

evaluation of ABgene® QPCR Master Mix for SNP scoring on the ABI PRISM® 7700

Sciona is Britain's leading developer of personalised healthcare services based upon the unique genetic profiles of individual clients. Sciona tests for Single Nucleotide Polymorphisms (SNPs), and by screening select genes (genes are not typically considered biomarkers) is able to offer personalised healthcare advice to clients.

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Introduction

One example of a SNP analysed at Sciona is the polymorphism MTHFR C667T. This consists of a C to T substitution on the 5,10-methylenetetrahydrofolate-reductase gene, giving rise to a thermolabile form of the enzyme, which in turn is linked with hyperhomocysteinemia. Homocysteine is a sulphur-containing amino acid produced from methionine during processing of dietary protein. This amino acid has gained considerable attention because an elevated concentration of total homocysteine (tHcy) has been demonstrated to be associated with an increased risk of cardiovascular disease and other disorders. These include coronary artery, cerebrovascular, and peripheral vascular disease^{1,2} as well as a greatly increased risk of neural tube defects during pregnancy^{3,4}. Several vitamins function as cofactors and substrates in methionine and homocysteine metabolism, including folate, vitamin B₆, and vitamin B₁₂. It has been shown that an increased intake of folic acid and B complex vitamins can compensate for the reduced activity associated with the MTHFR SNP and so reduces the propensity for these conditions. The MTHFR C667T mutation is fairly common with the heterozygotic genotype accounting for around 35-40% and the homozygotic mutant genotype accounting for around 10% of all Caucasians screened.

Method

Sciona uses the ABI TaqMan® Allelic Discrimination Assay⁵, a PCR-based assay that is able to distinguish between homozygotic wild types, heterozygotes and homozygotic mutant types. Two ABI TaqMan® probes are used in this allele discrimination assay, one probe for each allele in a two-allele system. Each probe consists of an oligonucleotide with a 5'-reporter dye and a 3'-quencher dye. 6-FAM™ (6-carboxyfluorescein) is covalently linked to the 5' end of the probe for the detection of allele 1 (wildtype). VIC™ is covalently linked to the 5' end of the probe for the detection of allele 2 (mutant). Fluorescence from each of the reporter molecules is quenched by a quencher molecule attached via a linker arm located at

the 3' end of each probe.

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer (FRET)⁵. During PCR, forward and reverse primers hybridise to specific sequences of the target DNA. The ABI TaqMan® probe hybridises to a target sequence within the PCR amplicon and is cleaved by the 5'-3' nuclease activity of the Taq DNA polymerase as it extends from the primer along the template. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter. The 3' end of the ABI TaqMan® probe is chemically blocked to prevent extension of the probe during PCR. The cleavage process occurs every cycle and results in an increase in fluorescence from each of the 6-FAM™ and VIC™ reporters, proportional to the amount of PCR product. Both primer and probe must hybridise to the exact target sequence for amplification and cleavage to occur. Therefore, fluorescence signals are generated only if the target sequences for the probe are amplified during PCR. Because of these requirements, non-specific amplification is not usually detected.

Experimental Design

The assays are set up in a 96-well PCR plate, with each reaction containing a 25µl total volume. Human genomic DNA was isolated from buccal cell swabs using a proprietary silica gel technology and quantified using the PicoGreen® (Molecular Probes) assay. All DNA samples were then normalised to 5ng/µl with

dH₂O. Wild type controls (3), mutant type controls (3) and no template controls (3) were aliquoted in triplicate, and then triplicate repeats of client samples (3) were used. The PCR plate was then subjected to a standard ABI TaqMan® thermal cycle (50°C for 2 min, 95°C for 10 min (hot-start), followed by 40 cycles of 95°C for 15 sec, 60°C for 60 sec).

Fluorescence normalisation was achieved by dividing the emission intensity of the probe fluorophore by the emission intensity of the ROX™ passive reference to give a ratio, R_n. The change in fluorescence (ΔR_n) was obtained by calculating R_{n+} (with template DNA) minus R_{n-} (without template DNA). During the thermocycling process the ΔR_n was constantly recalculated. At the point in the reaction when a statistically significant ΔR_n was first detected, the Ct (threshold cycle) value was determined. This value is the average standard deviation (SD) of R_n multiplied by an adjustable factor, as automatically calculated by the ABI PRISM® 7700.

Results

Raw spectral data (figure 1) demonstrates a clear distinction between the three fluorophores used in the system. This allows a reliable comparison to the internal ROX™ standard and thus reaction consistency between wells.

For the wildtype SNP detection with 6-FAM™ labelled probe (figure 2), the first significant change in probe fluorescence (Ct) was observed at 23 cycles (SD = 0.0029), with the exponential phase of the reaction lasting for

Reagent	Volume (µl)	Concentration
ABgene® 2x QPCR Master Mix (CM-125)	12.5µl	1x
Forward primer	1.13µl	20pmol/µl
Reverse primer	1.13µl	20pmol/µl
6-FAM™ probe (minor groove binder)	0.33µl	15pmol/µl
VIC™ probe (minor groove binder)	0.33µl	15pmol/µl
Template DNA	5µl	1ng/µl
Distilled Water	4.58µl	
TOTAL VOLUME	25µl	

approximately 5 cycles. This demonstrates high reaction efficiency, with a typical reaction profile for this probe. No background fluorescence from the second probe (VIC™) could be detected. For the mutant SNP detection with VIC™ labelled probe (figure 3), a later Ct at around 29 cycles and a far steeper exponential phase lasting around 10 cycles was observed for the positive control reactions. Again, this demonstrates a typical reaction profile for this probe. For client samples where no PCR amplification was observed, Ct values are 40, indicating no probe was detected throughout the reaction.

