Figure 2.5a Allele specificity of ARMAS-PCR using primers A8MS and A8NAS.

EB 2% of results from amplification using A8MS and A8NAS (lanes 2 to 5), A8MS and ESAS (lanes 6 to 9) and A8MS and A9MAS (lanes 10-13) DNA samples used as template are listed.

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: SAO-2 p53 null cell line: no PCR product seen (negative control)
Lane 3: HUVEC DNA: no PCR product seen
Lane 4: Leukocyte DNA: a non-specific PCR product seen at 129bp.
Lane 5: SW480 cell DNA: a 129bp product is seen
Lane 6: SAO-2 DNA: no PCR product seen
Lane 7: HUVEC DNA: no PCR product seen
Lane 8: Leukocyte DNA: no PCR product seen
Lane 9: SW480 cell DNA: no clear band observed (PCR using A8MS and A8NAS were repeated see Fig. 2.5b)
Lane 10: SAO-2 DNA no PCR product seen
Lane 11: HUVEC DNA no PCR product seen
Lane 12: Leukocyte DNA no PCR product seen (some non-specific amplification seen producing a 100bp size product)
Lane 13: SW480 cell DNA a 258bp product is seen
Lane 14: Negative control with omission of template

Figure 2.5b Repeat of ARMAS-PCR with primers A8MS and A8NAS detailed in 2.5a EB 2%.

Lane 1: mw IX
Lane 2: SAO-2 DNA no PCR product seen
Lane 3: HUVEC DNA no PCR product seen
Lane 4: Leukocyte DNA no PCR product seen
Lane 5: Fibroblast DNA no PCR product seen
Lane 6: SW480 cell DNA showing a 258bp product

Figure 2.6 Optimisation of ARMAS-PCR using primers A9NS and A9MAS at 64°C annealing temperature and varying MgCl₂ concentration.

EB2% showing 125bp products using wildtype and mutant SW480 DNA as template for amplification.

Lane 1: mw IX
Lanes 2 to 6: Mutant template
Lane 2: 1.5mmol MgCl₂ a 125bp product is produced
Lane 3: 2.0mmol MgCl₂ a 125bp product is produced
Lane 4: 2.5mmol MgCl₂ a 125bp product is produced
Lane 5: 3.5mmol MgCl₂ a 125bp product is produced
Lane 6: 5.0mmol MgCl₂ a 125bp product is produced
Lane 7: Negative control with omission of template
Lanes 8 to 12: wildtype allele
Lane 8: 1.5mmol MgCl₂ a faint band is seen at 125bps
Lane 9: 2.0mmol MgCl₂ a faint band is seen at 125bps
Lane 10: 2.5mmol MgCl₂ a 125bp product is produced
Lane 11: 3.5mmol MgCl₂ a 125bp product is produced
Lane 12: 5.0mmol MgCl₂ a 125bp product is produced

Figure 2.7 Titration of primer concentration for the use of A8MS and A9MAS using SW480 DNA as template.

EB 2%:

Lane 1: mw IX
Lane 2: Negative control with omission of template
Lane 3: 0.15µM primers with a 258bp product
Lane 4: 0.10µM primers with a 258bp product
Lane 5: 0.05µM primers with a 258bp product
Lane 6: 0.02µM primers no PCR product observed
Lane 7: 0.01µM primers no PCR product observed

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Primer set number 4: A8MS and A9MAS (Table 2IV). with both sense and antisense primer complementary to a mutation point at its 3’ end, produced good amplification products, both with wildtype and mutant DNA. Optimal primer concentrations were titrated by lowering the concentration in a stepwise manner. The optimal primer concentration was observed to be 0.15 μM (Fig. 2.7). The combination of A8MS and A9MAS was specific for mutation detection (see section 2.4 Assessment of sensitivity of ARMAS-PCR using various primer combinations for further details). This technique has previously been coined the “Double ARMS-PCR” (Lo et al., 1991). This can be compared with primer set number 2: A8NS and A8NAS which was less specific than the combination of A8MS and A9MAS (Fig. 2.5a lane 4).

A primer (A8N2AS) placed further downstream on the p53 gene was used to generate a larger PCR product. The aim of the use of a larger PCR product is later described in Chapter 3.

ARMAS-PCR amplification was also optimised for primers of either A8MS or A8NS and A8N2AS. Improvements were achieved when primer concentrations were lowered.

2.3.2.2 Primers for CEM CELL exon 5 or 7 mutation

Table 2VI Primers for ARMAS-PCR for mutations in CEM cell DNA.

<table>
<thead>
<tr>
<th>Primer number</th>
<th>set</th>
<th>Exon</th>
<th>Mutant Sense</th>
<th>Wildtype sense</th>
<th>Mutant antisense</th>
<th>Wildtype antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>A5MS</td>
<td>A5NS</td>
<td>A5NS</td>
<td>A5NAS</td>
<td>A5NAS</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>A5M2S</td>
<td>A5NS</td>
<td>A5NS</td>
<td>A5NAS</td>
<td>A5NAS</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>A5M3S</td>
<td>A5NS</td>
<td>A5NS</td>
<td>A5NAS</td>
<td>E6AS</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>A5MS</td>
<td>A5NS</td>
<td>A7NAS</td>
<td>A7MAS</td>
<td>A7NAS</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>A7NS</td>
<td>A7MAS</td>
<td>A7NAS</td>
<td>A7NAS</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5 &amp; 7</td>
<td>A5MS</td>
<td>A5NS</td>
<td>A7MAS</td>
<td>A7NAS</td>
<td></td>
</tr>
</tbody>
</table>
A5MS had a deliberate T to T (a pyrimidine to pyrimidine) mismatch 5 nucleotides upstream from the 3' end but with homology with the mutation in exon 5 at the 3' end. When PCR amplification parameters were optimised for primers A5MS and A5NAS, and assessed for specificity of mutation detection, A5MS was unable to discriminate between mutant and wildtype allele (Fig. 2.8 lane 3). Similar results were obtained when a different downstream primer E6AS was used in place of A5NAS. A signal was generated using mutation specific primers on leukocyte DNA, but no signal was generated with fibroblast DNA, or HUVEC DNA. Primer set number 5 (Table 2.VI): A7MS and A7MAS with the antisense primer homologous to the mutation at its 3' end and a deliberate mismatch 9 nucleotides upstream from the 3' end was also unable to distinguish between wildtype and mutant alleles.

A similar strategy of “Double ARMS-PCR” for the detection of exon 5 and 7 mutations in CEM cells as SW480 cells was attempted using primers listed as primer set number 6 (Table 2.VI) (A5MS and A7MAS). However, amplification consistently produced extraneous bands. Wildtype primers to amplify the corresponding wildtype allele did not generate a good PCR product. Bands evident at about 70bp were prominent, suggestive of primers predominantly forming primer dimers during amplification. Even lowering annealing temperature to 56°C failed to produce an obvious band. Increase of temperature to 68°C did not improve amplification. Use of a different upstream primer (E6S) produced similar results suggestive of non-specific binding particularly of primer A7MAS.
Figure 2.8a Optimisation of ARMAS-PCR for the detection of mutations in CEM cell DNA by changing the concentration of primers: A5MS and A5NAS, dNTPs and MgCl₂.

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2 to 4: Change of primer concentration from 2µM to 0.25µM
Lane 2: Mutant allele CEM DNA as template generating a 252bp product
Lane 3: Wildtype leukocyte DNA as template generating a non-mutant-specific product
Lane 4: Wildtype HUVEC DNA as template, no product seen
Lane 5: Negative control with omission of template
Lane 6 to 8: Change of dNTP concentration from 0.2mM to 0.1mM
Lane 6: Mutant allele CEM DNA as template generating a 252bp product
Lane 7: Wildtype leukocyte DNA as template generating a non-mutant-specific product
Lane 8: Wildtype HUVEC DNA as template generating a non-mutant-specific product
Lane 9: Negative control with omission of template
Lane 10 to 12: Change of MgCl₂ concentration from 1.5mmol to 0.75mmol
Lane 10: Mutant allele CEM DNA as template, no product seen
Lane 11: Wildtype leukocyte DNA as template, no product seen
Lane 12: Wildtype HUVEC DNA as template, no product seen
Lane 13: Negative control with omission of template

Figure 2.8b Optimisation of ARMAS-PCR for detection of mutation in exon 5 of CEM cell DNA using A5MS and A5NAS by changing concentration of primers.

EB 2% The optimal primer concentration was 0.075µM
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: Mutant CEM cell DNA with 0.2µM primers
Lane 3: Wildtype leukocyte DNA with 0.2µM primers
Lane 4: Mutant CEM cell DNA with 0.125µM primers
Lane 5: Wildtype leukocyte DNA with 0.125µM primers
Lane 6: Mutant CEM cell DNA with 0.075µM primers
Lane 7: Wildtype leukocyte DNA with 0.075µM primers
Lane 8: Mutant CEM cell DNA with 0.05µM primers
Lane 9: Wildtype leukocyte DNA with 0.05µM primers
Lane 10: Mutant CEM cell DNA with 0.025µM primers
Lane 11: Wildtype leukocyte DNA with 0.025µM primers
To optimise the mutation detection, dNTP, MgCl₂, and primer concentrations were lowered according to Huang et al. (1992) and Newton et al. (1989). Fig. 2.8 (a and b) summarises the changes employed to obtain an optimal mutation detection parameter. After extensive optimisation of amplification parameters, specificity was achieved with A5MS/A5NAS (Fig. 2.8b lane 6 and 7) and A5MS/A7MAS (results not shown) by lowering primer concentration. Changes in MgCl₂ alone did little to improve specificity. Even at the lowest concentration of MgCl₂ which provides the most stringent conditions, there was a failure to amplify the mutant allele specifically. Detection specificity was achieved at the expense of amplification production; only a very faint band was observed.

2.3.2.2.1 Modification of primers

Additional deliberate mismatches were added to the original A5MS primer. The deliberate mismatch was moved from the 5ᵗʰ nucleotide upstream from the 3′ end of the primer to the penultimate position; this primer was designated as A5M2S. Another primer designated as A5M3S was modified by placing an additional deliberate T/C mismatch 3 nucleotides upstream from the 3′ end of the primer. Both A5M2S and A5M3S amplified with A5NAS were easily able to discriminate between wildtype and mutant (Fig. 2.9).
Figure 2.9 Optimisation of ARMAS-PCR for detection of mutation in exon 5 of CEM cell DNA, increasing specificity by modifying the deliberate mismatch design in mutant-specific primers (Fig 2.2b). EB 2%:
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lanes 2 and 3: Primers A5M3S and A5NAS were used
Lane 2: Mutant CEM cell DNA template producing a 252bp product
Lane 3: Wildtype leukocyte DNA template, no product is seen
Lane 4: Negative control with omission of template
Lanes 5 and 6: Primers A5M2S and A5NAS were used
Lane 5: Mutant CEM cell DNA template producing a 252bp product
Lane 6: Wildtype leukocyte DNA template, no product is seen
Lane 7: Negative control with omission of template.

Figure 2.10a Assessment of sensitivity of ARMAS-PCR for detection of p53 mutations in exon 8 in SW480 cell DNA. A8MS and A8NAS primers were used to amplify DNA extracted from cell dilutions as listed. EB 2%:
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X10^5 SW480 cells, a 129bp product seen
Lane 3: DNA extracted from SW480:fibroblast in ratio of 1:1, a 129bp PCR product seen
Lane 4: DNA extracted from SW480:fibroblast in ratio of 1:10, a 129bp PCR product seen
Lane 5: DNA extracted from SW480:fibroblast in ratio of 1:100, a faint 129bp PCR product seen
Lane 6: DNA extracted from SW480:fibroblast in ratio of 1:10 000, no PCR product seen
Lane 7: DNA extracted from SW480:fibroblast in ratio of 1:10 000, no PCR product seen
Lane 8: DNA extracted from SW480:fibroblast in ratio of 1:100 000, no PCR product seen
Lane 9: DNA extracted from 2 X10^5 fibroblasts, no PCR product seen.
Lane 10: Negative control with omission of template

Figure 2.10b Assessment of sensitivity of ARMAS-PCR for detection of p53 mutations in exon 8 and 9 in SW480 cell DNA dilution into leukocyte DNA measured by spectrophotometry using primers A8MS and A9MAS.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lanes 2 to 7: See Fig 2.18a
Lane 8: Mutant DNA to wildtype DNA dilution in ratio 1:10, a 258bp PCR product seen
Lane 9: Mutant DNA to wildtype DNA dilution in ratio 1:100, a 258bp PCR product seen
Lane 10: Mutant DNA to wildtype DNA dilution in ratio 1:1 000, an extremely faint 258bp PCR product seen
Lane 11: Mutant DNA to wildtype DNA dilution in ratio 1:10 000, no amplification
Lane 12: Mutant DNA to wildtype DNA dilution in ratio 1:100 000, no amplification
Lane 13: Leukocyte DNA, no amplification
Lane 14: Negative control with omission of template
Fig. 2.9

252bp

Fig. 2.10a

129bp

Fig. 2.10b

258bp

See Fig. 2.18a
<table>
<thead>
<tr>
<th>Primers</th>
<th>Reagents</th>
<th>Primer concentration</th>
<th>Salt concentration MgCl₂</th>
<th>PCR denaturation temperature and time</th>
<th>Denaturation and annealing temperature and time</th>
<th>extension</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8MS</td>
<td>E8AS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>144</td>
</tr>
<tr>
<td>A8NS</td>
<td>E8AS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>144</td>
</tr>
<tr>
<td>A8MS</td>
<td>A8NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>129</td>
</tr>
<tr>
<td>A8NS</td>
<td>A8NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>129</td>
</tr>
<tr>
<td>A8NS</td>
<td>A8N2AS</td>
<td>0.2μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>561</td>
</tr>
<tr>
<td>A8MS</td>
<td>A9MAS</td>
<td>0.15μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>258</td>
</tr>
<tr>
<td>A8NS</td>
<td>A9NAS</td>
<td>0.15μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>258</td>
</tr>
<tr>
<td>A9NS</td>
<td>A9MAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>72°C 10min</td>
<td>125</td>
</tr>
<tr>
<td>A9NS</td>
<td>A9NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>72°C 10min</td>
<td>125</td>
</tr>
<tr>
<td>A5MS</td>
<td>A5NAS</td>
<td>0.75μM</td>
<td>1.5Mm</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 65°C 20sec</td>
<td>nil</td>
<td>252</td>
</tr>
<tr>
<td>A5NS</td>
<td>A5NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>252</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
<td>-----------</td>
<td>-------------------------</td>
<td>------------</td>
<td>-----</td>
</tr>
<tr>
<td>A5MS</td>
<td>E6AS</td>
<td>1μm</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>314</td>
</tr>
<tr>
<td>A5MS</td>
<td>E6AS</td>
<td>1μm</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>314</td>
</tr>
<tr>
<td>A5MS</td>
<td>A7MAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>nil</td>
<td>928</td>
</tr>
<tr>
<td>A5NS</td>
<td>A7NAS</td>
<td>(unamplifiable)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A5MS2</td>
<td>A5NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min C</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>72°C 10min</td>
<td>252</td>
</tr>
<tr>
<td>A5MS3</td>
<td>A5NAS</td>
<td>0.25μM</td>
<td>1.5mM</td>
<td>94°C 3min C</td>
<td>94°C 20sec &amp; 64°C 20sec</td>
<td>72°C 10min</td>
<td>252</td>
</tr>
</tbody>
</table>

Table 2. VII Summary of PCR amplification parameters for ARMAS-PCR (cont.).
2.4 Assessment of sensitivity of ARMAS-PCR using various primer combinations

2.4.1 Materials and Methods

2.4.1.1 Cells and DNA preparation

SW480 or CEM cells were diluted in mixtures with normal fibroblasts in mutant: normal cell ratios varying from 1:1 to 1:100 000. The DNA was extracted as above by the proteinase K method (Towner, 1993) from a total of 0.2 X 10^6 of the cell mixture and submitted to ARMAS-PCR, using various primer combinations.

