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CRYOPRESERVATION AND GENETIC DAMAGE

A thesis presented in partial fulfilment of
the requirements for the degree of
Masters of Science in Genetics
at Massey University, Palmerston North,
New Zealand

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2005

ABSTRACT

Blood analysis is time consuming and laboratories may require methods of storing samples until time permits analysis. The effects of storage on the sample are mostly unknown, yet some laboratories commonly store blood samples to allow processing of samples in batches. Cryopreservation is proposed as a convenient means of preserving blood samples, as the associated cold temperatures render cryopreservation an ideal storage method for tests requiring viable cells. In the literature, few studies have explored whether cryopreserved *whole* blood samples can be utilised effectively for cytogenetic testing.

This study extended the work on cryopreservation of blood to observe the cytogenetic effects of storing *whole* blood samples for an extended period. In this study three cytogenetic tests: Sister Chromatid Exchange (SCE), Micronucleus Assay (MN) and Fluorescence *in situ* Hybridisation (FISH) were conducted on whole blood samples from ten participants to observe whether the results from the cytogenetic tests are statistically consistent over a prolonged period of cryopreservation (fresh, one month, three months and six months). These tests were conducted on a single blood sample cryopreserved from each participant.

The results indicated that cryopreservation of whole blood is not a reliable method for storing blood samples prior to cytomolecular tests. The culturing of lymphocytes from cryopreserved blood was found to be inconsistent and the lymphocyte viability after cryopreservation reduced. When lymphocytes were successfully cultured, SCE and MN demonstrated increased genetic damage after a period of cryopreservation ($P = <0.050$ and $P = 0.016$ respectively) but FISH was not successfully performed on cryopreserved blood samples. It is unclear from the results obtained whether cryopreservation actually induces genetic damage or if the observed damage was the result of the specific storage technique.

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge and give my sincere thanks to my research supervisor, Dr Al Rowland. Over the last two years your assistance, input and moral support have been invaluable to me. Thank you.

I am indeed blessed with encouraging parents and friends. I would especially like to thank my Parents, Brad, Divya and Sarah for their full support throughout the highs and lows of this thesis and for tolerating all my 'thesis talk'. I love you all.

This research would not have been possible without the support of numerous skilled individuals. I would particularly like to thank the following people for their contributions:

Liz Nickless, Mohammed Abdul Wahab, Chad Johnson and Louise Edwards for technical advice, problem solving sessions and your company in the lab.

Vicki Scott and Chris Kendrick for the loan of equipment, endless assistance, valuable advice and for drawing numerous blood samples (even after some very difficult patients!).

The team at LA2 radiotherapy, especially Wendy for often squeezing me into your lunch break.

Clive Felix and his team at Wellington Hospital and Janine Gunderson at Palmerston North Hospital for your willing experimental assistance.

The New Zealand Nuclear Test Veterans Association and their president Roy Sefton.

Associate Professor John Podd for your assistance with experimental design.

Ted Drawneck for the statistical analysis of my results.

The William Georgetti Scholarship, New Zealand Federation of Graduate Women Scholarships, Massey Scholarship, Massey Masterate Scholarship, Goodman Family Scholarship, Molecular Genetics Research Scholarship and the Palmerston North Medical Foundation for funding.

ABBREVIATIONS

BrdU-	5-bromo-2-deoxyuridine
CB-	Cytokinesis-blocked cells
cm-	Centimeter
Cu-	Copper
dH_2O-	Distilled Water
DMSO-	Dimethyl Sulfoxide
DNA-	Deoxyribose Nucleic Acid
EBV-	Epstein Bar Virus
et al-	*Latin*, and others
EthD-1-	Ethidium Bromide Homologue
ex/em-	Excitation/Emission spectra
FISH	Fluorescence *in situ* Hybridisation
γ-	Gamma
g-	Gram
G-	Gauge
GHz-	Gigahertz
Gy-	Gray
H_2O-	Water
HCl-	Hydrogen Chloride
HCR-	Host-Cell Reactivation Assay
HIV-	Human Immunodeficiency Virus
Hrs-	Hours
KCl-	Potassium Chloride
L-	Litre
L N_2-	Liquid Nitrogen
μg-	Microgram
μL-	Microlitre
μm-	Micrometer
μM-	Micromolar concentration in moles/litre
MB-	Megabytes
M-	Molar concentration in moles/litre

M_a-	The mean number of micronucleated cytokinesis-blocked cells per 100 cytokinesis blocked cells
M_b-	The mean number of micronuclei per 100 cytokinesis-blocked cells
mg-	Milligram
mins-	Minutes
ml-	Millilitre
mm-	Millimeter
Mn-	Manganese
MN-	Micronucleus Assay
MqH_2O-	Milli-Q water
mRNA-	Messenger Ribose Nucleic Acid
N-	Sample Size
NaCl-	Sodium Chloride
NaOH-	Sodium Hydroxide
Nm-	Nanometer
No.-	Number
NZNTV-	New Zealand Nuclear Test Veterans
PBMC-	Peripheral Blood Mononucleate Cells
PHA-	Phytohaemagglutinin
PVP-	Polyvinylpyrrolidone
RAM-	Random Access Memory
rpm-	Revolutions per minute
secs-	Seconds
SCE-	Sister Chromatid Exchange
Std. Dev.-	Standard Deviation
Std. Error-	Standard Error
TM-	Thawing Media
UV-	Ultra Violet Light
W-	Watt
WBC-	White Blood Count
Zn-	Zinc

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CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

Researchers at Massey University are currently conducting an investigation into whether New Zealand military personnel who witnessed the British atomic bomb tests at Christmas Island and Malden Island in 1957-58, known as Operation Grapple, have incurred any genetic damage. All cytogenetic tests are currently conducted on fresh blood samples in accordance with the accepted international protocols. It is vital to determine if these tests can be performed on cryopreserved blood samples, as many of the test veterans are ill; most notably with blood cancers, and of the 551 men who witnessed the bomb tests fewer than half are alive today. Time is therefore crucial to collect and analyse all samples. For this purpose and other similar studies it would be beneficial to store the blood samples with the guarantee that the results from the tests will be identical to tests performed on fresh blood samples. Another advantage of storing blood samples for this form of study is that samples can be collected in the field where sample processing may not be appropriate due to unsatisfactory facilities.

This study explores cryopreservation as a method for storing whole blood samples prior to the application of cytomolecular techniques. The main aim is to determine whether cryopreserved whole blood samples maintain statistically similar results to the cytomolecular test performed on fresh (non-cryopreserved) blood samples. The following sections of this chapter explore the previous international research performed in this area of cryopreservation and genetic damage.

1.2 STORAGE BENEFITS

The analysis of blood samples is often time consuming and laboratories may require methods of storing samples until time permits analysis. As the effects of storage on the sample are mostly unknown, current tests are generally performed on fresh blood cells. In some laboratories though, the storage of blood samples, or subsets of blood

cells, is a common practise. This storage is beneficial to allow processing and analysis of the samples in batches.

Sample storage is not only beneficial for ease of analysis in genetic research facilities, but also to assist with medical research. In the medical field it is often difficult to obtain matched samples of ill and healthy patients at the same time, resulting in a delay. In this situation, the blood samples must be stored in an appropriate manner until the matched sample is also obtained. The storage of samples also allows numerous tests to be performed on the same batch of cells to ensure consistent results.

Recently, various methods of storage have been explored in an attempt to allow expanding cellular therapies to be more manageable to patients' schedules (Celluzzi & Welbon 2003). Adequate storage of specimens is able to accommodate sufficient repeated treatments with the same sample, delivered at flexible times, using single batch preparation. Utilising single batch preparations ensures consistency of the cellular therapy.

1.3 STORAGE CONDITIONS

There are numerous methods available for storing blood samples. A method is selected depending on cost, availability and the suitability for the subsequent analysis. The varying storage conditions are found to be adequate for some assays involving blood samples, but unsuccessful for others. Therefore storage conditions must be tested and proven suitable for the required purpose, before research is performed on any variety of stored blood.

Many medical laboratory tests are performed on stored blood samples. Some further testing has explored the cellular effects of the different storage methods. Variations in the levels of blood subsets have been the focus of the majority of these studies, but often the results are conflicting.

In two such medical laboratory studies, the determined optimal storage conditions differed, demonstrating that storage conditions acceptable for one assay are not

necessarily acceptable for all other assays. Gulati *et al* (2002) discovered that haemoglobin and red blood cell counts were stable for up to seven days when the samples were stored at room temperature, but in the same conditions the white blood cell count was not considered stable. Ekong *et al* (1993) found that the optimum storage temperature for the test conditions was 17°C, and at other temperatures changes were observed in the lymphocyte subsets. The combined results from Ekong *et al* (1993) and Gulati *et al* (2002) illustrates the requirement for adequate investigation into the effect of a storage method, before utilising a stored blood sample.

1.3.1 Important Factors in Blood Sample Storage

A variety of aspects must be considered prior to the storage of a blood sample. As cells differ in their requirements for storage, the optimum storage conditions must be decided after testing *all* factors. These factors include temperature, length of storage, cryopreservation solutions, and blood cell selection.

1.3.1.1 Storage Temperature

The temperature at which samples are stored is only limited by the equipment available. Some of the most common methods for storage are refrigeration (4°C), room temperature (~18°C), Electric freezer (-20°C to -80°C), and the liquid and vapour phases of liquid nitrogen (-196°C and -150°C - respectively). This range of storage temperatures offers scope for optimisation of the storage conditions. Duvigneau *et al* (2003) determined that the undesired effects of prolonged storage can be selectively suppressed by choosing the appropriate storage temperature. A number of studies have been performed to optimise storage temperatures.

An extremely comprehensive study performed by Fowke *et al* (2000) explored the effects of storage at different temperatures on isolated peripheral blood mononuclear cells (PBMC). The effects of storage on whole blood and PBMCs at -30°C, -70°C and -150°C were investigated. They concluded (after the analysis of cell viability,

apoptosis and cell function) that the lower the temperature, the more reliable the yield of cells from both whole blood and PBMCs. The results obtained by Valeri *et al* (2003) reinforce the observation that lower temperatures are often necessary for appropriate viability and function. It was found that platelet recovery was improved simply by reducing the temperature from -80°C to -135°C, without performing additional complicated procedures.

1.3.1.2 Length of Storage

The length of storage is an important factor in determining the correct storage condition. The period of storage must be determined after considering the type of study to be performed on the blood sample. Previous studies have indicated that not all cell factors are stable over extended storage periods.

VenKataraman *et al* (1992) demonstrated that frozen whole blood, thawed after one day in storage, had a remarkable 98% viability. This excellent viability dramatically decreased after 30 days of storage to 56%. The storage conditions of this sample must be modified before an extended storage period occurred. Beritino *et al* (2003) found that platelets stored for transfusions also lose viability with storage. This loss of viability increases over a one to two day storage period at 37°C and signs of apoptosis could first be determined after only one hour. Therefore, if viability is vital, a shorter storage period may be required.

An extended storage period may also affect the expression of cellular components. Duvigneau *et al* (2003) explored whether a delay in processing whole blood (1, 2, 4, 6 and 24 hours) affected cytokine expression. A delay in sampling greater than one hour resulted in a significant change of the cytokine mRNA levels. The conclusion was reached that blood samples should be processed within two hours to prevent undesired stimulatory effects on the cytokine expression pattern. Pieters *et al* (2002) discovered changes in the expression of fibrins in blood plasma over a four month frozen storage period. Therefore, before beginning a study on stored blood cells, it must be determined that the storage period will not affect the conclusions of the study.

1.3.1.3 Cryopreservation Solutions

The first cryopreservation solution was discovered, purely by accident, in the laboratory of Polge, Smith and Parkes in the early 1950's. At the time, their experiments explored whether spermatozoa from roosters could be frozen for future use. Previously, saline had been used as a solution to freeze the samples, and very poor results were obtained (5% mobility post thaw). After a labelling mistake, glycerol was used to freeze the samples and an increase of spermatozoa mobility to 50% was observed. In present times, a storage solution is usually utilised to maintain viability of the cells during storage.

The common cryopreservation solutions utilised today fall into two categories: those that have the ability to penetrate the cells plasma membrane (internal cryoprotectants), and those that cannot penetrate cells (external cryoprotectants). Although the functional mechanism of cryopreservation solutions is not well understood, a number of factors are known to be tightly regulated: reduction of the osmotic damage during the addition and removal of the cryoprotectant, the chemical toxicity of the cryoprotectant to the target cell, and the interrelationship between cryoprotectant concentration and cooling rate (Hunt *et al* 2003).

Cryoprotectants include glycerol, polyvinylpyrrolidone (PVP), methanol, sucrose, proline, glycine, trehalose, fructose, lactose, galactose, betaine, hydroxy ethyl starch, dextran, and dimethyl sulfoxide (DMSO) (Natarajan *et al* 1991; Tomkins & Scheid 1986). The most common cryoprotectant for mammalian cells is DMSO. Due to its polar nature and small, compact structure, DMSO is able to penetrate large cells without causing significant damage to the cell.

Hunt *et al* (2003) investigated the effects of freezing and thawing over a range of cooling rates and cryoprotectant concentrations. The results determined that the optimal recovery of CD34(+) cells requires serial addition of DMSO, slow cooling rates and elution of the cryoprotectant upon thawing. Their team determined that 10% DMSO is optimal for storage, as this concentration is unlikely to be of practical importance with regard to chemical toxicity regardless of equilibriation and temperature. Some contention within the literature over the optimal cryoprotectant

concentration is evident. While many support a 10% concentration, Bakken *et al* (2003) and Abrahamsen *et al* (2004) determined that storage of blood cells with 5% DMSO produced cells with significantly higher viabilities than those cells cryopreserved in 10% DMSO (as determined by Trypan Blue Exclusion test). These contradictory results highlight the importance for a case-by-case analysis of cryoprotectant concentration.

1.3.1.4 Blood Cell Selection

Blood consists of a suspension of cells in a liquid called plasma. In a medical and research sense, the majority of these blood cells are isolated before use. The blood cells are classified as erythrocytes (red blood cells), leukocytes (white blood cells) and platelets (although these are not considered genuine cells). The Leukocytes consist of many elements: Granulocytes; Monocytes; Neutrophils; Eosinophil; Basophil and Lymphocytes. Lymphocytes are a commonly utilised cell as they are easy to isolate, contain a large nucleus and proliferate rapidly.

As a large variety of blood cells are available, the blood cell/s stored may also affect subsequent test results. For example, in some instances it is appropriate to store isolated lymphocytes, isolated peripheral blood mononuclear cells (PBMC), isolated erythrocytes, and in others to store whole blood. Nomura *et al* (2003) and Bailey *et al* (2002) found that freezing isolated PBMC was advantageous over whole blood storage when performing assays for cytokine response. Isolated lymphocytes have also been determined as an ideal blood cell to store when interested in mRNA, DNA and chromosomal damage.

Aside from storing isolated components of blood, the cryopreservation of whole blood has been described as a cost-effective approach in the large-scale storage of viable cells for epidemiological studies (Hayes *et al* 2002). The storage of whole blood is suitable when staff time is low, when collecting in the field and where standardized and cost-effective sample collection, processing, and storage procedures are required.

Cheng *et al* (2001), during a study on the use of lymphocytes for molecular epidemiological studies, determined that the cryopreservation of isolated lymphocytes resulted in a considerable loss of viable cells. A mutagen sensitivity assay (HCR) found that whole blood and isolated lymphocytes responded in a similar manner to the mutagen but the lymphocytes in the whole blood sample maintained a higher viability.

Hayes and his team (2002) furthered the investigation into the effects of cryopreservation of whole blood. These tests, conducted to evaluate the potential uses of cryopreserved whole blood, displayed successful Epstein Bar Virus (EBV) transformation (>90%, after 20 months of storage) which is a demonstration of cell function. In addition, within the whole blood sample, the lymphocytes maintained a high viability, cells had a strong proliferation response and a stable T-cell:B-cell ratio was observed. They did notice, in contrast to Cheng *et al* above, that lymphocytes from stored whole blood appeared to have a lower viability when compared to isolated lymphocytes. The lower viabilities though were attributed to difficulties with flow cytometry. This slight loss in viability was considered an adequate trade-off for the reduced sampling costs and sample processing time.

1.4 CRYOPRESERVATION

Cryopreservation is the storage of samples at extremely low temperatures. While freezing can be lethal to many systems, it can also preserve cells in their natural state. Cryopreservation of blood has been proposed as a convenient means of preserving blood samples until such time as the cells can be processed and analysed (Kondo & Sasaki 1981) and has been determined to be an adequate method of storage for many other purposes such as embryos, tissues and cell lines.

Long term cell viability generally improves with decreasing temperatures of storage (Trummer *et al* 1998), so the cold temperatures associated with cryopreservation make it an ideal method of storage for tests requiring viable cells. Within a cryopreservation chamber, samples are stored in the liquid or vapour phase of liquid nitrogen. This results in a range of storage temperatures depending on where the

vials are placed in the chamber. Direct immersion in the liquid nitrogen provides a temperature of -196°C, and in the gaseous phase the temperature is -150°C to –178°C (Figure 1.1). Although cryopreservation is costly over other methods of storage (Host *et al* 1999), cryopreservation may be necessary in some instances to maintain viable cells.

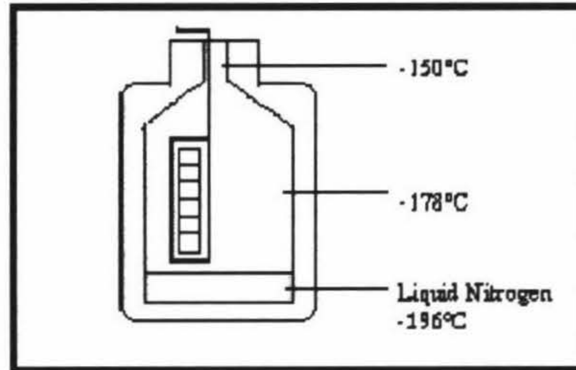


Figure 1.1. The recommended range of temperatures in a liquid nitrogen storage system. Figure adapted from the Nalge Nunc cryopreservation catalogue.

The main difficulties associated with cryopreservation are avoiding contamination of the samples and maintaining viability of cells after thawing. Other issues debated include whether cryopreservation increases the rate of apoptosis, damages the cell or has a clastogenic effect. Weinberg *et al* (1998) even suggested in their study that successful cryopreservation is related to technical expertise. In a study of various facilities they observed that the facilities with experienced staff obtained higher viability of cells after cryopreservation, compared to facilities with inexperienced staff.

1.4.1 Cryopreservation and Contamination

Contamination is a common difficulty with cryopreservation, especially microbial contamination. The main source of contamination is upon thawing of the samples but other sources include blood donor bacteraemia, the collection pack, or contamination during the sample collecting and processing procedures (it may be virtually impossible to completely decontaminate human skin (Blajchman 2004). All procedures must be performed in a sterile environment to protect samples from contamination.

Rollig *et al* (2002) explored two different methods of thawing cryopreserved blood and the resulting contamination level. The two thawing methods involved either submersion in a 37°C water bath or an electric dry warming device. Both these methods have similar viability and apoptosis rates, but use of the dry warming device was found to lower the chance of a bacterial contamination. It was suggested that while in the water bath, water may enter pores in the cryogenic vial and contaminate the sample. This contamination is largely avoided with the dry warming device. Despite this research many laboratories utilise a hot water bath for thawing, without an increase in contamination levels.

1.4.2 Cryopreservation and DNA Damage

There is some evidence that cryopreservation causes damage to the cell and to the DNA. Men *et al* (2003) designed a study to examine the effect of three cryopreservation protocols on the integrity of bovine oocyte DNA. Morphological evaluation and the Comet Assay were applied to detect cryoinjury at a DNA level. It was found that significant morphological damage and DNA damage was present in the frozen samples compared to the unfrozen controls.

Damage to the cells occurs through two main mechanisms, the formation of intracellular ice crystals and dehydration of the cells due to osmotic imbalance. Mazur (1970) proposed that the use of cooling rates below the optimal level results in extracellular ice formation. This extracellular ice creates a chemical water potential

difference between the unfrozen intracellular space and the extracellular space. This difference drives the dehydration of cells, increasing the intracellular concentration of solutes and causing cellular damage. Conversely, if freezing occurs at rates above its optimal level, intracellular ice forms and is considered responsible for the reduced cell survival. Bischof and Rubinsky (1993) noted that ice crystals forming within the nucleus are actually larger than those that form in the cytoplasm. These larger ice crystals may cause considerable damage to the DNA.

To combat the damaging effects of freezing, a cooling rate of -1°C per minute is generally preferred, as it allows the cryoprotectant to shield the cells from solute concentration and ice crystal formation. For the majority of cells, warming from the frozen state should occur as rapidly as possible at 37°C. This returns the cells swiftly to physiological temperature, and reduces the observed cellular damage.

Park *et al* (1998) demonstrated the involvement of another factor in cryoinjury. The role of oxidative stress in freeze-thaw injury to yeast cells was analysed using mutants defective in a range of antioxidant functions, including Cu, Zn superoxide dismutase, Mn superoxide dismutase, Catalase A, Catalase T, glutathione reductase, gamma-glutamycysteine synthase and *Yap1* transcription factor. The colonies with mutations affecting superoxide dismutase function were most affected, indicating superoxide anion formation during freezing and thawing. Free radicals were shown to be generated as a result of freezing and thawing by electron paramagnetic resonance spectroscopy.

The combined results from the Park *et al* paper indicates oxidative stress as a cause of major injury to cells during freezing and thawing. This oxidative stress may be initiated in the cytoplasm by an oxidative burst of superoxide radicals formed from the oxygen and electrons leaked from the mitochondrial electron transport chain. It has been shown that cryopreservation can slow or stop some biochemical reactions, but it may accelerate others. Additional damaging pathways may exist that have not yet been identified.

1.4.3 Cryopreservation and Apoptosis

Apoptosis (programmed cell death) is a multi step process involving a cascade activation of caspase proteases and nucleases. These nucleases result in the destruction of chromatin structure, membrane blebbing and a reorientation of the plasma membrane phospholipids and ultimately cell death.

It is contested in the literature whether cryopreservation increases the apoptosis rate of cryopreserved cells. Lombet *et al* (1998) raises concerns that apoptosis may occur at a higher rate in a cryopreserved sample than in fresh samples. To address the concern that cryopreservation may affect cell viability, Tomkins and Scheid (1986) examined the consequences of storing blood samples at 22°C and 4°C for 24 hours, and cryopreserving isolated lymphocytes for one week. The cell recovery and viability of the thawed cells exceeded 90%. Storage at 22°C or 4°C for 24 hours though, affected the mitotic index but did not alter the cytogenetic endpoints. It is possible that during cryopreservation cells receive certain physical or physiological signals that prime for apoptosis but this is not initiated until later cultured in the presence of a mitogen.

In support of Tomkins and Scheids' view, Riccio *et al* (2002) evaluated the effect that cryopreservation has on the spontaneous apoptosis rate of peripheral blood mononuclear cells (PBMC). Their results indicated that although cryopreservation can to some extent affect lymphocyte membrane integrity rates, flow cytometry analysis revealed that the frequency of spontaneous apoptosis in cryopreserved cells was not significantly modified after 24 hours. This analysis of 24 hours though, is not a considerable time frame to conclude that cryopreservation will not increase spontaneous apoptosis rates.

In a study of cell viability after a longer period of cryopreservation, Kleeberger *et al* (1999) found that PBMC cryopreserved for twelve years had no general tendency towards cell loss over time. A simple linear regression model of the trends in recovery and viability over time, showed the stability of the stored cells (Figure 1.2). The relatively flat line of the graph demonstrates the success of storing these cells for an extended period of time. By the outliers on the graph though, it is clear that the storage success was not 100%.

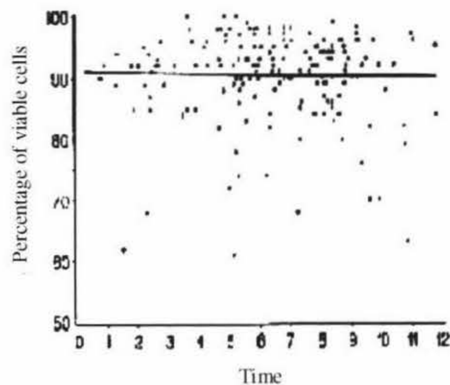


Figure 1.2. The linear regression model of viability over time from Kleebergs' lab. Each point represents the percent of viable cell at each period of storage. This graph shows the stability of viability (90%) and recovery observed for PBMCs that were cryopreserved for twelve years.

Some cryopreservation studies though have not been successful. Celluzzi and Welbon (2003) found that isolated lymphocytes stored for one year, resulted in a significant decrease in viability from the pre-freeze to post-thaw condition. As variation between the lots before freezing remained after thawing, Celluzzi and Welbon suggest that loss of viable cells may not be a setback in the assay providing an increased quantity of the sample is stored, ensuring sufficient viable cells to execute the experiments.

Despite the conflicting apoptosis data, Host *et al* (1999) demonstrated that the loss of a few cells by apoptosis during cryopreservation is trivial to the success of most studies. It is suggested that experiments should be controlled to reduce spontaneous apoptosis rates but some cell loss cannot be avoided. In Host's study to find storage conditions to minimise apoptosis occurrence, cells were stained with bis-benzimide and apoptosis detected through the observation of nuclear morphology under a fluorescence microscope. It was found that whole blood stored at –150°C demonstrated less apoptosis than when stored at –70°C. Similar results were also obtained by Fowke *et al* (2000).

Cheng *et al* (2001), in an attempt to reduce apoptosis rates, identified a disputed problem that cryopreservation of *isolated lymphocytes* may result in considerable loss of viable cells. As it is beneficial to store blood but also retain viability of cells, they explored the use of cryopreserved *whole* blood as a source of lymphocytes. Viabilities of lymphocytes between those isolated and those remaining as whole blood were not significantly different. Therefore Cheng *et al* concluded that cryopreserved whole blood is a good source of viable lymphocytes for epidemiological studies.

1.5 CYTOGENETIC TESTS

The tests discussed in previous sections have largely been haematological tests on blood storage conditions. Only a few studies have examined whether cytogenetic tests are appropriate on cryopreserved blood. Common cytomolecular tests include, Sister Chromatid Exchange (SCE), Micronucleus Assay (MN) and Fluorescence *in situ* Hybridisation (FISH).

1.5.1 Sister Chromatid Exchange

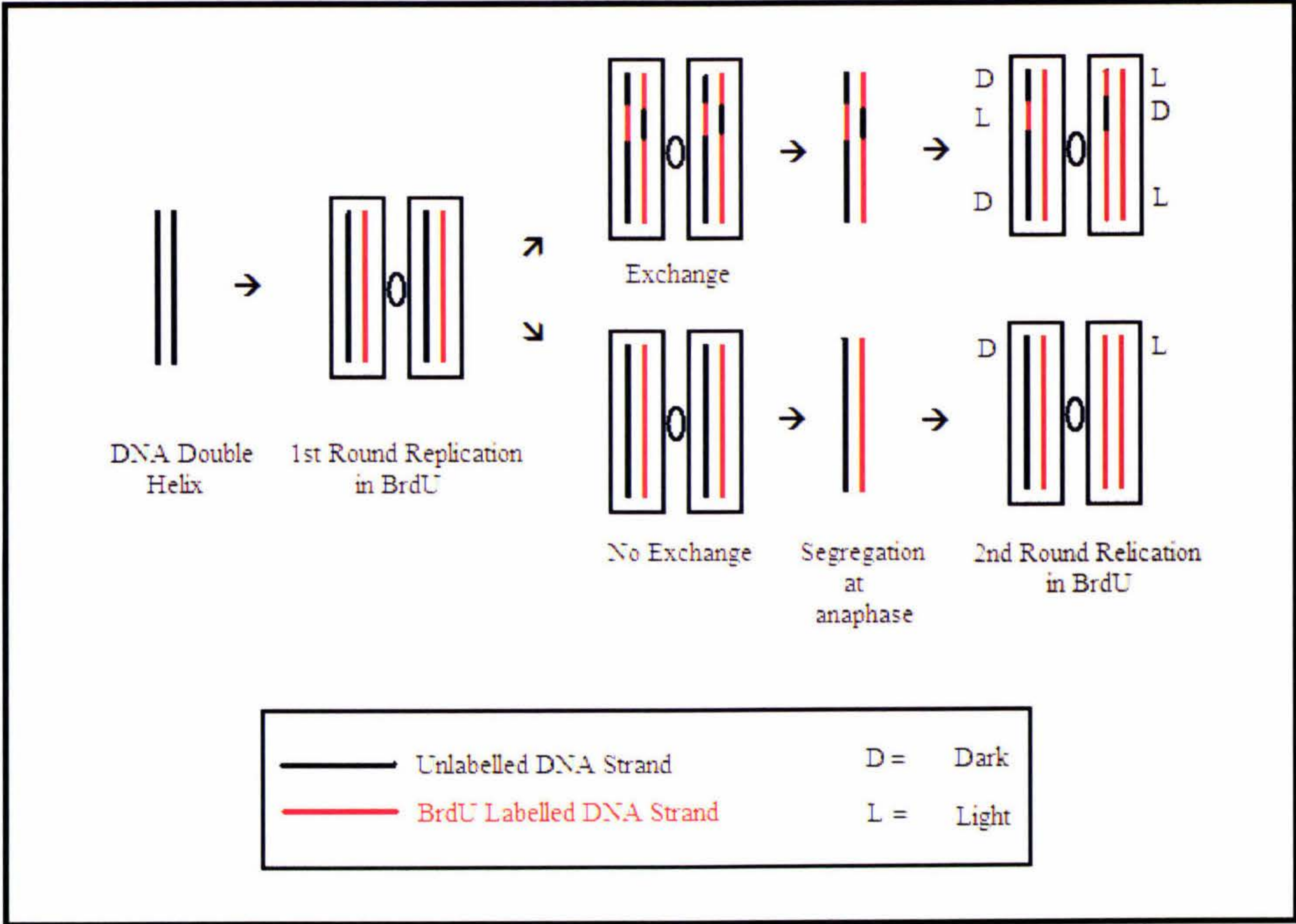
J.H. Taylor (1958) was the first to observe an apparent recombination event between two sister chromatids, during his autoradiographic work. He grew cells in the presence of radioactive thymidine, resulting in genetically identical sister chromatids that were physically different. Changes in the sister chromatids were observed by the exchange between the radioactive chromatid and the non radioactive chromatid. This exchange therefore, was determined to have occurred after the replication that created them. The resolution afforded by autoradiography though, did not allow the nature of this exchange to be studied with precision.

