finalreport

Value Added Products

Project code: A.BIT.0008

Prepared by: Dr. Julian Adams

Authors Julian Adams, Chris Franco, Lisa Schmidt, Chris Sentance, Raymond Tham, Fiona Young,

Wei Zhang

Flinders University

Date published: July 2011

ISBN:

PUBLISHED BY

Meat & Livestock Australia Limited

Locked Bag 991

NORTH SYDNEY NSW 2059

Production of bioactives using *in vitro* culture of cells from organs sourced from the meat processing industry

Abstract

The production of bioactives using *in vitro* culture of cells from organs sourced from the meat processing industry provides a unique value-adding process. The first phase of this project achieved its aim to provide a proof-of-concept for a new technology that could amplify the production of target bioactive compounds from cells isolated from animal organs. The second phase of the project focused on identification of marketable target bioactives with a quick route to market for the commercialisation of this technology. Disaggregated primary cells and minced organ culture were examined for their ability to produce bioactives through an investigation of amplification signals and scale-up in a bioreactor system. Alkaline Phosphatase (AP) was identified as a champion product from an initial business case that was developed using data obtained in the laboratory-scale experiments. However the identification of a new recombinant AP competitor manufactured in bacteria resulted in the business case for the production of AP using minced organ culture being non-viable. A proteomics approach was used to identify new bioactive targets produced during the minced organ culture process. It is recommended that this project directs its focus on these newly identified bioactive targets as a quick route to market for commercialisation of this technology. The diversification of this technology to alternative organs would give a greater range of bioactive targets, therefore we recommend applying the successful proteomics approach to the identification of new bioactives to alternative organs such as the heart, spleen and pancreas.

Executive Summary

Extraction of bioactive products from animal organs has been used to produce valuable co-products. This is a one-off process that results in low level yields of product with associated expensive purification costs. Alternatively, genetically modified recombinant technology for the generation of bioactives can be used. This process is highly reproducible and scalable but has a high capital cost in the growth of cells for the production of the bioactive product and in meeting regulatory requirements.

The aim of this project is to develop new *in vitro* cell culture bioprocesses that amplify the yields of commercially valuable bioactive products from farmed animal organs to commercially competitive levels. The advantage of using the *in vitro* cell culture approach is the production of high yields of target bioactives in a highly reproducible system. Additionally, this concept uses relatively low cost raw materials, involves relatively low production costs and allows for the production of multiple bioactives in a unitary process. It is well documented that *in vitro* cellular production of bioactives is largely dependent upon the culture system. The proof-of-concept of production of bioactives for future commercial

`

application was demonstrated in Phase 1 of this project. The strategy of Phase 2 was to further demonstrate the feasibility and scalability of this technology through process optimisations and scale-up from the proof-of-concept demonstrated in Phase 1 towards a commercial scale. We focused on demonstrating the feasibility of bioactive production using minced organ culture and disaggregated cell culture in a bench-scale bioreactor system. Optimisation and scale-up of the *in vitro* cell culture technology was performing in parallel to the development of a business case to attract industry investment.

In collaboration with MLA, five target bioactives were identified; alkaline phosphatase (AP), fibrinogen, basic fibroblast growth factor (bFGF), ferritin and heparin. The Phase 2 literature review of commercially viable bioactives and the use of bioreactor systems for scale-up of the technology were performed. The Phase 2 literature review demonstrated that heparin was unlikely to be a successful target for this project as there is no direct evidence that heparin is synthesized in liver cells. There was also a lack of information on the use of stimulatory factors and/or amplification signals that could increase production of heparin; therefore heparin was removed from the list of bioactive targets. Analytical methods were successfully developed for bFGF, fibrinogen and AP.

The ability to disaggregate organs for the collection of cells, the culturing of the cells *in vitro* and amplification of target bioactives was demonstrated in the proof-of concept Phase 1 of this project. The next phase of this project focused on using the established techniques in the production of liver cells to develop a commercially viable target bioactive and fast-track the transfer of this technology to industry. Transfer of this technology from ovine to bovine tissue sources was a key aim to enable a greater range of meat process to access this technology. The ability to add value to the meat industry by product diversification into the bioactive market will provide the opportunity for the meat processor and the bioactive market to access this technology for the production of valuable bioactives.

Specific Aims

- To demonstrate the feasibility and scalability of the integrated process in a 1-2 litre laboratory-scale proof-of-concept bioreactor and the bench-scale demonstration bioreactor for stimulated bioactive production from minced organ culture and/or in vitro cell culture of sheep hepatocytes.

- To develop analytical methods and biological assays for target bioactives as indicated from market research conducted by MLA.

- To transfer technology to bovine organs at laboratory scale, demonstrating disaggregation, viability and bioactive amplification.

`

- To test the ability to amplify 1-2 selected bioactives in crude organ slices or clumps for fast tracking to commercialisation.

- To establish bioassays to test the efficacy and stability of the target bioactives produced.

- To assist MLA for the development of a comprehensive business and market analysis of the target bioactives, enabling successful industrial engagement in the project.

Contents

`

`

Background

`

Introduction

1.1.1 Organ Culture

The overall aim of this project is to amplify the yield of commercially valuable bioactive products from farmed animal organs. The project aims to demonstrate a proof-of-concept that it is feasible to use animal organs for the efficient and economic amplification of high value bioactive compounds.

Organ culture often refers to the cultivation of whole organs *in vitro* however it is not likely to be a viable choice for the efficient production of bioactives. The primary reason is that solid organs of large size prevent efficient gaseous diffusion and the exchange of nutrients and metabolites, therefore the capacity for cell survival and synthesis of bioactives will be largely discounted.

Animal organs produced as by-products of the meat processing industry are important sources of valuable bioactives, with potential uses in the pharmaceutical, cosmetic and nutraceutical industries. The spectrum of these bioactives includes proteins, glycoproteins, polypeptides, growth factors, lipids, carbohydrates, and secondary metabolites. While the organs harvested after slaughter could be directly used for bioactives extraction, great potential exists to add significant value by increasing production of bioactives using *in vitro* culture.

The cells from animal organs obtained after slaughter can be viable and metabolically active for several days. Considerable effort was devoted to establishing *in vitro* whole organ culture before immortalised mammalian cell lines became widely available in early 1980's. A wide range of organs from bovine, ovine, mouse, rat and human sources have been successfully cultured *in vitro* (Guinazu, Richter et al. 2002; Placzek, Jessel et al. 1993; Schobitz, Gonzalez et al. 2001; Lee, Latridis et al. 2006; Gahwiler 1989; Saitoh, Yasaka et al. 1999; Moseley, Waddington wt al. 2003; Kosazuma, Hashimoto et al. 2004; Kolesnichenko & Popova 1976; Colon, Bhol et al. 2001; Tavakkol, Varani et al. 1999). However, the majority of these organ cultures were used at the laboratory scale to study organ responses to hormones and chemicals in medical or toxicological applications. Therefore, the commercial potential of using *in vitro* culture of cells from harvested organs remains to be demonstrated for the production of mammalian tissue-derived bioactives.

There is evidence in the scientific literature demonstrating that bioactive compounds can be synthesized *in vitro*. For instance, the isolation and *in vitro* culture of bovine luteal cells has demonstrated an increase in progesterone production of 2 to 50 fold in culture (Young, Luderer et al. 1995; Young, Menadue et al. 2005; Rodgers, Lavranos et al. 1995; Rodgers, Vella et al. 1995). Though this success is limited to a research exercise, it provides strong evidence that the amplification of bioactive yield is feasible.

`

An alternative method for the production of bioactives is to use immortalised mammalian cell lines in bioreactors. The current best immortalised mammalian cell culture systems begin the culture with 1 $\times 10^5$cells/ml with an increase in cell density to 1×10^7cells/ml over 2-3 weeks. However, the volume of 1×10^7cells/ml cells in a bioreactor corresponds to only 2-3% of the total culture volume, and the capital costs to achieve this high density are extremely high (Wurm 2004). It would be far more cost-effective to start the bioreactor process with a high density of isolated cells from harvested organs. Millions of tonnes of mammalian organs are produced as low-value by-products of the meat processing industry annually. For instance, Australia produces ca. 27,400 tonnes liver from beef and veal each year at a market price of less than $2/kg. Therefore, the cost of these metabolically active cells from harvested organs is only a tiny fraction of immortalised mammalian cell culture. As these organs are available all year round, significant economic advantage can be expected to accrue to the meat processing industry if high-value bioactive products can be produced by culturing organ-derived cells.

1.1.2 Using Organs for Bioactive Production

Cells that make commercially valuable bioactives, are specialised, differentiated, and tend not to divide or proliferate. The lack of proliferation is not a problem in this project, because there is a constant supply of cells from the meat processing industry. The *in vivo* cellular production of bioactives with commercial potential is technically possible, for example liver hepatocyte cells can make the commercially valuable bioactive compound, transferrin (Goldsworthy, McCartor et al. 1970). The key aim of the Phase 2 of this project is to determine whether the amount of bioactives produced is sufficient for using in a commercial process.

1.1.3 Amplification of Bioactives

One of the main aspects of this project involves identifying methods that promote the amplification or stimulation of bioactives. In the corpus luteum of the ovary, luteal cells make the steroid hormone progesterone. In a fertile cow, the brain-derived luteinizing hormone (LH) stimulates the luteal cells to amplify production of progesterone more than two-fold. In a pregnant cow, embryo-derived chorionic gonadotrophin (CG) stimulates luteal cells to secrete 10 to 20 times more progesterone. These amplification mechanisms are employed routinely in *in vitro* research-based cell culture systems (Young, Luderer et al. 1995; Young, Menadue et al. 2005; Rodgers, Lavranos et al. 1995; Rodgers, Vella et al. 1995). In addition, the starter substrate molecule from which progesterone is synthesised can be obtained cheaply from Sigma and provided to luteal cells in culture systems. This has been demonstrated to increase progesterone production by 2 to 50-fold. In Phase 1 we demonstrated that the principle of stimulation could be applied to chosen bioactives, resulting in an increased the production of a high value bioactive through the addition of a cheaper stimulating substance. In phase 2 we focused on the stimulation of top 5 bioactives identified by MLA.

Objectives

Overall Phase 2 was primarily aimed to further demonstrate the feasibility and scalability of this technology through process optimisations and scale-up from proof-of-concept Phase 1 towards a commercial scale, together with the development of a business case to attract industry investment.

- Compile a comprehensive literature and patent review discussing the top five bioactive targets identified by MLA and review the bioreactors that can be used for producing bioactives using *in vitro* cell culture.

- To establish bioassays to test the efficacy and stability of the target bioactives produced.

- To develop analytical methods and biological assays for target bioactives as indicated from market research conducted by MLA.

- To transfer technology to bovine organs at laboratory scale, demonstrating disaggregation, viability and bioactive amplification.

- To test the ability to amplify 1-2 selected bioactives in crude organ slices or clumps for fast tracking to commercialisation.

- Indentify alternative bioactives and optimise amplification signals using a proteomics approach

- Complete laboratory scale downstream purification of bioactives selected for scale-up in the bioreactor system.

- To demonstrate the feasibility and scalability of the integrated process in a laboratory-scale proof-of-concept bioreactor and the bench-scale demonstration

`

bioreactor for stimulated bioactive production from minced organ culture and/or in vitro cell culture of sheep hepatocytes.

(a) Determine the culture format that maximizes production of bioactives and enables easy scale-up.

(b) Selection of a suitable bioreactor system.

(c) Establish a bench-scale bioreactor system for proof-of-concept demonstration

- To assist MLA for the development of a comprehensive business and market analysis of the target bioactives, enabling successful industrial engagement in the project.

The completing of these specific aims help to demonstrate the proof of concept that producing bioactives using *in vitro* cell culture can add value to the meat processing industry and that the process is scalable.

Methodology

The detailed protocols and developed Research Operating Procedures for each stage of the process are presented in Appendix 1 of this report.

Liver

1.1.4 Organ Collection

The collection of liver was performed at the meat processing facility, T&R, following guidelines and Occupation Health and Safety Policies as per Research Operating Procedure 1: T&R Protocols. The collection of liver required the sterilisation of equipment, such as scissors, scalpel blades, forceps and collection pots. Sterile transport medium (RPMI) was prepared and 10-150ml was chilled to 4^{o}C overnight in the collection pots, depending on the amount of liver required. The liver was collected in sterile trays and liver segments removed using scissors and immediately placed into the collection medium, which was stored on ice. The unused organ was returned to the line and the organ transported to the laboratory on ice as detailed in Research Operating Procedure 2: Collection of Animal Tissue.

`

1.1.5 Disaggregation of liver tissue

Liver tissue was disaggregated using both small and large scale disaggregation methods, which are outlined in Research Operating Procedure 11: Mincing of liver samples and Research Operating Procedure 12: Disaggregation of liver tissue.

1.1.6 Isolation and plating of liver cells

Liver cells were isolated from contaminating red blood cells by using two centrifugations at 50xg and resuspending the cells in serum-free HMM. Any remaining liver tissue was removed by passing the cell suspension through a cell filter mesh as detailed in Research Operating Procedure 13: Isolation of liver cells. Cell numbers and viability were assessed by trypan blue exclusion assay using a haemocytometer as per Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

Liver cells were plated into 96 well or 6 well plates or T25, T75 or T175 flasks at a cell density of approximately 2.1 x 10^5 cells per cm^2 as per Research Operating Procedure 14: Liver cell plating.

1.1.7 Amplification of target bioactives from liver cells

Stimulatory factors were dissolved in the correct solvent and master mix solutions of the correct medium containing the stimulatory factor at the correct concentration were prepared following Research Operating Procedure 15: Stimulation of liver cells. The fresh medium containing the stimulatory factor was added to the flask and cells incubated for 48 hours at 37^{o}C, 5% CO_2. The medium was collected and assayed by ELISA.

Analytical Methods

1.1.8 ELISA

ELISA (Enzyme-linked Immunosorbent Assay) was the optimal method for the detection and quantification of target bioactives as it is a highly specific method that allows for high through-put and accurate quantification, particularly at low concentrations. ELISAs for the detection of progesterone, fibroblast growth factor-2 and platelet derived growth factor were developed in the laboratory as no commercial kits were available to detect the sheep isoforms.

The FGF2 ELISA utilises the R&D FGF2 ELISA kit using a bovine standard to generate a standard curve. The ELISA is a sandwich ELISA which involves the use of two antibodies that detect different regions of the same protein, one antibody captures the protein, the other

`

allows for detection using a chemical reaction, as described in Research Operating Procedure 16: basic fibroblast growth factor ELISA.

1.1.9 Enzymatic AP assay

An enzymatic assay was used for the detection of alkaline phosphatase activity in samples from both ovine and bovine. The principle of the assay is to measure the conversion of a substrate into a product that can be measured by a spectrophotometer at 405nm. The assay measures the change in absorbance over time and the slope of the line is used to calculate the activity of alkaline phosphatase. See Research Operating Procedure 18: Enzymatic AP assay.

Bioreactors

The goal of this project task is to develop an integrated system for bench-scale demonstration, ready for further scale-up. The key to demonstrating the proof-of-concept production of bioactives with commercial potential will be determined by the development of a bioreactor system that stimulates the production of bioactives and enables scale-up. The techniques established throughout this project were oriented for use in such a bioreactor system.

1.1.10 Stirred tank

The first system tested was a traditional stirred tank bioreactor that consisted of 2 sets of rotating flat blade impellers in a 2 litre vessel. Temperature was monitored though a temperature probe and heating achieved through a computer controlled heating rod. Sterile air was bubbled through the chamber using a sparger. Minced organ culture was performed in this traditional stirred tank bioreactor See ROP 19: Stirred tank bioreactor.

1.1.11 Wave

The WAVE Bioreactor (GE Healthcare) consists of a disposable chamber called a cellbag that is placed on a special rocking platform. The rocking motion of this platform induces Waves in the culture fluid. These Waves provide mixing and oxygen transfer, resulting in a perfect environment for cell growth. The speed and angle of the rocking platform can be altered to obtain the optimal conditions of culture survival and mixing in a low shear stress environment. Temperature in the cellbag is controlled using a heating element and temperature sensor that are located within the rocking platform. Air and carbon dioxide were mixed and pumped into the bag through a sterile filter. The bioreactor requires no cleaning or sterilization, providing the ultimate ease in operation and protection against cross-contamination. Minced organ culture and disaggregated cell cultures were performed using the WAVE bioreactor, see ROP 20: WAVE bioreactor.

`

1.1.12 FibraStage

FibraStage from New Brunswick Scientific consists of 500mL culture bottles, pre-filled with Fibra-Cel disks and operates by compressing and expanding bellows at the base of each bottle. This ensures constant exchange of nutrients and removal of waste. The temperature and carbon dioxide level were maintained by placing the FibraStage into a cell culture incubator. Disaggregated cell culture was performed in using the FibraStage, see ROP 21: FibraStage bioreactor.

Protein Purification

1.1.13 Ammonium sulphate precipitation

A common first step in the purification of proteins is precipitation with ammonium sulfate $(NH_4)_2SO_4$. This is performed by adding increasing amounts of ammonium sulfate and collecting the different fractions of precipitated protein. One advantage of this method is that it can be performed inexpensively with very large volumes. For details of this method see ROP 22: ammonium sulphate precipitation.

1.1.14 DEAE ion-exchange chromatography

Ion-exchange chromatography separates compounds based upon charge. Molecules can be either anions (having a negative charge) or cations (having a positive charge). Diethylaminoethyl cellulose (DEAE) is a positively charged resin used in ion-exchange chromatography, and binds negatively charged molecules into the matrix, until released by increasing the salt concentration in the column elution buffer. Binding to and elution from DEAE can be optimised by changing the buffer, pH and salt concentration. The optimised conditions for the purification of AP are presented in ROP 23: DEAE chromatography.

Proteomics

The use of proteomic methods for the detection, quantification and identification of proteins is a method we utilised for the detection of unknown, or non-target bioactives.

1.1.15 Depletion of BSA

Due to the large amount of albumin present in the medium, we removed the albumin by a number of methods, including precipitation and column purification. Minced organ cultures could be analysed using an unprocessed sample. See Research Operating Procedure 24: Depletion of BSA.

`

1.1.16 In-solution digestion

Protein samples of interest can be directly subjected to an enzymatic digestion and analysed via mass spectrometry. This is an approach to get a rough idea of all the proteins present in the sample, without having to run any 2D gels first. See Research Operating Procedure 25: In-solution digestion.

1.1.17 2D gel electrophoresis (2D GE)

Conventional 2D gel electrophoresis was used to characterize the proteome of the minced organ culture. 2D GE is a powerful technique capable of resolving several thousand proteins based on their charge in the first dimension and size in the second. See Research Operating Procedure 26: 2D gel electrophoresis (2D GE).

1.1.18 Two-dimensional differential gel electrophoresis (2D-DIGE)

2D-DIGE enables us to resolve two different samples on the same 2D gel by labelling them prior to electrophoresis with spectrally resolvable fluorescent CyDyes (Cy2, Cy3, and Cy5). This overcomes gel-to-gel variations, which can lead to problematic detection and quantification of differences in protein expression. This approach was used to directly compare the differences in protein expression after 0hrs compared to 48hrs culture time, 48hrs non-stimulated compared to 48hrs stimulated minced organ culture, as well as 48hrs minced organ culture compared to a total protein extract of the bovine minced tissue. Proteins of interest could then be subjected to peptide mass fingerprint analysis by mass spectrometry. See Research Operating Procedure 27: Two-dimensional differential gel electrophoresis (2D DIGE).

Results and Discussion

General Achievements

1.1.19 Phase 2 literature Review

The Phase 2 literature review focused on gaining greater knowledge about the top 5 bioactives that were identified by MLA as candidates for production using the in vitro cell culture process. The literature was examined to determine stimuli that could used to amplify the production of each bioactive target, protocols that could be used to detect and quantify bioactive production, and identification of methods for purifying the target bioactive from organs and cell culture. A comprehensive review of bioreactor systems that could be used for liver cell culture and their scalability to commercialisation was also performed.

Heparin

Heparin is a highly-sulphated glycosaminoglycan that inhibits the blood clotting factor thrombin by affecting the activity of the co-factor antithrombin (Rosenberg 1975). There was no evidence of heparin synthesis in liver tissue or hepatocytes in the scientific literature. The production of heparin appears to be limited to mast cells or vascular endothelial cells (Marcum, Fritze et al. 1983; Andrade-Gordon, Wang et al. 1992).

Stimulation of Heparin

The amplification of heparin associated factors has been relatively well reported in the scientific literature however the amplification of heparin itself has not been published (Kaji, Ohkawara et al. 1994).

Detection of Heparin

There are many heparin detection and quantification kits available on the market. All kits utilise a similar method of binding with antithrombin and measuring coagulation efficiency.

Purification of Heparin

A modern method for the purification of heparin from beef intestinal mucosa was discovered that used tissues extraction with papain and alkaline conditions followed by anion-exchange chromatography to purify the glycosaminoglycans (Volpi 1996).

This literature review of bioactives demonstrated that Heparin was an unsuitable target for this project as there is no direct evidence for its synthesis in liver cells and a lack of information available on the amplification of its expression. Therefore heparin was removed from the list of possible targets because it was not a viable bioactive target that could demonstrate the feasibility of this project.

Alkaline Phosphatase

Alkaline phosphatase (AP) catalyses the hydrolysis of organic phosphate esters, thereby releasing inorganic phosphate. APs have been implicated in processes such as the regulation of protein phosphorylation, collagen phagocytosis, cell growth, apoptosis, defence against bacterial infection and cellular migration during embryonic development (Chan and Stinson 1986; Kaplan 1986; Zackson and Steinberg 1988; Hui, Hu et al. 1993; Hui, Tenenbaum et al. 1997; Poelstra, Bakker et al. 1997; Poelstra, Bakker et al. 1997; She, Mukherjee et al. 2000).

Stimulation of Alkaline Phosphatase

AP activity is often dependent upon the cell type tested and has been shown to vary between different cell lines of similar origin. Many well established factors were identified that can increase production of AP as single agents or combination, for example retinoic acid and granulocyte-cell stimulating factor resulted in a 197-fold increase in AP above baseline (Gianni, Terao et al. 1994).

Detection of Alkaline Phosphatase

The detection and quantification of alkaline phosphatase is generally conducted using an enzymatic assay that hydrolyses phosphate esters in an alkaline environment, resulting in the formation of an organic radical and an inorganic phosphate. The Department of Medical Biotechnology has an established protocol for the measurement of AP activity (Appendix ROP 18).

Purification of Alkaline Phosphatase

Purification of alkaline phosphatase from human liver had previously been reported whereby the liver tissue was homogenised in a water:butanol mix, followed by acetone treatment with precipitate collection via dialysis and the enzyme was purified by column chromatography (Sugiura, Hirano et al. 1975). The method we developed used a similar strategy with the omission of the solvent extraction step (see appendix ROP 22 and 23).

Fibrinogen

Fibrinogen is a complex dimeric glycoprotein constituted by six chains of three pairs of non-identical proteins that is predominantly synthesised by hepatocytes in the liver and secreted into the plasma. Fibrinogen is the final component in the coagulation cascade where thrombin converts it into fibrin that ultimately leads to the formation of a fibrous clot in response to vascular disruption (Koenig 2003).

Stimulation of Fibrinogen

Fibrinogen can be amplified by a number of different stimuli, with the most studied compounds being interleukin 6 (IL6) and dexamethasone. Dexamethasone induction has been shown to cause a 10 to 30-fold increase in mRNA levels after 60 hours (Bhattacharya and Holland 1991).

Detection of Fibrinogen

`

The most efficient way to detect and quantify fibrinogen from cell culture medium is via an ELISA kit. There are several ELISA kits available on the market however there are no kits available for the specific detection of the ovine or bovine protein. The human ELISA fibrinogen kit are available, from GenWay (catalogue number 40-288-22856) and this adapted for uses with bovine/ovine samples.

Purification of Fibrinogen

The purification of fibrinogen from plasma is simplified because of its relatively high concentration and unusual solubility characteristics. Precipitation techniques used for the purification of fibrinogen includes the use of Ammonium sulphate, sub zero ethanol (Ware and Lanchantin 1954) and cationic detergents (Kurioka, Inoue et al. 1975) .

basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF) is one of the best characterised members of a family of at least nine structurally related heparin-binding growth factors of 18 kDa in size (Burgess and Maciag 1989; Baird 1994). bFGF stimulates the proliferation and modulates the differentiation of a variety of cell types and plays a major role in wound healing and angiogenesis (Gospodarowicz, Ferrara et al. 1987; Gospodarowicz 1991). Basic FGF is synthesised in multiple parts of the body, with the liver being a major site. Both the mRNA and protein are readily detected in isolated liver cells, including hepatocytes and hepatic stellate cells (Peng, Wang et al. 2005).

Stimulation of bFGF

bFGF has been demonstrated to be induced by retinoic acid (RA) inducing mRNA expression 2 to 3-fold and increased protein secretion into the cell medium by approximately 10-fold (Gaetano, Catalano et al. 2001).

Detection of bFGF

The amount of bFGF present in a sample can be detected by ELISA (R and D Systems, catalogue number DY233). We have previously shown in Phase 1 that this kit can detect both the ovine and bovine isoforms of this protein.

Purification of bFGF

Techniques used for the purification of bFGF exploit its heparin binding properties, using a combination cation exchange CM-sephadex chromatography and heparin-sepharose columns (Presta, Rusnati et al. 1988, Brigstock, Heap et al. 1990).

`

(Apo)ferritin

Ferritin is a ubiquitous iron-storage protein with a molecular mass of approximately 440 kDa (Lawson, Artymiuk et al. 1991). Ferritin is found to be ubiquitously expressed in animals however most research has focussed on the expression of ferritin in heart and liver tissue with the liver showing a higher level of mRNA (Cairo, Rappocciolo et al. 1991).

Stimulation of Ferritin

The amplification and mechanisms involved in the amplification of ferritin synthesis has been extensively studied, the addition of iron to adult primary hepatocytes has been shown to cause a 40-fold increase in ferritin production (Lescoat, Hubert et al. 1991).

Detection of Ferritin

The detection of ferritin levels in blood and serum is predominantly performed by enzyme-linked immunosorbent assay (ELISA). There are currently no ELISA kits on the market for the detection of bovine or ovine ferritin therefore using the Genway human ferritin ELISA kit (40-052-115015) was attempted.

Purification of Ferritin

Purification of ferritin from homogenised tissues has been achieved using simple methanol precipitation (Cham, Roeser et al. 1985) or a combination of gel filtration and anion exchange chromatography columns (Suryakala and Deshpande 1999).

Bioreactors

To sustain this high cell number and productivity, a steady supply of nutrients and continuous removal of metabolic by-products must accompany the cell propagation and production phases (Liu, Liu et al. 2006). To enable the generation of high density cultures of hepatocytes, a substrate must be utilised. The currently used micro and macro carriers were comprehensively reviewed.

Single-use systems have recently been identified as having benefits over re-useable systems. The accepted benefits include the decreased risk of contamination, elimination of clean-in-place and steam-in-place procedures and decreased facility start-up times. In a study (LoMonaco 2006) it was found that it was economically more beneficial to use single-use

`

systems over re-useable systems, particularly because of the elimination of the clean-up and sterilisation steps, there was a reduction in costs and cycle times. A further advantage is that because the system is enclosed, it can be used in unclassified areas and are cGMP compliant. Furthermore, these systems are more beneficial to the environment and lower a companies carbon footprint (Sinclair 2008). The two systems identified for testing where the Wave (GE Healthcare) and the FibraStage (New Brunswick Scientific).

The Wave Bioreactor consists of cells and culture medium that are contained within a disposable chamber, the CellBag, which is placed on a rocking platform. The rocking motion induces waves to mix and transfer oxygen to the culture medium to create an optimal environment for cell growth. The system is suitable for cGMP commercial production without the use of an incubator and can be easily connected to other closed system cell process devices. The system is also scalable from volumes of 100ml to 500 litres using up to 1 x 10^7 cells/ml.

The FibraStage system is a new, high-yield disposable cell culture system that has been shown to produce high yields of proteins, virus or cell mass from anchorage-dependent or suspension cultures. The FibraStage can hold up to 4 bottles, each containing 500ml of medium and contain 10g of Fibra-Cel disks for the cells to adhere to. Each bottle provides enough Fibra-Cel disks to provide a surface area of 12,000cm^2 and a yield of up to 2.4 x 10^{10} cells (equivalent to 70 x T175 flasks). Gas and nutrient exchange occur by placing the bottles into the FibraStage which slowly raises and lowers the media level in the bottles.

1.1.20 Collaborations and Organ Transport

In Phase 1 of this project a formal approach was made to T&R Pastoral Pty Ltd, Murray Bridge through their Operations Manager to establish their collaboration with this project and allow the research team regular access to the raw materials. Prior to access to the processing floor, all collection staff were required to be tested and if necessary vaccinated against Q-fever and to undergo induction under the Occupational Health and Safety guidelines of T&R Murray Bridge.

In Phase 1 of this project we established collection methods that used a sterile medium (HBSS or RPMI). This medium facilitated a high cell viability through an even temperature distribution, the prevention of cell dehydration and the maintenance of physiological conditions, such as osmolarity and pH. The aim of collection and transport was to cool the cells quickly to reduce metabolic activity. The inclusion of serum or growth factors was not required. Cells that were disaggregated from tissue collected and transported in this way were observed to maintain viability and remain sterile (Milestone 1.2).

`

1.1.21 Commercialisation Support

Throughout this project we have provided MLA with commercialisation support for the continuation of this project as a new value adding process for the meat processing industry. We prepared documents and presentations for industrial engagement with Flinders and this research project and have initiated industry commitment to the technology through a meeting with MLA and T&R. We have also engaged with MLA and a patent attorney Tom Gumley for discussions on the intellectual property within the project. It was suggested that we proceed with the technology as a trade secret as the process had no definitively identifiable inventive steps that would be required for the technology to be successful, therefore patents would need to be sought as novel processes for the production of each bioactive.

1.1.22 Research Operating Procedures

During this project we have standardised many protocols for the collection, isolation and culturing of liver and luteal cells. When standardising collection protocols we have considered the need to integrate future commercial activities with existing meat processing procedures. We have also developed methods for the detection and quantification of target bioactives. Each of these protocols has been established into a Research Operating Procedure and we have developed 32 protocols throughout this project. These protocols are shown in Appendix 1 of this report.

Development of Analytical assays

1.1.23 Alkaline Phosphatase

An enzymatic assay was used to detect and quantify alkaline phosphatase activity from ovine and bovine samples. The Department has established an alkaline phosphatase assay that measures the conversion of a substrate to a product by spectrophotometer and the alkaline phosphatase concentration can be calculated. This assay was used to detect alkaline phosphatase activity in medium from ovine cell samples available (see appendix ROP 18).

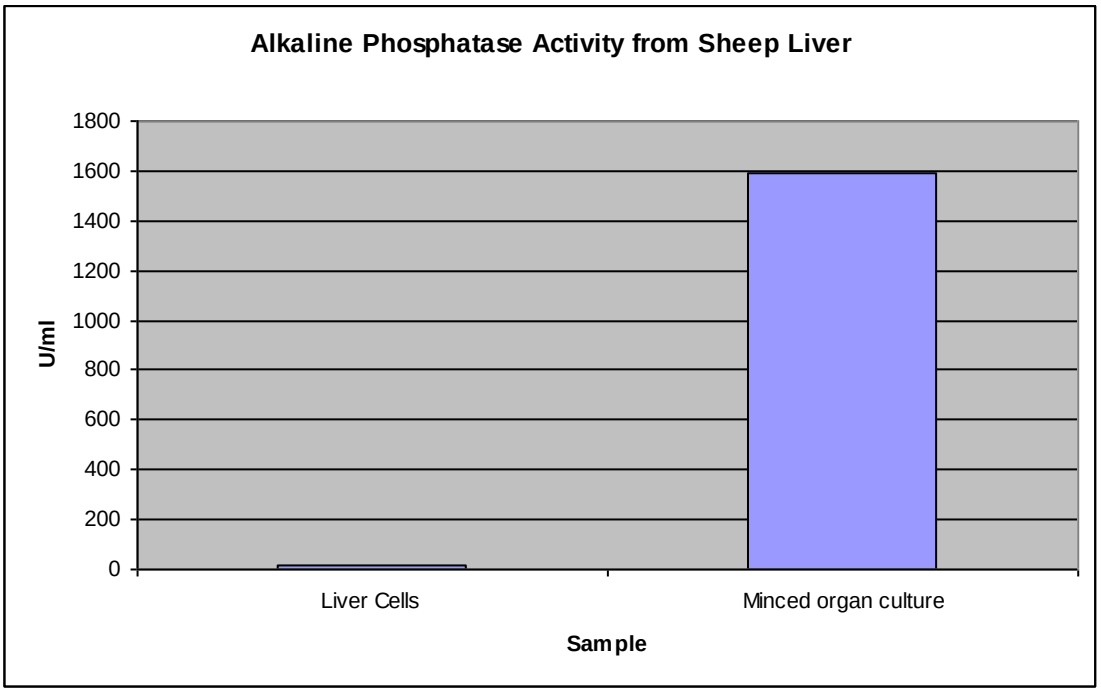


Figure 1. Sheep liver cells were isolated by standard methods and plated at a density of 2.5 x 10^6 cells per well of a 6 well plate 48hrs hours. 1.5g of minced was cultured in 2ml of HMM Medium for 48hrs. Medium was collected and assayed for alkaline phosphatase using the AP activity assay (ROP 18). The conversion of the slope from the change in absorbance over time was calculated to Units/ml. Data is presented as the mean from a single measurement of each sample.

