

**DEVELOPMENT OF A ZINC-BASED
FIXATIVE FOR DNA, RNA AND
PROTEIN MOLECULAR STUDIES**

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DECLARATION

All the work contained in this thesis was conducted by me unless otherwise stated in the text.

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ABSTRACT

Fixation is a chemical process by which tissue and cell components are 'stabilised' temporally and spatially, preserving morphology, nucleic acids, proteins and other cell constituents. There is a wide range of fixatives, based on their chemical function, but none of the compounds used is ideal, i.e. for preserving both morphology and integrity of DNA, RNA and protein. This thesis aimed to develop a reliable, cost-effective and non-toxic fixative to meet the needs of contemporary molecular pathobiology research, particularly in respect of RNA and DNA integrity.

The effects of 30 different fixative recipes on the fixed quality of tissues from C57Bl6 mice colon, liver and spleen were investigated. Results from immunohistochemistry (IHC), polymerase chain reaction (PCR), Reverse transcription PCR, RNA Agilent Bioanalyser and Real-Time PCR showed that a novel zinc-based fixative (Z7) containing zinc trifluoroacetate, zinc chloride and calcium acetate was significantly better than the standard zinc based fixative (Z2) and neutral buffered formalin (NBF) for DNA, RNA and protein preservation. DNA sequences up to 2.4Kb in length and RNA fragments up to 361 bp in length were successfully amplified from Z7 fixed tissues, as demonstrated by PCR, RT-PCR and Real-Time PCR. Total protein analysis was achieved using 2-D gel electrophoresis. In addition, nucleic acids and proteins were very stable in fixed tissue blocks over a 12 month period. Fresh frozen samples were also included in this experimental model as controls for DNA and RNA integrity.

Using Affymetrix exon microarrays it was shown that Z7-fixed samples had no statistical significant difference in the exon alternative splicing pattern compared to fresh-frozen samples, and could be used for further human cancer array genome studies. Mass Spectroscopy studies using synthetic peptides and their reaction with zinc trifluoroacetate showed that zinc is binding to at least one amino acid, either cysteine or histidine. Future work is required to elucidate the exact chemical mechanism of zinc fixation.

In conclusion, this novel, non-toxic and economical tissue fixative could be applied for routine laboratory use to permit subsequent genomic/proteomic studies.

DEDICATION

This PhD Thesis is dedicated with all my love to my father Aristotle and to the loving memory of my mother Anastasia.

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LIST OF ABBREVIATIONS

ABC	Avidin biotin complex
AGCC	Affymetrix GeneChip® Command Console™
ANOVA	Analysis of variance
APE 1	Apyrimidinic endonuclease 1
ARK	Animal research kit
CSC/IC	Clinical Sciences Centre/Imperial College
CEL	Cell intensity
cDNA	Complementary DNA
cRNA	Complementary RNA
CHP	Chip files
CT	Crossing threshold
Cys	Cysteine
DAB	Diaminobenzidine
ddH ₂ O	Double-distilled water
DEPC	Diethyl pyrocarbonate
dH ₂ O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPX	Mounting medium
DTT	Dithithrietol
dUTP	Deoxyuridine triphosphate
His	Histidine
EtBr	Ethidium bromide

EtOH	Ethanol
EDTA	Ethylenediaminetetracetic acid
FDAS	Fixation dependent alternative splicing
FIAS	Fixation independent alternative splicing
FF	Fresh frozen
FLIM	Fluorescent lifetime imaging
GAPDH	Glyceraldehyde phosphate dehydrogenase
GCOS	GeneChip® operating system
H&E	Haematoxylin and eosin
HCl	Hydrochloric acid
HOPE	Hepes-glutamic acid buffer organic solvent protection effect
IHC	Immunohistochemistry
IgG	Immunoglobulin G
IVT	In vitro transcription
MALDI	Matrix-assisted laser desorption-ionisation
MS	Mass Spectrometry
NBF	Neutral buffered formaldehyde (3.7% v/v formaldehyde)
PBS	Phosphate buffer saline
PCA	Principle component analysis
PCR	Polymerase chain reaction
PLIER	Probe logarithmic intensity error estimation
QA	Quality assessment
RIN	RNA integrity number
RLE	Relative log expression
RMA	Robust multichip analysis
rRNA	Ribosomal RNA

RPT	Report files
RT-PCR	Reverse-transcription polymerase chain reaction
RT	Room temperature
SEM	Standard error mean
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TdT	Terminal deoxynucleotidyl transferase
UDG	Uracil DNA glycosylase
WT	Whole transcript

Units/

cm	Centimetres
g	grams
<i>g</i>	centrifuge force
l	litre
M	Molarity (moles/l)
m	Milli (10^{-3})
mg	Milligram
min	Minutes
mM	Millimolar
ml	Millilitre
μ	Micro (10^{-6})
μ l	Microlitre
μ g	Microgram
p	Pico (10^{-12})

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CHAPTER 1

INTRODUCTION

1.1 History of Fixation

During the course of history, people have employed a variety of chemical methods to preserve organic matter. In ancient Egypt, the inorganic salt natron (sodium sesquicarbonate) was used as a tissue desiccant along with organic preservatives, although the nature of the organic chemical treatments remains unknown due to the lack of written records by the Egyptians (Buckley & Evershed 2001). Traces of bitumen have been found in several mummies which may be an explanation for the blackened appearance of preserved bodies (Bahn 1992). Many centuries later, around 400 BC, Hippocrates in his medical writings discussed the biological effects of mercury and alcohol as fixatives (Srinivasan, Sedmak, & Jewell 2002). However, the histological study of tissues only began with the invention of microscopy by Hooke in 1655. In order to generate sections of soft tissues thin enough for microscopic examination, the tissues had to be preserved and stiffened sufficiently.

Once tissues are removed from the body, they undergo a process of self-destruction or autolysis which is initiated soon after cell death by the action of intracellular enzymes causing the breakdown of protein and eventual liquefaction of the cell. Autolysis is independent of any bacterial action, retarded by cold, greatly accelerated at temperatures of about 30°C and almost inhibited by heating to 50°C (Bancroft & Gamble 2002).

Autolysis is more severe in tissues rich in enzymes, such as the liver, pancreas and kidney, and is less rapid in tissues such as elastic fibre and collagen. By light microscopy, autolysed tissue presents a 'washed-out' appearance with swelling of cytoplasm, eventually converting to a granular, homogeneous mass which fails to take up biological stains. The nuclei of autolytic cells may show some of the changes of necrosis including condensation (pyknosis), fragmentation (karyorrhexis) and lysis (karyolysis) but these are not accompanied by an inflammatory or cellular response. There may be diffusion of intracellular substances of diagnostic significance, such as glycogen which is lost from the cells in the absence of prompt and suitable fixation. Autolysis also causes desquamation of epithelium which separates from its basement membranes.

When tissues are removed from a living organism deterioration of structure and loss of chemical constituents follow. The rate at which these occur in an individual tissue will depend on its susceptibility to putrefaction and autolysis (Warford Anthony 1999). Putrefaction describes tissue destruction caused by microorganisms while autolysis is the self-digestion of cells by enzymes that are always present within them (Baker 1958).

Fixation is defined as a series of complex chemical modifications of macromolecules present in tissues and cells, in order to: a) inhibit autolysis and bacterial decay in tissues, b) preserve their structural and functional components as closely as possible to the living state (Bancroft & Gamble 2002). Despite the fact that it is used by so many laboratories, most of the chemical reactions that occur during the process of fixation are largely obscure. The increasing numbers of molecular pathological techniques that are currently available, require material with good long-term preservation of nucleic acid and protein integrity.

However, pathological investigations of tissues performed at the molecular level are inevitably compromised by the effects of the fixatives and subsequent processing used. Remedial action is often needed to retrieve the original reactive properties of the macromolecules. In light of recent developments in molecular biology, and a requirement for rapid high throughput diagnosis, it is essential to optimise tissue fixation so that microscopy and tissue imaging can be combined with molecular biology techniques and genetic 'profiling'. An ideal fixative would enable researchers to do all this on tissue sections, thus allowing molecular studies on archival histological material.

A large number of fixatives is nowadays available but still no single substance or known combination of substances has the ability to preserve and allow the demonstration of every tissue component. The selection of a suitable fixative is based on considerations such as structures and entities to be demonstrated and the short and long-term effects of storage (Srinivasan, Sedmak, & Jewell 2002). Also, the requirements of a busy high throughput histopathology laboratory are quite different from those of a molecular research laboratory receiving a small number of specimens that require specialised molecular analysis but without the need for urgency.

1.2 Types of fixatives

Over the years, various fixatives fixative classifications have been proposed, some according to function, others according to their chemical nature. In this Chapter, we will use a modification of Hopwood's classification (Bancroft & Gamble 2002) (Table 1.1). This section of Chapter 1, aims to provide the reader with a useful introduction of the most commonly used fixatives, their applications, disadvantages and mode of action where it is known.

Table 1.1: Classification of fixatives.

Fixative type	Examples
Cross-linking	Formaldehyde, glutaraldehyde
Protein-denaturing	Methanol, ethanol
Oxidising agents	Osmium tetroxide, chromic acid
Unknown mechanism	Picric acid, zinc salts
Combined reagents	Methacarn, Carnoy's
Commercial compounds	HOPE, UMFIX
Alternative ways of preservation and fixation	Fresh-freezing, microwaving

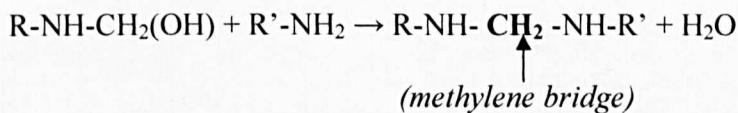
Fixation is dependent upon several parameters such as pH, temperature, osmolarity, concentration, duration and depth of penetration. All these parameters are of importance for a satisfactory result during fixation. The rate of penetration for each fixative is based on the formula: $d = K\sqrt{t}$, where d = depth penetrated, t = time of fixation and K = coefficient of diffusibility of the fixative and is specific for each fixative. Below is an overview of some of the current fixatives and some of

their most common features and limitations. This is not a complete list of all the existing fixatives, the intention being to provide the reader with an insight into different types of fixatives.

1.2.1 Cross-linking fixatives

Cross-linking fixatives include aldehydes such as formaldehyde and glutaraldehyde. Formaldehyde ($H_2C=O$) is soluble in water to 37%. It is better known as formalin (a 10% solution of formaldehyde) and is probably the most commonly used fixative for routine histopathology. Ferdinand Blum was the first person to use formaldehyde as a tissue fixative in 1893 (Puchtler & Meloan 1985). The most important reaction during formaldehyde fixation is the formation of methylene bridges between lysine side chains of proteins at pH between 6 and 8, as seen in the chemical formulations below:

$R-NH_2 + HCHO \rightarrow R-NH-CH_2(OH)$, which is followed by



Similar reactions with arginine and sulphydryl groups of cysteine (Pearse 1980) have also been demonstrated but at a $pH > 8$. Used alone morphological preservation is not optimal but can be improved if sodium chloride is added. Also formalin buffered to neutral pH with sodium phosphate favours additive reactions with proteins. It has also been reported that the amount of DNA that can be extracted from tissues fixed in neutral buffered formalin (NBF) is up to 50 times more than those fixed in unbuffered formalin (Zsikla, Baumann, & Cathomas 2004).

Paraformaldehyde (PFA) is the polymerised form of formaldehyde ($(OH-(HCHO)_n-H)$). It is mostly used for fixing tissue samples for electron microscopy (EM). In aqueous solutions it reverts to its monomeric form. Nevertheless paraformaldehyde needs to be made fresh as it has a tendency to precipitate (Bancroft & Gamble 2002a).

Glutaraldehyde ($CHO-CH_2-CH_2-CH_2-CHO$), another aldehyde, is a faster fixative since it has more reactive groups ($-CH_2$) in its chemical composition, but penetrates tissues more slowly than formaldehyde because it is a larger molecule, necessitating the use of very small pieces of tissue. The fixation reaction is not reversible by addition of water and it cross-links histones (Luft 1992). It also needs to be made up fresh. It is mainly used for fixing tissue samples for EM.

Periodate-lysine-paraformaldehyde (PLP)

Periodate-Lysine-Paraformaldehyde (PLP) is a variant of PFA that preserves ultrastructure as well as glutaraldehyde and antigenicity as well as paraformaldehyde (McLean & Nakane 1974). Other less commonly used cross-linking fixatives include acrolein, carbodiimide and diethyl pyrocarbonate (DEPC). DEPC is also an RNase- and trypsin inhibitor. It reacts with free protein amino groups and preserves good immunoreactivity of peptides. Periodate oxidizes macromolecules containing carbohydrates such as cell surface glycoproteins, that cross-link by the two amino groups of lysine (Hopwood 1969).

Interactions of formaldehyde and glutaraldehyde with proteins.

Both formaldehyde and glutaraldehyde act as fixatives by cross-linking proteins in cells, but the chemistry differs for the two aldehydes. Glutaraldehyde reactions have not been completely elucidated and are complicated by the fact that these aldehydes

exist in many polymerised forms. Chemical studies so far, have indicated that glutaraldehyde reacts with amines to produce pyridine derivatives. This reaction is considered to be the cause of cross-linkage of proteins within the cell and is also irreversible (Hopwood 1969; Sewell, Bouloukos, & Holt 1984).

On the other hand, formaldehyde cross-links cellular proteins by the formation of methylene bridges. In contrast to glutaraldehyde, formaldehyde fixation is reversible. Furthermore, glutaraldehyde seems to cross-link only the protein moiety of chromatin, whereas formaldehyde cross-links both protein and DNA (Sewell, Bouloukos, & Holt 1984). Studies by Hopwood indicate that at temperatures up to 64°C no reaction occurs between native DNA and glutaraldehyde. Above 75°C, reactions occur and they proceed more rapidly at higher temperatures. Similar reactions between glutaraldehyde and RNA also occur except that they start above 45°C. The possible reason for this could be that the nucleic acid tertiary structure is maintained by hydrogen bonds which are weakened or broken at higher temperatures (Hopwood 1975). The different fluorescent lifetime imaging (FLIM) properties of glutaraldehyde and formaldehyde have also been indicative of possible different chemical interactions of those two aldehydes (Elson et al. 2004).

Genipin

Genipin is a naturally occurring cross-linking agent which has been shown to react spontaneously with amino acids to form a dark blue pigment, which could be a major disadvantage in microscopic work. After fixation with genipin, the resistance of the tissue against collagenase degradation increased significantly (Sung et al. 2001). Genipin may form intramolecular and intermolecular cross-links with a cyclic structure within collagen in tissues (Sung et al. 2001). This fixative was

developed for use in biological implants such as heart valves because it allows the tissue to retain its elasticity while sterilising it. Thus it may not be suitable alone as fixative for histology, but rather be tried as an additive.

Reuterin

A natural compound produced by *Lactobacillus reuteri*, reuterin (β -hydroxypropionaldehyde) ($OH-CH_2-CH_2-CHO$) was tested as a tissue fixative. Results showed that reuterin is significantly less cytotoxic than glutaraldehyde and is an effective agent in the sterilisation and fixation of biological tissues (Chen et al. 2002; Sung et al. 2002; Sung et al. 2003). Like genipin, reuterin has also been used in biological implants and could be tested as an additive in fixation protocols. This aldehyde has not been used for molecular studies and is not commercially available.

1.2.2 Precipitant fixatives

Precipitant fixatives include alcohols such as methanol and ethanol, acetone and acetic acid. Their mode of action is to denature proteins by breaking the hydrophilic bonds that make up the tertiary structure of protein molecules. However, the primary and secondary structures of the protein molecules are left intact, thus the amino acid residues that act as antigenic sites remain available to applied antibodies. This is because the sites for antibody presentation are not altered. Tissue preservation is usually inferior to that achieved by formalin fixation, probably due to shrinkage (Srinivasan, Sedmak, & Jewell 2002). The exact chemical reactions that take place during alcohol fixation are not established.

(Alcohols) - 70% Ethanol

Methanol and ethanol are the only alcohols which have a role as fixatives. Methanol is closely related in structure to water and it competes almost as effectively as the latter for hydrogen bonds. Ethanol contains one more carbon atom, but is also closely related in structure and replaces water molecules in the tissues, unbound and bound, during fixation. While 100% ethanol preserves glycogen, it will cause distortion of nucleic detail and shrinkage of the cytoplasm. If fixation is prolonged, alcohols will remove histones from the nuclei (Bancroft & Gamble 2002). Fixation of human prostate and kidney using 70% ethanol showed that it allows for molecular analysis, although there were adverse effects on the protein and nucleic acid quality (Gillespie et al. 2002). Another study has shown that 70% ethanol can be useful for molecular studies, despite a significant drop in the number of transcripts detectable (Perlmutter et al. 2004).

Acetone

Acetone is a clear, colourless, inflammable liquid which is miscible with water, ethanol and most organic solvents. It has a rapid action but causes brittleness in tissue if exposure is prolonged and because it is volatile and inflammable, acetone is not used in automated processing schedules. However, it has a greater solvent action on lipids and is rapidly removed by most clearing agents, making it very useful in manual processing procedures (Bancroft & Gamble 2002). Acetone is used mostly as a post-fixative for cryostat sections. It has also been used as a fixative in the acetone-methylbenzoate-xylene technique (AMEX) (Sato et al. 1986).

1.2.3 Oxidising agents

Chromic acid (chromium trioxide)

Chromium acid (chromium trioxide) is a strong oxidiser that is used with other components. It has no effect on fats, penetrates slowly and leaves tissues in a state where shrinkage may occur during subsequent tissue processing. Chromium salts interact with water molecules to bring about complexes that combine with reactive groups of protein chains. That creates a cross-linking effect similar to that of formalin (Bancroft & Gamble 2002). When potassium dichromate reacts with adrenal medullary catecholamines a black or brown water-insoluble precipitate is formed. For that reason, potassium dichromate is never used alone, and if it is used other than for the demonstration of amines, the tissue should be washed thoroughly to remove the oxide that forms, since it cannot be removed later in tissue processing.

Osmium tetroxide

Osmium tetroxide is a non-coagulant fixative and its chemical reaction with phenols with a hydroxyl group (-OH) give rise to a stable chelate. Amino acids such as cysteine, histidine, lysine, methionine and tryptophan all form adducts with osmium tetroxide. Its main use is to confer electron density on tissues to be studied by electron microscopy. It should be noted, though, that osmium tetroxide is a toxic compound (Hopwood 1985).

1.2.4. Unknown mechanism

Picric Acid

Picric acid, when it is combined with other chemicals penetrates well, precipitating all proteins. It will continue reacting with tissue components unless the specimen is thoroughly washed after fixation (Hopwood 1969). It is also a component of Bouin's fixative.

1.2.5. Combinations of fixatives

The utilisation of more than one chemical for the purpose of fixation has been a logical evolution in the history of tissue fixation. There is obviously a very long list of combinations of fixatives and the aim is to present below some of the most commonly used combinations that are of value. The rationale for using combinations of fixatives is to combine the advantages of each compound in order to have an improved fixative.

Methacarn

Methacarn consists of 60% methanol, 30% chloroform and 10% glacial acetic acid. As a precipitant fixative its mode of action is similar to alcohol but it is enhanced by the presence of chloroform and acetic acids, creating a more acidic environment that allows faster penetration. Nevertheless, the use of chloroform makes this a toxic compound. It partially preserves RNA (Bancroft & Gamble 2002; Kim et al. 2003; Shibutani et al. 2000) and immunoreactive antigenic sites minimising the need for antigen retrieval in tissues fixed in it. Methacarn causes precipitation of ribosomal proteins in the cells inactivating endogenous RNases and/or protecting the mRNA from RNase action (Srinivasan, Sedmak, & Jewell 2002).

Carnoy's fixative

It consists of 60% ethanol, 30% chloroform and 10% glacial acetic acid. It is used for preserving DNA and RNA but again the use of chloroform makes it a toxic compound, despite the fact that it removes lipid and thus gives better staining.

Bouin's fixative

It consists of saturated aqueous picric acid (70%), formaldehyde (25%), and glacial acetic acid (5%), but alternative formulations can be found as well. These components all react with each other; therefore it should not be used after 40 days of preparation (Hopwood 1985). The use of Bouin's solution is labour intensive, requiring multiple alcohol rinses to remove picric acid for optimal preservation and immunohistochemical detection. Tissues are damaged when the picric acid is not removed properly and remains in the tissue throughout the entire processing. In effect, the harmful reaction continues within the embedded specimen for several years (Bonin et al. 2005). This fixation method is also used for specific purposes because it better conserves some morphological details, such as nuclear conformation. This property makes Bouin's solution the elective fixative for testis and lymphoma specimen. Molecular analysis from Bouin's fixed samples is very difficult since this acidic fixative, heavily damages both DNA and RNA.

1.2.6. Commercial Fixatives

In the past few decades, the introduction of commercially available fixatives has increased significantly. Most of these components are alcohol-based, due to the growing need for fixatives that preserve the molecular integrity of samples. There are also other chemical solutions that claim to provide good molecular preservation and tissue integrity. Some of the most known compounds are discussed below.

1.2.6.1. Alcohol based (FineFix, UMFIX)

UMFIX (Universal Molecular Fixative)

The potential value of a new universal molecular fixative (UMFIX) for preservation of macromolecules in paraffin-embedded tissue has been tested. UMFIX is a mixture of alcohol (originally methanol but now isopropanol) and polyethylene glycol. It has been demonstrated that UMFIX can preserve morphology and macromolecules in paraffin-embedded tissue (Vincek et al. 2003), although it requires a specific tissue processor not currently available in the UK.

FineFix

Another commercial compound containing a predetermined mixture of ethanol and polyethylene glycol, with the commercial name FineFix has been used recently as a fixative (Stanta et al. 2006). Results showed that this alcohol-based compound, produced promising results, although for DNA and RNA amplification, 45-60 cycles were used in the PCR protocols, which is unusually high.

1.2.6.2. Other (DSP, HOPE)

HOPE (Hepes-glutamic acid buffer mediated Organic solvent Protection Effect)

The use of the recent HOPE fixation technique (Olert et al. 2001) has allowed researchers to preserve and extract high molecular weight DNA and RNA of >20Kbp claimed to be suitable for polymerase chain reaction (PCR) and (reverse transcriptase PCR (RT-PCR) from paraffin-embedded tissues up to 5 years old (Wiedorn et al. 2002). Also HOPE-fixed cultured cells and tissue sections can be used for immunohistochemistry (IHC) and *in situ* hybridisation (ISH) (Umland et al. 2003). More recently another report showed that HOPE fixation can be applied

to Western blotting (Uhlir et al. 2004). A potential problem is the need to use low temperature melting point wax for embedding. The major drawbacks are the cost of product, the fragility of tissues, the use of acetone in dehydration and the requirement for hand processing. In addition, there are some indications that not all RNA and protein molecules are preserved.

DSP (reversible cross-linker)

A recent study, showed that a reversible cross-linker dithio bis[succinimidyl propionate] (DSP) can be used to fix mouse fibroblast cells and rat brain tissue successfully for the purpose of immunostaining, microdissection and expression profiling (Xiang et al. 2004).

1.2.7. Alternative ways of tissue preservation and fixation

Snap freezing

This method involves freezing the tissue quickly in melting *isopentane*. *Isopentane* is pre-cooled in liquid nitrogen until it is solid and is then allowed to melt at room temperature (RT) until the tissue can be immersed. *Isopentane*, at its melting point (-160°C), is an excellent heat conductor so that tissue freezes instantly and formation of ice crystals is minimised. Sections of frozen tissue can be cut in a cryostat, or tissue components can be extracted from the frozen block. One advantage is that enzyme activity is well preserved. However, sections have distinctive morphology to formalin-fixed paraffin sections and frozen material is difficult to store and to handle (Naber, Smith, & Wolfe 1992).

Microwave

Microwave irradiation for the purpose of tissue fixation was first introduced by Mayers (1970), who showed that direct exposure to microwaves generated by a 630 Watt heating device, produced adequate fixation in both mouse and human tissues. Microwaves are a form of non-ionising radiation, commonly generated by domestic ovens at a frequency of 2450 MHz. Fixation by heat is a well-established technique (Bancroft & Gamble 2002). Heat can effect the partial denaturation of proteins (Mayers 1970). Microwave energy interacts with dipolar molecules, causing their oscillation at a frequency of 2450 MHz. The optimum temperature for human tissue fixation is about 45°C-55°C (Hopwood 1985; Login & Dvorak 1985).

While microwave irradiation may serve as the primary method of fixation, microwaves can also accelerate the fixing action of aldehydes or alcohols. In a study by Leong et al., the heating to 58°C of tissue (<2mm² thick) submerged in normal saline resulted in fixation of a quality comparable with that produced by conventional fixation in 10% formalin. Microwave irradiation applied to tissues which had up to 1 h prior immersion in 10% formalin also produced excellent preservation of morphology, the time required for microwave fixation being no more than 150 s (Leong, Daymon, & Milios 1985). Microwave fixation was also found to be applicable to electron microscopy. Tissue samples immersed in 2.5% glutaraldehyde and fixed in 90 s by irradiation to 50°C showed excellent preservation of ultrastructural morphology (Login & Dvorak 1988). Nevertheless, microwaves have limited penetration in solid tissues and temperature gradients of up to 15°C were observed between the surface and core of large pieces of solid tissue such as the spleen, kidney, breast and liver (Leong & Duncis 1986). Since there is no significant protein cross-linking by microwave fixation further chemical fixation may be required (Kayser et al. 1988).

RNAlater

The use of snap freezing and storage at -80°C is costly and shipping of well preserved tissue samples from one laboratory to another on dry ice is inefficient and expensive. An alternative to snap freezing for preserving RNA is a new stabilization medium *RNAlater*, a non-toxic solution that is stored at ambient temperature. The contents of *RNAlater* are: sodium citrate, EDTA and ammonium sulphate at pH 5.2. Normal and tumour tissue stored in *RNAlater* for up to 7 days at (RT), has been successfully used for extraction of high-quality RNA (Florell et al. 2001; Grotzer et al. 2000). *RNAlater* is a tissue preservative and not a fixative, and for that reason tissues treated with *RNAlater* require post-fixation in order to maintain the morphological structure (Florell et al. 2001).

1.3 Zinc Biochemistry

Numerous enzymes and proteins utilise transition and group IIB elements to carry out their biological function. Zinc is the most widely used of these metals in biology, although not the most available, since it is only the 27th most abundant element in the earth's crust (Vallee & Falchuk 1993). In biological systems, very little if any, zinc is free in solution. Zinc carries out its biochemical function as a divalent cation primarily when bound to enzymes and other proteins. The chemical properties of zinc are particularly favourable to a multiplicity of functionally significant interactions, since under physiological conditions, it does not undergo reduction or oxidation. This lack of redox change renders zinc stable in a biological medium whose oxidoreductive potential is subject to continual flux (Berg & Shi 1996; Vallee & Falchuk 1993).

Zinc has been known to be an essential trace element in eukaryotes for more than a century (Berg & Shi 1996). There are approximately 2 to 3 grams of zinc in adult humans, making it one of the most prevalent “trace” elements. It wasn’t until 1940 though, that a specific biological function for zinc was identified, when it was shown that it was required for the catalytic conversion of carbonic anhydrase (Berg & Shi 1996). The enzyme carbonic anhydrase II from erythrocytes is a well-characterized enzyme that has a zinc ion bound to three histidine (His) residues in a tetrahedral geometry, which catalyzes efficiently the reversible hydration of carbon dioxide to form bicarbonate with discharge of a proton, and have important physiological and physiopathological functions (Thompson, Maliwal, & Fierke 1999). To date, 16 different carbonic anhydrase isoforms have been described in higher vertebrates, including humans, and some of them have been considered as important targets for inhibitors with therapeutic applications (Winum et al. 2007).

Currently, the number of zinc-containing enzymes known has blossomed to over 300, and in most of these enzymes, zinc is directly involved in catalysis or interacting with the substrate molecules undergoing transformation. But in a few enzymes zinc has a clearly structural function. Such a structural role for zinc in transcriptional factors was proposed in 1983 for the protein transcription factor IIIA (TFIIIA) (Berg & Shi 1996). The primary structure of TFIIIA contains highly conserved sequences comprised of 2 cysteine (Cys) and 2 histidine (His) residues separated by variable numbers of amino acids in 9 repeat units of 30 amino acids. Further analysis revealed the existence of 30 amino acid long zinc-based domains, termed “zinc fingers” in TFIIIA and in a wide variety of proteins involved in gene regulation (Berg & Shi 1996).

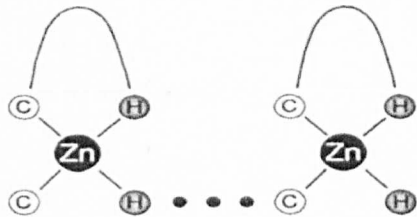
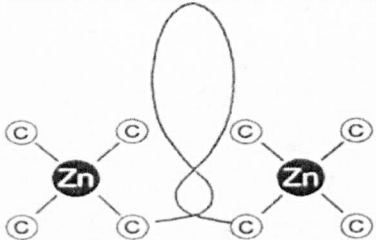
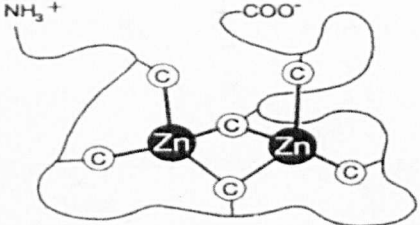
DOMAIN	STRUCTURE	PROTEIN	$d_{\text{Zn-Zn}}$
ZINC FINGER		TFIIIA	—
ZINC TWIST		Glu Rec	13
ZINC CLUSTER		GAL4	3.5

Figure 1.1: Zinc binding sites in gene regulatory proteins are shown. Three distinct zinc sites are presently known. In the zinc finger motif, zinc is coordinated to 2 Cys and 2 His residues. In both the zinc twist motif found in the glucocorticoid receptor and estrogen receptor and the zinc cluster motif found in the GAL4 protein, zinc is coordinated by 4 Cys residues. The structure of resultant DNA binding domain differs for each type. Taken from Vallee et al. 1991 (Vallee, Coleman, & Auld 1991).

Many nucleoproteins directly involved with replication and transcription of DNA are now known to contain functionally important zinc atoms. This is probably one of the best times to study the biology of zinc. After decades of obscurity, zinc has emerged from the list of elements in biochemical studies and in the last decade the importance of zinc in a range of biological processes has been revealed. These include brain function and pathology, gene transcription, immune function and mammalian reproduction (Thompson 2005), as well as a host of disease processes, such as Alzheimer's disease, epilepsy, ischemic stroke and infantile diarrhoea (Thompson 2005).

Still, important questions about the zinc function remain unanswered. What properties of zinc allow this ion to play structural roles in a series of important proteins? Zinc exists as a divalent cation Zn^{2+} , which has a completely filled d shell with 10 d electrons (zinc atomic number is 30). This electron configuration has 4 important consequences. First, because of the filled d shell, Zn^{2+} has no ligand field stabilisation energy when coordinated by ligands in any geometry. For ions with partially filled d shells, this electronic energy term can favour certain arrangements of ligands over others. Second, in terms of hard-soft acid-base theory, Zn^{2+} is regarded as a borderline acid. Because of this, zinc can interact strongly with a variety of ligand types including sulphur from cysteine, nitrogen from histidine, and oxygen from glutamate, aspartate, and water. Third, divalent zinc is not redox active; neither the potential oxidised form, Zn^{3+} , nor the potential reduced form, Zn^+ , is accessible under physiological conditions. Finally Zn^{2+} is relative labile in kinetic terms; it undergoes ligand exchange reactions relatively rapidly (Berg & Shi 1996).

Biological functions of Zn^{+2} have been reported for the protein-bound form. However, the biological function of free loosely bound (labile, chelatable) Zn^{+2} is less certain (Kikuchi, Komatsu, & Nagano 2004).

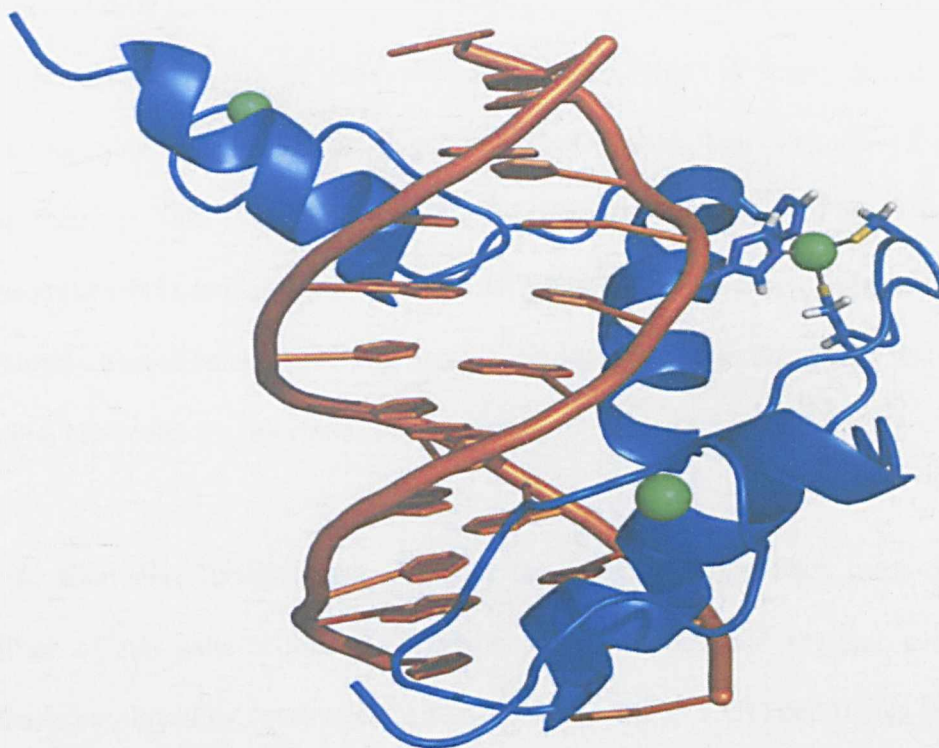


Figure 1.2: 3-dimensional representation of the transcription factor protein *Zif268* (blue) containing three zinc fingers in complex with DNA (orange). The coordinating amino acid residues of the middle zinc ion (green) are highlighted. This image was reproduced from Wikipedia online encyclopedia.

Zinc in Histopathology and Zinc-based fixatives

Zinc in the form of the salt zinc chloride has been used successfully against bacterial infection in dental hygiene products for years (Auschill et al. 2007; Fine et al. 2005). Therefore, since zinc salts are cheap and have known antimicrobial properties, it could be the reason why zinc salts were at some point tested as additives in already known fixatives and eventually used without any other fixative additives. The use of zinc solutions as primary fixative appears to be a new idea, but their use as additives to fixative solutions is not recent. Examination of the 1954 edition of the *Microtomists Formulae and Guide* reveals six fixative/preservative solutions containing zinc compounds, the oldest which appears to be Gilson's

solution (1890) (Beckstead 1994). Beckstead in his report from 1994, stated that Sheinin and Davenport in 1931, tested a large number of heavy metal salts, including some zinc compounds, as potential fixatives, but they categorised them as poor fixatives with the exception of ZnCl_2 , which they rated a “fair” fixative (Beckstead 1994). Under experimental conditions, zinc ions promote renaturation of previously heat-denatured DNA by holding the separated strands together while the original base pairs are reformed (Jia & Marzilli 1991).

Of the alternative fixatives proposed over the years, there has been focus on the addition of zinc salts to formalin (Dapson 1993). The original 1% zinc sulphate formalin employed by Jones et al. (Jones, Banks, & Caron 1981) and Banks (Banks 1985) was used to improve morphological detail. In a study by Herman et al. (Herman et al. 1988) zinc-formalin demonstrated very good immunoreactivity. Other zinc additives to formalin that have generated satisfactory immunohistological results include 2% zinc salicylate (Mugnaini & Dahl 1983), and 2% zinc sulphate (Tome et al. 1990). In 1994, Beckstead et al. for the first time demonstrated that a simple buffer containing zinc chloride, zinc acetate and calcium acetate could be used to fix tissue. More specifically, in this study, paraffin-embedded tissues fixed with a simple buffer containing zinc as the primary fixative were compared with tissues fixed with routine formalin, zinc-formalin, paraformaldehyde, ethanol, a variety of commercial (non-formalin-containing) fixatives that have been recommended for reduced toxicity and improved antigen survival, and frozen sections. Human lymphoid tissues and a group of antibodies to antigens (CD1, CD4, CD7, CD8, CD19) usually preserved only in frozen tissue were used as a model system.

Fixation in this solution of zinc acetate and zinc chloride in a Tris-Ca acetate buffer resulted in antigen preservation comparable to that in frozen sections with antibodies to these cell surface markers. Morphological preservation was comparable to formalin-fixed sections. This study presents a new method that represents the closest approach yet to a technique that combines optimal antigenic survival with the convenience and morphological preservation of traditional formalin-fixed tissue embedded in paraffin (Beckstead 1994).

A report in 2001, described a similar fixation method followed by paraffin wax-embedding and immunolabelling for the demonstration of several immune system cell epitopes (CD1, CD2, CD4, CD8, CD14, CD21, CD45R, WC-1, 28 kDa surface antigen, immunoglobulins and MHC II antigens) in ovine lymph nodes collected at necropsy. Cell surface epitopes considered to be sensitive to processing methods were successfully demonstrated by a procedure that included the use of a non-aldehyde-containing, zinc salts-based fixative, coupled with a sensitive system of immunolabelling. This novel method had the advantage of avoiding antigen-retrieval steps and of providing consistently good morphological definition (Gonzalez et al. 2001).

More recently, a zinc-based fixative (ZBF) was evaluated regarding its effects on tissue morphology, quality of genomic DNA, and preservation of protein immunoreactivity in a broad spectrum of tissues. Four different modes of fixation were analyzed: ZBF-paraffin embedding, NBF-paraffin embedding, ZBF-fixation prior to snap-freezing, and immediate snap-freezing. Laser-assisted microdissection, allowing retrieval of a defined number of cells for PCR, was used to study DNA quality. Genomic DNA was analyzed using primers for beta2-

microglobulin and the transferrin receptor. Immunohistochemistry was performed using nine antibodies (Ki67, Cyclin B1, p53, p27, Epithelial-cadherin, CD31, EGFR, HER-2, Pan-Cytokeratin). Only slight impairment of morphologic qualities was found after ZBF-paraffin embedding, whereas ZBF prior to freezing resulted in a crisper morphology compared with routine cryosections. A significantly higher DNA yield was observed in samples isolated from ZBF-paraffin-embedded tissues compared with NBF-paraffin-embedded tissues. Both yield and quality of DNA was comparable in frozen tissues irrespective to prior ZBF fixation. Immunoreactivity in paraffin-embedded tissue was superior in ZBF-fixed tissue compared with NBF-fixed for a majority of tested antibodies. Furthermore, for seven out of nine antibodies, antigen retrieval pretreatment proved unnecessary in ZBF-fixed tissue. Thus, despite a slight impairment of morphology, ZBF preserves protein structures well. The authors concluded that ZBF was superior to NBF for analysis of DNA and protein expression and suggested that fixation of tissues in ZBF may also be an alternative strategy to freeze storage of tissue specimens, for example, in future biobanks (Wester et al. 2003).

Ismail and co-workers (2003) fixed mouse normal and infarcted heart and small intestine in 4% formaldehyde, methyl Carnoy's and zinc (zinc acetate and zinc chloride in a Tris-Ca acetate buffer) and embedded the tissues in paraffin. They compared sections of these tissues with frozen sections for IHC identification of endothelial cells, using a wide variety of markers (Isolectin B(4), thrombomodulin, vWF, Tie-2, VE-cadherin, CD34, and CD31) and concluded that zinc-fixed paraffin-embedded tissue provided superior morphology. The CD31 immunostaining in this preparation was best for identifying proliferating endothelial cells during infarct repair and the reaction did not require antigen retrieval (Ismail et al. 2003).

In a 2006 study, tissue samples from RIII mice with immune mediated lesions, *Mycobacterium bovis* infected mice, and uninfected control mice were fixed in either zinc salt fixative or buffered formalin, then tested for IHC using a panel of antibodies (CD3, CD4, CD8, CD45, CD54, F4/80, Interferon-gamma and MIP2). In contrast to buffered formalin, zinc salt fixation preserved processing-sensitive murine cell markers (CD4, CD8 and CD54) and improved the intensity of immunolabelling for CD45, macrophage marker F4/80 and CD3 (Hicks et al. 2006). The molecular interactions involved in zinc fixation are not known. Zinc compounds are not suspected carcinogens and from that point of view zinc might appear to be a novel fixative candidate. To date there have not been any published studies using microwave heat with zinc-based fixatives for the purpose of tissue fixation, and subsequent analysis on the effect on tissue molecular integrity as well as morphology.

Tables 1.2 and 1.3 below summarise the key features of commonly used fixatives and the recipes for some of the most common combination fixatives.

Table 1.2: Properties of commonly used fixatives.

Name	Mode of action	Nucleic Acid Preservation	Morphological preservation	Protein preservation	Overall Comment
NBF	Cross-linking, methylene bridges, penetrates fast	Degrading DNA heavily	Retains best structural quality	Requires antigen retrieval, but good	Most common fixative for routine histology
Glutaraldehyde	Cross-linking, penetrates slow	Degrading nucleic acids	Preserves ultrastructure (best for electron microscopy)	Loss of α -helix structure of proteins	Needs to be made fresh, non-reversible fixation
Ethanol	Precipitant, penetrates slower than formalin	Good for DNA and RNA (up to 900bp)	Shrinks cytoplasm, distorts nuclear details	Good protein preservation	Not suitable for morphology
Acetone	Precipitant	Good for DNA (up to 300bp)	Causes brittleness if over exposed	Not good	Used for post fixation in frozen tissue, highly flammable
Methacarn	Precipitant	Good for DNA and RNA (up to 700bp)	Good (but not as good as NBF)	Good	Needs to be made fresh, toxic
HOPE	Unknown	Low degradation of nucleic acids	Good (but not as good as NBF)	Good protein preservation	Expensive product, makes tissue fragile, acetone is used
UMFIX	Precipitant	Good for DNA (up to 500bp)	Not tested	Not good	Requires specific processing method, contains ethanol
ZBF	Unknown	Preserves DNA well (up to 600bp)	Shrinkage is observed, but still good	Improved staining without antigen retrieval	Non-toxic, cheap, could be used for routine work

Table 1.3: Some fixative mixtures used.

Name of fixative	Components
Acetic Zinc Formalin	1. Zinc chloride, 12.5 g 2. Formaldehyde, 150 ml 3. Glacial acetic acid, 7.5 ml 4. Distilled water, to 1000 ml *Fixation time: up to 24 hours
Bouin's	1. Saturated aqueous picric acid solution 75 ml 2. 40% formaldehyde 25 ml 3. Glacial acetic acid 5 ml Prepare, using quantities indicated. *Fixation may vary from a few hours to 18 hours. Washing with 70% ethanol after fixation will remove most of the yellow colour. Sections can also be washed after removal of paraffin wax.
B5	Stock reagent A 1. Mercuric chloride 60 g 2. Sodium acetate 12.5 g 3. Distilled water 1lt Stock reagent B 1. 10% buffered neutral formalin To prepare a working solution mix 90 ml stock reagent A with 10 ml stock reagent B. *Fixation time: 5-8 hours.
Carnoy's	1 Absolute ethanol 60 ml 2 Chloroform 30 ml 3 Glacial acetic acid 10 ml *Fixation time: 5-6 hours.
Davidson's	1. Ethyl alcohol (33%), 2. 37-39% Formalin (22%), 3. Glacial Acetic Acid (11.5%) 4. Distilled water to 100% *Fixation time: up to 24 hours
Methacarn	1. Absolute methanol 60 ml 2. Chloroform 30 ml 3. Glacial acetic acid 10 ml Fixation time: 5-6 hours.
Zenker's	1. Distilled water 950 ml 2. Potassium dichromate 25 g 3. Mercuric chloride 50 g 4. Glacial acetic acid 50 g *Fixation is normally for 4-24 hours followed by an overnight wash.
ZBF	1. Zinc chloride 5gr 2. Zinc acetate 5gr 3. Calcium acetate 0.5gr 4. Make up to 1lt with Tris-HCl buffer *Fixation time: 24 hours

*Fixation time= time in fixative prior to paraffin wax processing and embedding

1.4 The Use of Fixed Tissues for Molecular Analysis

Molecular biology techniques have been utilised extensively in the study of human disease in areas as varied as: inherited disease diagnosis and screening, diagnosis and monitoring of patients with malignancies, detection of pathogenic organisms (bacteria, viruses, and fungi), epidemiologic surveillance, genetic predisposition to certain disorders, forensic pathology applications, and paternity testing. These techniques require the isolation of DNA and/or RNA from a variety of cell preparations or fresh tissue (Crisan & Mattson 1993;Mies 1992;Mies 1994).

Rearrangements of chromosomes, such as translocations, insertions or deletions may result in oncogene activation. DNA deletions or single base-pair substitutions may also activate an oncogene or suppress a tumour suppressor gene (Strachan & Read 2004).

Many research groups have attempted to extract nucleic acids and protein from different tissues (human and animal) fixed in various fixatives. However, a single 'ideal' fixative as defined earlier has not been found. This may be due to a number of factors. One reason is that the type of fixative and the time of fixation are crucial for the preservation of intact nucleic acids in the tissue (Ben Ezra et al. 1991;Foss et al. 1994;Goldsworthy et al. 1999). Another reason seems to be the effect of the post fixation processing step. During processing, the fixative is replaced by alcohol which in turn is replaced by xylene and finally embedded in wax (Bancroft & Gamble 2002).

1.4.1 The Effect of Fixatives on Protein Quality

Localisation of proteins with good antigenic properties from fixed and processed tissue is a useful tool for immunohistochemists and pathologists. Fixation denatures proteins, causing enzyme molecules and antigenic sites to be altered or destroyed. This is obviously a major consideration for the successful application of IHC techniques. Formaldehyde, which is the most common routine fixative cross-links proteins and 'masks' the antigenic sites of proteins. In order to reverse that reaction, protease treatment of tissue sections was introduced in 1976 (Huang, Minassian, & More 1976). More effectively, the use of heat combined with alkaline buffer (antigen retrieval) has been in use since 1991 (Shi, Key, & Kalra 1991), (Leong 2004; Miller, Swanson, & Wick 2000). An 'ideal fixative' would not require antigenic retrieval.

1.4.2 The Effect of Fixatives on DNA and RNA Quality

The tissue fixation process and the paraffin embedding procedure have a profound effect on the molecular arrangement of nucleic acids in the tissue and result almost invariably in DNA and RNA degradation. The ease with which DNA can be extracted and purified from fixed tissues and its quality depend on a multitude of factors. Some of the most important ones are thought to be: the chemical composition of the fixative, the duration of fixation, the duration of tissue hypoxia determined by the length of time elapsed between the surgical removal of the specimen and its fixation, the size of the specimen and its permeability to the fixative, and the period of storage in paraffin blocks (Crisan & Mattson 1993).

Fixation-induced DNA degradation is due to a range of mechanisms, some of which are only partially understood (Crisan & Mattson 1993; Mies 1994). The major mechanism, especially for formaldehyde-based fixatives, appears to be the cross-linking of proteins to DNA. The reaction of formaldehyde with polynucleotides is a two-step reaction; first, there is a reversible and rapid hydroxymethylation of the amino and imino groups of the nucleic acid bases, followed by a slow reaction resulting in methylene bridge formation between amino acids such as lysine, which can proceed over several days. These two steps are modulated by the reaction temperature, and the concentrations of the reactants. Temperature is a critical factor affecting the structure of native DNA in the tissue; double stranded DNA is present at 37°C, but when heated to 65°C the hydrogen bonds between bases start to break and at $\geq 90^\circ\text{C}$ single stranded DNA molecules are present. Formaldehyde reacts rapidly with denatured DNA by hydroxymethylation and inhibits reannealing of the DNA strands when the reaction temperature is lowered (Crisan & Mattson

1993;Mies 1992). This single-stranded DNA produced is unavailable for restriction endonuclease digestion. This could be an important mechanism of DNA degradation in paraffin-embedded tissues because of the high temperature sustained during wax impregnation (Warford Anthony 1999).

Fixatives containing mercuric chloride, such as B-5 and Zenker's, result in more extensive DNA degradation due to the additive effects of mercuric chloride and formalin (B-5) or mercuric chloride, potassium dichromate, and acetic acid (Zenker's pH 2.0), with Zenker's resulting in acid depurination of DNA as well. That is the case for other acid fixatives, such as Bouin's containing picric acid, acetic acid, and formalin (pH 2.0).

Fixation and processing of tissue usually degrades the RNA and makes extraction difficult. From the available methods, frozen tissues are preferred over paraffin-embedded since they yield higher quality RNA and more RT-PCR product than paraffin embedded samples (Goldsworthy et al. 1999). Cross-linking agents such as buffered formaldehyde limit the efficiency of extraction and the quality of extracted RNA (Finke et al. 1993;Rupp & Locker 1988;Stanta & Schneider 1991), and genomic DNA (Frank, Svoboda-Newman, & Hsi 1996;Poncin et al. 1999;Shibata 1994).

A greater problem than for DNA, RNA isolated from paraffin-embedded tissue blocks is of poor quality due to extensive degradation during formaldehyde fixation (Masuda et al. 1999). Moreover, formaldehyde fixation causes cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of

mono-methylol groups to the bases, making subsequent RNA extraction, reverse transcription and quantitative analysis problematic (Masuda, Ohnishi, Kawamoto, Monden, & Okubo 1999). As reported in several studies, the use of proteinase K and guanidinium thiocyanate in the isolation process was essential for obtaining a high yield of intact RNA with acceptable purity (Bencheikroun et al. 2004; Chirgwin et al. 1979; Chomczynski & Sacchi 1987; Masuda et al. 1999; Specht et al. 2001).

PCR has revolutionised the field of molecular biology by allowing exponential amplification of single target DNA molecules from a background of genomic DNA (Loda 1994). PCR is defined as an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be replicated exponentially using sequence-specific oligonucleotide primers synthesized *in vitro*. DNA extracted from fresh-frozen tissue can easily be used for PCR amplification whereas DNA extracted from archival fixed and paraffin-embedded material is achievable but has limitations and special requirements. The most important limitation is the size of the fragment to be amplified, which should not exceed 500bp. Also, for any PCR using fixed and embedded material, PCR conditions must be optimised, including low annealing temperature, high number of cycles (>35) and additives to the PCR master mix, such as formamide and dimethyl sulfoxide.

1.4.3 The Effect of Processing on Fixed Tissues

Tissue processing is divided into 4 steps: a) dehydration, where water is removed from the fixed tissue and replaced with a dehydrating liquid such as alcohol, b) clearing, where the dehydrating liquid is replaced with a fluid that is miscible with both the dehydrating agent and the embedding medium, usually an organic solvent such as xylene c) impregnation, where the clearing fluid is replaced by the embedding medium (commonly paraffin wax) at a temperature where the wax is infiltrated into the tissue and d) embedding and solidifying the tissue block (Bancroft & Gamble 2002).

Wax embedding causes structural damage and precludes the use of some histochemical methods (e.g., for most lipids and enzymatic activities) that require cryostat sections. Nevertheless, wax embedding is preferred for most purposes due to its technical simplicity, which is simplified further by the use of automatic tissue processing machines (Conway & Kiernan 1999).

Little is known of the effect that processing has on the morphology and the molecular quality of fixed tissue. Wester et al (2003) indicated that processing and/or storage in paraffin is responsible for the degradation of RNA. Designing and testing novel processing protocols could be a useful tool for improving the preservation of sensitive organic molecules such as DNA and RNA that might be lost through the conventional processing method.

