Appendix I Molecular biological methods

I.1 An optimised protocol for rapid DNA extraction from European flounder tissues.

Extensive optimisations were carried out to find a DNA extraction method which would produce good quality (high molecular weight) DNA from a variety of European flounder (*Platichthys flesus*) tissues including liver and fin clips. Investigations were carried out based on two protocols for DNA extraction, one from Atlantic salmon (Taggart *et al*., 1992), and another from mouse tail (Mullins traditional DNA extraction). The following protocol was therefore optimised, and all DNA extractions used throughout this study used this method of extraction (with the exception of larval DNA extraction – details provided in Chapter 3). Provided all materials and solutions are sterile and autoclaved where appropriate, high molecular weight DNA was regularly extracted and was suitable for PCR and RE digest etc.

I.1.1 DNA Extraction protocol – European flounder

Materials required:

Solution:
1. 50mM Tris HCl; pH8.0, 10mmM EDTA, 100mM NaCl, 1% v/v SDS
2. Proteinase K, 20mg/ml
3. RNase (Dnase free), 20mg/ml
4. Phenol, pH 8.0 equilibrated
5. Chloroform:isoamyl alcohol 24:1
6. 70% Ethanol
7. TE pH 8.0 (100mM Tris, 1mM EDTA) - Sterile
8. Isopropanol

Method

Day 1:

1. Add 375µL solution 1 + 12µL proteinase K to a clean eppendorf
2. Add fin clip / tissue (ca. 5-10mm) to each tube
3. Incubate overnight at 55°C in a shaking incubator or rottiserie oven with constant agitation.

Day 2:

1. Add 10µl RNase to each tube and invert gently to mix. Incubate for one hour at 37°C (waterbath or oven).
2. Add 400µL phenol to each tube. Mix by over-end turning for 15-20 mins.
3. Add 400µL chloroform:isoamyl alcohol to each tube. Mix by over-end turning for 15-20 mins.
4. Centrifuge the tubes at 10,000 x g for 5 mins.
5. Remove 300µLs of the top aqueous layer to a clean microfuge tube using pre-cut wide bore Gilson tips.
6. Add 400µLs cold isopropanol (or 600µLs absolute ethanol) to precipitate the DNA. Invert several times, and incubate at –20°C for 30 mins (if using ethanol to precipitate DNA, leave at room temperature for 30mins).
7. Centrifuge microfuge tube at 12,000g for 10mins to pellet the DNA.
8. Decant off most ethanol.
9. Add 1ml 70% ethanol to wash the DNA. Mix by gentle over-end turning for 30mins. Centrifuge at 12,000g for 10mins, and remove ethanol.
10. Allow DNA to air dry at room temperature for 5-10mins until all remaining ethanol has evaporated.
11. Resuspend pellet in 100µL molecular biology grade water. Total dissolution often takes several days at 4°C. Store at 4°C, or –20°C for long term storage.

I.2 Spectrophotometric quantification of nucleic acids

Nucleic acids were quantified using a DNAamp spectrophotometer (Pharmacia). An aliquot of the nucleic acid (RNA or DNA) was removed from the extracted sample, and diluted to the appropriate factor in molecular biology grade water (BDH). The same water was used as a blank to calibrate the spectrophotometer prior to first use. After every 10^{th} sample quantified, a reading was taken from the molecular grade water. Assuming a value of zero was returned, the machine was accurate, and the results were accepted. Quantity of dsDNA or RNA was calculated using the following calculation:

Abs_{260} x nucleic acid quantification coefficient* x dilution factor = Concentration of DNA RNA in original sample

*(DNA = 50, RNA = 40)

Quality of the DNA and RNA was checked using the Abs_{260} / Abs_{280} ratio.

I.3 First strand cDNA synthesis (reverse transcription)

Reverse transcription was carried out to synthesise first strand cDNA from an RNA template. The method presented below using Moloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase (Promega Inc.), and poly T primers (to select all mRNA) is based on standard protocols (Promega technical data as supplied; & (Sambrook et al 1996)). Poly T primer was synthesised by MWG Biotech, sequence $T_{(16)}V$.

- To a 200µl PCR tube, add:

	Stock	Volume
1µg total RNA	1µg.µl stock	1µl
500ng (76pmols) poly T primer	7pmol.µl stock	11µls
Total		12µls

- Incubate at 70°C for 5 mins, then place on ice. Add the following reagents:

	Stock	Volume
5x RT buffer	5x	4µls
dNTP	10mM	1µl
dH_20		2µls
Total		19µls

- Incubate at 42 deg for 5 min
- Add 1µl (200 U) MMLV H- Reverse Transcriptase (200 U.µl)
- Incubate for a further 50 mins at 42°C.

Place on ice if using immediately, otherwise store at –40°C.

I.4 1st strand cDNA Purification using QIAquick spin columns (Qiagen)

The protocol follows manufacturers instructions as detailed below. All samples for comparison were prepared using the same batch of kit & reagents to ensure consistency of reaction.

1. Five volumes (50µL) of binding buffer were added to 10µl of RT reaction, and the whole mix transferred to a purification column.
2. Columns were spun at 12,000 RPM for one minute to allow binding of the solutions to the column matrix.
3. 0.75ml of Wash buffer were added to remove impurities from the sample, and re-spun at 12,000 RPM for two minutes.
4. Columns were transferred to clean Eppendorfs, and an appropriate volume (100µL) of TE (pH8.0 1/10th EDTA) was added to the centre of the column. Samples were eluted in the maximum possible volume to increase yield efficiencies (see Qiagen data). At 100µL elution volume, efficiency is consistently 90% (QIAgen).
5. Columns were re-spun at 12,000 RPM for 2 mins to recover the sample.
6. Samples were then stored at –20^{o}C until required.

I.5 Agarose gel electrophoresis of RNA prior to Northern Blotting

A midi-gel (13×15×1cm, Amersham Pharmacia, UK) casting tray was prepared, and the desired number of 16-well combs inserted. To a conical flask was added:

1.3g Agarose

4.8 ml 25x MOPS buffer

126 ml dH2O

The above was then heated in a microwave at full power for two minutes, and allowed to boil to dissolve the agarose. The gel was then allowed to cool to 50°C, before addition of 40µl 10mg/ml ethidium bromide. The gel was immediately cast into the prepared tray, and allowed to set for approximately 20 minutes. Once the gel was fully set, combs were removed. The gel was then immersed in 700mls of 1× MOPS running buffer, samples were loaded, and electrophoresed for the appropriate time (between 30mins – 1hour) at 4V/cm.

I.6 Northern (& Southern) blotting

A plastic tray was half filled with 20xSSC. Onto the plastic tray was placed a glass sheet, with Whatman 3mm blotting paper soaked in 20xSSC wrapped around to act as a wick. Either end of the blotting paper was placed into the tray containing 20xSSC to allow the buffer to soak up by capillary action. The agarose gel (RNA or cDNA PCR) was laid onto the blotting paper face down, and a plastic mask (Saran wrap) was then placed around the gel to prevent any short-circuiting between the wick and the absorbent material above. Hybond 'N' membrane was used as the filter. The filter was marked on the top side with pencil, and the top right hand corner cut off to verify orientation. This was cut to size, then laid over the gel, being careful to avoid getting any air bubbles trapped between the filter and gel which would cause inefficient, and possibly uneven, transfer of RNA. Once the filter was in place, eight sheets of Whatman 3mm paper, equal in size to the gel, were placed on top, and a stack of paper tissues added above that. The absorbent stack was to allow capillary diffusion of the RNA from the gel onto the Nylon filter. Finally, a second glass plate, and a 500gm weight were placed onto the top of the stack to compress the layers sufficiently to allow soaking up of the SSC.

Once assembled, the blot was left overnight (ca 20hrs) to allow complete transfer of the RNA / DNA. After blotting was complete, the assembly was dismantled, and the gel visualised under UV light to ensure total transfer had occurred.

Filter preparation

Filters were removed and washed in 2x SSC to remove any agarose debris and excessive salts. Filters were then placed between fresh Whatman 3MM paper and air dried. Once dry, filters were baked for 60mins at 80^{o}C to fix the RNA. Once fixed, filters remain stable for several months if stored in dry, dark conditions (Dyson, 1991).

I.7 Probing / hybridisation of filters - Northern or Southern blot

I.7.1 Probe preparation and labelling

Generation of probe fragment

Plasmid containing the required probe was transformed into Top 10 competent cells according to standard protocols (see I.8). Colonies were grown up over night on LB (100μg/ml ampicillin) plates at 37^{o}C. Single colonies were then picked, and cultures grown up over night in 3ml of LB Amp broth at 37^{o}C. Plasmids were then purified out of the cultures using GFX plasmid preparation kits (Amersham Pharmacia) according to the manufacturer's protocol. Plasmids were checked by restriction digest, and sizing of fragments on 1.2% agarose (1xTAE gels). Once sizing was confirmed as correct, probe fragments were generated by cutting the required DNA out of the appropriate plasmid using the relevant restriction enzymes (see individual probe descriptions for details). Restriction digests were stopped with heat denaturation of the enzyme at the relevant temperature, and aliquots of the reaction were run on low melting point agarose gels. Bands were excised from gels using a scalpel, and purified using GFX gel band purification kits (Amersham Pharmacia) according to manufacturers protocol. The purified products were then eluted in dH_2O, and quantified spectrophotometrically, and on agarose. Twenty five nanograms of probe fragment was then used in the labelling reaction.

Probe labelling reaction

All probes were labelled with α^{32}P dCTP (4000 Ci / mmol; 10μCi/μl) using a 'random primers DNA labelling kit' (Gibco BRL) according to manufacturers protocol. Briefly:

- Twenty five nanograms of probe DNA was diluted in 5-25μl BDH dH_2O in a screw-cap 1.5ml microcentrifuge tube. DNA was denatured for 5 min in a boiling water bath, then immediately transferred to ice.

- While on ice, the following components were added to the microcentrifuge tube from the 'Random primers DNA labelling kit':

2µl dATP soln.
2µl dGTP soln
2µl dTTP soln
15µl random primers buffer mix
5µl (50µCi) α^{32}P dCTP (4000 Ci / mmol; 10µCi/µl)
dH_2O to a total vol. Of 49µl

- The solution was mixed briefly, and 1µl Klenow fragment (as supplied) added. Solution was then mixed again, and spun down in centrifuge to collect it at the base of the tube. The reaction mix was then incubated for 1 hr at 25^{o}C.
- After one hour incubation, 5 µl stop buffer was added, then the probe purified using ProbeQuant^{TM} G-50 Micro Columns (Amersham Pharmacia) according to manufacturers protocol to remove any unincorporated isotope.

Prior to hybridisation, the probe was denatured for 5 mins in a boiling water bath.

