Chapter II

Materials and Methods

2.1 Gene identification

2.1.1 DNA manipulation methods

2.1.1.1 Polymerase chain reaction

PCR reactions were performed in 96-well microtitre plates (Costar Thermowell^{TM} C- or M-type) in an Omnigene (Hybaid) (C-type) or a PTC-225 (MJ Research) (M-type). For most applications, 15 µl reactions were prepared.

1. A premix sufficient for all reactions was prepared, allowing for a 1 X reaction mix once the DNA template was added (10 µl of mix and 5 µl of template).
2. The standard reaction mix contained 2 µl of Buffer 1, 2 µl of 5 mM dNTPs, 0.28 µl of 1/20th βME, 0.07 µl of 5 mg/ml BSA, 5.165 µl of 40% sucrose, 0.325 µl of primer mix (each primer at 100 ng/µl) and 0.16 µl (0.8 U) of Taq DNA polymerase (AmpliTaq). All reaction mixture variants are listed in Table 2.1.
3. Amplifications were performed under the following cycling profile (unless specified otherwise): 94^{o}C for 5 minutes, 35 cycles at 94^{o}C for 30 seconds, annealing temperature (specific to each primer) for 30 seconds and 72^{o}C for 3 minutes, and finally 1 cycle at 72^{o}C for 10 minutes. All cycling reaction mixtures used are listed in Table 2.1.
4. Reaction products were visualised by agarose gel electrophoresis and stained with ethidium bromide (section 2.1.1.2).

DNA templates

The templates used were:

1. cDNA pools.
2. DNA excised from agarose gels in 100 µl $T_{0.1}E$ and left overnight; 5 µl used directly.
3. Human genomic DNA at 12.5 ng/µl.

2.1.1.2 Gel electrophoresis

1. An agarose gel was prepared (2.5% for most PCR amplified products and 1% for fragments over 1 Kb) in 1 X TBE and ethidium bromide (250 ng/µl).
2. PCR reaction products were loaded directly. For purified DNA samples, the appropriate amount of 6 X loading buffer was added prior to loading (e.g. 5 µl of purified DNA and 1 µl of 6 X loading buffer).
3. Size markers (100 bp or 1 Kb ladder) were also loaded.
4. Minigels were run at 80 volts for 10-15 minutes and larger gels were run at 200 volts for approximately one hour.
5. DNA was visualised under UV on a transilluminator and photographed with a Polaroid camera.

2.1.1.3 DNA purification

2.1.1.3.1 Gel purification

The DNA fragment was excised from the agarose gel with a clean scalpel.

1. The gel slice was weighed in a 1.5 ml Eppendorf tube.
2. The gel slice was then purified using a Qiaquick Gel Extraction Kit™ (Qiagen) according to the manufacturer's instructions.
3. Recovery was tested by gel electrophoresis (section 2.1.1.2).

2.1.1.3.2 Ethanol precipitation

1. In a 1.5 ml microcentrifuge tube, 0.1 volumes of 3 M sodium acetate and either one volume of isopropanol or two and a half volumes of ethanol were added to the DNA.
2. The samples were mixed well by vortexing and incubated for 20 minutes at –20°C.
3. DNA was pelleted in a microcentrifuge at 13,000 rpm and washed with 70% ethanol.
4. The pellet was left to dry and then resuspended in the appropriate amount of $T_{0.1}E$.
5. Recovery was tested by gel electrophoresis (section 2.1.1.2).

2.1.1.4 Restriction enzyme digests of DNA

1. Up to 10 µg of DNA was digested in a reaction containing the appropriate 1 X buffer, 1 mM spermidine, 100 µg/ml BSA and 20-50 units of the appropriate enzyme.
2. The DNA was digested for 2 hours or overnight at the appropriate temperature for the enzyme.
3. The DNA was subjected to agarose gel electrophoresis and visualised (section 2.1.1.2).

Table 2.1: PCR mixes (A) and cycling programs (B, next page). All primer concentrations are at 100 ng/μl unless specified otherwise.

A. PCR mixes

Default		SSP-PCR1		SSP-PCR2		Vectorette	
Buffer 1	2μl	Buffer 1	2μl	Buffer 1	2μl	Buffer 1	1.5μl
5mM dNTPs	2μl	5mM dNTPs	1.1μl	5mM dNTPs	1.1μl	5mM dNTPs	1.5μl
1/20 βME	0.28μl	1/20 βME	0.28μl	1/20 βME	0.28μl	1/20 βME	0.21μl
5mg/ml BSA	0.07μl	5mg/ml BSA	0.66μl	5mg/ml BSA	0.66μl	0.5mg/ml BSA	0.495μl
40% sucrose	5.165μl	40% sucrose	5.11μl	40% sucrose	5.11μl	40% sucrose	4.545μl
Primer mix	0.325μl	$T_{0.1}E$	1.85μl	$T_{0.1}E$	2.85μl	Primer 224	0.375μl
Amplitaq	0.16μl					Primer specific	0.375μl
Total	10μl	Total	11μl	Total	12μl	Total	9μl

RACE1		RACE2		Vectorette/RACE enzyme mix		SSP-PCR enzyme mix	
Buffer 1	1.5μl	Buffer 1	1.5μl	AmpliTaq	0.12μl	Buffer 1	0.1μl
5mM dNTPs	1.5μl	5mM dNTPs	1.5μl	Taq Extender	0.12μl	AmpliTaq	0.2μl
1/20 βME	0.21μl	1/20 βME	0.21μl	Perfect Match	0.12μl	$T_{0.1}E$	0.7μl
5mg/ml BSA	0.495μl	5mg/ml BSA	0.495μl	40% sucrose	0.64μl		
40% sucrose	4.745μl	40% sucrose	4.32μl				
Primer specific	0.45μl	Primer specific	0.65μl				
Primer AP1	0.1μl	Primer AP2	0.325μl				
Total	9μl	Total	9μl	Total	1μl	Total	1μl

B. Cycling programs

Default		SSP-PCR		Vectorette	
95°C	5min	95°C	3min	95°C	3min
		Add 1μl SSP-PCR enzyme mix	Pause PCR machine	Add 1μl vectorette enzyme mix	Pause PCR machine
		95°C	2min	95°C	2min
94°C	30sec	94°C	30sec	94°C	5sec
60°C	30sec 30cycles	60°C	30sec 25cycles	68°C	30sec 17cycles
72°C	3min	72°C	3min	72°C	3min
				94°C	5sec
				60°C	30sec 18cycles
				72°C	3min
72°C	10min	72°C	10min	72°C	10min

RACE		PCR product re-amplification	
95°C	3min	95°C	5min
Add 1μl RACE enzyme mix	Pause PCR machine		
95°C	2min		
94°C	5sec	94°C	30sec
68°C	30sec 25cycles	60°C	30sec 20cycles
72°C	3min	72°C	3min
72°C	10min	72°C	10min

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2.1.1.5 Primer design, synthesis and storage

1. Primers were designed using the Primer3 program (Rozen and Skaletsky, 2000; http://www.genome.wi.mit.edu/genome_software/other/primer3.html) from http://www.sanger.ac.uk/cgi-bin/primer3.cgi.

2. Primers were synthesised at the Sanger Institute by David Fraser and Diane Gibson. A subset of the primers were synthesised by GenSet (http://www.genxy.com/index.html).

3. Primers were stored at –20°C and working dilutions were prepared at 100 ng/µl for each primer.

2.1.2 Clone resources

Different types of clone resources have been used throughout this project. The Sanger Institute clone resource and the gene identification groups maintain the clone resources.

2.1.2.1 cDNA libraries used

The cDNA libraries used during the course of this project are described in Table 2.2.

