

CHAPTER 5

RESULTS AND DISCUSSION

5.1. The loading test

The loading test did not only involve the distribution of the adulterated juice and collection of urine, but explanations and convincing volunteers were necessary to obtain maximum participation and compliance in a study of this nature. The major challenge in performing an exercise like the loading test is not handing out of the juice. It is actually the collection of urine and/or blood, both of which most people find very sensitive to give or collect. The second challenge, even more difficult to overcome was that after explaining and convincing the volunteers to take the adulterated juice, the bad fish-like smell and the unpleasant taste of trimethylamine in the juice was almost always met with some complaints, even from the most interested volunteer. A greater part of the time and effort was therefore understandably spent in discussions with large target volunteer groups.

Trimethylamine is a highly volatile amine, and a more accurate quantification of the compound from the urine sample would only be possible if the samples were quantified immediately after collection. In cases where it was unavoidable to keep the samples for an extended period before analysis, the urine samples were kept chilled in the refrigerator to minimise loss of TMA. This fact complicated the timing of sample collection as well as quantification time due to the four-hour waiting period between administration of the TMA dose and the collection of the second urine sample. In summary, the loading test is not a straightforward procedure, but one that involves other factors such as good human relations and moral judgement rather than simply pure biochemistry.

Results of the loading test were as shown in table 5.1. Most of the volunteers that participated in this study projected a normal TMA to TMAO metabolic conversion which was over 95%. There were a few volunteers whose conversion ratios were barely above 95% but were also grouped with the defect free type of volunteers. A less stringent boundary at the top limit (95%) was introduced to compensate for any fluctuations due to different diets taken by volunteers before the loading test was performed. The mean age of all volunteers was 21 years.

Table 5.1: Results of the loading test.

Individual type	No. of individuals	Percentage	Comments
Suspected heterozygote (Subject 3) ^a	1 (Male)	0.73%	Trimethylaminuria symptoms after the loading test. Pre-loading urine sample was negative.
Suspected heterozygotes (Subject 1) ^b	1 (Female)	0.73%	Consistently produced a TMA/TMAO ratio of 89.1%.
Suspected homozygotes	0	0%	
Defect-free	135	98.7%	Their TMA/TMAO ratios were above 96% after loading with 600mg TMA.
Overall result = 1.46% (mild trimethylaminuric individuals)			

^b (Subject 1): According to table 4.2

^a (Subject 3): According to table 4.2

One of the volunteers, subject 1 in table 4.2, invariably showed a TMA / TMAO ratio of 89.1% when 600mg TMA was administered. The verification-loading test gave the same result as the initial loading test for subject 1 as shown in figure 5.1. Subject 1 was thus assumed to be a heterozygote or at least a homozygote of a mutation that affects the function of the FMO3 enzyme marginally. In the verification-loading test, the maximum effect of the FMO3 enzyme malfunction was projected overtly in the first hour after drinking the juice containing 600 mg TMA. The type of mutation causing the enzyme incapacity could only be confirmed after genetic screening has been completed.

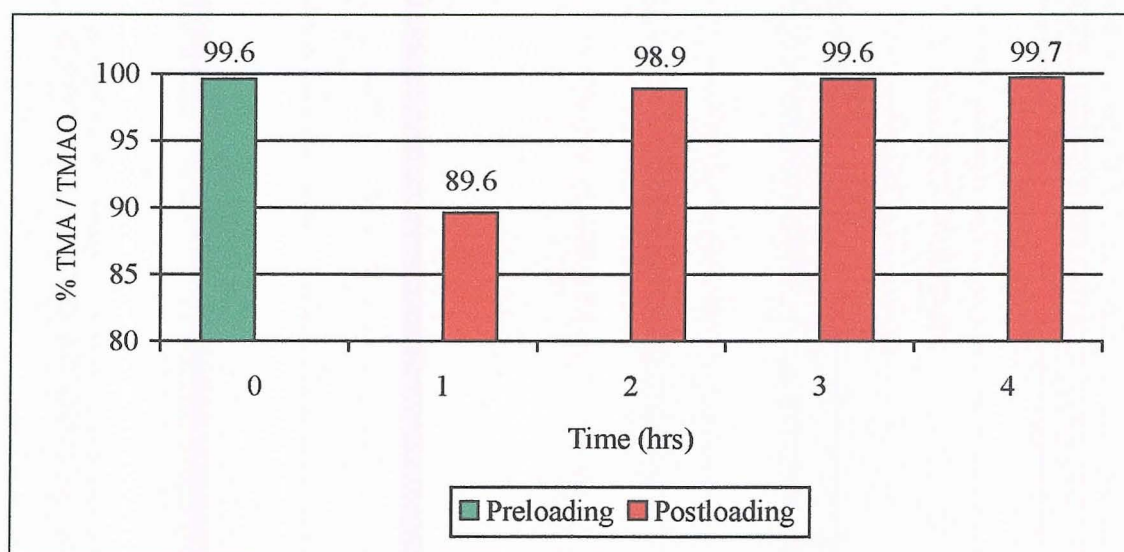


Fig 5.1: The TMA and TMAO ratios versus time of a suspected heterozygote (Subject 1). Preloading test represents the urine sample before oral administration of the adulterated juice. Postloading test represents the urine sample after the oral dose. The values above each bar show the percent conversion of TMA to TMAO.

The second suspected trimethylaminuric volunteer (subject 3) got sick after the loading test and could not collect the urine sample for further analysis. Symptoms ranging from excessive sweating, vomiting, minor rash and high protein concentration in the urine were observed. Vomiting has been noted to be induced by toxicity resulting from high concentrations of trimethylamine in pigs (Christensen, 1999).

Vomiting due to excess amounts of TMA has never been observed in humans before because it is unethical to deliberately give humans toxic amounts or exceed the prescribed dose of any chemical for the purpose of observing the chemical's toxicity symptoms. It is worth noting that this phenomenon of vomiting and related symptoms which was noted in subject 3 is apparently a direct consequence of toxic amounts of TMA in the human body and was accidentally induced in the subject involved. It is thus possible that the vomiting could be a result of the malfunctioning FMO3 enzyme hence, 600 mg of trimethylaminuria could have toxic effects on such an individual. Yet, the vomiting phenomenon can only be confirmed to be due to mild trimethylaminuria only after genetic screening has been completed.

Subject 3 had also taken a meal containing broccoli the previous day before the loading test. Broccoli is known to contain FMO3 enzyme inhibiting chemical compound known as goitrin (Shelley and Shelley, 1984). This could have had a major contribution in the violent reaction the subject showed after the loading test. Since the subject had, by virtue of the A52T mutation (shown in section 5.2), limited capacity to metabolise 600 mg of TMA, the presence of an inhibitor of the FMO3 enzyme could have prolonged the time taken to metabolise TMA by the liver. Consequently, the extended period resulting in accumulation of TMA in the blood stream hence the toxic effects. This raises an important question in the ethical context, whether continuous use of TMA as a loading probe should be allowed if it can possibly induce toxic effects in volunteers that have specific mutations. Further studies with regard to the pharmacology of TMA and its toxic effects in humans would have to be conducted before this question can be answered unambiguously.

