CHAPTER 1 Construction of the Panel

Introduction

Mouse material was obtained from the Anatomy Department, Cambridge University and brought to the Sanger Centre on cardice for subsequent RNA extraction, reverse transcription, panel construction and analysis by PCR.

1.1. Special Equipment and Suppliers

Dissecting kit	
Dissecting microscope	
Powergen 700 homogeniser	Fisher Scientific
Metal block and hammer	made in-house
Multichannel pipettors	Matrix range
RNA- and DNA-free eppendorf tubes and pestles	CP labs
15 ml falcon tube (individually wrapped)	Falcon # 2059
UV Spectrophotometer	Amersham Pharmacia
Flatbed electrophoresis equipment	Flowgen and Hybaid
UV transilluminator	Genetic Research Instrumentation
Polaroid camera	Genetic Research Instrumentation
Film for Polaroid camera	Genetic Research Instrumentation
Image capture software	developed by Alan Flook
Microtitre plates	CostarCorning # 6511
Hybaid rubber mats	Hybaid
MJ tetrad thermocyclers	Genetic Research Instrumentation

1.2 Materials and Solutions

Chemicals and Suppliers

All reagents are either analar or molecular biology grade and made up in double deionised water at 18 ohms, unless otherwise stated.

1 kb DNA ladder	GibcoBRL # 15615-024
8-hydroxyquinoline	Sigma # H6878
agarose	GibcoBRL # 15510-019
amplitaq	Perkin Elmer #
boric acid	Sigma # B 7660
bovine serum albumin fraction V	Sigma # A8022
bromophenol blue	Sigma # B5525
chloroform	Sigma # C 5312
citric acid	Sigma # C 3674
Cresol Red	ALDRICH # 11448-0
deoxyribonuclease 1 amplification grade (Dnase 1)	GibcoBRL # 18068-015
diethyl pyrocarbonate (DEPC)	Sigma # D 5758
dithiothreitol	Sigma # D9779
ethanol	BDH # 10107
ethidium bromide solution 10 mg/ml (EtBr)	Sigma # E1510
ethylene diamine tetraacetic acid (EDTA)	Sigma # ED2SS
glycerol	Sigma # G 7757
human placenta DNA	Sigma # D3160
isopentane	Sigma # 27,041-5
mixed bed resin TMD-8	Sigma # M-8157

moloney murine leukemia virus	
reverse transcriptase (M-MLV)	GibcoBRL # 28025-021
mouse gDNA	Clontech # 6650-1
oyster glycogen	Sigma # G 8751
phenol ultra pure	GibcoBRL # 5509UB
phosphate buffered saline (PBS)	Oxoid #BR14
primers	Sanger Institute
rat gDNA	Promega # G313A
RNAce total pure purification system	Bioline # BIO-28054
Rnase Inhibitor (RNAguard)	Pharmacia # 27-0815-01
sodium acetate	Sigma # S 2889
"T17 primers (T17A,G,C)"	Sanger Institute
trizma base	Sigma # T 1503
ultrapure dNTP 2'-deoxyribonucleoside 5'-triphosphate	
100 mM	Pharmacia # 27-2035-
01xylene cyanol FF	Sigma # X 4126

1.2.1. depc-treated water

Diethylpyrocarbobate (depc) is an alkylating agent and inactivates any proteins including RNAses found in solutions. It is a potential carcinogen and all manipulations should be performed in a fume hood.

- Add 1.8 ml depc to 1.8 L of nanopure (18 ohm) millipore water in a 2 L duran bottle.

- Shake vigorously, cover the lids with foil, autoclave tape and label allow to stand overnight in the fume-hood.
- Autoclave. Upon autoclaving, depc is broken down to carbon dioxide and ethanol.

1.2.2. 1 kb ladder

1 ml 1 kb DNA ladder (GibcoBRL)

7.6 ml depc treated water

1.68 ml 6x LB

Dispensed as 400 µl aliquots, store at –20°C

Fragments sizes (bp): 12,216, 11,198, 10,180, 9,162, 8,144, 7,125, 6,108, 5,090, 4,072, 3,054, 2,036, 1,635, 1,018, 516/506, 394, 344, 298, 220, 200, 154, 142, 75

1.2.3. 6 x LB	fc (final concentration)
6 ml glycerol	33%
14 ml depc treated water	66%
0.05 g bromophenol blue	0.25%

filtered through 0.22um filter,

Store frozen as 2 ml aliquots

1.2.4. T0.1E	fc
121.1 g Trizma base	1 M
37.22 g EDTA	0.1 M

pH 8 with hydrochloric acid and made up to 1 L with depc treated water

1.2.5. TBE 10 x Stock fc

108 g Trizma base	890 mM
55 g Boric acid	890 mM
40 ml 0.5M EDTA	20 mM

pH 8 with hydrochloric acid and made up to 1 L with depc treated water

1.2.6. Cresol Red Solution fc

0.845 mg/ml Cresol Red sodium salts in T0.1E	2mM

1.2.7. 10 x PCR Rxn buffer (3.5 mM $MgCl_2$fc) fc 50 x 2 ml

4.50 mls 1 M Tris-HCl pH 8.8	100 mM	45 ml
5.00 mls Cresol Red solution	0. 1mM	50 ml
0.15 ml depc treated water		1.5 ml
0.35 ml 1 M $MgCl_2$	3.5 mM	3.5 ml
0.1454 g $(HN_4)_2SO_4$	10 mM	1.454

Store frozen in 2 ml aliquots

1.2.8. Dilution buffer 48 x 3.1 ml

16.00 mls depc treated water	100 ml
8.00 mls T0.1E	50 ml
0.13 ml Cresol red solution	0.8125 ml
14 l 1 M NaOH (to pH 8.1)	0.0875 ml

Store frozen in 3.1ml aliquots

1.2.9. Sucrose Solution fc

121.1 g sucrose in 350 ml depc treated water 34.6%

Store frozen in 7ml aliquots

1.2.10. PCR Standards: fc

Blank: oyster glycogen (Sigma) 2 □g/□l

Genomic mouse DNA 4 ng/□l

Genomic human placenta DNA 4 ng/□l

Genomic rat DNA 4 ng/□l

1.2.11. 10 mM Deoxynucleotides fc

Ultra pure dNTP set PHARMACIA # 27-2035-01

4 x 250 □l 100 mM 2'-deoxyadenosine 5'-triphospate 10 mM

4 x 250 □l 100 mM 2'-deoxycytidine 5'-triphospate 10 mM

4 x 250 □l 100 mM 2'-deoxyguanosine 5'-triphospate 10 mM

4 x 250 □l 100 mM 2'-deoxythymidine 5'-triphospate 10 mM

6 ml depc treated water

Store frozen in 1ml aliquots

1.2.12. Phosphate Buffered Saline (PBS)

1 tablet (Oxoid Dulbecco A) to 100 ml double distilled water

autoclaved, 115^0C, 10 mins

1.3. Methods

1.3.1. Preparation and maintenance of Animals

BLJ6 females and stud males, obtained from Harlan, were maintained in a twelve hour light/dark cycle with controlled temperature and allowed free access to food and water, and were naturally mated in the Combined Biofacility of the School of Biological Sciences, Cambridge University.

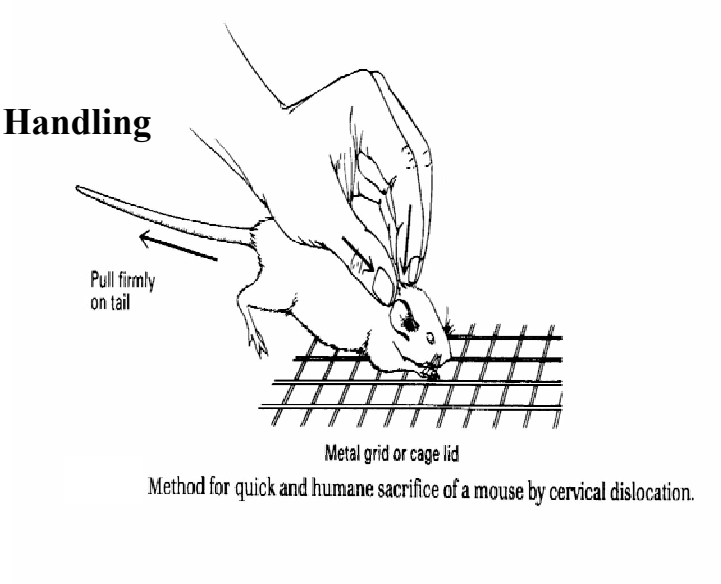
1.3.2. Tissues Collection

Mouse embryo dissections were performed by Professor Martin Johnson and Dr Anne Ferguson-Smith. Dissections were carried out over a period of 12 months to collect sufficient material for the panel, using gloved hands and thoroughly cleaned dissecting instruments. All mice were culled between 20.00 and 22.00 hours.

In brief:

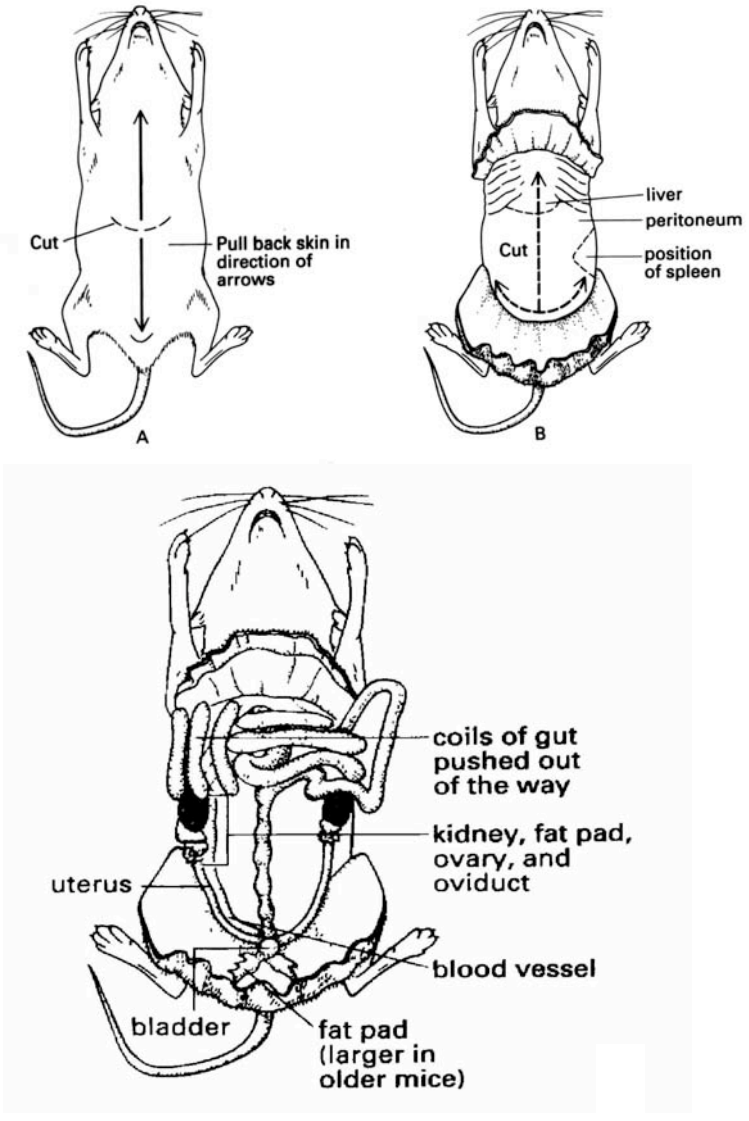
- Females naturally mated with male mice were identified by the presence of a vaginal plug (= d0.5), and killed by cervical dislocation (see Figures 6) at the following stages: 8.5d, 9.5d, 10.5d, 11.5d, 12.5d, 13.5d, 15.5d, 17.5 days post-coitum (dpc), and their uterine horns excised for dissection (see Figure 7).
- The foetuses were removed into sterile phosphate buffered saline (PBS) at room temperature. And photographed (see appendix 1).
- They were then dissected under the microscope, using flame polished forceps into cold sterile PBS.
- Tissues were placed into isopentane held on cardice and finally stored in 0.5 ml sterile tubes in liquid nitrogen prior to RNA extraction.

Figure 6 : Mouse Handling



Method for quick and humane sacrifice of a mouse by cervical dislocation.

Figure 7: Dissection of Mice



1.3.3. Dissection Procedure

- Taking one foetus at a time, the outer uterine musculature was removed keeping the placenta intact and the amnion/yolk sac was peeled away before taking a photographic record of the developmental stage.
- The placenta/yolk sac was separated from the foetus proper, cutting away the umbilicus leaving the fetal gut intact; the placenta and yolk sac were then removed separately.
- Cutting near the shoulder and hip joints the limbs were removed.
- The abdomen was then cut open to remove the liver, the duodenum, then the stomach, spleen and lastly separating the intestines. The mesentery was removed in all cases.
- The heart was removed from the thoracic cavity, followed by the release and separation of the oesophagus and lungs.
- Gonads were then removed, together with the urogenital tract (mesonephros) and where possible, in later stages, differentiated into male and female gonads.
- Next the bladder, adrenals, and kidneys were removed.
- The brain was exposed through making an incision at the back of the neck and gently peeling back the skin over the head, to remove the cranium. The whole brain and spinal cord, where then teased out and separated into the forebrain, midbrain, hindbrain and spinal cord.
- The rest of the head was then taken, and finally the remainder of the body was collected as a single sample.

