GENE EXPRESSION PROFILES OF

3D GENE-EDITED BIOPRINTED CELLS

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ABSTRACT

Cancer is the biggest cause of death in New Zealand each year and places a large economic strain on the health system. Previously, two-dimensional (2D) cell models have provided insight into the development of cancer but now, three-dimensional (3D) models provided more accurate information on how cell-cell interactions in the body affect the efficacy of different drug therapies. This research aims to investigate the gene expression profiles of 3D gene-edited bioprinted lung cancer cells to understand how mutations and the vital trace element Selenium (Se) affects gene expression. The human A549 lung cancer cell line was bioprinted into a set dome shape made of sodium alginate, gelatine, and cell media. The cells were then cultured for 21 days before a six-hour drug exposure with 2μM and 10μM Methylselenic Acid (MSA).

RNA was also extracted from 18 bioprints and the spectrophotometer data suggested high quality with a total RNA concentration range of 9 – 671μg/μL, respectively. The RNA integrity was further examined using the RNA tape station. The solvent control (n=2) and the 2μM MSA treatment (n=3) from the second independent experiment resulted in intact ribosomal bands. The protein concentration was determined using a Bradford assay and ranged from 0.1 – 3.42mg/mL. The electrophoresed protein showed a molecular weight size range from 120kDa – 10kDa. The protein was then transferred onto a western blot membrane and detected by two primary antibodies; (1) HSP70 - correctly fold proteins that are under cellular stress and (2) GAPDH – a loading control reference antibody. The HSP70 protein expression was measured for the drug-treated bioprints for three bioprinting experiment. We hypothesised an upregulation in HSP70 expression based on previous studies. However, the data set could not be replicated to conclusively downregulation in response to 2 and 10μM MSA. Thus, further optimisation into drug accessibility, cell and protein loading concentration is required.

Gene-editing using lipofectamine and CRISPR-Cas9 was attempted using A549 cells but no conclusive evidence that a mutation had been introduced into the *Cyclin Dependent Kinase 4* (*CDK4*) gene was observed (*n*=5). Three research optimisations have been recommended for gene-editing and establishing a stable gene-edited cell line for future use in 3D bioprinting and gene expression analysis.

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Lastly, to my late grandfather Graeme Ginn, who passed in early 2023. While you may no longer be with us you always believed and supported me. I learnt from all your family and friends that you told every single one of them about how you did not understand what my research was but that I was helping find a cure for cancer and that I killed all my cells (which was not my intention). While that may not be entirely true, what was true was how proud you were of me. Thank you for your belief, I hope that I can continue to make you proud.

In honour of his memory, I would like to dedicate this thesis to Graeme Ginn.

TABLE OF CONTENTS

TABLE OF TABLES

TABLE OF FIGURES

ABBREVIATIONS

1°	Primary
2°	Secondary
2D	Two-dimensional
3’	Three-prime direction of the DNA strand
3D	Three-dimensional
5’	Five-prime direction of the DNA strand
A	Absorbance
aa	Amino acid
Ab	Antibody
bp	Base pairs
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
Cas9	CRISPR-associated (protein) 9
CO_2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
FBS	Fetal Bovine Serum
g	G-force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GFP	Green Fluorescent Protein
GPX1	Glutathione peroxidase 1
HDR	Homology Directed Repair
HRP	Horse Radish Peroxidase
HSP60	Heat Shock Protein 60
HSP70	Heat Shock Protein 70
IgG	Immunoglobulin G

IPA	Isopropyl alcohol
kDa	Kilodalton
MEM	Minimum Essential Media
mRNA	Messenger RNA
MSA	Methylselenic acid
MSC	Se-methylselenocysteine
mt	Mitochondrial
NaCit	Sodium citrate
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
NHEJ	Non-Homologous End Joining
nt	Nucleotide
P53	Tumour protein P53
PAGE	Polyacrylamide Gel Electrophoresis
PAM	Protospacer-Adjacent Motif
PBS	Phosphate Buffer Saline
PC1/2	Physical Containment 1 or 2
PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
PVDF	Polyvinylidene difluoride
R2	Coefficient of determination
rcf	Relative centrifugal force
rep	Replicate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT+	Reverse Transcriptase
RT-qPCR	Real time quantitative P
SDS	Sodium dodecyl sulphate
Se	Selenium
SeCys	Selenocysteine
sgRNA	Single guide RNA

SLM	Selenomethionine
SNP	Single Nucleotide Polymorphism
SNV	Single Nucleotide Variant
SS	Sodium selenite
TAE	A buffer that consists of Tris base, acetic acid and EDTA
Taq	*Thermus aquaticus*
TBS	Tris-buffered Saline
TBST	Tris-buffered Saline mixed with Tween 20
TE	Tris-EDTA solution
Tm	Melting Temperature
TME	Tumour Microenvironment
TP53	Tumour Suppressor protein 53
TUBA4A	Tubulin Alpha 4A
UV	Ultraviolet
WB	Western blot
WT	Wild type
βME	Beta-mercaptoethanol
α	Alpha
β	Beta
°C	Degrees Celsius

CHAPTER ONE - Introduction

The National Cancer Institute (NCI) in the United States of America defines cancer as "*a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body*", causing the cells to then cluster and form tumours that can become cancerous[1]. Cancer is New Zealand's biggest cause of death each year and will affect most New Zealanders in their lifetime, whether it is through personal experience or by knowing a who has experienced it[2]. The overall aim of this thesis was to study the gene expression effects of Selenium (Se) on *in vivo* 3-Dimensional (3D) gene-edited cancerous bioprints. A range of methodologies can be used to study gene expression such as RNA-seq and western blotting, providing further insight into the mechanisms of gene action and aid in the development of a pharmacogenomic model to study specific gene mutations with respect to cancer treatment.

For the purposes for this thesis, there are five chapters. This chapter outlines the structure of the research thesis presented. Chapter 2 will provide a literature review on cancer, the development of cancerous cells, the genetics of *CDK4* gene, the chemo-preventative effects of Se, the history of bioprinting, and finally the technique of gene editing.

Chapter 3 will investigate how bioprinting can be used as an *in vitro* model for studying cancer. This will include the methodology to isolate and extract the pEF-GFP plasmid, the transfection of that plasmid into the human lung carcinoma epithelial (A549) cell line and establishment of a stable cell line. In addition with, bioprinting, Se exposure studies, RNA and protein extraction and expression.

