

Cranfield University

Elizabeth E.A. Bull

Towards a Low-Cost Clinical Multiple Mutation Diagnostic:

Cystic Fibrosis as a Model

Institute of BioScience and Technology

PhD Thesis

ProQuest Number: 10832250

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10832250

Published by ProQuest LLC (2018). Copyright of the Dissertation is held by Cranfield University.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Cranfield University

Institute of BioScience and Technology

PhD Thesis

Academic Years 1998-2001

Elizabeth E.A. Bull

Towards a Low-Cost Clinical Multiple Mutation Diagnostic:

Cystic Fibrosis as a Model

Supervisors- Dr. David C. Cullen, Dr. Stan Evans, Dr. John

O'Donnell and Professor Phil Warner

May 2002

*This thesis is submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy*

Abstract

Cystic fibrosis is used in this work as an example of a genetic disease where early diagnosis and medical intervention can improve quality of life. Current methods of cystic fibrosis diagnosis rely heavily on the sweat test, a biochemical method of measuring the concentration of sodium and chloride in sweat. A repeat test is required for sodium levels between 40 and 70 mmol per litre, which may occur in babies with either mild or no cystic fibrosis. The advantage of a DNA test is that a cut off value is not necessary, this reduces false positive and negative results. A mutation is either present in a person's DNA or it is absent. Cystic fibrosis has been associated with over 500 mutations, therefore a type of mutation detection was investigated which could potentially examine a large proportion of these in one test. A simple, low cost method of mutation detection is required for use within the National Health Service. The reverse dot blot hybridisation allows a known sequence of DNA to be placed on a membrane (mutant and wild type) and hybridised with an unknown sequence (patients' DNA). Covalent coupling of oligonucleotides to membrane produced increased attachment over physical attachment as examined via radioactive labels. Polystyrene slides were chemically modified (using 5% potassium permanganate in 1.2M H₂SO₄) to provide carboxyl groups for the covalent attachment of amino terminated oligonucleotides. Human DNA was amplified by PCR, labelled with biotin and detected via chemiluminescence. Genomic DNA was extracted from blood samples with the Qiagen QIAamp system and both wild type and mutant sequences were amplified. Hybridisations were performed on nylon membranes and modified polystyrene slides. Hybridisation was specific at high stringency (0.1%SDS/0.1xSSC). Further work is required to produce a prototype diagnostic device.

Acknowledgements

I would like to thank my supervisors, Professor Phil Warner and Dr. David Cullen at Cranfield University and Dr. Stan Evans and Dr. John O'Donnell at Northampton General Hospital for their support over the last three and a half years. I would like to thank the staff at the Cranfield and Silsoe Medical centres and the Paediatric Nurse at Northampton General Hospital for their assistance with blood collection. Thank you to everyone who donated blood for use in this project. I would also like to thank Dr. Wim Tachelet and Walter Hofer at Innogenetics, Belgium for their assistance with the radioactive labelling and detection.

I would like to thank Dr. Sarah Stephens at Genetix for her invaluable assistance with the arraying of DNA and scanning for fluorescent signals.

Thank you to Heidi Johnson for the AFM images, Reinhard Fend for his technical support using computers and to everyone in the Molecular Biology Lab for helping to make most of the past few years fun!

I would like to thank my Dad for his sound advice on all things, thesis related and otherwise!

Finally, thanks are due to Dr. Karen James and her husband Alistair for putting up with me camping out in their spare room while I was writing up.

Contents

Abstract.....	ii
Acknowledgements.....	iii
Contents	iv
List of Figures.....	viii
List of Tables	x
Abbreviations.....	xi

1 INTRODUCTION AND LITERATURE REVIEW.....1

1.1 OVERVIEW.....	1
1.1.1 AIMS	1
1.2 REVIEW OF DNA DIAGNOSTICS.....	2
1.2.1 OVERVIEW OF GENETIC DISEASE	2
1.2.2 PAST AND CURRENT APPROACHES TO DIAGNOSTICS.....	2
1.2.2.1 Biochemical-based diagnosis	2
1.2.2.2 Cystic fibrosis diagnosis by sweat testing	3
1.2.2.3 Cystic fibrosis diagnosis by measuring meconium albumin	4
1.2.2.4 Cystic fibrosis diagnosis by measuring Immunoreactive Trypsinogen	5
1.2.2.5 Cystic fibrosis diagnosis by measuring Pancreatitis-associated protein.....	5
1.2.2.6 Summary of biochemical methods of cystic fibrosis diagnosis.....	6
1.2.2.7 DNA-Based Diagnostics.....	8
1.2.2.8 Neonatal Screening for cystic fibrosis.....	14
1.2.2.9 Current Genetic Testing of Cystic Fibrosis	16
1.2.2.10 Commercially Available DNA-Based Tests.....	21
1.3 GENETIC TESTING OF CYSTIC FIBROSIS	22
1.3.1 THE CHOICE OF CYSTIC FIBROSIS AS A MODEL	22
1.3.2 THE GENETICS OF CYSTIC FIBROSIS	23
1.4 TECHNOLOGY OF DNA DIAGNOSTICS.....	25
1.4.1 POLYMERASE CHAIN REACTION AND RELATED TECHNIQUES.....	25
1.4.2 DNA CHIPS	25
1.4.3 DNA MICROARRAYS	27
1.4.4 DETECTION METHODS	27
1.4.5 HYBRIDISATION CONDITIONS.....	30
1.4.6 NUCLEIC ACID IMMOBILISATION.....	32
1.4.7 MICRO-FABRICATION.....	35
1.5 SUMMARY	36
1.6 PROJECT AIMS AND OBJECTIVES.....	38

2 IMMOBILISATION AND MATERIALS ASPECTS OF SOLID-PHASE DNA DIAGNOSTICS40

2.1 INTRODUCTION.....	40
2.2 GENERATION OF DNA MATERIALS AND REAGENTS	41
2.2.1 LACTOBACILLUS PLANTARUM DNA EXTRACTION AND PCR	41
2.2.2 HUMAN DNA EXTRACTION	43
2.2.3 PCR OF CYSTIC FIBROSIS SEQUENCES	44
2.2.3.1 Digoxigenin (DIG) labelling of PCR.....	44

2.2.3.2	Biotin labelling of PCRs.....	45
2.2.4	RESULTS AND DISCUSSION.....	46
2.2.4.1	<i>Lactobacillus plantarum</i> DNA extraction and PCR.....	46
2.2.4.2	Human gDNA extraction.....	47
2.2.4.3	PCR DIG labelling of cystic fibrosis sequences.....	52
2.2.4.4	Biotin labelling of cystic fibrosis sequences via end labelled primers	52
2.2.5	SUMMARY.....	58
2.3	PHYSICAL IMMOBILISATION OF DNA TO NYLON MEMBRANES.....	58
2.3.1	INTRODUCTION	58
2.3.2	METHOD OF PROBE LABELLING AND HYBRIDISATION.....	59
2.3.3	OLIGONUCLEOTIDE ATTACHED BY BAKING IN AN OVEN AND SUBSEQUENT HYBRIDISATION.....	60
2.3.4	POLY(T) TAILED OLIGONUCLEOTIDES ATTACHED TO MEMBRANES USING UV- CROSSLINKING	60
2.3.4.1	Method of adding a Poly(T) tail to an oligonucleotide.....	60
2.3.4.2	Method of UV-crosslinking poly(T) tails to nylon membranes	60
2.3.5	RESULTS AND DISCUSSION.....	61
2.3.5.1	DNA attachment to nylon membrane by baking.....	61
2.3.5.2	DNA attachment to nylon membrane by UV-crosslinking	64
2.3.6	SUMMARY.....	65
2.4	COVALENT IMMOBILISATION OF DNA TO CARBOXYLATED NYLON MEMBRANES	66
2.4.1	INTRODUCTION	66
2.4.2	OLIGONUCLEOTIDE COVALENTLY ATTACHED TO CARBOXYLATED NYLON MEMBRANE	66
2.4.2.1	Activation of carboxyl groups and covalent coupling of oligonucleotides	67
2.4.2.2	'Mock' hybridisation	67
2.4.3	DETERMINATION OF QUANTITATIVE AMOUNTS OF OLIGONUCLEOTIDE COVALENTLY ATTACHED TO MEMBRANE	68
2.4.3.1	Method for radioactive labelling of oligonucleotides.....	69
2.4.3.2	Method to test the efficiency of radioactive labelling	69
2.4.3.3	Method for determination of probe yield.....	70
2.4.3.4	Mock hybridisation experiment.....	70
2.4.3.5	Detection of radioactivity in order to measure absolute amounts of oligonucleotide attached to membrane	71
2.4.4	EVALUATION OF COVALENT COUPLING OF OLIGONUCLEOTIDE TO MEMBRANE VIA HYBRIDISATION TO LABELLED TARGET	71
2.4.4.1	Method of pre-hybridisation, hybridisation and DIG detection on nylon membrane...	72
2.4.5	RESULTS AND DISCUSSION.....	72
2.4.5.1	Radioactive labelling and detection of oligonucleotides	75
2.4.5.2	Hybridisation of DIG labelled PCR product to oligonucleotides covalently coupled to carboxylated nylon membrane	82
2.4.6	SUMMARY.....	86
2.5	COVALENT IMMOBILISATION TO ACTIVATED POLYSTYRENE	87
2.5.1	INTRODUCTION	87
2.5.2	COMPARISON OF THE SURFACE CHEMISTRY OF MODIFIED AND UNMODIFIED POLYSTYRENE SLIDES	88
2.5.2.1	Method of modification of polystyrene slides to produce carboxyl groups	88
2.5.2.2	Method of determining contact angle.....	89
2.5.2.3	Method of taking AFM images of polystyrene slides	91
2.5.3	COVALENT BINDING OF OLIGONUCLEOTIDES TO MODIFIED POLYSTYRENE SLIDES	91
2.5.3.1	Method of the activation of carboxyl groups on polystyrene slides	91
2.5.3.2	Covalent attachment of amino terminated oligonucleotides to carboxyl groups on polystyrene slides.....	91

2.5.3.3	Mock hybridisation and detection of DIG and biotin labels.....	92
2.5.4	HYBRIDISATION TO COVALENTLY COUPLED OLIGONUCLEOTIDES ON POLYSTYRENE SLIDES	92
2.5.4.1	Hybridisation of PCR product to covalently coupled oligonucleotides on polystyrene slides	93
2.5.5	RESULTS AND DISCUSSION.....	93
2.5.5.1	Contact angle measurements on polystyrene slides.....	93
2.5.5.2	AFM analysis of polystyrene slides.....	96
2.5.5.3	Covalent coupling of oligonucleotides to modified polystyrene slides.....	98
2.5.6	SUMMARY	104
2.6	THE EFFECT OF DNA SPACERS ON HYBRIDISATION EFFICIENCY	105
2.6.1	INTRODUCTION AND MATERIALS	105
2.6.2	INVESTIGATION OF THE EFFECT ON HYBRIDISATION OF A POLY(T) TAILED PROBE ATTACHED BY UV-CROSSLINKING TO A SURFACE.....	105
2.6.3	INVESTIGATION OF THE EFFECT OF A POLY(T) TAIL ON COVALENTLY COUPLED OLIGONUCLEOTIDE USED IN HYBRIDISATION.....	105
2.6.4	RESULTS AND DISCUSSION.....	106
2.6.5	SUMMARY	108
2.7	SUMMARY AND CONCLUSIONS	109

3 DETECTION AND PROBE DEPOSITION FOR SOLID-PHASE DNA DIAGNOSTICS

3.1	INTRODUCTION.....	112
3.2	GENERAL MATERIALS AND METHODS	115
3.2.1	LABELLING OF TARGET DNA	115
3.2.1.1	Cy5 labelling by PCR amplification with Cy5 labelled primers	115
3.2.1.2	Cy5 labelling via aminoallyl dUTP incorporation into PCR product.....	116
3.2.2	HYBRIDISATION DETECTION VIA SA-HRP AND CHEMILUMINESCENCE ON SLIDES.....	118
3.2.3	USE OF COLOURED STREPTAVIDIN BEADS AS A READOUT SYSTEM	119
3.2.4	FLUORESCENT LABELLING OF PCR PRODUCT VIA END-LABELLED PRIMERS	119
3.2.5	PCR INCORPORATION OF AMINOALLYL-DUTP AS AN ALTERNATIVE METHOD OF FLUORESCENT LABELLING	120
3.2.6	BioDOT DEPOSITION OF OLIGONUCLEOTIDE PROBE ONTO MODIFIED POLYSTYRENE SLIDES	121
3.3	RESULTS AND DISCUSSION.....	124
3.3.1	BIOTIN LABELLED PROBES HYBRIDISED TO OLIGONUCLEOTIDES ON POLYSTYRENE SLIDES	124
3.3.2	COLOURED BEADS AS A DETECTION SYSTEM	125
3.3.3	FLUORESCENT LABELLING OF PCR PRODUCT BY END-LABELLING OF PRIMERS	128
3.3.4	FLUORESCENT LABELLING BY PCR INCORPORATION OF AMINOALLYL DUTP	132
3.3.5	COMPARISON OF CY5 LABELLING METHODS.....	133
3.3.6	DEPOSITION OF OLIGONUCLEOTIDE USING THE BioDOT SYRINGE SYSTEM	134
3.4	SUMMARY AND CONCLUSIONS	140

4 DEMONSTRATION OF A DNA DIAGNOSTIC FOR CYSTIC FIBROSIS

4.1	INTRODUCTION.....	142
4.2	MATERIALS AND METHODS.....	142
4.2.1	GENOMIC DNA EXTRACTION FROM PATIENTS BLOOD	143

4.2.2	PCR AMPLIFICATION AND BIOTIN LABELLING OF PATIENTS' GENOMIC DNA.....	143
4.2.3	MODIFICATION OF POLYSTYRENE SLIDES AND HYBRIDISATION OF PATIENT SEQUENCES	143
4.3	RESULTS AND DISCUSSION.....	144
4.3.1	GENOMIC DNA EXTRACTION FROM PATIENT BLOOD AND PCR AMPLIFICATION	144
4.3.2	HYBRIDISATION OF AMPLIFIED PATIENT SEQUENCES TO OLIGONUCLEOTIDES ON POLYSTYRENE	145
4.4	SUMMARY AND CONCLUSIONS	154
5	<u>FINAL DISCUSSION, FUTURE WORK AND CONCLUSIONS</u>	155
5.1.1	INTRODUCTION	155
5.1.2	REITERATION OF AIMS AND OBJECTIVES	155
5.1.3	GENOMIC DNA EXTRACTION	155
5.1.4	PHYSICAL IMMOBILISATION OF OLIGONUCLEOTIDES TO NYLON.....	155
5.1.5	POLY(T) TAILING	157
5.1.6	COVALENT COUPLING OF OLIGONUCLEOTIDES TO CARBOXYLATED MEMBRANE	158
5.1.7	HUMAN GENOMIC DNA EXTRACTION, AMPLIFICATION AND HYBRIDISATION TO COVALENTLY COUPLED OLIGONUCLEOTIDE	161
5.1.8	SPACERS.....	162
5.1.9	COVALENT IMMOBILISATION OF OLIGONUCLEOTIDE TO ACTIVATED POLYSTYRENE ...	164
5.1.10	HYBRIDISATION TO COVALENTLY IMMOBILISED OLIGONUCLEOTIDE ON POLYSTYRENE	166
5.1.11	DEPOSITION SYSTEMS.....	167
5.1.12	DETECTION SYSTEMS.....	169
5.1.13	DEMONSTRATION OF DIAGNOSTIC.....	171
5.2	RECOMMENDATIONS FOR FUTURE WORK.....	173
5.3	CONCLUSIONS.....	177
	References.....	179
	Appendix A. Primer sequences.....	200
	Appendix B. Control sample consent forms	201
	Appendix C. oligonucleotide/probe sequences, Lactobacillus plantarum and cystic fibrosis ...	202
	Appendix D. Table showing the amount of oligonucleotide put down in each spot during radioactive studies.....	204
	Appendix E. Radioactive data- CPM and calculations and probe yield	205
	Appendix F. Contact angle measurements on polystyrene slides	209
	Appendix G. Diagram to show the position of droplets on the slide during contact angle measurements.....	211
	Appendix H. Cy5 absorbance levels of PCR products	212
	Appendix I. Blood collection protocol, patient information and sample consent form	213
	Appendix J. List of solutions and suppliers	220

List of Figures

Figure 1. <i>Lactobacillus plantarum</i> genomic DNA extract	48
Figure 2. PCR product from primers 1Ab and R4s	49
Figure 3. Human genomic DNA extracted from wild type blood samples	50
Figure 4. PCR product from DIG labelling exons 10, 11 and 21	54
Figure 5. PCR product from biotin labelling	56
Figure 6. Oligonucleotides were attached to Hybond N+ membrane by baking.....	62
Figure 7. Oligonucleotides were attached to Nylon membrane by UV-crosslinking	63
Figure 8. DIG labelled oligonucleotides were covalently coupled to Biotin C membrane and detected using chemiluminescence.	74
Figure 9. Graphs showing the amount of oligonucleotide remaining attached to Hybond N+ membrane (UV-crosslinking) and Biotin C membrane (covalent attachment).	78
Figure 10. Oligonucleotides representing the mutant and wild type sequences of four cystic fibrosis mutations (Δ F508, G542X, G551D and N1303K) were covalently coupled to Biotin C membrane and hybridised with the appropriate PCR product.	84
Figure 11. A representation of a droplet as a sector of a circle.	90
Figure 12. An example of a CCD image of a water drop on a modified polystyrene slide from which contact angle measurements were taken	95
Figure 13. AFM images of unmodified and modified slidesAn example of a CCD image of a water drop on a modified polystyrene slide from which contact angle measurements were taken.....	97
Figure 14. DIG oligonucleotides covalently coupled to modified polystyrene slides....	99
Figure 15. Biotin oligonucleotides covalently attached to modified polystyrene slides.....	99
Figure 16. Oligonucleotides were covalently coupled to modified polystyrene slides and hybridised to the appropriate biotin labelled PCR product.	102
Figure 17. Poly(T) tailed oligonucleotide was attached to nylon membrane by UV- crosslinking and hybridised to DIG labelled <i>Lactobacillus plantarum</i> DNA.	107
Figure 18. Poly(T) tailed oligonucleotide was attached to Biotin C membrane via covalent coupling and hybridised to <i>Lactobacillus plantarum</i> PCR product.	107
Figure 19. The BioDot CV1000 syringe system with additional pump and three-way switch.	123
Figure 20. Red streptavidin beads used as a hybridisation detection method.	127

Figure 21. PCR products from Cy5 primer labelling.	130
Figure 22. Drops of oligonucleotide on a modified slide as printed using the BioDot CV1000 syringe system.....	137
Figure 23. The BioDot syringe system was used to print oligonucleotide and PCR product onto slides for hybridisation.	138
Figure 24. Genomic DNA extracted from patient blood samples using the Qiagen QIAamp extraction kit.....	146
Figure 25. PCR product from biotin labelling of patient DNA.....	147
Figure 26. Hybridisation of biotin labelled target (exon 10, 11 and 21) from patients 1 (a) and 2 (b) to cystic fibrosis sequences.....	153

List of Tables

Table 1. Summary of biochemical tests for cystic fibrosis.....7

Table 2. The advantages and disadvantages of specific methods of mutation
detection.....11

Table 3. Showing the mean contact angle measurements taken on modified and un-
modified slides.....96

Abbreviations

A	Adenine
AFM	Atomic force microscope
ARMS	Amplified refractory mutation detection system
ASO	Allele specific oligonucleotides
C	Cytosine
CCD	Charged coupled device
CCM	Chemical cleavage of mismatch
cDNA	Complementary DNA
CFTR	Cystic fibrosis transmembrane conductance regulator
CHT	Congenital hypothyroidism
CPSD	Disodium 3-(4-methoxy Spiro{1,2-dioxetane-3,2'-5'-chloro}tricyclo[3.3.1.1 ^{3,7}]decan}-4-yl) phenyl phosphate
Cy	Cyanine
DGGE	Denaturing gradient gel electrophoresis
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuracil triphosphate
DIG	Digoxigenin
ECL	Enhanced chemiluminescence
ECM	Enzyme cleavage of mismatch
EDC	1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide
ELISA	Enzyme linked immunosorbant assay
F-dUTP	Fluorescein labelled dUTP
F-dCTP	Fluorescein labelled dCTP
G	Guanine
gDNA	Genomic DNA
HRP	Horseradish Peroxidase
IRT	Immunoreactive Trypsin
LB	<i>Lactobacillus plantarum</i>

LB media	Luria-Bertani media
MALDI-TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MASPCR	Multiple allele specific PCR
MBL	Manose-binding lectin
MI	Meconium ileus
OLA	Oligonucleotide ligation assay
PAP	Pancreatitis-associated protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Poly(ethylene) glycol
PI	Pancreatic insufficiency
PKU	Phenylketonurea
PNA	Peptide nucleic acid
PON	Serum paraoxonase activity gene
PS	Pancreatic sufficiency
PTT	Protein truncation test
RCA	Rolling circle amplification
RDB	Reverse dot blot
RFLP	Restriction fragment length polymorphism
SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
SSC	Sodium citrate sodium chloride buffer
SSCP	Single stranded conformational polymorphism
STE	Salt-tris-EDTA buffer
T	Thymine
TE	Tris-EDTA buffer
TdT	Terminal deoxynucleotidyl transferase
T _m	Melting temperature
TMAC	Tetramethylammoniumchloride
UV	Ultra-violet

1 Introduction and Literature Review

1.1 Overview

Genetic diseases have a significant impact on the lives of people who have them and those around them. In some cases early diagnosis can allow early treatment to delay deterioration of the disease and improve prognosis. This thesis describes attempts to move towards a reliable, easy to use genetic diagnostic system for use in a District General Hospital. Cystic Fibrosis is used as a model throughout, although the work may apply equally well to other genetic diseases that are linked to specific defects at the molecular level.

1.1.1 Aims

The major aim of this project is to work towards a hybridisation based system for genetic mutation detection in human DNA. The system will detect different types of mutation (for example, deletions and point mutations) and will use cystic fibrosis sequences as a model, although the conclusions should be easily transferable to other sequences.

This work will investigate three areas, 1) methods of labelling target DNA and detection of hybridisation, 2) methods of attaching DNA to various surfaces prior to hybridisation and 3) hybridisation conditions to ensure specificity of hybridisation.

The final system should be easily used by someone with no specialised training in molecular biology. It should have a short run time and use few reagents, making it cheap to use. The test results should be reproducible, ensuring correct mutation detection and the test itself should be cheap to produce to make it a viable option for use in a District General Hospital.

1.2 Review of DNA Diagnostics

This section of the thesis will review present methods of using DNA to diagnose disease as a background to the work described later.

1.2.1 Overview of Genetic Disease

Genetic disease is caused by an alteration in the DNA of an individual, which is usually passed down from either one, or both parents. Such diseases may either be due to a single mutation in a single gene, or multiple mutations in the same or different genes.

Some diseases have a genotype-phenotype correlation, where the severity of the disease can be predicted based on the mutation present in the individual's DNA, in these cases it may be useful to identify the mutation present in order to adjust the patient's treatment.

Disease causing mutations may be deletions, insertions or base changes. The type of mutation may affect the severity of the disease, deletions or insertions often lead to the production of a more deficient protein than single base changes.

1.2.2 Past and Current Approaches to Diagnostics

Until the specific mutations in the gene or genes causing a disease are known, the disease must be diagnosed on the basis of clinical and biochemical findings. Non-specific methods of mutation detection are available, however care must be taken when using these not to diagnose a harmless polymorphism (a variation of a sequence that has no effect on the final protein) as a mutation.

1.2.2.1 Biochemical-based diagnosis

Before DNA diagnosis was widely available, biochemical methods of diagnosis were used. This, and the following five sections consider the biochemical methods of diagnosis currently available as alternative methods to DNA diagnosis once again using

cystic fibrosis as a model disease. Biochemical methods of testing for diseases such as cystic fibrosis have been known for a relatively long time, although lack of detailed knowledge of the biochemical defects involved with such diseases has meant that the direction of test development is not obvious. Neonates have been tested for cystic fibrosis since 1959 (Gibson and Cooke) as early intervention with treatments such as vigorous antibiotic treatment for chest infections, physiotherapy and the use of enzyme supplements have been observed to improve quality and length of life for cystic fibrosis patients (Larsen *et al.* 1994). The earlier a diagnosis of cystic fibrosis is made, the sooner suitable treatment can be given.

Methods of diagnosis of cystic fibrosis originally relied solely on the fact that the sweat of cystic fibrosis patients contained increased sodium and chloride. The biochemical defect associated with cystic fibrosis is not fully understood, although it is known that levels of certain substances, for example, sodium, Immunoreactive Trypsin (IRT), and Pancreatitis-associated Protein (PAP) are usually altered in cystic fibrosis patients, and this knowledge can be utilised when developing tests (Iovanna *et al.*, 1994, Crossley *et al.*, 1981).

1.2.2.2 Cystic fibrosis diagnosis by sweat testing

The first sweat test was devised by Gibson and Cooke in 1959, it consisted of an electric current being used to introduce pilocarpine nitrate into the skin. The pilocarpine nitrate stimulates the sweat glands to produce sweat, which is absorbed onto filter paper. The sweat can then be tested for sodium and chloride concentration. Chloride alone may be tested for directly from the skin, although this may be an unreliable diagnostic indicator as it has a wider range of 'normal' values than sodium and some medications, such as the antibiotic Cloxacillin, are known to affect levels of chloride measured this way

(Heeley and Watson, 1983). 'Normal' values for sweat sodium are between 8 and 40 mmol per litre, and for sweat chloride are between 8 and 50 mmol per litre (Heeley and Watson, 1983). The levels of sweat sodium that are associated with cystic fibrosis are above 70 mmol per litre (Heeley and Watson, 1983). The concentration of sodium in sweat is normally higher than that of chloride although if a high sodium concentration is observed in conjunction with a low chloride concentration, this may indicate contamination by detergent. Values of sodium between 40 and 70 mmol per litre indicated the necessity for a retest, and may be observed in cases of milder cystic fibrosis (Mckendrick, 1962). These values are only representative of normal and abnormal values in infancy and early childhood. As the child gets older, the amount of sodium and chloride present in sweat increases regardless of disease. The most common cystic fibrosis mutation in the white European population is $\Delta F508$, a deletion of three bases. A second mutation (R553Q) has been associated with patients homozygous for $\Delta F508$ who have normal sweat electrolyte concentration, suggesting that the sweat test may be inconclusive (Stern, 1997). Several diseases other than cystic fibrosis, such as Addison's disease and Nephrotic syndrome, are known to increase the sodium in a sweat test, and whilst these are unlikely to be confused with cystic fibrosis, it is important not to rely on a sweat test alone for confirmation of cystic fibrosis diagnosis (Heeley and Watson, 1983).

1.2.2.3 Cystic fibrosis diagnosis by measuring meconium albumin

It was discovered that the meconium (intestinal contents of the neonate) of cystic fibrosis patients showed an increase in albumin compared to normal levels (Green *et al.* 1958). A simple test, using a colour change to indicate meconium albumin loads of over approximately 20 mg/g, was devised by Stephan *et al.* (1975) which was observed

to give positive results in 75-100% of babies with cystic fibrosis (Heeley and Watson, 1983). Unfortunately a high number of false negative results were also observed.

1.2.2.4 Cystic fibrosis diagnosis by measuring Immunoreactive Trypsinogen

Immunoreactive Trypsinogen (IRT) is normally present in low amounts in blood. When the pancreas does not work properly, as in cases of cystic fibrosis, it may allow small amounts of IRT to 'leak' into the blood stream. Crossley *et al.* (1977) designed the first neonatal screening test for cystic fibrosis; they measured IRT in faecal trypsin.

Unfortunately the method produced both false positive and false negative results. In 1979 Crossley *et al.* discovered high levels of IRT in the blood of babies with cystic fibrosis and by 1981 had developed a radioimmunoassay using polyclonal antibodies for IRT on samples taken from blood spots on Guthrie cards (Crossley *et al.* 1981).

Bowling *et al.* (1987) describe an alternative assay to identify levels of IRT in neonatal blood spots. The assay uses monoclonal antibodies to IRT, which are more specific than the polyclonal antibodies previously used by Crossley *et al.* (1981).

1.2.2.5 Cystic fibrosis diagnosis by measuring Pancreatitis-associated protein

Pancreatitis-associated protein (PAP) is a better marker of alteration of pancreatic function than IRT. In adults PAP is leaked into the blood only after prolonged pancreatic alteration, although trypsin may be released when there is a transient obstruction of the pancreatic duct (Iovanna *et al.* 1994). Elevated PAP is seen in all babies with cystic fibrosis. False positive results are only seen where there is a high level of IRT or when babies are heterozygous for cystic fibrosis. This method gives slightly better discrimination than IRT testing (Iovanna *et al.* 1994). PAP discriminates between babies with cystic fibrosis and those with a wild type genotype, however heterozygotes may or may not have elevated PAP levels. The levels of PAP are

measured in blood spots using an ELISA with monospecific polyclonal antibodies to PAP.

1.2.2.6 Summary of biochemical methods of cystic fibrosis diagnosis

In 1983 Heeley and Watson reviewed the different biochemical methods available for predicting whether or not a patient has cystic fibrosis, covering sweat tests, meconium ileus and methods of measuring trypsin. They concluded that accurate sweat testing remains the main method of biochemical diagnosis for cystic fibrosis. They also suggested that the centralisation of sweat testing would improve early diagnosis.

Table 1. Summary of biochemical tests for cystic fibrosis

Test	Brief description	Advantages	Disadvantages	References
Sweat Test	Pilocarpine nitrate is introduced to the skin via an electric current, which induces sweating. The sweat is collected on filter paper and levels of sodium and chloride concentrations are measured.	Non-invasive test.	'Normal' values for sodium may include those from patients with mild CF. Other diseases may increase sweat sodium. The test may not always be accurate.	Gibson and Cooke (1959)
Meconium Albumin Loads	Neonatal meconium albumin loads were observed to be higher in cystic fibrosis patients.	Meconium can be collected non-invasively.	High numbers of false negatives and false positives were observed	Green <i>et al.</i> (1958) Stephen <i>et al.</i> (1975)
Immunoreactive Trypsinogen (IRT)	Blood IRT levels were measured using a radioimmunoassay.	Blood spots on Guthrie cards simplified mass screening.	Blood collection is invasive. Radiolabels preferably avoided.	Crossley <i>et al.</i> (1981)
IRT detection with monoclonal antibodies	Monoclonal antibodies were used to identify IRT in blood.	Monoclonal antibodies are more specific than polyclonal. Colourimetric detection.	Blood collection is invasive.	Bowling <i>et al.</i> (1987)
Pancreatitis-associated protein (PAP)	PAP is a marker of altered pancreatic function.	Elevated PAP is seen in all babies with CF.	Heterozygotes for CF may or may not have elevated PAP levels.	Iovanna <i>et al.</i> (1994)

1.2.2.7 DNA-Based Diagnostics

There are several methods of mutation detection that can be used to detect genetic mutations; these methods have been categorised as specific and non-specific methods of detection. Non-specific detection methods identify the presence of an alteration to a sequence; these include denaturing gradient gel electrophoresis (DGGE) (Loumi *et al.*, 1999), single strand conformational polymorphism (SSCP) (Murakami *et al.* 1991), heteroduplex analysis (Schwarz, 1998), chemical or enzyme cleavage of mismatch (C or ECM) (Rodrigues *et al.* 1990 and Hsu *et al.* 1998) and the protein truncation test (PTT) (Schwarz, 1998).

A difference in sequence will cause a difference in annealing temperature. DGGE uses a gradient of temperature or chemical denaturing agents such as urea and formamide to denature the DNA duplex as it moves through a polyacrylamide gel. As denaturation occurs the mobility of the DNA will become retarded and therefore different sequences will cease migration at different points in their migration through the gel. A GC rich 'clamp' is often used at one end of the duplex in order to prevent complete denaturation (Loumi *et al.*, 1999).

SSCP uses the ability of single stranded DNA to form secondary structures which are dependant on the sequence of that DNA. Even a single nucleotide alteration may cause a different structure to be formed and so the DNA will migrate to a different place on a polyacrylamide gel (Murakami *et al.* 1991).

In heteroduplex analysis the unknown sequence is hybridised to a known sequence, this will either form a homoduplex (that is, a duplex of DNA where the strands are complementary) or a heteroduplex (where there is a mismatch. The mobility of a

heteroduplex will be retarded compared to the mobility of of a homoduplex (Schwarz, 1998).

Cleavage of mismatch can be carried out using chemicals (for example, as used by Tessitore *et al.*, 2002 for Rhodopsin mutation detection) or enzymes (as used by Hsu *et al.*, 1998 for cystic fibrosis mutation detection). The unknown sequence of DNA is hybridised to a known sequence. If the duplex is cleaved (as indicated by the presence of two small bands after gel electrophoresis, compared to one large one) then there was a difference between the sequences

In the PTT PCR products are used for the *in vitro* synthesis of RNA, which is in turn translated into protein. The protein produced in this way is analysed on a gel and compared to the normal protein. Where there has been a mutation causing premature termination of translation, the protein will be shorter than normal, and this will be identifiable after gel analysis (Schwarz, 1998).

Non-specific methods will only identify a change in sequence; they cannot distinguish between a mutation and a harmless polymorphism because they do not identify specific sequence changes.

In 1998 Köhler *et al.* described a method of detecting mutations which involved immobilising biotin labelled target DNA onto streptavidin coated microplates.

Oligonucleotides were labelled with 5'-fluorescein isothiocyanate and hybridised to the target. Correctly matched probe and target were cleaved using the restriction enzyme *HhaI*, and the probe label was washed away. Thus, a signal was seen only in those wells where the probe and target were incorrectly matched.

Brinson *et al.* (1999) performed multiplex PCR and then used the product in an Oligonucleotide Ligation Assay (OLA) with three probes. Each probe was labelled with a different fluorescent dye, one probe represented the wild type sequence, one represented the mutant sequence, and one would hybridise to both. When two probes hybridise adjacent to one another, they are joined by ligase and the presence or absence of a mutation can be identified depending on which label is observed when the probes are analysed on a gel. A size standard was labelled with a fourth colour. This method was found to give 100% reliability.

There are several methods of specific mutation detection including reverse dot blot hybridisation (RDB) (Kawasaki and Chehab, 1994), restriction enzyme digestion (Schwarz, 1998), rolling circle amplification (RCA) (Hatch *et al.* 1998), and amplification refractory mutation detection system (ARMS) (Newton *et al.* 1989). Many of these methods have already been investigated for use in cystic fibrosis diagnosis.

The RDB uses the principle of DNA hybridisation. A short oligonucleotide of known sequence is attached to a membrane (usually by UV-crosslinking or baking). If an unknown sequence can hybridise to the oligonucleotide (with adequate stringency conditions) it can be concluded that the unknown sequence contains the complementary sequence to the oligonucleotide.

Restriction enzyme digestion relies on the fact that restriction enzymes will only cleave DNA at a specific sequence. If the mutation being studied either creates, or destroys one of these sequences, the presence or absence of the digestion will indicate the presence or absence of a mutation when the digest is run on a gel.

The use of RCA for mutation detection was first published in 1999. Short oligonucleotides are immobilised on a support; the target is hybridised to these and then circularised using DNA ligase and amplified using the immobilised oligonucleotide as a primer. Radioactively labelled nucleotides are incorporated and the primers are washed off the support and run on a gel for detection. RCA is still a relatively new method and has been used to identify a known mutation in the breast cancer gene, BRACA 1 (Hatch *et al.*, 1998), to increase the sensitivity of microarrays (Nallur *et al.*, 2001) and in the genotyping of single nucleotide polymorphisms (Farugi *et al.*, 2001).

ARMS uses PCR to identify mutations (Newton *et al.* 1989). Primers are developed which will anneal to either the mutated or wild type sequence, with the altered base at the 3' end of the primer. If there is a product, the unknown sequence is complementary to the primer, if not, it is different. In commercial ARMS based cystic fibrosis tests (for example Elucigene CF20, Zeneca), each tube contains two sets of primers, one that will amplify the wild type version of mutation A and one that will amplify the mutated version of mutation B. The second tube contains primers that will amplify the mutated version of mutation A and the wild type version of mutation B. The PCR products are run on a gel and the presence and absence of bands indicates which mutations the individual carries.

Table 2. The advantages and disadvantages of specific methods of mutation detection.

Test	Brief description	Advantages	Disadvantages	Reference
Enzyme cleavage of mismatch (ECM)	DNA is immobilised onto a microtitre plate, hybridised to a labelled oligonucleotide and cleaved with Hha1. Label is washed away where there is mismatch.	For testing a single mutation, this is a relatively simple method.	Each mutation requires a different experiment. Not all mutations will have a sequence that a restriction enzyme will cut.	Kohler <i>et al.</i> (1998)
Oligonucleotide ligation assay (OLA)	Three different fluorescent-labelled probes hybridise to the wild type sequence, the mutant sequence and both. The sequence is determined by which probes hybridise. A fourth probe is labelled as a size marker.	100% reliability. Different probe can be designed to detect different mutations.	The cost of using four differently labelled probes in each experiment is prohibitive.	Brinson <i>et al.</i> (1999)
Reverse dot blot (RDB)	An oligonucleotide is attached to a surface and hybridised to a labelled target. The presence of a signal indicates the sequences are complementary.	Many mutations can be analysed in one experiment.	Membranes can be cumbersome, and require relatively large amounts of solutions such as hybridisation buffer.	Kawesaki <i>et al.</i> (1993) Southern (1996)

Table continued on next page

Continuation of Table 2

Rolling circle amplification (RCA)	An oligonucleotide is attached to a solid support and hybridised to the target, which is then circularised. Amplification incorporates radiolabelled nucleotides using the oligonucleotide as a primer.		New method, only tested using one mutation. Radiolabels are preferably avoided.	Hatch <i>et al.</i> (1998)
Amplified refractory mutation detection system (ARMS)	Sequence specific primers are used in PCR for both wild type and mutant sequences in separate PCR reactions.	Reliable system, commercially available.	Many PCR reactions needed to test each patient for many mutations.	Elucigene CF(20) Zeneca Diagnostic, UK.

1.2.2.8 Neonatal Screening for cystic fibrosis

Neonatal screening requires a test that can be carried out on every newborn baby to detect a particular disease. Both biochemical and genetically based tests can be used for screening, although the test must be easy to carry out, inexpensive and also reliable.

The first biochemical test for cystic fibrosis analysed faecal trypsin (Crossley *et al.* 1977), although false positive and false negative results were observed. Testing then moved on to look at IRT levels in blood (Crossley *et al.* 1981). Heeley *et al.* (1983) also detected IRT in blood and found a very low false positive rate of one in 19,500 babies tested, although they theorised that it would be possible to increase the specificity to 100% with no alteration to the sensitivity of the test. In order to make a screening test valid, it is important that the test is both sensitive (identifies as many affected people as possible) and specific (identifies only those with a particular disease).

In 1991 Elliott looked critically at measuring levels of immunoreactive trypsin (IRT) using radioimmunoassay. Several methods of error reduction were put forward, the two most viable being automation, and clinical follow up. Elliot (1991) suggested that each patient underwent two forms of testing. It was found that the use of both IRT and $\Delta F508$ testing (on the same patients), reduced false positive results from 5% to 3% in a sample population of 5,040 new-borns tested.

PAP testing gives better discrimination between affected and unaffected individuals than IRT testing and Iovanna *et al.* (1994) have proposed this method as a suitable candidate to be considered for neonatal screening for cystic fibrosis.

Genetic analysis is highly specific for identifying cystic fibrosis, although the large number of possible mutations makes screening difficult. This wide range of mutations

is currently the major obstacle to mutation screening of the population, closely followed by the risk of identifying babies as carriers of a mutation when they are too young to give their consent to testing (Beaudet, 1990; Ten Kate, 1990). Other concerns about screening include worries about the reliability and specificity of any test used and the possibility of discrimination against carriers of a mutation or people with an 'affected' genotype who show mild symptoms (Billings, 1990).

Genetic detection of the $\Delta F508$ mutation is useful for screening because 80% of cystic fibrosis patients carry this mutation (Riordan *et al.*, 1989; The Cystic Fibrosis Genetic Analysis Consortium, 1990). This mutation can be detected via PCR (see section 1.2.2.9.). An additional method of screening is required, however, to identify the remaining 20% of cystic fibrosis patients who do not have this mutation.

In 1991 Laroche and Travert used PCR to amplify the KM.19 marker from dried blood spots. Diagnosis was given after a sweat test, although only babies with high levels of IRT and an indicative KM.19 genotype were recalled for sweat tests. This method was seen to be slightly more sensitive than simply testing for the $\Delta F508$ mutation, which would only pick up a maximum of 80% of patients. KM.19 testing can identify up to 96% of patients with cystic fibrosis, depending on which alleles are chosen to indicate IRT testing should be performed. Larsen *et al.* (1994) used IRT tests, then sweat tests and found good sensitivity and specificity in the case of the IRT tests, although they suggested that the cut off point for the level of IRT in neonatal blood should be altered in order to identify more babies with cystic fibrosis. This would, however, increase the risk of babies being called for a retest when they do not have cystic fibrosis.

Currently in the UK 30% of babies are screened for cystic fibrosis. Each baby born in the UK has a heel prick blood spot taken to test for phenylketonuria (PKU) and

congenital hypothyroidism (CHT) at 6 days; it is relatively easy to take additional blood spots at this time. The use of these blood spots allows mass screening of all new-borns. It is known that IRT levels are elevated in 95% of babies with cystic fibrosis in the first four weeks after birth. An assay to detect levels of IRT from dried blood spots was developed by Crossley *et al.* (1981). At six days, levels of IRT are not as specific to cystic fibrosis as a second sample, taken at four weeks. If the second sample proves inconclusive, a sweat test is performed (Pollitt, 1996). Screening tests using monoclonal antibodies against trypsinogen have been proposed as they produce few false results (Bowling *et al.* 1987). Iovanna *et al.* (1994) identified the main disadvantage with using IRT levels to screen for cystic fibrosis to be the cut off point for classifying a result as representing 'elevated' IRT. The cut off point must be chosen so that all affected babies are identified whilst excluding the majority of unaffected. It is difficult to achieve this, and there is always a risk of missing an affected child due to the fact that IRT is not always elevated in affected babies. A study by Farrell *et al.* (2001) in Wisconsin (USA) concluded that a two tier method of screening (IRT and DNA testing) identified more affected babies than IRT testing alone.

1.2.2.9 Current Genetic Testing of Cystic Fibrosis

The cystic fibrosis transmembrane conductance regulator (CFTR) gene is located at 7q31 (Rommens *et al.* 1989). The cystic fibrosis mutation that is most commonly found in the Caucasian population is $\Delta F508$, a three base deletion (Riordan *et al.* 1989; The Cystic Fibrosis Genetic Analysis Consortium, 1990). The genetics of cystic fibrosis will be discussed in further detail in section 1.3. One simple way to detect $\Delta F508$ is to amplify the region of exon 10 around it and analyse the product by agarose gel electrophoresis. The DNA of a patient who has the $\Delta F508$ mutation will produce a

smaller PCR product (by 3 bases) than the wild type DNA (Schwarz, 1998). This method was used in Brazil to identify the percentage of $\Delta F508$ mutations in the population (Raskin *et al.* 1993). This simple technique has been modified in order to increase the difference in size between mutant and wild type PCR fragments. Veronina *et al.* (1991) used two upstream primers of different lengths. The primer that annealed to the wild type sequence was 26 base pairs and that which annealed to the mutated sequence was 22 base pairs. These different length primers increase the size difference between wild type and $\Delta F508$ containing PCR products to 7 bases. The larger the difference in PCR fragment size, the more quickly gel analysis may be carried out. Fortina *et al.* (1992) used fluorescence based, multiple allele specific PCR (MASPCR) to reduce the number of PCRs required per patient. Three different fluorescent labels were used, the normal primer was tagged with a blue label, the primer for the $\Delta F508$ mutation was tagged with a green label, and internal size standards were tagged with red labels. All PCR products were analysed in one lane of a gel, thus, only one PCR and one lane on a gel is required for each patient. These PCR based methods are capable of distinguishing between patients heterozygous and homozygous for the $\Delta F508$ mutation. Unfortunately the methods are only suitable for detecting deletions of three bases or more, for example, the $\Delta F508$ mutation, or another cystic fibrosis mutation which is a three base deletion, $\Delta I507$. PCR based methods are unable to identify point mutations as easily as a three base deletion. Costes *et al.* (1993) identified a method of using DGGE to detect cystic fibrosis mutations. The method analysed 15 exons of the CFTR gene and detected nearly 50% of all mutations described at the time. Costes *et al.* believed that the analysis of an additional four exons would increase the detection rate to 95% of possible mutations.

Mismatch cleavage, where enzymes or chemicals are used to cleave a mismatch in a duplex, has been used to detect the $\Delta F508$ cystic fibrosis mutation. Hsu *et al.* (1998) showed that mismatch cleavage is a possible method of identifying the $\Delta F508$ mutation. A target and a probe with only one base difference are annealed and an enzyme were used to cleave the mismatch. Stringency was adjusted so that mismatches of more than one nucleotide would prevent annealing. Thus, the sequence could be deduced depending on whether the DNA is cleaved when annealed to the wild type or mutant probe. Problems were identified with the sensitivity of this method, as large amounts of DNA were required. One milligram of DNA was needed from each patient. Hsu *et al.* (1998) were able to reduce the required amount of DNA by recycling the target DNA. This method used a radioactive label, however which should be avoided in mass screening.

One method of ensuring mutation identification is to sequence the whole of the cystic fibrosis gene, although this is time consuming, especially if it is uncertain that the patient has cystic fibrosis (Loumi *et al.* 1999). It is also possible that complete sequencing could miss rare or new mutations, or identify harmless polymorphisms as mutations.

