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Materials

2.1 Chemical reagents

All common chemicals were purchased from Sigma Chemical Co., BDH Chemical Ltd., and Difco Laboratories unless specified below or in the text.

Amersham Pharmacia Biotech	Dextran sulphate, Na^+ salt
Bio-Rad Laboratories	β-mercaptoethanol
Gibco BRL Life Technologies	Foetal bovine serum
	ultraPURE™ Ammonium sulphate, enzyme grade
	ultraPURE™ agarose
Roche Applied Science	Restriction Buffer B
Stratagene®	Perfect Match® (1 U/µl)
	Taq Extender

2.2 Enzymes and commercially prepared kits

All restriction endonucleases were purchased from New England Biolabs, unless listed.

Amersham Pharmacia Biotech	T4 DNA ligase (1 U/µl)
	*Sau*3A1
Bio101Inc	Geneclean II
Gibco BRL Life Technologies	M-MLV reverse transcriptase
New England Biolabs	T4 DNA ligase
PE Applied Biosystems	Amplitaq™
	Sequenase
	TaqFS
Qiagen	DNA gel purification
Roche Applied Science	Klenow enzyme (sequencing grade, 5 U/µl)
	T4 Polynucleotide kinase
Sigma	Ribonuclease A
USB	Shrimp Alkaline Phosphatase
	Exonuclease I

2.3 Nucleotides

Amersham Pharmacia Biotech	Redivue™[α-^{32}P]-dCTP (AA 005) aqueous solution (370 Mbq/ml, 10 mCi/ml)
	Redivue™[γ-^{32}P]-dATP (AG 1001) aqueous solution (370 Mbq/ml, 10 mCi/ml)
	[α-^{35}S]-dATP (Q11135) (370 Mbq/ml, 400 Ci/mmol)
PE Applied Biosystems	Fluorescently labelled (TET, HEX, NED) dideoxyadenosine triphosphate (ddA)
	Fluorescently labelled (ROX) dideoxythymidine triphosphate (ddT)
Amersham Pharmacia Biotech	2'-deoxynucleoside 5'-triphosphates (dATP, dTTP, dGTP, dCTP)
	dideoxyguanine 5' -triphosphate (ddGTP)
	Random hexanucleotides pd$(N)_6$, 5'-PO_4, Na^+ salt

2.4 Solutions

Solutions used in the present study are listed below, alphabetically within each section. Final concentrations of reagents are given for most solutions. Amounts and/or volumes used in preparing solutions are given in some cases. Unless otherwise specified, solutions were made up in autoclaved nanopure water.

2.4.1 Buffers

10x Ligase buffer	500 mM Tris-HCl (pH 7.4)
	100 mM dithiothreitol
	100 mM $MgCl_2$
10x PCR buffer	670 mM Tris-HCl (pH7.4)
	166 mM $(NH4)_2SO_4$
	67 mM $MgCl_2$
1x TE	10 mM Tris-HCl (pH 7.4)
	1 mM EDTA

1x $T_{0.1}E$	10 mM Tris-HCl (pH 8.0) 0.1 mM EDTA
SAP reaction buffer	20 mM Tris-HCl (pH 8.0) 10 mM $MgCl_2$

2.4.2 Electrophoresis and hybridisation solutions

6x Buffer II	0.25% bromophenol blue 0.25% xylene cyanol 15% ficoll
Denaturation solution	0.5 M NaOH 1.5 M NaCl
Formamide dyes	80% v/v deionised formamide 0.1% w/v bromophenol blue 0.1% w/v xylene cyanol 1 mM EDTA 50 mM Tris-borate (pH 8.3) (*i.e* 0.56x TBE)
Formamide dyes mix	0.0075% w/v SDS 3.75 mM EDTA 1.6x formamide dyes
6x Glycerol dyes	30% v/v glycerol 0.1% w/v bromophenol blue 0.1% w/v xylene cyanol 5 mM EDTA (pH 7.5)
Neutralisation solution	1.5 M NaCl 1 M Tris-HCl (pH 7.4)
20x SSC	3 M NaCl 0.3 M Trisodium citrate

10x TAE	400 mM Tris-acetate 20 mM EDTA (pH8.0)
10x TBE	890 mM Tris base 890 mM Borate 20 mM EDTA (pH 8.0)

2.4.3 Media

All media were made up in nanopure water and either autoclaved or filter-sterilised prior to use.

For agar used for bacterial growth 15 mg/ml bacto-agar was added to the appropriate media. Antibiotics were added to media as appropriate (see Table 2.1) to the following final concentrations: Ampicillin (sodium salt dissolved in 1 M sodium bicarbonate, stored at -20^{o}C), 100 μg/ml; Kanamycin (purchased as a solution, stored at 4 ^{o}C), 30 μg/ml; Chloramphenicol (stored at 4 ^{o}C), 12.5 μg/ml (all supplied by Sigma).

Table 2.1: Clones and appropriate antibiotics.

Clone type	**Library**	**Antibiotic**
Cosmid	LL22NC01	Kanamycin
PAC	RPCI1,3,4,5, 6	Kanamycin
BAC	RPCI-11, 13	Chloramphenicol
SIL clones	FS library	Ampicillin

LB	10 mg/ml bacto-tryptone 5 mg/ml yeast extract 10 mg/ml NaCl (pH 7.4)
2X TY	15 mg/ml bacto-tryptone 10 mg/ml yeast extract 5 mg/ml NaCl (pH 7.4)

2.4.4 DNA labelling and hybridisation solutions

100x Denhardt's	20 mg/ml Ficoll 400-DL
	20 mg/ml polyvinylpyrrolidine 40
	20 mg/ml BSA (pentax fraction V)
Hybridisation buffer	6x SSC
	1% w/v N-lauroyl-sarcosine
	10x Denhardt's
	50 mM Tris-HCl (pH 7.4)
	10% w/v dextran sulphate
OLB3	240 mM Tris-HCl (pH 8.0)
	75 mM β-mercaptoethanol
	0.1 mM dATP
	0.1 mM dGTP
	0.1 mM dTTP
	1 M HEPES (pH 6.6)
	0.1 mg/ml hexadeoxyribonucleotides (2.1 OD units/ml)

2.4.5 General DNA preparation solutions

GTE	50 mM glucose
	1 mM EDTA
	25 mM Tris-HCl (pH 8.0)
3 M K^+/5 M Ac^-	300 mM potassium acetate (pH 4.8)
	11.5 ml glacial acetic acid
	28.5 ml H_2O

2.5 Size markers

1 kb ladder (1 mg/ml) (Gibco BRL Life Technologies)
Contains 1 to 12 repeats of a 1,018 bp fragment and vector fragments from 75 to 1,636 bp to produce the following sized fragments in bp: 75, 142, 154, 200, 220, 298, 344, 394, 516/506, 1,018, 1,635, 2,036, 3,054, 4,072, 5,090, 6,108, 7,125, 8,144, 9,162, 10,180, 11,198, 12,216.

Lambda DNA/*Hin*d III (Gibco BRL Life Technologies)
Contains *Hin*d III restricted dsDNA fragments of the following sizes (kb): 23.13, 9.416, 6.557, 4.361, 2.322, 2.027, 0.564, 0.125

Analytical marker DNA wide range (Promega)
Provides an evenly spaced distribution of DNA fragments from 0.702 kb to 29.95 kb

DNA molecular weight marker V (Boehringer - Mannheim)

2.6 Hybridisation membranes and X-ray and photographic film

Amersham	Hybond-N™ Nylon (78 mm x 119 mm) (used for high-density clone gridding)
Polaroid	Polaroid 667 Professional film
X-ray film	Fuji RX medical X-ray film

2.7 Sources of genomic DNA

Human placental DNA for pre-reassociation (ready-sheared) was purchased from Sigma Chemical Co. Human placental DNA for PCR was purchased from Sigma Chemical Co. DNA from whole chromosome 1 hybrid cell line, GM13139, and chromosome 1p specific cell line, GM11526A, was kindly provided by Richard Wooster

2.8 Bacterial clone libraries

2.8.1 Cosmid libraries

Cosmid clones used for the development of the fluorescent fingerprinting were from the Lawrence Livermore flow-sorted 22 chromosome cosmid library (LL22NC01) (prefixed 'cE') were kindly provided by Ian Dunham and the chromosome 22 mapping group.

2.8.2 PAC and BAC libraries

The RPCI-1, RPCI-3, RPCI-4, RPCI-5 (prefixed 'dJ'), and the RPCI-11 (prefixed 'bA') BAC libraries were used as a source of human derived PAC clones and BAC clones respectively in this thesis. These libraries were all kindly provided by Pieter de Jong and Joe Catanese (see http://bacpac.med.buffalo.edu/), and imported and maintained by the Sanger Institute Mapping Core Group.

2.8.3 cDNA libraries

A range of up to 9 different cDNA libraries were used in this study (see Table 2.2). cDNA libraries were imported and maintained by Jacqueline Bye and Susan Rhodes. Each library contains 500,000 cDNA clones, divided into 25 pools of 20,000 clones. Five pools were combined to form a superpool containing 100,000 clones. Prior to their use in PCR, each superpool was diluted 1:100 and 1:1000 in $T_{0.1}E$.