5.2. PCR-RFLP

Mutations M66I, P153L, E305X and R492W are known to cause severe trimethylaminuria as a result of complete inactivation of the FMO3 enzyme (Sasche *et al.*, 1999). Since none of the volunteers showed symptoms of severe trimethylaminuria before and after the loading test, homozygous presence (presence of a mutation in both alleles of the gene) of these specific mutations was not expected. Heterozygous presentation (presence of a mutation in a single allele of the gene) was a possibility yet was proven absent.

Besides several silent mutations, four single nucleotide polymorphisms coding for amino acid exchanges namely, E158K, V257M, E308G (Sasche *et al.*, 1999) and A52T (Akerman *et al.*, 1999a) have been shown to cause only mild trimethylaminuria. The expected patterns of the mutant and normal fragment are shown in table 5.2.

Table 5.2: Common mutations' restriction fragment patterns in the FMO3 gene.

Mutation	Exon	Restriction enzyme	Normal (bp)	Mutant (bp)
A52T (Akerman <i>et al.</i> , 1999b)	3	<i>Nhe</i> I	229 80	309
P153L (Sasche <i>et al.</i> , 1999)	4	<i>Bam</i> HI	247 27	274
E158K (Sasche <i>et al.</i> , 1999)	4	<i>Hinf</i> I	227 47	274
E305X (Akerman <i>et al.</i> , 1999b)	7	<i>Eco</i> RI	365 164	529
E314X (Akerman <i>et al.</i> , 1999b)	7	<i>Aci</i> I	409 122	531
R387L (Akerman <i>et al.</i> , 1999b)	7	<i>Mse</i> I	315 112 102	315 102 88 24

Mutation E158K, V257M, E308G and A52T were the most likely to be present in subject 1 and 3 either as homozygous or heterozygous mutations since both subjects presented with mild trimethylaminuria symptoms after the loading test. However, of the screened mild TMAuria causing mutations, only A52T (Table 5.2) was apparently found in both subjects. Subject 1 presented with regular headaches, more than twice a week even before the loading test. It is difficult to tell if the headaches are the result of this mutation since FMO3 is suspected to be involved in biogenic amine metabolism or simply a coincidence. The headaches are also more intense towards her menstruation period. She has never noticed or had from family members of any unpleasant odour originating from her breath, sweat or urine. Yet, trimethylaminuria symptoms are known to be exacerbated during or towards menstruation.

Subject 3 is a healthy male that participates in athletics and rugby. He has never complained of any odour problems and none of his family members have either. Besides from 'athletic asthma', he does not suffer from any chronic disease.

It is not yet clear if this mutation was the only one responsible for all the symptoms displayed by subject 1 and subject 3. It is too early to say if mutation A52T is predominant in the South African population since the number of samples screened are statistically insignificant, but it is worth noting that these two unrelated subjects have both presented with the same mutation. It would be necessary to perform nucleotide sequencing of exon 3 before one can conclusively confirm the A52T mutation without any ambiguity. Since this study is a pilot study towards a bigger study that would involve all family members of the suspected heterozygotes, more exhaustive methods such as nucleotide sequencing will be conducted in the subsequent study.

Figure 5.2 and 5.3 show the fragmentation pattern of exon 3 when digested with *Nhe* I. In subject 1 and 3, the fragmentation show the following fragment sizes: 309bp, 229bp and 100bp and 80bp. The 309bp is the unrestricted amplified exon 3 while the 100bp fragment is most probably the result of non-covalent aggregation of primers. The 229bp and the 80bp fragments arise from the digestion of exon 3 by *Nhe* I endonuclease.

The presence of an unrestricted fragment as well as the presence of restriction fragments (229bp and 80bp) signals the presence of a single mutant allele. The same result was obtained for both subject 1 and 3 (shown as insert in figure 5.2) in two different PCR products and this confirms the presence of the A52T mutation as the possibility of PCR contamination is eliminated.

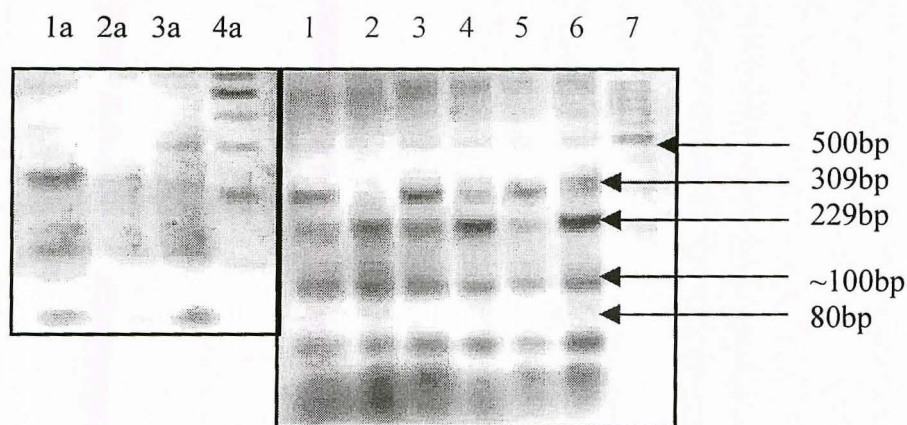


Fig 5.2: **The fragment pattern of exon 3 after digestion with *Nhe* I.** Lane 2 (subject 2), lane 4 (subject 1), lane 6 (subject 3) and lane 7 shows the 100bp ladder with the brightest band representing the 500bp fragment. Lanes 1, 3 and 5 in the main picture are not part of the present discussion. Lanes 1a, 2a and 3a (picture insert) are a different PCR exon 3 product digested with *Nhe* I of subject 2, 1 and 3, respectively. The bands in lane 1a and 2a are 309bp, 229bp, ~100bp and 80bp, respectively. Lane 3a has similar bands to 1a and 2a plus a 309bp band. Lane 4a represents the 100bp ladder with the brightest band being the 500bp. The 80bp fragment is not very clear in both pictures due to the scanning resolution power but was much more visible in the original photograph.

All known mutations and/or polymorphisms and their metabolic effects are listed in Appendix V. Subject 2 did not show any signs of a mutant allele as compared to her sister (subject 1). There is absence of fragment 309bp in lane 2 in figure 5.2. This absence shows that the A52T mutation is absent in subject 2's exon 3. Fragment 309bp in lane 4 & 6 shows the presence of an uncut exon 3 PCR product and this means there is an A52T mutation in both subjects, respectively.

Before nucleotide sequencing has been performed on the exon 3 fragments of subject 1 and 3 one cannot conclusively confirm the presence of the A52T mutation. This is due to the fact that one cannot completely rule out the possibility of incomplete endonuclease digestion even though the rest of the samples were completely digested in similar experimental conditions. Yet, the clear presence of the 309bp fragment in the second exon 3 PCR product of subject 3 can be considered to be in favour of the presence of a single allele of the mutant A52T mutation.