Scobie *et al.* (1996) used ARMS to identify five common cystic fibrosis mutations. The first PCR amplifies the regions of the gene containing the five mutations; a second round of PCR will amplify any of these first PCR products using ARMS. The method was further refined to allow amplification from a single cell, for example, for use in pre-implantation diagnosis. The presence or absence of a PCR product indicates the presence or absence of a mutation. Scobie *et al.* (1996) point out that it is necessary to optimise the ARMS method for each mutation. A 70% contamination rate of the

negative controls of PCR reactions was noted by Lui *et al.* (1992) which indicated the necessity of controls and strict adherence to procedures implemented in order to reduce contamination.

One popular method of detecting cystic fibrosis mutations in the past few years has been the reverse dot blot (RDB) method using allele specific oligonucleotides (ASO) (Chehab and Wall, 1992; Cronin *et al.* 1996; Cuppens *et al.* 1992; Cuppens *et al.* 1993; Serre *et al.* 1991; Shuber *et al.* 1993; Wall *et al.* 1995). A dot blot usually has a PCR product of approximately 500 bases immobilised on a membrane, and a short piece of labelled DNA 'probe' is hybridised to this. The RDB is the reverse, the short 'probe' is attached to a surface and a larger 'target' (the 500 bp PCR product) is labelled. This technique can reduce the number of hybridisations required when testing a small number of patients for a large number of mutations, because it is the patients' DNA that is amplified and labelled and may be hybridised to many probes at once. Previous to the use of RDB for detection of cystic fibrosis mutations, this method had been used in several ways. RDB was used to identify the presence of a sequence in DNA (Merl *et al.* 1984), to detect point mutations in the N-Ras gene (Baens *et al.* 1990), to identify microorganisms (Dattagupta *et al.* 1989), to diagnose cancer (Zhang *et al.* 1991) and to diagnose β thalassemia (Cai *et al.* 1994; Maggio *et al.* 1993). Cystic fibrosis and β thalassemia present similar diagnostic problems due to the large number of different mutations that may cause each disease (Chehab and Wall, 1992).

When performing RDB there are several important considerations, (i) the surface to which the DNA will be attached; (ii) the method of attachment; (iii) which mutations to study; (iv) the hybridisation conditions; and (v) the method of detection. The RDB

technique lead to the development of microarrays, which use nonporous, rigid surfaces, rather than nylon or nitrocellulose membranes (Southern *et al.* 1999).

Arrays are becoming a more popular method of mutation analysis. Several 'chips' are now commercially produced for applications such as HIV detection (Gerhold *et al.* 1999). Chips are usually made of glass, and the oligonucleotides can either be synthesised *in situ* by photolithography, or covalently attached to the glass.

Light-generated arrays have been used to identify cystic fibrosis mutations (Cronin *et al.* 1996). Methods of producing arrays quickly, and at low cost are being investigated, particularly by companies wishing to produce 'chips' (Stimpson *et al.* 1998; Wallace, 1997). Robotics-assisted systems for producing arrays have been used for carrier detection in cystic fibrosis. A robotic workstation was used to produce the dot blots and found to be readily applied to any type of allele specific oligonucleotide (ASO) assay (DeMarchi *et al.* 1994)

One concern about the use of RDBs and arrays is that the results are qualitative not quantitative, however, improvements in image analysis have made it much easier to produce quantifiable results, without using radioactivity (Granjeaud *et al.* 1996). The lack of quantifiable results is not a significant problem if wild type oligonucleotides are included in the RDB unless the patient is homozygous for a mutation.

Hybridisation has been detected using surface plasmon resonance (SPR) (Nilsson *et al.* 1999) (specifically to look for $\Delta F508$ mutations in cystic fibrosis (Feriotto *et al.* 1999)), and Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF) (Blecinski and Richert, 1998).

Peptide nucleic acid (PNA) has been suggested as an alternative to oligonucleotides for use in arrays as it has a neutral backbone, reducing the need for ions, which are required to stabilise DNA/DNA duplexes in hybridisation buffers. PNA was found to behave in a similar way to oligonucleotide probes in hybridisation, as well as reducing hybridisation interference due to intramolecular structures (Egholm *et al.* 1993; Geiger *et al.* 1998). PNA can be very expensive to produce and this would suggest that it is not suitable for cheap, easy to use arrays.

1.2.2.10 Commercially Available DNA-Based Tests

Kits are currently available from a number of commercial companies to detect cystic fibrosis mutations. These include the CF(12) kit from Zeneca Diagnostics, to identify four or twelve mutations and an oligonucleotide ligation assay (PE Applied Biosystems) that identifies 31 mutations. The CF(12) kit uses ARMS to amplify the DNA, and the products are separated by gel electrophoresis. The presence or absence of bands indicate which mutations are present. The PE Applied Biosystems (Foster City, California, USA) OLA mutation detection method uses two probes with different tail lengths, one that hybridises to the normal sequence and one which hybridises to the mutant sequence. A third probe is labelled and hybridises next to the other probes. The two probes that have hybridised to the patient's DNA are ligated, and the product is denatured and run on a gel to detect the size of the product, and by inference, the sequence (Brinson *et al.* (1999). The CF(12) kit has been evaluated (Houdayer *et al.* 1998) and was found to be reliable; it allows at least ten samples to be screened for twelve mutations in one day. One shortcoming identified by Houdayer *et al.* was that heterozygotes could only be distinguished from homozygotes if the mutation is $\Delta F508$ as this is the only mutation for which the normal allele is included. It would be possible

to detect compound heterozygotes, providing the kit covered both mutations. It is suggested that the kit would be used in conjunction with a biochemical method of screening for example pancreatitis associated protein (PAP) (see section 1.2.2.6.) or immunoreactive trypsinogen (IRT) testing. Biochemical screening techniques could identify patients who have mutations that are not included by the kit. Choosing which mutations to test for is difficult, especially now that people from such a mixture of races are present in each city in England. The Elucigene CF(12) kit (made by Cellmark Diagnostics, Zeneca) uses the 12 mutations most common in the Caucasian population as set out by Welsh *et al.* (1990). They calculated that 78.8% of cystic fibrosis mutations would be identified in the French population using the Elucigene kit.

1.3 Genetic Testing of Cystic Fibrosis

1.3.1 The choice of cystic fibrosis as a model

Many diseases other than cystic fibrosis are known to be due to more than a single mutation. There may be more than one disease causing mutation found within a gene, or the mutations may be spread across several genes. The antithrombin gene, for example, may carry one of many different mutations that either prevent the synthesis or reduce the activity of its protein. This gene affects a person's susceptibility to deep vein thrombosis. Protein C can be used as a biochemical marker to test for the mutations, but the levels can overlap between affected and unaffected people (Bertina, 2001). In a case such as this a DNA diagnostic capable of testing for many different mutations at once would be useful. Other examples of multi-mutation disease include renal disorders with over 50 mutations reported (Khoshnoodi and Tryggvason, 2001), and the low-density lipoprotein receptor gene, which has over 150 mutations that result in

hypercholesterolaemia (Pocsai *et al.* 2001). There are many other similar diseases where the sheer number of possible mutations is currently prohibitive to DNA testing.

Cystic fibrosis has been chosen as a model system for this work because of its relatively high prevalence in the Caucasian population, and thus the relative ease of obtaining blood samples from affected people. Cystic fibrosis is a good model because it contains a range of types of mutation (Wall *et al.* 1995). For example, $\Delta F508$ is a deletion, G542X is a point mutation, 3849+10kb is a large insertion, and 3905insT is a small insertion.

1.3.2 *The Genetics of Cystic Fibrosis*

This section describes the background to the discovery of the size and position of the cystic fibrosis gene, as well as a brief discussion of the mutations found there. The number of mutations in that gene and their frequency are an important consideration when designing a DNA based test for cystic fibrosis. The mutations causing cystic fibrosis are found in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Rommens *et al.* 1989). In 1985 a new marker DOCRI-917 was linked to the serum paraoxonase activity gene (the PON locus) which itself had previously been linked to the cystic fibrosis locus (Tsui *et al.* 1985). The physical position of PON was, however, still unknown, although linkage of DOCRI-917 to PON was able to reduce the possible location of cystic fibrosis to 1% of the human genome. The same year, Knowlton *et al.* (1985) located DOCRI-917 on chromosome 7 using hybridisation to a human genomic library. The cystic fibrosis gene was linked to two other markers in 1985, the *met* gene and pJ3.11. White *et al.* (1985) found tight linkage between the oncogene *met* and the cystic fibrosis gene. The position of the *met* gene was known, and so the location of the cystic fibrosis gene was proposed to be between q21 and q31

of chromosome 7. Wainwright *et al.* (1985) found tight linkage between marker pJ3.11 and cystic fibrosis. PJ3.11 had previously been located to 7q22. An even tighter linkage was seen between the cystic fibrosis locus and a new marker, pMP6d-9 that reduced the possible position of the cystic fibrosis gene by 160 Kb (Estivill *et al.* 1989). Restriction Fragment Length Polymorphism (RFLP) analysis was used by Kerem *et al.* (1989) to try and refine the position of the cystic fibrosis gene. The cystic fibrosis gene was eventually cloned using chromosome walking and jumping and comparison of cystic fibrosis and non-cystic fibrosis genomes. The final position of the cystic fibrosis gene was given by Rommens *et al.* (1989) as 7q31.

The CFTR gene is known to have 27 exons, the DNA is 250kb, with a cDNA sequence of 6,129 bp (Riordan *et al.* 1989; Rommens *et al.* 1989). The CFTR protein has five domains. Two of the domains are membrane-spanning, two are nucleotide-binding domains and one is a regulatory domain. The CFTR protein is known to be a cAMP-dependant protein kinase and an ATP regulated chlorine channel. It has recently been discovered that two CFTR molecules are required to form each pore (Zerhusen *et al.* 1999). Although $\Delta F508$ (a deletion of a phenylalanine at position 508) is the most common mutation in the Caucasian population (Riordan *et al.* 1989; The Cystic Fibrosis Genetic Analysis Consortium, 1990), over 650 mutations causing cystic fibrosis have been identified to date. The prevalence of each mutation varies between ethnic groups and $\Delta F508$ is not always the most frequent mutation. This is an important consideration when deciding which genetic mutations to include in a test. The most suitable mutations will depend on the area of the world for which the test is intended.

This thesis aims to contribute towards a genetically based test for cystic fibrosis mutations. The test should be able to identify all varieties of genetic mutation (for

example, point mutation, deletion, insertion) in order to cover the variety of potential mutations which may lead to cystic fibrosis.

1.4 Technology of DNA Diagnostics

This section looks at systems that have been developed to allow the identification of many mutations in one experiment.

1.4.1 Polymerase Chain Reaction and Related Techniques

Kary Mullis developed polymerase chain reaction (PCR) as a method of amplifying specific regions of DNA (Mullis and Faloona, 1987). PCR is useful in mutation detection to amplify the DNA of interest so that there is a sufficient quantity to be observable on a gel, or be used as a probe in a hybridisation. Labels can be added to DNA during amplification, either as labelled nucleotides added to the reaction mix, or as labelled primers. The addition of labelled primers or nucleotides to a PCR mixture may necessitate optimisation of the PCR. The annealing temperature may need to be reduced or the magnesium chloride concentration increased. Most PCRs should require little adjustment to allow amplification with a label (McPherson and Møller, 2000).

1.4.2 DNA chips

There are many different terms used for similar devices. These include DNA chip, microarray, DNA array and GeneChip (GeneChip is a registered trademark of Affymetrix Inc.). Here, DNA chip is used to describe an array produced on glass, where the DNA is synthesised directly onto the surface, and microarray is used to describe the situation where DNA is synthesised and then attached by some method to the surface.

The general objective of DNA chips and microarrays are the same, the miniaturisation of the hybridisation process. This miniaturisation requires less DNA, both on the chip and in solution, less hybridisation solution and usually shorter hybridisation times than a standard hybridisation. DNA which is already attached to a chip (either by synthesis directly onto the chip or attachment after synthesis) is easier to handle, and glass slides are easier to handle than membranes.

DNA chips are used on the same basis as a reverse dot blot. In the same way that a blot can be hybridised with a labelled sequence of interest, so can a DNA chip. The scale is the major difference between a blot and a chip, a blot may be 10cm^2 , where the active surface on a chip may be as small as 1cm^2 . There is some confusion over the nomenclature used when talking about the DNA which is attached to the surface of the chip and the DNA that is being hybridised to the DNA on the chip. This thesis will follow the recommendation made in *Nature Genetics*, which says that the 'probe' is the DNA attached to the chip, and the 'target' is the labelled DNA being detected (Phimister, 1999). Chips can be used for sequencing unknown DNA, the probes on the chip are short, known sequences of DNA, and by matching up the sequences of the oligonucleotides the target hybridises to, the sequences can be inferred (O'Donnell-Maloney *et al.* 1996). DNA chips can also be used for many other purposes, such as mutation detection, for example, Affymetrix produces a Cancer array, which holds over 1,700 cancer genes.

Oligonucleotides may be synthesised directly onto glass slides using photolithography where areas of the chip are masked from light. Light sensitive protecting groups in the exposed areas are washed off. A base, for example adenine is added to the whole chip and will couple to the deprotected areas (Blanchard, 1998, Maskos and Southern, 1991).

In this way specific oligonucleotides may be synthesised in specific places on the chip (Fodor *et al.* 1993; Ramsay, 1998). The method has been used by Cronin *et al.* (1996) to scan exon 11 of the CFTR gene. It produced reliable results, although the photolithographic masks are expensive to produce (Blanchard, 1998).

1.4.3 DNA Microarrays

The difference between a DNA chip and a microarray is that the DNA is synthesised onto a DNA chip, whereas a pre-synthesised piece of DNA is attached to a microarray.

Many microarrays are made from cDNA and used in order to look at gene expression in different tissues. By labelling the mRNA from a tissue and hybridising it to a microarray of known genes it is possible to see which genes are being expressed in the tissue studied. Comparisons of gene expression can be made on a single gene between different tissues and between people. Comparisons may also be made between the expression of two or more different genes in the same person.

1.4.4 Detection Methods

There are several methods of carrying out the detection of DNA annealing, both in hybridisations and in OLAs, all involve the patients' DNA being amplified and labelled in some way. Radioactivity is the most sensitive method of detection, although from a Health and Safety point of view, it is preferable to use non-radioactive methods.

Dendrimeric oligonucleotide probes have been labelled using a radioactive label and shown to produce an increased signal compared to a normal oligonucleotide (Shchepinov *et al.* 1997b). This dendrimetic approach could be applied to other methods of labelling. An example of a non-radioactive method is the streptavidin-horseradish peroxidase method where the patients' DNA is biotinylated and then hybridised to immobilised oligonucleotides. An avidin-enzyme conjugate is added, the

avidin part of which binds to the biotin. A substrate for the enzyme is added after the hybridisation washes. The enzyme converts a colourless substrate into a coloured product that can be observed by eye. (Cook *et al.* 1988).

Chemiluminescent methods of labelling are available as kits, for example the ECL 3' labelling kit is available from Amersham Pharmacia Biotech (Bucks, UK) which uses an antibody-horseradish peroxidase conjugate, the antibody of which will bind to fluorescein (acting in this case as an antigen), which is used to end-label the probe. Luminol is added which is cleaved by horseradish peroxidase and emits blue light detectable by X-ray film. Kawasaki and Chehab (1994) used chemiluminescent detection to perform sequencing by hybridisation. Probes can be labelled directly with alkaline phosphatase, which cleaves its substrate, producing the chemiluminescence (Narayanan, 1992). Digoxigenin has been used as a label, with an antibody conjugated to an enzyme (alkaline phosphatase) for either chemiluminescent or colourimetric detection (Roche, UK).

A study has been carried out comparing probes labelled with biotin at either the 5' or 3' end. It was found that labelling at both ends increased sensitivity, although neither 3' nor 5' labelling alone conferred an advantage. Although the study was carried out on probes used for *in situ* hybridisation, there is no theoretical reason why this conclusion can not be transferred to probes used for membrane hybridisation (Bardin *et al.* 1993).

Fluorescent dyes can be used as labels; these are available in different colours (as utilised in experiments described earlier). Fluorescent dyes have been more frequently used to label proteins in the past, rather than DNA (Szewczyk *et al.* 1987; Zuk *et al.* 1979). Fluorescence has also been used to label probes for hybridisation (Guo *et al.* 1994) and in OLAs (Eggerding *et al.* 1995). The dyes may be attached to a single base,

which is then incorporated into the fragment of DNA at the PCR stage. Alternatively, a pre-labelled base may be added to the end of the oligonucleotide using an enzyme such as Terminal deoxynucleotidyl Transferase (TdT). In 1979 Roychoudhury *et al.* identified the influence of the sequence at the end of a fragment of DNA as being important when using TdT to add nucleotides to the end of that DNA. It was observed that a G:C base pair at the exposed end would reduce incorporation of nucleotides. The more G:C pairs are at the end of the oligonucleotide, the greater the reduction in labelling efficiency (Roychoudhury *et al.* 1979). Methods such as random prime labelling and nick translation labelling have been used to incorporate pre-labelled nucleotides (Harvey *et al.* 1994; Harvey and Wheeler, 1994).

Fluorescent dyes require excitation at specific wavelengths. Methods of detection of the emitted light include CCD cameras and laser confocal microscopy (Fodor *et al.* 1993). Glass supports have been seen to work well in conjunction with fluorescence detection (Guo *et al.* 1994), where a Molecular Dynamics FluorImager 575 was used to scan the slide and record the results. There is some evidence to show that fluorescent signals may be quenched by the 3MM filter paper (Whatman, Kent, UK) used in these experiments (Woolford and Dale, 1992).

Molecular beacons are probes which have sequences at each end which are complementary, allowing the ends to hybridise, making the oligonucleotide hairpin shaped. This brings the fluorophore on one end into the proximity of a quenching moiety on the other so that no signal is given off. Before hybridisation the probe is heated to allow denaturation. If the probe hybridises to its target (via a complementary sequence in the centre of the probe), the beacon will no longer have a hairpin structure so the fluorophore will fluoresce (Tyagi *et al.* 1998). Stimpson *et al.* (1995) used a

particulate label on the DNA to act as a light scattering source when illuminated. The results were comparable to fluorescence detection with a confocal microscope (Stimpson *et al.* 1995).

Cyanine (Cy) dyes are fluorescent dyes that can be used to label proteins and DNA covalently (Ernst *et al.* 1989). Cy dyes can be excited at wavelengths between 500nm and 750nm. New derivatives of the Cy dyes have been created, which have absorbance in the red and near infra-red spectrum. These new derivatives have increased water solubility making them easier to work with (Mujumdar *et al.* 1989). One particular Cy dye, Cy5, can be excited with a HeNe laser at 633nm, or a Kr laser at 647nm, HeNe lasers are readily available. The photostability of Cy5 is better than that of fluorescein (Mujumdar *et al.* 1993).

A comparison of several red-emitting fluorophores concluded that Cy3.18 gave the best signal, which was brighter than fluorescein. It was thought that this might have been due to Cy3.18 having a higher extinction coefficient than the other dyes (that is, it captures more of the excitation light). Fluorescein was also seen to stain least brightly in this study, despite having the highest concentration (Wessendorf and Brelje, 1992).

1.4.5 Hybridisation Conditions

Hybridisation conditions must be stringent enough to distinguish between single base changes because many cystic fibrosis mutations are point mutations. A problem with RDB can be that different oligonucleotides are likely to have different annealing temperatures due to their different G:C content. If the annealing is performed at too low a temperature, non-specific annealing may occur, at the other extreme, too high a temperature will not allow annealing at all. To avoid the problems this causes, tetramethylammoniumchloride (TMAC) may be added to the hybridisation and wash

buffers (Marky *et al.* 1988). In the presence of TMAC, annealing temperature becomes a function of oligonucleotide length rather than G:C content (Shuber *et al.*, 1993). It is easy to ensure that all oligonucleotides on one chip are the same length. Stringency can be altered using the annealing temperature of the hybridisation. The closer the annealing temperature is to the T_m of the probe (the point at which 50% will be annealed) the more likely it is that any hybridised target DNA will have hybridised to an exactly complementary sequence. The T_m of a sequence can also be lowered by increasing the formamide concentration of the hybridisation buffer (Narayanan, 1992).

In order to improve the signal from hybridisations, it is important to understand the hybridisation kinetics. Several studies have been carried out in order to identify the method of duplex formation. In one study by Forman *et al.* (1998) probes were synthesized using photolithography and a target was hybridised. Longer probes were seen to adsorb more target DNA than shorter probes. Melting temperature was not dependent on probe length or target concentration. It was suggested that the high density of probes might interfere with the hybridisation, due to probes associating with each other due to steric effects (Forman *et al.* 1998). Matrix-assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry can be used to produce hybridisation curves which show hybridisation of oligonucleotides to DNA on a support as a function of temperature and could be used to study hybridization of complementary sequences (Blecinski and Richert, 1998).

Surface diffusion of oligonucleotides has been studied and conditions to improve the rate of hybridisation and minimise the amount of probe required, have been suggested. Probes should be chemically coupled to the surface, rather than attachment by physical adsorption to minimize adsorption of the target DNA to the surface. In theory, the

three-dimensional diffusion of the probe out of solution is slow, but the two-dimensional diffusion is relatively quick. The theory was originally proposed to explain the binding of ligand to cell surfaces by Adam and Delbruck (1968).

A study carried out by Chan *et al.* (1997) suggests that single stranded oligonucleotides of 80 nucleotides or less lie along the surface they are attached to. An increase in target or probe DNA was seen to reduce the diffusion coefficient, that is, it slows the diffusion of the target DNA across the surface. The work was carried out on silanised glass, and the authors suggest oligonucleotides may behave differently on other surfaces (Chan *et al.* 1997).

It is important to ensure that the immobilised oligonucleotides are not too close together as this can interfere with their binding properties (Blanchard, 1998). Conversely, it is desirable to make the best use of the space available by immobilising as many oligonucleotides as possible onto the surface. In practice, only the concentration of oligonucleotides deposited can be controlled, not their precise placement.

1.4.6 Nucleic Acid Immobilisation

The surfaces most commonly used for RDB are nitrocellulose or nylon membranes; the nylon may be positively charged (for example, Hybond N+, Amersham Pharmacia Biotech, Bucks, UK or Biodyne B, Pall Life Sciences, Hants, UK). There are several ways to attach DNA to these membranes. DNA may be put onto the membrane in a drop of water and baked onto the membrane over one or two hours. This method was shown to produce good retention of DNA on both nylon and nitrocellulose membranes (Nierzwichi-Bauer *et al.* 1990). An alternative to attachment by baking is UV-crosslinking of the DNA where it is placed on the membrane (as for baking) and then exposed to UV light for an appropriate period of time. This exposure activates the

thymine bases (T) in the DNA, which become covalently attached to primary amines in nylon membrane (Saiki *et al.* 1989). When performing RDB, very short oligonucleotides are attached to the membrane, for example 20 bases. Saiki *et al.* (1989) raised concerns that such a short piece of DNA would not be able to bind adequately to the membrane and specifically to its target DNA simultaneously. Poly(T) tails have been added to oligonucleotides using the enzyme Terminal deoxynucleotidyl Transferase (TdT) before UV-crosslinking. An alternative to this is to synthesise the oligonucleotide with a tail (Kawasaki *et al.* 1993). Theoretically, the poly(T) tail would be preferentially bound to the membrane, allowing the oligonucleotide to be free to bind to its target DNA (Saiki *et al.* 1989). It was found that oligonucleotides with long poly(T) tails (up to 800 bp) hybridise more efficiently than those with shorter tails, and a spacer effect was suggested. An optimum amount of UV exposure was observed, it was thought that this was due to DNA damage caused by prolonged exposure, as well as longer exposure attaching more of the tail to the membrane reducing the spacer effect (Saiki *et al.* 1989). Spacer molecules increased hybridisation signals, with an optimal spacer length of 40 atoms giving 150 times increase in hybridisation yield. Positive and negatively charged groups on the spacer interfered with hybridisation (Shchepinov *et al.* 1997a). Spacers are now routinely used to increase hybridisation efficiency (Zhang *et al.* 1991). Poly(Ethylene Glycol) (PEG) can be used as a spacer, it is commercially available in various lengths, with a range of different chemical groups at the termini to aid attachment (Shearwater Polymers, 1999). PEG is a non-DNA spacer, which reduces the chance of interaction with the target DNA, it is water soluble and allows small molecules to remain active when covalently bound to them (Milton Harris, 1992) indicating that it should act as a spacer without restricting access of the target DNA.

Positively charged nylon membranes increase the attraction of the negatively charged DNA (Oss, 1987). Studies that have compared different nylon membranes as solid phases for hybridisation have been performed showing that Nytran nylon+ and NC nitrocellulose produced weaker hybridisation signals than hydrophobic polyvinylidene difluoride (PVDF) (Williams, 1990) although the availability of a wide range of nylon membranes used for RDB implies that each may be more suitable for some procedures than others.

Biodyne C membrane (Pall Life Sciences, Hants, UK) has free surface carboxyl groups that can form covalent bonds with amino groups, which can be added to the end of an oligonucleotide during synthesis. This produces a specific attachment between one end of the oligonucleotide and the membrane, leaving the entire oligonucleotide free to hybridise to its target (Ivanovskaya *et al.* 1995; Kawasaki and Chehab, 1994; Maggio *et al.* 1993; Zhang *et al.* 1991). Zhang *et al.* (1991) studied different spacer lengths in conjunction with covalent attachment to Biodyne C membranes and found that spacers improved hybridisation signals. Covalent attachment of DNA to a surface is more readily transferable to different surfaces than baking or UV-crosslinking.

DNA has been attached to many different surfaces, including glass (Guo *et al.* 1994), glass via disulphide bonds (Rogers *et al.* 1999), gold (Caruso *et al.* 1997), optical fibers using various linkers (Henke *et al.* 1997) and polystyrene microwells (Hirayama *et al.* 1996; Zammattéo *et al.* 1996). Methods have also been developed to attach oligonucleotides to polystyrene microwells (Rasmussen *et al.* 1991) and glass via covalent bonds (although utilising succinylated oligonucleotides rather than amino termination). It has been observed that 40-50% of oligonucleotide put onto glass was bound, and 90% of the bound oligonucleotide was available for hybridisation (Joos *et*

al. 1997). Beier *et al.* (1999) have investigated the use of dendrimeric linkers in order to increase the capacity of silanised glass to hold oligonucleotides, without hindering the hybridisation efficiency. The most suitable surface will depend on the application of the array and also the detection method to be used. Modification of polystyrene is required to produce chemical moieties to allow the coupling of oligonucleotides.

Waveguides have been used as a support for DNA (Stimpson *et al.* 1995; Stimpson and Gordon, 1996), a microscope slide may be used as a waveguide, improving the signal emitted when using fluorescent labelled probes. Light is directed into the slide at such an angle as to allow reflection of the light down the slide in the same way that light moves down fibre optics. This means that more light reaches each fluorophore on the slide. DNA may be immobilised onto several different types of beads (O'Donnell-Maloney *et al.* 1996), for example, streptavidin coated beads capture biotinylated oligonucleotides. This method does not use a covalent bond, although it is a strong bond and stable enough to withstand stringent washing conditions.

1.4.7 Micro-fabrication

When designing a device for detection of genetic disease, it is important to consider the final product, and how easy it is to mass-manufacture. For example, in the case of the reverse dot blot, the oligonucleotides must be spotted onto the membrane or an alternative surface. Depending on the size of spot of probe required, there are several different options available for the deposition of DNA onto a surface. The simplest method is to pipette the DNA solution by hand, using a normal laboratory pipette. This method is adequate for spots of approximately 1-5µl, however it is impractical to print smaller spots in this way, and the accuracy of pipettes may be questioned. In terms of reliability and aesthetics, it may be difficult to place spots in a straight line, or in the

same position each time. The BioDot CV1000 syringe pump dispenser is an advanced stepper motor driven syringe pump which can accurately dispense drops onto a surface. The system allows rows of identical spots to be placed accurately and reproducibly (BioDot, 2002). Commercially produced microarraying robots use pins that dip into many stock DNA solutions and place the drops gathered onto the slide in a reproducible manner.

The method of probe printing used very much depends on the size of the drop of liquid containing the DNA and the reproducibility required. The cost of each method increases as the accuracy and reproducibility increase.

1.5 Summary

In some cases biochemical tests cannot give a definitive answer to the question ‘does this person have that disease?’ due to overlapping values of the protein in question between the ‘normal’ and disease state. Genetic testing will give a more reliable diagnosis in these situations. There are cases where a person has been found to have two cystic fibrosis mutations and be only mildly affected (Lester *et al.* 1994) but the undoubted benefits of testing to discover those with the disease, to allow early treatment, outweighs the possible harm done by diagnosing ‘healthy’ people with a disease.

There are many methods of mutation detection currently available and they all have advantages and disadvantages. For this project’s purposes it is important to use a system where the ability to detect new mutations is easily incorporated because new cystic fibrosis mutations are still being discovered. It is also important that the system is easy to use in order to reduce the chances of user error when detecting mutations;

because the system should be capable of being used in a district general hospital by people without specialist knowledge of molecular biology. Reverse dot blots can take time setting up, deciding which probes and targets to use, and optimising stringency conditions. Once these problems have been resolved, it is a relatively easy task to carry out multiple mutation analysis. If the reverse dot blot was miniaturised and carried out on a microscope slide, the ease of handling would be improved, and such slides could be mass-produced. The oligonucleotides must be immobilised on the slides in such a way as to minimise their removal during hybridisation, whilst allowing hybridisation to take place. End labelling of oligonucleotides with poly(T) or with an amino group allows the attachment of the end of the oligonucleotide to the surface, leaving the rest of the oligonucleotide free to hybridise. The use of covalent attachment of amino terminated oligonucleotides to a surface requires carboxyl groups on that surface and also allows specific attachment of the end of the oligonucleotide to the surface. Carboxyl groups are found on commercially produced nylon membranes (for example, Biotyne C, Pall Life Sciences, Hants, UK) and can also be produced on polystyrene slides by chemical modification (Zammetto *et al.* 1996).

Hybridisation conditions must be optimised in order to reduce false positive and negative signals, and to give a high degree of reliability in the hybridisation. It is also preferable that this be carried out with as few washes as possible, in order to speed up mutation detection.

Hybridisation detection methods must be chosen carefully, again, with the end user in mind. The detection method should be inexpensive on a day to day basis, and also should also be easy to use and read.

1.6 Project Aims and Objectives

This project aims to produce a pre-prototype of a working system that will provide information about a person's genetic cystic fibrosis status from a blood sample. The system will be designed for the genetic testing of neonates, however, it would not require adjustment for adult testing. The system will be simple enough to be used in a District General Hospital by staff with the minimum of training in molecular biology. The device will have a low production cost and the diagnosis will be carried out in as short a time as possible.

The reverse dot blot (RDB) method of multiple mutation detection will be utilised as it is a convenient method of multiple mutation analysis. The RDB will allow one patient's DNA to be screened for many mutations in one experiment. A solid phase is required for attachment of the probe DNA prior to hybridisation, the surface used will determine the methods which can be used to attach the oligonucleotides. Different surfaces will be investigated to try and identify useful methods of attaching DNA to various surfaces. Miniaturisation of the surface will be explored to incorporate the ease of handling of microarrays and DNA chips. A smaller surface area for hybridisation will also reduce the amount of solutions such as hybridisation buffer which are required. Several methods of hybridisation detection are currently available, the most suitable method for the system used will be investigated.

Four cystic fibrosis mutations will be used in this project as a model, and then up to one hundred mutations will be chosen for inclusion in the final device, based on their frequency in the population. The device will then be tested by being used to provide a 'diagnosis' for patients whose cystic fibrosis mutations are already known.

For simplicity the following three chapters of the thesis report on the different aspects of this work, i) the immobilisation and materials aspects of solid-phase DNA diagnostics, ii) the detection and probe deposition for solid-phase DNA diagnostics and finally, iii) the demonstration of a DNA diagnostic for cystic fibrosis. Each of these chapters is essentially independent with its own introduction, methods, results and discussion. This provides a convenient mechanism for evaluating each part of the project separately. The overall project is assessed through the discussion and conclusions that are presented in Chapter 5.

2 Immobilisation and Materials Aspects of Solid-Phase DNA Diagnostics

2.1 Introduction

This study set out to produce a diagnostic device based on the reverse dot blot method of hybridisation (RDB). In an RDB a series of short oligonucleotides are attached to a surface and a patient's DNA is amplified, labelled and hybridised to the oligonucleotides. The specific hybridisation of oligonucleotides to different mutation sequences allows identification of the patient's mutations from the hybridisation signals produced. A standard dot blot consists of a large piece of target DNA (approximately 300-500 bases) attached to a membrane, which is hybridised to a short, labelled probe oligonucleotide. A reverse dot blot consists of a short probe oligonucleotide attached to a membrane, hybridised to a large, labelled DNA target (usually PCR product). This work aims to combine the ability of the reverse dot blot to test for several mutations at once, with the ease of handling and small size of the microarray. The number of oligonucleotides required in each test will be approximately 100, meaning that the size of each spot of oligonucleotide may be larger than those normally found on a microarray.

This chapter reports the investigation of the extraction of genomic DNA from cells to identify the mutations present in the extracted DNA. In order to be used in a hybridisation the extracted DNA must be amplified and labelled. Different methods of labelling DNA to allow detection after hybridisation were examined. Methods of immobilising oligonucleotides on to a series of solid phases were compared to discover the most suitable surface and method of attachment for use in this work.

Current methods of attaching DNA to nylon membranes include baking (Nierzwichi-Bauer *et al.* 1990), UV-crosslinking (Saiki *et al.*, 1989; Kawasaki *et al.* 1993) and covalent attachment (Ivanovskaya *et al.* 1995; Kawasaki and Chehab. 1994; Maggio *et al.* 1993; Zhang *et al.* 1991). Reverse dot blots are usually carried out on nylon membranes whilst microarrays are generally attached to glass slides. Joos *et al.* (1997) have investigated the use of covalent attachment to secure oligonucleotides to glass slides, however, glass can be inconvenient to use as it may chip or break, representing a health risk. Polystyrene microtitre plates have been chemically modified to produce carboxyl groups on the surface that will covalently bind to an amino group which can be added to an oligonucleotide (Rasmussen *et al.* 1991). This chapter reports on both the use of nylon membranes as a solid phase and the possibility of using oligonucleotides covalently attached to polystyrene slides in hybridisations.

2.2 Generation of DNA Materials and Reagents

2.2.1 *Lactobacillus plantarum* DNA extraction and PCR

Initially *Lactobacillus plantarum* cells were used as a model system for genomic DNA extraction, labelling and hybridisation as the principles being investigated should work equally well on any DNA sequence and the ethical approval for the collection of human blood takes time to obtain. *Lactobacillus plantarum* cells were chosen because they were readily available in the molecular biology laboratory at Cranfield University (UK). A full list of reagents used can be found in Appendix J.

Genomic DNA was extracted from a single colony of *Lactobacillus plantarum* inoculated into LB media and grown at 30 °C to an optical density of 0.7-0.8 measured at a wavelength of 600nm (OD₆₀₀). The culture was centrifuged for 10 minutes at 7000 x g and the pellet was resuspended in 9 ml STE (0.14% w/v NaCl, 25 mM Tris-HCl, 10

mM EDTA, adjusted to pH 8). Lysozyme (100 mg in 1ml TE (pH 8)) was added to the resuspended pellet and the mixture was incubated at 37 °C for 30 minutes. Proteinase K (20 mg in 1ml) was added and the mixture was incubated for 1 hour at 60 °C. SDS was added to a final concentration of 0.1% and incubated at 60 °C for 15 minutes. The mixture was cooled to room temperature.

DNA was extracted three times using phenol:chloroform (1:1, equilibrated with Tris, adjusted to pH 8) and three times with chloroform:IsoAmyl Alcohol (24:1). Solvent was added, the tube was inverted to mix the contents, and centrifuged (10 minutes at 10,000 x g), the upper phase was retained.

Nucleic acid was precipitated by adding 0.1 volumes of sodium acetate (3M at pH 5.2) and 1 volume of cold isopropanol, mixing by inversion and incubating on ice for 30 minutes. DNA was curled out of the mix using the hooked tip of a sterile Pasteur pipette, washed in 70% ethanol, air dried and re-suspended in TE (pH7.5). DNAase free RNAase (50 µg ml⁻¹, Sigma-Aldrich, Dorset, UK) was added and incubated for 1 hour at 37°C. 5µl of the DNA solution was analysed by agarose gel electrophoresis (Witt, 1996; Sambrook *et al.* 1989).

Primers 1Ab and R4S (see Appendix A for sequence) were used to amplify a 334 bp region of *Lactobacillus plantarum* genomic DNA. The PCR reaction mix consisted of 10µl of 10x buffer (Perkin Elmer, Massachusetts, USA), 16µl of dNTPs (1.25 mM of each dNTP, Promega, Southampton, UK), 20 pmol of each primer (MWG-Biotech, Milton Keynes, UK), 16µl 25 mM solution of MgCl₂ (Perkin Elmer, Massachusetts, USA), 0.9 nmol of the target (*Lactobacillus plantarum* genomic DNA), and 5 units of *Taq* polymerase (Promega, Southampton, UK), in a total volume of 100µl. The cycles consisted of a hot start at 95°C for 6 minutes, then 30 cycles of denaturing (95°C, 30

seconds), annealing (55°C, 30 seconds), and extension (72°C, 1 minute). Final extension was at 72°C for 4 minutes. The PCR product was analysed by agarose gel electrophoresis.

2.2.2 *Human DNA extraction*

This project was concerned with human cystic fibrosis sequences. Although DNA from human placenta can be bought (Sigma-Aldrich, Dorset, UK), the final diagnostic device aims to use DNA extracted from blood, therefore, it is essential to check that methods of blood collection will not interfere with the gDNA extraction and running of the test.

All work with blood samples was carried out on a tray, in a flow hood, waste blood was mixed with disinfectant (10x Virkon (Merck, Dorset, UK)), and disposed of down the sink with a copious quantity of water.

Genomic DNA was extracted from human blood using the Qiagen QIAamp blood kit as the instructions suggested (Qiagen, UK). The blood (200 µl) was added to 20 µl Proteinase K and mixed, 200 µl of Buffer AL was added and mixed by vortexing for 15 seconds. The mixture was incubated at 56°C for 10 minutes and briefly centrifuged to remove drops from the lid. The sample was mixed with 200µl of 99.9% ethanol by vortexing for 15 seconds, then briefly centrifuged. The mixture was spun through a QIAamp spin column at 6,000 x g for 1 minute, the filtrate was discarded. Buffer AW1 (500µl) was added to the column, and spun at 6,000 x g for 1 minute. Buffer AW2 (500µl) was added to the column and spun at 20,000 x g for 3 minutes. The column was placed in a clean 1.5ml tube, 200µl molecular biology grade water (Fisher, UK) was added to the column and incubated for 5 minutes at RT before centrifuging at 6,000 x g for 1 minute. The genomic DNA was analysed by running 10µl of each DNA solution on a 0.3% agarose gel.

2.2.3 PCR of cystic fibrosis sequences

Amplification of the areas of interest in the cystic fibrosis transmembrane regulator (CFTR) gene was carried out in order to provide DNA to be used in the following work.

PCR amplification were carried out on human genomic DNA, on three different exons of the CFTR gene; exon 10 (covering the $\Delta F508$ mutation), exon 11 (G542X and G551D) and exon 21 (N1303K). The PCR of each exon was carried out with two variations, PCR incorporated digoxigenin label and biotin labelled primers. All cystic fibrosis PCR primer sequences were as shown in Zielenski *et al.* (1991) and Wall *et al.* (1995) (see Appendix A).

2.2.3.1 Digoxigenin (DIG) labelling of PCR

PCR products were labelled with digoxigenin (DIG) in order to allow their detection after hybridisation. DIG labelling was carried out using a PCR DIG labelling kit (Roche, UK). The directions from the kit were followed, however optimisation of the proportion of DIG labelled dUTP added to the PCR was required. All solutions (except template and primers) were supplied from the kit.

The PCR reaction mix consisted of the same components for each exon, in a total reaction volume of 50 μ l, 5 μ l of 10x buffer, 1 μ l of DIG dNTP mix, 4 μ l of dNTP stock (concentration as supplied), 0.23 pmol of template, 5 units of *Taq* polymerase and 20 pmol of each primer were used. The PCR for each exon was carried out under different cycling conditions, which were as shown by Zielenski *et al.* (1991) (exons 10 and 11) and Wall *et al.* (1995) (exon 21).

The cycles used to amplify exon 10 consisted of a hot start at 94°C for 6 minutes, then denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds), extension (72°C, 2.5 minutes), repeated for 30 cycles, then final extension at 72°C for 7 minutes

The cycles used to amplify exon 11 were a hot start at 94°C for 6 minutes, then denaturation (94°C, 30 seconds), annealing (52°C, 30 seconds), extension (72°C, 1 minute), repeated for 30 cycles, then final extension at 72°C for 7 minutes

The cycles used to amplify exon 21 were a hot start at 94°C for 6 minutes, then denaturation (96°C, 30 seconds), annealing (55°C, 30 seconds), extension (72°C, 30 seconds), repeated for 35 cycles, then final extension at 72°C for 7 minutes.

The success of each PCR was analysed by agarose gel electrophoresis and the amount of PCR product produced was estimated using EZ load precision molecular mass standard (Bio-Rad, California, USA).

2.2.3.2 Biotin labelling of PCRs

The biotin-streptavidin bond has commonly been used as part of methods of detection due to its strength and reliability. Labelling of a PCR product can be carried out during PCR by incorporation of labelled primers, with little modification of protocols required, making it a simple way to incorporate a biotin label for use in hybridisation detection.

The PCR reaction mix consisted of the same components for each exon. *Taq* polymerase, 10x buffer, dNTPs and MgCl₂ were from Qiagen (UK).

In a final volume of 100µl, 10µl of 10x buffer, 2µl of dNTPs, 8µl of MgCl₂, 5 units of *Taq* polymerase, 0.23pmol template and 20pmol of each primer (MWG-Biotech, Milton Keynes, UK) were used. Primers were synthesised by MWG-Biotech with a biotin label on the 5' end, and used as normal. The cycles for each PCR are shown below, the cycling conditions are as described by Zielenski *et al.* (1991) (exons 10 and 11) and Wall *et al.* (1995) (exon 21) except for the annealing step and number of cycles for exon 11 which required optimisation.

Exon 10 cycling conditions were 6 minutes at 96°C, then 30 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds), extension (72°C, 2.5 minutes) and a final extension at 72°C for 7 minutes.

Exon 11 cycling conditions were 6 minutes at 94°C, 34 cycles of denaturation (94°C, 30 seconds, 94°C), annealing (46°C, 40 seconds), extension (72°C, 1 minute) and a final extension at 72°C for 7 minutes.

Exon 21 cycling conditions were 6 minutes at 96°C, then 30 cycles of denaturation (96°C, 1 minute), annealing (55°C, 30 seconds), extension (72°C, 30 seconds) and a final extension at 72°C for 7 minutes.

The PCR reactions were analysed by agarose gel electrophoresis and the amount of PCR product was estimated using EZ load precision molecular mass standard (Bio-Rad, California, USA).

2.2.4 Results and discussion

2.2.4.1 *Lactobacillus plantarum* DNA extraction and PCR

Genomic DNA was successfully extracted from *Lactobacillus plantarum* (see Figure 1) and amplified using primers 1Ab and R4S. The PCR product was approximately the correct size (334 bp) as estimated in comparison to the molecular marker run on a gel alongside it (see figure 2). The concentration of PCR product produced was between 0 and over 10 ng/μl (as compared to the molecular mass standard, Bio-Rad). The PCR product was labelled and found to be of sufficient quality to be used as a target in a hybridisation.

2.2.4.2 Human gDNA extraction

Genomic DNA was successfully extracted from control samples of human blood using the Qiagen QIAamp extraction kit (see figure 3). Blood samples used as wild type controls were collected by staff at the Silsoe and Cranfield medical centres (Cranfield University, Bedfordshire, UK). The samples were taken anonymously from 6 volunteers, no anti-coagulant was used, and the genomic DNA was extracted within 30 minutes of collection. Volunteers signed a consent form (Appendix B) which explained the purpose of blood collection, and the anonymity of the results of the experiments. Figure 3 clearly shows that genomic DNA has been extracted from the blood cells.

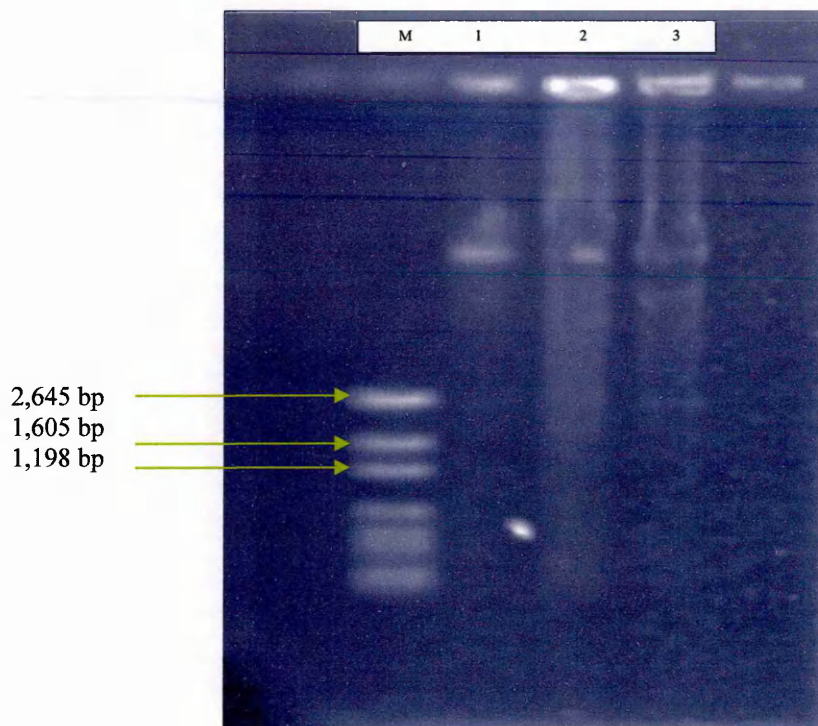


Figure 1. *Lactobacillus plantarum* gDNA extract

Genomic DNA was extracted from *L. plantarum* cells using phenol:chloroform extraction. Two lanes of 5 μ l of extract from a single extraction were run on a 0.6% agarose gel in order to confirm extraction, a 1/100 dilution was run in the third lane in order to confirm the presence of DNA at this dilution. The bands in the pGEM marker used were not entirely separated due to the short running time of the gel. Lanes: M-marker. 1-gDNA1. 2-gDNA2. 3-1/100 gDNA1.