Table 2.2: cDNA libraries used.

cDNA lib. code	cDNA library description	Vector	Source/ Reference
1. U*	(Monocyte NOT activated-from a patient with promonocytic leukaemia) (U937+)	pCDM8	Simmons (1993)
2. AK	Adult kidney	pcDNA3.1	Invitrogen
3. AB	Adult brain	pcDNA3.1-Uni	Invitrogen
4. HeLa	Cervical carcinoma cell	pcDNA3.1-Uni	Invitrogen
5. SK	Neuroblastoma cells	pCDNA1	Invitrogen
6. T	Testis	pCDM8	Clontech
7. FLU	Fetal lung	pCDNA1	Invitrogen
8. HPB*	T cell from a patient with acute lymphocytic leukaemia (HPBALL)	pH3M	Simmons (1993)
9. AH	Adult heart	pcDNA3-Uni	Invitrogen

* Generously provided by Dr Simmons, Oxford (Simmons, D., et al., 1993).

2.9 Primer sequences

All primers were synthesised in house by Dave Fraser or externally by Genset. Table 2.3 lists the universal primer. Tables 2.4 and 2.5 list the primer pairs used in this thesis for cDNA library screening and exon amplification, the sequence and size in base pairs (bp) of each PCR product. The 110 STSs used to screen large insert bacterial clone libraries (and the resultant 878 positive PAC clones) can be found in 1ace within STS pools pool_1pc-1p13_STS1 – 5. Where appropriate, the clones, or genes from which the STSs were derived are also listed.

Table 2.3: Vector-specific primer used in vectorette PCR.

Primer Name	Primer Sequence
224*	CGAATCGTAACCGTTCGTACGAGAATCGCT
BPHI*	CAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGACAG AAGGGAGAG
BPHII*	CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCT CTCCTTG

* (Riley, J., et al., 1990)

Table 2.4: STSs designed for cDNA screening and Link PCR product synthesis. S=Sense, A=Anti-sense; a 60^{o}C annealing temperature was used for all PCR reactions. stSG # corresponds EMBL accession numbers assigned to individual STSs.

Gene	Exon	stSG #	Primer 1 (S)	Primer 2 (A)	bp
bA483I13.C1.1.mRNA	e2	452926	GGTATCTGCCGACCCTTTGT	GAGTAGGCAGTAGCTTGAGT	147
bA483I13.C1.3.mRNA	e2	452927	GCAGTCTGGAGATTGGTGGA	TGCATCATGACTTTCAAGCG	102
	e5	452928	GGGATTATTGATTGTGGCAA	CGGCATAAGGTACAATGCCT	100
	e7	452929	GGTTTCACCTCAAACATCAT	ACATCTCTTTATAACACAGG	164
bA475E11.C1.2.mRNA	e1 5'UTR	452930	TGACGGCTGAAGAAACAGTG	CTCCAGGGCCAGCATACTAA	104
	e4	452931	GCTTTTGACTTTGCCTCGTC	GCTTCCTATCAGCAGGGATG	128
	e7	452932	CAGCACTCAACCAGCAATGT	TCCAGGATTACGAGGAGTGC	149
	e10	452933	ATGAGACTCCTAAGCAGCCG	GGGCAGCACTTTGACGTATT	137
	e12	452934	ATACCTGGAGTGGCTGGATG	GTTGTGCCAACAACACGAAC	100
	e14 3'UTR	452935	CGAAGAGGCCCCTTATTACC	GGAGTGCACACCAACAACTG	166
bA475E11.C1.1	e1 5'UTR	452936	AGGCTATGCATAGTGAGACT	GCTTGACTTAGAAGCGTCTC	155
	e5	452937	ACTGCAGGGACACCTTGAAC	CCAACGATTGTTGATTCGTG	105
	e6	452938	TTGGAGATGCTGCTTGAAGA	AGAGAAGGTGGAGGCCAAGT	117
	e10	452939	GAAGCAAAACGTGGAGAAAA	TTGAATCTGAGTGTGGTGCC	92
	e13	452940	CTTCCAAATCCAGCCCTACA	ATGGGTTGCTACCAACTTGC	127
bA297O4.C1.1.mRNA	e1 5'UTR	452941	GCCACTATTGGGAGACCAAG	GTAGAGCCAGAGGTTCGACG	124
dJ831G13.C1.1.mRNA	e1	452942	CTTTGCTATTTTCGCCTTCG	CTGAAGGGATAGCCAAATGC	123
	e3	452943	CCTTCACCTTCTTCTGGCTG	CTTCCTCTCCATGGCACACT	120
	e4	452944	GCTCAGTGCTTCTTGTGCAG	TGGCTGCTAGGAACCAGTCT	146
bA180N18A.C1.2.mRNA	e1	452945	CCCACTGATCGTGAACAACA	CTCTGAGTCTTTGCGCTGGT	154
dJ773N10.C1.1.mRNA	e2	452946	CAGCCTGCATCTTCCTCTTT	AGACCTTCTCCAGCTCCTCC	125
dJ1003J2.C1.1	e2	452947	CCCTGAATGAGAAGGAGCTG	CACGGACTCAGTGACATGCT	148

	e4	452948	CTGTCTCTTTGTGGGGCTGT	ACAGGACATTCACTCCAGGG	102
	e7	452949	ACCCAGGTCTTCTTTGCCTT	GCCAACACTGACGTGAAGAA	134
	e9	452950	CCTTCATCGCCTTCACTGAG	GTGAACATCTCCTTGGGCAC	169
	e12	452951	CGCTACCTGTATTTCCCCAA	CCCTTCTTGTAGGACACGGA	149
bA470L19.C1.2.mRNA	e1	452952	CACCAAGCATTCCATACGTG	GAACCCAATGGGGATTCTTT	150
bA284N8.C1.2/.3	e2	452953	ATGCTCGGCTGTCTTCAAGT	AATGGTGAGTCATTCTGGGC	128
bA165H20.C1.3.mRNA	e3	452954	TGTTACTTCACCAACTGGGC	TGGTGATCTCGTTGTTCTGC	127
	e5	452955	TTCCACTCCTGAGAACCACC	CTGCACCAGGACAGTGAAGA	151
	e7	452956	CTATGACCTCCATGGCTCCT	ACATTGAGGTAGGCGTTGCT	96
	e10	452957	AACAACTTTGGAGGTGCCAT	TTGTACTCTGCAGGCCCAGA	124
dJ1125M8.C1.1mRNA	e2	452958	GCGGATAACTACCCTTTTGG	AAATAACACCCAGGCCCTCT	128
	e4	452959	ATGCAGGCAGGTACCAGAAA	TTCTTAAATCGAGGCACCAA	91
	e6 3'UTR	452960	ACGTTACTGTGGCCCTCTTG	ACAGAAACCCACAGACCCAG	154
dJ1125M8.C1.2	e1	452961	GAATGGAGGAGCAGGGTGTA	TCCAGGTAGTTGGTGAAGGG	121
	e3	452962	ACAACAGGTTCAATCCCAGC	TGTCATAGCCCAGGAACACA	105
	e5	452963	ACCCGCCAGTATTGTGGAGA	GGCAATCTGCCAGTACAGTT	107
	e8	452964	AACAATGGCTACTGCAGGCT	CTAGGCAGAGAAGGCAAAGC	153
bA552M11.C1.4.1/.2	e2 .2	452965	ACCACGTGGGATTTGATGTT	GGATGCCAAATTAAGAGCCA	137
	e3/4 .1	452966	CCTTTTGTGCTGGGGTTCTA	GCTGGAGGATCTGAGTGAGG	121
	e5	452967	TGAGGCTTGAATCCATTTCC	CTCTGGCCAGGAAAAGACTG	175
bA552M11.C1.5	e1/2	452968	TGCTTCCTTCCAGTCATGTG	TGGTCAGGCAGGACATAGTG	120
	e3/4	452969	CAGGCAACAAAACCAGAAGC	CCCAAACCCGTGATCAGTAT	103
	e5	452970	ATCATTTGCAGCCAGGTAGC	GTCCCCAATCCAGATTCTCC	154
dJ836N10.C1.1	e2/3	452971	GGAAGAACAAGGAAAAGGGC	CTCAATGCTTCCCCTCACTG	176
	e4	452972	AAAAGCCAGAGCTTCCTGAC	TGTGGTCCCTTTCTTCTTGT	120
dJ1073O3.C1.3	e1	452973	CTGGGCTGAAAACTGCTTGT	GTTGGGCTCAAGAAGTCCAT	134
	e3	452974	GACCTGGTGTGCTCAGGATT	TTCCCATTGATCATACCCGT	144
dJ1037B23.C1.1.mRNA	e2	452975	TCCCTCTTCTGCTAATCCCC	ACCTCAGCTGGGATATCTGG	122
	e4	452976	AGCGTGGACTTGGGAGAGAT	GTGATGTCCATCGCCTTGAG	107
	e6	452977	TAGGAGTCTGTCGTGTGGGG	TTACCTCCACCAAGGAGTGC	114
	e8 3'UTR	452978	AAACAGTGTTGTGCAGTCGC	CATCACCTTGGGAGACACAA	144
dJ1156J9.C1.1	e1 5'UTR	452979	ACCTTGGAGCGGGATCTTAT	TGCCAGGGAATTGTTGTATG	127
dJ929G5.C1.1.mRNA	e2	452980	TCCTGTTGAAGAGTGGCTCC	TCCAGAATAAGTGGATTCGG	157
	e4	452981	GTTTGTGTTTCGTGCCCTTT	TATTGCACAATGCCCTGGTA	120
	e6	452982	CAGTAACAATGCCACTGGCC	CTTCTTTACTCGCCGTTCAT	118
	e8	452983	GCAATATGACAAGGACCGCT	TACGAGGCTGAAGTCCAAGC	121
bA12L8.C1.1.mRNA	e2	452984	CATCCTCATTGCACTGGTTG	TGCACGTGCTTATGGATCTC	159
dJ655J12.C1.2.mRNA	e1	452985	AAGACAAGGAAGAGCACCTG	GAGTCCTTGAAGTGGTCGGA	120
	e2	452986	GCTCTGTTCAGGAAAATGCC	TGATCATCAGTGAGCCAAGC	165
dJ655J12.C1.3.mRNA	e2	452987	AGTCCTCCCTGAACTGTTGC	TGGGCATGAGATAAAACACG	106
dJ686J16.C1.1.mRNA	e1/2	452988	GGGCAGTGTCCAATTTATGG	GGAAGGAGGACTGATGGTGA	116
bA39H13.C1.1.mRNA	e1	452989	AAAAACCCAGCTGGACAATG	TCAGCAAGATTCCTCGGTCT	142
	e2	452990	ATCTGGAAGCAGAGCCAGTA	CCATTTCAGAGCTTCTGTGC	90
bA42I21.C1.1.mRNA	e1	452991	CACATGCGTCGGCTTAAATG	CCTCCACGATCGATGTTTCT	95
	e2	452992	CCCTCGCTGGGAAAGACATA	TGCTGGGGGAAAAGATTACT	139
dJ776P7.C1.1	e1	452993	GGCACTCTATTCGCACGTCT	CTCCATCATCCCAGGACACT	99
	e1/2	452994	GCTGAGAGGATTATGGAGGC	CTGAACTCTGCCCCTTACCA	101
	e4	452995	CTGTCCTCCCACTGGAATGT	TTCCGAGGTGAAGGAGAAAG	145
dJ832K2.C1.1.mRNA	e1	452996	AAAAACTCCAGGACCTCCGT	ACCTGCAGCCTCAGTTTCAC	171
	e6/7	452997	CCGCATAATACCACCCTTTT	CAGCTGTTTCGTTTGCATCT	131
dJ832K2.C1.2.mRNA	e2	452998	CCTCCAAACACAGGCTCTCT	CATGATGTACCTGCCAGCTC	126
dJ832K2.C1.3.mRNA	e2	452999	CCTTCAAGAAGCCCATAAGC	CAACATTGGAGTGGAGAGCA	146

