Chapter II - Materials and Methods

All solutions and media used are listed at the end of this chapter.

2.1 DNA manipulation methods

2.1.1 Polymerase Chain Reaction (PCR)

PCR was performed in a 96-well microtitre plate (ABgene) in a PTC-225 (MJ Research) thermal cycler or in 0.5 ml microcentrifuge tubes in a DNA Thermal Cycler (Perkin Elmer). 15 μl reactions were prepared except where noted.

1. A premix sufficient for the number of planned reactions was prepared, allowing for a 1X reaction mix once the DNA template was added (usually 10 μl of mix and 5 μl of template).
2. The final reaction contained 1X buffer (Buffer 1 unless otherwise specified), 200 μM of each of the four nucleotides (Pharmacia), 40 ng of each primer, and 0.5 units/μl of DNA polymerase (*Taq* (Applied Biosystems Amplitaq) or *KOD* Hot-start (Novagen)).
3. PCR amplifications using *Taq* polymerase were performed under the same cycling profile (except where specified): 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, annealing temperature (specific to each primer pair) for 30 seconds and 72°C for 30 seconds, and finally followed by 1 extension cycle at 72°C for 5 minutes.
4. PCR amplifications using KOD polymerase were performed with the cycling profile: 94°C for 2 minutes (to activate the Hot-start KOD polymerase), followed by 35 cycles at 94°C for 15 seconds, 60°C for 30

seconds and 68°C for 3 minutes, and finally followed by 1 extension cycle at 68°C for 5 minutes.

5. Reaction products were visualised by agarose gel electrophoresis and staining with ethidium bromide (see below).

2.1.2 DNA templates

The templates used were:

1. Bacterial colonies picked into 100 µl of sterile water and 5 µl used directly.
2. 2 µl of overnight bacterial culture inoculated into 100 µl of sterile water and 5 µl used directly.
3. cDNA or bacterial pools.
4. DNA excised from an agarose gel into 100 µl sterile water, left overnight at 4°C and 5 µl used directly.
5. Human (Sigma D-3035), mouse (Coriell and Babraham Institute), wallaby (kind gifts of Marilyn Renfree and Jenny Graves), opossum (kind gift of Guillaume Smits), platypus (kind gift of Marilyn Renfree) or chicken (Novagen 69233) genomic DNA at 10 ng/µl.

2.1.3 Agarose gel electrophoresis

1. An agarose gel was prepared (2.5% for most PCR amplified products and 1% for fragments over 1 kb) in 1X TBE and ethidium bromide (250 ng/µl).
2. DNA was added to the appropriate amount of 6X loading buffer (e.g. 5 µl of PCR product and 1 µl of 6X loading buffer) and loaded. In the case of Buffer 2, the samples were loaded directly.
3. Size markers (100 bp or 1 kb ladders, Invitrogen) were also loaded.

4. Mini-gels (50 ml) were run at 80 Volts for 10-15 minutes and larger gels (250 ml) were run at 190 Volts for the time required to obtain satisfactory separation, typically 45 minutes.
5. DNA was visualised under UV light on a transilluminator and photographed with a Polaroid camera (Kodak) or digital system (UVP).

2.1.4 Size markers

100 bp and 1 kb DNA ladders for agarose gel electrophoresis were supplied from Invitrogen (15628-019 and 15615-024 respectively).

A wide-range analytical marker DNA was used in fingerprinting (Promega DG1931). This marker provides an evenly spaced distribution of 32 DNA fragments ranging from 702 bp to 29,950 bp and 4 smaller fragments (498, 525, 536 and 645 bp) resulting from a mixture of restriction enzyme digests of Lambda and θX174 DNAs.

2.1.5 Restriction enzyme digests

2.1.5.1 Liquid DNA

1. Up to 10 μg of bacterial clone, or plasmid, DNA was used in a reaction containing the appropriate 1X 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 temperature recommended by the supplier of the enzyme (typically NEB).
3. The DNA was subjected to agarose gel electrophoresis and visualised.

2.1.5.2 PCR products

1. After PCR amplification, the required amount (usually 5-10 µl) was transferred to a new 0.5 ml microcentrifuge tube, and 5 units of the restriction enzyme added.
2. DNA was digested for 1 hour at the recommended temperature (obtained from the NEB catalogue) and visualised by gel electrophoresis.

2.2 DNA extraction

2.2.1 Phenol/chloroform extraction of plasmids

1. To 100-700 µl of sample was added an equal volume of Tris-buffered phenol. Sample volumes smaller than 100 µl were made up to 100 µl with water to ease subsequent steps.
2. The samples were mixed thoroughly by vortex then the two phases separated by centrifugation for 1 minute at 13,200 rpm.
3. The aqueous (upper) layer was carefully removed to a fresh 1.5 ml eppendorf tube by pipetting.
4. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed and spun.
5. To the carefully removed aqueous layer was added an equal volume of chloroform/isoamyl alcohol (24:1). Following mixing and centrifugation the aqueous layer containing DNA was removed for ethanol precipitation (see below).