Zakharov and Egolina in 1972, worked to increase the observable resolution of sister chromatid exchanges. It was found that growth of lymphocytes in bromodeoxyuridine (BrdU) produced chemically distinct sister chromatids. BrdU is a thymidine analogue which is incorporated into the DNA at replication in a similar manner to Taylor's radioactive thymidine. A powerful technique, now called Sister

Chromatid Exchange (SCE), has been developed in order to study these exchanges with greater precision, using BrdU. With the greater resolution afforded by this technique, it has now been determined that SCEs occur by a reciprocal exchange of DNA between assumed identical loci of two sister chromatids of a duplicated chromosome, presumably at the replication fork (Rodriguez & Morales 2003).

The Sister Chromatid Exchange technique relies on BrdU incorporation into the replicating DNA. BrdU quenches the reaction of chromatids with the fluorescent dye, Hoechst. Visually this is observed as a decrease in fluorescence, which can be visualised also as a decrease in staining with Giemsa (Figure 1.3).

Figure 1.3. A diagrammatic illustration of The Sister Chromatid Exchange Assay and its theoretical aspects. The first round of replication in the presence of BrdU results in incorporation into one strand of each DNA duplex. When BrdU is incorporated into only one DNA strand of a sister chromatid it is labelled unifilar. When both strands are the same for their BrdU incorporation it is termed bifilar. A Sister Chromatid Exchange may occur after the first or second round of replication before separation at anaphase. This exchange can be detected after a second round of replication in BrdU when treated with Hoechst solution and then Giemsa, as the chromatid with BrdU incorporated into both strands is much lighter than the unifilar chromatid. A sister chromatid exchange is seen as an exchange of label between the two chromatids.



D
L
D
L
D
L
Exchange
DNA Double Helix
1st Round Replication in BrdU
D
L
No Exchange
Segregation at anaphase
2nd Round Relication in BrdU
Unlabelled DNA Strand
BrdU Labelled DNA Strand
D = Dark
L = Light

The trigger for SCEs is still largely debated. SCEs appear to be a consequence of errors in DNA replication on a damaged template at the replication fork, although the exchange process is not fully understood. Some autoradiographic work has suggested that with thymidine incorporation, the decay of tritium may cause exchanges. SCEs occur naturally within the cell, but BrdU is utilised to increase the spontaneous level of SCEs. The primary determination of genetic damage is therefore established by whether the SCEs observed in the study group and control group are statistically different.

A wide variety of agents that are known to cause chromosome breaks, have also been found to induce SCEs. Numerous studies have utilised SCE analysis to explore the extent of genetic damage caused by an environmental agent. A study by Rowland and Harding (1999) found that SCE frequency increased in women smokers between the ages of 16 and 25, compared to non smoking controls. Exposure to pesticides, iodine-131, wood dust and many other environmental factors have also shown increases in SCE frequency compared to matched controls (Zeljezic & Garaj-Vrhovac 2002; Sonmez *et al* 1997; Elavarasi *et al* 2002). These studies highlight the usefulness of SCE analysis in the biomonitoring of human populations exposed to a variety of agents.

1.5.2 Micronucleus Assay

The Micronucleus Assay (MN) utilises the micronuclei that arise in mitotic cells during anaphase. This technique is usually applied to measure the efficiency of a DNA repair system. Micronuclei can be defined as small extranuclear bodies formed during mitosis as a result of chromosomal fragments that are not included in either daughter nucleus. These can be formed by direct DNA breakage, replication of a damaged DNA template and by inhibition of DNA synthesis. When whole chromosomes are contained within the micronuclei there has most likely been a vast mechanical disruption. These disruptions in the DNA are usually corrected by the DNA repair systems but when DNA repair systems are dysfunctional, an increase in the number of micronuclei is observed.

DNA repair systems are often reduced in cells exposed to clastogenic agents, so an increase in the frequency of micronuclei can be an accurate representation of exposure to clastogenic agents (a clastogen is any environmental agent that causes damage to genetic material). The greater the number of micronuclei the greater the genetic damage that has been incurred on the individual and their DNA repair system is functioning less. The reduction in a DNA repair mechanism is dangerous, as uncorrected mistakes in replication can result in a plethora of diseases including cancer.

In the same manner as SCE analysis, the MN Assay has been utilised extensively to monitor the exposure to clastogenic agents. Vlastos *et al* (2004) were able to find that the exposure to pesticides used commonly on tobacco farms (metalaxyl and imidacloprid) were not harmful to the DNA repair system of the farmers. Unfortunately, not all tests demonstrate a lack of damage from exposure. Hadjidekova *et al* (2003) studied the extent of genetic damage in nuclear power plant workers. A dose-related association between MN frequency and the level of ionizing radiation was evident and the lowered DNA repair capability was supported by the increased frequency of cancers within the focus group. Similar results were obtained by Vaglenov *et al* (2001) with exposure to lead. Lead-exposed workers had significantly higher levels of genetic damage as measured by the MN Assay compared to non lead exposed matched controls. The Micronucleus Assay has therefore been well utilised and characterised method to study genotoxic effects.

1.5.3 Fluorescence *in situ* Hybridisation

A well-known effect of radiation damage and of genotoxic agents is the translocation of genetic material from one chromosome to another. These translocations may have disastrous effects on a person's health, especially if an oncogene is placed under the control of a strong promoter or a tumour suppressor gene is inactivated. Small translocations are often difficult to detect, but Fluorescence *in situ* Hybridisation (FISH) is able to locate genetic exchanges, even between only a few kilobases of DNA. The rapid detection of numerical chromosomal anomalies is made possible

through centromere-specific repetitive-DNA probes and chromosome-specific unique-sequence probes.

The type of FISH utilised can differ greatly, depending on the probes. The most common form of FISH in biomonitoring is the labelling of whole chromosomes to observe large translocations. Burim *et al* (2004) utilised the FISH method with DNA library probes for chromosomes 1, 3, and 6 to test the induction of chromosomal aberrations by ethanol, in alcoholics and recovering alcoholics. The results showed that the chromosomal aberrations for chronic alcoholics and recovering alcoholics were significantly higher than those obtained for control individuals. The mean translocation frequency was discovered to be two times higher in alcoholics than controls, which is a clear indication that excess alcohol has damaging effects. FISH had been used to monitor several disasters, such as the radiation disaster in Brazil (Natarajan *et al* 1991), demonstrating that FISH is a well established and trusted technique to monitor DNA damaging events.

1.6 CYTOGENETIC TESTING ON CRYOPRESERVED BLOOD

Cryopreservation of blood has been proposed as a convenient means of preserving peripheral blood lymphocytes until such time as the cells can be processed and analysed (Kondo & Sasaki 1981). Unfortunately, past research in this area of cytogenetic testing is sparse and the results of studies have been contradictory, allowing scope to debate the suitability of cryopreservation for cytomolecular tests.

The frequency of micronuclei from cryopreserved lymphocytes may accurately indicate the level of damage sustained before freezing (Prosser *et al* 1994) but debate in this area demonstrates that the link between increased micronuclei and cryopreservation is uncertain. Prosser and his research team noticed a change only in the micronuclei yield for lymphocytes that had been irradiated prior to freezing. Schmezer *et al* (2001) also concluded that cryopreservation of lymphocytes for up to 12 months did not affect the sensitivity to mutagens for DNA repair tests. There are though, contradictory results in this area (Rudd *et al* 1998, Duthie *et al* 2002). Rudd and his team observed elevated frequencies of micronuclei in cultured cryopreserved

fibroblasts in 64% of the cell lines examined. Interestingly, they discovered that the freezing-related increase in micronucleus frequency was donor dependent, suggesting a genetic influence on the response to storage. This donor dependent increase has not been studied further.

Duthie *et al* (2002) assessed the influence of cryopreservation on DNA strand breakage, especially on DNA repair mechanisms. It was concluded that cryopreservation of blood samples is satisfactory for DNA damage biomonitoring, but not appropriate for tests that assess DNA repair. A study on the suitability of performing MN assay on cryopreserved lymphocytes for determining breast cancer, found the use of frozen lymphocytes to be unsatisfactory (Burrill *et al* 1999). Burrill observed that with the use of cryopreserved lymphocytes the experimental failures were higher, the inter-experimental variability was higher, and the ability to distinguish between breast cancer cells and normal cells using the MN Assay was lower.

Cheng *et al* (2001) compared the results from the host-cell reactivation (HCR) assay and mutagen sensitivity assay when performed on cryopreserved whole blood, cryopreserved lymphocytes and on fresh blood samples. The DNA repair capacities of the two stored samples were shown to be similar to the fresh samples. The mutagen sensitivity assay (using γ-radiation to induce chromatid breaks) revealed no significant difference between the baseline level of chromatid breaks between lymphocytes from frozen whole blood and fresh blood. These contesting results from Duthie *et al* (2002) and Cheng *et al* (2001) suggest DNA repair tests may not be appropriate on stored blood. As the MN assay is based on DNA repair mechanisms, cryopreserved blood may not give results similar to fresh blood samples.

Hinninger *et al* (2004) developed an optimised protocol for the evaluation of DNA damage in frozen whole blood. Using their optimised technique, no statistically significant differences in the level of DNA damage between fresh blood samples and cryopreserved samples were observed, as assessed by the Comet Assay. Hinninger and his team were very confident that cytogenetic tests could be performed on cryopreserved blood without affecting the results, once the process had been optimised for use on stored blood.

Littlefield *et al* (1986) concluded that freezing in liquid nitrogen does not induce unstable *chromosomal* aberrations in human separated lymphocytes, even though an increase in the number of metaphases containing *chromatid* aberrations was observed. Gardner *et al* (1986) also observed an increase in chromatid aberrations after a period of cryopreservation by an increase in mutagen-induced SCEs in isolated lymphocytes. In disagreement with Littlefield's and Gardner's observations, no obvious consequences of cryopreservation were observed on the frequency of SCEs when whole blood was cryopreserved for one week (Tomkins & Scheid 1986). Murli *et al* (1987) tries to explain these contradictory claims, as cryopreserved isolated lymphocytes were shown to have a higher baseline SCE than the cryopreserved whole blood. Murli *et al* therefore suggest that SCE levels may be increased not by cryopreservation, but by the process of lymphocyte separation. Cheng *et al* (2001) further supports the view that cryopreserved whole blood is an ideal lymphocyte source indicating that the process of lymphocyte separation may be unnecessary for cryopreservation prior to cytogenetic testing.

1.7 CRYOPRESERVED BLOOD AND GENETIC DAMAGE

In biomonitoring genetic damage, it is important to assess whether the damage recorded is the result of the monitored disaster, or if the storage method is affecting the results. The prior tests mentioned have generally been performed on preserved blood from healthy subjects. Only a handful of studies have explored if irradiated blood, such as that exposed to a radiological accident, is also stable during cryopreservation. The majority of the previous work on blood from ill subjects is not conclusive as the storage conditions are not well described. A disaster concluded to have a detrimental effect on genetic information may not actually be as severe, if the blood samples were stored incorrectly prior to conducting the tests. The method of blood storage may actually influence the results. For example in a cytogenetic study of victims involved in a radiation accident in Brazil, lymphocytes were cryopreserved from radiation exposed subjects without knowledge as to whether damaged blood could be stored in the same way as normal healthy samples (Natarajan *et al* 1991).

The results from a Lymphocyte Proliferation Assay were greatly affected by shipment and storage when the assay was performed on normal controls and HIV patients (Weinberg *et al* 1998). It was found that storage of the blood samples did not affect the results from healthy cells but did affect the results from HIV infected whole blood. Therefore it was concluded that the effectiveness of blood storage depends on the person's health.

Chambrette *et al* (1996) investigated whether cryopreservation can defer the analysis of blood samples from radiological accidents to assess the degree of exposure. The authors found no modification of SCE after 1-6 months of cryopreservation and also observed no effect on the result of the micronucleus assay. Lee *et al* (1999) results support Chambrette *et al* findings. Lee and his team compared the micronucleus frequency between cryopreserved blood samples of cancer patients and normal, healthy controls and fresh blood samples. Non-significant differences were observed between the fresh blood samples and those stored for 96 hours for *both* study groups. This is a short storage period though, and no micronucleus assays appear to have been performed on samples stored for an extended period.

1.8 CRYOPRESERVATION PILOT STUDY

1.8.1 Cryopreservation and NZNTV

In the literature much debate is evident as to whether cryopreserved blood samples can be used effectively for cytogenetic testing. As established earlier, the method of storage may differ depending on the assay to be performed carried out on the blood sample. It must first be demonstrated that cryopreserved blood obtains similar results to the fresh sample. As current studies on storage conditions are few, and test storage times short, research is needed to discover if storage over a longer period of time (up to one year) provides reproducible results. The previous short trials are not satisfactory to accurately determine that no clastogenic effects will occur. As lymphocyte separation prior to cryopreservation has been shown to reduce viability of the cells and to increase chromatid aberrations, the cryopreservation of whole blood must be considered as an alternative source of viable lymphocytes. It also must be

determined whether cryopreserved blood samples from radiation-exposed individuals provide the same results as fresh blood. This project addresses these issues of the suitability of whole blood use, extended storage, the use of radiation exposed blood samples in the context of cryopreservation.

As cryopreservation itself may have a clastogenic effect, this project determines whether cryopreservation of whole blood for an extended period of time is an acceptable form of storage prior to performing three cytogenetic tests on potential radiation exposed subjects and non radiation exposed control subjects. The three cytomolecular tests performed are Sister Chromatid Exchange (SCE), Micronucleus assay (MN) and Fluorescence *in situ* Hybridisation (FISH). The tests are performed on a single blood sample collected from New Zealand Nuclear Test Veterans (NZNTV) and non-exposed matched controls to observe whether the results of the cytomolecular tests are statistically consistent over a prolonged period of cryopreservation (fresh, one month, three months and six months). If a long period of storage has an effect on the radiated and/or control blood then a higher frequency of SCE, micronuclei and translocations in FISH would be observed than the fresh control samples.

1.8.2 Objectives

Hypothesis

That Sister Chromatid Exchange, Micronucleus Assay and Fluorescence *in situ* Hybridisation can be performed on whole blood of radiation exposed veterans and non-exposed controls after a period of cryopreservation without causing an increase in genetic damage.

Aims

- To determine whether cryopreserved whole blood can be utilised as a source of viable lymphocytes for cytogenetic tests (SCE, MN and FISH).

- To optimise a procedure for the cryopreservation and successful culturing of lymphocytes from whole blood prior to cytogenetic testing.

- To determine whether whole blood samples from individuals potentially exposed to radiation in the Operation Grapple atomic bomb tests, and non-exposed whole blood can be cryopreserved while maintaining statistically similar results to the cytomolecular tests performed on fresh blood samples.

CHAPTER TWO: MATERIALS AND METHODS

The materials utilised in this study were stored at room temperature, unless otherwise stated. The various requirements for sterility are discussed separately for each material.

2.1 COMMON BUFFERS AND SOLUTIONS

Bromodeoxyuridine Solution

Bromodeoxyuridine (BrdU) (Sigma)	0.00614g
Milli-Q Water (MqH_2O)	2ml

This solution was made fresh on the day of use. The BrdU mass was carefully weighed before placing in an autoclaved 5ml Duran. Autoclaved water was delivered to the Duran by a sterile syringe (Terumo), avoiding contamination. The resulting solution was mixed on a magnetic stirrer for one hour or until completely dissolved.

Dimethyl Sulfoxide (DMSO)

DMSO (Life Technologies Inc.) was used undiluted. DMSO was stored in the dark at room temperature.

Colchicine (0.05%)

Colchicine	0.05g
Distilled Water (dH_2O)	100ml

The Colchicine solution was stored refrigerated at $4^{\circ}C$ and could reliably be stored at this temperature for one year.

Cytochalasin-B Stock Solution

Cytochalasin-B powder (Sigma)	2mg
Dimethyl Sulfoxide (DMSO) (Life Technologies Inc.)	1ml

The DMSO was added to the Cytochalasin-B powder through a 0.22μm syringe tip filter to ensure sterility (Acrodisc). Once the Cytochalasin-B powder was dissolved in the DMSO it was stored at –20°C. Cytochalasin-B stock solution is light sensitive so was stored covered with foil in the dark.

Ethanol Solution (70%)

Absolute Ethanol	7 Parts
dH_2O	3 Parts

Fixative

Glacial Acetic Acid	1 Part
Methanol	3 Parts

Fixative was prepared fresh on the day of harvesting and kept on ice, as the fixative was required chilled.

Hoechst Stock Solution

Hoechst 33258 (bisbenzimide) (Sigma)	10mg
dH_2O	20ml

The stock solution was prepared to a concentration of 0.5mg/ml. This stock solution can be stored for up to six months in the dark at 4°C. The Hoechst Stock solution is very sensitive to light so the Duran was wrapped in foil to avoid light exposure.

Hoechst Working Solution

Before use, the Hoechst Stock Solution was diluted 100 fold in Sorensen's Buffer to obtain the working solution. This dilution was performed in one step with 0.1ml stock solution diluted in 10ml Sorensen's Buffer, obtaining a final Hoechst concentration of 5 µg/ml. The working solution could be stored for up to 3 months in the dark at 4°C. This solution is also very light sensitive and the Duran was wrapped in foil to avoid light exposure.

KCl

0.075M KCl	2.785g
dH_2O	500ml

The solution was mixed well on the magnetic stirrer until completely dissolved.

MacIlvaines Buffer (pH 7.0)

0.1M $C_4H_6O_7 .1H_2O$	10.5g
dH_2O	1L
0.2M $Na_2 HPO_4 .2H_2O$	17.8g

The citric acid ($C_4H_6O_7 .1H_2O$) and 500ml of dH_2O were mixed well to form solution A. The di sodium phosphate ($Na_2 HPO_4 .2H_2O$) and the remaining 500ml dH_2O were mixed well to form solution B. The two solutions were then mixed to form MacIlvaines Buffer and the pH adjusted to 7.0 through the use of HCl and NaOH.

Phytohaemagglutinin (PHA)

Phytohaemagglutinin (Dehydrated) (Gibco)	
dH_2O	10ml

10ml of autoclaved dH_2O was delivered to the PHA by a sterilised syringe and mixed. The solution could be stored frozen at -20°C for one year.

Sorensen's Buffer (pH 6.8)

6.25×10^{-2} M KH_2PO_4	
6.25×10^{-2} M $Na_2HPO_4 \cdot 2H_2O$	
MqH_2O	1L

Sorensen's Buffer was made using Sorensen's Buffer tablets (BDH). The tablets were added to 1L MqH_20 and mixed on a magnetic stirrer until completely dissolved. These tablets ensured correct buffer formation and sterility was not required.

2 x SSC (pH 8.0)

0.3M NaCl	8.766g
dH_2O	1L
0.3M $Na_3C_6H_5O_7 \cdot 2H_2O$	44.115g

The Sodium Chloride (NaCl) and 500ml of dH_2O were mixed well to form solution A. The tri sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 500ml dH_2O were mixed well to form solution B. The two solutions were then mixed to form 2 x SSC and the pH adjusted to 8.0 by using HCl and NaOH.

2.2 STAINS

Giemsa Stain

Giemsa Stain solution (BDH)	8ml
Sorensen's Buffer	72ml

The Giemsa Stain solution was stored refrigerated at 4^0C. To make the stain, the Giemsa Stain solution was removed from the refrigerator and allowed to warm to room temperature. The bottle was swirled to ensure homogeneity of the solution and

an 8ml aliquot removed. The Sorensen's Buffer was added to the 8ml aliquot and mixed well before immediate use.

2.3 CELL CULTURE MEDIA

Culture media were prepared in bulk and stored frozen at -20°C. In these conditions the culture media could be stored for up to six months.

Fluorescence *in situ* Hybridisation (FISH) Cell Culture Media

Wellcome Media (Media 199) (Gibco)	5ml
AB Serum (Gibco)	1ml
Phytohaemagglutinin (PHA) (Gibco)	0.1ml

To ensure sterility all steps were performed in a biohazard hood used only for blood work. The media was made to the instructions of Media 199 and mixed for a minimum of 30 minutes on a magnetic stirrer at room temperature until all traces of powder were dissolved. The resulting media was vacuum filtered through a positive flow filter to sterilise (Stericup/steritop filter unit, Millipore, 0.22μm pore size). The AB Serum was also sterilised through a positive flow filter after heat inactivation. To heat inactivate the AB Serum, the thawed solution was placed in a 57°C water bath for 45 minutes. All required components were placed in sterile 8ml Falcon™ culture tubes and stored frozen at -20°C until required. To use the culture tubes, the tubes were placed in a 37°C hot water bath until completely thawed.

Micronucleus Assay (MN) cell culture media

Wellcome Media (Media 199) (Gibco)	5ml
AB Serum (Gibco)	1ml
Phytohaemagglutinin (PHA) (Gibco)	0.1ml
Cytochalasin-B (Sigma)	16.5μL

The welcome media and AB Serum were prepared and placed in Falcon™ culture tubes as for the FISH cell culture media. The PHA and Cytochalasin-B solutions were not initially added. PHA was added by sterile pipettes 6 hours after irradiation had occurred. Cytochalasin-B was added by sterile pipettes 30 hours after irradiation.

Sister Chromatid Exchange (SCE) Cell Culture Media

Wellcome Media (Media 199) (Gibco)	5ml
AB Serum (Gibco)	1ml
Phytohaemagglutinin (PHA) (Gibco)	0.1ml
Bromodeoxyuridine solution (BrdU) (Sigma)	0.05ml

This media is prepared in the same way as for FISH cell culture media but includes BrdU. The BrdU was not added prior to freezing but was added fresh upon use. The BrdU was delivered to the culture tubes through a 0.22μm syringe tip filter (Acrodisc) to ensure sterility.

2.4 CRYOPRESERVATION MEDIA

Freezing Media

Wellcome Media (Media 199) (Gibco)	2 Parts
AB Serum (Gibco)	2 Parts
Dimethyl Sulfoxide (DMSO)	1 Part

The Wellcome Media and AB Serum were prepared as for cell culture media (section 2.3). The Freezing Media was generally prepared as 5ml aliquots in 8ml Falcon™ tubes and stored frozen at -20°C until required, excluding DMSO. To thaw, the freezing media was placed in a 37°C hot water bath until only a few ice crystals remained and then left at room temperature. DMSO was added fresh at a concentration of 20% so that upon the addition to the blood samples, DMSO was at a final concentration of 10%.

Thawing Media

Wellcome Media (Media 199) (Gibco)	3 Parts
AB Serum (Gibco)	1 Part

The Wellcome Media and AB Serum were prepared as for the cell culture media (section 2.3). The Thawing Media was prepared in 12ml Aliquots and stored frozen at -20^{0}C until required. The thawing media was thawed on the day of use by placing in a 37^{0}C hot water bath until it reached this temperature. This was important to ensure that the thawed blood's first contact was with its physiological temperature.

2.5 SELECTION OF PARTICIPANTS

To determine the appropriate selection criteria, professional researchers and the current literature were consulted. It was clear from this consultation that the study was of a non-comparative nature, as no comparison would occur between individuals. Due to the studies' non comparative nature no inclusion or exclusion criteria were required for the participants' selection.

However, some inclusion and exclusion criteria were utilised to simplify the sample collection. As this study stemmed from the New Zealand Nuclear Test Veterans (NZNTV) Study, the participants were selected from a list of veterans and controls used in the current trials. The veterans and controls from this study were familiar with the cytogenetic tests and our laboratory processes, so explaining the study and its benefits was simplified. The project also utilised blood samples from NZNTV as this cryopreservation technique would potentially be performed on similar sub-groups.

The participants were also selected preferentially on their proximity to Palmerston North and their availability for a blood sample. This reduced sampling costs by minimising the travel expenses.

Ten participants were chosen for this study to allow a range of sampling. Ten participants was also the maximum number of samples that could be processed in the

time constraints of this study. In the event of an alarming difference in response to cryopreservation, five nuclear test veterans and five controls were selected to achieve the total of ten participants. Although the numbers of participants in this study are not sufficient for this control/veteran comparison, it was suspected that features may be highlighted during the project for potential future research.

2.6 SAMPLE COLLECTION AND ENCODING

From each participant a 10ml blood sample was drawn by venipuncture (21G$^3/_4$ Vacutainer™) into heparinized tubes (BD Vacutainer™) by a qualified phlebotomist. Although it was scheduled to collect the blood samples from five participants one week and the remaining five participants a fortnight later, the nature of working with people resulted in the blood being collected from three participants on one day, three participants the following week and from the other four participants two weeks later (Schedule for blood collection shown in Appendix I). From the total blood taken, the cytogenetic tests were performed immediately, and fifteen samples were frozen for each individual. For the following analysis, the first five individuals were grouped together and the second five individuals were processed together. Due to the long periods of storage this slight discrepancy in storage period was not considered a complicating factor.

Each tube of blood was coded to identify the individual without revealing the samples original source (veteran or control). This code (A-J) was also written on the relevant samples for storage. To ensure blind analysis and to avoid bias when scoring the assays, the code was modified by a researcher (not associated with the project) after thawing a sample but before the analysis was conducted. This code was not decoded until all analysis was completed for that time period.

2.7 BLOOD MANIPULATIONS

For the three assays (Sister Chromatid Exchange Assay, Micronucleus Assay and Fluorescence *in situ* Hybridisation) sterility was essential. To reduce contamination all manipulations were performed in the sterilised biohazard hood shown in Figure 2.1. The Biohazard hood was sterilised prior to use, by swabbing with 70% ethanol and exposed to UV light for 1 hour. The Biohazard hood was reserved solely for blood work which removed the risk of bacterial contamination from microbial work.

Figure 2.1. A photograph of the Biohazard Hood used for all blood manipulations to avoid contamination. The system can be sealed off by the UV light unit which sterilises the surface.

Extreme care was taken during the initial whole blood manipulations to avoid bacterial contamination: gloves were swabbed with 70% ethanol, the surface of the blood vial was swabbed with 70% ethanol skin preparation pads (BDH) prior to the removal of blood using a sterilised 1ml syringe (Terumo).

When the samples were prepared for a period of culturing, the blood samples were all placed in the same 37°C incubator illustrated in Figure 2.2. This incubator was

custom built by Massey University physicists for other research on electromagnetic fields. The incubators' heat is generated by numerous 150W light bulbs and the temperature controlled by an external thermostat. The chamber for the incubating samples is blackened out to prevent light from affecting the samples growth. The samples are placed on a rack in the incubator at a 30° angle as this provides the optimal surface area for growth.

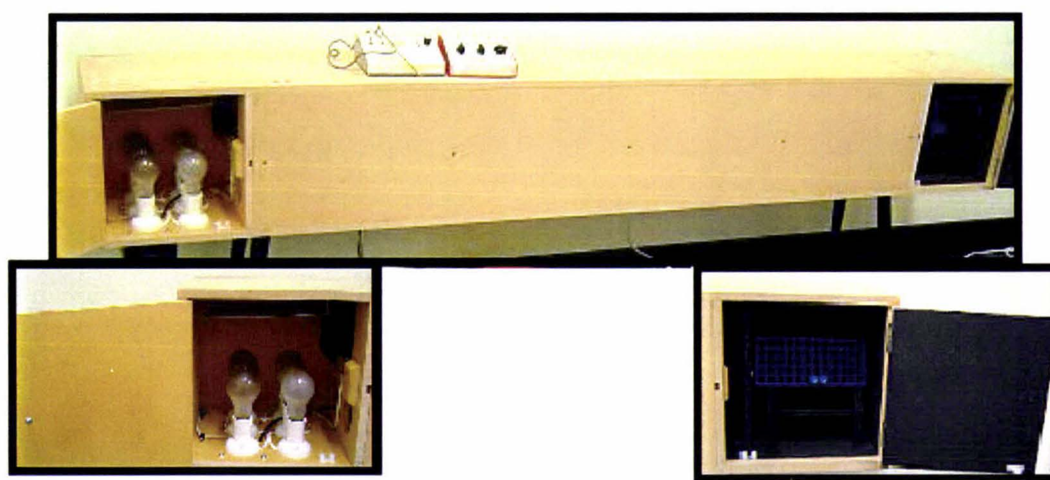


Figure 2.2. The 37°C incubator apparatus. The light bulbs are situated on the left hand side and the samples were placed at the opposite end for the period of incubation. The enlarged sections display the two openings in the incubator, one for the heat generating light bulbs, the other with a rack for the samples.

