY364535.1| Homo sapiens coagulation factor V ...	40.1	0.028
gi|23296276|gb|AY136818.1| Homo sapiens coagulation factor V ...	40.1	0.028
gi|20146843|gb|AY046060.1| Homo sapiens mutant coagulation fa...	40.1	0.028
gi|11095906|gb|AF285083.1|AF285083 Homo sapiens coagulation fact	40.1	0.028
gi|182797|gb|M14335.1|HUMFVA Human coagulation factor V mRNA, co	40.1	0.028
gi|488093|gb|L32764.1|HUMF510 Human coagulation factor V gene, e	40.1	0.028

***Figure 8B: BLAST results**. Here we show the ten most significant genes that are associated with the BLASTed sequence. The gene accession number, species type, gene and probability number are reported. The lower the probability numbers the more significant the match.*

Reaction Set-up

General Considerations for Amplification set-up

- Use primer concentrations less than 300 nM. This helps avoid primer-dimer formation.
- Use $MgCl_2$ concentrations of 1.5 mM (SYTO 9) or 3 mM (LCGreen, EvaGreen). Note that the concentration of magnesium will affect the observed T_M.
- Some recommended 3rd generation saturating dyes are:
    1. SYTO® 9, Invitrogen, http://www.invitrogen.com
    2. LC Green™ Plus+, Idaho Technologies, http://www.itbiochem.com
    3. EvaGreen™, Biotium, http://www.biotium.com
- Most commercial Taq DNA polymerases can be used. Hot-Start enzymes are advantageous as they reduce the possibility of primer-dimer and non-specific product amplification.
- Ensure that the same reagents and component concentrations are used for each run (such as KCl and Mg^{2+}). Any variation will affect the observed T_M.
- Run positive controls for each genotype. This will control for run-to-run variation.

General Considerations for HRM

- Ensure the starting amount of template is similar between samples. Large differences will affect the observed T_M.
- The thermal mass of the reaction vessels can influence the observed T_M. It is recommended that the same reaction vessels be used between runs. However, if positive controls are used this is not necessary.
- Use shorter hold-times for denature and anneal and there is usually no need for an extension step. A typical profile would be 5 sec denature at 95°C followed by 10 sec anneal at 60°C. Longer products (larger than about 150 bp) will require longer hold times.
- Optional: Insert a 2 min hold at 72°C following amplification to allow for complete amplicon extension and, if applicable to the enzyme, for proof reading.
- Optional: Insert a pre-hold temperature of 50°C for 30 sec prior to the melt. This will ensure that all products have re-associated and encourage heteroduplex formation.
- Determine the melt domain for each genotype being analyzed to span a larger melt domain (e.g. 75°C to 95°C). Then reduce the melt domain to span about 10°C. Ensure that the melt starts at a temperature at least 5°C cooler than the T_M.

Assay Set-up: Factor V Leiden (G1691A)

To setup the amplification reaction for the genotyping of Factor V Leiden, the reagent components should be mixed together in the volumes outlined in the table below.

Factor V Assay Components

Component	Concentration	Volume per 25 µL Rxn.
Diluent (Mol. Biol. Grade water)	-	14.75 µL
10X PCR Buffer	1X	2.5 µL
50 mM $MgCl_2$	1.5 mM	0.75 µL
2.5 mM dNTPs	0.2 mM	2.0 µL
5 µM Forward primer	300 nM	1.5 µL
5 µM Reverse primer	300 nM	1.5 µL
50 µM SYTO® 9	1.5 µM	0.75 µL
5 U/µL Platinum® Taq DNA polymerase	1.25 U	0.25 µL
DNA Template	3 x 10^3 copies/µL	1.0 µL

Primer Sequences for Factor V Leiden Assay

Forward: 5'- taa gag cag atc cct gga ca- 3'