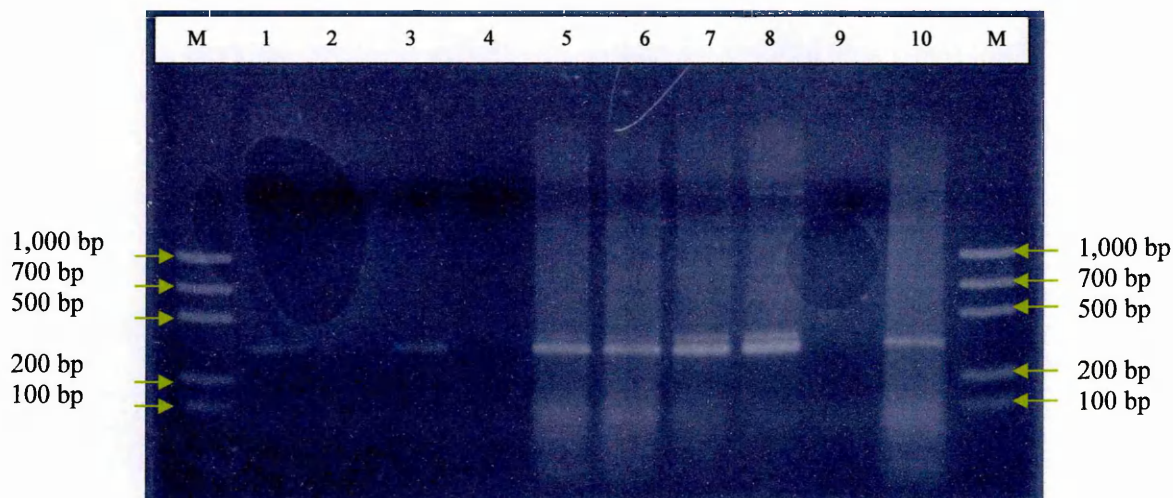


Figure 2. PCR product from primers 1Ab and R4s

L. plantarum gDNA was amplified with primers 1Ab and R4s (see Appendix A for sequence). 10µl of amplicon were analysed on a 1.2% agarose gel. PCRs a-h are all amplifications of gDNA1 (see figure 2). The expected product is approximately 334 bp. Bio-rad Molecular Mass marker was used with bands of (top down) 1000 bp, 700 bp, 500 bp, 200 bp and 100 bp. Lanes: M-marker. 1-LB PCRa. 2-LB PCRb. 3-LB PCRc. 4-LB PCRd. 5-LB PCRe. 6-LB PCRf. 7-LB PCRg. 8-LB PCRh. 9-negative control. 10-LB PCR (positive control).

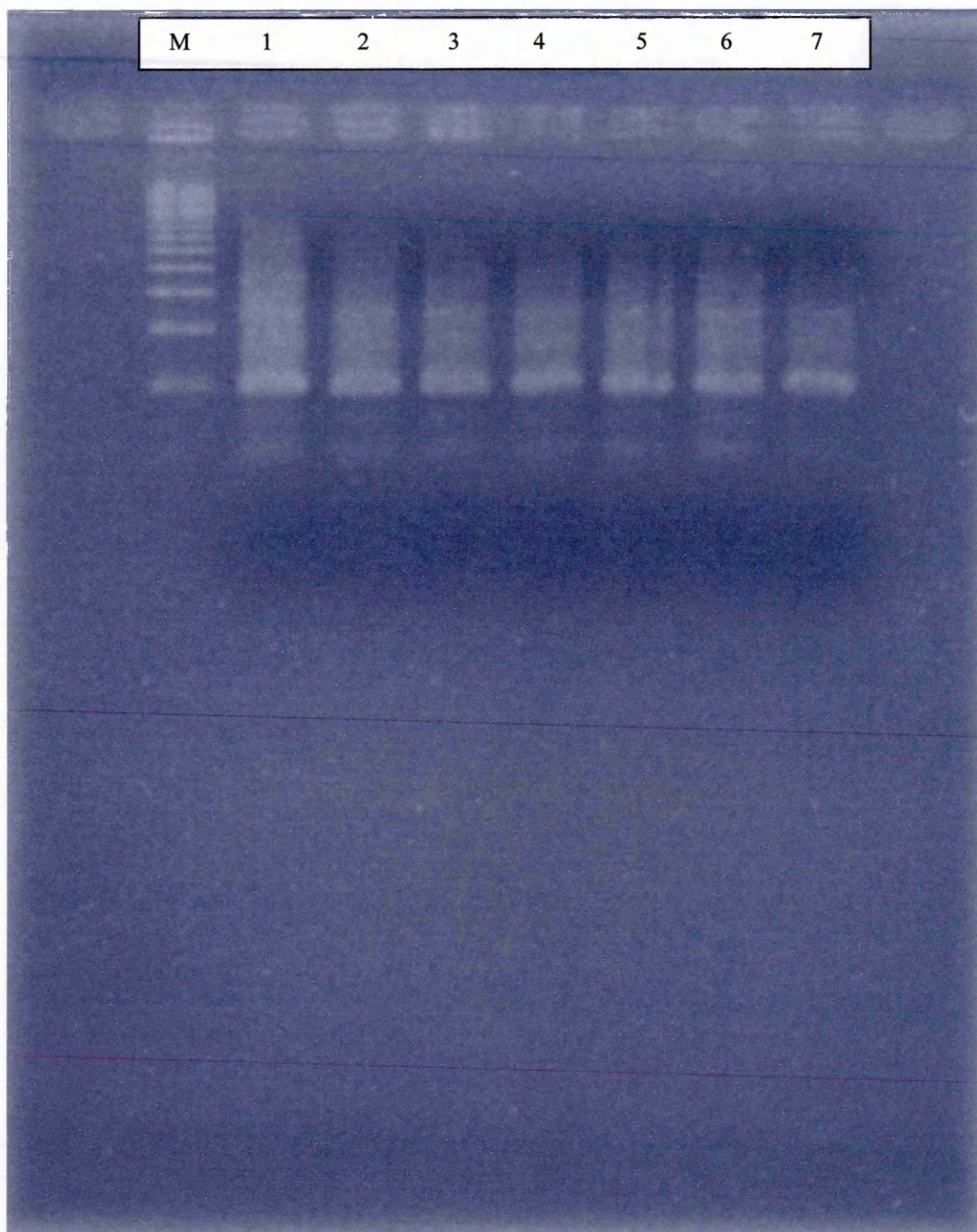


Figure 3. Human gDNA extracted from control blood samples

Genomic DNA was extracted from 7 control blood samples using the Qiagen ReadyAMP Extraction Kit. 10 μ l of extract were run at 50V on a 0.3% agarose gel in order to confirm extraction. The bands in the pGEM marker used were not entirely separated and it is not possible to distinguish their sizes due to the use of a low

(Figure 3. legend continued)

agarose percentage in the gel (0.3%). Lanes: M-marker. 1- gDNA1. 2-gDNA2. 3-gDNA3. 4-gDNA4. 5-gDNA5. 6-gDNA6. 7 gDNA7. (gDNAs from seven different volunteers).

2.2.4.3 PCR DIG labelling of cystic fibrosis sequences

Strong bands were seen when the PCR products were analysed by agarose gel electrophoresis (figure 4). The bands were observed to be approximately the correct sizes (exon 10-491bp, exon 11-425bp and exon 21-376bp) when compared to the molecular mass marker. The proportion of DIG labelled dUTP was reduced from that suggested in the protocol (Roche, UK) in order to reduce interference with the PCR. The DIG label on dUTP may interfere with the PCR if the labelled bases are present at too high a concentration. An experiment was performed to determine how much labelled dUTP could be added to the dNTP mix before the PCR was affected. The aim was to increase labelling without affecting the PCR. DIG labelled dUTP was provided as part of a dNTP mixture by Roche (UK) which could be diluted using an unlabelled dNTP mix. The final concentration of DIG labelled dNTPs used (a 1:4 ratio of labelled to unlabelled nucleotide mix (Roche, UK)) was the optimum amount to allow PCR to be carried out and to produce a good signal after hybridisations (see section 2.4). The DIG labelled PCR was successful with no need for modification of the thermal cycling conditions.

2.2.4.4 Biotin labelling of cystic fibrosis sequences via end labelled primers

Biotin labelling of PCR was carried out in an alternative manner to DIG labelling. Biotin labelled primers were ordered from MWG-Biotech (Milton Keynes, UK), which produced a PCR product with only one biotin label per strand of DNA, compared to the multiple DIG labels incorporated previously. The presence of a biotin moiety at the 5' end of primers may be expected to have an affect on how easily the primer anneals to the target DNA, necessitating some optimisation of the PCR in question. It is important to position the biotin label at the 5' end of the primer as if it were positioned at the 3'

end it may inhibit the PCR by interfering with the addition of nucleotides to the 3' end during replication. The length and temperature of the PCR cycles for amplifying exons 10 and 21 did not require adjustment in order to produce PCR product when the primers were end-labelled with biotin. The annealing temperature used to amplify exon 11 with biotin labelled primers, however, was reduced from that used in the DIG labelling amplification (from 52°C to 46°C).

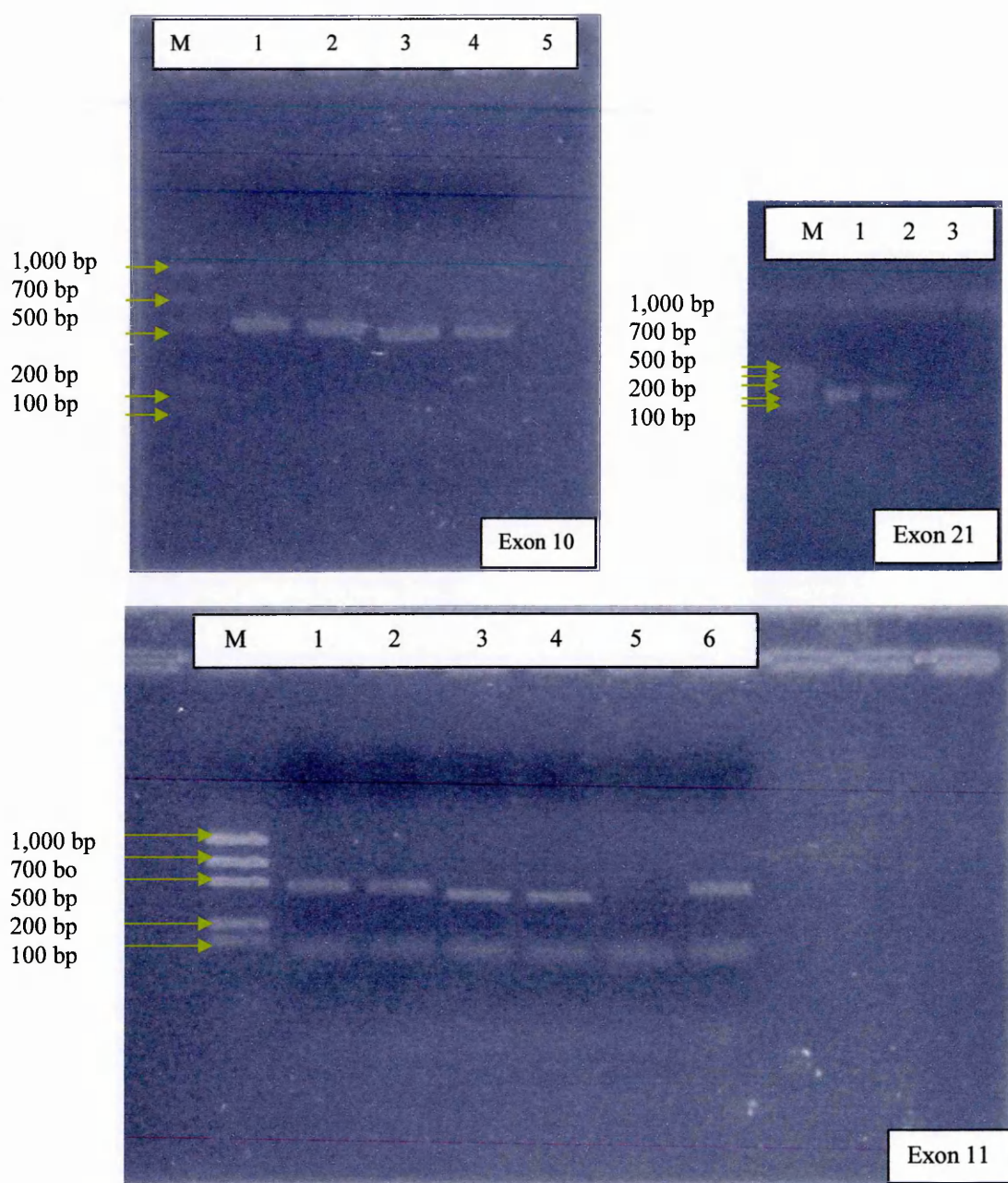


Figure 4. PCR product from DIG labelling exons 10, 11 and 21

Three exons from the CFTR gene in human gDNA were amplified using primers 10i-3, 10i-5, 11i-3, 11i-5, 21a and 21b (see Appendix A for sequence). The amplicon was labelled by incorporation of DIG labelled nucleotides. 10µl of amplicon were analysed on a 1.2% agarose gel. The expected band sizes were, exon 10-491 bp, exon 11-425 bp and exon 21-376 bp, however the DIG labels will affect the migration of the bands making them appear slightly larger than they are.

(Figure 4. legend continued)

Exon 10 lanes: M-marker, 1 and 2-DIG labelled PCR, 3 and 4 +ve control. 5- -ve control.

Exon 21 lanes: M-marker, 1-+ve control. 2-DIG labelled PCR. 3- -ve control.

Exon 11 lanes: M-marker, 1, 2 and 6 DIG labelled PCR, 3 and 4-+ve control. 5- -ve control.

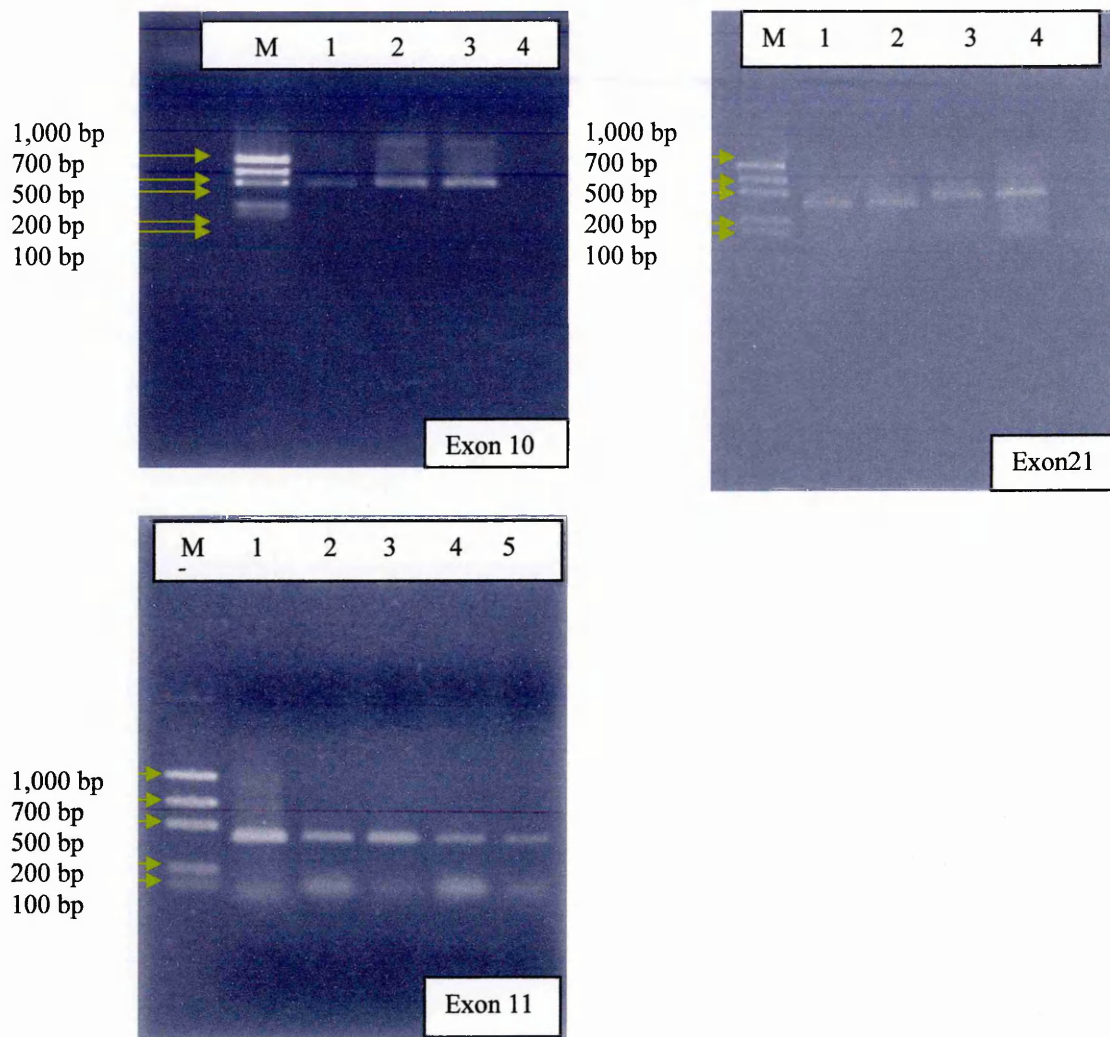


Figure 5. PCR product from biotin labelling

Three exons from the CFTR gene in human gDNA were amplified using biotin labelled primers 10i-3, 10i-5, 11i-3, 11i-5, 21a and 21b (see Appendix A for sequence). 10µl of amplicon were analysed on a 1.2% agarose gel. The expected band sizes were, exon 10-491 bp, exon 11-425 bp and exon 21-376 bp, however the biotin label reduced DNA migration time and make the PCR product appear larger than it is.

Exon 10 lanes: 1-+ve control. 2 and 3- biotin labelled PCR. 4- -ve control

Exon 11 lanes: 1- +ve control. 2-5 biotin labelled PCR. (-ve control not shown)

(Figure 5. legend continued)

Exon 21 lanes: 1 and 2-+ve controls. 3 and 4-biotin labelled PCR. 5- -ve control

2.2.5 Summary

Genomic DNA has been extracted and amplified from *Lactobacillus plantarum* and human blood cells. Amplification of sequences of interest has been successfully carried out on all genomic DNA extracted. The PCR amplification also included a method of labelling (DIG or biotin) for detection. This is a requirement for the preparation of the target to be used in hybridisations. The PCR products were found to be of sufficient quality for use as a target in a hybridisation, as shown by successful hybridisation (see sections 2.3 and 2.4).

2.3 Physical Immobilisation of DNA to Nylon Membranes

2.3.1 Introduction

Nylon membranes are commonly used as the solid phase for attachment of DNA in hybridisations. This section will look at methods currently available to physically attach oligonucleotides to nylon membranes. It describes this study's investigations of two commonly used methods of attachment, baking and UV-crosslinking using *Lactobacillus plantarum* sequences as a model.

Hybond N+ membrane was used because nylon membranes are stronger than nitrocellulose in solution and after baking (Amersham Pharmacia Biotech, Bucks, UK). The target DNA used was amplified from *Lactobacillus plantarum* bacteria, (see 2.2.1) because these bacteria were readily available in the laboratory. Detection of hybridisation requires observation of a signal from the hybridised target.

Chemiluminescence is a commonly used method of detection that relies on the catalysis of a substrate by an enzyme coupled to an antibody (specific to the label of choice) in order to produce light, which is detected via X-ray film. Hybridisation was detected via chemiluminescence using the ECL kit (Amersham Pharmacia Biotech, Bucks, UK) as a

convenient method of detection. Oligonucleotide probes were attached to the membrane and hybridised to the ECL labelled target.

Two different methods of attaching oligonucleotides to nylon membranes were studied, baking and UV-crosslinking. It has been suggested that the addition of a poly(T) tail will allow UV-crosslinking to attach the tail preferentially to the membrane, allowing the hybridising sequence to behave as if it were in solution (Saiki *et al.* 1989; Kawasaki *et al.* 1993). Both tailed and non-tailed oligonucleotides were used in the following experiments.

2.3.2 Method of probe labelling and hybridisation

Amplified *Lactobacillus plantarum* DNA (see section 2.2.1) was labelled using an ECL direct labelling kit (Amersham Pharmacia Biotech, Bucks, UK). The probe was diluted to a concentration of 10ng/μl, and 10μl were denatured by heating for 5 minutes in a boiling water bath. The DNA was cooled on ice and spun briefly to collect the liquid at the bottom of the tube. An equivalent volume of DNA labelling reagent was added and mixed thoroughly, 10μl of glutaraldehyde solution (from ECL kit) were added and mixed. The solution was spun briefly and incubated for 10 minutes at 37 °C. After 1 hour pre-hybridisation at 42°C, 0.23pmol of ECL labelled target were added to the hybridisation buffer. Hybridisation was carried out overnight at 42°C in a Hybaid minioven (Hybaid, UK). Washes (0.4% SDS/0.5xSSC) were carried out for 2 x 20 minutes as the protocol suggested, and 2 x 5 minute washes in 20 x SSC. ECL detection (Amersham Pharmacia Biotech, Bucks, UK) was used to observe the hybridisation. Equal volumes of detection reagents 1 and 2 were mixed and applied to the membrane. The membrane was incubated for 1 minute at room temperature,

drained of excess reagent and wrapped in clingfilm. The membrane was exposed to Kodak X-OMAT film (Sigma-Aldrich, Dorset, UK) for 1 hour.

2.3.3 Oligonucleotide attached by baking in an oven and subsequent hybridisation

Control sequences and oligonucleotide R4A (see appendix C) were pipetted (in 1 µl water) onto Hybond N+ membrane (Amersham Pharmacia Biotech, Bucks, UK), and baked at 80°C for 1 hour. Probe labelling and hybridisation were carried out as described in section 2.3.2.

2.3.4 Poly(T) tailed oligonucleotides attached to membranes using UV-crosslinking

2.3.4.1 Method of adding a Poly(T) tail to an oligonucleotide

A sequence from the *Lactobacillus plantarum* genome, (oligonucleotide R4A) was synthesised by MWG-Biotech, (Milton Keynes, UK) (See Appendix C for oligonucleotide sequence). A poly(T) tail was added to the oligonucleotide (50 pmol) by incubation at 37°C with Terminal deoxynucleotidyl transferase (TdT) (40 units), dTTP (10mM) and 5x buffer (made up to 50 µl with water), then the enzyme was inactivated by incubation at 80°C for 10 minutes. The length of the tailing reaction is proportional to the length of the poly(T) tail added (as tested by the supplier, Promega, UK). Approximately 25 nucleotides should be added to the 3' end of the oligonucleotide in 30 minutes.

2.3.4.2 Method of UV-crosslinking poly(T) tails to nylon membranes

A poly(T) tail was attached to oligonucleotide R4A as described in section 2.3.4.1. and was attached to Hybond N+ membrane by exposure to an UV source (CleneCab plus, Herolab, Wiesloch, Germany). Probes were deposited onto the membrane, allowed to dry and UV irradiated for 90 seconds. Hybridisation and ECL detection were carried out as described in section 2.3.2.

2.3.5 Results and discussion

These experiments using baking and UV-crosslinking to attach DNA to nylon membrane were carried out to confirm the results of previous experiments (Nierzwicki-Bauer *et al.* 1990) showing that hybridisation is possible when probe DNA has been attached to a nylon membrane using these methods.

2.3.5.1 DNA attachment to nylon membrane by baking

Figure 6 shows hybridisation results after probe DNA has been baked onto the membrane. The PCR amplified *Lactobacillus plantarum* target DNA has hybridised to both spots of genomic DNA, strongly to itself and also to tailed R4A oligonucleotide. Hybridisation has not occurred between the target and R4S or 1Ab oligonucleotides. The R4A sequence is found in the middle of the sequence amplified from *Lactobacillus plantarum*, oligonucleotides R4s and 1Ab are the primers used to amplify the sequence therefore their complementary sequences are found at the ends of the PCR product. The position of the oligonucleotide sequence in the target should not influence hybridisation, therefore it is likely that the hybridisation signal observed from the tailed oligonucleotide (R4A) is due to the addition of the poly(T) tail.

Baking of DNA onto membrane is a common procedure in hybridisations, it relies on non-specific attachment of the DNA to the membrane. It is probable that DNA attached to the membrane using this method does not always have an adequate number of bases free for hybridisation (Saiki *et al.* 1989). Whilst this method has been shown to allow hybridisation and production of a weak signal, it is likely that if the DNA were attached in a more controlled way, for example by one end, the amount of target which would be available to hybridise to the probe would be increased.

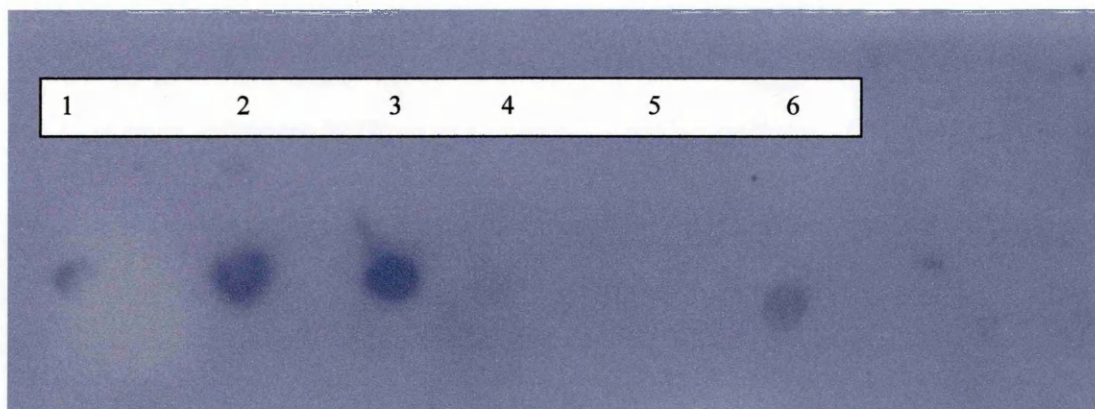


Figure 6. Oligonucleotides were attached to Hybond N+ membrane by baking Oligonucleotides were physically attached to nylon membrane (Hybond N+) by baking at 80°C for 60 minutes. LB PCR product was labelled using an ECL labelling Kit (Amersham Pharmacia Biotech, Bucks, UK), and hybridised to spots of: 1- LB gDNA. 2-LB gDNA. 3- LB PCR product. 4-oligonucleotide R4s. 5-oligonucleotide 1Ab. 6-PolyT tailed oligonucleotide R4A (see appendix C for oligonucleotide sequences). Each of these spots of DNA contains a complimentary sequence to LB PCR product.

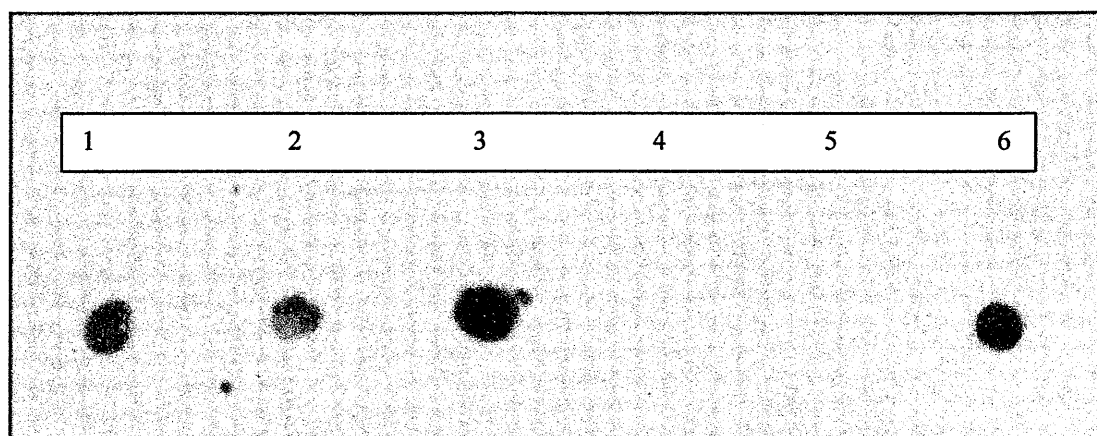


Figure 7. Oligonucleotides were attached to Nylon membrane by UV-crosslinking

Oligonucleotides were attached to nylon membrane (Hybond N+) by exposure to UV-light for 90 seconds. LB PCR product was labelled using an ECL labelling Kit (Amersham Pharmacia Biotech, Bucks, UK), and hybridised to spots of: 1- LB gDNA. 2-LB gDNA. 3- LB PCR product. 4-oligonucleotide R4s. 5-oligonucleotide 1Ab. 6-PolyT tailed oligonucleotide R4A (see appendix C for oligonucleotide sequences). Each of these spots of DNA contains a complimentary sequence to LB PCR product.

2.3.5.2 DNA attachment to nylon membrane by UV-crosslinking

Figure 7 shows the results of the hybridisation carried out after probes were attached to membrane by UV-crosslinking. The *Lactobacillus plantarum* target DNA hybridised to both gDNA spots, very strongly to itself, weakly to oligonucleotide R4s and very strongly to tailed R4A oligonucleotide. No hybridisation signal can be observed at the position of oligonucleotide 1Ab. A comparison can be made between the results of hybridisation after baking and UV-attachment of oligonucleotides as the control gDNA (spots 1 and 2) and PCR (spot 3) spots are of a similar intensity in the two figures (figures 6 and 7). Hybridisation is increased (indicated by an increase in the strength of the hybridisation signal) when UV-attachment is used, compared to baking.

Hybridisation has occurred to the R4S oligonucleotide after UV-crosslinking, but no signal was observed when baking was used to attach this oligonucleotide to the membrane.

There is obviously a much stronger signal from target hybridised to tailed R4A than the un-tailed R4s, implying that the addition of a poly(T) tail allows the oligonucleotide more freedom to hybridise. UV-crosslinking of DNA activates the Thymine (T) bases, which become covalently attached to the primary amines in the nylon membrane (Saiki *et al.* 1989). The purpose of the poly(T) tails added before UV-crosslinking the oligonucleotides to the membrane, was to allow the tail to be preferentially bound, leaving the probe sequence free to hybridise. There is still very little control over which part of the oligonucleotide attaches to the membrane, and the probe sequence as well as the poly(T) tail may be attached. It has been observed by Saiki *et al.* (1989) that the longer a poly(T) tail is, the stronger the signal, implying that more target had hybridised to the probe. It is possible that the longer the poly(T) tail is, the more likely it is that

only the tail will be attached to the membrane and the probe sequence will be left free to hybridise.

The addition of the poly(T) tail itself is not precise. The information given by the supplier indicates how many bases will be added by the TdT enzyme in a set time, under certain conditions. From this information it is possible to estimate the length of the poly(T) tail produced, although it is not possible to be certain this length of tail is added to each oligonucleotide. Tail length could be confirmed by running the tailed oligonucleotides on a gel, however this can be time consuming and therefore was not considered to be appropriate for use in the production of a high through-put test. An alternative to in-house tailing is to have the oligonucleotide synthesised with the tail, although this can be expensive, and there may still be problems with the accuracy of tail length if the total oligonucleotide is over 80 bases (A. Venables, Personal communication).

2.3.6 *Summary*

The hybridisation signals obtained after hybridisations using baking and UV-crosslinking as methods of attaching probe oligonucleotides to Hybond N+ membrane were not reproducible or easily visible enough for use in a diagnostic device.

UV-crosslinking and baking of DNA to membranes give similar results after hybridisation, however they are both unreliable methods of attaching DNA to a surface. The consistency of signal (for example, between spots 1 and 2 in figures 6 and 7) is not reproducible. The desired reproducibility for a clinical diagnostic test is of an extremely high standard, and physical absorption is not precise enough to meet that standard. In a later section (2.5) the issue of the amount of oligonucleotide deposited will be addressed.

2.4 Covalent Immobilisation of DNA to Carboxylated Nylon Membranes

2.4.1 Introduction

It is important that a diagnostic device always gives the same results with the same patient's DNA, under the same conditions. Physical attachment of oligonucleotides to membranes for hybridisation does not give the consistency and reliability required, as shown in section 2.3, therefore an alternative method of attachment must be examined.

A covalent bond is stronger than pure physical attachment, and may be more reliable and reproducible, therefore a method of covalently attaching oligonucleotides to a commercially produced carboxylated nylon membrane (Biodyne C, Pall Life Sciences, Hants, UK) was investigated in this section. Oligonucleotides were required to have an amino group at one end in order to perform the coupling chemistry, the amino group was added at the time of synthesis (MWG-Biotech, Milton Keynes, UK).

Covalent attachment was carried out in all cases as suggested in the literature (Chehab and Wall, 1992). Published protocols suggest that the covalent attachment of between 1 and 5 pmol of oligonucleotide to the membrane should be attempted (Kawasaki and Chehab, 1994; Pall Life Sciences, Hants, UK, Personal Communication). Experiments using radioactive labelling were carried out to estimate how much oligonucleotide is covalently attached to Biodyne C membrane. Both radioactive labels and DIG labels have been used in this section as alternative labelling methods to the ECL method of detection used previously.

2.4.2 Oligonucleotide covalently attached to carboxylated nylon membrane

Covalent attachment of amino terminated oligonucleotides to carboxylated nylon membrane was investigated as an alternative to physical attachment of oligonucleotides to nylon membrane.

An oligonucleotide was synthesised (MWG-Biotech, Milton Keynes, UK) with an amino termination at the 5' end and a DIG label at the 3' end and was covalently attached to Biotodyne C membrane (Pall Life Sciences, Hants, UK).

2.4.2.1 Activation of carboxyl groups and covalent coupling of oligonucleotides

The Biotodyne C membrane was activated by a brief incubation in 0.1M HCl, and a 15 minute incubation in 16% 1-Ethyl-3-(3-Dimethylaminopropyl)Carbodiimide (EDC) solution. The amino terminated oligonucleotides were spotted on to the membrane, air dried for 30 minutes and the membrane was chemically quenched by a 15 minute incubation with 0.1M NaOH.

2.4.2.2 'Mock' hybridisation

The membrane was incubated in DIG hybridisation buffer (5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% DIG block made up in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl adjusted to pH 7.5 with solid NaOH)) for 1 hour pre-hybridisation, as suggested in the protocol (Roche, UK). Fresh hybridisation buffer was used to replace the pre-hybridisation buffer, the 'mock' hybridisation was carried out without the addition of probe DNA at 42°C overnight in a Hybaid minioven. Washes in 2xSSC/0.1%SDS and 1xSSC/0.1%SDS were carried out for 2 x 5 minutes each. The membrane was then incubated in 1x DIG block solution (in Maleic acid buffer) for 30 minutes and anti-DIG-AP conjugate (Roche, UK) diluted 1/10,000 in 1x DIG block solution for 30 minutes. The membrane was washed for 2 x 15 minutes in DIG washing buffer (0.3% Tween 20 in Maleic acid buffer). The membrane was equilibrated in detection buffer (0.1 M Tris-HCL, 0.1 M NaCl, pH 9.5) and sealed in a hybridisation bag with disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl phenyl phosphate (CSPD) diluted 1/100 in

detection buffer for 5 minutes. The membrane was blotted (DNA side up) on to filter paper, sealed in a hybridisation bag and incubated at 37°C for 15 minutes before being exposed to film (Kodak X-OMAT Sigma-Aldrich, Dorset, UK) for 2 hours for signal detection.

2.4.3 Determination of quantitative amounts of oligonucleotide covalently attached to membrane

Physical attachment and covalent coupling methods of attaching oligonucleotides to membrane were compared by quantification of the amount of oligonucleotide attached. Radioactive labels were used to allow this quantification to be carried out.

The amount of hybridisation generally indicates the amount of DNA attached to the membrane. The amount of DNA observed through hybridisation to be physically attached to membrane (in figures 6 and 7, spots 1 and 2) was inconsistent and lower than expected, therefore, a radioactive label was used to directly quantify the amount of DNA which could be attached to the membrane by UV-crosslinking and covalent coupling. The use of a radioactive label allows a measurement of the radiation produced by a defined amount of oligonucleotide to be taken, and compared to a measurement of the radiation produced by oligonucleotide attached to membrane. It is possible to calculate how much oligonucleotide is left on the membrane after mock hybridisation and stringency washes.

Two sets of 7 oligonucleotides were radioactively labelled, one set had 5' amino termination in order to allow covalent attachment and the remaining set did not. See Appendix C for the sequence of these oligonucleotides (R4A and R4Aa), the number in the name refers to the length of poly(T) tail. The oligonucleotides without amino

termination were identical in sequence to those with, the only difference being the presence or absence of the amino group.

2.4.3.1 Method for radioactive labelling of oligonucleotides

Oligonucleotides and poly(T) tails were synthesised by MWG-Biotech (Milton Keynes, UK), and then labelled with [γ -P³²] ATP (Amersham Pharmacia Biotech, Bucks, UK) using T4 Polynucleotide kinase (Roche, Brussels, Belgium). The reaction mix consisted of 20 pmol of oligonucleotide, 20 units of kinase, 10x buffer and 156 pmol [γ -P³²] ATP made up to 20 μ l with water, incubated at 37°C for 30 minutes and inactivated at 68°C for 10 minutes.

Labelled oligonucleotide was purified from excess [γ -P³²] ATP using P30 spin columns (Bio-Rad, California, USA). The column was inverted several times, the tip broken off and placed in a 2ml tube. The column and tube were spun at 12,000 x g for 2 minutes to remove excess buffer, which was discarded. The column was placed in a 1.5ml tube, 20-75 μ l of labelled oligonucleotide were added to the centre of the column and centrifuged for 4 minutes.

2.4.3.2 Method to test the efficiency of radioactive labelling

A DE81 test was carried out after purification in order to test the efficiency of labelling, it is used to assess the amount of labelling present after unincorporated nucleotides have been removed. For the test purified oligonucleotide (1 μ l) was placed in the centre of each of 4 DE81 filters (Whatman, Kent, UK). The filters were dried under a lamp for 15 minutes and then two were washed in 2 x SSC at room temperature for 2 x 5 minutes. The washed filters were rinsed in ethanol and dried under a lamp for 15 minutes. All 4 filters were placed into scintillation tubes and 4ml of scintillation fluid

(Aqua Luma Plus, Lumac-LSC) were added to each. The radioactivity was counted using a Beckman scintillation system (LS6500, Beckman, High Wickham, UK) and the average counts per minute (CPM) of the two washed filters and that of the two unwashed filters was used to calculate the percentage of radioactivity washed off the membrane in the form of unincorporated [γ -P³²] ATP.

2.4.3.3 Method for determination of probe yield

Radioactively labelled oligonucleotide was diluted 1 part in 100 with water and 5 μ l were counted using Cherenkov counting. [γ -P³²] ATP was also diluted 1/100 and counted in the same way. These values were used to calculate the yield of labelled probe. The purity of the labelled probe is the percentage of the CPM remaining on the filter paper in the DE81 test after washing. The final yield of probe is calculated using the following equation:

$$\text{Final yield} = \frac{\text{CPM} / \mu\text{l of purified probe}}{\text{CPM} / \mu\text{l of } [\gamma - \text{P}^{32}] \text{ATP}} \times \% \text{ purity of labelled probe}$$

2.4.3.4 Mock hybridisation experiment

Labelled oligonucleotide was diluted 1 part in 100 with unlabelled oligonucleotide.

Amounts of between 18.24 pmol and 28 pmol of the hot/cold mixture of oligonucleotides were spotted onto membranes and attached either covalently (see section 2.4.2) or via UV-crosslinking. UV-crosslinking of oligonucleotides to nylon membrane strips was performed as described in section 2.3.4.2 using a hand held UV source (UV-hand lamp, L2766, Labcentre).

A dummy hybridisation was carried out as described in section 2.4.3, where all protocols were followed as normal except that no target was added and it was the oligonucleotides themselves that were labelled. All dummy 'hybridisations' were carried out in duplicate and 5 different concentrations of each oligonucleotide were used (see Appendix D).

2.4.3.5 Detection of radioactivity in order to measure absolute amounts of oligonucleotide attached to membrane

The strips of membranes were wrapped in clingfilm and exposed to film for 5 minutes. The film was analysed using a cyclone phosphoimager (Packard Bioscience, UK) to confirm the presence of radioactively labelled probe (results not shown). Each strip was cut in half to give one piece of membrane with two spots of the same oligonucleotide. The radioactivity on each half strip was counted using Cherenkov counting, to give the value for an average of the two spots the counts per minute (CPM) value was divided by two. The CPM value for each spot was used to calculate the total amount of oligonucleotide remaining on the membrane, and the values for the washed and unwashed strips were compared to see how much oligonucleotide had been washed off during the dummy hybridisation.

2.4.4 *Evaluation of covalent coupling of oligonucleotide to membrane via hybridisation to labelled target*

Covalent attachment of oligonucleotides to membrane is stronger than physical attachment. Therefore this section aims to show that covalent attachment, will allow a hybridisation signal to be observed, and show that it is a viable method for attaching oligonucleotides to membrane for hybridisation. The reverse dot blot can be carried out using physical attachment of DNA to the membrane, however the use of covalent

attachment is expected to increase the amount of oligonucleotide available to hybridise as the attachment occurs only at the 5' terminus of the oligonucleotide.

At this point in the project human blood samples became available, therefore human genomic DNA was used instead of *Lactobacillus plantarum* genomic DNA as it is human cystic fibrosis sequences that this project is concerned with. The PCR amplicons and oligonucleotides used in this experiment were sequences that were of relevance to detection of cystic fibrosis mutations. Four different cystic fibrosis mutation sequences were studied, $\Delta F508$, G542X, G551D and N1303K. These mutations are found in exons 10 ($\Delta F508$), 11 (G542X and G551D) and 21 (N1303K). PCR was used to amplify sequences from exons 10, 11 and 21, incorporating digoxigenin labels as described in section 2.2.3.1. Spots of oligonucleotides complementary to the wild type and mutated sequence of each mutation were covalently attached to carboxylated membrane (as in section 2.4.3.5). PCR product exactly complementary to the target, and labelled target itself were spotted down and physically absorbed to the membrane as positive controls for signal detection and hybridisation.

2.4.4.1 Method of pre-hybridisation, hybridisation and DIG detection on nylon membrane

The pre-hybridisation, hybridisation and detection of DIG labels were carried out as described in section 2.4.3, with the exception that labelled target (6 μ l) was added after pre-hybridisation.

2.4.5 Results and discussion

This work confirms previous experiments that showed covalent attachment of oligonucleotides to Biodyne C membrane to be possible (Chehab and Wall, 1992).

Figure 8 shows that oligonucleotide has been successfully bound to Biodyne C membrane and 20pmol can be easily detected using the DIG detection system from

Roche (UK). Covalent attachment should couple oligonucleotides to membrane in a more specific orientation than either baking or crosslinking as the amino termination on the oligonucleotide is the site of attachment. This specific attachment is expected to increase the availability of the oligonucleotide for hybridisation, thus increasing the hybridisation signal.

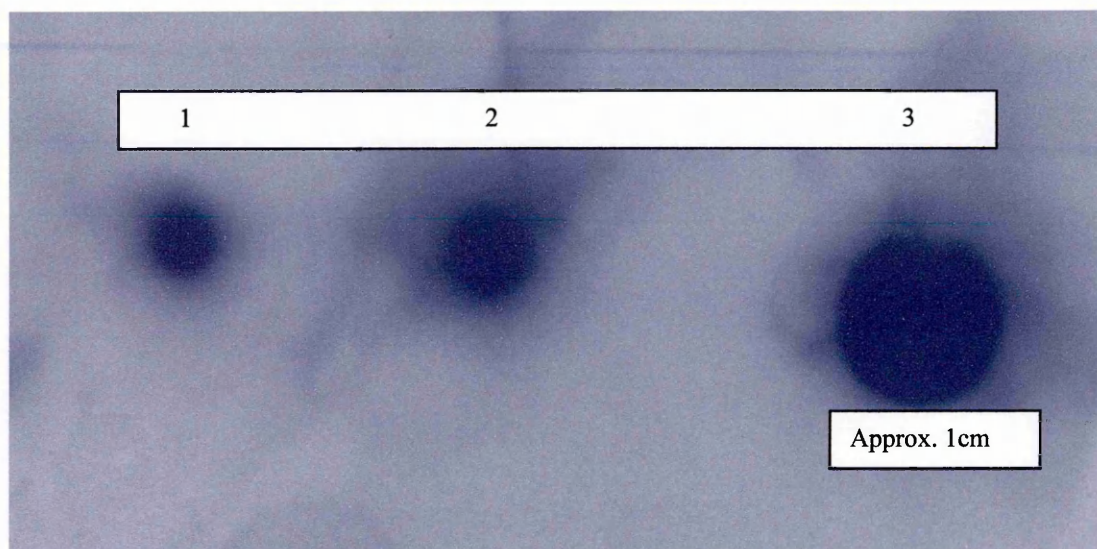


Figure 8. DIG labelled oligonucleotides were covalently coupled to Biodyne C membrane and detected using chemiluminescence.

DIG labelled oligonucleotides were covalently coupled to Biodyne C membrane and detected using a PCR DIG detection kit (Roche, UK). 1-1 μ l DIG test. 2-2 μ l DIG test. 3-10 μ l DIG test (see Appendix C for oligonucleotide sequence).

2.4.5.1 Radioactive labelling and detection of oligonucleotides

The amount of oligonucleotide deposited under each of the four experimental conditions was plotted against the amount detected on the membrane at the end of the experiment (shown in Figure 9, for raw data see appendix E). The amount of oligonucleotide remaining on the membrane after washes have been carried out is indicative of the amount of oligonucleotide undergoing covalent attachment.