1.3.4. Extraction of RNA from Tissues using Bioline RNace Kit

General notes: Skin surfaces and dust particles contain an abundance of RNAse, so it is important to wear gloves throughout and change them frequently, also to keep bottles/tip boxes/tubes covered as much as possible. Use RNAse free plastic and plugged tips, to prevent contamination. Chemicals used for RNA work should be kept separate from the general stock and weighed out without the use of spatulas, by carefully tapping the chemical out of the bottle.

Solutions and water should be treated with Diethyl PyroCarbonate (depc) see section 1.2.1. Tris solutions, however require making up in depc treated water, as they do not respond well to neat depc. Solutions, which cannot be autoclaved, can be filtered through a sterile 0.22 μm filter to reduce contaminating ribonucleases.

Equipment can be cleaned by soaking in 0.1 M NaOH + 1 mM EDTA for 1 hour followed by rinsing in depc treated water.

Small scale (5 – 50 mg)

- Place tissue in a sterile RNAse-free 1.5 ml eppendorf tube, add 350 μl of lysis solution homogenise using a sterile RNAse-free pestle.
- Add 25 μl of adsorbin solution (mix well before use) and vortex. Place on ice for 5 mins.
- Centrifuge for 2 mins to sediment the adsorbin pellet. Carefully remove the aqueous layer without disturbing the gelatinous pellet and place in a new sterile 2 ml eppendorf tube.
- Add 700 μl water saturated phenol plus hydroxyquinoline, then add 140 μl chloroform and 70 μl buffer A.
- Vortex for 10 secs place on ice for a further 5 mins.

- Spin 14,000 rpm for 10 mins at 4^{o}C, remove the upper aqueous layer being careful not to remove any of the interface. Place in a new sterile 1.5 ml eppendorf tube.
- Add an equal volume of isopropanol, mix and leave at –20^{o}C for a minimum of 20 mins.
- Spin at 14,000 rpm for 10 mins at 4^{o}C, remove the supernatant.
- Add 1 ml ice-cold 75 % ethanol, gently agitate through a tip to wash the pellet, spin at 14,000 rpm for 5 mins. Repeat twice, removing as much ethanol as possible on the last wash without dislodging the pellet.
- Allow the pellet to air dry for 15 mins, re-suspend in 50 µl depc treated water and measure the OD.
- Take 2 µl of the RNA solution to 1 ml T0.1E and measure at 260nm and 280nm. Take note of the 260/280 ratio and calculate the concentration of total RNA (1 OD at 260 nm is equivalent to 40 µg/ml, a ratio of 1.8-2 is preferred, though ratios as low as 1.5 are still worth pursuing).
- Add 150 µl 100 % ethanol to the original 50 µl RNA solution and make a new calculation of the concentration.
- Remove 2 µg to a sterile 0.5 ml eppendorf tube, add 1/10th the volume as 3 M sodium acetate, pH 5.2, leave at –20^{o}C for 30 mins. Pellet at 14,000 rpm for 5 mins remove the supernatant, air dry, add 7 µl of 1 X LB and run on a 1 % agarose + 0.004 % Ethidium Bromide gel, in 1 X TBE. Run at 30 milliamps, 65 volts for 1.5 to 2 hours in 1 X TBE + 0.004% Ethidium Bromide.

1.3.5. Deoxyribonuclease Treatment

Take 50 µg of total RNA from the ethanol stock add 1/10th the volume as 3 M sodium acetate, pH 5.2, leave at –20^{o}C for 30 mins. Pellet at 14,000 rpm for 5 mins, wash with 70 % ethanol, leave to air-dry.

		fc
50 µg	RNA pelletted, washed and dried	1 µg/µl
39.5 µl	depc treated water	
0.5 µl	RNA Guard 33 U/µl (Pharmacia)	0.3 U/µg
5 µl	10 X DNase I reaction buffer (BRL)	x 1
5 µl	Dnase 1 U/µl (BRL)	0.1 U/µg
50µl	Total	

leave at room temperature for 15 mins

add 5 µl 25 mM EDTA (BRL)

65^{o}C for 10 mins, 90^{o}C for 2mins, Chill on ice, spin

1.3.6. Reverse Transcription

		fc
50 µl	DNA-free RNA (50 µg see section 1.3.5.)	250 ng/µl
40 µl	5 x first strand buffer (BRL)	x1
5 µl	10 mM dNTPs	250 µM
20 µl	100 mM DTT (BRL)	10 mM
15 µl	200 U/µl M-MLV (BRL)	15 U/µl or 60 U/ng RNA
20 µl	40 mM Sodium Pyrophosphate	4 mM
50 µl	20 µM Primer T_{17}AGC	5 µM
200 µl		

37^{o}C for 90 mins, 60^{o}C for 5 mins, Chill on ice, spin

1.3.7. Stock and working solutions

cDNA currently at 250 ng/µl (50 µg/200 µl), was diluted (1/5) with 800 µl T0.1E to make a 1 ml stock solution of 50 ng/µl and stored as 50 µl aliquots at -20°C. 50 µl aliquots plus 1.2 ml (1/25) oyster glycogen solution at 2 mg/ml in depc treated water to provide a cDNA template working solution for PCR at 2 ng/µl.

1.3.8. Polymerase Chain Reaction

per reaction		fc
7.2 µl	34.6 % sucrose	12.45 %
0.187 µl	1/10 fresh β-mercaptoethanol in T0.1E	0.093 %
1.0 µl	10 mM dNTPs	0.5 mM
0.125 µl	AmpliTaq 5 U/µl	0.03125 U/µl
2.0 µl	10 x buffer 35 mM $MgCl_2$	3.5 mM
3.488 µl	Dilution buffer	0.228 µg/µl (cresol red)
1.0 µl	forward and reverse primers at 100 ng/µl	5 ng/µl
5.0 µl	RT product/cDNA template at 2 ng/µl	0.5 ng/µl
20 µl		

1.3.9. Typical Cycling Programme

Using hot lids

92°C 2 mins		
92°C 30 secs	denaturation)	
55°C 90 secs	annealing)	35 cycles
72°C 1 min	polymerasation)	
72°C 10 mins	extension	

1.3.10. Primer design

Primers were designed using the computer program PRIMER (version 0.5, Whitehead Institute for Biomedical Research, 1991) by Sarah Hunt, to regions 300 bp from the 3' end of the sequence, with a GC content of 40-60% and blast searched for complementarities to other known sequences. They were then synthesised by Geneset (Paris, France) or in-house at the Sanger Centre. Figure 8 identifies the primer sequences used in the purity testing of the panel.

Where primers were synthesised in-house at the Sanger Centre, they were supplied in ammonium hydroxide, dried down in a speedie vac and suspended in milliQ water to a concentration of 1 mg/ml as assessed by OD at 260 nm. Primer mixes of forward and reverse at 100 ng/μl were then made as working solutions and stored at –20^{o}C.

The planned dissection and tissue collection procedures were modified during the initial few dissections, ensuring that all dissectible tissues (defined as those tissues developed sufficiently to be unambiguously distinguished as those tissues) were collected in a given sequence.

The RNA extraction procedure was developed from Bioline's original protocol. RTPCR conditions were developed in-house originally for the adult mouse panel and subsequently used for a number of panels created in the Gene Expression Group. Similarly panel construction was a method devised in-house and applied to this foetal panel.

Figure 8: Primer Sequences

Sanger ID	Forward Primer	Reverse Primer	Symbol	Accession number
st95_22	ATGTGTGTGTGTGCACATG	TACACCCGCACTAATGGTCA	Slc17a2	L33878
st95_35	GCTTCTATCTGGCGGAAGG	TGTCATCTGGCTACCTTCCC	Calb1	M21531
st95_42	GCTTCTATCTGGCGGAAGG	TGTCATCTGGCTACCTTCCC	Calb1	M23663
st95_43	CTCTTTTCTCCCACCTCATCC	CTACCAGGCAGCAGGAGTTC	Hox5b	M26283
st95_58	AAGTCAACTTCTCAGAGCCTGG	GCTTTGACAAGGCTGGAGAC	Fsbpi	M65034
st95_69	CCCAGAAGTACTACGGGAAGG	CGAGTTGCCGTGTGTGAG	Adora1	U05671
st96_128	GCCAATGATTCATCTTGAGTTG	CCTTGATTCTCTCCGCTCAG	Csna	M36780
st96_153	CTCATGGTTTTCCCCTCTGA	GGTTCTGCTTTATTGACCTTGG	Fabph1	U02883
st96_273	GGGGAAGTGGAACACACGG	AGCAGGAGTTGGCTGGAATG	Si-s	X15546
st96_310	CTGATCCGCAAATACGGG	GCATGATCGGTTCCACTTG	Rps29	L31609
st96_428	GAAGAGTTCTGAGCATGCCC	TTCTTGGGGCCTATGGAAG	Cab45	U45978
st97_760	ATGGCTTGATTGGTACCAGTG	GACAAGTGGAAAAACAGGAAGC	bac 573K1a	
st97_761	TGCAGGCAGAGATGCTACTG	CGCTCAGAGAGAAAAAAATTGG	bac 573K1b	

1.4. Construction of the Panel

1.4.1. Tissue dissection

As mentioned in section 1.3.2., tissue was collected over a period of 12 months, stored frozen and transported to the Sanger Centre for RNA extractions. Figure 9 below illustrates the information collected at the time of tissue dissection.

Figure 9: Table of Tissues Dissected

Tissues	Plug date	Kill date	Kill time	Female no	Gestation/ Morphological age	Photo log no	sex	Tray code
Placenta								
Yolk sac*								
Liver								
Oesophagus/stomach*								
Intestines*								
Spleen*								
Bladder*								
Kidney*								
Female *Urogenital + gonad								
Male *Urogenital + gonad								
Heart*								
Lung*								
Forelimb								
Forebrain*								
Midbrain*								
Hindbrain*								
Spinal cord*								
Rest of head								
Remainder of body								

The asterisk, denote material combined from an entire pregnancy, therefore the amount of a given tissue was dependent on the foetal number in addition to the developmental stage. A total of 60 mice were culled providing 425 foetuses, with approximately 8000 dissected tissue samples. From this collection, RNA was

extracted from a single pregnancy at any one time, combining material where appropriate.

1.4.2. RNA extraction and assessment

A 50 μg, good quality RNA sample, as defined by ratio of ODs and strength of 18S/28S banding on an agarose gel, was selected. Figure 10 demonstrates a typical table showing this information for a 15.5 day foetus dissected on 19.11.97.

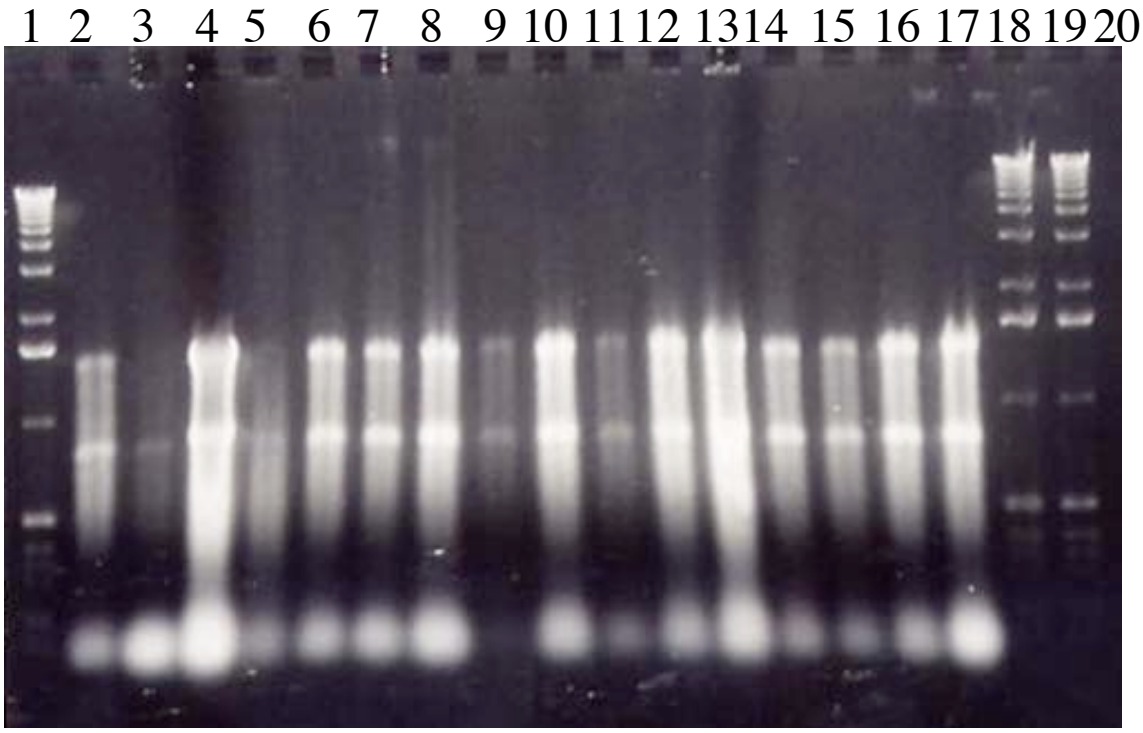
Figure 10: RNA extraction from 15.5d foetus