DNA from 10^5, 10^4, 10^3, 10^2, 10, and 1 SW480 cell was also extracted for ARMAS-PCR using primers A8MS, A8NAS and A9MAS to assess the amplification power of PCR.

Average DNA concentrations were calculated according to the Warburg-Christian formula given above (see Appendix A for DNA spectrophotometer readings). Average DNA concentration of DNA derived from 2 X 10^6 cells was 15 µg/ml. 50 nanograms were used for each ARMAS-PCR reaction.

To confirm DNA ratios achieved by dilutions of SW480 cells and fibroblasts, blood leukocyte DNA was also diluted into SW480 cell DNA, following DNA spectrophotometry and calculation using the Warburg-Christian formula.

Using the above optimised conditions (Table 2.VII) for mutation detection, PCR primers were selected to test the sensitivity of ARMAS-PCR. The primers used were designed so that at least one primer was complementary to the p53 mutation at its 3' end, and the other primer complementary to a normal segment of the p53 gene or
complementary to a second mutation point on the gene in the opposite direction (Double ARMAS-PCR).

2.4.2 Results

2.4.2.1 Non-nested ARMAS-PCR

Table 2.VIII Assessment of different primer pairs for the analysis of sensitivity of mutation detection of: SW480 cell DNA mutation in a) exon 8 b) exons 8 and 9 of the p53 gene.

<table>
<thead>
<tr>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A8MS</td>
<td>E8AS</td>
<td>1:100</td>
</tr>
<tr>
<td>A8MS</td>
<td>A8NAS</td>
<td>1:100</td>
</tr>
<tr>
<td>A8MS</td>
<td>A9MAS</td>
<td>1:1,000</td>
</tr>
</tbody>
</table>

Assessment of sensitivity of ARMAS-PCR of primer combinations A8MS/E8AS and A8MS/A9MAS were done in conjunction with Dr Alex Jones (refer to Jones (1998)).

Different detection limits were reached using different primer pairs (Table 2.VIII). A8MS/E8AS detected 1 SW480 cell in 100 normal fibroblasts, while the same was also achieved with A8MS/A8NAS (Fig. 2.10a), producing a smaller product. A8MS/A9MAS employing a “Double ARMS-PCR” was more sensitive, detecting 1 mutant cell in 1,000 normal cells.

Similar limits for detection of the exons 8 and 9 mutations were observed when the SW480 cell DNA was diluted with normal leukocyte DNA (Fig. 2.10b).
Table 2.IX Sensitivity of mutation detection of CEM cell DNA mutation in a) exon 5 b) exons 5 and 7.

<table>
<thead>
<tr>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5MS</td>
<td>A5NAS</td>
<td>1:1</td>
</tr>
<tr>
<td>A5MS</td>
<td>A7NAS</td>
<td>unable to detect</td>
</tr>
<tr>
<td>A5M2S</td>
<td>A5NAS</td>
<td>(1:10 000) see text</td>
</tr>
<tr>
<td>A5M3S</td>
<td>A5NAS</td>
<td>1:1 000</td>
</tr>
</tbody>
</table>

Using CEM cells diluted in fibroblasts, primer pair A5MS/A5NAS was limited to a detection of 1:1 ratio of mutant to normal cells (Fig. 2.11a). No improvements were gained from increasing the temperature from 64°C to 68°C. A5MS/A7MAS designed to generate a long PCR product of 925bp was unable to amplify the small quantity (50 ng) of DNA; PCR product was seen only when 200 ng was used as template.

Primer pair A5M2S/A5NAS with a single deliberate mismatch in A5M2S at the penultimate position produced very faint products and inconsistent products. A faint band was visible at a dilution ratio of 1:10 000 of mutant to wildtype DNA (Fig. 2.11b). Good sensitivity was achieved using primers A5M3S/A5NAS detecting 1 cell amongst 1 000 normal cells (Fig. 2.11c).
Figure 2.11a Assessment of sensitivity of ARMAS-PCR for detection of p53 mutations in exon 5 in CEM cell DNA.

ASMS and ASNAS primers were used to amplify DNA extracted from cell dilutions as listed. EB 2% showing amplification a products of 252bp size up to a cell dilution of (CEM:fibroblast of 1:1)

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X 10^5 CEM cells, PCR product evident
Lane 3: DNA extracted from CEM:fibroblast in ratio of 1:1, PCR product evident
Lane 4: DNA extracted from CEM:fibroblast in ratio of 1:10, no amplification seen
Lane 5: DNA extracted from CEM:fibroblast in ratio of 1:100, no amplification seen
Lane 6: DNA extracted from CEM:fibroblast in ratio of 1:1 000, no amplification seen
Lane 7: DNA extracted from CEM:fibroblast in ratio of 1:10 000, no amplification seen
Lane 8: DNA extracted from CEM:fibroblast in ratio of 1:100 000, no amplification seen
Lane 9: DNA extracted from 2 X 10^5 fibroblasts, no amplification seen
Lane 10: Negative control with omission of template

Figure 2.11b Assessment of sensitivity of ARMAS-PCR for detection of exon 5 in CEM cell DNA.

Using primers ASMS2S and ASNAS to amplify DNA from cell dilution ratios described below. EB 2% showing a 252pb PCR product up to a cell dilution of CEM:fibroblas, 1:10 000

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2X10^5 CEM cells
Lane 3: DNA extracted from CEM:fibroblast in ratio of 1:1, a 252bp product seen
Lane 4: DNA extracted from CEM:fibroblast in ratio of 1:10, a 252bp product seen
Lane 5: DNA extracted from CEM:fibroblast in ratio of 1:100, a 252bp product seen
Lane 6: DNA extracted from CEM:fibroblast in ratio of 1:1 000, a faint 252bp product seen
Lane 7: DNA extracted from CEM:fibroblast in ratio of 1:10 000, a 252bp product seen
Lane 8: DNA extracted from CEM:fibroblast in ratio of 1:100 000, no amplification seen
Lane 9: DNA extracted from 2 X 10^5 fibroblasts, no amplification seen
Lane 10: Negative control with omission of template

Figure 2.11c Assessment of sensitivity of ARMAS-PCR for detection of p53 mutations in exon 5 in CEM cell DNA.

Using primers ASMS3S and ASNAS to amplify DNA from cell dilution ratios described below. EB 2% showing 252bp product up to cell dilution ratio of CEM:fibroblast, 1:1 000

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X 10^5 CEM cells, a 252bp product seen
Lane 3: DNA extracted from CEM:fibroblast in ratio of 1:1, a 252bp product seen
Lane 4: DNA extracted from CEM:fibroblast in ratio of 1:10, a 252bp product seen
Lane 5: DNA extracted from CEM:fibroblast in ratio of 1:100, a 252bp product seen
Lane 6: DNA extracted from CEM:fibroblast in ratio of 1:1 000, a 252bp product seen
Lane 7: DNA extracted from CEM:fibroblast in ratio of 1:10 000, no amplification seen
Lane 8: DNA extracted from CEM:fibroblast in ratio of 1:100 000, no amplification seen
Lane 9: DNA extracted from 2 X 10^5 fibroblasts, no amplification seen
Lane 10: Negative control with omission of template
Nested ARMAS-PCR on extracted DNA

Amplification was attempted using a nested PCR (Simmonds et al., 1990) to increase sensitivity of detection of mutated DNA from both SW480 cells and CEM cells.

The sensitivity and specificity of PCR can be improved by using nested PCR. Two separate amplifications are involved. The first uses a set of primers that yields a large product, which is then used as a template for the second amplification. The second set of primers or a single primer (semi-nested) anneals to sequences within the initial product, producing a second smaller product. The specificity of the reaction increases because formation of the final product depends upon the binding of two separate sets of primers. The sensitivity is also increased because two sets of amplifications, both in the order of 25 cycles, are used (Kawasaki, 1990; Nelson et al., 1996). Nested PCR is commonly used when there is the presence of poor quality or low copy template nucleic acid (Baumforth et al., 1999).

2.4.2.1 SW480 cell DNA

Primers E8S and E9AS antisense (Table 2.1) were used for a first round amplification using previously established optimal conditions and parameters, followed by a second round of amplification with the ARMAS Primers (A8MS and A9MAS). Initial amplification of p53 fragment with E8S/E9AS primers cycled for 35 cycles followed by another 35 cycles with A8MS/A9MAS was associated with a loss of specificity; both mutant and wildtype were amplified. Eight cycles of A8MS/A9MAS did not effect an improvement of sensitivity. The greatest sensitivity without loss of specificity was achieved with 15 cycles of mutation-specific amplification using primers A8MS/A9MAS (Fig. 2.12).
2.4.2.1.2 CEM cell DNA

A semi-nested PCR was attempted for the detection of mutations in exon 5 and/or 7 in CEM cell DNA. An initial amplification of 35 rounds of PCR where performed using E5S and A5NAS, and a 421bp product was produced. When this was followed by 35 cycles using primers A5MS and A5NAS, extraneous bands were produced as well as a band corresponding to the size of the PCR product achieved with the first round of PCR amplification using E5S and A5NAS. This suggested that residual PCR primers from the initial rounds of amplification were available for further amplification during the second round of amplification. A similar strategy to that employed in the SW480 cell mutation detection by nested PCR was then attempted, by decreasing the number of cycles in the second round of PCR amplification. Twelve cycles were used with primers A5MS and A5NAS. However, similar non-specific amplifications were produced. Thirty five cycles of PCR using mutant-specific primers produced a band at the expected size of 252bp at a sensitivity of 1:1 000 which was not seen at dilutions of 1:10 000 and beyond, but all DNA dilution ratios resulted in non-specific amplification.
Figure 2.12 Nested ARMAS-PCR of mutation in exon 8 and 9 of the SW480 cell line.
Nested ARMAS-PCR using primers E8S/E9AS followed by A8MS and A9MAS cycled for 15 cycles. 
EB 2%:

Lane 1: Mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X 10^5 SW480 cells-a 258bp product seen
Lane 3: DNA extracted from SW480:fibroblast in ratio of 1:1-a 258bp product seen
Lane 4: DNA extracted from SW480:fibroblast in ratio of 1:10-a 258bp product seen
Lane 5: DNA extracted from SW480:fibroblast in ratio of 1:100-a 258bp product seen
Lane 6: DNA extracted from SW480:fibroblast in ratio of 1:1 000-a 258bp product seen
Lane 7: DNA extracted from SW480:fibroblast in ratio of 1:10 000-a faint 258bp product seen
Lane 8: DNA extracted from SW480:fibroblast in ratio of 1:100 000-a faint 258bp product seen
Lane 9: DNA extracted from 2 X 10^5 fibroblasts, no amplification seen
Lane 10: Negative control with omission of template

Figure 2.13a SSCP analysis of ARMAS-PCR using primers A8MS and A8NAS.
Lane 1: SW480 DNA as template: mutant banding pattern
Lane 2: Leukocyte DNA as template: very faint mutant bands present
Lane 3: Fibroblast DNA as template: no bands detected

Figure 2.13b SSCP analysis of ARMAS-PCR using primers A8MS and E8AS.
Lane 1: HUVEC DNA as template, no bands detected
Lane 2: Leukocyte DNA as template, no bands detected
Lane 3: Fibroblast DNA as template, no bands detected
Lane 4: SW480 cell DNA as template, mutant bands shown by arrows

Figure 2.13c SSCP analysis of ARMAS-PCR using primers A8MS and A9MAS.
Lane 1: Negative control using SAO-2 p53 null cell line DNA as template
Lane 2: HUVEC DNA as template, no bands detected
Lane 3: Leukocyte DNA as template, no bands detected
Lane 4: SW480 cell DNA as template, mutant bands shown by arrows
2.5 Specificity of primer combinations was assessed using PCR-SSCP

Supplementary analysis of PCR products was undertaken using PCR-SSCP on a 6% polyacrylamide gel. ARMAS-PCR products were analysed using the established method of SSCP to analyse mutation-specific products.

Polyacrylamide gel electrophoresis is used in instances where greater resolution of the gels is required and where there is the need to discriminate between even a single base. In these experiments, SSCP analysis was used to take advantage of the ability of the technique to discriminate between wildtype and mutant (see Section 1.6.1.6 for detailed description of the theory of the technique), combined with more sensitive methods to stain the amplified DNA.

The staining of DNA with ethidium bromide is often the first method of choice, since this method is simple, quick, and comparatively sensitive. Other stains are also available for example: acridine orange, methyl green, pyronin B and toluidine blue O. DNA and RNA in polyacrylamide gel electrophoresis (PAGE) may also be sensitively resolved by a silver stain (Bassam et al., 1991; Merril et al., 1979).

A silver stain is about 2-10 times more sensitive than the ethidium bromide method (0.1 ng/mm² {ethidium bromide} versus 0.03 ng/mm² {silver stain}) (Allen et al., 1989). Bassam et al. (1991) were able to detect 1 pg/ mm² by pre-exposure to formaldehyde during silver impregnation and by addition of sodium thiosulfate to the image developer to reduce background. Lo et al. in (1991) successfully employed the combination of ARMS-PCR and SSCP to distinguish HLA alleles.
2.5.1 Materials and Methods

SSCP was assessed using PCR fragments amplified from human cell lines containing the known p53 mutations indicated above.

2.5.1.1 The optimal conditions for SSCP

Ten microlitres of PCR product and ten microlitres of tracking dye (98% deionised formamide, 10mM EDTA, 0.025% Xylene Cyanol FF, 0.025% Bromophenol Blue) were denatured for 5 min at 95°C then plunged into ice for 3 min and loaded onto a 0.5% MDE™ gel (AT Biochem cat #1-500). The gel was polymerised with TEMED and fresh 10% ammonium persulfate and was equivalent to a 6% polyacrylamide gel. A thermostatically controlled refrigerated circulator (Julabo F40 ultratemp 2000) was connected to a heat exchanger Hoefer gel tank (model# SE600). Thus the 5 L of 0.6 X TBE running buffer (90 mM Tris, 92 mM boric acid, 2.5 mM EDTA) were maintained at a constant preset temperature throughout the run (15°C). The electrophoretic apparatus allowed two, 14 X 16 X 0.5 cm gels to be immersed completely in 0.6 X TBE buffer. Gels were run at 6 watts for five hours.

2.5.1.2 Gel staining

The single-stranded DNA was detected non-isotopically by silver staining (Dockhorn-Dworniczak et al., 1991). The gels were incubated in commercially available staining kit according to manufacturer’s guidelines (Silver Express™ NOVEX cat # LC 6100). Gels were then dried using a Novex large gel drier.

