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# *NF2* MUTATION SCREENING BY DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND HIGH RESOLUTION MELTING ANALYSIS

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**RUNNING TITLE:** DHPLC and HRM analysis in *NF2* gene

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**ABSTRACT**

Neurofibromatosis type 2 (NF2) is an autosomal dominant disorder caused by mutations in the *NF2* gene and predisposing to the development of nervous system. Identification of germline mutations is essential to provide appropriate genetic counselling in NF2 patients, but it represents an extremely challenging task, as the vast majority of mutations are unique and spread over the entire coding sequence. Moreover, about 30% of *"de novo"* patients are indeed mosaic and direct sequencing can undetect mutated alleles present in a minority of cells. As most screening techniques do not meet the requirements for efficient NF2 testing, we have developed a semi-automated denaturing high-performance liquid chromatography (DHPLC) method for point mutation detection combined with a MLPA approach to screen for gene rearrangements. In addition, we have evaluated high-resolution melting analysis (HRMA) as a exon scanning procedure to identify point mutations in the *NF2* gene.

The results obtained in 92 NF2 patients expand the *NF2* mutational spectrum and indicate DHPLC and HRMA as good systems to screen for point mutations in diseases with a heterogeneous spectrum of alterations.

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## INTRODUCTION

Neurofibromatosis type 2 (NF2; MIM 101000) is an autosomal dominant cancer syndrome that predisposes to the development of nervous system tumours (Evans et al., 1992). Bilateral vestibular schwannomas are pathognomonic of the disorder; furthermore, schwannomas at other locations, meningiomas, ependymomas and juvenile posterior subcapsular lenticular opacities are often associated with the disease (Evans et al., 1992; Parry et al., 1994; Mautner et al., 1996). About half of NF2 patients have no family history of the disease, representing *de novo* mutations.

The disease is caused by mutations in the *NF2* tumour-suppressor gene, located in 22q12 (Trofatter et al., 1993; Rouleau et al., 1993). Since its identification, different germline mutations have been found in up to 91% of NF2 patients with a positive family history using exon-scanning-based methods and Multiplex Ligation-dependant Probe Amplification (MLPA) to detect genomic rearrangements (Kluwe et al., 2005; Evans et al., 2007). Somatic mosaicism has been found in 25-33% of *"de novo"* NF2 patients (Kluwe et al., 2003; Moyhuddin et al., 2003; Evans et al., 2007). Somatic mosaicism may be even more common in sporadic NF2 patients with mild phenotypes, resulting from small proportions of cells harbouring mutant *NF2* alleles: it has been estimated that 60% of sporadic cases with unilateral vestibular schwannoma at presentation might indeed be mosaics (Evans et al., 2007).

Since the vast majority of mutations spread over the entire coding sequence, analysis of the *NF2* gene has been commonly carried out with PCR-SSCP as pre-screening method, followed by DNA sequence analysis of amplicons that show aberrant band shift. Although this strategy is effective; however, turnaround time is relatively long and the limited sensitivity of SSCP does not allow detection of all point mutations. The use of direct sequencing as diagnostic tool is reserved to familial NF2 patients,

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since it has been shown that this approach does not have a 100% sensitivity in mosaic patients, especially in cases with low proportion of mutated cells (Jones et al., 2001).

To overcome problems deriving from the limited sensitivity of SSCP and other screening techniques, denaturing high performance liquid chromatography (DHPLC) (Underhill et al., 1997) was developed as a suitable mutation scanning method in a large number of single-locus disease genes (Liu et al., 1998; Wu et al., 2001; Marsh et al., 2001; Hegde et al., 2005). DHPLC is a semi-automatic method, that entails limited post-PCR manipulation; it requires heteroduplex formation and need an accurate optimization of running setting prior to chromatography in order to predict conditions for each PCR product. The method allows identification of sequence variants through characteristic peak patterns and shows many advantages including high sensitivity, specificity and throughput. However, DHPLC requires the avalilability of dedicated and expensive instrumentation.

Recently, high-resolution melting analysis (HRMA) has been described as a new and highly sensitive mutation scanning method to identify point mutations in an amplicon without the need for gel or column separation or labeled oligonucleotides (Wittwer 2003**;** Liew et al., 2004; Reed et al., 2007). In this technique PCR is performed in the presence of saturating concentrations of double-stranded DNA binding dyes and then amplicons are submitted to high resolution melting analysis monitoring the fluorescence across a defined temperature range (Reed and Wittwer 2004). Data acquisition generates a change in melting curve shape (Graham et al., 2005) that can be used to identify the presence of sequence variation within the amplicon. HRMA has been shown to have near 100% analytical sensitivity and specificity when used on products up to 400 base pairs in length (Chou et al., 2005; Margraf et al., 2006; Krypuy et al., 2006). HRMA was already used to identified germline and somatic point mutations (Kemerson et al., 2007; Margraf et al., 2007; Pal et al., 2007; Dobrowolski et al., 2007; Krypuy et al., 2007; Takano et al., 2007).

