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**Expression of Mitochondrial Stress Protein
(Cpn60) in *in vitro* Cultured Neonatal Porcine
Islet Cells**

by

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ABSTRACT

Xenotransplantation of neonatal porcine islets have been demonstrated to be a viable alternative to exogenous insulin therapy for diabetes mellitus. The use of liberase has gained much success in islet isolation but factors such as batch-to-batch variation and deterioration of a batch with storage time have hampered the quality and reproducibility of tissue dissociation. Islet culture aims to optimise islet survival and insulin release in response to glucose challenge. However, it is difficult to recover and preserve islets *in vitro*.

Mitochondria play a key role in the secretion of insulin from pancreatic β islet cells in response to glucose stimulation. Mitochondrial dysfunction results in the induction (at mRNA and protein levels) of a molecular stress protein/heat shock protein called Cpn60. Since mitochondrial impairment will have a significant effect on the ability of *in vitro* cultured islet cells to function properly (i.e. release insulin in response to glucose stimulation), the expression of Cpn60 was investigated as a function of exposing neonatal porcine islet cells to various growth conditions.

The best choice of media to culture neonatal porcine islet cells was found to be not heated activated serum which showed the least levels of Cpn60 expression at mRNA levels suggesting that the cells had low levels of mitochondrial stress. Neonatal porcine islet cells would be best digested in cells digested with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS) as this gave the lowest levels of Cpn60 expression suggesting low levels of mitochondrial stress.

Although expression of Cpn60 at mRNA levels seems to be modulated during the growth of the porcine islet cells in media supplemented with different serum, heat treatment of serum and lipase content, no firm conclusion can be made with regard to the effect of the different treatments on mitochondrial health status until the porcine Cpn60 protein can be unequivocally identified.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ATP	adenosine-5'-triphosphate
β	beta
bp	base pair(s)
BSA	bovine serum albumin
$^{\circ}\text{C}$	degree Celsius
Cdna	complimentary deoxyribonucleic acid
Cpn60	chaperon 60
GA3PDH	Glycerol -3-
Da	daltons
DEPC	diethyl pyrocarbonate
DM	diabetes mellitus
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
EtBr	ethidium bromide
ETC	electron transport chain
kb	kilobase
kDa	Kilodaltons
mA	milli amps
min	minute
ml	millilitre
mRNA	messenger ribonucleic acid

ng	nanogram
pmol	picomole
rRNA	ribosomal ribonucleic acid
μ	micro (10^{-6})
MOPS	3-(N-morpholine)-propane- Sulfonic acid
N-terminal	amino-terminal
SB buffer	Sodium boric acid electrophoresis buffer
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RNA	ribonucleic acid
RNase	ribonuclease
Secs	seconds
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TFA	trifluoroacetic acid
TB	Tris-buffered saline
Tris	2-amino-2-(hydroxymethyl)-1,3- Propanediol
UV	Ultra Violet
W	watt

CHAPTER ONE

Introduction and Literature Review

1.1 Diabetes Mellitus

Diabetes Mellitus (DM) is one of the most common chronic diseases in both western and developing countries. DM is characterized by varying or persistent hyperglycaemia (high blood sugar levels) (Maki *et al.* 1992) resulting from defective secretion or action of the hormone insulin (Titus *et al.* 2000).

According to (British Medical Association 2004), DM has a prevalence of approximately 8% in Europe and the USA and exacts a high cost in terms of morbidity and mortality. British Medical Association (2004) has estimated that by 2010, the diabetic population will increase to 221 million from 110 million in 1994. In New Zealand, there are approximately 115,000 people diagnosed with diabetes, while 40,000-60,000 of population remain undiagnosed (Health Funding Authority NZ 2000). It is expected that over the next 20 years, the number of people with diabetes will increase up to 97% within Maori, 117% in Pacific Islanders, 47% in European New Zealanders (Health Funding Authority NZ 2000).

DM can be classified into four groups: type 1 diabetes, type 2 diabetes, other 'specific' types and gestational diabetes. Non- insulin dependent diabetes mellitus (NIDDM) also known as Type 2 Diabetes Mellitus (T2DM) is a multifactorial disease (Panunti *et al.* 2004). T2DM has been shown to result

from an interaction between a genetic predisposition and specific lifestyle factors (obesity, sedentary behavior and high caloric diets) (Mueckler 1993).

1.1.1 Non Insulin-dependent diabetes mellitus (IDDM)

A clustering of gene defects or polymorphisms may disadvantage some individuals to develop insulin resistance and therefore predispose to diabetes (Carulli *et al.* 2005). “Thrifty gene hypothesis” is applied to a population that appear to have genes that favour fat storage during the periods of food deprivation but produce obesity, hyperinsulinaemia, hyperglycaemia and diabetes when there is an excess of food supply (Zimmet 1997).

Pima Indians residing in Arizona live in an environment that promotes high caloric diets and minimal physical activity. Another tribe of Pima Indians living in Northern Mexico live in mountains, where their lifestyle consists of intense physical activity and low caloric diets (Zimmet 1997). Pima Indians living in Arizona have a greater prevalence of (T2DM) (54%) compared to Mexican Indians who have a prevalence of 37% (Carulli *et al.* 2005).

Obesity is epidemic in many developing countries particularly in the Pacific Islands. (T2DM) affects about 3% of all New Zealanders, with a very much greater prevalence amongst Maoris and Pacific Islanders (Mann & Lewis-Barned 1995). T2DM is a self managed condition which often requires stringent changes in the patient’s lifestyle (Simpson *et al.* 2003). A tailored program of diet and physical activity is the first toward managing diabetes. The goal of such

approach is to achieve glycemic control, weight loss and prevent microvasculature complications (Warren 2004).

Lifestyle intervention studies have consistently shown that modest changes can reduce the progression from impaired glucose tolerance to diabetes by 50-60% (Simpson *et al.* 2003). Short term effect on lifestyle intervention was carried out by (Brekke *et al.* 2004) on non-diabetic first degree relative (FDR) of T2DM patients, as they have an increased risk of developing T2DM during their lifetime. A 13% reduction in fasting insulin and a decrease in body weight by 2.1% were observed in the Diet-Exercise (D-E) groups compared to the control groups.

Gestational diabetes mellitus (GDM) is glucose intolerance (Cutchie *et al.* 2006) of variable severity with onset or first recognition during pregnancy (Hanna & Peters 2002) resulting in hyperglycaemia of variable severity, regardless of whether or not insulin is used and whether diabetes persists after pregnancy . Hyperglycaemia in gestational diabetes is not established until the late second trimester, well after organogenesis (Hsu-Hage & Yang 1999).

GDM is associated with adverse maternal and foetal outcome (Griffin *et al.* 2000). The obstetric complications of GDM are a result of the vaginal delivery of a large for gestational age infant (LGA) and the increased risk of late stillbirth (Dornhorst & Frost 2002). The neonatal metabolic complications of GDM, including hypoglycaemia and hypocalcaemia, are caused by foetal hyperinsulinaemia which occurs as a consequence of maternal hyperglycaemia

(Dornhorst & Frost 2002). In the long term, GDM is associated with diabetes in the mother and diabetes and obesity in the children (Dornhorst & Frost 2002).

Other 'specific' types of diabetes or Maturity onset diabetes of the young (MODY) is a familial condition with autosomal dominant inheritance. MODY is characterized by beta (β) cell dysfunction at a young age of diagnosis before 25 years. MODY has frequently been linked to the glucokinase gene on chromosome 7 and to the region of adenosine deaminase gene on chromosome 20. There are at least 6 genes implicated in the pathogenesis of MODY study carried out in 90 families by (Frayling *et al.* 2001), who found that 63% had mutations in the hepatocyte nuclear factor-1 α (MODY3), 20% had glucokinase mutations (MODY2) and 2% had HNF- α (MODY1) mutations. MODY only accounts for 1% of (T2DM) (Zimmet 1997). Variation in the variable number of tandem repeats (VNTR) regulatory polymorphisms to the insulin (INS) gene has been shown to the development of (T2DM) (Zimmet 1997).

As the focus of this study relates to the conditions used in the *in vitro* culturing of neonatal porcine islet cells, which are ultimately used to treat Type 1 diabetic patients, the following sections will review the biology and treatment options available for T1DM.

1.1.2 Insulin-dependent diabetes mellitus (IDDM)

Type 1 Diabetes mellitus (T1DM), formally called Insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes, is characterized by progressive destruction of β cells in the islets of Langerhans of the pancreas.

Destruction of the β cells is a consequence of an autoimmune process, the susceptibility to which is determined by genetic and environmental factors (Tisch & McDevitt 1996).

Once the β cells are destroyed, the pancreas is unable to produce insulin, a hormone that controls blood sugar levels. People with T1DM must take insulin shots several times a day in order to survive. People with this condition have a lifespan that is, on average, 15 years shorter than the norm (Mann & Lewis-Barned 1995).

The immune system exhibits tolerance towards self antigens, i.e. antigens of normal cells and tissues in the body fail to stimulate an immune response (Martini *et al.* 2001). The β cells autoantigens such as Insulin, I-A2, GAD, glucagon and somatostatin are expressed in the thymus. T-cells showing high affinity towards these antigens are eliminated through negative selection, while T-cells showing low affinity are left circulating in the periphery so that tolerance to β cell autoantigens are maintained (Bach & Chatenoud 2001)

Autoimmunity occurs when tolerance to self antigens is disrupted (Christen & von Herrath 2004). Hauben *et al.* (2004) describes autoimmunity as a physical

defense mechanism, a protective response that is triggered by tissue damage and degeneration to restore homeostasis. A malfunction of this system occurs when activated B and T- cells start making antibodies against normal body cells and tissues and begin their attack (Martini *et al.* 2001). The progression from autoimmunity to autoimmune disorder is not well understood, however several factors have been implicated to contribute to the onset of T1DM.

According to (Bresson & von Herrath 2004), T1DM occurs in a genetically pre-disposed environment. The inheritance is polygenic (Tisch & McDevitt 1996) with at least 60% of the genetic susceptibility being conferred by the major histocompatibility complex (MHC) also known as insulin independent diabetes mellitus 1 (IDDM1) found on chromosome 6 (Tisch & McDevitt 1996).

Studies (Salvetti *et al.* 2000) and (Barnett *et al.* 1981) have shown that the concordance rate for the development of T1DM is only 50% among monozygotic twins. This has led to believe that diabetes is a multi-factorial autoimmune disease where both genetic predisposition and environmental factors play significant roles (Hauben *et al.* 2004).

Environmental challenges such as diet, toxin or stressful life events and virus infections might trigger or accelerate auto-immune diseases when they occur in genetically pre-disposed individuals (Bresson & von Herrath 2004).

Infections induce strong immune response in T1DM diabetic prone individuals and have the ability to attract autoaggressive lymphocytes to the site of infection

(Christen & von Herrath 2004). Microbes and viruses mediate autoimmunity through several pathways. Strong inflammatory response induced by infections, activate bystander (non-specific) T-cells in an antigen non specific manner which not only cause damage but their activation potentiates the effect of other mechanisms; such as molecular mimicry (Christen & von Herrath 2004). Molecular mimicry occurs when immunity against viral antigens cross-reacts with self antigens that are exhibiting a conformational similar structure (Bresson & von Herrath 2004) such as a similar sequence homology has been observed between GAD and Coxsackie B3 virus protein (Bach & Chatenoud 2001).

T1DM is a T-cell mediated autoimmune disease where the pancreatic islets become successively infiltrated with both macrophages CD4⁺ and CD8⁺ T cells subpopulations. Activated macrophages produce interleukin (IL)-1 which activates resting T-cells (Poulsen 1996). The local release of cytokines such as (IL)-1 β , tumor necrosis factor α (TNF α) and gamma interferon in the surrounding area of the islets by T lymphocytes (Efrat 1998) affects the function of β cells before their destruction by apoptosis (Hostens *et al.* 1999; Harrison *et al.* 1992).

Kurrer *et al.* (1997) studied β cell death using BDC.2.5/ NOD Scid transgenic mice that lacked TCR- α β and TCR-gamma δ that lacked CD8⁺ T cell subpopulations but contained normal number of CD4⁺ T cells [**Figure 1.1**].

The first stage, known as peri-insulitis seen at day 5 and 6 (Kurrer *et al.* 1997) was characterized by recruitment of islet specific B and T lymphocytes (Bresson

& von Herrath 2004), macrophages, and dendritic cells which enter the periductal areas but remain outside of the islet proper (Harrison *et al.* 1992). Moderate insulinitis set in at day 7-10, becoming severe by day 22-30 (Kurrer *et al.* 1997). During this time, cytokines and macrophages began invading the islets, a process dependent on the recognition of β cell antigens. Autoantigens such as GAD (Napoli *et al.* 1996) and insulin are targeted in the early course of diabetes (Hauben *et al.* 2004). Autoantigens serve as diagnostic markers for progression of the disease process as autoantibodies (aAbs) directed against them are detected during the prediabetic phase (Nepom 1995). Anti insulin antibodies can be detected in \sim 50% of recent onset T1DM subjects (Tisch & McDevitt 1996).

Subjects became diabetic by day 30 (Kurrer *et al.* 1997). Examination of the pancreata revealed that β cell apoptosis was limited to islets affected by insulinitis. Apoptosis is generally induced by several pathways including the activation of Fas (CD95) pathway by Fas ligand (CD95L) interaction; however, (Allison & Strasser 1998) demonstrated that CD95 had a minor role to play on the progress to diabetes. Inflammatory cytokine production induces β cell apoptosis through raised expression of nitric oxide synthase (iNOS) and an intracellular increase in nitric oxide (NO) free radical (Efrat 1998). IL-1 β in combination with TNF α and IFN- γ causes both the synthesis of nitric oxide and of reactive oxygen species in pancreatic islets (Darville *et al.* 2000).

By the time non obese diabetic (NOD) mice show hyperglycaemia, $>90\%$ of the total β cell mass is destroyed (Kurrer *et al.* 1997). Destruction of β cells result in

the absence of endogenous insulin production and uncontrolled blood glucose levels. High blood glucose levels can damage blood vessels and nerves, predisposing the patient to a higher risk of blindness, heart disease, stroke, high blood pressure, kidney disease, dental disease, pregnancy complications, nervous system disease and limb amputations (Titus *et al.* 2000).

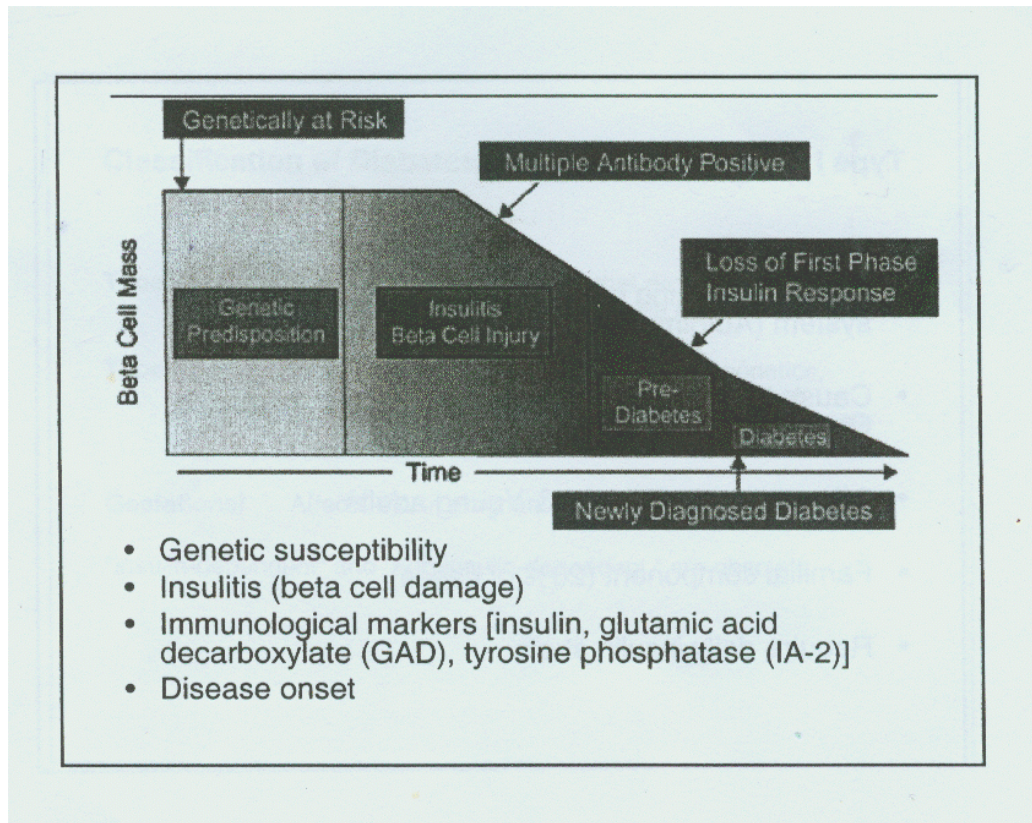


Figure 1.1 Timeline showing beta cell damage

Despite intensive insulin therapy, most individuals develop one or more complications of the disease (Lei *et al.* 2001). Therefore, the only way to ensure the long term health of patients with diabetes is to find an alternative to maintain constant normoglycaemia.

1.2 Islet Transplantation

Successful islet transplantation of pancreatic islets or whole pancreas from suitable human donors is an alternative to insulin injection (Kim 2003). In conjunction with a kidney graft, islet transplantation can be effected successively as the patient is already receiving immunosuppressants. There are two generally two types of islet transplantation.

The first is islet autotransplantation, where an organ or cells that has been removed from the patient is re-transplanted back into the same person (Titus *et al.* 2000). Autotransplantation is restricted to patients undergoing extensive pancreatectomy, to prevent the development of post operative diabetes mellitus because the graft is recognized as 'self' and would not induce an immune response (Lacy 1984; Titus *et al.* 2000). The second is islet allotransplantation, which has been prescribed for type one diabetic patients with a function solid organ graft.

Transplanting normal islets into diabetic patient would maintain normoglycaemia, give relief to the patient from daily injections and dietary restrictions, and prevent or arrest the development of secondary complications of diabetes without the requirement of constant blood sugar monitoring (Lacy 1984; Kim 2003; Maki *et al.* 1992) hence, improve the quality of life.

Following the success of the Edmonton trial carried out by (Shapiro *et al.* 2000) in 2000 who were able to achieve 100% insulin independence in seven patients after islet transplantation. The Edmonton group identified possible barriers to

successful transplantation. These were inadequate fusion of islet mass, islet isolation and islet processing (Kim 2003).

A healthy human pancreas is reported to contain 1,000,000 islets (White *et al.* 2001). The average yield of islets derived from a single pancreatic cadaver is only 250,000 with many islets lost during the process of purification (Kim 2003). As a result, multiple donors are needed to replace normal islet numbers in just one recipient but this is difficult as there are not enough cadaver pancreatic donors available to meet the demand for the increasing number of diabetic patients in need of a transplant (Vajkoczy *et al.* 1995; White *et al.* 2001).

1.2.1 Islet Isolation

The procurement technique has a major input on the outcome of islet isolation. A critical set of handling conditions determine the success with which islets can be harvested. Both warm and cold periods of ischemia are detrimental to islet yields. Another concern is the duration of ischemia (Tanioka *et al.* 1997). Cold storage of the pancreas for more than 12 hours reduces the human islet yield, but the islet yields are significantly compromised if they are kept in cold storage for longer than 16 hours (White *et al.* 2001). A two-layered method of preservation using University of Wisconsin (UW) solution and perfluorochemical has been designed by (Kuroda *et al.* 1988) for whole pancreas preservation. This method supplies sufficient oxygen to a pancreas graft during preservation by allowing production of ATP. Oxygen helps to maintain the cellular integrity and retain both the parenchymal and nonparenchymal viability for more than 24 hours successfully in canine pancreas transplants (Tanioka *et al.* 1997).

The development of an automated digestion and purification procedure for islet isolation by (Ricordi *et al.* 1988) was a breakthrough for clinical application involving pancreatic transplantation. Pancreas is the last organ to be excised from the cadaveric donor. Collagenase mixed with Hanks Balanced Salt Solution (HBSS) is administered intraductally into the pancreas to decrease the direct exposure of the islets to the digestive enzymes while increasing the rate of digestion of the exocrine pancreas (Gaglia *et al.* 2005). The choice of collagenase is critical, as reagents such as Ficoll that have high endotoxin levels have been shown to impair islet cell engraftment, activate cytokine expression and nitric oxide (White *et al.* 2001).

The distended pancreas is then incubated at 37°C in a closed stainless steel chamber with a screen filtering outlet. Pancreatic islets are then isolated using the automated collagenase digestion process. The automated collagenase digestion process slowly but progressively digests the organ into smaller size (Titus *et al.* 2000). As the solution circulates in the closed chamber, microscopic examination of aliquots taken from the chamber allow continuous monitoring of the enzyme digestion (Gaglia *et al.* 2005). When most of the islets become free from the exocrine tissue, digestion is stopped by diluting and cooling of the collagenase solution with HBSS at 4°C (Tanioka *et al.* 1997).

Further purification of islets from the exocrine tissue is achieved by cell separation based on the varying density of the different cell types. However, all of the islet cells do not separate completely and this leads to a reduced islet yield (Lacy 1984). The use of the continuous gradient centrifugation, with a standard

Ficoll density gradient allows greater separation of islet cells from the tissue (Titus *et al.* 2000).

After digestion and purification, the neonatal porcine islet cells are screened for β islet insulin producing cells. Dithizone (DTZ), a zinc-binding fluorescent dye, selectively stains islets of Langerhans *in vitro* staining them red. The islet yield is calculated under a phase-contrast microscope with a calibrated grid in the eyepiece, by counting random aliquots of the islet suspension. The number and diameter are determined using the ranges; (50-100, 101-150, 151-200, 201-250 and 251-300 μ m) (Sato *et al.* 2002). The islet function is determined by static glucose stimulation test, a test which assesses the rate of insulin secretion in response to glucose challenge (Balamurugan *et al.* 2004).

To improve the potential of cell replacement therapy for T1DM, studies are being done that focus on understanding the β cell and its regeneration and on the efforts to develop alternative sources of β cells.

1.3 Stem Cell Therapy

Stem cells have two important characteristics that distinguish them from other types of cells. They are unspecialized cells that renew themselves for long periods through cell division (Hussain & Theise 2004) and they can be induced to become cells with special functions such as the beating cells of the heart muscle or the insulin-producing cells of the pancreas (Blyszczuk & Wobus 2004) under certain physiologic or experimental conditions (Colman 2004).

1.3.1 From Embryonic Stem Cells to Pancreatic β Cells

(Colman 2004) and (Stoffel *et al.* 2004) extensively review protocol on generation of insulin producing cells from embryonic stem (ES) cells. (ES) cells are derived from the inner cell mass (ICM) of the blastocysts (Street *et al.* 2004). They are pluripotent (Blyszczuk & Wobus 2004) and have the ability to differentiate into cells of all three embryonic germ layers (Jones *et al.* 2004). The rationale of this protocol is to select for cells that are expressing the intermediate filament protein nestin (Colman 2004), a marker for intra-islet stem cells (Stoffel *et al.* 2004).

1.3.2 Pancreatic progenitors

The pancreas undergoes dynamic changes in response to growth, development and conditions such as obesity and pregnancy (Street *et al.* 2004). β cell neogenesis has been reported in the adult pancreas, particularly with pancreatic injury, partial pancreatectomy, partial duct obstruction and streptozin treatments (Peck & Ramiya 2004) suggesting the presence of adult stem/progenitor cells. This elusive progenitor cell is suggested to be located in islet, acinar, oval and duct cells (Montanya 2004).

Pancreas ductal cells have been implicated as progenitors for β cell neogenesis (Peck & Ramiya 2004). The formation of islets is closely associated with pancreatic duct epithelium (Colman 2004), through which sequential steps of differentiation gives rise to all pancreatic endodermal cell types (Blyszczuk & Wobus 2004).

In 1995, Peck and his colleagues were able to successfully grow functional islets in the long term cultures of islet producing stem cells, initiated from ductal epithelium cells (Peck & Ramiya 2004).

Ramiya *et al.* (2000) followed Peck's protocol to differentiate islets from pancreatic stem cells *in vitro* conditions. The islet progenitor cells (IPCs) that arose from islet producing stem cells (IPSCs) expressed glucagon, somatostatin, glucose transporter 2-receptor, responded *in vitro* to glucose challenge and reversed diabetes after implantation into female diabetic NOD mice. Colman (2004) is critical of (Ramiya *et al.* 2000) experiment stating that the islet progenitor cells were growing very slowly and insulin was expressed in very little quantity.

Street *et al.* (2004) discuss the possibility that the islets themselves contain progenitor cells that allow their prolonged survival and continual cell turnover. This suggests that expansion in β cells independent of any pancreatic stem cells (Jones *et al.* 2004).

1.3.3 Progenitors from other tissues

Progenitor cell from the tissues such as the liver might also be suitable for transdifferentiation as they have a common embryonic origin. Both liver and β cells are equipped to monitor changes in circulating glucose transporters and a high specificity, low affinity glucokinase (Jones *et al.* 2004).

Zalzman *et al.* (2003) were able to express pdx1 in foetal human (FH) progenitor liver cells by transduction via pdx1 lentivirus. Their finding demonstrated that pdx1 activated a number of β -cell genes. The cells expressing

pdx1 were able to produce, store and release insulin in response to glucose and were able to reverse hyperglycaemia.

Stem cells offer new possibility for the treatment of Type 1 diabetes. However, there still remains a problem in devising methods for efficient and reliable generation of insulin-secreting cells from pluri-potent progenitors.

1.4 Xenotransplantation

The scarce availability of human organs has influenced the need to utilize non-human donor from animals such as pigs and non-human primates by the means of xenotransplantation (Tisch & McDevitt 1996). Xenotransplantation is the transplantation of living cells, tissues, and/or organs from one species to another (Boneva *et al.* 2001).

Research involving xenotransplantation of cells and tissues provide evidence that xenogenic cellular replacement could be a potential therapy for disorders characterized by cellular dysfunction or cell death (Edge *et al.* 1998).

Transplanting porcine islets to treat diabetes is viewed as the most suitable approach to treat diabetes as the porcine islets are readily available (White *et al.* 2001), the amino acid sequences of pig insulin are similar to that of human insulin (Heneine *et al.* 1998) and there are no restrictions on the supply of the pancreases from these species (Korsgren *et al.* 2003).

Living Cell Technologies (LCT), an international biotechnology company is focused on providing a cure for a number of diseases such as diabetes,

haemophilia, Huntington's disease and stroke. Auckland based LCT hosts the worlds most advanced porcine herd bred in a gnotobiotic environment for the therapeutic treatment of type 1 diabetes via xenotransplantation.