2.5.2 Results

The specificity of the ARMAS-PCR of primer pairs:

A8MS and E8AS
A8MS and A8MAS
A8MS and A9MAS
A5MS and A5MAS

was assessed.

Analysis of ARMAS-PCR products was carried out on SSCP non-denaturing polyacrylamide gels. The SSCP made it possible to detect amplified products with greater sensitivity, and it was also possible to discriminate bands as wildtype or mutant by the difference in banding patterns.

Primer pairs A8MS and A8NAS as shown in Figure 2.5a were unable to specifically discriminate between mutant SW480 DNA and leukocyte DNA (lane 4). Consistent with this, SSCP analysis showed a mutant band (the same banding pattern as that derived from SW480 cells) (Fig. 2.13a lanes 1 and 2).

Primer pairs A8MS and E8AS as well as primer pairs A8MS and A9MAS were specific for the mutation and no nucleic acids were detected from the amplification of wildtype DNA (Fig. 2.13b lanes 2 and 3, Fig. 2.13c lanes 2 and 3), even with silver staining.

Primers A5MS and A5NAS were limited in their detection ability to a ratio of mutant to wildtype of 1:1, as demonstrated in Figure 2.13a. PCR products not observed on 2% agarose gels were analysed on SSCP polyacrylamide gels. Some non-specific products were seen on SSCP gels and pure fibroblast DNA showed the same banding pattern as positive control which were PCR products amplified from mutant p53 DNA.
2.6 Additional strategies: allele-specific

**competitive blocker PCR**

An allele-specific competitive blocker-PCR was used in an attempt to increase specificity and sensitivity. The strategy of Orou et al. (1995) was adopted. Figure 2.14 demonstrates the underlying rationale behind the system. The essential principle is that a primer homologous to the wildtype allele is 3' end labelled with a dideoxynucleotide to serve as a blocking oligonucleotide.

TAC TGG GAC GGA ACA GCT TTG AGG GGC oligonucleotide was synthesised.

This primer is homologous to the wildtype allele, with two modifications. This primer contains a different deliberate mismatch compared to the one in the mutant primer, and was labelled with a dideoxynucleotide at the 3' end. The mismatches cause a hybridisation difference between the competitive oligonucleotides of the mutant primer and the wildtype-dideoxylabelled primer. This also ensures that once the initial fragment is amplified, the melting temperature for the blocker oligonucleotide is lower than the homologous mutant oligonucleotide and this avoids the blockage of a newly synthesised fragment.
Figure 2.14 Schematic illustration of allele-specific competitive blocker PCR.

"U" and "D" denote primers of the first round PCR. Primer "M" contains a deliberate mismatch (○). "Y" denotes the wildtype allele-specific competitive blocker primer which contains a different deliberate mismatch (□). Mismatches which cause a hybridisation difference between the competitive oligonucleotides M and X are indicated (#). Modified from Orou (1995).
2.6.1 Methods for 3' end labelling an oligonucleotide

Labelling of dideoxynucleotide was done according to manufacturer's (Boehringer Mannheim) instructions for the terminal transferase enzyme used to ligate a nucleotide.

Purified oligonucleotides were dissolved in water and concentrations calculated following absorbance measurements and using the formula:

\[
\text{Abs}(260\text{nm}) \times 100 = \text{concentration of single strand nucleic acid } \mu\text{g}/\mu\text{l}
\]

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A 1 mM solution of ddGTP was prepared.

4 µl of 5 X concentrate reaction buffer (1M potassium cacodylate, 0.125 M Tris HCl, 1.25 mg/ml Bovine Serum Albumin; pH 6.6 (25°C)) was added to 4µl of 25 mM CoCl₂, 100pmole oligonucleotide, 1 µl of 1 mM ddGTP, 1 µl (50units) Terminal transferase. The rest was made up to 20 µl of water. The cocktail was mixed and centrifuged briefly. It was then incubated at 37°C for 15 min and placed on ice. The reaction was stopped with a mixture of 200 µl 0.2M EDTA (pH 8) with 1µl of glycogen solution (20 mg/ml) in water. 2 µl of this solution were added to the reaction mixture.

The labelled oligonucleotide was precipitated by adding 2.5 µl 4 M LiCl and 75 µl pre-chilled (-20°C) 100% ethanol and mixed. The mixture was left to precipitate at -70°C for 30 min. Then mixture was then centrifuged at 13 000 g for 5 min at 4°C. The supernatant was discarded and the pellet dried. The pellet was re-dissolved in 20µl of sterile water and stored at -20°C.

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2.6.2 Allele-specific competitive Blocker-PCR

2.6.2.1 Materials and Methods

A non-nested single round ARMAS-PCR as well as a two round nested-ARMAS-PCR was tested with the use of labelled blocker oligonucleotide.

A cocktail of reagents was made of 10 X Buffer (500 mM KCl, 100 mM Tris-HCl pH8.3), primers 20 μM sense and 20 μM antisense, dNTPs (1.25 mM diluted from 100 mM stock solutions, Boehringer Mannheim cat #277-049) and Taq polymerase (5 units/μl Boehringer Mannheim cat #1418-43) for non-nested ARMAS-PCR. In a 40 μl reaction volume, the final concentrations in the PCR were 50 mM KCl, 10 mM Tris-HCl, 0.25 μM of primer A8MS and A9MAS, 0.5 μM dideoxyoligonucleotide labelled primer (blocker oligonucleotide), and 0.2 M dNTPS, 1 unit/20μl of Taq was used to amplify 50 ng of DNA extracted from different cell dilution ratios. Amplification was carried out in a Corbett thermocycler (Corbett Research, Mortlake, NSW, Australia) at 94°C for 3 mins, followed by 94°C for 20 sec and annealed at 64°C for 20 sec for 35 cycles without an extension step.

Nested ARMAS-PCR was performed using a first round amplification with a 40 μl reaction volume and final concentrations in the PCR of 50 mM KCl, 10 mM Tris-HCl, 0.25 μM of primer E8S and E9AS, 0.2 M dNTPS, 1 unit/20μl of Taq and 50 ng of DNA extracted from the different cell dilution ratios. Amplification was carried out in a Corbett thermocycler at 94°C for denaturation for 3 mins, followed by 94°C for 20 sec and 64°C for 20 sec annealing time for 35 cycles without an extension step. This was followed by a nested ARMAS-PCR using the primers A8MS and A9MAS. In a 40 μl reaction volume, the final concentrations in the PCR were 50 mM KCl, 10 mM Tris-HCl, 0.25 μM of primer A8MS and A9MAS, 0.5 μM
dideoxyolignucleotide labelled primer (blocker oligonucleotide), 0.2 M dNTPS and 1 unit/20 µl of Taq was used. Amplification was carried out in a Corbett thermocycler at 94°C for denaturation for 3 mins, followed by 94°C for 20 sec and 64°C for 20 sec annealing time for 15 cycles without an extension step.

2.6.2.2 Results

The allele-specific blocker-PCR did not achieve a greater sensitivity of detection of the gene mutation. Non-nested ARMAS-PCR with allele-specific blocker was able to achieve sensitivity of 1:100 (Fig. 2.15a), while Nested-ARMAS-PCR with allele-specific blocker was able to generate a faint signal at 1:10 000 but no signal was seen at 1:1 000 (Fig. 2.15b).

2.7 Mutation-specificity of ARMAS-PCR

ARMAS-PCR was evaluated for its ability to discriminate between the mutant p53 gene and wildtype alleles, but it was also assessed for its capacity to distinguish between different p53 mutations.

This section of the investigation used primers A8MS and A9MAS specific for mutations in SW480 p53 gene, exons 8 and 9. When these primers were used to amplify CEM cell DNA mutated in p53 exons 5 and 7 the DNA was refractory to amplification (Fig. 2.16).
Figure 2.15a Assessment of sensitivity of detecting mutations in exon 8 and 9 in SW480 cells with allele-specific competitive blocker-PCR and non-nested ARMAS-PCR.

EB 2% gel:

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X 10^5 SW480 cells a 258bp product seen
Lane 3: DNA extracted from SW480:fibroblast in ratio of 1:1, a 258bp product seen
Lane 4: DNA extracted from SW480:fibroblast in ratio of 1:10, a 258bp product seen
Lane 5: DNA extracted from SW480:fibroblast in ratio of 1:100, a 258bp product seen
Lane 6: DNA extracted from SW480:fibroblast in ratio of 1:1 000, no PCR product seen
Lane 7: DNA extracted from SW480:fibroblast in ratio of 1:10 000, no product seen
Lane 8: DNA extracted from SW480:fibroblast in ratio of 1:100 000, no PCR product seen
Lane 9: DNA extracted from 2X10^5 fibroblasts, no PCR product seen
Lane 10: Negative control with omission of template, no PCR product seen

Figure 2.15b Assessment of sensitivity of detecting mutations in exon 8 and 9 in SW480 cells with allele-specific competitive blocker-PCR and nested-ARMAS-PCR.

EB 2% gel:

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: DNA extracted from 2 X 10^5 SW480 cells a 258bp product seen
Lane 3: DNA extracted from SW480:fibroblast in ratio of 1:1, a 258bp product seen
Lane 4: DNA extracted from SW480:fibroblast in ratio of 1:10, a 258bp product seen
Lane 5: DNA extracted from SW480:fibroblast in ratio of 1:100, a 258bp product seen
Lane 6: DNA extracted from SW480:fibroblast in ratio of 1:1 000, no obvious PCR product seen
Lane 7: DNA extracted from SW480:fibroblast in ratio of 1:10 000, a 258bp product seen
Lane 8: DNA extracted from SW480:fibroblast in ratio of 1:100 000, no PCR seen
Lane 9: DNA extracted from 2 X 10^5 fibroblasts, no PCR products seen
Lane 10: negative control with omission of template, no PCR product seen

Figure 2.16 Specificity of ARMAS-PCR using primers A8MS and A9MAS.

EB2%:

Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lanes 2 and 3: CEM cell DNA as template, no products seen
Lane 4: SW480 cell DNA as template, a 258bp product seen
Lane 5: Negative control with omission of template
2.8 Specificity of the ARMAS-PCR reaction: comparison of amplification of matched and mismatched DNA by Taq polymerase (Boehringer Mannheim cat # 1435094)

Extension of base mispairs by Taq DNA polymerase (Chien et al., 1976) from *Thermus aquaticus* BM was assessed. This enzyme was originally isolated from the thermophilic eubacterium *Thermus aquaticus* BM, a strain lacking Taq I restriction endonuclease and purified free of unspecific endo- or exonucleases. The enzyme consists of a single polypeptide chain with a molecular weight of approximately 95kD. It is a highly processive 5'-3' DNA polymerase that lacks 5'-3' and 3'-5' exonuclease activities (Tindall & Kunkel, 1988).

It has been shown that Taq polymerase extends mismatches much less efficiently than correct matches (Creighton et al., 1992; Kuchta et al., 1988; Kunkel & Alexander, 1986; Mendelman et al., 1990; Perrino & Loeb, 1989; Petruska et al., 1988; Yu & Goodman, 1992). One of the major concerns of ARMAS-PCR is the necessity for the concentration of template to be constant, as the Taq polymerase will extend a mismatched 3' end at a rate of $10^6$-fold less than the rate with the matched primer (Huang et al., 1992), but nevertheless, still does generate some DNA. Therefore if sufficient template is used, a band of amplified DNA will result even when there is a 3' mismatch. This experiment was designed to assess the limit of amplification of the Taq polymerase supplied by Boehringer Mannheim.
2.8.1 Materials and Methods

Increasing quantities of wildtype DNA were used as template for amplification using mutant-specific primers: A8MS and A9MAS.

Quantities used: 40ng, 80ng, 160ng, 360ng.

2.8.2 Results

At 360 ng of wildtype DNA a very faint band of non-specific PCR product was seen, showing that this was the limit reached by the kinetics of Taq polymerase (Fig.2.17).

2.9 Reverse transcriptase-ARMAS-PCR (RT-ARMAS) for detection of p53 mutations in mRNA

2.9.1 Materials and methods

Total RNA was extracted from SW480 cells diluted in fibroblasts.

2.9.1.1 Generation of double-stranded cDNA from total RNA using reverse transcriptase

Approximately 15 µg RNA in 5 µl of sterile diethyl phosphorocyanidate (DEPC) treated water were placed on ice and diluted in a 25 µl mix containing 20 pmol oligonucleotide primer A9MAS (Boehringer Mannheim cat# 1496062), transcriptase buffer (0.05 M Tris-HCl pH 8.0, 5.0 mM MgCl₂ and 0.05 M KCl), 40 units RNase inhibitor (Boehringer Mannheim cat #799 017), 10 mM dithiothreitol (DTT), 1.0 mM of each deoxyribonucleoside triphosphate (Boehringer Mannheim cat #1277049) and 25 units AMV reverse transcriptase (RT). This was then incubated at 42°C for 30 min.
2.9.1.2 ARMAS-PCR amplification of mutant cDNA

A cocktail of reagents was prepared with 10 X Buffer (500 mM KCl, 100 mM Tris-HCl pH8.3), primers 20 µM sense and 20 µM antisense, dNTPs (1.25 mM diluted from 100 mM stock solutions, Boehringer Mannheim catt #277-049) and Taq polymerase (5 units/µl Boehringer Mannheim cat #1418-43). A 40µl reaction volume was used and the final concentrations in the PCR were 50 ng of cDNA 50 mM KCl, 10 mM Tris-HCl, 1.0 µM of each primer, 0.2 M dNTP and 1 unit/20 µl of Taq.

2.9.2 Results

Mutant mRNA was successfully amplified via cDNA using ARMAS-PCR mutation-specific primers A8MS and A9MAS (Fig. 2.18a lanes 1-5). The same PCR parameters previously determined to be optimal for the amplification of genomic mutant DNA were adopted for the ARMAS-PCR of mutant cDNA initially. This did not permit selective amplification. ARMAS-PCR was optimised by changing primer concentrations (Fig. 2.18b). Specific discrimination between mutant and wildtype cDNA was achieved at 0.05 µM primers and 1.5 mM MgCl₂ (Fig. 2.18b lane 4), which produced a good positive control, using the wildtype primers (Fig. 2.18c lane 1 to 4).
Figure 2.17 Examination of Taq polymerase extension of base mispairs. Taq was able to extend a mismatch at 320ng of DNA.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: 40ng of wildtype HUVEC DNA, no amplification seen
Lane 3: 80ng of wildtype HUVEC DNA, no amplification seen
Lane 4: 160ng of wildtype HUVEC DNA, no amplification seen
Lane 5: 320ng of wildtype HUVEC DNA, a 258bp product seen
Lane 6: 60ng SW480 cell DNA, a 258bp product seen: positive control
Lane 7: Negative control with omission of template

Figure 2.18a ARMAS-PCR of mutant mRNA from SW480 cells using mutation-specific primers A8MS/A9MAS.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lanes 2 to 6: Mutant RT-PCR products using A8MS and A9MAS: a 165bp product.

Figure 2.18b Optimisation of RT-ARMAS-PCR for mutations in exon 8 and 9 of SW480 cells.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: 0.01μM primers used, no amplification evident
Lane 3: 0.025μM primers used, no amplification evident
Lane 4: 0.05μM primers used, no amplification evident, optimal for RT-ARMAS-PCR
Lane 5: 0.125μM primers used, a non-specific product seen.

