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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™
	TaqFS
Qiagen	DNA gel purification
Roche Applied Science	Klenow enzyme (sequencing grade, 5 U/µl)
	T4 Polynucleotide kinase
Sigma	Ribonuclease A

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 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

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; Tetracycline (dissolved in absolute ethanol, stored foil-wrapped at 4 ^{o}C), 5 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**
Plasmid	NA	Ampicillin or Tetracycline
Cosmid	LL0XNC01	Kanamycin
PAC	RPCI1,3,4,5, 6	Kanamycin
BAC	RPCI-11, 13	Chloramphenicol

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

2 X 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^-	60 ml 5 M 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 (Roche Applied Science)

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
Autoradiographs	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 hybrid Clone 2D (Cl2D) that contains the entire X chromosome was kindly provided by Adam Whittacker. DNA from two affected individuals from family MRX23 was kindly provided by Ron Gregg. DNA from a normal male control sample was kindly provided by Alison Coffey.

2.8 Bacterial clone libraries

2.8.1 Cosmid libraries

Cosmids from the Lawrence Livermore flow-sorted X chromosome cosmid library (LL0XNC01) (prefixed 'cU') were kindly provided by Dave Vetrie and Elaine Kendall. Cosmids from a library constructed from a male with 5 X chromosomes (Holland, J.*, et al.*, 1993) (prefixed 'cV') were also kindly provided by Dave Vetrie and Elaine Kendall.

2.8.2 PAC and BAC libraries

The RPCI-1, RPCI-3, RPCI-4, RPCI-5 (prefixed 'dJ'), and RPCI-6 (prefixed 'dA') PAC libraries, and the RPCI-11 (prefixed 'bA') and RPCI-13 (prefixed 'bB') BAC libraries were used as a source of human derived PAC clones and BAC clones respectively in this thesis. Mouse-derived BAC clones were obtained from the RPCI-23 (prefixed 'bM') library, and zebrafish-derived BAC clones were obtained from the RPCI-71 libraries (prefixed 'bZ'). 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 Clone Resources Group.

2.8.3 cDNA libraries

A range of up to 20 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 25,000 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 library code	cDNA library description	Vector	Source/ Reference
1. U	(Monocyte NOT activated-from a patient with promonocytic leukaemia) (U937+)	pCDM8	Simmons (1993)
2. H*	Placental, full term normal pregnancy (H9)	pH3M	Simmons (1993)
3. P	Adult brain	pCDNA1	Pfizer
4. DAU	B lymphoma (Daudi)	pH3M	Simmons (1993)
5. FB	Fetal brain	pCDNA1	Invitrogen
6. FL	Fetal liver	pcDNA1	Invitrogen
7. HL	Peripheral blood (HL60)	pCDNA1	Invitrogen
8. SK	neuroblastoma cells	pCDNA1	Invitrogen
9. T	Testis	pCDM8	Clontech
10. FLU	Fetal lung	pCDNA1	Invitrogen
11. AL	Adult lung	pCDNA1	Clontech
12. UACT*	(Monocyte PMA activated - from a patient with promonocytic leukaemia) (U937act)	pCDM8	Simmons (1993)
13. YT*	HTLV-1+ve adult leukaemia T cell	pH3M	Simmons (1993)
14. NK*	Natural killer cell	pH3M	Simmons (1993)
15. HPB*	T cell from a patient with acute lymphocytic leukaemia (HPBALL)	pH3M	Simmons (1993)
16. BM*	Bone Marrow	pH3M	Simmons (1993)
17. DX3*	Melanoma	pH3M	Simmons (1993)
18. AH	Adult Heart	pcDNA3-Uni	Invitrogen
19. SI **	Small Intestine	pcDNA3	Stammers

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

** Generously provided by Dr Stammers (Sanger Institute)

2.9 Primer sequences

All primers were synthesised in house by Dave Fraser or externally by Genset. Table 2.3 lists the vector-specific primers and sequences used in the vectorette method. Table 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9 list the STSs used in this thesis, the sequence and size in base pairs (bp) of each primer, and the optimal annealing temperature (AT – given in ^{o}C). Where appropriate, the clones, or genes from which the STSs were derived are also listed.

Table 2.3: *Vector-specific primer sequences and 'bubble' sequences for primers used in vectorette PCR and SSPCR (performed on clone DNA and cDNA)*

Primer Name	Primer Sequence
SP6PAC*	ATTTAGGTGACACTATAG
T7PAC*	TAATACGACTCACTATAGGGAGA
PACS2*	GCTAGGAGGGCTTAACTGAT
PACT2*	CTGGGTTGAAGGCTCTCAAG
224**	CGAATCGTAACCGTTCGTACGAGAATCGCT
BPHI**	CAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAACGGACGACAG AAGGGAGAG
BPHII**	CTCTCCCTTCTCGAATCGTAACCGTTCGTACGAGAATCGCTGTCCT CTCCTTG
pH3M1FP	CTTCTAGAGATCCCTCGA
pH3M2FP	GCTCGGATCCACTAGTAA
pH3M1RP	CTCTAGATGCATGCTCGA
pH3M2RP	CGACCTGCAGGCGCAGAA
pCDM8.RP	TAAGGTTCCTTCAGAAAG
T7.2FP	AATACGACTCACTATAG

* designed by John Collins (Sanger Institute)

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

Table 2.4: *STSs from Srivastava, A.K., et al., (1999) used for contig construction as described in chapter 3*

STS name	Primer 1	Primer 2	Size (bp)	AT (°C)
sWXD797	GCCTTGGAATATCTTCCTAC	AAACATTTGTGAGTCATCAGTGTC	83	55
sWXD940	CATGCATAATGCATAGCATGG	TGGTAAGAGCTTAAATTTGCTAAGGG	65	60
sWXD1199	GCGAAGAAAACATTACCTGG	CAGGATATCAAAAAACCTCAACTG	71	60
sWXD1259	GGGAAGAAATGAAAGGAGG	AGTCAGTCCCACTCTTGTC	65	60
sWXD1283	TTGGAGTAGACAACAGGAC	TAAATTAAAGGGAAGCACTAAGAG	140	60
sWXD1440	CCCTGCTCCTACTCATAGC	GGGCTTGGTAGTAGTGCTTG	100	60
sWXD1549	CAAACAGAATGATTAAATACAGGAC	GTAGGAGGCTACTAAGAAG	126	60
sWXD1805	CATATTTTGTCAGTGTGGTC	GCCTTAACTATAAGAACCAG	140	60
sWXD2782	CCCATTCCAGATATAGATTATCAG	TTTCATCACAACATCCCCAAAGAC	84	60
sWXD2783	GTGATGAGAGCTTAAAAATCCC	ACCAGCATTCAAGAAAGGTGAG	84	60
sWXD2789	ATTCCTTACCCACACAGTC	AATATAACGTGCATGTATGGTTTC	140	60
sWXD2804	CAATTCACATAGTCCTCAAACC	TCTCCTACAGATGATTAACCACC	160	60
sWXD2841	GGGAACAAGGAAGAAAGAATG	TCCCTAAGACCCTCCTATG	81	60
sWXD2843	GTGGGTCCTACTTTCAGGG	CTGACGAGTTCATGCAAGCC	69	60
sWXD2844	GAAGGTTTAGGTGGGATAATG	CATCTCTTGTCTGGACTATTTC	145	60
sWXD3375	TAGTTTAATGGAATGGTAGAATGG	TTCACATACAGCTTCCTGG	97	60
sWXD3381	GAAGTAAGATGGGACAATAGG	CAGTTTTACGGAAATGAACAGTAG	107	60

Table 2.5: *STSs derived from clone ends used for walking as described in chapter 3*

