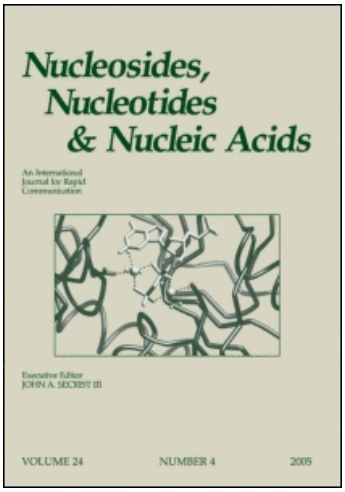

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Improved Allelic Differentiation Using Sequence-Specific Oligonucleotide Hybridization Incorporating an Additional Base-Analogue Mismatch

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ABSTRACT

Sequence-specific oligonucleotide hybridization (SSOH, 'dot-blotting') is a widely employed method of typing single nucleotide polymorphisms (SNPs), but it is often compromised by lack of allelic differentiation. We describe a novel improvement to SSOH that incorporates an additional mismatch into the oligonucleotide probe using the universal base analogue 3-nitropyrrole. This method greatly increases allelic differentiation compared to standard SSOH where oligonucleotides contain only SNP-defining base changes. Moreover, stringency of the hybridisation is predictably maintained over a wide range of temperatures, which can be calculated empirically, thus facilitating the genotyping of multiple SNPs using similar conditions. This

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improved method increases the usefulness of hybridisation-based methods of rapid genotyping of SNPs and may have implications for array methodologies.

Key Words: Sequence-specific oligonucleotide hybridization; Dot-blotting; Universal base analogue; Base mismatch.

INTRODUCTION

Sequence specific oligonucleotide hybridization (SSOH, dot-blotting)[1,2] is a commonly employed method for genotyping single nucleotide polymorphisms (SNPs). It has the virtue of being relatively inexpensive and using widely available equipment and reagents. The method is most useful when several SNPs are closely situated or when DNA template is scarce, as the same PCR product may be re-probed several times for neighbouring polymorphisms. SSOH relies on the binding of a labelled oligonucleotide probe to immobilised target sequences (usually PCR products). The probes contain a mismatch specific for each of the SNP alleles and allelic specificity is therefore determined by the efficient removal of mismatched probe from target/probe duplex. For certain sequences, the differential stability between a perfectly matched oligoduplex and a duplex mismatched at only one base pair may be small,[3] making allelic discrimination difficult. Moreover, as the melting temperature (Tm) for each allele varies with each oligonucleotide sequence, it is necessary to determine the optimum temperature for allelic discrimination for each SNP allele under investigation.

Incorporation of an additional mismatched base into the probe may enhance allelic discrimination, so that in the presence of a mismatched probe-target duplex, the area of sequence mismatch is increased and the mismatched probe is more easily removed from target sequence. Although a naturally occurring base can be used to provide the additional mismatch, results are unpredictable as they remain at the mercy of the surrounding sequence.[4] Incorporation of an additional mismatch with a universal base analogue into the probe potentially circumvents this problem.

A universal base analogue is capable of binding to each of the natural DNA bases within a duplex with little discrimination between each of the natural DNA bases. The most commonly used universal base is hypoxanthine,[5,6] but although capable of forming base pairs with each of the natural DNA (RNA) bases, it is not indiscriminate in its base-pairing properties.[7] More recently, a family of non-polar, aromatic, non-hydrogen bonding analogues have been described which behave as universal base analogues with little discrimination between the natural DNA bases.[8] Of these, the most commonly used analogues are 1-(2′-deoxy-β-D-ribofuranosyl)-3-nitropyrrole (3-nitropyrrole)[9] and -5-nitroindole.[10] Both analogues form stable duplexes when bound to the natural DNA bases, though both are destabilising, as there is a decrease in stability due to loss of hydrogen bonding and to solvophobic effects (loss of hydrating water molecules and negative interaction between solvent and hydrophobic bases). The extent of destabilisation is related to the size (amongst