Table 2.2 (next page): cDNA resources. Pools available for each technique are listed at the far right. Column A reports the number of SSP-PCR Super Pools available for each library (each Super Pool representing cDNA inserts from 100,000 clones). Column B reports the number of SSP-PCR individual Pools (each representing cDNA inserts from 20,000 clones). Column C reports the number of vectorette Super Pools (each representing cDNA inserts from 100,000 clones). Column D reports the number of individual plated pools (each representing cDNA inserts from 20,000 clones). Column E reports the number of liquid pools (each representing cDNA inserts from 250,000 clones grown in liquid media). Column F reports the number of liquid pools (each representing cDNA inserts from 100,000 clones grown in liquid media).

cDNA library code	cDNA library description	Supplier	Vector	Pools available					
				SSP-PCR		Vectorette			
				A	**B**	**C**	**D**	**E**	**F**
T	Adult testis	Clontech	pCDM8	5	25	-	-	-	10
FB	Fetal Brain	Invitrogen	pcDNAI	5	25	5	25	10	-
FL	Fetal Liver	Invitrogen	pcDNAI	5	25	5	25	10	-
FLu	Fetal Lung	Invitrogen	pcDNAI	5	25	5	25	10	-
HL60	Peripheral blood	Invitrogen	pcDNAI	5	25	5	25	10	-
AH	Adult Heart	Invitrogen	pcDNA3	5	25	5	25	10	-
ALu	Adult Lung	Clontech	pcDNAI	5	25	5	25	10	-
SK-N-MC	Neuroblastoma cells	Invitrogen	pcDNAI	5	25	-	-	-	-
PF	Adult brain	Pfizer	pcDNAI	3	15	-	-	-	-
U937+	(Monocyte, NOT activated, from a patient with promonocytic leukaemia)	DS*	pCDM8	5	25	-	-	-	-
U937ACT	(Monocyte, PMA activated, from a patient with promonocytic leukaemia)	DS*	pCDM8	5	25	-	-	-	-
H9	Placental, full term normal pregnancy	DS*	pH3M	5	25	-	-	-	-
YT	HTLV-1 +ve adult leukaemia T cell	DS*	pH3M	5	25	-	-	-	-
NK	Natural killer cell	DS*	pH3M	5	25	-	-	-	-
Daudi	B lymphoma	DS*	pH3M	5	25	-	-	-	-
HPBall	T cell from a patient with acute lymphocytic leukaemia	DS*	pH3M	5	25	-	-	-	-
BM	Bone marrow	DS*	pH3M	5	25	-	-	-	-
DX3	Melanoma	DS*	pH3M	5	25	-		-	-

*Libraries marked with an asterisk were generously provided by David Simmons, Oxford

2.1.2.2 Construction of cDNA pools

2.1.2.2.1 Library titration

1. 2 litres of LB agar were used to prepare 35 agar plates. Molten LB agar was left to cool down to 55°C and the appropriate amounts of antibiotics were added (ampicillin at 50 μg/ml and tetracycline at 10 μg/ml final volume). Approximately 50 ml of the mixture was used for each colony picker plate. The plates were left to set and stored at 4°C.

2. The cDNA library, consisting of bacteria stored in glycerol, was defrosted on ice and 2 μl were diluted in 198 μl LB. Six ten-fold serial dilutions were prepared and 100 μl of each was plated on LB agar plates with Hybond N+ filters, using an ethanol-flamed bent Pasteur pipette.

3. The plates were left inverted, at 37°C for 4 hours. They were then transferred to 30°C for 16 hours, and then back to 37°C for an additional 4 hours. The colonies of each plate were counted and the library titre estimated.

2.1.2.2.2 Low-density plated pools

1. The cDNA library was diluted in 20% glycerol/LB/antibiotics. 20,000 clones were plated out on each of 25 LB/antibiotics plates with Hybond N+ filters (500,000 clones in total).

2. The plates were left to dry for 3-5 minutes, and the clones were then left to grow (4 hours at 37°C, 16 hours at 30°C and 4 hours at 37°C).

2.1.2.2.3 High-density liquid pools

1. Ten 50 ml Falcon tubes containing 20 ml of LB/antibiotics were set up.
2. The cDNA library was diluted in LB/antibiotics and 250,000 clones were added to each tube (2,500,000 clones in total). The clones were left to grow at 37°C/240 rpm for 20 hours in a shaking incubator.
3. Dilutions of 1000, 100, 10 and 1 were plated out on colony picker plates/Hybond+ filters to check titration. The plates were inverted and left to grow overnight at 37°C.

2.1.2.2.4 Preparation of SSP-PCR pools

1. All SSP-PCR pools were prepared from low-density plated clones (section 2.1.2.2.2).
2. The contents of each filter were scraped into 3 ml of 20% glycerol in LB/antibiotics using a glass Pasteur pipette, and transferred to a 15 ml Falcon tube. The cells were shaken off and the filters were removed.
3. Super Pools were prepared by pooling 1 ml from each of a group of five tubes (Pools) in a new Falcon tube thus generating 5 Super Pools. The tubes were frozen in dry ice and stored at –70°C.
4. Pool templates for use in PCR screens and SSP-PCR reactions were prepared by transferring 0.3 ml of each Pool (or Super Pool) in screw-top Eppendorf tubes. The aliquots were boiled for 5 minutes and then quenched in ice. The contents were briefly spun, and stored at –20°C. 1/100 dilutions of the boiled template/$T_{0.1}E$ were prepared for the first and second steps of SSP-PCR (Super

Pool and individual pool screens), respectively. 1/10 dilutions of the boiled Pools template/$T_{0.1}E$ were prepared for the third step of SSP-PCR (cDNA-end recovery). All dilutions were stored at –20°C.

2.1.2.2.5 Preparation of vectorette pools

1. For plated vectorette pools, each Hybond N+ filter was removed from the colony picker plates, rolled-up and placed in a 50 ml Falcon tube containing 20 ml of SET. The cells were shaken off and the filters were removed.
2. The cells in the thirty-five, 50 ml Falcon tubes (25 plated pools/SET and 10 liquid pools/LB), were pelleted at 4,000 rpm for 10 minutes at room temperature, using a Beckman J6-MC centrifuge.
3. The media (or SET) was removed and the pellets were re-suspended in 200 µl of GTE on ice, transferred to a 1.5 ml tube and left to stand for 5 minutes.
4. 400 µl of freshly made 0.2 M NaOH/1% SDS (briefly cooled in ice) was added and the tubes were left to stand on ice for 5 minutes. 300 µl of 5 M acetate/3 M K^+ were added to each tube. The tubes were gently inverted once and left on ice for 10 minutes.
5. Cell debris was pelleted in a microcentrifuge at 13,000 rpm for 10 minutes and the clear supernatants were removed and put into clean tubes. These were spun for 5 minutes to remove any remaining debris.
6. 600 µl of isopropanol stored at –20°C was added in each tube. The tubes were then well shaken and left on ice for at least 10 minutes.

7. DNA was pelleted by spinning the tubes in a microcentrifuge at 4°C /13,000 rpm, for 15 minutes. The pellets were re-suspended in 200 µl of $T_{0.1}E$.
8. 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added in each tube. The tubes were shaken and spun for 5 minutes. The top (aqueous) layers were removed and placed in fresh tubes.
9. The DNA was ethanol precipitated (section 2.1.1.3.2).
10. The DNA was pelleted in a microcentrifuge and washed with 70% ethanol.
11. The DNA was resuspended in 30 µl $T_{0.1}E$ and stored at –20°C.
12. 1 µl of 10 mg/ml RNase was added to each tube and incubated at 37°C for 1 hour.
13. 1 µl of the DNA was run on a 0.8% agarose gel to check the extraction outcome.
14. 1 µg of each extracted DNA was digested with the appropriate enzyme in a total volume of 30 µl (section 2.1.1.4).
15. 70 µl of water was added and the DNA was extracted with 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
16. The DNA was ethanol precipitated (section 2.1.1.3.2).
17. The DNA was pelleted in a microcentrifuge, washed with 70% ethanol and left to dry for 5 minutes. The pellets were then re-suspended in 100 µl of ligation buffer.
18. 10 µl of 1 pmol/µl annealed vectorette bubbles, 1.1 µl adenosine 5'-triphosphate and 2.5 units of T4 DNA ligase were added to each tube and left at 16°C overnight.