Tissues 15.5 day female date:19.11.97	Weight mg	Sample number	260nm	280nm	260/280 ratio	μg/μl	Total amount of RNA μg	ng RNA per mg tissue	Quality post agarose gel
Placenta	400	4	0.016	0.009	1.77	0.32	64	160	Good
Yolk sac	505	9	0.049	0.03	1.63	0.98	49	97	Poor
Liver	120	4	0.086	0.049	1.75	1.72	86	716	Good
Oesophagus/stomach	60	9	0.066	0.043	1.53	1.32	66	1100	Poor
Intestines	122	9	0.177	0.078	1.5	2.34	117	959	Good
Spleen	30	9	0.002	0.001	2	0.04	2	66	
Bladder	50	9	0.007	0.008	0.87	0.14	7	140	Good
Kidney	60	9	0.03	0.025	1.2	0.6	30	500	Good
Female Urogenital + gonad	10	1	0	0.005					
Male Urogenital + gonad									
Heart	60	9	0.347	0.232	1.48	6.9	345	5750	Good
Lung	210	9	0.217	0.115	1.88	4.34	217	1033	Good
Forelimb	90	1	0.018	0.031	0.88	0.36	18	200	Good
Forebrain	240	9	0.125	0.062	2.01	2.5	125	520	Good
Midbrain	170	9	0.047	0.033	1.42	0.94	47	276	Good
Hindbrain	160	9	0.032	0.021	1.52	0.64	32	200	Good
Spinal cord	50	9	0.025	0.016	1.56	0.5	25	500	Good
Rest of head	160	2	0.251	0.148	1.7	5.02	251	1568	Good
Remainder of body	400	2	0.065	0.038	1.71	1.3	65	162	Good

A typical gel of 2 μg amounts of RNA as assessed by OD measurements of the above tissue extraction is shown below as Figure 11. Samples are in the same order as the above table, excluding spleen and urogenital samples. From this gel, the quality of the RNA is scored and an indication as to the accuracy of the OD measurements gained. Repeat gels are run for confirmation, especially where the lanes are overloaded (lanes 3, 5,6,7,9,11,12, 15 and 16). The sample in lane 3 (yolk sac) is a

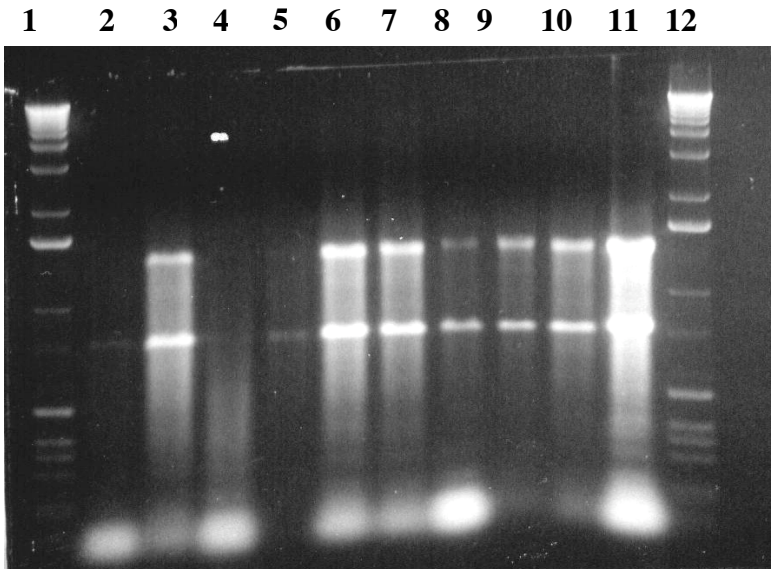
poor sample and was subsequently re-extracted. In this instance, the placental sample (first lane of gel) was given a score of 1, all other RNA volumes were then adjusted in relationship to this, to provide an approximately equivalent quantity of total RNA in each tissue sample.

Figure 11: Agarose gel of RNA extracted from 15.5d foetus.



A good quality RNA profile on a gel should show no contaminating DNA (near the gel origin), bold 18S and 28S bands with a stronger representation at the 28S level with minimal banding at the 5S level. The full range of tissues from a single gestation stage was extracted for total RNA, in this fashion on a single day. The process was repeated until there was sufficient material for the completed list of tissues and stages of animals [see above about numbers. of foetuses contributing to each sample]. Total RNA was stored at –70^{o}C in 75% ethanol as a suspension.

Figure 12: Illustration of differing qualities of RNA



Lanes 1 & 12: 1kb ladder

Lanes 2, 4 & 5: 18s band very faint, little RNA present - poor quality – not used.

Lane 8: 28s band much lighter than 18s, - degraded RNA at bottom of gel – not good, could be used if no other sample available.

Lanes 3, 6, 7 & 11: some degradation in lane 11, but generally strong 28s and 18s banding, good smearing between the two major bands where the message is expected to migrate – good quality.

Lanes 9 & 10: Clear 18s and 28s bands, no obvious degradation at bottom of gel – good quality.

1.4.3. Reverse transcription and PCR amplification

From earlier work in our lab, we knew the safest way to perform the reverse transcription (RT) step was to RT a small portion (2 μg) from all of the samples and check the resulting cDNA for it's ability to serve as a template for the PCR of housekeeping genes and to check for any contaminating DNA. cDNA contamination with residual genomic DNA was assessed by primers designed against non-coding sequences from a mouse bac generated in-house (a kind gift from Ruth Young [sts 760 and 761-Sanger notation for sequence tag sites]) (Figure 15). Any contaminated samples were discarded and replaced where possible. Major differences of RNA levels were assessed by use of primers to housekeeping genes. Housekeeping genes are those that are essential to the fully functioning cell, in particular those that are needed for ribosomal activity, and thus presumed to be at a similar level in all tissues. For this exercise, the Ribosomal Protein S29 (Acc.no. L31609) and calcium-binding protein Cab 45b (Acc.no. U45978)(Figure 16) were employed. Primers designed to the ribosomal proteins were used to normalise the samples to account for differences in the amount of total RNA present in each sample and to compensate for any variations in the efficiency of the RT reaction. RNA concentration was assessed by the amount of amplification of these housekeeping genes and only when these assays were satisfactory was a larger amount of RNA committed to the procedure. By introducing this valuable step, it was possible to eliminate samples that were sub-optimal and re-extract better quality RNA. Samples which were replaced due to genomic contamination at this stage included 15.5d forebrain, 17.5d bladder, kidney, and yolk sac. Through carefull examination of the small scale RTPCR, a definitive list of tissues with 50 μg amounts of DNA-free total RNA was compiled and reverse transcribed on a single day with known batches of DNase1 and M-MLV enzymes.

In addition to the 74 mouse foetal tissues and 11 adult mouse tissues, genomic DNA from mouse, rat and human plus glycogen blanks were included in the panel to allow for a direct comparison of known and novel genes.

The next step was to PCR the full range of cDNAs again to recheck for contaminating genomic DNA. Contamination was found in some samples (including 17.5d midbrain and spleen, 15.5d head, rest of body and placenta, 13.5d yolk sac, and 12.5d yolk sac). As we were unable to replace these samples with a genomic free sample, the minimal level of contamination was noted (Figure 15) - these samples were kept in preference to no representation of that tissue sample. This panel was originally designed as a resource to probe with a range of primers designed to within 300bp of the 3' end, a region not thought to contain introns, and so every precaution was taken to eradicate DNA prior to reverse transcription. Levels of cDNA were then adjusted to provide an even amplification of housekeeping genes when PCR'ed at a low cycle number (Figure 16), as assessed by eye from the ethidium bromide stained gels. These calculations are illustrated in the table below as the dilution factor in ul/ml.

1.4.4. Storage of panel

Once an even amplification of housekeeping genes could be demonstrated (see Figure 9) with a minimal degree of genomic contamination (see Figure 15) the panel was judged to be normalised and ready for production. Stocks of cDNAs were stored as 50 □g/ml at –70^{o}C. Working solutions at 2 ng/□l were plated out in 96 well format in a deep well microtitre plate.

Aliquoting was performed with the aide of a Robbins Hydra 96 set to dispense 5 □l into microtitre plates and rapidly frozen on a metal block held on cardice. These plates were then kept at -20^{o}C for up to one month.

Figure 13: Final format for The Mouse Foetal cDNA Panel

tissue order	tube number	ul /ml	gel ID	tissue order	tube number	ul /ml	gel ID
whole conceptuses 8.5d	1	39.5	1	urogenital/gonads 15.5d	46	39.6	49
whole conceptuses 9.5d	2	39.5	2	urogenital/male gonads 17.5d	47	39.6	50
whole foetus 10.5d	3	59.4	3	urogenital/female gonads 17.5d	48	39.6	51
whole foetus 11.5d	4	79.2	4	bladder 15.5d	53	79.2	52
whole foetus 12.5d	90	39.5	5	kidney 15.5d	54	39.6	53
whole foetus 13.5d	91	39.5	6	bladder 17.5d	88	39.6	54
whole foetus 15.5d	92	39.5	7	kidney 17.5d	56	39.6	55
whole foetus 17.5d	93	39.5	8	spleen 17.5d	57	39.6	56
Forebrain 12.5d	5	39.5	9	forelimbs 12.5d	49	39.6	57
Forebrain 13.5d	6	39.5	10	forelimbs 13.5d	50	39.6	58
Forebrain 15.5d	7	39.5	11	forelimbs 15.5d	51	39.6	59
Forebrain 17.5d	8	39.5	12	forelimbs 17.5d	52	39.6	60
midbrain 12.5d	9	39.5	13	rest of body 12.5d	58	39.6	61
midbrain 13.5d	10	39.5	14	rest of body 13.5d	59	39.6	62
midbrain 15.5d	11	39.5	15	rest of body 15.5d	60	59.4	63
midbrain 17.5d	12	39.5	16	rest of body 17.5d	61	59.4	64
Hindbrain 12.5d	13	39.5	17	extra embryonic 10.5d	62	39.6	65
Hindbrain 13.5d	14	39.5	18	extra embryonic 11.5d	63	39.6	66
Hindbrain 15.5d	15	39.5	19	placenta 12.5d	64	39.6	67
Hindbrain 17.5d	16	39.5	20	yolk sac 12.5d	65	39.6	68
spinal cord 12.5d	17	39.5	21	placenta 13.5d	66	39.6	69
spinal cord 13.5d	18	39.5	22	yolk sac 13.5d	67	59.5	70
spinal cord 15.5d	19	39.5	23	placenta 15.5d	68	39.6	71
spinal cord 17.5d	20	59.4	24	yolk sac 15.5d	69	39.6	72
head 12.5d	21	39.5	25	placenta 17.5d	70	39.6	73
head 13.5d	22	39.5	26	yolk sac 17.5d	70b	39.6	74
head 15.5d	23	39.5	27	whole brain - adult	75	39.6	75
head 17.5d	24	79.2	28	spinal cord - adult	76	39.6	76
heart 12.5d	25	79.2	29	skeletal muscle - adult	77	59.4	77
heart 13.5d	26	39.6	30	heart - adult	78	39.6	78
heart 15.5d	27	198	31	liver - adult	79	59.4	79
heart 17.5d	28	39.6	32	kidney - adult	80	79.2	80
lung 12.5d	29	26.4	33	fundus - adult	81	26.4	81
lung 13.5d	30	39.6	34	caecum - adult	82	39.6	82
lung 15.5d	31	59.4	35	testis - adult	83	79.2	83
lung 17.5d	32	39.6	36	ovary - adult	84	52.8	84
liver 12.5d	33	39.6	37	one day old mouse	85	52.8	85
liver 13.5d	34	39.6	38	no RT product	71		86
liver 15.5d	35	59.4	39	Mouse genomic	72		87
liver 17.5d	36	59.4	40	Human genomic	73		88
gut 12.5d	37	39.6	41	Rat genomic	74		89
intestine 13.5d	39	39.6	42	no RT product	71		90
oes/stom 13.5d	40	39.6	43				
intestine 15.5d	41	39.6	44				
oes/stom 15.5d	42	39.6	45				
intestine 17.5d	43b	59.4	46				
urogenital /kidney 12.5d	44	19.8	47				
urogenital/kidney 13.5d	45	59.4	48				

Of the original dissected samples, the following failed to provide sufficient RNA of good enough quality to be included in the panel: 6.5 d, 7.5 d, 15.5 d spleen, and 17.5 d oesophagus/stomach. The 12.5 d gut of embryonic stomach/oesophagus and intestine is a combined sample post RNA extraction. As mentioned earlier, there is included in this list a number of genomically contaminated samples, which we were unable to replace.

1.4.5. Prescreen

A prescreen cDNA panel (plus controls) of whole conceptuses at each of the developmental stages (8) was created to reflect the full range of the panel.

Figure 14: Prescreen

Order	tube number	µl for 1ml	prescreen letter
whole conceptuse 8.5d	1	39.5	A
whole conceptuse 9.5d	2	39.5	B
whole foetus 10.5d	3	59.4	C
whole foetus 11.5d	4	79.2	D
whole foetus 12.5d	90	39.5	E
whole foetus 13.5d	91	39.5	F
whole foetus 15.5d	92	39.5	G
whole foetus 17.5d	93	39.5	H
Glycogen			I
mouse genomic DNA			J

This prescreen panel was employed to check the PCR conditions prior to full profiling. Primers were routinely run at 35 and 45 cycles of PCR with annealing temperatures of 55^{o}C and 60^{o}C. The settings, which allowed the detection of amplicons by ethidium bromide on an agarose gel, at less than saturating conditions, ie before the components of the reaction became limiting and during the time of amplicon accumulation, were generally the most suitable to use for full profiling.

1.5. Panel Validation

Initially a range of specific genes was analysed to demonstrate the integrity and validity of the panel, as compared to data produced with the adult mouse panel.

Sucrase isomaltase (Acc.no. X15546): (Figure 17) an enzyme switched on at birth in response to a diet of lactose in the mother's milk, is expressed exclusively in the adult gut region (Figure 18). As expected, its cDNA was not found in the mouse foetal section of the panel.