Chapter 4 will examine how to introduce a mutation into the *CDK4* gene of a cancerous cell line using CRISPR.

Finally, Chapter 5 will discuss the future recommendations to achieve the overall research aim.

CHAPTER TWO – Literature Review

Overview of Cancer

Cancer is a classified as a genetic disease that is caused by mutation to the genes that control the way cells grow, divide and function[1]. The variability of these mutations means that cancer can occur in almost any cell in the body[1], with over 200 different types of cancers reported [23].

Cancer is not only a drastic health consequence, but also a large economic consequence that only continues to increase each year putting immense strain on the health system[9]. It was estimated that in 2020 $53.83 billion USD was spent worldwide on drug research and development with 30% of that going into anticancer treatments[22]. It is believed that by 2026 this cost will reach $66.66 billion USD. With the ageing population and high stress lifestyles becoming a societal norm the risk of developing cancer will only continue to increase.

By understanding where cancer begins, how it mutates, and spreads is crucial to acquiring the best method to treat that cancer. Researchers observed a large proportion of cancer-causing mutations in genes involved in the eukaryotic cell cycle[20].

2.1 The Eukaryotic Cell Cycle

The cell cycle is a complex process that allows cells to grow and divide, while also being involved in organismal development, regulation of DNA damage and repair, tissue hyperplasia in the response to an injury as well as diseases like cancer[20]. The cell cycle is composed of four phases (***Figure 1***). G_1, S, and G_2 are grouped together and known as interphase, where the cell spends the most of its time[21]. The G_1 and G_2 phases are known as the "gaps" in the cell cycle. The phases are where the cell prepares for two upcoming significant events[20]; (1) in G_1, the cell is preparing for DNA replication that occurs in the S phase; (2) in G_2, the cell is preparing the cell for the M phase. The M phase is where the cell replicates its DNA and prepares for cell division, when it completes its division, the cell produces two new daughter cells, and the cycle continues[21].

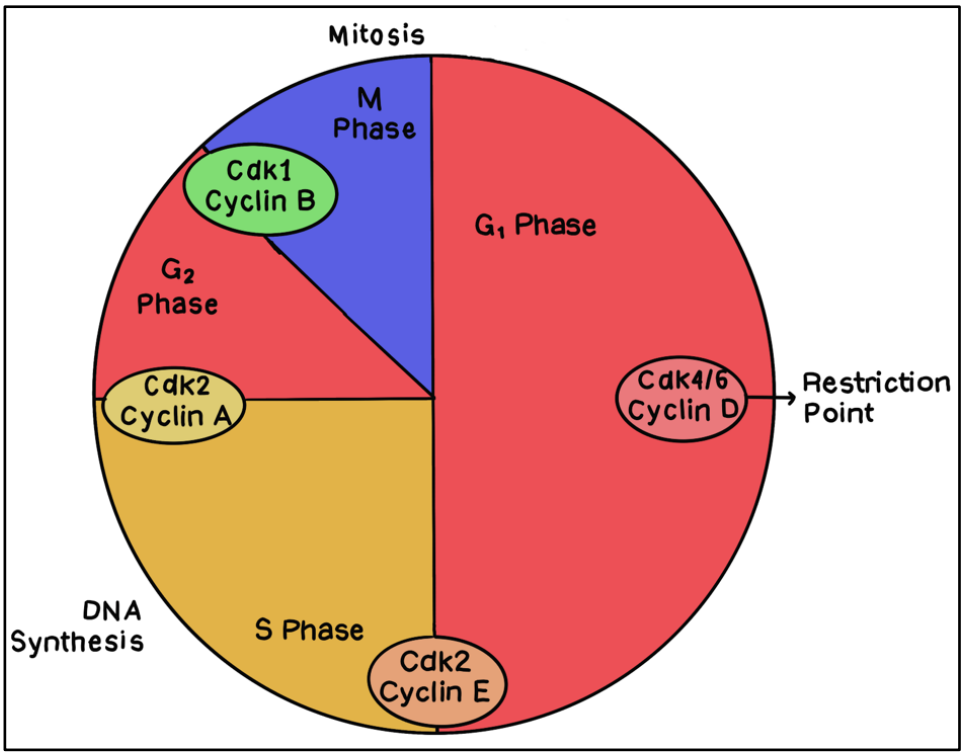

Figure 1 A schematic diagram of the progression of a eukaryotic normal cell cycle as well as the cyclin and CDKs that form the cyclin-CDK complexes involved in each phase of the cell cycle. This figure was modified from Schafer[20].

There are seven known cyclin-dependent kinases (CDK) complexes (serine/threonine protein kinases)[20] that are essential for the cell's progression into the next phase of the cell cycle. For the purposes of this thesis, we will be focussing on CDK4 as it was the positive gene editing control for introducing a gene mutation using CRISPR (***Chapter 4***).

CDKs must be activated by binding with the respective cyclin. The cyclic expression of the respective cyclins ensure that the activation of the CDKs can only occur at specific stages during the cell cycle. This cyclic expression is in part due to the regulated degradation that the cyclins undergo which is caused by a specific protein sequence in the cyclins called PEST sequences. These sequences are protein motifs that are rich in proline, glutamine, serine, and threonine. Ubiquitination then targets these sequences at the specific times and causes the degradation of the cyclin. Degradation and synthesis of these cyclins can regulate how the cells move in and out of cell phases. The cyclins are also responsible for targeting the CDKs to the nucleus as they contain nuclear localisation signals that are otherwise lacking in the CDKs.

2.1.1 Cyclin Dependent Kinase 4 (CDK4)

Mutations in the cell cycle affect the cell's regulation and cause normal healthy cells to become cancerous cells. The Cyclin Dependent Kinase 4 (CDK4) protein is an essential component in the eukaryotic cell cycle that works with D-type cyclins to help mediate the cells progression from the G_1 phase into the S phase of the cell cycle[7]. CDK4 or/and CDK6 are both used for the progression of the cell cycle from G_1 to S phase[83]. Current *in vitro* studies have found no major differences in the function of CDK4 and CDK6 in the progression of the cell cycle. It has been found that the dysregulation of the CDK4 pathways results in high proliferation in these cells and this characteristic is commonly seen in almost all forms of cancer[8]. The dysregulation of the CDK4 pathway can occur due to several factors and understanding how these affect the development and treatment of cancer is important due to its prevalence in cancer and its significance to normal cell cycle control.

