MOLECULAR DETECTION OF MINIMAL RESIDUAL DISEASE IN BREAST CANCER AND LEUKAEMIAS USING p53 TUMOUR SUPPRESSOR GENE MUTATIONS AS MARKERS

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CONTENTS

CONTENTS		**i**
DECLARATION		**viii**
ACKNOWLEDGEMENTS		**ix**
LIST OF ABBREVIATIONS		**xi**
ABSTRACT		**xv**

CHAPTER 1 INTRODUCTION		**Page**
1.1	**Haemopoietic progenitor cell transplantation (HPCT)**	**2**
1.1.1	HPCT in breast cancer	2
1.1.1.1	Theoretical considerations	3
1.1.1.2	Clinical experience	4
1.1.2	HPCT in acute leukaemias	6
1.1.2.1	HPCT in AML	7
1.1.2.2	HPCT in ALL	9
1.2	**Minimal residual disease detection in breast cancer**	**11**
1.2.1	Significance of MRD in bone marrow	11
1.2.2	Significance of MRD in haemopoietic grafts	12
1.2.3	Methodologies of MRD detection	13
1.3	**Minimal residual disease detection in acute leukaemia**	**17**
1.3.1	MRD detection in ALL	17
1.3.2	MRD detection in AML	21
1.4	**The p53 tumour suppressor gene**	**23**
1.4.1	p53 biology	23

1.4.1.1	Introduction	23
1.4.1.2	p53 and cellular proliferation - an oncogene?	23
1.4.1.3	p53 and cell cycle negative regulation - a tumour suppressor gene?	24
1.4.1.4	p53 protein structure	25
1.4.1.5	p53 protein function	25
1.4.2	p53 and cancer - general aspects	27
1.4.3	Profiles of p53 mutations in human cancers	30
1.4.4	p53 databases	31
1.4.5	p53 and sporadic breast cancer	32
1.4.6	p53 and acute leukaemias and myelodysplasia (MDS)	34
1.5	**Mutation detection**	**37**
1.5.1	Denaturing gradient gel electrophoesis (DGGE)	39
1.5.1.1	Principle	39
1.5.1.2	Use of GC-clamps	40
1.5.1.3	PCR primer design	42
1.5.1.4	Gradient concentrations	42
1.5.1.5	Constant denaturant gel electrophoresis (CDGE)	43
1.5.1.6	Temperature gradient gel electrophoresis (TGGE)	43
1.5.2	Single strand conformation polymorphism (SSCP)	43
1.5.3	Chemical cleavage of mismatch (CCM) / hydroxylamine-osmium tetroxide (HOT) technique	45
1.5.4	RNase a cleavage of heteroduplexes	46
1.5.5	Carbodiimide (CDI) modification	46
1.5.6	Heteroduplex analysis (HA)	46
1.5.7	Comparison of techniques	47
1.5.8	Amplification refractory mutation system (ARMS)	48
1.6	**Aims of the project**	**50**

CHAPTER 2 MUTATION DETECTION AND ANALYSIS 52

2.1 Mutation detection - general methods 52

2.1.1 Selection of control cell lines and DNA 52

2.1.2 Tissue culture techniques 53

2.1.2.1 Materials 53

2.1.2.2 General culture method 53

2.1.2.3 Re-plating of cells 54

2.1.2.4 Cryopreservation of cells 55

2.1.3 DNA extraction 55

2.1.3.1 Materials 55

2.1.3.2 DNA extraction from paraffin blocks 56

2.1.3.3 DNA extraction from haemopoietic tissues 57

2.1.3.4 Measurement of DNA concentration 57

2.1.3.5 DNA storage 58

2.1.4 Polyacrylamide and agarose gel electrophoresis 58

2.1.4.1 Materials 58

2.1.4.2 Methods 59

2.1.5 The polymerase chain reaction (PCR) 60

2.1.5.1 Materials 60

2.1.5.2 Preparation of reagents 60

2.1.5.3 p53 exons amplification 60

2.1.6 Denaturing gradient gel electrophoresis (DGGE) 63

2.1.6.1 Materials 63

2.1.6.2 Parallel DGGE 64

2.1.6.3 Perpendicular DGGE 67

2.2 Optimisation of DGGE 69

2.2.1 Suitability of tonsil DNA as a WT control 69

2.2.2 Sensitivity of DGGE in detecting mutations 71

2.2.3 Effect of electrophoresis time on DGGE 73

2.2.4 Effect of polyacrylamide cross-linker on DGGE 73

2.2.5	Effect of running temperature on DGGE	76
2.2.6	Effect of electrophoresis voltage on DGGE	77
2.2.7	Use of perpendicular dgge to assess effectiveness of denaturant gradient	77
2.2.8	Quality of the denaturant solutions and age of the gels	78
2.3	**Mutation detection in breast cancer patients**	**80**
2.3.1	Patient selection	80
2.3.2	Nature and source of materials	81
2.3.3	Results	81
2.4	**Mutation detection in acute leukaemia patients**	83
2.4.1	Patient selection and nature and source of materials	83
2.4.2	Results	83
2.5	**Mutation analysis - general methods**	**86**
2.5.1	Materials	86
2.5.2	Extraction of mutant homoduplex band from DGGE	86
2.5.3	Preparation of sample for sequencing	87
2.5.4	Use of TaqI restriction endonuclease in difficult samples	88
2.6	**Sequence results from breast cancer patients**	**90**
2.7	**Sequence results from leukaemia patients**	**94**
2.8	**Summary**	**94**
CHAPTER 3 AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)		**96**
3.1	**Materials**	**97**
3.2	**Design of ARMS primers**	**97**
3.3	**Optimisation of ARMS reactions - cell line model**	**100**

3.3.1	Annealing temperature (Ta)	100
3.3.2	Magnesium concentration ([Mg])	102
3.3.3	Presence of glycerol	102
3.3.4	Presence of formamide	104
3.3.5	Effect of cycle number	104
3.3.6	Primer concentrations ([primers])	107
3.3.7	dNTP concentrations ([dNTP])	107
3.3.8	Use of 1 versus 2 mismatches at 3' end of arms primer	112
3.3.9	Conclusions from optimisation experiments	112
3.4	**Mixing tumour cells in donor buffy coat white cells**	**114**
3.5	**ARMS model for MRD detection**	**115**
3.5.1	Use of internal control	115
3.5.2	Limits of detection	115
3.6	**Attempts at improving limits of detection**	**116**
3.6.1	Using nested PCR	116
3.6.2	Using 32P radiolabelled nucleotides in PCR	119
3.6.2.1	Using α-32P radiolabelled dCTP	119
3.6.2.2	Using γ-32P radiolabelled ARMS primer	123
3.7	**Results of ARMS optimisation and sensitivity in clinical samples**	**125**
3.7.1	ARMS in breast cancer samples	125
3.7.2	Use of 32P-labelled PCR in the 5 optimised systems	135
3.7.3	ARMS in acute leukaemia sample / case report of patient L11	139
3.8	**MRD detection in breast cancer patients**	**142**
3.9	**MRD detection in acute leukaemia patient**	**152**

3.10	**Summary**	**155**

CHAPTER 4 RESTRICTION SITE-GENERATING POLYMERASE CHAIN REACTION (RG-PCR) APPROACH **157**

4.1	**Introduction**	**157**
4.2	**Design of mutagenic primers**	**158**
4.3	**Use of RG-PCR in the T47D model**	**160**
4.4	**Use of RG-PCR in breast cancer samples**	**165**
4.5	**Summary**	**165**

CHAPTER 5 DISCUSSION **171**

5.1	**Use of p53 as disease marker**	**173**
5.2	**Mutation detection**	**174**
5.2.1	Choice of technique	174
5.2.2	Proportion of tumour tissue required	176
5.2.3	Choice of primers and DGGE conditions	177
5.2.4	Coverage of possible mutations	180
5.2.5	Presence of p53 mutations in clinical samples	181
5.3	**Amplification refractory mutation system (ARMS)**	**184**
5.3.1	Use of arms in the detection of minority clones	184
5.3.2	Optimisation of arms	185
5.3.2.1	Addition of a deliberate mismatch	186
5.3.2.2	Alterations of other parameters	187
5.3.2.3	Nature of primer-template mismatches	190
5.3.2.4	Sensitivity of ARMS	191
5.4	**Restriction site-generating polymerase chain reaction (RG-PCR)**	**194**

5.5	**Clinical relevance of MRD in PBPC harvests of breast cancer patients**	**197**
5.6	**Clinical relevance of MRD in PBPC harvests of leukaemia patients**	**200**
5.7	**Conclusions - are p53 mutations useful as markers of MRD?**	**200**

REFERENCES **203**

APPENDIX **226**

DECLARATION

I declare that this thesis is my own work and effort, planned, performed and written by myself, unless otherwise specifically stated in the acknowledgements.

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LIST OF ABBREVIATIONS

[......]	Concentration of ......
32P	32-Phosphorus
AAT	Alpha 1 anti-trypsin
ABMT	Autologous bone marrow transplantation
ACCOG	Anglo-Celtic Cooperative Oncology Group
AgT	Large T antigen
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
APS	Ammonium persulphate
ARMS	Amplification refractory mutation system
ASO	Allele-specific oligonucleotide
BM	Bone Marrow
BMT	Bone marrow transplantation
bp	Base pair
BuCy	Busulphan, cyclophosphamide
CCM	Chemical cleavage of mismatch
CDGE	Constant denaturant gel electrophoresis
CDI	Carbodiimide
cDNA	Complementary deoxyribonucleic acid
CEA	Carcinoembryonic antigen
CK 19	Cytokeratin 19
CLL	Chronic lymphocytic leukaemia
CMF	Cyclophosphamide, methotrexate, fluorouracil
CML	Chronic myeloid leukaemia
CNV	Cyclophosphamide, mitoxantrone, vincristine
CR	Complete remission
DATD	N,N'-diallytartardiamide
dATP	Deoxyadenine trisphosphate

dCTP	Deoxycytosine trisphosphate
DFS	Disease-free survival
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EMBL	European Molecular Biology Laboratory
FAB	French-American-British (subtype)
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FISH	Fluorescent in-situ hybridisation
g	Gravitational force
G-CSF	Granulocyte colony stimulating factor
HA	Heteroduplex analysis
HBSS	Hank's balanced salt solution
HCC	Hepatocellular carcinoma
HD-CNVp	High dose cyclophosphamide, mitoxantrone, etoposide
HDT	High dose therapy
HLA	Human leucocyte antigen
HOT	Hydroxylamine-osmium tetroxide
HPC	Haemopoietic progenitor cell
HPCT	Haemopoietic progenitor cell transplantation
HSR	Hot-spot region
ICC	Immunocytochemistry
IgH	Immunoglobulin heavy chain
IMDM	Iscove's Modified Dulbeco's Medium
kDa	Kilo-Daltons
LOH	Loss of Heterozygosity
M	Mutant
MDS	Myelodysplastic syndrome

MNC	Mononuclear cells
Moab	Monoclonal antibodies
MRC	Medical Research Council
MRD	Minimal residual disease
mRNA	Messanger ribonucleic acid
OCM	Optimum culture medium
OS	Overall survival
PAGE	Polyacrylamide gel electrophoresis
PB	Peripheral blood
PBPC	Peripheral blood progenitor cells
PBPCH	Peripheral blood progenitor cell harvest
PBPCT	Peripheral blood progenitor cell transplantation
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PR	Partial remission
RCLS	Red cell lysis solution
RFLP	Restriction fragment length polymorphism
RG-PCR	Restriction site-generating polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SCLC	Small cell lung cancer
SSCP	Single strand conformation polymorphism
SV40	Simian virus 40
Ta	Annealing temperature
TAE	Tris-acetate-EDTA (buffer)
TBI	Total body irradiation
TCR	T cell receptor
TdT	Terminal deoxynucleotide transferase
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	Melting temperature
TNM	Tumour, node, metastasis (classification)

UICC	International Union Against Cancer
UKALL X	The 10th trial on ALL by the MRC Working Party on Leukaemia in Adults
UV	Ultraviolet
WT	Wile type

ABSTRACT

Of recent advances in the treatment of breast cancer and acute leukaemias, peripheral blood progenitor cell (PBPC) transplantation is a very prominent milestone, and the reverse is also true, that these diagnoses are among the commonest indications for such procedures. Inevitably, there is concern that malignant cells may contaminate progenitor cell harvests and be re-infused during transplantation and cause disease relapse. Various methods are available for the detection of such minimal residual disease (MRD), and the key aim of this project was to evaluate the feasibility of using a tumour-specific marker, namely mutations within the p53 gene, for this purpose. This provided a useful model to assess the feasibility of using subtle genetic changes in the detection of MRD within PBPC harvests from patients with malignant diseases.

The first step involved the use of denaturing gradient gel electrophoresis (DGGE) to screen original tumour tissues for mutations in 5 individually PCR-amplified DNA fragments (A to E) covering exons 5 to 8 of p53, to be used as disease markers. The technique was first optimised using cell lines known to contain p53 mutations in each fragment. Optimisation was performed with respect to electrophoresis temperature, time, voltage and polyacrylamide cross-linker. The sensitivity of DGGE in detecting a mutation in a mixed cell population was determined by diluting tumour cells in wild type (WT) cells. Although the presence of a mutation could be demonstrated when tumour cells occured as 5% of total, a representation of at least 40% was required for the mutant homoduplex to be isolated for sequencing. Clinical samples studied were from 51 breast cancer patients, 38 of whom had metastatic disease or at high risk of metastasis, and 13 had high risk stage II/III disease randomised in a clinical study investigating PBPC transplantation as adjuvant therapy, and from 29 patients with acute leukaemias. A positive result was obtained in 14 of 51 primary breast cancer patients (1 was positive in 2 different fragments) and 3 of 29 patients with acute leukaemias. The second step was to isolate the mutant species from DGGE gels and to obtain the exact sequence. In the breast cancer series, sequencing failed in 2 of the positive samples, a further 2 had an identical mutation, and 2 others were silent polymorphisms. A total of 10 different somatic missense mutations were identified. In

the leukaemia samples, 2 were silent polymorphisms and only 1 somatic mutation was found, a novel 8 bp insertion sequence in codon 281 representing the more aggressive of 2 leukaemic clones in this patient.

The third step used the amplification refractory mutation system (ARMS) technique to screen PBPC harvests for MRD in patients whose original tumours contained p53 mutations. This involved allele-specific PCR in which the specific sequence of the ARMS primer and the unique reaction conditions allowed the mutant but not the WT DNA to be amplified. First, parameters important for the optimisation of an ARMS system were determined using the T47D cell line. Conditions studied included PCR annealing temperature, concentrations of magnesium, dNTP's and primers, the presence of glycerol and formamide, cycle number and the use of additional deliberate mismatches. Only 5 of 10 systems with missense mutations could be optimised. WT and mutant DNA could not be distinguished in a further 3, and in the remaining 2 the PCR products from the mutant DNA were too weak to be of value in MRD detection. MRD detection sensitivities for the 5 optimised systems were only from 1 in 10^2 to 10^3. ARMS primer design and optimisation were easier for the 8 bp insertion with sensitivity of 1 in 10^4. The use of nested PCR and radiolabelled ARMS to improve sensitivity were largely unsuccessful. A restriction site-generating (RG-) PCR approach was attempted as an alternative. All identified missense mutations either already resulted in abolition of a restriction enzyme recognition site, or a mutagenic primer could be designed to enable restriction of WT but not the mutant sequence. Although PCR itself was efficient, when products were treated with restriction enzymes after PCR, the detection senstivity of the mutant DNA was extremely low, at approximately 1 in 10. This approach as used in this project was therefore, not useful in MRD detection. Using ARMS, 3/5 breast cancer patients and 8/12 of their PBPC harvests demonstrated MRD. With such small numbers, little can be said of the correlation between MRD in PBPC harvests and outcome, but disease-free survival appeared to depend more on the clinical stage of disease. By studying sequential marrow and PBPC harvest samples, clonal evolution was shown in the leukaemia patient with the 8 bp insertion. He was re-infused with leukaemia-contaminated

PBPC's but the disease was aggressive and failure of disease eradication was also likely to be the cause of his relapse.

In conclusion, the use of single base changes of p53 in MRD detection is, therefore, laborious and insensitive and only applicable to a small number of patients and to certain mutations. They appear to be of little value in general use, although with very specific alterations, as in a long insertion, moderate sensitivity can be achieved.

CHAPTER 1
INTRODUCTION

Over the last 2 to 3 decades, the use of cytotoxic chemotherapy, in combination with advances in supportive care, has revolutionised the treatment and prognosis of patients with many type of malignancies. In the modern treatment of many of these diseases, despite the addition of other therapeutic approaches such as cytokines, monoclonal antibodies, immuno- or gene therapy, the ability to deliver a high dose of chemotherapy over a short period of time remains for many the key to prolonged survival. Inevitably, the higher the dose of chemotherapy given, the more likely for toxic events to occur. These may indeed be dose-limiting. Whatever the underlying disease, the bone marrow (BM) is a site very vulnerable to cytotoxic drugs, and toxicity to which often determines the maximum dose possible. Very high doses of chemotherapy have been made possible by the use of previously harvested autologous or allogeneic BM (and latterly peripheral blood progenitor cell) support. In the autologous setting, however, concerns have also grown for the re-infusion of malignant cells which have contaminated these haemopoietic grafts, as gene-marking studies have demonstrated that re-infused tumour cells can contribute to disease relapse (Rill et al, 1994), and occult tumour cells can be mobilised along with peripheral blood progenitor cells (PBPC) (Brugger et al, 1994). The presence of morphologically undetectable occult tumour cells is termed minimal residual disease (MRD) and depending on the disease in question, various techniques are available to identify them. The advent of the exquisitely sensitive polymerase chain reaction (PCR) technology (Saiki et al, 1988) opened up a whole new arena in the field of MRD detection, and in this project, it is my aim to assess the usefulness of a PCR-based molecular technique which employs mutations within the p53 tumour suppressor gene as markers of MRD. p53 is the single most commonly altered gene in human cancers and should serve as a patient- and tumour-specific marker of disease. In Edinburgh, as in many units nationwide and worldwide, PBPC have largely superseded BM as the preferred source of haemopoietic progenitor cells, and here acute leukaemias and breast cancer are amongst the diseases for which PBPC transplantation is most often

performed. For these reasons, these should provide a satisfactory platform on which to assess such a technique.

In this introduction, I shall first describe the current state of opinion on PBPC transplantation, focusing on the 2 diseases studied in this project. Then mention will be made of the issue of MRD and its clinical relevance, followed by a description of the p53 gene and the possible methods for mutation detection, which forms a key part of this project. Finally the aims of the project will be listed.

1.1 HAEMOPOIETIC PROGENITOR CELL TRANSPLANTATION (HPCT)

Breast cancer and the acute leukaemias are the diseases for which assessment of the techniques of MRD detection in this project are based, and the main 'tissue' in which MRD is sought, is PBPC harvests. As a background to the study of MRD for these diseases, I shall first review some of the clinical aspects of HPCT as applied to these diseases, and outline the findings of a number of controlled trials which reflect the current understanding of the usefulness of high dose therapy and transplantation in this context. In current literature, transplantation may be performed using BM or PBPC, and to encompass both, I have used the term HPC in the wider context, but specified the source of the graft where appropriate.

1.1.1 HPCT IN BREAST CANCER

Breast cancer is the most common non-cutaneous malignancy affecting women. In the UK, 1 in 12 women will be diagnosed with breast cancer during their lifetime, and 25,000 new cases and 16,000 deaths from the disease occur annually (ACCOG Clinical Trial Protocol, 1994). There is wide heterogeneity in the natural history of the disease, and although in many patients the disease can be aggressive, in others it has a very protracted course over many years. One important stratification of prognosis is according to clinical stage, and the tumour-node-metastasis (TNM) and International Union Against Cancer (UICC) classifications are most commonly used. As the stages of disease will be frequently referred to in this thesis, I shall first define the stages

according to these 2 systems (Table 1.1; Sainsbury et al, 1994). Stage IV or metastatic breast cancer is not curable by conventional dose chemotherapy; with chemotherapy, the overall response rates are 40 - 60% with a median time to relapse of 6 - 10 months (Leonard et al, 1994). Stage II and III disease if associated with many positive axillary lymph nodes, also have very poor outlook. In this latter group, 40 - 50% of patients with 4 - 9 positive nodes and 70 - 80% with 10 or more nodes will ultimately relapse (Cagnoni et al, 1996). This is the reason why alternative therapies, particularly high dose chemotherapy, are being intensively assessed.

1.1.1.1 THEORETICAL CONSIDERATIONS

The rationale of using high dose chemotherapy is based on experimental tumour models, and reviewed in recent commentaries by Peters and Dansey (1997) and Bezwoda (1997). The main consideration here is the phenomenon of (Skipper and Schabel) dose-response relationships. When tumours are treated with cytotoxic drugs, for a given dose, a constant proportion of cells are killed, and there is a logarithmic or exponential relationship between drug dose and cell kill. For some cytotoxic drugs, especially the alkylating agents, the curve is steep. For each drug in a combination regimen, exponential cell-kill exists, hence the effects are additive, and with a lack of cross-resistance provide the rationale of using combination high dose therapies. Another reason for using high dose therapy (HDT) is based on the Gompertzian model which states that tumour-doubling time increases with increasing tumour size, predicting more rapid proliferation in the early stage of disease and slowing of growth when the tumour reaches a clinically detectable size. This justifies the use of courses of cytoreductive induction chemotherapy, followed by a single course of ablative HDT, and this may be particularly relevant for adjuvant therapy as any putative micrometastasis is presumed to be rapidly proliferating. In the dose range of conventional chemotherapy, the benefit of dose intensification has been demonstrated for both metastatic breast cancer (Tannock et al, 1988) and as adjuvant therapy for stage II disease (Wood et al, 1994).

Table 1.1. TNM and UICC Staging Systems for Breast Cancer

TNM Classification

T: Tumour size

Tis	Cancer in situ
T1	≤ 2 cm
T2	> 2 cm - 5 cm
T3	> 5 cm
T4a	Involvement of chest wall
T4b	Involvement of skin

N: Axillary lymph node status

N0	No regional node metastasis
N1	Palpable ipsilateral axillary nodes
N2	Fixed ipsilateral nodes
N3	Ipsilateral internal mammary node involvement

M: Metastasis

M0	No metastasis
M1	Distant metastasis

UICC Staging System

UICC stage	TNM
I	T1, N0, M0
II	T1, N1, M0; T2, N0-1, M0
III	any T, N2-3, M0; T3, any N M0
IV	any T, any N, M1

1.1.1.2 CLINICAL EXPERIENCE

Early attempts at using HDT were performed in the 1980's where patients mostly had poor prognosis because of relapsed or refractory disease (Ayash, 1994). These authors found a response of 50%, and 25% achieving temporary complete remission (CR). Results improved with time, and CR rates of 50%, 25% being durable beyond 5 years, were reported (Eddy et al, 1992). Procedure-related mortality also fell from around 25% in early studies to under 5% currently (Leonard, 1998) with improved supportive care. Most studies have been non-randomised and used HDT as consolidation in chemosensitive disease. In this context, a 20 - 40% conversion rate from partial remission (PR) to CR, and 18 - 25% of patients disease-free at 2 years is

observed (reviewed by Bezwoda, 1997). Peters et al (1995b) described a survey of the experience of American bone marrow transplant centres. For metastatic disease, in 1106 patients from 37 centres, overall survival (OS) at 3 years of patients given HDT + PBPCT as first-line therapy after induction is 37% and compares favourably to 5 - 20% in historical controls, although disease-free survival (DFS) data were not available. Data on 662 patients with high risk primary disease (median of 13 positive lymph nodes) from 33 centres showed 3-year OS of 79% and event-free survival of 69%. Unfortunately, no comparison with controls was made. Our unit in Edinburgh has used high dose cyclophosphamide (4 gm/m^2) with G-CSF to mobilise progenitor cells from patients with stage IV breast cancer after a 12-week induction course of chemotherapy, and found 88% success in mobilisation, and rapid post-transplant haematopoietic recovery (Cameron et al, 1996). No procedure-related mortality was observed.

Despite the huge numbers of breast cancer patients treated with PBPCT worldwide, only 2 randomised studies have been published, both in the context of stage IV disease. Bezwoda et al (1995) compared the use of high dose cyclophosphamide, mitoxantrone and etoposide (HD-CNVp) with conventional dose cyclophosphamide, mitoxantrone and vincristine (CNV) as first-line therapy in stage IV patients. With the relatively small number studied (45 in each arm), a CR rate of 51% in the high dose group compares with 4% in the conventional group, and the median durations of response are 80 weeks and 34 weeks, respectively. A major criticism of this study is the particularly low survival of the conventional group patients, although the disease in many of these patients is particularly aggressive, so that these response rates are consistent with published figures and are believable (Kennedy, 1995). A further criticism is that follow-on tamoxifen given to only the responders may have accounted for the better survival by amplifying the benefit of a higher response rate in the HDT group. Peters et al (1996) randomised chemosensitive patients in CR to receive either immediate HDT or observation, the latter group offered HDT upon relapse. The HDT group had a significantly longer DFS, but paradoxically shorter OS than the observation group. This paradox may be explained in terms of the biology of a resistent clone of malignant cells whose proliferation in the 'observation' patients was

delayed by the observation period (Bezwoda, 1997). Both available randomised studies, therefore, demonstrated that high dose therapy is beneficial, although in very different contexts both in terms of patient selection and timing. Results of other on-going randomised trials are awaited.

Enthusiasm in the use of HDT in the adjuvant setting has been fuelled by non-randomised trials suggesting benefit. An influential study by Peters et al (1993) reported 85 women with 10 or more positive axillary nodes treated with 4 courses of induction chemotherapy then HDT and BM support. Event-free survival of 72% was noted at 2.5 years, and compares with 38 - 52% in historical controls. Bearman et al (1997) studied the safety and feasibility of HDT / PBPCT in patients with 4 - 9 positive nodes, and reported 15/54 (29%) pulmonary toxicity (1 fatality) and otherwise acceptable safety. Their 4-year DFS of 71% is within the historical range for standard dose therapy. A number of randomised studies assessing the efficacy of HDT and HPCT as adjuvant therapy in high risk stage II or III breast cancer are in progress worldwide, not least the Anglo-Celtic Cooperative Oncology Group study in the UK, comparing high dose cyclophosphamide and thiotepa + PBPCT, versus conventional dose cyclophosphamide, methotrexate and 5-fluouracil, after a common induction regimen, in patients with 4 or more positive axillary nodes. Accrural for this study (target of 300) is ahead of schedule at present.

1.1.2 HPCT IN ACUTE LEUKAEMIAS

Unlike breast cancer, HPC transplantation for acute leukaemias may involve cells derived from the patient him- or herself (autologous), or from a histocompatible donor (allogeneic) who may be a sibling or an unrelated person. The screening of the HPC graft for MRD is obviously only relevant in autologous transplants, and as a result, in the context of this project, this review will largely focus on the clinical aspects of autologous transplantation for acute leukaemias. In clinical trials that assess the benefit of transplantation as compared with conventional chemotherapy, data on patients who have undergone allogeneic transplantation are often included, and will therefore be mentioned here. In ALL, no data are available for the role of autologous

transplants, and for completeness, a description of the current status of allogeneic transplantation will be given.

1.1.2.1 HPCT IN AML

Despite advances in cytotoxic chemotherapy and supportive measures with the use of powerful antibiotics, the mortality from AML remains high. In the region of 65 - 80% of adults with AML will achieve first remission (Cassileth et al, 1998; Burnett et al, 1998), but most will relapse if no further post-remission treatment is given. As a result, a number of groups have undertaken to study the benefit, or otherwise, of high dose chemotherapy with either autologous or allogeneic BM support. As only one patient in 3 or 4 is likely to have an HLA-compatible sibling, autologous transplantation is more often performed for this indication. Several published studies are available to date, and all have used harvested BM rather than PBPC as haemopoietic support. All but one compared the merits of BMT with an alternative 'intensive' but not myeloablative course of therapy. There is a degree of heterogeneity in the design and treatment schedules of these studies, and some studies used marrow purged with 4-hydroperoxycyclophosphamide whilst others did not. Although randomisation to receive BMT or not took place for patients who did not have an HLA-compatible sibling marrow donor, those who had a suitable donor were usually offered allogeneic transplantation and included in statistical analyses.

Harousseau et al (1994) studied 470 adults with de novo AML. Patients who achieved CR were given either 2 further consolidative courses (course 1: high dose cytarabine and idarubicin or rubidazone; course 2: amsacrine and etoposide) or one consolidative course then autologous BMT with BuCy conditioning and unmanipulated BM. There was no significant difference in the relapse rate (30 - 44%), event-free (48 - 56%) and OS (56 - 59%) whether they received chemotherapy, ABMT or allogeneic BMT. Another large study (Zittoun et al, 1995, for the EORTC / GIMEMA groups) used a post-remission consolidative course of amsacrine and intermediate-dose cytarabine, then randomised patients without a suitable marrow donor to receive a second course of intensive chemotherapy with high-dose cytarabine

and daunorubicin, or unpurged BMT conditioned with cyclophosphamide and TBI or the BuCy regimen. A clear disease-free survival (DFS) advantage was demonstrated for the BMT groups over the chemotherapy group: 55%, 48% and 30%, repectively for allogeneic BMT, ABMT and chemotherapy. Although relapse rates were also highest in the chemotherapy group, no OS difference was shown in the 3 groups. Whilst the above studies were European initiatives, the American experience was published recently (Cassileth et al, 1998). A total of 740 adolescent and adult AML patients were given induction chemotherapy, 70% of whom achieved CR. A consolidative course of idarubicin and cytarabine was given, following which those with a compatible marrow donor were offered an allogeneic transplant, whilst the rest were randomised to receive high dose cytarabine or an autologous transplant conditioned with BuCy. With a median follow-up of 4 years, there was no difference in the DFS in all 3 groups, and in fact, there was a marginal OS advantage in the chemotherapy-only group.

Similar studies have also been performed in children. Ravindranath et al (1996) compared continuing intensive therapy with 6 courses and 5 drugs in varying combinations, with purged ABMT using BuCy conditioning. The main difference in the design of this study is, apart from age, high dose cytarabine was used pre-randomisation, and the numerous courses of 'alternative' therapy. DFS at 3 years was similar (36% and 38%), as was OS (44% and 40%) which balanced a lower relapse rate in the ABMT group by a higher procedure-related mortality.

In contrast to the above, patients in the MRC AML 10 study (Burnett et al, 1998), after 3 courses of intensive chemotherapy, and in complete remission, were randomised to receive, after a forth course of treatment, unpurged ABMT or no further therapy if a suitable marrow donor was not available. Superior DFS was demonstrated at 7 years for the ABMT group (53% versus 40%), with fewer relapses (37% versus 58%). The trend for OS for the ABMT group was better, although not statistically significant (57% versus 45%, p=0.2).

It seems that additional ABMT carries a survival advantage over 4 courses of standard chemotherapy, but it is still not clear if ABMT is definitely superior to an alternative intensive course such as with high dose cytarabine. The apparent discrepancy between better disease-free but not OS in the ABMT group has often been explained by the fact that patients randomised to receive the alternative regimen were more likely to be salvaged with ABMT when relapsed, in which situation a significant proportion still had sensitive disease and survived longer. Nevertheless, high dose chemotherapy and autologous transplantation, especially with PBPC, is very much in routine practice in most units for patients with AML. No conclusion can be drawn from the above studies regarding the role of MRD in the haemopoietic grafts, but those which demonstrate lower relapse rates in the ABMT group may suggest that residual disease in the patient, rather than the graft, is most important.

1.1.2.2 HPCT IN ALL

There is a relative paucity of randomised studies of high dose chemotherapy and HPC transplantation in ALL, by comparison with AML as outlined above. The majority of cases of ALL are in children. In childhood acute leukaemia, ALL makes up approximately 80% of cases, and the reverse (20%) is true for adults. Childhood ALL in general has a good prognosis. Results from the MRC UKALL X study (Chessells et al, 1995) demonstrated that a 5-year DFS of 71% was achieved if 2 blocks of intensification therapy were given, and even the arm with the worse outcome (no intensification) achieved a figure of 57%. As a result of this, there is a general reluctance to transplant children in first CR. On the other hand, children who relapse are deemed to have such poor prognosis that should a HLA compatible sibling donor be available, transplantation is usually carried out. For these reasons, to date, no prospective randomised study comparing conventional chemotherapy with autologous transplantation in first or second CR has been published and the role of autografting in childhood ALL is completely unknown. Attention has been focused on the role of allogeneic BMT in both first and second CR. Only 1 prospective study (also within the MRC UKALL X framework) has been published that investigated the role of allogeneic BMT in poor-risk childhood ALL, defined by a high leucocyte count at

presentation of > 100 x 10^9 / l (Chessells et al, 1992). Of 111 patients who were HLA-typed, 41 had a compatible sibling donor, of whom 34 received an allograft. Comparison of this group with 144 similar children treated with conventional chemotherapy showed no significant difference in event-free survival at 5 years, although no OS data were given. Treatment-related mortality was higher in the transplant group and relapse rate was higher in the chemotherapy group, however. With BMT in second or subsequent CR, again only 1 prospective study has been published (Johnson et al, 1981). Patient numbers in this early study were small, 24 in the transplant group and 21 in the conventional chemotherapy group. Crude survival data suggest a clear advantage for allogeneic transplantion. These 2 prospective studies aside, there have been a number of cohort studies looking at the role of allografting in second CR, and the study by Barrett et al (1994) is the largest, drawing cases from the International Bone Marrow Transplant Registry. The DFS at 5 years for the BMT group was 40%, versus 17% for the chemotherapy group, and the probabilties of relapse at 5 years were 45% and 80%, respectively. This has provided fuel for advocates of BMT, which currently seems to be common practice in this context.

Prospective studies in adult ALL are likely to be even more difficult to conduct, owing to the smaller number of cases, and like childhood ALL, firm conclusions in favour of either transplantation or conventional treatment cannot be drawn. For example, Sebban et al (1994) studied adult ALL patients in first CR, and in those with no compatible sibling donor, found no difference between autografting and conventional chemotherapy. Those with a sibling donor and therefore allografted also did not appear to enjoy any survival advantage over the autograft and chemotherapy group as a whole. In general clinical practice, because of the adverse prognosis associated with adult ALL, patients young enough and with a compatible sibling donor usually receive an allograft, whilst those without such a donor may receive an allograft from a matched but unrelated donor identified through national or international registries.

1.2 MINIMAL RESIDUAL DISEASE DETECTION IN BREAST CANCER

The issue of micrometastasis is not a new one and circulating malignant cells were recognised in the peripheral blood in cancer patients in the 1950's. There has been a surge of interest in the detection of MRD since the 1980's, both in the context of haematological malignancies and in solid tumours, especially breast cancer and neuroblastoma. In the management of breast cancer, 2 aspects of MRD research have emerged over the last decade. Before high dose therapy and PBPC transplant became popular for this disease studies were performed on the detection of micrometastases in BM and attempts were made to correlate these to clinical prognosis (Mansi et al, 1989; Cote et al, 1991). More recently, with the common use of BM / PBPC transplantation, many groups have focused their efforts on correlating transplant outcome to the presence of MRD in haemopoietic grafts (Sharp et al, 1992 and 1995a; Fields et al, 1996; Vredenburgh et al, 1997). Inevitably, in line with clinical interest is a large body of literature on ultra-sensitive techniques in detecting MRD.

1.2.1 SIGNIFICANCE OF MRD IN BONE MARROW

It has been known for some time that a proportion of patients with apparently early stage breast cancer can harbour micrometastatic disease in their BM. But the mere presence of these cells may not indicate that they are truly 'metastatic', and may not actually be viable or clonogenic. By immunocytochemistry, Mansi et al (1989) found that, in patients with primary breast cancer after surgery but prior to overt relapse, 2 - 3% had evidence of MRD in the marrow. This compares with 26% before surgery (albeit from a different study by the same authors), and 27% of BM positive patients if the tumour was left in situ. When patients relapsed, their likelihood of having BM micrometastasis progressively increased with the extent of relapse. They concluded that 'shedding' of non-viable cells could account for many observed cases of micrometastases. The presence of BM micrometastases before surgery, however, does seem to correlate with various poor prognostic factors such as vascular invasion, positive axillary lymph nodes, and primary tumour size, and predicts early relapse (Mansi et al, 1987 and 1991) and poor OS. Risk of relapse is also dependent on the

number of cancer cells in the marrow. Patients with higher micrometastatic tumour burden have a much higher (5.2x) risk of relapse than those with a low burden (Cotes et al, 1991).

1.2.2 SIGNIFICANCE OF MRD IN HAEMATOPOIETIC GRAFTS

The use of high dose therapy and haematopoietic cell transplantation is now commonplace in metastatic, and often high risk stage II and III breast cancer, and as a result much effort has focused on the detection of MRD in these grafts. There is concern that patients may suffer disease relapse as a result of re-infusion of malignant cells when their disease is otherwise eradicated. This phenomenon has been demonstrated in acute leukaemia, neuroblastoma and chronic myeloid leukaemia (CML) by using gene marking studies. For example, Rill et al (1994) retrovirally transfected the neomycin-resistant gene into target cells in PBPC harvests from children with neuroblastoma. Both the PBPCs and the contaminating neuroblastoma cells (if any) were labelled. The authors estimated that 1% of clonogenic neuroblasts would be labelled with the marker gene. When the labelled PBPCs, with the unlabelled aliquots, were returned to 8 patients, subsequent follow-up showed that, of 3 patients who relapsed, the marker gene was detectable in the recurrent tumours in all cases, thus offering direct evidence that re-infused tumour cells could lead to disease relapse. Tumour cells may be mobilised into the peripheral blood (PB) at the same time as PBPC after chemotherapy and G-CSF, and the kinetics of this process was reported for the first time by Brugger et al (1994). Peripheral blood samples from 46 patients, of whom 9 had stage IV or high risk stage II / III breast cancer, were studied with immunocytochemistry (ICC). 29% of stage IV breast cancer and 20% of small cell lung cancer (SCLC) patients had circulating tumour cells before PBPC mobilisation. 21% of patients without steady state circulating tumour cells, and all stage IV breast cancer patients and 50% of SCLC patients mobilised tumour cells at the same time as PBPC, at a concentration of 4 - 5,600 tumour cells / 1.6×10^6 mononuclear cells (MNC). Tumour cell mobilisation occurred early (days 1 - 7) in patients without marrow metastasis, but between 9 and 16 days in those with marrow disease, within the optimal period for PBPC harvesting. All 7 stage IV breast cancer

patients, but neither of the 2 with stage II / III disease, mobilised tumour cells. Although there is little doubt that cancer cells do mobilise along with PBPC, it is difficult to be certain of the patterns of mobilisation on the basis of this study owing to the small number of patients involved.

Using ultra-sensitive detection technologies, various authors have correlated clinical outcome with the presence of graft contamination. Using culture assays Sharp et al (1992) demonstrated 6 / 32 (18.7%) breast and solid tumour patients had contaminated PBPC, and 4 of these patients had very short survival times, while culture negative patients had a more favourable prognosis. The exquisitely sensitive ($1:10^7$ MNC) cytokeratin 19 RT-PCR of Fields et al (1996) showed an increasing likelihood of marrow MRD with advancing stage following initial chemotherapy: 52% for high risk stage II, 57% for stage III and 82% for stage IV. BM was used as haematopoietic support and survival benefit was demonstrated for stage IV patients in whom those positive for marrow MRD had a probability of relapse at 3 years of 94% versus 14% for those who were MRD negative. Although the corresponding figures of 32% and 10% did not reach statistical significance for stage II / III disease, Vredenburgh et al (1997) found, using ICC with 4 monoclonal antibodies (Moab), that in this group ($\geq$ 10 axillary nodes) disease-free and OS are also compromised in those with marrow MRD, and the larger the number of contaminating cells, the worse the prognosis.

1.2.3 METHODOLOGIES OF MRD DETECTION

Table 1.2 is a compilation of several studies using various methods to detect MRD in breast cancer, also illustrating the reported sensitivities for each technique and some data on detection rate and the quantity of malignant cells detected. There are 3 broad groups of techniques used (Pantel et al, 1994a; Ross, 1998):

(1) Moab-based methods. ICC, which has been the gold standard of MRD detection, employs one or a cocktail of Moabs to stain cells cytospun onto a glass slide, and the signal amplified and visualised by an APAAP or immunoperoxidase technique. These

Moabs are directed against surface membrane antigens, typically the cytokeratin family of proteins found on epithelial cells. Immunofluorescence is based on the same principle except the Moabs used are conjugated to a fluorochrome which can be directly visualised with a fluorescent microscope without further staining / counter-staining. Moab labelled cells may also be visualised by flow cytometry (FACS scanning), with the main advantage of being much less labour-intensive and a large number of cells may be analysed rapidly. Specificity of Moab's may vary (Thor et al, 1988), and cocktails of Moab's are often used (Ross et al, 1993 and 1995).

(2) Clonogenic / culture methods. These are the only assays that directly demonstrate the clonogenic potential of contaminating cancer cells, but are time-consuming. Culture may be performed in a liquid medium (Sharp et al, 1992 and 1995b) or a soft-agar based medium containing recombinant growth factors and serum (Ross et al, 1993). After cell culture, tumour cells are visualised by ICC. Some authors have reported extraordinary sensitivities with culture techniques, e.g., $1{:}10^5$ before culture and $1{:}10^7$ after culture (Joshi et al, 1990), and the presence of MRD correlated with poorer clinical outcome (Sharp et al, 1992).

(3) PCR-based methods. The majority of these methods are based on amplifying the epithelial cell-specific cytokeratin 19 (CK19) mRNA message by RT-PCR. The primers used vary between studies, but most include a nested system plus Southern hybridisation using an internal probe to maximise sensitivity, often in the region of $1{:}10^6$ - 10^7 (Fields et al, 1996; Traystman et al, 1997). Gerhard et al (1994) used carcinoembryonic antigen (CEA) as a marker, and reported a sensitivity of $1{:}2\text{-}5\text{x}10^7$ which is the highest yet reported, although the authors did caution the possibility of over-estimation.

Table 1.2a. Summary of several studies using various methodologies and their detection rate of MRD in breast cancer. Some also contain information on the level of contamination in clinical materials.

Author	Method / marker	Material studied / Disease (stage)	Sensitivity	Positivity Tissue	Positivity % positive	Conc of tumour cells (per normal cells)
Brugger (1994)	ICC - Moab anti CK x2, anti-epithelial x1	Breast ca (IV)	1: $4x10^5$	Blood	28	
				PBPC	100	
Mansi (1987)	ICC - EMA	Breast ca	n/a	BM	26.4	
Pantel (1994)	ICC - CK18	Breast / colon / prostate ca	1 : $8x10^5$	BM	43	
Ross (1993)	ICC - Moab x5 / TCA	Breast ca (III/IV)	1 : 10^5	BM harvest	62	$22.9 / 10^5$
				PBPC	10	$0.8 / 10^5$
Vredenburgh (1997)	ICC	Breast ca (II/III)	n/a	BM	36	
				PBPC	4	
Theocharous (1997)	ICC - Cell Tak glue A45-B/B3 Moab	Breast ca cell line / Daudi cells mix	1 : 10^6			
		Breast ca		BM	100	$1\text{-}62 / 10^6$
				PBPC	33	$6 / 2x10^6$
Franklin (1996)	ICC - Moab x4	CAMA cell line / MNC mix	1 : $2.5x10^5$			
		Breast ca (IV)		BM	37	
		Breast ca (III)		BM	0	
		Breast ca (II)		BM	7	
		Breast ca		PB	17	$1\text{-}97 / 10^6$
Molino (1991)	ICC - Mbr1 Moab	Breast ca cell line / MNC mix	1 : $4x10^5$	BM	85	
	FACS		1 : 100	BM	65	
	Morphology		1 : 2000	BM	40	
Vredenburgh (1996)	ICC - Moab x4	CAMA cell line / MNC mix	1 : 10^5			
	2-colour immunofluorescence		1: 10^6			
	FACS		1 : 10^5			
Leslie (1990)	FACS - Moab x5	CAMA cell line / MNC	1: 10^4			
	FACS + cytology		1 : 10^5			

ICC - immunocytochemistry; Moab - monoclonal antibodies; MNC - (normal) mononuclear cells; TCA - tumour clonogenic assay; BM - bone marrow; PBPC - peripheral blood progenitor cells; FACS - fluorescent activated cell sorting; CK - cytokeratin; EMA - epithelial membrane antigen.

Table 1.2b.

Author	Method / marker	Material studied / Disease (stage)	Sensitivity	Positivity		Conc of tumour cells (per normal cells)
				Tissue	% positive	
Simpson (1995)	FACS - Moab x1	CAMA cell line / MNC mix	1 : 2 x 10^5			
		Breast ca (IV)		BM	28	
				PB	39	
Racila (1998)	IME + FACS + ICC nucleic acid+/CK+/CD45-	Breast ca	1 cell / 1 ml blood			
Naume (1997)	IME + ICC - A45-B/B3 Moab	Breast ca cell line / MNC mix	> 1 : 10^6 3.7-4.2x detection for positive selection 3.1-3.8x detection for negative selection			
Easton (1997)	IME + ICC - Ber-EP4 Moab	Breast ca cell line/ whole blood mix	1 : 10^6			
Joshi (1990)	Culture + ICC - EMA	MCF7 cell line /	1 : 10^7			
	ICC only - EMA	MNC mix	1 : 10^5			
Sharp (1992)	Culture + ICC	Breast ca (IV)	n/a	BM	42	
				PBPC	16	
Datta (1994)	RT-PCR (nested) - CK19	Breast ca cell line / MNC mix	1 : 10^5			
		Breast ca (IV)		PB	21	
				BM (pre-BMT)	83	
				PBPC	0	
Gerhard (1994)	RT-PCR (nested) + probe - CEA	Breast, colon ca	1 : 2-5x10^7	BM	67	
Fields (1996)	RT-PCR (nested) + probe - CK19	Breast ca (II)	1 : 10^7	BM	52	
		Breast ca (III)		BM	57	
		Breast ca (IV)		BM	82	
Traystman (1997)	RT-PCR (nested) + probe - CK19	Breast ca cell line / MNC mix	1 : 10^6			

CEA - carcinoembryonic antigen; RT-PCR - reverse transcriptase polymerase chain reaction;
IME - immunomagnetic enrichment.

1.3 MINIMAL RESIDUAL DISEASE DETECTION IN ACUTE LEUKAEMIAS

The techniques involved in detecting MRD in acute leukaemias are totally different from those for breast or epithelial cancers, owing to their different surface markers and molecular genetic characteristics. Whilst epithelial characteristics such as cytokeratin 19 are not expressed in haematopoietic tissues and can be used as markers for breast cancer, leukaemias are of haematopoietic origin and share certain characteristics with normal haematopoietic progenitors, and it is essential that techniques for their detection employ features that are not shared and are able to differentiate between these populations. MRD in leukaemias have also been more extensively reviewed than for breast cancer (Campana and Pui, 1995; Lin and Cross, 1995). Most of the literature focuses on acute lymphoblastic leukaemia (ALL) in children, simply because various distinct molecular markers are available in this disease, enabling approximately 90% of cases to be studied (Campana and Pui, 1995; Steward et al, 1995), and ALL occurs predominantly in childhood. The same approaches cannot be used for AML, because as a whole, they lack consistent molecular and surface markers that can be use to distinguish disease from normal tissues. As a result MRD in AML is still not satisfactorily or as extensively studied. The body of literature on aspects of MRD in acute leukaemias is much more extensive than that for breast cancer, and a detailed review is beyond the scope of this Introduction. Here, some of the techniques available will be outlined.

1.3.1 MRD DETECTION IN ALL

Detection of MRD in ALL is generally focused on using molecular markers which reflect the lineage origin of the leukaemic progenitor cells. T-cell receptor (TCR) gene rearrangements and immunoglobulin heavy chain (IgH) gene rearrangements are most often used (Deane and Norton, 1990). The rationale in using these markers lies in the fact that rearranged genes for a particular clone of cells is unique to that clone, ensuring specificity, and also the incidence of detecting a rearrangement is high, making it possible for a molecular marker to be found in the great majority of cases.

In normal lymphoid maturation, these genes comprise 3 discontinuous germline segments, named variable, diversity and joining segments (V, D, J segments). IgH genes also contain a C_H segment which specify the particular class of immunoglobulin (ie. IgA, D, E, G or M). Different TCR (α, β, γ, δ) and IgH loci have different numbers of V, D and J segments. For example, the IgH locus on chromosome 14q contains 86 V, 30 D and 9 J gene segments (Strachan and Read, 1997). On the other hand, TCR γ and δ loci contain 0 and 3 D segments, respectively. Each lymphoid cell is committed to produce only one type of IgH or TCR, and during maturation, these segments are juxtaposed by splicing of the intervening sequences at the DNA level, and at the same time, non-template derived nucleotides (known as N regions) are inserted between V and D, and between D and J segments. Because the large numbers of gene segments for the V, D and J regions, the possible combinations of these segments as a result of DNA splicing is huge. In a normal lymphoid population, different gene rearrangements within individual cells mean that, as a whole, the molecular repertoire is a mosaic. In a leukaemic population, however, the clonal nature of the cells means that all the leukaemic blasts will have the same rearrangement. This characteristic may be detected by PCR using primers that span the rearranged junctions, and produce a single product seen as a single band on polyacrylamide gel electrophoresis. This contrasts with a normal lymphoid population, in which PCR will result in a polyclonal smear plus one or two faint bands representing unrearranged germline configurations in immunologically naive cells. IgH, TCRδ, TCRγ and TCRβ genes may be found to be rearranged, respectively, in 95%, 54%, 55% and 33% of B-lineage ALL, and 14%, 68%, 91% and 89% of T-lineage ALL (Campana and Pui, 1995). Hence it is possible to study the majority of cases of ALL with these markers. In general, for B-lineage ALL, IgH gene markers are most often used, and for T-lineage ALL, TCR γ and δ genes, which cover approximately 90% of cases (Cave et al, 1994).

In the IgH gene, V_H segments may be further grouped into 3 conserved framework (FR) regions, and 3 hypervariable 'complementarity-determining regions' (CDR). Clonal IgH rearrangement may be detected using PCR primers corresponding to

different V_H segments, in conjunction with a consensus J_H segment primer. Owen et al (1995) used a combination of a consensus FR3 as well as 6 CDR1 primers, visualised using a fluorescence based system, and reported a sensitivity of one ALL cell in 10^3 - 10^4 normal cells with FR3 PCR and a higher sensitivity by up to 1 log with CDR1 PCR. In general, the combination of PCR analysis of gene rearrangements with Southern blotting using clonospecific probes provide the highest sensitivity, possibly capable of detecting one leukaemic cell in up to 10^6 normal cells, but probably more reliably 10^4 or 10^5 cells (Bartram, 1993; Ouspenskaia et al, 1995).

Clonal evolution during the course of disease may be a potential problem, leading to false negative MRD detection. However, the incidence of this is relatively low and false negative results may be minimised by using a combination of markers. Steward et al (1994) found only 3 patients out of 52 studied had complete change in pattern of amplification between presentation and first relapse, despite the fact that some change was observed in 31% of cases with an IgH marker and 25% with a TCRδ marker at presentation.

The clinical relevance of MRD detection in childhood ALL is now in little doubt, and several recent publications highlight the prognostic implications. Cave et al (1998) used TCR γ and δ, and IgH rearrangements plus clonospecific probes with a detection sensitivity of 1 cell in $2x10^4$ normal cells, to study 178 patients within 6 months of complete remission and were able to assign patients to risk groups with respect to MRD. At the end of induction chemotherapy, those whose marrow contained ≥ 1 residual blast in 100 had a relative risk of relapse 16 times higher than those with < 1 blast in 1000. When assessed at 3 later time points, 'standard risk' patients had a significantly shorter time to relapse if they contained detectable MRD, and particularly if more than 1 blast in 1000 (>15 times higher risk). In reverse, Goulden et al (1998) reported that in standard risk patients who ultimately relapsed, a much higher incidence of MRD was detected at 1, 3, and 5 months into therapy, compared with those remaining in CR in whom all had cleared MRD from the marrow by 5 months. In the context of allogeneic BMT for ALL, Knechtli et al (1998) studied 71 children

using IgH and TCR rearrangements plus clonospecific probes, with a sensitivity of at least 1 in 10^4 cells. A significantly higher incidence of MRD (88%) was detected in a group of 32 patients who subsequently relapsed, when compared with 36 patients who remained in continuing CR in whom only 22% had MRD at any time after BMT. In the former group, more than half had detectable MRD at all times, and the remainder were initially negative but became positive at a median of 3 months. The authors concluded that any evidence at all of MRD after allogeneic BMT is a poor prognostic sign. This finding is supported by others who used immunological techniques for MRD detection with comparable sensitivity (Coustan-Smith et al, 1998).

Other molecular approaches are available, although, as indicated in the review by Campana and Pui (1995), candidate markers are very rare amongst ALL cases, except perhaps for deletion of the TAL1 gene from chromosome 1 in T-lineage ALL (up to 30%). As in AML, flow cytometry has also been studied, but the general applicability in a cross-section of cases is low, except in T-ALL in which the (extra-thymic) co-expression of terminal deoxynucleotide transferase (TdT) and T cell markers such as CD3 and CD5 on blasts in the majority of cases may serve as a marker. The generally accepted level of detection using flow cytometry is in the order of 1 cell in 10^4 (Lin and Cross, 1995; Coustan-Smith et al, 1998).

Yet another approach may be to use a combination of flow cytometry, cell sorting and colony-forming assay. Uckun et al (1993) studied the pre-transplant leukaemia burden in 83 patients with ALL with this technique, which has a detection sensitivity in the region of 5 in 10^5 cells. Not only does this technique provide functional information on the clonogenic ability of any leukaemic cell detected, the authors also demonstrated prognostic relevance in that patients with greater than 51 leukaemic cells per 10^6 MNCs in the BM just prior to transplantation were 3.5 times more likely to relapse than patients with lower burdens. The obvious disadvantage of this technique is the multi-step nature and the need for cell culture.

1.3.2 MRD DETECTION IN AML

MRD studies in AML is much less clearly defined than for ALL, largely because of the lack of consistent molecular markers in the majority of cases. Nevertheless, a range of techniques are available (Sievers and Loken, 1995). The most commonly used markers are certain unique chromosomal translocations resulting in juxtaposition of known genes, but unlike receptor and IgH markers for ALL, only a minority of cases of AML have such available markers. All these markers, when present, can be detected using RT-PCR. In acute promyelocytoic leukaemia (FAB M3), t(15;17)(q22;q11-22) results in the PML-RARα fusion transcript which can be detected using RT-PCR (Miller et al, 1993). Again, favourable clinical outcome is reported to be associated with molecular negativity after treatment. Eleven of 13 patients with BM RT-PCR positivity 4 months after clinical remission relapsed 1 to 4 months later, whilst all 22 patients who were negative at 4 months remained in CR 3 months to 5 years later (Lo Coco et al, 1992). This molecular marker has also been used to demonstrate the possibility that MRD in BM may be too low to be detected even with RT-PCR, but may reach detection level after enrichment by PBPC apheresis, as found in a patient who had been marrow RT-PCR negative for 20 months (Karlic et al, 1995). The t(8;21)(q22;q22) translocation is characteristic of AML FAB M2, and results in the AML1-ETO fusion gene. The clinical significance of detecting this transcript in patients in remission is less clear, and patients in continuing remission may often be found to contain the transcript, and it has been hypothesised that t(8;21) is an early step in leukaemogenesis, but insufficient in leading to frank leukaemia which may require other genetic changes (Sievers and Loken, 1995). AML FAB M4-Eo, associated with an eosinophilic predominance is associated cytogenetically with inv(16)(p13q22) which results in the CBFβ-MYH11 transcript. Again, patients in continuing remission have been found to be positive for the transcript, throwing into doubt the clinical usefulness of this assay (Poirel et al, 1995).