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In the present study, we evaluated DHPLC and HRMA-based approach for point mutation scanning of *NF2* gene.

## MATERIALS AND METHODS

### Patients and DNA samples

In the present study DHPLC and HRMA were used to evaluate 92 patients from 92 unrelated Italian probands who had been diagnosed with NF2 based on the presence of bilateral vestibular schwannomas. Sixteen NF2 patients in whom a pathogenic mutation had been identified by PCR-SSCP and characterized by cycle sequencing, were used as positive controls for DHPLC and HRMA (Table 1). The second group included 76 cases sporadic patients in which PCR-SSCP and Multiplex Ligation-dependant Probe Amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands), did not reveal single point mutations or genomic rearrangements in *NF2* gene. High molecular weight genomic DNA was extracted by standard phenol/chloroform method from blood samples collected from patients, following provision of informed consent.

### Primers and PCR Conditions

Mutation scanning of *NF2* gene requires the analysis of 15 exons, including intron /exon junctions. Genomic DNA was amplified using the intronic primer sets reported in Table 2. For DHPLC analysis, PCR reactions were performed in 30 µl, containing 1X PCR buffer (Applied Biosystems, Milan, Italy), 200 mM of each dNTP, 2mM $MgCl_2$, specific primers, 0.75 U TaqGold DNA polymerase (Applied Biosystems) and 50 ng of genomic DNA.

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**DHPLC Analysis**

DHPLC was performed using the WAVEs DNA Fragment Analysis System (Transgenomic, San Jose, CA). The PCR products were denatured at 95°C for 3 min, followed by gradual reannealing for 30 min with a temperature ramp of 11°C/min to optimize the formation of heteroduplexes and homoduplexes. Five µl were applied to a preheated C18 reversed-phase column of nonporous poly(styrene-divinyl-benzene) particles (DNA-Sep; Transgenomic). DNA was eluted within 2.5 min at a flow rate of 0.9 ml/min using a linear acetonitrile gradient from 52% to 67% buffer B (0.1M triethylammonium acetate [TEAA]; 25% acetonitrile) at the appropriate denaturing temperature. The temperature for heteroduplex detection was determined using WAVEMAKER software (Transgenomic), based on the melting profiles of the 15 *NF2* amplicons (Table 2). The predicted temperature was optimized by testing specimens of wild-type DNA and different positive controls. Regeneration of the column was achieved by washing with 100% buffer B for 30 sec followed by an equilibration time of 2 min. DNA elution was observed online at 260 nm.

**High-Resolution Melting Analysis**

DNA samples were amplified with the same primers and PCR conditions used for DHPLC analysis. For HRMA, PCR was performed in 20 µL with 50 µM Syto9® (Invitrogen Corp., Carlsbad, CA) as intercalating dye. Amplified products were denaturated at 95°C for 1 min and then rapidly cooled to 40°C for 1 min in order to facilitate heteroduplex formation. HRMA was then performed on a Rotor Gene™ 6000 Instrument (Corbett Research, Sydney, Australia). Melt curve data for each PCR product were acquired in a wide temperature range (75°C to 95°C), at a ramping rate of 0.1°C/sec. Results were analyzed as fluorescence versus temperature graphs as previously described (Krypuy M. et al., 2006).

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**DNA sequencing**

All cases that exhibited an abnormal melting curve at HRMA and/or aberrant elution profile on DHPLC chromatograms, were subjected to sequence analysis. PCR products were purified using a QIAquick PCR Purification kit (Qiagen Inc., Germany) and then subjected to cycle sequencing in both forward and reverse directions using a BigDye Terminator Cycle Sequencing version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. The products from each reaction were analyzed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Milan, Italy).

Mutation nomenclature follows the Human Genome Variation Society (URL: http://www.hgvs.org/mutnomen/) recommendations. The DNA mutation numbering is based on the *NF2* cDNA sequences (GenBank accession number NM_181832.1) with the A of the ATG translation-initiation codon numbered as + 1. Amino acid numbering starts with the translation initiator methionine as +1.