19. The contents of each tube were diluted to 500 µl with $T_{0.1}E$ to generate Stock Pools.

20. Equal volumes of sets of five plated Stock Pools were mixed to generate Stock Super Pools.

21. 1/100 dilutions of Stock Super Pools were prepared using $T_{0.1}E$, for Super Pool PCR screens.

22. 1/100 dilutions of the plated Stock Pools were prepared using $T_{0.1}E$, for the second step of the vectorette method (individual Pool screens).

23. 1/10 dilutions of the plated Stock Pools were prepared using $T_{0.1}E$, for cDNA-end recovery (vectorette PCR). 1/10 dilutions of the liquid Stock Pools were also prepared using $T_{0.1}E$, for PCR pool screening and cDNA-end recovery.

2.1.3 Isolation of cDNA fragments

Three methods (SSP-PCR, vectorette and RACE) were used to isolate expressed sequences from cDNA pools. The PCR mixes and cycling programs used for each technique are listed in Table 2.1. The various universal primers used are listed in Table 2.3.

Table 2.3: Universal primer sequences.

SSP-PCR	Primer Sequences	Comments
pH3M-1FP	CTT CTA GAG ATC CCT CGA	Amplifies inserts from pH3M and pCDM8 vectors
pH3M-2FP	GAT CCC TCG ACC TCG AGA T	Amplifies inserts from pH3M and pCDM8 vectors
pH3M-1RP	CGC AGA ACT GGT AGG TAT	Amplifies inserts from pH3M and pCDM8 vectors
pH3M-2RP	CGA CCT GCA GGC GCA GAA	Amplifies inserts from pH3M and pCDM8 vectors
T7-2FP	TAA TAC GAC TCA CTA TAG G	Amplifies inserts from pCDM8, pcDNA3 and pcDNAI vectors
pCDM8-RP	TAA GGT TCC TTC ACA AAG	Amplifies inserts from pCDM8 and pcDNAI vectors
SP6	ATT TAG GTG ACA CTA TAG	Amplifies inserts from pcDNA3 vectors
VECTORETTE	**Primer Sequences**	**Comments**
224	CGA ATC GTA ACC GTT CGT ACG AGA ATC GCT	Universal primer for all vectorette pools. Used for cDNA-end isolation
Xho-I	TCG AGC AAG GAG AGG ACC AAG GAG AGG ACG CTG TCT GTC GAA GGT AAG GAA CGG ACG AGA GAA GGG AGA G	Used for the construction of the Testis vectorette library
Xho-II	CTC TCC CTT CTC GAA TCG TAA CCG TTC GTA CGA GAA TCG CTG TCC TCT CCT TGG TCC TCT CCT TGC	Used for the construction of all vectorette libraries
RACE	**Primer Sequences**	**Comments**
AP1	CCA TCC TAA TAC GAC TCA CTA TAG GGC	Adaptor Primer 1
AP2	ACT CAC TAT AGG GCT CGA GCG GC	Nested Adaptor Primer 2

2.1.3.1 SSP-PCR

The technique of end-fragment isolation from cDNA libraries is an adaptation (Bye and Rhodes, unpublished) of the original SSP-PCR (Shyamala and Ames, 1989; Shyamala and Ames, 1993). cDNA pools for SSP-PCR were constructed by Jackie Bye, Suzan Rhodes and George Stavrides.

2.1.3.1.1 Identification of positive pools

1. 5 μl from each of the 88 available Super Pools (five Super Pools from seventeen cDNA libraries and 3 Super Pools from 1 cDNA library) were PCR screened.
2. For each positive Super Pool, the five individual constituent Pools (1/100 dilutions) were PCR screened.

2.1.3.1.2 Amplification of cDNA ends

1. The first part of the SSP-PCR cDNA-end isolation (SSP-PCR1) was performed using the 1/10 dilutions of the positive individual Pools. Because the orientation of the cDNA insert in the clone was unknown, two reactions were set-up for each gene-specific primer.
2. 1 μl from each positive pool and 4 μl of $T_{0.1}E$ were added to 11 μl of SSP-PCR1 buffer mix. 2 μl of the sense or antisense gene-specific primer (100 ng/μl), 1 μl (10 ng/μl) of either the forward or reverse vector primer and a drop of mineral oil were also added.
3. The PCR reaction was performed under the SSP-PCR cycling profile, which was briefly paused after the first step to add 1 μl of SSP-PCR mix in each well.

4. The second part of the SSP-PCR method of cDNA-end recovery (SSP-PCR2) was performed using 1/50 and 1/10 dilutions (in $T_{0.1}E$) of the SSP-PCR1 products as templates.

5. 5 µl from each dilution was added to 12 µl of SSP-PCR2 buffer mix. In agreement with the SSP-PCR1 step, 1 µl of either a sense or an anti-sense gene-specific nested primer was also added. 1 µl of either a forward or reverse nested vector primer and a drop of oil were also added.

6. The PCR reaction was performed under the SSP-PCR cycling profile, paused after the first step to add 1 µl of SSP-PCR mix in each well.

2.1.3.1.3 Isolation and re-amplification of cDNA ends

1. Reaction products were visualised by agarose gel electrophoresis (section 2.1.1.2).

2. The amplified DNA fragments were excised and the gel slices were placed in 1.5 ml tubes containing 100 µl $T_{0.1}E$ and left overnight at 4°C.

3. To obtain sufficient DNA for sequencing, liquid from around the gel slice was re-amplified by PCR (re-amplification PCR mix and cycling program). Four reactions were set-up to obtain sufficient DNA for sequencing. The amplified products were separated by gel electrophoresis (section 2.1.1.2) and gel purified (section 2.1.1.3.1). Recovery was checked by gel electrophoresis (section 2.1.1.2).

4. Elizabeth Huckle performed the DNA sequencing reactions.

2.1.3.2 Vectorette

The technique of vectorette cDNA end isolation is an adapted version (Collins, unpublished) of the original vectorette PCR (Riley et al., 1990; Arnold and Hodgson, 1991). cDNA pools for vectorette were constructed by John Collins, Melanie Goward and George Stavrides.

2.1.3.2.1 Identification of positive pools

1. 5 μl from each of the 30 available Super Pools (five Super Pools from six cDNA libraries) or the 60 liquid Pools (ten Pools from six cDNA libraries) were PCR screened.
2. For each positive Super Pool, the five individual constituent Pools (1/100 dilutions) were PCR screened.

2.1.3.2.2 Amplification of cDNA ends

1. Vectorette cDNA-end recovery was performed on 1/10 dilutions of positive Pools. 5 μl of each positive Pool were added to 9 μl of the vectorette reaction mixture that contained one gene-specific primer and a universal primer (224). One drop of oil was also added to each well.
2. The PCR reaction was performed under the vectorette cycling profile that was briefly paused after the first step in order to add 1 μl of vectorette enzyme mix.

2.1.3.2.3 Isolation and re-amplification of cDNA ends

1. The PCR products were isolated, re-amplified and sequenced, as above (section 2.1.3.1.3).

2.1.3.3 RACE

RACE was performed on either Human Brain or Testis Marathon-Ready™ cDNA.