Figure 2.18c Optimisation of RT-ARMAS-PCR. Positive control amplification of wildtype DNA with wildtype primers, all producing a 165bp product.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lane 2: 0.01μM primers used
Lane 3: 0.025μM primers used
Lane 4: 0.05μM primers used
2.10 Amplification capacity of PCR

DNA extracted from a decreasing number of cells ranging from $10^4$, $10^3$, $10^2$, 10, to 1 cell was amplified using ARMAS-PCR primer sets A8MS combined with A9MAS and primers A8MS combined with A8NAS. The primer pair A8MS and A8NAS was clearly able to amplify DNA from a single cell, however, there was a decrease in intensity with amplification of 100 cells (Fig. 2.19a). Amplification using A8MS and A9MAS was only able to produce a faint PCR product from the DNA of 10 cells.

PCR products generated using A8MS and A9MAS were also analysed using PCR-SSCP. Whereas agarose gel stained with ethidium bromide showed a very faint band with amplification of DNA from 100 cells, the corresponding PCR-SSCP revealed amplified DNA from 1 cell (Fig. 2.19b).

2.11 Comparison of sensitivity of ARMAS-PCR and PCR-SSCP for detection of mutations

In a collaborative assessment of sensitivity of different systems of mutation detection with Dr Alex Jones, the ARMAS-PCR was compared with PCR-SSCP. ARMAS-PCR was shown to be at least 10 times more sensitive, and with the use of nested-ARMAS-PCR, up to 1 000 times more sensitive than PCR-SSCP (Low et al., 2000).
Figure 2.19a Amplification capacity of PCR using primers A8MS/A9MAS and A8MS/A8NAS.
EB 2% agarose gel.
Lane 1: mw IX (Boehringer Mannheim cat # 1449460)
Lanes 2 to 7: primers A8MS and A9MAS were used generating a 258bp product
Lane 2: DNA from 100 000 cells
Lane 3: DNA from 10 000 cells
Lane 4: DNA from 1 000 cells
Lane 5: DNA from 100 cells
Lane 6: DNA from 10 cells
Lane 7: DNA from 1 cell
Lane 8: Negative control with omission of template
Lanes 9 to 14: Primers A8MS and A8NAS were used, generating a 129bp product
Lane 9: DNA from 100 000 cells
Lane 10: DNA from 10 000 cells
Lane 11: DNA from 1 000 cells
Lane 12: DNA from 100 cells
Lane 13: DNA from 10 cells
Lane 14: DNA from 1 cell
Lane 15: Negative control with omission of template

Figure 2.19b SSCP analysis of PCR products from Figure 2.19a lanes 2 to 7.
Lane 1: DNA from 100 000 cells, mutant bands evident (black arrows)
Lane 2: DNA from 10 000 cells, mutant bands evident
Lane 3: DNA from 1 000 cells, mutant bands evident
Lane 4: DNA from 100 cells, mutant bands evident
Lane 5: DNA from 10 cells, mutant bands evident
Lane 6: DNA from 1 cell, very faint mutant bands evident (blue arrows)
2.12 Controls for ARMAS-PCR

Amplification of the entire exon using intronic primers was used as control. DNA extracted from the SAOS-2 cells, a p53 null-mutant human cell line was used as a negative control. The omission of template was also used as a negative control against external contamination. All controls performed as anticipated.

2.13 Verification of PCR products produced during amplification by ARMAS-PCR

PCR products generated by ARMAS-PCR primers listed above were sequenced to confirm true amplification from the human p53 gene. Automated sequencing was used at the Westmead Hospital (Westmead, NSW, Australia) sequencing facility. The facility was equipped with a Perkin Elmer Applied biosystems (ABI) 373A DNA sequencing system.

PCR products were purified prior to sequencing, using Polyethylene Glycol (PEG) precipitation. 45 μl of PCR product were added to 45 μl of 2 X PEG precipitation mix (PEG 8000 26.7%, 0.6 M Sodium acetate pH5.2 6.5 mM MgCl₂). This mixture was left at 4°C for 10 min. DNA was pelleted by centrifugation for 15 min at 13 k in an Eppendorf tube. The supernatant was removed by gentle suction and respun for 10 sec. The last of the PEG was removed. DNA pellets were washed twice with 300 μl of 95% ethanol and spun for 15 min after each wash. Residual ethanol was removed and the pellets dried for 5-10min. Pellets were resuspended in 20 μl of water. 50-100 ng per 300 bp of PCR fragment were used in each sequencing reaction.
Sequencing was performed by the Westmead Sequencing facility, Westmead Hospital NSW, Australia. Dye terminator fluorescent sequencing was used, where the dideoxy terminators were labelled. AmpliTaq FS a mutant form of Taq DNA polymerase was used.

2.13.1 Results

Figure 2.20 shows an example of the results of the fluorescent-labelled sequencing for exon 9 using primers A8MS and E8NAS.
Figure 2.20 Fluorescent labelled sequencing data for PCR product generated by A8MS and E8NAS.
2.14 Discussion

The concept of the clonal nature of the evolution of human malignant tumours implies that due to the accumulation of mutations, a mutation or mutations must be present at early stages in a minority of cells in essentially normal tissue. Thus, the majority of the template DNA extracted for analysis may originate from the stromal tissue or tumour tissue lacking the mutation. Tumour molecules could be less than 5% of the wildtype which is often below the limits of mutation scanning methods (Cotton, 1997).

Sensitive techniques of detection are required when the mutation of interest is only present in a small population of cells in tumour samples. Analysis of mutations that contribute to the process of generation of uncontrolled growth in tumour cells may offer prognostic information, information about the causative mutagen, and assist in assessment of success of therapy. Other areas of need for mutation detection include: examination of one gene in a population to obtain the spectrum of mutations causing disease, mutation directly associated with inherited disease, screening a population for disease, assessing differences between species/strains of microorganisms/animals/plants.

With the advent of PCR, in vitro amplification of DNA has enormously increased the sensitivity of detecting mutations (O Leary et al., 1997). PCR is able to provide a large quantity of the DNA region of interest from the sample available. This not only aids the visualisation of the data, but aids mutation detection in systems in which the mutation might be only weakly differentiated from the wildtype, as is the case with some mutations in the gene of the p53 protein.

The p53 protein is a multifunctional transcription factor that plays a role in the control of cell cycle progression, DNA integrity and the survival of cells exposed to
DNA damaging agents. By 1998, over 9 300 different p53 mutations had been reported in human tumours as compiled in an electronic database: http://www.iarc.fr/p53/homepage.htm (Hainaut et al., 1998). 95% of the known p53 mutations fall within the DNA binding domain (Hernandez-Boussard et al., 1999), and the majority are point mutations.

Ideally, the procedure for mutation detection would be accurate, fast, inexpensive, provide precise information about the position and nature of the detected mutation, require relatively little effort, lend itself to automation, and avoid the use of dangerous reagents.

ARMAS-PCR, a subgroup of the techniques classified as allele-specific PCR amplification systems, was used in this study. ARMAS-PCR actually depends upon the actual process of amplification rather than analysis of the amplification product. The ARMAS-PCR was selected as a detection method because of its simplicity, requiring only a single PCR reaction. It also had the potential to be applied further in conjunction with other analysis techniques such as in situ hybridisation (to be discussed in Chapter 3).

The system relies on the characteristics of the PCR reaction including the primer design, which also influences the annealing temperature. The reagent concentrations and, importantly, the inability of the polymerase to extend a mismatched primer are significant.

The technique consists solely of PCR amplification; all technical considerations were the same as those for PCR (Innis, 1990; Kocher, 1991; Old, 1994; Rybicki, 1994; Taylor, 1992). Primers were designed to be about 30 base pairs to increase annealing temperature to between 55-80°C. The GC content of primers was greater than 50%. Other factors applicable to all PCR reactions were considered
during the designing of all primers. Primers with long runs with more than three or four of the same base were not considered ideal, as were primers with a secondary structure (for example, hairpin loops) or those containing sequences that are complementary to each other. This will avoid the annealing of primers to each other (primer-dimer formation). Palindromic sequences were avoided. The ARMAS-PCR placed certain restrictions on these designs in that it was necessary to position at least one of the primers with its 3' end at the point of mutation. Therefore the flanking sequence to the mutation determined the design of the primer. Designs were also aimed so that Double-ARMAS-PCR may have been used, therefore primers were designed to amplify the region between two known mutations.

2.14.1 Primer design:

Taq polymerase was the enzyme of choice because it does not possess a 3' to 5' exonuclease. However, despite the PCR primer having a mismatch at the 3' end, Taq polymerase will actually extend the primer, albeit at a very much lower rate than normal. This rate was found to be 10^6-fold less than the rate with the matched primer (Huang et al., 1992). Kwok et al. (1990) looked at the effect of mismatches at the 3'end of primers using Taq polymerase to amplify DNA using an HIV model system. Their purpose was to find conditions that allowed amplification deliberately in the presence of mismatches so that PCR conditions for finding rare variant virus genomes could be optimised. Using 29 base pair primers, a primer annealing temperature of 55°C and standard PCR reaction conditions including 200 μM of each dNTP, only 4 of the possible 12 mismatches (primer-template) were inefficiently extended: C-C, G-A, A-G and to a lesser extent A-A. They also found mismatches to be symmetrical that is, A-G had the same effect as G-A. PCR products were detectable after 30 cycles
of all other mismatches. Their results supported the work of Ehlen et al. (1989), who found that at 200 \( \mu\)M of dNTP, C-T mismatches were extended, but at 2 \( \mu\)M dNTP C-T, A-C, C-C mismatches were not extended. Wu et al (1989) used smaller-sized primers (14 base pairs) showing that A-A and T-T mismatches were not extended. According to Rhodes et al (1997) A-C and A-G provided the best mismatch pair. In contrast, work by Okayama et al. (1989) showed that 10 out of the 12 possible mismatches tested prevented significant amplification. Newton et al. (1989) demonstrated that G-T, T-G, A-C, C-A mismatches were extended by Taq polymerase, whereas A-A, T-T, and C-T were refractory to amplification contrary to results of Kwok et al. (1990).

The degree of destabilisation designed into the additional deliberate mismatches upstream from the 3' terminal end should be sufficient to prevent extension of the primer mismatched at its 3' terminal base but not so strong as to prevent extension of the primer matched at its terminal base. The ability of Taq polymerase to extend a 3' terminal mismatched primer probably depends not only upon the strength of the non-Watson-Crick base pair, but also on the level of stacking interactions between adjacent bases on the same strand. Thus it is the actual sequence of a particular DNA fragment, not just the GC content which determines the Tm and also the overall steric structure of the terminus to be extended. The amplification results presented in this study were comparable to studies mentioned above (summarised in Table 2.X). The sequence of the 3' terminal ends of primers and their non-Watson-Crick base pairs (Table 2.XI) were the determinants of specificity and sensitivity.
Table 2.X Base pair mismatches ideal for ARMAS-PCR: summary of results from different investigators.

<table>
<thead>
<tr>
<th>Best primer: allele mismatch to prevent significant amplification in ARMAS-PCR</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-G, T-C</td>
<td>(Rhodes et al., 1997)</td>
</tr>
<tr>
<td>C-C, G-A, A-G</td>
<td>(Kwok et al., 1990)</td>
</tr>
<tr>
<td>C-T, A-C, C-C</td>
<td>(Ehlen &amp; Dubeau, 1989)</td>
</tr>
<tr>
<td>A-A, T-T</td>
<td>(Wu et al., 1989)</td>
</tr>
<tr>
<td>A-A, T-T, C-T</td>
<td>(Newton et al., 1989)</td>
</tr>
</tbody>
</table>

Table 2.XI Efficiency of elongation of primer-target mismatches by Taq polymerase (Kwok et al., 1990).

<table>
<thead>
<tr>
<th></th>
<th>T</th>
<th>C</th>
<th>G</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>/</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>&lt;0.01</td>
<td>/</td>
<td>1.0</td>
</tr>
<tr>
<td>G</td>
<td>1.0</td>
<td>/</td>
<td>1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>A</td>
<td>/</td>
<td>1.0</td>
<td>&lt;0.01</td>
<td>0.05</td>
</tr>
</tbody>
</table>

/ Watson-Crick base pairs
Table 2.XII Base pair strengths and destabilising ability (Hawkins, 1997).

<table>
<thead>
<tr>
<th>Base pair</th>
<th>Base pair strength</th>
<th>Destabilising strength</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-C, A-T</td>
<td>Maximum</td>
<td>None</td>
</tr>
<tr>
<td>G-T, A-C</td>
<td>Strong</td>
<td>Weak</td>
</tr>
<tr>
<td>G-G, A-A</td>
<td>Medium</td>
<td>Medium</td>
</tr>
<tr>
<td>C-C</td>
<td>Weak</td>
<td>Strong</td>
</tr>
<tr>
<td>G-A, C-T, T-T</td>
<td>Very Weak</td>
<td>Maximum</td>
</tr>
</tbody>
</table>

Table 2.XIII SW480 and CEM cell mutations and primer mismatches to wildtype allele

Table 2.XIIIa SW480 cell line.

<table>
<thead>
<tr>
<th>c</th>
<th>exon 8 mutation</th>
<th>exon 9 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>G-C</td>
<td>C-G</td>
</tr>
<tr>
<td>mutant</td>
<td>A-T</td>
<td>T-A</td>
</tr>
<tr>
<td>mismatch</td>
<td>A-C</td>
<td>C-A</td>
</tr>
</tbody>
</table>

Italic letters correspond to 3’ terminal base of the designed mutant primer.

Table 2.XIIIb CEM cell line.

<table>
<thead>
<tr>
<th>c</th>
<th>exon 8 mutation</th>
<th>exon 9 mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wildtype</td>
<td>G-C</td>
<td>C-G</td>
</tr>
<tr>
<td>mutant</td>
<td>A-T</td>
<td>A-T</td>
</tr>
<tr>
<td>mismatch</td>
<td>A-C</td>
<td>G-T</td>
</tr>
</tbody>
</table>

Italic letters correspond to 3’ terminal base of the designed mutant primer.

Mutation in exon 8 of SW480 cells is a G to A transversion. The primer with its 3’ end terminating at the mutation creates a A–C mismatch which, according to
Hawkins 1997 (Table 2.XII), possesses a strong base pair strength. The same mismatch was created in exon 9.

The results demonstrated that specific and sensitive amplification was greatly assisted with the incorporation of a destabilising deliberate mismatch upstream from the 3' end of the primer, as was illustrated by Newton et al. (1989) and Kwok et al. (1990). Since both primers for the two different exons had the same mismatch, refractory amplification would have relied on the additional mismatch. For exon 9 no deliberate mismatch was used; amplification specificity was poor.

According to Hawkins (1997), the degree of destabilisation required depends upon the nature of the 3' terminal mismatch. Purine/pyrimidine mismatches form relatively strong non-Watson-Crick base pairs, whereas purine/purine or pyrimidine/pyrimidine mismatches form very weak pairs (See Table 2.XII). Use of a deliberate A-A mismatch 4 nucleotides upstream from the 3' end in the primer specific for exon 8 mutation was able to destabilise it sufficiently to generate good specific products. A-A mismatches were moderately extended in the above studies (Kwok et al., 1990).