When UV-crosslinking and covalent attachment to the membrane were compared, the amounts of oligonucleotide attached were noticeably different. If a comparison is made between unwashed controls, on first sight UV attachment appears to be the better method (Figure 9a and b). It should be noted, however, that in the case of covalent attachment, even unwashed membrane undergoes a 15 minute wash in NaOH solution after the addition of the oligonucleotides, making comparison between the two results impossible as oligonucleotides attached to membranes by UV-crosslinking undergo no such treatment.

Because the unwashed UV treated membrane undergoes no washes to remove the DNA, it would be expected that all of the DNA deposited would remain on the membrane, however this was not found to be the case. No more than 77% of UV-attached oligonucleotide remained on the membrane for detection. UV light is known to damage DNA, however, as the membrane did not undergo washing after UV-attachment, damaged DNA or single bases would be expected to remain on the membrane, and therefore be detected. It is not clear why this is the case.

The situation was very different with washed samples. Covalent attachment allowed an average of 4% of oligonucleotide to remain on the membrane after washes compared to an average of 0.4% after UV-attachment and washes. This shows that an increase of

approximately 10x the amount of oligonucleotide was bound to the membrane through covalent attachment.

The amount of oligonucleotide attached by both UV-crosslinking and covalent attachment was smaller than expected. In a previous experiment Joos *et al.* (1997) showed that 40-50% of oligonucleotide deposited by them was bound to glass via disulphide bonds. The difference between results may represent the different surfaces and attachment methods used.

These results were obtained from a single experiment and as such would need to be verified. It was not possible to repeat the experiment within this project as the radioactive experiments were carried out in a laboratory at Innogenetics B.V. (Gent, Belgium) in a three week period. Repetition of the experiment could confirm the amount of oligonucleotide which can be attached to nylon membrane by these methods. If the amount of oligonucleotide detected in this experiment is representative of that attached to membranes in a standard coupling procedure, then the efficiency of all methods of attachment should be studied, with a view to improving attachment efficiency in order to reduce the amount of oligonucleotide necessary to achieve an adequate signal strength.

An increase in the amount of oligonucleotide attached to the membrane by UV-irradiation was seen when a poly(T) tail of 0 or 90 bases was present at the 3' terminus of the oligonucleotide probe sequence. The amount of oligonucleotide attached did not increase proportionally to the length of the poly(T) tail as observed by Saiki *et al.* (1989). A longer poly(T) tail would be expected to allow more stable attachment for each oligonucleotide, with a resulting increase in total oligonucleotide attachment because more bases are available for the attachment. This was not seen in this work.

Covalently coupled oligonucleotides showed the greatest amount of attachment when poly (T) tails of 40 and 60 bases were present between the 3' end of the oligonucleotide probe sequence and the amino termination. This implies that there is an optimum tail length and that a tail that is too long may interfere with hybridisation. It is possible that this increase in attachment of oligonucleotides with medium length tails may be due to differences in the synthesis of the oligonucleotide or due to degradation of the amino groups on some of the oligonucleotides, or possibly due to the formation of secondary structures by the oligonucleotides. Again, this would require investigation by repetition of the experiment with new, freshly made oligonucleotides.

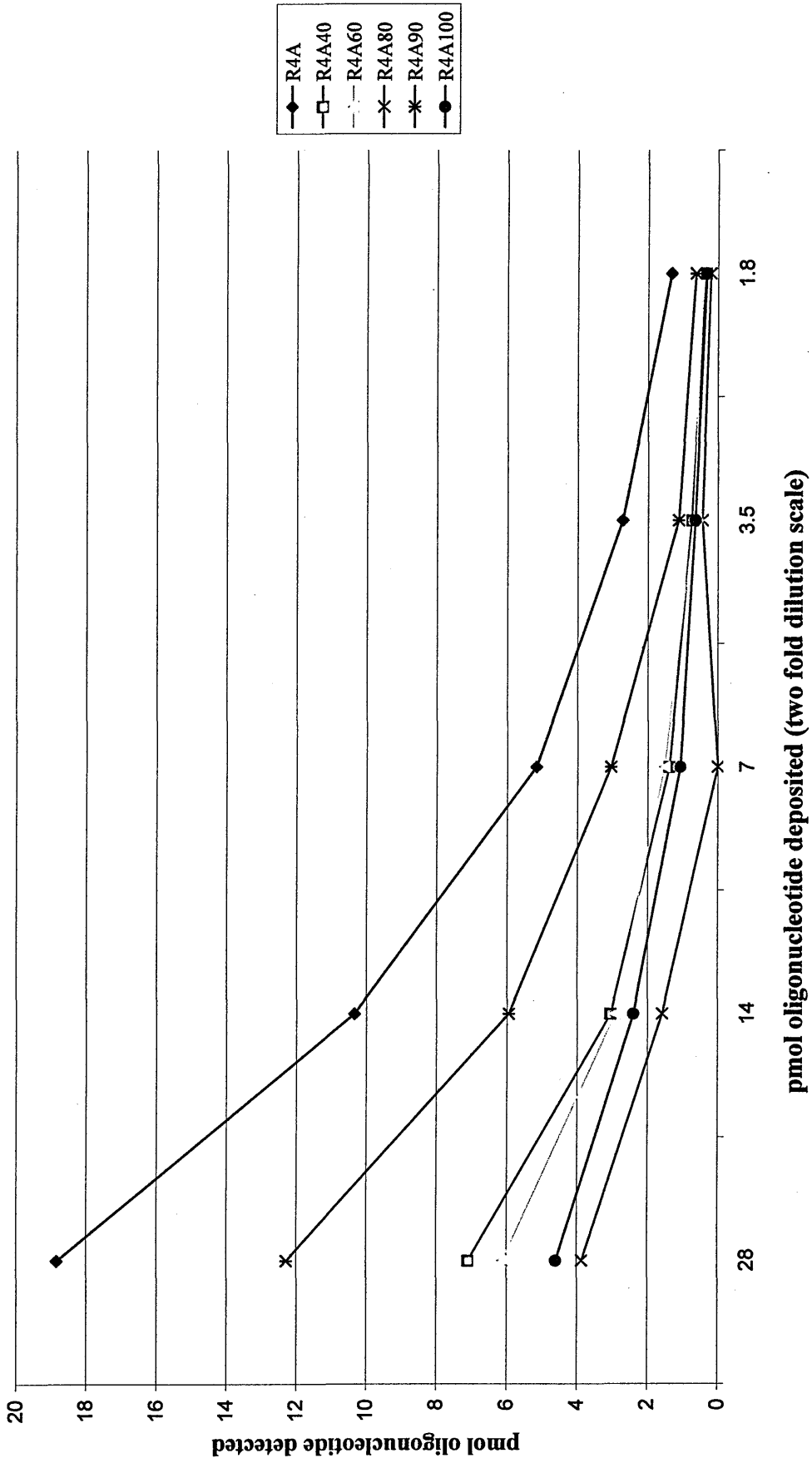


Figure 9a. Graph showing the amount of physically attached oligonucleotide detected on nylon membrane after no washes

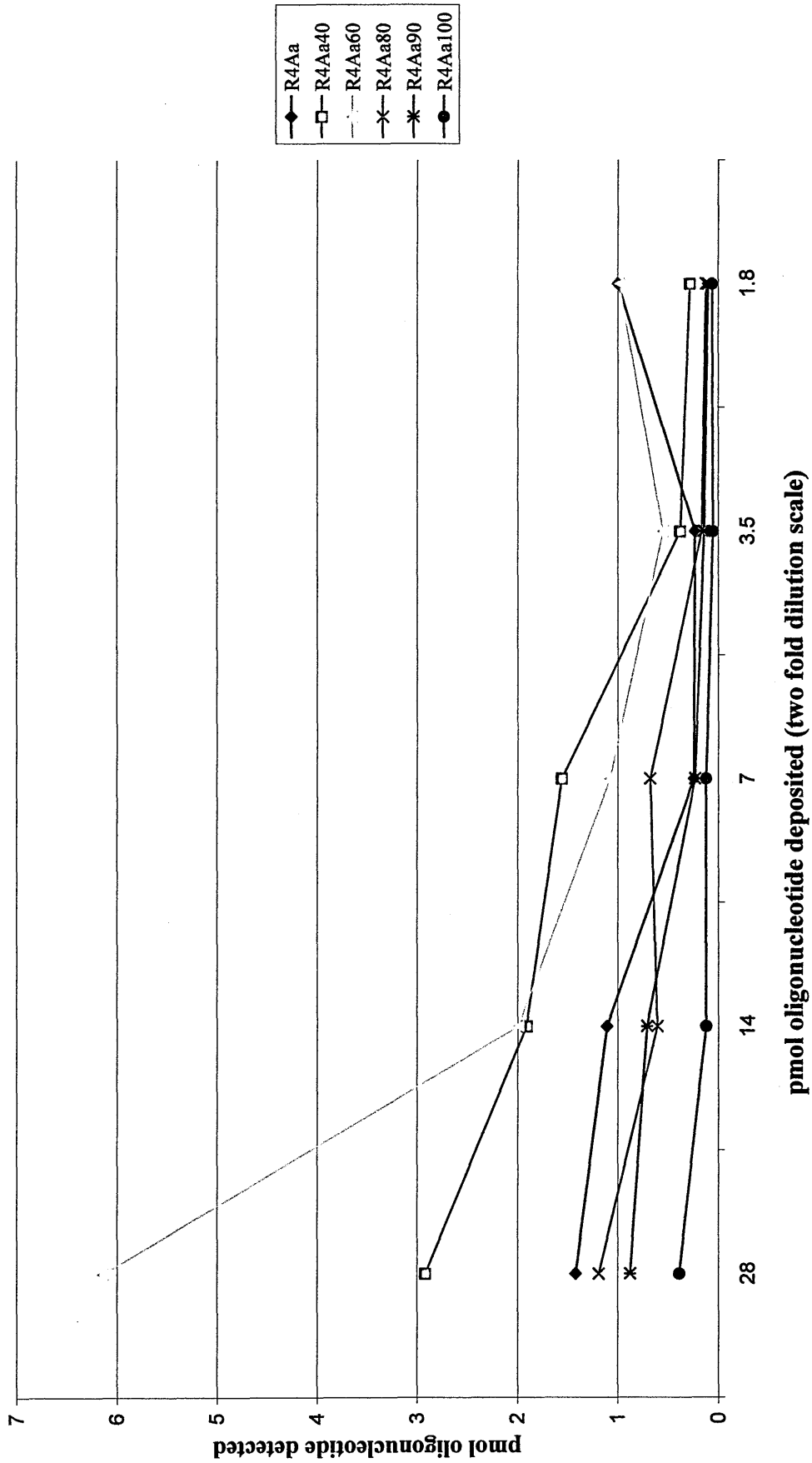


Figure 9b. Graph showing the amount of covalently coupled oligonucleotide detected on Biodyne C membrane after no washes

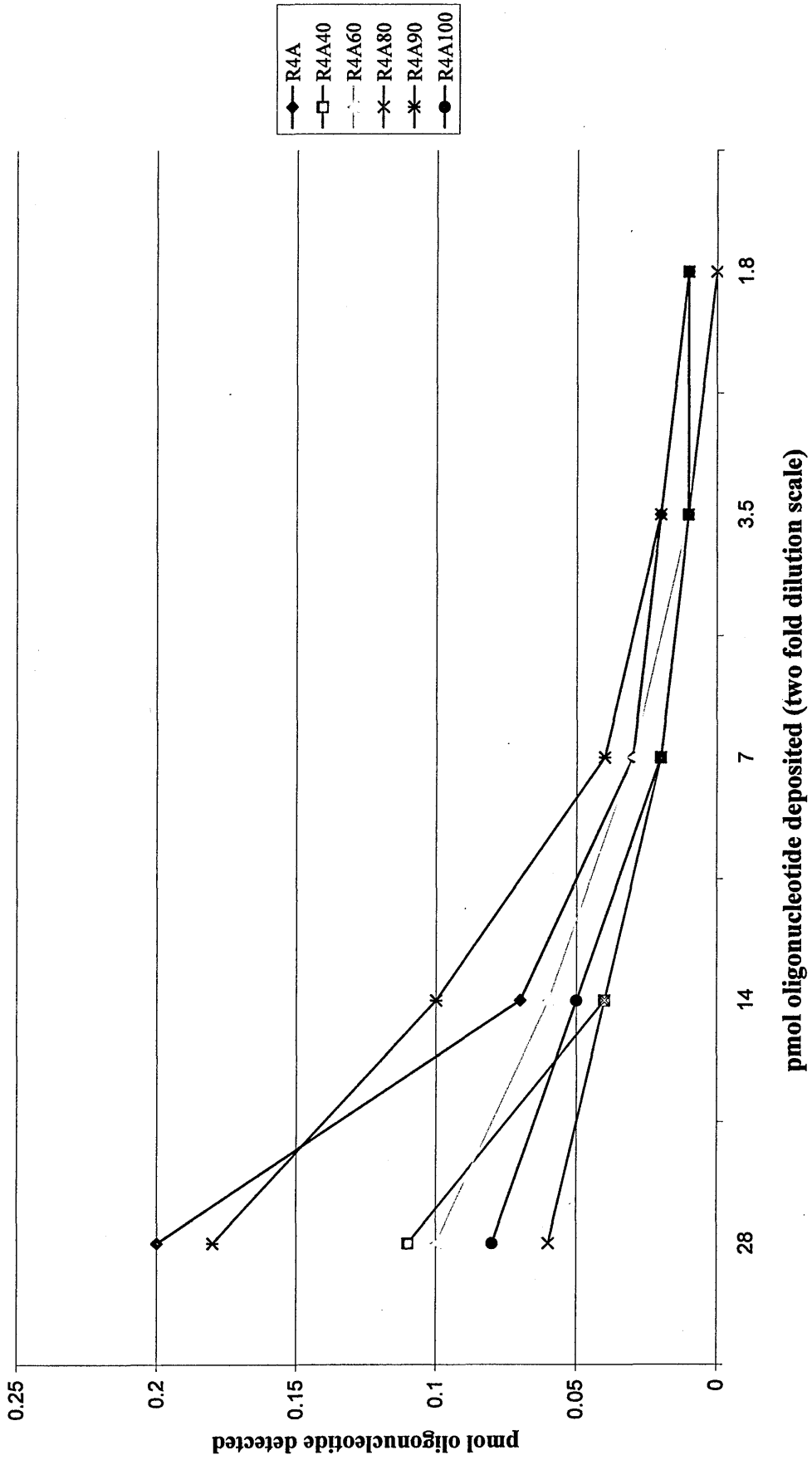


Figure 9c. Graph showing the amount of physically attached oligonucleotide remaining on the membrane after hybridisation and two five minute stringency washes

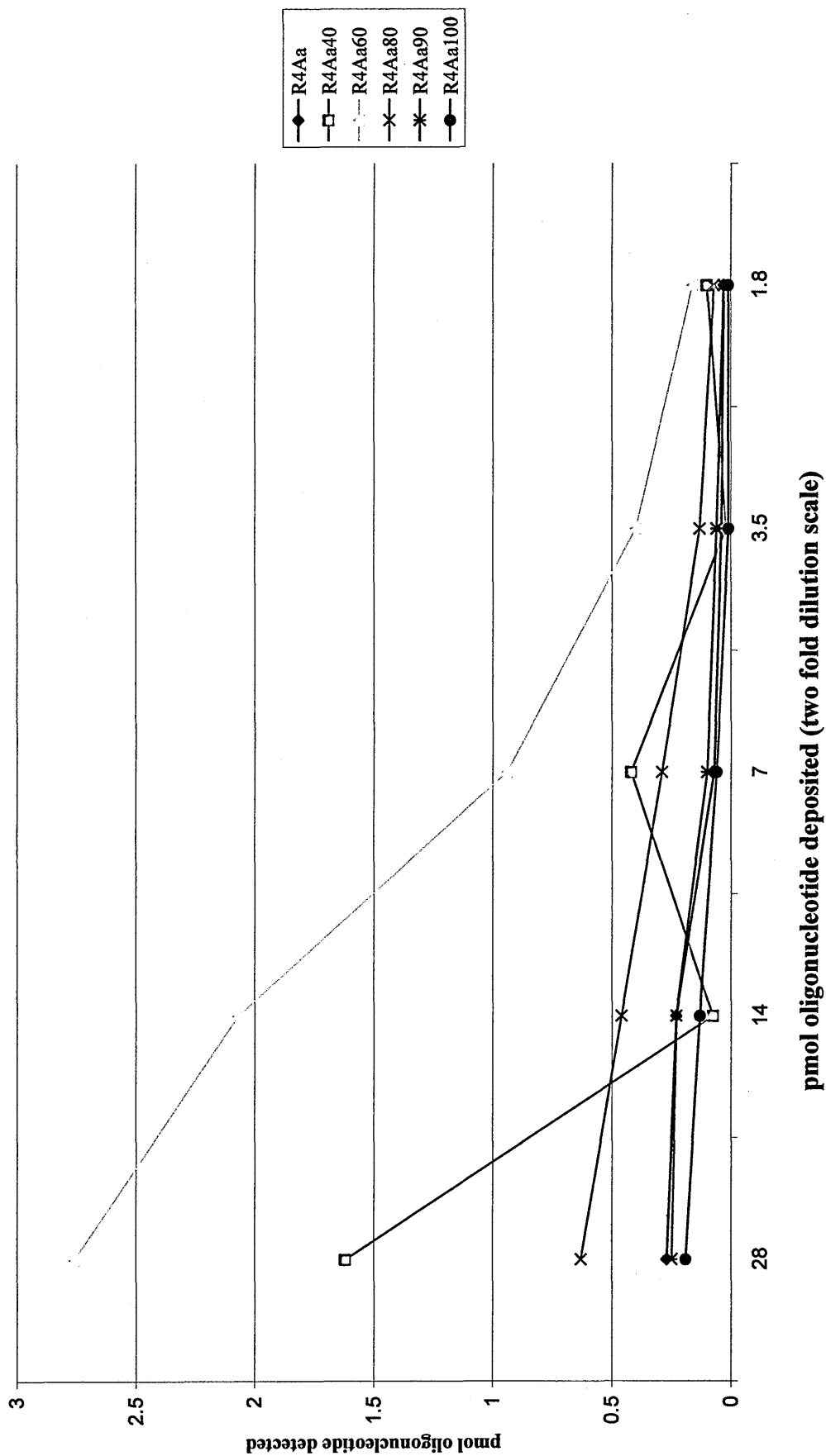


Figure 9d. Graph showing the amount of covalently coupled oligonucleotide remaining on the membrane after hybridisation and two five minute stringency washes

Figure 9 a-d. These graphs show the amount of radioactively labelled oligonucleotide remaining attached to Hybond N+ membrane (UV_crosslinking) and Biotin C (covalent attachment) both before and after washes.

Oligonucleotides were attached by the chosen method and the radioactivity on the membrane was measured without washes (9a and 9b) and after washes (9c and d).

The amount of oligonucleotide left after washes is indicative of the amount of oligonucleotide undergoing attachment.

For raw data see appendix E.

2.4.5.2 Hybridisation of DIG labelled PCR product to oligonucleotides covalently coupled to carboxylated nylon membrane

DIG labelled PCR product was hybridised to oligonucleotide covalently bound to Biotyne C membrane (see figure 10). Stringency conditions were explored to discover those that allowed hybridisation only between complementary sequences, as seen in figure 10. Final washes of 0.1x SSC/0.1% SDS gave sufficiently stringent conditions for use in this hybridisation.

Hybridisation produced the expected signals in all cases, although the signals corresponding to the lower concentrations of the $\Delta F508$ wild type sequence produced weaker signals than the lower concentrations of each of the other mutation sequences. The mutation sequence oligonucleotide was spotted onto the membrane at a ten times higher concentration than the wild type sequences in order to ascertain whether a high concentration of a non-complementary oligonucleotide would hybridise to the target non-specifically, this was not seen to be the case.

The signals produced from hybridisations of the exon 10 PCR product to the $\Delta F508$ oligonucleotides in figure 10 were not very strong. The longer an oligonucleotide is stored and the more often it undergoes freeze/thaw cycles and the more likely it is that there will be heat damage or enzyme contamination which will degrade the oligonucleotide. As the $\Delta F508$ oligonucleotides have been stored for over a year it is possible that the oligonucleotide has been damaged in this way and is therefore there is not a sufficient quantity of intact oligonucleotide to hybridise to the target.

It is not possible to be certain of the amount of oligonucleotide that has been covalently bound to the membrane in this experiment. If the results of the radioactive study (section 2.4.5.2) are indicative of the average percentage of oligonucleotide washed off

after covalent coupling, then approximately 4% of the oligonucleotide deposited remains on the membrane. Therefore, approximately 0.4 pmol of 10 pmol deposited will remain bound to the membrane. The proportion of oligonucleotide that is available for hybridisation after covalent attachment is unknown, however Joos *et al.* (1997) found that 90% of oligonucleotide covalently attached to glass was available for hybridisation.

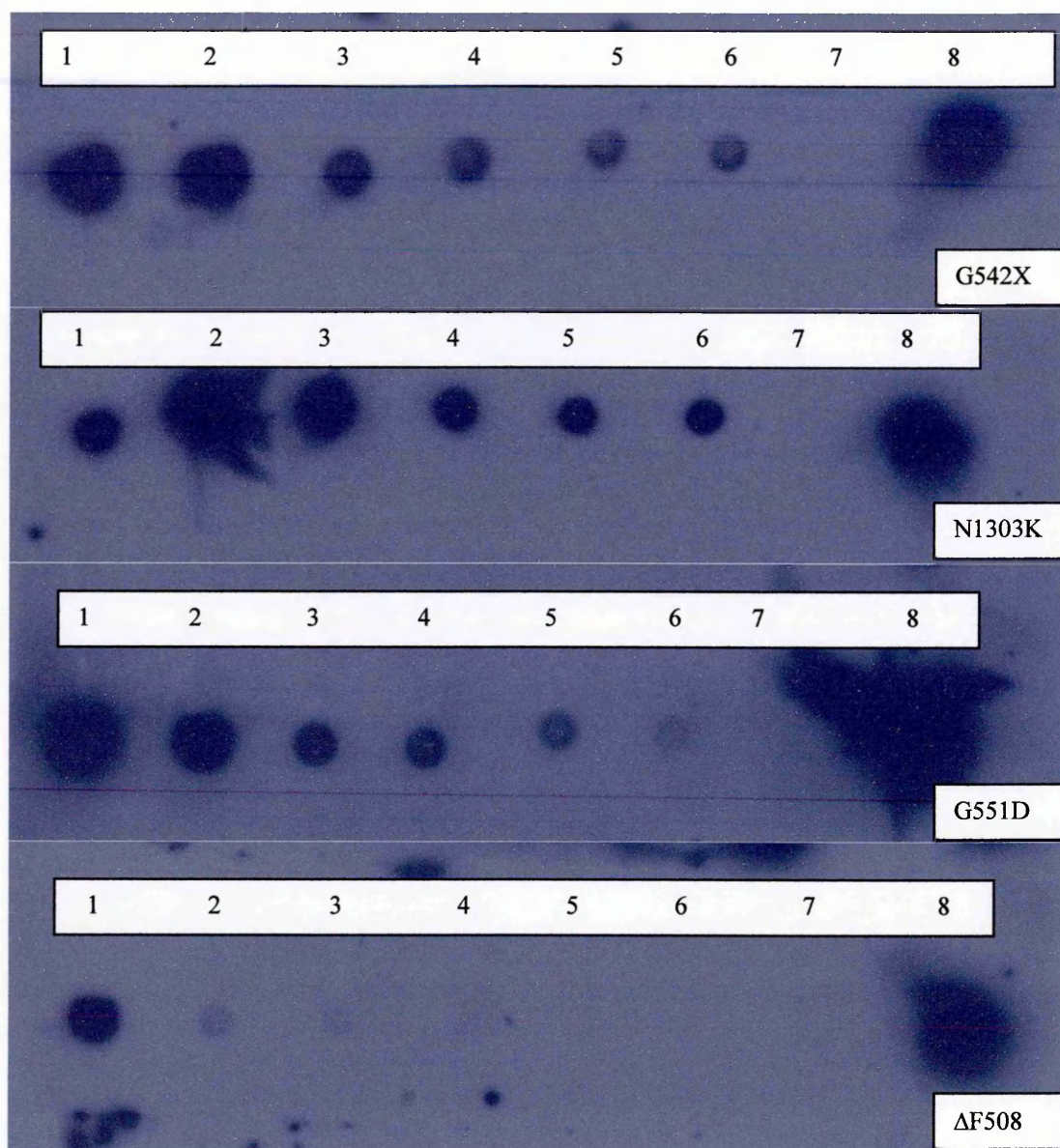


Figure 10. Oligonucleotides representing the mutant and wild type sequences of four cystic fibrosis mutations ($\Delta F508$, G542X, G551D and N1303K) were covalently coupled to Biodyne C membrane and hybridised with the appropriate PCR product amplified from control blood.

Hybridisation was carried out to mutant and wild type oligonucleotide sequences of four mutations found in the CFTR gene. Mutations G542X and G551D are found in exon 11, N1303K in exon 21 and $\Delta F508$ in exon 10. For each mutation, 1-PCR product. 2-100pmol wt. 3-20pmol wt. 4-10pmol wt. 5-5pmol wt. 6-1pmol wt. 7-20pmol mut. 8- DIG labelled PCR (see appendix C for oligonucleotide sequence).

(Figure 10. legend continued)

The control blood would be expected to contain wild type sequences for each mutation, and so hybridisation signals should be observed for spots 1-6 and 8. This was the case for the G542X, N1303K and G551D mutations, however, hybridisation only occurred with 100pmol of the wild type $\Delta F508$ oligonucleotide.

2.4.6 *Summary*

This section has shown that amino terminated oligonucleotides can be successfully coupled to carboxylated nylon membranes. It has also been found that covalent attachment of oligonucleotide to carboxylated nylon membrane will couple approximately 10x more oligonucleotide than attachment by UV-crosslinking. The final experiment in this section was to confirm that hybridisation could be performed with oligonucleotides covalently coupled to carboxylated nylon membrane. Hybridisation to oligonucleotides covalently coupled to carboxylated membrane was carried out successfully, using human cystic fibrosis sequences.

2.5 Covalent Immobilisation to Activated Polystyrene

2.5.1 Introduction

The work reported in this thesis attempts to combine the ease of handling and small surface area of a microarray with the efficiency of testing for multiple mutations of the reverse dot blot systems. In the case of the reverse dot blot, the smaller oligonucleotide is attached to the surface. The longer a piece of DNA is, the more chance there is that bases will be available for hybridisation after being used in surface attachment. A short oligonucleotide is unlikely to have many bases available for hybridisation after attachment to the surface, which may explain the faint signals observed after the physical attachment of oligonucleotide to membrane in section 2.3. Covalent attachment of amino terminated oligonucleotides to a surface allows the maximum number of bases in each oligonucleotide to be available for hybridisation.

Polystyrene slides can be produced by injection moulding and therefore, are compatible with high throughput manufacture of a device. Polystyrene slides can also be chemically activated in order to produce carboxyl groups on the surface (Zammatteo *et al.* 1996) producing a small, rigid surface to which amino terminated oligonucleotides can be covalently attached.

This section investigates the possibility of covalent attachment of amino terminated oligonucleotides to carboxyl groups on polystyrene slides using the principles explored in section 2.4.

Polystyrene slides (Nunc, Paisley, UK) were modified using a method based on that described by Zammatteo *et al.* (1996) to modify polystyrene microtitre plates.

Some modification to the DIG and biotin detection protocols were required in order to allow detection of these groups accurately when transferring the technology from flexible, absorbent membrane to the rigid, non-absorbent slides.

2.5.2 Comparison of the surface chemistry of modified and unmodified polystyrene slides

Contact angle measurements can be used to compare the hydrophobic characteristics of surfaces (Weston, 1999). The chemical modification of polystyrene slides is expected to alter the hydrophobicity of the surface, therefore a change in hydrophobic characteristics of the surface after modification will imply an alteration of the surface chemistry.

An atomic force microscope (AFM) will allow the observation of the polystyrene surfaces in detail. The AFM generates an image by using the force between the probe and the sample surface (Hansma *et al.*, 1988). The AFM has a tip, which is mounted on a cantilever spring that will move as the tip runs over the surface being examined. The most common method of detecting the movement of the cantilever is a laser beam which is directed onto the end on the cantilever and reflected to a photodetector. The AFM includes a compensation network or feedback loop that keeps the cantilever deflection constant by monitoring the cantilever deflection and adjusting the cantilever height via a piezoelectric positioner in order to allow scanning at very low forces. Data collected is presented typically as a topographic image. Differences between the modified and unmodified polystyrene surfaces should be observable using AFM.

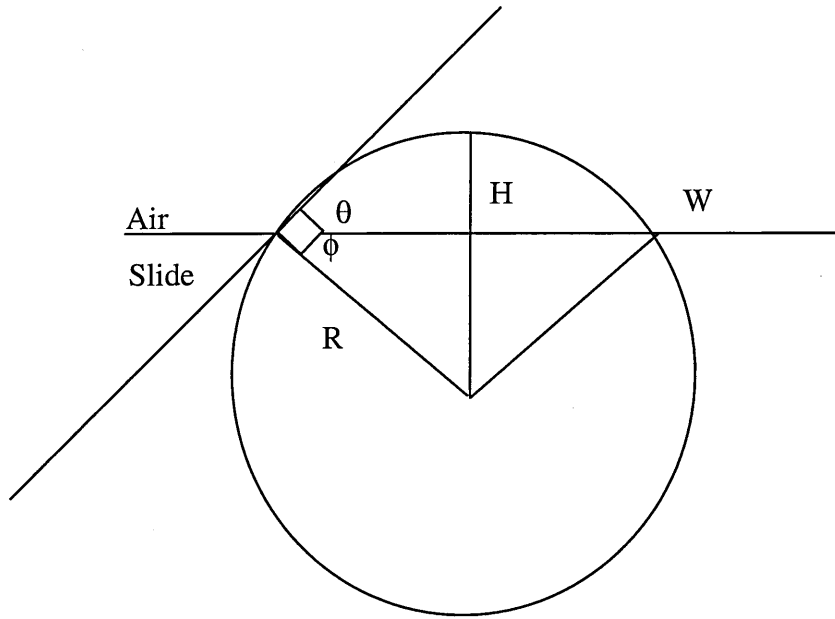
2.5.2.1 Method of modification of polystyrene slides to produce carboxyl groups

Polystyrene slides (Nunc, Paisley, UK) were modified by incubation in 5% potassium permanganate in 1.2 M H₂SO₄ in Coplin jars for 2 hours at room temperature (based on

Zammatteo *et al.* (1996)). The slides were rinsed in distilled water, washed in 6 M HCl for 5 minutes and water for 5 minutes, the latter two washes were repeated three times each. The slides were then dried by centrifugation at 25 x g for 6 minutes.

2.5.2.2 Method of determining contact angle

Contact angle measurements were taken by using the sessile drop method (Weston, 1999). A known volume of water was placed onto the slide, and the side view was captured with a CCD camera. The image obtained from the CCD camera was saved as a TIF file and analysed via Paint Shop Pro (version 3.11, JASC Software, Eden Prairie, MN 55344 USA). The height and width of the drop were measured by counting the number of pixels present and the contact angle was calculated from these as shown below. Between six and nine drops were measured for each slide so that an average contact angle measurement could be used.



By using the equation

$$\phi = \tan^{-1} \left(\frac{\frac{W^2}{4H} - H}{W} \right)$$

The contact angle, θ , can be calculated by

$$\theta = 90 - \phi$$

Figure 11. A representation of a droplet as a sector of a circle (Weston, 1999).

H is the height and W is the width of the droplet. R is the radius of the hypothetical circle created by extension of the droplet.

2.5.2.3 Method of taking AFM images of polystyrene slides

Atomic Force Microscope (AFM) images were by taken of both modified and unmodified slides (H. Johnson, Cranfield University). A Quescent Instruments Corp. (California, USA) Resolver™ scanning probe microscope was used to investigate the surface of both modified and unmodified polystyrene slides. Samples were prepared for atomic force microscopy by mounting on a stage with double-sided tape in order to prevent the movement of the sample during scanning. Images were achieved with a scan rate of 2Hz at a resolution of 500 scan lines per image with scan dimensions of 5 µm.

2.5.3 Covalent binding of oligonucleotides to modified polystyrene slides

2.5.3.1 Method of the activation of carboxyl groups on polystyrene slides

Polystyrene slides were modified as described in section 2.5.2.1. Activation of the carboxyl groups on the slides was carried out as described for nylon membrane in section 2.4.2.1. then slides were dried by centrifugation at 25 x g for 6 minutes.

2.5.3.2 Covalent attachment of amino terminated oligonucleotides to carboxyl groups on polystyrene slides

One microlitre of each oligonucleotide was spotted onto the slide. Two oligonucleotides with 5' amino termination, one with a DIG label at the 3' end and the other with a biotin label at the 3' end were covalently attached to different modified polystyrene slides via the amino group. A template showing where to place the spots was drawn on paper and placed under the slides before the oligonucleotides were spotted down. The spots were dried onto the slides for 20-30 minutes at 37°C and the slides were incubated in 0.1 M NaOH for 15 minutes.

2.5.3.3 Mock hybridisation and detection of DIG and biotin labels

Pre-hybridisation was carried out by pipetting 100µl of Sigma PerfectHyb Plus hybridisation buffer (Sigma-Aldrich, Dorset UK) onto the slide and covering with a Sigma Hybri-slip coverslip (Sigma-Aldrich, Dorset, UK). The slides were placed in a hybridisation chamber (Genetix, UK) and incubated at 42°C overnight. Washes were carried out in 0.1%SDS/0.1x SSC solution and the slides were incubated in DIG block buffer (Roche, UK) for 30 minutes. Streptavidin –HRP conjugate was diluted 1/500 in DIG block buffer and 0.5ml of antibody mixture was put onto the slides which were incubated in a humid chamber for 30 minutes. The slides were washed for 3x 5 minutes in biotin wash buffer (Southern detection kit, KPL, Maryland, USA).

Detection of biotin was carried out by mixing solutions A and B (Southern detection kit, KPL) together in equal parts and adding 0.5ml to each slide. A glass coverslip was placed on the slide and it was drained onto filter paper by placing the slide on its edge, lengthways along the filter paper.

In the case of the DIG detection protocol, after the addition of CSPD to the slides, a coverslip was placed on top. The slides were not blotted on filter paper, but the underside of the slide was dried.

After the slides were exposed to the appropriate detection reagents, they were then sealed in a hybridisation bag and exposed to Kodak X-OMAT film (Sigma-Aldrich, Dorset, UK) for 2 hours.

2.5.4 Hybridisation to covalently coupled oligonucleotides on polystyrene slides

Human CFTR exon 10, 11 and 21 PCR products were labelled with biotin as described in section 2.2.3.2. Polystyrene slides were modified as described in section 2.5.3.1.

Activation of the carboxyl groups on the slides was based on the method described by

Chehab and Wall, (1992) for the activation of Biodyne C membrane (Pall Life Sciences, Hants, UK), and was performed as described in section 2.5.3.1, oligonucleotides were covalently coupled as described in section 2.5.3.2.

2.5.4.1 Hybridisation of PCR product to covalently coupled oligonucleotides on polystyrene slides

Pre-hybridisation was carried out as described in section 2.5.3.3 Biotin labelled target (12µl of each exon) was mixed with 6µl of hybridisation buffer, the mixture was denatured at 100°C for 5 minutes, pulse spun and pipetted down the centre of the slide. The coverslip was replaced onto the slide and the slide was placed back into the hybridisation chamber for hybridisation at 42°C overnight.

Detection of biotin labels after hybridisation was carried out as described in section 2.5.3.3.

2.5.5 Results and discussion

2.5.5.1 Contact angle measurements on polystyrene slides

Static contact angle measurements were taken which show a small change in hydrophobicity of the surface of the slides. The larger the angle, the greater the hydrophobicity. The modified slide was less hydrophobic on average, than the unmodified slide, although the contact angles showed inconsistency between different areas on each slide and between different slides for both modified and unmodified slides (see appendix F). The mean contact angles for four slides, two modified and two unmodified, are shown in Table 3. There is a small difference between the mean contact angles of modified slides and unmodified slides, although the inconsistency of the contact angles across the surface of the slide implies that the modification is not occurring consistently in these experiments. This could be due to settling of the

potassium permanganate in sulphuric acid during modification, they were mixed briefly before being added to the slides in the Coplin jars. Over the two hour modification, potassium permanganate may settle onto the slides or to the bottom of the Coplin jar if inadequately mixed. There is no one area of the slides that consistently appears more or less modified than any other from the contact angle measurements, which may be expected the case if the potassium permanganate had settled out of solution. The inconsistency of modification may be due to differences in the slides themselves.



Scale bar approximately 2mm

Figure 12. An example of a CCD image of a water drop on a modified polystyrene slide from which contact angle measurements were taken.

Table 3. The mean contact angle measurements taken on modified and unmodified slides (for raw data, see appendix G).

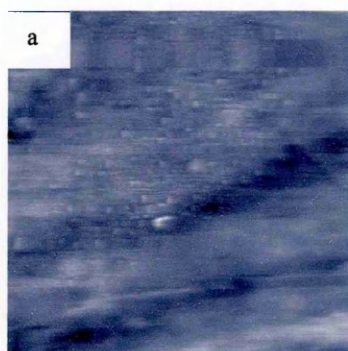
Slide number	Mean contact angle (taken from 7 or 9 measurements)
Unmodified 1	68° ±1°
Unmodified 2	66° ±1°
Modified 1	64° ±1°
Modified 2	57° ±1°

2.5.5.2 AFM analysis of polystyrene slides

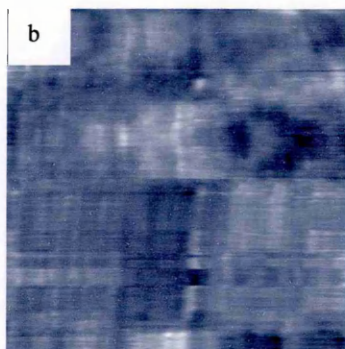
The AFM images show a difference between the modified and unmodified slides (Figure 13). It is possible to see streaking on the modified slides, which is indicative of a mobile layer on the top of the slides, produced when the surface is oxidised, this softer, mobile layer is thought to be the oxidised material (Gillespie, 2001), and allows the differentiation of modified and unmodified slides. It is important to check that the modification has had an effect on the surface of the slides in this way because it does not produce changes that are visible by eye.

All images were taken using the following parameters:

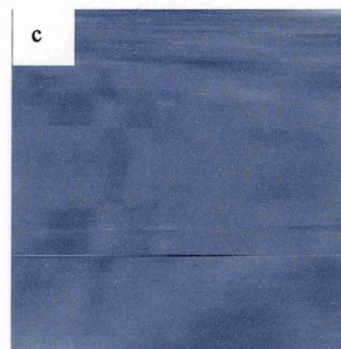
Scan rate	2Hz	Resolution	500
Integral gain	200	Proportional gain	200
Derivative gain	100	Image	Z height
Scan angle	0		



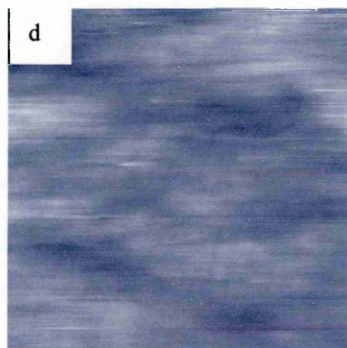
**Unmodified
polystyrene slide**
*Beam align 0.4
Set point offset -0.4
5 μ m x 5 μ m x 31.82nm*



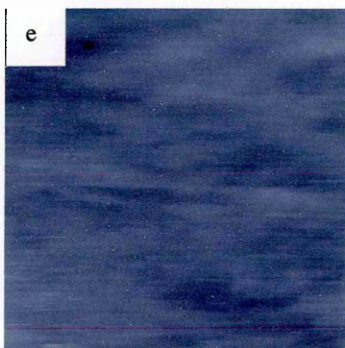
**Unmodified
polystyrene slide**
*Beam align 0.4
Set point offset -0.4
5 μ m x 5 μ m x 59.93nm*



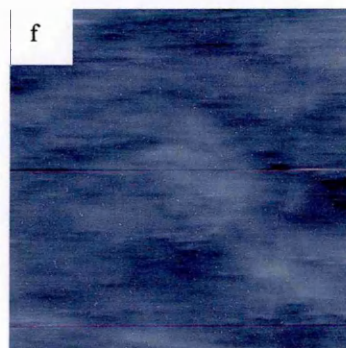
**Unmodified
polystyrene slide**
*Beam align 0.4
Set point offset -0.4
5 μ m x 5 μ m x 52.42nm*



**Modified polystyrene
slide**
*Beam align 0
Set point offset 0
5 μ m x 5 μ m x 51.06nm*



**Modified polystyrene
slide**
*Beam align 0.2
Set point offset -0.2
5 μ m x 5 μ m x 19.27nm*



**Modified polystyrene
slide**
*Beam align 0.2
Set point offset -0.2
5 μ m x 5 μ m x 115nm*

Figure 13. AFM images of unmodified and modified polystyrene slides

Polystyrene slides were chemically modified by incubation in a solution of 5% potassium permanganate in 1.2M Sulphuric acid in order to produce carboxyl groups on the surface. Unmodified and modified polystyrene slides were scanned using Quescent Instruments Corp. (California, USA) Resolver™ scanning probe microscope (H. Johnson).

2.5.5.3 Covalent coupling of oligonucleotides to modified polystyrene slides

The surface of the polystyrene slides has been altered by the modification as seen from both the AFM and contact angle results. The practical test of modification, however, is the covalent coupling of amino terminated oligonucleotides.

Short, labelled oligonucleotides were used to test the transfer of attachment and detection technology from nylon membrane to polystyrene slides. Both DIG labelled and biotin labelled oligonucleotides bound to polystyrene slides (see figures 14 and 15). Nylon membrane, is porous and will adsorb liquids, polystyrene slides are not and therefore will not adsorb solutions. The detection methods used in this work were designed for use with a porous membrane, therefore the transfer of the technology to non-porous polystyrene required some modification of the protocol used. In preliminary experiments coverslips were used only during pre-hybridisation and hybridisation incubations. These experiments produced no observable signal (not shown).

The introduction of the use of a coverslip on a polystyrene slide after the addition of the enzyme substrate during detection produced very weak signals using biotin-streptavidin compared to those seen on nylon (see figure 14). Further experiments using biotin detection also used a humid chamber for incubation of the slide in the streptavidin-HRP conjugate solution, which improved the signals and protected the solution from evaporation. In conclusion, coverslips were required in order to hold the enzyme substrate in place on the slide to allow its detection and a humid chamber was used to decrease the chance of evaporation of other solutions.

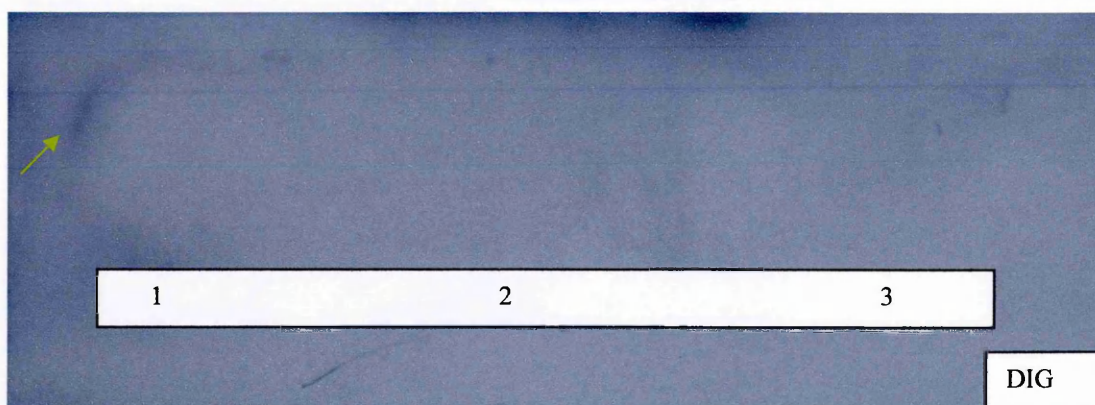


Figure 14. DIG labelled oligonucleotide covalently attached to a modified polystyrene slide.

All spots are DIG test oligonucleotide (see appendix C for sequence). 1-100 μ l (2 μ mol). 2-1 μ l (20 pmol). 3-0.5 μ l (10 pmol). The arrow indicates the edge of a spot.

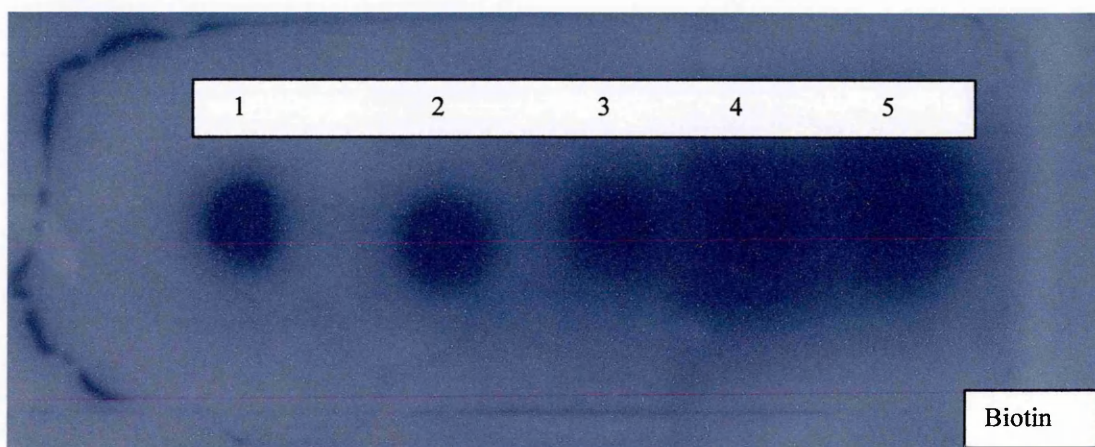


Figure 15. Biotin labelled oligonucleotides covalently attached to a modified polystyrene slide and detected using Southern detection kit (KPL, MD, USA).