STS name	Primer 1	Primer 2	Size (bp)	AT (°C)	Clone Name
stSG14873	GAAGATTGTTGCCAGAACTGC	GCCCAGCCAAAGAATTACAA	103	60	cU101D3
stSG14874	AATGGCCAGTAAAGACAGAAGA	ATGACTCAATGCCTAAAAAGGA	82	60	cU235H3
stSG14875	AATTGGCGATCATAGCTTTAGC	CTCTGGAATCACAAATGTGGG	104	60	dJ127B14
stSG14875	AATTGGCGATCATAGCTTTAGC	CTCTGGAATCACAAATGTGGG	104	60	dJ127B15
stSG14929	AAGGAAAAGCAAGAGAAGGACA	AGTGTGCATTGCATAGCTGG	118	60	cU105G4
stSG14930	AGCCGTGTTAATCTTGACACTC	TGGGGGAAAAACATTCATGT	104	60	cU116E9
stSG14933	CATGCTCATTTTAACACTTGCC	AACCCCTTTCCTAAGTAGTGGC	101	60	cU160A4
stSG14958	ATTTTACATGTCCAGGACAGGG	TCAAAAAGAACACCGCACC	119	60	cU105G4
stSG14962	ACACTGAAGCCTTTTGGAGG	TCATGGGGGTTTGTTGTACA	118	60	cU17A7
stSG14963	GTGTGTGTGTATTTAACAGGCG	GGCAGTTGTCAGCTAAATAGCC	183	60	cU212C1
stSG17548	GCACAGTGCTTGGCACAC	CTCCCTCAGGTACACTGGTAAG	127	60	cU235H3
stSG17555	ATTTATTGAGTTGGCATCCCC	TTTCCCCCCATACTGTCAAA	121	60	dJ334P19
stSG17563	AAAGGATGAAATGACTCTTGCC	TACCACCAGTTTAGCAGGCC	154	60	dJ82J11
stSG17563	AAAGGATGAAATGACTCTTGCC	TACCACCAGTTTAGCAGGCC	154	60	dJ334P19
stSG22771	GGTTGTAGGTGTGCATGTGC	GCAAAGCGTTCTGAATACCC	121	60	cU159B9
stSG22771	GGTTGTAGGTGTGCATGTGC	GCAAAGCGTTCTGAATACCC	121	65	cU159B9
stSG22772	TGCTAGCACAACAGGGTGAC	ATCATGGAGAATGGGGTATCC	135	60	cU159B9
stSG22773	TTGACAGCATAATCCACTTTGG	TGGTTCTTTCAGCATCTGTCA	181	60	cU160A4
stSG22774	CTCGCTTTTCCTTTTTGGC	TTTTATTACCCAATCAGCCCC	149	60	cU50F11
stSG22775	AAGGCCTTACCATTGTCCCT	TTTTTCTTGGGCAATTCCAG	170	60	cV602D8
stSG27821	AGCAATCCCACAGCTAGGC	TGTTGATGGACACTTAGGTTGC	133	60	cU232G2
stSG27821	AGCAATCCCACAGCTAGGC	TGTTGATGGACACTTAGGTTGC	133	60	cU232G2
stSG27822	TTTTTTTTTTGACGGAATCTCA	TGGTGGTGTGCACCTGTAGT	145	60	cU232G2
stSG38412	GCAAAAATAAATGGTTGGAAGG	TCCCAGAGGTAACCGTTATCC	101	60	dJ79P11
stSG41236	TTGAGACCTGAATAGCTCCCA	TATTGCTGAAACCACTTTGGG	167	65	dJ148H18
stSG41239	GCGAGTGGTGCAAGAGTGT	GTCAGGAGAGTGTGTGAAATGG	165	65	dJ306P24
stSG41240	TCCTAAGGGCCTTGATGATG	GAGATACTGGACAGCTATGGGG	138	65	dJ34L20
stSG45649	TGTGTTGCTTTTGCTTCCTG	AAGTCCTTACAGTCAGCAAGGC	140	60	cU35G3

stSG45650	TGGAGGGTCATAAGGCAAAG	TTGAACCTCTCAAGTCGCCT	140	60	cU35G3
stSG45651	CTGCAAAACTGGCAGTACCA	CACTGACCAGTGATTTTCAAGG	123	60	cV362H12
stSG45652	AGAACATGGGGTTCTTGGG	TTCACAAAACCAATAAAAGCCC	130	60	cV461C10
stSG45653	TTGCCTGATCATATGAATCACC	CACTCACGTTGCTGTGGG	141	60	cV461C10
stSG45654	AGCCTTTTCAGATTCTGAGCC	TGTCAGCAGAGTGTCATCCTG	137	60	cV698D2
stSG45655	TAGTGACATGTGGAAATGCCA	GGCATGGCTCTTTGTCCTAA	182	60	cV698D2
stSG45656	TCATTTTGACTTTGTGGAGGC	AGGAAAGCCCAGAAGAAAGG	163	60	dJ409J21
stSG45751	GTATGCCATTTCCAATCAGATG	TTGCCCCAGATTTGCTTC	86	60	cU157D4
stSG45752	CTTGGCACAAAGAATGGGAT	AGAAGATGGGTTTCTGGGCT	129	60	cU157D4
stSG45753	AGCTCCAAGCCAACTTGAAA	GTTCACTTAAAGGGTGGAGCC	120	60	cU19D8
stSG45754	CTGAATCTTTGCATGATTGCA	CATTCTAAACATGTGCTCAGGG	177	60	cU19D8
stSG45755	ACTATGGATTCTGTCCCCAGG	TCCAGGCTACTACCCAAATCC	197	60	cU237H1
stSG45756	AGCGCCATTAGATGAGCAAT	AGATGGCCCCTTCCTCTTAA	213	60	cU237H1
stSG45757	CTGGCTCACATTCAGGGC	TGTTAAAAACAACCCGCTCC	174	60	cU46H11
stSG45758	GTGCATCCTATGAAGCACATG	ATCTGCAGGTGATTCCTGTACA	133	60	cV351F8
stSG45759	TGAGCCACCGCTAATAAAGG	GAATAGGACAGCCCTTTCCC	180	60	cV351F8
stSG45761	TGAGAACCACTGCATCATAAGG	GTTTTCCCTTTTGAACTTGCC	102	60	cV389H8
stSG45762	TTCTGTCATTTGGGACACCA	TGGTTGTGTGTTTTTTAGGCA	135	60	cV521F8
stSG45763	GCCATTTTCACTTATTGTGGTT	GAGCAATAAAGGAAAAAATGCA	146	60	cV602D8
stSG45764	GGCAAAGTTCAGCTCAGGAC	CTAGGAAGTGCTTTGGCTGG	180	60	cU65A4
stSG49025	CAGCAGTCTTCTAGGTGCCC	TAATACCCAGCTGTTGGAACG	155	60	cU86H4
stSG49026	TGGGAAATGCTCCTCTGGTA	TTTCCGTGTCTTGGGAAAAG	122	60	cV618H1
stSG49027	GCCCAGAAGGTGTTAACTTCC	CAATGATGGCATTTCATATTGC	121	60	dJ1184O6
stSG49032	AGGGGAGAGAACAGCACTAGC	TGGGAAGGCACTAACATTCC	147	60	dJ198P4
stSG49032	AGGGGAGAGAACAGCACTAGC	TGGGAAGGCACTAACATTCC	147	60	dJ198P4
stSG49038	GTGCTTCCATAGCTTCATCTCC	CACAAAGGTTAGAGCACACAGC	123	60	dJ738A13
stSG49038	GTGCTTCCATAGCTTCATCTCC	CACAAAGGTTAGAGCACACAGC	123	60	dJ738A13
stSG50178	ATGGCAAACAGAGAGCTGGT	ACTGTGGCTGCAGGTTCTTT	170	60	cU65A4
stSG61723	ATCTGATCATTCTGGCCCAG	GATTCAGCCAAGCACATGAA	122	60	cU165H7
stSG61725	TCAACAGGGAACAACTTGACC	GGACTGTCTCTTCAAAGTTGCC	122	60	cU96H1
stSG61726	TAGAAGGGCCTCATGTGTCC	TGGGCAAAATGTCCCAAC	145	60	cV870H8

stbA191C22SP6	CACCATCACAATGCATACTGC	GGCAATTTGTTAGTATTTGGCA	140	60	bA191C22
stcU105G4.1	GCAGCTGTTTTTGCTAAGGG	AAAGCTGGTTTGTTCTCTCTGC	139	60	cU105G4
stcU105G4.1	GCAGCTGTTTTTGCTAAGGG	AAAGCTGGTTTGTTCTCTCTGC	139	60	cU105G4
stcU173H7SP6	GAGCTCCTTCTGATCTTGGTC	AGTTCCACATGGTCAAAGCC	99	65	cU173H7
stcU212C1T7B	AAGCTACTTTGAGTGCTTTGGC	AGTGTGGACAACATCTGGAGG	133	60	cU212C1
stdJ77O19.1	CAATGGGGTGCTAGTGGAGT	CTAGCATTTCCCAAGACCCA	167	60	dJ77O19
stdJ82J11	CTCCTTCAGATGCAATTGATTG	GAGGGTGTTCATTCAAAAAAGG	186	60	dJ82J11
stdJ148H18T7A	AGGGATCAGCAACATTGACC	AAAACAATTGCATCGAAGGG	172	60	dJ148H18
stdJ233G16T7	CTTTCCATTTCTACCGTCATCC	GTTCCGATTTAGGCTTTCAGG	158	60	dJ233G16
stdJ258H17SP6.224	CGTTTCAAAGTCCATGGGTT	GCCATTTAGAACCTCTGCCA	174	60	dJ258H17
stdJ258H17T7.224	AAAAAAAATTTTCTGCTGGTGG	AAATAGGCCTGCTCGTTCAA	122	60	dJ258H17
stdJ324P21SP6	AACTCCAGGTTCTGTAGCAAGC	GTACTGGCCCGGTTTACTAGC	177	60	dJ324P21
stdJ324P21SP6	AACTCCAGGTTCTGTAGCAAGC	GTACTGGCCCGGTTTACTAGC	177	60	dJ324P21i
stdJ341D10T7	TGCATTGGGTGCAAAATTTA	GCCTCAGTGAGCCTTACCTG	168	60	dJ341D10
stdJ400D4T7	CTGTTCCTCAAAGCTTGTAGCA	TAAGTACTGTGATGGGCATTGG	150	60	dJ400D4
stdJ421I20SP6	GGAAAGGAGAAGAAAAGGCC	TCTGTGCCTGCAACCATG	122	60	dJ421I20
stdJ479J7T7A	GACCACCTGGCCTAACTTCC	CCAAATTAGGAAAGACTCCATG	121	60	dJ479J7
stdJ479J7T7A	GACCACCTGGCCTAACTTCC	CCAAATTAGGAAAGACTCCATG	121	65	dJ479J7
stdJ519L22T7.224	TTCAAAGATTGGCAAGATTGG	GTGGATCCTTGAAAAACAAAGC	130	60	dJ519L22
stdJ663P11SP6	CGCAGTTTACTTAAGGGGACC	GGAACCTAAGGAGTGGGCTT	137	60	dJ663P11
stdJ664K17T7	GTATCAGAGGCCAAGCTCATG	GGAAGAAGTGCTGATGAGGG	142	60	dJ664K17
stdJ777L12T7	GTTTCCTGGCAGAAGCAGTC	CTTTCATCAGGGTGAACTGTTT	147	60	dJ777L12
stdJ823F3SP6	AACTGCTTTGTTAATGCCTGC	GCTTTACATGTGAGTGCTCAGG	123	60	dJ823F3
stdJ969N12T7	GCCTTTCATAATTTCTTCCAGC	TGGAATAAATGCTTGAAATTGC	126	60	dJ969N12

