

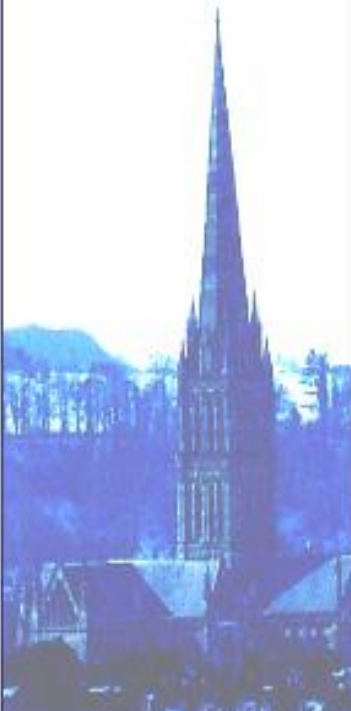


National Genetics Reference Laboratory  
(Wessex)

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**NHS**


**Technology  
Assessment  
Report**



**NGRL WESSEX**

Evaluation of MassCLEAVE™  
Chemistry for Diagnostic  
Screening using MALDI-TOF  
Mass Spectrometry.

June 2004

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### Peer Review and Approval

This document has been subject to internal review and Sequenom have had the opportunity to comment on the contents.

### Conflicting Interest Statement

The authors declare that they have no conflicting financial interests

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## 1. INTRODUCTION

A number of the UK molecular genetics laboratories carry out routine [diagnostic] screens for diseases associated with multiple or large genes. To screen a single patient would typically require between 30 and 100 separate tests. Currently such screens are largely restricted to cancer genes such as BRCA1, BRCA2, hMLH1 and MSH2, but this list is likely to grow. This type of testing requires a high throughput approach in order to achieve adequate patient turnaround time. This has become particularly pertinent since the publication of the recent white paper on genetics (DOH 2003: *Our Inheritance, Our future* available at [www.doh.gov.uk/genetics/whitepaper.htm](http://www.doh.gov.uk/genetics/whitepaper.htm)). Two general approaches may be considered when deciding on an appropriate strategy:

1. Direct sequencing of all fragments to identify and characterise variations. For diagnostic purposes all mutations identified would be confirmed in a second test, usually also by sequencing.
2. Pre-screen using an indirect method to determine which fragments harbour sequence variations, followed by sequencing of those fragments to characterise the variation present. In this case the sequencing is counted as the confirmatory test.

The advantage of sequencing is that variations are simultaneously identified and characterised: only pathogenic mutations need be confirmed. However in comparison with most pre-screen methods, sequencing is expensive and analysis can be extremely time consuming. Even if the pick up rate (mutations per patient) is 90%, a screen requiring 50 tests per patient would have a discovery rate (mutations per fragment tested) of <2%. This means that >98% of the sequencing carried out would be unnecessary. Moreover analysis of a normal (i.e. proving a fragment does not harbour a mutation) is substantially more troublesome than identifying mutations that are present.

A rapid and accurate pre-screen to eliminate normal fragments before confirmatory sequencing would be a major advantage. In general the problem with indirect mutation detection is the inability to distinguish polymorphisms from pathogenic mutations. Consequently all variants need to be sequenced regardless of pathogenicity. This is compounded by the fact that most pre-screen techniques will not give an unequivocal signal for all mutations. In some cases experimental variation can lead to a signal in a normal that resembles that of a mutation that only gives a subtle indication. In this case, in the interests of safety, the fragment in question would need to be sequenced. Excessive occurrence of polymorphisms and/or ambiguous signals can eliminate the advantage a pre-screen strategy has over direct sequencing.

The MassCLEAVE™ biochemistry from SEQUENOM has the potential to virtually eliminate the problems associated with pre-screen strategies. This method should be able to both identify and characterise point mutations allowing known polymorphisms to be excluded before confirmatory sequencing. Since insertion, deletion and complex mutations are almost invariably pathogenic they would all need to be sequenced anyway. It is therefore not necessary to characterise these classes of mutation; merely flag their existence. Because of the nature of the cleavage reactions MassCLEAVE™ is more likely to give an unequivocal signal for any given mutation than most current pre-screen strategies.

This study was carried out in order to evaluate the MassCLEAVE™ method in the context of a mutation screening strategy with the following aims:

1. To determine if MassCLEAVE™ would pick up all mutations in a given set (false negative rate).
2. To determine the false positive rate.
3. To determine the rate at which MassCLEAVE™ would characterise point mutations.
4. To make a preliminary evaluation of practical aspects of the technique.

