A Novel Assay for the Quantification of Active Transcription Factors

Isabel M. Goodhand

The thesis is submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy of the University of Portsmouth

Translational Oncology Research Centre
Queen Alexandra Hospital

March 2011
A Novel Assay for the Detection of Active Transcription Factors

Isabel M. Goodhand

Transcription factors are a family of DNA-binding proteins involved in the regulation of all cellular processes. One particular area of transcription factor function is the process of apoptosis, the deregulation of which is involved in cancer. The study of transcription factors may therefore provide a novel perspective on cancer aetiology, as well as insight into potential treatment opportunities. However, research into transcription factor activity has traditionally been hindered by technical limitations. Firstly, transcription factors being such potent molecules need only be present at a low concentration within a cell, thus requiring sensitive methods of detection, or a large cell sample for analysis. Secondly, their tertiary protein structures present problems for interactions with inhibitory pharmaceutical agents, limiting their clinical application.

The aim of this project was to develop a method to overcome these obstacles; a method for the quantification of active transcription factors, with the sensitivity for analysing endogenous transcription factors, and the high throughput adaptability as a screen for testing molecules altering a transcription factor’s DNA-binding activity. This method is based on the isolation of a synthetic oligonucleotide probe complexed with the transcription factor of interest, using Protein-A conjugated magnetic bead immunopurification. The synthetic oligonucleotide is subsequently quantified using quantitative Polymerase Chain Reaction. The complex of synthetic oligonucleotide, transcription factor and antibody was shown by the Electromobility supershift assay to form under a defined set of experimental conditions, and the synthetic oligonucleotide reliably detected by quantitative Polymerase Chain Reaction. Following optimisation, this method was able to quantify purified p53, with a sensitivity of 5 ng, However, in the scope of this project, performance was not sufficient for detection of endogenous p53 from LS174T cells. Further optimisations are required to reach this goal in order to compete with commercially available Enzyme Linked Immunosorbent Assay based methods.
Table of Contents

Abstract i
Table of contents ii
Declaration vi
List of figures and tables vii
Abbreviations x
Acknowledgements xii

Chapter 1

Introduction

1.1 General introduction 1
1.2 The transcription factor p53 in apoptosis and cancer 2
1.2.1 p53 biology in the regulation of apoptosis and cancer 4
1.2.2 Understanding apoptosis and cancer using systems biology 8
1.3 Methods for transcription factor study 10
1.3.1 Established methods for the study of transcription factors 10
1.3.2 Novel methods for the detection of transcription factors 12
1.3.3 The Probe Immunopurification assay under development in this project 15
1.4 Cancer diagnostics and drug development 17
1.4.1 Anti-cancer drug design and development 18
1.5 Drug targets 20
1.5.1 Molecular tumour profiling: Biomarkers in drug target identification 20
1.5.2 Transcription Factors as drug targets 21
1.6 Aims and Hypothesis 26
Chapter 2
Materials and Methods 27
2.1 Materials 27
2.1.1 List of solutions 27
2.1.2 Suppliers 28
2.2 Cell culture 29
2.3 Preparation of nuclear protein extracts 30
2.3.1 BCA assay 30
2.4 Western Blot 31
2.4.1 SDS-PAGE 31
2.4.2 Sample preparation 31
2.4.3 Transfer and processing 32
2.4.4 Developing 32
2.5 Electromobility Shift Assay 33
2.5.1 Radioactive labelling of oligonucleotide probe 33
2.5.2 EMSA gel 33
2.5.3 EMSA sample preparation and analysis 33
2.6 Magnetic bead immunopurification using Miltenyi Biotec µMACS system 35
2.7 qPCR 36
2.8 TransAM p53 ELISA 37

Chapter 3
Verification of DNA-Transcription Factor-Antibody complex formation 38
3.1 Introduction 38
3.2 Western blot analysis of cell extract 43
3.3 EMSA to determine experimental conditions for ternary complex formation 46
3.4 Discussion 54
Chapter 4

Initial development of ternary complex immunopurification and qPCR

4.1 Introduction
4.2 Initial method for the Probe Immunopurification Assay
4.3. qPCR optimisation and efficiency for DNA primers and Taqman probe
4.3.1 PCR equipment
4.3.2 qPCR efficiency using the ABI 7500
4.4 Effect of immunopurification components on qPCR
4.5 Magnetic bead immunopurification of ternary complex
4.5.1 EMSA using pure protein
4.5.2 Identifying the assay range of p53 detection
4.6 Verification of signal specificity
4.6.1 Protein specificity: replacing p53 with BSA
4.6.2 Binding specificity: oligonucleotide competition assay
4.6.3 Antibody dependence: omitting antibody in assay
4.7 Verification of assay reproducibility and sensitivity
4.7.1 Inter-assay variation
4.7.2 Intra-assay variation
4.7.3 Sensitivity
4.8 Discussion

Chapter 5

Optimisation of Probe-IP assay performance

5.1 Introduction
5.2 Titration of reaction components
5.2.1 Probe titration
5.2.2 Antibody titration
5.3 Blocking nonspecific probe interactions using BSA
5.4 Optimisation of column washing volume and stringency
5.5 Pre-binding PAb421 and p53
5.6 Discussion
Chapter 6

The detection of endogenous p53 activity in nuclear extracts 118
6.1 Introduction 118
6.2 BCA method for the quantification of total protein in nuclear extracts 119
6.3 Performance comparison with Active Motif ELISA based kit 120
6.4 Discussion 125

Chapter 7

Discussion 127
7.1 Aims and Achievements 127
7.2 Future work 129

8. References 130

9. Appendices see disc

9.1 DNA oligonucleotides
9.2 Raw qPCR data
Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.
List of Tables and Figures

Chapter 1
Figure 1.1. Modular primary structure and covalent modifications of the tumour suppressor p53. 5
Figure 1.2. p53 and the apoptosis network. 7
Figure 1.3. Key steps in the Probe-IP assay. 16

Chapter 3
Figure 3.1. Stages of the probe-IP assay. 39
Figure 3.2. Immunoglobulin structure. 41
Figure 3.3. Western blot analysis of transcription factor p53 in whole cell extracts from LS174T and MCF7 cells. 44
Figure 3.4. EMSA conditions for p53 in LS174T whole cell extract. 47
Figure 3.5. EMSA using buffer #2 at room temperature. 50
Figure 3.6. Effect of antibody titration on p53 supershift using nuclear extract. 52
Table 3.7. Greater supershift enrichment from whole cell extract relative to nuclear extract. 53
Figure 3.8. p53 and PAb421 EMSA supershift from McLure & Lee (1998). 55

Chapter 4
Figure 4.1. qPCR chemistry using the Taqman probe system. 58
Figure 4.2. Schematic structure of the human p53 protein. 59
Figure 4.3. Image of Miltenyi Biotec’s μMACS separation unit. 61
Figure 4.4. Interpretation of raw qPCR data to determine absolute quantities of DNA template, using the ABI 7500 machine. 63
Figure 4.5. Efficiency of qPCR reactions for the BioRad iCycler. 65
Figure 4.6. Standard deviations of CT values within qPCR triplicates compared between iCycler and ABI 7500 qPCR machines. 67
Figure 4.7. Efficiency of qPCR reactions for the ABI 7500. 69
Figure 4.8. qPCR inhibition from reaction components. 71
Figure 4.9. Effect of immunopurification components on probe detection by qPCR.

Figure 4.10. EMSA supershift using recombinant p53 protein (Calbiochem).

Figure 4.11. p53 concentration dependent signal response.

Figure 4.12. Protein specificity in the probe-IP assay.

Figure 4.13. Binding specificity.

Figure 4.14. Non-specific p53-DNA binding.

Figure 4.15. Antibody dependence in the absence or presence of 10 ng and 50 ng p53 protein.

Figure 4.16. Positively charged regions on the surface of a Mouse immunoglobulin IgG2a.

Table 4.17. Inter-assay variation of assay samples and p53+/- from section 4.6.

Figure 4.18. Signal to noise ratio (p53 +/-) in response to probe titration.

Figure 4.19. Change in probe capture over time in the absence of p53.

Figure 4.20. Aspects of EMSA buffer on assay values.

Table 4.21. Intra-assay variation of assay sample triplicates.

Figure 4.22. Sensitivity of Probe-IP assay detection of purified p53.

Chapter 5

Figure 5.1. Probe titration effect on signal to noise ratio.

Figure 5.2. Antibody titration effect on signal to noise ratio.

Figure 5.3. Presence of BSA affects antibody dependence.

Figure 5.4. Presence of BSA does not affect p53 +/- signal to noise ratio.

Figure 5.5. Increased column wash volume reduces signal to noise ratio.

Figure 5.6. Increased column washing stringency depletes signal to noise ratio.

Figure 5.7. Allowing PAb421 to bind p53 prior to the addition of probe improves signal to noise ratio.

Table 5.8. Inter-assay variation of the Probe-IP assay.
Chapter 6

Figure 6.1. BCA method for total protein quantification. 119
Figure 6.2. Binding specificity of the Active Motif TransAM kit determined from a competition experiment. 121
Figure 6.3. Linear range of the TransAM active p53 detection assay. 122
Figure 6.4. Detection of a Cisplatin-dependent increase in active p53 levels. 123
Figure 6.5. Range of LS174T nuclear extract for use in Probe-IP assay. 124
Figure 6.6. p53-DNA binding competition. 125
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABI</td>
<td>Applied Biosystems</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine Triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic Acid</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electromobility Shift Assay</td>
</tr>
<tr>
<td>FAM</td>
<td>6-carboxyfluorescein</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GRN</td>
<td>gene regulatory network</td>
</tr>
<tr>
<td>IP</td>
<td>immunopurification</td>
</tr>
<tr>
<td>MAB</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Mdm2</td>
<td>mouse double minute 2</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>nicotine amide adenine dinucleotide</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonidet P-40 (Igepal)</td>
</tr>
<tr>
<td>NRS</td>
<td>normal rabbit serum</td>
</tr>
<tr>
<td>NTC</td>
<td>no template control</td>
</tr>
<tr>
<td>PAb421</td>
<td>anti-p53 mouse monoclonal antibody</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBM</td>
<td>protein-binding microarray</td>
</tr>
</tbody>
</table>
PBS: phosphate buffered saline
PCR: Polymerase Chain Reaction
PDB: Protein Data Bank
PNK: polynucleotide kinase
Poly(dI-dC): poly(deoxyinosinic-deoxycytidylic) acid sodium salt
qPCR: quantitative (real time) Polymerase Chain Reaction
ROX: carboxy-X-rhodamine
RPM: revolutions per minute
RT-qPCR: reverse transcriptase quantitative (real time) Polymerase Chain Reaction
SDS: sodium dodecyl sulphate
SPIKE: signalling pathway integrated knowledge engine
ss: supershift
ssDNA: single stranded DNA
SPR: Surface Plasmon Resonance
STDev: standard deviation
TAMRA: tetramethylrhodamine
TEMED: $N, N', N', -$tetramethylethylenediamine
TF: transcription factor
Acknowledgements

I am grateful to the BBSRC and Vertex pharmaceuticals for financing this project.

I would like to thank my parents and grandparents for helping me through the destitution of my final year, and all my family & friends who saw me through some difficult times.

My thanks also go out to everyone at the Biophysics and TORC laboratories for all their help and ideas. Most importantly of all, I would like to thank my supervisors Matt Guille and Ian Cree for their continued scientific and moral support. I would not have completed this project without them.
Chapter 1

Introduction

1.1 General introduction

The cell is formed of distinct, but interconnected molecular systems. Whilst the genome carries the same information throughout all somatic cells of an organism, phenotypic heterogeneity between cell populations arises instead from the dynamic nature of the transcriptome and proteome. Functioning at the interface of these three molecular systems are a family of DNA-binding proteins known as transcription factors. Involved in all cellular processes, a transcription factor is able to modulate the expression of a target gene by directly binding regulatory elements of the genome.

One example of transcription factor function is in the process of apoptosis, also known as programmed cell death, which results in the removal of either unnecessary but harmless cells, or those cells exhibiting various functional abnormalities. Apoptosis presents a barrier to the proliferation of those cells whose genomes have become damaged beyond repair, and as a result may present a threat to the organism as a whole. Impaired regulation of apoptosis is a key characteristic of one of the most notorious pathologies currently affecting the developed world: Cancer.

Cancer is one of the leading causes of mortality in the developed world, responsible for a quarter of adult deaths in the United States and United Kingdom (Jemal et al., 2010; Cancer in the UK: July 2010, 2010). Consequently, treatment and prevention of cancer is a major area of scientific research including the main topic explored here: improving existing assays for transcription factor activity, thus translating scientific knowledge into clinical application for both diagnostics and drug development. With such a pivotal role in the molecular basis of cancer, to study transcription factors may yield a novel perspective on cancer aetiology, providing insight into potential treatment opportunities. However, due to technical limitations, this has not always been a productive area of
research. Firstly, transcription factors being such potent molecules need only be present at a low concentration within a cell, thus requiring sensitive methods of detection, or a large cell sample for analysis. Secondly, their tertiary protein structures generally present problems for interactions with inhibitory pharmaceutical agents. This project focuses on technology with a potential to overcome both limitations: by developing a novel method with the sensitivity to quantify the activity of endogenous transcription factors, and having the high throughput adaptability as a screen for testing molecules altering a transcription factor’s DNA-binding ability. The transcription factor p53 is used as a model for assay development.

These topics are discussed here in further detail, including the role of the transcription factor p53 in apoptosis and cancer, and the relationship between molecular oncology and clinical application. How further insight into the role played by transcription factors may facilitate cancer diagnostics and treatment strategies in the future will also be considered.

1.2 The transcription factor p53 in apoptosis and cancer

A cancer cell can be defined by a manifestation of six physiological traits not observed in a normal cell (Hanahan & Weinberg, 2000), these being:

- Self-sufficiency in growth signals
- Insensitivity to growth inhibitory signals
- Evasion of apoptosis
- Limitless replicative potential
- Sustained angiogenesis
- Tissue invasion and metastasis

Although these six processes are all interlinked, this section focuses on how evasion of apoptosis is integral to tumour development, and how the transcription factor p53 is in turn key to the control of apoptosis.
Apoptosis, also termed programmed cell death, is characterised by nuclear and cytoplasmic condensation, dilated endoplasmic reticulum and membrane blebbing (Kerr, Wyllie & Currie, 1972). This process of apoptosis is triggered by the release of cytochrome c from the mitochondrial inter-membrane space into the cytosol. Cytochrome c subsequently complexes with Apaf-1, forming the Apoptosome (see Figure 1.2), which catalyses the conversion of procaspase 9 into its fully functional form Casp9. This begins a proteolytic cascade, resulting in cleavage of cellular proteins by executioner caspases 3, 6 and 7; and consequently, disintegration of the cytoskeleton, nuclear membrane and mitochondria. Damage to the mitochondria releases reactive oxygen species into the cell, causing further cell damage, and finally apoptotic bodies are observed in place of the former cell, to be removed by neighbouring cells or macrophages (Weinberg, 2006). Although a role for apoptosis exists in routine tissue maintenance, removing cells when and where they are not needed, for example in the sculpting of features during embryonic development, apoptosis also functions to remove those cells found to be in some way faulty and potentially dangerous. Only the latter involves the transcription factor p53.

The transcription factor p53 was first identified in 1979 via two different approaches. Studies into the viral cause of cancer looking at the SV40 virus found that a 55 kDa antigen co-immunoprecipitated with the large T antigen from SV40-transformed cells (Lane & Crawford, 1979, cited by Kress et al., 1979). This 54-55 kDa antigen, initially thought to be a viral protein, was overexpressed in many different transformed cells, including those not infected with SV40 (Linzer & Levine, 1979). Furthermore, partial peptide maps showed sequence similarity between different cell types, but all were very different from the viral-encoded SV40 large T antigen (Linzer & Levine, 1979; Kress, 1979), indicating that this antigen was instead host-encoded. Published in the same year were serological studies identifying antibodies against a 53 kDa antigen in the sera of mice bearing several different tumour types (DeLeo et al., 1979). It was only realised later that this was in fact the same antigen identified in both sets of studies (Crawford 1983, cited by Soussi 2010), and it was subsequently termed p53. The function of p53 was initially misclassified as an oncogene, owing to the finding that transfection of p53 into normal cells led to transformation (Eliyahu et al., 1984; Jenkins et al., 1984; Parada
et al., 1984, cited by Soussi, 2010). The researchers were not aware that these observations were a result of mutant p53 exerting a gain of function activity, in the form of a dominant negative effect. At the time, this oncogene theory also explained the observed accumulation of p53 in tumour cells (Crawford et al., 1981). The oncogene theory of p53 was later contradicted by genetic studies, with the finding that p53 mutations and loss of p53 expression were present in many cases of both murine and human osteosarcoma (Matsuda et al., 1987; Chander et al., 1992). p53 was redefined as a tumour suppressor when it was shown that transfections of cDNA derived from healthy cells in fact suppressed transformation in oncogene-activated cells (Finlay et al., 1989), and that p53-inactivating genetic abnormalities were observed in a variety of lung and colorectal cancers (Takahashi et al., 1989, cited by Soussi, 2010; Baker et al., 1989). The function of p53 was first associated with apoptosis when this process was observed in cancer cell lines transfected with wild-type p53 (Yonish-Rouach, 1991, cited by Shaw et al., 1992; Shaw et al., 1992), and its central role in apoptosis became increasingly apparent from there on (reviewed by Vousden & Lou, 2002).

Since apoptosis poses a threat to cancer cells, inactivation of this process is highly advantageous to tumour progression, with apoptotic integrity posing a selective pressure to the cancer cell population. The importance of p53 function in tumour progression is indicated by the fact that missense mutations are observed in 50% of all human cancers (Soussi et al., 2006). Furthermore, 95% of these occur in the DNA-binding domain (Vousden & Lu, 2002), highlighting the importance of p53-DNA interaction in its function as a tumour suppressor. Thus, the p53 pathway presents a major potential route for anti-cancer therapy, particularly by reinstating apoptotic function, which will be discussed in section 1.5.2.

1.2.1 p53 biology in the regulation of apoptosis and cancer

Transcription factors function to regulate expression of a target gene by directly binding the upstream regulatory promoter sequences. This regulation can occur by directly affecting the ability of the basal transcriptional complex (including RNA polymerase and basal transcription factors) to bind DNA. Alternatively, histone modification can be
affected, which in turn leads to both a complex system of altered chromatin compaction for access of the transcription machinery to DNA, and recruitment of various proteins involved in transcription. Corepressor or coactivator proteins may also be recruited by the transcription factor as a further level of regulation (reviewed by Latchman, 1997; Fedorova & Zink, 2008). Transcription factors have specialised structural domains allowing direct interaction with DNA. Figure 1.1 shows the primary structure of the tumour suppressor p53 as an example. It can be seen that transcription factor structure is modular, with individual regions assigned to individual functions, such as sequence-specific DNA binding, and transactivation.

Figure 1.1. **Modular primary structure and covalent modifications of the tumour suppressor p53.** From the N-terminus: Transcription activation domains (TADs), the proline-rich domain (PP), the sequence-specific DNA-binding core, the linker region (L), tetramerisation domain (Tet). Specific post-translational modifications dictate interactions with specific proteins and hence outcome (Vousden & Prives, 2009).

P53 binds dsDNA as a tetramer (McLure & Lee, 1998) in both a sequence-specific and sequence-independent manner. Sequence-independent binding is conferred by the C-terminal domain (Wang et al., 1993), which enables p53 to slide along DNA constantly scanning the genome for specific sequences (Hinow et al., 2006; Tafvizi et al., 2011). Sequence-specific binding is conferred by the central DNA-binding domain (Wang et al., 1993; Bargonetti et al., 1993) (shown in green, Figure 1.1); the DNA consensus.
being defined as two or more copies of the 10 bp half-site \( 5'\)-PuPuPu-C(A/T)(A/T)G-PyPyPy-3', which can be adjacent or separated (el-Deiry et al., 1992; Funk et al., 1992). How the activity of p53 is regulated, and how this affects cell phenotype is also illustrated in Figure 1.1.

A transcription factor may itself be regulated at the level of transcription. It may be expressed in one cell type or developmental stage, but there is also the need for regulation at the level of the protein itself. Basal p53 levels are regulated by a negative feedback loop whereby the p53 antagonist protein Mouse double minute 2 (Mdm2) (Momand et al., 1992; Wu et al., 1993) constantly targets p53 for degradation via the ubiquitin-proteasome system. E3 ubiquitin ligase activity of Mdm2 catalyses the formation of polyubiquitin chains onto lysine residues of the p53 C-terminal domain, resulting in the translocation of p53 into the cytoplasm for proteasome-mediated degradation. With a half-life of only 15 to 25 minutes (Finlay, 1993), the high turnover rate of p53 therefore maintains the cell in a state primed for response. Once a cell is subject to stress stimuli including DNA damage (Maltzman & Czyzyk, 1984; Fritsche et al., 1993), imbalances of intracellular signalling pathways resulting from oncogene activity (Palmero et al., 1998; Zindy et al., 1998) and hypoxia (Graeber et al., 1994), an increase in active p53 concentration is quickly observed. This is due to a decrease in degradation rather than an increase in synthesis. For example, DNA damage is detected via the Ataxia telangiectasia mutated (ATM) kinase, resulting in phosphorylation at the N-terminus of p53, protecting it from Mdm2 binding and ubiquitylation. ATM also inactivates Mdm2 by direct phosphorylation (Weinberg, 2006). The resulting increase in p53 concentration can lead to either cytostasis or apoptosis, depending on a variety of factors including post-translational modifications dictating selectivity in target gene activation, as shown in Figure 1.1., although many more modifications have been mapped (reviewed by Meek & Anderson, 2009).

