Chapter 2

Materials and methods

2.1: Zebrafish husbandry and embryo techniques

Zebrafish (*Danio Rerio*) were maintained at 28°C on a 14 hours light/10 hours dark cycle. Embryos were collected by natural spawning, and staged following standard methods (Kimmel et al., 1995). Embryos were raised at 28°C in E3 solution, with distilled H_2O and 3 drops of methylene blue per litre, in petri dishes. Pigmentation was prevented by immersion in 0.2mM N-phenylthiourea.

2.1.1: Fish strains

All fish stocks used are maintained at the University of Sheffield.

The AB line of fish was used as the wild type fish, unless stated otherwise.

Homozygous *smu*^{b577} (Barresi et al., 2000), *smu*^{hi1640} (Chen et al., 2001) and *yot*^{ty119} (Brand et al., 1996) were identified from their siblings by the presence of U-shaped somites identified from 20 hours post fertilisation.

Ptc1^{hu1062} (Koudijs et al., 2008)/*ptc2*^{lep} (Koudijs et al., 2005) double mutant zebrafish were identified from their siblings by the presence of straight somites visible from 18 hours post fertilisation and a loss of the lens in the eye.

Homozygous *ace* mutant zebrafish (Reifers et al., 1998) were identified from their siblings by the loss of the cerebellum.

Homozygous *ubo* mutant zebrafish were first characterised by van Eeden et al., 1996.

Islet1 GFP transgenic fish were characterised by Higashijima et al., 2000.

Mylz2 GFP transgenic fish were characterised by Ju et al., 2003.

2.1.1.1: Fish identification

Tail clipping was used to extract DNA from fish anaesthetised in 4.2% tricaine. 40µl 1x tail mix (see *Table 2.4* for composition) was added to the tail tissue, and incubated at 95°C for 20 minutes. 40µl of 40mM tris HCl was added to neutralise the reaction. PCR was performed to amplify the desired fragment. PCR DNA was cleaned up using ExoSAP treatment, and sent for sequencing to confirm the presence or not of point mutation.

ExoSAP mix:

Exo (NEB, 20U/µl):	0.05µl
Shrimp Alkaline Phosphatase (SAP) (USB, 1U/l µl):	1µl
H_2O:	4µl

ExoSAP mixture is added to 5µl PCR product, and incubated for 45 minutes at 37°C. Incubation at 80°C for 15 minutes follows.

2.1.2: Cyclopamine/SU5402 treatment of embryos

Cyclopamine (cyclopamine, V.californicum; Calbiochem) dissolved in (ethanol) EtOH was added to E3 medium to a final concentration of 100μM. Cyclopamine E3 medium was added to a 24 well plate, containing about 15 fish at 80% epiboly per well. Embryos developed to the desired stage, at which point they were dechorionated, washed in E3 medium, and fixed. Control fish were treated with only EtOH dissolved in E3 medium.

SU5402 (Calbiochem) dissolved in DMSO was added to E3 medium to a final concentration of 20μM. SU5402 E3 medium was added to a 24 well plate containing about 15 fish per well for 6 hours, at which point the embryos were washed in E3 medium and fixed. Application of SU5402 therefore occurred 6 hours before the desired stage of zebrafish development. Control fish were treated with only DMSO dissolved in E3 medium.

2.1.3: Embryo micro-injection

Morpholino or nucleic acid solution was made by diluting the stock solution to the desired concentration, with Milli-Q water and phenol red (less than 10% of the final volume). A Flaming/Brown P-97 micropipette puller (Sutter Instruments) was used to prepare microinjection needles from glass capillaries. The volume injected was checked by microinjecting the morpholino/nucleic acid solution into a small petri dish containing mineral oil placed on top of a microscope graticule. Embryos were injected with 1-2nl of morpholino or nucleic acid solution at the 1-2 cell stage with a micromanipulator. Injected embryos were transferred to E3 solution and maintained at 28.5°C. Penicillin and Streptomycin were added to the culture medium to prevent infection. Dead or dying embryos were removed from the culture medium, identified by the presence of opaque or brown tissue.

2.1.3.1: Morpholinos

Morpholinos were designed and manufactured by Gene Tools, LLC (Philomath, OR, USA).

