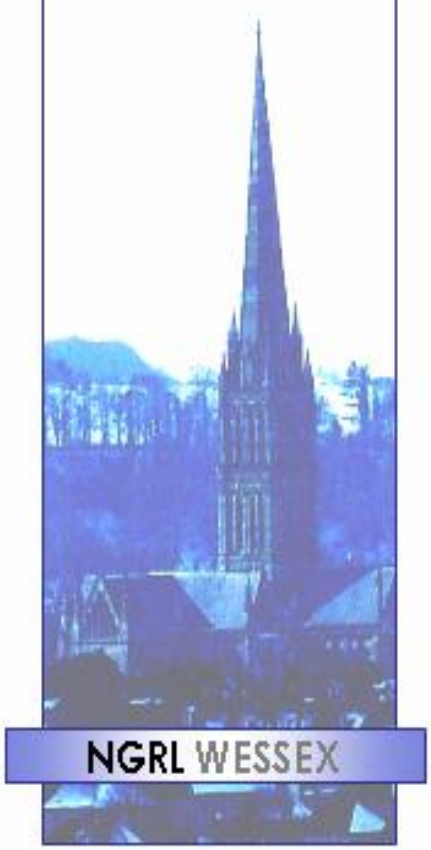
National Genetics Reference Laboratory (Wessex)

NHS

Technology Assessment

Mutation scanning by high resolution melt analysis.

Evaluation of Rotor-Gene™ 6000 (Corbett Life Science), HR-1™ and 384 well LightScanner™ (Idaho Technology)



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Peer Review and Approval

This document has been reviewed by two experts. Corbett Life Science and Idaho Technology have also been given the opportunity to comment on the content of the report. A letter from Corbett Life Science is attached in appendix 7. Idaho Technology did not provide comments.

Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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SUMMARY

- Recently, with the development of a new family of saturating double stranded DNA binding dyes, high resolution melt curve analysis (HRM) has been identified as a new and potentially useful method of high throughput mutation scanning.

- Recent publications suggest that HRM has a mutation detection sensitivity which is comparable or superior to currently available pre-screening techniques

- We have evaluated three machines that are specialised for HRM analysis: Rotor-Gene™ 6000 (Corbett Life Science), HR-1™ and 384 well LightScanner™ (Idaho Technology)

- Eleven different amplicons were analysed. Seven amplicons were generated from the NGRL (Wessex) panel of generic mutation detection control plasmids and 4 were generated from genomic DNA: hMLH1 Exons 1, 7 & 13 and hMSH2 Exon 10.

- The amplicons varied in size from 139 to 449bp and had GC contents ranging from 22 – 79% and the types of mutations analysed included all possible point mutation base substitutions and 1 and 2bp insertions and deletions.

- A total of 624 blinded samples (including controls) were amplified in the presence of the saturating ds DNA binding dye LCGreen® Plus (Idaho Technology) using the Rotor-Gene™ 6000 (Corbett Life Science).

- Identical PCR products were analysed using HRM on the Rotor-Gene™ 6000, HR-1™ and 384 well LightScanner™ platforms. Analysis of the Rotor-Gene™ 6000 and HR-1™ melt curves was undertaken manually by two operators and the LightScanner™ data was analysed with the software supplied using both high and normal sensitivity settings.

- Data were unblinded and the sensitivity and specificity of mutation detection were determined for each amplicon and platform.

- The overall sensitivity and specificities for each machine were 100% and 95% (Rotor-Gene™ 6000, Corbett Life Science), 98.4 % and 95% (HR-1™, Idaho Technology) and 99% and 88% (384 well LightScanner™, Idaho Technology).

- We conclude that HRM is an extremely sensitive and specific technique for mutation scanning which could be easily integrated into clinical diagnostic pre-screening strategies.

- The technique has the potential to allow large genes to be screened and reported within 6-8 weeks although further work is required to determine the feasibility of analysing many different exons for small batches of patients within the same HRM run.

1. INTRODUCTION

High resolution melt curve analysis (HRM) is a simple and cost effective post-PCR technique which can be used for high throughput mutation scanning and genotyping. The technique requires the use of standard PCR reagents only and the dsDNA binding dye LC Green® Plus. This closed tube pre-screening method has advantages over current mutation scanning techniques since it requires no post-PCR handling (minimising the risk of PCR contamination) and no separation step, which improves analysis time.

In the past, the success of heteroduplex detection from whole amplicon fluorescent melting curve analysis was limited due to technical constraints with data acquisition capabilities and sensitivity of temperature control of instruments as well as inadequacies of the fluorescent chemistry. However, with improvements in high resolution melt instrumentation and the development of a new family of double strand specific DNA (dsDNA) binding dyes that can be used at high enough concentrations to saturate all double stranded sites produced during PCR amplification, HRM has now become an extremely promising method for mutation scanning. Recent studies suggest that HRM has a mutation detection sensitivity which is comparable or superior to currently available scanning techniques (Wittwer *et al.*, 2004)

Many recent publications have documented the successful use of HRM on several platforms (Herrmann *et al.*, 2006) for mutation scanning / genotyping (e.g. Wittwer *et al.*, 2003; Liew *et al.*, 2004; Reed and Wittwer, 2004; Graham *et al.*, 2005; Willmore-Payne *et al.*, 2005 Dufresne *et al.*, 2006; Margraf *et al.*, 2006), simultaneous mutation scanning and genotyping (Zhou *et al.*, 2005), methylation profiling (Worm *et al.*, 2001) and genotyping with unlabeled probes (Zhou *et al.*, 2004).

1.1 Use of dsDNA dyes

The process of HRM relies on performing the PCR in the presence of DNA binding dyes that have the ability to distinguish double stranded DNA from single stranded DNA by a change in fluorescent signal intensity. Traditionally dyes such as SYBR® Green were used for melt analysis, but these inhibit PCR when used at concentrations sufficient to saturate the number of dsDNA molecules generated during the amplification reaction. It has been suggested that this can result in 'dye jumping' during amplicon melting which decreases the sensitivity of heteroduplex detection. Recently a new family of LCGreen® dyes have been developed that can be used at saturating concentrations without inhibiting PCR thereby increasing the sensitivity and specificity of mutation detection (figure 1; Wittwer *et al.*, 2003; Herrmann *et al.*, 2006).

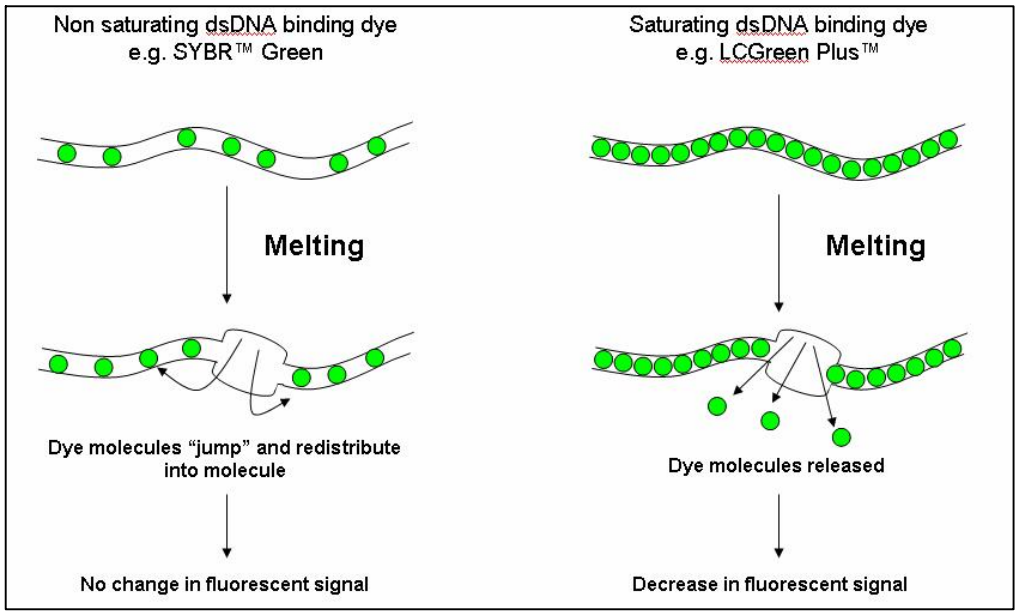

Figure 1: Dye saturation model. Use of non saturating dsDNA binding dyes such as SYBR Green may cause the redistribution of dye molecules ('dye jumping') from melted regions back into the dsDNA amplicon being analysed potentially resulting in no change in fluorescent signal even in the presence of a heteroduplex. Use of saturating dsDNA binding dyes such as LC Green® Plus eliminates this problem and increases the sensitivity of mutation detection allowing theoretical detection of all sequence changes.

1.2 Melt curve analysis

For a heterozygous sample, a PCR amplicon will be comprised of four molecular species: two heteroduplexes and two homoduplexes. However, a wild-type or homozygous mutant amplicon will comprise a single homoduplex species. When melted, each duplex will exhibit characteristic melting (disassociation) behavior. HRM instrumentation can monitor this melting behavior by plotting the changes in fluorescence that occur as amplicons disassociate with increasing temperature from dsDNA to ssDNA . This analysis produces high resolution melt curves that can reflect the particular combination of heteroduplex and homoduplex DNA species in a sample. The presence of a heteroduplex causes a change in the shape of the melt curve compared to a homozygous reference sample (Figure 2). However homoduplexes (resulting from homozygous samples from either wild-type or mutant samples) generally exhibit a simple Tm shift as opposed to an alteration in melt curve shape. Differences in melt curves arise from variations in an amplicons sequence, length, and GC content (assuming salt and buffer conditions as well as the volume of each tested sample remains constant). Overall changes in fluorescence intensity are small and need to be monitored efficiently over a tightly controlled temperature ramp. It is therefore necessary to use instrumentation specifically designed for high resolution melt analysis to ensure maximum sensitivity and specificity (Herrmann *et al.* 2006). This study investigates each of the instrument systems developed specifically for HRM analysis that are currently available.

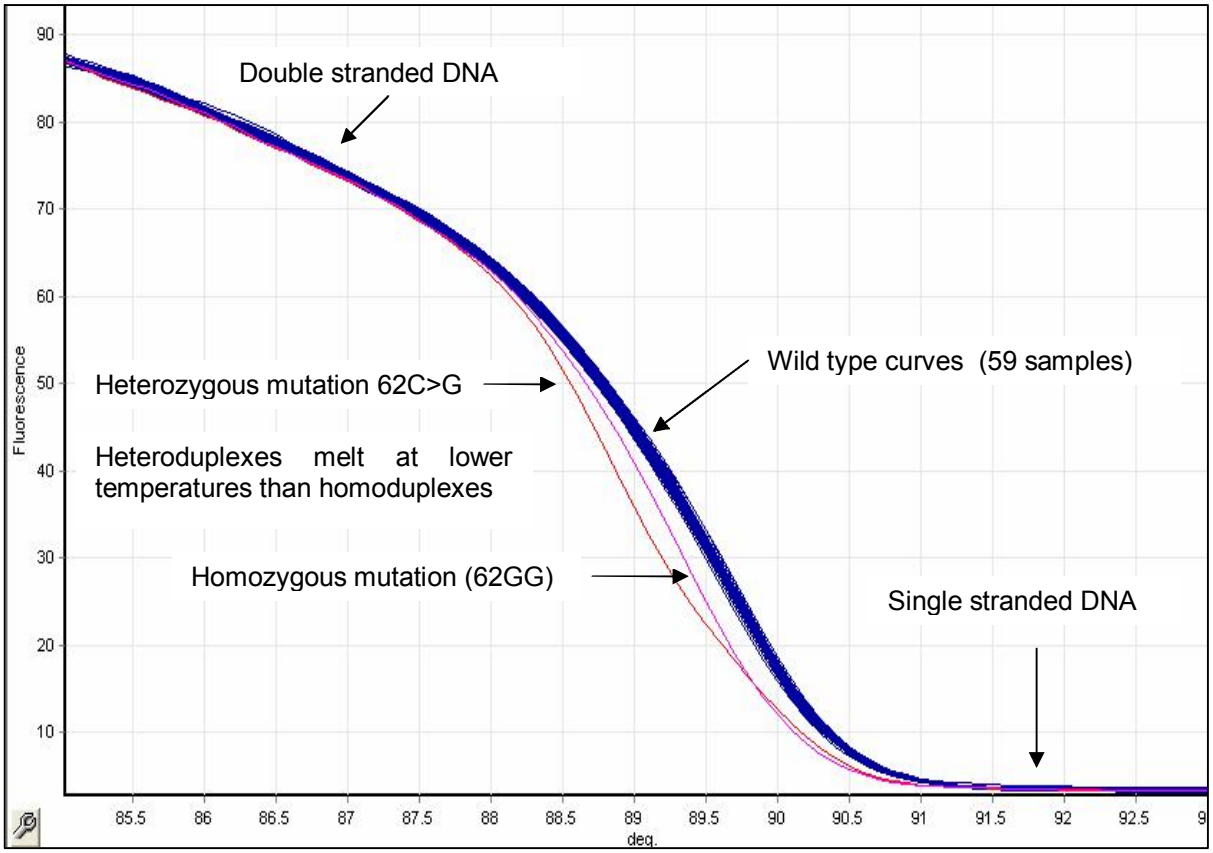

Figure 2: Example of high resolution melt curve for hMLH1 Exon 1 generated using the Rotor-Gene™ 6000 (Corbett Life Science). The blue lines are melt curves for wild type samples, the red line is the melt curve for a heterozygous samples 62C>G and the pink line is the melt curve for homozygous sample for the same mutation.