	e5	453000	AGGCAAGGATAACGCAGAGA	CTTAGGTTCTGGTTGGTGGG	133
	e8	453001	ATCCCTTCAGCACTCACTCC	TCTTTGGGTTTTTCTTTGCC	98
bA224F24.C1.1.mRNA	e1	453002	TTAGAGGCCAATGCTTCTCC	AGCGAGGGTTCCCATATCTT	95
	e4	453003	ACGGCAGCAAAAGCAATTAT	TTCTTTTCATTTTCCCGTCG	125
	e6	453004	GGGCTTTAACAATCCTCAGC	CTGGTTAACTGCTGCCAGGT	124
	e8	453005	TTGCCTGCTTGATGTATGAC	CTCTGAAGTTGGCATGGCTT	131
	e11	453006	AAGAAGATCTCGTCCCACCC	GACAGAGTGAGGGCAGAAGG	105
	e15	453007	AGCAACCGAGAACCTTCAGA	AGAGACTCATGTTGGGGCTG	149
dJ794L19.C1.1.mRNA	e1	453008	GGCGGCTAAAATGAGTGAAA	ATAGACAGGTCCAGCCCCTT	141
	e3	453009	AGGATGTTTCCTGCCATGAG	TTTTATTGTCCACAGGCACA	94
	e5	453010	CTCGAGTTCATGTGATTCGC	AGGCCGTAACTGTGGTGAAC	123
	e8	453011	TACCAACTCCTCCCTCGTTG	CATGTGTGGTGATGAGGAGC	137
dJ834N19.C1.1.mRNA	e1	453012	TACCGGTCAGACTCCAGGTC	AGGTCCTCTTCTTTGCCTCC	93
	e3	453013	CAGTAACTGAGGAGGGCCAC	GGCTGCGATAGAAAGCAAAG	143
dJ834N19.C1.2.mRNA	e2	453014	AGACGAGGTCTTGCCACATT	GCATGGTGGCTTATGCTGTA	108
dJ599G15.C1.5.mRNA	e1	453015	TCGCTAGCCATTATCCAACC	CCTGTCCTTGTAGTGGGCAT	129
dJ599G15.C1.6.mRNA	e1	453016	ACTCTTCAGGAGCCACATGC	TCTACTGGAAGAGCACCAGC	95
dJ1042I8.C1.4.mRNA	e2	453017	ATGCTGGCCACAATCTACCT	GATCACTCCCCACAGCACTT	127
	e3	453018	TGGTCCAGTGAGAAAGCAGA	CCGGCCATTTGAGTTACAAG	125
	e5	453019	GGAACGAGAGCTGATCCAGT	AGCTGTTCTCGGAAGTCCTG	138
	e7	453020	GGAGGAATGTGCCATCACTT	GAGCATCCTGCCATTCATCT	152
	e9	453021	AGGAAGCTGCAGGAGTCTGA	CCAAGAAAGTGCCTTCACAA	124

Table 2.5: Exon specific primer pairs designed to pharmacogenomic gene targets. An annealing temperature of 60^{o}C was used for PCR reactions except where listed in chapter 6.

Target	Chr	Exon	stSG #	Primer 1	Primer 2	bp
GSTM4	1	1	452701	TCCGGACCTTGCTCCCTGAA	CCTAGTCTACACTGCACTGC	528
		2	452702	TTCCCTCCTTAGGGCTATCT	TGTCATAGTCAGGAGCTGCC	724
		3/4	452703	TCACTTCTTCTTCCCCACGG	ACACAGACTCACTCTGAGCAT	566
		5	452704	TGGCTGGATTGGGGTGCTAT	GGTGCTATTACATCCCACTACA	527
		6/7	452705	GTGTAATAAATGCTGGTATG	AGGACTGACCCTCTCATTCAA	701
		8	452706	CCTCAGCACTTGAGCCCACG	AGCAAATAAGACAAGACTAT	689
GSTM2	1	1	452707	AGCCCCATGAGCGCGCTCCA	GGGGAGCCCCATCTCCTCCT	408
		2	452708	GCGGTGGGACGGGGGTGCGT	CCCATCATAATTACCCAGAC	534
		3/4	452709	GCCCCCGTCTGGGTAATTAT	CGCATCTTGCAACCAAATCT	719
		5	452710	TCGGCTTGGCTGGGCTGTGAG	TGTATTTTCTTTCACTCGTCA	614
		6/7	452711	CAGCTGGGGCCATGCACAAA	GGCGTGAGCCACCGCACATG	508
GSTM1	1	1	452712	CCTGGGAGGCGGGAGGAAGT	GACTTTGTCTGCACCAGGGA	448
		2	452713	TGGGACGAGGGCGCAGGGGA	AAGCCCTGAGGGACACCCGT	535
		3	452714	GCCCCCTGTCTAATTGGGAC	TCACATGAACGAATGCAGGT	484
		4/5	452715	TGTCCACCTGCATTCGTTCA	AGATGCAGCTCACTGGGGAC	468
		6/7	452716	CTCTGCCTTCTGATCAGTTT	GATAATTCTGTTACCTTACT	709
GSTM5	1	1	452717	ACTGGGAGGCGGGAGGGGGC	GACTTTCTCTGCACCAGGCC	448
		2	452718	TAAGCGAGGGTCCTCTGGTG	AACCCAATTAGACAGGGTGT	548
		3/4	452719	CTGGGGCGGGATGCTGGACA	TGACCAGCTCCATGTGGTTA	878