Mutation P153L and E158K were also tested for as shown in table 5.2 yet both mutations proved absent. The results were also performed in duplicate to assure validity of the restriction fragment profile. However, results for P153L and E158K are not shown in this chapter. The restriction patterns of exon 7 in all the three subjects for different mutations were identical in all tests performed. Since most mutations tend to cluster at exon 7, it was critical to target exon 7 for most of the tests performed in this study. The fact that all tests results were identical meant that there was no E305X mutation in any of the three subjects exon 7 samples. The absence of the E305X mutation was expected since its presence would have resulted in severe trimethylaminuria yet none of the subjects had shown such signs before or after the loading test. The fragments, 365bp and 164bp shown in figure 5.3 clearly confirms the absence of the E305X mutation. This result was confirmed in two independent PCR products of exon 7 from the same subjects.

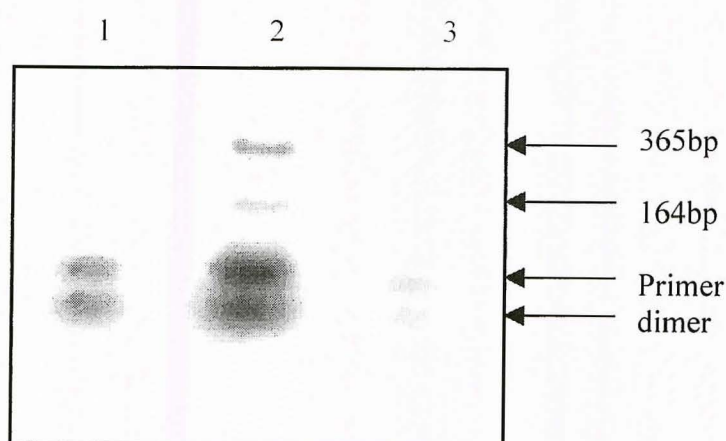


Fig 5.3: The fragmentation pattern of exon 7 after digestion with EcoRI.

Lane 1, 2 & 3 represent subject 1, 2 and 3. The 100bp marker was used to verify the sizes of the fragments but the lane is not shown in the picture.

Mutation E314X and R387L are both severe trimethylaminuria-causing mutations and their presence would not be expected to be present in any of the three subjects shown in table 4.1. This is due to the fact that subject 1 and 3 had presented with symptoms of mild trimethylaminuria after the loading test. The screening for all mild trimethylaminuria-causing mutations was even more relevant since the subjects had shown signs of mild trimethylaminuria.

The absence of the E314X and R387L mutations were confirmed in two separate PCR amplification products. In both sets of RFLP results there were clear fragments of sizes 409bp and 122bp for E314X. The exon 7 PCR fragment has more restriction sites for *Mse* I and when digested with this particular enzyme two characteristic fragments are produced in the absence of the R387L mutation. The characteristic fragments for mutation R387L absence are 315bp, 112bp and 102bp.

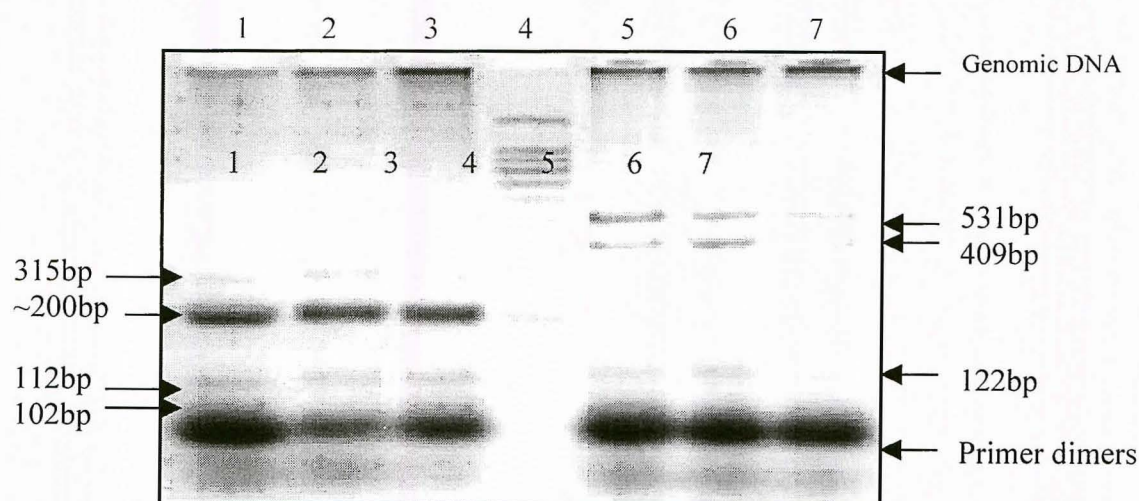


Fig 5.4: The fragmentation pattern of exon 7 after digestion with *Aci* I and *Mse* I. lane 1, 2 and 3 show restriction enzyme profile of exon 7 of subject 1, 2 and 3, respectively with *Mse* I. Lane 5, 6 and 7 show partial digestion of subject 1, 2 and 3 exon 7 with *Aci* I. Lane 4 shows the 100bp ladder with fragments ranging 100bp to 1100bp.

The presence of the ~200bp in the *Mse* I profile is apparently the non-covalent aggregation of the 112bp and the 102bp. The 102bp is not clearly depicted as it overlaps with the primer dimers. The fragmentation patterns shown after digestion with *Mse* I and *Aci* I are as expected in the absence of both mutations, respectively as described in table 5.2. The presence of a 531bp fragment in the E314X RFLP profile in figure 5.4 was deliberately induced for the purpose of validating the SSCP method as shown in section 5.3, yet in a separate result (not shown) the complete digestion of the fragment was evident.

5.3. PCR-SSCP/HA

In clinical diagnostic laboratories, scanning of large genes for private mutations is the rate-limiting step. Complete sequencing of large genes cannot be a routine procedure in clinical laboratories, not only because it is time-consuming, costly and tedious, but mainly because mutations can be missed (Claustres, 2001). The alternative approach is to use scanning methods that allow rapid analysis of exons and intron boundaries, and then use limited sequencing to confirm and identify the mutation in the fragment.

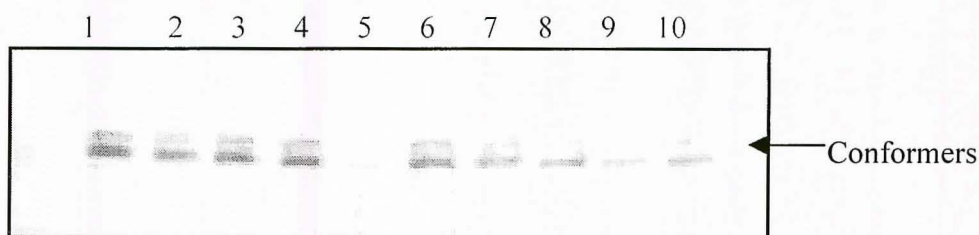


Fig 5.5: The single-strand conformation polymorphism profile of exon 2 and 4. Lane 1, 2 and 3 represent exon 2 of subject 1, 2 and 3, respectively. Lane 4 represents exon 2 of a control subject. Lane 6, 7 and 8 represent exon 4 of subject 1, 2 and 3. Lane 9 and 10 are exon 4 of control subjects.