2.1.3.3.1 Identification of positive Marathon pools

1. 5 µl of RACE template (2 µl of Marathon-Ready™ cDNA diluted in 3 µl $T_{0.1}E$) was PCR screened.

2.1.3.3.2 Amplification of cDNA ends

1. The first step of RACE (RACE1) was performed with 5 µl of RACE template (2 µl of Marathon-Ready™ cDNA diluted in 3 µl $T_{0.1}E$) added to 9 µl of RACE1 reaction mixture (which included one gene-specific primer and the adaptor primer AP1). PCR was performed under the RACE cycling profile, which was briefly paused after the first step to add 1 µl of RACE enzyme mix.
2. 1/50 and 1/10 dilutions of the RACE1 products were prepared using $T_{0.1}E$. 5 µl of each dilution was used as templates in a second round of RACE.
3. 9 µl of RACE2 buffer mix (which includes one gene-specific primer and the nested adaptor primer AP2) was added in each template and PCR was performed under the RACE cycling profile, which was briefly paused after the first step to add 1 µl of RACE enzyme mix.

2.1.3.3.3 Isolation and re-amplification of cDNA ends

The PCR products were isolated, re-amplified and sequenced, as above (section 2.1.3.1.3).

2.1.4 Northern Blots

2.1.4.1 Probe generation and labelling

1. Probes were generated by PCR (section 2.1.1.1) from cDNA templates (Table 2.2).
2. PCR products were separated by gel electrophoresis (section 2.1.1.2).
3. The expected-size band was excised and stored in 100 µl $T_{0.1}E$, at 4°C.
4. Labelling was performed (to be described in section 2.2.1.2).

2.1.4.2 Hybridisation

Labelled probes were hybridised to Multiple Tissue Northern (MTN®) Blots (Clontech). Each blot contains 2 µg of polyA mRNAs from different adult and fetal human tissues (Table 2.4).

Table 2.4: Northern Blots.

Human MTN Blot #7760-1	Human MTN Blot II #7759-1	Human Fetal MTN Blot II #7756-1
Heart	Spleen	Brain
Brain	Thymus	Lung
Placenta	Prostate	Liver
Lung	Testis	Kidney
Liver	Ovary	
Skeletal muscle	Small Intestine	
Kidney	Colon	
Pancreas	Peripheral Blood Leukocyte	

1. The blots were pre-hybridised for 1 hour and then hybridised for 18 hours at 65°C in ExpressHyb Hybridisation solution (Clontech).
2. The blots were washed twice in Northern Wash Solution I for 5 minutes at room temperature, then twice in Northern Wash Solution II for 3 minutes at 55°C.
3. The blots were subjected to autoradiography for an average of 4 days, at room temperature.

2.2 Mouse studies

The RPCI-23 female (C57Bl/6J) mouse BAC library (Osoegawa *et al*., 2000) was screened in this study. Library details are shown in Table 2.5.

Table 2.5: Details of the mouse genomic library.

Library	Library type	Library code	Antibiotic	Vector	Cloning site	Genomic digest
RPCI-23	BAC	bM	Chloramphenicol 12.5 μg/ml	pBACe3.6	*Eco*RI	*Eco*RI

2.2.1 Probe preparation

2.2.1.1 Primer testing and probe generation with PCR

1. 20 μl reaction mixtures were set-up in a 96-well plate containing 10 μl of dilution-buffer/primer-mix solution, 7.063 μl sucrose solution, 2 μl of PCR Buffer 2, 0.187 μl of 1/10 β-ME, 0.25 μl of 5mM dNTP solution, 0.4 μl of mouse genomic DNA (or $T_{0.1}E$ as negative control) and 0.1 μl (0.5 U) of AmpliTaq.
2. PCR was performed using a touch-down PCR program (Table 2.6).
3. PCR products were separated by gel electrophoresis (section 2.1.1.2). The expected-size band was excised and stored in 100 μl of $T_{0.1}E$ at 4°C.

Table 2.6: Touch-down PCR programs.

Steps	65T		60T		55T	
1	94°C	5min	94°C	5min	94°C	5min
2	93°C	30s	93°C	30s	93°C	30s
3	65°C	50s	60°C	50s	55°C	50s
4	-0.5 per cycle		-0.5 per cycle		-0.5 per cycle	
5	72°C	50s	72°C	50s	72°C	50s
6	repeat steps 2-5, 9 times		repeat steps 2-5, 9 times		repeat steps 2-5, 9 times	
7	93°C	30s	93°C	30s	93°C	30s
8	60°C	50s	60°C	50s	60°C	50s
9	72°C	50s	72°C	50s	72°C	50s
10	repeat steps 7-9, 29 times		repeat steps 7-9, 29 times		repeat steps 7-9, 29 times	
11	72°C	5min	72°C	5min	72°C	5min

2.2.1.2 PCR labelling

1. A 9.5 µl reaction mixture was set-up in a 0.5 ml tube, for each probe. The reaction mixture contained 1 µl of PCR Buffer 3, 0.4 µl of primer mix, 2.5 µl of the liquid surrounding the gel slice, 0.4 µl of d(ATG), 4.6 µl of H_2O and 0.1 µl of AmpliTaq.
2. A single drop of mineral oil was added on top of the reaction mixture, followed by 0.5 µl of α-^{32}P.
3. PCR was performed as follows: 94°C for 5 minutes, 20 cycles of 93°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by 1 cycle at 72°C for 5 minutes.
4. The PCR products were then denatured at 99°C for 5 minutes in the thermal cycler, snap chilled on ice and added to the hybridisation mix (unless competitive re-association was required, section 2.2.1.3).

2.2.1.3 Competitive re-association of radiolabelled probes

Where appropriate, the radiolabelled probes were competed using polyCA•GT to suppress the non-specific binding of PCR-labelled probes containing polyCA•GT.

1. The labelling reaction products (section 2.2.1.2) were transferred in a screw-top microcentrifuge tube containing 5 μl of 1 mg/ml polyCA•GT, 125 μl of 20 X SSC and 360 μl of H_2O.
2. The tube was left to boil for 5 minutes in a water bath.
3. The tube was snap chilled on ice and the contents were then added to the hybridisation mix.

2.2.2 Screening

2.2.2.1. Screening library filters

1. The probes were prepared as described above (section 2.2.1).
2. **A.** 2-30 library filters were placed in a 15 X 10 X 5 cm sandwich box containing approximately 150 ml of hybridisation buffer (enough to cover all filters). A plastic sheet was placed on top of the filters. The filters were then left to pre-hybridise at 65°C for at least 3 hours, in an orbital shaker. The filters and the plastic sheet were removed from the sandwich box and the denatured, labelled probe(s) added to the hybridisation solution and mixed well. The filters were added back to the box one by one and the plastic sheet was again placed on top.

B. When only one filter was to be screened, a 15 ml Falcon tube was used instead of sandwich boxes. The filter was placed in the 15 ml Falcon tube containing 13 ml of hybridisation buffer. The filter was left to pre-hybridise at 65°C for at least 3 hours, in an orbital shaker. The labelled probe was then added and mixed well.

3. The filters were left to hybridise overnight at 65°C, in an orbital shaker.
4. The filters were then rinsed twice in 2 X SSC at room temperature, followed by two 30 minute washes with 0.5 X SSC/1% N-Lauroyl Sarcosine at 65°C in an orbital shaker.
5. The filters were rinsed twice in 0.2 X SSC and wrapped in Saran™ wrap.
6. The wrapped filters were placed overnight in a cassette with an X-ray film and two intensifying screens.
7. The X-ray films were developed and data was entered in the 2musace database.

2.2.2.2 PCR screening of BAC DNA pools

1. BAC DNA pools were PCR screened with the STS of interest (section 2.1.1.1).
2. Positive pools were identified by gel electrophoresis (section 2.1.1.2).