		5	452720	AATGTGCGGGGGGAAGGTGA	GGTAGCAGATCATGACCAGT	409
		6/7	452721	GAAGAGCATCTCATTCTGAT	GTATAATGTGCTGGGCATGA	663
		8	452722	GCCTGCAGCAAAGCTACTTG	ACAGTCCTGAGTCAAGGGAG	621
GSTM3	1	1	452723	CGGCCCTGTGGAGCCGCGGA	CAGCGGTTCAGCGACTGCGC	520
		2/3	452724	CCCCGGGCCGGGGAACGTTA	GCAAGGATGGATATACTTGAA	622
		4/5	452725	TGTTCACTGCCCTGCAAGTGT	GCAGCAGAATGGAACAGAGA	463
		6/7	452726	GTGCTGGTGCCTCTTCTTTC	CCCATTAGGCAAAAGCCGGG	567
		8	452727	GGTTGGGGTCGTTATAAGAT	TCTCCTACCCCGTGGTCACA	748
GSTP1	11	1	158595	GGTCCTCTTCCTGCTGTCTG	CCCCTGAAAGCCGCTAAC	465
		2	158596	GTTAGCGGCTTTCAGGGG	GAGGGAACAGGGAAACAGGT	384
		3	158597	GCCCCAGTGTTGTGTGAAAT	AGGTCTCCGTCCTGGAACTT	385
		3/4	158591	TCTCGTACTTCTCCCTCCCC	GATTTAAACAAAAGGGCTCCG	399
		5	158592	ACATCCTCTTCCCCTCCTCC	AGGTTGTGTCTTGTCCCAGG	399
		6	158593	AAGGATGGACAGGCAGAATG	CATCCCCTAGGTCTGCTCTG	399
		7	158594	GCTTCCAGATGGACACAGGT	TGCTGGAGGAGCTGTTTTCT	498
GSTT1	22	1	140015	CTCCAAACCAGACCAGCAAT	CTAAAGAGTGTCCCAGGCGT	348
		2	140017	TGGAATAGCAGGAAGGCAAG	GTCTTTGCCAACCAGGAGTG	351
		3	452728	GACTATGTATGAAATACCCA	CTGGTGCCTGAACACCTTTG	276
		4	452729	GCTCAGCATCACTAATCATT	GATTTGGGGACCACAGATCT	406
		5	140020	GGGGGTTGTCTTTTGCATAG	CCTGCTTATGCTGCCACAC	659
CYP1A1	15	1a	452730	CTGACACTCTAGATATTGGCT	GTCAGAGGCAATGGAGAAAC	556
		1b	452731	ATGGTCAGAGCATGTCCTTC	CCCAGGCCCTGATGCCATCT	533
		2/3/4	452732	CCTGTGGACTTTCCCTACCT	CAGTGGCTCCATGGGGCCTT	564
		5/6	452733	TTGCCCTGAGCCTGACTGAG	GGTAGACAGAGTCTAGGCCT	637
		3' UTRa	452734	TTGAGAGCCCTGAGGCCTAG	CAGGACAGCAATAAGGGTCT	620
		3' UTRb	452735	CAGCAAGTTAGAACTAGCCA	GGCTACACCTCTTCACTGCT	697
CYP1A2	15	1a	452736	ATCTCAACCCTCAGCCTGGT	CTCATCGCTACTCTCAGGGA	700
		1b	452737	GCCCTCAACACCTTCTCCAT	TCTGAGGTGTCCAGAGCCTT	536
		2/3	452738	ACCTTGGAAGTGCCAGAGGT	TCAAGGCTTCTCCTGGCTAT	753
		4	452739	GACAGTCTTACATAAGAGTG	CAATAGGGTCATGCTTGTGA	511
		5	452740	TGCTGAAGTTAAAGAACAGG	GATTATAGGCTTGAGCCACT	484
		6	452741	CCATCTCCTCTGTTCCTCTT	GCCTCCTAAAATGCTGGGAT	464
CYP2A6	19	½	452742	GGCAGTATAAAGGCAAACCA	GAACACTGAGACCTTCGTGT	722
		¾	452743	TGTCTCCATTCCCGCGTTCA	GCAGTTGGCAGGTTGTGGTA	626
		5	452744	CAGCCTCGTTTAAATACCTG	GGATTACAGGCTGTTAGCCA	588
		6	452745	GAGCGAGTCTGGTAGATCTA	CCTGTCTCTGGACAGCAAGT	404
		7	452746	TGGCACAACCTGGTTAACAG	CAGGGTCTAGAAAGCTTCTA	467
		8	452747	CCTGTTTCAGAGATGTGAAC	GGTAGATTCTAACAGGAACT	443
		9	452748	TGCACTGAGAGTGGGCTTCA	ATTAGGTGAGCGTGCAATGG	538
CYP3A4	7	1	452755	ACTGCAGGCAGAGCACAGGT	GGCATGATCTCAGCTCACTG	613
		2	452756	GACCATGAAGACTTCAGCTG	GAGGTTCCTGAGAGTTAGCA	519
		3	452757	GACATCAGAAAGACAAAGAG	TCCCATTGCAATACTCTACA	510
		4	452758	CATATGAAGACTTGAGTGGC	GCACATAAAGCTGGTGAAAT	549
		5/6	452759	CCTCCACAACTGATGTAGGA	GGAATATCAGCTCCATGGCA	589
		7	452760	AGTGTCTCCATCACACCCAG	ATGATGACAGGGTTTGTGAC	592

		8	452761	GTCATAGAAGGAATGGCTTC	GCTGTCTCTGACTCATTCTC	476
		9	452762	CCATCTCACATGATAGCCAG	TCTAGCATGCCAGGTTTGCT	512
		10	452763	GCCCACATTCTCGAAGACCT	TTCAGAGCCTTCCTACATAG	523
		11	452764	ACATCTCAGTGGGCCACTGA	GGACATAACTGATGACCTTC	654
		12	452765	CAGCCCACAAAAGTATCCTG	GGCCTAATTGATTCTTTGGC	419
		13a	452766	CCTCAACACTGAAGGAGTGT	GTGCAGGAAAGCATCTGATA	669
		13b	452767	CACATGTTTTCTCTGGAGTA	GTGCTTTTAGGCTTATTGCT	791
NFE2L2	2	1	452768	AGAGCGCTGCCCTTATTTGC	TCCTGGCTCTGGCCAGACGT	589
		2	452769	CACTTCCCACCATCAACAGT	GTGTTTCCTTAAACCTGCCA	419
		3	452770	GTGCATCAAAGTGATCTCTG	CTTCGTTTATTGCCCAGCTG	577
		4	452771	GGGTCATGACTGGTTAGTAA	TCAGAGTTCCCAGATCAGAC	527
		5a	452772	GAGATAAGCCTGAAGATAAT	TCTTCCACTTCAGAATCACT	649
		5b	452773	TGTGGCATCACCAGAACACT	GCTGCAGGGAGTATTCACTA	695
		3' UTR	452774	GCATGCTACGTGATGAAGAT	TTATTTCTCTGTAACCCTGG	756

2.10 World Wide Web addresses

ACeDB	http://www.acedb.org/
ACT	http://www.sanger.ac.uk/Software/ACT
Baylor College of Medicine Search Launcher	http://searchlauncher.bcm.tmc.edu/
British Columbia Genome Sequence Centre	http://www.bcgsc.bc.ca/
Chromosome 1 Mapping Project - Sanger Institute	http://www.sanger.ac.uk/HGP/Chr1/
CHLC	http://chlc.org/
Coriell	http://locus.umdnj.edu/ccr
dbSNP	http://www.ncbi.nlm.nih.gov/SNP /index.html
DOTTER	www.cgr.ki.se/cgr/groups/sonn/ Dotter.html
EMBL	http://www.ebi.ac.uk/
Ensembl	http://www.ensembl.org/
GDB	http://gdbwww.gdb.org/
Généthon	http://www.genethon.fr/php/index.php
HGNC	http://www.gene.ucl.ac.uk/ nomenclature/
INTERPRO	http://www.ebi.ac.uk/interpro/scan.html
Incyte	http://incyte.com/company/news/1999 /genes.shtml
Locus Link	http://www.ncbi.nlm.nih.gov/LocusLink/
National Centre for Biotechnology Information	http://www.ncbi.nlm.nih.gov/

OMIM	http://www3.ncbi.nlm.nih.gov/Omim/
Primer 3	http://www.sanger.ac.uk/cgi-bin/primer3.cgi
PIX	http://www.hgmp.mrc.ac.uk/Registered/Webapp/pix/
PSI-BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
RepeatMasker	http://ftp.genome.washington.edu/RM/webrepeatmaskerhelp.html
The Institute for Genome Research	http://www.tigr.org/
The SNP Consortium	http://snp.cshl.org
The Wellcome Trust Sanger Institute	http://www.sanger.ac.uk/
Unigene	http://www.ncbi.nlm.nih.gov/UniGene/
University of California, Santa Cruz genome browser	http://genome.cse.ucsc.edu/
Washington University Center Genome Sequencing Center	http://genome.wustl.edu/
Whitehead Institute	http://www-genome.wi.mit.edu/

Methods

2.11 Isolation of bacterial clone DNA

2.11.1 Miniprep of cosmid

1. Ten ml of 2 X TY containing 30 μg/ml of appropriate antibiotic (kanamycin for cosmids and PACs, chloramphenicol for BACs) were inoculated with a scraping from the frozen glycerol stock of the chosen bacterial clone and incubated overnight at 37^{o}C with shaking.
2. The cells were collected by centrifugation at 4,000 rpm for 10 minutes at room temperature in a Beckman J6-MC, re-suspended in 200 μl of GTE in a 1.5 ml eppendorf tube, and left on ice for 5 minutes.
3. 400 μl of freshly prepared 0.2 M NaOH/1% SDS were added to the cells, mixed by gentle inversion, and the sample left on ice for another 5 minutes.
4. 300 μl of 3 M K^{+}/5 M Ac^{-} (pH 4.8) were added, mixed by gentle inversion and left on ice for 10 minutes. The sample was centrifuged for 10 minutes at 14,000 rpm in an eppendorf microfuge.