The *CDK4* (*cyclin dependent kinase 4*) gene is located on human chromosome 12q14.1[15], with eight exons (one noncoding and seven coding) and is 4,584 nucleotides long[16]. The *CDK4* gene encodes for the 303 amino acid CDK4 protein (33 kDa)[93].

Once the CDK/cyclin complex has been formed and has a phosphate bond attached, the complexes can move into the nucleus where the CDKs phosphorylate a variety of substrates (depending on the phase of the cell) which catalyses the cells move into the next phase[20].

In the G_2 and M phases of the cell cycle the substrates that are phosphorylated by CDKs are substrates that form the nuclear cytoskeleton of cells, like nuclear lamins and microtubules.

Like every important biological process, the cell cycle has important inhibitors that inactivate CDKs by binding to them before the cyclins can bind and form the CDK/cyclin complexes[20].

Known mutations of *CDK4* are seen in the germline having an autosomal dominance pattern of inheritance (***Table 1***). The mutation in this gene have been linked with familial malignant melanoma occurring in the epithelial tissue[82]. Mutations in the gene have been shown to promote the cells replicative immortality. Mice models have found that the ablation

of *CDK4* or *CDK6* are compatible with life (specifically in these models) due to the interchangeable nature of *CDK4/6*[83]. The mice models have also found that the inactivation of the *CDK4* gene affects the proliferation of certain cell types only, which in these studies where prevention of proliferation of postnatal pancreatic β cells. Similar was seen in the *CDK6* knockout models where only certain cell types were affected by the loss of expression. This data could suggest that cells the differences between *CDK4* and *CDK6* could play a crucial role in the cell's movements from the G_1 phase into the S phase.

Name	Protein Change	Conditions	Molecular Consequence	Clinical Significance
NM_000075.4(CDK4):c:70C>T (p.Arg24Cys)	R24C	Familial Melanoma, Hereditary Cancer-predisposing	Missense	Pathogenic
NM_000075.4(CDK4):c:71G>A (p.Arg24His)	R24H	Familial Melanoma, Hereditary Cancer-predisposing	Missense	Pathogenic
NM_000075.4(CDK4):c:279dup (p.Glu94Ter)	E94*	Gastric Cancer	Nonsense	Pathogenic
NM_000075.4(CDK4):c:71G>T (p.Arg24Leu)	R24L	Multiple Myeloma, Lung adenocarcinoma, Malignant melanoma of skin	Missense	Likely Pathogenic
NM_000075.4(CDK4):c:70C>A (p.Arg24Ser)	R24S	Malignant melanoma of skin, Lung adenocarcinoma, Multiple myeloma	Missense	Likely Pathogenic

Table 1 Current (as of 20/06/2023) pathogenic and likely pathogenic mutations seen in the CDK4 sourced from ClinVar[109] https://www.ncbi.nlm.nih.gov/clinvar as well as the protein change, conditions, and molecular consequence of these mutations.

2.2 Chemo-preventative effects of Selenium

There has been great interest over the potential chemo-preventative effects that Selenium (Se) may possess. Se is a trace element that is element for human health [26] and it can affect the immune system[25], digestion of toxins, and regulation of chemical reactions[67, 76]. Se has been shown to both boost and supress the immune system by enhancing activation and proliferation of B cells, promoting immune cell differentiation.[25]

Se was first discovered and isolated by the Swedish chemist Jöns Jacob Berzelius in 1817[25] and can be found in organic forms like selenocysteine (Sec) and Se-methylselenocysteine (MSC)[25] or inorganic forms like selenite ($HSeO_4^-$) and selenate (H_2SeO_3)[25].

Se is obtained through our diet, supplemental or from food like seafood, dairy, meat, cereals, grains and vegetables[67, 74, 75]. When Se is obtained from the foods rather than supplements the amount of Se content that is absorbed is varied. For animal products the level of Se changes depending on the availability in the animals diet. For plant-based products the amount of Se in the soil will affect this level[67, 75]. Dietary Se is found in different forms due to the bioavailability in the area. These forms can include Sec, selenite, selenate and selenomethionine (SLM)[73]. It has been found the insufficient amounts of Se can cause extreme harm to health but so can excessive intakes of Se, this balance is still vague[27].

What makes Se an essential micronutrient for humans and other mammal? Se is used to make up the rare 21st amino acid selenocysteine (Sec, U)[30]. Sec is instrumental in the production of Se-containing proteins (known as selenoproteins) that make up components of antioxidant enzymes like glutathione peroxidase (GPX) and thioredoxin reductase (TrxR) (selenoenzymes)[30]. Selenoproteins with Sec residues are used to mediate the effects of DNA synthesis, redox control, thyroid function, anti-inflammatory and immune response, and oxidative stress response[65(3)]. The selenoenzymes have been found to protect, defend, and safeguard the cells from oxidising damage that is caused by reactive oxygen species (ROS)[30].

2.2 The different forms of Selenium and dosage

The two most prevalent forms of Se are SLM (organic) and sodium selenite (SS) (inorganic). Both these forms of Se have been shown to have no signs of toxicity when studied as a potential chemo preventative therapy[67,73]. In deciding what Se form is better, a study suggested that for Se to have the desired therapeutic effects the Se metabolite needs to be methylated, seen only in the organic forms[73].

Recent observational studies have found that a higher dietary intake or circulating levels of Se have been associated with a lower risk of overall cancer (as well as several site-

specific cancers like breast, lung, and prostate cancer)[27]. Most clinical trials that have used Se have focussed on three forms, SS, MSC and SLM[77]. In these studies, it has been found that MSC is the most effective out of the three forms, this is due to its ability to generate methylselenol. Methylselenol is the hypothesised metabolite that interacts with the cancer therapies. This is contrary to the original beliefs by some researchers that SS would be the most effective due to the reactive oxygen species-driven cytotoxicity that is highly selective and induces the death of malignant cells over healthy cells. However, SS has a disadvantage compared to its organic counterparts when using comparable doses causing a higher rate of genotoxicity in the cells. These results are therapeutically significant when using DNA-damaging cancer therapies. These genotoxicities have been shown to include acute leukaemia, myelodysplasia and other malignancies which are unwanted consequences of the combination of Se and cancer therapies. This shows the importance of choosing a form of Se with the least genotoxicity.