Isolation and purification of neonatal porcine islet cells were performed by (LCT), Auckland using (Ricordi *et al.* 1988) method with some modification. A standard collagenase method was used. Briefly, using aseptic technique, the glands were distended with 1.5mg/ml collagenase. Excess fat, vessel and connective tissue were removed by trimming. The glands were then minced and digested at 37°C in a shaking water bath for 18 minutes at 120rpm. Following digestion, the cells were passed through a sterile 280µm mesh into a sterile beaker. A second digestion for 10min was repeated to ensure complete digestion of any undigested tissue.

The neonatal porcine islet cells were subjected to different conditions such as extracting islets from the pancreas using old and new liberase and culturing the islet cells in different serum-supplemented media to determine the most favorable condition that would allow greatest islet survival and function.

1.5 Liberase

Collagenase preparations are routinely used to extract the β islet cells from the pancreas tissue. Crude collagenase preparations are concentrated from bacterial *Clostridium histolyticum* culture supernatants. This impure mixture is heterogeneous, containing as many as 30 different enzymes (White *et al.* 2001), cellular debris, pigments and endotoxin (Bond & Van Wart 1984). Highly

purified collagenase is ineffective for islet isolation, and some of the other components of crude collagenase are therefore necessary (London *et al.* 1998). The primary enzyme constituent is collagenase (classes I and II) (Bond & Van Wart 1984). Collagenase is a member of the matrix metalloproteinase (MMP) family. They function at neutral pH and can digest synergistically all the matrix macromolecules (Reynolds 1996). All collagens or collagen-like proteins are susceptible to proteolysis by the enzyme obtained from *Clostridium histolyticum* (Harper 1980).

Liberase, an optimized blend of purified collagenase enzymes having a gentle enzymatic action (Linetsky *et al.* 1997) is designed to generate reproducible and high isolation yields of highly functional cell (Lakey *et al.* 1998). Islet recovery and function was significantly improved when Liberase CI, a purified enzyme blend was used during pancreatic digestion. On the other hand, digestion with crude collagenase resulted in highly variable islet yields, extensive islet fragmentation, and variable islet functionality (Lakey *et al.* 1998). Similar results were observed by (Linetsky *et al.* 1997) who found that the islets isolated with Liberase enzyme were of larger size and were much less fragmented.

The digestion phase of islet isolation by liberase is variable and unpredictable. Some of the problems associated with digestion using liberase are batch-to-batch variation and loss of activity with time (Chen & James 2001). A liberase batch that would work well for human islet isolation may not work well when isolating islets from either rat, pig or dog because there is a marked species variation in both total pancreatic collagen and collagen type and distribution

(London *et al.* 1998). Like any other enzyme, the enzymatic activity of liberase deteriorates with storage time. In their studies, (Cavanagh *et al.* 1997) found that traditional collagenase loses islet dissociation efficacy over time regardless of storage conditions.

Bacterial endotoxin is the most critical contaminate in collagenase as it has been shown to impair islet cell engraftment, activate cytokine gene expression (e.g. interleukin (IL-) β), tumor necrosis factor (TNF)- α , and IL-6, human complement and activation of nitric oxide (White *et al.* 2001). Vargas *et al.* (1998) detected high levels of endotoxin in the collagenase preparations used to disperse the pancreatic tissue before islet purification and transplantation. In another study, (Eckhardt *et al.* 1999) demonstrated reduced xenograft survival within the first three days after transplantation prepared under endotoxin conditions.

1.6 Cell Culture

Islet culture following isolation is important as it is during this period; the islets are allowed to recover from the traumatic stress of islet isolation. During the enzymatic digestion of porcine pancreas with liberase, the adhesion mechanism between pancreatic endocrine and exocrine tissue, as well as between endocrine cells and the extracellular matrix are destroyed (Sato *et al.* 2002). The destruction of these tissues may subject the islets to cellular stress that could impair islet survival and function.

A general finding, in rat, porcine and human foetal pancreatic β cells are that they exhibit a poor insulin secretory response to glucose. The onset and maturation of glucose-induced insulin secretion is more evident in the postnatal period (Korbitt *et al.* 1996), i.e. after 6-8 weeks of being in culture (Kim *et al.* 2004). Hence, it is vital to culture porcine islets until they are able to produce good insulin response to *in vitro* glucose stimulation.

The early tissue culture media used for growing mammalian cells were based on biological fluids such as plasma and embryonic extracts (Holman *et al.* 1995). There are several different types of serum such as; foetal bovine serum, foetal calf serum, neonatal porcine serum, human albumin serum. Serum is an effective growth promoting and cell-protecting supplement for all types of cells in culture.

Islets which had been cultured at a physiological glucose concentration (5.5 mmol/l) in the absence of serum had an impaired glucose-stimulated insulin biosynthesis and reduced insulin content, whereas insulin biosynthesis and release and insulin content markedly increase after culture in the presence of serum (Andersson 1978).

Serum contains growth factors, albumin, transferrin, anti-proteases and attachment factors (Cartwright & Shah 1994). Absence or removal of certain growth factors will result in cell death via apoptosis. Serum proteins such as albumin are involved in transport of substances such as steroids, lipids and fatty acids (Cartwright 1994). Transferrin, a metalloprotein (Martini *et al.* 2001)

makes up 3-6% of total serum protein (Cartwright & Shah 1994). Iron binds to transferrin, an iron transporter when released into the bloodstream. Transferrin is then absorbed by the red blood cells in the bone marrow for the synthesis of new haemoglobin molecules (Martini *et al.* 2001). Iron is an essential trace element for cells in culture (Cartwright & Shah 1994) but large amounts of free iron can be toxic to cells (Martini *et al.* 2001). Antiproteases, such as anti trypsin and α_2 macroglobulin which makes up 2% of the total serum prevents proteolytic damage to cells and cellular products (Cartwright & Shah 1994). Attachment factors such as fibronectin, laminin and feutin facilitate the binding of the anchorage dependent protein to the substratum.

Cartwright & Shah (1994) discuss the disadvantages associated with the use of serum-supplemented media. Disadvantages include dependency on its supply, lack of reproducibility due to variation in quality between batches, complications in protein purification and the risk of contamination of the product with viruses. Serum used in culture media critically depend on the characteristics of the source animals used, on the time of the year, and the type of feed they have been given. Certain factors present in some batches maybe absent in the other batches while it maybe present at inhibitory amounts in some batches.

Serum also has a risk of contamination with substances such as virus, bacteria, fungi, mycoplasma. Heat-labile serum factors could lead to non-specific activation of lymphocytes, resulting in elevated lymphocyte responses such as those observed by (Leshem *et al.* 1999). Heat inactivation of serum has always

been a compulsory step in cell culture in order to destroy contaminants such as bacteria, fungi and heat-labile proteins (Triglia & Linscott 1980).

Studies have shown that heat not only destroys the contaminants but could also cause aggregation of proteins (Leshem *et al.* 1999) and destroy other components such as amino acids, vitamins, growth factors. Heat inactivation (56 degrees C for 40 min) of bovine calf serum was shown to diminish its capacity to promote the attachment of cells to plastic or glass surfaces (Giard 1987). Foetal calf serum showed similar results but it was up to a smaller extent.

1.7 Mitochondria

Mitochondria play an important role in the life and death of all mammalian cells. They play a key role in many cellular functions including energy production, fatty acid metabolism, pyrimidine, heme and steroid biosynthesis (Van Hauten *et al.* 2005), calcium homeostasis and cell signaling (Gibson 2005). Mitochondria also play a significant role in cellular metabolism such as reactive oxygen and nitrogen species, regulation of apoptosis and glucose and calcium homeostasis (Enns 2003).

Mitochondria also play a key role in glucose stimulated insulin secretion from the β islet cells [**Figure 1.2**]. The high K_m glucose transporter Glut2 facilitates the movement of glucose into the pancreatic β cells (Brownlee 2003). Once inside the beta cell, the breakdown of glucose begins by the process of glycolysis. During glycolysis, glucose is phosphorylated by an enzyme called glucokinase to produce glucose-6-phosphate. Although this is a rate limiting

step in glycolysis, this step traps the glucose inside the cell and prepares it for further biochemical processes. Glucose-6-phosphate is phosphorylated again to form fructose-1,6-bisphosphate. The pyruvate is transported into the mitochondria where it is oxidized by tricarboxylic acid (TCA) cycle to produce NADH and FADH₂. NADH and FADH₂ from the TCA cycle are provided for ATP production via oxidative phosphorylation (OXPHOS). Each of the 8 molecules of NADH yields 3 ATP and 1 H₂O molecule while each of the FADH₂ molecule yields 2 ATP and 1 H₂O molecule (Martini *et al.* 2001). The increase in the ATP:ADP ratio closes ATP sensitive potassium channel in the β cell membrane preventing the positively charged potassium ion (K⁺) from leaving the cell. The rise of the positive charge inside the cell causes depolarization and as a result, activating a voltage gated calcium channel. The resultant influx of calcium triggers secretion of insulin via exocytosis.

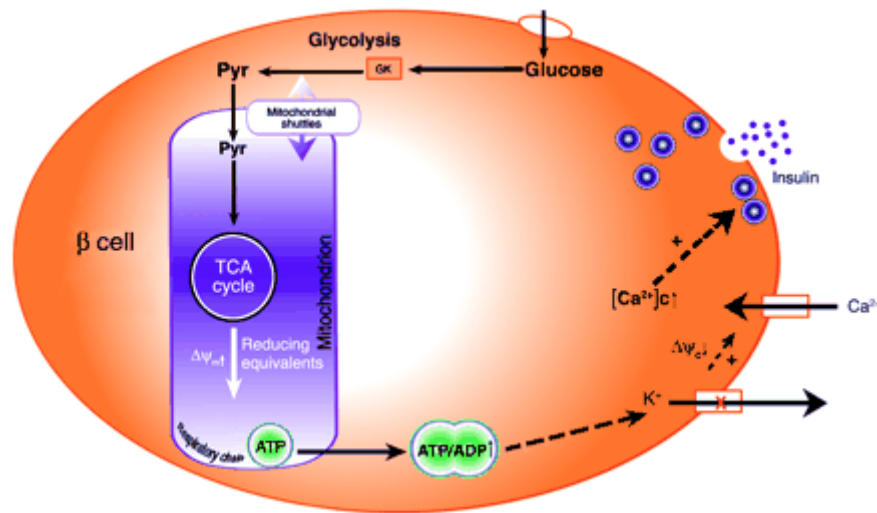


Figure 1.2 Model of glucose-stimulated insulin secretion in the pancreatic β cell (Adapted from (Brownlee 2003))

A characteristic feature of mitochondria with impaired function is the induction (at mRNA protein levels) of a molecular stress protein/heat shock protein called Cpn60 (Mallard *et al.* 1996).

Molecular stress proteins are found through out the cell and are induced in response to a variety of cellular stresses (Heather *et al.* 2004). They are involved in the re-folding of the denatured proteins, trafficking of intercellular proteins (Duchen 2004) and protecting cellular homeostatic processes from environmental and physiologic insult by preserving the structure of normal proteins and repairing or removing damaged ones (Tytell & Hooper 2001).

Since mitochondrial impairment will have a significant effect on the ability of *in vitro* cultured islet cells to function properly (i.e. release insulin in response to

glucose stimulation), the expression of Cpn60 was investigated as a function of mitochondrial health by exposing neonatal porcine islet cells to various growth conditions.

1.8 Outline of this study

This study aimed to determine the best (i) serum supplemented media to grow and culture neonatal porcine beta islet cells in, (ii) to determine what type of enzyme would be best for islet isolation (iii) and to determine whether heat inactivation of serum had an effect on the mitochondrial health of neonatal porcine β islet cells.

CHAPTER TWO

Materials and Methods

This chapter reviews the materials and methods employed through out the project. It includes all reagents, chemicals and stock solutions, as well as a detailed overview of all the methods used.

2.1 Materials

Section 2.1 of this chapter outlines specific laboratory equipment required, all the commercially available kits and reagents used during the course of this research.

2.1.1 Enzymes

Enzymes used and their sources are listed in Table 2.1:

Table 2.1 *Enzymes*

Enzyme	Source
Transcriptor Reverse Transcriptase	Roche
<i>Taq</i> DNA polymerase	Roche
DNase 1	Roche
Trypsin	Promega

2.1.2 Antibodies

Antibodies used and their sources are listed in Table 2.2:

Table 2.2 *Antibodies*

Antibody	Product #	Source
Mouse Anti-Hsp60 Monoclonal Antibody	SPA 807	Stressgen
Goat anti-Mouse IgG	SPA 101	Stressgen
Actin (C-11)-R sc-1615-R Rabbit polyclonal IgG	K1904	Santa Cruz

2.1.3 Primers

Primers were synthesized by Invitrogen (Auckland, New Zealand). The primers were resuspended in 1X TE buffer to a final amount of 20pmol

Table 2.3 *Primers*

Primers	Sequence	Product
Rat Cpn60	Forward-5'-GCGGATGCTCGAGCCTTAAT-3' Reverse- 5'-ATGACCAAGGGCTTCCGGTG-3'	702bp
Rat GA3PDH	Forward-5'-ACCACAGTCCATGCCATCAC-3' Reverse-5'- TCCACCACCCTGTTGCTGTA-3'	452bp

2.1.4 Antigen

Antigen used and its source are listed in Table 2.4

Table 2.4 *Antigen*

Antigen	Product #	Source
Recombinant Rat Hsp60 Protein	SPA 742	Stressgen

2.1.5 Stock Solutions

Stock solutions were made routinely in the labs. Stock Solutions and their composition are listed in Table 2.3.

Table 2.5 *Stock Solutions*

Solution	Composition
PBS	8g NaCl 0.2g KCl 1.44g Na ₂ HPO ₄ 0.24g KH ₂ PO ₄ Adjust to pH 7.4 and make up to 800ml with d H ₂ O
5X Electrode Buffer (SDS)	9g Tris 43.2g Glycine 3g SDS 600ml dH ₂ O

Loading Dye (RNA)	50% v/v glycerol 1mM EDTA 0.4% (w/v) Xylene (Cyanol) 0.4% (w/v) Bromophenol Blue
Ficoll Dye	0.75g Ficoll 6.25mg Bromophenol Blue 0.5ml TBEX10 4ml distilled H ₂ O Mix chemicals with small amount of water to a paste then stir for several hours
10 X MOPS Electrophoresis Buffer	400mM MOPS 100mM Sodium Acetate 10mM EDTA Make up to 1 litre using DEPC- treated water
TENT Buffer	50mM Tris-HCL (pH 7.4) 150mM NaCl 5mM EDTA 1% Triton
Dithiothreitol (DTT) Solution	250mg DTT in 0.5ml dH ₂ O

Stock Buffer (Westermeier's)	6.06g Tris 0.4g SDS
Westermeier's Sample Loading Buffer (Reducing)	1ml DTT Solution 1g SDS 0.003g EDTA 0.01g Bromophenol Blue (R250) 2.5ml Stock Buffer (Westermeier's) 10ml Glycerol
Towbin transfer buffer (SDS)	192mM Glycine 25mM Tris 1.3mM SDS 20% Methanol Made upto 500ml with dH ₂ O
Transfer buffer without SDS (Edman)	12.5mM Tris 96mM glycine, ph 8.3 10% Methanol
10% Tween	10g Tween Made to 100ml with PBS
0.2% Tween/PBS	20ml 10% Tween/PBS Mixed with 980ml PBS

10X SB Buffer	56g Boric Acid 9g NaOH Adjust to pH 8.5, Make up to 2 litre with dH ₂ O
TE Buffer (pH 8.0)	10 mM Tris-HCl 1 mM EDTA
Stain Solution (SDS-PAGE)	1g Coomassie Blue G-250 400ml Methanol 100ml Acetic Acid 500 ml dH ₂ O
De-stain Solution (SDS-PAGE)	400ml Methanol 100ml Acetic Acid 500 ml dH ₂ O
Colloidal Coomassie G-250	1.2g Coomassie Blue G-250 100G Ammonium Sulfate 200ml Methanol 100ml Phosphoric Acid 700ml MQ Water
Fixative Solution	500ml Methanol 2ml Phosphoric Acid 500ml MQ Water

2.2 Methods

2.2.1 Porcine Neonatal Islet Samples

Neonatal porcine islet samples utilised in this study were provided by Living Cell Technologies (LCT), Auckland. The isolated islet samples in each batch were subjected to different treatments and conditions. Each sample had a control which was not subjected to any treatment.

The Auckland Island Pigs (AIP) is a closed herd from a remote, Sub-Antarctic Island group to the south of New Zealand. Feral pigs from these islands have had essentially no contact with other pigs or humans for about 200 years, and have subsequently been bred in isolation in New Zealand. Their origin and isolation may have protected them from many of the common exogenous porcine viruses. Possible sources of zoonotic infections are monitored in the herd, sows, piglets, and subsequently in the isolated islets. The Pig Improvement Company, (PIC) herd is supported by three additional herds which operate as multiplier units, in addition to producing commercial pigs for market.

2.2.2 RNA Extraction

Total RNA was extracted from the samples using the High Pure RNA Tissue Kit (Roche) according to the manufacturer's instructions with simple modifications. The kit contained the following: Lysis/-binding buffer, DNase 1, DNase incubation

buffer, Wash buffer I, Wash buffer II, Elution buffer, high pure filter tubes and collection tubes.

Precautions were taken when handling reagents and RNA samples during the extraction process to avoid RNase H, microbial and nuclease contamination. It was ensured that work surfaces were thoroughly cleaned and disinfected with 70% ethanol prior to the start of each experiment. Sterile, disposable DNA LoBind tubes (Eppendorf, Global Science) and RNase free tips (Bio-Strategy) were used during the assay. DEPC-treated water was used in all RNA assays unless otherwise stated.

The following working solutions were made before the start of the experiment: DNase I was dissolved in 0.55ml of elution buffer. 10 μ l aliquots were made and were stored at -20°C. 20 ml of absolute ethanol was added to the bottle of Wash buffer I and mixed well. This was stable at room temperature. 40ml of absolute ethanol was added to the bottle of Wash buffer II and mixed well. This was also stable at room temperature. 30% Triton X 100 + Lysis buffer was made just before the start of the experiment by mixing 120 μ l of Triton X 100 (BDH Laboratory Supplies) with 280 μ l Lysis/-binding buffer in MS1 mini shaker (IKA, Global Science) at a speed of 1400/min until a uniform solution was formed.

1x10⁶ of neonatal porcine islet cells were pelleted by centrifuging using a Mini Spin Eppendorf Centrifuge (Global Sciences) for 7 seconds at 10 000 x g. The pellet

was then resuspended in 0.5ml sterile PBS and centrifuged for 3 seconds at 10 000 x g. The cells were resuspended in 200µl sterile PBS. 1µl of RNase Inhibitor was added to prevent RNA degradation by RNAases in the sample. 400µl of 30% Triton X 100 + Lysis buffer was added to the sample and mixed (MS1 mini shaker, Global Science) at room temperature for 4 minutes at 1400 l/min. *High pure* (Eppendorf, Global Science) filter tubes and collection tubes were assembled and the entire sample was transferred into the upper reservoir of the filter tube. The entire *High pure* filter tube assembly was placed in the Mini Spin Eppendorf Centrifuge (Global Sciences) which was then centrifuged at 13 000 x g for 30 seconds. The centrifugation was repeated if the glass fleece of the filter tube still looked wet. The flow through in the collection tube was discarded. In order to remove contaminating DNA, 90µl of DNase 1 incubation buffer was mixed with 10µl of DNase 1 in 1.5ml sterile, RNase free DNA LoBind tubes (Eppendorf, Global Science). The solution was then pipetted on the glass filter fleece in the upper reservoir of the same filter tube and was incubated for 15mins at room temperature. After the incubation period, 500µl of wash buffer I was added to the upper reservoir of the filter tube and centrifuged Mini Spin Eppendorf Centrifuge, (Global Science) at 8000 x g for 15 seconds. The flow through was discarded from the collection tube and the filter tube were re-assembled. 500µl of wash buffer II was added to the upper reservoir of the filter tube and the filter tube assembly was centrifuged Mini Spin Eppendorf Centrifuge, (Global Science) again at 13 000 x g for 2mins to remove any residual wash buffer. The collection tube was then discarded and the filter tube was inserted

into a 1.5ml sterile, RNase free DNA LoBind tube (Global Science). To elute RNA, 100µl elution buffer was added to the upper reservoir of the filter tube. The tube assembly was then centrifuged (Mini Spin Eppendorf Centrifuge, Global Science) again at 8000 x g for 1min. The RNA concentrations were measured using a ND-1000 Spectrophotometer (Nanodrop). The RNA samples were then frozen at -70°C for use later.

2.2.3 Reducing Formaldehyde Gels

The quality of an RNA preparation can be assessed by gel electrophoresis. Denaturing gels such as formaldehyde-agarose eliminate the effects of RNA secondary structure that can lead to aggregation and anomalous migration 1.

A key to good formaldehyde gels is to prepare a thin gel (~2-3mm). 1g of agarose (Roche) was added to 72ml DEPC-treated water. The gel solution was boiled on high using a 1000W microwave (Sanyo) until the agarose was completely dissolved. The solution was allowed to cool down to about 60°C. 10ml of 10X MOPS buffer and 18ml of 37% formaldehyde was added to the solution which was swirled gently to ensure a homogenous solution. The gel was poured into an assembled casting stand (Horizon, Gibco, BRL) in a fume hood and was allowed to set for 40mins. The gel was then put into the horizontal gel electrophoresis system (Horizon, Gibco, BRL) and 1X MOPS (900ml of DEPC-treated water added to 100ml of 10X MOPS buffer) buffer was poured in until the gel was completely

covered. A pre-electrophoresis was run for 1hr at 100V using the Mode 1000/500 power supply (Bio-Rad).

17 μ l of RNA sample was prepared containing, 2.5 μ l 10X MOPS buffer, 3.5 μ l 37% formaldehyde, 10 μ l deionized formamide and 1 μ l of (0.2mg/ml) ethidium bromide and 50 μ l of RNA sample that contains 1 μ g of RNA. The RNA sample was then incubated in a water bath (Grant) at 65°C for 10mins and immediately cooled on ice. 2 μ l of loading dye was added and 20 μ l of sample solution was run alongside 1.0 μ g of E.coli 18S + 28S Ribosomal RNA (rRNA) standard (Sigma) which was prepared exactly the same as the RNA sample. The electrophoresis was run for 1hr at 100V (Mode 1000/500 power supply, (Bio-Rad)). The gel was visualized using an UV illuminator (Life Technologies, TFX-35M) and photographed using a COHU High Performance CCD Camera.

2.2.4 cDNA Synthesis

First strand cDNA was generated by reverse transcription (RT) with the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the instructions. This kit contains the following:- 20U/ μ l Transcriptor Reverse Transcriptase, 1ml 5X Transcriptor RT Reaction Buffer, 40U/ μ l Protector RNase Inhibitor, (10mM each of dATP, dCTP, dGTP, dTTP) Deoxynucleotide Mix, 50 μ M Anchored-oligo(dT)₁₈ Primer, 600 μ M Random Hexamer Primer, 1ml PCR-grade Water, 50ng/ μ l Control RNA; total RNA fraction purified from an immortalized cell line

(K562) and (5 μ M forward and reverse primer) Porphobilinogen Deaminase (PBGD).

The kit was stored at -20°C, while the Control RNA was kept at -70°C. To minimize the risk of RNase contamination sterile, disposable thin-walled RNase- and DNase-free PCR tube (Eppendorf, Global Science) and RNase free tips (Bio-Strategy) were used during the assay. Gloves were worn at all times. All cDNA synthesis reactions were carried out in the conventional Peltier Thermal Cycler (PTC-200, Gene Works).

For each sample, 5 μ l of total RNA was reverse transcribed. To the RNA, 2 μ l (600 μ M) Random Hexamer Primer at a final concentration of 60 μ M was added and the volume was made up to 13 μ l by adding 6 μ l of PCR-grade Water. The sample was then incubated at 65°C for 10mins and then cooled immediately on ice. 4 μ l 5X Transcriptor RT Reaction Buffer, 0.5 μ l (40U/ μ l) Protector RNase Inhibitor at a final concentration of 20U, 2 μ l (10mM each of dATP, dCTP, dGTP, dTTP) Deoxynucleotide Mix at a final concentration of 1mM each and 0.5 μ l (20U/ μ l) Transcriptor Reverse Transcriptase at a final concentration of 10U was then added to the samples. The samples were mixed well by pipetting. Samples were incubated at 25°C for 10mins. They were further incubated at 55°C for 30mins. The Transcriptor Reverse Transcriptase was inactivated by heating to 85°C for 5mins. The samples were then placed on ice until further use.

2.2.5 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to amplify specific DNA sequences using the FastStart *Taq* DNA polymerase kit (Roche). This kit contains: 5U/ μ l FastStart *Taq* DNA polymerase, 10X PCR reaction buffer with 20mM MgCl₂, 10X PCR reaction buffer without 20mM MgCl₂, 25mM MgCl₂ stock solution, 5x GC-RICH solution.

The kit was stored at -20°C. Sterile, disposable thin-walled RNase- and DNase-free PCR tube (Eppendorf, Global Science) and RNase free tips (Bio-Strategy) were used during the assay. Sterile gloves were worn at all times.

PCR on the samples was performed as described in the manufacturer's protocol. PCR reactions were set up in 50 μ l volumes, consisting of 2 μ l of 10X PCR reaction buffer without 20mM MgCl₂ mixed with 3 μ l of 25mM MgCl₂ stock solution to give a final a concentration of 1.5mM MgCl₂, 1 μ l of 10mM PCR nucleotide mix (Roche), 1 μ l of reverse primers (Invitrogen), 1 μ l of forward primers (Invitrogen), 0.4 μ l *Taq* DNA polymerase (Roche) at a final concentration of 2U and 5 μ l of DNA template. 36.6 μ l of PCR-grade Water was added to the sample. The reagents were mixed well by pipetting. The PCR was carried out in the Peltier Thermal Cycler (PTC-200, Gene Works). PCR cycling conditions were specific to the housekeeping gene and the Cpn60 gene; however, they retained the same pattern of denaturation, primer annealing and extension. This pattern was repeated 35 times. Specific PCR

cycling temperatures and times are described in the Section 3.2.3 of Materials and Methods of Chapter 3, the relevant results chapters where PCR was used.

2.2.6 Non-Reducing Agarose Gels

Application of the agarose gels allows easy separation of DNA fragments into 100bp to 10,000bp range. Thin agarose gels are cast for optimal resolution (Maniatis *et al.* 1982).

1g of agarose (Roche) was added to 50ml of 1X SB buffer (100ml of 10X SB buffer mixed with 900ml of distilled H₂O). The gel solution was boiled on high using a 1000w microwave (Sanyo) until the agarose was completely dissolved. The solution was allowed to cool down to about 60°C and 2µl of (10mg/ml) ethidium bromide was added. The solution was swirled gently to ensure a homogenous solution. The gel was poured into an assembled casting stand (Horizon, Gibco, BRL) and was allowed to set for 40mins. The gel was then put into the horizontal gel electrophoresis system (Horizon, Gibco, BRL) and 1X SB buffer was poured in.