2.8 CYTOGENETIC ASSAYS

2.8.1 Sister Chromatid Exchange (SCE)

2.8.1.1 Sister Chromatid Exchange Culturing

To ensure the number of lymphocytes in each culture tube was constant, a lymphocyte count was taken using the Coulter JT Whole Blood Counter (Appendix II demonstrates a characteristic white blood count reading obtained). BrdU has the

capability to induce SCEs at high concentrations so a constant lymphocyte count standardises the results across the samples, reducing internal variation. The amount of blood added to the cell culture media by a sterile syringe was adjusted to ensure 3.25×10^6 lymphocytes were added to each culture tube.

Worked Example:

Sample White Blood Count = 8.4

8.4 WBC = 8.4×10^9 Leukocytes/1000ml

= 8.4×10^6 Leukocytes /ml

BUT require 3.25×10^6 per 6ml

Blood added to each tube = 3.25 / WBC

Therefore $3.25 \times 10^6 / 8.4 \times 10^6 = 0.3869$ml

= 0.39ml of whole blood added to the culture tube

After the blood was added to the prepared SCE culture tube, 0.05ml of BrdU was added to the culture tube through a 0.22μm pore syringe tip filter (Acrodisc). The culture tubes were inverted several times to ensure homogeny of the tube before placing in the 37°C incubator for 72 hours. Two culture tubes were established for each individual to cover the risk of accidental damage to one tube.

2.8.1.2 Sister Chromatid Exchange Harvesting

After 72hours of culturing, the cell culture tubes were removed from the incubator and 100μL colchicine added to each tube. The tubes were inverted several times to ensure the colchicine was evenly mixed. Although sterility is not important at this step, the colchicine addition was performed in the biohazard hood to protect both the sample and the researcher's safety. The cell cultures were placed in a 37°C room for a further 1hour of incubation to allow the cells to arrest chromosomes at metaphase.

This incubation occurred in the dark to prevent any inducement of SCEs through light exposure.

Once the hour incubation was completed, the cell cultures were removed and centrifuged at 1000rpm for 10 minutes. The resulting supernatant was withdrawn using a positive flow vacuum flask, before 5ml of 0.075M KCl (warmed to 37°C) was added to the remaining pellet to lyse the erythrocytes. The cell pellets resuspended in KCl were incubated for precisely 10 minutes in a 37°C water bath, prior to a second 10 minute centrifugation at 1000rpm. The supernatant was once again discarded using a vacuum flask, and while under constant agitation 5ml of 6% Acetic acid was added slowly to the pellet. 6% Acetic Acid is added as a prefixative step. This prefixative step is vital, allowing the cells to adapt to a low dose of acetic acid before a high dose is delivered. The culture tubes remained at room temperature for 5 minutes after the acetic acid addition. After prefixation, a centrifugation of 20 minutes at 1000rpm is required to pellet the lymphocytes.

After the prefixative step, fixative (1:3 glacial acetic acid/methanol) was added to the pellet of the last centrifugation (20 minutes), mixed, and centrifuged for 10 minutes at 1000rpm, followed by removal of the supernatant and the addition of more fixative. This addition of fixative and centrifugation occurs three times in total. After the final centrifugation with fixative, the supernatant is removed (without disturbing the lymphocyte pellet).

To prepare slides (frosted 25mm x 75mm, BioLAB) for staining, the resulting lymphocyte pellet was drawn up into a Pasteur pipette and dropped from a height of approximately 40cm onto five pre-cleaned microscope slides. This height ensured the metaphases were spread for visualisation.

2.8.1.3 Sister Chromatid Exchange Staining (Fluorescence-plus-Giemsa Staining)

The slides prepared from harvesting were completely set before staining occurred. The slides were generally stored at room temperature for three days or more, before

commencing the staining process. To begin the staining process the slides were soaked in Sorensen's buffer solution for 5 minutes and then rinsed with distilled water. Sorensen's buffer is used to "revive" the cells, ready to take up the Hoechst solution (M.A. Wahab, personal communication 2003).

To prevent light exposure, the staining was performed in the dark room. Each slide received 7 drops of the light sensitive reagent Hoechst working solution, and a cover slip (BioLAB) placed over each slide. The slides were left for 30 minutes allowing the Hoechst to intercalate. Still in the dark, the slides were washed with Sorensen's buffer and distilled water to remove the cover slips.

The slides were mounted with 8 drops of MacIlvaines buffer and a cover slip placed on top. Slides were exposed to UV light (356nm) for 2.5 hours, at a distance of 10cm. After the 2.5 hours exposure, the cover slips were removed by rinsing with Sorensen's buffer and distilled water. The samples were now no longer light sensitive and the remaining steps no longer needed to be performed in the darkroom.

Slides were incubated in 2x SSC for 20 minutes at a temperature of 65°C. Once the slides had dried they were stained with fresh Giemsa for approximately 8 minutes before rinsing in Sorensen's buffer. The duration of staining required often differed, and it was common practice for one slide to be stained first in order to correct the staining time. After the slides were dry they were mounted in DPX and allowed to set.

2.8.1.4 Sister Chromatid Exchange Analysis

After the Fluorescence-plus-Giemsa-staining had been completed, analysis of the slides could occur. The examination of fifty to one hundred metaphases is the conventional number of cells scored for SCE (Kasuba *et al* 1999, Villarini *et al* 1998 and Elavarasi *et al* 2002). In this study, fifty metaphases were examined for each individual to optimise the analysis time.

To analyse the metaphases, images were viewed using an Olympus BX51 microscope, under 1000X magnification. Metaphases were identified that contained the complete complement of 46 chromosomes, differentially stained sister chromatids, no overlapping chromosomes and no indistinguishable sister chromatids. When these metaphases were captured, the images were captured using an Optronics MagnaFIRE S99802 digital camera with MagnaFIRE frame-grabbing software on a 2GHz Pentium 4 computer with 256 MB of RAM and a 128MB video card, illustrated in Figure 2.3.

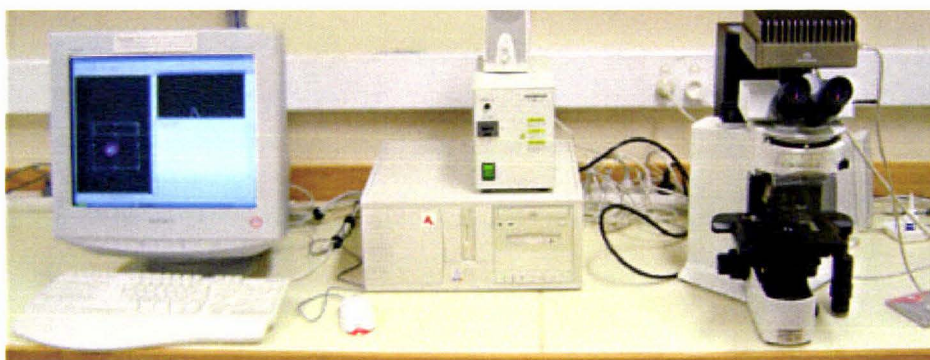


Figure 2.3. The Olympus BX51 fluorescent microscope is situated at the right hand side of the figure. On top of the microscope is the Optronics MagnaFIRE S99802 digital camera (Appendix III). The computer has the software required for the image analysis.

When scanning for metaphases, steps were taken to ensure no bias when deciding which metaphases were chosen for analysis. The scanning for metaphases commenced at one end of the slide and progressed sequentially to the other side, with images of all identified metaphases taken, until the required number was reached. This sequential method both ensured the lack of bias and prevented the occurrence of duplicated images.

Analysis was performed in Microsoft Paint, with a mark made on each chromosome when counted to ensure accuracy of scoring (Figure 2.4). If a metaphase was not complete no further analysis was performed. Fifty images were scored for SCE frequency.

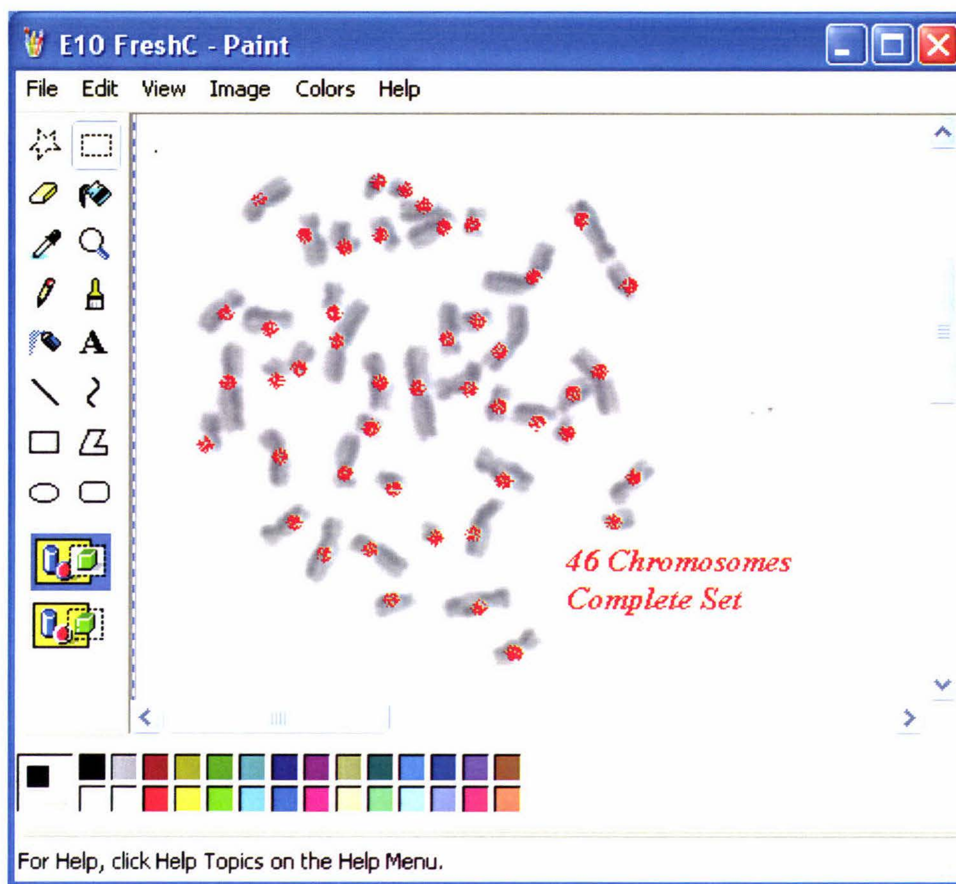


Figure 2.4. A representation of the method to count the chromosomes. The spray-paint function was used to mark each chromosome so that each was only counted once. If a full complementation of 46 non-overlapping chromosomes was present, the metaphase was analysed for SCEs.

The number of Sister Chromatid Exchanges were scored with the assistance of ImagePro Software. ImagePro Software is able to enhance the contrast and brightness of an image to make them easier to score. The ImagePro Software is demonstrated in Appendix IV. When scoring, each exchange between the dark appearing chromosomes and the light appearing chromosomes was counted (Figure 2.5) and the results recorded for future statistical analysis.

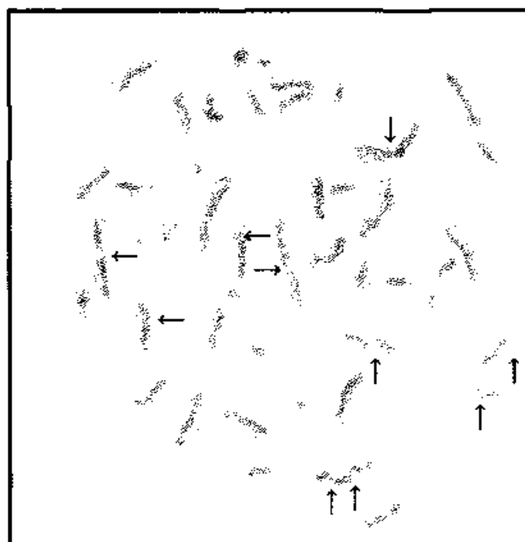


Figure 2.5. A demonstration of how the Sister Chromatid Exchanges were scored. Each arrow represents a SCE, an exchange between the light and dark stained chromatids.

2.8.2 Micronucleus Assay (MN)

2.8.2.1 Micronucleus Assay Culturing

Prior to blood collection or thawing, the required number of MN culture tubes were removed from the freezer and warmed to 37°C in the hot water bath. In the case of fresh blood samples a sterile 1ml syringe (Terumo) was utilised to deliver 0.5ml of whole blood to each culture tube. With thawed blood samples, 0.5ml of resuspended pellet was added to each culture tube using a sterile plastic transfer pipette.

The micronucleus assay procedure followed that of Scott *et al.* (1999). The culture tubes with the added blood samples were kept at 37°C for 1hour before irradiation with 3.5Gy at Palmerston North's radiotherapy unit. The samples were transported to Palmerston North Hospital in a thermos flask to prevent a rapid decrease in temperature. Prior to irradiation, the culture tubes were inverted to ensure solution homogeneity and placed in a holder tray with a diffraction plate placed over the

culture tubes to ensure even irradiation. After irradiation the culture tubes were placed into the thermos flask and returned to the laboratory.

A six hour incubation at 37°C followed the radiation treatment. After six hours of incubation 83.3μL of PHA was added to each culture tube using aseptic technique. This PHA addition stimulated mitosis before the samples were returned to the 37°C incubator. Twenty-four hours after PHA stimulation the cytokinesis-blocking agent Cytochalasin-B was added (16.5μL/tube) (again by aseptic technique) and the culture tubes returned to the incubator. After a total of 90 hours in the incubator the samples were harvested (refer to Appendix V for a week's schedule of the MN Assay).

2.8.2.2 Micronucleus Assay Harvesting and staining

The Micronucleus Assay harvesting technique varies from that described for Sister Chromatid Exchange as the technique is gentler to preserve the delicate binucleate cells. The initial harvesting step is the centrifugation of well mixed tubes for 10 minutes at 1000rpm. As in SCE harvesting, 5ml KCl (warmed to 37°C) is added to the resuspended cell pellet after the removal of the supernatant via a vacuum flask. The culture tubes were incubated at 37°C for precisely 4 minutes before a further 10 minute centrifugation step.

6% acetic acid is not used in the Micronucleus Assay, instead straight fixative is utilised. The majority of the supernatant was removed and the pellet resuspended. Fixative was added to the pellet, in three steps under constant agitation. The next centrifugation had a duration of 15 minutes at 1000rpm. Only one more 5ml aliquot of fixative was added to the resulting pellet before the final centrifugation.

The final lymphocyte pellet was dropped onto 5 slides (frosted 25mm x 75mm, BioLAB) per tube from a distance of 2cm. The cells are dropped in close proximity to the slides to prevent the binucleate cells breaking open upon contact, due to their fragile nature.

In order to visualise the binucleate cells the slides are allowed to dry before staining with 10% giemsa stain. For best visualisation, slides were mounted in DPX.

2.8.2.3 Micronucleus Assay Analysis

To obtain statistically accurate results, 500 to 1000 binucleate cells per sample were required in accordance with a recommendation from the developer of the Micronucleus Assay, Michael Fenech (personal communication, 2003). In this study, 1000 binucleate cells were scored. The Zeiss microscope was utilised to analyse the slides and is illustrated in Figure 2.6. As with the SCE analysis, the cells were sequentially counted, starting at one end of the slide and moving to the other end until 1000 binucleate cells were counted. If further slides were required to reach the full count they were counted in the same sequential manner.

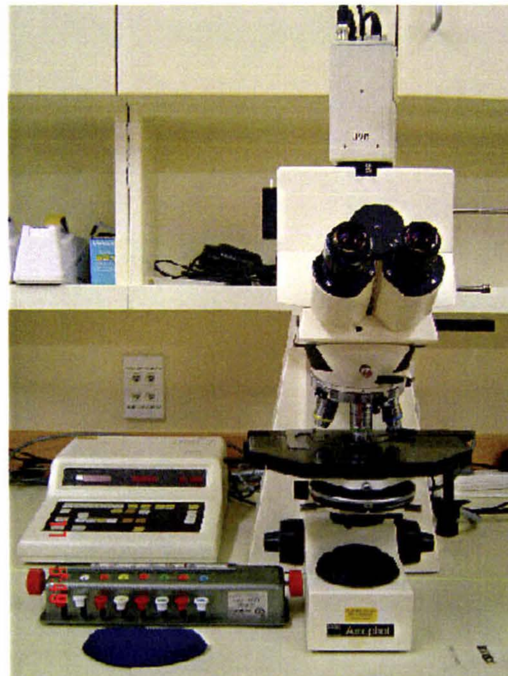


Figure 2.6. The Zeiss microscope and counter used for scoring the micronucleus assay. The counter is displayed on the left side of the picture and was used to keep a running total of the numbers of binucleate cells with each number of micronuclei.

The number of micronuclei were scored for each of the 1000 binucleate cells. The total number of cells containing each amount of micronuclei was recorded using a counter. There were several criteria to ensure standardisation of counting and these criteria were extracted from Bonassi *et al* (2001) and are explained with the assistance of Figure 2.7. The micronuclei counted were greater than 1/16 the size of the main nuclei; the cells were non-apoptotic (as visualised by granulation of the nuclei); and the cell membrane was complete to ensure all micronuclei were still contained.

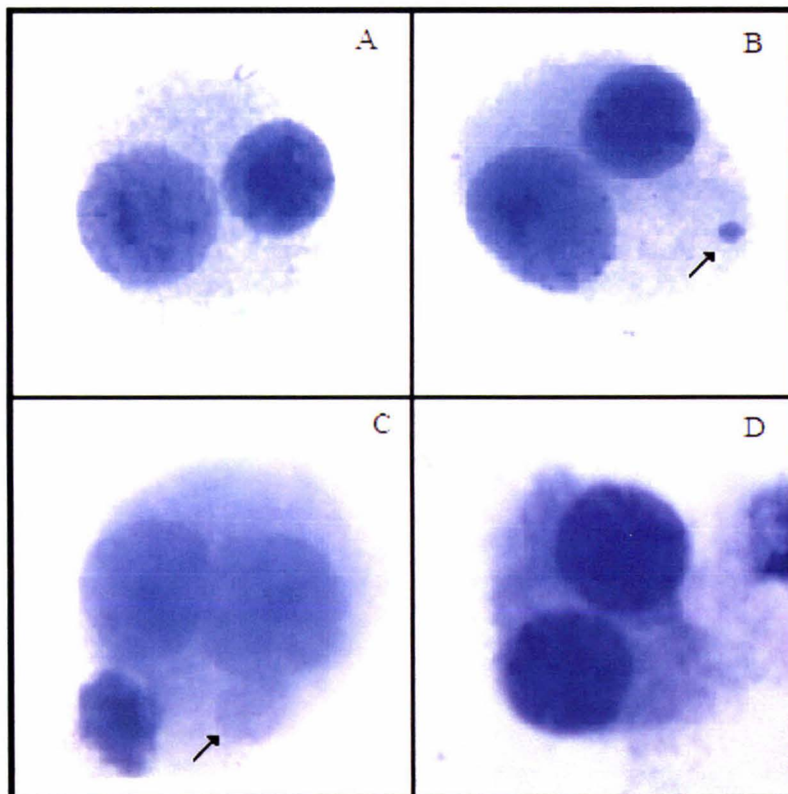


Figure 2.7. The standards for scoring the Micronucleus Assay. **A.** A normal binucleate cell with no micronuclei. **B.** A binucleate cell with one micronuclei as indicated by the arrow. **C.** The region indicated by the arrow is not counted as a micronucleus as it is still joined to a daughter nucleus. **D.** An unacceptable binucleate cell for scoring as the cell membrane is incomplete.

2.9 FREEZING THE BLOOD SAMPLES

Whole blood samples were cryopreserved using a modified version of the protocol outlined in "Human Cytogenetics. A Practical Approach" (Eds. Rooney & Czepulkowski. Oxford University Press. 1992). A total of fifteen cryogenic vials were frozen for each individual; six vials for SCE (two for each sampling period); six vials for MN (two for each sampling period); three for FISH (one for each sampling period).

The volume of blood to be frozen was adjusted so that after thawing, the same numbers of viable lymphocytes were cultured as when the tests were performed on fresh blood. This volume was calculated by following the same white blood count procedure as described in Section 2.8.1.1. with a predicted post thawing viability of 75%. This 75% viability was determined by the viability tests outlined in Section 2.13.

Worked Example:

Assume 0.39ml was required for a fresh sample as calculated in Section 2.8.1.1.

Need 25% more lymphocytes to obtain the same number of lymphocytes in both the fresh and cryopreserved tests.

0.39 x 0.25 = 0.098ml

0.39 + 0.098ml = 0.49ml blood to freeze

The required aliquot of blood was placed in a 1.5ml cryogenic vial (Nalge Nunc International) using a sterile 1ml syringe (Terumo). The freezing media was added to the blood in the cryogenic vials in equal amounts to the blood. The room temperature freezing media was delivered drop wise into the vial under constant agitation using a

sterilised plastic Pasteur pipette. Plastic Pasteur pipettes were utilised as glass Pasteur pipettes demonstrated limited success.

The viability of cryopreserved lymphocytes can be maintained by a controlled freezing rate of -1°C per minute. Controlled rate freezers were utilised in this study without success and are expensive so many laboratories have determined cheaper methods of controlled freezing. One of these methods (taken from Frenshneys' Culture of Animal Cells, Chapter 17) uses cotton wool and an insulated polystyrene box to control the cooling rate to -1°C per minute. This method has been determined as a reliable approximation but results between freezes vary slightly and no freezing method can be reproduced exactly.

In this study, once 15 cryogenic vials were prepared (the total for one individual), the vials were placed on a rack in the refrigerator (4°C) for half an hour. The time placed in the refrigerator was recorded on the freezing chart (refer to Appendix VI for a sample freezing chart) and the times recorded to reduce the sample temperature further. The samples were then placed in the freezing canes (Nalge Nunc 6-place) and wrapped in cotton wool before placed in a 1.5cm thick polystyrene box. These insulated samples were incubated in the –20°C freezer for 30 minutes, then placed in the –80°C for 1 hour before being unwrapped from the cotton wool and stored in the liquid nitrogen dewar demonstrated in Figure 2.8.

Figure 2.8. The 11L liquid nitrogen dewar utilised for cryopreserving the stored blood samples until thawing.

The cryovials were placed into the cryo-canes in the order that they were to be removed, with the samples to be first thawed at the top of the canes. The inventory listings for the cane layout can be observed in Appendix VII.

2.10 THAWING OF BLOOD

During the thawing procedure several safety precautions were observed. Insulated gloves were worn at all times, a face shield was worn to ensure eye protection in the event of a vial explosion, and the water bath was equipped with a lid.

The cryogenic vials for each individual were thawed at periods of either 1 month, 3 months or 6 months after freezing. The cryopreserved whole blood samples were thawed using a modified version of the protocol outlined in "Human Cytogenetics, A Practical Approach" (Eds. Rooney & Czepulkowski, Oxford University Press, 1992).

The required vials were removed from the liquid nitrogen carefully in pairs, and placed immediately into a beaker containing 37°C water. The beaker was sat in a 37°C hot water bath to keep its temperature constant. Once the vials were placed in the beaker to thaw, the lid was placed on the hot water bath. The vials were flashed-thawed in the water bath for approximately two minutes until only a small amount of ice crystals remained.

The thawed vials were taken to the biohazard hood and the exterior of the vials were swabbed with 70% ethanol to prevent contamination. Once sterilised, the vials were opened and each sample transferred to a 15ml conical centrifuge tube using a sterile plastic Pasteur pipette. Once the sample was in the centrifuge tube the thawing media was added drop-wise using a sterile plastic Pasteur pipette. One drop of thawing media was added every 10 seconds under constant agitation. After 2 minutes the number of drops were increased by one each time until the tubes were filled to 5ml. The tubes were then slowly topped up to 10ml.

Once the 10ml volume was obtained, the samples were centrifuged for 15 minutes at 1000rpm to pellet the blood cells. After centrifugation, the supernatant was removed and the thawing media added by the previous method described above. The tube was centrifuged for a further 15 minutes prior to the removal of the supernatant. The resulting pellet was added to the relevant culture tubes (pre-warmed to 37°C) using a sterile plastic Pasteur pipette.

2.11 BLOOD SMEARS; STAINING AND ANALYSIS

After thawing a blood sample, a drop of the resuspended pellet was removed using a capillary tube and placed on a microscope slide (frosted 25mm x 75mm, BioLAB). A blood smear was then formed by drawing another slide across the surface of the microscope slide, obtaining a single layer of blood cells as seen in Figure 2.9. Once the blood smear had dried at room temperature, a quick fix and staining kit was used (DIFF-QUIK (BDH)) to fix and stain the cells.

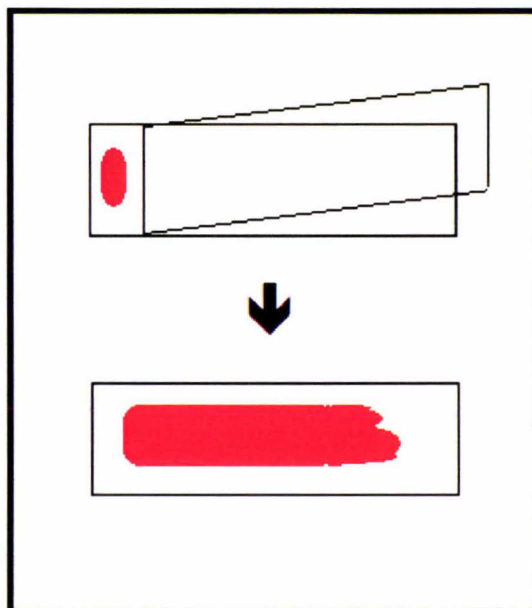


Figure 2.9. A schematic illustration of the process to obtain a blood smear for analysis. A small aliquot of blood is placed at the edge of a microscope slide. Another slide is placed at the front of the blood aliquot. As the slide is drawn across the surface of the microscope slide, the blood is drawn across by capillary action. This results in a single layer of blood cells.

Morphological analysis (with assistance from Vicky Scott, a medical laboratory scientist) was utilised to determine the numbers of lymphocytes when viewing the slide down a microscope. This analysis can confirm the presence of lymphocytes but not ascertain their viability. Blood smears were performed following many different freezing and thawing methods.

2.12 LYMPHOCYTE SEPARATION AND EVALUATION

During the optimisation of the cryopreservation technique, the use of whole blood was compared to the use of isolated lymphocytes. To separate lymphocytes, 2.5ml of fresh whole blood was gently mixed with 2.5ml of chilled Wellcome Media in an 8ml Falcon™ tube. 2.5ml of the resulting solution was layered onto 2.5ml of a Ficoll-

Paque Lymphocyte Separation Media (Amersham Bioscience) in a new 8ml Falcon™ tube. The blood-Ficoll-Paque solution was centrifuged at 1000rpm for 20 minutes using a Haraeus Megafuge 1.0 equipped with a 17cm rotor. If the separation of lymphocytes from erythrocytes was not complete, centrifugation was repeated until the lymphocytes were completely separated from the plasma and erythrocytes, as demonstrated in Figure 2.10.

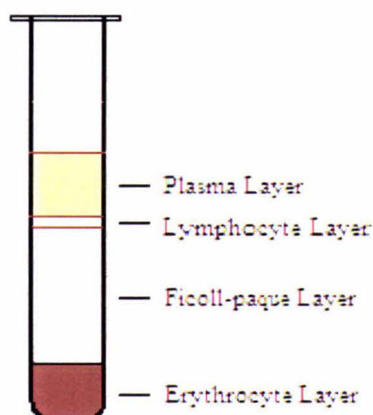


Figure 2.10. An illustration of the separated layers observed after the centrifugation of whole blood over a Ficoll concentration gradient.

The lymphocyte layer or 'buffy' layer was gently removed using the swirling motion of a glass Pasteur pipette. The use of glass at this stage did not affect the success of lymphocyte retention as glass appeared to have limited effect on non cryopreserved lymphocytes. This 'buffy' layer contained the majority of the lymphocytes. The lymphocytes were transferred into an 8ml Falcon™ tube that contained 1ml of chilled Wellcome Media, mixed by vortex and stored on ice.

To determine the lymphocyte concentration of the isolated sample, Trypan Blue staining and a haemocytometer was utilised (Weber Scientific International Ltd.). A Haemocytometer is a specialised slide with two gridded chambers. These gridded chambers are used to determine the concentration of cells in a sample. Each grid is composed of nine large squares (1mm in length) with the central square divided into

25 smaller squares (0.2mm in length). The slide layout is demonstrated in Figure 2.11.

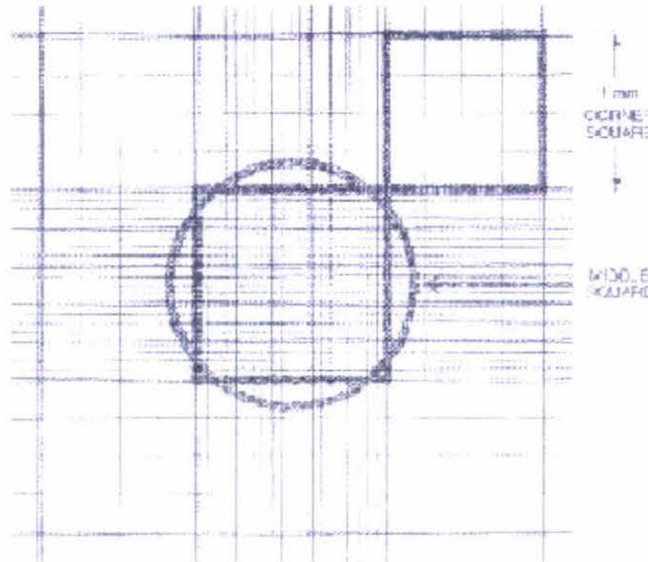


Figure 2.11. An illustration of the Haemocytometer counting chamber. The encircled central square is the square used for counting cells.