All spots contain 10pmol of biotin test oligonucleotide, (see appendix C for sequence).

Once it had been established that amino terminated oligonucleotides could be bound to the polystyrene slides, hybridisation with biotin labelled PCR product was attempted (see figure 16). Although both DIG and biotin detection systems were shown to work on polystyrene slides, further experiments were performed using biotin as this produced a stronger more consistent signal. Hybridisation was carried out on polystyrene slides with the covalently attached oligonucleotides corresponding to the mutant and wild type sequence of all four cystic fibrosis mutations studied ($\Delta F508$, G542X, G551D and N1303K). In general the target hybridised as expected to the oligonucleotides and gave visible signals. The G542X wild type oligonucleotide hybridised to the target when present at concentrations of 100, 20 and 10 pmol, the strongest signal was observed for hybridisation to the 20 pmol spot, the signal from the 10pmol spot was weak. The N1303K wild type oligonucleotide produced similar strong signals at concentrations of 100, 20 and 10pmol, with a reduced strength signal unexpectedly seen for hybridisation to the mutant sequence, present at 100pmol. The $\Delta F508$ wild type oligonucleotide showed a weak hybridisation signal for hybridisation when present at 10pmol, and also a stronger signal from 100pmol of mutant sequence oligonucleotide. It is unclear why this was the case as the target was known to contain only the wild type $\Delta F508$ sequence as shown in other hybridisations (figure 10). The G551D wild type oligonucleotide produced signals when hybridised at concentrations of 100, 20 and 10pmol, with the strongest signal present at 10pmol. There was no hybridisation to the mutant G551D oligonucleotide.

It appears the excess of mutant sequence oligonucleotide used in these hybridisations has had an adverse effect on the specificity of the hybridisation in the case of the $\Delta F508$ and N1303K mutant sequences. A reduced amount of mutant sequence oligonucleotide

should eliminate these signals. It was expected that the hybridisation signals would be strongest where the highest concentration of oligonucleotide was deposited, however this was not seen to be the case. This may be due to steric interference (Forman *et al.*, 1998), or possibly a lack of available carboxyl groups on the polystyrene slides for the covalent attachment of the oligonucleotides. Signals from hybridisations on polystyrene slides are not as strong as those seen from hybridisations on membrane, despite an increased concentration of oligonucleotide being placed onto the polystyrene for attachment. This is because the nylon membrane has a 3-dimensional structure that allows more oligonucleotide to be absorbed in a given area than in the same area on a flat slide. It has been found that a greater concentration of oligonucleotide is required on polystyrene slides in order to produce signals comparable to those seen on nylon membranes. This requirement must be considered in the context of this work, as increased use of oligonucleotide would increase the overall cost of a diagnostic device, however it is essential that the signals produced are easily identifiable. Biotyne C membrane costs approximately £2.50 per hybridisation (assuming 10cm x 10cm per hybridisation). Polystyrene slides cost £1 each. Without considering the reduced amount of solutions used, a significant increase in oligonucleotide used on polystyrene could be tolerated without it proving more cost effective to use Biotyne C membrane.

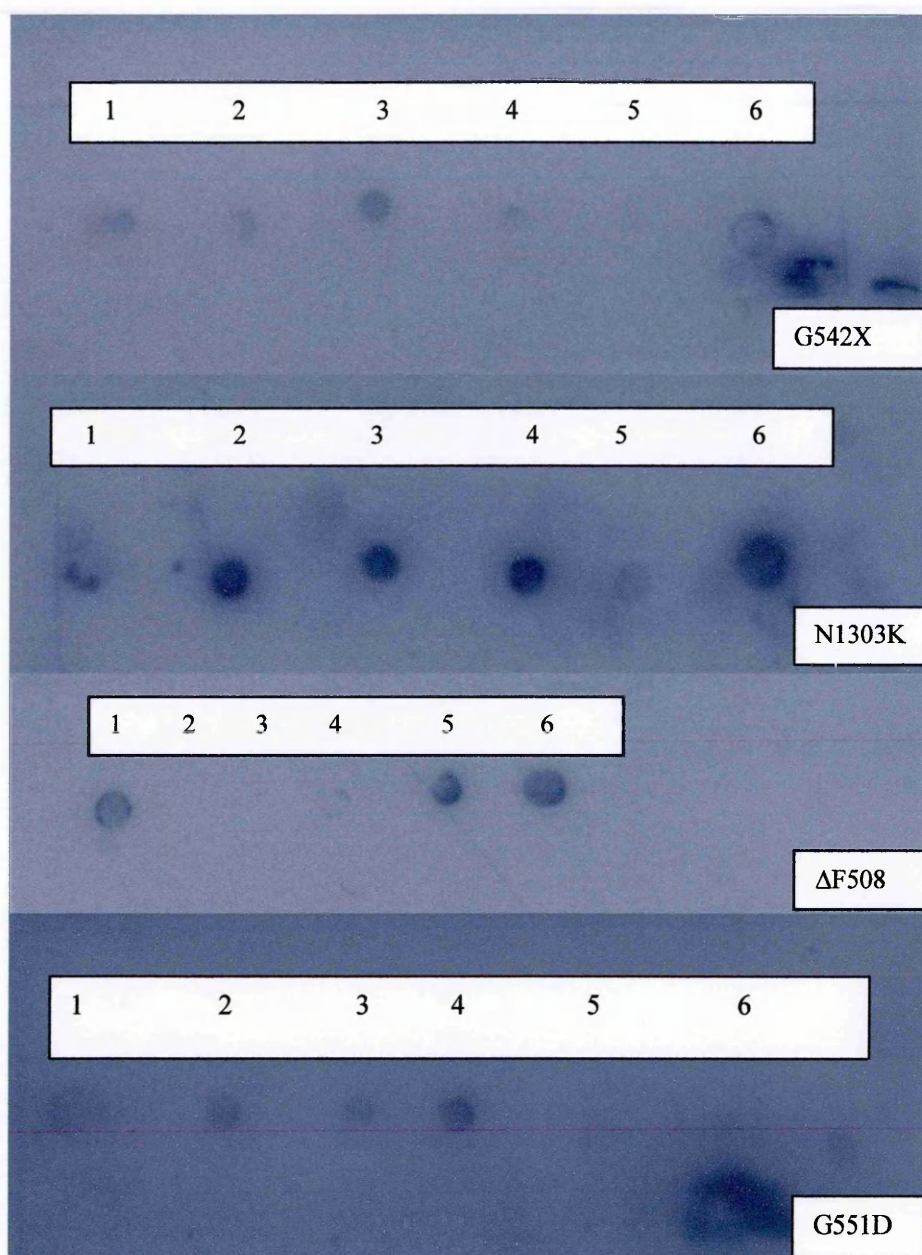


Figure 16. Oligonucleotides were covalently coupled to modified polystyrene slides and hybridised to the appropriate biotin labelled PCR product.

Mutation G542X and G551D are found in exon 11, Δ F508 is found in exon 10 and N1303K in exon 21. Each slide has oligonucleotides that are complementary to the wild type and mutant sequences of one of the cystic fibrosis mutations. 1-PCR product. 2-100pmol wt oligonucleotide. 3-20pmol wt oligonucleotide. 4-10pmol wt

(Figure 16. legend continued)

oligonucleotide. 5-100pmol mut oligonucleotide. 6- biotin labelled PCR product (see appendix C for oligonucleotide sequence).

2.5.6 *Summary*

This section has shown that the surface of modified polystyrene is slightly different to unmodified polystyrene. The AFM images showed streaking on the modified polystyrene, indicating a soft, mobile layer. The hydrophobicity of the modified slides, as determined using contact angle measurements was inconsistent over each slide and between slides. This implies that the modification of the slides is not occurring consistently, although there was a small difference in the mean contact angles from modified and unmodified slides. Oligonucleotides were covalently coupled to modified polystyrene slides and detected using chemiluminescent detection methods. Hybridisation was carried out to oligonucleotides covalently coupled to modified polystyrene, and the chemiluminescent signals produced were detectable, although an increase in the amount of oligonucleotide deposited was required when transferring the technology from nylon membranes to polystyrene slides.

2.6 The Effect of DNA Spacers on Hybridisation Efficiency

2.6.1 Introduction and materials

It has been suggested that the addition of a spacer between the oligonucleotide and the membrane would allow the oligonucleotide to behave more as if it was suspended in a solution than attached to a surface, increasing hybridisation due to availability of the oligonucleotide (Zhang *et al.*, 1991). Spacers may be DNA or non-DNA (for example polyethylene glycol (Joschke, 1997)). A non-DNA spacer is less likely to interfere with hybridisation, however a DNA spacer, such as a poly(T) tail, is more easily added 'in-house'. Several lengths of poly(T) tail have been studied, both with and without an amino termination. These experiments were carried out using oligonucleotides complementary to sequences from *Lactobacillus plantarum*, and DIG labelled PCR products as described in section 2.2.3.1.

2.6.2 Investigation of the effect on hybridisation of a poly(T) tailed probe attached by UV-crosslinking to a surface

A poly(T) tail was added to oligonucleotide R4A as described in section 2.3.4.1.

Hybridisation with DIG labelled target and subsequent detection were carried out as described previously (section 2.4.2).

2.6.3 Investigation of the effect of a poly(T) tail on covalently coupled oligonucleotide used in hybridisation

Oligonucleotides were synthesised with varying lengths of poly(T) tail and an amino termination (MWG-Biotech, Milton Keynes, UK). The oligonucleotides were covalently attached to Biodyne C membrane (Pall, UK), covalent attachment was carried out as suggested by Chehab and Wall, (1992) as described in section 2.4.2.1.

2.6.4 *Results and discussion*

A poly(T) tail at the end of an oligonucleotide is expected to allow it to move more freely and hybridise as if it were in solution. A longer poly(T) tail may be expected to increase the mobility of the oligonucleotide because the T bases are preferentially bound to the membrane by UV-crosslinking, however the poly(T) tail starts adjacent to the oligonucleotide regardless of how long it is. If the whole of the poly(T) tail and none of the hybridising sequence was bound to the membrane, the tail length would not provide a spacer effect and increase the mobility of the oligonucleotide. Addition of poly(T) tails to oligonucleotides, did not produce a significant increase in signal strength compared to oligonucleotides without (see figure 17), except when the oligonucleotide was covalently attached via an amino group at the end of the poly(T) tail, where the strength of signals was seen to increase (see figure 18). The actual tail lengths were not confirmed and this may be the cause of the weak signals. It is possible that the poly(T) tails added to the oligonucleotides 'in-house' were not as long as estimated and this may have had a significant impact on the number of bases attached by UV-crosslinking to the membrane, and subsequently, the strength of the attachment. This seems likely considering that the more strongly attached, covalently coupled oligonucleotides produced an increased signal. The poly(T) tail of covalently attached oligonucleotides is more likely to be the length expected as it was added at synthesis, although this was not confirmed. Long oligonucleotide may not always be synthesised accurately (MWG-Biotech, Milton Keynes, UK. Personal Communication)

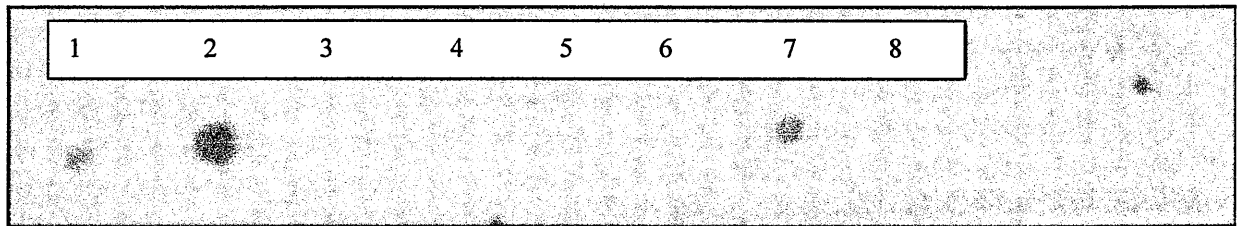


Figure 17. Poly(T) tailed oligonucleotide was attached to nylon membrane by UV-crosslinking and hybridised to DIG labelled *Lactobacillus plantarum* PCR.

Detection was via chemiluminescence (PCR DIG detection kit, Roche, UK). See

Appendix C for oligonucleotide sequences. 1-LB gDNA. 2-LB PCR (57 pmol). 3-

R4A30 (28 pmol). 4-R4A60 (36 pmol). 5-R4A90 (30 pmol). 6-R4A120 (30 pmol). 7-

3A60 (30 pmol). 8-DIG labelled LB PCR.

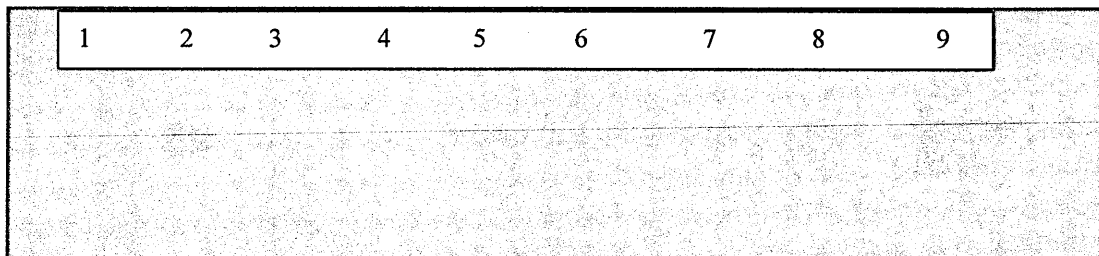


Figure 18. Poly(T) tailed oligonucleotides were attached to Biodyne C membrane via covalent coupling and hybridised to DIG labelled *Lactobacillus plantarum* PCR product.

Detection was via chemiluminescence (DIG PCR detection kit, Roche, UK). See

Appendix C for oligonucleotide sequences. 1-gDNA, 2-LB PCR (57pmol). 3-R4Aa

(57pmol). 4-R4Aa60 (42pmol). 5-R4Aa90 (40pmol). 6-2Ata60 (32pmol). 7-2Ata90

(32pmol). 8-3Ata (28pmol). 9-DIG labelled LB PCR.

Poly(T) tails on amino terminated, covalently attached oligonucleotides were seen to increase the hybridisation signal (and by inference the ability of the probe to hybridise) (see figure 18). The longer the spacer, the stronger the signal was observed to be. This was found to be the case for two different oligonucleotides sequences hybridised to DIG labelled PCR amplifications of *Lactobacillus plantarum* DNA, and confirms the results of experiments performed by Zhang *et al.* (1991). The increase in signal is due to the spacer allowing the oligonucleotide to behave more as if it was in solution than attached to the membrane (Zhang *et al.* 1991).

Although the addition of a poly(T) tail to a covalently attached oligonucleotide increases the hybridisation signal, the cost of the addition of this tail, the increase in manufacturing time, and the final low yield of oligonucleotide mean that it is not cost effective. Covalent attachment of non-tailed oligonucleotides to membranes gives a sufficient observable signal at a lower cost.

2.6.5 Summary

This section has shown that the addition of a DNA spacer increases the hybridisation signal when the oligonucleotides are covalently coupled to a carboxylated nylon membrane. The longer the spacer is, the stronger the signal. A DNA spacer did not affect the hybridisation signals produced when the oligonucleotide was attached to the membrane by UV-crosslinking in this work. It is not cost effective to use amino terminated oligonucleotides with a DNA spacer as sufficient signals can be achieved without spacer.

2.7 Summary and Conclusions

This chapter has described the investigation of immobilisation of DNA to solid phases. DNA has been successfully extracted and amplified from both *Lactobacillus plantarum* and human blood cells. The DNA was amplified and labelled using PCR in order to be used as a target in reverse dot blot hybridisations.

Two methods of physically attaching oligonucleotides to nylon membranes were studied, baking and UV-crosslinking. Both methods of physical attachment of oligonucleotides to nylon membranes investigated gave insufficient strength of hybridisation signal and reproducibility for use in a diagnostic device, therefore a more reproducible immobilisation method, covalent attachment was explored. Covalent attachment increased the strength of hybridisation signal due to specific attachment of only one end of the attached oligonucleotide producing an increase in its mobility during hybridisation.

Covalent coupling and UV-crosslinking were studied using radioactive labels as a method of quantifying the amount of oligonucleotide bound to the membrane. The amount of oligonucleotide bound to the membrane was lower than expected, although covalent coupling produced a 10-fold increase in the amount of oligonucleotide bound compared to UV-crosslinking.

Covalent coupling was considered to be the most suitable method of attachment and the possibility of using this method on polystyrene slides was explored as chemical modification may be performed directly on polystyrene and glass slides may break or become chipped, posing a health risk.

Polystyrene slides were chemically modified in order to produce carboxyl groups on the surface and investigations were carried out in order to ascertain that the surface had been altered. Contact angle measurements and AFM images were used to study the surface of modified and unmodified polystyrene slides. The polystyrene surface was observed to have slightly different characteristics after modification and so the covalent coupling of labelled, amino terminated oligonucleotides to modified polystyrene slides was performed. Some adjustment was required for the use of detection systems designed for use on porous nylon membranes, however both DIG and biotin detection systems produced signals after modification of the protocol. Hybridisations were successfully carried out on polystyrene slides with covalently coupled oligonucleotides using sequences relevant to cystic fibrosis.

DNA spacers at the 3' terminus of oligonucleotides were investigated as a method of increasing hybridisation signal. The addition of a poly(T) tail at the 3' terminus of oligonucleotide attached to nylon membrane by UV-crosslinking did not produce an increase in hybridisation signal, however, the use of DNA spacers in conjunction with an amino termination and covalent attachment did. The addition of a spacer to amino terminated oligonucleotides must be carried out at synthesis, which increases the cost of the oligonucleotide. The signals produced after hybridisation to amino terminated oligonucleotides without poly(T) tails are observable via chemiluminescent detection, therefore, it may not be possible to justify the use of a DNA spacer in the production of a low-cost diagnostic device.

The work described in this chapter has covered all of the immobilisation and materials aspects of hybridisation used in this project. It has shown that covalent attachment of oligonucleotides to carboxylated nylon or polystyrene is a more reproducible method of

attachment than physical attachment. This work has also shown that reverse hybridisation can be carried out on polystyrene slides, which are more convenient to work with as part of a diagnostic device than both nylon membranes, due to their size and rigidity and glass slides, due to their robustness. The use of a slide instead of nylon membrane allows a reduction of the amount of materials and solutions required when carrying out hybridisations. It also improves the ease of performing hybridisations with several patients DNA in parallel, with the use of slide racks and hybridisation chambers.

3 Detection and Probe Deposition for Solid-Phase DNA Diagnostics

3.1 Introduction

The development of a mutation detection device (which is the aim of this project) will require methods of hybridisation detection and probe deposition which are cost effective and adaptable to mass manufacture. This chapter looks in detail at different methods of hybridisation detection and probe deposition that are available, taking into account the way in which the final device is to be used.

It is important that the protocol for use of the device can be easily followed, and contains the minimum number of steps possible in order to reduce the chances of experimental or user error when detecting mutations. The final device must produce results which are both reliable and reproducible so that it may be used as a diagnostic test. The detection method used should be easy to read and inexpensive to allow its use in a District General Hospital with limited funding and staff with no specific training in molecular biology. An example of an easily read method of detection would be a colourimetric system, where one colour is present for a positive signal, and another for a negative one, (for example, red is positive and blue is negative) as this system would distinguish a negative result from a test where hybridisation has not taken place. A similar more easily used method would be to label the target in the hybridisation with a coloured label, which will produce coloured spots to indicate only a positive result. The result could be read off the membrane after the washes to remove target that has not hybridised to the probe, with no complicated read-out system, this method is investigated in this chapter. Other detection systems to be investigated in this chapter are fluorescent detection, (which requires an excitation source and detection system) and a streptavidin-HRP system with chemiluminescence. Chemiluminescent detection

takes longer to perform than a colourimetric or fluorescent detection system but may provide more accurate results.

The size of the hybridisation spots on the surface will depend on the size of the drops containing the oligonucleotide probe, which will depend on the method used to dispense them. The required size of the spots will vary depending on which method of detection is used. When fluorescence is used (for example in the analysis of microarrays), the spots are often very small, in the region of 100-200 μ m in diameter (Jordan, 2000). This reduces the amount of probe used, however it can increase the production cost of the array due to the requirement of an arraying system.

The simplest method of depositing spots onto a surface is to use a standard hand held pipette. Most pipettes cannot deposit accurately sized drops much smaller than 1 μ l, therefore this restricts the size of spot which can be used. A 1 μ l drop placed onto a porous membrane will have a diameter of approximately 1-2mm. A drop will have a smaller diameter on a solid, hydrophobic surface and a larger diameter on a hydrophilic surface due to the spread of the drop. Drops deposited by a hand held pipette may not be placed precisely. If more than 10 spots are required on one slide more precise deposition is required and more than 15 spots would require the use of a smaller volume of oligonucleotide solution to avoid the drops merging together on the slide, therefore, for the deposition of up to 100 wild type and mutation sequence oligonucleotides (as required in the device this project is working towards) an alternative to deposition by hand held pipette is required.

An arrayer is a robotic device that is used for the mass production of microarrays. They usually use pins to deposit oligonucleotide solution onto a slide and will produce spots of between 200 and 250nm in diameter. Companies such as Qiagen (UK) and Genetix

(UK) commercially produce arrayers, however the cost of buying an arrayer may be prohibitive for production of a test for a single disease in a District General Hospital. If many arrays were to be produced then it is possible that the purchase of an arrayer would be cost effective, however in this model, where slides are to be produced for cystic fibrosis testing in one hospital, the demand for slides would not justify the expense of an arrayer.

An alternative to these methods, which can deposit sizes of drops that are between the extreme ends of the scale, is the BioDot CV1000 syringe system (BioDot, UK). The syringe dispensing system uses a syringe to control the amount dispensed. Small volumes of the liquid of interest will form a droplet on the end of the nozzle. The drop is touched onto the surface in order for it to be transferred from the nozzle to the slide. The CV1000 system can be set to deposit spots of between 0.2 μ l and 1000 μ l. A disadvantage of using the CV1000 syringe dispenser for deposition is that it only has a single reservoir, which requires a large amount of solution to fill it. Several different oligonucleotides would need to be dispensed for production of a diagnostic device, therefore the single reservoir would require cleaning and refilling between each oligonucleotide. The BioDot system of deposition is the best for production of a DNA diagnostic on a small scale, as it combines accuracy of spot size with a relatively small outlay. This chapter assesses the effectiveness of a BioDot syringe system that was used in this project to produce arrays of oligonucleotides and control sequences with a view to final production of a diagnostic device. It also makes a comparison of three methods of hybridisation detection that were studied i) biotin-streptavidin with chemiluminescence, ii) biotin-streptavidin coloured beads and iii) fluorescence. It

reports on two different methods of labelling of target DNA and two methods that were used for fluorescence labelling.

3.2 General Materials and Methods

3.2.1 Labelling of target DNA

Target for use in hybridisation requires labelling to allow the detection of hybridisation.

This section examines two different methods of labelling (incorporation of labelled nucleotide and the use of labelled primers) to attach two different labels (biotin and Cy5). Biotin labels were added to PCR products as described in section 2.2.3.2.

3.2.1.1 Cy5 labelling by PCR amplification with Cy5 labelled primers

Cy5 labelled primers were used to label PCR product for detection after hybridisation.

End-labelled primers were considered to be a relatively simple method of labelling PCR product as they can be bought commercially.

Optimisation of PCR conditions was required in order for an amplicon to be produced when the 5' ends of the primers were labelled with Cy5 (MWG-Biotech, Milton Keynes, UK). In general, the annealing temperature was lowered, and the annealing time and cycles were increased. The PCR reaction mix was the same for each exon and consisted of 76µl of water, 10µl of 10x buffer, 14µl of MgCl₂ (exon 10 and 11) 20.5µl MgCl₂ (exon 21), 2µl of dNTPs, 20pmol of each primer, 0.32 pmol of target genomic DNA extracted from human blood (see section 2.2.2) and 3.75 units of *Taq* polymerase (10x buffer, MgCl₂, dNTPs and polymerase, from PCR core kit, Qiagen, UK).

Exon 10 cycling conditions consisted of a hot start at 94°C for 6 minutes, then 35 cycles of denaturing (94°C, 30 seconds), annealing (53°C, 40 seconds), extension (72°C, 2.5 minutes), then final extension at 72°C for 7 minutes.

Exon 11 cycling conditions consisted of a hot start at 94°C for 6 minutes, then 35 cycles of denaturing (94°C, 30 seconds), annealing (51°C, 45 seconds), extension (72°C, 1 minute), then final extension at 72°C for 7 minutes.

Exon 21 cycling conditions consisted of a hot start at 94°C for 6 minutes, then 35 cycles of denaturing (96°C, 30 seconds), annealing (52°C, 45 seconds), extension (72°C, 30 seconds), then final extension at 72°C for 7 minutes.

In order to confirm PCR amplification, 10µl of each PCR reaction were analysed by gel electrophoresis on a 1.2% agarose gel.

3.2.1.2 Cy5 labelling via aminoallyl dUTP incorporation into PCR product

The incorporation of a label into PCR product provides more label per amplicon than the use of end labelled primers. The Cy5 moiety itself is too large to be used in this way, however it can be coupled to aminoallyl groups after the incorporation of aminoallyl-dUTP.

Aminoallyl-dUTP (Sigma-Aldrich, Dorset, UK) was incorporated into the PCR product by replacing 60% of dTTP in the PCR reaction. The cycling conditions were carried out as described in section 3.2.1.1. The PCR reaction mix consisted of 76µl water, 10µl 10x buffer, 8µl MgCl₂, 2µl of dNTPs (10 mM aa-dUTP, 15 mM dTTP, and 25 mM of dATP, dCTP and dGTP), 20pmol of each primer, 0.32pmol of genomic DNA (see section 2.2.2) and 3.75 units of *Taq* polymerase (Qiagen, UK) (Modified from Hasseman, 2001). The PCR reactions were analysed by gel electrophoresis on a 1.2% agarose gel to confirm the generation of an amplicon. Unincorporated nucleotides were removed using Qiagen PCR clean-up kit with a modified protocol (Hasseman, 2001). The PCR mixture (60µl) was mixed with 300µl PB buffer (Qiagen, UK) and transferred

to a QIAquick column. The column was placed in a 2ml tube and spun at 8,000 x g for 1 minute. The collection tube was emptied and 750µl phosphate wash buffer (5 mM KPO₄, pH 8.5, 80% ethanol) was added to the column. The column was spun at 8,000 x g for 1 minute, the collection tube emptied and 750µl phosphate wash buffer was added. The column was spun at 8,000 x g for 1 minute, the collection tube emptied, and further spun for 1 minute at 8,000 x g. The column was transferred to a 1.5ml tube and phosphate elution buffer (30µl, 4mM KPO₄, pH8.5) was added. The tube was then incubated at room temperature for 1 minute and spun at 8,000 x g for 1 minute. A further 30 µl of phosphate elution buffer was added, incubation and centrifugation were repeated.

Cy5 coupling was carried out as described by Hasseman (2001). Cy5 ester (Amersham Pharmacia Biotech, Bucks, UK) was re-suspended in DMSO (Sigma-Aldrich, Dorset, UK) (1mg Cy5 ester in 73µl DMSO) aliquoted and stored at -80°C, in the dark. Cleaned up PCR product (5µl) was added to 5µl of 0.1M sodium carbonate buffer (pH 9), 5µl of Cy5 ester was added and mixed well. The mixture was incubated in the dark at room temperature for 1 hour.

The purification of the labelled PCR product from excess Cy5 ester was carried out as described by Hasseman (2001), using the QIAquick PCR cleanup kit. The Cy5/PCR solution was mixed with 100µl buffer PB, and the mixture was placed in the spin column. The column was spun at 8,000 x g for 1 minute. The collection tube was emptied and 750 µl buffer PE was added and centrifuged at 8,000 x g for 1 minute. The column was placed in a 1.5ml tube, buffer EB (30µl) was added, incubated at room temperature for 1 minute and spun at 8,000 x g for 1 minute. A further 30µl of buffer EB was added, incubation and centrifugation were repeated. Throughout the whole

purification, exposure of the samples to light was avoided in order to avoid photobleaching of the Cy5 dye. The incorporation of Cy5 molecules was estimated using the absorbance readings at 260nm and 650nm. The calculations used were

$$pmol\ Cy5 = \frac{OD_{650} \times volume(\mu l)}{0.25}$$

$$pmol\ nucleotide = \frac{OD_{260} \times volume(\mu l) \times 37\ ng/\mu l \times 1000pg/ng}{324.5\ pg/pmol}$$

$$nucleotide / dye\ ratio = \frac{pmol\ DNA}{pmol\ Cy5\ dye}$$

3.2.2 Hybridisation detection via SA-HRP and chemiluminescence on slides

This section compares the use of the biotin-streptavidin bond in chemiluminescent detection with fluorescent detection. Biotin labelling and detection is carried out as described in section 2.5.4.

Human CFTR exon 10, 11 and 21 PCR products were labelled with biotin as described in section 3.2.1.1. Polystyrene slides (Nunc, Paisley, UK) were modified as described in section 2.5.2.1. Activation of the carboxyl groups and deposition of oligonucleotide was carried out as in section 2.4.2.1.

Hybridisation and subsequent washes were carried out as described in section 2.5.3.3 with the addition of target after pre-hybridisation. An aliquot of each of the three biotin

labelled targets (exon 10, exon 11 and exon 21) were mixed together (12µl of each), the mixture was denatured at 100°C for 5 minutes, pulse spun and pipetted down the centre of the slide.

3.2.3 Use of coloured streptavidin beads as a readout system

A coloured read out system requires fewer washes and incubations after hybridisation than chemiluminescent detection, and may be cheaper than alternative detection methods, for example, the fluorescent scanner required to detect fluorescent signals. Oligonucleotides were covalently attached to Biodyne C membrane (Pall Life Sciences, Hants, UK). Covalent attachment was carried out as described in section 2.4.2.1. Hybridisation to biotin labelled PCR product was performed as described in section 2.4.2.2. Red streptavidin coated microspheres (Bangs laboratories, Fishers, Illinois, USA) were used to detect the biotin labelled PCR product after hybridisation. Two methods of allowing the streptavidin/biotin bond to form were used; in the first the beads were diluted 1/1000 in water and the membrane was incubated in the solution. In the second method beads were diluted ¼ in 1 M glucose solution and spotted onto the dry membrane in the same position that the oligonucleotide had been spotted previously. The beads were allowed to dry onto the membrane. The membranes were then washed for 2 x 15 minutes in DIG washing buffer (0.3% Tween in maleic acid buffer) in an ultrasonic water bath to remove any non-specifically bound beads and were observed by eye to detect the beads.

3.2.4 Fluorescent labelling of PCR product via end-labelled primers

Fluorescent detection requires only stringency washes after hybridisation and does not require antibody or conjugate incubations. When compared to the chemiluminescent

method of detection this can reduce the time necessary to carry out a test by 1-3 hours, depending on which chemiluminescent detection system is being used.

In this section an arrayer, a Genetix Q-Array, was made available courtesy of Genetix (UK). Oligonucleotide probes were deposited onto modified polystyrene slides by both manual pipetting and arraying using the Genetix Q-array equipped with 150µm tip diameter stainless steel microarraying pins courtesy of Genetix (UK). Hybridisations with Cy5 labelled target were carried out on polystyrene slides. The PCR products were labelled with Cy 5 using 5' end-labelled primers in the PCR (see section 3.2.1.2).

Hybridisations were carried out as described in section 3.2.2 except that modifications to the protocol, such as the covering of wash trays with foil, were made to avoid exposure of the slides and Cy5 labelled target to light. Washes were carried out (1 x 5mins 2xSSC/0.1% SDS and 2 x 5mins 0.5xSSC/0.1%SDS), the slides were dried by centrifugation, stored in a dark box and scanned by Dr. S. Stephens (Genetix, UK) using a GSI Lumonics Scanarray 4000 (Packard Bioscience, UK).

3.2.5 PCR incorporation of aminoallyl-dUTP as an alternative method of fluorescent labelling

PCR incorporation of a label will allow the addition of more labels per amplicon than end labelling. This should produce an increase in the final signal observed after hybridisation.

The PCR incorporation of aminoallyl-dUTP and subsequent coupling of Cy5 to produce a labelled target was carried out as described in section 3.2.1.3. Amino terminated oligonucleotides were covalently attached to modified polystyrene slides as described in section 3.2.2. Hybridisations and washes were carried out and the slides were dried by centrifugation and sent to Dr. S. Stephens at Genetix (UK) to be scanned. As in section

3.2.4, an attempt was made to reduce exposure of the Cy5 labelled target and the hybridised slides to light.

3.2.6 BioDot deposition of oligonucleotide probe onto modified polystyrene slides

The deposition of oligonucleotide using a hand held pipette limits the size and reproducibility of the spots produced. A mechanical system of deposition allows a smaller drop to be deposited and the spots to be placed in the same position on each slide.

Polystyrene slides were modified as described in section 3.2.4. After the slides had been activated with EDC and dried by centrifugation (section 3.2.2.), oligonucleotide was spotted onto the slides using the BioDot CV1000 syringe dispenser system.

Due to the large reservoir on the BioDot CV1000 syringe dispenser system, some modifications were required to allow deposition from a relatively small amount of oligonucleotide solution. The BioDot CV1000 syringe dispenser system was connected to a peristaltic pump via a 3-way switch (see figure 19). Oligonucleotide solution was drawn into the nozzle using the peristaltic pump and the T-switch. The flow was then turned back so that the oligonucleotide solution in the nozzle could be deposited onto the slide. The volume of the drops deposited was 150nl, the oligonucleotides were at a concentration of 100 pmol/ μ l (15 pmol per spot) and 20 pmol/ μ l (3 pmol per spot).

Identical spots were deposited in 5 rows of 15. In order to print multiple oligonucleotides and control sequences it was necessary to purge the syringe system tubes and change the nozzle between different control sequences and oligonucleotides. A pattern repeat was set up so that several slides could be printed at once. Four rows, with five spots of each oligonucleotide or PCR control sequence in each row were

spotted. Hybridisations with biotin labelled PCR product were carried out on the slides as previously described in section 2.5.4.

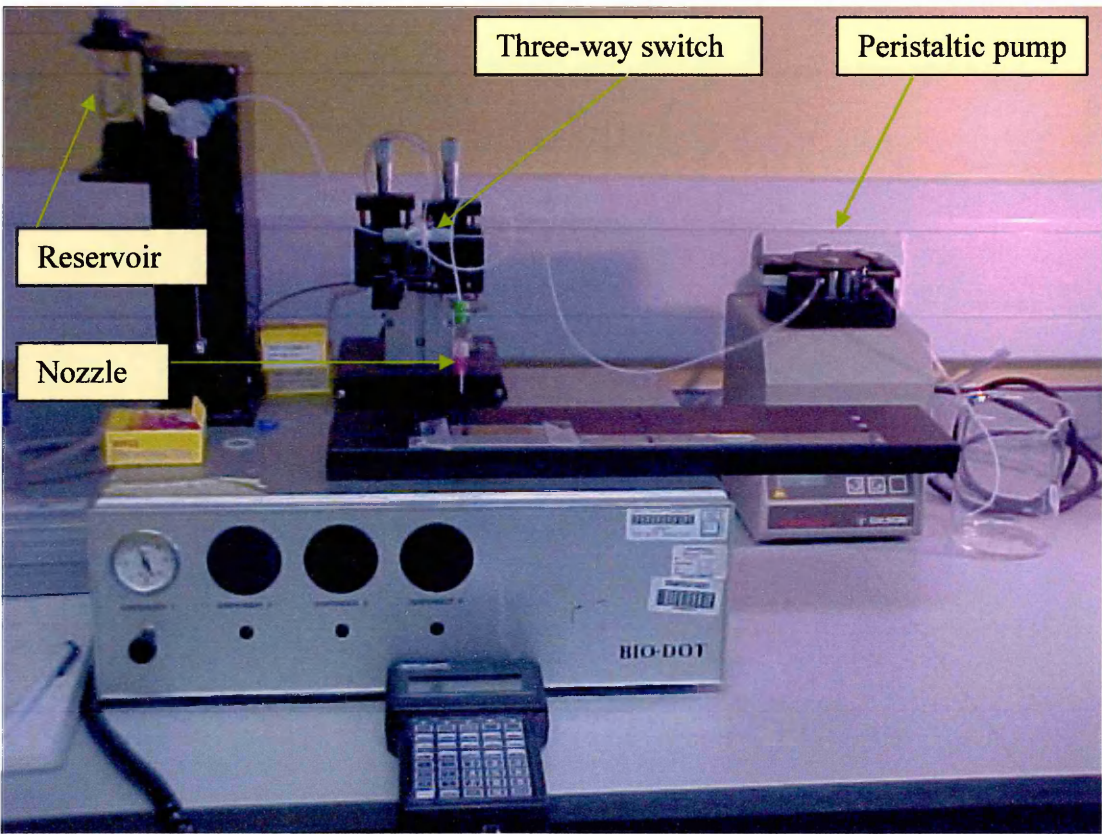


Figure 19. BioDot CV1000 syringe system with additional pump and three-way switch.

3.3 Results and Discussion

3.3.1 Biotin labelled probes hybridised to oligonucleotides on polystyrene slides

Oligonucleotides representing cystic fibrosis mutation and wild type sequences were covalently coupled to modified polystyrene slides in section 2.5. Biotin labelled target was hybridised to the oligonucleotides and a streptavidin-HRP conjugate was used to allow chemiluminescent detection (Southern detection kit, KPL, Maryland, USA). The chemiluminescent detection system used was optimised by the manufacturers for use with porous membrane therefore some alterations to the protocol were necessary in order to transfer the system to non-porous slides. The aim of this modification was to prevent the detection solutions from evaporating or pooling on one area of the slide. The use of glass coverslips on the slides after the addition of the detection solutions reduced evaporation and pooling and improved the signals observed. It can be seen from figure 16 that hybridisation can be carried out with oligonucleotides covalently coupled to modified polystyrene slides. Hybridisation has occurred between the target and the wild type oligonucleotide in the case of each mutation. The signal from hybridisation to the wild type $\Delta F508$ mutation is not strong, it is unclear why this is the case. There was some hybridisation to the oligonucleotide complementary to the mutant sequence in the case of the $\Delta F508$ and N1303K mutations. An amount of mutant sequence oligonucleotide in excess of that normally required to allow hybridisation of complementary sequences was deposited in order to observe the effect on hybridisation. It is likely that this non-specific hybridisation is due to the excess amount of oligonucleotide used. Although the chemiluminescent biotin-streptavidin detection system (modified from Southern detection kit, KPL, Maryland, USA) required optimisation for use on slides, once this had been performed, detection of mutations

appeared to be consistent and as expected between experiments (see figure 15). The chemiluminescent method of detection is not simple it requires several steps, many solutions, as well as the use of a dark room. This can make the procedure time consuming, however it does provide easily observable signals.

3.3.2 Coloured beads as a detection system

A colourimetric method of detection would not require antibody or conjugate incubations, or substrate incubations as the chemiluminescent method used above does. This could reduce the time required to perform a test. Coloured streptavidin beads are commercially available and should form a bond with biotin labels on PCR products in the same way as the streptavidin-HRP conjugate does. Initially streptavidin beads appeared to bind to biotin labels on hybridised PCR product (figure 20a), however this result was not reproducible. Streptavidin beads did not bind to biotin labels on the PCR product when the beads were added to a wet membrane, or if the membrane was exposed to a weak solution of beads. Parallel experiments were carried out using streptavidin-HRP conjugate and chemiluminescence to detect the biotin labelled PCR product after hybridisation. These control experiments showed that hybridisation had taken place (figure 20b), however it was not detectable via red streptavidin beads. An additional experiment added beads to dry membrane followed by a 30 minute incubation for the streptavidin-biotin binding to take place. This method allowed the beads to bind non-specifically to the membrane. Glucose has been previously used to facilitate the removal of non-specifically bound antibody to nitrocellulose membranes (Birk and Koepsell, 1987). In this work it was added to the bead solution in order to facilitate the removal of non-specifically bound beads. The use of glucose in conjunction with gentle washes was not sufficient to remove non-specifically bound beads. Washing was then

performed in an ultrasonic water bath in order to remove non-specifically bound beads.

When attempts were made to remove non-specifically bound beads by this method, however, all of the beads were removed. The biotin-streptavidin bond is strong and should be able to withstand these washes. These results suggest the washing method was too disruptive, either breaking streptavidin-biotin bonds or the hydrogen bonds holding the DNA duplex together. It is also possible that none of the beads were specifically bound to the biotin labels on the target due to their relatively large size.

In one case red streptavidin beads were thought to have provided a detection signal (figure 20). In this experiment gentle washes were carried out to remove beads which were non-specifically bound beads. A strong red spot is visible representing hybridisation to the wild type $\Delta F508$ oligonucleotide, and weaker spots are seen representing control hybridisations to exon 10 PCR product and biotin labelled exon 10 PCR product. This result was not reproducible, however and the conclusion of this work must be that streptavidin beads did not bind consistently to biotin labelled PCR product on nylon membrane.

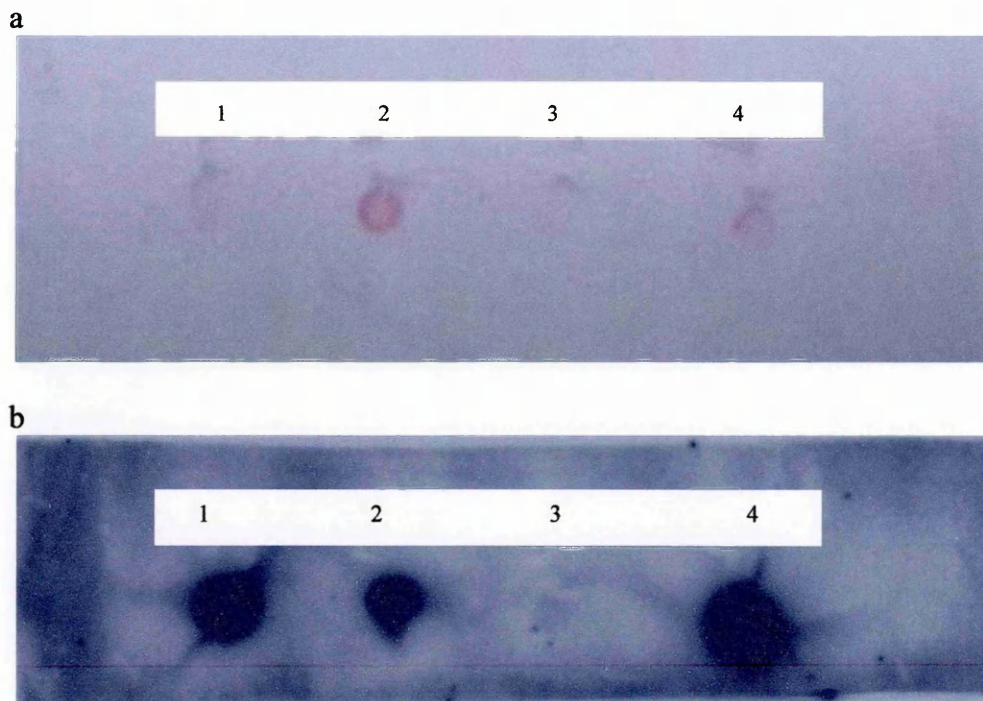


Figure 20. Red streptavidin beads used as a hybridisation detection method. Oligonucleotides were covalently coupled to Biodyne C membrane and biotin labelled Exon 10 PCR product was hybridised. a. red streptavidin beads were used for detection. b. chemiluminescent detection via streptavidin-HRP conjugate. 1-Exon 10 PCR product. 2- Δ F508 wt. 3- Δ F508 mut. 4-biotin labelled exon 10 PCR (see appendix C for oligonucleotide sequences). Hybridisation signals were expected to be visible for spots 1,2 and 4 as the PCR was amplified from control gDNA.

3.3.3 Fluorescent labelling of PCR product by end-labelling of primers

Fluorescent methods of hybridisation detection only require washes to remove unbound target and drying before results can be obtained. This allows hybridisations to be processed in a shorter time than using chemiluminescence. A fluorescently labelled target was required in order to investigate this method of detection. Fluorescent labelling was initially executed using Cy5 end-labelled primers in a PCR, giving only one label per strand of DNA as in the case of the biotin labelling in section 3.2.2. The Cy5 moiety interfered with the PCR significantly more than the biotin. The Cy5 PCRs required optimisation of annealing temperature and length, the annealing temperature for all three PCRs was reduced and the length of annealing was increased. This had the additional effect of decreasing the specificity of the PCR, which was seen by the presence of extra bands on the gel (for example, exon 21 in figure 21). The amplified target for use in a diagnostic device must be the same sequence as the patient's DNA, this means that the PCR carried out to produce target must amplify only the sequence of interest from the patient's DNA to avoid false positive signals. A series of PCRs were carried out in order to discover the conditions which produced the most specific PCR with minimum reduction of PCR product. Because of the requirement for specificity Cy5 labelled primers gave little PCR product, which produced weak bands when run on an agarose gel (figure 21).

Although only a limited quantity of the Cy5 labelled target was available, hybridisation was attempted with oligonucleotides covalently coupled to modified polystyrene slides. The hybridisation and washes were carried out in the dark to reduce photobleaching of the Cy5, however signals were not observed from these hybridisations.

One reason for failure of the experiment may be due to the Cy5 moiety. The Cy5 label may be the first part of the oligonucleotide to undergo degradation (MWG, Personal Communication). The Cy5 labelled primers were not aliquoted due to an attempt to minimise their exposure to light, this did, however, mean that they were subjected to more freeze/thaw cycles than a primer would normally undergo, possibly allowing contaminating enzymes to cause damage and degradation. In retrospect it may have been better to aliquot the primers as this could have been carried out in a dark room, reducing exposure to light.