1.5 Requirement for Novel Fixatives

The need for an 'ideal fixative' is now more than ever essential. This is due to the large amount of data that can be obtained from tissue using molecular biology techniques in order to aid prognosis, diagnosis and investigation of the mechanisms of human diseases such as cancer. It has been shown that formalin fixation produced up to one mutation artefact per 500 bp, most of them being C-T or G-A transitions (Williams et al. 1999). This might be an important finding considering the implication of molecular diagnostics from archival material, which in most cases is fixed with NBF. In another study by Quach et al 2004, where DNA human specimens extracted from fresh or formaldehyde fixed were amplified with Taq DNA polymerase and their PCR products were cloned and sequenced, it was shown that significantly more (3- to 4-fold) mutations were observed with fixed DNA samples. The majority of mutations were transitions, predominantly at A:T base pairs, randomly distributed along the template (Quach, Goodman, & Shibata 2004). This is a tremendous conclusion and one should seriously consider the possibility of a default during the sequencing process, and repeat the experiments before any meaningful conclusions can be drawn.

DNA isolated from paraffin-embedded tissues has been used for analysis of DNA alterations in disease states. A study by Wong et al. 1998, demonstrated that DNA alterations identified in formaldehyde preserved human tissues may be the result of chemical fixation and may not be present in unfixed tissues from the same person. Thus, the validity of sequence information obtained from DNA isolated from chemically fixed tissues must be questioned in the absence of corroborating data from untreated tissues. This finding compromises the reliable use of chemically

fixed tissues as a source of DNA for research as well as for clinical genetic testing in families for which a disease affected member is not alive. It also indicates the importance of setting up archives of fresh-frozen tissues, for use in future research and clinical genetic testing (Wong et al. 1998). In a 2005 study it was found that 4% unbuffered formaldehyde is unsuitable for telomere length determination by quantitative PCR providing another reason to re-evaluate the usage of formaldehyde as fixative (Koppelstaetter et al. 2005).

Another issue might be the degradation of DNA and RNA which is most common with NBF-fixed tissues. This prevents the application of high-throughput techniques such as real time-PCR and microarrays to a vast number of archival samples.

When compared to the DNA isolated from frozen tissues, formalin-fixed tissues exhibit a high frequency of nonreproducible sequence alteration. It has been speculated that the artifacts may be because of formalin cross-linking cytosine nucleotides on either strand. As a result, in PCR the Taq-DNA polymerase fails to recognize the cytosine and incorporates an adenine in the place of a guanosine, creating an artificial C-T or G-A mutation (Srinivasan, Sedmak, & Jewell 2002). While the requirement is for fixed samples to retain morphological features, there is a growing need to design new fixatives that will overcome all the above mentioned drawbacks.

1.5.1 Biochemical Techniques to Elucidate the Chemical Interactions of Fixatives

The mass of a protein can be precisely determined by mass spectrometry (MS)

In order to understand the chemistry of known fixatives but also to investigate the chemical interactions of novel development in fixation, the use of MS has been employed in recent years. This very powerful technique can separate molecules according to their relative mass and it is now possible to determine protein masses with an accuracy of one mass unit or less in favourable cases. This ability has opened up many new horizons in the study of proteins. Most powerfully, the mass of a peptide or protein can be used as a name tag for picking out a specific molecule in a vast database of amino acid sequences.

MS is a technique for analysing ionised forms of molecules in the gas phase. It is most readily applied to gases or to volatile liquids that easily release gas-phase ions. Mass measurements are obtained by determining how readily an ion is accelerated in an applied electric field. Consider two ions with the same overall charge but with different masses. In a given electric field, the field force will act on each ion. However, the acceleration of the more massive ion due to this force will be less according to Newton's third law, $F = ma$, where F is the force, m is the mass, and a is the acceleration. Thus a measurement of the acceleration in a known applied force provides the mass.

Because proteins and peptides are not volatile, generating a sufficiently high concentration of ionised but intact protein molecules in the gas phase is a great challenge. Two widely used methods, *matrix-assisted laser desorption-ionisation*

(MALDI) and *electrospray ionisation* (ESI) have been developed to solve this problem. In MALDI, the protein or peptide under study is coprecipitated with an organic compound that absorbs laser light of an appropriate wavelength (the “matrix”). The flash of a laser on the preparation expels molecules from the surface. These molecules capture electrons as they exit the matrix and hence leave as negatively charged ions. In ESI, solutions containing the protein or peptide flow through a fine metallic tip held at a nonzero electrical potential. Fine electrically charged droplets are released containing both the protein and the solvent. The solvent evaporates from droplet, concentrating the charge. The mutual repulsion of the like-charged molecules increases and individual molecular ions fly free.

After gas-phase ions have been generated, several approaches may be used to determine their mass. In *time of flight* (TOF) analysis, the ions are accelerated in an electric field toward a detector. The lighter ions are accelerated more, travel faster, and arrive at the detector first. Tiny amounts of biomolecules, as small as a few picomoles (pmol) to femtomoles (fmol), can be analysed in this manner (Berg, Tymoczko & Stryer 2006).

1.6 Rationale for this study

With the rapidly expanding base of genetic information from the Human Genome Project, and the development of high throughput technologies to obtain gene and protein expression profiles, it has become feasible to study the molecular basis of disease. The amount of molecular information that can be obtained from clinical tissue samples depends on how the samples are handled and processed. Future

efforts will focus on developing fixatives for maintenance of excellent histology and preservation of biomolecules (Leiva, Emmert-Buck, & Gillespie 2003). The application of imaging techniques such as Fluorescent Lifetime Imaging (FLIM), (Elson et al. 2004; Siegel et al. 2003; Tadrous et al. 2003) only make the demand stronger. It is evident from the literature data presented that such an 'ideal' fixative does not exist yet. Each category of known fixatives is useful for specific areas of research such as morphological studies or molecular studies with pitfalls including, toxicity, cost, ease of usage, limitation in molecular analysis. 10% buffered formalin and 2.5% glutaraldehyde are currently the most widely used fixatives for routine light microscopy and ultra-structural studies respectively, but they too, have inherent disadvantages including those of adverse effects on health and cost of safe disposal, which the user should be well conversant with (Arts, Rennen, & Heer 2006).

From the above it can be seen that there is an urgent need for a new, improved method of tissue fixation and preservation, that would provide good tissue morphology, preserve the molecular integrity of tissues, be of low cost and meet the health and safety criteria for everyday use, without the need for expensive chemical disposal.

The aim of this project therefore was to develop a chemical formula that can be used as a fixative for preserving tissue morphology, nucleic acid and protein. This was done in the following steps:

1. Identification of novel fixative candidates and comparative analysis of fixation properties; in terms of nucleic acid, and morphological preservation, and logistics.

2. Application of chemical modifications, alterations and substitutions in order to improve its fixation properties. Then evaluation of candidate fixatives by comparing DNA, RNA, protein and morphological preservation.
3. Detailed analysis of the ability to preserve DNA, RNA and proteins by comparison with NBF and fresh-frozen for the application of molecular techniques such as IHC, RT-PCR, Real-time PCR, Real-time RT-PCR, 2-dimensional protein gel electrophoresis, Affymetrix exon arrays. Also investigation of whether archival tissue fixed in this fixative can be used for molecular studies.
4. Investigation of the biochemical basis of the novel fixative using MALDI-MS.

CHAPTER 2

MATERIALS AND METHODS

Materials and chemicals used in this study were purchased from Sigma Chemicals unless otherwise stated.

All chemicals and solvents mentioned here were of analytical grade unless otherwise stated.

2.1 Mice

All the mice used in this study were C57Bl6 male/female mice 5-10 weeks of age. Mice were killed with CO₂ and dissected in a fume hood by animal house staff. Five types of tissue were used in this study. Colon was used for its variety of tissue type including epithelial, endothelial, nervous and muscular. Spleen was used as a type of lymphoid tissue and liver as a solid parenchymal tissue. Kidney was used as an organ that contains tubules, glomeruli and large vessels. Heart was used as a type of tissue that contains smooth muscle. Colon, liver and spleen tissues were removed, cut into pieces not more than 5mm thick and immediately immersed in the fixing solutions. Tissues were also fresh frozen using liquid N₂ for use as control samples for DNA and RNA quality. For every experiment, 3 different mice were used and then experiments from each mouse were repeated (3x2).

2.2 Fixatives

Standard tissue fixatives used in this study are given below: In all experiments, tissue samples were fixed in a shaking rotor (SANDREST, UK) at RT for 24 hours.

1) NBF

10% Formaldehyde (37%) (1.3M) in phosphate buffer, pH 7.4, always made fresh.

2) Zinc based fixative (Z2),

In 0.1 M Tris Buffer, pH 7.4 (1 l) add:

a) 0.5 g Calcium acetate (2.8 mM)

b) 5 g Zinc chloride (36.7 mM)

c) 5 g Zinc acetate (22.8 mM)

Mix to dissolve. Final pH: 6.4-6.7.

Do not readjust pH as it will cause zinc to come out of the solution

3) HOPE (Commercially available), pH 7.

4) 70% EtOH (700ml ethanol, 300ml dH₂O), pH 6.8.

4) Fresh frozen: place in liquid N₂ and then store at -80°C. This method was used as a control for comparison of DNA and RNA integrity.

2.3 Modifications to the Zinc Based Fixative

A range of chemical modifications were examined for their effect on morphology and nucleic acid preservation. These were:

a) Additions to the standard zinc based fixative (Z2).

1) DMSO: 2.5% (Z3), 5% (Z4), 10% (Z5) and 20% (Z6)

2) EDTA: 1.25% (Z9), 2.5% (Z10), 5% (Z11) and 7.5% (Z12)

3) DEPC: 0.5% (Z13) and 1% (Z14)

4) 0.5% DEPC + 5% DMSO (Z15)

b) Substitutions to the zinc based fixative (Z2), where zinc acetate from the Z2 fixative was replaced with an equal amount (5g in a 1 lt of fixative solution) of the following zinc salts:

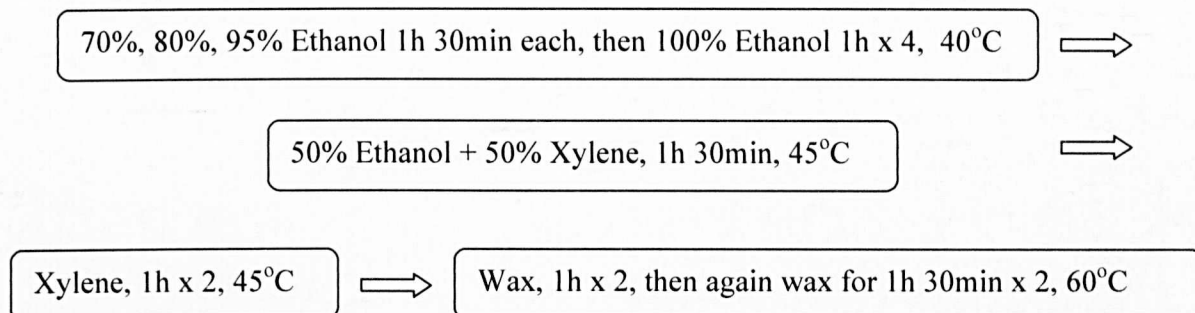
- 1) zinc trifluoroacetate (Z7), zinc citrate (Z8),
- 2) zinc trifluoroacetate + 5% DMSO (Z16)
- 3) zinc tartrate (Z17)
- 4) zinc tartrate + 5% DMSO (Z18)
- 5) zinc isovalerate
- 6) zinc trifluoromethylsulfonate
- 7) zinc perchlorate

c) Replacement of zinc in the zinc-based fixative with other metallic ions.

- 1) Manganese chloride (0.5%), manganese acetate (0.5%) and calcium acetate (0.05%) in 1 L of Tris-HCl Buffer, pH 6.5 (code name M2). In all experiments, tissue samples were fixed in a shaking rotor at room temperature (RT) for 24 hours.
- 2) Magnesium chloride (0.5%), magnesium acetate (0.5%) and calcium acetate (0.05%) in 1 L of Tris-HCl buffer, pH 6.5.
- 3) Gallium acetate (0.5%), calcium acetate (0.05%) in 1 lt of Tris-HCl buffer, pH 6.5.
- 4) Vanadium chloride (0.5%), calcium acetate (0.05%) in 1 lt of Tris-HCl buffer, pH 6.5.
- 5) Calcium chloride (0.5%), calcium acetate (0.05%) in 1 lt of Tris-HCl buffer, pH 6.5.

2.4 Tissue Processing and Embedding

Fixation in any one of the above solutions was followed by processing of tissues. Tissue processing was performed using an automated processing machine Vacuum Infiltrating Processor (VIP) by BAYER and program 3 (overnight) was used. Briefly this comprised:



When the processing program was finished tissue samples were embedded in paraffin wax. An appropriate size metal mould was chosen according to the size of tissue and was filled with molten wax. The processed tissue was placed in the metal mould with the surface to be cut facing down. Warm forceps were used, and the wax was hardened for 30 min at 4°C before removing from moulds.

2.5 Tissue Sectioning

Tissue sections (4 µm) from 5 types of mouse tissue (colon, liver, spleen kidney and heart) were cut using a rotary microtome (AS 325, SHANDON, UK). Sections immediately after cutting were floated on 20% alcohol and then on distilled H₂O at 37°C for 5 min. Sections were then picked up with a charged slide and dried at 39°C overnight. The blade was wiped with 70% ethanol to avoid cross-contamination.

2.6 Haematoxylin and Eosin Staining (H&E)

H&E staining was used for routine morphological assessment of fixed tissues due to its ability to demonstrate clearly a large number of different tissue structures. Briefly, 4 µm sections were taken through 3 changes of xylene, hydrated through 2 changes of absolute alcohol and 1 change of 70% alcohol and brought to running tap water for 1 min. Slides were stained with Harris' Haematoxylin for 30 sec, and washed in running tap water for 3 min. Slides were differentiated in 1% acid alcohol (1% HCL in 70% alcohol) for 5 sec and again washed in running tap water for 3 min.

Sections were stained with 1% eosin for 1 min and washed in running tap water for 3 min. Finally, sections were dehydrated through graded alcohols, cleared in xylene and mounted with DPX mounting media.

2.7 Immunohistochemistry (IHC)

IHC is a method for precise localisation of cellular or tissue constituents (antigens) using labelled antibodies. The antibody binding site can be recognized either by direct labelling of the antibody, by using a secondary labelled antibody, or a more sensitive three layer method as used here (Polak & Van Noorden 2003). IHC staining consists of applying a series of antibodies; the first one (primary) binds to the antigen in the tissue and then can itself act as an antigen for a second antibody, raised in an unrelated animal species to the IgG of the species providing the primary (Polak & Van Noorden 2003).

2.7.1 Standard avidin-biotin complex (ABC) immunostaining method

The following figure demonstrates the ABC immunostaining method.

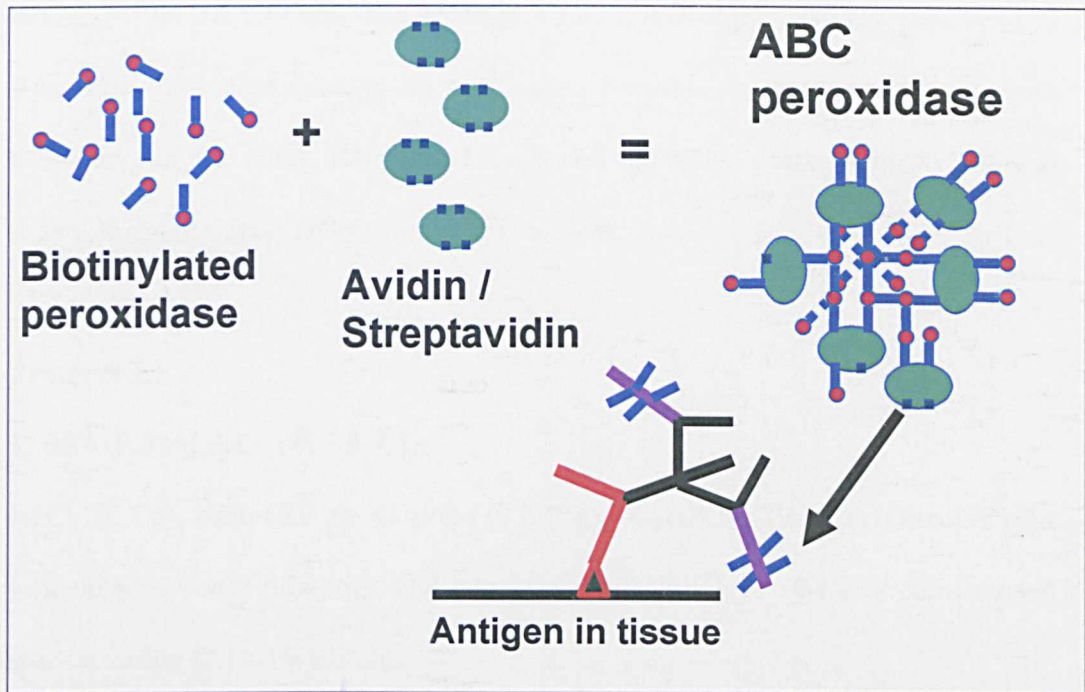


Figure 2.1: The ABC immunostaining method. Figure was kindly provided by Miss Susan Van Noorden.

In all experiments, slides were dewaxed in 3 changes of xylene, rehydrated through graded alcohols and brought to tap water. Slides were immersed for 15 min in 0.6% H_2O_2 in distilled water washed for 5 min in phosphate-buffered saline (PBS) (Protocol 2.1) and then transferred to Sequenza racks (Protocol 2.1). Appropriate normal serum (from the species providing the second antibody) diluted 1:20 in volume was applied for 10 min followed by a wash in PBS/Tween for 5 min. 100 μl of the primary antibody in PBS diluent was applied at 4°C overnight. Sections were washed in PBS/Tween for 5 min and incubated for 1h with appropriately diluted biotinylated secondary antibody. A negative control was included for every experiment where, instead of the primary antibody, PBS diluent was added to the slides. Sections were rinsed in PBS/Tween for 5 min and incubated for 30 min with

peroxidase-labelled ABC reagent (Protocol 2.1). Slides were rinsed in PBS/Tween for 5min. Diaminobenzidine/H₂O₂ (DAB) was prepared (Protocol 2.1), slides were developed for 10 min and then checked under a microscope. Slides were washed with PBS and then brought to tap water. Sections were counterstained with haematoxylin for 10sec, dehydrated in graded alcohols, cleared with xylene and mounted in DPX (permanent mountant) medium.

Protocol 2.1 :

1) PBS (0.01M PBS, pH 7.0-7.4):

NaCl (8.7 g), KCL (0.2 g), KH₂PO₄ (0.272 g), Na₂HPO₄ (1.136 g). Dissolve salts separately in water then mix, make to 1l and check pH. A 10-times concentrated stock solution (0.1M) was made.

2) PBS/Tween (0.01M PBS, pH 7.0-7.4 + 0.05% Tween-20).

3) PBS diluent (PBS containing 0.1% sodium azide and 0.1% Bovine Serum Albumin).

4) Sequenza racks (Thermo Shandon) were used in this semi-automated system, where slides are held in a rack against a coverplate. 100µl is applied over the slides from which 80µl are retained.

5) Peroxidase-ABC reagent (Vector "ELITE" kit: 18 µl solution A (avidin) in 1 ml of PBS, mix well and add 18 µl solution B (biotinylated peroxidase). Make more than 1 ml if necessary. Mix and leave for at least 30 min at room temperature (RT) before use.

6) DAB development solution: 50 mg (1 ml of stock frozen solution DAB solution contains 50 mg DAB tetrahydrochloride) per 100 ml PBS. Just before use add 33.67 µl 30% hydrogen peroxide per 100 ml solution and mix well.

2.7.2 Immunostaining using the DakoCytomation ARK-Biotinylated antibody method

The following figure demonstrates the ARK (Animal Research Kitt) immunostaining method.

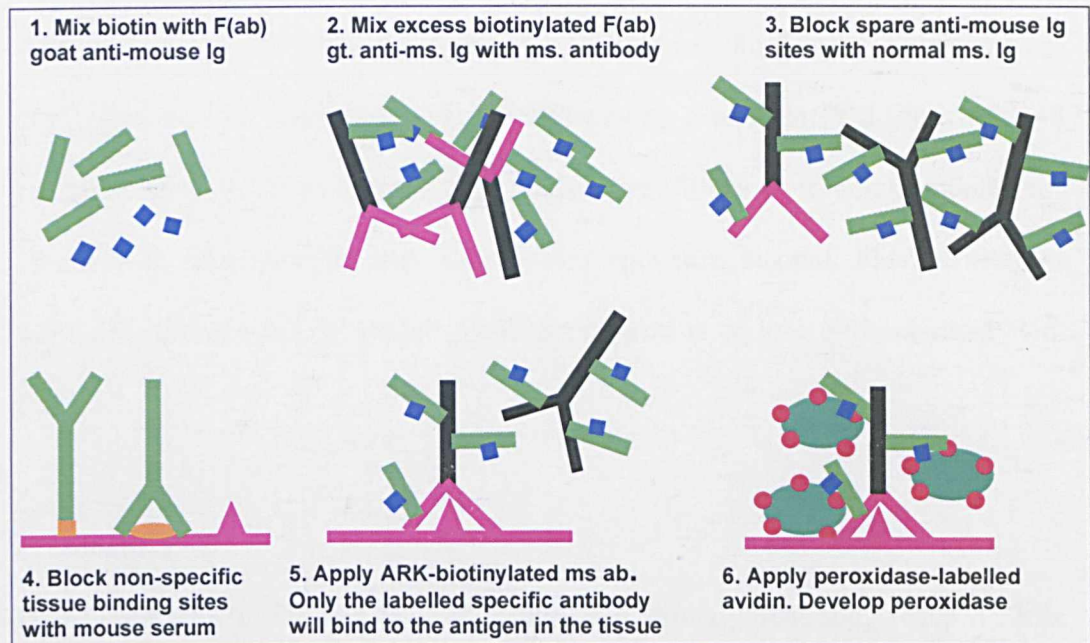


Figure 2.2: The ARK immunostaining technique. Figure was kindly provided by Miss Susan Van Noorden.

The ARK method was used when the primary antibody was raised in the same species as the tissue to be tested, i.e. mouse antibody on mouse tissue. The use of a biotinylated primary antibody followed by labelled avidin prevents the possibility of a second (anti-mouse Ig) layer binding to reactive mouse immunoglobulins within the tissue being tested. Briefly, sections were dewaxed in xylene, dehydrated through graded alcohol and brought to tap water. Endogenous peroxidase was blocked with 0.6 % H_2O_2 in distilled water for 15 min. Sections were washed in running tap water then washed in PBS for 5 min and placed in Sequenza racks. Background was blocked with 100 μl of normal goat serum diluted

1:20 in PBS diluent for 10 min. Endogenous biotin was blocked with 100 µl avidin for 15 min followed by PBS/Tween for 5min. Then, 100 µl biotin was applied for 15 min followed by PBS/Tween for 5 min. After that, 100 µl of ARK-biotinylated antibody (Protocol 2.2) was added and sections were incubated overnight at 4°C. Sections were rinsed in PBS/Tween for 5 min followed by 100 µl of peroxidase-ABC reagent for 30 min and a wash with PBS/Tween for 5 min. Sections were developed in DAB solution for 10 min followed by a wash in PBS for 2 min and examination of positive control under microscope. Slides were counterstained for 10 sec with haematoxylin and differentiated with acid alcohol. Finally, sections were dehydrated through graded alcohols, cleared in xylene and mounted with DPX.

Protocol 2.2

DAKO ARK Reagent Preparation: Biotinylate Primary Antibody. Step 1: Mix calculated volumes of diluent, concentrated primary mouse antibody and biotinylation reagent (biotinylated Fab fraction of goat anti-mouse IgG). Incubate 15 minutes at room temperature. Step 2: Add blocking reagent (normal mouse serum) to block unbound anti-mouse Ig. Incubate for 5 minutes at room temperature.

2.7.3 Imaging

All images of IHC and H&E were taken using a digital camera (Olympus DX70, Olympus, UK).

Table 2.1: Sources and dilutions of antibodies for IHC.

Antibody	Source	Manufacturer	Dilution Factor
Primary Antibodies			
Muscle specific actin (MSA) (M0635)	Mouse/Monoclonal	DakoCytomation	1: 100
Broad range cytokeratin (AE1/AE3) (M3515)	Mouse/Monoclonal	DakoCytomation	1:50
T-cell marker (CD3) (PC630)	Rabbit/Polyclonal	Oncogene	1: 100
Secondary Antibodies			
Biotinylated Goat anti-rabbit Ig (E0432)	Goat polyclonal	DakoCytomation	1: 400
FITC-labelled Donkey anti-mouse Ig (NC0513)	Donkey polyclonal	Novocastra	1: 300

2.8 DNA extraction from fixed and fresh- frozen tissues

DNA was extracted from fixed paraffin embedded tissues using a commercially available kit (QIAGEN DNA Tissue Kit, Cat. 69504). For each experiment, 25 mg of tissue was used as starting material and this was done in the following manner: Four or five 20 µm sections according to the size of the paraffin block, were cut using a rotary microtome (AS 325, SHANDON, UK) and placed in a 2.0 ml Eppendorf tube. Eppendorf tubes were weighed before and after addition of tissue.

1.0 ml of xylene was added and the sample was centrifuged for 5 min at 12,000 g. Xylene was cleared with 100% molecular grade ethanol twice. Samples were then incubated with lysis buffer and proteinase K according to the QIAGEN protocol at 55°C in a shaking waterbath (Shaker bath SBS30, Stuart Scientific, UK) overnight. The remaining steps were according to the manufacturer's protocol and the DNA was eluted with 100 µl TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA). Fresh-frozen tissue was used as a control for DNA integrity. DNA was extracted using the same commercially available kit (QIAGEN DNA Tissue Kit) according to the manufacturer's instructions for fresh-frozen tissue.

2.9 RNA extraction from fixed and fresh frozen tissues

RNA from fresh-frozen tissue was extracted using the RNeasy Tissue kit (Qiagen, Cat. 74104). The tissue was weighed and no more than 30 mg was used in all experiments. Each sample was placed in liquid nitrogen and grinded thoroughly with a mortar and pestle. After the liquid nitrogen was allowed to evaporate, 600 µl of RLT buffer was added into each sample and the sample was homogenized by passing the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase free syringe. The lysate was centrifuged for 3 min at 12,000 g and the supernatant was transferred to a new 2.0 ml Eppendorf tube. 600 µl of 70% ethanol was added and after mixing well, up to 700 µl of the sample was transferred to an RNeasy column placed in a 2 ml collection tube. The sample was centrifuged for 15 sec at 8,000 g, the flow-through was discarded and 700 µl of buffer RW1 was added to the RNeasy column. The sample was centrifuged for 15 sec at 8,000 g, the flow-through was discarded and 500 µl of buffer RPE was added to the RNeasy column. The sample was centrifuged for 15 sec at 8,000 g, the flow-

through was discarded and another 500 µl of buffer RPE was added to the RNeasy column. The sample was centrifuged for 3 min at 12,000 g. To elute, 50 µl of RNase-free water was directly added onto the silica-gel membrane and centrifuged for 1 min at 8,000 g.

RNA from fixed tissue was extracted using the RecoverAll Total Nucleic Acid Isolation extraction kit, (Ambion Ltd, Cat. 1975, UK). 15 - 20 mg of tissue was used as starting material. Two to three 20 µm sections (according to the size of the tissue block) were taken and placed in a 1.5 ml microfuge tube. Wax was removed with xylene and then the tissue was cleared with 100% alcohol twice. 400 µl of Digestion buffer and 4 µl of protease were added and the sample was incubated at 50°C for 3 hours. For nucleic acid isolation the steps according to the manufacturer's protocol were followed. For nuclease digestion, 6µl of 10X DNase buffer, 4 µl DNase and 50 µl nuclease-free water were added to each sample. The sample was then washed with both solutions Wash 1 and Wash 2/3 and finally the RNA was eluted with 2 x 30 µl RNase-free water.

2.10 RNA Measurements Using the 2100 Agilent Bioanalyser.

RNA analysis on a simple agarose gel can at best be only semi-quantitative. The Agilent 2100 Bioanalyser was therefore used for better quantitative evaluation of the RNA quality. This LabChip-based total RNA analysis integrates the quantitation of RNA samples with a quality assessment in one quick and simple assay and enables the reader to see how degraded an RNA sample is from different fixatives compared to the fresh frozen control. The RNA samples were kept on ice and their concentration was measured using a Nanodrop spectrophotometer (see Protocol 2.3) before use. The RNA samples were prepared according to the 2100 Agilent Bioanalyser protocol and were loaded into the NanoChip provided (up to 12

samples + ladder). Samples were then processed for 30 min and graphical results were printed.

An equal amount of RNA was used for each sample. The most intense and distinct features are the 18S and 28S ribosomal peaks. RNA degradation based on the electrophoregram is shown by:

- a) The shift of the peaks towards the left of the electrophoregram,
- b) The relative decrease of intensity of the 18S and 28S ribosomal peaks.

Protocol 2.3:

DNA and RNA yield and purity was measured using spectrophotometry, using 2 different protocols.

In the first protocol, A 1/100 dilution of the sample was prepared by adding of 5µl sample to 495µl TE (10mM TrisHCl pH 7.5, 1mM EDTA) buffer. Readings at 260 nm and 280 nm were taken and the ratio of 260/280 was given. The concentration (mg/ml) of nucleic acids was estimated according to the following formula:

- a) 260nm reading x dilution factor x 50 (DNA).
- b) 260 nm reading x dilution factor x 40 (RNA).

Alternatively, in the second protocol, the Nanodrop 1000 spectrophotometer was used. 1 µl or sample as added and a DNA/RNA reading including concentration was given. Following measurement of the RNA concentration, the RNA quality was tested using the RNA Bioanalyser.

RNA degradation produces a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescent signal. The 18S and 28S peaks can no longer be identified with certainty. With more severe degradation, the spectrum

shifts entirely toward the left of the electrophoregram. Figure 2.2 shows examples of different quality RNA produced using the Bioanalyser.

1) Severely degraded RNA 2) Partially degraded RNA 3) Intact RNA

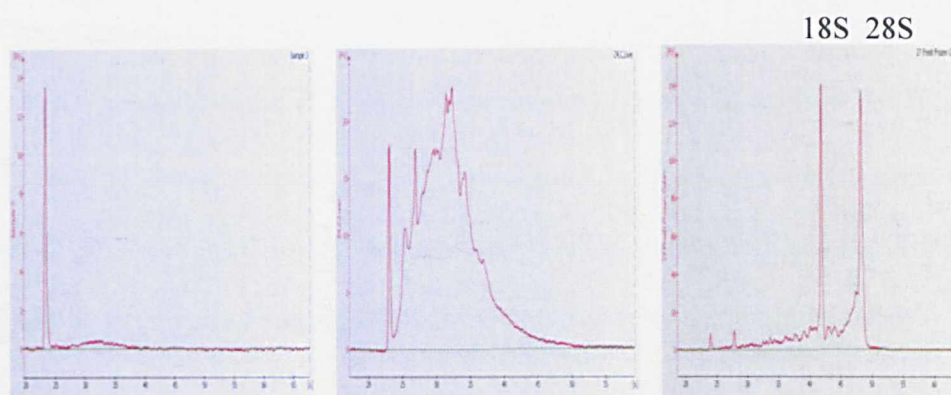


Figure 2.3: Representative images from the RNA Bioanalyser.

The RNA Integrity Number (RIN)

The RNA integrity number (RIN) is a tool designed to help scientists estimate the integrity of total RNA samples. The RIN extension automatically assigns an integrity number to a eukaryote total RNA sample analyzed on the Agilent 2100 bioanalyzer. Using this tool, sample integrity is no longer determined by the ratio of the ribosomal bands alone, but by the entire electrophoretic trace of the RNA sample, including the presence or absence of degradation products. In this way, interpretation of an electropherogram is facilitated, comparison of samples is enabled and repeatability of experiments is ensured.

What the RIN can do:

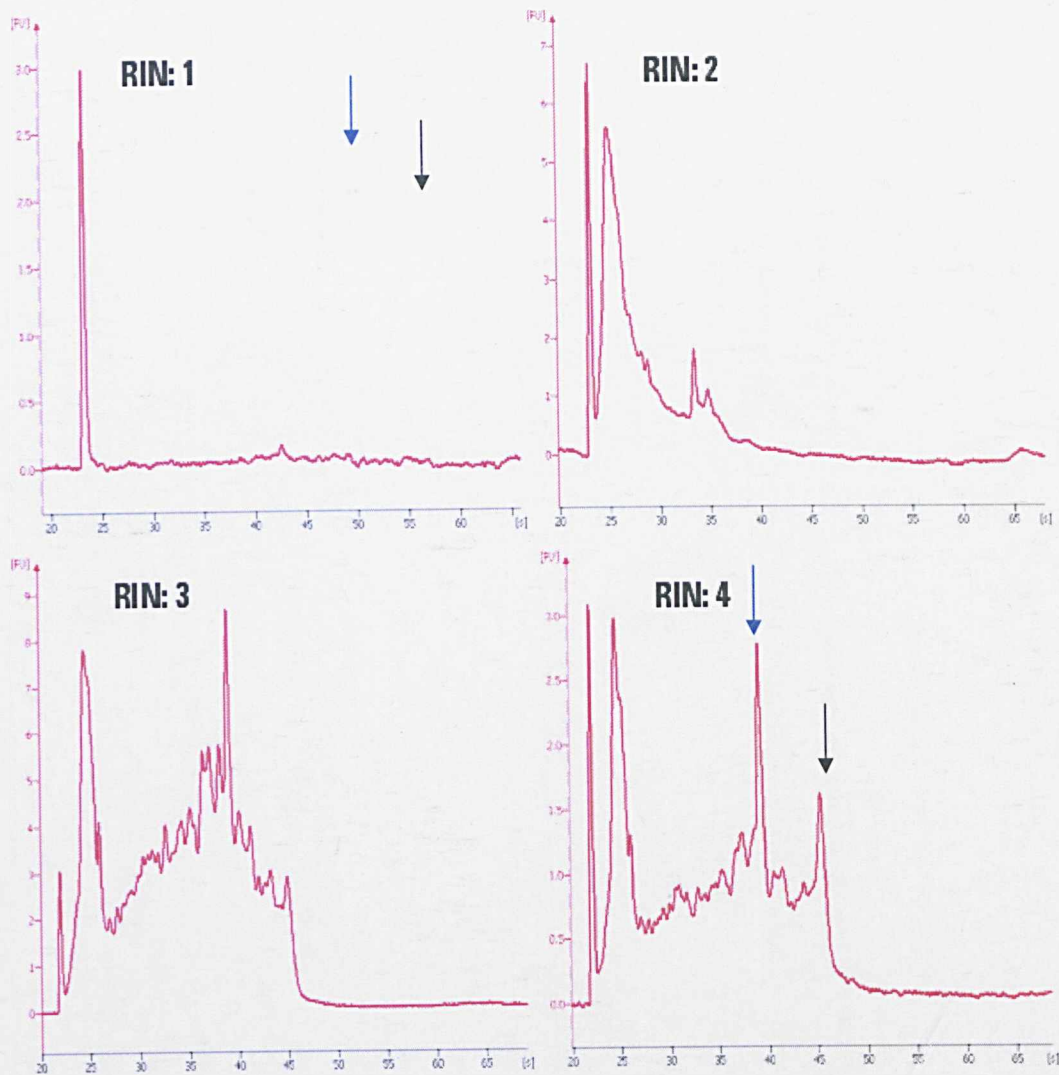
1. Obtain an assessment of the integrity of RNA.
2. Directly compare RNA samples (e.g. before and after shipment, compare integrity of same tissue across different labs, etc.).

3. Ensure repeatability of experiments (e.g. if RIN shows a given value and is suitable for microarray experiments, then the RIN of the same value can always be used for microarray experiments given that the same organism/tissue/extraction method was used).

What it cannot do:

1. Tell a scientist ahead of time whether an experiment will work or not if no prior verification was done (e.g. RIN of 5 might not work for microarray experiments, but might work well for an appropriate RT-PCR experiment. Also, an RIN that might be good for a 3' amplification might not work for a 5' amplification).

A database of about 1300 mammalian total RNA samples was created using the RNA 6000 Nano assay (Agilent, US). The samples came from different species (mainly human, rat and mouse), tissues, preparation methods, concentrations and degradation states. All samples were classified according to their degradation state. Numbers from '1' to '10' were used as labels. '10' stands for a perfect RNA sample without any degradation products, whereas '1' marks a completely degraded sample. The labels in-between are used to indicate progressing degradation states of the RNA sample. Figure 2.4 below shows typical representatives for each of the 10 RNA integrity classes.



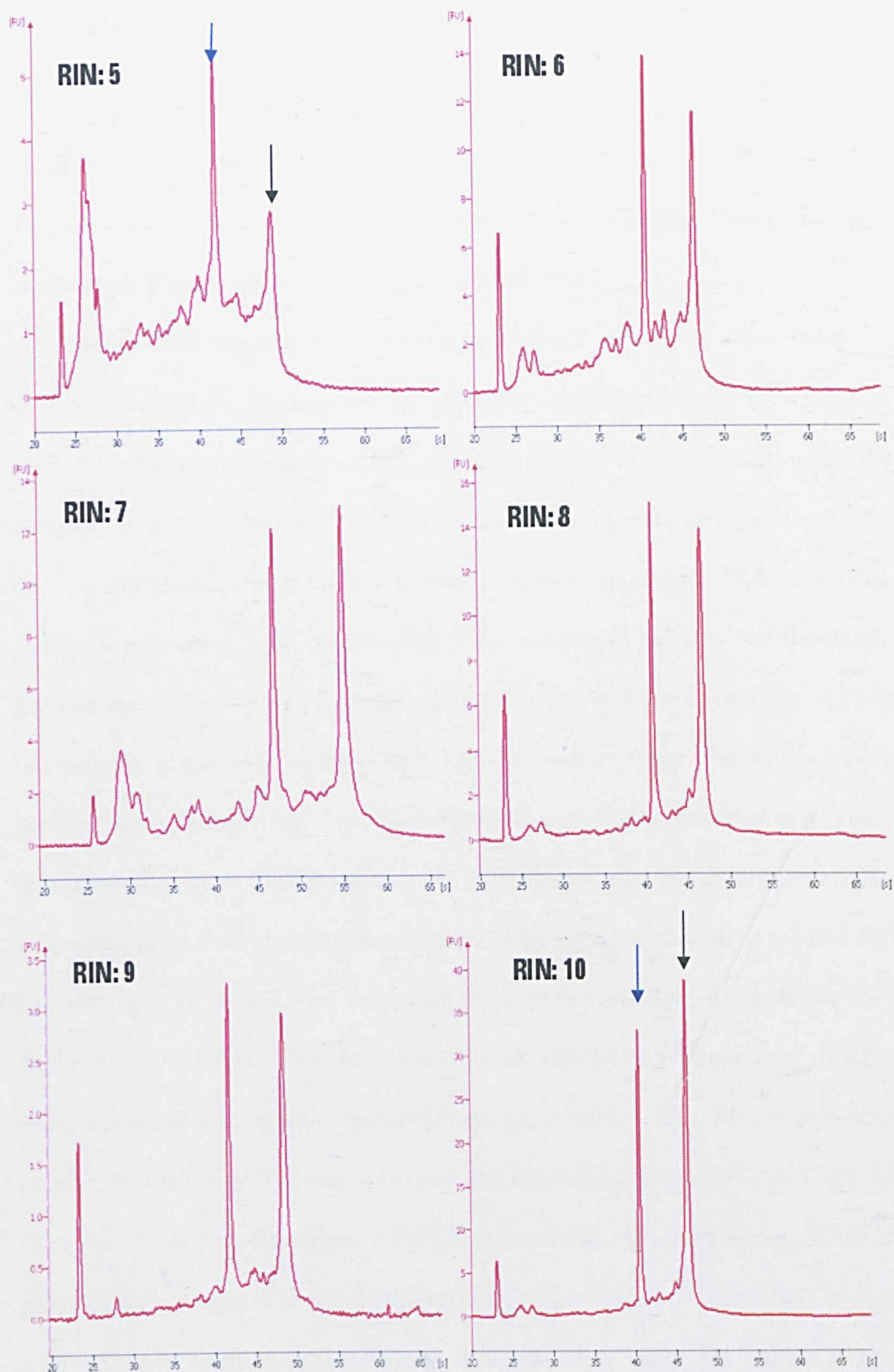


Figure 2.4: Typical representatives for each of the 10 RNA integrity classes. Blue arrows indicate the 18S ribosomal peaks and black arrows indicate the 28S ribosomal peaks.

2.11 PCR & RT- PCR

PCR is the molecular technique that enables the amplification of specific DNA fragments using the thermostable enzyme *Taq* polymerase. It was first developed by Kary Mullis and coworkers 1985 (Saiki et al. 1985) and has since been a milestone in molecular biology research (Strachan and Read, 2004).

PCR and RT-PCR was performed in order to amplify 2 universally expressed genes as illustrative models, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin. Specific primers were used for each gene. GAPDH and β -actin primers were designed using the software for custom made primers from the Invitrogen web site. For the amplification of RNA a commercial primer was chosen (R&D Systems, GenBank Accession Number: X03672). This was due to the fact that the mouse genome contains many pseudogenes and in order to avoid amplifying any of them this specific primer was selected. This specific β -actin primer also comes with a positive control sample. S15 is a small ribosomal gene that is also used as a control 'housekeeping' gene. The trefoil factor 2 (TFF2) was used for testing large DNA fragments. In all PCR experiments extracted DNA was diluted in RNAase-free H₂O and 100 ng of DNA was used from each sample (Protocol 2.4). Experiments were duplicated in order to verify the results. cDNA was prepared from 1 μ g of RNA using a reverse transcription system (Promega, A3800, USA). Mouse beta-actin transcripts were amplified using primers and conditions summarised in Table 2.2 (Reverse Transcriptase program, RT-PCR primers and PCR conditions). RT-PCR products were subjected to electrophoresis in 1% agarose gel (Protocol 2.5), stained with ethidium bromide and visualised using an ultra violet gel imager (Alpha Innotech, UK).

Protocol 2.4:

PCR mix was prepared in the following way:

H ₂ O:	17.25 µl
Buffer:	2.5 µl (1x)
Forward Primer:	0.5 µl (0.2 µM)
Peverse Primer:	0.5 µl (0.2 µM)
dNTP's:	0.5 µl (200 µM)
Taq:	0.25 µl (1.25 U)
MgCl ₂ :	2.5 µl (2.5 mM)
DNA:	<u>1 µl (100 ng)</u>
Total:	25 µl per sample

Protocol 2.5:

To make a (1%) agarose gel:

1. 50 ml of 1x TAE buffer (To make up 50x TAE buffer: 2M Tris-acetate, 50mM EDTA).
2. Add 0.5 g agarose (Promega, Cat. V3121, analytical grade, melting point 87–89°C).
3. Microwave at 850 Watt for 1 min 30 sec.
4. Add 1 µl Ethidium bromide (10 mg/ml, aqueous, Promega, Cat. H5041).
5. Let the solution cool under tap H₂O then carefully pour in the gel tank ensuring no bubbles in the gel.

Table 2.2: PCR, RT-PCR primers and PCR conditions.

Name	Primer Sequence	Size (bp)	PCR conditions Den, Ann, Exte,
β -actin*#	5'-TGTTACCAACTGGGACGACA-3' 3'-TCTCAGCTGTGGTGGTGAAG-5'	392	94°C, 55°C, 72°C, 25 cycles 30s, 30s, 30s
β -actin *	5'-GACGGCCAGGTCATCACTAT-3' 3'-CCTGGGCCATTCAGAAATTA-5'	936	94°C, 60°C, 72°C, 25 cycles 45s, 60s, 45s
GAPDH*#	5'-AACTTTGGCATTGTGGAAGG-3' 3'-TGTGAGGGAGATGCTCAGTG-5'	599	94°C, 55°C, 72°C, 25 cycles 30s, 30s, 30s
Trefoil Factor 2*	5'-GTCCCTTGGTGTTTCCACCC-3 3'-ACTGTGATCAGCAGTCATGCG-5'	2400	94°C, 59°C, 72°C, 30 cycles 45s, 1min 3min
β -actin **	Commercial Primer (R&D Systems)	302	94°C, 55°C, 72°C, 30 cycles 45s, 45s, 45s
S15 **##	5'-TTCCGCAAGTTCACCTACC-3' 3'-CGGGCCGGCCATGCTTTACG-5'	361	95°C, 55°C, 72°C, 30 cycles 30s, 30s, 30s

* PCR, # Real-Time PCR, ** RT-PCR, ## Real-Time RT-PCR

For every PCR reaction, the termination step was: 72°C for 8 minutes, followed by 4°C, end of PCR protocol.

2.12 Real-Time PCR and Real-Time RT-PCR

The advent of real-time PCR and real-time reverse transcription PCR (real-time RT-PCR) has dramatically changed the field of measuring gene expression. Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescent intensity (Wong & Medrano 2005). Reactions are

characterised by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (CT), the time at which fluorescent intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower CT (Heid et al. 1996). A difference between CT's by 1 means that there is double the amount of DNA or RNA, since this is a logarithmic correlation.

Real-time PCR was performed using the SYBR Green fluorescent dye and a real-time PCR system from BioRadTM (model MJU-200). A series of seven DNA and cDNA 3-fold dilutions ranging from 100ng to 1.5625ng were used in all the experiments. Real-Time PCR was performed using the SYBR Green Ready Taq Mix (Sigma-Aldrich Ltd, Dorset, UK, Cat. S4438), using the same primers as for PCR and RT-PCR. A series of seven 2-fold dilutions was prepared in each run starting from 100ng of DNA and gradually reducing to 1.5 ng of DNA. A reagent-only (no DNA) negative control sample was included in each run. DNA extracted from fresh-frozen samples was used as positive control for each experiment. Complementary DNA was prepared from 1 µg of RNA using the reverse transcription system (Promega) described above (Section 2.11) and Real-Time PCR was performed using the S15 primers. A sample that contained no reverse transcriptase was used as negative control in each experiment. Experiments were carried out in triplicate using three sets of tissue (nine tests per tissue in total) to ensure reliability.

2.13 Two-dimensional polyacrylamide gel electrophoresis

The main tool for protein separation is 2D-PAGE, which involves separation of proteins by isoelectric point in one dimension and molecular size in the second dimension. Each resultant “spot” on the two dimensional gel therefore constitutes a unique protein species. Each protein can be cut out of the gel, digested, and then identified by mass spectrometry (Leiva, Emmert-Buck, & Gillespie 2003).

Tissue samples fixed by different methods were subjected to 2-D PAGE analysis. For fresh frozen samples, 2-3 sections of 10 μm with an area size of 1cm^2 were placed in an Eppendorf tube with 100 μl Extraction Buffer II (Bio-Rad Laboratories, Hercules, CA, Cat. 163-2086). Samples were vortexed for 30 sec, transferred to dry ice for 5 minutes and then thawed at RT. The freeze-thaw-vortex step was repeated 3 times and samples were centrifuged at 12,000 g at 4°C for 8min. Supernatant (50 μl) from each sample was transferred to a fresh tube. For paraffin-embedded samples, thirty 10 μm sections were dissolved into 250 μl Extraction Buffer II (Bio-Rad Laboratories, Hercules, CA, Cat. 163-2086) and vigorously vortexed at room temperature. Samples were heated at 55°C for 30 min, 1ml of xylene was added and samples were centrifuged at 12,000 g for 10min. The supernatant was transferred to a fresh Eppendorf tube and centrifuged for 8 min at 12,000 g. The final supernatant of both fresh-frozen and paraffin-embedded tissue was combined with a rehydration buffer mixture containing rehydration buffer and bromophenol blue (Bio-Rad Laboratories, Cat. 163-2083), Immobilized pH gradient (IPG) buffer (Amersham Biosciences, Piscataway, NJ, Cat. 18100430), and subsequently rehydrated overnight with Immobiline Drystrips (pH 4/7, 11 cm; Amersham Biosciences, Cat. 18101660).

The isometric focusing for the first dimensional electrophoresis was performed with a Multiphore II Electrophoresis System (Amersham Biosciences, Cat. 18101806). The strips were subjected to high voltage at 3,500 V. IPG strips were equilibrated with Equilibration Buffer I (Bio-Rad Laboratories, Cat. 163-2107) and Buffer II (Bio-Rad Laboratories, Cat. 163-2108) for 15 minutes each. Precast ExcelGel SDS gels (Amersham Biosciences, Cat. 17123601) were used for the second dimension of protein separation by a Multiphor II Flated System (Amersham Biosciences, Cat. 18113005) under a constant voltage of 700 V for 3 hours. A silver staining kit (Amersham Biosciences, Cat. 17115001) was used according to the manufacturer's instructions to detect protein spots. All samples were run in duplicate to guarantee reproducibility.

2.14 Affymetrix Mouse Exon Array

cDNA microarrays are composed of a collection of DNA segments spotted, in a grid arrangement, onto a solid support ("chip"; glass slide or fibrous mesh membrane). The microarrays spots serve as hybridisation targets for cDNA, representing messenger RNA extracted from tissues samples or cell lysates. The test and reference samples are differentially labelled with fluorophores, and then combined and hybridised to the array under controlled conditions. After stringent washes to remove non-specific hybridisation, reference and sample cDNA will only hybridise to complementary sequences on the arrays (Reis-Filho, Westbury, & Pierga 2006). Vast amounts of data representing expression levels of many genes for any given sample can be generated by these techniques, and increasingly sophisticated analytical methods are being developed to process and analyse the data (Reis-Filho, Westbury, & Pierga 2006; Wildsmith & Elcock 2001).

The following figure 2.5 summarises the exon microarray procedure used in this Thesis.

DAY 1:	
	Experimental Time
Total RNA (1.5µg)	
rRNA reduction	1 hour
Clean-up of RNA ¹	0.5 hours
First cycle, first strand cDNA synthesis	1.5 hours
First cycle, second strand cDNA synthesis	2.5 hours
First cycle <i>in vitro</i> Transcription using unlabelled ribonucleotides	0.5 hours
DAY 2:	
First cycle, clean-up of cRNA ²	0.5 hours
Second cycle, first strand cDNA synthesis using random primers	2.5 hours
cRNA hydrolysis	1 hour
Clean-up of cDNA ²	0.5 hours
Fragmentation ¹	1.5 hours
Terminal labelling	1.5 hours
Hybridisation	

Figure 2.5: Summary of the exon microarray procedure.

¹ QC check 1:

- Bioanalyser trace

² QC check 2:

- Raw ODs at 260 nm and 280 nm within the linear range of the spectrophotometer
- Ratio of 260/280 nm within 1.9-2.1
- Yield of cRNA at least 16µg, Yield of ssDNA at least 5.5µg

Figure 2.5 is followed by figure 2.6, which is an overview of the GeneChip Whole Transcript (WT) Assay protocol.