Data presented in Figure 1 shows that the level of alkaline phosphatase was higher in medium from minced organ culture when compared to the concentration in the cell culture medium, indicating that minced organ culture may represent a superior method of AP production. This data indicates that the sheep isoform of alkaline phosphatase can be detected and quantified with the enzymatic assay.

1.1.24 Ferritin

The most common method of detecting and quantifying ferritin levels is via an ELISA. We purchased a human ferritin immunoassay kit from MP Biomedicals (Catalogue Number 07BC-1025) to determine whether ovine and bovine proteins could be detected. Both the human ferritin standard (from the kit) and a horse ferritin positive control (horse was used as there are no ferritin products available from bovine or ovine sources) were used to generate a standard curve with serial dilutions from a maximal concentration of 1000ng/ml. The ferritin ELISA kit is unable to detect the equine standard or ferritin from ovine or bovine serum. Without a suitable detection assay ferritin could not be continued as a target bioactive for this project.

`

1.1.25 Fibrinogen

The most common method of detecting and quantifying fibrinogen is via an ELISA. We have purchased a human Fibrinogen ELISA kit from GenWay (Catalog Number 40-288-22856) and tested the ability for a recombinant bovine protein, bovine serum, sheep serum and sheep liver cell culture medium to be detected in this assay.

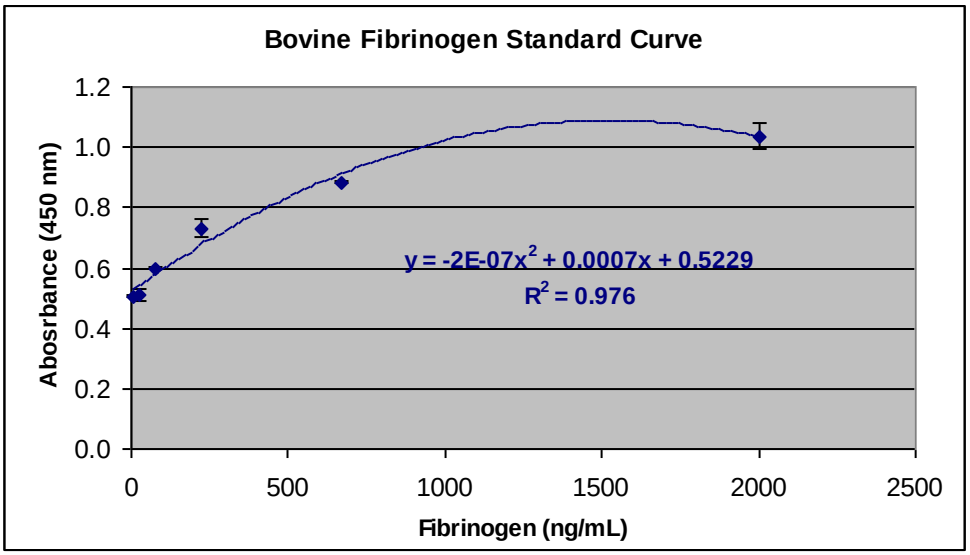


Figure 2. The bovine fibrinogen standard was prepared, serially diluted and subjected to the fibrinogen ELISA and plotted on its own axis. Data is presented as the mean ± standard deviation from duplicate samples.

Figure 2 shows that there is an increase in the absorbance as the fibrinogen concentration increases. Although the assay is not as sensitive at detecting the bovine standard as compared with the human standard, the polynomial standard curve has a consistent shape, indicating that the assay is able to detect and quantify the bovine standard.

We next tested whether we could detect fibrinogen in conditioned cell culture medium from sheep liver cells, cow liver cells and crude sheep liver medium. The crude sheep liver medium was liver tissue that was minced and immediately placed into the incubator in cell culture medium for 24 hours. All media was collected 24 hours post-seeding and assessed for fibrinogen levels by ELISA (Figure 3).

`

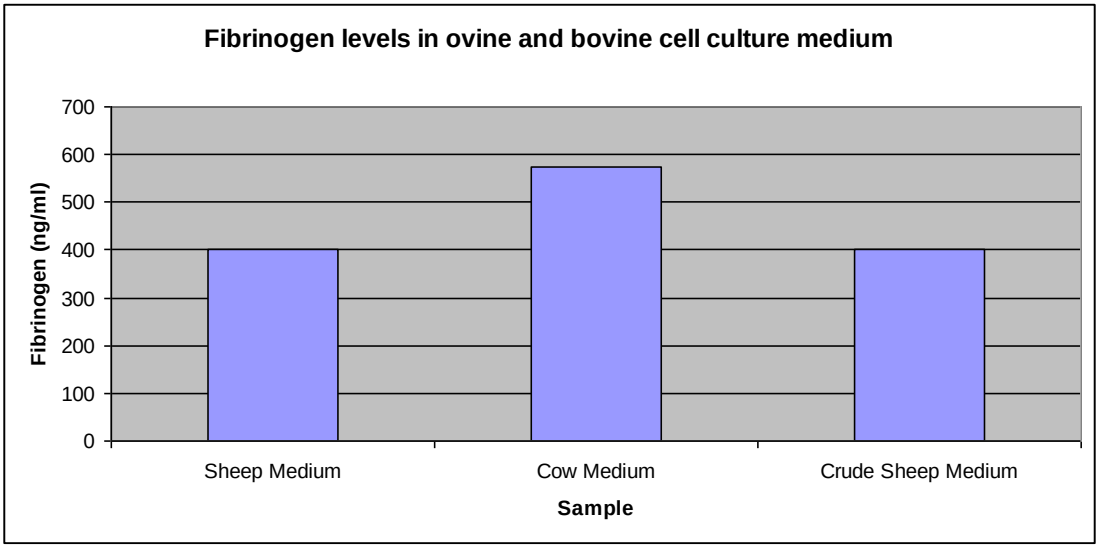


Figure 3. Bovine and sheep conditioned cell culture medium and sheep crude liver culture medium was collected after incubation with cells or liver tissue after 24 hours. The medium was then subjected to the fibrinogen ELISA. Data is presented as the mean from duplicate samples.

Absorbance values were calculated to give fibrinogen concentrations using the standard curve illustrated in Figures 2. In cell culture medium, fibrinogen was readily detected from both ovine and bovine cells at levels of approximately 400 and 575ng/ml, respectively. This data demonstrates the successful development of an analytical method for the detection and quantification of fibrinogen from ovine and bovine sources.

1.1.26 Basic Fibroblast Growth Factor

The detection of basic fibroblast growth factor (bFGF) will be detected by ELISA. In Phase 1 of this project we demonstrated the ability of a bFGF ELISA kit to detect and quantify ovine levels from serum samples and cell culture medium due to the lack of recombinant ovine bFGF in the market. The ability for bovine bFGF to be detected using the same kit was measured with recombinant bovine protein and with bovine serum.

`

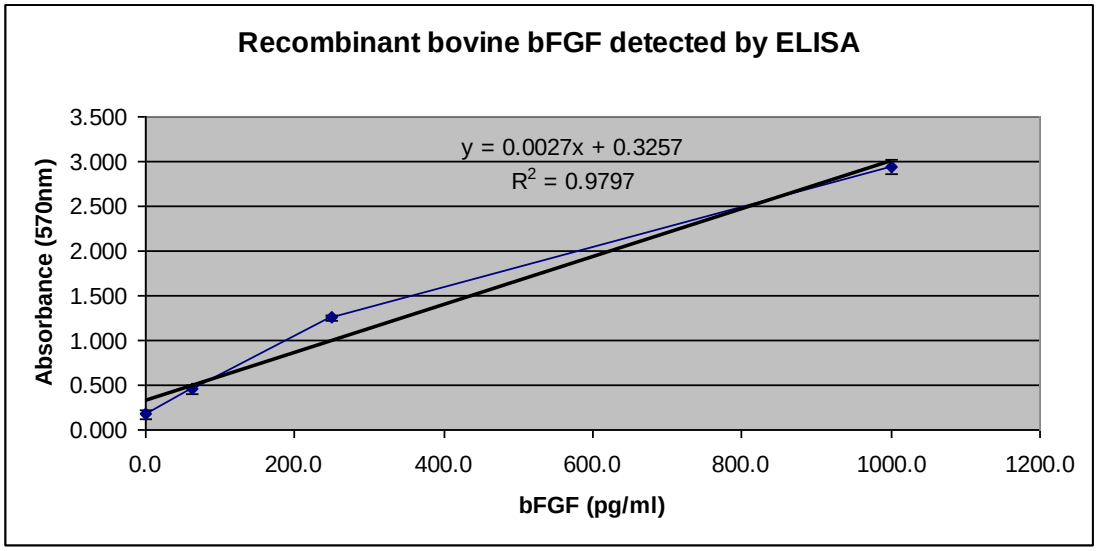
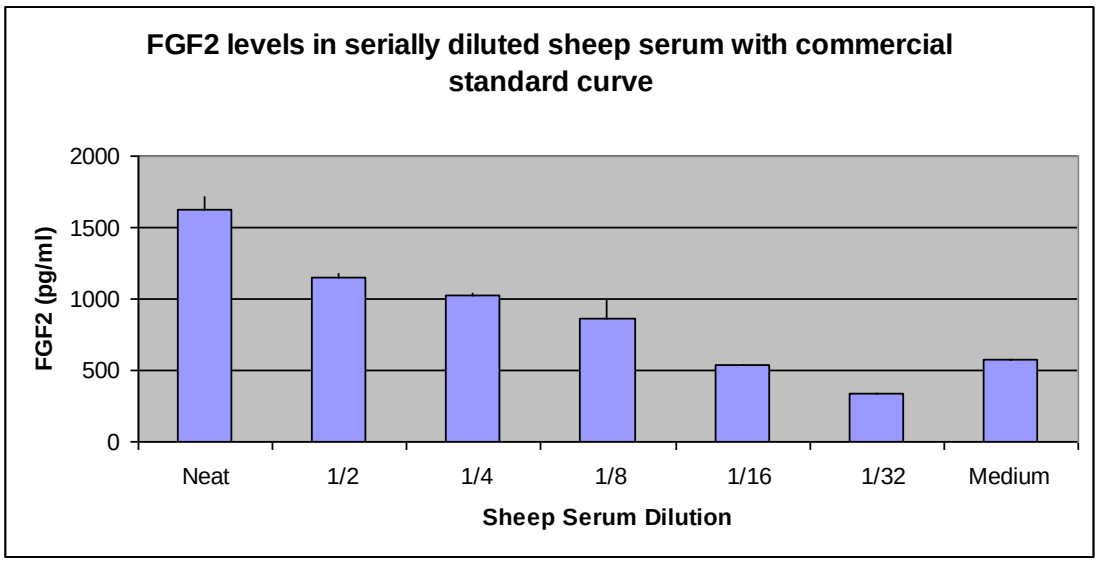


Figure 4. Recombinant bovine bFGF was serially diluted and subjected to ELISA. Data is presented as the mean ± standard deviation from three replicate wells.

Figure 4 shows that recombinant bovine bFGF protein results in a dose-dependent increase in absorbance when subjected to analysis in the ELISA assay. This result is consistent with the human protein standard supplied in the kit.

To determine whether bovine bFGF could be detected in foetal bovine serum, two-fold serial dilutions were made and subjected to ELISA (Figure 5).



`

Figure 5. Two-fold serial dilutions of foetal bovine serum were subjected to analysis by bFGF ELISA. Data is presented as the mean ± standard deviation from three replicate wells.

Figure 5 demonstrates that the larger the dilution of the bovine serum, the less detectable bFGF was measured. Notably, primary sheep liver cells cultured in medium for 24 hours showed similar levels of bFGF to that of the 1/16 dilution of bovine serum.

These data demonstrate that the ELSIA kit can detect both recombinant bovine bFGF and the presence of bFGF in serum. This indicates that the bFGF analytical method is established and viable for the detection and quantification of both ovine and bovine bFGF.

1.1.27 Transferrin

The transferrin detection method chosen utilises a radial immunodiffusion assay from IR Biologicals. This assay involves pipetting a small volume of sample into the prepared wells in the agar containing an anti-transferrin antibody and allowing the sample to diffuse into the agar. A “ring” appears and the larger the ring, the higher the concentration of transferrin in the sample.

In this assay, a positive human control was loaded into three wells as recommended by the manufacturer. We replicated these standard concentrations using the bovine transferrin standard and assessed the ability of the assay to detect transferrin in bovine and ovine serum and in cell culture medium conditioned by sheep liver cells. The immunodiffusion assay can detect the human transferrin protein, but is unable to detect the bovine or ovine proteins. Without a suitable analytical assay transferrin could not continue as a bioactive target

1.1.28 Overall progress with assay development

We demonstrated the successful development of three analytical methods for the target bioactives; bFGF, Fibrinogen and Alkaline Phosphatase. These methods provided us with robust and reliable methods to analyse the production of these three bioactives using the in vitro cell culture process using disaggregated cells and minced organ culture.

The development of bioactive stimulants

The development of methods to amplify the production of bioactives is a critical stage of this project. The ability to induce bioactive production above levels currently harvested from animal organs or blood gives this technology the competitive advantage. Before scaling up to a bioreactor system we needed obtain reliable and repeatable data in the lower cost cell culture systems and consolidating the data for the amplification signals is crucial for

`

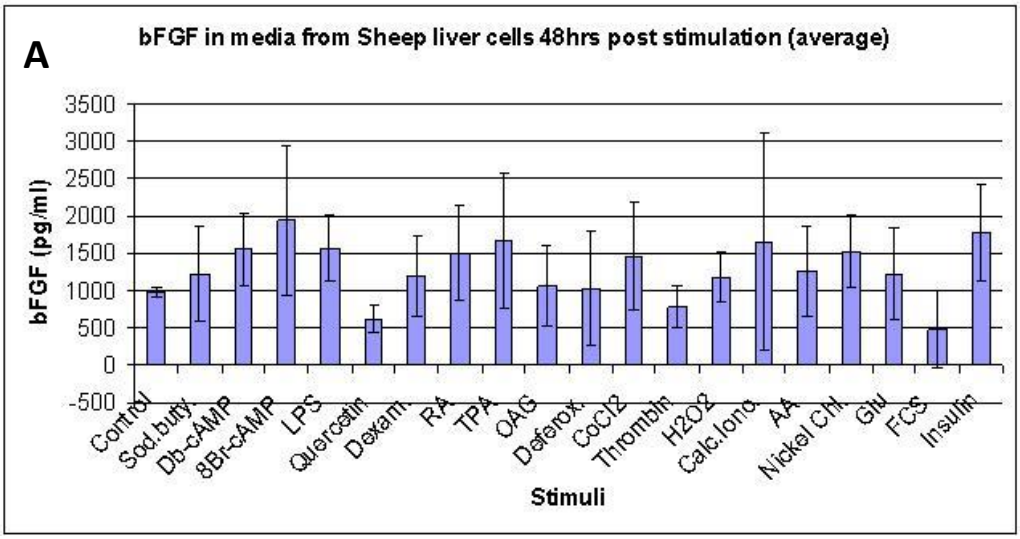
progressing into the more costly scaled-up bioreactor systems. We performed a large scale screen of stimulants using multiple preparations of disaggregated ovine liver cells.

Bioactive	Stimuli	Concentration
AP	Sodium Butyrate	2mM
	N6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (Db-cAMP)	1mM
	8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (8Br-cAMP)	1mM
	Lipopolysaccharides from *Escherichia coli* 055:B5 (LPS)	5 ug/mL
	Quercetin	50uM
AP / bFGF	Retinoic Acid	10uM
Fibrinogen	1-Oleoyl-2-acetyl-sn-glycerol (OAG)	200ng/ml
Fibrinogen/ bFGF	Deferoxamine	250uM
	Cobalt(II) chloride hexahydrate (CoCl2)	250uM
	Phorbol 12-myristate 13-acetate (TPA/PMA)	100nM
bFGF	Thrombin	3U/ml
	Hydrogen Peroxide	100 uM
	Calcium Ionophore	100nM
	Arachidonic Acid	5uM
	Nickel Chloride	250uM
	Glucose	25mM
	FCS	10%
	Insulin	100nM
AP / Fibrinogen/ bFGF	Dexamethasone	100nM

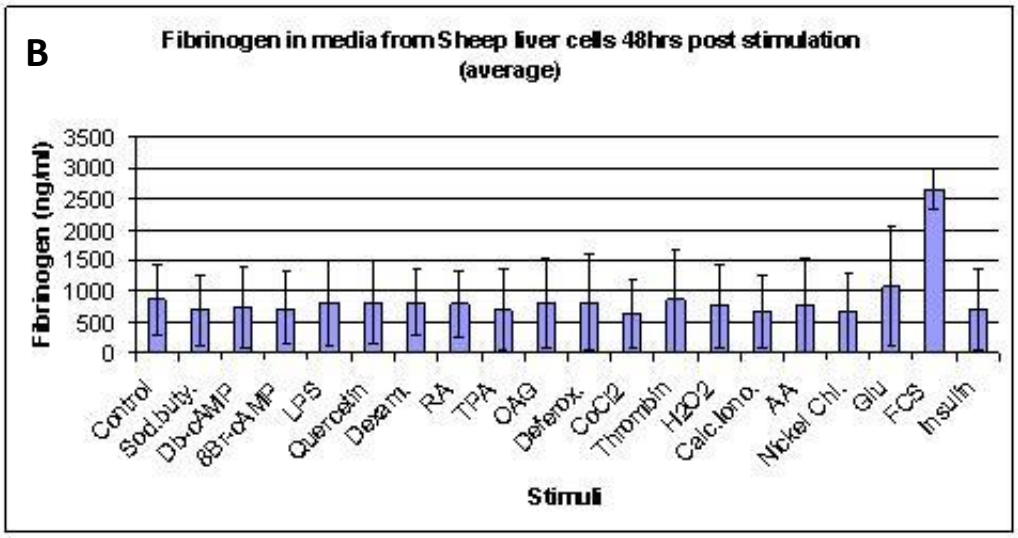
`

Table 1. Summary table showing the target bioactives and a sample of amplification signals that have been shown to induce the expression of AP – Alkaline phosphatase, bFGF2 – basic fibroblast growth factor and fibrinogen. The displayed concentrations are those used for the initial screen of bioactive stimuli (Fig 6.).

A


bFGF in media from Sheep liver cells 48hrs post stimulation (average)
bFGF (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
-500
Control
Sod.buty.
Db-cAMP
8Br-cAMP
LPS
Quercetin
Dexam.
RA
TPA
OAG
Deferox.
CoCl2
Thrombin
H2O2
Calc.Iono.
AA
Nickel Chl.
Glu
FCS
Insulin
Stimuli

B


Fibrinogen in media from Sheep liver cells 48hrs post stimulation (average)
Fibrinogen (ng/ml)
3500
3000
2500
2000
1500
1000
500
0
Control
Sod.buty.
Db-cAMP
8Br-cAMP
LPS
Quercetin
Dexam.
RA
TPA
OAG
Deferox.
CoCl2
Thrombin
H2O2
Calc.Iono.
AA
Nickel Chl.
Glu
FCS
Insulin
Stimuli

`

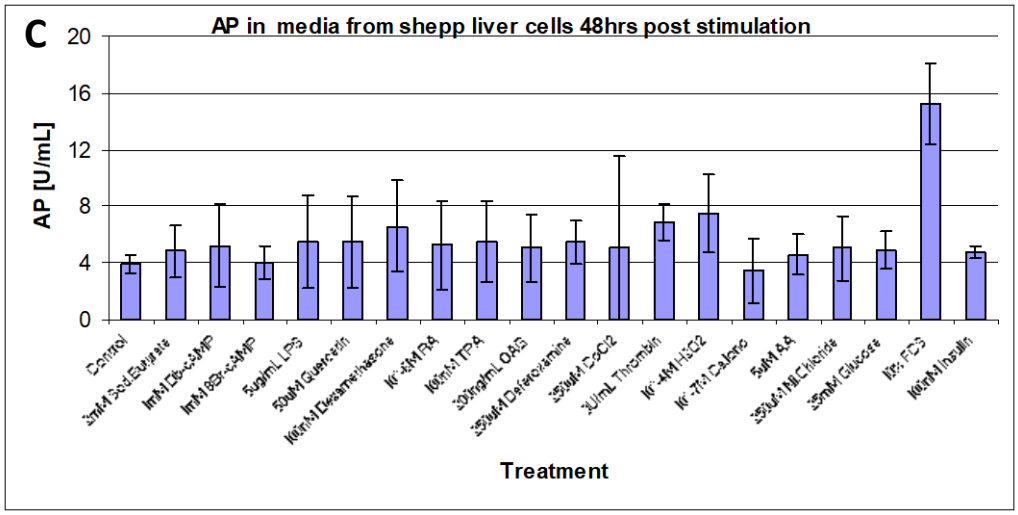
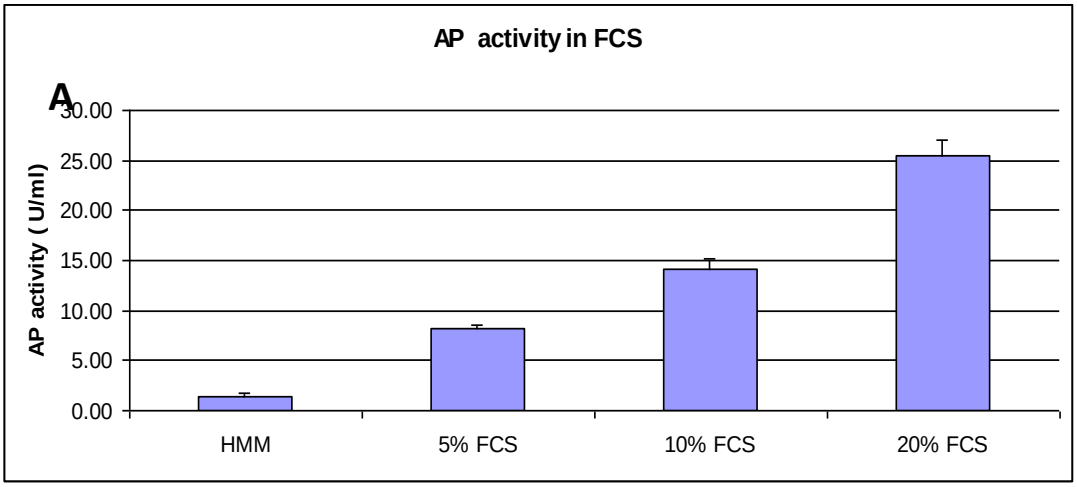
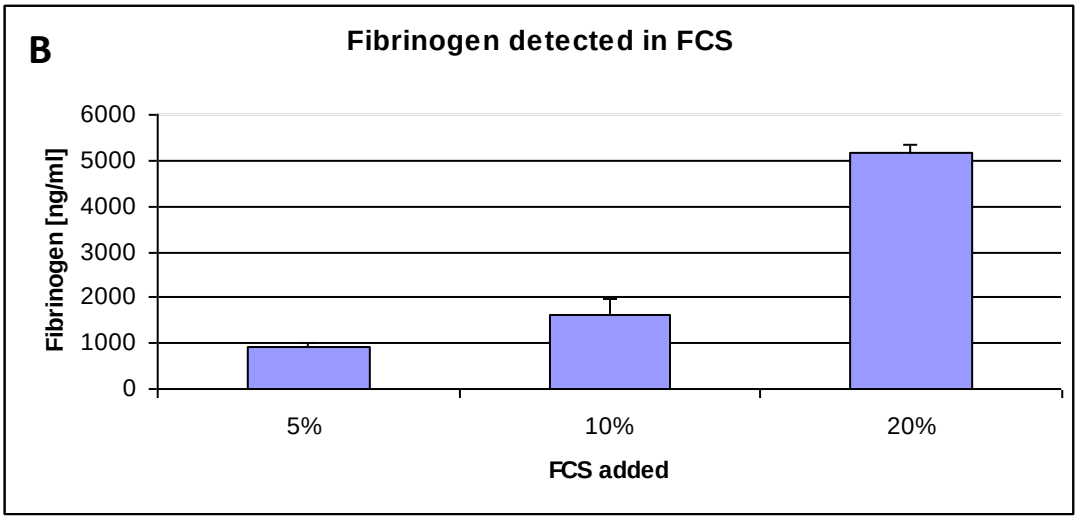


Figure 6: Disaggregated liver cells were plated into 6-well plates at 2.5x10^6 cells per well with HMM containing the appropriate stimulus and incubated for 48 hrs. Medium was collected and subjected to assays for bFGF (A), fibrinogen (B) and alkaline phosphatase (C). The large scale screen of stimuli was performed using a medium concentration of each stimulant as shown in table 1. Data is presented as the mean +/- standard deviation from 3 individual liver preparations that was plated into triplicate wells (n=3).

Figure 6 demonstrates that the production of bFGF was not significantly amplified by the range of stimuli tested (Fig 6A). Increased production of Fibrinogen (Fib 6B) and AP (Fig 6C) was observed when FCS was added to the culture medium. An investigation into the presence of fibrinogen and AP in FCS discovered high background levels in the serum (Figure 7). Therefore the incubation of medium on the cells showed apparent increases in the amount of bioactive produced; this actually represents the detection of bioactive that was present in the FCS added to the medium.

`


AP activity in FCS
A
AP activity (U/ml)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
HMM
5% FCS
10% FCS
20% FCS


B
Fibrinogen detected in FCS
Fibrinogen [ng/ml]
6000
5000
4000
3000
2000
1000
0
5%
10%
20%
FCS added

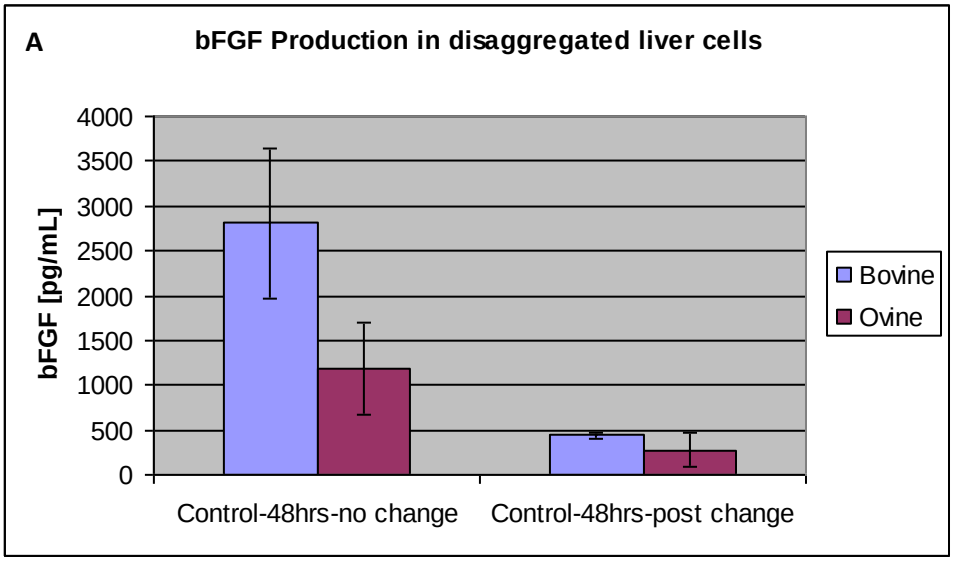
`

Figure 7: FCS was added to HMM that was not incubated on cells and it was assessed as a back ground control for FCS stimulated cells. This medium was subjected to the standard AP activity (A) and fibrinogen (B) assays. Data represents triplicate wells of the FCS used in these experiments.

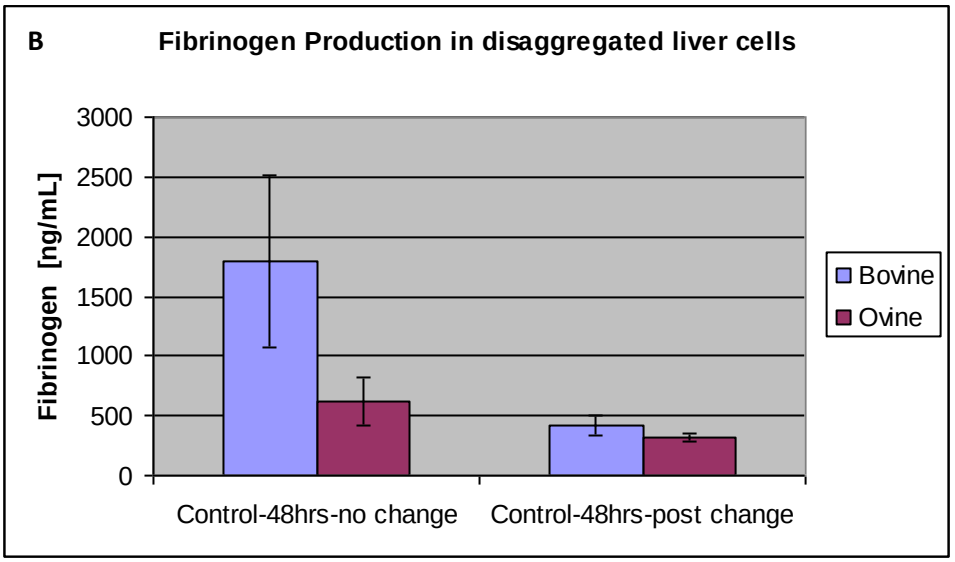
Figure 7 demonstrates that at the concentrations used none of the potential stimuli increased the production of AP above the amount produced by unstimulated control cells. FCS appears to increase the production of AP, however the amount of AP detected in the no cell control (Fig 7A) is similar to that present in the FCS stimulated cells (Figure 6C). Therefore when cells are "stimulated" with FCS the increase in AP detected represents the AP present in the FCS that was added to the medium rather than an increase in production of AP.

1.1.29 Residual Bioactives present in disaggregated cell preparations

In Phase 2 we discovered that the amount of bFGF produced in control unstimulated primary sheep liver cells had increased when compared to the amount reported in Phase 1. We further investigated this phenomenon by comparing the production of bioactives from both ovine and bovine cells that were either grown for 48 hours (no change of medium) or allowing the cells to settle overnight and then changing the media and allowing the cells to grown for 48hrs (48hrs post change).



`



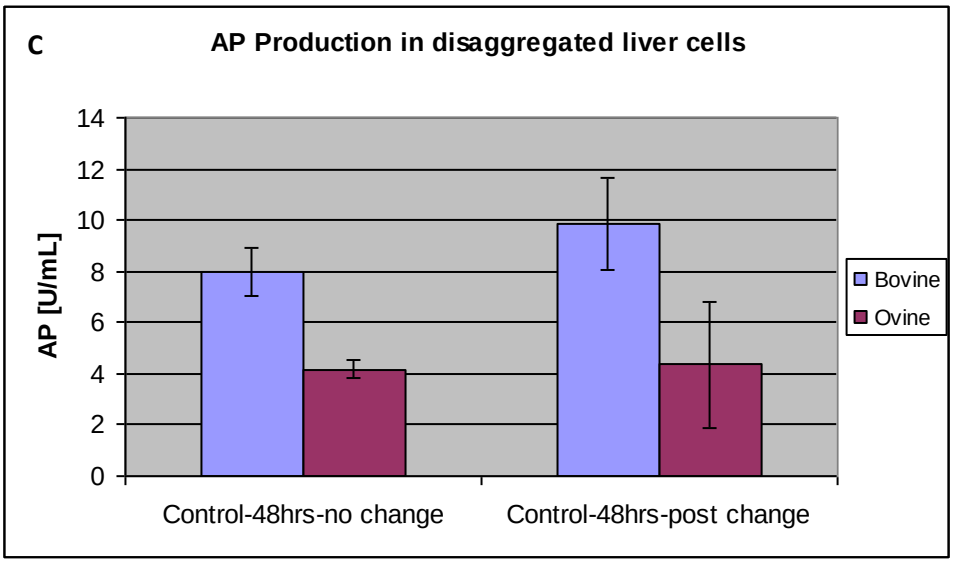


Figure 8. Bovine and Ovine liver cells were isolated through disaggregation and seeded in serum-free HMM at 2.5 x 10^6 cells/ well in 6-well plates. Medium was either collected after 48hrs or changed after 16hrs and incubated on the cells for a further 48hrs. Harvested media was subjected to (A) bFGF, (B) fibrinogen and (C) Alkaline phosphatase assays. The data is presented as the mean + standard deviation of assay performed on 3 separate cell preparations and triplicate wells per preparation (n= 3). Bovine data is shown in blue whereas ovine data is shown in maroon.