The use of Cy5 labelled primers interfered with PCR, making it impossible to obtain sufficient target, without compromising specificity. It goes without saying that specificity is critical in a diagnostic device. Therefore, an alternative method of labelling PCR products with Cy5 was investigated.

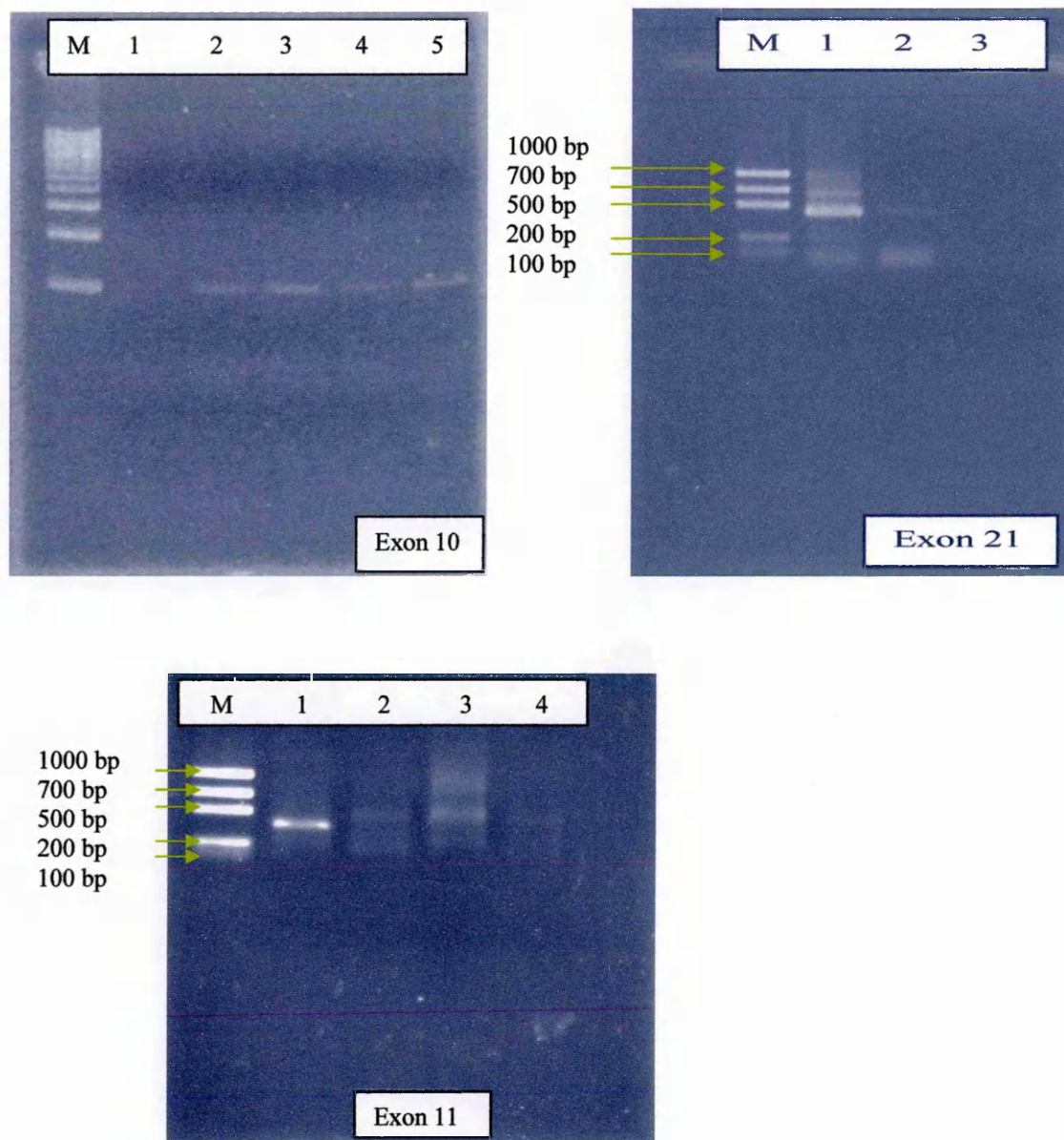


Figure 21. PCR product from Cy5 primer labelling

Three exons from the CFTR gene in human gDNA were amplified using Cy5 labelled primers 10i-3, 10i-5, 11i-3, 11i-5, 21a and 21b (see Appendix A for sequence). 10µl of each amplicon was analysed on a 1.2% agarose gel. The expected band sizes were, exon 10-491 bp, exon 11-425 bp and exon 21-376 bp, however the Cy5 label reduced DNA migration time making the PCR product appear larger than it was. Bio-Rad Molecular Mass Marker was used for exons 11 and 21, pGEM was used for exon

(Figure 21. legend continued)

10, however the bands did not separate adequately and their size can not be distinguished so they cannot be used as indicators of size. Multiple bands were observed when exon 21 PCR was analysed, this is discussed in more detail in the text.

Exon 10 lanes: M-Marker. 1- -ve control. 2 and 3 +ve controls. 4 and 5 Cy5 labelled PCR.

Exon 21 lanes: M-Marker. 1-+ve control. 2-Cy5 labelled PCR. 3- -ve control. Exon 11 lanes: M-Marker. 1-+ve control. 2, 3 and 4-Cy5 labelled PCR. 5- -ve control.

3.3.4 *Fluorescent labelling by PCR incorporation of aminoallyl dUTP*

Target labelled using end-labelled primers did not produce an observable hybridisation signal, therefore an alternative method of fluorescent labelling was carried out. A method of incorporating labels into the target during PCR was investigated to increase the amount of label per amplicon and so ensure that the target could be distinguished from the background.

PCR incorporation of 60% aminoallyl labelled dUTP (replacing dTTP) did not interfere with the PCR, the thermal cycling conditions used were the same as those used for DIG and biotin labelling. A good yield of PCR product was produced. The Cy5 label was coupled to aminoallyl groups after their incorporation into the PCR, and unincorporated nucleotides and Cy5 were removed from the labelled PCR product to reduce background signal. The calculations carried out in order to check incorporation of Cy5 showed that there was a variation between samples (see Appendix H for examples). Most samples however, had a ratio of less than 50 nucleotides per dye molecule which has been suggested to be adequate for signal detection in a hybridisation (Hasseman, 2001).

No fluorescent signals were observable after hybridisation with oligonucleotides covalently coupled to modified polystyrene slides when scanned by Dr. S. Stephens (Genetix, UK) using the GSI Lumonics Scanarray 4000 (Packard Bioscience, UK). Labelling by incorporation of aminoallyl-dUTP and subsequent coupling of Cy5 has increased the number of Cy5 molecules per target and should therefore increase the signal strength compared to the background fluorescence. As signals can not be detected it is likely that a weak signal is not the cause of the lack of observable signal.

It is possible that the lack of signal is due to photobleaching or degradation of the Cy5 dye.

3.3.5 *Comparison of Cy5 labelling methods*

Neither the use of end labelled primers nor aminoallyl incorporation to produce fluorescently labelled target produced a target that gave a signal when scanned after hybridisation. A possible cause of the lack of observable signal after hybridisation with the amplicon produced using Cy5 labelled primers was that it was not possible to distinguish the signals produced by the target, from the background signal produced from the natural fluorescence of the slide and non-specifically bound target. This could be due to reduced hybridisation, because of the reduced target production when using Cy5 labelled PCR primers. Incorporation of labels allows the addition of more labels per amplicon and should remedy this problem. The method of labelling PCR products by incorporation of dUTP labelled with aminoallyl groups and subsequent coupling of Cy5 has previously been used to label RNA for hybridisations (Hasseman, 2001). Modification of the protocol by labelling DNA instead of RNA should not affect the labelling or detection of the PCR product. The lack of hybridisation signal could be due to photobleaching of the Cy5 label (Molecular Probes, 2002), or a problem with the labelling reaction. It is unlikely that hybridisation did not occur as biotin labelled control hybridisations carried out at the same time, under the same conditions as the fluorescent hybridisations gave positive results. Much more work would need to be carried out on the fluorescent labelling and hybridisations before this detection method could be used in a final device, however, due to time constraints, this work could not be carried out in this project.

3.3.6 *Deposition of oligonucleotide using the BioDot syringe system*

The BioDot syringe system can be programmed to print multiple slides (up to 10) in one run. A pre-programmed volume of DNA solution will be deposited in a specific position and therefore rows of spots can be produced. The syringe system is able to print smaller spots than a hand held pipette, and larger spots than most arrayers, the cost of this system is less prohibitive than an arrayer. It is important to consider the cost of production of the printed slides as the end-user of this technology will be a District General Hospital, with limited funds. The reservoir on the BioDot syringe system requires approximately 10mls of liquid to fill it, making it both expensive and impractical to fill the reservoir with oligonucleotide solution. Modifications were made to the system to reduce the amount of oligonucleotide required and therefore reduce slide printing costs.

The hybridisation results produced when a single oligonucleotide was spotted by this method were not as consistent as expected from the uniformity of spotting. The drops of oligonucleotide solution were spotted onto the slide consistently (figure 22), however the hybridisation signals were not consistent over the slide (figure 23a). This may be due to a lack of immobilisation or hybridisation or a problem with detection. It seems likely that the inconsistency of hybridisation signals is due to the method of incubation with streptavidin-HRP conjugate after hybridisation that was carried out in hybridisation bags which touch the slide. When the method was altered to incubation in a humid chamber, with no bag or coverslip on top of the slide, the results were more consistent over the slide (this can be seen in figure 23).

Figure 23a shows hybridisation of DIG labelled exon 21 PCR target to N1303K wild type oligonucleotide (15 pmol per spot). The oligonucleotide was covalently attached

to a modified polystyrene slide. It can be seen that an air bubble has been present at some point during the processing of this slide, producing a large area where no signal is observable. The bubble is likely to have occurred during incubation with the streptavidin-HRP conjugate in a hybridisation bag because when a humid chamber was used for this incubation bubbles were not observed (see figure 23c). It is difficult to ensure that air is not preventing the even distribution of solution when using hybridisation bags to incubate non-porous slides. This problem has not been observed when performing hybridisation on porous membrane as seen from the lack of bubbles in figure 10.

Figure 23b shows hybridisation of DIG labelled exon 21 target to exon 21 PCR product on a modified polystyrene slide (attached by physical adsorption). This hybridisation was considered to be a positive control as the target and probe were the same sequence. The hybridisation signals on the slide in figure 23b showed an inconsistency which is likely to be due to the use of a hybridisation bag for incubation with streptavidin-HRP conjugate.

Figure 23c shows the results of a hybridisation with four duplicate sets of four different probes printed onto a slide. The streptavidin-HRP incubation on this slide was carried out in a humid chamber as opposed to the hybridisation bag used for the two previous slides (23a and b). It can be seen that there is more consistency between hybridisation signals over the slide. The oligonucleotides and PCR product on this slide were printed using the BioDot CV1000 syringe system, the tubes were purged with water and the nozzle was changed between each different DNA printed. The four probes are present in duplicate, rows 1,5 and 9 are exon 11 PCR product, rows 2,6 and 10 are 100 pmol wild type N1303K, rows 3, 7 and 11 are 20 pmol wild type N1303K and rows 4, 8 and

12 are mutant N1303K. Each row contains 5 duplicate spots. There has been some non-specific hybridisation to the control sequence in some cases (the fourth row of sets 2 and 3). The signals in the first set are not as strong as those in following rows, possibly due to dilution of the oligonucleotide because of mixing with the water used to flush the system between different DNA sequences. The signals in the fourth set are also weaker than other sets, this may be due to dilution of the oligonucleotide solution with water in the tubes of the syringe system as the oligonucleotide solution is used up. Up to 10 slides can be printed in one run, this method of printing would not be suitable for commercial printing of slides in the order of hundreds per day; however, it may be suitable for production of diagnostic devices within a single hospital. Setting up the BioDot syringe system and switching between oligonucleotides is time consuming, however once the BioDot syringe system has been set up, it is quicker and more accurate in terms of position of spots and reproducibility of that position from slide to slide than pipetting by hand. The BioDot syringe system also allows the deposition of smaller drops than can be deposited using a hand held pipette.

It is conceivable that the system could be modified to allow the deposition of several different oligonucleotides, from relatively small amounts of solution (approximately 50µl). If the manufacture of slides was undertaken for the production of a DNA diagnostic for use in a hospital, the production of a specifically designed system may be cost effective and reduce the manufacturing time of the slides. The purchase of an arrayer for the printing of slides may be justifiable because it would not require the time and expense of modification before slides could be printed.

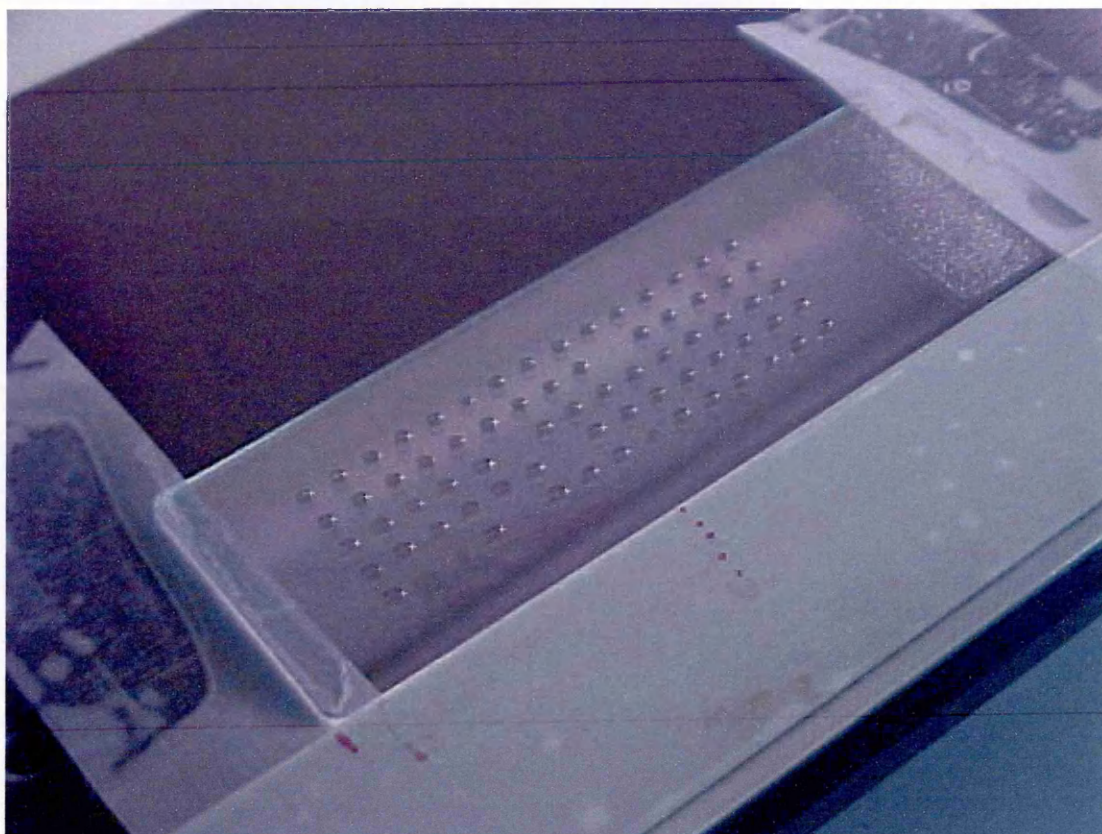


Figure 22. Drops of oligonucleotide on a modified slide as printed using the BioDot CV1000 syringe system.
A 5 x 15 array was printed, the drop volume was 500nl.

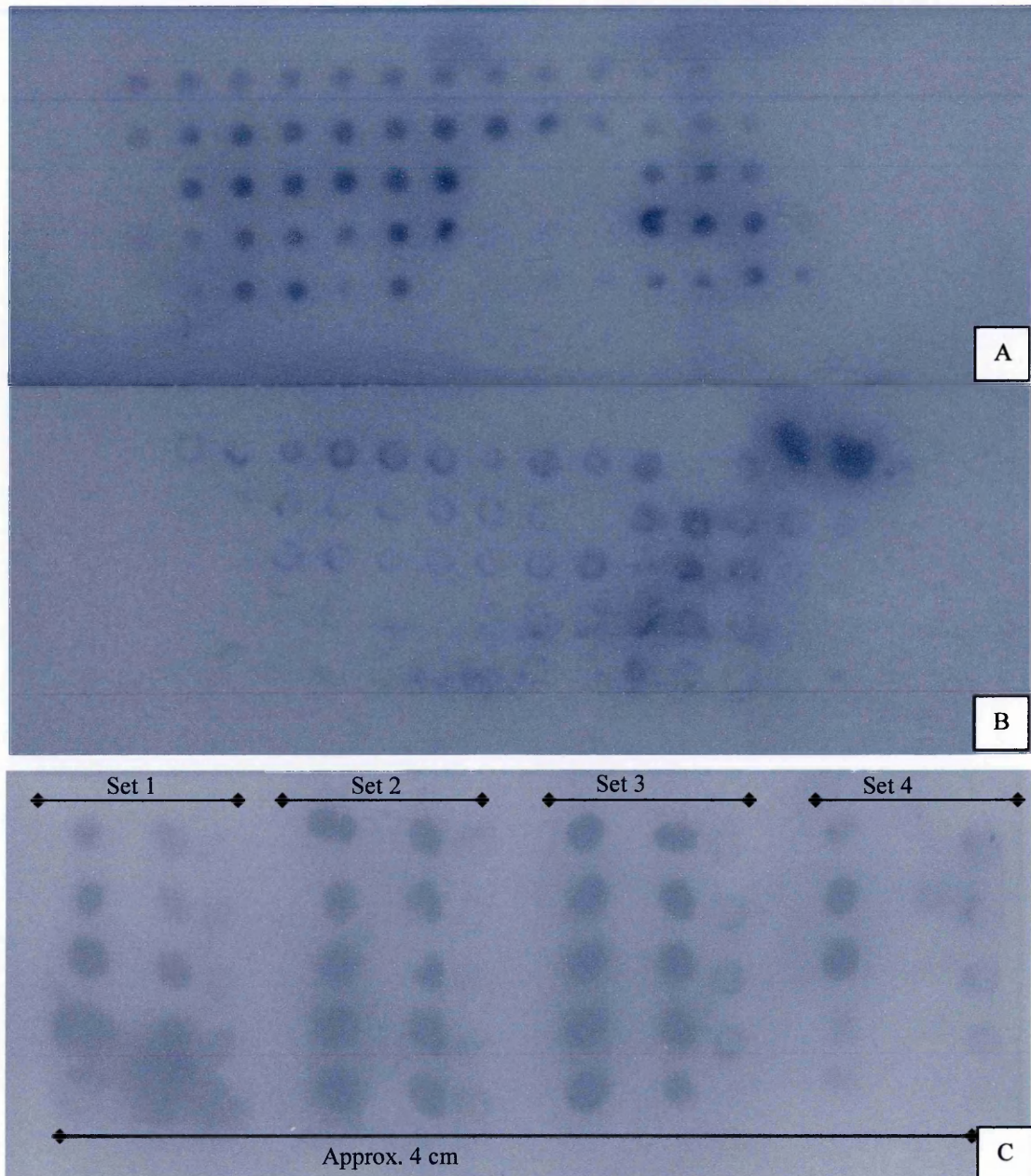


Figure 23. The Bio-dot syringe system was used to print oligonucleotide and PCR product onto modified slides for hybridisations.

A) N1303Kwt (100pmol/ μ l). B) exon 21 PCR. C) Sets 2-4 are duplicates of set 1.

Rows 1, 5 and 9- exon 11 PCR. Rows 2,6 and 10- N1303Kwt (100pmol/ μ l). Rows 3,7 and 11- N1303Kwt (20poml/ μ l). Rows 4,8 and 12-N1303Kmut (20pmol/ μ l). The oligonucleotide/PCR product on slides were hybridised to biotin labelled exon 21

(Figure 23. legend continued)

PCR product and detected via chemiluminescence. (see appendix C for oligonucleotide sequence).

3.4 Summary and Conclusions

This chapter has compared three methods of hybridisation detection, (i) biotin-streptavidin with chemiluminescence, ii) biotin-streptavidin coloured beads and iii) fluorescence. Chemiluminescent detection has been successfully carried out on both nylon membrane (see section 2.4.5.2) and modified polystyrene slides. The use of non-porous polystyrene as a surface for hybridisation required adjustment of the chemiluminescent detection protocol; however, with the use of coverslips to compensate for this, hybridisation detection was successful. The disadvantage of a chemiluminescent method of detection is that it requires several incubations and washes before the results can be observed, as well as a lengthy exposure to Kodak X-OMAT film (Sigma-Aldrich, Dorset, UK).

The use of coloured streptavidin coated beads as a hybridisation detection method would not require incubation and development of film, and therefore would be a faster method of hybridisation detection. The streptavidin beads produced non-specific positive signals and did not give a good indication of where biotin labelled PCR product had hybridised. Various methods of bead application and membrane washing were investigated in order to produce specific signals, however, conditions were not discovered which allowed the specific detection of hybridisation.

Two methods of fluorescent labelling were used. In the first, labelling was performed using end-labelled primers. In the second, PCR was used to incorporate aminoallyl labelled dUTPs, to which Cy5 ester was subsequently coupled. Neither method of fluorescent labelling produced a satisfactory hybridisation signal. Photobleaching and oligonucleotide degradation are believed to be the cause of this lack of signal.

This chapter has also investigated different methods available for the deposition of oligonucleotide solutions onto a surface. The BioDot CV1000 syringe system was found to be a consistent method of drop deposition, allowing the same amount of solution to be spotted each time. The disadvantage of this system was the single, large reservoir, which meant that the system had to be flushed through between oligonucleotides and an alternative to filling the reservoir was required in order to conserve oligonucleotide solution. A modification of this system, in order to produce a smaller reservoir requirement and allow the printing of more than one oligonucleotide at once would be cost effective if the production of slides was undertaken for use in a hospital.

A diagnostic device for use in an NHS hospital requires a method of detection that is simple to perform, cheap and accurate. The coloured bead and fluorescent detection methods were simple to perform and required fewer washes and incubations than chemiluminescence, however they did not give consistently accurate results. The chemiluminescent method of detection used here involved more steps and took a longer period of time than using beads or fluorescence, however it gave more consistent results.

It is more important that a diagnostic device provides reliable information rather than a quick result. Therefore the biotin-streptavidin chemiluminescent method was chosen to for use in further experiments.

4 Demonstration of a DNA Diagnostic for Cystic Fibrosis

4.1 Introduction

The testing of patient samples is necessary for the validation of a diagnostic device. In the final system DNA will be extracted from human blood for amplification, therefore samples of patients' blood were required for testing the device. In this chapter, the amplification and genetic testing on an 'array' of DNA containing cystic fibrosis mutations is described. The aim of these studies was to ensure that these mutations could be amplified and detected (and distinguished from normal control samples). Blood samples were collected from patients with cystic fibrosis mutations for the extraction of genomic DNA and amplification of specific sequences from the cystic fibrosis gene.

Patient samples were obtained through Northampton General Hospital. Patient blood was collected in tubes containing anti-coagulant in order to prevent clotting. This chapter also considers the effect this may have on amplification, labelling and hybridisation of DNA.

Amplified and labelled patient DNA underwent hybridisation to oligonucleotides covalently attached to modified polystyrene slides as a method of cystic fibrosis mutation detection. Although this hybridisation method has been tested using control DNA with wild type sequences (see section 2.5.4), it is important to ensure that it also works adequately using patient samples, containing cystic fibrosis mutations.

4.2 Materials and Methods

The collection of patient blood samples was carried out by staff at Northampton General Hospital. Ethical approval was obtained from the hospital ethics committee before the

use of patient samples in this research. The Northampton General Hospital ethics committee approved the use of patient blood for testing of the diagnostic device in this project (see Appendix I).

Blood samples were collected from cystic fibrosis patients with known mutations during routine medical treatment at the hospital or in the patient's home. Patients were from the Northampton General Hospital catchment area. Consent forms were signed by each patient and their parent or guardian (see Appendix I). Blood was collected in Heparin tubes to prevent coagulation, and stored at 4°C, for no longer than seven days before genomic DNA extraction was carried out.

4.2.1 Genomic DNA extraction from patients blood

The validity of the hybridisation system being investigated was confirmed using DNA extracted from patient blood. Blood samples were obtained as described above (section 4.2) and genomic DNA was extracted as described in section 2.2.2 in preparation for PCR amplification and subsequent hybridisation.

4.2.2 PCR amplification and biotin labelling of patients' genomic DNA

It was necessary to amplify and label the regions of interest from patients' genomic DNA in order to produce sufficient quantities of DNA to be used as a target in hybridisation.

Exons 10, 11 and 21 from the CFTR gene in patients' genomic DNA were amplified and labelled with biotin using PCR as described in section 2.2.3.2.

4.2.3 Modification of polystyrene slides and hybridisation of patient sequences

Modification of polystyrene slides was carried out as described previously in order to produce carboxyl groups on the surface (see section 2.5.3.1). Amino terminated oligonucleotides were covalently coupled to the polystyrene slides as described in

section . PCR amplification products of patient sequences were hybridised to the oligonucleotides and subsequently detected as wild type amplicons were previously (section 3.2.2).

4.3 Results and Discussion

It is important to test any potential diagnostic device with DNA containing both wild type and mutant sequences to ensure the test is able to discriminate between the two. In this work, it was considered essential to compare the hybridisation results from blood with both wild type and mutation sequences as the bloods were collected using different methods. Different methods were used for blood collection from volunteers and patients because the samples were collected in different locations. Blood was collected from volunteers without anti-coagulant by staff at the Cranfield University Medical centres (at Cranfield and Silsoe). Blood from cystic fibrosis patients was collected with anti-coagulant by staff at Northampton General Hospital. It is possible that the use of anti-coagulant at blood collection may interfere with genomic DNA extraction and subsequent PCR (Personal communication, Qiagen, UK).

4.3.1 Genomic DNA extraction from patient blood and PCR amplification

Anti-coagulant is required to prevent the blood clotting, if the genomic DNA is extracted shortly after the blood sample is taken then it is not required. Blood collected by Northampton General Hospital required collection in anti-coagulant tubes because it was stored for a number of days at 4°C before genomic DNA could be extracted.

Control blood samples were collected without anti-coagulant due to the expense of anti-coagulant blood tubes and the short period of time elapsed before genomic DNA was extracted (within 30 minutes of sample collection) which reduced the chance of the blood clotting before genomic DNA extraction.

Genomic DNA extraction was carried out successfully from blood collected both with and without anti-coagulant (Figures 24 and 3 respectively). Neither the anti-coagulant nor the difference in length of storage time before genomic DNA extraction appears to have influenced the final quantity or quality of the genomic DNA extracted.

PCR was carried out successfully on genomic DNA extracted from blood collected using both methods (Examples of PCR products are shown in figures 4 and 5 for wild type gDNA and figure 25 for patient gDNA). In a hospital setting it would be more prudent to use anti-coagulant as the time between the blood sample collection and the genomic DNA extraction could not be guaranteed. It has been shown here that the presence of Heparin anti-coagulant at blood collection does not have an effect on genomic DNA extraction or PCR by these methods. The PCR product produced in section 4.2.2 was used as a target for hybridisation to immobilised oligonucleotide in section 4.2.3.

4.3.2 Hybridisation of amplified patient sequences to oligonucleotides on polystyrene

The pre-prototype mutation detection device produced in Chapter 3 is tested in this chapter by using it to identify the presence or absence of four different cystic fibrosis mutations, $\Delta F508$, G542X, G551D and N1303K in a patient's DNA. These mutations were chosen as they occur most commonly in the cystic fibrosis population in the catchment area of Northampton General Hospital. Due to the geographical bias of the presence of different cystic fibrosis mutations throughout the world population, the sequence of the oligonucleotides used in a final device (and the cystic fibrosis mutations to which they correspond) should be adapted to the area where the device is used (Raskin *et al.*, 1993). The diversity of the population and the most common mutations in that geographical area must be taken into account in order to produce a useful test.

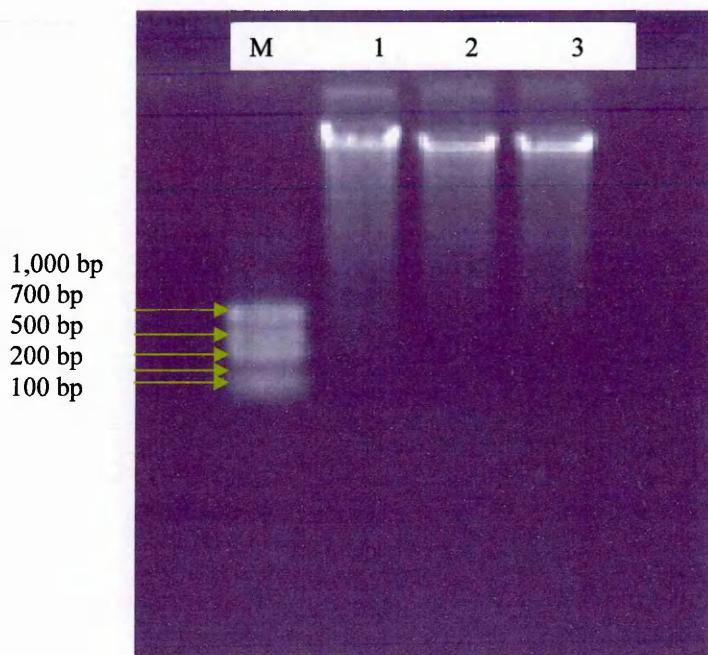


Figure 24. Genomic DNA was extracted from 3 samples of patient blood using the Qiagen ReadyAMP Extraction Kit.

10 μ l of extract were run at 50V on a 0.3% agarose gel in order to confirm extraction.

The bands in the Bio-Rad Molecular Mass Marker used were not entirely separated due to the use of a low agarose percentage in the gel. Lanes: M-marker. 1- gDNA1. 2- gDNA2. 3-gDNA3

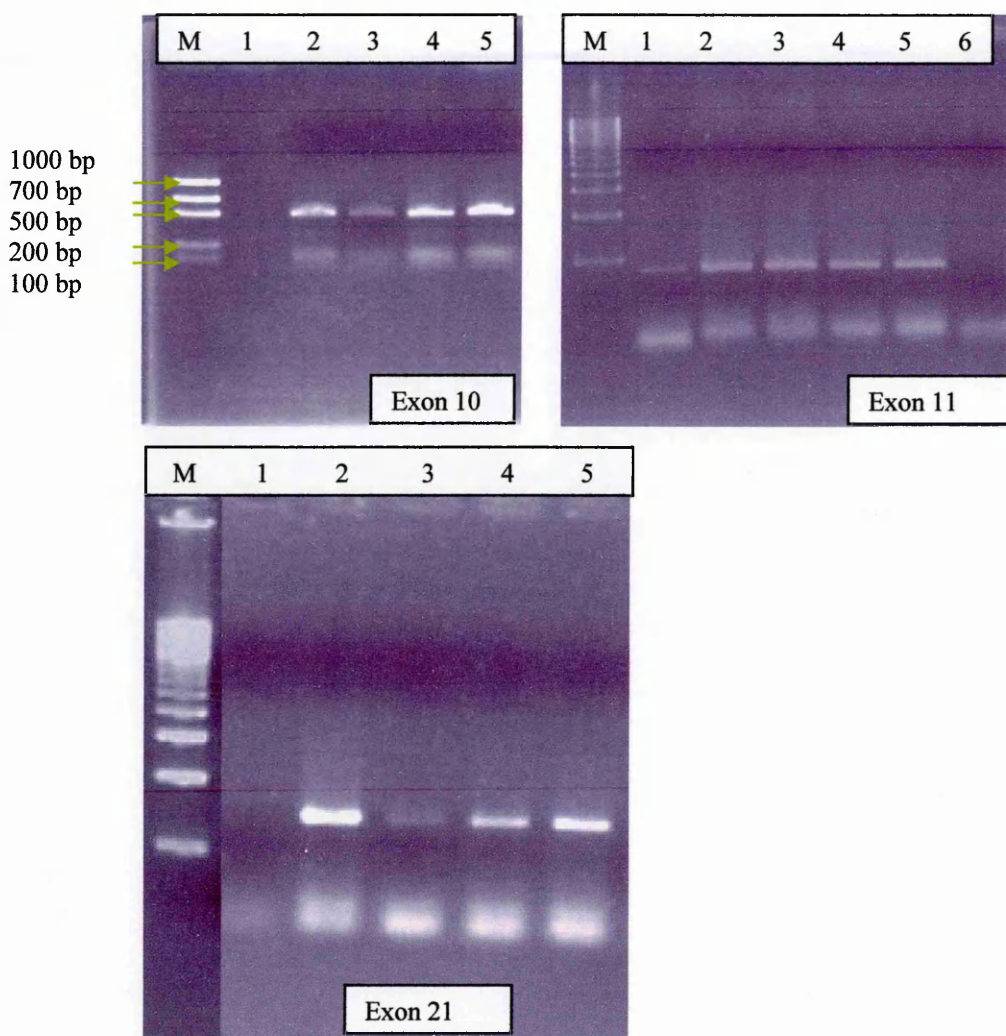


Figure 25. PCR product from biotin labelling of patient gDNA

Three exons from the CFTR gene in human gDNA were amplified using biotin labelled primers 10i-3, 10i-5, 11i-3, 11i-5, 21a and 21b (see Appendix A for sequence). 10µl of amplicon were analysed on a 1.2% agarose gel. The expected band sizes were, exon 10-491 bp, exon 11-425 bp and exon 21-376 bp, however the biotin label reduced DNA migration time and the PCR product appears larger than it is. pGEM marker was used for exons 11 and 21, however not all of the bands are visible, so the band sizes can not be distinguished. Bio-Rad Molecular Mass marker was used for exon 10.

(Figure 25. legend continued)

Exon 10 lanes: M-marker. 1--ve control. 2 and 3- patient 1 biotin labelled PCR, 4 and 5- patient 2 biotin labelled PCR.

Exon 11 lanes: M-marker. 1- +ve control. 2 and 3- patient 1 biotin labelled PCR, 4 and 5- patient 2 biotin labelled PCR, 6 --ve control.

Exon 21 lanes: M-marker. 1 -ve control. 2 and 3- patient 1 biotin labelled PCR, 4 and 5- patient 2 biotin labelled PCR.

Genomic DNA was extracted from blood collected from patients with known mutations. Hybridisation was carried out in order to identify the mutations present in each patient's DNA. Due to time constraints samples from only two patients were analysed.

Hybridisation between amplified DNA from patient 1 and the cystic fibrosis oligonucleotides (see figure 26a) shows positive spots representing hybridisation to the oligonucleotides representing the G542X, G551D and N1303K wild type oligonucleotides and the N1303K mutant oligonucleotide. Neither the mutant nor wild type $\Delta F508$ oligonucleotide hybridised to the patient's DNA. This would imply that the patient's DNA contains only wild type copies of the G542X and G551D mutations and is heterozygous for the N1303K mutation. It is not possible to confirm the presence or absence of the $\Delta F508$ mutation from this hybridisation because there was no hybridisation to either the wild type or mutant oligonucleotide. This result was not expected because either the mutant or wild type $\Delta F508$ should have hybridised to the patient DNA unless both patient copies contained a novel mutation. It is unlikely that two novel mutations are present in this patient. This theory is supported by the absence of a hybridisation signal from hybridisation between the DNA from patient 2 and the $\Delta F508$ oligonucleotides (see later). In summary, from prior knowledge, Patient 1 has a $\Delta F508$ mutation and an N1303K mutation in their CFTR genes. The N1303K mutation was identified by the hybridisation, as were the wild type versions of the G542X and G551D mutations, however the $\Delta F508$ wild type and mutated sequence were not.

Hybridisation was carried out between DNA extracted and amplified from the blood of patient 2 and the four cystic fibrosis sequences of interest. Hybridisations signals representing the wild type versions of N1303K, G542X and G551D were present (see

figure 26b). The hybridisation signal from the G542X oligonucleotide was less intense than the other signals in this hybridisation, it is unclear why this is the case.

Hybridisation signals were not observed in the areas representing the mutant version of any of these sequences, implying that the patient has only wild type copies of these three sequences. No signal is visible for either the $\Delta F508$ wild type or mutant oligonucleotide therefore a conclusion cannot be drawn about the presence or absence of this mutation in this patient. Patient 2 is known to be homozygous for the $\Delta F508$ mutation, therefore this test would not identify the patient's mutation due to the lack of hybridisation signal from either $\Delta F508$ oligonucleotide. In summary, three wild type sequences were correctly identified for the N1303K, G551D and G542X mutations, however the two copies of the $\Delta F508$ mutation known to be present were not identified.

There was a consistent lack of signal showing hybridisation to both the wild type and mutant $\Delta F508$ oligonucleotides throughout this experiment, this was not expected because previous hybridisations with this oligonucleotide have been successful (for example, Figure 16). The lack of signal may indicate a problem with the oligonucleotides themselves, or the PCR amplification of exon 10 (the relevant target). The exon 10 PCR was shown to produce a band of the expected size when analysed by agarose gel electrophoresis (see section 2.2.3) and hybridisation to an amplicon generated in the same way as this one has been previously successful (for example, figure 10), therefore a problem with the PCR product is unlikely. The product of one PCR provides target for two or three hybridisations, and as such is not stored for a long period of time. The oligonucleotides, however, were frozen at stock concentration, then thawed and diluted to working concentration, aliquoted and re-frozen until use. This thawing and re-freezing, coupled with storage of over a year may allow degradation of

the oligonucleotide, due to contamination with nucleases or exposure to heat (IDT, 2002). The most recently synthesised oligonucleotides (N1303K being the most recent) produced stronger hybridisation signals than the $\Delta F508$ oligonucleotides, supporting the hypothesis that the age of the oligonucleotide can have an effect on the hybridisation signal produced. These experiments were carried out towards the end of this project and therefore sufficient time was not available to obtain new oligonucleotide to confirm or deny this hypothesis.

It can be seen from figure 26 that the hybridisation conditions were sensitive enough to between the mutant and wild type sequences for three of the mutations (N1303K, G551D and G542X), however the $\Delta F508$ hybridisations did not produce any signal and could not be used in a diagnostic device at this time.

It is essential that for each mutation either a wild type or mutant oligonucleotide sequence, or both, show a strong signal after hybridisation otherwise it is not possible to be certain that the test has performed correctly. It may be prudent to include control sequences in the final system which would produce a hybridisation signal regardless of the mutations present. The wild type sequence for each mutation can be used as a control however, an additional sequence which does not cover a known mutation or polymorphism may be a more reliable control. Some further developmental work is required in order to increase the signals produced by hybridisation to the $\Delta F508$ oligonucleotides. It is possible that the use of a newly synthesised oligonucleotide would produce a sufficient increase in signal to produce a signal clear enough for use in a diagnostic device. Alternative optimisation methods such as an increase in oligonucleotide attached to the slide, or the use of a more sensitive detection system (for

example, fluorescence) may be required to produce the desired sensitivity and reliability of signal.

Further experiments would need to be performed to establish the reliability of PCR amplification of other regions of the cystic fibrosis gene and hybridisation to oligonucleotides other than those tested here. It is likely that PCR amplification of other areas of the cystic fibrosis gene would require optimisation, however the hybridisation conditions should be sensitive enough to distinguish any mutation. It is expected that all oligonucleotides complementary to the cystic fibrosis or other mutations would hybridise as efficiently as those tested, because the mutation which has the smallest impact on the wild type sequence is a point mutation, and these experiments have included three point mutations and a three base deletion, which were identified correctly. This method should have a similar level of success in detecting other mutations such as insertions or single base deletions as these would be expected to have a greater effect on the sequence than a point mutation because they cause a frameshift (with the exception of deletions or insertions of multiples of three bases). A frameshift mutation not only affects the base that is added or removed, it also shifts the following bases so that they will not be opposite their complementary base as hybridisation occurs.

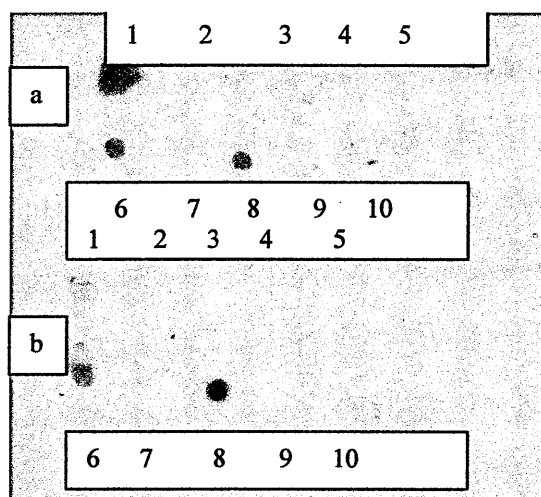


Figure 26. Hybridisation of biotin labelled target (exon 10, 11 and 21) from patients 1 (a) and 2 (b) to cystic fibrosis sequences

20pmol of each oligonucleotide was covalently attached to modified polystyrene slides (see appendix C for oligonucleotide sequence). 1-exon 10 PCR +ve control. 2- Δ F508 wt. 3- Δ F508 mut. 4-G542X wt. 5-G542X mut. 6-G551D wt. 7-G551D mut. 8-N1303K wt. 9-N1303K mut. 10-biotin labelled exon 10 PCR.

4.4 Summary and Conclusions

This chapter has confirmed that genomic DNA can be extracted from anti-coagulated blood using a commercially available kit, as claimed by the manufacturer (Qiagen, UK). Anti-coagulated blood could be stored for up to seven days at 4°C until a sufficient quantity of samples were available for testing, if required. This would allow more cost effective testing by reducing the amount of solutions needed for washing slides as the trays would be filled with solution whether one slide or ten were being processed.

This chapter has also shown that the basic principle of hybridisation of labelled PCR product, to oligonucleotides covalently coupled to modified polystyrene, is suitable for use in a diagnostic device. Some further work is required to establish the reproducibility of the hybridisation to the $\Delta F508$ oligonucleotide used here, however this oligonucleotide has previously given adequate hybridisation signal and re-synthesis would be expected to overcome these problems.

5 *Final Discussion, Future Work and Conclusions*

5.1 *Introduction*

This discussion draws on those at the end of Chapters 2, 3 and 4, bringing them together to assess how far this work has progressed towards the aims, and achieving the objectives, set out at the end of Chapter 1. It also gives an indication as to how further work might progress and draws final conclusions.

5.1.1 *Reiteration of aims and objectives*

The aims and objectives, set out in Section 1.6 stated that this project would create a pre-prototype of a system that could provide genetic information, using cystic fibrosis status in neonates, as an example. It was decided to use reverse dot blot technology as a simple method of screening neonatal DNA for multiple mutations. This project also set out to explore possible oligonucleotide immobilisation methods and hybridisation detection systems suitable for use in a final diagnostic device.

5.1.2 *Genomic DNA extraction*

Bacterial genomic DNA was used as a model system for the initial exploration of immobilisation and hybridisation issues because the use of human blood samples (more relevant to a test for detecting cystic fibrosis) required ethical approval which took time to obtain. *Lactobacillus plantarum* was used because of the convenient availability of that bacterium in the laboratory. Genomic DNA was successfully extracted from bacterial cells and amplified using PCR as previously described (Witt, 1996).

5.1.3 *Physical immobilisation of oligonucleotides to nylon*

Three methods of attaching oligonucleotides to a surface were investigated in this work and comprised of baking, UV-crosslinking and covalent coupling. Bacterial DNA was

used in these investigations before human DNA became available. There should be no general difference in the way that these two different DNAs behave in hybridisation as they are made up of the same basic nucleotides and any specific differences should be due to specific sequence issues. There is a possibility that a G:C rich sequence would hybridise more strongly than an A:T rich sequence due to there being three hydrogen bonds between complementary C and G bases and only two between A and T bases. As different sequences would be required to detect different cystic fibrosis mutations, this did not pose a problem for the use of bacterial DNA as a model for human DNA. Any methods investigated using bacterial DNA required repetition with the finalised human DNA sequences before inclusion in a final system. The use of baking and UV-crosslinking as methods of attaching DNA to nylon membranes was investigated as these methods are commonly used in current molecular biology approaches (Nierzwichi-Bauer *et al.* 1990) and therefore form a reference point for alternative immobilisation methods. These methods of attachment have been successfully used to immobilise short oligonucleotides for hybridisation (for example, Bsat and Batt, 1993 (baking) and Stothard, 2001 (UV-crosslinking)).

As it is not easy to directly quantify the amount of oligonucleotide immobilised on a membrane, the success of hybridisation, as shown by the strength of the hybridisation signal obtained after chemiluminescent detection, was used as an indication of how much immobilisation had taken place. The hybridisation signal may not be the best method of monitoring immobilisation however, as a decrease or lack of signal may be caused at several points during the immobilisation and hybridisation process and not only by a lack of oligonucleotide immobilisation. The hybridisations carried out in this project did not produce strong signals from hybridisation to oligonucleotides that had

been immobilised by baking or UV-crosslinking. Baking and UV-crosslinking of oligonucleotides to membrane does not allow control over which part of the oligonucleotide is attached to the membrane, this may be different for each oligonucleotide, leading to an inconsistency in the amount of oligonucleotide available for hybridisation. This does not confirm the results of Bsat and Batt (1993) who found that satisfactory signals could be obtained after baking oligonucleotides to Zeta-probe GT membrane (Bio-Rad, Richmond, CA, USA). It is likely that the weak signals observed in this work are due to a large part of the short oligonucleotide being involved in immobilisation to the membrane, leaving a minimal part available for hybridisation as discussed by Saiki *et al.* (1989). The stronger signals observed by Bsat and Batt (1993) after attachment by baking may be due to their use of a different membrane. The use of UV-crosslinking by Stothard (2001) to immobilise oligonucleotide probes, produced weak hybridisation signals, which was confirmed by the work carried out in this project. Stothard suggests that the weak signals are due to a low amount of target being applied for hybridisation, however, weak signals may also be fundamentally due to the immobilisation method involving a majority of the oligonucleotide sequence.