2.2.2 Bacterial clone DNA micro-preparations

1. A single colony was inoculated into 500 μl of 2xTY broth containing the appropriate antibiotic and grown overnight at 37°C with shaking at 300 rpm.
2. 250 μl aliquots of culture were dispensed into 96-well round-bottom plates (Greiner).
3. The cells were pelleted at 2500 rpm for 4 minutes. The plate was inverted to drain the supernatant.
4. The pellet was resuspended in 25 μl of solution I (GTE).
5. 25 μl of fresh 0.2M NaOH and 1% SDS was added and mixed by gently tapping.
6. 25 μl of 5M Acetate, 3M K^+ was added and mixed by tapping gently.
7. The well contents were transferred to a 96-well 0.2 μm filter-bottom plate (Corning #3504).
8. The filter plate was taped on top of a 96-well round-bottom plate (Greiner) containing 100 μl of isopropanol.
9. The plates were centrifuged at 2500 rpm for 2 minutes and the filter plate, containing precipitated protein and bacterial genomic DNA, was discarded.
10. The round-bottom plate was incubated at room temperature for 30 minutes and then spun at 3200 rpm for 20 minutes at room temperature.
11. Supernatant was removed by inverting the plate and dabbing onto tissue.
12. 100 μl of 70% ethanol was added to each well and the plate tapped gently. The plate was then spun at 3200 rpm, for 20 minutes at room temperature.
13. The supernatant was removed by inversion and the pellet dried at room temperature.
14. The DNA was resuspended in 5 μl of $T_{0.1}E$ with RNase (10 μl of 1 mg/ml RNase per 1 ml of $T_{0.1}E$).

2.2.3 Bacterial clone DNA mini-preparations

DNA mini-preparations from 3-5 ml bacterial cultures were performed using the QIAprep spin mini-prep kit (Qiagen) as per manufacturer's instructions. Quantification of DNA was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.2.4 Bacterial clone DNA midi-preparations

DNA midi-preparations from 50 ml bacterial cultures were performed using the Qiagen plasmid midi prep kit as per manufacturer's instructions. Quantification of DNA was performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

2.3 DNA purification

2.3.1 Ethanol precipitation

1. In a 1.5 ml microcentrifuge tube, 0.1 volumes of 3M sodium acetate (pH 5.2) and either 1 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. The DNA was then pelleted in a benchtop microcentrifuge at 13,200 rpm and washed once with 70% ethanol.
4. The pellet was left to air dry and then re-suspended in an appropriate amount of $T_{0.1}E$.

5. The DNA recovery was tested by agarose gel electrophoresis and/or NanoDrop spectrophotometry.

2.3.2 Gel purification

1. The DNA fragment was excised from the agarose gel with a clean scalpel.
2. The gel slice was weighed in a 1.0 ml (1 g) eppendorf tube.
3. The gel slice was then purified using a Qiaquick Gel Extraction Kit™ (Qiagen) according to the manufacturer's instructions.
4. The DNA recovery was tested by agarose gel electrophoresis and/or NanoDrop spectrophotometry.

2.3.3 Exonuclease/Shrimp Alkaline Phosphatase (ExoSAP) purification of PCR products

1. A premix sufficient for the number of planned reactions was prepared allowing for a 1X reaction mix once the PCR reaction was added (usually a 15 μl PCR reaction volume).
2. The final reaction contained 1X reaction buffer (SAP Buffer), 1X Dilution buffer, 1 unit/μl of Shrimp Alkaline Phosphatase (USB) and 1 unit/μl exonuclease I (USB).
3. The mixture was incubated at 37°C for 30 minutes, followed by denaturation of the enzymes at 80°C for 15 minutes.

2.4 Clone resources

2.4.1 Bacterial artificial chromosome (BAC) libraries

Table II-1. Whole genome BAC library details.

Species	*Ornithorhynchus anatinus*	*Macropus eugenii*	*Monodelphis domestica*	*Gallus gallus*	*Mus spretus*
Common name	Duck-billed platypus	Tammar wallaby	Grey short-tailed opossum	White leghorn chicken	Algerian mouse
Library source	Clemson University Genomics Institute (CUGI)	Arizona Genomics Institute (AGI)	Benaroya Research Institute at Virginia Mason	Wageningen	Children's Hospital Oakland Research Institute (CHORI)
Constructed by	Jeff Tomkins	M. Luo	Andrew Stuart	Richard Crooijmans	Baoli Zhu
Supplied by	CUGI	AGI	BACPAC Resources	Geneservices Ltd.	BACPAC Resources
Number of clones in library	230,400	239,616	364,800	49,920	165,888
Number of gridded filters	12	13	21	4	11
Average insert size (kb)	143	166	175	134	181
Genome coverage	10.8x	11.36x	15x	5.5x	11.1x
Cloning site	*Hin*dIII	*Hin*dIII	*Eco*RI	*Hin*dIII	*Eco*RI
Cloning vector	pCUGIBAC1	pCUGIBAC1	pCC1BAC	pECBAC1	pTARBAC2.1
Library code	OA_Bb	ME_KBa	VMRC-18	WAG	CHORI-35

2.5 Cloning

2.5.1 pGEM T-Easy cloning

1. 3 µl of purified PCR products were A-tailed using 1mM dATP and *Taq* polymerase (Applied Biosystems Amplitaq) in a 10 µl reaction buffered with NEB.
2. A-tailing reactions were incubated on a thermal cycler block at 70°C for 30 minutes.

3. 10 µl ligation reactions were performed containing 5 units of T4 DNA ligase (Roche), 5 ng pGEM T-Easy vector, 1X ligation buffer (Roche) and 2 µl A-tailed PCR product or appropriate controls. Reactions were mixed and incubated overnight at 4°C.

2.5.2 Gateway® cloning

Gateway® cloning (by recombination) was performed as described by the manufacturer (Invitrogen) except for the following modifications.