Figure 8 demonstrated that bovine cells produced more of the bioactives bFGF, fibrinogen and AP than the ovine cells. Figure 8 also demonstrates that the amount of bFGF and fibrinogen being produced by either bovine or ovine liver cells decreases after the media is

`

replaced. The decrease in fibrinogen production is greater in the bovine liver cells than in the ovine liver cells. AP activity was similar in either preparation of disaggregated cells form both ovine and bovine liver tissues. These result prompted the further investigation into the production of bioactives over time to determine why changing the media caused such a dramatic decrease in the detection of the bioactives fibrinogen and bFGF but not AP.

1.1.30 Post plating stimulation

Screening for substances that may stimulate the production of bioactives was performed using disaggregated liver cells that did not have their medium changed. After the discovery that residual bioactives present in the disaggregated cells that were carrying through into the bioactive assay (Fig. 8) we decided to repeat the stimulation screen using cells that had been allowed to settle and then had their media replaced with medium containing stimulants. Rather than directly repeating this time and resource consuming laborious screen, the 5 stimuli 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (8Br-cAMP), Retinoic Acid (RA), Insulin, Foetal calf serum (FCS) and Estradiol were choose for the media replacement stimulation experiments because they had previously shown the potential to stimulate production of these bioactives. Harvested media were subsequently assessed for the production of bioactive. At the concentrations used none of the potential stimuli increased the production of bFGF, fibrinogen or AP above the amount produced by control unstimulated cells.

1.1.31 Overall progress with the development of bioactive stimulants

The large scale screen for bioactive stimuli was repeated so that we have results using three independent preparations of liver cells. Using this more robust method there is no statistically significant increase in bioactive productions for all stimulants examined.

Previously we reported the discovery that there were increases in the basal production of bioactives in unstimulated cells, we can explain this result as a residual level of endogenous bioactive present within the disaggregated cell preparations and these increases did not represent real increases in the production of bioactives by the cells. This residual amount of endogenous bioactives bFGF and fibrinogen present within the disaggregated cell preparation explains why we no longer see the same extent of stimulation in our disaggregated cell preparations. The amount of residual endogenous bioactives in disaggregated cell preparations is greater than the amount previously reported to be produced by the stimulated cells. Thereby this residual bioactive present in the disaggregated cell preparation would mask the relatively smaller stimulation that was previously shown to occur.

`

Transfer of the technology to bovine

The transfer of the IVCC technology was completed by comparing morphology, viability, cell survival and response to stimuli for both the disaggregated cells and minced organ culture from ovine liver and bovine liver tissues. Demonstrating the ability to transfer this technology from ovine to bovine will broaden the scope of industrial engagement of beef processors, resulting in more opportunity to take this technology to industry and commercialisation.

1.1.32 Isolation of bovine liver cells

Applying disaggregation protocol research operating procedures 11-13 (see appendix) to bovine livers we were able to obtain a high yield of bovine liver cells that was consistent with what was achieved using the ovine livers.

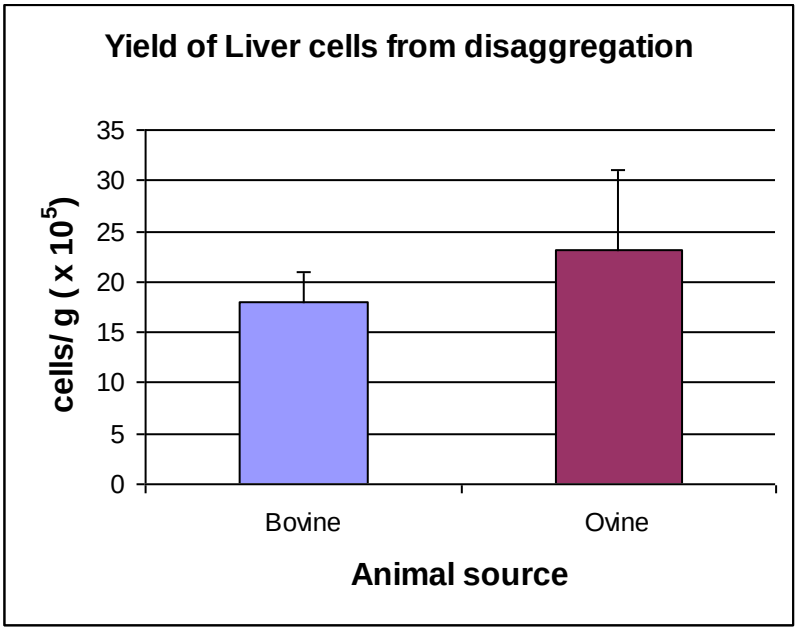


Figure 9 40 grams of 8 pooled sheep livers or 40g of 4 pooled cow livers were subjected to disaggregation following the research operating procedures 11 - 13. Briefly cells were disaggregated in 0.05% collagenase for 2 hours with cell collections every 30 minutes. Cells were collected by centrifugation and counted in a haemocytometer. Total cell numbers were calculated and the number of cells collected per gram of minced liver tissue was assessed. Data is presented as the mean + standard deviation from 3 independent pooled liver preparations (n=3). Bovine data is shown in blue whereas ovine data is shown in maroon.

`

Figure 9 demonstrates that the number of cells collected per gram of tissue disaggregated was consistent between the two animals, with ovine and bovine liver yielding approximately 2 x 10^6 cells per gram.

The morphology of the bovine collected cells was compared with cells collected from ovine sources and assessed by photography (Figure 10).

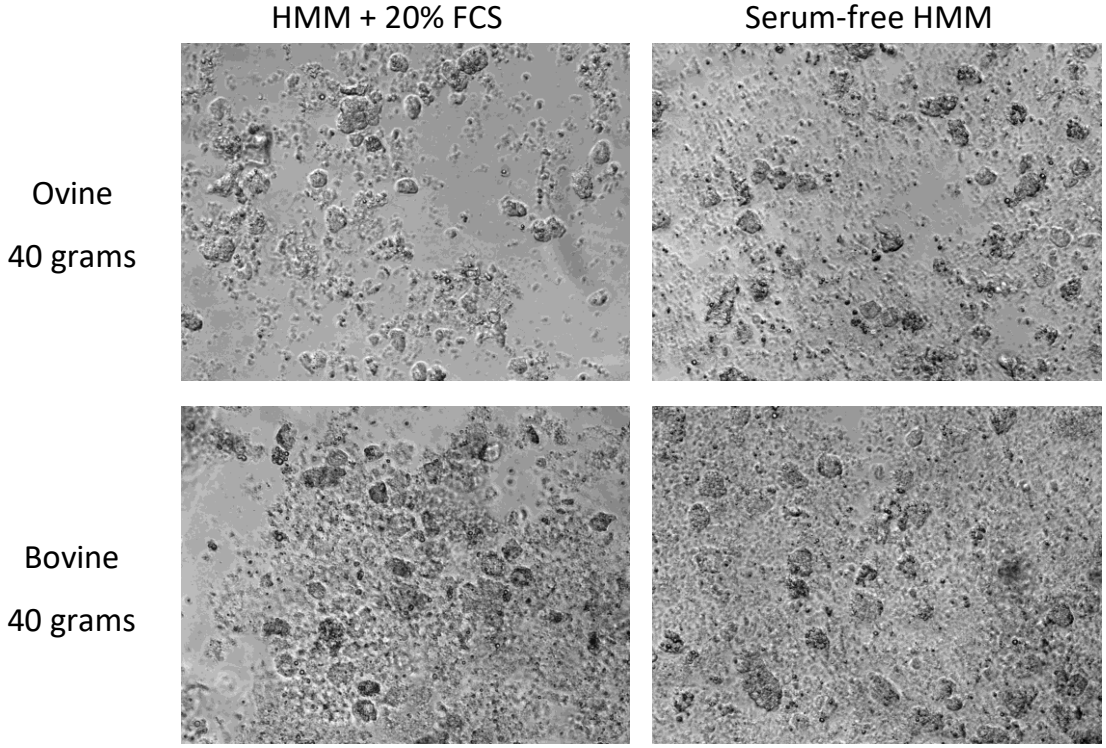


Figure 10. Cells were collected after disaggregation from 40g of ovine or bovine liver and plated into 96 well plates at a cell density of 20,000 cells per well in HMM containing 20% serum (left-hand column) or in serum-free HMM (right-hand column). Cells were allowed to adhere overnight and photographed using the Fluorescence Olympus IX71 Inverted Microscope at 200x magnification. Representative photos of at least 6 independent photos are shown.

Figure 10 shows that the morphology between the two liver samples was similar with both containing the large hepatocyte cells and smaller cell types. Both samples have the presence of red blood cells despite efforts to remove these cells from the culture with multiple organ washes and slow centrifugation steps. In the 96 well plates, there was only a small difference in cell number when comparing the use of serum and serum-free medium however a higher attachment of cells was observed for both the ovine and bovine liver preparations in serum-free medium when compared to the medium containing serum. This data demonstrates that

`

the morphology of the cells disaggregated from bovine liver tissue is consistent with that collected from ovine liver.

The survival of liver cells was measured in 96 wells plates using the MTT assay in ovine and bovine cells that were plated in serum-free HMM or HMM containing 20% serum. Viable cell numbers were compared over a one week period between the small- and large-scale disaggregation samples (Figure 11).

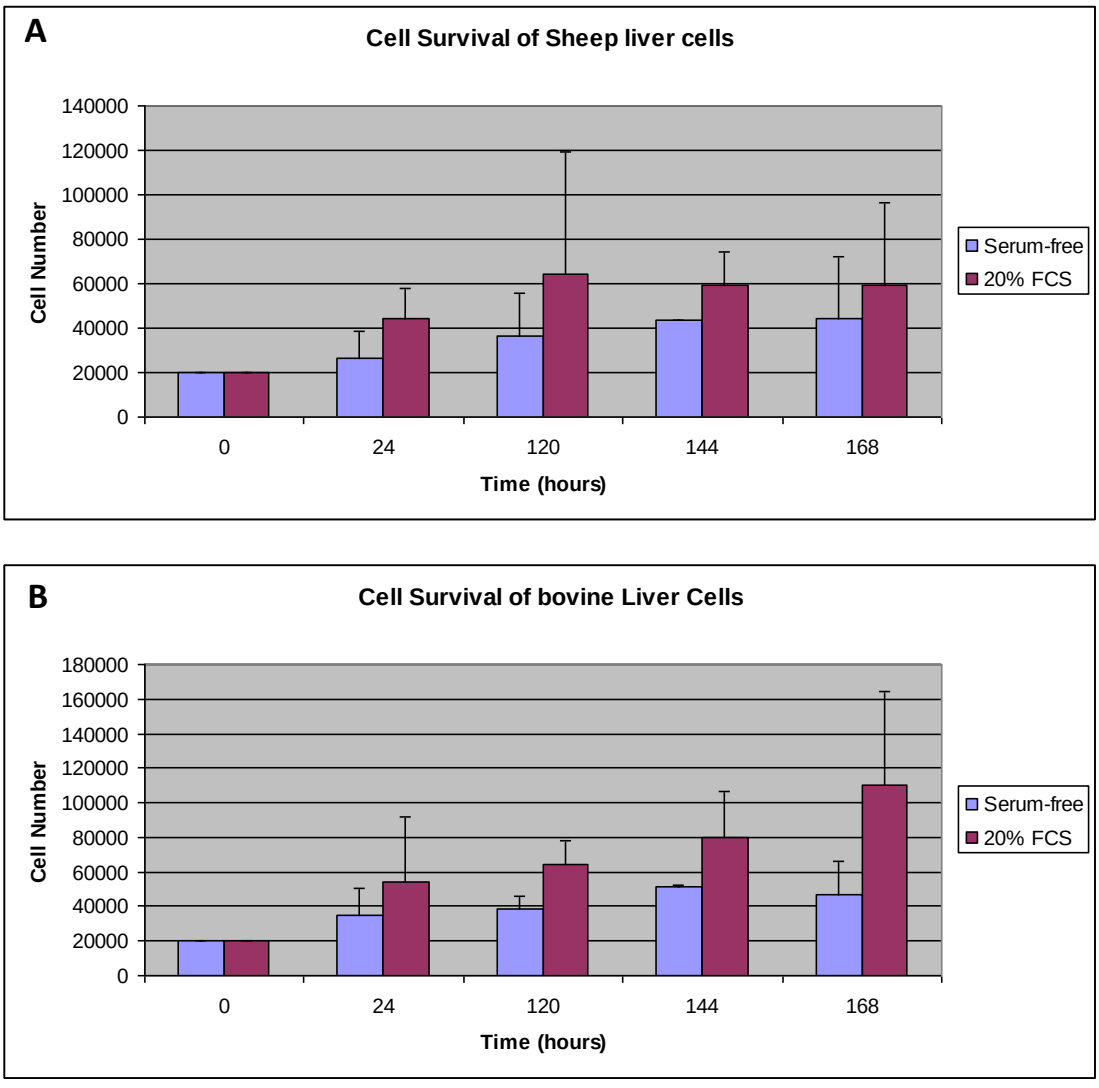


Figure 11. A. 40g of sheep liver cells or **B.** 40g of bovine liver cells were collected and plated at a cell density of 20,000 cells per well in a 96 well plate in serum-free HMM (Blue bars) or HMM containing 20% FCS (Maroon bars) in 4 replicate plates. MTT assays were performed on each plate after 24, 120, 144 and 168 hours post-seeding and cell number was calculated from a standard curve. Data is presented at the mean ± standard deviation from 6 replicate wells from at least three independent experiments

`

Figure 11 shows that cells isolated from sheep liver or bovine liver tissue remained viable for a period of at least one week in cell culture in both serum-free and serum containing medium. There was a notable increase in cell numbers across the 168 hours tested indicating that cell numbers had increased and this was more notable in the serum containing cells. This data suggests that non-parenchymal cells may be responsible for the increase in cell number (epithelial / fibroblastic). This data demonstrates that bovine cells survive for at least one week in culture and demonstrate a consistent cell number with ovine liver cells over time.

We next tested the efficiency of cell attachment and survival in 6 wells plates in both serum-free HMM and HMM containing 20% bovine serum (Figure 12).

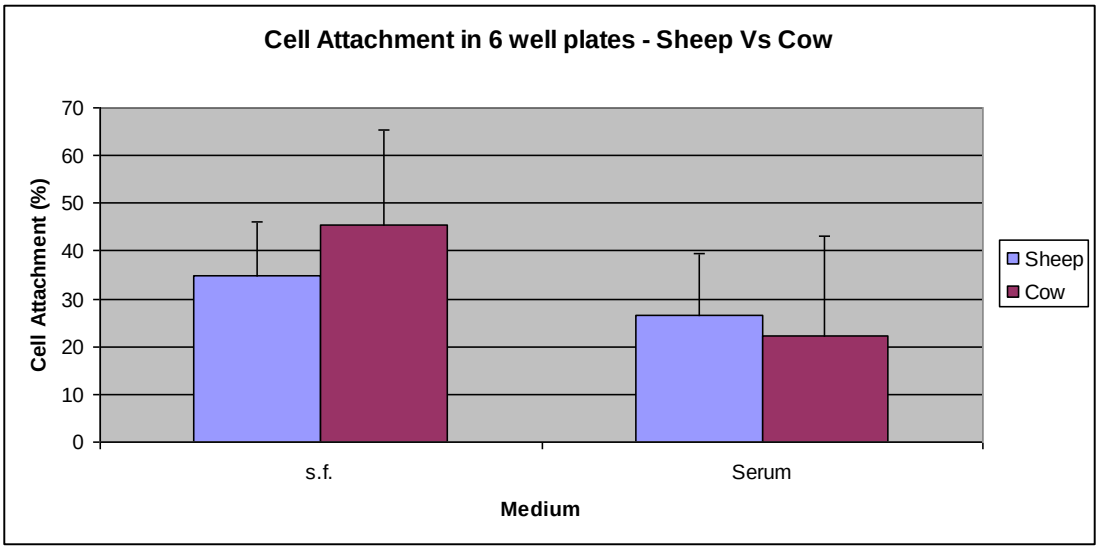


Figure 12. Sheep liver cells (blue bars) or bovine liver cells (Maroon bars) were collected from 40g of liver tissue and plated at a cell density of 2.5 x 10^6 cells per well in a 6 well plate in serum-free HMM (s.f.) or HMM containing 20% FCS (serum). 48 hours post-seeding cell numbers were calculated by trypsinizing the cells from the plate and counting cell numbers in a haemocytometer. Data is presented at the mean ± standard deviation from duplicate wells of three independent experiments.

Data presented in Figure 12 shows that there was a similar level of attachment between the ovine and bovine samples. Consistent with previous experiments, the attachment in serum-free medium was higher than that observed for the same samples seeded into medium containing serum, indicating that cell attachment is more efficient in serum-free medium. This data shows that there is little difference in cell attachment between ovine and bovine samples in regards to attachment in 6 well plates. Cell attachment was also assessed in flasks with surface areas of 75cm^2 (T75) and 175 cm^2 (T175) (Figure 22).

`

To ensure that the methods of IVCC technology could be transferred to bovine tissue the new method of post media change stimulation was performed using the bovine cells. Disaggregated bovine cells were plated, allowed to settle overnight and the simulated with media containing a single concentration of the stimulants 8Br-cAMP, RA, Insulin, FCS or Estradiol. Under the condition used no stimulation of bioactives occurred, this is consistent with the ovine cells.

1.1.33 Production of AP by minced organ cultures

Ovine minced organ cultures have previously identified in Phase 1 as a novel process that could be used to produce bioactives. To determine whether the minced organ culture technology could be transferred to bovine, the amount of AP production in bovine and ovine minced organ cultures was examined and the amount that could be extracted from both ovine and bovine tissues was also determined.

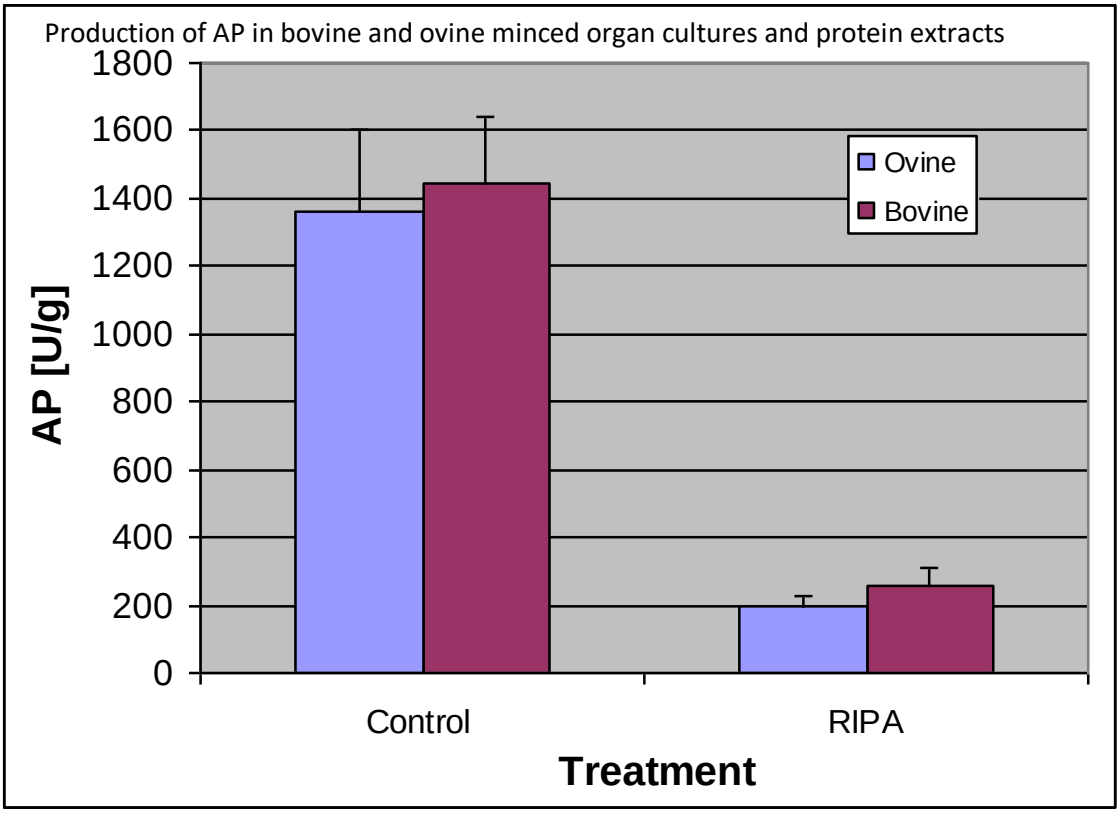


Figure 13 Minced ovine (blue) and bovine (maroon) livers were plated at 1.5 g/well in 6 well plates with the addition of 2mL HMM. Medium was collected after 48 hours (control). RIPA buffer extractions were performed on 1.5g of minced liver tissue in 2ml of RIPA buffer. Medium and RIPA buffer extracts were subjected to AP activity assays. The data is presented

`

as the mean + Standard deviation from 3 individual liver preparations of 8 pooled livers plated in triplicate (n=3).

Figure 13 demonstrates that when ovine or bovine organ cultures are maintained under the same conditions they give an equivalent yield of AP per gram of minced tissue. Figure 13 also demonstrates that both ovine and bovine minced organ cultures produce 7-fold more AP into their medium than was present in the RIPA extraction. This result suggests that there is additional production of AP in the minced organ culture than was present in the original tissue. These results indicate that minced organ cultures from bovine livers are consistent with ovine liver.

1.1.34 Stimulation of ovine and bovine mince liver cultures

The development of methods that can amplify the production of bioactives is a critical stage of this project. Therefore 5 stimulants were selected based upon what the literature suggested would increase the production of AP and were tested using minced organ culture of ovine and bovine livers.

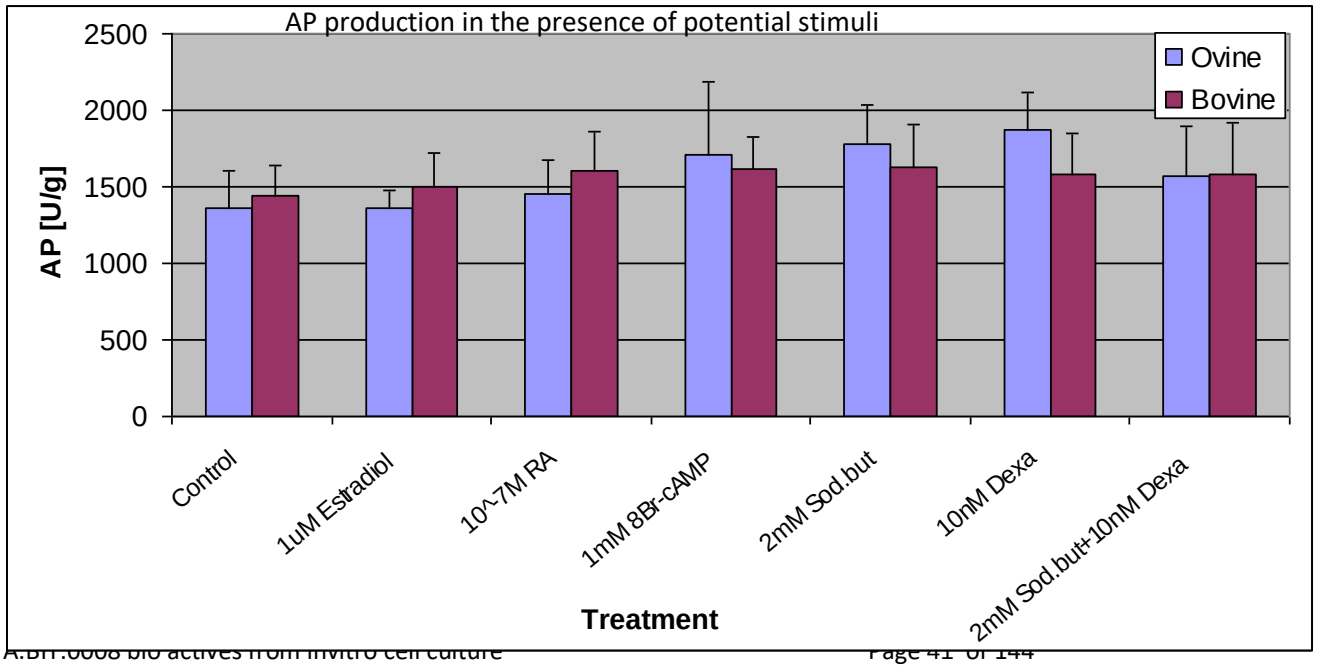


Figure 14 Minced ovine (blue) or bovine (marron) livers were plated at 1.5 g/well in 6 well plates. Minced tissues were maintained in control HMM or HMM with the addition of estradiol, retinoic acid, 8-bromo-cyclic AMP, sodium butyrate, dexamethsone, or dexamethsone and sodium butyrate. Medium was collected after 48hrs. RIPA buffer extractions were performed on 1.5g of minced liver tissue in 2ml of RIPA buffer. Medium and RIPA buffer extracts were subjected to AP activity assays. The data is presented as the mean + deviation from 3 individual liver preparations of 8 pooled livers plated in triplicate wells (n=3).

Figure 14 demonstrates that when ovine and bovine organ cultures were maintain under the same conditions they give an equivalent yield of AP per gram of minced liver tissue. This similarity in yield of AP indicates that it is acceptable to complete the optimisation of bioactive stimulation using bovine tissue rather than the unavailable ovine tissues. However none of these potential stimulants significantly increased the production of AP in minced ovine organ cultures. These experiments demonstrate that minced bovine liver organ culture give similar results to the ovine minced organ cultures.

1.1.35 Optimising the mince inoculums

The amount of mince added to the organ cultures was varied in an attempt to optimise the amount of inoculum that would give maximum AP production.

`

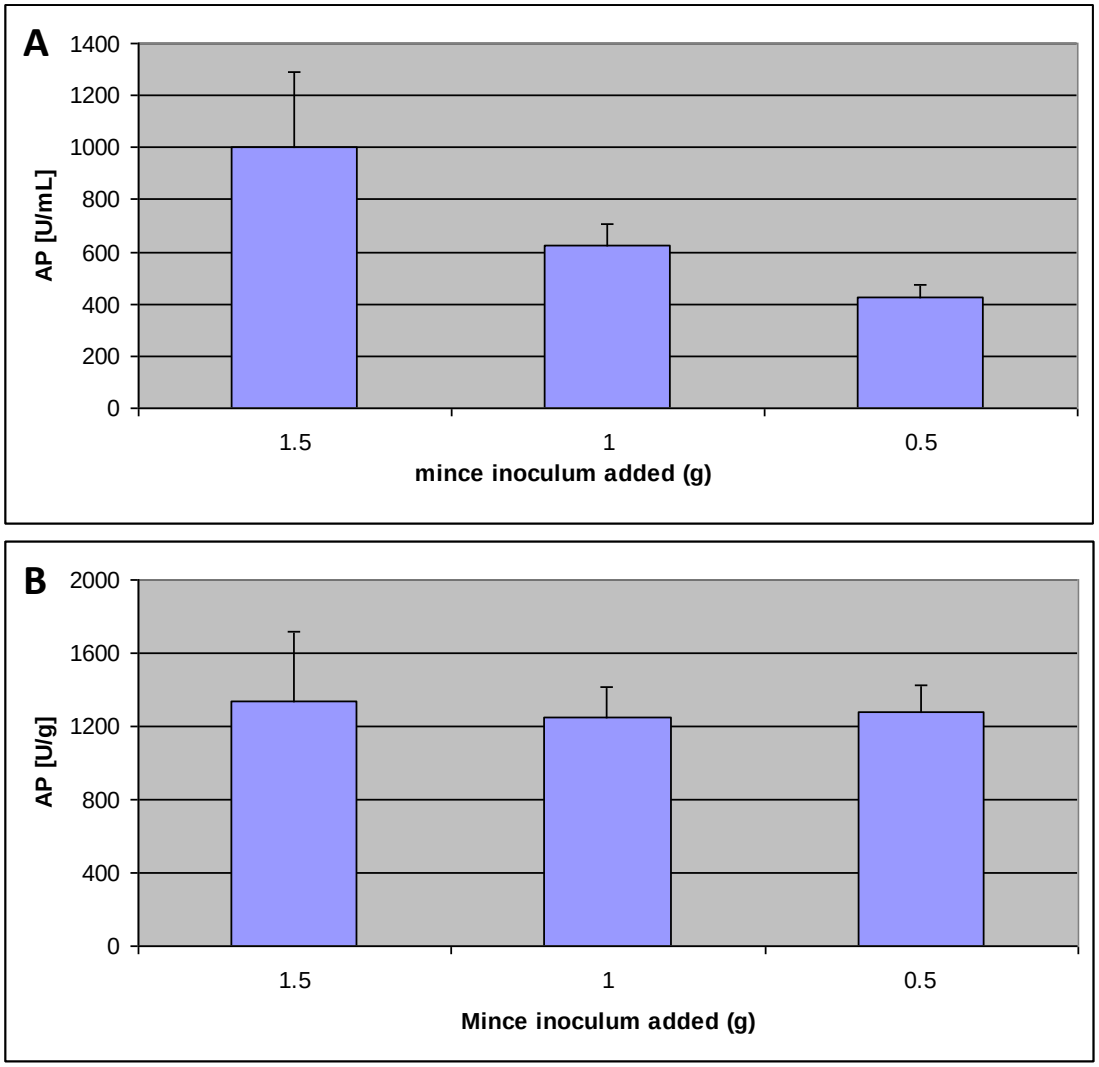


Figure 15 Minced bovine livers were plated at 1.5, 1 and 0.5 g/well in 6 well plates and maintained in HMM. At 48hrs medium was collected and subjected to AP activity assays. The data is expressed in terms of AP produced per ml of media (A) or per gm of mince inoculum (B). The data is presented as the mean + standard deviation of assay performed on 3 separate cell preparations plated in triplicate wells (n= 3).

Increasing the amount of mince inoculated into the wells increases the amount of AP produced per ml of medium (Fig 15A). Changing the amount of mince inoculated into the minced organ culture did not significantly alter the amount of AP produced per gm of tissue (Fig 15B).

`

1.1.36 Progress of transfer technology to bovine

We have demonstrates that the bovine liver cells are consistent with the ovine as they do not respond to stimuli and that a similar number of viable cells could be isolated from liver tissues from either ovine or bovine sources.

Using Ovine cultures it had been established that minced organ culture produces more AP than what is released from RIPA extraction of the same amount of tissue, indicating that new AP is being produced by the organ culture rather than this process being a simple extraction process. Bovine minced organ culture produced 7-fold more AP than what could be extracted using RIPA buffer; this is similar to the result that was found for ovine minced organ culture demonstrating that the minced organ technology can be transferred from ovine to bovine.

The addition of potential chemical stimulants to minced organ cultures did not significantly alter the production of AP in bovine or ovine culture. This again demonstrates similarities between ovine and bovine, with minced organ cultures from either animal failing to respond to potential stimulants.

Bioreactors

The goal of this project task was to develop an integrated system for bench-scale demonstration, ready for further scale-up. The key to demonstrating the proof-of-concept production of bioactives with commercial potential will be determined by the development of a bioreactor system that stimulates the production of bioactives and enables scale-up. The chosen culture formats were minced organs and cells isolated from disaggregated tissue. The first approach involved the culturing of minced organ in a bioreactor and evaluate for the production of AP as a bioactives. The second approach used disaggregated cells that were cultured on an adherent matrix. Attempts to stimulate bioactive production in the disaggregated cells did not increase the production of the chosen bioactives above the amount produced by non-stimulated cells. Therefore experiments involving disaggregated cells focused on the attachments and survival of cells rather than bioactive production.

The Flinders Team has sourced 2 disposable bioreactor systems that we recommend to be delivered and tested. The WAVE system from GE Healthcare is a mechanically driven, horizontally oscillating system with a disposable chamber that is positioned on a special rocking platform. The cell bags with a cell culture volume range up to were tested with and without FibraCells as the microcarriers lower the area to cell density ratio and therefore intensify the process in smaller volumes but with higher cell densities. The Wave system without microcarriers represented a suitable disposable bioreactor system for testing the

`

minced organ culture. The second chosen system, FibraStage from New Brunswick Scientific consisted of 4 x 500mL culture bottles, pre-filled with Fibra-Cel disks and operated by compressing and expanding bellows at the base of each bottle. This ensures constant exchange of nutrients and removal of waste. The culturing of minced organ was also performed in a traditional stirred tank bioreactor that consisted of 2 sets of rotating flat blade impellers in a 2 litre vessel with computer controlled heating rod and sterile air bubbled through the chamber using a sparger.

1.1.37 Comparison of yield between bioreactor systems

Bioreactor trials of minced organ culture were performed using the stirred tank and Wave that can be summarised for easy comparison (table 1). Trials were performed as direct scale-up of 6-well plate using the stirred bioreactor (B1-A) and Wave bioreactor systems (W2). B1-A was not showing sufficient mixing, therefore more medium was added and the trial continued as in trial B1-B. The first trial of the Wave bioreactor systems (W1) was performed using the same inoculum as the first trial in a stirred tank (B1-B); thereby the yields from these two trials can be used to directly compare the bioreactor systems.