5.1.4 *Poly(T) tailing*

Two approaches to address the problem of weak hybridisation signals were subsequently investigated with the objective being to allow the majority of the oligonucleotide sequence to be less involved in immobilisation and thus be freer to behave as if it was in solution. A more consistent method of immobilisation would be expected to produce a stronger and more reproducible signal between hybridisations. The first method investigated was the addition of a poly(T) tail to the 3' end of the oligonucleotide (as used by Saiki *et al.* 1989). A poly(T) tail was added to a short

oligonucleotide complementary to part of the genomic sequence of *Lactobacillus plantarum*, (Oligonucleotide R4A, see Appendix C for sequence) before UV-crosslinking or baking was carried out for attachment of oligonucleotide to a nylon membrane. This method of immobilisation produced a clearly visible hybridisation signal (spots on X-ray film produced by chemiluminescence) compared to the faint or absent signals from hybridisation to non-tailed oligonucleotides. This implies that the addition of a tail allows the oligonucleotide more freedom to hybridise. In retrospect, it would have been useful to have included a non-tailed R4A oligonucleotide in this hybridisation study in order to allow a direct comparison of the strength of hybridisation signal between tailed and non-tailed oligonucleotides of the same sequence and also act as a control. Therefore, the implication that an oligonucleotide with a poly(T) tail produces a clearly visible hybridisation signal should be confirmed by comparison with a non-tailed oligonucleotide of the same sequence before use in hybridisation. After this confirmation, the addition of a poly(T) tail can be used to improve the strength of signals obtained after hybridisation, although the consistency of attachment can not be guaranteed as any of the T bases in the oligonucleotide and poly(T) tail may be bound to the membrane.

5.1.5 Covalent coupling of oligonucleotides to carboxylated membrane

Because the signals observed after hybridisation to oligonucleotides immobilised by baking or cross-linking were not considered strong enough or consistent enough for use in a diagnostic device, (even after the addition of a poly(T) tail) an alternative method of immobilisation was investigated.

Covalent coupling of oligonucleotides with a 3' amino group to carboxylated nylon membrane was carried out, as reported by Ivanovskaya *et al.* (1995), Kawasaki and

Chehab, (1996), Maggio *et al.* (1993) and Zhang *et al.* (1991). This method of immobilisation was considered appropriate, as it should be more easily transferred to surfaces other than nylon membrane than either baking or UV-crosslinking. It is of interest to study hybridisation on solid surfaces in order to improve the ease of handling (and therefore the hybridisation process) of multiple hybridisations because standard methods use membranes, which are flexible and therefore difficult to manipulate during hybridisation. This would be valuable in a hospital where many tests may have to be carried out in one experiment, as slide racks and specially designed hybridisation chambers simplify the high throughput of hybridisation on slides. Nylon membrane was originally used in this work to investigate covalent coupling of oligonucleotides because carboxylated nylon membrane was readily available. It was also considered useful to use nylon membrane as it was the standard surface used for hybridisation, and had been previously used in this work to study the effect of immobilisation by baking and UV-crosslinking. Covalent immobilisation should produce attachment from a specific point on the oligonucleotide, that is, at the amino termination added during synthesis, allowing the rest of the oligonucleotide to remain free and in an appropriate configuration for hybridisation. This might be expected to increase the availability of the oligonucleotide for hybridisation and therefore increase the hybridisation signal observed. Kawasaki and Chehab (1994) carried out hybridisations to covalently immobilised oligonucleotides and found that easily visible, reproducible signals could be observed after hybridisation. The results observed in this project confirm that oligonucleotides can be successfully covalently coupled to carboxylated nylon, by the labelling and detection of the oligonucleotide itself rather than using a hybridisation signal as an implication of the presence of coupled oligonucleotide. Although the results

obtained here are able to provide information about the relative amounts of oligonucleotide attached to nylon membranes, it is not possible to obtain information about the absolute amount. Therefore, the use of radioactive labels was investigated in this context. Cranfield University does not have a license for the use of radioactive labels, therefore a study involving radioactive labels was carried out in laboratories at Innogenetics, Ghent, Belgium. A radioactive labelled oligonucleotide was used to determine the absolute amount of oligonucleotide remaining on the membrane after immobilisation by UV-crosslinking and covalent coupling, followed by standard hybridisation washes. Only a very small percentage of both UV-crosslinked, and covalently coupled oligonucleotide was retained on the membranes. The amount remaining covalently attached to the membrane (4%) was a ten-fold increase compared to UV-crosslinking, however, it was much less than expected as previous experiments (Joos *et al.*, 1997) showed retention of 40-50% of the oligonucleotide covalently immobilised on silanised glass. The results from this project would suggest that the immobilisation of oligonucleotide for the system studied in this work is much less efficient than shown in related reports. It is likely that the difference between the amount of oligonucleotide remaining on the surface in this work and that carried out by Joos *et al.* is due to the use of a different surface and coupling chemistry, suggesting that silanised glass should be investigated further as a surface for hybridisation. In the context of a final commercial diagnostics device, if the efficiency of hybridisation was increased, even from the 40-50% observed by Joos *et al.*, less probe would need to be deposited, allowing a reduction of production cost of a final device. It is important to note, however, that the experiment carried out here using radioactive labels was only a preliminary experiment. The results will need to be confirmed by repetition before

significant conclusions can be drawn regarding the amount of oligonucleotide remaining bound to the membrane.

5.1.6 Human genomic DNA extraction, amplification and hybridisation to covalently coupled oligonucleotide

Experiments were conducted to investigate hybridisation to covalently coupled oligonucleotide and compare the strength of hybridisation signal obtained after covalent coupling to that obtained after UV-immobilisation. At this point in the project, ethical approval was granted, and seven human blood samples were obtained from healthy volunteers. It is important to note that the method of blood collection (for example, the addition of anti-coagulant, or long periods of storage before DNA extraction) can affect the yield of genomic DNA. In this project DNA was extracted from control (healthy volunteer) blood within 30 minutes of sample collection, therefore anti-coagulant was not required.

Genomic DNA was extracted from the volunteer blood samples and sequences from the cystic fibrosis gene were amplified, labelled with an appropriate label for use in a chemiluminescent read-out system (Digoxigenin (DIG)) and used as target in hybridisations. Four cystic fibrosis mutations were studied as examples; these were three point mutations and a three base deletion. A point mutation has the least impact on the sequence of the DNA as only a single base is altered; therefore it is likely that if a mutation detection method can correctly identify a point mutation, it will be able to identify a deletion or insertion, (which will cause a frameshift unless it consists of a multiple of three bases). Hybridisation signals were expected only from hybridisation to the wild type sequence oligonucleotides because the DNA extracted from blood from volunteers was not expected to contain any cystic fibrosis mutations. However,

although the volunteers were all healthy, there was a possibility that they could be carriers of a cystic fibrosis mutation. The results of hybridisations carried out to examine the hybridisation of target amplified from human DNA to covalently immobilised probes showed that covalent attachment of oligonucleotides to nylon membrane produces a consistent increase in hybridisation signal (approximately three-fold, evaluated visually) compared to physical immobilisation methods. Hybridisation signal is used in this project to imply an increase in hybridisation and immobilisation of oligonucleotide. It is important to note, however, that without further work, preferably using radioactive labels to quantify the absolute amount of immobilisation and hybridisation, the increase in hybridisation signal cannot be clearly attributed to an increase in immobilisation or hybridisation. This could not be done in this study as there was only limited access to a laboratory licenced to handle radioisotopes.

It was observed from the hybridisation results, that none of the control DNA used contained any of the four cystic fibrosis mutations tested for in this work.

5.1.7 *Spacers*

It has been suggested that the use of poly(T) tails on immobilised oligonucleotides produces a spacer effect, allowing the oligonucleotide to move more freely and increase the hybridisation signal obtained (Zhang *et al.*, 1991). The use of UV-crosslinking to attach the oligonucleotide to the membrane may negate the spacer effect by immobilising the whole of the poly(T) tail, or even bases within the oligonucleotide sequence. It was decided to investigate the effect of a DNA spacer (Poly(T) tail) in conjunction with an amino termination to allow covalent coupling of the oligonucleotide to carboxylated membrane. An increase in the strength of hybridisation signals was observed after covalent immobilisation of tailed oligonucleotides when

compared to attachment by UV-crosslinking. Covalent immobilisation of tailed oligonucleotides allowed the hybridisation signals to be readily visible, whereas tailed oligonucleotides immobilised by UV-crosslinking did not produce consistently observable hybridisation signals. Some correlation was also seen between the length of the poly(T) tail (in conjunction with covalent attachment) and the strength of the hybridisation signal, with a tail of approximately 60 bases increasing the signal intensity approximately 3-fold compared to an oligonucleotide with no tail at all, repeating the pattern seen above when comparing UV and covalent immobilisation. An increase of a further 30 bases to the tail length produced a small increase the hybridisation signal intensity. Zhang *et al.* (1991) also observed that the longer the spacer tail on an oligonucleotide, the stronger the hybridisation signal produced, and investigated tails of up to 400 bases in length. In the work carried out in this project this effect was seen to a lesser extent than in the study by Zhang *et al.* (1991) although in the majority of cases an increase in signal was observed to correlate with an increase in spacer length. It is not clear why this was the case, although there may have been differences in the detailed experimental protocols, for example, exposure time of X-ray film to the chemiluminescent signal or the reagents used, which make it difficult to compare the density of spots produced in this work and by Zhang *et al.* For a final device, if the addition of a spacer to a commercially produced oligonucleotide was desired, this would significantly increase its cost. It should also be considered that it is not possible to be absolutely certain of the length of the spacer once it reaches approximately 80 bases (MWG-Biotech, Personal Communication), therefore the synthesised tail itself may not be consistent between batches of oligonucleotide. These disadvantages must be

balanced against the increase in hybridisation signal observed, depending on the priorities of the experiment, that is, cost or increased hybridisation signal.

5.1.8 Covalent immobilisation of oligonucleotide to activated polystyrene

Once it had been established that hybridisation could be successfully carried out to amino terminated probes covalently coupled to carboxylated nylon membrane, an investigation into the use of polystyrene as a surface for hybridisation was performed. Polystyrene was chosen as a surface because it is an easily obtainable, rigid surface, for which there are modification techniques available to produce carboxyl groups on the surface. In terms of a final device, polystyrene can also be injection moulded, which would simplify production of slides and more complex devices incorporating fluidics and other integrated assay components. The decrease in the area that hybridisation is carried out on when using a slide compared to a nylon membrane also allows a reduction in the amount of each solution used throughout the immobilisation and hybridisation steps. It is also important to note, however, that polystyrene provides a 2-D surface for the attachment of oligonucleotide, while nylon is a porous 3-D surface. A porous 3-D surface will allow the attachment of an increased amount of oligonucleotide compared to a 2-D surface. This was expected to be reflected as a decrease in the strength of hybridisation signal, and also to create problems for the use of chemiluminescent detection techniques.

Signals observed from hybridisations to oligonucleotides immobilised on polystyrene slides were as strong as those observed after hybridisation to oligonucleotides immobilised on carboxylated membrane. Once the use of coverslips and a humid chamber during incubation with small amounts of solutions had been introduced, chemiluminescent detection of hybridisation could be carried out satisfactorily on 2-D,

non-porous polystyrene slides. The washes carried out during immobilisation, hybridisation and detection can be carried out with the slides in slide racks, allowing the faster, easier throughput of more hybridisations than when using nylon membranes which require a separate hybridisation tube and wash tray for each hybridisation.

Chemical modification of commercially produced polystyrene slides was carried out and confirmed by the use of contact angle measurements (to detect changes in the hydrophobicity of the surface) and AFM imaging (to observe physical alterations to the surface). The results of these methods of identification of modification were inconclusive, with only small differences being observed between modified and unmodified slides. Thus these techniques are probably unable to aid a study of this type. Zammateo *et al.* (1996) used binding of $^3\text{[H]}$ lysine to indicate the presence of carboxyl groups on their modified polystyrene, however due to the lack of a radioactivity licence at Cranfield University it was not possible to use this method. It is likely that the polystyrene slides were unevenly modified because the covalent attachment of oligonucleotide was not observed to be consistent across the slide. The modifications carried out by Zammateo *et al.*, (1996) were carried out at a higher temperature (60°C) and for a shorter period of time (30 minutes) than the modification in this project. Zammateo *et al.* also used shaking during modification which may prevent the reagent used for oxidation of the polystyrene (potassium permanganate) settling out of solution, altering its concentration in that solution. The increase in temperature may increase the speed of modification, alternatively, there may be an optimum modification time. It would be useful to investigate these issues in further work. As the presence of carboxyl groups could be neither confirmed nor denied, covalent immobilisation of oligonucleotide attempted. The covalent coupling of oligonucleotide can, in itself,

confirm the presence of carboxyl groups. Immobilisation of labelled oligonucleotides was carried out to confirm that it was possible to do this before hybridisation was attempted. Labelled oligonucleotides were covalently coupled to modified polystyrene slides using an adapted version of the method used for immobilisation to carboxylated membranes. The label detection protocol also required modification to take into account that polystyrene slides are 2 dimensional compared to the 3 dimensional nature of porous membrane, for example, coverslips were required to prevent the evaporation of the small amounts of solutions used. Two different labelling and detection systems (DIG and biotin) were explored to identify which was more useful for work on polystyrene slides. Both systems were originally created for use with porous nylon membranes, and, as such, may not produce the required intensity of signal in a 2-D format. It was observed that oligonucleotide attachment had taken place, although the biotin labelling and detection system produced a stronger, more consistent signal when used to detect the presence of covalently immobilised oligonucleotides, therefore further work was carried out using only this system.

5.1.9 Hybridisation to covalently immobilised oligonucleotide on polystyrene

Confirmation of the covalent coupling of oligonucleotides to modified polystyrene was the first step towards carrying out hybridisation on these slides. Hybridisations of cystic fibrosis sequences amplified from control blood samples were carried out as a preliminary test of the hybridisation system before patient samples were examined. Hybridisation to the wild type and mutant sequences for each of four cystic fibrosis mutations were tested for, each on a different slide. A vast excess of mutant sequence probe was deposited onto the slide for hybridisation in order to identify whether this would affect the specificity of hybridisation. Hybridisation signals were not expected in

the positions corresponding to the mutant sequences of each mutation (as the DNA samples used were those found to contain no mutations previously in this work), however, signals were observed from the mutant sequences of two of the mutations studied (N1303K and Δ F508). These mutant sequence oligonucleotides were present at a concentration of 100 pmol per μ l. When the mutant sequence oligonucleotide was present at a 5 times more dilute concentration however, there was no non-specific hybridisation to the mutant sequence oligonucleotide. This suggests that the aberrant signals were due to the high concentration of oligonucleotide used. Work has been carried out previously to identify cystic fibrosis mutations via the reverse dot blot method (Kawasaki *et al.*, 1993) where hybridisation to oligonucleotides covalently coupled to membrane was performed, and mutations were correctly identified. The transfer of this technology to rigid plastic slides results in a device similar to the microarrays already in use. The advantages of polystyrene slides are that they are less likely to be dangerous if broken, and the possibility of injection moulding for production on a large scale (as mentioned above). Other advantages of polystyrene slides are identical to those for other slide-based microarrays which include the use of less of each of the solutions used during hybridisation and detection washes, including oligonucleotide probe and labelled target, as well as a greater ease of handling than nylon membrane.

Glass slides were an option which was not explored, however in retrospect, this may have been useful due to their widespread use in the field of microarrays (Sinclair, 1999).

5.1.10 Deposition systems

Once the principles of hybridisation on polystyrene slides had been established, different options for the deposition of oligonucleotide were considered. The use of a

hand-held pipette does not produce drops of a consistent size, and may not be accurate for the deposition of small volumes and wholly inappropriate for a mass-produced device. Two automated methods of drop deposition were considered, the BioDot CV1000 automated syringe dispense system and a robotic pin-based micro-arrayer (for example, Q-Array, Genetix, UK). The BioDot system was used in this project for convenience in the Research and Development situation. The BioDot syringe system was not designed for this use and therefore, there were some disadvantages in using it for oligonucleotide deposition. The BioDot has only one large reservoir, which would require cleaning between each oligonucleotide used. It would be impractical and very expensive to fill the reservoir (10mls) with oligonucleotide solution. A modification was made to the BioDot syringe system, which involved the attachment of an extra pump to allow oligonucleotide to be drawn, or back-filled, through the dispense nozzle instead of using the reservoir. This system was tested by the deposition of oligonucleotides for hybridisation. Although the drops on the slide were deposited consistently, the oligonucleotide did not appear to be evenly distributed, (identified by variations in hybridisation signals observed). The first and last drops deposited appear to contain little or no oligonucleotide (that is, they produce either a weak, or no, hybridisation signal), the drops between these produced consistent hybridisation signals, which are easily visible by eye. It is likely that this fluctuation in hybridisation signal is due to the flushing through of the tubes on the BioDot and the mixing of oligonucleotide solution with water (which is used to fill the tubes and reservoir rather than oligonucleotide solution itself). The addition of multiple pumps to dispense oligonucleotide and the reduction of variation in deposition of oligonucleotide would allow this system to be a more viable option for the deposition of oligonucleotide.

The BioDot system has been useful for the the Research and Development of the hybridisations in this project, however for large scale manufacturing, an arrayer would be the preferred option.. The use of a diagnostics company to perform the arraying would reduce the impact of the cost of the arrayer on the hospital. In this way the diagnostic device could be consistently produced and then supplied for use in a number of hospitals.

5.1.11 Detection systems

Although biotin labelling and chemiluminescent detection of target has been successfully used in this work, it was originally designed for use with three-dimensional porous membranes. Two alternative methods of detection were investigated. As the hybridisation is working towards a diagnostic test, rapid throughput of samples is desirable. A visual detection system would decrease throughput time and allow more tests to be analysed in a working day. As biotin labels and streptavidin-enzyme chemiluminescence detection had already been used successfully in this work, the use of coloured streptavidin beads for detection of hybridisation was investigated.

Although the initial hybridisation appeared to produce specific signals where hybridisation was expected (from experiment using chemiluminescent detection run in parallel), further hybridisations showed non-specific binding of coloured beads to the membrane. The coloured streptavidin beads produced by Bangs laboratories were designed to be used as a solid support for affinity separation, they have also been used as a surface for hybridisation, (Fry, *et al*, 1992; Tong and Smith, 1992; Fujita and Swaroop, 1995; Ji and Smith, 1993). It is likely that these beads are too large to remain bound to biotin labels on hybridised PCR product. This system was not found to

produce accurate results, although it is possible that under the correct conditions it could be a viable system for the visual readout of hybridisation results.

Fluorescent labelling of target is commonly used to label target for hybridisation to microarrays (Sinclair, 1999). The use of fluorescent detection requires only stringency washes after hybridisation compared to the multiple block and wash incubations required for chemiluminescent detection, therefore this method of detection would reduce the time taken to process samples. A simple method of fluorescent labelling is to use commercially produced oligonucleotide PCR primers with a fluorescent label attached for target amplification. Cy5 was used as a convenient label, and added to target DNA in this way. Hybridisations were carried out on polystyrene slides and the slides were scanned, however, no signal could be detected. As each piece of target DNA had only one fluorescent label it was considered that the hybridisation signals may be overpowered by background signal caused by the binding of unincorporated fluorescently labelled primers to the slide, therefore an alternative method of labelling was used. This method was based on one that incorporated aminoallyl-dUTP into the PCR for the subsequent attachment of Cy5 moieties (Hasseman, 2001). Hybridisation with this multiply labelled target did not produce observable hybridisation signals. If fluorescent labels are exposed to light, there is a possibility them of undergoing photo-bleaching, although this is unlikely to be the cause of the lack of signal as care was taken to avoid exposure of the fluorescent labels to light. The sequences used for the fluorescent hybridisation were identical to those used before with chemiluminescent detection, therefore hybridisation should have occurred. Therefore it must be concluded that the physical difference between the fluorescent label and the biotin label used previously altered the target so that the optimisation of hybridisation conditions was

required. This could be confirmed by the re-optimisation of the hybridisation conditions. In order to reduce possible photobleaching of the Cy5 the entire PCR amplification of target and hybridisation would be carried out in a dark room. If this did not produce an observable signal, the background could be reduced by increasing the stringency of the washes. SDS is known to fluoresce, therefore it cannot be used in the final washes, however, it can be used in the preliminary washes providing it is removed subsequently. If elimination of photo-bleaching does not allow fluorescent signals to be produced, investigations must be made into alternative explanations for the lack of signal, such as inadequate amount of target, or lack of hybridisation (which could be tested using an antibody to Cy5 conjugated to an enzyme for chemiluminescent detection). The amount of target added to the hybridisation could be optimised in order to identify that which produces the strongest hybridisation combined with the least background signal.

Time constraints meant that it was not possible to investigate this lack of signal further. Within this project, it must be concluded that fluorescent labelling is not a viable system for detection of hybridisation signals without further study.

As neither of the alternative methods of hybridisation detection investigated proved viable for use in this work, biotin labelling and chemiluminescence were used for the testing of patient DNA samples.

5.1.12 Demonstration of diagnostic

Due to the time constraints on this work, and the limitation of taking blood samples only when patients were being treated, only three patient blood samples were obtained. The blood samples were collected in heparin (anti-coagulant) tubes. The final test

produced would be used in a hospital where the length of time between blood collection and DNA extraction cannot be guaranteed; therefore blood samples would require collection with anti-coagulant to prevent clotting. Due to the cost of heparin tubes, only blood from cystic fibrosis patients was collected in these. Genomic DNA extraction and subsequent PCR amplification was not expected to be affected by the use of Heparin at blood collection, although it was necessary to confirm that this was the case (Personal communication, Qiagen, UK). It would be useful to check that Heparin does not interfere with the extraction and amplification of DNA from people who do not carry a cystic fibrosis mutation, as this has not been confirmed in this work. This should be confirmed before the system is used a diagnostic test. Heparin has not been found to affect DNA extraction using the QIAamp method in previous work (Steiner *et al.*, 2002; Zimmerman *et al.*, 2002).

Oligonucleotides corresponding to the mutant and wild type sequence of all four mutations to be tested for were immobilised on one slide as this is a similar format to the microarray system this study is working towards, where all of the mutations of interest are represented on a single slide. Because of this, the target PCR product was a mixture of all four targets produced. This mixture should not affect the hybridisation as the target was denatured before application to the slide. Genomic DNA was extracted and amplified from two patients with known mutations. Biotin was used to label the target and chemiluminescent detection of hybridisation was carried out. The hybridisation detected the wild type and mutant sequences as expected for each of the mutations, with the exception of the $\Delta F508$ mutation. This latter oligonucleotide was the first one purchased, and there does appear to be a correlation in this work between storage age of oligonucleotide and strength of hybridisation signal. This can be

observed where hybridisation to the oldest oligonucleotide produces weak signals in comparison to hybridisation to the newest oligonucleotide. This was seen for hybridisations carried out on both nylon membrane and polystyrene. If oligonucleotides are aliquoted and frozen, and not subjected to multiple freeze/thaw cycles then they would not be expected to degrade, however, there is clearly a difference between the strength of these hybridisation signals. In order to confirm that it is the age of the oligonucleotide causing the alteration in signal intensity, it is important to obtain freshly synthesised oligonucleotides for comparison in future studies.

With the exception of the $\Delta F508$ wild type and mutant sequences, hybridisation occurred to the expected oligonucleotides, once the concentration of mutant sequence oligonucleotide had been reduced to 20 pmol (see previously). Therefore, the main aims and objectives of the project were met in this device, as a pre-prototype of a multiple mutation diagnostic for use in a hospital for the detection of cystic fibrosis has been produced. The pre-prototype can detect the presence of the N1303K mutation and the wild type sequence of two other mutations (G551D and G542X). Some further work is required to detect the $\Delta F508$ mutant and wild type sequences.

5.2 Recommendations for Future Work

While this project has made significant steps towards the development of a suitable method of mutation detection for neonatal screening, there are areas of work that can be recommended for the future. These would confirm some of the results presented here and provide more information on the suitability of alternative methods of hybridisation detection to chemiluminescence.

(a) To confirm the results reported in Chapter 2, the experiments using radioactive labels should be repeated to determine absolute amounts of oligonucleotide remaining on membrane after washes. The confirmation of these results will show that a very low percentage of the deposited oligonucleotide is coupled to the membrane, even when using covalent attachment. Once this has been established, methods of increasing the amount of attached oligonucleotide could be investigated. A study looking at the increase of oligonucleotide concentration and optimisation of the percentage of EDC used to activate the membrane could be undertaken.

(b) A method of increasing the efficiency of covalent attachment would allow the reduction of wasted oligonucleotide and reduce the cost of carrying out hybridisation with oligonucleotides. It would be of interest to determine the absolute amount of oligonucleotide which remains coupled to modified polystyrene slides after washes as this would be relevant to the production of a mutation detection device in this work.

(c) It would be useful to use radioactive labels to study the efficiency of hybridisation to oligonucleotide by radioactively labelling the target and hybridising to a known amount of oligonucleotide. As above, if the efficiency of hybridisation could be improved the cost of hybridisation could be reduced.

(d) Chemiluminescent detection of hybridisation is a time consuming method of detection. Two alternative methods were investigated in this work, coloured streptavidin coated beads and fluorescence. Coloured beads would be the simplest method to use as the results can be observed by eye, rather than requiring a scanner. When coloured beads were used in this work it is believed that the streptavidin-biotin bond either did not occur, or was disrupted by the vigorous washing techniques used. It is necessary to test this by carrying out vigorous washes when using chemiluminescent

detection in order to confirm that it is the biotin-streptavidin bond which is being affected and not the hydrogen bonds involved in DNA hybridisation. Once this has been established, biotin-streptavidin bonding should be carried out under varying conditions, for example in buffers containing various salt concentrations in order to discover the optimum conditions to allow the bond to form. If a method cannot be found to ensure the specific binding of streptavidin coloured beads to biotin labelled PCR product, and alternative method of detection must be used. Fluorescence requires a scanner or other system in order to observe the hybridisation results, however it requires fewer incubations and is therefore less time consuming than chemiluminescent detection.

The lack of fluorescent signals observed in this work could be addressed as discussed in section 5.1.11 (page 170).

If these investigations do not produce an observable signal, it will be necessary to use chemiluminescent hybridisation detection.

Optimisation of the chemiluminescent method of detection to reduce incubation time and so reduce the time taken to produce a result would give a more useful test in a hospital situation

(e) Once the detection method has been established, the issue of lack of hybridisation to the $\Delta F508$ oligonucleotides when using target amplified from patient blood must be addressed. Freshly synthesised oligonucleotides should be obtained and used in hybridisation with patient target. If this does not resolve the problem then the hybridisation stringency conditions must be re-examined and optimised. It may also be necessary to investigate alternative oligonucleotide suppliers. Once the hybridisation

signals are satisfactory, more patient samples must be tested in order to establish the reproducibility of the hybridisation. As many patient samples as possible should be tested 'blind' so that the hybridisation is known to be a true reflection of the patients mutations without the experimenter being influenced by the knowledge of the patients mutations.

(f) Once the best detection method has been identified, and the reproducibility of the test is established, it will be necessary to create a prototype system and protocol. Ideally this would be tested by a technician from a Biochemistry Department in a District General Hospital as this is the setting the device is designed to be used in. The system will comprise of a printed slide, a 'PCR' mix, containing all of the PCR ingredients except the Taq polymerase, Taq, hybridisation buffer and a system of detection (preferably coloured beads if this system can be optimised). Genomic DNA extraction will be carried out using the Qiagen QIAamp kit.

(g) If the genetic test is to be used to test only neonates whose sweat test results are indicative of cystic fibrosis then only a few tests (1-3) each week will be required in a single hospital. If the test is to be carried out on each baby born, however, the demand will be much higher and mass manufacture of slides will be a more realistic option. Investigations into the stability of oligonucleotides on printed slides during storage should be investigated to show if there is deterioration of the oligonucleotides over time. Once the oligonucleotide is dried onto the slide it should not degrade, however studies have not been undertaken into how long the carboxyl groups produced by chemical modification of the slides will remain stable. If these investigations show that printed slides can be stored for a relatively long period of time (for example up to six months) then the use of a mass manufacturing system will be viable.

The BioDot CV1000 syringe system could be modified by the addition of extra pumps and shorter tubes and smaller oligonucleotide reservoirs, in order to allow the printing of several oligonucleotides in one run. This modification would facilitate the mass production of printed slides.

The hybridisations performed in this work will allow the testing of genomic DNA for four cystic fibrosis mutations. The test can be expanded to include other cystic fibrosis mutations or used to identify genetic mutations involved in other diseases. When new oligonucleotides are added to the hybridisation, optimisation may be necessary to ensure the correct stringency conditions are being used to allow discrimination between wild type and mutant oligonucleotides. It may also be necessary to optimise or create the PCR cycles used to amplify the new target of interest.

The establishment of this prototype could lead to production of a test for any genetic mutation of interest. Depending on the oligonucleotides used, several different disease mutations could be tested for in a single hybridisation.

5.3 Conclusions

This project aimed to produce a pre-prototype of a genetic test for use in a hospital, using cystic fibrosis diagnosis as a model. The constraints imposed by the use of the test in a hospital are that both setting up and running the diagnostic test should be as cheap as possible, and also that the test should be simple enough to be used by staff with no specialist molecular biology knowledge.

From the work carried out in this thesis, the most suitable system for use in a hospital would use an arrayer to deposit oligonucleotides onto modified polystyrene slides as this method of deposition is consistent and once purchased, an arrayer requires no

modification before use, as the BioDot syringe system would. Polystyrene slides would be used because of their ability to undergo modification to produce carboxyl groups on the surface. Chemiluminescence would be the most suitable method of hybridisation detection studied in this work as it has been found to be the most robust method, producing the most consistent hybridisation signals in our hands. The use of chemiluminescent detection adds 4-5 hours to the length of time take to process a test, however, the more consistent results obtained justify this increase.

The use of hybridisation on polystyrene slides and chemiluminescent hybridisation detection requires some further investigation in order to ensure that the hybridisation is reliable, however a pre-prototype has been established which may be useful as a basis for further work.

References

- Adam, G. and Delbruck, M. (1968) *Structural Chemistry and Molecular Biology*, San Francisco: W.H.Freeman and Co.
- Baens, M., Buyse, I., Stul, M., Decorte, R., Marynen, P. and Cassiman, J.-J. (1990) Rapid nonradioactive detection of point mutations an codon 12, 13, and 61 of the N-ras gene. *Technique* **2**, 268-272.
- Bardin, P.G., Pickett, M.A., Robinson, S.B., Sanderson, G., Holgate, S.T. and Johnston, S.L. (1993) Comparison of 3' and 5' biotin labelled oligonucleotides for in situ hybridisation. *Histochemistry* **100**, 387-392.
- Beaudet, A.L. (1990) Carrier screening for cystic fibrosis. *American Journal of Human Genetics* **47**, 603-605.
- Beier, M. and Hoheisek, J.D. (1999) Versatile derivatisation of solid support media for covalent bonding on DNA-microchips. *Nucleic Acids Research* **27**, 1970-1977.
- Bertina, R.M. (2001) Genetic aspects of venous thrombosis. *European Journal of Obstetrics and Gynecology and Reproductive Biology* **95**, 189-192.
- Biffi, A., Sersale, G., Cassetti, A., Villa, A., Bordinon, C., Assael, B.M. and Conese, M. (1999) Restoration of bacterial killing activity of human respiratory cystic fibrosis cells through cationic vector-mediated cystic fibrosis transmembrane conductance regulator gene transfer. *Human Gene Therapy* **10**, 1923-1930.

Billings, P.R. (1990) Mutation analysis in cystic fibrosis. *The New England Journal of Medicine* **323**, 62.

BioDot, www.biodot.co.uk. Accessed January 2002.

Birk, H.W. and Koepsell, H. (1987) Reaction of monoclonal antibodies with plasma membrane proteins after binding on nitrocellulose: renaturation of antigenic sites and reduction of non-specific antibody binding. *Analytical Biochemistry* **164**, 12-22.

Blanchard, A. (1998) Synthetic DNA arrays. *Genetic Engineering* **20**, 111-123.

Blecinski, C.F. and Richert, C. (1998) Monitoring the hybridization of the components of oligonucleotide mixtures to immobilized DNA via Matrix -assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry* **12**, 1737-1743.

Bowling, F.G., Rylatt, D.B., Bunch, R.J., Watson, A.R.A., Elliot, J.E. and Bundesen, P.G. (1987) Monoclonal antibody-based enzyme immunoassay for Trypsinogen in neonatal screening for Cystic Fibrosis. *The Lancet* **8537**, 826-827.

Brinson, F. C. Performance of a multiplex DNA test for cystic fibrosis.
<http://www2.perkin-elmer.com/ab/apply/dr/dra2c5.html>. Accessed September 1999.

Bsat, N and Batt, C A (1993) A combined modified reverse dot-blot and nested PCR assay for the specific non-radioactive detection of *Listeria monocytogenes*. *Molecular and Cellular Probes*, **7**, 199-207

Cai, S.-P., Wall, J., Wai Kan, Y. and Chehab, F.F. (1994) Reverse dot blot probes for the screening of β -Thalassemia mutations in Asians and American Blacks. *Human Mutation* **3**, 59-63.

Caruso, F., Rodda, E., Furlong, D.N. and Haring, V. (1997) DNA binding and hybridization on gold and derivatized surfaces. *Sensors and Actuators B* **41**, 189-197.

Chan, V., Graves, D., Fortina, P. and McKenzie, S.E. (1997) Adsorption and surface diffusion of DNA oligonucleotides at liquid/solid interfaces. *Langmuir* **13**, 320-329.

Chehab, F.F. and Wall, J. (1992) Detection of multiple cystic fibrosis mutations by reverse dot blot hybridization: a technology for carrier screening. *Human Genetics* **89**, 163-168.

Cook, A.F., Vuocolo, E. and Brakel, C.L. (1988) Synthesis and hybridization of a series of biotinylated oligonucleotides. *Nucleic Acids Research* **16**, 4077-4095.

Costes, B., Fanen, P., Goossens, M. and Ghanem, N. (1993) A rapid, efficient, and sensitive assay for simultaneous detection of multiple cystic fibrosis mutations. *Human Mutation* **2**, 185-191.

Cronin, M.T., Fucini, R.V., Mee Kim, S., Masino, R.S., Wespi, R.M. and Miyada, C.G. (1996) Cystic fibrosis mutation detection by hybridization to light-generated DNA probe arrays. *Human Mutation* **7**, 244-255.

Crossley, J.R., Berryman, C.C. and Elliott, R.B. (1977) Cystic fibrosis screening in the newborn. *The Lancet* **ii**, 1093-1095.

Crossley, J.R., Elliott, R.B. and Smith, P.A. (1979) Dried blood spot screening for cystic fibrosis in the newborn. *The Lancet* **1**, 472-474.

Crossley, J.R., Smith, P.A., Edgar, B.W., Gluckman, P.D. and Elliott, R.B. (1981) Neonatal screening for cystic fibrosis, using immunoreactive trypsin assay in dried blood spots. *Clinica Chimica Acta* **113**, 111-121.

Cuppens, H., Buyse, I., Baens, M., Marynen, P. and Cassiman, J.-J. (1992) Simultaneous screening for 11 mutations in the cystic fibrosis transmembrane conductance regulator gene by multiplex amplification and reverse dot-blot. *Molecular and Cellular Probes* **6**, 33-39.

Cuppens, H., Marynen, P., De Boeck, C. and Cassiman, J.-J. (1993) Detection of 98.5% of the mutations in 200 Belgian cystic fibrosis alleles by reverse dot-blot and sequencing of the complete coding region and exon/intron junctions of the CFTR gene. *Genomics* **18**, 693-697.

Dattagupta, N., Rae, P.M.M., Huguenel E.D., Carlson, E., Layga, A., Shapiro, J.A. and Albarella, J.P. (1989) Rapid identification of microorganisms by nucleic acid hybridization after labelling the test sample. *Analytical Biochemistry* **177**, 85-89.

DeMarchi, J.M., Richards, C.S., Fenwick, R.G., Pace, R. and Beaudet, A.L. (1994) A robotics-assisted procedure for large scale cystic fibrosis mutation analysis. *Human Mutation* **4**, 281-290.

Dezateux, C. (2001) Screening newborn infants for cystic fibrosis. *Journal of Medical Screening* **8**, 57-60.

Doull, I.J.M., Ryley, H.C., Weller, P. and Goodchild, M.C. (2001) Cystic fibrosis-related deaths in infancy and the effect of newborn screening. *Pediatric Pulmonology* **31**, 363-366.

Eggerding, F.A., Iovannisci, D.M., Brinson, E., Grossman, P. and Winn-Deen, E.S. (1995) Fluorescence-based oligonucleotide ligation assay for analysis of cystic fibrosis transmembrane conductance regulator gene mutations. *Human Mutation* 153-165.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B. and Nielsen, P.E. (1993) PNA hybridises to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365**, 566-568.

Elliott, R.B. (1991) Newborn screening for cystic fibrosis: a historical perspective. *Pediatric Pulmonary Supplement* **7**, 7-10.

Ernst, L.A., Gupta, R.K., Mujumdar, R.B. and Waggoner, A.S. (1989) Cyanine dye labelling reagents for sulfhydryl groups. *Cytometry* **10**, 3-10.

Estivill, X., McLean, C., Nunes, V., Casals, T., Gallano, P., Scambler, P. and Williamson, R. (1989) Isolation of a new DNA marker in linkage disequilibrium with cystic fibrosis, situated between J3.11 (D7S8) and IRP. *American Journal of Human Genetics* **44**, 704-710.

Farrell, P.M., Kosorok, M.R., Rock, M.J., Laxova, A., Zeng, L., Lai, H-C., Hoffman, G., Laessig, R.H., Splaingard, M.L., and the Wisconsin Cystic Fibrosis Neonatal Study Group. (2001) Early diagnosis of cystic fibrosis through neonatal screening prevents severe malnutrition and improves long-term growth. *Pediatrics* **107**, 1-13.

Faruqi, A.F., Hosono, S., Driscoll, M.D., Dean, F.B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., Du, Y., Kingsmore, S., Knott and Lasken, R.S. (2001) High-throughput genotyping of single nucleotide polymorphisms with rolling circle amplification. *BMC Genomics* **2**, 4.

Feriotto, G., Lucci, M., Bianchi, N., Mischiati, C. and Gambari, R. (1999) Detection of the (F508del) mutation of the cystic fibrosis gene by surface plasmon resonance and biosensor technology. *Human Mutation* **13**, 390-400.

Fodor, S.P.A., Rava, R.P., Huang, X.C., Pease, A.C., Holmes, C.P. and Adams, C.L. (1993) Multiplexed biochemical assays with biological chips. *Nature* **364**, 555-556.

Forman, J.E., Walton, I.D., Stern, D., Rava, R.P. and Trulson, M.O. (1998) Thermodynamics of duplex formation and mismatch discrimination on photolithographically synthesised oligonucleotide arrays. *ACS Symposium Series* **682**, 206-228.

Fortina, P., Conant, R. Parrella, T., Rappaport, E., Scanlin, T., Schwartz, E., Robertson, J.S., and Surrey, S. (1992) Fluorescence-based, multiplex allele-specific PCR (MASPCR) detection of the $\Delta F508$ deletion in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Molecular and Cellular Probes* **6**, 353-356.

Fry, G., Lachenmeier, E., Mayrand, E., Giusti, B., Fisher, J., Johnston-Dow, L., Cathcart, R., Finne, E. and Kilaas, L. (1992) A new approach to template purification for sequencing applications using paramagnetic particles. *BioTechniques* **13**, 124-131

Fujita, R. and Swaroop, A. (1995) Alu-vector PCR with biotinylated primers to isolate YAC ends ready for sequencing. *BioTechniques*, **18**, 796-800

- Geiger, A., Lester, A., Kleiber, J. and Ørum, H. (1998) PNA array technology in molecular diagnostics. *Nucleosides and Nucleotides* **17**, 1717-1724.
- Gerhold, D., Rushmore, T. and Caskey, C.T. (1999) DNA chips: promising toys have become powerful tools. *Trends in Biochemical Sciences* **24**, 168-173.
- Gibson, L.E. and Cooke, R.E. (1959) Test for concentration of electrolytes in sweat in cystic fibrosis of pancreas utilising pilocarpine by iontophoresis. *Pediatrics* **23**, 545-549.
- Gillespie, S. (2001) Design, construction and surface chemistry of a real-time microarray platform. PhD Thesis, Cranfield University
- Granjeaud, S., Nguyen, C., Rocha, D., Luton, R., and Jordan, B.R. (1996) From Hybridization image to numerical values: a practical, high throughput quantification system for high density filter hybridization. *Genetic Analysis: Biomolecular Engineering* **12**, 151-162.
- Green, M.N., Clarke, J.T. and Schwachman, H. (1958) Studies in CF: protein pattern in meconium ileus. *Pediatrics* **21**, 635.
- Grosse, S.D., Khoury, M.J., Hannon, W.H., and Boyle, C.A. (2001) Early diagnosis of cystic fibrosis. *Pediatrics* **107**, 1492.
- Guo, Z., Guilfoyle, R.A., Thiel, A.J., Wang, R. and Smith, L.M. (1994) Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. *Nucleic Acids Research* **22**, 5456-5465.

Hansma P.K., Elings V.B., Marti O. and Bracker C.E. (1988) Scanning Tunnel Microscopy and Atomic Force Microscopy: Application to Biology and Technology. *Science* 242 209-216

Harvey, B.M. and Wheeler, C.B. (1994) Hybridization and detection of fluorescein-labelled DNA probes using enhanced chemiluminescence. In: Isaac, P.G., (Ed.) *Protocols for Nucleic Acid Analysis by Nonradioactive Probes*, pp. 135-140. Totowa, NJ: Humana Press

Harvey, B.M., Wheeler, C.B. and Cunningham, M.W. (1994) Random prime labelling of DNA probes with fluorescein-11-dUTP. In: Isaac, P.G., (Ed.) *Protocols for Nucleic Acid Analysis by Nonradioactive Probes*, pp. 93-100. Totowa, NJ: Humana Press

Hasseman, J. [http://www.tigr.org/tdb/microarray/pdf/M004\(Amino-allylLabelling\).pdf](http://www.tigr.org/tdb/microarray/pdf/M004(Amino-allylLabelling).pdf). Accessed October 2001.

Hatch, A., Sano, T., Miasi, J. and Smith, C.L. (1998) Rolling circle amplification of DNA immobilised on solid surfaces and its application to multiplex mutation detection. *Genetic Analysis: Biomolecular Engineering* 15, 35-40.

Heeley, A.F., Heeley, M.E. and Richmond, S.W.J. (1983) Progress in perinatal medicine. In: *The value of blood trypsin measurement by RIA in the early diagnosis of cystic fibrosis*, Amsterdam: Elsevier Press.

Heeley, A.F. and Watson, D. (1983) Cystic fibrosis-its biochemical detection. *Clinical Chemistry* 29, 2011-2018.

Henke, L., Piunno, P.A.E., McClure, A.C. and Krull, U.J. (1997) Covalent immobilization of single-stranded DNA onto optical fibres using various linkers. *Analytica Chimica Acta* **344**, 201-213.

Hirayama, H., Tamaoka, J. and Horikoshi, K. (1996) Improved immobilization of DNA to microwell plates for DNA-DNA hybridization. *Nucleic Acids Research* **24**, 4098-4099.

Houdayer, C., Cazeneuve, C., Cougoureux, E., Magnier, C., Tredano, M., Aymard, P., Goossens, M. and Feldman, D. (1998) Clinical evaluation of the CF(12)m cystic fibrosis DNA diagnostic kit. *Clinical Chemistry* **44**, 1346-1348.

Hsu, I.-C., Highsmith, W.E., Xu, J. and Kong, D. (1998) Mismatch cleavage detects base deletion in cystic fibrosis gene. *BioTechniques* **25**, 692-696.

IDT, www.idtdna.com. Accessed March 2002.

Iovanna, J.-L., Férec, C., Sarles, J. and Dargon, J.-C. (1994) The pancreatitis-associated protein (PAP). A new candidate for neonatal screening of cystic fibrosis. *C.R.Acad.Sci.Paris, Sciences de la vie/ Life sciences* **317**, 561-564.

Ivanovskaya, M.G., Kozlov, I.A., Naryshkin, N.A. and Shabarova, Z.A. (1995) A method of oligonucleotide immobilization on carboxyl-containing nylon membranes for hybridization with nucleic acids. *Russian Journal of Bioorganic Chemistry* **21**, 461-463.

Jaschke, A. (1997) Oligonucleotide-poly(ethylene glycol) conjugates: synthesis, properties and applications. *ACS Symposium Series* **680**, 265-283.

Ji, H. and Smith, L.M. (1993) Rapid purification of double-stranded DNA by triple-helix-mediated affinity capture, *Analytical Chemistry*, **65**, 1323-1328

Joos, B., Kuster, H. and Cone, R. (1997) Covalent attachment of hybridisable oligonucleotides to glass supports. *Analytical Biochemistry* **247**, 96-101.

Jordan, B. GEM microarray reproducibility study.

<http://gem.incyte.com/gem/technology/reproducibility.shtml>. Accessed May 2000

Kawasaki, E. and Chehab, F.F. (1994) Analysis of gene sequences by hybridization of PCR-amplified DNA to covalently bound oligonucleotide probes. In: *Methods in Molecular Biology*, pp. 225-236. Totowa, NJ: Humana Press Inc.

Kawasaki, E., Saiki, R. and Erlich, H. (1993) Genetic analysis using polymerase chain reaction-amplified DNA and immobilized oligonucleotide probes: Reverse dot blot typing. *Methods in Enzymology* **218**, 369-381.

Kerem, B.-S., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M. and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: Genetic analysis. *Science* **245**, 1073-1080.

Khoshnoodi, J. and Tryggvason, K. (2001) Congenital nephrotic syndromes. *Current Opinion in Genetics and Development* **11**, 322-327.

Knowles, M.R., Gatzky, J.T. and Boucher, R.C. (1983) Relative ion permeability of normal and cystic fibrosis nasal epithelium. *Journal of Clinical Investigation* **71**, 1410-1417.