2.5.2.1 Creating Gateway® Entry (pENTR) clones

The donor vector (pDONR™223, kind gift from James Hartley) was used to generate all pENTR™ clones. Primers used to amplify sequence for cloning were synthesised with the following 5' adaptor (*att*B) sequences to increase the specificity of cloning orientation:

Oligo_1 5'-AAAGTTGGCATG<specific primer sequence>-3'

Oligo_2 5'-GAAAGTTGGGTA< specific primer sequence>-3'

To introduce full-length *att*B recombination sites into the PCR product a second round of PCR was performed using the stpDONR223.att.E primers:

Oligo_1 5'-GGGGACAACTTTGTACAAAAAAGTTGGCATG-3'

Oligo_2 5'-GGGGACAACTTTGTACAAGAAAGTTGGGTA-3'

1. The pDONR™223 plasmid in host *E. coli* DB3.1 cells was cultured in 5 ml LB broth containing Specintomycin (50 µg/ml).
2. Following overnight growth at 37°C with shaking at 280 rpm plasmid DNA was extracted (see section 2.2.3).
3. The sequences to be cloned were amplified in a 25 µl PCR reaction containing 1x KOD buffer (Novagen), 200 µM dNTPs, 1mM $MgSO_4$, 0.25

units KOD Hot-Start DNA polymerase, 200 nM sequence specific primers with *att*B adaptors (see above) and 50 ng DNA.

4. The amplifications were performed with the cycling profile: 94°C for 2 minutes (to activate the Hot-start KOD polymerase), followed by 35 cycles at 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 3 minutes, and finally followed by 1 extension cycle at 68°C for 5 minutes.
5. One microlitre of the above reaction product was diluted in 100 µl DDW and 5 µl used in a second-round KOD PCR reaction, set-up as above except the use of stpDONR223.att.E primers (see above).
6. Amplification of this second-round PCR utilised the cycling profile: 94°C for 2 minutes, followed by 5 cycles at 94°C for 15 seconds, 45°C for 30 seconds and 68°C for 3 minutes. This was followed by 20 cycles at 94°C for 15 seconds, 55°C for 30 seconds and 68°C for 3 minutes and finally followed by 1 extension cycle at 68°C for 5 minutes.
7. PCR products were loaded into wide-wells of a 1% agarose gel. Following electrophoresis (section 2.1.3) bands were excised and gel purified (QIAquick, Qiagen).
8. 10 µl BP reactions contained 1X BP3 buffer (see solutions), 1 µl BP ClonaseII enzyme, 50 ng *att*B PCR product and 150 ng pDONR223 vector DNA.
9. BP reactions were incubated overnight at 25°C in a thermal cycler (MJ Research) and terminated by the addition of Proteinase K (2 µg) and incubation at 37°C for 10 minutes.
10. Microtitre plate transformation of *E. coli* Mach1™ cells (Invitrogen) were performed as described in section 2.7.1.
11. pENTR clones were verified by sequencing (see section 2.13).

2.5.2.2 Creating Gateway expression clones

For optimal LR recombination between pENTR clones and destination vectors the pENTR clones were linearised with an appropriate restriction endonuclease (see section 2.1.5.1).

1. LR reactions containing 75 ng destination plasmid DNA, 150 ng linear pENTR plasmid DNA and 1 µl LR ClonaseII enzyme were made up to 10 µl with TE (pH 8.0). Positive (pENTR^{TM}-gus) and negative (DDW) controls were also performed.
2. LR reactions were performed in the wells of an ABgene 96-well plate and incubated overnight at 25°C in a thermal cycler (MJ Research). Reactions were terminated by the addition of Proteinase K (2 µg) and incubation at 37°C for 10 minutes.
3. Microtitre plate transformation of *E. coli* Mach1^{TM} cells (Invitrogen) were performed as described in section 2.7.1.

2.6 Making chemically competent cells

1. A single colony of the *E. coli*, T1 phage-resistant, Mach1^{TM} strain (Invitrogen) was picked into 5 ml LB broth for overnight growth at 37°C with shaking at 280rpm.
2. 1 ml of overnight culture was used to inoculate 110 ml LB broth in a 500ml conical flask and incubated at 37°C with shaking at 280 rpm.
3. After 1hr 45min a 1 ml aliquot of the culture was taken and an OD_{550nm} measured. The desired OD_{550nm} of 0.48 was generally achieved in less than 2 hours with Mach1^{TM} cells (doubling time of approximately 50min).
4. 25 ml of culture was added to pre-chilled 50 ml Falcon tubes and further chilled on ice for 15 minutes.

5. Cells were pelleted by centrifugation at 2500rpm in a Beckman J6-MC centrifuge cooled to 4°C.
6. Supernatant was discarded and excess media dabbed onto absorbent tissue paper.
7. 4 ml of cold (4°C) TfbI was added to the cell pellets on ice and the pellets re-suspended by vigorous tapping of the tubes.
8. Following a 15 minute incubation on ice the cells were re-pelleted at 2500rpm, 4°C for 10 minutes.
9. Supernatant was discarded and the pellets re-suspended in 1 ml cold TfbII solution added using pre-chilled 1 ml pipette tips.
10. Cells were again incubated on ice for 15 minutes before taking 250 µl and 100 µl aliquots into pre-frozen 1.5 ml eppendorf tubes.
11. Tubes were rapidly frozen in an ethanol/dry ice bath for storage at -70°C.