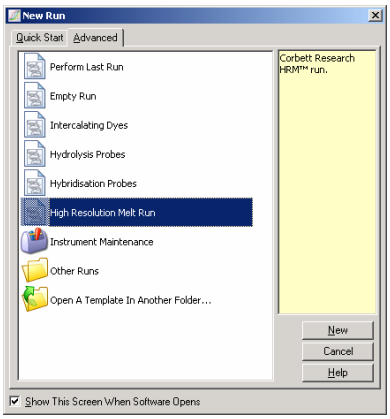
Reverse: 5'- tct gaa agg tta ctt caa gga caa- 3'

Standard Thermal Cycling Conditions

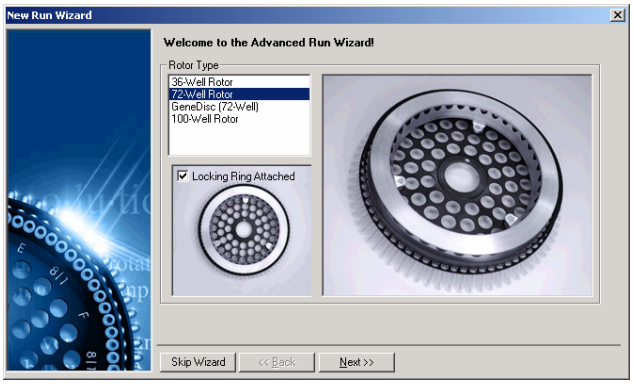
Condition	Temperature	Time	No. Cycles
Hold	95°C	2 min	1
Denature	95°C	5 sec	40
Anneal	60°C	10 sec	40
HRM	73–83°C 0.1°C increments	2 sec per step	1

Software Set-up: Amplification and HRM

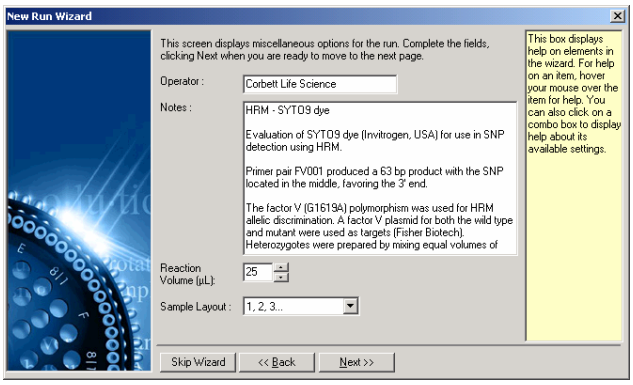
Step 1: Open a new run file
From the *File>New…* menu, select *High Resolution Melt Run* from the *Advanced* wizard.



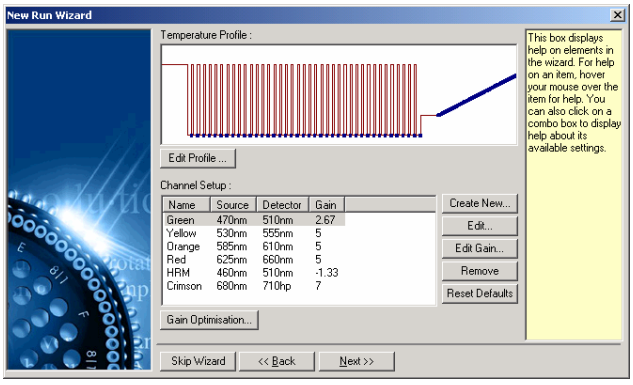
Step 2: Set the rotor type
For this example the 72-Well Rotor is used. Ensure that the locking ring is in place and the *Locking Ring Attached* checkbox is checked before proceeding to the next step.