PCR products were removed from -20°C and were diluted at a 1:1 ratio using 1x SB buffer. The 6µl diluted sample was mixed with 2µl of DNA loading dye and loaded into 20µl wells. 5µl of 100kb DNA ladder (Invitrogen) was run alongside the samples to allow the determination of the DNA product size. The DNA bands were visualized using an UV illuminator (Life Technologies, TFX-35M) and photographed using a COHU High Performance CCD Camera.

2.2.7 Quantification of PCR Products by Densitometry Analysis

PCR products separated on 2% agarose gels were quantified using the Quant Analysis Mode of Image Gauge Version 4.0 Software, which measures pixel intensity of the specific area. Triplicates were used.

The mouse was used to define a specific region of the PCR product bands. The pixel density within the area of the measured region is shown as Arbitrary Unit (AU). The Background (BG) is subtracted from the Arbitrary Unit i.e., AU- BG. The AU- BG results represent quantified result of the gene expression of Cpn60 genes.

The PCR products of house keeping gene (GA3PDH) gene was also quantified so that it could be used to normalise the heat shock protein (Cpn60) gene expression as the amount of GA3PDH in cells and tissues do not change when they are subjected to changes.

The Cpn60 expressions were normalised by calculating the mean ratio, where mean AU- BG of the house keeping gene was divided by the mean AU- BG of the Cpn60 gene. The mean ratio of Cpn60/ GA3PDH against treatment was plotted onto bar graphs.

2.2.8 Statistical Analysis

The Students T-test, the most commonly used method to evaluate the differences in means between two groups; treatment and control was employed to determine the significance of the levels of Cpn60 expression when the neonatal islet cells were subjected to different treatments. Student T-test was ideal because it allowed for the uncertainty associated with estimating the variance from a small size group (<30). Variance from a small sample size is more likely to be unrepresentative of the whole population.

The following formula was employed to determine if the conditions and treatments the neonatal porcine islets cells were exposed had an effect on the levels of Cpn60 expression.

$$T = \frac{\bar{X} - \bar{Y}}{\sqrt{\frac{S_X^2}{n_1} + \frac{S_Y^2}{n_2}}}$$

Where: - S^2X = Variance of population 1

S^2Y = Variance of population 2

X = Mean of sample population 1

Y = Mean of sample population 2

n_1 = Number of values in population 1

n_2 = Number of values in population 2

The value of t depends on the degree of freedom which is calculated by taking the sum of both samples and subtracting it by two, i.e. $d.f. = n_1 + n_2 - 2$. The probability calculated was used to determine the significance. If the probability calculated was <0.05 then it would be said that the levels of Cpn60 expressed is significant in comparison to the level of Cpn60 expressed in the control samples. However, if the probability calculated was >0.05 then it would be said that the levels of Cpn60 expressed is not significant in comparison to the level of Cpn60 expressed in the control samples.

2.2.9 Protein Extraction

Total protein was extracted from the piglet islet samples for Western Blot analysis. The samples were taken out of -20°C and thawed under running water. They were re-frozen at -20°C for 2 hours and re-thawed again under running water. The cell pellets were then re-suspended in $250\mu\text{l}$ or $500\mu\text{l}$ of TENT buffer and 2mM PhMeSO₂F which was added to the solubilisation buffer prior to extraction, depending on the quantity of samples available. The cell lysate was vortexed (Grant-Bio) for 30s and incubated at 4°C for 30mins. To pellet cell debris, the lysate was centrifuged in a Mini Spin Eppendorf Centrifuge (Global Sciences) at $10,000 \times g$ at room temperature for 5mins. The supernatant was then transferred to a new 1.5ml eppendorf tube and was kept at 4°C until required.

2.2.10 Protein Estimation

Bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Illinois, USA) was used to determine an estimation of the total protein concentration. This kit contains BSA Reagent A, BCA Reagent B, 10 x 1 ml ampules containing bovine serum albumin (BSA) at 2000 μ g/ml. BSA standards (2000-0 μ g/ml) were made using PBS. 25 μ l of standards and samples were pipetted into a 96- well micro plate. 200 μ l of BCA working reagent (50 parts of Reagent A to 1 part of Reagent B) was then added to each well. Duplicate wells were run for each sample and standard. The assay was covered and incubated (Hera Cell 240, Heraeus) at 37°C. After 30mins of incubation, absorbance was measured on a plate reader (Lab System Multiskan RC) at 570 nm. Delta Soft programme generated a 4-parameter standard curve from the BSA absorbance readings and calculated the concentration of the protein samples by comparing the standard curve.

2.2.11 SDS Polyacrylamide Gel Electrophoresis

The proteins were separated using SDS-PAGE gels. SDS-PAGE gels were run in the Mini-Protean[®] 3 Cell gel apparatus (BioRad Laboratories, Hercules, California, USA) in 1X electrode buffer (60ml of 5X stock electrode buffer mixed with 240ml distilled water) at room temperature at 200V for 90 minutes. The samples were separated on discontinuous polyacrylamide gels that were 0.75mm thick using the method of (Laemmli 1970).

The 10% polyacrylamide separating gel consisted of 4.1ml of distilled water, 2.5ml of 1.5M Tris-HCL (pH 8.8), 100 μ l of 10% SDS, 3.3ml acrylamide-bis stock (30% acrylamide, 2.67% bis-acrylamide; BioRad), 50 μ l of 10% Ammonium persulphate (BioRad) and 10 μ l of TEMED (BioRad). The 4% polyacrylamide stacking gel consisted of 6.1ml of distilled water, 2.5ml of 0.5M Tris-HCL (pH 6.8), 100 μ l of 10% SDS, 1.3ml acrylamide-bis stock (30% acrylamide, 2.67% bis-acrylamide; BioRad), 50 μ l of 10% Ammonium persulphate and 10 μ l of TEMED (BioRad).

Protein samples were prepared by diluting each sample with 20 μ l of (Westermeier 1993) sample loading buffer on the basis of its total protein estimate, so that all samples were at same protein concentration.

The samples containing the (Westermeier 1993) sample buffer were heated on the multi block heater (Lab Line) for 2mins at 95 °C to denature the proteins. 1 μ l of DTT solution was added to each sample. 10 μ l of sample containing 20 μ g protein was loaded onto each lane. 4 μ l of Kaleidoscope pre-stained standard (Bio-Rad) was always loaded in the first lane of the gel to allow estimation of the molecular weights of the protein samples. 0.2 μ g of Cpn60 antigen (Stressgen) was always loaded on the second lane for identification of the position of the Cpn60 band of the protein samples.

The gels were then stained with staining solution for 15mins and de-stained with fixative solution until the color was removed from background of the gel. The gels were then scanned using ScanWizard 05 software on ScanMaker 4900 (Microtek). Gels were arranged such that each gel contained samples from the same batch.

2.2.12 Western Blot

For western blot analysis, gels were transferred to nitro-cellulose membrane (Bio-Rad) using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad). The following was performed. At the completion of electrophoresis, the gel was removed from the gel apparatus. The stacking gel was removed and discarded, and the separating gel was equilibrated in Towbin transfer buffer with SDS for 15mins.

Nitro-cellulose membrane was incubated in 100% methanol for 15mins. Four extra-thick filter papers were also wetted in the same transfer buffer until well soaked. It was ensured that nitro-cellulose membrane was approximately the same size as the gel and the filter papers were slightly bigger.

For transfer, a gel sandwich was prepared as follows: - two filter papers were placed on the bottom platinum anode. The nitrocellulose membrane was then placed on top of the filter papers. The gel was gently placed on the membrane and the remaining two filter papers were placed on top of the gel. It was ensured that air bubbles were

removed between each layer by gently applying pressure while rolling a falcon tube on top of the stack. The stainless steel cathode top was then placed on top of the gel sandwich and the gel was transferred at 15V (Power Pac 200, Bio Rad) for 1hour. Once the electrophoresis transfer was completed, the membrane was air-dried at room temperature and frozen at -20°C until it was needed for use later.

2.2.13 Western Analysis

Non-specific binding sites were blocked overnight at 4°C in 2.5g of non-fat milk powder in 50 ml 0.2% PBS/Tween solution. The next day, the blocked membrane was washed thrice in 15ml of 0.2% PBS/Tween buffer for 15minutes to ensure complete removal of milk powder. The membrane was then incubated in the primary Mouse Anti-Hsp60 Monoclonal Antibody, (Stressgen) at a concentration of 1:250. After 90mins of incubation, the membranes were washed thrice with 5min washes in 15ml 0.2% PBS/Tween buffer. The membranes were then incubated in the Goat anti-Mouse IgG secondary antibody (Stressgen) at a concentration of 1:1000 which was conjugated to alkaline phosphatase. After 1hr of incubation, the membranes were once again washed thrice thoroughly with 5min washes in 15ml 0.2% PBS/Tween buffer. The membrane was then placed in a plastic bag gently with a pair of forceps. The membrane was incubated with 3mls of 1-STEP™ NBT/BCIP (Pierce, Rockford, Illinois, USA) until the bands started to become visible. The reaction was immediately stopped by rinsing the membrane in large amounts of distilled water.

2.2.14 Edman sequencing

N-terminal sequencing, also known as Edman sequencing is the preferred technique for determination of the N-terminal, identifying a proteolysis site, sequencing of proteins blotted to PVDF and analysis of radioactive samples (Fernandez *et al.* 1992). N-terminal sequence analysis of protein samples consists of repetitive cycles of the edman chemistry after which the peptides are isolated by microbore HPLC for sequence analysis (Fernandez *et al.* 1992).

20ug of total protein was separated by 10% SDS-PAGE as described in Section 2.2.11 of Materials and Methods, alongside 4µl pre-stained kaleidoscope standard to allow estimation of the molecular weights of the protein samples and 0.2µg/ml of Cpn60 antigen (Stressgen) for identification of the position of the Cpn60 band in the protein samples.

Proteins were separated out on a 10% SDS-PAGE gel as it allows better transfer of proteins on the membrane. The acrylamide gel was soaked in transfer buffer without SDS for 15mins to eliminate excess SDS as it would interfere with the protein transfer during Western blot. The proteins were then transferred onto a 0.2µm microporous polyvinylidene fluoride (PVDF) membrane (Immobilon™-P^{SQ}, Millipore) via a semi-dry transfer cell (Trans-Blot SD, Bio-Rad) as described in Section 2.2.12 of Materials and Methods. The small pore size of the membrane ensured that protein would be trapped in the membrane.

After the proteins were transferred, the membrane was removed from the sandwich and was briefly rinsed with MQ H₂O. The membrane was then stained with coomassie G-250 solution for 60 seconds. The membrane was then destained in 50% methanol with constant staining, changing the solution until the solution remained colorless. The membrane was then rinsed with MQ H₂O and the membrane was allowed to dry at room temperature. Once dry, the bands were cut out from the membrane and placed into eppendorf tubes to be sent to the University of Otago for sequencing.

2.2.15 MALDI -TOF-TOF

Matrix assisted laser desorption/ionization time of flight/time of flight (MALDI TOF/TOF) is a practical tool for measuring the mass of a variety of proteins and other high molecular mass compounds such as carbohydrates (Liu & Schey 2004). The mass of an ion can be measured by using its velocity to determine the mass to charge ratio (m/z) by TOF mass analysis (Hillenkamp & Karas 1991).

20 μ g of total protein from neonatal porcine islet cells were electrophoresed through 12% SDS-PAGE gels. The SDS-PAGE gel was run in PROTEAN[®] II xi Cell (BioRad Laboratories, Hercules, California, USA). The samples were separated on discontinuous polyacrylamide gels using (Laemmli 1970) method.

The 12% polyacrylamide separating gel consisted of 33.5ml of distilled water, 25.0ml of 1.5M Tris-HCL (pH 8.8), 1ml of 10% SDS, 40ml acrylamide-bis stock (30% acrylamide, 2.67% bis-acrylamide; BioRad), 500 μ l of 10% Ammonium persulphate (BioRad) and 50 μ l of TEMED (BioRad). The 4% polyacrylamide stacking gel consisted of 6.1ml of distilled water, 2.5ml of 0.5M Tris-HCL (pH 6.8), 100 μ l of 10% SDS, 1.3ml acrylamide-bis stock (30% acrylamide, 2.67% bis-acrylamide; BioRad), 50 μ l of 10% Ammonium persulphate and 10 μ l of TEMED (BioRad).

1.5 liters of electrode buffer was prepared, 350ml of electrode buffer was placed in the upper chamber while the rest was poured into the lower chamber. The samples were prepared as discussed in the Section 2.2.11 of the Materials and Methods chapter. 40 μ l of sample containing 20 μ g protein was loaded onto each lane using a 100 μ l Hamilton syringe. Molecular masses of the protein bands were determined by running a 10kDa protein marker (Bio-Rad). 0.2 μ g of Cpn60 antigen (Stressgen) was loaded on alongside the protein marker for identification of the Cpn60 of the protein samples. The cooling system was utilized to ensure uniform migration across the width of the gel, which was maintained at 15°C by circulating water through the cooling chamber using a water bath (Julabo). The water temperature was maintained at 15°C using frozen slicker pads which were changed hourly. The gel was run for 4hrs 30mins at a constant current of 35mA.

Before being stained, the gels were washed twice for 30mins in a fixative solution. The gels were then stained overnight in (Candiano *et al.* 2004) colloidal coomassie G-250 stain. The next day, the gels were destained in 5% methanol for 1 hour. The gel was scanned with ScanWizard 05 software on ScanMaker 4900 (Microtek).

The gels were then placed on a glass slab, on a light box (MediaLux). The bands labeled A, B, C, D, and E shown in Section 3.3.3.6 of the Results Chapter were excised from the gel using a clean scalpel blade and placed into a 1.5ml Eppendorf Protein LoBind Tubes. The blade was cleaned with 70% Ethanol between each use. The gel pieces were washed in 100 μ l of 25mM NH_4HCO_3 for 15mins. 100 μ l of 25mM NH_4HCO_3 : Acetonitrile at (1:1 ratio) was added to the Eppendorf tubes containing the gel pieces. The tube was then agitated in a MS1 mini shaker (IKA, Global Science) at a speed of 1400/min at room temperature for an hour to de-stain the gel pieces. This step was repeated two more times, to ensure the complete removal of colloidal coomassie G-250 stain.

After the de-stain process, 50 μ l of Acetonitrile was added to the eppendorf tubes and incubated at room temperature to dehydrate the gel pieces. At this point, the gel pieces appear whitish and smaller than their original size. The solution was removed after 10mins of incubation and the gel pieces were thoroughly dried in the fume hood for 90mins. 100 μ g of Trypsin Gold (Promega) was resuspended in 100 μ l of 50mM Acetic Acid. 1 μ l of the resuspended Trypsin Gold (Promega) at 1 μ g/ μ l

was diluted in 49 μ l of 25mM NH_4HCO_3 to 0.02 μ g/ μ l. The gels were incubated in 10 μ l of trypsin solution overnight at 37°C. Trypsin, a serine protease specifically cleaves at the carboxylic site of lysine and arginine residue unless followed by proline to yield peptides of molecular weight that can be analysed by mass spectrometry.

The following day, peptides were extracted from the gel slice digests with 7 μ l of 30%ACN/0.1% TFA by sonicating (Sonica) for 10mins at room temperature. Saturated solution of α -cyano-4-hydroxy-cinnamic acid (HCCA) (Bruker Daltonics) was made in 50 μ l TA solution (0.1% TFA and $\text{H}_2\text{O}/\text{ACN}$ at 2:1 ratio) by sonicating (Sonica) for 15mins. The saturated HCCA solution was centrifuged in a Mini Spin Eppendorf Centrifuge (Global Sciences) at 10,000 x g at room temperature for 30secs. The clear solution from the HCCA solution was used in the MALDI.

1 μ l of extracted peptides was mixed with 1 μ l of saturated HCCA solution by pipetting on paraffin. The peptide and HCCA mixture was directly applied onto a polished steel target (Bruker Daltonics). The peptide and the HCCA solution were gently dried with a hair dryer at a low temperature setting. The samples were washed once by adding and then removing 5 μ l of 0.1% TFA in 10mM $\text{NH}_4\text{H}_2\text{PO}_4$ and allowed to dry again.

The mass spectrometer used in this work was an AutoFlex II™ (Bruker Daltonics) operated using the FlexControl™ (Bruker Daltonics) software. The instrument was operated with an acceleration voltage of 19kV and a reflector voltage of 20kV, Pulsed Ion Extraction of 80ns was used build to up concentration of ions in the ion source. To avoid detector saturation, masses below 500Da were suppressed using a timed ion gate. Each spectrum was produced by accumulating data from 100 consecutive laser shots. An external calibration of the instrument was performed with the peptide calibration standard mixture (Bruker Daltonics), deposited on a neighbouring target spot in the same fashion as the samples. The peptide calibration standard mixture (Angiotensin I, Angiotensin II, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39, Somatostatin 28) had a mass range between ~1000 and 4000DA. Mass Spectra were interpreted with FlexAnalysis™ (Bruker Daltonics) software. Database searches, through Mascot, using combined spectra were performed via BioTools 3.0™ (Bruker Daltonics) software.

CHAPTER THREE

Results

3.1 Chapter Summary

Transplanting pancreatic islets of the Langerhans has been proposed as a promising clinical application for the treatment of insulin independent diabetes mellitus (IDDM); however the scarcity of human cadaver of islets poses a major obstacle to the use of the islets as treatments. The use of porcine islets for transplantation is seen as a viable alternative to the chronic shortage of donor human organs.

Pigs are the preferred organisms for obtaining donor tissue as they are easy to breed under high hygienic standards and the structure of the pig insulin is similar to that of human insulin. Technical factors such as maintenance of islets in culture and the choice of collagenase for pancreatic isolation have been identified as major obstacle that need to be addressed to achieve successful transplantation using porcine islets.

The integrity of mitochondrial function is fundamental to cell life. Although the main function of mitochondria is the generation of energy (ATP), they also play significant roles in cellular metabolism such as the generation of reactive oxygen and nitrogen species, regulation of apoptosis, glucose and calcium homeostasis (Maguire *et al.* 2002). Impairment to mitochondrial functions can lead to energy deficiency, disruption of cell function and eventual cellular death (Duchen 2004).

Mitochondrial stress proteins called heat shock protein (Hsp) 60 or Cpn60 are a good indicator of mitochondrial health. The levels of Cpn60 increase when mitochondria are exposed to high levels of cellular stress (Hickman-Miller & Heildebrand 2004).

This chapter looks at the Cpn60 expression at mRNA levels and at protein levels as the markers of stress when the neonatal porcine islets are grown in different media and collagenases. The levels of Cpn60 expressed when the neonatal porcine islets are exposed to different treatments will give an indication of the mitochondrial health these islets.

3.2 Methods

Isolation and purification of neonatal porcine islet cells were performed by Living cell Technologies (LCT), Auckland using (Ricordi *et al.* 1988) method with some modification as described in Section 1.4 of Introduction and Literature Review.

3.2 (a) Islet cells grown in different serum supplemented media

The cells were then cultured into the following media conditions for 6 days:-

1. 72A1- control islet cells cultured with RPMICPN (Porcine Serum Media)
2. 72A2- islet cells cultured with 50% NTCM (Choriod Plexus Media)
3. 72B1- islet cells cultured with RPMICAN (Human Serum Media)
4. 72B2- islet cells cultured with not heated activated serum

5. 72B3- islet cells cultured with 100% NTCM (Choriod Plexus Media)

3.2 (b) Islet cells isolated using different collagenase

The islet cells were isolated and purified as described in Section 1.4 of Introduction and Literature Review. The isolated islets were kept in culture for 8 days after which they were subjected to different isolation conditions as follows:-

1. 69A- control islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS)
2. 69B1- islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS)
3. 69B2- islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS)

Islet cell samples were sent for study to University of Waikato at 4°C via an overnight courier.

3.2.1 RNA Integrity

Upon arrival, islet samples were immediately placed at 4°C. A high Pure RNA tissue kit (Roche) was used to extract total RNA from samples as described in Section 2.2.2 of Materials and Methods.

A high RNA integrity is absolutely vital for a successful PCR analysis. Due to limited sample availability, the RNA sample with the highest RNA concentration

was electrophoresed through formaldehyde gels in order to determine the integrity of the RNA sample as described in Section 2.2.3 of Materials and Methods.

3.2.2 Reverse Transcriptase

The most important factor in the synthesis of cDNA is the quality of the reverse transcriptase (Maniatis *et al.* 1982). Before the start of cDNA synthesis and PCR reactions, control cDNA synthesis and control PCR reaction were performed to confirm the quality of transcriptase.

Control RNA that was kept at -70°C and other reagents were thawed and placed on ice at room temperature. $2\mu\text{l}$ of 100ng Control RNA, $1\mu\text{l}$ (50pmol/ μl) Anchored-oligo (dT)₁₈ Primer at a final concentration of $2.5\mu\text{M}$ was added in a thin-walled RNase- and DNase- free PCR tube (Eppendorf, Global Science). To make up the final volume to $13\mu\text{l}$, $10\mu\text{l}$ of PCR-grade Water was added. $4\mu\text{l}$ of 5X Transcriptor RT Reaction Buffer, $0.5\mu\text{l}$ (40U/ μl) Protector RNase Inhibitor, $2\mu\text{l}$ (10mM each of dATP, dCTP, dGTP, dTTP) Deoxynucleotide Mix at a final concentration of 1mM each, and $0.5\mu\text{l}$ Transcriptor Reverse Transcriptase was added. The reagents were mixed well by pipetting. cDNA synthesis was carried out in a conventional Peltier Thermal Cycler (PTC-200, Gene Works) with a heated lid. The tube was then incubated for 30mins at 55°C and the Transcriptor Reverse Transcriptase was inactivated by heating to 85°C for 5mins. The tube was stored at -20°C . Control PCR reaction was performed according to the manufacturer's protocol. PCR reactions were set up in $50\mu\text{l}$ volumes, consisting of $5\mu\text{l}$ 10X concentration of

FastStart buffer with 20mM MgCl₂, 1µl of 10mM PCR Nucleotide Mix (Roche) at a final concentration of 0.2mM, 2µl of 5µM Control Primer Mix PBGD, 5µl of cDNA from Control RT reaction, 0.4µl of 5U/µl FastStart *Taq* DNA polymerase at a final concentration of 2U, and 36.6µl of PCR-grade Water. The contents were mixed well by pipetting.

The PCR was carried out in a conventional Peltier Thermal Cycler (PTC-200, Gene Works) with a heated lid. The first cycle of initial denaturation of the enzyme was run at 94°C for 5mins. The denaturation was done at 94°C for 10s, Annealing was done at 59°C for 20s and elongation was done at 72°C for 30s. This cycle was performed 35 times. The final elongation cycle was done at 72°C for 7mins. The PCR product was then placed at -20°C for long-term storage.

3.2.3 The Polymerase Chain Reaction

The first step in Polymerase Chain Reaction (PCR) is generation of single strand DNA from mRNA by cDNA synthesis. The Transcriptions First Strand cDNA Synthesis Kit (Roche) was utilized for cDNA synthesis as described in section 2.2.4 of Materials and Methods.

A 50µl PCR 'mix' was prepared using the fast start *Taq* DNA Polymerase Kit (Roche) as described in Section 2.2.5 of Materials and Methods. In a typical PCR cycle, the PCR mix is heated at 94°C for 5mins to separate the DNA strands; which then becomes the DNA template. This step is known as the initial denaturation step.

The temperature is then reduced; to the optimum annealing temperature of the primer being used to allow the oligonucleotide primers to bind to the complement DNA sequence on the template. The temperature is brought up to 72°C, the optimal temperature of the heat stable Taq DNA polymerase. DNA polymerase begins the synthesis of DNA, adding nucleotides complementary to the DNA sequence. This is known as elongation. Once DNA synthesis is completed, the temperature is raised back to 94°C to separate the DNA strands. The newly synthesized DNA becomes template for the next round of DNA synthesis. This cycle of initial denaturation, annealing, the elongation can be repeated 30 to 60 cycles. For a successful PCR, it is important to determine factors such as primer annealing temperature, the number of PCR cycles and salt concentration as these are dependent on the sequence of the DNA being amplified.

In this experiment, PCR was used to study the pattern of gene expression of housekeeping gene; glyceraldehydy-3-phosphphate dehydrogenase (GA3PDH) and heat shock protein (Cpn60) as an indicator of mitochondrial stress. Their specific denaturing and annealing temperatures and the number of PCR cycles are described in [Table 3.1] and [Table 3.2] below.

The primers used for housekeeping gene were a rat forward primer: 5'-ACCACAGTCCATGCCATCAC-3' and a rat reverse primer: 5'-TCCACCACCCTGTTGCTGTA-3', which generated a 452bp product (Zawadzka & Kamiska 2004).

	Temperature	Time	Number of Cycles
Initial denaturation	95°C	4mins	1
Denaturation	95°C	1min	34
Annealing	64°C	1min	
Elongation	72°C	1min	
Final elongation	72°C	1min	10mins

Table 3.1 Denaturing and annealing temperatures and the number of PCR cycles for the housekeeping gene; glyceraldehydy-3-phosphphate dehydrogenase (GA3PDH).

The primers used for heat shock protein (Cpn60) gene were a rat forward primer: 5'- GCGGATGCTCGAGCCTTAAT -3' and a rat reverse primer: 5'- ATGACCAAGGGCTTCCGGTG -3', which generated a 702bp product (Meinhardt *et al.* 1995).

	Temperature	Time	Number of Cycles
Initial denaturation	95°C	4mins	1
Denaturation	95°C	1min	34
Annealing	66°C	1min	
Elongation	72°C	1min	
Final elongation	72°C	1min	10mins

Table 3.2 Denaturing and annealing temperatures and the number of PCR cycles for the heat shock protein (Cpn60)

3.2.4 Western Analysis

Total protein was extracted from neonatal porcine cells in 250µl of TENT buffer as described in Section 2.2.9 of Materials and Methods. Protein concentration was calculated using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) as stated in Section 2.2.10 of Materials and Methods. Total protein (20µg) was separated by 10% SDS-PAGE as in Section 2.2.11 of Materials and Methods and transferred to 0.45µm nitrocellulose membrane as described in Section 2.2.12 of Materials and Methods. The membranes were blocked in 2.5g of non-fat milk powder in 50ml of 0.2% PBS/TWEEN solution at 4°C overnight. The membrane was then incubated with primary antibody and secondary antibody as described in Section 2.2.13 of Materials and Methods.

3.3 Results

3.3.1.1 Viability and purity of neonatal porcine islet cell clusters

Before the neonatal porcine islet cell clusters (ICCs) were sent to University of Waikato, the ICCs were subjected to viability and purity assays [Table 3.3].