This section has outlined the function of p53 as a mediator of apoptosis in response to cell stress. However, the layers of regulation in this process generate such complexity that for full appreciation of studies looking at how the activity of a transcription factor such as p53 is involved in cancer progression, a different perspective is required.
Figure 1.2. **p53 and the apoptosis network.** The position of p53 in the apoptosis network is shown (TP53 node with black border). Regulatory relationships are indicated by blue directed edges: either arrows for activation or T-shape for inhibition. Green edges and nodes represent components of a complex. Violet nodes correspond to protein-coding genes, and yellow nodes to protein families. Red and green dots within a node indicate that not all known regulation and containment relationships are displayed (Paz, 2009: SPIKE software).
1.2.2 Understanding apoptosis and cancer using systems biology

As can be seen in Figure 1.2., apoptosis does not occur as an isolated event. On the contrary, to understand the regulation of this process as an aspect of neoplasia, the bigger picture must be taken into consideration. How the cell responds to its environment depends on the interaction of a vast number of intracellular component molecules forming the cell’s biological network. Although each component, be it protein, nucleic acid, metabolite, lipid or metal is interconnected, the network can be broadly separated into three sections: signalling, regulatory and metabolic (Hyduke & Palsson, 2010). Transcription factor activity is modulated mainly as an output of signalling networks. Although functioning at the interface of proteome and genome, transcription factors themselves are a critical part of gene regulatory networks (GRNs) (Davidson, 2008).

The advent of high throughput technologies for acquiring genome-wide data on these networks has facilitated the integration of biology with mathematical modelling and computational simulation, forming the discipline known as Systems biology. Models of biological networks are formed based on a variety of mathematical calculations (formalisms) and experimental observation to describe and subsequently predict the overall outcome in response to changes in upstream perturbations (i.e. gain of function or loss of function mutations, or environmental factors). For example, how does the perturbation profile of a cancer cell affect processes such as apoptosis and proliferation, and how does this determine the cell’s response to anti-cancer therapeutics?

Genome-wide experimental observations of GRNs are largely based on the cDNA microarray or RNA-seq providing data on mRNA concentrations, and the ChIP-on-chip or ChIP-seq methods (Johnson, 2007, cited by Ravasi, 2010), which map transcription factor occupancy throughout the genome (discussed in the next section). Regulatory relationships are inferred in response to a defined perturbation. In order to infer a GRN model from experimental data, elements are first determined to form a parts list. A topology model of these will then be constructed describing the connections between these elements (i.e. the
edges between the nodes). An example of this is Figure 1.2., formed using the Signalling Pathway Integrated Knowledge Engine (SPIKE) programme. It must be taken into account that since these parts are differentially expressed, their observation is dependent on experimental conditions. Various formalisms have been developed to describe the rules regulating interactions within a GRN (reviewed by Schlitt & Brazma, 2007), and which model to use presents a compromise between descriptive detail and computational cost. Boolean networks (Kauffman, 1969) represent one of the most simple formalisms, relying on binary functions (AND, OR, NOT) to describe discrete states, for example describing a gene as either expressed or silent. Although the computational cost of a simple binary system may allow for the description of larger systems, GRNs cannot be fully described by a discrete model. On the contrary, the outcome of cellular decisions arising from GRN activity depends on quantitative parameters, and the integration of quantitative data on transcription factor activity into formalisms may be valuable in order to aid predictions. Since there are many cases where protein and mRNA concentration changes exhibit discordance (Newman et al., 2006; Gygi et al., 1999; Wasburn, 2003), numerous studies have developed mathematical models to infer transcription factor activity from the mRNA profile of target gene expression (Liao et al., 2003; Boulesteix & strimmer, 2005; Sanguinetti, rattray & Lawrence 2006; Barenco et al., 2006; Rogers, Khanin & Girolami, 2007; Wang & Tian, 2010).

Sanguinetti, Rattray & Lawrence (2006) define transcription factor activity as “the concentration of the transcription factor at a certain experimental point and its binding affinity to its target genes”. This definition assumes active transcription factor concentration is directly related to target gene expression. However, owing to both transcription factor and promoter-specific regulatory mechanisms involved in transcription, the concentration of active transcription factor and the level of target gene expression must be treated as separate entities. For example, it has been noted that expression of p53 target genes does not uniformly correlate with the kinetics of p53-promoter binding (Szak et al., 2001). Further, following UV-C induced DNA damage, an increase in p53 occupancy at the promoter of its target and antagonist Mdm2 is observed, although the level of Mdm2
mRNA instead incurs a drop (Kaeser & Iggo, 2002). This indicates the activity of additional factors in the DNA damage signalling pathway functioning to repress Mdm2 synthesis and disrupt the p53 auto-regulatory loop, and therefore to model the p53 network based on inference of target gene expression alone would yield confounding data. On the other hand, to provide direct data on transcription factor activity may provide insight into the quantitative control of GRNs.

1.3 Methods for transcription factor study

The study of transcription factors relies on the performance of existing methods. This section discusses the technical basis of methods able to observe transcription factor concentrations; their strengths and limitations, and the advantages offered by a novel method developed in response to the challenges presented. Novel technologies emerging will also be discussed, including the method under development in this project.

1.3.1 Established methods for the study of transcription factors

The majority of established methods for detecting a specific protein fundamentally rely on a specific antibody against the protein of interest, which can be detected by labelling of the antibody. There are numerous techniques relying on antibodies for specific protein detection, including Western blot (Burnette, 1981) whereby the analyte is separated by SDS-PAGE and transferred to a nitrocellulose membrane for probing by a specific antibody, which in turn is probed with a labelled secondary antibody for detection. The Enzyme Linked Immunosorbant Assay (ELISA) is also a common method of specific protein detection, whereby a 96-well plate is coated with the analyte to be detected by antibody (Engvall & Perlmann, 1971). Alternatively, the plate can be coated by antibody used to capture the specific protein from the analyte, which is then detected using an antibody recognising a different epitope, thereby increasing the assay's specificity. These two assays can detect proteins of any property, but DNA-binding proteins can also be detected by Chromatin Immunoprecipitation (ChIP) (Solomon et al., 1988). The ChIP
assays endogenous chromatin fragmented by sonication. The analyte is mixed with antibody specific to the protein of interest, which is bound to protein A/G Sepharose beads, substantially increasing antibody mass. Fragments of antibody-bound chromatin containing the protein of interest are isolated from the remaining chromatin based on mass, and the DNA isolated from the chromatin. This DNA can then be quantified by PCR, detected and identified by microarray, known as ChIP-on-chip, or sequenced, which is known as ChIP-seq (Johnson, 2007, cited by Ravasi, 2010). The problem surrounding the ChIP is that it can only detect proteins bound to the endogenous DNA, whereas some transcription factors subject to upregulation may be unbound in the analyte, and so is not a direct representation of transcription factor concentration. Another widely used method for studying DNA-binding proteins is the gel-shift assay, also known as the Electromobility Mobility Assay (EMSA) (Fried & Crothers, 1981; Garner & Revzin, 1981). This semi-quantitative assay is commonly used in the analysis of DNA-protein interactions. The reasoning behind this assay is that, as radiolabelled DNA migrates through a native polyacrylamide gel under an electrical current, the binding of a protein will impede its migration toward the anode in two ways: increased mass and shielding of the negative charge conferred by the DNA phosphate backbone. This results in a higher band, known as a gel shift (or band shift). Furthermore, when an antibody specific to the protein of interest is added, under optimum conditions the binding of the antibody to the complex will impede migration to an even greater extent, resulting in an even lower mobility, which is known as a supershift.

An example of an assay not relying on an antibody is the Exonuclease protection assay (Galas & Schmitz, 78). Exonuclease III (Exo III) is a monomeric enzyme, which exhibits 3’-5’ exonuclease activity, catalysing digestion of ssDNA from the 3’ end of a blunt ended dsDNA molecule (Rogers & Weiss, 1980; Henikoff, 1984 cited by New England Biolabs, n.d.). The presence of this enzyme following a DNA-protein binding reaction will digest any unbound DNA, and the remaining DNA is analysed either by gel electrophoresis or by PCR.
1.3.2 Novel methods for the detection of transcription factors

The methodologies discussed so far are still used in the majority of studies, since they represent the gold standard of DNA-protein analysis, without which data may be criticised as unreliable. However, an increasing number of novel assay methodologies are being published; and this section will discuss the strengths and limitations of each, as well as how they compare to the assay theory under development in this project. These methods can be broadly separated into spectroscopic, immunological and enzymatic digestion based approaches.

Förster Resonance Energy Transfer (FRET) is a technique used to study the occurrence of biomolecular interactions. Each molecule is labelled with either a donor fluorophore or an acceptor chromophore. Interaction at a distance specific to each FRET pair causes the donor emission spectrum to overlap with the acceptor absorption spectrum. On excitation by light energy, this overlap allows energy to pass from donor to acceptor, resulting in fluorescence (Forster, 1948, cited by Wang et al., 2005). There are several methods based on FRET. The molecular beacon assay relies on two labelled probes, each encoding half the protein binding site. The annealing of the two probes is driven by the DNA-binding protein, bringing the donor and acceptor into close proximity resulting in a high FRET signal (Dummitt & Chang, 2006). Catabolite activator protein (CAP) and lacR proteins were detected at nanomolar concentrations in one study using pure protein (Heyduk & Heyduk, 2002). An advantage of this technique lies in the real-time detection of the FRET signal. Additionally, unlike alternative techniques, the molecular beacon approach uses a “one-step” protocol, i.e. it does not rely upon multiple incubations and washing steps; the DNA and protein are mixed, and the signal is detected, lending itself to high throughput application. However, FRET has a short dynamic range (10-100 Å), therefore a fluorophore and a quencher on either side of the binding site limits the length of DNA for analysis and results in a low FRET signal. An alternative technology to FRET is alternating-laser excitation (ALEX) spectroscopy. This technology differs from FRET, in that one half-site is labelled with a green fluorophore, and the other a red. Two lasers alternately excite either
the red or the green fluorophore, allowing detection of both half-sites, and their proximities to one another are measured by FRET occurrence to determine the proportion of those half sites bought together by protein binding, to those unbound. Lymperopoulos et al., (2010) used this technique to study CAP in HeLa cells, detecting an increase on plasmid-induced expression of 20-30% of total protein, which was consistent with SDS-PAGE detection at 40%. It is also interesting to note that as the oligonucleotides can permeate cells with use of electroporation, lipofectamine and liposomal transfection, this may also have application in vivo. However, despite the potential of FRET for in vitro applications, a barrier remains against the study of endogenous transcription factors. On one hand, it may be seen as an advantage not relying upon an antibody for detection, since antibodies are expensive and are not commercially available for all proteins. On the other hand, many proteins bind non-specifically to any given DNA sequence at sufficient concentrations, and therefore specificity in these assays may not be sufficiently reliable without the use of an antibody.

With this in mind, Surface Plasmon Resonance (SPR) can be used to study a wide range of biomolecular interactions (Liedberg et al., 1983; Flanagan & Pantell, 1984., cited by SPR History, 2009). SPR measurement is based on the observed change in angle of reflected light from a thin gold surface, which depends on the presence of molecules immobilised to the other side. The ligand (for example DNA or antibody) is first chemically immobilised to the surface, and the analyte (for example a DNA-binding protein) is then added in solution. The binding of the analyte to ligand is measured in real time, and the signal change is observed until equilibrium is reached. Signal drop is observed on washing the surface; and in this way, reaction kinetics and equilibrium measurements can thus be obtained. This method of detection therefore has the benefit of being label-free, but also the flexibility that if a lower limit of detection is necessary for the particular experiment, antibodies can also be used to increase the molecular weight (and therefore detection) of the specific complex (Su et al., 2008). However, SPR requires expensive specialist equipment, and heterogeneity in the concentration of immobilised ligand may affect results. Furthermore, if high ligand density is required for sufficient detection of binding, this can be problematic for the accurate calculation of reaction kinetics. Firstly, if the rate at which
the immobilised ligands bind the analyte exceeds the rate at which the analyte can be delivered to the surface (referred to as mass transport), then mass transport becomes the rate-limiting step, and the measured association rate constant ($k_{on}$) is superficially slower. Secondly, following dissociation of the analyte excess unoccupied ligand will be available for re-binding before being washed away, resulting in a superficially slower measured dissociation rate constant (apparent $k_{off}$).

The novel technologies discussed so far are also limited by cost, and for this reason, the most successful transcription factor assays seem to be those based on the conventional Enzyme Linked Immunosorbant Assay (ELISA), as an internet search of “Transcription Factor assay” indicates. Gubler & Abarzua (1995) initially developed a method to detect a complex of antibody, p53 and biotinylated dsDNA. This was then added to an anti-IgG coated microwell plate, and the biotinylated dsDNA detected using streptavidin-conjugated alkaline phosphatase. A second ELISA approach is commercially available as the TransAm kit marketed by Active Motif, which uses a sandwich ELISA system, but replaces the capture antibody with a capture oligonucleotide containing the transcription factor consensus sequence (Renard et al., 2001). This has a stated sensitivity of detecting 0.5 µg of nuclear extract per well, which is 5 times more sensitive than EMSA (Active Motif, retrieved 2010). In addition to kits that supply plates with pre-immobilised capture oligonucleotide, Flexi kits are now also available which allow the operator to use a sequence of choice for studying protein binding sites. This assay is amenable to automation since the whole assay is performed in a single well of a plate, and systems in place may not need much alteration from traditional ELISA set-ups. The one limitation of this assay is the detection system, which relies upon the enzyme activity of an HRP-conjugated antibody to produce a substrate for colourimetric analysis, thereby limiting assay sensitivity. Lin et al., (2010) have described a method using streptavidin-labelled Microspheres to capture a biotinylated dsDNA probe encoding a transcription factor consensus sequence, which in turn captures the transcription factor of interest. The transcription factor is detected using a fluorescently labelled antibody analysed by flow cytometry. Again, detection of nucleic acid offers greater sensitivity when compared to antibody detection.
The third main approach described in this section is the Exonuclease III protection assay, which detects DNA and therefore has the ability to take advantage of the sensitivity offered by qPCR, which in some systems has been shown to detect 10 copy numbers of a template (Applied Biosystems, 2004). Initially, Chen et al., (2006) describe a technique using the DNA intercalating dye SYBR Green I with the Exo III protection assay. DNA probe encoding the transcription factor consensus sequence is protected from Exo III digestion if protein is bound, resulting in a SYBR Green I fluorescent signal. This signal drops on probe digestion by Exo III in the absence of transcription factor. NF-κB was used as a model for this assay, which detected in 0.1 mg/ml of NF-κB in nuclear extract from TNF-α induced HeLa cells. The same research group then adapted this method to encompass qPCR (Hou et al., 2007), performing a standard Exo III protection assay, and quantifying the remaining probe by qPCR. Although specific and non-specific oligonucleotide competition experiments showed binding specificity, they were using TNF-α-induced expression of NF-κB in HeLa cells. The abundance of specific transcription factor relative to other nuclear proteins in a different system and analysing different transcription factors may be a lot lower. As with the FRET approach, this Exo III based method is limited by lack of specificity conferred by the use of an antibody based technique. Therefore, in this project we are investigating the combined specificity of an antibody-based approach with the detection sensitivity of qPCR.

1.3.3 The Probe Immunopurification assay under development in this project

Another approach described in the literature for the identification of transcription factor binding sequences, is known as the McKay assay (McKay, 1981, cited by el Deiry et al., 1992 and Szak et al, 1999). The transcription factor is captured by antibody cross-linked to Sepharose beads, to which the DNA probe is subsequently added. The captured probe is then isolated and analysed by gel electrophoresis. In this project, we adapt this assay for the application of quantifying active transcription factors either in a cell or nuclear extract, or in an in vitro system, with increased sensitivity of the detection system, and the potential for high throughput application. The method is outlined in Figure 1.3. The synthetic DNA
probe encodes sequences enabling subsequent amplification and quantification by qPCR. In addition, the probe/Transcription factor/Antibody complex will be purified by Protein-A magnetic beads instead of Sepharose, and an Exo III protection digest will be performed, potentially improving sensitivity. It is hypothesised that the concentration of transcription factor present in the original sample will be directly reflected by the concentration of probe detected by qPCR.

Figure 1.3. **Key steps in the Probe-IP assay.** Cell, nuclear extract or pure transcription factor (TF) is first mixed with DNA probe (step 1) and antibody (step 2), and incubated. Protein A/G magnetic beads are then added (step 3), and further incubated. After incubation, the solution is transferred to the magnetic bead µMACS protein A/G microbead kit column produced by Miltenyi Biotec (step 4). The column is washed, and the complex eluted on removal of the magnetic field; Exonuclease III (Exo III) is added to digest unbound probe, and remaining probe quantified by qPCR (step 5).
The transcription factor chosen as a model for this assay is p53. This is due to both interest and practicality. As previously discussed, p53 is a tumour suppressor whose cellular concentration is increased in response to cell stress, such as DNA-damage and hypoxia, leading to cytostasis or apoptosis (Giaccia & Kastan, 1998; Asker, Wiman & Selivanova, 1999). The high mutation rate observed in cancer affecting the DNA binding ability of p53 presents the possibility of various future studies using the transcription factor-DNA binding assay under development in this project (this is discussed in more detail in section 1.5.2). In addition, since it is such a well-studied protein, there are many antibodies available, including those verified for EMSA supershift, meaning the antibody does not interfere with DNA binding activity, which is central to this assay. Equally important, endogenous p53 levels in cultured cells can be easily increased to aid detection whilst the assay is under development, and there are many publications available detailing optimised p53-DNA binding conditions and binding buffers, giving us a head start in our assay development.

As with most technologies, there is considerable flexibility in how this can be applied. This will be discussed further in section 1.7 at the end of this chapter, once the context into which this assay fits has been discussed in the next two sections, and its importance thus highlighted.

1.4 Cancer diagnostics and drug development

The role of transcription factors in the molecular processes underpinning cancer, and their methods of study have been discussed. Sections below outline the clinical aspect of cancer: both established and novel strategies of diagnostics and treatment, and how a sensitive and high throughput method of transcription factor analysis may benefit clinical research and drug development.
1.4.1 Anti-cancer drug design and development

Many cancer treatments still used today evolved before the molecular basis of cancer was known. These were first developed in the 1940’s, when autopsy results of soldiers dying of nitrogen mustard in the First World War showed lymphoid hypoplasia and myelosuppression. From these observations, it was reasoned that measured doses of a similar agent could be used in the treatment of a lymphatic tumour (reviewed by Gilman, 2005). These first approaches to anti-cancer drug development were based solely on observations of effects on human physiology, with no insight into the mechanism of action.

However, with the advance in knowledge of signal transduction pathways in cancer, the focus is now shifting to mechanism-based rational drug design, which consists of the following steps (Druker & Lydon, 2000; Workman & Kaye, 2002; Weinberg, 2006).

1. Drugs should be targeted against specific proteins with a causative role in cancer pathology
2. Further candidacy of these proteins as attractive targets should be determined by their predicted “drugability”
3. Molecular structures of targets should be used to inform the design of chemical structures of drugs that are to be developed. These drugs should be developed to fit in a lock and key fashion into specific sites within the protein target.

The most notable success in this process is the drug Gleevec. CML (Chronic Myelogenous Leukaemia) is cytogenetically marked by what is known as the “Philadelphia” chromosome (Nowell & Hungerford, 1960, cited by Nowell, 2007), which is a translocation between chromosomes 9 and 22 (Rowly, 1973). This results in a fusion of the ABL gene (a nonreceptor tyrosine kinase) with the BCR gene, encoding the BCR-ABL protein, which is a tyrosine kinase with enhanced signalling activity. This is an example of a target that is both selective, since it is not expressed in a patient’s normal cells, and which also has a causative role in the pathology, responsible for approximately 95% of all CML cases (Witte, 1993). Random screening of compounds exhibiting BCR-ABL tyrosine kinase
inhibition led to the discovery of a selective inhibitor with affinity for the ATP-binding pocket of BCR-ABL (Druker, Tamura & Buchdunger, 1996).

Another example of a targeted therapy is the use of the monoclonal antibody as an anti-cancer agent. The EGFR-related receptor HER2 (ErbB2 or Neu) is overexpressed in 25-30% of cancers, and has been associated with poor clinical outcome (Slamon et al., 1987). The genomic amplification of the HER2 gene results in 10-100 times more receptor on the cell surface, which results in amplification of mitogenic signals. Because of the prevalence of HER2 mutations, and the accessibility of a receptor, being on the cell surface, this was considered a target for monoclonal antibody therapy. Several monoclonal antibodies with their epitope on the HER2 extracellular domain, which specifically inhibit growth of tumour cell lines with HER2 amplification were investigated (Fendley et al., 1990), and one was chosen for development, now named Trastuzumab (Herceptin). This development required the murine-derived antibody to be “humanised”, that is, the cDNA encoding the murine Fc region to be replaced with the human to prevent immune response (Carter et al., 2002). This humanised antibody was found to be active against breast cancers with HER2 amplification (Slamon et al., 1998; Pegram et al., 1998), and in 1998 was approved for clinical use.