Comparable results were seen with amplification of mutant fragments from exons 5 and 7 of the CEM cells. According to both Newton et al. (1989) and Kwok et al. (1990), G-T mismatch would be readily extended by Taq polymerase. Addition of a T-T deliberate mismatch, which, according to Hawkins (1997) would have produced maximum destabilisation, this was incorporated in primer A5MS. Better results were obtained when a T-G mismatch was placed at the penultimate position of the 3' end of the mutant-specific primer, compared with a T-T mismatch 5 nucleotides upstream (A5MS). The significance may be explained by the fact that mismatches closer to the 3' end of a primer have greater effect on Taq extension than do mismatches upstream.
Newton et al. (1989) and Kwok et al. (1990) found that single internal mismatches did not significantly affect PCR product yield. Similar results were seen when two mismatches were used in the same primer (A5M3S), one 3 nucleotides upstream and as well as the T-T mismatch 5 nucleotides upstream from the 3' end. Primer pairs A7NS and A7MAS could not generate specific products, where an additional mismatch was placed 9 nucleotides upstream from the 3' end. This supports the observation also that mismatches further from the 3' end are less useful for increasing specificity. The presence of multiple mismatches at least 8 bases from the 3' terminus did not have a significant effect on ARMAS-PCR (Kwok et al., 1990).

The ability of ARMAS-PCR to detect the lowest fraction of mutant cells diluted amongst wildtype cells was also dependent on primer design. A8MS and A9MAS along with A5M3S and A5NAS, achieved the greatest sensitivity (1:1 000). This was consistent with the primers' specificity for their respective mutations.

Nested ARMAS-PCR improved the sensitivity by another 100-fold for the detection of SW480 cell line mutations. Extraneous amplification products seen during the semi-nested PCR amplification were most likely to be due to the remnant primers from the first round of amplification. Stoichiometrically handicapping one primer in the first round of amplifications was suggested by Orou et al. (1995). The primers used by Orou for the first round of amplification were much longer in length and the annealing temperature was much higher than the annealing temperature for internal mutation-specific primers.

Orou et al. (1995) also used an allele specific blocker in their PCR system that utilised a primer synthesised with a dideoxynucleotide at the 3' terminal end. This was initially introduced by Seyama et al. (1992), who employed blocker oligonucleotides (18mers) located between two primers (24mers). Use of this in the present study did
not increase sensitivity. This may be mainly attributed to the use of different-sized primers.

The guidelines for allele-specific amplification suggested by authors (Dutton & Sommer, 1991; Sarkar et al., 1990; Sommer et al., 1992) emphasise the use of the lowest concentrations of reagents. Concentrations of MgCl₂ were kept to the minimum required for a product with the requisite specificity. It was found that lowering of primer concentration had the greatest effect, and this was apparent with multiple primer pairs: A8MS/A8N2AS, A5MS/A5NAS, and A5MS/A7MAS.

Specificity of amplification was affected by the use of different downstream primers designed to anneal to wildtype regions of the gene. E8AS was specific for detection of the mutation. It generated a 144 bp product and allowed a relatively good range of parameters. Use of A8NAS as a downstream primer did not prove to be specific. The reason for the non-specific amplification of a mutant PCR product using wildtype DNA for a shorter amplification product as compared with a primer 15 bp downstream has yet to be elucidated. Perhaps in the linear phase of amplification, sufficient mutant primer molecules were extended by Taq, and such small products were then primed and extended by the downstream wildtype primer, resulting in sufficient molecules produced to be detected on an agarose gel. The number of molecules found to be visible by gel electrophoresis is estimated as $10^{11}$ (Rhodes et al., 1997).

Inefficient amplification by some primer pairs (A5MS/A7MAS, A9NS/A9MAS, A8MS/A8N2AS) may have been due to the formation of primer dimers, not anticipated during the design of primers using the “Amplify” program. Dimers were observed on 2% agarose gel as strong staining bands evident at around 70bp.
The GAPDH primers were used in an attempt to amplify the GAPDH gene in the same amplification reaction as the p53 mutant gene. However this was not successful. Theoretical annealing temperature for the GAPDH primers were 56°C and 60°C while for ARMAS-PCR primers A8MS and E8AS they were 63°C and 53°C. However, the optimal annealing temperatures for the two sets of primers as determined in the laboratory actually differed by 10°C. A band corresponding to the size of amplified cDNA was detected. Presence of cDNA was possible as DNA extracted and purified from cells were not digested with RNases. The amplification of genomic DNA of the GAPDH gene should have produced a 679 bp product. Ideally the PCR product generated should be sequenced. Due to the mispriming and amplification, the use of GAPDH primers was abandoned and amplification of genomic DNA from the p53 gene using intronic primers was adopted as the control instead.

A double ARMAS-PCR was used to increase specificity by utilising the two mutations present on the same gene in the SW480 cell lines. This was initially used by Lo et al. (1991) also to increase specificity in their analysis of the 5’ region of the human delta-globin gene. They used the system to elucidate the relationship of polymorphic sites and allowed the determination of haplotypes. Sarker and Sommers (1991) also relied on a similar method with four specific primers to determine four possible haplotypes in the human dopamine D2 receptor. However, the use of this system in the CEM cell line in the present investigation was not as successful. Both the mutant-specific primers (A5MS and A7MAS) and wildtype (A5NS and A7NAS) primer sets were designed to produce a 928 bp product. In the case of longer products of amplification, there is a greater possibility of loops created in which the 3’ end of the product anneals somewhere within the product, and forms a primer structure.
Secondary structures could also be a problem. Reannealing is also more likely. Larger PCR products designed to be applied in a subsequent part of the project (Chapter 3) also proved less satisfactory. Mispriming and mutation-specificity was a problem. Lowering the annealing temperature although issuing more PCR product permitted erroneous extension of wildtype alleles.

Mutant mRNA was successfully amplified by ARMAS-RT-PCR. Mutant primers were used for the generation of cDNA and for the amplification of PCR product. However, other studies have proven that viral reverse transcriptases (AMV RT, HIV-1 RT) and Taq polymerases bind with about equal affinities to matched and mismatched primer-template 3'–termini (Creighton et al., 1992; Wong et al., 1991; Yu & Goodman, 1992), and reverse transcriptases, which lack proof-reading exonuclease activity, appear to extend most mismatched termini more efficiently than Taq polymerase (Creighton et al., 1992; Mendelman et al., 1990; Yu & Goodman, 1992). Therefore, the allele specificity relies on the mutant-specific amplification of cDNA by the Taq polymerase. To my knowledge this is the first report of the use of ARMAS-PCR for the detection of mutant mRNA.

In summary, as pointed out by Rolfs (1992), the specificity of an allele-specific amplification is determined by two kinetic variables: the rate at which the annealed primer dissociates from the template before the primer is elongated (P_off) and the rate at which the annealed primer is elongated by the DNA polymerase (P_elong). Therefore, ideally P_elong ≫ P_off for the matched primer and P_elong << P_off for the mismatched primer. The ability of Taq polymerase to extend a 3' terminal mismatched primer probably depends not only upon the strength of the non-Watson-Crick base pair, but also on the level of stacking interactions between adjacent bases on the same strand. Thus it is the actual sequence of a particular DNA fragment, not
just the GC content, which determines the Tm and also the overall steric structure of the terminus to be extended.

3 Chapter 3: Use of ARMAS-PCR for *in situ* detection of p53 mutations

3.1 Introduction

The polymerase chain reaction represents one of the most exciting developments in molecular pathology, but one important limitation is the inability to visualise and localise the amplified product within the cells and tissue specimens. *In situ* hybridisation (ISH) is a technique for the localisation of specific nucleic acid sequences within individual cells. It is based on the complementary binding of a nucleotide probe to a specific target sequences of DNA or RNA in the cell. These may be endogenous DNA, mRNA, viral sequences, or bacterial sequences.

*In situ* hybridisation allows the localisation of nucleic acids in morphologically preserved cells, but may be insufficiently sensitive to allow the detection of low copy nucleic acids by non-isotopic ISH. Lack of sensitivity also renders ISH alone unable to discriminate point mutations in genomic DNA from wildtype DNA. Extractive methods have therefore been essential for demonstrating and defining the specific mutation in most PCR-based techniques but the destruction of the tissue precludes specific identification of cells containing the mutation of interest. DNA extraction followed by PCR does not provide information on the proportion, location or clonal distribution of mutated cells, and whether there is a monoclonal or polyclonal proliferation of cells with specific mutations. *In situ* PCR was an important advance, allowing DNA amplified by PCR to be localised to individual cells while preserving their structure.
In this study we combined the sensitivity and selectivity of ARMAS-PCR (described in Chapters 1 and 2) with the localising power of in situ hybridisation to identify genomic mutations in situ in cultured cell lines. The sensitivity of in situ ARMAS-PCR was compared with that of in situ hybridisation. In situ amplified mutant PCR products were detected using digoxigenin labelled probes. The specificity of in situ ARMAS-PCR has also been assessed using the CEM cells as negative controls. In an extension of this approach, in situ ARMAS-PCR was used to amplify cDNA generated from mutant mRNA by reverse transcription (RT). ARMAS-PCR adapted for in situ use to distinguish specific DNA and mRNA mutations in structurally intact cells has not to our knowledge been reported previously.

3.1.1 In situ hybridisation and its applications

ISH was first described in 1969 (Gall & Pardue, 1969; John et al., 1969), and has now gained widespread acceptance and utility. It has many applications in basic research and in diagnostic pathology. ISH provides a high degree of spatial resolution that permits the localisation of genes to chromosome (Gray, 1993), the identification of translocations and other chromosomal abnormalities (Anastasi et al., 1991; Lee, 1991), and the study of gene expression at the cellular and subcellular levels (Gee & Roberts, 1983; Lawrence & Singer, 1986). It has also been used extensively for the identification of viruses and other micro-organisms (Chang et al., 1992).

Much of the work in the area of the study of gene expression by ISH has related to tumours. It has been useful in the study of endocrine tumours (McNicol et al., 1991), B-cell lymphomas (Speight et al., 1994) and hepatic tumours (Yamaguchi et al., 1993). The technique has been applied widely in the investigation of the expression of range of oncogenes (Gan et al., 1993), growth factors (Hashimoto et al.,
1995), growth factor receptors (Tuck et al., 1996), and adhesion molecules (Dorudi et al., 1995).

Viral infections have been widely investigated and the technique has proved useful both in the identification of specific viral infection and in defining the extent of systematised infection. ISH has been particularly useful in the study of the role of the human papillomavirus in neoplasia (Alonso et al., 1974), cytomegalovirus in encephalitis (Musiani et al., 1994) and Epstein-Barr virus in the pathogenesis of a wide range of human lymphoid and epithelial tumours (Anagnostopoulos & Hummel, 1996). Hybridisation to viral DNA and mRNA in the liver has aided the understanding of the complexity of hepatitis (Naoumov et al., 1993; Wu et al., 1996).

Genotypic changes have been identified by hybridisation to chromosome or single copy gene sequences in interphase nuclei. Aneuploidy, by the use of probes complementary to repeat sequences specific for individual chromosomes is identified by the use of the alpha repeats located close to the centromere. Translocations are investigated using probes hybridising to regions close to the known breakpoints (Taylor et al., 1993). This is a growing area that is translating to diagnostic practice.

3.1.1.1 Advances in detection methods

Recent advances have been developed that have substantially improved the detection of mRNA and DNA. The use of non-radioactive molecules to reveal the sites of hybridisation has been considered superior to radioactive molecules in terms of resolution, probe stability and personal safety (Koji & Nakane, 1996). Oligonucleotide probes have recently become popular because any sequence can be synthesised, whereas previously double-stranded DNA probes were the most commonly encountered for DNA detection and RNA riboprobes for RNA detection. The choice of label is governed to some extent by personal preference and
commercial availability. Isotopic labelling is now largely confined to RNA detection. The most commonly used non-isotopic labels used are biotin and digoxigenin, but fluorescein is also frequently chosen. One advantage of using a fluorochrome as hapten is that both direct fluorescent detection and indirect immunoenzymatic detection can be used for the same probe.

Labelling probes is most commonly performed using enzymatic methods. DNA probes are conveniently labelled by either nick translation or randomly primed extension, both of which introduce labelled nucleotides such as digoxigenin-11-dUTP into the probe molecules. RNA probes are generally labelled by \textit{in viro} transcription whereby the probe sequence is cloned into a vector containing RNA polymerase promoter sites, and probe molecules generated using phage RNA polymerase mediated incorporation of labelled nucleotides such as digoxigenin-11-dUTP. Oligonucleotides can be labelled during synthesis, when 5' labelled nucleotides can be incorporated directly into the molecule. Alternatively, enzymatic procedures can be used for either 5' or 3' labelling. An advantage of 3' end labelling is that multiple probe labels can be introduced to form a tail, thus increasing the sensitivity of detection. Probe labelling may also be carried out using polymerase chain reaction methods with either direct incorporation of labelled nucleotides or by using labelled primers (reviewed in Southern \& Herrington (1998)).

\textbf{3.1.2 \textit{In situ} PCR}

\textit{In situ} PCR is the marriage of two established technologies in molecular genetics, the polymerase chain reaction and \textit{in situ} hybridisation (ISH). It is based on the amplification within intact cells or tissue sections of specific gene sequences, or mRNA species to levels detectable by ISH and/or immunohistochemistry (Long, 1998).
3.1.2.1 Terminology

The notion of employing a PCR-based amplification step to increase the sensitivity of in situ hybridisation came to several laboratories independently in the late 1980s (Haase et al., 1990; Staskus et al., 1991). Many groups have now reported successful combination of these technologies using various names: in situ PCR, PCR in situ, PCR in situ hybridisation, in-cell PCR, PCR driven ISH or PRINS (Bagasra et al., 1993; Embleton et al., 1992; Nuovo et al., 1991a; Patterson et al., 1993; Staskus et al., 1991; Teo & Shaunak, 1995). More recently an intracellular reverse transcription (RT) step to generate cDNA from mRNA templates prior to in situ PCR has been used for the intra-cellular detection of low copy mRNA sequences. This has been termed in situ RT-PCR (Komminoth et al., 1994) or RT in situ PCR (Nuovo et al., 1992) or in situ cDNA PCR (Chen & Fuggle, 1993).

3.1.2.2 Principles of in situ PCR

The basic principles of in situ PCR are illustrated in Figure 3.1. In situ PCR may be performed either in a micro-Eppendorf tube, with fixed cells suspended in the PCR reaction mixture, or on glass slides, with the cell preparation or tissue section affixed. Cell suspensions are recovered after PCR amplification and cytocentrifuged onto glass slides for subsequent study by ISH and histochemistry or flowcytometric study (Embleton et al., 1992; Haase et al., 1990; Long et al., 1993; Patterson et al., 1993). Various groups have found that PCR performed on cells suspended in PCR reaction mixture provided optimal physical conditions and yielded maximal cellular target sequence amplification. It is postulated that nucleic acids were better preserved in single cell preparations and fixed cells act as “amplification sacks” with semi-permeable membranes which permit the primers, nucleotides and DNA polymerase to pass into the cell nucleus, yet sufficiently retarding the outward diffusion of the PCR
products, to allow for their detection \textit{in situ} (Long, 1998). However, the potential applications of \textit{in situ} PCR with cells in suspension are limited. More useful is the amplification of cells and tissues fixed to microscope slides. Slides are placed on purpose-built thermal cyclers and overlaid with PCR reaction mixture.