## RESULTS

Analytical sensitivity and specificity of HRMA were firstly validated on a series of 16 DNA samples with previously identified NF2 mutations, detected by PCR-SSCP and characterized by cycle sequencing. Exons harbouring these mutations were specifically targeted for screening by DHPLC and HRMA. Both methods showed a 100% concordance for the identification of all DNA alterations (Table 1). All samples were distinguishable by significant differences in the melt curves and DHPLC traces of the amplified DNA samples when compared to wild-type DNA (Figure1).

To evaluate the diagnostic sensitivity of DHPLC and HRMA analyses and their ability to identify mosaicism in DNA samples, compared to direct sequencing, we performed serial dilutions of a wild-type DNA with genomic DNA deriving from non-mosaic NF2 patients carring the following mutations:

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c.997C>T (exon 10), IVS14+2T>C, IVS15+1G>C. Dilutions were made corresponding to a 1:1, 1:4, 1:10, 1:20, 1:100 and 1:1000 ratio of patient:normal DNA, respectively. In our experimental conditions, DHPLC and HRMA were able to detect the c.997C>T and IVS15+1G>C mutations until a dilution 1:100 in which the mutant allele was present at 0.25% relative to the normal allele (Figure 2) while the IVS14+2T>C mutation was detectable until 1:20 dilution (1.25% of the mutant allele). Direct sequencing detected the c.997C>T and IVS15+1G>C mutations until dilution 1:4 (6.25% of the mutant allele) and the IVS14+2T>C alteration until dilution 1:1 (25% of the mutant allele) (Figure 3). To test *"in vivo"* this capability, DHPLC and HRMA were performed in one previously described mosaic patient with an inactivating mutation in *NF2* gene (Sestini et al., 2000). In this patient, both techniques indicated an abnormal profile related to the presence of a c.1416delC mutation in exon 13 (Figure 4).

Subsequently, we analysed 76 NF2 samples for which previous mutation screening by PCR-SSCP had not revealed sequence changes. As reported in Figure 5, we identified 7 samples with abnormal DHPLC peaks and showing differences in the melt curves in HRMA. Sequencing analysis of the involved exons demonstrated in all cases the presence of 6 different point mutations. These six different mutation included two nonsense (c.592C>T, p.R198X and c.1090A>T, p.K364X involving exon 6 and exon 11 respectively), one missense (c.447G>C, p.K149N in exon 4), one frameshift (c278delCCTT in exon 3) and two splice site mutations (IVS3+3A>C and IVS7 -1G>T).

## DISCUSSION

High sensitivity in detection of DNA sequence variations is essential for mutation analysis in genetic diseases. A wide range of methodologies for exon scanning have been proposed to select samples with

aberrant profiles to be submitted to DNA sequencing, with the aim to reduce laboratory costs and analysis time.

DHPLC has been extensively applied to single nucleotide polymorphism analysis and mutational screening for different disease associated genes (Gross et al., 1999; Nickerson et al., 2000; Dobson-Stone et al., 2000; Jones et al., 2000; Jones et al., 2001). This methodology allows a rapid separation and visualization of homo- and heteroduplex DNA targets by using a reverse-phase liquid chromatography system. Maximal sensitivity in the heteroduplex detection is obtained maintaining the HPLC column at an optimal melting temperature that allows partial strand denaturation in the presence of a single base-pair mismatching.

Recently, HRMA has been proposed as an alternative scanning method to identify changes in DNA sequence using melting-curve analysis of the amplicons. This technique allows characterization of the DNA samples according to their dissociation behaviour in transition from double stranded to single stranded DNA with increasing temperature. As reported for DHPLC, also HRMA identifies heteroduplexes by their lower thermal stability (Reed et al., 2004). However, unlike DHPLC, melting analysis scans through a wide range of temperatures rather than depending on a specific temperature, a feature that normally requires time-consuming optimization for each amplicon to be analyzed.

HRMA relies on the use of fluorescent dsDNA-binding dyes that can be added directly to the PCR mix at saturating concentrations without inhibiting amplification (Wittwer and Reed 2003). This allows the precise monitoring of very low changes in fluorescence, caused by the release of a intercalating dyes consequent to the increase of temperature (Liew et al., 2004). Thus, single base changes can be readily identified as a significant differences in the melting curves of DNA amplicons (Reed et al., 2007). Consequently, HRMA results to be a closed-tube mutation screening method, that can be performed immediately after the amplification and does not require post-PCR handling or separation steps. These

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features make HRMA particularly suitable for medium to high-throughput applications (Dobrowolski et al., 2007; Margraf 2006; Montgomery et al., 2007) .