From the successful examples of Gleevec and Trastuzumab given here, the direction of anti-cancer drug development towards the targeted approach is evident. This is an expensive strategy, in which more efficient High Throughput compound screening technologies will be of great benefit.

High throughput screening (HTS) is defined as screening 10,000 and 100,000 compounds per day, and is integral to finding lead compounds to provide a chemical structure as a starting point for further optimisations leading to development of a suitable drug compound. There are many different assays described for use in HTS, including enzyme reactions (e.g. kinases, transferases and proteases), receptor binding and macromolecular and immunological interactions. Cell-based assays are described, which have the advantage
of discovering compounds with the desired phenotype of inhibiting tumour cell growth compared with normal cell growth, and compounds identified will also be cell-permeable. Reporter gene assays are also possible in HTS format (Reviewed by Aherne, McDonald & Workman, 2002), so it is reasonable to hope that the Probe-IP assay developed in this project may also lend itself to HTS application.

1.5 Drug targets

The shift in drug development strategies toward a target-based approach poses the initial challenge of finding a target worth pursuing. This section looks at how the molecular profiling of cells aids the identification of these targets, and how transcription factors are increasingly being investigated as potentially specific and effective targets.

1.5.1 Molecular tumour profiling: Biomarkers in drug target identification

Profiling a cell at the molecular level seeks to define a set of “Biomarkers”, or molecules whose expression can be correlated with a specific phenotype (Khan et al., 2001), clinical outcome (Lin et al., 2007; Lopez-Rios et al., 2006), or response to therapy (Ganly et al., 2007; Ayers et al., 2004) i.e. diagnostic, prognostic and predictive. The majority of the studies in this area employ the cDNA microarray to identify Biomarkers at the level of the transcriptome (reviewed by Clarke, Poele & Workman, 2004; Liotta & Petricoin, 2000). For example, a prognostic study now approved by the US Food and Drug Administration is the MammaPrint (Agendia) diagnostic classifier. Van’t veer et al., (2002), identified 70 genes associated with poor prognosis (metastases and reoccurrence) from the cDNA analysis of 98 primary breast tumours. This classifier was further verified (van de Vijver et al., 2002; Buyse et al., 2006), and adapted for clinical use (Glas et al., 2006). Analysis of a biopsy classifies patients into high and low risk categories, informing decisions on treatment strategy.
Although the cDNA profile may contribute to identification of different disease states, transcriptome data alone provide little insight into the phenotypic traits of a cell. This is because the protein encoded by the mRNA, not the mRNA itself, are the functional components of the cell, and many studies have shown that significant differences can occur between the transcriptome and proteome (Chen et al., 2003; Humphery-Smith, Cordwell & Blackstock, 1997; Newman et al., 2006; Gygi et al., 1999; Washburn, 2003). This technology is further limited by variations observed depending on the statistical analysis used (Tan et al., 2007). The possibility of proteomic Biomarkers present in blood, serum or plasma has also been explored based on technologies such as mass spectrometry and the antibody array. However, to date these technologies have produced limited clinical application (reviewed in Kulasingam et al., 2010; Brennan et al., 2010).

An alternative to profiling the entire transcriptome or proteome is to focus on profiling transcription factors. Since a transcription factor can regulate the expression of hundreds of downstream genes, producing a profile of active transcription factor concentrations may also identify Biomarkers. Transcription factor combinations, rather than in isolation, have been shown as important for determining cell fate (Ravasi et al., 2010), and this would perhaps provide insight into the GRN control of the disease state. Furthermore, as a transcription factor Biomarker is more likely to play a causal role in cell fate, these may present themselves as potential drug targets. Although transcription factors have traditionally been thought of as “undruggable”, there are an increasing number of examples to negate this idea.

1.5.2 Transcription Factors as drug targets

Several problems have arisen with the increase in those targeted therapies discussed in section 1.4.1. PTKs pose problems as targets due to the level of redundancy present in signalling cascades, together with examples of Gleevec resistance due to further, acquired BCR-ABL mutations. Unlike PTKs which function within signalling pathways upstream of gene expression, transcription factors make attractive drug targets due to their position at
the interface of signalling networks and target gene expression; there are many more potentially oncogenic signalling proteins upstream of transcription factors than there are transcription factors themselves. Therefore altering the activity of a transcription factor is more likely to have a greater effect on cell physiology compared with other network components, which may have a level of functional redundancy with other pathways.

Traditionally, the general consensus within the literature and the pharmaceutical industry as a whole, was that however much transcription factors might be attractive targets, in practice their lack of ATP binding domain for interaction with drug compounds as competitive inhibitors makes this challenging (Hopkins & Groom, 2002; Overington, Al-Lazikani & Hopkins, 2006; Imming, Sinning & Meyer, 2006).

However, there are many different levels whereby the activity of a transcription factor can be disrupted. For example in the case of c-myc, as well as looking at blocking transcription factor-DNA binding, there are also studies looking into blocking translation of mRNA with siRNA, disrupting heterodimerisation with its partner protein max, and targeting c-myc for proteosome degradation. The two approaches that are of interest in the context of this project, i.e. that may benefit from a sensitive and quantitative assay to detect transcription factor-DNA interaction are the blocking of myc/max dimerisation, which prevents DNA binding, and direct prevention of myc/max binding to DNA.

Small molecules for the interference of myc/max heterodimerisation have been identified by compound screens based on FRET. This identified four compounds from a library of 7000, two of which were able to inhibit the growth of myc-transformed chicken fibroblast cells (Berg et al., 2002). A similar screen was also performed by Xu et al., (2006) from a credit-card library of 285 structures based on a planar aromatic scaffold, to fit between the interface of the myc and max interaction. They found several hits, which were also shown to inhibit growth of myc-transformed chicken fibroblasts. As for interfering with c-myc/DNA interaction, one method explored is dsDNA decoy oligonucleotides to compete with genomic DNA binding sites. They have been shown to inhibit the growth of human
breast carcinoma cells (MCF-7) and neuroblastoma cells within 24-48 hr of exposure (El-Andaloussi et al., 2005).

As a potential target for cancer therapy, c-myc is overexpressed in a broad range of different cancers, from a translocation in Burkitt’s lymphoma causing overexpression, to overexpression in response to upstream signalling via the EGFR pathway (reviewed by Vita & Henriksson, 2006). However, rather confusingly c-myc has a role in both apoptosis and proliferation, with studies producing conflicting results with regards to the effect of treatment with Topoisomerase II inhibitors doxorubicin and etoposide. In one study, apoptosis in c-myc<sup>-/-</sup> rat fibroblasts in response to doxorubicin and etoposide was found to be dependent on c-myc (Grassilli, 2004). Conversely, Bidwell & Raucher (2004) found anti-proliferative effects of these drugs were increased in human breast, cervical and uterine carcinoma cells after pre-treatment with a polypeptide inhibiting c-myc/max dimerisation and transcriptional function. These two studies may reflect the effect of cell type on proliferative and apoptotic pathways involving c-myc, and this may present problems with c-myc as a therapeutic target in cancer.

Another interesting example of a transcription factor currently under investigation as a drug target is the tumour suppressor p53. Missense mutations in p53 are observed in 50% of human cancers (Soussi et al., 2006). Furthermore, 95% of these occur in the DNA-binding domain (Vousden & Lu, 2002), highlighting the importance of p53-DNA interaction in its function as a tumour suppressor. From this, it was reasoned that reinstating the DNA-binding capability of p53 should result in tumour regression, and this was indeed found in two studies. Ventura et al., (2007) used a cre-loxp gene expression system in mice with lymphoma and sarcoma to control expression of wild type p53. Owing to a loxp flanked stop codon in the first p53 intron, these constructs lacked wild type p53 expression. Administration of Tamoxifen released cre recombinase from fusion with an oestrogen receptor domain, resulting in excision of the stop codon, and thus allowing expression of full-length wild type p53. On treatment with Tamoxifen, tumour regression was observed in both cancer types, but interestingly via apoptosis in lymphomas, and via cytostasis in
sarcomas. A similar study by Xue et al., (2007) used RNA interference to regulate wild type p53 expression in murine liver carcinoma, finding tumour regression also by cellular senescence, but that an innate immune response was also triggered resulting in complete tumour clearance. These studies provide support that reinstating p53 function may well result in a very effective treatment for many different cancer types.

Screening studies have discovered small molecules, including CP-31398, WR-1065, PRIMA-1, and MIRA-1, that reinstate DNA binding capability of mutant p53 (Bykov et al., 2002a, 2002b, 2005; Foster et al., 1999). Bykov et al., (2002a), discovered the molecule PRIMA-1, which can restore sequence-specific DNA binding. They found this is dependent on reinstating p53’s wild-type conformation. This compound was shown to induce p53-dependent apoptosis in human tumour cells, and suppressed the growth of human tumour xenografts expressing mutant p53. Lambert et al., (2009) found PRIMA-1 functions by being converted by hydrolysis to a product that reacts with nucleophiles, alkylating thiol groups on cysteine residues within the core domain of p53. Instead of merely conferring thermostability on mutant p53 to maintain the protein in a folded state, it seems that PRIMA-1 in fact reverses unfolded conformation of p53 molecules already accumulated within a cancer cell. This is thought to trigger a greater apoptotic effect, since cancer cells expressing mutant p53 are observed to have especially high p53 concentrations (Thomas et al., 1983), due to mutant p53’s inability to induce Mdm2 expression and the subsequent negative feedback loop (Wu et al., 93).

Rapid advances allowing transcription factors to become drug targets in oncology presents two problems. Firstly, selecting the transcription factor targets most effective in the control of cell growth and proliferation; and secondly, selecting an assay with HTS application to detect transcription factor-DNA interaction and interference in response to chemical agents. The discovery of these small molecules that reinstate p53’s DNA binding capability offers an interesting context for the Probe-IP assay design explored in this project.
PRIMA-1 was discovered by a cell-based screen for compounds that selectively inhibit growth of mutant-p53 expressing tumour cells. Whilst the DNA-binding assay approach discovers those compounds that modulate DNA binding regardless of the in vivo situation, the cell-based screen is unbiased toward the molecular mechanism of action. A cell-based screen will consider bioavailability and toxicity, but the molecular mechanism of action may also be indirect (for example involving co-factor function), and therefore be environment or cell-type specific. For a target such as p53, where 95% of mutations affect the DNA-binding domain (Vousden & Lu, 2002) our Probe-IP assay may offer the advantage in respect of time and high throughput adaptability.

As with all methodologies, the Probe-IP design has limitations. The detection relies on the ability of an antibody to bind the transcription factor. A chemical compound may be affecting the transcription factor’s conformation, or shielding the epitope to prevent antibody detection, but the DNA binding domain may retain the same function as in the absence of chemical modifier, and a screen may therefore yield false-positives. Furthermore, it provides a simplistic view on the concept of transcription factor activity; that this alone is reflected by DNA binding ability is not always the case. For example, the transcription factor complex AP-1 may comprise of a variety of transcription factors from the Fos and Jun families. Either c-Fos or Fra-1 can heterodimerise with any of the jun proteins, and although they have similar DNA-binding activity and specificity, the Fra-1 protein lacks a transactivation domain present on c-Fos (Suzuki et al., 1991). Furthermore, phosphorylation increases the transactivation potential of proteins such as c-Jun and c-Fos, with no effect on DNA-binding activity (Smeal et al., 1992; Deng & Karin, 1994). Despite these limitations, the Probe-IP assay may still narrow down the identification of compounds of interest for further investigation.
1.6 Hypothesis and aims

It is hypothesised that the Probe-IP method will enable the accurate and sensitive quantification of active transcription factors; and this project aims to develop the Probe-IP method for the detection of both purified and endogenous active p53.

To summarise this introduction, a niche has been highlighted for a quick, robust, high throughput assay in the screening of transcription factor-DNA interactions; and the possible applications of this assay are outlined below:

**In vitro assay system**

- Screening libraries for compounds altering protein-DNA binding activity. For example, finding an agent to reinstate mutant p53-DNA binding, or to block c-myc-DNA binding ability
- Testing antibodies against a DNA-binding protein
- Comparing DNA binding sites of DNA-binding proteins

**Endogenous transcription factor assay system**

- Understanding the quantitative nature of GRNs
- Understanding the role of post-translational modifications using specific antibodies
- Biomarkers for comparing disease states or response to therapies
Chapter 2

Materials and Methods

2.1 Materials

2.1.1 List of Solutions

Accugel: 40% (w/v) acrylamide: bisacrylamide 29: 1

Column Equilibration buffer: 1x Triton x-100 in EMSA Buffer

DNA dilution buffer: 1 mM MgCl₂, 75 mM NaCl

Elution buffer: 10 mM Bis-Tris-Propane-HCl (pH 7.0), 10 mM MgCl₂, 1 mM DTT

EMSA Buffer #1: 20 mM Tris-HCL pH 7.5; 10% Glycerol; 5 mM DDT; 1% NP40; 100 mM NaCl

EMSA Buffer #2: 20 mM HEPES-KCL pH 7.9; 10% Glycerol; 0.1 mM EDTA; 0.5 mM DTT; 0.025% NP40; 25 mM KCL; 2 mM MgCl₂

PBST: PBS (pH 7.4), 0.02% Tween-20

SDS-PAGE loading buffer (1x): 50 mM Tris-HCL pH 6.8, 2% (w/v) SDS, 10% (v/v) Glycerol, 0.05% (w/v) Bromophenol blue, 100 mM DTT

T4 Polynucleotide Kinase buffer (1x): 70 mM Tris-HCL (pH 7.6), 10 mM MgCl₂, 5 mM DTT
TBE buffer: 89 mM Tris Borate pH 8.3, 2 mM Na₂ EDTA

TBST: 150 mM Tris-HCL pH 7.5, 10 mM NaCl, 0.05% Tween-20

TNE: 10 mM Tris-HCL (pH 8.2), 1 mM EDTA, 0.1 mM NaCl

Tris-Glycine buffer: 25 mM Tris, 192 mM Glycine, 0.001% SDS

Western blocking solution: 5% (w/v) Marvel-TBST, 150 mM Tris-HCL pH 7.5, 10 mM NaCl, 0.05% Tween-20

Western developing solution A: 2.5 mM Luminol/DMSO; 100 mM Tris pH 8.5; 0.4 mM p-Coumaric acid

Western developing solution B: 100 mM Tris pH 8.5; 0.02% H₂O₂

Western transfer solution: 20 mM Tris, 150 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol

2.1.2 List of Suppliers

All chemicals and reagents were purchased from either Applied Biosystems, GE Healthcare (Amersham biosciences), Flowgen Bioscience, National Diagnostics, New England Biolabs, Pierce, Roche or Sigma Aldrich. Recombinant purified protein was purchased from Calbiochem (Merck), who also supplied antibodies together with Santa Cruz Biotechnology and Abcam. DNA oligonucleotides were purchased from Sigma genosys, and the magnetic beads were purchased from Miltenyi Biotec or Invitrogen. The nuclear protein extraction and TransAM p53 kit were purchased from Active Motif.
2.2 Cell Culture

MCF7 breast adenocarcinoma cells were cultured in RPMI-1640 medium supplied by Sigma-Aldrich, complemented with 2 mM L-glutamine (Sigma), 1% penicillin-streptomycin (Sigma) and 10% Foetal Bovine Serum (FBS; Sigma). LS174T colorectal adenocarcinoma cells were cultured in Minimum essential medium (MEM), supplied by Sigma-Aldich, complemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% v/v FBS and 1% v/v penicillin-streptomycin. Cells were grown at 37º C in the presence of 95% air; 5% CO₂, and media renewed every few days to maintain cells in a logarithmic phase of growth. Once a flask confluence of approximately 70% was reached, cells were harvested as follows: media was removed and cells rinsed with PBS. 3 ml of 0.25% w/v trypsin-EDTA solution (Sigma) was added per 75 cm² flask (Corning, Fisher), and incubated at 37° C until visibly detached. Cell suspensions were transferred to a 30 ml tube containing 10 ml of RPMI for MCF7 cells or MEM for LS174T cells, and centrifuged for 7 minutes at 217 g using the MSE Harrier 15/80 centrifuge. The cell pellet was resuspended in 10 ml of phosphate buffered saline (PBS). To determine viability and cell count, a sample of 20 μl was added to an equal volume of trypan blue (Sigma), mixed and counted using the automated Cellometer (Nexcelom). Cell suspension was then aliquoted in samples of 10⁶ cells and centrifuged for 7 minutes at 217 g. The supernatant was discarded and cell pellets either stored at -80° C until use, or nuclear extracts prepared as outlined in section 2.3.

For LS174T cells treated with Cisplatin (local pharmacy, Queen Alexandra Hospital, Portsmouth), once a confluence of 70% was reached, fresh media was added supplemented with none, 0.2, 2, 5 or 10 µM Cisplatin. Cells were incubated overnight as normal, and harvested after 24 hours as previously described.
2.3 Preparation of nuclear protein extracts

Nuclear protein extracts were prepared from either MCF7 or LS174T cells, using the Active Motif nuclear protein extraction kit. A pellet of $10^6$ cells was re-suspended in 5ml pre-chilled (to 4º C) PBS/phosphatase inhibitor, centrifuged at 217 g for 7 minutes at 4º C to pellet cells, and the supernatant removed, which was replaced with 3 ml of PBS/phosphatase inhibitor. The cells were again centrifuged at 217 g for 7 minutes at 4º C, and the cells re-suspended in 500 µl of Hypotonic buffer. After incubation at 4º C for 15 minutes, 25 µl of detergent was added, and the cells vortexed for 10 seconds. The cells were then centrifuged at 14000 g for 30 seconds at 4º C to pellet nuclei. The nuclei were re-suspended in 50 µl complete lysis buffer by gentle pipetting, vortexed for 10 seconds and incubated for 30 minutes at 4º C with gentle agitation on a grant plate shaker set to 150 rpm. The nuclei were then vortexed again for 30 seconds and centrifuged at 14000 g for 10 minutes at 4º C. The suspension of nuclear protein was transferred to a clean chilled microtube, and stored at -80º C until required.

2.3.1 BCA assay

Total protein concentration in the nuclear extract samples were determined by the Bicinchoninic Acid (BCA) assay kit purchased from Pierce (Perbio). One sample was analysed per batch of nuclear extractions, as samples are damaged by freeze-thaw cycles. Manufacturer’s instructions were followed. Working reagent was prepared for a sufficient number of samples by mixing reagents A and B together in a 50:1 v/v ratio. Nuclear extract samples were diluted ten-fold to ensure they were in the range of the standard curve. This standard curve was prepared using bovine serum albumin (BSA) at concentrations of: 0, 25, 125, 250, 500, 750, and 100 µg/ml. Each sample was mixed with working reagent with a ratio of 1:20 v/v, and a final volume of 1 ml. Samples were incubated at 37º C for 30 minutes, and allowed to cool to room temperature, before the absorbance was read at 562 nm using the Jenway Genova spectrophotometer. The plot of the BSA standard curve was used to interpolate the amount of total protein in nuclear extract samples.
2.4 Western Blot

2.4.1 SDS-PAGE

The SDS-PAGE apparatus (10 cm x 12 cm size, Atto) was set up, and a discontinuous gel prepared as follows: National Diagnostics Protogel resolving buffer containing 12% National Diagnostics Protogel (30% w/v acrylamide/methylene bisacrylamide solution 37.5:1 ratio) was polymerized with 0.1% w/v Ammonium persulphate and 1.2% v/v TEMED (Sigma). The solution was poured immediately between the glass plates of the gel apparatus, layered with isopropanol and left to polymerize fully. Once set, the isopropanol layer was removed and the stacking gel prepared. National Diagnostics Protogel stacking buffer, containing 6% acrylamide (National Diagnostics Protogel 30% w/v acrylamide/methylene bisacrylamide solution 37.5:1 ratio), was polymerized with 0.1% w/v Ammonium persulphate and 1.2% v/v TEMED (Sigma). This was immediately poured over the resolving gel, and left to set.

2.4.2 Sample preparation

Uniform sample loading onto the gel was made difficult by viscosity conferred by the high concentration of DNA present. Therefore, samples were first sonicated to fragment this DNA. Each pellet of $10^6$ cells was suspended in 100 μl of PBS/protease inhibitor (mini EDTA-free protease inhibitor tablets, Roche). The samples were then placed on ice and sonicated using the Vibra cell sonicator, with a 2 mm probe at 30% amplitude for 3x10 seconds. An equal volume of 2x SDS loading buffer was added, and samples were vortexed for 15 seconds, heated to 100° C for five minutes, and centrifuged for three minutes at 14000 g using the Eppendorf 5415D microcentrifuge. Samples were then loaded onto the gel and run at 150 V for two hours in Tris-Glycine buffer. A pre-stained protein marker (SeeBlue Plus2, Invitrogen) was run adjacent to the samples for determining the molecular weight of visualised proteins.
2.4.3 Transfer and processing

To transfer proteins from the gel to the Nitrocellulose membrane (H-Bond ECL membrane, Amersham Biosciences), the Trans-Blot transfer cell apparatus (Bio-Rad) was set up with the gel and membrane as per manufacturer’s instruction, surrounded by Western transfer solution. The transfer was run at 300 mA for two hours. Once complete, the membrane was incubated in Western blocking solution to prevent non-specific binding of the antibody, for two hours at room temperature or overnight at 4° C. The blocking solution was discarded, and the primary antibody added at appropriate concentration depending on each antibody used (see results section) in blocking solution containing either 5% w/v Marvel for two hours at room temperature if background was found to be high, or in blocking solution containing 0.5% w/v Marvel overnight at 4° C to increase signal intensity. The membrane was then washed four times for 15 minutes in Western blocking solution at room temperature, prior to addition of secondary antibody, diluted 4000-fold for anti-mouse, 10000-fold for anti-chicken and anti-rabbit in Marvel-TBST as for primary antibody. The membrane was then washed at room temperature twice in blocking solution for 15 minutes, once in TBST for 15 minutes and twice in PBS for 15 minutes.