1.3 NGRL Evaluation

In this study we have evaluated the sensitivity and specificity of mutation detection for three machines capable of HRM analysis: Rotor-Gene™ 6000 (Corbett Life Science), HR-1™ and 384 well LightScanner™ (Idaho Technology). We have analysed amplicons generated from plasmid reagents and genomic DNA to assess how effectively HRM can be used for genetic diagnostic testing. The amplicons analysed varied in size from 139 to 449bp and had GC contents ranging from 22 – 79% The types of mutations analysed included all possible point mutation base substitutions and 1 and 2bp insertions and deletions. The flow chart in figure 3 shows the experimental design.

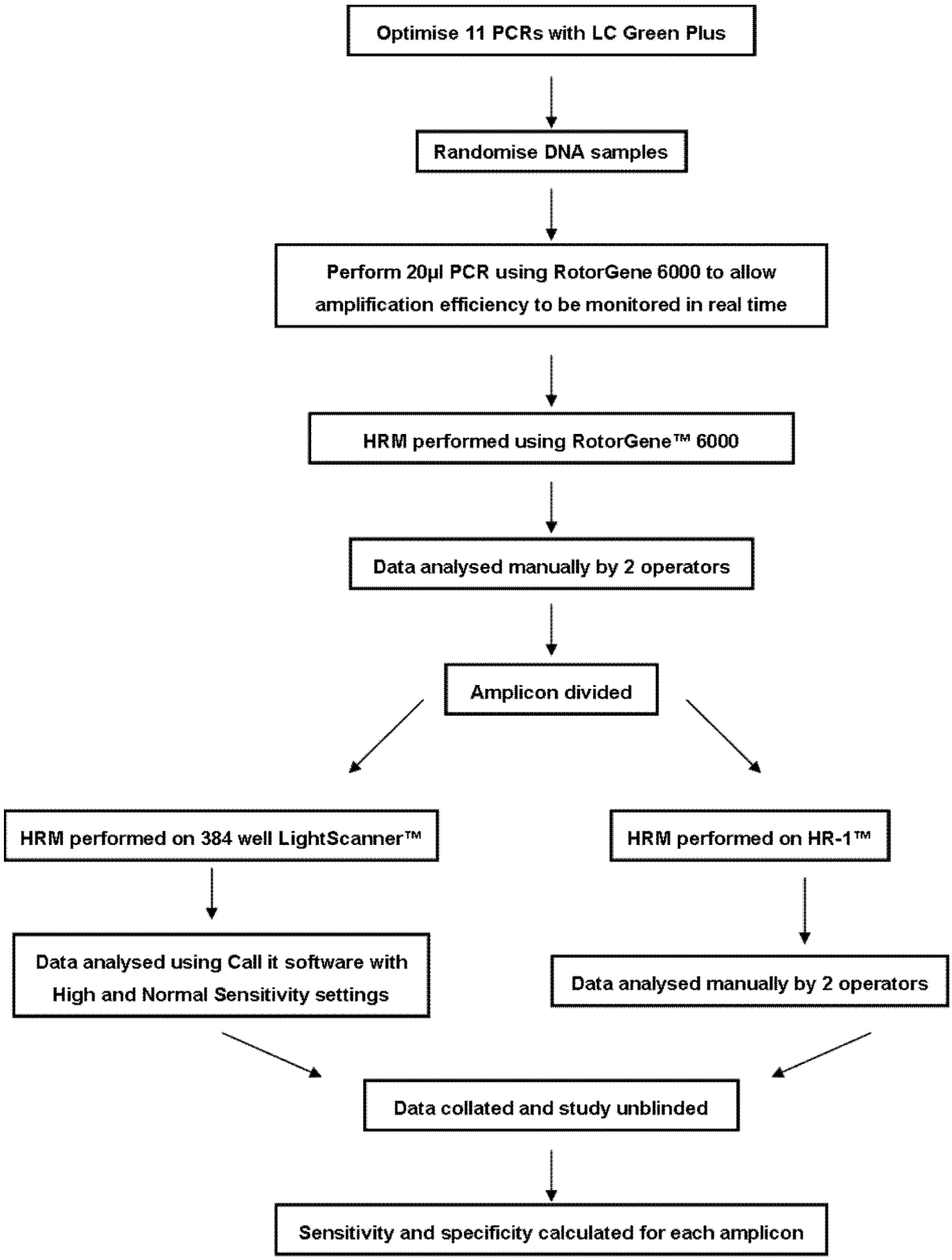

Figure 3: Flow chart of NGRL (Wessex) HRM Evaluation. All amplicons generated in this study were also verified by sequencing.

2. MATERIALS AND METHODS

2.1 Amplicons Analysed

2.1.1 Plasmid based template DNA (Figure 4)

Seven amplicons were derived from plasmid based DNA templates, details of which can be found in the NGRL (Wessex) reference reagent report "Plasmid based generic mutation detection reference reagents; production and performance indicator field trial" (www.ngrl.org.uk/Wessex/downloads.htm). Four wild type plasmids were constructed which contain inserts with 20%, 40%, 60% and 80% GC content. Each of the wild type plasmids has been mutated at either P1, P2 or P3 (figure 4) to introduce the changes shown in table 1. When the mutated plasmids are mixed 1:1 with the corresponding wild type plasmid the resulting 48 samples can be used to validate mutation detection techniques by analysing how effectively each of the possible heteroduplex configurations are detected at three different positions within amplicons of varying GC content.

Plasmid templates with GC contents of 20%, 40%, and 60% were amplified to produce products of 449, 437 and 433 bp respectively. We were unable to optimize the PCR conditions for the 80% GC rich 424bp amplicon in the presence of LC Green® Plus. Shorter amplicons derived from the same plasmid templates with GC contents of 20%, 40%, 60% and 80% were also produced: 272, 259, 260 and 258 bp respectively. Figure 4 shows a diagrammatical representation of the seven amplicons: GC and AT rich regions within the amplicons are represented by black and white shading respectively. The position of the mutations in the fragments are indicated by the pink (P1), blue (P2) and green bars (P3) the black and white shading shows the local sequence context surrounding each mutation.

Mutation created	Sequence generated	Heteroduplex produced
A > C	nnnCGnnn	C:T & G:A
A > T	nnnTGnnn	T:T & A:A
G > A	nnnAAnnn	A:C & G:T
G > C	nnnACnnn	C:C & G:G

Table 1: Four wild type plasmids have been constructed which contain inserts with a 20%, 40%, 60% and 80% GC content. Each of these plasmids has been mutated at three positions within the amplicon (Figure 4) to introduce the base changes listed in the table.

48 samples were analysed for each amplicon. These were randomised as shown in appendix 1a.

2.1.2 Genomic DNA (Figure 5)

Although the plasmid reagents are useful for studying the effects of GC content, base changes and positions of mutation in fragments they largely test the mutation scanning system rather than factors such as PCR optimisation which can be more problematic for genomic DNA targets. Use of genomic DNA also allows investigation of mutations which are more complex than point mutations. Four amplicons were generated from genomic DNA samples. For the purpose of this evaluation we selected three exons from hMLH1 (Exons 1, 7 and 13) one exon from hMSH2 (exon 10) for analysis. Figure 5 shows a diagrammatical representation of the four amplicons: GC and AT rich regions within the amplicons are represented by black and white shading respectively. The mutation types analysed are also indicated. We analysed DNA from patients who had previously characterized mutations for the HNPCC exons to be tested (n=35) and normal controls (n=32). We also analysed plasmid mutations controls for each exon with the mutation in both a heterozygous and homozygous form. Details of the plasmids controls can be found in the NGRL (Wessex) reference reagent report "Production and field trial evaluation of reference reagents for mutation screening of BRCA1, BRCA2, hMLH1 and MHS2" (www.ngrl.org.uk/Wessex/downloads.htm). hMSH2 exon 10 was selected as a representative polymorphic exon as it contains a common G/A polymorphism (dbSNP 3732183) with genotype frequencies of 0.13 (AA), 0.42 (GA) and 0.45 (GG).

DNA samples were randomised as shown in appendix 1b.

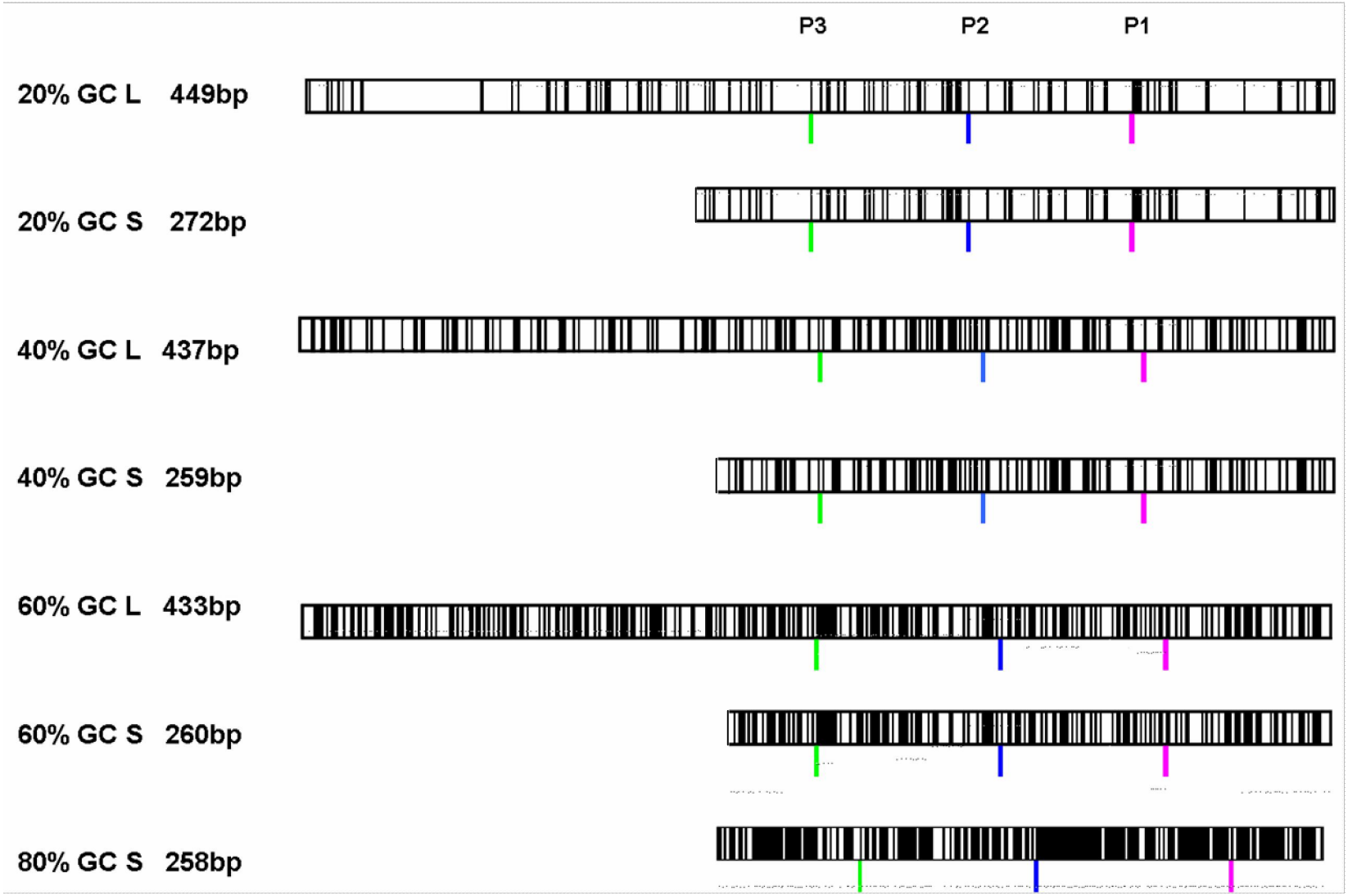

Figure 4: Diagrammatical representation of the seven amplicons produced from plasmid DNA templates: G/C and A/T bases within the amplicons are represented by black and white bars respectively. The position of the point mutations in the fragments are indicated by the pink (P1), blue (P2) and green bars (P3) the black and white shading shows the local sequence for each mutation. Key S = short amplicon, L = long amplicon, x% GC = percentage GC content of amplicon and indicates from which plasmid construct the amplicon was derived.