Detection of intracellular PCR products has been achieved by either of two methods: indirectly by ISH following the amplification step (indirect \textit{in situ} PCR) or through direct incorporation of labelled nucleotides (e.g. Digoxigenin-11-dUTP, fluorescein-dUTP, 3H-CTP) during the PCR process (direct \textit{in situ} PCR).

Direct \textit{in situ} PCR has proved less reliable, and has produced more false positive results, especially when working with tissue sections (Komminoth & Long, 1994; Komminoth et al., 1992; Long et al., 1993; Nuovo et al., 1991a; Sallstrom et al., 1993; Teo & Shaunak, 1995). The false positive signals result from primer-independent incorporation of labelled nucleotides into fragmented endogenous DNA undergoing "repair" by the DNA polymerase, or by "priming" of non-specific PCR products by fragments of DNA or cDNA. The indirect method by using ISH probes that recognise only the amplified sequences provides maximum specificity in detection of intracellular PCR products.

Reporter systems also affect the quality or applicability of \textit{in situ} PCR. Radioactively labelled probes offer high sensitivity, but have significant disadvantages related to cost, instability and biohazard. Enzyme-staining methods based on either alkaline phosphatase, or horseradish peroxidase enzymes have been most frequently employed. Fluorescence base detection systems have also been employed, but may not be practical when it is essential to visualise the cell and tissue context and do not provide a permanent preparations unlike immunoenzyme labelling techniques.
The efficiency of *in situ* DNA amplification is consistently lower than in solution phase PCR. The amplification has been estimated to be approximately 50-fold after 30 cycles in suspended cells and even lower in cytospin preparations and tissue sections (Komminoth et al., 1992).
Figure 3.1 Principles of in situ PCR (Gu, 1995).

Samples can be individual cells in suspension or solid tissue samples (A). In most cases, the samples can be made into cytopsins, smears or tissue sections. Chromosomal preparations may also be used (B). DNA in the nuclei is denatured (C & D) and is then bound by primers. The primers serve as the starting sites for initiation of the polymerisation reaction and form new complementary chains of nucleotides. The two chains extend beyond the boundary defined by the primer of the opposite strand of the DNA template (E). The second cycle repeats the first and uses the original DNA as well as the two newly synthesised strands as templates for making new complementary strands (F). The third cycle repeats the process to make more products and the reaction repeats in a thermocycler. By the end of n cycles, the sequence encased by the 5’ of the two primers (short products) becomes $2^{n-1}$ copies and the long products become $2 \times n$ copies (G and H). A possible mechanism for the amplified products to stay in situ is that the long products serve to anchor some of the short products as well as to form networks among themselves at the sites of the original templates (I). The amplified sequences and the original sequences are detected by specific probes (J). The label is recognised by specific antibodies and visualised by markers (K). The cells that contain the amplified DNA are detected (L).
Figure 3.1 Principles of in situ PCR (Gu, 1995).
3.1.2.3 Applications of \textit{in situ} PCR

Many groups have reported successful \textit{in situ} PCR detection of specifically amplified single copy nucleic acid sequences in single cells, and low copy DNA or RNA sequences in tissue sections. Most of the studies have focused on the detection of viral or proviral nucleic acid sequences, but \textit{in situ} PCR has also been applied to study endogenous DNA sequences. These have included human single copy genes, rearranged cellular genes (Embleton et al., 1992; Komminoth et al., 1992) and chromosomal translocation (Long et al., 1993) and mapping of low copy number genomic sequences in metaphase chromosomes. Successful amplification and detection of low copy number mRNA (Chen & Fuggle, 1993; Heniford et al., 1993; Martinez et al., 1995; Nuovo et al., 1995; Patel et al., 1994; Staecker et al., 1994) and viral RNA sequences have also been reported (Komminoth et al., 1994; Lau et al., 1994; Nuovo et al., 1993).

Detection of viral or foreign DNA within cells has been the major application of \textit{in situ} PCR. \textit{In situ} PCR has major potential in medicine and biology. The study of genetic determinants of tumourigenesis, including DNA mutations or chromosomal translocations, aims to increase our understanding of cause and effect as well as the latency period between DNA alteration/s and morphologic characteristics and morphologic characteristics of atypia or malignancy. Suggestions have been also for the use of monitoring radiation, or other treatment-induced mutations and presence of residual disease.

\textit{In situ} RT-PCR has an obvious application where the level of gene expression is below that detectable by ISH. This would be a good correlative technique for Northern blotting and solution-phase RT-PCR.
3.2 Materials and Methods

3.2.1 Cells

Pure cultures of SW480, CEM cell lines, HUVEC cells and fibroblasts were cultured separately on chamber slides (Nunc, Naperville, USA), washed with PBS and then fixed for 30 min in 4% paraformaldehyde (PFA) at 4°C, followed by 2 washes in PBS, prior to in situ amplification. Alternatively, 0.25 $\times\ 10^6$ cells were harvested, washed in PBS, fixed in 4% PFA, followed by washing twice in PBS and cytopspun at 150 000 rpm onto silane-coated slides for in situ amplification.

3.2.1.1 Direct comparison of normal and mutated cells fixed on slides

HUVEC cells were plated on chamber slides at $2\times 10^4$ cells/cm$^2$ and left to grow overnight. SW480 cells were then added to these chambers at concentrations of $2\times 10^4$ cells/cm$^2$, $4\times 10^3$/cm$^2$ and $2\times 10^3$/cm$^2$ to produce final anticipated ratios of mutated to normal cells of 1:1, 1:5 and 1:10 in culture chambers.

3.2.1.2 In situ ARMAS-PCR in fixed cells suspended in solution

ARMAS-PCR amplification was confirmed by performing in situ ARMAS-PCR on cells suspended in PCR solution in thin-walled Thermowell PCR tubes (Corning Costar Corporation, Cambridge). $2.5\times 10^5$ of each cell type were fixed and washed as above and resuspended initially in PBS in thin-walled PCR tubes.
3.2.2 Primers

The best performing primers were selected from previous studies (described in Chapter 2) examining extracted DNA for the detection of the mutated p53 gene of interest by ARMAS-PCR.

A8MS and A9MAS or E8AS were used for the detection of mutant p53 in SW480 cells. A8NS and A9NAS were used to amplify wildtype p53 in normal cells. A5M2S and A5NAS were used for CEM cells.

3.2.3 Synthesis of digoxigenin-labelled single stranded genomic DNA and cDNA probes for use in situ ARMAS-PCR

3.2.3.1 Background

Biotinylated and digoxigenylated probes can be generated by polymerase chain reaction as mentioned above. Such probes were initially used both for Southern blotting and FISH detection of multicopy sequences and were amplified from cloned sequences (An et al., 1992; Celeda et al., 1992; Emanuel, 1991; Link et al., 1992). In this study, probes were generated from total genomic DNA using PCR, an approach introduced by Richard et al. (1994).

3.2.3.2 Probe synthesis

Single-stranded digoxigenin (DIG) labelled DNA probes were prepared by amplified primer extension labelling (Paine 1995), using A8MS and A9MAS or E8AS primers and 250ng DNA or cDNA template. In summary, 100 μl of PCR solution was used that contained 250-500 μg genomic DNA or cDNA in the same protocol as that described in Section 2.3.1 and 2.9.1. RNA extracted from SW480 or HUVEC cells was obtained by the method of Chomczynski et al. (1987), as described in section
2.1.2.2. Primers A9MAS or A9NAS were used to generate cDNA from RNA. PCR products were purified by a commercially available purification kit according to the manufacturer's guidelines (Qiagen PCR purification kit cat# 28106, Qiagen, Hilden, Germany) or by Peg purification as described in Section 2.13.

RT-PCR and PCR products were used to generate sense and antisense DIG-labelled probes by asymmetric PCR. Asymmetric PCR was carried out for 50 cycles, using template, 5 µl 10 X dNTP/DIG-labelled dUTP and 20 pmole of either A8MS, A9MAS or A9NAS for each reaction. PCR products were then analysed on a 2% agarose gel to confirm that the labelled products were heavier than unlabelled products. Probes were dot blotted on nylon membranes (Hybond N+, Amersham, Buckinghamshire, UK) and labelling efficiency assessed using colorimetric detection with NBT and X-Phosphate according to the manufacturer's (Boehringer Mannheim, Basle, Switzerland) instructions.

3.2.3.3 Assessment of probe specificity by Southern analysis

PCR products were electrophoresed on 2% agarose gel and PCR products were transferred by Southern blotting on to Hybond™ N+ nylon membrane (Amersham) according to manufacturer’s instructions. Specificity of probe hybridisation was confirmed by membrane hybridisation according to the manufacturer's (Boehringer Mannheim, Basle, Switzerland) guidelines for filter hybridisation of digoxigenin labelled probes.
3.2.4 In situ amplification of mutant genomic DNA in whole cells

3.2.4.1 In situ ARMAS-PCR on cells fixed on slides

SW480, HUVEC or fibroblasts were washed in PBS, permeabilised with 0.3% Triton-X/PBS for 15 min, washed twice in PBS for 5 min and post-fixed in 4% PFA for 3 min at 4°C. The cells were then washed in PBS and equilibrated with 1 X PCR buffer (50 mM KCl, 10 mM TrisHCl, 2.5 mM MgCl₂). A hot start PCR was performed, using a 100μl PCR solution, containing 50 mM KCl, 10 mM TrisHCl, 5.0 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP, dTTP and 0.8 μM primers (A8MS and A9MAS) preheated to 75°C, while cells were also pre-warmed to 75°C on an Omnislide thermocycler (Hybaid, Integrated Sciences Australia). Five units of Taq was added to the pre-warmed PCR solution and applied to the cells on slides. PCR solutions were confined on the slides with a Gene frame (Integrated Sciences, Sydney, Australia). PCR was performed on the Omnislide thermocycler, with an initial denaturation of 3 min at 94°C, followed by 1 min at 94°C, 1 min 30 sec at 64°C (annealing) and 1min 30 sec at 74°C (extension) for 35 cycles.

3.2.4.2 In situ ARMAS-PCR on cells in suspension

SW480, HUVEC or fibroblasts in Thermowell tubes were resuspended in 100 μl PCR solution containing 50 mM KCl, 10 mM Tris HCl, 5.0 mM MgCl₂, 0.2 mM dATP, dGTP, dCTP, dTTP, 0.8 μM A8MS and A9MAS primers and 5 units of Taq. Amplification was carried out in a Corbett thermocycler at 94°C for denaturation for 3 mins, followed by 94°C for 1 min, 64°C for 1 min 30sec annealing time and 74°C for 1 min 30 sec extension time for 35 cycles. Following amplification, cells were pelleted by centrifugation (5 000rpm). The post-amplification supernatant was
collected for analysis. The pellets of cells were washed twice in PBS and DNA extracted from the nuclei as according to Towner et al. (1993) to detect PCR products that had been generated in the cells in situ. The supernatant collected from the PCR reaction with the cells and the DNA extracted from the cells following PCR were electrophoresed separately on a 2% agarose gel and visualised by ethidium bromide staining.

3.2.4.3 In situ reverse transcription-ARMAS-PCR of mutant or wildtype mRNA in whole cells

SW480 and HUVEC cells, cultured and fixed on slides were permeabilised and post-fixed as above, washed in PBS, then equilibrated with 100μl reverse transcriptase (RT) buffer (50 mM Tris HCl, 8 mM MgCl₂ 30 mM KCl 1 mM dithiothreitol pH8.5). RT-PCR generating cDNA from mRNA was performed using 100 μl volume containing 50 mM Tris HCl, 8 mM MgCl₂, 30 mM KCl, 1 mM dithiothreitol pH 8.5, 0.05 mM dATP, dTTP, dCTP, dGTP, 0.8 uM downstream primer (A9MAS for mutant mRNA amplification or A9NAS for wildtype mRNA amplification), 40 units AMV reverse transcriptase and 160 units of RNase inhibitor. Slides were incubated at 42°C for 30 min. Hot start PCR was performed to amplify mutant cDNA as described above for DNA amplification. To amplify wildtype cDNA the A9MAS primer was replaced by A9NAS and the same protocol as above followed.

3.2.4.4 In situ hybridisation for in situ ARMAS-PCR

After thermocycling, cells were washed in 2 X SSC (1 X SSC =0.15 M NaCl, 0.015 M Sodium citrate, pH7). A new Geneframe (Integrated Sciences, Sydney, Australia) was attached to contain solutions applied. 100μl of prehybridisation solution (50% deionised formamide, 6 X SSC, 5 X Denhardt’s solution, 0.1 mg/ml salmon sperm

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DNA, 10% dextran sulfate, 5 mg/ml sodium pyrophosphate, 0.5% SDS and 1 mM levamisol) were applied gently to each slide and incubated in a humidified chamber at 37°C for 2 hours. Slides were removed from the hybridisation chamber, drained and 100 µl hybridisation solution applied comprising the prehybridisation solution with digoxigenin labelled probe at 1 ng/µl. Slides were heated to 95°C for 10 minutes to denature amplified products and hybridisation was performed for 12 hours at 37°C. This was followed by a digoxigenin detection system as previously described (Paine et al., 1995) to visualise amplified products. *In situ* hybridisation alone followed by digoxigenin detection was also performed on a sample without prior amplification.

### 3.2.4.5 Controls

As a positive control, wildtype p53 was amplified in normal HUVEC cells, using primers A8NS (normal-sense) & A9NAS (normal-antisense) and detected as described above. As negative controls, Taq polymerase or primers were omitted from the PCR step.

### 3.3 Additional strategies

Additional strategies were attempted to increase amplification *in situ*.

### 3.3.1 Longer PCR products

Various groups (Komminoth et al., 1992; Teo & Shaunak, 1995) found that successful detection of amplified DNA in cells relied on the retention of PCR products *in situ*. It was hypothesised that a larger product may increase the chance of retention within the cell. Primer A8N2AS described in section 2.3.2.1.1 was designed to anneal further downstream of the mutation point in exon 8 of the p53 gene in SW480 cell line.
3.3.2 Use of agarose to limit diffusion of PCR product

According to Chiu et al. (1992) preheated PCR reagents (described in sections 3.2.4.1 and 3.2.4.3) were added with 2.5% molten SeaKem GTG agarose. This was used to attempt to contain the PCR products in situ.

3.3.3 Modification of primers

A strategy according to Chiu et al. (1992) was adopted, in which the upstream mutant primer A8MS was modified at its 5' end with a sequence abbreviated as "T" (Fig. 3.2), while the downstream primer A8NAS was also modified at its 5' end with a sequence that is complementary to "T". These complementary sequences were added to the PCR primers to result in perfect direct repeats at each end of the primary product, which in turn acts both as primer and template for further secondary polymerisation that generates high molecular weight concatamers illustrated in Figure 3.2.

![Diagram](image)

Figure 3.2 Formation of larger in situ PCR products.

Complementary primer tails "T" result in the formation of tandem repeat sequences at each end of the primary PCR products. The products themselves become primers as well as templates for secondary polymerisation (Chiu et al., 1992).
3.4 Results

3.4.1 Assessment of probe generation and labelling

Probe generation and labelling was confirmed first by electrophoresis and then by Southern blotting and filter hybridisation. Concentrations of the probe were calculated using dot blot analysis against standards supplied by the manufacturer.