## 2. METHODS AND DATA ANALYSIS

The study was set up using 12 different PCR amplicons, six from exon 11 of BRCA1 five from hMLH1 and one from MSH2. For each amplicon 15 samples containing various mutations and one water blank were screened. This represents two 96 well micro-titre plates of samples. Although existing primer sets were used it was noted that this does not represent an ideal situation as amplicons should be specifically designed to suit the MassCLEAVE™ biochemistry. PCR reactions were carried out in the NGRL in 40ul volumes. These were then split into two 20ul aliquots; one for direct sequencing in the NGRL (Wessex), UK and one for blind analysis by SEQUENOM in Hamburg, Germany using MassCLEAVE™ biochemistry and SNP Discovery software. Care was taken to avoid cross contamination of samples but the possibility of this could not be entirely eliminated as a result of the transportation between the two labs.

The MassCLEAVE™ methodology involves PCR amplification of the fragment of interest using a modified primer that incorporates a T7 promoter site into the one end of the PCR product. This product is then transcribed *in vitro* to yield an RNA copy of one strand of the fragment. Two separate base specific cleavages are then performed using RNase A; one cleaving the product at every C base and one at every U (the U cleavage is named after its DNA equivalent, i.e. T cleavage). This whole sequence of reactions is also carried out to produce the equivalent cleavage products from the opposing strand. This process yields four separate fragment populations (each representing cleavage of the fragment of interest at a different base A,C,G or T) that are individually analysed using a MALDI-TOF mass spectrometer. Theoretically the observed cleavage patterns are unique for each different target composition so that even a single heterozygous change will be detectable. The observed patterns are compared to a theoretical cleavage of the known 'normal' sequence of the fragment and complex algorithms are used to de-convolute any discrepancies and report both the position and character of any variations (see figure 1). Two types of indicators can be generated: entirely new peaks and intensity changes to existing peaks. New signals are the primary consideration for mutation identification as they deliver solid and reliable information. Intensity changes, termed as 'silent', are only used to back up information gained from new peaks.

The analysis was carried out by SEQUENOM using SNP Discovery software. The version of the program used was specifically designed to analyse target sequences in a (large) sample sets for SNP discovery. Consequently the software is designed to report a summary of single base variations, including substitutions, but was not [at the time of writing] designed to investigate insertions and deletions longer than one base pair. Moreover genotype data was not automatically generated for each individual sample. As a result it was necessary to review all spectra manually in order to assign genotypes.

**(a) Normal fragment**

5' -CATTACAGCATGAGAACAGCAGTTTATTAC-3'  
 3' -GTAATGTCGTACTCTTGTTCGTCAAATAATG-5'

**(b) Mutant fragment – A>C at base 13 fragment**

5' -CATTACAGCATG**C**GAAACAGCAGTTTATTAC-3'  
 3' -GTAATGTCGTAC**G**CTTGTTCGTCAAATAATG-5'

<p>5' -CAT ACAGCAT T AT AC-3'                      T <span style="border: 1px solid orange; padding: 2px;">GAGAACAGCAGT</span> T T</p>	T forward cleavage	<p>5' -CAT ACAGCAT T AT AC-3'                      T <span style="border: 1px solid green; padding: 2px;">GCGAACAGCAGT</span> T T</p>
<p>5' -C ATTAC <u>AGC</u> <span style="border: 1px solid orange; padding: 2px;">ATGAGAAC</span> <u>AGC</u> AGTTTATTAC-3'</p>	C forward cleavage	<p>5' -C ATTAC <u>AGC</u> <span style="border: 1px solid green; padding: 2px;">GAAC</span> <u>AGC</u> AGTTTATTAC-3'                      ATTAC <span style="border: 1px solid green; padding: 2px;">ATGC</span> <u>AGC</u></p>
<p>3' -G TG <span style="border: 1px solid grey; padding: 2px;">TAC</span> T TCG TAA  <u>TAA</u> TCG <span style="border: 1px solid grey; padding: 2px;">TC</span> TG TCAA TAA TG-5'</p>	T reverse cleavage	<p>3' -G TG <span style="border: 1px solid green; padding: 2px;">TACGC</span> TG TCAA TG-5'  <u>TAA</u> TCG T TCG TAA</p>
<p>3' -GTAATGT <u>CT</u> CGT                      CGTA CTTGT CAAATAATG-5'</p>	C reverse cleavage	<p>3' -GTAATGT <u>CG</u> CGT                      CGTA CTTGT CAAATAATG-5'</p>

**Figure 1:** Example of MassCleave™ analysis.

(a) shows the cleavage products obtained from the four cleavage reactions of a normal amplicon. For the purposes of analysis, cleavage products smaller than four bases (grey) are excluded since they are likely to be ambiguous i.e. many different cleavage products can have the same mass – examples of this are the AGC cleavage products in the C forward reaction and the TTA cleavage products in the T reverse (single underlined).