2.7 Transformation

1. 0.5 µl of ligation reactions were added to the wells of an ABgene microtitre plate, frozen at -20°C and transferred to a cold block (Stratagene).
2. A 250 µl or 100 µl aliquot of competent Mach1™ cells was transferred from a -70°C freezer to a 1.5ml cold block (Stratagene).
3. After 2 minutes the cells were flick mixed and 10 µl added to each of the ligation reactions and controls.
4. Cells were kept cold for 20 minutes before heat shocking at 42°C for 50 seconds in a pre-heated thermal cycling block (MJ Research).
5. Heat shocked cells were replaced on the cold block for 2 minutes before adding 90 µl of LB broth.

6. Cells were incubated at 37°C for 1.5 hours without shaking.
7. The entire transformation was plated onto blue/white selective agar plates and incubated overnight at 37°C. Colonies generally containing inserts (white) and those generally without (blue) were scored.

2.7.1 Microtitre plate transformation

Steps 1 to 6 above were performed.

1. Large square 48-well Bioassay plates (Genetix) were poured with 250 ml LB agar containing appropriate antibiotic selection.
2. 90 µl and 10 µl aliquots of transformed Mach1™ cells were plated and spread onto the plates using 2-4 roll-and-grow plating beads (Q-biogene) per well.
3. Plates were left to dry then inverted and incubated at 37°C overnight.

2.8 Tissue Culture

2.8.1 Resuscitating frozen human Caucasian hepatocyte carcinoma (HepG2) cells

1. A Nunc vial containing a frozen aliquot of HepG2 cells (ECACC Number 85011430; CB number 04A014) was taken from liquid nitrogen and defrosted at 37°C.
2. A few drops of pre-warmed (37°C) EMEM plus (see solutions and media) was added to the Nunc vial and the re-suspended cells transferred to a 15 ml Falcon tube. An additional 12 ml EMEM plus was added to this tube.
3. Centrifugation was performed at 1200 rpm for 5 minutes to pellet the cells.

4. The supernatant (containing freeze media) was poured off into 1% Virkon solution.
5. Cells were resuspended by pipetting in 5 ml EMEM plus media and the cells transferred to a 25 cm^2 flask for overnight growth at 37°C and 5% CO2.

2.8.2 Splitting adherent HepG2 cells

When a monolayer of cells was observed covering more than 80% of a 75cm^2 flask (approximately 1x10^7 cells) cells were split according to the following procedure. Note that HepG2 cells grow relatively slowly in islands. Cells in a 75cm^2 flask typically require splitting every 5 or 6 days.

1. EMEM plus media was aspirated off into 1% Virkon and the cells washed twice with 10 ml PBS.
2. 3 ml of pre-warmed trypsin, 0.5% EDTA solution was added to the cell monolayer, poured off and repeated.
3. The flask was incubated at 37°C for two minutes until the cells could be seen flowing down the flask bottom on tapping.
4. 10 ml of pre-warmed EMEM plus media was added to the cells with repeated pipetting to resuspend the cells.
5. To 13 ml of EMEM plus in one flask and 14 ml in a second was added 2 ml and 1 ml of cell suspension respectively. These flasks were labelled with cell type (HepG2), passage number, cell dilution (e.g. 1 in 5 and 1 in 10) and date.
6. The flasks were incubated in a humidified 37°C incubator with 5% CO_2.

2.8.3 Freezing cells for storage

1. Steps 1 to 4 of the splitting procedure above were performed and the resuspended cells transferred to a 50 ml Falcon tube.

2. Cells were pelleted by centrifugation at 1200 rpm for 5 minutes and the media poured off into 1% Virkon solution.
3. Cells were washed with 10 ml PBS, re-pelleted and the solution poured off.
4. Cells were resuspended in 1 ml freeze media (FBS and 10% DMSO).
5. 550 µl of cells were added to labelled cryo vials (Nunc), placed in a cryo 1°C freezing container (Nalgene, Cat. No. 5100-0001) and frozen at -70°C.
6. Once frozen the cells were transferred into liquid nitrogen for long-term storage.

2.9 Transient transfection of HepG2 cells

Steps 1 to 4 of the splitting procedure (2.8.2) were performed.

Cells were counted in the 25 x 25 gridded area (0.0025mm^2) of a haemacytometer to determine cell concentration. For example:

Cell count	Cell concentration (cells/ml)	Required dilution
66	66x10^4	1 in 10 (2 ml in 20 ml)
100	100x10^4	1 in 15 (1.32 ml in 20 ml)
130	130x10^4	1 in 20 (1 ml in 20 ml)

1. Per 96-well plate to transfect a pre-mix sufficient for 140 wells was prepared containing 560 µl EMEM (without additives) and 31.5 µl GeneJuice transfection reagent (Novagen 70967). This pre-mix was incubated for 5 minutes at room temperature.
2. 21.1 µl of the pre-mix above was added to each of the 24 wells containing 400 ng pGL3 test-construct and 8 ng pRL-CMV co-reporter plasmid DNA, mixed and incubated for 5 minutes.

3. An appropriate volume (see table above) of pre-warmed EMEM plus media was added to a 50 ml tube (Falcon). A volume of cells to give a final concentration of 6.6 x 10^4 cells/ml was added to the tube and mixed.
4. Cells were poured into a sterile multi-channel chamber and 150 µl (1 x 10^4 cells) pipetted into each well of a 96-well plate (Falcon #3072).
5. 6.2 µl of the DNA/GeneJuice complex (from step 1) was added to the cells and mixed by pipetting.
6. The 96-well plate was placed into a plastic box (containing water in circular Petri dishes to maintain humidity) and incubated at 37°C with 5% CO_2 for 48 hours. Cells should not be more than 95% confluent prior to dual luciferase reporter assays.