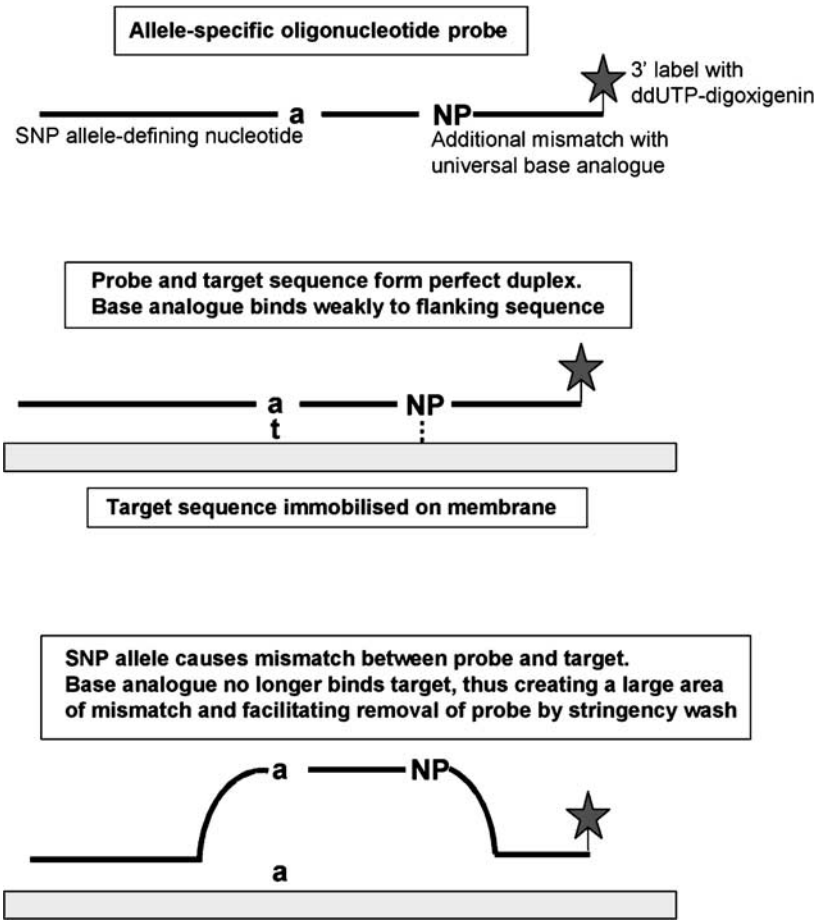

Figure 1. Principle of SSOH genotyping using a digoxigenin-labeled probe containing an additional mismatch introduced by the universal base analogue 3-nitropyrrole (NP). In this example an 'a/t' SNP is probed with an oligonucleotide specific for the 'a' allele.

other parameters) of the analogue, as the main stabilising effect is through stacking interactions.

Thus a universal base analogue may not cause significant disruption of matched probe-target duplexes, but a mismatch between the probe and the target sequence at the allele-defining base results in severance of the weak bonds between the analogue and target. By this destabilisation the probe is more easily removed by the stringency wash (Fig. 1). Therefore the incorporation of universal base analogues into short synthetic oligonucleotides increases destabilisation of mismatched duplexes dramatically and predictably.[11,12] Here we report that the incorporation of the universal base analogue 3-nitropyrrole significantly increases the allelic discrimination of SSOH genotyping when compared to conventional methods.

```
GACTACTTCTCTCCAGCACATGCAAGGTTA
   GTGAAGCGCGGTCGTGTACGTTCC        80°C
   GT3AAGCGCGGTCGTGTACGTTCC        69°C
   GT5AAGCGCGGTCGTGTACGTTCC        75°C
```

Figure 2. Melting temperature of duplexes with G:T mismatch compared with those containing either 3-nitopyrrole (**3**) or 5-nitroindole (**5**). Tms were measured in 3 M TMAC, 30 mM Tris HCl, 2 mM EDTA.