5. The supernatant was transferred to a fresh tube and mixed with 600 µl of cold isopropanol and left on ice for at least 10 minutes. The tube was subjected to centrifugation for 15 minutes at 14,000 rpm in an Eppendorf microfuge at 4°C to pellet the DNA, the supernatant removed and the pellet re-suspended in 200 µl of $T_{0.1}E$.
6. 200 µl of 50:50 (v/v) phenol/chloroform were added to the sample, which was vortexed and briefly centrifuged. 20 µl of 3 M sodium acetate (pH 5.2) and 200 µl of isopropanol were added to the aqueous layer, and the sample placed at -20°C for at least 10 minutes. The tube was subjected to centrifugation at 14,000 rpm in an Eppendorf microfuge for 15 minutes at 4°C to pellet the DNA. The pellet was washed with 70% ethanol and re-suspended in 50 µl of $T_{0.1}E$.
7. 1 µl of 10 mg/ml RNase was added and the sample incubated at 37°C for 1 hour prior to storage at -20°C.

2.11.2 Microprep of cosmid, PAC and BAC DNA for restriction digest fingerprinting

1. 500 µl of 2 X TY containing 30 µg/ml of appropriate antibiotic (kanamycin for cosmids and PACs, chloramphenicol for BACs) were added to a 96-well deep-well microtitre plate (COSTAR).
2. Each well was inoculated from a glycerol stock with either a 96-well inoculating tool, or a sterile cocktail stick. A plate sealer (Dyntax) was placed on top of the plate to seal the wells, and the cultures grown for 18 hours at 37°C with gentle shaking.
3. For each well, 250 µl of the overnight growth were transferred to a clean microtitre plate. The cells were collected by centrifugation (Sorvall RT7, Du Pont Company Sorvall, Delaware US) at 1550 g for 4 minutes.
4. For each well, the supernatant was removed and the pellet re-suspended in 25 µl of GTE, by vortexing gently (a cocktail stick was used for resuspending pellets still attached to the plate).
5. 25 µl of GTE were added to each well and gently mixed. 25 µl of freshly prepared 0.2 M NaOH/1% SDS were added, mixed and left to stand for 5 minutes at RT.
6. 25 µl of 3 M K^+/5 M Ac^- (pH5.0) were added, mixed and left at RT for 5 minutes. A plate sealer was placed on top of the plate and the plate was vortexed gently for 10 seconds, prior to centrifugation of the samples in a microtitre plate in a Sorvall RT7 centrifuge at 1800 g for 10 minutes at 4°C (Sorvall RT7, Du Pont Company Sorvall, Delaware US).

7. 75 µl of the supernatant from each well were transferred to a fresh microtitre plate containing 150 µl of 96% ethanol (or 100 µl of isopropanol) and left at -20 °C for 30 minutes.
8. The microtitre plate containing the samples was centrifuged at 1800 g in a Sorvall RT7 centrifuge for 10 minutes at 4°C. The supernatant was discarded and the samples drained on a tissue.
9. 25 µl of ddH_20 were added to each well and the pellet resuspended by tapping or with a sterile toothpick. 25 µl of 4.4 M lithium chloride were then added to each well mixed by tapping and left for 60 minutes at 4°C.
10. After centrifuging the samples in a Sorvall RT7 centrifuge at 1800 g for 10 minutes at 4°C, the supernatant from each well was added to 100 µl of 96% ethanol in a fresh plate and left for 60 minutes. The samples were then spun in a Sorvall RT7 at 1800g for 10 minutes at 4°C, the supernatant discarded and 200 µl of 70% ethanol added. The pellets were then air dried until transparent.
11. 10 µl of $T_{0.1}E$ were added to each well and mixed by careful pipetting up and down. The resuspended DNA was then used directly for fingerprinting or stored at -20 °C.

2.11.3 Filterprep of cosmid, PAC and BAC DNA for restriction digest fingerprinting

1. Steps 1 – 6 from the microprep procedure (*2.11.2*) were followed before progressing to step 2 of the filterprep procedure.
2. A microtitre plate containing 100 µl of isopropanol was taped to the bottom of 2 µm filter-bottomed plate (Millipore cat. no. MAGVN2250). The total well volume of the sample was transferred to the filter-bottomed plate and the sample was filtered by centrifugation in a Sorvall RT7 at 1550 g for 2 minutes at 20°C.
3. The filter-bottomed plate was removed and the microtitre plate was left at RT for 30 minutes, before samples were centrifuged in a Sorvall RT7 at 1500g for 20 minutes at 20°C.
4. The supernatant was tipped from the microtitre plate and the DNA dried by inverting the plate and placing it on clean tissue paper, ensuring no disruption of the pellet.
5. 100 µl of 70% ethanol were added to the dried DNA to wash the pellet, mixed gently, and the DNA precipitated by centrifugation in a Sorvall RT7 at 1500g for 10 minutes at 20°C. For restriction digest fingerprinting the wash was repeated.
6. 5 µl of freshly prepared $T_{0.1}E$ / 1 µg/ml RNase were added and mixed gently to resuspend the DNA. Samples were stored at -20°C.

2.12 Bacterial clone fingerprinting

2.12.1 Radioactive fingerprinting

1. For each well of a 96-well microtitre plate of sample DNA, a premix containing 1x NEB2 buffer (as supplied by New England Biolabs), 0.72 U *Hin*d III, 1.3 U *Sau*3AI, 0.4 U Reverse Transcriptase, 0.07 µl [alpha-^{32}P]dATP (3000Ci/mmol), 0.04 µl 10 mM ddG was prepared in a 1.5 ml microfuge tube.
2. 2 µl of premix were added to the sample DNA using a Hamilton repeat dispenser. The reaction was mixed by gentle agitation and the samples were centrifuged in a Sorvall RT7 at 150 *g* for 10 seconds.
3. The reaction was incubated for 1 hour at 37^{o}C. The reaction was then stopped by the addition of 2 µl formamide dye.
4. The sample DNA was denatured at 80^{o}C for 10 minutes and loaded in groups of 6 onto a 4% polyacrylamide gel, leaving the first well and every subsequent seventh well of empty (see Section 2.14.2). Marker DNA (see Section 2.13.1) was denatured by boiling for 5 minutes and 2 µl were loaded in the first well and every seventh well. Fragments were resolved by running the gel at 74 W for 1.5 hours (or until the bromophenol blue dye front reached the bottom of the gel).
5. Following electrophoresis, the back plate was removed and the gel was fixed in a 10 % glacial acetic acid solution for 10 minutes then washed in water for 25 minutes. The gel was dried onto the front plate by incubation at 80^{o}C for 45 minutes in an oven. Autoradiography was for 72 hours at RT.
6. The autoradiograph was scanned using a flat bed scanner (Amersham) and the digitised version imported to IMAGE.

2.12.2 Fluorescent fingerprinting

1. For one 96-well microtitre plate of sample DNAs, three digest premixes were prepared, one for each fluorescent label, in three 1.5 ml microfuge tubes labelled TET, HEX and NED. Each premix contained 25.5 µl $T_{0.1}E$, 24.5 µl NEB2 buffer, 5.0 µl *Hin*d III (20 U/µL), 8.0 µl Taq FS, (32 U/µl) and 3.0 µl *Sau*3AI (30 U/µl) and 4.0 µl of the appropriate ddA-dye. Each premix was mixed prior to being aliquoted.
2. 2 µl of the TET premix were added to wells A1-H4 of the microtitre plate containing sample DNAs using a Hamilton repeat dispenser. Similarly, 2 µl of the HEX premix were added to wells A5-H8 and 2 µl of the NED premix were added to wells A9-H12. The plate was covered with a plate sealer, the reaction mixed by gentle agitation on a vortex. In order to ensure the sample was in the bottom of the wells the plate was centrifuged at 150 *g* for 10 seconds (Sorvall RT7, Du Pont Company Sorvall, Delaware US).
3. The reaction was incubated for 1 hour at 37^{o}C.
4. To precipitate the DNA, 7 µl of 0.3 M sodium acetate (pH 5.2) and 40 µl 96% ethanol were added to each well. For multiplexing the samples, rows 5 and 9 were added to row 1, rows 6 and 10 were added to row 2, rows 7 and 11 were added to row 3, and rows 8 and 12 were added to row 4 respectively, using a multichannel pipette.
5. The samples were incubated at RT for 30 minutes in the dark.
6. The samples in the microtitre plate were centrifuged in a Sorvall RT7 at 1550 *g* for 20 minutes at 20^{o}C to pellet the DNA.
7. The supernatants were discarded and the pellets dried by tapping the plate face down onto tissue paper.
8. The pellets were washed by adding 100 µl of 70% ethanol to each well, mixed gently tapping the plate, and the samples centrifuged in a Sorvall RT7 at 1550 *g* for 10 minutes at 20^{o}C.
9. The supernatants were discarded and the pellet dried as in step 7.
10. The fingerprinted DNAs were re-suspended in 5 µL $T_{0.1}E$.
11. Prior to loading, 2 µl of the marker DNA (see Section 2.13.2) were added to each sample using a Hamilton repeat dispenser. The samples were denatured for 10 minutes at 80^{o}C. 1.25 µl of each sample were loaded on a 5% denaturing acrylamide gel and resolved on an ABI377 Automated DNA sequencer using a 0.2 mm, 12 cm well-to-read 4.5% denaturing polyacrylamide gel (prepared by Sanger Institute Gel Production Team). Data were collected using the ABI Prism Collection Software v1.1.
12. After data collection, the gel image was transferred to a UNIX workstation for entry into IMAGE.