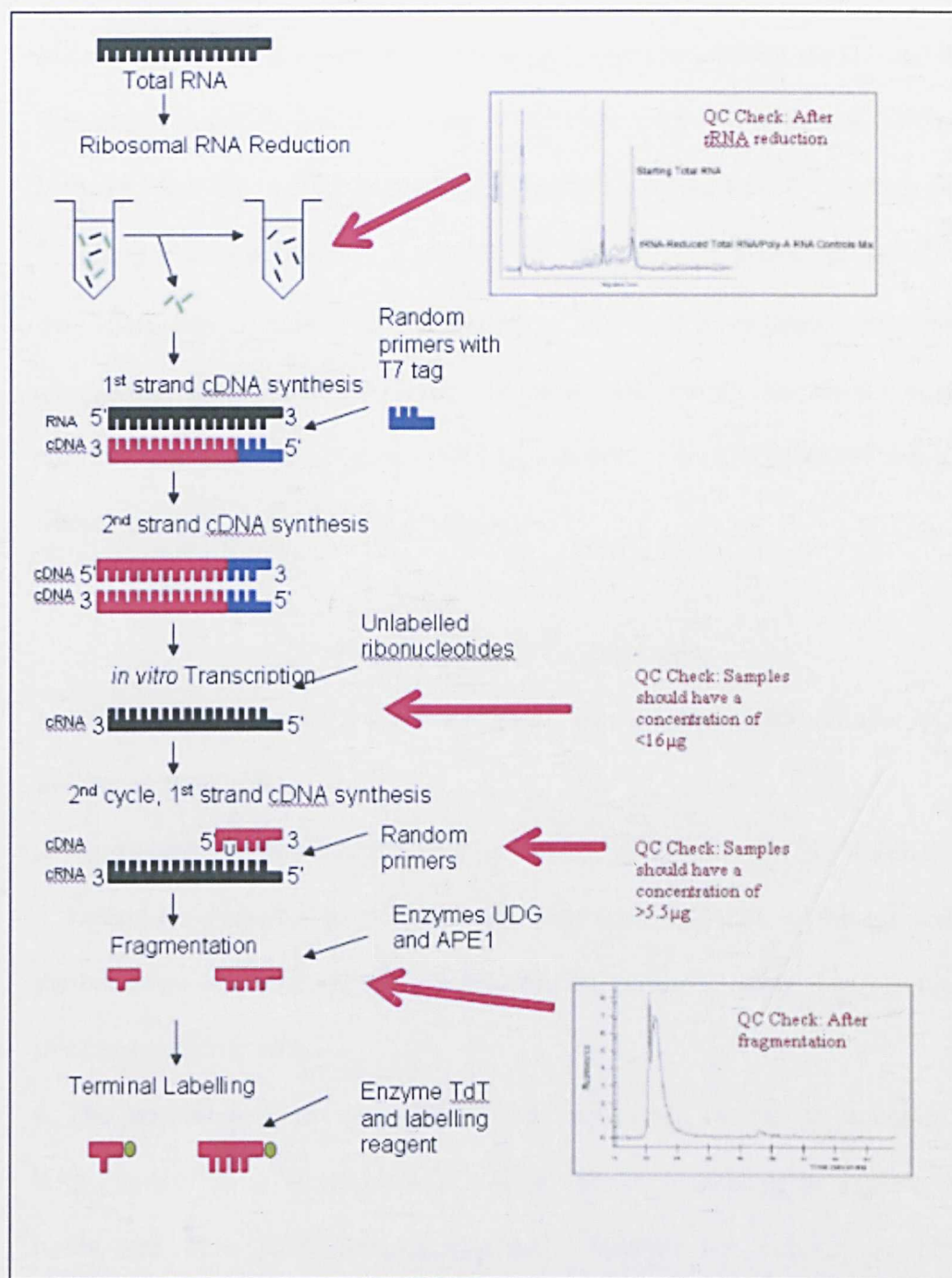


Figure 2.6: Overview of the GeneChip Whole Transcript Assay protocol.

WT Labelling Assay

These protocols are based on those provided by Affymetrix and include modifications made by the CSC/IC (Clinical Sciences Centre/Imperial College) Microarray Centre. It is very important to start with good quality, clean, total RNA. Acceptable ratios for the absorbance of the total RNA at 260nm and 280nm are between 1.9 and 2.1. This protocol is optimised for 1.5 µg total RNA (there should be 1.5 µg in a maximum of 3.2 µl solution). The Poly A⁺ Controls are provided by the Microarray Centre and are stored at -80°C. All reagents were thawed completely, mixed thoroughly and kept on ice until used. Solutions containing enzymes were mixed by gently flicking the tube. Clean RNase-free tubes and filtered pipette tips were used throughout.

Protocol 2.6: rRNA Reduction

1. This protocol uses the RiboMinus Transcriptome Isolation Kit (Human/Mouse) Invitrogen (K1550-02).
2. Magna-Sep Magnetic Particle Separators were from Invitrogen (K1585-01).
3. During the preparation of the magnetic beads (steps 7- 14), it was ensured that the beads are hydrated and did not dry. Mix by vortexing rather than pipetting as this can result in bead loss.
4. The preparation of the beads (from step 7 up to step 14) can be processed in a large batch of up to 10 reactions in a single tube by increasing the volume of the beads and wash solutions proportionately. Increase the volume by 10% if processing a batch of beads. If processing multiple samples in a single tube, mix the beads by vortexing after each step.

Method:

1. The Betaine Hybridisation Buffer was prepared. For each reaction was added:

54 μ l (5M) Betaine, (Sigma, B-0300)

126 μ l Invitrogen Hybridisation Buffer

180 μ l final volume
2. The Total RNA/Poly A control mix was prepared. 1 μ l of Poly A to 1.5 μ g of Total RNA was added and RNase free water up to a volume of 5.2 μ l was added.
3. To a 1.5ml PCR tube for each reaction was added:

5.2 μ l Total RNA/Poly A mix

0.8 μ l (100 pmol/ μ l) RiboMinus Probe

30.0 μ l Betaine Hybridisation Buffer (from step 1)

Up to 36.0 μ l final volume
4. Tubes were gently flicked to mix.
5. Samples were incubated at 70°C in a thermal cycler for 5 minutes.
6. Spin the tubes briefly and place samples back on ice for at least 2 minutes while preparing the magnetic beads.
7. Spin the tubes briefly and place samples back on ice while preparing the magnetic beads.
8. The magnetic beads were re-suspended by flicking the bottle until no deposit was observed at the bottom of the bottle.
9. For each reaction, 50 μ l of the beads suspension was transferred into a 1.5 ml non-stick RNase-free tube (The volume can be increased to prepare a

batch of beads for up to ten samples. Increase the volume by 10% for each sample e.g. for 10 reactions add 550 μ l).

10. Spin briefly and the tubes were placed with the beads suspension, on the magnetic stand for 1 minute.
11. Gently aspirate and discard the supernatant whilst the tube is in the rack.
12. 50 μ l of RNase-free water was added (for 10 reactions add 550 μ l) to the beads and the tubes were flicked to re-suspend the beads. The tubes were placed on the magnetic stand for 1 minute. The supernatant was gently aspirated and discarded.
13. For a second time, 50 μ l of RNase-free water was added (for 10 reactions add 550 μ l) to the beads and the tubes were flicked to re-suspend the beads. The tubes were placed on the magnetic stand for 1 minute. The supernatant was gently aspirated and discarded.
14. For the third wash, 50 μ l of Betaine Hybridisation Buffer was added (for 10 reactions add 550 μ l) to the beads and the tubes were flicked to re-suspend the beads. The tubes were placed on the magnetic stand for 1 minute. A thin film of beads may coat the inside of the tube and the solution may appear yellow. The supernatant was gently aspirated from the centre of the tube, which should be clear, and was discarded.
15. 20 μ l Betaine Hybridisation Buffer was added to the beads and the tubes were flicked to re-suspend the beads. The tubes were incubated in a heating block at 37°C for 2 minutes.
16. The total RNA samples were transferred to the prepared beads. Mix well and spin briefly if necessary.

17. Incubated at 37°C in a heating block for 10 minutes. Gently flick the tubes to mix half-way through the incubation.
18. Spun briefly and the tubes were placed on the magnetic stand for 2 minutes.
Do not discard the supernatant.
19. For the first sample, the supernatant was transferred to a 1.5 mL non-stick RNase free tube and placed on ice.
20. 50 µl Betaine Hybridisation Buffer was added to the beads and vortexed to resuspend. Steps 19 and 20 for each sample were repeated in turn.
21. Incubated at 50°C in a heating block for 5 minutes.
22. The tubes were placed on the magnetic stand for 2 minutes. Do not discard the supernatant.
23. The supernatant was transferred to the tube containing the supernatant from Step 18. The total volume of the rRNA-reduced sample should be ~100 µl.
24. The tubes were placed on ice and proceeded immediately to “Concentrating the rRNA-reduced Sample”.

Protocol 2.7: Concentrating the rRNA-reduced Sample

1. This protocol uses the GeneChip IVT cRNA Cleanup Kit, Affymetrix 900547.
2. Ensure 20 ml 100% ethanol has been added to the cRNA Wash Buffer before use.

Method:

1. 350 µl cRNA Binding Buffer was added to each rRNA reduced sample.
Mixed thoroughly by pipetting.
2. 250 µl (100%) ethanol was added and mixed by pipetting.
3. Each sample was applied to an IVT cRNA Cleanup Spin Column.

4. Spun at 12,000 g for 15 seconds.
5. Each spin column was transferred to a fresh 2 ml collection tube.
6. 500 μ l of cRNA Wash Buffer was added onto the spin column.
7. Spun at 12,000 g for 15 seconds.
8. The collection tubes were emptied.
9. 500 μ l (80%) ethanol was added onto the spin column.
10. Spun at 12,000 g for 15 seconds.
11. The collection tubes were emptied.
12. The cap of the spin columns was opened and centrifuged at 12,000 g for 5 minutes to completely dry the membrane.
13. Each spin column was transferred into a new 1.5 ml collection tube.
14. Eluted by placing 11 μ l of RNase-free water directly onto the middle of the spin column membrane and incubated at room temperature for 1 minute.
15. Spun at maximum speed for 1 minute. There should be $\sim$ 9.8 μ l of eluate.
16. To assess the quality, an aliquot of the concentrated sample and the original total RNA sample was visualised, on the Agilent 2100 Bioanalyser, as seen below in figure 2.7.

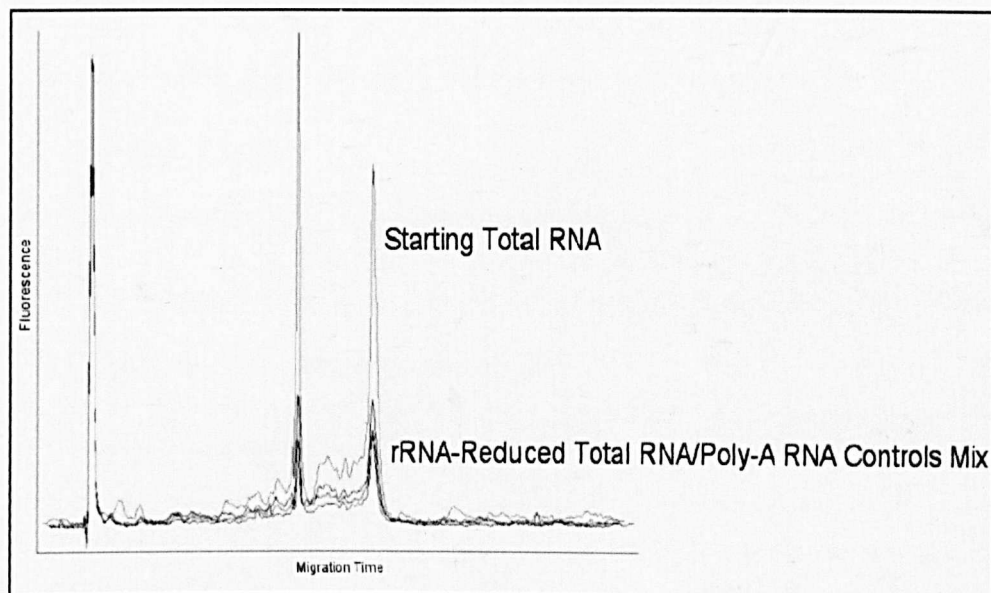


Figure 2.7: An example of a 2100 Bioanalyser trace of total RNA before and after the rRNA reduction treatment. This picture is taken from the Affymetrix WT Sense Target Labelling Assay Manual.

Protocol 2.8: First Cycle: First Strand cDNA Synthesis

1. This protocol uses the GeneChip WT cDNA Synthesis and Amplification Kit, Affymetrix 900673 (30 reactions) or 900672 (10 reactions).
2. The T7-(N)₆ primer provided (2.5 µg/µl) must be diluted to the appropriate concentration of 500ng/µl (1:5) with RNase free dH₂O in a clean RNase free tube. Make as much as required, 1 µl of the diluted T7-(N)₆ primer (500ng/µl) is needed for 1 reaction.
3. At the end of this protocol, the tubes were placed on ice and proceeded immediately to "First Cycle: Second Strand cDNA Synthesis".
4. Keeping the reaction at 4°C longer than 10 minutes in step 7 may result in reduced cRNA yields.

Method:

1. To a 0.2ml PCR tube for each reaction was added:
 - 4 µl rRNA-reduced Total RNA/Poly A mix
 - 1 µl (500 ng/µl) diluted T7-(N)₆ primer
 - 5 µl final volume
2. Mix by pipetting and spin briefly as necessary.
3. The reaction tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (1) 70°C for 5 minutes
 - (2) 4°C for 2 minutes

(3) 4°C hold

4. Spun briefly and placed on ice.

5. Prepared a master mix on ice. For each reaction was added:

2 µl 5X First Strand Buffer

1 µl (0.1M) DTT (dithiothreitol)

0.5 µl (10mM) dNTP Mix

0.5 µl RNase Inhibitor

1 µl Superscript II

5µl final volume

Master mix was mixed by pipetting.

6. 5 µl of the master mix was transferred to each of the reaction tubes. Mix by pipetting and spin briefly if necessary.

7. The tubes were placed in a thermal cycler and the following programme was used (with heated lid):

(1) 25°C for 10 minutes

(2) 42°C for 1 hour

(3) 70°C for 10 minutes

(4) 4°C for 2 minutes

(5) 4°C hold

8. Spun briefly.

9. The tubes were placed on ice and proceed immediately to " First Cycle: Second Strand cDNA Synthesis ".

Protocol 2.9: First Cycle: Second Strand cDNA Synthesis

1. Make a fresh dilution of MgCl_2 each time. Dilute 2 μl MgCl_2 (1 M) with 112 μl RNase free dH_2O . Mix by pipetting and spin briefly.
2. Programme the thermal cycler in advance. Step 3 requires the thermal cycler to be run without the heated lid option and step 4 requires the thermal cycler to be run with the heated lid option.
3. At the end of this protocol, the tube were placed on ice and proceeded immediately to "First Cycle: cRNA Synthesis".
4. Keeping the reaction at 4°C longer than 10 minutes at step 7 may result in reduced cRNA yields.

Method:

1. A master mix was prepared. At least 4 reactions were prepared to prevent pipetting small volumes. For each reaction it was added:
 - 4 μl (17.5 mM) diluted MgCl_2
 - 0.4 μl (10mM) dNTP Mix
 - 0.6 μl DNA Polymerase I
 - 0.2 μl RNase H
 - 4.8 μl RNase free dH_2O
 - 10 μl final volumeMix by pipetting, spin briefly if necessary.
2. 10 μl of the master mix was transferred to each of the first strand reaction tubes. Mix by pipetting and spin briefly if necessary.
3. Incubated at 16°C in a thermal cycler for 2 hours without the heated lid option.
4. Incubated at 75°C in a thermal cycler for 10 minutes with the heated lid option.

5. Cool the samples to 4°C in a thermal cycler for 2 minutes.
6. Spun briefly.
7. The tubes were placed on ice and proceeded immediately to "First Cycle: cRNA Synthesis".

Protocol 2.10: First Cycle: cRNA Synthesis

Method:

1. A master mix was prepared at room temperature. For each reaction it was added:
 - 5 µl 10X IVT (in vitro transcription) Buffer
 - 20 µl IVT NTP Mix
 - 5 µl IVT Enzyme Mix
 - 30 µl final volumeMixed by pipetting.
2. 30 µl of the master mix were transferred to each of the second strand reaction tubes. Mix by pipetting and spin briefly if necessary.
3. Incubated at 37°C in a thermal cycler for 16 hours with the heated lid option.
4. Spun briefly.
5. Tubes were placed on dry ice to freeze and then stored at -80°C or proceeded directly to "First Cycle: Clean-up of cRNA".

Protocol 2.11: First Cycle: Clean-up of cRNA

1. This protocol uses the GeneChip Sample Cleanup Module, Affymetrix 900371.
2. Ensure ethanol has been added to the cRNA Wash Buffer before use.

Method:

1. Samples were transferred to a 1.5 ml PCR tube.
2. 50 µl RNase free dH₂O was transferred to the cRNA reaction tubes.
3. 350 µl cRNA Binding Buffer was added to the cRNA tubes and mixed thoroughly by pipetting.
4. 250 µl (100%) ethanol was added and mixed by pipetting.
5. Each sample was applied to an IVT cRNA Cleanup Spin Column.
6. Spun at 12,000 *g* for 15 seconds.
7. The spin columns were transferred to a fresh 2 ml collection tube.
8. 500 µl of cRNA Wash Buffer was added onto the spin columns.
9. Spun at 12,000 *g* for 15 seconds.
10. The collection tubes were emptied.
11. 500 µl (80%) ethanol was added onto the spin columns.
12. Spun at 12,000 *g* for 15 seconds.
13. The collection tubes were emptied.
14. The cap of the spin columns was opened and centrifuged at 12,000 *g* for 5 minutes to completely dry the membrane.
15. Each spin column was transferred into a new 1.5 ml centrifuge tube.
16. Eluted by placing 12 µl of RNase-free water directly onto the middle of the spin column membrane. Incubated at room temperature for 3 minutes.
17. Spun at 12,000 *g* for 1 minute.
18. Measure the volume of eluate, there should be ~10.5µl.
19. The absorbance at 260nm and 280nm of the eluate was measured and ensured that the readings are in the linear range for the spectrophotometer. A ratio of the absorbance at 260 and 280 nm should be around 2.0, with an acceptable range being 1.9 to 2.1. The first cycle cRNA yield (concentration

x eluate volume) should be $> 16 \mu\text{g}$ (concentration $\sim 1.54 \mu\text{g}/\mu\text{l}$), however this may vary depending on tissue type. A lower yield indicates there may be a problem with the reaction.

20. The appropriate volume of cRNA to be used was calculated in the second round of amplification using $10 \mu\text{g}$ of cRNA. It is important that the starting amount is the same for all of the samples.

Protocol 2.12: Second Cycle: First Strand cDNA Synthesis

Method:

1. The following were placed in a 0.2ml tube for each reaction:
 - $_\mu\text{l}$ ($10\mu\text{g}$) single stranded cRNA (from previous step)
 - $1.5 \mu\text{l}$ Random Primers ($3 \mu\text{g}/\mu\text{l}$)
 - $_\mu\text{l}$ RNase-free dH_2O
 - $8\mu\text{l}$ final volume
2. Mixed thoroughly.
3. The tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (1) 70°C for 5 minutes
 - (2) 25°C for 5 minutes
 - (3) 4°C for 2 minutes
 - (4) 4°C hold
4. Spun the tubes briefly and placed on ice.
5. A master mix was prepared on ice. For each reaction it was added:
 - $4 \mu\text{l}$ 5X First Strand Buffer
 - $2 \mu\text{l}$ (0.1M) DTT

1.25 μ l (10mM) dNTP + dUTP (deoxyuridine triphosphate)

4.75 μ l Superscript II

12 μ l final volume

Mixed by pipetting.

6. 12 μ l of the master mix was transferred to each of the reaction tubes. Mix by pipetting and spin briefly if necessary.
7. The reaction tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (1) 25°C for 10 minutes
 - (2) 42°C for 90 minutes
 - (3) 70°C for 10 minutes
 - (4) 4°C for 2 minutes
 - (5) 4°C hold
8. The tubes was spun briefly, placed on ice and proceeded immediately to "Hydrolysis of cRNA".

Protocol 2.13: Hydrolysis of cRNA

Method:

1. 1 μ l of RNase H was added to the single stranded cDNA tubes. Mixed by pipetting and spun briefly as necessary.
2. The tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (1) 37°C for 45 minutes
 - (2) 95°C for 5 minutes

(3) 4°C for 2 minutes

(4) 4°C hold

3. The tubes were spun briefly.
4. The tubes were placed on ice and proceeded immediately to "Clean-up of Single Stranded cDNA".

Protocol 2.14: Clean-up of Single-Stranded cDNA

1. This protocol uses the GeneChip Sample Cleanup Module, Affymetrix 900371.
2. Ensure 24 ml ethanol has been added to the cDNA Wash Buffer before use.

Method:

1. Each sample was transferred to a new 1.5ml PCR tube.
2. 80 µl RNase-free dH₂O was added to the single stranded cDNA tubes.
3. 370 µl cDNA Binding Buffer was added and mixed thoroughly by pipetting.
4. Each sample was applied to a cDNA Spin Column.
5. Spun at 12,000 g for 1 minute.
6. The spin columns were transferred to a fresh 2 ml collection tube.
7. 750 µl of cDNA Wash Buffer was added onto the spin columns.
8. Spun at 12,000 g for 1 minute.
9. Empty the collection tubes.
10. Open the cap of the spin columns and centrifuge at maximum speed for 5 minutes to completely dry the membrane.
11. Spin columns were transferred into a new 1.5 ml centrifuge tube.
12. Eluted by placing 15 µl of cDNA Elution Buffer directly onto the middle of the spin column membrane.
13. Incubated at room temperature for 3 minute.

14. Spun at maximum speed for 1 minute.
15. 15 µl of cDNA Elution Buffer were added directly onto the middle of the spin column membrane.
16. Incubated at room temperature for 3 minute.
17. Spun at 12,000 g for 1 minute.
18. The volume of eluate was measured (should be ~28µl).
19. The absorbance at 260nm and 280nm of the eluate was measured and ensured that the readings are in the linear range for the spectrophotometer. A ratio of the absorbances at 260 and 280 nm should be around 2.0, with an acceptable range being 1.9 to 2.1. The yield of single stranded cDNA (concentration x eluate volume) was calculated. The single stranded cDNA yield should be > 5.5 µg.
20. Tubes were placed on dry ice to freeze and then stored at -80°C or proceeded directly to “Fragmentation of single stranded cDNA”

Protocol 2.15: Fragmentation of Single Stranded cDNA

This protocol uses the GeneChip WT Terminal Labelling Kit, Affymetrix 900671 (30 reactions) or 900670 (10 reactions).

Method:

1. Single stranded cDNA (5.5 µg) was transferred from the previous step to a 0.2 ml PCR tube. RNase-free dH₂O was added to make a total volume of 41.2 µl.
2. A master mix was prepared. For each reaction it was added:
4.8 µl 10X cDNA Fragmentation Buffer
1 µl (10 U/µl) UDG (uracil DNA glycosylase)

1 µl (1,000 U/µl) APE 1 (apyrimidinic endonuclease 1)

6.8 µl final volume

Mixed by pipetting.

3. 6.8 µl of the master mix was transferred to each of the reaction tubes. Mixed by pipetting and spun briefly if necessary.
4. The tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (1) 37°C for 60 minutes
 - (2) 93°C for 2 minutes
 - (3) 4°C for 2 minutes
 - (4) 4°C hold
5. Spun the tubes briefly.
6. 1 µl aliquot of the fragmented cDNA sample was used for size analysis using a Bioanalyser. Fragment size should be ~40-70 bp. 1 µl of the unfragmented samples was run on the Bioanalyser as controls, as seen below in figure 2.6.
7. Tubes were placed on dry ice to freeze and then stored at -80°C or proceeded directly to "Labelling of Fragmented Single-Stranded DNA".

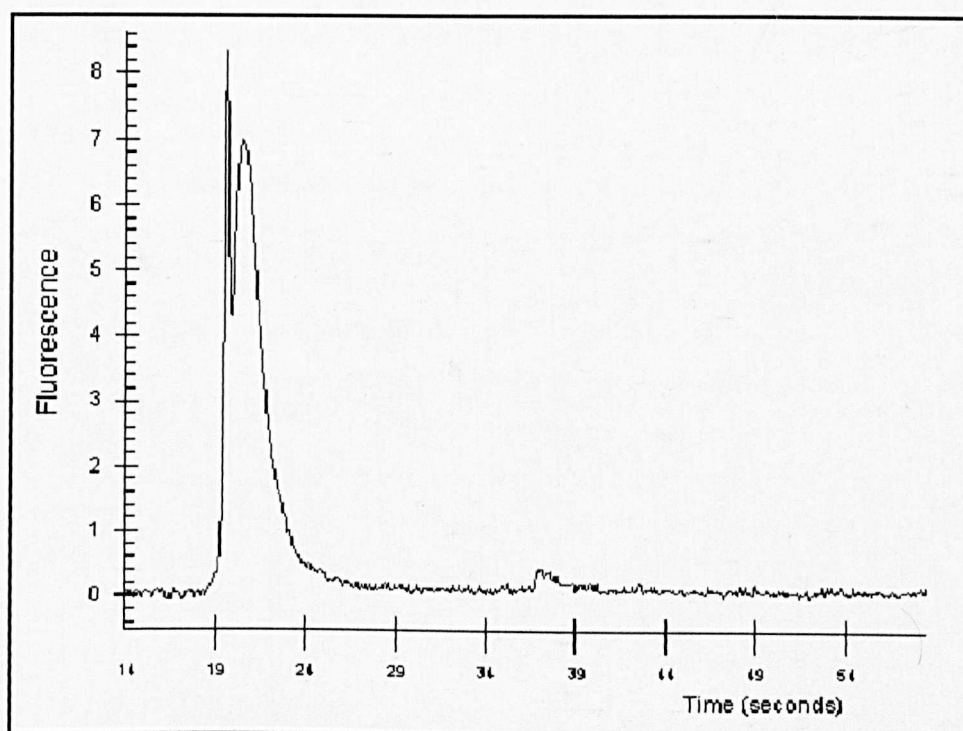


Figure 2.8: An example of a 2100 Bioanalyser trace of fragmented single stranded DNA. This picture is taken from the Affymetrix WT Sense Target Labelling Assay Manual.

Protocol 2.16: Labelling of Fragmented Single Stranded cDNA

This protocol uses the GeneChip WT Terminal Labelling Kit, Affymetrix 900671 (30 reactions) or 900670 (10 reactions).

Method:

1. 45 μ l of the fragmented cDNA sample was transferred to a new 0.2ml PCR tube.
2. A master mix was prepared. For each reaction it was added:
12 μ l 5X TdT (Terminal Deoxynucleotidyl Transferase) Buffer
2 μ l TdT
1 μ l (5 mM) DNA Labelling Reagent
15 μ l final volume
Mixed by pipetting.
3. 15 μ l of the master mix was added to each of the reaction tubes. Mixed by pipetting and spun briefly if necessary.
4. The tubes were placed in a thermal cycler and the following programme was used (with heated lid):
 - (4.1) 37°C for 60 minutes
 - (4.2) 70°C for 10 minutes
 - (4.3) 4°C for 2 minutes
 - (4.4) 4°C hold

5. The tubes were spun briefly and the 60µl of labelled extract was transferred in a clean nuclease free 1.5 ml microfuge tube.
6. Tubes were placed on dry ice to freeze and stored at -80°C.

For the final two protocols 2.17 (Hybridisation) and 2.18 (Array wash and stain) it must be noted that they were both performed in the CSC/IC microarray centre by authorised personnel and included the following:

Protocol 2.17: Hybridisation

1. This protocol uses the GeneChip Hybridization, Wash and Stain Kit, Affymetrix 900720, Wash A (900721), Wash B (900722).
2. The arrays were equilibrated at room temperature for 30 minutes before use.

Method:

1. RNase-free water was added to the labelled extract to give a total volume of 60µl.
2. Prepare a hybridisation cocktail master mix on ice. For each reaction add:
3.7 µl (3 nM) Control Oligonucleotides
11 µl 20X Hybridization Controls,
15.4 µl DMSO, (Sigma D5879)
110 µl 2X Hybridisation Buffer,
19.9µl RNase-free dH₂O
160 µl
3. 160 µl of the master mix was added into 60 µl of labelled extract to give a total volume of 220 µl. Mixed by pipetting.

4. 220 µl of hybridisation cocktail was denatured by incubating at 99°C in a heating block for 5 minutes.
5. Cool fragmented cRNA at 45°C for 5 minutes.
6. Spun at 12,000 g for 1 minute.
7. Pipetting solution from the top of the tube, 200 µl of the denatured was added to the hybridisation cocktail into the Exon 1.0 ST Array.
8. Hybridise in the rotisserie oven at 45°C for 16 hours at 60 rpm.

Protocol 2.18:

Array Wash and Stain

The streptavidin-phycoerythrin in stain cocktail 1 is light sensitive. Do not expose it to light more than necessary, store in the dark at 4°C and aliquot into an amber microfuge tube or a foil wrapped tube.

Note that the same vial containing 600 µl stain cocktail 1 is used for the 1st and 3rd stain.

Method:

1. Once hybridisation is completed, remove the hybridisation cocktail from array and place in a microcentrifuge tube. Freeze by placing the tube on dry ice and stored at -80°C.
2. Fill array completely with 250 µl Wash A Buffer. Ensure that the array is equilibrated to room temperature before washing and staining.
3. Remove stain cocktail 1, stain cocktail 2 and array holding buffer from hyb, wash and stain kit. Tap bottles to mix
4. For each array aliquot 600µl of stain cocktail 1 into a 1.5ml amber microfuge tube.

5. For each array aliquot 600µl of stain cocktail 2 into a 1.5 ml clear microfuge tube.
6. For each array aliquot 800µl of array holding buffer into a 1.5 ml clear microfuge tube.
7. Open “Fluidics” dialogue box of appropriate station. Select the correct experiment name in the “Experiment” list.
8. Select experiment “FS450_0001” wash protocol. Select “Run”, and follow instructions in fluidic station LCD window:
 - “Eject washblock” – move lever to eject position, insert array, close washblock and move lever back to engage position.
 - “Remove vial” – remove microfuge tube from sample holder.
9. “Load stains” – for each array place the stain cocktail 1 vial in position 1
place the stain cocktail 2 vial in position 2
10. When wash is complete, LCD window displays "Eject cartridge". Remove array and check array window for bubbles. If bubbles are present, return the array to the array holder. Engage the washblock and follow the instructions on the LCD window. The fluidics station will drain the array and fill it with Array Holding Buffer. When finished the LCD window displays "Eject cartridge" again. If there are still bubbles present, manually remove by filling with Array Holding Buffer.
11. Apply Tough-Spots to each of the two septa on the back of the array. Keep the stained array covered with foil before scanning. Make sure that the window is clean and free from dust, if necessary, wipe with the window using a lens cleaning cloth.

12. Open “scanner” dialogue box, select experiment and then “start”. When instructed, load array into scanner. Make sure that arrays are in randomised order and that the “first 4 arrays at room temperature” option is not selected. When scanning is finished complete remove array from scanner.
13. Once all arrays are scanned proceed to ‘Shutdown of GeneChip Station’.

Introduction to the Expression Console™ Software

The Affymetrix® Expression Console™ software is a stand-alone application. It can be installed on computers that have GeneChip® Operating System (GCOS) software, Affymetrix GeneChip® Command Console™ (AGCC) software, or neither. The Expression Console™ software is an application that will generate the summarized expression values for individual or collections of Affymetrix 3' Expression Arrays (e.g., U133 Plus 2.0) or Exon Arrays (e.g., HuEX-1_0-st). In addition, the application will also capture a standard set of metrics for evaluating the success of the individual hybridizations and identification of outlier samples. Today, this functionality is provided in two separate applications, GCOS for 3' Expression Arrays and ExACT for Exon Arrays. Development of the Expression Console software has enabled the unification of the workflow for both types of assays in a single application. At the same time additional features for assessing the quality of hybridizations and the integration of additional probe summarization algorithms have been added to the Expression Console software. It is important to note that the Expression Console software is not intended to replace secondary analysis packages for the statistical examination of expression results and the creation of gene lists. The intended workflow is to run expression GeneChips, then use the Expression Console software to determine if the hybridization results are of

sufficient quality for analysis and create the CHP (chip) files for secondary analysis in the Affymetrix GeneChip Compatible Software package of choice.

One of the two major functions of the Expression Console software is the creation of the summarized probe signals. Today in GCOS, MAS 5.0 is the only algorithm available for this purpose. As the state of the art of expression analysis has advanced it has lead to the development of new probe set summarization methods like RMA (Robust Multichip Analysis) and PLIER (Probe Logarithmic Intensity Error Estimation) which are unavailable within the GCOS architecture. With the development of the Expression Console software, these additional algorithms have been added to the supported workflow. By default the summarized expression values are written to a CHP file for each CEL (cell intensity) file analyzed, but it is also possible to create a tab-delimited version of the summarized expression values for all GeneChips using the “Export Pivot table” functionality.

The second function of the Expression Console software is to enable scientists to evaluate the success of the individual hybridizations and identification of outlier samples. Historically GCOS has provided the report or “RPT” file that contained a collection of signal and quality metrics for hybridizations one assay at a time. In the Expression Console software these same metrics are available, but they are now provided in a single table for multiple assays. It is still possible to generate the historical RPT file, but the majority of users will find the full report table or a custom table containing only the metrics they are interested in to be more informative since it will contain all of the metrics for a collection of arrays in a single table. In addition, scientists can define thresholds for these metrics so that

GeneChips and the metrics themselves that are outside of the defined thresholds will be highlighted in the tables. It is also possible to display line graphs for individual or collections of metrics or probe sets for those who prefer to evaluate the data graphically. Beyond examining the quality metrics the application provides functionality to display the signal distributions for the arrays by plotting the signals before and after normalization enabling scientists to identify outlier arrays.

2.15 Mass Spectrometry (MALDI-MS)

Data acquisition

All mass spectra were recorded with an Applied Biosystems 4700 Proteomics Analyzer (Foster City, CA) with both the MS and the MS/MS default calibration being updated prior to sample data acquisition. MS data was acquired in MS Reflector Positive mode with a fixed laser intensity of 4150, scan range of 800-4000 m/z and a focus mass of 1800 m/z. 0.3ul of sample was spotted along with 0.3 µl spotting buffer containing matrix [50% (v/v) Acetonitrile/Water (Rathburn), 0.1% (v/v) trifluoroacetic acid (Pierce matrix [α -cyano-4-hydroxycinnamic acid (Sigma) 10 µg/µl in spotting buffer] with 3.3 nm of ammonium monophosphate to suppress matrix adducts. Using Applied Biosystems Data Explorer software (version 4.6 [build 111]) the spectra were initially Advanced Baseline Corrected with a Peak Width of 32, Flexibility of 0.5 and Degree 0.1. Masses that were of interest were subjected to MS/MS analysis via collision induced disassociation (CID) acquisition using a fixed laser intensity of 5101, and absolute window of 1 Dalton $\pm$.

Preparation of samples for MS

All tested peptides were diluted in ddH₂O and the concentration that was used was 11.7 mM. Zinc trifluoroacetate was also diluted in ddH₂O and the concentration that was used was 11.7 mM.

Data analysis

In silico fragmentation was generated using MS-Product on UCSF's public access website (<http://prospector.ucsf.edu/prospector/4.0.8/html/msprod.htm>)

Figure 2.7 below illustrates the main components of the Applied Biosystems 4700 Mass Proteomics System that was used in this research project. It is a rather complicated image, but its purpose is to give the reader a general idea of what is comprised such a powerfull system.

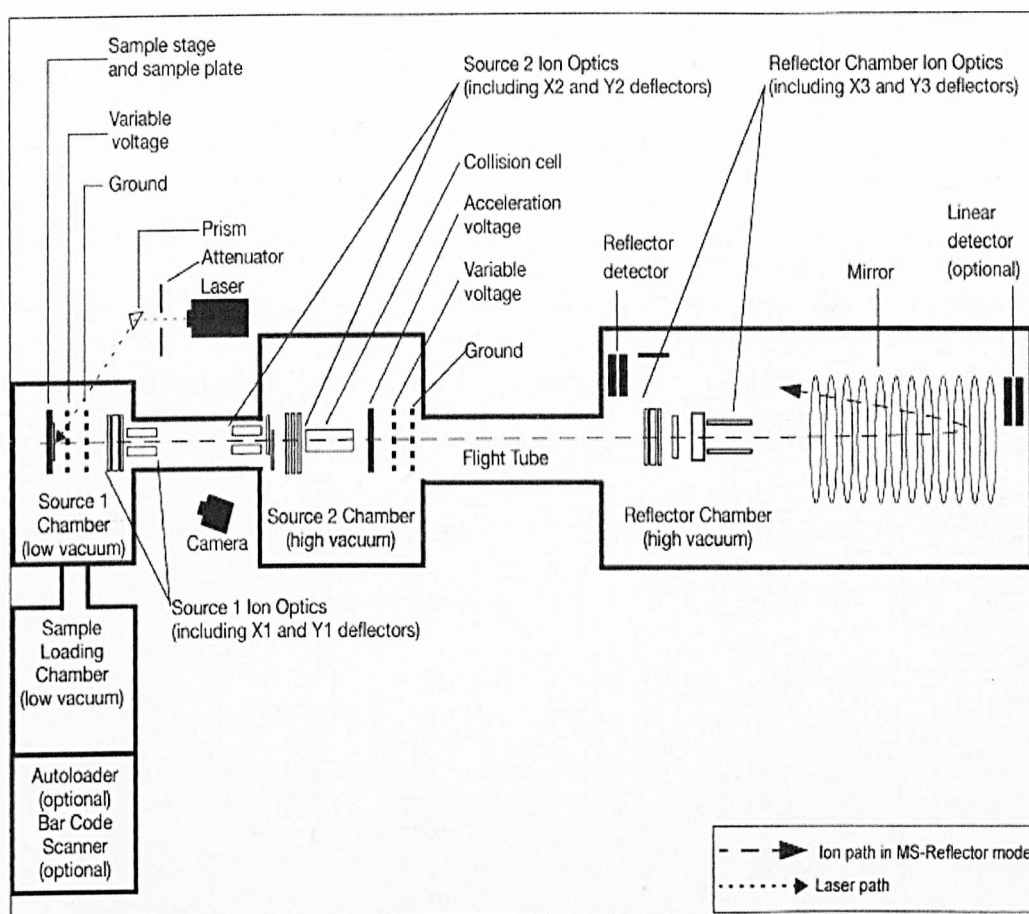


Figure 2.9: 4700 Proteomics Analyser Mass Spectrometer.

2.16 Assessment of data

Tissues used during this study were assessed for their morphological structure and the integrity of their proteins and nucleic acids. Three independent and experienced histopathologists graded H&E and IHC results. Grades ranged from 1 (lowest) to 10. For the H&E, assessment was based on criteria such as shrinkage, cell morphology, nuclear structure and fixative penetration. For the IHC, criteria for assessment were the intensity and localisation of immunostaining. The PCR and RT-PCR products were also scored on their intensity in the agarose gel. A score was also given for the RNA Bioanalyser graphs compared to the fresh-frozen

control samples. For all the data produced scores were given in the following manner: 1-3 = poor, 4-6 = adequate, 7-9 = good and 10 = excellent.

Statistical analysis of results comprised the Student's t-test which was carried out between pairs of fixatives for each of the 3 organs (liver, spleen and colon). The 2-tailed paired Student's t-test (assuming equal and unequal variances) was used for comparison of the mean CT values between fixatives for each tissue. A 95% level of confidence ($p = 0.05$) was set for each comparison to show significant difference and 99% level of confidence ($p=0.01$) to show strong significant difference.

CHAPTER 3

EVALUATION OF NOVEL CANDIDATE FIXATIVES

3.1 Introduction

The search for a universal fixative that would preserve all the crucial tissue components, such as DNA, RNA and proteins, while maintaining optimum tissue architecture for the pathologist, is today far from over. Since formaldehyde was first used as a fixative, there have been many alternatives, depending mostly on the type of histological work that needs to be carried out. NBF is the routinely used fixative in many histopathology laboratories. The main reason for that is that NBF preserves tissue morphology better than most of the other fixatives. But formaldehyde has many limitations and drawbacks for routine use. Formaldehyde heavily degrades RNA, DNA and proteins, making problematic any molecular analysis of formalin fixed samples. Also formaldehyde is now classified as a carcinogenic substance (Arts, Rennen, & Heer 2006), making its handling more difficult and increasing sufficiently the risk of an accident in any lab, plus the high cost of safe removal and disposal.

3.2 Aims:

The aim of the initial part of this project was to identify possible novel fixative candidates from the scientific literature and to test them against NBF, as the current working standard.

1. The identified candidates were tested for their ability to preserve protein, DNA, and their morphological quality, ease of use, and overall cost.
2. The next step was to improve the candidate fixative in order to improve its ability to preserve DNA, RNA and tissue morphology.
3. Finally from the compounds tested the overall best candidates were further evaluated against NBF and the published fixative (zinc-based fixative-Z2).

3.3 Experimental design:

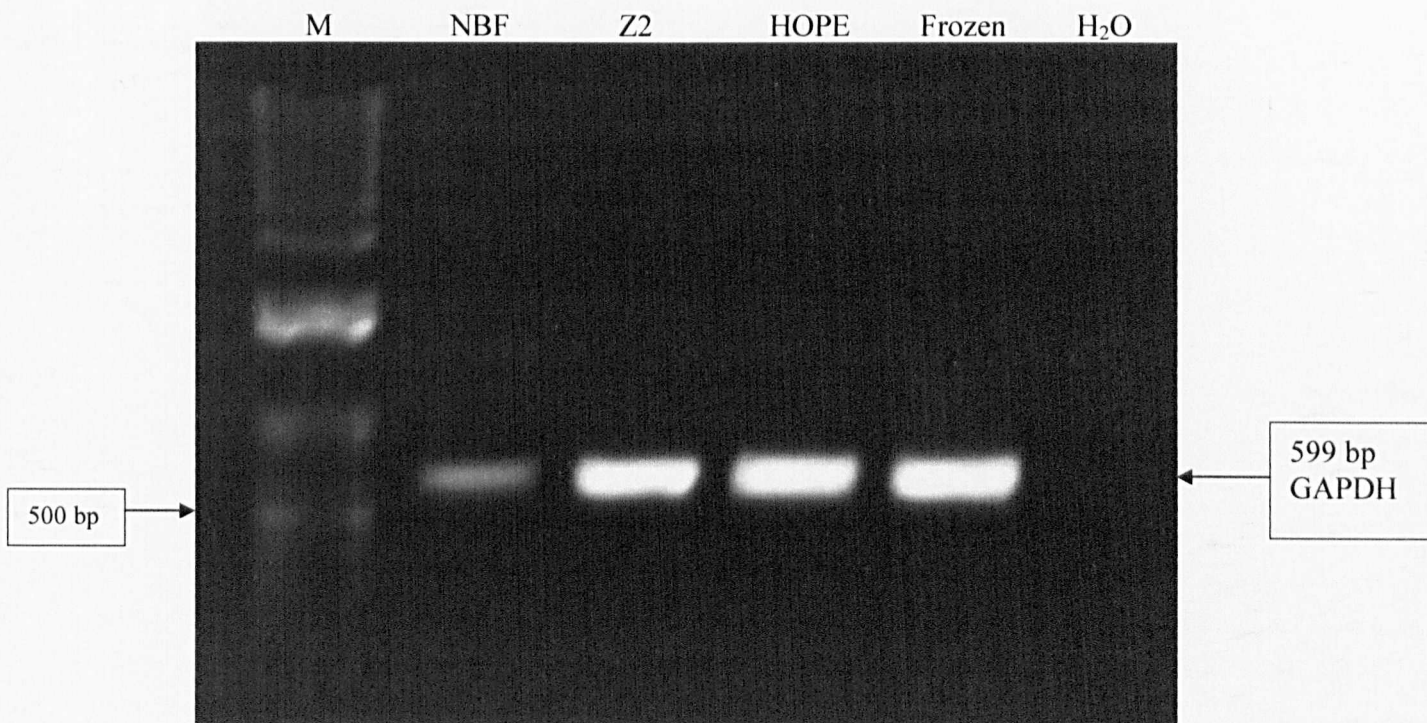
Healthy wild type mice C57Bl6 were euthanised and 3 organs were removed and immediately placed in the various fixatives. Comparisons were done in terms of morphology, DNA/RNA integrity and protein preservation. Of the candidates tested, 2 were further investigated, Z4 and Z7, using further morphological, protein, DNA and RNA evaluation. Further zinc combinations were tested in order to improve the morphology quality of zinc based tissues. At the same time other metallic solution were tested for possible fixation capabilities.

3.4 Results:

3.4.1 Preliminary work for identification of candidate fixative

Preliminary experiments were performed with neutral buffered formalin used as a standard fixative with two non-formalin based fixatives, Z2 and HOPE. Both these are non-toxic and in particular the zinc-based fixative is very simple to use. Tests included morphological evaluation (H&E), (IHC) for 1) a widely distributed antigen not requiring antigen retrieval in formalin-fixed material, such as muscle-specific actin. 2) Antigens requiring pre-treatment in formalin-fixed material such as cytokeratin which is an epithelial marker and CD3 which is a T-lymphocyte marker. Preservation of nucleic acids was demonstrated by extraction and gel electrophoresis with and without PCR.

A)



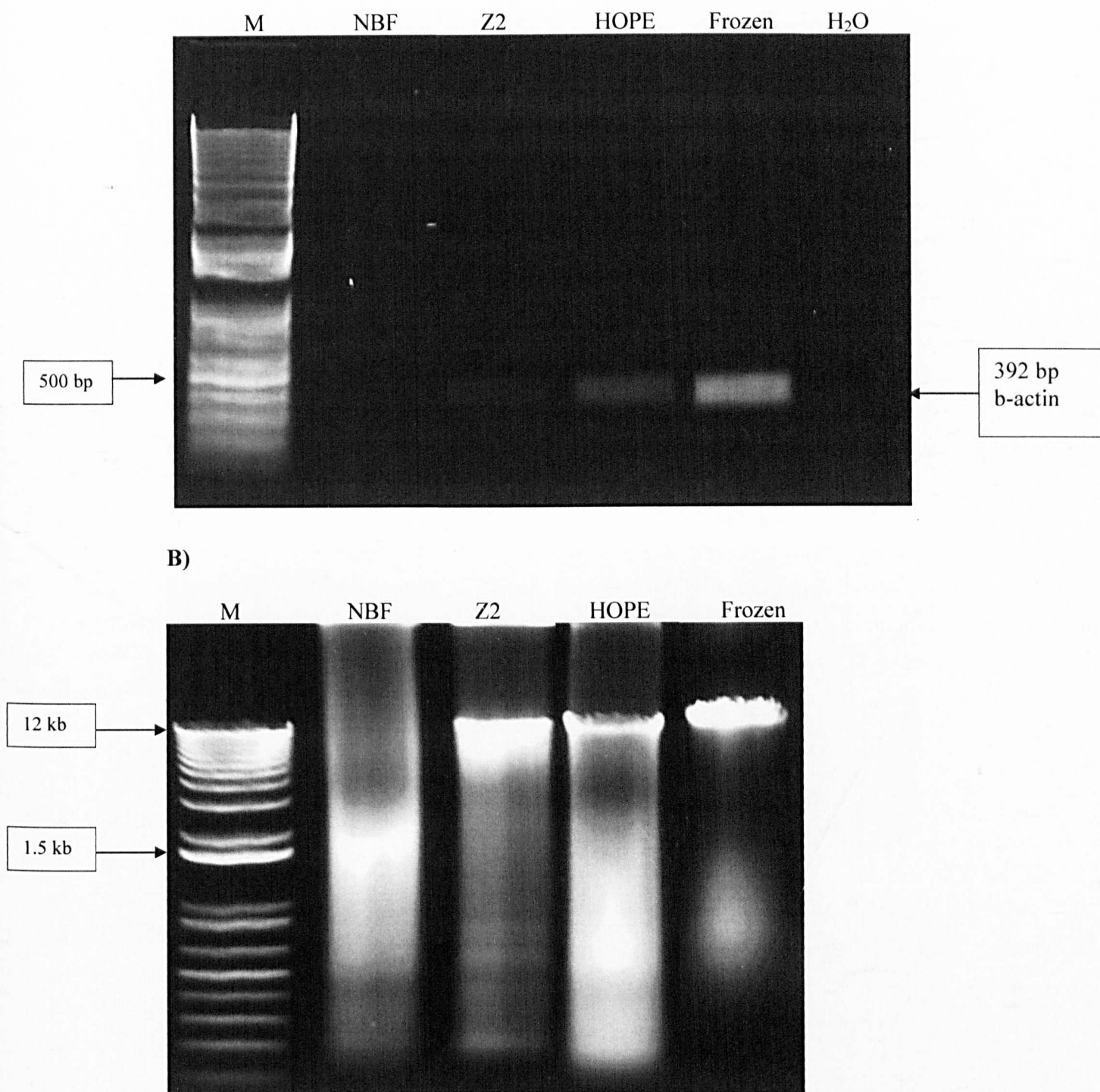


Figure 3.1: A) PCR using β -actin and GAPDH primers. Mouse colon tissue was fixed with NBF, Z2 and HOPE fixatives. A fresh frozen sample was used as control. 100ng of DNA was loaded for each sample. B) DNA samples extracted using various fixation methods, run on a 1% agarose gel. Each experiment was duplicated.

From the results in figure 3.1, NBF shows little preservation of DNA, while Z2 and HOPE both preserve DNA, and the intensity of the PCR bands generated was equal. The intensity of the PCR products from fresh-frozen samples was also shown to be overall slightly higher. Regarding the overall DNA quality shown by agarose gel electrophoresis it can be seen that the DNA extracted from NBF-fixed samples was fragmented and most of it was around 1.5 kb. On the other hand DNA from both Z2-fixed and HOPE-fixed mouse colon appeared less fragmented and gave a band of around 12 kb. The same ofcourse was observed for the control sample (frozen), but with even higher intensity in the gel and less fragmentation.

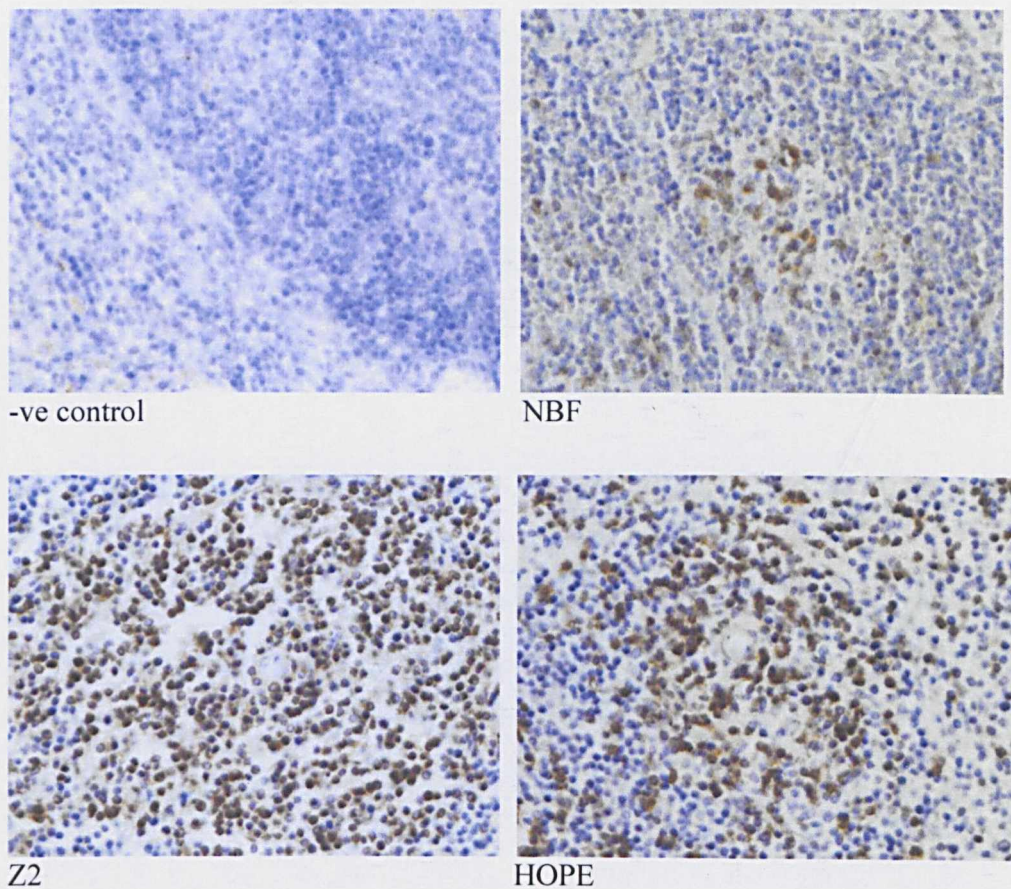


Figure 3.2: Representative IHC results from spleen tissue and staining for CD3. Magnification is set at x 40.