C125 ATG	5’–gga ttc ttt gtt ttt ccc att gtt a-3’
C125 ATG 2	5’-tta aac tcg cgt aga ctc aca aca c-3’
C125 splice ex1	5’–ggt gtc agc tta ctt ttt cca gta g-3’
C125 splice ex4	5’–taa gtt cat tct tac cag agg ttc c-3’
C125 splice ex7	5’-cat ggc aga tta taa ctc aca ggc a-3’
Standard Control (human β-globin)	5’-cct ctt acc tca gtt aca att tat a-3’

2.1.3.2: RNA synthesis

5µg of vector DNA was digested in a 100µl reaction volume, at 37°C. RNAses were removed by addition of proteinase K (150µg/ml) (Roche) and 0.5% SDS to the digestion reaction, with incubation for 30 minutes at 50°C. Following phenol/chloroform extraction and precipitation, cut DNA was resuspended in 10µl nuclease-free H_2O. Capped RNA was synthesised using the Sp6 mMESSAGE mMACHINE Kit Instruction Manual (Ambion) (*Table 2.1*). RNA was purified using phenol/chloroform extraction, and resuspended in 30µl nuclease-free H_2O. Aliquots of RNA are stored at -80°C.

RNA	Vector	Restriction enzyme	Polymerase
c125	pExpress1	Nhe1	Sp6
dnPKA	pCS2MT	Not1	Sp6
Transposase	pCS2	Not1	Sp6

Table 2.1: *Vector, restriction enzyme and polymerases used to synthesis mRNA for injection into the one-cell stage zebrafish embryo.*

2.1.3.3: RNA injection

Capped mRNA was injected into the cell at the one-cell stage at a concentration of 200-500pg. For rescue experiments, RNA was co-injected with morpholino.

2.1.4: Fixation of embryos

Embryos were collected and raised in E3 solution until the desired stage. They were washed twice with qPBS, and fixed in fish fix *(Table 2.4* for composition) overnight at 4°C. Embryos were then washed twice in qPBS, and dechorionated with forceps. Alternatively, dechorionation took place before fixation. Fish were then put through a methanol series with 25% increments until 100% was achieved, with 5-minute intervals. Fish were washed twice in 100% methanol and then stored in 100% methanol at -20°C.

GFP embryos were fixed at room temperature for 90 minutes in fish fix, and then slowly equilibrated (2, 5, 10, 15, 20, 25, 35, 50, 65, 80% glycerol/PBS washes) into 80% glycerol/PBS for imaging.

2.1.5: In situ hybridisation

2.1.5.1: Digoxygenin-labelled RNA probe synthesis

Antisense digoxygenin (DIG)-labelled RNA probes were synthesised by in vitro transcription, in a 20µl volume (*Table 2.2*). Each reaction contained 1µg of linearised DNA template, 4µl 5x transcription buffer (Promega), 2µl 10x DIG RNA labeling mix (Roche), 2µl RNA polymerase (T7 or Sp6 depending on the vector, and insert orientation) (Promega), 1µl RNAsin RNase

Inhibitor (Promega) and DEPC-treated H_2O. Reactions were incubated at 37°C for 2 hours, and then for a further 30 minutes with 2µl RNase-free DNase I (Promega) to remove the template DNA. Riboprobes were precipitated with the addition of 130µl DEPC-treated water, 26.35µl 4M LiCl and 375µl RNAse free ethanol for 1 hour at -80°C. The RNA was pelleted by centrifugation at 13,000RPM for 30 minute at 4°C, and washed with 200µl 70% RNAse free ethanol. This was centrifuged again at 13,000RPM for 10 minutes at 4°C, the supernatant removed, and the RNA pellet air-dried. RNA probe was resuspended in 50µl DEPC-treated water and 50µl deionised formamide (Sigma), and stored at -20°C.