2.2.2.3 Colony PCR

1. The colony of interest was picked in 70 µl of H_2O and boiled at 95°C.
2. 5 µl aliquots were used as templates in PCR screens with the STS(s) of interest (section 2.1.1.1) and PCR products were visualised by gel electrophoresis (section 2.1.1.2).

2.2.3 Fingerprinting

2.2.3.1 Clone picking and microprepping

1. BAC clones were picked in 96-deep-well plates containing 1.5 ml LB broth/antibiotics, using a wooden cocktail stick.
2. The picked clones were grown at 37°C/300 rpm for 16 hours.
3. Eight plate copies were prepared by aliquoting 170 µl from each well into 96-well plates. Each plate copy was frozen on dry ice and stored at –70°C.
4. Bacterial clones were microprepped (Marra *et al*., 1997) by Carol Carder (Sanger Institute).

2.2.3.2 Restriction enzyme digestion

1. Simultaneous digestion of 96 clones was achieved with the use of 96-well plates.
2. The restriction digest mixture for each BAC DNA consisted of 2.6 µl of ddH_2O, 0.9 µl of 10 X enzyme buffer B, and 0.5 µl of *Hind*III. The mixture was delivered in each well using a Hamilton combitip dispenser.
3. The 96-well plate was sealed, gently agitated using a whirlimixer, briefly centrifuged at 1,000 rpm and incubated at 37°C for 2 hours.
4. The reaction was terminated by the addition of 2 µl of 6 X loading dye. The plate was resealed, and briefly centrifuged at 1,000 rpm.

2.2.3.3 Agarose gel electrophoresis and data acquisition

1. 450 ml of molten agarose were used to prepare 1% agarose gels in 1 X TAE.
2. Each solidified gel was placed in an electrophoresis unit containing 2-3 litres of 1 X TAE.
3. 0.8 µl of marker mix was added in the first well and every fifth well. 1 µl from each restriction enzyme digestion/loading dye mix was then loaded.
4. Samples were electrophoresed at room temperature for 30 minutes at 90 volts. The electrophoresis apparatus was then moved into the cold room where the gels were left to run for 15 hours at 90 volts.

5. Following electrophoresis, the gels were trimmed to ~19 cm, placed in plastic trays containing Vistra Green stain mix and agitated in the dark, on an orbital flatform shaker, for 45 minutes.

6. The gels were then briefly rinsed with de-ionised H_2O and imaged using a FluorImager SI.

2.2.3.4 Fingerprint analysis and contig construction

1. Fingerprint analysis was performed interactively using the Image 3.10 software (Sulston *et al*., 1980; Platt and Wobus, unpublished; also see section 4.2.3).

2. Band data was collected and used to perform an automatic contig assembly using FPC V4 (Soderlund *et al*., 2000). The parameters used were an overlap statistic of $3x10^{-12}$ (about 75% clone overlap) and 0.7 mm tolerance.

3. To identify potential joins, fingerprints of clones at the extreme ends of contigs were used to query the FPC database at a lower fingerprint overlap stringency (overlap statistic of $1x10^{-8}$ or about 50% clone overlap).

4. Joins were incorporated into the map if the fingerprint data was logically consistent with the proposed map order.

2.3 Human variation

2.3.1 DNA samples

The Caucasian, Asian and African American samples were obtained from the Coriell Cell Repository (http://locus.umdnj.edu/ccr/). The Caucasian panel consists of 95 DNA samples and is drawn from the UTAH CEPH pedigree collection. The Asian panel consists of twelve Japanese DNA samples from unrelated individuals. The African American panel consists of twelve African American DNA samples from unrelated individuals. Specific sample identifiers are listed in Table 2.7.

All DNA samples were diluted to 3.5 ng/µl using $T_{0.1}E$ (working DNA solutions).

2.3.2 SNP selection and primer design

Publicly available SNPs from various discovery efforts were utilised. SNPs were selected in a hierarchical way so as to generate a polymorphic SNP map of increasing density. Genotyping was performed after each round of SNP selection followed by additional SNP selection and genotyping. Where possible, SNPs that mapped outside repeats were selected.

Sarah Hunt designed the primers and probes in a quadruplex format, using the SpectroDesigner software (Sequenom, San Diego, CA). GenSet (http://www.genset.fr/) synthesised all SNP primers and probes.

Table 2.7: (A) The Caucasian family samples. (B) and the Caucasian, Asian and African American samples (twelve unrelated individuals from each ethnic group). PGF, paternal grandfather; PGM, paternal grandmother; MGF, maternal grandfather; MGM, maternal grandmother; F, father; M, mother; S, son; D, daughter.

A.

Family ID	Relation	DNA name	Family ID	Relation	DNA name	Family ID	Relation	DNA name
1331	PGF	NA07007	1341	PGF	NA07034	1408	PGF	NA12154
1331	PGM	NA07340	1341	PGM	NA07055	1408	PGM	NA12236
1331	MGM	NA07016	1341	MGM	NA06993	1408	MGM	NA12155
1331	MGF	NA07050	1341	MGF	NA06985	1408	MGF	NA12156
1331	F	NA07057	1341	F	NA07048	1408	F	NA10830
1331	M	NA06990	1341	M	NA06991	1408	M	NA10831
1331	S	NA06983	1341	S	NA07020	1408	S	NA12148
1331	D	NA06988	1341	D	NA07006	1408	D	NA12149
1333	PGF	NA07049	1346	PGF	NA12043	1416	PGF	NA12248
1333	PGM	NA07002	1346	PGM	NA12044	1416	PGM	NA12249
1333	MGM	NA07017	1346	MGM	NA12045	1416	MGM	NA12250
1333	MGF	NA07341	1346	F	NA10857	1416	MGF	NA12251
1333	F	NA07038	1346	M	NA10852	1416	F	NA10835
1333	M	NA06987	1346	S	NA12039	1416	M	NA10834
1333	S	NA07009	1346	D	NA12040	1416	S	NA12243
1333	D	NA07011	1347	PGF	NA11879	1416	D	NA12244
1334	PGF	NA12144	1347	PGM	NA11880	1420	PGF	NA12003
1334	PGM	NA12145	1347	MGM	NA11881	1420	PGM	NA12004
1334	MGM	NA12146	1347	MGF	NA11882	1420	MGM	NA12005
1334	MGF	NA12239	1347	F	NA10858	1420	MGF	NA12006
1334	F	NA10846	1347	M	NA10859	1420	F	NA10838
1334	M	NA10847	1347	S	NA11871	1420	M	NA10839
1334	S	NA12138	1347	D	NA11870	1420	S	NA12007
1334	D	NA12139	1362	PGF	NA11992	1420	D	NA11997
1340	PGF	NA06994	1362	PGM	NA11993	1423	PGF	NA11917
1340	PGM	NA07000	1362	MGM	NA11994	1423	PGM	NA11918
1340	MGM	NA07022	1362	MGF	NA11995	1423	MGM	NA11919
1340	MGF	NA07056	1362	F	NA10860	1423	MGF	NA11920
1340	F	NA07029	1362	M	NA10861	1423	F	NA10842
1340	M	NA07019	1362	S	NA11984	1423	M	NA10843
1340	S	NA07040	1362	D	NA11985	1423	S	NA11909
1340	D	NA07053				1423	D	NA11910

B.

No.	Asian	African American	Caucasian[1]
1	NA17051	NA17109	NA11879
2	NA17053	NA17111	NA11880
3	NA17056	NA17114	NA11881
4	NA17057	NA17115	NA11882
5	NA17058	NA17117	NA12248
6	NA17060	NA17119	NA12249
7	NE00251	NA17122	NA12250
8	NE00374	NA17124	NA12251
9	NE00904	NA17125	NA07340
10	NE00810	NA17132	NA07016
11	NE00299	NA17134	NA07050
12	NE00744	NA17136	NA07007

[1]Also part of the family panel

2.3.3 Working PCR primer mix and probe dilutions

1. For each set of 384 SNPs to be genotyped, 375 nM (for each primer) quadruplex primer mix dilutions were prepared in either a 96-well V bottom plate or a 0.5 ml Costar Assay Block on a Genesis RSP Tecan, using ddH_2O.