(b) shows the cleavage products for the same amplicon with a single A>C base change (red) at base 13. Cleavage products coloured orange in the normal analysis represent those that will not be present in the homozygous mutant analysis or drop in height by ~50% in a heterozygous mutant analysis. Cleavage products coloured green represent those only present in the mutant analysis (whether homozygous or heterozygous). The analysis software compares the actual mass spectra obtained with the theoretical cleavage pattern calculated using the normal sequence. Possible mutations are examined until the most likely cause of any discrepancies is identified. Note that a single mutation normally causes at least two linked indicators in each reaction: a heterozygous mutation in this example generates a new peak in the T forward reaction (green - boxed) combined with a drop in height by ~50% of a second peak (orange - boxed) that has a different mass owing to the A>C base change. Similarly the ATGAGAAC in the C forward reaction drops by ~50% combined with the generation of two new peaks (double underlined). There are several exceptions to this rule: if two cleavage products less than 4 base pairs long are no longer cleaved as a result of the mutation (T reverse reaction – double boxed) or if the base change only affects a cleavage product less than four bases long (C reverse reaction – overlined). It is also possible that a new cleavage product generated by a mutation may have the same mass as a cleavage product elsewhere in the fragment. In this case the only changes to the mass spectra would be height alterations which are not considered to be unequivocal indicators.

### 3. RESULTS

Tables 1 and 2 summarize the results of this study. In each case the MassCLEAVE™ results are compared with results obtained by fluorescent sequencing. The left panel (grey) displays the numbers of mutations correctly flagged, false negative and false positive results. The right panel describes how many mutations were correctly characterized, how many were correctly located within the amplicon and whether the correct zygosity was assigned. Although exon 12 of hMLH1 was included in this study no results were obtainable as a result of sub-optimal fragment design. Consequently this fragment has been excluded from the analysis.

Mutation type	Seq. results	Mutations identified with MassCLEAVE (SNP discovery software aided analysis)					
		flagged	missed	False +ve	position	nature	Zygosity (hom/het)
Insertion	5	4	1*	7	0	2	3
Deletion	32	26	6*	1	15	7	26
Het. point	34	34	0	0	20	34	34
Hom. point	60	60	0	0	45	60	60
Total	131	126	7*	8+1 <sup>#</sup>	80	103	123

**Table 1:** Summary of results for six different BRCA1 exon 11 amplicons (15 samples each) divided by mutation type.

\*These 7 insertions and deletions were not reported by the software. However retrospective inspection of spectra clearly displays the mutation identifying signals (figure 1).

<sup>#</sup>one unclassified variant was called in a normal sample.

Mutation type	Seq. results	Mutations identified with MassCLEAVE (SNP discovery software aided analysis)					
		flagged	missed	False +ve	position	nature	Zygosity (hom/het)
Insertion	5	4	0	0	4	4	5
Deletion	12	10	2*	0	1	1	8
Het. point	25+1 <sup>#</sup>	25+1 <sup>#</sup>	0	0	25+1 <sup>#</sup>	25	25+1 <sup>#</sup>
Hom. point	4	4	0	0	4	4	3
Total	46+1 <sup>#</sup>	44+1 <sup>#</sup>	2*	0	34+1 <sup>#</sup>	34	41+1 <sup>#</sup>

**Table 2:** Summary of results for four different hMLH1 amplicons and one hMSH2 amplicon (15 samples each) divided by mutation type.

\*These 2 deletions were not reported by the software. However retrospective inspection of spectra clearly displays the mutation identifying signals.

<sup>#</sup>The 'extra' mutation is a two base pair substitution, AA>GC



### 3.1 BRCA1 analysis

For the purposes of detailed discussion this report is focussed on the BRCA 1 analyses since these raised to most interesting and pertinent points. The term mutation has been used to describe any sequence variation regardless of pathogenicity.

The sample panel for BRCA1 analysis comprised 90 samples (some of which were repeated) containing a total of 131 sequence variations from the sequence provided. Because of sample repeats and multiple occurrences of the same mutation there was a total of 23 different mutations represented. Sixteen fragments had no mutations (normal), 37 had one mutation, 17 had two mutations and 20 had three mutations.

