Chapter 2. Materials and General Methods

2.1 Equipment

2.1.1 25cm^2 cell culture flasks

Bibby Sterilin. Product no. 3103-025.

2.1.2 75cm^2 cell culture flasks

Bibby Sterilin. Product no. 3123-075.

2.1.3 12 well cell culture plates

Bibby Sterilin. Product no. 3815-012.

2.1.4 96 well cell culture plates

Bibby Sterilin. Product no. 612F96.

2.1.5 96 well ELISA plate (high binding)

Bibby Sterilin. Product no. 3801-096.

2.1.6 15 ml tubes

Bibby Sterilin. Product no. 15PPB.

2.1.7 50 ml tubes

Bibby Sterilin. Product no. 36050CPG.

2.1.8 PCR tubes (0.2 ml)

Thermo Life Sciences. Product no. MTP3.

2.1.9 Thermocycler

Biometra Personal Cycler. Anachem.

2.1.10 Microplate reader

Dynex Technologies. Model MRX II.

2.1.11 Horizontal Electrophoresis unit

Anachem. Model H2-SET.

2.1.12 High Performance Ultraviolet Transilluminator

UVP (Ultraviolet Products).

2.1.13 Digital Camera

Kodak DC290 Zoom. Used with Kodak EDAS290.

2.1.14 Gel Imaging Software

Kodak 1D Scientific Imaging System used with Digital Camera (section 2.1.13).

2.1.15 Haemacytometer

Weber. Depth 0.1 mm. 1/400mm^2

2.1.16 Mini-Protean II Vertical Electrophoresis System

Bio-Rad.

2.1.17 Mini Trans-Blot Cell

Bio-Rad. Product no. 170-3930.

2.1.18 Trans-Blot Transfer Medium (Nitrocellulose Membrane)

Bio-Rad. Product no. 160-0115

2.2 Reagents

2.2.1 RPMI 1640 Medium

Sigma. Product no. R-8758.

2.2.2 Alpha MEM Medium

Sigma. Product no. M-8042.

2.2.3 Medium 199 with Hanks Salts

Invitrogen, Paisley, UK.

2.2.4 Alpha MEM Medium (phenol free)

Invitrogen. Product no. 2257-020.

2.2.5 HEPES

Invitrogen, Paisley, UK.

2.2.6 L-Glutamine (200 mM)

Sigma. Product no. G-7513.

2.2.7 Antibiotic/Antimycotic Solution (100 x)

Sigma. Product no. A-5955.

2.2.8 Benzylpenicillin

Glaxo.

2.2.9 Streptomycin

Glaxo.

2.2.10 Fetal Calf Serum (Fetalclone I)

Perbio. Product no. SH30080.2.

2.2.11 Heat inactivated Fetal Calf Serum

PAA Laboratories. A15-144.

2.2.12 Trypsin-EDTA Solution

Sigma. Product no. T-4049.

2.2.13 Supplemented RPMI 1640 Medium

RPMI medium (section 2.2.1) supplemented with 10% Fetal Calf Serum (section 2.2.10), 1% 200mM L-Glutamine solution (section 2.2.6), and 1% Antibiotic/Antimycotic solution (section 2.2.7).

2.2.14 Supplemented Alpha MEM Medium

Alpha MEM medium (section 2.2.2) supplemented with 10% Fetal Calf Serum (section 2.2.10), 1% 200mM L-Glutamine solution (section 2.2.6), and 1% Antibiotic/Antimycotic solution (section 2.2.7).

2.2.15 Supplemented Medium 199

Medium 199 with Hanks Salts (section 2.2.3) supplemented with 25 mM HEPES (section 2.2.5), 2 mM L-glutamine (section 2.2.6), 10% heat inactivated fetal calf serum (section 2.2.11), 100 IU benzylpenicillin (section 2.2.8), and 100 µg/ml streptomycin (section 2.2.9).

2.2.16 Supplemented Alpha MEM Medium (phenol free)

Alpha MEM medium (phenol free) (section 2.2.4) supplemented with 25 mM HEPES (section 2.2.5), 2 mM L-glutamine (section 2.2.6), 10% heat inactivated fetal calf serum (section 2.2.11), 100 IU benzylpenicillin (section 2.2.8), and 100 µg/ml streptomycin (section 2.2.9).

2.2.17 Phosphate Buffered Saline (PBS)

NaCl (8.0 g/L), KCl (0.2 g/L), KH_2HPO_4 (1.44 g/L) in dH_2O. Adjusted to pH 7.2.

2.2.18 Histopaque 1077

Sigma. Product no. 1077-1.

2.2.19 OPG ELISA Sample Buffer

10% skimmed milk (Sainsbury's) in PBS (section 2.2.17).

2.2.20 OPG ELISA Wash Buffer

0.05% Tween 20 in PBS (section 2.2.17).