Dosage is the next important question in understanding where the line between effectiveness ends and toxicity begins. The current recommended Se dosage for is based on the levels that are needed to maximise the activity of the GPX family[73]. This does not consider ingested Se's chemical form, the individuals genotype and other selenoproteins. The current average daily intake for Se recommended by the European Food Safety Authority is 60μg for men and 53μg for women[66,80]. In a clinical trial, cancer patients were given 400μg/day of SS, SLM or MSC in conjunction with cancer therapies (chemotherapy, radiotherapy or angiogenesis targeting agents) for eight weeks[78]. The results showed that there were no significant changes to patients when looking at the pharmacodynamic (PD) parameters set by the researchers. This result confirmed preclinical analysis studies that showed that higher doses of Se exist as there is a threshold for activation of pathways in normal and malignant cells. SS showed the greatest single- and double-stranded DNA breaks, and other types of DNA damage at low levels of exposure in cultured human lymphocytes which when compared to the organic Se forms which appear to be safer than SS. However, SS and SLM have both been shown to have antigenotoxic properties enhancing DNA repair in cell lines and cultured lymphocytes.

Evans et al (2019)[77] showed that all three of these Se compounds (SS, MSC, and SLM) were safe and well tolerated at the dose of 400μg/day and with no clinically significant

adverse effects to the treatment[77]. The study aim was to focus on the genotoxicity of these forms of Se and it showed that at 400μg/day, in all forms of Se genotoxicity were all insignificant compared to patients without Se exposure. Out of the three forms of Se used in this trial MSC had a greater efficiency of methylated Se metabolites formed *in vivo*, followed by SS and then SLM, these results however vary depending on the enzyme and cell type of interest.

Another form of Se, methylselenic acid (MSA) has been shown to share antigenotoxic like SS and SLM[77]. MSA is comparable to MSC and is directly metabolised to methylselenol. MSA is one of the most potent *in vitro* Se forms in activating the PD mechanisms and can mimic the actions of how MSC is *in vivo*[78].

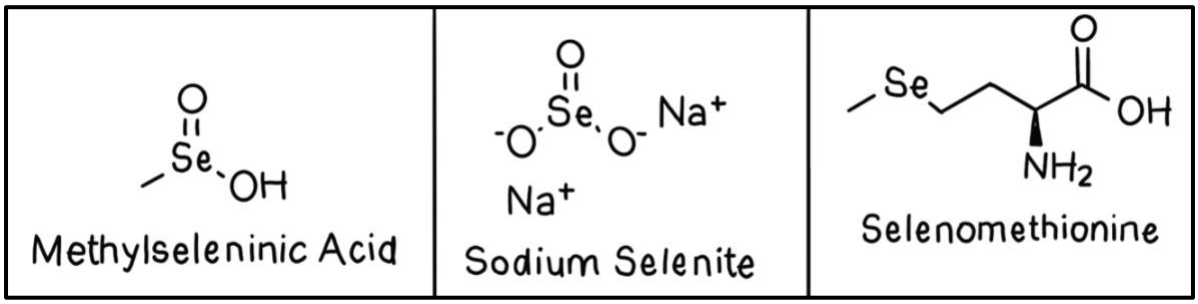

Figure 2 Chemical structure of methylselenic acid (MSA) (left), and sodium selenite (SS) (middle) and selenomethionine (SLM) (right). Image sourced from Saywer[67].

2.3 Developing a 3D *In vitro* Cancer Model Using Bioprinting to Investigate Gene Expression Effect of Selenium

Bioprinting is an additive manufacturing (AM) biotechnique[35]. 3D bioprinting has expanded and evolved the biotechnology field especially in biomedical engineering (tissue engineering), drug exposure and disease models by creating 3D cell cultures or engineered organ structures[35,37]. 3D bioprinting has allowed traditional biological questions to be studied and answered, that once could only be answered by two-dimensional (2D) cell cultures or animal models[37]. Bioprinting can create high reproducibility and the precise control during the fabrication of the 3D bioprints, enabling high-throughput production which is need for tissue engineering[38]. The main goal of bioprinting, particularly 3D cell models, is to make them clinically available for patients so that they can benefit from these techniques[43].

2.3.2 Bioprinting techniques

To ensure that bioprinting creates biologically functional yields, bioprints must contain bioactive molecules, living cells, and cell aggregates (biomaterials or hybrid cell-material constructs) followed by a tissue maturation period[38]. The hydrogel is a critical component of 3D bioprints as must maintain the 3D structure, hold all the essential molecules and biomaterials needed. Once the living cells are added to the hydrogel it becomes a "bioink". A Bioink is defined as a formulation of cells that are suitable for processing through bioprinting that may contain biologically active components and biomaterials[47,48] and should possess the important physiochemical properties like; mechanical, rheological, chemical, and biological properties.

Extrusion bioprinting uses pressure to extrude the bioink through the nozzle[44]. This pressure can be pneumatic (air-force pump) or mechanical (mechanical screw plunger) pressure[44,45]. With extrusion bioprinting there is a continuous force which allows uninterrupted printing to form consistent shapes rather than a single droplet like the other available bioprinting techniques[45]. The pressure used can be adjusted to suit the viscosity of the bioink and the amount of bioink that is needed to be dispensed[46]. After printing the hydrogels need to be cross-linked either physically (using UV light) or chemically[46].

2.3.3 Gene Expression and Selenium

To investigate the gene expression changes in the 3D bioprints with respect to different concentrations of MSA, protein will be extracted and analysed using a western blot protocol. The transferred protein will be probed with different primary antibodies (1º Ab).

Heat Shock Protein 70

Se has been hypothesised to induce oxidative stress in cancer cells, this stress causes the expression of Heat Shock protein 70 (HSP70) to help correctly fold critical proteins[138]. Studies have shown that under Se-induced oxidative stress, HSP70 has been upregulated. We hypothesise upregulation in HSP70 expression.