One of the purposes of this study was to validate the application of SSCP and heteroduplex analysis for the purpose of screening mutations in the FMO3 gene. For the purposes of this objective only, the SSCP and heteroduplex analysis were successful as all the expected fragments could be identified in specific profiles such as in figure 5.5 and figure 5.6.

For the purposes of screening mutations, the SSCP method was only partially successful since not all possible optimisation environments were applied towards the analysis of the DNA fragments. The SSCP results in all three subjects screened showed no difference in their exon nucleotide sequence conformation pattern as shown by their similar mobility in the polyacrylamide gel.

This result does not necessarily mean that there are no differences in the nucleotide sequences of the three subjects. A more sensitive mutation detection method (e.g. DGGE or direct sequencing) would need to be employed or more SSCP runs at different gel conditions have to be performed before one can assume that there are no mutations in the exons screened for.

Yet, the presence of expected bands means that the SSCP technique is applicable for the screening of the FMO3 gene. However, the lack of absolute confirmation of the absence of mutation remains one of the short falls of this technique.

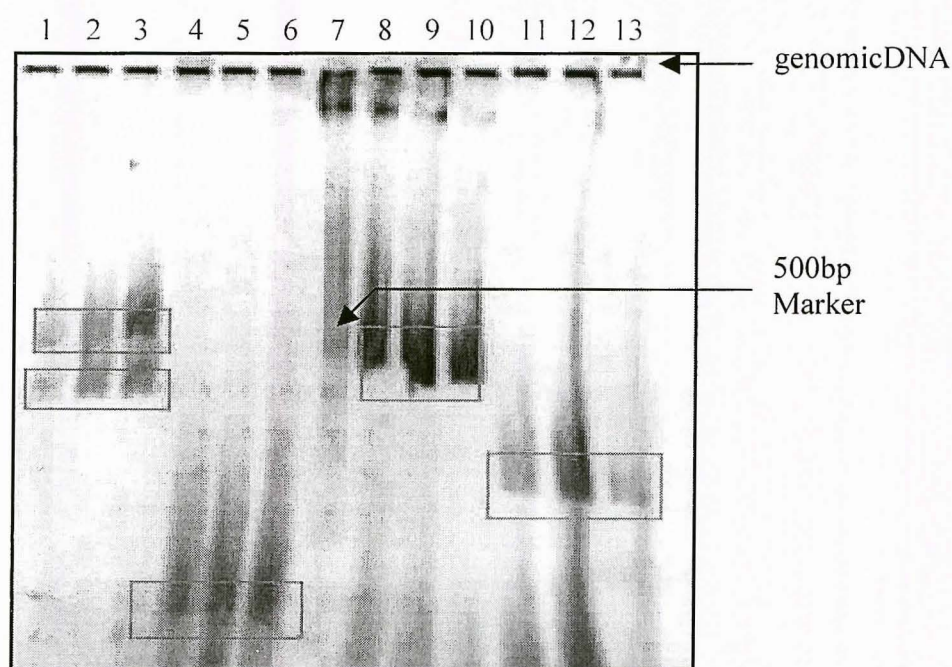


Fig. 5.6: The PCR-RFLP-HA profile of exon 2, 4 and 7. Lane 1, 2 and 3 represent exon 7 of subject 1, 2 and 3, respectively restricted with *Aci* I. The two fragments of double stranded DNA in purple box are 529bp and 409bp respectively. Lane 4, 5 and 6 represent exon 2 (266bp) of subject 1, 2, and 3. Lane 7 shows the 100bp ladder which was much more visible in the original photo. Lane 7, 8 and 9 represent exon 6 (402bp) of subject 1, 2 and 3, respectively. Lane 11, 12 and 13 represent exon 4 (274bp) of subject 1, 2 and 3 respectively.

There was no evidence for differences in the nucleotide sequences of exon 2, 4, 6 and 7 of subject 1, 2 and 3 in comparison to each other as shown in the HA profile in figure 5.5. In the restricted exon 7 HA profile, the two double stranded DNA fragments can be spotted in figure 5.6 lane 1 – 3.

A much clearer picture is depicted when using a more sensitive camera (photograph not shown) such as the gel documentation system from Bio-Rad. An enlarged version of the partially digested fragment of exon 7 with *Aci* I to produce a profile similar to that of a mutation present in one allele only is depicted in figure 5.7.

In this figure, three distinct bands can easily be identified and the fourth band is probably embedded with the bottom broader band. The four bands are a result of partial digestion with *Aci* I as shown in figure 5.4. All subjects did not show presence of the E314X mutation when digested with *Aci* I, yet partial digestion was induced to validate the SSCP technique coupled with RFLP for detection of heterozygote subjects. This presents a perfect example of the application of PCR-RFLP-SSCP technique in screening for possible mutations in a known nucleotide sequence. The presence of all the expected fragments and strands shows that PCR-RFLP-SSCP combination can be used for the effective screening of mutations in the FMO3 gene. Although no mutation was observed up using this technique, performing these tests at different conditions would most probably increase the sensitivity to close to 100% mutation detection.

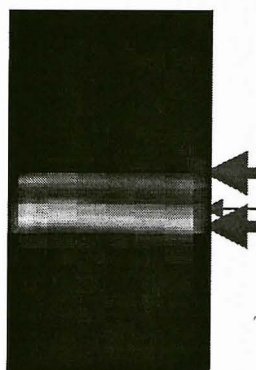


Fig. 5.7: The SSCP profile of a heterozygous mutation in exon 7. The arrows point to the three distinct fragments produced by partial digestion of exon 7 with endonuclease *Aci* I.

5.4. Denaturing gradient gel electrophoresis

5.4.1. Computer simulation

The figures 5.8 through to 5.13 illustrate the melting profile of each exon with or without an attached GC-clamp, respectively. Each profile exhibits different melting domains as shown by differences in temperature as compared to each other. The number of melting domains as well as the temperature range differences determines the amount of resolution with regards to percent mutation detection. All the graphs were plotted using the Bio-Rad MacMelt™ programme. The reproducibility and resolution of mutation detection using the DGGE results depend mainly on the introduction of at least one high melting domain.