Trial	**6 well plate**	**B1-A**	**B1-B**	**B2**	**W1**	**W2**
Reactor type	6 well plate	Stirred tank	Stirred tank	Stirred tank	Wave	Wave
Inoculum (g)	1.5	500	500	150	150	375
Volume (ml)	2	666	1666	1000	500	500
Mince/Medium ratio (g/ml)	0.75	0.75	0.3	0.15	0.3	0.75
Air source	5% CO2	air	air	air	5% CO2	5% CO2
air flow rate (L/min)	NA	1	1	0.6	0.1	0.1
Stir speed	NA	120 rpm	120 rpm	107	NA	NA
Rock speed	NA	NA	NA	NA	24 rpm/7º	24 rpm/7º
AP (U/ml) at 48hrs	1615	NA	635	313	1997	1975
AP (U/g) at 48hrs	1211	NA	187	63	599	1481

Table 1. Bioreactor conditions and yields

Table 1 summarises the key reaction conditions and bioactive yield after 48hrs from various bioreactor trials. Table 1 demonstrates that the amount of AP produced in a small scale 6-well plate can be replicated in a Wave bioreactor (W2) by adding the same ratio of mince to

`

medium (m/m= 0.75). The Wave bioreactor system (W1) produces 3-fold more AP than a stirred tank bioreactor (B1-B) that was inoculated with the same ratio of mince to medium (m/m= 0.3 g/ml).

The amount of AP produced in each bioreactor trial has been compiled into a graph with the AP production presented as a percentage of the yield obtained from a small scale 6 well plate. This gives the ability to easily compare the effectiveness of each bioreactor system in producing AP.

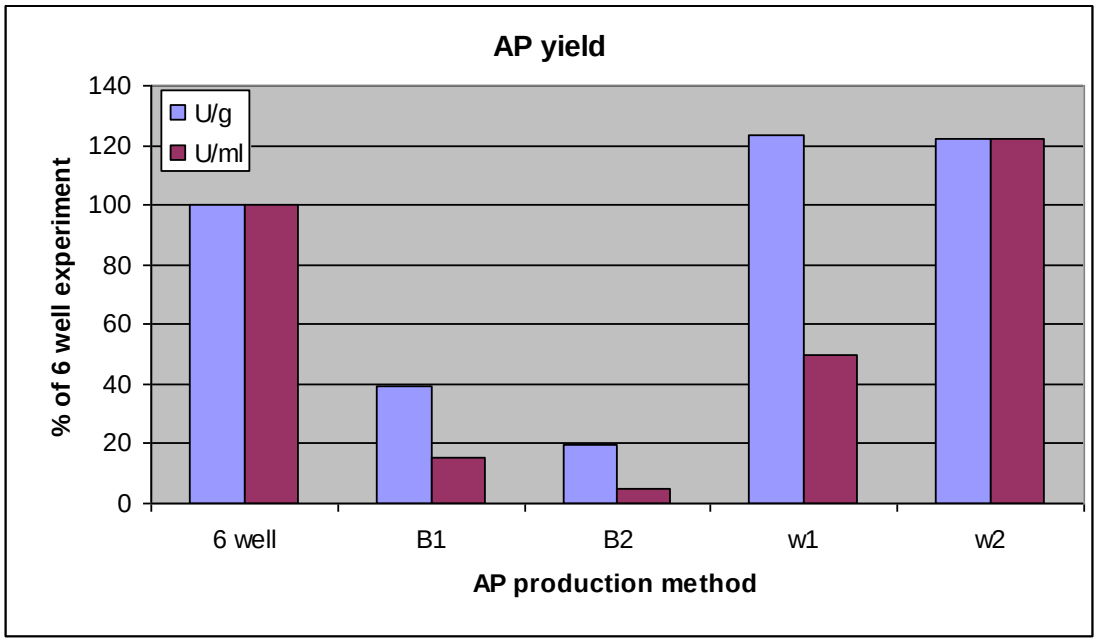


Figure 16. Production of AP in bioreactor trials

Small scale minced organ culture in a 6 well plate (6-well), stirred tank bioreactor trial 1 (B1), stirred tank bioreactor trial 2 (B2), Wave bioreactor trial 1 (W1) and Wave bioreactor trial (2) were performed as detailed in table 1. Cultures were maintained in HMM for 48 hours and subjected to AP activity assays. The data is expressed as a % of the AP production of a 6 well plate in terms of AP produced per gm of mince inoculum (Blue) or per ml of media (maroon).

Fig 16 demonstrates that the stirred bioreactor trials produced much less AP than what was produced by small scale minced organ culture performed in a 6 well plate. Both trials of the Wave bioreactor system produced similar amounts per g as were produced in the 6 well plate cultures. However increasing the production of AP per ml to the similar levels produced in the small scale cultures was only achieved in Wave trial 2 (W2) when the mince/medium ratio was increased to the same amount as what was used in the 6-well plates (m/m= 0.75 g/ml).

`

1.1.38 Bioreactor trials using disaggregated cells

The aim of this task was to scale-up the culture of disaggregated cells from 6-well plates (2 ml) into a laboratory-scale bioreactor (0.5-1 Litre) to evaluate their ability for adherence and survival. Wave and FibraStage bioreactor systems were chosen for testing scaling-up. It is not known whether the production and stimulation of bioactives will require cells to be bound to a surface. Therefore liver cells were cultured in the Wave and FibraStage system with Fibra-Cel microcarriers. The wave system was also trialled without microcarriers. The bioreactor trials were run for 48hrs and monitored for changes in pH, temperature and production of bioactives of bFGF and Fibrinogen. Cells in the bioreactor where also analysed for adherence to microcarriers, metabolic activity and survival. During trials Wave 4, Wave 5, FibraStage 1 and FibraStage 2 the medium was replaced at 48hrs, and the trials continued for an additional 48hrs.

Table 2. Bioreactor conditions and yields for culturing disaggregated cells

Table 2 summarises the key culture conditions from various bioreactor trials using 1x10^9 cells per culture. We completed scale-up test of integrated process in a laboratory-scale bioreactor system for disaggregated cells testing cell adherence and survival after 16hrs and 48hrs. Wave bioreactor trial 3 demonstrated that in the short term, adherence of cells to the microcarriers occurred in the Wave bioreactor system (56%) however this adherence became more moderate with time and at 48hr this was similar to what occurred in the FibraStage bioreactor trials 1 and 2 (~20%). Optimization of the Wave bioreactor operation was performed by increasing the volume of medium in Wave bioreactor trial 4 (500ml to 1000ml), this increased the survival of cells in the medium but also caused a decrease in adherence of liver cells to the microcarriers. When culturing cells in Wave bioreactor system without Fibra-Cel microcarriers 45% - 65 % of cells survived for 48hrs, indicating substantial variability in the data generated using the same condition and significant cell loss. Further optimisation of the rocking speed, pH control, dissolved oxygen and nutrient supply could be performed to increase this cell survival.

In the first 16hrs of culture the FibraStage trial 1 demonstrated a low adherence (bound and associated) of the liver cells to the microcarriers (6%), however adherence had increased to 24% that was similar to the amount achieved at 48hr in the most successful bioreactor trial (Wave trial 3). The FibraStage was optimised by altering the amount of time spent in bottom hold position during the culturing phase (60min to 10min). However altering the bottom hold time did not increase the adherence of cells to the microcarriers. One of the key problems with the FibraStage system was that mixing in the system was not sufficient to maintain the relatively thick disaggregation inoculums in suspension.

`

Contamination of the bioreactor was confirmed in 3 trials and suspected in another 2. Therefore we focused on trying to identify the source of the contamination. Mince organ culture and the HBSS that pre-disaggregated tissues where stored in did not show any contamination after 24hrs of incubation, whereas the samples from the cells in a 6 well plate and bioreactor samples were contaminated. This demonstrates that the bacterial contamination was coming from the disaggregation process. Quality control / quality assurance regimes where developed to examine this process and the use of alternative antibiotic such as combination of doxycline and ciprofloxacin investigated, however no further contamination occurred therefore we were unable to fully test the ability of alternative antibiotics in the control of the microorganism contamination.

1.1.39 Progress of Bioreactors

The stirred tank bioreactor was an unsuccessful system for the minced organ culture, the lack of mixing in the first trial of the stirred tank bioreactor was suspected to be a critical factor that caused the low yield of AP. Therefore in the second trial of the stirred tank bioreactor the amount of mince added was reduced with the aim of getting better mixing. To achieve sufficient mixing the stirred tank bioreactor would have to be modified. The

Bioreactor Trial		**Wave3**	**Wave4**	**Wave5**	**Wave 6**	**FibraStage 1**	**FibraStage 2**	**FibraStage 3**
Volume		500ml	1000ml	1000ml	1000ml	500ml	500ml	500ml
5% CO_2 Flow rate (L/m)		0.1	0.2	0.2	0.2	N/A	N/A	N/A
Culture Bottom hold (min)		N/A	N/A	N/A	N/A	60	10	10
Fibra-Cel (g)		20	20	0	0	10	10	10
% of cell at 16hr	Medium	40	64	130	113	99	88	93
	Associated	20	13			4	4	2
	Bound	34	12			2	6	4
% of cell at 48hr	Medium	8	72	45	65	114	73	88
	associated	9	1			10	10	3
	Bound	12	11			14	10	6
Cell source		Bovine	Ovine	Ovine	Ovine	Ovine	Ovine	Ovine
Contamination		No	No	Possible	Yes	Possible	Yes	yes

`

modified bioreactor would need to have a better internal design that had fewer areas in which the mince could get caught and remain stationary. The flat blade impellers could also be replaced with pitched blades that are located closer to the bottom of the vessels. These modifications should aid the mixing of the mince but would require further testing.

The Wave bioreactor system performed much better than the stirred tank, maintained temperature throughout the bioreactor trials and the rocking motion caused sufficient mixing to keep the majority of the mince moving. Wave trial 2 was inoculated with the same ratio of mince to medium as would be used for a 6 well plate (m/m=0.75 g/ml) and production of AP per ml and per gm were similar in both the bioreactor and 6-well plate. This indicates that production of the bioactive AP can be scaled-up in a Wave bioreactor system.

Bioactive Purification

A three step purification strategy was employed to isolate the AP from other proteins that were release into the medium during minced organ culture. Purification experiments were performed using conditioned medium contained proteins released during the minced organ culture of bovine liver in Wave bioreactor trial 2.

1.1.40 Three step purification strategy for the purification of AP

Ammonium sulphate (AS) precipitation was first performed to provide a crude separation based upon solubility. AS precipitation resulted in 113% of AP activity being recovered in proteins precipitated between 70%-80% AS. 12% of the total protein was present in the AP enriched protein precipitation and AP activity per mg of protein was 883 U/mg. This represented a 10-fold purification from the starting material. The second stage of AP purification was anion exchange chromatography to separate the proteins based upon charge using the weak anionic resin DEAE. The elution of AP from the DEAE with a gradient of 0.25 -0.5 M NaCl resulted in an increase the specificity activity of AP 6-fold when compared the AS precipitation and 60 fold when compared to the original starting material from minced organ culture. The AP enriched fractions also contain 43% of the activity that was present in the stating material. These results indicate that the purity has increased while the yield has decreased, with further refinement to increase yield this may represent a commercially viable method of purification.

`

Method	Sample	Total Protein (ug)	Total activity (U)	Specificity activity (U/mg)	Yield (% activity)	Fold purification
Minced organ culture	**Starting material**	42243	3648	86	100	1
Ammonium sulphate precipitation	**70-80% AS**	5417	4018	883	110	10
DEAE (0.25, 0.5 and 1.5M elution)	**fr 59-60**	298	1565	5176	43	60

Table 3 Summary of AP purification

Table 3 summarises the result from the purification of AP from the medium of a minced organ culture. These data represent the yields obtained from a single column run. The first step in Purification was successfully in obtaining 10 fold in the Ammonium sulphate precipitation, retaining 110% of the activity. The most successful column purification strategy was DEAE using successive elution at 0.25, 0.5 and 1.5M elution resulting in a 44% recovery of activity and a 60 fold purification of AP.

Mass spectrometry was performed on an in-gel digestion of the upper band 75kDa band in fraction 59 eluted from the DEAE column. Results are presented in Table 4.

Accession	Coverage	# PSMs	# Peptides	# AAs	MW [kDa]	calc. pI	Score	Description
Q29443	81.53	412	63	704	77.7	7.08	1590.21	Serotransferrin OS=Bos taurus GN=TF PE=2 SV=1 - [TRFE_BOVIN]
Q05927	28.57	25	14	574	62.9	6.95	81.23	5'-nucleotidase OS=Bos taurus GN=NT5E PE=1 SV=2 - [5NTD_BOVIN]
P09487	22.33	16	11	524	57.2	6.77	64.79	Alkaline phosphatase, tissue-nonspecific isozyme OS=Bos taurus GN=ALPL PE=1 SV=2 - [PPBT_BOVIN]
P02769	29.00	19	15	607	69.2	6.18	63.43	Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4 - [ALBU_BOVIN]

`

Q2HJ49	19.24	12	9	577	67.9	6.16	47.29	Moesin OS=Bos taurus GN=MSN PE=2 SV=3 - [MOES_BOVIN]
Q5EA20	25.19	10	8	393	44.9	6.73	30.73	4-hydroxyphenylpyruvate dioxygenase OS=Bos taurus GN=HPD PE=2 SV=3 - [HPPD_BOVIN]
O46421	7.95	6	3	566	62.5	6.65	24.07	Liver carboxylesterase 1 OS=Macaca fascicularis GN=CES1 PE=2 SV=1 - [EST1_MACFA]
Q9TTE1	12.41	5	4	411	46.2	5.99	23.81	Serpin A3-1 OS=Bos taurus GN=SERPINA3-1 PE=1 SV=3 - [SPA31_BOVIN]
Q3SZV7	13.73	6	5	459	52.2	7.80	22.55	Hemopexin OS=Bos taurus GN=HPX PE=2 SV=1 - [HEMO_BOVIN]
O43280	9.61	4	4	583	66.5	5.68	16.60	Trehalase OS=Homo sapiens GN=TREH PE=1 SV=2 - [TREA_HUMAN]
Q9N0V4	11.47	3	2	218	25.6	7.39	14.36	Glutathione S-transferase Mu 1 OS=Bos taurus GN=GSTM1 PE=1 SV=3 - [GSTM1_BOVIN]
P00432	10.25	4	4	527	59.9	7.28	14.24	Catalase OS=Bos taurus GN=CAT PE=1 SV=3 - [CATA_BOVIN]

Table 4. Mass-spectrometry profile of proteins present in the 75kDa band of fraction 59 of DEAE purification (Fig. 7). Enzymatic digestion of the excised protein bands was performed with 12.5ng/uL of trypsin and 100mM ammonium bicarbonate and incubated overnight at 37C. Following enzymatic digestion, the resulting peptides were extracted twice with 10uL of 5% formic acid and concentrated to 4-5uL in the same solvent. The entire sample was then analysed on a Thermo LTQ XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corp, San Jose, CA).

Table 4 demonstrates that the 75kDa band in fraction 59 from the DEAE column (lane 4 Figure 9) contained AP. Alkaline phosphatase was not previously detected through in solution digestion of the medium obtained from minced organ culture, indicating that specific purification and enrichment of AP has occurred though the use of AS precipitation and DEAE chromatography. Table 4 also shows that the 75kDa band is not pure AP, therefore the planed third step of purification using size exclusion will not remove the remaining non-AP proteins from this preparation.

1.1.41 Progress on the purification of Bioactives

In this task we initiated the laboratory scale downstream purification of bioactives selected (AP) for the progression to a bioreactor system. A three step purification strategy was developed to isolate the AP from other proteins that were released into the medium during minced organ culture. The strategy developed was 1) Ammonium sulphate precipitation to provide crude separation based upon solubility 2) ion-exchange chromatography with DEAE

`

resin to separate protein based on charge and 3) a final polishing step using size exclusion chromatograph to separate the proteins based upon size.

AP precipitation gave a specific AP 10-fold enrichment between 70- 80% AS. The DEAE purification was successful in further increasing purification of AP, increasing the specific activity of AP a further 6 fold to a total of 60 fold gain from the starting material. There was a decrease in total activity recovered and the 75kDa band that was suspected to be AP contained multiple proteins. Therefore this second stage of purification requires further optimisation. The third stage of the purification strategy, size exclusion chromatograph, was not attempted because of the failure in obtaining sufficient recovery and purity of AP from the DEAE ion-exchange chromatography.

Scale up of minced organ culture

The aim of this milestone was establish conditions to scale-up minced organ culture in an integrated bench-scale demonstration system. We plan to scale-up from 375g of tissue (0.5 Litre) to 2kg (2.7 litres), a 5-fold scale-up.

Each stage of the minced organ culture process was analysed to determine the appropriate method of scale-up of bioactive production. Doxycycline and ciprofloxacin (Dox/Cip) were tested as an alternative antibiotic to penicillin and streptomycin (Pen/Strep) to control the contamination that had been found to occur in previously preformed bioreactor trials. However when testing the Dox/Cip, contamination did not occur in Pen/Strep controls, therefore the effectiveness of Dox/Cip could not be determined. The contamination of the 6 well plates has been found to be inconsistent, occurring in only 1 of the last 4 experiments. Through these experiments we develop quality control / quality assurance regimes to test for the contamination of cultured cells and minced organ cultures (ROP 28: Assaying cell/mince cultures for microorganism contamination).

The yield of mince from bovine liver was found to decrease in the latest series of experiments. An investigation into the collection, chopping, rinsing and mining of bovine liver was performed and it was determined that 1 litre Schott bottles are suitable for collection and eliminating the chopping and washing, resulted in an increase in yield of minced organ without decreasing AP activity or the amount of protein obtained. Quality controls were established to analyses the minced organ culture that included contamination sampling, small scale 6 well culture and RIPA buffer analysis of the minces. Scale-up of mince preparation from 3 kg of organ gave a yield 1.338 kg (44%), indicating that 5 kg of starting material with be required to produce 2kg of minces for the bioreactor scale-up (See appendix Research Operating Procedure 29: Scale up protocol of mince preparation for bioreactor).

`

All the conditions necessary for establishing bench-scale demonstration system have been completed. The Biostat cultibag bioreactor system has been organised for demonstrating the scale-up to 2kg of minced organ culture. Economic process modelling analysis at first seemed to give a promising business case (Gareth Ford, SEME report). However this trial was abandoned because of the discovery of a recombinant Bovine AP expressed in *Pichia pastoris* (Sigma-Aldrich catalogue number ,P8361-5000U), this altered the competitive environment such that AP was no longer suitable bioactive target because the business case for AP would no longer be viable (personal communication, Gareth Forde, SEME).

Proteomics

A proteomics approach was applied to identify potential stimuli and new bioactives of commercial value. Samples from minced organ culture media was analysed using 2-D protein gel electrophoresis coupled with Mass Spectrometric analysis in the Flinders Proteomics Facility. This approach was taken as it allowed us to identify bioactive targets of commercial significance that can be stimulated rather than attempting to stimulate chosen bioactive targets.

1.1.42 Screening BSA depletion methods

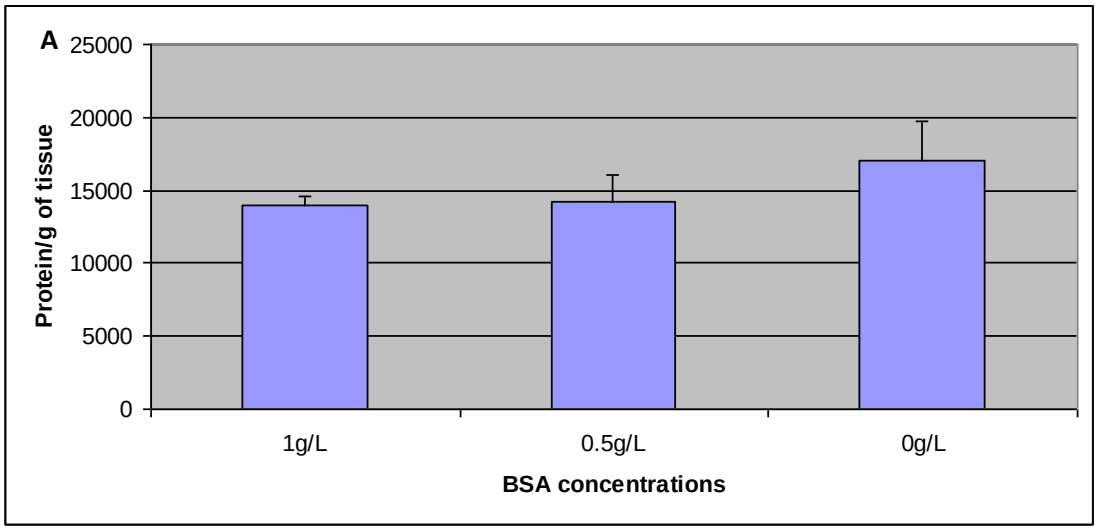
Due to the high BSA content of 1g/L in the culture media used for the minced organ culture, the bands visible on a 1d-gel show a very bright BSA band at around 66kDa and this would effect the ability to load more novel proteins on the gel and detect less abundant proteins. Depletion techniques were also tested to obtain better resolution of 2D gel when examining proteins from the medium of minced organ culture. The five depletion methods trialled included ethanol precipitation, acetone precipitation, Enchant^{TM} albumin depleting column, Amicon^{TM} filtration and Blue Trisacryl M AcroSep column. Only the Acetone/TCA method depleted some of the albumin from the WAVE#2 sample.

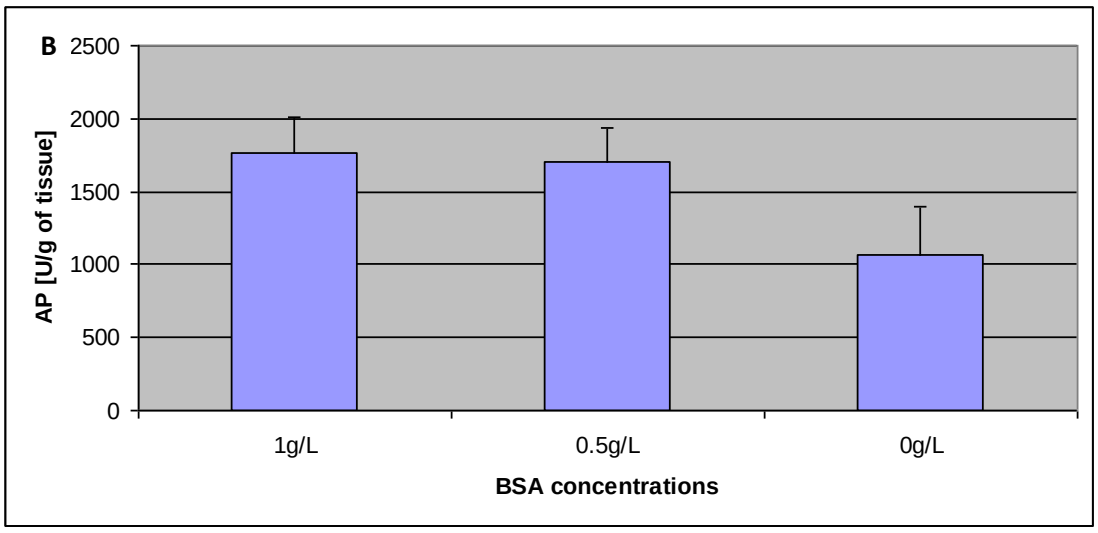
The screening of several different albumin depletion methods for the minced organ culture sample showed no BSA depletion for the Ethanol method, the Enchant Centrifugal column, 50kDa Amicon filter cut-off and AcroSep chromatography column method. The Acetone/TCA depletion method resulted in depletion of BSA present in the initial sample, whereas the Amicon Ultracentrifugation method with a MWCO of 50 and 100kDa was only successful for the Permeates (<50 and <100kDa).

`

1.1.43 The effect of BSA addition on the Production of Alkaline Phosphatase in the minced organ culture

To test whether the addition of bovine serum albumin has any effect on total protein and/or AP-activity, different amounts of BSA were added to the culture media and total protein as well as AP-activity was measured.





`

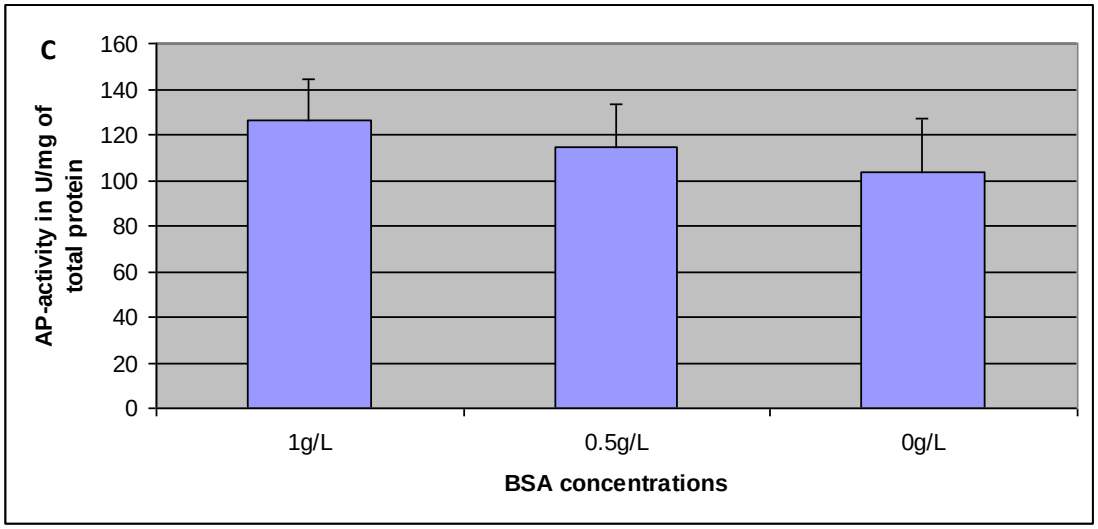


Figure 17. Minced bovine livers were plated at 1.5 g/well in 6-well plates with the addition of 2mL HMM at 37C. Minced organ cultures were incubated under stable controlled conditions for 48hrs and subjected to protein assay and AP activity assays. The data is presented as the mean + deviation from 3 individual liver preparations plated in triplicate wells (n=3). Protein/g of tissue **(A)**, Units/g of tissue **(B)** and U/mg of total protein **(C)**.

Figure 17 demonstrates that adding different concentrations of BSA (1, 0.5 and 0g/L) to the minced organ culture shows no significant impact on protein production or AP-activity. Therefore, adding less BSA to the minced organ culture does not affect specific activity of AP obtained. The effect of BSA addition on 1D proteomic profiling was also examined (Figure 18)

`

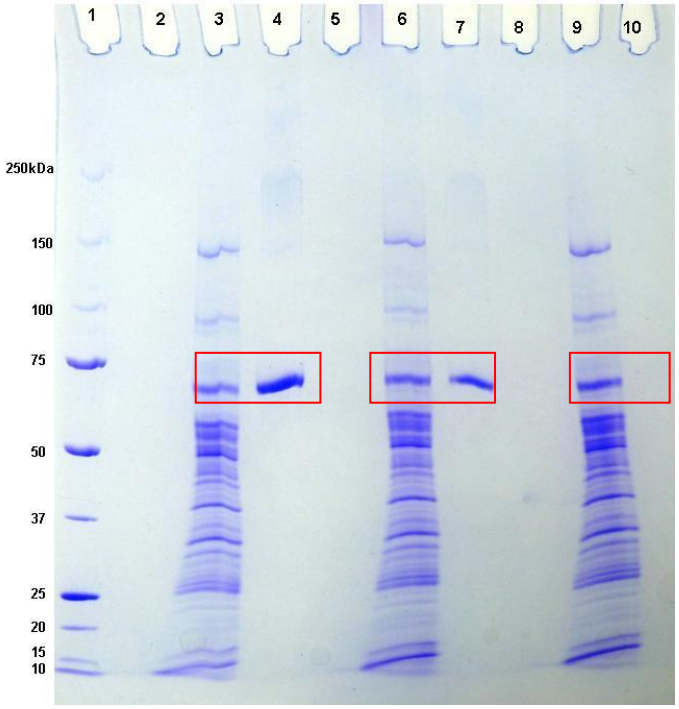


Figure 18. Coomassie stained 4-20% PAGE of different BSA concentrations in the minced organ culture (Disaggregation #34) in 6-well plate with the addition of 2mL HMM. Samples were denatured by the addition of DTT and heating to 95C for 2min. 15 ug of each sample in a total volume of 20uL was run at 200V for 1hr and 10min and then stained with Coomassie blue. Lane 1: unstained molecular weight marker (10-250kDa): lane 3: 1g/L BSA containing minced organ culture; lane 4: 1g/L BSA Control; lane 6: 0.5g/L BSA containing minced organ culture; lane 7: 0.5g/L BSA Control; lane 9: 0g/L BSA containing minced organ culture; lane 10: 0g/L BSA Control.

Figure 18 demonstrates that BSA maybe produced by the minced organ culture. The culturing performed without the addition of BSA to the medium still has a prominent band at 48hrs (Fig. 11, lane 9). There is also decrease in intensity between control (lane 4) and minced organ culture with 1mg/ml BSA (lane 3) which may indicate that either the cells are consuming the BSA during the culture process or it gets degraded, therefore removing of the BSA may effect bioactive production other than AP.

1.1.44 In-solution enzymatic digestion of minced organ culture wave trial 2 and RIPA extracted liver tissue

Protein samples from the wave trial 2 and RIPA extracted tissues were directly subjected to an enzymatic digestion and analysed via mass spectrometry. This is an approach to get a rough idea of all the proteins present in the sample, without having to run expensive 2D gels.

`

Protein mixtures were identified using the in-solution tryptic digestion and analysed with a Thermo LTQ XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corp, San Jose, CA). 137 proteins were identified in the WAVE#2 sample and 228 proteins in the RIPA extracted bovine minced liver sample. Keratins were excluded from the samples as they are the principal proteins found in skin, nails and hair and represent an artefact often found in mass spectrometry samples. The ranking the protein within a sample tends to give an indication about their abundance in the sample, although this is not quantitative (Table 5). The bioactive ranked higher in the wave sample than the RIPA extraction is listed below.

Protein disulfide-isomerase is ranked 7 in the WAVE#2 samples relative to all the other proteins found in that sample and 27 in the RIPA sample. Protein disulfide isomerase (PDI) catalyses thill/disulphide interchange reactions and, depending on the nature of the polypeptide substrate and the imposed redox potential promotes protien disulphide formation, Isomerization or reduction. PDI does not determine the polypeptide's folding pathway, but rather facilitates formation of the correct set of disulphide bonds by promoting rapid reshuffling of incorrect dulphide pairings. Price from Sigma is $147 / 1mg for lyophilized powered from bovine liver, 100-400 units/mg protein (Cat# P-3818) (Gething and Sambrook, 1992).

Fatty acid-binding protein is ranked 20 in the WAVE#2 and 40 in the RIPA sample relative to the other proteins found in the sample. Human Fatty Acid Binding Protein-s exhibit high affinity for small lipophilic ligands. Studies suggest that FABPs are involved in the uptake and metabolism of fatty acids, maintenance of cellular membrane fatty acids levels, intracellular trafficking, modulation of specific enzymes of lipid metabolic pathways, as well as in the modulation of cell growth and differentiation. Recombinant human FABP-1 was expressed from *E. coli* and purified through proprietary method. The price for this protein from Sigma is $576.45 per 100ug for human recombinant protein derived from E coli (SRP 4501-100UG)

Superoxide dismutase (SOD) is ranked 23 and in the WAVE#2 versus Rank 76 in the RIPA sample. Superoxide dismutase catalyses the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Plays a critical role in the defence of cells against the toxic effects of oxygen radicals. Competes with nitric oxide (NO) for superoxide anion (which reacts with NO to form peroxynitrite), thereby SOD promotes the activity of NO. SOD has also been shown to suppress apoptosis in cultured rat ovarian follicles, neural cell lines, and transgenic mice [4-8]. It is sourced at a concentration of 120-200mg/kg tissue including from blood, liver, heart and kidney. Actual market size is not known, however several companies supply Superoxide Dismutase at retail level (MLA, 2009). Superoxide Dismutase is valued at $426.30 / 5mg for a bovine liver derived product (Sigma, cat # S8160-75kU).

		Rank

`

Number	Protein	Wave#2	RIPA
1	Serum Albumin	1	1
2	Catalase	2	2
3	Glutamate dehydrogenase 1, mitochondrial	4	8
4	Aldehyde dehydrogenase, mitochondrial	5	12
5	Haemoglobin subunit beta	6	15
6	Protein disulfide-isomerase	7	27
7	3-hydroxyacyl-CoA dehydrogenase type-2	8	5
8	Glutathione S-transferase A1	9	24
9	Haemoglobin subunit alpha	10	21
10	3-ketoacyl-CoA thiolase, mitochondrial	11	13
11	Glycogen phosphorylase, liver form	12	11
12	Glycine N-acyltransferase	13	7
13	Serotransferrin	14	17
14	Regucalcin	15	6
15	Retinal dehydrogenase 1	16	10
16	Alpha-actinin-4	17	25
17	Calreticulin	18	22
18	60 kDa heat shock protein, mitochondrial	19	28
19	Fatty acid-binding protein, liver	20	40
20	78 kDa glucose-regulated protein	21	38
21	Fructose-bisphosphate aldolase B	22	36
22	Superoxide dismutase [Cu-Zn]	23	76
23	Glycine amidinotransferase, mitochondrial	24	4
24	4-hydroxyphenylpyruvate	25	32

	dioxygenase		
25	Glutathione S-transferase Mu 1	26	34

Table 5. List of the first 25 protein hits detected in the WAVE#2 samples and its correspondent rank versus their Rank in the RIPA extracted minced liver sample.