RESULTS

Non-hydrogen bonding universal base analogues are generally destabilising, though the destabilisation is related to the size of the base analogue.[13] For example, the analogue 5-nitroindole destabilises a 17-mer duplex by 2°C when located towards either 3′- or 5′-end of the duplex, whereas the smaller 3-nitropyrrole causes a 6°C destabilisation in the same duplex.[10] This is more marked when the universal base analogue is positioned close to a mismatch site. For the mismatch duplex (Fig. 2) the Tm is 80°C, and the presence of a 5-nitroindole residue (**5**) causes a 5°C destabilisation, whereas 3-nitropyrrole (**3**) causes an 11°C destabilisation. This destabilisation is more pronounced when the universal base is incorporated towards the middle of a sequence.[8]

The results clearly demonstrate that the 3-nitropyrrole-containing probes for the *NOS2A* -277 SNP gave superior allele discrimination compared to equivalent conventional probes. Moreover this discrimination was maintained over a range of stringency wash temperatures of at least 5°C (Fig. 3). High-throughput genotyping using the 3-nitropyrrole-containing oligonucleotides allowed ready discrimination of the *NOS2A*—a 277 g SNP alleles in a population based study (Fig. 4). Subsequent SSOH of six further SNP alleles in the proximal *NOS2A* promoter demonstrated that 3-nitropyrrole-containing oligonucleotide probes consistently gave clear genotyping results and that discrimination was maintained over a similar range of stringency wash temperatures (data not shown).

The range of stringency wash temperatures that permitted optimal allelic discrimination generally lay between the calculated Tm of the oligonucleotide probe and 5°C below the calculated Tm; no increase in the calculated Tm is necessary for the addition of the 3-nitropyrrole due to its destabilising effect. SSOH employing 3-nitropyrrole-containing oligonucleotides permitted allelic discrimination of SNPs that had previously proved impossible using conventional probes (data not shown).

The incorporation of the universal base analogue 3-nitropyrrole into oligonucleotide probes used for SSOH appreciably improved the resolution power of this method. The use of a tetramethylammonium chloride (TMAC)-based protocol further reduces the influence of probe sequence on Tm,[14] making stringency wash temperatures more predictable empirically. As the range of stringency wash temperatures was generally predictable from the calculated Tm, accurate genotyping of different SNPs was possible without the need for time-consuming optimisation experiments. The data obtained by the stringency wash temperatures was compared to measured Tm measurements (Fig. 5) and the two sets of data were found to be in good agreement. Thus the predicted Tm values can be taken as a reasonably accurate method.