I.7.2 Filter preparation

Pre-hybridisation of filters

Filters were soaked in 2x SSC, then rolled out into a Techne hybridisation tube. Where necessary, two to three filters were probed per tube. In this case, a nylon mesh was placed between the filters, and between the outer filter and the tube to prevent air bubbles from becoming trapped, and the filters from sticking together and cause non-uniform probe binding. Twenty-five ml of modified Church buffer (hybridisation solution) (3.5g SDS; 49mls 0.5M Na phosphate buffer, pH 7.2; 1ml 0.5M EDTA) was added to each tube, and hybridisation tubes were transferred to a rotisserie style oven (Techne). Filters were pre-hybridised at appropriate temperature (see individual probe details) in the hybridisation tubes for a minimum of three hours in order to reduce background noise during probe hybridisation.

Hybridisation protocol

Once the filters were suitably pre-hybridised, the pre-hybridisation wash fluid was discarded. The purified and denatured labelled probe was added to 25ml Church buffer, and the solution added to the hybridisation tube containing the membranes. The probe was then hybridised to the filter by overnight incubation at appropriate temperature in a Techne rotisserie oven.

Post hybridisation washes of filters

Each of the following solutions was made up prior to washing of filters. As the stringency of washes varied between probes, counts per second (cps) readings were taken after each wash using a hand-held mini-monitor.

200 mls	2X SSC; 0.1% SDS
200 mls	1X SSC; 0.1% SDS
100 mls	0.5X SSC; 0.1%SDS

Washes were carried out specific to each probe (see chapters for details).

Autoradiographic exposure

After washes were completed, transfer membranes were blotted dry on Whatman 3MM paper and wrapped in Saran wrap™. The wrapped transfer membranes were placed in a film cassette with an intensifying screen and autoradiography film (Kodak) (orientation marked by cutting the top right hand corner). The cassette was then placed at -70°C for overnight exposure, and was removed thirty minutes prior to developing film. Autoradiograms were developed, and dried for thirty minutes in a drying cabinet. After developing autoradiograms, images were scanned using an Epson GT2000 flatbed scanner. Images were analysed via densitometry as e.g. (Kaplan, et al.,(1995)); (Courtenay et al., (1999)) using 'gel capture' macros in Scion Image analysis (based on NIH Image analysis software (Bethesda, MD, USA)) and the intensities recorded as relative mean expression, as described by (Courtenay *et al*., (1999)).

Strip filters:

Filters were then stripped by removing from SARAN wrap, and placing in a 0.1% SDS solution at95°C. The solution containing filter was then allowed to cool to room temperature.

I.8 Transformation protocol using 'Top 10' competent cells

1. Thaw one tube (125µl) of cells per transformation for 5-10 mins on ice.
2. Add 1µl of ligation reaction or pre-prepared plasmid (template) to each tube (whilst on ice).
3. Mix thoroughly, but gently, and incubate tubes on ice for 30 mins.
4. Heat shock cells for 45s at 42°C, ensuring all ice is removed from bottom of tube, to allow cells to take up the template (phagemid construct).
5. Transfer tubes to ice.
6. Add 250µl of LB Broth.
7. Cap tube and incubate at 37°C for 1 hour at 225-250rpm in a shaking incubator to allow cells to grow.
8. Plate out aliquots of cells according to protocol I.9. If required, freeze down remainder of transformation at –80°C: add an equal volume of LB Broth (including 30% v/v filter sterile glycerol) to make the final solution 15% v/v glycerol to preserve cells, then place in freezer.

I.9 Plating out of colonies – from (Sambrook *et al.*, 1999)

I.9.1 Day one

- Pour 30ml of prepared agar (100µg/ml ampicillin), per 90mm plate.
- Dry for 5mins upright with lid removed. Invert and dry for 40mins with lid removed (at 37°C).
- 100µl of 100mM IPTG and 1.2µl 40mg/ml X-Gal were spread onto the top surface of the plates to allow colour selection.
- Plates were dried for 5mins upright, and 40mins inverted in a 37°C oven.
- Transformation were then plated out by pipetting the required amount onto the centre of the prepared agar plate, and spread uniformly across the plate using a sterile plate spreader: Initially using dilutions;

  1µl transformation in 99µl NZY^+ broth

  10µl transformation in 90µl NZY^+ broth.
- Plates were dried upright for 5 mins at 37°C, and wrapped in Parafilm™. Plates were inverted, and incubated at 37°C for *c.*17hrs.

I.9.2 Day two

- Incubate plates at 4°C for 2 hrs to enhance blue colour (if using blue / white selection).
- Colonies without inserts will appear blue, colonies with inserts will remain white.
- Once required colonies have been identified, single examples can be picked and transferred to 3ml of LB Broth (100µg/ml ampicillin) in sterile universals. These colonies are then grown up overnight (approx 16hrs) in a shaking incubator at 37°C with constant rotation.

I.9.3 Day three

- Once adequate growth was observed, universals were removed from the incubator, and cells pelleted. The aqueous LB Broth was then removed, and plasmid preparations carried out as required.

I.10 Probing / hybridisation of filters – bacterial colony screening

Colony screenings were performed in order to detect positive colonies during microsatellite enrichment (i.e. those colonies containing a microsatellite insert)(see Chapter Three). The method presented below was used for both colony lifts, and colony screenings from 96 well plates, and involve fixing the bacterial (plasmid) DNA onto Hybond N (Amersham, UK) membrane.

Lysing of bacterial colonies and fixing of DNA to membrane

Filters were removed from agar plates, and denatured by placing them on Whatman 3MM paper (soaked in denaturing solution) for 8mins. The filters were then transferred to clean Whatman 3mm paper (soaked in neutralising solution) for 2 x 4 minute washes to neutralise them. The filters were then baked at 80^{o}C for 2 hours to fix the DNA to the membrane. (For full details of denaturing and neutralising solution, please see 'Recipes' section).

Filter preparation

Prior to hybridisation, filters were soaked overnight in 6X SSC (0.1% SDS) to remove bacterial debris. Filters were washed in 5X SSC (0.1% SDS) for 2hours at 37^{o}C, then wiped with soft tissue (Kleenex) soaked in 6X SSC.

Pre-hybridisation washes

Filters were placed into hybridisation tubes and 30ml of hybridisation buffer (see recipes, Appendix IV) was added. The tubes were sealed, and incubated in a Techne rotisserie-style hybridisation oven for 5hrs at 42°C.

Probe hybridisation

Pre-hybridisation buffer was removed from the tubes and was replenished with fresh hybridisation solution containing the appropriate probes. Colony blots were hybridised with the appropriate cocktails of end-labelled α^{32}P ATP oligonucleotide probes (see

tables in Chapter three) over-night at 42°C in a Techne rotisserie-style hybridisation oven.

Post-hybridisation washes

Post-hybridisation washes were carried out as follows in hybridisation tubes:

6× SSC (0.2% SDS) for 5' at 42°C ×1

5× SSC (0.2% SDS) for 10' at 42°C ×3

After over-night autoradiographic exposure, positive recombinants were identified and graded from 1-10 (10 = highest intensity). Grading was carried out by eye, to an arbitrary scale. Positive colonies were then sequenced.

I.11 Pouring and running a Polyacrylamide gel, and gel electrophoresis.

Denaturing gel electrophoresis on 6% denaturing polyacrylamide sequencing gels was carried out to manually genotype microsatellites isolated in this study (Chapter Three). The following details the method of polyacrylamide gel electrophoresis.

Preparation:

Prior to running the polyacrylamide gel, it was essential to ensure that the gel rig and plates were clean. The lower plate was cleaned with a 70% Ethanol wash, then 'Repelcoat' was spread evenly over the plate (siliconisation to allow easy removal of gels), and left for two mins. A final 100% ethanol wash was carried out, and the plate rinsed with dH_2O. The top plate was cleaned by addition of a few drops of NaOH to remove grease, then wiped over. A soak in water, then rinse with dH_2O was included to remove any remaining NaOH. After 2mins a 70% Ethanol wash was carried out, and the plates left to dry. The gel rig was assembled by placing the lower (rabbit eared) plate into the cassette. 0.2mm spacers were placed at either edge of the plate, and the top plate placed on top. Clamps were tightened, and gel was then prepared and applied as follows.

All mixing of the gel was carried out on ice, and components were added in the following order. APS was added immediately before pouring to prevent premature polymerisation.

Polyacrylamide gels	
64ml	Sequagel XR
16ml	Sequagel complete
640µl	10% APS

The gel mix was then poured into the assembled gel rig plates, and left to polymerise for 2 hours. The gel rig was fully assembled, and pre-electrophoresed in 1xTBE for 45mins at 70W until it reached a temperature of 50°C. Prior to electrophoresis, wells were flushed with 1× TBE to remove any excess ammonia build up.

Prior to loading of samples, 2.5µl of formamide 'Stop' dye was added to each PCR reaction. Samples were then denatured at 98°C for 5mins, and immediately placed on ice. Gel wells were washed out again after pre-run by flushing with buffer, then 1.5 – 2.0µl of each sample was loaded (depending on how 'hot' the samples were). Samples were then electrophoresed for 1-2hrs at ~ 35V/cm (~ 2,500 to 3,000 V*H).

Post run, the gel rig was dismantled, and the gel transferred to Whatman 3mm paper. The 3mm paper with fixed gel was dried for 2hrs at 85°C under vacuum in a gel drier. Once dried, the gel was loaded into an autoradiography cassette, and exposed for an appropriate amount of time to visualise the amplified products (from 24 – 72 hrs at -70°C with or without an intensifying screen, as required). In some cases it was necessary to shorten or lengthen the exposure time depending on signal strength.

I.12 Sequencing of plasmid construct

Reactions were set up as follows:

Component	Volume	Amount
100-250 ng/µl template	2µl	[200-500ng]
Big Dye™ Ready Reaction Mix	2µl	[as supplied]
3.2µM primer	1µl	[3.2pmol]

Sequence reactions were then run on a Biometra T-Grad thermocycler using the standard cycle (25 cycles of: 95^{o}C denaturation for 20s, 50^{o}C annealing for 10s, 60^{o}C extension for 4mins, followed by a hold at 4^{o}C).

Samples were cleaned up using a standard ethanol precipitation for 96 well plates (see I.14), and products run on an ABI PRISM 377™ Automated DNA sequencer (PE Applied Biosystems). Sequences were aligned using Autoassembler (PE Applied Biosystems), and clones identified via Blast searching (BLASTN Version 2.2.1) (Altschul, *et al*., 1997).