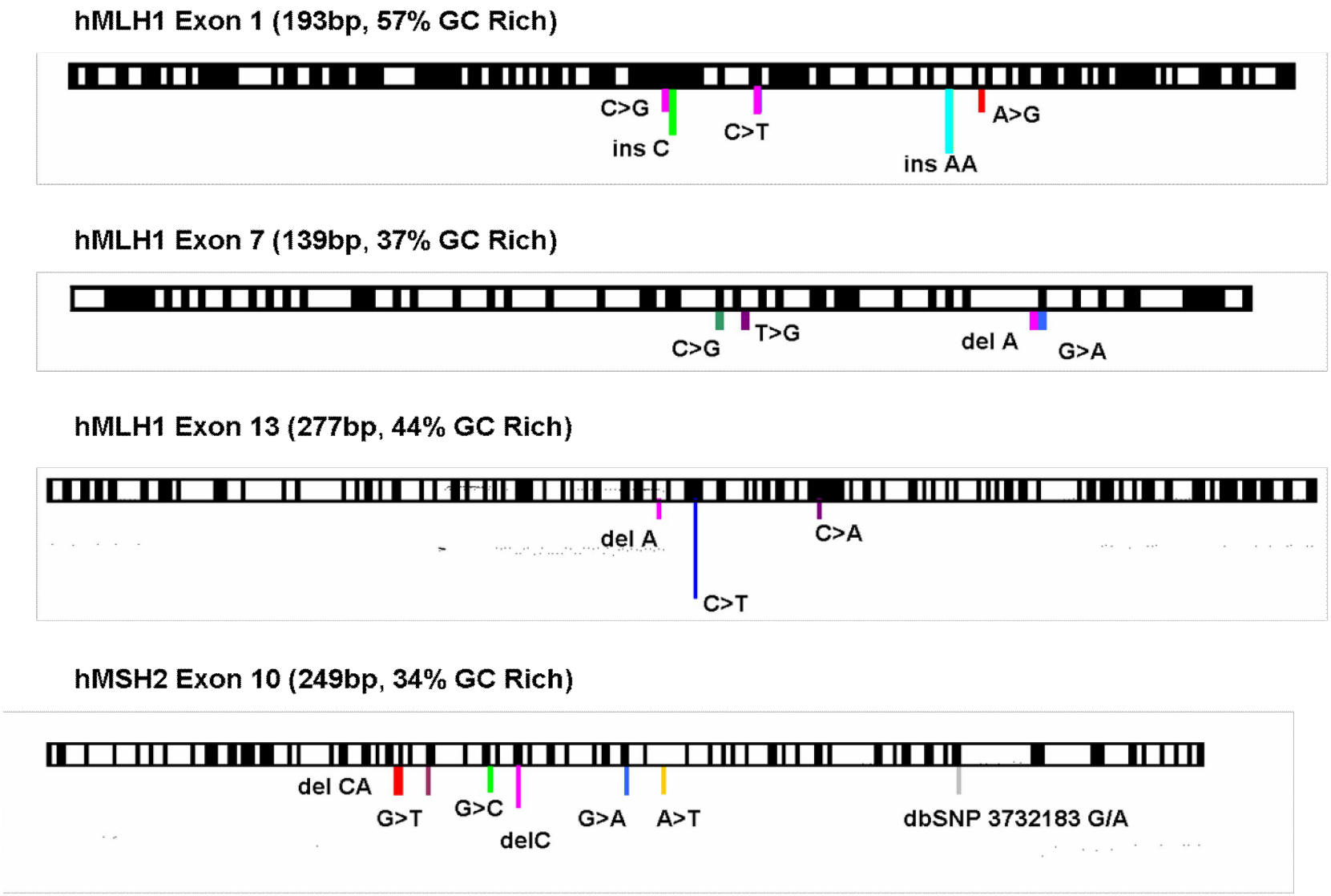

Figure 5: Diagrammatical representation of the four amplicons produced from genomic DNA templates G/C and A/T bases within the amplicons are represented by black and white bars respectively. The coloured bars indicate the position of the different types of mutations analysed within the different amplicons.

2.2 PCR optimisation

Successful HRM is almost entirely dependent on meticulous optimisation of PCR conditions as the presence of non-specific bands and primers dimers can significantly reduce HRM performance. In general the following guidelines may be applied to help achieve a robust amplification in the presence of LC Green® Plus:

Template DNA	5-10 ng	
$MgCl_2$	2 mM	} Final concentration
Primer concentration	0.2 / 0.3 µM	
LC Green® Plus	1X	

The PCR can be optimised using a 10-12°C gradient to determine optimal annealing conditions using 45 cycles. The annealing temperature of the reaction may have to be raised significantly (2-10°C) above that used in the absence of LC Green® Plus. The NGRL evaluation did not specifically cover an analysis of different *Taq* polymerases however we did observe that reactions performed using Ampli*Taq* Gold (Applied Biosystems) were more subject to PCR inhibition whereas those performed using Platinum *Taq* (Invitrogen) were more robust. Optimisation of more troublesome PCR templates can be achieved by using the LC Green® Plus master mix (Idaho Technology) although this is a more expensive option (£1 / 10µl PCR).

2.2.1 Real Time PCR Amplification

To help optimize PCR conditions and to monitor amplification efficiency in real time, all amplifications were carried out using the Rotor-Gene™ 6000 (Corbett Life Science). Eleven amplicons (20µl reaction volume) were generated using the primer sequences and amplification conditions shown in appendix 2.

2.2.2 Effect of DNA Quality

To determine the effect of DNA quality on HRM analysis we analysed hMLH1 Exon 1 using DNA samples of variable DNA quality (n=70). We assessed the quality and amplifiability of the DNA samples using a multiplexed control gene primer set which amplifies products of 100, 200, 300, 400 and 600bp with equal intensity (White *et al.,* 2003). Loss of the higher molecular weight products indicates that the DNA sample is of poor quality. HRM data was correlated with the DNA quality and amplification plot data (see figure 6).

2.3 HRM Machine specifications

The Rotor-Gene™ 6000 is a real time PCR machine which also has HRM capability. The HR-1™ and 384 well LightScanner™ have HRM capability only. More detailed machine specifications are given in Appendix 3. Further details can be obtained from the respective company websites:

Corbett Life Science: http://www.corbettlifescience.com
Idaho Technology: http://www.idahotech.com

2.4 HRM Analysis

HRM was performed on the same PCR reactions using three HRM platforms. Melting conditions are shown in table 2.

Amplicon	Melting conditions
20L	73°C – 83°C
20S	73°C – 83°C
40L	77°C – 87°C
40S	78°C – 88°C
60L	85°C – 95°C
60S	85°C – 95°C
80S	84°C – 95°C

Amplicon	Melting conditions
hMLH1x1	85°C – 95°C
hMLH1x7	75°C – 85°C
hMLH1x13	82°C – 89°C
hMSH2x10	76°C – 86°C

Table 2: Melting temperatures ranges used to analyse PCR amplicons on the Rotor-Gene™ 6000, HR-1™ and 384 well LightScanner™

The rate of melting for the HR-1™ and 384 well LightScanner™ were 0.15°C / sec and 0.3°C / sec respectively. The Rotor-Gene™ 6000 melting conditions can be altered by the user. Our reactions were melted using 0.1°C increments with a 2 second hold at each step. Denatuation was performed before analysis on all machines but cooling parameters were not standardized.

2.5 Data Analysis

HRM data from the Rotor-Gene™ 6000 and HR-1™ were analysed manually by two independent operators. HR-1™ data were normalised and temperature shifted prior to analysis by each operator independently. LightScanner™ data were analysed by one operator using the software provided with high and normal sensitivity settings. Manual analysis of the LightScanner data was not performed and therefore results may not be directly comparable with manual analysis data from the Rotor-Gene™ 6000 and HR-1™.

Once all data analysis was complete the samples were unblinded and the sensitivity and specificity of mutation detection for each amplicon was determined.

3. RESULTS

3.1 PCR optimisation, real time amplification and effect of DNA quality

PCR optimisation plays a critical role in successful HRM analysis. We found that variable amplification resulting either from DNA quality or sub-optimal amplification increased the number of false positive results and in some cases analysis was not possible as the data became too noisy to reliably discriminate wild type and mutated samples. The use of real-time PCR amplification meant that PCR optimisation was more easily achieved as detailed information about the dynamics of amplification for each sample could be monitored. This enabled detection of poor quality samples before HRM analysis and also allowed the efficiency of each PCR to be determined. The real time amplification plots and agarose gel images for all amplicons are shown in appendix 4.

Figure 6 shows the amplification plot, agarose gel of the PCR products and the control gene PCR analysis for the DNA samples of variable quality. The agarose gel of the control gene PCR products (figure 6a) shows that samples C1, C4, D6, C8, D11, F9, H4, H8 and G10 amplified products between 100 and 200bp only and are thus of poor quality. When these samples were amplified using the hMLH1 exon 1 assay the PCR products, when viewed on an agarose gel, all appeared to have amplified successfully (figure 6b). However, the amplification plot (figure 6c) shows that there was considerable variation in amplification efficiency between samples. The 9 poor quality DNA samples only reached plateau at above 35 cycles and when HRM data were analysed these 9 samples were identified as false positive results (data not shown). Therefore poor DNA quality may result in increased false positive rates when using HRM.

3.2 HRM Analysis

The sensitivity and specificity of mutation detection for each amplicon on each platform are shown in table 3. Full details of the data analysis are given in Appendix 5. The Rotor-Gene™ 6000 had a sensitivity of 100% for all amplicons with specificities ranging from 82.6 – 100% and the HR-1™ had a sensitivity range of 89.6 – 100% with specificities ranging from 84.4 – 100%. The LightScanner™ data were analysed using normal and high sensitivity software settings. Using the normal sensitivity setting the sensitivity ranged from 50% - 100% with specificities of 73.7 – 100%. However, using the high sensitivity setting the sensitivity improved to 100% for all amplicons (except 20%GC long) with specificities ranging from 80 to 100%. All melt curves are shown in appendix 6.

4. DISCUSSION

As can be seen from table 3, HRM is a mutation detection technique which has high sensitivity and specificity. The amplicons used for this evaluation were not designed specifically for HRM and time constraints meant that PCR conditions for some amplicons could have been improved. It is possible that the false positive rates observed for some amplicons could be lowered following further optimisation. Overall it appears that HRM has a mutation detection sensitivity which is comparable or superior to currently available pre-screening techniques and that each of the platforms evaluated produced comparable results.

4.1 Effect of DNA quality and amplicon quality

DNA and amplicon quality appear to have the most significant effect on HRM data quality. Figure 6 shows the control gene PCR results for a panel of DNA samples. Samples which only amplified products up to 200bp (using the DNA quality multiplex assay) were detected as false positive results when analysed by HRM. However, poor quality samples can be readily identified and excluded from further analysis if the PCR is monitored in real time. DNA extracted using different methods may produce melt curves which have subtle differences and which in some cases may results in increased false positive rates (Idaho Technology, personal communication). It is important to be aware of this when analysing archival samples or samples from other laboratories.

PCR optimisation is critical as the presence of primer dimers or non specific products may alter the melt curve characteristics causing the generation of false positive results. Amplicon design is also important to ensure that maximum information can be obtained from the melt curve data. Complex melt curves which have two or more melting domain may be more difficult to analyse. In this evaluation the 60% GC long amplicon, which had two melting domains, was difficult to analyse and could not be analysed successfully using automated mutation calling with either high or normal sensitivity settings. It appears that single melting domains, as seen in the shorter amplicon, are more easily analysed. Optimal amplicon design and subsequent optimisation are therefore crucial to the success of HRM.

4.2 Effect of Amplicon Length

The length of the amplicon does appear to have an effect on the sensitivity and specificity of mutation detection. In general, we observed that mutations were more difficult to detect in amplicons which were greater than 400bp which is concordant with a previous study (Reed and Wittwer, 2004). The shorter amplicons tended to have less complex melt profiles as compared with longer amplicons and therefore data could be more easily analysed. Mutations were generally more easy to discriminate in smaller amplicons as the overall change in fluorescent intensity for mutated samples was greater than that observed in the longer amplicons.

The effect of amplicon length was particularly evident for the 20% GC rich plasmid based amplicon (table 3). The P1 mutations, located 84bp from the end of the amplicon, were difficult to detect in the long amplicon (449bp) but were successfully detected in the shorter form of the amplicon (272 bp). Mutated plasmid templates were amplified in duplicate for each mutation for both the long and short forms of the 20%GC amplicon (appendix 5). When data from the Rotor-Gene™ 6000 were analysed, the P1 A-T mutation in the 449bp amplicon was identified as ambiguous in both cases by operator 1 and in one case by operator 2. However both operators detected the mutation unequivocally in the shorter amplicon. Using the HR-1, the P1 A-T and P1 G-C mutations were not detected by either operator for one replicate in the 449bp amplicon but were detected successfully in the shorter amplicon (both replicates). When the LightScanner™ data for the 449bp amplicon were analysed using normal sensitivity software settings the P1 A-T, G-C and G-A mutations were not detected using normal sensitivity conditions (both replicates). Using high sensitivity settings single replicates of the P1 A-T, G-C and G-A mutations were not called as mutated. However, all mutations were successfully called with normal and high sensitivity settings for the shorter amplicon. Examples of melt curves for each platform for the long and short forms of the 20% GC amplicons are shown in appendix 6.

4.3 Effect of mutation type or local sequence context surrounding mutation

In some amplicons the type of mutation or perhaps more plausibly the local sequence context surrounding the mutation appeared to have an effect on mutation detection. For example, when analysing the short and long amplicons generated from the 40% GC rich plasmid based template the P3 mutations were most subtle and the difficult to detect (appendix 6). The resolution of detection of the mutations at this position did not significantly improve when the amplicon length was shortened and therefore we assume that the context of sequence surrounding the mutation may be contributing to this effect. For the long amplicon (437bp) the P3 A-C mutation was called as ambiguous by one operator for one replicate when analysed using the Rotor-Gene™ 6000 and for the shorter amplicon (259bp) mutations P3 A-C and P3 A-T were called as ambiguous by operator 2 for both replicates. When the amplicons were analysed using the HR-1™ the P3 A-T was undetected by both operators for one replicate and called as ambiguous by both operators for the second replicate in the long amplicon. When data from the shorter amplicon were analysed the P3 A-T mutation was detected successfully but P3 G-C was called as ambiguous by operator 1 for both replicates and was undetected by operator 2 for one replicate; P3 G-A was also undetected by both operators for one replicate. When data from the LightScanner™ were analysed using the normal sensitivity settings P3