2. Similarly, 10 μM (for each probe) quadruplex probe dilutions were also prepared.

2.3.4 PCR amplification

Reactions were performed in 384-well microtitre plates. Each microtitre plate was used to genotype 384 SNPs assays across four DNA samples (1,536 assays).

1. The quadruplex PCR reaction mixtures (5 μl final volume) consisted of 2 μl of the appropriate primer mix, 0.75 μl of 10 X PE buffer, 0.2 μl of 5mM dNTP mix,

1.01 µl of ddH_2O, 0.04 µl (2 X) of Titanium Taq and 1 µl of the DNA to be tested.

2. Each 384-well plate was sealed with Microseal 'A' film and PCR was performed under the following cycling profile: 95°C for 1 minute, 45 cycles of 95°C for 20 seconds, 56°C for 30 seconds and 72°C for 1 minute, followed by 1 cycle at 72°C for 5 minutes.
3. The plates were spun at 1,000 rpm for one minute, their seals replaced with ScotchPad™ Tape Pads, and stored at –20°C until the next step.

2.3.5 SAP

After the PCR reaction, the un-incorporated dNTPs were inactivated using Shrimp Alkaline Phosphatase (SAP).

1. SAP deactivation of dNTPs was performed in a total volume of 7 µl consisting of the PCR reaction products (5 µl), 1.5 µl of ddH_2O, 0.2 µl of 10 X TS buffer and 0.3 µl (0.3 U) of SAP enzyme.
2. Each 384-well plate was sealed with Microseal 'A' film. The SAP reaction was performed at 37°C for 20 minutes, followed by inactivation of the SAP enzyme at 80°C for 5 minutes.
3. The plates were spun at 1,000 rpm for one minute, their seals replaced with ScotchPad™ Tape Pads, and stored at –20°C until the next step.

2.3.6 Extension of primer probe

1. Primer probe extension was performed in a total volume of 9 µl consisting of the SAP reaction products (7 µl), 0.5 µl of the appropriate extend primer probes, 0.382 µl of ddH_2O, 0.2 µl of 10 X TS buffer, 0.9 µl of the appropriate dNTP/ddNTP combination and 0.018 µl (0.58 U) of Thermosequenase.
2. Each 384-well plate was sealed with Microseal 'A' film and reactions were cycled as follows: 94°C for 2 minutes and 55 cycles of 94°C for 5 seconds, 52°C for 5 seconds and 72 °C for 5 seconds, followed by 1 cycle at 72°C for 5 minutes.
3. The plates were spun at 1,000 rpm for one minute, their seals replaced with ScotchPad™ Tape Pads, and stored at –20°C until the next step.

2.3.7 Water and resin addition

1. After the extension of primer probes, the 384-well plates were spun at 1,000 rpm for 1 minute.
2. 16 µl of Milli-Q water was delivered in each well using a BECKMAN Multimek-96 automated 96-Channel Pipettor.
3. To remove residual salt from the reactions, a cation exchange resin (SpectroCLEAN™) was added. The resin was initially applied on specially adapted trays and then delivered in each well. Approximately 1 g of resin was used for each 384-well plate.

4. The plates were rotated at medium speed on a Roto-Shake Genie™ rotator for 4 minutes.
5. The plates were then spun for 4 minutes at 4,000 rpm.

2.3.8 Mass spectroscopy

1. 7 nl of the purified primer extension reaction was loaded on to a matrix pad (3-hydroxypicoloinic acid) of a SpectroCHIP.
2. SpectroCHIPs were analysed using a Bruker Biflex III Maldi-TOF mass spectrometer (SpectroReader, Sequenom).
3. Spectra were processed using SpectroTYPER (Sequenom).

2.4 Bioinformatics and computational support

The software used in these studies is listed in Table 2.8. The names of people involved in database management and sequence analysis are reported in Table 2.9. Table 2.10 gives the URLs of web sites used.

Table 2.8: Software used in this study.

Software	Description	Reference
ACeDB	Data storage/graphical display	Durbin and Thierry-Mieg, 1994
Ensembl	Genome browser	Hubbard *et al*., 2002
UCSC GB	Genome browser	Kent *et al*., 2002
Genscan	Gene prediction	Burge and Karlin, 1997
FGENESH	Gene prediction	Salamov and Solovyev, 2000
RepeatMasker	Repeat sequences prediction	Smit and Green, unpublished
CPGFIND	CpG island prediction	Micklem, unpublished
PromoterInspector	Promoter prediction	Scherf *et al*., 2000
Eponine	TS site prediction	Down and Hubbard, 2002
BLAST	Similarity searches	Altschul *et al*., 1990, 1997
InterProScan	Protein motif analysis	Zdobnov and Apweiler, 2001
Dotter	Dot plot DNA comparisons	Sonnhammer and Durbin, 1995
CLUSTAL W	Sequence alignments	Thompson *et al*., 1994
Belvu	Formatting of aligned sequences	Sonnhammer, unpublished
FPC 4V	Contig building	Soderlund *et al*., 2000
Image 3.10	Processing of raw fingerprint data	Sulston *et al., 1980*
PipMaker	Comparative sequence alignments	Schwartz *et al*., 2000
Spectro Designer	SNP assay design	Sequenom™, unpublished
SpectroTYPER RT	Genotype analysis	Sequenom™, unpublished
SpectroTYPER DB	SNP data storage	Sequenom™, unpublished
SpectroCHECK	Genotype Quality Control check	Sequenom™, unpublished

Table 2.9: People involved in sequence analysis and data storage and management.

James Gilbert	Automated sequence analysis and chromosome 20 Ensembl database curator
Michele Clamp Guy Slater	Exonerate analysis of chromosome 20 sequence and WGS homologous mouse reads
Sarah Hunt	SNP analysis and data management
Carol Scott	Management of chromosome-specific fingerprint and sequence databases
Jilur Ghori	Oligo ordering and management of primace database
Jennifer Ashurst Laurens Wilming Andrew King Kerstin Jekosch	Sequence analysis and annotation
Panos Deloukas George Stavrides	Chromosome 20 gene annotation group
Lisa French Ian Mullenger	Manual curation of mouse FPC database

Table 2.10: URLs used in this study.