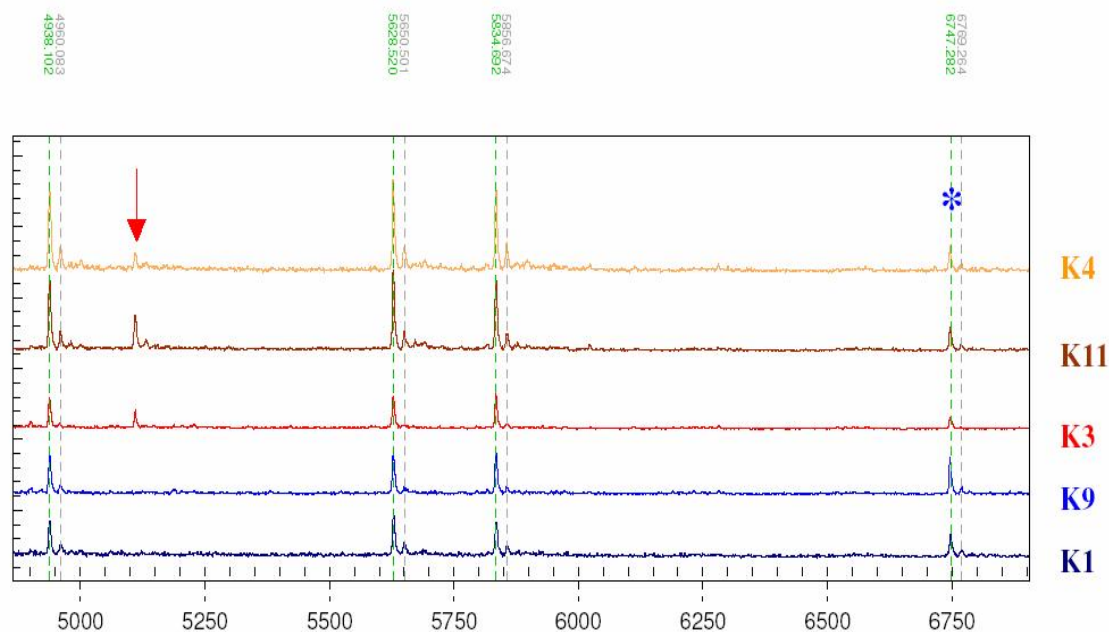
Analysis of the results can be conveniently split into two groups: Insertions and deletions (frameshifts), which need to be flagged but not necessarily fully characterised, and point mutations (including both heterozygous and homozygous changes), which need full characterisation in order to be excluded from confirmatory sequencing as necessary.

#### 3.1.1 Frameshift mutations.

Of the 37 frameshift mutations present five (14%) were not detected. In terms of unique mutations this group represented two of the 12 different frameshifts present. The first, heterozygous 3819 del GTAAA, was not detected in any of the fragments in which it occurred (K3, K4 and K11). This deletion represents one of two tandem repeats of this sequence. Of the four theoretical indicators for this mutation (see table 3) only one represents a new peak (5110Da in C forward cleavage). Retrospective examination of the spectra revealed that this indicative peak was in fact present in samples containing the 3819 del GTAAA mutation (Figure 2). The remaining indicators were all relative intensity changes (Table 3). This raises the question of how stringently the spectra need to be analysed in order to pick up all mutations.

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	1658	1	0.5	AAAGT
CF	6747	1	0.5	TTGTTATTTGGTAAAGTAAAC
CF	5110	0	0.5	TTGTTATTTGGTAAAC
CR	1566	2	1.5	TTTAC

**Table 3:** Theoretical analysis of heterozygous 3819 del GTAAA.



**Figure 2:** Analysis of heterozygous 3819 del GTAA.

The C forward cleavage spectra for mutant samples K4, K11 and K3 are compared with the spectra for wild type samples K9 and K1. Although they were not picked up by the current software the new signal at 5110Da can clearly be seen in all the mutant samples (highlighted with a red arrow). The relative height of the corresponding peak at 6747Da (asterisk) has dropped in the mutant samples but this is difficult to detect as the signal is so far away from the nearest peak.