Sample ID	Total IEQ	Purity DTZ	Viability AO/PI
BR69A - Day 8 Islet cells isolated with old liberase in 2% heat inactivated porcine serum (NPS)	15,143	98 %	99%
BR69B1- Day 8 Islet cells isolated with new liberase in 10% not heat activated porcine serum (NPS)	9,487	96 %	100%
BR69B2- Day 8 Islet cells isolated with new liberase in 2% not heat inactivated porcine serum (NPS).	13,691	98 %	100%
BR72A1- Day 3 Islet cells cultured in Control RPMICPN	203,570	99%	100%
BR72A2- Day 3 Islet cells cultured in RPMICPN + 50% NTCM	68,613	99%	100%
BR72B1-Day 3 Islet cells cultured in RPMICAN (Human Serum Media)	115,984	97%	100%
BR72B2- Day 3 Islet cells cultured with not heated activated serum	69,089	98%	100%
BR72B3- Day 3 Islet cells cultured with 100% NTCM (Choriod Plexus Media)	69,121	99%	100%

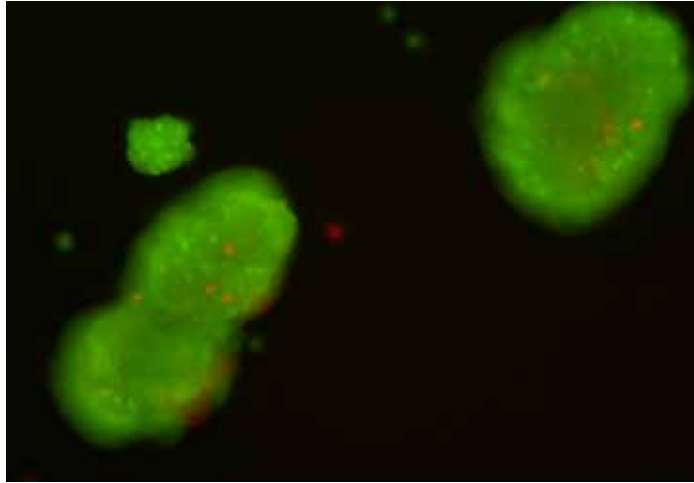
Table 3.3 Viability and purity of neonatal porcine islet cell clusters was assessed using AO/PI and DTZ at LCT, Auckland

Acridine orange and propidium iodide (AO/PI) dye was used to study the islet viability.

ICCs showed more than 99% viability [Figure 3.1 A]. Acridine orange is a fluorescent stain that readily passes through all cell membranes to stain the cytoplasm and nucleus. Bright green fluorescence in both the cytoplasm and nucleus on exposure to ultraviolet (UV) light denotes intact cells. Conversely, propidium iodide is a fluorescent stain that cannot pass through an intact membrane. Presence of propidium iodide in a cell nucleus emits a red fluorescence when exposed to ultraviolet (UV) light, indicating severe damage to the cell or even cell death.

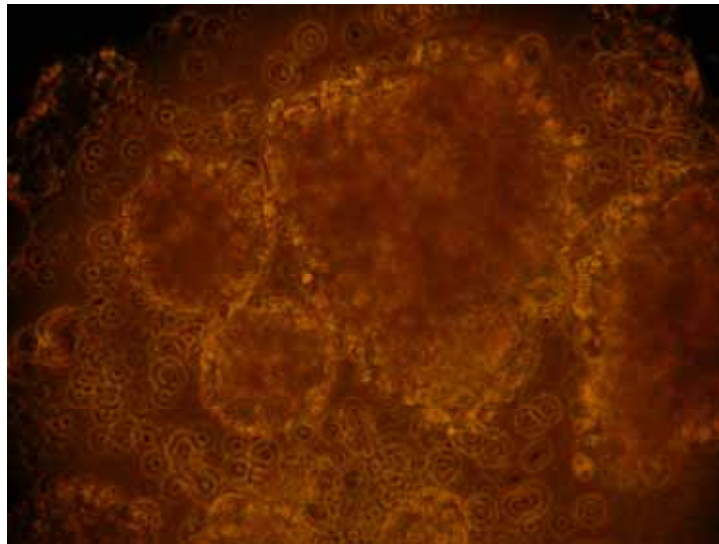
ICCs were screened for β -islet insulin producing cells by staining the cells with Dithizone (DTZ), a zinc-binding fluorescent dye [Figure 3.1 B]. Insulin is stored in pancreatic islets as a zinc–insulin complex, and stimulating the islets results in the release of insulin and zinc (Maghasia *et al.* 2003). Dithizone binds to the zinc resulting in a distinctive red appearance. More than 95% ICCs gave red appearance when DTZ bound to ICCs indicating that these clusters were indeed the β islet insulin producing cell clusters.

A



Magnification = 200X

B



Magnification = 200X

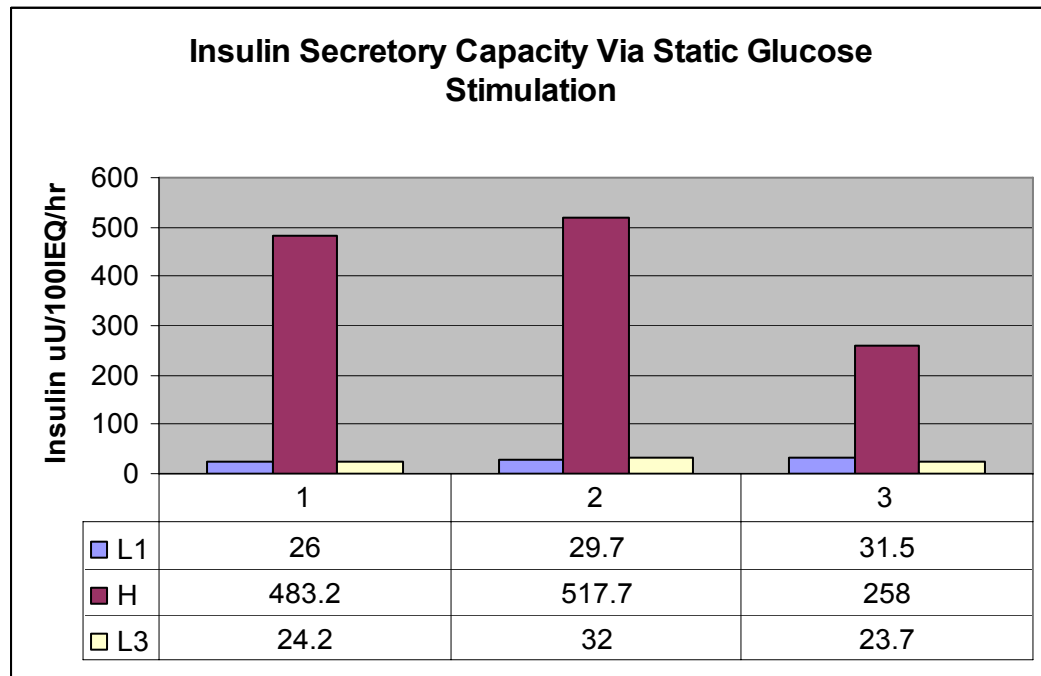
Figure 3.1 Neonatal Porcine Islet Cell Clusters stained with **A:** AO/PI and **B:** DTZ

3.3.1.2 Static Glucose stimulation (SGS) of Neonatal porcine islet cells

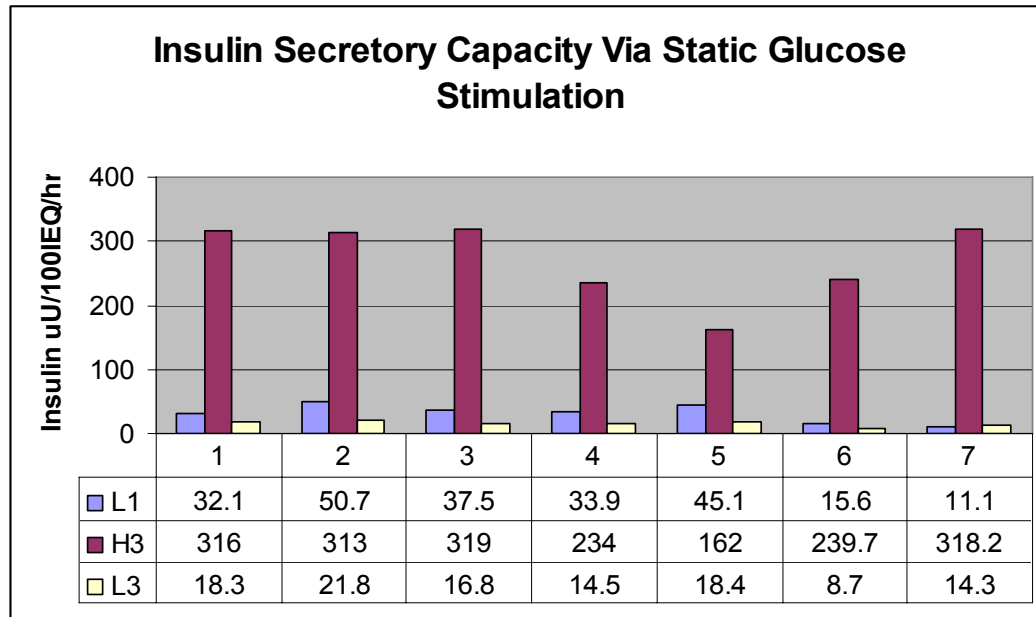
Neonatal porcine islet cell's functionality was measured through a static glucose stimulation test [Graph 3.1] and [Graph 3.2]. This assay measured the functionality of the islets in culture as a measure of their viability.

Radioimmunoassay, a technique developed by Berson and Yalow is used to measure the hormone levels, such as insulin in the blood *in vitro* (Franchimont *et al.* 1973).

The level of insulin released from the neonatal islet cells (L1) was measured before glucose was added to the culture. The islets were incubated with glucose and the levels of insulin released from the neonatal porcine islet cells (H) were again measured. The levels of insulin secreted were much higher than the first measurement. After 1 hour of incubation, a third reading of released insulin was measured and the results showed lower levels of insulin secreted indicating that the neonatal porcine islet cells were still viable.



Graph 3.1 Representative measurements of the levels of insulin released before (L1) the porcine neonatal islet cells were incubated in glucose, (H) when the neonatal islet cells were incubated in glucose and (L3) 1 hour after the neonatal islet cells were incubated in glucose. Track: **1** (69A) control, islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS), **2**: (69B1) islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS), **3**: (69B2) islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS).



Graph 3.2 Representative measurements of the levels of insulin released before (L1) the neonatal porcine islet cells were incubated in glucose, (H) when the neonatal islet cells were incubated in glucose and (L3) 1 hour after the neonatal porcine islet cells were incubated in glucose. Track **1**: (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media), **2**: (72A2) islet cells cultured with 50% NTCM (Choriod Plexus Media), **3**: (72B1) islet cells cultured with RPMICAN (Human Serum Media), **4**: (72B2)- islet cells cultured with not heated activated serum, **5**: (72B3) islet cells cultured with 100% NTCM (Choriod Plexus Media).

3.3.1.3 Quality of RNA extracted from neonatal porcine islet cells

A typical mammalian cell contains about 10^{-5} μ g of RNA, 80-85% of which is made up of ribosomal (28S, 18S and 5S subunits), whereas 10-15% is made up of a variety of low molecular species such as tRNA and small nuclear RNAs (Maniatis *et al.* 1982)

Neonatal porcine islet cells were cultured for 8 days under the following conditions:-

1. Islet cells isolated with old liberase (QC 0696/1) while in culture with 2% heat inactivated serum-media
2. Islet cells isolated with new liberase (QC 1050) while in culture with 2% not heat inactivated serum-media

In order to determine if the RNA extracted from neonatal porcine islet cells had a good preparation, total RNA from neonatal porcine islets subjected to conditions 1-2 were extracted using the High Pure RNA Tissue Kit (Roche) as described in Section 2.2.2 of Materials and Methods. RNA concentrations and purity of the samples were measured using a ND-1000 spectrophotometer (Nanodrop). [Table 3.4]

Extraction	OD 260/280 N=3 (\pmS.E.M)	Concentration ng/ul
Islet cells isolated with old liberase (QC 0696/1) while in culture with 2% heat inactivated serum-media	1.87 \pm 0.01	66.9
Islet cells isolated with new liberase (QC 1050) while in culture with 2% not heat inactivated serum-media	1.88 \pm 0.01	69.1

Table 3.4 Total RNA concentration and purity of neonatal porcine islet cells after being isolated with different collagenase (old and new liberase) were measured using the ND-1000 spectrophotometer (Nanodrop).

A good RNA preparation is determined by three factors; (a) Concentration, (b) purity and (c) integrity. A high concentration (0.3-3ug) of total RNA per mg tissue is desirable as it demonstrates a successful mRNA isolation, which has not yet been exposed to RNase and nuclease activity. The purity of RNA samples were determined by OD260/280 ratio. A highly pure RNA sample is expected to have an OD260/280 ratio between 1.8 and 2.0. Ratio lower than 1.8 may indicate presence of protein, phenol that may absorb strongly at or near 280nm.

To confirm their integrity, RNA sample was electrophoresed through a reducing formaldehyde gel. The RNA sample was run against 1.0 μ g of E.Coli 18s+28s ribosomal RNA (rRNA) standard (Roche) as described in

Section 2.2.3 of Materials and Methods. The bands were visualised using UV illuminator and were photographed using COHU High Performance CCD Camera [Figure 3.2]. The 18s and 28s bands were clearly visible against the rRNA standard. Absence of smears and multiple bands indicated that the extracted RNA was not subjected to degradation during the extraction procedure.

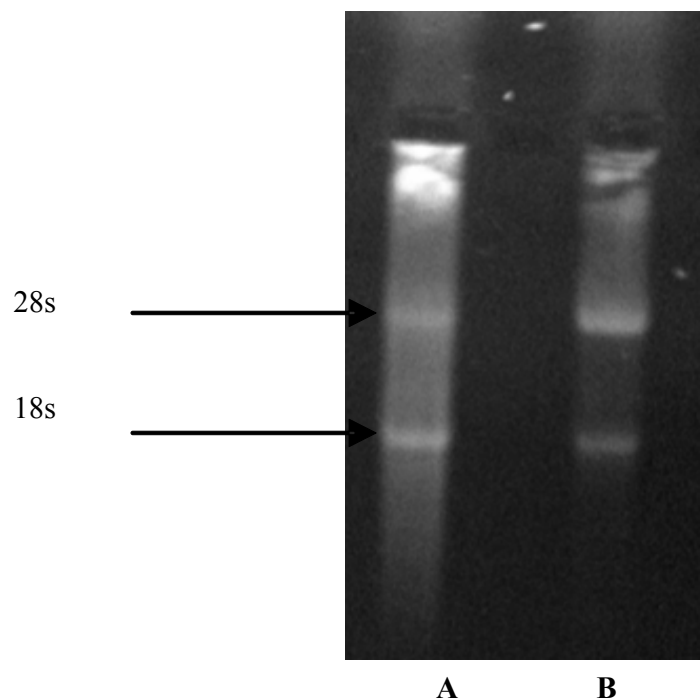


Figure 3.2 Reducing formaldehyde 2% agarose gel showing the integrity of the RNA extracted from neonatal porcine islet cells. The distinct 28s and 18s RNA bands confirm the integrity of RNA. Track **A**: 1.0µg of E.Coli 18s+28s ribosomal RNA (rRNA) standard **B**: 1.0µg RNA sample

3.3.1.4 Confirming the quality of Reverse Transcriptase

Before the start of PCR, the quality of the reverse transcriptase was confirmed by performing cDNA synthesis and PCR on control RNA (Roche) using the Fast Taq Polymerase as described in Section 3.2.2 of Chapter 3.

The PCR product generated was electrophoresied through 2% non-reducing agarose gel against a 100bp DNA ladder (Roche) as in Section 2.2.6 of Materials and Methods. The bands were visualised using UV illuminator and were photographed using COHU High Performance CCD Camera. The control RNA and primers provided in the kit (Roche) should give rise to a 151bp product. cDNA synthesis and PCR of the control RNA gave the expected 151bp fragment [**Figure 3.3**]

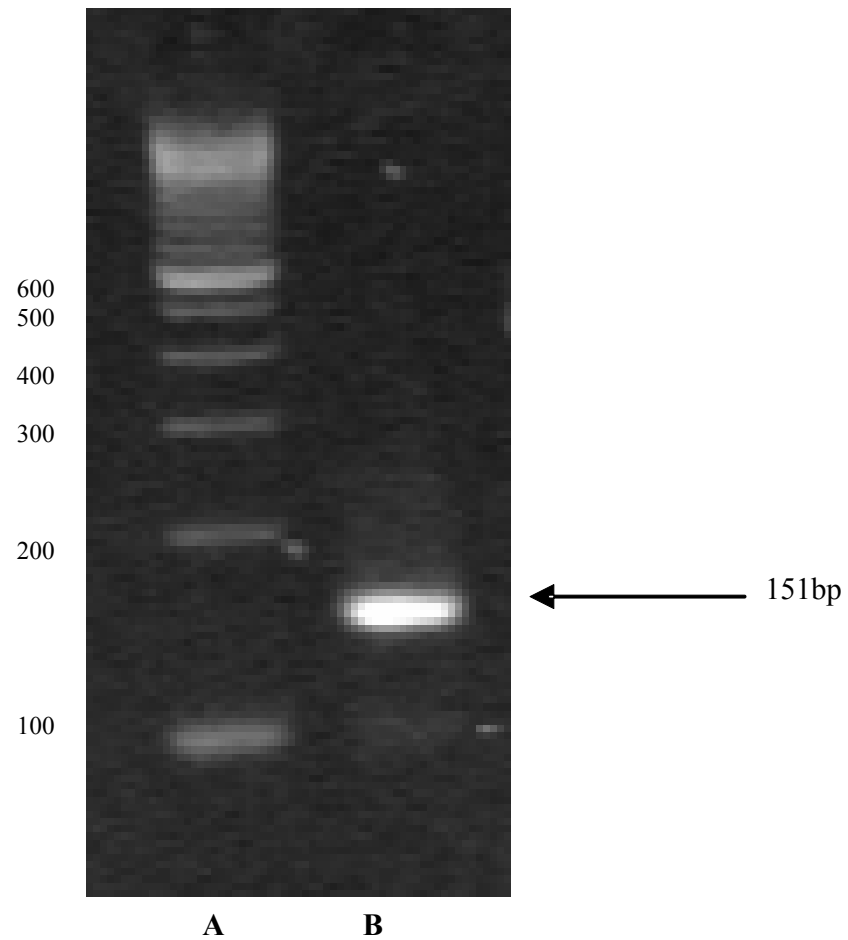


Figure 3.3 The PCR product from Control RNA was electrophoresied on 2% non-reducing agarose gels and an expected PCR product size of 151bp was generated, confirming the quality of the Reverse Transcriptase and the correct use of the cDNA and PCR synthesis protocol as described by the manufacturer (Roche). Track A: 100bp DNA ladder B: Control PCR product at 151bp.

3.3.2.1 Expression of Cpn60 from isolated neonatal porcine islet cells via PCR using rat primers

During isolation, the endotoxin free collagenase is administered intraductally into the pancreas. Collagenase begins the isolation of the pancreas by destroying the adhesion mechanism between pancreatic endocrine and exocrine tissues as well as between endocrine cells and the cellular matrix to separate the islet cells. Islet cells are then separated from the exocrine and endocrine tissue density gradient columns, which rely on the density of the islet tissue that is slightly different from exocrine tissue. Destruction of the adhesion molecules between endocrine cells and extracellular matrix may subject the islets to a cellular stress that would impair β cell function and survival.

Porcine pancreatic islets have been difficult to preserve in culture (Lacy 1984), as they disintegrate into single cells and lose insulin secretory functions under culture conditions (Heiser *et al.* 1994), therefore improving the isolation technique is important in order to increase the number of islet cells that are able to maintain their secretory functions *in vitro* (Titus *et al.* 2000).

In order to enhance the process of growth and proliferation of neonatal porcine islet cells, attention has been focused on factors such as growth factors and nutrients (Rayat *et al.* 1999). Incubating islet cells allows the viability and maintenance of the islets to be assessed until transplantation (White *et al.* 2001). Neonatal porcine islet cells can be kept in culture for up to 14 days after which its viability and functions

begin to decline. Essential nutrients, vitamins and co-factors, metabolic substrates; such as amino acids, inorganic ions, trace elements and growth factors are needed for proliferation and survival *in vitro*.

In order to culture mammalian cell in the laboratory, they require various growth factors to be present in the growth medium. The use of serum-supplemented media has become widely used. Serum has a cocktail of factors; such as growth factors, albumin, anti-proteases, transferrin and attachment factors needed for cell growth and maintenance. Different cells types have different nutrient requirement. Serums used in culture media critically depend on the characteristics of the source animals used.

The culture media must be designed in such a way that it would promote optimal growth of a particular cell type and support cellular function and synthesis of the new cells. Heat inactivation of the serum is considered necessary to destroy, bacteria, fungi, mycoplasma and viruses. However, heat inactivation could also be detrimental to the growth promoting capacity of the serum and destroy important components such as amino acids, vitamins and growth factors.

Emerging studies suggest that choroid plexus cells could be used for transplantation purposes. Choroid plexus are located within the ventricles of the brain. The most recognized function of choroid plexus is the production of cerebrospinal fluid (CSF) and the formation of the part of the structure of the blood brain CSF barrier. They

are also involved in the production of proteins such as TTR (transthyretin, (formerly prealbumin) is one of 3 thyroid hormone-binding proteins found in the blood of vertebrates (Duan *et al.* 1991)), Transferrin, ceruloplasmin, cytokines and growth factors including TGF- α , TGF- β . In their research, (Li *et al.* 2003) demonstrated choroid plexus proliferation and differentiation after being transplanted into adult rats with stroke.

In order to observe whether the growth of neonatal porcine islet cells in different serum-supplemented media had any impact on their mitochondrial health, the expression of the mitochondrial specific stress protein Cpn60 was investigated using RT-PCR.

Neonatal porcine islets were cultured in different serum supplemented media for 6 days:-

1. 72A1- control, islet cells cultured with RPMICPN (Porcine Serum Media)
2. 72A2- islet cells cultured with 50% NTCM (Choriod Plexus Media)
3. 72B1- islet cells cultured with RPMICAN (Human Serum Media)
4. 72B2- islet cells cultured with not heated activated serum
5. 72B3- islet cells cultured with 100% NTCM (Choriod Plexus Media)

Total RNA from neonatal porcine islets grown under conditions 1-5 were extracted from the neonatal porcine islet cells using the High Pure RNA Tissue Kit (Roche) as discussed in Section 2.2.2 of Materials and Methods. The RNA purity and

concentration were measured using the ND-1000 spectrophotometer (Nanodrop) [Table 3.5]. cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche).

Islets grown in different serum supplemented media	OD 260/280 N=3 (±S.E.M)	Concentration ng/ul
Control islet cells cultured with RPMICPN (Porcine Serum Media)	1.90±0.02	162.0
Islet cells cultured with 50% NTCM (Choriod Plexus Media)	2.13±0.02	150.2
Islet cells cultured with RPMICAN (Human Serum Media)	1.99±0.05	178.8
Islet cells cultured with not heated activated serum	2.05±0.01	148.0
Islet cells cultured with 100% NTCM (Choriod Plexus Media)	2.05±0.03	236.6

Table 3.5 Total RNA concentration and purity of neonatal porcine islets grown under different serum supplemented media were measured using the ND-1000 spectrophotometer (Nanodrop).

The porcine Cpn60 gene has not yet being sequenced, however, since there is a high degree of homology between Cpn60 of different species, the rat primers were used to amplify up the porcine mRNA gene. A housekeeping gene, (GA3PDH) known to have consistent levels of expression under different treatments and conditions was used to normalize the Cpn60 expression observed under the different growth conditions.

Fast Taq Polymerase Kit (Roche) was used to carry out PCR reactions as in Section 2.2.5 of Materials and Methods according to their specific cycling temperature and time as described in Section 3.2.3 of Materials and Methods.

The PCR products generated after the cDNA synthesis and PCR reactions were electrophoresied on 2% non reducing agarose gels using a 100bp DNA ladder (Roche) as a marker as described in Section 2.2.6 of Material and Methods. PCR bands were visualised using UV illuminator and were photographed using COHU High Performance CCD Camera [**Figure 3.4**].

The rat primers used should give a 702bp fragment and a 452bp fragment in the presence of cpn60 mRNA and GA3PDH mRNA respectively. The presence of expected bands at 702bp and 452bo from neonatal porcine islet cells indicated that the rat primers were indeed able to detect the porcine Cpn60. The bands were quantified using densitometry analysis as described in Section 2.2.7 of Materials and Methods [**Graph 3.1**].

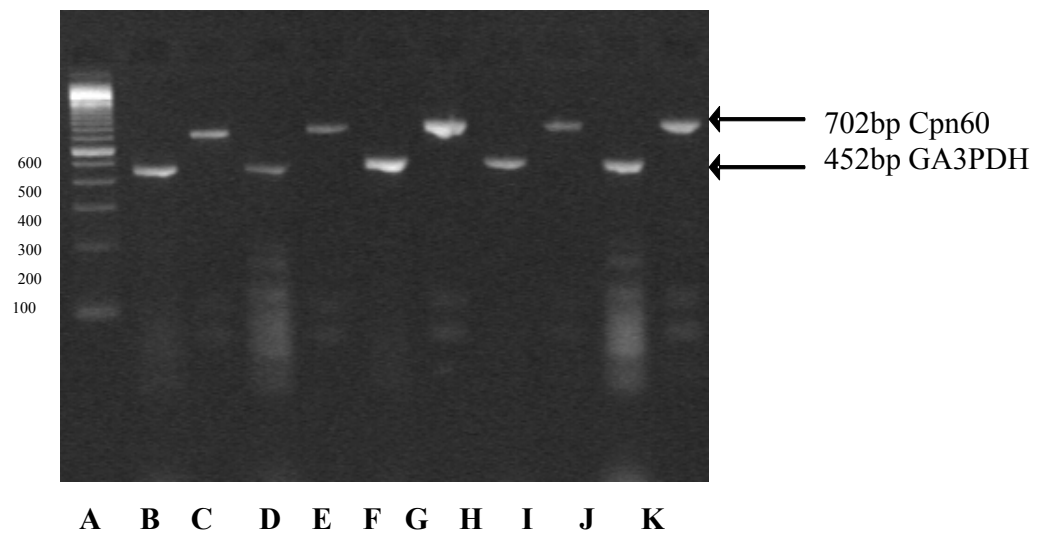
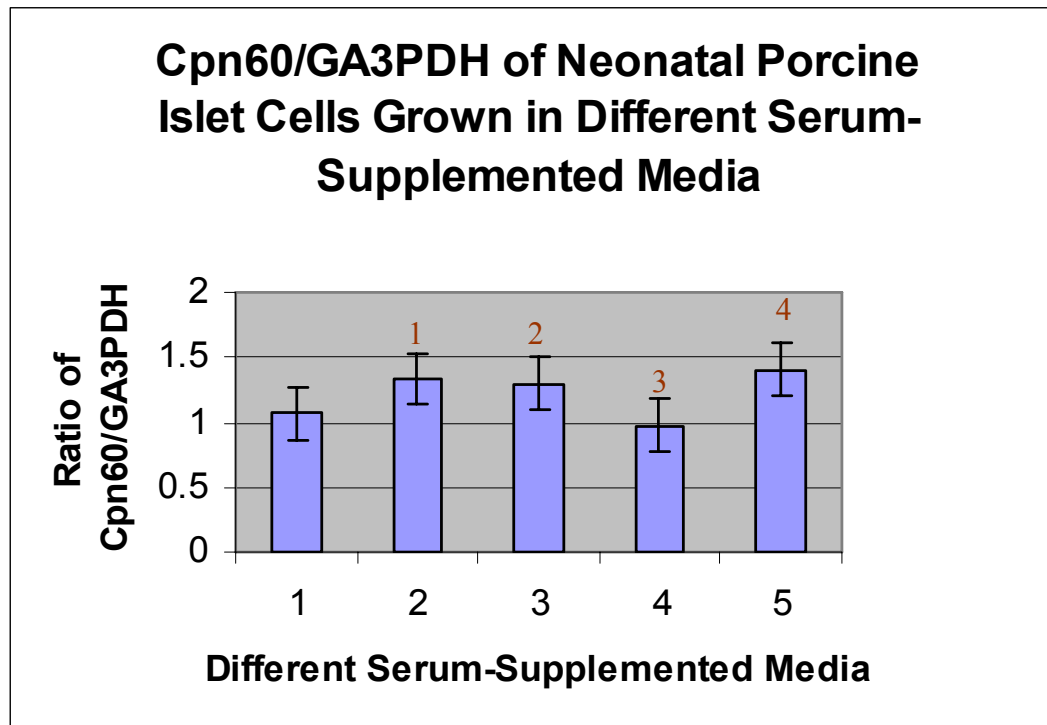


Figure 3.4 The expected PCR product size of 702bp and 452bp were generated for Cpn60 and GA3PDH respectively. Different levels of Cpn60 expression were observed in different serum supplemented media. Track **A**: 100bp ladder, **B-C**: (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media), **D-E**: (72A2) islet cells cultured with 50% NTCM (Choriod Plexus Media), **F-G**: (72B1) islet cells cultured with RPMICAN (Human Serum Media), **H-I**: (72B2)- islet cells cultured with not heated activated serum, **J-K**: (72B3) islet cells cultured with 100% NTCM (Choriod Plexus Media).