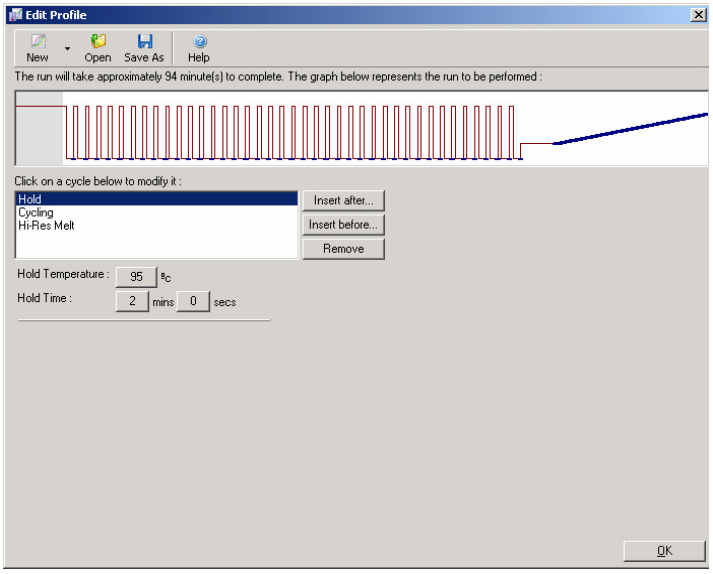
Step 3: Set run details
Type in the *Operator* name (optional) and add any *Notes* about the experiment to be recorded in the run file and incorporated into a post-run report (optional). Select the *Reaction Volume* (required) and *Sample Layout* desired (default is consecutive 1, 2, 3…)



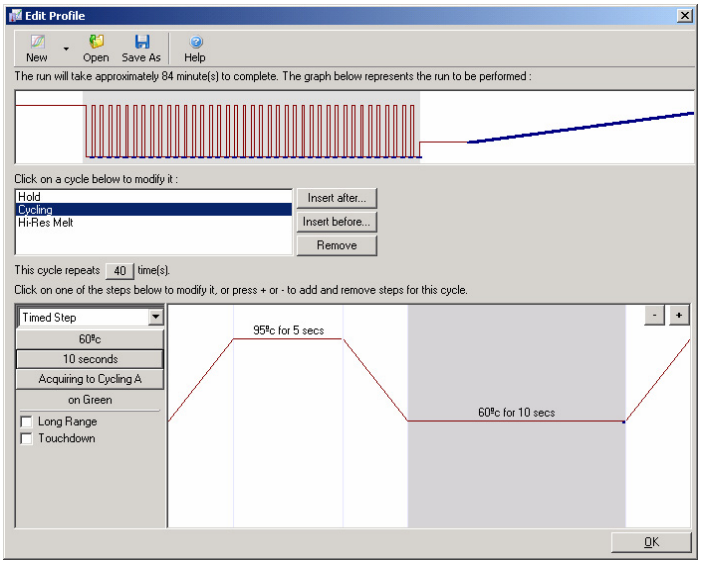
Step 4: Open *Edit Profile…* to modify the programmed times and temperatures for the reaction.



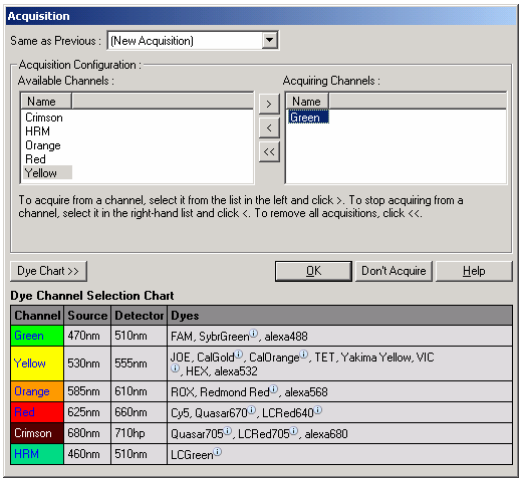
Step 5: Set an appropriate initial hold time
This time depends on the type of DNA polymerase used. For this assay we used Platinum® Taq DNA polymerase from Invitrogen Corp, which requires a 2 min activation time only. The default activation time is 10 min.