Glutathione Peroxidase 1

Glutathione Peroxidase 1 (GPX1) is a selenoprotein that reduces the cellular presence of hydrogen peroxide[66]. Se is necessary for the biosynthesis of the GPX protein. Research has

found that Se deficiency results in a reduced synthesis of GPX1. Therefore, it is hypothesised that the protein expression of GPX1 should be upregulated in both drug-treated sample replicates.

Tumour Suppressor Protein 53

Tumour Suppressor Protein 53 (TP53) is a transcription factor that activates different genes involved in the cell cycle and death[137]. It is one of the most mutated genes in cancer. In a study done by El-Bayoumy and Sinha (2005)[136], Se supplementation was found to induce the expression of TP53. For the protein expression of the drug-treated 3D bioprinted samples the same expectation of upregulation is hypothesised.

2.4 Developing a Gene-Edited 3D Cancer Cell Model

Clustered Regularly Interspaced Short Palindromic Repeats, (CRISPR), is a technique that is used by researchers genetically modify or edit DNA or RNA sequences in the genome[85]. Any genome whether it is cultured cells, plants, animals, bacteria and even viruses[86] can be edited using CRISPR. CRISPR was discovered in microbes. It is hypothesised that the system evolved to provide the microbes a defence against viruses by giving them an RNA-guided adaptive immunity that can remove the foreign genetic material that a virus inserts. This was achieved by directing the nuclease (Cas protein) to bind and cut at the specific nucleic acid sequence, preventing the viruses from being able to take over the cells' replication functions. They could also add that foreign nucleic acid sequence into their genomic CRISPR array.

The essential component of the CRISPR-Cas complex with the RNA-guided nuclease known as CRISPR-associated nuclease (Cas)[86]. Cas is responsible for the target cut or interference of the desired genomic region. There are many different types of Cas proteins[86]. This research utilises the Cas9 nuclease. There are also two other components; the CRISPR RNA (crRNA) paired with a trans-activating crRNA (tracrRNA), guide the Cas nuclease to the correct region of the sequence where cutting will take place. In engineered CRISPR complexes, the two RNA strands can be combined to create a single guide RNA (sgRNA), creating a two-component complex[86]. Finally, the complex has a recognition sequence that helps target the specific area of interest; this is known as the protospacer adjacent motif (PAM)[86], this three-nucleotide recognition sequence is usually depicted by “NGG”[90].

Without the PAM sequence the Cas protein is unable to cut the target sequence even if the sgRNA is completely complementary to the target sequence[88].

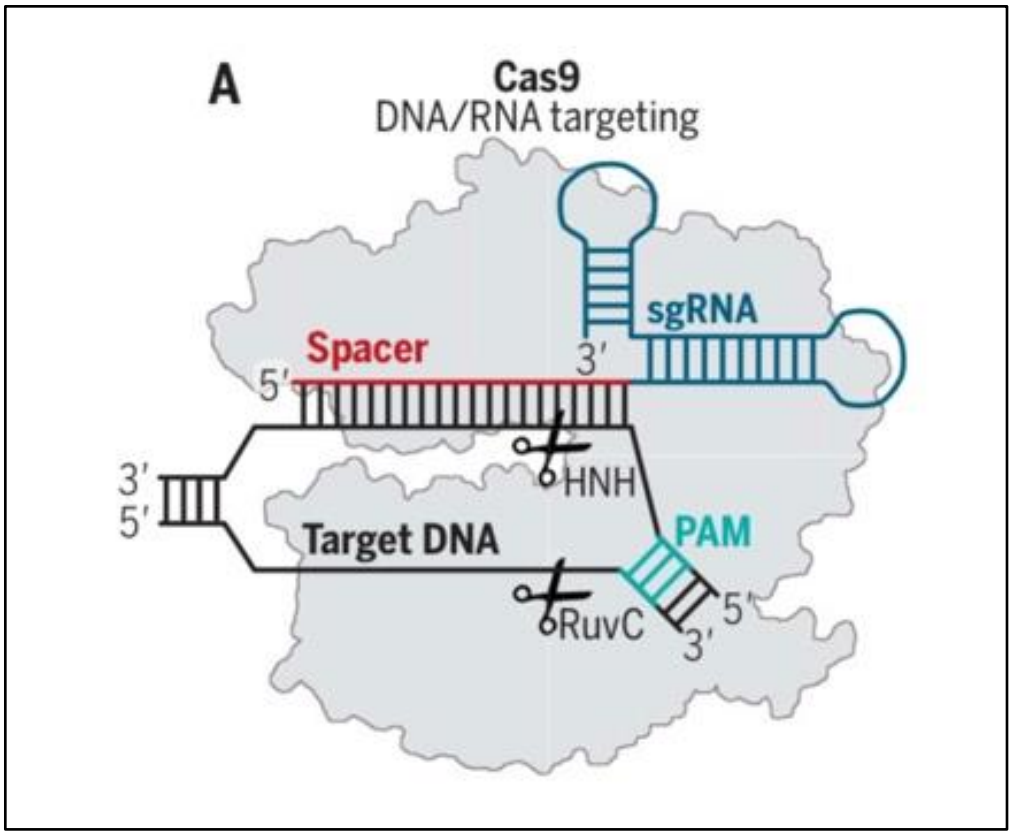

Figure 3 Schematic of the CRISPR-Cas9 System.Cas9 enzyme (grey) contains the sgRNA (blue) encoding a spacer (red) bound to a target dsDNA (black) near the PAM (teal) sequence. Image sourced[86]

The CRISPR-Cas9 system uses the sgRNA to guide the Cas9 to cleave the target genetic sequence causing a double stranded DNA break (DSB) [90]. The cleavage occurs at the PAM sequence. The DSB is causes deletions or small insertions (indels) through the process of non-homologous end joining (NHEJ) or homology directed repair (HDR). HDR is used to create precise genetic modification using a donor sequence.