The second missed frameshift, heterozygous 4184 del TCAA, was present in nine fragments but only detected in seven (missed in L3 and L12). Theoretical analysis of this mutation shows new peaks in the T forward, C forward and C reverse cleavages, although the 6913Da fragment in C forward is beyond the analysis range (Table 4). The 2479Da fragment was very T rich which tends to give a low signal strength. In fact this peak was not picked up by the software in any of the samples tested although it was present on visual inspection. The only remaining unequivocal indicator is the 5247Da peak in the T forward reaction. In contrast to the cases where the mutation was detected, both cases where this mutation was missed gave weak or poor spectra for the T forward cleavage. In one case (L3) the PCR failed, whereas in the other (L12) the PCR was deemed to be of satisfactory quality suggesting a problem arose during the subsequent processes. The characterisation of this mutation was also complicated by the presence of a second mutation (4193 A>G) in close proximity.

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	5520	1	0.5	CAAGAAGAACAAAGCAT
TF	5247	0	0.5	AAGAAGAACAAAGCAT
CF	2990	1	0.5	AAGAAGAAC
CF	5210	1	0.5	TTGGAAGAAAATAATC
CF	6913*	0	0.5	TTGGAAGAAAATAAGAAGAAC
CR	3761	1	0.5	TTGATTATTTTC
CR	2479	0	0.5	TTATTTTC

**Table 4:** Theoretical analysis of heterozygous 4184 del TCAA.

\*Beyond analysis cut off range

The main issue here is one of robustness. If detection of certain mutations is reliant on a single reaction and that reaction has to be of good quality to ensure detection, any samples with a single reaction not up to optimal quality would need to be either re-analysed or put forward for confirmatory sequencing to be sure of the correct result. In the current study 41/90 (46%) samples attained optimal spectra quality for all four cleavages. By extrapolation 54% of normal samples screened would therefore need confirmatory sequencing. Theoretical analysis of all the mutations in this study suggests that unequivocal indicators should be present in more than one reaction. By adjustment of the analysis parameters it may be possible to make a confident normal call based on three cleavages or four with non-optimal spectra. More stringent control of DNA quantity and quality, particularly at the PCR stage should improve robustness.

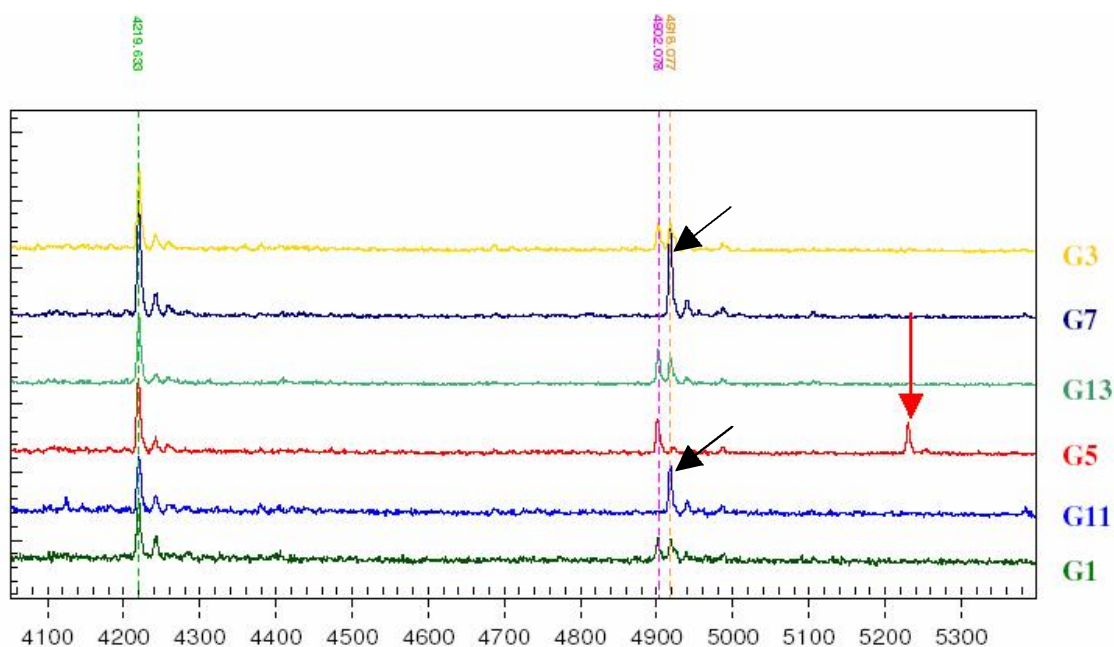
Two other mutations are noteworthy in the context of frameshifts. Eight of the samples in amplicon G contained the heterozygous 2731 C>T polymorphism. Sample G5 contained both the homozygous T/T form of 2731 C>T and a heterozygous 2731 ins T but the insertion was not called. Both PCRs and all the cleavage spectra were of satisfactory quality. Although this is flagged as a mutation, in this case confirmatory sequencing would not have been deemed necessary since 2731 C>T is not pathogenic. Although the spectra are similar for these mutations they should be distinguishable on the basis of the T reverse and C reverse reactions (Tables 5 and 6). In retrospect the unique identifier 5247Da peak for the 2731 ins T mutation is only detected in sample G5 containing this mutation (figure 3).

