Chapter 2:

General Materials and Methods

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GENERAL MATERIALS AND METHODS

This chapter describes the materials and methods common to much of the work contained within this thesis, more specific methods can be found within the chapter to which they are unique.

Unless otherwise stated, all materials and reagents were obtained from Sigma.

2.1 MICE

Mice carrying the *bv* mutation were originally obtained from Professor M.S Deol at University College London in 1982. Details of the genetic background were unknown and the mice were maintained at the MRC Institute of Hearing Research in Nottingham on their original background. In 2003 the stock was successfully rederived at the Wellcome Trust Sanger Institute in Cambridgeshire. However, poor breeding performance necessitated the use of IVF using oocytes from the inbred strain CBA/Ca and as a result the *bv* mutation is currently maintained on a 50% CBA/Ca background. In addition, to ensure the survival of the *bv* allele, sperm samples from *bv* affected males have been frozen and are currently stored at the MRC Mammalian Genetics Unit, Harwell, UK.

2.2 DNA PREPARATION FROM TISSUE

Mouse DNA samples were prepared by phenol/chloroform extraction and sodium acetate precipitation. Tissue for DNA extraction was obtained from the tail and pinnae. Tail tissue was removed from the tail bone by cutting down the length of the tail and peeling away the skin. This tissue, together with the pinnae, was cut into fragments of approximately $5mm^2$ and divided between two 1.5ml tubes and one screw-capped microcentrifuge tube (Eppendorf), with the latter snap frozen in liquid nitrogen and later removed for storage at -70°C. During tissue collection from backcross mice, the liver,

spleen, kidneys, heart, lungs and testis were also removed. These were divided between two 2ml cryotubes (Nunc), snap frozen in liquid nitrogen and stored at -70°C.

To the tissue in each of the two 1.5ml microcentrifuge tubes (Eppendorf) containing tail/ear tissue was added 1.0ml tail mixture (100mM EDTA, pH 7.5; 0.5% SDS; 50mM Tris-HCL, pH 7.5; 1.0mg/ml Proteinase K) and they were incubated at 55°C overnight. One of these tubes was then stored long term at 4°C while the other was processed immediately for DNA extraction. After being spun at 14,000 rpm in an Eppendorf 5415C microcentrifuge for three minutes in order to remove undigested hair and tissue, the supernatant was removed to a fresh tube. An equal volume of equilibrated phenol (pH 7.9) was added and gently mixed by inversion for one minute, then centrifuged for a further three minutes at 14,000 rpm and the aqueous layer removed to another fresh tube. This step was repeated twice more, then an equal volume of 24:1 chloroform:isoamyl alcohol (IAA) was added, mixed by inversion for one minute and centrifuged at 14, 000 rpm for three minutes. The aqueous layer was again removed and this step repeated once more. DNA was precipitated with two volumes of 100% ethanol and one tenth volume of 3M sodium acetate, pH 4.8. The precipitate was spooled out using the sealed and hooked end of a glass Pasteur pipette, washed in 70% ethanol and allowed to air-dry before being resuspended in 100µl TE and melted at 65°C for one hour.

2.3 ESTIMATING NUCLEIC ACID CONCENTRATIONS

DNA concentrations of prepared samples were estimated using first a Cecil 2020 spectrophotometer and later an Eppendorf Biophotometer. In both cases optical density (OD) readings were taken at wavelength (λ) 260nm to measure DNA concentration, and λ 280nm to measure protein concentration. The ratio between these two readings was calculated as a means of assessing the purity of the sample, with an $OD_{\lambda 260}/OD_{\lambda 280}$ ratio of 1.8 to 2.0 being considered adequately pure for subsequent PCR reactions. DNA

concentration was calculated on the basis that an $OD_{\lambda 260}$ of 0.1 corresponds to 1μg/μl of double stranded DNA. Samples were generally diluted 1:500 for this measurement, and the final concentrations adjusted to reflect this dilution factor.

2.4 POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a method which allows the amplification of a specific region of DNA (Saiki *et al*; 1985). Two short oligonucleotides, or primers, are designed to match DNA sequence at either end of the region of interest. One of these is designed to complement the + (plus) strand of the DNA molecule, whilst the other matches the – (minus) strand. In the presence of the thermostable enzyme *Taq* polymerase I, derived from the thermophilic bacterium *Thermus aquaticus*, and under the correct conditions, the portion of DNA lying between these two primers is amplified.