2.2.21 Polyclonal IgG anti-human OPG antibody

R & D Systems. Product no. AF805.

2.2.22 Biotinylated IgG anti-human OPG antibody

R & D Systems. Product no. BAF805.

2.2.23 Recombinant human OPG/Fc Chimera

R & D Systems. Product no. 805-05-1000.

2.2.24 ExtraAvidin peroxidase.

Sigma. Product no. E-2886.

2.2.25 Substrate Buffer

Guildhay (Biovet). Product no. 0701349.

2.2.26 TMB Chromogen

Guildhay (Biovet). Product no. 0701360.

2.2.27 QuickPrep Micro mRNA Purification Kit

Amersham Biosciences. Product no. 27-9255-01.

2.2.28 First-Strand cDNA Synthesis Kit

Amersham Biosciences. Product no. 27-9261-01.

2.2.29 Ready To Go You Prime First-Strand Beads

Amersham Biosciences. Product no. 27-9264-01.

2.2.30 pd$(N)_6$ Random Hexamer

Amersham Biosciences. Product no. 27-2166-01

2.2.31 Taq DNA Polymerase with PCR Buffer (10x)

Amersham Biosciences. Product no. 27-0799-61.

2.2.32 DNA Polymerisation mix (dNTP) (20mM)

Amersham Biosciences. Product no. 27-2094-01.

2.2.33 Magnesium Chloride ($MgCl_2$)

Fisher Chemicals. Product no. M/0600/53.

2.2.34 Magnesium Chloride Solution (35mM)

A 35 mM $MgCl_2$ (2.2.33) solution was prepared in dH_2O. The solution was then sterilised by autoclaving, and frozen in aliquots at –20°C.

2.2.35 Ready-To-Go PCR Beads

Amersham Biosciences. Product no. 27-9555-01.

2.2.36 Restriction Enzymes: Hind II (10 u/µl), Bcl I (10 u/µl), Bam H1 (10 u/µl), Nhe 1 (10 u/µl), Ava 1 (5 u/µl). Enzyme Buffer (10x).

Sigma. Product no. R-1382, R-8631, R-0260, R-5634, R-3379.

2.2.37 Bovine Serum Albumin (BSA)

Sigma. Product no. A-9056.

2.2.38 Agarose

Appligene oncor. Product no. 130022.

2.2.39 Ethidium Bromide (diluted to 1mg/ml in dH_20)

Sigma. Product no. E-8751.

2.2.40 TAE Buffer (50x)

Tris base (242 g), Glacial Acetic Acid (57.1 ml), 0.5 M EDTA (100 ml). Made up to 1 L with dH_2O. Adjusted to pH 8.

2.2.41 Agarose Running Buffer

TAE Buffer (50x) (section 2.2.40) (6 ml), dH_2O (294 ml).

2.2.42 Agarose Sample Buffer

Sucrose (4 g), Bromphenol blue (2.5 mg), TAE buffer (6 ml) (section 2.2.40). Made up to 10 ml with dH_2O.

2.2.43 100 bp DNA Ladder (0.05 mg)

MBI Fermentas. Product no. SM0241.

2.2.44 Trypan Blue Solution (0.4%)

Sigma. Product no. T-8154.

2.2.45 Hsp60 ELISA Kit

Stressgen Biotechnologies. Product no. EKS 600.

2.2.46 Hsp70 ELISA Kit

Stressgen Biotechnologies. Product no. EKS 700.

2.2.47 Phenylmethylsulphonylflouride (PMSF)

Sigma. Product no. P-7626.

2.2.48 Acrylamide/Bis solution

Acrylamide (30g) and N,N'-Methylenebisacrylamide (0.8g) dissolved in 100 ml dH_2O.

2.2.49 Electrode Buffer

Tris (1.8 g), Glycine (8.64 g), 10% SDS (3 ml) made up to 300 ml with dH_2O.

2.2.50 SDS Sample Buffer

0.5 M Tris/HCl (2 ml), Glycerol (2.5 ml), 10% SDS (2 ml), β-mercaptoethanol (0.2 ml), 0.05% Bromphenol Blue (0.4 ml), dH_2O (8.9 ml).

2.2.51 Precision Plus Protein^{TM} Standards – All Blue

Bio Rad. Product no. 161-0373.

2.2.52 Transfer Buffer

Tris (3.03 g), Glycine (14.40 g), Methanol (200 ml) made up to 1 L with dH_2O. pH 8.1 – 8.4. Transfer buffer used at 4°C.

2.2.53 Tris Buffered Saline (TBS)

Tris (2.85 g), NaCl (29.24 g), dH_2O (900 ml). Adjusted to pH 7.5 and then made up to 1 L with dH_2O

2.2.54 Tween + Tris Buffered Saline (TTBS)

TBS (section 2.2.53) (500ml), Tween 20 (0.25 ml).