I.13 Direct sequencing of PCR product

Eluent from a purified PCR product was used as a template in a direct PCR sequencing reaction using Big Dye™ Terminator Cycle Sequencing Ready Reaction mix (PE Applied Biosystems). Each sample was sequenced, using the appropriate forward and reverse primer, as detailed below. The following components were added to each sequencing reaction:

Component	Volume	Concentration / Amount
6-18ng/µl template	5µl	[30-90ng]
Big Dye™ Diluent	2µl	[2µl]
Big Dye™ Ready Reaction Mix	2µl	[as supplied]
3.2µM primer	1µl	[3.2pmol]

Thermal cycling was then carried out on a Biometra T-Grad using a standard cycle sequencing cycle (30 cycles of: 95^{o}C denaturation for 20s, 50^{o}C annealing for 10s, 60^{o}C extension for 4mins, followed by a hold at 4^{o}C).

Samples were purified using a standard Ethanol / NaOAc precipitation for 96 well plates (see I.14). Products were sequenced on an ABI PRISM 377™ Automated DNA sequencer (PE Applied Biosystems). Post-run, gel lanes were tracked manually, and sequence data extracted using ABI PRISM 377™ sequence analysis package version 3.4. Forward and reverse sequences were then aligned using Autoassembler (PE Applied Biosystems) and combined to produce consensus sequences which were then subjected to a BLAST search (BLASTN Version 2.2.1) (Altschul, *et al*., 1997), and verified as the correct products.

I.14 Big Dye sequencing clean up method – Sanger Centre

BigDye Terminator Clean up Method.

This clean-up method is good for removing excess dye from reactions, so you should see less "dye blobs" in the sequences. The EDTA in the precipitation mix reduces the precipitation of smaller DNA fragments and so also reduces the precipitation of un-incorporated dye. HOWEVER, it is very important not to exceed the recommended centrifugation time (step 3) as this will increase the dye blob problem. It is also important to keep the precipitation mix at room temperature as both sodium acetate and EDTA may precipitate out of the mixture if it is stored in the fridge or freezer....and then the precipitation will not work at all!

Precipitation Mix:

100ml 96% ethanol

2ml 3M sodium acetate soln

4ml 0.1mM EDTA

Keep precipitation mix at room temperature and make up fresh solns regularly.

Protocol 96-well plates

1) For a 10µl reaction volume, add 10µl water to each well (->20µl).

2) Add 50µl pptation mix, at room temperature, to each well.

3) Spin at 4000rpm/4^{o}C for 20-25 mins.

Note: do not exceed spin time it is not necessary to pre-chill the centrifuge

4) Tip off the supernatant, drain plates upside-down on tissue.

5) Add 100µl chilled 70% ethanol to each well.

6) Spin at 4000rpm/4^{o}C for 2-4 mins.

n.b. for 3700 templates, repeat steps 5,6 once more.

7) Tip off the supernatant, spin plate upside-down on a tissue at 250rpm for 30-60 secs.

8) Allow plate to dry before loading.

http://www.sanger.ac.uk/Teams/Team51/BDC.shtml

I.15 Protocol for colony PCR.

PCR mix:

Component	Volume	Final concentration / amount
10 × buffer	2 µl	1 ×
25mM $MgCl_2$	1.2 µl	1.0mM
5mM dNTPs	0.8 µl	200µM
10 ng/µl T3	3 µl	1.5ng/µl
10 ng/µl T7	3 µl	1.5ng/µl
5 units/µl Taq polymerase	0.1 µl	0.5 U
dH_20	9.9 µl	to final 20µl

The appropriate mastermix volume was made up, and aliquoted this into PCR tubes on ice. Part of the colony was picked using a cocktail stick or sterile pipette tip, then placed into a PCR tube and swirled gently to mix. Post-PCR, results were checked on agarose gel.

PCR programme:

Initial denaturation at 96°C for 3min, followed by 28 cycles of: 94°C for 70s, 50°C for 75s, 72°C for 90s, followed by 1 cycle of 72°C for 4 min

Appendix II Microsatellite data: Chapter 3

II.1 Microsatellite sequences

Repeat (Rep) regions are underlined, and primer bind sites (forward and reverse, PF and PR respectively) are given in **bold.** Sequence length is given at end of each sequence.

StPfl001

ACGACTGTGTGTTT**CATCAAAGCATGAAACCC**TGTGTGTGTGTGTCTCTATGTCAATCTATCTATCTATC
TATCTATCTATCTATCTATCTATCTATCTCTCTCTATCTCTCTCCCTATCTGTCTATCTATCTATCCATA
TATTAATCCATCCATCCGTCTTTTTACTGATTTGCATTACTCTTTTCTTACTTTCACCATCTTTCAGATC
AATACAGAGAAGTTGAGCTATTTTGAGAGCATTTC**ATGCTCCACTTGGGCCAG**ATGTTCTATAAAAATCC
CCTTCCTAGATAAACCTAAAAATATGATTG 310
F 15-32, R 263-310, Rep 58-113

StPfl002

TGTATTGATATATGAGAGGGGG**ACCCGATCAAGTTGTAGTCAT**GTCTGTGTGTGTTAGTGCTGCTGTCTG
TCTGTCTGTCTGTCTGTCTGTTCTACTCGCTGACAGTGGACCTGCATTGTTGCTTATCCTGTTTGCCAGA
AGTTGCACCCAAAATACTGCCAGGAGGGC**AACACGCCGAGAGGAGAAATG**TGCTTCTACATGTGCTTCTA
CATGTGCTT 219
F 23-43, R 190-170, Rep 64-91

(CTGT)$_7$

StPfl003

CTTTCTGGCGTTTCTCTTTTGCTGTGCACTGAATACCAATCACGTGTCACACCGAATGGCAGTTGGTAAC
AGCAAATNGTAGGTGGTTGATTTAAGTTAATGTCATGCG**GTCAAATTAGGGAGGGCAGTG**TGAGCACTGG
TCAAGGACCCTCCTTCGGGTTGATCCATCAATCTATCCATCCATCCACCATCTATCTATCTATCTATCCA
TCCATCCATCTATCCATCCATCCATCCATCATAGCACAAACCA**ATTTATCCTGTGCAAGGTCAGAG** 276
F 110-130, R 276-254, Rep: 173-239

StPfl004

TTAATGAACGGACTCTTTCACCGGCTCCAGCTTCCTCTGTGC**ATGAGGACGTGGATGTTCTTC**CGCTCAG
CGACGGGGACTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGTGTCACATGGCTCCGTTCTGCCAC
AGCCAGCTAATTAGTGGTGGCTTA**GTGAACATTAAGCAGAGATAGGGG**TTTAACG 195
PF 43-63, PR 165-188, Rep: 80-115

StPfl005

GTGAGCAACATGCCTGA**TGCTGCCGAGCTGGGCTTATTATC**ATGGTGTGTGTGTTAGTGTGTGTGTGTGT
GTGTGTGCATGCCTGCAGACACGTGTGTGCATATGT**ATGTGACAGTTGGgATGCcGTGTG**GAGCGTcCTG
CTAATGTGGAATTATGAGCTTCAACAATGACCA 173
PF 18-41, PR 107-130, Rep 45-76

StPfl006

CTGTCATGCAAAAGAAAAGAGAGAGAAGAAAGGAAACAAAGAGAAACTGATTTATAAATGATGTAGAT
ACTCCGCTATAAAAAGTATCTAGATGGATGGATAGATAAATAGTTAGATAGATAGATAGATGGATAAATA
GATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGA
TAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATGGATAGATAGATA
GACACCTACCCAATGGTCCACAGACGCTGCTCAGGGGGCTGGTTATCAGGATCATCGACAGCAGCAGCAT
ACACAGGGGAGAGGAGCTAAGGCTGAGCCCCAGGGTAGGCCTCCTGGACTGGGCCAGAGCGCCACCTGTC
CTGGGCAGAGGCATGGCAGGCACCGNNTGTGGGCCGGGTCGGANATGACGGACACTCAGGTANGGCTCCA
CTCAACANGGATAGGCACTCTGCAGACCAGANAGANNNAAAGCATGAACGTCAG 543
PF , PR , Rep 93-280

Stpfl007

L1GCCAACAAACATCCGTTGTGAGACGAAAA**CAGACCAAAGGCCAAGAGTG**GTGTGTGTGTGTGTGTGAG
AGAGAGAGAGAGACAGACGGACAGACAGACAGACAGACAGACAGACAGACAGACAGACAGATAGACAGAC
AGACAGATAGACAGACAGACAGACAGACAGAGACAGACAGGTCAGAGACAGACAGGTGAGAGACAGACAG
AGACAGACAGAGACAGACAGAGACAGACAGAGACAGACAGGTGAGAGACAGGTCAGAGACAGACAGGTGA
GAGACAGACAGAGACTTGTTCTGGTTCTACTTTTANATCTAATCCAGGGTTA**CATTCTTATCCCGCTTCT**
GTTCTCACTTCACATCCTCGTACTTTTTGTAGNTCTGGTTCTGATTCTACTTCTAGTACCACTCCTTGTT
CTGATTTTCTTTCTGCTGCTGTTGCTGGT 447bp
PF 30-49, PR 352-331, Rep: 41-292

Stpfl008

CGGCGCTCGGGGAGCAGATGTCTGGGGAGTAAGGTGCCTTGCTCAGGGGCACTAGACAGGGTAGGGAGAC
TCTTGGATTTTTGGACAGATCAATCCAGGTTATTCTTTTGTTGTCTCTCCGTGGAGTC**GAACCAGAGACG**
AACCAGAGACCTTCTCTGCCCATANTCCAAGTTTCTATAGATAGATAGATAGATAGATAGATAGATAGAT
GGATGGATAGAAATAAAGATAGAgATGGATGGATATATGGATGGATGGATAAGGGTGAAAACTCAGTTGT
ATATGTGTCTATCCCTTTAAGACTCCATTAAAATAAATAAAATACTTtACTTATCTNATCTCCCCCCACA
TGTCATCTTATTAAACTTTCCAGNCTCAACAT**TTCTGTCTCAAATCTGTCCCCTGT**ATNCANTCATCATC
ACCCATGCTCA 431bp
PF 129-151, PR 406-383, Rep:180-260

Stpfl009

CTGCTCAATGCCTTGATCATTTTAAGAAGGAATCTTTGAGACAn**GGAGGAGTTTTCTTTGTTTTAG**CACA
ACATGAAACTTGTAAAGtaCAAGTTAGATAgATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATA
GaCAGATAGATAGATAGAGAGATAGATAGATAGATAGATAGATATAGATGGATCGATGGATAGATAGACA
GATAGATaGATaGATAGATAGATAGATaGATaGATAGATAGATAGATAGATAGATAGATAGATagATAGA
TTGATaGAAGCAcTTGtTAGATCA**CTAaTaATATAGTCATAACACTGAC**mAACCATaATATTGCATCAAT
AAAGAATTAGAACATTACATTGCCTTGCATTACTATTAAACAATTACATTGtAaCACCAGTAATAAGCAA
TTCTGGCTTAAATGCTGTATTTyGAAATGCTTGACTTGACaAGATGTCCCACTGTCCTGTCACCAAAGGG
AGCCGGGTGACAGCCAAwAAGTACAAGTCmaTTTAAATGCTGTTAACCAACCCCAAAAGGCTCTTAGCcA
AAGCATTGAATGG
PF 45-66, PR 330-306, Repeat: 98-287