2.10 Dual luciferase reporter assays

All assays were carried out using the Dual-Luciferase Reporter Assay Kit (Promega #E1960). Approximately 6 ml reagents (LARII and Stop & Glo) were required for each 96-well plate to be assayed, 3 ml for the plate and 3ml for the luminometer (Berthold LB96V) injection lines.

1. Media from each well of a 96-well plate was aspirated into 1% Virkon solution.
2. Cells were washed with 250 µl PBS.
3. 23 µl 1 X passive lysis buffer (PLB – Promega #E1941) was added to the cells followed by incubation at room temperature for 1 hour with shaking (The Belly Dancer, Stovall Life Science, Inc).
4. 20 µl of the cell lysates were transferred to a white 96-well PE Optiplate (Perkin Elmer #6005290).

5. Firefly and renilla luciferase levels were assayed using a luminometer (Berthold LB96V) equipped with dual injectors, one for each of the two luciferase substrates. The injectors were programmed to dispense 30 µl of luciferase assay reagent II (LARII) and 30 µl Stop & Glo reagent. Each injection was followed by a 1.8 second delay and a 10 second measurement time.
6. Data was collected using the WinGlow (Berthold Technologies) software package and analysed in Microsoft® Office excel.

2.11 Library screening

2.11.1 PCR radiolabelling of STSs

DNA probes were radiolabelled by PCR (Feinberg and Vogelstein. 1983, Hodgson and Fisk. 1987).

1. The required fragment was amplified from either genomic DNA or cDNA as appropriate.
2. The fragments were separated on a 2.5% agarose gel, cut out and transferred to a 0.5 ml microcentrifuge tube containing 100 µl of sterile water. The DNA was allowed to diffuse out of the gel slice (at least one hour).
3. Using Guy's buffer, 9.5 µl reactions were set up containing the required primers, 2.5 µl of the liquid surrounding the gel slice, nucleotides (except dCTP) and *Taq* (Applied Biosystems) DNA polymerase. A single drop of mineral oil was placed on top of the reaction mixture.
4. 0.5 µl of ^{32}P-dCTP (3000 Ci/mol, Amersham Pharmacia Biotech) was added.
5. PCR was performed in a DNA Thermal Cycler (Perkin Elmer) under the following cycling profile, 94°C for 5 minutes, 25 cycles of 93°C for 30

seconds, 55°C for 30 seconds, 72°C for 30 seconds and 1 cycle of 72°C for 5 minutes.

6. After completion, the probes were denatured in the thermal cycler at 99°C for 5 minutes and then snap chilled in ice water.
7. The probes were then added to the hybridisation mix.

2.11.2 Screening of library filters by hybridisation of PCR-labelled probes

High-density gridded library filters of mouse, wallaby, opossum, platypus and chicken BAC clones were imported (Table II-1). Filters were screened as follows:

1. STSs were radiolabelled as described above.
2. Up to 13 (22x22 cm) filters were sequentially placed in a 22x22x5 cm sandwich box with sufficient hybridisation buffer to cover the filters. A plastic sheet, cut to size, was placed on top to reduce evaporation. The filters were pre-hybridised at 65°C for 2 hours with shaking at 50 rpm (Innova 4000 orbital shaking incubator, New Brunswick Scientific).
3. The filters were removed and the denatured probe was added to hybridisation solution in the box and mixed.
4. The filters were added back into the box and carefully submerged under the hybridisation mix. The plastic sheet was replaced on top.
5. After hybridisation overnight at 65°C and 50 rpm shaking, the filters were washed by rinsing twice in 2X SSC at room temperature for 5 minutes. The filters were then washed twice in 0.5X SSC and 1% N-lauroyl-sarcosine at 65°C for 30 minutes, before rinsing twice in 0.2X SSC at room temperature for 5 minutes.

6. The washed filters were wrapped in Saran wrap (Dow Chemical Co.) and exposed to pre-flashed Fuji Medical X-ray film (036010) (or equivalent) overnight. If longer exposure was required the wrapped filters were sandwiched between intensifying screens and stored at –70°C.
7. Occasionally filters were re-washed to 0.2X SSC with 1% N-lauroyl-sarcosine at 65°C for 30 minutes if required (e.g. to remove high background signals).
8. The autoradiographs were developed and labelled with the name of the filter and the data entered into implementations of ACeDB.

Positive BACs resulting from this screening were received from distribution centres (Table II-1) in agar stabs and subsequently inoculated into LB broth containing 7.5% glycerol and chloramphenicol. Four identical aliquots of each BAC were stored at -70°C in 96-well microtitre plates, representing Archive, Backup, Working and Gridding copies. Filters corresponding to region-specific subsets of bacterial clones were gridded by the Sanger Institute clone resource group. These "polygrid" filters were screened as above except that (pre-)hybridisations were performed in 15 ml tubes (Falcon) and only one probe was screened against one filter to establish the STS content of arrayed BACs. For all other BAC clone experiments the Working copy was used.

2.12 Landmark production

2.12.1 Primer design

Primers were designed using the web-based Primer3 program (Rozen and Skaletsky. 2000) (http://www.genome.wi.mit.edu/genome_software/other/primer3.html). Additional primers for amplification and subsequent cloning of full-length cDNA were designed using the perl script Expresso (Dave Beare). For cloning of PCR

products *att*B adaptor sequences (Gateway®) or restriction endonuclease recognition sites were added to the 5' end of oligonucleotide primers.