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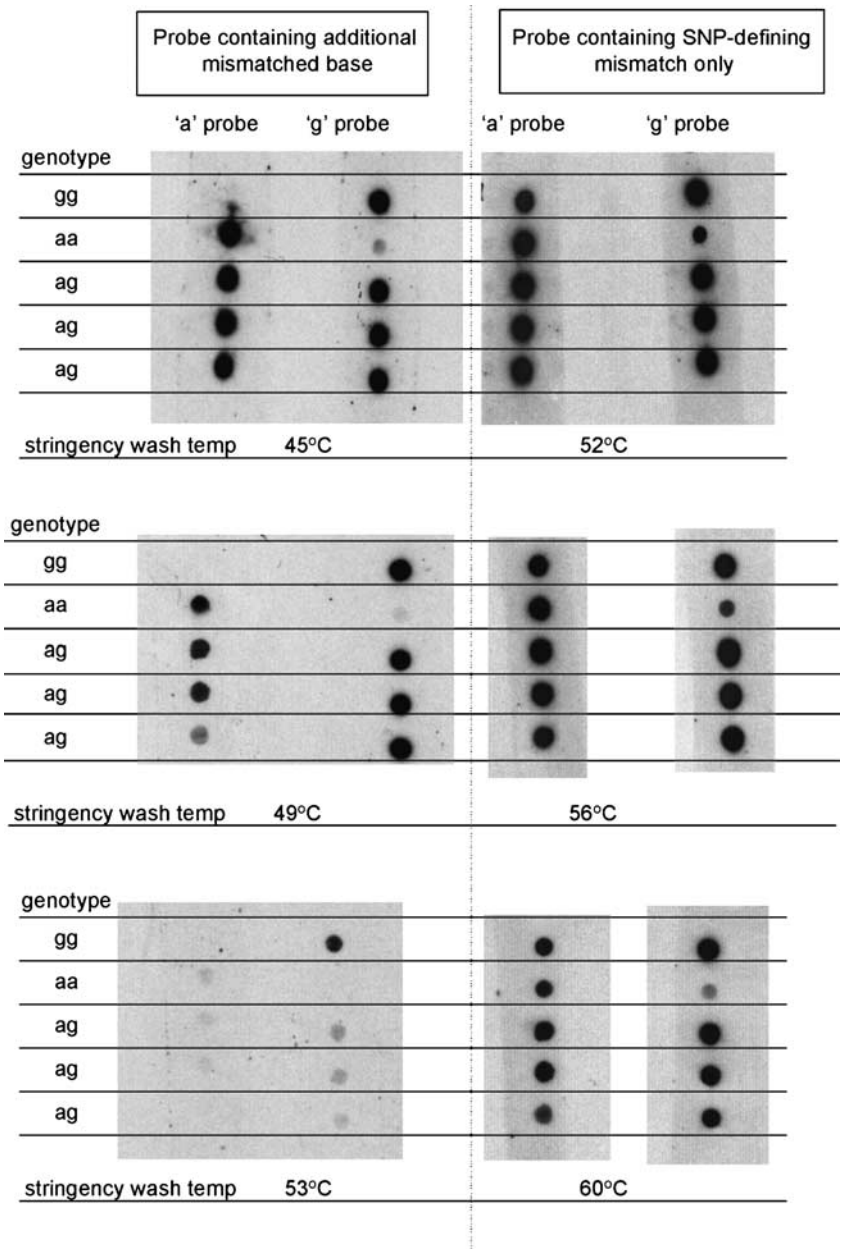
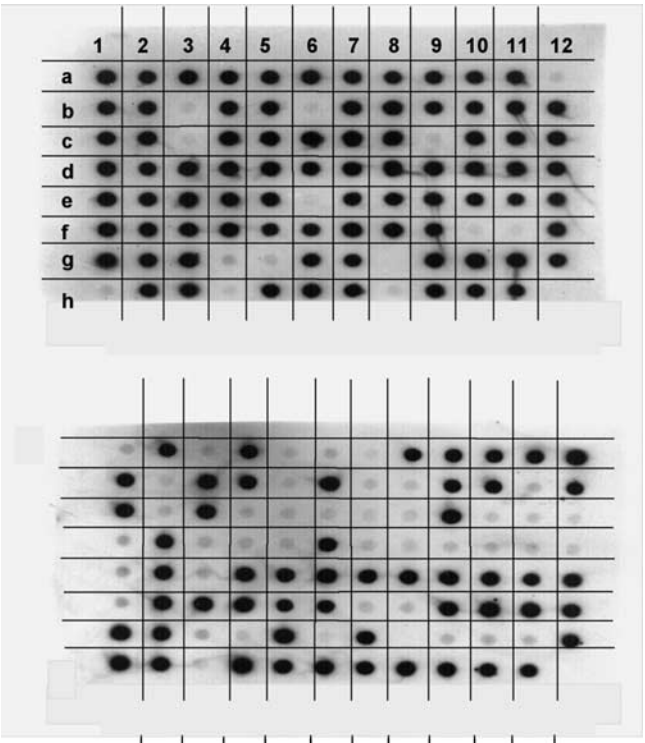


Figure 3. Comparison of SSOH-based allelic discrimination for the *NOS2A*—a 277 g SNP using conventional probes (with only a SNP-defining base change) and probes containing an additional base mismatch created with the universal base analogue 3-nitropyrrole. Data is shown using three equivalent stringency wash temperatures for each allele.