Graph 3.3 Culturing islet cells in different serum-supplemented media led to varied expression of Cpn60 levels relative to GA3PDH. Track **1**: (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media), **2**: (72A2) islet cells cultured with 50% NTCM (Choriod Plexus Media), **3**: (72B1) islet cells cultured with RPMICAN (Human Serum Media), **4**: (72B2) islet cells cultured with not heated activated serum, **5**: (72B3) islet cells cultured with 100% NTCM (Choriod Plexus Media). N=3, **1**, **3** and **4** = the levels of Cpn60 expression are significant in relation to the control (Track 1) $p < 0.05$ **2** = the levels of Cpn60 expression are insignificant in relation to the control (Track 1) $p < 0.05$

The quantification of the densitometry analysis on the resulting heat shock protein (Cpn60) PCR products relative to house keeping (GA3PDH) PCR products showed a varying degree of Cpn60 expression in islets grown in different media.

Neonatal porcine islet cells cultured 100% NTCM (Choroid Plexus Media) had the highest ratio (1.5403), which was higher than the neonatal porcine islet cells cultured with 54% NTCM (Choroid Plexus Media) and RPMICAN (human Serum Media) at ratios 1.33 and 1.3 respectively. Neonatal porcine islet cells cultured with not heat inactivated serum showed the lowest ration of 0.978 while the control; neonatal porcine islet cells cultured with RPMICPN (Porcine Serum Media) had a ratio of 1.07.

Student t-test results revealed that neonatal porcine islet cells cultured with 50% NTCM (Choroid Plexus Media) (72A2) had significant levels of Cpn60 expressed ($p < 0.05$) in relation to (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media). Neonatal porcine islet cells cultured with not heated activated serum (72B2) and neonatal porcine islet cells cultured with 100% NTCM (Choroid Plexus Media) (72B3) also showed significant levels of Cpn60 expressed ($p < 0.05$) in comparison to the neonatal porcine islet cells cultured with RPMICPN (Porcine Serum Media) (72A1). However, neonatal porcine islet cells cultured with RPMICAN (Human Serum Media) (72B1) showed insignificant levels of Cpn60 expressed ($p > 0.05$) relative to the neonatal porcine islet cells cultured with RPMICPN (Porcine Serum Media) (72A1).

The high levels of cpn60 expression observed in neonatal porcine islet cells cultured in choroid plexus media suggest that these islet cells may be under stress under these growth conditions. These media may not have the optimal concentration of growth factors required for favorable growth of islet cells. Neonatal porcine islet cells cultured with not heated activated serum were least stressed. Islet cells cultured in porcine serum media (control) showed higher levels of Cpn60 expression than islet cells cultured in not heated activated serum, suggesting that heating the serum may be detrimental to the viability of the neonatal porcine islet cells as heat would also destroy the growth factors needed for cell proliferation *in vitro*.

3.3.2.2 Investigation of the islet cell proteomes as a function of different treatment regimes.

PCR reactions performed on RNA from neonatal porcine islet cells that were grown in different serum-supplemented media showed varied levels of Cpn60 expression. Western blot analysis was carried to determine if the levels of Cpn60 expression observed at the mRNA levels were also translated to be observed in the levels of expressions at the protein level.

10,000 IEQs of neonatal porcine islet cells were solubilised in TENT buffer as described in Section 2.2.7 of Materials and Methods. The concentrations of the extracted proteins [Table 3.6] were determined by BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) as described in Section 2.2.8 of Materials and Methods.

The proteins were quantified against a 4-parameter BSA standard curve that was generated using the Delta Soft program.

Extraction	Absorbance ($\lambda=540\text{nm}$) N=3	Concentration $\mu\text{g/ml}$
islet cells cultured with RPMICPN (Porcine Serum Media)	1.309 \pm 0.01	2685.833
islet cells cultured with 50% NTCM (Choriod Plexus Media)	2.296 \pm 0.02	5532.277
islet cells cultured with RPMICAN (Human Serum Media)	0.652 \pm 0.01	1380.343
islet cells cultured with not heated activated serum	1.636 \pm 0.02	3445.44
islet cells cultured with 100% NTCM (Choriod Plexus Media)	2.305 \pm 0.02	6315.257

Table 3.6 Total protein concentration of neonatal porcine islets grown under different serum-supplemented media were measured using the BCA protein Assay kit

20 μg of total protein was separated by 10% SDS-PAGE as described in Section 2.2.11 of Materials and Methods, alongside 4 μl pre-stained kaleidoscope standard to allow estimation of the molecular weights of the protein samples and 0.2 $\mu\text{g/ml}$ of Cpn60 antigen (Stressgen) for identification of the position of the Cpn60 band in the protein samples. There was considerable variation in number and intensity of

the protein bands from islet cells subjected to different serum-supplemented media

[Figure 3.5].

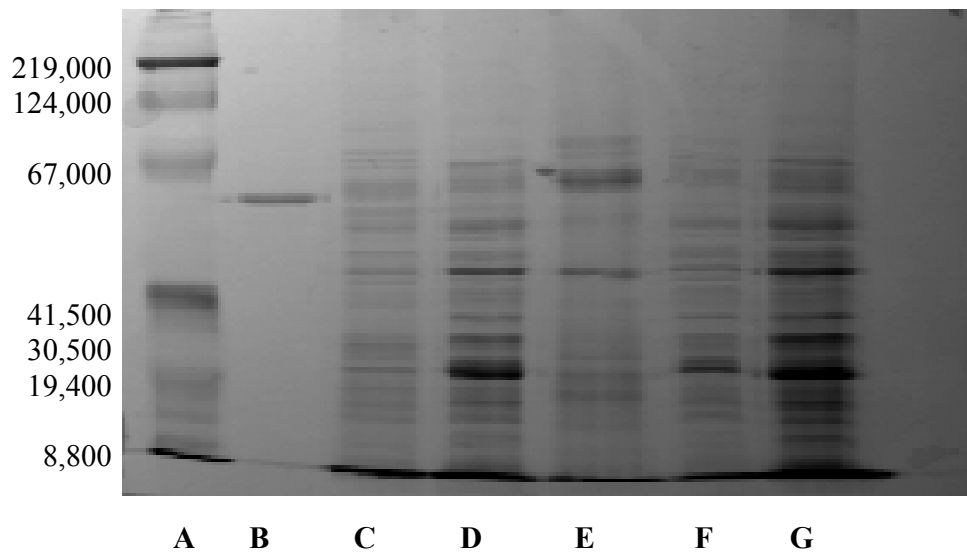


Figure 3.5 Showing a 10% SDS-PAGE of total cellular protein (20ug) expressed from neonatal porcine islet cells subjected to the different serum supplemented media. Track **A**: pre-stained kaleidoscope standard, **B**: 0.2ug/ml Cpn60 antigen, **C**: (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media), **D**: (72A2) islet cells cultured with 50% NTCM (Choriod Plexus Media), **E**: (72B1) islet cells cultured with RPMICAN (Human Serum Media), **F**: (72B2) islet cells cultured with not heated activated serum, **G**: (72B3) islet cells cultured with 100% NTCM (Choriod Plexus Media).

Islet cells cultured in (72A1) control; RPMICPN (Porcine Serum Media) had very low levels of protein band intensities with a large number of protein band clusters between 10,000 and 60,000da. Islet cells cultured in (72A2); cells 50% NTCM (Choriod Plexus Media) showed strong protein band intensities at ~ 40,000da, 48-30,000da, ~25,000da, ~22,000da, ~20,000da, ~19,000da, ~15,000da and ~10,000da. Islet cells cultured in (72B1) RPMICAN (Human Serum Media) had faint protein band intensities at 65,000kda, and 48-30,000da while Islet cells cultured in (72B2) not heated activated serum protein band clusters ~48-30,000da, ~25,000da, ~20,000da, and ~19,000da. Islet cells cultured in (72B3) islet 100% NTCM (Choriod Plexus Media) showed the strongest amount of protein bands intensity with the dominant bands at ~ 40,000da, 48-30,000da, ~25,000da, ~22,000da, ~20,000da, ~19,000da, ~15,000da and ~10,000da. Protein band at ~ 40,000da was present at the lowest levels in islet cells cultured in (72A1) control; RPMICPN (Porcine Serum Media) while it was present at the highest concentration islet cells cultured in (72B3) islet 100% NTCM (Choriod Plexus Media).

The presence of multiple bands around 60,000da molecular weight made it impossible to identify the Cpn60 band from the 10% SDS-PAGE. It was necessary to determine if one of these bands was in fact Cpn60.

The SDS-PAGE also showed protein bands that had strong band intensities indicating that there are proteins that seem to be modulated with varying concentrations when the neonatal porcine islet cells are subjected to different

conditions. The different serum-supplemented media may have an effect on the levels of expression of other proteins. Identifying these proteins would give important clues that would aid in improving media constituents which would in turn increase the rate of survival of neonatal porcine islets cells while in culture.

3.3.2.3 Investigation of the levels of Cpn60 expression at protein levels (via western blot analysis) during the growth of the neonatal porcine islets in different serum-supplemented media.

SDS-PAGE of extracted proteins from the neonatal porcine islet cells indicated that there was a band migrating at ~60,000da corresponding to the migration of the Cpn60 antigen. In order to determine if this band was indeed Cpn60, a western blot analysis was carried out.

Proteins separated out on a 10% SDS-PAGE gel were transferred onto a 0.45 μ m nitrocellulose membrane (Bio-Rad) via a semi-dry transfer cell (Trans-Blot SD, Bio-Rad) as described in Section 2.2.12 of Materials and Methods. Probing the nitrocellulose membrane with mouse anti-Cpn60 antibodies was able to pick up the Cpn60 antigen at 60,000da [**Figure3.6**] and a band from the neonatal porcine protein islet samples at ~ 55-57,000da.

In order to confirm that this band was indeed Cpn60 that was migrating differently, arrangements were made to send the protein samples to the Microchemistry Facility at the University of Otago to perform N-terminal sequencing.

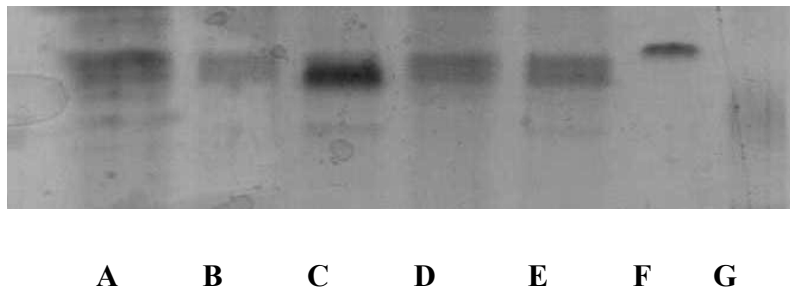


Figure 3.6 Showing a western blot analysis of total cellular protein (20ug) expressed from neonatal porcine islet cells subjected to the different serum-supplemented media. Track **A:** (72B3) islet cells cultured with 100% NTCM (Choriod Plexus Media), **B:** (72B2) islet cells cultured with not heated activated serum, **C:** (72B1) islet cells cultured with RPMICAN (Human Serum Media), **D:** (72A2) islet cells cultured with 50% NTCM (Choriod Plexus Media), **E:** (72A1) control, islet cells cultured with RPMICPN (Porcine Serum Media), **F:** 0.2ug/ml Cpn60 antigen **G:**. Pre-stained kaleidoscope standard

3.3.2.4 Identification of the unknown protein bands on the nitrocellulose membrane Via N-terminal Sequencing

Proteins samples for identification from the neonatal porcine islet cells were prepared as described in Section 2.2.14 of Materials and Methods. The prepared samples were sent out to University of Otago at room temperature for identification using N-terminal sequencing. The results from sequencing revealed that the bands appearing at ~55-57,000da was not Cpn60.

In order to confirm the findings, MALDI TOF-TOF was carried out.

3.3.2.5 Confirming that the unknown bands are indeed not cpn60 Via MALDI TOF-TOF

The purpose of this experiment was to confirm the results from N-terminal sequencing that the band at ~55-57,000da that is being picked up by western blot analysis is not a cpn60 band migrating at different molecular weight.

20µg of total protein from neonatal porcine islet cells were run out on high resolution discontinuous 12% polyacrylamide SDS-PAGE gels as described in Section 2.2.15 of Materials and Methods. After the staining and destaining process, the bands labeled A, B, C, D, and E [**Figure 3.7**] were excised from the gel.

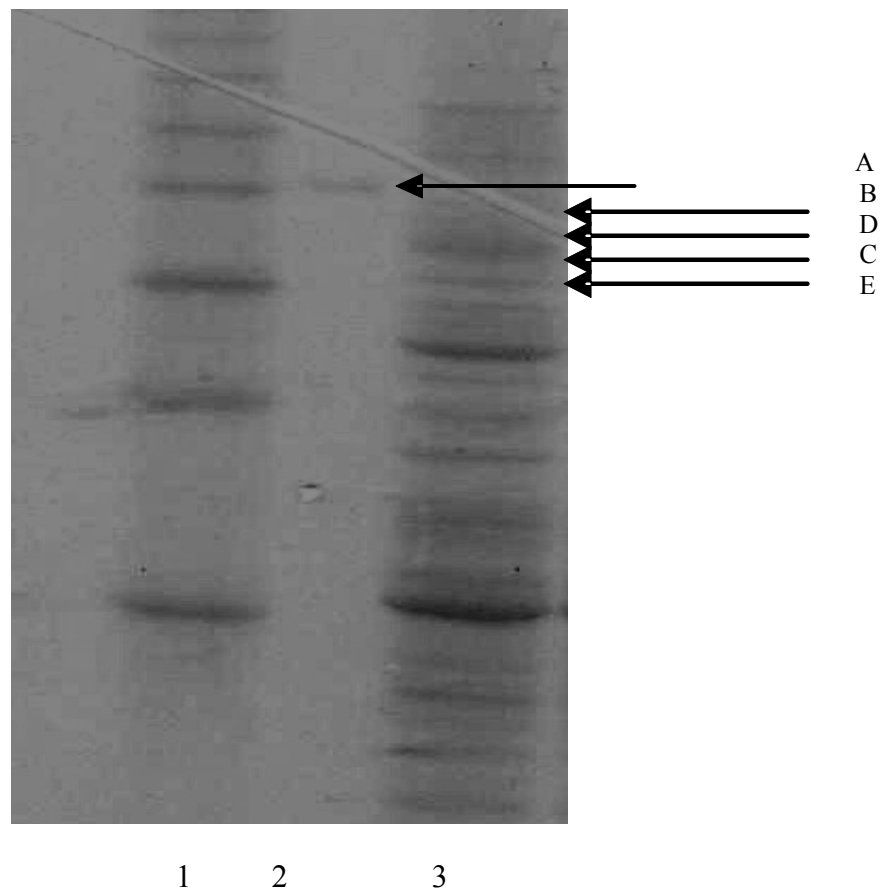


Figure 3.7 Showing a 12% SDS-PAGE of total cellular protein (20ug) expressed from neonatal porcine islet cells for MALDI TOF-TOF analysis. **A** is the Cpn60 antigen while **B**, **C**, **D**, and **E** are unknown protein bands that have a molecular of ~55-57,000da. Track **A**: Protein ladder, **B**: Cpn60 Antigen, **C**: 20ug of total cellular protein from neonatal porcine islet cells.

The gel pieces were then thoroughly washed to ensure complete removal of colloidal coomassie G-250 stain dehydrated with Acetonitrile and incubated in 10 μ l of the trypsin solution overnight at 37°C as described in Section 2.2.14 of Materials and Methods. The next day, the peptides yielded after incubation in trypsin and saturated HCCA solution were loaded onto a steel plate (Bruker Daltonics) that was loaded with α -cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) matrix thin layer as described in Section 2.2.15 of Materials and Methods. Ultraflex™ TOF/TOF (Bruker Daltonics) operated in the LIFT mode for MALDI TOF/TOF with a fully automated mode using the FlexControl™ software mass spectrometer was used to obtain mass spectra.

The resulting mass spectra were analysed with the Mascot Version 2.1.02 software (Matrix Science Ltd, London, UK). Database searches, through Mascot, using combined spectra were performed via BioTools 2.2 software (Bruker Daltonics).

In this study, unknown proteins; B, C, D and E [Figure 3.7] were identified. [Table 3.7] lists the unknown proteins with their SWISS-PROT/TREMBL, relative molecular weights, pI values, mass spectroscopical data (number of peptide matches, masses of matching peptides, peptide sequences and sequence coverage).

Protein A was positively identified as Chaperonin 60 [Rattus norvegicus] as the sample for this preparation was taken from the Cpn60 antigen.

Proteomic analysis failed to identify unknown proteins as the Cpn60 but revealed their identities. **Unknown protein B** was identified as Chain A, crystal structure of pig phosphoglucose isomerase (gi|21730305) is a glycolytic enzyme that catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate (Sun *et al.* 1996) by transfer of a carbon bound hydrogen between C1 and C2.

Chapter 3- Results

Sample Number	Accession no. NCBIInr.	N a m e	relative molecular weights (da)	pI values	Number of peptide matches	Mascot Score	Sequence coverage MS
A	gi 1778213	Chaperonin 60 [Rattus norvegicus]	60858.340	5.7	18	187	41.0%
B	gi 21730305	Chain A, Crystal structure of Pig Phosphoglucose Isomerase	63086.260	8.7	10	80.60	24.2%
C	gi 73968064	PREDICTED: similar to 78kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca)	50923.710	6.0	18	152	43.3%
D	gi 14250251	Prolyl 4 hydroxylase, β polypeptide (Mus Musculus)	57022.770	4.6	14	120	35.8%
E	gi 73968064	PREDICTED: similar to 78KDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca)	50923.710	6.0	18	181	43.3%

[Table 3.7] Showing the list of unknown proteins that were picked up via MALDI TOF-TOF.

Unknown proteins C and E was identified as GRP78, also known as immunoglobulin heavy chain binding protein (Morris *et al.* 1997), which has shown to be a molecular chaperone and Ca^{2+} binding protein. This protein is predicted similar to 78kDA glucose regulated protein precursor. Transcription of GRP78 is highly induced on response to cellular stress. Potent inducers of GRP78 Transcription include glucose starvation and oxygen deprivation (Huo *et al.* 2004).

Unknown protein D was identified as Prolyl 4 hydroxylase, β polypeptide (Mus Musculus) gi| 14250251. Prolyl 4 hydroxylase catalyses the formation of 4 hydroxyprolin in collagen by the hydroxylation of the praline residues on peptide linkages (Kivirikko *et al.* 1989)

3.3.3.1 Investigating the effect of different collagenase (old/new) in different serum on the mitochondrial health (via Cpn60 expression) of neonatal porcine islet cells

The intraductal delivery (for detailed refer to Chapter 1 of Literature Review) of collagenase is the ‘gold standard’ method for pancreatic digestion (Titus *et al.* 2000). Collagenase is used to digest islet cells from the pancreatic tissue. The choice of collagenase is critical as factors such as high endotoxin levels in the collagenase can have an impact on the immune response in host when the islet cells are eventually transplanted (White *et al.* 2001). Endotoxin may induce islet graft failure when present beyond safety levels because islets adsorb endotoxin through the LPS receptor which sets off inflammatory events at the transplantation site.

(White *et al.* 2001) have demonstrated an improvement in islet yield and secretory function when liberase, a type of collagenase that doesn't have such high endotoxin levels was used for isolation. Batch to batch variation in collagenase and deterioration of the enzyme activity of a particular batch over a period of time can also lead to inconsistent results (Titus *et al.* 2000).

3.3.3.2 Expression of Cpn60 from isolated neonatal porcine islet cells via PCR using rat primers

In this experiment, the expression of Cpn60 mRNA was investigated when porcine islets were isolated using old liberase and new liberase. High Pure RNA Tissue Kit (Roche) was used to extract total RNA from 1×10^6 neonatal porcine islet cells [Table 3.8] as described in Section 2.2.2 of Materials and Methods.

Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to perform cDNA synthesis as described in Section 2.2.4 of Materials and Methods. PCR reactions were performed using Fast Taq Polymerase Kit (Roche) in Section 2.2.5 of Materials and Methods according to their specific cycling temperature and time as described in Section 3.2.3 of Materials and Methods.

Extraction	OD 260/280 N=3 (\pmS.E.M)	Concentration ng/ul
Islet cells isolated with old liberase while in culture with 2% not heat inactivated serum-media	2.02 \pm 0.01	10.1
Islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS)	1.94 \pm 0.01	15.6
Islet cells isolated with new liberase while in culture with 2% not heat inactivated serum-media	2.12 \pm 0.01	13.3

Table 3.8 Total RNA concentration and purity of neonatal porcine islets after being isolated using different collagenase (old and new liberase) were measured using the ND-1000 spectrophotometer (Nanodrop).

The PCR products generated were separated on a 2% non reducing agarose gels against a 100bp DNA ladder (Roche) as in Section 2.2.5 of Material and Methods and visualised using UV illuminator and were photographed using a COHU High Performance CCD Camera. Variations in the levels of Cpn60 expression were observed when the islet cells were subjected to either old or new collagenase during isolation [**Figure 3.8**]. The bands were quantified using densitometry analysis as described in Section 2.2.7 of Materials and Methods [**Graph 3.4**].

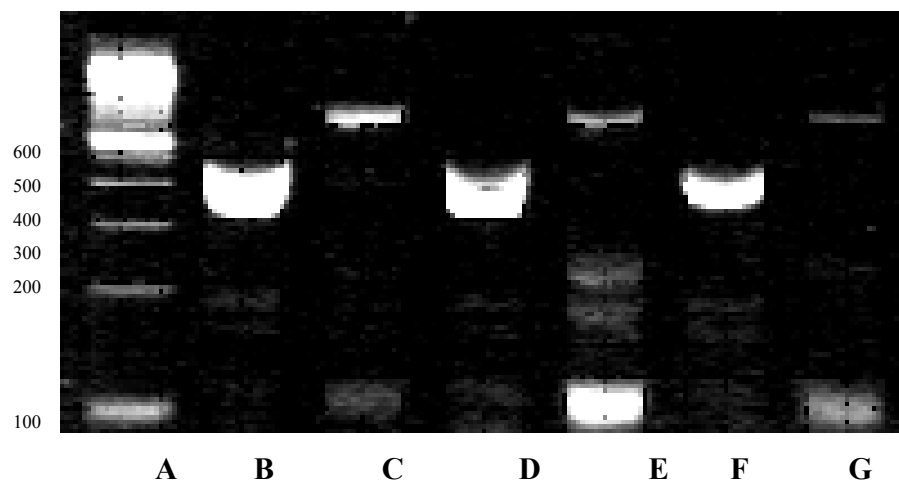
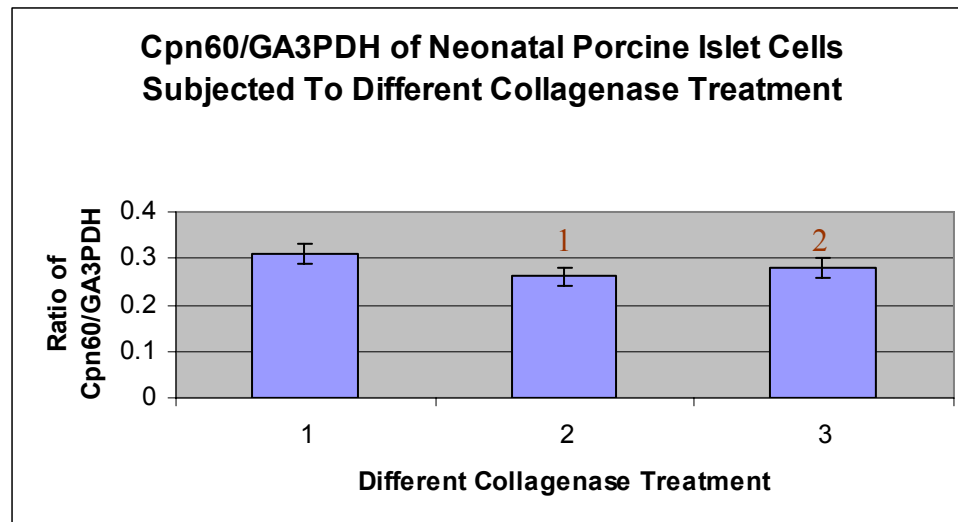


Figure 3.8 The expected PCR product size of 702bp and 452bp were generated for Cpn60 and GA3PDH respectively. Different levels of Cpn60 expression were observed in islets cells that were subjected to old and new liberase in different serum media. Track **A**: 100bp ladder, **B-C**: (69A), islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS), **D-E**: (69B1) islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS), **F-G**: (69B2) islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS).