In this study we compared for the first time DHPLC and HRMA for mutation scanning of the *NF2* gene in two groups of patients. The first one, included patients with a known pathogenetic mutation. The second group included 76 *NF2* sporadic patients where PCR-SSCP screening was unable to reveal point mutations and MLPA analysis excluded genomic rearrangements. In both groups, HRMA results were compared in blind with those obtained with DHPLC. Our results indicated that both DHPLC and HRMA show a 100% sensitivity in detecting mutations previously characterised by PCR-SSCP, without any false positive samples. In addition, both DHPLC and HRMA detected 7 new samples with abnormal peaks at DHPLC analysis and anomalous melting profile at HRMA. Sequencing analysis revealed mutations in the involved exons including nonsense, missense, frameshift and splice site mutations.

Another very important aspect in the evaluation of methods for *NF2* mutational scanning is their capability to detect mosaicism. The identification of somatic mosaicism in a genetic syndrome is clinically relevant and this information has important implications for genetic counselling. According to our data, DHPLC and HRMA, performed on serial diluition of mutated:normal DNA samples, were able to detect the presence of 1.25% - 0.25% mutated alleles, a theorical threshold able to identify patients with a very low percentage of mutated cells in blood; this percentage is well under the direct sequencing threshold of detection .

In summary, in our experience that both DHPLC and HRMA are reliable techniques for mutation scanning in *NF2* gene. However, due to its simplicity, rapidity, and lower costs, together with its high sensitivity in detecting germline mutations and mosaicisms and to the absolute concordance with DHPLC results, we propose that HRMA could be used as a diagnostic screening technique for NF2 patients.

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**Table 1**: Known mutations in the *NF2* gene detected with DHPLC and HRMA.

| | **MUTATION** |
|---|---|
| EXON 1 | c.71-72insT |
| EXON 2 | c.169C>T p.R57X<br>c.169C>T p.R57X<br>c.169C>T p.R57X<br>c.169C>T p.R57X<br>IVS2+2T>C |
| EXON 5 | c.463delCCinsT |
| EXON 7 | c.656T>A p.V219E<br>c.663C>G p.Y221X |
| EXON 8 | c.773G>A p.W257X |
| EXON 10 | c.997C>T p.Q333X |
| EXON 12 | c.1279G>T p.E427X |
| EXON 13 | c.1416delC<br>c.1396C>T p.R466X |
| EXON 14 | IVS14+2T>C |
| EXON 15 | IVS15+1G>C |

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**Table 2**: Primers for PCR amplification of NF2 gene and temperatures for heteroduplex detection in DHPLC analysis

| EXON | PRODUCT SIZE (bp) | PRIMER SEQUENCE | | Tm PCR* | DHPLC ANALYSIS** |
|---|---|---|---|---|---|
| | | FORWARD | REVERSE | | |
| 1 | 235 | 5'-GGG CTA AAG GGC TCA GAG TG-3' | 5'-AAC CTC TCG AGC TTC CAC CT-3' | 59°C | 64.5°C |
| 2 | 265 | 5'-CCT GAG AGT GGA GAG TGC AG-3' | 5'-GGG AAA AAC TGG AAA GCT CA-3' | 62°C | 58.5°C - 60.9°C |
| 3 | 241 | 5'-TGA GGG TAG CAC AGG AGG AA-3' | 5'-CAA CTC TGC AAC CAC TCC TG-3' | 57°C | 58.7°C |
| 4 | 237 | 5'-CAC AGA GTA TCA TGT CTC CCT TG-3' | 5'-CAC TTT CTG GGG CAA GTC TC-3' | 58°C | 57.5°C - 58.3°C - 61.2°C |
| 5 | 188 | 5'-AGC TGG GAG GGA ATG AGA TT-3' | 5'-TCC TTC AAG TCC TTT GGT TAG C-3' | 59°C | 56.5°C - 59.5°C |
| 6 | 192 | 5'-AAA AGT GGC AAA CAA TAC CAA A-3' | 5'-AAG CCC ATA AAG GAA TGT AAA CC-3' | 61°C | 54.7°C - 59.5°C |
| 7 | 177 | 5'-GCTCTCCACCCATCTCACTT-3' | 5'-TTT AGC AGT CTG GCC CTC AC-3' | 59°C | 59.7°C - 60.6°C |
| 8 | 258 | 5'-TGC CAG ATT CTT TGG AAG GT-3' | 5'-GCA GAC AGG GAA AGA TCT GC-3' | 57°C | 58.8°C - 60.5°C |
| 9 | 248 | 5'-GGT AAC ATT CCA GGC TGT CG-3' | 5'-GCG CCA AGT GAG ATA CCA TT-3' | 59°C | 55.9°C - 56.5°C |
| 10 | 234 | 5'-TAG TGG GCC AGT AGG CAG TG-3' | 5'-GCC AGG ACT GAC CAC ACA G-3' | 60°C | 57.2°C - 58.8°C - 60.0°C |
| 11 | 212 | 5'- CTC GAG CCC TGT GAT TCA A -3' | 5'-AGT CCC CAA GTA GCC TCC TG-3' | 62°C | 60.5°C - 61.8°C - -63.0°C |
| 12 | 284 | 5'- CCC ACT TCA GCT AAG AGC AC -3' | 5'-CTC CTC GCC AGT CTG GTG-3' | 64°C | 62.1°C - 63.8°C |
| 13 | 227 | 5'- GGT GTC TTT TCC TGC TAC CT -3' | 5'-ACC ACT ACA AGA GAA AGG AGG G-3' | 60°C | 60.7°C - 61.9°C - 64.5°C |
| 14 | 246 | 5'- AGG ATC GGT TGT CAA CAC AG -3' | 5'-GGC CCC AAT CAC TCA GTC TA-3' | 61°C | 58.9°C - 60.0°C |
| 15 | 255 | 5'- TGT CTC ACT GTC TGC CCA AG--3' | 5'-TGG TCC TGA TCA GCA AAA A-3' | 60°C | 56.6°C - 60.5°C |