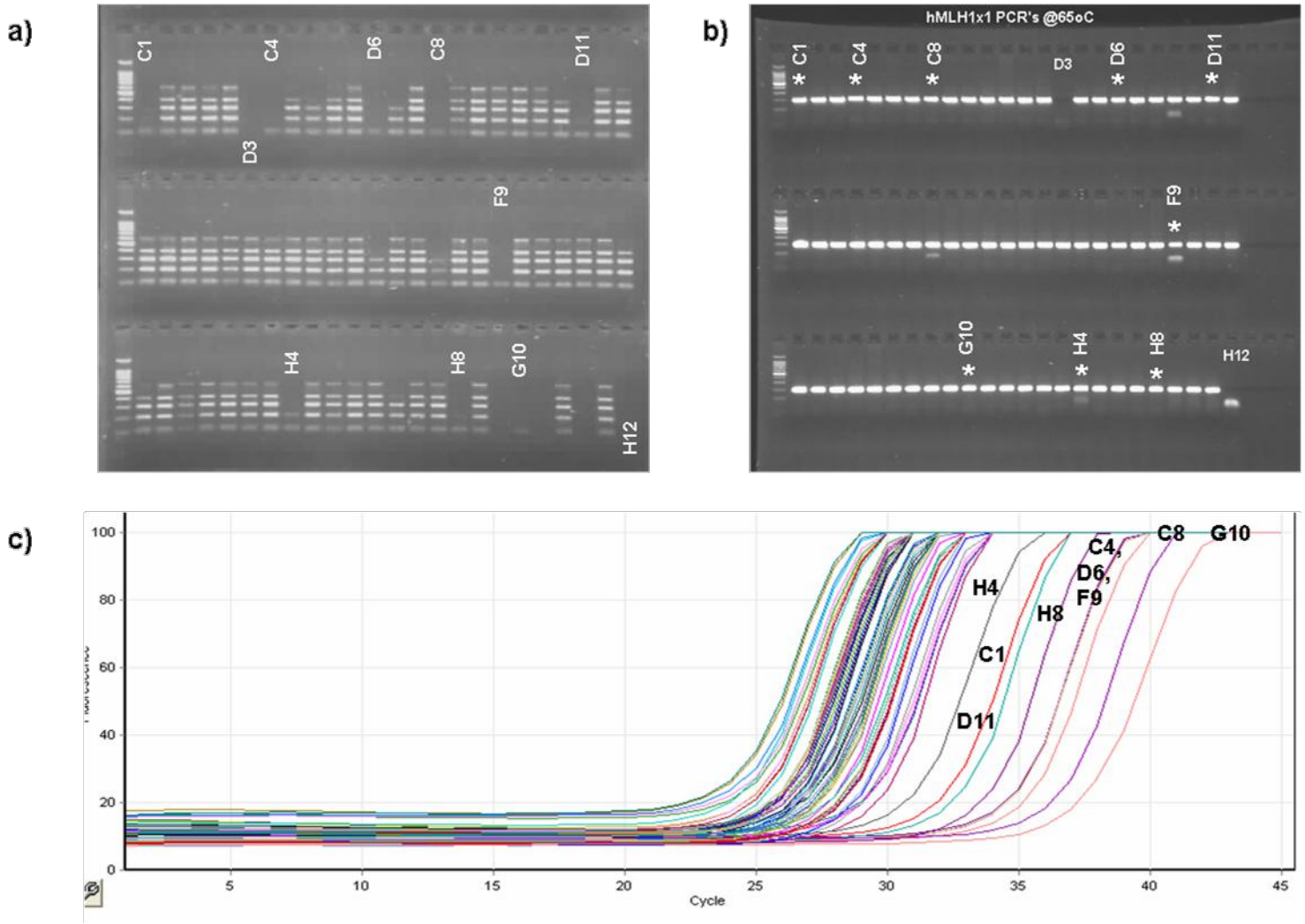

Figure 6: Effect of DNA quality. a) Agarose gel of control gene PCR products for DNA samples of variable quality. Good quality DNA samples amplify bands of 100, 200, 300, 400 and 600bp. Low quality samples (labeled in white) have only amplified products of 100-200bp. b) Agarose gel of hMLH1 Exon 1 products for the variable quality DNA samples. All samples appear to have amplified well. Samples marked with an asterisk are those of poor DNA quality. c) the PCR amplification plot shows that the asterisked samples have amplified poorly with samples only reaching plateau at 36 cycles. These samples were called as false positives when analysed with HRM (data not shown). Samples D3 and H12 are water controls.

				Rotor-Gene™ 6000 (Corbett Life Science)		HR-1™ (Idaho Technology)		LightScanner™ 384 well (Idaho Technology)			
								High Sensitivity		Normal Sensitivity	
Amplicon	**No. of wild type samples**	**No. of mutated samples**	**No. of unique mutations**	**Sensitivity (%)**	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**	**Sensitivity (%)**	**Specificity (%)**
20L	23	24	12	100	82.6	89.6	97.8	87.5	100	75	100
20S	23	24	12	100	100	100	91.3	100	100	100	100
40L	23	24	12	100	100	95.8	97.8	100	100	75	100
40S	23	24	12	100	100	95.8	97.8	100	100	71	100
60L	23	24	12	100	90.9	95.8	84.4	Not analysed	Not analysed	Not analysed	Not analysed
60S	23	24	12	100	100	100	97.7	100	100	69.6	100
80S	23	24	12	100	87	100	93.5	100	87	98.5	87
hMLH1 x1	60	9	5	100	96.7	100	94.2	100	80	80	90
hMLH1 x7	62	7	5	100	100	100	95.2	100	90.5	71.4	96.8
hMLH1 x13	55	14	5	100	98.2	100	85.5	100	96.4	50	100
hMSH2 Combined	55	14	6	100	96.4	100	96.3	100	80	100	90.9

Table 3: % Sensitivity (true positive/ (true positive + false negative)) and specificity (true negative / (true negative + false positive)) of mutation detection for eleven amplicons analysed using the three HRM platforms. LightScanner™ figures are given for the two software settings: high sensitivity and normal sensitivity. Homozygous mutations have been excluded from the analysis. Full details of data analysis can be found in appendix 5.

A-T, G-A and G-C were undetected for both replicates in both the long and short forms of the amplicon. However, when the data were analysed with the high sensitivity settings the P3 mutations were successfully detected in all cases.

The overall GC content of the amplicon did not appear to have a significant effect on the sensitivity of mutation detection as mutations were detected successfully in amplicons with a wide range of global GC content: 20 -79%.

For the genomic DNA samples the most subtle (but detectable) mutations were :

hMLH1 Exon 1:	Rotor-Gene™ 6000	110 A>G
	HR-1	110 A>G
	LightScanner	110 A>G
hMLH1 Exon 7:	Rotor-Gene™ 6000	588 del A
	HR-1	588 del A
	LightScanner	588 del A (not detected with normal sensitivity)
hMLH1 Exon 13:	Rotor-Gene™ 6000	All detected unambiguously
	HR-1	1451 del A, 1459 C>T
	LightScanner	1451 del A, 1409+14G>A polymorphism

The fact that the 110A>G and 588delA mutations in exons 1 and 7 respectively were subtle for each platform suggests that these mutations are intrinsically difficult to detect by HRM e.g. they are sited in regions which, when melted, produce only subtle overall changes in fluorescent intensity. Apparently, differences in data handling between the platforms meant that the 1459 C>T, 1451 delA and 1409+14G>A sequence variants in exon13 were subtle when analysed using the HR-1™ and LightScanner™ but were easily detected using the Rotor-Gene™ 6000. Data from the HR-1™ and LightScanner™ is first normalised and then temperature shifted. It appears that mutations such as 1451 delA changes the temperature at which the amplicon melts rather than altering the overall shape of the melt curve. When the data are temperature shifted these curves become superimposed on the wild type samples making detection difficult. Data from the Rotor-Gene™ 6000 is normalized, but not temperature shifted, and therefore such mutations can be more easily detected as the melt curves do not become overlaid on the wild type curve. Melt curves are shown in appendix 6.

4.4 Effect of position of mutation in fragment

The position of a mutation within a fragment did not appear to have a significant effect on mutation detection. Mutations were detected successfully in positions close to the end of the amplicon e.g. 24bp (delA and G>A mutations in hMLH1 Exon 7) and 23bp (G/A polymorphism hMLH1 Exon 13) from the end of the fragment; central positions e.g. 551 C>G in hMLH1 Exon 7, 1601 G>A in hMSH2 Exon 10 and intermediate positions between the middle and end of the amplicon e.g. 1551_2 delCA in hMSH2 Exon 10 and 105ins AA in hMLH1 Exon 1. Examples of melt curves can be seen in appendix 6.

4.5 Mutation scanning in polymorphic exons

To determine whether polymorphic exons could be analysed by mutation scanning using HRM, hMSH2 exon 10 was included in the analysis. hMSH2 exon 10 was selected as a representative polymorphic exon as it contains a common G/A polymorphism (dbSNP 3732183) with genotype frequencies of 0.13 (AA), 0.42 (GA) and 0.45 (GG). For this study the genotype of each sample was determined by sequencing. Several diagnostic labs that use mutation scanning approaches for analysis of large genes are beginning to investigate the use of polymorphism screens e.g. SNPlex (Applied Biosystems) so that samples with defined non-pathogenic polymorphisms and no pathogenic mutation can be reported as normal without the need for confirmatory sequencing. In this evaluation the genotype at the polymorphic site was characterized and melt curves were then analysed in genotype groups (using genotype specific controls for reference). Mutations were successfully discriminated for all three genotype groups on each HRM platforms. Therefore HRM may be suitable for mutation scanning polymorphic exons (also see section 6.2).

4.6 Detection of homozygous/ hemizygous mutations

Plasmid control samples were included as positive controls for the analysis of hMLH1 Exons 1, 7, 13 and hMSH2 Exon 10. Three controls were included: a wild type control, a heterozygous mutation

control and a homozygous mutation control. Data from the homozygous controls were not included in the final analysis shown in table 3. The Rotor-Gene™ 6000 detected the homozygous mutations for all exons whereas only the homozygous controls for hMLH1 Exons 1 and 13 were detected using the HR-1™ and LightScanner™ (see melt curves in appendix 6). The detection of homozygous controls using the HR-1™ and LightScanner™ is more difficult as the data produced is first normalised and then temperature shifted. Homozygous changes appear to alter the temperature at which the amplicon melts rather than altering the shape of the melt curve. When the data is temperature shifted the homozygous curves become superimposed on the wild type samples making detection difficult in some cases. Data from the Rotor-Gene™ 6000 is normalized but not temperature shifted and therefore homozygous mutations appear to be more easily detected as the melt curves do not become overlaid on the wild type curve.

4.7 Software

For automated calling it is important that wild type samples cluster very tightly to allow the unambiguous detection of subtle mutations and reduce the number of false positive calls. Software is continually developing and improving and this should ultimately allow the full automation of data analysis. During the evaluation period the LightScanner™ was the only platform equipped with mutation calling software and data were analysed using normal and high sensitivity settings. For diagnostic use it appears that the high sensitivity setting is most appropriate to use since this gave 100% sensitivity i.e. no false negative results. In some cases the false positive rate was high (e.g. hMLH1 exon 1) however this is most likely due to sub optimal assay optimisation as this exon shows primer dimers in some samples (appendix 4). The hMLH1 exon 1 samples were more successfully analysed using the Rotor-Gene™ 6000 and HR-1™ where calls were made manually and operators were able to discriminate 'noise' from true variation.

When using the HR-1™ and LightScanner™ certain operator interventions are required during the data analysis. To normalize the data vertical cursors can be moved and placed in a linear region of the melting curve prior to and following the melting transition. The cursors are initially paced at default positions and then require moving by the operator to generate normalised melt curves which have horizontal regions before and after the melt. Positioning of the cursors can alter the appearance of the normalized curves and cause differences in both qualitative manual calling and automated mutation calling. This led to variation in the scoring of samples by different operators. In a diagnostic setting criteria for positioning the cursors for different amplicons would need to be incorporated into standard operating procedures to ensure that there was no variability in data analysis between operators.

4.8 Sensitivity and Specificity compared to other techniques

Data from this evaluation and published studies suggest that HRM has a mutation detection sensitivity which is comparable or superior to currently available pre-screening techniques. In our recent field trial of the generic mutation detection controls the sensitivity and specificity of mutation detection of existing mutation detection techniques ranged from 75% - 100% and 68 – 100% respectively (NGRLW report: (www.ngrl.org.uk/Wessex/downloads.htm). In this evaluation the sensitivity and specificity of HRM was 98 - 100% and 88 - 95% respectively.

4.9 Costings

HRM is a cost effective technique which requires the use of standard PCR reagents and a dsDNA binding dye. The cost price of LC Green® Plus is 22.5p per 10µl reaction (Cadama Medical). HRM machine prices are available from company representatives or distributors:

www.corbettlifescience.com
www.cadama.co.uk
www.idahotech.com

5. OVERALL SUMMARY

We conclude that HRM melt analysis is a simple and cost effective post-PCR technique which can be used for high throughput mutation scanning and genotyping. The technique requires the use of only PCR reagents and the dsDNA binding dye LCGreen® Plus and reactions requires no post-PCR handling (which reduces post-PCR contamination risk) and no separation step, which improves analysis time.

HRM has a mutation detection sensitivity and specificity which is comparable or superior to currently available pre-screening techniques although good amplicon design and PCR optimisation are

essential. In this study, the overall sensitivity and specificities for each HRM machine platform evaluated were highly comparable at 100% and 95% (Rotor-Gene™ 6000, Corbett Life Science), 98 % and 95% (HR-1, Idaho Technology) and 99% and 88% (384 well LightScanner, Idaho Technology).

HRM is capable of detecting some homozygous/hemizygous mutations and appears to be useful for mutation scanning fragments with defined non-pathogenic sequence variants. Although further work is required to determine the feasibility of analysing many different exons for small batches of patients within the same HRM run the technique has the potential to allow large genes to be screened and reported within the 6-8 weeks recommended in the Genetics White Paper (2003) "Our inheritance, our future - realising the power of genetics in the NHS".

6. FUTURE WORK

6.1 Detection of homozygous/hemizygous mutations

To investigate how effectively homozygous / hemizygous mutations can be detected using HRM on the Rotor-Gene™ 6000 and 384 well LightScanner™ we will analyse the NGRL (Wessex) panel of generic mutation detection controls. The controls will be amplified as homozygous samples and will be analysed in a blinded fashion.

6.2 Simultaneous SNP detection and mutation scanning

HRM has the potential to be used for mutation scanning polymorphic exons as has been outlined in section 4.5. However, using HRM it may also be possible to simultaneously type a known polymorphism and mutation scan in a single reaction using asymmetric PCR and unlabeled oligo probes (Zhou *et al*., 2005). We will investigate whether this approach could be used in clinical diagnostic practice to reliably exclude samples containing only non-pathogenic sequence variants from further analysis.