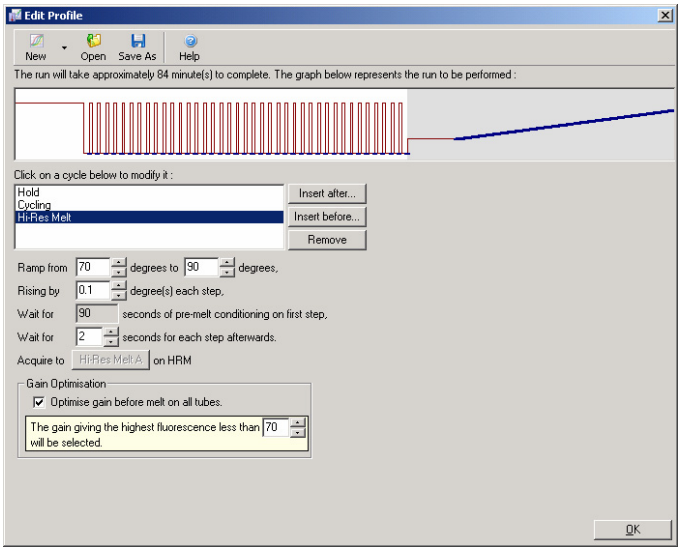
Step 6: Modify cycling to suit the amplicon
For short products use the default of 5 sec denature at 95°C and 10 sec anneal at 60°C (annealing temperatures may vary between assays).



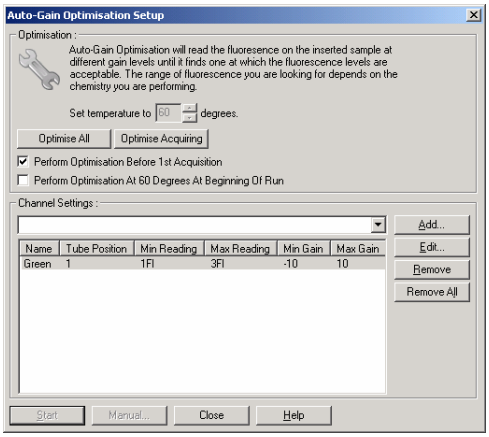
Step 7: Ensure fluorescence data will be acquired
Acquire amplification data to the Green channel at the end of the anneal step.



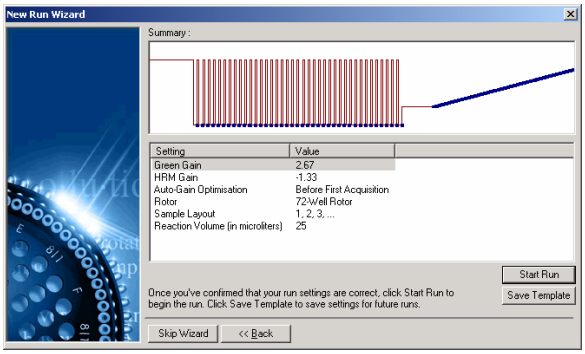
Step 8: Set HRM run conditions
Modify the HRM conditions to suit the amplicon. For the first set of experiments allow for a wide melt domain. Use the theoretical T_M to guide you to a suitable range. Once you have determined where the product will melt, reduce the melt domain to no greater than 10°C. 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.



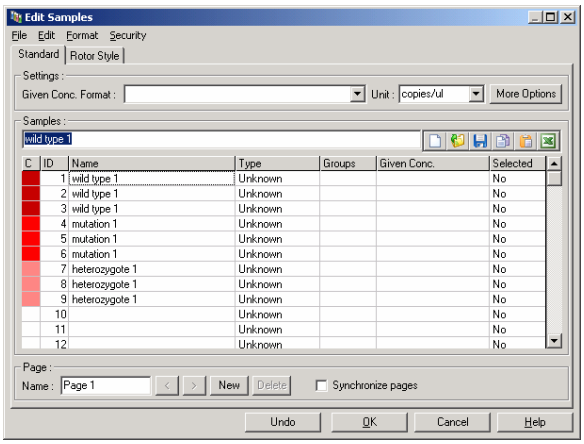
Step 9: Set Auto-Gain Optimization (Optional)
This applies to the real-time amplification step only and is set for the Green channel. Click the Optimize Acquiring button (to optimize only those channels actually used by a run). Optimization is best performed just prior to the first acquisition step, 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.