* Annealing temperature for PCR amplification

**Optimum temperatures for DHPLC analysis were empirically determined using the predicted fragment melting profile generated by WAVEMAKER software.

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**Figure legends**

**Figure 1**

DHPLC chromatograms and HRMA melting profiles for known *NF2* gene mutations in exons 2, 7, 12 14, 15. The arrows indicates variant elution profiles and change in melting curve shape.

MUT – mutant

**Figure 2**

Evaluation of the sensitivity in detecting mosaicism of DHPLC and HRMA. Serial dilutions of a wild-type DNA with genomic DNA carring a c.997C>T mutation in exon 10, deriving from a non-mosaic NF2 patient were prepared. Dilutions corresponding to a 1:1, 1:4, 1:10, 1:20, 1:100 and 1:1000 ratio of patient : normal DNA were amplified in PCR and analysed by DHPLC and HRMA.

A) DHPLC chromatograms showing the double peak corresponding to the mutation in dilutions 1:1, 1:4, 1:10, 1:20, 1:100.

B) HRMA difference plots of patient : normal DNA dilutions. All dilutions were compared to the median wild-type control sample to produce the plot. The mutated allele was easily detectable in all the dilution tested except 1:1000.

**Figure 3**

Evaluation of the sensitivity in detecting mosaicism of HRMA and direct sequencing. HRMA difference plots (on the left) and direct sequencing (on the right) of patient : normal DNA dilutions.

A) Mutation IVS15+1G>C. The mutated allele was detectable, until dilution 1:100 and 1:20 respectively by HRMA and direct sequencing.

B) Mutation IVS14+2T>C. The mutated allele was detectable, until dilution 1:20 and 1:1 respectively by HRMA and direct sequencing.

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**Figure 4**

DHPLC chromatograms and HRMA melting curves representative of the mutation detected in a mosaic patient carrying a mutation in *NF2* exon 13, namely c.1416delC.

**Figure 5**

DHPLC (left) and HRMA (right) profiles of the mutations lost at SSCP analysis.

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**Table 1**: Known mutations in the *NF2* gene detected with DHPLC and HRMA.

| MUTATION | |
|---|---|
| EXON 1 | c.71-72insT |
| EXON 2 | c.169C>T p.R57X<br>c.169C>T p.R57X<br>c.169C>T p.R57X<br>c.169C>T p.R57X<br>IVS2+2T>C |
| EXON 5 | c.463delCCinsT |
| EXON 7 | c.656T>A p.V219E<br>c.663C>G p.Y221X |
| EXON 8 | c.773G>A p.W257X |
| EXON 10 | c.997C>T p.Q333X |
| EXON 12 | c.1279G>T p.E427X |
| EXON 13 | c.1416delC<br>c.1396C>T p.R466X |
| EXON 14 | IVS14+2T>C |
| EXON 15 | IVS15+1G>C |