2.2.55 Blocking Solution

TBS (section 2.2.53) (100ml), Bovine Serum Albumin (BSA) (1 g).

2.2.56 Antibody Buffer

TTBS (section 2.2.54) (200 ml), BSA (2 g).

2.2.57 Mouse Anti-Hsp60 Monoclonal Antibody

Stressgen Biotechnologies. Product no. SPA-806

2.2.58 Mouse Anti-Hsp70 Monoclonal Antibody

Stressgen Biotechnologies. Product no. SPA-810

2.2.59 Goat Anti-mouse IgG (Whole Molecule) Peroxidase Conjugate

Sigma. Product no. A-5278.

2.2.60 Metal Enhanced DAB Substrate Kit

Pierce. Product no. 34065.

2.2.61 Dynal T-cell Negative Isolation Kit.

Dynal Biotech. Product no. 113.11.

2.2.62 Dynal B-cell Negative Isolation Kit.

Dynal Biotech. Product no. 113.13.

2.2.63 GroEL (recombinant)

Stressgen Biotechnologies. Product no. SPP-610-10.

2.2.64 Recombinant murine sRANKL

Peprotech EC. Product no. 315-11.

2.2.65 Recombinant murine M-CSF

Peprotech EC. Product no. 315-03.

2.2.66 Dexamethasone

Sigma. Product no. D-1756.

2.2.67 Prednisolone

Sigma. Product no. P-6004.

2.2.68 1 α 25 – Dihydroxyvitamin D_3

Sigma. Product no. D-1530.

2.2.69 Polymyxin B Sulfate Salt

Sigma. Product no. P-4932.

2.2.70 LPS (Lipopolysaccharides from E.coli 0127:B8)

Sigma. Product no. L-4516.

2.2.71 Recombinant Human Hsp60

Stressgen Biotechnologies. Product no. NSP-540.

2.2.72 Recombinant Human Hsp70

Stressgen Biotechnologies. Product no. NSP-555.

2.2.73 MTS solution

Promega. Cell titer 96.

2.2.74 Glycine Buffer Solution

Sigma Diagnostics. Product no. 826-3.

2.2.75 Lactate Standard

Sigma Diagnostics. Product no. 826-10.

2.2.76 β-Nicotinamide adenine dinucleotide

Sigma Diagnostics. Product no. 260-110.

2.2.77 LDH standard

Sigma Diagnostics. Product no. 826-6.

2.2.78 TEMED

Sigma. Product no. T-7024.

2.2.79 PHA

Sigma. L-9017

2.2.80 Bafilomycin A1

Sigma. Product no. B-1793.

2.2.81 Methyl-B-cyclodextrin.

Sigma. Product no. C-4555.

2.2.82 Monensin

Sigma. Product no. M-5273

2.2.83 Brefeldin A

Sigma. Product no. B-6542

2.2.84 Methylamine

Sigma. Product no. 180467

2.3 General Methods

2.3.1 MG63 (osteosarcoma) cell line culture

MG63 cells were cultured in supplemented α-MEM medium (section 2.2.14). The cells were seeded at a density of ~2 x 10^5 cells per ml, and were incubated at 37°C in 5% CO_2. Cells were sub-cultured every four days, or when confluent; the media was removed from the cell layer using a pipette, and 1 ml of 0.25 % Trypsin/EDTA solution (section 2.2.12) per 25 cm^2 flask area was pipetted onto the cells. After 1 minute the trypsin was removed from the cell layer using a pipette, and the cells were incubated for 3 minutes at 37°C. Following incubation, the cells were resuspended in 30 ml of supplemented α-MEM (section 2.2.14) per 25 cm^2 flask (section 2.1.1). The resuspended cells (density ~ 2 x 10^5/ml) were then divided into new flasks (10 ml per 25 cm^2 flask) and grown to confluence in the culture conditions previously described in this section.

2.3.2 Giant Cell Tumour (GCT) cell line culture.

GCT cells were cultured in supplemented α-MEM medium (section 2.2.14). The cells were seeded at a density of ~2 x 10^5 cells per ml, and were incubated at 37°C in 5% CO_2. GCT cells were sub-cultured (1:3 ratio) every 8 to 10 days, or when confluent. Sub-culture was carried out as described for MG63 cells (section 2.3.1).

2.3.3 U937 (human monocytic leukaemia) cell line culture

U937 cells were cultured in supplemented RPMI 1640 medium (section 2.2.13) after seeding at a density of ~ 1 x 10^5 cells/ml. The cells were incubated at 37°C, 5% CO_2. Sub-culture was carried out every 3 to 4 days by adding 1 ml of the cell suspension that had been cultured for 3 to 4 days to every 10 ml of new supplemented RPMI 1640 medium (section 2.2.13). The cells were cultured in either 25cm^2 or 75cm^2 tissue culture flasks (section 2.1.2) in the conditions previously described.