6.3 Software developments

As new versions of software become available for automated calling of samples we will evaluate these using our panel of generic mutation controls.

6.4 dsDNA binding dyes

Other saturating dsDNA binding dyes are now available for use in HRM *e.g.* EvaGreen™ (Biotum), SYTO®9 (Invitrogen). We will investigate their utility for HRM analysis on two platforms: Rotor-Gene™ 6000 and 384 well LightScanner™.

6.5 Amplicon design

Good amplicon design is essential to obtain robust and reproducible HRM assays. At present it is difficult to predict the quality of the melt curve that will be produced from DNA sequence alone. We hope to work with other labs using HRM to develop amplicon design criteria.

6.6 Batching of different amplicons

In this evaluation many samples were analysed for a few amplicons. However, in diagnostic labs it is usually the case that fewer samples are analysed for many exons. Further investigation is now required to determine whether analysis of many exons in the same HRM run is feasible and to determine whether this has an effect on the overall quality of data produced.

6.7 Evaluation of other HRM platforms

We will evaluate other HRM platforms as they become available.

7. ACKNOWLEDGMENTS

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APPENDIX 1: RANDOMISATION OF DNA SAMPLES

Appendix 1a: 96 well plate map showing randomisation of 20, 40, 60 and 80% template plasmid DNA samples and NTC

20% GC

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	P2(A to C)	P2(A to T)	P1(A to T)	P3(G to C)	wt	P3(A to T)	wt	P1(A to C)	P1(G to C)	P2(G to A)
B	wt	wt	wt	P3(A to C)	wt	Water	wt	wt	P2(G to C)	wt	P1(G to A)	P3(G to A)
C	wt	P1(G to A)	wt	P3(G to C)	P1(A to C)	wt	wt	wt	wt	wt	wt	P3(A to C)
D	wt	P2(A to T)	P2(G to C)	P3(A to T)	P2(A to C)	wt	P3(G to A)	wt	P1(G to C)	wt	P2(G to A)	P1(A to T)

40% GC

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	P2(G to C)	P3(A to T)	P3(A to C)	P1(G to C)	P3(G to A)	P2(A to T)	wt	P1(A to T)	wt	wt	wt
B	wt	P1(A to C)	wt	P1(G to A)	wt	Water	P2(G to A)	wt	wt	wt	P3(G to C)	P2(A to C)
C	wt	wt	P1(G to C)	wt	wt	P1(A to T)	P2(A to T)	P2(G to A)	wt	P1(G to A)	wt	wt
D	wt	wt	wt	P3(A to C)	P3(A to T)	P3(G to A)	P2(A to C)	P2(G to C)	P1(A to C)	wt	P3(G to C)	wt

60% GC

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	wt	P2(G to C)	P1(G to A)	wt	wt	P3(G to A)	wt	P3(A to C)	P2(A to T)	wt
B	wt	wt	P1(G to C)	P2(G to A)	wt	Water	P3(A to T)	wt	P1(A to T)	P3(G to C)	P2(A to C)	P1(A to C)
C	wt	wt	wt	P3(A to C)	wt	wt	P3(G to C)	P1(G to A)	wt	P2(A to T)	wt	wt
D	wt	wt	P3(A to T)	P2(A to C)	P1(A to C)	P2(G to C)	P1(A to T)	wt	P2(G to A)	P3(G to A)	P1(G to C)	wt

80% GC

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	P1(A to T)	wt	P3(G to C)	P2(A to T)	wt	wt	P3(G to A)	P1(A to C)	wt	P3(A to C)	P2(G to C)
B	wt	wt	wt	P1(G to A)	P2(A to C)	Water	P2(G to A)	wt	P1(G to C)	P3(A to T)	wt	wt
C	wt	wt	wt	P1(G to A)	P1(G to C)	wt	wt	P3(A to C)	P2(A to T)	wt	P3(A to T)	P2(G to A)
D	wt	P3(G to C)	P2(G to C)	wt	P1(A to C)	P1(A to T)	P3(G to A)	wt	wt	wt	wt	P2(A to C)

Appendix 1b: 96 well plate map showing randomisation of genomic DNA samples and NTCs

hMLH1 Exon 1

	1	2	3	4	5	6	7	8	9	10	11	12
A			110 A>G						110 A>G		63 insC	
B			Water									
C	105insAA							105insAA				
D		105insAA		76 C>T					63insC			
E												
F										62C>G	62GG	Water

hMLH1 Exon 7

	1	2	3	4	5	6	7	8	9	10	11	12
A					554 T>G							
B			Water					588 delA				588+1 G>A
C												
D			588 delA									
E								551 C>G		588+1 G>A		
F										588+1 G>A	588+1 AA	Water

hMLH1 Exon 13

	1	2	3	4	5	6	7	8	9	10	11	12
A						GA	1459C>T					
B		1451 delA	Water	GA		AA					GA	
C				1459C>T								AA
D						1459C>T						1459C>T
E		GA	GA									
F				1459C>T						1486C>A	1486AA	Water

hMSH2 Exon 10

	1	2	3	4	5	6	7	8	9	10	11	12
A	1578 del C	g/a	g/a	1551_2 delCA	g/g	g/g	g/a	1601 G>A	g/a	g/g	g/g	g/g
B	g/a	g/a	Water	g/g	1578 delC	a/a	1578 delC	g/g	g/g	g/g	1609 A>T	g/a
C	g/g	g/g	g/a	g/g	g/g	g/a	g/a	a/a	1609 A>T	1578 delC	g/g	a/a
D	g/g	a/a	g/g	g/g	g/a	g/g	g/g	g/a	g/g	g/a	g/g	g/a
E	1578 delC	g/a	g/a	1571 G>C	g/g	1578 delC	g/a	g/a	g/g	g/a	g/g	g/g
F	g/g	g/g	1578 del C	g/g	a/a	g/g	g/g	1578 del C	g/g	1558 G>T	1558 TT	Water

Genotype of mutated samples:	AA	GA	GG

APPENDIX 2: PCR PRIMERS AND AMPLIFICATION CONDITIONS

Primer sequences

Amplicon Name	Size (bp)	%GC content	PCR primers
20L	449	22	F: TGATAAAATGAGTTGAGTATCTTTC R: ACTATCCTTTTGTTGTTAATACCTTA
20S	272	24	F: TTCAGACATTTTTCTTTAGTT R: ACTATCCTTTTGTTGTTAATACCTTA
40L	437	39	F: TGAGATGATGGGGTTTTCTA R: GGATGCAAGGCTGGTTC
40S	259	42	F: TGAGATGATGGGGTTTTCTA R: AATAATAAGAGCTATCTATGACAA
60L	433	60	F: AATTTGGCCTCTGGGATGAA R: CCCTTTCTCCTTTGGCAATG
60S	260	56	F: AATTTGGCCTCTGGGATGAA R: CACATTGTCCCACAGGAAGTC
80S	258	77	F: GCTGTCGGTGCTGTTGG R: CTAGCGCCCAGCGAGAG
hMLH1x1	193	57	F: GACGTTTCCTTGGCTCTTCTG R: CCGTTAAGTCGTAGCCCTTAAGT
hMLH1x7	148	37	F: AAAAGGGGGCTCTGACATCT R: AAACAAAACCATCCCCCATA
hMLH1x13	247	44	F: CAAGAATAATAATGATCTGCACTTCC R: CAAAACCTTGGCAGTTGAGG
hMSH2x10	249	34	F: TGGAATACTTTTTCTTTTCTTCTT R: CACATCATGTTAGAGCATTTAGGG

Amplification conditions

1. Plasmid DNA Templates

All PCRs were performed using 1X LightScanner™ PCR Master mix (Idaho Technology), 0.2µM each primer, 10^5 copies plasmid template DNA. The 80%S amplicon required the addition of 10% DMSO.

Cycling conditions:

94°C	1 min	
94°C	10 sec	} 40 cycles
x°C	20 sec	
72°C	20 sec	

Annealing temperatures (x°C):

60°C	20%GC L
65°C	20%GC S
	40%GC S
	40%GC L
68°C	60%GC L
	80%GC S
70°C	60%GC S (two step PCR 30 sec anneal and extend)

2. Genomic DNA Templates

PCR Reaction components	hMLH1 Exon 1	hMLH1 Exon 7	hMLH1 Exon 13	hMSH2 Exon 10
Platinum Taq Buffer (Invitrogen)	1X	1X	1X	1X
$MgCl_2$	2mM	1.5mM	2mM	2mM
dNTPs	0.2mM	0.2mM	0.2mM	0.2mM
Platinum Taq (Invitrogen)	1 Unit	1 Unit	1 Unit	1 Unit
LC Green® Plus (Idaho Technology)	1X	1X	1X	1X
F Primer	0.2µM	0.2µM	0.2µM	0.2µM
R primer	0.2µM	0.2µM	0.2µM	0.2µM
DNA	10ng	10ng	10ng	10ng

Cycling conditions:

94°C	1 min	
94°C	10 sec	} 40 cycles
x°C	20 sec	
72°C	20 sec	

Annealing temperatures (x°C):

60°C	hMLH1 Exon 7
63°C	hMLH1 Exon 1
	hMSH2 Exon 10
65°C	hMLH1 Exon 13

APPENDIX 3: HRM MACHINE SPECIFICATIONS

1. Rotor-Gene™ 6000 (Corbett Life Science)

Instrument Options	P/N 62H0: 2-Plex (Green, Yellow) + HRM (High Res. Melt) P/N 65H0: 5-Plex (Green, Yellow, Orange, Red, Crimson) + HRM (High Res. Melt)
Rotor Configurations	30 µL x 100-wells: GeneDisc™ 100 (heat-sealed plate) (AVAILABLE SOON) 0.1 mL x 72-wells: GeneDisc™ 72 (heat-sealed plate) 0.1 mL x 72-wells: 0.1 mL strip tubes (strips of 4 with matching caps) 0.2 mL x 36-wells: 0.2 mL tubes with attached cap (flat or domed)
Channel Information	**Colors** / **Excite/Detect** / **Some Example Fluorophores Detected** Blue 365/470 BiosearchBlue™, Marina Blue®, Bothell Blue®, Alexa Fluor® 350… Green 470/510 FAM™, SYBR® Green 1, Fluorescein, EvaGreen™, Alexa Fluor® 488… Yellow 530/555 JOE™, VIC™, HEX™, TET™, Yakima Yellow®, CAL Fluor® Gold 540... Orange 585/610 ROX™, Cy®3.5, Redmond Red®, Alexa Fluor®568, CAL Fluor®Red 610… Red 625/660 Cy®5, Quasar®670, LCRed650®, Texas Red®, CAL Fluor® Red 635… Crimson 670/710 Quasar705™, LCRed705®, Alexa Fluor® 680 HRM 460/510 SYTO®9, LC Green®, LC Green® Plus+, EvaGreen™...
Thermal Performance	Uniformity: ± 0.01°C Resolution: ± 0.02°C Range: ambient–99°C Maximum Ramp Rate: 10°C/sec (air)
Optical System	Fixed path length Separate color high-intensity LED (light-emitting diode) excitation source per channel Separate emission filter per channel
Detector	Photomultiplier (PMT) detector with variable or automatic gain setting (sensitivity control), user selected
Dimensions	H 275 mm (10.8") , W 370 mm (14.6") , D 420 mm (16.5") door closed, 560 mm (22") door open
Weight	14 kg (31 lbs)
Electrical	100-120VAC @ 60Hz, 200-240VAC @ 50Hz Power consumption: 8 VA (idle/standby), 560 VA (peak)
Communications	USB or RS232 Serial Port
Computer	Pentium™ IV, 2 GHz, 512 MB RAM , Windows XP® operating system
Software	Extensive analysis, graphing and statistical functions built-in Unlimited use software license included Free upgrades (by web download)
40 Cycle Run	Typically 40 minutes to 1.5 hours (protocol dependant)
Supported Volumes	5 µL to 100 µL (protocol dependant) Typical reaction volume 20 µL
Instrument Color	Outback Red high-gloss metallic Bondi Blue high-gloss metallic
High-Resolution Melt	Dedicated high-performance optical subsystem, additional hardware and specific HRM analysis software (fully integrated). Thermal uniformity ±0.01°C, Resolution ±0.02°C, Range ambient–99°C HRM data acquisition (read) rate: 20 reads for each 0.02°C increment (=1000 reads/°C)
Concentration Measurement	For direct measurement of nucleic acid concentration using fluorescent dyes (e.g. PicoGreen®, RiboGreen®, etc). Comprises dedicated analysis software (standard on all models

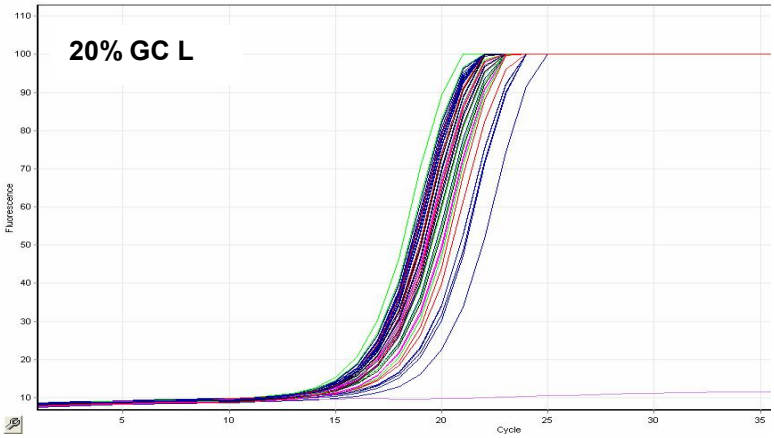
2. HR-1™ (Idaho Technology)