2.4.4 Developing

Developing solutions A and B were mixed together and poured onto the membrane, which was agitated for 30 seconds and left for a further 30 seconds, before exposure for 10 minutes with the Fujifilm LAS-3000 imager.
2.5 Electromobility Shift Assay

2.5.1 Radioactive labelling of oligonucleotide probe

A reaction of 10 µl containing 15 pmol of dsDNA probe, 1 µl T4 Polynucleotide kinase (New England Biolabs), 1 µl T4 polynucleotide kinase buffer (New England Biolabs), and 1 MBq \( \gamma^{33} \text{P} \) ATP (Perkin Elmer) was incubated for 30 minutes at 37° C. The labelling reaction was terminated by the addition of 90 µl TNE. The sample was then divided between two G-50 Sepharose spin columns (GE Healthcare), and unincorporated radioisotope removed from the labelled DNA by centrifuging for 30 seconds at 12,000 g.

2.5.2 EMSA gel

The gel apparatus was assembled, the glass plates cleaned with 70% ethanol, and secured with clips. Native 0.25x TBE, 6% polyacrylamide gels were made with 6% Accugel (National Diagnostics) and 0.25x TBE, which was polymerized with 0.1% w/v Ammonium persulphate and 1.2% v/v TEMED. The solution was immediately poured between the gel plates, and the comb added to form the sample wells once set. The gels were pre-run at 190 V in 0.25x TBE buffer for two hours at 4° C to remove free radicals and small molecules from the gel, whilst also allowing a constant current and temperature to be reached.

2.5.3 EMSA sample preparation and analysis

For optimising conditions of ternary complex formation, different approaches to sample preparation were performed. Factors affecting the occurrence of supershift (the ternary complex formation), and which were altered in different experiments will be briefly mentioned here, but are covered in more detail throughout Chapter 3.

- The comparison of different EMSA buffers; the components and concentrations of which affect the ternary complex formation in different ways
• The comparison of using whole cell or nuclear extract as analyte
• The order of adding the DNA probe and antibody to the analyte
• The incubation temperature

A pellet of either 10^6 cells, or 10^6 cells worth of nuclear proteins was resuspended in 10 µl of PBS/protease inhibitor (complete mini EDTA-free, Roche). Enough EMSA 1x buffer was added to ensure 10 µl of cell extract, or 5 µl nuclear extract per lane (120 µl and 60 µl per gel, respectively). EMSA 1x buffers used were named EMSA Buffer #1 and EMSA Buffer #2.

Samples were centrifuged at 14,000 g for three minutes at 4° C using the Eppendorf 5417R microcentrifuge, and added to a clean 1.5 ml tube. Negative controls contained EMSA 1x buffer alone. From this point, different protocols were used by adding components to the reaction in a different order. For the protocol whereby the antibody is added before the probe, antibody or normal rabbit serum (NRS) negative control was added at a concentration of 0.1 µg, 0.2 µg, 0.5 µg or 1 µg, and incubated for 45 minutes either on ice (at 4° C) or at room temperature. Enough probe and poly(dI-dC) (Sigma) were mixed to allow approximately 0.15 pmol of probe in a volume of 2 µl and 100 ng of poly(dI-dC) in a volume of 0.5 µl per lane, and 1.5 µl added to each sample. All samples were incubated for a further 15 minutes either on ice or at room temperature, before being loaded onto the gel. For the protocol whereby the probe is added to the analyte before the antibody: enough probe and poly(dI-dC) were mixed to allow approximately 0.15 pmol of probe in a volume of 2 µl and 100 ng of poly(dI-dC) in a volume of 0.5 µl per lane. To samples containing either whole cell extract, nuclear extract or EMSA buffer alone, 1.5 µl of this probe/poly(dI-dC) mix was added, and the samples gently mixed. Antibody (or NRS as a negative control) was added at a concentration of 0.1 µg, 0.2 µg, 0.5 µg or 1 µg, and all samples incubated for 15 minutes either on ice at (4º C) or at room temperature.

The samples were loaded onto the gel and run for either two or four hours at 190 V, depending whether it was important for the free probe to be visualised on the gel, or more
important to have improved separation of the DNA-protein or ternary complex. The gels were laid on two sheets of 3 mm paper, covered in saran wrap and vacuum dried for 90 minutes. The saran wrap was removed and the gels expose to a Fujifilm BAS-MS 2325 imaging plate placed inside a Fujifilm BAS cassette overnight. The imaging plate was read with the Fujifilm FLA-5000, IPS 653 nm red laser and IP filter.

2.6 Magnetic bead immunopurification using Miltenyi Biotec µMACS

This forms the main basis of the project. The method has undergone modification, which is discussed in the results chapters 4 and 5. This section will therefore give a description of the method following the protocol modifications later discussed.

The method was modified from the manufacturer’s instructions to fit the requirements of the assay, which is to purify an antigen complex of protein-DNA as opposed to protein alone. The µMACS Protein A kit was purchased from Miltenyi Biotec, containing the apparatus, columns and protein A conjugated magnetic beads required for the immunopurification. All buffers used were prepared using Sigma ultra-pure dH₂O. Due to viscosity, igepal was weighed to make a 10% v/v solution for adding to buffer, whilst glycerol was weighed directly. All solutions were filter sterilised through a 2 µm filter and de-gassed prior to the assay by sonication for 15 minutes at 30 kHz with the Ultrawave sonicator bath.

For the Miltenyi Biotec immunopurification procedure, samples were prepared as found by EMSA to be the most effective protocol for ternary complex formation, discussed in Chapter 3. All components are prepared and kept on ice. A mix is prepared to contain those components present in the same quantities in all samples, reducing error arising from handling of small volumes. This contains:

- 14 µl EMSA 2x buffer,
- 2 µl of 14x protease inhibitor (Roche mini EDTA free)
• 1 µg of anti-p53 antibody PAb421, at a concentration of 100 ng/µl
• Desired concentration of LS174T nuclear extract or pure p53 in 2 µl volume (diluted in EMSA 1x cooled on ice)

This mixture is aliquoted into Eppendorf DNA LoBind 1.5 ml tubes (Sigma), and incubated for 15 minutes at room temperature on a rocking platform. 0.054 pmols of probe (re-annealed overnight and diluted in DNA dilution buffer) is then added in a volume of 2 µl, and incubated for a further 45 minutes at room temperature on a rocking platform. As instructed in the Protein A magnetic bead protocol, samples are diluted to 1 ml with 1x EMSA buffer, to which 50 µl magnetic beads are added. Samples are then incubated for 30 minutes on a rocking platform at 4º C. The μMACS columns are placed in the magnetic holder, and washed with 100 µl of degassed equilibration buffer, followed by 300 µl of 1x EMSA buffer. 1600 µl of the sample is then applied to the column, and sample tubes rinsed with 100 µl of wash buffer, centrifuged on pulse for 12 seconds and added to column once initial sample has flowed through. Column is washed with 900 µl 1x EMSA buffer containing 120 mM NaCl, followed by 100 µl of 20 mM Tris pH 7.5 buffer and 100 µl of elution buffer (NEBuffer #1). For sample elution, column is removed from magnet, and placed over a clean 1.5 ml tube (Eppendorf DNA LoBind). 100 µl of NEBuffer #1 elution buffer is applied to column and allowed to flow through. This is the antibody-bound sample.

2.7 qPCR

The probe of the ternary complex captured in the immunopurification procedure was detected using qPCR. The probe in the assay is also termed the qPCR “template” (not to be confused with the Taqman probe used in the qPCR method). The template was designed from a random DNA sequence, and analysed by Applied Biosystems Primer Express software to ensure suitability of the amplicon, primers and Taqman probe for efficient amplification by qPCR (see Appendices for analysis). The Taqman probe consisted of a
FAM reporter fluorophore, and TAMRA quencher fluorophore. See Appendices for oligonucleotide sequences.

To a 96 well plate, 10 µl of master mix (Applied Biosystems) and 5 µl of primer/probe mix containing 600 nM of primer and 200 nM Taqman probe were added, the concentrations of which were determined by a checkerboard assay (see results section 4.2). The plate was centrifuged at 2000 g for two minutes to remove any air bubbles. For the BioRad iCycler, the machine was calibrated for use of FAM/TAMRA fluorophores by running a well factor plate, each well to be used in the assay contained 20 µl of 1x well factor solution (Applied Biosystems), and was run prior to the assay plate. Using Applied Biosystem’s 7500 machine, no well factor plate was required. 5 µl of template DNA, or eluent from the microbead column was added to the corresponding assay well, and was spun again at 2000 g. The assay plate was run on either the BioRad iCycler, or the Applied Biosystem’s 7500 or 7900, using a programme of 95º C for 10 minutes followed by 40 cycles of: 95º C for 15 seconds followed by 55º C for 1 minute.

2.8 Active Motif TransAM p53 DNA-binding assay

Manufacturer’s instructions were followed (Active Motif, n.d.). The optical density was read at 450 nm, with a reference reading at 620 nm, using the Anthos Labtec HT2 microplate reader.
Verification of DNA-Transcription factor-Antibody complex formation

3.1 Introduction

This project aims to develop a novel assay for the detection of active transcription factors, as discussed in section 1.3.3 of the Introduction. The assay protocol can be broken down into three main sections, which will be investigated and optimised individually in this project. These sections are covered in Figure 3.1. on the following page, and comprise the first step, which is the association of the DNA probe and Transcription factor (in the case of this project, the tumour suppressor p53) with the anti-p53 monoclonal antibody PAb421. This complex of DNA, protein and antibody will be termed the ternary complex. The second step is the immunopurification of the ternary complex, to which an Exonuclease III digestion of unbound DNA probe will also be carried out, followed by the third step, which is the qPCR detection and quantification of the DNA probe.

This chapter focuses on the first step of the probe-immunopurification (probe-IP) assay, which is to ensure the formation of the ternary complex under a defined set of experimental conditions. This ternary complex is formed by the occurrence of two macromolecular interactions: the protein-DNA interaction of the transcription factor with the DNA probe; and the protein-protein interaction of the transcription factor with the antibody. A variety of non-covalent forces contribute to varying extents toward a particular macromolecular interaction, and therefore each requires specific conditions for optimum formation. Here we seek to optimise conditions for these two different types of interaction simultaneously.
There are a variety of conserved tertiary structures found in the DNA binding domains of transcription factors. Close proximity is required between these and the DNA, as the strength of non-covalent forces is dependent on the distance between the two interacting groups. The non-covalent forces involved in the interaction between the transcription factor and DNA are as follows (described in Berg, Tymoczko & Stryer, 2001, p. 9-10):
• Hydrogen bonds form when a donor hydrogen atom, which is covalently bonded to a relatively electronegative atom (such as nitrogen or oxygen) has an attraction to another acceptor electronegative atom. The energy of this bond is usually in the region from 5-30 kJ/mole.

• Van der waals interactions result from interactions between electron orbitals of adjacent atoms.

• Hydrophobic interactions occur between non-polar, hydrophobic amino acid side chain groups. Water molecules hydrophilically exclude non-polar groups.

• Electrostatic (or ionic) bonds occur between oppositely charged side chains of amino acids. Together with hydrogen bonding, they form what are known as salt bridges. They are relatively rare interactions, but the strength of a bond can approach that of a covalent bond.

Antibodies, or immunoglobulins, as shown in Figure 3.2, consist of two main regions: the Fc and Fab regions. The Fab region is responsible for antigen binding, relying on all four of the previously mentioned non-covalent forces. Hydrophobic forces are the predominant force of this antibody-antigen interaction, and may contribute up to half of the interaction’s total strength (Roitt, 2001, p. 71-74).

In addition to attractive forces between molecules, repulsive forces also play an important part in defining the specificity of antibody-epitope interaction. The overlap of electron orbitals results in steric clash, the strength of which is inversely proportional to the 12th power of the distance between orbitals. The antibody has some flexibility in the fit of the epitope, and some amino acids may be displaced, however the overall energy of attraction must be greater than that of repulsion.
Figure 3.2. **Immunoglobulin structure.** The N terminal consists of the variable heavy ($V_H$) and variable light ($V_L$) chains, whilst the rest of the structure is relatively constant, consisting of constant heavy ($C_H$) and constant light ($C_L$) chains. The different domains are stabilized by disulphide bonds. Proteolytic digestion yields the Fc (fraction crystallization) region, and the antibody binding Fab (fraction antigen binding) regions. (Roitt *et al.*, 2001).

The effects of different experimental factors on macromolecular interactions include:

- **Nuclear or Cell extract, or purified protein**
  Excluding non-nuclear proteins from the reaction decreases the amount of background in the reaction, due to a decrease in nonspecific protein-DNA interactions. Purified proteins may be less active than endogenous ones due to the purification process.

- **Reaction buffer components**
  Concentrations of detergent, NaCl, KCl and MgCl$_2$ are optimum for different interactions, with the buffer depending on the protein of interest. In the case of p53, the concentration of univalent cations (NaCl or KCl) is important to consider, as increasing the concentration above 5 mM reduces access of the antibody PAb421 to its epitope, but p53-DNA interaction also decreases (Butcher *et al.*, 1994). Detergents, such as igepal (NP-40) are
therefore also usually present in binding buffers in order to unfold the protein tertiary structure enough to expose the epitope.

- Order of adding components to reaction
Antibodies are raised to protein without DNA bound. Bearing in mind that protein conformation alters on DNA binding, pre-forming the protein-DNA complex may induce conformational change in the protein, sufficient to shield the epitope from the antibody. On the other hand, pre-forming the protein-antibody complex risks interfering with DNA-protein interaction. However, each antibody-epitope interaction is different, and returning to the example of p53, despite low salt conditions Hainut et al. (1994) found that PAb421 was able to bind p53 once specifically bound to DNA.

- Incubation temperature
Greater movement of molecules occurs at room temperature, and is closer to the physiological temperature of DNA-protein interactions. However, proteins may be sensitive to degradation and remain better intact kept on ice at 4º C. This is particularly true of p53, which has a short half life of approximately 30 minutes at physiological temperatures (Ogretmen & Safa, 1997).

For both the protein and DNA, and protein and antibody interactions to occur simultaneously only a limited set of reaction conditions are likely to be suitable. EMSA (the theory of which was described in section 1.3.1) was used to observe the experimental conditions required for ternary complex formation. Prior to this it was decided to verify the cell extracts contained sufficient levels of p53 protein for analysis by EMSA, using Western blot.
3.2 Western blot analysis of cell extract

The Western blot method was used to determine the presence of the transcription factor p53 in whole cell extracts from the cell lines LS174T and MCF7. This ensured the protein of interest was present in the analyte at levels sufficient for further analysis by EMSA.

The Western blot method is a common method used for detecting a specific protein with its corresponding antibody. All proteins within the whole cell extract are first denatured and separated by their MW using SDS-PAGE. The proteins within the PAGE matrix are then transferred to a nitrocellulose membrane using an electric current to pull the proteins toward the anode (proteins will have a net negative charge conferred from the SDS). This nitrocellulose membrane is then blocked for non-specific antibody interaction, and probed with a specific antibody against the protein of interest.

Since TFs are present in the cell at relatively low concentrations, specificity was compromised for increased sensitivity in the Western blots performed in this section. Incubating the primary antibody overnight instead of two hours, but using a lower concentration (0.5% w/v) of milk protein resulted in increased non-specific binding of the antibody to the nitrocellulose membrane, but increased intensity of the p53-specific band.
**Figure 3.3.** Western blot analysis of transcription factor p53 in whole cell extracts from LS174T and MCF7 cells. MCF7 and LS174T cell extracts were subject to SDS-PAGE, and proteins transferred to a nitrocellulose membrane using the Western blot method. The membrane layered with proteins present in both cell extract types was probed with anti-p53 PAb421 (Calbiochem). A chemiluminescence detection system probed for primary antibodies with HRP-conjugated secondary antibody. SeeBlue plus 2 pre-stained protein molecular weight (MW) marker from Invitrogen was used for protein identification by size. On a single gel, the p53-specific band is visible in LS174T cell extract, at approximately 53 kDa, but is not visible in MCF7 extract.
In LS174T whole cell extract, a thick band of signal is observed at approximately 53 kDa when probed with the anti-p53 antibody PAb421, as shown in Figure 3.3. It should be noted that although the observed molecular weight suggests a protein of approximately 53 kDa, the molecular weight of p53 as calculated from amino acid composition (NCBI Accession BAC16799.1; UniProt primary Accession P04637) is actually 43.69 kDa. It is thought this discrepancy is due to the proline-rich primary structure of p53, which prevents binding of the standard amount of SDS (Segrest & Jackson, 1972; Hames 1990, cited by Simpson, 2003), resulting in a less negative charge of the protein, slowing migration through the SDS-PAGE gel and hence giving the impression of a protein with the larger molecular weight of 53 kDa.

The band visible at 53 kDa in Figure 3.3 can therefore be identified as p53 specific, and shows the presence of p53 in LS174T, but not MCF7 whole cell extract. Although MCF7 cells are known to express wild type p53 (Ogretmem and Safa, 1997), the cell types were not cultured in parallel. It may be that slight differences in confluence at harvest, timings of media changes before cell harvesting, or possibly the process of washing and harvesting the cells, rendered the L2174T cells more stressed, stimulating a rise in p53 concentration; or rendered p53 protein degraded in the MCF7 cells. Whichever one of these possibilities was the cause of this difference in p53 presence between the two cell extracts, they were aliquots from batches of extracts treated under the same conditions. Therefore, it was decided to pursue the EMSA supershift using the LS174T cells as analyte, to study the optimum conditions for ternary complex formation.
3.3 EMSA to determine experimental conditions for ternary complex formation

Since Western blot analysis exhibited detection of sufficient levels of p53 in the LS174T cell extract, EMSA will now be used to determine the experimental conditions for ternary complex formation. As a starting point for these conditions described in section 3.1, a search on Pubmed with the term “p53 Supershift” yielded buffer conditions under which supershifts have been successfully observed, which we have termed Buffer #1 and Buffer #2:

Buffer #1: 20 mM Tris-HCl pH 7.5; 10% Glycerol; 5 mM DDT; 1% NP40; 100 mM NaCl (Gubler & Abarzua, 1995)

Buffer #2: 20 mM HEPES-KCL pH 7.9; 10% Glycerol; 0.1 mM EDTA; 0.5 mM DTT; 0.025% NP40; 25 mM KCL; 2 mM MgCl₂ (Pitkänen et al., 1998; Zupnick & Prieves, 2006)

This search also yielded an antibody able to interact with DNA-bound p53: the monoclonal PAb421 purchased from Calbiochem (Merck). The antibody to be used for both the EMSA supershift and the probe-IP assay is extremely important, which is why the choice to use PAb421 was based on its wide use in published research articles (Pitkänen et al., 1998; Zupnick & Prieves, 2006). Hanson et al. (2005) also found that PAb421 enhances the sequence-specific DNA binding ability of p53, in the presence of nonspecific competitor DNA. PAb421 binding to its epitope on the C-terminus of p53 interferes with its nonspecific DNA binding activity (Tafvizi et al., 2011), reducing the time p53 is spent sequestered on nonspecific DNA. With knowledge of EMSA supershift buffers and a reliable antibody source, the first experiment compared the two buffers, incubation temperature, and order of adding the antibody and probe.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Buffer 1</th>
<th>Buffer 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>temperature</td>
<td>4°C</td>
<td>RT</td>
</tr>
<tr>
<td>Serum addition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>order</td>
<td>first</td>
<td>last</td>
</tr>
<tr>
<td>Serum type</td>
<td>NRS</td>
<td>Ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image of gel electrophoresis with bands labeled SS and free probe]
Figure 3.4. **EMSA conditions for p53 in LS174T whole cell extract.** Samples were prepared as described in Chapter 2: Materials and Methods: to approximately 65,000 cells equivalent of crude cell extract, either 15 µg of NRS or 0.5 µg PAb421 polyclonal antibody was added to corresponding samples (serum addition order first; either NRS or Ab respectively), and incubated at either room temperature or 4º C on ice for 45 minutes. [³³P]-γ ATP end-labelled dsDNA probe mixed with poly(dI-dC) was then added, and incubated at either room temperature or 4º C on ice for 15 minutes. Samples were separated on a 0.25x TBE 6% native polyacrylamide gel for 80 minutes at 4º C. In all conditions, the samples containing PAb421 antibody each have a noticeably lower mobility than those samples containing NRS (denoted by ss). Excess free probe is visible at the bottom of the gel.
The EMSA gels were initially electrophoresed for 80 minutes, as in Figure 3.4, ensuring any unbound DNA probe was still present within the gel as a separate band at the bottom. This therefore ensures the probe concentration is not the limiting factor in the reaction, and is in excess for favourable reaction kinetics. When comparing those samples containing 0.5 µg of PAb421 with the normal serum controls, there are two visible differences. Firstly, the amount of free probe is greater in the presence of PAb421, which could result from the PAb421-p53 complex interfering with the DNA-binding ability of p53. However, this disagrees with the literature, which finds PAb421 has the opposite effect (Hanson et al., 2005), and another reason may be the contribution from the high concentration of NRS. The second difference, subtle but reproducible, is a visible difference in migration between samples containing PAb421, and normal serum controls. This indicates a lower mobility and therefore potential supershift, but further electrophoretic separation of the complexes is required to visualise any supershifted ternary complex as a separate band. Therefore, in later EMSA experiments, the gels were electrophoresed for 240 minutes to better separate the complexes, despite this resulting in the excess unbound DNA probe migrating off the end of the gel. With respect to different experimental conditions, none yielded any substantial difference in EMSA supershift occurrence.
Figure 3.5. **EMSA using buffer #2 at room temperature.** Samples were prepared as described in Chapter 2, Materials and Methods: to approximately 65,000 cells equivalents of crude cell extract, $[^{33}\text{P}]-\gamma$ ATP end-labelled dsDNA probe mixed with poly(dI-dC) was added. 15 µg of NRS or 0.5 µg PAb421 polyclonal antibody was added to corresponding samples (NRS and 5 µl Ab samples, respectively), and incubated at room temperature for 45 minutes, prior to separation on a native polyacrylamide gel for 240 minutes at 4°C. A) Samples either contain no serum (-), 0.5 µg of anti-p53 PAb421 antibody (0.5 µg Ab), or 15 µg Normal Rabbit Serum (NRS). A) Possible supershift band visible, denoted by ss. Signal was quantified using Fujifilm Image gauge signal quantification (B), showing enrichment in samples containing specific PAb421 antibody relative to negative controls.
It was found that using Buffer #2, and with better separation of the complexes by an increased gel running time of 240 minutes in Figure 3.5, a band was observed which was significantly enriched in all samples containing PAb421 antibody, relative to the negative controls. It is highly likely this is the ternary complex since it contains the radioactive DNA probe, and enrichment level is dependent on PAb421 concentration. Buffer #2 was used, since this is has been used in two more recent publications.