2.3.4 Jurkat (human T-cell leukaemia) cell line culture

Jurkat cells were cultured as U937 cells (section 2.3.3).

2.3.5 Isolation and culture of osteoclast precursors from bone marrow

Osteoclast precursors were obtained from 5 to 8 week old Balb/c mice using the methods described in section 2.3.5.1, section 2.3.5.2 and section 2.3.5.3. Guidelines as set by the HO Animals (scientific procedures) Act 1986 were followed for the housing and care of the animals. The environment was maintained at an ambient temperature of 19 to 21°C with air replaced 20 times per hour. There was a daily cycle of 12 h of light and 12 h of darkness and food and water was constantly available.

2.3.5.1 Removal of murine femora and tibiae

A 5 to 8 week old Balb/c mouse was killed with diethyl ether inhalation (schedule 1 killing). The mouse was immersed for 1 minute in 75% IMS to minimise bacterial contamination. An incision was made into the skin surrounding the pelvic area of the mouse, and the skin was pulled back to reveal the hind legs. Using a scalpel blade, the femur was cut below the femoral head. The tibia was cut below the fibula and the knee joint was dislocated. The femora and tibiae were removed and were washed in a small petri-dish containing 5 ml of supplemented Medium 199 (section 2.2.15)

2.3.5.2 Isolation of osteoclast precursors

The marrow cavities of the bones were flushed into a sterile 15 ml centrifuge tube. This was carried out by slowly injecting supplemented Medium 199 (section 2.2.15) into the distal end of the femora and the proximal end of the tibiae using a sterile 25-gauge needle. The marrow cells were disaggregated and suspended in the media using a fine pipette. The suspended cells were then centrifuged at 150 *g* for 4 minutes, and the

supernatant was removed from the cell pellet. The marrow cells were resuspended in 3 ml 0.83% ammonium chloride in 10 mM Tris buffer (pH 7.2), and the cells were incubated for 5 minutes at room temperature (to allow lysis of erythrocytes). Following incubation the cells were centrifuged at 150 *g* for 4 minutes and the supernatant was removed. The cells were resuspended in 3 ml supplemented Medium 199 (section 2.2.15) and centrifuged at 150 *g* for 4 minutes. The supernatant was removed from the cells.

2.3.5.3 Culture of osteoclast precursors

The prepared marrow cells were resuspended in supplemented phenol free α-MEM medium (section 2.2.16) to give a cell density of 1 x 10^6 cells/ml. MCS-F (5 ng/ml) (section 2.2.65) was added to the cells, and the cells were incubated at 37°C, 5% CO_2 for 24 hours prior to use in further experiments.

2.3.6 Isolation and culture of Peripheral Blood Mononuclear Cells (PBMCs)

Informed consent was obtained from blood donors prior to blood collection. Immediately after collection, venous whole blood was placed in heparinized tubes. The blood was transferred to 15 ml centrifuge tubes (2.5 ml in each tube), and was diluted 1:1 in PBS (section 2.2.17). After dilution, 2.5 ml of Histopaque solution (section 2.2.18) was pipetted slowly into the base of each centrifuge tube containing the diluted blood and the tubes were centrifuged at 400 *g* for 30 minutes. A polythene dropping pipette was used to remove the interface layer containing the peripheral blood mononuclear cells (PBMCs). The PBMCs were transferred into a 15 ml centrifuge tube, and PBS (section 2.2.17) was added to the tube to make the volume up to 15 ml. The tube was centrifuged at 300 *g* for 10 minutes, and after centrifugation, the supernatant was carefully removed, leaving the pelleted PBMCs at the base of the tube. The PBMCs were either used directly for mRNA extraction (section), or they were resuspended in supplemented RPMI 1640 media (section 2.2.13) and incubated at 37°C, 5% CO_2 prior to use in cell culture experiments.

2.3.7 Cell counting

Cell counts were carried out using a haemocytometer (section 2.1.15) according to the manufacturer's instructions. Counts to determine cell viability were carried out by diluting the suspended cell sample 1:1 with Trypan Blue (section 2.2.44); cells that took up the blue stain were counted as non-viable.

2.3.8 MTS Assay

For adherent cell types, the culture media was removed from cells that had been cultured in 96 well plates. New media of a type normally used with the cells was added to the plate (100 µl/well), followed by 20 µl of MTS solution (section 2.2.73). For non-adherent cell types, 100 µl/well of the cell suspension was added in triplicate to a 96 well plate for each sample, followed by 20 µl of MTS solution (section 2.2.73).
After MTS addition, the plate was incubated at 37°C for 30 minutes (or until adequate colour had developed). The plate was read at 490 nm using a microplate reader (section 2.1.10).