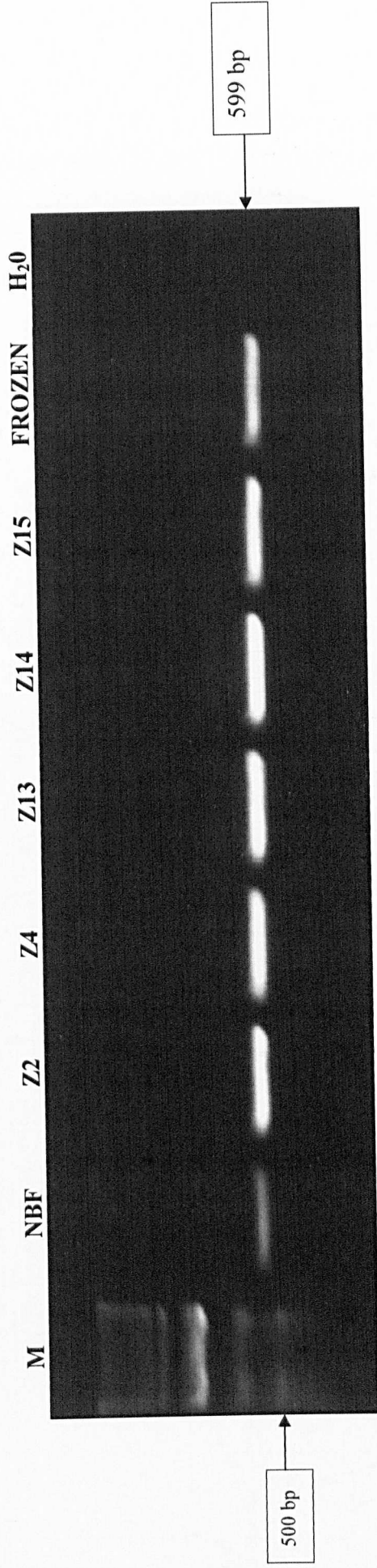
From the results in figure 3.2 we can see that there is no staining in the –ve control sample. In all other images CD3 staining is seen in dark brown colour within the tissue. More specific, there is some positive staining in the NBF sample, where as in both Z2 and HOPE samples, the CD3 staining is more spread throughout the whole tissue section. Z2 appears to have overall better CD3 staining with no visible background.

Other results from Z2 and HOPE showed that Z2 was better for structural and protein preservation, when looking at the results from H&E staining and IHC. Therefore, in view of the expense of HOPE and the cheapness of the zinc-based fixative the zinc-based fixative was chosen for further investigation. Also the fragility of HOPE fixed samples and the requirement for hand processing was a disadvantage.

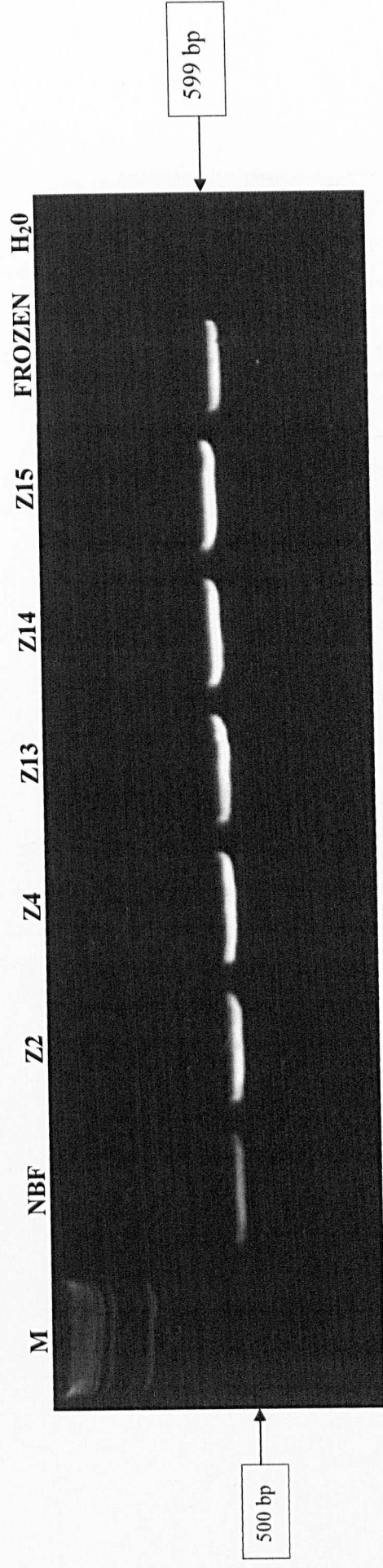
3.4.2 Optimisation of zinc based fixatives

After showing that the zinc-based fixative preserved morphology, IHC and DNA better than HOPE, the next step was to chemically optimise the Z2 fixative. This was done using a series of modifications and substitutions using as a basis the Z2 fixative formula as described before (Chapter 2, Section 2.3). These modifications were aimed at improving the function of zinc as a fixative and allowing zinc ions to penetrate tissue faster and to move more freely. Below is a summary from the data produced using different chemical modifications. Code names were given for simplicity and the details are given in (Chapter 2, Section 2.3).

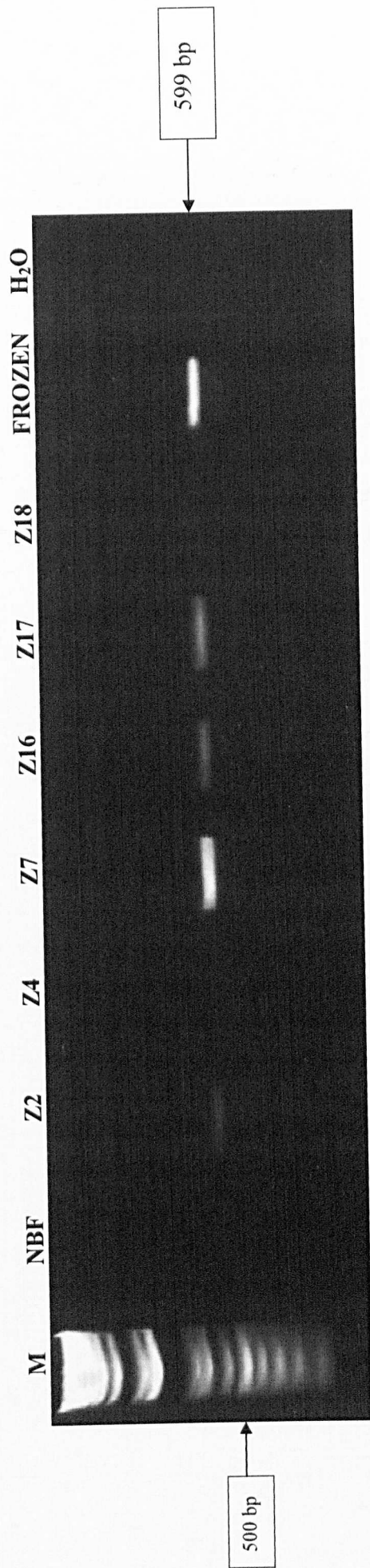
PCR Liver



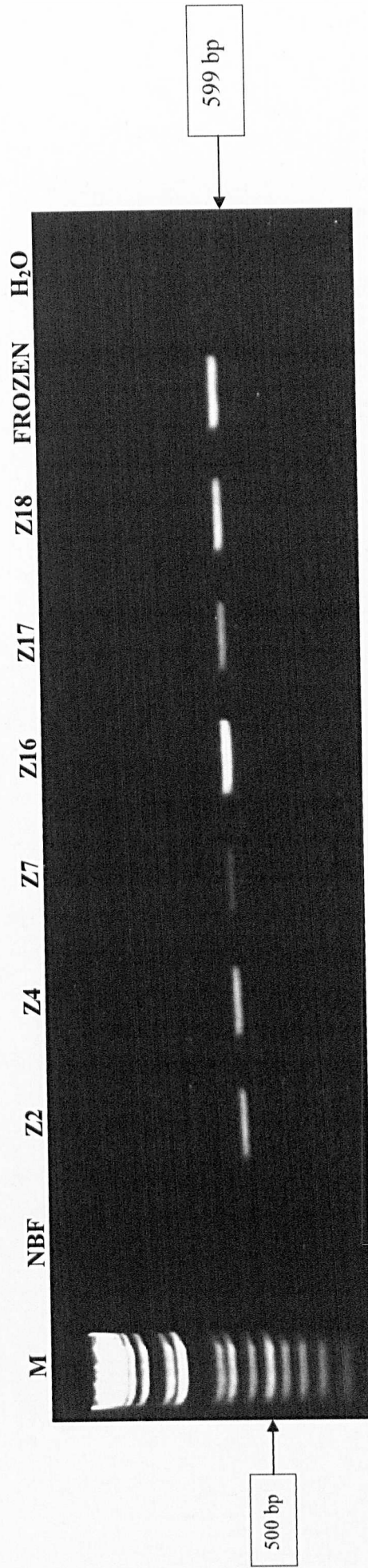
PCR Colon



PCR Liver



PCR Colon



Bioanalyser (Liver)

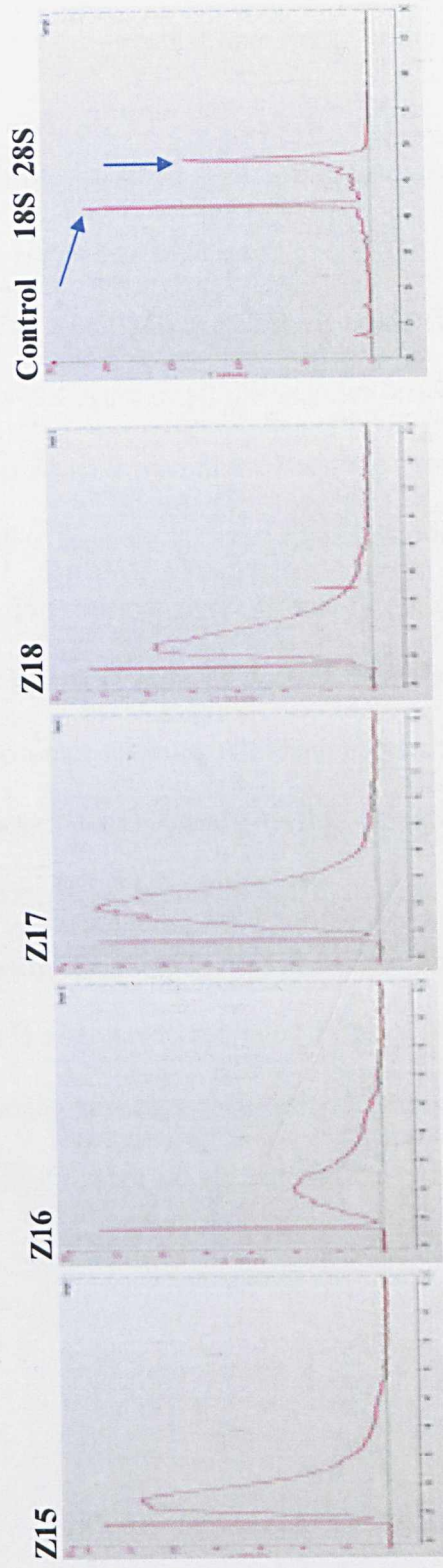
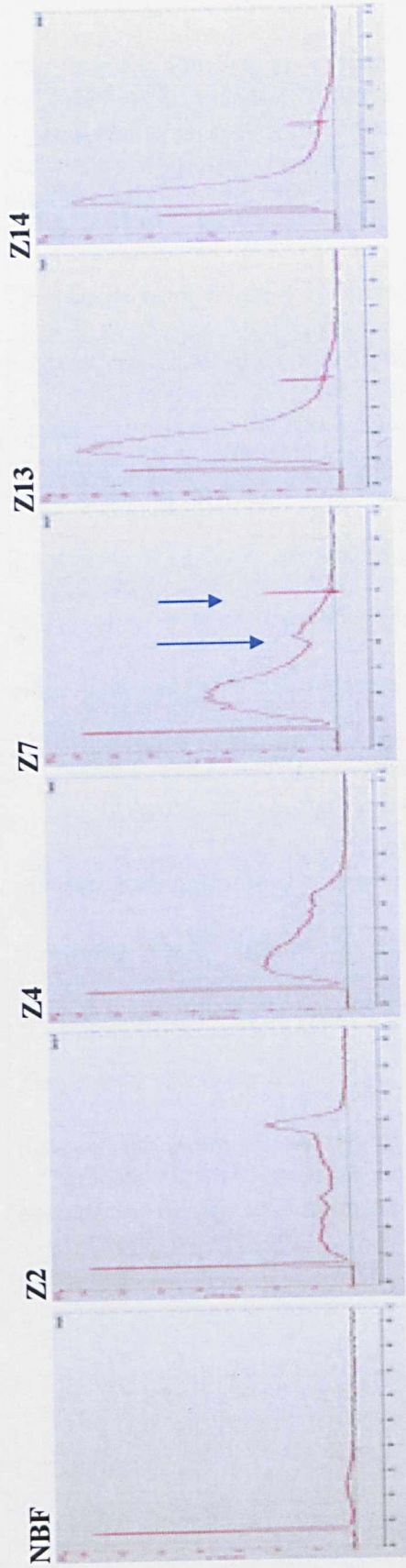


Figure 3.3: Representative results from 8 chemical modifications to the Z2 fixative. PCR was performed with GAPDH primers using liver and colon tissue to assess the DNA quality. Fresh-frozen tissue was used as control for each PCR experiment and for every PCR reaction 100ng of each sample was loaded. The Agilent 2100 Bioanalyser was used to evaluate the RNA quality. Mouse liver samples from 8 chemical modifications to the Z2 fixative were tested. Fresh-frozen samples were used as control.

The results from figure 3.3 showed that NBF was consistently giving the worst PCR product, compared to the rest of the fixatives. Z4 and Z7 showed overall the best PCR results compared to the rest of the modified zinc fixatives. There is some variability in the intensity of the Z4 and Z7 products but again in all the samples tested and all the repeats of each experiment both Z4 and Z7 gave overall the best PCR products.

The quality of RNA extracted from tissues fixed in Z7 was superior compared to other fixatives. This can be seen clearly in the Bioanalyser results from figure 2.3, where only RNA extracted from Z7-fixed samples produced both characteristic peaks corresponding to the 18S and the 28S ribosomal subunits. Although none of the fixed samples had only the 2 characteristic peaks corresponding to the 18S and 28S of ribosomal RNA, without any degradation, these results were a step in the right direction and showed improved RNA quality compared to the NBF fixed sample.

Zinc-based solutions (Z13, Z14, and Z15) also gave promising PCR results, but in light of the more crucial RNA assessment and also their inferior morphological evaluation, finally only solutions Z4 and Z7 were chosen for further analysis and development.

3.4.3 Assessment of the two optimised fixative solutions.

This part of the project was done in order to assess the 2 best fixative candidates. These were the Z4 (Z2 + 5% DMSO) and the Z7 (with zinc trifluoroacetate instead of zinc acetate). NBF, Z2 and fresh frozen tissues were used as controls. The tests that were performed included H&E staining for morphological structure, PCR for DNA integrity, RNA Agilent Bioanalyser and RT-PCR for RNA quality. Experiments were repeated three times and the results were assessed by three independent assessors. For IHC, negative control staining was included in each experiment and no staining was observed.

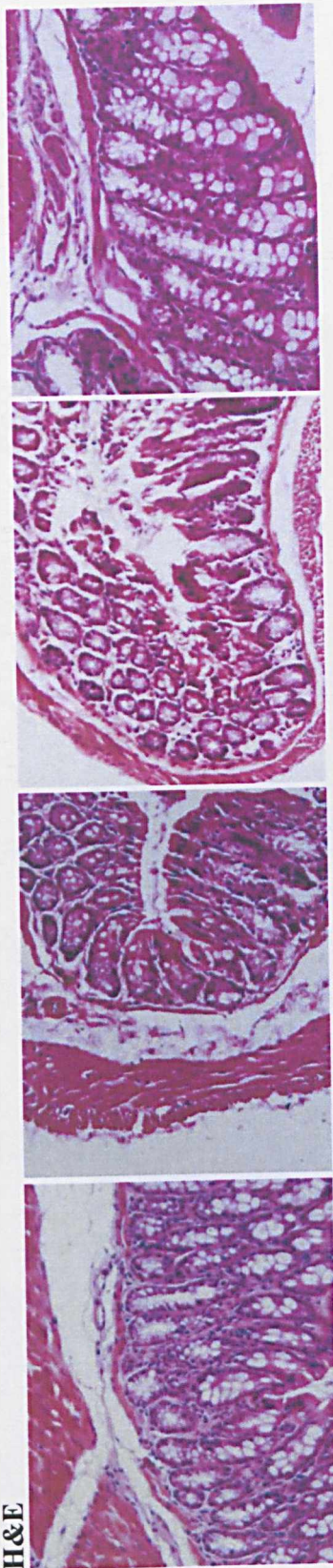
Z7

Z4

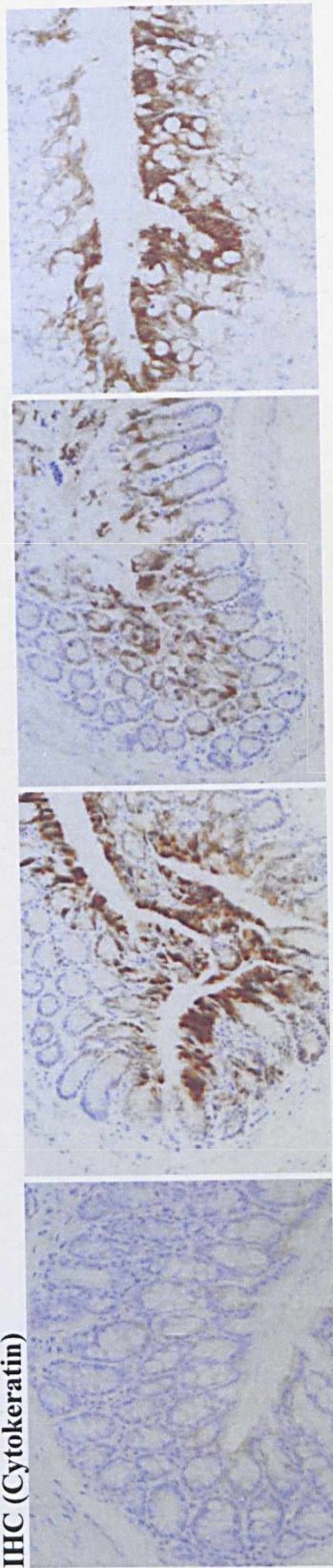
Z2

NBF

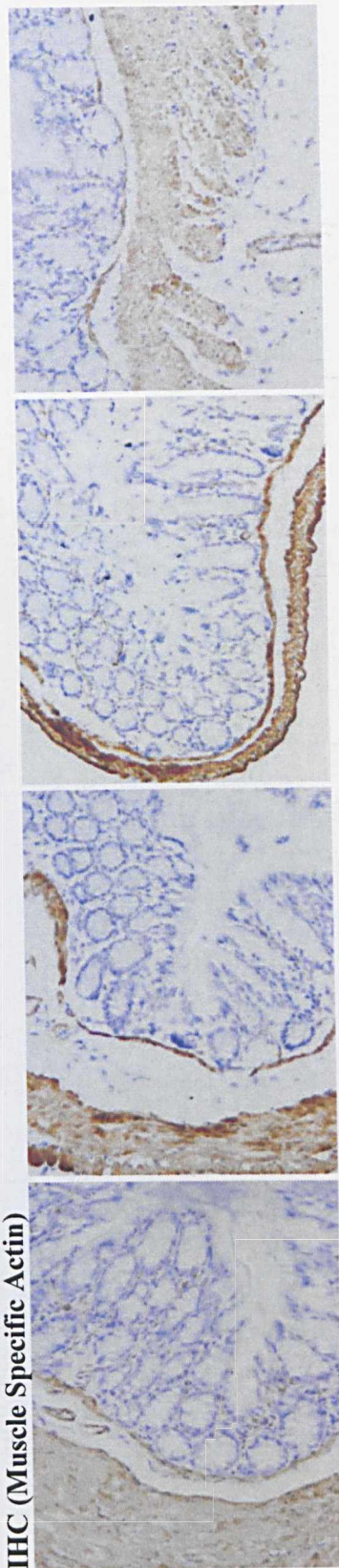
H&E

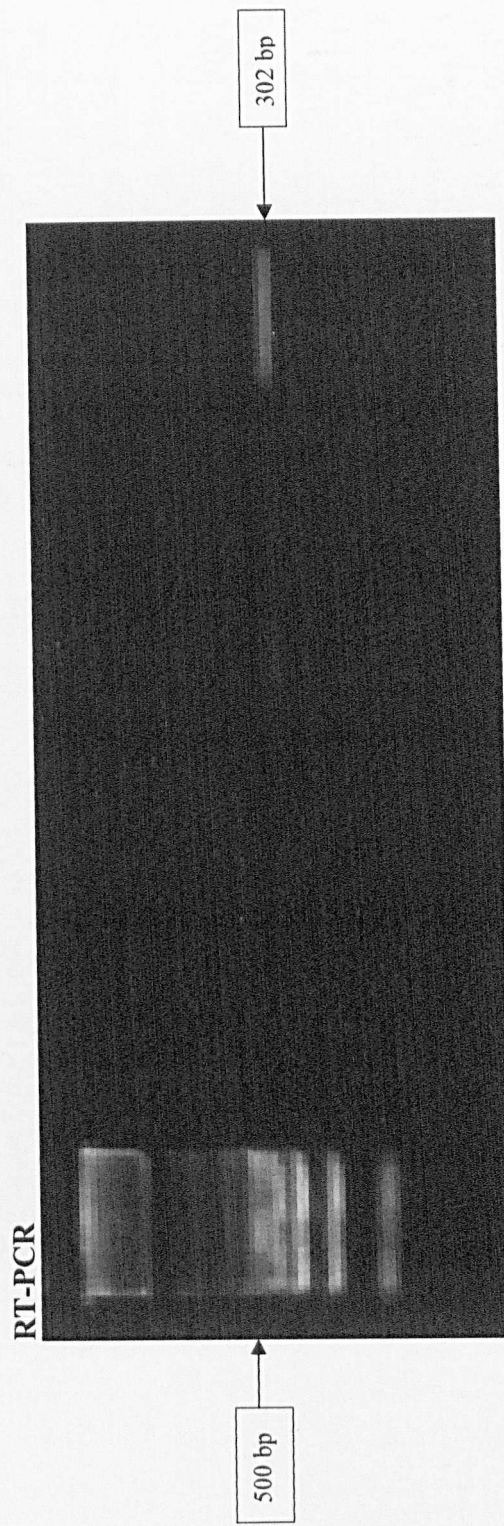
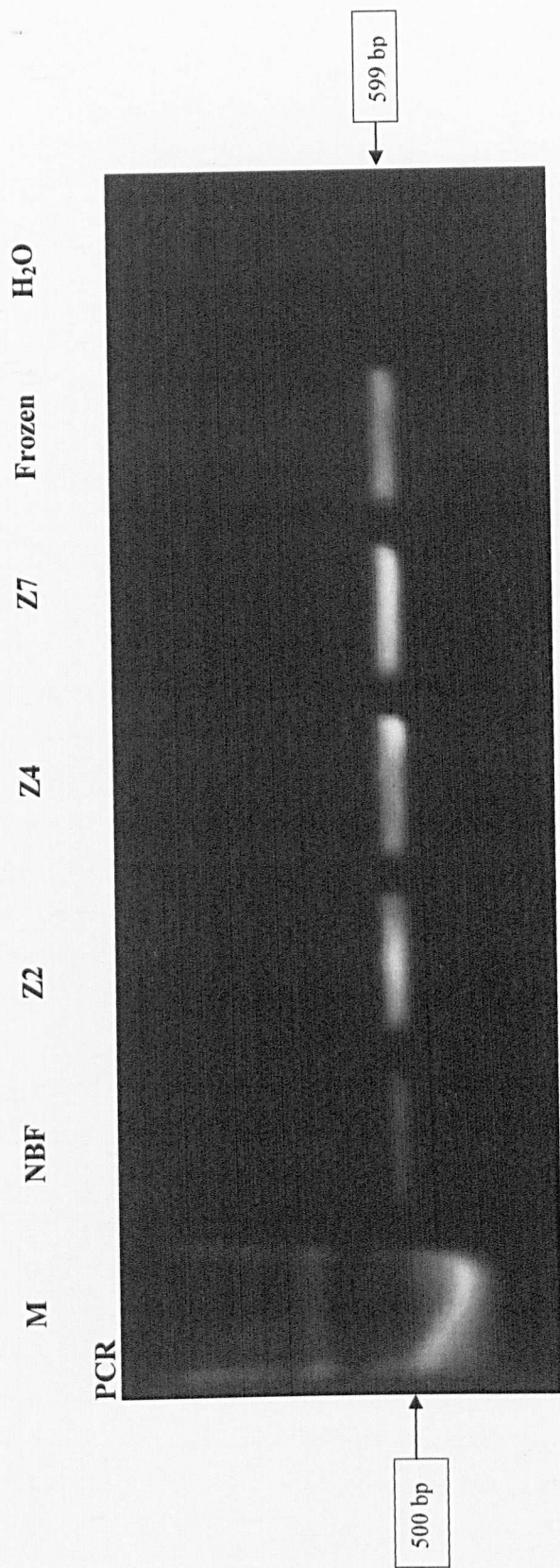


IHC (Cytokeratin)



IHC (Muscle Specific Actin)





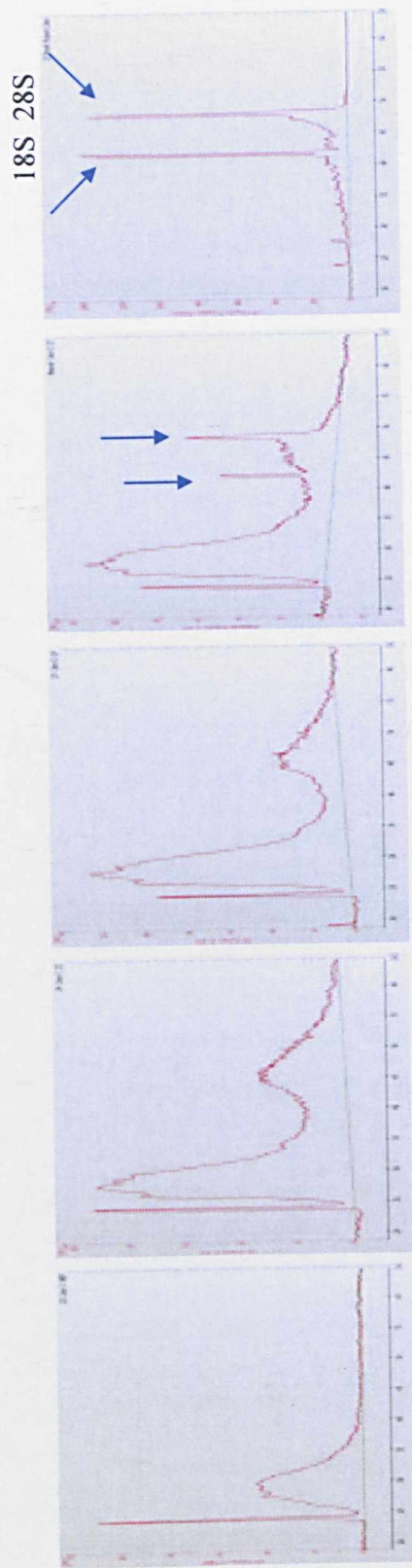


Figure 3.4: Results showing mouse colon tissue fixed in NBF, Z2, Z4, Z7 and fresh frozen tissue as a control sample for DNA and RNA quality. Morphology was assessed by H&E, protein preservation was examined by IHC for Cytokeratin AE1&AE3 and MSA. The magnification on all images was x20. The DNA quality was assessed by PCR with GAPDH primers and 100 ng of DNA from each sample was used. The RNA quality was examined by a) RT-PCR with β -actin primers using 1 μ g of RNA from each sample, b) the Agilent 2100 Bioanalyser using 250 ng of RNA from each sample. Blue arrows indicate the 18S and 28S ribosomal units. Note that the green line shown in some of the electrophoregrams was a digital by-product of the computer graph and has no value to the analysis. For simplicity results from one experiment are shown.

All the above data using colon tissue are summarised in the **Table 3.1**. Marks were given from 1 to 10.

Table 3.1: Results on the assessment of mouse colon tissue.

Test ↓	Fixative →	NBF	Z2	Z4	Z7	Frozen (Control)
H&E		9	6	7	8	-
IHC (mean)		5	8	7	7	-
(Cytokeratin)		3	9	6	9	
(MSA)		8	7	8	5	
DNA (PCR)		3	7.5	8	8.5	7
1) RNA (RT-PCT)		0	0	4	3	9
2) RNA Bioanalyser		1	3	3	5	10
Total (50)		18	24.5	29	31.5	26/30*
Percentage (%)		36%	49%	58%	63%	87%

* The fresh frozen samples were only assessed for DNA and RNA quality and therefore had a much higher percentage score.

From the scores on table 3.1 it can be said that NBF (36%) was inferior to the rest of the fixatives tested although it had the best marks for morphological architecture. Fresh frozen samples showed high quality of both DNA and RNA in all tests performed. The Z7 fixative was better than NBF and the rest of the zinc-based fixatives for nucleic acid preservation and morphological structure. More specific, it must be noted that Z7 gave again a very promising result regarding the RNA quality, were both ribosomal bands were visible, indicating a higher quality of RNA than the rest of the fixatives tested.

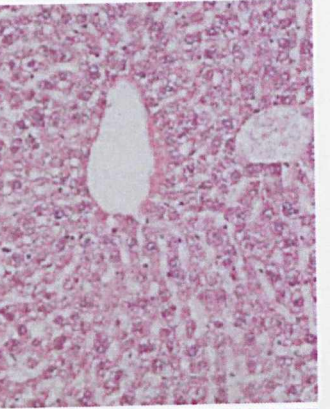
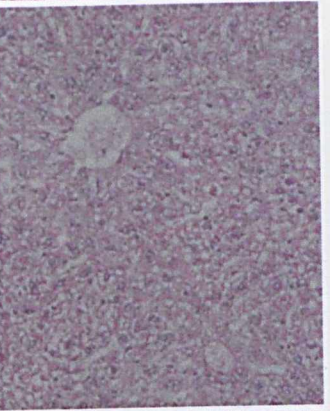
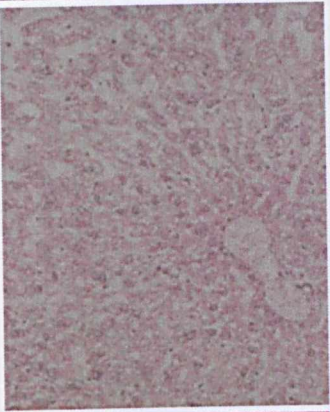
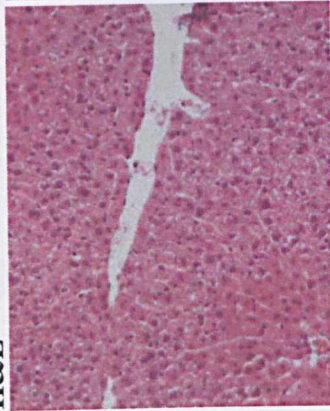
NBF

Z2

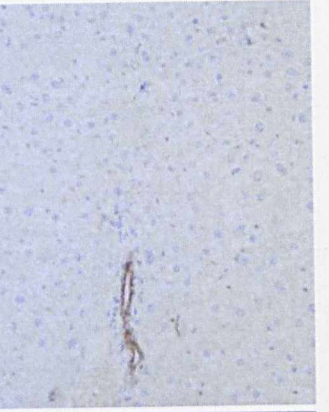
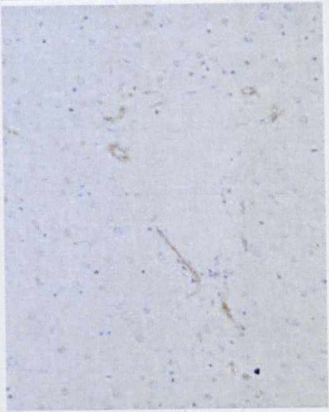
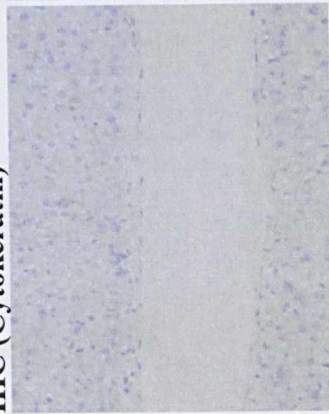
Z4

Z7

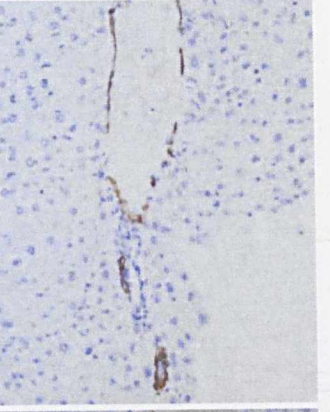
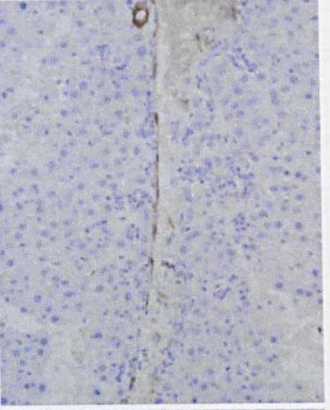
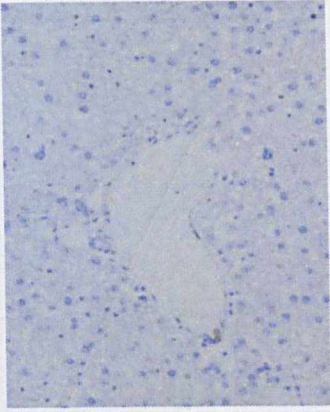
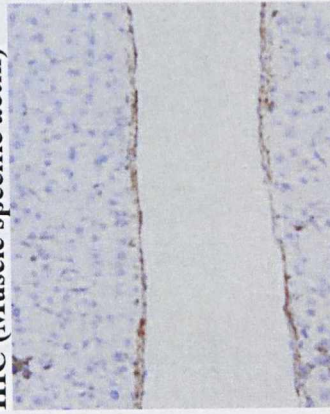
H&E

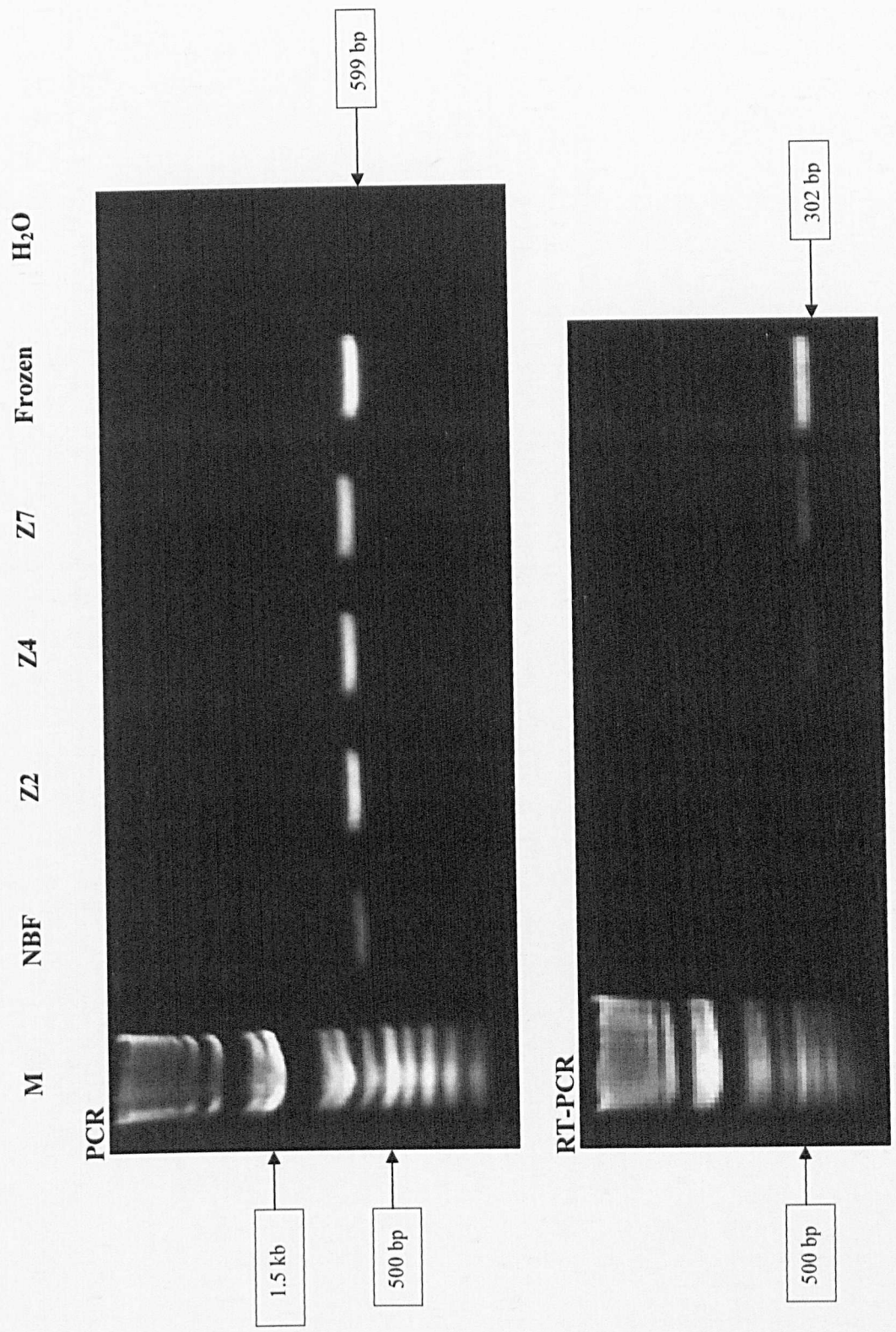


IHC (Cytokeratin)



IHC (Muscle specific actin)





Bioanalyser

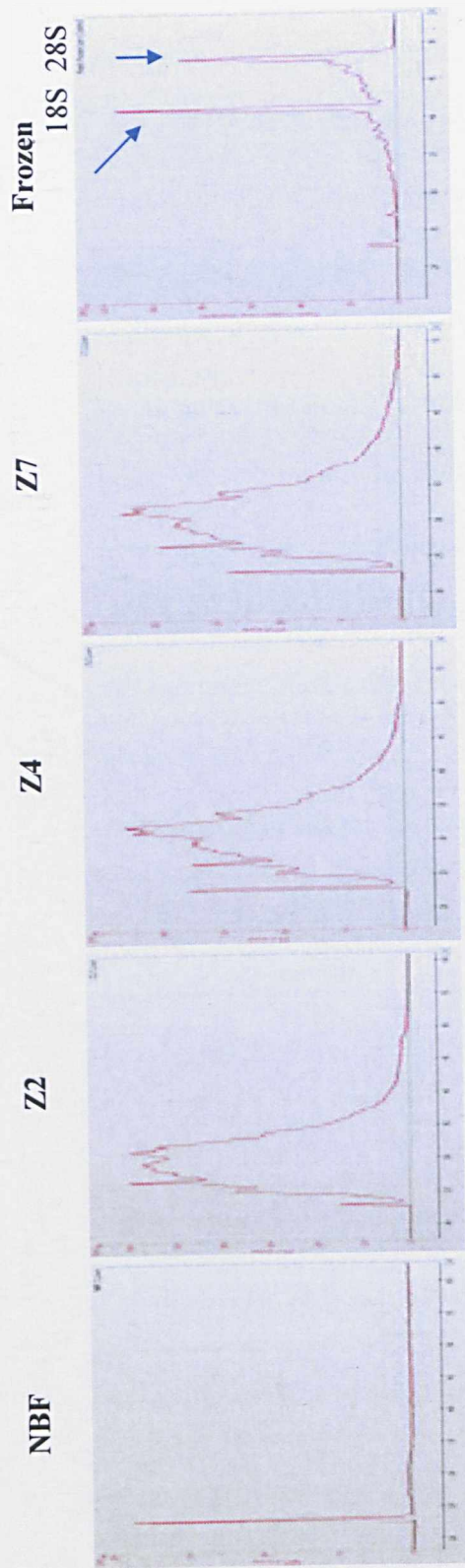


Figure 3.5: Results showing mouse liver tissue fixed in NBF, Z2, Z4, Z7 and fresh frozen tissue as a control sample for DNA and RNA quality. Morphology was assessed by H&E, protein preservation was examined by IHC for Cytokeratin AE1&AE3 and MSA. The magnification on all images was at x20. The DNA quality was assessed by PCR with GAPDH primers and 100 ng of DNA from each sample was used. The RNA quality was examined by a) RT-PCR with β -actin primers using 1 μ g of RNA from each sample, b) the Agilent 2100 Bioanalyser using 250 ng of RNA from each sample. Blue arrows indicate the 18S and 28S ribosomal units. Note that the green line shown in some of the electrophoregrams was a digital by-product of the computer graph and has no value to the analysis. For simplicity results from one experiment are shown.

Regarding the IHC staining seen in figures 3.4 and 3.5 it can be said there are many cytokeratins of differing molecular weight. They are found in epithelial cells and, depending on the type of cytokeratin used as the immunogen, are markers of carcinomas of various organs. The antibody used in this work consists of mixed clones AE1/AE3, designed to react with a wide range of cytokeratins, and will label normal liver and kidney cells to some extent. In the colon, cytokeratins are localised on the baso-lateral membranes of the glandular epithelial cells. Spleen cells are not of epithelial origin and will not be labelled. Furthermore, muscle-specific actin (MSA), as its name implies, is a marker of muscle fibres, both smooth and striated. In the colon the two muscle layers will be labelled as well as the muscularis mucosae layer and muscle fibres surrounding the glands and some blood vessels. In the kidney, liver and spleen, only the vessel muscle fibres will be labelled.

All the above data using mouse liver tissue are summarised in table 3.2. Marks were given from 1 to 10.

Table 3.2: Results on the assessment of mouse liver tissue.

Test ↓ Fixative →	NBF	Z2	Z4	Z7	Frozen (Control)
H&E	9	6	7	8	-
IHC (mean) (Cytokeratin) (MSA)	4 1 7	3 4 2	7 9 5	8 8 8	-
DNA (PCR)	4	8	7.5	7.5	10
1) RNA (RT-PCT) 2) RNA Bioanalyser	0 0	1 2	3 3	4 3	9 10
Total (50)	17	20	27.5	30.5	29/30*
Percentage (%)	34%	40%	55%	61%	96%

* The fresh frozen samples were only assessed for DNA and RNA quality and therefore had a much higher percentage score.

From the scores on table 3.2 it can be said that NBF (34%) was inferior to the rest of the fixatives tested although it had the best marks for morphological architecture. Fresh frozen samples showed high quality of both DNA and RNA in all tests performed. The Z7 fixative was better than NBF and the rest zinc-based fixatives for protein preservation, morphological structure and was given the highest total score.

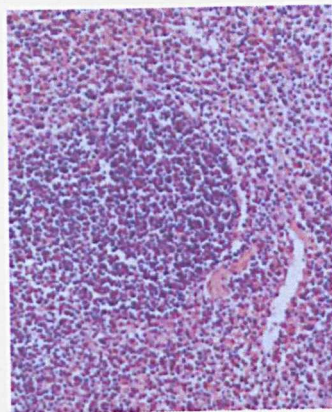
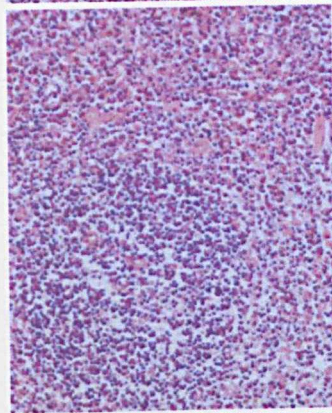
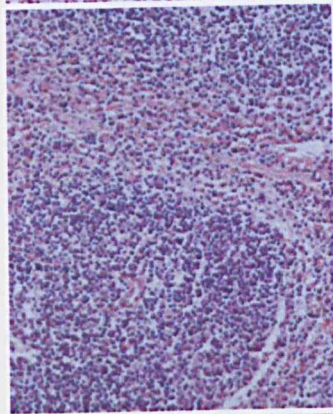
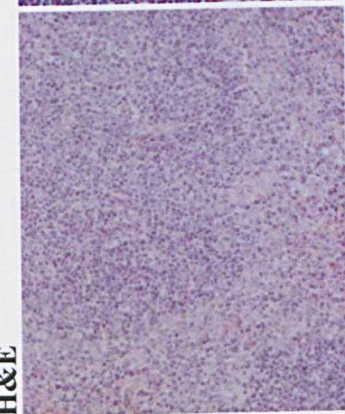
NBF

Z2

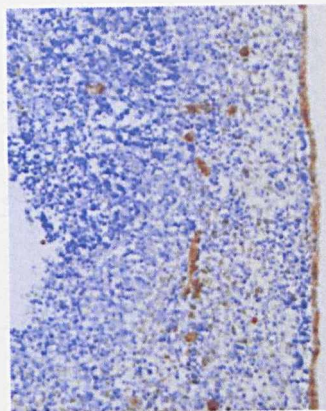
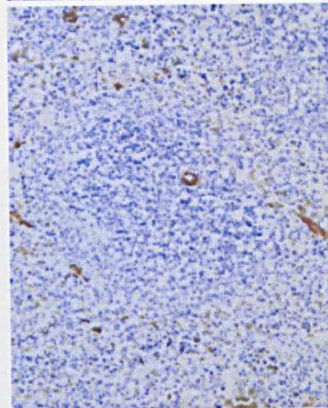
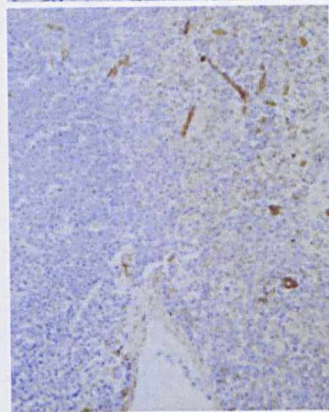
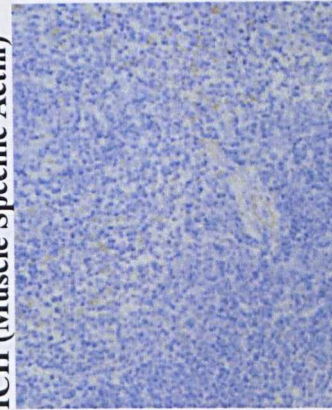
Z4

Z7

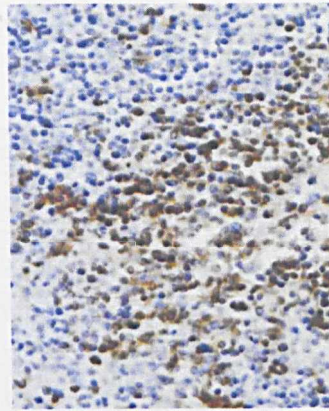
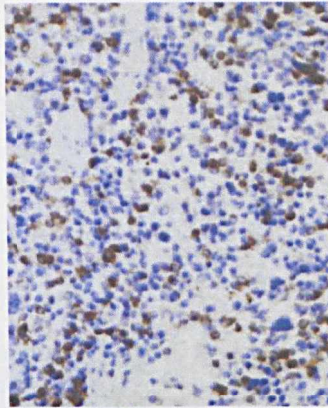
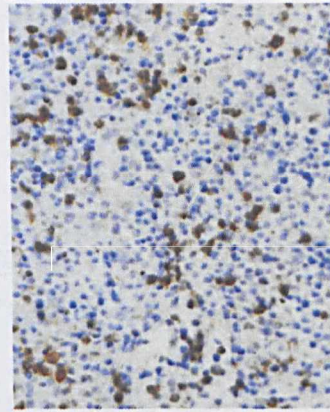
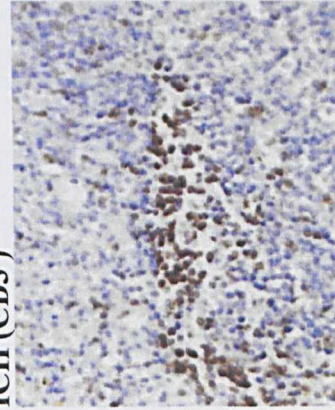
H&E

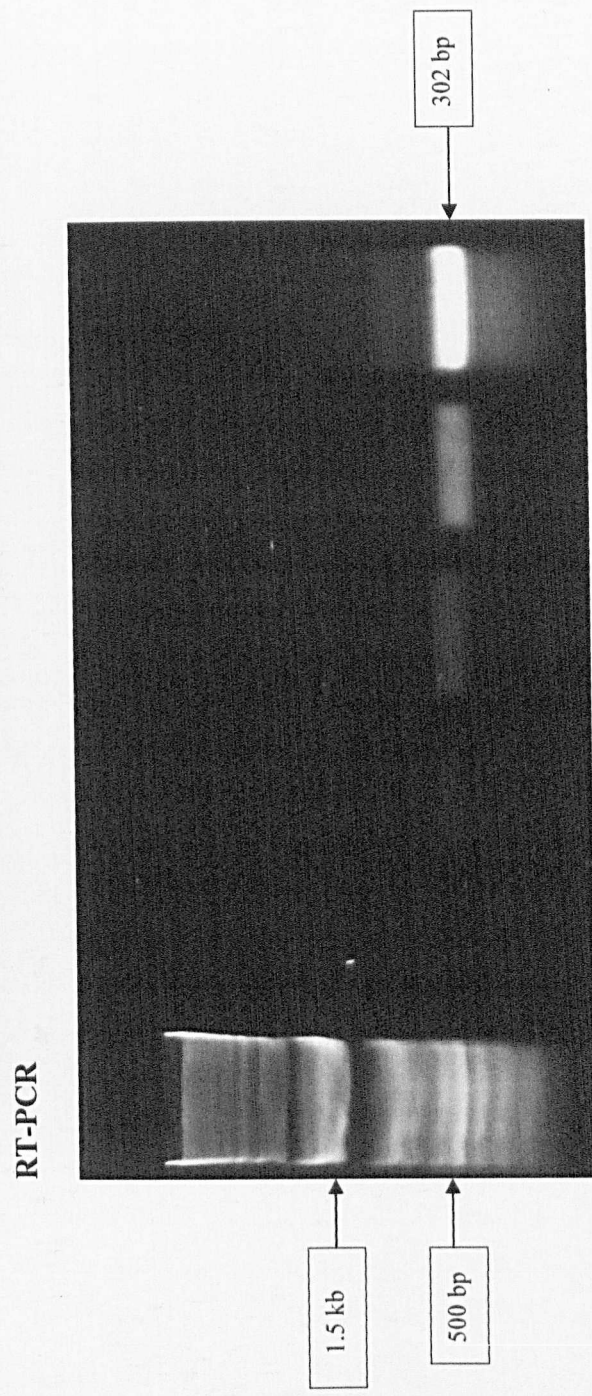
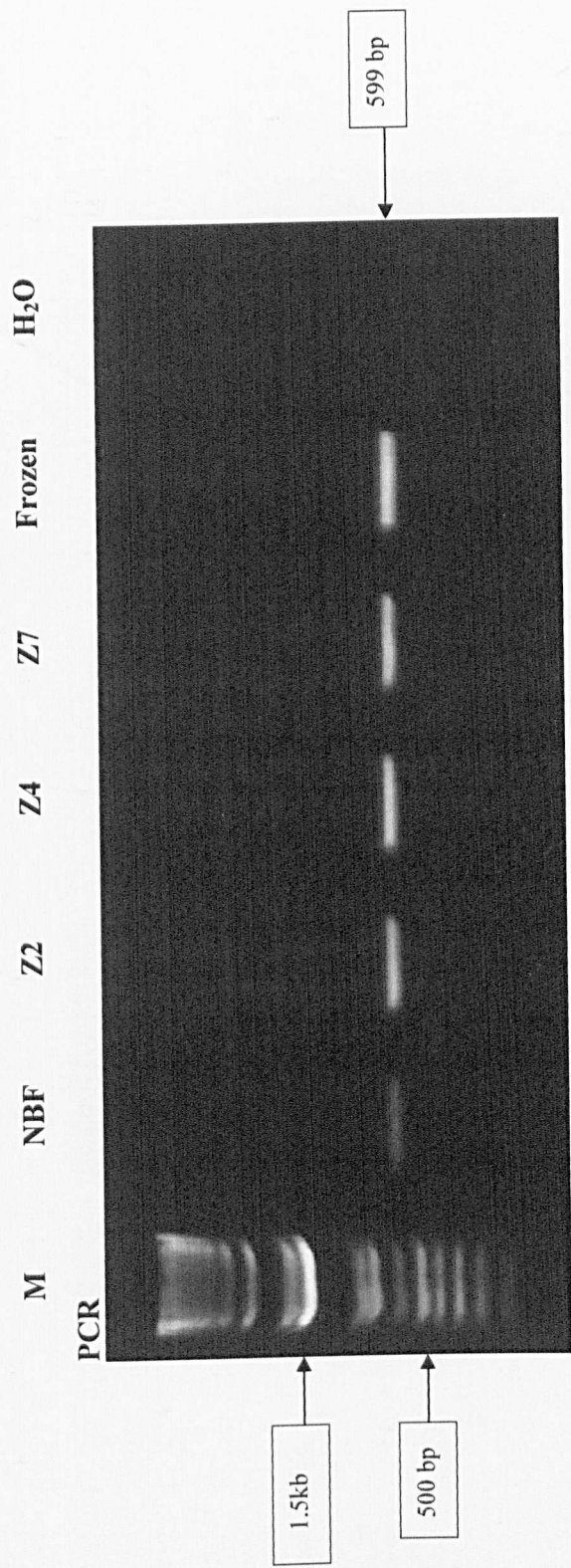


ICH (Muscle Specific Actin)



ICH (CD3)





Agilent Bioanalyser

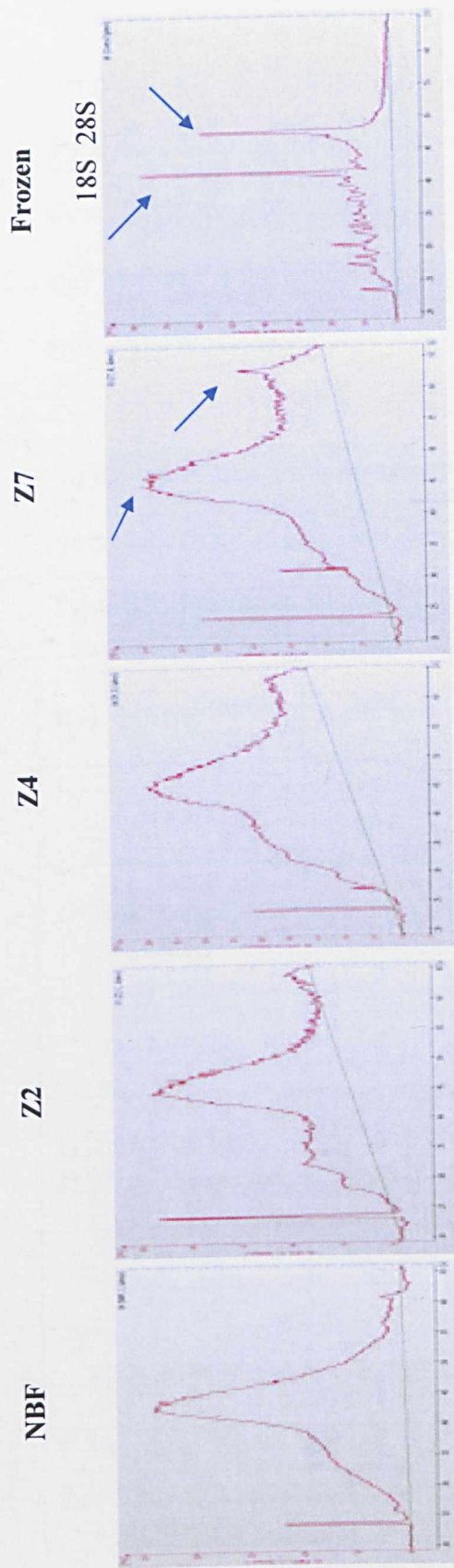


Figure 3.6: Results showing mouse spleen tissue fixed in NBF, Z2, Z4, Z7 and fresh frozen tissue for DNA and RNA quality. Morphology was assessed by H&E, protein preservation was examined by IHC for Cytokeratin AE1&AE3 and MSA. The magnification on all images was at x 20. The DNA quality was assessed by PCR with GAPDH primers and 100 ng of DNA from each sample was used. The RNA quality was examined by a) RT-PCR with β -actin primers using 1 μ g of RNA from each sample, b) the Agilent 2100 Bioanalyser using 250 ng of RNA from each sample. Blue arrows indicate the 18S and 28S ribosomal units. Note that the green line shown in some of the electrophoregrams was a digital by-product of the computer graph and has no value to the analysis. For simplicity results from one experiment are shown.