Casein, alpha (Acc.no M36780): (Figure 19) was found from the adult panel to be expressed solely in the mammary gland and mid term foetus (Figure 20): interestingly, this gene showed positive in all stages of the head and 17.5 day hindbrain, the 17.5 day midbrain and spleen both showed as positive, but may be due to contamination

Solute Carrier family 17, Na/H exchanger (Acc.no. L33878): (Figure 21) this gene is expressed exclusively in the kidney of the adult panel (Figure 22), and in the foetal panel its cDNA is found in the 17.5 day kidney and spleen samples, this latter finding may be due to the contamination (see figure 15) of the spleen sample.

Calbindin 28K (Acc.No. M23663): (Figure 23) expression in the adult panel is predominantly in the brain, spinal cord, female gonads and kidney (Figure 24) and this pattern is mirrored in the foetal panel, even at the level of 17.5 day uro. + female gonads.

Fatty Acid Binding Protein (Acc.no. M65034): (Figure 25) is expressed exclusively as an intestinal gene in the adult panel (Figure 26), and has strong expression in a similar region in the foetal panel.

Other genes, which had shown very specific patterns in adult mouse tissues, were less easy to interpret when run on the mouse foetal panel. These include ***Adora 1*** (Figure 27) and ***Hox b5*** (Figure 29). Figures for mouse adult panels of these genes are

included (28 and 30). The *Adora1* adult profile illustrates expression to be widely found in the brain and gonads; with the foetal panel, expression is found in parts of the brain, developing gonads and interestingly also the gut regions. This may be a result of contamination during the processing of this tissue or a true result for this gene. *Hoxb5* shows clear expression in the region of the adult cerebellum, spinal cord and gut regions; in the foetal panel, expression is found in the mid/hind brain, spinal cord, gut regions, and developing gonads. Expression found in the midbrain is clearly a result of contamination as the gene *Hoxb5* is well documented as a gene expressed in the upper rhombomers only during developement, this would include the hindbrain only. Expression also found in the 17.5 day spleen (see figure 15) is due to contamination.

In order to thoroughly examine the band intensities of a selection of the gels, images were photographed directly from the UV light box, relayed to a computer and the specific band intensities recorded. Ethidium bromide, the commonly used molecular biology stain, at a concentration of 2ug/ml forms a complex with DNA. The fluorescence emission for the DNA-ethidium bromide complex peaks at 300nm and is between 10-50 times that of the uncomplexed dye. With a UV transilluminator light source of 302nm, the DNA-ethidium bromide complex fluoresces in proportion to the amount of complex present [1]. Through the use of densitometric measurements, the digital image of the fluorescence was measured in a similar way to commercially available software http://www.uvp.com/. The lane containing the band from mouse genomic DNA was used as a reference and given a score of 100, to which all other bands measurements were related to. It should be noted that the fluorescent intensities of the ethidium bromide-DNA complexes can be reduced by as much as 40% over a 10 minute period and so it was important that image capturing was performed in a timely manner. In this way the profiles for housekeeping genes from

Figure 16 are displayed graphically as Figure 31 to illustrate the level of evenness obtained through normalisation and to show how these two genes vary in their expression patterns (Figure 35 is included in this chapter to allow identification of tissues). The *Ribosomal Protein 29* is the profile labelled 310 and *Stromal Cell derived factor 4 (Sdf4)* (previously known as *Cab45 [2])* is the profile labelled 428. Ribosomal proteins are regarded as genes essential for the cell's maintenance as they are required for translation. *Stromal cell derived factor 4* is a calcium ion protein, found to be expressed in all adult mouse tissues [3]. Use of this approach highlighted the inaccuracies undetected by eye from the ethidium bromide stained gels, in particular it demonstrated the lack of concordance between the two presumptive housekeeping genes and the inter-tissue variations. The agarose gels displayed have expression levels marked strong through to trace, presenting a broad range of scoring.

1.6. Concluding Remarks

It was through validating the panel against these genes (Figures 17 – 29), which had shown reproducibly specific expressions in the adult panel, that a representation of adult mouse tissues was subsequently included in the final panel format (Figures 15 and 16). Human and rat genomic samples were also included to allow inter species comparison. The panel was considered sufficiently robust to continue with the test by applying primers to the family of *Sox* genes.

Legend for Figures 15 through to 30

Ethidium bromide stained 2.5% agarose gels, run at 125 volts, 30 milliamps, 2 hours and photographed with a Polaroid camera. Photographs were scanned on an Epson scanner, opened in Adobe Photoshop, where defined regions were then cut and pasted into MacDraw and scored through viewing the original photographs.

Gene/Est: Gene or EST name
Symbol: Acronym for gene/est
EMBL AcNo: Accession number from EMBL database
Primers: Sanger Centre identifying number

PCR: Polymerase Chain Reaction
Date: date of lab. work
$MgCl_2$: final concentration
Temp: annealing temperature
Cycles: number of cycles

Amplification signal: four catagories
Strong – dark blue – major signal from both duplicates.
Moderate-weak – blue – broadest range of signal.
Trace – green – faint signal.
None – yellow – no signal from either duplicate.
Discrepancy – red – signal from one only, of the duplicates.