Table 2.6: *STSs used for gene identification as described in chapter 4.STSs are named with the prefix 'st' followed by the clone name from which the STSs was derived. The number distinguishes multiple STSs designed in the same clone. The 'n' indicates nested primers for SSPCR. These were not used as primer pairs and so no annealing temperature is given.*

STS name	Primer 1	Primer 2	Size (bp)	AT (^{o}C)
stbA45J1.1.1	CTCTCAGCTCTCGGAAGGAC	TGCTGAGTCAGGGACTGATG	142	60
stbB125M24.1.1	TTGCAAGCATCACTTCTTGG	GCTGGTATCTTGTGTCAAATGC	140	60
stbK421I3.1	GCCCAGGACTCTTCTTCCTC	GGGATACTGAGAGCATCGGA	123	55
stbK421I3.2	GAGAACCAGAAGGGCGGT	AATGCTGTCTAGCTCCTTCAGG	162	60
stbK421I3.2n	TGGCCACCAGGAGCCCTG	GCTGGGGCTGAATAGACG	-	-
stbK421I3.3	TTCCAGCAGCCTGTGTTTC	ATAACAAAAGGGAATGGGCC	123	65
stbK421I3.3n	CACCTATGCCACCCGCTG	GCATGAGTGGAAGGGGCAAG	-	-
stdA39H21.1	TGTGCTGGTTCTGGCAGC	TTGTTGACTGAGGCAGATAAGC	124	60
stdA155F9.1	GTGACGAATCCACATCCTG	GTTCTGCACAGTGTGTAATG	82	60
stdA155F9.1	GTGACGAATCCACATCCTG	GTTCTGCACAGTGTGTAATG	82	60
stdA155F9.1n	GCCAGAATTGAAAAGGTAC	GTACCTTTTCAATTCTGGC	-	-
stdA155F9.2	CAAAGTTGTTGAGCCCCTG	GATATATCTTCCATTGGGAAC	108	60
stdA155F9.2n	GACTATGAGAATGTTATTG	CTTTGGGCAATAACATTCTC	-	-
stdJ29I24.1	CTCCGGCTCAGTCTTACAGG	AAGTTTGCTAGCCACGCG	127	60
stdJ57A13.1	TGTGATAGAAGCACGCAAGG	TATTCACCAAAATCAGCTGTGG	160	60
stdJ57A13.2	GAGCCCACTTTGGTGGTG	GTAAAGGGAGAAGTGCAACCC	129	60
stdJ57A13.2n	GCAAGGCCTGGCTGGGTTCT	TTATTACCTCCAGCACAGGA	-	-
stdJ93I3.1	TCCTGAAGACAGCTGCCC	TGGTTTTTCCTCCAATTTTTC	87	60
stdJ93I3.2	TCTTTGCAGCTGTGGCTCTA	CACCCAGTTGATGTGACAGG	152	60
stdJ169K13.1	GACCACTTCACCCTGTCCTG	GCCGCAGTAGCTCAGCTC	120	60
stdJ169K13.2	AGGACATGGAGTTCACCGAG	CTAGGCCATCTCCTCCTCG	124	60
stdJ169K13.3	AGAAGGTACTGCCTCAGTCTGG	CCCTGGATCTGTCTCCAGAA	127	60
stdJ170D19.1	GATGATGGATACCCAGTGGC	TCATCATCTACCACTGGGCA	92	60
stdJ170D19.1n	GATGATGGATGCCCAGTG	ATCCATCATCTGCCACTG	-	-
stdJ170D19.2	CAAAAATGGAGCTTTGTCAGC	TCAAAAGAAAAGCGCATGC	163	60

stdJ222H5.1	CTCCAAGATTCAACTATGTGGG	TCCCAAACAACTCAAGCTCC	125	60
stdJ222H5.2	TCCTTGCCCATGCAAATC	GATCCCATGGAACTGAAGGA	119	60
stdJ278D1.1	TCACTAGCAGATGCCATCATG	ACCAGCTTCGACTTGAAGGA	103	60
stdJ278D1.1n	TCTATGCACAGGGAAAGC	GTGATGTGACCCTGATGC	-	-
stdJ318C15.1	ATTTTAGGGACATGGGACTGG	CAGACAGCATGCTTAAAAGGC	161	60
stdJ318C15.1n	GAGTTATTTAGGGCTCATATT	TTCGAAGTAAACTTCTATCA	-	-
stdJ321E8.1	CTCATACCTGCCTCCTGCTC	TCCAGTCAGATGGAGATTTGG	128	60
stdJ321E8.2.1	CGAAAAGTGGGATGAAGAGG	TGGATTTTCTTGGCTTCACC	136	60
stdJ321E8.3.1	ATGCCTGTGGGAATTGTAAC	TTGAACACTGTCACATACATCCA	102	60
stdJ327A19.1	TCCAGCGATGCAGCTTTAC	ATGGCTAATACCACTTTCCTGC	120	60
stdJ327A19.1n	CAACCAGGAGCTCGAAGCCG	TGCTGCTGAATCTCCAGACT	-	-
stdJ327A19.2	CAAGCCAGAAAGCAATGGAT	GATGACATCCTCAACCAGAGC	121	60
stdJ327A19.3	TGCTGATGAGAAGAGAGCCC	TTTGTCATCCTTCCCTTTGG	120	60
stdJ327A19.3n	CTTGCATCCTTTAACCAAGA	GTCTTTCTGTAAACCATTTC	-	-
stdJ327A19.4	GCCATGCTCTGTTACCTGGT	ACCTGAGCATTCAAATGATGC	124	60
stdJ327A19.4n	TGCAGCTATGGCTGAATATG	ATTTCTTCACTTGTCAAGCC	-	-
stdJ327A19.5	CTACTTGTTCGATCCTTCCAGG	GATGCCCTCTAACATAGGAAGA	170	60
stdJ327A19.5n	ATTTTCCAGAAACTCTTCTT	TTAAGTGAATTTGTATCA	-	-
stdJ327A19.6	AGTTCGTCGAAGAGTCCGAA	AGAGGGCCGCTCTCTAGAAC	188	60
stdJ327A19.6n	AGCCCAGCAAATCTGCCCGC	CGACCGCGAGCGTGAGCGGT	-	-
stdJ327A19.7	GATCGAACAAGTAGGTTC	ATCATCTTGAGAGGTAAG	170	60
stdJ378P9.1	TGAAGGATTTTCAAAGTCTCCA	CATACAAATAGCAACACTGGGC	85	60
stdJ378P9.1n	ATGTTTTGTTGCATTTAG	CATTGTTAATCCTAAATG	-	-
stdJ394H4.1	TTCCAGCAGCCAGTCAAAG	AGGCATGCTGTAGCAGGTG	128	60
stdJ404F18.1	TCAGAGCCCCTACCTCCC	ATTGGCTGTCAATCCATTCC	123	60
stdJ404F18.1n	CACACAGTGGAGGAGTAG	TGGGCCTTCACTATCTGC	-	-
stdJ404F18.2	GTGGACTGCCGCTCTTCTAC	TTAGGAGGCTCTTGTTTCTGAG	132	-
stdJ404F18.2n	CAGCCAAAGAATGCTCCTGT	TGTTCGTTTTCCCAAAGC	-	-
stdJ404F18.3	AGCCAAGAAAGCGAAAATGA	ACGTAAAGCCTTCTGCTAGGG	105	60
stdJ404F18.3n	TGAAGGGGACCTTGATTG	AATAGGAATCCGTCTATG	-	-
stdJ404F18.6	GCATACACGATGCAAGAGGA	GCAGGAGGCACCACTTCTT	135	60