Regarding the IHC results from figure 3.6 it can be said that CD3 is a marker of the cell membrane of T-lymphocytes. In the spleen these cells predominantly surround the B-cells zone of the germinal centres but may also be scattered through these areas. In the liver and kidney occasional cells may be seen and in the colon T cells may be found in the lamina propria and also in aggregations of lymphoid cells in the muscularis mucosae.

All the above data using mouse spleen tissue are summarised in **Table 3.3**. Marks were given from 1 to 10.

Table 3.3: Results on the assessment of mouse spleen tissue.

Test ↓ →	Fixative →	NBF	Z2	Z4	Z7	Frozen (Control)
H&E		9	7	7	8	-
IHC (mean)		4	7.5	8.5	9.5	-
(MSA)		1	7	8	9	
(CD3)		7	8	9	10	
DNA (PCR)		6	9	9	9	10
1) RNA (RT-PCT)		0	0	2	3	10
2) RNA Bioanalyser		2	3	3	4	9
Total (50)		21	26	29.5	33.5	29/30*
Percentage (%)		42%	53%	59%	67%	93%

* The fresh frozen samples were only assessed for DNA and RNA quality and therefore had a much higher percentage score.

From the scores on table 3.3 it can be said that NBF (42%) was inferior to the rest of the fixatives tested although it had the best marks for morphological architecture. Fresh-frozen samples showed high quality of both DNA and RNA in all tests performed. The Z7 fixative was better than NBF and the rest of the zinc-based fixatives for protein preservation, morphological structure and RNA and equal to Z2 and Z4 fixatives for DNA. Overall, again Z7 was given the highest score from the fixatives tested.

The results obtained on the assessment of each organ separately are summarised in Table 3.4. The mean score from all above assessments is shown below, and scores were given from 1 to 10. In cases where the mean number had decimals it was rounded to the nearest whole number.

Table 3.4: Results on the assessment of all 3 types of mouse tissue.

Test ↓ Fixative →	NBF	Z2	Z4	Z7	Frozen (Control)
H&E	9	6	7	8	-
IHC (mean)	4	7	7	8	-
DNA (PCR)	4	8	8	8	9
1) RNA (RT-PCT)	0	0	3	3	9
2) RNA Bioanalyser	1	2	3	4	10
Total (50)	18	23	28	31	28/30*
Percentage (%)	36%	46%	56%	62%	93%

* Again fresh frozen samples were only assessed for DNA and RNA quality and therefore had a higher percentage score.

From the 3 zinc-based fixatives the best candidate was shown to be the Z7 fixative, therefore it can be summarised that: **(NBF < Z2 < Z4 < Z7 < Fresh-frozen).**

3.5 Replacing zinc with other metallic ions

In order to investigate whether zinc could be replaced by other metallic ions for the purpose of fixation, 5 different metals were tested. These were: calcium, gallium, manganese, magnesium and vanadium. All these metals were chosen on the basis that they: a) belong to the same family (periodic table), b) have a 2+ charge, c) are non-toxic, d) have a similar atomic mass, and e) are of low cost. Manganese was replaced in the zinc-based fixative solution (manganese chloride, manganese acetate, calcium acetate in Tris buffer, pH 6.7) which was named compound M2. Experiments were performed to assess tissue morphology and nucleic acid preservation. Morphological structure was very poor for the M2 fixed samples. RNA was extracted from colon and liver mouse tissue fixed in NBF, Z2 and M2. The RNA concentration was measured with a Nanodrop 1000 spectrophotometer. The RIN values were also read from each measurement and were used to assess the overall RNA quality of each sample tested.

COLON

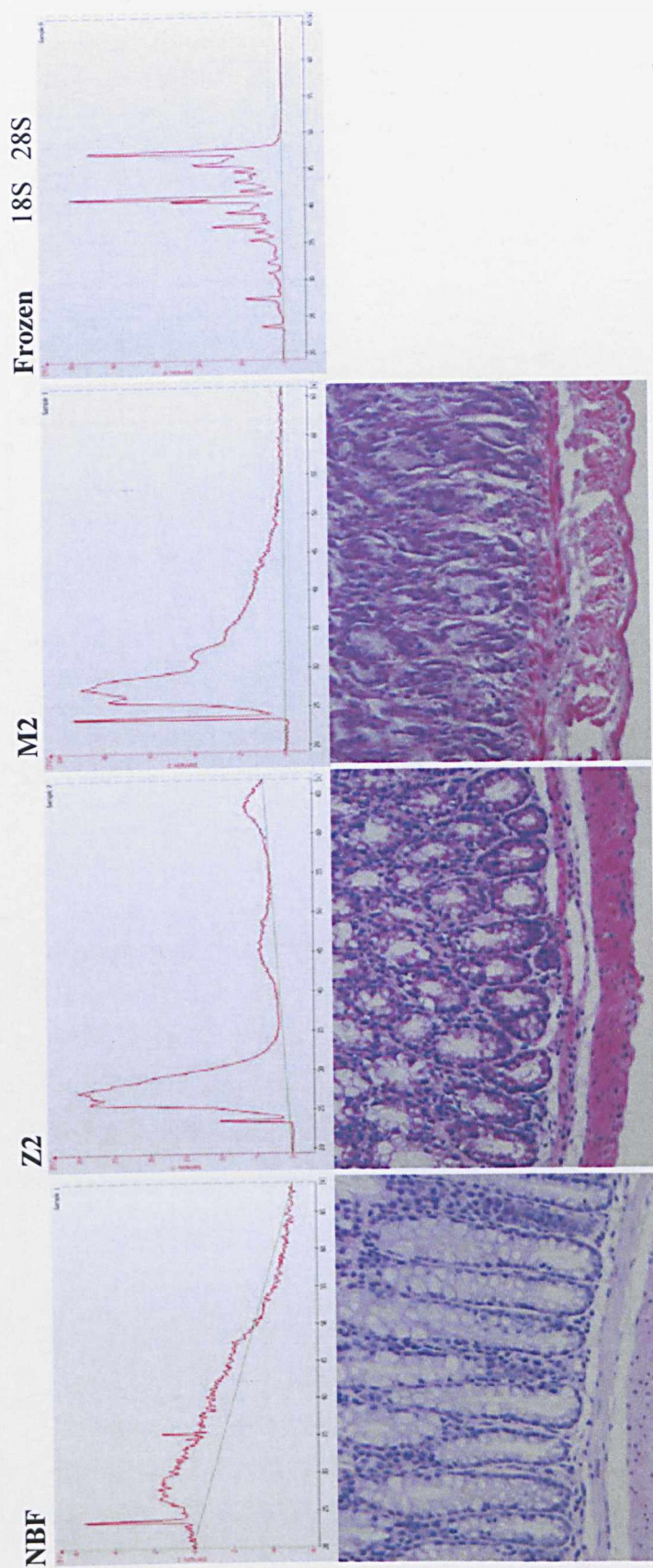


Figure 3.7: Agilent 2100 Bioanalyser electropherograms and H&E staining showing RNA quality and morphological structure of mouse colon samples using 3 different fixatives. The magnification at the H&E images was set at x20. Fresh-frozen samples were used as control for RNA quality. Please note that the green line shown in some of the electrophoregrams was a digital by-product of the computer graph and has no value to the analysis.

LIVER

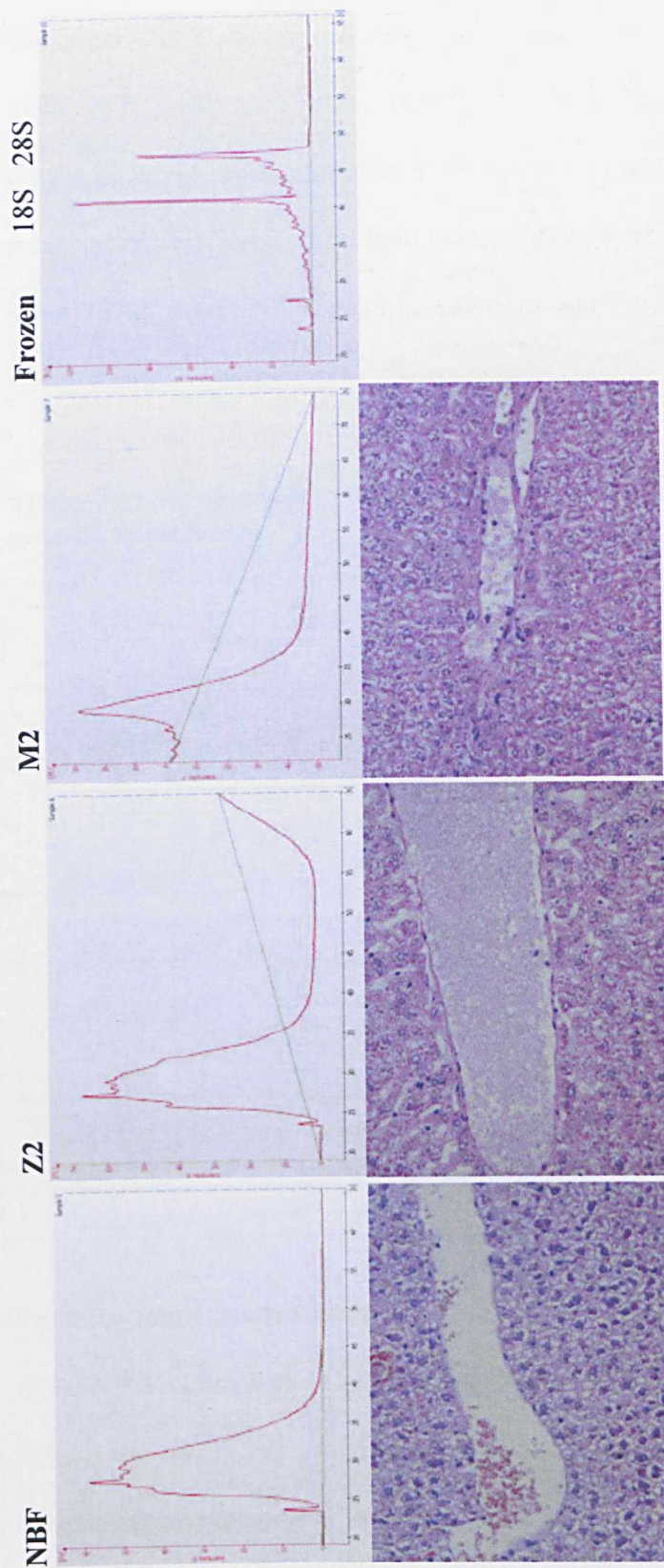


Figure 3.8: Representative Agilent 2100 Bioanalyser electropherograms and H&E staining showing RNA quality and morphological structure of mouse colon samples using 3 different fixatives. The magnification at the H&E images was set at x20. Fresh-frozen samples were used as control for RNA quality. Please note that the green line shown in some of the electropherograms was a digital by-product of the computer graph and has no value to the analysis.

The visible results on morphology using H&E assessment and RNA quality using the RIN value from the 2100 Agilent Bioanalyser shown in figure 3.8, have become numerical values and are shown in Table 3.5. For the purpose of comparison NBF was used as a reference control samples and for RNA quality, fresh-frozen samples were used as reference control samples. Student's t-test was performed using all the mean values from each assessment to test if there were significant differences in the mean values between the metallic solutions and NBF for morphology, and fresh frozen for RNA quality.

Table 3.5: Summary of morphological and RNA quality assessment of other metallic salt solutions.

Name	H&E Mean of 5 organs	RIN Mean of 5 organs and p-value
Calcium	3.2	1.8, $p < 0.01$
Gallium	4.1	2.1, $p < 0.01$
Magnesium	4.3	2.2, $p < 0.01$
Manganese	4.5	1.9, $p < 0.01$
Vanadium	3.4	2.2, $p < 0.01$
Z7	8.4	5.9, $p < 0.05$
NBF	9.2	2.5, $p < 0.01$
Fresh-Frozen	-	9.1, control sample

From the results shown on table 3.5, it can be concluded that for the five different metallic ions that were tested using five different organs (colon, liver, spleen, kidney and heart), none of them was considered to be as good as Z7 in terms of morphological preservation and RNA quality.

These results showed that replacing zinc with other metallic ions such as gallium, magnesium, manganese, vanadium and calcium did not provide adequate morphological preservation when assessed by H&E. Also, results from the Agilent 2100 Bioanalyser showed that the quality of extracted RNA from manganese-based solution was inferior to the RNA extracted from the zinc-based fixative.

3.6 The effect of various zinc concentrations and zinc combinations on the morphological quality of mouse tissue.

For this part of the project we evaluated the effect of a series of different zinc salt concentrations on tissue morphology using five different tissues. These were: liver, spleen, colon, kidney and heart. Colon was used for its variety of tissue type including epithelial, endothelial, nervous and muscular. Spleen was used as a type of lymphoid tissue and liver as a solid parenchymal tissue. Kidney was used as an organ that contains tubules, glomeruli and large vessels. Heart was used as a type of tissue that contains smooth muscle. The different concentrations used are summarised in Table 3.6. The aim of this experimental work was to identify whether an increase, decrease or change between the zinc salts has a significant effect on tissue architecture.

Table 3.6: Assessment of different zinc concentration and zinc salts on tissue morphology.

Zinc salt + mass (in 500 ml solution)*	Mean Score of 5 different organs tested	p value (95% & 99% significance level)
0.25 g Zinc trifluoroacetate	5.2	p<0.01
0.5 g Zinc trifluoroacetate	6.1	p<0.01
1g Zinc trifluoroacetate	6.3	p<0.01
4g Zinc trifluoroacetate	8.2	p<0.05
6g Zinc trifluoroacetate	8.1	p<0.05
7g Zinc trifluoroacetate	8.0	p<0.05
1.25g Zinc Trifluoroacetate + 1.25gr Zinc Acetate	7.0	p<0.01
2g Zinc trifluoroacetate + 1gr Zinc acetate	7.1	p<0.01
3g Zinc trifluoroacetate + 1gr Zinc acetate	7.4	p<0.01
2.5g Zinc isovalerate	5.1	p<0.01
2.5g Zinc perchlorate	6.2	p<0.01
2.5g Zinc trifluoromethylsulfonate	6.3	p<0.01
Standard Zinc (Z2), 2.5g zinc acetate	7.6	p<0.01
Z7, 2.5g zinc trifluoroacetate	8.5	p>0.05
70% ethanol	7.5	p<0.05
NBF	9.2	Control

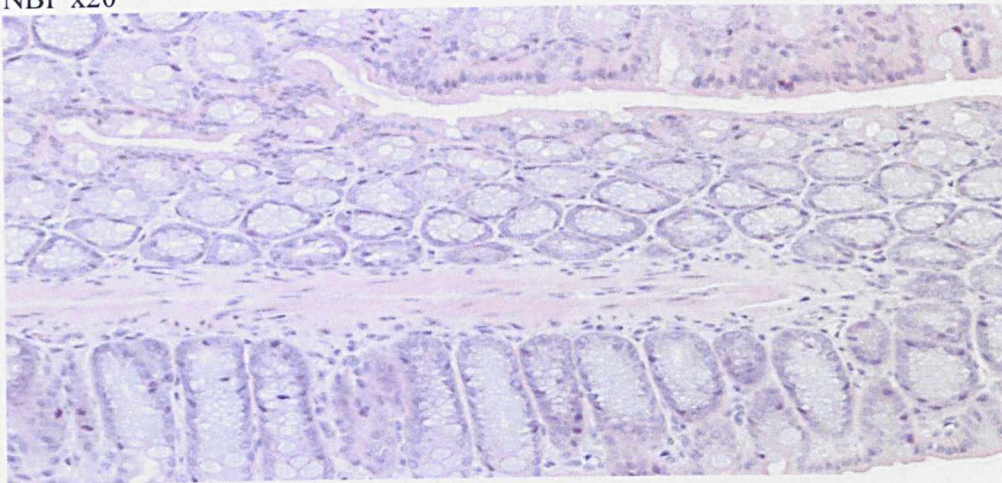
*In all the recipes shown in table 3.6, the following were added: 2.5 g zinc chloride and 0.25 g calcium acetate, in 500 ml 0.1M Tris-HCl buffer, pH 6.4-6.7.

All the results presented are from experiments that were done in triplicate. Although the quantification of the quality of fixed tissue is subjective at best, there is no other method to achieve an assessment. For that reason we used the experience of 3 histopathologists and for the scoring we included parameters such as those mentioned in Chapter 2 (Section 2.16, Materials and Methods). 70% ethanol was also used as fixative in this experimental work in order to compare it with all the zinc-based solutions tested. These investigations were done using healthy mouse tissue, which included liver, spleen, colon, kidney and heart. From all the above different modifications and zinc salts we have selected a number of H&E samples which are shown in figure 3.9 below. All the samples were compared against NBF and Z7. From the results shown it can be seen that overall Z7 was the best recipe for tissue morphology preservation compared to all the other zinc salts tested and the different zinc salt concentrations. Nevertheless, overall, NBF achieved the highest scores.

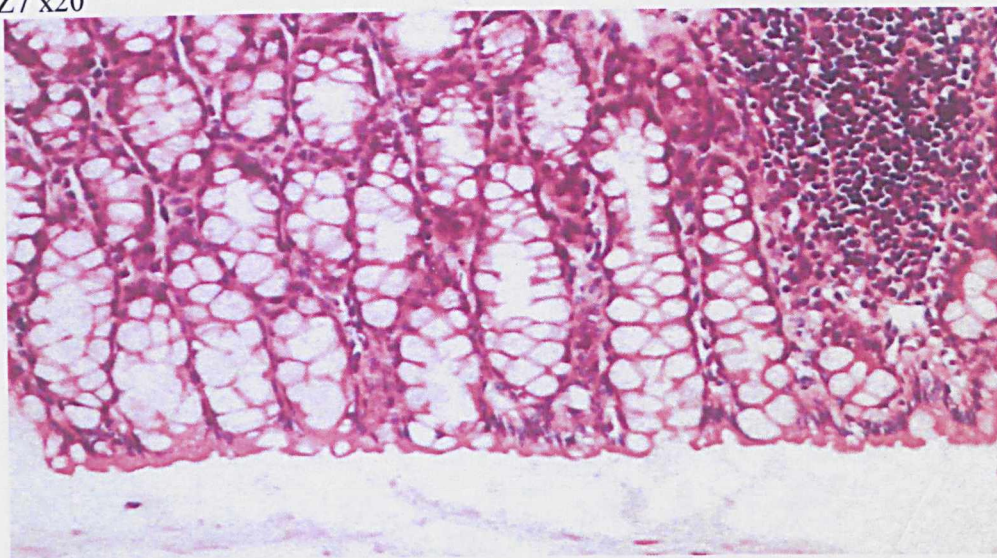
The effect of heat during Z7 fixation was also tested. Mouse liver, spleen and colon were immersed in Z7 and then placed in a waterbath at 37°C, 45°C and 55°C. Samples were also fixed in Z7 at 4°C. All samples were fixed for 24 hours. From the H&E results it was shown that there was no significant difference between room temperature (20-22°C) Z7-fixed tissues and the different temperature Z7-fixed samples. Raising the temperature above 55°C for Z7 fixation was considered but was abandoned at this stage, since it is known that raising the temperature above during fixation and processing has negative effects on the RNA quality of fixed tissues (Bancroft & Gamble 2002).

In figure 3.9 below we present a number of H&E figures using some of the modifications done, with Z7 and NBF as reference.

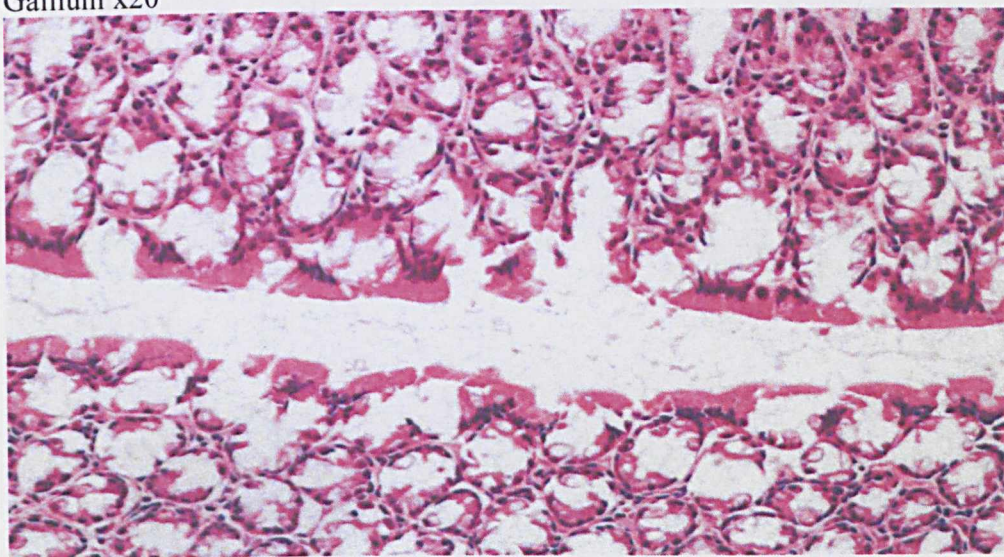
NBF x20



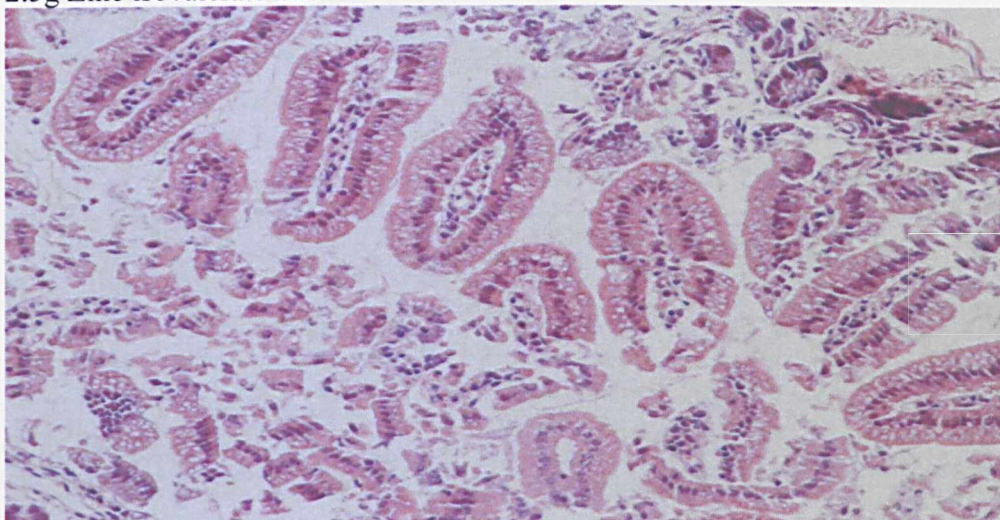
Z7 x20



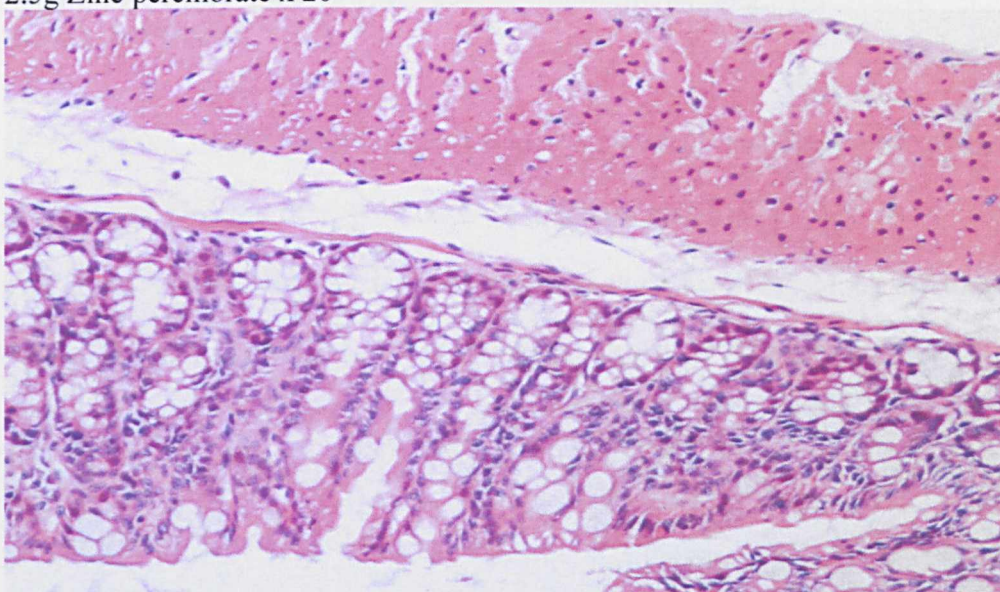
Gallium x20



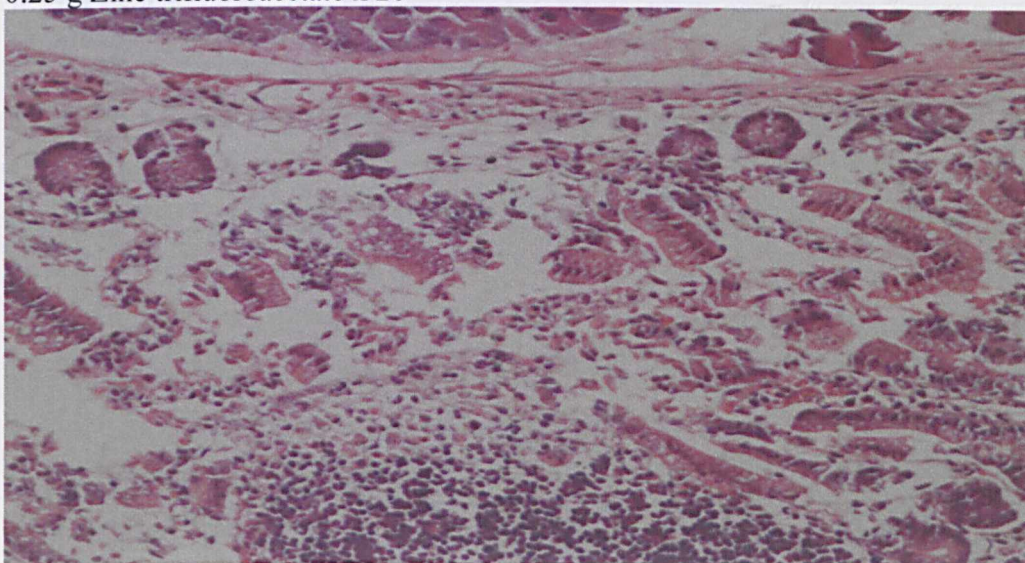
2.5g Zinc isovalerate x20



2.5g Zinc perchlorate x 20



0.25 g Zinc trifluoroacetate x 20



7g Zinc trifluoroacetate x 20

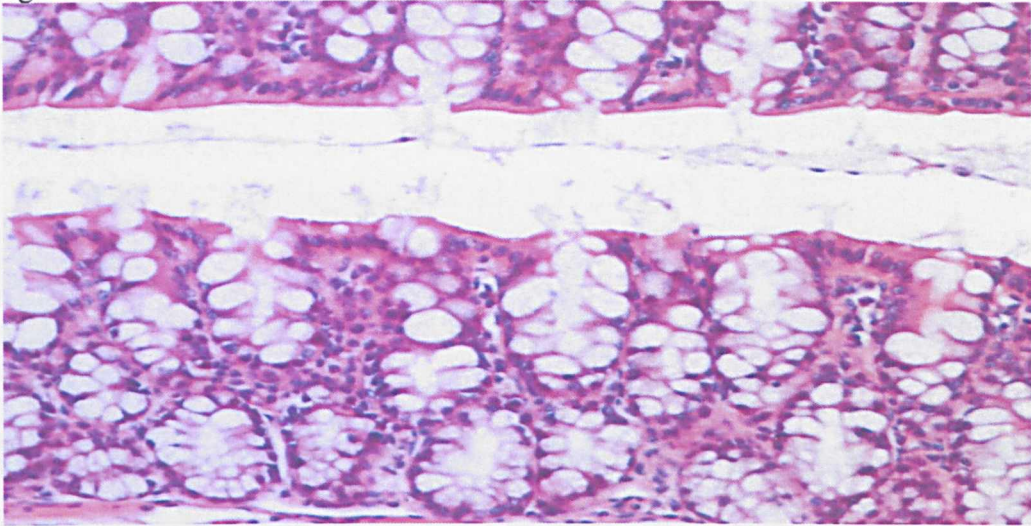


Figure 3.9: Representative H&E images from different modified fixative solutions using colon tissues.

In conclusion, morphological integrity was considered inadequate for diagnostic purposes in the case of gallium, manganese and magnesium fixed tissues. Some zinc modifications such as zinc perchlorate showed barely adequate morphology. Again the best fixatives were Z7 and the commonly used NBF (Figure 3.9).

CHAPTER 4

MOLECULAR STUDIES USING THE NOVEL ZINC-BASED FIXATIVE Z7

4.1 Introduction

In the second phase of the project we evaluated the Z7 fixative for its ability to preserve high quality DNA, RNA and protein. For that reason we employed molecular techniques such as real-time PCR and real-time RT-PCR, 2-D PAGE. Using the above techniques we also tested whether archival fixed samples would be useful for molecular studies compared to fixed and fresh-frozen samples. The type and length of fixation determine the degree of preservation of intact nucleic acids in tissue (Ezra et al. 1991; Foss et al. 1994; Goldsworthy et al. 1999).

4.2 Aims & Experimental Design:

Three types of tissue from C57BL/6 male/female mice 8 - 10 weeks of age were used in this study. Colon was used for its variety of tissue types including epithelial, endothelial, nervous and muscular tissues and its susceptibility to putrefaction and autolysis. Spleen was used as a solid reticuloendothelial organ and liver as a solid parenchymal organ. This selection provided a range of tissue density and structure to assess physical properties such as penetration of fixatives in different tissue types. For every experiment, 3 mice were used and experiments from each mouse were repeated once.

Assessment of data

Tissues used during this study were assessed for their integrity of their proteins and nucleic acids. The PCR and RT-PCR products were scored for their intensity in the agarose gel as a means of quantifying amount of product. An RIN score was also given for the RNA Bioanalyser graphs compared to the fresh-frozen control samples. For all the data produced scores were given in the following manner:

1-3 = poor, 4-6 = adequate, 7-9 = good and 10 = excellent.

Statistical analysis of results comprised the Student's t-test which was carried out between pairs of fixatives for each of the three organs (liver, spleen and colon). The 2-tailed paired Student's t-test (assuming equal and unequal variances) was used for comparison of the mean CT values between fixatives for each tissue. For each of the tests a level of $p = 0.05$ was taken to show significant difference and $p = 0.01$ to show highly significant difference.

4.3 Results

4.3.1 Assessment of DNA, RNA and protein quality following tissue fixation with Z7

RNA was extracted from mouse liver sections fixed in Z2, Z7 and NBF and from fresh-frozen (FF) samples and was assessed for integrity using the Agilent 2100 Bioanalyser (see Figure 4.1). Typical traces that can be obtained from RNA in various degrees of degradation are shown for purposes of comparison. It can be seen that Z7 has two peaks corresponding to 18S and 28S RNA and that it is significantly better than both Z2 and NBF at preserving RNA structure and integrity.

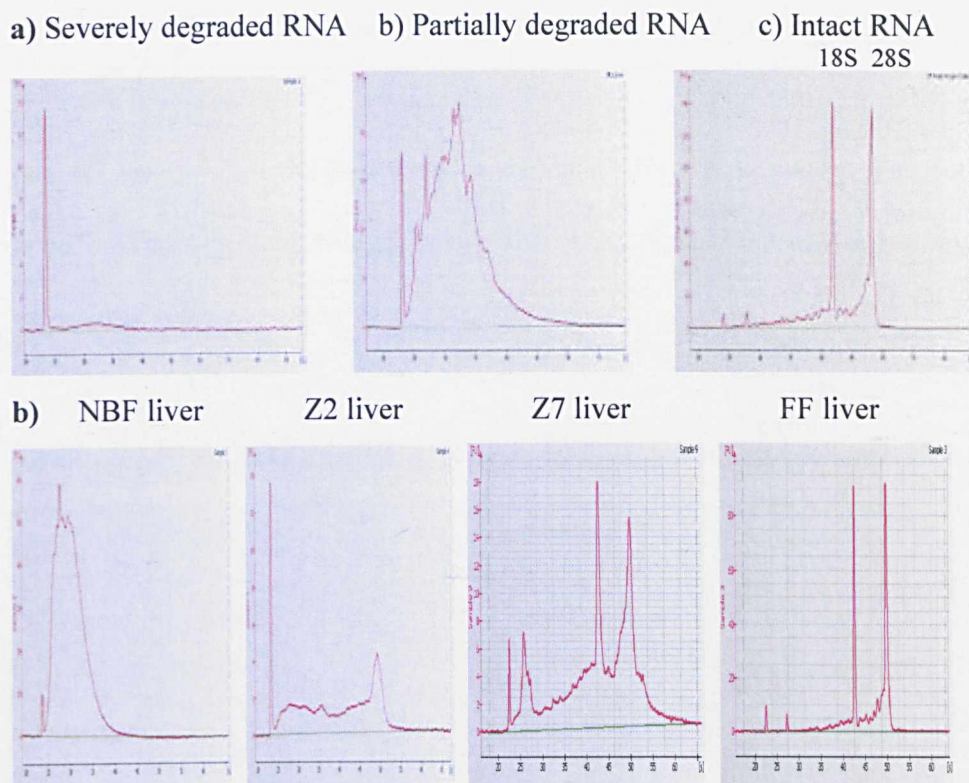


Figure 4.1: Assessment of mouse liver RNA quality using the Agilent 2100 Bioanalyser.

a) Typical graphs showing severely degraded, partially degraded and intact RNA.

b) Representative graphs showing RNA quality from tissues fixed in: NBF, Z2, Z7 and also FF mouse liver.

The RIN mean values of the 3 organs tested were:

Table 4.1: Mean RIN values of RNA extracted from fixed and FF tissues.

Fixative name	RIN mean values of 3 organs tested
NBF	2.6
Z2	3.6
Z7	5.9
FF	9.1

These mean values were also compared for statistical differences between the different fixatives and FF samples. The results from all the samples tested showed that Z7 was overall the best fixative candidate for RNA quality. The following table summarises these findings using the results from Student's T-test and the p values that were generated from each comparison.

Table 4.2: Statistical comparison of mean RIN values of three different tissues.

RIN value	<i>p-value</i>
Z2 vs NBF	$p < 0.01$
Z7 vs NBF	$p < 0.01$
FF vs NBF	$p < 0.01$
Z7 vs Z2	$p < 0.05$
FF vs Z7	$p < 0.05$
FF vs Z2	$p < 0.01$

DNA was extracted from Z7-fixed, Z2-fixed, NBF-fixed and fresh frozen mouse tissues and was assessed for the maximum size of fragment that could be amplified by Real-Time PCR using GAPDH primers which yield a product of 599 base pairs (bp). Figure 4.2 shows results obtained from comparing the mean CT values of liver, spleen and colon tissues depending on fixation process and gene sequence amplified.

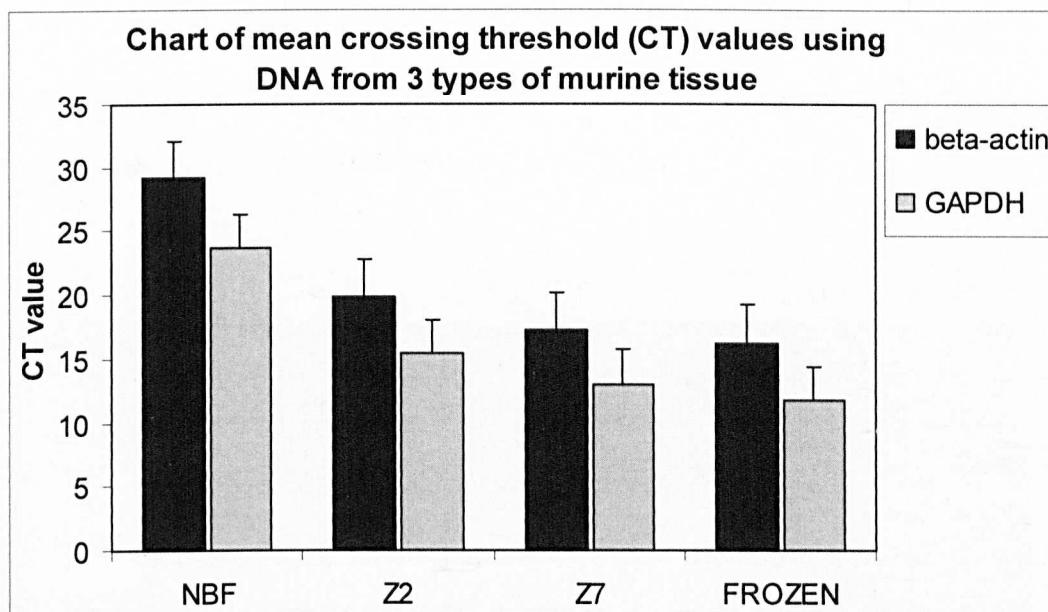


Figure 4.2: Representative Real-Time PCR figure, summarising the results from mouse liver, spleen and colon tissue fixed in NBF, Z2, Z7 and FF tissue as a control sample for DNA quality. Bars indicate the standard error of the mean ($\pm$ SEM).

Fragments up to 2.4 (kb) in length could be amplified from the Trefoil Factor 2 gene following Z7 fixation but could not be obtained from amplification of DNA fixed in NBF. These results are shown below in figure 4.3.

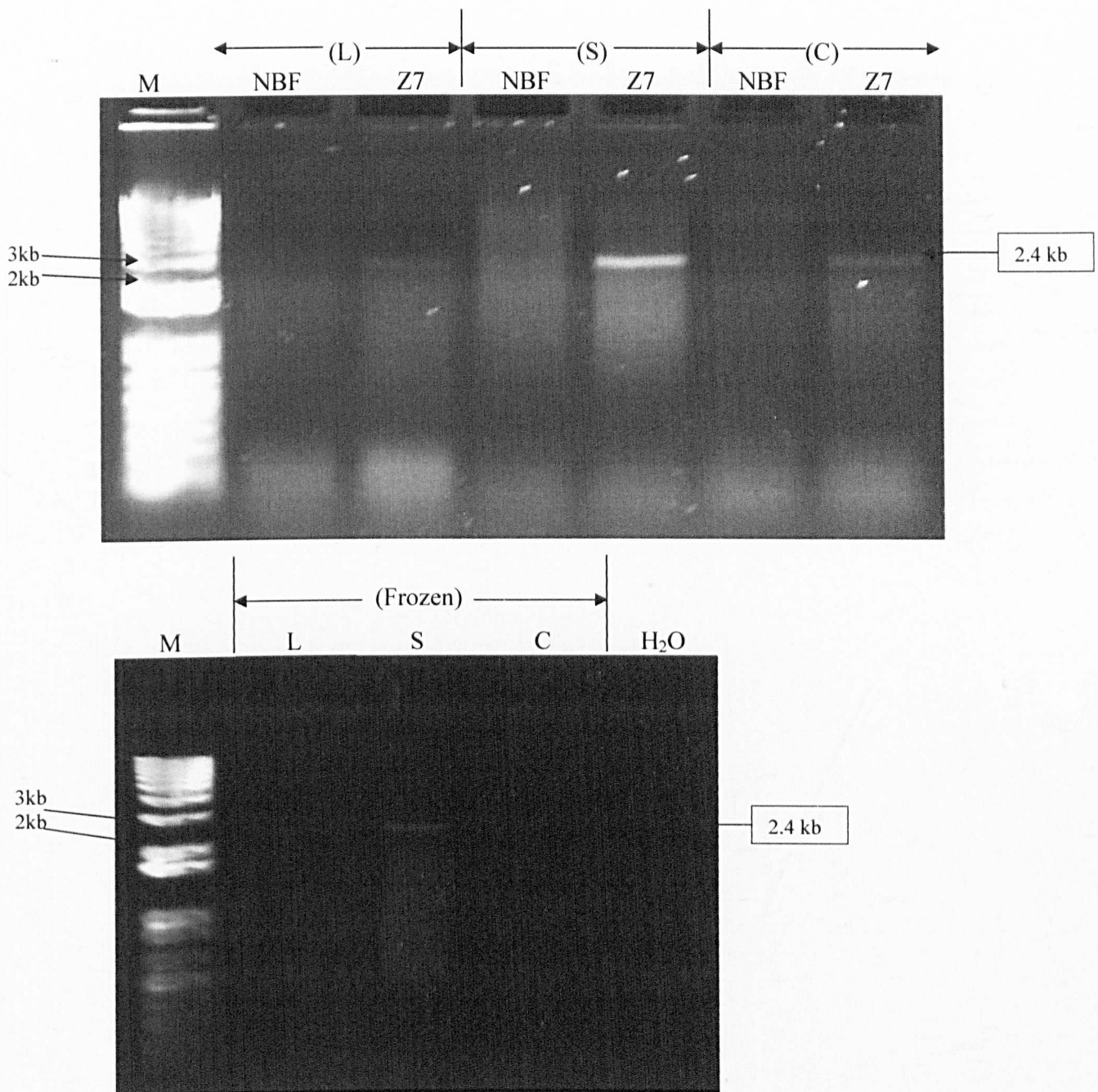


Figure 4.3: PCR amplification of a 2.4 kb Trefoil Factor 2 gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF and Z7. FF samples (Frozen) tissues were used as positive control and a water only sample (H₂O) as negative control.

Figure 4.4 below shows the results from comparing the mean CT values from real-time RT-PCR using S15 primers, which yield a product of 361 bp, on mouse liver, spleen and colon tissue fixed in NBF and Z7 with FF tissue as a control for RNA quality. After reverse transcription the samples were amplified by conventional PCR using commercial β -actin primers and the results from figure 4.5 showed that there was no contamination with genomic DNA.

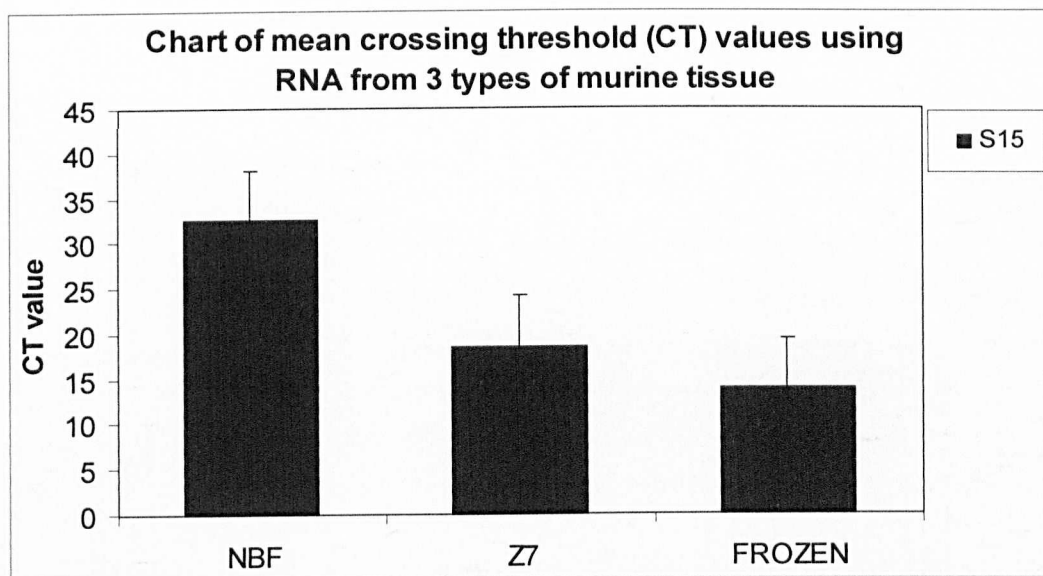


Figure 4.4: Representative Real-time RT-PCR figure, summarising the results from the pooled mean of mouse liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 with FF (Frozen) tissue as positive control and a no-RT negative control sample. Bars indicate the $\pm$ SEM.

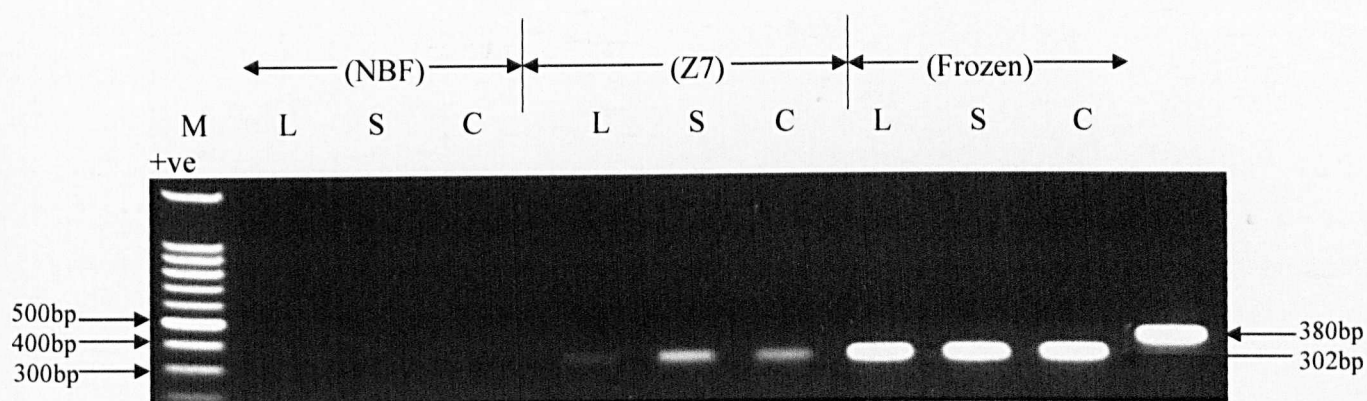


Figure 4.5: Genomic DNA contamination control using RT-PCR with commercial β -actin primers.

Proteins were extracted from liver fixed with Z7 and NBF and subjected to 2-D PAGE. Fresh frozen liver tissue was used as positive control. Results are shown in Figure 4.6.