StPfl010

ACTGTTGCTTGTNGCATTTATATCTATGTTTACAGGGTCATGCAAAAATGATGTTGTTTATGTGGTTCTC
TTAACAAATCCCTAAGTGTTCTTTATAGAAACTGATCCAAACAAAGCGCAGTG**CTTCACCTTGTTTTTGC**
TCTCATTGGGGTGACCTATCTACCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCGGTCTGTCTG

TCTGCCTGTCTGTCGGTCTGTCTGTCTCTCGTTTCAGATACAACCTACAGTCATANCATNTGAAANAAGT
NCAACACCCATNTGAACTGANTCTCTGT 308
PF: 124-144, Rep: 166-242

Stpfl011

CTGGAGTACATNCNGTATCCATCCATCCATCCCTCCATCCACTCATCCATCCATCCATCCCTCCATATAT
CAATCTGTTCGTAACCCTCATCCATCCATCCATTCATCTATCCATCCATCCATTTAGTCGTAACCCTCAT
CCATCCATCCATCATCCATCTATTCATCCATCA**ATCCATCTGTCAGTAAACCTAACC**CTCATCCATCCAT
CTGTCTGTCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTGTCTGACTGTTTGTCT
GTCTGTCTGTCTGTCTGTCTGTCTGNCTGTCTGTGTGTCTGTCTATCTGACATCTGTCCAAACGTTCAAG
TNTTT**CTGCCCCCTTCTGGACAC**ACAGTGCAATGCCTGCTGTCTTTGGCAAGTTGCAACACAAAGGTTTC
TGAGGTATTACATTATATA 439
PF 174-197, PR 373-356, Rep:203-324

Stpfl012

CTAACATGGAATCTTAATGTCAGCCACAGTTCAATGAAATGAAACAATAATTATTTCATAATGTAAAATT
TCAATGT**GTATTATTTCAAACCCCTACAT**GATCCCCTCACAGCCTGGATGGATGGATGGATGAATGGATG
GATACTTACAAATGAGTGTAGTTCTAATACA**GTATTTGCGTGAgGTCTTGTTAA**AAATGTCTAACATG
208
PF 78-99, PR 194-172, Repeat:116-143

Stpfl013

TTGTGGTCATCACACAGCATGTGGTGCTCACAAACAGACACCAGCCCTGTAGCAGGCTATTGACTAATTC
CTGGTGCTAATGGAAAACCCTATCAAATCCACACTATCAGGCCCAGATTGAGTCTAAACACCAGAACAAC
TCACGTCAGAGGACTCTGCGGAATAAAATGGCTGCAGGTGTGTTTGTGTTGTATGTGTTCGTGACATCGT
CCATAATGAG**TTGGGAAGTTAAACAGGGAGTATC**TCCTCCAGCAGACAGACAGACAGACAGAGAGACAGG
CAGACAGAGAGATAGGCAGACAGAGAGACATGCAGACAGGCGACGAGCGGCGGTTCCG**TAGACAAACAAC**
CAAACGCTCATGTTAAAGTCAAG **373**
PF 221-244, PR 360-339 , Rep 252-310

Stpfl014

CGGCGCATACAGCGCGCGCTCCGCTCCAAATACTTCAAGCAGCTGGGGCGCGAGGAGGGGCAGAGTCGTG
CGCGTCTCACTGAACTGCGCGCGTAGCCGCAGGGGGGCGTGACGGAAACAGACTGATC**TGCAAGAAGAGC**
GGAGGAATGGAAGCAGACAGAGATACAGAGAGACAGACGGGCAGAGTGATAGACACGCAGAGAGAGGCGG
AGAGAGGCAGACAGACAGACAGACAGAAATACTTACAGACAGACAGGCAAGCAAACAGATAGATAGACAG
GCAGACAGACAGGTAAACAGAGAGGCAGGCAAACAGACTGTTCAGTAATGTGTTTGTCAACCACCTGCCC
TGTGAGCAGCTTCGTTTCTGTTCACTCAAGTCAGTGGAAGTGCACTGGACAGATTCTTCTTACTCTTGAC
AGTGGTGGTGGTAAAGAGAGCCCAGTATCCACCCAAAGTCTCCCAGGTG 469
PF 129-149, Rep: 156-255

Stpfl015

CAGAATATGTCTGTGTGTGTGTGTTCCAGATGGCTTCTCCAGCTAACCGGGGAAAAGAACCCAGCATCAC
ATTACAGCCTGCCTTTGTCTCACCTCCCACAGCAGAGTGTGTGTGTGTCCTGTCTAGGTTGACTT**GAGGG**
TTTAGTCTTGTGGTCATTGTTTCCCCTGGTCAGGAAATGGGAGACAGAGTTACACAGACAGACAAACAAA
CGGACGGATGGACAGACAGACAGACAGACAGACAGAC**AGAAAGCAAGCTGATGGCCT**TGT 270
PF: 136-159, PR 267-248, 221-248 rep

Stpfl016

ACATACTGTGCTGTGCGGACTGATTATCTCCAGCCATCAACTAATTGTGTGCATGGTGTGAGGCAAGTCA
TTAACCCCTTAAAGCCTAGAGATTGACGTGTGCCCCACAGTTTACCAGCGCAGC**CTTTAATTGCGCCAGA**
CTGACAGTGACGATTTGTTTTAAATGAAAAAGCAACCAGATCTGATCTAGATAGATAGATAGATAGATAG
ATAGATAGATAGATAGATAGTTAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATT**ACT**
TTATTCATCCCCGAAGGGAAATTAAGTCGTCATAGG **316**
PF 125-147, PR 298-278, Rep: 190-273

Stpfl017

CTCAGTATCTCCTTGGGCGAGAGCTCGTGTGCGGAGTGTGCGTACGAACGTGCACGTGTCTCCAAAGGAG
GATATCAGAGGACATTTAGGGGTAAAGACATGGTGTTAATGACGTGGTTTCATCTTGGACACTGCAATGA
GATTGAAATGGATGCTCATCATAGAAAGAGCAGATTTGTTTATGTCCTTTT**TCCCTTTCTGCATGTAGTG**
ATTCACAGGATAAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAG
ATAGATAGATAGATGGATTAGATTCAACTATCTAGATGATAGATAGATAGATAGATAGATGGATGGATAG
ATTTGACAGTTGACTTTTATCCGTTGATCATATAACACCAGACTCTCTCTCTCGTGGTTTGACAGATGTT
TTCATTCTATATTATTTAGTGGAACCCGTCACGGCCCTGACTCCATGTTGGTGTTAGCTTAGTTGCACCT
CAAGCGGAAAG**TCTCTTTGTCTTCCCTCATCC**CTTCACTGTAATCACnTGATATCCCAGGGATTATGCTT
CCCCTTTGACTTTGGCAAATTCTCTCCTGCAGGAGAATGTAATGACTTGAAACTGTCCCTTGTGAGACAG
nCTAGTTGGAGGGnCTTnTCGGCCnCCnCCGCGGGGAGTCCAAAGGCTTCAATCCAA **687**
PF: 192-213, PR: 522-502, Rep:224-340

Stpfl018

ACTTCCCAGGACCTCTAAGACCACACATCCCAAGTGTTAAACTGATATGTTGAA**TGGTTTGCAAGATATT**
CATTCAACATACAGACAGATAGAAAGACAGACAGACAGACAGACAGACAGACATACATCTGTGGAATTAG
TAGGTAGTTTGAATT**GACAAACGGGCAGTAGGGCT**GTGCAGTGGTTAGTGCTGTCGCTGTTGCTCAGTAG
CAAGAAGGTTCTGGGTTCAAACCTGATGGCCAACCAGGGCTGTTATTTATGGAGTTTGAATGTGCCCCAT
GTCTGTGGG 289
PF: 55-76, PR: 175-156, Rep: 96-123

Stpfl019

CTGCCAGCGGGGGTGAAATTACCCCATCCACCATCGGCAGCAGTGCGGATGGAGTGTCCGGTGAGACGGG
TTGCATGGCGGGAGGAGCATCCGGGCAAAGTATGGAGGAAGGTGCTAGCAAAGCGCCCAAAACTCCCTCA
GTCCGGGCGAGT**GAACGAGGAGGCGACCGACCAG**GAGAGGTGGAGGCCCCGGATTGCAGTGAGGCTCCAA
GGGACGGCGGGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCGGCTGCTGCTGCTGC
GGCTGCGGCTGCTGCTGCTGCTGCTGCTGCTTCACCTAAGGAGCCCAGACNATTCGAGCATGGAGNGTGG
CGGANACAA**GAACATCGGGAGAAGGAGAGTCAA**TGGACTACGAGGCNGTGAAAGCCANCANGAANANAAT
GAGCNNACAGACANGTGAAANAGCCTACACNAACNAGATTAANAAAACAAAAGTGAAAGTCTTGNTTACA
CTCCATCTTCCTTGGCAAACAGAAACAAAAAACAAACATTTAGTCCTTAACCGGNCCGTCACTCGCTNGA
GGAGCTCCANCNTTTTGTTACCCTTTTAATGAGGGTAAAATTGC 604
PF 153-174, PR 383-360, Rep: 222-311

Stpfl020

CCGGTCTCGGGTCTTTGGGCACCGACTCCGCCCACTCGCTCAGAAGAGAGGGGGAAAAATGTCTCTTCTG
ATAGTAAAGGTTTCCTCCCCCTGCTCACTATGGAGAATACATTGCCTCACAAACAGGGGCTTGGTTGTTA
GCTCTGTTTATTCAGTTTAACAGGAGAAGACGGCATGTCTCTCG**ATCGGACCATCTTCAGAACTCAG**AGG
TCACTGTGGCTCTCTCCGAGTGAGTGGGCGGGGTCGGAGCCCCGGTGCGCTGGCAACACACAAACACACA
CACACACACACACGCACACACACACACACACACACACACACACACACACACACTTTGAATTGTTGTGC
AGCAGGACAATGTGGTTAAAAACCTCCTGTTTGTGCCGTTTTGTGCATCAGATNGTGGATGAATTA**ATAA**
TCTAATGTTGGAAGGAGGCCTTCACACAGTTTAACCCCCAGCGTGTGTCAG 471