To verify both this potential supershift and its occurrence using nuclear extract, the next experiment examines the effect of an antibody titration, comparing nuclear extract to whole cell extract (the preparations of which are described in Materials and Methods, Chapter 2). Nuclear extract may decrease non-specific background on the EMSA gel, since nuclear extracts will not contain cytoplasmic and membrane proteins, which may interfere with DNA binding. However, the majority of DNA-binding proteins (which cause the most interference) are present within the nucleus, so this may have little effect on the non-specific bands.
Figure 3.6. **Effect of antibody titration on p53 Supershift using nuclear extract.**

Samples were prepared as described in Chapter 2, Materials and Methods: to approximately 65,000 cells equivalent of nuclear extract, $[^{33}P]$ DNA probe mixed with poly(dI-dC) was added. 15 µg of NRS, or 0.2, 0.5, or 1 µg of PAb421 polyclonal antibody was added to corresponding samples, and incubated at room temperature for 45 minutes. Samples were separated on a native polyacrylamide gel for 240 minutes at 4º C. A) Samples either contain no serum (-), 0.2, 0.5, or 1 µg of PAb421, or 15 µg Normal Rabbit Serum (NRS). A) Possible supershift band visible, denoted by ss; the intensity of which increases with PAb421 concentration. Signal was quantified using Fujifilm Image gauge signal quantification (B), showing enrichment in samples containing specific anti-p53 antibody relative to negative controls.

As observed on both the original EMSA gel (A), and after quantification (B), in all cases the supershift band intensity is increased in comparison with the negative controls of extract only (-) and Normal Rabbit Serum (NRS), whilst also increasing in response
to antibody concentration. Signal to noise ratio (defined by the supershift enrichment in the presence of 0.5 µg PAb421 divided by the mean enrichment in the presence of NRS), was compared between whole cell and nuclear extract analyte. These data are shown in Table 3.7., and a slightly higher signal to noise ratio is observed when using whole cell extract, indicating a greater level of ternary complex formation. This is consistent with the notorious instability of p53, which may be sensitive to degradation during the nuclear extraction process.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Whole cell</th>
<th>Nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 0.5 µg PAb421</td>
<td>26000</td>
<td>513</td>
</tr>
<tr>
<td>Mean NRS</td>
<td>12500</td>
<td>410</td>
</tr>
<tr>
<td>PAb421/NRS ratio</td>
<td>2.08</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Table 3.7. Greater supershift enrichment from whole cell extract relative to nuclear extract. From Figures 3.5 and 3.6, mean levels of supershift enrichment were determined relative to the mean NRS negative control, and expressed as the signal to noise ratio. The supershift was more enriched when analysing p53 from whole cell extracts.

The presence of 1 µg of PAb421 provides the most intense supershift. It was not deemed necessary to try higher concentrations of antibody for two main reasons. Firstly, this antibody is expensive, with 1 µg costing £2.36. This will incur a higher cost per sample for the eventual probe-IP assay. Secondly, too much excess antibody in liquid phase may result in the problem of non-specific interaction with other components of the assay, contributing to a high background. Figure 3.6 therefore provides sufficient evidence of the DNA-protein-antibody complex formation for the conditions used in this EMSA to be used in the assay protocol.
This chapter has used EMSA to verify the formation of the ternary complex under a defined set of experimental conditions, which will be used to ensure the formation of the ternary complex in the probe-IP assay.

Initially, Western blot analysis confirmed that the p53 protein is present in LS174T cell extract at levels sufficient for detection for further analysis by EMSA. Secondly, a search on Pubmed yielded buffer recipes and an antibody PAb421 used in p53 supershift experiments published in research articles. Finally, using the EMSA supershift we have determined the conditions under which the ternary complex is able to form. This chapter has provided the evidence of ternary complex formation of the PAb421 anti-p53 antibody, with endogenous p53 transcription factor present in LS174T whole cell or nuclear extracts, and a dsDNA oligonucleotide containing the consensus sequence from the gadd45 promoter 5’-GAACATGTCTAAGCATGCTG-3’.

The conditions for ternary complex formation have been shown to be relatively robust, as in Figure 3.4, a uniform level of supershift is observed over two buffers, incubation at 4°C and room temperature, and adding PAb421 to either DNA-bound or unbound p53. Further observation of the supershift, with incubation at room temperature, using Buffer #2, adding PAb421 to DNA-bound p53, showed very similar levels for both whole cell extract and nuclear extract. Supershift enrichment was seen to be antibody-dependent by a titration in Figure 3.6.

It was only decided to use p53 as a model for this project after cell culture was performed, and so the unstressed cells contained only basal levels of p53, and although this was found sufficient for detection by Western blot, only EMSA reflects DNA-binding activity. For p53 detection in the literature, cells were stressed by the addition of actinomycin D. From Figure 3.8 it can be seen that cell stress significantly enriches the supershift (McLure & Lee, 1998), and the ternary complex has a much lower mobility.
Figure 3.8. p53 and PAb421 EMSA supershift from McLure & Lee (1998). Balb/c 3T3 total cell extract was prepared from cells with or without prior DNA damage by actinomycin D. Cell extract was added to [\(^{32}\)P]DNA either with or without 0.5 µg of PAb421, as indicated. Gel shift (denoted by s) occurs only under conditions of DNA damage, with supershift (denoted by ss) also in presence of PAb421.

Although a protocol and antibody have been found under which the ternary complex can form, further experiments to look at specificity could have been performed. However, instead of optimising conditions for supershift, it was decided to use the above conditions as a starting point for the optimisation of the second and third steps of Figure 3.1: the immunopurification of the ternary complex using the Protein A magnetic bead kit purchased from Miltenyi Biotec, and the qPCR detection of the DNA probe, which is explored in the next chapter.
Chapter 4

Initial development of ternary complex immunopurification and qPCR

4.1 Introduction

In the previous chapter it was verified, using Western blot and EMSA that the transcription factor p53 was both present in the LS174T analyte, and that under defined experimental conditions could form a complex with a DNA probe encoding a specific consensus sequence and the anti-p53 monoclonal antibody PAb421 purchased from Calbiochem.

This chapter moves on from evidence of ternary complex formation in Chapter 3, to analyse and verify the two remaining steps of the assay. Firstly, quantification of the assay probe by qPCR; and secondly, the capture of the ternary complex by immunopurification using Miltenyi Biotec’s magnetic bead system (third and second steps of Figure 3.1, respectively). Although this may seem back to front, qPCR is an established technique and therefore whether the experimental conditions are suitable to quantify the probe in a reliable manner can quickly be determined. The immunopurification of the ternary complex on the other hand, is likely to require more optimisation. Using conditions under which the ternary complex is known to form, this optimisation will be possible once the probe output can be reliably quantified.

The PCR method relies on thermocycling of a DNA polymerisation reaction. This reaction contains the starting DNA sample, known as the template. In the case of this assay, this template is the DNA probe of the ternary complex eluted from the immunopurification procedure. The reaction temperature is raised to denature the template, and dropped to allow annealing of ssDNA primers complementary to the 3’ ends of each template strand; which is subsequently extended from the 5’ end by Taq polymerase. This process is outlined in Figure 4.1, which also describes the particular
type of PCR used in this assay: quantitative real time PCR (qPCR), where a fluorogenic probe complementary to a region within the amplicon is also present. This is known as the Taqman probe, and is labelled at the 5’ end with a reporter dye (in this case FAM), and on the 3’ end with a quencher (in this case TAMRA) to suppress the reporter signal while the Taqman probe is intact. The Taqman probe will anneal to its complementary sequence on the amplicon, and once elongation from the primers and along the DNA template results in hydrolysis of the probe, the reporter signal is separated from the quencher. Fluorescent signal is therefore dependent on both the specific target sequence, and the action of DNA polymerisation, improving the specificity of this PCR method.

To avoid confusion, it should be noted that throughout this chapter (and elsewhere in this thesis), the DNA probe used in the Probe-IP assay is referred to as simply “probe”; whereas the qPCR Taqman probe is referred to as the “Taqman probe”.
Figure 4.1. **qPCR chemistry using the Taqman probe system.** 1. The primers and Taqman probe anneal to the ssDNA template. 2. The Taq polymerase 5’ nuclease activity degrades the probe during extension from the forward primer, and the reporter and quencher molecules are released. In the case of the Probe-IP assay, FAM is used as a reporter dye, whilst TAMRA is used as the quencher. 3. Fluorescence of FAM is proportional to number of amplicon extensions (adapted from Applied Biosystems [ABI], n.d.).

Initially, pure p53 protein is used instead of nuclear extract in the development of this assay. Whilst as discussed in Chapter 1, this assay has many possible applications as a pure protein system, another reason for this is the likelihood of nuclear proteins interacting non-specifically with the DNA probe, which is thought could be a possible interference in this technique. Therefore optimising assay performance with pure protein first allows for improvement without the added complication of using nuclear extract.
The purchased recombinant p53 protein (Calbiochem), with a MW of 46.42 kDa is His-tagged on the N-terminal of the protein. This is the opposite end of the primary structure to that containing the epitope for the PAb421 anti-p53 antibody (amino acid residues 376-378), which resides at the C-terminal regulatory domain of the protein. The protein–antibody interaction should therefore not be affected by the presence of the His-tag, but this will be checked using EMSA.

Figure 4.2. Schematic structure of the human p53 protein. The His-tag of the recombinant p53 protein purchased from Calbiochem is located at the N-terminal end of the protein; the opposite end of the epitope for the PAb421 antibody (also purchased from Calbiochem), located at amino acid residues 376-378 of the p53 protein.

This chapter describes the initial development and performance of this Probe-IP method as a novel approach for the quantification of purified active p53 transcription factor for potential application in both biological research and drug development. Since this project, and in particular this chapter is focused on method development, the initial protocol is described below to provide a reference point together with the diagrammatic view given in Figure 3.1. Chapter 2 describes the protocol in greater detail.
4.2 Initial method for the Probe Immunopurification Assay

**Buffer preparation**

1. All buffers are prepared and degassed to remove any air bubbles which may interfere with column flow

**Association of ternary complex**

2. Ternary complex is formed with 0.15 pmols of probe, 1 μg PAb421 and the desired amount of pure protein in a final concentration of 1x EMSA buffer and 1x protease inhibitor, with a total volume of 30 µl
3. Samples are incubated on a plate shaker at the lowest setting (100 rpm) for one hour at room temperature

**Addition of magnetic beads**

4. Samples are diluted to 1 ml with 1x EMSA buffer
5. 50 µl magnetic beads are added, and samples are incubated for 30 minutes on ice on a plate shaker at the lowest setting (100 rpm)

**Immunopurification of complex**

6. The columns are placed in the magnetic holder (see Figure 4.3), equilibrated and washed with EMSA 1x buffer
7. The sample is immediately applied to the column, and owing to the Protein A conjugated magnetic beads, the ternary complex will now be immobilised within the column.
Washing

8. Column is washed once with 33x column volume’s worth of 1x EMSA buffer, followed by 3x column volume of 10 mM Tris pH 7.5 and elution buffer

Elution

9. Column is removed from magnet, and placed over a clean 1.5 ml tube (Eppendorf DNA LoBind)
10. Ternary complex immobilised to the magnetic bead is eluted in 3x column volume of elution buffer
11. Excess unbound DNA is digested with 10 units of Exonuclease III (Exo III), incubated at 37°C for 10 minutes. The reaction is terminated by incubation at 70°C for 20 minutes.
12. Samples analysed by qPCR (see section 4.3)

Figure 4.3. Image of Miltenyi Biotec’s μMACS separation unit. Showing a set of magnetic bead columns held to the stand by the magnetic column holder. Rack containing collection tubes can be placed directly beneath the column outlets.

4.3 qPCR optimisation and efficiency for DNA primers and Taqman probe

The data output from the qPCR assay consists of the template amplification plot, tracing the increase of Taqman probe fluorescence signal (in this case, FAM), against the number of amplification cycles on the x-axis. An example is given in Figure 4.4. From this plot, in the logarithmic view, a threshold is marked half way between the baseline and plateau (horizontal line shown in pink at a fluorescence signal of 0.35), in the centre
of the logarithmic phase of DNA template amplification. From this threshold, each amplification reaction is given a value known as the Threshold Cycle (C_T), which is the amplification cycle number at which the level of fluorescence crosses the Threshold. The number of cycles required to reach this defined level of fluorescence is dependent on the starting concentration of template DNA. A series of 10-fold dilutions of known template concentration are used to construct a standard curve, plotting C_T against log [absolute quantity], and the absolute quantities of unknown samples are subsequently interpolated from C_T values.
A. Amplification plot of FAM fluorescence normalised to the carboxy-X-rhodamine (ROX) reporter dye ($\Delta Rn$), against amplification cycle number. Threshold is marked in the centre of the logarithmic phase of amplification, at $\Delta Rn$ 0.35, and this is used to determine the Threshold Cycle ($C_T$) value of each sample: the cycle number at which the fluorescence level crosses the Threshold. B: 10-fold serial dilutions of known template concentrations (grey points) form the standard curve, plotting $C_T$ value against log [absolute quantity]. Quantity of unknown samples (coloured points) interpolated from the standard curve. Graph slope, coefficient of determination ($R^2$) and Efficiency given as measures of qPCR performance.

Figure 4.4. Interpretation of raw qPCR data to determine absolute quantities of DNA template, using the ABI 7500 machine. A: Amplification plot of FAM fluorescence normalised to the carboxy-X-rhodamine (ROX) reporter dye ($\Delta Rn$), against amplification cycle number. Threshold is marked in the centre of the logarithmic phase of amplification, at $\Delta Rn$ 0.35, and this is used to determine the Threshold Cycle ($C_T$) value of each sample: the cycle number at which the fluorescence level crosses the Threshold. B: 10-fold serial dilutions of known template concentrations (grey points) form the standard curve, plotting $C_T$ value against log [absolute quantity]. Quantity of unknown samples (coloured points) interpolated from the standard curve. Graph slope, coefficient of determination ($R^2$) and Efficiency given as measures of qPCR performance.
One of the ways in which the performance of the amplification reaction, and thus quantification of template DNA by qPCR is evaluated, is by the reaction efficiency. The efficiency is a measure of how close to a perfect logarithmic reaction occurs, and is determined for each standard curve, such as that shown in Figure 4.4. (B), by the equation:

$$E_X = 10^{(-1/\text{slope})} - 1$$

The level of efficiency is dependent on the concentration and composition of reagents used, including the primers and Taqman probe, as well as the template DNA itself. The DNA sequences for this assay were designed using Applied Biosystem’s Primer Express software (see Appendices). To determine the optimum concentrations of primer and Taqman probe, a checkerboard assay was first performed, and efficiency determined for each combination of primer/Taqman probe concentration.
Figure 4.5. **Efficiency of qPCR reactions for the BioRad iCycler.** PCR reactions were performed in a checkerboard fashion varying concentration of primers and probe. Using template DNA concentrations of 1000 M$^{-15}$, 100 M$^{-15}$, 10 M$^{-15}$, 1 M$^{-15}$, 0.1 M$^{-15}$, and 0.01 M$^{-15}$ with each primer/Taqman probe combination, reaction efficiency was determined as follows: Figure A plots a graph of ΔC$_T$ for these reactions against log template concentration. B shows Efficiency (E) determined by the slope of the graph (y) using the calculation $E= 10^{(-1/slope)}-1$. Efficiency is expressed as a percentage (%E), and is highest using 200 nM of Taqman probe, with either 450 or 600 nM primer.
4.3.1 PCR equipment

The fact that qPCR is an extremely sensitive method for the quantification of DNA is hypothesised to provide main strength within the assay design, potentially improving the lower limit of detection of active transcription factor. However, this sensitivity brings with it a greater potential for operator error leading to internal variations greater than variations observed between samples. This error is also linked to equipment used for the PCR, and this section highlights the importance of validation in the equipment used as well as modifications to the method itself.
Figure 4.6. **Standard deviations of Cₜ values within qPCR triplicates compared between iCycler and ABI 7500 qPCR machines.** Two Probe-IP assays (sample set A and B) were performed with a titration of 0, 50, 75 and 100 pg of p53 (samples 1-4), and ternary complex eluted from the magnetic bead column in NEBuffer #1. The probe content of eluent was quantified in triplicate samples on the BioRad iCycler and the ABI 7500 fast PCR machines in parallel. The standard deviations (STDev) of Cₜ values within the triplicate repeats are shown, with the acceptable STDev level of 0.5 Cₜ in red. All samples analysed on the ABI 7500 exhibit a STDev below 0.5, in contrast to samples analysed on the iCycler, where only sample B1 is below this level.
The ABI 7500 was a new purchase made by the Cancer laboratory in October 2009, three years into the project, where previously only the iCycler was available for use. From Figure 4.6, it is apparent that the standard deviation within triplicates is higher on the iCycler for each sample. In addition, for all samples except B1, the iCycler is above the acceptable variation of 0.5 $C_T$, whilst the ABI 7500 is consistently below this level. This difference is most likely due to the passive reference ROX, which normalises signal fluctuations between wells. The accurate quantification of DNA allows observation of much subtler differences between samples, which is especially important where PCR is a logarithmic reaction; a difference of one $C_T$ equals a two-fold difference of starting material in absolute terms (described by the equation $2^{\Delta C_T}$, where $\Delta C_T$ is the difference between two $C_T$ values).

4.3.2 qPCR efficiency using the ABI 7500

Owing to the improved standard deviations observed in data analysed using the ABI 7500 machine compared to the BioRad iCycler, it was decided to use the ABI 7500 for the qPCR detection step. The qPCR amplification efficiency of the template, and its dependence on primer and Taqman probe concentration were then determined.
### A

<table>
<thead>
<tr>
<th>Primer [Probe]</th>
<th>Slope (y)</th>
<th>$R^2$</th>
<th>E%</th>
</tr>
</thead>
<tbody>
<tr>
<td>125nM</td>
<td>-3.426</td>
<td>0.999</td>
<td>95.844</td>
</tr>
<tr>
<td>450nM</td>
<td>-3.429</td>
<td>0.998</td>
<td>95.702</td>
</tr>
<tr>
<td>250nM</td>
<td>-3.421</td>
<td>0.998</td>
<td>96.01</td>
</tr>
<tr>
<td>125nM</td>
<td>-3.435</td>
<td>0.999</td>
<td>95.481</td>
</tr>
<tr>
<td>600nM</td>
<td>-3.437</td>
<td>0.998</td>
<td>95.397</td>
</tr>
<tr>
<td>250nM</td>
<td>-3.481</td>
<td>0.999</td>
<td>93.764</td>
</tr>
</tbody>
</table>

### B

#### Figure 4.7. Efficiency of qPCR reactions for the ABI 7500.

qPCR reactions were performed in a checkerboard fashion varying concentration of primers and probe. Using template DNA concentrations of 1000 M$^{-15}$, 100 M$^{-15}$, 10 M$^{-15}$, 1 M$^{-15}$, 0.1 M$^{-15}$, and 0.01 M$^{-15}$; with each primer/probe combination, reaction efficiency was determined as follows: Figure A plots a graph of ΔC$_T$ for these reactions against log template concentration. B shows Efficiency (E) determined by the slope of the graph (y) using the calculation $E = 10^{(-1/slope)} - 1$. Efficiency is expressed as a percentage (E %).