2.3.9 mRNA extraction from cells

mRNA extraction from cells was carried out using the QuickPrep Micro mRNA Purification Kit (section 2.2.27)

2.3.9.1 Binding of mRNA to Oligo dT cellulose.

Extraction buffer (0.4 ml) was added to the micro centrifuge tube containing the cell or blood sample, and the sample was vortexed to suspend the cells. Elution buffer (0.8 ml) was then added to the sample. A 1 ml aliquot of Oligo (dT) cellulose (cellulose) was

added to another micro centrifuge tube. Both the tube containing the sample and the tube containing the cellulose were centrifuged for 1 minute at 15000 *g*. The supernatant from the cellulose was removed, and 1 ml of cleared homogenate from the centrifuged cell sample was added to the cellulose. The tube containing the cellulose and cleared homogenate was vortexed for 3 minutes and the tube was centrifuged for 10 seconds at 15000 *g*.

2.3.9.2 Washing Steps

After centrifugation, the supernatant was discarded and 1 ml of high salt buffer was added to the cellulose. The cellulose was suspended in the buffer, and the sample was centrifuged for 10 seconds at 15000 *g*. This step was repeated five times using high salt buffer and twice using low salt buffer. A third low salt wash was carried out using the method described above, except during the third wash the cellulose was resuspended in 0.3 ml low salt buffer. This cellulose slurry was transferred to a MicroSpin column fitted in a 1.5 ml centrifuge tube. The slurry was pelleted by centrifugation for 5 seconds at 15000 *g*, the eluent was discarded, and 0.5 ml of low salt buffer was added to the spin column. This wash in the MicroSpin column was repeated a further two times.

2.3.9.3 Elution of the mRNA

After pelleting the cellulose for the third time, the microspin column was placed into a clean 1.5 ml micro centrifuge tube. Pre-warmed (0.2 ml, 65°C) mRNA elution buffer was then pipetted onto the cellulose in the MicroSpin column. The MicroSpin column was centrifuged for 5 seconds at 15000 *g*, and the mRNA containing eluent was placed in a 65°C water bath for 10 minutes. After 10 minutes the eluent was cooled immediately on ice in preparation for the cDNA synthesis step.

2.3.10 cDNA synthesis using the First-Strand cDNA Synthesis Kit

cDNA synthesis was carried out using the First-Strand cDNA Synthesis Kit (section

2.2.28). For each sample the following reaction mix was prepared in an RNAase free tube:

	Quantity
Bulk 1st Strand reaction mix	11µl
DTT Solution	1µl
Primer pd(N)$_6$	1µl

A 20µl volume of the prepared mRNA solution (section 2.3.9.3) was then added to above reaction mix and the sample was incubated for 1 hour at 37°C. Following incubation the sample was either used directly for PCR, or resuspended in 200 µl of 95% ethanol, and stored at –20°C.

2.3.11 cDNA synthesis using Ready To Go You Prime First-Strand Beads

Two cDNA synthesis beads (section 2.2.29) were placed in an RNAase free tube, followed by 33µl of the prepared mRNA solution (section 2.3.9.3), and 1µl of the primer pd(N)$_6$ (section 2.2.30). The sample was then incubated for 1 hour at 37°C. Following incubation the sample was either used directly for PCR, or resuspended in 200 µl of 95% ethanol, and stored at –20°C.

2.3.12 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.3.12.1 Primer Sequences

Hsp 27 (Wang *et al* 1999).

Sense 5'-ATG GCG TGG TGG AGA TCA CC -3'
Anti-sense 5'-CAA AAG AAC ACA CAG GTG GC -3'

Hsp 60 (Wang *et al* 1999).

Sense	5'-ATT CCA GCA ATG ACC ATT GC -3'
Anti-sense	5'-GAG TTA GAA CAT GCC ACC TC -3'

Hsp 70 (Wang *et al* 1999).

Sense	5'-TTC CGT TTC CAG CCC CCA ATC -3'
Anti-sense	5'-CGT TGA GCC CCG CGA TGA CA -3'

Hsp 90α (Wang *et al* 1999).

Sense	5'-AAA AGT TGA AAA GGT GGT TTG -3'
Anti-sense	5'-TAT CAC AGC ATC ACT TAG TA -3'

Hsp 90β (Wang *et al* 1999).