185-207, 440-417, 267-334

Stpfl021

CTGCTTCCTCCTGCCCCGGCGGCCGGAGAGAGACGCGCTCCAGTGAGAAGGTCTTACCGTGGGGAGTGTG
GTGATGTTGTGTTTCCTTTGGTGGGTTTTAAACAAGATTAAAGTTTCTGAATGTCT**GTGCGCACTCCGCG**
GCTCATCATCCTCCTCATCATCATCATCATCATCATCCTCCTCCTCCTCCCAGCAAGCANG**CGTGTCCCC**
CGCCCCAAACTGAGCCTTATTTGGTAAAGAGCAGGAACCTTCTCCCGATGAAGCCTCCACTCACTACACC
CAAGTCCACCCAGTCCACCACAGCCCTGCTGCATCGAGGACCATCAGGCGTGCAG 335
PF 127-147, PR 222-202, Rep 156-176

Stpfl022

CTGCACCAATCAGCGACTTCCTCCGTGTCTGGCTTCTSTCTCAACCCCTCTCATTTCACCCTGCGGCCGC
TTCACTGCGTCACAACACCTGCTCCCCTGGTCGCCACACAGATCCAT**GCACTCACCTTTCACTTCAACCT**
TATCTGTGTTKGAAATCACAACTCTTTTTTCATCTTTCTACAAAGTATCCATCTATCTATCTATCTATCT
ATCTATCTATCTATCTATCTATCTATCTATCAGTTCAAACTCACATCYTGCCRCTTGTCATCGGTAAC**CT**
TTATCTTCTCGCGCTGCTTCTGCAGATCCATCTATCT 317
PF 118-141, PR 302-279, Rep 193-240

Stpfl023

CCGGTGACAAAACACACCAGCCGGTCAAACTGTGTCACTCGCTAATCCTCCCCAGTTCAAAACCAGGGAG
AATGGACTGGTTTGGTTTGGAGATAACTAATTAGTAACTTCTCTGTGGT**GATTAGGGCTTCAGGGCAGAG**
TGTGTCGCTCGGTGTTTATCGCAGGTGAGGCTCTCT**CACACACACACACACACACACACACACAAACACA**
CATACACACACCATGATG**CTGTAATGATCCCCGGTGCTC**AGGACCGACAGCAGACTCTTGGGTCGCCCCT
CTGTAAGTGATGACCTTTGGCTGAACAGAGACGAGGTTCTTATTTTGTCCCTGAACATCAGATTGACAGA
CGCTGGCCGGTTGATGCCTGATGACATCATCCTCAAACAGATTTACAGTAACAGANAGCGACAGGTTCTA
CACCGTGACAGAGGTGTGGAGTTAAACATGTGCAGATCAAGCTCGATCTCTGCGCATACACTTNGCCCAT
AGATCACCCATGAATGGCCCACTGTCTCTCCCCTGTNTGTNCCCTGTCTGTNCACCCCGTCTTGTCCCCC
TGTNTCTTAACCTGTCTTGTCCNCCTGTCTGTCCCTNTGTCTGTCCCG 608
PF: 120-141, PR: 249-229, Rep: 177-220

Stpfl024

CTTTCTGTGTTTCCAGTGAAACCTCGTGACCGGGCTCACATGTCGGACCTGCTCGTTGCTCGTGGACGAG
ATGCTCGACTCGGCTTCGTCTGTCCGCTGTGGTCTGTCTGGTCTGTCTGGTCTGTCTGGTCTGTCTGGTC
TGTCTGTCCGCTCTGGTCTGTCGGGCCTC**GCGGCGCCTCCACCACCTC**CACCGCCCTCCTCTTCCTCCTC
CACCACATCGT**CCTCCTCCTCCTCCTCCT**CCACTTCCTGCAGCGGCTGCTGCTGGCTCTGG**CTCTGTTGA**
CGGTGCGGCAGGCTGCTTTCCTCCATCTTTGTTTTCCAGTAGCCGTCCCTCCCTCAGGAAACGCTGGTAG
ACCTCAAG 358
PF: 170-188, PR: 294-272, Rep: 222-239

Stpfl025

GAATTATGAGC**TTCAACAATGACCAAGGGGCATCA**GATCTGTAATANAGTTATCTGTAGCGTTGTGTGTG
TGTGTGTGTGTGTCTGTCTGTCTGTGTGTGTGTTGGAGAGAAGTTGTATGAAGGTGTTGCACATTATCAC
CATGAACAAAAAGG**TCTCAGGTGTTACAAGATTGCAAA**AACTGCATTAGTT
PF 12-35, PR 178-155, Rep: 64-103

Stpfl026 (minisatellite repeat)

CTGATCGACTCCCGCTTCN**AGACTTCACATGCCAAGGTCAA**ACACTGTMTGTCCCTCTGTC
TGTCCCTCTGTCTGTCCCTCTGTCTGTCCCTCTGTCTGTCCCTCTGCCCGTCCGCCCCTCTGTC

TGTCACTCTGTCTGTCCGTCCCTCTGTCTGTCTCTCTCTGCTTGTCCCTCTGCCCGTCCGCCCC
TCTGTCTGTCCCTCTGTCGGCCTGTCCCTCTGTCCGTCTGTCCCTCTGTCTGTCTCTCTGTCTG
TCCGTCCCTCTGTCCGTCTGTCTGTCCCTCTGTCTGTCCCTCTGTCTGTCCCTCTCTGCTTGTC
CCTCTGTCTGTCTGTCCCTCTGTCTGTCCCTCTGTCTGTCCCTCTGTCTGTCTGTCTGTCCCTG
ATGGTTCTAACGTC**TCTCTACCTCAGGGTTCTCTGA**GCGTCTCTMAG 428
PF 20-41, PR 417-396 Rep 53-374

Non-Enriched libraray

Stpfl027

CTGACTGGACGTCTCTCTGAGCATGAACCT**GGTGACGAACTGACCCAACACA**CTGGAGCCCTGGTACTCC
TGAGGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAAACAAAGA**AGCAGATGTCAAAGCCCGA**
ACTGAAGCAACGGCAAAGTAAAAGCATCTCAGGGACAAAACAGAAGGGAAAGATAACTTTGAATAACTTT
CAACCAAAACAAATATTCCAGGCACCTTTGTGATGTGTCAGCCGGTATGAAACCCTACTAATTAATTYTC
TTGACACCCACATGTATAATTCATTAGTACAAATCCAAATTCCAAGGTGAAGGAGCTCAGCGGGGCATCC
CTCATGACTGAGGGCCCTCGTCTGTGTGGTAACGACTGGGTTTTTCAGCAGGACAACGGCTCCAACTTCA
CAATGCCCGTCTGACCAAGACTTTTTTCCAGGAGAATAACATCACTCTTTTGGACCATCCTGCGTGTTCC
CCTAATCTTAATCCAATTGAGAACATTTGGGGATGGATGGCAAGGGAATTTTACAAAAATGGACTTCAGT
TCCAGACAGTGGATGCCCTTCATGAAGCCATCTTCACCACTTGGCGCAACATTCGCACTAGCCnTTTTGG
AAACACTTGCATYAAGCATGCCAAAACGAATTTTTTGATGTGATCAACMATAATGGTGGAGA 692
PF: 31-52, PR: 143-122, Rep: 76-113

Stpfl028

CTGCTCCTCACAAAGAGAGACACACACACACACACACACACACTCAATACCAACACCAGCACCATCCTGGCC
TTCGCTTCCAATTTGATGAATTTTTTCCATTTGAGTTCTTTTAAAAATTCATCCAGTGAGAAAAATCTTGT
TTTTTGTGACCCGACTGCTGGAGGGAGGAGGGAAATGAGAGCGGTCTTCTGTGRAGTTGG**ACCGGAGGAA**
CTCGCTTTTTAAAGCGCGGCTCTTTGYTCCCACATGCAGCGCCATGGGACACGTGTGTGTCTGTGTGTGT
TTGTGTGTCTGTGTGTGAGTGTCTTACAGATG**TATATGTTCATGTCAGCAGGCATC**TTGAGTCGG 345
PF 201-224, PR: 336-313 Rep 263-296

Stpfl029

CTWCGTCTTCTSKCTACTAACACATTTTAnCAGGTGTGYATCACATCAATGTTCC**TTTATCTAGCAACAC**
CTCAATGTnTAGTGWCATTGYTCCAATTGTGWGATAGTAGAGCCSAGAGWTAGAGAKTASATAGAGTAAn
GAGAGAGAGAGAGAGAGACATTGCAAGACATA**AAGATTCCTTTGATCTAGGACC**ACTGACAATGTCACTG
CAGGAGTGGAGTTGTTTGTGTGACATAAAATCGATCCCTGAGTCAGTTTTTATCTCCCATCTTGCTGAGA
TGACACTCATCTCAATTTGAAG 302
PF: 56-77, PR: 194-173 Rep 141-158

II.2 Inheritance of microsatellites – Allele data

Inheritance of microsatellite loci was tested in Chapter three by genotyping parents and offspring from known families. Allele data of these genotypes are presented below from four families tested at three loci. For further details see Chapter three – Isolation and characterisation of microsatellites in the European flounder.

Table II.1: Inheritance of microsatellites – allele results from Family One

Locus:	3B3		3F10		4H4	
Male 1	244	248	146	153	166	170
Female 1	248	290	144	146	166	170
Offspring 1	244	290	144	153	166	170
Offspring 1	244	290	144	146	166	166
Offspring 1	244	290	144	153	166	170
Offspring 1	244	248	146	146	166	170
Offspring 1	0	0	146	153	166	166
Offspring 1	244	290	144	153	166	166
Offspring 1	244	290	144	146	166	166
Offspring 1	244	290	146	153	166	170
Offspring 1	244	248	146	146	166	166
Offspring 1	248	290	144	153	170	170
Offspring 1	248	248	146	146	170	170

Table II.2 Inheritance of microsatellites – allele results from Family Two

Locus:	3B3		3F10		4H4	
Male 2	248	260	146	153	162	166
Female 2	252	252	144	146	162	166
Offspring 2	248	252	144	146	162	166
Offspring 2	252	260	146	153	162	166
Offspring 2	252	260	146	146	162	162
Offspring 2	252	260	144	153	162	162
Offspring 2	248	252	144	146	162	166
Offspring 2	252	260	144	146	162	166
Offspring 2	252	260	146	146	166	166
Offspring 2	252	260	146	146	162	162
Offspring 2	248	252	146	153	162	166
Offspring 2	248	252	144	146	166	166
Offspring 2	248	252	146	153	162	166
Offspring 2	252	260	146	146	166	166