Flow cytometric techniques have been used in determining MRD in AML. Many AML blast cells aberrantly express antigens such as terminal deoxynucleotide

transferase (TdT) or CD7, antigens associated with early lymphoid, especially T cell, differentiation. One early study reported 19.1% and 9.8% expression, respectively, of these antigens co-expressed with myeloid markers in AML (Campana et al, 1990), but this technique obviously lacks universal application. A more detailed study of the patterns of aberrant antigen expressioin in AML by Terstappen et al (1991) demonstrated 4 different categories, covering the majority of cells, thus potentially allowing most patients to be studied. Features identified here include expression of non-myeloid antigens, asynchronous expression of myeloid antigens (representing different stages of maturation), and over- or non-expression of myeloid antigens. Subsequently, another study reported the identification of aberrant phenotypes in 85% of AML blasts, using a panel of 22 Moab's and multi-parameter flow cytometry (Reading et al, 1993). Significant correlation between cytogenetic abnormalities and coexpression of surface antigens was also noted. When 16 AML patients in remission were studied, aberrant expression of antigens reflecting residual leukaemia was found to the proporton of 0.03 - 1.4% of nucleated BM cells. Quantification of the residual blasts enabled the authors to attach some prognostic value to their MRD analysis, as all of 6 patients with more than 0.2% of cells with aberrant expression relapsed within 1-7 months, in contrast to 9 of 10 patients with less than this number remaining in remission. The follow-up was short, however. Although seeding experiments were not reported by these authors to determine the sensitivity of detection, this is illustrated by the detection down to 0.03%, equivalent to 3 AML cells in 10^4 normal cells, a level comparable to other technologies. The need for so many Moab's to achieve the 85% applicability obviously limits the general usefulness of this approach.

Other techniques have been studied in AML, including fluorescent in-situ hybridisation (FISH) which is increasingly used in routine diagnostic service. However, the sensitivity of this is only in the region of 1%, making it unsuitable of ultra-sensitive detection of MRD. The sensitivity of FISH is mainly hampered by the presence of background non-leukaemic aneuploid cells, the potential total number of cells that can be studied, and technical artefacts (Gray et al, 1990). The additional step of short-term culture is another requirement.

1.4 THE p53 TUMOUR SUPPRESSOR GENE

1.4.1 p53 BIOLOGY

1.4.1.1 INTRODUCTION

p53 is a 53 kDa nuclear phosphoprotein which, since its original description in the 1970's, has emerged as having a vital role in the regulation of the cell cycle, implicated in DNA repair, cell differentiation, genome plasticity and apoptosis (programmed cell death). Over the years it has been extensively studied, and the gene controlling it is now known to be a major tumour suppressor gene, and is the single most frequently mutated gene in human cancers. The p53 protein was first described in 1979 by groups using 2 different experimental approaches: virological (Lane et al, 1979) and serogical (DeLeo et al, 1979). The former approach, which is better known, demonstrated that, in simian virus-40 (SV-40) transformed hamster or mouse fibroblasts, the SV-40 large T antigen (AgT) co-precipitated immunologically (using monoclonal antibody Pab419) with a 53 kDa cellular protein. As this protein was demonstrated to accumulate in the nuclei of various virus transformed cell lines as well as chemically-induced tumours (DeLeo et al, 1979) but not in normal cells, it was felt likely to be an oncoprotein and associated with malignant transformation. An extensive body of literature followed over the ensuing years, first pointing to p53 being associated with cellular proliferation and tumour formation, and then others demonstrated the opposite and hinted that p53 might actually have anti-tumour effects.

1.4.1.2 p53 AND CELLULAR PROLIFERATION - AN ONCOGENE?

Various evidence suggested that p53 was required for progression of the cell cycle. First, an increased p53 mRNA transcript and protein synthesis in induced murine Swiss 3T3 cells, maximally at the late G1/S phase junction prior to DNA replication, was found, suggesting a role for p53 in the progression of cells from a growth-arrested to an actively dividing state (Reich et al, 1984). Mercer et al (1984)

performed microinjection of Moab's against p53 (Pab122 and 200-47) into the nuclei of quiescent 3T3 cells and found that entry of the cells into S phase was inhibited if given at the time of serum stimulation of DNA synthesis, but no inhibition was observed if given at other times, and the progression through G1 was not inhibited. The authors concluded that there appeared to be a role for p53 in the transition of cells from a resting to a growing stage. The various pieces of evidence taken together suggested that p53 was a positive cell cycle regulator. Other evidence to implicate p53 as an oncogene includes the finding of a cooperation between p53 and an activated H-ras oncogene in cellular transformation (Eliyahu et al, 1984). Later studies, however, provided much evidence to suggest that wild-type p53 had, in fact, anti-proliferative properties.

1.4.1.3 p53 AND CELL CYCLE NEGATIVE REGULATION - A TUMOUR SUPPRESSOR GENE?

Mowat et al (1985) studied spleen cells obtained from mice in whom erythroleukaemia was induced by the Friend erythroleukaemia virus, and found that the provirus genome was integrated into the p53 gene, causing rearrangement of the latter and expression of a truncated p53 protein or its total absence. Another example is the finding of a mutated p53 gene leading to a 46 kDa truncated protein in a similar erythroleukaemia cell line (Munroe et al, 1988). The authors reported that the deletion, in exons 7 and 8, involved a highly conserved and therefore functionally important region of the gene. When other groups demonstrated that, in such situations, the second allele was usually deleted, it appeared that p53 loss conferred a growth advantage to these malignant cells. Two further important pieces of evidence came separately in 1989. Finlay et al (1989) reported that DNA clones of wild type p53, co-transfected either with a combination of activated oncogenes like E1A and ras, or of mutant p53 and ras, inhibited the ability of such oncogenes to transform rat embryo fibroblasts. Most of the ones that did transform, even though they contained p53 in their genome, failed to express the protein; and the cell lines that did express p53 had the mutant conformation. Eliyahu et al (1989) confirmed these findings and also found that plasmids carrying mutant p53 totally failed to inhibit oncogene-

induced transformation and often even slightly stimulated it. These effects were more marked in early-passage rather than established tumour cells, suggesting a role for mutant p53 in tumourigenesis. p53 was therefore becoming established as a tumour suppressor gene.

1.4.1.4 p53 PROTEIN STRUCTURE

Three separate structural regions of p53 are recognised: the amino and carboxy terminals and the central region. The amino terminal contains acidic residues and is proline-rich. It is the transcriptional transactivator domain, amino acids 20 - 42 being functionally most important. It is the binding site of many viral and cellular proteins including the E1B protein from adenovirus type 5 and the murine double minute 2 (MDM2) protein which is an up-stream regulator of p53. The carboxy terminal is hydrophilic and contains the 3 nuclear localisation signals of the p53 protein (Soussi, 1995), mutations of which may render p53 cytoplasmic. Some non-specific and specific DNA-binding functions have also been assigned to this region. The carboxy terminal also contains a region required for tetramer formation, and residue 315 which is the phosphorylation site for cyclin-dependent kinases (cdk), and a role for the latter in nuclear localisation has been suggested though not proven (Soussi, 1995). The central region of p53 appears to be the most important functional domain of the protein. Spanning amino acids 91 to 301, it contains the sequence-specific DNA-binding domain of p53. By a process of immunoprecipitation and PCR amplification of DNA co-precipitated with wild type p53 (El-Deiry et al, 1993; Vogelstein and Kinzler, 1992), a consensus DNA-binding motif consisting of 2 repeats of a 10 bp sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0 - 13 bp. Through this motif, p53 binds to DNA as a tetramer. The central region is also reported to contain 86% of mutations affecting p53, as well as 4 of the 5 of highly conserved domains of the protein (Keith and Russell, 1996). The SV-40 AgT also binds to this region.

1.4.1.5 p53 PROTEIN FUNCTION

p53 is a nuclear phosphoprotein which, in wild type conformation, has a very short half-life of approximately 20 minutes and is not usually detectable in normal cells.

Mutant p53, however, has a much longer half-life of 24 hours or so, and may be detected by immunocytochemistry. In normal cells, p53 occurs in the cytoplasm during G1 phase of the cell cycle, and enters the nucleus during G1/S transition, until the end of the G2/M phase. Described as a 'guardian of the genome' (Lane, 1992), p53 is now known to have a major role in protecting the integrity of DNA from damage by arresting the cell cycle in G1 hence enabling DNA repair, or else mediate apoptosis to prevent continued growth of an abnormal cell. In response to DNA damage, for example by UV light, radiomimetic drugs (Maltzman et al, 1984), γ-radiation or cytotoxic drugs (Fritsche et al, 1993), G1 arrest is induced by an elevation of the level of p53 protein, which is in turn mediated by a post-translational stabilisation mechanism. Several down-stream effectors are recognised to mediate this process. p53 acts as a transcription factor for these genes. WAF1 is expressed in the nuclear p21 protein, an inhibitor of cyclin-dependent kinases (cdk) whose normal association with G1 cyclins allows progression of the cell cycle. Up-regulation of p21 by increased p53 therefore, leads to cycle arrest. GADD45 (growth arrest and DNA damage-inducible) is also transcriptionally activated by p53 and may be involved in DNA repair. The murine double minute 2 (MDM2) gene is a cellular proto-oncogene which is transcriptionally activated by p53, but rather than being a down-stream effector, is a negative regulator of p53 function, acting in a feed-back loop. It is proposed that, if DNA damage is repairable, MDM2 continues to suppress the up-regulated p53; but if damage is too severe, p53 is so much up-regulated as not be suppressible by MDM2, and an irreversible apoptotic response ensues (Haines, 1997). One mechanism by which over-expressed MDM2 is associated with human malignancies is via its suppressive effect on p53.

As mentioned, p53 may mediate apoptosis in cells whose DNA is too severely damaged to be repaired. Bax is an apoptosis gene which is up-regulated by p53, in response to, for example, ionising radiation (Miyashita et al, 1994), and is likely to be a down-stream effector of apoptosis for p53. On the other hand, the product of the bcl-2 gene, which has anti-apoptotic effect, may form heterodimers with bax, and the ratio of the two determines cell survival or death (Oltvai et al, 1993). High levels of

bcl-2 may inhibit p53-mediated apoptosis (Wang et al, 1993), and in human malignancies, an inter-relationship between the 3 genes may exist, with p53 loss, reduced expression of bax and increased expression of bcl-2 (Miyashita et al, 1994).

1.4.2 p53 AND CANCER - GENERAL ASPECTS

The p53 gene is situated within approximately 20 kb of DNA on the short arm of chromosome 17, mapped to 17p13.1. The entire gene contains 11 exons, the first of which is non-coding and is 8 - 10 kb upstream of exon 2. The remaining exons are spliced together during transcription to produce an mRNA of 393 codons. Much effort has been spent in understanding the relationship between biology and function of p53, and its DNA structure. Of the entire p53 cDNA sequence, 5 regions (domains) are described to be highly conserved, by comparing sequences across different species as diverse as humans, mouse and X. Laevis. These 5 domains contain more than 90% identity between species. 4 of them correspond to the central, DNA binding region of the p53 protein as described above, while domain I is in the amino terminal. These are (Soussi et al, 1990; Hollstein et al, 1991):

Domains	Codons	Exons
I	13 - 19	exon 2
II	117 - 142	exons 4 and 5
III	171 - 181	exon 5
IV	234 - 258	exon 7
V	270 - 286	exon 8

Fig. 1.1 illustrates the lay-out of the p53 gene, showing the number of bases associated with each exon and intron. The lower panel maps the conserved domains to the corresponding exons. Because of the reported high level of conservation, these domains are likely to be functionally important to the p53 protein, and have been at the heart of studies of p53 mutations in human cancers. p53 is the most frequently mutated gene in human cancers. The great majority of p53 mutations associated with malignancies are somatic mutations, but in certain families with strong histories of cancers, p53 mutations may occur in the germline, giving rise to the Li-Fraumeni syndrome. Affected family members have an elevated risk of early-onset breast

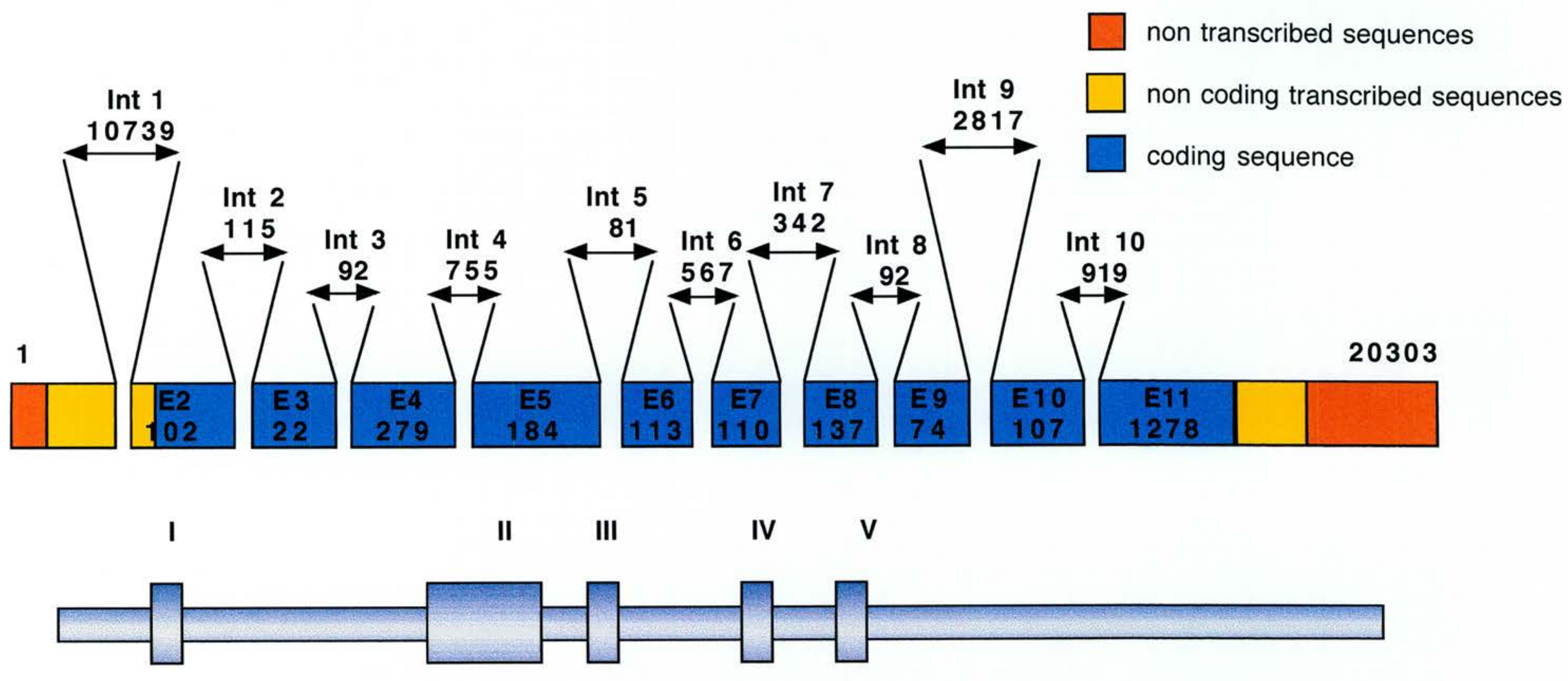


Fig 1.1 Layout of p53 gene. E denotes exon and Int denotes intron. The numbers denote the number of bases. The lower figure represents the conserved domains.

cancer, childhood sarcomas, brain tumours, osteosarcoma, leukaemia and adrenocortical carcinoma (Harris and Hollstein, 1993; Malkin et al, 1990). They have a 50% chance of having cancer by the age of 30, and 90% chance by the age of 65, and this high-lights the crucial role of p53 in keeping check on the cell cycle to prevent the development of cellular immortality. Mutations of p53 have been found in most types of malignancies, and taken together, the frequency is 45 - 50% of cases (reviewed in Soussi, 1995), the highest frequency being found in squamous carcinoma of the oral mucosa (81%), SCLC (70%) and anaplastic thyroid cancer (68%). In general, mutation of one allele is associated with loss of the second. This implies that it is the loss of a functional gene product that leads to tumour formation. 95% of mutations in p53 are found in the hydrophobic central region, and 68% in domains II, III, IV and V, between codons 117 and 286 (Levine et al, 1991). These mutations are not randomly distributed and, in particular, 6 hot-spots have been identified in codons 175, 245, 248, 249, 273 and 282, accounting for 30% of all p53 mutations found in malignancies. Also, 44 codons are relative hot-spots, being arbitrarily defined as having > 12 reports in the database. Overall about 80% of p53 in human cancers are missense mutations (Harris and Hollstein, 1993; Greenblatt et al, 1994). These are results from an extensive study by Greenblatt et al (1994), who compiled a list of 2567 mutations from > 300 papers. From these, of 560 mutations reported in 50 studies in which the entire coding region of p53 were sequenced, 87% of mutations are in exons 5 - 8, 8% in exon 4 and 4% in exon 10. Because of the concentration of mutations to these domains of p53, many (if not most) mutational studies focus on exons 5 to 8 of the gene.

As mentioned, loss of heterozygosity (LOH) in which loss of the normal allele is seen with a mutated one, is often the case in most tumours studied, and is a hallmark of tumour suppressor genes. LOH at 17p, however, may be found without a p53 mutation, for example in cases of breast cancer, and explanations, apart from the fact that a mutation may exist outside the region of the gene studied, may include the possibility of another tumour suppressor locus in 17p (Coles et al, 1990). These authors reported a 58% incidence of allelic loss mapped to 17p13.3 which is

approximately 20 Mb telomeric to p53. From the opposite perspective, Nigro et al (1989) examined 19 tumours of various histological types with 17p allelic loss, and only 3 did not have a mutated gene on the other allele.

1.4.3 PROFILES OF p53 MUTATIONS IN HUMAN CANCERS

Mutational spectrum analysis is the study of the numbers, types and locations of DNA alterations, and since there can be a great diversity of a combination of these parameters, spectra may be very different from one type of tumour to the next. These spectra may describe footprints of specific DNA alterations by endogenous or exogenous mutagenic processes, and form the basis of the important area of molecular epidemiology in cancer research (Jones et al, 1991). Hollstein et al (1997) compiled the most recently updated mutation pattern in the 5 commonest cancers in the world, based on the European Bioinformatics Institute database, shown in Table 1.3. Of the 6 hot-spot codons, 5 contain CpG dinucleotides (codons 175, 245, 248, 273 and 282). These are endogenous mutagenic sites where the cytosine residue is methylated and may undergo spontaneous deamination to thymine, resulting in a G:C > A:T transition. Certain malignancies not known to be related to exogenous carcinogens, such as colonic cancer, have a particularly high incidence of mutation at CpG dinucleotides indicating that these mutations arise from endogenous processes (Caron de Fromentel and Soussi, 1992).

Table 1.3. p53 point mutation pattern in the 5 commonest cancers

Rank	Site	Total mutations	Base insertions/ deletions	Substitutions at G:C				Substitutions at A:T (to T:A, G:C or C:G)
				to A:T at CpG	non-CpG	to T:A	to C:G	
1	Lung	437	11%	10%	16%	39%	10%	16%
2	Stomach	146	13%	26%	24%	6%	4%	26%
3	Breast	388	14%	21%	19%	13%	9%	24%
4	Colon/ rectum	369	7%	47%	14%	9%	6%	12%
5	Skin	206	8%	8%	37% + dincleotide mutations 17%	10%	6%	12%

Mutational analyses have also revealed associations between various external carcinogens and particular p53 mutations. The association between hepatocellular

carcinoma and aflatoxin B1 was realised when a predominance of G:C > T:A transversions in the third base of codon 249 (Arg to Ser), mainly in countries, such as Mozambique and Qidong province of China, where the diet involves foods contaminated by aflatoxin B1, produced by Aspergillus flavis. The incidence of G:C > T:A in HCC in this part of China is an overwhelming 95% compared with 26% in Taiwan and Japan (Greenblatt et al, 1994). This contrast is again high-lighted in Mozambique with a 50% incidence of this transition in HCC, compared with 10% in neighbouring countries without aflatoxin B1 but a similar hepatitis B carrier rate, another risk factor for HCC.

Other examples of exogenous mutagen footprints include the association between squamous cell skin cancer and UV radiation, in which the hallmark is mutations in pyrimidine dimers, particularly tandem mutations of CC > TT (Dumaz et al, 1994), and G:C > T:A transversions (mainly on non-transcribed strand) in tobacco-related lung cancers in which bezo(a)pyrene has been implicated.

1.4.4 p53 DATABASES

A number of databases of reports of p53 mutations are available to the scientific community, the most extensive of which is probably the one deposited in the European Molecular Biology Laboratory (EMBL) in Heidelberg (Hollstein et al, 1994), which is being intermittently updated. As of April 1997, there were reports of 5867 p53 mutants, and these are openly available on the internet. Also widely available on the internet is a large database compiled at the Curie Institute, Paris, by T. Soussi, who regularly updates mutational spectrum analyses on a variety of malignancies (http://perso.curie.fr/thierry.soussi/p53_database.html). Another database is that described by De Vries et al (1996) also available through the internet via EMBL, and contains mutations reported to the English world literature. The printed publication by these authors contains an abbreviated summary of p53 mutations reported to September 1993, from which I estimated the coverage of possible p53 mutations by primers used in this project (Section 5.1.4).

1.4.5 p53 AND SPORADIC BREAST CANCER

Much work can be found in the literature regarding p53 in sporadic breast cancer, particularly with respect to the following: incidence of p53 mutations or increased expression, prognostic value of p53 alterations, and molecular epidemiology. The incidence of p53 gene mutations in breast cancer is 15 - 40%, and more closely in the 20 - 25% range in most studies. Most studies have used SSCP or DGGE to screen individual exons, mainly exons 5 - 8, but some have sequenced the entire coding region (Bergh et al, 1995). In an up-to-date analysis (Novermber, 1997) by Soussi on the Curie Institute database (internet, see above) of 869 breast tumours and cell lines, the key hot-spot codons for mutations are 273, 248, 175 and 245, which together account for 20.8% of all p53 mutations in breast cancer. Most tumours that contain mutations have also lost the remaining allele by restriction fragment length polymorphism (RFLP) analysis (loss of heterozygosity, LOH). Immunocytochemical (ICC) studies tend to reveal a rather higher incidence of p53 protein accumulation, but the range is also wide, eg, 22% (Davidoff et al, 1991), 33% (Thorlacius et al, 1995) and 52% in the largest series to date (Allred et al, 1993). ICC and mutation analysis do not always correlate and reasons may include mutations occurring outwith the regions screened, or the presence of contaminating normal tissue making mutation detection difficult. Alternatively, p53 protein accumulation may be due to factors other than mutations.

p53 alterations (genetic or ICC) have relevance both in tumour biology and in clinical prognosis. Cattoretti et al (1988) studied 200 primary breast tumours with p53 Moab's Pab421 and Pab1801 and found a significantly higher proliferative index, as measured by the expression of Ki-67 antigen, in tumours that stained positive. This is supported by data from Allred et al (1993) who measured proliferation in terms of the percentage of cells in S phase of the cell cycle. p53 alterations are also correlated with negative oestrogen receptor (ER) and progestogen receptor (PgR) status, suggesting more advanced disease (Mazars et al, 1992; Andersen et al, 1993; Thorlacius et al, 1995). Various authors have studied the correlation between p53 status and clinical or pathological parameters. It is apparent that breast tumours containing p53 mutations

carry adverse prognostic implications and associated with shorter DFS and an increased risk of relapse (Elledge et al, 1993). Andersen et al (1993), who found 21% and 22% of 163 tumours positive for mutation (in exon 5 through 8) or ICC, respectively, performed multivariate analyses, and reported a positive correlation between p53 mutations and/or positive ICC with positive axillary lymph node status, a tumour size (T) score of > 1 and invasive ductal histology, all with known adverse prognostic implications. These authors also performed univariate survival analyses and found strong negative correlation between p53 alterations and both overall and DFS. Bergh et al (1995) studied the prognostic implications of p53 mutations anywhere in the coding sequence. Direct sequencing of tumours from 298 informative patients detected a 21.8% incidence of mutations. Axillary node positive patients whose tumours had a p53 mutation had significantly shorter survival than if the tumours were p53 negative. Statistical significance was not achieved in node-negative patients. p53 alteration as measured by ICC, however, correlated with survival in node-negative patients in the study of Allred (1993). Another prognostic variable that emerged from Bergh's study is that tumours whose p53 mutations occurred in the conserved regions II and V are associated with significantly poorer OS than those with mutations in other regions. Because of the small numbers these data must be interpreted with caution, particularly when set again the finding of Borresen et al (1995) who reported poor prognosis associated with mutations in sequences more closely related to regions III and IV, the zinc-binding domains L2 (codons 163 - 195) and L3 (codons 236 - 251). To extend their observation on the clinical relevance of these mutations, the latter authors (Aas et al, 1996) demonstrated that breast cancer patients harbouring a p53 mutation are much more likely to show primary resistance to doxorubicin chemotherapy by having progressive disease during treatment - 4 / 18 patients (22.2%), versus 2 / 45 (4.4%) patients without mutations. Also the mutations of 3 of these 4 patients occurred in the L3 domain.

Close scrutiny of the nature of p53 mutations in breast cancer may shed light on its epidemiology. Although no carcinogen has been conclusively implicated, some features of the mutation spectrum may suggest involvement of exogenous factors:

nearly half of mutations are either G:C > T:A transversions, G:C > A:T transitions at non CpG sites, or A:T > G:C transitions, all with heavy non-transcribed strand bias (Greenblatt et al, 1994). DNA repair on the non-transcribed strand tends to be slower than the transcribed strand, and such strand bias tends to indicate DNA damage by exogenous physical factors. To support this is the observation that breast cancer incidence increases in Oriental immigrants to America (Ziegler et al, 1993), even within one generation. When the nature of mutations between different racial and geographical groups are compared, differences emerge that would suggest the possibility of different aetiologies in these groups. For example, a high frequency of G:C > T:A transversions (88%) is observed in a Scottish population (Coles et al, 1992), A:T > G:C transitions (31.2%) in black Americans (Blaszyk et al, 1994), A:T > T:A tranversions in Austrians, and microdeletions in white Americans (Sommer et al, 1992a). An exceptionally high incidence (55.5%) of p53 mutations has been observed in a certain Japanese population (which has a low incidence of breast cancer), suggesting p53 alterations may be aetiologically important in this group (Hartmann et al, 1996).

For this project, I have chosen p53 as a marker for MRD studies largely because of **(1)** the relatively high incidence of mutations compared to other candidate genes, **(2)** the choice of patients who have come to have PBPC harvesting and transplantation, or with metastastic disease means that they are already in an adverse prognostic group and p53 mutations may therefore be more prevalent, and **(3)** the heterogeneity of mutations should provide a good system to test the feasibility of using diverse single base changes as disease markers.

1.4.6 P53 AND ACUTE LEUKAEMIAS AND MYELODYSPLASIA (MDS)

Much work has also been done to elucidate the relationship between p53 and the haematological malignancies, in which mutations of this gene are relatively infrequent except for certain subgroups of disorders. Those in which p53 mutations are relatively more prevalent are: blast crisis of chronic myeloid leukaemia (CML), Burkitt's lymphoma or its leukaemic equivalent (L3 sub-type of acute lymphoblastic leukaemia,

ALL) and Richter's immunoblastic transformation of chronic lymphocytic leukaemia (CLL). Of most haematological malignancies studied, several general conclusions may be drawn regarding p53. Mutations of p53 are associated with: **(1)** more advanced-stage or terminal disease, **(2)** adverse clinical outcome, and **(3)** resistance to cytotoxic chemotherapy. Here, I shall describe what is known in the acute leukaemias and MDS, as relevant to the cohort of patients that I am studying in this project . Table 1.4 illustrates results from some of the studies on these disorders. In AML, the incidence of p53 mutations is usually less than 10%, but evidence suggests that these are much more frequent in relapsed disease than at presentation. Zhu et al (1996) found 10 mutations of 31 samples (32.3%) at relapse, whilst when diagnostic samples from 8 of these patients were evaluated, only 3 were positive. The use of more sensitive allele-specific PCR methods demonstrated that a minority clone was present at diagnosis in some of these patients, indicating that the p53 mutation containing cells had undergone clonal evolution to become the majority clone, allowing them to be demonstrated by single strand conformation polymorphism (SSCP) (with sensitivity of 5 - 10%). The same observation was made by Hsiao et al (1994) in T cell ALL. None of 15 samples at diagnosis had a p53 mutation, but mutations were demonstrable in 10 of 38 samples (28%) at relapse. In this series, 6 patients from whom relapse samples were available also had diagnostic samples, and in 3 of these, a missense mutation was present only in the relapse sample, indicating that relapse had arisen with the presence of a p53 mutation. The fact that CML blast crisis and Richter's syndrome have higher frequencies of p53 mutations emphasises the fact that such genetic changes are hallmarks of aggressive disease. In a landmark paper, Wattel et al (1994) set out clearly some of the prognostic and biological implication of various haematological malignancies containing p53 mutations. In AML, 3 / 9 (33%) of cases with mutations achieved complete remission (CR) when treated by intensive anthracycline-cytosine arabinoside base regimens, compared to 66 / 81 (81%) of the non-mutated cases. Likewise, for patients treated by low dose cytosine arabinoside, CR rate was also lower for the mutated patients - 0 / 5 - compared to non-mutated ones - 3 / 6 (50%), although just failing to achieve significance (p=0.06). Improvement in median survival in the non-mutated group (15 months) compared to

Table 1.4 Incidence of p53 mutations in some haematological malignancies

Author	Disease	Pos for p53 mutation / no. of cases	(%)	Exons studied	Methods
Pignon (1994)	AML	2 / 40	(5%)	1 - 11	DGGE + sequencing
Fenaux (1992)	AML	8 / 112	(7.1%)	5 - 8	SSCP + sequencing
Trecca (1994)	AML	5 / 70	(7.1%)	5 9	SSCP + sequencing
Wattel (1995)	AML	16 / 107	(15%)	5 - 9	SSCP + sequencing
Mori (1992)	AML	0 / 3	(0%)	5 - 8	SSCP + sequencing
Slingerland (1991)	AML	1 / 5	(20%)	1 - 11	RTPCR + sequencing
Preudhomme (1994)	AML	8 / 72	(11%)	4 - 10	SSCP + sequencing
Misawa (1990)	MDS	10 / 70	(14%)	5 - 8	SSCP + sequencing
Sugimoto (1993)	MDS	3 / 44	(7%)	1 - 11	RTPCR + sequencing
Jonveaux (1991)	MDS	5 / 151	(3%)	5 - 8	SSCP + sequencing
Mori (1992 + 1995)	MDS	3 / 17	(17.6%)	5 - 8	SSCP + sequencing
	MDS > AML	2 / 12	(16.7%)	5 - 8	SSCP + sequencing
Wattel (1994)	MDS	20 / 182	(22%)	5 - 9	SSCP + sequencing
Adamson (1995)	MDS	4 / 26	(15.4%)	5 - 8	SSCP + sequencing
Preudhomme (1994)	MDS	2 / 11	(18%)	4 - 10	SSCP + sequencing
Fenaux (1992)	ALL (adult)	1 / 51	(2%)	5 - 8	SSCP + sequencing
	L3 ALL (adult)	2 / 9	(22%)	5 - 8	SSCP + sequencing
Pignon (1994)	ALL (adult)	0 / 21	(0%)	1 - 11	DGGE + sequencing
Marks (1997)	ALL (childhood):			4 - 9	SSCP + sequencing
	- poor outcome	2 / 17	(11.8%)		
	- good outcome	0 / 17	(0%)		
Wada (1993)	ALL (childhood)	5 / 312	(2%)	5 - 8	SSCP + sequencing
	L3 ALL / Burkitt's lymphoma (childhood)	2 / 8	(25%)	5 - 8	SSCP + sequencing
Hsiao (1994)	T-ALL (diagnosis)	0 / 15	(0%)	4 - 9	SSCP + sequencing
	T-ALL (relapse)	10 / 36	(28%)		
Gaidano (1991)	L3 ALL (adult)	5 / 9	(55.5%)	5 - 9	SSCP + sequencing
Marasca (1996)	CML blast crisis	4 / 27	(14.8%)	4 - 9	SSCP + sequencing
Wada (1993)	CML blast crisis (ch)	0 / 2	(0%)	5 - 8	SSCP + sequencing

the mutated group (2.5 months) was highly significant. Similar trends were observed in MDS patients. When all chemotherapy and remission status (CR and partial remission (PR)) were taken together, response rate was 1 / 13 (8%) for the mutated group versus 23 / 38 (60%) of the non-mutated group. One argument against these observations is that the p53 mutated patients were significantly more likely to have complex cytogenetic abnormalities, which could have been the cause of the adverse prognosis. The authors argued, however, that the role of DNA repair of wild type p53 would be lost with mutations, which in turn, could have contributed to the other genetic and cytogenetic abnormalities rather than being the results of them. Activation of the multidrug resistance (mdr1) gene promotor by mutant p53 has been reported, although, at least in MDS, has not been proven to be of relevance in the adverse clinical outcome of these patients (Preudhomme et al, 1993).

p53 may seem an unlikely candidate gene as a marker for MRD detection in acute leukaemias, given the low incidence, but reports of the more frequent occurance in relapsed disease (to a level comparable to breast cancer) makes it worth studying, and may also shed light on the prevalence of the phenomenon of clonal evolution of one leukaemic clone over another.

1.5 MUTATION DETECTION

The abililty to detect genetic mutations is important in many areas of medicine and biology. This is only likely to become increasingly important as the rate of identification and characterisation of genes that cause inherited diseases in man gains momentum, and more and more mutations are identified in particular genes. More importantly this trend will be fuelled further by the Human Genome Project. Cancer genetics and the molecular biology of cancer is another area where there is a huge amount of interest, and with the increasingly central role molecular biology is playing in the diagnosis, detection of residual disease, and sometime treatment of these diseases, to be able to detect both unknown and previously described mutations has now become essential rather than being a luxury for the research laboratory. For these

reasons, a number of techniques have emerged over the last decade or so, that enable mutations to be detected or screened more readily. Prior to this era, it was only possible to screen for gross genetic lesions such as deletions, insertions and rearrangements by Southern blotting or RFLP's. RFLP's, although a powerful technique, however, are often limited by the fact that many mutations known to cause human diseases do not lead to alterations in restriction enzyme cleavage sites (for example β-thalassaemias) or that long stretches of the genome may be devoid of polymorphisms, making it unsuitable for the study of these. The real 'dawn' of the mutation detection era when small alterations, for example single base changes, came in 1985 when two independent methods were described for the detection of previously unknown mutations: denaturing gradient gel electrophoresis (DGGE) and RNase cleavage of mismatched heteroduplexes. Since this time, a host of other technique have been described by workers in the West and in Japan. Examples are chemical cleavage of mismatch (CCM), single-strand conformation polymorphism (SSCP), heteroduplex analysis (HA), carbodiimide chemical modification of mismatch (CDI), and variations of some of the above, such as dideoxy finger printing (ddF) and constant denaturant gel electrophoresis (CDGE). To enable more efficient detection of mutations by these techniques, specially designed apparatuses, improved polyacrylamide gels (e.g. the Hydrolink MDE), and computer software to help determine DNA fragment melting characteristics and design primers for the polymerase chain reaction (PCR) have become available over the last few years. Apart from unknown mutations, methods are available also to screen for previously described mutations. All these out-lined methods have been revolutionised by the advent of the PCR.

This review will concentrate on the 2 main techniques in this project, namely DGGE and the amplification refractory mutation system (ARMS), and other available technologies will be touched on. It will be divided broadly into methods used for screening unknown mutations in a DNA fragment, and those which screen and detect previously described mutations.

1.5.1 DENATURING GRADIENT GEL ELECTROPHOESIS (DGGE)

DGGE was one of the first techniques to be introduced to allow the detection of single base changes within DNA fragments, described by Fischer and Lerman in 1983, who with Fischer published their studies on the theoretical basis of DGGE two years previously.

1.5.1.1 PRINCIPLE

DGGE distinguishes DNA fragments of identical size and differ only in changes as small as a single base substitution by virtue of the fact that they often have different melting temperatures (Tm), and migrate differently through a standard polyacrylamide gel containing a linear increasing gradient of denaturants such as urea and formamide. Much work was done in the 1970's on the area of DNA melting, and Fischer and Lerman (1983) first described their study of using denaturing gradient polyacrylamide gels to separate DNA fragments differing by single base pair substitutions, and demonstrated a correlation with predictions made with melting theories. While the original Fixman melting map only takes into account 2 stability values, one for G.C and one for A.T base pairs, Lerman and Fischer found the best correlation with the actual behaviour of λ strain DNA fragments in a denaturing gradient gel is by devising maps using a set of 10 temperature values for a set of 10 nearest-neighbour doublets. Within a double-stranded DNA fragment in solution, base sequences melt not individually but in blocks known as domains, which are characteristically between 25 and several hundred base pairs in length. Adjacent domains may have very different Tm's and have sharply defined borders. DNA fragments from 100 to 1,000 base pairs usually have between 2 and 5 domains, with Tm of approximately 65 - 80°C. Within the DNA double helix, stacking interactions between bases contribute greatly to its thermodynamic stability, help maintain the fragment in a helical form, and contribute more energy to stabilisation than hydrogen bonds that link complementary bases in opposing strands. The degree of stacking, and hence the Tm of a fragment, is determined by the order in which the bases are arranged in a strand. Small changes, even single base substitutions, can alter stacking interactions sufficiently to change the Tm by 1°C. These differences in Tm may be translated into different migration

patterns between wild type and single-base mutated DNA fragments on a polyacrylamide gel with a linear gradient of denaturants. The electrophoretic apparatus is set at a high temperature but below the Tm of the domain to be studied. The polyacrylamide gel is standard, and contains a cross-linker, polymerisation initiated by the addition of 10% ammonium persulphate and N,N,N,N'-tetramethylethylenediamine (TEMED), but contains a linear gradient of urea and formamide from top to bottom of the gel. As a DNA fragment, e.g. in the form of a PCR product, is electrophoresed, at some point down the gel it encounters a concentration of denaturant which, in addition to the high temperature, causes the double-stranded domain with the lowest Tm within that fragment to melt and the 2 strands to separate. Owing to the fact that the adjacent domain still remains double-stranded, the molecule assumes a Y-shape as the first domain 'forks'. Mobility of this entire fragment through acrylamide is then dramatically slowed down. Indeed, further migration is often neglible and the band tends to be sharply focused as a result of this mobility arrest. Mutant DNA fragments melt at a slightly different denaturant concentration to wild type, and produce bands on the gel at different levels, enabling them to be identified. Fig. 1.2 illustrates the principle of DGGE.

1.5.1.2 USE OF GC-CLAMPS

DGGE is therefore able to examine DNA fragments with a minimum of 2 domains. When the domain with the highest Tm within that fragment melts, single strands will result which are able to migrate through the gel with ease and mutant and wild type sequences can no longer be seperated. For a standard PCR product, therefore, mutations within the highest melting domain cannot be screened. In general, approximately 50% of single-base changes in DNA fragments ranging from 50 to 1000 bp can be detected (Sheffield et. al., 1989). Myers et al (1985a) attached a 300 bp GC-rich (80% GC) DNA fragment to the sequence studied, and found that nearly all single-base substitutions can be detected (Myers et al, 1985b). With the advent of PCR, this technique was made much easier (Sheffield et al, 1989). During PCR this region is also amplified, and the resulting PCR product contains a double-stranded GC tail. GC base pairs have much higher Tm's that AT base pairs, and such a long

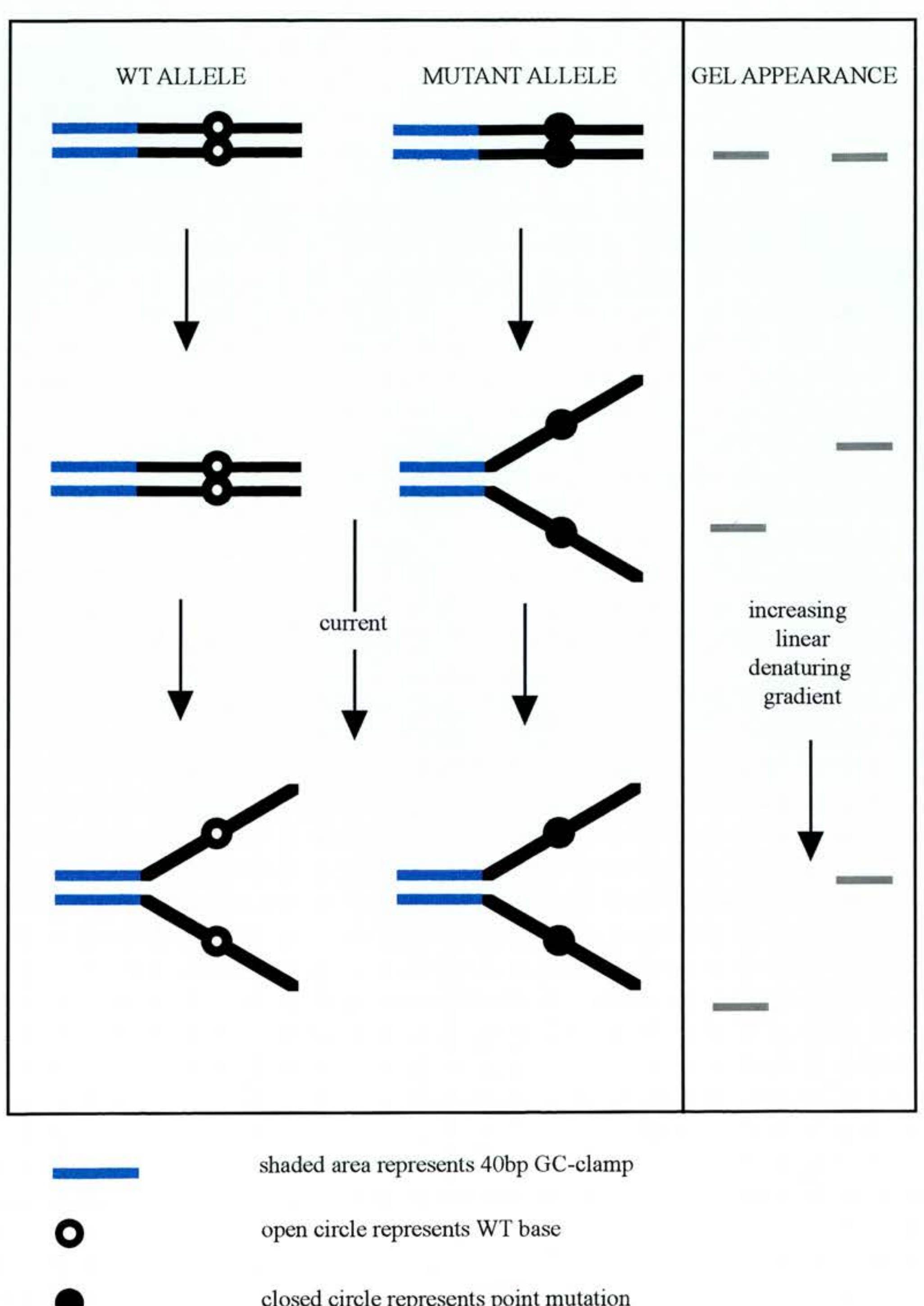


Fig. 1.2 Schematic diagram of the principle of DGGE.

GC-rich region (GC-clamp) predictably has a much higher Tm than the rest of the product, such that it remains double-stranded throughout DGGE and enables the whole of the original fragment to be studied. The application of GC clamps enables the sensistivity of detection to increase from 40% to approximately 95% (Myers et al, 1985b). Further refinement suggested that a clamp as short as 30 bp would suffice (Myers et al, 1989), but most authors would now probably use a 40 bp clamp.

1.5.1.3 PCR PRIMER DESIGN

Although DGGE is able to study DNA fragments up to approximately 1kb in length, 100-500 bp fragments are optimal (Myers et al, 1988). The main reasons are that the longer the fragment, the lower the resolution between mutant and wild type, and the electrophoretic times involved are too long to be practical. Longer fragments should be screened in separate sections, each with a GC-clamp added during PCR. Although the Tm of the GC-clamp is likely to be higher than the rest of the fragment, primers should not be selected at random, and there are means to predict the melting behaviour of DNA fragments based on nucleotide sequences such that the best sequence to be studied and hence the best primers to produce it can be determined. Primers should be 20 - 25 bp, with at least 50% GC and not contain indirectly repeated sequences (Myers et al, 1989). Computer programmes that help determine melting behaviours of DNA fragments are available (e,g, MacMelt, Melt87, SQHTX) and plot the Tm at each base pair position against the bp number from the end of the fragment. Some can translate this Tm data into differences in migration on a gel. Ideally the Tm of the domains studied should be as far below that of the GC-clamp as possible. The optimal length of the GC-clamp is 40 bp. Although shorter clamps may work with certain DNA fragments, this does not apply to others. The sequence may be any random GC combinations, and should be 100% GC in content to maximise the Tm.

1.5.1.4 GRADIENT CONCENTRATIONS

It is important to have information on the melting profile of a DNA fragment in order to design the optimal concentrations of denaturants to use in the gel, in order to

maximise the separation between mutant and wild type bands. Computer algorithms such as the programmes mentioned above can be used to determine the Tm's, and the gradient concentrations calculated as follows. Take a DNA fragment that melts as one domain, with the GC-clamp as the second domain. The concentration of the denaturants in the gel should represent approximately 10°C around the Tm of the first domain. The conversion factor for Tm and percent denaturant is roughly 1°C for each 3% denaturant. Therefore, for this fragment with a Tm of 75°C (GC-clamp Tm 95°C), the percent denaturant should represent 70 - 80°C, and with the gel heated to 60°C, percent denaturants required is therefore (70-60)x3 = 30% at the top, and (80-60)x3 = 60% at the bottom of the gel. Although satisfying these theoretical parameters is likely to produce the best results, this need not always be the case. In this project, I found that for simplicity, a common gradient of 30 - 80% denaturants worked well for all the fragments screened.

1.5.1.5 CONSTANT DENATURANT GEL ELECTROPHORESIS (CDGE)

This variation on DGGE utilises the same principle, except that denaturing conditions are optimised such that only the concentration of denaturant most likely to cause the widest seperation between wild type and mutant (Borresen et.al., 1991). This simplifies the procedure as gradients are not required, and because wild type and mutant DNA migrate differently from the beginning of electrophoresis, seperation is likely to be wider.

1.5.1.6 TEMPERATURE GRADIENT GEL ELECTROPHORESIS (TGGE)

Again the same principle is applied, except that the gradient is a steadily increasing temperature rather than a chemical one (Rosenbaum and Reissner, 1987). This has been applied in the study of mutations of the p53 gene (Ke et.al., 1993), and the androgen receptor gene (Tincello, 1995).

1.5.2 SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

SSCP is currently among the most commonly used screening methods for the detection of unknown mutations (Orita et al, 1989a and b). It is one of the few

techniques where single rather than double stranded DNA is examined. The sequence of interest is amplified by flanking primers in the presence of a 32P labelled deoxynucleotide triphosphate (dNTP), usually α-32P-dATP or -dCTP, which renders the PCR product radiolabelled. Samples are then denatured by heating and immediately electrophoresed in a polyacrylamide gel under non-denaturing conditions. Migration of single stranded DNA product is highly dependent on their tertiary structure, and SSCP is said to be able to detect subtle changes caused by as small a change as a missense mutation. Unlike DGGE which has a sound theoretical basis and where the melting behaviour of DNA fragments may be predicted with computer programmes, no such model is available to predict the three-dimensional structure of single-stranded DNA. As intra-molecular interactions are important in determining DNA folding, mobility in the gel is often different even between complementary strands. Different physical conditions within the polyacrylamide gel, such as temperature and ionic strength, may affect the precise tertiary structure of single-stranded DNA, and so the conditions at which electrophoresis is run may need to be adjusted for each fragment of DNA studied, and rigidly controlled for reproducibility. Usually electrophoresis at room temperature with 5-10% glycerol or at 4°C without glycerol is employed as a starting point. A low level of polyacrylamide cross-linking (e.g. < 2%) is desirable, and the shorter the fragment of DNA to be studied the more sensitive the technique. From analysis of a number of studies using SSCP, mutations in fragments of less than 200 bp have an over 90% likelihood of being found, and the figure for fragments of 300 - 350 bp is over 80% (Hayashi and Yandell, 1993). Larger PCR products may also be studied with SSCP after restriction enzyme digestion and each product screened separately. Characteristically, DNA containing wild type alleles gives 2 bands on the gel corresponding to the 2 complementary strands, and mutant alleles produce bands with different mobility. The total number of bands in a lane is not predictable, however, as some fragments may have more than one stable conformation. Modifications of the original description of SSCP include the use of non-radiotopic labels and silver-staining which may simplify the technique although the ultimate sensitivity may be compromised. Sarkar et.al. (1992) described using DNA-derived RNA for SSCP with the rationale that RNA can assume a larger

number of conformations than DNA. By comparing the detection rates of DNA and RNA-SSCP on a number of mutated samples, they demonstrated a clear advantage of the latter (80% vs 59%), although the detection rate of 59% with DNA-SSCP is low. SSCP remains one of the most commonly used technique in mutation screening because of its simplicity and the fact that no specialist equipment is required.

1.5.3 CHEMICAL CLEAVAGE OF MISMATCH (CCM) / HYDROXYLAMINE-OSMIUM TETROXIDE (HOT) TECHNIQUE

This method is based on the observation (Cotton et.al., 1988) that, in double-stranded DNA, mismatched thymine and cytosine (hence presence of heteroduplexes) are particularly sensitive to chemical modification by osmium tetroxide and hydroxylamine, respectively, and after subsequent cleavage of these fragments by piperidine, the resulting fragments may be analysed on denaturing polyacrylamide gels alongside an end-labelled molecular weight marker, so that not only the presence but also the location of the mutation may be determined. This procedure involves first preparing a radio-labelled 'probe' which is the 32P end-labelled PCR product of normal (control) DNA. Mutant DNA is separately amplified by PCR. Heteroduplexes between mutant and wild type PCR products are produced by boiling them together, precipitated, the radioactivity determined with a β-counter and then resuspended in water to 1,000 cpm/ul. Aliquots are treated separately with hydroxylamine and osmium tetroxide at 37°C, and cleaved by 1M piperidine at 90°C prior to electrophoresis in an 8% denaturing polyacrylamide gel. An aberrant band produced by the cleaved, mismatched fragment is visible. The sensitivity of CCM is high as all T and C mismatches are detected. However, because certain GT mismatches are unreactive to osmium tetroxide, labelling mutant DNA as the probe for hybidisation with wild type ensures that the mutation is detected by the reciprocal AC mismatch with hydroxlamine. Apart from the theoretical 100% detection sensitivity, another advantage of CCM/HOT is able to screen large DNA fragments, up to 2kb. The major disadvantages are the need for multiple steps, and the use of toxic chemicals in the fumehood. Modifications such is non-isotopic labelling may increase its application, but no other (safer) candidate chemical has thus far been identified to substitute HOT.

1.5.4 RNASE A CLEAVAGE OF HETERODUPLEXES

Radio-labelled wild type RNA is heteroduplexed with potentially mutant RNA or DNA, and cleaved by RNase A. Fragments are separated on a denaturing gel by size to help locate the site of the mutation. Although a single step is required, preparing an RNA probe is time-consuming. Detection efficiency is also low as RNase A cleaves well only at pyrimidines. Using a probe for the complementary strand circumvents this but creates extra work. Overall sensitivity is about 70% only, accounting for the lack of appeal.

1.5.5 CARBODIIMIDE (CDI) MODIFICATION

The bulky reagent CDI reacts more rapidly with the imino sites of mismatched thymine and guanine bases than with fully matched ones, and such treated heteroduplexes are refractory to primer extension, and can therefore be detected by the presence of a smaller than full size product on electrophoresis (Ganguly and Prockop, 1990). Although only one relatively non-toxic chemical is used, two sets of incubation conditions are required for complete detection. Apart from the original laboratory, wide-spread use of this method has not been found. One remarkable feature, though, is that the largest heteroduplex successfully screened, albeit with the aid of anti-CDI antibodies and immuno-electron microscopy, is 7.2 kb.

1.5.6 HETERODUPLEX ANALYSIS (HA)

Heteroduplexes are created during the latter cycles of PCR when the template consists of both mutant and wild type species of DNA. Alternatively, they may be produced by boiling a mixture of mutant and wild type PCR products, followed by slow cooling. They consist of a mutant strand of DNA annealed onto a wild type strand, and may be differentiated on a denaturing polyacrylamide gel from homoduplexes by having different mobilities. The concept of HA is similar to SSCP except that double-strands are involved (White et al, 1992; Soto and Sukumar, 1992; Glavac and Dean, 1995; Prosser, 1993; Cotton, 1993). There is no theoretical basis to predict the influence of environmental factors such as buffer ionic concentrations and temperature of electrophoresis, and like SSCP, these also have to be optimised.

Electrophoretic retardation of heteroduplexes is dependent on the nature of the mismatch: large insertions or deletions give stable heteroduplexes leading to greater retardation. Base substitutions, however, create 'bubble'- type heteroduplexes with looser structures and are less easily discernable. The sensitivity of mutation detection by HA is around 80% (Cotton, 1993) and the optimal length of fragment screened is 200-300 bp. HA and SSCP may be used in combination, thus increasing the sensitivities of the techniques. As heteroduplexes formed between wild type DNA and single-base-mismatched mutant may be practically undetectable, mixing suspected mutants with another deletion-mutant before HA renders these samples positive (van den Akker, et. al., 1992). Thus HA is easy to perform, and although not 100% sensitive, is a popular technique for mutation detection.

1.5.7 COMPARISON OF TECHNIQUES

Surprisingly few pieces of published work have systematically compared the detection sensitivity between various techniques. Condie et.al. (1993) compared the efficacy of SSCP, CDGE and the HOT technique in detecting point mutations of the p53 tumour suppressor gene. Three laboratories specialised in each of these techniques, participated, and each sent DNA (9 or 10 mutant, 2-5 normal controls) to the other two for analysis by their own techniques. SSCP detected 90% of mutations, CDGE detected 88% although further optimisation of conditions after the presence of mutations was known picked up 100%, and HOT detected 100%. This study has been criticised for its fundamental design flaw of using three separate laboratories to test three different techniques, and using each of the techniques as the basis for labelling a sample 'positive' to be screened by the other two.