2.12.2 Primer synthesis

1. Primers were synthesised at the Sanger Institute by David Frazer and Di Gibson or externally by Sigma-Genosys (Haverhill, UK). Primer concentrations were supplied in both cases.
2. Primers were stored at –20°C and working dilutions for PCR prepared at 100ng/µl for each primer in pairs.
3. The primers were tested at three different annealing temperatures, 55°C, 60°C and 65°C, using the standard cycling on thermal cyclers to establish optimal PCR conditions.

2.12.3 Primer sequences

All primer sequences used are available in the Appendix B (CD at the back of this thesis).

2.13 Plasmid and PCR product sequencing

All plasmid end-sequencing or PCR product sequencing was performed by the Sanger Institute Faculty Small Sequencing Projects (FSSP) team. Sequence-ready reactions were supplied containing 5 µM primer, 5-100 ng purified DNA (depending on size of PCR product or plasmid) in a total volume of 7 µl.

2.14 Bacterial clone fingerprinting

*Hin*dIII fingerprinting of bacterial clones was performed using the standard protocol below (Marra et al. 1997).

2.14.1 Restriction endonuclease digestion

1. Bacterial clones were micro-prepped as described in section 2.2.2 above.
2. 2.6 μl of water, 0.9 μl of NEB buffer 2 and 20 units of *Hin*dIII (NEB) were added to each well, mixed by gentle tapping and then the plate centrifuged up to 1000 rpm to collect the contents.
3. The plate was incubated at 37°C for 2 hours.
4. The reaction was terminated by addition of 2 μl of 6X Dye Buffer II and the plate centrifuged up to 1000 rpm to collect the contents.

2.14.2 Gel preparation and loading

1. A 1% gel mix was prepared using 450 ml of 1X TAE and 4.5 g agarose and poured at 4°C. A 121-well comb was placed in the gel and allowed to set for 45 minutes. The comb was then removed.
2. 3-4 litres of 1X TAE was added to the gel tank.
3. 0.8 μl of the marker (Promega, DG1931) was loaded in the first well and then in every subsequent fifth well.
4. 2.0 μl of each sample was then loaded into the empty wells between markers.
5. The gel was run in a coldroom (4°C) at 85 V for 16 hours.

2.14.3 Gel staining

1. The gel was trimmed to ~19 cm and stained with vistra green stain for 45 minutes. The gel was covered whilst staining to prevent light degradation of the vistra green.
2. The gel was then rinsed with 0.5 litres of deionised water. The gel was visualised and the image recorded using a Molecular Dynamics scanner.

2.15 Computational analysis

2.15.1 ACeDB

For each species an implementation of ACeDB (http://www.acedb.org) was used to track mapping, sequencing and analysis data:

Chicken 'gallusace'

Platypus 'platypace'

Wallaby 'wallabase'

These databases are curated by me but the underlying code maintained by Carol Scott.

For other species and/or test data my own 'Imprintace' database was used.

2.15.2 Sequence analysis and annotation

Clones selected for sequencing are uploaded into ChromoView using the perl script tpf2oracle. The ChromoView web interface links to gull to track sequencing

progress and displays pre-computed sequence overlaps. Finished sequence assemblies, from ChromoView, are exported as an AGP file for sequence analysis.

Finished BAC sequences were analysed in the standard HAVANA (Human And Vertebrate Analysis aNd Annotation) pipeline (http://www.sanger.ac.uk/HGP/havana/). The sequences were analysed for repeats using RepeatMasker (Smit, AFA., Hubley, R. and Green, P., "RepeatMasker" at http://www.repeatmasker.org) and tandem repeats finder (trf, Benson. 1999). The repeat masked sequences were subsequently screened in similarity searches against the public domain DNA and protein databases using the BLAST suite of programs. The exon and gene prediction programs, Genscan (Burge and Karlin. 1997), Fgenesh (Solovyev et al. 1994), tRNAscan (Fichant and Burks. 1991, Lowe and Eddy. 1997) and Eponine TSS (Down and Hubbard. 2002) were used to predict possible gene structures. The unmasked sequence was used in C+G content analysis and prediction of CpG islands. The completed sequences were visualised in the DNA map display of ACeDB ('otterlace', Searle et al. 2004) and this database used for the manual annotation of gene structures.

2.15.3 Multi-species comparative sequence analysis

Web based multi-species comparative sequence analyses were performed using the zPicture server (Ovcharenko et al. 2004a) (http://zpicture.dcode.org).

With assistance from Carol Scott and Paul Bevan, a SAVOIR consortium webpage showing comparative sequence-ready maps for each region and species being studied was implemented (http://www.sanger.ac.uk/PostGenomics/epicomp). Clone map and sequence data was regularly updated using MySQLMan tables (v1.09, Gossamer Threads Inc).

2.15.4 BLAST and BLAT

Web based BLAST analyses were performed at NCBI (http://www.ncbi.nlm.nih.gov/BLAST), EnsEMBL (http://www.ensembl.org/) or Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast). A local UNIX installation of BLASTN was run on the command line as follows. A BLASTN database of FASTA format sequences was created using the 'pressdb <seq.fa>' command. The command 'blastn <database> <query.fa> > <output>' was used to redirect the BLASTN output to a file.

BLAT (Kent. 2002) was performed at the UCSC Genome browser (http://genome.ucsc.edu/).