Sense	5'-AGA AGG TTG AGA AGG TGA CAA -3'
Anti-sense	5' –AAG AGT AGA GAG GGA ATG GG -3'

OPG

Sense	5' GTG CGC CCC TTG CCC TGA CC -3'
Anti-sense	5' TGA GCT GTG TTG CCG TTT TAT CCT -3'

RANKL (Menna *et al*, 2000)

Sense	5'-ACT GGA TCC GGA TCA GGA TG - 3'
Anti-sense	5'-AGC TGC GAA GGG GCA CAT GA - 3'

RANKL

Sense	5'- CGG TCT GGA GAG GAA ATC AG - 3'
Anti-sense	5'- CAA AAG AAT TTG CCC CTT CA - 3'

Osteocalcin

Sense	5'- CCA TGA GAG CCC TCA CAC TC - 3'
Anti-sense	5'- ATC CAT AGG CGT GGG AGG T – 3'

Beta Actin

Sense	5'-TGC TAT CCC TGT ACG CCT CT -3'
Anti-sense	5'-AGT ACT TGC GCT CAG GAG GA -3'

TNFα

Sense	5'-GGC TCC AGG CGG TGC TTG TTC -3'
Anti-sense	5'-AGA CGG CGA TGC GGC TGA TGT -3'

CD25

Sense	5'-GCC TAC AAG GAA GGA ACC A-3'
Anti-sense	5'-CCG GCT TCT TAC CAA GAA A-3'

IFN-β

Sense	5'-ATC TAG CAC TGG CTG GAA T-3'
Anti-sense	5'-AGG TTC AGG TCC CCT TAT T-3'

All primers unless otherwise referenced were designed for this work. mRNA sequences were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/), and the online primer design software Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/) was used to select the primer sequences. All primers listed were used at a working concentration of 20 pM.

2.3.12.2 RT-PCR Reaction Mix

The PCR reaction mix was prepared using the following formulation:

	Quantity
PCR Buffer (10x) (2.2.31)	18µl
dNTP Mix (20mM) (section 2.2.32)	4.5µl
MgCl (35mM) (section 2.2.34)	18µl
Taq Polymerase (section 2.2.31)	4.5µl
cDNA solution (section 2.3.11)	18µl
Distilled H_2O	105µl

The PCR reaction mix was then transferred into six PCR tubes (28µl per tube) and 1µl of the required sense and anti-sense primer was added to each tube.

RT-PCR using Ready-To-Go PCR Beads (section 2.2.35)
The following mix was prepared for each set of primers:

	Quantity
PCR bead	1 bead
Water	25µl
cDNA solution (section 2.3.11)	3µl
Sense primer	1µl
Anti-sense primer	1µl

2.3.12.3 PCR cycles

RT-PCR was carried out in a thermocycler (section 2.1.9) using the appropriate PCR cycle for the primers being used.

PCR cycle 1

94°C for 1 minute
92°C for 1 minute
56°C for 1 minute } x 30 cycles
72°C for 1 minute
72°C for 10 minutes

PCR Cycle 2

94°C for 1 minute
92°C for 1 minute
60°C for 1 minute } x 30 cycles
72°C for 1 minute
72°C for 10 minutes

PCR Cycle 3

94°C for 1 minute
92°C for 1 minute
60°C for 1 minute } x 35 cycles
72°C for 1 minute
72°C for 10 minutes

PCR Cycle 4

94°C for 1 minute
92°C for 1 minute
60°C for 1 minute } x 45 cycles
72°C for 1 minute
72°C for 10 minutes

2.3.13 Agarose Electrophoresis

A 2% agarose gel was prepared using the following formulation:

	Quantity
Agarose (section 2.2.38)	0.56 g
TAE Buffer (50x) (section 2.2.40)	0.56 ml
d.H_2O	27 ml

The above mix was heated in a microwave using high power for 40 seconds, 1 µl of 1 mg/ml ethidium bromide (section 2.2.39) was then added, and the gel was poured into a casting chamber (section 2.1.11). The appropriate combs were added and the gel was allowed to set for 30 minutes. After the gel had set, running buffer (section 2.2.41) was poured over the gel until it filled the casting and running chamber. The combs and sides of the casting chamber were removed.

The PCR products were prepared for loading by adding 1µl of agarose sample buffer (section 2.2.42) to 30 µl of PCR product. 10 µl of DNA ladder (section 2.2.43) was added to the well on the far left hand side of each row, and 10 µl of each PCR product were loaded into the designated wells. The samples were run at 200 volts for 30 minutes, or until the bands had run to the required point on the gel. The gels were visualised using a UV illuminator (section 2.1.12) and photographed using a digital imaging system (section 2.1.14).

2.3.14 SDS-PAGE gels

The apparatus used in the preparation and running of the SDS-PAGE gels was the Mini-Protean II system. (section 2.1.16).

2.3.14.1 Denaturing gel preparation

An 8% SDS-PAGE separating gel solution (sufficient for 4 gels) was prepared by mixing 9.4 ml dH_2O, 5.4 ml Acrylamide/Bis solution (section 2.2.48), 5 ml 1.5 M Tris-HCl, and 0.2 ml 10% SDS. The solution was de-gassed for 15 minutes, after which 100 µl of 10% Ammonium Persulphate, and 20 µl of TEMED (section 2.2.78) were added. The solution was pipetted immediately between the glass plates in the casting chamber to a level of 1 cm below the level of the combs. The gels were overlaid with 1 ml of dH_2O, and were left to polymerise at room temperature for 1 hour.