Theophilus et.al. (1989) compared RNase cleavage, CCM and DGGE in screening mutations in the human acid B-glucosidase gene, responsible for Gaucher's disease. GC-clamped DGGE emerged as the best of the three, being the only method successfully detecting the five mutations used. CCM was effective in detecting C mismatches but high background with osmium tetroxide prevented T mismatches to be identified. RNase cleavage only picked up 3/5 mutations. One further study (Moyret et.al., 1994) directly compared SSCP and DGGE in detecting 52 different

mutation of exons 5-8 of the p53 gene. 100% detection was achieved by DGGE, compared to 90% by SSCP.

1.5.8 AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

The detection of known or characterised mutations generally is more straigtforward that screening unknown ones, and a totally different set of techniques is applied. These techniques are also more likely to be used for medical diagnostic purposes, which makes it even more important for them to be highly sensitive as well as specific, and be simple enough to perform for everyday diagnostic use. Most of these are PCR-based. ARMS was first described in 1989 (Newton et.al., 1989a) when it was used in the differentiation of disease carrier status in patients with α1-antitrypsin (AAT) deficiency with respect to the S and Z loci, each of which differs from normal by a single base substitution. In general, ARMS employs 2 PCR reactions with one common primer between them, and the other primer of each pair is specific for each of the 2 alleles in question. In this way, one can differentiate these genotypes by observing which reaction with which specific primer produces amplification products. It is expected that genomic DNA with the opposite allele would not amplify. This specificity is dependent on the nucleotide at the 3' end of these primers being complementary to one allele. Taq DNA polymerase is used in these reactions owing to its lack of a 3' to 5' exonucleolytic proof-reading activity. Enzymes with this activity are able to recognise mismatches between primer and template, and excise such mismatches and insert the correct nucleotide, and hence not suitable for ARMS application.

Primers with one single base mismatch will not necessary fail to amplify, and may create problems with poor specificity. Newton et.al. (1989a) described the technique of introducing further deliberate mismatchs near the 3' end of the ARMS primer, and makes DNA with the opposite allele even more refractory to amplification. The closer the second mismatch is to the 3' end, the more the annealing is destabilised, and the less likely for a product to form. Pyrimidine/pyrimidine and purine/purine mismatches have greater destabilising effect then pyrimidine/purine mismatches. The rank order of

destabilisation is therefore: CC>CT>GG=AA=AC>GT. Frameshift mutations do not require such a manoeuvre as any deletion or insertion already produces further mismatching.

ARMS, also known as allele-specific PCR (ASPCR) and PCR with sequence-specific primers (PCR-SSP), has found wide application and been used in the prenatal diagnosis and carrier assignment of cystic fibrosis (Newton et.al., 1989b), identification of specific strains of bacteria (Lampel et al, 1996; Cebula et al, 1995), detection of 3 of the common genetic polymorphisms of the apolipoprotein E gene (Wenham et.al., 1991a), diagnosis of familial defective apolipoprotein B-100 (Wenham et.al., 1991b), diagnosis of sickle cell anaemia (Wu et. al., 1989), HLA typing of the DR locus (Patel et.al, 1993), and typing of the HPA-1 platelet antigens (Metcalfe and Waters, 1993). Lo et.al. (1991) also described the 'double ARMS' technique where 2 allele-specific primers (instead of one allele-specific and one common) were used to study a highly polymorphic region of the human δ-globin gene, and found it more sensitive and specific than single ARMS. This was applied in the detection of chimaerism after allogeneic BMT (Lo et.al., 1993). In cancer diagnostics, Takeda et.al. (1993) described the application of ARMS (or MASA, mutant-allele specific amplification) in the detection of Kirsten-ras oncogene mutations in sputum of lung cancer patients, and this technique was adopted in the use of K-ras and p53 gene mutations to detect the presence of sub-microscopic deposits of colonic cancer in intra-abdominal lymph nodes (Hayashi et.al., 1995). The authors of the latter study were able to demonstrate survival benefit in patients without evidence of MRD as detected by MASA. Althought these authors claimed that MASA is capable of detecting one tumour cell amongst 'thousands' of normal cells, they did not present evidence of the precise sensitivity and specificity of their method.

Other modifications of ARMS have been described, including the introduction of a new enzyme restriction site near the 3' end (ARMS with restriction enzyme cleavage or restriction site-generating PCR, RG-PCR). This is achieved by the fact that a

primer mismatch, not being at the 3' base and therefore allows amplification, may create a recognition site in one but not the other allele.

In the current project, ARMS is one of the major techniques where tumour tissue carrying a mutation may be recognised.

1.6 AIMS OF THE PROJECT

Primary aim:

To assess the feasibility of using subtle genetic alterations, e.g., single base changes, associated with human cancers as markers for the detection of minimal residual disease (MRD) in peripheral blood progenitor cell (PBPC) harvests. The models used are the p53 tumour suppressor gene, and patients with breast cancer and acute leukaemias who have undergone PBPC harvesting / transplantation.

Secondary aims:

(1) To assess the sensitivity and optimisation parameters for a denaturing gradient gel electrophoresis (DGGE) technique employed for mutation detection, using cell lines with known p53 mutations.

(2) To determine the incidence of mutations of the p53 gene in primary breast cancers and acute leukaemias in a local cohort of patients, such that these may be characterised by sequencing and be used as disease markers.

(3) To determine the optimisation parameters and sensitivity of the amplification refractory mutation system (ARMS) technique employed in MRD detection, using cell lines with known p53 mutations.

(4) To determine the incidence of MRD in PBPC harvests of patients whose tumours or leukaemic blast cells contain a p53 mutation.

(5) To correlate, if possible, clinical outcome of patients whose PBPC harvests can be assessed for MRD.

CHAPTER 2

MUTATION DETECTION AND ANALYSIS

2.1 MUTATION DETECTION - GENERAL METHODS

2.1.1 SELECTION OF CONTROL CELL LINES AND DNA

As 5 DNA fragments of the p53 gene (covering 4 exons) were screened, at least one cell line was selected as a positive (mutant) control for each of the fragments. Information was obtained from the extensive p53 database of the European Molecular Biology Laboratory (EMBL) housed in Heldelberg, Germany (Hollstein et.al., 1994 and 1996). Negative (wild type) control DNA was extracted from a pool of human tonsils taken from normal children undergoing tonsilectomy. One human breast cancer cell line (MCF7) documented to contain wild type p53 sequence in the entire coding region was also available. The characteristics of positive controls are listed in Table 2.1.

Table 2.1 Characteristics of cell lines known to contain p53 mutations used as positive controls in this project.

Name	Tissue	Codon	Exon	Mutation	Amino Acid Change	LOH?
BT-20	Breast carcinoma	132	5	AAG > CAG	Lys > Glu	Yes
H69	Lung carcinoma	175	5	GAG > TAG	Glu > End	
T47D	Breast carcinoma	194	6	CTT > TTT	Leu > Phe	Yes
Raji	B-cell lymphoma	213	6	CGA > CAA	Arg > Glu	
Ovcar-3	Ovarian carcinoma	248	7	CGG > CAG	Arg > Glu	Yes
5637	Bladder carcinoma	280	8	AGA > ACA	Arg > Thr	Yes
HT-29	Colorectal carcinoma	273	8	CGT > CAT	Arg > His	

As described below, 5637, T47D and Raji cell lines were grown in our laboratory. DNA from H69, HT-29 and Ovcar-3 were kindly donated by Dr. Hamish Phillips, Medical Research Council Human Genetics Unit, Edinburgh. DNA from BT-20 was kindly donated by Dr. Sigrid Lystad, Norwegian Radium Hospital, Oslo, Norway. Several of the cell lines have documented loss of heterozygosity (LOH) in the allele concerned, and can be considerd as a pure source of mutant DNA sequence, and hence produce only the single mutant homoduplex band on DGGE.

2.1.2 TISSUE CULTURE TECHNIQUES

2.1.2.1 MATERIALS

Iscove's Modified Dulbeco's Medium (IMDM) (Sigma, Poole, UK)
Penicillin/Streptomycin solution (Sigma, Poole, UK)
Gentamicin solution (Sigma, Poole, UK)
Foetal calf serum (FCS) (Sigma, Poole, UK)
L-glutamine (Sigma, Poole, UK)
Hank's Balanced Salt Solution (HBSS) (Sigma, Poole, UK)
Trypsin-EDTA solution (0.25%)
Dimethylsulphoxide (DMSO) (Sigma, Poole, UK)
25cm^2 Tissue culture flasks (Costar, High Wycombe, UK)
75cm^2 Tissue culture flasks (Costar, High Wycombe, UK)
5637 bladder carcinoma cell line
T47D breast carcinoma cell line (ECACC, Porton Down, UK)
Raji non-Hodgkin lymphoma cell line

2.1.2.2 GENERAL CULTURE METHOD

Of the 7 positive control DNAs for the 4 exons of p53, 3 were obtained from cell lines cultured during this project, whose p53 genes are documented to contain point mutations in the relevant exons: exon 8 for 5637, exon 6 for T47D and Raji. 5637 and Raji cell pellets were already available in our laboratory, stored in the vapour phase of liquid nitrogen. One viable T47D cell pellet was obtained from the European Collection of Cell Cultures (ECACC). All 3 cell lines were cultured and propagated,

excess pellets again stored in liquid nitrogen for future use. During repeated cultures, a number of flasks were taken out for DNA to be extracted from the cells. The initial culture was performed in a 25 cm^2 tissue culture flask. Optimal culture medium (OCM) for all 3 cell lines consisted of IMDM with 10% FCS, 1% penicillin/streptomycin, 0.1% gentamicin, and 1% L-glutamine. The presence of the antibiotics, penicillin and streptomycin with half-life in culture of 2 to 3 days prevented infection of the cell culture. Gentamicin, with a longer half-life of 5 days, was synergistic and covered mycoplasma. A viable cell pellet was taken from liquid nitrogen storage and thawed in a 37°C water bath. It was washed once with HBSS in a 15 ml tube by centrifugation at 1,000 g for 5 min. HBSS supernatant was removed, the cell pellet resuspended in 1 ml of prepared OCM, and added to a further 9 ml of OCM in a 25 cm^2 culture flask. The lid was loosened 1/4 turn and place in a tissue culture incubator at 37°C in air with 5% CO_2. Epithelial carcinoma cells adhered to the bottom of the flask, spreading along the surface in a single-cell layer. Raji lymphoma cells grow in suspension. The flasks were checked daily by observing with a light microscope, and the cells re-plated when either the cells fully covered the flask surface, or when the medium had acquired an orange colour indicating exhaustion of essential components. Raji, being non-epithelial and non-adherent, was re-plated empirically when numbers increased or when the medium changed colour.

2.1.2.3 RE-PLATING OF CELLS

Cells were re-plated to a 75 cm^2 flask. Culture medium was poured off, and 5 ml of HBSS added and left for several minutes at room temperature. The HBSS was then poured off and 0.5 ml of trypsin-EDTA solution added to strip the cells from the plastic. After 1 min excess was removed to leave a wet cellular film. The flask was incubated at 37°C for a few minutes to loosen the cells from the plasticware. With gentle tapping of the flask, a film of cells could be seen to run down the culture surface. Two ml of OCM was then added to resuspend and transferred to a 75 cm^2 flask where a further 23 ml of OCM was introduced. The flask was incubated with the lid loosened 1/4 turn. Further re-plating was performed in 75 cm^2 flasks, by splitting

the cells from a fully confluent culture into two. Trypsin-EDTA was not required for Raji, which was centrifuged and resuspended in OCM.

2.1.2.4 CRYOPRESERVATION OF CELLS

From a fully confluent culture in a 75 cm^2 flask, cells were stripped from the plasticware with trypsin-EDTA, washed once in HBSS, resuspended in 1 ml FCS and split into 2 equal (0.5 ml) portions. 0.5 ml of 10% DMSO was added to each aliquot, so that the final concentration of DMSO was 5%. The pellets were frozen in the vapour phase of liquid nitrogen (-170°C) until required.

2.1.3 DNA EXTRACTION

2.1.3.1 MATERIALS

QIAamp Tissue Kit (Qiagen GmbH, Crawley, UK), each designed for 50 extractions of up to 25 mg of tissue each, includes the following:

QIAamp spin columns	Wash buffer AW
2 ml collection tubes	Elution buffer AE
Lysis buffer ATL	Proteinase K
Buffer AL	

Proteinase K was supplied in lyophilised form, and was reconstituted by added 1.4 ml of sterile distilled water. This was divided into aliquots and stored frozen at -20°C until use.

Buffer AL was reconstituted by mixing the 2 components, buffer AL1 and buffer AL2, and was stable for 1 year stored at room temperature.

40 ml of 100% ethanol was added to the concentrate of wash buffer AW, which washed off impurities after DNA binding to the spin column.

100% ethanol (BDH, UK)

Xylene (BDH, Poole, UK)

1.5 ml Eppendorf tubes (Costar, High Wycombe, UK)

50 ml Falcon centrifuge tubes (Costar, High Wycombe, UK)

Red Cell Lysis Solution (RCLS) containing the following:

Ammonium chloride (150 mM)

Potassium carbonate (6 mM)

Disodium EDTA (10 μM)

2.1.3.2 DNA EXTRACTION FROM PARAFFIN BLOCKS

The Qiagen Tissue Kit was chosen for its simplicity and speed. It used the adsorption / elution priniciple where genomic DNA, extracted from cells lysed with proteinase K, was bound to a silica membrane mounted on a spin column, washed of impurities, and eluted with a buffer of pH > 9.

Two 15 μm sections of breast tumours cut with a clean microtome blade were placed in a 1.5 ml Eppendorf tube. Paraffin was first removed by extraction with 1.2 ml of xylene, and vortexed vigorously to mix. The tube was centrifuged at 16,000 g for 5 min, and the supernatant removed. Care was taken not to remove any of the tissue pellet as it often remained suspended rather than spun to the bottom of the tube. Residual xylene was removed by 2 washes with 1.2 ml of ethanol (5 min at 16,000 g). The opened tube was warmed to 37°C for 15 min to evaporate residual ethanol, and the tissue pellet resuspended in 180 μl of buffer ATL. 20 μl of proteinase K was added, mixed thoroughly and incubated at 55°C, with regularly vortexing 2-3 times per hour, until the tissue was completely lysed. Lysis was often continued over-night in a water-bath at 37°C. Buffer AL was then added, vortexed and incubated at 70°C for 10 min. Ethanol (210 μl) was added and vortexed thoroughly. The resulting extraction mixture (610 μl + tissue) was placed in a QIAamp spin column within a 2 ml collection tube, and centrifuged at 6,000 g for 1 min. Care was taken not to moisten the rim of the column and to ensure adequate sealing to prevent aerosol formation. Extracted DNA was now bound to the membrane, and the column was placed in a clean 2 ml collection tube. Washing was achieved by adding 500 μl of buffer AW, centrifuging at 6,000 g for 1 min, and repeating this step, with a longer 2 min spin at 16,000 g at the end. Bound DNA, now free of impurities, was eluted twice with the same 200 μl of buffer AE warmed to 70°C, incubated in the spin column for 2 min and centrifuged for 1 min at 16,000 g. The eluate collected in the final Eppendorf tube contained the purified genomic DNA.

2.1.3.3 DNA EXTRACTION FROM HAEMOPOIETIC TISSUES

The procedure for DNA extraction from peripheral blood and PBPC harvests was similar to the procedure for paraffin sections, and could be performed with the QIAamp Tissue Kit and proteinase K supplied. The starting material was a washed cell pellet containing up to 10^7 cells. A peripheral blood sample of 10 ml, for example, was transferred to a 50 ml Falcon tube and 40 ml of RCLS added. Red cell lysis was achieved by gently rotating the tube until a clear red solution was obtained. Centrifugation was performed at 1,000rpm for 5 min, after which the supernatant was removed. Residual red cells above the cell pellet were lysed by repeating this step once. The cell pellet was washed with PBS once, resuspended in an empirical small volume of PBS, divided into aliquots, placed in 1.5 ml Eppendorf tubes, centrifuged at 16,000 g in a microfuge for 3 min, and the supernatant removed. The resulting cell pellet was stored at -80°C until required for DNA extraction. The Qiagen Blood and Body Fluid Protocol was used. Twenty five microlitres of proteinase K and 200 µl of buffer were added to the cell pellet and mixed immediately by vortexing for 15 seconds followed by incubation at 70°C for 10 min followed. Ethanol (210 µl) was then added and vortexed. The sample (435 µl + pellet volume) was transferred to a QIAamp spin column, and binding, washing and elution steps were as for solid tissues above. DNA was eluted in 200 µl of buffer AE.

2.1.3.4 MEASUREMENT OF DNA CONCENTRATION

The concentration of extracted DNA was measured with a spectrophotometer with in-built ultraviolet lamp (Unicam Helios). Each sample to be measured was diluted 100 fold in distilled water to a total volume of 1 ml. The optical densities (OD) at wavelength of 260 nm and 280 nm were measured, calibrated against a blank of distilled water. OD260 is a factor of the amount of DNA, and OD280 is a factor of the amount of protein present. The ratio of the 2 values (OD260/OD280) should ideally be between 1.8 and 2.0. Too high a ratio would suggest the presence of excess RNA, whilst too low a ratio would suggest excess protein contamination. The DNA concentration ([DNA]) was calculated as follows:

[DNA] ng/µl = OD260 x 50 x dilution factor (i.e. 100)

2.1.3.5 DNA STORAGE

Samples whose [DNA] were greater than 100 ng/μl were diluted with sterile distilled water to this concentration, divided into aliquots, and stored frozen at -20°C until required. Longer term storage of samples was at -70°C.

2.1.4 POLYACRYLAMIDE AND AGAROSE GEL ELECTROPHORESIS

2.1.4.1 MATERIALS

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Hoefer SE 600 Electrophoresis Unit, with internal heating unit connected to a water bath via rubber tubings

Power Pack, max capacity 500 V or 200 mA (Biorad, Hemel Hempstead, UK)

16x18 cm glass plates

1.5 mm spacers

15-well combs

40% Acrylamide solution with 19:1 acrylamide to N,N'-methylene-bisacylamide (Scotlab, Paisley, UK)

Ammonium persulphate (APS) powder (BDH, Poole, UK)

N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, Poole, UK)

20X Tris-Acetate-EDTA (TAE) buffer (per litre containing: Tris base 98.8 gm, glacial acetic acid 22.84 ml, 0.5 M disodium EDTA (pH 8) 80 ml)

Ethidium bromide (10 mg/ml) (Sigma)

Ultra-violet (UV) light box

AGAROSE GEL ELECTROPHORESIS

Biorad 10x15 cm gel size horizontal electrophoresis tank

Nusieve 3:1 agarose powder (Sigma, Poole, UK)

30-well comb

Power pack

20X TAE buffer as above

2.1.4.2 METHODS

POLYACRYLAMIDE GEL ELECTROPHORESIS

In this project, PAGE was mainly used to confirm the presence or absence of PCR products after ARMS reactions (Chapter 3), but was also sometimes used to confirm or purify PCR products from secondarily amplified mutant homoduplex bands cut from denaturing gradient gels (Section 2.6). It is described here for completeness. For sizes of PCR products relevant to this project, a 6% polyacrylamide gel allowed good resolution and was routinely used. With the restriction-site-generating PCR (RG-PCR) approach described in Chapter 4, some of the products were smaller, and gels of higher concentrations were used. Gels were 1.5 mm thick. After diluting 40% acrylamide solution to 6% and 20X TAE to 1X TAE with distilled water, 10% APS (w/v) solution (70 μl per 10 ml) and TEMED (10 μl per 10 ml) were added to initiate polymerisation. Gels were left to polymerise for 30 min before use. Three litres of TAE buffer was used in the lower tank, and 500 ml in the upper tank. The large volume in the lower tank ensured full immersion of the gels with even temperature preventing lane distortion at the ends of the gels. After loading of samples, electrophoresis was carried out at a constant current of 45 mA per gel for 1 hr 15 min. Staining with ethidium bromide (0.1 mg/l) for 20 min was followed by de-staining in distilled water for 20 min, and gels were viewed under UV light.

AGAROSE GEL ELECTROPHORESIS

Electrophoresis using agarose was performed to confirm PCRs had been successful prior to analysis of these products by DGGE. High-resolution Nusieve 3:1 agarose was uniformly dissolved in 1X TAE buffer by heating in a microwave oven and poured onto the platform of a standard Biorad horizontal electrophoresis tank to approximately 5 mm thick and allowed to set for 30 min with a 30-well comb in situ. The gel was immersed in 1X TAE buffer and, after loading of samples, electrophoresed at a constant voltage of 100 V for 45 min. Staining and de-staining with ethidium bromide was carried out for 10 min each, and viewed under UV light.

2.1.5 THE POLYMERASE CHAIN REACTION (PCR)

2.1.5.1 MATERIALS

Hybaid Omnigene Thermocycler (Hybaid, Life Sciences, Basingstoke, UK)

Deoxynucleotide triphosphates (dNTPs) : 100mM solution of each of dATP, dCTP, dGTP and dTTP (Promega, Southampton, UK)

Taq DNA Polymerase (5 units / ul) in storage buffer consisting of 50% glycerol, 50mM Tris-HCl (pH 8.0), 100mM NaCl, 0.1mM EDTA, 1mM DTT and 1% Triton X-100 (Promega, Southampton, UK)

10X Reaction buffer: 500mM KCl, 100mM Tris-HCl (ph 9.0), 1% Triton X-100 (Promega, Southampton, UK)

$MgCl_2$ 25mM (Promega, Southampton, UK)

Oligonucleotide primers (Oswel, Southampton, UK). *See Table 2.3*

Sterile distilled water

Sterile light mineral oil (Sigma, Poole, UK)

Glycerol (Molecular biology grade) (BDH, Poole, UK)

Eppendorf tubes (Costar, High Wycombe, UK)

PCR 0.5ml reaction tubes (Costar, High Wycombe, UK)

2.1.5.2 PREPARATION OF REAGENTS

The 4 dNTPs were mixed together, in equal volumes, to a concentration of 25 mM. The mixture was further diluted 1:12.5 to a working concentration of 2 mM. Oligonulceotide primers were diluted to a working concentration of 2.5 pMol/μl (i.e. 2.5 μM). During hot-start of PCR, Taq DNA polymerase was diluted 1:10 to 1U/μl, to a volume appropriate for the number of reaction tubes.

2.1.5.3 p53 EXONS AMPLIFICATION

Five fragments of p53 (fragments A-E), covering 4 hot-spot regions (HSR) in exons 5 to 8, where mutations are most commonly found, were amplified separately with 5 sets of primers (Borresen A-L et.al., 1991). Fragment E was introduced to cover exon 6 which was omitted from Boressen's description. The relationship of each designated

fragment to the exons and codons is outlined in Table 2.2. The properties of all primers used in exon amplifications are outlined in Table 2.3.

Table 2.2 Relationship between the designated DNA fragments A - E in this project and codons and exons of p53.

Fragment	Codons	Exons
A	124 - 148	5
B	155 - 185	5
E	187 - 222	6
C	237 - 253	7
D	264 301	8

Every PCR reaction mixture contained the following: 10X buffer, deoxynucleotide triphophates (dNTPs), magnesium chloride, a pair of oligonucleotide primers, Taq DNA polymerase, sterile distilled water, and a DNA template (usually high molecular weight genomic DNA) or water for 'blank' control reactions. For each set of reactions in which the same fragment was being amplified, the quantity of each reagent in each tube was determined, and each volume was multiplied by the number of tubes plus one (to allow for wastage) to give the volumes required in a 'master mix'.

e.g. For 9 amplifications of fragment E, a master mix for 10 was made up:

	Each tube (μl)	Master mix (μl x 10)
10X Buffer	5	50
dNTPs (2mM)	5	50
$MgCl_2$ (25mM)	4 (2 mM)	40
E-GC primer (2.5μM)	5 (12.5 pmol)	50
E-Bio primer (2.5μM)	5 (12.5 pmol)	50
Taq polymerase (0.5U/μl)	2 (1U)	---
DNA template (100ng/μl)	3 (300 ng)	---
Sterile water	21	210
Total volume	50	450

45 μl of master mix was aliquoted to each of 9 reaction tubes, DNA template was added to each separately, and layered with 45ul of sterile light mineral oil. For hot-start, reaction mixtures were heated to 94°C and held at this temperature for 7 min during which all DNA double strands were fully dissociated. Two μl (1 unit) of Taq

Table 2.3 List of primers used in PCR amplification of p53 fragments A-E for use in DGGE.

Fragment Amplified	Primer Name	%GC	Sequence
A	A-GC	81	5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG
			CCG CCC CCG CCC GTG TGA CTG CTT GTA
			GAT G-3'
A	A-NB	50	5'-CTC TGT CTC CTT CCT CTT-3'
B	B-GC	90	5'-CGC CCG CCG CGC CCC GCG CCC GTC
			CCG CCG CCC CCG CCC GTT CCA CAC
			CCC CGC CCG GCA-3'
B	B-Bio	70	5'-GCC CCA GCT GCT CAC CAT CG-3'
C	C-GC	80	5'-GCG GGC GGC GCG GGG CGC GGG CAG
			GGC GGC GGG GGC GGG CAG TCT TCC
			AGT GTG ATG ATG-3'
C	C-NB	45	5'-CAC CAT CCA CTA CAA CTA CA-3'
D	D-GC	83.3	5'-GCG GGC GGC GCG GGG CGC GGG
			CAG GGC GGC GGG GGC GGG CTA
			CCT CGC TTA GTG CTC CCT-3'
D	D-Bio	40	5'-ATC CTG AGT AGT GGT AAT CT-3'
E	E-GC	85	5'-CGC CCG CCG CGC CCC GCG CCC
			GTC CCG CCG CCC CCG CCC GGA
			GAG ACG ACA GGG CTG GTT-3'
E	E-Bio	54.5	5'-AGT TGC AAA CCA GAC CTC AGG C-3'

polymerase was then added to each tube and cycling continued. Standard cycling conditions for exon amplification are based on the 3 step PCR, consisting of a denaturing step, an annealing step, and an extension step. Based on published data (Borresen et al, 1991) fragments C-E shared the same cycling conditions, whilst fragment B required longer denaturing and annealing steps, and fragment A, after some optimisation, required a higher annealing temperature to produce a clear, clean product. Table 2.4 outlines cycling conditions for the 5 fragments.

A total of 35 cycles were run. PCR products were kept at 4°C until electrophoresed to confirm presence of amplification products, or otherwise. Prior to DGGE, 4% agarose gel electrophoresis was carried out on a 5 μl aliquot of each reaction product to confirm successful amplification.

Table 2.4 PCR cycling conditions for p53 fragments as defined and used in this project. For all PCR's, hot-start was performed with the denaturation step of the first cycle held for 7 min, and the extension step of the final cycle held for 10 min.

	Fragment A	Fragment B	Fragments C,D,E
Denaturing temperature (°C)	94	94	94
Denaturing duration (sec)	75	75	45
Annealing temperature (°C)	59	55	55
Annealing duration (sec)	75	75	45
Extension temperature (°C)	72	72	72
Extension duration (sec)	75	75	45
[Mg] mM	2	1	2

2.1.6 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

2.1.6.1 MATERIALS

Hoefer SE 600 Electrophoresis Unit, with internal heating unit connected to a water bath via rubber tubings

Water bath delivering water at 60°C

Power pack with max capacity 500 V or 200 mA (Gibco, Paisley, UK)

Electrophoresis apparatus (as described in Section 2.4.1) consisting glass plates, spacers, combs

Gravitational gradient gel former (Biorad, UK)

Magnetic stirring system (Stuart Scientific)

40% acylamide solution (BDH, Poole, UK)

N,N'-diallyltartardiamide (DATD) (BDH, Poole, UK)

Urea (BDH, Poole, UK)

Formamide solution (BDH, Poole, UK)

Ammonium persulphate powder (BDH, Poole, UK)

N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, Poole, UK)

AG501-X8 resin (Biorad, Hemel Hempstead, UK)

20x'DGGE Running Buffer': 800 mM Tris base, 400 mM sodium acetate, 20 mM EDTA, pH 7.4

80% denaturant stock solution for 12.5% polyacrylamide gel

0% denaturant stock solution for 12.5% polyacrylamide gel

(100% denaturants correspond to 7M urea and 40% formamide; denaturant stock solutions contain acrylamide 12.5%, 0.93 gm DATD / 100 ml and 1X 'Running Buffer')

6X gel loading buffer (bromophenoblue, xylene cyanol, ficoll, BDH, Poole, UK)

2.1.6.2 PARALLEL DGGE

Virtually all the DGGE performed during this project were of the parallel variety, i.e., the denaturant gradient was in the same direction as the electric current, in order to screen primary tumour tissues for the presence of p53 mutations. Perpendicular DGGEs, the principle of which is outlined in Chapter 1, helped to visually demonstrate the presence of a denaturant gradient, will be described below. During this project, a universal gradient of 30 - 80% denaturants was used for all 5 DNA fragments screened. The optimal denaturant concentrations for each of these fragments was demonstrated to fall within this range (Borresen et.al., 1991; Busby-Earle, 1992). Glass plates, cleaned with 100% ethanol on the inner aspects in contact

with the gel, were set up and clamped as per usual procedure. Acrylamide gel was poured from a Biorad gradient gel former at a height slightly above the vertical glass plates via a thin rubber tubing, the distal end of which was inserted between the plates, half-way from each end. The gel former consisted of 2 interconnected chambers which could be isolated from each other, and the first chamber contained the outlet which is controlled by a 3-way tap. The gel former was rested on a magnetic rotator, with a stirrer within the first chamber to enable even mixing of the denaturant solutions. 80% denaturant solution was placed in the first chamber, and 0% denaturant solution in the second, and 10% ammonium persulphate (50 μl / 10 ml) and TEMED (15 μl / 10 ml) are added to each chamber to initiate polymerisation. When the 3-way tap was opened and the 2 chambers connected, 80% denaturant solution flowed out first, and was the denaturant concentration at the bottom of the gel. As the gel was cast, with mixing from the non-denaturing solution, concentration of the denaturant in the first chamber fell. The amount of denaturant solution added to each chamber was determined graphically (Fig. 2.1). A line was drawn through the coordinates [0 ml, 80%] and [33.6 ml, 30%], 33.6 ml being the volume of the gel cast (14 x 16 x 0.15 cm). The X-axis was intercepted at 54 ml giving the total volume of denaturant solutions used, hence 27 ml of 80% and of 0% denaturant solutions were added to each of the 2 chambers. The gradient gel should not be cast in less than 5 min (when the gradient may disperse) or longer than 10 min (when acrylamide may set within the apparatus). By trial and error, the optimum was approximately 8 min. A 2 cm gap was left at the top of the gel. Immediately after the gel was cast, a thin film of water-saturated butan-1-ol was gently applied onto the surface of the gel to prevent oxygen interfering with the polymerisation process. The gel was allowed to set for at least 20 min. The butan-1-ol was then removed and the 2 cm gap washed with distilled water before a 7% acrylamide stacking gel was poured, when a 15-well comb was inserted, clearing the interface by 1 - 2 mm. This prevented contact between PCR products in the wells and denaturants in the main gel body before electric current was applied. The lower concentration of acrylamide in the stacking gel prevented the set gel swelling into the wells thus reducing volume. When the stacking gel was set, the wells were washed with 1X running buffer. If the gradient gel was not used

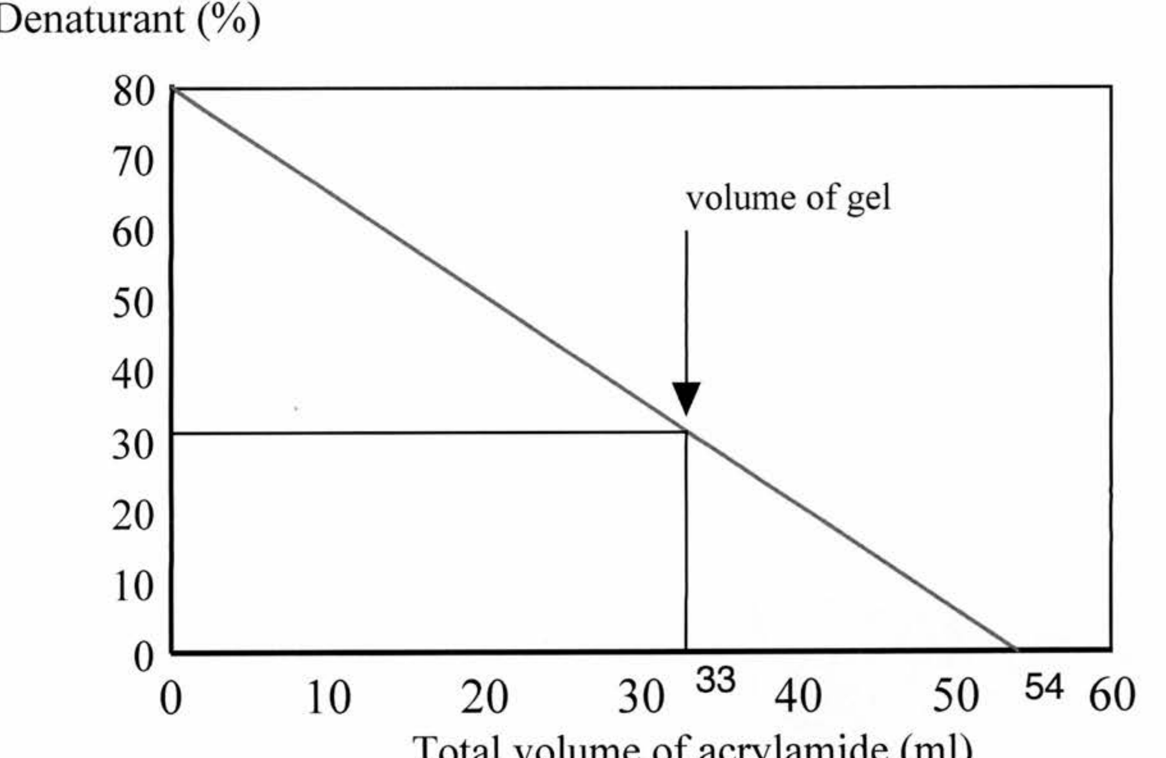


Fig. 2.1 Determination of volume of acrylamide required to cast a gradient gel. Volume of gel is 14x16x0.15 cm = 33 ml. Gradient required is 30-80%. A line is extrapolated from 80% on the Y-axis through the coordinate 33 ml / 30%, giving total volume required to be 54 ml, ie, 27 ml of 0% and 80% denaturant solutions.

immediately, the sandwich was wrapped in cling film and stored at 4°C for not more than 48 hr.

To perform DGGE, the Hoefer SE600 vertical tank was set up with the heating element (inter-connected glass tubes) in the centre connnected to a water bath heated to 62.5°C. The tank sat on a magnetic stirring base, and the stirrer within the tank ensured the entire volume of buffer was heated to a uniform temperature of 60°C. The extra 2.5°C allowed for heat loss. Four litres of 1X running buffer was placed in the tank, in which the denaturing gradient gel set-up was immersed. The large volume of buffer ensured that the gels were electrophoresed at a uniform 60°C. Six hundred milliltres of 1X running buffer was placed in the top chamber, and the gels were electrophoresed at a constant voltage of 185 V which produced a current of 80 - 90 mA across each gel of dimensions described above. Fig. 2.2 is a schematic representation of the apparatus.
Forty microlitres of PCR product was mixed with 8 μl of 6X loading buffer and loaded onto each lane. Running time was approximately 6 hr when the xylene cyanol band (dark blue colour) reached the bottom of the gel.

2.1.6.3 PERPENDICULAR DGGE

Perpendicular DGGE was not a regular technique in this project, but was performed to confirm that a linear gradient was satisfactorily achieved by gel casting with the gravitational caster, and to show that denaturation occured within a relatively narrow range of denaturant concentration. The 30 - 80% gradient was poured as described above, but 3 spacers were required, arranged in a U-shape. Petroleum jelly, bull-dog clips and electrical tape were use to ensure a tight seal between spacers. The gel was poured in the usual manner through the fourth side of the plates, leaving a gap equivalent to the width of a spacer. After polymerisation, the 2 vertical spacers were removed, the plates turned 90°, and a new spacer inserted into the gap through which the gel was poured. The result was an apparatus with 2 vertical spacers and a gradient gel with a gradient running left to right rather than top to bottom. There was also a 1 - 2 cm space at the top of the gel into which the PCR product to be studied was

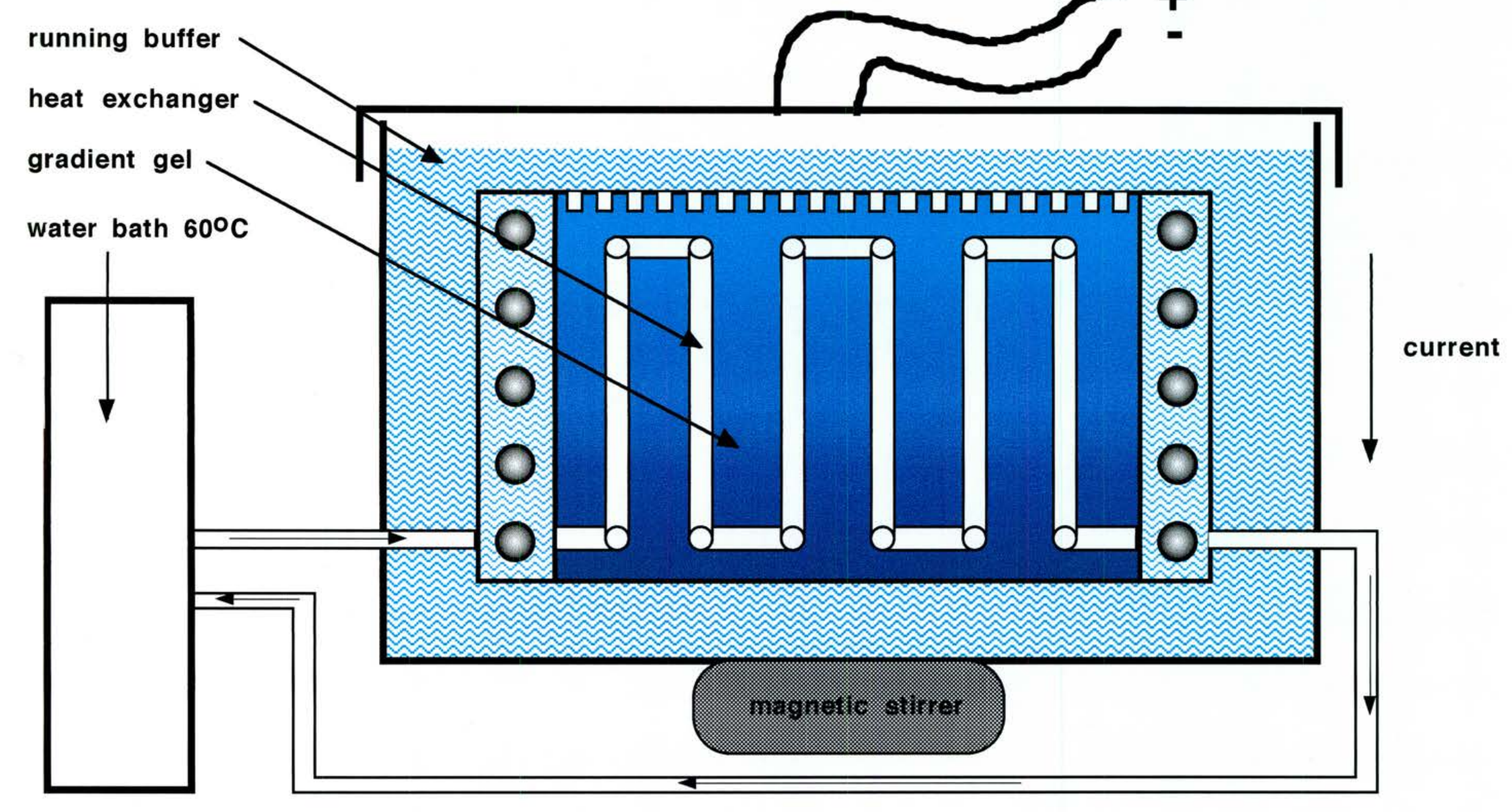


Fig 2.2 Schematic representation of electrophoresis tank apparatus for DGGE

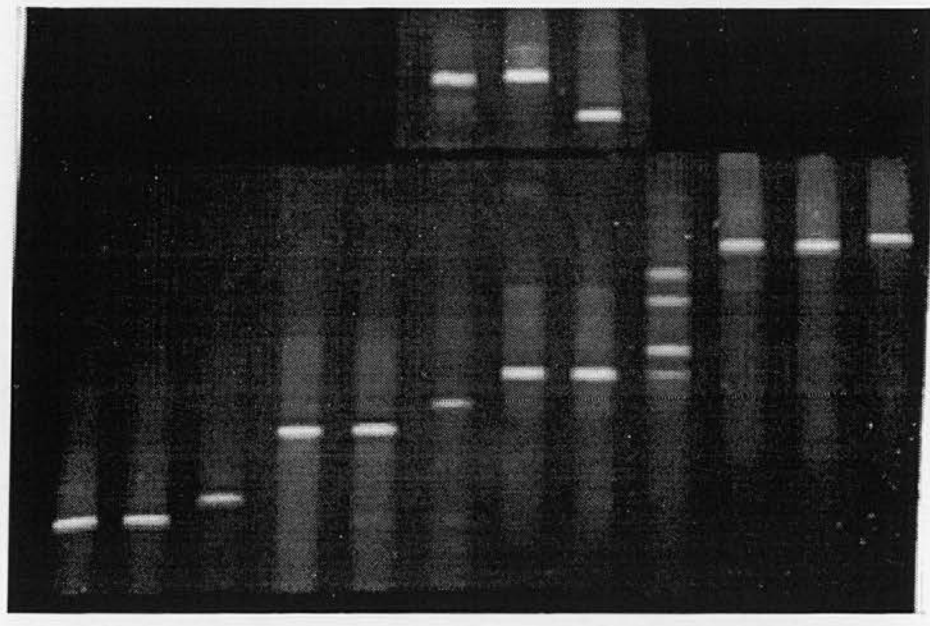
placed. Electrophoresis time required was determined empirically and was shorter than for parallel DGGE, approximately 3.5 hr. PCR product travelling through the 30% denaturant end of the gel did not denature and migrated furthest, whilst that at the 80% end denatured as soon as electrophoresis started and hardly migrated. Somewhere in between was a denaturant concentration at which denaturation first took place and represented an abrupt slowing down of migration. The overall result was a sigmoid curve (See Section 2.2.7).

2.2 OPTIMISATION OF DGGE

2.2.1 SUITABILITY OF TONSIL DNA AS A WT CONTROL

A large stock of human tonsil DNA was previously prepared in 1989 in our department from a pool of tonsils obtained from children undergoing elective tonsillectomy at the Royal Hospital for Sick Children, Edinburgh (J.Craig, personal communication). DNA was extracted using standard proteinase K / phenol / chloroform methods, and was kept at -70°C over the years. It was decided to use this supply of DNA simply because of its ready availability. DNA concentration was measured by recording the optical densities (260 and 280 nM) of DNA diluted 1:100 in water. Tonsil DNA was diluted to a working concentration of 100 ng/µl and aliquoted. OD260/OD280 ratios were confirmed to be within the optimal range of 1.7 - 2.0. It was important to determine if tonsil DNA contained any mutations of p53 in the fragments to be studied. For this purpose, tonsil DNA, DNA from a breast cancer cell line, MCF7, known to contain wild type sequence in the entire coding sequence of p53, and a positive mutant control, were amplified with primers of each of the five p53 fragments studied. PCR products were electrophoresed in DGGE (Fig. 2.3), and the results clearly showed tonsil DNA co-migrated with MCF7 DNA in each fragment, and all 5 positive controls migrate differently. This confirmed the legitimacy of tonsil DNA as a wild type control, and that, with a universal gradient of 30 - 80% denaturants, mutations in all 5 fragments of DNA were identified.

FRAGMENT	A	A	A
DNA	WT	t	M
LANES	1	2	3



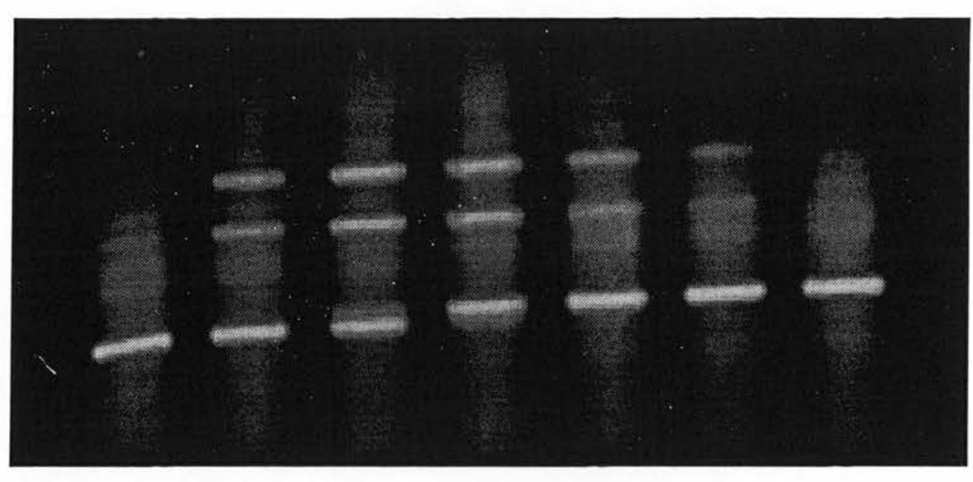
LANES	1	2	3	4	5	6	7	8	9	10	11	12
DNA	WT	t	M	WT	t	M	WT	t	M	WT	t	M
Fragment	B	B	B	C	C	C	D	D	D	E	E	E

Fig. 2.3 Suitability of tonsil DNA (t) as WT control (Section 2.2.1). For each of the five DNA fragments (A-E), PCR products were studied with DGGE for a) MCF7, a breast cancer cell line known to contain WT sequence, b) tonsil, and c) a positive control cell line known to contain a mutation. For all 5 fragments, PCR product of tonsil DNA co-migrated with MCF7 DNA, indicating that it contained WT sequence. Both migrated differently from the positive control.

WT MCF7
t tonsil
M mutant

2.2.2 SENSITIVITY OF DGGE IN DETECTING MUTATIONS

The primary starting point of mutation screening for a particular patient was, for breast cancer patients, either a frozen section or archival paraffin-embedded section of primary tumour, and for acute leukaemia patients, an aliquot of diagnostic bone marrow aspirate. It is conceivable that such biological samples 'never' entirely consisted of cells of the malignant clone, and invariably normal stromal tissues were interspersed amongst the malignant cells, to varying proportions. In order to account for sample variability, it was important to determine the approximate sensitivity of DGGE, which might have a bearing on the choice of material to be screened. A model was based on cells of a bladder carcinoma cell line, 5637, known to contain a point mutation within fragment D (exon 8) of p53. 5637 cells were spiked in normal blood donor buffy coat white cells in serial dilutions in the following proportions: 80%, 40%, 20%, 10% and 5%. DNA was extracted from a 10^7 cell pellet of each of these dilutions, subjected to fragment D PCR as previously described, and electrophoresed by DGGE. Fig. 2.4 illustrates the pattern of such PCR products on DGGE. Mutant homoduplex of 5637 migrated marginally faster (or denatures later) than WT homoduplex, and as the proportion of tumour cells decreased, a transition was seen, whereby the mutant homoduplex band was no longer visible in proportions of 40% or less. It was, however, still possible to see that a mutation was present on the basis of the presence of heteroduplexes, down to a malignant cell fraction of 5%. Therefore, tumour tissue with as high a proportion of malignant cells as possible should be used as a clear and distinct mutant homoduplex band was required for sequencing.



LANES	1	2	3	4	5	6	7
M DNA(%)	100	80	40	20	10	5	0
WT DNA(%)	0	20	60	80	90	95	100

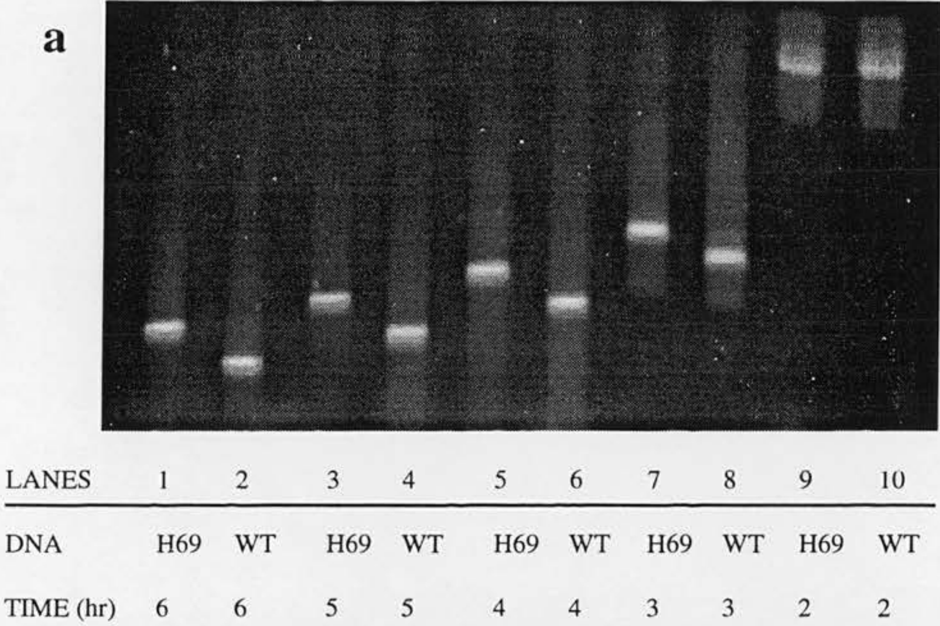
Fig. 2.4 Sensitivity of DGGE in mutation detection (section 2.2.2). 5637 cells were spiked in buffy coat cells in various dilutions expressed as a percentage and extracted DNA studied with DGGE. The slightly faster mutant homoduplex band is no longer clearly visible (and be physically isolated) when the proportion of tumour cells is 40% or less, although heteroduplex bands indicate the presence of a mutation down to at least 5%.

2.2.3 EFFECT OF ELECTROPHORESIS TIME ON DGGE

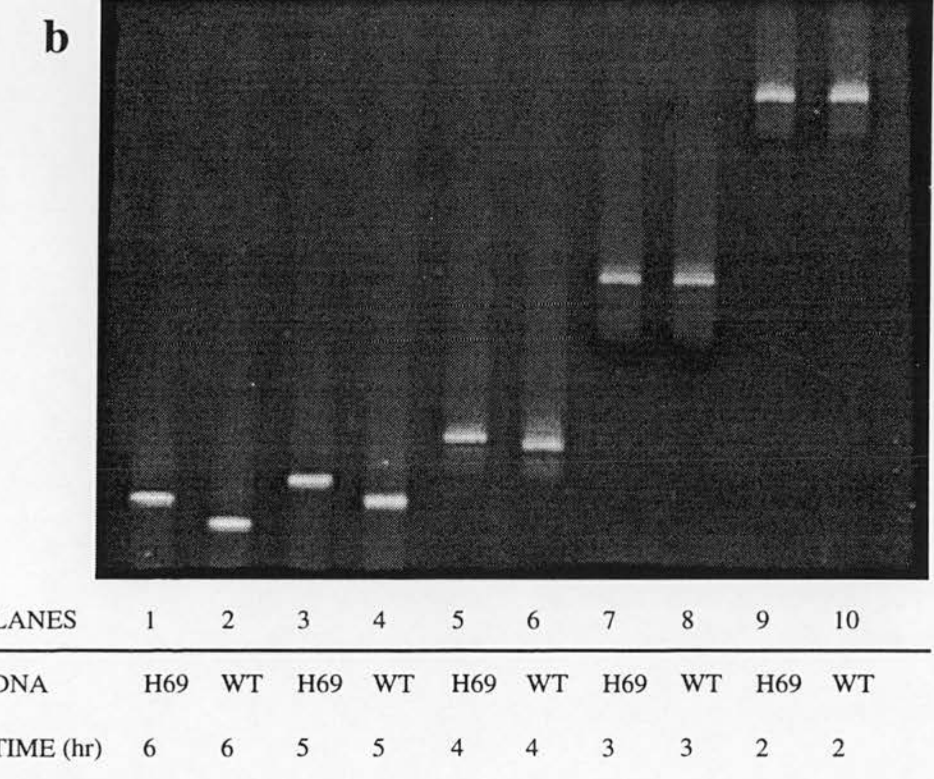
Duration of electrophoresis was a factor of the power delivered through , or the voltage across, the polyacrylamide gel. A 'travel schedule' DGGE had to be performed for each fragment studied to determine the duration of electrophoresis. In this set up, paired wild type and mutant PCR products were loaded onto the gradient gel sequentially, and electrophoresed for varying lengths of time. Fig. 2.5(a) shows such a gel. Fragment B PCR products were used in this model. Paired mutant (H69 colonic carcinoma, left lane of each pair) and WT (tonsil) fragment B PCR products were electrophoresed at a constant 200V, 60°C, at hourly increments from 2 to 6 hr. After 3 hr of electrophoresis, migration of the mutant PCR product was noticably retarded compared to that of WT. Further eletrophoresis up to 6 hr contributed little in the resolution of mutant DNA from wild type. Comparing the distance travelled from 2 to 3 hr, and subsequent time intervals, it was clear that the migration of both DNA species was markedly retarded owing to denaturation of the double strands. Empirically, to ensure that any difference between mutant and wild type bands were maximised, all subsequent DGGE's were performed at 200 - 220V for 5 to 6 hr , when the xylene cyanol component of the loading buffer reached the bottom of the gel. This was found to be a reliable indicator for the duration of electrophoresis.