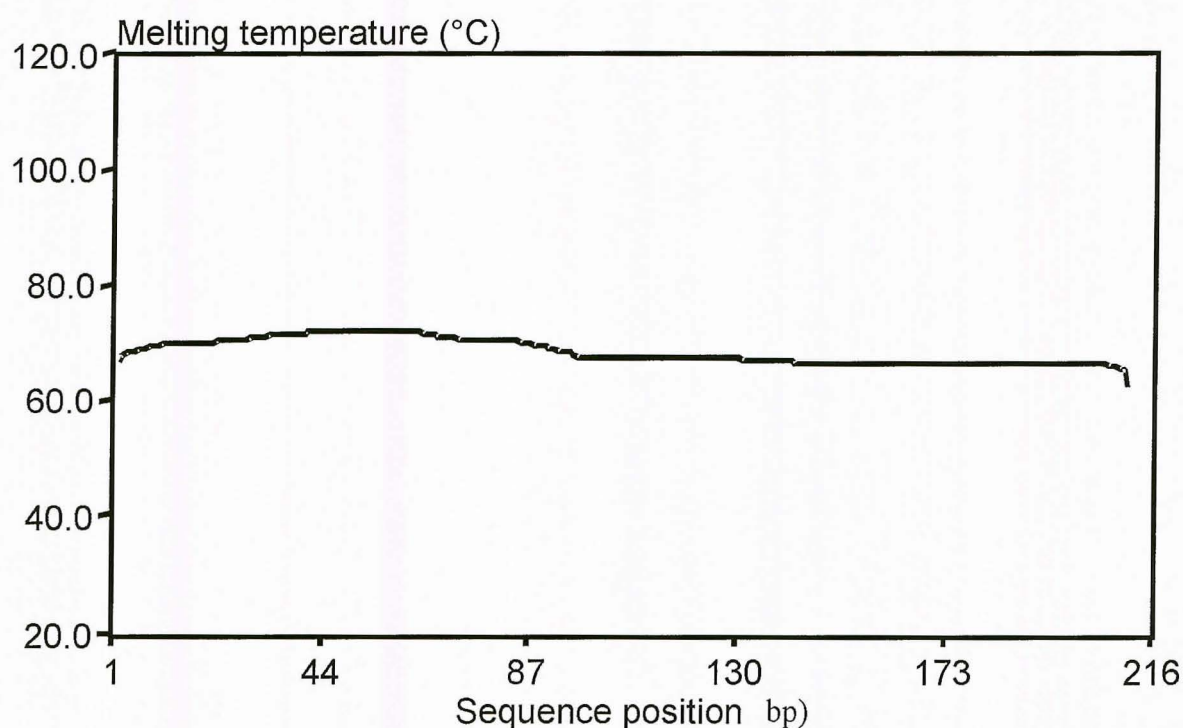


Fig. 5.8: The MacMelt™ melting profile of FMO3 exon 3 without a GC-clamp. The length of the nucleotide fragment is 212bp.

The melting profile of exon 3 is relatively linear. Without at least one high melting domain, the fragment will completely denature when subjected to denaturing conditions applicable for DGGE or TTGE. The introduction of a GC-clamp would aid in rendering the fragment incomplete denaturation. The melting profile of FMO3 Exon 3 with a GC-clamp attached on the 5'-end primer and on the 3'-end primer is shown in figure 5.9.

For the 3'-end primer: Two melting domains have been introduced, namely, the 67°C domain (nucleotide 1 – 205) and the GC-clamp domain (nucleotide 206 – 256). This profile is the best possible compared to the introduction of a GC-clamp at the 5'-end primer. The GC-clamp on the 3'-end provides a much smoother curve compared to the 5'-end clamped primer. The two melting domains created by the 5'-end clamped primer reduce the sensitivity of the DGGE method in comparison with its 3'-end counterpart.

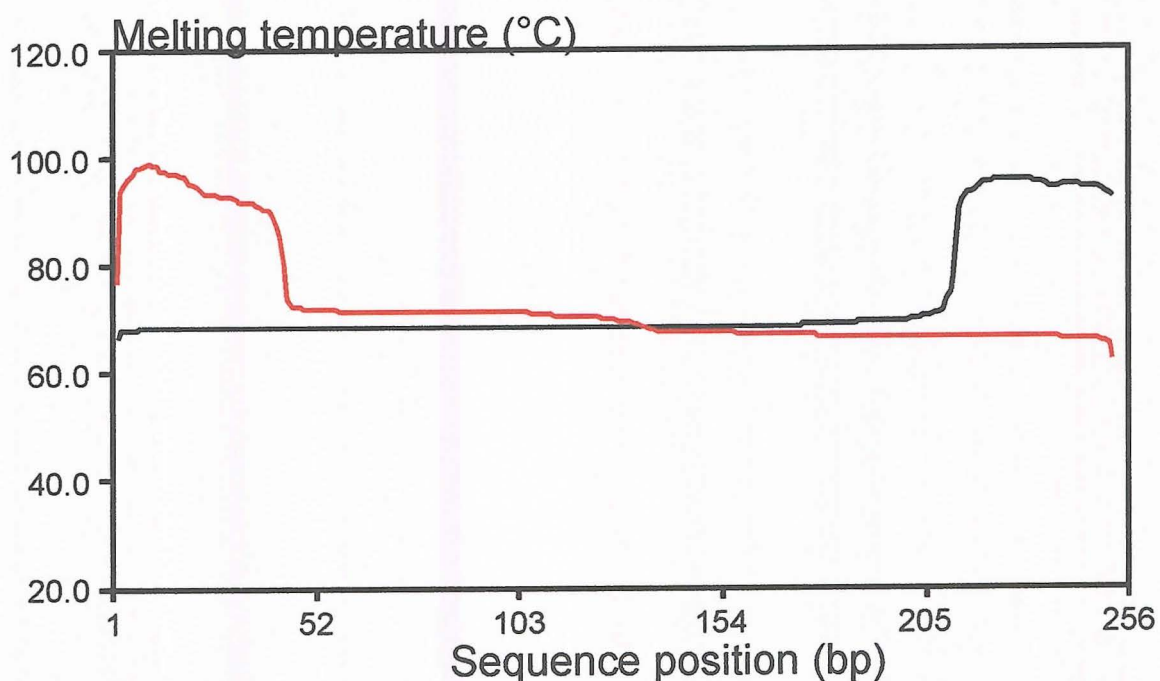


Fig. 5.9: The MacMelt™ melting profile of FMO3 exon 3 with a GC-clamp. The red curve represents exon 3 with the GC-clamp at the 5'-end. The black curve shows exon 3 with the GC-clamp appended at the 3'-end. The length of the nucleotide fragment is 252bp.

The exon 4 melting profile has no high melting point domain as shown in figure 5.10. It has two relatively linear domains yet the temperature difference between the two domains is not large enough to prevent complete strand dissociation in DGGE or TTGE denaturation environments. Any attempt to perform the DGGE technique to detect nucleotide sequence differences on such a fragment would be unsuccessful. The failure to resolve any sequence variations by the DGGE technique would be enforced by complete dissociation or denaturation of the double strand.