2.12.3 *Hin*d III and *Eco* RI fingerprinting

1. For one 96-well microtitre plate of sample DNA, a premix containing 231 µl H_20, 99 µl Boehringer buffer B (50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl2, 1 mM DTE, pH 7.5), 55 µl *Hin*d III 10U/µl (or *Eco* RI 10U/µl), was prepared in a 1.5 ml microfuge tube, and mixed using a vortex. 4 µl of the premix were added to each well of a 96-well microtitre plate containing previously prepared DNA (see Section 2.11.2), and the plate covered with a plate sealer (Dynex).
2. The reactions were mixed gently on a vortex and incubated at 37 ^oC for 2 hours.
3. The reactions were terminated by the addition of 2 µl of buffer II and either loaded straight away or stored at 4 ^oC.
4. 0.8 µl of the marker (see Section 2.13.3) were added to the first well and then every sixth well of a freshly prepared 1% agarose/1x TAE gel (see Section 2.14.1 for preparation). 1 µl of each sample was loaded (i.e. wells 2-5, 7-10 *etc*.) between the marker lanes. Fragments were resolved by electrophoresis through the gel at 4^oC in a cold room for 15 hours at 90 volts.
5. Following electrophoresis, the gel was cut down so the length was 19-20 cm and stained in Vista Green (mix 5 ml 1 M Tris HCL, 0.5 ml 0.1 M EDTA, 50 µl Vista Green, make up to 500 ml with H_20) for 30-45 minutes on a shaker. The gel was washed with ddH_20 to remove excessive stain.
6. The gels were scanned on a FluorImager SI. The parameters were set to 530 nm for emission filter, the pixel size was 100 microns, detection sensitivity was normal, digital resolution was at 16 bits, dye was single label, excitation filter was 488 nm, Em filter 1530 nm and PMT voltage was 800.
7. The gel image was transferred to a UNIX workstation for entry into IMAGE.

2.13 Marker preparation

2.13.1 Radioactive fingerprinting marker

1. 171 µl of $T_{0.1}E$, 25 µl of 10 X NEB2 buffer (as supplied by New England Biolobas), 16.5 µl of lambda DNA (500 µg/ml) and 5 µl of *Sau*3AI (50 U/µl) was added to a 1.5 ml Epperndorf microfuge tube and incubated at 37^{o}C for 1 hour
2. To a 43.5 µl aliquot of digested lambda DNA (from step 1) in a 1.5 ml Epperndorf microfuge tube was added 2.0 µl 10 mM dGTP, 2.0 µl 10 mM ddTTP, 4.0 µl [alpha-^{35}S] dATP (3000Ci/mmol), 1.0 µl AMV reverse transcriptase (10 U/µl) and incubated at 37^{o}C for 1 hour.
3. The reaction was stopped by the addition of 53 µl of 1:15 dilution of formamide dye.
4. The marker was stored at -20^{o}C.

2.13.2 Fluorescent fingerprinting marker

1. 70 µl $T_{0.1}E$, 10 µL NEB2, 6 µl lambda DNA (500 ng/µl), 6 µl *Bsa*J 1 (2.5 U/µl), 4 µl TaqFS (32 U/µl), 4 µl of 10 mM ddC-ROX were added to a 1.5 ml microfuge tube and incubated for 1 hour at 60^{o}C.
2. 100 µl of 0.3 M sodium acetate (pH 5.2) and 400 µl 96% ethanol were added to the reaction mix and incubated at RT in the dark for 15 minutes, then at -20^{o}C for 20 minutes. The Eppendorf microfuge tube was subjected to centrifugation in a bench top centrifuge at maximum speed for 20 minutes to pellet the DNA.
3. The supernatant was discarded and the DNA pellet dried by tapping the tube gently onto tissue paper. The pellet was washed by adding 200 µl 70% ethanol and spun in a bench top centrifuge at maximum speed for 5 minutes, the supernatant discarded and the pellet dried as described in step 2.
4. The DNA was re-suspended in 120 µl $T_{0.1}E$ and 120 µl blue dextran formamide dye.
5. The marker was stored at -20^{o}C.

2.13.3 For *Hin*d III fingerprinting

1. 19.2 µl $T_{0.1}E$, 1.5 µl Analytical Marker DNA wide range, 0.2 µl Molecular Weight Marker V and 4.2 µl 6x loading dye were added to a 1.5 ml microfuge tube.
2. The marker was stored at -20^{o}C.

2.14 Gel preparation and electrophoresis

2.14.1 Agarose gel preparation and electrophoresis

1. Agarose gels were prepared in 1x TBE (or 1x TAE, for *Hin*d III fingerprinting) containing 250 ng/µl ethidium bromide and the appropriate percentage of agarose according to the size of fragments being separated: 2.5 % agarose gels were used for electrophoresis of fragments below 1 kb; 1.0% agarose gels were used for analysis of larger fragments. Electrophoresis was performed at 50 - 90 V for 15 - 45 minutes depending on the separation required.

2.14.2 Polyacrylamide gel preparation for radioactive fingerprinting

1. 42.0 g of urea were dissolved in 10 ml 10x TBE and 35 ml ddH_20 by warming to 37^oC, and stirring.
2. A large glass plate (back plate – Gibco BRL) was washed on both sides and one side was treated with 2 % dimethyldichlorosilane, and left to dry.
3. A small glass plate (front plate – Gibco BRL) was washed on both sides with detergent and water and one side was treated with freshly prepared bonding solution (3 ml 96 % ethanol, 50 µl 10 % Acetic acid, 5 µl methacryloxypropyl-trimethoxysilane; Sigma-Aldrich) and left to dry.
4. The front and back plates were taped together along three edges (treated sides facing inwards) separated by 4 mm spacers.
5. 10 ml 40% acrylamide, 800 µl 10 % ammonium persulphate and 80 µl TEMED (KODAK) were added to the dissolved urea solution, mixed and poured in between the glass plates using a 50 ml syringe. A 4 mm, 60 well comb (IBI) was placed in the top of the gel (the edge not taped) and the glass plates clamped with bulldog clips. The gel was left to set for up to 3 hours.

2.15 Construction of small insert library

2.15.1 Library preparation

1. 1 µl 4 mg/ml PMSF was added per every 100 µl of flow-sorted chromosome preparations (previously treated with Proteinase K and sarkosyl) for a 40 µg/ml final concentration and incubated at room temperature for 40 minutes.
2. 20 µl 5 M NaCl was added to a concentration of 0.2 M. Two volumes of absolute ethanol were added, and the DNA precipitated overnight at -20^{o}C.
3. DNA was pelleted in a microfuge at maximum speed (14,000 rpm) at RT for 15 minutes.
4. The supernatant was removed with a P1000 Gilson pipette and the remainder removed with Gilson P200 pipette, avoiding the pellet. 1 ml 70% ethanol was added and the eppendorf tube centrifuged again at maximum speed for 7 minutes and the supernatant removed as before. Pellets were air-dried for approximately 10 minutes.
5. 17.0 µl of sterile TE were added directly to the pellet to give approximately 8.0 ng / µl of flow sorted DNA. DNA was mixed by very gentle flicking and allowed to dissolve for at least 2 hours.
6. 2.0 µl 10x buffer 2 (as supplied by New England Biolabs), 1.0 µl HindIII (20 U/µl) and 2.0 µl ddH_20 were added to 15 µl of flow-sorted DNA which was digested for 3 hours at 37^{o}C.
7. The *Hin*d III was inactivated by incubation of the digest at 65^{o}C for 20 minutes.
8. 2.5 µl (15 ng) of digested flow-sorted DNA, 1 µl (5 ng) of phosphatased pBluescript II (SK+) vector DNA (5 ng/µl) (kindly supplied by Dr. Mark T. Ross), 1 µl of 10x ligase buffer (NEB), 0.5 µl of T4 DNA ligase (NEB; 400 U/µl) and 6.5 µl of water (a total reaction volume of 10 µl) were incubated for 16 hours at 16^{o}C
9. The ligase was inactivated by incubation at 65^{o}C for 10 minutes.
10. 10 µl of TE were added and the ligation stored at -70^{o}C.
11. 1 µl of each of the samples were electroporated into *E. coli* XL1-blue electrocompetent cells (see 2.15.2)

2.15.2 Electroporation and library plating.

1. LB agar plates (containing 50 µg/ml ampicillin) were poured and left to dry before 40 µl of Xgal and 4 µl of 200 mg/ml of IPTG were spread on the surface of the plates and left for 2 hours at 37^{o}C to dry.