To use the haemocytometer, a coverslip was placed on a clean haemocytometer. A slight rainbow appearance was observed on the edges of the coverslips which indicated that the distance between the slide and coverslip was exactly 0.1mm, as required for an accurate cell count. The central large square encircled in Figure 2.11 was used for all cell counts. The volume contained in the large central square is $0.1mm^3$ (1mm x 1mm x 0.1mm). As there is $1cm^3$ in 1ml, there are $1x10^4$ volumes of the counting square in 1ml. Therefore to determine the cell concentration in cells/ml the number of cells counted in the central square was multiplied by $1x10^4$.

To determine cell viability trypan blue exclusion staining was performed. A single drop of Trypan Blue stain was mixed with a single drop of cell suspension. After 1 minute the trypan blue/cell suspension mix was taken up by a Pasteur pipette and

slowly added to the edge of the haemocytometers' coverslip. The solution is taken up under the coverslip by capillary action until the chamber was filled.

Under a microscope at 400X magnification the live and dead lymphocytes can be evaluated. Non-viable cells have damaged membranes and so appear as dark blue structures due to the penetration of the Trypan Blue into the cell. Viable cells remain relatively clear against the light blue background.

To determine an accurate figure for viability both the viable cells and the non-viable cells contained within the central square were counted. A dilution factor of 2 was considered and the calculation to a cell/ml concentration performed. Cell viability was determined by the concentration of viable cells divided by the total cellular concentration and multiplied by 100 to achieve a percentage.

2.13 VIABILITY KIT; STAINING AND ANALYSIS

Viability upon thawing was important to establish, so lymphocyte viability was confirmed with the use of the Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes). This viability kit is based on the simultaneous determination of live and dead cells with two probes that measure two parameters of cell viability- intracellular esterase activity and plasma membrane integrity. Live cells are distinguished by the presence of esterase activity, determined by the enzymatic conversion of the cell-permeant calcein AM to the intensity of fluorescent calcein. The calcein dye is well retained within live cells and produces a uniform green fluorescence in live cells (ex/em ~495nm/~515nm). Dead cells are detected by the penetration of EthD-1 into the cells through damaged membranes. Within the cell, EthD-1 binds to nucleic acids and therefore undergoes a 40-fold enhancement of fluorescence, producing a bright red fluorescence (ex/em ~495nm/~635nm).

The best results were obtained by first optimising the dye concentrations to achieve distinct labelling of the live and dead cells. To optimise the EthD-1 concentration dead cells were prepared by treating a slide of cells with 70% methanol for 30

minutes. The correct dye concentration was discovered to be 4μM as this stained the nucleus bright red without staining the cytoplasm significantly. 0.4μM of calcein was found to not stain the cytoplasm of the dead cells significantly, but did stain live cells.

To analyse the viabilities, blood smears were made using the cell pellet of thawed samples as in Section 2.11. These smears were allowed to settle onto the slides in a humidity chamber at 37°C. This settling process took approximately one hour. After the cells had settled onto the slides, 150μL of the optimised fluorescent probes and a cover slip were added to the cells. The slides were then incubated for a further 45 minutes in a dark humid environment for the probes to penetrate the cells. The cover slip was then sealed to the glass slide with clear nail polish.

Analysis was performed with care as the blood cells on the slides were not fixed. The fluorescent images were viewed using the Olympus BX51 fluorescent microscope and MagnaFIRE digital camera. The calcein and EthD-1 were viewed simultaneously with a conventional fluorescein longpass filter.

The analysis was assisted by the use of two computer programs. MagnaFIRE Software was used to capture 10 random fields of view from each slide (two slides prepared per test). Random fields were selected by choosing random coordinates: using a random number generator, numbers between 100-160 were chosen for the x axis and numbers between 0-20 were chosen for the y axis (Appendix VIII).

Once the images were obtained ImagePro software was utilised for analysis. Each fluorescent colour was selected for counting and the software counted the red cells and the green cells separately to give the total numbers present of each colour (Figure 2.12). This random software-based scoring ensured accurate, non biased analysis. On occasion non-lymphatic cells were observed on the slide, as determined by morphological analysis (eg. red blood cells) and these cells were manually excluded from the viability analysis. The percentage viability was determined by the average viability from the images for each sample.

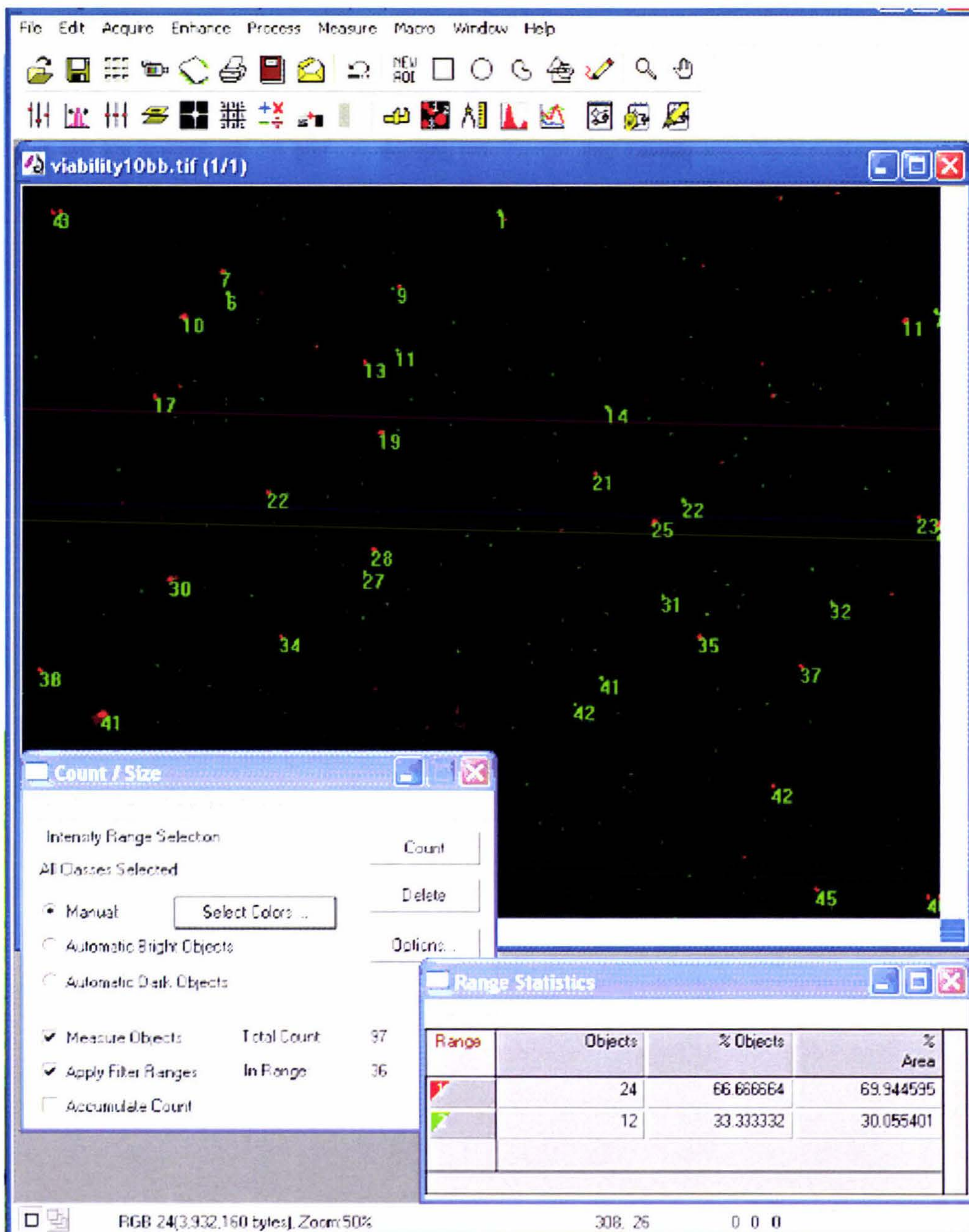


Figure 2.12. A view of the ImagePro software used to analyse the viabilities. Each lymphocyte is labelled red or green and numbered. The count window (bottom left) counts the live and dead cells separately to produce the range statistics window (bottom right).

CHAPTER 3: RESULTS

3.1 OPTIMISATION OF THE CRYOPRESERVATION TECHNIQUE

The cryopreservation of blood is a well characterised technique for the study of blood cell subsets. The cryopreservation of whole blood for *cytogenetic* testing though is not well described. Before the suitability of cryopreserved whole blood as a source of lymphocytes for genetic testing could be explored, an optimised method for the cryopreservation of whole blood was required. Successful culturing of lymphocytes from fresh whole blood is routinely achieved and this acted as the control upon which the success, or otherwise, of the cryopreservation attempts were compared.

The initial attempt to culture lymphocytes from cryopreserved whole blood followed a method described in the 'Daids Virology Manual for HIV Laboratories' (1997). This method was performed on three different blood samples, on two separate occasions, but was found to be an unsuccessful method for the requirements of this study. The slides resulting from the harvesting procedure were devoid of any blood cells despite three days of culturing conditions (for captured images of a slide refer to Appendix IX). The results indicated that either the lymphocytes were not preferentially selected or cryopreservation conditions were too harsh causing damage to the lymphocytes.

To obtain the optimised cryopreservation technique outlined in the Materials and Methods Chapter (Sections 2.9, 2.10), several different aspects of the freezing, thawing, culturing and harvesting procedures were explored. The results obtained after each adjustment, the implications of the findings on the overall method and their role on the success of culturing cryopreserved blood samples, are described in the following sections. Once this optimised cryopreservation method was established, the cryopreservation and genetic damage study proceeded.

3.1.1 Thawing Procedure

The blank slides obtained after the initial cryopreservation method suggested the removal of blood cells at a point prior to cell visualization. Samples were therefore analysed throughout the cryopreservation procedure for the presence of lymphocytes to determine the point of lymphocyte removal. To ascertain the presence of lymphocytes prior to cryopreservation, the blood/freezing media mix was tested for the presence of lymphocytes by Trypan Blue Staining (Section 2.12). Lymphocytes were determined by morphological analysis and their presence was confirmed in the samples for storage.

After assessment, it was determined that the blood cells could be removed either during the thawing procedure or the harvesting technique, as both of these manipulations involve the sample removal (the supernatant). Trypan Blue Staining was utilized to determine the point of lymphocyte removal. Lymphocyte counts were performed on five blood samples from one individual before cryopreservation (on the blood/freezing media mix), and at four different points after thawing. The observation points included immediately after thawing and after the supernatant had been removed in each of the three wash steps. The lymphocyte counts from this analysis can be observed in Table 3.1, and the mean lymphocyte counts are displayed graphically in Figure 3.1.

Table 3.1. Lymphocyte counts during the processing of five cryopreserved whole blood samples from one individual as determined by Trypan Blue Staining.

Observation Point	Lymphocyte Count (cells/ml) 1	2	3	4	5	Mean	Std. Dev	Std. Error
Before Cryopreservation	$6.5x10^6$	$6.5x10^6$	$6.5x10^6$	$6.5x10^6$	$6.5x10^6$	$6.5x10^6$	0.0	0.0
After Thawing	$4.2x10^6$	$3.8x10^6$	$4.3x10^6$	$4.4x10^6$	$3.9x10^6$	$4.1x10^6$	0.3	0.1
After 1st Wash	$8.0x10^5$	$8.2x10^5$	$8.1x10^5$	$8.1x10^5$	$7.8x10^5$	$8.0x10^5$	0.2	0.1
After 2nd Wash	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
After 3rd Wash	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

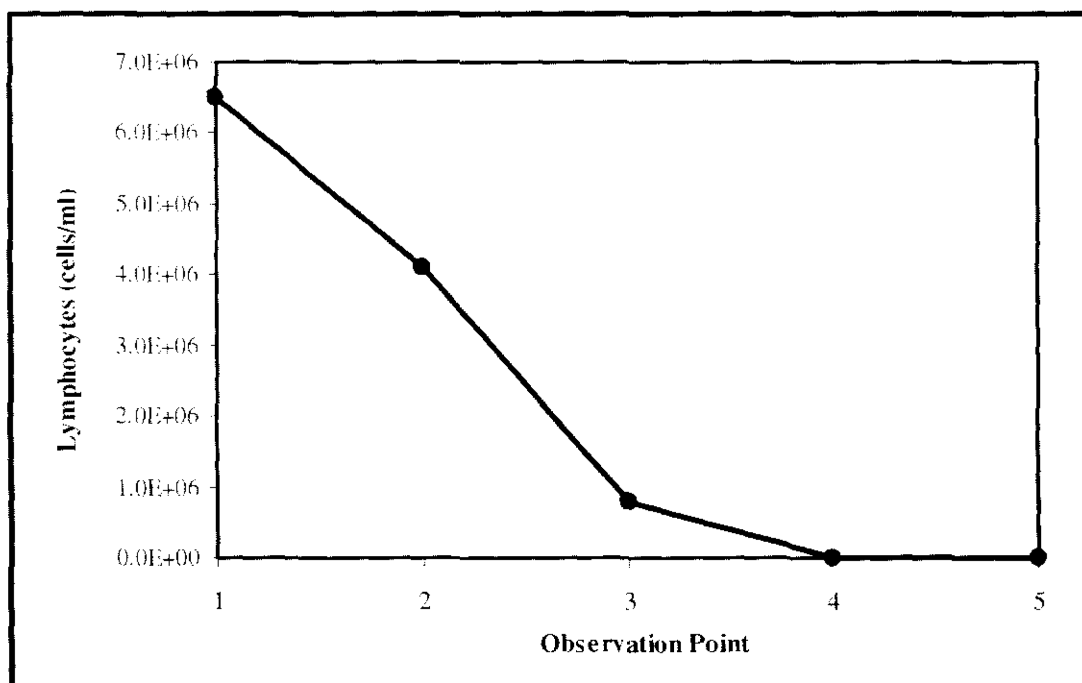


Figure 3.1. Graph of lymphocyte concentration (cells/ml) over the thawing period, as measured by Trypan Blue Staining Analysis. (Observation Points: 1= before cryopreservation, 2= after thawing, 3= after 1st wash, 4= after 2nd wash, 5= after 3rd wash).

Initially, the mean lymphocyte counts from the cryopreserved blood samples were only slightly decreased from the lymphocyte counts prior to cryopreservation. As Trypan Blue Staining was performed after each stage of manipulation, the numbers of lymphocytes were noted to reduce gradually throughout the procedure until their sudden removal after the second wash step. Attempts to culture these samples were unsuccessful. These data confirmed the removal or loss of lymphocytes during the thawing procedure.

The gradual reduction in the lymphocyte count portrayed in Figure 3.1, before the anticipated decline, suggested the loss of lymphocytes was occurring at each centrifugation rather than at one unique step. To combat this overall lymphocyte loss, various adjustments were made throughout the procedure to improve the lymphocyte retention.

The possible effects of dimethyl sulfoxide (DMSO) on lymphocyte retention during centrifugation were explored. DMSO was possibly altering the lymphocyte weight thereby reducing their sedimentation rate during centrifugation. A reduced sedimentation rate would result in the removal of lymphocytes with the supernatant as a proper pellet is not formed. Increased centrifugation periods and speeds were tested for their ability to increase the numbers of lymphocytes retained.

Qualitative morphological analysis was performed on the cultured cryopreserved samples as a quantitative method was not available. Analysis occurred after each adjustment to the centrifugation method. Modifications to the centrifugations included: increasing the thawing centrifugation to 20 minutes, increasing the centrifugation after acetic acid addition to 25 minutes, increasing the centrifugation to 15 minutes after KCl addition and a 5 minute increase on all centrifugations. These modifications did not result in the successful culturing of cryopreserved lymphocytes as demonstrated in Table 3.2. Illustrations of the observed morphologies are displayed in Appendix IX.

Table 3.2. The lymphocyte morphologies observed after modifications to the centrifugation periods.

Centrifugation at 1000g	Trials	Lymphocyte Morphology
Thawing centrifugation 20mins	3	Cell debris and fragmented lymphocytes
25mins centrifugation after acetic acid addition	3	Cell debris
15mins centrifugation after KCl addition	3	Cell debris
5mins increase on all centrifugations	5	Cell debris and intact lymphocytes

Overall the observed lymphocyte morphology was indicative of cellular damage. Lymphocytes were fragmented and a vast amount of cell debris was observed. A five minute increase on all centrifugations throughout the thawing and harvesting procedures did appear to improve the condition of the lymphocytes and their retention (as some intact lymphocytes were observed), but did not render the lymphocytes suitable for culturing.

As lymphocytes were not successfully cultured after these adjustments, further factors influencing lymphocyte retention were investigated. All following manipulations, though, involved a five minute increase on all centrifugation steps to improve the retention of intact lymphocytes.

3.1.2 Cryopreservation Solution Concentration

The increased cellular damage and cell debris observed after cryopreservation indicated the potential role of cellular stress during the freezing and thawing processes. Damage to the cells occurs through two main mechanisms, the formation of intracellular ice crystals and dehydration of the cells due to osmotic imbalance. The effects of ice crystal formation and osmotic balance though, are not the only potential causes of cellular stress. DMSO is also a toxic substance and has been

observed to cause cellular damage especially at room temperature or elevated temperatures. In an effort to reduce the freeze/thaw stress on the lymphocytes the effects of different DMSO concentrations were explored. The study of DMSO effect was coupled with exploring the effects of additional cryopreservation solutions, including polyvinylpyrrolidone (PVP) and Glycerol.

The DMSO concentrations explored included 5%, 10% and 20% with the DMSO kept on ice. The effects of the DMSO concentration on lymphocyte morphology were determined by morphological examination of the lymphocytes after 72 hours of culturing. Other common cryoprotectants (glycerol and PVP) were also analysed for their effect on the lymphocyte morphology. Glycerol was analysed due to its frequency of use for cryopreservation and the penetrating cryoprotectant PVP was explored to determine if increased intercellular protection was required to reduce cellular damage (Table 3.3). Repeat experiments were performed with each different cryoprotectant.

Table 3.3. Morphological results from the differing cryopreservation solutions at their differing concentrations

Cryoprotectant	Trials	Bacterial Contamination	Lymphocyte Morphology
None	2	Mild	Cell Debris
20% DMSO	5	None	Cell Debris
10% DMSO	3	None	Mix of fragmented and intact
5% DMSO	3	None	Mix of fragmented and intact
10% DMSO + 10% PVP	2	None	Mix of fragmented and intact
5% DMSO + 10% PVP	2	None	Mix of fragmented and intact
10% Glycerol	2	None	Cell Debris

Table 3.3 indicates a considerable morphological improvement when using 5% and 10% DMSO concentrations compared to the cells cryopreserved using 20% DMSO. Intact lymphocytes were more numerous, less dehydrated and the numbers of fragmented lymphocytes and cell debris was reduced. 5% and 10% DMSO therefore appeared to be the appropriate concentrations for cryopreservation.

The use of 10% PVP in addition to 5% and 10% DMSO revealed no difference in lymphocyte culturing ability to the use of DMSO alone. PVP therefore appeared to be an unnecessary addition. The use of Glycerol as a cryoprotectant resulted in an increase of cell debris so was not deemed suitable for the cryopreservation of whole blood and is generally considered a cryopreservation solution for smaller cells such as bacterial stocks. The results confirmed DMSO as the appropriate cryoprotectant for the cryopreservation of whole blood, when lymphocytes are required.

To determine the extent of DMSO toxicity over the other cellular stresses incurred during cryopreservation, the extent of DMSO toxicity on fresh whole blood (samples that were not cryopreserved) was also evaluated. DMSO (5%, 10% and 20%) was added to culture tubes containing 0.5ml of fresh blood sample and incubated at 37°C for 72hours. After four trials at each concentration, the concentrations of 5% and 10% DMSO were found not to hinder the growth of fresh lymphocytes at 37°C (Table 3.4) while 20% DMSO prevented lymphocyte proliferation (images in Appendix X). The toxicity of DMSO may therefore be irrelevant to the lymphocyte's cellular damage observed in Table 3.3 as non cryopreserved blood had the capacity to proliferate in the presence of DMSO. This indicates a greater role for cellular freezing stress in the cellular damage observed after cryopreservation.

Table 3.4. The morphological effects from differing DMSO concentrations on non cryopreserved lymphocytes during culturing.

DMSO Concentration	Trials	Lymphocyte Morphology	Culturing Success
5%	4	Normal	Metaphases observed
10%	4	Normal	Metaphases observed
20%	4	Fragmented	No metaphases observed

3.1.3 DMSO Temperature

DMSO is reported to be toxic above room temperature (Sperling & Larsen 1979). In addition to observing the effects of DMSO concentration, the effects of the DMSO working temperature were also explored. The DMSO working temperature may be the cause for lymphocyte cellular damage as its effectiveness can be constrained to a narrow range of temperatures.

Manipulations involving DMSO, such as the addition of freezing media and thawing media, were performed at several different temperatures: on ice (less than 0°C), 4°C, 0°C, room temperature (18°C) and physiological temperature (37°C). Giemsa-staining after the harvesting procedure allowed analysis of the lymphocyte morphology for each of the DMSO working temperatures. Each temperature was tested four times to ensure consistent results. The data from this morphological analysis is shown in Table 3.5.

Table 3.5. The effects of different DMSO temperatures on the resulting lymphocyte morphology after culturing cryopreserved whole blood samples.

DMSO Temperature	Trials	Lymphocyte Morphology
On Ice	4	Mix of fragmented and cell debris
0°C	4	Cell debris
4°C	4	Mix of fragmented, intact and cell debris
18°C	4	Mix of fragmented and intact
37°C	4	Cell debris

None of the attempted temperatures demonstrated in Table 3.5 provided dividing lymphocytes for analysis. The working temperatures of ~18°C (room temperature) and 4°C resulted in the most intact lymphocytes, so room temperature was selected as the future working temperature, over 4°C, for ease of manipulations.

Despite the selection of room temperature working conditions for biohazard hood manipulations, it was a possibility that the centrifugations during the thawing procedure may increase the temperature of the sample, rendering DMSO toxic to the cells. This potential heat increase during centrifugation and the information obtained from DMSO toxicity analysis on fresh blood (section 3.1.2), resulted in the attempt to culture lymphocytes that were not washed after thawing. DMSO was found to be non toxic on fresh cells at 37°C, so it was suspected that unwashed cells could grow diluted in culture media at 37°C avoiding the toxic effects of DMSO. This concept was tested by the addition of the entire cryovial content to the culture media immediately after thawing, without complicated thawing procedures. After 72 hours of incubation at 37°C this method was found to be unsuccessful as no divided lymphocytes were observed, only fragmented lymphocytes (Appendix XI). The

failure to culture at this point demonstrated the insignificant role that DMSO was playing in regard to cellular damage during the cryopreservation process. The insignificant role of DMSO suggested that freeze/thaw cellular stresses may play a more vital role in the cellular damage observed than originally anticipated.

3.1.4 Freezing Rate

A critical step in the cryopreservation of whole blood is the rate at which the samples are frozen. The freezing rate must be optimized to reduce the intracellular ice formation and osmotic gradients, which if incorrect, can cause damage to the cells. It is well noted that the optimal freezing rate is -1°C per minute. To explore the effect of the freezing rate, several methods of rate controlled freezing were attempted with many repeat trials (Section 2.9). These methods involved different periods of refrigeration (4°C), freezing (-20°C and -80°C) and placing in Liquid Nitrogen (L N_2) (Table 3.6). At each of the freezing rates analysed, two different forms of insulation were utilised to reduce the freezing rate. Both methods used cotton wool to insulate the cryovials before placing them in either a 1mm thick polystyrene box or a 2mm thick polystyrene box. The 2mm thick polystyrene box consistently gave a higher proportion of intact lymphocytes then the 1mm thick polystyrene box so the former was utilised for further rate controlled freezing (results not shown).

The attempted methods of rate controlled freezing did not result in increased lymphocyte survival and proliferation. The rapid freeze showed compete absence of cells after all four trials demonstrating the requirement for a slow rate controlled cooling system. The other methods of rate controlled cooling attempted all displayed a similar level of fragmented and intact lymphocytes (Table 3.6) but still did not result in cultured lymphocytes with metaphases for analysis. Illustrations of the scored lymphocyte morphologies are featured in Appendix IX.

Table 3.6. The different methods of rate controlled freezing using 2mm thick polystyrene box for insulation and the resulting lymphocyte morphologies.

Freezing Method	Trials	Lymphocyte Morphology
2hrs -80oC, Liquid N_2	4	Absent
1hr -20oC, 1hr -80oC, LN_2	4	Mix of fragmented and some intact
1hr -20oC, 2hs -80oC, LN_2	4	Mix of fragmented and some intact
30mins -20oC, 1hr -80oC, LN_2	4	Mix of fragmented and some intact
10mins 4°C, 30mins -20oC, 1hr -80oC, LN_2	4	Mix of fragmented and some intact
30mins 4°C, 30mins -20oC, 1hr -80oC, LN_2	4	Mix of fragmented and some intact

These forms of rate controlled freezing are not exactly reproducible but the method has been measured at approximately -1°C per minute. To provide a more accurate cooling rate of -1°C, the rate controlled freezer at Palmerston North Hospital was utilised. This system of cryopreservation is accurate and reproducible and the cooling rate is demonstrated in Figure 3.2. The first attempt to use this rate controlled freezer was unsuccessful due to bacterial contamination, but upon the second attempt, dehydrated, fragmented lymphocytes were observed. The use of a rate controlled freezer was therefore also found to be unsuccessful for reducing the observed lymphocyte cellular damage.

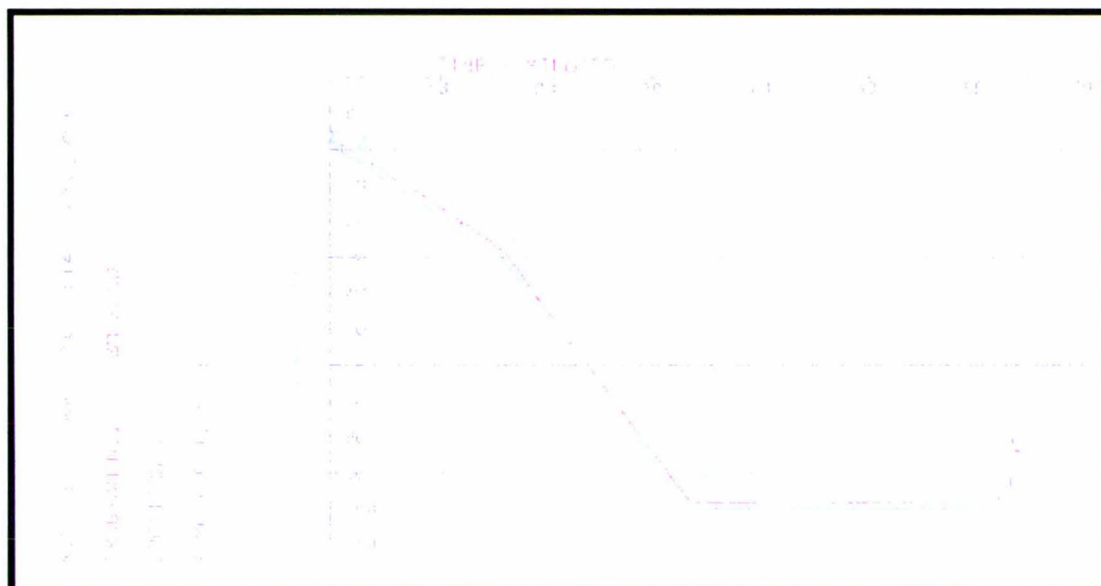


Figure 3.2. The graph of cooling rate from the rate controlled freezer at Palmerston North Hospital. The blue line is the expected cooling rate and the red line is the actual cooling curve observed.

There was the potential that DMSO was not allowed a period long enough to fully protect the lymphocytes before cooling commenced. It was therefore determined whether an equilibration time was required to allow the DMSO to take effect before cooling. Different periods of standing at room temperature and on ice were trialed (5mins, 10mins, 15mins, 30mins) but none resulted in improved lymphocyte morphology or culturing ability (results not shown). The method of cooling the samples therefore appeared to be irrelevant to the cellular lymphocyte damage observed so further potential causes for the damage were explored.

3.1.5 Thawing Rate

Another critical step where cellular damage may occur in the cryopreservation process is the thawing rate. A variety of thawing rates were performed on the cryopreserved samples to determine an optimal thawing rate, in terms of morphological characteristics after culturing. For all samples, the thawing media was

added after swiftly thawing the samples at 37°C (Section 2.10). The rates of thawing media addition were slow, medium, fast and instant. Morphologies observed for each of these thawing rates are shown in Table 3.7.

Table 3.7. The effects of different thawing media (TM) addition rates on the observed lymphocyte morphology.