1.1.45 Quantitative two-dimensional differential gel electrophoresis

Proteomic analysis of minced bovine liver organ cultures was performed using quantitative two-dimensional differential gel electrophoresis (2D DIGE) to determine relative abundance of target bioactives. Mass spectrometry was then used to determine the identity of amplified bioactive targets to find out whether any of the amplified proteins are of commercial interest. Proteomics techniques were applied to determine whether the addition of chemical stimulants, the process of minced organ culture or time in culture increased the production of bioactive targets. Selection of new bioactives for this research program can now to be based upon relative abundance of the bioactives and economic analysis of the targets' commercial viability.

Sample preparation of the 0 hr non-stimulated 48 hr stimulated and non-stimulated bovine minced organ culture

Bovine minced organ culture was obtained by collection of approximately 8 different bovine livers. Livers were rinsed, minced and 1.5 g of pooled liver tissue was inoculated in triplicate into 6-well plates in a total volume of 2 mL HMM (see appendix ROP 30 for details). A stimulant combination of Insulin at a final concentration of 10^{-4}M and Retinoic acid at a final concentration of 1 μM was added to one row of three replicates and minced organ tissue was then cultured for 48 hr at 5% CO_2, 37°C. Samples were drawn and tested negative for bacterial growth. Total protein determination was carried out using a colorimetric protein-assay. Samples were then subjected to 1-DE, followed by 2-DE and DIGE.

Two-dimensional differential gel electrophoresis

Three experiments were carried out to determine the upregulation of new bioactives in different ways. Gel#1 examined 0 hr minced organ culture and 48 hr non-stimulated to examine the production of bioactives over time. Gel#2 48 hr minced organ culture non-stimulated and 48 hr minced organ culture stimulated (Retinoic Acid/Insulin). This experiment looked at the effect of the stimulants on bioactive production. Gel#3 compared RIPA total protein and 48 hr minced organ culture non-stimulated. This experiment looked at bioactives that were up regulated in the minced organ culture compared to what could be

`

extracted from the tissue. This experimental plan including the fluorescent labelling of proteins is summarised in Table 6.

Gel	Cy2	Cy3 - Control	Cy5 – "Treated"
1	Pooled standard	0 hr (A)	48 hr (A)
2	Pooled standard	48 hr (A)	Stimulated 48 hr (A)
3	Pooled standard	RIPA (A)	48 hr (A)

Table 6 Experimental design DIGE

Image Acquisition and Analysis

DIGE gels were visualized by scanning the gels directly between the glass plates with a fluorescence Typhoon 9400 scanner (GE Healthcare). Images were analysed using the DeCyder software (v7.0 Amersham Biosciences), Cy-2 images (pooled samples) were compared with Cy-3 or Cy-5 images that were alternatively minced organ culture for the first analysis, 48 hr minced organ culture non-stimulated and 48 hr minced organ culture stimulated (Retinoic Acid/Insulin) for the second analysis and RIPA and 48 hr minced organ culture non-stimulated for the third analysis. Inter-gel spot detection and quantification was performed using differential in-gel analysis (DIA), see appendix ROP#27 for details).

After 2DE, the analysis of the Cy2, Cy3, and Cy5 gel images with the DIA module of the DeCyder software revealed changes in the relative abundance of 588 proteins out of 1279 detected in Gel#1, 22.8% were up-regulated and 23.2% were down-regulated. In Gel#2, only 15 proteins out of 1259 proteins showed changes in the relative abundance, 0.4% showed up-regulation and 0.8% showed down-regulation. 1140 proteins were detected in Gel#3, among these proteins, 16.6% showed an increase in the relative abundance and 26.6% were decreased. Figure 19 shows graphically a comparison between the amount of protein spots that have increased and decreased within the different gels.

`

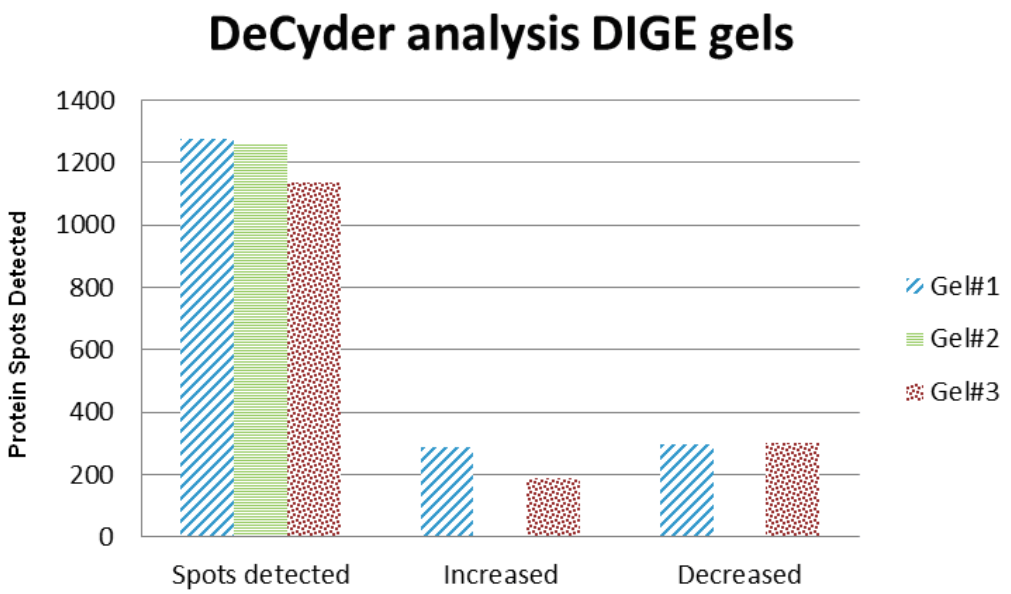


Figure 19 Spot detection DeCyder DIGE gel analysis

Figure 19 demonstrates there were very little differences in protein abundance between proteins obtained from 48 hr n.s. minced organ culture and 48 hr stimulated. minced organ culture, therefore no proteins were chosen from Gel#2 (48 hr non-stimulated vs 48 hr stimulated). Gel#1 (0 hr vs 48 hr n.s.) and Gel#3 (RIPA vs 48 hr n.s.) revealed both proteins that showed either up-regulation in proteins located on the same position in the gel or a significantly high total volume of the peaks, which gives an indication about the total protein amount in the sample. Therefore we chose 10 proteins (Table 7) that showed either at least a two-fold increase in protein abundance or a significant high maximum volume in either of the gels. A suite of different parameters influenced the choice of spots for further analysis: proteins that were the same in both the gels, proteins that showed a high volume ratio and/or maximum volume or whether the spot could be located on the gel. Proteins that were located in the middle of a protein crowded area (~pI 8 and Mr~60kDa) and or close to bovine serum albumin proteins (~pI 5-6 and Mr~66kDa) could not be chosen.

`

Spot Number	Volume Ratio Gel#1 (48 hr n.s. vs 0 hr)	Max Volume Gel#1 (48 hr n.s. vs 0 hr)	Total Volume Ratio Gel#1 0 hr vs 48 hr n.s. Factor: 1.96 (conc.48 hr n.s. / conc.0 hr) Fold change	Volume Ratio Gel#3 (48 hr n.s. vs RIPA)	Max Volume Gel#3 (48 hr n.s. vs RIPA)	Total Volume Ratio Gel#3 48 hr n.s. vs RIPA Factor: 0.43 (conc.48 hr n.s. / conc.RIPA) Fold change
1	53.60	2770936.0	105.06	20.02	2135101.0	8.61
2	34.20	1154789.0	67.03	18.65	1117649.0	8.02
3	18.20	715445.0	35.67	1.24	801822.0	0.53
4	10.90	3628807.0	21.36	1.76	1005576.0	0.76
5	10.40	2166801.0	20.38	2.01	2039876.0	0.86

`

6	9.00	11852796.0	17.64	3.24	10442066.0	1.39
7	7.60	5731884.0	14.90	3.07	4222071.0	1.32
8	2.80	6482141.0	5.49	0.51	7605044.0	0.22
9	8.50	5700230.0	16.66	1.97	5180457.0	0.85
10	3.30	4660425.0	6.47	0.72	5356192.0	0.59

Table 7 Ratio volumes for each spot chosen for MS analysis in Gel#1 (0 hr vs 48 hr n.s.) and Gel#2 (RIPA vs 48 hr n.s.)

Table 7 shows the 10 protein spots chosen for mass spectrometric analysis with their corresponding Volume Ratios in Gel#1 (0 hr vs 48 hr n.s.) and Gel#3 (48 hr n.s. vs 48 hr stim.). Volume Ratios were calculated by DeCyder v7.0 (GE Healthcare). The gels where loaded with equal amount of protein, therefore the volume ratio gives us the relative amount a given protein spot. To calculate the total fold change of each protein spot, these relative volume ratios were multiplied (if increased) or divided (if decreased) by a factor that takes into consideration the difference in protein concerntration for each of the samples. This factor was calculated by dividing the original protein concentration of the "treated" sample by the original protein concentration of the corresponding "Control" (see Table 6). This resulted in the total fold change of each protein in relation to the total protein content in the control sample. Spot numbers 1-4 show a volume ratio of at least 10-fold in Gel#1 and Gel#2 and, therefore, were picked for MS analysis. Spot number 5 shows a 10-fold increase as well as a high max volume (11million) in Gel#1 and at the same time it resulted in a 2-fold increase in Gel#3. Spot number 6 shows over 2-fold increase in protein expression and a high max volume in both gels. Spot number 7 resulted in an over 2-fold increase in protein expression in both of the gels. Protein numbers 8-10 show at least 2-fold increase in protein expression in Gel#1 and even though they resulted in a decrease in volume ratio, they showed a relatively high max volume with at least 5 million in Gel#3.

MS analysis and database searching of in-gel tryptic digested proteins

Spots of interest were localized on the gel by comparing the Silver stained spot pattern with the 2D-DIGE protein pattern (Figure 2). The digested peptides were analysed with a Thermo LTQ XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corp, San Jose, CA). The spectra were searched with Bioworks 3.3 (Thermo Electron Corp, San Jose, CA) using the Sequest algorithm against the IPI rat database v3.39 with the following filters: 1) the cross-correlation scores (Xcorr) of matches were greater than 1.5, 2.0 and 2.5 for charge state 1, 2 and 3 peptide ions respectively, 2) peptide probability was greater than 0.001 and 3) each protein identified has at least 2 different peptides sequenced.

Table 8 shows the names of the 15 proteins that were identified via mass spectrometry from the 10 chosen protein spots of interest. Due to the sensitivity of the ion trap several hits were given for one protein picked from the gel (Figure 8). Molecular weight (Mr) and pI of the hits were compared manually with the real spot that was chosen from the gel. Therefore some of the protein spots resulted in a mixture consisting of several proteins (e.g. Proteins number 4 and 6).

`

Number	MLA Bioactive Compendium	Name	Protein AC	Coverage	Score	Mr	pI	Production	Price as given by manufacturer in US dollar	Price per mg in US dollar	Supplier
1	No	Cytochrome b5 (CYB5)	P00171	47.76	51.48	15.3	5.03	active human recombinant produced in E.coli).	1mg - $331.00	$331.00	*Sigma-Aldrich* http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=C1427|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC
2		Cytochrome b5 (CYB5)	P00171	74.76	25.28	15.3	5.03				
3	No	Thioredoxin-dependent peroxide reductase, mitochondrial (PRDX3)	P35705	18.29	17.74	28.2	7.58	(human recombinant produced in E.coli; cross reacts with human).	1mg - $2,500	$2,500.00	*GenWay* http://www.genwaybio.com/product_info.php?products_id=5153
4.1	Yes	L-lactate dehydrogenase B chain (LDHB)	Q5E9B1	42.81	246.53	36.7	6.44	(human recombinant produced in E.coli)	20ug - $129.00	$6,450.00	*American Research Products, Inc.™* http://www.arp1.com/content/ldhb-l-lactate-dehydrogenase-b-chain-

`

											1-334aa-his-tagged-human
4.2	Yes	Malate dehydrogenase,cytoplasmic (MDH1)	Q3T145	32.63	59.06	36.4	6.58	(human recombinant produced in E.coli)	20ug - $159.00	$ 7,950.00	*American Research Products, Inc.™* http://www.arp1.com/node/6419
4.3	No	Delta-aminolevulinic acid dehydratase (ALAD)	Q58DK5	40.43	39.80	36.1	6.98	(human recombinant in vitro in wheat germ expression)	10ug – $299.00	$29,900.00	*Abnova* https://www.abnova.com/Products/products_detail.asp?Catalog_id=H00000210-Q01
4.4	No	Fructose-1,6-bisphosphatase 1 (FBP1)	Q3SZB7	27.51	21.55	36.7	6.99	(human recombinant in vitro in wheat germ expression)	10ug - $299.00	$29,900.00	*Abnova* http://test.abnova.com/Products/products_detail.asp?Catalog_id=H00002203-P01&PageID=1&classid=&OwnClass=&SL=EN
5	Yes	Superoxide dismutase [Mn], mitochondrial	P41976	19.37	33.27	24.6	8.54	(recombinant expressed in E.coli)	100ug - $290.00	$2,900.00	*Sino Biological Inc.*

`

		(SOD2)									http://www.sinobiological.com/SOD2-Protein-g-5226.html
6.1	Yes	Glutamate dehydrogenase 1, mitochondrial (GLUD1)	P00366	51.79	605.94	61.5	7.59	(Human recombinant produced *in vitro* in wheat germ expression system)	10ug - $299.00	$29,900.00	*Abnova* http://www.abnova.com/products/products_detail.asp?Catalog_id=H00002746-P01
6.2	No	UTP--glucose-1-phosphate uridylyltransferase (UGPA)	Q07130	44.69	138.24	56.9	7.88	---	---	---	---
6.3	No	Aldehyde dehydrogenase, mitochondrial (ALDH2)	P20000	42.50	105.66	56.6	7.65	(human recombinant produced in E.coli)	1mg – $2,145.00	$2,145.00	*GenWay* http://www.genwaybio.com/product_info.php?products_id=47078&osCsid=2146429fd51522b6c62099b7313a7f9
6.4	no	Serine hydroxymethyltransferase, cytosolic (SHMT1)	Q5E9P9	35.74	85.68	52.9	8.03	(Human recombinant produced *in vitro in* wheat	10ug - $299.00	$29,900.00	*Abnova* http://www.abnova.com/P

`

								germ expression system)			roducts/products_detail.asp?Catalog_id=H00006470-Q01&PageID=1&classid=&OwnClass
7	No	60 kDa heat shock protein, mitochondrial (HSPD1)	P31081	66.67	655.57	61.1	5.74	(Human recombinant produced *in vitro in* wheat germ expression system)	10ug - $299.00	$29,900.00	*Abnova* http://www.abnova.com/products/products_detail.asp?Catalog_id=H00003329-P01
8	No	Protein disulfide-isomerase (PDIA1)	P05307	45.29	416.70	57.2	4.91	(human recombinant in E.coli)	1mg – $93.00	$93.00	*Sigma* http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=P3818|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC
9	No	Aspartate aminotransferase, mitochondrial (AATM)	P12344	59.77	291.48	47.5	9.07	(human recombinant in E.coli)	100ug – $260.00	$2,600.00	*Abcam* http://www.abcam.com/Aspartate-Aminotransferase-protein-

`

											ab99147.html
10	No	Dipeptidyl peptidase 3 (DPP3)	Q99KK7	10.57	29.99	82.9	5.34	(human recombinant produced in vitro in wheat germ expression system)	10ug - $299.00	$29,900.00	*Abnova* http://www.abnova.com/products/products_detail.asp?Catalog_id=H00010072-Q01

Table 8 Differential protein expression in DIGE experiment (bovine liver minced organ culture)

Function of proteins

1,2 CYB5 is a membrane bound hemoprotein which functions as an electron carrier for several membrane bound oxygenases. **[1]**

3 PRDX3 is involved in redox regulation of the cell. It protects radical-sensitive enzymes from oxidative damage by a radical-generating system. **[2]** It functions as an antioxidant and is localized in the mitochondrion. **[3]**

4.1 LDHB, is a member of the lactate dehydrogenase family. It is an oxidoreductase which catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD+. As this protein can also catalyze the oxidation of hydroxybutyrate, it is occasionally called Hydroxybutyrate Dehydrogenase. The LDH family consists of three members, LDH-A, LDH-B and LDH-C. LDHs function as powerful markers for germ cell tumors. **[4]**

4.2 MDH1 is one of the two malate dehydrogenases. MDH catalyzes the reversible oxidation of malate to oxaloacetate, utilizing the NAD/NADH cofactor system in the citric acid cycle. In particular, MDH1 is localized to the cytoplasm and may play pivotal roles in the malate-aspartate shuttle that operated in the metabolic coordination between cytosol and mitochondria. This protein also regulates p53-dependent cell-cycle arrest and apoptosis in response to glucose deprivation. **[5]**

`

4.3 ALAD catalyzes an early step in the biosynthesis of tetrapyrroles. Binds two molecules of 5-aminolevulinate per subunit, each at a distinct site, and catalyzes their condensation to form porphobilinogen. **[6]**

4.4 FBP1 is an enzyme in the liver that converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis (the synthesis of glucose from smaller substrates). This enzymes only catalyse the reaction in one direction each and is regulated by metabolites such as fructose 2,6-bisphosphate. **[7]**

5 SOD2 destroys superoxide anion radicals which are normally produced within the cells and which are toxic to biological systems. **[8]**

6.1 GLUD1 may be involved in learning and memory reactions by increasing the turnover of the excitatory neurotransmitter glutamate. **[9]** GLUD1 is a mitochondrial matrix enzyme, with a key role in nitrogen and glutamate metabolism and energy homeostasis. GLUD1 is expressed at high levels in liver, brain, pancreas and kidney, but not in muscle. In the pancreatic cells, GLUD1 is thought to be involved in insulin secretion mechanisms. In nervous tissue, where glutamate is present in concentrations higher than in the other tissues, GLUD1 appears to function in both the synthesis and the catabolism of glutamate and perhaps in ammonia detoxification. **[10]**

6.2 UGPA plays a central role as a glucosyl donor in cellular metabolic pathways. **[11]**

6.3 ALDH2 belongs to the aldehyde dehydrogenase family of proteins. Aldehyde dehydrogenase is the second enzyme of the major oxidative pathway of alcohol metabolism. This gene encodes a mitochondrial isoform, which has a low Km for acetaldehydes, and is localized in the mitochondrial matrix. **[12]**

6.4 SHMT1 is an enzyme which plays an important role in cellular one-carbon pathways by catalyzing the reversible, simultaneous conversions of L-serine to glycine (retro-aldol cleavage) and tetrahydrofolate to 5,10-methylenetetrahydrofolate (hydrolysis). This reaction provides the largest part of the one-carbon units available to the cell. **[13]**

7 HSPD1 is implicated in mitochondrial protein import and macromolecular assembly. It may facilitate the correct folding of imported proteins. It may also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix. **[14]**

8 PDIA1 is a multifunctional protein which catalyzes the formation, breakage and rearrangement of disulfide bonds. At the cell surface it seems to act as a reductase that cleaves disulfide bonds of proteins attached to the cell. It may therefore cause structural modifications of exofacial proteins. Inside the cell, it seems to form/rearrange disulfide bonds of nascent proteins. At high concentrations, it functions as a chaperone that inhibits aggregation of misfolded proteins. At low concentrations, it facilitates aggregation (anti-chaperone activity). It may be involved with other chaperones in the structural modification of the TG precursor in hormone biogenesis. It also acts as a structural subunit of various enzymes such as prolyl 4-hydroxylase and microsomal triacylglycerol transfer protein MTTP. **[15]**

9 AATM plays a key role in amino acid metabolism. It is important for metabolite exchange between mitochondria and cytosol. It facilitates cellular uptake of long-chain free fatty acids. **[16]**

10 DPP3 is a member of the S9B family in clan SC of the serine proteases. This cytoplasmic protein binds a single zinc ion with its zinc-binding motif (HELLGH) and has post-proline dipeptidyl aminopeptidase activity, cleaving Xaa-Pro dipeptides from the N-termini of proteins. Increased activity of this protein is associated with endometrial and ovarian cancers. Alternate transcriptional splice variants have been characterized. **[17]**

1 http://www.uniprot.org/uniprot/P00171

2 http://www.uniprot.org/uniprot/P35705

3 http://www.en.wikipedia.org/wiki/PRDX3

4 http://www.arp1.com/content/ldhb-l-lactate-dehydrogenase-b-chain-1-334aa-his-tagged-human

5 http://www.arp1.com/node/6419

6 http://www.uniprot.org/uniprot/Q58DK5

7 http://www.en.wikipedia.org/wiki/Fructose_1,6-bisphosphatase

8 http://www.uniprot.org/uniprot/P41976

9 http://www.uniprot.org/uniprot/P00366].

10 http://www.en.wikipedia.org/wiki/Glutamate_dehydrogenase_1

11 http://www.uniprot.org/uniprot/Q07130

12 http://www.biomart.com/symbolsearch_ALDH2.htm

13 http://www.en.wikipedia.org/wiki/Serine_hydroxymethyltransferase

14 http://www.uniprot.org/uniprot/P31081

15 http://www.uniprot.org/uniprot/P05307

16 http://www.uniprot.org/uniprot/P12344

17 http://www.en.wikipedia.org/wiki/DPP3

`

1.1.46 Progress in proteomics analysis of minced organ culture

The screening of several different albumin depletion methods for the minced organ culture sample showed Acetone/TCA was a suitable method for the depletion of BSA present in medium from disaggregated cell.

In-solution tryptic digestion of the WAVE#2 and the RIPA extracted minced organ tissue resulted in 137 proteins for the WAVE#2 and 228 protein for the RIPA sample. All proteins from the WAVE#2 samples that have a high score are also present in the RIPA sample but in a different rank. This gives an indication about the protein abundance in the sample, as the higher ranked samples tend to be more abundant. Through comparing the ranks of the proteins in the solution digested RIPA sample to the minced organ culture medium we identified the proteins disulfide isomerase, fatty acid binding protein and super oxide dismutase as potential bioactive targets found within the minced organ culture medium.

The addition of BSA to minced organ culture medium did no significantly change the protein production and AP activity, therefore BSA is not required for AP production. However removing the BSA from in the media did not lower the amount of BSA in the media after 48hrs culturing. When the addition of 1mg/ml BSA to the minced organ culture was analysed on a one-dimensional SDS-PAGE a decrease in BSA could be observed to the corresponding control, indicating that the BSA might get either consumed by the cells or degraded. Therefore although BSA is not required for the production of AP, because it is may be consumed it could be required for the production of other bioactives.

we have established a 2D proteomics profile of a total protein extract of bovine minced organs, as well as a bovine minced organ liver culture, cultured for 48 hr in a small-scale experiment with and without any stimulation. To identify new target bioactives, proteomics analysis was performed using quantitative 2D DIGE. In this approach relative abundance of proteins was determined that resulted in a 2-fold increase or more in expression and that showed a higher total protein volume, indicating a greater amount of protein in the starting material, compared to the corresponding control.

2D DIGE analysis revealed up-regulation in protein expression in 291 (22.8%) proteins out of 1279 detected in Gel#1 (48 hr n.s. vs 0 hr). In Gel#2 (48 hr stim. vs 48 hr n.s.), only 5 (0.4%) proteins were up-regulated out of 1259 proteins. Gel#3 (48 hr n.s. vs RIPA) resulted in a total detection of 1140 proteins, 189 (16.6%) of these proteins were up-regulated.

We identified the protein composition of 10 protein spots that showed an increase in expression of either 2-fold or more or a high total volume in either Gel#1 (48 hr n.s. vs 0 hr) or Gel#3 (48 hr n.s. vs RIPA). No proteins were chosen from Gel#2 (48 hr stim. vs 48 hr n.s.) as none of them showed any increase in protein expression or abundance. The proteins were classified according to their commercial availability and how they are produced (e.g. recombinant), their cost as stated by the manufacturer and their approximate cost per 1mg of product.

`

Cytochrome b5 was the best target identified (Table 2 and 3, proteins 1 and 2) in terms of fold increase in total abundance over time (Gel#1 volume ratio) and fold increase in total abundance when minced organ culture is compared to a RIPA extraction (Gel#3 volume ratio). However this bioactive has a relatively low price of $331/mg (Table 3) therefore market analysis and the development of analytical assays to quantify the amount of Cytochrome b5 produced would be required before it can be selected as a new bioactive target.

The proteomics analysis of the bovine minced organ culture and total protein extract of minced bovine livers resulted in 15 proteins identified by mass spectrometric analysis. All these proteins are commercially available at a high prices and show many different biological and biochemical applications. The majority of these proteins are currently being produced recombinantly in either *E.coli* or via wheat germ expression.

To make a final decision about a new target bioactive, more market analysis is required to see how this process can be competitive with recombinant bioactive production. From our proteomic analysis cytochrome b5 has been scientifically selected as the bioactive target with the highest fold increase in this series of experiments. Dependent on the market information, the research team would test commercially available assays to detect proteins of interest, determine its quantity and then proceed with purification of the product.

Success in Achieving Objectives

General Achievements

- Completed a comprehensive literature and patent review that achieved the following:
    - Review the top 5 bioactives selected by MLA to determine there suitability in terms of :
        - Function
        - Ability to be amplified
        - Availability of Detection methods
        - Availability of a Purification
- Provided information and knowledge for the development of a process model prepare by MLA industry consultant (Gareth Forde).
- Provided commercialisation support with MLA and aided in the engagement of industry into the bioactives value-adding projects

`

Specific achievement using liver

- **The successful development of three analytical methods for the target bioactives**

    - The challenge was to establish methods that are robust and reliable and can deal with both bovine and ovine samples that have significant biological variations and complex biochemical profiles.
    - We were successfully in developing analytical methods for alkaline phosphatase, Fibrinogen and basic Fibroblast growth factor.
    - The development of these assays enabled us to analyse the production of these bioactives using the in vitro cell culture technology.

- **Production of Bioactives from minced organ culture**

    - The amount of Alkaline Phosphatase produced in the mince medium was greater than the amount present in an extract of total proteins from the liver tissue.
    - A successful purification strategy was used to enrich for AP in the medium from minced organ culture.
    - Economic analysis for the process model prepared by Gareth Forde indicated that a successful business case could not be made for AP because of the emergence of a recombinant AP product into the market.
    - Minced organ culture represents a promising alternative to disaggregation that should enable fast tracking to commercialisation if a suitable economically viable bioactive can be identified.

- **Transfer the technology from Ovine to bovine**

    - Bovine cells show consistent yield, viability, morphology and cell survival to that of cells isolated from ovine tissues.
    - Bovine cells also produced more bioactives per gram of tissue than the equivalent ovine cells.
    - Transferring this technology to bovine broadens the scope of industrial engagement of beef processors, resulting in more opportunity to take this technology to industry and commercialisation.

- **Scale-up development of ovine liver disaggregation**

    - To demonstrate that scale-up to an integrated process was possible, we investigated the effects of scaling-up each individual step in the whole process and performed further optimisation, with the aim of achieving the maximal bioactive production.
    - This was a critical step to enable the progression to a bioreactor system and ensure the process could be further scaled-up to a commercial operation.
    - The development of quality control / quality assurance regimes for the investigation of microorganism contamination.

- **Testing of bioreactor systems**
    - The FibraStage (New Brunswick Scientific) and Wave (GE healthcare) disposable bioreactor systems that use the Fibra-Cel microcarriers technology where used to demonstrate attachment and survival of the disaggregated liver cells in a bioreactor.
    - Minced organ culture was also performed in a traditional stirred tank and disposable Wave bioreactor.
    - Scaled-up of AP production using minced organ culture was successfully performed using the Wave bioreactor, achieving a direct scale-up in the amount of bioactive produced in the small scale 6 well plate.

- **Identification of alternative bioactives using a proteomics approach**
    - Insolution digestion and DIEG were used to identify a range of bioactive targets
    - Market and economic analysis of these targets is required to determine their suitability as a commercial product

Impact on Meat and Livestock Industry – now & in five years time

For this project to have a significant impact on the meat and livestock industry the identification of a commercially viable target bioactive that has a route to market is of paramount importance. The identification of a target with a suitable business case will allow the successful uptake and implementation of this technology by industry. The selection of a bioactive target will need to be based upon the market and industry research will need to be conducted in conjunction with MLA.

Successful implementation of the technology to the target bioactive(s) will result in this process being ready for commercialisation within two years. The uptake of this technology by industry will add value to the meat processing industry via product diversification and to the bioactive producers by offering competitive advantages of price, quality and supply volumes over competitors.

Within five years, we envisage that this technology will be in commercial production. The production of bioactives from animal organs would be sourced from the less technical cell culture process producing bioactives from a minced organ cultures.

Conclusions and Recommendations

To obtain successful industry engagement we would need to developed commercial process within one to two years of investment. To enable a fast-track to commercialisation, several key tasks must be completed to enable the success of this technological opportunity:

- **Analysis of new bioactives identified through proteomics**
- **Transfer technology to alternative organ sources**
- **Scale-up bioreactor bioactive production to a pilot scale**

Proteomics studies of minced organ culture will be used to identify new bioactive targets in liver and other organs. This will demonstrate the technical feasibility of transferring the minced organ culture technology to other bioactives. Newly indentified bioactives will be included into the program after the completion of a preliminary market and economic analysis. Commercially viable targets will need to have suitable analytical assays developed and undergo initial evaluation through small scale bioactive production. Then this process will be scaled up to a pilot scale production of bioactive and examining the ability to scale-up the production of newly identified bioactives.

The identification of a target bioactive that has a quick route-to-market and is economically viable with a good market size is critical for the commercialisation of this technology. This will require analysis of the bioactives identified through our proteomics analysis. We recommend as part of the next phase of this project working closely with MLA in the development of a Market Size and Feasibility Study to aid in the identification of these bioactives and assist in commercialisation of this product to market. This bioactive target will require the development of analytical methods for the detection and quantification from ovine and bovine sources.

Recommendations

We recommend performing an initial marketing and economic analysis of the bioactives identified using the proteomics approach to determine whether any of these bioactives can demonstrates a commercially viable business case. Once a suitable bioactive target is identified we recommend moving forward by applying the following seven key recommendations.

1. *Generation of a computer model of minced organ culture technology*

We recommend that an accurate computer model of the minced organ culture technology be developed to:

- Establish a model of the production processes, productivities and yields for the project
- Simulate optimisation of the scale of production of a pilot plant to maximise yield for capital outlay

2. *Economic and Market analysis of new bioactives*

MLA has already prepared a database of economic data relating to a wide range of animal sourced bioactives. We recommend conducting a cost benefit analysis of the bioactives identified in this project through proteomics to determine which has the greatest potential for commercialisation and financial viability against current prices and likely competitor response variations in market prices and volumes. Marketing information that should be sourced includes: current market volumes, market values, SWOT, producers and techniques, potential markets, demand and market growth..

3. *Develop analytical assays*

We recommend developing analytical methods for the characterization and quantification of the newly selected bioactives. The development of a specific assay will be determined by the characteristics of the chosen bioactives.

4. *Test small scale bioactive production and scalability*

We recommend that the production of new bioactives identified from proteomic analysis should be investigated in a small scale (e.g. 6 well plate) experiment. This will require the establishment of new analytical methods and culture conditions for any new organs analysed. The initial feasibility for the scale-up of production will be analysed using laboratory techniques; such as increasing the size of the culture vessel (10-fold increase in size, from 2ml to 20ml) and the addition of a rocking platform. Newly identified

bioactives that are shown to be technically feasible and a have strong business case would then progress into bioreactor scale experiments.

5. *Transfer technology to other organs*

We recommend transferring this technology to other organs as this will further diversify the range of products. Organs chosen would include the heart, spleen and pancreas. These organs where chosen for their size, range of potentially valuable bioactives that they produce, and low cost. The first stage of transferring the minced organ culture technology to other organs would be the establishment of harvesting, mincing and culturing methods based upon the literature and what has previously been established for minced liver organ cultures in this project. New bioactives would be identified in these organs using a proteomics approach. New bioactives would require the development of analytical methods and need to undergo an economic and market evaluation.

6. *Identify novel bioactives using a proteomics approach*

The proteomics approach in Phase 2 has successfully expanded the number of bioactive targets, therefore we recommend that this same proteomics approach be applied to other organs. This will allow us to identify bioactive targets of commercial significance that are present in the minced organ culture medium that have been stimulated rather than attempting to stimulate or detect chosen bioactive targets. New bioactives identified would require the development of analytical assays, economic and market analysis. Selection of new bioactives into the research program could then be based upon commercial viability, abundance in media and level of stimulation.