stdJ404F18.6	GCATACACGATGCAAGAGGA	GCAGGAGGCACCACTTCTT	135	60
stdJ452H17.1	ATGCACCTCTGAAACCCTTG	CGACCTCTCTCGGGATATTT	140	60
stdJ452H17.1.1	TTTCCACCACTGGCATTACA	CTCAATAGCCAGGCAGAAGC	159	60
stdJ452H17.1.1n	TGAAACCCTTGCTAAGTA	GGTTGAACTGGAACATAC	-	-
stdJ452H17.1n	AAGTAGAGTATGTTCCAG	AGGCAGAAGCGAATATTG	-	-
stdJ525N14.1	AAAGTTAAAGTCGGCAGGAGC	TCTGGTCGCTGTCCTCAAC	154	60
stdJ525N14.1n	GCCACCTATGGGAAGGAGAC	AACTCCGGCGCCGCCGCCAT	-	-
stdJ525N14.2	TGCCATACACTGGCACTGAT	AAAGAAAGAGCTGGCATCCA	161	60
stdJ525N14.2n	CAACTGGCACACCTCGTTGG	GGCATCCATAGTCGTGGAAG	-	-
stdJ525N14.3	CTTTGGCTTCAGCGCTTC	AACGTCGCTCCAGTCTGG	120	-
stdJ525N14.4	AATGAGCAACGTGGCCAT	GCAACAGAGAAGAGCTGATGG	124	60
stdJ525N14.4n	GACTCTTCTGTGATGTTACC	AGTAGGTACTAGAAGCTGAA	-	-
stdJ525N14.5	AAAAGAACCTCCAGTAGGGACC	TCAAACTCAGTACTGCCATCTG	128	60
stdJ525N14.5n	ACTACATCTACTCAGAACAA	TTACATTTTGTTTAAAAATT	-	-
stdJ525N14.6	AAAGAAGGGGCAGAATCG	TGCTTCCCGGCGCCGCCG	101	60
stdJ525N14.7	ACCCAGACTGGAGCGACG	CTCCTCCGGACGCGCGGAAG	98	60
stdJ525N14.10	ATAGCAATGCCAGTGGAACC	GAGAACACCAGTCTCCGCTC	158	60
stdJ555N2.1	CAGCACCTCTACCTCAAGCC	TGGAGAGCTGAACTGTGGTG	154	60
stdJ555N2.2	TATGGGGTCTTTGCTGGAAG	CTGGGCAGCAGTGAGGTCAG	111	60
stdJ555N2.3	ACAAAAGATTTGGAGGGGCT	AAACTGCTTCCATCCCTGC	141	60
stdJ555N2.3	ACAAAAGATTTGGAGGGGCT	AAACTGCTTCCATCCCTGC	141	60
stdJ555N2.4	GGGCTTGTCAGTGAAATCAA	GAAGATGAGTGAGAGCAAAGGG	142	60
stdJ555N2.4	GGGCTTGTCAGTGAAATCAA	GAAGATGAGTGAGAGCAAAGGG	142	60
stdJ562J12.1	ACATGAAGTTGTTCTCGGGG	CCCTAAGGGTTTTCATCAAGC	144	60
stdJ562J12.1	ACATGAAGTTGTTCTCGGGG	CCCTAAGGGTTTTCATCAAGC	144	60
stdJ562J12.1n	CTGGCTGACCCAAGTCAATG	CAGACATATTCCAATCTGGC	-	-
stdJ655L22.1.1	CTATGCTAGGACACATTAG	GAACTCTGCTTTGTCACAG	130	60
stdJ655L22.1.1n	GATTGGTCATGGAAATAG	GCAGCCCAAAGACTCACATC	130	-
stdJ755D9.1	GAAAACACCGGGGTACACTG	AATGCTGCATGAGAGACATG	120	60
stdJ755D9.1n	GCTGTCACAGACGTCCCA	AATCATGGAGTGCAGTAG	-	-
stdJ755D9.2	AGATGTAAACTAGGAGCAGCCG	CACCAGTGTGAAAGTGAAGAGC	168	60

stdJ755D9.2n	CAGAATGCCGTGGTAGTG	GCTGCTGACTGTCCTCAG	-	-
stdJ755D9.3	ATGGCATCCCCTTAGCTTCT	CTGCCACAGGCTCTCCTC	122	60
stdJ755D9.3n	GTCCGCCAATTATGGCAG	TTATTGAGGAGGCTAGGC	-	-
stdJ755D9.4	TGTTTGTCGGACAAGCTCAG	TGCCTCTTCTTCCTGGCTTA	128	60
stdJ808P6.1	ATGATGAGAATCAGGACCGTG	CCTCCACCATTGCTGTAGGT	127	60
stdJ808P6.1n	CTATGATTGGATACGCAG	AGGTCAGATATGGAAATC	-	-
stdJ808P6.2	ATGGAAAGATGCTGCCACTC	CAAATCCAGCAAACACGCTA	158	60
stdJ808P6.2n	CTTGGGGTGGCTTAGTTG	CGCTAGTGAGACAGTTTG	-	-
stdJ808P6.3	CGCCTTATAAGTTGCTGCAG	AGTAGGTATTTCATGGTCAGCC	120	60
stdJ808P6.3n	ACCTGGAACAACATCGTG	TCTTTCCCCACGATGTTG	-	-
stdJ876A24.1	CTGCCTAGCTCGCGGTCCG	GAAGTCGCCCCCCAACAG	98	60
stdJ876A24.1n	TCGTCCCTGGGGTCCCTG	CTCCAGCCATCTTCTCCG	-	-
stdJ876A24.2	ATGCACAATTCGAGGCCTAC	AGAGACTTCAGGGAATGACCC	127	60
stdJ876A24.2n	CTGCAGGAAGAGCTAAAG	ACAAGCAGACATGGATTCG	-	-
stdJ876A24.3	GAGACCTCGTTTTGAGCCTG	TCTTGGTAGATGTCTCTTGGCA	138	60
stdJ876A24.4	ACACATTTTGCCAGCATG	TGAGAATGAATCCTGATG	109	60
stdJ876A24.4n	TACTCAAGACTCTTTCAG	CTGAGAATTCCCATCTTG	-	-
stdJ878I13.1	GAGAAAGTGGGAGCAGCAAG	ATGCTTCTCTTTCCCTCTTGC	181	60
stdJ1139I1.1	CAGCACCTCTACCTCAAGCC	TGGAGAGCTGAACTGTGGTG	154	60
stdJ1139I1.2	AATGATGGACTCTTCCCGC	ACTTCGTAGGGGTTGACGG	183	60
stdJ1139I1.3	GACGCTCAGCCTCAGCCT	GTTCCCCTTCCACAGGGC	148	60
stdJ1139I1.4	CGTAGACGGGGCTTCCCG	ACCGCGTGGCTCGGGCCTG	109	60
stdJ1139I1.8	GTTCAAAGGAACATTGCCAA	ATAAAGAGTACTTCCTTGGGGG	81	60
stdJ1139I1.8	GTTCAAAGGAACATTGCCAA	ATAAAGAGTACTTCCTTGGGGG	81	60
stdJ1152D16.1	GTTAGAGAAATTGCCCATGAGG	CCGCAGATACATAGTCTCCTCA	109	60
stdJ1152D16.1n	GACCGGTCCCGTGTGATTG	ACTTATGGGCATGTTTGGCAG	-	-
stdJ1189B24.1	GGCGATGGTCCAGATAGAAA	TGGAACCATGAATCCTATTGC	81	60

Table 2.7: *STSs used for mouse mapping as described in chapter 5. STSs are named with the prefix 'st' followed by the clone name from which the STSs was derived. The suffix (SP6, T7 etc.) indicates the vector specific primer used to generate the product.*

STS name	Primer 1	Primer 2	Size (bp)	AT (°C)	Gene/BAC
stAA386485.1	AGGAAACCGGAAAAAGGAGA	TCAGTTCGACATCAGAACGC	141	60	EST
stAA597301.1	GTGGACTGCCGCTCTTCTAC	TATTGGTTTTCCCAAAGCCT	105	60	EST
stAB023622.1	AGTCCACGCTCATGGATACC	GTGAGTTTCAACCCCACGTT	129	60	Septin6
stAB023622.2	GGCGAAGATTGTCGAACTGT	GCACAGGATGTTGAAGCAGA	111	60	Septin6
stAB023622.3	CTGCATGAGAAATTTGACCG	CTTTCTCTGCTTGAAGGCGT	111	60	Septin6
stAF042491.1	CCTGCTCTACAAGATCGTTCG	GAACACCTTGCCGTTGATG	175	60	Mapr
stAF089812.1	CGGGAATATGAAAAGCGTGT	TGTCTCTGATGCTCCACAGG	237	60	hHr6a
stAF097416.1	GTGGCCATGGTCTCTTTTGT	AGTTCAACAACTTGCCCAGC	133	60	Znf-kaiso
stU27316.1	TTCGTATCCCCAAGGAACAG	GAACTGGGTCCTCTTGTCCA	216	60	Ant2
stbM65I16SP6	AGCAACCACACTTTGGCTG	ACTCTGCCCTCTTGTGGCTA	170	60	bM65I16
stbM65I16T7	TCCCATGAGTTTACATCTGGC	TTTCACGCACCAACATTCAT	129	60	bM65I16
stbM110K19SP6	AGTGCTGCTGTGTAAAGCAGG	TTTTCTGAGGGACTAAAGGGTT	80	65	bM110K19
stbM110K19T7	TTTGATGCCAGCAGAAAGC	ATGCACCCTGCAGAGTTTCT	150	65	bM110K19
stbM167L6SP6	CAGAGTTGGGAGTCAGTGCA	CTTGGCCTCAAATTAACTCTGG	157	65	bM167L6
stbM193O17SP6	GAATGCTTCATTGGAGGGAA	TGTGACATTTGTCTTACAGCCA	167	60	bM193O17
stbM193O17T7	TGGAACATGGGTCTATCAAGC	GTCAGCCTGTACCCACCATT	120	60	bM193O17
stbM260F21T7	TCCAAACCCTCTGAACCAAC	CACTAAGCAATGGGCCTGAT	266	60	bM260F21
stbM279D19SP6	ATGTCTCTCTGAGGGGCAGA	TGCACTGACTCCCAACTCTG	163	60	bM279D19
stbM286I5SP6	ACTGAGAGGCTAGGAAGCACC	GAAGAATCTTGACTGGGAGGG	125	65	bM286I5
stbM286I5T7	GACATCATGCCCAGGAGG	TTATGCTGCTGCATGACACA	145	65	bM286I5
stbM302H8T7	CAGAGCTTTGCTGTGCAGAG	CTAGCACTGCAAAGGCAGC	135	65	bM302H8
stbM322E15T7	TAGTCTGTAGGGAGGCTCAAGG	CCTCAGGACCACTCTTGAGC	169	65	bM302H8
stbM343N5SP6	GAGACCATATCCAGTGGCTAGG	GCAGTCTCTGGATGGCTTTC	152	65	bM343N5
stbM461E19T7	GTCATGCATGGTAGTTGCATG	CAGTAAGCTGAACTTAGGTGCA	160	65	bM461E19