2.3.14.2 Stacking gel preparation

A stacking gel solution (sufficient for 4 gels) was prepared by combining 12.6 ml dH_2O, 2 ml Acrylamide/Bis solution (section 2.2.48), 5 ml 0.5 M Tris-HCl, and 0.2 ml 10% SDS. The solution was de-gassed for 15 minutes, after which 200 µl of 10% Ammonium Persulphate, and 20 µl of TEMED (section 2.2.78) were added. The water that overlaid the polymerised stacking gels was poured off, and the stacking gel solution was pipetted between the plates with the combs in place. The stacking gel was left to polymerise for 1 hour.

2.3.14.3 Running SDS-PAGE gels

The samples were diluted 1:1 in SDS sample buffer (section 2.2.50) and were heated to 80°C for 5 minutes. The gels were placed into the running tank, and electrode buffer

(section 2.2.49) was poured into the tank and between the gels until it was level with the top of the glass plates. The combs were removed from the gels, and the samples (20µl per well) and the selected molecular weight markers (section 2.2.51) were loaded into the designated wells. The gels were run at 200V until the samples had run to the base of the gels.

2.3.15 Western Blotting

The apparatus used in the method described for western blotting was the Mini Trans-Blot Cell (section 2.1.17).

2.3.15.1 Protein transfer

Cell extracts and media samples were run on an SDS gel (section 2.3.14). Sponges (2 per gel), filter paper (3 pieces per gel) and the nitrocellulose membrane were cut to size and soaked in transfer buffer (section 2.2.52) at 4°C for 30 minutes.
After the SDS gel had run, the stacking gel was removed from the separating gel. The items were assembled in the transfer chamber from the black panel upwards in the order of a sponge, 2 pieces of filter paper, the separating gel, the nitrocellulose membrane, 1 piece of filter paper and a sponge. The transfer chamber was closed and placed into the running chamber. An ice unit was added to the running chamber, and the chamber was filled with transfer buffer (section 2.2.52). The transfer was carried out at 100V for 60 minutes. After 60 minutes the nitrocellulose membrane was carefully removed from the chamber, and was placed in blocking solution (section 2.2.55) prior to immunostaining.

2.3.15.2 Immunostaining of Western Blots

Following protein transfer (section 2.3.15.1) the nitrocellulose membrane was placed in blocking solution (section 2.2.55) for 1 hour at room temperature. After 1 hour the blocking solution was removed and the first antibody, diluted to the required concentration in antibody buffer (section 2.2.56), was added to the membrane. The membrane was incubated for one hour at room temperature. Following incubation with

the first antibody the membrane was washed three times with TTBS (section 2.2.54) for 5 minutes per wash. The second antibody (peroxidase conjugated, and specific to the first) was diluted 1 in 1000 in antibody buffer (section 2.2.56) and was added to the membrane. The membrane was than incubated for a further 1 hour at room temperature. Following incubation, the membrane was washed three times with TTBS (section 2.2.54) for 5 minutes per wash. After washing, metal enhanced DAB substrate (section 2.2.60) was added to the membrane, and the membrane was incubated at room temperature until bands appeared. The reaction was stopped by removing the DAB substrate and washing the membrane in dH_2O.

2.3.16 Quantification of OPG using ELISA

Goat anti-human OPG (section 2.2.21) (200ng/ml in PBS) was adsorbed (100 µl/well) onto a 96 well plate (section 2.1.5). The plate was incubated overnight at 4°C. After overnight incubation, the plate was blocked using sample buffer (section 2.2.19) (200 µl/well), and incubated for 1 hour at room temperature. Following incubation the plate was washed three times using wash buffer (200 µl/well) (section 2.2.20). The standards (doubling dilutions of rhOPG/Fc Chimera (section 2.2.23) from 100 ng/ml to 0.781 ng/ml in sample buffer), and the samples were added in duplicate to the plate (100 µl/well), and the plate was incubated at 4°C for 1 hour. Following incubation the plate was washed three times using 200µl/well of wash buffer (section 2.2.20). Biotinylated goat anti-human OPG (section 2.2.22) (200 ng/ml in sample buffer) was added to the plate (100 µl/well) and the plate was incubated for 1 hour at 4°C. After incubation, the plate was washed three times using wash buffer (200 µl/well) (section 2.2.20), and a 1 in 1000 dilution of avidin peroxidase (section 2.2.24) in sample buffer was added to the plate (100 µl/well). The plate was incubated for 1 hour at 4°C, and after incubation the plate washed three times using 200µl/well of wash buffer (section 2.2.20). A 2 in 10 dilution of TMB (section 2.2.26) in substrate buffer (section 2.2.25) was added to the plate (100µl/well) and the plate was incubated for 15 minutes at 25°C. After incubation, 50µl of 1M phosphoric acid was added to each well to stop the reaction. The plate was read at 450nm using a microplate reader (section 2.1.10).