The first stage of PCR is a denaturation step at about 94°C in order to melt apart the two strands of the DNA template and so render them accessible for the primers to adhere. Second is an annealing step, carried out at around 55°C, which allows the primers to bind to the section of template DNA they have been designed to complement. Third is an extension phase at 72°C which enables the *Taq* enzyme to synthesise new strands of DNA complementary to the template, beginning at the points where primers have bound. This is followed by further cycles of the three temperature phases, resulting in an exponential increase in the number of copies of the amplified DNA fragment

2.4.1 Design and synthesis of oligonucleotides for PCR

Oligonucleotides were designed using the Primer3 design package hosted by the Whitehead Institute for Biomedical Research at http://frodo.wi.mit.edu/primer3/. Criteria for choosing primers were set as follows: Primer size – minimum 18, optimum 20, maximum 27; Melting point – minimum 57, optimum 60, maximum 63; GC content – minimum 20,

optimum 50, maximum 80. In addition, a GC clamp of one base at the internal end of each primer was preferred and the mispriming library for rodent and simple sequences was used to check for repetitive sequences which might anneal in multiple locations. All the remaining options were left as defaults, including those designed to discard primers with self-complementarity which might form hairpins, and those to check complementarity between primer pairs which could give rise to primer dimers.

Primers were synthesised initially by MWG Biotech (Milton Keynes, Bedfordshire, UK) and later by Sigma-Genosys (Haverhill, Essex, UK). In both cases, primers arrived lyophilised and were diluted according to their supplied optical density readings to a stock concentration of 100μm. Working dilutions were then made to a concentration of 10μm.

2.4.2 General PCR protocols

Throughout the project two different PCR systems were utilised. The first, ABgene Thermoprime Plus PCR ReddyMix™ was used for the majority of PCR reactions carried out. The second, BioLine BIO-X-ACT™ High Fidelity Long DNA Polymerase was used for reactions where a larger fragment was to be amplified or where proofreading capability in the enzyme was thought to be beneficial.

ABGene ReddyMix™ is supplied at 2X final concentration, with the final reaction concentrations as follows: 0.025units/μl Thermoprime Plus DNA Polymerase; 75mM Tris-HCL (pH 8.8 at 25°C); 20mM $(NH_4)_2SO_4$; 0.01% Tween 20; 200μm of each dATP, dCTP, dGTP, dTTP; 1.5mM $MgCl_2$. To this were added forward and reverse primers at a final concentration of around 1mM each, and template DNA at approximately 5ng/μl. The exact concentrations of primers and templates were adjusted to optimise the reaction. The remainder of the reaction volume was made up with sterile

ddH_2O. An example of the volumes used a typical reaction using ABGene ReddyMix™ is shown in Table 2.1.

Stock Solution	**Volume per 10µl reaction (µl)**	**Final reaction concentration**
2X ReddyMix™	5	1X
Forward primer (10µM)	1	1µM
Reverse primer (10µM)	1	1µM
DNA template (25-100ng/µl)	1	2.5-10ng/µl
Sterile ddH_2O	2	-

Table 2.1: Preparation of a 10µl PCR reaction using ABGene ReddyMix™

Bioline BIO-X-ACT™ reactions were comprised of 10X OptiBuffer (constituents not disclosed), with the addition of 200µM each of dATP, dCTP, dGTP, dTTP; 1.1mM $MgCl_2$; 300nM of each forward and reverse primer and 0.5-2ng/µl template DNA. A typical reaction using Bioline BIO-X-ACT™ is shown in Table 2.2.

Stock Solution	**Volume per 50µl reaction (µl)**	**Final reaction concentration**
10X OptiBuffer	5	1X
DNA template (25-100ng/µl)	1	0.5-2ng/µl
Forward primer (10µM)	1.5	300nM
Reverse primer (10µM)	1.5	300nM
dNTP (2mM)	5	200µM
$MgCl_2$ (50mM)	2.2	1.1mM
BIO-X-ACT Long DNA Polymerase (4u/µl)	0.5	0.04u/µl
ddH_20	33.3	-

Table 2.2: Preparation of a 50µl PCR reaction using Bioline BIO-X-ACT™

PCR reactions were performed in ABGene 96-well polypropylene PCR plates, heat-sealed with foil, and run on an M.J. Research PTC-225 Tetrad DNA Engine thermocycler. PCR conditions were adjusted to optimise different reactions but all were based on the standard protocol shown in Table 2.3.