The MacMelt™ melting profile of FMO3 Exon 4 without a GC-clamp is as depicted below:

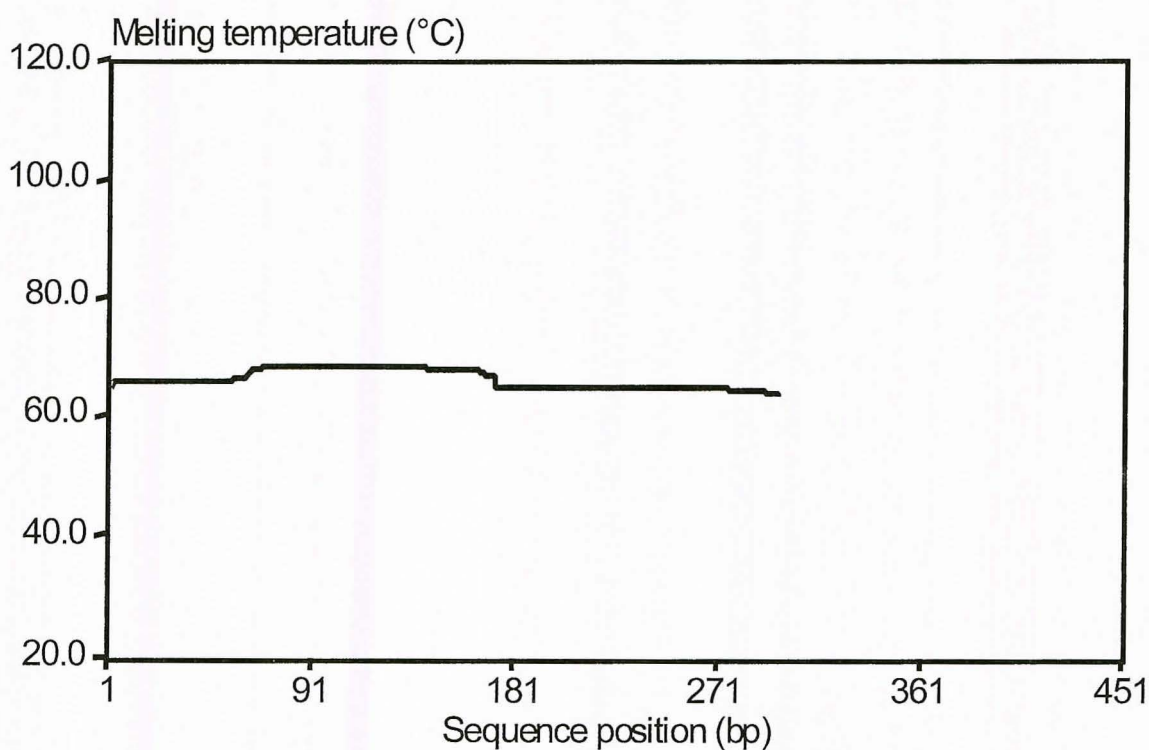


Fig. 5.10: The MacMelt™ melting profile of FMO3 exon 4 without a GC-clamp. The nucleotide fragment size is 274bp.

The two opposite attachment sites, namely, the 3'-end and the 5'-end both produce relatively linear profiles. The difference in melting temperature between the two domains produced are within the DGGE working limits, that is, not more than 10°C. The 3'-end attachment seems to be the better choice of the two sites because it smoothes down the two domains to approximately the same temperature range whereas the 5'-end clamp retains two melting domains that differ by a wider temperature margin. The 5'-end primer also presents a low temperature domain that would be difficult to resolve due to its irregular temperature profile.

The figure below shows the Macmelt™ profile of FMO3 exon 4 with an attached GC-clamp:

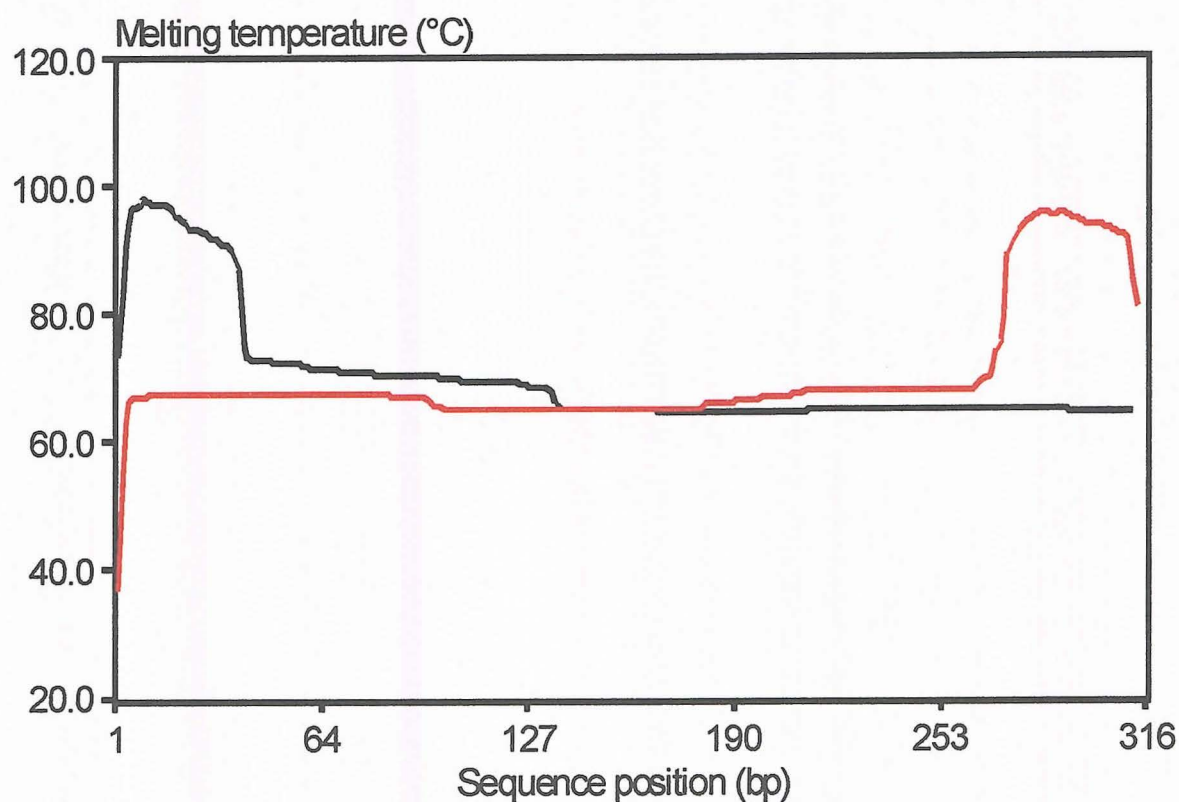


Fig 5.11: The MacMelt™ melting profile of FMO3 exon 4 with a GC-clamp. The red curve shows the 3'-end GC-clamped fragment whereas the black curve shows the 5'-end GC-clamped fragment. The fragment size is 314bp.

Exon 7 produced a high melting domain in the centre of the nucleotide sequence. Apart from the high melting domain, there are other melting domains that are awkward to characterise as they differ by wide temperature ranges. The DGGE technique would not be able to fully resolve the sequence differences in this case due to many different domains present. It will also prove difficult to smooth out these varying temperature ranges with a GC-clamp. This will thus translate to a reduced sensitivity especially in regions with much higher melting temperatures. The vast melting temperature differences in exon 7 poses the major challenge with regards to levelling out the melting domains to a similar temperature. The MacMelt™ melting profile of FMO3 Exon 7 with and without a GC-clamp is shown in figure 5.12 below.