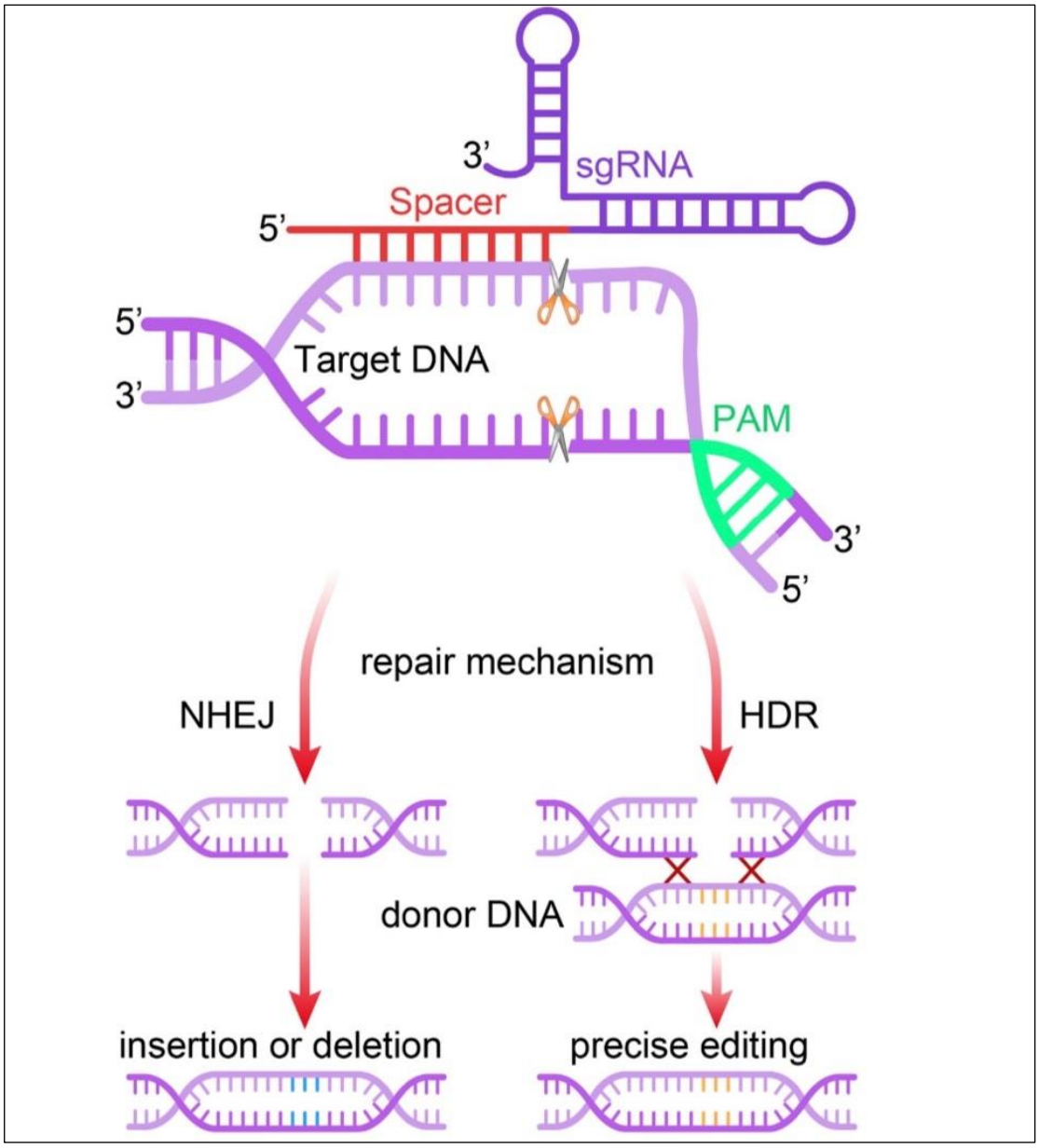

Figure 4 The mechanism of the CRISPR-Cas9 system. The sgRNA directs the Cas9 nuclease to the target sequence with the correct PAM sequence "5'-NGG- 3'" which will then initiate the DSB. These DSB are then repaired using NHEJ or HDR (if there is the presence of a repair DNA template). Image sourced[90]

The CRISPR-Cas complex is an interesting technology in which all areas of the complex are interchangeable depending on the nucleic acid sequence[86]. The Cas protein can be changed depending on what the PAM sequence is or whether your target sequence is DNA or RNA.

Because CRISPR is the easiest and most effective of the gene-editing technologies it shows the most promise for clinical applications. The first clinical trial to use CRISPR/Cas9 gene-editing was conducted in 2016, using CRISPR-gene-edited immune cells to a patient

with advanced lung cancer[88]. *Nature* reported on the mechanism and current progress of the research[89]. The researchers took immune cells from the patients' blood and then knocked out the *PDCD1* gene that codes for the PD-1 (programmed cell death protein 1) protein which is responsible for inhibiting the cells' immune response. However, in cancer it is mutated and allows the cancer to proliferate[89]. The *PDCD1* gene-edited cells were injected back into the patient, the hope that the cells with the knocked-out *PDCD1* gene will attack and defeat the cancer cells. The researching team told the *Nature* journalist that the first injections went smoothly and that second injections would be undergone. Due to the early stages of development for this treatment no further comments could be made. The aim of this trial is to show that cell therapy is a better option than antibody therapy[126]. Dr Naiyer Rizvi (2016)[89], commented on the treatment saying, "*He doubts this treatment will be superior to the use of antibodies*". Whether the trial is or is not successful does not dispute the fact that CRISPR will play a critical part in the future of cancer drug research.

2.5 Hypothesis, Aim and Objectives:

The aim of this research was to investigate the gene expression profiles of 3D gene-edited bioprinted A549 cells that have been exposed to Se compounds. The six objectives of the research were:

1. Print and grow 3D cancer A549 cell models for 21 days in a six-well tissue-culture plates using the Allevi 2 desktop 3D bioprinter using established in-house methodologies;
2. Develop and optimise a new methodology to extract correctly folded proteins from 3D A549 bioprints;
3. Set-up 3D bioprint exposure assays using a range of different Se concentrations;
4. Gene-edit the *CDK4* gene using CRISPR/Cas 9 and establish a stable gene-edited A549 cell line;
5. Extract and measure RNA expression of the Se-treated A549 cells in the wild-type and gene-edited 3D bioprints;
6. Measure the resulting protein expression of the Se-treated A549 cells in the 3D wild-type and gene-edited bioprints using western blot analyses.