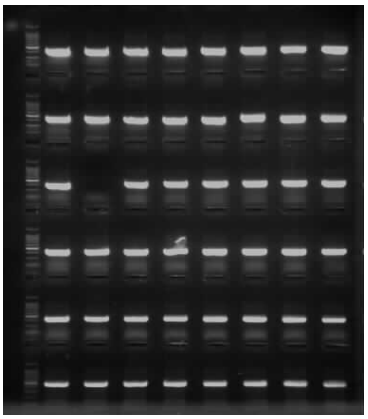
Linear Ramp Rate	0.01ºC to 1ºC per second
Temperature Range	Ambient to 100ºC
Data Acquisition	24 bit temperature and fluorescence analogue to digital conversion
Sample Volume	5-20 µl
Capacity	Single sample melting using glass capillary tubes. Typical Throughput: 35 samples per hour with a 0.3°C ramp rate
Laptop Computer	Dell Notebook Computer with Ethernet capabilities
Software	Windows based software includes control and analysis modules
Size	4 x 5.5 x 12 inch / 10.2 x 14 x 30.5 cm (w x h x d)
Weight	5.8 lbs. / 2.65 kg
Power Supply	100 to 240 VAC, 1.3 Amp, 50/60 Hz universal power

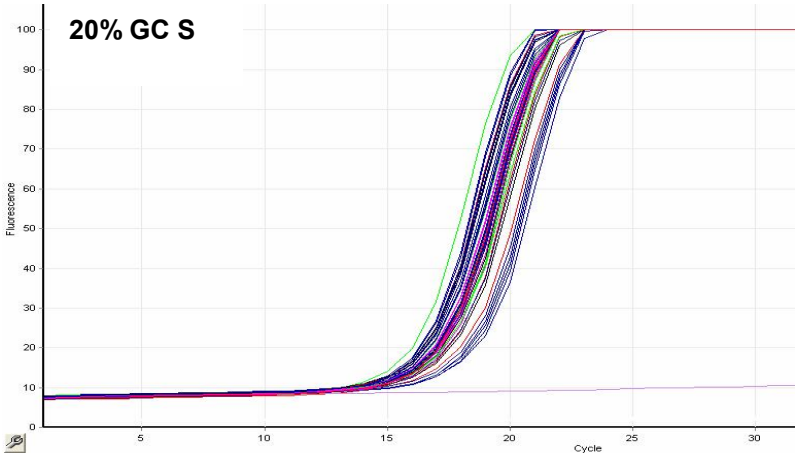
3. LightScanner™ (Idaho Technology)

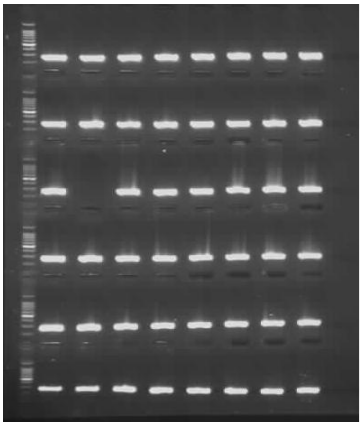
Linear Ramp Rate	0.15 °C per second
Temperature Range	Ambient to 100 °C
Sample Volume	Standard 96 or 384 microtitre plate
Capacity/Throughput	15 minutes per run, over 20,000 samples per 8 hour day (96 wells)
Melting Cycles	1 to 100
Desktop Computer	Windows XP, Pentium 4 Computer with 17” Monitor
Software	Windows based software includes control and analysis modules
Size (w x h x d)	7 x 31 x 16.5 inch / 20 x 81 x 42 cm
Weight	55.1 lbs / 25 kg
Power Supply	100 to 240 VAC, 2.5 Amp, 50/60 Hz

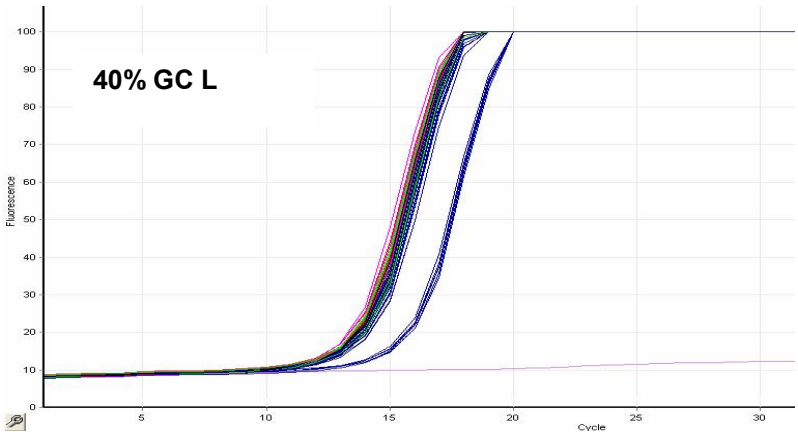
APPENDIX 4: AMPLIFICATION PLOTS AND AGAROSE GEL IMAGES