Graph 3.4 Use of different collagenase during isolation led to varied expression of Cpn60 levels relative to GA3PDH. Track: **1:** (69A) control, islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS), **2:** (69B1) islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS), **3:** (69B2) islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS). N=3, **1**= the levels of Cpn60 expression are significant in relation to the control (Track 1) $p < 0.05$ **2** = the levels of Cpn60 expression are insignificant in relation to the control (Track 1) $p > 0.05$

The quantification of the densitometry analysis on the resulting heat shock protein (Cpn60) PCR products relative to house keeping (GA3PDH) PCR products showed a varying degree of Cpn60 expression in porcine islet cells that were subjected to isolation using old and new liberase.

Neonatal porcine islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS) had a ratio of 0.261. Neonatal porcine islet cells isolated with old liberase (QC 0696/1) while in heat inactivated porcine serum (NPS) had a ratio of 0.31 and neonatal porcine islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS) had a ratio of 0.28.

Students T-Test showed that neonatal porcine islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS) had significant levels of Cpn60 expression ($p < 0.05$) in comparison to the neonatal porcine islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS). Neonatal porcine islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS) did not show significant levels of Cpn60 expressed ($p > 0.05$) relative to neonatal porcine islet cells isolated with old liberase (QC 0696/1) while in heat inactivated porcine serum (NPS).

Liberase™, a type of collagenase, is made up of a mixture of enzymes including collagenase I and II from *clostridia hystolyticum* and non- clostridial neural

protease (Gaglia *et al.* 2005). An improved islet yield was observed when liberase was used for enzymatic low temperature porcine pancreas isolation compared with crude collagenase (Titus *et al* 2000).

Neonatal porcine islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS) showed lowest levels of Cpn60 expression in comparison to the islet cells isolated using the old liberase (QC 0696/1). This could be because the liberase's enzymatic action and pH becomes inconsistent as it ages causing it to have an effect on pancreatic isolation.

Islet cells isolated using new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS) showed a lower levels of Cpn60 expression in comparison to islet cells isolated with new liberase (QC 1050) while in 2% not heat activated porcine serum (NPS). The results from the experiments seem to be consistent with the results of the previous experiments which demonstrated that the method used for the inactivation of complements present in the serum may influence the viability of islets while in culture. Heat inactivation of serum would not only destroy the complement proteins but could also destroy components such amino acids, vitamins and growth factors as they are subjected to degradation at high temperatures.

3.3.3.3 Investigating the level of Cpn60 expressed via Western Blot Analysis on islets that are subjected to isolation using different collagenase treatment.

PCR performed on RNA from neonatal porcine islet cells were subjected to different collagenase showed varied levels of Cpn60 expression. Western blot analysis was carried to determine if these neonatal porcine islet cells would express Cpn60 at protein levels.

10,000 IEQs of neonatal porcine islet cells were solubilised in TENT buffer as described in Section 2.2.7 of Materials and Methods. The concentrations of the extracted proteins [Table 3.9] were determined by BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) as described in Section 2.2.8 of Materials and Methods. The proteins were quantified against a 4-parameter BSA standard curve that was generated using the Delta Soft program.

Extraction	Absorbance N=3 (± S.E.M)	Concentration µg/ml
islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS)	0.542±0.01	496.923
islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS)	0.596±0.01	543.036
islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS)	0.626±0.01	568.427

Table 3.9 Total protein concentration of neonatal porcine islets after being isolated using different collagenase (old and new liberase) was determined using the BCA protein Assay kit

20µg of total protein was separated by 10% SDS-PAGE as described in Section 2.2.11 of Materials and Methods, alongside pre-stained kaleidoscope standard to allow estimation of the molecular weights of the protein samples and 0.2µg/ml of Cpn60 antigen (Stressgen) for identification of the position of the Cpn60 band in the protein samples. There was considerable variation in number and intensity of the protein bands from islets that were subjected to isolation using old and new collagenase [**Figure 3.9**].

Islet cells (69A) isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS) and (69B1) islet cells isolated with new liberase (QC 1050) while in 10% not heat inactivated porcine serum (NPS) had similar levels of protein expressions. This is evident by the similar intensity of cluster of protein bands. The dominant protein bands of these two treatments occur at ~67,000da, 62,000da, ~55-57,000da, 52,000da and ~40,000da. (69B2) islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS) show increased levels of protein expression. This is apparent by increased protein intensity. Intense protein bands were observed at ~67,000da, 62,000da, ~55-57,000da, while similar protein band intensities were observed at ~40,000da. Another protein expression is seeing at ~30,000da which is not shown in the two treatments.

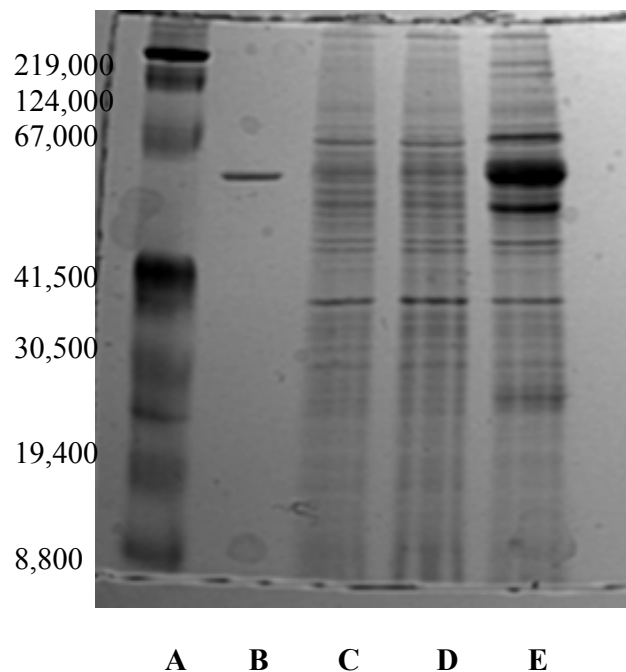


Figure 3.9 20ug of total protein separated on a 10% SDS-PAGE via electrophoresis shows the different levels of protein expressed when the islet cells are subjected to different liberase action. Track **A**: pre-stained kaleidoscope standard, **B**: 0.2ug/ml Cpn60 antigen, **C**: (69A) control, islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS), **D**: (69B1) islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS), **E**: (69B2) islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS). SDS-PAGE of extracted proteins of the islet cells being subjected to different collagenase indicate that there are bands at ~60kda present alongside the Cpn60 antigen.

Collagenase, like serum has an effect on the levels of protein intensities of protein bands being expressed. Identification of these unknown proteins is important to determine if technical factors such as the type of collagenase used during isolation and the sera conditions in which islets were cultured in before transplanted have adverse effects on the viability and functionality of the islet cells.

10% SDS-PAGE gels were transferred onto a 0.45 μ m nitrocellulose membrane (Bio-Rad) via a semi-dry transfer cell (Trans-Blot SD, Bio-Rad) as described in Section 2.2.12 of Materials and Methods. The mouse anti-Cpn60 antibodies were able to pick up a band that was at the same position as the Cpn60 antigen on the SDS-PAGE [Figure 3.10] indicating that the band on the nitrocellulose membrane was indeed the Cpn60 antigen. However, the band from the porcine protein islet samples were at a different position to the Cpn60 antigen of \sim 55-57,000da.

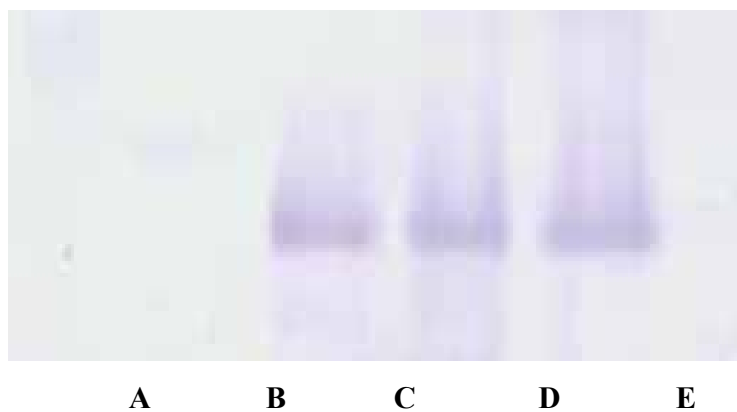


Figure 3.10 Showing a western blot analysis of total cellular protein (20ug) expressed from islet cells subjected to the different liberase action. Track **A**: pre-stained kaleidoscope standard, **B**: 0.2ug/ml Cpn60 antigen, **C**: (69A) control, islet cells isolated with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS), **D**: (69B1) islet cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS), **E**: (69B2) islet cells isolated with new liberase (QC 1050) while in 10% not heat activated porcine serum (NPS).

These results confirmed that the method of inactivation whether by heat or not plays a role in the expression levels. The results also showed that old liberase was the preferable collagenase as BR70A showed lower levels of Cpn60 expression when old liberase was used in culture media that had 2% heat inactivated NPS.

CHAPTER FOUR

Discussion

4.1 The effect of culturing neonatal porcine islet cells in different serum supplemented media on mitochondrial stress at mRNA levels

The choice of serum in media is critical as it provides the cells with all the essential nutrients and growth factors to support its growth and development. Islet culture aims to optimize the islet survival (Trivedi *et al.* 2005) until the neonatal porcine beta islet cells are capable of secreting significant quantities of insulin in response to an *in vitro* glucose challenge (Juang *et al.* 2004).

According to [Graph 3.3] of Section 3.3.2.1 of Results, varying levels of Cpn60 mRNA was expressed when the neonatal porcine islet cells were cultured in different serum supplemented media. The best choice of media to culture neonatal porcine islet cells was found to be not heated activated serum which showed the least levels of Cpn60 expression at mRNA levels suggesting that the cells had low levels of mitochondrial stress. On the other hand, the worst choice of media to culture neonatal porcine islet cells was found to be 50% NTCM (Choriod Plexus Media) as culturing islets cells in this media gave the highest levels of Cpn60 expression, suggesting high levels of mitochondrial stress. The findings of this experiment contradict with the preliminary results of LCT (data not shown); who have found that choroid plexus conditioned media support and enhance the survival of neuronal cells *in vitro*. Another research performed by the same group demonstrated that conditioned media obtained from rat choroid

plexus protected primary cortical neurons against death from serum deprivation (Borlongan *et al.* 2004).

Studies done by (Miao *et al.* 2006) demonstrated comparable results with LCT, Auckland in that media containing neurotrophic factors improve the survival of cells *in vitro*. Nerve growth factors (NGF) have been shown to play an important regulatory role in pancreatic beta-cell function. Murine islets cultured with NGF displayed improved viability and survival, had more insulin secretion than islets cultured without NGF in response to 2.8 mmol/L glucose ($P < 0.05$), and 20 mmol/L glucose conditions. 67% of recipients with a submarginal number of islets cultured in NGF attained normoglycemia for more than 120 days, whereas transplanted islets without NGF treatment survived a maximum of 13 days in control mice and at post transplant day 4, recipients of NGF-cultured islets showed significant improvement of glucose tolerance.

Choroid plexus have been shown to secrete potent neuroprotective factors such as TTR, transferrin, ceruloplasmin, cytokines and growth factors such as; TGF- α , TGF- β , basic fibroblast growth factor, glial cell line derived neurotrophic factor, neurotrophin (NT)-3, NT-4, tumor necrosis factor and insulin-like growth factor *in vitro*. Culturing neonatal porcine beta islet cells in such rich media may enhance the mitochondrial metabolic rate which could lead to an over production of ROS and hydrogen peroxide; a natural by-product of oxidative phosphorylation placing the islet cells under oxidative stress. Elevated ROS levels could affect the function and survival of β cells through direct oxidization

of cellular macromolecules such as DNA and lipids and activation of cellular stress-sensitive signalling pathways (Evans *et al.* 2003).

4.1.1 N-Terminal Sequencing

During the N-terminal sequence analysis of a protein, the polypeptide is degraded stepwise by using di-methyl-aminoazobenzene isothiocyanate/phenyl isothiocyanate double coupling method, and the released dimethylaminoazobenzene thiohydantoin of amino acids were identified by reversed-phase high-pressure liquid chromatography (Chang 1981). Repeating this process gives a protein sequence.

Edman sequencing requires a free amino group at the N-terminal of the protein. However, it may not always be easy to identify what is blocking the N-terminus as a protein could be blocked by pyroglutamic acid, acetyl group, formyl group or modified naturally. Majority of soluble proteins have blocked N-termini (Brown & Roberts 1976). Having a blocked N-terminal will not yield edman sequencing data. If the protein has been N-terminally blocked by pyroglutamic acid, then treating the blocked pyroglutamate residue with pyroglutamate aminopeptidase will unblock the N-terminus (Podell & Abraham 1978).

Many proteins acquire blocked N-termini by acetylation (Yet *et al.* 1988). Even though several enzymatic and chemical procedures have been reported for the removal of the acetyl-group from the N-terminus, alcoholic deacetylation is preferred (Gheorghe *et al.* 1997). Gheorghe *et al.* (1997) in their study found that incubating N-terminally blocked proteins with trifluoroacetic acid (TFA) in

methanol (1:1) at an elevated temperature (~47°C) for 2–3 days would give efficient deacetylation. The deacetylation yield of peptides can then be determined through capillary electrophoresis.

Internal sequencing can also be used to unblocked N-terminally blocked proteins. In their study, (Deng *et al.* 1997) used internal sequencing to find the amino acid of a new type of fish antifreeze protein. Briefly, the N-terminally blocked protein was subjected to proteolytic digestion. The fragments generated were then separated by reverse-phased HPLC. Molecular mass measurements of peptides and the protein were determined by MALDI-TOF.

4.1.2 MALDI-TOF TOF

Mass spectrometry has become the primary tool for the analysis of protein primary structure. MALDI TOF TOF can provide structural information for proteins as large as 12kDa with sufficient sensitivity for protein identification and analysis of post translational modifications (Liu & Schey 2004).

MALDI TOF TOF on the protein samples were carried out as alternative in an attempt to identify the Cpn60 band. Even though, identification of Cpn60 was unsuccessful other proteins (b, c, d and e) were identified.

The protein bands separated out on the 12% SDS-PAGE gels that was electrophoresed in a PROTEAN[®] II xi Cell (BioRad Laboratories, Hercules, California, USA) ([**Figure 3.7**] of Section 3.3.2.5 of Results) showed very poor resolution. There was a large number of proteins, most of them were appearing

as faint protein bands on the SDS-PAGE gels were migrating in a cluster at ~ 50,000- 60,000da.

The use of 2D electrophoresis would give higher resolution that would overcome the poor separation of protein bands during an electrophoresis. 2D gel electrophoresis is a routine technique used in proteomics. The proteins are separated according by SDS PAGE and isoelectric focusing. The SDS PAGE separates the protein according to their molecular weight while the isoelectric focusing separates the protein by their charge (pI) (Gorge *et al.* 2000).

4.2 The effect of digesting neonatal porcine islet cells with different liberase (old/new) on mitochondrial stress at mRNA levels

Porcine-specific difficulties of islet isolation are attributed to the intrinsic fragility of islets during pancreas digestion (Brandhorst *et al.* 1999). Since very little peri-insular capsule is present, especially in pigs, and most peripheral islet cells are in direct contact with exocrine cells, islet cells are severely damaged during the digestion and purification processes (Sato *et al.* 2002).

Liberase, a highly purified blend of collagenase has been specifically developed to eliminate the numerous problems associated with the conventional use of crude collagenase when isolating islet-like cell clusters (ICCs) from pancreases of different species (Georges *et al.* 2002).

According to [**Graph 3.4**] of Section 3.3.3.1 of Results, varying levels of Cpn60 was expressed when the neonatal porcine islet cells were isolated using different

types of collagenase, i.e. old and new liberase. From the results, neonatal porcine islet cells would be best isolated in cells isolated with new liberase (QC 1050) while in 2% not heat inactivated porcine serum (NPS) as this gave the lowest levels of Cpn60 expression suggesting low levels of mitochondrial stress, whereas, digestion of neonatal porcine islets with old liberase (QC 0696/1) while in 2% heat inactivated porcine serum (NPS) would be the worst choice as this condition had the highest levels of Cpn60 expressed indicating high levels of mitochondrial stress. The results of this experiments would be influenced by (i) the type of liberase used, i.e. old or new liberase and (ii) the type of serum i.e. heat activated or heat inactivated serum.

4.2.1 Choice of Liberase

Liberase, a highly purified blend of enzymes has been developed to improve the quality and reproducibility of tissue dissociation (Lakey *et al.* 1998). A good enzyme will release intact islets that are entirely free of exocrine tissue (cleaved). Unfortunately, batch-to-batch variation and deterioration of a good batch with storage are a common experience.

The specific collagenase activity of enzyme decreases with time. Liberase has been found to lose its islet isolating efficacy with increased duration of storage (Linetsky *et al.* 1997). Isolating islets using liberase that has altered specific collagenase activity will result in porcine islet recovery loss. Cavanagh *et al.* (1997) demonstrated that the islet recovery was greatly impaired when the islet cells were isolated with collagenase stored for >16months after lot production.

The islet cells that were isolated using new liberase showed lower Cpn60 expression in comparison to the islet cells that were isolated using old liberase; aliquots of liberase were kept frozen for 1 year, in comparison to the islet cells isolated with new liberase; which was kept for 1 month. The high levels of Cpn60 expressed when the islets were isolated using the old liberase would have been due to the impaired enzymatic activity of the enzyme. The porcine pancreas would have been subjected to a harsher digestion, resulting in islet cells that are a damaged severely.

4.2.2 Choice of Serum

Serum is routinely added to culture medium for a wide range of cells and tissues. This is because serum contains many components which are beneficial for cell survival and growth. Serum is also known to have contaminants like viruses, mycoplasma and bacteria. Heat inactivation of serum has always been a routine step in a cell culture lab to destroy complements and heat labile proteins. Routine culturing of porcine islet cells involve the use heat-inactivated serum. This study indicates that cells growing in the presence of non-heat inactivated serum have a lower level of mitochondrial impairment (i.e. indicated by levels of cpn60 mRNA expression). This suggested that non-heat inactivated serum be considered for future culture of β islet cells.

In a study, (Imoedemhe *et al.* 1994) found that oocytes handled in serum-supplemented medium that had not been heat-treated was significantly better. The proportion of pronucleate oocytes that cleaved was also significantly better in the non-heat-treated serum group. Their results demonstrated that the absence

of heat treatment of serum used to supplement culture medium has no adverse effect on the fertilization rate and short-term embryo development in vitro. In another study, (Otonkoski *et al.* 1999) studied the effect of serum free media on porcine foetal islet cell survival in comparison to the survival of islet cells in serum supplemented media. With the analysis on DNA content of the islet cells, cell survival in serum-free was identical with serum-supplemented medium. However, the insulin content was about twice as high in the serum-free as compared with serum-containing medium, both with and without nicotinamide.

Difficulties with recovering and preserving islets have hampered progress in islet transplantation. Further research needs to be done to compose a defined media that has eliminated components that are toxic to pancreatic beta cells while in culture and at the same time is enriched with protective agents that would promote islet survival and proliferation.

To investigate if cells cultured in choroid plexus supplemented media were in fact causing mitochondrial ROS generation due to hyperstimulation of mitochondrial functions, the levels of ROS could be measured by using hydroethidine (HE) and dihydrorhodamine 123 (DHR) (Molecular Probes) as described by (Wu *et al.* 2004).

To determine if the higher levels of Cpn60 expression had any effect on the proliferative capacity of the neonatal porcine islet cells, 5-bromo-2'-deoxyuridine (BrdU) double staining could be used to study the proliferation of β islet cells. BrdU is incorporated into the cellular DNA (during S phase) in place of

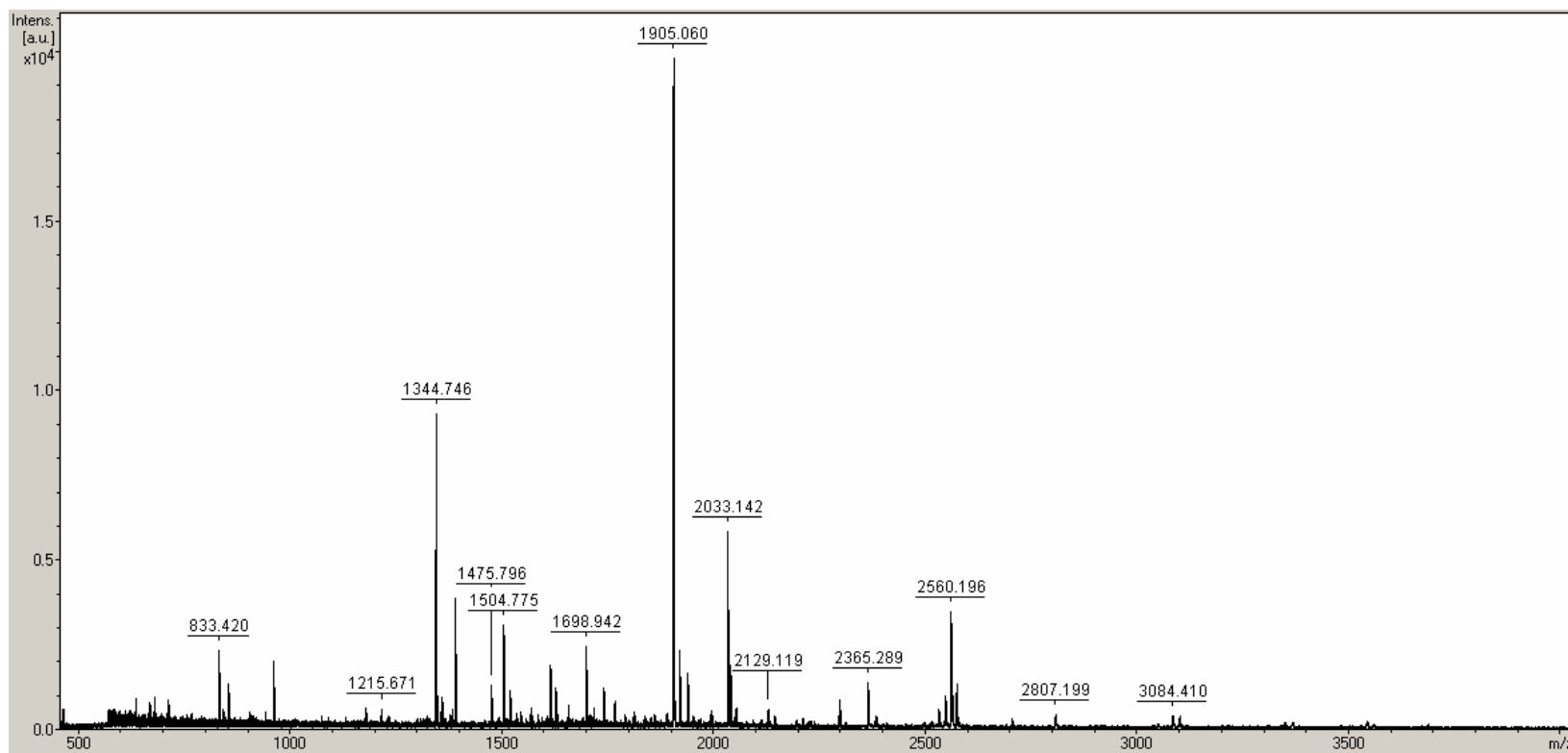
thymidine. Cells that have incorporated BrdU into their DNA are easily detected using a monoclonal anti-body against BrdU. The binding of the antibody is achieved by denaturation of the DNA. This is usually obtained by exposing the cells to acid, or heat (Weisgerber *et al.* 1993).

This investigation has failed to identify the porcine Cpn60 at protein level using Cpn60 specific antibodies (western blots). This was surprising as the antibody used was known to be able to recognise the porcine Cpn60 protein. N-terminal sequencing and mass spectra analysis of bands separated by SDS-PAGE also failed to identify this protein.

Since Cpn60 is known to be induced at protein level by heat stress (Martinus *et al.* 1996), subjecting neonatal porcine islet cells to heat stress (such as growth at 42°C for 6-12 hours) should be undertaken in any future studies as a positive control for identifying the porcine Cpn60 protein by SDS-PAGE and Western Blot analysis.

The use of 2D gel electrophoresis instead of 1D gel electrophoresis would give higher resolution as mentioned earlier. The samples could then be analysed using MALDI TOF TOF. The possibility that N-terminal sequencing failed to show Cpn60 was due to N-terminal blocking during experimental treatment. To overcome this problem, the N-terminal end could be de-blocked by either treating the blocked N-terminus residue with pyroglutamate aminopeptidase or using enzymatic and chemical procedures to remove the acyl-group from the N-terminus as mentioned earlier.

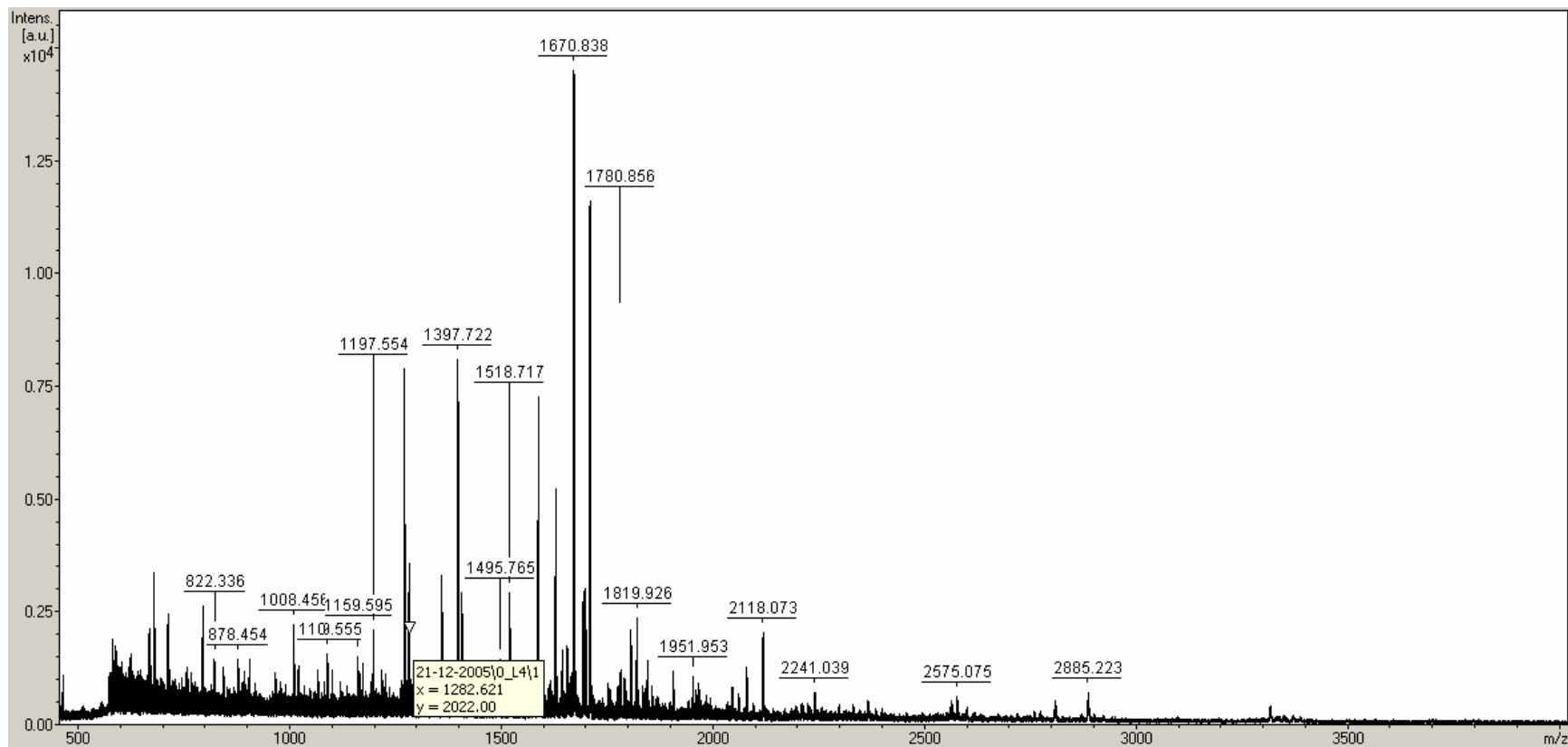
It should be noted that although expression of Cpn60 at mRNA levels seems to be modulated during the growth of the porcine islet cells in media supplemented with different serum, heat treatment of serum and liberase content, no firm conclusion can be made with regard to the effect of the different treatments on mitochondrial health status until the porcine Cpn60 protein can be unequivocally identified.