Table 2.8: *STSs for conserved sequence analysis in human and mouse described in chapter 5*

STS name	Primer 1	Primer 2	Size (bp)	AT (°C)
stdJ404F18.6	GCATACACGATGCAAGAGGA	GCAGGAGGCACCACTTCTT	135	60
stdJ555N2.3	ACAAAAGATTTGGAGGGGCT	AAACTGCTTCCATCCCTGC	141	60
stdJ555N2.4	GGGCTTGTCAGTGAAATCAA	GAAGATGAGTGAGAGCAAAGGG	142	60
stdJ1139I1.7	TATTACTGAGGGAAACAGCTGG	ATGTGAAGCTGTCCAGTTTTTT	89	60
stdJ1139I1.8	GTTCAAAGGAACATTGCCAA	ATAAAGAGTACTTCCTTGGGGG	81	60

Table 2.9: *STSs used for zebrafish mapping as described in chapter 6*

STS name	Primer 1	Primer 2	Size (bp)	AT (°C)	Gene
stbG421I3.4	GGCAATATGCATGGATGTGA	CAAGGTCTGAGGCAGGTTTC	100	60	bG421I3.CX.2
stbG421I3.5	GAACCTGGGCAGCAGTATTC	AAAATGCACTCCAGCTCCTG	122	60	dJ525N14.CX.1
stbK38K21.3	ACTTTCAGGATTAAGCGATTCC	TCCAGTTTTCATCCGAATCC	81	65	RPL39
stdJ327A19.10	AGCCAAGAAATTGGACAAAG	ATCTTTAAGTTCGCTATCAG	94	60	UPF3B
stdJ327A19.11	TGGCCATGCTCTGTTACCTG	TCACTTGTCAAGCCAATTTC	99	60	dJ327A19.CX.3
stdJ327A19.12	GGTCACAGACATAGCAGCGC	CGTTCAGATTACAAGCATG	235	60	ZNF183
stdJ327A19.14	CTGTGTCCAGCTCATAGACAGC	CGACTTGAGCAGCGAAGAG	131	65	ZNF183
stdJ327A19.13	GTTAGTGAACCTGTGGATG	GTAACGGGGCAGAGATGTG	107	60	NDUFA1
stdJ404F18.4	AGATCTTCCTGGGTGGTGTG	TGCACAGACACGTTAAAGCC	256	60	ANT2
stdJ404F18.5	GTGGACTGCCGCTCTTCTAC	TATGTTCGTTTTCCCAAAGC	107	60	dJ876A24.CX.1
stdJ525N14.11	TGTGGAAGACCGAAAATTCC	TCAGTTCAACAACTTGCCCA	101	60	KAISO
stdJ555N2.2	TATGGGGTCTTTGCTGGAAG	CTGGGCAGCAGTGAGGTCAG	111	60	HPR6.6
stdJ876A24.2	ATGCACAATTCGAGGCCTAC	AGAGACTTCAGGGAATGACCC	127	60	SEPTIN2
stdJ876A24.11	ACAGATTGCAAGGCAACG	TGAATTCTCCAGGGGAAG	368	60	SEPTIN2
stdJ876A24.16	TTCTCCAAATGGCTGAAGGT	ACATGTTGAGAGGTGGCGTT	101	60	dJ876A24.CX.3
stdJ876A24.17	CTCTGTTGGATGAACCCAATC	CAATCACGCCAGCTTTGTTC	124	60	UBE2A
stdJ1139I1.6	ACAGACCTCATCAAAACCCG	AAGGAACCCTTCCTGTTGGT	105	60	dJ1139I1.CX.1
stwz3779.1	ACACTGCTGCTTTGCTGAGA	GGAGGCAGTGAAGAAGTTGC	156	60	dJ876A24.CX.3
stwz8217.1	TAGCTTGGCTCGTTCTTGGT	GTGTCGTGATTTGTGCTCGT	247	60	dJ327A19.CX.3

2.10 World Wide Web addresses

Baylor College of Medicine Search Launcher	http://dot.imgen.bcm.tmc.edu:9331/
Baylor College of Medicine Sequencing Center	http://www.hgscbcm.tmc.edu/
British Columbia Genome Sequence Centre	http://www.bcgsc.bc.ca/
CHLC	http://lpg.nci.nih.gov/CHLC/
DOTTER	http://www.cgr.ki.se/cgr/groups/sonhammer/Dotter.html
EMBL	http://www.ebi.ac.uk/
GDB	http://gdbwww.gdb.org/
Généthon	http://www.genethon.fr/genethon_en.html
Genome Sequencing Center, St Louis	http://www.ibc.wustl.edu/cgm/jcgm.html
Genome Sequencing Center, Jena	http://genome.imb-jena.de/
Genome Sequencing Center, Naples	http://hpced.area.na.cnr.it/grsl/
INTERPRO	http://www.ebi.ac.uk/interpro/scan.html
MPIMG, Berlin (X sequencing)	http://www.mpimg-berlin-dahlem.mpg.de/~xteam/
National Centre for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
OMIM	http://www3.ncbi.nlm.nih.gov/Omim/
PIPMAKER	http://bio.cse.psu.edu/cgi-bin/pipmaker
RepeatMasker	http://ftp.genome.washington.edu/RM/webrepeatmaskerhelp.html
The Institute for Genome Research	http://www.tigr.org/
The Wellcome Trust Sanger Institute	http://www.sanger.ac.uk/
TRANSFAC	http://transfac.gbf.de/TRANSFAC/
Washington University Center for Genetics in Medicine (CGM)	http://www.ibc.wustl.edu/cgm/
Whitehead Institute	http://www-genome.wi.mit.edu/
X Chromosome Mapping Project at the Sanger Institute	http://www.sanger.ac.uk/HGP/ChrX/
VISTA	http://www-gsd.lbl.gov/vista
Zebrafish RH mapping	http://www.genetics.wustl.edu/fish_lab/cgi-bin/human_int_map.cgi

Methods

2.11 Isolation of bacterial clone DNA

2.11.1 Miniprep of cosmid, PAC and BAC DNA

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, resuspended 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^{o}C to pellet the DNA, the supernatant removed and the pellet resuspended 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^{o}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^{o}C to pellet the DNA. The pellet was washed with 70% ethanol and resuspended in 50 μl of $T_{0.1}E$.
7. 1 μl of 10 mg/ml RNase was added and the sample incubated at 37^{o}C for 1 hour prior to storage at -20^{o}C.

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

1. 500 μl of 2 X TY containing appropriate antibiotic (see Section 2.11.1) 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 culture grown for 18 hours at 37^{o}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 resuspended 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.
7. 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 at 1550 g for 2 minutes at 20^{o}C.
8. The filter-bottomed plate was removed and the microtitre plate was left at RT for 30 minutes, before being centrifuged at 1500g for 20 minutes at 20^{o}C.
9. The supernatant was removed and the DNA was dried by inverting the plate and placing it on clean tissue paper, ensuring no disruption of the pellet.
10. 100 µl of 70% ethanol were added to the dried DNA, mixed gently, and DNA precipitated by centrifugation at 1500g for 10 minutes at 20^{o}C. For restriction digest fingerprinting the wash was repeated. The supernatant was removed and the DNA dried as before.
11. 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^{o}C.