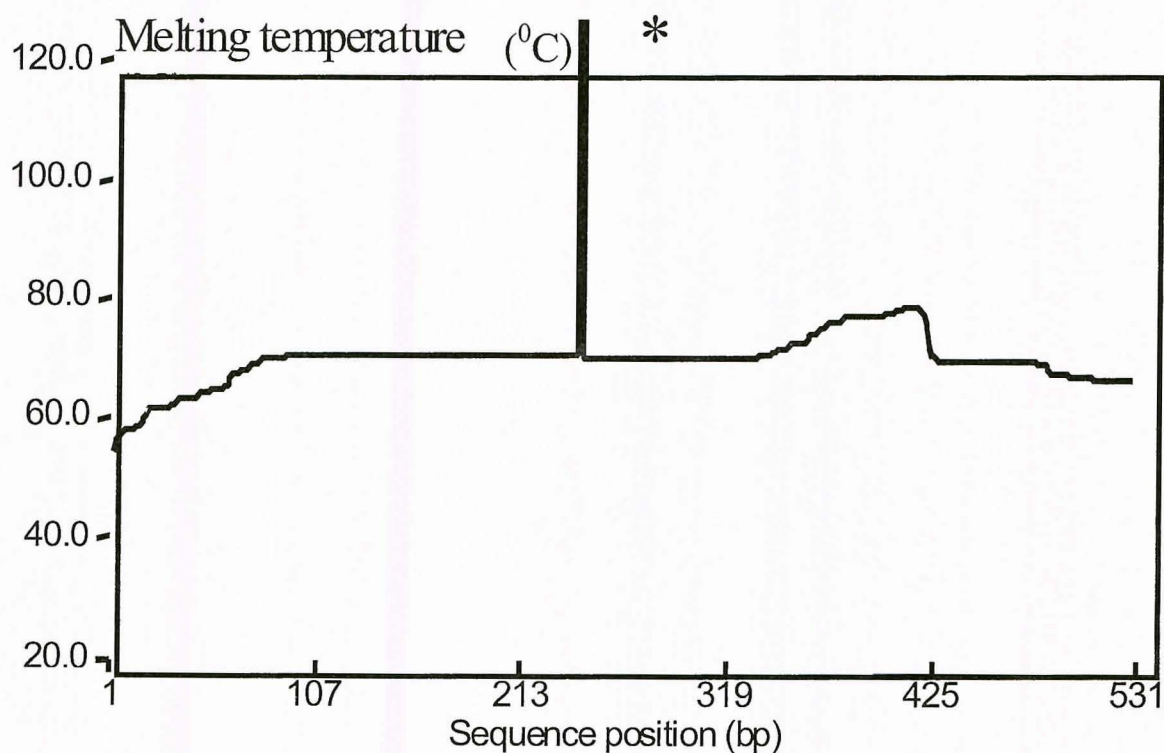


Fig 5.12: The MacMelt™ melting profile of FMO3 exon 7 without a GC-clamp. The nucleotide fragment size is 529bp. * represents the highest melting domain, which is 140°C.

It is conceivable that although the GC-clamps normalise the melting domains to a somewhat workable region, the sensitivity of the DGGE technique would be severely reduced. The only consolation is that at least some mutations can be resolved, rather than no detection at all. Although the two curves have more than two domains each, the 5'-end clamped primer curve is the best option to use the DGGE mutation detection system on. Its melting domains are numerous yet the temperature ranges are close in comparison to each other in contrast to the 3'-end GC-clamped primer fragment domains. The best option would be to cut this large exon into two and analyse each fragment separately. The MacMelt™ melting profile of a GC-clamped exon 7 is shown in figure 5.13

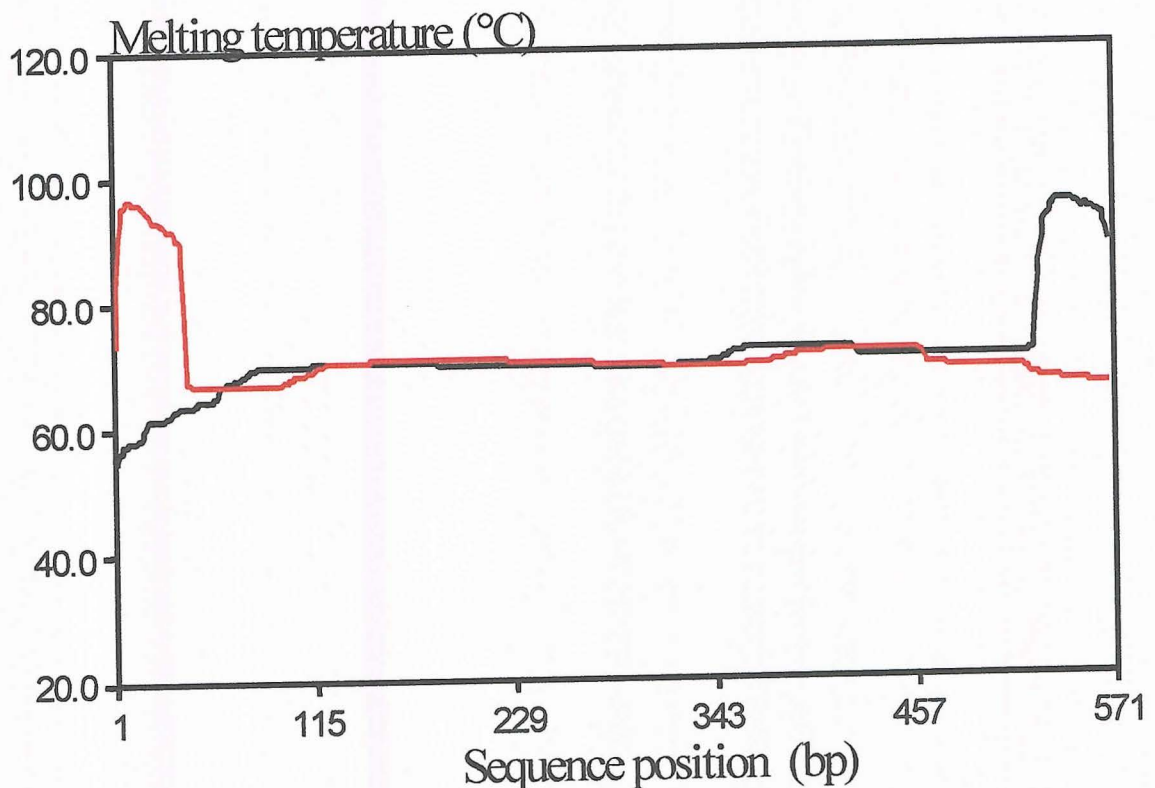


Fig 5.13: The MacMelt™ melting profile of FMO3 exon 7 with a GC-clamp. The red curve shows the 5'-end GC-clamped fragment whereas the black curve shows the 3'-end GC-clamped fragment. The size of the fragment is 569bp.