2.2.4 EFFECT OF POLYACRYLAMIDE CROSS-LINKER ON DGGE

In any PAGE, bisacrylamide is most commonly used as a cross-linker to achieve polymerisation between acrylamide molecules. However, in the method described by Borressen, et.al. (1991), N,N'-diallyltartardiamide (DATD) was routinely used. After initial failure in mutation screening using acrylamide/bisacrylamide, It was decided to assess the difference between using bisacrylamide and DATD in DGGE under defined conditions. Figs. 2.5(a) and 2.6 illustrate the result. Both of these denaturing gradient gels were performed in the 'travel schedule' manner, with paired mutant and WT PCR products of fragment B loaded at hourly intervals, and electrophoresed for 2, 3, 4, 5 and 6 hr, respectively, as described in Section 2.2.3 above. Fig. 2.6 shows a gel with bisacylamide as cross-linker, and Fig. 2.5(a) has DATD. Apart from this, the 2 gels were identical, with 12.5% acrylamide, and a 30 - 80% denaturant gradient from top

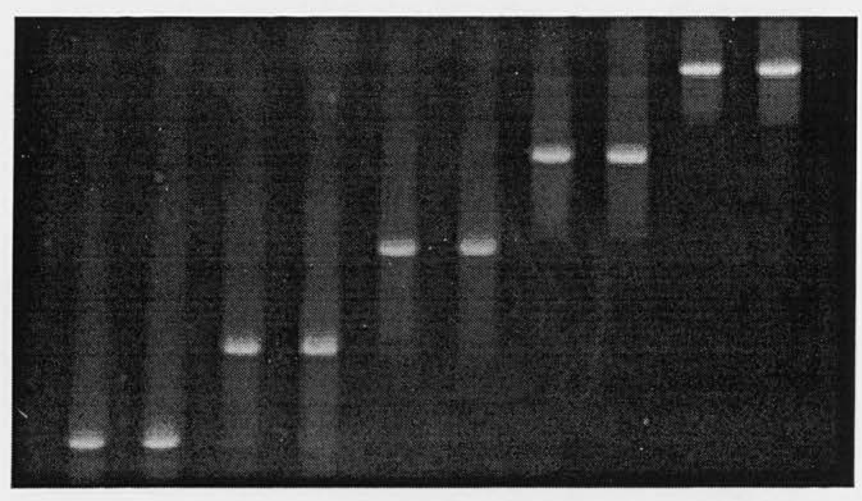


LANES	1	2	3	4	5	6	7	8	9	10
DNA	H69	WT	H69	WT	H69	WT	H69	WT	H69	WT
TIME (hr)	6	6	5	5	4	4	3	3	2	2



LANES	1	2	3	4	5	6	7	8	9	10
DNA	H69	WT	H69	WT	H69	WT	H69	WT	H69	WT
TIME (hr)	6	6	5	5	4	4	3	3	2	2

Fig. 2.5 a & b Effect of running temperature on DGGE, with DATD as cross linker (section 2.5.5). (a) Travel schedule DGGE of H69 cell line and WT DNA fragment B PCR products, electrophoresed at 60°C. DGGE was at a constant voltage of 200V. (b) is identical except the gel was electrophoresed at 55°C. At lower running temperature, differential mobility retardation is noted after 5 hours of electrophoresis, while much earlier retardation at 3 hours is observed when electrophoresed at 60°C.



LANES	1	2	3	4	5	6	7	8	9	10
DNA	H69	WT	H69	WT	H69	WT	H69	WT	H69	WT
TIME (hr)	6	6	5	5	4	4	3	3	2	2

Fig. 2.6 Effect of crosslinker on DGGE (section 2.2.4). Travel schedule DGGE of H69 cell line and WT DNA fragment B PCR products with bisacrylamide as cross linker. DGGE was at 60°C at constant voltage of 200V. This is contrasted with Fig 2.5a which is identical except with DATD as cross linker. This experiment showed no mobility loss during electrophoresis when bisacrylamide was used, but differential retardation of mobility between WT and mutant PCR products was apparent after 3 hours of electrophoresis when DATD was used.

to bottom. Both gels were electrophoresed simultaneously in the same tank at 60°C. The DNA bands in the gel with bisacrylamide showed no sign of retardation with increasing electrophoresis time, and no difference was apparent between mutant and wild type DNA. The gel with DATD, in contrast, showed that migration of both mutant and wild type DNA were markedly retarded after approximately 3 hr of electrophoresis, when the difference in migration (and hence denaturation properties) between them became apparent. Experiments with the other p53 fragments, comparing bisacrylamide and DATD yielded the same results. In the p53 system, therefore, bisacrylamide, at least at a acrylamide/bisacrylamide ratio of 37.5:1, was unsuitable as a cross-linker for DGGE, whilst DATD was able to confirm the mutations in all 5 positive controls.

2.2.5 EFFECT OF RUNNING TEMPERATURE ON DGGE

'Travel schedule' DGGEs were performed as described, using buffer temperatures of 55°C and 60°C, in order to assess the running temperature at which denaturation of DNA was most reliably achieved when all other variable factors were kept constant. As an increase of 1°C in the running temperature is equivalent to an increase of 3% in denaturant concentration, it was hypothesized that raising the running temperature of the buffer, e.g., from 55°C to 60°C should reduce the distance a GC-clamped DNA fragment had to travel before denaturation took place, and hence reduce the time of electrophoresis required. Fig. 2.5(a) shows a travel schedule gel run at a buffer temperature of 60°C, and indicates that DNA denaturation began after approximately 3 hr. Fig. 2.5(b), the same reactions run at 55°C, indicates that DNA continued to migrate down the gel for much longer and did not begin to denature until 4 to 5 hr after electrophoresis began. They also ran further into the gel before denaturation took place, indicating that a higher denaturant concentration was required to compensate for the lowered temperature. The quality of the separation and sharpness of the bands did not appear to be impaired at a higher temperature, and, therefore, 60°C became the standard buffer running temperature for this project.

2.2.6 EFFECT OF ELECTROPHORESIS VOLTAGE ON DGGE

During this project, a range of electrophoresis voltages was tested to study the effect on the appearance of the bands. Some have reported the superiority of using a lower voltage and longer electrophoresis time (B.Cohen, personal communication), and to enable electrophoresis to be performed within the scope of a working day. A narrow range of voltages, from a constant 160V to 220V, which created a current varying between approximately 70mA and 100mA across each 1.5 mm thick, 14x16 cm polyacrylamide gel was tested. A higher voltage had no undesirable effect on the appearances of the bands, but very conveniently shortened electrophoresis time from 7 hr to 5 hr, by the criteria indicated in Section 2.2.3 above. Care was taken not to create too high a current across the gel, and a maximum current of 100 mA was not exceeded.

2.2.7 USE OF PERPENDICULAR DGGE TO ASSESS EFFECTIVENESS OF DENATURANT GRADIENT

As described by Busby-Earle et al (1992) a universal denaturant gradient of 30 - 80% was adequate in screening the 5 p53 fragments described, and this was the experience in this project. However, to maximise the sensitivity of DGGE, the profile of the melting domain in question should be determined, such that the denaturant range should represent a *temperature* gradient of 5°C on either side of the optimal temperature, as described in Section 1.5.1. This may be achieved by one of 2 methods. Computer programmes (e.g. Lerman and Silverstein, 1987; Melt87; SQHTX) are now available to help design PCR primers to produce double-stranded products suitable for DGGE, and determine the optimal denaturant range. They may also predict, as a function of electrophoresis time, the expected difference in gradient level (separation) between a WT homoduplex DNA molecule, and a related heteroduplex created with a mutant species differing by a single-base mismatch at each selected position along the sequence. An alternative is to determine the denaturant range experimentally, by performing a perpendicular DGGE, in which the direction of the denaturant gradient is at right angles to the direction of current. The set up of this apparatus is described in Section 2.1.6.3. Because of reproducible

success with the parallel DGGE system with the universal gradient to detect mutations in all 5 p53 fragments, it was not necessary to perform perpendicular DGGE for each fragment to determine the effective range. Nevertheless, it was important, and interesting, to confirm that the technique of linear gradient gel casting did produce a linear denaturing gradient. Fig. 2.7 shows the resultant appearance of such a gel on which 600 μl each of fragment B PCR products, using H69 colonic cancer positive control and WT tonsil DNA as templates, amplified separately, were electrophoresed. Electrophoresis time was 3.5 hr, at a constant 200V. The denaturant gradient, from left to right, was 30-80%. Samples were loaded across the top of the gel. At the low denaturant concentration end of the gel, no denaturation of PCR products occurred and products travelled to the bottom. At the high concentration end, PCR products were denatured immediately upon entering the gel and stayed near the top. Between these 2 extremes, there occured a denaturant concentration at which the double-stranded, GC-clamped products denatured suddenly, resulting in a sigmoid courve. Here, assuming a linear gradient, this occured at approximately 64.4%.

2.2.8 QUALITY OF THE DENATURANT SOLUTIONS AND AGE OF THE GELS

Although not formally assessed over the course of the project, the quality of duplex bands on DGGE was significantly influenced by the age of the 80% denaturant solution used in the gels. It is conceivable that, with high concentrations of denaturants like urea and formamide in solution, the quality of the cross-linking of acylamide molecules would be influenced. The interactions between these chemicals may be more pronounced with time, affecting the quality of electrophoresis. Quality of DGGE was especially poor if the 80% denaturant was over 4 weeks old, and routinely, it was ensured that the solution was used within 2 weeks of preparation, or otherwise discarded. As the 0% denaturant stock solution contained only acrylamide, DATD, buffer and water, and without denaturants, the age of this solution was not relevant.

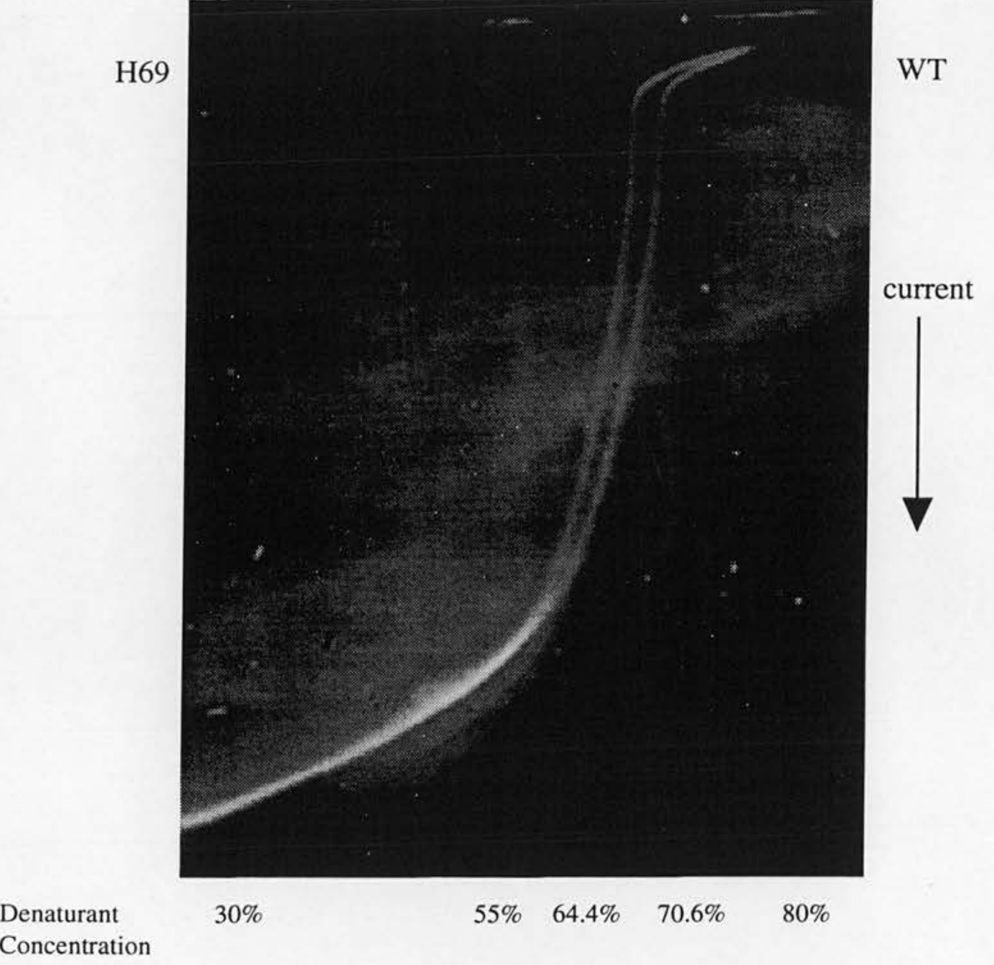


Fig. 2.7 Perpendicular DGGE with fragment B PCR products from WT and H69 cell line DNA which contains a point mutation (Section 2.2.7). Current is from top to bottom and 30% - 80% denaturant gradient from left to right of the gel. Mixture of WT and mutant PCR product was loaded in a well spaning the whole width of the gel and the sigmoid curve confirmed the presence of a gradient from left to right. The 2 split bands in the steep slope represent mutant DNA on the left and WT DNA on the right. Assuming a linear gradient, by measuring the distances from each end, the denaturant concentration range in which sharp mobility retardation occurs during electrophoresis is 50 - 70.6%, with the centre of the slope, 64.4%.

Denaturing gradient gels were routinely poured the day before use, wrapped in cling-film and kept at 4°C overnight, and it was ensured that the polymerised gel was used within 24 hr of preparation because of the potential risk of diffusion of the denaturants from the higher to the lower concentrations.

2.3 MUTATION DETECTION IN BREAST CANCER PATIENTS

2.3.1 PATIENT SELECTION

All patients studied were or are being treated at the Department of Medical Oncology, Western General Hospital, Edinburgh, and were referred either locally or from other hospitals in Lothian or Fife. A total of 51 patients were studied and were divided into two broad groups: **(1) Group 1**: 38 patients, the great majority with metastatic breast cancer who had undergone PBPC harvesting at the Scottish National Blood Transfusion Service Cell Separator Unit, Royal Infirmary of Edinburgh. Most of these patients were also treated with high dose chemotherapy and the progenitor cell harvests returned. Whilst it is noted that in the early to mid 1990's the policy was to offer PBPCT to eligible patients with metastatic disease, occasional patients with relapsed disease were also treated this way. In this project, to distinguish patients in this group from Group 2 described below, they are designated the 'metastatic' group. **(2) Group 2**: 13 patients with high risk stage II or III breast cancer (4 or more positive axillary lymph nodes but not known to have distant metastases) who took part in the Anglo-Celtic Cooperative Oncology Group (ACCOG) study comparing adjuvant conventional chemotherapy with adjuvant high dose therapy and PBPC support (see Section 1.1). Having earlier stage albeit high risk disease, these patients should provide a contrast to those in Group 1 above, and as half were randomised to HDT and had PBPCH cryopreserved, it would be of interest to study these aliquots for MRD in those in whom a disease marker is available. Patient characteristics are summarised in Table 2.5.

2.3.2 NATURE AND SOURCE OF MATERIALS

A total of 51 primary breast tumours were studied and came from one of 3 different sources: **(1)** 22 frozen sections taken at the time of primary surgery, courtesy of Prof. W.R. Miller, Imperial Cancer Research Fund (ICRF); 17 patients had metastatic disease and 5 in the ACCOG study; **(2)** 7 paraffin-embedded sections, courtesy of Dr. K. McLaren, Department of Pathology, University of Edinburgh. All patients had metastatic disease; and **(3)** 22 paraffin-embedded sections, courtesy of Dr. T. Anderson, Department of Pathology, Western General Hospital, Edinburgh; 14 patients had metastatic disease and 8 in the ACCOG study. All frozen were kept in the vapour phase of liquid nitrogen since the initial surgery, and aliquot sections (approximately 25 gm) were cut for DNA extraction. Any remaining tissue was re-frozen in the liquid nitrogen tank. Paraffin-embedded sections were retrieved from the archive, and two to three 15 μm sections cut from each block with a clean microtome blade and placed in an Eppendorf tube until DNA extraction. Procedures are described in Section 2.1.

2.3.3 RESULTS

Of the entire group of 51 patients, an abnormal banding pattern was detected in 14 individual patients (27.5%), one of whom had an abnormal pattern in 2 different p53 fragments. Of these 15 positive lanes on DGGE, 13 had the clear-cut 4-band pattern, representing mutant and WT homoduplexes and 2 mutant-WT heteroduplexes. The remaining 2 cases showed a clear band which co-migrated with WT on DGGE, and 2 extra bands with markedly different mobility (earlier retardation) suggesting that they were likely to be heteroduplexes. Section 2.5.4 describes the use of TaqI restriction endonuclease to resolve these samples.

The distribution of mutations among the 5 fragments are: 5 in fragment B (exon 5), 2 in fragment C (exon 7), 2 in fragment D (exon 8) and 6 in fragment E (exon 6). 12 of these were found in the metastatic group, and 3 in the ACCOG group. As described in Section 2.6, sequence information suggests that 2 of the fragment E positive results were constitutional silent polymorphisms in codon 213, including patient B15 who had

Table 2.5 Characteristics of breast cancer patients studied.

Metastatic Disease Patients									
Patient ID	Patient Initials	Sample ID	Source	Age	date surg	Type surg	TNM at diag		
B1	LBl	LBl	ICRF	27	Dec-91	ans/wle	T2		
B2	EMcC	EMcC	ICRF	33	Dec-92	met	T3		
B3	BD	BD	ICRF	34	Jun-88	mx/anc	T2		
B4	JPh	JPh	ICRF	45	Aug-92	wle/ans	T2N0M0		
B7	MW	MW	ICRF	37	Oct-83	mx/anc	T2		
B8	PY	PY	ICRF	35	Dec-87	ans/wle/mx	T1N0M0		
B9	JD	JD	ICRF	42	Nov-89	mx/anc	T2N0M0		
B10	LMcL	LMcL	ICRF	31	Mar-92	nil	T4		
B11	HB	HB	ICRF	41	Nov-92	mx/anc	T2N2M1		
B12	JR	JR	ICRF	29	Jul-91	mx/anc	T2N0M0		
B13	JS	JS	ICRF	34	Jun-91	wle/ans	T2N0M0		
B15	VK	VK	ICRF	39	Jul-90	wle/anc	T2N0M0		
B16	LB	LB	ICRF	31	Dec-92	wle/ans	T2N0M0		
B17	BB	BB	ICRF	54	90	mx	T2N1M1		
B20	JF	JF	ICRF	51	Jul-91	mx/anc	T1N0M0		
B21	ED	ED	ICRF	27	Mar-91	wle/rt	T2N1		
B22	JL	JL	ICRF	55	May-92	wle/ans	T2N1M0		
B23	AD	23046/90	RIE	34	Jul-89	mx	T2		
B24	LA	15380/90	RIE	38	Jul-90	ans/wle/mx	T1		
B25	MJ	30155/89	RIE	34	Oct-89	mx/anc	T2		
B26	JF	25590/90	RIE	37	Nov-90	mx/ans	T2		
B27	KE	7071/90	RIE	43	Apr-91	wle/rt/mx	T2N0M0		
B28	EM	8375/91	RIE	39	Apr-91	wle/ans	T1		
B29	MM	5346/89	RIE	43	Mar-89	mx/anc	T2N1M0		
B31	CM	6623/93	WGH	43	n/a	n/a	T2/met		
B32	EL	11925/95	WGH	32	n/a	n/a	T4N1M0		
B33	SF	3757/94	WGH	28	Apr-94	mx/anc	T1N0M0		
B34	BS	4048/94	WGH	51	Apr-94	wle/anc	T2N1M0		
B35	PC	2694/95	WGH	35	n/a	nil/met	T4N2M1		
B36	ER	4552/97	WGH	39	Sep-92	wle/anc	T3N1M0		
B39	GM	9267/93	WGH	31	Sep-93	wbx	T4N1M0		
B42	ID	331/94	WGH	50	Feb-94	wle/ans	T2N1M0		
B43	NT	6556/92	WGH	28	May-92	wle/ans	T2N0M0		
B44	MMcH	6137/94	WGH	42	Jul-94	wle-mx/anc	T1N0M0		
B45	MPr	1232/94	WGH	40	n/a	n/a	T2M1		
B46	DJG	6342/93	WGH	43	Apr-91	mx/anc	T2N1M0		
B47	IOC	2515/96	WGH	51	Mar-96	wle/anc	T1N0M0		
B49	EA	3576/96	WGH	53	Apr-96	wbx	T4N2M0		
Anglo-Celtic Cooperative Oncology Group Patients									
Patient ID	Pt Initials	Sample ID	Source	Age	date surg	Type surg	TNM at diag	Pos nodes (total)	Therapy
B5	JP	JP	ICRF	52	Feb-96	mx/anc	T1N0M0	9 (14)	HDT
B6	SP	SP	ICRF	43	Dec-95	wle	T1N0M0	17 (20)	CON
B14	MP	MP	ICRF	51	Aug-95	wle/anc	T2N1M0	15 (18)	HDT
B18	LT	LT	ICRF	35	Jul-95	wle/ans/mx	n/a	5 (20)	HDT
B19	JC	JC	ICRF	46	Jul-95	mx/anc	T1N1M0	4 (9)	HDT
B30	CL	2668/96	WGH	35	Mar-96	wle/anc/mx	T2N0M0	8 (28)	HDT
B37	SM	2477/96	WGH	34	Feb-96	wle/anc/mx	T2N0M0	6 (11)	HDT
B38	JA	1442/97	WGH	32	Feb-97	wle/anc	T1N0M0	13 (25)	HDT
B40	CMcD	1441/97	WGH	41	Feb-97	mx/anc	T3N1M0	7 (21)	HDT
B41	MP	11277/96	WGH	52	Nov-96	mx/anc	T2N0M0	9 (22)	HDT
B48	VF	4785/97	WGH	54	May-97	mx/anc	T2N0M0	12 (16)	HDT
B50	JW	6098/97	WGH	51	Jun-97	mx/anc	n/a	12 (27)	CON
B51	MC	3701/96	WGH	51	Apr-96	mx/anc	n/a	14 (16)	CON

wle - wide local excision, ans - axillary node sampling, anc - axillary node clearance, mx - mastectomy, wbx - wedge biopsy, rt - radiotherapy, HDT - high dose therapy, CON - conventional therapy.

this polymorphism and a fragment B mutation. Therefore, 10 of 38 patients (26.3%) in the metastatic group and 3 of 13 patients (23%) in the ACCOG group had a somatic mutation in exons 5 to 8 of p53. Fig. 2.8 illustrates DGGE demonstrating tumour DNA with a positive pattern.

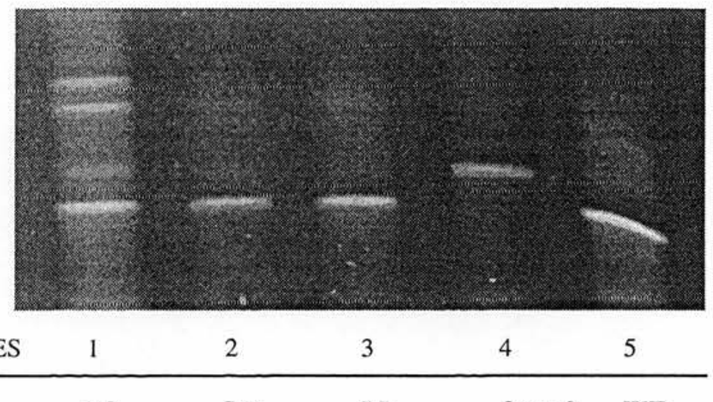
2.4 MUTATION DETECTION IN ACUTE LEUKAEMIA PATIENTS

2.4.1 PATIENT SELECTION AND NATURE AND SOURCE OF MATERIALS

All patients with acute leukaemia studied were referred to and treated at the Department of Haematology, Royal Infirmary of Edinburgh. A total of 29 consecutive patients were studied. Studied patients were unselected with regard the nature of their disease or treatment, but were chosen on the basis of having available archival extracted DNA from routine bone marrow or peripheral blood samples taken during the course of their treatment, as has been the policy of our department for several years. DNA from aliquots of these cellular samples was extracted by standard proteinase K / phenol / chloroform methods, and stored at -70°C. A total of 34 representative bone marrow samples, or peripheral blood samples in which leukaemic blast cells form a significant proportion of the white blood cell population, were studied. This information was obtained by studying all bone marrow aspirate reports or the case notes of the patients concerned, to ensure that the majority of the cells were leukaemic blasts. Of these, 16 were taken at disease relapse, and 18 at diagnosis. In 5 patients both diagnostic and relapse samples were screened. Fourteen patients had de novo AML, 10 had de novo ALL, 1 biphenotypic leukaemia, 1 chronic myeloid leukaemia (CML) transformed to AML, 1 CML transformed to ALL, 1 secondary AML from Hodgkin's disease, and 1 secondary AML transformed from MDS. Table 2.6 illustrates the characteristics of these patients.

2.4.2 RESULTS

It is noted that in patients with acute leukaemia, fragment A was not studied by DGGE. The reason for this is that during this project, leukaemia samples were first studied, and fragment A PCR using published primer sequences (Borresen et al, 1991)



LANES	1	2	3	4	5
DNA	BR	BR	BR	Ovcar3	WT

Fig. 2.8 An example of fragment C DGGE (Section 2.3.3). The breast tumour in lane 1 is positive while those in lanes 2 and 3 are negative. Ovcar3 is a positive cell line. In lane 1, the 4 bands from bottom to top are respectively, WT homoduplex, mutant homoduplex and 2 WT/mutant heteroduplexes.
BR - breast tumours

Table 2.6 List of acute leukaemia patients studied.

Patient ID	Pt initial	Sex	Age	Diagnosis	Subtype	Sample	Blasts %	Clinical Info
L1	KP	M	58	AML		BM	50%	Relapse
L2	AMS	F	37	AML	M1	BM		Relapse
L3	HI	F	50	CML>AML		BM	55%	Transformation
L4	VT	F	39	AML/ALL	Biphen	BM	>90%	Relapse
L5	DC	M	47	AML		BM	70%	Relapse
	" "			AML		BM	90-95%	Presentation
L6	JM	F	52	AML		BM	100%	Relapse
L7	EG	M	52	ALL		BM	81%	Relapse
L8	AC	F	53	AML	M5b	BM	'Sheets'	Residual disease
	" "					BM	100%	Relapsed post BMT
	" "					BM	Majority	Presentation
L9	MS	F	20	ALL		BM	Gross infiltration	Relapsed ALL
L10	DP	M	17	ALL	B-cell	BM	95%	Relapse
	" "					BM	Sheets' (trephine)	Presentation
L11	DT	M	17	ALL	L1	BM	70%	Relapse
	" "					BM	Marrow blood	Presentation
L12	RA	F	38	AML	M5	BM	10-15%	Relapse
L13	GG	M	40	ALL	CALL	BM	27%	Relapse
	" "					BM		Presentation
L14	KD	M	30	AML		BM	75%	Relapse.
L15	JP	M	50	CML>ALL		BM	60%	Relapse
L16	SR	M	20	ALL		BM	64%	Relapse post MUD
L17	EE	F	65	AML	M2	BM		
L18	DB	F	15	AML		BM	'Sheets'	Presentation
L19	RC	F	30	ALL	L2	PB	Majority	Presentation
L20	DJ	M	27	ALL		BM	76%	Presentation
L21	RR	M	66	AML		BM		Presentation
L22	ACa	F	22	AML(2ndary)	M2	BM	60%	Presentation
L23	AT	M	45	MDS>AML	M6	BM	35%	Presentation
L24	CM	F	57	AML		BM	'Sheets'	Presentation
L25	KP	M	43	AML	M4	BM	95%	Presentation
L26	JH	M	33	APML		BM		Presentation
L27	PM	M		B-ALL		BM		
L28	WL	M	22	ALL		PB		Presentation
	" "					BM		Presentation
L29	IM	M	39	AML	M4	BM		Presentation

BM - bone marrow, Biphen - biphenotypic, CALL - common ALL, MUD - matched unrelated donor transplant, PB - peripheral blood.

did not result in consistent amplification of this part of exon 5. Screening for fragment A was abandoned at this point, while subsequently, new primer sequences for this fragment were obtained from this author's group in Oslo (S. Lystad, personal communication). These were successfully applied to study breast tumours, but owing to time constraints, I was unable to repeat fragment A DGGE for the leukaemia samples. When all the relapse samples were screened by DGGE for fragments B to E, 3 samples demonstrated abnormal banding suggesting the presense of a mutation. Two of these were in fragment E (patients L8 and L13) and one in fragment D (patient L11). When all the diagnostic samples were screened, patient L11 was negative for a fragment D mutation, while the other two patients remained positive in fragment E. Again, in Section 2.6, patients L8 and L13 were found also to have a silent constitutional mutation (CGA > CGG) in codon 213. Therefore, only 1 of 16 relapse samples from 16 individuals (6.25%) was positive for a somatic p53 mutation, and none of 18 diagnostic samples was found to have a mutation. This incidence is disappointingly low and I was unable to confirm the much higher incidence reported by some authors (Zhu et al, 1996).

2.5 MUTATION ANALYSIS - GENERAL METHODS

2.5.1 MATERIALS

1.5 ml Eppendorf tubes (Costar, High Wycombe, UK)
0.5 ml PCR reaction tubes (Costar, High Wycome, UK)
Spin-X columns (Costar, High Wycome, UK)
23 gauge sterile hypodermic needles
Clean razor blades
QiaQuick PCR Purification Kit (Qiagen, Crawley, UK)
TaqI restriction endonuclease and buffer (Promega, Southampton, UK)

2.5.2 EXTRACTION OF MUTANT HOMODUPLEX BAND FROM DGGE

As described in the principles of DGGE in Chapter 1, DGGE usually separates 4 different species of DNA from a PCR product according to their melting properties, namely, mutant and WT homoduplexes, and 2 heteroduplexes formed by hybridisation

of WT and mutant strands. Heterduplexes are usually characterised by their high degree of instability, tend to melt first in a denaturing gradient gel, and appear highest in the lane. The WT homoduplex can be recognised when a wild type control is run alongside in the same gel. The mutant homoduplex may run faster or slower than the wild type, and is usually recognised by exclusion. To obtain sequence information of the mutant (Langlands et. al., 1995), this homoduplex band was cut out with a clean blade, cropping tightly, and placed in a 0.5 ml PCR tube where 3 evenly spaced perforations had been created close to the tip by puncturing with a 23 gauge sterile hypodermic needle. This tube was, in turn, place inside a 1.5 ml Eppendorf tube where 50 μl of sterile distilled water had been placed. The tubes were centrifuged at 14,000 rpm for 5 min during which the polyacrylamide gel was forced through the perforations into the distilled water. The surface area of contact between gel and water was therefore maximised, and the tube floated in a water bath at 37°C overnight when DNA was eluted out from the gel into the water. The Eppendorf tube was centrifuged and the contents place in the column of a Spin-X column, centrifuged at 14,000 rpm for 5 min. The spin column contained the remains of the polyacrylamide gel and was discarded. The collection tube contained a solution of the mutant homoduplex PCR product which was used as template for a second round PCR amplification prior to sequencing.

2.5.3 PREPARATION OF SAMPLE FOR SEQUENCING

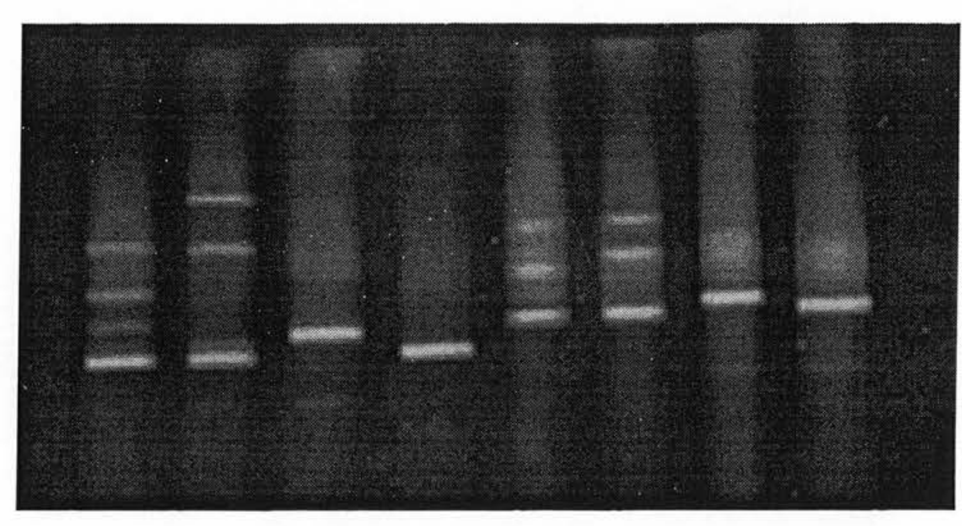
The extract of the mutant homoduplex band, hopefully free of wild type sequence, was secondarily amplified by PCR using essentially the same primers which produced the product for DGGE, except an alternative primer equivalent to the GC-clamped primer but without GC-rich region was used. After confirming successful secondary amplification with 4% agarose gel electrophoresis the product was purified with the QiaQuick PCR Purification Kit which consisted of spin columns, 2 ml collection tubes, Buffer PB and Buffer PE. Five volumes of Buffer PB is added to 1 volume of PCR product and mixed well. This was transferred to a spin column where DNA binding to the membrane was allowed to occur. After centrifugation in an Eppendorf micro-centrigfuge for 1 min at 10,000 g, bound DNA was washed with 0.75 ml of

Buffer PE by centrifugation, first for 1 min at 10,000g, then at maximum speed (14,000 g) for a further minute. The spin column with the DNA still in situ was placed in a 1.5 ml Eppendorf tube, and the DNA eluted with 50 μl of distilled water by centrifugaton after standing for 1 min.

The purified product was sent for sequencing commercially (Oswel, Southampton, UK). Sequencing was performed by the Sanger dideoxy technique on an automated ABI Prism sequencer, and the result expressed in a 4-colour chromatogram. I originally attempted to perform manual sequencing myself, using the Sequenase Version 2 Kit (Amersham, UK), but soon realised that the labour-intensiveness, the time involved in sequencing all positive samples and the lack of reproducibility made this unfeasible.

2.5.4 USE OF TaqI RESTRICTION ENDONUCLEASE IN DIFFICULT SAMPLES

Not all samples positive for a mutation on DGGE show a distinct 4-band pattern, and the mutant homoduplex may not appear sufficiently separated from the WT homoduplex to enable a pure mutant species to be obtained for sequencing. This was the case in 2 of the positive samples (patients B16 and B30), both with a mutation in fragment E (exon 6). I reasoned that a GC-clamped DNA fragment slightly shorter than the original fragment used in DGGE might have a significantly different melting profile, and the same mutation might produce quite different banding patterns on DGGE. The hypothesis of different melting profiles could not be verified without the appropriate Lerman-type computer programme, but for fragment E, a common restriction endonuclease, TaqI (recognising 5'-TCGA-3'), cuts once at 51 bases from the non-GC-clamped end of the fragment (192 bp). Hence the majority of the fragment would still be amenable to study by DGGE. For these 2 patients, a GC-clamped fragment E produced by PCR in the usual way was incubated with 10 U TaqI at 65°C as per manufacturer's protocol for 3 hr, to complete digestion, and the resulting product seperated in DGGE. Fig. 2.9 illustrates the result. Lanes 1 - 4 represent the same samples as lanes 5 - 8, respectively, the former after TaqI digestion and the latter without such treatment. Lanes 1 and 5 are fragment E from patient B30,



LANES	1	2	3	4	5	6	7	8
TaqI digest	+	+	+	+	-	-	-	-
DNA	B30	B16	T47D	WT	B30	B16	T47D	WT

Fig. 2.9 Use of TaqI enzyme digestion of GC-clamped PCR products prior to DGGE enhances mutant homoduplex bands in some cases (Section 2.6.3). For both patients (B30 and B16), the previously indistinct or invisible mutant homoduplex band (second band from bottom) became visible (and could be physically isolated) when the PCR products were treated with TaqI prior to DGGE, thus altering the melting profile of the remainder fragment. T47D positive control and WT negative control are included

showing a much more distinct mutant homoduplex band (second from bottom) after TaqI digestion. Lanes 2 and 6 are patient B16. The formerly invisible mutant homoduplex could be seen after TaqI digestion, migrating slightly more slowly than the WT but sufficient separation to excise it from the gel. Lanes 3 and 7 are T47D cell line positive control, and lanes 4 and 8 are WT control. The mutant bands from patients B16 and B30 were isolated as described, although, as the primary products had been cut with TaqI, secondary PCR prior to sequencing was not possible. Fortunately, with the amount of product from the primary reactions, a mutant sequence was obtained for both samples, and later used successfully in the design of ARMS primers.

2.6 SEQUENCE RESULTS FROM BREAST CANCER PATIENTS

As indicated in Section 2.3.3, 15 positive results were detected in 14 patients. Attempts were made to sequence all these samples, but unfortunately, despite repeated sequencing, 2 samples, from patients B1 and B15, both for fragment B, gave inconclusive results, and analysis could not proceed. Of the remaining 13, sequencing revealed that patients B12 and B15, both containing fragment E mutations in their tumours, had an identical constitutional silent polymorphism in codon 213 (CGA > CGG, arginine > arginine) which is a known polymorphism (Carbone et al, 1991) and the only reported one in the 5 fragments of p53 studied in this project. This base change results in the loss of a TaqI restriction enzyme recognition site, and when fragment E PCR products from tumours of these patients and from their PBPCH's (taken to represent normal tissues) were incubated with TaqI, failure of digestion in all these tissues confirmed the presence of this base change in the germline and not a somatic event.

Therefore, there were 11 positive samples which could serve as markers for MRD, and sequencing of each of these revealed the information indicated in Table 2.7. The sequence chromatographs are illustrated in Fig. 2.10. Two patients had an identical mutation in codon 248, hence 10 individual mutations were recognised. All 10 mutations were single-base missense mutations resulting in an amino acid change.

Table 2.7 Sequence information of samples positive for a p53 mutation on DGGE.

Patient	Sample ID	Fragment	Exon	Codon no	Codon change	WT Sequence	Amino acid change
B3	BD	B	5	171	GAG > TAG	ATGACG G AGGTTG	Glu > End
B11	HB	B	5	175	CGC > CAC	TGAGGC G CTGCCC	Arg > His
B16	LB	E	6	193	CAT > GAT	CCTCAG C ATCTTA	His > Asp
B27	7071/90	D	8	267	CGG > CAG	TGGGAC G GAACAG	Arg > Glu
B28	8375/91	C	7	248	CGG > CAG	TGAACC G GAGGCC	Arg > Gln
B30	2668/96	E	6	198	GAA > TAA	CGAGTG G AAGGAA	Glu > End
B33	3757/94	C	7	248	CGG > CAG	TGAACC G GAGGCC	Arg > Gln
B37	2477/96	B	5	179	CAT > CGT	CCCACC A TGAGCG	His > Arg
B45	1232/94	D	8	273	CGT > TGT	GAGGTG C GTGTTT	Arg > Cys
B46	6342/93	E	6	216	GTG > TTG	CATAGT G TGGTGG	Val > Leu
B50	6098/97	E	6	216	GTG > ATG	CATAGT G TGGTGG	Val > Met
L11	2007	D	8	281	CCGGGGGG insertion	GAGAGA (ins) CCGGCG	Frameshift
B12	JR	E	6	213	CGA > CGG	TTTTCG A CATAGT	Arg > Arg
B15	VK	E	6	213	CGA > CGG	TTTTCG A CATAGT	Arg > Arg
L8	AC	E	6	213	CGA > CGG	TTTTCG A CATAGT	Arg > Arg
L13	GG	E	6	213	CGA > CGG	TTTTCG A CATAGT	Arg > Arg

The mutated base is in the centre of the column 'WT Sequence'.

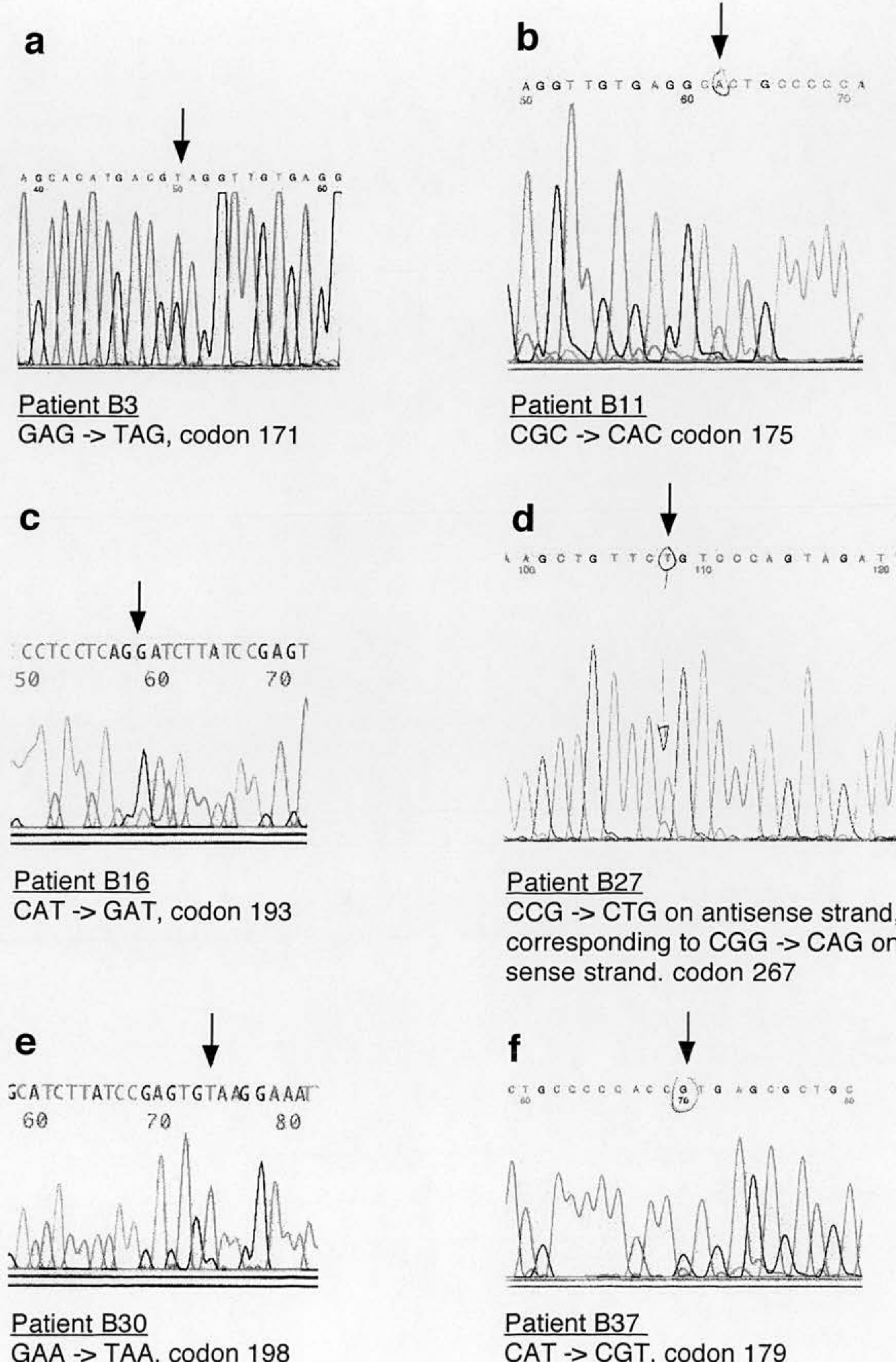


Fig. 2.10 a-f Sequence chromatographs showing single base mutations in patients B3, B11, B16, B27, B30, and B37. Mutations are indicated with an arrow.

g

Patients B33 & B28
CCG -> CTG on antisense strand.
Corresponding to CGG -> CAG on sense strand. codon 248

h

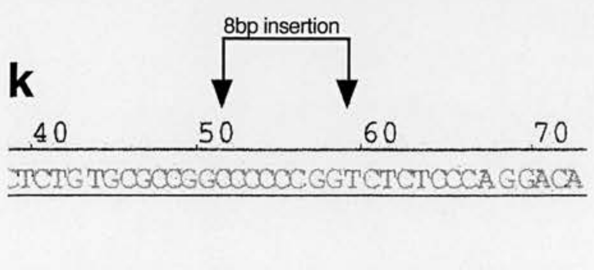
Patient B45
ACG -> ACA on antisense strand.
Corresponding to CGT -> TGT on sense strand, codon 273.

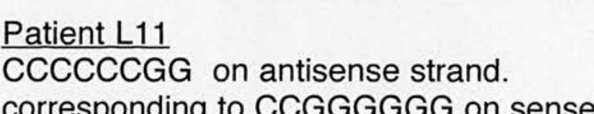
i

Patient B46
GTG -> TTG, codon 216

j

Patient B50
GTG -> ATG, codon 216





Patient L11
CCCCCCGG on antisense strand.
corresponding to CCGGGGGG on sense strand, codon 281.

Fig. 2.10 g-k Sequence chromatographs showing single base mutations in patients B33 & B28, B45, B46, B50 and 8bp insertion in patient L11. Mutations and insertion are indicated with an arrow.

Four mutations occurred in one of the 6 hot spot codons (Section 1.4.2) - codons 175, 248 and 273. Five mutations occurred at CpG dinucleotides (codons 175, 193, 248, 267, 273) and 4 of these resulted in a G:C > A:T substitution.

2.7 SEQUENCE RESULTS FROM LEUKAEMIA PATIENTS

In the cohort of 29 patients with acute leukaemia, 2 (patients L8 and L13) also had the silent constitutional polymorphism (CGA > CGG) in codon 213. This was demonstrated by screening normal and leukaemic samples with TaqI restriction endonuclease as described in the previous section. Apart from these patients, only patient L11 was positive on DGGE in fragment D (exon 8). Despite a clear and distinct mutant homoduplex band on DGGE, obtaining a pure double-stranded mutant product proved more difficult than expected. Secondary amplification of the homoduplex extract (Section 2.5) repeatedly yielded a significant WT product suggesting contamination with WT. Purity was much improved when extract of the mutant homoduplex was first electrophoresed on a polyacrylamide gel and the resulting band physically cut out again before secondary PCR. With this extra step, a clear mutant double-stranded PCR product was obtained, as evidenced by a larger product than WT. Sequencing indicated a novel 8 bp (5'-CCGGGGGG-3') insertion between the second and third bases of codon 281 (Table 2.7). Chapter 3 describes the use of ARMS in detecting MRD, and because of the specific nature of this insertion, ARMS primer design was straightforward, and Sections 3.2, 3.7.3 and 3.9 are descriptions of the design, clinical association and application of the system for this patient.

2.8 SUMMARY

This chapter describes the first of the 2 main stages in this project, namely the detection and sequence analysis of p53 mutations in the studied clinical samples, the other stage being the use of ARMS in detecting MRD (Chapter 3). Having chosen the DGGE technique for mutation screening of the clinical samples, the technique was set

up by studying various parameters which might influence the working of such gels. Using primer sequences determined by Borresen et al (1991), exons 5 to 9 of the p53 gene were individually amplified. Cell lines known to contain mutations in each of these DNA fragments (designated A to E) were used to confirm that the technique was able to yield a positive result for each of these. Previously extracted DNA from normal human tonsils was used as WT control. The sensitivity of DGGE was determined by dilutions of DNA from the bladder cancer cell line 5637 in WT DNA. It was demonstrated that, although the presence of a mutation can be detected when tumour cells were out-numbered by normal cells 19:1, the proportion of tumour cells should be much higher than this (approximately 40% or more) if the sequence was to be determined by excising the mutant homoduplex band from the gel. This helped with the choice of leukaemia samples to be studied, and in many cases with that of breast cancer sections. Various optimisation parameters were analysed, such that an optimal running temperature, voltage, cross-linker, electrophoresis duration, etc., were used. Instead of 'fine-tuning' the conditions for each DNA fragments with regards the denaturant concentrations, a universal gradient of 30 - 80% was found to be satisfactory.

Having established a satisfactory set of running conditions, DGGE was used to screen malignant tissues from 51 breast cancer patients and 29 acute leukaemics. Amongst the breast tumours, 15 showed an abnormal banding pattern on DGGE. Two of these were previously recognised as a codon 213 silent polymorphism (CGA > CGG), and a mutant sequence could not be obtained from a further 2. Of the remaining 11, 2 had an identical mutation (codon 248, CGG > CAG). As a result, 10 different point mutations could potentially be used in ARMS for detection of MRD. Amongst the acute leukaemia samples, 3 showed abnormal banding on DGGE. Again, 2 were the same codon 213 polymorphism described. Of the remainder, only 1 sample showed a genuine somatic mutation, which on sequence analysis was a hitherto unreported 8 bp insertion sequence (5'-CCGGGGGG-3') between the second and third bases of codon 281. Therefore, at the end of this stage of the project, a total of 11 mutations (10 of which were single-base missense mutations) were found, which could be assessed for their suitability as markers for MRD. This is described in Chapter 3.

CHAPTER 3

AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

In order to use p53 mutations as markers for MRD detection, allele-specific primers (ARMS primers) have to be designed, based on the exact nature of the mutation as determined by sequence analysis. The main characteristic of such primers is that they allow amplification with the mutant but not the WT allele, and it is crucial that they are 100% specific, i.e., at a particular set of optimal conditions, no detectable product be produced with the WT allele, where the detection system may be ethidium bromide staining with UV light viewing, or PCR with radioactive 32-phosphorus (32P)-labelled dCTP with autoradiography. It is important to understand that, unlike using tissue-specific markers such as CK 19 for MRD detection, ARMS involves a difference in the efficiencies of amplification using a mutant versus a WT template (differing usually by a single base), under identical conditions. A product is still likely to be produced with a WT template, albeit in much smaller quantities, and the optimisation process is an attempt to maximise this difference between WT and mutant for a particular detection system used.

In this chapter, a high *specificity* of an ARMS system refers to its ability to produce no detectable product with a WT DNA template, while a high *sensitivity* refers to its ability to produce a strong product with a mutant DNA template at low concentration. I shall **(1)** first describe the principles behind the design of ARMS primers to be used in PCR, then **(2)** methods of optimising the specificity of such reactions by varying each of a number of reaction parameters, **(3)** attempts at improving the sensitivity of such reactions to detect minimal disease, **(4)** the results of efforts to optimise individual ARMS systems for the patients found to have markers in Chapter 2, and **(5)** finally, the results of screening PBPCH's with optimised ARMS systems. With all ARMS reactions that did not involve incorporation of 32P, 20 μl of each 50 μl PCR product was mixed with 4 μl of 6X gel loading buffer, and

electrophoresed in a 1.5 mm thick 6% polyacrylamide gel at a constant 45 mA current for approximately 1 hr 15 min, stained with ethidium bromide (0.1 mg/l) for 20 min, de-stained with distilled water for 20 min, viewed under UV light and photographed with Polaroid '665' ISO 80 black and white film.

3.1 MATERIALS

Fresh T47D and 5637 cell lines in culture

Blood donors' buffy coat white blood cells (SEBTS, Edinburgh)

Phosphate Buffered Saline (PBS)

'Red Cell Lysis Solution' (RCLS, see Section 2.1.3.1)

Coulter STKS Cell Counter (Coulter Ltd, UK)

Hybaid Omnigene Thermocycler (Hybaid, Life Sciences, Basingstoke, UK)

PCR primers described in Table 3.1, and PCR reagents described in Section 2.1.5.1.

Electrophoresis apparatus (Hoefer SE 600 tank) and accessories (as described in Chapter 2)

For 32P-labelled PCR:

Mini electrophoresis tank and accessories (Biorad, UK)

Kodak X-omat X-ray film (Sigma, Poole, UK)

α-32P-dCTP (3000 Ci/mmol) (ICN, Basingstoke, UK)

γ-32P-ATP (3000 Ci/mmol) (ICN) - see Section 3.6.2.2

T4 polynucleotide kinase and 10X buffer (Promega, Southampton, UK) (see Section 3.6.2.2)

3.2 DESIGN OF ARMS PRIMERS

All ARMS primers were approximately 20 bp in length. All mutations found in the breast cancer patients were missense mutations. The 3' base of an ARMS primer incorporates the mutation itself. A deliberate mismatch was routinely introduced in the neighbouring base in order to increase specificity of reactions, making WT DNA even more refractory to amplification. Hence, a WT template was mismatched to the primer in the last 2 bases at the 3' end, while a mutant template was mismatched to

the primer only in the penultimate 3' base but fully matched in the 3'-most base. Refractoriness was maximised by choosing the second mismatch such that a purine-purine or a pyrimidine-pyrimidine mismatch resulted, and using guidelines described by Newton and Graham (1994) (see Section 1.5.8). The cell line T47D was studied most extensively, and a second ARMS primer for its mutation, involving only the single 3' mismatch to WT but fully complementary to the mutant, was also studied. The 8 bp insertion associated with the patient with ALL (patient L11, Section 2.8) was more straightforward with regards ARMS primer design. The anti-sense strand sequence was used for ARMS primer design, as this primer sequence involves only 2 matched bases with a WT template (4 and 6 bases from the 3' end) which was unlikely to allow strand extension, whilst an ARMS primer placed on the sense strand would have 5 matched bases to the WT template and much more likely to allow strand extension, as indicated here.

ARMS primer on anti-sense strand:

WT template: 5'-TCTGTGCGCCGGT **C** T **C** TCCCAGGACAGG-3'

ARMS primer: 5'-TCTGTGCGCCGGC **C** C **C** CGG-3'

ARMS primer on sense strand:

WT template: 5'-CCTGGGAGAGA **CCGG** C **G** CACAGAG-3'

ARMS primer: 5'-CCTGGGAGAGA **CCGG** G **G** G-3'

Bases underlined are the insertion sequence and bold type indicates bases matched to a WT template. It was noted that an error in primer design was made in that only 7 bases (instead of 8) were inserted, with the last 5' C missing. However, in practice this did not appear to affect efficiency or specificity of PCR, and so it was decided to continue with this primer sequence (2007-ARMS1).

A list of all the ARMS primers used in this project appears in Table 3.1. Included are GC content and Tm of each ARMS primer and the opposite primer in PCR for comparison.

In this project, I have elected to use one of the exon primers with each ARMS primer in PCR, rather than designing another new primer. The reason for this is in order to

Table 3.1 List of ARMS primers used.
Characteristics listed include melting temperature (Tm), % GC content, and the opposite primer and its Tm.
The control AAT gene primers are also listed.

Patient ID	Primer Name	%GC	Tm	Sequence	3' mismatch	2nd mismatch	3rd mismatch	Use with	%GC	Tm	Product size
Cell line	T47D-ARMS1	60	55.8	5'-TCT GGC CCC TCC TCA GCA AT-3'	T - G	A - A	n/a	E-Bio	54.5	56.6	127 bp
Cell line	T47D-ARMS2	60	55.8	5'-TCT GGC CCC TCC TCA GCA TT-3'	T - G	n/a	n/a	E-Bio	54.5	56.6	127 bp
Cell line	5637-ARMS1	61.9	74.7	5'-TGT TTG TGC CTG TCC TGG GCC-3'	C - C	C - T	n/a	D-NGC	55	53.7	104 bp
Cell line	RAJI-ARMS1	52.2	56.9	5'-ATA GGG CAC CAC CAC ACT ATG	G - T	A - G	n/a	E-NGC	60	55.8	165 bp
B3	BD-ARMS1	65	57.8	5'-GGG CAG CGC CTC ACA ACC AA-3'	A-G	A-A	n/a	B-NGC	75	61.9	87 bp
B11	HB-ARMS1	65	57.8	5'-GCG CTC ATG GTG GGG GCA TT-3'	G - T	C - T	n/a	B-GC	90	n/a	140 bp
B16	LB-ARMS1	70	59.9	5'-AGG TCT GGC CCC TCC TCA CG-3'	G - G	C - C	n/a	E-Bio	54.5	56.6	130 bp
B27	7071/91-ARMS1	38.1	48.4	5'-TAG TGG TAA TCT ACT GGG AAA-3'	A - C	A - G	n/a	D-NGC	55	53.7	143 bp
B30	2668/96-ARMS1	45	49.6	5'-TCA GCA TCT TAT CCG AGT CT-3'	T - C	C - C	n/a	E-Bio	54.5	56.6	115 bp
B33	3757/94-ARMS1	56	60.9	5'-GTG ATG ATG GTG AGG ATG GGC-3'	G - T	G - G	n/a	C-NB	45	49.6	78 bp
B37	2477/96-ARMS1	65	57.8	5'-GCT ATC TGA GCA GCG CTC CC-3'	A - C	C - T	n/a	B-NGC	75	61.9	112 bp
B45	1232/94-ARMS1	50	51.7	5'-ACG GAA CAG CTT TGA GGT CT-3'	T - G	C - C	n/a	D-NGC	55	53.7	125 bp
B45	1232/94-ARMS2	50	51.7	5'-ACG GAA CAG CTT TGA GGA CT-3'	T - G	C - C	A - A	D-NGC	55	53.7	125 bp
B46	6342/93-ARMS1	65	57.8	5'-GGC TCA TAG GGC ACC ACC CA-3'	A - G	C - T	n/a	E-NGC	60	55.8	170 bp
B50	6098/97-ARMS1	65	57.8	5'-GGC TCA TAG GGC ACC ACC CT-3'	G - T	T - C	n/a	E-NGC	60	55.8	170 bp
L11	2007-ARMS1	78.3	67.6	5'-TTC CTC TGT GCG CCG GCC CCC GG-3'	n/a	n/a	n/a	D-Bio	40	47.6	94 bp
Control	AAT1	63.3		5'-CCC ACC TTC CCC TCT CTC CAG							
				GCA AAT GGG-3'							
Control	AAT2	60		5'-GGG CCT CAG TCC CAA CAT GGC							
				TAA GAG GTG-3'							

limit the number of steps involved in this ARMS system for MRD detection, which is already a multi-step process. Table 3.1 compares the GC content and Tm characteristics of these primers.