TM Speed	Method of TM addition	Lymphocyte Morphology
Slow	Drop-wise for 10 secs for 2mins and slowly up to 5ml and then to 10ml	Cell debris
Medium	Drop-wise for 10 secs for 2 mins and slowly to 10 ml	Fragmented
Fast	Drop-wise for 10 secs for 1 min and slowly to 10ml	Normal
Instant	Thawing media added instantly	Cell debris

The fast method, of one drop every 10 seconds for 1 minute and then topping up slowly to 10ml, resulted in the highest number of visual whole lymphocytes, while the instant and slow methods yielded only cell debris. This fast method of thawing media addition appeared to reduce the effects of osmotic shock upon thawing. Despite the appearance of intact lymphocytes the attempts of culturing these lymphocytes were still unsuccessful.

The results from this experiment indicated that the whole blood/freezing media mix must be diluted quickly to avoid damage to the cells. To reduce this damage, samples were processed swiftly. This swift processing though, did not further improve the number of intact lymphocytes or their ability to be cultured so further modifications to the cryopreservation method were required.

3.1.6 Isolated Lymphocytes

As lymphocytes from whole blood samples were not successfully cultured after cryopreservation, the success of cryopreserved isolated lymphocytes was assessed. Isolated lymphocytes, although requiring greater processing time, have an advantage in that the other cells (less amenable to cryopreservation) are removed and are unable to inhibit the growth of the lymphocytes.

This method of lymphocyte separation was attempted several times to ensure that its success was not dependent on the expertise of performing this technique (Section 2.12). The isolated lymphocytes were stored at the same, two times and three times the concentration of lymphocytes found in the cryopreserved whole blood samples. All other thawing and freezing procedures, previously found to be optimal, were followed. To ensure lymphocytes had been isolated prior to cryopreservation, their presence was determined by Trypan Blue Staining (Section 2.12). The success of culturing cryopreserved isolated lymphocytes is outlined in Table 3.8.

Table 3.8. The lymphocyte morphology observed after cryopreservation of isolated lymphocytes.

Lymphocyte Concentration	Lymphocyte Morphology		
	Trial 1	**Trial 2**	**Trial 3**
Isolated Lymphocytes	No cells	No cells	Cell Debris
2x Isolated Lymphocytes	Fragmented	Fragmented	Fragmented
3x Isolated Lymphocytes	Fragmented	Intact	Intact

The culturing successes of cryopreserved isolated lymphocytes improved with each attempt for all lymphocyte concentrations but overall were still as unsuccessful in

obtaining cells undergoing cell division as cryopreserved whole blood. Therefore whole blood was continued as the sample of choice for this project.

3.1.7 Length of Culture

In many cases, the lymphocytes obtained after culturing were intact but had simply not divided. Their lack of replication suggested that a longer recovery time to re-establish, grow and divide after cryopreservation may be required. To determine if the lymphocytes from cryopreserved whole blood required an extended recovery period in the culture media, the lymphocytes were cultured after cryopreservation (Section 2.8.1.1) for 72hours, 84hours and 96 hours. These culturing times did not significantly improve the appearance of the lymphocytes nor did they promote lymphocyte growth and cell division (results not shown).

An increased level of mitotic stimulant may be required to promote cell growth after cryopreservation. To assess the role of the mitotic stimulant, twice the normal level of PHA was added to the culture tubes prior to culturing. This increased mitotic stimulant though, showed no modification in the lymphocyte culturing ability (results not shown).

3.1.8 Non Adhesion

A potential reason for the lack of lymphocytes and their altered morphology was the use of glass transfer pipettes. Lymphocytes may adhere to glassware and can be either damaged or lost entirely. To combat this adhesion, sterile plastic transfer pipettes were utilised to transfer the blood cell pellet into the culture tubes. Five blood samples from an individual were cryopreserved and thawed for analysis. The numbers of lymphocytes were evaluated after creating five blood cell smears and stained with the DIF QUICK fixative and staining solution (Section 2.11). The lymphocyte counts from pellets processed with plastic pipettes are shown in Table 3.9.

Table 3.9. The lymphocyte counts after each wash using plastic pipettes to transfer the blood pellet.

Observation Point	Lymphocyte Count (cell/ml) 1	2	3	4	5	Mean	Std. Dev	Std. Error
After 1st Wash	$1.27x10^9$	$1.03x10^9$	$1.33x10^9$	$1.25x10^9$	$1.41x10^9$	$1.26x10^9$	1.14	0.06
After 2nd Wash	$1.51x10^9$	$1.42x10^9$	$1.39x10^9$	$1.43x10^9$	$1.67x10^9$	$1.48x10^9$	0.11	0.05
After 3rd Wash	$2.10x10^9$	$1.87x10^9$	$1.64x10^9$	$1.91x10^9$	$2.12x10^9$	$1.92x10^9$	0.22	0.99

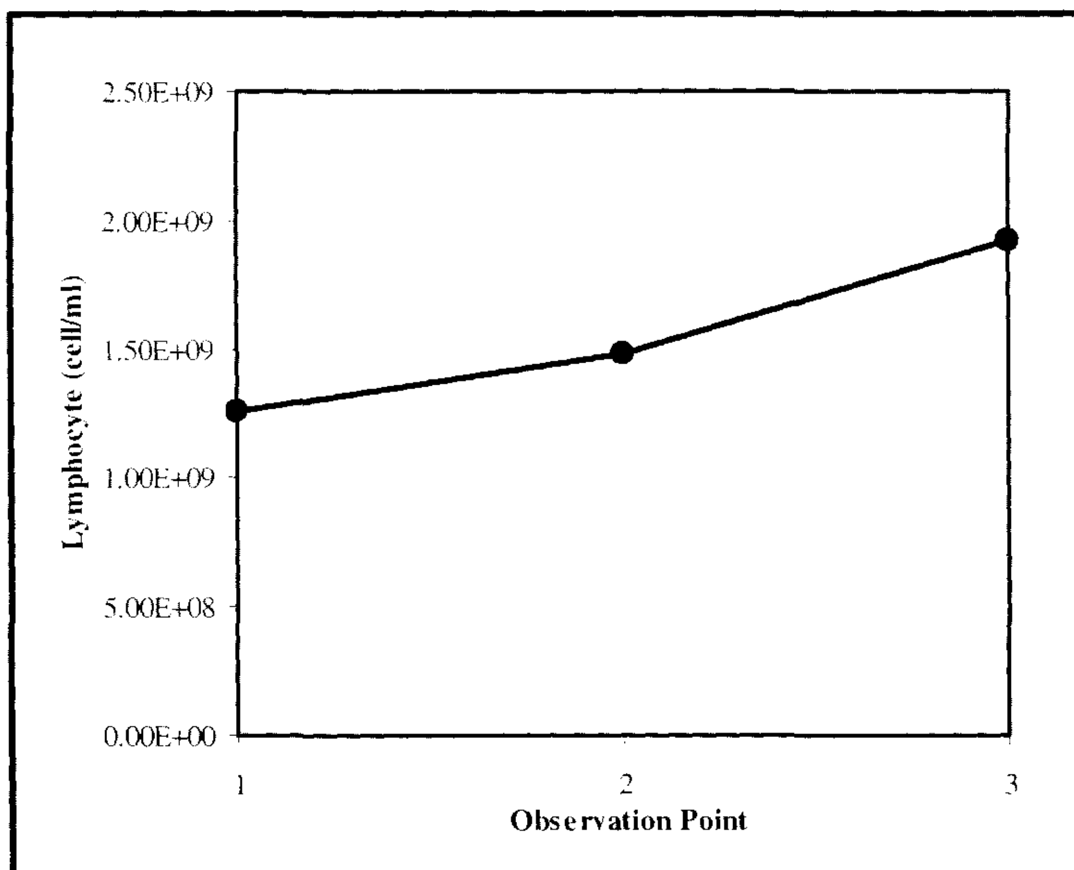


Figure 3.3. Graph of the mean lymphocyte counts (cell/ml) observed over the three wash steps when utilising plastic Pasteur pipettes.

The use of plastic Pasteur pipettes instead of the previously utilised glassware maintained a higher lymphocyte count throughout the thawing process. When comparing these to the values obtained from glass (results not shown) an increase in intact healthy lymphocytes were observed, along with less fragmented cells and cell debris. It is interesting to note that the lymphocyte concentrations actually increase with each thawing wash step. This increase may be due to the excellent retention of lymphocytes throughout the thawing process, resulting in the pellet becoming increasingly concentrated as the supernatant is removed. These data demonstrate the usefulness of plastics for blood work.

After determining that the utilisation of plastic Pasteur pipettes results in increased healthy lymphocyte counts, a blood sample was frozen using 10% DMSO (at room temperature), frozen at a medium rate and thawed at a swift rate with fast thawing media addition and two washes (all performed with plastic ware) (Section 2.9 and Section 2.10). Under these conditions numerous metaphases were observed after a period of 72 hours culturing at 37°C, illustrating that a method had been obtained to culture cryopreserved lymphocytes from a whole blood sample (Figure 3.4).

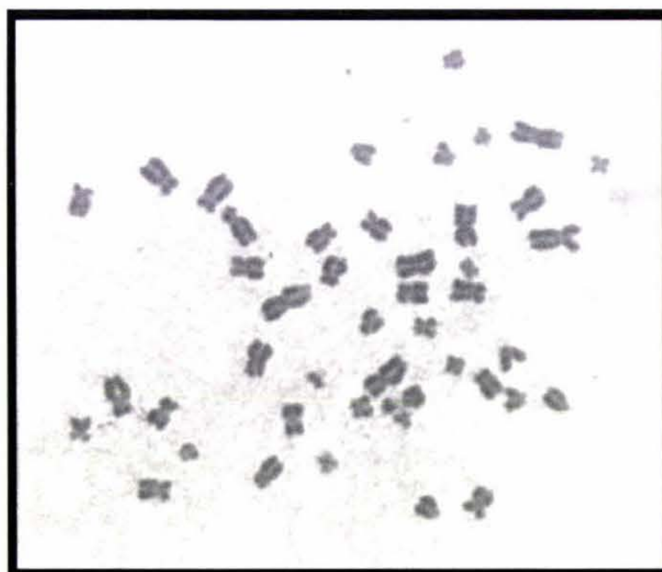


Figure 3.4. An example of a metaphase obtained when culturing lymphocytes after cryopreservation using the optimised technique.

3.2 VIABILITY DATA

To accurately assess the potential levels of cell death occurring during cryopreservation, lymphocyte viability was assessed after several methodological modifications. The Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes) was utilized rather than Trypan Blue Staining as Trypan Blue Staining does not indicate cells entering the early stages of apoptosis, only those in the late stages of programmed cell death. The Live/Dead Viability/Cytotoxicity Assay provides a more accurate level of cell viabilities and was performed as described in Section 2.13. An accurate read of cell viability was required to enable compensation for this cell loss when cryopreserving blood aliquots.

The variations to the cryopreservation method were: (1) the media used for freezing (Wellcome Media or RPMI), (2) the thawing media addition rate (slow, medium or fast) and (3) the number of thawed samples processed at one time (two or four). After these variations, the most successful method was selected for optimised viabilities and the final optimised viabilities after thawing was determined before proceeding with the Cryopreservation and Genetic Damage study.

3.2.1 Media

The majority of studies in the literature have utilised RPMI as the media for freezing media, thawing media and culture media. Viabilities of samples cryopreserved and thawed with RPMI and the media utilised in this study, Wellcome Media, were compared to determine the optimal media for use. A blood sample from an individual was frozen as 20 aliquots, 10 aliquots with RPMI as the freezing media and 10 aliquots with Wellcome media as the freezing media (refer to Section 2.9 for method). Upon thawing, the samples frozen with RPMI were thawed with RPMI containing thawing media while those samples frozen with Wellcome Media were thawed with thawing media containing Wellcome Media. The results for this experiment are shown in Table 3.10.

Table 3.10. The descriptive statistics for the viabilities of samples treated with either RPMI or Wellcome Media.

Media	N	Mean	Std. Dev	Std. Error
RPMI	10	44.84	13.47	4.26
Wellcome	10	83.36	14.46	4.57

A Paired *t*-test of these data was conducted and the difference between RPMI use and Wellcome Media utilisation was found to be highly statistically significant (P = < 0.050). Wellcome Media consistently obtained higher viabilities after thawing and had a mean viability of approximately double the mean viability of RPMI. These results allowed confidence in the use of Wellcome Media over RPMI for the cryopreservation and thawing of whole blood samples.

3.2.2 Thawing Media Addition Rate

To assess the importance of the thawing media addition rate on lymphocyte viability, cryopreserved whole blood samples from one individual were thawed in the same slow, medium and fast methods as in section 3.1.5. In prior experiments the morphological effects of the thawing media addition speed were explored, but these morphological differences were not statistically relevant so more accurate viabilities were required. To obtain these data, thirty 0.5ml aliquots of an individuals' blood were cryopreserved and ten samples thawed at each of the differing rates before viability analysis was performed. The descriptive statistics from these trials are demonstrated in Table 3.11.

Table 3.11. The descriptive statistics for lymphocyte viability after different thawing speeds.

Speed	N	Mean	Std. Dev	Std. Error
Slow	10.0	89.5	8.5	2.68
Medium	10.0	80.9	11.4	3.61
Fast	10.0	72.4	18.0	5.69

The data demonstrated a decrease in the mean viability with the increasing thawing speeds but overall this difference was not significant ($P = 0.63$) when multivariate statistics were applied. The trend though, for increased viability with lower speeds was a significant effect when within-subject contrasts were applied ($P = 0.027$). These results confirm the slow thawing rate as the rate with best lymphocyte retention.

These results dispute those found with morphological analysis (section 3.1.5) which found the fast thawing rate to be optimal but as the Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes) is a more accurate measure of cell viability, than the original methods of morphological analysis, the results from this analysis are more statistically reliable. Therefore the slow method of thawing media addition was confirmed as the most appropriate rate for thawing media addition.

3.2.3 Total Numbers of Samples Processed

Standard experimental procedures are to process a single blood sample at a time. To simplify the procedure it was determined whether multiple samples could be processed simultaneously whilst maintaining viability. To investigate the optimum sample number, forty 0.5ml aliquots of whole blood were cryopreserved from one individual and thawed in pairs, or in sets of four. The descriptive statistics for the viabilities of these samples are displayed in Table 3.12.

Table 3.12. The descriptive statistics for the mean viabilities when processing blood samples in simultaneous batches.

Processed	N	Mean	Std. Dev	Std. Error
2 Samples	20.0	88.31	7.32	1.64
4 Samples	20.0	87.60	6.34	1.42

The mean viabilities obtained from the processing of two samples was not significantly different to the viabilities obtained from the processing of four samples ($P = 0.706$) when compared in a paired *t*-test. Despite the high viabilities, there is variation within the viabilities, above that for reliability. The determined lymphocyte viabilities though, are overall adequate for culturing and analysis. Without a significant statistical difference, either batch of two or four samples could be processed in an optimised method. For this project it was decided to process two samples simultaneously as it improved the efficiency and processing time while reducing the potential for experimental error by processing too many samples simultaneously.

3.2.4 Overall Viability

After these optimal viabilities for each stage were established, viabilities were determined for the overall optimised method. These optimised viability data is required to adjust the volume of blood sample stored to correct for lymphocyte loss during cryopreservation. In each case, eleven 0.5ml aliquots of blood were cryopreserved from three individuals (Sample 1, Sample 2 and Sample 3). The viabilities were determined post thawing after following the optimised method and the data pooled for the means. From the three different sample means (Table 3.13) the average percentage of lymphocyte viability after cryopreservation was determined to be ~70%. This viability was used to adjust all lymphocyte figures for cryopreservation.

Table 3.13. The descriptive statistics for mean viabilities obtained from the performed optimised method.

Sample	N	Mean	Std. Dev	Std. Error
Tube 1	11.0	76.2	14.1	4.25
Tube 2	11.0	66.6	30.7	9.26
Tube 3	11.0	63.0	24.4	7.36

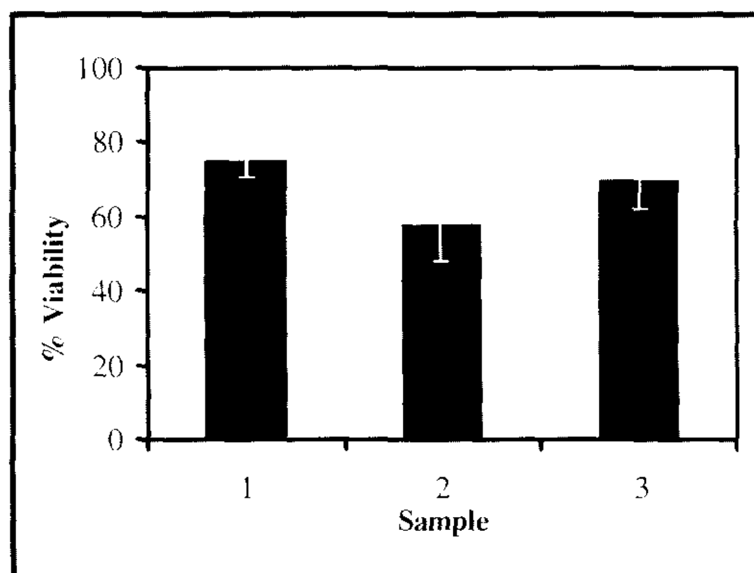


Figure 3.5. The percentage viabilities of each of the three samples tested. The error bars represent the standard error.

No statistically significant differences were identified between the three samples (P = > 0.050) after pair-wise comparisons, multivariate tests and within-subject contrasts. These data indicated the technique was reliable and reproducible for the lymphocyte viability. Further cryopreservation of whole blood could then be performed accounting for a 30% loss of lymphocyte viability over a cryopreservation period.

As the cryopreservation and thawing of these samples was successful in terms of lymphocyte viability, the cryopreservation and culturing of lymphocytes was expected to consistently work, with only a few exceptions due to the variability displayed in the standard deviation. This analysis therefore completed the first two aims of this project: To determine if cryopreserved whole blood is a good source of viable lymphocytes for cytogenetic tests (SCE, MN and FISH); to optimise a procedure for the cryopreservation of whole blood prior to cytogenetic testing. Whole blood appears to be a good source of viable lymphocytes for cytogenetic testing having maintained an approximate 70% viability during cryopreservation. A method for the cryopreservation of whole blood was now optimised and the remaining aim to perform the cytogenetic testing on the cryopreserved whole blood could be explored.

3.3 CRYOPRESERVATION PILOT STUDY

Blood samples from ten participants were studied to determine whether observed genetic damage increases with cryopreservation when compared to the fresh blood samples. The participants were selected from the list of participants involved in the current New Zealand Nuclear Test Veterans study. Over the course of a month, a 10ml blood sample was drawn from each participant by venipuncture into heparinised tubes (Vacutainer™). Each sample was analysed fresh and the excess samples prepared for cryopreservation (Section 2.9). Samples were cryopreserved and analysed after one month, three months and six months of cryopreservation for each participant.

These cytogenetic tests were performed on the both fresh samples and after thawing the samples at each of the cryopreservation periods (Section 2.10). The tests, Sister Chromatid Exchange and Micronucleus Assay, are sensitive and reliable indicators of genetic damage induced through clastogenic effects. These tests have the potential to highlight any genetic damage induced by cryopreservation.

3.3.1 Viability Data

Measures of lymphocyte viability were collected for each of the samples when initially collected (fresh), and also after one month, three months and six months of cryopreservation, before culturing. These data were collected to determine the extent of lymphocyte viability maintenance during cryopreservation and to act as complementary data to the genetic damage assays (SCE, MN and FISH) as DNA damage may induce cell death.

At each phase of determining lymphocyte viability, a blood smear was created after the full thawing process had been performed, prior to the culturing of lymphocytes (Section 2.11). The Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes) was utilised to determine cell viability (Section 2.13). These data from the lymphocyte viabilities were analysed by two methods. Initially the data was analysed grouped, with the mean (%) viability of all fresh samples compared to the mean (%) viability of all samples cryopreserved for one month, three months and six months. The results from this grouped analysis of lymphocyte viability are displayed in Table 3.14. The second method of analysis was to observe the individual lymphocyte viability from each participant. The data from individual samples is graphically represented in Figure 3.6 and the complete data is detailed in Appendix XII.

Table 3.14. The descriptive statistics for the grouped mean viabilities from all samples, for each time period.

Sample	N	Mean (%)	Std. Dev	Std. Error
Fresh	10	85.09	2.71	0.85
1 Month	10	65.02	8.45	2.67
3 Months	10	0.00	0.00	0.00
6 Months	10	0.00	0.00	0.00

The mean viability of the freshly processed blood samples was statistically significantly higher than the mean viability of blood samples processed after one month, three months and six months cryopreservation (P= <0.050). These data demonstrate a trend of reduced lymphocyte viability with extended periods of storage. The decrease in viability after one month of cryopreservation, although significant, is not great enough to entirely prevent analysis. The complete lack of viable cells after three and six months of cryopreservation however, entirely prevented analysis at these time periods as viable cells are required for SCE, MN and FISH. It is therefore likely that the cells have incurred either cellular or genetic damage causing cell death, resulting in reduced viability.

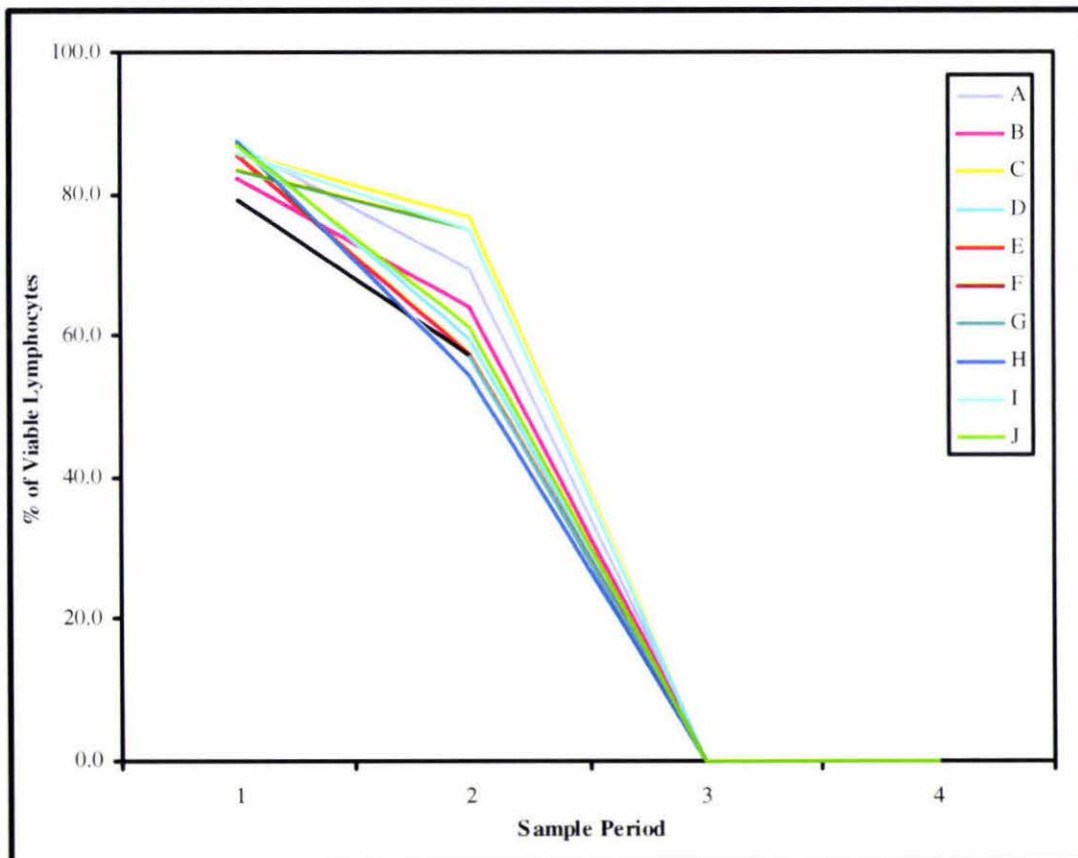


Figure 3.6. Graphical representation of the different blood samples maintenance of lymphocyte viability over the cryopreservation period (Sample period 1= fresh, Sample period 2= one month, Sample period 3= three months, Sample period 4= six months).

Individually, the fresh samples were consistently above the 95% confidence interval for the one month samples (except for samples E, I and J) demonstrating that the significant difference in lymphocyte viability throughout cryopreservation is a common effect. Samples E, I and J though, maintained lymphocyte viability (within the 95% confidence interval) for one month of cryopreservation but rapidly declined with further cryopreservation. Lymphocyte viability is therefore not well maintained during cryopreservation of whole blood and was found to decrease with increasing periods of cryopreservation until complete cell death. Possible causes for this cell death are proposed during the discussion (Section 4.2.2).

3.3.2 SCE Analysis

Sister Chromatid Exchange Assay (SCE) is a sensitive assay to explore the extent of genetic damage induced by an environmental agent. In the current study, the aim was to explore whether cryopreservation affects this SCE assay.

SCE was performed on all blood samples (Section 2.7) when the blood was fresh, and after one month, three months and six months of cryopreservation. The cell pellets were cultured at 37^{0}C for 76 hours in the presence of BrdU prior to harvesting. To eliminate bias, an independent researcher coded the samples differently so the samples' origin was unknown during the analysis involving metaphase image capture and scoring of SCEs.

Prior to the complicated Fluorescence Giemsa Plus staining, a single slide was stained with Giemsa and the morphology of the cultured lymphocytes in the cell pellet was recorded. Morphologies were recorded into five categories as in the morphological analysis in Appendix IX; Normal, fragmented, dehydrated, divided, absent. The morphology of the resulting cell pellet after culturing from each participant is tabulated in Table 3.15. These morphologies indicated prior to the SCE staining process whether the lymphocytes were successfully cultured.

Table 3.15. The morphological observations from each sample, after culturing lymphocytes for SCE analysis.

		Observed Lymphocyte Morphology				
Sample	**Month**	**Normal**	**Fragmented**	**Dehydrated**	**Divided**	**Absent**
A	0	✓			✓	
	1		✓			✓
	3					✓
	6					✓
B	0	✓			✓	
	1		✓	✓		✓
	3					✓
	6					✓
C	0	✓			✓	
	1		✓	✓		✓
	3					✓
	6					✓
D	0	✓			✓	
	1		✓	✓		✓
	3					✓
	6					✓
E	0	✓			✓	
	1	✓	✓		✓	
	3					✓
	6					✓
F	0	✓			✓	
	1		✓	✓		
	3		✓			✓
	6					✓
G	0	✓	✓	✓	✓	
	1		✓			✓
	3					✓
	6					✓

Sample	Month	Observed Lymphocyte Morphology				
		Normal	Fragmented	Dehydrated	Divided	Absent
H	0	✓			✓	
	1					✓
	3					✓
	6					✓
I	0	✓			✓	
	1					✓
	3					✓
	6					✓
J	0	✓			✓	
	1		✓			
	3					✓
	6					✓
A Repeat	0	✓			✓	
	1		✓			✓
B Repeat	0	✓			✓	
	1					✓
D Repeat	0	✓			✓	
	1	✓	✓	✓		
E Repeat	0	✓			✓	
	1	✓	✓	✓		
G Repeat	0	✓			✓	
	1		✓	✓		

Most cultured samples demonstrated poor lymphocyte morphology after a cryopreservation period of one month and after a longer period of cryopreservation (three months and six months), lymphocytes were predominately absent. Only two samples (E and F), maintained a relatively normal morphological condition after cryopreservation compared to the other cryopreserved samples.

The morphologies obtained after cryopreservation were therefore not consistent and demonstrated sample variation. Overall the maintenance of healthy lymphocyte morphology after cryopreservation was poor. The experiment repeated on retaken blood samples did not produce results that were consistent with the morphologies observed from the original participants sample, demonstrating that the difficulties may arise due to experimental errors or a change in that individuals health between the different collection times. This donor effect will be returned to in more detail in the discussion.

Only one of the two samples with improved morphology after cryopreservation provided metaphases from the cultured lymphocytes for analysis. The microscope slides from sample E were found to contain metaphases so Fluorescence Giemsa Plus staining was performed.

After staining, the slides were analysed but a range of stainings were observed, not all satisfactory for cytogenetic analysis. For SCE analysis, the metaphase complements that were scored contained 46 non-overlapping chromosomes and each chromosome was differentially stained with one chromatid appearing light and the other sister chromatid appearing dark when viewed under the light microscope. The types of metaphases rejected were those comprising less that 46 chromosomes, BrdU incorporated into all strands, mixed BrdU incorporation, and normal block staining (chromatids with no BrdU incorporation). Appendix XIII details these metaphase staining patterns. The metaphases observed are outlined in Table 3.16.

Table 3.16. The appearance of the metaphases obtained after cryopreservation for one month of blood sample E.

Metaphase Appearance	No. of Metaphases
SCE stained and complete metaphase	13
All BrdU incorporated	4
Mixed BrdU incorporation	3
Normal block staining	20
Incomplete metaphase	4
Total Metaphases Observed	44

A relatively low number of metaphases were obtained (44) but this is not unusual for even fresh blood samples, as on occasion fresh samples may not provide sufficient metaphases for analysis. Unfortunately the lack of SCE staining effectiveness resulted in only 13 metaphases being analysed in depth.

Despite obtaining only 13 SCE metaphases rather than the required 50, statistical analysis was performed to compare the differences in the mean SCE rate between Sample Es' fresh results and results obtained after one month of cryopreservation. The statistical description of these means is displayed in Table 3.17 and the complete SCE results for E and the remaining samples are detailed in Appendix XIV.

Table 3.17. The descriptive statistics for SCE performed on fresh blood samples and those cryopreserved for one month.