APPENDIX**A: - MALDI TOF TOF**

Mass Spectra of Chaperonin 60 [Rattus norvegicus] gi|1778213; Sample Number A

Protein: chaperonin 60 [Rattus norvegicus] gi 1778213										Peak threshold: 0.0	
Intensity coverage: 72.8 % (51187 cnts)		Sequence coverage MS: 41.0 %		Sequence coverage MS/MS: 0.0 %		pI: 5.7		MW (kDa): 60.9			
10	20	30	40	50	60	70	80	90			
MLRLPTVLRQ	MRPVSRALAP	HLTRAYAKDV	KFGADARALM	LOGVDLLADA	VAVTMGPKGR	TVIIEQSWGS	PKVTKDGVTV	AKSIDLKDKY			
100	110	120	130	140	150	160	170	180			
KNIGAKLVQD	VANN ^T NEEAG	DGTTTATVLA	PSIAKEGF EK	ISKGANPVEI	RRGVMLAVDA	VIAELKKQSK	PVTTPEEIAQ	VATISANGDK			
190	200	210	220	230	240	250	260	270			
DIGNIISDAM	KKVGRKGVIT	VKDGKTLNDE	LEIIEGMKFD	RGYISPYFIN	TSKGQKCEFQ	DAYVLLSEK	ISSVQSIVPA	LEIANHRKP			
280	290	300	310	320	330	340	350	360			
LVIIAEDVDG	EALSTLVLR	LKVGLQVVAV	KAPGFGDNRK	NQLKDMAIAT	GGAVFGEEGL	NLNLEDVQAH	DLGKVGIVIV	TKDDAMLLKG			
370	380	390	400	410	420	430	440	450			
KGDKAHIEKR	IQEITEQLDI	TTSEYEKEKL	NERLAKLSDG	VAVLKVGGTS	DVEVNEKKDR	VTDALN ^A TRA	AVEEGIVLGG	GCALLRCIPA			
460	470	480	490	500	510	520	530	540			
LDSLKPANED	QKIGIEIIKR	ALKIPAMTIA	KNAGVEGSLI	VEKILQSSSE	VGVDAMLGDF	VNMVEKGIID	PTKVVRTALL	DAAGVASLLT			
550	560	570	580								
TAEAVVTEIP	KEEKDPGMGA	MGMGGGGMGG	GMF								

Sequence of Chaperonin 60 [Rattus norvegicus] gi|1778213; Sample Number A

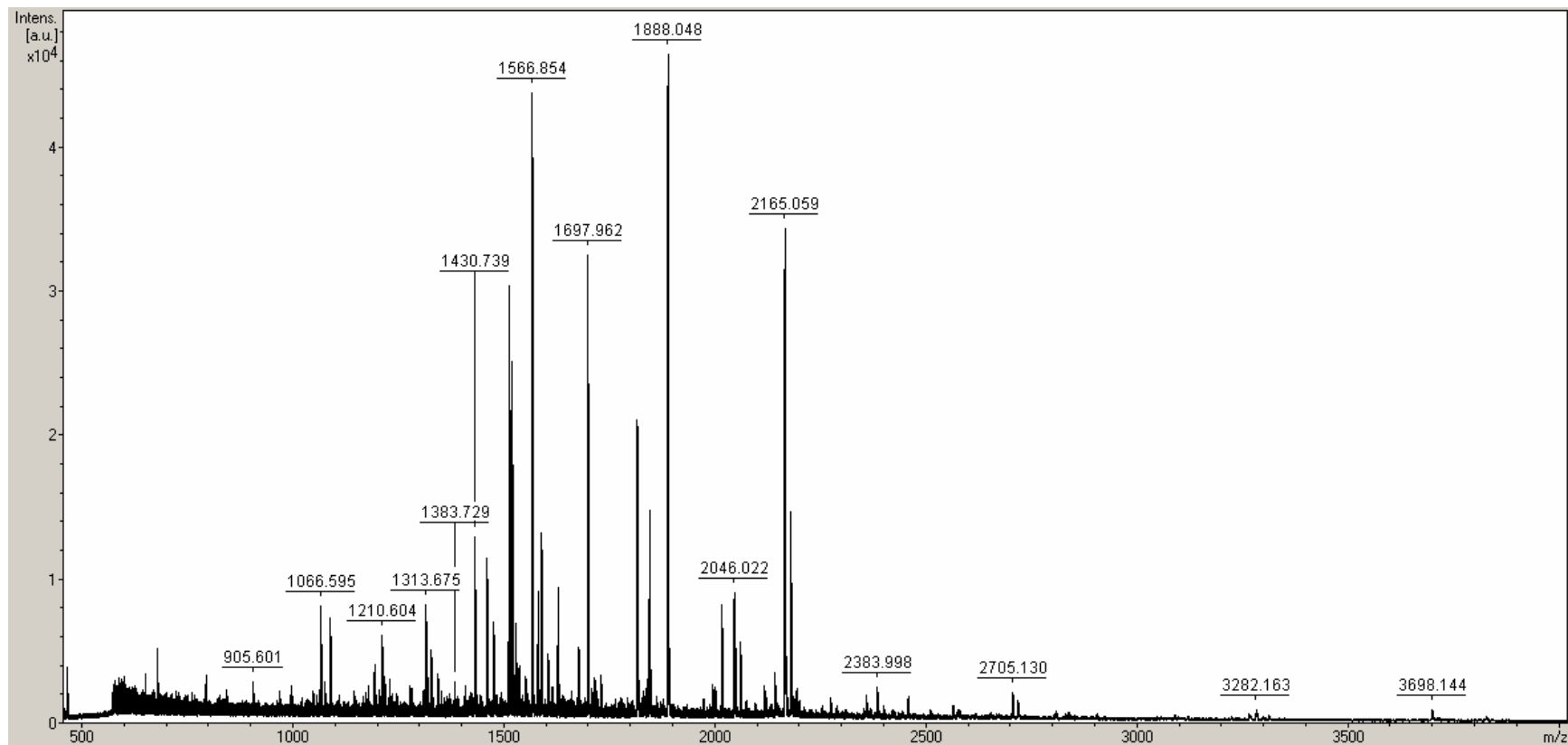


Mass spectra of Chain A, Crystal structure of Pig Phosphoglucose Isomerase gi|21730305; Sample B

Appendices

Protein:		Chain A, Crystal Structure Of Pig Phosphoglucose Isomerase gi 21730305							Peak threshold:		0.0
Intensity coverage:		56.6 % (57009 cnts)		Sequence coverage MS:		24.2 %		Sequence coverage MS/MS:		0.0 %	
		pl:		8.7		MW (kDa):		63.0			
10	20	30	40	50	60	70	80	90			
AALTONPQFK	KLQTWYHEHR	SDLNLRRLF	GDKDR FNHFS	LNLNTNHGRI	LLDYSKNLVT	EAVMQMLVDL	AKSRGVEAAR	ERMFNGEKIN			
100	110	120	130	140	150	160	170	180			
F TEDRAVLHV	ALR NRS NTPI	LVDGKDVMPE	VNRVLEKMKS	FCKRVRSGEW	KGYSGKSITD	VINIGIGGSD	LGPLMVTEAL	KPYSAE GPRV			
190	200	210	220	230	240	250	260	270			
WFVSNIDGTH	I AKTLATLNP	ESSLFIIASK	TFTTQETITN	AETAK EWFLQ	SAK DPSAVAK	HFVALST NTT	KVKEFGIDPQ	NMFEFWDWVG			
280	290	300	310	320	330	340	350	360			
GRYSLWSAIG	LSIALHVGFD	NFEQLLSGAH	WMDQHFRTTP	LEKNAPVLLA	LLGIWYINFF	GCETHAMLPY	DQYLHR FAAY	FQQGD MESNG			
370	380	390	400	410	420	430	440	450			
KYITKSGTRV	DHQTGPIVWG	EPGTNGQHAF	YQLIHQGTKM	IPCDFLIPVQ	TQHPIRKGLH	HKILLANFLA	QTEALMKGKS	TEEAR KELQA			
460	470	480	490	500	510	520	530	540			
AGKSPEDFEK	LLPHK VFEGN	RPTNSIVFTK	LTPFILGALI	AMYEHKIFVQ	GVIWDINSFD	QWGV ELGKQL	AKKIEPELDG	SSPVTSHDSS			
550	560										
TNGLINFIKQ	EREARSQ										

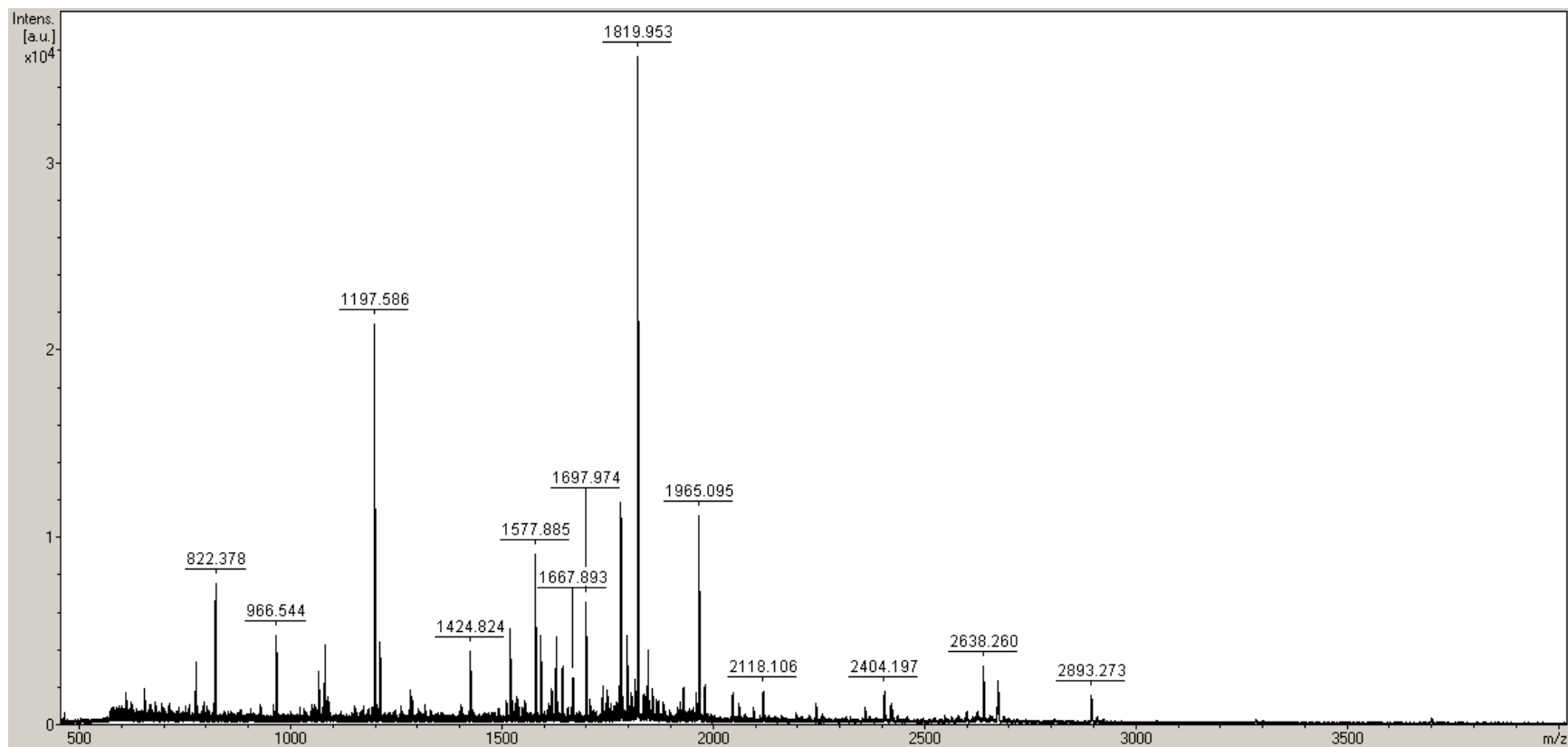
Sequence of Chain A, Crystal structure of Pig Phosphoglucose Isomerase gi|21730305; Sample B



Mass spectra for PREDICTED: similar to 78kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca) gi|73968064; Sample C

Protein:	PREDICTED: similar to 78 kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP)						Peak threshold:	0.0	
Intensity coverage:	49.8 % (219545 cnts)	Sequence coverage MS:	43.3 %	Sequence coverage MS/MS:	0.0 %	pI:	6.0	MW (kDa):	50.9
10	20	30	40	50	60	70	80	90	
MKLSLVA AVL	LLLSAARAEE	EDKKEDVGTV	VGIDLGTTYS	CVGVFKNGRV	EIIANDQGNR	ITPSYVAFTP	EGERLIGDAA	KNQLTSPEN	
100	110	120	130	140	150	160	170	180	
TVFDAKRLIG	RTWNDPSVQQ	DIKFLPFKVV	EKKTKPYIQV	DIGGGQTKTF	APEEISAMVL	TKMKETAEAY	LGKKVTHAVV	TVPAYFNDAQ	
190	200	210	220	230	240	250	260	270	
RQATKDAGTI	AGLNVMRIIN	EPTAAAIAYG	LDKREGEKNI	LVFDLGGGTF	DVSLLTIDNG	VFEVVATNGD	THLGGEDFDQ	RVMEHFIKLY	
280	290	300	310	320	330	340	350	360	
KKKTGKDVRK	DNRAVQKLRR	EVEKAKRALS	SQHQAIEIE	SFYEGEDFSE	TLTRAKFEEL	NMDLFRSTMK	PVQKVLEDS	LKKSIDEIV	
370	380	390	400	410	420	430	440	450	
LVGGSTRIPK	IQQLVKEFFN	GKEPSRGINP	DEAVAYGAAV	QAGVLSGDQD	TGDLVLLDVC	PLTLGIETVG	GVMTKLIPRN	TVVPTKNLPR	
460	470								
KTSSRSALI	LEMN								

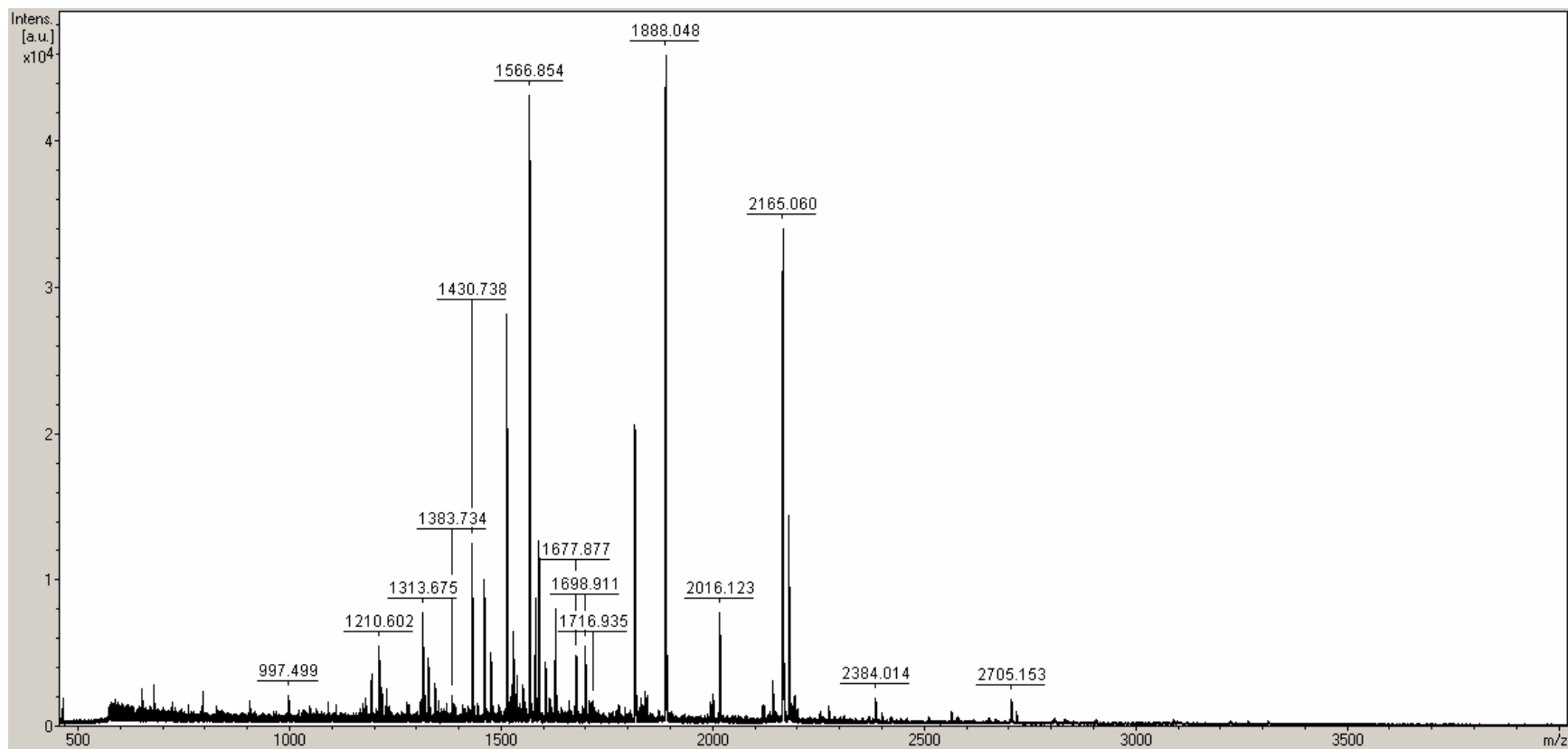
Sequence for PREDICTED: similar to 78kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca) gi|73968064; Sample C



Mass Spectra for Proyl 4 hydroxylase, beta polypeptide (Mus Musculus) gi|14250251; Sample D

Protein:	Prolyl 4-hydroxylase, beta polypeptide [Mus musculus] gi 14250251						Peak threshold:	0.0	
Intensity coverage:	49.1 % (87765 cnts)	Sequence coverage MS:	35.8 %	Sequence coverage MS/MS:	0.0 %	pI:	4.6	MW (kDa):	57.0
10	20	30	40	50	60	70	80	90	
MLSRALLCLA	LAWAARVGAD	ALEEEDNVLV	LKKSNFEEAL	AAHKYLLVEF	YAPWCGHCKA	LAPEYAKAAA	KLKAEGSEIR	LAKVDATEES	
100	110	120	130	140	150	160	170	180	
DLAQYGVRG	YPTIKFFKNG	DTASPKEYTA	GREADDIVNW	LKKRTGPAAT	TLSDTAAAES	LVDSSEVTVI	GFFKDVESDS	AKQFLAAEA	
190	200	210	220	230	240	250	260	270	
IDDIPFGITS	NSGVFSKYQL	DKDGVVLFKK	FDEGRNMFEG	EITKEKLLDF	IKHNQLPLVI	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	
280	290	300	310	320	330	340	350	360	
DGKLSSFKRA	AEGFKGILF	IFIDSHTDN	QRILEFFGLK	KEECPAVRLI	TLEEEMTKYK	PESDELTAEK	ITEFCHRFLF	GKIKPHLMSQ	
370	380	390	400	410	420	430	440	450	
EVPEWWDKQP	VKVLVGANFE	EVAFDEKKNV	FVEFYAPWCG	HCKQLAPIWD	KLGETYKDHE	NIIIAKMDST	ANEVEAVKVH	SFPTLKFFPA	
460	470	480	490	500	510				
SADRTVIDYN	GERTLDGFKK	FLESGGDGA	GDEDLLEE	ALEPDMEEDD	DQKAVKDEL				

Sequence for Prolyl 4 hydroxylase, beta polypeptide (Mus Musculus) gi|14250251; Sample D



Mass Spectra for PREDICTED: similar to 78kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca) gi|73968064; Sample E

Protein:	PREDICTED: similar to 78 kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP)								Peak threshold:	0.0	
Intensity coverage:	72.3 % (233487 cnts)		Sequence coverage MS:		43.3 %		Sequence coverage MS/MS:		0.0 %		
	pl:		6.0		MW (kDa):		50.9				
	10	20	30	40	50	60	70	80	90		
	MKLSLVAAVL	LLLSAARAE	EDKKEDVGT	VGIDLGTTYS	CVGVFKNGRV	EIIANDQNR	ITPSYVAFTP	EGERLIGDAA	KNQLTSNPEN		
	100	110	120	130	140	150	160	170	180		
	TVFDAKRLIG	RTWNDPSVQQ	DIKFLPFKVV	EKKTKPYIQV	DIGGGQTKTF	APEEISAMVL	TKMKETAEEY	LGKKVTHAVV	TVPAYFNDAQ		
	190	200	210	220	230	240	250	260	270		
	RQATKDA GTI	AGLNVMRIIN	EPTAAAIAYG	LDKREGEKNI	LVFDLGGGTF	DVSLLTIDNG	VFEVVA TNGD	THLGGEDFDQ	RVMEHF IKLY		
	280	290	300	310	320	330	340	350	360		
	KKKTGKDVRK	DNRAVQKLR	EVEKAKRALS	SOHQARIEIE	SFYEGEDFSE	TLTRAKFEEL	NMDLFRSTMK	PVQKVLESD	LKKSDIDEIV		
	370	380	390	400	410	420	430	440	450		
	LVGGSTRIPK	IQQLVKEFFN	GKEPSRGINP	DEAVAYGAAV	QAGVLSGDQD	TGDLVLLDVC	PLTLGIETVG	GVMTKLIPRN	TVVPTKNLPR		
	460	470									
	KTKSSRSALI	LEMN									

Sequence for PREDICTED: similar to 78kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding protein) (BiP) (Endoplasmic reticulum luminal ca) gi|73968064; Sample E

APPENDIX

B: - N-Terminal Sequencing

Uncorr																					
	A	D	E	F	G	H	I	K	L	N	M	P	Q	R	S	T	V	W	Y		
1	0.68	1.27	0.97	0.33	1.48	0.44	0.33	0.25	0.63	0.11	4.59	0.49	0.31	1.92	0.88	0.67	0.54	0.11	0.13		
2	0.79	0.95	4.93	0.56	1.61	0.27	0.64	0.14	0.97	0.00	1.37	1.29	0.37	1.90	0.58	0.53	0.87	0.00	0.48		
3	0.82	0.99	1.62	0.61	1.82	0.20	0.61	0.16	0.94	0.08	1.26	0.89	0.49	1.92	0.61	0.84	3.82	0.00	0.37		
4	0.94	0.98	1.28	0.73	1.88	0.33	0.61	0.00	1.09	0.00	1.47	1.03	0.44	1.66	0.53	0.74	1.39	0.00	0.43		
5	0.98	1.05	1.20	0.78	1.89	0.27	0.58	0.11	0.82	0.11	1.23	1.04	0.58	1.36	0.61	0.83	1.35	0.00	2.67		
6	1.05	0.93	1.00	0.71	1.61	0.26	0.57	0.00	1.00	0.12	1.27	1.03	0.50	1.85	0.57	0.76	1.00	0.00	1.04		
7	1.01	1.89	0.98	0.84	1.77	0.26	0.82	0.18	0.97	0.10	1.44	0.99	0.51	1.67	1.20	0.90	0.97	0.00	0.82		
8	0.78	1.17	0.74	0.51	1.63	0.23	1.32	0.00	0.71	0.00	0.90	0.79	0.32	1.15	0.73	0.78	0.79	0.00	0.37		
9	0.62	1.09	0.70	0.52	2.61	0.09	0.62	0.00	0.60	0.00	0.85	1.15	0.28	0.98	0.45	0.49	0.70	0.00	0.25		
10	0.87	1.03	0.71	0.50	2.41	0.15	0.54	0.00	0.88	0.09	1.00	1.05	0.44	1.05	0.42	0.56	0.62	0.07	0.32		
11	0.80	1.02	0.88	1.13	2.11	0.19	0.53	0.00	0.94	0.08	1.12	0.92	0.49	1.24	0.42	0.70	0.76	0.00	0.39		
Skcpd																					
	A	D	E	F	G	H	I	K	L	N	M	P	Q	R	S	T	V	W	Y		
1	0.00	0.30	0.00	0.00	0.00	0.09	0.00	0.07	0.00	0.00	3.19	0.00	0.00	0.00	0.20	0.00	0.00	0.08	0.00		
2	0.00	0.00	3.49	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.01	0.29	0.00	0.07	0.00	0.00	0.00	0.00	0.02		
3	0.01	0.00	0.27	0.00	0.14	0.00	0.00	0.02	0.08	0.00	0.00	0.00	0.05	0.00	0.00	0.11	2.96	0.00	0.00		
4	0.13	0.00	0.01	0.09	0.14	0.04	0.02	0.00	0.25	0.00	0.20	0.03	0.00	0.02	0.00	0.02	0.54	0.00	0.00		
5	0.17	0.05	0.02	0.10	0.03	0.00	0.00	0.01	0.00	0.00	0.00	0.03	0.13	0.00	0.03	0.12	0.51	0.00	2.26		
6	0.24	1.91	0.11	0.00	0.00	0.03	0.00	0.00	0.18	0.04	0.08	0.01	0.04	0.40	0.02	0.05	0.18	0.00	0.84		
7	0.20	0.87	0.00	0.10	0.00	0.04	0.25	0.13	0.17	0.02	0.20	0.00	0.05	0.32	0.67	0.19	0.16	0.00	0.43		
8	0.00	0.14	0.00	0.00	0.00	0.02	0.76	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.08	0.00	0.00	0.00			
9	0.00	0.05	0.00	0.00	0.56	0.00	0.06	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
10	0.00	0.00	0.00	0.00	0.29	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00		
11	0.00	0.00	0.25	0.26	0.00	0.04	0.00	0.02	0.19	0.00	0.15	0.00	0.02	0.26	0.00	0.02	0.01	0.00	0.06		
Lag																					
	A	D	E	F	G	H	I	K	L	N	M	P	Q	R	S	T	V	W	Y		
1	0.00	0.30	0.00	0.00	0.00	0.09	0.00	0.07	0.00	0.00	3.19	0.00	0.00	0.00	0.20	0.00	0.00	0.08	0.00		
2	0.00	0.00	3.75	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.29	0.00	0.07	0.00	0.00	0.00	0.00	0.02		
3	0.01	0.00	0.00	0.00	0.14	0.00	0.00	0.02	0.08	0.00	0.00	0.00	0.05	0.00	0.00	0.11	2.90	0.00	0.00		
4	0.13	0.00	0.01	0.09	0.14	0.04	0.02	0.00	0.25	0.00	0.20	0.03	0.00	0.02	0.00	0.02	0.00	0.00	0.00		
5	0.17	0.05	0.02	0.10	0.03	0.00	0.00	0.01	0.00	0.00	0.00	0.03	0.13	0.00	0.03	0.12	0.51	0.00	2.26		
6	0.24	1.91	0.11	0.00	0.00	0.03	0.00	0.00	0.18	0.04	0.08	0.01	0.04	0.40	0.02	0.05	0.18	0.00	0.84		
7	0.20	0.87	0.00	0.10	0.00	0.04	0.25	0.13	0.17	0.02	0.20	0.00	0.05	0.32	0.64	0.19	0.16	0.00	0.36		
8	0.00	0.14	0.00	0.00	0.00	0.02	0.81	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.08	0.00	0.00	0.00			
9	0.00	0.05	0.00	0.00	0.70	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
10	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00		
11	0.00	0.00	0.25	0.26	0.00	0.04	0.00	0.02	0.19	0.00	0.15	0.00	0.02	0.26	0.00	0.02	0.01	0.00	0.06		
Call																					
	First	Second	Third	Match	Known																
1	N	D	R	+++	N																
2	E	P	R	+++	E																
3	V	T	G	+++	V																
4	N	L	V																		
5	Y	O	T	+++	Y																
6	D	R	L	+++	D																
7	S	D	I	+++	S																
8	L	N	K	+++	L																
9	G	P	E	+++	G																
10	G	N	Q	+++	G																
11	F	R	G	+++	F																