3.3 OPTIMISATION OF ARMS REACTIONS - CELL LINE MODEL

Three cell lines, T47D (breast carcinoma), 5637 (bladder carcinoma) and Raji (non-Hodgkin's lymphoma), were used as models in the work-up to indicate the various factors that can be altered to make ARMS more sensitive and specific. 100% specificity is defined as the absence of a detectable PCR product when WT DNA is used as template for amplification. Whilst all 3 cell lines were used to study the effect of Ta on the specificity of ARMS PCRs, T47D was the most extensively studied, with respect to all of the following conditions:

(1) Annealing temperature of the ARMS PCR.

(2) Magnesium concentration.

(3) Presence of glycerol.

(4) Presence of formamide.

(5) Total number of PCR cycles.

(6) Primer concentration.

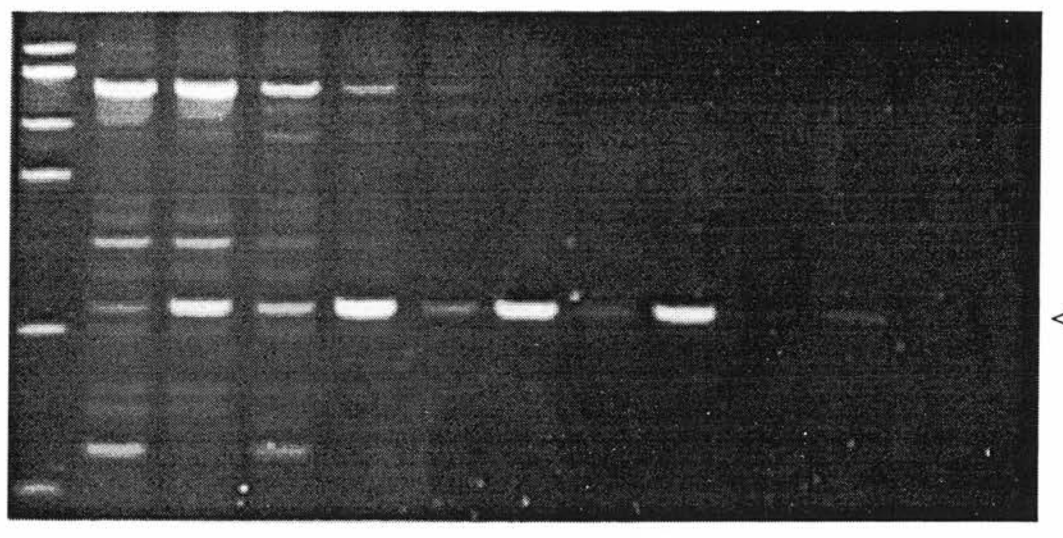
(7) dNTP concentration.

(8) One or 2-base mismatch with WT sequence at the 3' end of ARMS primer.

PCR's were performed as described, using WT or mutant DNA as template. Reactions were performed in duplicates, always including one tube in which water substituted DNA as a blank. 200 - 500 ng of genomic DNA was used per 50 μl reaction mixture. Molecular weight markers were ϕX174 DNA digested by HaeIII (Promega, Southampton, UK).

3.3.1 ANNEALING TEMPERATURE (Ta)

The Ta of the ARMS PCR appeared to be the single most important factor in achieving specificity of the system. Using each of the 3 systems with cell lines T47D, 5637 and Raji, Tas were increased from 55°C in 3 - 4°C steps. Fig. 3.1 shows the

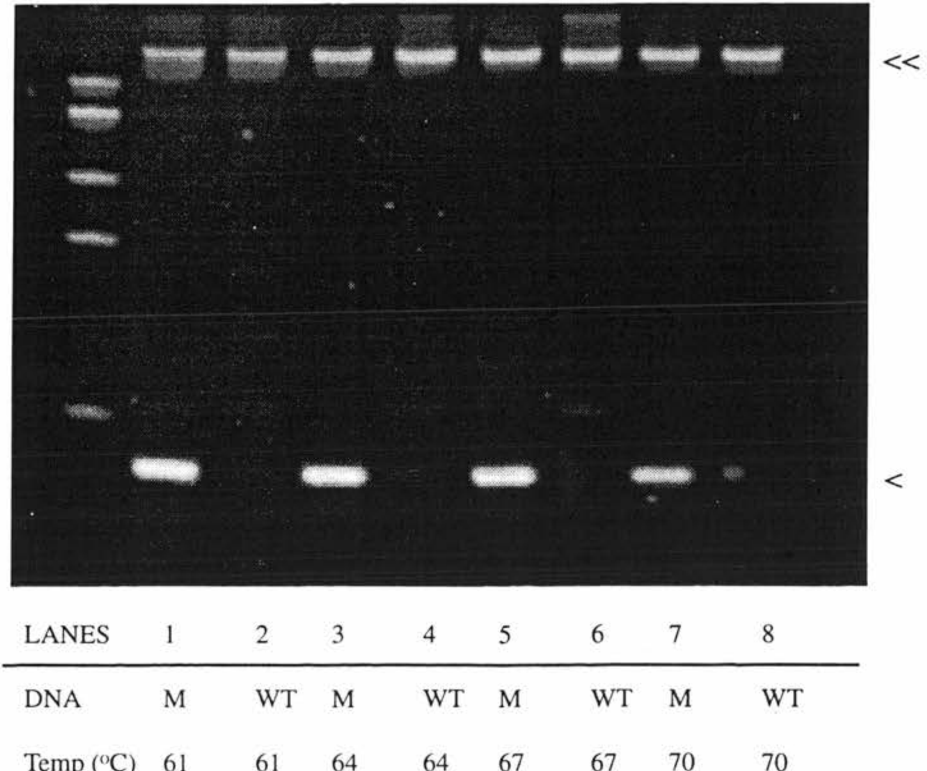


LANES	1	2	3	4	5	6	7	8	9	10	11	12
DNA	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M
Ta(°C)	55	55	58	58	61	61	64	64	67	67	69	69

Fig. 3.1a Effect of annealing temperature (Ta) on T47D ARMS (Section 3.3.1). At this [Mg] 64°C is closest to being optimal, and further optimisation is required by lowering [Mg].

< 127bp ARMS product

Lane in far left is ϕX174/HaeIII molecular weight marker



LANES	1	2	3	4	5	6	7	8
DNA	M	WT	M	WT	M	WT	M	WT
Temp (°C)	61	61	64	64	67	67	70	70

Fig. 3.1b 5637 cell line annealing temperature titration (Section 3.3.1). ARMS is specific across the annealing temperature range.

< 104bp ARMS product

<< 360bp AAT control product

Lane in far left is ϕX174/HaeIII molecular weight marker

effect this had on T47D and 5637 systems. Denaturing and extension temperatures were 94°C for 45 seconds and 72°C for 1 min, respectively. The magnesium concentration ([Mg]) was fixed at 2.0 mM and the total number of cycles at 35. A 127 bp product was produced with the T47D ARMS primers. Whilst at a low Ta of 55°C, a difference in efficiency of amplification between WT and mutant templates was seen, this was not specific (i.e., produce no product with WT). Many spurious products were also visible. As the Ta increased, less product was seen with WT until 64°C when only a faint band is discernable. At higher temperatures, the product with the mutant template also became much reduced so that the system was useless for detecting minimal quantities of template (MRD). There was, therefore, a window in which the Ta was optimal. With T47D, ARMS was still not specific at 64°C and other factors had to be altered. The 5637 system, on the other hand, appeared much more robust. Mutant DNA gave a much more intense 104 bp product, and ARMS was specific across a wide range of Ta's. This system allowed maximum sensitivity and specificity and was most suited for MRD detection.

3.3.2 MAGNESIUM CONCENTRATION ([Mg])

Using the T47D system, the effect of altering [Mg] was investigated. Ta was kept at 64°C (see above), total cycle number at 35 cycles, and [Mg] was increased from a final concentration in reaction of 1.0 mM, in 0.5 mM steps, to 5.0 mM (Fig. 3.2). The higher the [Mg], the less specific the system, i.e., the stronger the product amplified from WT template, and more spurious products were obtained. Specificity was achieved when [Mg] was 2.0 mM or lower, optimally at 1.75 mM as repeated experiments showed. Sensitivity was severely compromised with [Mg] of 1.5 mM or less. The window of specificity with [Mg], therefore, was again very narrow for the T47D system.

3.3.3 PRESENCE OF GLYCEROL

Some authors report improved specificity of ARMS by the addition of glycerol, when the quantity of DNA template is minimal (Cha et al, 1992). The effects of adding 5, 10, 15 or 20% of molecular biology grade glycerol to the ARMS reactions were

LANE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
DNA	H_2O	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M
[Mg]mM	2.0	1.0	1.0	1.5	1.5	2.0	2.0	2.5	2.5	3.0	3.0	3.5	3.5	4.0	4.0	4.5	4.5	5.0	5.0

FIG 3.2 Effect of [Mg] on T47D ARMS (Section 3.3.2). [Mg] of 2mM resulted in specific ARMS reaction with mutant DNA only (lanes 6 and 7).

< 127bp ARMS product

Lane on far left is ϕX174/HaeIII molecular weight marker.

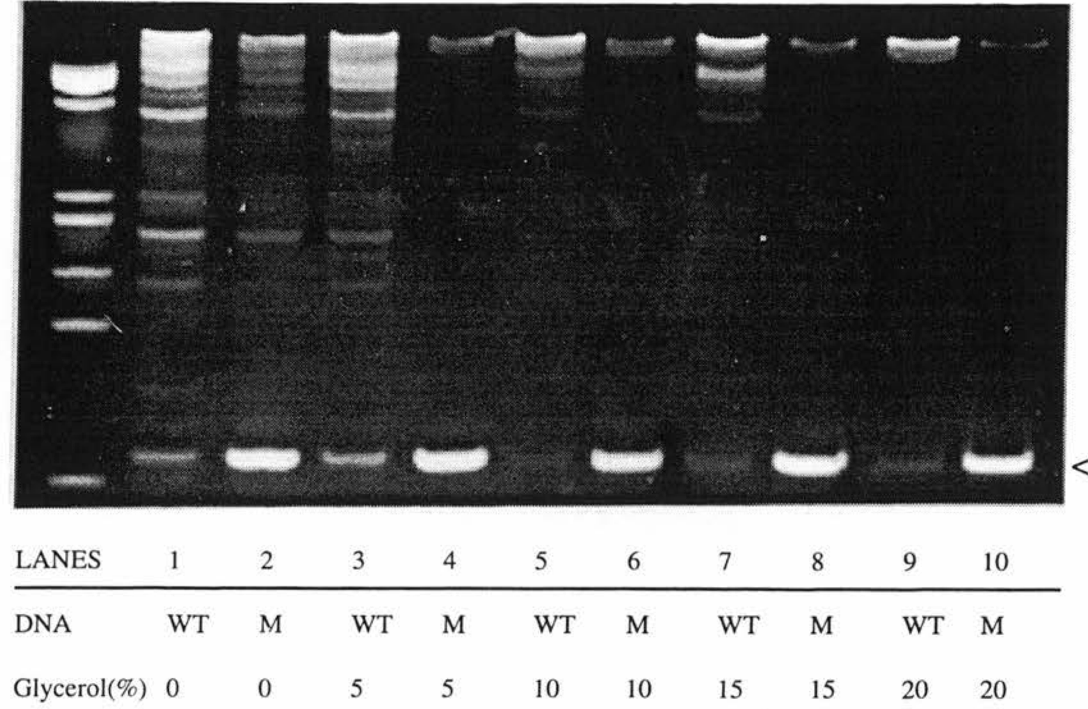
studied (Fig. 3.3). Five percent glycerol did not have any effect on improving specificity, while at higher concentrations specificity was improved. Glycerol at concentrations of up to 20% did not significantly affect reactions using a mutant DNA template, but higher concentrations did not improve specificity better than lower concentrations (10%). One major drawback of using glycerol was the viscosity of this chemical which led to inaccurate pipetting. This was at least partially remedied by heating an aliquot of glycerol to 70 - 80°C prior to pipetting. Because of the potential inaccuracies in pipetting and the slight effect on amplification of the mutant product, glycerol was not used routinely in ARMS that did not involve radio-nucleotides. It was reasoned that when 32P-labelled PCR was used, the potential problem of a lower sensitivity might be solved by increasing exposure time to the X-ray film (see below).

3.3.4 PRESENCE OF FORMAMIDE

Formamide has been used to enhance specificity of PCR, although not in the context of ARMS. The effects of formamide in the ARMS reaction was studied by using 2.5%, 5%, 7.5% or 10% in ARMS PCR reactions (Fig. 3.4). The results show that formamide has a marked suppressive effect on ARMS reactions even at lower concentrations. Amplification with both WT and mutant was suppressed, even at 2.5% and no product was observed at concentrations higher than 7.5%. Formamide, therefore, had no apparent role in ARMS reactions as assessed here.

3.3.5 EFFECT OF CYCLE NUMBER

As the intensity of a band corresponding to a PCR product on a polyacrylamide gel is proportional to the amount of product present, which, in turn, has an exponential relationship to the number of cycles of amplification performed, both the ability of an ARMS system to be specific and sensitive may depend on the number of cycles used. For the T47D system, a relatively non-specific set of conditions - a low Ta of 61°C and a [Mg] of 2.0 mM were used to study the effect of reducing cycle number. As shown in Fig. 3.5, using 30 instead of 35 cycles abolished the WT product, but also reduced the intensity of the mutant product. Lowering to 25 cycles almost abolished the mutant product as well. This suggested that lowering the number of cycles of PCR

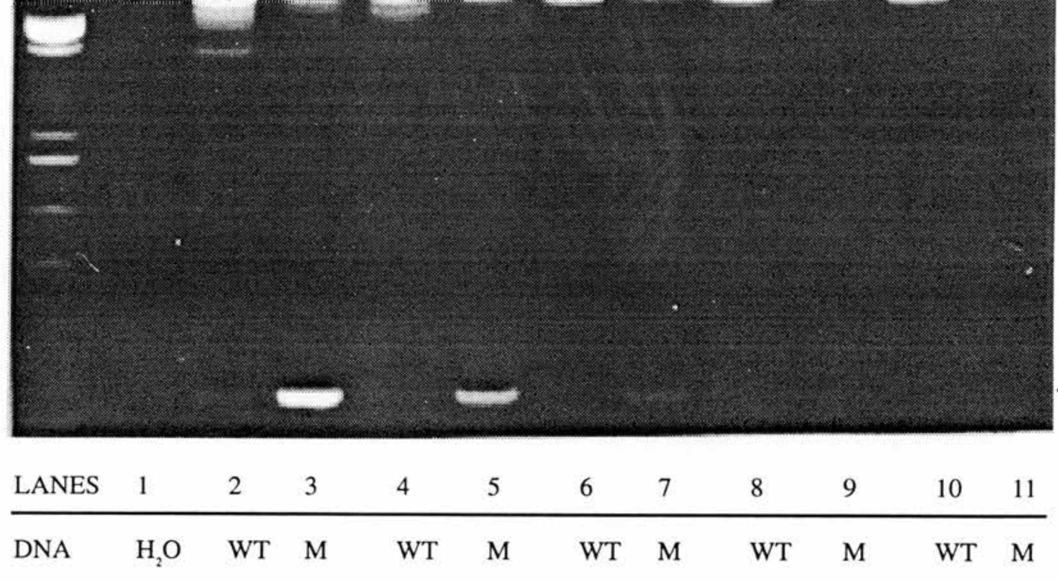


LANES	1	2	3	4	5	6	7	8	9	10
DNA	WT	M	WT	M	WT	M	WT	M	WT	M
Glycerol(%)	0	0	5	5	10	10	15	15	20	20

Fig. 3.3 Effect of glycerol on T47D ARMS (Section 3.3.3). 10% or more of glycerol appears to improve specificity of ARMS.

< 127bp ARMS product

Lane in far left is ϕX174/HaeIII molecular weight marker



LANES	1	2	3	4	5	6	7	8	9	10	11
DNA	H_2O	WT	M	WT	M	WT	M	WT	M	WT	M
formamide(%)		0	0	2.5	2.5	5	5	7.5	7.5	10	10

Fig. 3.4 Effect of formamide on T47D ARMS (Section 3.3.4). Formamide has a strong inhibitory effect on ARMS PCR.

< 127bp ARMS product

H_2O water control

Lane in far left is ϕX174/HaeIII molecular weight marker

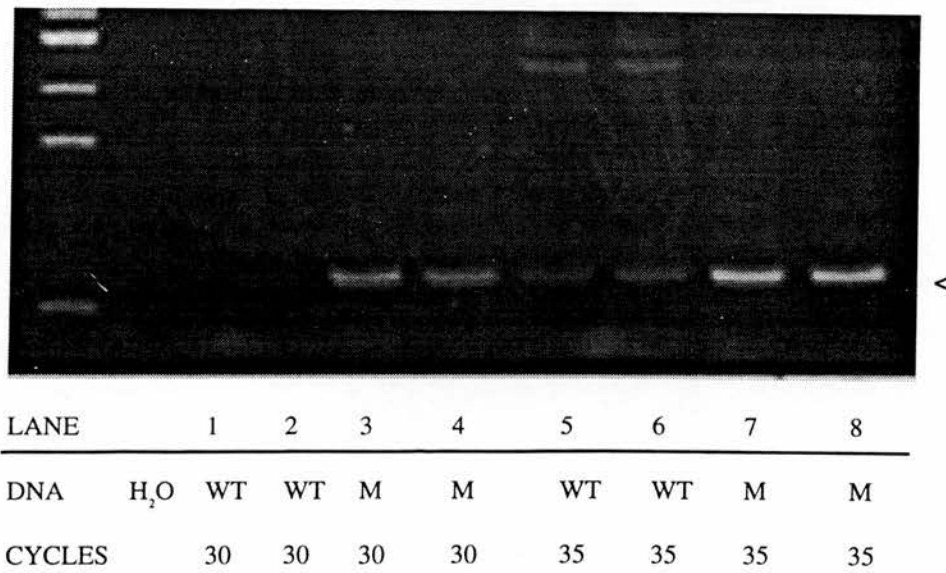
improved specificity, but at the expense of sensitivity, and again, there was only a fairly narrow window in which a balance of the two was achieved. Increasing cycle number under more stringent PCR conditions in which ARMS is normally specific were also studied. A Ta of 67°C and [Mg] of 2.0 mM were chosen (Fig. 3.6). After 35 cycles, a clear product with mutant DNA but none with WT DNA was seen. Under the same conditions but increasing the cycle number to 40, produced a clear product with WT DNA. Increasing further to 45 cycles gave an even stronger product with other spurious bands appearing as well.

3.3.6 PRIMER CONCENTRATIONS ([PRIMERS])

The effect reducing the concentrations of both primers in ARMS has on specificity of the T47D system was studied. Again, a less specific set of conditions, using a Ta of 61°C, 2.0mM [Mg] and 35 cycles were used. Standard concentration of each primer in the reaction mixture was 0.25 μM. In addition, concentrations of 0.15, 0.075 and 0.025 μM were used, under otherwise identical conditions. These represented 60%, 30% and 10% of the full concentration, respectively. Fig 3.7 shows that amplification efficiency with both WT and mutant template was reduced with decreasing [primers], and at 0.025 μM the mutant product was too weak. This showed that reducing [primers] did not differentially affect the WT reaction, and sensitivity could be compromised.

3.3.7 dNTP CONCENTRATIONS ([dNTP])

[dNTP] has been reported to be an important parameter in achieving a high specificity. The effect of 5 other [dNTP] on ARMS specificity in the T47D system was studied. Standard concentration of each dNTP used was 200 μM. In addition, [dNTP] of 50, 25, 12.5, 6.25 and 3.125 μM were tested in reaction mixtures (Fig. 3.8). It was clear that, like many of the other parameters above, lowering [dNTP] reduced amplification ofboth WT and mutant templates, and when full specificity was achieved, the mutant ARMS product was too weak to be detected.



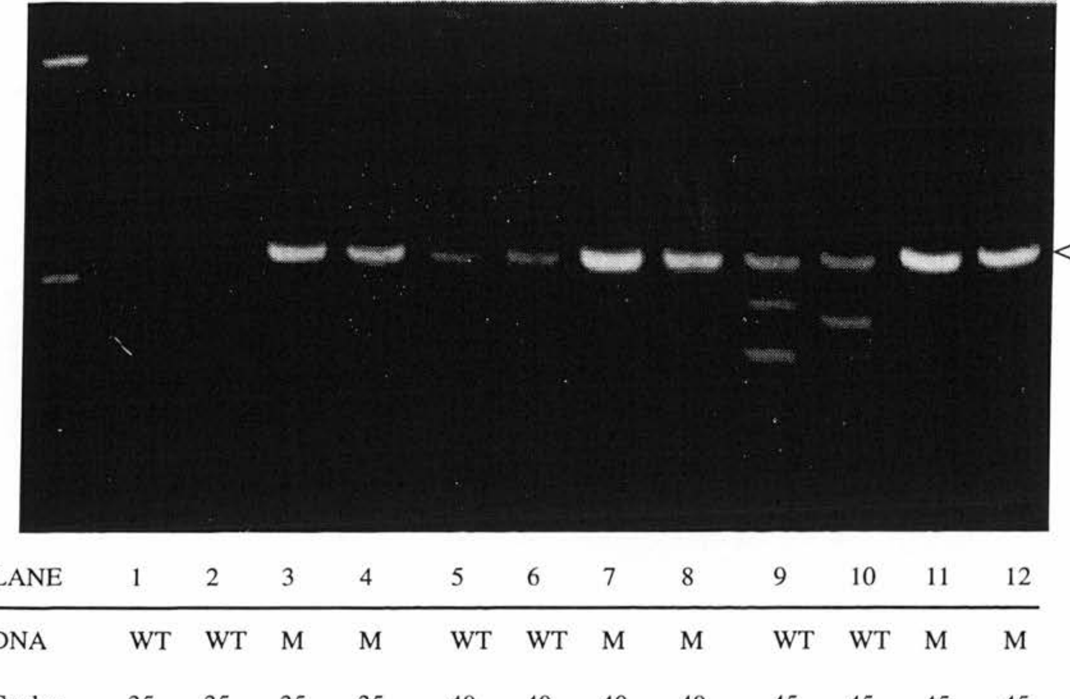
LANE		1	2	3	4	5	6	7	8
DNA	H_2O	WT	WT	M	M	WT	WT	M	M
CYCLES		30	30	30	30	35	35	35	35

Fig. 3.5 Effect of lower cycle number on T47D ARMS (Section 3.3.5). Less stringent conditions (Ta 61°C, [Mg] 2.0mM) were used to assess the effect of lowering cycle number.

< 127bp ARMS product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

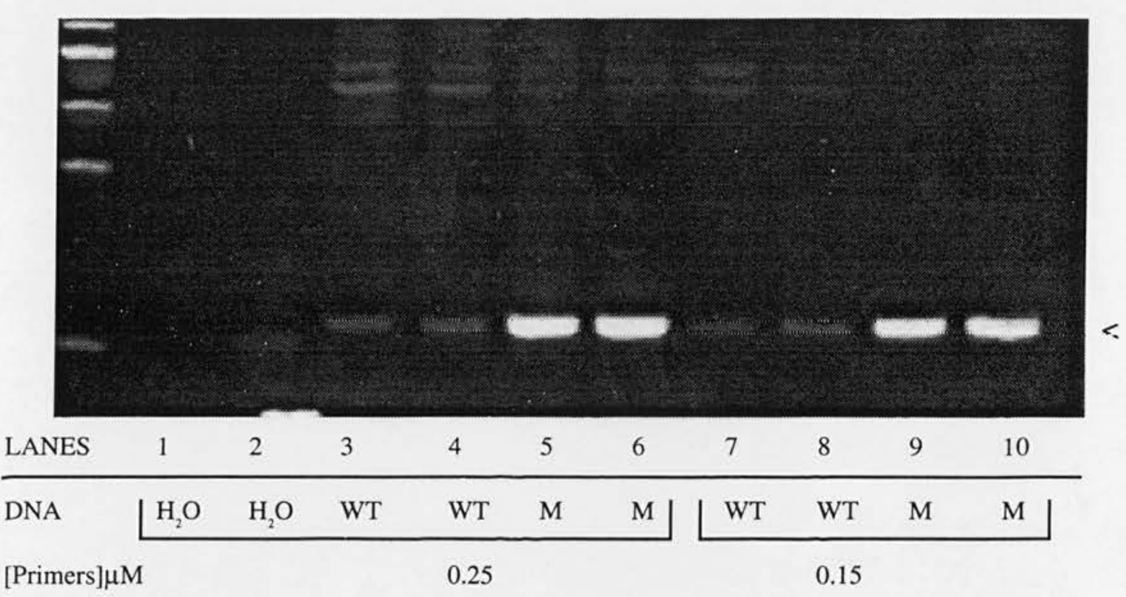


LANE	1	2	3	4	5	6	7	8	9	10	11	12
DNA	WT	WT	M	M	WT	WT	M	M	WT	WT	M	M
Cycles	35	35	35	35	40	40	40	40	45	45	45	45

Fig. 3.6 Effect of higher cycle number on T47D ARMS. More stringent conditions (Ta 67°C, [Mg] 2.0mM) were used to assess the effect of increasing cycle number.

< 127bp ARMS product

Lane in far left is ϕX174/HaeIII molecular weight marker



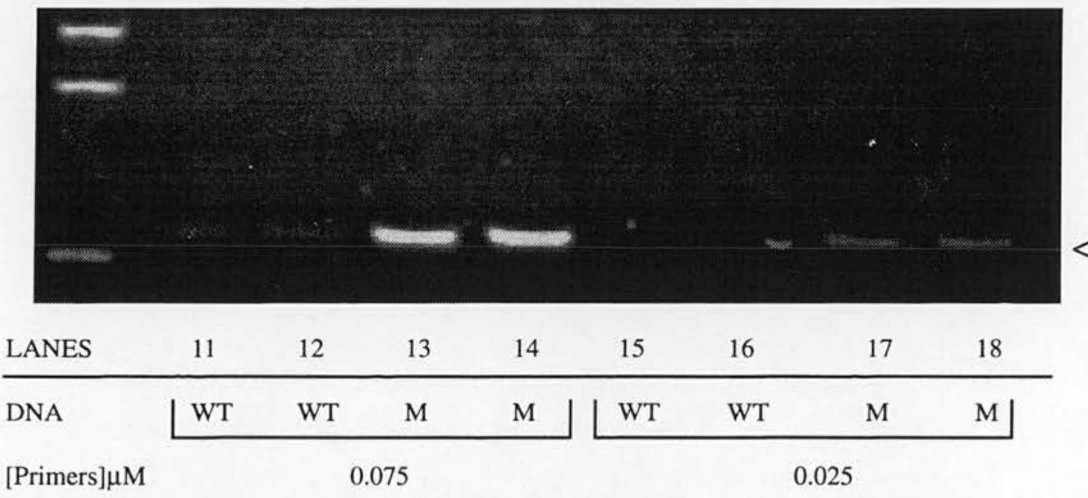
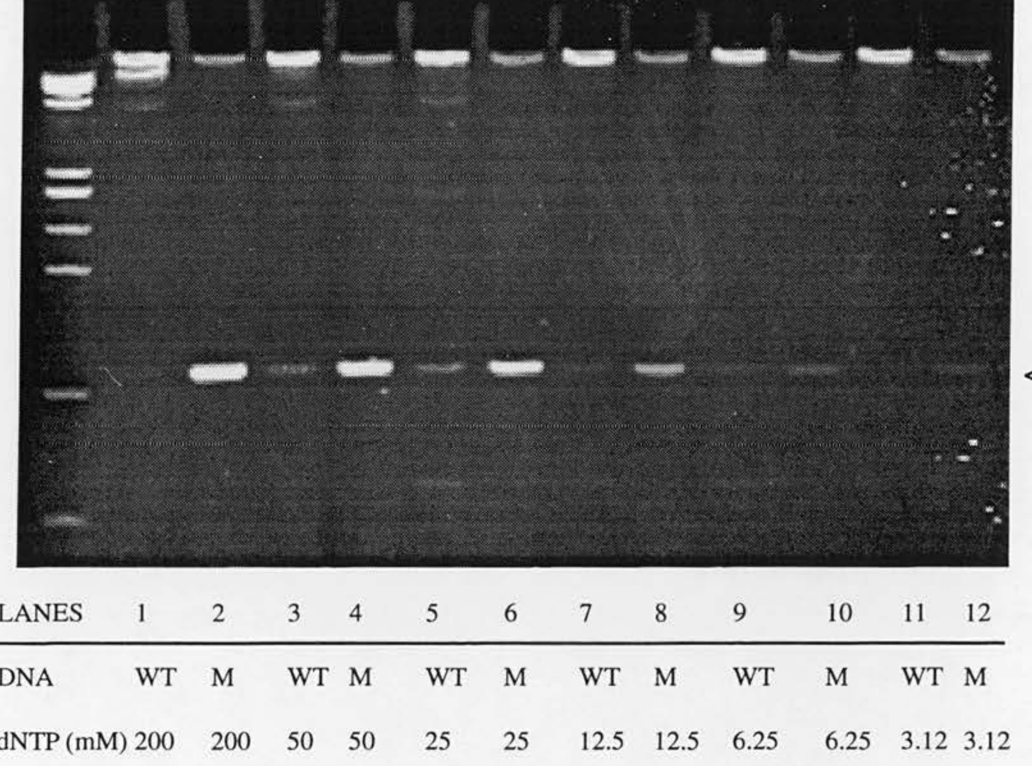


Fig 3.7 Effect of [primer] on T47D ARMS (Section 3.3.6). Lowering the [primer] reduces efficiency of both WT and M amplifications.

< 127bp ARMS product

H_2O water control

Lanes in far left are φX174/HaeIII molecular weight markers



LANES	1	2	3	4	5	6	7	8	9	10	11	12
DNA	WT	M	WT	M	WT	M	WT	M	WT	M	WT	M
dNTP (mM)	200	200	50	50	25	25	12.5	12.5	6.25	6.25	3.12	3.12

Fig. 3.8 T47D ARMS [dNTP] titration (section 3.3.7). Reducing [dNTP] appeared to have affected amplification with both WT and mutant DNA equally. No product was produced with either at the lower extreme of [dNTP].

< 127bp ARMS product

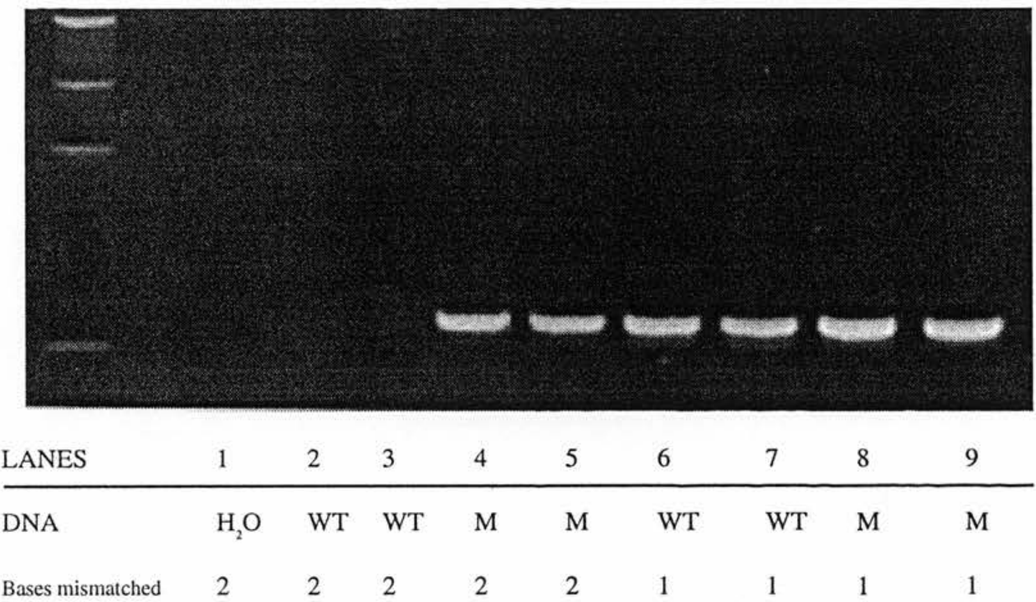
Lane in far left is ϕX174/HaeIII molecular weight marker

3.3.8 USE OF 1 VERSUS 2 MISMATCHES AT 3' END OF ARMS PRIMER

The sequence of the ARMS primer (T47D-ARMS1) used above corresponds to the sense strand, and in PCR, is used with the opposing primer on the anti-sense strand known as E-Bio. Apart from having thymine in the 3' position, resulting in a T:G mismatch with WT template, a deliberate mismatch A:A was also introduced in the penultimate base by substituting adenine for thymine. Hence this primer was mismatched to WT at the last 2 bases, and mismatched to the mutant sequence in the penultimate base only, i.e., fully matched in the 3' position and should allow strand extension to occur during PCR. To determine if it was important for a second mismatch to be inserted in order to improve ARMS specificity and ease of optimisation, primer with one- ('ARMS2') or two- ('ARMS1') base mismatches were compared in the T47D system. PCR conditions were otherwise identical. Fig 3.9 shows that, with Ta of 64°C, [Mg] of 2.0 mM, and 35 cycles of amplification, PCR with T47D-ARMS1 was specific, with no visible product amplified from a WT template, while a clear product was observed with T47D-ARMS2. Further optimisation experiments (data not shown) suggested that optimisation with T47D-ARMS2 was more difficult and more stringent conditions, e.g., higher Ta's, were required. Therefore, ARMS primers with 2 mismatches to WT were routinely used in all the ARMS systems in this project.

3.3.9 CONCLUSIONS FROM OPTIMISATION EXPERIMENTS

From optimisation experiments above, assessing the effect of 8 different parameters, it appeared that the most important parameter to optimise, is the Ta. If an optimal temperature can be achieved within the range used in PCR, i.e., lower than the extension temperature (72°C in this case), no further optimisation is required. If full specificity is not achieved, the next step would be to reduce the [Mg] until no product is produced with WT template. From these experiments, reducing the [primers] and [dNTP's] affected the WT and mutant reaction equally, and did not appear to have a critical role in optimisation. It would appear, therefore, that the third step to achieve higher specificity was either to add glycerol (10% optimal) or reduce the total number of PCR cycles. In this project, as explained above, glycerol was not used routinely in



LANES	1	2	3	4	5	6	7	8	9
DNA	H_2O	WT	WT	M	M	WT	WT	M	M
Bases mismatched	2	2	2	2	2	1	1	1	1

Fig. 3.9 Effect of ARMS primer with 1- or 2- base mismatch on T47D ARMS reaction (section 3.3.8). Under identical conditions, the ARMS primer mismatched to WT in 2 bases produced a highly specific ARMS system, whilst the primer with only 1 base mismatched was non-specific.
H_2O water control
Lane in far left is φX174/HaeIII molecular weight marker

non-radioactive reactions owing to potential errors with pipetting inaccuracies, although glycerol may have a role in ARMS reactions in which the best conditions achieved are just not specific enough. As indicated below, the possibility of improving sensitivity of ARMS by using radio-labelled nucleotides or primers in PCR, or using a nested system was investigated.

3.4 MIXING TUMOUR CELLS IN DONOR BUFFY COAT WHITE CELLS

To investigate the limit of detection of ARMS using p53 point mutations as markers, cell mixing experiments were performed with the cell lines T47D and 5637, mixed at varying dilutions in normal blood donors' peripheral blood buffy coat white cells. These 2 lines were kept in constant culture for the duration of this project. Mixing experiments were performed by harvesting each cell line fresh, washing them once in HBSS, and resuspending in PBS, ensuring that single-cell suspensions were achieved, usually by gentle aspiration up and down with a wide bore glass pipette which reduced the possibility of lysing the cells. Resuspended cells were temporarily kept on ice. Blood donors' buffy coats were obtained from the Components Section, SE Scotland Blood Transfusion Service, Edinburgh, as unwanted fractions after platelets had been extracted from them, and were between 24 and 48 hr old. Each volume (typically 10 ml) of buffy coat was red-cell-lysed twice with 4 volumes of RCLS, washed once and resuspended in (approximately 5 ml) PBS. Both cell line and buffy coat cells were counted with a Coulter STKS counter, and the numbers expressed in millions per ml. The number of buffy coat cells were invariably several times higher that those of either cell line, and were diluted down with PBS to the same concentration as that of the cell line to be spiked in them. Tumour cells were then spiked in buffy coat cells to a ratio of 1:10, and further 10-fold serially diluted in buffy coat cells to 1:100, 1: 10^3, 1:10^4, 1:10^5, 1:10^6 and 1:10^7. The total number of cells for each dilution was between $5x10^6$ and $1x10^7$. The cells were centrifuged and resuspended in 200 µl of PBS. DNA was then extracted from each pellet as per the Qiagen protocol described in Section 2.1.3. DNA concentration was determined and cryopreserved at -20°C until required.

3.5 ARMS MODEL FOR MRD DETECTION

3.5.1 USE OF INTERNAL CONTROL

In these experiments ARMS PCR's were performed on DNA extracted from cell pellets in which tumour (5637 and T47D lines) cells were spiked in normal buffy coat DNA prepared above. These DNA samples were also separately amplified, under identical conditions, by a pair of primers (AAT1 and AAT2) corresponding to a fragment of exon 3 of the α1-antitrypsin (AAT) gene giving a product of 360 bp (Wenham et al, 1991a). Amplification of this product confirmed the absence of factors inhibitory to PCR in the DNA extracts. Moscinski et al (1994) using β-actin as internal control in one of the most sensitive systems for detecting cytokeratin 19 message by RT-PCR, performed the reactions separately and mixed the product with CK19 PCR product prior electrophoresis.Separate reactions were performed for the AAT controls, rather than multiplexing PCRs in the same tube, in order to avoid the potential risk of the control reaction (which is a far more efficient system) reducing the detection limit of the ARMS system by competing for reagents and Taq polymerase. In the experiments below, 20 μl of each ARMS PCR product was mixed with 2 μl of product from the corresponding AAT control reaction, and 4 μl of 6X loading buffer, and loaded onto each lane prior to electrophoresis.

3.5.2 LIMITS OF DETECTION

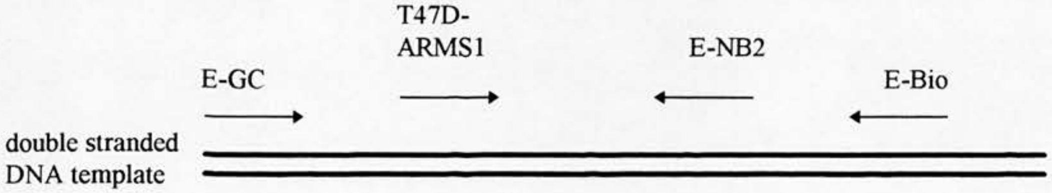
As can be seen above, the ease of achieving a high specificity with ARMS varies even between cell lines with different point mutations, when good quality, fresh DNA is used. The efficiency of amplification also varied, and under optimal conditions, a stronger PCR product was seen on PAGE for the 5637 cell line than for T47D. This was reflected in the different limits of detection in these 2 systems. Fig. 3.10 illustrates the titration for 5637. This experiment demonstrated that, when cycling conditions were optimal, mutant DNA could be detected when one 5637 cell existed amongst 10^5 normal cells, although on repeated experiments, $1:10^4$ was much more reproducible. The AAT controls worked equally well with all DNA samples. With T47D, however, ARMS with mutant DNA appeared less efficient and at optimal

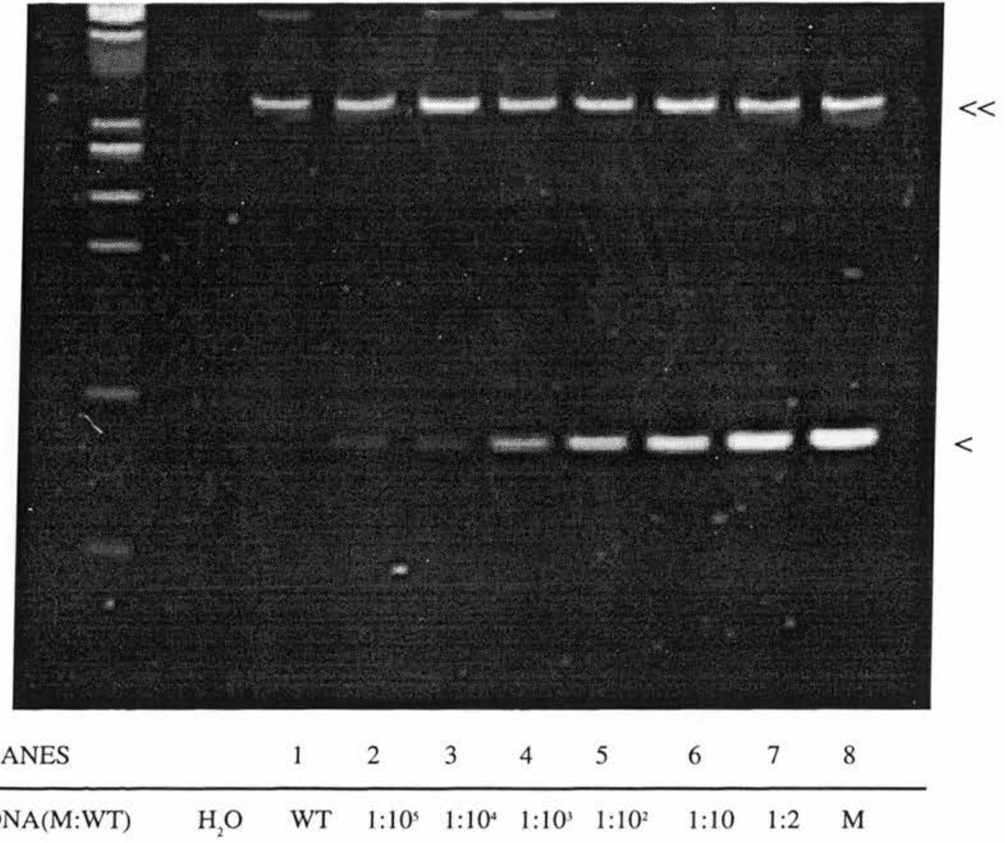
cycling conditions (Ta 64°C, [Mg] 2.0 mM, 35 cycles) a detection limit of 1 cell in 10^3 was achieved (Fig. 3.11). These 2 cell lines indicate that, with different point mutations, there can be at least a one log difference between limits of detection.

3.6 ATTEMPTS AT IMPROVING LIMITS OF DETECTION

3.6.1 USING NESTED PCR

Nested PCR systems involve 2 separate PCR reactions, with the second set of primers internal to the first set, and an aliquot of product from the first reaction was used as template for the second reaction. In general terms, nesting improves specificity of PCR as non-specific products from the first round amplification are unlikely to be sufficiently complementary to the nested primers to be used as template (Newton and Graham, 1994). Sensitivity of PCR is also enhanced as the copy number of template for the inner nest reaction is much higher. Outer nest PCR was performed on 500 ng of genomic WT and mutant T47D DNA, and 5 µl (10%) of outer nest PCR product used as template for the inner nest reaction, under identical cycling conditions (Ta 64°C, [Mg] 1.75 mM, 35 cycles, normally specific for the T47D system). Primers for the outer nest reaction were E-GC and E-Bio as used for fragment E amplification as described in Section 2.1.5.3. For the inner nest, a semi-nested system versus a fully nested system were studied. In the former, primers T47D-ARMS1 and E-Bio were used, i.e., only one end of the inner nest product was internal to the outer nest product. In the latter, T47D-ARMS1 and another primer on the anti-sense strand, E-NB2, were used, and the resulting 100 bp product was fully internal to the first. The relative positions of these primers are indicated in the diagram below.





LANES		1	2	3	4	5	6	7	8
DNA(M:WT)	H_2O	WT	$1:10^5$	$1:10^4$	$1:10^3$	$1:10^2$	1:10	1:2	M

Fig. 3.10 5637 cell line ARMS detection limit dilution experiment (Section 3.5.2). ARMS product visible down to 1 in 10^5.

< 127bp ARMS product

<< 360bp AAT control product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

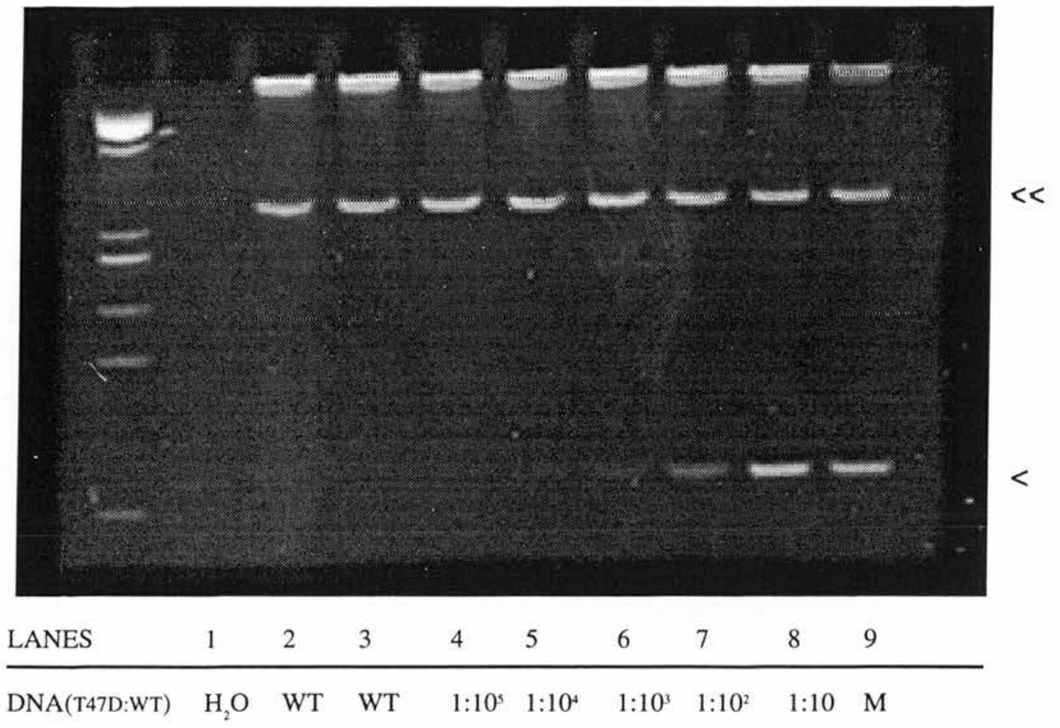


Fig. 3.11 T47D ARMS detection limit dilution experiments (Section 3.5.2). Detection limit is 1 cell in 10^3 buffy coat cells.

< 127bp ARMS product

<< 360bp AAT control product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

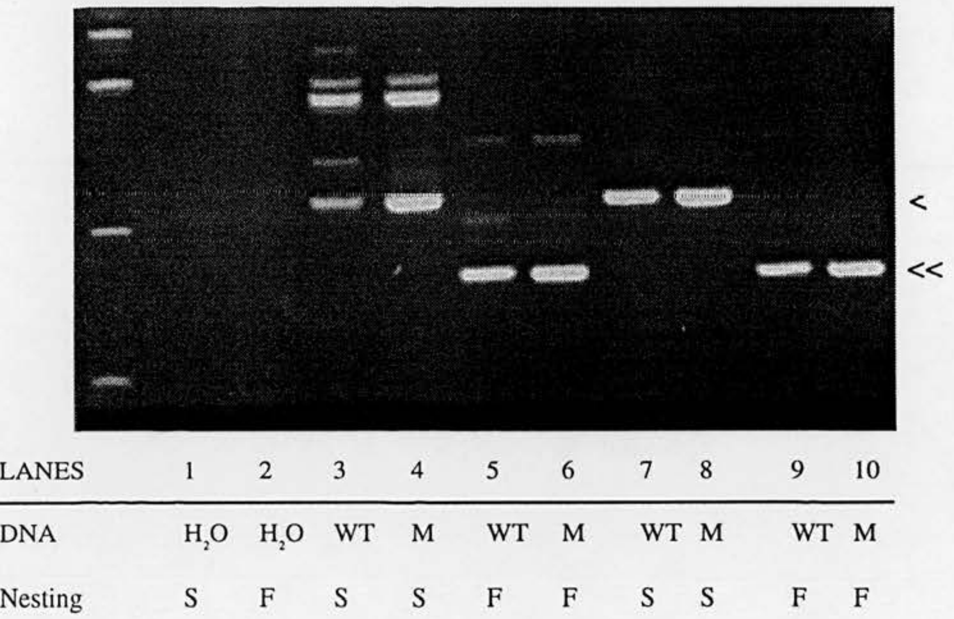
The sequence of E-NB2 was chosen empirically, ensuring similar GC content and Tm to T47D-ARMS1. As expected, for both systems, a much more intense product was produced with T47D DNA. However, specificity was completely lost and an intense product was also produced with WT template. Many spurious products were amplified on direct transfer of outer nest PCR product to the second reaction, and diluting the outer nest product 1 in 500 in water removed these products but did not alter the specificity. Fig. 3.12 illustrates these results.

3.6.2 USING 32P RADIOLABELLED NUCLEOTIDES IN PCR

ARMS PCR incorporating 32P was studied as an alternative means of product detection to ethidium bromide and UV light. The rationale for this was two-fold: **(1)** a small quantity of a PCR product may be made more readiy visible by prolonging the exposure time of the polyacrylamide gel to the X-ray film, and **(2)** because of this, cycling conditions may be made more stringent such that specificity of ARMS is maximised, and although under these conditions mutant DNA is also less efficiently amplified, their product may be visualised by increasing film exposure time. PCR products were radiolabelled by each of two means: **(1)** using α-32P-labelled dCTP in the PCR reaction mix such that it was incorporated into multiple positions on the product where cytosine occurred, and **(2)** using γ-32P-labelled ATP to radio-label the ARMS primer by means of T4 polynucleotide kinase, in which case each molecule of PCR product contained only one label.

3.6.2.1 USING α-32P RADIOLABELLED DCTP

ARMS PCRs were performed along the general principles of PCR described in Section 2.1.5, and similar cycling conditions to the corresponding non-radiolabelled reactions were used. The major difference is a reduced concentration of non-radiolabelled dCTP in the reaction mix from 200 μM to 20 μM, and 0.5 μl of α-32P-dCTP (3000 Ci/mmol), or 5 μCi was added to each reaction. Concentration of all other dNTPs was 200 μM. As explained in Section 3.3.3, 10% glycerol was routinely included in these reactions to improve specificity. To avoid excessive handling of radioactive material, the entire 50 μl of PCR product was mixed with 10 μl of 6X



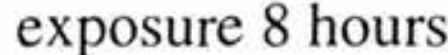
LANES	1	2	3	4	5	6	7	8	9	10
DNA	H_2O	H_2O	WT	M	WT	M	WT	M	WT	M
Nesting	S	F	S	S	F	F	S	S	F	F

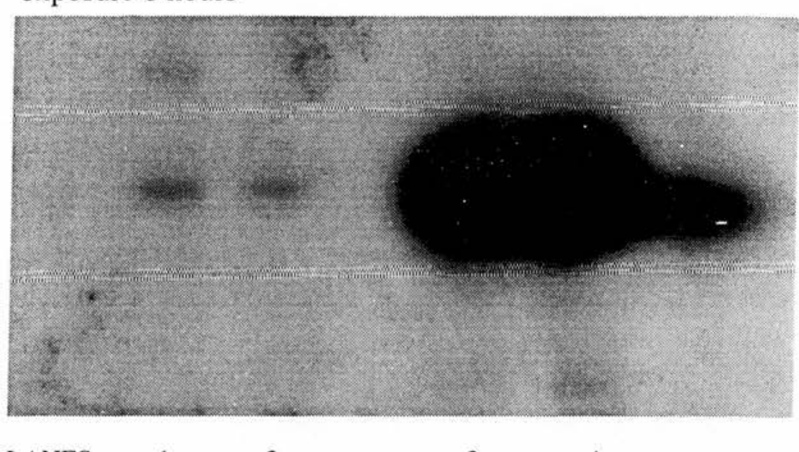
Fig 3.12 Effect of nesting on T47D ARMS (Section 3.6.1). ARMS using WT and tumour DNA were performed both with a semi-nested (S) system giving a 127bp product and a fully nested system (F) giving a 100bp product. Outer nest PCRs were performed with fragment E primers, and the outer nest products were either subject to inner nest PCR immediately (lanes 3-6) or diluted in water 1:500 first (lanes 7-10). PCR nesting results in loss of specificity of ARMS.

< 127bp product
<< 100bp product
H_2O water control
Lane in far left is φX174/HaeIII molecular weight marker

loading buffer, and 6 µl of the mixture loaded onto each lane of a 8% mini polyacrylamide gel and electrophoresed in a Biorad mini tank for approximately 1 hr. The gel was placed on blotting paper, wrapped in cling film and exposed to Kodak X-omat film in a cassette with intensifying screen for 1 to 16 hr at -70°C using counts from a hand-held Geiger counter as a guide to exposure time. In the T47D system, while ARMS was specific with Ta of 64°C and [Mg] of 1.75 mM when the ethidium bromide stained gel was viewed under UV light, with 32P-labelled PCR a product was readily visible when WT DNA was amplified. Increasing Ta to 67°C significantly improved specificity, exposure of X-ray film to the gel for 1 hr 20 min produced no product with WT template. However, a WT PCR product was evident on exposure for 8 hr (Fig. 3.13). This high-lights the fact that the heart of ARMS systems used in this project is the distinction of a PCR product with a mutant template over and above that with WT, ie, a difference in the efficiencies of the two reactions, and hence when radio-labelled PCR products are visualised, it is important to adjust the exposure time to X-ray film (largely empirically) so as to maximise sensitivity of the system.