Step	PCR phase	Temperature	Time
1	Initial denaturation	95°C	2 minutes
2	Denaturation	95°C	30 seconds
3	Annealing	2°C below T_M of primers	30 seconds
4	Extension	72°C – ReddyMix™ 68°C – BIO-X-ACT™	1 minute/1Mb of expected product
5	Thermocycling	Repeat steps 2-4, 34 times	–
6	Cooling	4°C	Indefinitely

Table 2.3: PCR conditions used for DNA amplification

2.5 AGAROSE GEL ELECTROPHORESIS

Size determination of DNA fragments was carried out by agarose gel electrophoresis using Hybaid Electro4 gel tanks. Agarose (UltraPURE, GIBCO BRL) was added to 0.5X TBE buffer (44mM Tris, 45mM boric acid, 1mM EDTA, pH 8) at concentrations varying from 2-4% depending on the expected product size. This was melted using a microwave on medium power and ethidium bromide was added at a concentration of 0.4μg/ml in order to allow visualisation of DNA fragments under long-wave ultraviolet light. Gels were cast in perspex gel trays with appropriate combs inserted for the loading of samples. In general, 2% gels were used to resolve fragments ranging from 100-600bp in length. Where higher resolution was required for small fragments or when screening for small differences in product size, 3-4% gels were used. To provide a reference for size and for concentration, DNA molecular weight marker was loaded on each row of samples to be run. Marker VIII (Boehringer Mannheim) was used initially, later being replaced by Hyperladder IV (Bioline). PCR samples from ReddyMix reactions could be loaded directly into agarose gels since the 2X master mix includes a loading buffer. Samples from BIO-X-ACT reactions were prepared for loading by the addition of 6X loading buffer (0.25% bromophenol blue, 12.5mM EDTA,

40% sucrose). Electrophoresis was carried out at 80-150 volts for 1-2 hours depending on the percentage of the gel and the size of products being resolved. Lower voltages were used for lower percentage gels, higher voltages for higher percentage gels. Following electrophoresis gels were visualised on a UV transilluminator and images captured using initially a video capture system (Herolab E.A.S.Y. 429K) and later a digital capture device (SynGene).

2.6 RNA PREPARATION

Preparation of RNA was achieved using TRIzol (Invitrogen), a solution of phenol and guanidine isothiocyanate which maintains the integrity of the RNA, while disrupting cells and dissolving cell components. In the case of mouse and fish tissue, samples were homogenised before the addition of 1ml TRIzol. In the case of ES cells, TRIzol was added directly and homogenisation achieved by passing the lysate through a pipette several times. Samples were incubated at room temperature for 5 minutes to allow full lysis to occur, then 200µl chloroform was added, mixed vigorously by hand for 15 seconds and incubated for a further 2-3 minutes at room temperature. Following centrifugation at no more than 12,000g for 15 minutes at 2 to 8°C, the colourless aqueous upper phase was transferred to a fresh tube. RNA was precipitated from the supernatant by addition of 500µl isopropyl alcohol, mixing and incubation at room temperature for 10 minutes before centrifugation at no more than 12,000g for 15 minutes at 2 to 8°C. The supernatant was then removed and discarded, and the RNA pellet washed with 1ml of 75% ethanol (prepared using RNase-free water). This was mixed by vortexing and centrifuged at no more than 7.500g for 5 minutes at 2-8°C before being allowed to partially air-dry. Resuspension was carried out by the addition of RNase-free water, mixing by pipette and incubation at 55-60°C for 10 minutes. Quantification of RNA was carried out using an Eppendorf Biophotometer with readings being taken at $OD_{\lambda 260}$ and a conversion factor of 1 OD unit being equal to 38µg of single-stranded DNA or RNA used to calculate concentration.

Optionally, the extracted RNA could be treated with DNase in order to remove genomic DNA contamination prior to reverse transcription. In this case, 1 unit of amplification grade DNase I, (Invitrogen) was added for each 1 μg of RNA sample to be treated. To this was added an appropriate volume of 10X DNase I reaction buffer, and the remaining volume made up with DEPC-treated water. Samples were incubated at room temperature for 15 minutes and a one tenth volume of 25mM EDTA was then added to inactivate the enzyme. Heat inactivation was carried out at 65°C for 10 minutes, after which the samples were ready to be used for reverse transcription.

