

DOE Grant Final Report

Title: The spectrum of mutation produced by low dose radiation
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Reference No. DE-FG02-00ER62906
Funding Body: Department of Energy, USA

This grant funding was spread out over three years. During the first year the two personnel supported were Dr Sarah Swinburne and Mrs Ann Rogers. During the final two years, only Dr Swinburne was funded.

The aim of the grant was to develop a PCR-based system for detecting point mutations. During the first 1-1½ years, the strategy used was to develop a linker approach to create a stem loop structure which incorporated both DNA strands involving the mutation site and which could then be amplified by PCR. Using a model mutation system for the ras gene, it was possible to synthesise this structure but the efficiency of amplification proved to be inadequate, despite numerous and varied technical modifications to the PCR. We surmise that the snap-back of the stem of the stem-loop structure was sufficiently favoured to prevent efficient primer access and thus prevent efficient amplification.

During the final 1½ years of the grant, we investigated a different approach which was directed towards detection of dinucleotide CC→TT mutations. The C>T transition is by far the most common base substitution mutation, arising from oxidative damage to DNA. Oxidised cytosine may undergo deamination to form a uracil derivative which is poorly repaired and is strongly miscoding during replication¹. The CC>TT mutation is also relatively common. It is the usual outcome of pyrimidine photodimer production by UV radiation² and also occurs as a consequence of oxidative damage³.

Results

Dr Sarah Swinburne developed a highly sensitive assay for detection of CC>TT mutations. The assay is PCR-based and is designed to detect mutation of a DNA target sequence which is present in multiple copies per cell, thus enabling very low mutation frequencies to be detected. The preliminary results have been obtained with human sequences. One sequence incorporates the (wild-type) CC sequence at nucleotides 1279-1280 in human mitochondrial DNA and the second incorporates the CC sequence at nucleotides 1745-1746 in the human 18S ribosomal gene. Both sites contain restriction enzyme recognition sites which can be used preferentially to cleave the wild-type sequence and they also have sequences spanning the mutation site for which hybridising peptide nucleic acid (PNA) oligonucleotide can be synthesised. The purpose of using both sites for detecting the signature mutation has been that the cytoplasmic mitochondrial genome is subject to a high flux of ROS as a by-product of oxidative phosphorylation and possibly has a low capacity for DNA repair, whereas the ribosomal genes are nuclear and are subject to the full range of DNA repair mechanisms operating in the particular cell. Mutation at the mitochondrial sequence is expected to reflect primarily cumulated endogenous oxidative phosphorylation induced damage, whereas mutation in the nuclear genes will reflect both endogenous and exogenous damage which has escaped faithful DNA repair and has been fixed into the genome following replication. Development is most advanced for the mitochondrial system.

To develop the system she used a synthesised mutated sequence which also contains a small insert. When used as a template for PCR, the amplicons of the mutated sequence can be readily distinguished by length from those of the wild-type sequence.

The PCR strategy for detection of mutated sequences essentially involves two phases, both of which are designed to specifically amplify mutated sequences with minimal or no amplification of wild-type sequences. The first phase involves a PCR primer which ends just

3' to the -CC- dinucleotide and a 16 mer PNA which is centred on the dinucleotide and has a sequence complementary to the wild-type sequence. The PNA binds strongly to the wild-type sequence, blocks primer access and prevents amplification during the PCR. By contrast, owing to the dinucleotide mismatch, the PNA binds weakly to the mutated sequence, the primer can obtain access, and amplification can occur. The table shows the extent of amplification, measured as the ratio of the number of sequences at the end of the PCR to the number of sequences at the beginning of the PCR, when 100 mutant sequences were mixed with various numbers of wild-type sequences and amplified for 20 PCR cycles in the presence of PNA.