Sample E	N	Mean	Std. Dev	Std. Error
Fresh	52	5.85	2.75	0.38
One Month	13	11.64	3.72	1.12

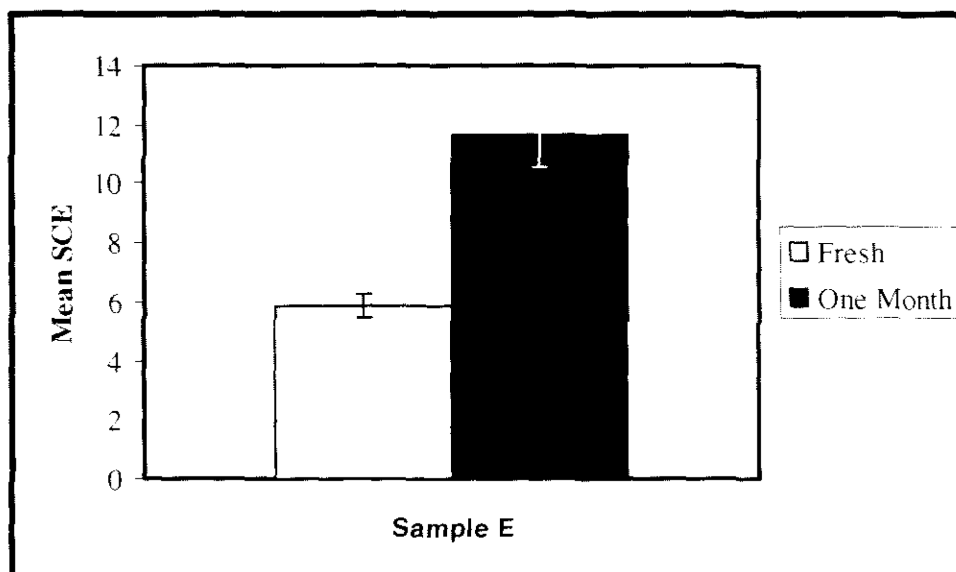


Figure 3.7. Graphical representation of the mean SCE observed for sample E at fresh and after one month of cryopreservation. The error bars represent the standard error.

The difference between the fresh and one month means of SCE frequency is highly statistically significant (P= <0.050) when a paired *t*-test was applied, despite the small sample size for the one month samples. These data demonstrate an increase in the sister chromatid exchange rate with cryopreservation and therefore suggests cryopreservation may increase the genetic damage of a cell.

The effect of cryopreservation on the number of SCEs counted cannot be established with certainty due to the small sample size and limited success of obtaining cultured lymphocytes after cryopreservation. SCE analysis was successful only once out of fifteen cryopreserved samples which indicates that some hindrance to cell cycle progression and growth is occurring due to the cryopreservation process. This cell cycle hindrance may also be a causative factor in genetic damage. Once this cell cycle block is determined SCE analysis may be able to proceed and genetic damage may not be induced by cryopreservation.

In summary, SCE can be performed on stored blood samples using this method of cryopreservation but not reliably or without a significant increase in the observed genetic damage. This does not however, prevent SCE from being performed on cryopreserved whole blood but merely indicates a shortcoming in this method of cryopreservation. More research is required to optimise a technique that results in consistent success when culturing cryopreserved whole blood.

3.3.3 MN Analysis

The MN assay was performed on all blood samples from the study group at the same time periods as SCE (fresh, one month, three months and six months). The same optimised cryopreservation conditions were used and analysis was once again conducted blind (Section 2.9). The Micronucleus Assay also assesses cells for genetic damage but measures a different form of genetic damage than SCE. MN is usually applied to measure the efficiency of a DNA repair system which when reduced can have disastrous effects on the fidelity of DNA. A reduced DNA repair capacity is determined by an increase of micronuclei within binucleate cells.

As with SCE, the morphology of each cell pellet cultured for the micronucleus assay was recorded to complement the MN results. The morphologies were scored into four groups; binucleate cells, fragmented cells, cell debris and non replicated cells (normal lymphocytes). These morphological groups were chosen to encompass the frequent morphologies observed. The observed morphologies of the binucleate cells are described in Table 3.18.

Table 3.18. The observed morphology of binucleate cells after culturing for the micronucleus assay before and after cryopreservation.

		Observed Lymphocyte Morphology			
Sample	Month	Binucleate	Fragmented	Non replicated	Cell Debris
A	0	✓		✓	
	1	✓	✓	✓	✓
	3				✓
	6				✓
B	0	✓		✓	
	1	✓	✓	✓	✓
	3				✓
	6				✓
C	0	✓		✓	
	1	✓		✓	✓
	3				✓
	6				✓
D	0	✓		✓	
	1	✓		✓	✓
	3				✓
	6				✓
E	0	✓		✓	
	1	✓		✓	✓
	3				✓
	6				✓
F	0	✓		✓	
	1				✓
	3				✓
	6				✓
G	0	✓		✓	
	1	✓		✓	✓
	3				✓
	6				✓

Sample	Month	Observed	Lymphocyte	Morphology	
		Binucleate	Fragmented	Unreplicated	Cell Debris
H	0	✓		✓	
	1				✓
	3				✓
	6				✓
I	0	✓		✓	
	1			✓	✓
	3				✓
	6				✓
J	0	✓		✓	
	1				✓
	3				✓
	6				✓
A Repeat	0	✓			
	1			✓	✓
B Repeat	0	✓			
	1			✓	✓
D Repeat	0	✓			
	1			✓	✓
E Repeat	0	✓			
	1		✓		✓
G Repeat	0	✓			
	1			✓	✓

The samples that were cryopreserved for one month were in the most part replicated to a binucleate cell allowing MN analysis. Despite being replicated, the numbers of binucleate cells were low and the levels of cell debris and fragmented lymphocytes were often high compared to the levels obtained when analysis is conducted on fresh blood samples. Those that did not replicate were samples F, H, I, J and the repeats. These samples were cryopreserved at a separate stage to the samples that were successful and the failure to replicate may be due to errors in cryopreservation or thawing procedure. None of the samples were successful at replicating after three months and six months of cryopreservation, probably due to the lack of viable lymphocytes determined in Section 3.3.1.

There were discrepancies with the numbers of binucleate cells that could be counted for micronuclei, with few samples allowing full analysis of 1000 binucleate cells (Michael Fenech, personal communication 2003) after cryopreservation. Despite the sometimes low values of binucleate cells obtained, Scott *et al* (1999) found that using a high dose of radiation (3.5Gy) induces relatively high yields of MN so only 100-200 binucleate cells are required. To obtain working values for the analysis of the micronucleus assay results two figures were calculated, the M_a and the M_b so that the experimental condition (cryopreservation) could be compared to the controls (fresh blood samples).

The M_a is the *mean number of micronucleated cytokinesis-blocked (CB) cells per 100 CB cells*. M_a essentially measures the percentage of damaged cells. Table 3.19 summarises the data for the number of cells analysed (N), M_a and the standard deviations and standard errors for both the fresh and one month samples. The descriptive statistics obtained for M_a are detailed in Table 3.19 and Figure 3.8.

Table 3.19. The descriptive statistics for the M_a values for fresh samples and when analysis was performed after one month's cryopreservation.

Sample	N	Mean M_a	Std. Dev	Std. Error
Fresh	14,031	22.85	4.77	1.95
1 Month	2063	38.67	8.71	3.56

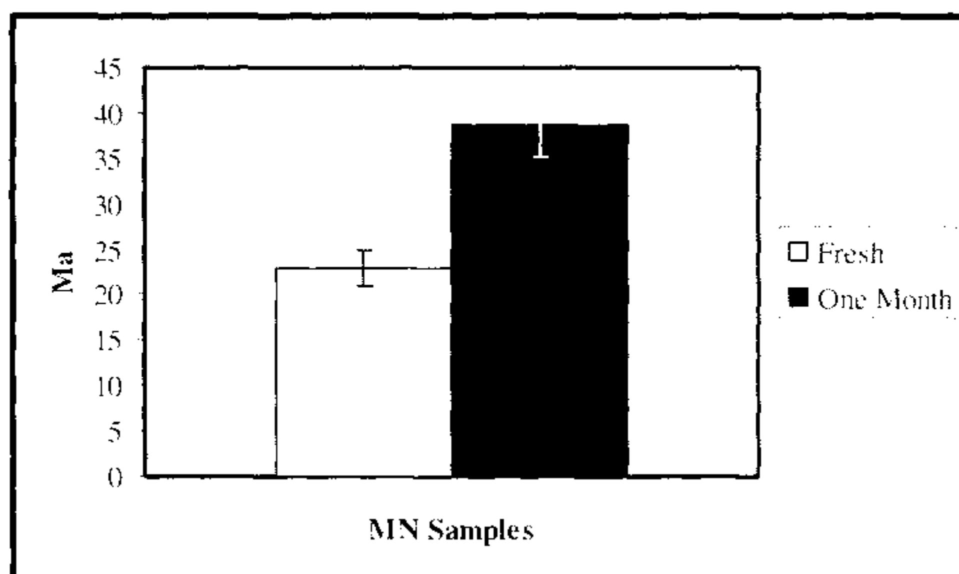


Figure 3.8. Graph of M_a values for both the combined results from the fresh blood samples and those cryopreserved for one month. The error bars represent the standard error.

The difference between the M_a values for the fresh and one month samples were explored through a paired samples *t*-test. A significant difference between the M_a values was determined (P= 0.023). This significant difference shows that the increased genetic damage observed after cryopreservation is statistically significant. It can be said with confidence then that cryopreservation increases the mean number of micronucleated cytokinesis-blocked (CB) cells per 100 CB cells, which is an indication of genetic damage.

The M_b is calculated as the *mean number of micronuclei per 100 CB cells*. M_b is therefore an indication of the extent of damage. The results for M_b from the combined fresh and one month samples are described in Table 3.20. and represented graphically in Figure 3.9.

Table 3.20. The descriptive statistics for the M_b values of the fresh samples and those analysed after one month's cryopreservation.

Sample	N	Mean M_b	Std. Dev	Std. Error
Fresh	14,031	26.52	5.54	2.26
1 Month	2063	48.17	11.72	4.78

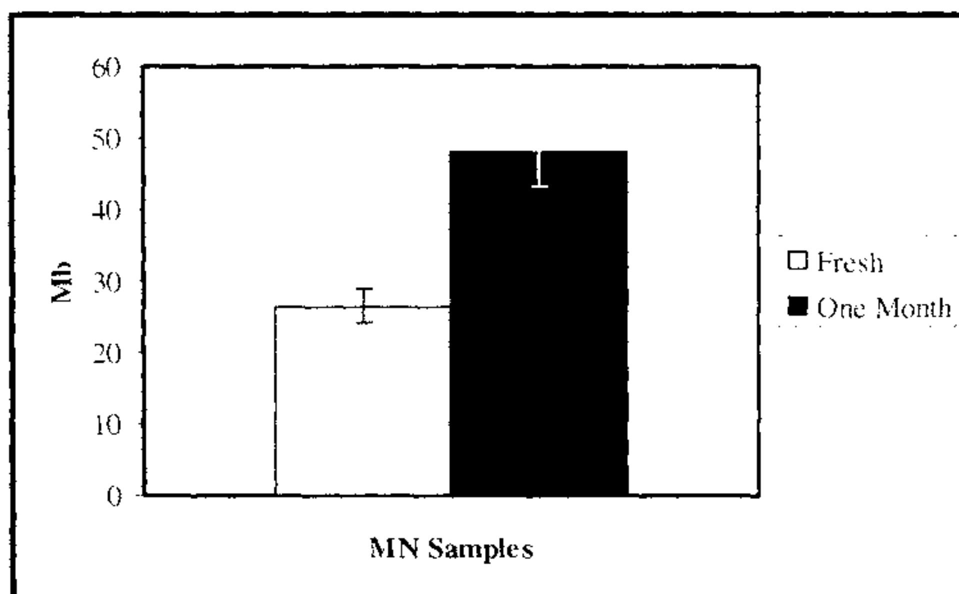


Figure 3.9. A graphical representation of M_b values for both the combined results from the fresh blood samples and those cryopreserved for one month. The error bars represent the standard error.

The difference between the M_b values for the fresh and cryopreserved samples was explored through a paired samples *t*-Test. A significant statistical difference was observed (P= 0.016) for the mean numbers of micronuclei per cell damage between the fresh samples and those cryopreserved for one month. This demonstrated a potential increase in genetic damage after cryopreservation as an increase in micronuclei indicates a reduced DNA repair mechanism.

The significant differences observed for both the Ma values and Mb values between fresh blood samples and cryopreserved whole blood samples demonstrate the clastogenic effect of cryopreservation on human peripheral blood lymphocytes. It can therefore be said with confidence, that cryopreservation increases the genetic damage of cryopreserved lymphocytes as observed by the Micronucleus Assay. The full tables of analysis for all Micronucleus Assay results can be seen in Appendix XV.

3.3.4 FISH Analysis

A well-known effect of genotoxic agents is the translocation of genetic material from one chromosome to another. These translocations may have disastrous effects on a persons' health, especially if an oncogene is placed under the control of a strong promoter or a tumour suppressor gene is inactivated. FISH is a well established and trusted technique to monitor DNA damaging events and was planned to be performed on the fresh blood samples and those cryopreserved.

Unfortunately due to the problems of reliably obtaining metaphases from a thawed cryopreserved whole blood samples, FISH was unable to be performed on the blood samples. FISH is a very expensive technique and as the SCE Assay was relatively unsuccessful, the FISH assay was not attempted to avoid a waste of resources. FISH could only be attempted with an increase in the success of culturing lymphocytes after cryopreservation.

3.4 CONTROLS VS VETERANS

The success of MN performed after cryopreservation was also compared between the control and radiation exposed subjects used in this study. Only a handful of studies have explored if irradiated blood, such as that exposed to a radiological accident, is also stable during cryopreservation. Although the sample size is not large enough for full reliable statistical analysis, the comparison of controls and experimental subjects could give an indication of effect for future research.

The M_a and M_b values obtained for the controls (A, B, E, F and J) were compared to the values obtained from the Veterans (C, D, G, H and I). The descriptive statistics for these comparisons are displayed in Table 3.21. and graphically displayed in Figures 3.10. and 3.11.

Table 3.21. The descriptive statistics for the experimental participants and control participants individually.

Sample		N	Mean	Std. Dev.	Std. Error
Experimental	**M_a Fresh**	3	22.53	6.90	3.99
	M_a 1 Month	3	34.03	7.40	4.27
	M_b Fresh	3	25.93	8.20	4.73
	M_b 1 Month	3	43.86	8.93	5.16
Controls	**M_a Fresh**	3	23.12	2.97	1.71
	M_a 1 Month	3	43.30	8.40	4.85
	M_b Fresh	3	27.10	2.91	1.68
	M_b 1 Month	3	52.46	14.42	8.33

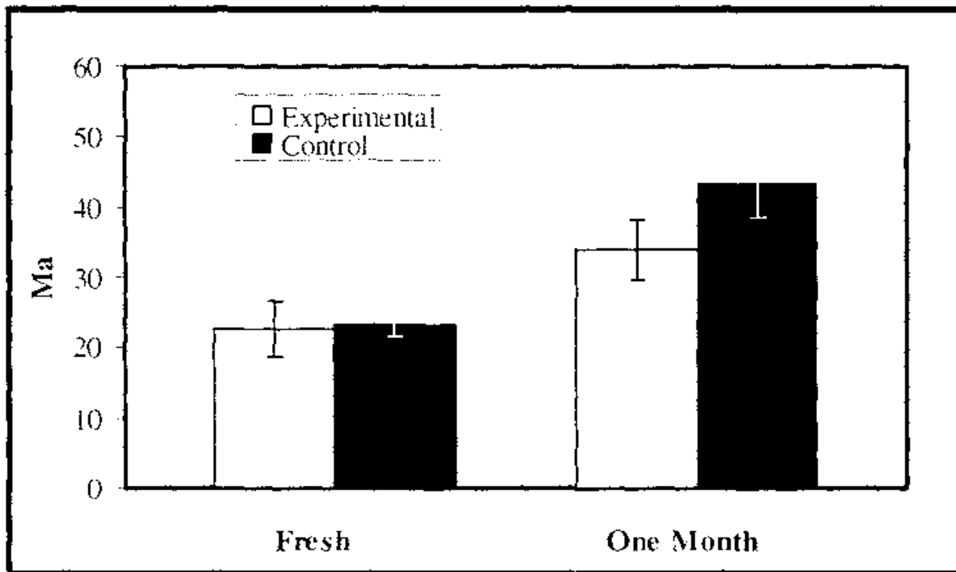


Figure 3.10. Graphical representation of the comparison of fresh M_a values and one month M_a values for control subjects and experimental subjects. The error bars represent the standard error.

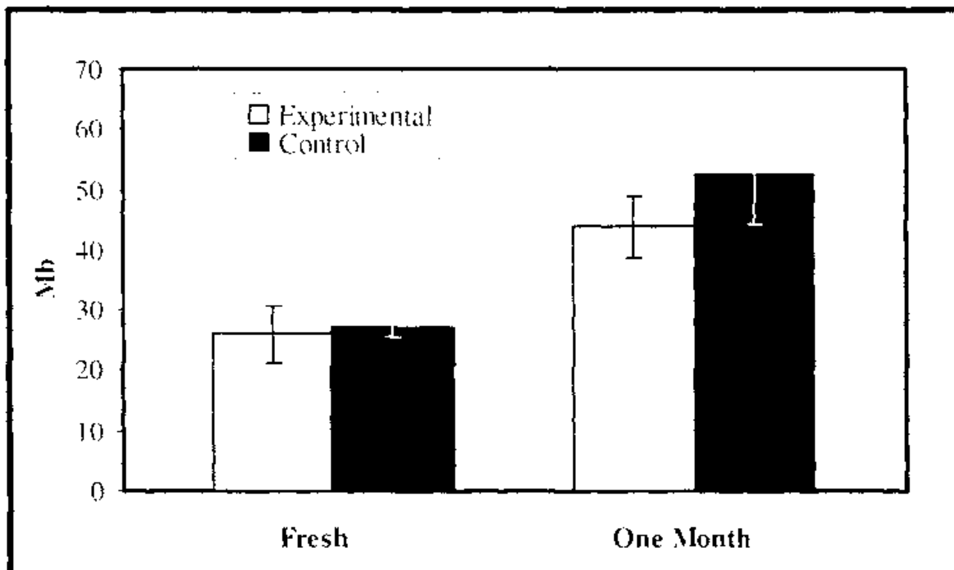


Figure 3.11. Graphical representation of the comparison of fresh M_b values and one month M_b values for control subjects and experimental subjects. The error bars represent the standard error.

Statistical analysis of the variance between the control and experimental group reaction to cryopreservation by an increase in damage observed by the Micronucleus Assay (ANOVA) showed non significant differences between the control and experimental subjects. The difference is not significant for both the Ma values (P= 0.435) and the Mb values (P= 0.600). Therefore although it appears that the control group shows a greater effect than the experimental group this effect is not statistically significant. More samples for both the control and experimental group are required to be studied before conclusions can be made as to whether cryopreservation affects different groups differently.

CHAPTER 4: DISCUSSION

The storage of whole blood prior to performing cytomolecular tests for genetic damage is a little studied area. For many laboratories the storage of blood samples or blood subsets is a common practice to allow the processing of samples in batches. Several studies have explored the effects of storing blood subsets but until now, only a handful of studies (with conflicting results) have explored the use of cryopreserved *whole* blood for cytogenetic tests.

This project was conducted to determine whether cryopreservation of *whole* blood for an extended period of time is an acceptable form of storage prior to performing three cytogenetic tests, Sister Chromatid Exchange (SCE), Micronucleus Assay (MN) and Fluorescence *in situ* Hybridisation (FISH). The main goal of this study was to determine whether or not there was an increase in genetic damage after cryopreservation as observed by the data obtained from performing SCE, FISH and MN on fresh and cryopreserved whole blood. The feasibility of using cryopreserved lymphocytes for such studies was investigated.

4.1. PRELIMINARY MORPHOLOGICAL ANALYSIS

4.1.1 Experiments

Before whole blood samples were utilised for the Cryopreservation and Genetic Damage study, a method to achieve viable, replicating lymphocytes after cryopreservation was determined. The initial attempts to cryopreserve whole blood were not successful so further modifications, mainly in the areas of freezing and thawing, were applied until proliferating lymphocytes were obtained.

4.1.2 Freezing Method

The freezing method was first considered for modification, due to its crucial role in the cryopreservation process. The freezing method was explored by the examination of lymphocyte morphology after several adjustments: the concentration of the cryopreservation solution (DMSO); the temperature of the cryopreservation solution; and the overall freezing rate of the samples. Lymphocytes were found to retain normal morphology when 5% and 10% DMSO were used as the cryopreservation solution (Table 3.3), when the DMSO working temperature was 18°C (Table 3.5) and when a slow rate of cooling was utilised (Table 3.6). However, despite the morphologically normal lymphocyte observed, replicating lymphocytes were not obtained, despite lymphocytes being well noted to be restored after cryopreservation (Singh *et al* 1990).

The observation of intact lymphocytes suggested that these adjustments prevented the cellular damage that cryopreservation is known to inflict (Mazur 1970, Bischof & Rubinsky 1993, and Stead & Park 2000). The potential for vast amounts of cellular damage to occur during the freezing process is well recorded. To reduce the cellular damage, the freezing rate and protective environment must be optimised (as performed here). This optimisation sought to reduce the intracellular ice formation and osmotic gradients, which if incorrect, can cause damage to the cells. These osmotic gradients and ice formations are controlled by a combination of an optimal freezing rate of -1°C per minute and mild levels of DMSO. This level of control is supported by this current study research.

Another common cause for damage that was investigated was the level of DMSO toxicity. The reduction in DMSO temperature and concentration (to 18°C and 10% respectively), improved the lymphocyte morphology immensely. The effects of DMSO are supported by many other studies such as a study by Sperling and Larsen (1979) that found DMSO to cause latent cell damage at 31°C but not at 4°C when diluted to a lower concentration in Eagles medium and Dextran.

Despite the methodological improvements to minimise these damaging effects, replicating lymphocytes were still not obtained. This research demonstrated that more

subtle causes, other than freezing damage, may be involved in the observed cellular damage and lack of lymphocyte growth.

4.1.3 Thawing Method

Modifications to the freezing method were not successful in obtaining replicating lymphocytes so the thawing procedure was also modified to optimise the storage conditions. The experiments clearly determined that the lymphocytes were decreasing in concentration throughout the thawing process until their complete disappearance (Table 3.1). Overall increases in the centrifugation periods counteracted the lymphocyte loss and improved lymphocyte retention (Table 3.2). Variations to the thawing rate demonstrated optimal morphological health of the cells when the whole blood/freezing media was diluted quickly to avoid damage to the cells (Table 3.7). Despite the swift processing of samples by these thawing methods the lymphocytes still did not replicate in premium culturing conditions, even after obtaining morphologically normal lymphocytes.

The standard convention for thawing blood cells is the swift return to a physiological temperature of 37°C prior to the slow elution of DMSO by the addition of thawing media and centrifugation. The centrifugation times were adjusted to determine DMSO's effect on the lymphocyte sedimentation rate. DMSO was found to reduce the sedimentation rate of lymphocytes as the lymphocyte pellet was larger after longer centrifugation periods. Despite the increase in lymphocyte yield, no improvement in lymphocyte replication was observed.

4.1.4 Subtle Adjustments

An indication that the errors exceeded the cryopreservation procedure for only *whole* blood was noted by the fact that *isolated lymphocytes* were also unable to be cryopreserved, thawed and cultured despite publications of this method having been utilised (Murli *et al* 1987, Littlefield *et al* 1986). Therefore other more subtle aspects of blood manipulations were explored for their effect on lymphocyte viability. The

use of plastic Pasteur pipettes instead of the previously utilised glassware were found to result in lymphocytes that were morphologically normal and were able to replicate and divide (Figure 3.4).

Plastics were successful as it prevented the adhesion of lymphocytes to the transfer pipette, unlike untreated glass that lymphocytes will adhere to, causing either cell removal or the shearing of cells. This adhesion can be avoided by using either plastic apparatus, as in this project, or trypsinising the glassware to form a less adhesive surface.

4.1.5. Conclusions

The morphological data gathered from these preliminary experiments determined whole blood as a suitable source of viable lymphocytes after cryopreservation, as after several modifications to the cryopreservation method, proliferating lymphocytes were obtained. The experiments isolated a method specific for the cryopreservation of *whole blood* which was simple and cost effective. The successful conclusion of this projects first aim to determine if lymphocytes can be cultured from cryopreserved whole blood allowed the Cryopreservation and Genetic Damage study to proceed.

4.2. PRELIMINARY VIABILITY ANALYSIS

The conditions for maximum retention of viable lymphocytes were explored through analysis of lymphocyte viability using The Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes). The initial method for obtaining dividing lymphocytes from cryopreserved whole blood was determined through morphological study, and did not include statistically significant viability analysis. This viability analysis using the viability kit accurately assessed the potential levels of cell death occurring during cryopreservation. This research was performed to explore the second aim of this project; to obtain an optimised method for the cryopreservation and recovery of lymphocytes from whole blood.

4.2.1 Optimisation of Viability

The optimised method for retaining viable lymphocytes was determined by testing the viabilities obtained from each adjustment to the cryopreservation method. The optimal freezing media, thawing media and culture media was found to be Wellcome Media (Table 3.10). This statistically significant support for the use of Wellcome Media, opposes the use of RPMI utilised in the majority of papers exploring blood cryopreservation. The optimal thawing rate for maintaining lymphocyte viability was found to be different to the rate that optimised healthy lymphocyte morphology. With the viability kit, the viabilities obtained by the slow addition of thawing media were found to be statistically significantly greater than the other thawing rates (Table 3.11). Viabilities for the processing of multiple samples simultaneously did not reduce upon thawing. It was determined that up to two samples could be processed simultaneously allowing ease of processing while reducing sampling errors.

The most interesting discovery from this aspect of the study was the different results obtained from the morphological data and the viability data. The results for optimal thawing rate differed between the two tests. This could have arisen because of the increased accuracy and lack of subjectivity that the viability analysis involved compared to the qualitative morphological data. Also, for the viability analysis, plastic Pasteur pipettes were utilised instead of the glass Pasteur pipettes used for the morphological analysis. This subtle difference in apparatus may have caused the differences between the results of the two experiments. The use of the slow thawing method is in agreement with the common methods published in international journals.

It was very pleasing, productivity wise, to discover that more than one blood sample could be processed simultaneously as the processing of samples simultaneously reduces experimental and staff time. A cautionary procedure is wise to prevent an excess of samples processed simultaneously as experimental error can ensue.

4.2.2 Final Viabilities

After determining the optimised method for cryopreservation, samples were processed (entirely by the optimised method) to establish the mean lymphocyte viability after cryopreservation. This mean viability was determined to be 68.6% (Table 3.13). This lymphocyte viability, although not ideal, is a substantial retention of viability for consequent cytomolecular analysis. Despite this adequate mean lymphocyte viability, a significant level of variation in the lymphocyte viability was observed (Table 3.13), with some samples not obtaining acceptable lymphocyte viabilities. This variation in lymphocyte viability may have affected the results from this study as viable lymphocytes were required for all the cytomolecular tests and without sufficient lymphocyte levels the tests were not successful.

The effect of cryopreservation on lymphocyte viability is debated widely in the literature. Lombet *et al* (1998) and Celluzzi and Welbon (2003) raised concerns that apoptosis may occur at a higher rate in a cryopreserved sample than in fresh samples, with significant decreases in viability from the pre-freeze to post-thaw condition observed. On the other hand, Tomkins and Scheid (1986) and Riccio *et al* (2002) found in their studies that cell recovery and viability of thawed cells exceeded 90%. Furthermore, in a study of cell viability after a longer period of cryopreservation, Kleeberger *et al* (1999) found that PBMC cryopreserved for twelve years had no general tendency towards cell loss over time. In yet another study by Hayes and his team (2002) the reduction in lymphocyte viability to 69.7% was found to be irrelevant if an increased sample is cryopreserved to compensate for the loss. This agrees with research performed by Cheng *et al* (2001) who also concluded that cryopreserved whole blood is a good source of viable lymphocytes for epidemiological studies. It could be concluded from the analysis performed for this thesis that cryopreserved whole blood, although not ideal, is a suitable source of viable lymphocytes.

4.2.3 Conclusions

The optimised cryopreservation method was found to result in a mean lymphocyte viability of 68.6%. This viability value was not reliable due to the large variation in the measured values. But generally the viabilities were found to be suitable for this project when the cryopreserved lymphocyte concentration was increased to compensate for this decrease in viability. Therefore the aim for an optimised method for the cryopreservation of whole blood samples prior to cytomolecular testing on the resulting viable lymphocytes was obtained.

4.3 CRYOPRESERVATION AND GENETIC DAMAGE

The three cytogenetic tests utilised to explore the effects of cryopreservation on genetic damage were Sister Chromatid Exchange, Micronucleus Assay and Fluorescence *in situ* Hybridisation. Once it had been determined that viable lymphocytes were obtained after cryopreservation of whole blood samples and were suitable for cytomolecular analysis, the final aim could be explored. The final aim of this project was to determine whether cryopreservation of whole blood over a long period of time (one month, three months and six months) changes the observed genetic damage when cytomolecular tests are conducted on the blood samples.