2.7 REVERSE TRANSCRIPTION

Reverse transcription (RT-PCR) utilises the ability of the enzyme reverse transcriptase to synthesise DNA from an RNA template. The resulting complementary DNA (cDNA) fragments represent the population of genes which are expressed in the tissue from which the RNA was extracted. Since the messenger RNA molecules will have been processed and introns excised from the sequence, cDNA can be used to study the transcripts present in a given tissue type.

cDNA was prepared using the SuperScript™ II First-Strand Synthesis System for RT-PCR (Invitrogen). To 5μg of RNA sample was added 1μl 10mM dNTP and 1μl 50mM Oligo$(dT)_{20}$ primers. The reaction volume was made up to 10μl with RNase-free water and the sample incubated at 65°C for five minutes in order to allow annealing of primers to the template RNA, before being placed on ice for at least one minute. A cDNA synthesis mix consisting of 2μl 10X RT buffer, 4μl 25 mM MgCl2, 2μl 0.1M DTT and 1μl RNaseOUT™ (40 U/μl) was made up and added to the RNA/primer mixture. This was gently mixed and collected by brief centrifugation before being incubated ate 42°C for two minutes. At this point 1μl SuperScript™ II RT (50 U/μl) was added and the sample incubated at 42°C for a further 50 minutes before the reaction was terminated at 70°C for 15 minutes and chilled

on ice. RNA was then removed by the addition of 1µl RNase H (2 U/µl) and incubation at 37°C for 20 minutes. The reaction volume was made up to 50µl by the addition of 30µl ddH_2O and 1µl used as the template in subsequent PCR reactions.

2.8 DNA SEQUENCING

Sequencing of DNA samples was carried out using the dideoxy chain termination method described by Sanger *et al.* (1977). In this process, DNA polymerase I is utilised to synthesise complementary copies of a template DNA molecule in the presence of an ample quantity of deoxy-NTPs and a limited amount of differently fluorescence-labelled dideoxy-NTPs. DNA synthesis continues until a dideoxy nucleotide residue is incorporated, at which point the chain is terminated. In a given sequencing reaction, different chains will be terminated at different points, giving a population of DNA molecules carrying fluorescent labels denoting which nucleotide constitutes the terminal residue. When these are separated on a polyacrylamide gel, the differently-sized molecules migrate at different rates, giving a series of fluorescent bands which can be read as the sequence of the DNA molecule.

DNA fragments to be sequenced were obtained by PCR and their specificity verified by agarose gel electrophoresis before proceeding. They were then purified using an AMPure™ PCR Purification kit (Agencourt) which utilises magnetic beads to separate PCR products from remaining reaction ingredients which might disrupt subsequent processes. To each reaction in a 96-well PCR plate was added 1.8X the reaction volume of AMPure™ and the solution mixed thoroughly before being placed onto a SPRIplate96-R magnetic plate. After 5-10 minutes separation time, cleared solution was removed and discarded while the magnetic beads to which PCR products have adhered remained attached to the sides of the well. The beads were washed twice by the addition of 90µl of 70% ethanol and after the removal of the second wash they were allowed to air-dry. Elution was carried out by the addition of 25µl 1X T0.1E (10mM Tris-HCL; 0.1mM EDTA) and the plate

sealed and vortexed before collection by brief centrifugation. The PCR plate was then returned to the magnetic plate and separation of the beads allowed to occur once more, at which point the eluate was removed to a fresh plate. A small amount of the purified product was run out on an agarose gel in order to determine concentration before being used as the template for sequencing reactions.

Sequencing reactions were set up using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Each reaction consisted of 1.5µl Sanger BigDye Dilution buffer (0.3M Tris HCl, pH9; 6mM MgCl2; 10% tetramethylene sulphone), 0.5µl BigDye terminator v3.1, 5-15ng template DNA, 1µM forward or reverse primer, and was made up to a total volume of 9µl with ddH2O. Cycle sequencing was then carried out on an M.J. Research PTC-225 Tetrad DNA Engine thermocycler using the conditions shown in Table 2.4.

Step	**Cycle**	**Temperature**	**Time**
1	Initial denaturation	96°C	30 seconds
2	Denaturation	92°C	15 seconds
3	Annealing	52°C	15 seconds
4	Extension	60°C	2 minutes
5	Thermocycling	Repeat steps 2-4, 44 times	-
6	Cooling	10°C	Indefinitely

Table 2.4: Thermocycling conditions used for sequencing reactions

DNA precipitation was used to purify the amplified samples from the remaining reaction components. This was achieved by the addition of 50µl precipitation mix (100mM sodium acetate in 96% ethanol) and 10µl water. Following centrifugation at 4000rpm and 4°C for 40 minutes, the supernatant was removed and the pellet washed with 100µl cold 70% ethanol. Samples were again centrifuged at 4000rpm and 4°C for 10 minutes, the supernatant was removed and the pellet allowed to dry. They were then loaded onto an ABI 3700 capillary sequencer (Applied Biosystems). Analysis of the resulting

sequence files was carried out initially using the software suite Vector NTI (InforMax) and later the software package Gap4 (Staden).