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**Table 2**: Primers for PCR amplification of NF2 gene and temperatures for heteroduplex detection in DHPLC analysis

| EXON | PRODUCT SIZE (bp) | PRIMER SEQUENCE | | Tm PCR* | DHPLC ANALYSIS** |
|---|---|---|---|---|---|
| | | FORWARD | REVERSE | | |
| 1 | 235 | 5'-GGG CTA AAG GGC TCA GAG TG-3' | 5'-AAC CTC TCG AGC TTC CAC CT-3' | 59°C | 64.5°C |
| 2 | 265 | 5'-CCT GAG AGT GGA GAG TGC AG-3' | 5'-GGG AAA AAC TGG AAA GCT CA-3' | 62°C | 58.5°C - 60.9°C |
| 3 | 241 | 5'-TGA GGG TAG CAC AGG AGG AA-3' | 5'-CAA CTC TGC AAC CAC TCC TG-3' | 57°C | 58.7°C |
| 4 | 237 | 5'-CAC AGA GTA TCA TGT CTC CCT TG-3' | 5'-CAC TTT CTG GGG CAA GTC TC-3' | 58°C | 57.5°C - 58.3°C - 61.2°C |
| 5 | 188 | 5'-AGC TGG GAG GGA ATG AGA TT-3' | 5'-TCC TTC AAG TCC TTT GGT TAG C-3' | 59°C | 56.5°C - 59.5°C |
| 6 | 192 | 5'-AAA AGT GGC AAA CAA TAC CAA A-3' | 5'-AAG CCC ATA AAG GAA TGT AAA CC-3' | 61°C | 54.7°C - 59.5°C |
| 7 | 177 | 5'-GCTCTCCACCCATCTCACTT-3' | 5'-TTT AGC AGT CTG GCC CTC AC-3' | 59°C | 59.7°C - 60.6°C |
| 8 | 258 | 5'-TGC CAG ATT CTT TGG AAG GT-3' | 5'-GCA GAC AGG GAA AGA TCT GC-3' | 57°C | 58.8°C - 60.5°C |
| 9 | 248 | 5'-GGT AAC ATT CCA GGC TGT CG-3' | 5'-GCG CCA AGT GAG ATA CCA TT-3' | 59°C | 55.9°C - 56.5°C |
| 10 | 234 | 5'-TAG TGG GCC AGT AGG CAG TG-3' | 5'-GCC AGG ACT GAC CAC ACA G-3' | 60°C | 57.2°C - 58.8°C - 60.0°C |
| 11 | 212 | 5'- CTC GAG CCC TGT GAT TCA A -3' | 5'-AGT CCC CAA GTA GCC TCC TG-3' | 62°C | 60.5°C - 61.8°C - -63.0°C |
| 12 | 284 | 5'- CCC ACT TCA GCT AAG AGC AC -3' | 5'-CTC CTC GCC AGT CTG GTG-3' | 64°C | 62.1°C - 63.8°C |
| 13 | 227 | 5'- GGT GTC TTT TCC TGC TAC CT -3' | 5'-ACC ACT ACA AGA GAA AGG AGG G-3' | 60°C | 60.7°C - 61.9°C - 64.5°C |
| 14 | 246 | 5'- AGG ATC GGT TGT CAA CAC AG -3' | 5'-GGC CCC AAT CAC TCA GTC TA-3' | 61°C | 58.9°C - 60.0°C |
| 15 | 255 | 5'- TGT CTC ACT GTC TGC CCA AG--3' | 5'-TGG TCC TGA TCA GCA AAA A-3' | 60°C | 56.6°C - 60.5°C |

* Annealing temperature for PCR amplification

**Optimum temperatures for DHPLC analysis were empirically determined using the predicted fragment melting profile generated by WAVEMAKER software.