Description	URL
ACeDB	http://www.acedb.org/
BACPAC resources	http://www.chori.org/bacpac/home.htm
BLAST at NCBI	http://www.ncbi.nlm.nih.gov/BLAST/
BLAT	http://genome.ucsc.edu/cgi-bin/hgBlat?db=hg7
DKFZ	http://mbi.dkfz-heidelberg.de/
Dotter	http://www.cgr.ki.se/cgr/groups/sonnhammer/Dotter.html
Ensembl	http://www.ensembl.org/Docs/
Ensembl Trace server	http://trace.ensembl.org/
European Bioinformatics Institute	http://www.ebi.ac.uk/
European Molecular Biology Laboratory	http://www.embl.org/
FPC	http://www.sanger.ac.uk/Software/fpc
GeneMap '99	http://www.ncbi.nlm.nih.gov/genemap/
Genoscope	http://www.genoscope.cns.fr
Human BLAST server at the Sanger Institute	http://www.sanger.ac.uk/HGP/blast_server.shtml
Human Chromosome 20	http://www.sanger.ac.uk/HGP/Chr20/
Image	http://www.sanger.ac.uk/Software/Image
Mouse BAC end sequences	http://www.tigr.org/tdb/bac_ends/mouse/bac_end_intro.html
Mouse BAC fingerprints	http://www.bcgsc.bc.ca/projects/mouse_mapping/
Mouse genome sequence FTP at the Sanger Institute	ftp://ftp.sanger.ac.uk/pub/mouse/
PipMaker	http://bio.cse.psu.edu/pipmaker/
PromoterInspector	http://www.genomatix.de/cgi-bin/promoterinspector/promoterinspector.pl
PubMed	http://www.ncbi.nlm.nih.gov/PubMed/
RIKEN Genomic Sciences Centre	http://www.gsc.riken.go.jp/
SMART	http://smart.embl-heidelberg.de/help/smart_about.shtml
SSAHA	http://www.sanger.ac.uk/Software/analysis/SSAHA/
The Baylor College of Medicine search launcher	http://searchlauncher.bcm.tmc.edu/
The Coriell Cell Repository	http://locus.umdnj.edu/ccr/
The dbSNP database	http://www.ncbi.nlm.nih.gov/SNP/

The EMBL nucleotide sequence database	http://www.ebi.ac.uk/embl/
The Ensembl Human genome server	http://www.ensembl.org/Homo_sapiens/
The Ensembl Mouse genome server	http://www.ensembl.org/Mus_musculus/
The EST database dbEST	http://www.ncbi.nlm.nih.gov/dbEST/
The GenBank DNA sequence database	http://www.ncbi.nlm.nih.gov/Genbank/index.html
The Genome Sequence Centre (BCGSC)	http://www.bcgsc.bc.ca/
The Institute for Genomic Research	http://www.tigr.org/
The InterPro database	http://www.ebi.ac.uk/interpro/
The InterProScan package	http://www.ebi.ac.uk/interpro/scan.html
The Jackson Laboratory mice web site	http://jaxmice.jax.org/index.shtml
The LocusLink query interface	http://www.ncbi.nlm.nih.gov/LocusLink/
The Mouse Sequencing Consortium	http://www.sanger.ac.uk/Info/Press/001006.shtml
The MRC Mouse Genome Centre	http://www.mgc.har.mrc.ac.uk/
The National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
The Online Mendelian Inheritance in Man database	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
The Pfam collection of protein sequence alignments	http://www.sanger.ac.uk/Software/Pfam/
The PRINTS compendium of conserved protein motifs	http://www.bioinf.man.ac.uk/dbbrowser/PRINTS/
The ProDom (protein domain database)	http://prodes.toulouse.inra.fr/prodom/doc/prodom.html
The PROSITE database of protein families and domains	http://www.expasy.ch/prosite/
The Reference Sequence project	http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html
The Sanger Institute	http://www.sanger.ac.uk/
The Sequence Retrieval System (SRS) at the EBI	http://srs.ebi.ac.uk/
The SNP Consortium	http://snp.cshl.org/
The SWISS-PROT protein sequence database	http://www.expasy.org/sprot/
The TIGRFAMs collection of protein families	http://www.tigr.org/TIGRFAMs/Explanations.shtml
The TrEMBL computer-annotated protein database	http://www.ebi.ac.uk/swissprot/
The UniGene human sequences collection	http://www.ncbi.nlm.nih.gov/UniGene/Hs.Home.html
The Whitehead Institute Center for genome research	http://www-genome.wi.mit.edu/
The Whole Mouse Catalog	http://www.rodentia.com/wmc/toc.html

2.5 Materials

Culture plates
(Genomic Solutions #CONS1001)

Ampicillin sodium salt (Sigma #A9518)

Hybond N+ filters (Amersham #NK9655)

1.2 ml screw-top propylene tubes
(Costar #2027)

Beckman J2-MC centrifuge

RNase solution, 500 μg/ml
(Boehringer Mannheim #119 915)

100 X NEB BSA, 10 mg/ml
(New England Biolabs #B9001S)

10 X Ligation buffer
(Boehringer Mannheim #1243 292)

Phenol:chloroform:isoamyl alcohol,
25:24:1 v/v
(GIBCO BRL #15593-031)

Taq Extender PCR additive 5 U/μl
(Stratagene #600-148-81)

BSA (Albumin, Bovine), 5% solution
(Sigma A-4628)

Tetracycline hydrochloride
(Sigma #T3383)

Glucose (BDH #10117)

EDTA (IBI #IB70182)

Innova 4000 Incubator Shaker
(New Brunswick Scientific)

Isopropanol (BDH #102246L)

*Eag*I, 10,000 U/ml
(New England Biolabs #505L)

5 U/μl T4 DNA ligase
(Boehringer Mannheim #799 009)

Glacial acetic acid
(Mallinckrodt Baker #9507-02)

100 mM adenosine 5'-triphosphate solution
(Amersham Pharmacia
Biotech #27-2056-01)

Perfect Match 1 U/μl
(Stratagene #600-129-81)

Ammonium sulphate, enzyme grade
(GIBCO BRL #5501UA)

Ultrapure dNTP set 100 mM solution (Amersham Pharmacia Biotech #27-20-35-01)	Cresol red sodium salt (Sigma #C-9877)
Sucrose (BDH #102744B)	Tris base (Anachem #0826)
Human Brain Marathon-Ready™ cDNA (Clontech #7400-1)	Human Testis Marathon-Ready™ cDNA (Clontech #7414-1)
Light white oil (Sigma #M3516)	MJ thermal cycler
BAC genomic filters	Hybaid OmniGene cycler
Costar 6511 96-well plates (M-type)	Omni seals (Hybaid #HB-TD-MT-SRS-5)
AmpliTaq DNA polymerase, 5 U/µl (Roche #N808-0145)	Mouse genomic DNA, 0.1 µg/µl (Clontech #6650-1)
2-mercaptoethanol (Bio-Rad #161-0710)	BSA (Sigma A-2153)
Perkin Elmer DNA thermal cycler	HAAKE SW20 waterbath
Kodak M35I film processor	Decon 90 (Decon Laboratories)
Beta cabinets, shields, bins, racks and boxes (Anachem-Scotlab)	Innova 4080 Incubator shaker (New Brunswick Scientific)
N- Lauroyl Sarcosine (Sigma #L-5125)	Saran™ wrap (Dow Chemical Co.)
Poly (dA-dC)•Poly (dG-dT) (Amersham Pharmacia Biotech #27-7940-01)	Redivue [α-^{32}P]dCTP 10 mCi/ml, 3000 Ci/mmol (Amersham Pharmacia Biotech #AA0005)
Sodium chloride (BDH #301237S)	Super RX Fuji X-Ray film (#03G010)
Tri-sodium citrate (BDH #301287F)	Whatman filter paper (#1001 240)

Sodium dodecyl sulphate (SDS, BDH #442444H)	4N Sodium hydroxide solution (BDH 191373M)
Whatman 3MM chromatography paper (#3030 931)	Dextran sulphate (Amersham #17-0340-02)
Polyvinylpyrrolidone (Sigma #PVP-40)	Ficoll (Sigma #F-9378)
Innova 4000 Incubator Shaker (New Brunswick Scientific)	2ml deep 96-square-well titre plates (Beckman #140504)
96-well Microtest, flat bottom plates (Falcon #353072)	Glacial acetic acid (Mallinckrodt Baker #9507-02)
HindIII, 40 U/µl (Roche Molecular Biochemicals #798983)	10 X SuRE/Cut Buffer B (Roche Molecular Biochemicals)
DNA molecular weight marker V (Roche Molecular Biochemicals #821705)	Gel tanks (Owl Scientific Gator Wide Forma System model A3-1 #008100191)
Analytical marker DNA, wide range (Promega #DG1931)	Seakem LE Agarose (FM Bioproducts #50004)
Mylar plate sealers (Dynex Technologies #5701)	Hamilton repeat dispenser (Hamilton Company)
Benchtop centrifuge (Eppendorf #5415C)	Wooden cocktail sticks
Ficoll Type 400-DL (Sigma #F-9378)	Cold room regulated to 4°C
Vistra Green (Amersham Life Sciences #RPN5786)	FluorImager SI Vistra Fluorescence (Molecular Dynamics)
Tabletop centrifuge (Sorvall #RT6000D)	Eppendorf Combitip Repeat Dispenser
Xylene cyanol (BDH #44306)	Bromophenol blue (BDH #20015)