2.3.17 Quantification of Hsp60 using ELISA

Quantification of Hsp60 was carried out using an Hsp60 ELISA kit (section 2.2.45) according to the method described in the manufacturer's instructions. In summary, samples (100 µl/well, in triplicate) and a standard curve of recombinant Hsp60 (in duplicate) were added to the Anti-Hsp60 immunoassay plate provided. The plate was incubated at room temperature for 1 hour. After incubation the plate was washed 6 times with wash buffer (300µl/well). Diluted Anti-Hsp60 (100µl/well) was added to the plate, and the plate was incubated at room temperature for 1 hour. Following incubation, the plate was washed 6 times with wash buffer (300µl/well) and diluted Anti-Goat IgG:HRP conjugate was added to each well (100µl/well). The plate was incubated at room temperature for 30 minutes. After incubation the plate was washed 6 times with wash buffer (300µl/well), and TMB substrate (100µl/well) was added to the plate. The plate was then incubated for 15 minutes at room temperature. The reaction was stopped by adding 100µl Acid Stop Solution to each well. The plate absorbance was measured at 450nm using a microplate reader (section 2.1.10).

2.3.18 Quantification of Hsp70 using ELISA

Quantification of Hsp70 was carried out using an Hsp70 ELISA kit (section 2.2.46) according to the method described in the manufacturer's instructions. In summary, samples (100 µl/well, in triplicate) and a standard curve of recombinant Hsp70 (in duplicate) were added to the Hsp70 immunoassay plate provided, and the plate was incubated at room temperature for 2 hours. After incubation, the plate was washed 6 times with wash buffer (300µl/well). Diluted Biotin Anti-Hsp70 (100µl/well) was added to the plate, and the plate was incubated at room temperature for 1 hour. Following incubation, the plate was washed 6 times with wash buffer (300µl/well) and diluted Avidin-HRP conjugate was added to each well (100µl/well). The plate was incubated at room temperature for 1 hour. After incubation the plate was washed 6 times with wash buffer (300µl/well), TMB substrate (100µl/well) was added to the plate and the plate was incubated for 10 minutes at room temperature. The reaction was stopped by adding

100 µl Acid Stop Solution to each well. The plate absorbance was measured at 450nm using a microplate reader (section 2.1.20).

2.3.19 LDH Assay

A reaction mixture was prepared consisting of 2 ml glycine buffer (section 2.2.74), 2 mM lactate (section 2.2.75), 10 mg NAD (section 2.2.76), and 3.4 ml dH_2O. Media samples (50 µl/well) and cellular samples (50 µl/well) and LDH standards (section 2.2.77) were added to a 96 well plate. The plate was read at 340 nm at 2 minute intervals up to 30 minutes.

2.3.20 Trap activity assay

Two reagents were separately prepared; 2 ml of the first (0.94 g KH+tartrate, 0.6 ml glacial acetic acid, 40 ml dH_2O, 4 M NaOH to give pH 5.5, followed by 80 ml dH_2O) was combined with 8 ml of the second reagent (11.3 mg Naphthol ASBI phosphate, 100 µl methyloxyethanol, 200 µl NP40, 1.7 ml dH_2O) to make the assay reagent.
Culture media was removed from the 96 well plate containing the cells, and 100 µl/well of the assay reagent was added. The plate was incubated at 37°C for 35 mins. Following incubation the reaction was stopped using 0.3 M NaOH (100 µl/well). The plate was read using dual filters of 405 nm and 650 nm. TRAP activity (m/IU) was calculated by mean – blank x 8.38 (extinction coefficient).

2.3.21 Trap Staining

A TRAP staining reagent was prepared (2 ml acetic acid buffer, 4 ml KH+tartrate, 25 mg Naphthol ASBI phosphate, 50 ml dH_2O, 15 mg Fast Garnet) and mixed for 30 seconds. Cells were prepared by washing with PBS (100 µl/well) and 50 µl/well of fixative (95% IMS, 5% acetic acid) was added to the cells, which were incubated for 5 minutes. The fixative was removed and the cells were washed once more with PBS (100 µl/well), and 50 µl/well of the TRAP staining reagent was added to the cells. The cells were incubated for 8 mins, after which the reagent was removed and 100µl/well of PBS was added to the stained cells.

2.3.22 Statistical Analysis

Statistical analysis on three or more matched groups was carried out using a one-way ANOVA. The Bonferroni post-test was using following the one-way ANOVA. In experiments where there were only two matched groups two-tailed unpaired t-tests were used to determine if there was a statistically significant difference.