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TR	4918	1	0	GAAAACGGAGCAAAT
TR	4902	0	1	GAAAACAGAGCAAAT
CR	1689	1	0	GGAGC
CR	1673	0	1	AGAGC

**Table 5:** Theoretical analysis of homozygous 2731 C>T.

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TR	4918	1	0.5	GAAAACGGAGCAAAT
TR	5247	0	0.5	GAAAACAGGAGCAAAT
CR	1689	1	0.5	GGAGC
CR	2018	0	0.5	AGGAGC

**Table 6:** Theoretical analysis of heterozygous 2731 ins T.

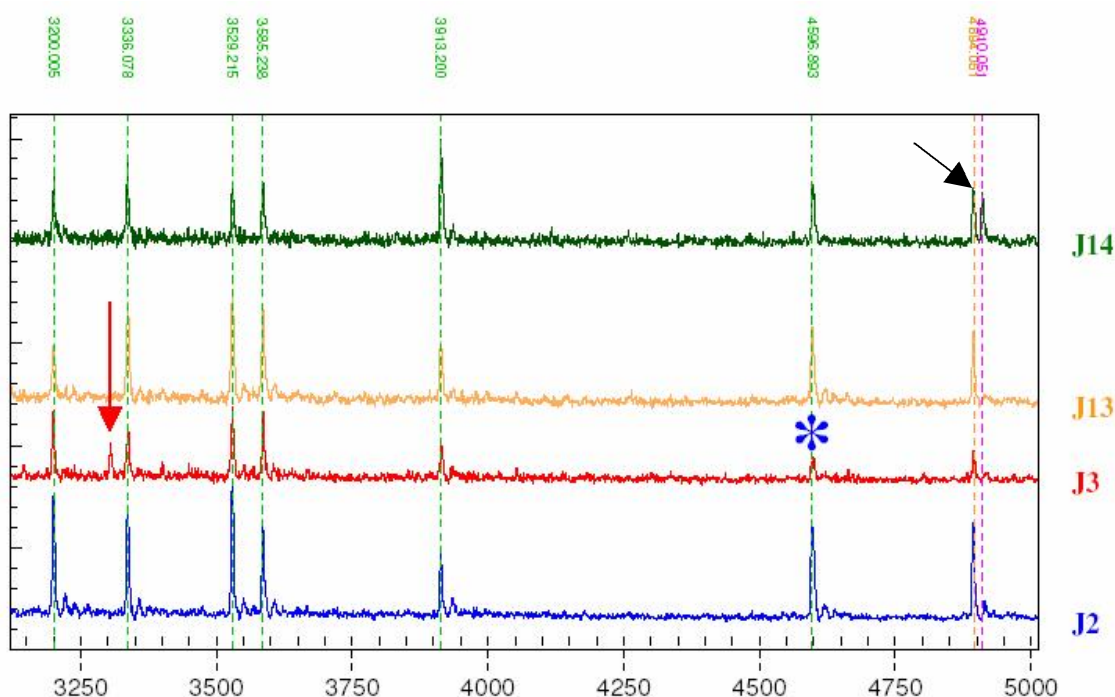


**Figure 3:** Analysis of sample G5.

The spectra shown represent the T reverse cleavage reaction for BRCA 1 exon 11 fragment G. Samples G7 and G11 are wild type (black arrows highlight the indicative peak at 4918Da), whilst samples G3, G13 and G1 contain the 2731 C>T mutation in heterozygous form (the 4918Da peak has dropped in height and a new peak at 4902Da has appeared). Sample G5 does not contain the 4918Da peak and was correctly called as a 2371 T/T homozygote. Although the software did not call the ins T mutation in sample G5 manual examination clearly shows the presence of the indicative 5247Da peak (red arrow).