2. 40 µl of XL1 blue cells (previously thawed at room temperature and stored on ice) and 1 µl of *Hin*d III digested DNA were added to a 0.1 cm cuvette and mixed. After cells were electroporated at 25 µFD, 1.8 KV and 200 Ω, 1ml of LB media was added, mixed then the whole sample added to a 50 ml Falcon tube and incubated in a shaking incubator for 45 minutes. at 37^{o}C.
3. 50 µl were plated on the previously prepared selection agar plates and grown overnight at 37^{o}C and then stored at 4^{o}C for 1 hour to accentuate the blue non-recombinant cells. Recombinant cells were picked into flat bottom microtitre plates containing 150 µl LB broth containing 50 µg/ml ampicillin and grown overnight at 37^{o}C . Glycerol was added to the cultures to a final concentration of 7.5% v/v, aliquotted and then stored at -70 ^{o}C.

2.16 Applications using the polymerase chain reaction

2.16.1 Primer design

Primers were designed manually using the following guidelines:

1. Primer 3 (http://www.sanger.ac.uk/cgi-bin/primer3.cgi) was used to design primers sequences that were 20 bp in length, were possible beginning and ending with a C or G, and with an optimal annealing temperature of 60^{o}C.
2. Where possible sequences were chosen to avoid areas of simple sequence showing non-representative use of the bases and obvious repetitive sequence i.e., runs of single nucleotide (e.g. TTTT) or double nucleotide (CGCGC) motifs.
3. Sequences were chosen to exclude palindromes which will form inhibitory secondary structure, especially at the 3' ends (e.g. GACGTC).
4. As far as possible, sequences were chosen with a GC content of at least 50%.
5. Sequences were chosen to avoid complementarity between pairs of primers, especially at the 3’ end, which could result in primers annealing to each other and forming primer dimers.
6. If possible, sequences were chosen which would generate products of at least 100 bp in length.

216.2 Oligonucleotide preparation

All oligonucleotides used were synthesised in house by David Fraser or supplied as working dilutions from Genset. The concentration of the primer in ng/µl was determined by measuring the absorbance at 260 nm (Abs_{260}) and multiplying this by 33 and any necessary dilution factor.

2.16.3 Amplification of DNA by PCR

1. 1-3 ng/µl of genomic DNA were amplified in a reaction volume of 15 to 50 µl as required. Reactions contained approximately 1.3 µM of each oligonucleotide primer, 67 mM Tris-HCl (pH 8.8), 16.6 mM $(NH_4)_2S0_4$, 6.7 mM $MgCl_2$, 0.5 mM of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 1.5 U of Amplitaq™ (Cetus Inc.); 10 mM β-mercaptoethanol and 170 µg/ml of BSA (Sigma Chemical Co., A-4628) were added to the reactions from freshly made stock solutions as the reactions were set up.

2. Unless specified otherwise, cycling conditions were as follows: all reactions were preceeded by an initial denaturing step of 5 minutes at 94°C, followed by 35 cycles of: 94°C for 30 seconds, (primer-specific annealing temperature) for 30 seconds, 60°C for 30 seconds, 72°C for 30; followed by a final extension step of 5 minutes at 72°C. Primer-specific annealing temperatures are given for each primer pair in the text or in Tables 2.3 – 2.9.

3. PCR products were separated on 2.5% agarose minigels as described in Section 2.14.1 and visualised by ethidium bromide staining.

2.17 Radiolabelling of DNA probes

2.17.1 Radiolabelling of PCR products by PCR

PCR products were radiolabelled essentially as described in Bentley et al. (1992).

1. 5 - 10 µl of PCR product were separated on a 2.5% agarose minigel and visualised by ethidium bromide staining.
2. The gel was rinsed in deionised water to remove excess buffer. The desired band was excised from the gel and placed in 100 µl of $T_{0.1}E$ at 4°C overnight.

3. 2 µl of the $T_{0.1}E$ were used as template in the PCR-labelling reaction containing 40 ng of each primer, 1 µl of 10x PCR buffer, 0.5 µl of [α-^{32}P]-dCTP (3,000 Ci/mmol), 0.5 U of *Taq* polymerase (Cetus) and 0.375 mM each of dATP, dTTP and dGTP. Reactions were performed in a 0.5 ml microfuge tube and overlaid with mineral oil (Sigma) in a DNA thermal cycler (Perkin Elmer, USA).
4. PCR cycling conditions were as follows: 94°C for 5 minutes; followed by 20 cycles of: 93°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; followed by 72°C for 5 minutes.
5. Probes were pre-reassociated (as described in Section 2.17.2) prior to use if necessary. All probes were boiled for 5 minutes and snap-chilled on ice prior to use.

2.17.2 Pre-reassociation of radiolabelled probes

1. Radiolabelled probe was mixed with 125 µl of 20x SSC and 250 µl of the sheared 10 mg/ml human placental DNA (Sigma) in a final volume of 500 µl.
2. The mix was boiled for 5 minutes, snap-chilled in ice-water, and then added directly to the hybridisation reaction.

2.18 Hybridisation of radiolabelled DNA probes

2.18.1 Hybridisation of DNA probes derived from STSs

1. Filters were prehybridised flat in sandwich boxes for 3 hours in 10-25 ml of hybridisation buffer at 65°C with gentle shaking.
2. Radiolabelled probe was added and hybridised to the filters.
3. Filters were washed twice at RT in 2x SSC for 5 minutes, twice at 65°C in 0.5 x SSC, 1.0% Sarkosyl for 30 minutes. Filters were rinsed at RT in 0.2x SSC prior to draining the excess liquid, wrapping in Saran Wrap (Dow Chemical Co.) and exposing to autoradiograph film.

2.18.2 Stripping radiolabelled probes from hybridisation filters

1. Filters were washed in 0.4 M NaOH for 30 minutes at 42°C followed by 30 minutes in 0.2 M Tris-HCl (pH 7.4), 0.1x SSC, and 1.0% w/v Sarkosyl at 42°C with gentle shaking. Successful removal of radiolabelled probe was assessed by autoradiography.

2.19 Restriction endonuclease digestion

1. 4 µl (approximately 150 ng) of prepared cosmid DNA (described in Section 2.11.1) were digested with *Hin*d III using commercial buffers according to manufacturers' instructions in a final volume of 10 µl.
2. 5 µl of each digest were checked for complete digestion by electrophoresis on a 1% agarose minigel and visualised by ethidium bromide staining.

2.20 Clone library screening

2.20.1 cDNA library screening by PCR

The strategy used to screen the cDNA libraries by PCR is illustrated in Figure 2.1.

1. Nine different cDNA libraries were subdivided into 25 subpools of 20,000 clones, which were then combined to produce 5 superpools of 100,000 clones by J. Bye and S. Rhodes. Details of the cDNA libraries are given in Table 2.2.
2. Aliquots of the superpools of each library were arranged in a microtitre plate to facilitate subsequent manipulations with a multi-channel pipetting device.
3. In the primary screen, 5 µl of each superpool were used as template in a 15 µl final volume PCR using buffer and PCR conditions as described in Section 2.16.3.
4. PCR products were loaded on 20 cm x 20 cm 2.5% agarose horizontal slab gels using an 8-way multi-channel pipetting device, separated by electrophoresis and visualised by ethidium bromide staining.
5. In the secondary screen, 5 µl of each of the 5 subpools of 20,000 clones corresponding to the superpool that were positive in the first round, were screened by PCR with the same primer pair as used in step 2. PCR products were separated by electrophoresis through 2.5% agarose minigels and visualised by ethidium bromide staining.

2.20.2 Vectorette PCR on cDNA libraries

1. Vectorette PCR was performed on the superpools of the cDNA libraries (figure 2.1). PCR was performed using 5 µl of the diluted superpools as template in a 15 µl final volume using buffer conditions as described in Section 2.16.3. Primer combinations were as follows: 224 and specific primer A, 224 and specific primer B.
2. PCR was performed in a DNA thermocyle (Omingene) using hot start. Cycling conditions were as follows: an initial denaturation step of 5 minutes at 95^{o}C, followed by 17 cycles of: 94^{o}C for 5 seconds, 65^{o}C for 30 seconds and 72^{o}C for 3 minutes, followed by 18 cycles of: 94^{o}C for 5 seconds, 65^{o}C for 30 seconds and 72^{o}C for 3 minutes, followed by 72^{o}C for 5 minutes. The PCR was paused after 4 minutes of the initial denaturation and 2 µl of Taq premix (containing 0.12 µl Amplitaq, 0.12 µl TaqExtender, 0.12 µl Perfect Match, 0.5 µl 40% sucrose + cresol red, 1.14 µl $T_{0.1}E$) were added to each reaction (pipetting underneath the oil).
3. Products were separated by electrophoresis through 2.5% agarose gels and visualised by ethidium bromide staining. Products were gel-purified using gel extraction kits and following manufacturer's instructions from either GeneClean^{TM} or Qiagen^{TM} prior to sequencing directly.