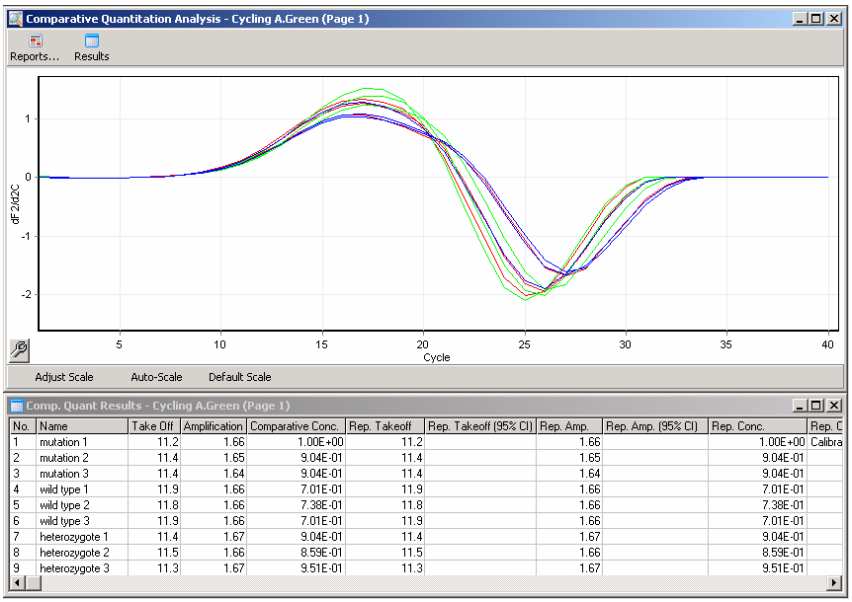
Real-Time Data Analysis

Pre-analysis of the quantitative real-time amplification data prior to HRM data analysis is of great benefit. Real-time data can easily highlight any poorly-performing individual assay. Identifying these outliers and filtering them out of subsequent HRM analysis will greatly improve the overall effectiveness of any HRM data set. Fundamentally, analyzing poor quality amplification product will result in similarly poor HRM results. We recommend analyzing quantitative real-time amplification data as follows:

1. **Assess C_T values**. First, analyze the real-time data using the Quantitation analysis module. If any C_T values are 30 or higher, the corresponding reactions are considered to have amplified too late. These samples must be analyzed with suspicion or removed from the analysis as an outlier. Late amplification is usually due to too little starting template amount and/or high levels of sample degradation.

2. **Assess the end point fluorescence level**. If end point fluorescence on any of the real-time amplification plots is low compared to the majority of plots in the data set then omit those samples from the analysis, even if their C_T value is less than 30. Low end-point fluorescence can indicate incorrect dye amount, incorrect levels of reaction components (such as primers) or the action of inhibitors.

3. **Assess amplification efficiency**. Use the Comparative Quantitation analysis module to obtain the individual reaction efficiency of each sample. If the efficiency is not similar to other reactions in the experiment or is less than about 1.4 then omit the reaction as an outlier.



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	

***Example Comparative Quantitation Results**. The individual reaction efficiency is shown in the* Amplification *data column in a score out of 2 (2=100% efficiency).*

Following these guidelines allows you to make an objective decision about the quality of the starting sample material and the reaction set-up. With outliers removed, the resulting analyses are typically all successful and easy to interpret.

NOTE

If you suspect primer dimers or non-specific products then assess reactions by drawing a derivative plot using the standard *Melt* analysis software module. Ensure there is a single peak indicative of a single product. If possible, also run a gel and check there is a single amplification product. If there is more than one product the reaction will need to be repeated or re-optimized.

HRM Data Analysis

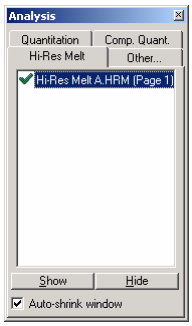
HRM analysis allows for both visual- and auto-calling of genotypes. Results can be viewed as either a normalized melt plot or a difference plot. Normalized curves provide the basic representation of the different genotypes based on curve shifting (for homozygotes) and curve shape change (for heterozygotes).