The levels of efficiency shown in Figure 4.7 all lie within the acceptable range of 90-110% (ABI, 2008). It is evident that 450 nM primer yields consistently higher levels of efficiency, together with 250 nM of probe. These concentrations will therefore be used in qPCR reactions from here on. Raw qPCR data are included in the Appendices chapter.
4.4 Effect of immunopurification components on qPCR

Following immunopurification, the DNA probe is part of the ternary complex retained in the magnetic bead column. On the one hand, elution of the whole complex on the removal of the magnetic field lends the assay methodology more to high throughput application (the fewer steps the better). However, qPCR is susceptible to inhibitors interfering with the reaction, one example of which is IgG antibody (Al-Soud et al., 2000), such as the IgG2a monoclonal antibody PAb421 used for the immunopurification of p53 in this assay. To address this, the qPCR amplification plots of probe eluted by two methods were compared, these methods being:

1. Elution of whole complex in NEBuffer #1
2. Elution of DNA probe only using NaCl 300 mM, and subsequent desalting through a Sepharose microspin column (GE Healthcare)

These were both also compared to the amplification plot of purified probe diluted in dH₂O alone.
Figure 4.8. **qPCR inhibition from reaction components.** A. The linear amplification plots are shown for the probe-IP in the presence and absence of 50 ng of p53, and the standard curve of the probe diluted ten-fold in dH2O (blue). Threshold of 0.395819 shown in pink. The whole complex was eluted in NEBuffer #1 (red), or the DNA probe eluted alone by 300 mM NaCl/NEBuffer #1 (yellow). The NaCl was removed using a Sephadex GE-spin column. The amplification plots show a lower C_T value and plateau level in the presence of all components of the ternary complex. B. Signal to noise ratio (p53 +/-) for absolute values of probe as determined from the Standard curve method.
The probe-IP assay was performed as detailed in section 4.2., in the absence or the presence of 50 ng of p53, and the elution methods previously described were compared. It is indicated in Figure 4.7 that the elution of the whole complex results in increased $C_T$ values and a lower level of reaction plateau, suggesting PCR inhibition. However, the elution of DNA alone produced greater DNA amplification, with lower $C_T$ values than amplification in the presence of the whole complex. The reaction plateau is also similar to that of the standard curve samples diluted in dH$_2$O alone (shown in blue). Although the sensitivity of the qPCR step is much improved, when comparing the signal to noise ratio, the samples where the whole complex was eluted yielded 4.67, compared to 6.22 for the DNA alone. This indicates a slight improvement, but at the cost of an additional assay step, requiring both cost and time.

Since the qPCR step is vital to the performance of the Probe-IP assay, it was deemed important to elucidate which assay components have the greatest negative impact on the qPCR reaction. Individual components (PAb421 antibody, p53 protein, magnetic beads and Exonuclease III) were added to pure probe diluted in dH$_2$O to concentrations identical to that expected in the eluent of an assay containing 10 ng p53.
Figure 4.9. **Effect of immunopurification components on probe detection by qPCR.** Components of the immunopurification reaction at concentrations identical to assay eluent were mixed with 100 M$^{-15}$ probe, and the effect on qPCR amplification plot observed relative to standard curve of 1 M$^{-12}$ probe diluted ten-fold (blue). The $C_T$ is unchanged in the presence of p53 or antibody, but the amplification plateau is lowered in the presence of both antibody and p53 (A). Magnetic beads visibly interfere with qPCR, reducing the plateau and increasing $C_T$ (B), whilst the presence of 0.1 units/µl Exo III increases $C_T$ (C).

Most importantly, no alteration of $C_T$ value is observed in the presence of p53 at concentrations tested in Figure 4.9 (A). The remaining components are present in each sample at uniform concentration, and will therefore affect the qPCR to the same degree. The magnetic beads are shown to cause a lowered plateau of the qPCR reaction, and increased $C_T$ value. The most likely explanation for this is due to the (slightly) opaque nature of the beads, a portion of the energy may be lost between fluorescent excitation and emission, causing a drop in signal. Even though in this experiment, Exo III activity is inhibited prior to mixing with the probe, an increased $C_T$ is observed in Figure 4.8(C), perhaps by retention of some activity.
Due to the presence of Exo III and magnetic beads, the probe quantities determined from the standard curve will be relative rather than absolute. However, the main purpose of this conversion is to unmask the vast differences observed when looking at logarithmic \( C_T \) values alone; if accurate probe concentration must be known, for example in the analysis of \( k_d \), probe can be eluted and purified alone as illustrated in Figure 4.8.

4.5 Magnetic bead immunopurification of ternary complex

Once it was verified that the ternary complex was forming under known experimental conditions by EMSA, and that the Probe-IP assay’s DNA probe could be efficiently amplified and quantified under optimum qPCR conditions, these two steps were coupled together with the magnetic bead immunopurification kit purchased from Miltenyi Biotec.

4.5.1 EMSA using pure protein

Before attempting the assay using pure protein, EMSA was used to show that pure protein purchased from Calbiochem was indeed active and able to form a complex with DNA probe and antibody. This section also provides data to the sensitivity of EMSA using the same conditions of ternary complex formation as the assay under development.
Figure 4.10. **EMSA supershift using recombinant p53 protein (Calbiochem)**. Samples were prepared as described in Chapter 2, Materials and Methods: ternary complex was formed by associating $3 \times 10^5$ of $[^{33}P]$-labelled probe with either 10, 25, 50, 100, or 200 ng of p53 protein diluted in EMSA 1x buffer, prior to adding 1 µg of PAb421 antibody. Ternary complex was incubated for 30 minutes at room temperature, and separated on a 6% native polyacrylamide gel for 80 minutes at 4° C. Although aggregated in the wells, a supershift formation of ternary complex is visible, as well as a depleted concentration of free probe with increasing protein concentration.
Ternary complex formation using pure p53 protein is observed in the EMSA supershift experiment in Figure 4.10., although the complex has become aggregated and unable to enter the gel matrix, perhaps due to the length of the probe (83 bp compared to 39 bp used in Chapter 3). Formation of ternary complex is evident both from the increased enrichment of the supershifted band, and the decreased enrichment of the free probe in response to an increase in protein concentration. This experiment also demonstrates that the sensitivity of EMSA to detect pure p53 protein is approximately 50 ng.

4.5.2 Identifying the assay range of p53 detection

Since the detection system of the assay relies on qPCR, it was expected this assay would have an increased sensitivity when compared to EMSA. Therefore, initial conditions analysed 0.015 – 1.5 ng (0.08 fmol to 8 fmol) of p53 tetramer (186 kDa). This gave approximately a 20x molar excess of probe (0.15 pmols, or 7.7 ng) to 1.5 ng of p53 tetramer, required for the equilibrium constant of a DNA-protein binding reaction to favour formation of the protein-DNA complex. However, these conditions were found to be insufficient for detection (data not shown). Higher concentrations of protein, as used in the EMSA, were therefore tested. The amount of pure p53 protein used in the assay was increased, and a titration performed of 10, 50 and 100 ng.
Figure 4.11. **p53 concentration dependent signal response.** 0, 10, 50 and 100 ng of protein were used in a reaction of 31 µl, with 0.15 pmols of DNA probe. CT values were converted to absolute quantity of DNA detected using a standard curve of 100 M$^{-15}$ to 1 M$^{-15}$, with a calculated PCR efficiency of 102%. Error bars represent qPCR standard deviation of triplicate samples.

Using a higher concentration of p53 in the reaction produces a concentration-dependent response for the Probe-IP assay performed using purified p53 protein, as indicated in Figure 4.11. Although 0.15 pmol of probe is only 2.8x molar excess to 10 ng (0.053 pmol) of p53 tetramer, and is limiting in an assay of 50 and 100 ng of p53 tetramer, this is not reflected in the data and suggests a proportion of the p53 is perhaps inactive. The data resulting from these assay conditions now provide a foundation to verify initial assay performance, as judged by signal specificity, sensitivity and reproducibility (intra- and inter-assay variation). These are explored in the following sections.
4.6 Verification of signal specificity

Although a signal increase in response to p53 concentration has been observed, it is possible these data reflect nonspecific signal arising from probe interaction to various components within the assay, and excess levels of protein are merely pulling down an increased level of probe non-specifically. To verify the specificity of this signal, further experiments were performed to check the following: protein specificity of the assay, binding specificity of p53 to its consensus sequence, and anti-p53 antibody signal dependence. This section describes this set of three experiments used to verify the level of signal specificity.

4.6.1 Protein specificity: replacing p53 with BSA

The first approach to verify that the signal observed in the assay is specific to p53 was to perform a p53 titration parallel to a titration with the same quantity of nonspecific protein, since it is possible that the probe may be captured by nonspecific interaction with protein in a concentration dependent manner.

In addition, to ascertain whether the Exo III digestion step in the protocol is of benefit to assay performance, the eluent was split; half being digested with Exo III, and the other half being kept at 4°C.
A

Protein specificity Exo+

B

Protein specificity Exo-

C

Signal to noise ratio in response to Exo III
Figure 4.12. **Protein specificity in the probe-IP assay**. Samples were treated as described in section 4.3, containing no protein (blue); 10, 50 or 100 ng of p53 (red) or BSA protein (green). Eluent was split, and treated with 10 units of Exo III (A) or not (B). Ct values from PCR were converted to M-15 based on E=98.7%. All samples containing BSA yielded signal no different from the no protein control (0 ng p53), independent of concentration, compared to the concentration-dependent signal increase observed with p53. Error bars signify qPCR standard deviation within triplicates. C: signal to noise ratio (p53 +/-) in response to Exo III treatment. The signal to noise ratio is consistently higher in the absence of Exo III.
Replacing p53 protein with the same amount of Bovine serum albumin (BSA, a nonspecific protein of 67 kDa), and following digestion by Exo III, levels of probe capture are similar to that observed in the absence of protein. In the absence of Exo III, no significant BSA concentration-dependent increase is observed in probe capture, although levels remain significantly lower than in the presence of p53. This indicates that the presence of nonspecific protein, despite not being a DNA-binding protein, reduces the specificity and may be a consideration when using endogenous analyte. A solution to this may be increasing column washing volume and stringency, studied in the next chapter.

Another factor of assay optimisation tested in this experiment is the effect Exo III digestion has on assay performance. It was hypothesised that the presence of 10 units of Exo III would remove unbound (i.e. unprotected) probe from the reaction, thereby increasing the signal to noise ratio, defined as the ratio of p53 presence to the no protein control (p53 +/-).

It is possible that in addition to digesting the unbound probe pulled down non-specifically by reaction components into the eluent, this step is also removing specific signal. This is indicated by the fact that the presence of Exo III treatment causes a drop in the p53 signal to noise ratio (Figure 4.12 C). This is consistent with the fact that p53 is unstable at 37° C, exhibiting loss of tetrameric formation and DNA-binding activity that is not protected by the binding of PAb421 (Hansen et al., 1996). Another factor to consider is that in any protein-DNA complex, DNA does not remain bound indefinitely, but instead periodically dissociates and re-associates. In a study of endogenous transcription factor, the dissociation of PAb421-bound p53 from its consensus site, as determined from EMSA has a reported half-life of 25 minutes (McLure & Lee, 1998). Another study using purified transcription factor to investigate the dissociation of PAb421-bound p53 from its consensus DNA site of the p21 promoter found an even shorter half-life of one minute (Cain et al., 2000). In either case, both factors help to explain the loss of specific signal, as previously p53-bound probe is degraded in the presence of Exo III. It has also been shown in Figure 4.9(C) that this concentration of Exo III reduces the qPCR signal. Exo III will therefore be omitted from the assay whilst
analysing initial performance, but it may be that in the analysis of a more stable protein, Exo III treatment would be improve signal to noise ratio.

This section has shown that the signal from the Probe-IP assay is p53 protein specific, and that the Exo III digestion step can be omitted from the assay for the time being, with the benefit of assay time and cost. The specificity of p53-DNA interaction will now be analysed using a competition experiment for 10 ng and 50 ng of p53.

4.6.2 Binding specificity: oligonucleotide competition assay

The specificity of a DNA-protein interaction can be analysed by a competition experiment. Oligonucleotides lacking the qPCR primer and probe annealing regions are added to the assay mixed with the assay DNA probe. These oligonucleotides are either the wild type p53 binding region, and therefore compete with the sequence-specific DNA binding of p53 to the assay probe; or they are a mutant binding site, and therefore will not interact with the protein in a specific manner or cause a drop in specific signal. The level of p53 binding non-specifically to DNA will be reflected in the level of change in signal observed in the presence of mutant oligonucleotide.
Figure 4.13. **Binding specificity.** Competition assay of 0 ng, 10 ng and 50 ng of p53 purified recombinant protein. CT values converted to absolute DNA concentration based on 96.7% efficiency. Competitor oligonucleotides were mixed with probe to a final quantity of 7.5 pmols to 0.15 pmols of probe, which taking molecular weight into consideration equals 100x excess of competitor binding site to probe. Probe and competitor mixes (Self or Mutant; red and green, respectively) or probe alone (None, blue) was then added to p53 protein prior to the addition of 1 µg PAb421 anti-p53 antibody. A signal drop is observed with the addition of competitor oligonucleotide, which is less for mutant than self competitor in the presence of p53. Error bars indicate standard deviation of qPCR triplicate samples.
Whilst the drop in signal in the presence of 100x excess of self competitor oligonucleotide is consistent with the known affinity of p53 for its consensus sequence, the observed signal drop in the presence of mutant competitor oligonucleotide indicates nonspecific p53-DNA interaction (Figure 4.13). The incidence of nonspecific p53-DNA interaction is lower than specific interaction, as illustrated by the increased signal drop in the presence of self competitor compared to mutant competitor. To rule out the possibility that the presence of residual competitor oligonucleotide in the qPCR is interfering with probe amplification, and to test the idea that the drop in signal in the presence of nonspecific DNA is due to the binding of p53, poly(dI-dC) was included in an assay of 50 ng p53. The results shown in Figure 4.14 are consistent with those of 4.13, whereby both the signal and signal to noise ratio (p53 +/-) drops in the presence of nonspecific DNA.
Figure 4.14. **Nonspecific p53-DNA binding.** 0 and 50 ng of p53 was assayed with 0.054 pmol of probe and 1 µg of PAb421 antibody. Either 1 µl of DNA dilution buffer alone, or containing 0.5 or 1 ng of poly(dI-dC) was mixed with the probe. The presence of poly(dI-dC) in the assay causes a signal drop in both the absence and presence of p53 (A), resulting in a total decrease in p53 +/- (B). Error bars show SD of qPCR triplicates in (A). In (B), SD is expressed as a mean percentage of the signal/noise ratio calculated in (A).

The presence of nonspecific DNA, both mutant competitor in Figure 4.13, and poly(dI-dC) in Figure 4.14 also reduces probe capture in the absence of p53 (Figure 4.14). Therefore, part of this signal drop observed in the presence of nonspecific DNA is also
p53-independent, due to the blocking of probe capture from nonspecific interaction with reaction components.

It is known that the C-terminal domain of p53 exhibits sequence-independent DNA binding (Wang et al., 1993). However, PAb421 with its epitope lying within this domain (see Figure 4.2) is known to repress this function (Hupp et al., 1992, cited by Ahn & Prives, 2001). The observed drop in signal in the presence of p53 and nonspecific DNA is thought to partly represent the nonspecific binding of p53 prior to the addition of PAb421. Although the half-life of p53 bound to nonspecific DNA is reported as 0.5 minutes, with a small portion of p53 remaining bound after 5 minutes (McLure & Lee, 1998), there could be two reasons for the apparent interaction of p53 with nonspecific DNA. Firstly, whilst the C-terminal domain of p53 is occupied by DNA, this may prevent the binding of PAb421; and secondly, the ternary complex does not remain intact over the incubation time, and since the competitor is present in 100x excess this allows for further interaction of p53 with nonspecific DNA. Furthermore, it must be taken into account that these findings were discovered using EMSA, and these data may reflect disparity between the two methods.

Nonspecific p53-DNA interaction poses a problem in the detection of specific DNA binding ability, such as whether mutant p53 is restored to wild-type conformation. It would be preferable to have PAb421-bound p53 prior to the addition of probe to increase specificity, and this will be studied in the next chapter. Another approach toward an increase in specificity is the addition of a nonspecific protein such as BSA, which is commonly used in pure protein systems as a carrier molecule. The next section looks at antibody dependence in the assay as another measure of assay performance.
4.6.3 Antibody dependence: omitting antibody in assay

This experiment looks at assay specificity conferred by the anti-p53 monoclonal antibody PAb421. Omitting the antibody should result in a signal drop of 100%, since the antibody is essential in the specific detection of p53. The remaining signal in the absence of antibody represents background noise resulting from nonspecific probe capture.

Figure 4.15. Antibody dependence in the absence or presence of 10 ng and 50 ng p53 protein. 1 µg of PAb421 anti-p53 antibody was either included or replaced with PBS pH7.4. The contribution of antibody to probe capture in the absence of p53 was also subtracted. A drop in signal is observed in the absence of antibody, the level of which is reflected in the ratio of antibody presence to absence: +/- (B).
In the absence of p53, all probe detected is due to nonspecific probe interactions with assay components. Furthermore, in these samples, the difference between the presence and absence of antibody represents the contribution of the antibody itself to background. This is thought to arise from positively charged regions of the antibody attracting the negatively charged DNA backbone. Although the structure for PAb421 has not been solved, observing the regions of positively charged amino acids (His, Lys and Arg) on the Mouse IgG2a immunoglobulin Mab231 (Figure 4.16) highlights areas of positive charge on the surface of the antibody, and this seems to fit with those data in Figure 4.15.

![Figure 4.16. Positively charged regions on the surface of a Mouse immunoglobulin IgG2a. Using the Jmol programme, positively charged regions of His, Lys or Arg amino acids of an IgG2a immunoglobulin are shown in red on a CPK Space-filling model. PDB Structure: 1IGT (Harris et al., 1997).](image)

Any direct interaction between antibody and probe will be uniform between samples, and as shown in Figure 4.15, does not contribute a great amount to background noise. However, it is possible this may be reduced by decreasing the concentration of antibody or probe used in the assay or washing the column more stringently, which will be studied in the next chapter. Figure 4.15, however, also highlights a more concerning problem with this assay. In the absence of antibody, a p53 concentration-dependent increase in captured probe is observed. This indicates the presence of p53-probe complexes, which are not being removed sufficiently during column washing, resulting in antibody-independent probe capture. Although under these conditions the probe is
only binding p53 protein, since no other proteins are present, analysing p53 levels in nuclear extracts may be problematic with the insufficient removal of protein-DNA complexes unbound by antibody. Of course with the probe encoding a p53 binding site, it is expected that p53 has a higher affinity for the probe than other nuclear proteins may, it would still be highly advantageous to ensure removal of all nonspecific probe-protein complexes prior to sample elution. The problem identified here is the interaction of nonspecific protein-probe complexes with assay components (as opposed to free probe), and therefore it may be possible to reduce this by the addition of BSA to the reaction, which will be explored in the next chapter.

4.7 Verification of assay reproducibility and sensitivity

Signal specificity has been investigated in the previous section by three separate experiments. This section now examines the aspects of sensitivity and reproducibility in assay performance.

4.7.1 Inter-assay variation

The levels of inter-assay variation reflect the reproducibility of the assay over separate experiments. The values observed throughout the experiments so far in section 4.6., and the associated variations are shown in Table 4.17. When analysed using a Student’s t-test, the data also show a statistically significant (P<0.05) increase in probe capture in the presence of 10 ng p53, with a P-value of 0.04. Therefore, 10 ng of p53 will be the concentration of p53 used to investigate different assay parameters.
Table 4.17. Inter-assay variation of assay samples and p53 +/- from section 4.6. The Mean and standard deviation (STDev) of assays containing 0, 10 and 50 ng of p53 in Figures 4.11B, 4.13, and 4.14 were used to determine the coefficient of variation (CV%), which reflects inter-assay variation.