Sample J3 contained heterozygous 3450 del CAAG. A range of mutations were called for this sample (del A @70, ins G @ 196 and C/T @197, 199 or 200, ins A@ 346 and G/C @ 349 and ins C @ 354) although none in the region where the mutation is located (154bp into amplicon). The same spectra of mutations was also called for sample J13, with the addition G>T @ position 223 of the fragment; the correct call for this sample. Therefore, it is questionable whether this spectra of mutations is an artefact (false positive) or truly arose from the presence of the 3450 del CAAG mutation. In fact a unique identifier for the 3450 del CAAG mutation (3304Da) was detected in retrospect although the signal strength was low and calling of the mutation was confounded by the presence of numerous spurious peaks (figure 4). In practice this sample would have been sequenced anyway as more than one call was made. In this respect the methodology fulfilled the requirements of a diagnostic screen.

The software also detected the heterozygous 2731 C>T mutation (non-pathogenic polymorphism) in sample J3, a call that was not originally made when the sample was sequenced.



**Figure 4:** Analysis of sample J3.

The spectra shown represent the T forward cleavage reaction for BRCA 1 exon 11 fragment J. Sample J3 contains a unique identifier for the 3450 del CAAG mutation although the signal strength is low (red arrow). The corresponding affected wild type signal is marked with an asterisk. For comparison two wild type traces are shown (J13 and J2). Sample J14 contained a heterozygous 3667 A>G mutation (the affected pair of peaks for this mutation in the T forward cleavage is highlighted with a black arrow).

### 3.1.2 Point mutations.

All the point mutations in this study were flagged, the correct base change identified and the correct zygosity given. However the positioning of mutations was ambiguous in 31% of cases. The location of homozygotes (75% correct) was slightly better than that of heterozygotes (59% correct) as might be expected. In terms of unique mutations, four of the 11 point mutations were ambiguously located. Homozygous 1184 A>G in amplicon B and heterozygous 3667 A>G in amplicon J were both narrowed down to one of two bases, and in both cases one of the calls was correct. Heterozygous 2731 C>T in amplicon G was called 1 bp 5' of the true location. The C forward reaction contains the only unequivocal indicator (2200Da) for differentiating between the two positions (tables 7 and 8). This peak is very T, which produces poor signal strength and was not detected by the software. In fact both positions were given equal likelihood by the software and the call was made manually. It is also possible that location of this mutation was confounded the presence of heterozygous 2687 C>T in the same samples.

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	1249	1	0.5	CCGT
CF	1894	1	0.5	GTTTTTC
CF*	2200	0	0.5	TGTTTTTC
TR	4918	1	0.5	GAAAACGGAGCAAAT
TR	4902	0	0.5	GAAAACAGAGCAAAT
CR	1689	1	0.5	GGAGC
CR	1673	0	0.5	AGAGC

**Table 7:** Theoretical analysis of heterozygous 2731 C>T (position 294 – true position).

\*This peak is the only unequivocal (i.e. not silent) indicator differentiating heterozygous 2731 C>T from heterozygous 2730 C>T (see table 8)

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	1249	1	0.5	CCGT
TR	4918	1	0.5	GAAAACGGAGCAAAT
TR	4902	0	0.5	GAAAACGAAGCAAAT
CR	1689	1	0.5	GGAGC
CR	1673	0	0.5	GAAGC

**Table 8:** Theoretical analysis of heterozygous 2730 C>T (position 293 – called position).

Heterozygous 4158 A>G in amplicon L was called 5 bp 3' of the true location. Only silent (i.e. peak height alterations) differentiate between these two positions (Tables 9 and 10). Again the software gave a number of equally likely positions for the mutation but a manual call was made.

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	6299	1	0.5	GAAGAAAGAGGAACGGGCT
TF	6315	0	0.5	GAAGAAGGAGGAACGGGCT
CF	6974	1	0.5	AGATGATGAAGAAAGAGGAAC
CF	6990	0	0.5	AGATGATGAAGAAGGAGGAAC
CR*	1243	2	1.5	TTTC

**Table 9:** Theoretical analysis of heterozygous 4158 A>G (position 205 – true position).

\* differentiating indicator

Rxn	mass	Relative frequency in normal	Relative frequency in mutant	Sequence
TF	6299	1	0.5	GAAGAAAGAGGAACGGGCT
TF	6315	0	0.5	GAAGAAAGAGGGACGGGCT
CF	6974	1	0.5	AGATGATGAAGAAAGAGGAAC
CF	6990	0	0.5	AGATGATGAAGAAAGAGGGAC
TR*	1193	1	0.5	CCCT
CR*	1282	1	0.5	GTTC

**Table 10:** Theoretical analysis of heterozygous 4163 A>G (position 210 – called position).

\* differentiating indicators

In this context it is more problematic to unambiguously call mutations. The called mutation could be a non-pathogenic polymorphism, whereas the true mutation could be significant. However it is likely that a policy of sequencing rare or novel mutations would be adopted even if they had no apparent protein consequence. Even so, there is still the possibility that a significant mutation could mimic a common polymorphism and thus be excluded from sequencing. In all cases more stringent criteria as to what can be unambiguously called would have led to multiple calls and consequently the samples would have been sequenced.