3.4.2 In situ ARMS PCR - in situ hybridisation for mutated genomic DNA

Using primers A8MS and A9MAS, mutant genomic DNA was amplified in situ in SW480 cells prepared by plating and culturing or as cytopsin preparations on glass slides. PCR products were successfully visualised as an intense purple coloured precipitate using the digoxigenin-labelled probe for the region of exon 8 to exon 9 in the p53 gene (Fig. 3.3A). Greater than 80% of the SW480 cells were labelled; some cells showed greater nuclear staining than others. It was also noted that some cells showed a distinct perinuclear staining (red arrow), suggesting an accumulation or entrapment of PCR product at the nuclear membrane. In contrast, the normal fibroblasts with wildtype p53 gene were refractory to amplification using the same primers (Fig. 3.3B). The concentrations of primer pairs, Taq polymerase and MgCl₂ were increased compared with solution phase DNA amplification, from 0.2μM to 0.8 μM, 1 unit to 5 units, and 2.5 mM to 5.0 mM respectively. Reaction conditions were also changed, increasing denaturation and annealing times and addition of an extension step which had not been required for solution phase amplification.
3.4.2.1 Specificity of ARMAS-PCR amplification products

Mixtures of mutant cells (SW480) and normal HUVEC plated onto slides were used to assess the specificity ratios of in situ ARMAS-PCR followed by in situ hybridisation. HUVECs were chosen to take advantage of the obvious distinction in size and morphology from SW480 as the basis for an accurate identification of cell types. In situ ARMAS-PCR amplification was able to detect a majority of the mutant cells, demonstrated in Figures 3.4A to C showing the detection of mutant cells amongst normal HUVEC cells in different dilution ratios. The sensitivity of detection was however not 100% (Fig. 3.4D). A number of SW480 cells recognisable by their morphology stained less intensely (Fig. 3.4D, red arrow), consistent with in situ ARMAS-PCR amplification of pure cultures of SW480 cells where a small percentage of SW480 cells remained unstained or lightly stained (Fig. 3.3A, yellow arrow). HUVEC DNA was refractory to amplification and the nuclei of these remained unstained. The HUVEC with their characteristic abundant cytoplasm and ill-defined cell membrane were readily distinguished morphologically from SW480 cells.

3.4.2.2 In situ ARMAS-PCR is mutation-specific

In situ ARMAS-PCR amplification was not detected in CEM cell lines using the above primers designed for detecting mutations in exons 8 and 9 of the SW480 cells (Fig. 3.5). CEM cells have a mutated p53 gene but the mutations are in exons 5 and 7, distinct from those of the SW480 cell lines.
3.4.2.3  *In situ* ARMAS-PCR increases sensitivity of mutation detection

Hybridisation alone without prior amplification was unable to detect single copies of the p53 gene in any of the cells (Fig. 3.6A and B).
Figure 3.3 *In situ* amplification of mutated genomic exons 8 and 9 DNA in the p53 gene.

(A) Shows the strong positive signal of PCR products visualised by *in situ* hybridisation with digoxigenin–labelled single stranded probe in SW480 cells as seen by the purple staining. A small percentage of cells were unstained (yellow arrow) or with a perinuclear staining suggestive of entrapment of PCR products at the nuclear membrane (red arrow). (B) Normal Fibroblasts cytospun onto glass slides show no amplified signal.
Figure 3.4 Specificity of in situ ARMAS-PCR ISH for mutated genomic DNA in mutant SW480 cells.

SW480 cells diluted amongst normal HUVECs in varying dilution ratios (SW480:HUVEC). (A) dilution ratio of 1:1, (B) dilution ratio of 1:5, (C) dilution ratio of 1:10 each showing stained cells with amplified mutant p53 gene, black arrows showing unstained HUVECs. (D) A small proportion of SW480 cells identified by morphology were not strongly positive by in situ ARMAS-PCR, followed by in situ hybridisation (red arrow). These were seen in each of the different cell dilution assays. SW480 cells positive by in situ ARMAS-PCR indicated by yellow arrow. Normal HUVECs indicated by blue arrow.
Figure 3.5 Mutation specific in situ ARMS-PCR.

CEM cells tested for in situ ARMS-PCR amplification using primers A8MS and A9MAS specific for SW480 cell p53 mutation. CEM cells showed lack of in situ amplification.
Figure 3.6 In situ hybridisation alone without amplification

In situ hybridisation of SW480 cells (A) and fibroblasts (B) without amplification showed no signal.

Morphology of the cells is better preserved than after ARMAS-PCR (compare with Fig. 3.3).
3.4.3 In situ ARMAS-PCR in cells suspended in solution

*In situ* ARMAS-PCR was performed in cells in suspension, without extracting the DNA from the cells initially (Fig. 3.7). Supernatant removed at the end of thermocycling and electrophoresed in a 2% agarose gel showed a distinct band at the appropriate size (Fig. 3.7A, lane 5 and 7, Fig. 3.7B, lanes 2 to 5), indicating either the presence of template DNA in the supernatant or a leakage of excess PCR products into the surrounding solution during the PCR reaction. The same amplified product was also present in DNA extracted from these same cells post *in situ* PCR but the band was less intense, reflecting dilution with other unrelated genomic DNA (Fig. 3.7A, lane 4). No PCR product was seen in the supernatant of the normal cells (Fig. 3.7A, lane 3, Fig. 3.7B, lanes 7 and 8) or in negative controls where Taq or primer was omitted from the reaction mixture (Fig. 3.7A, lanes 9 and 11, Fig. 3.7B, lane 6) or the unreacted template DNA (Fig. 3.7A, lane 10).

3.4.4 In situ ARMAS-PCR-*in situ* hybridisation for mutant mRNA

Mutant mRNA was successfully converted into cDNA *in situ* and amplified *in situ* in the SW480 cells. Cytoplasmic, but not nuclear, staining demonstrated expression of mutant mRNA in these cells (Fig. 3.8A). Normal HUVEC cells were refractory to amplification with the ARMS primers for this mutation in the SW480 cells (Fig. 3.8B).

Wildtype mRNA of exons 8 to 9 of the p53 gene was amplified *in situ* using primers A8NS (normal-sense) and A9NAS (normal-antisense) in normal HUVEC cells. Signal was detectable in the cytoplasm of the HUVEC cells, but at a reduced
intensity compared with that of the mutant p53 mRNA detected in the mutant SW480 cells.
Figure 3.7 Leakage of DNA into supernatant during \textit{in situ} ARMAS-PCR of cells in suspension.

(A) DNA extracted from SW480 cells showed a faint 258bp PCR product (lane 4) but distinct PCR products in supernatants of PCR mixture (lanes 5 and 7) indicating leakage of the product from the cells during the PCR. Negative controls: extracted DNA with omission of Taq (lane 10) and the supernatant with omission of Taq (lane 11) did not show PCR products. Supernatant of PCR reaction with the omission of primer in lane 9. HUVEC cell DNA was refractory to amplification (lane 3). Molecular weight marker IX (lane 1).

(B) Post-ARMAS-PCR supernatants (lanes 2 to 10). SW480 cells (lanes 2 to 5, 9 and 10). Negative control with omission of Taq polymerase (lane 6). HUVECs (lanes 7 and 8). DNA extracted from HUVEC cells (lane 11). Molecular weight marker IX (lane 1).
Fig. 3.7

A

B
As negative controls for both *in situ* ARMAS-PCR and *in situ*-RT-ARMAS-PCR, Taq polymerase or primer were omitted from the amplification ARMAS-PCR cycle. No staining resulted (Fig. 3.8C).

### 3.4.5 Additional strategies

No improvements were found with the use of primers to generate longer PCR products, nor were improvements found with the use of agarose in the *in situ* PCR mixture.

Modified primers were used to amplify extracted DNA from SW480 cells to assess the effect of complementary primer tails.

A 165 bp product was produced from primers A8MS and A8NAS with complementary tails at the 5' end after 35 cycles. After 50 cycles a larger product was seen at greater than 310 bp. However no improvements were seen in the *in situ* amplification and retention of products during *in situ* ARMAS-PCR. No increase in the proportion of cells detected with PCR products was seen.
Figure 3.8. *In situ* amplification of mutated exons 8 and 9 mRNA in p53.

(A) Strong cytoplasmic staining in SW480 cells that express the mutated p53 gene as detected by *in situ* hybridisation with digoxigenin-labelled single stranded cDNA probe following *in situ* RT-ARMAS-PCR. (B) Shows the lack of staining in normal HUVEC cells under the same conditions. (C) Negative Control. SW480 cells subjected to *in situ* ARMAS-PCR using primers A8MS and A9MAS without Taq polymerase followed by *in situ* hybridisation (unstained) (D) The same negative controls as seen in Figure 3.8C following staining with nuclear fast red.
Figure 3.8
3.5 Discussion

3.5.1 The technique

Since the first report in 1990 (Haase et al., 1990) in situ PCR has undergone rapid development. In situ PCR is still at its incipient stage. There is continuing evolution of the whole technique or of individual steps in the procedure. Various components of the technique have been considered particularly important in the success of in situ amplification.

3.5.1.1 Technical aspects of the in situ amplification

Special treatment of slides by coating with aminosilane or poly-L-lysine has been recommended to avoid the detachment of sections during proteolytic digestion (Chiu 1992). Aminosilane was found to be superior to poly-L-lysine in this study.

To avoid evaporation, a variety of methods has been attempted. Sections with PCR solution have been sealed with nail polish or rubber cement. Special designed cones have also been suggested (Long et al., 1993; Teo & Shaunak, 1995). Use of Gene frames (Integrated Sciences, Sydney, Australia) in these experiments was found to give optimal results, due to the ease of application.

The goal of fixation is to maintain optimal tissue morphology while preserving the integrity of the nucleic acids. Aldehyde-based fixatives, such as 10% neutral buffered formalin have been recommended (Greer et al., 1991; O. Leary et al., 1994). However, they may induce single-stranded breaks that, when repaired by the DNA polymerase, result in false positivity. Cell suspensions and cytology preparations have been routinely fixed in 95% alcohol. Paraformaldehyde has produced good results and
is the fixative of choice for many laboratories (Embretson et al., 1993). This also proved to be the best choice of fixative for the present experiments.

Fixatives cross-link proteins that diminish the accessibility of reagents to the target; pretreatment with proteases is then necessary. A balance has to be achieved to maintain preservation of morphology and false negativity due to poor penetration of reagents in underdigested samples. Various proteolytic agents suggested are proteinase K 10–100 micrograms/ml, trypsin 0.1% and pepsin 0.4%. The present investigation found digestion to be unnecessary for single cell preparations, but it was necessary for tissue sections (Chapter 4).

The polymerase chain reaction was initially optimised using DNA extracted from sample tissue prior to in situ PCR. Similar to the results from other studies, it was found that increases in concentrations of DNA polymerase and Mg\textsuperscript{2+} were required. The concentration of ions was a critical factor in PCR (Wu et al., 1991). Cycling profiles requiring longer annealing times and extension times have also been suggested, while other workers omit the extension step (Nuovo, 1992).

It is believed that in situ DNA amplification occurs at a low efficiency. It is estimated that in 30 cycles, even under the best conditions, amplification of DNA does not exceed 50-100 fold in suspended cells, and may be lower on tissue sections and cytospins (Embretson et al., 1993). The reasons are not clearly understood; although cross-linking of histones DNA, single-stranded DNA breaks and sequestration of DNA polymerase and other reagents on the surface of slide have been proposed as causes (Wu et al., 1991).
3.5.2 In situ ARMAS-PCR for detection of mutant cultured cells

This study introduced the use of ARMAS-PCR for the in situ demonstration of point mutations in the p53 gene. This use of the in situ ARMAS-PCR amplifies and localises a genetic mutation of interest in intact cells and potentially overcomes problems encountered by sequencing studies that analyse extracted DNA from tumour tissue; an approach that lacks the ability to precisely localise mutated cells in the tumour. Microdissection of tissue sections in sequencing studies is routinely used to try to overcome this restriction. The use of non-isotopic in situ hybridisation alone still lacks the sensitivity to reveal cells with point mutations in single copy genes and this is confirmed by the present investigation.

The exquisite sensitivity of in situ ARMAS-PCR is shown by the selective amplification of the mutated DNA of interest in SW480 cells and not CEM cells which carry the p53 mutations at different sites in the gene. In situ ARMAS-PCR has the ability to define the anatomical distribution and proportion of cells with particular DNA sequences in tissues, which is beyond the scope of other methods such as PCR-SSCP, restriction fragment length polymorphism and allele-specific oligonucleotide probe analysis which require extraction of nucleic acids and therefore destruction of the tissue architecture.

Various parameters were modified from the extractive solution phase ARMAS-PCR to be applied to in situ ARMAS-PCR. Additional pretreatments were also required for in situ amplification. Cells were fixed in paraformaldehyde which has been found to give good results by many laboratories (Embreton et al., 1993). The successful adaptation of the ARMAS-PCR for probing intact cells in situ remains dependent upon the allele specificity of the PCR reaction. The lowered kinetics of Taq
polymerase to extend a mismatched primer may be assisted by the physical barriers provided by cellular structures during *in situ* ARMAS-PCR, particularly the cytoplasmic and nuclear membrane. These could restrict access of PCR primers, dNTP and MgCl₂ to the target DNA, and the results simulate a decrease in concentration of these reagents at the reaction site. For this reason the conditions for ARMAS-PCR were modified from those used with extracted DNA, and the concentrations of primer and Taq were increased. The same annealing temperature was used, but the time for denaturation, annealing and extension was extended. The conditions for *in situ* PCR where the DNA is enclosed within the cell and nuclear membranes are far more stringent than those of a solution-phase PCR, where the nucleic acid is freely accessible.

*In situ* ARMAS-PCR has proved to be a very technique-sensitive assay. There are various causes of false negativity and false positivity. Poor thermal conduction by the slide or block, uneven convection, adsorption of reagents to the glass, presence of DNA polymerase inhibitors, evaporation, excessive washing and leakage of reagents can all account for false negative results (Crisan & Mattson, 1993; Grafstrom et al., 1984; Kellogg et al., 1994; Nuovo et al., 1995; Sallstrom et al., 1993). Non-specific incorporation of labelled nucleotides may occur as a consequence of “repair” of DNA breaks by DNA polymerase (Grafstrom et al., 1984). Excessive protease digestion or poor fixation could lead to DNA breaks. Amplification of unwanted sequences according to various groups depends on the specificity of primers, pH, ion concentration in the PCR mixture and annealing temperature (Chen & Fuggle, 1993; Embleton et al., 1992; Komminoth & Long, 1993; Long et al., 1993; Nuovo et al., 1992; Patterson et al., 1993; Teo & Shaunak, 1995). Mispriming occurs at lower melting temperatures and withholding the Taq polymerase until 55°C is reached.
reduced non-specific amplification without compromising the specific primer-target annealing. This is the “hot start” procedure (Kellog 1994, Nuovo 1995, Zaki 1994) which was applied in this study. Other alternatives developed are the Taq antibody system that initially blocks the activity of the enzyme, but the complex dissociates at higher temperatures freeing the enzyme at the appropriate time for specific amplification. Excessive protease digestion and excessive number of amplification cycles may contribute to the diffusion of the amplified product (Long 1993). A product which has diffused from its original location may be preferentially amplified, producing a signal in a different cellular compartment or even in an extracellular location with the possibility of labelling adjacent negative cells (Embroton et al., 1993; Long et al., 1993; Yap & McGee, 1991).