	1	2	3	4	5	6	7	8	9	10	11	12
a	aa	ag	aa	ag	aa	aa	aa	ag	ag	ag	ag	gg
b	ag	aa	gg	ag	aa	gg	aa	aa	ag	ag	aa	ag
c	ag	aa	gg	aa	aa	aa	aa	aa	gg	aa	aa	aa
d	aa	ag	aa	aa	aa	ag	aa	aa	aa	aa	aa	aa
e	aa	ag	aa	ag	ag	gg	ag	ag	ag	ag	ag	ag
f	aa	ag	ag	ag	ag	ag	aa	aa	ag	gg	gg	ag
g	ag	ag	aa	xx	gg	aa	ag	xx	aa	aa	aa	ag
h	**gg**	**ag**	**aa**	gg	ag	ag	ag	gg	ag	**ag**	**ag**	**nc**

Figure 4. Representative ASO hybridization output for a 96 well plate probed for the—a 277 g SNP. Each sample represents DNA from a different individual. The radiograph for the same samples probed with the 'a'-specific allele is shown at the top of the page and that for the 'g' allele below. The assigned genotype is shown at the foot of the page. Sequenced control samples are shown in bold. 'xx' represents PCR failure and 'nc' a PCR negative control.

DISCUSSION

Effect of Universal Base Analogue Mismatch on the Properties of the Oligonucleotide Probe

A number of universal base analogues may be incorporated into synthetic oligonucleotides. These include not only 3-nitropyrrole and 5-nitroindole, but also those

277C	CACGGAGATAGAATCGT	
277A	GTGCCTCT**A**TCTTAGCA	70
277G	GTGCCTCT**G**TCTTAGCA	63
277A5	GTGCCTCT**A**TC**3**TAGCA	62
277G5	GTGCCTCT**G**TC**3**TAGCA	54
954T	CTCAAGCTCTGGTCGTA	
954G	GAGTTCGA**G**ACCAGCAT	72
954C	GAGTTCGA**C**ACCAGCAT	59
954G5	GAGTTCGA**G**AC**3**AGCAT	63
954C5	GAGTTCGA**C**AC**3**AGCAT	50
1175W	ACAACCCTGCCACTCTA	
1175C	TGTTGGGA**C**GGTGAGAT	71
1175T	TGTTGGGA**T**GGTGAGAT	63
1175C5	TGTTGGGA**C**GG**3**GAGAT	61
1175T5	TGTTGGGA**T**GG**3**GAGAT	53
2447W	CGTACGGTGGTATGGGT	
2447C	GCATGCCA**C**CATACCCA	72
2447G	GCATGCCA**G**CATACCCA	65
2447C5	GCATGCCA**C**CA**3**ACCCA	63
2447G5	GCATGCCA**G**CA**3**ACCCA	56

Figure 5. Measured melting temperatures (Tms) of selected probe sets used in SSOH. Tms were measured in 3 M TMAC, 30 mM Tris HCl, 2 mM EDTA.

that are specifically degenerate for either purines or pyrimidines [such as the P and K analogues that together behave as a universal base[15] (Fig. 6)]. The incorporation of 3-nitropyrrole causes the greatest reduction in the melting temperature of the duplex than other base analogues, and is more destabilising if more than one base substitution is used.[16]

A recent report[11] studied the effect on mismatch discrimination of introducing two 3-nitropyrrole residues into a duplex. They reported that as the two nitropyrrole residues were moved further apart along the duplex that a bell-shaped curve was observed for the melting temperatures (Tm) of these duplexes. The lowest Tm was observed when the two 3-nitropyrrole residues were 10 bases apart, i.e. when they were separated by one helical turn of the DNA duplex.[11] We have repeated this work using the universal base analogues 3-nitropyrrole and 5-nitroindole and observed a similar effect with both analogues as described by Guo et al. (data not shown).[11] Whilst the duplexes containing the 3-nitropyrrole pairs showed the lower Tms (ΔTm for two nitropyrrole residues ten bases apart -11°C, for 5-nitroindole -8°C), there was no significant difference observed between the matched and mismatched duplexes.