The detection of multiple mutations in a single sample was generally good with up to three being detected and in most cases any point mutations present were fully characterised. There was a correlation between the number of mutations in a fragment and the accuracy of calling. Of the 14 point mutations miss-located within the fragment of interest 11 were in fragments with three mutations two in fragments with two mutations and only one in a fragment with one mutation. It should be noted that all of these 14 mutations were characterised correctly in every other way.

### 3.1.3 False positives.

Only three different, clear false positives were detected. An ins A at base 275 of fragment C was detected 7 normal samples. In all cases the distinguishing reaction was deemed to be weak. This may have arisen from cross contamination in transit. An unidentified mutation was detected at an unknown location in sample J9, which was in fact a normal sample. This call was only made with the use of more sensitive analysis and may also be due to cross contamination. Finally an del T was detected in sample J14 but was noted to be of 'uncertain affected genotype'. This call may be an artefact of the A>G mutation present in this sample as it was not seen in any other samples.

## 4. CONCLUSIONS

The software used for analysis was originally designed for SNP discovery. It was therefore not expected that the software alone would be capable of genotyping all the mutations in this study. This blind study has highlighted areas where the current version of the software needs to be improved to suit mutation detection analysis. Such improvements are currently under investigation within R&D in SEQUENOM.

Despite some problems of location the pick up rate for point mutations was good. Even with the location ambiguities, 69% of point mutations were fully characterised. This should allow the exclusion of 69% of polymorphisms from confirmatory sequencing, although in practice each known polymorphism would be analysed to determine if the signals given were unequivocal enough for identification at the pre-screen level. From a diagnostic point of view procedural aspects of the analysis would need to be clearly defined in order to make the process as sensitive and as objective as possible.

Generally the data obtained facilitate a higher information yield compared to other screening technologies. However we are slightly concerned about the robustness of the assay in the context of confidently calling 'normals'; a crucial, and substantial part of diagnostic work. However, a more detailed and closely controlled study would be required to determine the limits of the technology in this respect (i.e. how many reactions and of what quality are required to confidently call a normal).

There is evidence that the unequivocal characterisation of certain mutations may have been confounded by low level cross contamination during transit of PCR products between the two labs. Some of the false positive results could also be explained in this way. A more realistic diagnostic scenario would carry out the entire process at a single location thereby reducing this possibility.

We do not yet have a feel for the hands on time required for this procedure, in particular for analysis. Obviously we would like this to be as simple as possible and this could in part be achieved through standardisation of procedure as mentioned above. Further development of the analysis software could also simplify the process, for example the ability to apply a different reference sequences to a single analysis. The time required for analysis strongly affects the suitability of an assay for a pre-screen role in diagnostics.

In all cases where specific problems arose, alterations to the software (e.g. in stringency of making unambiguous calls, analysis range or peak height threshold) would have rectified the error. Currently the technique can only be viewed as a screening methodology since not all mutations can be fully characterised. However, with suitable changes to the analysis protocol and software, all mutations are flagged and a very low false positive rate is observed.

No technique has been shown to be 100% sensitive and problems often arise in analysis. Bearing in mind the crude nature of this study and the fact that the PCR protocols were not specifically designed for the MassCLEAVE approach the system performed very well and is certainly worthy of further study and development.



## 5. FURTHER WORK

A larger blind study is being planned to address the issues raised by this study. In particular we will be looking to develop an analysis protocol suitable for diagnostic screening by applying software developments from SEQUENOM and a more realistic work flow scheme. The issue of robustness in terms of the number of cleavage results and their quality that are required for an unequivocal 'normal' result to be called will also be addressed. Finally work flow and costing analyses will be carried out to assess the performance of MassCLEAVE™ compared to other methodologies suitable for diagnostic screening.

## 6. ACKNOWLEDGMENTS

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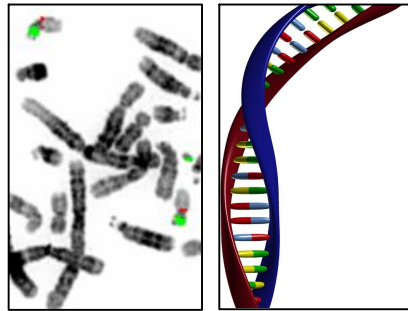
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