2.1.5.2: Whole-mount in situ hybridisation

Whole-mount in situ hybridisations (WISH) were based on the protocol from (Thisse and Thisse, 2008). Embryos were re-hydrated into PTW from methanol, using 75%, 50%, and 25% methanol, 5 minutes each time. Embryos were then washed 4 times in PTW, and permeabilised in 0.01mg/ml proteinase K (Roche) in PTW for a duration dependent on their stage of development:

1 cell-1 somite:	30 seconds
1-8 somite:	1 minute
9-18 somite	3 minutes
19-24 somite	10 minutes
24hpf	15 minutes
30hpf	22 minutes
36-48hpf	30 minutes
60hpf	40 minutes
72hpf	50 minutes

This was followed by fixation for 20 minutes at room temperature, and then 5 washes in PTW. Embryos were rinsed in 250µl hybridisation solution without tRNA/heparin, and then incubated in pre-hybridisation solution containing tRNA/heparin at 69°C for 3-5 hours. This was replaced with 200µl hybridisation solution containing 0.5µl-2µl DIG-labelled RNA probe, and incubated overnight at 69°C. The probe is removed (and re-used), and the embryos washed in 75%, 50%, and 25% hybridisation solution without tRNA/heparin: 2x SSC, at 69°C for 10 minutes each time. Embryos were washed in 2x SSC for 10 minutes at 69°C, and then twice in 0.2x SSC for 30 minutes each time at 69°C. The embryos were then left to cool, and washed progressively into PTW in 25% increments for 10 minutes each time. Blocking in PBT for 3-4 hours at room temperature followed, and then overnight incubation at 4°C with anti-DIG antibody (Roche, 1:10,000) in PBT. Next day, embryos were washed 6 times in PBT, 15 minutes each time, at room temperature. They were then equilibrated in staining buffer by washing 3 x 15 minutes, and

then stained in staining solution with 3.5µl/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (X-phosphate, Roche) and 4.5µl/ml 4-Nitro blue tetrazolium chloride (NBT, Roche) in the dark. When staining was complete, embryos were washed with PTW 3 x 15 minutes, and then fixed for 30 minutes at room temperature. Embryos were then cleared by putting them through a methanol series as previously described, and left in 100% methanol for about 2 hours or until sufficiently cleared. Embryos are then left in 50% glycerol:PBS for 15 minutes, and then placed into 80% glycerol:PBS, ready for imaging.

Probe name	**Vector**	**Restriction enzyme/Polymerase**	**Reference**
GFP	pBluescript	BamH1/T7	Gift from S.Elworthy
Olig2.2	pCMV-SPORT6.1	SmaI/T7	Cunliffe, V.T, Casaccia-Bonnefil, P. 2005
Islet1	pBluescript SK(-)	EcoRI/T7	Lewis, K.E. et al 2001
Pax6a		SmaI/T7	MacDonald, R. et al. 1994
C125 EST12	pExpress1	EcoRI/T7	IMAGE: 7001280
C125	pCRII-TOPO	NotI/Sp6	Antonio Milano (Borycki lab)
C125 sense	pCRII-TOPO	BamH1/T7	Antonio Milano (Borycki lab)
Ptc1	pGEM-T Easy	ApaI/Sp6	Gift from Rob Wilkinson
Pax7a	pT7 blue	EcoRI/T7	Seo, H.C. et al. 1998
MyoD		XbaI/T7	Weinberg, E.S. et al 1996
Pax3b		NotI/Sp6	Gift from Claudia Seger
Prdm1		BamH1/T7	Baxendale, S. et al. 2004
Six1	pCRII-TOPO	EcoRV/Sp6	This thesis
Myog		BamH1/T7	Gift from S. Baxendale
Myf5		NotI/T7	Gift from S. Baxendale
Luciferase	pCRII-TOPO	HindIII/T7	This thesis
Lama1	pCRII-TOPO	HindIII/T7	This thesis
Lama1 sense	pCRII-TOPO	EcoRV/Sp6	This thesis
Sox6	pCRII-TOPO	BamH1/T7	Von Hofsten, J. et al 2008
Tnni2	pBluescript	EcoRI/T7	Von Hofsten, J. et al 2008
Mrf4	pGEM-T Easy	Spe1/T7	Hinits, Y. et al. 2007

Table 2.2: *RNA probes used for in situ hybridisation*

2.1.6: Immunohistochemistry

Fixed embryos in methanol were progressively rehydrated into PTW (75:25, 50:50. 25:75, 5 minutes each), and permeabilised by incubation in 0.05% Trypsin-EDTA 1x (Gibco), at 37°C. Embryos at the 10 somite stage were incubated for about 5 minutes, and 24hpf fish were incubated for about 15 minutes. The trypsin was washed off with PTW washes (3 x 5 minutes). Embryos were blocked in PBT for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibody in 500µl PBT (*Table 2.3*). Embryos were washed in PBT (3 x 5 minutes) and then incubated with secondary antibody in 500µl PBT for 90 minutes at room temperature or overnight at 4°C, in the dark. Excess antibody was removed by washing in PTW (6 x 30 minutes) at room temperature, and the embryos equilibrated (30%, 50%) to 80% glycerol/PBS, ready for mounting. GFP expressing embryos were processed differently. These fish were fixed for 90 minutes at room temperature, and then washed 3 times in PTW. The normal immunohistochemistry protocol was followed from this point onwards.