5.4.2. Exon amplification

For exon 3, 4 and 7, none of the amplification produced the desired products when the primers shown in table 4.6 were used. Trouble shooting using some of the techniques described in Roche PCR applications manual (Steffen, 1999) showed that there was nothing wrong with the concentrations of ingredients necessary for amplification. The main culprit to the problem leading to failed fragment amplification was the primer design. When genomic DNA is used for the amplification of a specific exon (e.g. exon 7), the specificity of the primer is of utmost importance. The lack of product amplification was probably due to the fact that the GC-clamp is almost twice as big as the primer itself yet its sequence is not complementary to the targeted sequence adjacent to the primer. The absence of complementarity caused by the GC-clamp reduces the specificity of the whole primer (i.e. FMO75GC2 in table 4.6) which comprises of a 40-nucleotide GC-clamp appended to the 23-nucleotide original complementary primer) to less than 40%. The reduced specificity essentially lead to less specific binding in different areas of the genomic DNA which may not necessarily be FMO3 gene. This seems to be a plausible mechanism why none of the desired product (shown in figure 5.14) was produced when PCR was performed using a primer with an appended GC-clamp. The sequence of the appended GC-clamp and the complementary primer are shown in table 4.6.

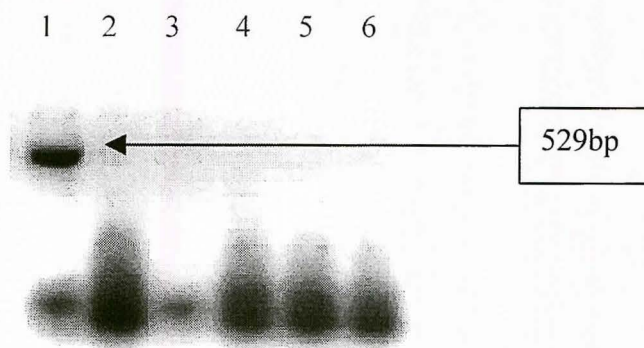


Fig 5.14: Failed PCR-GC-clamp amplification of FMO3 exon 7. Lane 1 shows an amplified exon 7 using primers FMO13 and FMO14 with no GC-clamp attachment. Lane 2 – 6 shows amplification products using primers FMO14 and FMO75GC2 (No product present).

Exon 7 was then amplified using normal primers (FMO13 & FMO14) without an attached GC-clamp. The PCR product was then separated on 2% agarose gel and cut out under long wavelength UV light to reduce the risk of introducing mutations. The excised DNA material was purified using QIAquick gel extraction kit (Qiagen) purchased via Southern Cross Biotechnology in Cape Town. The purified product was re-amplified using the GC-clamped primer (FMO75GC2) and its 3'-end counterpart (FMO14). The results were successful as can be seen in figure 5.15. The intensity in lane 2, 3, 4 and 6 proves that there was definite amplification. This can be attributed to increased specificity of the FMO75GC2 primer.

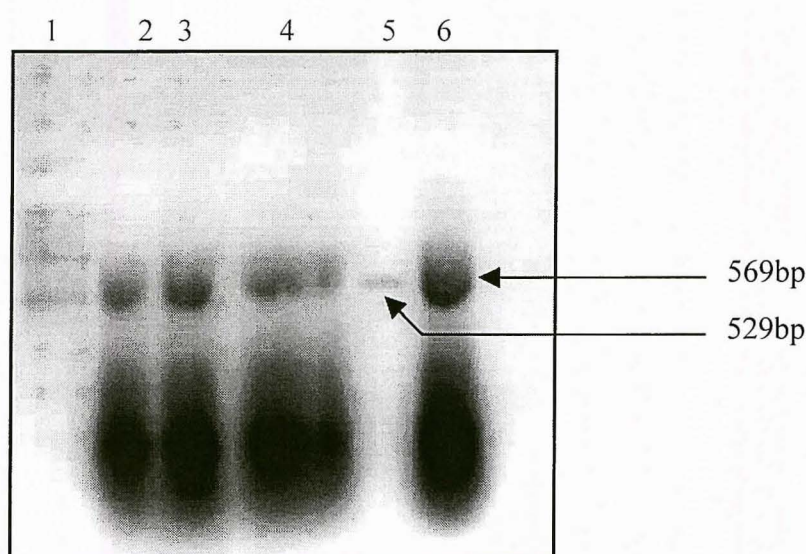


Fig 5.15: Successful PCR-GC-clamp amplification of FMO3 exon 7. Definite amplification results are shown in lane 2, 3, 4 and 6. Lane 5 shows the purified exon 7 without re-amplification. Lane 1 represents the 100bp ladder standard and the brightest fragment is the 500bp size fragment.

Exon 7 has a limited number of sequences that are complementary to the FMO75GC2 primer except for the intended position. Although the difference in size cannot easily be ascertained in figure 5.15 (40bp difference) because a high percentage agarose gel (3%) was used, a less dense ($\leq 2\%$) run for few hours would be able to resolve the size difference. Polyacrylamide gel would be an even better option to use for this purpose since it resolves bands more distinctly compared to agarose gel especially for such small size differences.

5.4.3. The gel conditions for exon 3, 4 and 7

Using the equation in section 2.3.3.5, the gel running conditions are illustrated in table 5.2. The table shows the melting domain temperature, which is the mean temperature at which the double strand of the specific domain is 50% denatured. The temperature range is the temperature at which the specific domain will start melting (minimum range temperature) and completely denature (maximum range temperature). The denaturant concentration corresponding to the minimum and maximum temperature ranges are calculated using the formula in section 2.3.3.5. The effect of the temperature and a chemical denaturant are essentially the same.

Table 5.3: **Minimum gel conditions for DGGE.**

Domain			Exon	Denaturant	Denaturant range
T _m (°C)	Range (°C)	Corrected range (°C)	No.	%	%
63	62 – 64	48 – 50	4	19	10 – 30
67	66– 68	52 – 54	4 & 7	32	20 – 40
68	67-69	53 – 55	3	35	20 – 50
70	69-73	55 – 60	7	42	30 – 60
71	69-73	55 – 60	7	45	30 – 60
72	69-73	55 – 60	7	48	30 - 60
100% denaturant = 7M urea and 40% formamide					

To ensure uniform and constant maintenance of the chosen temperature during electrophoresis, the gel is placed in an aquarium tank and submerged with electrophoresis buffer kept at the desired temperature by a combination of heater/stirrer thermostat (Bio-Rad lab Manual, 1996; Lerman *et al.*, 1984).

In summary, amplification of a GC-clamped product proved to be the major challenge in the process leading to DGGE analysis. Although it is possible to amplify a specific exon after having to amplify it twice and purify it, this method is tedious, expensive and is likely to introduce mutations in the DNA material in question due to exposure of the amplified DNA material to UV rays. For the purposes of mutation screening, it is desirable to develop a method through which there is zero chance of mutation induction through to the end of the mutation identification process.

One of the methods that can be applied for this purpose is described below:

- Synthesis of primers shown in appendix IV with a built in T4 ligase site.
- Synthesis of a universal GC-clamp with a T4 ligase site complementary to that in the primer sequences.
- Amplification of specific exons using the primers with a built-in T4 ligase site.
- Ligation of amplified PCR products with the GC-clamp.

These four steps will essentially produce the required GC-clamped product without exposure to UV light. This method is yet to be successfully performed.