Table II.3: Inheritance of microsatellites – allele results from Family Three

Locus:	3B3		3F10		4H4	
Male 3	248	253	146	148	167	167
Female 3	248	260	144	153	165	167
Offspring 3	252	260	146	153	167	167
Offspring 3	253	261	144	148	167	167
Offspring 3	248	260	144	146	167	167
Offspring 3	248	260	144	146	167	167
Offspring 3	248	248	146	153	167	167
Offspring 3	253	260	146	153	167	167
Offspring 3	248	248	148	153	167	167
Offspring 3	248	260	144	146	167	167
Offspring 3	248	253	146	153	167	167
Offspring 3	248	248	146	153	165	167
Offspring 3	248	248	144	146	165	167
Offspring 3	248	260	148	153	167	167
Offspring 3	248	253	144	148	167	167
Offspring 3	248	253	148	153	167	167
Offspring 3	0	0	0	0	0	0
Offspring 3	248	260	148	153	167	167
Offspring 3	248	253	146	153	167	167
Offspring 3	248	260	148	153	167	167

Table II.4: Inheritance of microsatellites – allele results from Family Four

Locus:	3B3		3F10		4H4	
Male 4	260	266	148	153	165	167
Female 4	256	260	153	153	167	167
Offspring 4	256	266	153	153	167	167
Offspring 4	0	0	153	153	167	167
Offspring 4	256	260	148	153	167	167
Offspring 4	260	266	148	153	165	167
Offspring 4	256	260	148	153	167	167
Offspring 4	256	266	148	153	165	167
Offspring 4	260	260	148	153	167	167
Offspring 4	256	266	153	153	167	167
Offspring 4	256	260	153	153	167	167
Offspring 4	0	0	148	153	167	167
Offspring 4	260	260	153	153	167	167
Offspring 4	0	0	153	153	167	167
Offspring 4	260	260	153	153	167	167
Offspring 4	256	260	153	153	167	167
Offspring 4	260	266	153	153	167	167
Offspring 4	256	260	153	153	167	167
Offspring 4	260	260	153	153	167	167
Offspring 4	256	266	153	153	167	167

Appendix III The European flounder CYP1A gene

III.1 *Platichthys flesus* cDNA for Cytochrome P4501A (annotated)

From (Williams, Lee, Sheader & Chipman, 2000)(EMBL Acc. No. AJ 132353)

Annotations are detailed below sequence. Exons are coloured differently to differentiate (i.e. Exon1|Exon2|Exon3|Exon4|Exon5|Exon6|Exon7)

attaagagagtaagctcagacaataactcaactgaactctttctatttttctgcaaaccacttttgggg
atcacgtcatcgctcctcactgaccgcttcaccgag|attgtgttgaaggcagaaaaaaaagtcgtcatt
atgatgctaatgatgctaccgttcattggatctgtgtctgtatctgagagtttggtggccatgacaacga
tgtgtctggtctacctgattcttaagtttttccaaactgagattcctgaagggctccttcggcttcctgg
gccgaagcccctgcctatcattggcaatgtgcttggattgggcagcaaaccttacctgagcctcactgac
atgagcaagcgatacggccacgtcttccagatccagatcggcatgcgtcctgtggtggtgctgagcggca
gcgaaacggttcgacaggcgctcatcaagcaaggggacgactttgcaggcagacccgacctgtacagctt
ccgcttcatcaatgccggcaagagtcttgccttcagcacggaccaggccggcgtctggcgtgcccgcaga
aagctggcctacagtgccctccgctccttctccaacctggaggggacaactccagaatactcctgtgttc
tggaggaacacatctgcaaagagggggagtatctcatcaaacagctcaacactgtcatgaaggccgatgg
cagctttgacccattccgccacattgttgtgtctgtggccaacgtgatctgcggaatgtgctttggccga
cgctacgaccacgacgatcaggagctggtcggcttggtgaccctcagtgatgagtttggccgagtggtgg
gcagtggaaaccctgcagacttcatccccatccttcagtacctgcccagcgcagcaatgaagaacttttt
gcgcattaatagccgcttcaccgagttcgtgcaaaagatcgtcaccgagcactacaccacttttgacaag
|gacaacatccgagacatcacagactcccttattgatcactgtgaggacaggaagctggatgagaactcg
aatgtccagatgtcagacgagaagattgtaggaattgtcaacgacctgtttggagctg**g|attcgatacc**
gtctctactgccctgtcatggtcggtcatgtaccttgtggcgcacccagagatacaggagagactttatc
aagaaatag|aggacaaagtgggtctggatcgcatgcctcttctctctgataaacccaatttgcctttcc
tggaagcctttatcctggagatccttcgccactcttcattcctgcccttcaccatcccacactg|cacca
ctaaagacacatctctgaatggctactttattcccaaagacacatgtgtcttcatcaatcagtggcagat
caaccatgatcc|agagctgtggaaagatccatcttc**cttcaacccagatcgcttcct**gagcgccgatgg
ctccgaggtcaacaagcttgatggcgagaaggtgatggcc*ttcggcatgggaaagcggcgctgcattggc*
*gaggtcatcgca*cggaatgaagtctacctcttcttggcgatcatcattcagaagctgcacttcctcccga
tcccgggcgagaagctagacatgacacctgaatatggtctcacaatgaagcacaaacgctgccacctgaa
agccacgatgcgagcaaggaacgagcat**tga**agctcatcgccacgtacaatacgtgactcagcaggtcag
attaagagtcagtggaggaagaataatctctctctgaatgtagaaggcttgattccagatttgatgttca
gagtgtgtggcattgaagcatataagtgacatgtgtctgttgcggagtgtcagaggtgtctgggtttttg
tgaacatatctctgctatgatttaagtcaaagagatattcctgcacgaggcagattcttctcagtgaagt
ataaggagatccttggttcatctgttgtgttgtccactaatctgcttccattcagaagttgtcttgttca
actttgggacagacagtatatggtttaattggtaggcattatcattgcaacatatttttttctattgaga
acaaatatcaccttatgacaccatttatacttgtaagctacttttaattccattataatgtcgacgtaa
gacactgaagatatatttgtatcccagaatgtgattttgtgtgtatatcaagttctttcgtatttttgga
aaataactatttttttgtgctcagtgtttgtatatatttgaggtgaaagtgtttttctgtgcatctacatg
atttatgagaataccaaaagagttccagggatgttttgaatatattagtatgcaacacatttttgtattt
ctctctctaatgtattgatgattgtctatgatgtaaggatggagcattctttaatcagccatacaaataa
agtaaaacaaatgtttgaattaatcctcctcattgtattctttgcttaatttaaaaaacaaacaacatcc
ccgtgcactgcag

Sequence	Note
atg	Initiation codon
gattcgataccgtctctactgc	Real-time PCR forward primer binding region
cttcaacccagatcgcttcct	Real-time PCR reverse primer complement
tga	Termination codon
ttcggcatgggaaagcggcgctgc attggcgaggtcatcgca	Heme binding region

III.2 Statistical results for chapter 5

III.2.1 Regression analysis of physical characteristics and *Ah* battery gene expression

All genes studies were correlated with physical characteristics to ensure that no variability in gene expression was caused by length, weight or condition factor. Values of the regression analysis (R^2) are provided in Table III.1 below.

Table III.1: Regression analysis of physical characteristics and expression levels. Values given are correlation coefficients (R^2), all are not significant.

Gene	Length	Weight	Condition factor
CYP1A	0.026	0.028	0.001
GSTA	0.060	0.067	0.022
AhR	0.135	0.152	1e-05

III.3 Raw data for Aroclor 1254 trial

III.3.1 Aroclor exposed flounder

ID	Family	Group	CYP1A	GSTA	AhR2	ARNT2	Length	Weight	Condition factor
A01	3	c	19.6	14	103	150	5.9	2.1	1.022
A02	2	c	8.5	38	0	24	8.4	7.5	1.265
A03	2	c	10.6	68	192	107	5.6	2.7	1.537
A04	2	c	41.8	23	174	82	7	5.3	1.545
A05	7	d	31.9	30	128	114	4.7	1.8	1.734
A06					145	160	5.3	2.17	1.458
A07	2	c	32.3	16	169	171	5.4	2	1.270
A08	2	c	17.1	57	178	137	6.3	4.2	1.680
A09	7	d	15.0	36	164	89	5.7	3.4	1.836
A10					141	89	5.2	1.9	1.351
A11					205	152	7.4	4.8	1.185
A12					198	127	7.1	4.9	1.369
A13	2	c	67.3	29	139	99	4.4	1.3	1.526
A14	2	c	28.5	35	135	94	7	5.7	1.662
A15	2	c	39.1	23	166	146	6.9	4.6	1.400
A16					0	21	4.7	1.3	1.252
A17	1	c	13.1	43	181	101	6.5	4.4	1.602
A18	2,8	c	39.7	44	229	123	8	6.5	1.270
A19	3	c	27.0	73	132	81	6.1	3.1	1.366
A20	2	c	22.3	13	106	206	5.9	1.9	0.925
A21	5	d	17.6	28	173	92	4.2	1.1	1.485
A22	3	c	29.1	31	117	75	4.4	0.8	0.939
A23	2	c	29.2	51	122	86	7.3	4.3	1.105
A24	2,3,8	c	19.9	10	102	75	5	1.5	1.200
A25	2	c	39.7	52	148	109	5.4	2.4	1.524
A26	3,8	c	13.4	6	176	102	5	1.7	1.360
A27	1,2,3	c	10.4	11	118	94	5.2	1.8	1.280
A28	1	c	25.1	11	137	113	4.5	1.1	1.207
A29	2	c	11.4	24	112	135	4.2	0.9	1.215
A30	1	c	38.9	7	73	90	5.2	2.1	1.494
A31	3	c	34.2	41	116	96	8.2	5.6	1.016
A32	7	d	5.6	32	78	92	4.9	1.8	1.530
A33	3	c	16.3	18	207	109	5.5	1.9	1.142
A34	3,8	c	15.2	65	151	89	6	3.5	1.620
A35	2,8	c	6.6	41	171	118	6.8	4.3	1.368
A36	2	c	30.5	53	191	110	6.8	4.5	1.431
A37	1	c	13.4	14	123	79	5.6	2.3	1.310
A38	2	c	11.3	12	87	94	5.6	2.4	1.367
A39	2	c	11.8	22	106	127	4.4	1.1	1.291