7. *Pilot scale production of bioactives*

The key to demonstrating the production of bioactives with commercial potential will be determined by the development of a bioreactor system that produces bioactives with sufficient yield to be economically viable. Therefore we recommend developing an integrated system for pilot scale production of a bioactive, ready for industrial production. The establishment of a pilot scale production will require the engagement of an industry partner.

Bibliography

Andrade-Gordon, P., S. Y. Wang, et al. (1992). "Heparin-like activity in porcine follicular fluid and rat granulosa cells." Thromb Res **66**(5): 475-87.

Baird, A. (1994). "Fibroblast growth factors: activities and significance of non-neurotrophin neurotrophic growth factors." Curr Opin Neurobiol **4**(1): 78-86.

Bakala, A.W., Karlik, et al. (2003) Preparation of equine isolated hepatocytes. *Toxicol In vitro* **17**(5-6): 615-21.

Bhattacharya, A. and L. J. Holland (1991). "Coordinate regulation of fibrinogen subunit messenger RNA levels by glucocorticoids in primary cultures of Xenopus liver parenchymal cells." Mol Endocrinol **5**(4): 587-97.

Brigstock, D. R., R. B. Heap, et al. (1990). "Purification and characterization of heparin-binding growth factors from porcine uterus." Biochem J **266**(1): 273-82.

Burgess, W. H. and T. Maciag (1989). "The heparin-binding (fibroblast) growth factor family of proteins." Annu Rev Biochem **58**: 575-606.

Cairo, G., E. Rappocciolo, et al. (1991). "Expression of the genes for the ferritin H and L subunits in rat liver and heart. Evidence for tissue-specific regulations at pre- and post-translational levels." Biochem J **275 (Pt 3)**: 813-6.

Cham, B. E., H. P. Roeser, et al. (1985). "A procedure for the purification of ferritin from human liver by heating a methanol-treated homogenate." Anal Biochem **151**(2): 561-5.

Chan, J. R. and R. A. Stinson (1986). "Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatases." J Biol Chem **261**(17): 7635-9.

Gaetano, C., A. Catalano, et al. (2001). "Retinoids induce fibroblast growth factor-2 production in endothelial cells via retinoic acid receptor alpha activation and stimulate angiogenesis in vitro and in vivo." Circ Res **88**(4): E38-47.

Gahwiler, B. (1989) Slice cultures of nervous tissue in *A dissection and tissue culture manual of the nervous system* (Shahar, A., Vellis, J. D., Vernadakis, A. & Haber, B., eds) pp. 65-67, Liss, New York.

Gianni, M., M. Terao, et al. (1993). "Retinoic acid and cyclic AMP synergistically induce the expression of liver/bone/kidney-type alkaline phosphatase gene in L929 fibroblastic cells." Biochem J **296 (Pt 1)**: 67-77.

`

Goldsworthy, P. D., McCartor, H. R., McGuigan, J. E., Peppers, G. F. & Volwiler, W. (1970) Relative albumin, transferrin, and fibrinogen synthesis rates in perfused bovine liver, *Am J Physiol. 218*, 1428-33.

Gospodarowicz, D. (1991). "Biological activities of fibroblast growth factors." Ann N Y Acad Sci **638**: 1-8.

Gospodarowicz, D., N. Ferrara, et al. (1988). "Basic fibroblast growth factor: expression in cultured bovine vascular smooth muscle cells." Eur J Cell Biol **46**(1): 144-51.

Hui, M. Z., H. C. Tenenbaum, et al. (1997). "Collagen phagocytosis and apoptosis are induced by high level alkaline phosphatase expression in rat fibroblasts." J Cell Physiol **172**(3): 323-33.

Hui, M., M. Hu, et al. (1993). "Changes in cell adhesion and cell proliferation are associated with expression of tissue non-specific alkaline phosphatase." Cell Tissue Res **274**(3): 429-37.

Kaji, T., S. Ohkawara, et al. (1994). "Cadmium-induced alteration of glycosaminoglycans with an enhancement of heparin-like activity in cultured vascular endothelial cells." Toxicology **94**(1-3): 161-71.

Kajio, T., K. Kawahara, et al. (1992). "Quantitative colorimetric assay for basic fibroblast growth factor using bovine endothelial cells and heparin." J Pharmacol Toxicol Methods **28**(1): 9-14.

Kaplan, M. M. (1986). "Serum alkaline phosphatase--another piece is added to the puzzle." Hepatology **6**(3): 526-8.

Koenig, W. (2003). "Fibrin(ogen) in cardiovascular disease: an update." Thromb Haemost **89**(4): 601-9.

Kurioka, S., F. Inoue, et al. (1975). "Purification of fibrinogen using cationic detergent." J Biochem **77**(2): 457-61.

Lawson, D. M., P. J. Artymiuk, et al. (1991). "Solving the structure of human H ferritin by genetically engineering intermolecular crystal contacts." Nature **349**(6309): 541-4.

Lescoat, G., N. Hubert, et al. (1991). "Iron load increases ferritin synthesis and secretion in adult human hepatocyte cultures." Liver **11**(1): 24-9.

Liu, X. M., H. Liu, et al. (2006). "Suspended aggregates as an immobilization mode for high-density perfusion culture of HEK 293 cells in a stirred tank bioreactor." Appl Microbiol Biotechnol **72**(6): 1144-51.

LoMonaco, J., Rumsey, T. (2006). "The economics of single-use systems." BioProcess International **4**(9): 2.

Marcum, J. A., L. Fritze, et al. (1983). "Microvascular heparin-like species with anticoagulant activity." Am J Physiol **245**(5 Pt 1): H725-33.

MJ Gething, J Sambrook. Protein folding in the cells. Nature 1992 Jan 2; 355 (6355):33-45.

MLA , Bioactives Compendium, , July 2009; ISBN – 1 74036 425 2

Peng, X., B. Wang, et al. (2005). "Expression of basic fibroblast growth factor in rat liver fibrosis and hepatic stellate cells." J Huazhong Univ Sci Technolog Med Sci **25**(2): 166-9, 222.

Poelstra, K., W. W. Bakker, et al. (1997). "A physiologic function for alkaline phosphatase: endotoxin detoxification." Lab Invest **76**(3): 319-27.

Poelstra, K., W. W. Bakker, et al. (1997). "Dephosphorylation of endotoxin by alkaline phosphatase in vivo." Am J Pathol **151**(4): 1163-9.

Presta, M., M. Rusnati, et al. (1988). "Purification of basic fibroblast growth factor from rat brain: identification of a Mr 22,000 immunoreactive form." Biochem Biophys Res Commun **155**(3): 1161-72.

Rosenberg, R. D. (1975). "Actions and interactions of antithrombin and heparin." N Engl J Med **292**(3): 146-51.

She, Q. B., J. J. Mukherjee, et al. (2000). "Growth factor-like effects of placental alkaline phosphatase in human fetus and mouse embryo fibroblasts." FEBS Lett **469**(2-3): 163-7.

Sugiura, M., K. Hirano, et al. (1975). "Purification and properties of human liver alkaline phosphatase." Chem Pharm Bull (Tokyo) **23**(10): 2369-75.

Suryakala, S. and V. Deshpande (1999). "Purification and characterization of liver ferritins from different animal species." Vet Res Commun **23**(3): 165-81.

Volpi, N. (1996). "Purification of heparin, dermatan sulfate and chondroitin sulfate from mixtures by sequential precipitation with various organic solvents." J Chromatogr B Biomed Appl **685**(1): 27-34.

Ware, A. G. and G. F. Lanchantin (1954). "Purification of fibrinogen, prothrombin and thrombin." Physiol Rev **34**(4): 714-21.

Zackson, S. L. and M. S. Steinberg (1988). "A molecular marker for cell guidance information in the axolotl embryo." Dev Biol **127**(2): 435-42.

Appendices

Research Operating Procedures

Research Operating Procedure 1: T&R Protocols

1. Enter T&R at second gate with stone wall.
2. Press button for entry.
3. Park in visitor's car park – directly to the right.
4. Go to the main office and sign in.
5. Ask receptionist for a visitor's coat, boots, ear protection and hair net.
6. Return to the car to collect equipment and esky.
7. Leave all valuables in the car hidden in car (Jewellery not permitted in meat processing room).
8. Go to the entrance to the meat processing floor.
9. Wash boots first.
10. Wash hands with green soap, followed by blue ethanol wash.
11. Go to collection area.
12. Upon leaving, wash boots and hands with green soap and blue ethanol wash.
13. Return boots and coat to Reception.
14. Discard hair net and ear protection in the bin.
15. Sign out at Reception.
16. Press button to speak to receptionist to leave.

Research Operating Procedure 2: Collection of Animal Tissue

1. Sterilise equipment several days prior to sample collection by autoclaving:
    a. Scissors (x2)
    b. Scalpel blades (several)
    c. Tweezers (x2)
    d. Collection pots
2. Prepare sterile solutions for the collection of tissue:

`

   a. Liver - RPMI medium + antibiotics
   b. Corpus Luteum - HBSS medium + antibiotics

3. Preparation of visit – Collection of equipment needed:
   a. Sterilised equipment
   b. Gloves – several pairs each
   c. Paper towel
   d. 30 ml or 200 ml sample pots
   e. Collection tray
   f. Ethanol Spray
   g. Tissues
4. Sample pots require the addition of 10 ml of RPMI or HBSS per 30 ml collection tube or 150 ml per 200 ml sample pot.
5. Set up number of sample pots per tissue samples required.
6. Sample pots containing RPMI or HBSS must be stored on ice at 4^0C at least overnight to ensure the collection solution is evenly chilled.
7. On the day of sample collection, fresh ice is placed into an esky containing the sample pots and sealed with the esky lid.
8. At T&R Pastoral, follow Research Operating Procedure 1: T&R Protocols.
9. Upon entering the meat processing room, alert the line manager of the organ you wish to collect.
10. Collect the organ directly from the line in a pre-ethanol sprayed and wiped container.
11. Cut approximately 1-2 gram segments of tissue from the organ and place directly into the sample pot containing ice-cold RPMI (liver) or HBSS (Corpus Luteum), opening the lid for as little time as possible.
12. Return the unused organ to the line and rinse container under tap.
13. Transport samples on ice to the laboratory.

RPMI

To make one litre add the following:

RPMI 1640 powder 10.40g/L (Thermo 50-020-PC)

Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Adjust pH to 7.2

HBSS

To make one litre add the following

1 bottle of HBSS powder (Sigma H1387)

Sodium bicarbonate solution 4.1ml/L (Pfizer DW99)

Phenol Red 0.011g/L (Sigma P3532)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 3: Disaggregation of Corpus Luteum

1. Place the collected Corpus luteum (CL) into a sterile biohazard cabinet
2. Excise the CL by slicing the CL through the centre with a sterile scalpel blade and push it away from the surrounding tissue into a sterile petri dish
3. Slice the CL as thinly as possible using a scalpel blade and place into a pre-weighed, sterile 50ml conical flask
4. Record the exact weight of CL
5. Add 10ml of 400U/ml Type II Collagenase (Invitrogen 17101015) per gram of tissue
6. Incubate at 37^{o}C for 40 mins with shaking
7. Remove the collagenase from the CL tissue and replenish with fresh collagenase at 400U/ml
8. Incubate for 2 hours at 37^{o}C with shaking
9. Collect cells using a transfer pipette to a 10ml centrifuge tube and pellet cells at 200xg for 5 mins.
10. Wash cells with DMEM medium containing 10% FCS and pellet cells at 200xg for 5 mins.
11. Repeat Step 10, for a total of two washes.
12. Store cells on ice until ready to plate.

DMEM

`

Follow the Instructions on the DMEM bottle to make up one litre of medium

To the medium, add:

Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)

Sodium pyruvate 2.2g/L (Sigma P5280)

L-glutamine 0.5g/L (Sigma G8540)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 4: Isolation of Luteal Cells

1. Luteal cells are isolated by using a ficoll density gradient
2. Place 3ml of lymphoprep (ficoll – GE Healthcare 17-1440-03) into a 10ml centrifuge tube
3. Carefully layer 2ml of luteal cell suspension on top of the ficoll layer
4. Centrifuge the density gradient at 1500rpm in a benchtop centrifuge for 5 minutes
5. Carefully remove the cell layer and transfer to a fresh centrifuge tube
6. Add 10ml DMEM containing 10% FCS to the centrifuge tube and pellet the cells at 150xg for 5 mins.
7. Repeat Step 6 for a total of 2 washes
8. Resuspend the cell pellet in 2ml medium and perform cell counts (See Research Operating Procedure 5: Cell Counts using a Haemocytometer)

DMEM

Follow the Instructions on the DMEM bottle to make up one litre of medium

To the medium, add:

Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)

Sodium pyruvate 2.2g/L (Sigma P5280)

L-glutamine 0.5g/L (Sigma G8540)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 5: Cell and Viability Counts using a Haemocytometer

1. Take a 20µl sample of cell suspension and place into a clean well of a 96 well tray

`

2. Clean the surface of the haemocytometer with 70% ethanol and dry with Kleenex tissue
3. Mix 20µl of trypan blue solution (Sigma T6146) with the cell sample and mix thoroughly
4. Collect 20µl of sample into a yellow pipette tip
5. Transfer the cell suspension to the edge of the haemocytometer chamber and expel the suspension and allow it to be drawn under the coverslip by capillarity. IMPORTANT – Do NOT overfill or underfill the chamber.
6. Count all cells in at least 2 large squares of a haemocytometer depending on the number of cells present.

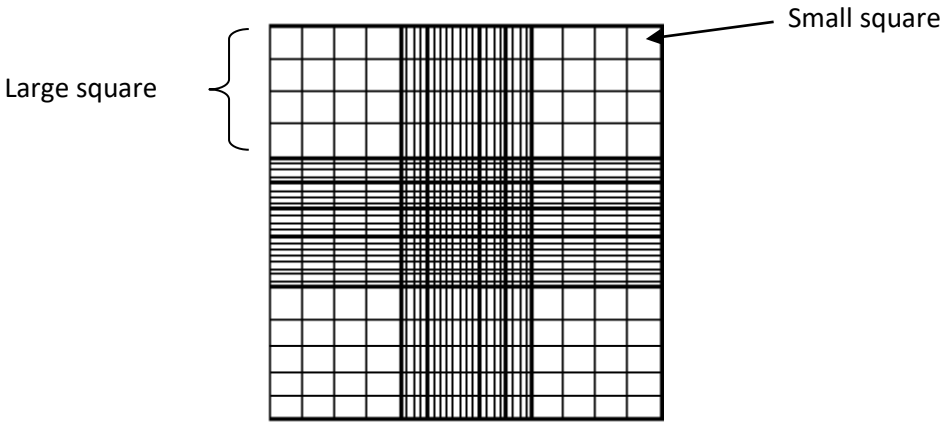


Figure 1. View of a haemocytometer under a microscope. (Figure modified from http://149.170.199.144/new_rd/contents/poisson.htm)

7. Calculate the number of cells by dividing the number of cells counted by the number of squares counted.
8. Multiply the number calculated in step 7 by the dilution factor (x2)
9. Multiply the number of cells calculated in step 8 by 1×10^4
10. This number is the number of cells present in 1ml of sample
11. Count the blue cells (dead) in the same square as total cell counts
12. Calculate viability by subtracting the number of dead cells counted from the total number of cells counted and divide the answer by the total number of cells counted. Express the answer as a percentage of total cells counted. Note: This count is based on numbers counted under the haemocytometer, not on the calculated number of cells per ml of sample

Research Operating Procedure 6: Plating of Luteal Cells into Cell Culture Flasks

1. Luteal cells are to be plated into DMEM containing 10%FCS and antibiotics

`

2. To seed cells into 96 well trays, cells are plated at densities from 10,000 to 20,000 cells per well in 200µl of medium
3. To seed cells into 6 well trays, 6 x 10^5 cells per well into 1.5ml medium
4. To seed T25 flasks, 1.67 x 10^6 cells per flask added to 5ml medium
5. To seed T75 flasks, 5 x 10^6 cells per flask added to 12ml medium
6. To seed T175 flasks, 1.17 x 10^7 cells per flask added to 40ml medium

Incubate cells in a CO_2 incubator at 37°C and 5%CO_2 overnight to allow cells to adhere to the tissue culture dish or flask

DMEM

Follow the Instructions on the DMEM bottle to make up one litre of medium

To the medium, add:

Sodium bicarbonate solution 17.8ml/L (Pfizer DW99)

Sodium pyruvate 2.2g/L (Sigma P5280)

L-glutamine 0.5g/L (Sigma G8540)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Research Operating Procedure 7: Stimulation of Progesterone from Sheep Luteal Cells

1. Prepare the stimulatory factors by dissolving them in the appropriate solvent (Luteinizing hormone – MQ water; 22-Hydroxy Cholesterol – ethanol; dibutyryl cyclic-AMP – MQ water).
2. Calculate the volume of medium required for each treatment and prepare a mastermix of each stimulant at the desired concentration in DMEM containing 10% FCS.
3. Concentrations of each stimulant should be in the following range:
    a. Luteinizing hormone (Sigma L5269) – 10-100ng/ml
    b. 22-Hydroxy Cholesterol (Sigma H9384) – 100 – 1000µM
    c. Dibutyryl cyclic-AMP (Sigma 34988) – 0.1 – 10mM
4. Remove luteal cells from the incubator that have adhered overnight following Research Operating Procedure 6: Plating of luteal cells into cell culture flasks.
5. Label the wells or flasks with the concentration and stimulant added
6. Aspirate the medium from the wells or flasks
7. Carefully add the medium containing the correct concentration and stimulant to each well / flask

`

8. Return the trays or flasks to the incubator and incubate the cells at 37°C and 5%CO_2 for 48 hours.
9. Collect the medium and transfer to a freshly labelled 96 well tray or 10ml centrifuge tubes and store at -20°C until ready to assay.

Research Operating Procedure 8: Analysis of Progesterone by ELISA

Day 1

Plate Coating

1. Use NUNC Maxisorp plates (44-2404 - Level 4 compactus)
2. Dilute primary progesterone antibody (Rabbit anti-progesterone) 1:20,000 in coating buffer
3. Add 50µl per well of antibody to plate
4. Tap plates gently to ensure that the coating covers the bottom of the well
5. Label, cover with acetate plate sealer and incubate overnight at 4°C (at least 12 hours)

Day 2

Standards

1. Standards used are 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12ng/well.
2. Dilute standard working stock (800ng/well or 16ng/ml) serially (2-fold) by using 200µl stock plus 200µl cell culture medium

Samples

1. Prepare media samples by diluting (as required) in medium to an appropriate dilution (approximately 1:100 to 1:1000)

HRP

1. Progesterone HRP conjugate working dilution is 1:40,000
2. Add 25µl of HRP working stock (1:200) to 5ml of EIA buffer to make the working dilution. Store this solution on ice.

Plate Washing

`

1. Wash the plate 5 times with wash solution
2. Blot the plate on paper towel to remove excess wash solution

Plate Loading

1. Pipette 50µl of standard or sample per well as quickly and accurately as possible, following the plate map
2. Add 50µl of diluted Progesterone HRP to all wells that contain standard, control or sample. Avoid Splashing!
3. Note: No more than 10 minutes should pass during this process.
4. Cover plates with acetate plate sealer and incubate at room temperature for exactly 2 hours

Plate Washing

1. Wash the plate 5 times with wash solution
2. Blot the plate on paper towel to remove excess wash solution
3. Plates are fairly stable at this point and can be left upside down on bench top until all plates are washed (no longer than 1 hour).

Substrate

Prepare ABTS substrate immediately before use (within 20 mins)

1. Combine 40µl 0.5M H_2O_2, 125µl 40mM ABTS and 12.5ml substrate buffer and mix well
2. Add 100µl ABTS substrate to all wells that contain standard, control or sample
3. Cover with plate sealer and incubate at room temperature with shaking
4. Plate colour development will vary based on the age of HRP and/or antibody, but should be less than one hour.

Plate Reading

1. Optical density (OD) of 0 wells should read 1 or less
2. Optimal readings for 0 wells: >0.7 to <1.0 OD.
3. Read plate at 405nm (reference 540 nm)

Progesterone Stock Preparation

`

Antibody

1. Dilute primary antibody (CL425) at a dilution of 1:50 by adding 100µl of stock to 4.9ml of coating buffer
2. Aliquot 300-400µl into vials and store at -20°C.
3. Store Antibody stock at -80°C.

HRP Conjugate

1. Dilute progesterone-3CMO-horseradish peroxidise 1:200 by adding 25µl of stock to 4.975ml EIA buffer for a working stock and store at 4°C.
2. Store HRP stock at -80°C.

Standards

1. Weigh out 0.5mg of progesterone (Sigma Cat. # P0130) and add 5ml ethanol in a scintillation vial for stock solution of 100µg/ml.
2. Dilute the 100µg/ml primary stock 1:5 by adding 1ml to 4ml ethanol for a 20µg/ml secondary stock (1000ng/well). A well is equal to 50µl.
3. Dilute the 20µg/ml secondary stock 4:5 by adding 4ml to 1ml ethanol for a 16µg/ml (800ng/well) working stock.
4. Aliquot working stock and store all stocks at -20°C.

Assay Buffers

Coating Buffer

0.795g Na_2CO_3 (Anhydrous) (Sigma S2127)

1.465g $NaHCO_3$ (Sigma S7561)

H_2O to 500ml

pH to 9.6 and store at 4°C.

EIA Buffer

`

Stock A Solution (0.2M NaH_2PO_4)

13.9g NaH_2PO_4 (Sigma S9638)

500ml H_2O

Stock B Solution (0.2M Na_2HPO_4)

14.2g Na_2HPO_4 (Sigma S7907)

500ml H_2O

97.5ml Stock A

152.5ml Stock B

4.35g NaCl (Sigma S9625)

0.5g BSA (Sigma A7906)

250ml H_2O

pH to 7.0 and store at 4°C

10x Wash Solution

43.83g NaCl (Sigma S9625)

2.5ml Tween 20 (Sigma P1379)

500ml H_2O

Store at 4°C

Dilute 10-fold for working wash solution (100ml wash solution plus 900ml H_2O). Store at room temperature

Substrate Buffer

`

4.805 g Citric Acid (Sigma C0759)

500ml H_2O

pH to 4.0, store at 4°C.

ABTS (40mM)

0.55g ABTS (Sigma A1888)

pH to 6.0

Make up to 25ml H_2O total volume

ABTS is light sensitive – use brown glass or foil for storage at 4°C.

Hydrogen Peroxide (0.5M)

500µl 30% H_2O_2 solution

8ml H_2O

Store at 4°C

Research Operating Procedure 9: Freezing and Thawing of Sheep Luteal Cells

Freezing of Cells

1. Collect sheep ovaries by following Research Operating Procedure 2: Collection of Animal Tissue.
2. Disaggregate the corpus luteum by following the Research Operating Procedure 3: Disaggregation of the corpus luteum.
3. Isolate luteal cells following Research Operating Procedure 4: Isolation of luteal cells.
4. Calculate the number of cells collected following Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

`

5. Prepare the freezing medium and place on ice
6. Pellet the cells at 1000rpm in a benchtop centrifuge for 5 minutes
7. Aspirate the medium and resuspend the cells in freezing medium to a concentration of 1 x 10^6 cells/ml.
8. Place 1ml of cell suspension into a labelled cryotube, secure the lid and place immediately on ice.
9. Incubate the cells in a -20^{o}C freezer for 1 hour
10. Transfer the cells to a -80^{o}C freezer and incubate overnight
11. Transfer the cells to liquid Nitrogen the next day

Thawing of Cells

1. Collect cryovial from liquid Nitrogen storage and place cells on ice
2. Transfer cells to tissue culture laboratory
3. Thaw cells as quickly as possible in a 37^{o}C water bath
4. Once the cells are liquid, add 1ml of DMEM containing 10% FCS to the cells dropwise
5. Transfer the cells to a 10ml centrifuge tube and add medium to 10ml total volume
6. Pellet the cells by centrifugation at 100 x g for 5 mins
7. Resuspend the cells in 1ml DMEM containing 10% FCS and count the cells using Research Operating Procedure 5: Cell Counts using a haemocytometer
8. Plate the cells into the desired tissue culture dishes / flasks following Research Operating Procedure 6: Plating of luteal cells into cell culture flasks

Freezing Medium:

Prepare DMEM as per Research Operating Procedure 6: Plating of luteal cells into cell culture flasks

To the DMEM add

FCS to 40%

DMSO (Sigma D2650) to 10%

Store on ice

Research Operating Procedure 10: MTT Assay

`

1. Plate cells into 96 well tissue culture trays
2. Add MTT (Sigma M2003) to a final concentration of 0.5 mg/ml (stock @ 5 mg/ml)
3. Incubate cells in MTT for 24 hours
4. Add 80µl per well of 20% SDS (Sigma L4390) in 0.2M HCl
5. Mix with a pipette gently
6. Ensure there are no bubbles present in each well as they will interfere with the spectrophotometer readings
7. Incubate plate in the dark at room temperature for 1 hr
8. Read on spectrophotometer at 560 nm with the lid removed

Research Operating Procedure 11: Mincing of Liver Samples

Small Scale

1. Measure the weight of a sterile 50ml conical flask
2. Place approximately 1g liver tissue dissected from at least 3 independent liver samples into a sterile petri dish in a biohazard cabinet.
3. Slice each of the liver samples as thinly as possible using a scalpel blade
4. Dice the liver sample into small cubes (approximately $1mm^3$) and transfer to the sterile conical flask using sterile forceps.
5. Measure the weight of the conical flask and calculate the exact weight of the liver sample
6. Add 10ml of RPMI to the liver samples and store overnight at 4^oC.

Large Scale

1. Measure the weight of a sterile 200ml conical flask
2. Set up the mincer by placing the blade and plate with the larger holes into the mincer
3. Place approximately 10g liver tissue dissected from at least 3 independent liver samples into a sterile petri dish in a biohazard cabinet.
4. Place the liver tissue into the mincer

`

5. Turn the mincer on and collect the minced liver tissue in a sterile petri dish
6. Transfer the mincer liver tissue into the conical flask using sterile forceps
7. Measure the weight of the conical flask and calculate the exact weight of the liver sample
8. Add 30ml of RPMI to the liver samples and store overnight at 4°C.

RPMI

RPMI-1640 Powder 10.4g/L (Thermo 50-020-PC)

Sodium Bicarbonate Solution 17.8ml/L (Pfizer DW99)

Penicillin / Streptomycin 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 12: Disaggregation of Liver Tissue

1. Preheat Shaking incubator and 0.05% collagenase (Invitrogen 17101015) solution to 37°C.
2. Remove liver samples from 4°C and transfer to a sterile biohazard cabinet.
3. For small scale disaggregation, remove the medium with a transfer pipette
4. For large scale, pass the liver and medium mixture through a metal strainer and transfer the tissue to the conical flask with a sterile metal spoon
5. To each flask add 0.05% collagenase solution (small scale: 6ml per 5g liver and large scale: 30ml per 30g liver tissue)
6. Transfer conical flasks to the shaking incubator and shake at 37°C for 30 mins
7. Transfer the flasks to the biohazard cabinet
8. Small Scale: Collect the cells in the disaggregation solution using a plastic transfer pipette. Transfer the cells to a 10ml centrifuge tube
9. Large Scale: Pass the liver tissue and disaggregation solution through a metal strainer and collect the cells into a clean, sterile beaker. Transfer the liver tissue back to the conical flask using a metal spoon. Transfer the cells into a 10ml centrifuge tube
10. Collect the cells by pelleting at 800rpm for 10 mins in a benchtop centrifuge
11. Remove the supernatant and resuspend the cells in 10ml HMM containing 20% FCS.
12. Incubate the cells on ice until the disaggregation process is complete

13. Add fresh 0.05% collagenase solution to the liver tissue as per step 5.
14. Repeat steps 6 to 12 for another 3 collections (total of 4 collections, or 2 hours)

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)

Medium 199 – 2.14g/L (Thermo 50-049-PA)

Sodium Pyruvate – 2.29 g/L (Sigma P5280)

L-Glutamine – 0.58g/L (Sigma G8540)

Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)

Bovine Serum Albumin – 1 g/L (Sigma A1470)

Penicillin / Streptomycin – 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 13: Isolation of Liver Cells

1. Liver cells are disaggregated from liver tissue as described in Research Operating Procedure 12: Disaggregation of liver tissue.
2. Pellet the cells at 800rpm for 10 mins in a benchtop centrifuge
3. Remove the supernatant and resuspend the cells in 10ml serum-free HMM
4. Pellet the liver cells by centrifugation at 50 x g resulting in the red blood cells staying in the supernatant
5. Remove the supernatant and resuspend cells in 10ml serum-free HMM
6. Repeat steps 4 and 5 for a total of two centrifugations at 50 x g.
7. To remove any liver tissue chunks that remain in the sample, set up the following:
    a. A sterile beaker (sterile)
    b. Small metal strainer (sterile)
    c. Cell filter mesh (sterile)
8. Gently pass the cell suspension through the cell filter mesh and collect the cells into the beaker below
9. Transfer the cells into a centrifuge tube

`

10. Count the number and viability of the cells following Research Operating Procedure 5: Cell and viability counts using a haemocytometer.

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)

Medium 199 – 2.14g/L (Thermo 50-049-PA)

Sodium Pyruvate – 2.29 g/L (Sigma P5280)

L-Glutamine – 0.58g/L (Sigma G8540)

Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)

Bovine Serum Albumin – 1 g/L (Sigma A1470)

Penicillin / Streptomycin – 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 14: Liver Cell Plating

1. Liver cells are to be plated into serum-free HMM
2. To seed cells into 96 well trays, cells are plated at densities from 10,000 to 20,000 cells per well in 200µl of HMM
3. To seed cells into 6 well trays, plate 2.5 x 10^6 cells per well into 1.5ml HMM
4. To seed T25 flasks, plate 6.51 x 10^6 cells per flask added to 5ml HMM
5. To seed T75 flasks, plate 1.56 x 10^7 cells per flask added to 12ml HMM
6. To seed T175 flasks, plate 3.65 x 10^7 cells per flask added to 40ml HMM
7. Incubate cells in a CO_2 incubator at 37^{o}C and 5%CO_2 overnight to allow cells to adhere to the tissue culture dish or flask

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)

`

Medium 199 – 2.14g/L (Thermo 50-049-PA)

Sodium Pyruvate – 2.29 g/L (Sigma P5280)

L-Glutamine – 0.58g/L (Sigma G8540)

Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)

Bovine Serum Albumin – 1 g/L (Sigma A1470)

Penicillin / Streptomycin – 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 15: Stimulation of Liver Cells

1. Prepare the stimulatory factors by dissolving them in the appropriate solvent to an appropriate concentration (See Table 1).
2. Calculate the volume of medium required for each treatment and prepare a mastermix of each stimulant at the desired concentration in serum-free HMM.
3. Concentrations of each stimulant should be within the range summarised in Table 1.
4. Remove liver cells from the incubator that have adhered overnight following Research Operating Procedure 14: Liver Cell Plating.
5. Label the wells or flasks with the concentration and stimulant added
6. Aspirate the medium from the wells or flasks
7. Carefully add the medium containing the correct concentration and stimulant to each well / flask
8. Return the trays or flasks to the incubator and incubate the cells at 37^{o}C and 5%CO_2 for 48 hours.

Collect the medium and transfer to a freshly labelled 96 well tray or 10ml centrifuge tubes and store at -20^{o}C until ready to assay.

Stimulant	Solvent	Stock Conc	Minimum Stim Conc	Maximum Stim Conc	Company	Product Number
Calcium Ionophore	DMSO	1mM	10^{-9}M	10^{-5}M	Sigma	C9400
Retinoic Acid	DMSO	0.1M	10^{-8}M	10^{-6}M	Sigma	R2625
Phorbol Ester (PMA)	DMSO	1mM	50nM	200nM	Sigma	P148
Arachidonic Acid	Ethanol	100mM	0.5µM	5µM	Sigma	A3555
Desferrioxamine Mesylate Salt	Water	500mM	100µM	500µM	Sigma	D9533

`

Cobalt Chloride	Water	0.5M	50µM	500µM	Sigma	15862
Insulin	Acidified water	10mM	250nM	1000nM	Sigma	I6634
Glucose	Water	1M	10mM	50mM	Sigma	G7528
Dexamethasone	Methanol	100mM	1nM	100nM	Sigma	D6645

Table 1. List of liver stimulants with the solvent used, stock concentration and range of concentration used for stimulating bioactive production. The ordering information is also provided

HMM (Hepatocyte Minimal Medium)

MEM – Eagle 6.27 g/L (Sigma M3024)

Medium 199 – 2.14g/L (Thermo 50-049-PA)

Sodium Pyruvate – 2.29 g/L (Sigma P5280)

L-Glutamine – 0.58g/L (Sigma G8540)

Sodium Bicarbonate solution – 17.8ml/L (Pfizer DW99)

Bovine Serum Albumin – 1 g/L (Sigma A1470)

Penicillin / Streptomycin – 10ml/L (Thermo SV30010)

Adjust pH to 7.2

Research Operating Procedure 16: basic Fibroblast Growth Factor ELISA

1. R&D Systems ELISA Kit (DY233)
2. Reconstitute **Capture Antibody** in 1ml PBS (results in a concentration of antibody at 360µg/ml)
3. Aliquot **Capture Antibody** and store at -20^{o}C
4. Reconstitute **Detection Antibody** in 1ml Reagent Diluent (1% BSA in PBS, filter sterilised) (results in a concentration of antibody of 45µg/ml)
5. Aliquot **Detection Antibody** and store at -20^{o}C
6. Reconstitute 1 vial of FGF-2 Standard with 0.5ml of Reagent Diluent (results in a concentration of standard of 80ng/ml).