The high inter-assay variation might be accounted for by the dilution of probe prior to each experiment from a highly concentrated stock solution of 75 µM. Slight operator error will translate, once diluted 1000-fold, to a much greater error in the assay itself. The standard curve on each qPCR run used to interpolate absolute quantity from C_T values also presents a potential source of inter-assay variation. This standard curve is prepared by initially diluting stock probe 1x10^{-7} prior to creating a serial 10-fold dilution series and so again slight error will be amplified to result in variation. Some qPCR software allows the import of a standard curve for data analysis, but this is not possible for the ABI 7500. However, looking at CV% between the ratio of p53 presence to absence (p53 +/-) in Figure 4.1(B) it is evident that variation also occurs from other sources, and therefore limited comparisons can be made between individual experiments at this stage in assay development.
Another source of inter-assay variation was found to occur in relation to the EMSA binding buffer. The preparation of a new stock of EMSA binding buffer coincided with a substantial increase in total probe capture and depletion of the signal to noise ratio. From this it was reasoned that perhaps batch variation between buffer stocks altered conditions, and a probe titration experiment indicated that a decreased probe concentration recovered the signal to noise ratio (Figure 4.18) in the freshly prepared buffer.
Figure 4.18. **signal to noise ratio (p53 +/-) in response to probe titration.** Increasing probe input concentration in the presence and absence of 10 ng p53 in the Probe-IP assay using 1 μg PA421 for capture (A). Probe concentration is expressed as molar excess to 10 ng of p53 in a tetrameric complex, which is 53 fmols. Signal to noise (p53 +/-) exhibits no significant difference (B). Probe concentrations are given in ng and fmols in C. Error bars indicate STDev of qPCR triplicates. In (B) SD is expressed as a mean percentage of the signal/noise ratio calculated in (A).
From the data shown in Figure 4.18, it was decided that instead of using 0.15 pmol of probe in the assay, 0.054 pmol will be used. However, over time values of both signal and background increased, depleting the signal to noise ratio once more, as can be seen in Figure 4.19.

**Figure 4.19. Change in probe capture over time in the absence of p53.** New buffer stock was prepared from recipe in section 3.3 (Pitkänen *et al.*, 1998). In five separate assays from 6 to 27 days after buffer was prepared, each containing 0.054 pmol of probe and 1 µg PAb421; probe capture in the absence of p53 is plotted over time (A), where an increase is visible. The signal to noise ratio, as defined by 10 ng p53/0 p53, exhibits a decrease over time (B). Error bars signify standard deviation of qPCR triplicate samples. In (B), SD is expressed as a mean percentage of the signal/noise ratio calculated in (A).
To investigate the contribution of EMSA buffer to the observations shown in Figure 4.19., new stocks were prepared and tested after two days stored at 4° C. In addition to testing a new batch of buffer, two different batches of the detergent Igepal (NP-40) were also tested. Although the source of the changes observed in Figure 4.19 suggested a gradual change over time as opposed to batch-to-batch variation, it was observed that turbidity forms over time in the presence, but not the absence of NP-40. However, as can be seen in Figure 4.20., batch variation between NP-40 was minimal, and the signal to noise ratio was instead recovered by preparing EMSA 2x buffer filtered through a 0.2 µm filter and used the same day.
Figure 4.20. **Aspects of EMSA buffer on assay values.** Probe-IP assays were performed using different EMSA 1x and EMSA 2x buffers. Reactions contained 1 µg of PAb421 used to capture 0 or 10 ng of p53 in the presence of 0.054 pmol of probe. Buffer prepared two days prior to assay containing different igepal CA-630 batches, old (over 4 years old) and new (two months old) caused no difference in assay values (A). Filtering buffer through a 0.2 µm filter and using buffer in assay on same day with no storage below room temperature resulted in an overall drop in signal, but increased signal to noise ratio of 3.95 (B).
A probe amount of 0.054 pmols has been used in Figure 4.18 – 4.20., instead of 0.15 pmols used previously. The optimum probe concentration will be determined in the next chapter. It remains unknown how exactly the buffer is affected, although the most likely explanation is that the visible turbidity is a result of NP-40 precipitating on storage at 4º C, and reduced detergent concentration in solution reduces efficiency of probe removal during column washing. Preparing a stock of buffer for each experiment risks introducing an additional source of inter-assay variation, and therefore buffer will be filter sterilised and stored at room temperature for up to a week.

4.7.2 Intra-assay variation

The intra-assay variation reflects reliability of the assay excluding the effects of probe preparation and standard curve variation discussed in the previous section. This was analysed by calculating the coefficient of variation (CV%) of triplicate repeats performed in the same experimental set. The CV is a statistical measure of variation expressed as a percentage of the mean.

<table>
<thead>
<tr>
<th>p53 ng</th>
<th>sample #</th>
<th>PCR mean</th>
<th>PCR STDev</th>
<th>CV%</th>
<th>Probe-IP assay triplicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>11.74</td>
<td>1.09</td>
<td>9.31</td>
<td>mean</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>STDev</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>9.76</td>
<td>0.54</td>
<td>5.56</td>
<td>10.40</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>9.71</td>
<td>0.46</td>
<td>4.77</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CV%</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td></td>
<td>11.81</td>
<td>0.47</td>
<td>3.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13.52</td>
<td>0.51</td>
<td>3.78</td>
<td>12.62</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12.53</td>
<td>1.08</td>
<td>8.62</td>
<td>6.82</td>
</tr>
</tbody>
</table>

Table 4.21. Intra-assay variation of assay sample triplicates. The Mean and standard deviation (STDev) within triplicate repeat samples containing 0 or 10 ng of p53 were used to determine the coefficient of variation (CV%), which reflects intra-assay variation.
As an interpretation the CV%, values of intra-assay variation within triplicates of the Probe-IP assay as shown in Table 4.21 are comparable to those within qPCR triplicate samples. In Chapter 6, these data will also be compared to Active Motif’s ELISA-based TransAM kit for the quantification of active p53.

4.7.3 Sensitivity

Sensitivity is an important measure of assay performance, as purified p53 is expensive to buy or produce, and an *in vitro* screen would benefit from the use of less. From the point of view of utilising this assay to quantify DNA binding activity of endogenous transcription factors, sensitivity must be sufficient to detect the low concentration present *in vivo*.

![Sensitivity of Probe-IP assay detection of purified p53](image)

Figure 4.22. **Sensitivity of Probe-IP assay detection of purified p53.** Probe-IP assay was performed with 0.054 pmol of probe input and 1 µl of PAb421, in the presence of 0, 1, 2, 5, or 10 ng of p53. Lower limit of detection is shown as approximately 5 ng of p53.

The lower limit of detection for this assay was found to be 5 ng of p53 protein, as indicated in Figure 4.22. This is approximately 10x more sensitive than similar conditions tested using EMSA (see section 4.4.1). Although the use of qPCR as a
detection system is a sensitive method in itself, sensitivity is limited by the immunopurification step prior to this. The reported sensitivity of commercially available ELISA-based methods is much lower than 5 ng, and the TransAM kit from Active Motif is quoted to detect 0.3 ng. In order to improve sensitivity, modifications to the protocol will be explored in the next chapter to decrease the level of background probe capture, improving signal to noise ratio and sensitivity.

4.8 Discussion

This chapter has outlined the initial development and performance of a novel assay for active transcription factor quantification. This has followed on from the previous chapter, which outlined the optimum conditions for ternary complex formation analysed by EMSA supershift, and has adapted this new protocol for the immunopurification of the ternary complex and subsequent detection of the probe by qPCR. Although making use of the established methods of magnetic bead immunopurification and qPCR, the development of this method has presented challenges in relation to the protocol, reagents and the equipment itself, as discussed in this section.

This chapter has shown that as hypothesised, the qPCR signal increases in response to p53 concentration (Figure 4.11). Signal is also shown to increase even once p53 is the limiting factor in the reaction, which suggests that either a proportion of purified protein is not active for DNA binding, or the increase in p53 concentration reduces the portion of probe lost through column washing.

This increase in qPCR signal was found to increase despite the observation that the magnetic beads affect the qPCR detection (Figure 4.8), and thus probe concentrations will instead be only relative values. However, the main purpose of this conversion to absolute quantities of probe is to unmask the vast differences observed when looking at logarithmic C\textsubscript{T} values alone, and if accurate probe concentration must be known, for example in the analysis of kinetics, probe can be eluted and purified alone as illustrated in Figure 4.9.
This chapter has also found that the signal is specific to the presence of p53-probe binding, as replacing p53 with the same concentration of BSA depletes probe capture to that of background levels (Figure 4.12 C). However, despite a reported $k_d$ of $7.7 \pm 1.2$ nM for p53 with the gadd45 DNA sequence used as the assay probe (Weinberg et al., 2005), under present conditions there is a level of sequence-independent p53-DNA interaction, as indicated by a competition assay in Figure 4.13, and the presence of poly(dI-dC) in Figure 4.14.

This could present a problem for the detection of sequence-specific p53-DNA binding activity. Whilst the site-specific DNA binding ability of p53 is conferred from the core domain of p53, the sequence-independent binding ability is conferred from the C-terminal domain; and the latter may be active despite absence of the former, yielding false-positive data. Associating PAb421 to p53 prior to addition of probe may increase specificity by blocking the C-terminal domain, and increased washing stringency of the column may be effective at removing nonspecific lower affinity interactions. However, it must be taken into account that nonspecific DNA is present in excess of probe, making interaction with p53 more likely. For this reason, pure protein-DNA binding studies commonly include BSA in the reaction (for example, Cain et al., [2000] use 2 µg BSA). It is also possible that the presence of BSA will alleviate a problem identified in the absence of antibody as illustrated in Figure 4.15., where protein-probe complexes contribute to background probe capture in a concentration dependent manner. As this may hinder the application of this assay in an endogenous system, it is hoped the presence of BSA will block the interference of protein-probe complexes.

Whilst the intra-assay variation is comparable to those of qPCR triplicates, the inter-assay variation is susceptible to changes occurring within the EMSA buffer used as both an environment for ternary complex formation, and for column washing to remove unbound probe. Although the composition of this buffer consists of stable components (the exception being DTT, which is added fresh prior to each assay), changing the buffer stock after 8 months had dramatic effect on assay conditions. Furthermore, change was observed over time both of background probe capture in the absence of p53 (see Figure 4.19), and visibly in the turbidity of buffer solution dependent on the
detergent igepal. As a precaution, buffer is filter sterilised and stored at room temperature no longer than a week, which has restored the signal to noise ratio.

With assay sensitivity in mind, the utilisation of qPCR for end-point detection seemed like the logical choice to provide an advantage over commercial ELISA-based kits available from a range of suppliers. However, this chapter has found that although qPCR is a sensitive method in itself for detection of DNA, the immunopurification assay upstream is limiting, and the qPCR detects high background presence of probe. It was thought this excess could be removed by Exo III, but the concentration tested was found to have a negative effect on the signal to noise ratio (Figure 4.11). The lower limit of detection of this assay is currently 5 ng of p53, but it is hoped this will be improved by further assay optimisation.

In conclusion, this chapter has confirmed the main hypothesis underpinning this method: that the qPCR signal reflects the concentration of p53 within the sample. However, limitations have been highlighted in the areas of specificity, sensitivity and reliability, and these problems will be addressed by exploring optimisation in the next chapter.
Chapter 5

Optimisation of Probe Immunopurification Assay performance

5.1 Introduction

The previous chapter verified initial assay performance, as defined by signal specificity, sensitivity and reproducibility, and established that all three areas require improvement. This chapter explores optimisation of some of those aspects thought to affect assay performance, which are as follows:

- Probe concentration
- Antibody concentration
- Column washing volume
- Column washing stringency
- Concentration of blocking component
- Order of adding antibody and probe to reaction

Further aspects of this assay which may also have an effect on performance, some of which were observed to make little difference in EMSA, but may make a difference in this system, or which are beyond the scope of this project to investigate are as follows:

- Antibody type
- Magnetic bead system
- Magnetic bead concentration
- Incubation times
- Incubation temperatures
- EMSA binding buffer recipe
5.2 Titration of reaction components

The concentration of reaction components is important in assay optimisation, in terms of both sensitivity and signal to noise ratio. As well as the concentration of transcription factor itself, the assay range of which was identified in section 4.3.2., the probe and antibody are predicted to have the greatest effect, and this is addressed in this section by altering the concentrations of both. A fixed amount of p53 protein at 10 ng was also decided on for use in the comparison of different optimisations, as this consistently provides a sufficient signal to noise ratio, and more optimisation parameters can be tested in parallel when testing only one p53 concentration. Assay performance is judged by the signal to noise ratio (p53 +/-).
5.2.1 Probe titration

![Probe titration graph]

Figure 5.1. **Probe titration effect on signal to noise ratio.** Probe concentrations of 0.054 pmols, 0.15 pmols or 0.3 pmols were used in assays containing 1 µg of PAb421 and either 10 ng or 0 ng p53. Both signal and background values rise with an increased probe concentration, but there is no significant difference observed in the signal to noise values (p53 +/-). Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.

There was found to be no significant change in the signal to noise (p53 +/-) ratio in the range of probe concentrations tested, although background probe capture does increase. Since it is preferential to limit background probe capture, 0.054 pmols of probe will still be used in further experiments.
5.2.2 Antibody titration

The concentration of the antibody in the reaction is thought to be important, since excess is required to ensure that the captured DNA-binding protein is not the limiting factor in the reaction. However, an excess of free antibody may contribute to more probe background as well as competing with the ternary complex for binding to the magnetic beads. Furthermore, financial considerations favour the use of less. In experiments performed so far, 1 µg of PAb421 has been used per sample, as this was shown to result in the largest EMSA supershift in Figure 3.6. The molecular weight of the PAb421 IgG2a is 148 kDa, and therefore assuming a valency of one antibody molecule to two molecules of p53 monomer, the presence of 1 µg of this immunoglobulin will be fully occupied by 628 ng of His-tagged recombinant p53 (MW 46.42 kDa). In these assays, since only 10 ng is being used this incurs a large excess of antibody and therefore it may be beneficial to decrease the concentration. In this section, the amount of antibody added to the assay is decreased to 0.5 µg, and the optimum amount of antibody will be used in further experiments.
Figure 5.2. *Antibody titration effect on signal to noise ratio*. Probe-IP samples were assayed containing 0, 0.5 or 1 μg of PAb421, in the absence or presence of 10 ng p53. Signal to noise (p53 +/-) increases with PAb421 concentration. Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.

Despite the excess of PAb421 to the concentration of p53 present under these assay conditions as previously discussed, an increase in sensitivity and the signal to noise ratio is observed with 1 μg of PAb421, as shown in Figure 5.2. It is possible that although in excess, doubling the concentration of antibody reduces loss of p53-probe complexes within the column due to present washing conditions, which will be investigated in section 5.4.
Also evident in Figure 5.2 is increased antibody dependence (1 µg PAb421 +/- ratio) in the presence of 10 ng p53. This is currently 9.1, compared to 2.5 in section 4.6.3 (Figure 4.15). Probe capture in the presence of 10 ng p53, but in the absence of antibody indicates interference from p53-probe complexes within the column resulting in nonspecific probe capture. It may be the lower concentration of probe now used, or the fresh EMSA buffer (for both binding and column washing) with NP-40 detergent fully in solution, is more conducive to the removal of these p53-probe complexes. However, the addition of BSA will still be tested, since it may improve assay performance further.

5.3 Blocking nonspecific probe interactions using BSA

The previous section found that under present conditions, using a lower concentration of probe and fresh binding and wash buffer, the antibody dependence was improved, meaning that fewer p53-probe complexes were being retained in the column in nonspecific probe capture. However, it is hypothesised that the presence of BSA might act to further block the nonspecific interaction of protein-DNA complexes with assay components, which will be important when using an endogenous analyte. Therefore, this section looks at how including BSA in the reaction affects antibody dependence and the signal to noise (p53 +/-) ratio.

When deciding on the range of BSA to use, it was reasoned that as it is the interference of 10 ng of p53 protein looking to be blocked in this experiment, a range of 50 and 100 ng should be sufficient. It has previously been shown that this concentration range of BSA protein contributes little toward nonspecific probe capture in the absence of p53 (section 4.6.1; Figure 4.11). Whether this is the case under altered experimental conditions (lower probe concentration and fresh EMSA buffer), and whether BSA affects the signal to noise ratio (p53 +/-) will also be studied.
Figure 5.3. **Presence of BSA affects antibody dependence.** With the addition of: 0, 50 and 100 ng of BSA to assays of 10 ng p53, using 0.054 pmol of probe and either 0 or 1 µg PAb421, probe capture increases in both the presence and absence PAb421, causing a drop in PAb421 +/- Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the PAb421 +/- ratio.  

With increasing concentration of BSA present in the reaction, a reduction in antibody dependence is observed (see Figure 5.3), due to increased probe capture in the absence of antibody. This is contrary to the hypothesis that the presence of BSA would decrease probe capture in the absence of antibody, which indicates the presence of BSA is contributing to nonspecific probe capture in the presence of p53-probe complexes. These data suggest that under current conditions the specificity of this assay would not be sufficient for use with endogenous analyte.
Figure 5.4. **Presence of BSA does not affect p53 +/- signal to noise ratio.** In an assay containing 0.054 pmols of probe, 1 µg of PAb421 and either 0 or 10 ng p53, the presence of 50 and 100 ng of BSA depleted probe capture in the presence of p53, also depleting the p53 +/- . Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.

The presence of BSA results in no significant effect on the p53 +/- ratio, as shown in Figure 5.4. Comparing data between Figures 4.3 and 4.4, it can be seen that BSA does not contribute to nonspecific probe capture in the absence of p53, but does in the absence of antibody. This is consistent with the idea that probe is removed less efficiently from the column when bound to p53; the presence of 2x and 10x more BSA protein than p53 interferes more with the removal of p53-probe complexes (in the absence of antibody), than with the removal of unbound probe (in the absence of p53).
Contrary to the hypothesis that the presence of BSA would decrease the interference caused by protein-probe complexes capturing probe non-specifically, its presence seems to interfere more with the removal of p53-probe complexes from the column. Therefore, instead of trying to prevent background probe or protein-probe complexes from binding within the column, increased column washing will now be investigated as a way to increase their removal.

5.4 Optimisation of column washing volume and stringency

The removal of unbound probe from the column can be altered by wash volume and stringency. This is important to optimise, as too little may result in high background noise, although too much threatens to break apart the specific ternary complex being measured in addition to removing nonspecific interactions.

From previous observations of assay performance suffering when probe was captured independently of p53 (see sections 4.6.3 and 5.2.1), it was hypothesised that increasing wash volume would increase removal of unbound probe and therefore improve the signal to noise ratio. Therefore, wash volume was increased from the 1 ml of EMSA 1x buffer used in the Probe-IP assay so far, to 2 and 3 ml. The results are shown in Figure 5.5.
Figure 5.5. **Increased column wash volume reduces signal to noise ratio.** In an assay containing 0.054 pmols of probe, 1 µg of PAβ421 and either 0 or 10 ng p53, all values decrease when column washing volume is increased to 2 and 3 ml of EMSA 1x buffer. Signal to noise (p53 +/-) exhibits no significant difference. Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.

It can be seen in Figure 5.5 that as expected, increasing the volume of column wash to 2 and 3 ml of EMSA 1x buffer reduces probe capture. However, this increased washing volume has also removes specific probe capture, suggestive of the removal of p53-dependent signal. Although the half-life of p53 binding specifically to its consensus sequence is 25 minutes (McLure & Lee, 1998), samples are incubated for 90 minutes in total prior to immunopurification, and therefore some p53 will be dissociating from the probe, if only momentarily. A wash of 1 ml takes approximately 3 minutes to flow through, and increasing wash volume increases the time of washing, hence the amount of probe potentially lost from the ternary complex.
As an alternative to changing the volume and hence time of washing, the salt concentration of wash buffer (EMSA 1x) was increased with NaCl to a concentration of 120 mM. As a charged compound, NaCl interferes with ionic bonds between basic amino acid residues (such as arginine and lysine), and the negatively charged phosphate backbone of the DNA. For example, if:

\[
\begin{align*}
\text{B}^+ & : \text{a cation of a basic amino acid (lysine or arginine) within the primary structure of p53} \\
\text{P}^- & : \text{an anion of the phosphate backbone of the DNA molecule} \\
\text{X}^+ & : \text{a cation in solution, i.e. the Na}^+ \text{ of the NaCl in the wash buffer} \\
\text{Y}^- & : \text{an anion in solution, i.e. the Cl}^- \text{ of the NaCl in the wash buffer}
\end{align*}
\]

Then dissociation of a protein-DNA complex by NaCl can be described by:

\[
\begin{align*}
\text{B}^+\text{P}^- + \text{X}^+ + \text{Y}^- & \rightleftharpoons \text{B}^+\text{Y}^- + \text{X}^+\text{P}^- \\
(\text{Shiffman et al., 1978})
\end{align*}
\]

It can be seen that the monovalent cations in solution effectively compete out ionic interactions between protein and DNA. Furthermore, these interactions are predominantly relied upon for nonspecific protein-DNA interaction; specific interactions tend to rely upon hydrogen bonding and hydrophobic interactions between DNA bases and amino acids, although ionic bonds are still important for stability (Molley, 2006).
Figure 5.6. **Increased column washing stringency depletes signal to noise ratio.** In an assay containing 0.054 pmols of probe, 1 µg of PAb421 and either 0 or 10 ng p53, adding 130 mM NaCl to column wash buffer reduced probe capture, and depleted p53 +/- Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.

As hypothesised, increasing the stringency of column washing has been shown to reduce probe capture, as shown in Figure 5.6. However, contrary to expectations, the signal to noise ratio has been reduced. It may be that with increased NaCl concentration, any probe associated with p53 via the C-terminal domain (with a lower affinity interaction) is washed away, resulting in a signal drop as this NaCl concentration is too low to affect specific interaction in EMSA (Butcher *et al.*, 1994).