Knowlton, R.G., Cohen-Haguenauer, O., Van Cong, N., Frézal, J., Brown, V.A., Barker, D., Braman, J.C.S.J.W., Tsui, L.-C., Buchwald, M. and Donis-Keller, H. (1985) A polymorphic DNA marker linked to cystic fibrosis is located on chromosome 7. *Nature* **318**, 380-382.

Kohler, T. (1998) Genotyping of human apolipoprotein E alleles by the new qualitative, microplate-based CASSI-detection assay. *BioTechniques* **25**, 80-85.

Laroche, D. and Travert, G. (1991) The application of PCR amplification and the polymorphic marker KM.19 to dried blood spots: Comparison with deletion 508 for the confirmation of the neonatal screening test for cystic fibrosis. *Pediatric Pulmonary Supplement* **7**, 19-22.

Larsen, J., Campbell, S., Faragher, E.B., Götz, M., Eichler, I., Waldherr, S., Dobianer, K. and Spona, J. (1994) Cystic fibrosis screening in neonates-measurement of immunoreactive trypsin and direct genotype analysis for Δ F508 mutation. *European Journal of Pediatrics* **153**, 569-573.

Lester, L.A., Kraut, J., Lloyd-Still, J., Karrison, T., Mott, C., Billstrand, C., Lemke, A. and Ober, C. (1994) Δ F508 genotype does not predict disease severity in an ethnically diverse cystic fibrosis population. *Pediatrics* **93**, 114-118.

Loumi, O., Baghriche, M., Delpech, M., Kaplan, M. and Bienvenu, T. (1999) Analysis of the complete coding region of the CFTR gene in ten Algerian cystic fibrosis families. *Human Heredity* **49**, 81-84.

Lui, J., Lissens, W. and Derovey, P. (1992) Efficiency and accuracy of the polymerase-chain-reaction assay for cystic fibrosis allele Δ F508 in single cells. *The Lancet* **339**, 1190-1192.

Maggio, A., Giambona, A., Cai, S.P., Wall, J., Kan, Y.W. and Chehab, F.F. (1993) Rapid and simultaneous typing of Hemoglobin S, Hemoglobin C and seven Mediterranean β -thalassemia mutations by covalent reverse dot-blot analysis: Application to prenatal diagnosis in Sicily. *Blood* **81**, 239-242.

Marky, L.A., Blumenfeld, K.S. and Breslauer, K.J. (1988) Differential effect of tetramethylammonium chloride and sodium chloride on duplex melting temperatures of deoxyoligonucleotides: resolution of a salt effect into specific and non-specific components. *Canadian Journal of Chemistry* **66**, 836-838.

Maskos, U. and Southern, E. (1991) Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesised *in situ*. *Nucleic Acids Research* **20**, 1679-1684.

Mckendrick, T. (1962) Sweat sodium levels in normal subjects, in cystic fibrosis patients and their relatives and in chronic bronchitic patients. *The Lancet* **I** 183-186.

McPherson, M.J. and Møller, S.G. (2000) *PCR*. BIOS Scientific Publishers Ltd. Oxford

Merl, S., Kloster, B., Moore, C., Hubbell, C., Tomar, R., Davey, F., Kalinowski, D., Planas, A., Ehrlich, G., Clark, D., Comis, R. and Poiesz, B. (1984) Efficient transformation of previously activated and dividing T lymphocytes by Human T Cell Leukemia-Lymphoma Virus. *Blood* **64**, 967-974.

Milton Harris, J. (1992) Introduction to biotechnical and biomedical applications of Poly(Ethylene Glycol). In: Milton Harris, J., (Ed.) *Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, pp. 1-14. New York: Plenum Press.

Molecular Probes, www.molecularprobes.com/handbook. Accessed January 2002.

Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R., Lewis, C.J. and Waggoner, A.S. (1993) Cyanine dye labelling reagents: Sulfoindocyanine Succinimidyl Esters. *Bioconjugate Chemistry* **4**, 105-111.

Mujumdar, R.B., Ernst, L.A., Mujumdar, S.R. and Waggoner, A.S. (1989) Cyanine dye labelling reagents containing Isothiocyanate groups. *Cytometry* **10**, 11-19.

Mullis, K.B. and Faloona, F.A. (1987) Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Methods in Enzymology* **155**, 335-350.

Murakami, Y., Hagashi, K., and Sekiya, T. (1991) Detection of aberrations of the p53 alleles and the gene transcript in human tumour cell lines by single-strand conformational polymorphism analysis. *Cancer Research* **51**, 3356-3361.

Nallur, G., Luo, C., Fang, L., Cooley, S., Dave, V., Lambert, J., Kukanskis, K., Kingsmore, S., Lasken, R. and Schweitzer, B. (2001) Signal amplification by rolling circle amplification on DNA microarrays. *Nucleic Acids Research* **29**, 118.

Narayanan, S. (1992) Overview of principles and current uses of DNA probes in clinical and laboratory medicine. *Annals of Clinical and Laboratory Science* **22**, 353-376.

Newton, C.R., Graham, A., Heptinstall, L.E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C., and Markham, A.F. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Research* **17**, 2503-2515.

Nierzwichi-Bauer, S.A., Gebhardt, J.S., Linkkila, L. and Walsh, K. (1990) A comparison of UV-crosslinking and vacuum baking for nucleic acid immobilization and retention. *BioTechniques* **9**, 472-478.

Nilsson, P., Larsson, A., Lundberg, J., Uhlén, M. and Nygren, P.-Å. (1999) Mutational scanning of PCR products by subtractive oligonucleotide hybridization analysis. *BioTechniques* **26**, 308-316.

O'Donnel-Maloney, M.J., Smith, C.L. and Cantor, C.R. (1996) The development of microfabricated arrays for DNA sequencing and analysis. *Trends in Biotechnology* **14**, 401-407.

Oss, C.J.V. (1987) Mechanism of DNA (Southern) and protein (western) blotting on cellulose nitrate and other membranes. *Journal of Chromatography* **391**, 53-65.

Phimister, B. (1999) A note on nomenclature. *Nature Genetics* **21**, 1.

Packard BioScience. <http://www.packardbioscience.com/products/805.asp>. Accessed April 2002.

Pocsai, Z., Paragh, G., and Adany, R. (2001) Multiplex PCR assay for screening deletions in the low density lipoprotein receptor gene. *Clinica Chimica Acta* **309**, 7-12.

Pollitt, R.J. (Feb, 1996) *Metabolic Disease Newsletter* Department of Paediatric chemical pathology and neonatal screening, Children's Hospital, Sheffield NHS Trust.

Ramsay, G. (1998) DNA chips: State-of-the-art. *Nature Biotechnology* **16**, 40-44.

Raskin, S., Phillips, J.A., Krishnamani, M.R.S., Vnencak-Jones, C., Parker, R.A., Rozov, T., Cardieri, J.M., Marostica, P., Abreu, F., Giugliani, R., Reis, F., Rosario, N.A., Ludwig, N. and Pilotto, R.F. (1993) DNA analysis of cystic fibrosis in Brazil by direct PCR amplification from Guthrie cards. *American Journal of Medical Genetics* **46**, 665-669.

Rasmussen, S.R., Larsen, M.R. and Rasmussen, S.E. (1991) Covalent immobilization of DNA onto polystyrene microwells: The molecules are only bound at the 5' end. *Analytical Biochemistry* **198**, 138-142.

Riordan, J.R., Rommens, J.M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M.L., Iannuzzi, M.C., Collins, F.C. and Tsui, L.-C. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**, 1066-1073.

Rodrigues, N.R., Rowan, A., Smith, M.E.K., Kerr, I.B., Bodmer, W.F., Gannon, J.V., and Lane, D.P. (1990) p53 mutation in colorectal cancer. *Proceedings of the National Academy of Sciences USA* **87**, 7555-7559.

Rogers, Y.-H., Jiang-Baucom, P., Haung, Z.-J., Bogdanov, V., Anderson, S. and Boyce-Jacino, M.T. (1999) Immobilization of oligonucleotides onto a glass support via disulfide bonds: A method for preparation of DNA microarrays. *Analytical Biochemistry* **266**, 23-30.

Rommens, J.M., Iannuzzi, M.C., Kerem, B.-S., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J.R., Tsui, L.-C. and Collins, F.S. (1989) Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science* **245**, 1059-1065.

Roychoudhury, R., Tu, C.-P.D. and Wu, R. (1979) Influence of nucleotide sequence adjacent to duplex DAN termini on 3' terminal labelling by terminal transferase. *Nucleic Acids Research* **6**, 1323-1333.

Saiki, R., Walsh, P.S., Levenson, C.H. and Ehrlich, G. (1989) Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. *Proceedings of the National Academy of Sciences USA* **86**, 6230-6234.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Schwartz, M.J. (1998) DNA diagnosis of cystic fibrosis. *Annals of Clinical Biochemistry* **35**, 584-610.

Scobie, G., Woodroffe, B., Fischel, S. and Kalsheker, N. (1996) Identification of the five most common cystic fibrosis mutations in single cells using a rapid and specific differential amplification system. *Molecular Human Reproduction* **2**, 203-207.

Serre, J.L., Taillandier, A., Mornet, E., Simon-Bouy, B., Boué, J. and Boué, A. (1991) Nearly 80% of cystic fibrosis heterozygotes and 64% of couples at risk may be detected through a unique screening of four mutations by ASO reverse dot blot. *Genomics* **11**, 1149-1151.

Shchepinov, M.S., Case-Green, S.C. and Southern, E.M. (1997a) Steric factors influencing hybridization of nucleic acids to oligonucleotide arrays. *Nucleic Acids Research* **25**, 1155-1161.

Shchepinov, M.S., Udalova, I.A., Bridgman, A.J. and Southern, E.M. (1997b) Oligonucleotide dendrimers: synthesis and use as polylabelled DNA probes. *Nucleic Acids Research* **25**, 4447-4454.

Shearwater Polymers, <http://www.swpolymers.com>. Accessed August 1999.

Shuber, A.P., Skoletsky, J., Stern, R. and Handelin, B.L. (1993) Efficient 12-mutation testing in the CTFR gene: a general model for complex mutation analysis. *Human Molecular Genetics* **2**, 153-158.

Sinclair, B. (1999) Everything's great when it sits on a chip *The Scientist* **13** 1-7

Southern, E.M. (1996) DNA chips: analysing sequence by hybridisation to oligonucleotides on a large scale. *Trends in Genetics* **12**, 110-115.

Southern, E., Mir, K. and Shchepinov (1999) Molecular interactions on microarrays. *Nature Genetics* **21**, 5-9.

Steiner, M., Ocran, K., Genschel, J., Meier, P., Gerl, H., Ventz, M., Schneider, M.L., Buttner, C., Wadowska, K., Kerner, W., Schuff-Werner, P., Lochs, H. and Schmidt, H. (2002) A homozygous hfe gene splice site mutation (ivs5+1 g/a) in a hereditary hemochromatosis patient of vietnamese origin. *Gastroenterology* **122**, 789-95

Stephan, U., Bush, E.W. and Killberg, H. (1975) Cystic fibrosis detection by means of a test strip. *Pediatrics* **55**, 35-38.

Stern, R. (1997) The diagnosis of cystic fibrosis. *The New England Journal of Medicine* **336**, 487-491.

Stimpson, D.I., Cooley, P.W., Knepper, S.M. and Wallace, D.B. (1998) Parallel production of oligonucleotide arrays using membranes and reagent jet printing. *BioTechniques* **25**, 886-890.

Stimpson, D.I. and Gordon, J. (1996) The utility of optical waveguide DNA array hybridization and melting for rapid resolution of mismatches, and for detection of minor mutant components in the presence of a majority of wild type sequence: statistical model and supporting data. *Genetic Analysis: Biomolecular Engineering* **13**, 73-80.

Stimpson, D.I., Hoiyer, J.V., Hsieh, W.T., Jou, C., Gordon, J., Theriault, T., Gamble, R. and Baldeschwieler (1995) Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical waveguides. *Proceedings of the National Academy of Sciences USA* **92**, 6379-6383.

Stothard, D R (2001) Use of a reverse dot blot procedure to identify the presence of multiple serovars in *Chlamydia trachomatis* urogenital infection. *Journal of Clinical Microbiology*, **39**, 2655-2659

Szewczyk, B., Bienkowska-Szewczyk, K. and Kozloff, L.M. (1987) Use of different fluorochromes for monitoring protein elution and transfer. *Electrophoresis* **8**, 25-28.

Ten Kate, L.P. (1990) Carrier screening for cystic fibrosis and other autosomal recessive diseases. *American Journal of Medical Genetics* **47**, 359-361.

Tessitore, A., Toniato, E., Gulino, A., Frati, L., Ricevuto, E., Vadala, M., Vinglo, E. and Martinotti, S. (2002) Prenatal diagnosis of a rhodopsin mutation using chemical cleavage of the mismatch. *Prenatal Diagnosis* **22**, 380-384.

The Cystic Fibrosis Genetic Analysis Consortium (1990) Worldwide survey of the Δ F508 mutation-report from the Cystic Fibrosis Genetic Analysis Consortium. *American Journal of Human Genetics* **47**, 359-361.

Tong, X; Smith, L M (1992) Solid-phase method for the purification of DNA sequencing reactions. *Analytical Chemistry* **64**, 2672-2677

Tsui, L.-C., Buchwald, M., Barker, D., Braman, J.C., Knowlton, R., Schumm, J.W., Eiberg, H., Mohr, J., Kennedy, D., Plavsic, N., Zsiga, M., Markiewicz, D., Akots, G., Brown, V., Helms, C., Gravius, T., Parker, C., Rediker, K. and Donis-Keller, H. (1985) Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. *Science* **230**, 1054-1057.

Tyagi, S., Bratu, D.P. and Kramer, F.R. (1998) Multicolour molecular beacons for allele discrimination. *Nature Biotechnology* **16**, 49-53.

Voronina, O.V., Gaitskhoki, V.S., Potapova, O.J. and Schwartz, E.I. (1991) A simple test for the detection of the Δ F508 cystic fibrosis mutation. *Biochemical Medicine and Metabolic Biology* **46**, 271-273.

Wainwright, B.J., Scambler, P.J., Schmidtke, J., Watson, E.A., Law, H.-Y., Farrall, M., Cooke, H.J., Eiberg, H. and Williamson, R. (1985) Localization of cystic fibrosis locus to human chromosome 7cen-q22. *Nature* **318**, 384-385.

Wall, J., Cai, S. and Chehab, F.F. (1995) A 31-mutation assay for cystic fibrosis testing in the clinical molecular diagnostics laboratory. *Human Mutation* **5**, 333-338.

Wallace, R.W. (1997) DNA on a chip: serving up the genome for diagnostics and research. *Molecular Medicine Today* **September**, 384-389.

Welsh, M.J., Tsui, L.-C., Boat, T.F. and Beaudet, A.L. (1990) Cystic Fibrosis. In: *The Metabolic and Molecular Bases of Inherited Disease*, pp. 3799-3876. New York: McGraw-Hill.

Wessendorf, M.W. and Brelje, T.C. (1992) Which fluorophore is brightest? A comparison of the staining obtained using fluorescein, tetramethylrhodamine, lissamine rhodamine, Texas Red, and cyanine 3.18. *Histochemistry* **98**, 81-85.

Weston, D. (1999) Molecular engineering of the biosensor interface. PhD Thesis, Cranfield University, UK.

White, R., Woodward, S., Leppert, M., O'Connell, P., Hoff, M., Herbst, J., Lalouel, J.-M., Dean, M. and Vande Woude, G. (1985) A closely linked genetic marker for cystic fibrosis. *Nature* **318**, 382-384.

Williams, D.L. (1990) The use of a PVDF membrane in the rapid immobilization of genomic DNA for dot-blot hybridization analysis. *BioTechniques* **8**, 14-15.

Witt, V. (1996) Characterisation and molecular analysis of Plantaricin S, a two-peptide bacteriocin from Olive-fermenting *Lactobacillus plantarum* LPCO10. First Year Report, Cranfield University, UK.

Woolford, A.J. and Dale, J.W. (1992) Simplified procedures for detection of amplified DNA using fluorescent label incorporation and reverse probing. *FEMS Microbiology Letters* **99**, 311-316.

Zammatteo, N., Girardeaux, C., Delforge, D., Pireaux, J.-J. and Remacle, J. (1996) Amination of polystyrene microwells: Application to the covalent grafting of DNA probes for hybridization assays. *Analytical Biochemistry* **236**, 85-94.

Zerhusen, B., Zhao, J., Xiet, J., Davis, P.B. and Ma, J. (1999) A Single Conductance Pore for Chloride Ions Formed by Two Cystic Fibrosis Transmembrane Conductance Regulator Molecules. *The Journal of Biological Chemistry* **274**, 7627-7630.

Zhang, Y., Coyne, M.Y., Will, S.G., Levenson, C.H. and Kawasaki, E.S. (1991) Single base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides. *Nucleic Acids Research* **19**, 3929-3933.

Zielenski, J., Rozmahel, R., Bozon, D., Kerem, B.S., Grelczack, Z., Riorden, J.R., Rommens, J., and Tsui, L.C. (1991) Genomic sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* **10**, 214-228.

Zimmermann, B., Holzgreve, W., Wenzel, F., and Hahn, S. (2002) QIAamp DNA Blood Mini was used to isolate DNA from 400µl of cultured amniotic cells as well as blood. PCR analysis was performed on TaqMan. *Clinical Chemistry* **48**, 362-363

Zuk, R.F., Rowley, G.L. and Ullman, E.F. (1979) Fluorescence protection immunoassay: A new homogeneous assay technique. *Clinical Chemistry* **25**, 1554-1560.

Appendices

Appendix A. Primer sequences

Primer name	Primer sequence (5'-3') and label	Reference
1Ab (<i>L. plantarum</i>)	TAT TCT TCG CGA ACG GTT GAT T	
R4s (<i>L. plantarum</i>)	GAT AAT ATC CGG ATC TTT TGA	Witt, (1996)
10i-3 (CFTR)	CAT TCA CAG TAG ATT ACC CA	Zielenski <i>et al.</i> (1991)
10i-5 (CFTR)	GCA GAG TAC CTG AAA CAG GA	Zielenski <i>et al.</i> (1991)
11i-3 (CFTR)	GCA CAG ATT CTG AGT AAC CAT AAT	Zielenski <i>et al.</i> (1991)
11i-5 (CFTR)	CAA CTG TGG TTA AAG CAA TAG TGT	Zielenski <i>et al.</i> (1991)
21a (CFTR)	Biotin-AG GCT TCT GTA CAT GGG TGT TTC	Wall <i>et al.</i> (1995)
21b (CFTR)	Biotin-CA AAA GTA CCT GTT GCT CCA	Wall <i>et al.</i> (1995)

PCR name	Product size
<i>Lactobacillus plantarum</i> PCR	334 bases
Exon 10 PCR (CFTR)	491 bases
Exon 11 PCR(CFTR)	425 bases
Exon 21 PCR (CFTR)	376 bases

Appendix B. Control sample consent forms

Donation of blood to aid the optimisation of a molecular biology based method of Cystic Fibrosis mutation detection

- I give my consent for the Medical Centre at Cranfield University to take 5mls of my blood and for it to be used in molecular biology research to improve cystic fibrosis detection.
- I consent for the DNA to be extracted from this blood and amplified. I understand that the amplified DNA will be hybridised to DNA containing known cystic fibrosis mutations in order to optimise these hybridisations.
- I understand that I will not be informed in the event of a mutation being discovered in my DNA and that the samples are analysed anonymously.

..... Date.....

Name of donor

.....

Signature of donor

.....

Signature of Medical Centre Representative

.....

Signature of scientist receiving the blood

Written by Lizzie Bull (1999)

Appendix C. oligonucleotide/probe sequences, Lactobacillus plantarum and cystic fibrosis

Oligonucleotide name	Oligonucleotide sequence (5'-3')	Tail length/ 5' Amino termination	Reference
R4A (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	n/a	Witt, 1996
R4Aa (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	n/a /Amino termination	
R4Ata40 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	20 bases/ Amino termination	
R4Ata60 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	40 bases/ Amino termination	
R4Ata80 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	60 bases/ Amino termination	
R4Ata90 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	70 bases/ Amino termination	
R4Ata100 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	80 bases/ Amino termination	
R4A40 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	Approx. 40 bases/ none	
R4A60 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	Approx. 60 bases/ none	
R4A80 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	Approx. 80 bases/ none	
R4A90 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	Approx. 90 bases/ none	
R4A100 (<i>L. plantarum</i>)	GAA ATA GAT GCA GTT CCA CTA	Approx. 100 bases/ none	
415N (Δ F508 wt, CFTR)	GAA ACA CCA AAG ATG ATA	Amino termination	Wall <i>et al.</i> , 1995
416M (Δ F508 mut, CFTR)	GGA AAC ACC AAT GAT ATT	Amino termination	Wall <i>et al.</i> , 1995
542N2+ (G542X wt, CFTR)	TA TAG TTC TTG GAG AAG GTG	Amino termination	Wall <i>et al.</i> , 1995
542M1 (G542X mut, CFTR)	TA TAG TTC TTT GAG AAG GTG	Amino termination	Wall <i>et al.</i> , 1995
551N (G551D wt, CFTR)	TC GTT GAC CTC CAC T	Amino termination	Wall <i>et al.</i> , 1995
551M (G551 mut, CFTR)	AG TGG AGA TCA ACG A	Amino termination	Wall <i>et al.</i> , 1995

1303N (N1303K wt, CFTR)	AG AAA AAA CTT GGA TCC	Amino termination	Wall <i>et al.</i> , 1995
1303M (N1303K mut, CFTR)	GG GAT CCA ACT TTT TTC T	Amino termination	Wall <i>et al.</i> , 1995
DIG test	ATG CCG ATA GAC GCA GTA CTG	5' Amino termination, 3' DIG label	
Biotin test	ATG CCG ATA GAC GCA GTA CTG	5' Amino termination, 3' biotin label	
LB-ve	GAT AAT ATC CGG ATC TTT TGA	5' Amino termination	

Appendix D. Table showing the amount of oligonucleotide put down in each spot during radioactive studies

	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
R4Aa	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4Aa40	19.5 pmol	9.75 pmol	4.875 pmol	2.438 pmol	1.219 pmol
R4Aa60	19.1 pmol	9.55 pmol	4.775 pmol	2.388 pmol	1.194 pmol
R4Aa80	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4Aa90	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4Aa100	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4at60	18.24 pmol	9.12 pmol	4.56 pmol	2.28 pmol	1.14 pmol
R4A	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4A40	22.22 pmol	11.11 pmol	5.55 pmol	2.78 pmol	1.39 pmol
R4A60	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4A80	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4A90	28 pmol	14 pmol	7 pmol	3.5 pmol	1.75 pmol
R4A100	24.6 pmol	12.3 pmol	6.15 pmol	3.08 pmol	1.54 pmol
R4At60	20.3 pmol	10.15 pmol	5.08 pmol	2.54 pmol	1.27 pmol

Appendix E. Radioactive data- CPM and calculations and probe yield

Membrane (w-washed)	Oligonucleotide	Amount deposited (pmol)	Average CPM of two spots	Pmol oligonucleotide detected (%)
Biodyne (w)	R4Aa	28	7,753.2	0.27 (0.98%)
Biodyne (w)	R4Aa	14	6,708.2	0.23 (1.67%)
Biodyne (w)	R4Aa	7	1,883.8	0.07 (0.94%)
Biodyne (w)	R4Aa	3.5	1,005.2	0.04 (1.00%)
Biodyne (w)	R4Aa	1.75	848.8	0.03 (1.69%)
Biodyne (w)	R4Aa40	28	34,580.5	1.62 (5.77%)
Biodyne (w)	R4Aa40	14	16,052.0	0.75 (5.36%)
Biodyne (w)	R4Aa40	7	8,933.0	0.42 (6.00%)
Biodyne (w)	R4Aa40	3.5	4,190.9	0.20 (5.60%)
Biodyne (w)	R4Aa40	1.75	2,147.9	0.10 (5.73%)
Biodyne (w)	R4Aa60	28	27,725.5	2.76 (9.80%)
Biodyne (w)	R4Aa60	14	20,787.6	2.07 (14.76%)
Biodyne (w)	R4Aa60	7	9,505.6	0.94 (13.50%)
Biodyne (w)	R4Aa60	3.5	4,045.7	0.40 (11.49%)
Biodyne (w)	R4Aa60	1.75	1,653.1	0.16 (9.39%)
Biodyne (w)	R4Aa80	28	13,222.3	0.63 (2.24%)
Biodyne (w)	R4Aa80	14	9,588.2	0.46 (3.25%)
Biodyne (w)	R4Aa80	7	6,120.7	0.29 (4.15%)
Biodyne (w)	R4Aa80	3.5	2,811.0	0.13 (3.82%)
Biodyne (w)	R4Aa80	1.75	1,374.8	0.07 (3.73%)
Biodyne (w)	R4Aa90	28	5,531.7	0.25 (0.89%)
Biodyne (w)	R4Aa90	14	5,125.9	0.23 (1.64%)
Biodyne (w)	R4Aa90	7	2,327.5	0.10 (1.49%)
Biodyne (w)	R4Aa90	3.5	1,245.5	0.06 (1.60%)
Biodyne (w)	R4Aa90	1.75	742.7	0.03 (1.90%)
Biodyne (w)	R4Aa100	28	3,451.9	0.19 (0.66%)
Biodyne (w)	R4Aa100	14	2,370.7	0.13 (0.91%)
Biodyne (w)	R4Aa100	7	1,195.7	0.06 (0.92%)

Biodyne (w)	R4Aa100	3.5	222.4	0.01 (0.34%)
Biodyne (w)	R4Aa100	1.75	232.0	0.01 (0.71%)
Biodyne	R4Aa	28	40,743.1	1.42 (5.08%)
Biodyne	R4Aa	14	31,650.0	1.11 (7.90%)
Biodyne	R4Aa	7	7,011.0	0.24 (3.50%)
Biodyne	R4Aa	3.5	6,722.7	1.23 (6.70%)
Biodyne	R4Aa	1.75	28,959.0	1.01 (57.80%)
Biodyne	R4Aa40	28	62,445.1	2.92 (10.42%)
Biodyne	R4Aa40	14	40,978.9	1.91 (13.68%)
Biodyne	R4Aa40	7	33,393.2	1.56 (22.30)
Biodyne	R4Aa40	3.5	8,085.5	0.38 (10.80%)
Biodyne	R4Aa40	1.75	6,095.6	0.28 (16.28%)
Biodyne	R4Aa60	28	61,729.4	6.13 (21.91%)
Biodyne	R4Aa60	14	20,057.4	1.99 (14.24%)
Biodyne	R4Aa60	7	10,897.0	1.08 (15.47%)
Biodyne	R4Aa60	3.5	5,484.7	0.55 (15.58%)
Biodyne	R4Aa60	1.75	9,951.7	0.99 (56.52%)
Biodyne	R4Aa80	28	25,109.2	1.19 (4.26%)
Biodyne	R4Aa80	14	12,777.5	0.61 (4.34%)
Biodyne	R4Aa80	7	14,309.8	0.68 (9.71%)
Biodyne	R4Aa80	3.5	3,313.6	0.15 (4.50%)
Biodyne	R4Aa80	1.75	2,607.7	0.12 (7.07%)
Biodyne	R4Aa90	28	19,638.8	0.88 (3.14%)
Biodyne	R4Aa90	14	15,907.8	0.71 (5.10%)
Biodyne	R4Aa90	7	5,179.3	0.23 (3.32%)
Biodyne	R4Aa90	3.5	3,045.6	0.14 (3.90%)
Biodyne	R4Aa90	1.75	2,318.9	0.10 (5.94%)
Biodyne	R4Aa100	28	7,312.0	0.39 (1.40%)
Biodyne	R4Aa100	14	2,305.1	0.12 (0.89%)
Biodyne	R4Aa100	7	2,142.1	0.12 (1.65%)
Biodyne	R4Aa100	3.5	1,014.4	0.05 (1.56%)
Biodyne	R4Aa100	1.75	1,055.1	0.06 (3.25%)
Hybond (w)	R4A	28	2,622.8	0.20 (0.71%)

Hybond (w)	R4A	14	972.7	0.07 (0.53%)
Hybond (w)	R4A	7	457.2	0.03 (0.50%)
Hybond (w)	R4A	3.5	249.2	0.02 (0.54%)
Hybond (w)	R4A	1.75	192.5	0.01 (0.84%)
Hybond (w)	R4A40	28	4,019.8	0.11 (0.38%)
Hybond (w)	R4A40	14	1,631.8	0.43 (0.31%)
Hybond (w)	R4A40	7	765.4	0.02 (0.29%)
Hybond (w)	R4A40	3.5	429.3	0.01 (0.33%)
Hybond (w)	R4A40	1.75	231.2	0.01 (0.35%)
Hybond (w)	R4A60	28	3,844.6	0.10 (0.35%)
Hybond (w)	R4A60	14	2,393.5	0.10 (0.44%)
Hybond (w)	R4A60	7	1,189.5	0.03 (0.43%)
Hybond (w)	R4A60	3.5	564.1	0.01 (0.41%)
Hybond (w)	R4A60	1.75	342.4	0.01 (0.50%)
Hybond (w)	R4A80	28	2,888.4	0.06 (0.23%)
Hybond (w)	R4A80	14	1,564.0	0.04 (0.25%)
Hybond (w)	R4A80	7	733.8	0.02 (0.24%)
Hybond (w)	R4A80	3.5	272.1	0.01 (0.17%)
Hybond (w)	R4A80	1.75	165.5	0.00 (0.21%)
Hybond (w)	R4A90	28	2,337.0	0.18 (0.63%)
Hybond (w)	R4A90	14	1,278.8	0.10 (0.69%)
Hybond (w)	R4A90	7	510.1	0.04 (0.55%)
Hybond (w)	R4A90	3.5	222.9	0.02 (0.48%)
Hybond (w)	R4A90	1.75	185.1	0.01 (0.79%)
Hybond (w)	R4A100	28	2,370.0	0.08 (0.29%)
Hybond (w)	R4A100	14	1,513.7	0.05 (0.37%)
Hybond (w)	R4A100	7	589.8	0.02 (0.29%)
Hybond (w)	R4A100	3.5	319.0	0.01 (0.31%)
Hybond (w)	R4A100	1.75	196.8	0.01 (0.39%)
Hybond	R4A	28	246,885.0	18.84 (67.30%)
Hybond	R4A	14	135,404.0	10.34 (73.82%)
Hybond	R4A	7	67,698.9	5.17 (73.81%)
Hybond	R4A	3.5	35,605.7	2.72 (77.65%)

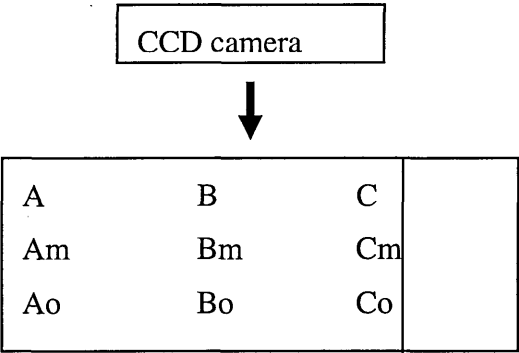
Hybond	R4A	1.75	17,266.4	1.32 (75.31%)
Hybond	R4A40	28	266,812.1	7.09 (25.32%)
Hybond	R4A40	14	114,730.3	3.05 (21.78%)
Hybond	R4A40	7	51,860.4	1.38 (19.68%)
Hybond	R4A40	3.5	27,950.0	0.74 (21.22%)
Hybond	R4A40	1.75	12,272.8	0.33 (18.63%)
Hybond	R4A60	28	240,012.1	6.12 (21.87%)
Hybond	R4A60	14	114,631.4	2.92 (20.89%)
Hybond	R4A60	7	59,558.6	1.52 (21.71%)
Hybond	R4A60	3.5	27,071.8	0.69 (19.74%)
Hybond	R4A60	1.75	15,492.4	0.40 (22.59%)
Hybond	R4A80	28	171,565.0	3.86 (13.78%)
Hybond	R4A80	14	70,301.4	1.58 (11.29%)
Hybond	R4A80	7	59.0	0.00 (0.02%)
Hybond	R4A80	3.5	19,676.2	0.44 (12.64%)
Hybond	R4A80	1.75	9,028.4	0.20 (11.60%)
Hybond	R4A90	28	163,676.0	12.27 (42.84%)
Hybond	R4A90	14	79,391.4	5.95 (42.53%)
Hybond	R4A90	7	40,606.9	3.05 (43.51%)
Hybond	R4A90	3.5	14,911.5	1.12 (31.95%)
Hybond	R4A90	1.75	8,239.5	0.62 (35.31%)
Hybond	R4A100	28	133,414.8	4.60 (16.43%)
Hybond	R4A100	14	69,680.7	2.40 (17.16%)
Hybond	R4A100	7	31,151.9	1.07 (15.35%)
Hybond	R4A100	3.5	18,620.0	0.64 (18.34%)
Hybond	R4A100	1.75	9,235.3	0.32 (18.20%)

Appendix F. Contact angle measurements on polystyrene slides

Slide number	Position (see Appendix G)	Contact angle all $\pm 1^\circ$
Unmodified 1	A	74.8°
Unmodified 1	Ao	53.5°
Unmodified 1	B	59.0°
Unmodified 1	Bo	58.2°
Unmodified 1	Bm	83.5°
Unmodified 1	C	69.3°
Unmodified 1	Co	78.8°
Unmodified 2	A	75.6°
Unmodified 2	Ao	60.1°
Unmodified 2	B	59.2°
Unmodified 2	Bo	58.8°
Unmodified 2	Bm	55.7°
Unmodified 2	C	80.1°
Unmodified 2	Co	76.2°
Modified 1	A	62.0°
Modified 1	Am	68.8°
Modified 1	ao	64.1°
Modified 1	B	59.3°
Modified 1	bm	63.1°
Modified 1	Bo	66.6°
Modified 1	C	66.2°
Modified 1	Cm	63°
Modified 1	Co	65.5°
Modified 2	A	59.7°
Modified 2	Am	60.5°
Modified 2	Ao	50.7°
Modified 2	B	59.3°
Modified 2	Bm	58.1°
Modified 2	Bo	67.4°

Modified 2	C	54.7°
Modified 2	Cm	55.9°
Modified 2	Co	55.2°

Appendix G. Diagram to show the position of droplets on the slide during contact angle measurements



Appendix H. Cy5 absorbance levels of PCR products

PCR product	Pmol dye	Pmol nucleotide	Nucleotide: dye ratio
Exon 10 (patient 1)	3200	74114.02	23.16
Exon 10 (patient 2)	10500	62711.86	5.97
Exon 10 (patient 3)	2400	62711.86	26.13
Exon 11 (patient 1)	3400	74114.22	21.80
Exon 11 (patient 2)	6200	205238.83	33.10
Exon 11 (patient 3)	58000	359167.95	6.19
Exon 11 (patient 1)	3600	108320.49	30.08
Exon 11 (patient 2)	2400	74114.22	45.13
Exon 11 (patient 3)	4600	68412.94	14.87
Exon 21 (patient 1)	2800	165331.28	59.05
Exon 21 (patient 2)	4600	336363.63	73.12
Exon 21 (patient 3)	4800	114021.57	23.75

According to Hasseman (2001) a value of over 200 pmol of dye incorporation per sample and a ratio of less than 50 nucleotides/dye molecule is optimal for hybridisation.

Appendix I. Blood collection protocol, patient information and sample consent form

PROTOCOL

DONATION OF BLOOD SAMPLES TO INVESTIGATE MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE DIAGNOSIS OF CYSTIC FIBROSIS

The samples should be kept at 4°C where possible

Samples required

Blood samples from Cystic Fibrosis patients with one or two known mutations, preferably including $\Delta F508$, G542X, G551D and N1303K. 1-2ml of blood, collected in a heparin or EDTA tube.

Collection of samples

1. Inform the patient and/or guardian about the project and blood sample donation.
2. Obtain signed consent to obtain a blood sample for research.
3. Fill in patient details (including the CF mutations) in a designated book (or use a sticker from their notes) a patient identification number and the date of collection.
4. Take the sample during routine procedure. Label the tubes with patient number.
5. Store the blood at 4°C and send to Cranfield University as soon as possible.

The samples collected as detailed above will be used in the following way: -

Genomic DNA will be extracted from the blood sample, and areas containing the cystic fibrosis mutation will be amplified. The amplified product will be used to check the reliability of a novel test for cystic fibrosis developed at Cranfield University in conjunction with Northampton General Hospital.

PATIENT INFORMATION

DONATION OF BLOOD SAMPLES TO INVESTIGATE MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE DIAGNOSIS OF CYSTIC FIBROSIS

BACKGROUND

The staff of Northampton General Hospital and scientists at Cranfield University are working together in order to improve the methods of diagnosis of cystic fibrosis in newborn babies.

The cystic fibrosis test that is being developed will provide information on the patients' cystic fibrosis mutations from a blood sample. It is crucial that the test is accurate. In order to check that the test will correctly diagnose cystic fibrosis mutations, it must be used on the blood of patients who have known mutations.

Please help us

During the normal course of your/your child's treatment it is necessary to take blood samples. If you are willing to allow us, we can use some of this blood to help develop a new diagnostic test.

Your care and treatment will not be altered in any way by your decision to donate or otherwise. Furthermore, no additional procedures will be undertaken as a result of your consent.

Thank you for your help and co-operation. If you have any questions please ask your doctor or contact Dr J. O'Donnell, Clinical Biochemistry, Northampton General Hospital. Tel 01604 634700 (ext. 5401).

CONSENT FORM

DONATION OF BLOOD SAMPLES TO INVESTIGATE MOLECULAR BIOLOGY TECHNIQUES TO ADVANCE THE DIAGNOSIS OF CYSTIC FIBROSIS

- I have read the attached information leaflet, discussed it with my doctor and fully understand what is required. YES/NO
- I request that any blood samples taken during the course of my care at Northampton General Hospital may be made available for research after all procedures required for my clinical management have been completed. YES/NO
- I understand that my care will be unaffected by my consent YES/NO

.....

Name of patient	Hospital Number
-----------------	-----------------

.....

Signature _____ Date _____

Your doctor will retain this form.

CONFIDENTIAL

Written by Dr. John O'Donnell (2000).

**NORTHAMPTON MEDICAL RESEARCH/
ETHICS COMMITTEE**

Deputy Chairman: Dr Chris Frerk

Secretary: Mrs Michelle Spinks

Northampton



Primary Care Trust

Our Ref: FE/MS/01/72

17 September 2001

Highfield
Cliftonville Road
Northampton
NN1 5DN

Tel: 01604 615000

Fax: 01604 615010

Dr John O'Donnell
Consultant Clinical Biochemist
Northampton General Hospital NHS Trust
Cliftonville
NORTHAMPTON
NN1 5BD

Dear Dr O'Donnell

01/72 Development of a screening test for the identification of cystic fibrosis

The Northampton Medical Research/Ethics Committee reviewed your application in relation to the above study at their meeting on Thursday 13 September 2001. I am writing to inform you that approval in principle was granted by the Committee, subject to the following amendment:-

The Committee noted that you intend to include patients between the ages of 5 - 35 years. Members were of the opinion that any children under the age of 16 who are sufficiently mature and intelligent to be able to understand what is involved in the treatment (i.e., those who are Gillick competent), should be able to give consent for themselves. The Committee have therefore requested that a separate Patient Information Sheet and Consent Form be prepared for children who are able to give consent.

The Committee have delegated authority to the Deputy Chairman to give final approval to your study following the receipt of a satisfactory Patient Information Sheet and Consent Form.

I look forward to hearing from you in due course.

Yours sincerely

Michelle Spinks
Secretary, Northampton Medical Research/Ethics Committee

c.c. Anna Fox, Research & Development Centre - Knightley Ward

20th September 2001

Dr John O'Donnell
Consultant Clinical Biochemist
Northampton General Hospital NHS Trust
Cliftonville
Northampton
NN1 5BD

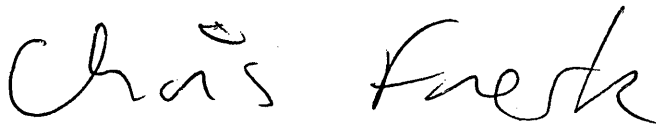
Dear Dr O'Donnell

01/72 Development of a screening test for the identification of cystic fibrosis

Thank you for sending the additional patient information sheet and consent form directly to me. On behalf of the Medical Research Ethics Committee, I now give you final approval of your study.

I hope this is satisfactory and I wish you well with your study.

Yours sincerely



Chris Frerk
Deputy Chairman, Northampton Medical Research/Ethics Committee

**Northampton General Hospital
Clinical Biochemistry
Patient Sheet (U16)**

Hello. I understand that you have a condition called Cystic Fibrosis. You have had this condition since birth. You may not have been unwell from the beginning, but you may have had to go to hospital since then, and you may have had visits at home from nurses looking after you because of this condition.

In Northampton, we carefully test all newborn babies for Cystic Fibrosis. This test we do is good, and we find babies who have the condition. It is better to find them at this time because we can the doctors and nurses can then plan treatments to help the baby with this condition. The test is not perfect, and very occasionally, the test does not tell us about a baby with the condition.

In Northampton, we are doing some research to try and find a better test to look at newborn babies. This involves using a material in the babies called DNA. The babies' DNA is made up from DNA from their mum and dad. It can possibly also tell us if the child has DNA that gives it Cystic Fibrosis.

We would like you to help us with this research. We would like to take a sample of blood from you. This will be just a simple blood test taking about 5mls of blood. It will be no different from any other sample you have given. We can then check if the new test can spot whether you have Cystic Fibrosis. This will let us know if we have to change the test to make it better.

We hope you will help us, but if you do not want to be involved; that is ok. You do not have to agree to this at all. None of the doctors or nurses will mind, and will give you the same care and attention as always.

Thank you for reading this.

**Northampton General Hospital
Clinical Biochemistry
Consent Form (U16)**

I have read and understand the patient information sheet. Yes/No

I understand that I have been asked to give a sample of blood to be used in research. Yes/No

I understand that I will NOT benefit from this research Yes/No

I know that I do not have to take part in this research if I choose not to. Yes/No

I have discussed this with a nurse/doctor. Yes/No

I have discussed this with my mum/dad/guardian Yes/No

I am happy to consent for myself Yes/No

Name

Sign

I have discussed this with
and I am satisfied that there is good understanding.

Name of nurse/doctor

Sign

Appendix J. List of solutions and suppliers

Solutions made in-house

Detection Buffer	-0.1M Tris, 0.1M NaCl. PH 9.5
DIG Hyb Buffer	-5xSSC, 0.1% N-laurosarcosine, 0.02% SDS, 1% DIG block (made up in Maleic Acid buffer) (from DIG hybridisation protocol, Roche, UK)
DIG Wash Buffer	-0.3% Tween in Maleic Acid buffer (from DIG hybridisation protocol, Roche, UK)
Maleic Acid Buffer	-0.15M NaCl, 0.1M Maleic Acid. pH 7 with solid NaOH (from DIG hybridisation protocol, Roche, UK)
Phosphate Elution Buffer	-4mM KPO ₄ . pH8.5 (1M KPO ₄ in made from 9.5ml K ₂ HPO ₄ and 0.5ml KH ₂ PO ₄ (Hasseman, 2001)
Phosphate Wash Buffer	-5mM KPO ₄ , 80% ethanol. pH8.5 (Hasseman, 2001)
20x SSC	-3M NaCl, 0.3M Sodium Citrate. pH 7
STE buffer	- 0.14% w/v NaCl, 25mM Tris-HCl, 10mM EDTA. pH 8
Stringency buffer	-0.4% SDS/0.5xSSC
TE buffer	-0.1M Tris, 0.1mM EDTA. pH 8
50x TAE buffer	-M Tris, pH8, EDTA, 57.1ml Glacial Acetic Acid (make up to 1 litre with water)

Kits used

DNADetector™ HRP Southern Blotting Kit (KPL, Maryland, USA)
ECL Labelling and Detection Kit (Amersham Pharmacia Biotech, Bucks, UK)
PCR Core Kit (Qiagen, UK)
PCR DIG Detection Kit (Roche, UK)
PCR DIG Labelling Kit (Roche, UK)
QIAquick PCR Clean Up Kit (Qiagen, UK)
Qiagen QIAamp Extraction Kit (Qiagen, UK)
Southern Detection Kit (KPL, Maryland, USA)

Chemicals and Products from Sigma-Aldrich, Dorset, UK

Chloroform
DMSO

DNAase free RNAase
Glucose
Hybrislip coverslips
IsoAmyl Alcohol
Kodak X-OMAT
Lysozyme
Maleic Acid
NaCl
N-laurosarcosine
PerfectHyb Plus
Phenol
Potassium Permanganate
Proteinase K
SDS
Sodium Acetate
Sodium Carbonate
Tris
Tween 20

Chemicals and product from other suppliers

Agarose (Helena Biosciences, Sunderland, UK)
Biodyne C membrane (Pall Life Sciences, Hants, UK)
Cy5 Ester (Amersham Pharmacia Biotech, Bucks, UK)
DE81 filter paper (Whatman, Kent, UK)
dNTPs (Promega, Southampton, UK)
EDC (Pierce, Illinois, USA)
Ethanol (Merck, Dorset, UK)
EZ load Precision Mass Molecular Standard (Bio-Rad, California, USA)
 γ -P³² ATP (Amersham Pharmacia Biotech, Bucks, UK)
HCl (Merck, Dorset, UK)
H₂SO₄ (Merck, Dorset, UK)
Hybond N+ Nylon Membrane (Amersham Pharmacia Biotech, Bucks, UK)
MgCl₂ (Perkin Elmer, Massachusetts, USA)
Molecular Biology Grade Water (Fisher, UK)

Oligonucleotides (MWG-Biotech, Milton Keynes, UK)

P30 spin columns (Bio-Rad, California, USA)

Polystyrene slides (Nunc, Paisley, UK)

Red streptavidin coated microspheres (Bangs Laboratories, Fishers, Illinois, USA)

T4 Polynucleotide Kinase (Roche, Brussels, Belgium)

Taq Polymerase (Promega, Southampton, UK)

Terminal Deoxynucleotidyl Transferase (Promega, Southampton, UK)

Virkon (Merck, Dorset, UK)