2.15.5 Electronic polymerase chain reaction (ePCR)

To establish the STS content of a sequence an in-house implementation of the program electronic PCR (ePCR, Schuler. 1997) was performed. Usage:

e-PCR STS_database Sequence [options]

Primer sequences used to create the STS database were exported from ACeDB and formatted in the text editor 'emacs' (http://www.gnu.org/software/emacs). The database is of the format:

STSname Oligo_1(5' ->3') Oligo_2(5'->3') Amplicon_size(bp)

The interrogated sequence is in FASTA format and ePCR options were:

M=50. The margin of discrepancy between the observed and expected amplicon sizes.

N=0. The number of mismatches allowed.

W=7. The word size

In addition the option –mid was used when the mid-point of the STS match was required and not the range (default).

2.15.6 Perl and EMBOSS scripts

The following perl scripts were generated by Dave Beare (unless stated otherwise) and can be found in the directory /nfs/chr22/gid22/perl/

scf2fa Converts sequence SCF files to FASTA format sequences

Expresso Used for ORF oligo design

MatchReport Batch BLAST and output processing tool (original software written by Luc Smink).

tpf2oracle Submits species specific tile path format to Oracle tracking database (written by James Gilbert)

CpGcount Calculates the frequency of CpG dinucleotides in a sequence

cpgplot Plots CpG rich regions (EMBOSS script)

newcpgreport Predicts CpG islands (EMBOSS script)

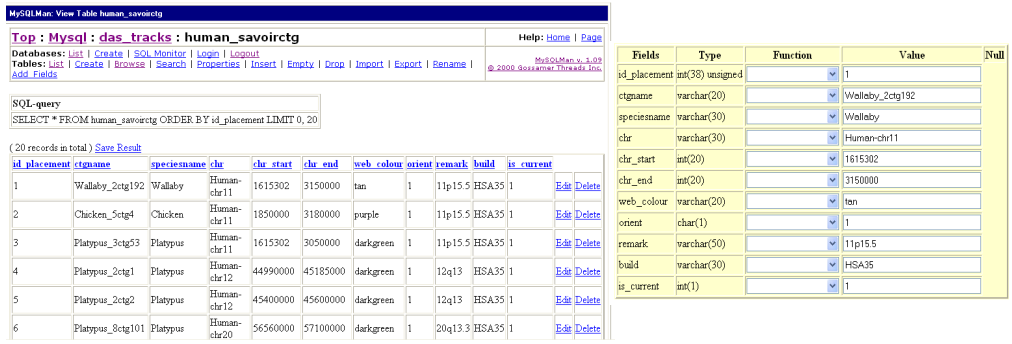
tracey Tool to retrieve data from the internal trace archive

2.15.7 MySQL tables

Updates to the SAVOIR website mapping displays (chapter IV) were made using MySQL tables that were regularly updated using a web-based database manager tool (MySQLMan v1.09, Gossamer Threads Inc). Four tables exist; savoirctg, savoirclone, savoireg and savoirer. The first two describe the mapped contigs and clones, respectively (Figure II.1). The third table (savoireg) contains details of the known EnsEMBL genes and the final table lists ENCODE pilot project regions (ENCODE Project Consortium. 2004). As new mapped clone contigs were generated the savoirctg table was updated with the contig name in the format

<species>_<chr>ctg<FPCid> (e.g. Wallaby_2ctg192 for the wallaby chromosome 2 region orthologous to human 11p15.5). Approximate coordinates of the contigs in the human genome assembly (NCBI Build35) were provided, using the gene content of the vertebrate sequences as a guide. Individual clones within the maps were entered into the savoirclone table with their international clone name, species name, sequencing status, sequence accession number (if available) and approximate position relative to human. Like the contigs, the position of clones was determined by gene content and/or sequence length. In total the MySQL database contains information on 99 BAC clones in 20 contigs across the 9 SAVOIR regions and 5 species.

A



B

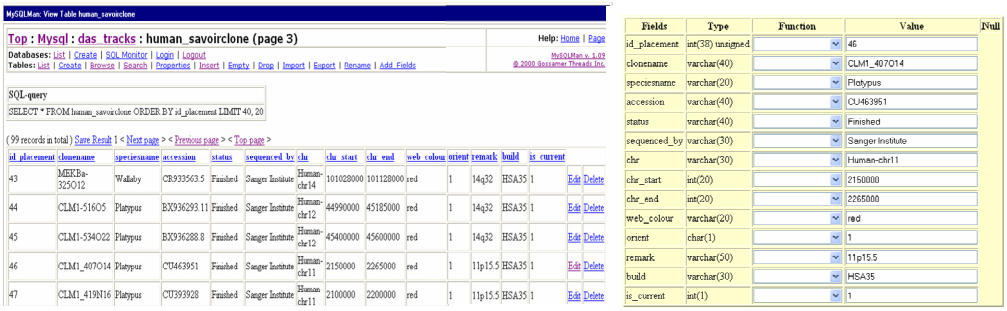


Figure II.1. SAVOIR contig and clone MySQL tables.

Tables containing data for the mapped contigs (A) and sequenced clones (B) are shown. Data is entered manually into the web forms on the right and include features such as contig or clone name, species of origin, orthologous human genomic region, feature coordinate (in the NCBI build 35 assembly of the human genome) and sequencing status of the clone.

2.16 URLs

Table II-2. URLs visited.