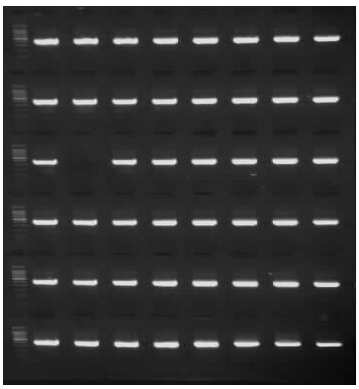


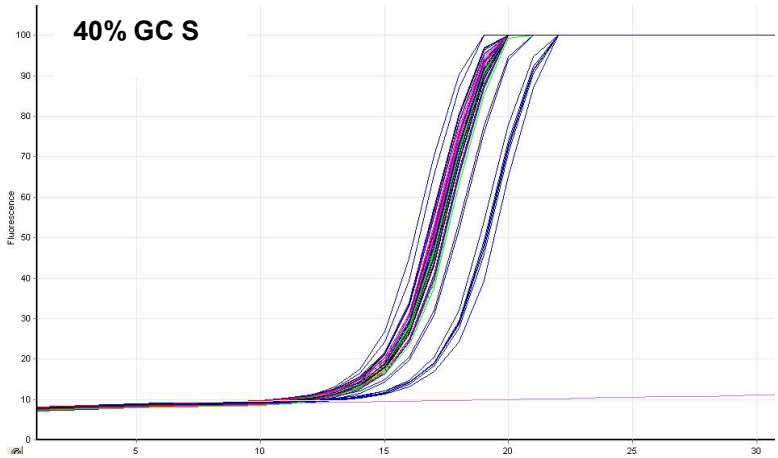


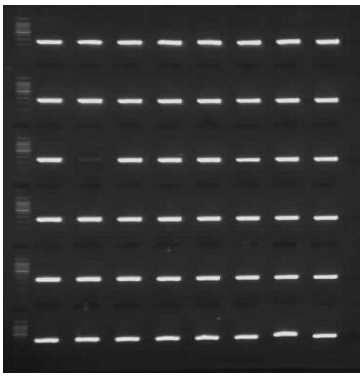


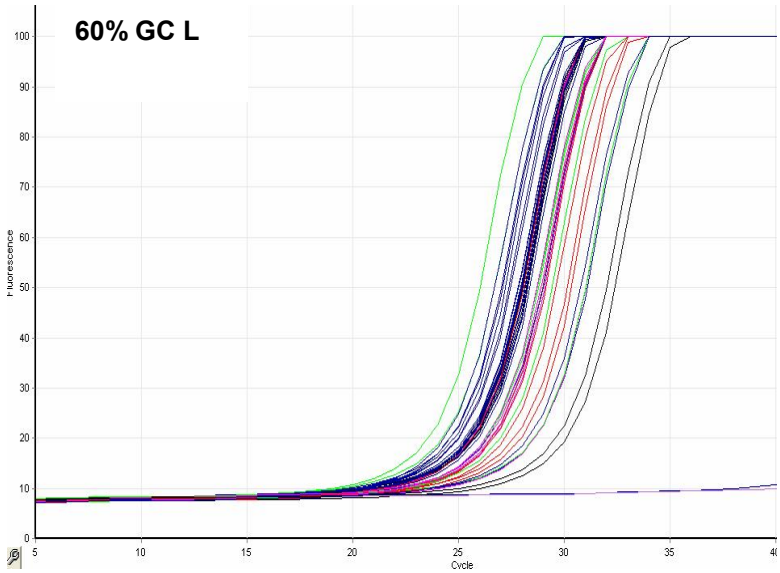


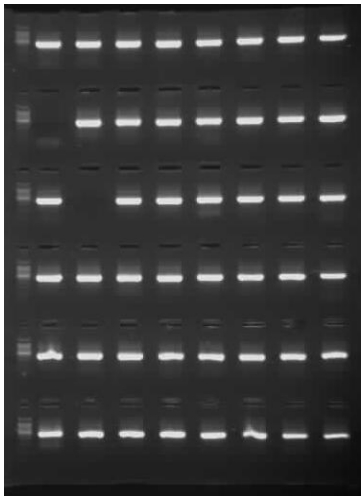


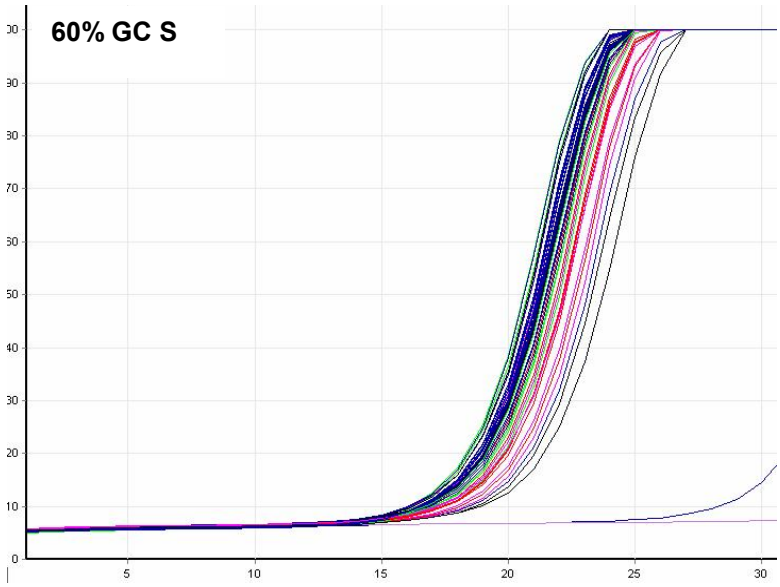


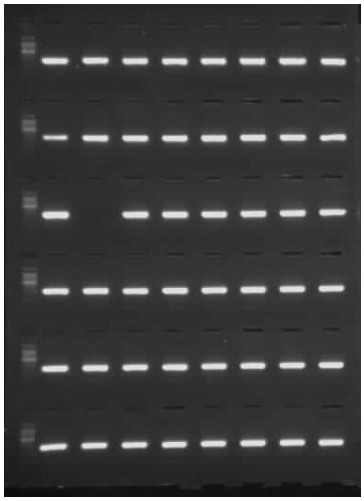


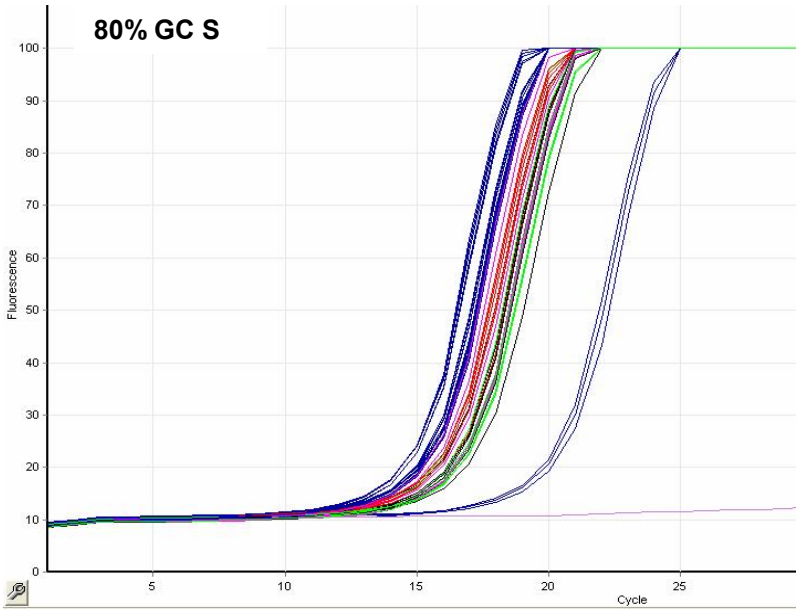


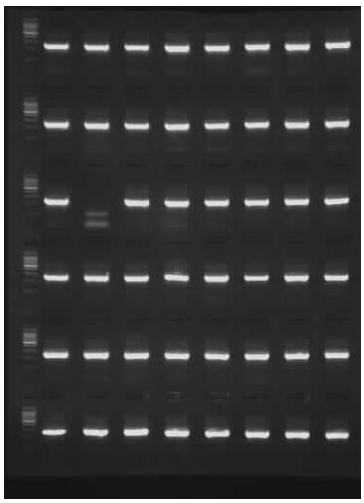

60% GC L
Fluorescence
Cycle

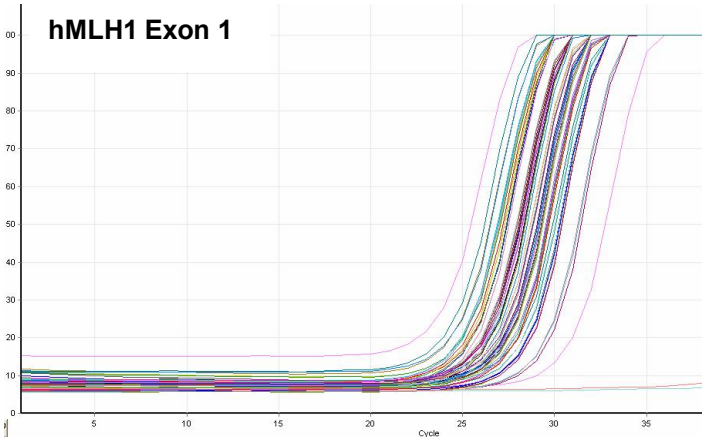


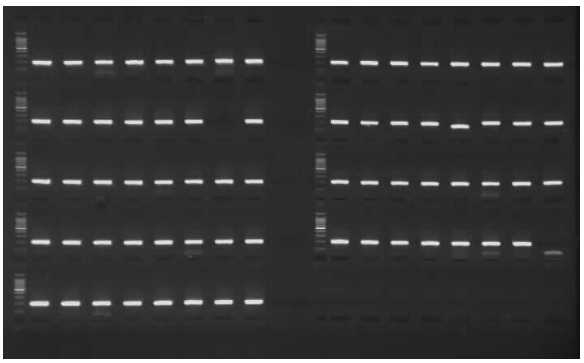

60% GC S

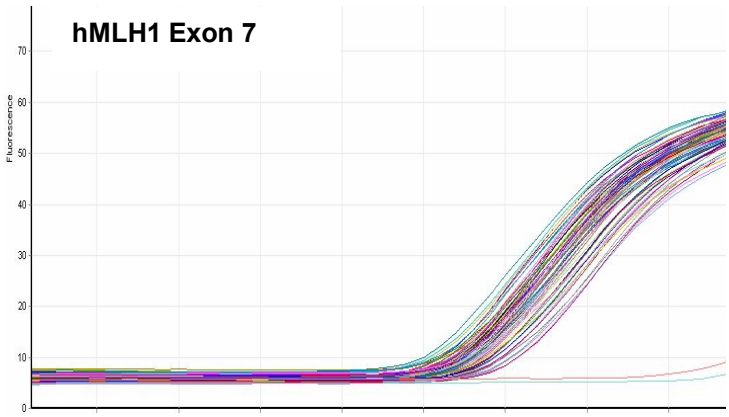


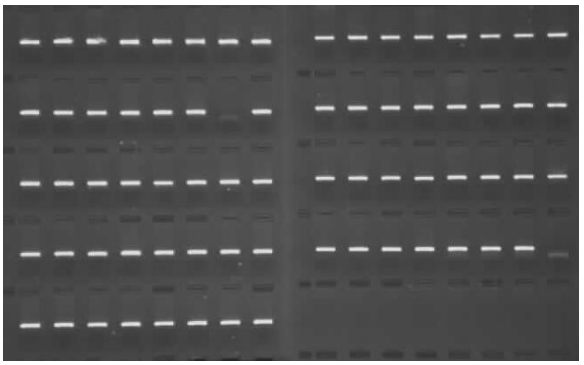

80% GC S
Fluorescence
Cycle

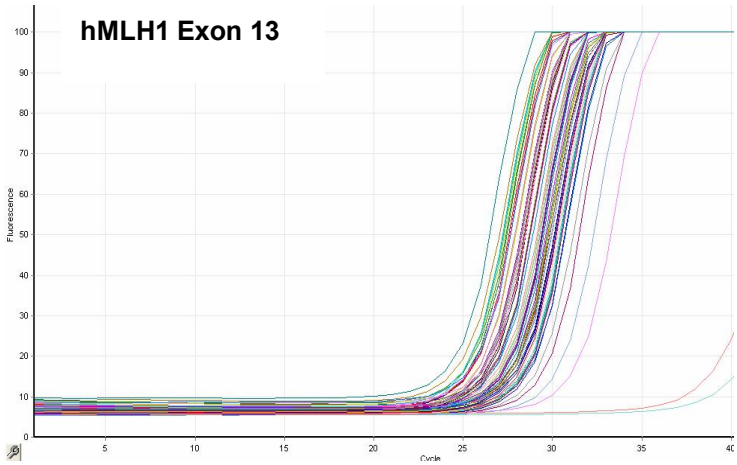


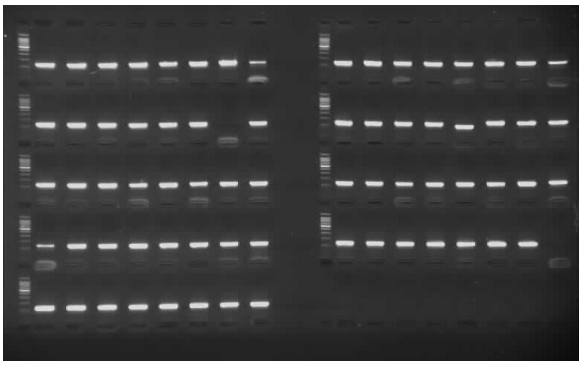

hMLH1 Exon 1
Cycle

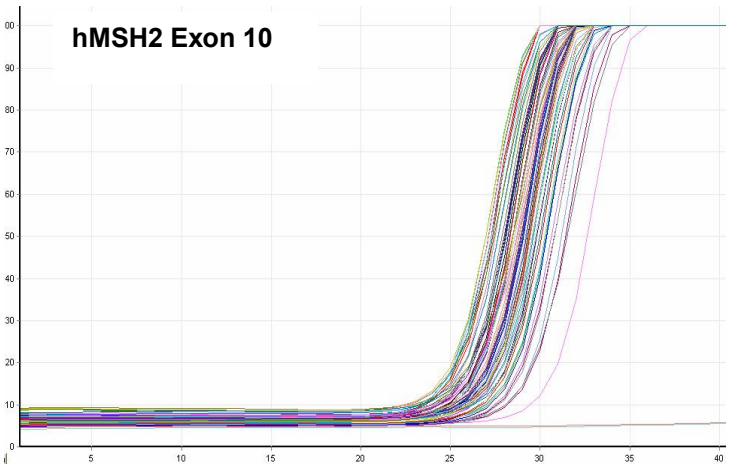


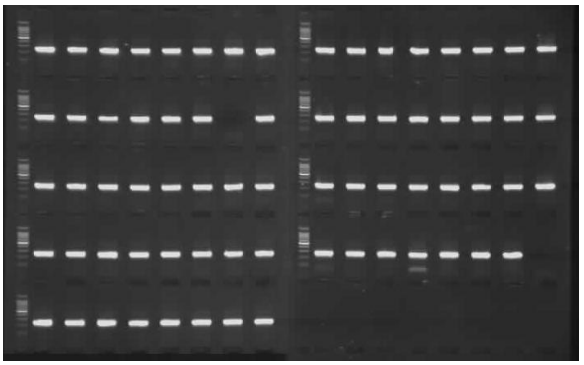

hMLH1 Exon 7
Fluorescence




hMLH1 Exon 13
Fluorescence
Cycle




hMSH2 Exon 10



APPENDIX 5: Data Analysis

20%GC Long: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	P2(A - C)	P2(A - T)	P1(A - T)	P3(G - C)	wt	P3(A - T)	wt	P1(A - C)	P1(G - C)	P2(G - A)
B	wt	wt	wt	P3(A - C)	wt	Water	wt	wt	P2(G - C)	wt	P1(G - A)	P3(G - A)
C	wt	P1(G - A)	wt	P3(G - C)	P1(A - C)	wt	wt	wt	wt	wt	wt	P3(A - C)
D	wt	P2(A - T)	P2(G - C)	P3(A - T)	P2(A - C)	wt	P3(G - A)	wt	P1(G - C)	wt	P2(G - A)	P1(A - T)

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	FP	TP	FP	Water	FP	TN	TP	TN	TP	TP
C	TN	TP	FP	TP	TP	TN	FP	TN	TN	FP	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

Operator 2

A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	FP	TN	TP	TN	TP	TP
C	TN	TP	FP	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	FN	TP	TN	TP	FP	TP	FN	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	FN	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

Operator 2

A	TN	TN	TP	TP	FN	TP	TN	TP	TN	TP	FN	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	FN	TP	TN	TP	TN	TP	FN	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	FN	TP
C	TN	FN	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	FN	TN	TP	FN

High Sensitivity

A	TN	TN	TP	TP	FN	TP	TN	TP	TN	TP	FN	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	FN	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

20%GC Short: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	P2(A - C)	P2(A - T)	P1(A - T)	P3(G - C)	wt	P3(A - T)	wt	P1(A - C)	P1(G - C)	P2(G - A)
B	wt	wt	wt	P3(A - C)	wt	Water	wt	wt	P2(G - C)	wt	P1(G - A)	P3(G - A)
C	wt	P1(G - A)	wt	P3(G - C)	P1(A - C)	wt	wt	wt	wt	wt	wt	P3(A - C)
D	wt	P2(A - T)	P2(G - C)	P3(A - T)	P2(A - C)	wt	P3(G - A)	wt	P1(G - C)	wt	P2(G - A)	P1(A - T)

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

Operator 2

A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	FP	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	FP	TP	TN	TP	TP

Operator 2

A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	FP	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	FP	TP	TP

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

High Sensitivity

A	TN	TN	TP	TP	TP	TP	TN	TP	TN	TP	TP	TP
B	TN	TN	TN	TP	TN	Water	TN	TN	TP	TN	TP	TP
C	TN	TP	TN	TP	TP	TN	TN	TN	TN	TN	TN	TP
D	TN	TP	TP	TP	TP	TN	TP	TN	TP	TN	TP	TP

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

40%GC Long: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	P2(G - C)	P3(A - T)	P3(A - C)	P1(G - C)	P3(G - A)	P2(A - T)	wt	P1(A - T)	wt	wt	wt
B	wt	P1(A - C)	wt	P1(G - A)	wt	Water	P2(G - A)	wt	wt	wt	P3(G - C)	P2(A - C)
C	wt	wt	P1(G - C)	wt	wt	P1(A - T)	P2(A - T)	P2(G - A)	wt	P1(G - A)	wt	wt
D	wt	wt	wt	P3(A - C)	P3(A - T)	P3(G - A)	P2(A - C)	P2(G - C)	P1(A - C)	wt	P3(G - C)	wt

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

Operator 2

A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

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Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	FP	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	FN	TP	TP	TP	TP	TN	TP	TN

Operator 2

A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	FP	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	FN	TP	TP	TP	TP	TN	TP	TN

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	FN	TP	TP	FN	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	FN	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	FN	FN	TP	TP	TP	TN	FN	TN

High Sensitivity

A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

40%GC Short: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	P2(G - C)	P3(A - T)	P3(A - C)	P1(G - C)	P3(G - A)	P2(A - T)	wt	P1(A - T)	wt	wt	wt
B	wt	P1(A - C)	wt	P1(G - A)	wt	Water	P2(G - A)	wt	wt	wt	P3(G - C)	P2(A - C)
C	wt	wt	P1(G - C)	wt	wt	P1(A - T)	P2(A - T)	P2(G - A)	wt	P1(G - A)	wt	wt
D	wt	wt	wt	P3(A - C)	P3(A - T)	P3(G - A)	P2(A - C)	P2(G - C)	P1(A - C)	wt	P3(G - C)	wt

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

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Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	FP	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	FN	TP	TP	TP	TN	TP	TN

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	FN	TP	TP	TP	TN	FN	TN

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	FN	TP	TP	FN	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	FN	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	FN	FN	FN	TP	TP	TP	TN	FN	TN

High Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN	TN	TN
B	TN	TP	TN	TP	TN	Water	TP	TN	TN	TN	TP	TP
C	TN	TN	TP	TN	TN	TP	TP	TP	TN	TP	TN	TN
D	TN	TN	TN	TP	TP	TP	TP	TP	TP	TN	TP	TN

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

60%GC Long: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	wt	P2(G - C)	P1(G - A)	wt	wt	P3(G - A)	wt	P3(A - C)	P2(A - T)	wt
B	wt	wt	P1(G - C)	P2(G - A)	wt	Water	P3(A - T)	wt	P1(A - T)	P3(G - C)	P2(A - C)	P1(A - C)
C	wt	wt	wt	P3(A - C)	wt	wt	P3(G - C)	P1(G - A)	wt	P2(A - T)	wt	wt
D	wt	wt	P3(A - T)	P2(A - C)	P1(A - C)	P2(G - C)	P1(A - T)	wt	P2(G - A)	P3(G - A)	P1(G - C)	wt

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	FP	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	FP

Operator 2

A	TN	FP	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	FP

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TP	TP	FP	FP	TP		TP	TP	TN
B	TN	FP	TP	TP	TN	Water	TP	FP	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	FP

Operator 2

A	TN	TN	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	FP	TP	TP	TP	TP
C	TN	TN	TN	FN	TN	TN	TP	TP	FP	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	FN	TN	TP	TP	TP	TN

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A		TN	TN	FN	FN	TN	TN	TP		FN	TP	TN
B	TN	TN	TP	TP	TN	Water	FN	TN	TP	FN	FN	TP
C		TN	TN	FN	TN	TN	FN	TP	TN	FN	TN	TN
D	FP	TN	FN	FN	TP	FN	FN	TN	TP	TP	TP	FP

High Sensitivity

A		TN	TN	TP	TP	FP	FP	TP		TP	TP	TN
B	TN	FP	TP	TP	TN	Water	FN	FP	TP	FN	TP	TP
C		FP	TN	TN	FP	TN	FN	TP	FP	TP	TN	TN
D	FP	FP	FN	FN	TP	TP	TP	FP	TP	TP	TP	FP

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

60%GC Short: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	wt	wt	P2(G - C)	P1(G - A)	wt	wt	P3(G - A)	wt	P3(A - C)	P2(A - T)	wt
B	wt	wt	P1(G - C)	P2(G - A)	wt	Water	P3(A - T)	wt	P1(A - T)	P3(G - C)	P2(A - C)	P1(A - C)
C	wt	wt	wt	P3(A - C)	wt	wt	P3(G - C)	P1(G - A)	wt	P2(A - T)	wt	wt
D	wt	wt	P3(A - T)	P2(A - C)	P1(A - C)	P2(G - C)	P1(A - T)	wt	P2(G - A)	P3(G - A)	P1(G - C)	wt