5.5 Pre-binding PAb421 and p53

By performing a competition experiment in the previous chapter, the occurrence of nonspecific p53-DNA interaction was observed (see Figures 4.13 and 4.14), and is problematic to the ability of this assay to detect site-specific DNA binding of p53. To
address this, it is reported in the literature that the binding of PAb421 interferes with the nonspecific binding properties of p53 by blocking the ability of the C-terminal domain to bind DNA in a nonspecific manner (Wang 93; reviewed by Ahn & Prives, 2001). Therefore, it is hypothesised that pre-binding p53 and PAb421 will improve specificity. Preventing nonspecific p53-DNA interaction will enable the addition of nonspecific DNA to the assay, which has been shown to decrease background probe capture in the absence of p53 (see Figures 4.13 and 4.14). Furthermore, the binding of PAb421 is also reported to increase the affinity of specific binding via the core domain in the presence of nonspecific DNA (Anderson et al., 1997), potentially improving assay sensitivity.

Figure 5.7. **Allowing PAb421 to bind p53 prior to the addition of probe improves signal to noise ratio.** Assays containing 0.054 pmol of probe, 1 µg of PAb421 and either 0 or 10 ng of p53 were compared between mixing probe and p53 prior to adding PAb421 and mixing p53 with PAb421 for 15 minutes at room temperature prior to adding probe. Pre-mixing PAb421 and p53 increases p53 +/- due to both a drop in probe capture in the absence of p53 and increase in probe capture in the presence of p53. Error bars represent standard deviation of qPCR triplicates, which is subsequently expressed as a mean percentage of the p53 +/- ratio.
As hypothesised, an improved p53 +/- signal to noise ratio is observed adding the probe fifteen minutes after incubating p53 and PAb421, as opposed to adding PAb421 to the p53-probe mix for an hour incubation at room temperature. Although a slight increase in specific signal is observed, the improved signal to noise ratio owes more to the decreased probe capture in the absence of p53. It is thought that reducing the time antibody is in contact with the probe reduces antibody contribution to background probe capture observed as in Figure 4.15.

5.6 Discussion

A range of optimisations were initially explored in order to improve assay performance, but with limited positive effect. There was no significant response to the concentrations of probe tested, but owing to background probe capture considerations, the lowest probe concentration tested, 0.054 pmols, was used in further assays. However, if it were possible to optimise with more samples available it would be advantageous to test probe concentration in parallel with washing stringency, as it may be that a compromise exists between these two parameters. A higher probe concentration is advantageous to drive the reaction toward the formation of the ternary complex, whilst column washing stringency must be optimised to remove excess probe, and thus decreasing background noise. The use of 1 µg of PAb421 was found to yield the highest p53 +/- signal to noise ratio, but the use of higher concentrations is limited by financial considerations. It is possible that other antibodies available may improve performance, and this is something to consider in the future. Owing to the inter-dependent nature of optimisation parameters, to improve assay performance further, the 96 well plate format of this assay is necessary to explore multiple factors in parallel.

Levels of inter-assay variation have also remained high. For example, in the experiments shown in this chapter for assays containing 0.054 pmols of probe, 0 or 10 ng of p53 and 1 µg of PAb421, inter-assay variation is calculated with a CV% for 0, 10 ng of p53, and the p53 +/- of 120, 77 and 42.5%, respectively, as shown in Table 5.11. This is increased from values in the previous chapter achieved when using the same
EMSA buffer stock in each experiment (50%, 30% and 68% for 0, 10 ng p53 and p53 +/-, respectively; see Figure 4.16).

<table>
<thead>
<tr>
<th>p53 ng</th>
<th>Figure 5.1</th>
<th>Figure 5.2</th>
<th>Figure 5.4</th>
<th>Figure 5.5</th>
<th>Figure 5.6</th>
<th>Figure 5.7</th>
<th>Mean</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.63</td>
<td>0.38</td>
<td>0.38</td>
<td>4.86</td>
<td>0.53</td>
<td>2.06</td>
<td>1.5</td>
<td>120.7</td>
</tr>
<tr>
<td>10</td>
<td>1.63</td>
<td>2</td>
<td>1.65</td>
<td>8.05</td>
<td>2.13</td>
<td>4.28</td>
<td>3.3</td>
<td>77</td>
</tr>
<tr>
<td>p53 +/-</td>
<td>2.6</td>
<td>5.22</td>
<td>4.33</td>
<td>1.66</td>
<td>4.05</td>
<td>2.08</td>
<td>3.3</td>
<td>42.5</td>
</tr>
</tbody>
</table>

Table 5.8. Inter-assay variation of the Probe-IP assay. In an assay of 0.054 pmols of probe, 1 µg of PAb421 and either 0 or 10 ng of p53, values for apparent probe capture were analysed for the coefficient of variation

In addition to the inter-assay variation of those experiments shown in this thesis, many experiments have failed to provide meaningful or reliable data. Although a likely contributor to this occurrence is the inherent error involved in the handling of small sample volumes (to which automation of the protocol may provide a solution), another observed effect on levels of probe capture is the rate of column flow, where a column running noticeably slower yields significantly higher levels of probe capture. This is not a factor that can be controlled, other than to limit its occurrence by ensuring all buffers are filtered and sonicated. Any sample where a column runs at a rate visibly different to the rest of the set is discarded.

Emerging from these data, a likely explanation for high levels of variation, as well as a higher limit of detection than expected, seems to lie in the $k_{off}$ of the p53-probe interaction. The ternary complex is dynamic in nature, and a study by Cain et al. (2000), using a time course EMSA in the presence of excess unlabelled specific competitor found that after 5 minutes, less than 20% of p53 remains bound to probe. Although the $K_{on}$ rate is very fast, in the order of seconds (Bargonetti et al., 1992) during the column washing process, once probe has dissociated from the complex it will be immediately washed away resulting in the depletion of specific probe capture. This is reflected in the data from Figure 5.5., where increasing the washing volume has no significant effect on
the signal to noise ratio, but does deplete p53-specific signal. This idea is consistent with the observed effect of variable column flows, especially when the average column flow rate of 300 µl/minute is compared to the 10-30 µl/minute rate used for washing the antibody-p53 or DNA-p53 complex immobilised to the sensor chip of a typical SPR experiment (Wang et al., 2009). Throughout Chapter 3 (and in Figure 4.10) ternary complex formation is visible by EMSA, and this discrepancy between these two methods can be explained by the effect of “caging”, whereby the gel matrix retains protein and probe in close proximity promoting rapid re-association unlike what occurs in free solution. Furthermore, this idea is also supported in the context of p53-antibody interaction, by the observation that even in conditions of antibody excess, an increase in concentration increases both signal and signal to noise ratio (Figure 5.2), suggesting the occurrence of p53-antibody recapture within the column. Together, these data and observations suggest that the column format of the Probe-IP assay generates flow speeds too fast for the stability of the ternary complex, with small variations in flow resulting in greater variations in probe capture.

One of the aims of this project was to optimise performance sufficiently for the detection of endogenous p53 activity. It may be that endogenous p53 is more active than purified His-tagged protein, and therefore the use of this assay to detect endogenous p53 will be investigated in the next chapter, as well as comparing performance with a commercially available kit.
Chapter 6

The detection of endogenous p53 activity in nuclear extracts

6.1 Introduction

The previous chapter outlined the development of the probe-IP assay to detect the binding of a purified transcription factor to its DNA consensus sequence. As a pure protein system, this has potential application as a tool for drug development and the \textit{in vitro} study of protein-DNA interactions. However, an equally important aspect of this project is the application of this assay in the study of endogenous transcription factors from cells derived from tissue culture, and eventually even primary tumour biopsies themselves.

The endogenous transcription factors analysed in this chapter were derived from tissue culture. LS174T colorectal adenocarcinoma cells were used, as these were shown by western blot to have levels of wild type p53 sufficient for detection (see Chapter 3). Levels of p53 were increased further by treatment with Cisplatin. The aim of this section is to detect increasing levels in endogenous p53 in response to this cytotoxic drug.

Another feature of this chapter is the comparison with a commercially available ELISA-based kit. In addition to setting a performance benchmark to the probe-IP approach, this also serves as a positive control for both the purified p53 and nuclear extracts used as analyte with the probe-IP assay.

Nuclear extracts were prepared using a Nuclear Extract kit purchased from Active Motif, and total protein levels were determined using the BCA (Bicinchoninic Acid) assay.
6.2 BCA method for the quantification of total protein in nuclear extracts

Levels of total protein extracted from the nuclei of cells were determined using the BCA method. A standard curve of BSA was constructed, and used to interpolate the total protein concentration of unknown samples. Different batches of nuclear extract will contain different levels of total protein, therefore four samples from different batches were tested and an average taken which will be used throughput this chapter unless otherwise stated.

![BCA method for total protein quantification](image)

Figure 6.1. **BCA method for total protein quantification.** A standard curve was constructed using BSA concentrations of 0, 25, 125, 250, 500, 750, and 100 µg/ml in the BCA assay. The absorbance of unknown samples was measured at 562 nm, and the total protein determined by interpolation from the graph.

At a wavelength of 562 nm, the four samples exhibited an absorbance of 1.096, 0.785, 1.1, and 1.19, which were converted to 820 µg/ml, 585 µg/ml, 820 µg/ml, and 880 µg/ml, respectively. From figure 5.2 an average of 776 µg/ml was determined for the samples, which were initially diluted 1/10 for the BCA method. Therefore, it was found that the nuclear protein samples extracted from LS174T cells contain on average 7.8 µg/µl of total protein. This average will be assumed for all verification and assay development experiments, but batches for individual analysis of assay sensitivity and performance, each batch will be individually quantified.
Alternative methods for quantification of active transcription factors are commercially available. One such kit was purchased from Active Motif for the quantification of active p53. This method is based on a sandwich ELISA format, although the capture antibody is instead replaced with a synthetic oligonucleotide. Unlike a conventional ELISA, this method therefore detects only those transcription factors with DNA binding capability.

This section is included within this project for two reasons. The first is to compare the performance of a commercially available method with that of the probe-IP. The second is to test the active p53 concentration in nuclear extract samples collected from LS174T cells treated with the cytotoxic agent Cisplatin, with the aim of detecting an active p53 increase in response to genomic damage, and to determine whether p53 is present at a concentration feasible for detection using the probe-IP method.

The p53-DNA binding specificity as evaluated by a competition experiment was tested, together with the sensitivity of detection for both purified and endogenous p53 present in nuclear extracts.
Figure 6.2. **Binding specificity of the Active Motif TransAM p53 kit determined from a competition experiment.** Free probes encoding either a wild type p53-binding sequence (self, red), or a mutated binding site (mutant, blue) were added to the p53-DNA binding reaction in the presence of 2 and 5 µg of LS174T nuclear extract. The addition of self competitor has diminished the signal, whereas the addition of mutant competitor has had no effect.

The synthetic oligonucleotide immobilised to the ELISA plate is of the sequence 5’-GGACATGCCGGCATGTCC-3’, forming a palindromic binding sequence for p53. In the presence of free oligonucleotide of this same sequence in the reaction, the signal drops 86%, as less p53 is available for binding the immobilised probe. Conversely, when this oligonucleotide bearing a single point mutation is added to the reaction no such drop is observed, as p53 has no specific affinity for this mutant probe (see Figure 6.2). This shows a high level of binding specificity between p53 and the oligonucleotide capture probe for this assay, despite association of p53 and probe prior to the addition of antibody. The washing stringency must be optimum to remove nonspecific p53-probe interaction via the C-terminal domain, but retain stronger specific interactions from the core domain.
Figure 6.3. **Linear range of the TransAM active p53 detection assay**. Lower limit of detection is 0.3 ng of p53 protein. The reaction begins to plateau above 2.5 ng of p53.

A titration of recombinant p53 (Calbiochem) formed the standard curve, which was used to determine the concentration of active p53 in nuclear extract samples. This also indicated the linear range of the assay as between 0.3 and 2.5 ng of p53 per sample, which agreed with the supplier's claims. The reaction was saturated above this level.
Figure 6.4 Detection of a Cisplatin-dependent increase in active p53 levels. Cisplatin was added to the growth media of LS174T cells once at approximately 70% confluence, to a final concentration of 0, 0.2, 2 or 10 µM, and left for 24 hours. Cells were harvested and nuclear extracts prepared. Using Active Motif’s TransAM p53 detection kit, an increase of p53 levels in 5 µg of nuclear extract is observed in response to Cisplatin treatment.

Specificity of this assay when analysing nuclear extracts is also shown, where Figure 6.3 shows p53 levels in cells treated with Cisplatin are shown to increase, using the same amount (5 µg) of nuclear extract. It is widely known that p53 levels increase with genotoxic stress caused by the DNA damaging agent Cisplatin.

In addition to this section serving as a comparison for ELISA-based methods for active transcription factor quantification, this has also highlighted two other points. Firstly, that in order for the probe-IP assay to detect endogenous transcription factors the assay must be able to detect at least 0.3 ng/µl of p53. Secondly, this section has also verified both the DNA-binding activity of purified p53 together with the presence of active p53 in the LS174T nuclear extract samples, and that as expected the levels do show a concentration-dependent response to Cisplatin treatment.
Figure 6.5. **Range of LS174T nuclear extract for use in Probe-IP assay.** Assays were performed binding 1 µg of PAb421 to either 0, 0.5, 1, 2 or 4 µg of nuclear proteins extracted from LS174T cells previously treated with 10 µM Cisplatin. After incubation for 15 minutes at room temperature, 0.054 pmol of probe was added and incubated for a further 45 minutes at room temperature. Probe capture drops in the presence of more than 1 µg of nuclear protein. Error bars indicate standard deviation of qPCR triplicates.

From Figure 6.4 it is evident that in 5 µg of nuclear extract treated with 10 µM Cisplatin, 3.23 ng of p53 could be detected. The lower limit of detection using the Probe-IP assay is 5 ng of purified p53. Therefore, approximately 7.7 µg of LS174T nuclear extract would be needed to detect 5 ng of p53. From Figure 6.5 it is evident that the assay becomes saturated with the use of more than 1 µg of extract, and therefore it is unlikely that probe capture in 6.5 is p53-specific. This was tested using a competition binding experiment with 1 µg of extract (data shown in Figure 6.6), and a similar drop in probe capture is observed in presence of both self and mutant competitor. In fact, probe capture in the presence of mutant competitor is lower than in presence of self, indicating no evidence of p53-specific detection.
Figure 6.6. **p53-DNA binding competition.** Assays were performed binding 1 µg of PAb421 to 1 µg of nuclear proteins extracted from LS174T cells previously treated with 10 µM Cisplatin. After incubation for 15 minutes at room temperature, 0.054 pmol of probe and 1 ng poly(dI-dC) was added either alone or with 100x self (s) or mutant (m) competitor oligonucleotide, and incubated for a further 45 minutes at room temperature. A drop in signal is present in presence of both self and mutant competitor oligonucleotides. Standard error in qPCR triplicates are shown. When looking at the ratio of mutant to self competitor signal as a measure of specificity, it can be clearly seen that the Probe-IP assay specificity of 0.7 is absent when compared with a ratio of 10.1 and 8.1 for 2 and 5 µg of LS174T extract as detected by the TransAM ELISA kit.

6.4 Discussion

The Active Motif TransAM kit for the quantification of p53 has shown that the same batches of nuclear extract from the LS174T cells used for the Probe-IP assay have detectable levels of active p53 present (the EMSA experiments in Chapter 3 were performed using different batches of both LS174T cells and nuclear extract). It has also been verified that the purified His-tagged p53 is active for p53 binding. As a comparison to the current performance of the Probe-IP assay, the ELISA-based kit has the ability to detect 0.3 ng of purified p53, has the specificity to detect a concentration increase in p53 in response to Cisplatin treatment of LS174T cells, and shows a high level of specificity of p53-DNA binding using a competition experiment.
In contrast, the Probe-IP assay does not demonstrate specificity in p53-probe binding using a competition experiment, as probe capture in the presence of 100x excess mutant DNA decreases more than in the presence of 100x self. In Figure 6.5 it can be seen that the detection of p53 is saturated at 2 µg of LS174T extract, which was found by the TransAM kit to contain 1.4 ng p53 (Figure 6.2), and this is below the lower limit of detection of 5 ng p53 for the Probe-IP assay (see Figure 4. 22).

In conclusion, the nuclear extract samples have been shown to contain levels of p53 which can be detected by the commercially available ELISA-based kit purchased from Active Motif. However, in the scope of this project is has not been possible to optimise the sensitivity and specificity of the probe-IP assay sufficiently for the detection of endogenous p53 in LS174T nuclear extract samples.
Chapter 7

Discussion

7.1 Aims and achievements

The aim of this project was to develop a suitable method for the quantification of both purified and endogenous active p53. During this project, attempting to develop two methods for the sensitive and high throughput quantification of transcription factors, both have been found to incur limitations in sensitivity and reliability. Initially it was hypothesised that an antibody array approach would be suitable, but this was found to be insufficient for the detection of tumour markers (Wainright, 2007). In the second approach, named the Probe-IP, it was hypothesised that the level of probe capture as quantified by qPCR, would reflect the initial concentration of DNA binding protein present within the analyte, with a sensitivity and specificity better than that of a commercially available ELISA-based kit.

It was found that the Probe-IP assay could detect the presence of purified His-tagged p53 with a lower limit of detection at 5 ng. This is considerably less sensitive than the 0.3 ng of p53 that was found can be detected by Active Motif’s TransAM ELISA-based kit.

The Probe-IP signal was shown to be specific for p53, as replacing p53 with BSA protein failed to enhance background probe capture. It was attempted to determine protein-probe binding specificity using a competition binding experiment, although it was found that using pure protein incurs nonspecific interaction between p53 and the probe DNA backbone, and adding BSA to the reaction decreases the signal to noise ratio further. Specificity was also evaluated by antibody dependence, which identified a significant contribution to background probe capture from protein-probe complexes.

When attempting to quantify active p53 levels from endogenous analyte, binding specificity was far greater for the TransAM kit, with a mutant/self ratio of 10.1 when detecting p53 from 2 µg LS174T nuclear extract, compared with 0.7 when performed
with 1 µg extract using the Probe-IP assay. Taken together, the current level of sensitivity and specificity were found to be inadequate for the detection of p53 from LS174T nuclear extract.

Another measure of assay performance is the level of intra- and inter-assay variation. Levels of intra-assay variation were found to be significantly affected by variation in column flow due to blockage resulted in a higher than expected value. Levels of inter-assay variation are sensitive to multiple factors, such as the standard curve used to convert C_T to absolute values, the large dilution factor in probe concentration, handling of small volumes of probe and protein, and changes in binding and wash buffer over time.

Since the three years when work began to develop this approach, increasing numbers of companies now supply commercial ELISA based kits. The fact that qPCR and immunopurification have been established methods for a number of years suggests commercial ventures are more likely to continue in the use of ELISA technology, which is potentially high throughput and offers adequate sensitivity for active transcription factor quantification at a lower cost than this probe-IP approach may. Under the current experimental conditions, the probe-IP costs approximately twice that (£12, compared to £6) of Active Motif’s TransAM plate. So far, no commercial transcription factor profiling kits are based on qPCR detection; and in this project we have perhaps identified a reason for this.

The inherent limitation appears to arise from the k_off of p53 from DNA, where it has been reported by EMSA that after 5 minutes less than 20% of p53 remains bound to probe (Cain et al., 2000). During the 6 minutes of column washing time, it could be reasonably expected that specific probe capture will be depleted by 85%. This idea is especially supported by the observed decrease in signal to noise ratio when increasing wash volume (and hence time), as well as the increased signal to noise ratio when increasing antibody levels far above excess, suggestive of re-binding within the column. The fact that the column flow rate is over 10-30x higher than that used in SPR, and that slowed column rates due to blocking increase probe capture significantly all point
toward this conclusion, and this may also help to explain low levels of sensitivity observed when compared to an ELISA-based method where washing is not a continuous flow.

Furthermore, in the detection of endogenous transcription factors using the Probe-IP design, the antibody will bind both active and inactive transcription factors, which in some cases may lead to saturation of antibody, reducing assay sensitivity. The ELISA method removes inactive transcription factor from the reaction prior to the addition of antibody, resolving this problem.

7.2 Future work

As described throughout the introduction to this thesis, a sensitive method for the quantification of transcription factors has much potential application. However, in order to detect and quantify endogenous transcription factors, further optimisation is required. This has been hindered under the current format, as the sample number in each assay is limited to twelve. With so many interdependent factors affecting assay performance, moving onto a higher throughout system (such as the multiMACS 96 well plate version) would facilitate the parallel optimisation of these factors necessary for the quantification of endogenous transcription factors.

To improve performance, it is also required to understand exactly how different parameter alterations in turn affect the composition of the column eluent, not just the apparent probe content of the sample as quantified by qPCR. For example, the antibody or p53 content as detected by ELISA or western blot. As an alternative approach, SPR could be used with the same format (PAb421> p53> probe) and buffer to compare washing rates in order to ascertain whether the $K_{off}$ rate of the protein-probe interaction is really the root cause of poor sensitivity and variability within the assay.

In conclusion, investigating new technologies is crucial for scientific progress, but the possibility of unforeseen challenges remains an inherent risk of such investigations. Although straightforward in theory, it seems that this particular assay format requires further modifications for the detection of endogenous transcription factor activity.
8. References