Table 1

PNA	<u>Starting Material</u>		<u>Amplification Factor</u>	
	mutant	wild-type	mutant	Wild-type
+	100	5×10^9	6×10^4	8
+	100	10^9	1.6×10^5	10
+	100	10^8	2.5×10^5	17
+	100	10^7	4.3×10^5	29
+	100	10^6	2.9×10^5	-
-	100	-	6.5×10^5	-

Based on these data with PNA, we designed the first phase, which involved PNA as follows

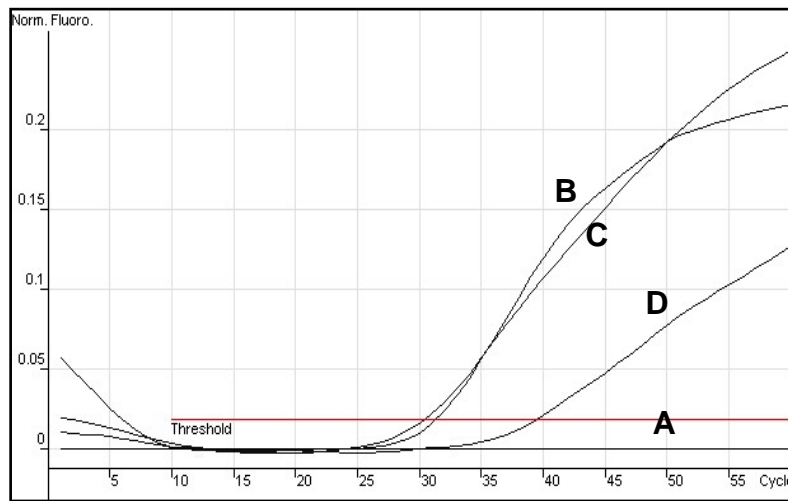
- an initial 20 cycle PCR involving 4 μ M of PNA
- a 10^{-3} dilution of product
- a second 20 cycle PCR involving 4 μ M of PNA
- a 10^{-5} dilution of product

As an indicative calculation, the data from Table 1 would suggest that, at the end of this first phase, 1 mutant copy would be represented by 210 copies, whereas 5×10^9 wild-type copies would be represented by 8000 copies. Another indicative calculation suggests that Taq-induced CC>TT PCR mutations would be eliminated by this protocol. Given the 10^{-4} - 10^{-5} frequency of Taq mutation/nucleotide/round of PCR, and the very limited amplification of wild-type sequences, we calculate that the first PCR round would result in approximately 15 - 40 such mutations. However these would all be lost after the first 10^{-3} dilution

The second phase of the amplification involved PCR primers terminating in TT. The two 3' bases of the primer were perfectly matched to the mutant sequence but mismatched to the wild-type sequence thus inhibiting mispriming from the latter. In practice, we found that mispriming would only occur if $\geq 10^6$ wild-type sequences were present in the PCR. With this final PCR, the number of mitochondrial sequences was quantified by real-time PCR using fluorescence resonance energy transfer for detection.

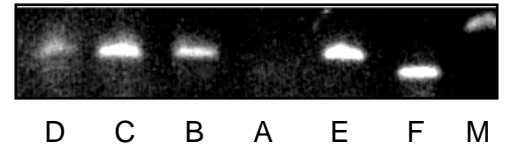
Using this protocol we have been able to detect 10 mutant sequences mixed in with 5×10^9 wild-type sequences, giving a level of detection of 2×10^{-9} . The amplified material was positively identified as mutant material, rather than misprimed wild-type material by its length characteristic as shown on gel electrophoresis.

Real time PCR



A - 5×10^9 WT copies, no spiking
 B - 5×10^9 WT copies, 100 mutants spiked
 C - 5×10^9 WT copies, 50 mutants spiked
 D - 5×10^9 WT copies, 10 mutants spiked

Gel electrophoresis



D - 5×10^9 + 10 Mutants
 C - 5×10^9 + 50 Mutants
 B - 5×10^9 + 100 Mutants
 A - 5×10^9 alone
 E - Mutant positive control
 F - Wild type positive control
 M - Marker

Conclusion

Preliminary results of this approach were promising. However, owing to time constraints, it was not possible to further develop the system and apply it to detecting CC→TT dinucleotide mutations produced in cells by ionising radiation. Further work will resume if further funding is obtained.

References

1. Kreutzer DA & Essigmann JM. Oxidized, deaminated cytosines are a source of C to T transitions *in vivo*. *Proc. Natl. Acad. Sci. USA* **95**: 3578-3582 (1998).
2. Kawasaki K. *et al.* CC to TT mutation in the mitochondrial DNA of normal skin: relationship to ultraviolet exposure. *Mutat. Res.* **468**: 35-43 (2000).
3. Newcomb TG. *et al.* Detection of tandem CC to TT mutations induced by oxygen radicals using mutation specific PCR. *Mutat. Res.* **427**: 21-30 (1999).