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.

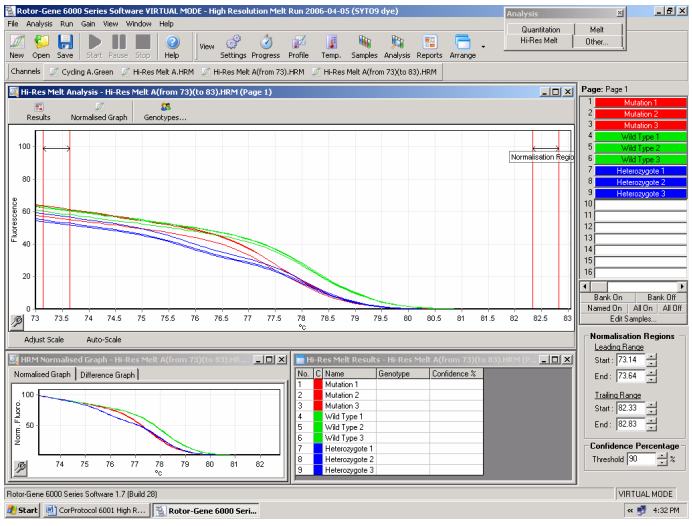
> ***NOTE***
>
> First derivative melt curve analysis (as used by the standard Melt analysis software module) is considered inappropriate for HRM analysis. This is because any derivation of the data adds artificial noise effects and makes data interpretation more difficult.

The following steps describe the analysis of HRM results using Rotor-Gene 6000 software:

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

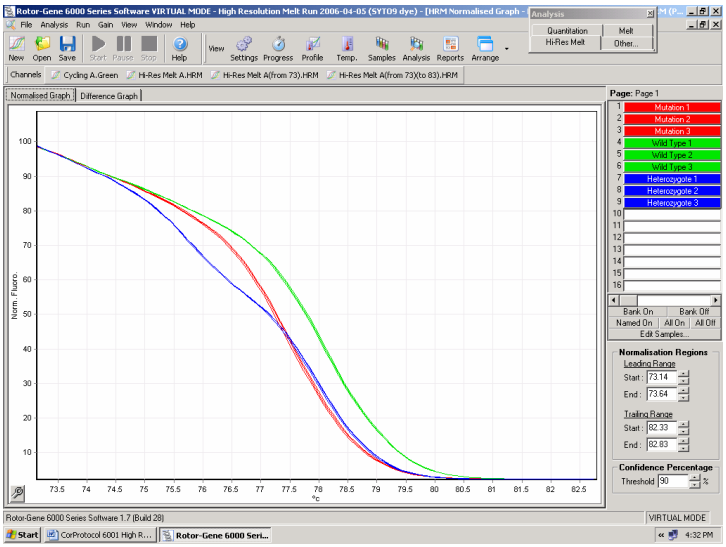


Step 2: The software will present three windows; raw data, normalized graph and results (shown opposite). The raw data window allows you to adjust the regions of normalization. Normalization allows all the curves to be compared with the same starting and ending fluorescent signal level to aid interpretation 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.

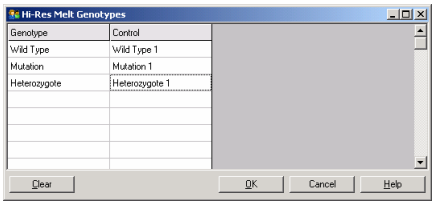


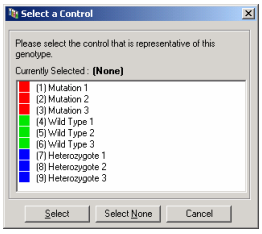
> ***NOTE***
>
> We recommend cursors are only moved if you wish to avoid areas of the melt curve. Movement of the cursors toward the melt phase transitions can affect subtraction plots and confidence percentages.