ExpressHyb Hybridisation solution (Clontech #8015)

RoboDesign SpectroPOINT (Sequenom)

Titanium Taq DNA Polymerase, 50 X (Clontech #8434)

Clontech MTN® Blots (#7756-1, #7759-1, #7760-1)

Bruker Biflex™ III MALDI-TOF mass spectrometer

Thermo-Fast® 384-well plates (Abgene #TF-0384)

96-well V bottom plates with lids (SARSTEDT #82.1583.001)

0.5 ml Assay block (Costar #3956)

Genesis RSP (Robotic Sample Processor) 100/8 Tecan with MβP® 50µl tips (BioRobotic Molecular BioProducts #902-262), linked to an AcerPower4100

MATRIX technologies 100 ml disposable reagent reservoir (MATRIX #8086)

TOMTEC THINLID ™ plate sealers (Costar #3095)

ScotchPad™ Tape Pads (3M #0212-61618)

BECKMAN Multimek-96 automated 96-Channel Pipettor

Microseal 'A' film (MJ Research #MSA-5001)

Impact multichannel pipettes by MATRIX

Benchtop centrifuge (Eppendorf #5403)

Shrimp alkaline phosphatase 1 U/µl (Amersham #70092)

SpectroCLEAN™ (Sequenom™ #10053)

Roto-Shake Genie™ (Scientific Industries Inc.)

Thermosequenase DNA polymerase, 32 U/µl (Amersham #79000)

3-Pt Calibrant (Sequenom #335)

SpectroCHIP (Sequenom #000153)

2.6 DNA ladders

2.6.1 1 Kb ladder (Gibco-BRL #15615-024). This contains 1 to 12 repeats of a 1,018 bp concatenated fragment and vector fragments from 75 to 1,636 bp, producing the following sized fragments (bp):

12,216	6,108	1,018	201
11,198	5,090	517/506	154
10,180	4,072	396	134
9,162	3,054	344	75
8,144	2,036	298	
7,126	1,636	220	

2.6.2 100 bp DNA ladder (Gibco-BRL #15628-019). This consists of 15 blunt-ended fragments between 100 and 1500 bp in multiples of 100 bp and an additional fragment at 2072 bp, producing the following sized fragments (bp):

2,072	1,000	400
1,500	900	300
1,400	800	200
1,300	700	100
1,200	600	
1,100	500	

2.6.3 Wide Range Analytical Marker DNA (Promega)

The Analytical Marker DNA, Wide Range, provides an evenly spaced distribution of 37 DNA fragments ranging from 702 bp to 29,950 bp in size and was used for band sizing in fingerprint experiments. This marker is composed of a mixture of restriction enzyme digests of Lambda DNA and ϕX174 DNA.

2.7 Solutions

$T_{0.1}E$

10 mM Tris-HCl (pH8.0)

0.1 mM EDTA

1 X GTE

50 mM glucose

25 mM Tris-HCl (pH8.0)

1 mM EDTA

Vectorette bubbles (1 ml)

1 nmole of XhoI or EagI primer

1 nmole of XhoII primer

25 µl of 1 M NaCl

H_2O up to 1 ml

Heated to 65°C for 5 minutes and left to cool at room temperature

5 M acetate-3M K^+ (100 ml)

60 ml 5 M potassium acetate

11.5 ml glacial acetic acid

28.5 ml H_2O

SET

10 mM Tris-HCl (pH8.0)

0.1 mM EDTA

100 mM NaCl

PCR buffer 1 (1 litre)

80 g Tris Base

22 g $(NH_4)_2SO_4$

65 ml 1 M $MgCl_2$

Made up to 1000 ml with ddH_2O

40% sucrose/cresol red (1litre)

400 g sucrose

0.1 g cresol red

Made up to 1000 ml with ddH_2O

34.6% sucrose/cresol red (1 litre)

346 g sucrose

0.1 g cresol red

Made up to 1000 ml with ddH_2O

Northern wash solution I

2 X SSC

0.05% SDS

Northern wash solution II

0.1 X SSC

0.1% SDS

Nucleotide mix (PCR labelling)

dATG, dGTP and dTTP at a concentration of 5 mM each.

Nucleotide mix (PCR amplification)

dATG, dCTP, dTTP and dGTP at a concentration of 5 mM each

Primer mix

Sense and antisense primers at a concentration of 100 ng/µl each

PCR buffer 2 (10 ml)

4.5 ml 1 M Tris-HCl (pH8.8)

0.15 ml 1 M $MgCl_2$

0.1453 g $(NH_4)_2SO_4$

0.35 ml H_20

5 ml cresol red solution

Cresol red solution

0.1 g/l cresol red in T0.1E

Dilution buffer (pH8.5)

8 ml $T_{0.1}E$

0.13 ml cresol red solution

14 µl NaOH

16 ml H_2O

PCR buffer 3

500 mM KCl

100 mM Tris-HCl (pH 8.3)

15 mM $MgCl_2$

Northern strip solution

H_2O/0.5% SDS

Dilution buffer/primer mix solution

2.5 ng/µl of primer mix in dilution buffer

20 X SSC

3 M NaCl

0.3 M Tri-sodium citrate

0.2 X SSC

0.03 M NaCl

0.003 M Tri-sodium citrate

2 X SSC

0.3 M NaCl

0.03 M Tri-sodium citrate

Wash solution

0.5 X SSC

1% N-Lauroyl Sarcosine

50 X TAE (1litre)

242 g Tris

0.1 M EDTA

57.1 ml glacial acetic acid

Made up to 1,000 ml with milli-Q water

6 X loading dye

0.25% bromophenol blue

0.25% xylene cyanol

15% Ficoll

Hybridisation buffer

6 X SSC

2 mg/ml polyvinylpyrrolidone

2 mg/ml Ficoll

2 mg/ml BSA

50 mM Tris-HCl (pH7.4)

1% N-Lauroyl Sarcosine

10% w/v dextran sulphate

LB broth

10 mg/ml bacto-tryptone

5 mg/ml yeast extract

10 mg/ml NaCl

pH 7.4

LB culture medium (for BAC clones)

92.4 ml LB broth

7.5 ml 100% glycerol

0.1 ml 25 mg/ml chloramphenicol

Strip solution I

0.4 N NaOH

Strip solution II

0.1 X SSC

0.2 M Tris-HCl (pH 7.4)

1% N-Lauroyl Sarcosine

Marker mix

1.5 µl Analytical marker DNA wide range

0.1 µl DNA molecular weight marker V

4.2 µl 6 X loading dye

19.2 µl $T_{0.1}E$

10 X TS buffer

260 mM Tris-HCl (pH 9.5)

65 mM $MgCl_2$

PE 10 X PCR buffer

500 mM KCl

100 mM Tris-HCl (pH 8.3)

15 mM $MgCl_2$

0.01% (w/v) gelatine

CGT stop mix

ddCTP, ddGTP, ddTTP and dATG
at a concentration of 5 mM each

ACT stop mix

ddATG, ddCTP, ddTTP and dGTP
at a concentration of 5 mM each

ACG stop mix

ddATG, ddCTP, ddGTP and dTTP
at a concentration of 5 mM each