For SSOH 3-nitropyrrole may therefore give greater discrimination by its more marked effect on Tm. The position of the base analogue in the oligonucleotide probe has a significant effect on the difference in Tm between an equivalent native and analogue-containing oligonucleotide. In short oligonucleotides containing a natural (i.e., SNP-defining) mismatch at the centre, positioning the universal base analogue 3 or 4 bp off-centre gives a maximal change in Tm of about 10–15°C.[11]

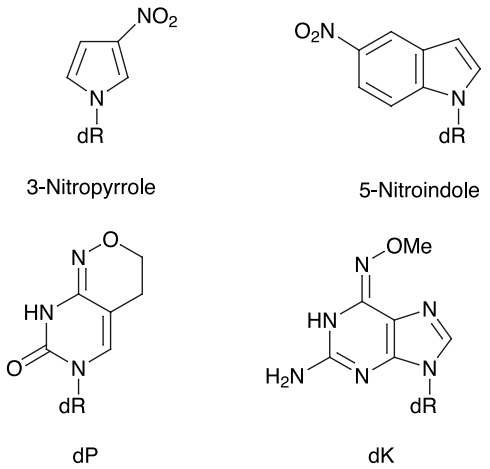


Figure 6. Structure of 3-nitropyrrole used in these experiments, and selected other universal base candidates.

The use of tetramethylammonium chloride buffer (TMAC) is known to equalise the difference between an A:T and a G:C base pair, thus in TMAC the melting temperatures of oligonucleotide duplexes are independent of the G + C content,[14] but rather are dependent on the length of the duplex. Jacobs et al.[17] have compared the melting temperatures of various length duplexes in 0.4 M Na^+ and 3 M TMAC buffers, and demonstrated the levelling effect on melting temperatures of TMAC buffer. They also showed that the longer the duplex, the lower the contribution to melting temperature per base pair. For example, in a decamer, the contribution per base pair is $\sim$3.2°C, whilst in a 32-mer, the contribution decreases to 2.3°C. Other groups have studied this effect, and whilst there are variations for the contribution per base pair varies slightly, the levelling effect remains. In this study we have used 17-mer duplexes, and the contribution per base pair is $\sim$4.1°C, and in the absence of a mismatch the melting temperatures remain unchanged.

The effect of incorporating 3-nitropyrrole into a duplex causes an average decrease in melting temperature of 9°C, and the presence of a mismatch causes an average similar drop in Tm, though it is dependent upon the particular mismatch. For example, a C:C mismatch caused an 11°C drop in Tm, whereas G:T (which is a more favourable mismatch) only depresses the Tm by 8°C. In the TMAC buffer, these decreases in Tm are again levelled out, and the presence of 3-nitropyrrole serves only as a mismatch site. However, the presence of 3-nitropyrrole three nucleotides away from a mismatch site gives a reproducible further decrease in Tm of $\sim$9°C, i.e., it is similar to a further mismatch. It is this effect that enables the use of 3-nitropyrrole to be used to further depress the melting temperature of a duplex containing a SNP. In these experiments, based on the melting of 17-mer duplexes, the contribution towards melting temperature per base pair is reduced from 4.1 to 3.1°C. The use of an artificial base analogue, such as 3-nitropyrrole, also offers the advantages over methods which use a mismatch to obtain the discrimination required for SNP detection. This is because some mismatches

are less discriminating than others. For example, a G:T mismatch will cause only a slight decrease in the duplex stability compared to a G:A mismatch.