2.12 Bacterial clone fingerprinting

2.12.1 Radioactive fingerprinting

1. For each 96-well microtitre plate of sample DNA, a premix containing 1x NEB2 buffer (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 plate was spun 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, and the reaction 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, leaving the first well and every subsequent seventh well of a 4% polyacrylamide gel 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), 4.0 µl of the appropriate ddA-dye. Each premix was mixed prior 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°C.
4. To precipitate the DNA, 7 µl 0.3*M* sodium acetate 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 plate was subjected to centrifugation at 1550 *g* for 20 minutes at 20°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 plate was subjected to centrifugation at 1550 *g* for 10 minutes at 20°C.
9. The supernatants were discarded and the pellet dried as above.
10. The DNAs were resuspended 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°C. 1.25 µl of each sample were loaded on a 5% denaturing acrylamide gel and resolved on a ABI377 Automated DNA sequencer using a 0.2 mm, 12cm, 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 Hind III fingerprinting

1. For one 96-well microtitre plate of sample DNA, a premix containing 231 µl H_20, 99 µl buffer B, 55 µl *Hin*d III, 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 reaction was mixed gently on a vortex and incubated at 37 ^{o}C for 2 hours.
3. The reaction was terminated by the addition of 2 µl of buffer II and either loaded straight away or stored at 4 ^{o}C.
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 running the gel at 4^{o}C 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 H_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 For radioactive fingerprinting

1. 68 µl of T0.1E, 10 µl of NEB2 buffer, 6.6 µl of Sau3AI (50 U/µl), 4 µl dGTP, 5 µl ddTTP, 0.07µl [alpha-^{35}S]dATP (3000Ci/mmol), 2 µl Reverse Transcriptase were added to a 1.5 ml microfuge tube and incubated at 37^{o}C for 1 hour.
2. The reaction was stopped by the addition of 106 µl of 1:15 dilution of formamide dye.
3. The marker was stored at -20^{o}C.

2.13.2 For fluorescent fingerprinting

1. 70 µl $T_{0.1}$E, 10 µL NEB2, 6 µl lambda DNA (500 ng/µl), 6 µl *Bsa*J1 (2.5 U/µl), 4 µl TaqFS (32 U/µl), 4 µl ddC-ROX were added to a 1.5 ml microfuge tube and incubated for 1 hour at 60^{o}C.

2. 100 µl 0.3 *M* sodium acetate and 400 µl 96% ethanol were added to the reaction mix and incubated at room temperature in the dark for 15 minutes, then at -20°C for 20 minutes. The tube was subjected to centrifugation in a bench top centrifuge at maximum 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 for 5 minutes, the supernatant discarded and the pellet dried as described in step 2.
4. The DNA was resuspended in 120 µl $T_{0.1}E$ and 120 µl blue dextran formamide dye.
5. The marker was stored at -20°C.

2.13.3 For Hind 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°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 urea were dissolved in 10 ml 10x TBE and 35 ml ddH_20 by warming to 37°C, 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 ethanol/acetic acid/WAKKER solution (3 ml 96 % ethanol, 50 µl 10 % Acetic acid, 5 µl Walker soln) and left to dry.
4. The front and back plates were taped together along three edges (treated sides facing inwards) separated by 4mm 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 4mm, 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 Applications using the polymerase chain reaction

2.15.1 Primer design

Primers were designed manually using the following guidelines:

1. As far as possible, sequences chosen were 18 - 25 bp in length, beginning and ending with a C or G.
2. 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.

215.2 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.

2.15.3 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^{TM} (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^{o}C, followed by 35 cycles of: 93^{o}C for 30 seconds, [primer-specific annealing temperature] for 30 seconds, and 72^{o}C for 30 seconds; followed by a final extension step of 5 minutes at 72^{o}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.

2.15.4 Colony PCR of STSs from bacterial clones

1. Colony PCR on bacterial clones was performed by touching a sterile toothpick onto the surface of a colony and stirring this into 200 µl of $T_{0.1}E$, and using 5 µl of the resulting suspension in a 15 µl final volume PCR.

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

2.16 Radiolabelling of DNA probes

2.16.1 Random hexamer labelling

adapted from Feinberg, A. P*., et al.*, 1983

1. Approximately 10 ng of DNA were boiled in a total volume of 13.5 µl of $T_{0.1}E$ for 5 minutes and snap chilled on ice-water.
2. Following the addition of 5 µl of OLB3, 1 µl of 10 mg/ml BSA, 2.5 U of Klenow enzyme (Boehringer Mannheim, sequencing grade) and 1 to 5 µl of [α- ^{32}P]-dCTP (3,000 Ci/mmol; 10 Ci/ml) and $T_{0.1}E$ to a total volume of 25 µl, the reactions were mixed and left at room temperature for a minimum of 3 hours and up to overnight.
3. All probes were boiled for 5 minutes and snap-chilled on ice-water prior to use.

2.16.2 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^{o}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^{o}C for 5 minutes; followed by 20 cycles of: 93^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 30 seconds; followed by 72^{o}C for 5 minutes.
5. Probes were pre-reassociated (as described in Section 2.16.3) prior to use if necessary. All probes were boiled for 5 minutes and snap-chilled on ice prior to use.

2.16.3 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, then added directly to the hybridisation reaction.

2.17 Hybridisation of radiolabelled DNA probes

2.17.1 Hybridisation of DNA probes derived from whole cosmids

1. Filters were prehybridised flat in sandwich boxes in 50 ml of hybridisation buffer at 65^{o}C with gentle shaking.
2. Radiolabelled probe was denatured by boiling for 5 minutes, added to the sandwich box, and hybridised to the filters at 65^{o}C for 18 hours.
3. Filters were washed twice at RT in 2x SSC for 5 minutes, twice at 65^{o}C in 0.5 x SSC, 0.1% Sarkosyl for 30 minutes, twice at 65^{o}C in 0.2x SSC, 0.1% Sarkosyl for 30 minutes, and twice at 65^{o}C in 0.1x Sarkosyl, 0.1% Sarkosyl for 30 minutes. All washes were carried out with gentle shaking. Filters were rinsed at RT in 2x SSC to remove Sarkosyl.
4. Excess liquid was removed from the filters by laying them briefly on Whatman 3MM paper. Filters were then wrapped in Saran Wrap (Dow Chemical Co.) and exposed to autoradiograph film under the appropriate conditions.

2.17.2 Hybridisation of DNA probes derived from STSs

1. Filters were prehybridised tightly rolled in 15 ml Sterilin tubes or flat in sandwich boxes for 3 hours in 10-25 ml of hybridisation buffer at 65^{o}C with gentle shaking.
2. Radiolabelled probe was added and hybridised to the filters as described in step 2 of Section 2.17.1
3. Filters were washed twice at RT in 2x SSC for 5 minutes, twice at 65^{o}C in 0.5 x SSC, 0.1% SDS 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.

2.17.3 Hybridisation of DNA probes to gridded zebrafish library

1. Filters were prehybridised in sandwich boxes for 3 hours in 10-25 ml of hybridisation buffer at 50°C with gentle shaking.
2. Radiolabelled probe was denatured by boiling for 5 minutes, added to the sandwich box, and hybridised to the filters as described in step 2 of Section 2.17.1
3. Filters were washed twice at room temperature in 6x SSC for 5 minutes, twice at 50°C in 6 x SSC, 0.1% SDS for 30 minutes, followed by similar sequential washes reducing the concentration of SSC to 4x, 2x, and 1x. Washes were stopped when the amount of the signal remaining on the filters reached less than 5 cpm (tested using a Gieger counter). Filters were given two final rinses in 1 x SSC prior to draining the excess liquid, wrapping in Saran Wrap (Dow Chemical Co.) and exposing to X-ray film.

2.17.4 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 0.1% w/v SDS at 42°C with gentle shaking. Successful removal of radiolabelled probe was assessed by autoradiography.

2.18 Restriction endonuclease digestion

2.18.1 Restriction endonuclease digestion of cosmid DNA

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. The remaining 5 µl were either used immediately for whole cosmid hybridisation (see Section 2.17.1) or stored at –20°C.

2.18.2 Restriction endonuclease digestion of PAC or BAC DNA

1. 5 µl (approximately 200ng) of prepared PAC or cosmid DNA (described in Section 2.11.1) were digested with *Rsa* I using commercial buffers supplied according to manufacturers' instructions, but with the addition of 1 mM spermidine, in a final volume of 20 µl.
2. 10 µl of each digest were checked for complete digestion by electrophoresis on a 1% agarose minigel and visualised by ethidium bromide staining. The remaining 10 µl were either used immediately for vectorette library construction (see Section 2.19) or stored at –20°C.

2.19 Generation of vectorette library of PACs and BACs

Adapted from vectorette PCR of YACs (Riley, J.*, et al.*, 1990)

1. *Rsa* I-digested PAC or BAC DNA (see Section 2.11.1) was precipitated by adding 40 µl of ddH_20, 5 µl of 3M sodium acetate and 100 µl of cold (–20°C) 96% ethanol, and leaving for 1 hour at –20°C. The microfuge tube was subjected to centrifugation in a bench top microfuge for 15 minutes at 14,000 rpm to pellet the DNA. The supernatant was discarded and the DNA pellet washed in 70% ethanol, and air dried.
2. The DNA pellet was resuspended in 100 µl freshly made ligation buffer. 10 µl of annealed vectorette bubbles (BPBI and BPHII, 1pm/µl), 1.1 µl rATP and 2.5 units T4 DNA ligase were added to the sample which was incubated at 37°C for 1 hour.
3. The vectorette library was diluted with 400 µl with $T_{0.1}E$, and was stored at –20°C.