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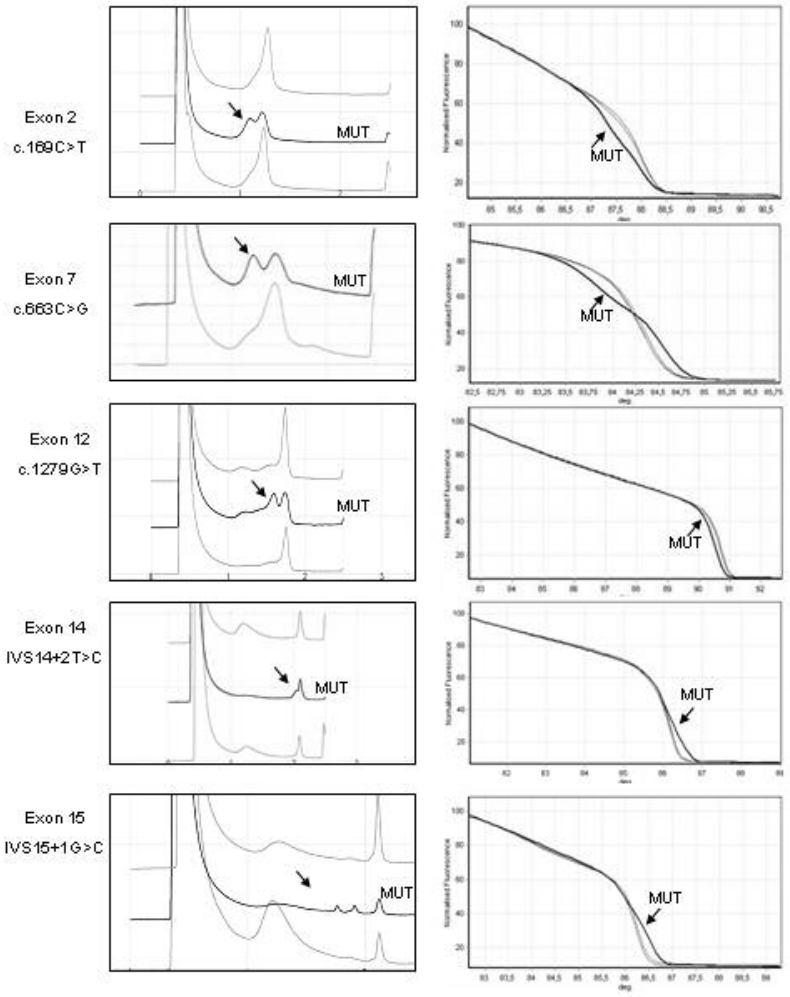


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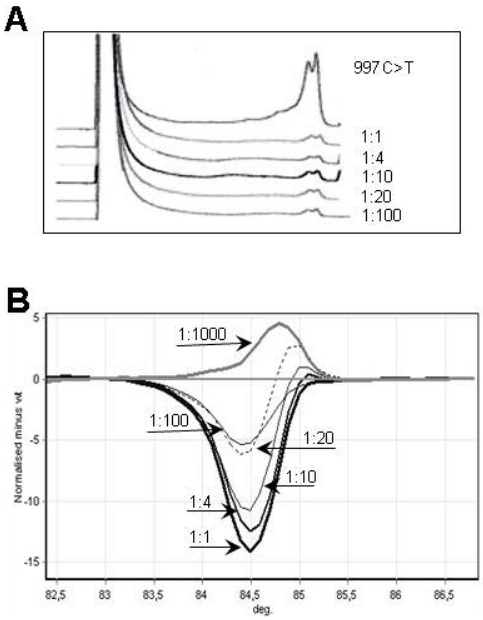


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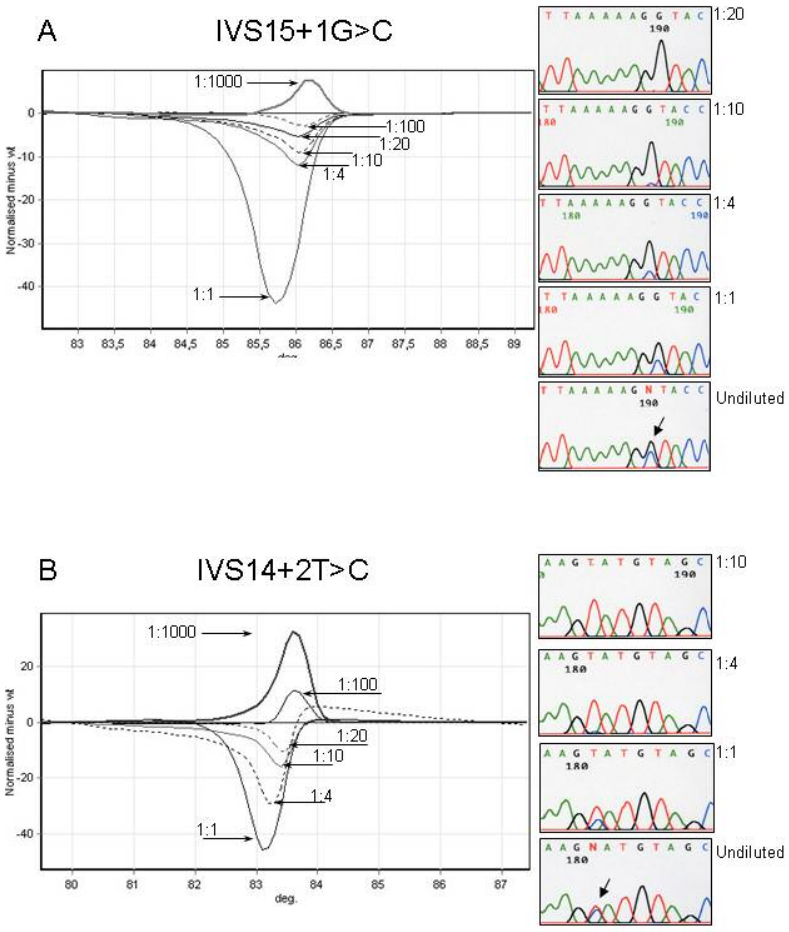