`

7. Allow the standard to sit for 15 minutes with gentle agitation prior to making dilutions.
8. Store reconstituted standard at -70^{o}C.

Plate Preparation

1. Dilute the capture antibody to the working concentration (2µg/ml) in PBS without carrier protein.
2. Immediately coat the 96 well microplate with 100µl per well.
3. Seal the plate and incubate at room temperature overnight
4. **The Next Day:** aspirate each well and wash with wash buffer (0.05% Tween 20 in PBS).
5. Repeat wash 2 times for a total of 3 washes
6. Blot paper on clean towels to remove any remaining wash buffer
7. Block plates by adding 300µl of reagent diluent to each well
8. Incubate at room temperature for at least 1 hour
9. Repeat aspiration and wash step as per steps 4 and 5.
10. Plates are ready for addition of samples

Assay Procedure

1. Add 100µl of sample or standards in reagent diluent per well (Standards should consist of 2-fold dilutions of stock standard, utilising 7 points of a standard curve with a high standard of 1000pg/ml)
2. Cover plate with adhesive strip and incubate for 2 hours at room temperature
3. Repeat the aspiration and wash steps as per steps 4 and 5 above
4. Dilute detection antibody to a working concentration of 0.25µg/ml in reagent diluent and add 100µl of detection antibody to each well
5. Cover with a new adhesive strip and incubate at room temperature for 2 hours
6. Repeat the aspiration and wash steps as stated in steps 4 and 5 above
7. Add 100µl of the working dilution of Streptavidin-HRP to each well (as stated on the bottle) to each well. Cover the plate and incubate at room temperature for 20 minutes in the dark
8. Repeat the aspiration and wash steps as stated above in steps 4 and 5

9. Add 100µl of substrate solution (1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) to each well and incubate for 20 minutes at room temperature in the dark
10. Add 50µl of Stop Solution (2 Molar Sulphuric Acid) to each well. Gently tap the plate to ensure thorough mixing
11. Determine the OD of each well immediately using a microplate reader set to 450nm with wavelength correction set to 540 or 570nm.
12. Calculate results by plotting standard curve

Research Operating Procedure 17: Platelet Derived Growth Factor ELISA

1. R&D Systems ELISA kit (DY220)
2. Reconstitute vial of PDGF Standard with 0.5ml of Reagent Diluent (results in a concentration of standard of 130ng/ml). Mix thoroughly.
3. Wash Buffer: to make 1 litre, add 50ml of 20x PBS to 950 ml RO Water. Add 500µl of Tween20 (Level 5 chemical store).

Plate Preparation

4. Dilute the capture antibody (abcam (ab38561); stored at 4^{o}C) to 1/5000 (i.e. 1µl of antibody per 5ml PBS) in PBS without carrier protein.
5. Immediately coat the 96 well microplate with 100µl per well.
6. Seal the plate and incubate at room temperature overnight

7. **The Next Day:** Remove capture antibody and wash plate 3 times in wash buffer (0.05% Tween 20 in PBS).
8. Blot plate on clean absorbent paper to remove any remaining wash buffer
9. Block plates by adding 300µl of reagent diluent to each well
10. Incubate at room temperature for at least 1 hour
11. Remove reagent diluent and wash plates 3 times with wash buffer.
12. Plates are ready for addition of samples

`

Assay Procedure

13. Add 100µl of sample or standards in reagent diluent per well (Standards should consist of 2-fold dilutions of stock standard, utilising 7 points of a standard curve with a high standard of 2000pg/ml)
14. Cover plate with adhesive strip and incubate for 2 hours at room temperature
15. Remove standards and samples and wash plate 3x with wash buffer
16. Dilute detection antibody to a working concentration of 400ng/ml in reagent diluent (stock at 72µg/ml) and add 100µl of detection antibody to each well
17. Cover with a new adhesive strip and incubate at room temperature for 2 hours
18. Remove detection antibody and wash plate 3x with wash buffer
19. Add 100µl of the working dilution of Streptavidin-HRP to each well (1/200 dilution) to each well. Cover the plate and incubate at room temperature for 20 minutes in the dark
20. Remove streptavidin-HRP and wash plate 3x with wash buffer
21. Add 100µl of substrate solution (1:1 mix of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) to each well and incubate for 20 minutes at room temperature in the dark
22. Add 50µl of Stop Solution (2 Molar Sulphuric Acid) to each well. Gently tap the plate to ensure thorough mixing
23. Determine the OD of each well immediately using a microplate reader set to 450nm with wavelength correction set to 540 or 570nm.
24. Calculate results by plotting standard curve

***** Keratin represents a class of structural proteins found in all vertebrates. Alpha keratins are the principal proteins found in skin, nails, hair and hoofs. In low concentrations compared to the proteins of interest, keratins are not a problem at all. Therefore Keratin was excluded from the samples.

Research Operating Procedure 18: Enzymatic AP assay

Determination of Alkaline Phosphatase activity

Tips:

- The recombinant alkaline phosphatase stock will need to be diluted a lot (start from 1:100
- The assay contains:
    - A. (sample + diethanolamine buffer) = 500µL

`

- B. (substrate in diethanolamine buffer) = 500μL

- Solutions A and B should be made up fresh and immediately prior to use. Solutions A and B should be mixed immediately prior to placing it in the spectrophotometer.

- A graph appears on the screen of the spectrophotometer as the assay progresses. If the line is horizontal and at zero, then there is no activity present. If the line is horizontal but up high, then there was activity present but the substrate has been consumed -> dilute the sample as necessary.

Assay Instructions

1. Prepare Sample A
    a. Add an appropriate volume of diluted sample to diethanolamine buffer to make a total volume of 500ul.

2. Prepare Sample B.
    a. Dissolve one 5mg pNPP tablet in 2.5ml of diethanolamine buffer

Diethanolamine buffer with 0.5mM Magnesium Chloride

97 ml Diethanolamine

101.65mg $MgCl_2.6H_2O$

0.2g sodium azide

Add 800ml RO water and pH to 9.8 using HCl

Make up to 1 litre and Autoclave to sterilise.

3. Set-up Spectrophotometer
    a. Press “return” to get to main menu
    b. Choose option 2: Prog Pack

`

c. Use 1st holder only for cuvette and make sure using the slim cuvettes as only using a small sample volume.

4. Add Sample A and B together, place immediately into cuvette holder and start readings
5. Spectrophotometer will measure absorbance at 405nm every 10 seconds for 3 mins.

6. The consumption curve should be linear but it will usually be linear and then plateau due to exhaustion of the substrate. When the spectrophotometer calculates ΔA/min, it does so for the entire measurement time, not just the linear portion. Therefore, look at the curve and decide where the linear region is. The range on the spectrophotometer can then be adjusted so that it recalculates the slope just for the linear region. To do this:
    a. Use F2 to move between the curve and the data list
    b. From the curve, identify the time at which the linear range ends
    c. On the data list page, choose 'Re-calc' (F1) from the menu at the bottom of the screen
    d. The lag time should be zero (you should only need to press Enter)
    e. The Rate time =
        i. input the time that the linear period ends; press Enter
    f. The slope is automatically adjusted and the value is recorded (ΔA/min)

1.1.47 How to calculate the activity in the APase assay

unit of activity U = μmol/min

A = absorbance at 405nm

C = concentration of product formed

ε = molar extinction coefficient = 18.7 L/mol/cm, = 18.7 μL/μmol/cm

l = light path = 1cm

Beer Lambert Law : $C = \frac{A}{\varepsilon l}$

Differentiate this with respect to time

`

$$\frac{\Delta C}{\Delta t} = \frac{1}{\varepsilon l}\frac{\Delta A}{\Delta t}$$

The spectrophotometer calculates $\frac{\Delta A}{\min}$ for you.

For example, imagine if the result is $\frac{\Delta A}{\min}$ = 0.446 min^{-1}

$$\frac{\Delta C}{\Delta t} = \frac{1}{18.7\,\mu L\,\mu mol^{-1}cm^{-1}\times 1cm}$$ x 0.446 min-1

= 0.0239 μmol$min^{-1}$$\mu L^{-1}$

= 0.0239 U/μL

The volume of the assay was 1mL = 1000μL

Therefore, the activity in the assay = 1000μL x 0.239 U/μL = 23.9 U

But the activity in the assay came from the volume of sample that you added.

For example, imagine that you used 5μL of sample in the assay.

The activity in the sample would be = $\frac{23.9U}{5\mu L}$ = 4.78 U/μL = 4780 U/mL

Shortcut:

Activity (U/mL of sample) = 1000 x ΔA/min

`

18.7 x Vol of sample in the assay (mL)

Research Operating Procedure 19: Stirred Tank bioreactor

A, Preparations:

1. Assemble Bioreactor

a. Cooler with hose and filter on top

b. Calibrate right pH Electrode according to protocol and attach to Reactor

c. Attach Sampling hose to sampling port and Clamp approx. 15cm above the end of the hose

d. Attach hose with filter on the end to Air Input

e. Close all the other outlets with Aluminiumfoil prior to Autoclaving

f. cover filers with alumininumfoil

2. Innoculum flask

a. Clamp hose of Innoculum flask and wrap with Aluminiumfoil

b. Insert self-made plug and wrap with Aluminiumfoil on top. fix with blue autoclave sticky tape

3. Autoclave Bioreactor and Innoculum flask in dry cycle

B. Preparation of the sample

1. Sample Preparation

a. Cut 100g of each liver into pieces and mix together in a 1L beaker

b. Wash 3x with 300mL of HBSS for each wash. Strain between washes.

d. Mince with industrial mincer

e. Wash 4x with 300mL of HBSS per wash. Strain well in between.

f. Weigh out 500g or how much needed of mince

`

g. Add 667mL of pre-warmed (37C) HMM

2. Innoculum

a. Transfer beaker content (mince and medium) into Innoculum flask under sterile conditions

b. Clamp with scissors clamp above the clamp and pour content into Innoculum port of Bioreactor. which is closed with blue silicon plug

c. Put blue silicon plug back in and transfer bioreactor to teaching lab

3. Start of Bioreaction

a. Connect Bioreactor to stirrer. switch stirrer on and adjust to about 120rpm. till mince is moving around and no dead volume visible

b. Insert the heater element into the heater port and adjust temperature (right) on Computer to 37C

d. Connect pH probe with right pH

d. Connect air flow to air inlet and set to 1L/min

4. Sampling and Recording

a. Time zero start as soon as system has reached it's final temperature (37C). make a record of Air-flow. pH and Temperature

b. Draw Sample #1

c. Draw Sample #2 4 hours later

d. Draw Sample #3 8 hours later

d. Draw Sample #4 16 hours later

f. Draw Sample #5 24 hours later etc.

5. Sampling

a. Clamp with scissors clamp before the first clamp

b. Attach syringe

c. Take clamp off

d. Release scissor clamp and draw sample

e. Remove dead volume first (about 8mL. if sample has been drawn before)

f. Draw "real" sample into 1.5mL eppendorf tube x 3

`

g. Clamp again. detach syringe and pout hose back into 70% Ethanol

6. Harvesting

a. Switch rotor. heater element. pH etc. off

b. Remove motor. heater element and pH probe

d. Transfer bioreactor to sink area and unsrew lid diagonally (otherwise glass can crack)

d. Pour content into a strainer that sits on top of a beaker

e. Discard meat tissue in a biohazard bag and transfer media into 50mL tubes

f. Spin @ 1.400rpm for 10min and transfer supernatant into a new tube

g. Freeze samples @ -20C until further analysis

h. Clean bioreactor and let stir @ RT over weekend

i. Clean all the ports by flushing with MilliQ water and let dry when clean

Reseach Operating produced 20: Wave bioreactor

, Sample Process:

1. Weigh out 50 x 8 Bovine livers (400g total) and cut into small pieces.

2. Rinse all liver tissue 3 times prior to mincing in a defined volume of HBSS (75mL/200g per wash → 450mL total per batch)

3. Mince the 400g batch

4. Rinse the minced liver samples 4 times in HBSS for blood removal (75mL/200g per wash → 600mL total per batch)

`

1. 5. Set up the following inoculum for the WAVE trial 1:

   150g liver mince + 500ml HMM (Ratio:0.3)

2. Set up the following inoculum for the WAVE trial 2:

   375g liver mince + 500ml HMM (Ratio:0.75)

3. Alternatively cells can be disaggregated using research operating procedure 11, 12, 13 and seeded into the bioreactor , wave trial 3 and wave trial 4 : 1x 10 9 cells in 500ml

C, Inccolating bag

1. Connect CO2 line to mixer with regulator set at 0.7 bar. Turn on CO2 mixer , first switch Pump then CO2 . Ensure Flow rate is set at 0.1 L/min and 5% Co2 mixture. Ensure that CO2 mix has stabilised.

2. Prepare sterile biological safety cabinet (turn on, ensure flow is stable, clean with ethanol)

3. The wave system has three bags 1 cover bag

   2 sterile bag

   3 wave bag (the bioreactor unit)

4. Remove the sterile bag from the cover bag, spray with ethanol. Open the sterile bag at the edge of the cabinet and in one fluid motion place the wave bag inside the cabinet.

5. Seal all hoses using clips and Remove foam from filters .

6. Remove the cap to inoculum port, raise from bench by holding the port and add inoculums to bag.

7. Replace the cap to seal the inoculum port.

8. Move the bag to the wave unit, ensure that the sample port on the left side. Connect the blue rods under the clips to fasten the bag to the unit.

`

9 . Open air hoses by unclipping clamps. Connect the C02 mixer to the shorter filter inlet port. Ensure that the bag inflates and that air is flowing from the longer air outlet port (should make a hissing sound). If you unsure whether air is flowing connect some tubing to the air outlet port and place into a beaker of water, it should have a constant flow of bubbles.

10. Connect the heating sock over the air outlet filter to stop condensation.

11. Set wave to 20 rpm/ 6° and start rocking, ensure that the mince is mixing , this can be increased to 28 rpm/8°.

12 Set temperature to 34°C and start heater (note the machine must have a media filled bag attached and rocking before the heater is engaged) .

13. Once temperature has reached 34°C increased to 36.5°C

14 Once temperature has reached 36.5°C take the first sample

C, sampling

1. Take samples at:

- 4pm (11/08/10) 0 hours (once temperature has reached 37C)
- 8pm (11/08/10) 4 hours
- 12am (12/08/10) 8 hours
- 9am (12/08/10) 16 hours
- 5pm (12/08/10) 24 hours
- 9am (13/08/10) 40 hours
- 4pm (13/08/10) 47 hours

2. Stop bag on angle

`

3. Spray ethanol on paper towel, remove lid from sample port and place on ethanol paper

4. Attach sterile syringe to sample port, push down on the bag and draw sample (7ml). Release bag and draw in some air, spray with ethanol and replace cap.

5. Spin 3 samples at 1400min for 10 min, take pH measurement and note temperature of unit and flow rate of the unit. Check the colour of the media and whether the mince is moving when rocking.

If you want disk/meat sample

1disconnect heating sock and CO2 in

Close flitter ports using clips

Carry to steril hood

Remove inoculum ports

Sample

Replace inoculum ports and connect bioreactor as B step 8 -14

Research Operating Produced 21 : Fibra stage Bioreactor

Inoculation of the FibraStage system (7.1, p.10 Operation guide)

1. Remove a fresh FibraStage bottle from its bag.
2. Aseptically remove the cap and add **500 L** of pre-warmed media (HMM).
3. Tilt the bottle to send all the media down into the bellow chamber.
4. Aseptically open the cap, and directly on top of the Fibra-Cel disks, working in a spiral from the centre, dispense of a cell suspension containing **1x10^9** live cells.
5. Open the cap and add the media with the inoculum.
6. Close and tighten the cap, then place the bottle in the FibraStage unit in the incubator, making sure the blue ring lock in place.
7. Turn the controller on by pressing the red ON/OFF button.
8. Adjust the FibraStage controls as shown in table below:

`

	Time	Up (Rise) Rate	Top Hold Time	Down Rate	Bottom Hold Time
Inoculation	3 hrs	2.0 mm/sec	20 sec	2.0 mm/sec	O sec
		Up (Rise) Rate	**Top Hold Time**	**Down Rate**	**Bottom Hold Time**
Cell culture	45hrs	1.0 mm/sec	10 sec	1.0 mm/sec	1 min

9. During the run, monitor the substrate and metabolite concentrations including glucose, glutamine, ammonia and lactate once a day. Media and glucose should be added as required.

10. Adjust the pH in the culture medium to a range of between 7.2 to 7.4

11. Measure the pH regularly, and adjust the CO2 concentration accordingly in the incubator. A good rule of thumb is that while the glucose concentration in the bottle is still sufficient and the media color has turned orange-yellow, it is time to adjust the CO2 concentration to a lower percentage.

12. To take a sample, press the STOP key to stop the platform and following point 8.3.1 in the FibraStage operation guide (p.15)

Sampling:

- 4pm (Wednesday) hours (Inoculation conditions)
- 8pm (Wednesday) 4 hours (Culture conditions
- 8am (Thrusday) 16 hours
- 4pm (Thrusday) 24 hours
- 8am (Friday) 40 hours
- 4pm (Friday) 48 hours
- 5pm (Friday) Change medium 0 hrs
- 5pm (Saturday)24hrs (72hrs)
- 5pm (Sunday)48hrs (96hrs)

`

13. Media Replenishment: replenish media once every day or two, beginning on the third day following inoculation. (8.3.3 operation guide, p. 16).

14. Harvest the first batch of media 48 hours after inoculation (Thursday, the 16th of April) by freezing the media and replacing it with fresh serum-free HMM. Then harvest again 48 hours later until cells stop producing the bioactive.

Trypsinize disk

1) using sterile tweezers remover 20 disk to 2 ml PBS , gently agitate by rocking. (wash , associated cells)

2) Remove wash and spin at 400g for 5 min. Resuspend wash in 1ml HMM

3) trypsinize disk in 500ul PBS 500ul trypsin for 15min

4) remove trypsin and replace disk into into 1ml of HMM + 10% FCS.

5) Combine trypsin and HMM + 10% FCS wash , spin cells at 400g for 5min.

6) Resuspend into 2ml HMM and spin cells at 400g for 5min.

7) Mix cells 1 in 10 with trypan blue for a cell count.

Research Operating Produced 22 : Ammonium Sulphate precipitation

1. Wear the personal protective equipment gloves, glasses and lab coat when preparing all solution.
2. Place protein sample in a beaker on ice with stirring.
3. Removal Add saturated Ammonium sulphate solution drop wise until desired concentration of 60% is achieved. This concentration must be determine for each protein through successive precipitation. Currently 60% then 80% ammonium sulphate is used for the preparation of minced organ culture alkaline phosphatase (APase).
4. Centrifuge at 5000 rpm for 20min.
5. Resuspend pellet in 30mM Tris –HCL pH 8.
6. Measure volume of supernatant and add ammonium sulphate saturated solution to 80% .
7. Centrifuge at 5000 rpm for 20min.
8. Resuspend pellet in 30mM Tris –HCL pH 8.
9. Dialysis resuspend pellets against 30mM Tris –HCL pH 8.

`

10. Perform Alkaline phosphatase activity assay and BCA assay on resuspended pellets. The alkaline phosphatase should be present in the 60—80% ammonium sulphate precipitation.

Research Operating Produced 23 : DEAE column chromatography

1. Reagents

A- Running buffer 30mM Tris –HCL pH 8

B- Elution buffers

0.25 M NaCl 30mM Tris –HCL pH 8 *

0.5 M NaCl 30mM Tris –HCL pH 8 *

1.5 M NaCl 30mM Tris –HCL pH 8 *

C - washing buffer 3 M NaCl 30mM Tris –HCL pH 8

D- Storage buffer 20% Ethanol

E-30% isopropanol

F- salurated ammonium sulphate

1- 10 M HC for pH adjustment

1-10 M NaOH for pH adjustment

* NaCl concentration in elution buffer can vary between 0-3M NaCl

** Note- all buffer should be filtered 0.45 uM

Rinse all connectors and tubing with MilQ H2O before placing into new buffers

Safety glasses when preparing solutions and adjusting pH with acids and bases or handling concentrated solvents (100% Ethanol and isopropanol).

2. Equipment

`

1 ml DEAE column PALL (S20067-C001)

ISCO pump , fraction collector and UV-vis

Magnetic stirrer

pH meter

Pipette tips

Pipette

Dialysis tubing

Centrifuge

3 . Method

Tubing preparation line

11. Column and tubing is stored in 20% Ethanol, column disconnected.
12. lock the pump , place the unlabeled pump tubing input into running buffer, switch to the left counter clockwise to rinse the line with 5ml running buffer. Pump setting of 25 = is approximately 0.5ml/min.

Equilibrate column

13. Connect the column. Metal from pump screws in top, plastic tubing to fraction collector pushes in bottom, cover the joints with parafilm. Connect tubing directly to the pump and NOT through the gradient mixer.

Note : pump and fraction collector have moving parts, keep fingers away if on .

14. Equilibrate a prepacked column DEAE column (packed bed vol ~ 1ml) with running buffer (30 mM Tris, pH 8). Start the pump , fraction collector and UV monitor . 5x bed volumes of running buffer (5ml) , collect 0.5 ml fractions.
15. First time a column was used 5 bed volumes of elution buffer was first run through the column , then 5 volumes of running buffer.
16. Equilibrate the peristaltic pump to flow rate of 0.5 ml/min before adding sample - monitor using volumetric test tube.

17. Apply 5 mg of partially purified alkaline phosphates from (ammonium sulphate precipitation/ dialysis sample step 10) at a column at a flow rate of 0.5 ml/min. Start the pump , fraction collector and UV monitor.
18. Wash column with 5 bed vol. of 30 mM Tris (pH 8) or until OD returns to baseline on chart recorder. Stop the pump , fraction collector and UV monitor.

Gradient elultion

19. Use gradient programmer – default settings.
20. Tubing of the Gradient mixer is stored in 30% isopropanol , this needs to be flushed out.
21. connect wash tubing to the waste port on the front right hand side of the gradient mixer.
22. Place the gradient inputs, A, B and C into 30mM tris running buffer .
23. Press primer and run 10ml of running buffer through the lines of the gradient mixer.
24. Remover the waste line and replace the cap.
25. Connect the pump input to the left hand side port labeled solvent.
26. Place Tube A running buffer, B into elution buffer and store C in water.
27. Start gradient mixer , pump , fraction collector and UV monitor. Gradient will run from 30mM Tris / 30mM Tris 0.25M NaCl in approximately 30min or once protein UV/vis has droped down to background levels .
28. Repeat steps 14 and 15 with A= 30mM Tris 0.25M NaCl B= 30mM Tris 0.5M NaCl.
29. Repeat steps 14 and 15 with A= 30mM Tris 0.5M NaCl B= 30mM Tris 1.5M NaCl.
30. Stop gradient mixer, pump, fraction collector and UV monitor. Disconnect the pump input to the left hand side port (labeled solvent) and replace its cap.

Wash the column, tubing and gradient mixer

31. Place the pump input into 3M NaCl , 30mM Tris. Start the pump , fraction collector and UV monitor. Wash column with 5 volumes of 3 M NaCl 30 mM Tris pH 8, (5 ml).
32. Wash column with 5 volumes of 20% Ethanol storage solution , recap and store column at 4C.
33. Connect wash tubing to the waste port on the front right hand side of the gradient mixer.
34. Place the gradient inputs, A, B and C into 30% isopropanol.
35. Press primer and run 20ml of 30% isopropanol through the lines of the gradient mixer.
36. Remover the waste line and replace the cap on the on right hand side of the gradient mixer.
37. Replace cap on pen (paraflim). Ensure all equipment is turned off , replace dust cover.

Fraction	volume (total)	content
1-6	3.2ml	sample flow though
7-17	5ml	running buffer
18-65	23.5ml	elution
66-75	10ml	High salt wash

Desalting

38. Do quick APase assay on the fractions that contain protein.
39. **Pool eluted fractions containing APase.**
40. Desalt by overnight dialysis against 30 mM Tris pH 8.

`

41. Store the final aliquot at -20C.

Research Operating Procedure 24: Depletion of BSA

a, Ethanol precipitation method:

1. Prepare 95% pre-chilled Ethanol

2. Add 95% Ethanol to a final concentration of 42% to the unprocessed minced organ culture sample

3. Incubate the Ethanol/protein sample mixture for 1 hour at 4C with gentle mixing

4. Collect the proteins by centrifuction at 16,000xg for 45min at 4C

5. Remove the supernatant (albumin-enriched fraction) and store at -80C

6. Centrifuge the microfuge tube containing the pellet briefly for a second time and remove any leftover residual supernatant

7. Pellet and supernatant can be stored at -80C until further analysis

8. For further proteomics analysis, thaw frozen pellets and resuspend in desired volume of resuspension-buffer (1% SDS, 10mM HEPES, pH 7.4)

b, TCA/Acetone precipitation method:

1. Add four volumes of ice-cold acetone containing 10% (w/v) TCA to the unprocessed minced organ culture sample

2. Mix immediately by gentle vortexing

3. Incubate the mixture at -20C for 90min and centrifuge at 15,000xg for 20min at 4C.

4. Remove and collect the supernatant

5. Add 1mL of ice-cold acetone to the protein pellet to wash the precipitate

6. Incubate the sample on ice for another 15min and centrifuge again at 15,000xg for 20min at 4C

7. Remove the acetone containing supernatant and lyophilize the precipitate

8. Add 1mL of ice-cold acetone to the 10% TCA/acetone-containing supernatant to completely precipitate the proteins in the supernatant

9. Resuspend the precipitated proteins prior to proteomics analysis in resuspension-buffer (1% SDS, 10mM HEPES, pH 7.4)

`

c, PALL Scientific Albumin depletion kit:

Kit contains a Nanosep centrifugal device containing albumin depletion discs.

1. Rehydrate the discs according to the manufacturer's recommendations
2. Place the unprocessed minced organ culture sample into the column and incubate for 2min at RT
3. Centrifuge the column at 12,000xg for 1min at RT and collect the filtrate
4. Add the filtrate back into the column and incubate for a further 2min at RT
5. Centrifuge at 12,000xg for 1min at RT and retain the filtrate for downstream analysis.
6. Add 50uL of binding/wash buffer (25mM Tris, pH 7.2) to the depleting column to release any unbound proteins
7. Centrifuge the column for 1min at 12,000xg at RT and retain the filtrate

d, Ultrafiltration via 50kDa molecular weight cut-off Millipore Centricon filter device

1. Add the unprocessed minced organ culture sample to the filter and centrifuge in a swinging bucker rotor at 4,000xg for 9min at RT
2. To recover the concentrate solute, insert a pipette (200uL) into the bottom of the filter unit and withdraw the Retentate using a side-to-side sweeping motion to ensure total recovery
3. The Permeate, less than 50kDa containg the BSA depleted proteins

e, Blue Trisacryl M Afffinity column:

Blue Trisacryl M is an affinity resin that contains a resin that binds albumin and does not interfere with downstream applications and proteomic analysis. The Blue Trisacryl M used in these experiments was the AcroSep column from PALL.

1. Apply unprocessed minced organ culture sample to the column and collect fractions
2. Measure UV/VIS of the different fractions individually and analyse correspondent fractions with Coomassie stained 1D SDS PAGE

Research Operating Procedure 25: In-solution digestion

`

- DTT 50mM
- Iodoacetamide 100mM
- Ammonium bicarbonate 100mM
- Trypsin 100ng/uL

* Perform digestions in 96 well digestion plate or low binding Eppendorf brand microfuge tubes. Other microfuge tubes may give polymer contamination.

* Trysin stock is stored at -80C at 1ug/uL in 3uL aliquots. For use in step 5, dilute 1/10 with 100mM ammonium bicarbonate. Any unused trypsin must be disregarded.

Process:

1. Precipitate or dialyse sample.

- For precipitation use the 2D Clean up kit (GE Healthcare or BioRad)

  Re-suspend the precipitated protein in 20uL of 100mM ammonium bicarbonate.
- For dialysis, dialyse sample against 100mM ammonium bicarbonate. The final volume of the sample should ideally be around 20uL.

Check pH of sample prior to the addition of trypsin by spotting a small amount onto pH paper. The sample must be between pH of 7.5 and 8.5. If necessary adjust with either Hydrochloric acid or Sodium hydroxide (make sure you have read and understood the MSDS).

2. Reduce the sample by adding 2uL of 50mM DTT. Incubate at 65C for 5min.

3. Alkylate the sample by adding 2uL of 100mM Iodoacetamide, incubate for 30min at RT.

4. Add 1uL or 2uL of 100ng/uL trypsin. The amount of trypsin added should be dependent on the amount of protein in the sample (for low protein loads, add 1uL).

5. Incubate sample at 37C for 4 hours to overnight.

`

6. Sample are now ready to inject into the Mass spectrometer.

Research Operating Procedure 26: 2-D gel electrophoresis (2D GE)

Reagents:

a, 2D Cleanup kit:

- Wash Buffer (2D Cleanup kit)
- Wash Additive (2D Cleanup kit)
- Precipitant (2D Cleanup kit)
- Co-Precipitant (2D Cleanup kit)
- Thiourea (TUC-Buffer)
- Urea (TUC Buffer)
- CHAPS (TUC Buffer)

b, EZQ protein-assay:

- Ovalbumin (chicken egg) for preparing standard solution
- Methanol (for fixing and washing protein spots)
- EZQ protein quantitation reagent
- Rinse buffer (10% Methanol, 7% Acetic acid)

c, Rehydration of the sample(s):

- Ampholytes
- DTT
- Bromophenolblue
- Mineral Oil

`

d, Isoelectric Focusing:

- Mineral Oil

e, 12.5% linear gels preparation:

- 40% Acrylamide:Bis (37:5:1)
- 1.5M Tris-HCl pH 8.8
- 10% SDS
- 10% APS
- TEMED

f, 2D gel running:

- **Equilibration-buffer #1**:
- 1M Tris,
- 6M Urea,
- 30% (w/w) Glycerol and
- 2% (w/w) SDS and
- 1% DTT (0.05g DTT + 5mL Equilibration-buffer)
- pH 8

- **Equilibration #2**:
- 1M Tris,
- 6M Urea,
- 30% (w/w) Glycerol

`

- 2% (w/w) SDS
- 4% Iodoacetamide (0.2g Iodoacetamide + 5mL Equilibration buffer).

- Unstained molecular weight marker
- Running – buffer:

a, Tris	0.25M
b, Glycine	1.92M
c, high purity SDS	0.6%

g, Silver staining:

- Fixative/stop solution:
  - 30% Ethanol, 10% acetic acid in dH2O

- Sensitiser solution:
  - 0.0006% Eriochrome Black (EBT) in 30% Ethanol in dH2O

- Destain solution:
  - 30% Ethanol in dH2O

- Silver staining:
  - 0.25% (w/v) silver nitrate
  - 0.037% (w/v) formaldehyde in dH2O

- Developer solution:
  - 2% w/v potassium carbonate
  - 0.04% w/v sodium hydroxide
  - 0.002% w/v sodium thiosulphate

`

- 0.007% w/v formaldehyde in dH2O

2D Clenaup-kit procedure:

1. Subject the sample to a 2D Cleanup-kit to get rid of any interfering substances, such as salts, lipids, phenolic acids etc.
2. Transfer between 1-100ug of protein sample into a 1.5mL eppendorf microcentrifuge tube.
3. Add 300uL of Precipitant #1. Mix well by vortexing or inversion. Incubate the tube on ice for 15min.
4. Add 300uL of Precipitant #2 to the mixture of protein and precipitant. Mix well by vortexing briefly.
5. Position the tubes in a microcentrifuge with cap-hinges facing outwards. Centrifuge at maximum speed (at least 12,000xg) for 5min. Remove the tubes from the microcentrifuge as soon as centrifugation has finished. A small pellet should be visible.

Proceed rapidly to the next step to avoid resuspension or dispersion of the pellet.

6. Remove as much of the supernatant as possible by decanting or careful pipetting. Do not disturb the pellet.
7. Carefully reposition the tubes in the microcentrifuge with the cap-hinges and pellets facing outwards. Centrifuge the tubes briefly to bring any remaining liquid to the bottom of the tubes. Use a pipette to remove the remaining supernatant. There should be no visible liquid remaining in the tubes.
8. Without disturbing the pellet, layer 40uL of Wash Reagent #1 on top of each pellet. Vortex.
9. Carefully reposition the tubes in the centrifuge with the cap-hinges facing outwards. Centrifuge at 6,000xg for 5min at RT. Use a pipette to remove the supernatant.
10. Pipette 25uL of distilled or deionized water on top of each pellet. Vortex each tube for 10-20sec. The pellet should be dispersed, but not dissolved in the water.
11. Add 1mL of Wash Reagent #2 (pre-chilled for at least 1hour at -20C) and 5uL of wash additive #2 to each tube. Vortex until the pellets are fully dispersed.