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	TN

Operator 2

A	TN	TN	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	TN

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TP	TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	TN

Operator 2

A	TN	TN	TN	TP	TP	FP	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	TN

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN		TP	TN	TN	FN		TP	FN	TN
B	TN	TN	TP	TP	TN	Water	FN	TN	FN	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	FN	TP	TP	TP	FN	TN	TP	FN	TP	TN

High Sensitivity

A	TN	TN	TN		TP	TN	TN	TP		TP	TP	TN
B	TN	TN	TP	TP	TN	Water	TP	TN	TP	TP	TP	TP
C	TN	TN	TN	TP	TN	TN	TP	TP	TN	TP	TN	TN
D	TN	TN	TP	TP	TP	TP	TP	TN	TP	TP	TP	TN

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

80% GC Short: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	wt	P1(A - T)	wt	P3(G - C)	P2(A - T)	wt	wt	P3(G - A)	P1(A - C)	wt	P3(A - C)	P2(G - C)
B	wt	wt	wt	P1(G - A)	P2(A - C)	Water	P2(G - A)	wt	P1(G - C)	P3(A - T)	wt	wt
C	wt	wt	wt	P1(G - A)	P1(G - C)	wt	wt	P3(A - C)	P2(A - T)	wt	P3(A - T)	P2(G - A)
D	wt	P3(G - C)	P2(G - C)	wt	P1(A - C)	P1(A - T)	P3(G - A)	wt	wt	wt	wt	P2(A - C)

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	TN	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	TN	TP	TP	TP	FP	TN	FP	FP	TP

Operator 2

A	TN	TP	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	TN	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	TN	TP	TP	TP	FP	TN	FP	FP	TP

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TP	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	TN	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	TN	TP	TP	TP	TN	TN	TN	TN	TP

Operator 2

A	TN	TP	FP	TP	TP	TN	TN	TP	TP	TN	FP	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	FP	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	FP	TP	TP	TP	TN	TN	TN	TN	TP

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	FP	TP	FP	TP	TP	FP	TN	TP	TP	TN	FN	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	TN	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	TN	TP	TP	TP	TN	TN	TN	TN	TP

High Sensitivity

A	FP	TP	FP	TP	TP	FP	TN	TP	TP	TN	TP	TP
B	TN	TN	TN	TP	TP	Water	TP	TN	TP	TP	TN	TN
C	TN	TN	TN	TP	TP	TN	TN	TP	TP	TN	TP	TP
D	TN	TP	TP	TN	TP	TP	TP	TN	TN	TN	TN	TP

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

hMLH1 Exon 1: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A			110 A>G						110 A>G		63 insC	
B			Water									
C	105insAA							105insAA				
D		105insAA		76 C>T					63insC			
E												
F										62C>G	62GG	Water

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TN	TN	TN	TN	TN	TP	TN	TP	TN
B	TN	TN	Water	TN	TN	TN	TN	TN	TN	TN	TN	TN
C	TP	TN	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN
D	TN	TP	TN	TP	TN	TN	TN	TN	TP	TN	TN	TN
E	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	FP	TN	TN	TN	TN	TN	TN	FP	TP	TP	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TN	TN	TN	TN	TN	TP	TN	TP	TN
B	TN	TN	Water	TN	TN	TN	TN	TN	TN	TN	TN	TN
C	TP	TN	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN
D	TN	TP	TN	TP	TN	TN	TN	TN	TP	TN	TN	TN
E	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	FP	TN	TN	TN	TN	TN	TN	FP	TP	TP	Water

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Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TN	TN	TN	TN	TN	TP	TN	TP	TN
B	TN	TN	Water	TN	TN	TN	TN	TN	TN	TN	TN	TN
C	TP	TN	TN	TN	TN	TN	FP	TP	TN	TN	FP	TN
D	TN	TP	TN	TP	TN	TN	TN	TN	TP	TN	TN	TN
E	FP	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TP	TN	TN	FP	TN	TN	TP	TN	TP	TN
B	TN	TN	Water	FP	TN	TN	TN	TN	TN	TN	TN	FP
C	TP	TN	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN
D	TN	TP	TN	TP	TN	TN	TN	TN	TP	TN	TN	TN
E	FP	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	FP	FP	TP	FP	TN	TN	TN	TN	TP	FP	TP	TN
B	FP	TN	Water	TN	FP	TN	TN	FP	TN	TN	TN	FP
C	TP	TN	TN	TN	TN	TN	FP	TP	TN	TN	TN	TN
D	TN	TP	TN	FN	TN	TN	TN	TN	FN	TN	TN	TN
E	FP	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

High Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	FP	FP	TP	FP	FP	TN	TN	TN	TP	FP	TP	TN
B	FP	TN	Water	TN	FP	TN	TN	FP	TN	TN	TN	FP
C	TP	TN	TN	TN	TN	TN	FP	TP	TN	TN	TN	TN
D	TN	TP	TN	TP	TN	TN	TN	TN	TP	TN	TN	TN
E	FP	TN	TN	TN	FP	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

hMLH1 Exon 7: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A					554 T>G							
B			Water					588 delA				588+1 G>A
C												
D			588 delA									
E								551 C>G		588+1G>A		
F										588+1G>A	588+1AA	Water

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	TN	TN	Water	TN	TN	TN	TN	TP	TN	TN	TN	TP
C	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
D	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	TN	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	TN	TN	Water	TN	TN	TN	TN	TP	TN	TN	TN	TP
C	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
D	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	TN	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	TP	Water

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	FP	TN	Water	TN	TN	TN	TN	TP	TN	TN	TN	TP
C	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
D	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	FP	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	FN	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	TN	TN	Water	TN	TN	TN	TN	TP	TN	TN	TN	TP
C	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
D	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	FP	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	FN	Water

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	TN	TN	Water	TN	TN	TN	TN	FN	FP	TN	TN	TP
C	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
D	TN	TN	FN	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	TN	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	FN	Water

High Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN
B	TN	TN	Water	TN	TN	TN	TN	TP	FP	TN	FP	TP
C	TN	TN	TN	TN	TN	TN	FP	TN	TN	TN	TN	TN
D	FP	TN	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
E	TN	TN	TN	TN	FP	TN	TN	TP	TN	TP	TN	TN
F	TN	TN	TN	TN	TN	TN	TN	TN	TN	TP	FN	Water

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

hMLH1 Exon 13: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A						GA	1459C>T					
B		1451 delA	Water	GA		AA					GA	
C				1459C>T								AA
D						1459C>T						1459C>T
E		GA	GA									
F				1459C>T						1486C>A	1486AA	Water

RotorGene 6000

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	FP	TP	TP	TN	TN	TN	TN	TN
B	TN	TP	Water	TP	TN	TP	TN	TN	TN	TN	TP	FP
C	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP
E	TN	TP	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP	TP	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TN	TP	TP	TN	TN	TN	TN	TN
B	TN	TP	Water	TP	TN	TP	TN	TN	TN	TN	TP	TN
C	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP
E	TN	TP	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP	TP	Water

HR-1

Operator 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TN	TP	TP	TN	TN	TN	TN	TN
B	TN	TP	Water	TP	TN	TP	TN	FP	TN	TN	TP	TN
C	FP	TN	TN	TP	TN	TN	FP	TN	FP	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	FP	TN	TN	TN	TN	TP
E	TN	TP	TP	TN	TN	TN	TN	TN	TN	TN	FP	TN
F	TN	FP	FP	TP	TN	TN	TN	TN	TN	TP	TP	Water

Operator 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TN	TP	TP	FP	TN	TN	TN	TN
B	TN	TP	Water	TP	TN	TP	TN	FP	TN	TN	TP	TN
C	FP	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	FP	TN	TN	TN	TN	TP
E	FP	TP	TP	TN	TN	TN	TN	TN	FP	TN	FP	TN
F	TN	TN	FP	TP	TN	TN	TN	TN	TN	TP	TP	Water

LightScanner

Normal Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TN	FN	TP	TN	TN	TN	TN	TN
B	TN	FN	Water	FN	TN	TP	TN	TN	TN	TN	FN	TN
C	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP
E	TN	FN	FN	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	TN	TN	TP	TN	TN	TN	TN	TN	FN	FN	Water

High Sensitivity

	1	2	3	4	5	6	7	8	9	10	11	12
A	TN	TN	TN	TN	TN	TP	TP	TN	TN	TN	TN	TN
B	TN	TP	Water	TP	TN	TP	TN	TN	TN	TN	TP	TN
C	TN	TN	TN	TP	TN	TN	TN	TN	TN	TN	TN	TP
D	TN	TN	TN	TN	TN	TP	TN	TN	TN	TN	TN	TP
E	FP	TP	TP	TN	TN	TN	TN	TN	TN	TN	TN	TN
F	TN	FP	TN	TP	TN	TN	TN	TN	TN	TP	FN	Water

Wild type call
Mutated call
Ambiguous call

TN: true negative
TP: true positive

FP: false positive
FN: false negative

hMSH2 Exon 10: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1578 del C	g/a	g/a	1551_2 delCA	g/g	g/g	g/a	1601 G>A	g/a	g/g	g/g	g/g
B	g/a	g/a	Water	g/g	1578 delC	a/a	1578 delC	g/g	g/g	g/g	1609 A>T	g/a
C	g/g	g/g	g/a	g/g	g/g	g/a	g/a	a/a	1609 A>T	1578 delC	g/g	a/a
D	g/g	a/a	g/g	g/g	g/a	g/g	g/g	g/a	g/g	g/a	g/g	g/a
E	1578 delC	g/a	g/a	1571 G>C	g/g	1578 delC	g/a	g/a	g/g	g/a	g/g	g/g
F	g/g	g/g	1578 del C	g/g	a/a	g/g	g/g	1578 del C	g/g	1558 G>T	1558 TT	Water

AA	GA	GG

RotorGene 6000

Operator 1 (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

Operator 2 (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

Operator 1 (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g	g/g	g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

Operator 2 (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g	g/g	g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

Operator 1 (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

Operator 2 (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

hMSH2 Exon 10: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1578 del C	g/a	g/a	1551_2 delCA	g/g	g/g	g/a	1601 G>A	g/a	g/g	g/g	g/g
B	g/a	g/a	Water	g/g	1578 delC	a/a	1578 delC	g/g	g/g	g/g	1609 A>T	g/a
C	g/g	g/g	g/a	g/g	g/g	g/a	g/a	a/a	1609 A>T	1578 delC	g/g	a/a
D	g/g	a/a	g/g	g/g	g/a	g/g	g/g	g/a	g/g	g/a	g/g	g/a
E	1578 delC	g/a	g/a	1571 G>C	g/g	1578 delC	g/a	g/a	g/g	g/a	g/g	g/g
F	g/g	g/g	1578 del C	g/g	a/a	g/g	g/g	1578 del C	g/g	1558 G>T	1558 TT	Water

AA	GA	GG

HR1

Operator 1 (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

Operator 2 (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

Operator 1 (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g	g/g	g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

Operator 2 (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g	g/g	g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

Operator 1 (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

Operator 2 (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

hMSH2 Exon 10: Master Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	1578 del C	g/a	g/a	1551_2 delCA	g/g	g/g	g/a	1601 G>A	g/a	g/g	g/g	g/g
B	g/a	g/a	Water	g/g	1578 delC	a/a	1578 delC	g/g	g/g	g/g	1609 A>T	g/a
C	g/g	g/g	g/a	g/g	g/g	g/a	g/a	a/a	1609 A>T	1578 delC	g/g	a/a
D	g/g	a/a	g/g	g/g	g/a	g/g	g/g	g/a	g/g	g/a	g/g	g/a
E	1578 delC	g/a	g/a	1571 G>C	g/g	1578 delC	g/a	g/a	g/g	g/a	g/g	g/g
F	g/g	g/g	1578 del C	g/g	a/a	g/g	g/g	1578 del C	g/g	1558 G>T	1558 TT	Water

AA	GA	GG

LightScanner

Normal Sensitivity (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

High Sensitivity (GA)

	1	2	3	4	5	6	7	8	9	10	11	12
A		g/a	g/a				g/a		g/a			
B	g/a	g/a	Water		g/a							g/a
C			g/a			g/a	g/a		g/a			
D					g/a			g/a		g/a		g/a
E	g/a	g/a	g/a	g/a		g/a	g/a	g/a		g/a		
F			g/a					g/a				Water

Normal Sensitivity (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g		g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

High Sensitivity (GG)

	1	2	3	4	5	6	7	8	9	10	11	12
A				g/g	g/g	g/g		g/g		g/g	g/g	g/g
B			Water	g/g				g/g	g/g		g/g	
C	g/g	g/g		g/g	g/g						g/g	
D	g/g		g/g	g/g		g/g	g/g		g/g		g/g	
E					g/g				g/g		g/g	g/g
F	g/g	g/g		g/g		g/g	g/g		g/g	g/g	g/g	Water

Normal Sensitivity (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

High Sensitivity (AA)

	1	2	3	4	5	6	7	8	9	10	11	12
A	a/a											
B			Water			a/a	a/a					
C								a/a		a/a		a/a
D		a/a										
E												
F					a/a							Water

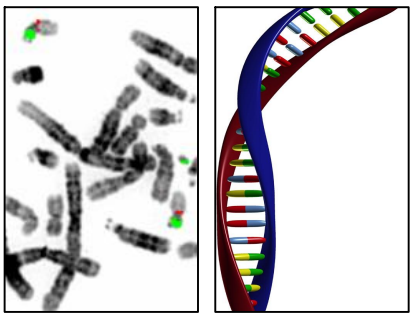
APPENDIX 6:
High Resolution Melt Curves

(file can be downloaded from www.ngrl.org.uk/Wessex)

APPENDIX 7:

Company comments

Letter from Corbett Life Science can be downloaded or viewed at www.ngrl.org.uk/Wessex



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