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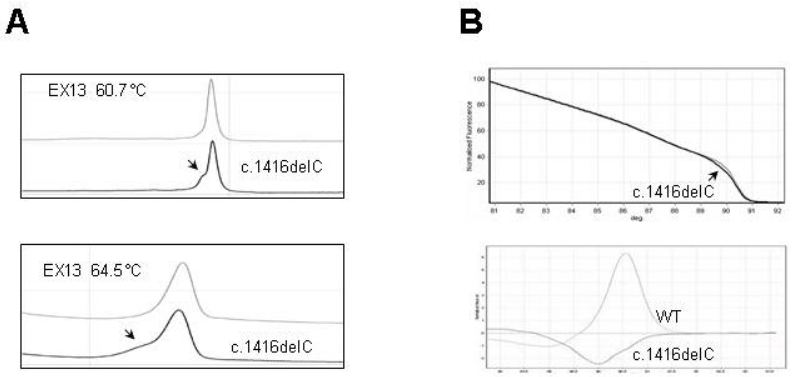


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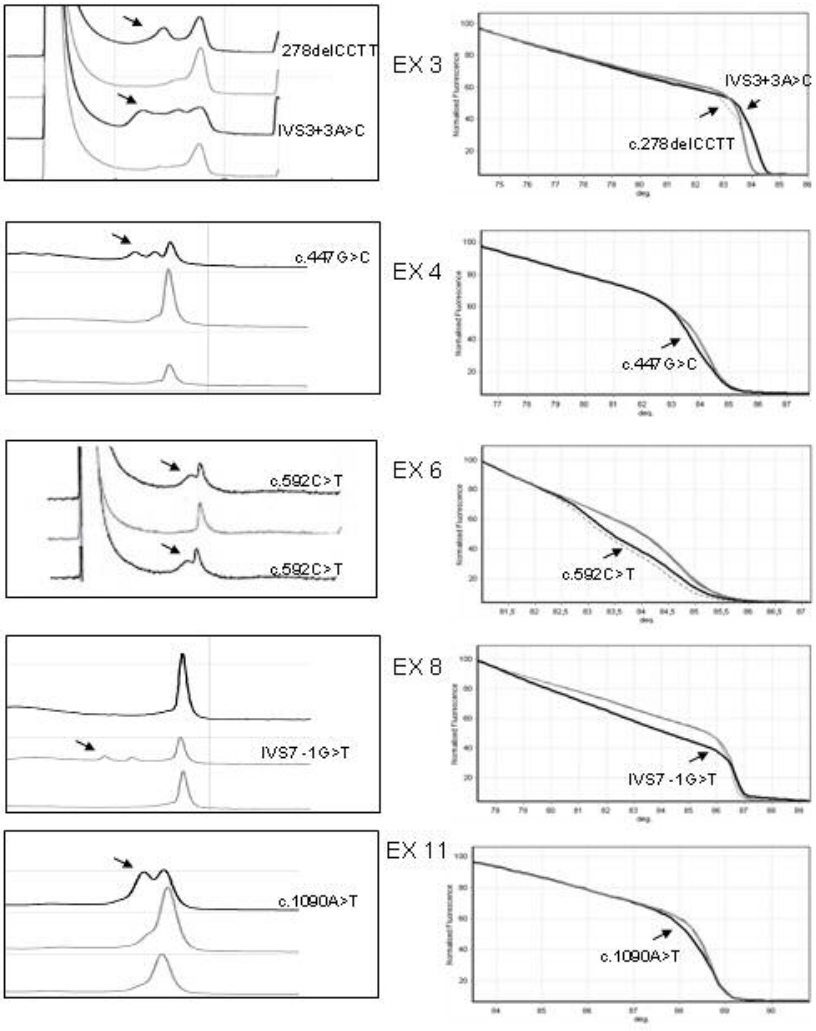


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