BR72A1 Electroblot: 2 Lanes

Appendices

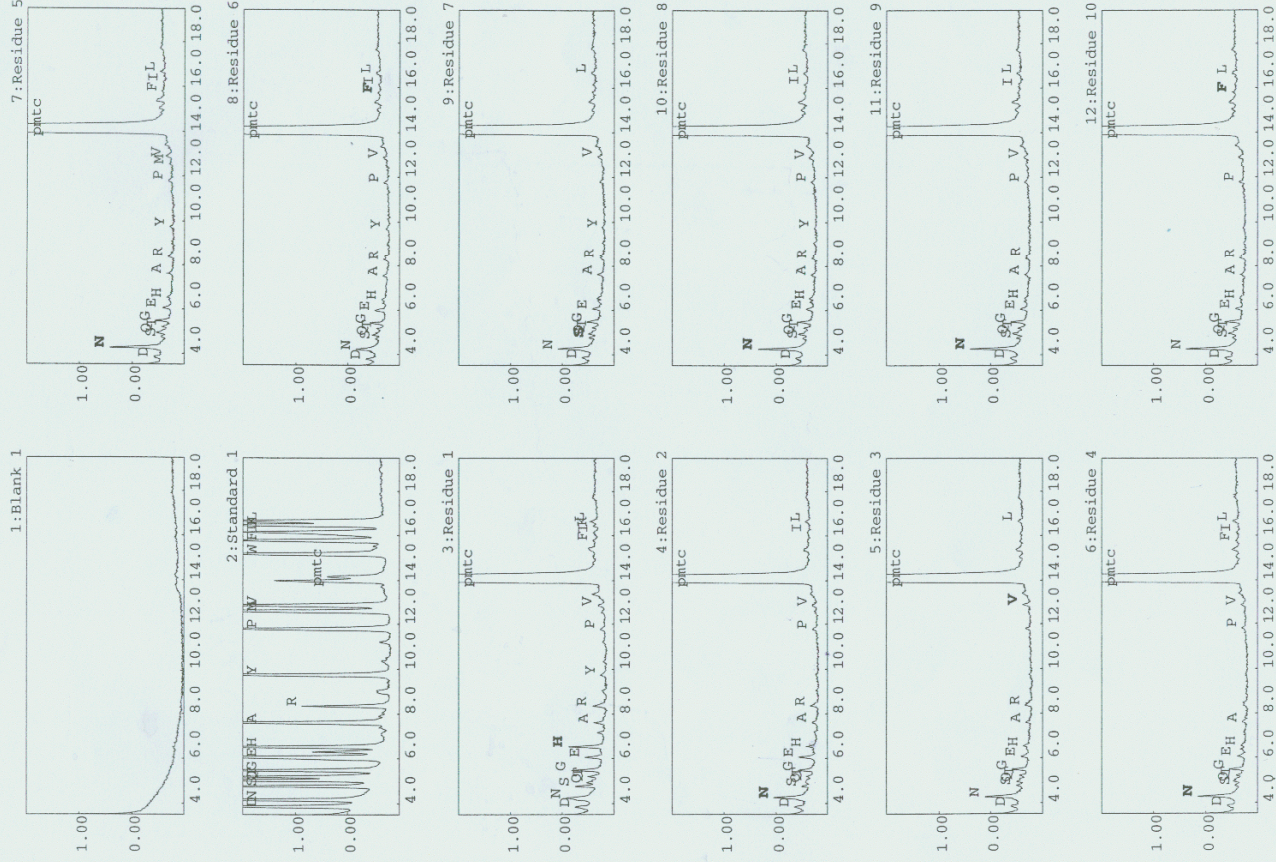
Uncorr																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.44	0.51	0.60	0.14	1.37	1.35	0.10	0.28	0.37	0.00	0.87	0.16	0.33	1.48	1.01	0.58	0.19	0.00	0.24	
2	0.11	0.29	0.60	0.00	0.71	0.34	0.12	0.00	0.23	0.00	1.12	0.25	0.08	0.68	0.37	0.24	0.13	0.00	0.00	
3	0.19	0.26	0.24	0.00	0.71	0.21	0.00	0.00	0.17	0.00	1.15	0.00	0.30	0.46	0.33	0.35	0.32	0.00	0.00	
4	0.17	0.12	0.27	0.14	0.68	0.16	0.20	0.00	0.23	0.00	1.32	0.17	0.28	0.00	0.23	0.31	0.20	0.00	0.00	
5	0.22	0.12	0.30	0.09	0.65	0.16	0.09	0.00	0.21	0.07	1.79	0.14	0.39	0.39	0.11	0.36	0.24	0.00	0.19	
6	0.19	0.30	0.37	0.12	0.59	0.17	0.11	0.00	0.16	0.00	0.73	0.16	0.23	0.53	0.17	0.27	0.17	0.00	0.09	
7	0.23	0.27	0.29	0.00	0.59	0.00	0.00	0.00	0.09	0.00	1.18	0.00	0.29	0.47	0.29	0.33	0.22	0.00	0.06	
8	0.18	0.15	0.24	0.00	0.66	0.13	0.20	0.00	0.19	0.00	1.64	0.18	0.38	0.44	0.21	0.32	0.11	0.00	0.07	
9	0.14	0.26	0.22	0.00	0.71	0.11	0.12	0.00	0.17	0.00	1.72	0.12	0.38	0.24	0.26	0.34	0.21	0.00	0.00	
10	0.15	0.28	0.24	0.16	0.79	0.14	0.00	0.00	0.10	0.00	1.73	0.22	0.26	0.40	0.15	0.35	0.00	0.00	0.00	

Bkgnd																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.18	0.22	0.11	0.07	0.65	1.09	0.00	0.19	0.07	0.00	0.00	0.02	0.02	0.75	0.62	0.30	0.00	0.00	0.07	
2	0.00	0.00	0.14	0.00	0.01	0.10	0.02	0.00	0.00	0.00	0.07	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.05	0.13	0.00	0.00	
4	0.00	0.00	0.00	0.07	0.00	0.00	0.09	0.00	0.00	0.00	0.11	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
5	0.01	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.07	0.51	0.00	0.09	0.00	0.00	0.05	0.05	0.00	0.10	
6	0.00	0.03	0.05	0.06	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.00	0.02	
7	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.05	0.00	0.02	0.00	0.00	
8	0.01	0.00	0.00	0.00	0.00	0.00	0.08	0.01	0.04	0.00	0.12	0.04	0.09	0.02	0.00	0.00	0.00	0.00	0.04	
9	0.00	0.00	0.00	0.00	0.05	0.00	0.01	0.03	0.04	0.00	0.12	0.00	0.09	0.00	0.08	0.00	0.00	0.00	0.00	
10	0.00	0.02	0.06	0.10	0.14	0.04	0.00	0.04	0.00	0.00	0.05	0.08	0.00	0.08	0.00	0.00	0.00	0.00	0.00	

Lag																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.18	0.22	0.11	0.07	0.65	1.10	0.00	0.19	0.07	0.00	0.00	0.02	0.02	0.75	0.62	0.30	0.00	0.00	0.07	
2	0.00	0.00	0.14	0.00	0.01	0.10	0.02	0.00	0.00	0.00	0.07	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.05	0.13	0.00	0.00	
4	0.00	0.00	0.00	0.07	0.00	0.00	0.09	0.00	0.00	0.00	0.11	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
5	0.01	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.07	0.51	0.00	0.09	0.00	0.00	0.05	0.05	0.00	0.10	
6	0.00	0.03	0.05	0.06	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.00	0.02	
7	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.05	0.00	0.02	0.00	0.00	
8	0.01	0.00	0.00	0.00	0.00	0.00	0.08	0.01	0.04	0.00	0.12	0.04	0.09	0.02	0.00	0.00	0.00	0.00	0.04	
9	0.00	0.00	0.00	0.00	0.05	0.00	0.01	0.03	0.04	0.00	0.12	0.00	0.09	0.00	0.08	0.00	0.00	0.00	0.00	
10	0.00	0.02	0.06	0.10	0.14	0.04	0.00	0.04	0.00	0.00	0.05	0.08	0.00	0.08	0.00	0.00	0.00	0.00	0.00	

Cell					
	First	Second	Third	Match	Known
1	H	R	G	+++	H
2	N	P	E	+++	N
3	V	M	Q	++	N
4	N	I	W	+++	N
5	N	Y	R	+++	N
6	F	D	E	-	-
7	S	N	A	++	N
8	N	I	Q	+++	N
9	N	S	D	+++	N
10	F	N	G	++	N

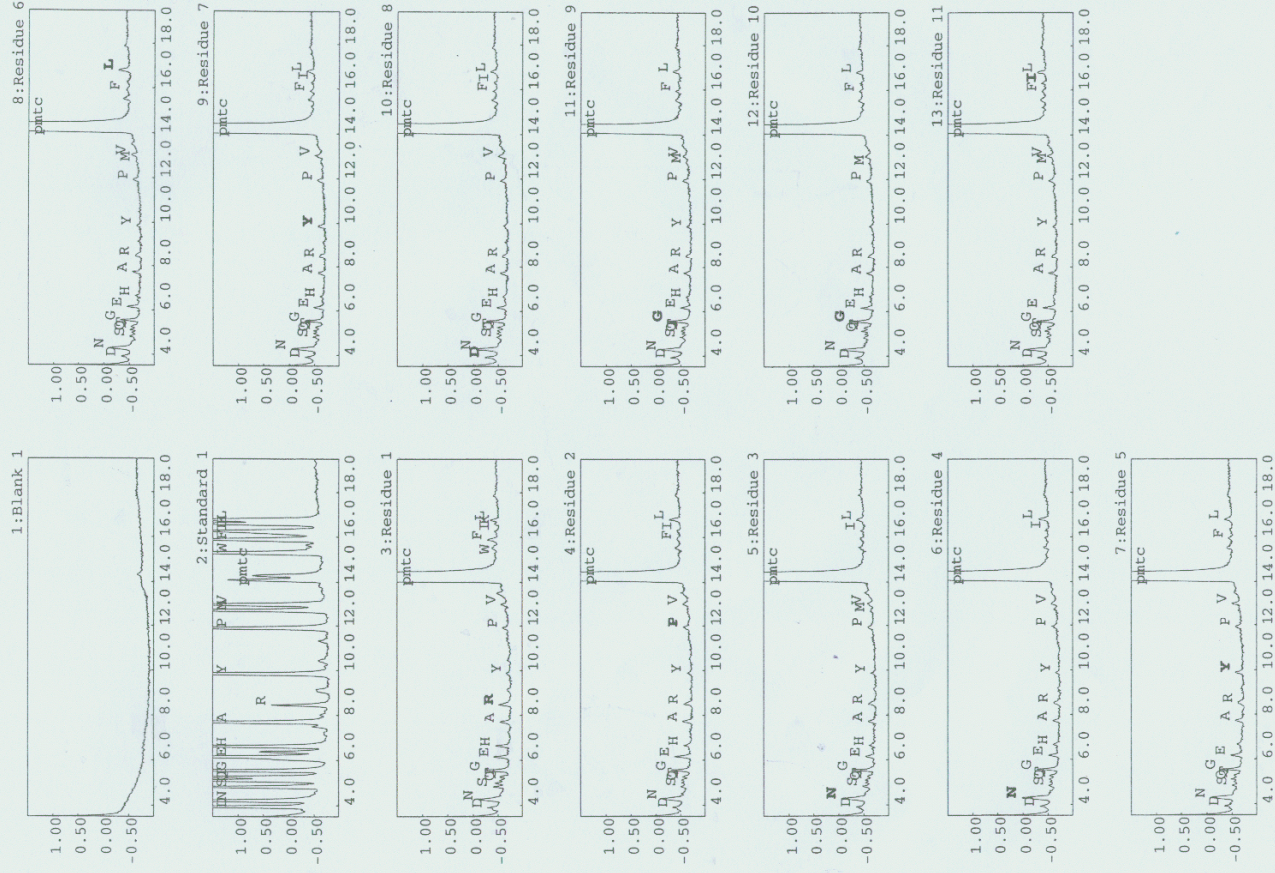
BR72A2 Electroblot: 2 Lanes



Applied Biosystems Procise - PROCISE

Uncorr																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.50	0.33	0.60	0.60	1.20	0.62	0.25	0.32	0.50	0.00	0.88	0.23	0.27	1.54	0.65	0.43	0.32	0.08	0.19	
2	0.43	0.25	0.55	0.18	0.93	0.30	0.17	0.00	0.59	0.60	0.83	0.14	0.25	1.01	0.28	0.31	0.29	0.00	0.16	
3	0.33	0.33	0.35	0.00	1.00	0.23	0.21	0.00	0.36	0.59	0.99	0.28	0.20	0.51	0.37	0.28	0.00	0.19	0.16	
4	0.36	0.25	0.39	0.00	1.03	0.24	0.11	0.00	0.53	0.00	1.10	0.22	0.25	0.70	0.33	0.37	0.21	0.00	0.18	
5	0.31	0.31	0.45	0.14	0.97	0.00	0.00	0.00	0.39	0.00	0.90	0.32	0.28	0.42	0.25	0.27	0.36	0.00	0.36	
6	0.33	0.33	0.38	0.13	0.85	0.20	0.00	0.00	0.59	0.10	0.93	0.18	0.24	0.72	0.26	0.32	0.30	0.00	0.18	
7	0.31	0.51	0.38	0.21	0.95	0.14	0.06	0.00	0.43	0.00	0.92	0.20	0.29	0.69	0.31	0.33	0.36	0.00	0.36	
8	0.32	0.40	0.39	0.23	1.09	0.16	0.50	0.00	0.34	0.00	0.89	0.33	0.25	0.65	0.29	0.38	0.36	0.00	0.60	
9	0.30	0.28	0.47	0.16	1.08	0.11	0.00	0.00	0.36	0.07	0.98	0.14	0.34	0.75	0.00	0.32	0.00	0.00	0.20	
10	0.32	0.35	0.50	0.14	0.90	0.00	0.31	0.00	0.45	0.05	0.85	0.29	0.31	0.74	0.23	0.31	0.27	0.00	0.14	
11																				
Bgnd																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.10	0.00	0.05	0.43	0.17	0.30	0.09	0.22	0.00	0.00	0.00	0.00	0.02	0.82	0.27	0.09	0.00	0.06	0.00	
2	0.04	0.00	0.11	0.01	0.00	0.00	0.02	0.00	0.09	0.00	0.00	0.10	0.00	0.29	0.00	0.00	0.50	0.00	0.00	
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	
4	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.01	0.10	0.05	0.00	0.00	0.00	0.00	0.00	0.00	
5	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	
6	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.10	0.01	0.00	0.00	0.00	0.06	0.00	0.18	
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.02	
8	0.00	0.18	0.00	0.05	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.02	0.00	0.07	0.00	0.19	
9	0.01	0.05	0.00	0.08	0.13	0.03	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.06	0.08	0.00	0.00	
10	0.00	0.00	0.11	0.02	0.12	0.01	0.00	0.03	0.00	0.02	0.08	0.00	0.04	0.03	0.00	0.00	0.00	0.00	0.05	
11	0.03	0.01	0.16	0.00	0.00	0.00	0.23	0.04	0.08	0.00	0.00	0.09	0.00	0.02	0.00	0.00	0.01	0.01	0.00	
Lag																				
	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	
1	0.10	0.00	0.05	0.43	0.17	0.30	0.09	0.22	0.00	0.00	0.00	0.00	0.02	0.84	0.27	0.09	0.00	0.06	0.00	
2	0.04	0.00	0.12	0.01	0.00	0.00	0.02	0.00	0.09	0.00	0.00	0.10	0.00	0.28	0.00	0.00	0.00	0.00	0.00	
3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	
4	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	
5	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	
6	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.10	0.01	0.00	0.00	0.00	0.06	0.00	0.19	
7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	
8	0.00	0.18	0.00	0.06	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.07	0.19	
9	0.01	0.05	0.00	0.08	0.13	0.03	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.01	0.06	0.08	0.00	0.00	
10	0.00	0.00	0.11	0.02	0.12	0.01	0.00	0.03	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	
11	0.03	0.01	0.16	0.00	0.00	0.00	0.23	0.04	0.08	0.00	0.00	0.09	0.00	0.04	0.03	0.00	0.00	0.01	0.00	
Call																				
	First	Second	Third	Match	Known															
1	R	M	F	+++	X															
2	P	R	L	+	L															
3	N	M	S																	
4	N	T	L	+	L															
5	Y	V	P	+++	Y															
6	L	R	H	+++	L															
7	Y	R	N	+++	Y															
8	D	R	P	+++	D															
9	G	R	Y	+++	G															
10	G	R	N	+++	G															
11	I	R	S		L															

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Ubcscr	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	0.48	0.33	0.85	0.23	1.35	0.56	0.34	0.34	0.50	0.14	0.61	0.19	0.00	1.58	0.84	0.55	0.32	0.08	0.20
2	0.48	0.32	0.93	0.00	1.06	0.21	0.00	0.00	0.20	0.06	0.99	0.55	0.26	0.76	0.42	0.45	0.19	0.00	0.20
3	0.15	0.34	0.46	0.11	1.11	0.15	0.17	0.21	0.59	0.00	1.44	0.35	0.00	0.75	0.40	0.43	0.32	0.00	0.15
4	0.22	0.33	0.53	0.15	1.08	0.29	0.14	0.00	0.59	0.07	1.44	0.34	0.30	0.67	0.39	0.44	0.28	0.00	0.21
5	0.20	0.26	0.72	0.13	1.19	0.25	0.14	0.00	0.34	0.00	1.67	0.38	0.30	0.34	0.33	0.44	0.56	0.00	0.20
6	0.28	0.32	0.69	0.00	1.00	0.26	0.00	0.00	0.52	0.07	1.53	0.18	0.00	0.27	0.33	0.39	0.34	0.00	0.00
7	0.30	0.35	0.48	0.15	1.07	0.22	0.31	0.00	0.45	0.00	1.67	0.45	0.40	0.45	0.44	0.42	0.33	0.12	0.19
8	0.29	0.32	0.44	0.27	1.30	0.22	0.14	0.00	0.37	0.00	1.72	0.35	0.35	0.22	0.34	0.42	0.50	0.11	0.00
9	0.33	0.38	0.41	0.18	1.36	0.15	0.24	0.00	0.38	0.00	1.21	0.50	0.33	0.57	0.33	0.44	0.39	0.00	0.15
10	0.28	0.38	0.44	0.15	1.50	0.27	0.24	0.10	0.43	0.00	1.99	0.37	0.33	0.36	0.34	0.47	0.37	0.00	0.00
11	0.30	0.38	0.53	0.21	1.09	0.11	0.16	0.09	0.56	0.06	2.18	0.32	0.33	0.68	0.37	0.36	0.34	0.20	0.00

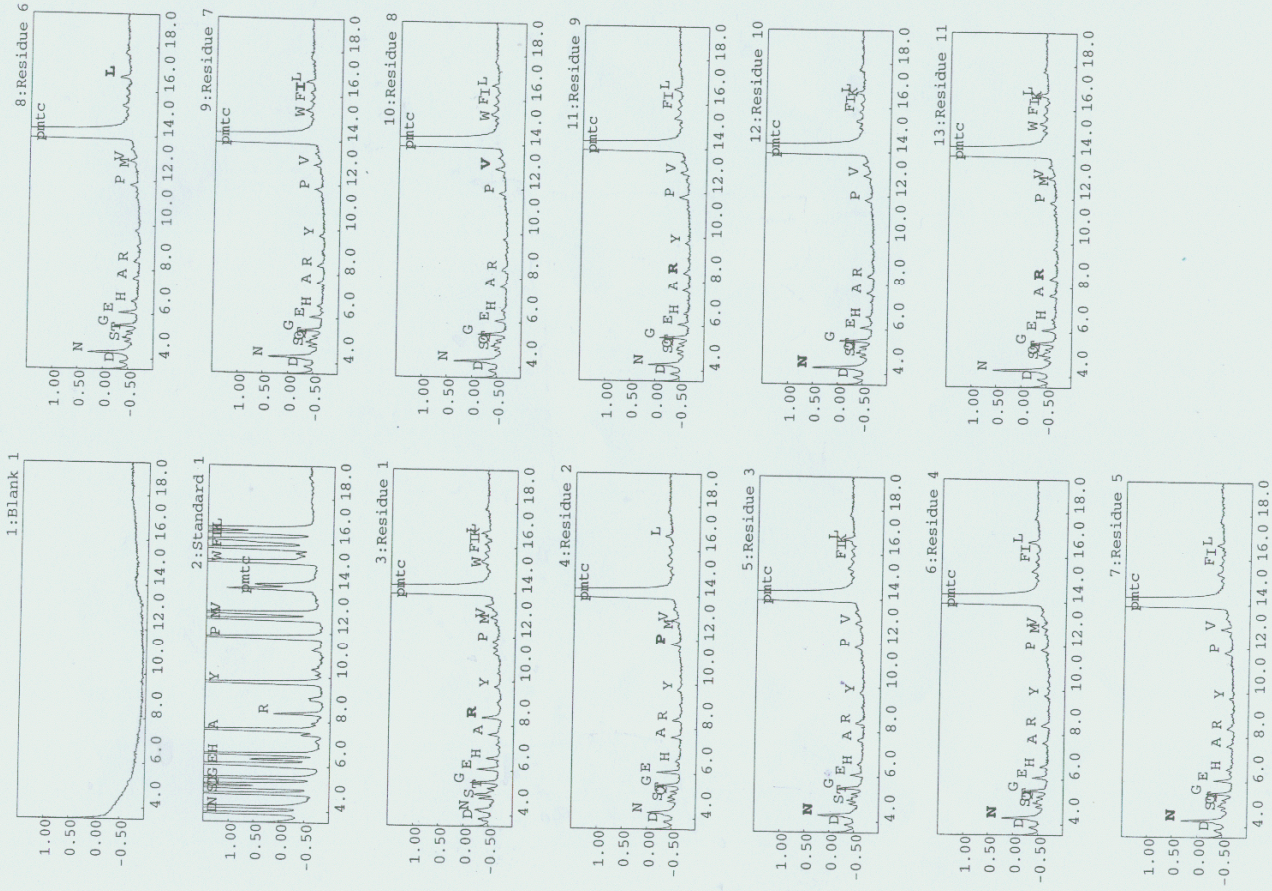
Bkgnd	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	0.25	0.03	0.04	0.14	0.31	0.21	0.21	0.11	0.00	0.07	0.00	0.80	0.00	0.76	0.42	0.09	0.02	0.08	0.00
2	0.23	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.02	0.00	0.00	0.00	0.00
3	0.00	0.02	0.00	0.00	0.00	0.00	0.04	0.10	0.11	0.00	0.25	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.12	0.02	0.14	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.06
5	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.06
6	0.00	0.00	0.08	0.00	0.00	0.02	0.00	0.00	0.07	0.04	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0.02	0.01	0.00	0.04	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.10	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.14	0.04	0.00
9	0.03	0.02	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.17	0.00	0.03	0.02	0.00	0.00
10	0.00	0.02	0.00	0.00	0.13	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.11	0.06	0.00	0.00	0.00
11	0.00	0.02	0.13	0.00	0.00	0.00	0.00	0.00	0.13	0.04	0.07	0.80	0.00	0.36	0.05	0.00	0.00	0.11	0.00

Leg	A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
1	0.25	0.03	0.04	0.14	0.31	0.21	0.21	0.11	0.00	0.07	0.00	0.80	0.00	0.76	0.42	0.09	0.02	0.08	0.00
2	0.23	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.02	0.00	0.00	0.00	0.00
3	0.00	0.02	0.00	0.00	0.00	0.00	0.04	0.10	0.11	0.00	0.25	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.12	0.02	0.14	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.06
5	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.06
6	0.00	0.00	0.08	0.00	0.00	0.02	0.00	0.00	0.07	0.04	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
7	0.02	0.01	0.00	0.01	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.10	0.01	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.10	0.00	0.15	0.04	0.00
9	0.03	0.02	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.18	0.00	0.03	0.01	0.00	0.00
10	0.00	0.02	0.00	0.00	0.13	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.06	0.00	0.00	0.00
11	0.00	0.02	0.13	0.00	0.00	0.00	0.00	0.00	0.13	0.06	0.07	0.80	0.00	0.36	0.05	0.00	0.00	0.11	0.00

Call	First	Second	Third
1	R	S	G
2	P	N	A
3	M	L	K
4	N	Q	Y
5	M	V	F
6	L	N	E
7	I	N	O
8	V	N	H
9	R	P	G
10	N	G	H
11	R	N	W

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