SSOH genotyping is widely used for SNP analysis in studies of human diseases and is still the method of choice for many diagnostic DNA-based tests. The insertion of 3-nitropyrrole in the oligonucleotide probes enhances the specificity of DNA hybridization and allows an easier set up of experimental conditions, thus making this technique more suitable for routine laboratory purposes. Moreover 'reverse dot-blotting,' where known target sequence is immobilised and hybridised with unknown oligonucleotides, underpins the new array methodology. Extension of this method may therefore have implications for the simultaneous hybridization of several thousand probes and may be useful in increasing the stringency of oligonucleotide-based microarray techniques.

MATERIAL AND METHODS

The universal base analogue 3-nitropyrrole (Cambio, Cambridge UK) was incorporated into 17 bp native oligonucleotide probes. In order to compare 3-nitropyrrole containing probes with 'conventional' probes (with only a SNP-defining base change), pairs of oligonucleotide probes were synthesised to identify a common novel SNP (−a277 g) in the proximal promoter of the inducible nitric oxide synthase gene (*NOS2A*; MIM 163730).[18] Probe sequences (5′ to 3′) were as follows (SNP shown in bold): GTGCCTCT**A**TCTTAGCA (conventional −277 a probe), GTGCCTCT**G**TCTTAGCA (conventional −277 g probe), GTGCCTCT**A**TCT**NP**AGCA (3-nitropyrrole (NP) −277 a probe) GTGCCTCT**G**TCT**NP**AGCA (3-nitropyrrole (NP) −277 g probe). To investigate the efficacy of this approach on other SNPs, 3-nitropyrrole-containing probes were synthesized for six other common *NOS2A* promoter SNPs.[18] All probes were labelled at their 3′ end with digoxigenin-11-dUTP according to the manufacturer's instructions (Boehringer Mannheim, Lewes, UK).

Prior to hybridization, a ∼1 kb fragment of the NOS2 promoter was amplified by PCR as previously described.[18] Briefly ∼50 ng of genomic DNA was mixed with 1μM each of sense (5′-CATGTCTTTTTTGTGGCTC-3′) and antisense (5′ CCTCTTTCTGGTCCCAAGTC-3′) primers (Gibco, Paisley, UK), 200 μM of each dNTP (Boehringer Mannheim), 2.5 mM $MgCl_2$, 1 mM Tris HCl, 5 mM KCl and 1 U Amplitaq Gold (all Perkin-Elmer, Beaconsfield, UK) in a final volume of 20 μl. Cycling conditions were 94°C for 12 minutes, followed by 30 cycles of 94°C for 30 seconds, annealing for 30 seconds at 58°C, elongation at 72°C for 90 seconds and a final step of 72°C for 5 minutes.

Sequenced controls of known genotype were immobilised on positively charged nylon membranes (Boehringer Mannheim) by a suction manifold and UV-cross-linking. Hybridization was performed using a standard tetramethylammonium chloride (TMAC)-based protocol[19] in accordance with the manufacturer's instructions. The labelled probe is removed from mismatched probe/target duplexes by washing and stringency is realised by setting the stringency wash temperature so that allelic discrimination is observed for the control samples of known genotype. As the addition of the 3-nitropyrrole reduces the overall melting temperature of a 17 base pair (bp) oligonucleotide by 5–10°C,[10] the hybridization and washing temperatures employed

for the 3-nitropyrrole-containing probes for the—a 277 g SNP were set 5–7°C lower than those used for conventional probes. Similarly, hybridization and washing temperatures 5–10°C lower than the predicted Tm were used for other 3-nitropyrrole-containing probes. Digoxigenin was detected by autoradiography following application of a specific anti-digoxigenin antibody and a chemiluminescent substrate, CSPD® (both Boehringer Mannheim).

Thermal melting experiments were carried out on a Perkin Elmer Lambda 40 spectrometer fitted with a peltier cell. Tms were carried out in 3 M TMAC, 30 mM Tris HCl, 2 mM EDTA at 0.5°C per minute, with an error of ± 0.5°C.

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