Antibody	**Expression in zebrafish**	**Supplier**	**Species derivation**	**Concentration used**
Primary				
F59	Slow muscle	DSHB	Mouse anti-chicken	1:10
F310	Fast muscle	DSHB	Mouse anti-chicken	1:50
Keratan sulphate	Notochord	DSHB	Mouse anti-rat	1:100
GFP		BD Bioscience	Rabbit anti-GFP	1:200
Secondary				
alexa m594		Invitrogen	Goat anti-mouse	1:500
alexa rb594		Invitrogen	Goat anti-rabbit	1:500
alexa rb488		Invitrogen	Goat anti-rabbit	1:500

Table 2.3: *Antibodies used for immunohistochemistry*

2.1.7: Mounting of zebrafish embryos

Embryos in 80% glycerol/PBS were de-yolked and dissected using a syringe needle and an eyebrow hair needle. 1 to 3 layers of electrical tape were placed on a microscope slide, and a well cut into the middle of the tape. The embryo was placed in the well in 80% glycerol/PBS and covered with a coverslip.

2.1.8: Embedding and sectioning

2.1.8.1: Vibratome sectioning

Embryos were embedded in 2% agarose in PBS, and incubated at 4°C for between 2 hours and 2 days. Sections of 80µm thickness were taken using a Vibratome 1500 sectioning system (Vibratome), and mounted in Glycergel (DakoCytomation) on a slide.

2.1.8.2: Cryostat sectioning

Post-ISH embryos were fixed in Solution 1 containing 0.2% PFA overnight at 4°C, and then washed overnight in Solution 1 without PFA at 4°C. Embryos were then washed and incubated overnight at 4°C in Solution 2. Embryos were then equilibrated for 1 hour in Solution 3 at 37°C, and embedded in the same solution by freezing in a dry-ice chilled isopentane bath. The resulting embryo blocks were stored at -80°C until needed for sectioning (Bajanca et al., 2004). Prior to sectioning, blocks were mounted onto chucks using OTC compound (BDH), and 15µM cryosections were taken using a Bright cryostat (Bright Instruments). Sections were collected on superfrost slides (Menzel-Glaser), rehydrated for 1 hour in PHT, and mounted in glycergel (DakoCytomation). GFP-expressing embryos were fixed in fish fix for 90 minutes at room temperature, rinsed 3 times in PTW and then incubated overnight at 4°C in a 30% sucrose/PBS solution, before being embedded in OCT. Frozen blocks were mounted onto chucks using OCT. Sections were rehydrated for 15 minutes with PBS, and mounted in glycergel (DakoCytomation).

2.1.9: Image capture and manipulation

2.1.9.1: Whole-mount images

Whole-mount embryo images were captured under a MZ12.5 stereomicroscope (Leica) using a SPOT INSIGHT Colour camera, and SPOT Advanced digital image capture software (Diagnostic Instruments).

2.1.9.2: Post-ISH sections and flat-mount images

Sectioned embryo and flat mounted images were captured on a Leica DMR microscope (Leica) with a DMR DC300FX digital camera (Leica). Images were captured using Leica IM50 Image capture software v1.20 (Leica).

2.1.9.3: Fluorescent embryos

Embryos used for immunodetection or embryos expressing GFP were visualised and photographed using a Leica MZ160F fluorescence stereomicroscope (Leica), with a DFC300FX camera (Leica) with Leica FireCam Mac V3.1.0 (Leica).

2.1.9.4: Confocal imaging of GFP/immunohistochemistry treated embryos

Confocal images were captured using the Olympus FV-1000 confocal microscope, with Olympus FluoView FV-1000 ASW 1.6 confocal software.

2.1.9.5: Image manipulation

All images were manipulated in Adobe Photoshop CS3 version 10.0.1 and ImageJ 64 version 10.2. The magnification used to capture each image is indicated on each panel, although the image sizes may be altered during the process of image manipulation.