Two main drawbacks of using 32P were recognised. **(1)** Previously optimised conditions based on ethidium bromide staining may no longer be optimal, and cycling conditions may need to be re-adjusted, as demonstrated here with T47D. **(2)** 32P-labelling of PCR products performed as described was much less reproducible than non-radioactive reactions, and repeated optimisation experiments often yielded inconsistent results, so much so that some of the systems here became unworkable. This was the case with the T47D model system. Although optimising 32P-labelled ARMS appeared relatively straightforward, dilution experiments to determine the new level of detection using the same set of DNAs in Section 3.5.2 gave inconsistent results, showing loss of specificity, poor amplification with T47D DNA, and irregular amplifications in the series of dilutions. The situation was not remedied by repeated experiments, taking care to ensure correct pipetting and mixing, and inevitably the assessment had to be abandoned.

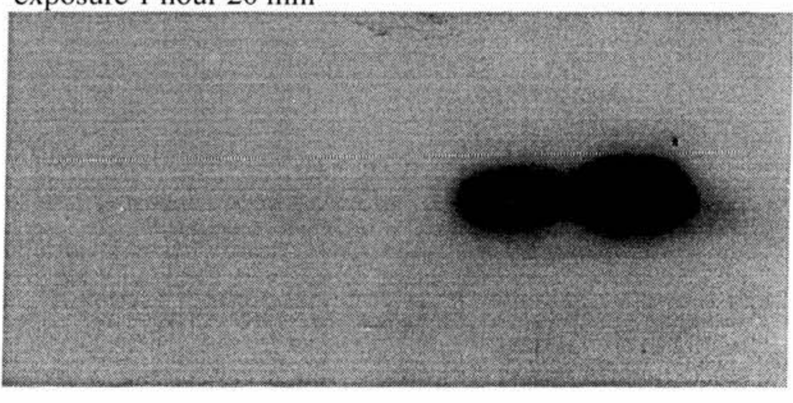




a

LANES	1	2	3	4
DNA	WT	WT	M	M

exposure 1 hour 20 min



b

LANES	1	2	3	4
DNA	WT	WT	M	M

Fig. 3.13 a & b T47D ARMS using 32P radiolabelled dCTP. ARMS is specific for mutant DNA but some products are made with WT DNA which become visible on prolonged exposure of X-ray film (8 hours). These are not visible when exposure is 1 hour 20 min.

3.6.2.2 USING γ-32P RADIOLABELLED ARMS PRIMER

An alternative method of radiolabelling PCR products by 32P-labelling the ARMS primer was attempted. A reason for using this approach was that each molecule of primer, and hence PCR product, was labelled only once, whilst in PCR using radiolabelled dCTP multiple radiolabelled nucleotides were inserted into each molecule of product, and depended on the efficiency of this incorporation and the relative competition with unlabelled dCTP in the reaction mix. The labelled primer approach could, therefore, produce a more uniform signal and more consistent results. This involved 2 separate steps: **(1)** radiolabelling the ARMS primer, and **(2)** the PCR itself. Reagents for step 1 are as follows:

γ-32P-ATP (3000 Ci/mmol)

T4 polynucleotide kinase (10 U/μl)

10X kinase buffer with 700 mM Tris-HCl (pH 7.6), 100 mM $MgCl_2$, 50 mM DTT

T47D-ARMS1 primer (14.5 pmol/μl from manufacturer)

Labelling was performed as follows. Each 10 μl of labelling mix contained:

T47D-ARMS1 primer	5.17 μl	(total 75 pmol)
T4 polynucleotide kinase (5 U/μl)	1 μl	(total 5 U)
10X kinase buffer	1 μl	
32P-ATP	1 μl	(total 10 μCi)
H_2O	1.83 μl	
Total	10 μl	

Volume of T47D-ARMS1 primer was calculated from the weight per μl and concentration, based on 1 μg of primer per 10 μl labelling mix. T4 kinase was diluted 1 in 2 with water before use. The labelling mix was incubated at 37°C for 20 min and heated to 95°C for 5 min to inactivate the enzyme. After brief centrifugation, aliquots of the labelled primer was used in PCRs which were otherwise performed exactly as the unlabelled reactions. A volume of 1.66 μl of T47D-ARMS1 primer was used per 50 μl reaction, equivalent to the usual quantity of 12.5 pmol. Because of the lower signal intensity from each molecule of PCR product, the entire product was mixed

with 6X loading buffer and electrophoresed in a standard 1.5 mm thick 6% polyacrylamide gel. Autoradiography was as in the preceeding section.

In multiple labelling experiments, this approach unfortunately suffered from the same inconsistency and lack of reproducibility with the radiolabelled dCTP approach, if not more so, and as each ARMS primer was radiolabelled, background signals were produced from occasional spurious products and unused primers. This approach also had to be abandoned.

3.7 RESULTS OF ARMS OPTIMISATION AND SENSITIVITY IN CLINICAL SAMPLES

3.7.1 ARMS IN BREAST CANCER SAMPLES

Base on the conclusions of Section 3.3, attempts were made to systematically optimise ARMS PCR's for those breast tumours found to contain a point mutation in p53 (Chapter 2). Of 51 primary breast tumours, 13 (25.5%) were found to contain mutations in p53. In 2 of these, repeated sequencing was unable to yield a sequence in the DNA fragment concerned different from the WT. Of the remaining 11 tumours, 2 had an identical missense mutation, CGG > CAG in codon 248. Hence there were 10 ARMS systems to be optimised, all involving a different single base missense mutation (Table 2.7).

Optimising these ARMS systems proved to be much more difficult in practice than in theory. Three of them were not able to be optimised (patients B33, B46, B50). Essentially, amplification with WT and mutant DNA templates behaved similarly and the ARMS system was unable to differentiate between the two with a single base difference. ARMS primers for patients B46 and B50 both involved E-NGC as the opposite primer, and close scrutiny of the GC content and Tm's of these primers suggests that they are similar and should otherwise be well-matched. Also, the second mismatch deliberately introduced was a C:T mismatch in each case and, being fairly high on the Newton and Graham (1994) ranking of destabilising mismatches, should make WT DNA refractory to amplification. There is also no obvious complementarity between the primers to enable primer-dimer formation. The ARMS system for patient B33 involved the use of primers (3757/94-ARMS1 and C-NB) with moderately different Tm's (60.9°C versus 49.6°C) and GC content (56% versus 45%). There is a run of 6 bases 5' in C-NB, plus 5 isolated bases downstream that are complementary to 3757/94-ARMS1, and anealing of these primers could potentially occur during PCR, and might have contributed to the failure of optimisation. This is indicated below, with the complementary bases in bold.

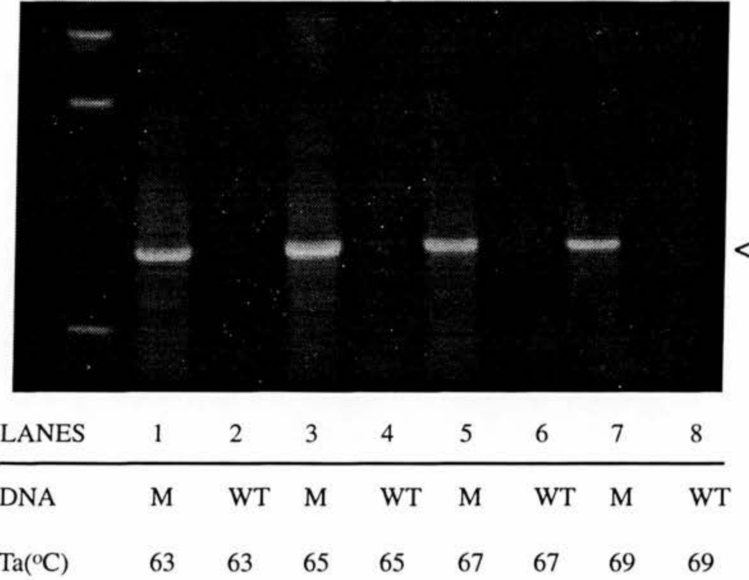
C-NB: 3'-A **C** AT **C** A **AC** AT **C** A **CCT ACC** AC-5'

3757/94-ARMS1: 5'-GT **G** AT **G** A **TG** GT **G** A **GGATGG** GCCTGT-3'

Of the remaining 7 samples, only 5 could be reasonably optimised to be of use in detecting minority mutant DNA species. Optimisation of the other two, patients B45 (primers 1232/94-ARMS1 and B-NGC) and B27 (primers 7071/90-ARMS1 and E-Bio), was difficult, and WT and mutant PCRs could not be differentiated sufficiently, such that when specificity was optimised, PCR product from the mutant template was too weak to be of use in MRD detection. The mutation involved in patient B27 was subsequently shown to be a germ-line mutation (Chapter 4), which made it unsuitable as an MRD marker. This mutation was found in all tissues, malignant or normal, from this patient.

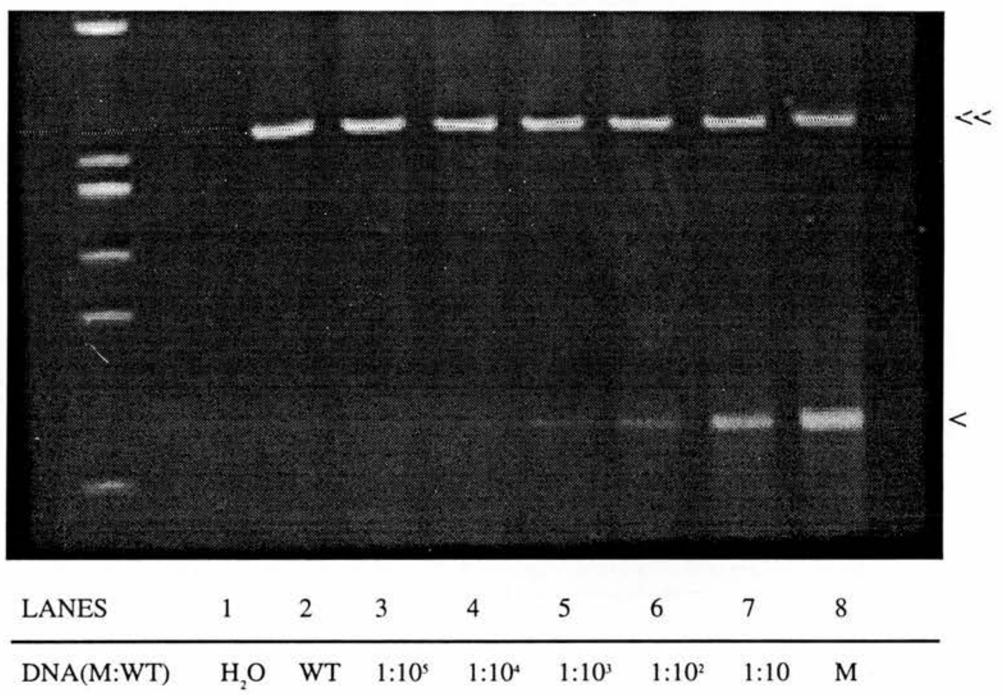
There remained patients B3, B11, B16, B30 and B37 in whom the ARMS systems were optimised, and could be used to screen PBPCH's. In the account below, the ARMS systems are identified by the name of the ARMS primer used in each case (see Table 3.1).

(1) Patient B11 (HB-ARMS1 system). The ARMS system for this patient was probably the most satisfactorily optimised. Using [Mg] of 1.5 mM, only the Ta needed to be adjusted. Specificity was achieved at 63°C, but it could be increased to 67°C without compromising the intensity of the mutant PCR product. Therefore, the optimised conditions were annealing at 67°C and [Mg] of 1.5 mM, amplified in a total of 35 cycles (Fig. 3.14). Tumour DNA was then spiked in WT control DNA in serial dilutions to determine the detection limit of this system (Fig. 3.15). With repeated experiments, it was shown that 1 part of tumour DNA in 10^3 parts of WT DNA could be reliably detected using ethidium bromide staining and UV light viewing of the gel. AAT control PCR indicated that no inhibitory factors were present. It was noted that the opposing primer used in PCR with HB-ARMS1 was B-GC, which is the GC-clamped primer used for initial amplification in DGGE. This primer was a 60-mer with the 40 bases 5' made up entirely of guanine and cytosine residues. As a result, the overall GC content of B-GC was much higher than HB-ARMS1, and the Tm was at



LANES	1	2	3	4	5	6	7	8
DNA	M	WT	M	WT	M	WT	M	WT
Ta(°C)	63	63	65	65	67	67	69	69

Fig. 3.14 Patient B11 ARMS optimisation of annealing temperature (Ta) (Section 3.7.1). Each pair of lanes represents PCR with mutant (M) and WT DNA respectively. ARMS is efficient at a wide range of Ta. 67°C was chosen in this project.
< 140bp ARMS product
Lane on far left is φX174/HaeIII molecular weight marker



LANES	1	2	3	4	5	6	7	8
DNA(M:WT)	H_2O	WT	$1:10^5$	$1:10^4$	$1:10^3$	$1:10^2$	1:10	M

Fig. 3.15 Patient B11 ARMS detection limit dilution experiment (Section 3.7.1). ARMS product visible only down to 1 in 10^3.

< 140bp ARMS product

<< 360bp AAT control product

H_2O water control

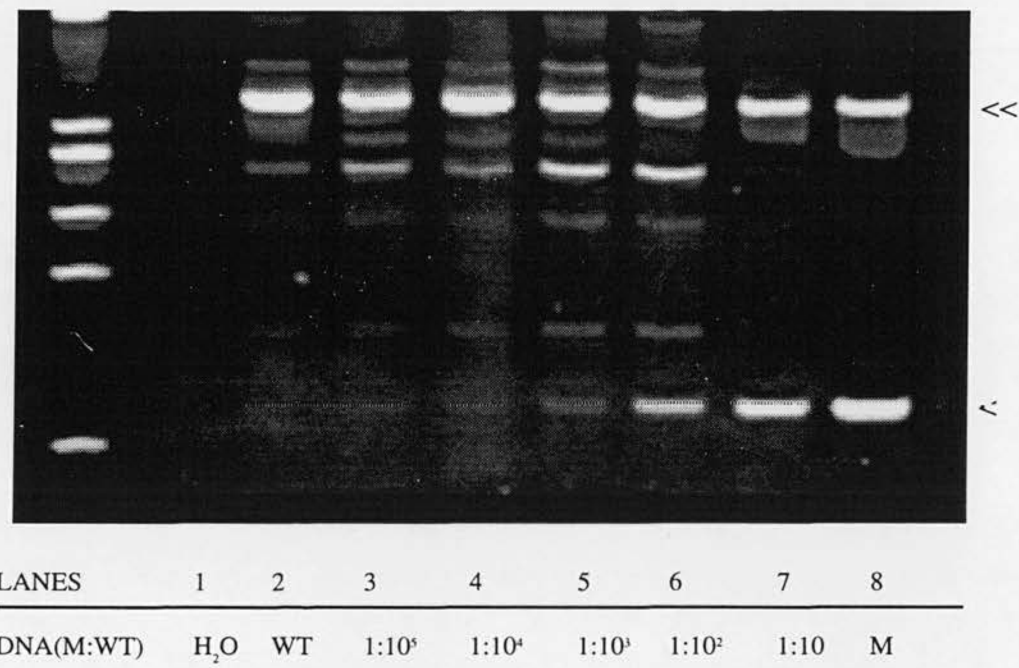
Lane in far left is ϕX174/HaeIII molecular weight marker

least 30°C higher. The ARMS system using this pair of primers worked surprisingly well, and paradoxically when the 20 bp equivalent of primer B-GC without the GC-rich region (B-NGC) was used instead, PCR efficiency was much reduced.

(2) Patient B16 (LB-ARMS1 system). This system was also relatively easily optimised. With [Mg] of 2.0 mM and Ta of 64°C, 35 cycles of PCR achieved a high specificity. Fig. 3.16 indicates the detection limit was also approximately 1 in 10^3 when tumour DNA was serially diluted in WT DNA.

(3) Patient B3 (BD-ARMS1 system). Optimal conditions were achieved with a Ta of 64°C and [Mg] was 2.0 mM (Fig. 3.17). Dilution experiments, however, indicated failure of detection beyond 1 in 100, which was surprising, as optimisation was straightforward and the mutant product appeared relatively intense. Judging by the above results, the sensitivity would have been expected to be at least another log higher. Sampling error would be the most likely cause for this, despite considerable care in ensuring DNAs were thoroughly mixed (Fig. 3.18).

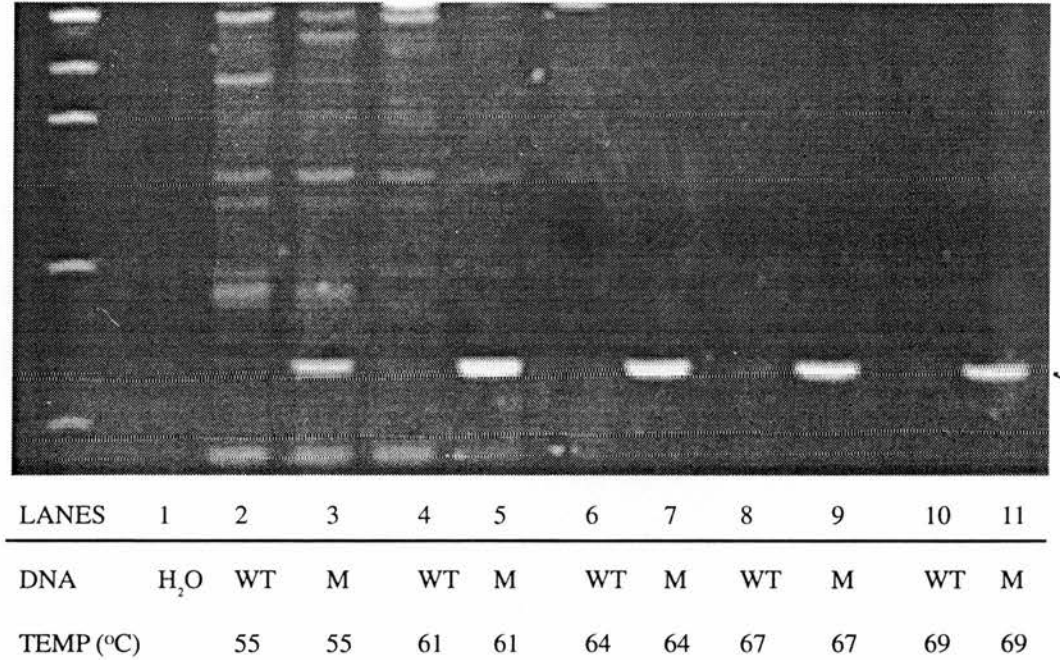
(4) Patient B37 (2477/96-ARMS1 system). Optimisation was more difficult. The highest tolerated Ta was 69°C whilst [Mg] was 2.0 mM. When [Mg] was lowered, specificity did not improve sufficiently before the mutant DNA product became too weak to be visualised. Hence, with a compromised set of conditions at Ta 69°C and [Mg] 1.75 mM, cycle number titration was performed (Fig. 3.19) There was a sharp drop in intensity of the mutant product at 33 cycles or lower, but the WT was no longer visible. Therefore, with the above Ta and [Mg], 33 cycles were optimal. Alternatives were tried, by titrating the [dNTP] and [primers], but satisfactory optimisation could not be achieved. This patient, as patient B11, had a point mutation in fragment B (exon 5) of p53. The ARMS primers used for both had 65% GC content and a Tm of 57.8°C. The second mismatch introduced was C:T for both, and the 3' mismatch was G:T for B11 and A:C for B37. As the GC-clamped B-GC primer worked well with primer HB-ARMS1, this was substituted for B-NGC with 2477/96-ARMS1 for patient B37. Unfortunately, unlike the B11 system, this pair of primers



LANES	1	2	3	4	5	6	7	8
DNA(M:WT)	H_2O	WT	$1:10^5$	$1:10^4$	$1:10^3$	$1:10^2$	1:10	M

Fig. 3.16 Patient B16 ARMS detection limit dilution experiment (Section 3.7.1). ARMS product visible only down to 1 in 10^3.

< 130bp ARMS product
<< 360bp AAT control product
H_2O water control
Lane in far left is ϕX174/HaeIII molecular weight marker



LANES	1	2	3	4	5	6	7	8	9	10	11
DNA	H_2O	WT	M	WT	M	WT	M	WT	M	WT	M
TEMP (°C)		55	55	61	61	64	64	67	67	69	69

Fig. 3.17 Patient B3 ARMS annealing temperature titration (section 3.7.1). Ta of 64°C or higher is optimal.

< 87bp ARMS product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

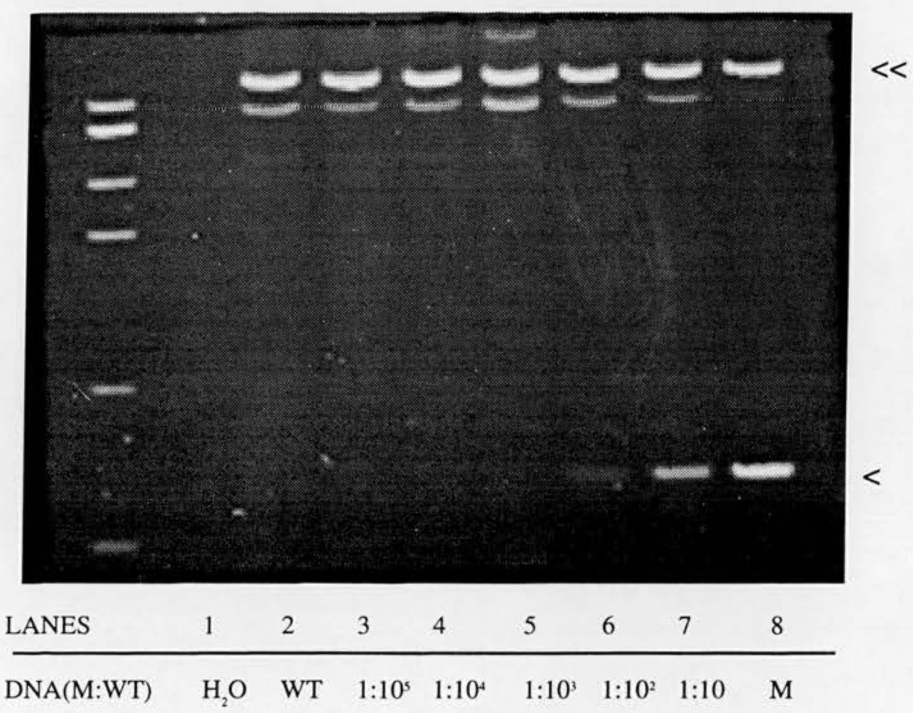


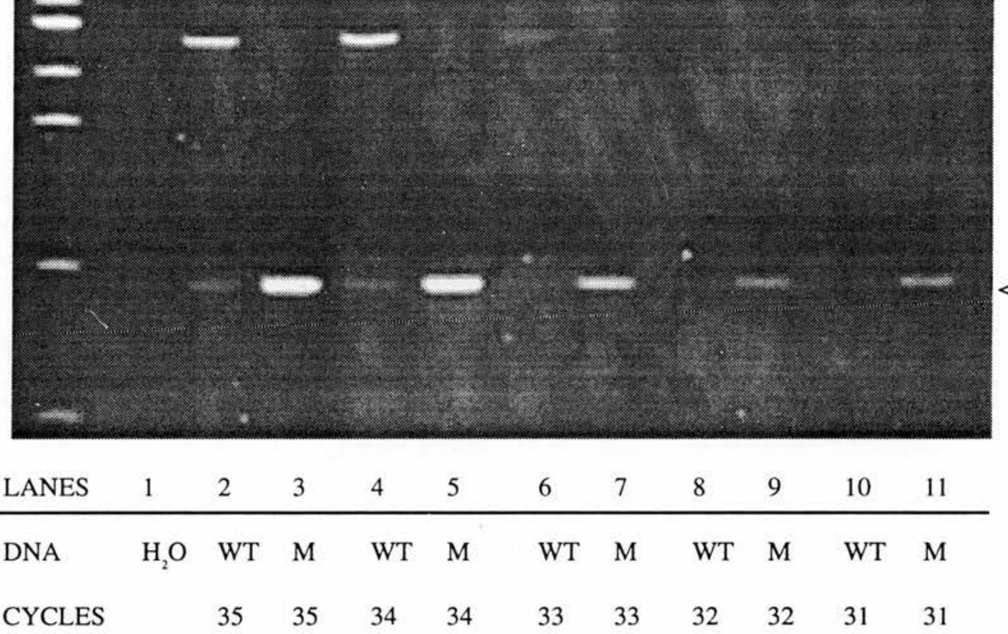
Fig. 3.18 Patient B3 ARMS detection limit dilution experiment (Section 3.7.1). ARMS product visible only down to 1 in 10^2.

< 127bp ARMS product

<< 360bp AAT control product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker



LANES	1	2	3	4	5	6	7	8	9	10	11
DNA	H_2O	WT	M	WT	M	WT	M	WT	M	WT	M
CYCLES		35	35	34	34	33	33	32	32	31	31

Fig. 3.19 Patient B37 ARMS cycle number optimisation (Section 3.7.1). ARMS is specific with 33 cycles of PCR. At 34 or more cycles, a product is seen with WT DNA template, and specificity is lost.

< 112bp ARMS product

H_2O water control

Lane in far left is ϕX174/HaeIII molecular weight marker

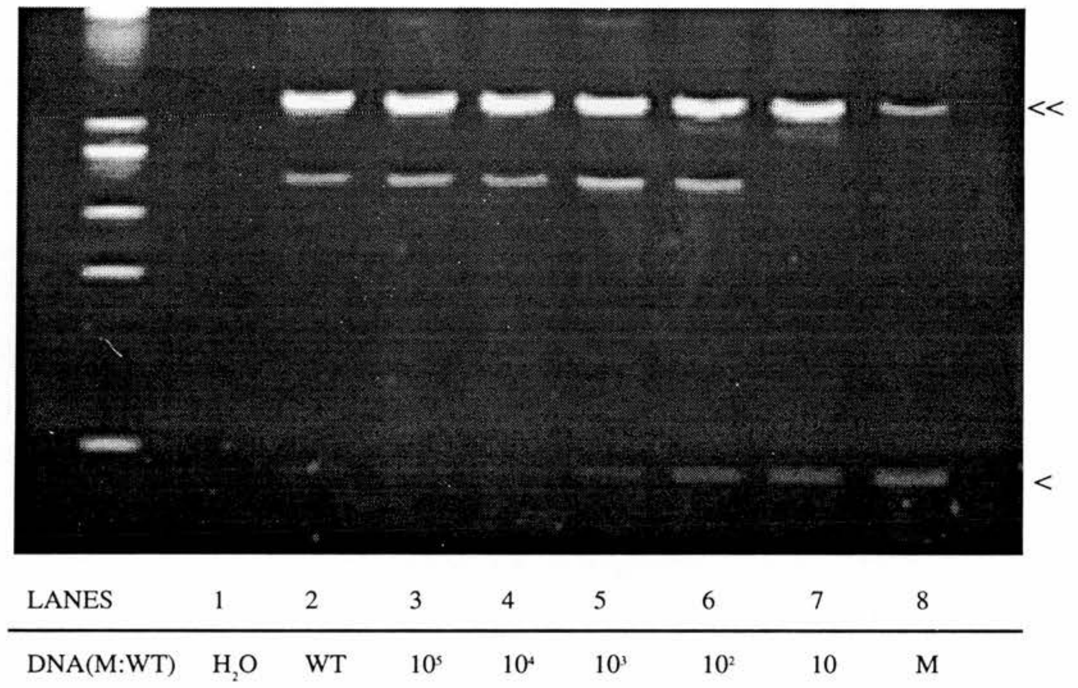


Fig. 3.20 Patient B37 ARMS detection limit dilution experiment (Section 3.7.1). ARMS product visible only down to 1 in 10^3.

< 112bp ARMS product

<< 360bp AAT control product

H_2O water control

Lane on far left is φX174/HaeIII molecular weight marker.

did not produce efficient amplification across a wide range of Ta's, and was abandoned. Serial dilution experiments indicated a detection limit of $1:10^3$ for this ARMS system (Fig. 3.20).

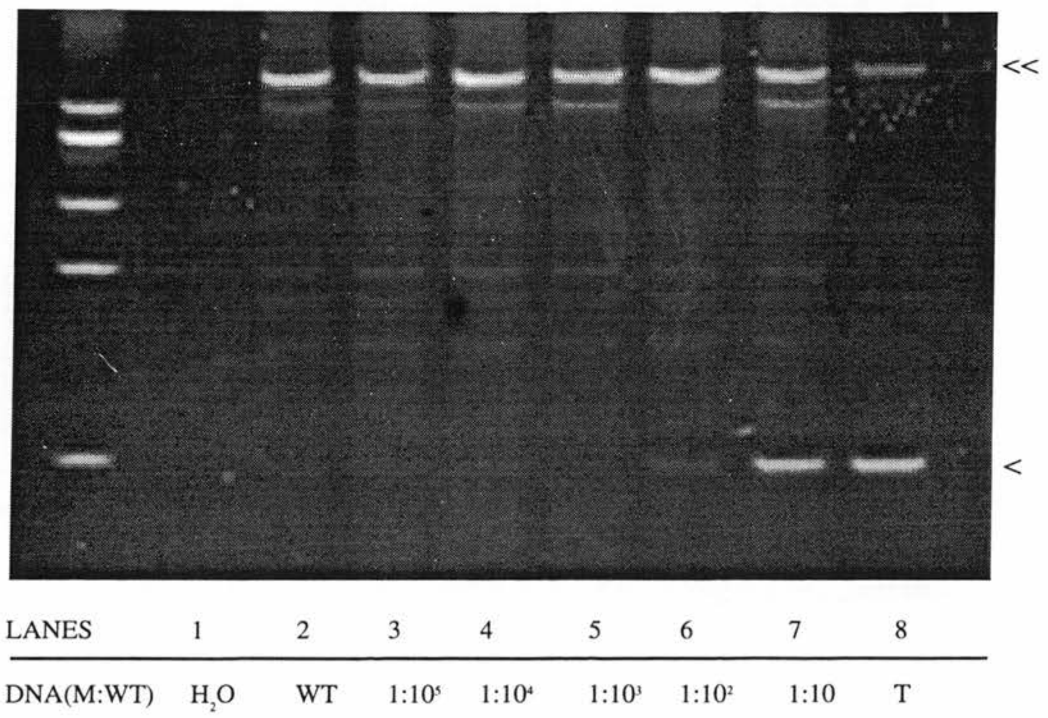
(5) Patient B30 (2668/96-ARMS1 system). The optimal Ta was much lower than the rest, at 56°C, [Mg] was 2.0 mM and cycle number was 35 in total. Disappointingly, DNA spiking and dilution experiments suggested a detection limit of only 1:100 for this system (Fig. 3.21).

Table 3.2 lists the optimal conditions achieved for all the ARMS systems assessed in the breast cancer patients, regardless of the intensity of the signal produced with a mutant template.

For patient B45, in whom optimisation was not possible because ARMS using a primer with a deliberately mismatched penultimate base was unable to differentiate WT or mutant DNA, another primer (1232/94-ARMS2) involving 2 instead of 1 additional deliberate mismatches was attempted in order to make ARMS with a WT template more refractory. In this situation, this primer was mismatched to a WT template at all of the last 3 bases, whilst fully matched to mutant at the last base but mismatched at the next two. Fig 3.22 confirms that this approach did improve specificity even at low Ta's, but not surprisingly, products given with a mutant template were too weak on ethidium bromide fluorescence to be of value in MRD detection. Furthermore, many spurious products were also made. Further attempts at using such a mismatching approach in other tumours were, therefore, not made.

3.7.2 USE OF 32P-LABELLED PCR IN THE 5 OPTIMISED SYSTEMS

The following observations were made with the 5 optimised ARMS systems described above. For patient B16, the original optimised conditions (Ta 64°C, [Mg] 2.0 mM) remained optimal, as was the case with patient B11 (Ta 67°C, [Mg] 1.5 mM). For patient B3, Ta was increased to maintain specificity (Ta 67°C, [Mg] 2.0 mM). In addition, all had 10% glycerol added. For patient B37, although lowering [Mg] from 1.75 mM to 1.5 mM in initial experiments improved specificity, repeated experiments



LANES	1	2	3	4	5	6	7	8
DNA(M:WT)	H_2O	WT	$1:10^5$	$1:10^4$	$1:10^3$	$1:10^2$	1:10	T

Fig. 3.21 Patient B30 ARMS detection limit dilution experiment (Section 3.7.1). ARMS product visible only down to 1 in 10^2.

< 115bp ARMS product

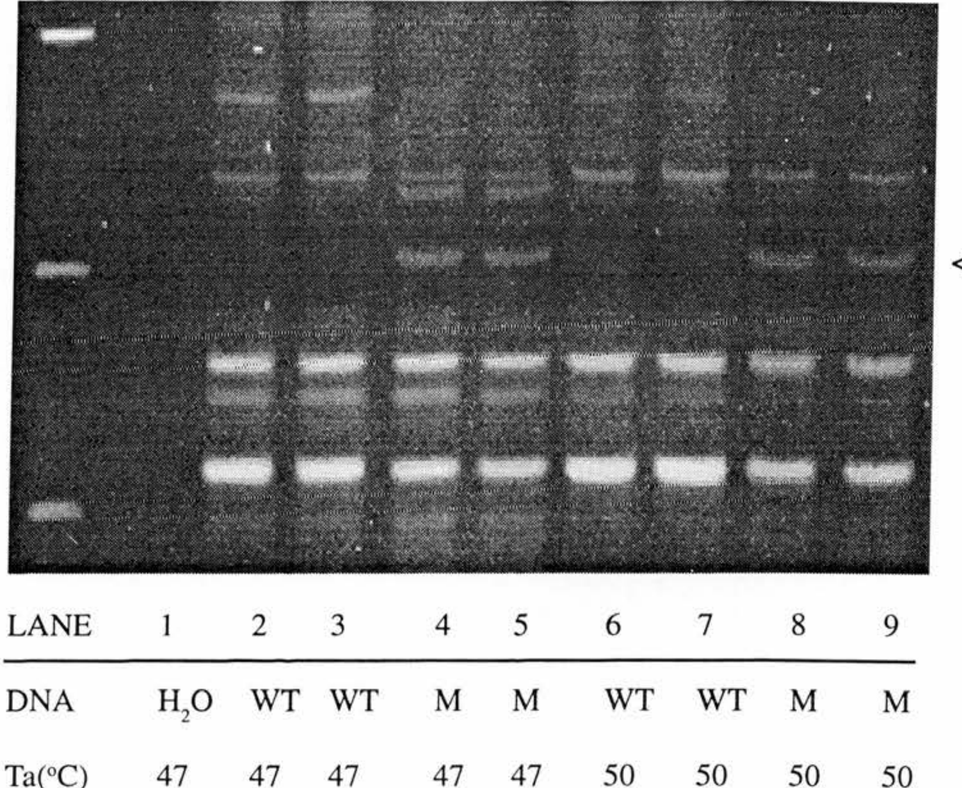
<< 360bp AAT control product

H_2O water control

Lane on far left is φX174/Hae molecular weight marker

Table 3.2 List of ARMS primers characteristics and optimised conditions of each system.

Patient	Primer Name	%GC	Tm (deg C)	3' mismatch	2nd mismatch	Other primer	%GC	Tm (deg C)	Product size	Ta (deg C)	[Mg] mM	Cycle no
Cell line	T47D-ARMS1	60	55.8	T - G	A - A	E-Bio	54.5	56.6	127 bp	64	1.75	35
Cell line	T47D-ARMS2	60	55.8	T - G	n/a	E-Bio	54.5	56.6	127 bp	72	1.75	35
Cell line	5637-ARMS1	61.9	58.1	C - C	C - T	D-NGC	55	53.7	104 bp	64	2	35
B3	BD-ARMS1	65	57.8	A - G	A - A	B-NGC	75	61.9	87 bp	64	2	35
B11	HB-ARMS1	65	57.8	G - T	C - T	B-GC	90	n/a	140 bp	67	1.5	35
B16	LB-ARMS1	70	59.9	G - G	C - C	E-Bio	54.5	56.6	130 bp	64	2	35
B27	7071/90-ARMS1	38.1	48.4	A - C	A - G	D-NGC	55	53.7	143 bp	60	2	35
B30	2668/96-ARMS1	45	49.6	T - C	C - C	E-Bio	54.5	56.6	115 bp	56	2	35
B33	3757/94-ARMS1	56	60.9	G - T	G - G	C-NB	45	49.6	78 bp	Failed to optimise		
B37	2477/96-ARMS1	65	57.8	A - C	C - T	B-NGC	75	61.9	112 bp	69	1.75	33
B45	1232/94-ARMS1	50	51.7	T - G	C - C	D-NGC	55	53.7	125 bp	60	1.75	35
B46	6342/93-ARMS1	65	57.8	A - G	C - T	E-NGC	60	55.8	170 bp	Failed to optimise		
B50	6098/97-ARMS1	65	57.8	G - T	T - C	E-NGC	60	55.8	170 bp	Failed to optimise		



LANE	1	2	3	4	5	6	7	8	9
DNA	H_2O	WT	WT	M	M	WT	WT	M	M
Ta(°C)	47	47	47	47	47	50	50	50	50

Fig. 3.22 Patient B45 ARMS using a primer (1232/94-ARMS2) which involves 2 additional deliberate mismatches at the penultimate positions. The primer is mismatched to WT DNA at the two penultimate bases. PCRs were performed in duplicates at 2 different annealing temperatures (47°C and 50°C). The reactions are specific for mutant DNA, giving a 125bp product, but the intensity of the products is too weak and many spurious products are also obtained.

< 125bp ARMS product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

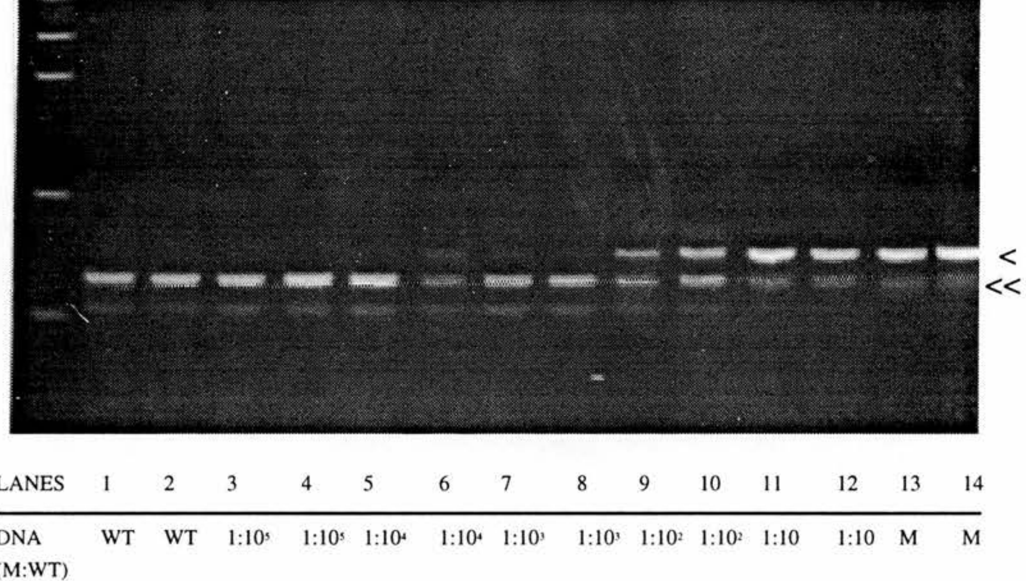
gave inconsistent results and the system could not in the end be optimised. Inconsistent results upon repeated experiments were also obtained for patient B30, and this again had to be abandoned.

3.7.3 ARMS IN ACUTE LEUKAEMIA SAMPLE / CASE REPORT OF PATIENT L11

As indicated in the previous chapter, the incidence of p53 mutations in this cohort of acute leukaemia patients was disappointingly low. The incidence of over 30% in relapsed disease (Zhu et al, 1996) was not confirmed here. Apart from 2 patients with consititutional silent polymorphism in codon 213, only one patient was found to contain a somatic mutation in fragment D (exon 8) of p53. Interestingly, this patient had previously been studied in our department by IgH gene and TCRσ gene rearrangement studies and was demonstrated to have 2 separate leukaemic clones assuming different importance during his disease (Langlands et al, 1993). This 16 year old male presented with anaemia, fundal haemorrhages and hepatosplenomegaly, and was found to have a white blood cell count of 151 x 10^9 /l. TdT, HLA class II and CD19 positive pre-B cell ALL was diagnosed. A t(4;11) translocation was detected. Remission was achieved with induction chemotherapy, after which PBPC were collected. A second collection of PBPC was made after intensification therapy. Relapse occured soon after, treated with re-induction chemotherapy, followed again by a third PBPC collection. High dose chemotherapy ensued with PBPC transplantation. All 3 collections were represented in the reinfusion. Interleukin 2 (IL2) was used to enhance graft-versus-leukaemia effect, but despite all the treatment, early relapse was observed and he subsequently died.

IgH and TCRσ gene rearrangement studies demonstrated 2 separate clones at presentation and at relapse. Radiolabelled probes corresponding to each of these clones were designed and used to screen a selection of samples collected during the course of his disease. Clone A was found at presentation, first remission marrow and the first and second PBPC collections. Despite first relapse, this clone was not found in any subsequent sample. Clone B, however, produced an intense signal at final relapse, but also present in all the samples screened, including marrow at diagnosis,

albeit less intensely, indicating its presence as a minority clone which became resistant to therapy, developed into the main clone and caused death of the patient. The p53 data in this study showed the presence of the 8 bp insertion at final relapse (Section 2.7). After searching 2 large p53 databases (Hollstein et al, 1994; Beroud and Soussi, http://perso.curie.fr/thierry.soussi/p53_database.html), it appeared that this insertion had not previously been reported. An ARMS primer (2007-ARMS1) was designed and used in PCR to screen earlier marrow and PBPC samples. Primer design is described in Section 3.2. Because of the highly specific nature of this insertion and of the resulting ARMS primer sequence, no specific optimisation of PCR was required. Standard conditions with Ta of 55°C and [Mg] of 1.5 mM were used. The opposite primer was D-Bio, and a 94 bp product was produced with a mutant DNA template. However, a spurious product slightly smaller than the ARMS product was produced with WT DNA, but this did not interfere with the use of this system as the 94 bp product was highly specific for mutant DNA sequence. Serial dilutions of leukaemia DNA collected at final relapse (marrow containing 75% blast cells) in WT control DNA demonstrated the limit of detection to be of the order of 1 leukaemia cell in up to 10^4 normal cells (Fig. 3.23).



LANES	1	2	3	4	5	6	7	8	9	10	11	12	13	14
DNA (M:WT)	WT	WT	$1:10^5$	$1:10^5$	$1:10^4$	$1:10^4$	$1:10^3$	$1:10^3$	$1:10^2$	$1:10^2$	1:10	1:10	M	M

Fig. 3.23 Patient 11 ARMS detection limit dilution experiments (Section 3.7.3). DNA from leukaemia at relapse (75% marrow blasts) spiked in WT DNA. Detection limit is of the order of 1 part leukaemia DNA in 10^4 parts WT DNA.

< 94bp ARMS product

<< spurious product serves as internal control

Lane on far left is φX174/HaeIII molecular weight marker.

3.8 MRD DETECTION IN BREAST CANCER PATIENTS

Section 3.7.1 described the optimisation of ARMS for 5 patients. Using these systems, a total of 12 PBPCH's were screened for the presence of MRD. PBPCH's were available in the form of cell pellets aliquoted at the time of collection and stored at -70°C. Each pellet was from PBPCH collected on consecutive days. Genomic DNA was extracted from each pellet by the Qiagen spin column technique described in Section 2.1.3. For all MRD screening of PBPCH's, 1 μg of DNA was amplified by ARMS in triplicates each in a 50 μl reaction mix. Separately, control PCRs were performed on each DNA sample using AAT gene exon 3 primers (Section 3.5.1). Two microlitres of the AAT control PCR product was mixed with 20 μl of the ARMS product and 4 μl of 6X loading buffer, loaded onto each lane of a 1.5 mm 6% polyacrylamide gel, electrophoresed at constant 45 mA for approximately 1 hr 15 min, stained with ethidium bromide, destained and viewed under UV light (Section 2.1.4). Below is a description of the results for each patient in detail, which are summarised in Table 3.3. Three of 5 patients and 8/12 PBPCHs demonstrated MRD.

(1) Patient B11 (HB-ARMS1 system). Cell pellets from 3 PBPCH's were available from this patient for study. ARMS PCRs demonstrated that, albeit faint, a product was produced from each harvest in all triplicate reactions, whilst WT control remained unequivocally negative (Fig. 3.24). AAT products were present in all samples. Therefore, there is evidence of tumour cell contamination in all 3 PBPCH's from this patient. α-32P-labelled ARMS reactions using newly optimised conditions confirmed the result. Exposure of the polyacrylamide gels over-night showed clear products with each of the 3 harvests but not with WT (Fig. 3.25).
Clinical Details: Age 41 years at diagnosis. Poorly differentiated breast adenocarcinoma was diagnosed in October 1992, and treated by right mastectomy. Many positive axillary nodes were found on both sides. Nodules were found on the chest wall, around mastectomy scar and in the liver in November 1992. Stage T2N2M1. She then received 11 courses of adriamycin and 5-flucytosine, associated with grade 3 mucositis. PBPC mobilisation was performed after treatment with high-

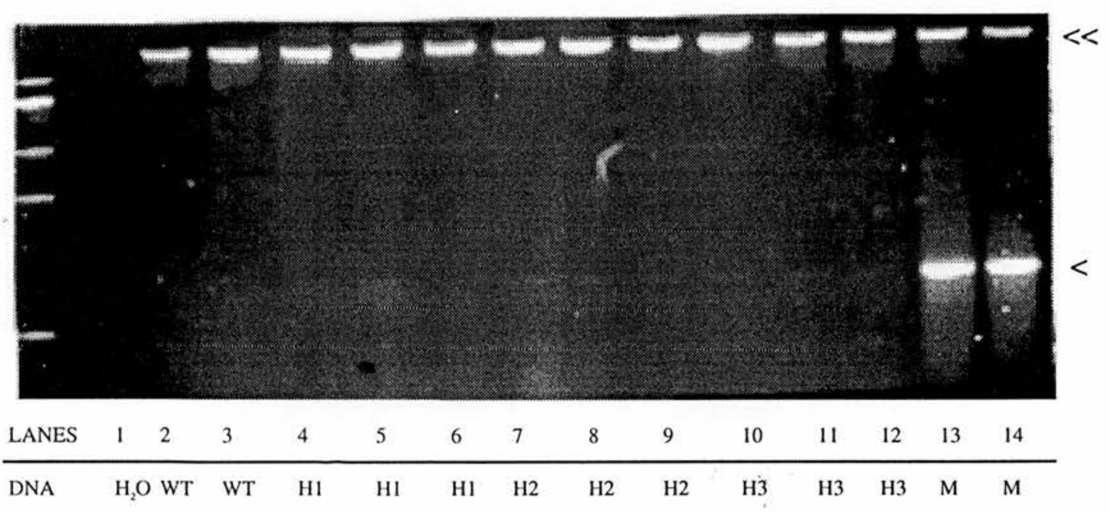


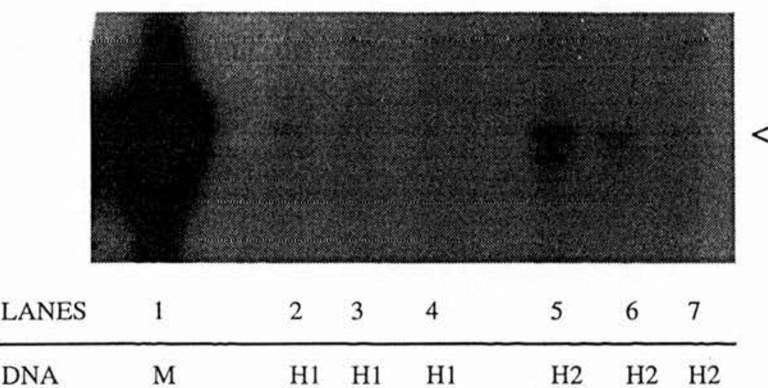
Fig. 3.24 Patient B11 ARMS screening of PBPC harvest (section 3.8). Faint bands across all 3 PBPC harvests in triplicates indicate the presence of MRD.

< 140bp ARMS product

<< 360bp AAT control product

H_2O water control

Lane in far left is ϕX174/HaeIII molecular weight marker



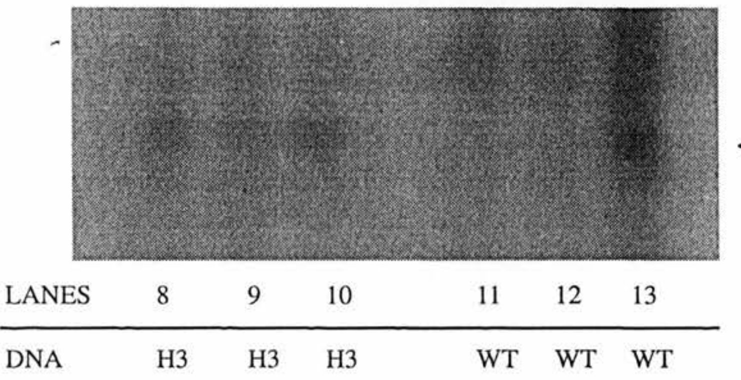


FIG. 3.25 Screening of PBPC harvests (H1, H2, H3) from patient B11 using 32P radiolabelled ARMS. A strong signal is produced with tumour DNA (M) and weak signals from each of 3 harvests in triplicates. One of 3 reactions using WT DNA produced a product (lane 13) but overloading of the well may have contributed.

< 140bp ARMS product

dose cyclophosphamide and G-CSF, following which she was conditioned with high-dose etopide and melphalan and received PBPC rescue in April 1993. Consolidative radiotherapy was given in June 1993. By January 1994, a cerebral metastasis was diagnosed, causing left hemiparesis and disturbance of balance. This was surgically excised and she was given cranial radiotherapy. In February 1994, further metastatic deposits were found in the thoracic spine in the ribs, both treated with radiotherapy, and CMF chemotherapy with cyclophosphamide, methotrexate and fluorouracil. Further spinal and liver metastases developed and jaudice and confusion ensued, leading rapidly to death. Disease-free survival was 9 months.

(2) Patient B16 (LB-ARMS1 system). 3 PBPCH's were studied, and at the detection limit possible, no evidence of MRD was detected (Figure 3.26). Again this finding was confirmed with α-32P-labelled ARMS. With this latter technique, ARMS appeared very specific, and on prolonged exposure to X-ray film, no product was visible with WT DNA or any of the 3 PBPCH's (results not shown).
Clinical Details: Age 31 at diagnosis. T2N0M0, oestrogen receptor negative right breast carcinoma was diagnosed in November 1992 and treated with wide local excision and axillary node sampling which was negative. She was given radical radiotherapy post-operatively. A right axillary nodule developed in December 1993 and axillary node clearance was carried out, showing one positive node out of 12. Six courses of cyclophosphamide, adriamycin and fluorouracil were given from March to June 1994 with good response. This was followed by PBPCH and transplantation in July 1994, which achieved complete remission. On follow-up in January 1998, she was still well and remained in CR. At the time of this follow-up, disease-free survival was 3.5 years.

(3) Patient B3 (BD-ARMS1 system). 3 PBPCH's were studied. Despite screening these harvests with ARMS at a higher Ta (67°C) than optimal (64°C), repeated experiments showed that 2 of 3 reactions using WT DNA had very faint products visible. However, all triplicate samples of all 3 harvests had slightly more intense products visible, especially one of 3 reactions with Harvest 1 and Harvest 3 (Fig.

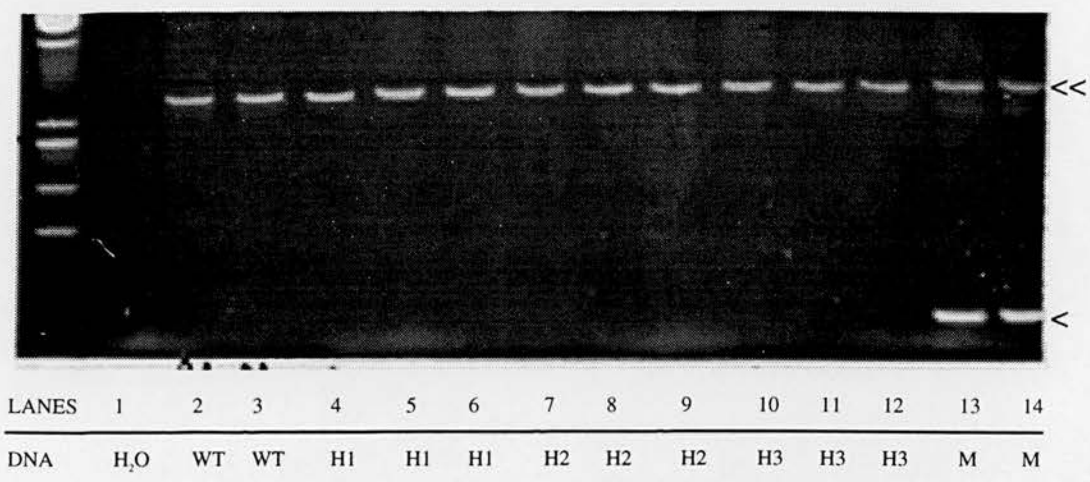


Fig. 3.26 Patient B16 ARMS screening of PBPC harvests (section 3.8). No evidence of MRD is seen in all 3 harvests in triplicates.