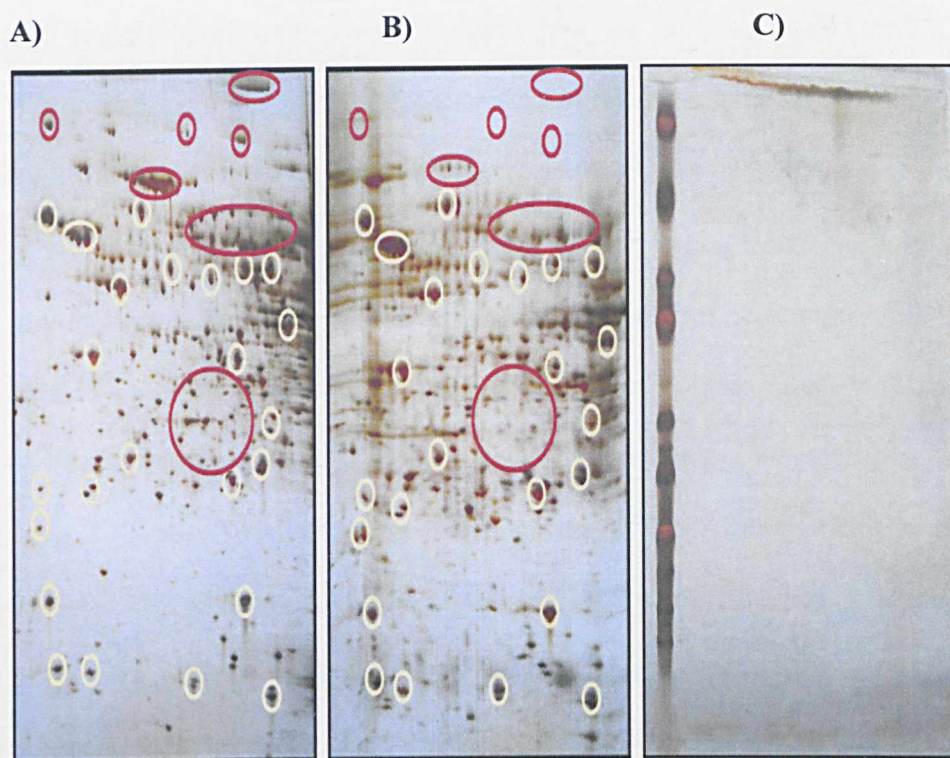


Figure 4.6: Proteomic comparison between fresh frozen and zinc-fixed liver tissue by 2-D PAGE. A) FF control, B) Z7-fixed sample and C) NBF-fixed sample. Three representative 2-D PAGE images are shown. Yellow and red circles were assigned to indicate similarities and differences respectively in stained protein spots between the two samples.

The results from figure 4.6 showed that the NBF-fixed sample was overall the worse fixative used for 2-D PAGE electrophoresis. On the other hand, the Z7-fixed samples showed a comparable spot pattern to the FF samples. This result could be very encouraging in future proteomic studies using fixed materials, since NBF-fixed and paraffin embedded samples are not suitable at all for use with this technique.

However, there were some differences in separation of proteins extracted from frozen and Z7-fixed tissues were noted. First, fewer protein spots were detected from Z7-fixed tissue. By comparing silver stained gels, we detected an average of 700 protein spots from ten 10µm frozen tissue sections, whereas approximately 600 protein spots were detected when extracted from an equal-sized, Z7-fixed, tissue sections. This differential detection of proteins is attributed to the amount of total protein extracted from the tissue preparations. For example, 1–1.8 µg/mm² of total proteins is typically extracted from a fresh-frozen tissue section; whereas, approximately 25% less total protein (0.75–1.4 µg/mm², respectively) is extracted from Z7-fixed tissue sections. In order to optimize protein extraction, we evaluated several technical modifications including: (1) increasing time of protein extraction from 5 min to 2 h; (2) heating of samples at 70°C for 2 h;(3) addition of 0.25% SDS to the protein extraction buffer; or (4) lysing of sample in the protein extraction buffer (excluding urea and thiourea), boiling, then adding both components to the cooled sample. However, these treatments either did not result in increasing protein yield or had deleterious effects on protein separation.

Another difference of the 2-D gels of fresh-frozen versus Z7-fixed tissues was separation quality. For example, protein spots in the 2-D gels of frozen tissues were sharp, in contrast to those from Z7-fixed tissues, which appeared slightly fuzzy. The presence of artefacts in the form of vertical smears was also more prominent in 2-D gels of Z7-fixed tissues than of frozen tissues

Real-Time PCR analysis for DNA and RNA quality

For DNA and RNA quality evaluation, a 2-tailed paired Student's t-test (assuming equal and unequal variances) was carried out for comparison of the mean CT values between fixatives, after real-time PCR of mouse liver, spleen, colon and the results are shown in Table 4.3.

Table 4.3: Statistical comparison of CT values from real-time RT-PCR of three different tissues (for both DNA and RNA quality).

DNA	<i>p-value</i>
Z2 vs NBF	<i>p < 0.01</i>
Z7 vs NBF	<i>p < 0.01</i>
FF vs NBF	<i>p < 0.01</i>
Z7 vs Z2	<i>p < 0.05</i>
FF vs Z7	<i>p < 0.05</i>
FF vs Z2	<i>p < 0.05</i>
RNA	
Z7 vs NBF	<i>p < 0.01</i>
FF vs NBF	<i>p < 0.01</i>
FF vs Z7	<i>p < 0.05</i>

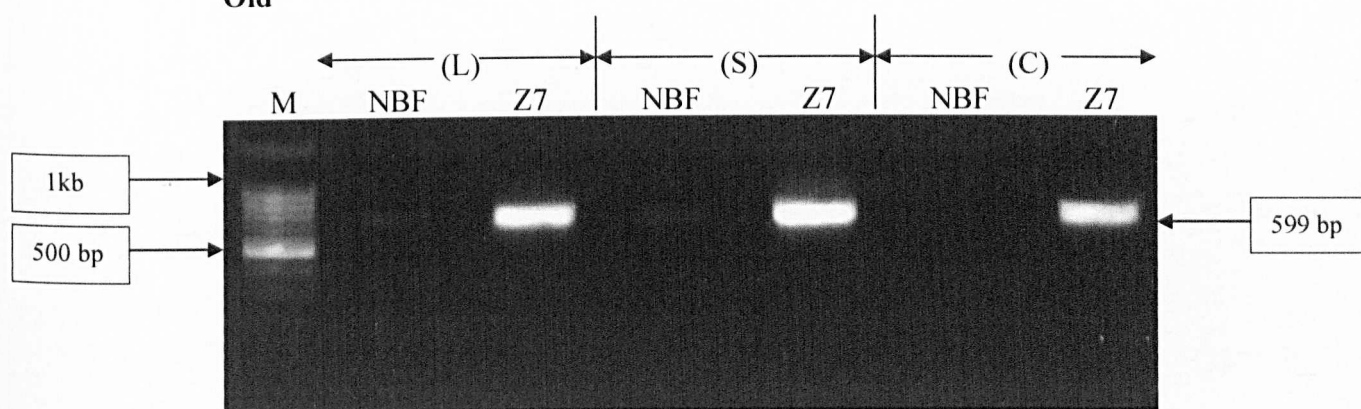
From the above data in Table 4.3 it can be seen that Z7 was very significant ($p < 0.01$) for DNA quality compared to NBF, Z2 and very significant for RNA quality compared to NBF. When Z7 was compared with FF, there was significant difference ($p < 0.05$) for RNA quality, but again Z7 was overall the best fixative.

4.3.2 Comparison of quality of archival DNA extracted from Z7- and NBF-fixed paraffin blocks

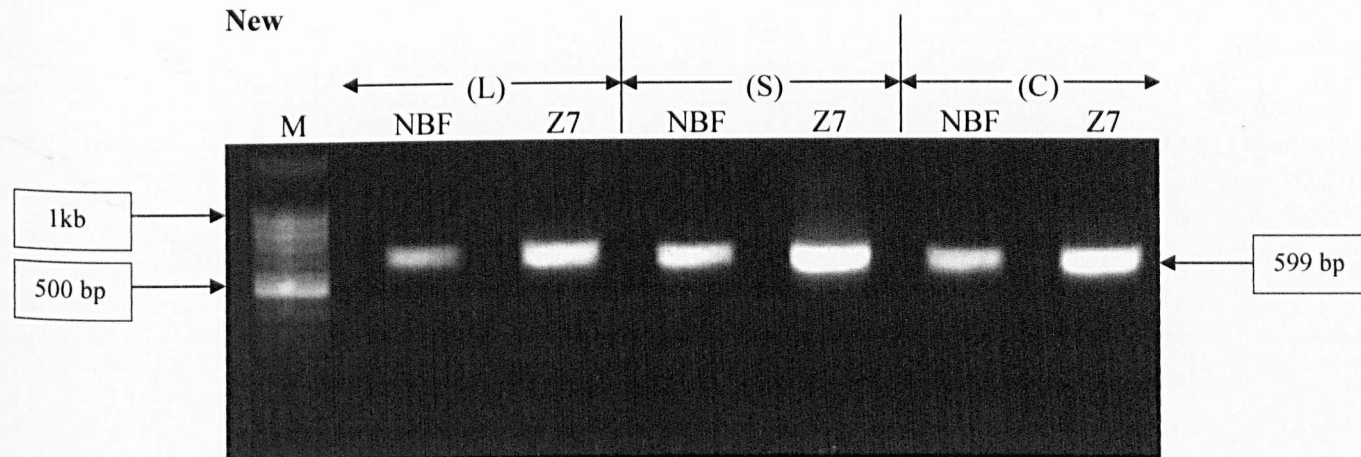
DNA was extracted from paraffin blocks fixed with NBF and Z7 stored at ambient temperature for 14 months and compared with DNA samples from paraffin blocks stored for less than a week. Conventional PCR results using two housekeeping genes, GAPDH and β -actin, showed that DNA quality from Z7-fixed archival and newly stored tissues was significantly better than from all NBF fixed tissue (see Figures 4.7 and 4.8). No significant difference was observed between archival stored Z7-fixed and newly stored Z7-fixed samples.

PCR (GAPDH)

Old



New



Frozen

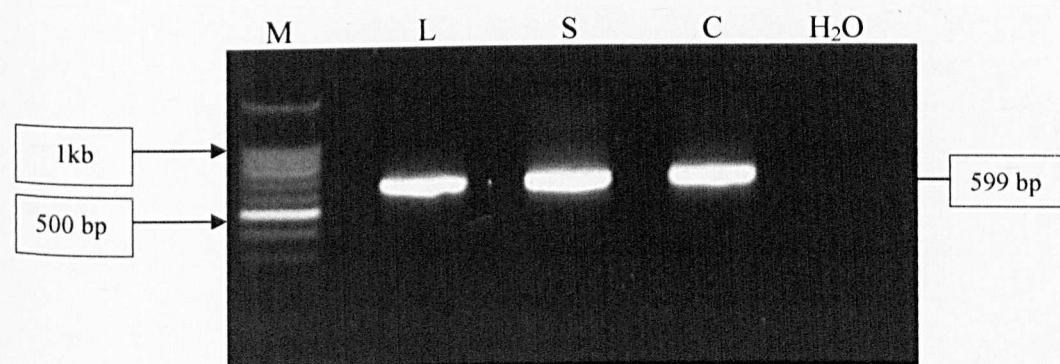


Figure 4.7: PCR of the GAPDH gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for 14 months (Old), compared with DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H₂O) as negative control.

Results from figure 5.7 showed that Z7 was overall better than NBF in all the organs tested and in both new and old samples.

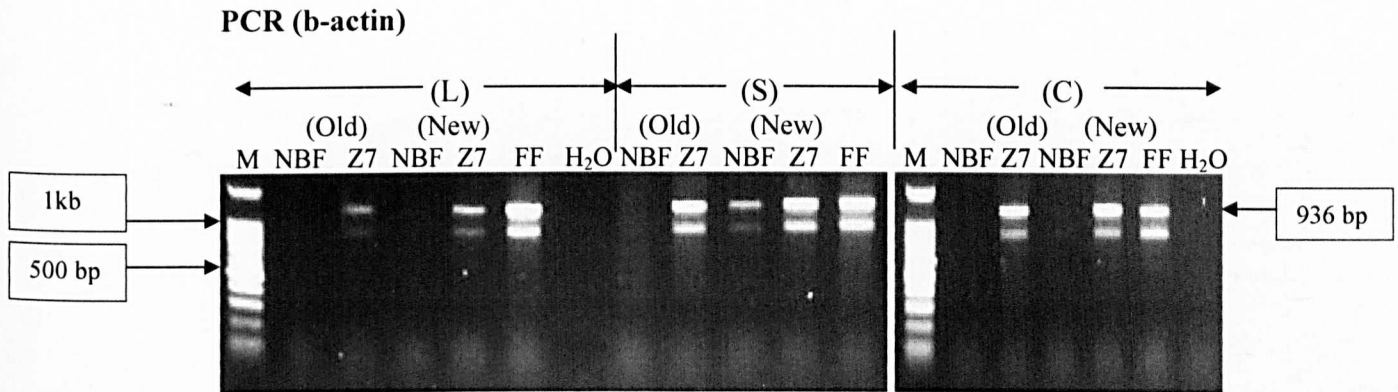


Figure 4.8: PCR amplification of the β -actin gene using DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and paraffin blocks stored at RT for 14 months (Old), compared with DNA extracted from liver (L), spleen (S) and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). Fresh-frozen samples (Frozen) tissues were used as positive control and a water only sample (H₂O) as negative control.

Again, results from figure 5.8 showed that Z7 was overall better than NBF in all the organs tested and in both new and old samples.

Real-time PCR

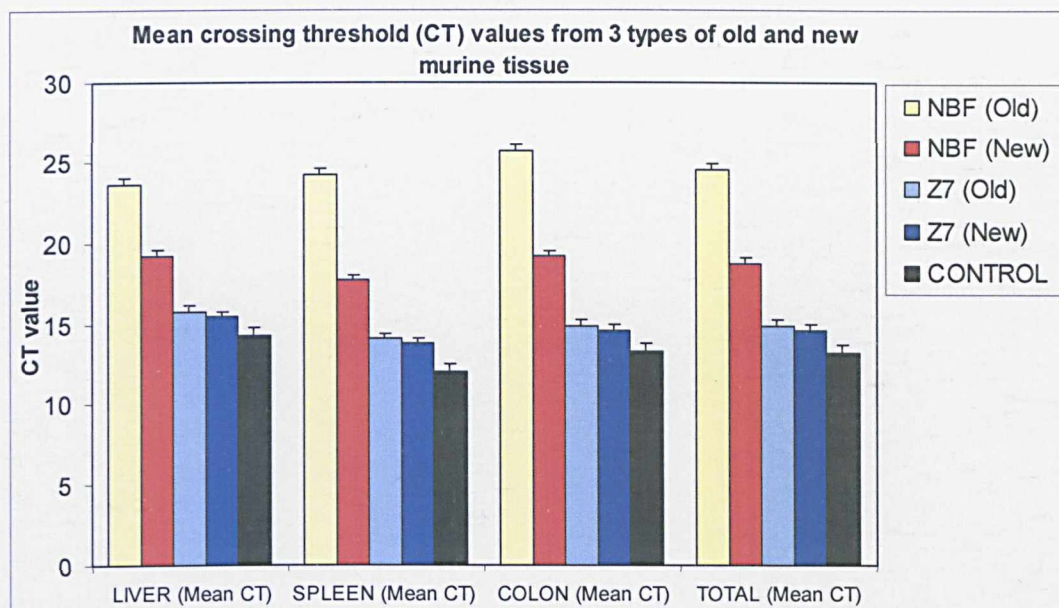


Figure 4.9: Real-Time PCR showing amplification of the GAPDH gene using DNA extracted from liver, spleen and colon tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for 14 months (Old), compared with DNA extracted from liver, spleen and colon (C) tissue fixed in NBF, Z7 and stored in paraffin blocks at RT for less than a week (New). FF samples (CONTROL) from each tissue were used as positive control. Bars indicate the $\pm$ SEM.

Table 4.4 below, shows the results from statistical analysis of mean CT values from real-time PCR of mouse liver, spleen and colon tissue fixed in NBF and Z7 when paraffin blocks were stored for 14 months and for less than a week, with fresh-frozen tissue as a control sample for DNA quality. There was no significant difference between the quality of DNA in Z7 fixed material between archived stored paraffin blocks and blocks stored for less than two weeks ($p = 0.497$). No significant difference in DNA quality was shown between the Z7-fixed and FF samples ($p = 0.1608$).

Table 4.4: Statistical comparison of mean CT values from real-time PCR of tissues fixed with three different fixatives and stored for 14 months (Old) or less than a week (New).

	Z7 vs NBF	FF vs NBF	FF vs Z7
Old vs Old	p < 0.01	p < 0.01	p > 0.05
New vs New	p < 0.01	p < 0.01	p > 0.05
	NBF vs NBF	Z7 vs Z7	
Old vs New	p < 0.01	p > 0.05	

From the data in Table 4.4, it can be seen that there was no significant difference between Z7-fixed (old or new) and FF samples from all the tissues tested. On the other hand there was very significant difference between NBF and Z7-fixed samples (both new and old) in all the tissues tested.

CHAPTER 5

EXON MICROARRAY USING Z7-FIXED

SAMPLES

5.1 Introduction

After showing that our novel zinc-based fixative enabled us to perform successfully various molecular biological techniques such as RT-PCR, Real-Time PCR, Proteomic analysis and archival studies, we wanted to test the hypothesis of whether zinc-fixed paraffin-embedded tissues were suitable for whole genome analysis. This was done mainly for two reasons. The first was to evaluate the whole genome RNA quality of Z7-fixed samples against fresh frozen ones. The second was to compare the exon and gene alternative splicing patterns between fixed and fresh frozen samples and test if there are significant differences caused by the mode of the tissue preservation. In other words we wanted to see whether Z7 fixed samples would be applicable for future large-scale human cancer studies using the exon array platform. NBF-fixed samples were not used for this part of the results.

5.2 Aims & Experimental Design

We therefore decided to compare the RNA quality of Z7-fixed tissue compared to fresh-frozen, using the newly developed exon-microarray platform from Affymetrix. By doing so, we attempted to evaluate the gene level and also the exon level quality of zinc-fixed samples against the ‘control’ frozen samples. It is known that formalin-fixed samples are not particularly useful for whole genome studies using methodologies such as microarray analysis (Paik et al. 2005; Reis-Filho, Westbury, & Pierga 2006).

5.3 Quality Assessment Results

In order to establish quality control processes for gene expression analyses, Affymetrix has developed several controls. Researchers are encouraged to monitor these controls on a regular basis to assess assay data quality. Many of these control metrics are generated as a product of the primary analysis by the respective analysis algorithms. To help with monitoring these values, a collection of tools for viewing and graphing metrics associated with the array and the analysis are provided in the Affymetrix® Expression Console™ software. These metrics include but are not limited to:

- Hybridization controls
- Labelling controls
- Internal control genes (Housekeeping controls)
- Global array metrics
- Algorithm parameters

Evaluation of particular samples should be based on the examination of all sample and array performance metrics. A good, general rule for the examination of these types of quality control data is to look for outliers when compared to other highly related samples. For example, tissue A may have an overall low level of gene expression so that the percent of probes detected may normally be between 10–15%. Therefore, a %Present = 11 would not be an indication of a problem. However, if tissue B normally has a %Present between 45–50, and one sample has %Present = 11, that is an indication of a problem. Examination of which metrics or controls are outliers will provide insight into the source of the problem and possible solutions.

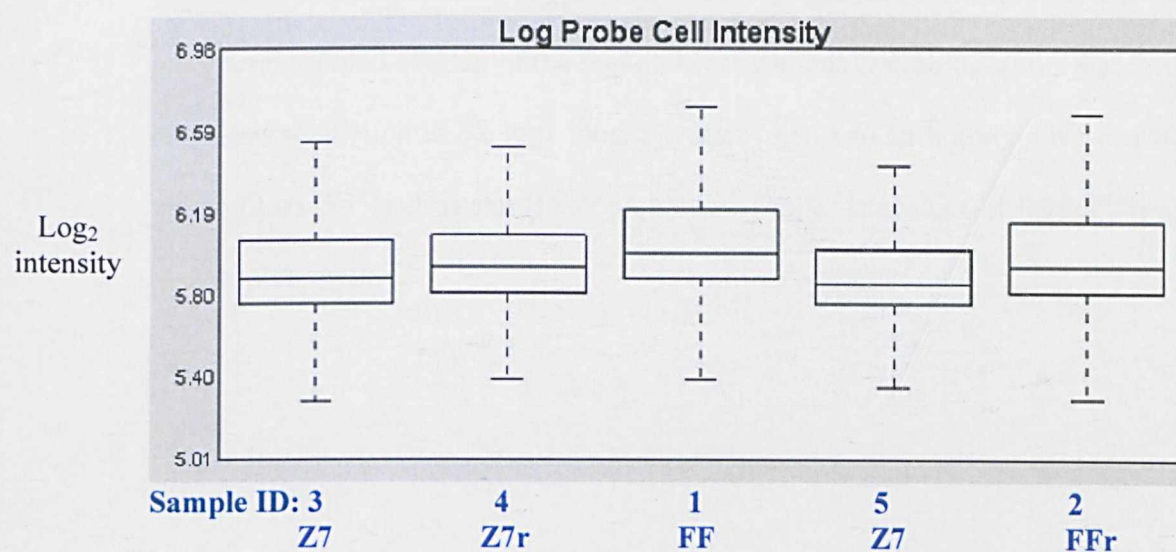
The quality assessment metrics described in this chapter are based on CEL intensity data from GeneChip® Affymetrix Exon Arrays generated in the CSC/IC Microarray Centre using the Z7-fixed and fresh-frozen samples. These procedures do not aim to provide guidelines to assess the overall quality of arrays but to aid the investigator in identifying outlier arrays within the set processed that can then be flagged and adjusted or excluded from further analysis. It is for this reason that specific thresholds are not provided as these need to be developed in the context of particular applications with specific types of samples.

Table 5.1: Correlation of hybridisation files to the sample name.

Hybridisation	Sample Name
CCT2007010401Aaa	Z7 Fixed (3)
CCT2007010402Aaa	Z7 Fixed (4)
CCT2007010403Aaa	Fresh Frozen (1)
CCT2007010404Aaa	Z7 Fixed (5)
CCT2007010405Aaa	Fresh Frozen (2)

Table 5.1 above provides the actual array codes used in this experimental work and are correlated (to the right) with the actual sample that was used in each array. Exploratory visualisation is essential in detecting quality problems. The diagnostic plots described below are in aid of this purpose and were generated using the Affymetrix® Expression Console™ software.

(A)



(B)

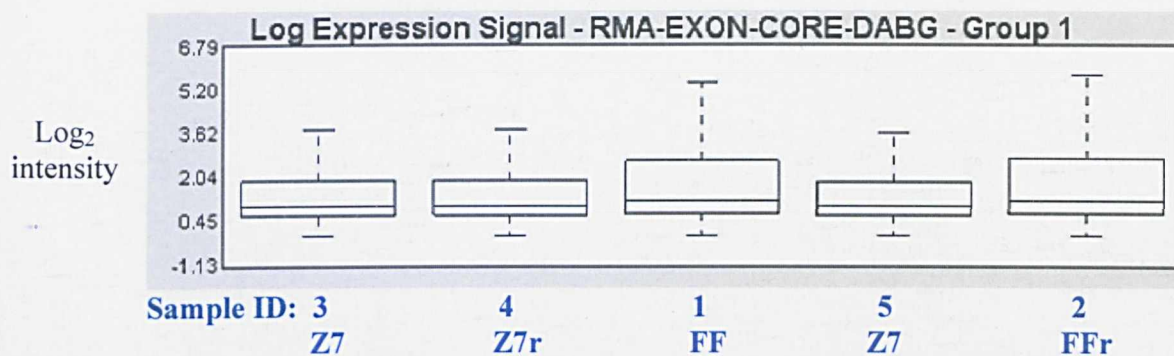


Figure 5.1: Box plots of the raw log₂ probe cell intensities for each array before normalisation (A) and after RMA normalisation (signal) at the Exon level using the core annotation (B).

In figure 5.1, the top and bottom sides of the box indicate the 75th and 25th percentiles of the log₂ intensity distribution while the line within is the median. In general the intensity distributions should be similar across arrays. However, this does not mean that an array with an inconsistent distribution should be removed from analysis as normalisation can bring back the aberrant array into line with the other arrays. Good overlap of the box plots after normalisation indicates that there is not large variation in the log₂ intensity values between each array. We can see that in figure 5.1, both A and B box plots show similar intensity distribution for all the samples tested.

RLE is the log₂ scale estimate of expression on each array relative to the median value across arrays on a probeset by probeset basis. Each box represents 1 array. Ideally these boxes would have small spread and be centred at 0 (indicated by the red line). An array that has quality problems may result in a box that has greater spread or is not centred near 0. Overall it represents a good way to identify arrays with divergent signal distributions relative to the other arrays in the study. From the results shown in figure 5.3 it can be said that all three Z7-fixed samples show similar signal distribution and the same applies for the two fresh-frozen control samples.

Table 5.2 below summarises the important assessment metrics and quality control parameters associated with assay and hybridisation performance generated in the Affymetrix® Expression Console™ software. Also an explanation of what each value stands for is given in the text below.

Table 5.2: Quality assessment metrics.

CEL File	#P	%P	Pos vs. Neg AUC	All Probeset Mean	MAD Residual Mean	RLE Mean	Negative Control Mean	Positive Control Mean
Sample 3 (Z7)	20181	8.8	0.762	1.63	0.286	0.303	0.95	1.972
Sample 4 (Z7 repeat)	23758	10.4	0.776	1.65	0.292	0.304	0.96	1.999
Sample 1 (FF)	56898	24.9	0.877	2.03	0.285	0.538	0.88	3.052
Sample 5 (FF)	19292	8.5	0.754	1.61	0.314	0.327	0.97	1.925
Sample 2 (Z7)	58047	25.4	0.884	2.06	0.293	0.570	0.88	3.141

#P: The number of probe sets present – The higher this value, the more exon pairing is occurring in each sample tested. It is seen that the higher values here occur in the fresh-frozen samples. This could be therefore a useful indication that the Z7-fixed samples need optimisation in order to achieve similar exon pairing values.

%P: The percent of probe sets present – For the exon level analysis, this is defined as the Detection Above Background (DABG) probe level p-value is less than or equal to 0.01.

Signal (P): The average signal for the probe sets defined as present (Exon-level DABG ≤ 0.01). Similar results is seen here, were fresh-frozen samples have a higher number of probes present compared to the Z7-fixed samples.

Pos vs. Neg AUC: The area under the curve (AUC) for a receiver operator curve (ROC) comparing how well the probe set summary separates the positive controls from the negative controls. The assumption is that the negative controls are a measure of false positives and the positive controls are a measure of true positives. An AUC of 1 reflects perfect separation whereas as an AUC value of 0.5 would reflect no separation. Again here, fresh-frozen samples have values closer to the ideal value of 1 compared to the Z7-fixed samples.

All Probesets Mean: The mean signal value for all the probe sets.

MAD Residual Mean: The mean absolute deviation of the residual for a chip versus all chips in the data set.

All Probeset RLE Mean: The mean absolute RLE. This metric is generated by taking the probe set summary for a given chip and calculating the difference in log base 2 from the median value of that probeset over all the chips. The mean is then computed from the absolute RLE for all the probe sets for a given CEL file. Finally, again fresh-frozen samples gave a higher RLE value compared to the Z7-fixed samples.

Hybridisation Controls

A mixture of biotin-labelled cRNA transcripts of bioB, bioC, bioD (genes in the biotin synthesis pathway of E.coli) and cre (recombinase gene from P1 bacteriophage), is prepared in staggered concentrations (1.5 pM, 5 pM, 25 pM, and 100 pM final concentrations for bioB, bioC, bioD, and cre, respectively). These are spiked into the hybridization cocktail and are thus used to evaluate sample hybridisation efficiency. bioB is at the level of assay sensitivity. bioC, bioD, and cre should always generate increasing Signal values, reflecting their relative concentrations.

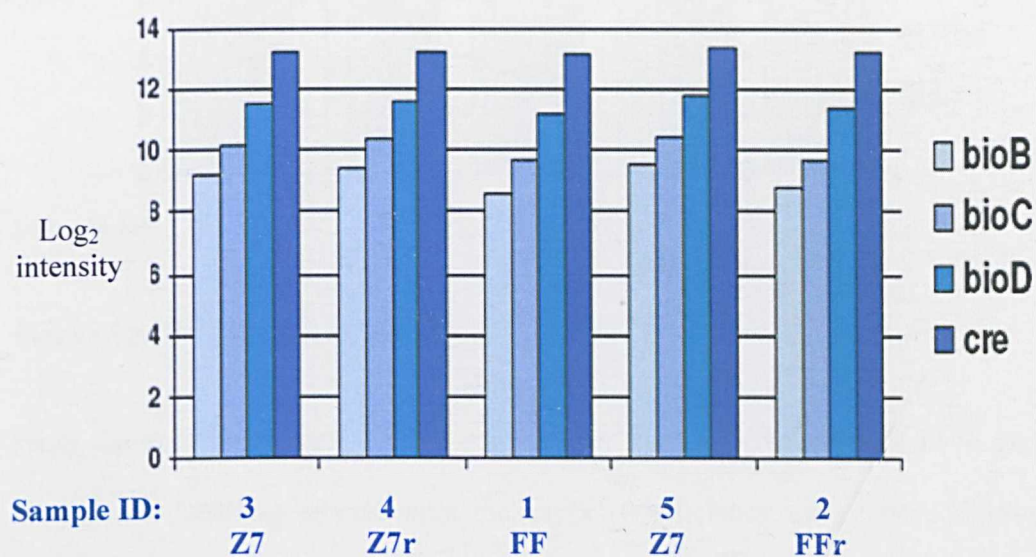


Figure 5.4: Hybridisation Controls.

From the results on figure 5.4 it can be seen that all five samples have hybridised with the expected efficiency under the conditions used.

Poly A+ Controls

A pre-synthesized mixture of specially modified B. subtilis genes as RNA controls (commercial acronyms used: lys, phe, dap, thr and trp) is spiked into each RNA sample in staggered concentrations. These are carried through the entire sample preparation process, and evaluated like internal control genes. The polyA+ controls

generate increasing signal values in the following order: Trp>Thr>Dap>Phe>Lys. They can be used to monitor the entire target labelling process. A bar graph is plotted to check for consistency of signal.

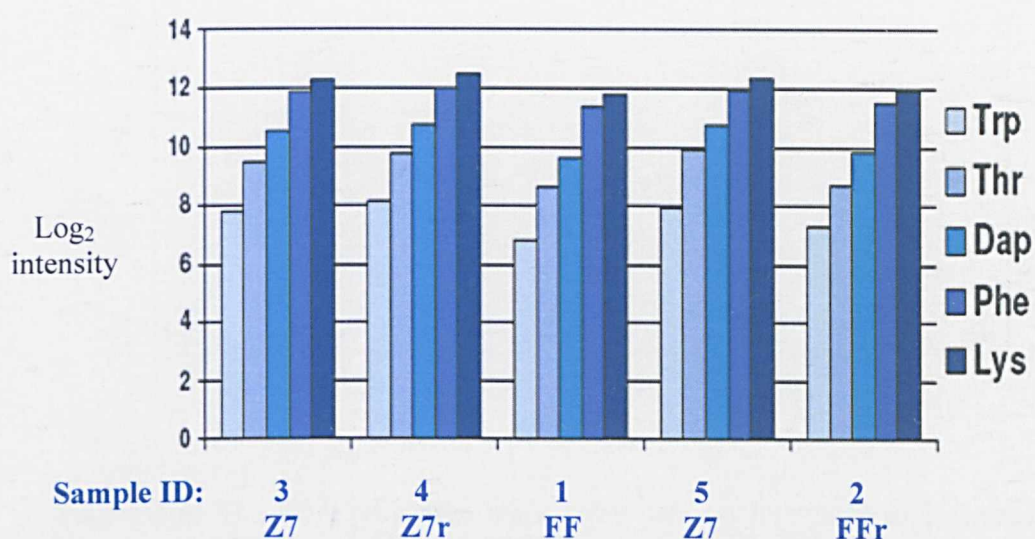


Figure 5.5: Poly A+ Controls.

From the results on figure 5.5 it can be seen that all five samples have gone through the labelling process with the expected efficiency under the conditions used.

The principal component analysis (PCA) was then used as an exploratory technique to describe the structure of high dimensional data (like microarray) data by reducing its dimensionality. In effect, all variation in the data is explained by a few variables (principal components) ordered by the amount of variance. Samples and/or experiments may be grouped due to the sources of variability that should ideally be due to biological factors rather than technical ones. However in the case of technical variability one can assess and account for it in the downstream analysis.

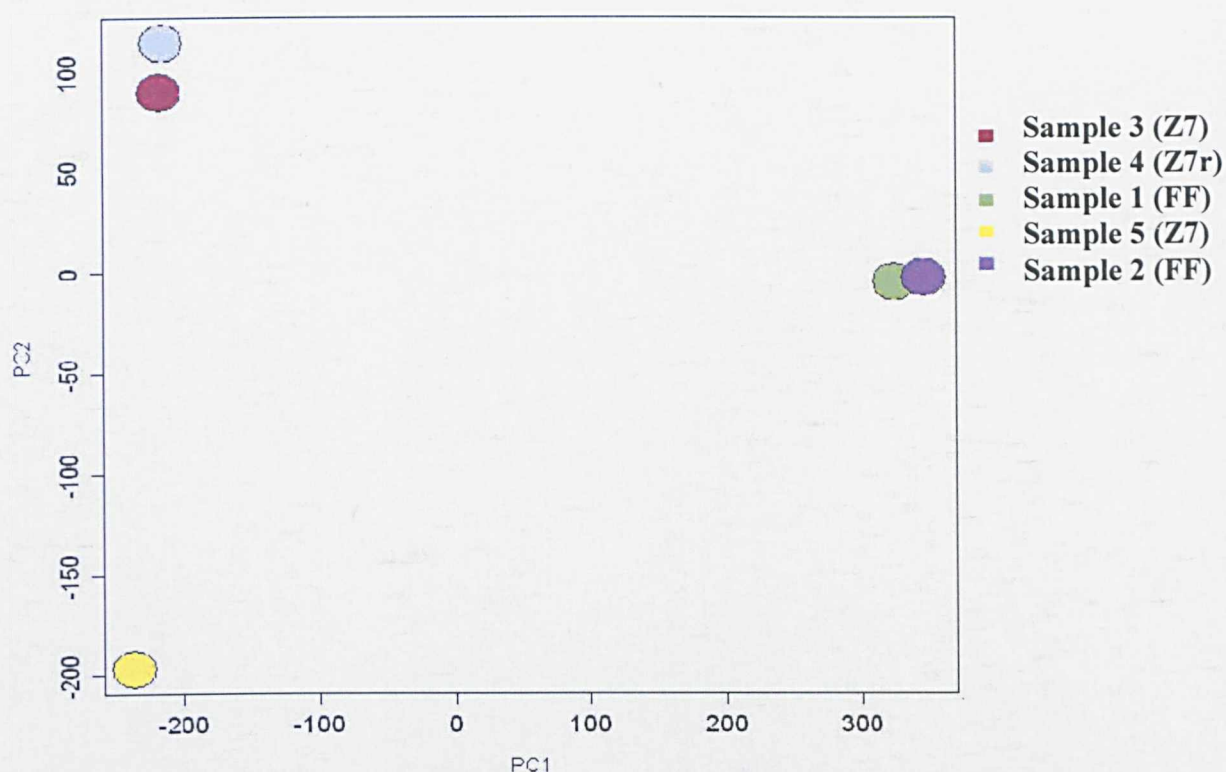


Figure 5.6: The PCA projection maps array data (in this case) to 2 dimensions. The x and y, axes are PC#1 and PC#2 respectively. The PCA plot here has been conducted on RMA normalised data using the Partek® analysis software.

PCA is a technique used to reduce multidimensional data sets to lower dimensions for analysis. The applications include exploratory data analysis data and for generating predictive models. It is a linear transformation that converts n original variables into n new variables (or principal components) which have 3 important properties: a) are ordered by the amount of variance explained, b) are uncorrelated and c) explain all the variation in the data.

PCA is a principal axis rotation of the original variables that preserves the variation in the data. Therefore, the total variance of the original variables is equal to the total variance of the principal components. The *eigenvectors* and the *eigenvalues* define the rotation and variation and are described as follows: The *eigenvalues* are the variances of the principal components. The *eigenvectors* are the direction cosines of

the new axes (PCs) relative to the old (original variables). Thus they define the rotations of the original axes. The values on the axis therefore are the values of those new variables.

The Robust Multichip Analysis (RMA) algorithm fits a robust linear model at the probe level to minimize the effect of probe-specific affinity differences. This approach increases sensitivity to small changes between experiment and control samples, and minimizes variance across the dynamic range, but does compress calculated fold change values.

Therefore, from figure 5.6, one can say that the 2 fresh-frozen samples show little variation between them and thus are of good quality. The same applies to two of the Z7-fixed samples, whereas the the third Z7-fixed sample was far from the other two Z7-fixed samples, and that indicates variability between those samples. Obviously in order to verify the above preliminary results further work using a larger number of samples and replicates is required.

5.4 Exon level analysis

The aim of this part of the thesis was to conduct a preliminary comparison of microarray performance of RNA extracted from Z7-fixed and unfixed frozen tissue samples. Liver samples were obtained from two C57BL/6 mice. From each mouse half of the liver tissue sample was fixed with a zinc-based fixative (Z7) and the other half tissue was snap frozen with liquid N₂ and then stored at -80°C. This resulted in a total of 2 paired samples plus 1 technical replicate for one of the Z7-fixed samples.

All five samples were successfully hybridized on GeneChip® Mouse Exon 1.0 ST Arrays. This is a new type of array produced by Affymetrix that enables two complementary levels of analysis—gene expression and alternative splicing.

In this study analysis was restricted to probesets in the “core” level annotation. These probesets correspond to exons that have high confidence annotation, such as RefSeq transcripts and full length mRNAs. The Affymetrix® Expression Console™ software was used to generate metrics for the quality assessment of the data before proceeding with downstream analysis that was included in QA Report. PCA was also used for identifying outliers and major effects in the data (included in the QA report). The Pearson correlation for the normalised exon level and gene level expression signal was estimated for all pair-wise sample comparisons.

Table 5.3: Exon level Pearson correlation analysis.

		Z7 1	Z7r 2	Frozen 3	Z7 4	Frozen 5
Z7	1	1	0.905	0.800	0.897	0.791
Z7r	2	0.905	1	0.798	0.896	0.791
Frozen	3	0.800	0.798	1	0.788	0.947
Z7	4	0.897	0.896	0.788	1	0.779
Frozen	5	0.791	0.791	0.947	0.7791	1

Table 5.4: Gene level Pearson correlation analysis.

		Z7 1	Z7r 2	Frozen 3	Z7 4	Frozen 5
Z7	1	1	0.957	0.884	0.952	0.878
Z7r	2	0.957	1	0.885	0.952	0.881
Frozen	3	0.884	0.885	1	0.877	0.979
Z7	4	0.952	0.952	0.877	1	0.871
Frozen	5	0.878	0.881	0.979	0.871	1

Pearson's correlation coefficient r , measures the strength of the linear relationship between two variables. The closer Pearson correlation is to 1 the better the linear relationship between the two variables. It can be concluded from tables 5.3 and 5.4 that there is correlation between all the 5 samples in both categories, Z7-fixed and fresh-frozen. This correlation occurs in both the gene level and the exon level, but is only an indication and not an absolute measurement.

Array data normalization and downstream analysis were performed in the commercial software Partek[®] software. The RMA method (Robust Multichip Analysis) (Irizarry et al. 2003) was used to normalize the data. Normalised data from the Z7 technical replicates were averaged and then used together with the rest of the data for statistical analysis.

A 4-way mixed model ANOVA (analysis of variance) ("Alternative Splice ANOVA") was fitted to the normalized exon level expression measurements for each transcript (implemented in the Partek[®] software). The ANOVA factors were: (1) Fixation Type, as a fixed effect with 2 levels (Z7 and frozen), (2) Mouse ID as random effect with 2 levels, (3) Exons as a fixed effect with as many levels as exons for each transcript, (4) Sample ID as a random factor with 4 levels. The interaction effect of Fixation Type with Exon was also fitted to the model. Multiple correction testing was performed using the false discovery rate (FDR) step down method (Benjamini et al. 2001) with a cut off of less or equal to 0.01.

Alternative Splicing Results

The "Alternative Splice" ANOVA model was used to identify differentially expressed *genes* between Z7-fixed *versus* unfixed fresh-frozen samples. After correction for multiple testing there were no genes identified as differentially expressed in these 2 groups.

The same model was also used to identify exons that were expressed differently between the 2 fixation methods (fixation dependent alternative splicing, FDAS) as well as exons that were expressed differently from other exons on the same gene regardless of the fixation type (fixation independent alternative splicing, FIAS).

Fixation independent alternative splicing was detected in 7295 transcripts while fixation dependent alternative splicing was identified in 329 gene transcripts after correction for multiple testing (FDR p-value ≤ 0.01). Table 5.5 below shows the alternative splicing results.

Table 5.5: Alternative splicing results.

	Cutoff Value	# of Significant p-values
p-value(FIAS)	2.19E-06	7295
p-value(FDAS)	6.61E-07	329

Table 5.6: A complete list of the 329 gene transcripts that showed fixation dependent alternative splicing. The number of exons and the p-value are given for each gene.

Number of Exons	Gene assignment	Exon level		Exon level		Gene level		Gene level	
		FIAS	p-value	FDAS	p-value	Fixation method (Z7 vs. FF)	p-value	Fixation method (Z7 vs. FF)	Fold Change
51	NM_010233 // Fn1 // fibronectin 1 // 1 C1-C5[1 36.1 cM // 14268 // BC051082 //	1.00E-50	3.68E-23	3.68E-23	0.032		0.032		-2.2
39	NM_030084 // Gpr108 // G protein-coupled receptor 108 // 17 E1.1 // 78308 // NM	1.00E-50	4.31E-17	4.31E-17	0.010		0.010		-2.3
34	NM_010309 // Gnas // GNAS (guanine nucleotide binding protein, alpha stimulating	1.00E-50	3.56E-16	3.56E-16	0.601		0.601		-1.0
71	NM_011261 // ReIn // reelin // 5 A3-B1[5 8.0 cM // 19699 // AK082447 // ReIn //	3.12E-32	1.49E-15	1.49E-15	0.004		0.004		-2.0
28	NM_024222 // Stt3b // STT3, subunit of the oligosaccharyltransferase complex, ho	4.02E-32	4.98E-15	4.98E-15	0.014		0.014		-3.4
35	NM_023617 // Aox3 // aldehyde oxidase 3 // 1 C1.3[1 23.2 cM // 71724 // AF17227	1.00E-50	2.27E-13	2.27E-13	0.014		0.014		-1.9
15	NM_009656 // Aldh2 // aldehyde dehydrogenase 2, mitochondrial // 5 F-G1 // 11669	1.65E-18	2.96E-13	2.96E-13	0.004		0.004		-3.1
26	NM_001005863 // Mtus1 // mitochondrial tumor suppressor 1 // 8 A4 // 102103 //	3.91E-39	3.81E-13	3.81E-13	0.049		0.049		-1.2
31	NM_146217 // Aars // alanyl-tRNA synthetase // 8 E1 // 234734 // NM_028274 // E	5.61E-31	6.79E-13	6.79E-13	0.048		0.048		-2.6
35	NM_007376 // Pzp // pregnancy zone protein // 6 F1-G3[6 62.0 cM // 11287 // AK1	1.00E-50	1.32E-12	1.32E-12	0.007		0.007		-3.0
33	NM_145830 // Ehmt2 // euchromatic histone lysine N-methyltransferase 2 // 17 B1	7.96E-34	1.51E-12	1.51E-12	0.050		0.050		1.3
22	NM_027902 // Tmprss6 // transmembrane serine protease 6 // 15 E2 // 71753	1.39E-33	1.78E-12	1.78E-12	0.004		0.004		-2.3
18	NM_194054 // Rtn4 // reticulon 4 // 11 A3.3 // 68585 // NM_194051 // Rtn4 // re	2.63E-20	3.29E-12	3.29E-12	0.032		0.032		-2.2
78	NM_007868 // Dmd // dystrophin, muscular dystrophy // X CIX 32.0 cM // 13405 //	1.12E-38	3.76E-12	3.76E-12	0.164		0.164		1.1
13	NM_021282 // Cyp2e1 // cytochrome P450, family 2, subfamily e, polypeptide 1 //	4.93E-28	3.82E-12	3.82E-12	0.007		0.007		-3.4
36	NM_029600 // Abcc3 // ATP-binding cassette, sub-family C (CFTR/MRP), member 3 //	1.20E-23	8.08E-12	8.08E-12	0.023		0.023		-3.1
28	NM_145506 // Epb4.1[5 // erythrocyte protein band 4.1-like 5 // 1 E2.3 // 226352	1.87E-21	8.25E-12	8.25E-12	0.111		0.111		-1.7
10	NM_021459 // Isl1 // ISL1 transcription factor, LIM/homeodomain // 13 D2.3 // 16	1.03E-12	8.34E-12	8.34E-12	0.095		0.095		1.8
21	NM_080556 // Tm9sf2 // transmembrane 9 superfamily member 2 // 14 E5[14 67.0 cM	3.19E-16	8.51E-12	8.51E-12	0.017		0.017		-5.6
18	NM_133981 // Alg9 // asparagine-linked glycosylation 9 homolog (yeast, alpha 1,2	3.90E-19	1.14E-11	1.14E-11	0.104		0.104		-1.9
20	NM_008408 // Stt3a // STT3, subunit of the oligosaccharyltransferase complex, ho	9.89E-19	1.23E-11	1.23E-11	0.000		0.000		-8.1
65	NM_010586 // Itp2 // inositol 1,4,5-triphosphate receptor 2 // 6 G3[6 73.0 cM /	3.20E-42	1.38E-11	1.38E-11	0.800		0.800		-1.0
25	NM_010074 // Dpp4 // dipeptidylpeptidase 4 // 2 C2-D[2 35.0 cM // 13482 // AK16	2.16E-24	1.41E-11	1.41E-11	0.024		0.024		-3.7

21	NM_028787 // Slc35f5 // solute carrier family 35, member F5 // 1 E3 // 74150	5.24E-14	1.48E-11	0.009	-3.4
35	NM_172606 // March6 // membrane-associated ring finger (C3HC4) 6 // 15 B2 // 223	3.10E-33	1.82E-11	0.049	-4.1
30	NM_021022 // Abcb11 // ATP-binding cassette, sub-family B (MDR/TAP), member 11 /	5.96E-29	1.84E-11	0.007	-3.0
25	NM_008292 // Hsd17b4 // hydroxysteroid (17-beta) dehydrogenase 4 // 18 D1 // 154	7.82E-32	1.91E-11	0.026	-5.7
28	NM_178888 // Gaml3 // GTPase activating RANGAP domain-like 3 // 2 B // 99326 //	1.12E-32	2.40E-11	0.105	1.4
15	NM_172759 // Ces5 // carboxylesterase 5 // 8 D3 // 234673 /// NM_133960 // Ces6	7.31E-16	2.59E-11	0.031	-7.3
22	NM_007940 // Ephx2 // epoxide hydrolase 2, cytoplasmic // 14 D14 32.5 cM // 138	3.65E-24	2.67E-11	0.018	-5.2
17	NM_144520 // Sec14l2 // SEC14-like 2 (S. cerevisiae) // 11 A1 // 67815	3.62E-25	3.18E-11	0.009	-3.5
95	NM_021523 // Huwe1 // HECT, UBA and WWE domain containing 1 // X F3 // 59026 ///	1.00E-50	3.45E-11	0.141	-1.4
45	NM_181277 // Col14a1 // procollagen, type XIV, alpha 1 // 15 D // 12818 // AK05	1.46E-15	3.53E-11	0.005	-2.1
24	NM_007752 // Cp // ceruloplasmin // 3 D // 12870 /// AK014835 // Cp // cerulopla	6.99E-25	3.79E-11	0.048	-5.5
14	NM_016906 // Sec61a1 // Sec61 alpha 1 subunit (S. cerevisiae) // 6 D1 // 53421 /	2.83E-19	3.83E-11	0.002	-6.1
32	NM_016965 // Nckap1 // NCK-associated protein 1 // 2 C3J2 48.0 cM // 50884 /// B	4.34E-29	4.46E-11	0.034	-4.2
21	NM_008466 // Kpna3 // karyopherin (importin) alpha 3 // 14 D1 // 16648	2.29E-15	4.96E-11	0.012	-2.7
14	NM_019760 // Serinc1 // serine incorporator 1 // 10 B4 // 56442 /// AK160498 //	3.13E-17	5.72E-11	0.020	-17.2
14	NM_146214 // Tat // tyrosine aminotransferase // 8 D3 // 234724	5.14E-20	5.91E-11	0.001	-2.9
18	NM_008960 // Pten // phosphatase and tensin homolog // 19 C1119 24.5 cM // 19211	2.69E-22	6.24E-11	0.084	-2.3
37	NM_207530 // Osbp1a // oxysterol binding protein-like 1A // 18 A2 // 64291 ///	6.53E-40	7.42E-11	0.029	-1.3
21	NM_001005385 // Gprasp1 // G protein-coupled receptor associated sorting protein	3.40E-16	8.94E-11	0.069	1.3
15	NM_053176 // Hrg // histidine-rich glycoprotein // 16 B1116 14.1 cM // 94175	4.64E-22	1.09E-10	0.012	-2.7
42	NM_007955 // Ptpnv // protein tyrosine phosphatase, receptor type, V // 1 F-G //	4.17E-33	1.31E-10	0.015	1.2
33	NM_010612 // Kdr // kinase insert domain protein receptor // 5 C3.3J5 42.0 cM //	3.96E-18	1.33E-10	0.051	-2.0
52	AK087690 // A1314180 // expressed sequence A1314180 // 4 B3 // 230249 /// AK0415	1.00E-50	1.52E-10	0.054	-2.1
66	NM_199476 // Rrm2b // ribonucleotide reductase M2 B (TP53 inducible) // 15 B3.1	1.00E-50	1.72E-10	0.008	-1.7
29	NM_138595 // Glc // glycine decarboxylase // 19 C119 25.0 cM // 104174 /// AK13	1.17E-28	1.77E-10	0.096	-3.3
13	NM_009202 // Slc22a1 // solute carrier family 22 (organic cation transporter), m	1.34E-19	1.84E-10	0.038	-4.4
17	NM_008281 // Hpn // hepsin // 7 B1 // 15451 /// AK002694 // Hpn // hepsin // 7 B	1.60E-21	2.37E-10	0.019	-1.8
16	NM_025899 // Uqcrc2 // ubiquinol cytochrome c reductase core protein 2 // 7 F2 /	2.57E-19	2.96E-10	0.016	-8.6
12	NM_013492 // Clu // clusterin // 14 D114 28.0 cM // 12759 /// XM_975431 // Clu	3.92E-20	3.42E-10	0.005	-4.1
16	NM_025407 // Uqcrc1 // ubiquinol-cytochrome c reductase core protein 1 // 9 F2 9	1.63E-15	4.46E-10	0.068	-4.5
28	NM_207636 // Fndc3a // fibronectin type III domain containing 3a // 14 D2 // 319	3.61E-26	4.79E-10	0.010	-1.7
13	NM_008101 // Gcgr // glucagon receptor // 11 E2 // 14527 /// BC012718 // Gcgr //	1.07E-19	5.10E-10	0.421	1.1