2.2: Molecular biology techniques

2.2.1: Solutions

Solution or buffer	Composition
E3	15mM NaCl, 0.5mM KCl, 1mM $CaCl_2$, 1mM $MgSO_4$, 1.5mM KH_2PO_4, 0.05mM Na_2HPO_4, 0.7mM $NaHCO_3$ and 3 drops per litre of methylene Blue
Tail mix	10mM Tris pH 8.2, 10mM EDTA, 200mM NaCl, 0.5% SDS, 200µg/ml Proteinase K
Phosphate buffered saline (QPBS)	1.25mM $MgSO_4$, 0.14mM $CaCl_2$, 137mM $NaCl_2$, 5.37mM KCl, 1.1mM KH_2PO_4, 1.1mM Na_2HPO_4 in DEPC-treated H_2O
Fish Fix	0.1M Phosphate (PO) buffer, 0.12M $CaCl_2$, 4% paraformaldehyde (PFA) powder (w/v) 4% sucrose (w/v)
Diethlpyrocarbonate (DEPC) treated water	1ml of 0.1% Diethlpyrocarbonate (DEPC) in 1000ml H_2O
PTW	QPBS with 0.1% Tween 20 (v/v)
Hybridisation solution	50% Formamide, 5x SSC, 0.1% Tween 20 (v/v), 50µg/ml heparin, 500µg/ml tRNA, ph 6.0 with citric acid, in DEPC-treated H_2O
PBT	PBS with 0.1% Tween 20, 2% heat inactivated goat serum, 0.2% BSA
Staining buffer	100mM Tris–HCl ph9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v)
Staining solution	100mM Tris–HCl pH9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween 20 (v/v), 4.5µl/ml NBT, 3.5µl/ml BCIP
Phosphate (PO) buffer	0.02M NaH_2PO_4, 0.08M Na_2HPO_4
Solution 1 (50ml)	4% Sucrose (w/v), 2µl 1M $CaCl_2$, 19.25ml 0.2M Na_2HPO_4, 5.75ml 0.2M NaH_2PO_4
Solution 2	0.12M Phosphate buffer, 15% sucrose (w/v)
Solution 3	0.12M Phosphate buffer, 15% sucrose (w/v), 7.5% gelatin (w/v)
PHT	1x PBS, 0.05% triton X, 1% heat inactivated goat serum
LB broth	10g/L Tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0 using NaOH, deionised H_2O to final volume
LB agar	10g/L Tryptone, 5g/L yeast extract, 10g/L NaCl, pH 7.0 using NaOH, 15g/L agar, deionised H_2O to final volume

Table 2.4: *Ingredient composition of solutions*

2.2.2: cDNA synthesis

2.2.2.1: RNA isolation

Total RNA was isolated from zebrafish embryos using 1ml TRIzol reagent per 60 embryos (Gibco). 0.2ml of chloroform/isoamylic alcohol (24:1) was added, and then shaken. Following incubation at room temperature for 3 minutes, the sample was centrifuged for 15 minutes at 13,000 rpm at 4°C. After centrifugation, the upper aqueous phase was precipitated, and resuspended in 20µl DEPC-treated H_2O. RNA was stored at -20°C.

2.2.2.2: Reverse transcription

cDNA was prepared using Superscript II First-Strand Synthesis System (Invitrogen) for RT-PCR, following the protocol supplied.

2.2.3: PCR

2.2.3.1: Primer design

All primers were designed using Primer Premier 5, version 5 (www.PremierBiosoft.com). Primers are designed to be between 18-25bp in length and have a fairly equal amount of A/T vs C/G. They usually contain at least 1 C/G at the 5' and 3' ends, and have a Tm of 55-65°C.

Primers including restriction sites for amplification of sequences intended for specific cloning were designed as above, with reference to Stratagene technical tools; Cleavage Activity Near DNA Termini.

2.2.3.2: Standard PCR protocol

Standard PCR reactions for cloning products into vectors were performed in a 20µl volume, as follows:

2x BioMix Red (Bioline)	10µl
MilleQ H2O	7.9µl
Forward primer (20µM)	0.8µl
Reverse primer (20µM)	0.8µl
Template DNA	0.5µl

In some cases, multiple primer pairs were added to the reaction mix, and the volume of water adjusted accordingly.