< 130bp ARMS product

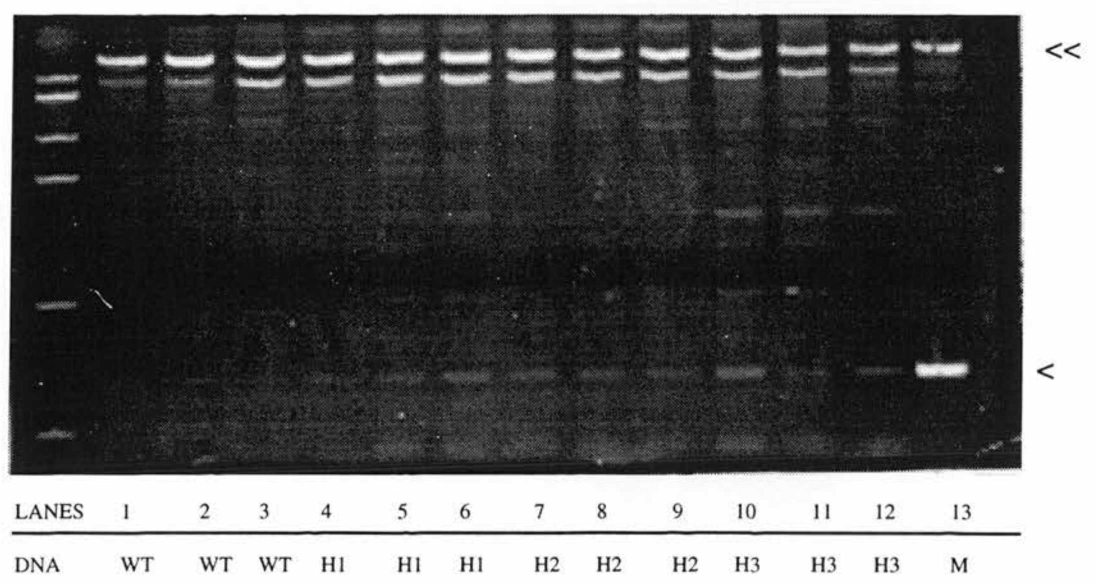
<< 360bp AAT control product

H_20 water control

Lane in far left is ϕX174/HaeIII molecular weight marker

3.27, lanes 6 and 10). α-32P-labelled reactions were less ambiguous regarding specificity of ARMS - all 3 WT reactions were clearly negative. With prolonged overnight exposure, one of 3 reactions from each of the 3 harvests yielded a clear product. This suggests that all 3 harvests may contain MRD, although at such a low level as to be present in only one of 3 samples (total 3 μg DNA) by random chance (Fig. 3.28).
Clinical Details: Age 34 at diagnosis. A right breast carcinoma was treated in June 1988 with mastectomy and axillary node clearance followed by tamoxifen. Stage T2N0M0. In July 1989 metastases were found in the right axilla and she received bilateral oophorectomy and radiotherapy to the right chest wall and shoulder area. A hot spot was noted on bone scan in January 1992 for which radiotherapy was given. By October 1992, widespread metastases were clinically noted in the liver, skin and axilla. Eleven courses of adriamycin and fluorouracil were given, and in February 1993, PBPC mobilisation was performed with cyclophosphamide and G-CSF. PBPC transplant was performed in April 1993 following high-dose chemotherapy conditioning with etoposide and melphalan. Maintainance with Megace was started in May 1993. A complete remission was achieved after transplant, but by October, she had relapsed with liver metastases. Three courses of CMF chemotherapy were given, but her disease progressed, leading to her death in December 1993. Disease-free survival after transplant was 6 months.

(4) Patient B37 (2477/96-ARMS1 system). Two PBPCH's were available. Using optimised conditions, both harvests produced weak products with ARMS in triplicates, detected with ethidium bromide (Fig. 3.29). It would appear that this patient also had tumour contamination of both harvests. Unfortunately, radiolabelled ARMS was unable to be optimised to confirm this.
Clinical Details: Age 34 years at diagnosis. She was found in February 1996 to have a 5 cm right breast mass, and wide local excision and axillary node clearance confirmed oestrogen receptor negative carcinoma. Clinical stage was T2N0M0. Six of 11 axillary nodes were positive with visible lymphatic invasion. She fulfilled the criteria for and was randomised on the ACCOG study to subsequently receive high dose therapy and PBPCT. She was first treated with 4 courses of adriamycin, to June 1996.



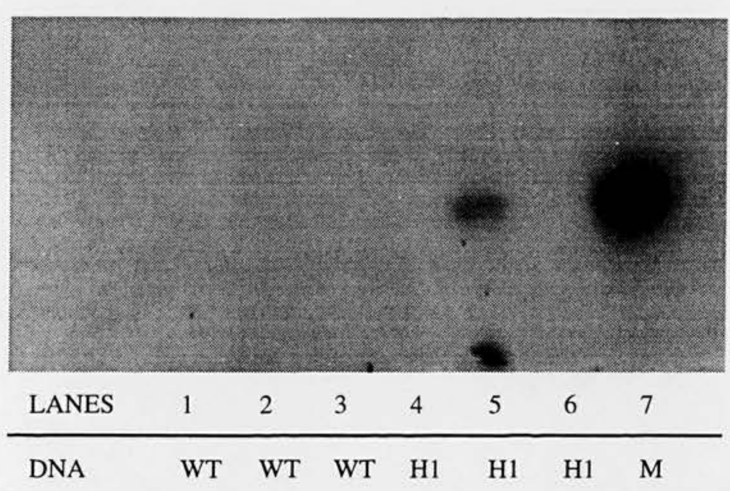
LANES	1	2	3	4	5	6	7	8	9	10	11	12	13
DNA	WT	WT	WT	H1	H1	H1	H2	H2	H2	H3	H3	H3	M

Fig. 3.27 Patient B3 ARMS screening of PBPCHs (H1, H2, H3) (section 3.8). Unexpectedly, despite prior optimisation, very faint hint of a product was seen in 2 of 3 WT DNA.

< 87bp ARMS product

<< 360bp AAT control product

Lane in far left is ϕX174/HaeIII molecular weight marker



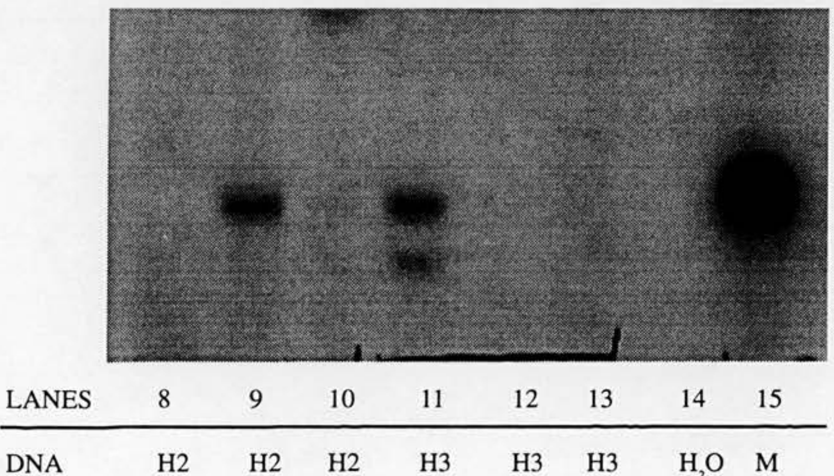
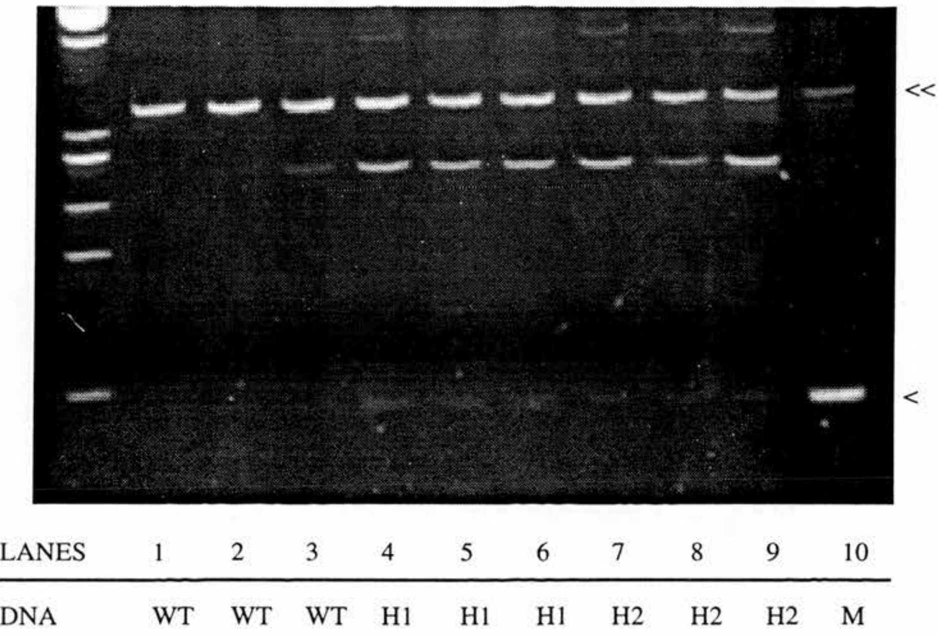


Fig. 3.28 Screening PBPCHs (H1, H2, H3) from patients B3 using 32P radiolabelled ARMS. A strong signal is produced with tumour DNA (M) and moderate signals in one of each set of triplicates from the 3 harvests. No signal is seen with WT DNA.



LANES	1	2	3	4	5	6	7	8	9	10
DNA	WT	WT	WT	H1	H1	H1	H2	H2	H2	M

Fig. 3.29 Patient B37 ARMS screening of PBPCHs (H1, H2)(Section 3.8). A faint product corresponding to the presence of MRD is seen in triplicates in each harvest.

< 112bp ARMS product

<< 360bp AAT control product

Lane in far left is ϕX174/HaeIII molecular weight marker

The following month, PBPC were successfully mobilised with cyclophosphamide and G-CSF. High dose chemotherapy with cyclophosphamide and thiotepa followed, with PBPC rescue. Consolidative radiotherapy was given to the chest wall in August 1996. Over a year later, in September 1997, routine mammogram showed micro-calcification in the right breast and mastectomy was carried out. She had remained in CR on follow-up in February 1998. However, in May 1998, disease relapse was observed on the skin, on the chest wall within a previously irradiated area. Outlook was therefore extremely poor, and palliative chemotherapy was offered in the form of infusional fluorouracil and vinorelbine. Disease-free survival after transplant was 14 months.

(5) Patient B30 (2668/96-ARMS1 system). Only one harvest was performed in this patient, and it appeared that this was unequivocally negative for MRD as detected by this optimised ARMS system. Again, radiolabelled ARMS could not be optimised to confirm this finding.
Clinical Details: Age 35 years at diagnosis. She presented in March 1996 with a large right breast mass confirmed on fine needle aspiration to be malignant. She underwent wide local excision and axillary node clearance which showed that 8 of 28 nodes were positive and there was extensive replacement by malignant cells. A mastectomy was performed. Clinical stage was T2N0M0. She was treated on the ACCOG study and after 4 courses of induction chemotherapy with adriamycin between May and August 1996, cyclophosphamide and G-CSF mobilisation of PBPC was carried out in September 1996, followed immediately by high dose cyclophosphamide and thiotepa conditioning and PBPCT. Consolidative radiotherapy was also given between December 1996 and January 1997 and initial follow-up confirmed CR. In March 1998, she presented with 3 weeks of cough and breathlessness. Chest X-ray showed diffuse extensive mottling in mid and lower lung fields, suggestive of lymphangitis. Liver function tests were also found at the same time to be abnormal, with the liver palpable 4 finger-breadths below the costal margin. The clinical picture was of extensive disease relapse, and histological confirmation was not possible because of

her poor clinical condition. She died rapidly following this event. Disease-free survival after transplant was 16 months.

Table 3.3 Summary of the screening of PBPCH's of the 5 patients described using ARMS. The detection sensitivity of each system is indicated. The numbers against each PBPCH for each patient represent the number of positive reactions out of triplicate tests.

	Patient B11	Patient B16	Patient B3	Patient 37	Patient B30
MRD detection sensitivity	1:1000	1:1000	1:100	1:1000	1:100
PBPCH 1	3	0	1	3	0
PBPCH 2	3	0	1	3	--
PBPCH 3	3	0	1	--	--

3.9 MRD DETECTION IN ACUTE LEUKAEMIA PATIENT

For patient L11 described in Section 3.7.3, primer 2007-ARMS1 was used to screen BM and PBPCH's taken at various time points during his disease (Fig. 3.30). At final relapse just prior to death, the BM contained 75% blast cells, and ARMS produced an intense 94 bp specific product associated with this clone. This signal was also noted in the BM 2 weeks previously when the sample was unsatisfactory for morphology, but relapse was evident on the basis of this ARMS product, albeit less intense presumably reflecting a smaller population of blasts. A BM sample at first relapse several months previously also gave this product, but when BM at diagnosis was screened, only a very faint band was noted. Consistent with the IgH and TCRσ studies described above, the data here indicates that the chemoresistant leukaemic clone which led to death of the patient was present at diagnosis as a minority, but was responsible for the first and second relapses. Comparing the intensity of the ARMS product at diagnosis and the dilution experiment, the proportion of the resistent clone might be as low as 1 cell in 10^4. ARMS also indicated the presence of MRD in the third PBPCH and in BM subsequent to this procedure, although the first and second harvests appeared free of this clone (Figure 3.30). High dose therapy appeared to have eradicated detectable

leukaemia from the marrow temporarily as the post-PBPCT BM was negative for MRD. His subsequent relapse may therefore be due either to re-infused malignant cells during PBPCT or to residual disease in the bone marrow occuring in minute quantities beyond the detection limit of this ARMS system.

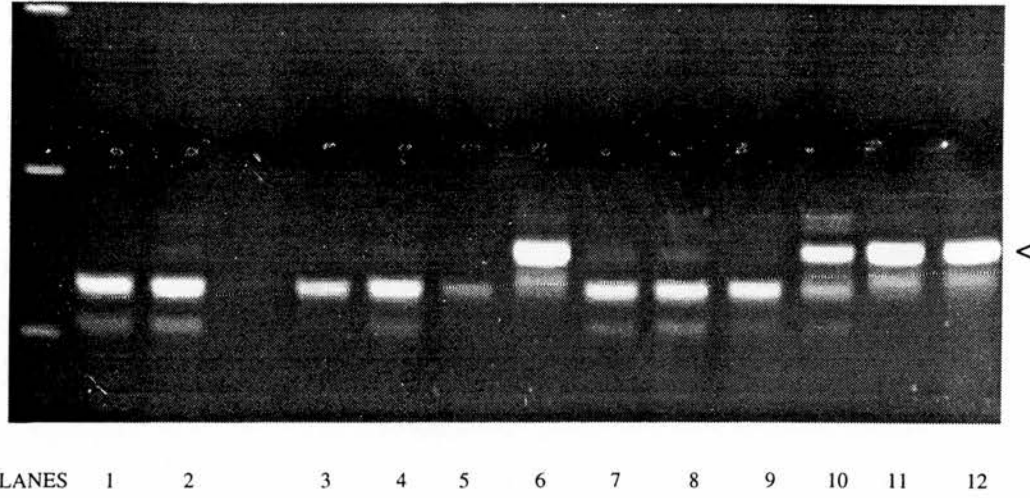


Fig. 3.30 Allele-specific PCR screening of index patient's samples. Note that a 94bp product is produced with mutant allele (<) and a slightly smaller spurious product produced with wild type allele. **lane 1**, wild type control DNA; **lane 2**, presentation peripheral blood; **lane 3**, first PBPCH; **lane 4**, bone marrow after induction chemotherapy; **lane 5**, second PBPCH; **lane 6**, first relapse marrow; **lane 7**, third PBPCH; **lane 8**, pre-PBPCT marrow; **lane 9**, immediately post-PBPC transplant marrow; **lane 10**, 4 weeks post-PBPCT marrow; **lanes 11 and 12**, 6 weeks post-PBPCT marrow (75% blasts). Evidence of minimal quantities of the mutant clone are detected in lanes 2,4,7 and 8, indicating the presence of this clone at diagnosis and in the 3rd PBPCH.

> 94bp PCR product

Lane in far left is ϕX174/HaeIII molecular weight marker

3.10 SUMMARY

This chapter has described my experience in optimising ARMS for 2 tumour cell lines, a cohort of breast cancers carrying point mutations in the p53 gene, and the one case of ALL with a novel 8 bp insertion. Used as potential markers in detecting minority clones, p53 mutations appears to be a relatively insensitive and unreliable marker. Cell line systems were easier to work with and produced more consistent results. It is clear that optimising a set of reaction conditions for ARMS is multi-factorial. The key parameters to be optimised as determined using the T47D cell line model are annealing temperature and magnesium concentration. Although altering most other conditions influence the amplification efficiency in ARMS, reactions with both WT and mutant DNA appear to be affected more or less equally. Also crucial is the use of an addtional deliberate mismatch near the 3' end of the primer which enhanced specificity substantially. The work-up for each individual point mutation suggests that optimisation of ARMS primers for such a heterogeneous system is very difficult, and here, only about half of the available systems could be optimised with a degree of satisfaction, using the guidelines in Section 3.3, albeit more difficult for some of them such as patient B37 where the cycle number had to be dropped to achieve specificity. Of 10 point mutations in this breast cancer cohort, 3 could not be optimised at all as the intensity of the mutant and WT products were too similar under all conditions. Of the remaining 7, 5 could be optimised with a degree of satisfaction. In the other two, ARMS was unable to distinguish between WT and mutant DNA sufficiently to enable the systems to be used for MRD detection - in each of these, the band produced with mutant DNA was too faint such that further dilutions with WT DNA in the MRD setting would make the system unworkable. One of these was subsequently found to be a germ-line mutation (see Chapter 4) which would, in any case, make it unsuitable as a tumour marker for MRD studies.

Detection limit is very poor by current standards using other markers, and although capable of studying the phenomenon of clonal evolution in certain malignancies, is unlikely to be useful in detecting MRD in tissues such as PBPCH's where the level of

contamination by tumour cells is often much lower. Using ethidium bromide staining of the polyacrylamide gels, the level of detection in the best ARMS systems is of the order of 1 cell in 10^4. This was achieved here with the 5637 cell line and a highly specific alteration like the insertion in patient L11. In most other systems, the limit is probably of the order of 1 in 100 to 10^3. 32P radiolabelled ARMS did not work consistently in this project, but results from 3 of the 5 optimised patients confirmed the findings of the non-radiolabelled assessment. It was not possible to demonstrate an improvement in detection sensitivity by using radiolabelled PCR.

Of the 5 optimised ARMS systems, 3 patients were in the 'metastatic' group (B3, B11, B16), and 2 given PBPCT as adjuvant therapy (B30, B37). Two of the metastatic group had evidence of MRD in all 3 PBPCH's (6 of 9 harvests, 66.7%), and one of the adjuvant (ACCOG) group had MRD in both harvests (2 of 3 harvests, 66.7%). It is notable that, on studying the clinical details of patient B16 in the metastatic group, she did not in fact have metastatic disease, but was considered to be at high risk of systemic relapse on the basis that she had axillary node relapse despite radical radiotherpy, and that her tumour was oestrogen receptor negative. She was also young and thought to be a good candidate for high dose therapy. Both patients who were transplanted because of known metastatic disease (B3, B11), had evidence of MRD in the PBSCH, and despite achieving CR, both had short DFS of 6 and 9 months, respectively. On the other hand both patients treated on the ACCOG study and given PBPCT as adjuvant therapy also had adverse outcomes despite achieving CR. Patient B37 had evidence of MRD in both harvests, and had DFS of 14 months, whilst patient B30 had no evidence of MRD in her single harvest, and had DFS of 17 months.

The situation with patient L11 is more straightforward, because of the relatively higher sensitivity of detection ($1{:}10^4$) and specificity, and that serial samples were available at various time points. This enabled the demonstration of the phenomenon of clonal evolution of the second (chemo-resistant) leukaemic clone, and its presence in 2 or the 3 different sets of PBPCH's albeit as a minority.

CHAPTER 4

RESTRICTION SITE-GENERATING POLYMERASE CHAIN REACTION (RG-PCR) APPROACH

4.1 INTRODUCTION

The ARMS approach described in Chapter 3 is based on allele-specific PCRs in which reactions with an ARMS primer are dependent on the higher efficiency of amplification using a mutant DNA template compared to a WT one. As noted, this relative difference in amplification efficiencies is variable and varies from one mutation to another. In this study, the ARMS systems for almost half of the mutations studied could either not be optimised at all or not sufficiently to be of any value in detecting MRD. Therefore, a lack of specificity and sensitivity are major drawbacks. For this reason, I attempted to examine a PCR approach in which a deliberate mismatch positioned in up to several bases 5' to the mutation is introduced during PCR by using a 'mutagenic' primer containing this mismatch, such that an artificial restriction enzyme recognition site is generated for the WT but not the mutant (tumour) sequence (Haliassos, et al, 1989a and b; Mullen et al, 1997; Ng et al, 1991, Gasparini et al, 1992). Because the mismatch introduced is not adjacent to the 3' end of the primer, strand extension is allowed at the same efficiency with WT and mutant templates. With this, a PCR product is made with both templates, to be distinguished from each other by the ability of a restriction enzyme to specifically recognise the unique sequence to allow digestion of the DNA. Therefore, 2 separate steps, PCR and restriction enzyme digestion, are required. The main advantages of this approach are that **(1)** a PCR product is always available and the issue is not of the presence or absence of a product, **(2)** unlike ARMS, no laborious optimisation is needed, and (3) the recognition sequence for a particular restriction enzyme is highly specific and is conceivably much more reliable than the relative non-specificity of ARMS.

4.2 DESIGN OF MUTAGENIC PRIMERS

A mutagenic primer introduces a recognition site by incoporating the 3' portion of its own sequence and the first couple of bases 3' to it in the template, where the base change in question is positioned. Two approaches are available. The mutagenic primer may introduce a recognition site in which the WT sequence is digested, leaving the mutant sequence intact, or it may introduce a recognition site for the mutant sequence, leaving the WT intact. Of these two, I chose the former approach. The reason for this is that it is dependent on the *disappearance* of a product (WT) with restriciton enzyme digestion, when any remaining product is mutant, while the latter approach is dependent on the *appearance* of one or more smaller products resulting from digestion of the mutant sequence, and can potentially suffer from the same problem as ARMS in that, in the MRD setting, the bands on PAGE gel may be so faint as to cause difficulty in determining if they are really present.

Mutagenic primers are designed according to the following principles:

(1) WT sequence around the point mutation is compared with recognition sequences of a list of restriction enzymes. If a recognition site is already lost as a result of the point mutation, no further mutagenic primer is required, and the appropriate enzyme may be used to digest PCR products of the exon amplified with primers described in Table 2.3;

(2) mutagenic primers are approximately 20 bp, stopping just short of the point mutation in question, such that the artificial recognition site generated encompasses the WT base at the position of the mutation;

(3) a mutagenic primer may stop just 1 base short of the mutation, but the 3' base of the primer should not be chosen to be the mismatch as this reduces amplification efficiency;

(4) not more than one mismatch is to be introduced if possible, as this may make amplification refractory;

(5) the DNA strand on which the primer is designed is chosen in order to produce a product of appropriate size when one of the exon primers are used in PCR.

Table 4.1 List of mutagenic primers used in RG-PCR.

Patient	Primer	Codon	Mutation	WT/Primer sequence	Restriction enzyme	Recognition site	Opposite primer	Product size	Cut fragments
Cell line	T47D-MP	194	CTT > TTT	5'-CAGCAT**C**TT-3'	MboI	^GATC	E-Bio	127 bp	WT - 111+16 bp
				5'-CAG**G**AT-3'					
B27	7071/91-MP	267	CGG > CAG	5'-CTGGGAC**G**G-3'	HpAII	C^CGG	D-NGC	143 bp	WT - 19+45+79 bp
				5'-CTGGG**C**C-3'					Mut - 64+79 bp
B45	1232/94-MP	273	CGT > TGT	5'-GAGGTG**C**GT-3'	HhAI	GCG^C	D-NGC	126 bp	WT - 20+32+74 bp
				5'-GAGG**C**G-3'					Mut - 52+74 bp
B46	6342/93-MP	216	GTG > TTG	5'-CACCACCA**C**ACT-3'	Bsp1286I	G(G/A/T)GC(C/A/T)^C	E-NGC	171 bp	WT - 151+20 bp
B50	6098/97-MP	216	GTG > ATG	5'-CAC**G**A**G**CA-3'					
B37	2477/96-MP	179	CAT > CGT	5'-CCCCACC**A**TGA-3'	Hsp92II	CATG^	Fragment B	131 bp	WT - 37+27+31+36 bp
				no mutagenic					Mut - 37+27+67 bp
				primer needed					
B33	3757/94-MP	248	CGG > CAG	5'-ATGAACC**G**G-3'	HpaII	C^CGG	Fragment C	89 bp	WT - 37+52 bp
B28	8375/91-MP	248	CGG > CAG	no mutagenic					
				primer needed					
B30	2668/96-MP	198	GAA > TAA	5'-CGAGTG**G**AA-3'	BsrSI	ACTGGN(1/-1)	E-Bio	116 bp	WT - 94+22 bp
				5'-CGA**C**TG-3'					
B11	HB-MP	175	CGC > CAC	5'-GTGAGGC**G**CT-3'	HaeII	(A/G)GCGC^(T/C)	Fragment B	131 bp	WT-82+18+31 bp
				no mutagenic					Mut - 100+31 bp
				primer needed					
B16	LB-MP	193	CAT > GAT	5'-CCTCAG**C**AT-3'	Bst71I	GCAGC(8/12)	E-Bio	131 bp	WT - 102+29 bp
				5'-CC**G**CAG-3'					
B3	BD-MP	171	GAG > TAG	5'-ATGACG**G**AG-3'	HpaII	C^CGG	B-Bio	84 bp	WT - 18+66 bp
				5'-ATG**C**CG-3'					

^ - restriction site, Mut - mutant, N - any nucleotide. For each, WT sequence is listed on top row and 3' end of primer on bottom row. High-lighted bases are positions of mutations in WT and deliberate changes in primers.

Table 4.1 lists the sequences of such primers for each point mutation in this project, the corresponding restriction enzyme used and its recognition sequence, and characteristics of the products and fragments resulting from digestion. All mutagenic primers are suffixed 'MP' after the respective sample identity. T47D again was used as the model system, and it can be seen that, for each of the mutations in question, it was possible to design a mutagenic primer for PCR, to be used with a restriction enzyme identified from a collection of scientific companies' catalogues. Three of the mutations naturally result in loss of recognition sites, and no mutagenic primers are required for these. Some of the systems contain more than one recognition site for the enzyme and generate more than one fragment.

4.3 USE OF RG-PCR IN THE T47D MODEL

The T47D system was used to assess if this approach would be feasible, and also to determine the detection limit of minority clones. The enzyme MboI recognises the sequence

- ^GATC -
- CTAG^ -

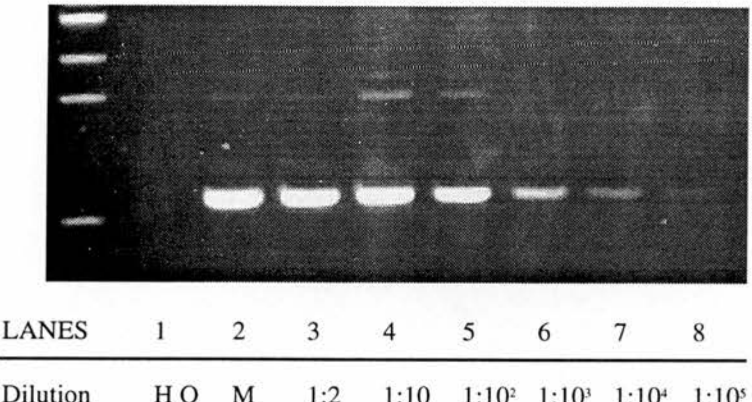
where ^ represents the restriction site. Incubations of PCR products with restriction enzymes were carried out along manufacturer's guidelines. In general, 20 μl of PCR product was mixed with 3 μl 10X enzyme buffer, 3 μl 1:10 diluted bovine serum albumin, 3 μl distilled water and 1 μl restriction enzyme (10 U/μl) (total 30 μl). The mixture was incubated at 37°C for a minimum of 3 hr, at the end of which time the mixture was heated to 60°C for 5 to 10 min prior to loading onto polyacrylamide gel for electrophoresis. For the size of the fragment concerned, 12.5% acrylamide was used to improve resolution.

The 127 bp PCR product from primers T47D-MP and E-Bio using WT DNA as template was digested to 111 bp and 16 bp fragments, easily visible on PAGE. If the enzyme-treated sample was subject to electrophoresis immediately, a weak residual product of 127 bp remained, indicating incomplete digestion. However, increasing the number of units of MboI per 30 μl digestion mix from 4 U to 12 U, or increasing incubation time (at 37°C) from 3.5 hr to over-night did not produce any improvement.

The problem was resolved when the digestion mix was heated to 60°C or higher for 5 to 10 min after digestion, just prior to loading and electrophoresis, and the most likely reason was that the staggered ends of the digestion fragments with a 4-base single strand had allowed the fragments to re-anneal. The MboI digestion is also specific, as no evidence of digestion of the product made with mutant DNA was observed, regardless of enzyme dose and time of incubation.

The most significant limitation of the RG-PCR technique is the lack of sensitivity when mutant DNA is dispersed among WT. Fig. 4.1 shows that the T47D-MP PCR reaction itself is efficient, and a product is visible when T47D DNA is diluted serially in distilled water down to $1{:}10^5$, or 10 pg of genomic DNA. However, when T47D DNA is likewise mixed in serial dilutions with WT DNA, amplified by PCR and digested with MboI, a product can barely be seen beyond 1 in 10 (100 ng DNA) (Fig. 4.2). This was confirmed with repeated experiments, and although sensitivity of other systems may be better (1 in 100 for patient B11), it would appear to be unsuitable for MRD detection. It is unclear what the cause of the discrepancy may be. Preferential amplification of WT DNA over mutant could account for it but as the mutated base is not involved in the annealing of the mutagenic primer onto template, this would seem unlikely to be the explanation.

A potential solution to the lack of sensitivity is to enrich the mutant DNA species from a mixed WT / mutant population by using a restriction enzyme to remove the WT species by cleavage, *followed* by PCR amplification, which should only amplify the mutant species (Pourzand and Cerutti, 1993; Kahn et al, 1991). An attempt was made with the T47D model and MboI enzyme. As described above, standard RG-PCR was performed on genomic T47D DNA using primers T47D-MP and E-Bio (Table 4.1). 20 µl of the PCR product was digested with 10 U of MboI, and an aliquot of 5 µl was reamplified with the same primers, and electrophoresed in a polyacrylamide gel. Fig. 4.3 shows the result of such second-round PCR reactions, using 2 different Ta's (64°C and 67°C) and for each, 2 different [Mg]. As in the attempts with nested ARMS PCR (Chapter 3), many spurious products were produced in all reactions, and disappointingly, a correct product (127 bp) was



LANES	1	2	3	4	5	6	7	8
Dilution	H_2O	M	1:2	1:10	$1{:}10^2$	$1{:}10^3$	$1{:}10^4$	$1{:}10^5$

Fig. 4.1 Efficiency of T47D RG-PCR. 1μg of T47D DNA was serially diluted in water and amplified by RG-PCR. A PCR product is visible down to $1{:}10^5$ (10pg) indicating a very efficient PCR system.

M neat tumour (mutant) DNA

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

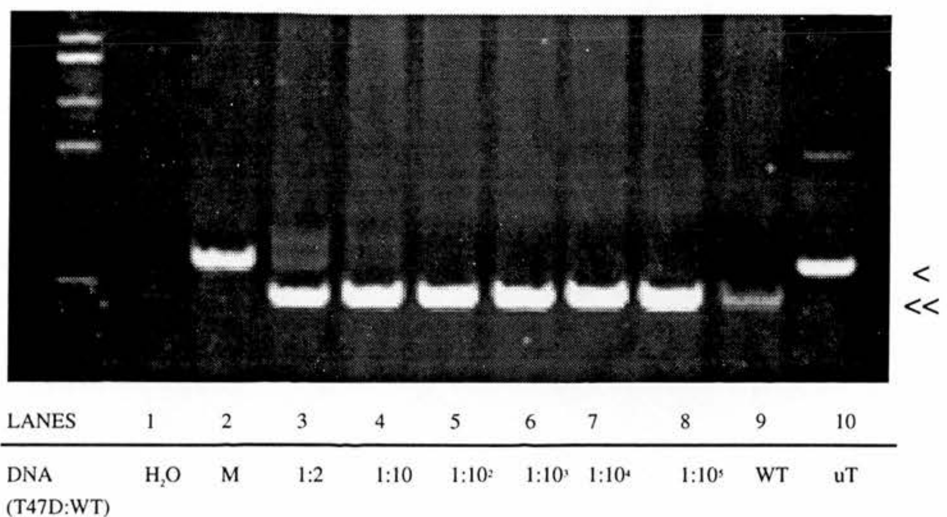


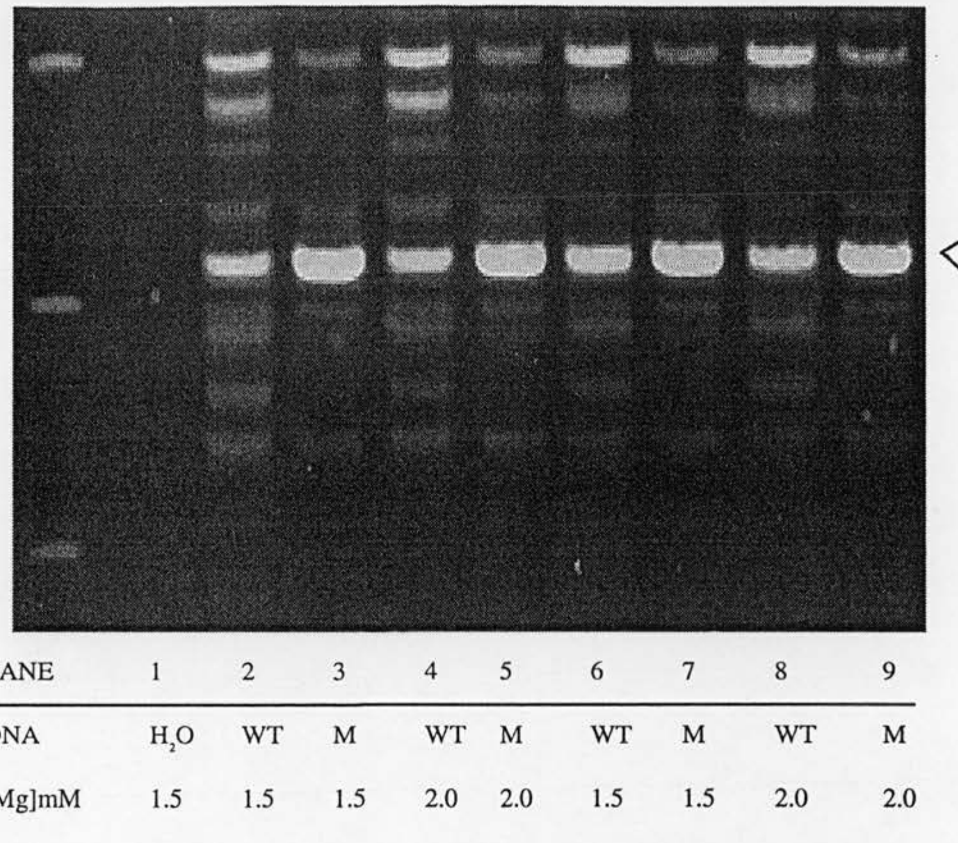
Fig. 4.2 RG-PCR detection limit (Section 4.3) using T47D cell line. PCR products in lanes 2 to 9 had been digested by MboI enzyme, lane 10 was untreated. A 127bp product with a mutant sequence would be uncut by MboI (<), whilst WT sequence would be cut to 16 + 111bp(<<). This experiment indicates that the RG-PCR approach is insensitive to the presence of tumour DNA which has been mixed with WT DNA and 1 tumour cell in 10 normal cells could hardly be detected.

< 127bp

<< 111bp

H_2O water control

Lane in far left is ϕX174/HaeIII molecular weight marker



LANE	1	2	3	4	5	6	7	8	9
DNA	H_2O	WT	M	WT	M	WT	M	WT	M
[Mg]mM	1.5	1.5	1.5	2.0	2.0	1.5	1.5	2.0	2.0
Ta(°C)	64	64	64	64	64	67	67	67	67

Fig. 4.3 Mutant enriched RG-PCR using T47D model. RG-PCR was performed using primers T47D-MP and E-Bio. An aliquot was digested with MboI enzyme and then subjected to the same RG-PCR for 35 cycles. This shows a lack of specificity as a visible 127bp product (<) is still obtained with WT template. Reactions were performed at two different [Mg] and Ta.

< 127bp ARMS product

H_2O water control

Lane in far left is φX174/HaeIII molecular weight marker

observed even with WT templates after digestion with MboI. The fact that a product was yielded with WT DNA template would suggest that the DNA was not completely digested with the restriction enzyme. Alternatively, the multi-step process of PCR - enzyme digestion - second PCR opened up considerable risk for contamination. It would be expected that 10 U of MboI should fully cleave the first round WT PCR product in 3 hr of incubation, and at least when visualised with ethidium bromide and UV light, 10 U of MboI appeared to be as effective as higher doses in cleaving WT PCR products in the one-step RG-PCR (work-up results not shown). Unfortunately, owing to time constraint, I was unable to further investigate the possibility of increasing the dose of enzyme for mutant enrichment or alternative strategies.

4.4 USE OF RG-PCR IN BREAST CANCER SAMPLES

Breast tumour DNAs from 5 patients (B3, B11, B27, B37, B45) were analysed by RG-PCR.

In 4/5 of these patients, RG-PCR as used in this manner lacked specificity as undigested product always resulted from digestion of WT PCR product. In these cases, higher enzyme dose or heating the digestion mix to 60°C did not produce any improvement. Fig. 4.4 shows the example of patient B11, whose ARMS system was optimised. RG-PCR was disappointing as a weak residual 131 bp product was visible on PAGE gel which made it unsuitable for MRD detection, which relies on 100% digestion of the WT. Fig 4.4 also indicates that despite an efficient PCR in amplifying the template, detection sensitivity is poor when mutant DNA is the minor population with WT.

The main value of RG-PCR used in this project is that it facilitated the detection of a germ-line mutation in patient B27. Previous sequencing detected a point mutation in codon 267 (CGG > CAG) which resulted in an arginine to glutamine amino acid change, but unfortunately, ARMS could not be adequately optimised for MRD detection. RG-PCR using a mutagenic primer generated 2 recognition sites on WT for enzyme HpaII but 1 site for the mutant sequence. The appearance of a 64 bp product on PAGE suggested the presence of mutant sequence (Fig. 4.5). Although such a

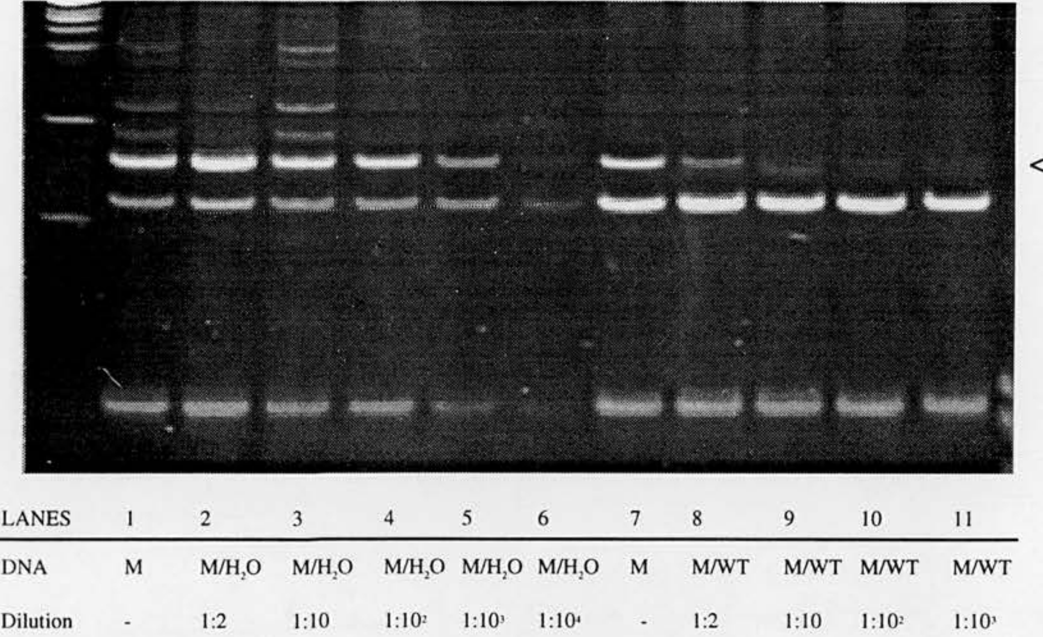


Fig. 4.4 Comparison of sensitivity of Patient B11 RG-PCR with detection limit using HpaII digestion (Section 4.4). All PCR reactions contained 1μg of DNA. With dilutions of tumour DNA in water, a 131bp PCR product can just be seen at $1:10^4$ (lanes 1 - 6). When tumour DNA was diluted in WT DNA and the product digested with HpaII, a digestion product can be reliably distinguished from WT control only down to dilution of 1:10 (lanes 7 - 11).

< 131bp ARMS product

Lane in far left is φX174/HaeIII molecular weight marker

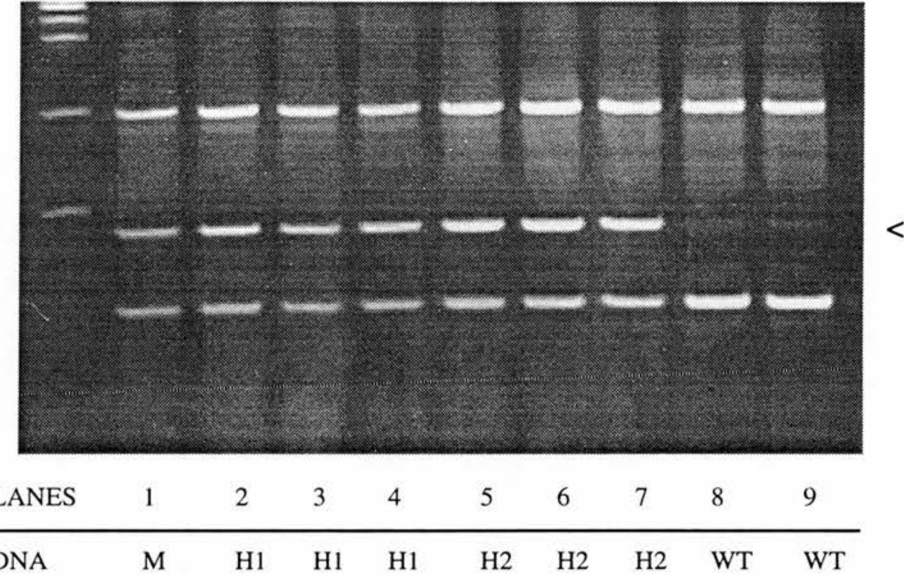


Fig. 4.5 Germline mutation in codon 267 (CGG>CAG) demonstrated by HpaII restriction enzyme digestion of primary tumour (M), PBPCHs (H1, H2) and WT control DNA (Section 4.4). After PCR using the 7071/91-MP mutagenic primer a mutant PCR product is characterised by the failure of HpaII digestion of a 64bp product (<) owing to loss of a recognition site.
Lane in far left is ϕX174/HaeIII molecular weight marker

band was also present as a weak band with WT control, when tumour DNA and DNA from each of the 2 PBPC harvests were studied, all samples showed a pattern indicative of mutant sequence. The intensity of the 64 bp band excludes tumour contamination being the cause, and a germ-line mutation was the most likely explanation. When DNA from these tissues were re-amplified and screened by DGGE, positive results were obtained (not shown), confirming that the mutation was in the germ-line. Of interest in this case is that a search of the EMBL p53 database revealed that a germline mutation in codon 267 has been reported only once, by Prosser et al (1991) of the MRC Human Genetics Unit, Edinburgh. They reported an identical mutation to that found here in patient B27, in a non-Li-Fraumeni cancer family. The proband was a 53 year-old woman with breast cancer. Four cancer deaths were reported in the 5-generation pedigree: breast cancer at age 53 (proband's maternal grandmother), breast cancer at age 67 (sister of grandmother), lung cancer at age 66 (proband's mother) and ovarian cancer at age 63. Materials were not available from these 4 relatives, but the same constitutional mutation was found in the proband's 37 year-old sister and a 74 year-old first cousin of the proband's mother, both of whom were unaffected by cancer. The authors argued that, owing to the late onset of cancers in members of this family, and the fact that the 74 year-old carrier was still cancer free, the mutation was a 'weak' one from the cell's point of view, not conferring a particularly strong growth advantage. A pedigree for patient B27 was kindly composed by Mrs. Alison Fordyce of the Registry Office, MRC Human Genetics Unit, Edinburgh (Fig. 4.6). This shows that B27 also was one of a breast cancer family. The 'weakness' or low penetrance of the codon 267 mutation was again demonstrated by the late onset of cancer; breast cancer deaths were recorded at the age of 63 (mother) and 87 (great-grandmother), the age characteristically becoming progressively younger with successive generations. Study of tumours from these individuals was beyond the scope of this project, although it would not be surprising to find these tissues also contain the same codon 267 mutation. Of even greater interest is that both patient B27's family and that described by Prosser et al (1991) originated from Fife, and with the rarity of codon 267 germ line mutations, the 2 families are likely to be related. However, in the composition of the pedigree, no

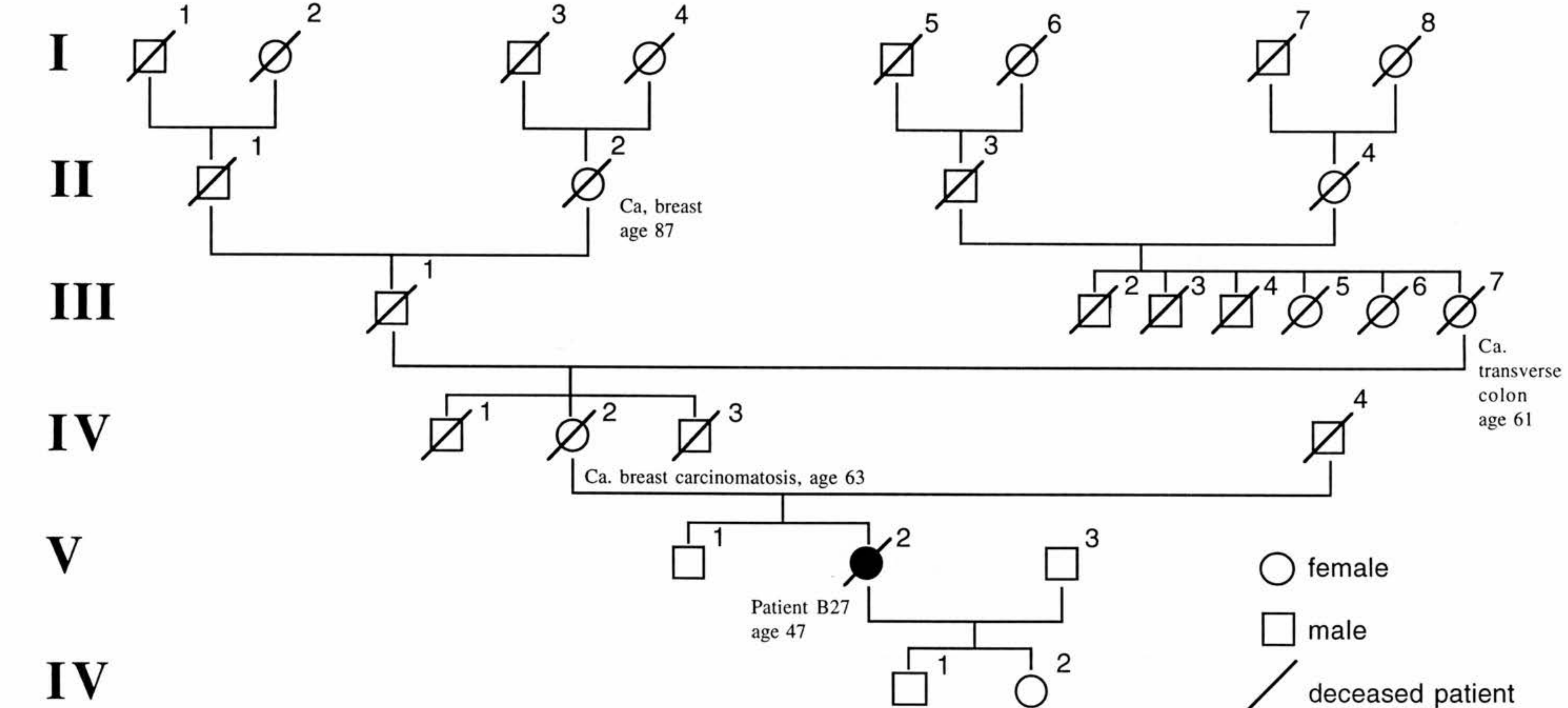


Fig. 4.6 Pedigree of patient B27 (V2) indicating the familial nature of breast cancer on the maternal side. Characteristically the age of the affected patients becomes progressively younger with successive generations (age indicated at death).

obvious link between the families was made. Potentially, the use of polymorphic markers for this region of chromosome 17p may help to link these families.

4.5 SUMMARY

In contrast to the optimism inspired by the report of Haliassos et al (1989b) on the sensitivity of RG-PCR, my work here has not demonstrated any usefulness of this approach in the detection of MRD, at least when ethidium bromide was used to visualise PCR products. The first major problem was the lack of specificity when a system involving restriction of the WT allele was used. This was not due to incomplete digestion of PCR products by restriction enzymes but may be due to re-annealing of cut ends especially where over-hanging bases exist. With hind-sight, the opposite approach, creating recognition sites for the mutant allele may have been preferable. The other problem is the lack of sensitivity. The figure of 10% found here is consistent with the comment of Haliassos ($\geq$ 5%), and the use of radio-labelled nucleotides in PCR or the use of Southern blotting may improve this situation. With the brief attempts at enriching the mutant DNA species using restriction enzymes, production of a PCR product with WT template was a major disappointment, and the lack of time here prohibited further attempts with this approach.

CHAPTER 5

DISCUSSION

The key purpose of this project was to assess the feasibility of using single-base mutations, specifically in the p53 tumour suppressor gene, as molecular markers for MRD detection. Rather than exclusively advocating p53 as a potential marker for this purpose, I set out to use p53 simply as a model, and the findings of this project could potentially be applied to other genes in which subtle alterations are present in malignant cells, and which may be the only markers available to distinguish these cells from normal ones. As outlined in Chapter 1, in both the diseases studied in this project, other markers are available, achieving high levels of sensitivities and specificities, and the usefulness as MRD markers of small alterations in cancer-associated genes would therefore have to be set against the gold standards set by these techniques. The purpose of this project is not to find an alternative ultra-sensitive technique to supercede those available, for instance as outline in Section 1.2.3, but to attempt to answer the question of whether, with the remarkable ability of PCR to amplify DNA, one can take advantage of certain relatively common genetic changes in malignancies to serve as tumour- and patient-specific markers of MRD. In a disease such as AML where often no consistent marker is available, this may potentially be of greatest interest.

p53 was chosen to be studied in this project for 3 main reasons:

(1) It is the single most frequently mutated gene in human cancers. Although in most cancers, p53 alterations are still found in only a minority of cases, one can expect to find abnormal p53 in one fifth or more of sporadic breast cancers (Bergh et al, 1995 - 21.8%; Saitoh et al, 1994 - 39.6%; Blaszyk et al - 34%, 1994; Sommer et al, 1992a - 32.6%; Coles et al, 1992 - 40%; Andersen et al, 1993 - 21%; Mazars et al, 1992 - 18.7%; Osborne et al, 1991 - 46%; Prosser et al, 1990 - 13%), and up to one third of relapsed acute leukaemias (Zhu et al, 1996), but in less than 10 - 15% of acute leukaemias at diagnosis. The incidence in AML at diagnosis is uniformly low (Pignon et al, 1995 - 5%; Fenaux et al, 1992 - 7.1%, Wattel et al, 1994 - 15%; Preudhomme

et al, 1994 - 11%) whilst that of ALL seeems slightly more variable, and is particularly high in FAB subtype L3 in adults (Fenaux et al, 1992 - 22%; Gaidano et al, 1991 - 55.5%; Wada et al, 1993 - 25%). With the sample size used in this project, It was expected that approximately 12 positive breast tumours and 5 or more acute leukaemia samples would be found and make a reasonable platform on which to assess the key aim.

(2) In significant subgroups of the cohort of patients studied, the process of selection meant that they were already in an adverse prognostic group or had demonstrated aggressive or extensive disease, so that the likelihood of finding p53 mutations (themselves an adverse prognostic marker) might be higher. For example, because PBPC harvests were the main 'tissue' to be assessed for the presence of MRD, patients were selected because they were known to have had PBPC harvested. This included a large number of patients with metastatic breast cancer (38 of 51 patients studied). In the ACCOG patient group, as the choice of PBPCT was made by randomisation, this rationale would obviously not apply. In contrast, for patients with acute leukaemia, it was customary in our department at the Royal Infirmary to carry out back-up harvests as a matter of routine, and furthermore, the selection of patients was made simply on the basis that we had prospective DNA samples stored and they were, therefore, more or less consecutive patients treated at our department. Nonetheless, 16 of 29 patients studied had samples taken at relapse, and carried a higher likelihood of a p53 mutation (Zhu et al, 1996; Hsiao et al, 1994).

(3) p53 mutations are heterogeneous in location and nature. The majority (79%) of mutations are single-base missense mutations (Greenblatt et al, 1994), and scattered amongst much of the exonic and intronic sequences of the gene, although hot-spot regions, roughly corresponding to exons 5 - 8, have been identified. 87% of mutations are located within these exons (Greenblatt et al, 1994), and the conserved domains II to V contain approximately 2/3 of all mutations (Levine et al, 1995; see Section 1.4.2). Both transversions and transitions are represented (Hollstein et al, 1997) with very different neighbouring base sequences and probably secondary and tertiary DNA

structures. This heterogeneity posed a major challenge in this project, as the basis for using p53 (missense) mutations is the use of allele-specific PCR or ARMS, and it can be anticipated that the design of primers and the optimisation process to achieve a high degree of specificity are likely to be very difficult, and very different from one mutation to another. Paradoxically, because of these potential difficulties, p53 is actually a 'good' system to test the feasibility of using such subtle alterations as markers to detect MRD. This contrasts with ras oncogenes where the codons in which mutations can take place in malignancies are much more limited and theoretically may be a more robust system.

5.1 USE OF p53 AS DISEASE MARKER

p53 mutations have often been used for the study of minority tumour clones in the areas of clonal expansion or evolution of these tumours (Pollock et al, 1996; Sidransky et al, 1992; Wada et al, 1993). Using p53 and allele-specific PCR to detect MRD within other non-tumour tissues has been much less often performed and even more uncommonly on a relatively large scale (Hayashi et al, 1995). The ras oncogene family has been more often used in this context, but nevertheless, examples of the use of p53 in this way are available (Mao et al, 1994; Hayashi et al, 1995; Rhodes et al, 1995). To utilise p53 as a tumour and MRD marker, bearing in mind the very subtle alterations in question in the majority of cases, the allele-specific PCR (or ARMS) must be both specific and sensitive, i.e., being able to amplify only the mutant but not the WT DNA template, and to have a high detection limit for minority mutant species amongst a large excess of WT species. There would be many steps and hurdles involved, but I felt that this system was achievable. Here I shall discuss the many issues raised throughout the stages of this project.

5.2 MUTATION DETECTION

5.2.1 CHOICE OF TECHNIQUE

After obtaining the appropriate clinical materials, the first objective was to determine which primary breast tumour or leukaemia had a mutation in p53 to be used as markers. Because the location of p53 mutations are diverse, a screening method was required to identify samples to be sequenced. As outlined in Section 1.5, there is a wide selection of techniques available. The choice of detection technique would have to fulfill several criteria: **(1)** it should be widely used and accepted as a hallmark of its efficacy; **(2)** it should be sensitive and is capable of detecting the great majority of mutations present in the fragments of DNA studied; **(3)** it should be able to detect the presence of a mutation even if the malignant cells are a relative minority amongst normal tissues; **(4)** it should be relatively simple to set up using materials readily obtainable within financial constraints; **(5)** it should be relatively easy to learn to use. A review of the available options identified 3 feasible methodologies, namely, SSCP, DGGE and HA. Most of the other techniques were either not in popular use (such as carbodiimide modification, Ganguly et al, 1990), or involved hazardous chemicals (such as HOT, Cotton et al, 1988).