4.3.1 Viability

Again The Live/Dead Viability/Cytotoxicity Assay Kit (L-3224) (Molecular Probes) was utilised to determine the stability of lymphocyte viability over the course of the cryopreservation period. It was determined that the mean viability from the freshly processed blood samples was statistically significantly higher than the mean viabilities obtained from blood samples processed after one month, three months and six months cryopreservation. Overall, the lymphocytes retained sufficient viability for cytogenetic analysis after one month of storage (65.02%, Table 3.14) despite a large decline from the lymphocyte viabilities established for the fresh blood samples. This viability loss is reflected in the individual sample results, as the fresh samples

were consistently above the 95% confidence interval determined for the cryopreserved samples. No viable lymphocytes were obtained after three and six months of cryopreservation. Lymphocyte viability is therefore not well maintained during cryopreservation of whole blood and was found to decrease with increasing periods of cryopreservation until complete cell death.

There are many potential reasons for the decrease in lymphocyte viability observed in this study and others. The reduction in viability of cryopreserved cells is a highly researched area especially with the constantly expanding field of cryogenics. Some of the common debated causes for increased cell death after cryopreservation are discussed here.

As noted previously by Celluzzi and Welbon (2003), the viabilities of cells may reduce with increasing periods of cryopreservation. If this reduction in viability is affected by the period of storage then potentially the lymphocyte concentrations were not adjusted correctly in the present study for the longer storage periods of three months and six months. The compensation of concentration may have been adequate for the one month period but the cell concentration may not have been sufficient to endure the longer cryopreservation periods without significant cell loss. This of course conflicts with Kleeberger *et al's* (1999) findings of no general tendency of cell loss over time.

The freeze/thawing stress inflicted upon cryopreserved cells may have also had a role in the observed reduced viability. Membranes appear to be the chief target of freezing damage (Mazur 1970), and damaged membranes are no longer able to perform normal duties such as membrane-mediated effects. Fowke *et al* (2000) suggests that during the cryopreservation process damaged cells receive physiological or physical signals that prime for apoptosis but do not initiate the process until in the presence of a mitogen. Their research using isolated PBMCs and whole blood suggests that cells are subjected to activation induced apoptosis rather than spontaneous apoptosis. This theory of freeze/thaw stress activating apoptosis though, does not account for the observed results where one month samples still had adequate viability. If freeze/thaw stress was the main initiator of reduced lymphocyte viability it could be assumed that this damage would occur regardless of the storage period.

Holland *et al* (2003) determined that the loss of cell viability is observed when cells experience an increase in temperature during cryopreservation, even mild fluctuations ($5^{o}C$). This increase in temperature can occur even temporarily such as moving boxes from one freezer to another. This is likely to be the most plausible explanation for lymphocyte loss in the present study, as the vials for the three months and six months samples were stored on the same cryocane as one month samples, so temperatures may have increased in the three and six month samples while removing the one month samples for analysis, resulting in the reduced viability. The liquid nitrogen dewar was also not thermostat controlled so mild fluctuations in temperature may have occurred during the storage period.

In Weinberg *et al's* (1998) extensive study on blood processing centres, it was found that there was a direct correlation between the viabilities obtained after cryopreservation and the technical expertise of the research staff. Inexperience could also have played a role in the lymphocyte reduction in the present study as researchers were not experienced in cryogenics prior to this project.

4.3.2 Sister Chromatid Exchange

For each sample collected, Sister Chromatid Exchange (SCE) was performed on the samples while fresh and also after the three different periods of cryopreservation. The majority of the cultured samples demonstrated poor lymphocyte morphology after a cryopreservation period of one month. After a longer period of cryopreservation (three months and six months), lymphocytes were predominately absent. Only one of the two samples with improved morphology after cryopreservation provided metaphases from cultured lymphocytes for analysis. From this sample, the difference between the mean SCE frequencies for the fresh and one month sample periods is highly statistically significant, with a greater level of SCEs observed after storage. SCE analysis was not successful on whole blood samples cryopreserved for the longer periods of three months and six months. These data demonstrate an increase in Sister Chromatid Exchange frequency after cryopreservation and therefore suggests cryopreservation may increase the genetic damage of a cell.

In the literature, there are again contradictory claims as to the consequences of cryopreservation on the frequency of SCEs. Tomkins and Scheid (1986) found that when whole blood was cryopreserved for one week, no obvious differences were observed in the frequency of SCE. In opposition to Tompkins and Scheid's view, Littlefield *et al's* (1986) results agreed with the conclusions of this study that freezing blood samples in liquid nitrogen increases genetic damage, as observed by studying the levels of chromatid aberrations.

It was interesting that despite the adequate viabilities of the samples cryopreserved for one month, lymphocyte proliferation was low, with only one successful cultured sample from a total of ten participants. In one study conducted by Holland *et al* (2003) it was found that cell viability does not always correspond to successful growth after cryopreservation. In their study, deliberate sabotage of the stored umbilical cord blood (UB) samples (by thawing and refreezing) resulted in the absence of UB growth but the viability measured by Trypan blue staining was still 68-98%. Therefore the viabilities measured in this project may be falsely reassuring and not an indication of cell function.

The reduced lymphocyte proliferation observed in this project is supported by Tomkins and Scheid (1986). In their research it was found that cryopreservation had a substantial effect on cellular proliferation resulting in significantly reduced mitotic activity in cultures with or without BrdU. The reduced cellular proliferation was off set by increasing the amount of blood sample stored.

In contrast to a common theory that freezing is a process of total destruction, cells do not burst solely as a result of cryopreservation. Intracellular freezing inducing the expansion of water causes only a 10% increase in cell volume and cells can survive a greater change in cell volume, even up to 50% (Mazur 1970). It is known that ice does not actually form within the cells during freezing, it occurs between the cells, and water actually travels out of the cell causing shrinkage and dehydration rather than bursting (Mazur 1970). Cell death after freezing therefore may be related to changes in the membrane after shrinkage but this is not an irreversible effect if thawing is controlled. Lymphocytes can still be intact, and appear viable after freezing even though they are not functional. The cryopreservation method, therefore,

needs to be optimised not necessarily only for structural cell preservation but also for function preservation.

Unfortunately, although the difference between the fresh and one month samples here are statistically significantly different, the results cannot be considered conclusive overall due to the small sample size. Whilst the results support the view that cryopreservation may cause genetic damage, it cannot be said with conviction that cryopreservation increases the frequency of Sister Chromatid Exchanges.

4.3.3 Micronucleus Assay

The majority of the whole blood samples cryopreserved for one month were replicated to binucleate cells during the culture period, allowing MN analysis. The numbers of binucleate cells obtained were in some instances low, compared to the recommendation from Michael Fenech (personal communication, 2003), but comparable to numbers of cells counted when a higher dose of radiation is used (Scott *et al* 1999) the procedure followed in this study. For analysis, two values were calculated as described in Section 3.3.3, the M_a and the M_b. The M_a value indicates the percentage of cells that are genetically damaged. In this project the mean cells with genetic damage after cryopreservation was 38.67 which is statistically significantly greater than the cells observed with genetic damage before cryopreservation (22.85, P= 0.023). It can therefore be said with confidence that cryopreservation increases the mean number of micronucleated cytokinesis-blocked (CB) cells per 100 CB cells, which is an indication of genetic damage. The M_b value is an indication of the extent of genetic damage. Again a significant difference was observed between the amounts of genetic damage seen in fresh lymphocytes to those after cryopreservation (P=0.016). This demonstrated an increase in genetic damage after cryopreservation, as an increase in micronuclei indicates a malfunction in the DNA repair mechanism.

The literature is somewhat inconsistent as to the effects of cryopreservation on the results of the Micronucleus Assay. Burrill *et al* (1999) found that the use of frozen lymphocytes is not satisfactory for MN analysis. In their study where blood samples were cryopreserved for 6-24 months it was found that experimental failures were

higher, the inter-experiment variability was higher and a poorer repair capacity was observed compared to the fresh samples. The proportion of cryopreserved samples that failed to yield results was higher than fresh samples where failure is rare. In opposition to this, Visvardis *et al* (1997) determined no difference in the DNA repair capacity between cryopreserved isolated lymphocytes and fresh isolates. Visvardis *et al's* results are not directly comparable to this current study as isolated lymphocytes were stored not whole blood samples as utilised here. It is interesting that Visvardis obtained similar results after cryopreservation because some studies have reported an increase in genetic damage by the process of lymphocyte isolation alone (Murli *et al* 1987). Again though, Prosser *et al* (1994) did not observe an increase in genetic damage after cryopreservation as determined by the Micronucleus Assay performed on both cryopreserved whole blood and isolated lymphocytes. They determined that the frequency of micronuclei from cryopreserved lymphocytes may accurately indicate the level of damage sustained before freezing. These studies suggest that the success of cryopreserving whole blood for MN analysis is dependent on the technique employed and the experience of the researcher.

In the current study, most samples, although showing some cellular proliferation, demonstrated reduced growth after cryopreservation. Again the problems associated with freeze/thaw stress, cellular damage, reduced mitotic index and incorrect temperature maintenance may have resulted in this reduced growth. The reduced numbers of binucleate lymphocytes may have resulted from an efficient apoptotic process. Perhaps cells demonstrating too much damage were initiated into activated apoptosis reducing the numbers of lymphocytes for analysis. The responses of each sample after thawing are very different, suggesting different blood sample donors may respond differently to cryopreservation. This donor effect was seen in this project as all samples were frozen and stored in presumably identical manners but some succeeded in maintaining viability and growth potential while others did not. It can then be concluded from the MN analysis that this method of cryopreservation reduces the DNA repair system resulting in increased genetic damage, or that so much genetic damage occurs as a response to cryopreservation that the DNA repair system is overloaded or stalled.

4.3.4 Conclusions

From these results the conclusion is drawn that this method of cryopreservation is not an appropriate technique for the storage of whole blood samples prior to cytomolecular testing. FISH analysis was not successful and in the two successful tests, SCE and MN, genetic damage was observed to be increased after a period of cryopreservation. This increase in genetic damage was coupled with a decrease in lymphocyte viability indicating the roles of both genetic and cellular damage during cryopreservation.

4.4 DONOR EFFECT

The donor effect mentioned in the previous section was explored to determine whether personal history and experiences played a role in cryopreservation-induced genetic damage. For this, the results from the New Zealand Nuclear Test Veterans participants were compared to the results from the non radiation exposed controls. Overall a non significant difference was found in response to cryopreservation between the two groups. This analysis though is not statistically significant due to the small sample size, but merely sought as an initial indicator of donor effect. If no differences were observed with a larger sample size it would allow the storage of any blood sample for cytogenetic analysis regardless of personal history.

Unfortunately in several other studies, donor dependence has been established. A study by Rudd *et al* (1988) and Schmidt-Preuss *et al* (1990) demonstrated a freezing related increase of micronuclei frequency that was dependent on the donor. Barale *et al* (1998) also determined that females show an increased effect to genetic damaging agents such as cigarette smoking. This suggests a genetic influence on the response to cryopreservation and genetic damage. Other studies have recognised environmental influences on cryopreservation success with blood samples from alcoholics and smokers having increased genetic damage after cryopreservation over non alcoholics and smokers (Maltei *et al* 2000, and Barale *et al* 1998). This donor effect needs further investigation before whole blood can be routinely cryopreserved.

4.5 OVERALL SUMMARY

The key findings of this study 'Cryopreservation and Genetic Damage' are that: (1) cryopreserved whole blood is a suitable source of viable lymphocytes, (2) the cytomolecular techniques MN and SCE can be successfully conducted on cryopreserved whole blood but with reduced cellular proliferation, (3) FISH is not successfully performed on cryopreserved whole blood samples, (4) lymphocyte viability reduces with longer periods of cryopreservation, and (5) genetic damage increases after cryopreservation of whole blood samples as measured by MN and SCE. Cryopreservation of whole blood prior to the application of cytomolecular techniques is therefore not yet a reliable technique, although with more research into this area it is likely that this process will be routinely used in the future.

CHAPTER 5: FUTURE WORK

5.1 CRYOPRESERVATION METHOD

The first part of this thesis involved an investigation to optimise the method for the cryopreservation of whole blood. A method was determined which allowed the replication of lymphocytes in a culture media after thawing from cryopreservation. Unfortunately this method was not consistently reliable in providing viable, replicating lymphocytes. Viabilities were reduced after one month of cryopreservation and were found to be 0% viable cells after three months of cryopreservation. Sister Chromatid Exchange and FISH demonstrated only limited success due to a lack of lymphocyte replication, and while MN replication was successful, it did not replicate to the extent of fresh blood samples.

Future work in this area would require additional modifications to the cryopreservation procedure to obtain a reliable method. This could include the exploration of cellular damaging factors, apoptosis triggers and optimum culturing conditions. An investigation into the cellular damaging factor during cryopreservation would potentially allow modification to prevent the vast cellular damage observed in the results of this thesis. As an increase in the apoptotic rate has been observed in many studies, the causes and effects of apoptosis need to be studied in an attempt to reduce both the spontaneous apoptotic rate and the activated-apoptosis rate. If these factors are explored and the cellular damage and death is reduced, the cryopreservation of whole blood may become a more viable method for laboratories to adopt.

A main confusing factor discovered in this research was the apparent lack of consistency between the viabilities of lymphocytes after cryopreservation and their culturing ability. This confusing affect has been noted in additional research but not fully explored. Further research could explore the factors that allow cells to remain viable but not replicate after cryopreservation.

5.2 CRYOPRESERVATION AND GENETIC DAMAGE

Once a reliable method for cryopreservation of whole blood prior to cytogenetic testing has been obtained further research can explore the relationship between cryopreservation and genetic damage. This research investigated only three cytogenetic tests and many more cytogenetic tests are utilised in genetics laboratories. These tests also need a comparison between the results obtained from fresh blood samples and the results obtained form cryopreserved blood samples to observe whether the recorded genetic damage changes with storage. Other cytogenetic tests for evaluation are the G2 Assay, the Comet Assay (Pilot study performed by Chad Johnson and found to be unreliable performed on cryopreserved isolated lymphocytes (Personal Communication – MSc Thesis 2004)), Fluorescence *in situ* Hybridisation, Karyotyping, Premature Chromosome Condensation Assay. An analysis of these tests, and further analysis of the Sister Chromatid Exchange and Micronucleus Assay with greater sample sizes, will allow a true representation of the levels of genetic damage obtained during cryopreservation.

A further interesting idea arising from the work of this thesis is the concept of donor effect. Once the cryopreservation of whole blood prior to cytogenetic testing is more reliable, the donor effect (that is the differences in results based on the blood donor) can be further explored. It is already established that age, sex, smoking, alcohol and some diseases have an effect on the ability to store blood samples. Further diseases, medications and clastogen exposures can be explored to determine their effect on the results of genetic damage after cryopreservation by exploring the differences between control groups and those exposed groups.

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APPENDIX I: BLOOD COLLECTION SCHEDULE

Table 1.1. The schedule for blood collection and analysis to demonstrate the dates for thawing stored blood samples from each participant.

Collection Date	Samples	One Month Thaw Week	Three Month Thaw Week	Six Month Thaw Week
Monday 19/04/04	**A**	Monday 17/05/04	Monday 12/07/04	Monday 04/10/04
	C	Monday 17/05/04	Monday 12/07/04	Monday 04/10/04
	D	Monday 17/05/04	Monday 12/07/04	Monday 04/10/04
Tuesday 27/04/04	**B**	Monday 17/05/04	Monday 12/07/04	Monday 04/10/04
	E	Monday 17/05/04	Monday 12/07/04	Monday 04/10/04
	F	Monday 31/05/04	Monday 26/07/04	Monday 18/10/04
Monday 03/05/04	**G**	Monday 31/05/04	Monday 26/07/04	Monday 18/10/04
	H	Monday 31/05/04	Monday 26/07/04	Monday 18/10/04
	I	Monday 31/05/04	Monday 26/07/04	Monday 18/10/04
	J	Monday 31/05/04	Monday 26/07/04	Monday 18/10/04

Table 1.2. The schedule for blood collection and analysis from the repeat stored samples demonstrating the dates for thawing samples from each participant.

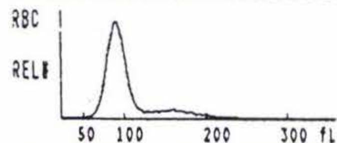
Collection Date	Samples	One Month Thaw Week
Monday 23/08/04	**A**	20/09/04
	B	20/09/04
	G	20/09/04
	D	20/09/04
Tuesday 21/08/04	**E**	11/10/04

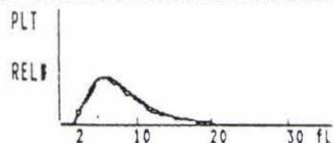
APPENDIX II: WHITE BLOOD COUNT

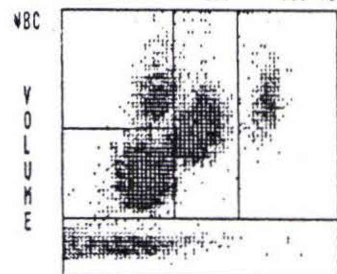
Figure 2.1. A printout of the characteristic results obtained from the differential blood count performed by the Coulter JT Whole Blood Counter and analysis computer. The White Blood Count (WBC) underlined is the number of interest.

21/09/04 09:37:56
V2901492 OPR

MASSEY BMLS
TENNENT DRIVE
PALMERSTON NORTH







Cass/Pos		ID# 1	DATE:	TIME:
S	CBC+Diff	ian metcalfe	21/09/04	09:37:23
				Reagent Sensor OFF

ID# 2

Sequence #

Normal WBC Pop			Normal RBC Pop		Normal PLT Pop	
WBC	5.8		RBC	5.01	PLT	229
NE%	52.1		HGB	150	MPV	8.3
LY%	33.5		HCT	0.452		
MO%	9.4		MCV	90.2		
EO%	4.9		MCH	29.9		
BA%	0.1	L	MCHC	332		
NE#	3.1		RDW	12.3		
LY#	1.9					
MO#	0.5	L				
EO#	0.3	L				
BA#	0.0	L				

APPENDIX III: MagnaFIRE SOFTWARE

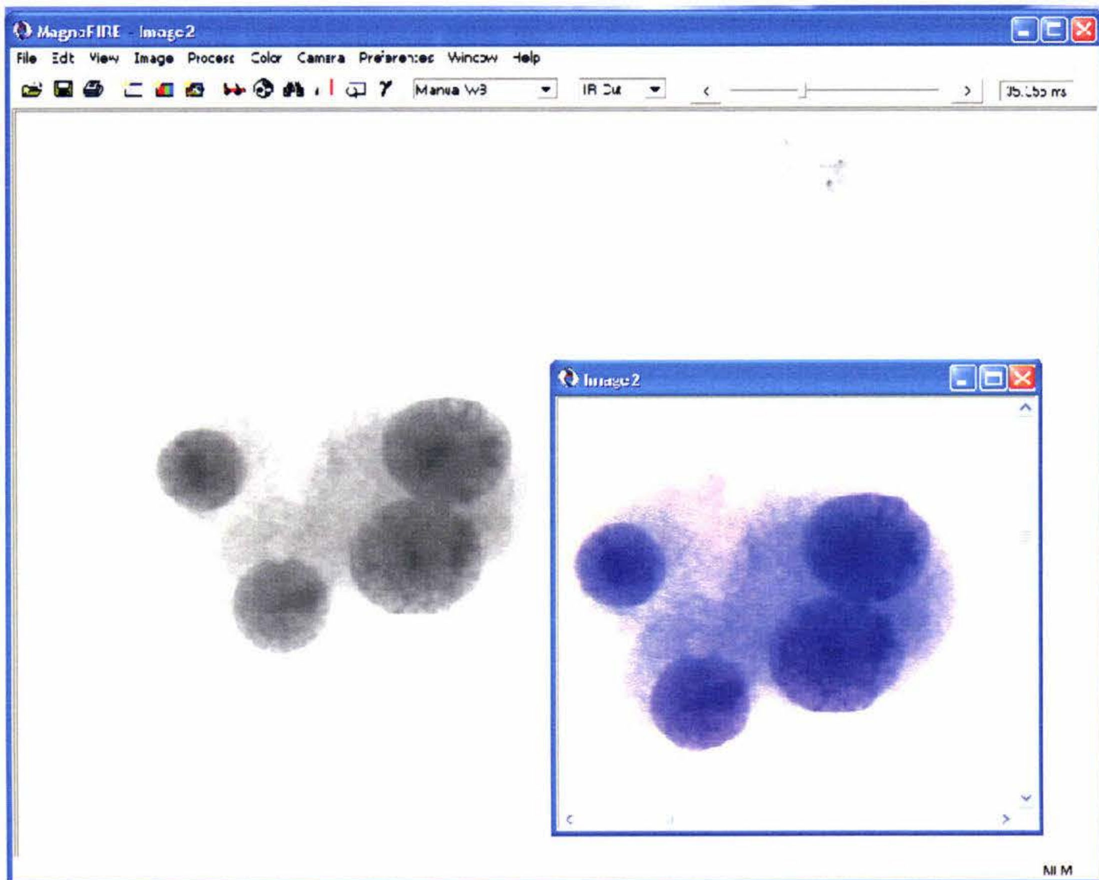


Figure 3.1. A screen view of the MagnaFIRE software utilised to obtain digital images of slides. This screen shows the black and white 'Live Window' (a window of what is viewed through the microscope), and a colour image captured using the MagnaFIRE s99802 digital camera.

APPENDIX IV: ImagePro SOFTWARE

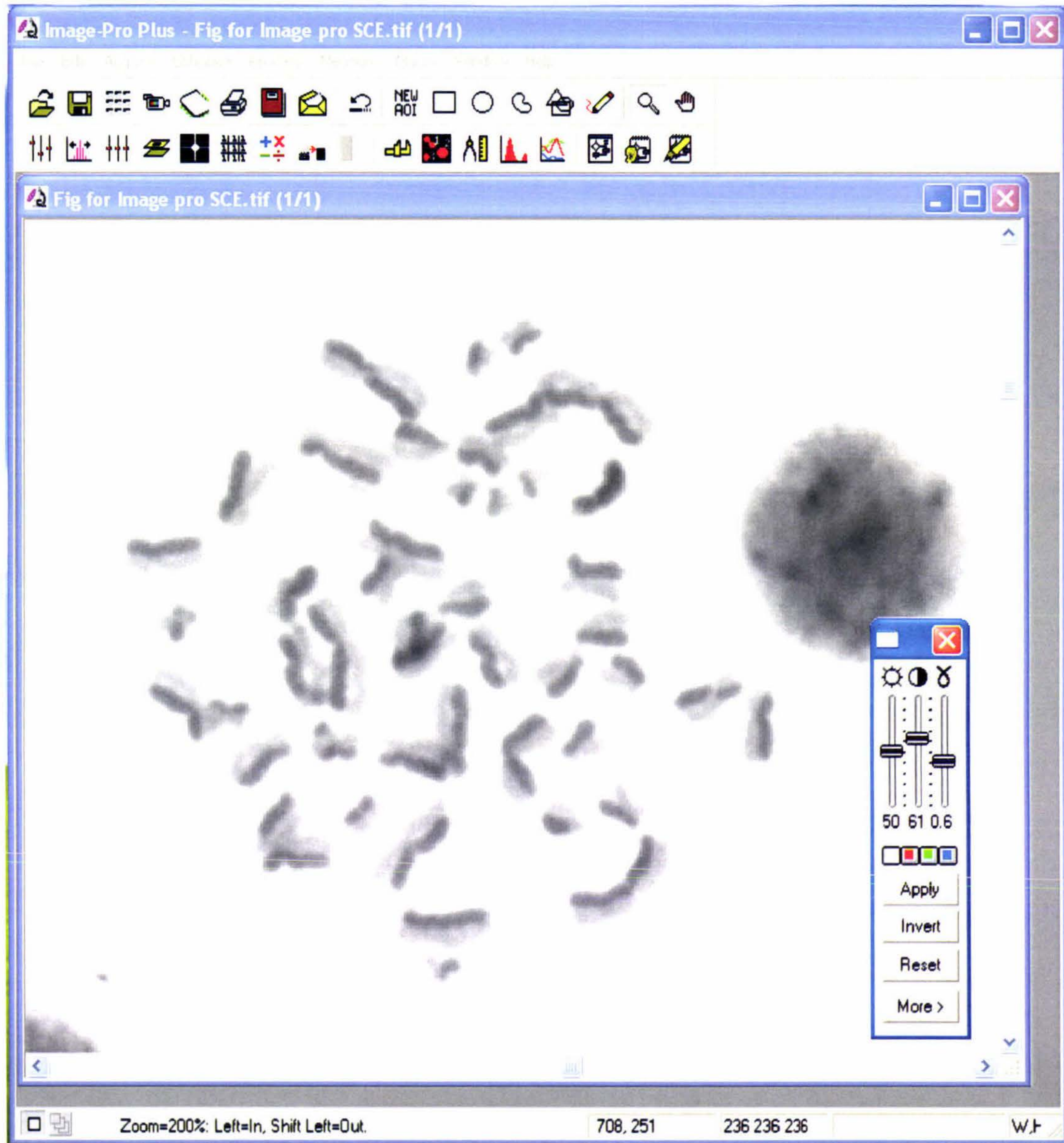


Figure 4.1. A screen view of the ImagePro Software when utilised to enhance the SCE images for scoring. An image taken using the MagnaFIRE software was opened in ImagePro. To clarify the exchanges, the contrast adjustment toolbar was used (shown in the bottom right corner) to reduce background exposure and to increase the contrast between the light and dark chromatids.

APPENDIX V: MN SCHEDULE

Table 6.1. The weekly schedule for the manipulation of blood samples when performing the Micronucleus Assay.

Day	Time	Process
Monday	**11:30**	Add 0.5ml blood, or cell pellet, to MN Cell Culture Media and sit at 37°C
	12:30	Place culture tubes in thermos flask and start for hospital
	13:00	3.5Gy Irradiation
	13:10	Return from hospital and place tubes in 37°C incubator
	19:00	Add PHA and return tubes to incubator
Tuesday	**19:00**	Add Cytochalasin-B and return samples to 37°C incubator
Friday	**13:00**	Commence Harvesting

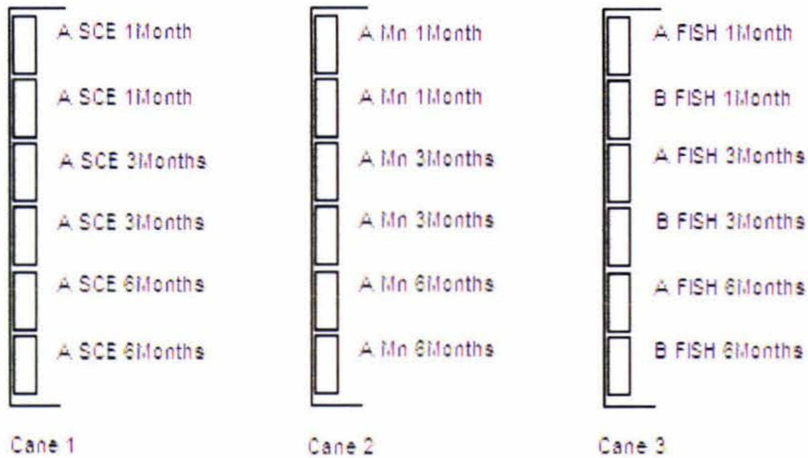
APPENDIX VI: FREEZING CHART

Table 6.1. A sample freezing chart used for monitoring the cooling progress of the whole blood samples. The times for reducing the temperature were recorded and followed.

Sample	Refrigeration -4°C	Freezer –20°C	Freezer -80°C	Liquid Nitrogen
A	11:00	11:30	12:30	13:30
B	11:35	12:05	13:05	14:05
C	12:10	12:40	13:40	14:40
D	12:50	13:20	14:20	15:20
E	13:30	14:00	15:00	16:00

APPENDIX VII: CRYOVIAL STORAGE

A



B

Holder 1	A SCE; A Mn; A/B FISH; B SCE; B Mn
Holder 2	C SCE; C Mn; C/D FISH; D SCE; D Mn
Holder 3	E SCE; E Mn; E/F FISH; F SCE; F Mn
Holder 4	G SCE; G Mn; G/H FISH; H SCE; H Mn
Holder 5	I SCE; I Mn; I/J FISH; J SCE; J Mn

Figure 7.1. These illustrations detail the organisation of sample storage. **A.** The samples were stored in the canes as shown in this detailed diagram using sample A as an example. The SCE cryovials were grouped together, the MN cryovials were grouped together and the FISH cryovials of two participants were grouped together in the canes. The remaining samples (B-J) were stored in the canes in the same way. **B.** Each holder contained five canes, and canes were stored in the holders as shown for holders 1-5.

APPENDIX VIII: RANDOM NUMBERS

Table 8.1. The random number pairs generated for viability analysis.