PCRs were performed in an Eppendorf mastercycler gradient PCR machine (Eppendorf). The standard program was as follows:

1. Denaturation	94°C	5 min
2. Denaturation	94°C	1 min
3. Annealing	see table 2.5	1 min
4. Elongation	72°C	40 secs
5. Repeat step 2-4	34x	
6. Final elongation	72°C	10 min
7. HOLD	10°C	

6-10µl of PCR product was run on a 1% Agarose/TAE gel, alongside 3µl of GeneRuler 1Kb Ladder (Fermentas).

Primer Name	Sequence	Supplier	Length (bp)	Tm (°C)	GC %
C125 Project					
C125 ex1 F	GCAGTTTTCTCCCGACGGTC	MWG	20	63.1	60
C125 int1 R	CATTGAGTGGGGTCTTCATCTTTAG	MWG	25	58	44
C125 ex4 R	GGAGAGTTCACGCCAAAGCC	MWG	20	62.9	60
C125 ex2 F	GCGAGTATGCCTGTGCCAGTATC	MWG	23	64.4	56.5
C125 int4 R	ACCTATTTGTGACTTGGTCCTTGC	MWG	24	63.1	45.8
C125 ex6 R	GAGTTCTCCTCTGTTACTGTGTGC	MWG	24	61.3	50
Lama1 Project					
12.7kb up F	GGTGGCTGCTGAGGTGTTGC	MWG	20	64.2	65
12.7kb up R	CCTTAGCAACCACTGAGAACAACC	MWG	24	64.7	50
Ex 37/38 F	GATTGGCTGCTGTGTAAGTCAC	MWG	22	58.2	50
Ex 37/38 R	CAGTTCTAACCACTGAGCCACC	MWG	22	61.6	54.5
Ex30/31 F	CTGCTGTCGTATGGAGGAAGACTC	MWG	24	62.2	54.2
Ex30/31 R	GCTCACGGCTTCATCAGACACTG	MWG	23	61.5	56.5
Lama1 F1	GGGGGTTCAGTTGAGTGTAGCG	MWG	22	66.2	59.1
Lama1 R1	CATCTAAGAAGTGTAAAAGAGCGAAG	MWG	26	60.5	38.5
Lama1 F2	GTGACGCAACAGACGCTACG	MWG	20	63.2	60
Lama1 R2	ACCCGTTTACACATGATTTGCC	MWG	22	60.4	45.5
GFP F	GCCTCGGTGAGTTTTCTCCTTC	MWG	22	63	54.5
GFP R	GATGCCGTTCTTCTGCTTGTCG	MWG	22	61.8	54.5
Cm F	ATAAATCCTGGTGTCCCTGTTG	MWG	22	58.3	45.5
AMP R	CGACACGGAAATGTTGAATACTC	MWG	23	56.8	43.5
Ex63 F	GACCTCTGACCCCGACACACG	MWG	21	65	66.7
Ex63 R	CGTCCAGGAGGATTAGAGTCGC	MWG	22	64	59.1
BAC backbone R	CGTTCGCTAACTCAGCATCGC	MWG	21	63.4	57.1
Ex4 F	GCAGGTGTTCCAGGTGGCG	MWG	19	64.1	68.4
Ex2 F	GCCCTGGTGTTGACCTTATCC	IDT	21	61.7	57.1
Intron1 F	CGCCTCACAGCAAGATGGTCG	IDT	21	62.1	61.9
Min prom. F	CACATCGGGGAAAGTATTAAAAACG	MWG	25	63	40
Min prom. R	CGTAGCGTCTGTTGCGTCAC	IDT	20	63.2	60
3.3Kb F	CGGACGGATGCATGGACGTGG	MWG	21	64.3	66.7
Primers for probes					
Myf5 F	GCAACTTGCGCTTCGTCTCC	MWG	20	64.1	60
Myf5 R	CATCGGCAGGCTGTAGTAGTTCTC	MWG	24	64.9	54.2
Lama1 F	GAAGTTTGAATGAGAGAAAGACGGAC	MWG	26	60.8	42.3
Lama1 R	CCTTGATGGAGTAATAATACCGTCG	MWG	25	61	44

Table 2.5: *Primer sequences. Primer sequences are in the 5' to 3' orientation.*