ARMAS-PCR of genomic DNA in situ by using intact cells in suspension confirmed the results of the in situ amplification of mutant p53 in cells fixed on slides. Specific PCR products were obtained when supernatants and extracted DNA from the suspended cells following PCR were electrophoresed on agarose gel. The reduced intensity of the bands derived from DNA extracted from cells post-PCR and the presence of specific PCR product in the supernatant, suggests that a proportion of PCR products escapes from the cell nucleus into the cytoplasm and into the surrounding solution. In addition, escape of minute amounts of PCR products out of the cell in the initial phase of gene amplification may result in rapid amplification of the same product extracellularly, using the readily available reagents with the potential to generate a high background signal. Detection of PCR products with in situ hybridisation is therefore limited by the retention of products in the cell as seen by other studies (Kommitch et al., 1992; Teo & Shaunak, 1995) and by restricted access of the reagents for the PCR.
The retention of PCR product within the cell was also important for the specificity of amplification. This was demonstrated when cells with the mutated gene of interest were scattered amongst normal cells, and in situ ARMAS-PCR used to amplify the mutated gene of interest. A number of mutant cells identified by their morphology were either unstained or lightly stained. Leakage of products may result in the lighter stained effects. This was consistently seen in all cell dilution ratios used. In situ ARMAS-PCR is as yet unable to detect 100% of mutant cells.

In the combined in situ RT-ARMAS-PCR, wildtype p53 mRNA was amplified in situ in normal cells by the appropriate normal p53 primers, but the intensity of signal was diminished compared with that of mutated cells. Relative to the normal cells tested, the SW480 cells, a mutated cell line, may be actively overexpressing mutant p53, resulting in a greater intensity of signal. In situ PCR may provide a simple semi-quantitative way of assessing the amount of intracellular RNA present and other studies have attempted to use in situ PCR in a quantitative manner (Bagasra et al., 1992; Nuovo et al., 1991b).

ARMAS-PCR applied in situ has the potential to selectively amplify genes containing mutations in individual cells. The potential of the approach presented in these experiments is indicated by the results obtained when cultured malignant cells with defined p53 mutations scattered amongst normal cells could be located by in situ ARMAS-PCR. The combination of in situ RT-ARMAS-PCR with in situ hybridisation can be used to discriminate between cells harbouring and expressing genetic mutations from normal cells expressing the wildtype gene.

The polymerase chain reaction has been used extensively to amplify specific gene sequences in extracted DNA for the molecular analysis of many diseases (Wright & Wynford-Thomas, 1990). It is evident from these results that ARMAS-
PCR may have the added potential to be able to be used in preserved tissues from malignant or premalignant lesions with known mutations and this potential was explored (see Chapter 4). The sensitivity and specificity of the method provides a powerful tool for pinpointing and quantitating the cells that contain defined genomic mutations, and the RT-PCR step for generating cDNA from messenger RNA also gives information on active gene transcription. ARMAS-PCR is not, however, suitable for screening for unknown mutations as it requires the mutation to be known in order to design the specific primers. The in situ ARMAS-PCR procedure should be capable of wide application for disclosing and localising any known mutation, whether in cell preparations from cytology samples, fine needle aspiration or tissue specimens. The use of this technique to identify clones of specifically mutated cells in tissues has the potential to become a powerful tool in studies of neoplastic progression.

The adverse effect of the in situ ARMAS-PCR method on the morphology of at least some cells, particularly the normal HUVEC cells is another disadvantage particularly where more than one cell type is under scrutiny. The time consuming or technically demanding nature of in situ ARMAS-PCR will also limit its general use in research or molecular diagnosis.
4 Chapter 4: In situ analysis of tumour clonality and p53 mutations in oral squamous cell carcinoma

4.1 Literature review

4.1.1 Incidence of oral and pharyngeal cancer

4.1.1.1 Incidence and mortality of oral and pharyngeal cancer worldwide

The World Health Organisation (WHO) published the Tenth Revision of the International Statistical Classification of Diseases and Related Health Problems in 1992. For most head and neck cancer studies prior to 1999, sites were mainly coded according to the International Classification of Diseases 9th revision (ICD-9). Certain publications, including those from the NSW Cancer Council, have more recently used the coding system of according to the International Classification of Disease for Oncology, 2nd edition (ICD-O-2)(World Health Organisation, 1978). Statistical comparison of the occurrence and mortality of oral cancer is often made difficult by the grouping of different sites by various authors. Over 80% of head and neck cancers (excluding skin tumours) are squamous cell carcinomas of the oral mucosa, tongue and lips (Cancer research campaign, 2000). “Oral cancer” has been described as sites defined by ICD-9 140-146 and 149 (Hindle & Nally, 1991) or ICD-9 140-149 (La Vecchia et al., 1997), ICD-C00-06, C09-10 (Llewellyn et al., 2003) while others have included nasopharyngeal cancers (ICD-9 147) (Brennan et al., 1995). “Oral and pharyngeal cancers” have also been described by ICD-9 140-149, while “Head and neck cancer” usually includes cancers of the larynx and paranasal sinuses (Brennan et
al., 1995). However cancers of the lip (ICD-9 140), salivary glands (ICD-9 142) and nasopharynx appear to have a different aetiology and incidence and mortality to cancers of the tongue, oral cavity, mouth, oropharynx and hypopharynx. Therefore, for the purpose of the current dissertation, "oral cancer" refers to cancers of the tongue, mouth or oral cavity, oropharynx and hypopharynx in accordance with Boyle et al. (1995). In the 9th Revision, cancer of the oral region was classified as follows (World Health Organisation, 1978):

**Tongue cancer**  
ICD-9 141, ICD-10 C01 and C02

**Mouth cancer:**

- **Gum cancer**  
ICD-9 143, ICD-10 C03

- **Cancer of the floor of the mouth**  
ICD-9 144, ICD-10 C04

- **“Other mouth cancer”**  
ICD-9 145, ICD-10 C05 C06

- **Oropharyngeal cancer**  
ICD-9 146, ICD-10 C09 and C10

- **Hypopharyngeal cancer**  
ICD-9 148, ICD-10 C13

- **Oral cavity cancer**  
ICD-9 149, ICD-10 C14

Estimates by Parkin et al. (1999) of the global total of new cases of oral cancers per annum including lip, salivary gland as well as the mouth, gum and tongue (ICD-9 140-145) were 212 000 in 1990, without including pharyngeal cancers. The incidence for cancer of the oral cavity as a proportion of all malignancies varies greatly ranging between 2.4% in a developed country such as Japan to 33% in Mumbai, India, a developing country (Sortino & Milici, 1998). Oral cancer (including cancers of the pharynx and nasopharynx) was ranked the third most common malignant disease in developing countries, while in developed countries it is the seventh (Parkin et al., 1999). Approximately three-quarters of all cases of oral cancer occur in the developing countries (Macfarlane et al., 1994a). In Central and Southeast
Asia, it accounts for up to 40% of all cancers, whereas in most industrialised countries it is relatively uncommon, accounting for less than 4% (Johnson, 1991). In India, about 56 000 new cases (ICD-9 140-145) are estimated to occur each year, and there are approximately 100 000 individuals suffering from oral cancer in the Indian population, in any given year (Sanghvi, 1981). To date India still has the highest incidence rates in the world for mouth cancers (ICD-9 143-145).

4.1.1.2 Oral cancer in Australia: incidence and mortality

In 1994, 1906 new oral cancers (all sites) were registered in Australia, with 804 new intra-oral cancers (ICD 9 141, 143-145). The tongue is the most common site for intra-oral cancers in Australia, and the majority of intra-oral cancers are in men. Three thousand one hundred new cases of oral and pharyngeal cancer (ICD-9 140-149) were recorded in Australia and New Zealand in 1990, an increase from 2 200 in the year 1985 and 1 900 recorded in 1980. The crude incidence rates during these periods also rose from 17.2 to 17.7 per 100 000 for males for the years 1980 to 1990 and for females from 5.0 to 6.4 per 100 000 (Parkin et al., 1988; Parkin et al., 1993; Parkin et al., 1999).

In NSW, oral cancer (ICD-9 141, 143-145, 146, 148 and 149) incidence rose both for men and women. The rates increased in men from 6.5 per 100 000 in 1974-1976 to 9.3 per 100 000 in 1984-86, while for women, the rates increased from 2.1 to 3.0 per 100 000. Inner Sydney and Western regions had greater rates than the average for NSW (Macfarlane et al., 1994b). In 1997, the NSW Cancer Council reported 499 new cases of oral cancers including tongue, mouth, oropharynx, hypopharynx and pharynx unspecified (ICD-O-2: C01-02, C03-06, C09-10, C12-13, C4) (Coates & Tracey, 2000).
4.1.1.3 Changes in incidence and mortality of oral cancer worldwide

In 1991, 1,815 new registrations of oral cancer (ICD-9 140, 141, 143-145) were recorded in England and Wales. This was 15.6% higher than in 1971 (Worrall, 1995). A study by La Vecchia et al. (1997) found that between 1990 and 1992, the mortality rate for oral cancer (ICD-9 140-149) in men was highest in Hungary (17/100 000), followed by France (13/100 000). In most European countries studied, the mortality rate was higher during this period than between 1955 and 1959. In Finland, Iceland, U.K. and Ireland there was some decline in mortality between 1990 and 1992.

In the U.S.A. between the period of 1988-1992, the incidence of oral and pharyngeal cancer (ICD-9 140-149, including lip, pharynx: nasopharynx, hypopharynx, oropharynx and tonsil and pharynx NOS and ill-defined sites) was 10.7 per 100 000. The age-adjusted rate for oral cavity sites (ICD-9 140-145) was 7.7 cases per 100 000. In the 20-year period (1973-92), the estimated overall age-adjusted incidence rates for oral and pharyngeal sites combined for races and sexes in the U.S.A. decreased by 0.4% per year, for the oral cavity sites, although the incidence trends varied with specific site. Increases of 1% in tongue cancer and 0.8% in cancers of “other sites” ICD-9 145 were recorded (Swango, 1996). Similar results were reported by Shiboski et al. (2000). Oral cancer (ICD-O codes C00.0 – C08.9) among white men declined from 13/100 000 in 1973 to 10/100 000 in 1996. Both white men and women experienced a significant 7% increase in age-adjusted incidence rate of tongue cancer. A recent issue of concern has been the increase in incidence of oral cancer in a cohort of patients of a younger age group, (Llewellyn et al., 2001) with for example, among white men aged 30-34 years and among white women aged 25 (Shiboski et al., 2000).
According to Pisani et al (1999) 197 000 deaths were recorded per year in 1990 due to cancer of the oral cavity and pharynx (ICD-9 140-145, 147,146,148, 149). Mortality from mouth cancer is particularly high in Melanesia followed by south-central Asia where the habit of chewing tobacco and betel quid is popular. Men in Europe are also particularly affected with 16 500 deaths per year compared with females of the same area of 4 500 deaths per year (Pisani et al., 2002).

The mortality resulting from intra-oral cancers was 299 in 1994 in Australia, an estimated 95% of which are squamous cell carcinomas (SCC). Intra-oral SCC therefore accounted for approximately one per cent of all cancer-related deaths in Australia (AIHW & AACR, 1998).

4.1.1.4 Risk factors for oral cancer

Collectively the use of tobacco, alcohol abuse and a deficient diet probably account for more than 90% of head and neck cancers (Johnson, 2001; Wynder et al., 1957).

Tobacco

Tobacco smoke in cigarettes, cigars, pipes or bidis or used as snuff is carcinogenic to humans (International agency for research on cancer, 1986). In excess of 300 carcinogens and pro-carcinogens have been found in tobacco smoke or in its water-soluble compounds, which will contaminate saliva.

Alcohol

Heavy alcohol consumption, irrespective of which beverage is taken is another important risk factor for oral cancer (Blot et al., 1988; Brugere et al., 1986). Tobacco and alcohol in combination have a super multiplicative rather than merely an additive effect on the risk of oral cancer (Franceschi et al., 1990).
Betel quid

The chewing of betel quid is certainly carcinogenic if tobacco is included (IARC 1986), (Daftary et al., 1992), and probably also if tobacco is omitted.

Ultraviolet light radiation

Ultraviolet light radiation predisposes to lip cancer especially of the vermillion margin of the lower lip (de Visscher & van der Waal, 1998). And increased exposure to sunlight is associated with high incidence rates of lip cancer (Moore et al., 1999).

Dietary factors.

A low intake of fresh fruit and vegetables is linked to an increased risk of oral cancer (De Stefani et al., 1999; McLaughlin et al., 1988). Although protective individual micronutrients have not been formally identified, patients with iron deficiency resulting in oral epithelial atrophy and the Patterson-Brown-Kelly (Plumer-Vinson) syndrome had a higher incidence of upper aero digestive tract carcinoma (Wynder & Bross, 1957).

Viruses

There is evidence that the herpes simplex virus (HSV) under particular circumstances may be oncogenic (Scully, 1983; Scully, 1992). It has been reported that HSV can act synergistically with chemical carcinogens in causing oncogenic transformation (Johnson, 2001). HSV-2 interacts with oncogenic types of HPV to induce tumours in vitro (Dipalo et al., 1990; Iwasaka et al., 1988).

High risk oncogenic types of human papilloma virus such as HPV-16 and 18, which are responsible for most carcinomas of the cervix, may also be involved in head and neck cancers (Miller & Johnstone, 2001; Scully, 2002). HPV DNA has been identified in a proportion of oral cancers, especially basaloid squamous cell carcinomas of the base of the tongue and tonsil (Gillison et al., 2000). However, such
studies need to be carefully controlled as oncogenic HPV has been found in normal oral mucosa.

**Candida**

Chronic hyperplastic candidosis has a significant risk of malignant change but allowance should be made for the fact that many patients are tobacco smokers (Arendorf & Walker, 1979). *In vitro*, candida is capable of metabolising pro-carcinogens to carcinogens (Krogh et al., 1987).

**Syphilis**

Tertiary syphilitic glossitis may undergo malignant change (Meyer & Shklar, 1967) and tertiary syphilis may be a risk factor for oral cancer when concurrent major risk factors such as tobacco and alcohol have been taken into account (Wynder et al., 1957; Wynder & Bross, 1957).

**Genetic factors**

People with a family history of head and neck squamous cell carcinoma were at increased risk for oral cancer, even when smoking habits and alcohol consumption were considered as confounding factors (Copper et al., 1995; Foulkes et al., 1995).

However, to exclude the possibility that non-genetic environmental factors could be responsible, concordance studies in twin pairs are useful. The study of Lichenstein et al. (2002) of over 44 000 pairs of monozygotic and dizygotic twins found no evidence of inherited factors in cancer of the lip, oral cavity or pharynx. Although genetic polymorphism for metabolising and activating enzymes for metabolising or activating carcinogens or pro-carcinogens or DNA repair may play a part in susceptibility to oral cancer (Scully et al., 2000), it seems that environmental factors are overwhelmingly important in most sporadic cancers (Lichtenstein et al., 2002).