The protein pellet will not dissolve in the wash buffer.

12. Incubate the tubes at -20C for at least 30min. Vortex for 20-30sec once every 10min. At this stage, the tubes can be stored at -20C for up to one week with minimal protein degradation of modification.
13. Centrifuge the tubes at 6,000xg for 5min to form a tight pellet.
14. Carefully remove and discard the supernatant. A white pellet should be visible. Allow the pellet to air dry briefly (no more then 1-2min).

Do not over-dry the pellet. If it becomes too dry, it will be difficult to resuspend.

`

15. Resuspend each pellet in an appropriate volume of rehydration or IEF sample loading solution for first-dimension IEF.

16. Vortex the tubes for at least 30sec. Incubate at RT. Vortex or aspirate and dispense using a pipette to fully dissolve.

If the pellet is large or too dry, it may be difficult to resuspend fully. Sonication or treatment with the Sample Grinding kit can speed resuspend.

17. Centrifuge the tubes at max. speed (at least 12,000xg) for 5min at RT to remove any insoluble material and to reduce any foam. The supernatant maybe loaded.

EZQ protein-assay for protein determination:

1. Spot sample in a volume of 1uL onto the membrane provided in triplicates.

2. Use Ovalbumin from chicken egg to prepare the standard curve.

Standard curve covers a range of 0.02 – 0.2mg/mL

3. Follow the manufacturer's instructions for Washing and fixation steps.

Rehydration of the samples:

1. Mix an appropriate amount of protein sample dissolved in TUC (Thiourea-Urea-CHAPS)-buffer with 0.5% Ampholytes, 0.5% DTT and 0.6uL of Bromophenolblue just prior to Rehydration.

2. Load the sample evenly down the coffin.

3. Peel the back of the strip carefully place with forceps in the coffin with gel side down and the positive end facing to the shaped coffin end.

4. Cover the strip with Mineral Oil (1-2mL) and place the lid on the coffin.

5. Place the coffin in the IPGphor with the positive end in the positive section of the apparatus. Two rubber bars are pressing onto the lid of the coffin. Set IPGphor on O/N rehydration, 20C and 50V for unlimited time.

6. Record the total volt hours the next morning and switch off IPGphor.

Isoelectric Focusing:

1. Remove coffin and place on tissue paper. Carefully remove the hydrated strip with forceps and place on tissue paper. Wash coffin and lid with MilliQ and dry with tissue.
2. Cut out wick from Whatmann filter paper number 1 and cover electrodes in the coffin.
3. Replace the strip again into the coffin and cover with Mineral Oil, replace the coffin lid.
4. Place coffin into IPGphor and set appropriate protocol for O/N, then record total volt hours.

Check if strip is completely rehydrated, it has to be evenly swollen everywhere

Pouring 2D gels (24cm, 12.5% linear):

Equipment:

- Gel caster (front and back sections), incl. 2 black screws
- Rubber triangular wedge for bottom of caster
- Plastic spacers
- 4 or 6 front gel plates (flat)
- 4 or 6 gel plates (with built-in spacers)
- 2 plate blanks (if pouring 4 gels)
- 6 butterfly clips
- Clamp
- Plate rack
- Gel dryer

Procedure:

1. Clean/rinse the gel caster, rubber wedge, plastic spacers and plate blanks in deionised water.
2. Clean/rinse the plates in deionised water, making sure plates are meticulously clean, then spray with 70% Ethanol. Take care not to touch glass plates to the metal sink (use plate rack),

   If performing DIGE labelling, use low fluorescent plates ('clear' when viewed from the edge). Otherwise, use regular plate ('blue' when viewed from the edge).

`

3. Repeat the water/ethanol wash cycle twice more and leave plates in plate rack in front of gel dryer to dry.
4. Place rubber triangular wedge in the bottom of the gel caster.
5. Starting at the back of the gel caster, place a spacer (rounded edges up), followed by a back gel plate, then a front gel plate.

   Put plates hard against the bottom and left edges, taking care that plates are very carefully aligned with each other.

6. Repeat this spacer/gel plate sequence until either four or six sets of plates have been loaded, finishing with a spacer. If four gels are being poured, replace the missing gel plates with plate blanks.

7. Fill any space left in the caster with plastic spacers until plate stack is level with the edges of the caster.
8. Put the front section on the gel caster, hard down against the black screws. Tighten the black screws (n=2) to hold the front of the gel caster in place and butterfly clip down the sides of the caster (n=3 either side). Clamp off inlet at bottom in case of leaks.

Preparation of gel solution:

Equipment:

- Degassing flasks (500mL conical flasks with side arms)
- Magnetic flea
- Degassing apparatus
- Magnetic stirrer

Recipe:

			4x24cm	**6x24cm**	
	50mL	**250mL**	**350mL**	**400mL**	**500mL**

`

40% Acrylamide:Bis (37:5:1)	15.62	78.1	109.3	125	156.2
1.5M Tris-HCl pH 8.8	12.5	62.5	87.5	100	125
10% SDS	21.06	105.3	147.4	168.5	210.6
10% APS	0.5	2.5	3.5	4.0	5.0
TEMED	0.28	1.4	2	2.2	2.8
	5uL	25uL	35uL	45uL	50uL

Procedure:

1. Add the Acrylamide:Bis, Tris and dH2O into degassing flask.
2. Add a magnetic flea and connecting to the degass for a minimum of 20mins.
3. Decant into a beaker (for ease of pouring).
4. Add 10% SDS AFTER degassing (it will froth if added prior)
    - 10% APS and TEMED are the catalysts that make the gel set, so they are only added immediately prior to puring.
5. Make up 10% APS solution (e.g. by adding 0.3g to 3mL dH2O), vortex to dissolve.

Pouring the gels:

1. Add the 10% APS to the solution, and the TEMED at the last possible minute and mix well
2. Pour the solution slowly into the channel at the back of the gel caster (a channel is cut through the rubber wedge so that the level for each gel will equilibrate).
3. Fill the gel caster to about 0.5cm below the front plates (the gels will shrink slightly when set).
4. Cut the end of a 1mL pipette tip and quickly and evenly lay 1mL of water saturated butanol over the top of each gel. It is important that the same amount of butanol is added to each, or the gels will equilibrate to different heights prior to setting.5. Leave gels to set for two hours.
6. Wash off the butanol with deionised water and disassemble the gels from the caster (put in gel rack). Put some 1x running buffer over the top of the gels

`

7. Wrap each gel in Gladwrap, label with the gel type (12.5% linear), name, date and store in the cold room until use.
8. Clean the gel caster and all other equipment thoroughly and leave to dry.

Second dimension – Gel running:

1. Thaw 10mL Equilibration-buffer from freezer
2. Equilibration #1: Place strip for 15min in an aqueous solution (pH 8) containing 1M Tris, 6M Urea, 30% (w/w) Glycerol and 2% (w/w) SDS and 1% DTT (0.05g DTT + 5mL Equilibration-buffer).
3. Make up 1% Agarose by adding 1g of Agarose to 100mL of dH2O. Heat up in microwave till it's bubbling away.
4. Equilibration #2: Place strip for 15min in an aqueous solution (pH 8) containing 1M Tris, 6M Urea, 30% (w/w) Glycerol, 2% (w/w) SDS and 4% Iodoacetamide (0.2g Iodoacetamide + 5mL Equilibration buffer).
5. Load the gel into the gel holder and make up empty spaces with spacers.
6. Load strip into the gel plate, leaving room for the molecular marker on one side.
7. Using a transfer pipette, place a layer of 1% Agarose over the strip, filling to the top of the gel plates.
8. Insert the gel comb for the marker lane on the end of the strip (if not enough space, cut edge of strip off).
9. Place gel in holder in cold room for a few minutes
10. Remove gel comb by pulling to the side slighty (away from strip) and then up (stops agarose from cracking).
11. Load 5uL of unstained molecular weight marker into the marker lane
12. Put a layer of 1x running buffer over the gels to get rid of any air bubbles.
13. Dampen the top component of the gel tank with 1x high grad SDS running buffer and attach on the gel tank on top of the gels.
14. If running less than six gels, fill up the empty space in the gel tank with thick spacers before dipping in the tank in the Ettan Dalt apparatus.
15. Run gels @ 1000V, 400mA and 2W/gel for 45min, then increase Watts to 17W/gel and run till Bromophenolblue reaches the bottom of the gel(s).

Silver staining:

`

1. Remove the gel(s) from the electrophoresis apparatus and transfer into Fixative/stop solution (30% Ethanol, 10% acetic acid in dH2O) and fix twice for 20min, or once overnight. For 100mL, add 30mL Ethanol, 10mL Acetic acid and fill up to 500mL with dH2O.
2. Place into solution for 2min with 0.0006% Eriochrome Black (EBT) in 30% Ethanol in dH2O. For 100mL add 30mL Ethanol, 0.006g EBT and fill up to 1L with dH2O.
3. Destain for 2min in 30% Ethanol in dH2O. 15mL Ethanol and fill up with dH2O to 500mL.
4. Wash the gel(s) with dH2O, 2min each.
5. Place into silver staining solution, consisting of 0.25% (w/v) silver nitrate 0.037% (w/v) formaldehyde in dH2O for 5min (Add the formaldehyde just prior before use). 0.625g silver nitrate, 92.5uL Formaldehyde and fill up to 250mL with dH2O.
6. Perform another two washing steps with dH2O, each of 20sec.
7. Place the gel(s) into developer solution (2% w/v potassium carbonate, 0.04% w/v sodium hydroxide, 0.002% w/v sodium thiosulphate and 0.007% w/v formaldehyde in dH2O).

The Developer step is completed when no more spots appear on the gel. 5g Potassium Carbonate, 0.1g Sodium hydroxide, 0.005g Sodium thiosulphate and 0.0175g Formaldehyde and fill up to 250mL with dH2O).

8. Transfer gel(s) into fixative/stop solution (30% Ethanol, 10% acetic acid in dH2O) until further analysis. (15mL Ethanol, 5mL Acetic acid and fill up to 500mL with dH2O).

Research Operating Procedure 27: Two-dimensional differential gel electrophoresis (2D DIGE)

Minimal labelling reaction:

1. Perform 2D-Cleanup kit as described in ROP#26. Resuspend the samples used for 2D DIGE in Resuspension-buffer containing Tris pH 9.0.
2. Check pH of the samples by adding a little amount (0.5-1uL) on a pH strip. PH should be ideally between 8-9. Adjust if necessary.
3. Add the appropriate volumes (50uL for each Cy3, Cy5 and Cy2) of the correspondent samples into a labelled 500uL eppendorf tube, containing 400pmol of CyDye in each tube.
4. Mix by pipetting and centrifuge briefly in a microcentrifuge.
5. Leave on ice for 30min in the dark.
6. Add 1uL of 10mM Lysine to stop the labelling reaction. Mix by pipetting and spin briefly in a microcentrifuge.
7. Leave for 10min on ice in the dark.

`

Samples can now be stored for at least 3 months at -70C in the dark.

8. Dilute the samples by adding an equal amount of Rehydration-buffer (7M Urea, 2M Thiourea and 4% CHAPS).
9. Leave on ice in the dark for 10min.
10. Pool the protein samples that are going to be separated on the same gel.
11. Make the volume up to 460uL with Rehydration-buffer, including 0.4% DTT, 1% Pharmalytes pH 3-11 and 1.2% DeStreak solution.
12. Continue with the Rehydration step as described in ROP#26.

Note: Pour 2D gels in low-fluorescent glass plates and run the gels in the dark.

Image Acquisition and Analysis:

1. For Visualisation of the gels, scan DIGE gels directly between the glass plates with a

fluorescence Typhoon 9400 scanner (GE Healthcare) at the wavelengths corresponding to

each CyDye, namely Cy2 (480nm), Cy3 (532 nm), and Cy5 (633 nm).

The Typhoon imager allows to adjust sensitivity by tuning the voltage setting of the photomultiplier tube (PMT) that captures the fluorescent image. The starting point for the PMT was 600V. All gels were scanned at 200um resolution.

2. Crop Images to remove areas extraneous to the gel image using Image Quant (Amersham Pharmacia) prior to analysis. Gel images are stored as .gel file.

3. Using the DeCyder software (v7.0 Amersham Biosciences), compare Cy-2 images (pooled samples) with Cy-3 or Cy-5 images that were alternatively 0 hr minced organ culture and 48 hr non-stimulated minced organ culture for the first analysis, 48 hr minced organ culture non-stimulated and 48 hr minced organ culture stimulated (Retinoic Acid/Insulin) for the second analysis and RIPA (total protein extract) and 48 hr minced organ culture non-stimulated for the third analysis. Inter-gel spot detection and quantification was performed using differential in-gel analysis (DIA).

The Image Acquisition is summarised in the table below

`

Gel	Strip number	Filter	Laser	Sensitivity	PMT
#1_Cy2	#12149	670BP 30	Yellow (633nm)	normal	580
#1_Cy3	#12149	580BP 30	Red (532nm)	Normal	515
#1_Cy5	#12149	520BP 40	Blue (488nm)	Normal	510
#2_Cy2	#12150	670BP 30	Yellow (633nm)	Normal	580
#2_Cy3	#12150	580BP 30	Red (532nm)	Normal	515
#2_Cy5	#12150	520BP 40	Blue (488nm)	Normal	510
#3_Cy2	#12151	670BP 30	Yellow (633nm)	Normal	590
#3_Cy3	#12151	580BP 30	Red (532nm)	Normal	525
#3_Cy5	#12151	520BP 40	Blue (488nm)	normal	515

Research Operating Procedure 30: Sample preparation of the 0 hr and 48 hr stimulated and non-stimulated bovine minced organ culture

1. Bovine minced organ culture was obtained by collection of approximately 100g of eight different bovine livers, each in a separate container filled with 125mL of RPMI-1640 medium (containing Pen/Strep and Sodium Bicarbonate).
2. Tissue samples were transported on ice back to the laboratory. Under sterile conditions 15g of each liver tissue was cut and tissues were pooled together in a total volume of 120g in 45mL of HBSS medium containing Phenol Red, Pen/Strep and Sodium Bicarbonate.
3. The tissue was minced in an Industrial mincer using a fine disk (5mm) and collected in a beaker. Minced liver tissue was washed five times in HBSS using a fine strainer to drain the liquid between each wash.
4. Yield was determined after the last wash (22.4%) and 1.5g of pooled liver tissue was inoculated in triplicates into 6-well plates in a total volume of 2mL HMM (MEM-Eagle, Medium-199, Sodium Pyruvate, L-Glutamine, Pen/Strep, Bicarbonate, BSA).
5. A stimulant in a combination of Insulin at a final concentration of 10^{-4}M and Retinoic acid at a final concentration of 1000nM was added to one row of three replicates and minced organ tissue was then cultured for 0 hr and 48 hours at 5% CO_2, 37°C.
6. Samples were drawn and tested negative for bacterial growths and pH was monitored throughout the whole culture period in the supernatant. Total protein determination was carried

`

out using a colorimetric protein-assay. Samples were then subjected to 1-DE, followed by 2-DE and DIGE.

Research Operating Procedure 28: Assaying cell/mince cultures for microorganism contamination

1. Chemicals/reagents

ATTC S22 Medium + 10% FBS (200ml)

Agar (pasta)	5g (omit if making liquid cultures)
Lablemco Beef extract	2g
Peptone	2g
Sodium Chloride (NaCL)	1g
Water	200 ml

Autoclave on fluid cycle

Once cool add:

Foetal Bovine Serum	22ml

70% Ethanol

2. Equipment/materials

Magnetic stirrer

Auto calve

Bunsen burner

Biological safety cabinet/laminar flow

Pipette

Pipette tips

Orbital shaker

37C Incubator

-80C Freezer

4C Fridge

`

3. Method

Basic Microbiological techniques include A) Pouring plates, B) Sampling from cell and minced cultures – C) Streaking plates and D) Glycerol stocks and F) Waste Disposal.

A) Pouring Plates - in PC2 laboratory

Risk of burning from hot agar and autoclave and use of flammable 70% ethanol.

1) A lab coat and glove must be worn throughout the procedure (PPE) and the entire procedure involving bacteria is carried out in an isolated room using dedicated pipettes. All waste tubes, tips and plates that have come in contact with Microorganism are disposed of in the yellow Biohazard bags. (step 16-19).

2) Insure you have had adequate training in the use of the biological safety cabinet and laminar flow Put on PC2 dedicated laboratory coat and new gloves.

3) Turn off any UV light, remove covers, turn on "normal visible" light and turn on air flow of the biological safety cabinet or laminar flow.

4) Wait until the air flow has stabilised (no-longer beeping).

5) Put on safety glasses when handling ethanol.

6) Wipe down all surfaces, equipment, reagents and gloved hands with 70% Ethanol before they are placed into the biological safety cabinet.

7) ATTC S22 agar is prepared and autoclaved.

8) Once **cool** Foetal bovine serum is added to the agar (+10%, ie 22 ml to 200). 15ml of ATTC S22 +10% FBS is poured per petri dishes and allowed to set.

9) Put on safety glasses when handling ethanol (refer MSDS and RA Ethanol).

10) Wipe down any equipment with 70% ethanol and remove from the cabinet. Wipe down all surfaces with 70% Ethanol.

Turn off "normal visible" light and turn off air flow of the biological safety cabinet or laminar flow. Replace covers, Turn on UV light.

11) Lab coat and gloves must be remove and Hands should be washed before leaving the PC2 facility .

B) Sampling from cell and minced cultures – cell culture laboratory

12) See steps 1-6 for set up of biological safety cabinet/ laminar flow.

13) Retrieve culture vessel from cell culture incubator.

`

14) Remove liquid from culture vessel (1ul – 1ml) and mix with 1ml – 5ml ATTC S22 medium.

15) Replace culture vessel into the cell culture incubator.

16) See steps 9-10 for shut down of biological safety cabinet / laminar flow.

17) Lab coat and gloves must be remove and Hands should be washed before leaving the cell culture laboratory to enter the PC2 facility.

18) Transport tubes to PC2 laboratory.

19) Put on PC2 dedicated laboratory coat and new gloves.

20) Place tubes in to 37C incubator for 16hrs.

21) Risk of moving parts on the orbital shaker, do not open the door to the shaker while turned on

22) Place tubes in to 37C orbital shaker for 24hrs.

23) Cultures stored in PC2 lab fridge at 4C.

24) Steps 9 and 11.

D) Streaking Plates – In PC2 lab

Risk from Ethanol, burning from naked flame and biological risk from microorganisms

25) See steps 1-6 for setting up biohazard hood/ laminar flow.

26) Ignite the Bunsen burner.

27) Spreader dipped in ethanol, heated red hot to sterilise.

28) Spread the microorganism from the growth culture on to the ATTC S22 + 10% FBS plate.

29) Repeat step 27-28 between different cultures.

30) ATTC S22 + 10% FBS plates are then grown over night at 37°C.

31) See steps 9 -11.

E) Glycerol stocks – PC2 lab

Risk of extreme cold from -80°C and biological risk from bacteria, and use of flammable 70% ethanol.

32) See steps 1-6.

`

33) Colonies from plates are picked into and grown in ATTC S22 +10 FCS for 16hrs at 37°C with aeration.

34) Bacterial stocks can be made by diluting the bacterial over night culture 1 to 1 with 100% sterile Glycerol and freezing at -80°C (see -80°C SOP).

35) See steps 9-11.

F) Waste Disposal – in PC2 lab

Biological risk from bacteria and use of flammable 70% ethanol.

36) All waste tubes, tips and plates that have come into contact with bacteria are disposed of in the yellow Biohazard autoclave bags.

37) Bacterial supernatants and contaminated containers are soaked with bleach. The liquid waste can now be rinsed down the sink with water.

38) After soaking with bleach re-usable containers are rinsed with water, washed with detergent , rinsed three times with RO water and three times milliQ water and autoclave for further bacterial only use.

Research Operating Procedure 29: Scale up protocol of mince preparation for bioreactor

1. Reagents

1. RPMI, pH 7.2 – 4L (470mL in each of the 8 Schott bottles):

Material	Material	Amount
		4 liter
RPMI powder	HyQ RPMI-1640 HyClone, Cat# SH30011.03, Lot# AVF70369A	41.6g
Pen/Strep	HyClone, 10,000 U/mL, Cat# SV300-10, Lot# JTJ32977, Thermo Scientific	40g
Bicarbonate	Pfizer	71.2ml

2. HBSS (Hanks Balanced Salt Solution), pH 7.2 – 5L:

Material	Material	Amount
		5 liter
1 HBSS	HBSS, H1387-10L, Lot#	47 g

`

	020M8306, Sigma	
Phenol red	P5530, Lot# 87H3625, Sigma	0.055 g
Pen/Strep	HyClone, 10,000 U/mL, Cat# SV300-10, Lot# JTJ32977, Thermo Scientific	50 ml
Bicarbonate	Pfizer	20.5 ml

3. HMM (Hepatocyte Minimal Medium), pH 7.2 – 100mL:

Material	**Material**	**Amount**
	Material	100ml
MEM-Eagle	M3024-10L, Lot# 018K8318, Sigma	0.627 g
Medium 199	M5017-1L, Lot# 059K1516, Sigma	0.214 g
Sodium Pyruvate	>=99%, P5280-100g, Lot# 105K07232, Sigma	0.229 g
L-glutamine	99% (TLC), G3126-100g, Lot# 072K0181, Sigma	0.058 g
BSA	Albumin bovine serum Cohn Analog, min98%, A1470, Lot# 019K1278, Sigma	0.1 g (filter through 2.2 um filter)
Pen/Strep	HyClone, 10,000 U/mL, Cat# SV300-10, Lot# JTJ32977, Thermo Scientific	1 mL
Bicarbonate	Pfizer	1.78 mL

4. ATTC S22 Medium + 10% FBS (200ml)

Agar (pasta) 5g (omit if making liquid cultures)

Lablemco Beef extract 2g

`

Peptone	2g
Sodium Chloride (NaCL)	1g
Water	200 ml

Autoclave on fluid cycle (see RA EquipRA Doc#BT48 and SOP EquipSOP Doc#BT48 for autoclave)

Once cool add:

Foetal Bovine Serum	22ml

Set up 14 10ml tubes with 2ml of ATTC S22 medium + 10% FBS

5. Set up 8 X 1L schott bottle plastic collection 470mlRPMI (total)

Method

1. Liver Collection:

Collect 8 bovine livers and fill containers to 900ml (~ 430g) cut long strips that can slide into the bottle . Store on ice for transport back to the lab.

2. Sample Process:

1. Weigh out 375g of each liver (3 kg total) , **do not cut** and mince with out rinsing
2. Wash mincer with first wash by pouring the media into the top of the mincer (1125 ml HBSS-medium).
3. Divide into two batches of 1kg and rinse 4x with 563ml for a total of 5 rinses.
4. Weigh rinsed mince to determine yield (this can be used to load bioreactor).
5. 1.5g of minced bovine liver tissue into 50mL tubes and Freeze at -20C.Thaw sample and add 15mL of RIPA extraction buffer containing 1x protease inhibitors. Homogenise liver tissues with lab homogenizer and put on ice to complete lysis process for 10min. Transfer to 1.5mL Eppendorf tubes and spin @ max.speed at 4C for 20min. Take off SN, transfer into fresh 1.5mL Eppendrof tube and store at -20C until further analysis. Discard pellets and label SN's, as described above.
6. 1.5g minced tissue in 2 ml HMM seed triplicate wells for contamination and AP/ protein determination

`

7. take 50ul sample for contamination at 16hr and 48hrs, grow for 24hrs in ATTC-22S + 10% FCS, 37C with shaking for contamination studies.

Research Operating Procedure 30: Sample preparation of the 0hrs and 48hrs stimulated and non-stimulated bovine minced organ culture

7. Bovine minced organ culture was obtained by collection of approximately 100g of eight different bovine livers, each in a separate container filled with 125mL of RPMI-1640 medium (containing Pen/Strep and Sodium Bicarbonate).
8. Tissue samples were transported on ice back to the laboratory. Under sterile conditions 15g of each liver tissue was cut and tissues were pooled together in a total volume of 120g in 45mL of HBSS medium containing Phenol Red, Pen/Strep and Sodium Bicarbonate.
9. The tissue was minced in an Industrial mincer using a fine disk (5mm) and collected in a beaker. Minced liver tissue was washed five times in HBSS using a fine strainer to drain the liquid between each wash.
10. Yield was determined after the last wash (22.4%) and 1.5g of pooled liver tissue was inoculated in triplicates into 6-well plates in a total volume of 2mL HMM (MEM-Eagle, Medium-199, Sodium Pyruvate, L-Glutamine, Pen/Strep, Bicarbonate, BSA).
11. A stimulant in a combination of Insulin at a final concentration of 10^{-4}M and Retinoic acid at a final concentration of 1000nM was added to one row of three replicates and minced organ tissue was then cultured for 0hrs and 48 hours at 5% CO_2, 37°C.
12. Samples were drawn and tested negative for bacterial growths and pH was monitored throughout the whole culture period in the supernatant. Total protein determination was carried out using a colorimetric protein-assay. Samples were then subjected to 1-DE, followed by 2-DE and DIGE.

Research Operating Procedure 31: Preparation of the total protein extract

1. Total protein was extracted from 1.5g minced bovine liver as **Research Operating Procedure 30** and homogenised for 1min/sample at 24,000min^{-1} in 2mL of RIPA extraction buffer, containing 50mM HEPES, pH 7.6, 1mM EDTA, 0.7% (w/w) Sodium Deoxycholate, 1% Nonidet P-40, 0.5M Lithium Chloride and Protease Inhibitors (Roche, Australia).
2. Extracts were lysed on ice for 30min with occasional shaking and spun at 10,400 rpm in a Beckmann Coulter Centrifuge at 4°C for 30min.
3. Supernatants were taken off and kept at -20°C until further analysis.

`

Research Operating Procedure 32: MS analysis and database searching of in-gel tryptic digested proteins

1. Spots of interest were localized on the gel by comparing the Silver stained spot pattern with the 2D-DIGE protein pattern. Proteins of interest were excised manually for digestion.
2. Gel pieces were partially dehydrated in a 50/50 mixture of acetonitrile and MilliQ water and incubated at room temperature for 15min under constant shaking.
3. Gel spots were covered with neat Acetonitrile for complete dehydration of the gel plugs. Acetonitrile was removed from the samples and the gel plugs were washed for 5min with 100mM ammonium bicarbonate under shaking.
4. An equal volume of acetonitrile was added to the samples and further incubated at RT for 15min. All the liquid was removed from the samples and the gel plugs were dried over night at 37°C in an oven.
5. To re-break any disulphide bridges that have re-formed, the gel plugs were covered with 10mM Dithioerythritol in 100mM ammonium bicarbonate buffer and incubated for 45min at 65C under shaking.
6. For Carbamidomethylation of the cysteine residues, all the liquid was removed from the samples and an equal volume of 100mM Iodoacetamide in 100mM ammonium bicarbonate was added and incubated for 30min at 30°C under shaking in the dark. Iodoacetamide solution was removed from the samples and gel plugs were washed with a 50/50 solution of acetonitrile and MilliQ water. This was repeated a further 2 times.
7. Gel pieces were dried at 37C. To extract the peptides, the polyacrylamide gel plugs were digested for 45min on ice in a solution, containing 100mM ammonium bicarbonate, 0.5mM Calcium chloride and 12.5ng/uL Trypsin Gold.
8. The excess digestion solution was removed and 100mM ammonium bicarbonate was added and the samples again incubated at 37°C overnight. Sufficient volume of 100mM ammonium bicarbonate was added to the samples the next day to cover the gel pieces and incubated for 15min at RT.
9. An equal volume of neat acetonitrile was added and incubated for 15min at RT. Under clean conditions, the supernatant was removed into a fresh low-binding eppendorf tube.
10. The extraction was repeated twice using 5% formic acid pooling all extraction into the same eppendorf tube. Samples were concentrated down to 1-2uL using a rotary evaporator and peptides were resuspended in 5% formic acid.
11. The digested peptides were analysed with a Thermo LTQ XL linear ion trap mass spectrometer fitted with a nanospray source (Thermo Electron Corp, San Jose, CA).
12. The samples were applied to a 300 µm i.d. x 5 mm C18 PepMap 100 precolumn and separated on a 75 µm x 150 mm C18 PepMap 100 column using a Dionex Ultimate 3000 HPLC (Dionex Corp, Sunnyvale, CA) with a 55 minute gradient from 2% acetonitrile to 45% acetonitrile containing 0.1% formic acid at a flow rate of 200 nl/min followed by a step to 77% acetonitrile for 9 minutes.

13. The mass spectrometer was operated in positive ion mode with one full scan of mass/charge (m/z) 300-2000 followed by product ion scan of the 3 most intense ions with dynamic exclusion of 30 seconds and collision induced dissociation energy of 35%.
14. The spectra were searched with Bioworks 3.3 (Thermo Electron Corp, San Jose, CA) using the Sequest algorithm against the IPI rat database v3.39 with the following filters: 1) the cross-correlation scores (Xcorr) of matches were greater than 1.5, 2.0 and 2.5 for charge state 1, 2 and 3 peptide ions respectively, 2) peptide probability was greater than 0.001 and 3) each protein identified has at least 2 different peptides sequenced.

Abbreviations

°C	Degree Celsius
1-DE	One-dimensional
2-D	2-dimensional
2D-DIGE	Two-dimensional differential gel electrophoresis
2-DE	Two-dimensional
3-DE	Three-dimensional
8Br-cAMP	8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt
A	Amperes
AATM	Aspartate aminotransferase, mitochondrial
Ac	Accession
ACN	Acetonitrile
ALAD	Delta-aminolevulinic acid dehydratase
ALDH2	Aldehyde dehydrogenase, mitochondrial
AP	Alkaline Phosphatase
B1 - A	Stirred tank bioreactor trial 1 A high inoculum (m/m = 0.75g/ml)
B1 - B	Stirred tank bioreactor trial 1 B low inoculum (m/m = 0.30g/ml)
B2	Stirred tank bioreactor trial 2 very low inoculum (m/m = 0.15g/ml)
BCA	Biocinchoninic-assay
BSA	Bovine serum albumin
cDNA	Complementary deoxyribose nucleic acid

`

cG	Chorionic gonadotrophin
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonic acid
CL	Corpus luteum
cm^2	Centimetres squared
CO2	Carbon dioxide
$CoCl_2$	Cobalt Chloride
Coll	Collagenase
Cy	Cyanine dyes
CYB5	Cytochrome b5
D2	diameter squared
db-cAMP	Dibutyryl cyclic adenosine monophosphate
DIA	Differential in-gel analysis
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPP3	Dipeptidyl peptidase 3
DTT	Dithiothreitol
E.coli	Escherichia coli
e.g.	Exempli gratis (for example)
EBT	Eriochrome Black T
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno-sorbent assay
Epo	Erythropoietin
FBP1	Fructose-1,6-bisphosphatase 1
FCS	Foetal Calf Serum
FGF2	Fibroblast Growth Factor -2
Fig.	Figure
g	Grams
g/l	Grams per litre
Glu	Glucose

`

GLUD1	Glutamate dehydrogenase 1, mitochondrial
H202	Hydrogen Peroxide
HBSS	Hank's Buffered Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMM	Hepatocyte Minimal Medium
HPLC	High Pressure Liquid Chromatography
hrs	hours
HSPD1	60 kDa heat shock protein, mitochondrial
IEF	Isoelectric Focusing
IGF2	Insulin-like Growth Factor-2
IPG	Immobilized pH gradient
kDa	kilo daltons
LC	Liquid chromatography
LDHB	L-lactate dehydrogenase B chain
M	Molar
m/m	Ratio of mince to mince to medium (g/ml)
m/z	Mass-to-charge ratio
mA	Milli amperes
MALDI-TOF-MS	Matrix Assisted Laser Desorption Ionisation – Time of Flight – Mass Spectrometry
max	Maximum
MDH1	Malate dehydrogenase, cytoplasmic
mg	Milligrams
mg/ml	milligrams per millilitre
min	minutes
mL	Milli litres
mm	Millimetres
mM	Milli molar
Mr	Molecular weight

`

MS	Mass Spectrometry
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
mW	Milli watts
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng/ml	nanograms per millilitre
nM	Nanomolar
Nm	Nanometres
^{o}C	Degrees centigrade
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
pg/ml	picograms per millilitre
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic Acid
rAP	recombinant Alkaline Phosphatase
RIA	Radioimmunoassay
RNA	Ribo-Nucleic Acid
ROP	Research operating procedure
RT	Reverse Transcription
RT-PCR	Reverse Transcription – Polymerase Chain Reaction
s.f.	Serum-free
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
Sod. Buty.	Sodium Butyrate
SPE	Solid Phase Extraction
TE	Trypsin-EDTA
TNF-alpha	Tumour Necrosis Factor - alpha
Tris	tris(hydroxymethyl)aminomethane
U/ml	Units/millilitre

`

ug/ml	Micrograms per millilitre
ul	Microlitres
W1	Wave bioreactor trial 1 low inoculum (m/m = 0.30g/ml)
W2	Wave bioreactor trial 2 high inoculum (m/m = 0.75g/ml)