HA may be slightly less sensitive than the other two, with figures of around 80% reported (Cotton, 1993). The sensitivity, however, is dependent on the nature of the substitution, and for some, there may be little or no difference in the mobility between WT and mutant. The type of gel matrix may help to improve this and the use of Hydrolink and MDE (Soto and Sukumar, 1992) gels have been described to this end. There is no theoretical basis to predict the behaviour and property of heteroduplexes, and HA is very much dependent on the ionic strength, concentration, and type of buffer used (Glavac and Dean, 1995). One advantage of HA is that it may be used without radioisotopes, and furthermore, manoeuvres such as hybridisation with an artificial deletion-mutant instead of WT DNA may improve sensitivity (van den Akker et al, 1992). Of the other 2, SSCP is said to be easy to use in most reports (Prosser, 1993), and has a high level of sensitivity (Moyret et al, 1994; Condie et al, 1993). The

sensitivity of DGGE is even higher, near 100% in most cases (Macek et al, 1997; Beck et al, 1993) when properly optimised. Both are popular techniques, perhaps more so with the former, and apart from the slight edge DGGE has in sensitivity, a number of factors influenced my decision to choose DGGE. SSCP requires the use of radioisotopes which, though not prohibitory, adds to the inconvenience, relative hazard and expense. Once a mutation is identified by SSCP, the isolation of the mutant DNA strand for sequencing requires the extra step of further autoradiography to identify the position of the correct band to be cut from the gel (H.A.Phillips, personal communication). On the contrary, mutant bands on DGGE can be visualised directly by UV light illumination of ethidium bromide-stained gel, hence isolation of the mutant species is achieved more rapidly. Like HA, there is no theoretical model to predict the 3-dimensional structure of single-stranded DNA, and all optimisation steps are largely empirical. Usually, not only does the gel matrix need to be optimised, for example, the addition of glycerol, electrophoretic temperature may have to vary between 4°C and room temperature, necessitating electrophoresis in the cold room and pre-cooling of the gel. In general, electrophoresis at room temperature with 5-10% glycerol or at 4°C without glycerol are good starting points (Hayashi and Yandell, 1993).

Although the optimisation and the set-up of DGGE are not by any means less laborious, there are key advantages.

(1) Sensitivity may be higher as has been mentioned. One of the mechanisms for this is that DGGE can take advantage of the formation of heteroduplexes between a mutant strand and a WT strand during the later cycles of PCR. Because of their reduced stability, heteroduplexes tend to have lower melting temperatures than homoduplexes formed by WT or mutant sequences, and hence bands that are far removed from the homoduplexes, making visualisation easier. Destabilisation by up to 6°C is possible by a single base mismatch (Myers et al, 1988). In the context of screening tumour samples, WT strands are derived either from the opposite allele within the same tumour cell, or from contaminating normal tissues which are almost invariably present.

(2) DGGE is non-radioactive, thus reducing the hazards of chemical handling and costs.

(3) DGGE enables the separation of pure mutant species of DNA which are usually easily distinguished from species containing WT, making it straightforward to re-amplify and sequence the mutant sequence without any confusion caused by WT strands.

(4) As the melting property of a double-stranded piece of DNA of known sequence can be calculated, computer programmes are available to predict the melting map, optimal gel running time, and the expected effects of any base change on the Tm. (Fodde and Losekoot, 1994). Programmes such as SQHTX can even predict the expected separation distance between bands on the gel (Hamelin et al, 1993). I have not taken advantage of this last factor during this project largely because of the expense involved in obtaining such programmes, when balanced against the fact that the fragments of p53 studied here are already well known and investigated, and it would be much easier to use published parameters and primer sequences. There are also a number of disadvantages with DGGE. Setting up the apparatus was labour-intensive, and much time had to be spent on learning to use it to be proficient (especially, for example, casting linear denaturing gradient gels). Again, custom-made apparatus is available from Biorad, but the cost is prohibitive. I improvised using equipment already available (such as the Hoefer SE600 electrophoresis tank, water bath and heat exchanger) which worked out to be quite satisfactory (Myers et al, 1988).

5.2.2 PROPORTION OF TUMOUR TISSUE REQUIRED

Unlike cell line models, the study of clinical materials almost always involves the presence of contaminating normal tissue, whether the primary tissue is frozen section of breast tumours, paraffin-embedded sections or bone marrow at the time of diagnosis of leukaemia. As can be expected, the higher the proportion of tumour tissue, the higher the copy number of amplicons of the mutant sequence after PCR, and the corresponding band on DGGE may be expected to be more intense. Mutation screening techniques are not ultra-sensitive in this regard, and for most techniques, the

proportion of mutant tissue require is of the order of 5-20% (Prosser, 1993). For example, SSCP can detect a mutant when tumour makes up 5-10% (H.A.Phillips, personal communication), DGGE 12.5% (Beck et al, 1993) and TGGE 10% (Ke et al, 1993). Section 2.2.2 describes the use of 5637 bladder carcinoma cell line in serial dilutions with normal blood white cells to determine this sensitivity. This cell line contains a AGA to ACA missense mutation in codon 280 (exon 8, 'fragment D'). By mixing experiments, I was able to demonstrate that DGGE as used in the way described, is able to demonstrate the presence of the mutation down to 5% of tumour cells by the appearance of heteroduplexes. Although I did not study the other 4 p53 fragments, all 4 positive controls were clearly demonstrable and there was no reason to suspect that the sensitivity should be significantly different from the 5% noted here. However it must be noted that below 40%, the mutant homoduplex band had faded so much that it was not possible to cut this out for reamplification. For practical purposes, therefore, samples containing more that 50% tumour content were used in this project. For the acute leukaemia diagnostic samples, this was easier to ensure, as a blast count was routinely given in the bone marrow reports. For the paraffin-embedded breast tumours, many were checked by colleagues in the Pathology departments to contain a substantial amount of tumour before being sectioned for DNA extraction. The frozen sections, however, were not studied by microscopy.

5.2.3 CHOICE OF PRIMERS AND DGGE CONDITIONS

Because of the fact that the great majority of p53 mutations are known to exist in hot-spot regions which correspond to the conserved domains of the gene, roughly representing exons 5-8, most studies concentrate on these exons (Beck et al, 1993; Nigro et al, 1989). For studies that describe the use of DGGE in p53 screening, there are a host of different conditions and primer sequences used. In this project, I selected the technique used by Borresen et al (1991) in their original description of CDGE as there was local experience with the use of this system which had proved to be efficient. Modifications were made, however, as the Borresen primers did not cover exon 6 which also contained mutations though not one of the 5 domains (Caron de Fromentel and Soussi, 1992). I empirically added primers to cover this exon

(fragment E), which turned out to be quite satisfactory. A second modification was a change in the primers that covered fragment A (exon 5) as the Borresen primers did not consistently amplify and in fact, the authors had changed to a new primer set more recently (S.Lystad, personal communication). Owing to time constraints, I was unable to screen the leukaemic samples using these fragment A primers. Therefore, all acute leukaemic samples were screened for mutations in fragments B, C, D and E only.

In order for a particular DGGE system to achieve the highest sensitivity of detecting all possible point mutations within the particular DNA fragment studied, the specific set of conditions ought to be optimised. This enables the separation between mutant and WT species to be as great as possible. Ideally the denaturant concentrations in the gel should represent approximately 10°C round the Tm of the studied fragment, ie, 5°C on either side (Myers et al, 1989). Each degree Celsius in Tm is represented by approximately 3% in denaturant concentration. The denaturants have, therefore, to make up the deficit in Tm between the desired range of temperature and the temperature at which the gel is run in the electrophoresis tank.. Higher running temperatures mean lower denaturant concentrations are required. Given the manufacturer's highest recommended temperature for the Hoefer tank is 55°C, I attempted a maximum running temperature of 60°C which did not produce any adverse effect on the apparatus, but conveniently shortened the electrophoresis time to enable gel run and staining to be performed within the same working day. 60°C, therefore, became the routine temperature used. In order to detect all possible mutations in a DNA fragment, it is important that the fragment melts as a single low-melting domain when attached to a GC-clamp. The presence of more that one such domain would mean that only the one with the lowest Tm is studied. Ensuring that this is the case may be achieved either by using computer programmes to plot the melting map, or by running a perpendicular DGGE which should show a simple sigmoid curve, the steep slope of which represents the optimal range of denaturant concentrations. The latter can be estimated by measuring the distance between each end of the slope and the left or right edge of the gel, and knowing the lowest and highest denaturant concentrations, assuming the gradient is linear. Section 2.2.7 and

the accompanying figure describe such a gel in which p53 fragment B (exon 5) PCR products are electrophoresed. Fragment B melts as a single domain, and the 2 lines at the slope of the sigmoid represent WT and mutant (H69 cell line) sequences co-electrophoresed. For this fragment, a denaturant range of 50 to 70% seems most appropriate, with 64.4% being the central point of the sigmoid. This differs from the original description (Borresen et al, 1991) in which 50% denaturants was optimal. The likely reason for this is the inconsistency of obtaining a perfect linear gradient gel in which concentrations along the gradient can be estimated. As noted in Section 2.1.6.2, outflow from the gravitational gel caster is variable, largely dependent on the smooth operation of the 3-way tap, and the time spent varies between 3 to 10 minutes or longer. For this reason, I elected to use a broad gradient of 30-80% which, with the relatively high electrophoresis temperature of 60°C, should enable detection of all point mutations in the studied fragment (Busby-Earle et al, 1994). For the same reason, I did not feel that perpendicular DGGE assessment of the remaining 4 fragments was worthwhile and applied these same conditions to them.

The use of DATD as cross-linker is unique to Borresen's description of CDGE and Busby-Earle's report (1994) which followed the Borresen protocol, and all other reports identified described the use of bisacrylamide as cross-linker, with an acrylamide: bis ratio of 37.5:1. This contrasts with the 19:1 ratio which I used for ordinary PAGE, which seems to require a higher degree of cross-linking than DGGE. As seen in Section 2.2.4, the polymerised DGGE gel with acrylamide:bis of 37.5:1 was unable to demonstrate the presence of mutations in positive controls. Gels with the lower ratio of 19:1 were also ineffective (data not shown). Furthermore, gels cross-linked with bis also appeared not to retard migration of the DNA fragments as shown in the travel schedule gels. One possible explanation might be that the high- and low-concentration denaturants in the gradient were allowed to mix between the plates prior to polymerisation, resulting in a uniform denaturant concentration throughout the gel with an equivalent Tm lower than that of the DNA fragment studied. I felt this was unlikely as meticulous care was taken to ensure that the gel was poured at such a speed that the acrylamide was not allowed to stand in liquid

phase for too long before it polymerised, and the optimal gel-pouring time was 8 to 10 minutes. It would seem, therefore, that under the electrophoretic conditions and primer sequences used here, DATD somehow provided a unique molecular environment for the retardation of migration of denatured double-stranded DNA fragments.

5.2.4 COVERAGE OF POSSIBLE MUTATIONS

In Borresen's description of CDGE (1991) from which my primer sequences were derived, it was remarked that the regions screened covered over 80% of mutations reported. This study did not cover exon 6 which, although not within the conserved domains, is known to contain a significant proportion of mutations (14.5% for breast cancer, see below). I have, therefore, empirically added a set of primers for exon 6 (fragment E) containing some 5' intronic sequences, which improved the coverage of possible mutations to 90.6% (Table 5.1). This figure is derived from a database of p53 mutations as described by De Vries et al (1996). In this database, 1965 p53 mutations are listed. These were reported in the English world literature up to September 1993. From the abbreviated listing of mutations, a total of 145 missense, nonsense, silent and complex exonic point mutations are identified for breast cancer. The hot-spot codons 175, 245, 248, 249, 273 and 282 cover 30.3% of all reports for this disease. Codons 175 and 248 cover 9% each. The merits of adding the exon 6 primers can be seen in my cohort of cases, as p53 mutations in exon 6 (fragment E) are found in 4/13 tumours. Table 5.1 compares the regions covered by the conserved domains where most mutations of p53 are clustered around domains II to V, and the regions screened in this project. For each of the 5 fragments (A - E) and 5 domains (I - V), the proportion of mutations in breast cancer likely to be found in it is listed (De Vries et al, 1996). It is noted that the p53 fragments covered by the primers used in this project are likely to contain a larger proportion of mutations than the conserved domains themselves. In this project, the incidence of non-polymorphic mutations in breast cancer is 13/51, or 25.5%. If this represents 90.6% of all mutations likely to be present in the cohort of breast cancers studied, the total incidence here is 28.1%, within the accepted range for this disease.

Table 5.1 Calculations based on p53 database of De Vries et al (1996). See Section 5.2.4.

Domain	Codons	Exon	Proportion of all mutations in breast cancer
I	13 - 19	2	0%
II	117 - 142	4 and 5	6.9%
III	171 - 181	5	12.4%
IV	234 - 258	7	29.6%
V	270 - 286	8	17.9%
			66.8% total

Fragment	Codons	Exon	Proportion of all mutations in breast cancer
A	124 - 148	5	9.6%
B	155 - 185	5	20%
E	187 - 222	6	14.5%
C	237 - 253	7	26.2%
D	264 - 301	8	20%
			90.6% total

5.2.5 PRESENCE OF p53 MUTATIONS IN CLINICAL SAMPLES

As noted in the above section, the incidence of p53 mutations in the studied breast cancer population is within expected limits, including 4 cases (of 11 sequenced) whose mutations are in the hot-spot codons (codon 175 - patient B11, codon 248 - patients B28 and B33, codon 273 - patient B45). The pattern of mutations in the breast cancer cohort, therefore, conforms to what is known and implies that the patients and tumours studied are representative of sporadic breast cancers generally. Of the 11 sequenced tumours, only 5 could be optimised adequately to be used in ARMS. It is suspicious, therefore, if the other 6 tumours had been correctly sequenced. If the mutations did occur elsewhere, the ARMS PCR would not be expected to amplify WT sequences whether tumour or control template was used. I surveyed the EMBL p53 database (Hollstein et al, 1994) to confirm that each of the 5 un-optimisable sequences (1 sequence occurred in 2 tumours) had been reported in breast or other cancers. The following is a list of these mutations and the tumours in which they have been detected in.

Codon	Mutation	Cancers in which mutations reported
216	GTG > TTG	Breast, gastric.
216	GTG > ATG	Breast (many reports), head and neck, lung, liver, oesophageal, leiomyosarcoma, MDS, ovarian.
248	CGG > CAG	Endometrial, CLL, bladder, nasopharyngeal, glioma, NHL, colorectal, head and neck, gastric, Burkitt's lymphoma, ALL, breast, oesophageal, urothelial, ovarian, plasma cell leukaemia.
267	CGG > CAG	Breast, prostate, Li-Fraumeni syndrome.
273	CGT > TGT	Breast, bladder, thyroid, head and neck, glioblastoma, astrocytoma, basal cell, liver, cervix, uterine, colorectal.

It would seem that the sequences obtained are believable. Moreover, the chromatograph appearances at these mutations are always unequivocal (Fig. 2.10). It must be admitted, however, that the fact that these mutations have been reported in association with cancers and in particular breast cancer would not necessarily confirm that they are correct for the samples whose ARMS were not optimisable. The only means of confirming would be to sequence the opposite strand which, with hind-sight would certainly have been worthwhile had it not been for time constraints.

The incidence of mutations in the leukaemia samples, on the other hand, is disappointingly low. Because of the reported higher incidence of such mutations in relapsed disease (Zhu et al, 1996), of 16 samples taken at relapse, approximately 5 could be expected. Of the remaining 18 patients, 1 or 2 would be present. Instead, one single mutation was detected (excluding the 2 constitutional polymorphisms in codon 213) in a relapse sample. This low incidence may be due to the fact that fragment A (5' portion of exon 5) was not screened in any of the leukaemia samples because of initial technical difficulties with PCR amplification. However, a study of the EMBL database shows that only approximately 6% of reports of acute leukaemias

fall within this fragment, and hence unlikely to contribute to the number of mutations detected here.Technical factors might be involved, but care was taken to ensure that materials studied contained a high proportion of leukaemic blasts, and a positive control was alway run on the same gel, so these would also seem unlikely. It remains possible that the incidence of p53 mutations in this cohort of patients is genuinely low.

The 8 bp insertion (5'-CCGGGGGG-3') between the second and third bases of codon 281 of the ALL patient (L11) is novel and not previously been reported. A survey of the EMBL database, that of De Vries et al (1996) and that of Beroud and Soussi of the Curie Institute (http://perso/curie.fr/thierry.soussi/p53_database.html) did not reveal this insertion in any malignancy. In this patient, clonal evolution is implicated. As described in Section 3.7.3, a previous study of this patient (Langlands et al, 1993) confirmed the presence of 2 leukaemic clones. The major clone at diagnosis appeared to have responded to chemotherapy such that there was no molecular evidence of it after therapy, but the more aggressive clone, which was a minority at diagnosis and which contained the 8 bp insertion in p53, ultimately resulted in death of the patient. This comes as no surprise, as it is now widely recognised that in many human malignancies, the presence of a p53 mutation carries adverse prognostic implication in terms of survival and resistance of the tumour to cytotoxic chemotherapy (Aas et al, 1996). In acute leukaemias, blast cells carrying p53 mutations are more resistant to both high dose chemotherapy and low dose cytosine arabinoside (Wattel et al, 1994). Clonal evolution in such leukaemias over a background of blasts not containing p53 mutations has also been implicated (Wada et al, 1994; Zhu et al, 1996), and this phenomenon is also recognised in lung cancer (Mao et al, 1994), malignant brain tumours (Sidransky et al, 1992) and soft tissue sarcomas (Pollock et al, 1996). Patient L11 again confirms this. Although the great majority of p53 alterations are missense mutations leading to conformation changes and often increased half-life of the p53 protein, over 280 short insertions or deletions have been reported to the EMBL database (Greenblatt et al, 1994). 90% are monotonic runs of 2 or more identical bases in tandem or with a short intervening sequence, and codons, 151-159 are most frequently involved. Although our mutation occurs in codon 281, it is adjacent to a

short run of G and C repeats, and the mechanism of slipped mispairing, or template misalignment, may be involved. There is no identifiable mutagen in this young patient of 16 years that may be implicated. From this case, it can be seen that as well as missense mutations, frameshift mutations of p53 may also be associated with a more aggressive phenotype, leading to therapy resistance and clonal evolution of acute leukaemias. Unlike single-base mutations, the specific nature of this insertion makes allele-specific PCR to detect MRD much more feasible.

5.3 AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)

5.3.1 USE OF ARMS IN THE DETECTION OF MINORITY CLONES

ARMS-based techniques (in their various synonyms) have frequently been used to distinguish one allele from another in inherited conditions, and in the analysis and characterisation of acquired mutations associated with a disease process, as outlined in Section 1.5.8. Prior to the introduction of such techniques in the late 1980's, PCR techniques coupled with dot-blot and allele-specific oligonucleotide hybridisation had been used but sensitivity was relatively low, approximately 5% (Halliassos et al, 1989b). ARMS has the advantage of being rapid; potentially highy sensitive (Kirby et al, 1996); non-radioactive and does not require hybridisation; amenable to manoeuvres such as multiplexing (Patel et al, 1993), detection with fluorochromes (Chehab and Kan, 1989), and use of oligonucleotide or peptide nucleic acid blockers (Seyama et al, 1992; Rhodes et al, 1997) to enhance sensitivity; and applicable to sites across a wide variety of genes (Bottema and Sommer, 1993). Because of the highly subtle nature (often a single base change) of the genetic alterations studied by ARMS, considerable optimisation of the PCR system was required to achieve the best cycling conditions for each site (Sommer et al, 1992b; Cha et al, 1992; Rychlik, 1995; Rhodes et al, 1997). All the studies that describe the optimisation process or the use of ARMS to detect minority clones of cells have one feature in common - they have all used models of one or a small number of single-base genetic changes. The ras oncogenes have often been studied along these lines with ARMS, but ras differs from p53 in that

mutations of this family of genes largely occur in a small number of codons, primarily codons 12, 13 and 61 (Takeda et al, 1993), such that it has even been possible to use a mixture of primers corresponding to mutations of the first or second nucleotide of codon 12 in the MASA technique (Takeda et al, 1993). This approach is clearly not applicable to p53 whose mutations are scattered over a large number of codons. A very small number of studies are available where heterogeneous disease markers such as p53 mutations are used in a large scale for detecting minority clones (Hayashi et al, 1995; Mao et al, 1994). Both of these studies used a combination of p53 and K-ras mutations as markers. Neither study described the optimisation procedure. Whilst Mao et al achieved a sensitivity of 1 tumour cell in 10^4 normal cells by cloning the PCR products and hybridising with a radio-labelled ASO probe, Hayashi et al did not use probes but then did not specify the sensitivity of their technique. In order for heterogeneous markers to be employed in MRD detection in an already multi-step protocol, steps should be simplified as much as possible and individual hybridisation and probing is likely to be too cumbersome. Hence I hoped to assess the feasibility of a PCR-based technique that did not require Southern hybridisation. Radio-labelling of either primers or dCTP was tried in an attempt to improve sensitivity, however.

5.3.2 OPTIMISATION OF ARMS

The principle of ARMS lies with the different efficiencies in which matched and mismatched primer-template pairs are amplified. Because of the large number of times a piece of DNA is amplified, differences in such efficiencies are magnified, to produce a detectable difference between the templates. For instance, if the amplification efficiency of a matched pair is 90% and a mismatched pair is 60%, after 25 cycles of amplification, there will be 73 times more product from the matched than the mismatched reactions (Wu et al, 1989). Optimisation is still required, however, to ensure that the difference in amplification efficiencies is maximal. Such differences are most important in the initial cycles of PCR as, once a product has been formed, subsequent cycles will produce perfectly matched products. Unlike the use of ARMS in screening inherited conditions or determining tissue types, in which the allele in question is in the germ-line and present in all cells studied, MRD detection has the

additional requirements of absolute specificity (no detectable PCR product with WT template) and a reasonably intense specific ARMS product such as to be visible when mutant DNA species are heavily out-numbered by WT species. Whilst a minor degree of non-specificity may be tolerated when ARMS is used for other applications, it is imperative that no detectable product is obtained with WT DNA when ARMS is used in the study of MRD. Therefore, the aim of the optimisation work-up for any mutation site in this project is not simply to ensure that PCR products are not produced with a WT template, but also to avoid reducing the intensity of the product with the mutant template. Much effort in this project was spent in determining the factors which would make an ARMS reaction specific and sensitive, using DNA from the cell lines T47D and 5637. Such optimisations were along the lines suggested by previous published work.

Because of the requirement of a lack of 3' to 5' exonuclease activity, Taq polymerase is usually used. Similar results can also be obtained with the Stoffel fragment of Taq (Rhodes et al, 1997). Apart from the choice of Taq which is not in dispute, numerous factors associated with PCR have been studied in optimisation experiments by various authors. In this project, the parameters assessed individually whilst keeping other variables constant were: annealing temperature, magnesium concentration, dNTP concentration, primer concentration, the presence of glycerol and of formamide, cycle number, and the use of an ARMS primer with the 3' base corresponding to the mutant sequence versus one in which the penultimate base was also deliberately mismatched.

5.3.2.1 ADDITION OF A DELIBERATE MISMATCH

As indicated in Chapter 3, using DNA from the breast cancer cell line T47D which contains a missense mutation CTT to TTT in codon 194, the first conclusion was that, as well as replacing the 3' base of the ARMS primer with the mutation, deliberately introducing a mismatch in the penultimate base did improve specificity, and at least in the T47D model, did not significantly compromise sensitivity. Based on this finding, all ARMS systems studied in this project used ARMS primers which mismatched a WT template at the 2 bases 3', and mismatched a mutant template at the penultimate

3' base only. The mismatch was chosen according to the destabilisation ranking as determined by Newton et al (1989). In descending order of destabilisation, mismatches are: CC > CT > GG = AA = AC > GT. For each ARMS system, the most destabilising mismatch was chosen, with the assumption that any other single base mismatch introduced would not improve specificity greater. The further from the 3' end of a primer the deliberate mismatch is introduced, the lower the destabilising effect, and the more likely for a product to result with a WT template (Newton et al, 1989; Newton and Graham, 1994), hence all mismatches were introduced in the penultimate base only. Internal mismatches are often very well tolerated, even with lower Ta's (Christopherson et al, 1997). Obviously, introducing 2 deliberate mismatches (first and second penultimate bases) would be even more destabilising for the WT template and improve specificity, and this was attempted with patient B45 (using primer 1232/94-ARMS2). The result was that, though no specific product was obtained with WT template, little amplification was obtained with the mutant template even at the lower Ta's of 47 and 50°C, and none at all at the higher Ta's (Section 3.7.1). Furthermore, a lot of spurious products were amplified. From this, it can therefore be concluded that, for certain ARMS systems, mismatching strategy may either produce too little or too much destabilisation of primer-template annealing, with nothing to bridge the gap to produce an operable system. Several mutation sites could not be optimised (patients B27, B28, B33, B45, B46, B50). Invariably, in each of these the intensity of the WT and mutant products were too similar, and any alterations in PCR conditions affected both to a similar degree.

5.3.2.2 ALTERATIONS OF OTHER PARAMETERS

Reviewing available literature on the optimisation of ARMS identified a number of studies in which PCR conditions were adjusted to make ARMS specific. Bottema and Sommer (1993) reviewed their experience of using ARMS at 41 different sites (69 allele-specific assays) for a variety of applications including population screening for variants of the coagulation factor IX gene, haplotyping of the transthyretin gene and carrier testing for genetic conditions. In the majority of cases, magnesium concentration (1.5 - 4.5 mM) and primer concentration (0.05 - 1.0 μM) titrations

were adequate in producing a robust and specific system. Using a G to A missense mutation of the factor IX gene as a model, [Mg] of < 3.5 mM and [primers] of around 0.05 μM produced specific reactions (Sommer et al, 1992b). It is widely known that specificity of a PCR reaction improves with lower [Mg]. Magnesium ions form a soluble complex with dNTPs, essential for their incorporation in a PCR. Magnesium also has the effect of stimulating DNA polymerase activity and increasing the melting temperature of the primer-template structure (Newton and Graham, 1994). The benefit of lowering [Mg] was demonstrated in this project and was in fact a key parameter to be optimised. The main issue here is that amplification of a doubly mismatched primer-template pair (i.e. WT template) was more susceptible to the effect of lower [Mg] than if the 3' bases were perfectly matched (mutant template), a feature much utilised in this project. On the other hand, the effect of lowering [primers] was not obvious, as amplifications with both WT and mutant DNA were affected simultaneously.

Increasing Ta can improve specificity and prevents priming of mismatches (Rychlik, 1995), although not found to be important by some authors (Sommer et al, 1992b). Ta was a most important condition to be optimised in this project. Because of the relatively higher melting temperature and stability of a matched (or less mismatched) primer-template pair (Huang et al, 1992), a higher Ta allows preferential annealing of matched primer to template and hence more efficient amplification. Generally, a high Ta of 64 - 67°C was required for optimal specificity, and the experience here is that Ta should be the first parameter optimised, and other factors may then be adjusted after the best Ta is selected. All other factors previously reported to enhance specificity, in particular [dNTP], were not found to contribute significantly. The main problem with all these factors appeared to be that they simultaneously reduced amplification with both WT and mutant templates. This was the case with [dNTP] which at 25 - 50 μM has been found to decrease spurious products (Bottema and Sommer, 1993), and very low concentrations of around 6 μM enhanced specificity, giving 10 - 20 fold less products than reactions at higher [dNTP] (Kwok et al, 1990). The discrimination between extension efficiencies of matched and mismatched

sequences is maximum when the concentration of the next correct dNTP is low (Huang et al, 1992). Although the range tested in this project (3.125 - 200 μM) covered these concentrations, amplification with the mismatched WT template was not preferentially inhibited by low [dNTP]. Some authors have found that extension of certain mismatches (C:T, A:C, C:C) were susceptible to even lower [dNTP], as low as 2 μM (Ehlen and Dubeau, 1989). Addition of other solvents have been found to be of some benefit to ARMS. Formamide at 2 - 5% increases signal strength, eliminates spurious bands and enhances allele specificity (Bottema and Sommer, 1993). Here, the addition of formamide was strongly inhibitory to amplification. The 2.5% of formamide used here produced a significant reduction of intensity of mutant product. Conceivably, a lower concentration might have produced less inhibition and might have been worth trying in sites where ARMS was difficult to optimise. Glycerol at 5 - 10% enhances specificity (Cha et al, 1992; Rychlik, 1995). This was tested here. 10 - 20% glycerol added to the ARMS reaction produced slight benefit by reducing the signal from the amplicon of the WT template. 5% did not make a difference. The main drawback of glycerol was the difficulty of accurate pipetting owing to its viscosity, but this was improved by prior warming. For this reason glycerol was not routinely used here.

Various other manoeuvres have also been reported in the optimisation of ARMS reactions. For example, a shorter ARMS primer enhances the difference with which extension takes place on a WT or mutant template (Huang et al, 1992) as the destabilising effects of mismatches are relatively greater. Primers as short as 14 nucleotides may be successfully used (Wu et al, 1989). Other authors advocate longer primers of around 30 nucleotides (Newton and Graham, 1994). As a compromise and in line with most reports of ARMS, I selected 20 nucleotides as the standard length. Sometimes, even though the design of an ARMS primer from the physico-chemical point of view appears optimal, the nature of the opposite (non-specific) primer may have bearing on the success of the reaction. Surprisingly, replacing the opposite primer with a different non-specific primer at another location, keeping the product size between 300 and 600 bp, has been reported to be beneficial in difficult cases

(Sommer et al, 1992b). In this project, I was reluctant to attempt this approach for various reasons. **(1)** There is no formula to help predict which sequence should be chosen, other than generally accepted guidelines for choosing primer sequences for PCR (Newton and Graham, 1994). Efforts in trying out various non-specific sequences randomly may well not pay off, and certainly if half of all systems require such optimisation as is the case here, the whole approach of using point mutations as MRD markers would be much too inefficient for clinical or even research application. It might be that some ARMS systems here were unoptimisable because of primers having different characteristics. On close scrutiny of the characteristics of the various ARMS / non-specific primer pairs (Table 3.2), 3 of the 5 unoptimisable systems (patients B45, B46, B50) are comparable within their primer pairs in terms of GC content and melting temperature (Tm). The other 2 systems have rather larger differences: Tm's for ARMS and non-specific primers for patient B27 are 48.4°C and 53.7°C, respectively, and for patient B33 are 60.9°C and 49.6°C, respectively. However, of the 5 optimised systems, large Tm differences are also observed. Patient B30's primers have Tm's 49.6°C and 56.6°C, and more remarkably, ARMS for patient B11 was very successfully optimised with the original GC-clamped primer used in DGGE (Section 2.1.5.3). **(2)** using p53 as a marker for MRD is already quite laborious and involves numerous steps, and I was keen to restrict the number of primers used and to take advantage of sequences already available.

5.3.2.3 NATURE OF PRIMER-TEMPLATE MISMATCHES

A number of studies have looked at the ability of mismatched sequences to extend in PCR. Although perfectly matched bases at the 3' end of a primer enables maximum efficiency of extension, mismatched bases may extend more or less efficiently depending on the nature of the mismatch. Studying the thermodynamics of mismatching to thymine, Petruska et al (1988) reported that a matched A:T terminus extended 200 times faster than a G:T mismatch, 1400 times faster than C:T, and 2500 times faster than T:T, using purified Drosophila DNA polymerase α. Contrasting results were reported by Kwok et al (1990) who found that any 3' mismatch involving T was extended efficiently, even at low [dNTP] and when coupled with an additional

deliberate mismatch. A:A mismatch reduced yield by 20 fold, whilst A:G, G:A and C:C mismatches reduced yield by 100 fold. Of note is that these authors used relatively long primers (30 bp), and the destabilising effects of these mismatches might be greater if the primers were much shorter. Another detailed studied by Huang et al (1992) showed that A:A, G:G, G:A, A:G, C:C mismatches were the most difficult to extend, whilst other mismatches could be extended slightly more efficiently but 100 - 10000 fold less than perfect matches. Overall, it seems clear that purine:purine or pyrimidine: pyrimidine mismatches are extended much less efficiently than purine:pyrimidine or pyrimidine:purine mismatches.

With this information in mind, a study of the 5 optimised ARMS systems here shows that the 3' mismatches are A:G, G:T, G:G, T:C and A:C, for patients B3, B11, B16, B30 and B37, respectively (Table 3.1). Only 2/5 of these are mismatches from the same class of nucleotide. On the other hand, the unoptimisable ones are A:C, G:T, T:G, A:G and G:T, for patients B27, B33, B45, B46 and B50, respectively. Only 1/5 are mismatches from the same class. In conclusion, although it is useful to have theoretical guidelines to help design ARMS primers or to assess the likelihood of success (Petruska et al, 1988; Ikuta et al, 1987), each system needs to be optimised individually and in practice, ARMS may work quite differently from what one might expect in theory. Apart from considerations at the 3' end of a primer, ARMS specificity is also influenced by the overall sequence context (Cha et al, 1992; Kwok et al, 1989).

5.3.2.4 SENSITIVITY OF ARMS

With a degree of heterogeneity of assay systems and optimisation criteria, a range of sensitivities have been reported for the use of ARMS in detecting minority DNA species. Many applications of ARMS are not for MRD detection (Section 1.5.8) and so a high sensitivity, or the ability to detect minority sequences is not important. In the application of ARMS in MRD detection, it is the 'discrimination sensitivity' between WT and mutant that is most important. The ARMS PCR itself may well detect a small quantity of mutant DNA, but much of this sensitivity is of no significance when the

system is optimised sufficiently to remove any trace of product with the WT template. For instance, Horikoshi et al (1994) reported that allele-specific PCR with the ras gene was capable of detecting 55 molecules of DNA, but the discrimination sensitivity was only 1:500. Other reported sensitivities range from 1:100 (Stork et al, 1991) to 1:1000 (Wada et al, 1993), $1{:}10^4$ (Cha et al, 1994) or $1{:}10^5$ (Ehlen and Dubeau, 1989) when radioisotopes are used, or glycerol added. In this project, my range of sensitivities also fall within this broad bracket, from 1:100 - 1000 in patient samples, to 1:1000 - 10^4 with cell line models (5637 and T47D). The lower sensitivities with patient samples may be due at least in part to the quality of DNA extracted from paraffin embedded sections, and that these are highly likely to be contaminated with normal surrounding tissues whilst cell lines provide a pure source of tumour DNA. Sensitivity is much more easily achieved in situations where the alteration is less subtle than a single base change, as in the leukaemia patient L11, in which system the mutation was an 8 bp insertion. This makes ARMS primer design and optimisation much easier as there is virtually no chance of a specific product of the correct size to be produced from a WT template. Indeed, here no optimisation was required, and simply standard PCR conditions were used and a moderately high sensitivity of $1{:}10^4$ was achieved.

Thus far discussed, all ARMS products were visualised by ethidium bromide staining of polyacrylamide gels. As described in Section 3.6, attempts were made to improve the detection sensitivity, based on the T47D breast cancer cell line model. The first attempt was to use a nested PCR, employing primer sequences with which the individual exons were amplified. It rapidly became apparent that a loss of specificity was limiting to this approach, owing to the much larger number of template molecules available for the inner nest (ARMS) reaction. This was not improved by diluting the product from the outer nest reaction 500 fold. I next attempted to use 32P radiolabelled dCTP, with the quantity of the non-radioactive dCTP counterpart reduced. This approach produced a slightly different set of optimised conditions from the non-radioactive ARMS, but it was perfectly feasible to have the system optimised. For reasons not apparent, attempts at obtaining detection sensitivity by serial dilutions

of mutant in WT DNA were unfortunately unsuccessful. The main difficulties appeared to be inconsistencies in the generation of products for each dilution. Despite repeated experiments, it was not unusual for higher dilutions to yield a product when lower dilutions did not, and loss of specificity was also observed. These problems could not be remedied and this approach had to be abandoned. Further attempts were made by 32P-radiolabelling the ARMS primer instead of dCTP in the hope of obtaining products with a more consistent signal as each molecule of the PCR product would contain one radiolabel, whilst the former dCTP approach would yield products with signal strength dependent on the number of C's incorporated. Unfortunately, the same inconsistencies were observed, and this too had to be abandoned. A possible explanation for the inconsistencies in the dilution experiments was of course sampling error, but it is to be noted that aliquots of these dilutions were also used in experiments with the non-radioactive approach which did not suffer the same difficulties. Since the initial optimisation using WT and mutant DNA only had not suffered these problems, I would imagine technical or sampling errors were still the likely reasons for failure of these experiments.

One limitation of detection sensitivity is obviously the quantity of DNA used in an ARMS reaction. In this project 1 μg of genomic DNA was used in each 50 μl reaction mixture as standard. Each 1 μg of mammalian genomic DNA contains approximately $3x10^5$ copies of an autosomal gene (Cha et al, 1992). p53 alterations in malignancies may occur either as a heterozygous phenomenon (i.e. on one of two alleles) or, more commonly, in a hemizygous state in which the normal allele is deleted (loss of heterozygosity). Hence, 1 μg of tumour DNA should contain $1.5x10^5$ copies of mutated p53. Even assuming that ARMS can amplify one single molecule of mutated p53 within 1 μg of template, the maximum sensitivity, whatever the visualisation method, should be of the order of 1 cell in 10^5, and probably much lower. I would, therefore, be satisfied with a sensitivity of around 1: 10^4. To increase the detection sensitivity one order of magnitude would require 10 μg of DNA, or 10 separate reaction tubes. Even then, with one tube out of 10 yielding a product, it would be impossible to conclude that MRD was present. Furthermore, the amount of DNA

required might well be prohibitive, depending on the source and quantity of study clinical material available. When compared to ultra-sensitive RT-PCR techniques using RNA as starting material (Datta et al, 1994; Gerhard et al, 1994; Fields et al, 1996) in which weight for weight many more copies of nucleic acids are examined, it would appear from here that methods using DNA may well not have a significant role in the detection of MRD, at least in the context of breast cancer in which RNA-based technologies are increasingly available.

5.4 RESTRICTION SITE-GENERATING POLYMERASE CHAIN REACTION (RG-PCR)

The short Chapter 4 describes my attempts at using the RG-PCR approach as an alternative to ARMS in detecting MRD. PCR is a powerful technique and as well as amplifying DNA with a specific defined sequence, can modify the sequence of the product one wishes to obtain by deliberately changing the primer sequence. This has already been described in the design of ARMS primers to enhance specificity. In RG-PCR, whilst the 3' end of the primer is perfectly matched both to WT and mutant (and stops short of the mutation itself), the deliberate mismatch is slightly further upstream such that an artificial restriction enzyme recognition site is generated for the WT sequence as seen here, or for the mutant sequence if desired. RG-PCR has more often been used for indications other than MRD detection. Examples include mutations in the CFTR cystic fibrosis gene such as the ΔF508 deletion of 3 bp, and 2 other relatively common mutations (Friedman et al, 1991); population studies of the 5382insC mutation of the BRCA1 gene using the enzyme BstO1; and detection of ras oncogene mutations (Jiang et al, 1989).

There are key advantages of RG-PCR over ARMS. A PCR product is always available, whether WT or mutant DNA is used, and internal control with another gene is not required. Because the mismatched base is slightly further upstream, extension of primer should not be impaired and amplification is efficient as a result. Optimisation of PCR should, therefore, be relatively straightforward. Also, recognition sequences of

restriction enzymes are usually highly specific and should reliably cleave double-stranded WT DNA only, leaving mutant DNA intact (or vice versa). This compares with the relatively non-specific nature of ARMS which is totally dependent on the discrimination sensitivity between WT and mutant (Section 5.3.2.4 above). RG-PCR has a wide applicability, as a restriction enzyme site can be generated for all 10 mutations detected in the breast cancer samples, 3 of which already altered restriction sites on their own (Table 4.1). With these positive features, I thought RG-PCR would be well worth assessing as an alternative to ARMS.

As with any technique used to detect minimal quantities of abnormal DNA amongst a large excess of normal DNA, sensitivity is a key requirement. In this project, RG-PCR, in which visualisation of PCR products was by ethidium bromide and UV light, has failed in this important regard. Serial dilution experiments showed that the mutant could barely be detected if it existed in less than 1:10 mixture. This is, however, not dissimilar to the sensitivities previously reported as determined by serial dilutions of mutant in WT DNA. Using K- and H-ras oncogene models, dilution experiments of Jiang et al (1989) obtained sensitivities of 1:16 and 1:9, respectively. Other earlier studies (Haliassos et al, 1989a and 1989b) gave a sensitivity of approximately 5%. These reports all involved the use of ethidium bromide and UV light to visualised products. The sensitivity was improved dramatically to 1: 2.5×10^4 when the PCR product was blotted onto a solid phase support and visualised with an internal radio-labelled ASO probe not corresponding to the mutation (Haliassos et al, 1989b). With the reasoning in the Section 5.3.2.4 above, this is bordering on the theoretical limit of detection when 1 μg of genomic DNA is used, although these authors did not specify the quanitity of DNA used in each reaction. This method was obviously worth attempting here, but because of time and resource constraints, was considered outwith the scope of this project.

Another method of potentially improving the detection sensitivity of RG-PCR is to use restriction enzyme to enrich the mutant DNA selectively for PCR. Kahn et al (1991) described this approach in the detection of codon 12 mutations in K-ras,

achieving a sensitivity of identifying one copy of the mutation amongst 10^4 copies of WT. This 'enriched' PCR was based on the use of a mutagenic primer which created a restriction site for the enzyme BstN1 if the template was WT. After first round PCR and enzyme digestion, another mutagenic primer also creating a BstN1 site further downstream was used for the second round PCR and this served as a control for the enzyme action. Because of cleavage of the WT DNA, only mutant DNA remained to be amplified in the second round of PCR. As a result of these 3 steps, products of different sizes representing WT and mutant species were obtained. Dilution experiments confirmed the marked improvement in sensitivity, and this was with non-radioactive reagents and visualisation by ethidium bromide staining. One drawback of using multiple steps is the risk of contamination, but some restriction enzymes, though active at lower temperatures, may withstand the high temperatures of PCR for short periods, and it may be possible to have a one-step procedure in which the enzyme is added at the outset (Huber et al, 1998). This is the case with BstN1, but obviously for different mutations, each enzyme needs to be evaluated for this individually, and with a heterogeneous set of mutations as with p53, this may not be feasible. Attempts here in using enriched RG-PCR were not successful (Section 4.3) because, despite enzyme digestion which should remove all WT DNA, a product was still produced after the second round PCR. Although 10 U of MboI enzyme would be expected to digest to completion after 3 hours of incubation, residual WT strands were likely to have remained. Time and resource constraints prevented a more complete evaluation of this technique, but probable avenues would include **(1)** using a higher dose of enzyme, **(2)** digesting for longer, or **(3)** reducing the number of cycles in the first round of PCR such that enough template molecules are available for the second round, whilst the smaller number of WT DNA to be digested by the enzyme means more complete digestion.

As described above, screening genomic DNA for MRD usually means that the limit of detection is of the order of 1: 10^5, if 1 μg of DNA is examined. This assumes the amplification and subsequent product detection of 1 copy of the mutation. In practice the actual detection limit is likely to be much lower, and figures from the techniques

described by both Haliassos et al (1989b) and by Kahn et al (1991) would suggest that they were approaching what was theoretically possible. When compared with the sensitivities reported for RT-PCR techniques used in MRD detection of breast cancer (Tables 1.2a and 1.2b), this high-lights the insensitivity of using DNA as study material.

5.5 CLINICAL RELEVANCE OF MRD IN PBPC HARVESTS OF BREAST CANCER PATIENTS

For epithelial tumours such as breast cancer, immunocytochemistry (ICC) was traditionally the method of choice for MRD detection (Mansi et al, 1987; Molino et al, 1991). Attempts have also been made to use ICC in the liquid phase, i.e., by flow cytometry (Leslie et al, 1990; Simpson et al, 1995) but this does not seem to have gained as much popular appeal. With the advent of the PCR in the 1980's (Saiki et al, 1988), naturally much interest was generated in the use of this technique as a ultra-sensitive means of MRD detection. Momentum in this development gathered only relatively recently (Datta et al, 1994; Gerhard et al, 1994) with the use of CK19, and perhap CEA as markers, and since that time a significant body of literature has gathered, both describing the laboratory development of such techniques (Traystman et al, 1997; Moscinski et al, 1996) and their actual clinical application, correlating the presence of MRD with clinical outcome (Fields et al, 1996). To date, ICC using immunostained cells visualised on fixed slides with a fluorescent microscope (Cooper et al, 1998) and RT-PCR detecting the CK19 message remain the 2 most sensitive 'gold standards' most widely employed.

Very few controlled studies have examined the contribution of MRD in PBPC harvests to clinical outcome. Sharp et al (1992) studied MRD using a cell culture technique with a sensitivity of 1 cell in 10^4 normal cells, and found that in patients with stage IV breast cancer at 10 to 28 months of follow-up, only 1 of 6 paitents who received contaminated PBPCs remained alive, which contrasted with 7 of 8 patients who received culture-negative collections. The fact that culture techniques were used

could mean that any malignant cells detected were more likely to grow and metastasise in vivo, unlike other techniques which are unable to distinguish between live and dead cells. The only other large study on PBPC (Cooper et al, 1998) in breast cancer came to a very different conclusion. Using a sensitive ICC technique capable of detecting 1 cell in 5×10^5 cells, these authors reported no significant difference in the outcome of patients with stage IV breast cancer who received MRD-positive or negative PBPCs. If the tumour dose received was $\geq 2/10^5$ tumour cells (which occurred in only 6 patients), there was reduced survival but not time to disease progression. Similar studies have also been performed for BM harvests, with results suggestive of favourable outcome if patients were transplanted with MRD-free products (Fields et al, 1996; Brockstein et al, 1996).

In this current project, overall results are hard to interpret. **(1)** Firstly, the number of patients in whom a disease marker is available is very small, and in even fewer could the ARMS systems be optimised sufficiently for MRD detection. No meaningful statistics could be performed on such a sample. Of the 5 patients described in detail in Section 3.8, 2 were known to have metastatic disease (B11, B3), 2 had high risk stage II/III disease on the basis of 4 or more axillary nodes (B30, B37), and the remainder (B16) was considered to have poor prognosis because of relapse after radical radiotherapy. After PBPCT, patients B11 and B30 relapsed in new sites, while B3 and B37 relapsed in previous sites of disease. Relapsing in new sites may potentially imply a role for re-infused tumour cells contaminating PBPCs. Here, patient B30 had no evidence of MRD determined by ARMS, whilst both B3 and B37 did. The fact that the latter 2 patients relapsed in previous sites indicate failure of disease eradication, and patient B30 relapsing with widespread lymphangitis may represent re-infused disease at a concentration of less than 1 cell in 1000. Cooper et al (1998), however, concluded that regardless of the incidence and quantities of tumour cells re-infused, the majority of patients did relapse in previously involved sites. **(2)** Another, more serious, difficulty in results interpretation is the level at which MRD was detected. Of the 3 patients whose PBPCH had evidence of MRD (B11, B3, B37), the corresponding detection sensitivities by ARMS were 1:1000, 1:100 and 1:1000,

respectively. It is somewhat doubtful if MRD can really exist in PBPCH in such high concentrations. These levels contrast with those reported in a number of studies in which the highest recorded level of MRD is in the region of $1{:}2x10^4$ (Cooper et al, 1998) and $1{:}2.3x10^4$ (Ross et al, 1993). With the ARMS system, as noted before, a major problem is with specificity and difficulties with optimisation. MRD is said to be present when there is a clear difference between the amplification of DNA extracted from the harvests and that from control WTcells (normal tonsils). This usually means no detectable product with WT, although as described in Section 3.8, some difficulty was encountered with the screening of PBPCHs from patient B3. Because each set of PBSCH screening was performed with WT negative control and tumour positive control, and each in triplicates (except positive control which was performed once), I can only conclude that using this ARMS approach, under given conditions and sensitivity as determined by serial dilution of tumour DNA in WT DNA, that MRD was present in these samples in at least those concentrations. A potential source of error is, of course, in the serial dilution of tumour in WT DNA. Dilutions were made in factors of 10, such that if the first dilution was inaccurate, this would be exaggerated 10-fold with each dilution. Against this explanation are the observations that, firstly, all 5 sets of serial dilutions for the 5 patients produced sensitivities from 1:100 to 1:1000, and secondly, Stork et al (1991) also reported a sensitivity of 1:100 when PCR products were visualised with ethidium bromide fluorescence. Another possibility may be that the sensitivities were under-estimated because the original tumour sections had significant amounts of surrounding normal tissues. This also seems unlikely because, in order for the mutation to be sequenced, the mutant homoduplex band had to be cut from the DGGE gel and as demonstrated by the study of DGGE sensitivity (Section 2.2.2), a significant proportion of cells (in the region of 20 - 40%) had to be mutant. Therefore, low fraction of tumour cells in the tissue section would not be expected to account for a one log reduction in the detection sensitivity of ARMS.

Whether directly re-infusing tumour cells accounted for the relapse of the 3 patients here is not possible to determine. The 2 patients who had metastatic disease relapsed

much faster (6 and 9 months) than the 2 with stage II/III disease (14 and 17 months), and this difference may well simply reflect the natural history of the disease itself at different stages.

5.6 CLINICAL RELEVANCE OF MRD IN PBPC HARVESTS OF LEUKAEMIA PATIENTS

In this project the MRD study on patients with acute leukaemias was much more straightforward than on breast cancer patients, except for the extremely low incidence of p53 mutations amongst these patients. Apart from 2 patients with a known constitutional polymorphism in codon 213, only 1 other patient had a genuine somatic mutation associated with clonal evolution of his leukaemia as already discussed (Section 3.7.3). Because of the specific nature of this 8 bp insertion, optimisation and screening were easier and a reasonable sensitivity of $1:10^4$ was achieved. During the course of his treatment, PBPCs were harvested on 3 separate occasions after courses of chemotherapy. One of these 3 harvests had evidence of MRD, and his PBPCT involved re-infusion of all of the 3 harvests. It is likely that his disease relapse was partly caused by leukaemia re-infusion, but equally likely is failure of eradication as his leukaemia was extremely aggressive, being refractory to a number of courses of chemotherapy.

5.7 CONCLUSIONS - ARE p53 MUTATIONS USEFUL AS MARKERS OF MRD?

The primary aim of this project was to assess the feasibility of using subtle genetic alterations, e.g., single base changes, as markers for MRD detection, and along with this aim several secondary aims arose (Section 1.6). The answer to the title question of this section may be arrived at by sequentially looking at the steps required in using p53 as an MRD marker. The theoretical basis of using a tumour-specific marker to study MRD is sound. If a cell containing a copy of the mutant gene found in the original tumour is present in another tissue such as PBPCH, tumour contamination is

present within that tissue. What cannot be concluded is that the cell is viable, which is also a drawback of most other MRD techniques except those using cell culture (Sharp et al, 1992). The advantage of tumour-specific markers over lineage-related markers is that false positives arising from other tissues expressing the marker do not occur. For example, RT-PCR for CK19 which is now commonly used may give false positive results in chronic myeloid leukaemia bone marrow (Moscinski et al, 1996) or even healthy controls (Krismann et al, 1995). But the main problem here is the lack of universal application of a single marker. Whilst CK19 can be used as a disease marker for all breast cancers, p53 mutations can only be found in a minority, approximately a quarter in this project. Because the mutations in different tumours are likely to be different, in the main each marker can only be used for the patient in whom it is found. The identification of mutations is also laborious, because 5 different DNA fragments (4 exons) of the gene have to be screened in order to cover over 90% of possible mutations. Although the successful use of a universal gradient of 30 - 80% denaturants does help to simplify the procedure, further steps involving direct sequencing are required to determine the exact nature of each mutation before ARMS primers can be designed to be used in a sensitive MRD assay. This again contrasts with CK19 in which one set of techniques can be applied to all patients studied, without the need for 'tailor-made' conditions for each patient.

The disadvantages of using a multi-step approach aside, ARMS as used in this project suffers badly from a lack of sensitivity and specificity. Optimisation is difficult, on account of the subtlety of the genetic alteration, resulting in the successful optimisation of only 5 of 10 missense mutations. Using ethidium bromide fluorescence to visualise PCR products, sensitivity is poor - in the region of 1:100 to 1:1000. Here, attempts at improving the sensitivity with 32P radiolabelled ARMS or nesting were not successful, but such alternative methods as using radiolabelled internal probes may improve the sensitivity 2 logs (Ehlen and Dubeau, 1989). With the already complex methods, I felt that the addition of dot-blot and probing techniques were unacceptably cumbersome in such a heterogeneous system. The question of sensitivity also throws into some doubt the reliability of the positive MRD results. With the very small

number of patients with a optimisable marker, it is not possible to comment on any correlation between positive MRD screens in PBPCH and survival.

Taken in all, therefore, p53 (single base) mutations are not of value as markers to be used in MRD detection, at least not with the level of tumour contamination one can expect to encounter in PBPC harvests of breast cancer patients. On the other hand, what this project has high-lighted also is that there are situations, such as with the 8 bp insertion of leukaemia patient L11, where less subtle p53 changes can be used as disease markers with a very reasonable degree of sensitivity and specificity. ARMS or allele-specific PCR used in this way can be a very useful technique in the study of cancer biology where ultra-sensitivity is not crucial. Meanwhile, as far as molecular detection of MRD is concerned, the keys to success appear to remain with techniques using large DNA changes or gene rearrangements, or those using RNA as starting material which gives a much higher copy number of the genetic change per unit of weight.

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APPENDIX

Published Works and Presentations

Dang R.K.B., Anthony R.S., Craig J.I.O. and Parker A.C. (1998)
A novel 8-bp insertion in codon 281 of p53 in a patient with acute lymphoblastic leukaemia and 2 separate leukaemic clones.
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Dang R.K.B., Craig J.I.O., Hendry L., Horton Y., Anthony R.S. and Parker A.C. (1997)
Mutations of the p53 tumour suppressor gene in acute leukaemias studied with PCR-DGGE.
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Dang R.K.B., Anthony R.S., Craig J.I.O., Parker A.C. and Leonard R.C.F. (1998)
Potential use of p53 point mutations as markers for minimal residual disease (MRD) detection in the breast cancer model.
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Dang R.K.B., Anthony R.S., Massie C.E., Craig J.I.O., Leonard R.C.F. and Parker A.C. (1998)
Optimisation of p53 allele-specific primers - potential application in minimal residual disease (MRD) detection.
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Dang R.K.B., Anthony R.S., Leonard R.C.F. and Parker A.C. (1998)
The use of p53 gene mutations as markers of minimal residual disease (MRD) detection in breast cancer and leukaemias.
Presented at the Scottish Society for Experimental Medicine meeting, 13th November 1998.