Figure 15: Contamination Profile

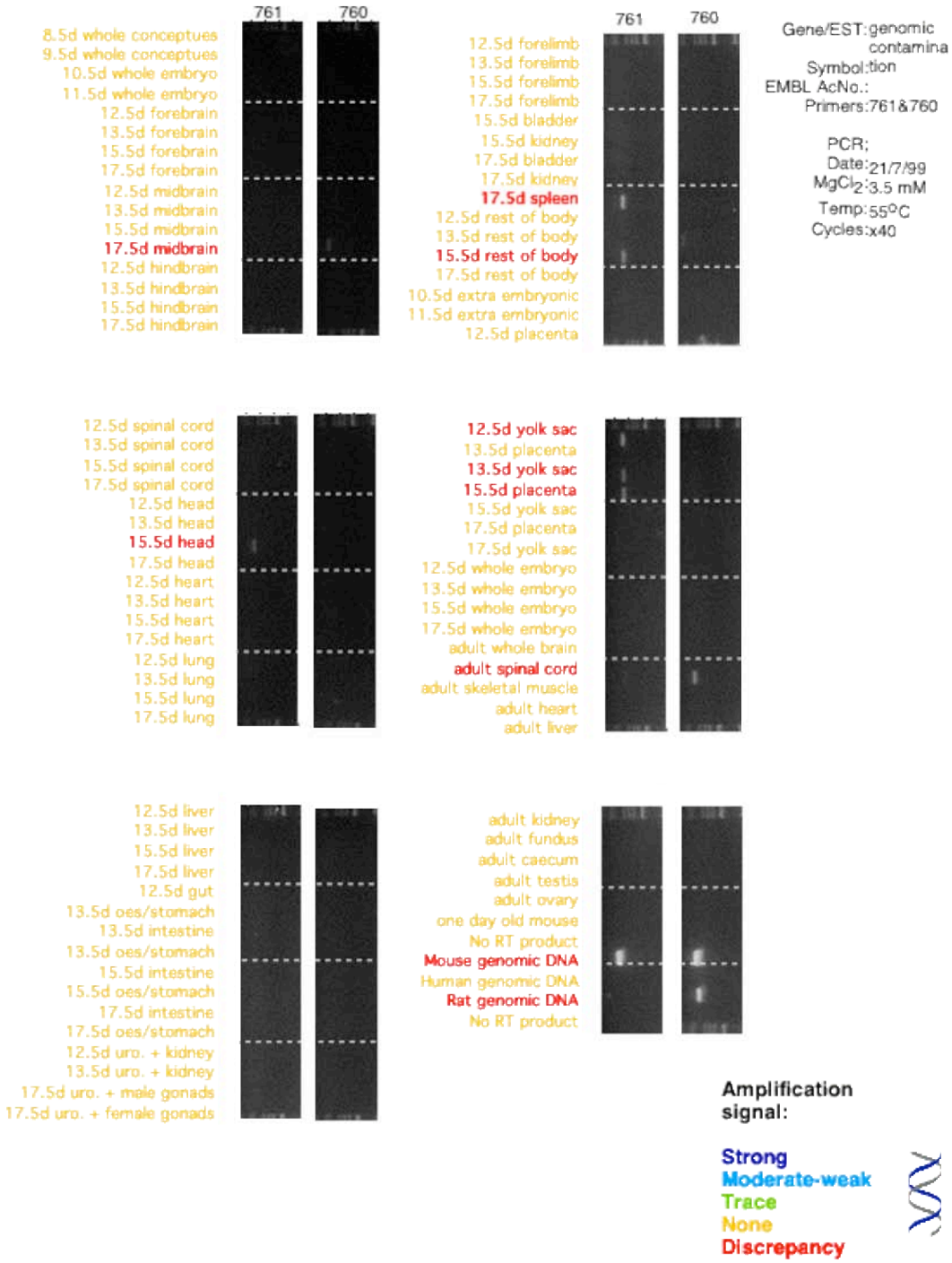


Figure 9: Normalisation Profile

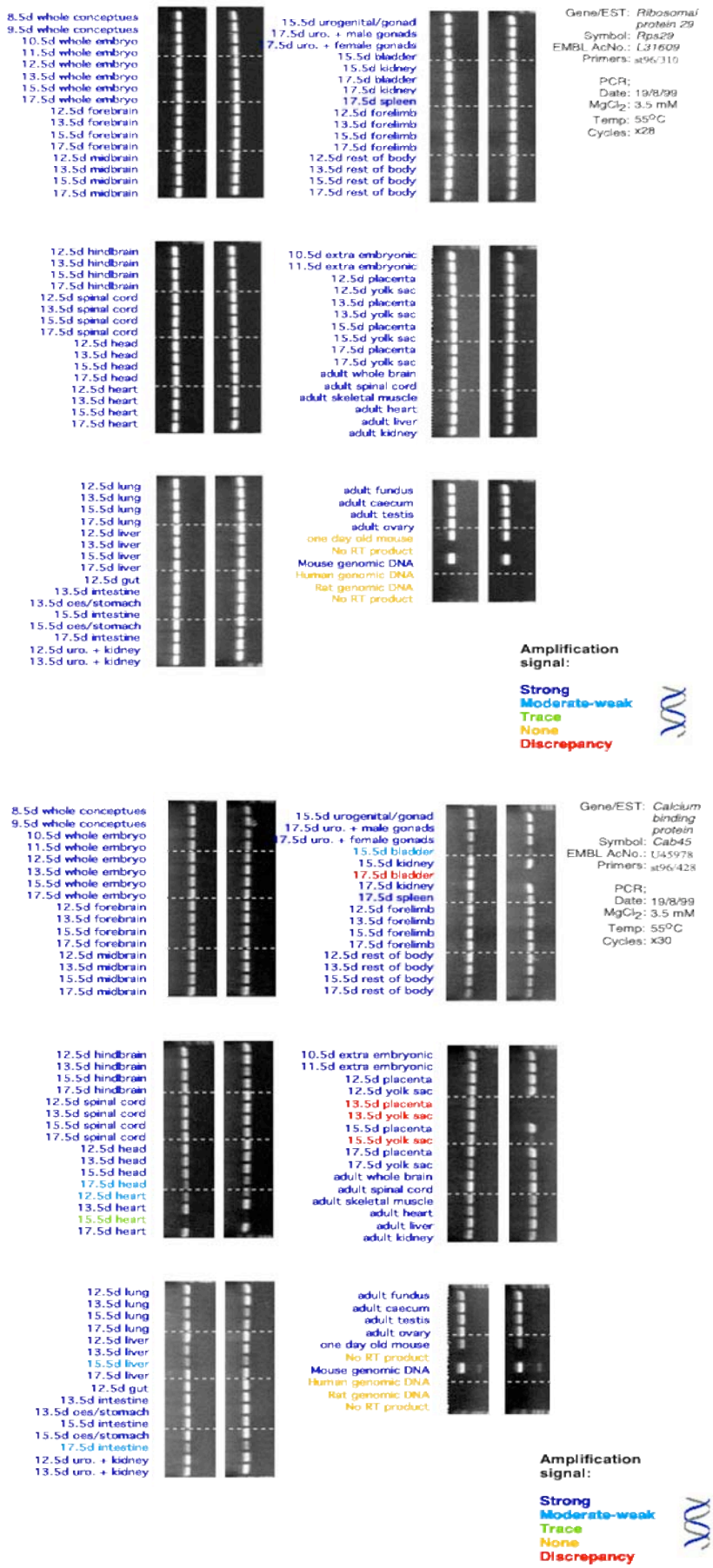


Figure 17: Expression Profile of Sucrase Isomaltase in Mouse Foetal Panel

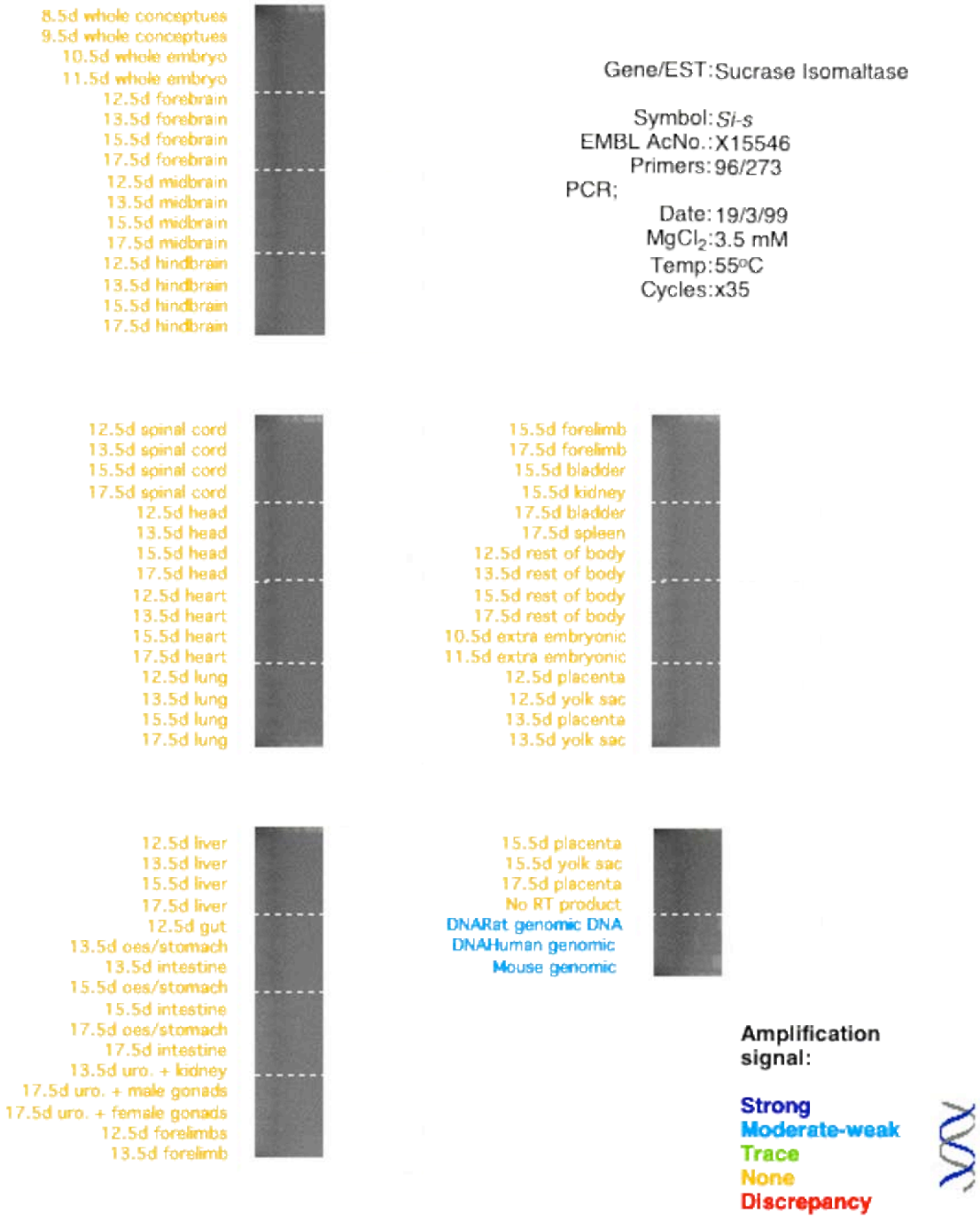


Figure 18: Expression Profile of Sucrase Isomaltase in Mouse Adult Panel

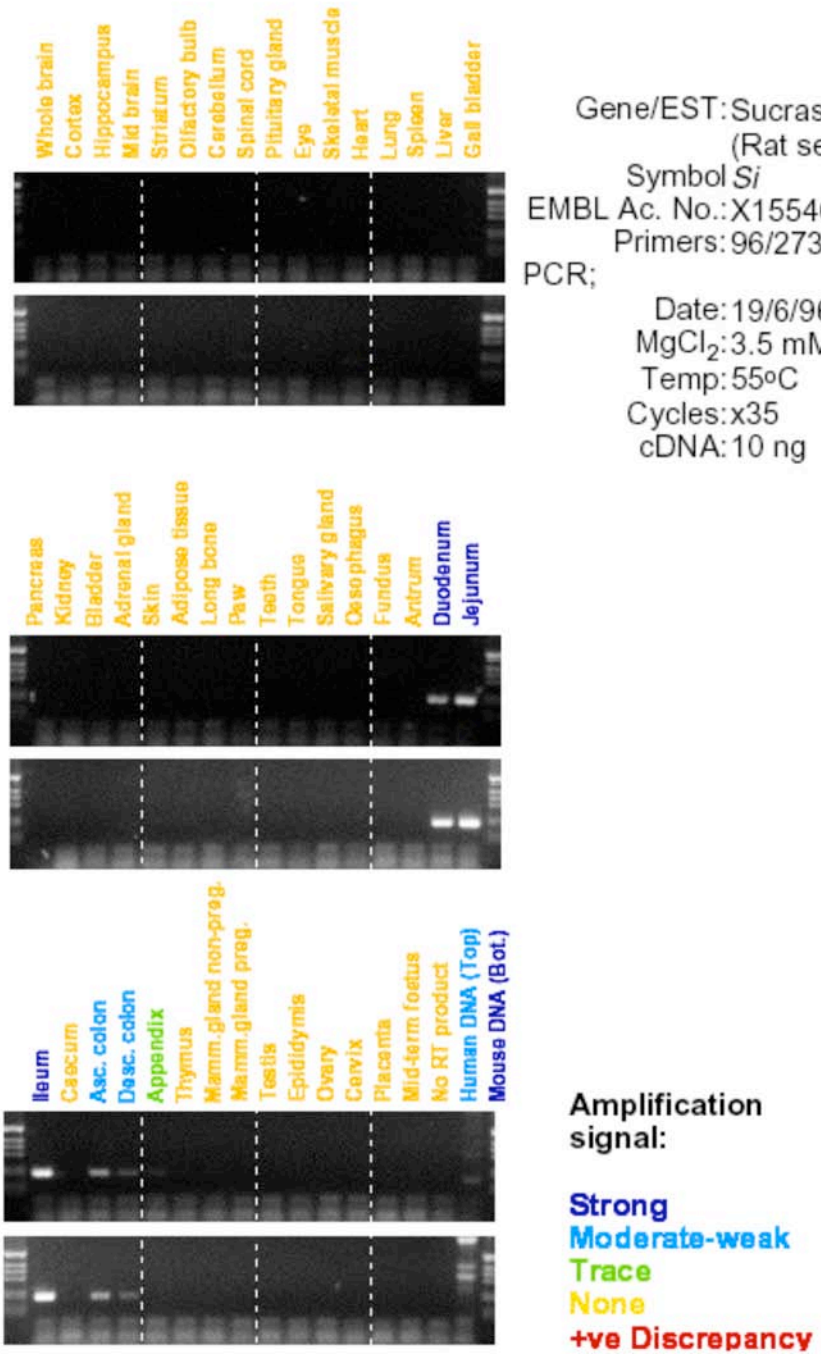


Figure 19: Expression Profile of Casein, alpha in Mouse Foetal Panel

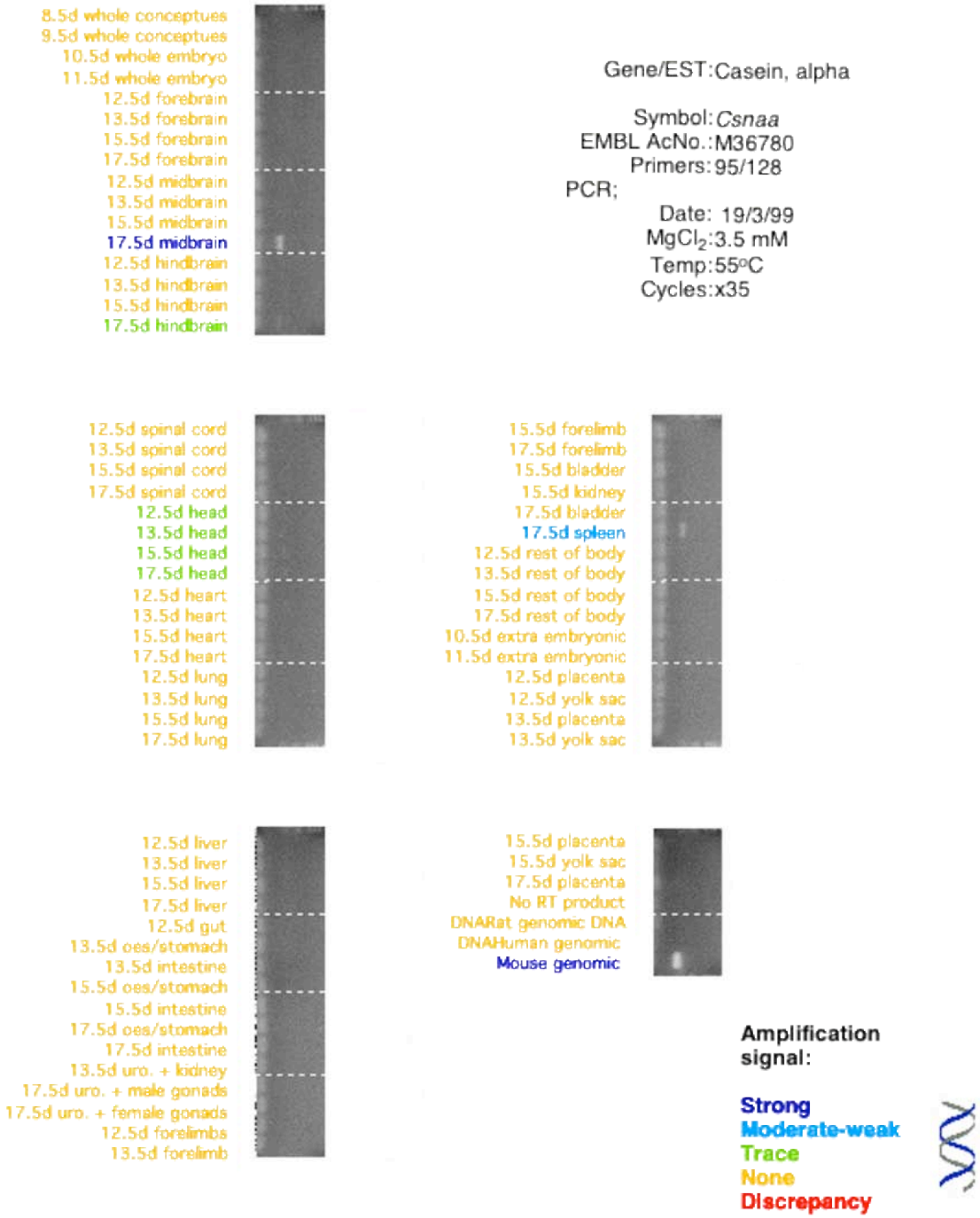
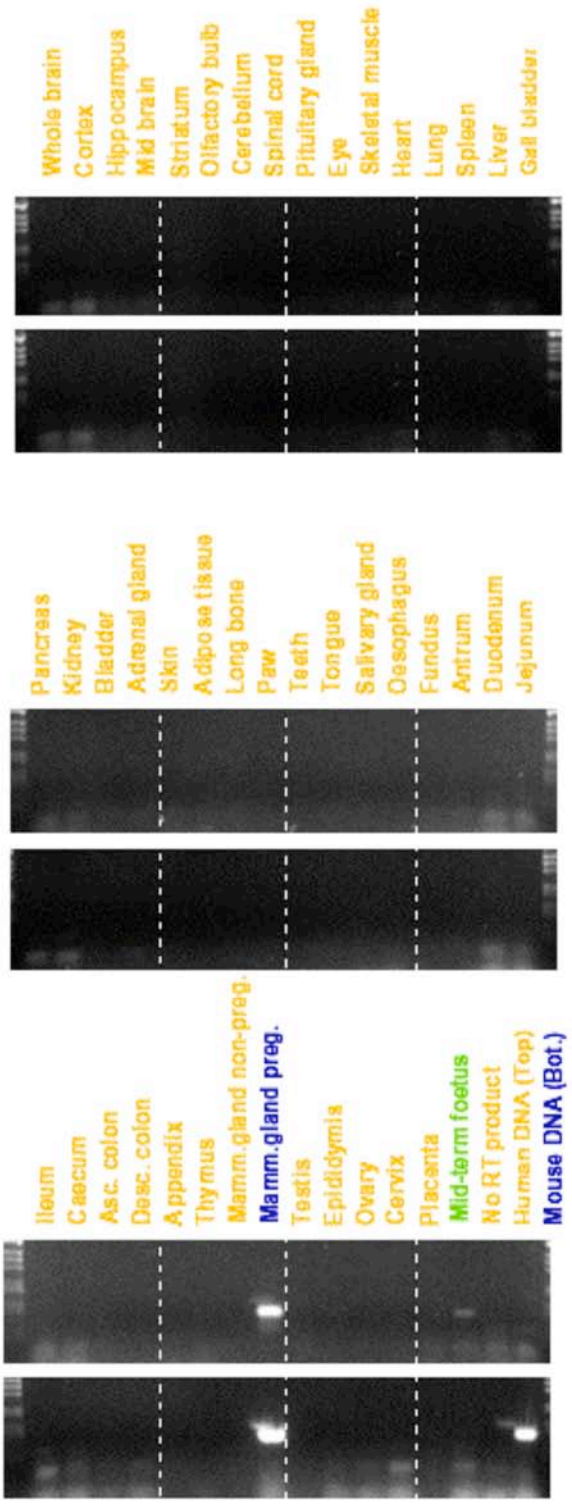


Figure 20: Expression Profile of Casein, alpha in Mouse Adult Panel



Gene/EST: Casein alpha
Symbol: *Csna*
EMBL Ac. No.: M36780
Primers: 96/128
PCR;
Date: 3/4/96
$MgCl_2$: 3.5 mM
Temp: 55°C
Cycles: x 35
cDNA: 10 ng

Amplification signal:

Strong
Moderate-weak
Trace
None
+ve Discrepancy

Figure 21: Expression Profile of Solute Carrier 17a2 in Mouse Foetal Panel

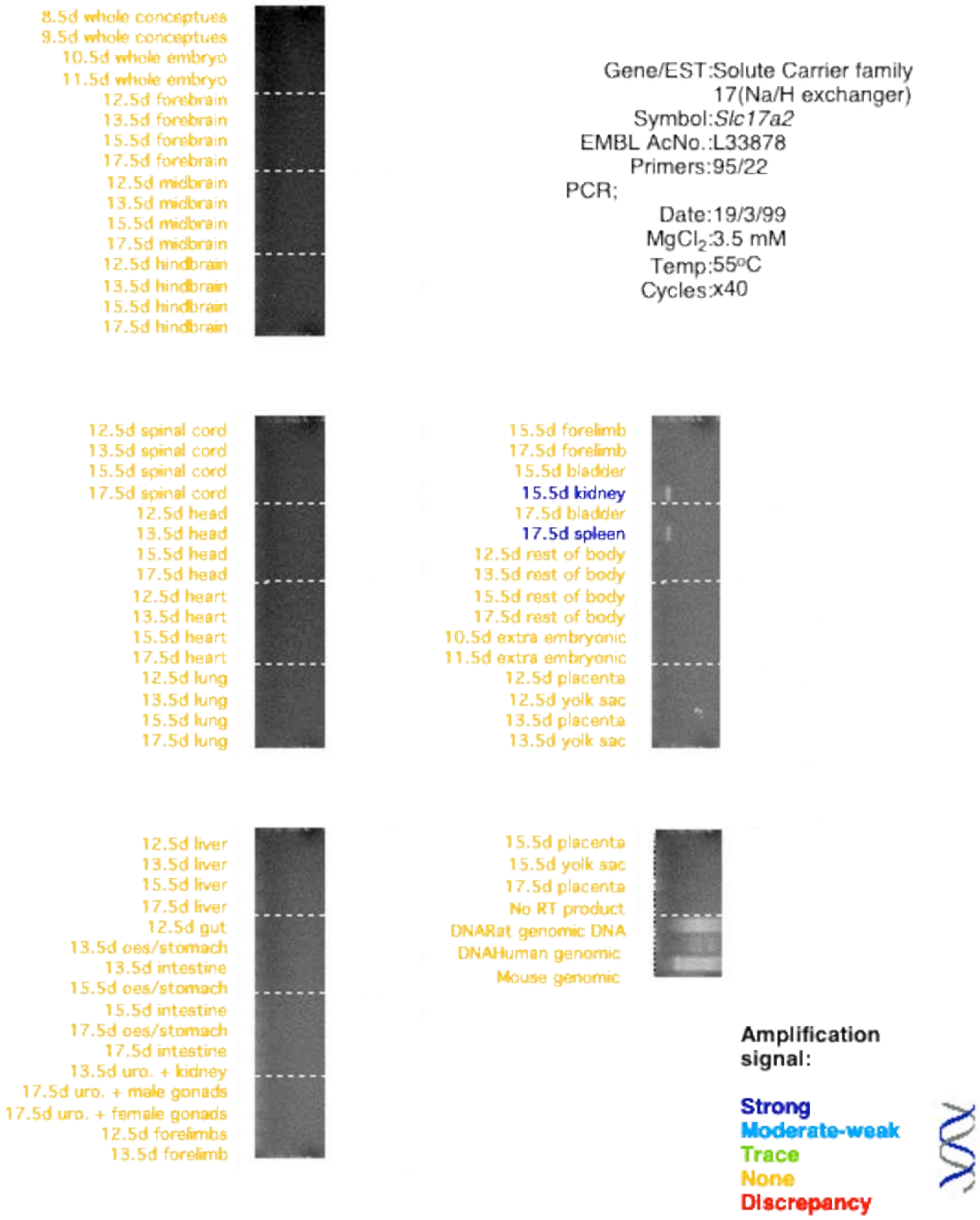
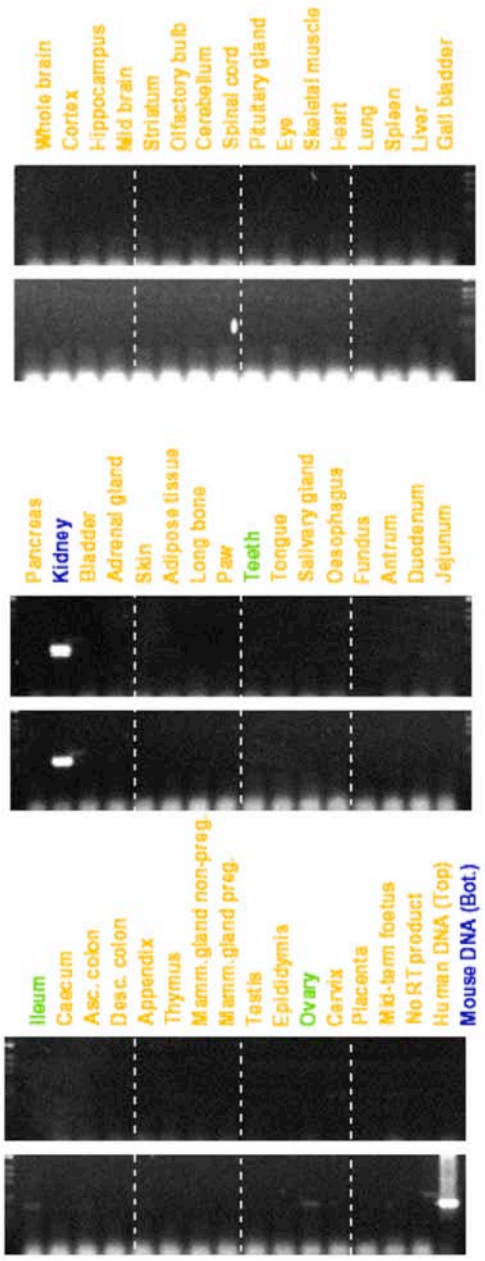


Figure 22: Expression Profile of Solute Carrier 17a2 in Mouse Adult Panel



Gene/EST: Solute carrier family 17 (sodium/hydrogen exchanger) member 2
Symbol: *Slc17a2*
EMBL Ac. No.: L33878
Primers: 95/22
PCR;
Date: 8/2/96
$MgCl_2$: 3.5 mM
Temp: 55°C
Cycles: x40ex

Amplification signal:

Strong
Moderate-weak
Trace
None
+ve Discrepancy

Figure 23: Expression Profile of Calbindin 28K in Mouse Foetal Panel

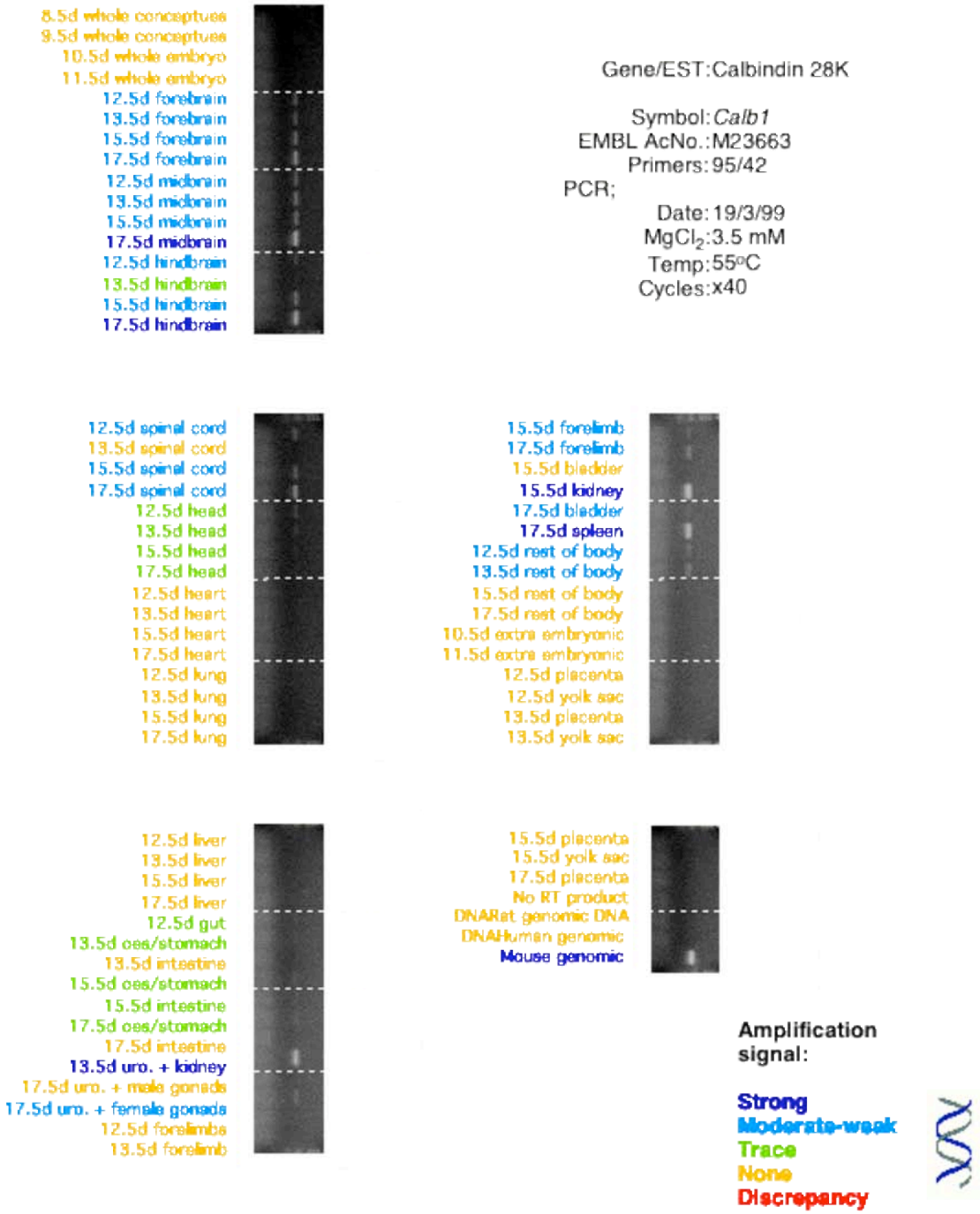
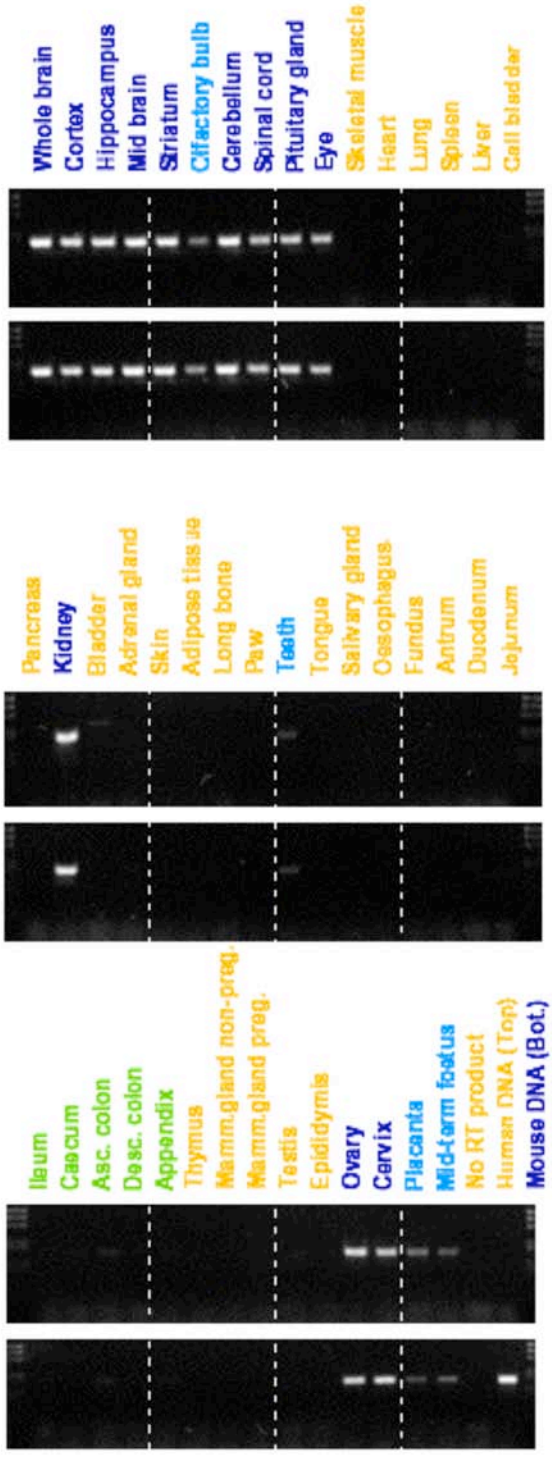


Figure 24: Expression Profile of Calbindin 28K in Mouse Adult Panel



Gene/EST: Calbindin-28K
Symbol: *Calb1*
EMBL Ac. No.: M21531
Primers: 95/35
PCR;
Date: 31/1/96
$MgCl_2$: 3.5 mM
Temp: 55°C
Cycles: x50ex

Amplification signal:

Strong
Moderate-weak
Trace
None
+ve Discrepancy

Figure 25: Expression Profile of Fatty Acid Binding Protein in Mouse Foetal Panel