35	NM_001003908 // Cltc // clathrin, heavy polypeptide (Hc) // 11 C // 67300 /// BC	3.92E-35	5.49E-10	0.004	-4.7
13	NM_145953 // Cth // cystathionase (cystathionine gamma-lyase) // 3 H4 // 107869	2.49E-18	6.18E-10	0.015	-5.4
11	NM_023792 // Pank1 // pantothenate kinase 1 // 19 G1-G3 // 75735 /// AF347700 //	6.84E-14	6.67E-10	0.118	-2.1
19	NM_173763 // Ccb12 // cysteine conjugate-beta lyase 2 // 3 H1 // 229905 /// BC03	9.12E-23	6.81E-10	0.068	-3.3
12	NM_029565 // Tmem59 // transmembrane protein 59 // 4 51.8 cM // 56374 /// AF1169	1.21E-14	7.00E-10	0.028	-14.9
14	NM_008640 // Laptm4a // lysosomal-associated protein transmembrane 4A // 12 A1.1	9.45E-16	7.50E-10	0.010	-5.6
31	NM_008149 // Gpam // glycerol-3-phosphate acyltransferase, mitochondrial // 19 D	2.58E-31	8.06E-10	0.015	-3.5
29	NM_018797 // Plxnc1 // plexin C1 // 10 C3 // 54712 /// AK033998 // Plxnc1 // ple	7.37E-24	9.32E-10	0.046	-1.2
92	NM_008512 // Lrp1 // low density lipoprotein receptor-related protein 1 // 10 B2	1.00E-50	9.45E-10	0.053	-1.4
18	NM_019975 // Hact1 // 2-hydroxyacyl-CoA lyase 1 // 14 B // 56794 /// NM_009937 /	9.94E-27	9.89E-10	0.048	-4.2
20	NM_029364 // Gns // glucosamine (N-acetyl)-6-sulfatase // 10 D2 // 75612 /// BC0	3.77E-20	1.01E-09	0.008	-2.6
24	NM_170778 // Dpyd // dihydropyrimidine dehydrogenase // 3 G1 // 99586 /// AK1494	1.10E-18	1.02E-09	0.039	-2.7
28	NM_008720 // Npc1 // Niemann Pick type C1 // 18 A1 18 4.0 cM // 18145 /// AK0871	1.63E-15	1.46E-09	0.016	-3.5
54	NM_207682 // Kif1b // kinesin family member 1B // 4 E 4 70.9 cM // 16561 /// NM_	1.00E-50	1.49E-09	0.041	-1.9
96	NM_023868 // Ryr2 // ryanodine receptor 2, cardiac // 13 A1-A2 13 7.0 cM // 2019	1.00E-50	1.56E-09	0.168	1.2
29	NM_022886 // Scel // scellin // 14 E // 64929 /// BC025142 // Scel // scellin	2.09E-31	1.57E-09	0.090	1.4
28	NM_144900 // Atp1a1 // ATPase, Na+/K+ transporting, alpha 1 polypeptide // 3 F3	3.92E-24	1.59E-09	0.061	-4.6
19	NM_023732 // Abcb6 // ATP-binding cassette, sub-family B (MDR/TAP), member 6 //	1.82E-22	1.62E-09	0.224	-1.2
50	NM_009931 // Col4a1 // procollagen, type IV, alpha 1 // 8 A1.1 8 5.0 cM // 12826	1.00E-50	1.62E-09	0.128	1.2
10	NM_133862 // Fgg // fibrinogen, gamma polypeptide // 3 E3 3 41.3 cM // 99571 ///	2.85E-22	1.69E-09	0.002	-2.9
28	NM_134005 // Enpp3 // ectonucleotide pyrophosphatase/phosphodiesterase 3 // 10 A	7.72E-26	1.71E-09	0.063	-3.5
7	NM_024287 // Rab6 // RAB6, member RAS oncogene family // 7 C4 // 19346 /// BC019	1.33E-11	1.92E-09	0.081	-2.3
31	NM_207655 // Egfr // epidermal growth factor receptor // 11 A1-A4 11 9.0 cM // 1	3.83E-35	1.94E-09	0.048	-1.5
23	BC023155 // Dnahc1 // dynein, axonemal, heavy chain 1 // 14 B 14 8.3 cM // 11008	3.99E-12	2.01E-09	0.063	1.2
	AK040838 // A530016L24Rik // RIKEN cDNA A530016L24 gene // 12 F1 // 319942 ///				
9	X				
23	NM_016902 // Nphp1 // nephronophthisis 1 (juvenile) homolog (human) // 2 F3 // 5	2.95E-09	2.08E-09	0.095	1.7
39	NM_153145 // Abca8a // ATP-binding cassette, sub-family A (ABC1), member 8a // 1	3.38E-36	2.12E-09	0.058	1.3
24	NM_199079 // Ddx17 // DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 // 15 E1 // 6704	7.82E-33	2.17E-09	0.067	-3.2
31	NM_133957 // Nfat5 // nuclear factor of activated T-cells 5 // 8 D2 8 53.0 cM //	4.50E-27	2.27E-09	0.187	1.6
21	NM_008959 // Ptdss1 // phosphatidylserine synthase 1 // 13 B-C 13 39.8 cM // 19	4.46E-23	2.27E-09	0.049	1.8
29	AK210771 // 1300001101Rik // RIKEN cDNA 1300001101 gene // 11 B4 // 74148 /// AK	1.03E-13	2.50E-09	0.090	-1.8
		1.37E-33	2.59E-09	0.009	-2.0

85	NM_007566 // Birc6 // baculoviral IAP repeat-containing 6 // --- // 12211 /// AK	1.00E-50	2.67E-09	0.071	-2.0
18	NM_133916 // Eif3s9 // eukaryotic translation initiation factor 3, subunit 9 (et	1.11E-21	2.70E-09	0.079	-2.8
29	NM_008676 // Nbr1 // neighbor of Brca1 gene 1 // 11 D11 60.5 cM // 17966 /// AF	2.03E-32	2.77E-09	0.074	-1.7
52	NM_008569 // Anapc1 // anaphase promoting complex subunit 1 // 2 F3 // 17222 ///	9.09E-33	2.82E-09	0.025	-1.4
34	NM_023066 // Asph // aspartate-beta-hydroxylase // 4 A1 // 65973 /// NM_133723 /	7.07E-34	3.23E-09	0.132	-1.1
16	NM_008175 // Grn // granulin // 11 D11 60.0 cM // 14824	4.83E-23	3.55E-09	0.050	-6.7
10	NM_019411 // Ppp2ca // protein phosphatase 2 (formerly 2A), catalytic subunit, a	6.41E-14	3.79E-09	0.025	-9.0
26	NM_080467 // Atp6v0a4 // ATPase, H+ transporting, lysosomal V0 subunit A4 // 6 B	1.93E-34	3.85E-09	0.096	1.2
18	NM_134042 // Aldh6a1 // aldehyde dehydrogenase family 6, subfamily A1 // 12 D1 /	3.23E-19	3.97E-09	0.002	-11.1
26	NM_008830 // Abcb4 // ATP-binding cassette, sub-family B (MDR/TAP), member 4 //	2.35E-24	4.03E-09	0.012	-2.6
43	NM_010406 // Hc // hemolytic complement // 2 cen-C12 23.5 cM // 15139	1.00E-50	4.09E-09	0.022	-2.9
	AK030074 // C920006C10Rik // RIKEN cDNA C920006C10 gene // 15 D1 // 76740 ///				
28	AK				
35	XM_898060 // Nek1 // NIMA (never in mitosis gene a)-related expressed kinase 1 /	1.26E-26	4.24E-09	0.114	-2.2
15	NM_011951 // Mapk14 // mitogen activated protein kinase 14 // 17 A3.317 13.5 cM	8.22E-26	4.36E-09	0.042	1.3
19	NM_023733 // Crot // carnitine O-octanoyltransferase // 5 A1 // 74114 /// AK0379	1.30E-16	4.74E-09	0.088	-1.5
13	NM_010232 // Fmo5 // flavin containing monooxygenase 5 // 3 F2.2 // 14263 /// XM	2.76E-28	4.85E-09	0.026	-5.2
22	NM_010195 // Lgr5 // leucine rich repeat containing G protein coupled receptor 5	1.29E-19	5.02E-09	0.042	-4.2
30	NM_024267 // Ipo4 // importin 4 // 14 C314 20.0 cM // 75751 /// BC085150 // Ipo	3.66E-26	5.35E-09	0.344	1.1
25	NM_153103 // Kif1c // kinesin family member 1C // 11 B3111 37.0 cM // 16562 ///	5.51E-23	5.44E-09	0.199	-1.4
31	BC043674 // 9030624J02Rik // RIKEN cDNA 9030624J02 gene // 7 F3 // 71517 /// BC0	1.23E-31	5.54E-09	0.557	-1.1
44	NM_026376 // Plxd1 // plexin D1 // 6 E3 // 67784 /// BC019530 // Plxd1 // plex	1.29E-20	5.56E-09	0.006	-1.5
18	NM_182990 // Ssrp1 // structure specific recognition protein 1 // 2 D12 47.8 cM	1.52E-42	5.76E-09	0.800	1.0
14	NM_020577 // As3mt // arsenic (+3 oxidation state) methyltransferase // 19 D1 //	4.24E-18	5.80E-09	0.002	-1.7
15	NM_133653 // Mat1a // methionine adenosyltransferase I, alpha // 14 C1 // 11720	8.80E-18	6.09E-09	0.007	-2.2
14	NM_009632 // Parp2 // poly (ADP-ribose) polymerase family, member 2 // 14 C1114	6.01E-22	7.76E-09	0.037	-3.0
	AK018637 // 9130019P16Rik // RIKEN cDNA 9130019P16 gene // 6B36 // 378878 ///	3.85E-14	7.77E-09	0.325	1.2
18	A				
28	NM_001040131 // Eif4g2 // eukaryotic translation initiation factor 4, gamma 2 //	7.84E-33	7.84E-09	0.004	1.4
19	NM_010766 // Marco // macrophage receptor with collagenous structure // 1 E4-F /	2.74E-31	8.16E-09	0.109	-3.8
39	NM_013851 // Abca8b // ATP-binding cassette, sub-family A (ABC1), member 8b // 1	4.93E-24	8.38E-09	0.047	1.7
30	NM_010890 // Nedd4 // neural precursor cell expressed, developmentally down-regu	3.60E-28	8.41E-09	0.035	-1.8
		6.40E-23	8.63E-09	0.008	-3.4

17	NM_144784 // Acat1 // acetyl-Coenzyme A acetyltransferase 1 // 9 30.0 cM // 1104	3.33E-23	9.11E-09	0.082	-3.4
14	NM_175194 // Slc25a16 // solute carrier family 25 (mitochondrial carrier, Graves	1.21E-14	1.00E-08	0.093	-3.0
39	AK209918 // Wdr62 // WD repeat domain 62 // 7 B1 // 233064 /// XM_001001114 // W	1.40E-45	1.14E-08	0.007	1.3
13	NM_019911 // Tdo2 // tryptophan 2,3-dioxygenase // 3 E3 // 56720	1.51E-15	1.26E-08	0.026	-6.1
18	NM_015729 // Acox1 // acyl-Coenzyme A oxidase 1, palmitoyl // 11 E2 // 11430 ///	2.49E-23	1.27E-08	0.021	-2.8
39	NM_144847 // Nrpb2 // nuclear receptor binding protein 2 // 15 D3 // 223649 ///	8.41E-45	1.28E-08	0.040	1.5
25	NM_009722 // Atp2a2 // ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	4.98E-26	1.34E-08	0.032	-3.1
26	NM_009457 // Ube1x // ubiquitin-activating enzyme E1, Chr X // X A2-A3 X 5.7 cM	5.60E-23	1.34E-08	0.008	-6.2
18	NM_018887 // Cyp39a1 // cytochrome P450, family 39, subfamily a, polypeptide 1 /	1.63E-16	1.39E-08	0.052	-1.8
20	NM_027052 // Slc38a4 // solute carrier family 38, member 4 // 15 F1 // 69354 ///	1.35E-30	1.53E-08	0.012	-3.3
14	NM_010481 // Hspa9 // heat shock protein 9 // 18 C 18 15.0 cM // 15526 /// XM_99	4.45E-20	1.53E-08	0.003	-2.9
16	NM_021881 // Qk // quaking // 17 A 17 5.9 cM // 19317 /// AF090403 // Qk // qua	4.49E-21	1.56E-08	0.120	-1.6
15	NM_008548 // Man1a // mannosidase 1, alpha // 10 B3 // 17155 /// AK172376 // Man	3.99E-11	1.71E-08	0.001	-7.2
16	NM_001024945 // Qscn6 // quiescin Q6 // 1 G3 // 104009 /// NM_023268 // Qscn6 //	3.50E-23	1.73E-08	0.027	-2.8
12	NM_009837 // Cct4 // chaperonin subunit 4 (delta) // 11 A3.2 11 13.0 cM // 12464	2.72E-12	1.75E-08	0.044	-3.2
12	NM_020559 // Alas1 // aminolevulinic acid synthase 1 // 9 F1 // 11655 /// AK1332	7.13E-21	1.75E-08	0.459	1.1
43	NM_153417 // Trpm6 // transient receptor potential cation channel, subfamily M,	2.57E-26	1.83E-08	0.062	1.1
15	NM_146013 // Sec14l4 // SEC14-like 4 (S. cerevisiae) // 11 A1 // 103655 /// NM_0	2.38E-18	1.92E-08	0.112	-2.3
22	NM_145614 // Dlat // dihydrolipoamide S-acetyltransferase (E2 component of pyruv	1.06E-23	1.98E-08	0.094	-1.7
30	NM_001003910 // Gnn // Grp94 neighboring nucleotidase variant 4 // 10 C1 // 1032	1.58E-22	2.14E-08	0.072	1.3
10	NM_010306 // Gnai3 // guanine nucleotide binding protein, alpha inhibiting 3 //	1.98E-15	2.19E-08	0.016	-3.0
15	NM_019930 // Ranbp9 // RAN binding protein 9 // 13 A4 // 56705 /// XM_977383 //	1.29E-16	2.20E-08	0.006	-1.5
53	NM_016919 // Col5a3 // procollagen, type V, alpha 3 // 9 A3 // 53867 /// AB04049	1.00E-50	2.20E-08	0.039	2.6
15	NM_009275 // Srpb // signal recognition particle receptor, B subunit // 9 F1 //	2.42E-24	2.21E-08	0.019	-1.7
12	NM_178143 // Prkaa2 // protein kinase, AMP-activated, alpha 2 catalytic subunit	1.74E-16	2.28E-08	0.079	-2.3
18	NM_001038995 // EG214321 // predicted gene, EG214321 // 12 D1 12 // 214321 /// N	2.20E-14	2.40E-08	0.028	-1.4
22	NM_172628 // Sh3tc2 // SH3 domain and tetratricopeptide repeats 2 // 18 E1 // 22	3.13E-32	2.43E-08	0.128	1.3
13	NM_023429 // Ociad1 // OCIA domain containing 1 // 5 C3.2 // 68095 /// AK078102	1.82E-14	2.45E-08	0.031	-3.4
8	NM_010106 // Eef1a1 // eukaryotic translation elongation factor 1 alpha 1 // 9 E	2.74E-11	2.48E-08	0.058	-3.3
15	NM_011179 // Psap // prosaposin // 10 B4 10 35.0 cM // 19156 /// AK182220 // Ps	2.53E-13	2.70E-08	0.021	-6.8
32	NM_153122 // Oplah // 5-oxoprolinase (ATP-hydrolysing) // 15 D3 // 75475 /// AK0	3.84E-26	2.82E-08	0.016	-2.1
15	NM_010344 // Gsr // glutathione reductase 1 // 8 A4 8 18.0 cM // 14782 /// XM_90	1.03E-16	2.84E-08	0.005	-2.9

28	NM_134037 // Acly // ATP citrate lyase // 11 D // 104112 /// BC065805 // Acly //	4.46E-28	2.87E-08	0.004	-4.5
22	NM_174857 // Mamdc2 // MAM domain containing 2 // 19 B // 71738 /// AK004794 //	5.76E-33	3.00E-08	0.040	1.2
28	NM_027135 // Sec24d // SEC24 related gene family, member D (S. cerevisiae) // 3	3.82E-18	3.01E-08	0.051	-2.8
22	NM_207625 // Acs14 // acyl-CoA synthetase long-chain family member 4 // X F2 //	6.70E-18	3.02E-08	0.007	-2.7
8	NM_016764 // Prdx4 // peroxiredoxin 4 // X F3 X 67.7 cM // 53381 /// AK157829 //	1.86E-10	3.14E-08	0.015	-7.1
12	NM_183294 // Cdk1 // cyclin-dependent kinase-like 1 (CDC2-related kinase) // 12	3.29E-12	3.18E-08	0.188	1.5
16	NM_015812 // Rgs6 // regulator of G-protein signaling 6 // 12 D1 12 36.5 cM // 5	2.49E-15	3.25E-08	0.038	1.4
15	NM_178604 // Txnl4 // thioredoxin-like 4 // 18 E3 // 27366 /// NM_001042408 // T	7.04E-18	3.27E-08	0.146	-1.5
20	NM_007883 // Dsg2 // desmoglein 2 // 18 A2 18 7.05 cM // 13511 /// BC020144 // D	1.01E-17	3.28E-08	0.060	-1.9
47	NM_027144 // Arhgef12 // Rho guanine nucleotide exchange factor (GEF) 12 // 9 B	1.45E-36	3.35E-08	0.129	-1.5
25	NM_009071 // Rock1 // Rho-associated coiled-coil containing protein kinase 1 //	1.25E-19	3.38E-08	0.017	-1.6
18	NM_026228 // Slc39a8 // solute carrier family 39 (metal ion transporter), member	1.24E-14	3.56E-08	0.012	-1.6
17	NM_031258 // Chrdl1 // chordin-like 1 // -- // 83453 /// BC066832 // Chrdl1 //	2.06E-19	3.59E-08	0.033	1.4
10	NM_008288 // Hsd11b1 // hydroxysteroid 11-beta dehydrogenase 1 // 1 H6 // 15483	1.44E-14	3.72E-08	0.025	-7.3
23	NM_010401 // Hal // histidine ammonia lyase // 10 C2-D1 10 51.0 cM // 15109 ///	2.10E-29	3.74E-08	0.034	-2.7
30	NM_001007220 // Adam22 // a disintegrin and metalloproteinase domain 22 // 5 A1 5	4.20E-40	3.75E-08	0.125	1.3
10	NM_019817 // Copz1 // coatomer protein complex, subunit zeta 1 // 15 F3 15 6.0 c	7.84E-17	3.87E-08	0.006	-2.5
18	NM_011920 // Abcg2 // ATP-binding cassette, sub-family G (WHITE), member 2 // 6	6.57E-17	3.95E-08	0.151	-2.1
12	NM_176942 // Gabra5 // gamma-aminobutyric acid (GABA-A) receptor, subunit alpha	2.10E-14	4.04E-08	0.043	1.4
25	NM_177236 // Atp2b3 // ATPase, Ca++ transporting, plasma membrane 3 // X A7.3 //	1.45E-27	4.19E-08	0.064	1.4
23	NM_212444 // Gyk // glycerol kinase // X C-D X 33.0 cM // 14933 /// NM_008194 //	5.08E-14	4.21E-08	0.187	-2.2
19	NM_028780 // Tm9sf1 // transmembrane 9 superfamily member 1 // 14 C3 // 74140 //	2.06E-20	4.34E-08	0.203	-1.6
18	NM_008489 // Lbp // lipopolysaccharide binding protein // 2 H1 2 83.0 cM // 1680	2.50E-13	4.38E-08	0.010	-6.5
13	XM_001006463 // EG209380 // predicted gene, EG209380 // 7 E3 // 209380 /// NM_00	5.75E-17	4.44E-08	0.176	1.4
16	NM_011188 // Psmc2 // proteasome (prosome, macropain) 26S subunit, ATPase 2 // 5	5.01E-19	4.45E-08	0.014	-2.3
12	NM_010562 // Ilk // integrin linked kinase // 7 E1 // 16202 /// AK154207 // Ilk	5.63E-13	4.47E-08	0.010	-2.6
9	NM_015780 // Cfrh1 // complement factor H-related 1 // 1 F // 50702 /// XM_88882	4.82E-16	4.66E-08	0.016	-2.9
24	NM_029766 // Dtl // denticleless homolog (Drosophila) // 1 H6 // 76843 /// AB095	2.95E-26	4.68E-08	0.133	1.3
15	NM_019447 // Hgfac // hepatocyte growth factor activator // 5 B2 // 54426	2.55E-22	4.69E-08	0.030	-1.7
17	NM_026163 // Pkp2 // plakophilin 2 // 16 B1 // 67451 /// BC068279 // Pkp2 // pla	3.32E-21	4.73E-08	0.047	-1.7
20	NM_199473 // Col8a2 // procollagen, type VIII, alpha 2 // 4 D2.2 // 329941 /// A	4.75E-22	4.87E-08	0.079	2.1
37	NM_001005331 // Eif4g1 // eukaryotic translation initiation factor 4, gamma 1 //	7.56E-34	4.90E-08	0.136	-2.0

16	NM_001005509 // Eif2a // eukaryotic translation initiation factor 2a // 3 D 3 37	1.15E-10	4.91E-08	0.035	-2.8
14	NM_181411 // Aftph // aftphilin // 11 A3.1 // 216549 /// AK136815 // Aftph // a	2.14E-16	5.00E-08	0.068	-2.1
21	NM_007471 // App // amyloid beta (A4) precursor protein // 16 C3-qter 16 56.0 cM	1.01E-16	5.01E-08	0.087	-3.0
16	NM_145512 // Sft2d2 // SFT2 domain containing 2 // 1 H2.3 // 108735 /// BC051069	9.21E-10	5.04E-08	0.002	-3.5
13	NM_025978 // Ttc14 // tetratricopeptide repeat domain 14 // 3 B // 67120 /// NM_	7.67E-17	5.75E-08	0.140	1.6
12	NM_028247 // Slc16a10 // solute carrier family 16 (monocarboxylic acid transport	5.71E-09	5.95E-08	0.050	-3.9
32	NM_010863 // Myo1b // myosin IB // 1 C1.1 1 24.8 cM // 17912 /// L00923 // Myo1b	1.91E-26	6.41E-08	0.052	-3.8
14	NM_133869 // Cept1 // choline/ethanolaminephosphotransferase 1 // 3 F2.3 // 9971	6.76E-16	6.75E-08	0.013	-3.2
34	NM_010365 // Gtf2i // general transcription factor II // 5 G2 5 75.0 cM // 148	3.24E-27	6.84E-08	0.057	-1.9
12	NM_025968 // Ltb4dh // leukotriene B4 12-hydroxydehydrogenase // 4 C1 // 67103 /	4.31E-20	6.93E-08	0.031	1.3
120	NM_007767 // Pcdha6 // protocadherin alpha 6 // 18 B2-B3 // 12937 /// NM_009957	1.00E-50	6.97E-08	0.133	1.2
9	NM_026428 // Dcxl // dicarbonyl L-xylulose reductase // 11 E2 // 67880 /// BC012	1.78E-16	7.27E-08	0.098	-2.1
10	NM_013592 // Matn4 // matrilin 4 // 2 H3 2 94.0 cM // 17183 /// AK160786 // Matn	2.51E-15	7.51E-08	0.092	1.5
13	NM_007598 // Cap1 // CAP, adenylate cyclase-associated protein 1 (yeast) // 4 D1	2.17E-13	7.55E-08	0.064	-3.5
21	NM_145629 // Pls3 // plastin 3 (T-isoform) // X A7.3 // 102866 /// AK165540 // P	1.04E-14	7.56E-08	0.021	-3.8
15	NM_130884 // Idh3b // isocitrate dehydrogenase 3 (NAD+) beta // 2 F3 // 170718 /	5.75E-16	7.92E-08	0.046	-4.8
	BC038044 // A530016006Rik // RIKEN cDNA A530016006 gene // 12 A3 // 319660 ///				
11	A	1.57E-12	7.96E-08	0.067	-4.6
44	NM_025685 // Col27a1 // procollagen, type XXVII, alpha 1 // 4 C1 // 373864 /// B	1.00E-50	8.04E-08	0.028	2.2
	NM_023220 // 2010106G01Rik // RIKEN cDNA 2010106G01 gene // 2 F3 // 66552 ///				
19	BC	5.95E-18	8.06E-08	0.060	-8.1
16	NM_025382 // Tmem57 // transmembrane protein 57 // 4 D3 4 65.69 cM // 66146 ///	3.65E-14	8.16E-08	0.046	-1.3
18	NM_013505 // Dsc2 // desmocollin 2 // 18 A2 18 7.0 cM // 13506 /// AK172168 // D	5.33E-18	8.20E-08	0.057	-2.8
31	NM_198018 // Abr // active BCR-related gene // 11 B5 11 45.0 cM // 109934 /// NM	1.68E-26	8.25E-08	0.041	1.4
20	NM_033314 // Slco2a1 // solute carrier organic anion transporter family, member	5.53E-15	8.50E-08	0.031	-4.3
58	NM_001009947 // Dock11 // dedicator of cytokinesis 11 // X A3.1-A3.2 // 75974 //	5.70E-42	9.59E-08	0.044	1.2
13	NM_016737 // Stip1 // stress-induced phosphoprotein 1 // 19 A // 20867 /// AK080	1.17E-09	9.62E-08	0.030	-3.7
15	NM_019464 // Sh3glb1 // SH3-domain GRB2-like B1 (endophilin) // 3 H2 // 54673 //	2.28E-14	9.74E-08	0.050	-2.3
18	NM_177614 // 4632413K17Rik // RIKEN cDNA 4632413K17 gene // 10 D3 // 216440 ///	5.48E-12	9.98E-08	0.028	-2.4
12	NM_011458 // Serpina3k // serine (or cysteine) peptidase inhibitor, clade A, mem	7.87E-23	1.03E-07	0.020	-4.5
15	NM_009543 // Rnf103 // ring finger protein 103 // 6 C1 6 30.5 cM // 22644 /// AK	1.22E-16	1.09E-07	0.156	-2.2
20	NM_011246 // Rasgrp1 // RAS guanyl releasing protein 1 // 2 E5 2 65.0 cM // 1941	6.17E-24	1.09E-07	0.155	1.4

17	NM_009840 // Cct8 // chaperonin subunit 8 (theta) // 16 C3.3 // 12469 /// AK2145	5.73E-14	1.10E-07	0.071	-3.4
18	NM_008621 // Mpp1 // membrane protein, palmitoylated // X A7.3 X 30.48 cM // 175	2.13E-16	1.14E-07	0.022	-2.2
16	NM_145507 // Dars // aspartyl-tRNA synthetase // 1 E4 // 226414 /// NM_177445 //	4.34E-16	1.14E-07	0.011	-3.9
12	NM_181816 // Ccdc67 // coiled-coil domain containing 67 // 9 A2 // 234964 /// BC	1.38E-13	1.14E-07	0.130	1.5
19	NM_020495 // Scl1b2 // solute carrier organic anion transporter family, member	6.73E-19	1.18E-07	0.056	-7.6
	NM_177775 // D9Erd280e // DNA segment, Chr 9, ERATO Doi 280, expressed // 9 E3.				
23		2.92E-31	1.19E-07	0.144	1.3
14	NM_011791 // Ash2 // ash2 (absent, small, or homeotic)-like (Drosophila) // 8 A	6.01E-16	1.20E-07	0.169	-1.5
25	NM_016747 // Dlg3 // discs, large homolog 3 (Drosophila) // X C2 // 53310 /// B	8.33E-28	1.21E-07	0.948	-1.0
15	NM_010067 // Trdm1 // tRNA aspartic acid methyltransferase 1 // 2 A1 // 13434	1.38E-25	1.21E-07	0.049	1.3
9	NM_016892 // Ccs // copper chaperone for superoxide dismutase // 19 A 19 4.0 cM	4.33E-17	1.22E-07	0.183	-1.6
32	NM_007496 // Atbf1 // AT motif binding factor 1 // 8 E1 // 11906 /// AK142804 //	7.13E-26	1.22E-07	0.045	1.6
23	NM_029413 // Morc4 // microorchidia 4 // X F1 // 75746 /// AK053895 // Morc4 // m	1.28E-19	1.24E-07	0.124	1.2
8	NM_146077 // Trim31 // tripartite motif-containing 31 // 17 B1 // 224762	6.55E-14	1.33E-07	0.011	1.6
44	NM_013719 // Eif2ak4 // eukaryotic translation initiation factor 2 alpha kinase	4.50E-36	1.34E-07	0.177	1.2
8	NM_080555 // Ppap2b // phosphatidic acid phosphatase type 2B // 4 C7 4 52.7 cM /	6.56E-10	1.35E-07	0.056	-3.3
15	NM_178854 // Cnot6l // CCR4-NOT transcription complex, subunit 6-like // 5 E3 //	3.50E-12	1.37E-07	0.065	-2.9
15	NM_178050 // Arl6ip2 // ADP-ribosylation factor-like 6 interacting protein 2 //	4.54E-13	1.38E-07	0.074	-5.5
9	NM_022996 // Ndfip1 // Nedd4 family interacting protein 1 // 18 B3 // 65113 ///	1.69E-11	1.38E-07	0.051	-13.0
22	NM_009794 // Capn2 // calpain 2 // 1 H5 // 12334 /// AK053832 // Capn2 // calpai	5.35E-21	1.38E-07	0.374	-1.3
17	NM_010414 // Hdh // Huntington disease gene homolog // 5 B2 5 20.0 cM // 15194 /	4.22E-17	1.39E-07	0.070	1.2
42	NM_028093 // Entpd8 // ectonucleoside triphosphate diphosphohydrolase 8 // 2 A3	1.00E-50	1.40E-07	0.246	-1.2
17	NM_016772 // Ech1 // enoyl coenzyme A hydratase 1, peroxisomal // 7 B1 // 51798	5.79E-13	1.43E-07	0.047	1.6
21	NM_172857 // Exdl1 // Vexonuclease 3'-5' domain-like 1 // 2 E5 // 241624 /// BC0	1.66E-28	1.44E-07	0.111	1.2
8	NM_022026 // Aqp9 // aquaporin 9 // 9 D // 64008 /// BC024105 // Aqp9 // aquapor	3.42E-08	1.45E-07	0.003	-3.8
19	NM_016985 // Mtmr1 // myotubularin related protein 1 // X A7.2 // 53332 /// AY09	1.34E-16	1.47E-07	0.023	-2.0
18	NM_021489 // F12 // coagulation factor XII (Hageman factor) // 13 B2 // 58992	2.39E-12	1.51E-07	0.087	-2.6
21	NM_017366 // Acadvl // acyl-Coenzyme A dehydrogenase, very long chain // 11 B2-B	6.61E-20	1.52E-07	0.075	-2.4
10	NM_133926 // Camk1 // calcium/calmodulin-dependent protein kinase I // 6 E3 6 48	2.24E-13	1.53E-07	0.024	1.8
26	NM_008813 // Enpp1 // ectonucleotide pyrophosphatase/phosphodiesterase 1 // 10 A	7.63E-14	1.55E-07	0.053	-2.5
12	NM_183022 // Accn4 // amiloride-sensitive cation channel 4, pituitary // 1 C4 //	1.64E-20	1.56E-07	0.096	1.7
25	NM_011714 // Baz1b // bromodomain adjacent to zinc finger domain, 1B // 5 G2 //	3.35E-26	1.58E-07	0.397	-1.1

11	NM_007972 // F10 // coagulation factor X // 8 A1.1 8 7.0 cM // 14058 /// BC00387	7.11E-20	1.61E-07	0.067	-2.0
14	NM_001042634 // Ctk1 // CDC-like kinase 1 // 1 C1.3 1 30.1 cM // 12747 /// NM_00	9.78E-20	1.64E-07	0.007	1.8
13	NM_178645 // Blmh // bleomycin hydrolase // 11 B5 // 104184 /// BC027362 // Blmh	6.90E-11	1.68E-07	0.153	-3.0
19	NM_175193 // Golph4 // golgi phosphoprotein 4 // 3 E3 // 73124 /// BC023824 // G	2.31E-20	1.70E-07	0.425	-1.2
29	NM_030722 // Pum1 // pumilio 1 (Drosophila) // 4 D2.3 // 80912 /// AK052145 // P	4.37E-18	1.71E-07	0.100	-1.5
12	NM_013932 // Ddx25 // DEAD (Asp-Glu-Ala-Asp) box polypeptide 25 // 9 A3-A5 // 30	3.87E-19	1.73E-07	0.178	1.3
13	NM_139297 // Ugp2 // UDP-glucose pyrophosphorylase 2 // 11 A3.1 11 12.0 cM // 21	7.38E-16	1.74E-07	0.085	-4.2
19	NM_145528 // D2Etd391e // DNA segment, Chr 2, ERATO Doi 391, expressed // 2 E1	6.04E-10	1.75E-07	0.033	-3.7
32	NM_031881 // Nedd4l // neural precursor cell expressed, developmentally down-reg	2.35E-29	1.76E-07	0.023	-1.2
10	NM_010120 // Eif1a // eukaryotic translation initiation factor 1A // 18 C // 136	4.95E-09	1.84E-07	0.399	-1.3
32	NM_175025 // Atp2c1 // ATPase, Ca++-sequestering // 9 F1 // 235574 /// AK028393	5.13E-26	1.84E-07	0.149	-1.8
8	NM_134249 // Timd2 // T-cell immunoglobulin and mucin domain containing 2 // 11	1.39E-09	1.84E-07	0.063	-4.0
45	NM_022410 // Myh9 // myosin, heavy polypeptide 9, non-muscle // 15 E1 15 43.3 cM	2.44E-41	1.85E-07	0.346	-1.3
10	NM_007443 // Ambp // alpha 1 microglobulin/bikunin // 4 C1-C3 4 30.6 cM // 11699	1.06E-15	1.86E-07	0.038	-2.1
16	NM_133882 // C8b // complement component 8, beta subunit // 4 C6 4 49.4 cM // 11	1.30E-18	1.97E-07	0.036	-4.9
21	NM_011122 // Plod1 // procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 // 4 E2	3.45E-21	2.08E-07	0.041	-1.8
16	NM_001003930 // Rtn3 // reticulon 3 // 19 A // 20168 /// NM_001003934 // Rtn3 //	3.20E-15	2.19E-07	0.068	-4.1
15	NM_173006 // Pon3 // paraoxonase 3 // 6 A1 6 0.5 cM // 269823 /// BC099416 // Po	4.75E-13	2.21E-07	0.069	-5.4
32	NM_019459 // Nphs1 // nephrosis 1 homolog, nephrin (human) // -- // 54631 /// N	1.36E-26	2.27E-07	0.073	1.4
29	NM_139144 // Ogt // O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-ace	5.66E-24	2.37E-07	0.032	2.1
29	NM_019490 // Vdp // vesicle docking protein // 5 E3 // 56041 /// AK172425 // Vdp	1.19E-26	2.38E-07	0.094	-2.1
10	NM_008133 // Glud1 // glutamate dehydrogenase 1 // 14 B 14 15.5 cM // 14661 ///	1.77E-15	2.38E-07	0.057	-4.8
22	NM_173189 // Mcph1 // microcephaly, primary autosomal recessive 1 // 8 A3 // 244	2.39E-23	2.39E-07	0.107	1.8
14	NM_008611 // Mmp8 // matrix metalloproteinase 8 // 9 A1 // 17394	8.73E-16	2.49E-07	0.101	1.4
27	NM_021455 // Mixl1 // MLX interacting protein-like // 5 G2 // 58805 /// BC06405	1.10E-30	2.55E-07	0.039	4.2
29	NM_008156 // Gpld1 // glycosylphosphatidylinositol specific phospholipase D1 //	2.74E-20	2.63E-07	0.000	-4.3
17	NM_011787 // Amfr // autocrine motility factor receptor // 8 C5 8 45.0 cM // 238	6.88E-16	2.63E-07	0.012	-3.4
19	NM_027350 // Nars // asparaginyl-tRNA synthetase // 18 E1 // 70223	4.87E-17	2.64E-07	0.066	-2.8
13	NM_025934 // Rik2 // RIO kinase 2 (yeast) // 17 A3.1 // 67045 /// BC080771 // R	2.42E-10	2.65E-07	0.121	-1.6
24	NM_029402 // Cul2 // cullin 2 // 18 A1 // 71745 /// BC027428 // Cul2 // cullin 2	5.92E-14	2.70E-07	0.056	-2.0
18	NM_026861 // Phgdh1 // phosphoglycerate dehydrogenase like 1 // 14 E5 // 68889	1.01E-18	2.71E-07	0.068	-1.3
12	BC003999 // Dnajb11 // DnaJ (Hsp40) homolog, subfamily B, member 11 // 16 B1 //	1.37E-13	2.73E-07	0.008	-2.3

21	NM_153055 // Sec63 // SEC63-like (S. cerevisiae) // 10 B2 10 25.0 cM // 140740 /	4.20E-19	2.76E-07	0.029	-3.0
45	NM_198899 // Ugc11 // UDP-glucose ceramide glucosyltransferase-like 1 // 1 B //	1.52E-24	2.78E-07	0.064	-2.8
14	NM_198659 // B930007M17Rik // RIKEN cDNA B930007M17 gene // 3 H1 // 381476 ///				
13	X				
25	NM_011978 // Slc27a2 // solute carrier family 27 (fatty acid transporter), membre	2.19E-25	2.87E-07	0.023	1.2
8	NM_009933 // Col6a1 // procollagen, type VI, alpha 1 // 10 41.1 cM // 12833	4.52E-18	2.88E-07	0.046	-1.8
21	NM_008624 // Mras // muscle and microspikes RAS // 9 E3.3 // 17532 // BC024389	8.56E-24	2.94E-07	0.080	1.9
17	NM_177178 // Lmbrd2 // LMBR1 domain containing 2 // 15 A1 // 320506 /// AK020173	1.07E-07	2.98E-07	0.159	1.4
8	NM_010431 // Hif1a // hypoxia inducible factor 1, alpha subunit // 12 C3 12 31.0	1.57E-11	3.00E-07	0.004	-5.6
23	NM_144854 // ORF63 // open reading frame 63 // 16 C3.3 // 224419	5.66E-20	3.06E-07	0.082	-2.3
19	NM_011588 // Trim28 // tripartite motif protein 28 // 7 A2 // 21849	1.20E-14	3.06E-07	0.110	2.0
26	NM_007937 // EphA5 // Eph receptor A5 // 5 E1 5 43.0 cM // 13839 // BC057401 //	4.53E-20	3.08E-07	0.016	-2.2
29	NM_001039089 // Sel1 // sel-1 suppressor of lin-12-like (C. elegans) // 12 E //	9.69E-31	3.09E-07	0.164	1.3
9	NM_175506 // Adamts19 // a disintegrin-like and metalloproteinase (reprolysin typ	4.05E-20	3.10E-07	0.050	-3.5
17	NM_007409 // Adh1 // alcohol dehydrogenase 1 (class I) // 3 G3 3 71.2 cM // 1152	1.30E-29	3.11E-07	0.007	1.5
9	NM_023805 // Slc38a3 // solute carrier family 38, member 3 // 9 F1 9 63.0 cM //	3.37E-13	3.17E-07	0.021	-4.2
26	NM_007482 // Arg1 // arginase 1, liver // 10 A4 // 11846 /// U51805 // Arg1 // a	2.27E-28	3.22E-07	0.005	-2.6
17	NM_022882 // Lpin2 // lipin 2 // 17 E1.3 // 64898 /// AF286723 // Lpin2 // lipin	6.26E-16	3.34E-07	0.050	-2.9
13	NM_181280 // Bre // brain and reproductive organ-expressed protein // 5 B1 // 10	2.94E-26	3.36E-07	0.058	-1.2
52	NM_145364 // Akr1d1 // aldo-keto reductase family 1, member D1 // 6 B1 // 208665	9.94E-25	3.45E-07	0.009	-2.8
13	NM_134013 // Psme4 // proteasome (prosome, macropain) activator subunit 4 // 11	1.75E-15	3.47E-07	0.028	-3.9
24	NM_080465 // Kcnn2 // potassium intermediate/small conductance calcium-activated	2.98E-33	3.52E-07	0.039	-2.9
11	NM_009691 // Aplp2 // amyloid beta (A4) precursor-like protein 2 // 9 A2-B 9 13.	3.36E-16	3.63E-07	0.016	-1.9
13	NM_031197 // Slc2a2 // solute carrier family 2 (facilitated glucose transporter)	1.39E-20	3.68E-07	0.014	-4.7
14	NM_010269 // Gdap2 // ganglioside-induced differentiation-associated-protein 2 /	7.47E-14	3.69E-07	0.023	-5.1
18	NM_026780 // Syf2 // SYF2 homolog, RNA splicing factor (S. cerevisiae) // 4 D3 4	2.17E-13	3.85E-07	0.065	-2.8
17	NM_019702 // Hbs1 // Hbs1-like (S. cerevisiae) // 10 A3 // 56422 /// NM_0010425	1.99E-15	3.86E-07	0.018	3.5
76	NM_011942 // Lypla2 // lysophospholipase 2 // 4 D3 // 26394	4.95E-15	3.87E-07	0.087	-2.2
13	NM_007738 // Col7a1 // procollagen, type VII, alpha 1 // 9 F2 9 61.0 cM // 12836	1.25E-13	3.87E-07	0.005	-1.4
17	NM_172296 // Dmrt2 // doublesex and mab-3 related transcription factor like fam	1.00E-50	3.96E-07	0.054	1.7
19	NM_008407 // Itih3 // inter-alpha trypsin inhibitor, heavy chain 3 // 14 A2-C1 /	2.26E-25	4.03E-07	0.133	1.8
	NM_008198 // Cfb // complement factor B // 17 B1 17 18.85 cM // 14962 /// NM_013	3.08E-19	4.04E-07	0.082	-1.4
		7.80E-18	4.05E-07	0.032	-5.1

40	NM_147218 // Abca6 // ATP-binding cassette, sub-family A (ABC1), member 6 // 11	7.04E-32	4.09E-07	0.012	-4.0
8	NM_009696 // Apoe // apolipoprotein E // 7 A3 7 4.0 cM // 11816	3.74E-07	4.12E-07	0.014	-2.0
4	NM_016783 // Pgrmc1 // progesterone receptor membrane component 1 // X A3.2 // 5	9.53E-09	4.22E-07	0.017	-6.1
26	NM_001001144 // Scap // SREBP cleavage activating protein // 9 F2 // 235623 ///	3.37E-26	4.26E-07	0.012	-1.5
13	NM_010279 // Gfra1 // glial cell line derived neurotrophic factor family receptor	1.61E-10	4.28E-07	0.039	-3.9
29	NM_007976 // F5 // coagulation factor V // 1 H2.2 1 86.6 cM // 14067	3.69E-24	4.36E-07	0.044	-4.1
11	NM_008410 // Itm2b // integral membrane protein 2B // 14 D2 14 32.5 cM // 16432	1.32E-15	4.42E-07	0.003	-4.3
17	NM_009804 // Cat // catalase // 2 E3 2 57.0 cM // 12359 /// AK183520 // Cat // c	9.57E-11	4.75E-07	0.010	-3.9
23	NM_010601 // Kcnh3 // potassium voltage-gated channel, subfamily H (eag-related)	6.26E-31	4.91E-07	0.146	1.6
15	NM_016805 // Hnrpu // heterogeneous nuclear ribonucleoprotein U // 1 H4 // 51810	1.44E-16	5.23E-07	0.215	-1.5
18	NM_177616 // 4930562D19Rik // RIKEN cDNA 4930562D19 gene // 11 A1 // 216516 ///	8.78E-15	5.25E-07	0.109	1.4
11	NM_027153 // Pir // pirin // X F5 // 69656 /// BC024062 // Pir // pirin // X F5	8.74E-16	5.25E-07	0.456	-1.2
12	NM_010448 // Hnrpab // heterogeneous nuclear ribonucleoprotein A/B // 11 B1.3 //	3.23E-14	5.25E-07	0.077	-2.3
17	NM_011112 // Papola // poly (A) polymerase alpha // 12 F1 // 18789 /// U58134 //	2.59E-18	5.30E-07	0.018	-3.4
8	M84563 // Pnp // purine-nucleoside phosphorylase // 14 B-C 14 19.5 cM // 18950	2.74E-12	5.44E-07	0.026	-2.0
15	NM_024229 // Pcyt2 // phosphate cytidyltransferase 2, ethanolamine // 11 E2 //	3.16E-20	5.45E-07	0.044	1.6
19	NM_199469 // Nploc4 // nuclear protein localization 4 homolog (S. cerevisiae) //	8.86E-17	5.54E-07	0.041	-2.3
12	NM_007954 // Es1 // esterase 1 // 8 C5 8 43.0 cM // 13884 /// AK050444 // Es1 //	9.87E-20	5.65E-07	0.098	-3.4
16	NM_025959 // Psmc6 // proteasome (prosome, macropain) 26S subunit, ATPase, 6 //	6.00E-12	5.76E-07	0.028	-5.0
12	NM_010476 // Hsd17b7 // hydroxysteroid (17-beta) dehydrogenase 7 // 1 H3 // 1549	2.46E-12	5.80E-07	0.119	-3.0
18	NM_013799 // Ate1 // arginine-tRNA-protein transferase 1 // 7 F3 // 11907 /// NM	1.00E-09	5.90E-07	0.429	-1.2
5	NM_019444 // Ramp2 // receptor (calcitonin) activity modifying protein 2 // 11 D	6.35E-11	6.00E-07	0.347	1.1
20	NM_133198 // Pygl // liver glycogen phosphorylase // 12 C2 12 30.0 cM // 110095	1.87E-23	6.20E-07	0.046	-4.1
16	NM_007514 // Slc7a2 // solute carrier family 7 (cationic amino acid transporter,	2.29E-20	6.37E-07	0.018	-3.0
10	NM_007809 // Cyp17a1 // cytochrome P450, family 17, subfamily a, polypeptide 1 /	3.54E-10	6.56E-07	0.021	-1.9

Table 5.6 shows analytical results for all 329 fixation dependent alternative splicing gene transcripts. This is quite a big list and there various types of gene products present (eg. transcription factors, nuclear proteins), but in order to verify this data more experimental work is required.

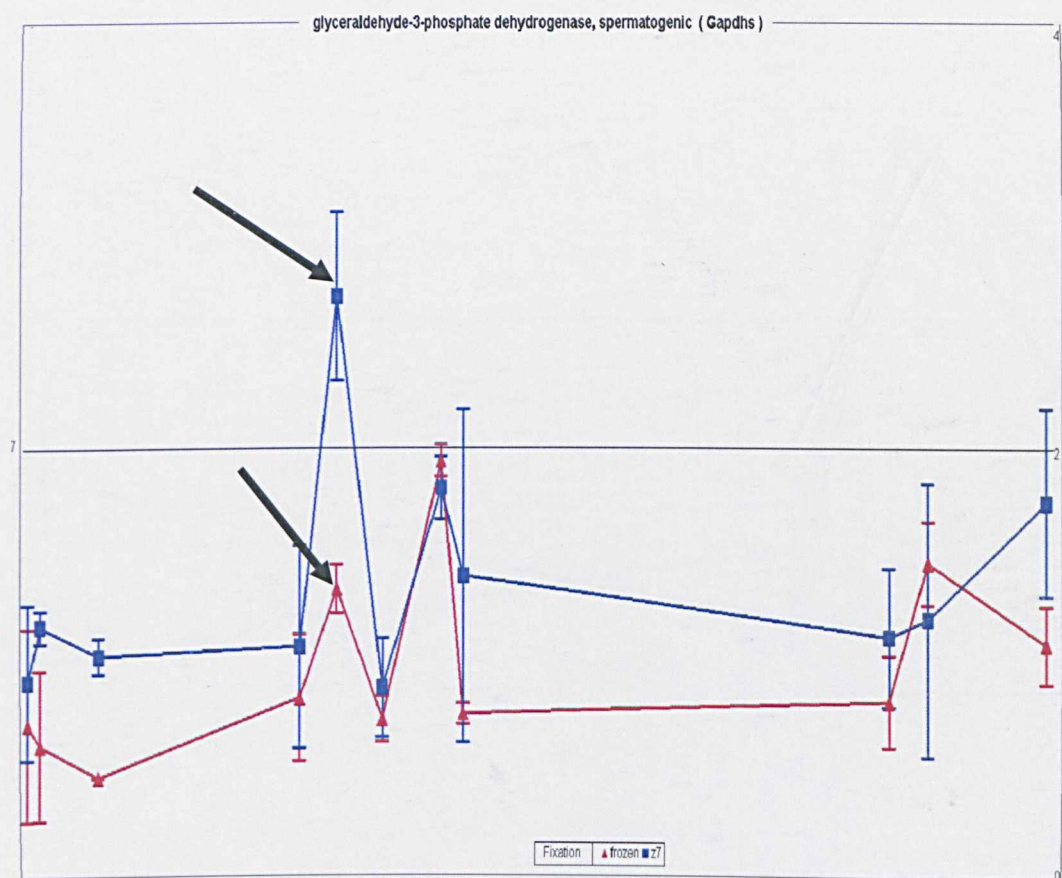
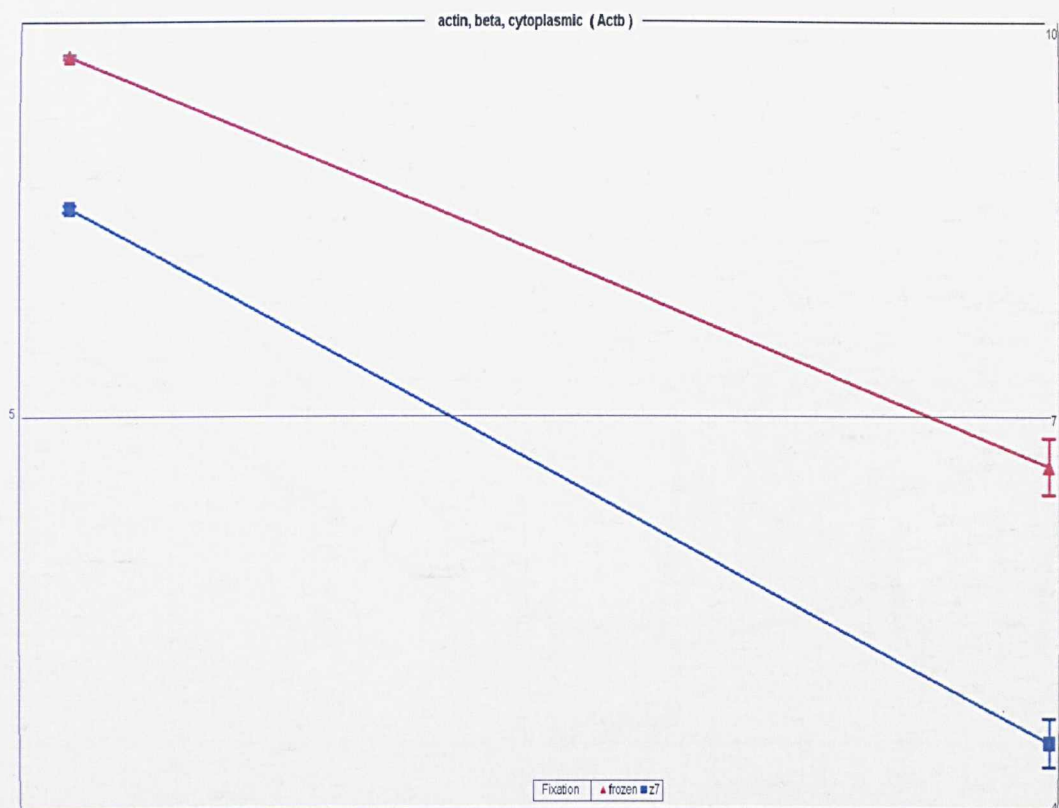
Housekeeping genes (Actb, Gapdhs and Mrps15) were not found to be differentially expressed between Z7-fixed *versus* unfixed frozen samples. From these 3 transcripts only Mrps15 showed significant FIAS. Table 5.8 below summarises the data generated by those three housekeeping genes and also gene p53.