2.20 Rescue of clone ends by PCR amplification of vectorette libraries

Adapted from vectorette PCR of YACs (Riley, J.*, et al.*, 1990)

1. PCR was performed using 1 µl of vectorette library as template. The primers used were either PACT2 (T7 end) or PACS2 (SP6 end) in conjunction with 224. Reactions were carried in standard buffer conditions described in Section 2.15.3.
2. PCR was performed in a DNA thermocycler (MJ). Cycling conditions were as follows: an initial denaturation step of 5 minutes at 94°C, followed by 35 cycles of: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 3 minutes; followed by a final step of 10 minutes at 72°C.

3. 5 µl of PCR product were separated by electrophoresis through 2.5% or 1% agarose gels and visualised by ethidium bromide staining. Bands were excised from the gel and placed in 100 µl of $T_{0.1}E$ and stored at 4°C until required. Products for direct sequencing were gel purified using either GeneClean™ or Qiagen™.

2.21 Preparation of colony grids

1. Clones were spotted onto Hybond N filters using sterile cocktail sticks. The filters were incubated at 37°C overnight.
2. Filters were transferred sequentially onto Whatman 3 MM paper soaked in the following solutions for the times given: 10% SDS (4 minutes), denaturation solution (5 minutes), neutralisation solution, (5minutes), 2x SSC/0.1% SDS (5 minutes), 2x SSC (5 minutes).
3. Filters were air dried on Whatman 3 MM paper. Prior to use in hybridisations the DNA was UV-cross-linked colony side down for 2 minutes on a transilluminator (320 nm).

2.22 Clone library screening

2.22.1 Clone library screening with STSs

1. Pools of bacterial clone DNA, each containing DNA from 3072 clones from 8 x 384-well-microtitre plates, were prepared by E. Sotheran and D. Pearson from the RPCI-1, 3, 4, 5, 6, 11 and 13 libraries.
2. The DNA pools were arranged in microtitre plates for screening. In the primary screen, 5 µl of each pool were used as template in a 15 µl final volume PCR using buffer and PCR conditions as described in Section 2.15.3. 50 ng of genomic DNA and $T_{0.1}E$ were included as positive and negative controls respectively. 10 µl of the 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.
3. In the secondary screen, the PCR product was radiolabelled as described in Section 2.16.2 and hybridised to individual filters of gridded arrays of bacterial clones representing the pools in which a positive signal had been observed, as described in Section 2.17.2

4. Positive clones were picked from the library and streaked to single colonies on LB agar plates with appropriate antibiotic and grown overnight at 37^{o}C. Single colonies were confirmed by colony PCR as described in Section 2.15.3

2.22.2 cDNA library screening by PCR

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

1. Twenty 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 and gel-loading post PCR 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 2.15.3.
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.

2.22.3 Single-sided specificity PCR (SSPCR) on cDNA libraries

The principle of SSPCR (Huang, S.-H.*, et al.*, 1993) is illustrated in Figure 2.1.

1. SSPCR was performed on the subpools of the cDNA libraries, each containing 20,000 clones. Prior to their use in PCR, the subpools were diluted 1:10 in $T_{0.1}E$ and boiled. Dilutions were stored at -20°C until required. On removing from -20°C, tubes were centrifuged briefly in a microfuge to settle the contents and then mixed carefully when thawed.
2. In the first round, PCR was performed using 1 µl of the diluted subpools as template in a 15 µl final volume using buffer conditions as described in Section 2.15.3. The primer combinations used are given in Table 2.10.
3. PCR was performed in microtitre plates in a DNA thermocycler (Omnigene) using hot-start. Cycling conditions were as follows: an initial denaturation step of 5 minutes at 95°C, followed by 25 cycles of: 94°C for 30 seconds, 60 °C for 30 seconds, and 72°C for 3 minutes; followed by a final step of 10 minutes at 72°C.
4. For the second round of PCR, products from the first round were diluted 1 in 50 and 1 in 500 in $T_{0.1}E$. 5 µl of each dilution was used as template in 15 µl final volume PCR using buffer conditions as described in Section 2.15.3. Cycling conditions were as described in step 3. The primer combinations used are given in Table 2.10.

Table 2.10: *Primer combinations used in SSPCR**

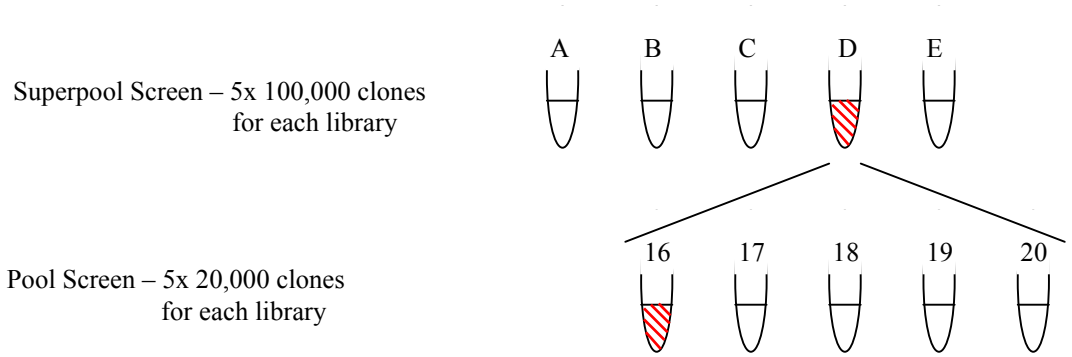
First round SSPCR	**Second round SSPCR**
Specific primer A and FP vector primer	Specific primer B and FP vector primer
Specific primer A and RP vector primer	Specific primer B and RP vector primer
Specific primer C and FP vector primer	Specific primer D and FP vector primer
Specific primer C and RP vector primer	Specific primer D and RP vector primer

*Primer sequences are given in Table 2.3

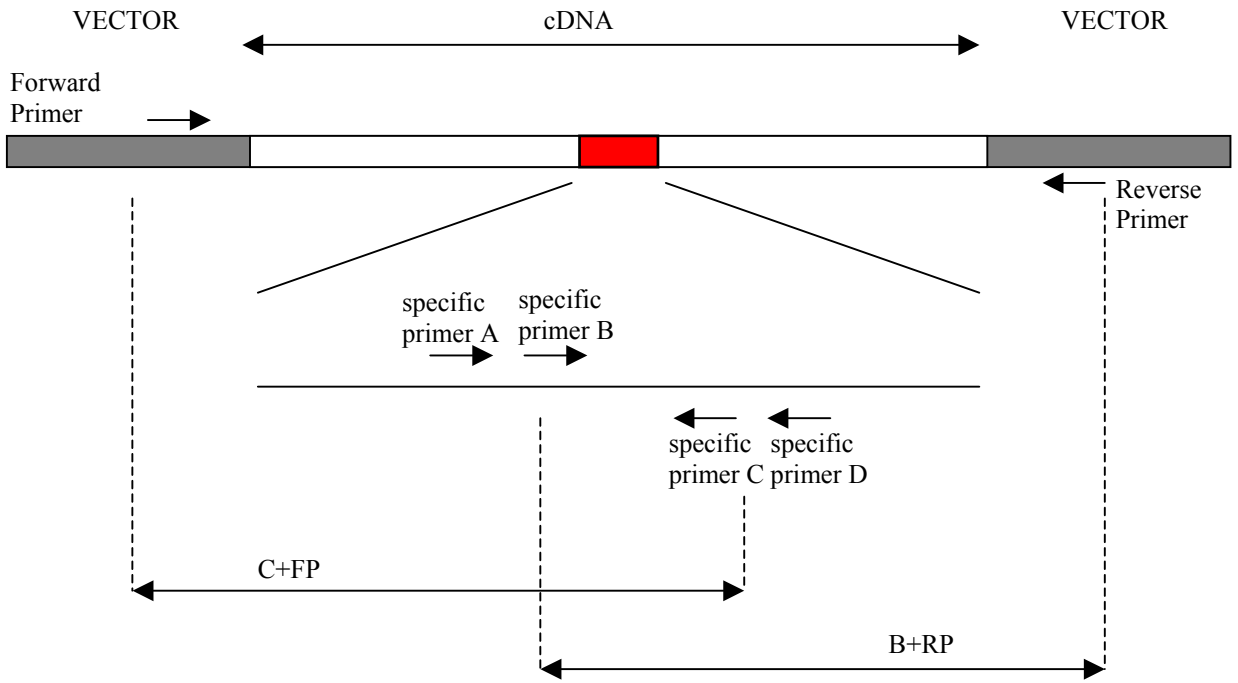
5. 5 µl of the second-round PCR products were separated by electrophoresis through either 1% or 2.5% agarose minigels depending on product size and visualised by ethidium bromide staining. Products were gel purified using the Qiaquick gel extraction kit (Qiagen™) prior to sequencing directly.