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.

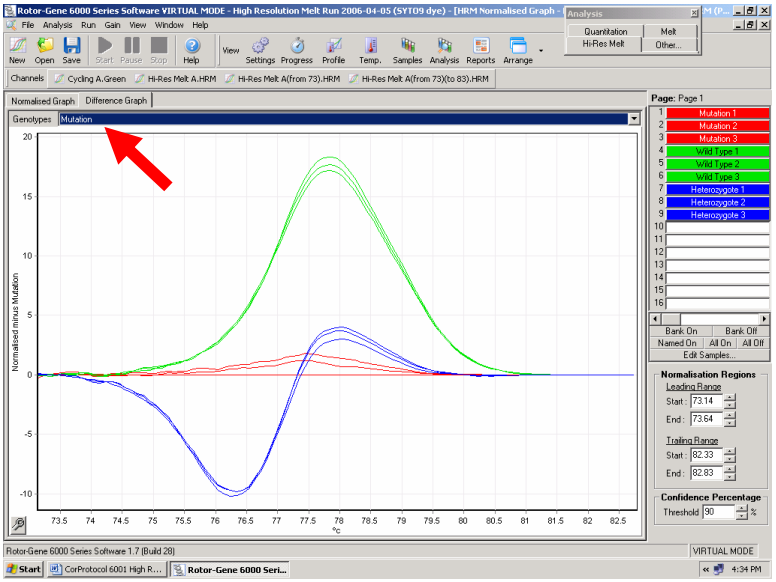


Step 4: In order to view the difference plot, representative genotypes must be defined. By clicking the *Genotypes…* button (top of the raw data window). Input each genotype category name and select a representative sample for each from the sample list, as shown in the example opposite.

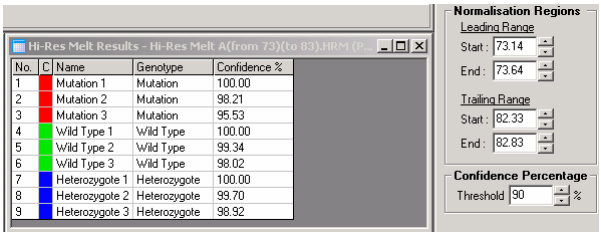




Step 5: View the difference plot by selecting the *Difference Graph* tab. Then select the *Genotype* you wish to compare all other samples against using the drop-down menu (arrowed). In the example shown, all samples are compared to the first sample "Mutation 1".



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.



Troubleshooting

Problem	Cause	Solution
Cannot run HRM	Rotor-Gene model is not HRM-equipped	Contact your local Corbett Life Science representative
No result	Incorrect setup	Check filter settings Check rotor type is correct Check reagents Check assay set-up Run any known good assay (i.e. as a control experiment)
Jagged plots	Poor or no amplification	Check reagents Check assay set-up Revise cycling conditions Check template starting quality and quantity
Saturated amplification or melt plots	Gain set too high	Use auto-gain optimization
Confidence percentages changed	Moving normalization regions	Only move normalization regions to avoid parts of the melt curve
Outliers	Inconsistent reaction setup Inhibitors present in sample Too little or degraded template	Check reagents Check tube uniformity Check master mix uniformity Check template starting quality and quantity

Disclaimers

Protocol Use

This protocol is for *in vitro* use only. Information contained herein is subject to change without notice. The content is believed to be true and complete at the time of publication. It is the user's responsibility to validate performance of this protocol for any particular application. Corbett Research or its affiliates shall not be liable for any damages that may arise from the use of this document.

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Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High resolution genotyping by amplicon melting analysis using LCGreen. Clinical Chemistry 2003; 49: 853-860.

White H and Potts G. Mutation scanning by high resolution melt analysis. Evaluation of Rotor-Gene 6000 (Corbett Life Science), HR-1 and 384 well LightScanner (Idaho Technology). National Genetics Reference Laboratory (Wessex, 2006). (http://www.ngrl.org.uk/Wessex/downloads.htm)

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