Arizona Genomics Institute – BAC orders	http://www.genome.arizona.edu/orders/
Harwell Mouse Imprinting	http://www.mgu.har.mrc.ac.uk
Catalogue of imprinted genes	http://cancer.otago.ac.nz/IGC/Web/home.html
Zebrafish EST BLAST site	http://134.174.23.160/zfBlast/PublicBlast.htm
Fugu genome project	http://fugu.hgmp.mrc.ac.uk
University of California, Santa Cruz (UCSC) genome browser	http://genome.ucsc.edu
Chicken EST project	http://www.chick.umist.ac.uk
CpG island identification	http://www.ebi.ac.uk/cpgplot/
Clemson University Genomics Institute – BAC orders	https://www.genome.clemson.edu/groups/bac/
ClustalW multiple sequence alignment tool	http://www.ebi.ac.uk/clustalw/index.html
EMBOSS	http://emboss.sourceforge.net/
ENCODE	http://www.genome.gov/10005107
EnsEMBL human blastview	http://www.ensembl.org/Homo_sapiens/blastview
EnsEMBL genome browser	http://www.ensembl.org/
Entrez Gene - Online catalogue of gene loci	http://www.ncbi.nlm.nih.gov/LocusLink
IMAGE 3.10b – Fingerprint image analysis	http://www.sanger.ac.uk/Software/Image/
NCBI Basic sequence alignment tool	http://www.ncbi.nlm.nih.gov/BLAST
Online Mendelian Inheritance of Man (OMIM)	http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM
GeneDoc – Multiple sequence alignment editor for windows	http://www.psc.edu/biomed/genedoc/
Geneservice Ltd	http://www.geneservice.co.uk/home/
Pipmaker	http://bio.cse.pse.edu/pipmaker
Primer 3.0	http://www.genome.wi.mit.edu/genome_software/other/primer3.html
Sanger Institute BLAST	http://www.sanger.ac.uk/cgi-bin/blast
The Human Epigenome Project	http://www.epigenome.org
The Epigenome Network of Excellence	http://www.epigenome-noe.net
The ENCODE project	http://www.genome.gov/ENCODE
The SAVOIR consortium	http://www.sanger.ac.uk/PostGenomics/epicomp
Trace archive (NCBI)	http://www.ncbi.nlm.nih.gov/Traces/trace.cgi
zPicture - Comparative sequence analysis tool	http://www.zpicture.dcode.org

2.17 Solutions and media

Table II-3. Solutions and media used

1X TE 10 mM Tris-HCl (pH 8.0) 1 mM EDTA	1X $T_{0.1}E$ 10 mM Tris-HCl (pH 8.0) 0.1 mM EDTA
10X NEB PCR buffer 670 mM Tris-HCl (pH 8.8) 166 mM $(NH_4)_2SO_4$ (enzyme grade) 67 mM MgCl (pH 8.8)	Guys Buffer 500 mM KCl 100 mM Tris pH 8.3 15mM $MgCl_2$
28% Sucrose/cresol red solution 1X $T_{0.1}E$ 28% w/v sucrose 0.008% w/v cresol red	6X Glycerol loading dyes 30% v/v glycerol 0.1% w/v bromophenol blue 0.1% w/v xylene cyanol 5 mM EDTA (pH 7.5)
6X Dye Buffer II 0.25% bromophenol blue 0.25% xylene cyanol 15% Ficoll (Type 400: Pharmacia)	Vistra Green stain For 1 gel: 0.01M Tris HCl 0.0001M EDTA (pH 7.4) 50 µl Vistra green (Amersham)
10X TAE 890mM Tris base 0.05M EDTA 5.71% glacial acetic acid	10X TBE 890 mM Tris base 890 mM Borate 20mM EDTA (pH 8.0)
	LB broth 10 mg/ml bacto-tryptone 5 mg/ml yeast extract 10 mg/ml NaCl (pH 7.4)
Hybridisation buffer 6X SSC Denhardt's solution 1% N-lauroyl-sarcosine 50mM Tris-HCl (pH 7.4) 10% w/v dextran sulphate	2 x TY 16 mg/ml bacto-tryptone 10 mg/ml yeast extract 5 mg/ml NaCl
20X SSC 3M NaCl 0.3M Trisodium citrate	SAP buffer 200 mM Tris HCl (pH 8.0) 100 mM $MgCl_2$

ExoSAP dilution buffer 50mM Tris HCl (pH 8.0)	100X Denhardt's solution 20 mg/ml Ficoll 400-DL 20 mg/ml polyvinylpyrrolidine 40 20 mg/ml BSA (pentax fraction V)
Solution I (GTE) 50 mM glucose 10 mM EDTA 25 mM Tris-HCl (pH 8.0)	Solution II 5M NaOH (0.2M final conc.) 10% SDS (1% final conc.)
Solution III 3M KOaC (pH5.5)	EMEM plus Minimal Essential Medium Eagles including 1% non-essential amino acids (Sigma M5650) 10% Foetal bovine serum 2mM L-Glutamine 100 units/ml penicillin 100µg/ml streptomycin
Antibiotics: Ampicillin 50 mg/ml (final conc. 100 µg/ml) Gentamycin 50 mg/ml (final conc. 7 µg/ml) Kanamycin 10 mg/ml (final conc. 50 µg/ml) Spectinomycin 10 mg/ml (final conc. 100 µg/ml)	1X PBS 137mM NaCl 10mM phosphate 2.7mM KCl pH 7.4
1X BP3 buffer 20mM Tris-Cl (pH7.5) 4mM EDTA 6mM spermidine-HCl 5% glycerol 45mM NaCl	