A scatter graph was generated for allelic calling (figure 4). The three clusters that can be seen are allele 1 in red (including the allele 1 wild type control), allele 2 mutant type control in blue and no template controls in black. No heterozygotes were present. The graph

demonstrates excellent discrimination between alleles, with very tight allele clusters indicating low well-to-well coefficient of variation and highly reproducible results. In summary, this reaction shows excellent amplification and good discrimination using ABgene®'s QPCR Master Mix. Based upon these results it can be concluded that all of the subjects carried a wild genotype for MTHFR C677T.

References

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level and polymorphism of homocysteine metabolism related enzymes in ischemic cerebrovascular or cardiovascular diseases, *Zhonghua Xue Ye Xue Za Zhi*, **23** (3):126-9.

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5. Applied Biosystems (2000). *TaqMan® Allelic Discrimination Demonstration Kit Protocol* (#4303267).

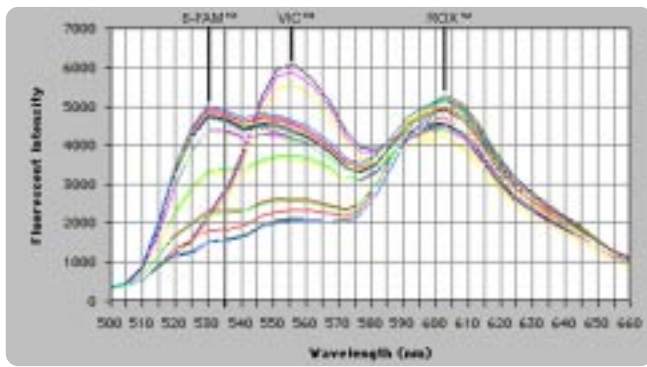


Figure 1: Raw spectral data for the reaction, in which three distinct peaks can be observed: 530nm (6-FAM™ probe); 555nm (VIC™ probe); 605nm (passive ROX™ internal standard). The fluorescence intensity ranged between 5000 and 6000 units.

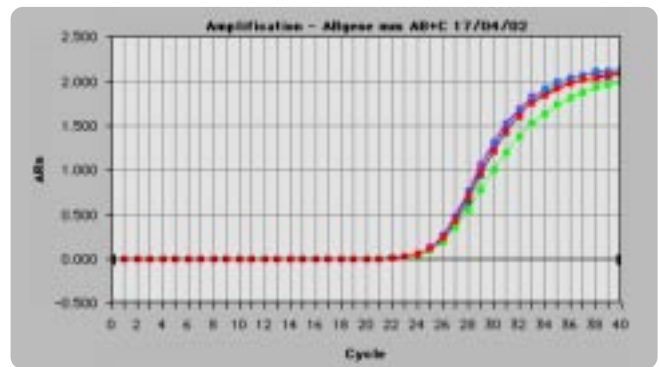


Figure 2: Wildtype allele detection by real-time PCR using a 6-FAM™ labelled probe.

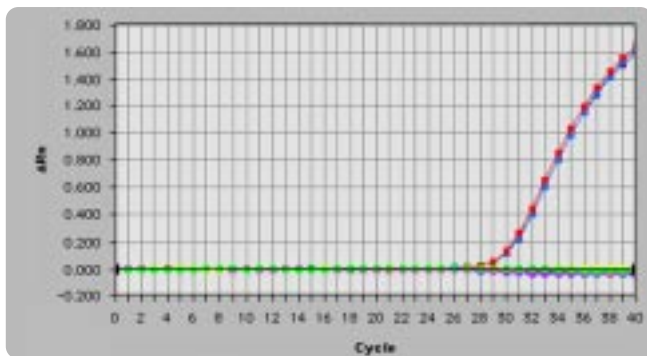


Figure 3: Mutant allele detection by real-time PCR using a VIC™ labelled probe.

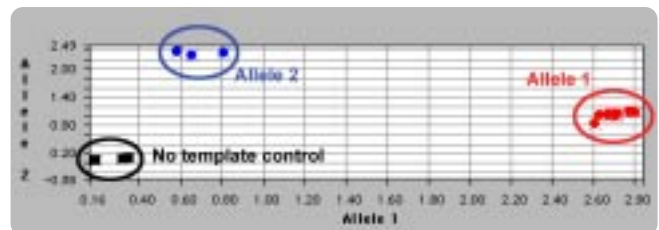


Figure 4: Scatter graph for allelic discrimination.

Cat. No.	Description	Quantity
CM-138	2x Thermo-Start® QPCR Master Mix	100 x 50µl rxns
CM-138/b	2x Thermo-Start® QPCR Master Mix	800 x 50µl rxns
CM-125	2x Thermo-Start® QPCR Master Mix with ROX	100 x 50µl rxns
CM-125/b	2x Thermo-Start® QPCR Master Mix with ROX	800 x 50µl rxns
CM-145	2x Thermo-Start® QPCR Master Mix with dUTP	100 x 50µl rxns
CM-145/b	2x Thermo-Start® QPCR Master Mix with dUTP	800 x 50µl rxns
CM-146	2x Thermo-Start® QPCR Master Mix with ROX and dUTP	100 x 50µl rxns
CM-146/b	2x Thermo-Start® QPCR Master Mix with ROX and dUTP	800 x 50µl rxns



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