ID	Family	Group	CYP1A	GSTA	AhR2	ARNT2	Length	Weight	Condition factor
A40	2	c	46.8	43	182	115	6.4	3.9	1.488
A41	2	c	10.0	13	169	89	5.5	2.6	1.563
A42	2	c	19.6	0		14	5.4	2.8	1.778
A43	3,8	c	11.7	17	315	132	8.5	7.3	1.189
A44	1	c	5.6	44	266	143	6.5	3.8	1.384
A45	2,8	c	0.0	532		11	4.6	1.3	1.336
A46	1	c	11.4	9	108	76	5.6	2.3	1.310
A47	1	c	36.3	43	129	73	5.6	2.1	1.196
A48	3	c	29.4	34	89	82	6.2	2.9	1.217
A49	2	c	41.4	33	315	81	6.8	4	1.272
A50	3,8	c	24.7	8	325	67	6.5	3.2	1.165
A51	3	c	55.9	74	319	84	6.8	3.9	1.240
A52	2,8	c	15.9	24	245	64	5.6	3	1.708
A53	1	c	63.2	18	172	66	7	3.7	1.079
A54	2,8	c	54.8	65	243	76	5.5	2	1.202
A55	3	c	39.6	11	204	47	5.6	2.7	1.537
A56	1	c	10.6	4	203	71	7	5.4	1.574
A57	2	c	24.1	50	331	84	6.7	4.3	1.430
A58	2	c	45.1	22	252	70	6.8	4	1.272
A59	3	c	36.9	15	275	93	6.4	3.4	1.297
A60	2,8	c	18.8	92	318	97	5.5	2.9	1.743
A61	3	c	31.7	30	310	98	5.5	2	1.202
A62	5	d	10.6	13	213	82	5.8	3.1	1.589
A63	1	c	14.1	9	65	76	4.6	1.6	1.644
A64	7	d	16.7		180	45	6.7	3.6	1.197
A65	7	d	9.5		179	81	6.4	3.3	1.259
A66	1	c	115.1		330	87	6.2	3	1.259
A67	5	d	49.7		172	77	5.4	1.6	1.016
A68	1	c	7.2		318	92	6.4	3.5	1.335
A69	5	d	29.6		299	75	4.3	0.9	1.132
A70	3	c	72.6		290	68	5.7	2.2	1.188
A71	3	c	19.1		207	53	8.7	7.3	1.109
A72	1	c	31.2		285	70	7.2	6.6	1.768
A73	1	c	18.0		0	22	4.5	0.96	1.053
A74	2	c	76.3		49	39	5.2	1.9	1.351
A75	5	d	41.7		344	111	6.2	2.8	1.175
A76	3	c	40.6		238	94	7.2	6.1	1.634
A77	4,5	c,d	72.8		179	98	8.2	2.3	0.417
A78	1	c	27.6		148	83	9.2	3.1	0.398
AT3	2,3	c	9.5						
AT4	2,3,8	c	49.6						
AT5	1	c	24.6						
AT6	2	c	14.2						
AT7	5	d	21.6						
AT8	2,8	c	66.0						

ID	Family	Group	CYP1A	GSTA	AhR2	ARNT2	Length	Weight	Condition factor
AT9	5	d	23.1						
AT10	8	c	23.8						
AT11	1	c	70.2						
AT12	2	c	50.6						
AT13	5	d	13.1						
AT14	2	c	89.3						
AT15	2	c	17.4						
AT16	2	c	19.4						
AT17	7	d	12.5						
AT18	3	c	15.4						
AT19	1	c	11.5						
AT20	5	d	11.1						

III.3.2 Control flounder

ID	Family	Group	CYP1A	GSTA	AhR2	ARNT2	Length	Weight	Condition factor
AC01	2,8	c	24.0		0	0	6	2.8	1.296
AC02	2	c	39.5		108	110	7.5	5.5	1.304
AC03	0	0	31.3		225	137	8.2	5.5	0.998
AC04	2	c	16.3		247	116	6	2.9	1.343
AC05	2	c	22.1		190	119	6.6	3.4	1.183
AC06	2	c	24.9		202	126	7.2	4.2	1.125
AC07	3	c	20.0		198	111	7	4	1.166
AC08	3	c	7.3		155	91	6.5	2.6	0.947
AC09	1	c	22.9		200	95	5.1	1.8	1.357
AC10	0	0	18.0		357	122	5.6	2.5	1.424
AC11	4,5	c,d	21.6		178	92	4	0.8	1.250
AC12	2,8	c	10.9		0	0	7.7	5.6	1.227
AC13	1	c	11.3		304	115	5.5	2	1.202
AC14	2	c	7.7		329	104	4.6	1.4	1.438
AC15	8	c	19.0		715	234	5.4	2.2	1.397
AC16	3	c	30.0		173	69	7	3.4	0.991
AC17	7	d	27.7		25	51	5.8	2.8	1.435
AC18	3	c	28.1		174	89	5.2	2.1	1.494
AC19	2	c	15.1		100	67	5.6	2.9	1.651
AC20	2	c	9.9		118	80	5	1.87	1.496
AC21	2	c	9.8		232	107	6.5	4	1.457
AC22	2	c	6.6		89	105	5.4	2.3	1.461
AC23	4	c			233	91	5.5	2.3	1.382
AC24	2	c			107	70	4.9	1.9	1.615
AC25	1	c	8.7	19	122	67	7.4	5.8	1.431
AC26	3	c	7.5	21	531	108	6.7	3.9	1.297
AC27	1	c	6.6	18	431	92	6.4	3.1	1.183
AC28	2,8	c	6.7	6	242	65	4.5	1.1	1.207

ID	Family	Group	CYP1A	GSTA	AhR2	ARNT2	Length	Weight	Condition factor
AC29	0	0	2.0	6	291	94	4.7	1.8	1.734
AC30	0	0	3.0	14	254	96	7	4	1.166
AC31	2,8	c	14.0	34	391	125	6	3.6	1.667
AC32	0	0	9.0	14	425	127	4.3	1	1.258
AC33	2,8	c	4.9	4	0	2	4	0.6	0.938
AC34	5	d	3.2	14	193	79	8.6	7.2	1.132
AC35	2	c	11.2	22	162	65	7.2	4	1.072
AC36	2	c	10.9	40	294	131	6	2.9	1.343
AC37	0	0	4.5	9	216	116	5	1.4	1.120
AC38	1	c	8.8	30	190	97	5	3.3	2.640
AC39	2	c	7.6	40	151	63	6.4	3.3	1.259
AC40	2	c	5.2	26	209	84	6.5	4.8	1.748
AC41	2	c	8.0	48	251	79	7.4	5.3	1.308
AC42	3,8	c	4.9	35	129	70	8.1	6.3	1.185
AC43	3	c	5.2	13	80	100	5	1.4	1.120
AC44	2	c	2.2	12	172	112	4.9	1.5	1.275
AC45	5	d	3.3	17	0	0	4	0.7	1.094
AC46	2	c	0.5	18	184	95	7.1	4.4	1.229
AC47	2	c	4.4	36	314	151	5.7	2.8	1.512
AC48	1	c	3.5	13	256	71	7.1	4.5	1.257
AC49	2	c	4.2	28	92	48	5.2	3	2.134
AC50	1	c	4.5	13	113	85	4.8	1.7	1.537
AC51	3,8	c	9.4	40	261	119	6.9	3.8	1.157
AC52	0	0	9.2	23	187	117	5.4	2.7	1.715
AC53	0	0	8.3	0	292	155	6	2.8	1.296
AC54	1,2	c	5.9	16	118	71	8.2	6.1	1.106
AC55	2	c	8.3	56	190	59	7.2	4.6	1.232
AC56	0	0	12.7	63	416	88	8.3	7.4	1.294
AC57	0	0	23.6	55	131	39	7.2	4.7	1.259
AC58	3	c	15.4	58	76	24	5.8	2.6	1.333
AC59	0	0	9.7	68	372	126	7	4.1	1.195
AC60	0	0	12.3	165	142	72	6.8	3.9	1.240
AC61	2	c	9.4	38	279	102	6	2.9	1.343
AC62	0	0	9.0	7	185	99	5.7	2.2	1.188
AC63	0	0	5.5	18	203	119	6.6	3.3	1.148
AC64	4,5	c,d	13.2	28	404	116	7.5	5.5	1.304
AC65	7	d	11.4			72	6.6	4.1	1.426
AC66	0	0	16.9			91	6.8	4.1	1.304
AC67	1,2	c	17.5			55	6.2	3	1.259
AC68	0	0	10.1			92	7.4	4.7	1.160
AC69	3,7	c,d	5.2			158	5.9	2.5	1.217
AC70	0	0	7.9			0	4.5	1.2	1.317
AC71	1,2	c	20.8			135	6.8	4.1	1.304
AC72	2	c	20.6			145	6.6	3.6	1.252

Appendix IV Recipes

IV.1 Buffers

DNA extraction buffer

1mM Tris-HCl pH 7.0

1mM EDTA

0.1mM 2-Mercapthoethanol

Cell lysis buffer (DNA extraction)

Puregene®, supplied with kit

0.5M EDTA

186.1g disodium ethylenediaminetetra-acetate.$2H_2O$

Add 800mls nanopure water with constant stirring.

Adjust pH to 8.0 with NaOH pellets (~20g) and adjust volume to 1L with nanopure water. Sterilise by autoclaving.

20X SSC

175.3g NaCl

88.2g Sodium citrate

Make up to 1L with nanopure water. Adjust pH to 7.0 with 10N NaOH. Sterilise by autoclaving.

10% SDS

Add 10g SDS powder to 100mls nanopure water.

1M Tris·Cl

121.1g Tris base

Make up to 1L with nanopure water. Adjust pH by adding concentrated HCl:

PH	Volume HCl
7.4	70ml
7.6	60ml
8.0	42ml

Allow solution to cool to room temperature prior to checking pH. Adjust volume to 1litre with nanopure water. Sterilise by autoclaving.

50X TAE (Tris Acetic acid EDTA)

working concentration = 0.5 – 1.0 X

242g Tris base

Dissolve in nanopure water. Add:

57.1mls glacial acetic acid

100mls 0.5M EDTA (pH 8.0).

Make up to 1 litre with nanopure water and sterilise by autoclaving.

5X TBE (Tris Boric acid EDTA)

working solution = 0.5X

108g Tris base

27.5g boric acid

20mls 0.5M EDTA (pH 8.0).

Make up to 1 litre with nanopure water. Sterilise by autoclaving.

NB TBE is stored at 5X concentration to prevent any precipitate forming.

TE Buffer

0.1mM EDTA

10mM Tris

Dissolve in nanopure water. Adjust pH to 8.0 with dilute HCl. Sterilise by autoclaving.

Modified TE λ Buffer (1/10th EDTA, pH 8.0)

0.01mM EDTA

10mM Tris

50pg/µl λ *Hind*III

Dissolve in nanopure water. Adjust pH to 8.0 with dilute HCl. Sterilise by autoclaving.

10× Taq polymerase buffer (Promega)

50mM KCl

10mM Tris-HCl pH 9.0

0.1% Triton X-100

Neutralising solution

1M Tris, pH 7.4,

1.5M NaCl

Denaturation solution

1.5M NaCl

0.5N NaOH

Hybridisation / pre-hybridisation solution (bacterial colony lift filters)

6X SSC

0.1% SDS

6X Denhardts solution.