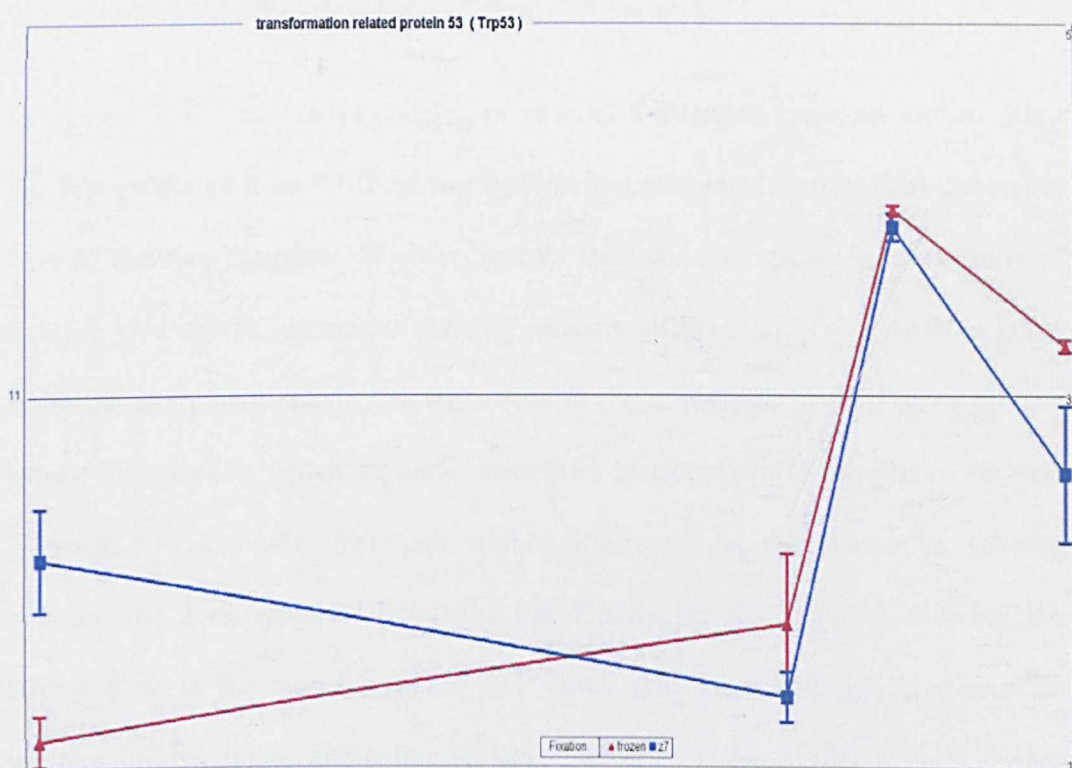
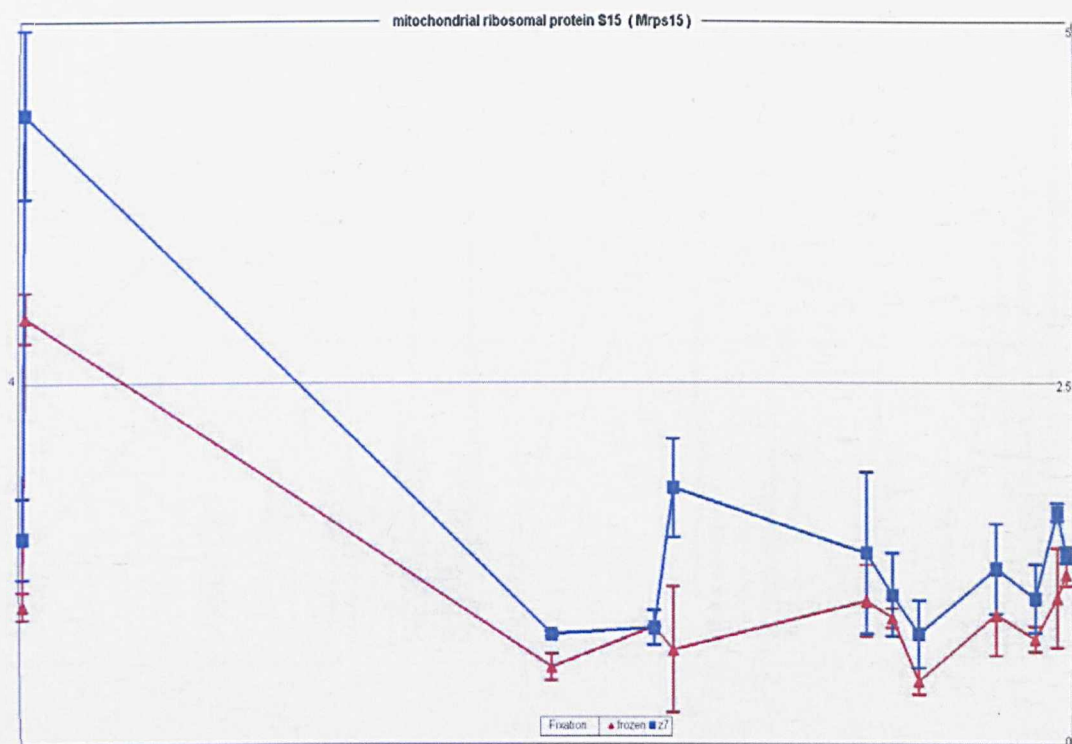
Table 5.7: Alternatively splicing from 3 housekeeping genes and p53.

Gene Symbol	#Probe sets	Transcript ID	Fixation Method p-value	FIAS p-value	FDAS p-value
Actb	2	6942854	1.69E-02	3.50E-03	1.57E-01
Gapdhs	11	6966309	1.58E-01	6.85E-04	2.59E-01
Mrps15	12	6917125	2.13E-01	3.03E-09 *	2.23E-01
Trp53	4	6782031	1.41E-01	1.16E-04	4.22E-02

* significant after multiple correction

When looking at the strict performance specifications, a drop in performance was seen in the gene expression of the Z7 versus the fresh-frozen (eg. lower %P). Although some transcriptional information is expected to be lost or inaccurately captured, due to Z7 tissue's compromised RNA compared to that of fresh frozen tissue, the microarray results in this small pilot indicate that there is still useful biological information that can be obtained from these samples. This is supported by both the fact that there were no genes identified as significantly differentially expressed between the Z7-fixed and fresh-frozen samples, and the fact that 95.5% of the transcripts that were alternatively spliced were detected regardless of their fixation method.





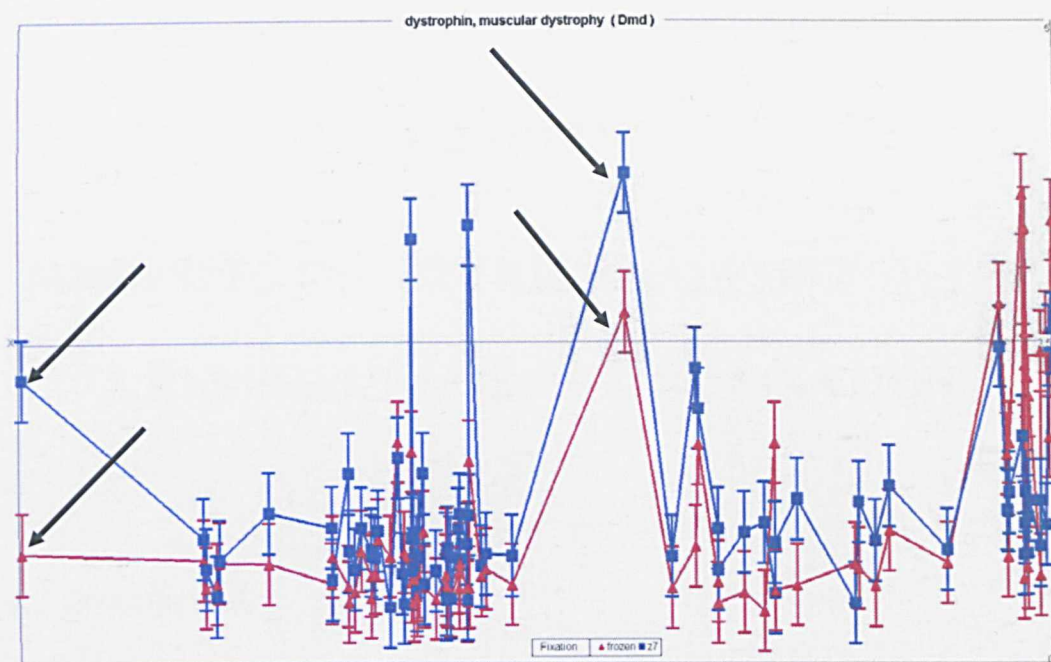


Figure 5.7: Alternative splicing patterns of 3 housekeeping genes, p53 and dystrophin from both Z7-fixed and fresh-frozen samples.

In figure 5.7, the alternative splicing patterns of 5 different genes are shown using the data generated from Z7-fixed and fresh-frozen samples. It can be seen that in the case of the first diagram, which represents the beta actin gene, both methods of fixation give similar alternative splicing patterns. In the case of the GAPDH gene, again the alternative splicing pattern between the 2 methods of fixation is similar, except for one exon, which is pointed with thick black arrows. In the case of the next 3 genes, S15 and p53, there are visible differences in the alternative splicing between the 2 different Z7-fixed and FF. Finally the last diagram showing the largest gene in the mouse genome and shows that there both Z7-fixed and FF samples follow similar alternative splicing patterns. These results warrant further investigation with a better designed study using a larger number of samples that will assess not only how well the Z7 fixation method can differentiate between biological states/levels of gene expression but also how well it can differentiate disease from normal states in comparison to fresh-frozen and NBF-fixed samples.

CHAPTER 6

MASS SPECTROMETRIC ANALYSIS FOR THE CHEMICAL BASIS OF ZINC FIXATION

6.1 Introduction

MALDI-MS is a powerful tool for the analysis of various biomolecules. Lehmann et al, showed that MALDI-MS can serve as a fast method suitable for studying the metal binding properties of zinc-finger peptides (Lehmann, Zenobi, & Vetter 1999).

6.2 Aims and Experimental Design

Zinc trifluoroacetate salt, was the key ingredient in the Z7 fixative. The Z7 fixative produced the best results in terms of tissue morphology compared to all other zinc-based salts and other metallic ions and nucleic acid preservation compared to NBF and all the other zinc-based fixatives. The aim of this experiment was to test whether the addition of this salt to a solution containing a synthetic peptide would generate any interaction between zinc and any of the amino acids of the peptide.

For that reason a synthetic peptide was chosen which consisted of 7 amino acids and its sequence was: N terminus- **Arg-Cys-His-Gly-Lys-Met-Phe**- terminus C.

The molecular formula of the custom peptide was $C_{37}H_{59}N_{13}O_8S_2$ with a relative molecular mass of 878.32. This peptide was custom made by BachemTM(UK), with

a purity >96% after high performance liquid chromatography (HPLC), and was delivered in a white powder that was soluble to water.

This synthetic peptide was designed using a number of different types of amino acids, and also including a cysteine and a histidine, since it is known that zinc binds those 2 amino acids in zinc-binding proteins. For purpose of ease the above custom peptide with a mass of 878 will be called: **peptide A**. Also the salt zinc trifluoroacetate will be termed: **Zinc** in the rest of this Chapter.

Peptide A was diluted in ddH₂O and Zinc was added to the solution containing the peptide. The reaction between the peptide and the zinc trifluoroacetate was for 24hours, 48hours, 72hours and 1 week. This was done in order to investigate the effect of time on the zinc-peptide chemistry. All reactions took place in 0.2ml Eppendorf tubes at room temperature (22°C-25°C). The reactions were stopped by placing the Eppendorf tubes at -20°C. All experiments were done in triplicates in order to verify the results.

The mixture of peptide A and zinc trifluoroacetate was in 1:1 molar ratio. The concentration of zinc trifluoroacetate was 11.7mM. This is the same concentration of zinc trifluoroacetate that was used for preparing the Z7-fixative solution and was therefore chosen for the mass spectrometry experimental work. So, in order to have a 1:1 molar ratio, the concentration of peptide A was also 11.7 mM. This was done so that all the zinc trifluoroacetate would react with peptide A. Furthermore, in order to improve on the reaction between zinc-peptide, we increased the molar ratio from 1:1, to 1:5, 1:10 and 1:20. This was done in order to investigate the effect of excess zinc on the zinc-peptide interactions.

6.3 Results

Figure 6.1 below shows a Mass Spectrum of peptide A without any Zinc added, which was used as a reference (control) sample in all the experiments. It can be seen that a main peak around 878 which corresponds to the mass of peptide A.

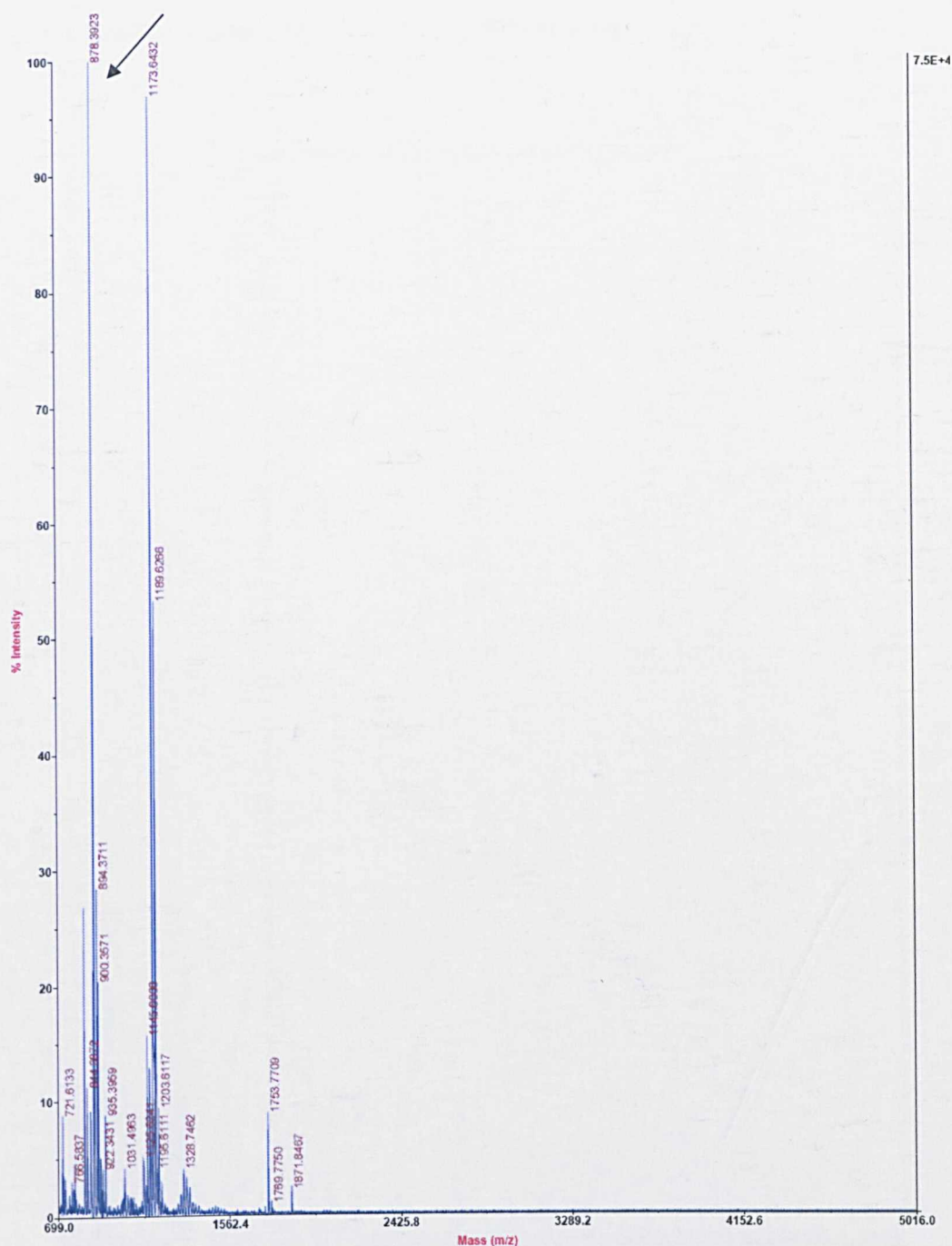


Figure 6.1: Control sample (only peptide A). Black arrow indicates the peptide molecular weight mass (878).

From figure 6.1, the peak that is indicated with a black arrow represents the mass of the peptide, which is approximately 878. There are also other peaks each with a different mass shown in figure 6.1. These peaks could be due to different isoforms of peptide A, that are present. Also it could be due to the fact that peptide A is not

100% pure and is possible that other chemical groups become attached to it and thus give a number of peaks each one with a different mass.

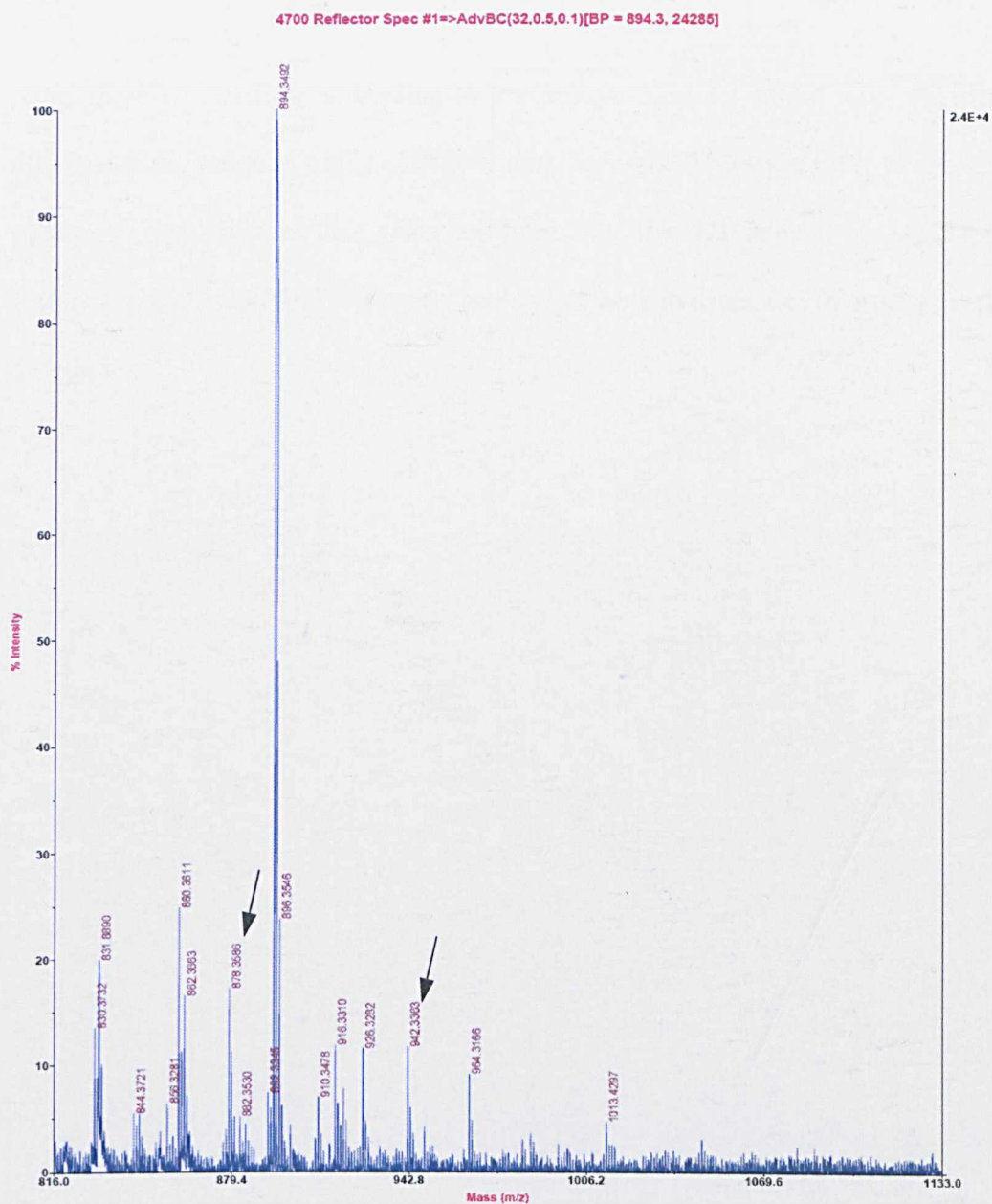


Figure 6.2: Mass Spectrometry analysis using an Applied Biosystems 4700 Proteomic Analyser (Foster City, CA), after 24 hours incubation at room temperature and using a 1:1 molar ratio. Note the two important peaks: One which is peptide A (with a mass of 878.3566) and the second peak is the peptide A + Zinc, which gives a mass of 942.3363.

In figure 6.2 there are two peaks indicated by black arrows. One represents again the mass of peptide A only (negative control) and the other peak with a mass of approximately 941 represents the mass of peptide A with the mass of a zinc atom.

After showing that Zinc is binding to the peptide A at 1:1 molar ratio we tested different molar ratios as well as different time intervals. Below are some of the mass spectrum graph showing data generated from 24h, 48h, 72h and 1:1 (1x), 1:5 (5x), 1:10 (10x), and 1:20(20x). All experiments were done in triplicates in order to verify the results.

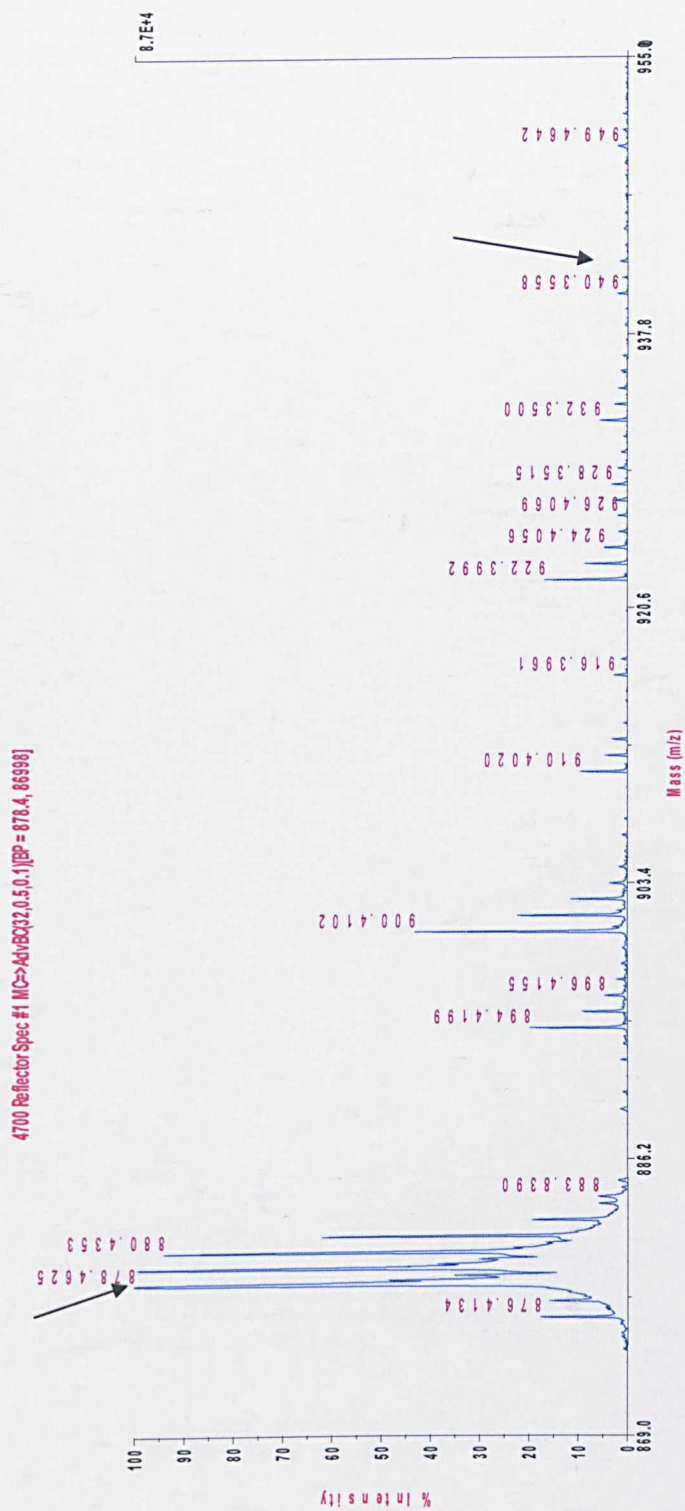


Figure 6.4: Representative mass spectrum graph with peptide A + Zinc at 1x and 24h. Note the two peaks, corresponding to the peptide A only and the peptide+Zn.

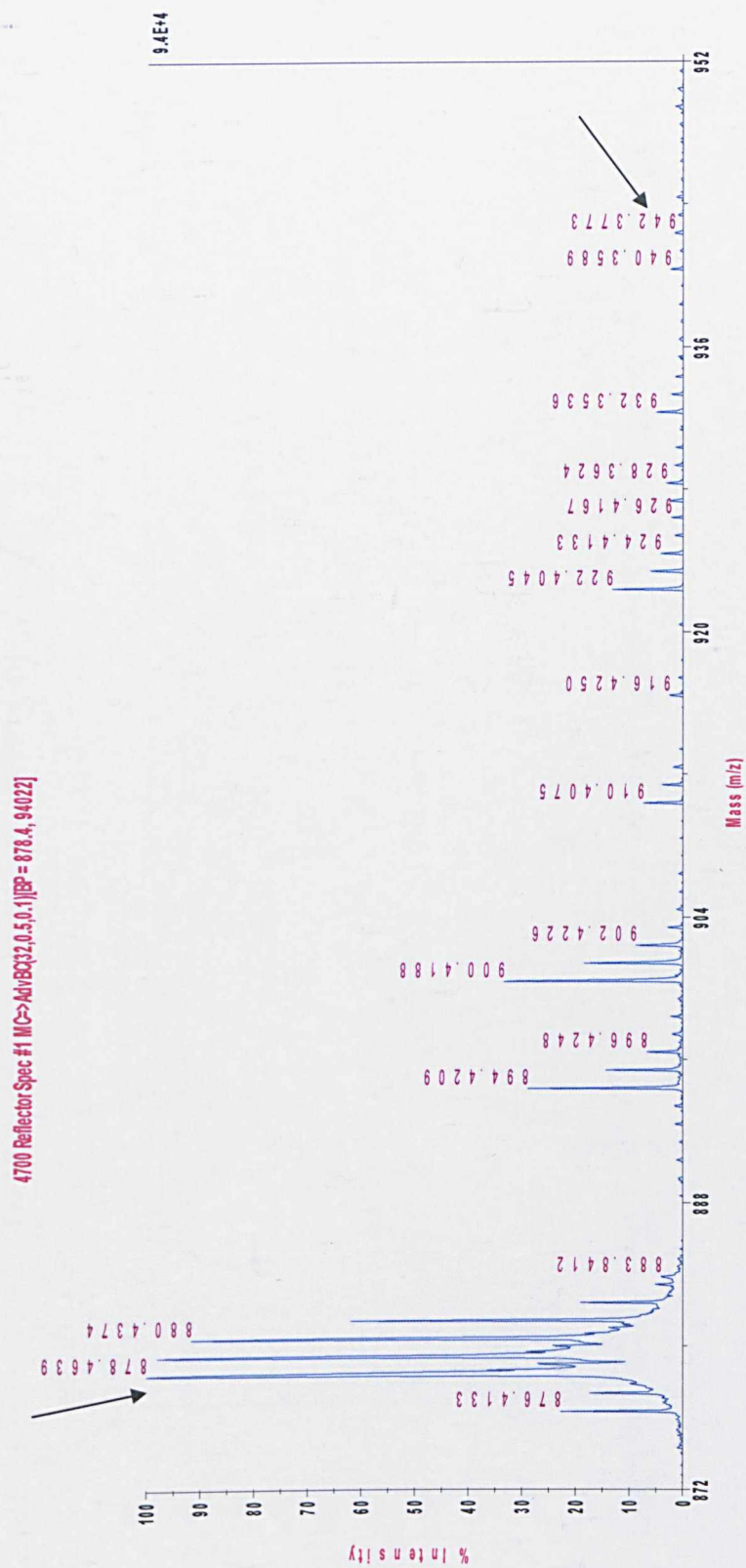


Figure 6.5: Representative mass spec graph showing peptide A + Zinc x 5, after 24h reaction.

4700 Reflector Spec #1 MC=>AdvBC(32,0,5,0,1)[BP=878.4,81868]

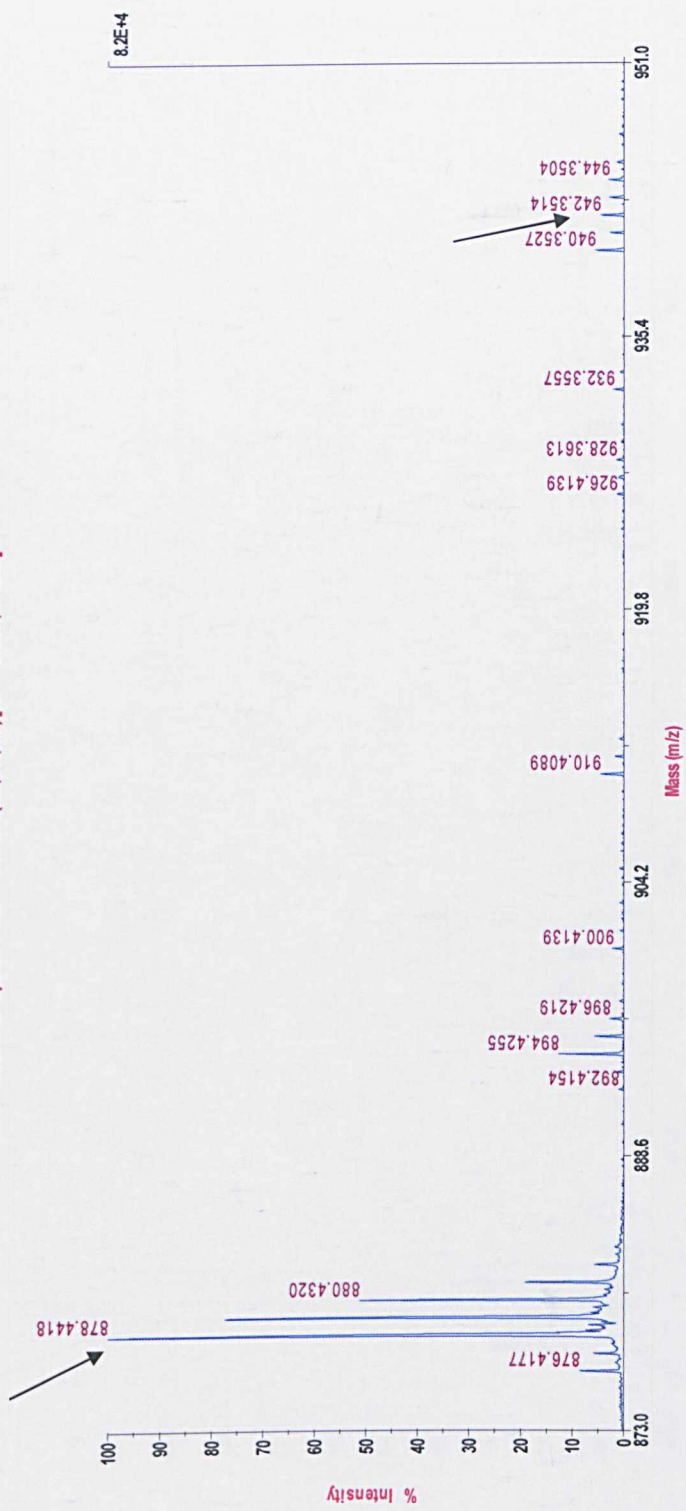


Figure 6.6: Representative mass spec graph showing peptide A with Zinc x 10, after 24 h reaction.

4700 Reflector Spec #1 MC=>AdvBC(32,0,5,0.1)[BP=878.4,87745]

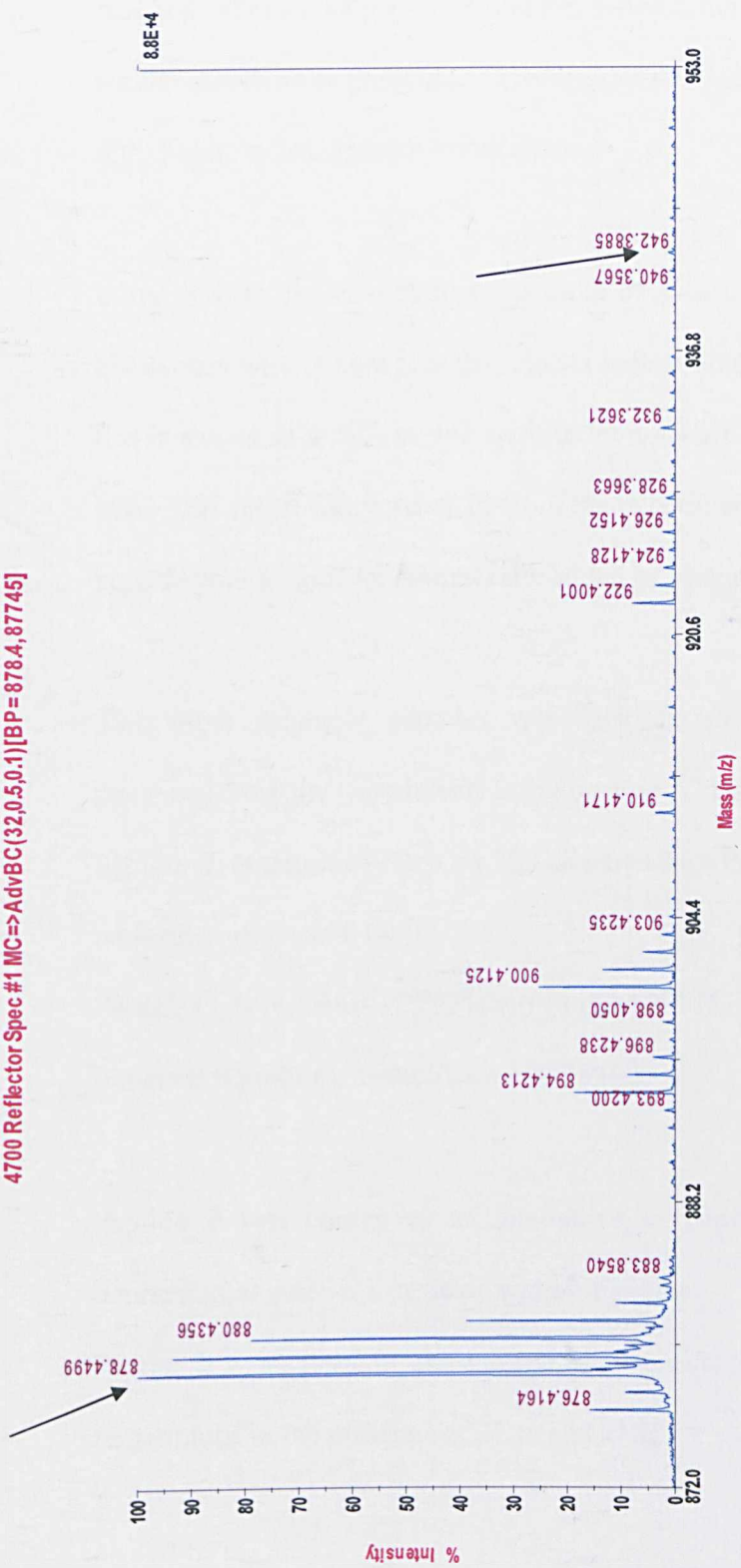


Figure 6.7: Representative mass spectrum graph showing peptide A + Zinc at x 20, after 24h reaction.

All experiments were duplicated and 3 sets of samples were tested in order to verify the results. From the above mass spectrometry graphs shown in figures 6.3-6.7, it can be said that the increase of Zn molarity did not have any visible effect on the reaction between the peptide A and Zn. When different time intervals were tested similar results were produced. This suggests that increasing the time of the reaction does not have any positive effect either.

Initial data so far showed that one atom of Zinc binds to peptide A. From all the above data we can conclude that zinc is indeed binding to the synthetic peptide, and this is shown as a peak at 942 addition of a mass of 64.5 to the mass of the peptide 878. This result was verified in all of the experiments performed using the synthetic peptide with no zinc trifluoroacetate added as a negative control sample.

Two more synthetic peptides were also tested following the positive results generated from the experiments using peptide A. These peptides were:

Peptide B: N terminus-Ala-Cys-His-Gly-Lys-Met-Phe-Arg- terminus C,
molecular mass: 948.44

Peptide C: N terminus-PPPPPHisPPPPPCysPPPPHisPPPPCysPPPPP-C terminus
(where P = proline), molecular mass: 2596.3.

Peptide B was chosen as an alternative to **peptide A**, that could enhance the interaction of zinc with cysteine and/or histidine

Peptide C was chosen in order to see whether zinc trifluoroacetate can create a zing-finger motif in the presence of 2Cys and 2His.

The preliminary results for this part of the MS analysis, showed no binding of zinc with any of the amino acids of peptide B and C. Possible reasons for this are discussed in Chapter 7.

When we combined an increase in reaction time with an increase in the zinc molar ratio to peptide A, again no increase was shown in any of the peaks produced. The reason for these findings could be due to the fact that the nature of the zinc interaction with the peptide is quite weak and so zinc is easily lost during the preparation of the samples for the MS experiments.

In conclusion, it was not possible to demonstrate from there results to which specific amino acid the zinc is binding, but calculations from the fragmentation pattern of peptide A after MS/MS, showed that zinc is either binding to cysteine or histidine. Further work is required to verify which amino acid zinc is binding. There is also the possibility of zinc binding both amino acids, creating a Cysteine-Zn-Histidine motif.

CHAPTER 7

DISCUSSION & FUTURE DIRECTIONS

7.1 Discussion

The aim of this thesis was to develop a tissue fixative that would preserve tissue architecture, nucleic acid and protein integrity, be low cost, non-toxic and applicable for routine molecular biology and histology protocols. This project was therefore initiated by comparing two fixatives (Z2, HOPE) that were shown according to the literature to be the most promising candidates for a 'universal fixative'.

Fixation is a series of complex chemical modifications of macromolecules present in tissues and cells, to preserve structural and functional components as closely as possible to the living state while inhibiting autolysis, bacterial and fungal decay (Bancroft & Gamble 2002). Short DNA and RNA sequences can be retrieved from conventionally NBF-fixed pathology material, but good, long-term preservation of intact nucleic acids and of protein integrity is necessary to meet the increasing number of molecular diagnostic and research techniques which are becoming available.

HOPE is a commercially available compound and it has been shown that it enables the extraction of good quality nucleic acids (Wiedorn et al. 2002) and protein (Uhlig et al. 2003). The exact components of the HOPE and their representation in this fixative are not known. On the other hand, zinc ions have been used as additives for formalin based fixatives in the past, but not until 1994 was it shown that a simple solution containing zinc salts alone could be used successfully for IHC of several antigens (Beckstead 1994). More recently, Wester et. al. (2003) have shown that a zinc based fixative preserved good morphology and DNA integrity in human tissue.

Results from the comparison between Z2 and HOPE, showed that Z2 was a better candidate for further investigation. This was because morphology was better preserved with Z2, IHC gave better results without antigen retrieval and the DNA integrity was equal between the 2 fixatives. Also the cost of HOPE (£200 per lt) is much higher than the cost of Z2 fixative (£5 per lt), and the fact that HOPE fixed samples are fragile and require hand processing was a further disadvantage.

In the next phase of the project a range of chemical modifications was tested. The chemicals added to the Z2 fixative were namely DMSO, DEPC, and EDTA at different concentrations. DMSO has been shown to be a valuable non cryogenic tissue preservative for DNA extraction (Kilpatrick 2002) and was tested on that basis. DEPC is a mild cross-linker and was suggested by personal communication with Miss Susan Van Noorden as a way to improve fixation. EDTA is a chemical that is present in *RNAlater* and was suggested on the basis of improving the preservation of RNA from fixed tissue. EDTA is a chelating agent and therefore the percentages at which it was used were low (<7.5% v/w).

As well as additions to the zinc formula, modifications were also tested. These included replacing zinc acetate with other zinc salts such as zinc trifluoroacetate, zinc citrate or zinc tartrate at the same concentration. This was done in collaboration with Prof. Alan Armstrong from the Chemistry Department of Imperial College. The rationale for these modifications was the fact that a negatively charged compound such as trifluoroacetate, citrate or tartrate would bind with the double positively charged zinc more consistently and allow it to act faster and with better penetration in tissue.

From the data generated in Chapter 6 of this Thesis, where the tandem mass spectrometry technique was used to identify possible chemical interactions between small synthetic peptides and zinc trifluoroacetate, I can hypothesize how Z7 fixative works in a biochemical level. My hypothesis is that zinc atoms bind specifically to two amino acids, namely, cysteine and histidine. It is possible that zinc is either binding with one of those two amino acids, or it could be that zinc is binding to both of them at the same time, generating a cysteine-zinc-histidine motif.

All the modifications to the zinc-based fixative recipe showed improved quality in terms of morphology and greater quality and quantity of DNA and RNA preservation compared to the standard zinc-based fixative (Z2). The addition of 5% DMSO to the Z2 fixative (Z4 recipe) markedly improved the morphological structure and nucleic acid and protein preservation. However, the addition of DMSO to the Z7 recipe did not produce any improvement in morphological quality and molecular preservation. Modifications to the zinc-based recipes included replacing zinc acetate with zinc trifluoroacetate (Z7 recipe), zinc citrate, zinc tartrate, zinc isovalerate and zinc trifluoromethylsulfonate at the same concentration. The rationale for these modifications was the fact that the different counterions were expected to lead to different degrees of binding to the zinc and different levels of hydrophobicity, effectively allowing zinc to act faster and with better penetration into the tissue. When Z4 and Z7 were compared, Z7 was found to be a better overall fixative in terms of improved nucleic acid, protein integrity and morphology, although the quality of RNA obtained from the RNA Bioanalyser was not as good as that obtained from fresh-frozen samples. This is likely to be due to the fact that RNA is degraded throughout the processing stage. Wester et al (2003) indicated that it is the processing and/or storage in paraffin that is responsible for the degradation of RNA. When zinc was replaced with the metallic ions calcium, magnesium, manganese, gallium and vanadium to investigate potential fixative properties, poor results were obtained for morphology and RNA quality and these recipes were abandoned.

Although zinc fixation caused shrinkage in all organs tested so far, none of the zinc-based solutions penetrated the tissue as fully as NBF. This resulted in well fixed edges but apparently not equally well fixed interiors. In view of the fact that tissues fixed in Z7 and taken through to paraffin blocks are significantly better for molecular studies from archival blocks compared to NBF, we believe that this reduction in observed penetration of the fixative does not result in any harm to the constituents of the preserved tissues and may actually reflect normal fixation in the interiors and possible over-fixation at the edges.

Personal communication with Ken Blight from the electron microscopy unit of Cancer Research UK, Lincoln's Inn Fields, where a small number of Z7-fixed samples were tested against NBF-fixed samples, showed that the rate of penetration of the Z7 is equal to NBF (1mm²/hour, at 20°C).

Results from the second part of this project (Chapter 4), showed that our Z7 recipe provided the best fixative in terms of DNA, RNA and protein preservation and stability, for morphology and for archiving. DNA fragments up to 2.4 kb were successfully amplified from Z7-fixed samples, compared to 0.6 kb from NBF-fixed samples. In addition, the ability to amplify cDNA fragments up to 361 bp in length was demonstrated from Z7 fixed samples, while from NBF fixation no product was detectable. Total protein could be analysed successfully by 2-dimensional gel electrophoresis following Z7, whereas NBF-fixed samples were heavily degraded and produced no visible results.

The DNA quality of Z7-fixed archival stored samples was similar to freshly fixed and processed samples, and significantly better than that found with NBF fixative. This could be a very important finding, because it demonstrates the ability of Z7-fixed tissues for archival use, since NBF-fixed archival material is not always suitable for nucleic acid/genomic analysis.

In the next phase of our project the new Affymetrix platform for exon arrays (GeneChip Mouse Exon 1.0 ST) was employed. For the experimental design, Z7-fixed samples of mouse liver were tested and compared with fresh-frozen samples. From the results generated it was shown that although the RNA quality from Z7-fixed samples is reduced compared to fresh-frozen samples, it is still possible successfully to perform whole genome arrays. Meanwhile, conducting microarray analysis on NBF-fixed samples can be problematic (Paik et al. 2005).

Alternative splicing is a major source of protein diversity for higher eukaryotic organisms, and is frequently regulated in a developmental stage-specific or tissue-specific manner. Current estimates suggest that 50 to 75 percent (or more) of human genes have multiple isoforms. Splice variants from the same gene can produce proteins with distinct properties and different (even antagonistic) functions. In addition, a number of genetic mutations involved in human disease have been mapped to changes in splicing signals or sequences that regulate splicing. Thus, an understanding of changes in splicing patterns is critical to a comprehensive understanding of biological regulation and disease mechanisms (Gardina et al. 2006)

In addition to gene expression regulation, it is evident that alternative splicing plays a key role in biology:

a) Tissue-specific and developmental stage-specific alternative splicing contributes to significant protein diversity.

b) Disease-related deregulation of splicing may be critical in pathogenesis and contribute to disease diversity and complexity.

Also by showing that there was no significant difference in the alternative splicing pattern between Z7 and fresh-frozen samples it was shown that this improved fixative could prove useful in answering important biological questions such as gene and exon rearrangements.

One must bear in mind that due to the high cost of this new platform, we tested only a small number of samples. To generate publishable results, 4 samples from 5 different tissues, for each preservation method i.e. Z7, NBF and fresh-frozen, would be required. That translates to a total of 60 samples and the cost for such experimental work would be around £30,000 with current prices for microarray reagents, hybridisation and mouse exon chips.

The results from mass spectrometric analysis using the MALDI Applied Biosystems 4700 Proteomics Analyser with TOF optics, have opened a new dimension in the understanding of how the Z7 fixative works. There are several reports demonstrating that in zinc finger motifs, zinc molecules interact with the amino acids histidine and cysteine, generating distinct 3-dimensional structures (Berg & Shi 1996; Kikuchi, Komatsu, & Nagano 2004; Thompson 2005), but the question of whether zinc-based fixatives generate similar chemical interactions remains very much unanswered. Our preliminary results showed that when adding zinc trifluoroacetate into a ddH₂O solution containing a custom peptide (mass approximately 878 daltons) using a 1:1 molar ratio, zinc is seen indeed binding a 7 amino acid peptide and, more specifically, we demonstrated that zinc is binding either to cysteine or to histidine. The failure of our subsequent trial with peptides B and C is probably due to two

main reasons: First both of the purity of these peptides was around 70% and therefore required further purification before re-testing. Second, the specific conditions that these peptides might require in order to bind with zinc ions might be different from the conditions that were used in the case of peptide A.

Knowledge of the specificity of zinc for binding specific amino acid residues could enable researchers to chemically improve zinc-based fixatives. In addition, we might be able to predict by knowing the amino acid sequence of specific proteins of interest especially for IHC diagnosis, how well zinc fixation will work for the specific protein. In addition, by knowing the amino acid sequence and the tertiary structure of specific proteins of interest, especially for IHC diagnosis, we might be able to predict how well zinc fixation will work for those proteins.

7.2 Future Directions

The work described in this thesis is the story of the scientific development of a new zinc-based fixative, followed by the evaluation of its potential to preserve morphological, nucleic acid and protein integrity of fixed-tissues. Furthermore, the application of the Z7 fixative to state-of-the-art techniques such as exon arrays for examining the whole genome was attempted, followed by mass spectrometric work to elucidate the possible chemical reactions between zinc trifluoroacetate and synthetic peptide/s. Some of the results from this thesis have been published in the Journal of Nucleic Acids Research (Lykidis et al. 2007). To follow up on those findings the following questions should be attempted in future studies:

1. What is the exact chemical reaction between zinc and peptides/proteins?

To answer this question, the technique of MALDI-TOF MS (Canas et al. 2006; Lehmann, Vetter, & Zenobi 1998; Lehmann, Zenobi, & Vetter 1999) would be required using at least 20 synthetic peptides of different molecular weight. It is expected that this would shed light on the biochemical interaction between zinc salts and important tissue constituents like proteins.

2. Can we employ Z7 for human genome microarray studies?

The Affymetrix Exon platform (GeneChip Human Exon 1.0 ST) should be applied to a range of human normal and tumour samples that are fixed in NBF, Z7 and fresh-frozen. This experimental model would enable the direct comparison between the different modes of preservation, as well as a comparison for each preservation method between normal and tumour samples.

3. Can we improve on the morphological preservation after Z7 fixation?

The process of histopathology, comprising tissue staining and morphological pattern recognition, has remained largely unchanged for over 140 years. Although it is integral to clinical and research activities, histopathologic recognition remains a time-consuming, subjective process to which only limited statistical confidence can be assigned because of inherent operator variability. Although immunohistochemical approaches allow limited molecular detection, significant challenges remain in using them for quantitative, automated pathology.

In the last decade, advances in methodologies for histopathology diagnosis have included techniques such as FLIM (Elson et al. 2004; Munro et al. 2005; Requejo-Isidro et al. 2004; Siegel et al. 2003; Tadrous et al. 2003) and Fourier transform infrared (FTIR) (Fernandez et al. 2005). Vibrational spectroscopic approaches, by contrast, directly provide non-perturbing molecular descriptors, but a practical spectroscopic protocol for histopathology is lacking. High-throughput FTIR spectroscopic imaging of tissue microarrays coupled with statistical pattern recognition of spectra indicated endogenous molecular composition and demonstrated histopathologic characterization of prostatic tissue. This automated histologic segmentation was applied to routine archival tissue samples, incorporating well-defined tests of statistical significance and eliminating any requirement for dyes or molecular probes. It was also possible to differentiate benign from malignant prostatic epithelium by spectroscopic analyses (Fernandez et al. 2005). Z7-fixed samples could possibly be used with FTIR for even better histologic analysis of normal and malignant human tissues.

These innovations, along with continuous improvements in molecular diagnostics, such as Real-Time PCR and microarrays, indicate that tissue fixation for the purpose of dye staining alone will be outdated in the future.

In animal material, perfusion fixation should be tried for better penetration of the fixative. For human material, possibly the use of gentle heat combined with the fixative might help. Studies on zinc fixation of cultured whole cells could also provide more data on the effect of fixation on morphology.

4. Rapid processing systems, fast turnover time, can zinc be used for it?

The advance of new rapid processing systems such as PelorisTM from Vision Biosystems, could be a very useful way to test the application of our Z7 recipe for rapid processing experiments. These protocols might provide a way of further reducing the effect of processing on the RNA quality or of testing the effect of processing on RNA quality compare to standard processing protocols.

5. What is the effect of processing on RNA quality?

Another problem during the tissue fixation is to do with the effect of processing on DNA and RNA integrity. There are very few studies to date that have investigated the effect of processing on tissue integrity and especially on RNA and DNA quality. We propose to investigate this by comparing the effect of processing on RNA quality of Z7, NBF, 70% EtOH fixed samples from 3 different organs with fresh-frozen samples. For that reason we will have two ranges of fixed samples: fixed processed samples and fixed un-processed samples. We have generated preliminary data using mouse liver and gut after 4h, 8h, and 24h of fixation where half of the samples were processed after fixation while the other half tissue was fixed, frozen in liquid N₂ and stored at -80°C. Further work would be required to verify this preliminary study.

In conclusion, this Thesis presents the development of a novel zinc-based fixative that offers significant advantages over current fixation methods. An 'ideal fixative' suitable for preserving nucleic acid, protein integrity and tissue morphology is a highly desirable requirement for molecular biological analysis of tissues and cells. Our results show that Z7 is a cheap, easily prepared and very effective fixative that

provides significantly improved preservation of DNA, RNA and proteins and allows improved PCR, RT-PCR and protein analysis.

It enables the use of fixed and paraffin-embedded tissues for high quality DNA, RNA and protein studies as well as preserving the morphological integrity of tissue. It is a cost- effective formula, without any toxic effects and can be used routinely with standard histological procedures (Lykidis et al. 2007). The applications of such a novel fixative could be numerous, especially considering the high cost of frozen tissue banks and the carcinogenicity of formaldehyde. This new fixative may provide an excellent alternative to NBF for contemporary molecular pathobiology research.

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