We hypothesised that three proteins, HSP70, GPX1 and TP53 would be upregulated in response to 2 and 10μM MSA.

CHAPTER THREE - Developing a 3D *In vitro* Cancer Model Using Bioprinting to Investigate Gene Expression Effect of Selenium

3.1 Introduction

Former MSc students, Allan Hardaker[29] (2021) and Renata Sawyer[67] (2021) established the starting point for all the bioprinting experiments conducted in this thesis. The composition, concentration and ratio of Sodium Alginate and Gelatin to human *in vitro* cells and cell growth media was optimised. The cell viability of 3D bioprinted breast cancer cells (MDA-MB-231) week two, four, six and eight was investigated. At week 4, Hardaker (2021), had cell viability of 76% compared to Sawyer (2021), with 55%. It was determined that the difference in the cell viability was due to size, duration of cell media change and human error.

Sawyer (2021)[67], was then able to extract high quality RNA and protein from the bioprints that had been incubated for three to 15 weeks. Sawyer found a large difference in the RNA concentrations between samples from the same bioprinting session, the range being 75 – 400ng/μL, respectively. The conclusion was that due to the randomisation of the cell distribution within the bioink, cells were able to be densely clustered in one bioprint but not in another. The duration of incubation of the bioprints also varied the RNA concentration. Week 15 bioprints had an RNA concentration range of ~10 – 25ng/μL whereas week 7 had an RNA concentration range of ~ 6 – 77ng/μL, respectively. Week 3 had the highest RNA concentration range of ~75 – 431ng/μL. Due to these results the culture periods for the bioprints of this research was decided to be for three weeks (21 days).

Sawyer (2021)[67], was also the first of our research team to successfully extract protein from the bioprints with a concentration range of 0.7mg/mL – 3.2mg/mL. The protein was able to be visualised on a PAGE-Gel using Coomassie blue staining but when the gel was transferred onto a membrane for western blotting the proteins were unable to be detected by the HSP60 primary (1°) antibody (Ab) (Abcam, ab46798). It was postulated that the

extracted proteins were not folded correctly, and the methodology needed further optimisation.

Thus, the starting point for this research was research objectives 1 – 4 with a major focus to improve the extraction and detection of protein from 3D bioprints.

3.2 METHODS

All the experimental work for this research project was carried out at the University of Waikato, Hamilton, New Zealand. All solutions were prepared using double-distilled Milli-Q (MQ) water (Barnstead double distilled/deionisation system at a resistivity of 18MΩ•cm). Before any work commenced, the work area was cleaned and wiped down with 70% ethanol to minimise microbial contamination. Reagents, buffers, media, and equipment that were used for cell culturing were brought into the ESCO Class II BSC Airstream (ESCO) cell culture hood and sprayed with 70% ethanol before being placed in the hood. All warmed reagents and media were heated in a 37°C water bath.

3.2.1 Obtaining ethical and regulatory approvals

Approval was also granted by the Environmental Protection Authority, NZ (EPA) (approval numbers; GMD101170, GMD101146, and GMD101157) for the generation and use of genetically modified organisms (GMOs) in this thesis project. A list of the GMOs is found in the Supplementary Appendix 1 (***SA1***)

3.2.2 Culturing mammalian cells

The A549 human cancer cell lines used in this research were provided by Dr Linda Peters (University of Waikato, Hamilton, NZ). The A549 cell line was used in this study as a positive control for CRISPR experiments and is a well-studied model for non-endocrine tissue (human lung carcinoma). The A549 cells were also used for bioprinting and the selenium drug exposure. For both experiments the passage numbers were between 10 and 20. The media bases for cell culturing were ordered from ThermoFisher Scientific (NZ) and on arrival were stored at 4°C. ***Table 2*** shows the media bases (and category numbers) used for the cell line including Opti-MEM™ that was used for transfecting cells. This table also shows the original passage number when the cells were first revived for the cell lines. Different media bases were also supplemented sera and antibiotics and when the media was fully supplemented it was deemed as *complete* media. Recipes for each complete media can be found in Supplementary Appendix 2 (***SA2***). All supplements were ordered from ThermoFisher Scientific (NZ) and on arrival were aliquoted and stored at -20°C. Before storage, sera were first filtered with a Terumo® syringe and a 0.22μM Minisart filter (Sartorius, Germany). ***Table 3*** shows the supplements used to complete media (this includes

trypsin-EDTA used to detach cells). All flasks and plates used for cell culturing were surface-treated and vented (JET BIOFIL®, China).

Cell line	Passage number	Media	Category Number
A549	5	Ham's F-12 Nutrient Mix	11765054
All cell lines (transfection media)	N/A	Opti-MEM™	31985062

Table 2: Media bases used for each cell line. Recipe can be found in Appendix 1

Supplement	ThermoFisher Scientific Category Number
Foetal Bovine Serum (FBS)	A4766801
Penicillin-Streptomycin (10,000U/mL)	10378-16
Trypsin-EDTA (0.25%)	25200056

Table 3: Supplements used in complete media.

3.2.3 Reviving/thawing frozen mammalian cells.

A 15mL centrifuge tube was prepared with 4mL of the warmed complete media. Frozen A549 cells that were stored in cryotubes in the -80°C freezer was thawed in the 37°C water bath for 1 minute and then added dropwise to the 15mL tube containing prewarmed media avoid osmotic stress to the cells. The cell suspension was then gently pipetted up and down to mix. The centrifuge tube was then centrifuged in the Heraeus Megafuge 1.0 at 200g (1100rpm) for 5 minutes at room temperature (RT) to pellet the cells. The supernatant was removed, and the cell pellet was resuspended in 3mL of warmed complete media and transferred to a T25 tissue-treated cell culture flask. The presence of cells in the flask was confirmed under a microscope and then incubated at 37°C/5% CO_2 in the Heraeus Hera Cell 240.