NB: Critical that pH of solution = 7

Ethidium Bromide (10mg/ml stock)

1g of ethidium bromide

Make up to 100mls with nanopure water by stirring for several hours. Store at room temperature in light proof container.

ABI 377 Sequencing Gel Loading Buffer

98% deionized formamide,

10mM EDTA (pH 8.0)

0.025% xylene cyanol FF

0.025% bromophenol blue

10% APS (Ammonium Persulphate)

1g APS

Make up to 10mls with nanopure water.

Solution can be stored for a 2-3 weeks at 4^{o}C.

Proteinase K

Dissolve proteinase K in deionised water to a concentration of 20mg/ml. Store at -20^{0}C.

RNAase A (DNAase free)

Dissolve pancreatic RNAase A at a concentration of 10mg/ml in 0.01M sodium acetate (pH 5.2). Heat to 100^{0}C for 15 minutes. Allow to cool, slowly, to room temperature. Adjust the pH by adding 0.1 volumes of 1M Tris·Cl (pH 7.4). Store at -20^{0}C.

IV.2 Bacterial Media

1M Glucose solution

18g glucose

Adjust the volume to 100 mls with nanopure water. Sterilise by filtration through a 0.22 μM filter.

LB Medium (Luria-Bertani medium)

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

Make up to 1L with nanopure water. Adjust pH to 7.0 with 5N NaOH. Sterilise by autoclaving.

LB Agar (1L)

10g NaCl

10g Bacto-tryptone

5g Bacto-yeast extract

20g agar

Make up to 1L with nanopure water. Adjust pH to 7.0 with 5N NaOH. Sterilise by autoclaving.

NZY Broth

10g NZ amine (casein hydrolysate)

5g Bacto-yeast extract

5g NaCl

Make up to 1L with nanopure water. pH to 7.5 with 5N NaOH. Sterilise by autoclaving.

NZY$^+$ Broth

To a 100ml aliquot of NZY Broth (see above) add the following supplements immediately before use:

1.25mls 1M MgCl2 filter sterilized

1.25mls 1M MgSO4 f/s

1ml 2M filter sterile glucose

X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)

Dissolve X-gal in dimethylformamide to make a 20mg/ml solution (use a glass or polypropylene tube). Wrap in aluminium foil or store in light-tight bottle at –20^{0}C.

IPTG (Isopropylthio-β-D-galactoside)

Dissolve 2g of IPTG in 8 mls distilled water. Adjust volume to 10mls with distilled water and sterilise by filtration through a 0.22-micron filter. Store at –20^{0}C.

Ampicillin

Dissolve in deionised water to a concentration of 100mg/ml. Sterilise by filtration through a 0.22 micron filter. Store at –20^{0}C.

Tetracycline

Dissolve in ethanol to a concentration of 5mg/ml. Store at –20^{0}C in a light-tight container.

IV.3 Formulae:

Primer Tm calculation:

69.3 + 0.41 x %GC content - (650 / length) = melting temperature

Vector : insert ratio

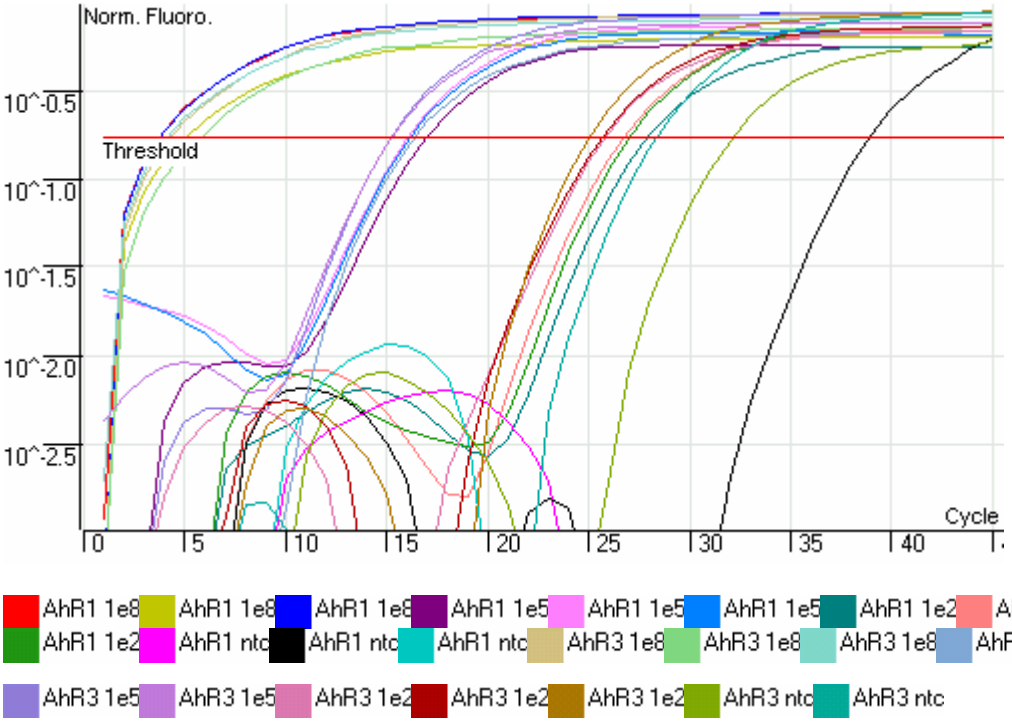
$$\frac{\text{ng vector x Kb size of insert}}{\text{Kb size of vector}} \times \text{Molar ration of } \frac{\text{insert}}{\text{vector}}$$

$$(\text{Xg} / \mu\text{l DNA} / [\text{plasmid length in bp} \times 660]) \times 6.022 \times 10^{23} = \text{y moles} / \mu\text{l}$$

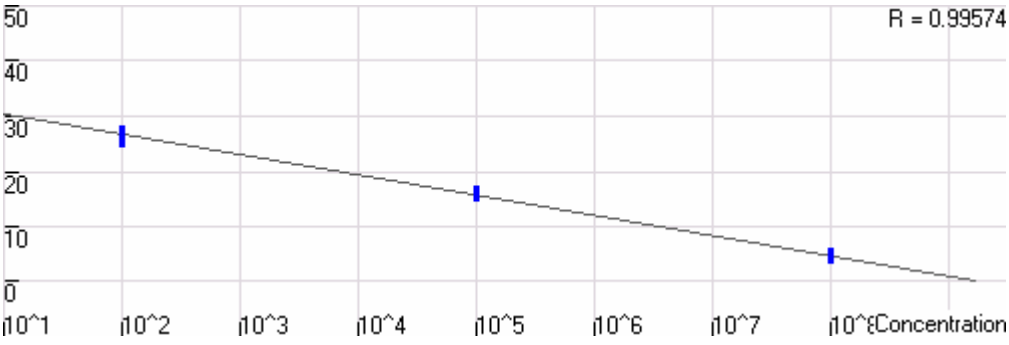
Equation 1: Calculation of plasmid construct copy number

Appendix V Real-time PCR

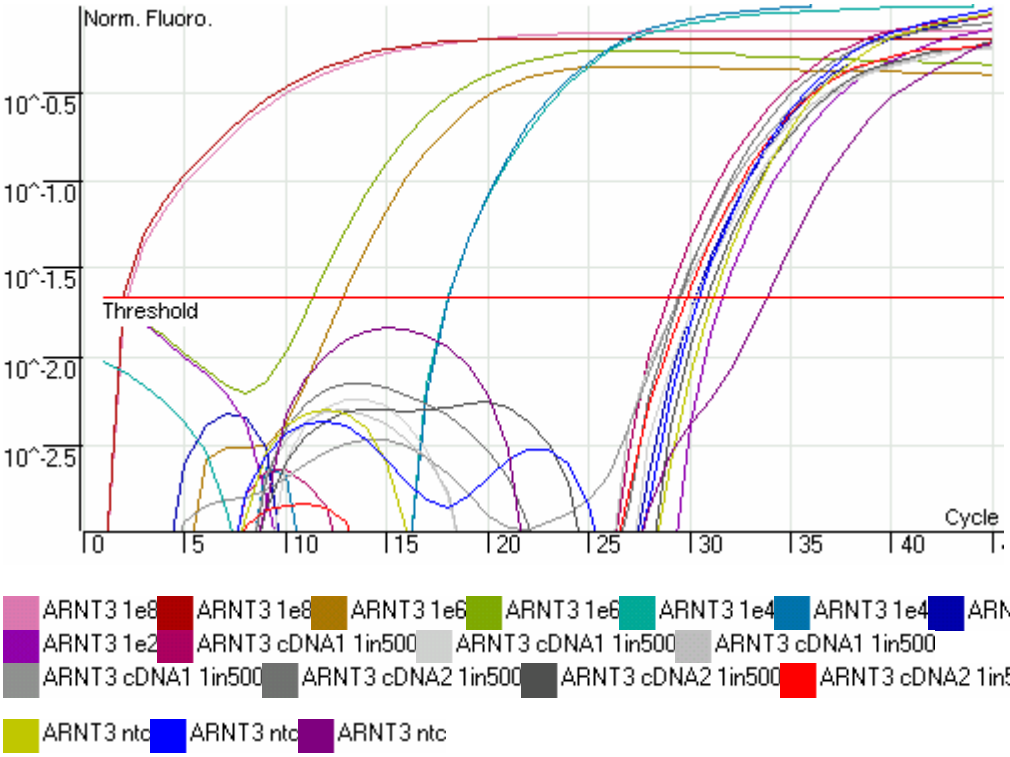
V.1 AhR2 standard curve



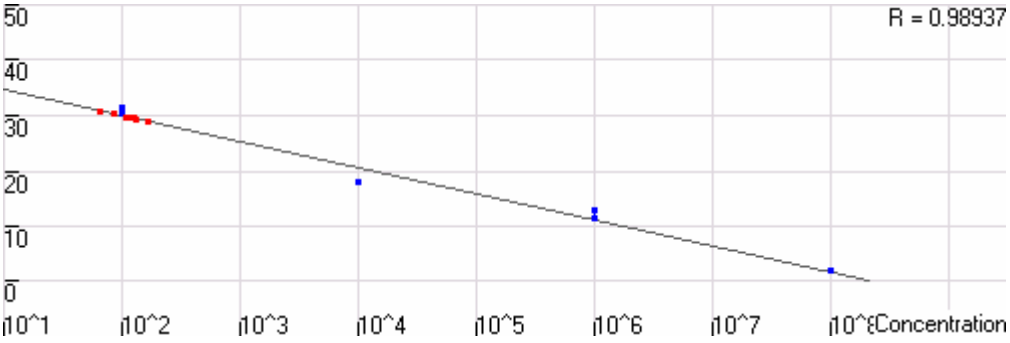
Standard curve



V.2 ARNT2 standard curve



Standard Curve



This report generated by Rotorgene Real-Time Analysis Software