Figure 2.1: *(see over) Strategy for SSPCR on 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).The five pools(containing 20,000 clones) that were combined to form the positive superpool (e.g. 16-20) were screened and positive pools recorded (e.g.16, shown in red).(b)Rescue of the insert of the cDNA of interest by SSPCR. cDNA is shown as white rectangle representing insert, vector shown as grey rectangles. Original position of primers for pool screening is indicated by a red rectangle. A combination of vector primers (e.g.FP and RP) and insert specific primers (e.g A and D) were used in the first round of the SSPCR. A second round of SSPCR was carried out with nested sequence specific primers (B and C) to generate two products (e.g. C+FP, B+RP) representing the entire insert of the cDNA of interest.*

(a)


Superpool Screen – 5x 100,000 clones for each library
A
B
C
D
E
Pool Screen – 5x 20,000 clones for each library
16
17
18
19
20

(b


VECTOR
cDNA
VECTOR
Forward Primer
Reverse Primer
specific primer A
specific primer B
specific primer C
specific primer D
C+FP
B+RP

2.22.4 Vectorette PCR on cDNA libraries (illustrated in Figure 2.2)

1. Vectorette PCR was performed on the superpools of the cDNA libraries. 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 2.15.3. 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 T0.1E) 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 from either GeneClean^{TM} or Qiagen^{TM} prior to sequencing directly.

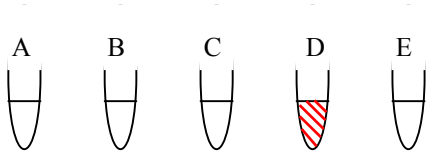
2.22.5 Reamplification of vectorette PCR products

1. In cases where multiple bands or weaker bands were observed, bands were excised and placed in 100 µl of $T_{0.1}E$. Reamplification of each band was carried out by PCR using 5 µl $T_{0.1}E$ taken from the 100 µl containing the excised band and by adding 1.5 µl 10x NEB Buffer, 1.5 µl 5 mM dNTPs, 0.495 µl 5 mg/ml BSA, 0.21 µl 1:20 βME, 3.17 µl 40 % sucrose + cresol red, 0.375 µl 224pure primer, 0.75 µl 100ng/µl specific primer, 0.12 µl Amplitaq, 0.12 µl TaqExtender, 0.12 µl Perfect Match, 0.5 µl 40% sucrose + cresol red, 1.14 µl $T_{0.1}E$ to a well of a 96-well microtitre plate.
2. PCR was performed on a DNA thermocycler (MJ). Cycling conditions were are follows: an initial denaturation step of 5 minutes at 95^{o}C followed by 35 cycles of 94^{o}C for 5 seconds, 60 ^{o}C for 30 seconds and 72^{o}C for 3 minutes, followed by 72^{o}C for 5 minutes.
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 (either GeneClean^{TM} or Qiagen^{TM}) prior to sequencing directly.

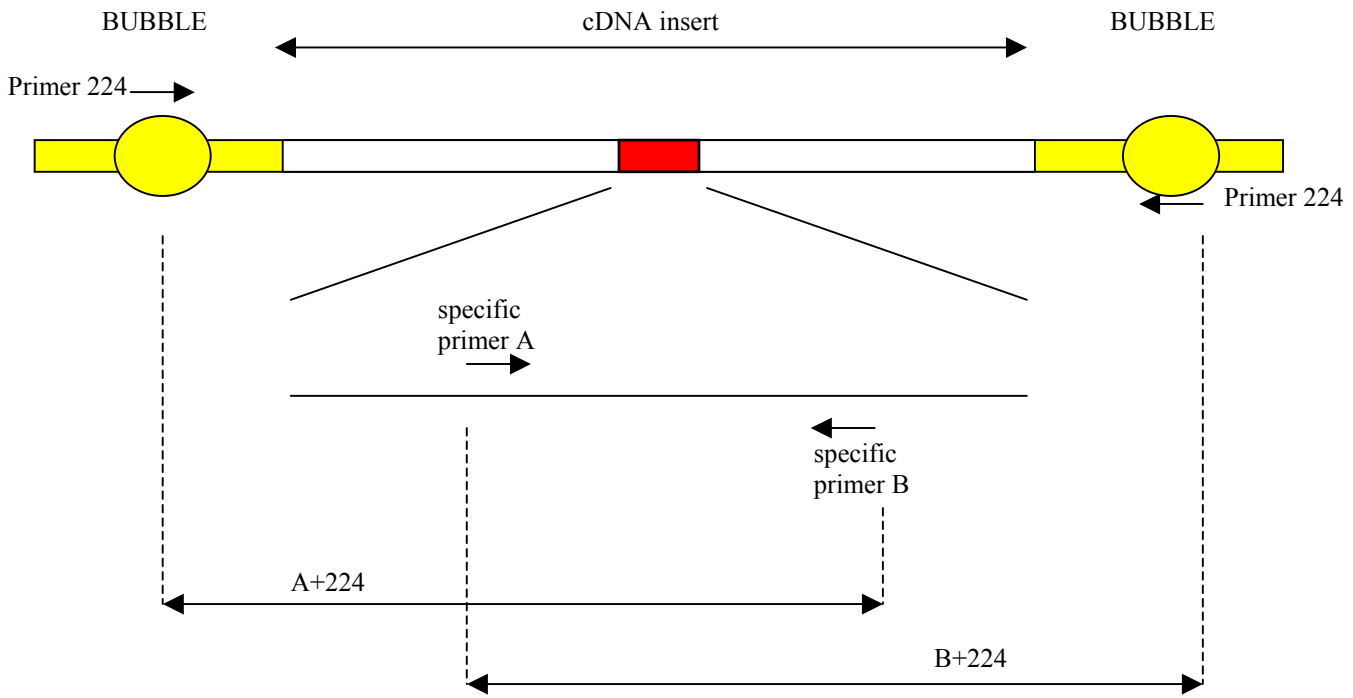
Figure 2.2: *(see over) Strategy for vectorette PCR on cDNA libraries (a) Superpools representing 100,000 clones (A-E) were screened by PCR and positive super pools recorded (e.g. D, shown in red). (b)Rescue of the insert of the cDNA of interest by vectorette PCR. Insert of cDNA is shown as white rectangle, 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.*

(a)

Superpool Screen – 5x 100,000 clones
for each library



(b)



2.23 Mapping and sequence analysis software and databases

2.23.1 IMAGE

All processing of fingerprinting gels was carried out using IMAGE. IMAGE processed gels from radioactive, fluorescent and *Hin*d III fingerprinting and extracted a normalised band pattern for each lane on a gel. Several procedures were run on each gel in turn:
Lane tracking – a grid was superimposed on the gel image and the grid manually edited to ensure it exactly matched the lanes on the gel.
Band calling – an analysis module traced the band pattern along the lanes and tried to identify the bands. Manual editing ensured the correct bands are chosen.
Marker locking – in order to compare band patterns from one gel to another all band positions were normalised to one master gel. A set of DNA fragments of known length or migration distance was loaded as a marker lane (see Section 2.13 for specific marker patterns for each method of fingerprinting used). Manual editing ensured the standard pattern matched to the pattern from the master gel.
Normalisation – once the marker lane patterns were locked onto the standard lane, the band positions of the sample lanes were normalised so that each lane appeared to have been run on the master gel with all distortions cancelled out. IMAGE finally generated a 'Bands' file for each gel containing normalised migration distances for all selected bands in each clone lane.

2.23.2 FPC

All contig construction described in this thesis was carried out in FPC. FPC took as the input a set of clones and their restriction fragments (called Bands) from IMAGE. Each fingerprint pattern for each clone is compared to the fingerprint patterns of all other clones in the database. The relationship between two clones was reported as a probability of coincidence, i.e. the probability that 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 coincdence is less than or equal to the cut off. When analysing matches between cosmids, the tolerance was set to 1e –04, and when analysing larger insert PAC and BAC clones, the tolerance was decreased to 1e –08.)

Tolerance – two bands 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 automatically and contigs were constructed manually using the available editing tools provided by FPC. Initially, two clones were positioned overlapping by the number of bands they had in common. Subsequent clones were positioned in the contig based on the number of bands they shared with the existing clones in the contig. Marker data was imported from Xace and integrated into the FPC contigs. 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, allowing for estimates to be obtained of contig sizes. For each method of fingerprinting, a kilobase/band figure was derived. *For radioactive fingerprinting of cosmids*, one band was the equivalent of 2 kb, based on the fact that the average size of a cosmid is 40 kb and the average number of fingerprint bands for each cosmid was 20. *For fluoresecent fingerprinting of PACs and BACs*, and average figure for each clone type was calculated based on the number of bands observed in clones whose insert sizes were known by genomic sequencing. For regions in a contig covered by PACs, a figure of 3.6 kb/band was used, and for regions of a contig covered by BACs, a figure of 4.4 kb/band was used. *For Hin*d III *fingerprinting of PACs and BACs*, a figure 4.4 kb/band was used and was based on the average number of bands observed in clones whose insert sizes were known from genomic sequencing.

2.23.3 Xace

All mapping and sequencing data generated in this thesis were stored in Xace, a chromosome-specific implementation of ACeDB. ACeDB was originally developed for the *C. elegans* genome project (Richard Durbin and Jean Thierry-Mieg, 1991) A. *C. elegans* Database.

ACeDB works using a system of windows and presents data in different types of windows according to the type of data. 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 Xace. 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 X chromosome mapping group, Xace also contains displays published X 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.

Xace can be accessed by following the instructions at http://www.sanger.ac.uk/HGP/ChrX.

2.23.4 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 “efetch” retrieves the record from an external database (*e.g.* EMBL, SWISSPROT).

2.23.5 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 datbases screened by RepeatMasker are based on the repeat databases (Repbase Update) copyrighted by the Genetic Information Research Institute.