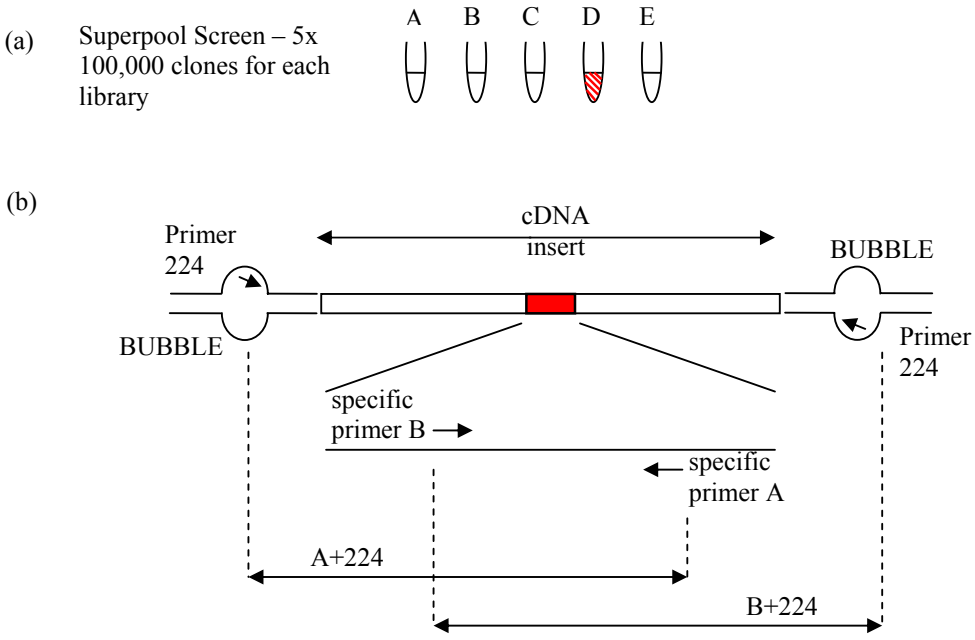

Figure 2.1: Strategy for vectorette PCR screening of cDNA libraries. a) Superpools representing 100,000 clones (A-E) were screened by PCR and positive super pools recorded (e.g. D, shown in red). (b)Rescue of the insert of the cDNA of interest by vectorette PCR. Insert of cDNA is shown as white rectangle; ligated 'bubble' is shown in yellow. Original position of primers for pool screening is indicated by a red rectangle. A combination of the 'bubble' primer (224) and insert specific primers (e.g A and B) were to generate two products (e.g. 224+A, 224+B) representing the entire insert of the cDNA of interest.

2.21 Exon Amplification

1. Exon-specific primers were diluted from 1 mg / ml to a stock 100 ng / µl and a working dilution of 8 ng / µl in $T_{0.1}E$.
2. 1 µl 10 X NEB buffer, 1 µl dNTPs (5mM), 0.33 µl BSA, 0.14 µl ßME, 0.12 Amplitaq and 1.41 µl of T0.1E were added to 1 µl of individual CEPH DNA (5 ng / µl) aliquotted in a 384-well PCR plate.
3. Following PCR samples were separated on an agarose gel as described in 2.14.3.
4. Residual primers and dNTPs were removed by the addition of 2 µl of a premix containing 0.66 µl of shrimp alkaline phosphatase, 0.66 µl of reaction buffer, 0.66 µl of double distilled H_2O and 0.066 µl of exonuclease I. The 384-well microtitre plate

containing the exon amplified PCR products was incubated at 37°C for 30 minutes, and then 80°C for 15 minutes.

5. Exon specific PCR products were then sequenced by the Research and Development Group or The Sanger Institute Sequencing facility.

2.22 Mapping and sequence analysis software and databases

2.22.1 IMAGE

Gel images from radioactive, fluorescent and *Hin*d III gels were processed using IMAGE. Band patterns for each lane were extracted and the data manually edited and normalised by marker locking prior to data entry into another program, fingerprinted contigs (FPC) (Soderlund *et al*., 1997), for analysis. Within IMAGE several procedures were run on each gel in turn:

Lane tracking – a grid was superimposed and manually edited on each gel image to ensure it exactly matched the lanes on the gel.

Band calling – an analysis module then identified labelled restriction digest fragments within each lane. Manual editing ensured the correct bands are chosen.

Marker locking – A set of DNA fragments of known length or migration distance was loaded as a marker to facilitate normalisation of restriction fragments and permit accurate clone to clone comparisons to be made (see Section 2.13 for specific marker patterns for each method of fingerprinting used). Manual editing of each marker lane ensured the observed experimental marker matched the previously established standard marker pattern.

Normalisation – once marker lanes were locked onto standard marker positions, the bands within the sample lanes were normalised to account for differences in gel to gel run conditions. IMAGE finally generated a 'Bands' file for each gel containing normalised migration distances for all selected bands in each clone lane.

2.22.2 FPC

FPC, which was used for all contig construction described in this thesis, utilises the bands file output from IMAGE as the digitised set restriction fragments for each clones. Fingerprint patterns for each clone were compared to those of all clones in the database. The relationship between two clones was reported as a probability of coincidence, i.e. the probability that bands

in common between two clones overlap by chance. Two variables can be set to filter the reported overlaps:

Cut off – a match between two clones will only be reported if the probability of coincidence is less than or equal to the cut off. When analysing matches between larger insert PAC and BAC clones the tolerance was 1e –08.

Tolerance – two bands of equal size are considered as their migration distances differ by less than tolerance. For the analysis carried out in this thesis the tolerance was set to 7.

Overlapping clones were identified by automatic analysis and contigs were constructed manually using the available editing tools within FPC. Clones were overlapped by iterative pair-wise analysis by determining the number of bands in common between clones. Subsequent clones were added positioned within the initial clone assembly based on the number of bands they shared with the existing clones in the contig. Marker data was imported from 1ace and integrated into the FPC contigs to identify clone overlaps that could not be identified using pre-determined analysis parameters. A minimum set of clones for sequencing was chosen based on a combination of shared bands and shared marker data.

Contig Sizing –one unit in the contig display represents one fingerprint band which permits for estimates to be made for contig sizes. For each fingerprinting method, a kilobase/band figure was derived. For radioactive cosmid fingerprinting, one band was the equivalent of 3.3 kb, based on the fact the average number of fingerprint bands for bacterial clones in comparison to finished sequence. For fluorescent fingerprinting of PACs and BACs an average figure for each clone type, based on the number of bands observed in clones that had generated finished sequence, was 4.0 kb/band. *For Hin*d III *fingerprinting of PACs and BACs*, 4.4 kb/band was used based on the length of finished genomic sequence.

2.22.3 1ace

All mapping and sequencing data generated in this thesis were stored in 1ace, a chromosome-specific implementation of ACeDB (Richard Durbin and Jean Thierry-Mieg, 1991).

ACeDB uses a flat file format with data being accessed using a series of windows to represent various data types. All windows are linked in a hypertext fashion, so that clicking on an object will display further information about that object. For example, clicking on a region of a chromosome map will highlight landmarks mapping to that part of the chromosome; clicking on a landmark will display information about that landmark including landmark-clone associations, etc.

All PAC, BAC and cosmid library filters and polygrids are represented graphically in 1ace and data were entered directly. Data were then saved in the database establishing landmark-to-clone associations which can be displayed as text windows relating to either the landmark or the clone. Data can also be entered via text windows or via an internal web page. PCR library pool screening and colony PCR results were entered via the text windows.

In addition to the data generated by the 1 chromosome mapping group, 1ace also contains displays published 1 chromosome maps, which have been used as part of the project. This greatly facilitates integration of maps from different sources. Genomic sequence data is also displayed in ACeDB along with the collated results from the computational sequence analysis performed by the Sanger Institute Human Sequence Analysis Group. 1ace can be accessed by following the instructions at http://www.sanger.ac.uk/HGP/Chr1.

2.22.4 Blixem

Individual matches identified as a result of similarity searches using the BLAST algorithm, or matches between sequences of cDNA clones or PCR products amplified from genomic DNA generated as part of the project, were viewed in more detail using Blixem. Blixem, (Blast matches In an X-windows Embedded Multiple alignment) is an interactive browser of pairwise Blast matches displayed as a multiple alignment. Either protein or DNA matches can be viewed in this way at either the amino acid or nucleotide level. Blixem contains two main displays: the bottom display panel shows the actual alignment of the matches to the genomic DNA sequence, and the top display shows the relative position of the sequence being viewed within the context of the larger region of genomic DNA. A program “pfetch” retrieves the record from an external database (*e.g.* EMBL, SWISSPROT).

2.22.5 RepeatMasker

Human repeat sequences were masked using RepeatMasker, a program that screens DNA sequence for interspersed repeats and low complexity DNA sequence (Smit, AFA & Green, P RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker.html). The output of the program is a detailed annotation of the repeats that are present in the query sequence. Sequence comparisons are performed by the program cross_match, an implementation of the Smith-Waterman-Gotoh algorithm developed by P. Green. The interspersed repeat databases screened by RepeatMasker are based on the repeat databases (Repbase Update) copyrighted by the Genetic Information Research Institute.