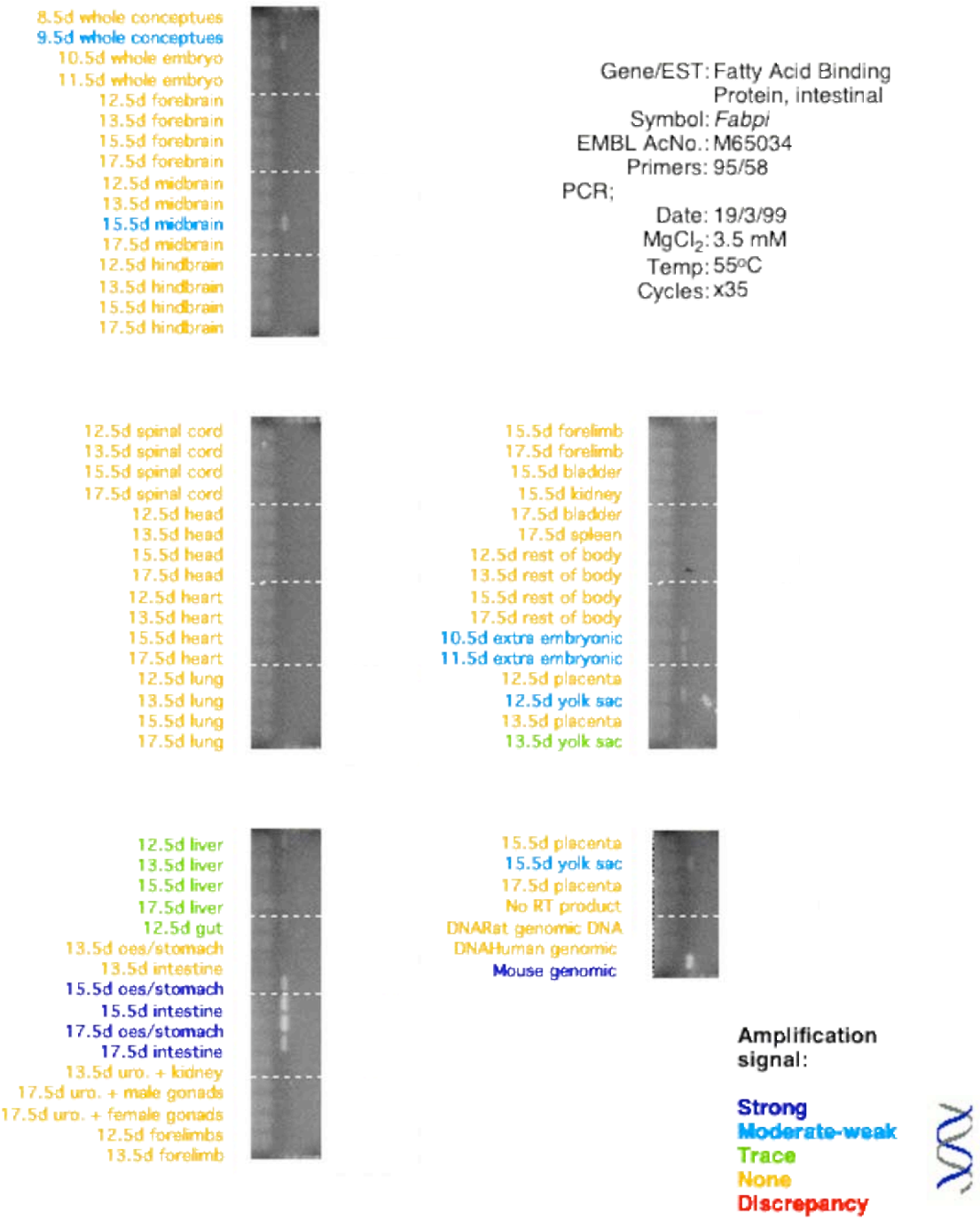


Figure 25: Expression Profile of Fatty Acid Binding Protein in Mouse Adult Panel

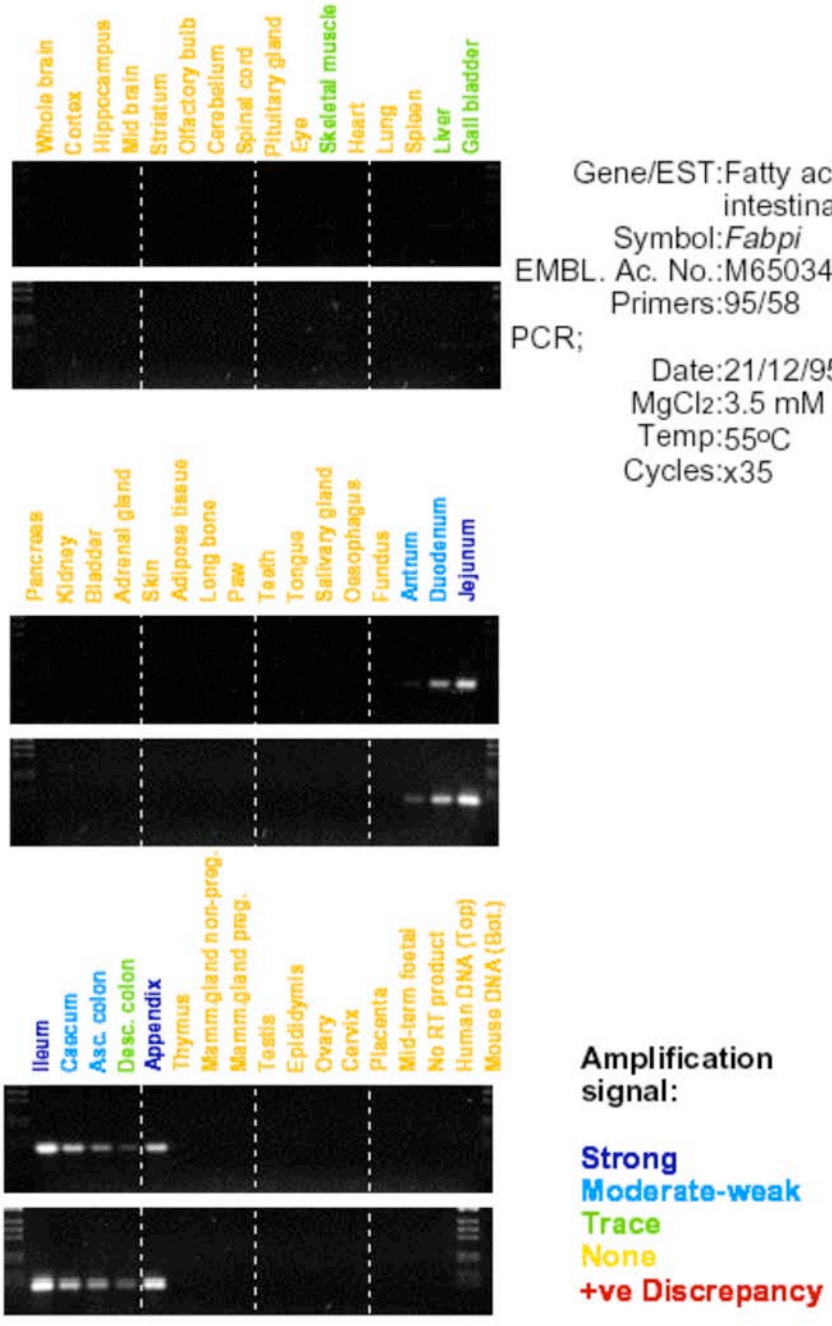


Figure 27: Expression Profile of Adenosine A1 in Mouse Foetal Panel

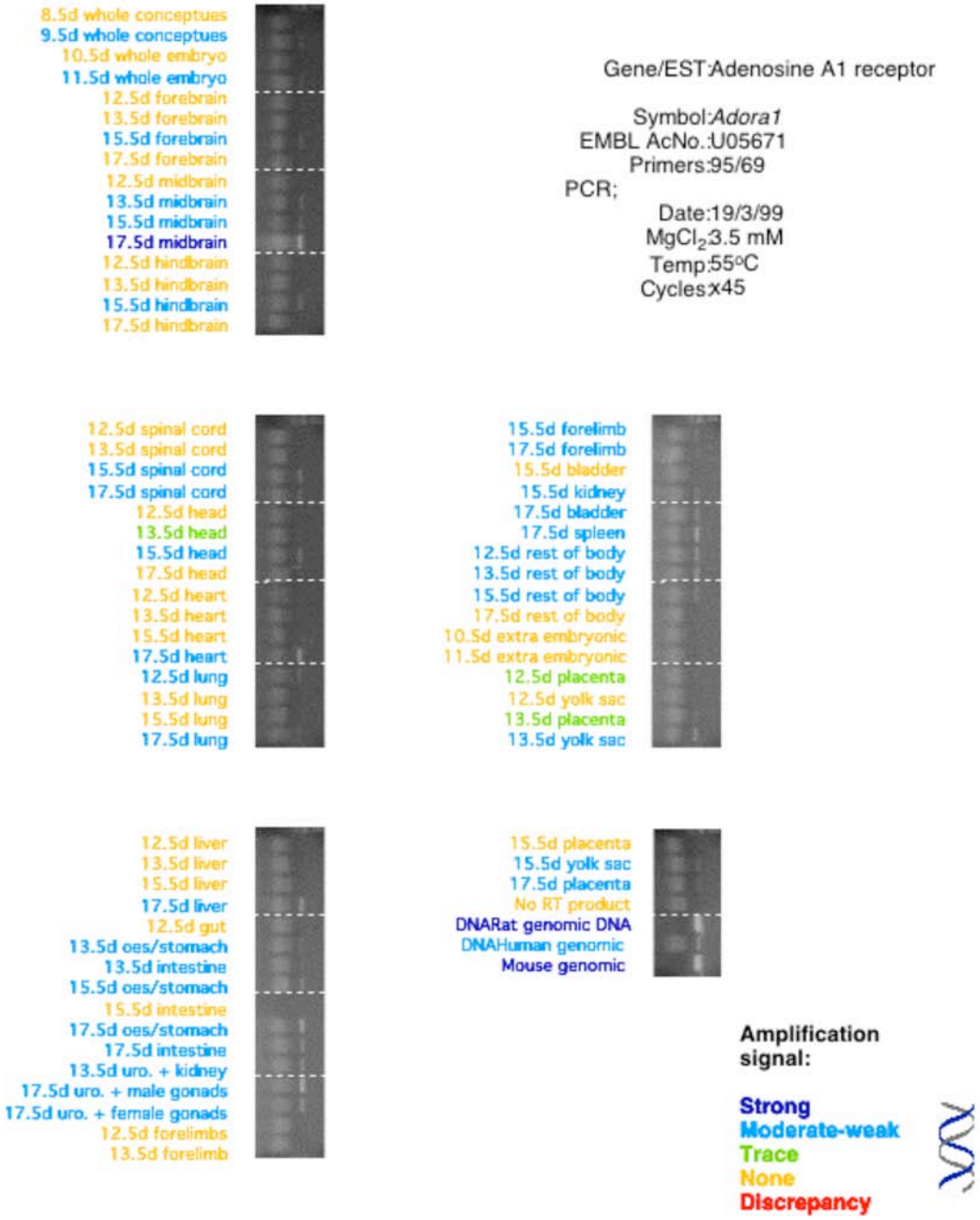
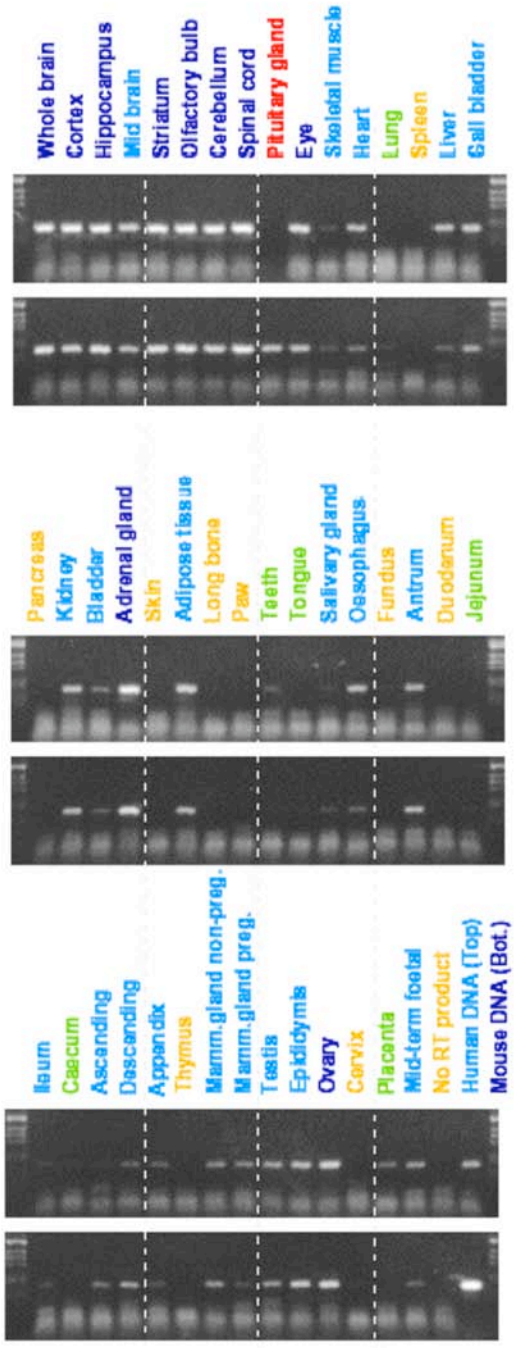


Figure 28: Expression Profile of Adenosine A1 in Mouse Adult Panel



Gene/EST: Adenosine A1 receptor
Symbol: *Adora1*
EMBL Ac. No.: U05671
Primers: 95/69
PCR;
Date: 14/3/96
$MgCl_2$: 3.5 mM
Temp: 55°C
Cycles: x45ex

Amplification signal:

Strong
Moderate-weak
Trace
None
+ve Discrepancy

Figure 29: Expression Profile of Homeo box B5 in Mouse Foetal Panel

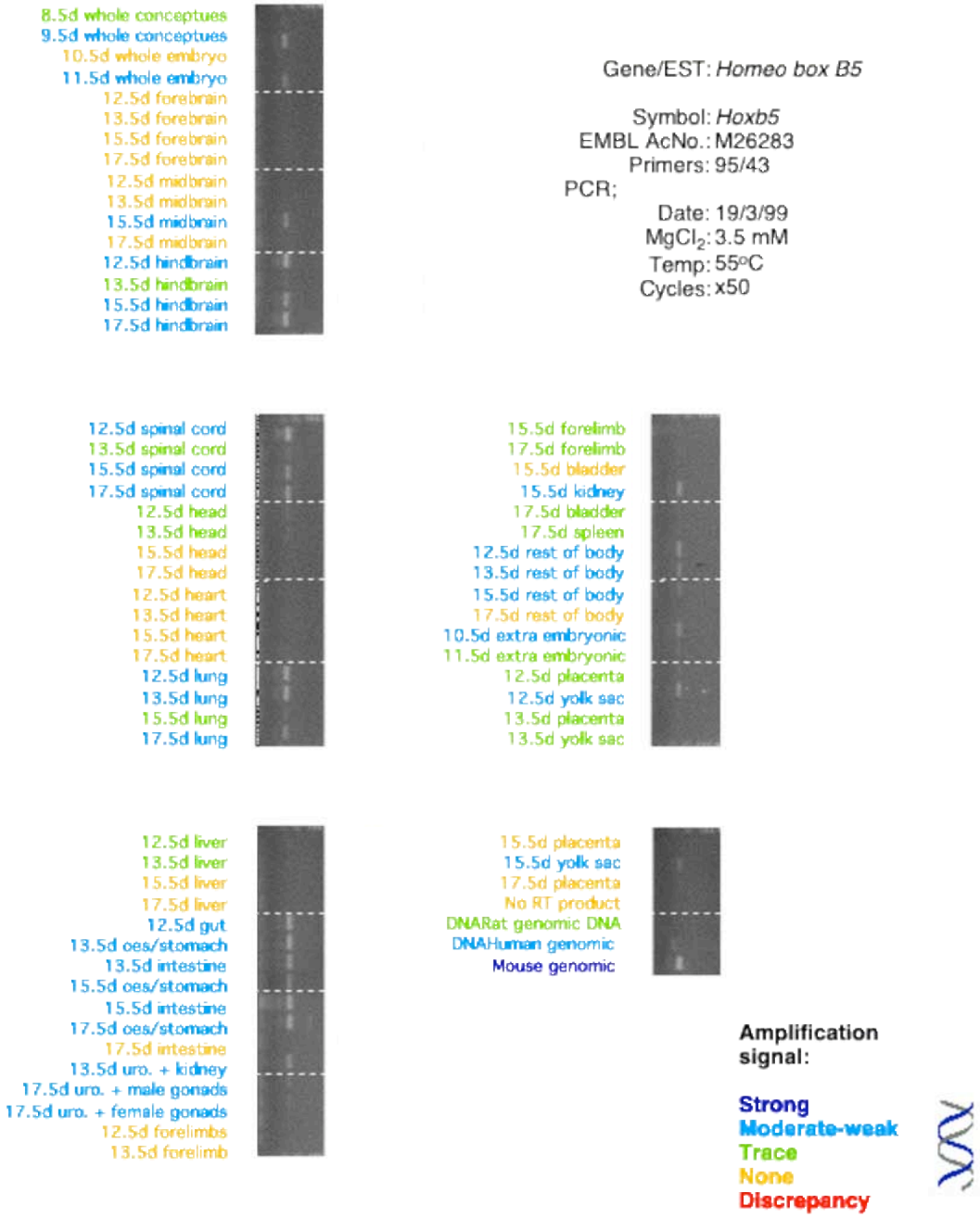
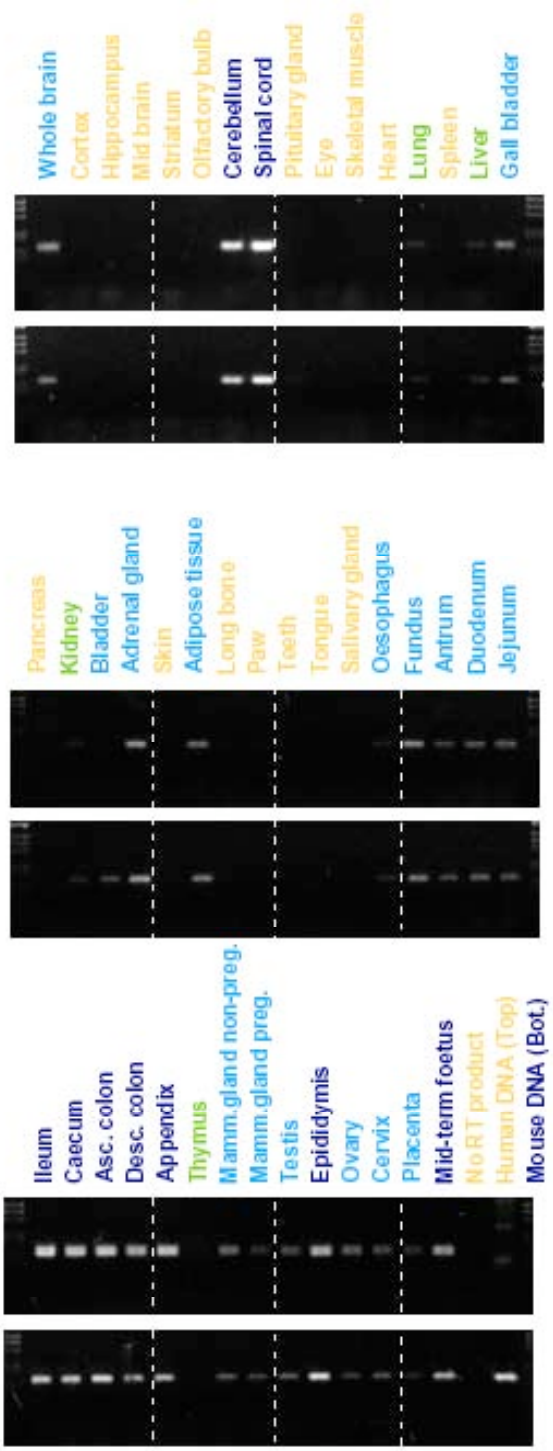


Figure 30: Expression Profile of Homeo box B5 in Mouse Adult Panel



Gene/EST:Homeo box B5
Symbol:*Hoxb5*
EMBL Ac. No.:M26283
Primers:95/43
PCR;
Date:31/1/96
$MgCl_2$:3.5 mM
Temp:55°C
Cycles:x50ex

Amplification signal:

Strong
Moderate-weak
Trace
None
+ve Discrepancy

Figure 31: Graphical representation of gel images in Figure 16

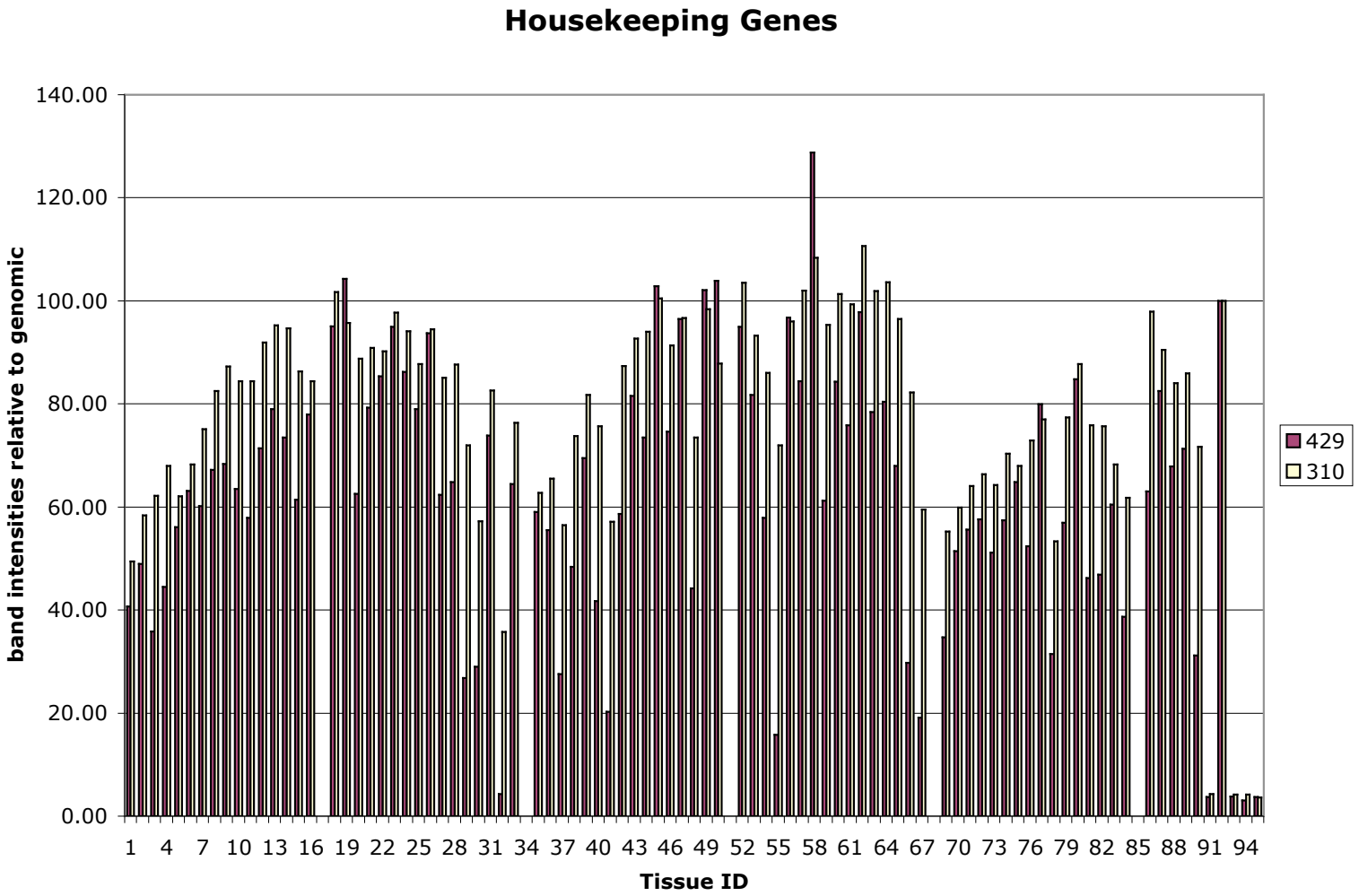


Figure 35: Tissue identification of graph position

ID	tissue
1	whole conceptuses 8.5d
2	whole conceptuses 9.5d
3	whole embryo 10.5d
4	whole embryo 11.5d
5	whole embryo 12.5d
6	whole embryo 13.5d
7	whole embryo 15.5d
8	whole embryo 17.5d
9	forebrain 12.5d
10	forebrain 13.5d
11	forebrain 15.5d
12	forebrain 17.5d
13	midbrain 12.5d
14	midbrain 13.5d
15	midbrain 15.5d
16	midbrain 17.5d
18	hindbrain 12.5d
19	hindbrain 13.5d
20	hindbrain 15.5d
21	hindbrain 17.5d
22	spinal cord 12.5d
23	spinal cord 13.5d
24	spinal cord 15.5d
25	spinal cord 17.5d
26	head 12.5d
27	head 13.5d
28	head 15.5d
29	head 17.5d
30	heart 12.5d
31	heart 13.5d
32	heart 15.5d
33	heart 17.5d
35	lung 12.5d
36	lung 13.5d
37	lung 15.5d
38	lung 17.5d
39	liver 12.5d
40	liver 13.5d
41	liver 15.5d
42	liver 17.5d
43	gut 12.5d
44	intestine 13.5d
45	oes/stom 13.5d
46	intestine 15.5d
47	oes/stom 15.5d
48	intestine 17.5d
49	urogenital/kidney 12.5d
50	urogenital /kidney 13.5d

ID	tissue
52	urogenital/gonads 15.5d
53	urogenital male gonads 17.5d
54	urogenital female 17.5d
55	bladder 15.5d
56	kidney 15.5d
57	bladder 17.5d
58	kidney 17.5d
59	spleen 17.5d
60	forelimbs 12.5d
61	forelimbs 13.5d
62	forelimbs 15.5d
63	forelimbs 17.5d
64	rest of body 12.5d
65	rest of body 13.5d
66	rest of body 15.5d
67	rest of body 17.5d
69	extra embryonic 10.5d
70	extra embryonic 11.5d
71	placenta 12.5d
72	yolk sac 12.5d
73	placenta 13.5d
74	yolk sac 13.5d
75	placenta 15.5d
76	yolk sac 15.5d
77	placenta 17.5d
78	yolk sac 17.5d
79	whole brain - adult
80	spinal cord - adult
81	skeletal muscle - adult
82	heart - adult
83	liver - adult
84	kidney - adult
86	fundus - adult
87	caecum - adult
88	testis - adult
89	ovary - adult
90	one day old mouse
91	glycogen
92	mouse genomic
93	human genomic
94	rat genomic
90	glycogen

References

1. Prunell, A., *A photographic method to quantitate DNA in gel electrophoresis*. Methods in Enzymology, 1980. **65**(1): p. 353-358.
2. Ringwald M, et al., *The mouse gene expression database*. Nucleic Acids Research, 2001. **29**: p. 98 - 101.
3. Freeman, T.C., et al., *Expression Mapping of Mouse Genes*. MGI Direct Data Submission, 1998.