Y Axis	X Axis
5	114
8	102
3	105
1	107
5	119
9	113
15	101
6	100
7	104
5	110
12	103
0	100
7	117
12	115
11	103
14	119
10	107
4	115
20	101
8	117
5	104
19	119
7	104
20	118
18	105
1	107
1	104
15	111
10	112
4	103
6	110
5	119
7	117
3	106
19	108
10	102
13	113
1	102
0	103
11	105
6	106
8	112
10	108
1	105

Y Axis	X Axis
18	107
0	119
10	102
7	104
1	101
1	108
20	113
1	102
17	112
8	111
15	112
10	114
12	101
6	107
10	110
7	113
2	102
2	119
14	105
16	120
6	112
5	117
15	101
0	108
10	100
16	110
8	104
11	107
12	106
13	101
6	105
10	103
0	116
15	117
7	115
15	117
6	103
1	104
16	103
1	105
16	117
1	114
10	106
19	110

Y Axis	X Axis
12	119
16	117
11	119
5	104
10	100
17	102
2	104
11	119
13	104
20	107
8	114
4	106
2	111
6	106
4	111
7	118
13	115
5	110
5	118
8	114
13	114
10	113
9	103
6	107
18	113
1	115
15	102
14	108
16	117
8	104
18	102
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3	111
6	106
9	111
1	104
15	111
6	100
6	101
16	106
13	103
12	112
13	113
11	110

16	108
10	108
6	103
16	102
3	109
5	107
11	119
9	108
2	107
9	104
8	104
8	116
2	120
18	110
17	112
6	107
10	111
11	104
14	115
7	111
8	117
8	105
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16	116
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7	109
7	117
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12	118
8	112
19	116
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15	105
12	100
9	109
18	115
2	109
4	105
3	105
7	104
17	110
4	117
8	100
6	112
11	119
19	119
2	101
12	108
13	108
15	105

4	118
4	112
11	111
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19	109
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7	115
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12	109
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APPENDIX IX: LYMPHOCYTE MORPHOLOGY

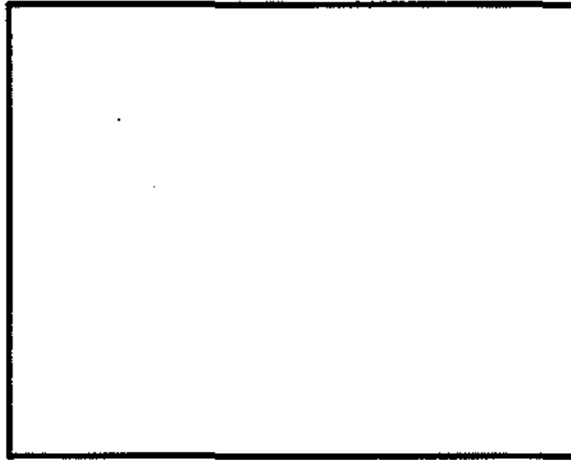

Figure 9.1. A captured image resulting from the first attempt to culture lymphocytes after cryopreservation (400X). No blood cells were observed.

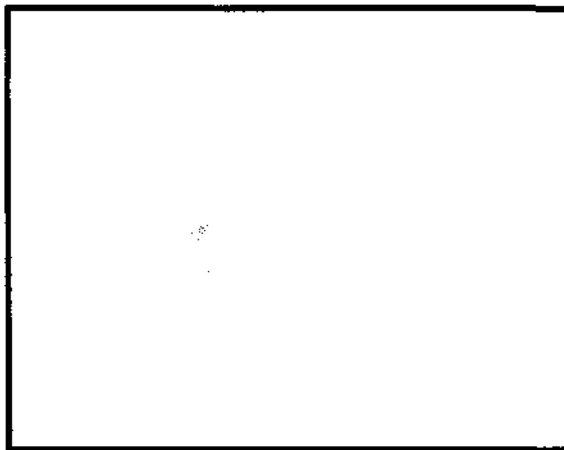

Figure 9.2. An image of a fractured lymphocyte from a cryopreserved blood sample (1000X).

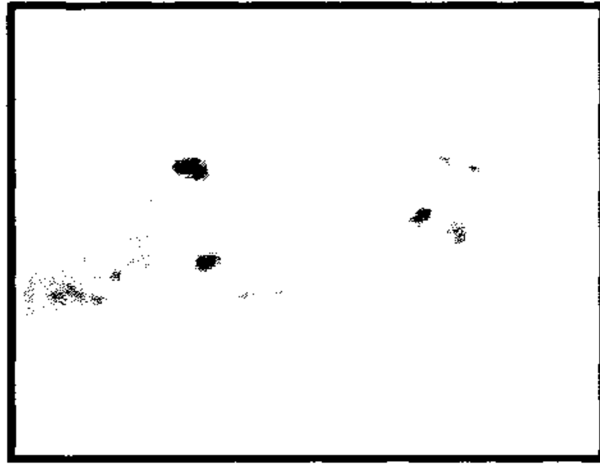

Figure 9.3. Cell debris observed after cryopreservation (1000X).

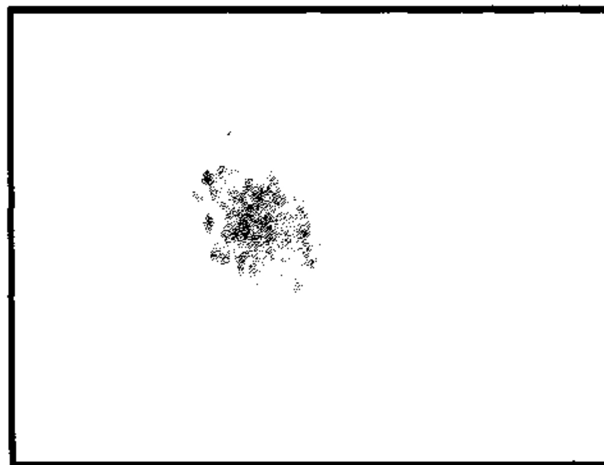

Figure 9.4. A dehydrated lymphocyte observed after cryopreservation (1000X).

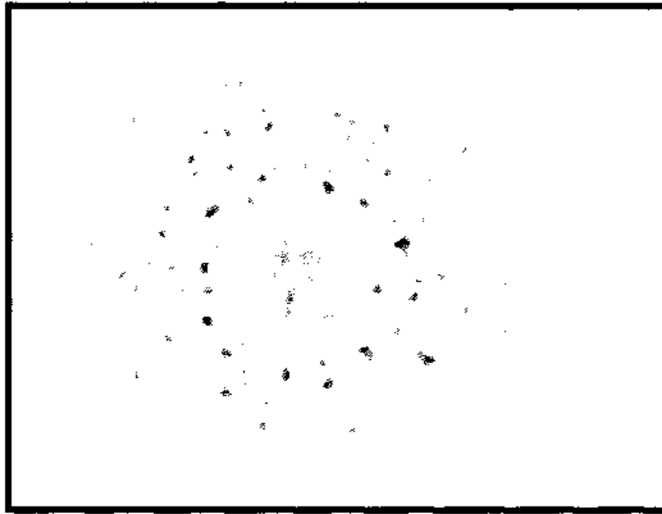

Figure 9.5. An apoptotic cell observed after cryopreservation (1000X). Although the rate of apoptosis was not measured casual observations indicated an increase in apoptotic cells after cryopreservation.

APPENDIX X: LYMPHOCYTES CULTURED IN DMSO

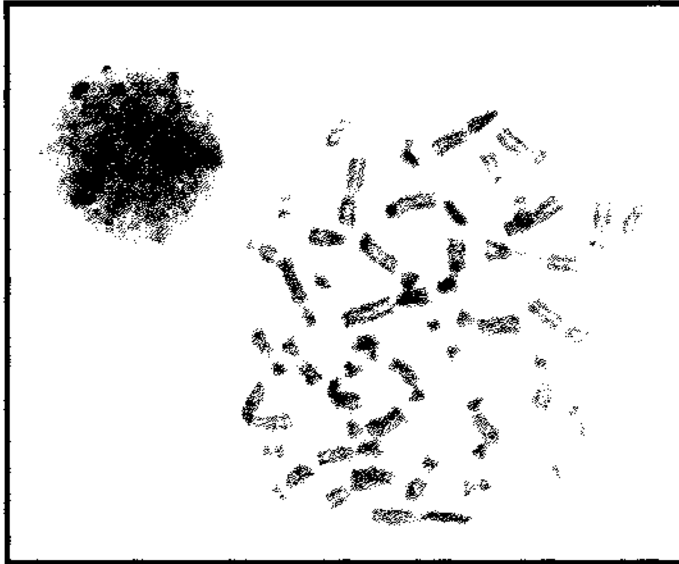

Figure 10.1. Success at culturing non cryopreserved lymphocytes in the presence of 5% DMSO. A normal number of metaphases were observed (1000X).

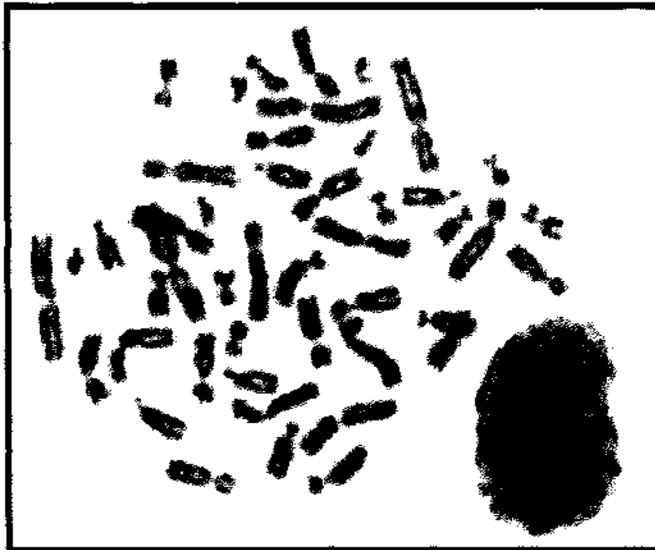

Figure 10.2. Success at culturing lymphocytes in the presence of 10% DMSO. A normal number of metaphases were observed (1000X).

APPENDIX XI: UNWASHED CELL PELLET

Figure 11.1. The undivided white blood cells observed after culturing the unwashed cryopreserved cell pellet (400x).

APPENDIX XII: CRYOPRESREVATION VIABILITIES

Table 12.1. The full viability data for the Cryopreservation and Genetic Damage Study. Each sample has values for analysis fresh, at one month, three months and six months. The values for each of the ten samples were recorded and the mean and standard deviation calculated. The viabilities are recorded in terms of percentage.

		A	B	C	D	E	F	G	H	I	J	E Repeat
Fresh		86.9	82.4	85.9	87.7	85.6	83.3	79.1	87.4	85.7	86.9	—
1 Month	**Sample 1**	82.9	13.3	89.6	57.1	72.8	—	42.1	41.7	93.2	45.5	0.0
	Sample 2	42.2	55.9	80.2	72.5	88.0	30.0	57.2	64.0	87.5	94.7	0.0
	Sample 3	18.4	90.1	82.2	33.4	40.0	98.0	47.1	60.7	67.9	15.2	0.0
	Sample 4	81.6	78.1	82.9	75.8	—	77.8	54.5	58.3	85.6	62.5	0.0
	Sample 5	70.2	79.8	61.5	43.5	38.2	50.3	75.0	50.7	55.9	81.3	0.0
	Sample 6	68.1	85.3	67.3	53.4	79.4	94.8	67.3	51.0	60.4	66.7	0.0
	Sample 7	73.7	63.2	75.2	67.3	83.7	—	—	—	—	—	0.0
	Sample 8	83.5	32.7	73.9	64.7	53.4	—	—	—	—	—	0.0
	Sample 9	79.3	84.4	86.1	56.3	49.2	—	—	—	—	—	0.0
	Sample 10	94.2	59.2	69.2	72.3	68.4	—	—	—	—	—	0.0
Mean		69.4	64.2	76.8	59.6	57.3	75.2	57.2	54.4	75.1	61.0	0.0
Std. Dev		22.6	25.0	9.0	13.6	21.2	28.9	12.3	8.2	15.7	28.0	0.0

3 Months	**Sample 1**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
Mean		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
Std. Dev		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—

6 Months	**Sample 1**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
	Sample 10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
Mean		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—
Std. Dev		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	—

APPENDIX XIII: SCE METAPHASES

Figure 13.1. A metaphase suitable for SCE analysis. The metaphase is complete (46 chromosomes) and the differential staining has been successful resulting in one chromatid appearing dark and the other light when viewed down a microscope (1000X).

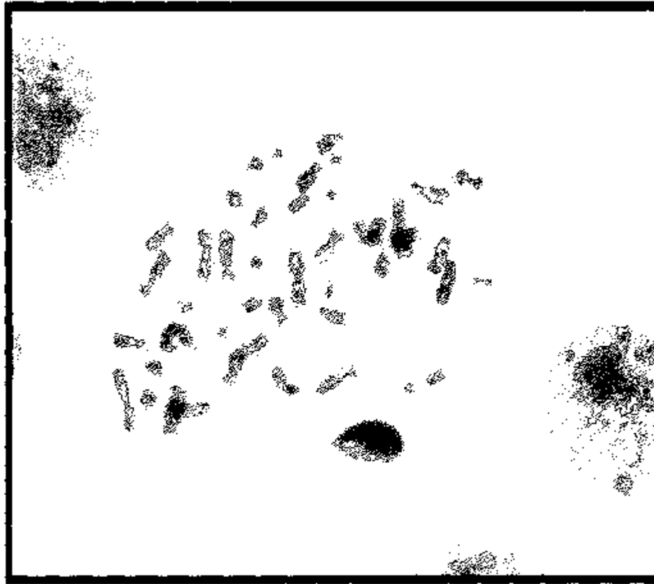

Figure 13.2. Metaphases sometimes appeared as normal Giemsa stained metaphases as shown in this figure. This can arise by the lack of incorporation of BrdU during culturing or the lack of differential staining during the staining procedure (1000X).

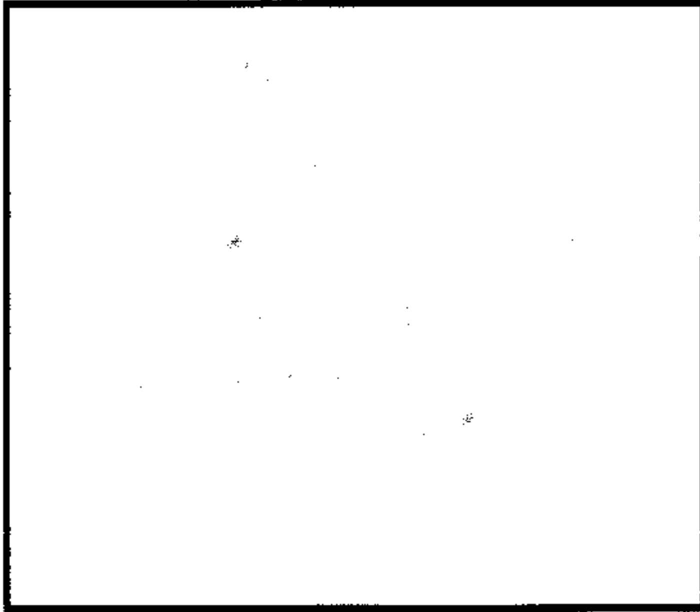

Figure 13.3. The chromatids were often differentially stained but lacking the complete chromosome complement, this metaphases contains only 43 chromosomes. When this occurred the metaphase could not be scored for SCEs. This may have resulted from the dispersal of chromosomes during slide preparation (1000X).

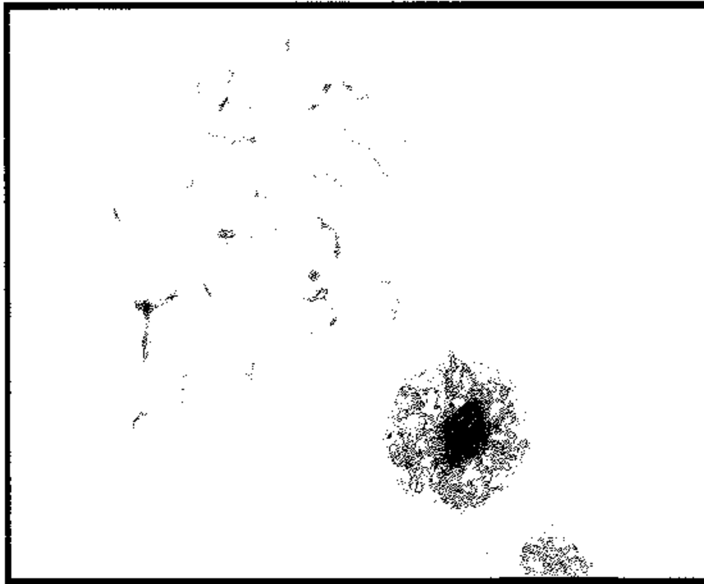

Figure 13.4. A mixed complement of BrdU incorporation is determined by the metaphase containing chromosomes that are not uniformly stained. Note some chromatid pairs are differentially stained, while others are similarly stained (1000X).

Figure 13.5. When the cells undergo DNA replication more than twice during the culturing period in the presence of BrdU, BrdU may be incorporated into both strands of the chromosomes as illustrated here (1000X).

APPENDIX XIV: COMPLETE SCE DATA

Table 14.1. The complete SCE results from sample A.

Metaphase	Fresh	1 Month	3 Months
1	15	—	—
2	15	—	—
3	9	—	—
4	9	—	—
5	8	—	—
6	1	—	—
7	1	—	—
8	5	—	—
9	3	—	—
10	9	—	—
11	16	—	—
12	5	—	—
13	11	—	—
14	7	—	—
15	3	—	—
16	10	—	—
17	6	—	—
18	5	—	—
19	9	—	—
20	6	—	—
21	10	—	—
22	10	—	—
23	17	—	—
24	5	—	—
25	8	—	—
26	4	—	—
27	4	—	—
28	9	—	—
29	3	—	—
30	11	—	—
31	4	—	—
32	9	—	—
33	3	—	—
34	7	—	—
35	6	—	—
36	9	—	—

37	5	—	—
38	4	—	—
39	6	—	—
40	5	—	—
41	2	—	—
42	6	—	—
43	7	—	—
44	2	—	—
45	2	—	—
46	7	—	—
47	5	—	—
48	9	—	—
49	11	—	—
50	6	—	—
Mean	6.98	—	—
Std. Dev	3.78	—	—

Table 14.2. The complete SCE results from sample B.

Metaphase	Fresh	1 Month	3 Months
1	7	—	—
2	9	—	—
3	6	—	—
4	9	—	—
5	4	—	—
6	4	—	—
7	7	—	—
8	3	—	—
9	1	—	—
10	2	—	—
11	10	—	—
12	4	—	—
13	5	—	—
14	5	—	—
15	12	—	—
16	9	—	—
17	7	—	—
18	6	—	—
19	8	—	—
20	11	—	—

21	5	—	—
22	6	—	—
23	1	—	—
24	3	—	—
25	4	—	—
26	0	—	—
27	7	—	—
28	4	—	—
29	5	—	—
30	2	—	—
31	5	—	—
32	8	—	—
33	4	—	—
34	6	—	—
35	3	—	—
36	3	—	—
37	4	—	—
38	6	—	—
39	8	—	—
40	2	—	—
41	4	—	—
42	5	—	—
43	4	—	—
44	2	—	—
45	3	—	—
46	1	—	—
47	7	—	—
48	7	—	—
49	6	—	—
50	4	—	—
Mean	5.2	—	—
Std. Dev	2.7	—	—

Table 14.3. The complete SCE results from sample C.

Metaphase	Fresh	1 Month	3 Months
1	6	—	—
2	5	—	—
3	5	—	—
4	6	—	—

5	8	—	—
6	11	—	—
7	6	—	—
8	3	—	—
9	4	—	—
10	5	—	—
11	8	—	—
12	4	—	—
13	7	—	—
14	9	—	—
15	3	—	—
16	16	—	—
17	9	—	—
18	12	—	—
19	7	—	—
20	5	—	—
21	4	—	—
22	9	—	—
23	12	—	—
24	4	—	—
25	9	—	—
26	6	—	—
27	2	—	—
28	10	—	—
29	7	—	—
30	7	—	—
31	4	—	—
32	7	—	—
33	13	—	—
34	9	—	—
35	12	—	—
36	6	—	—
37	3	—	—
38	8	—	—
39	6	—	—
40	7	—	—
41	7	—	—
42	8	—	—
43	7	—	—
44	5	—	—
45	9	—	—
46	14	—	—
47	3	—	—
48	9	—	—

49	6	—	—
50	1	—	—
Mean	7.1	—	—
Std. Dev	3.2	—	—

Table 14.4. The complete SCE results from sample D.

Metaphase	Fresh	1 Month	3 Months
1	5	—	—
2	4	—	—
3	9	—	—
4	6	—	—
5	7	—	—
6	6	—	—
7	6	—	—
8	9	—	—
9	6	—	—
10	4	—	—
11	3	—	—
12	5	—	—
13	6	—	—
14	11	—	—
15	5	—	—
16	13	—	—
17	18	—	—
18	7	—	—
19	4	—	—
20	6	—	—
21	14	—	—
22	4	—	—
23	7	—	—
24	10	—	—
25	9	—	—
26	8	—	—
27	3	—	—
28	9	—	—
29	9	—	—
30	6	—	—
31	9	—	—
32	10	—	—
33	12	—	—

34	4	—	—
35	12	—	—
36	10	—	—
37	9	—	—
38	5	—	—
39	9	—	—
40	4	—	—
41	7	—	—
42	8	—	—
43	6	—	—
44	12	—	—
45	11	—	—
46	3	—	—
47	11	—	—
48	8	—	—
49	7	—	—
50	5	—	—
51	3	—	—
Mean	7.6	—	—
Std. Dev	3.2	—	—

Table 14.5. The complete SCE results from sample E.

Metaphase	Fresh	1 Month	3 Months
1	5	15	—
2	5	13	—
3	10	9	—
4	5	6	—
5	3	17	—
6	12	9	—
7	13	18	—
8	12	9	—
9	3	11	—
10	6	11	—
11	6	10	—
12	6	—	—
13	6	—	—
14	3	—	—
15	4	—	—
16	3	—	—
17	4	—	—

18	10	—	—
19	8	—	—
20	8	—	—
21	3	—	—
22	9	—	—
23	7	—	—
24	6	—	—
25	4	—	—
26	4	—	—
27	2	—	—
28	7	—	—
29	4	—	—
30	4	—	—
31	3	—	—
32	9	—	—
33	8	—	—
34	6	—	—
35	1	—	—
36	9	—	—
37	7	—	—
38	6	—	—
39	5	—	—
40	6	—	—
41	4	—	—
42	10	—	—
43	5	—	—
44	3	—	—
45	7	—	—
46	7	—	—
47	7	—	—
48	6	—	—
49	2	—	—
50	2	—	—
51	5	—	—
52	4	—	—
Mean	5.9	11.6	—
Std. Dev	2.8	3.7	—

Table 14.6. The complete SCE results from sample F.

Metaphase	Fresh	1 Month	3 Months
1	3	—	—
2	4	—	—
3	7	—	—
4	3	—	—
5	6	—	—
6	3	—	—
7	1	—	—
8	7	—	—
9	3	—	—
10	12	—	—
11	10	—	—
12	7	—	—
13	4	—	—
14	8	—	—
15	3	—	—
16	2	—	—
17	6	—	—
18	3	—	—
19	2	—	—
20	3	—	—
21	1	—	—
22	3	—	—
23	3	—	—
24	3	—	—
25	7	—	—
26	10	—	—
27	3	—	—
28	9	—	—
29	2	—	—
30	3	—	—
31	6	—	—
32	7	—	—
33	5	—	—
34	4	—	—
35	2	—	—
36	2	—	—
37	6	—	—
38	1	—	—
39	3	—	—

40	3	—	—
41	7	—	—
42	6	—	—
43	3	—	—
44	5	—	—
45	8	—	—
46	3	—	—
47	4	—	—
48	3	—	—
49	6	—	—
50	7	—	—
51	6	—	—
52	4	—	—
53	6	—	—
Mean	4.6	—	—
Std. Dev	2.6	—	—

Table 14.7. The complete SCE results from sample G.

Metaphase	Fresh	1 Month	3 Months
1	10	—	—
2	8	—	—
3	23	—	—
4	7	—	—
5	8	—	—
6	6	—	—
7	12	—	—
8	2	—	—
9	5	—	—
10	4	—	—
11	4	—	—
12	10	—	—
13	10	—	—
14	8	—	—
15	7	—	—
16	5	—	—
17	7	—	—
18	7	—	—
19	12	—	—
20	8	—	—
21	8	—	—

22	3	—	—
23	9	—	—
24	8	—	—
25	10	—	—
26	9	—	—
27	16	—	—
28	7	—	—
29	10	—	—
30	4	—	—
31	5	—	—
32	12	—	—
33	6	—	—
Mean	8.2	—	—
Std. Dev	4.0	—	—

Table 14.8. The complete SCE results from sample H.

Metaphase	Fresh	1 Month	3 Months
1	4	—	—
2	11	—	—
3	2	—	—
4	14	—	—
5	2	—	—
6	7	—	—
7	3	—	—
8	6	—	—
9	9	—	—
10	5	—	—
11	7	—	—
12	7	—	—
13	3	—	—
14	9	—	—
15	9	—	—
16	11	—	—
17	6	—	—
18	14	—	—
19	4	—	—
20	6	—	—
21	6	—	—
22	5	—	—
23	11	—	—

24	8	—	—
25	6	—	—
26	10	—	—
27	1	—	—
28	6	—	—
29	9	—	—
30	10	—	—
31	8	—	—
32	7	—	—
33	2	—	—
34	2	—	—
35	8	—	—
36	8	—	—
37	8	—	—
38	6	—	—
39	13	—	—
40	11	—	—
41	7	—	—
42	9	—	—
43	13	—	—
44	11	—	—
45	4	—	—
46	5	—	—
47	6	—	—
48	6	—	—
49	12	—	—
50	4	—	—
Mean	7.2	—	—
Std. Dev	3.3	—	—

Table 14.9. The complete SCE results from sample I.

Metaphase	Fresh	1 Month	3 Months
1	5	—	—
2	8	—	—
3	4	—	—
4	13	—	—
5	14	—	—
6	7	—	—
7	21	—	—
8	8	—	—
9	8	—	—

10	6	—	—
11	4	—	—
12	8	—	—
13	6	—	—
14	6	—	—
15	5	—	—
16	4	—	—
17	6	—	—
18	4	—	—
19	7	—	—
20	8	—	—
21	5	—	—
22	11	—	—
23	10	—	—
24	13	—	—
25	11	—	—
26	8	—	—
27	6	—	—
28	12	—	—
29	17	—	—
30	11	—	—
31	3	—	—
32	10	—	—
33	5	—	—
34	9	—	—
35	4	—	—
36	13	—	—
37	3	—	—
38	5	—	—
39	13	—	—
40	6	—	—
41	9	—	—
42	9	—	—
43	6	—	—
44	14	—	—
45	7	—	—
46	6	—	—
47	4	—	—
48	6	—	—
49	7	—	—
50	9	—	—
51	8	—	—
Mean	9.7	—	—
Std. Dev	11.9	—	—

Table 14.10. The complete SCE results from sample J.

Metaphase	Fresh	1 Month	3 Months
1	7	—	—
2	11	—	—
3	7	—	—
4	5	—	—
5	9	—	—
6	8	—	—
7	8	—	—
8	9	—	—
9	12	—	—
10	9	—	—
11	6	—	—
12	7	—	—
13	6	—	—
14	13	—	—
15	7	—	—
16	13	—	—
17	9	—	—
18	13	—	—
19	10	—	—
20	2	—	—
21	11	—	—
22	9	—	—
23	14	—	—
24	5	—	—
25	12	—	—
26	10	—	—
27	6	—	—
28	8	—	—
29	6	—	—
30	4	—	—
31	5	—	—
32	6	—	—
33	10	—	—
34	8	—	—
35	12	—	—
36	10	—	—
37	6	—	—

38	7	—	—
39	12	—	—
40	8	—	—
41	8	—	—
42	11	—	—
43	7	—	—
44	8	—	—
45	7	—	—
46	5	—	—
47	10	—	—
48	6	—	—
49	4	—	—
50	11	—	—
Mean	8.4	—	—
Std. Dev	2.8	—	—

APPENDIX XV: COMPLETE MN DATA

Table 15.1. The complete data for the analysis of the Micronucleus Assay for each of the samples at fresh and one month periods.

		No. of Binucleate Cells	No. of Binucleate cells with MN	Total MN	Ma	Mb
A	**Fresh**	1000	228	260	22.8	26.0
	1 Month	40	14	17	35.0	42.5
B	**Fresh**	1001	155	177	15.5	17.7
	1 Month	208	85	111	40.9	53.4
C	**Fresh**	1013	207	246	20.4	24.3
	1 Month	45	23	28	51.1	62.2
D	**Fresh**	1000	263	301	26.3	30.1
	1 Month	509	226	302	44.4	59.3
E	**Fresh**	1000	293	341	29.3	34.1
	1 Month	1001	262	357	26.2	35.7
F	**Fresh**	1000	196	214	19.6	21.4
	1 Month	0	0	0	0.0	0.0
G	**Fresh**	1003	229	270	22.8	26.9
	1 Month	64	22	23	34.4	35.9
H	**Fresh**	1010	362	479	35.8	47.4
	1 Month	0	0	0	0.0	0.0
I	**Fresh**	1004	211	236	21.0	23.5
	1 Month	0	0	0	0.0	0.0
J	**Fresh**	1000	446	618	44.6	61.8
	1 Month	0	0	0	0.0	0.0
A Repeat	**Fresh**	1000	442	596	44.2	59.6
	1 Month	34	19	21	55.9	61.8
B Repeat	**Fresh**	1000	216	262	21.6	26.2
	1 Month	0	0	0	0.0	0.0
D Repeat	**Fresh**	1000	293	348	29.3	34.8
	1 Month	1000	238	280	23.8	28.0
E Repeat	**Fresh**	0	0	0	0.0	0.0
	1 Month	0	0	0	0.0	0.0
G Repeat	**Fresh**	1000	198	228	19.8	22.8
	1 Month	162	29	30	17.9	18.5