3.2.4 Passaging mammalian cells

Once the cells have reached a high confluency (~90%), the cells must be split into two new T25 flasks to prevent cell death due to accumulation of waste products and nutrient deprivation. Cell media was first removed first, before 2mL of warmed Phosphate Buffered Saline (1X PBS, pH7.4) was added to the cells to wash off any remaining media waste. Next, 3mL of 0.25% Trypsin-EDTA was added to the cells, the flask was then placed in the

incubator at 37°C/5% CO_2 for 10 minutes. Trypsin is used to prompt the cells to detach from the bottom of the flask. The flask was observed under microscope to check cells were detached from the bottom with cells floating and moving freely when the flask is gently shaken from side to side. Equal complete media (3mL) was added to cell suspension to neutralise the trypsin, the entire contents of the flask was then transferred into a 15mL centrifuge tube. The tube was centrifuged at 200g (1100rpm) at RT for 5 minutes. The supernatant was then removed and discarded, ensuring that the cell pellet was undisturbed. Warmed complete media was added to the two new T25 flasks (that were labelled with the date, initials, cell line, and subsequent passage number), ensuring the whole bottom of the flask was completely covered in media. Three mL's of warmed complete media was then added to the 15mL centrifuge tube to gently resuspend the cell pellet. The resuspended cells were then transferred to the new flasks, 1.5mL to one and 1.5mL to the other. The flasks were gently swirled to ensure that the media was well mixed together and that the cells were evenly distributed throughout the flask. The flasks were then placed into the incubator at 37°C/5% CO_2.

3.2.5 Mammalian cell culture expansion – upgrading from a T25 to a T75 flask.

Method 3.2.5 is also used for the cell culture expansion. The cells have their media is removed, are washed, trypsinised, neutralised and centrifuged. The cell pellet was then resuspended in 3mL of warmed media. The cell suspension was then added to the new T75 flask that contained a 2mL of warmed complete media. The T75 flask was gently swirled to mix the media with the cells and that the liquid was evenly spread in the flask. The flask was placed back into the incubator at 37°C/5% CO_2.

3.2.6 Cell counting

Cell counting is used to accurately determine the number of cells that are in each cell culture flask. Two methods can be used for cell counting. Manual cell counting using the Fortuna haemocytometer and automated cell counter Countess 3 FL Automated Cell Counter (Invitrogen). For both cell counting methods, a volume of 10μL of cell suspension and 10μL of 0.4% Trypan Blue stain solution (Sigma-Aldrich, NZ) is combined well in a 1.5mL centrifuge tube.

Manual cell counting

Manual cell counting uses a 0.0025mm^2 haemocytometer (0.100mm deep, FORTUNA®) under a 22 x 22 cover slip (LabServ®). The haemocytometer with a clear coverslip placed on top has 10µL of the cell suspension added to each side. Cell counting was accomplished by viewing the cells on two grids that contained four corner squares on each side of the haemocytometer under the Nikon Eclipse TS100 inverted microscope. The cell counts of all eight squares (the four corner squares of each of the two girds) were then averaged ($\bar{x}$).

$$cells\ per\ mL = \bar{x} \times 2 \times 10{,}000 \times supension\ volume(mL)$$

Equation 1 Cells/mL is calculated with the average($\bar{x}$), the trypan blue: cell suspension ratio (2), 10,000 and the suspension volume that the cell pellet is resuspended in (mL)

Equation 1 calculates the total number of cells that are present in the cell suspension.

Automated cell counting

Automated cell counting for brightfield was accomplished by following the instruction manual of the Countess 3 FL Automated Cell Counter (Invitrogen). A reusable cell counting slide with the reusable cover slip was used. It was then placed in the cell counter and was automatically counted. Parameters were able to be changed depending on personal needs, but for standard cell counting the default settings were maintained.

3.2.7 Cryopreserving mammalian cells

When freezing cells each cryotube should contain 1×10^{-6} cells/mL resuspended in freezing media. Cells have the media removed, washed, trypsinised, centrifuged. The cells were resuspended in 1mL of freezing media before being counted. The cells suspension was then adjusted to the desired volume (1×10^{-6} cells/mL). The cell suspension was then aliquoted into cryotubes with a volume of 1mL. The tubes were placed into a Biocision CoolCell® LX at RT allowing the cells to be slowly frozen down once they were placed in the -80°C freezer.

3.2.8 Extraction of the positive transfection plasmid control DNA

The 5054 bp pEF-GFP plasmid (Addgene, 11154) was used as the positive plasmid transfection control as it contains the green fluorescent gene under the transcription control of

the EF1a promoter. Thus, allowing visualisation of GFP protein under ultra violet (UV) light. Plasmid DNA was extracted from a -80°C bacterial stock of transformed *Escherichia coli* DH5-α cells by streaking the frozen cells aseptically with an inoculation loop onto a Luria Broth (LB) 90mm agar plate containing 100μg/mL ampicillin. The streaked agar plate was incubated upside down overnight at 37°C. The following day, a single colony was selected and aseptically inoculated in a sterile culture flask containing 30mL of LB Broth supplemented with 200μg/mL ampicillin. The flask was incubated overnight in a shaking incubator GallenKamp shaking incubator at 180rpm and 37°C.

3.2.9 'Mini-prep' extraction of plasmid DNA from the DH5-α *E. coli*

Six 5mL rapid plasmid extractions were carried out using the inoculated overnight LB Broth. Following the "DNA-spin^{TM} Plasmid DNA Purification Kit" (iNtRON Biotechnology, 17096) protocol. All the centrifuge speeds were performed at 15,800g and at RT. The volume of elution buffer used was 40μL and was eluted over the column twice to ensure all the plasmid DNA was transferred into the centrifuge tube. The plasmid DNA quality was measured using absorbance (A) ratios of 260/280nm and 260/230nm and concentration using a DeNovix DS-11 FX Spectrophotometer/Fluorometer (DeNovix).

3.2.10 'Clean and Concentrator' of the plasmid DNA extracted from the DH5-α *E. coli*

To increase the purity and concentration the extracted plasmid DNA was "cleaned" using the "Zymo Research DNA Clean and Concentrator^{TM} – 5" (Zymo Research, D4013) protocol. Two mini-prep samples were combined into one, thus resulting in three tubes with a starting volume of 80μL for all three samples. All the centrifuge speeds were conducted at 13,500g and at RT. The volume of elution buffer used was 40μL and was eluted over the column twice. The plasmid DNA quality and concentration was also measured.

3.2.11 Confirmation of the pEF-GFP plasmid DNA by Restriction digest

To confirm the extracted plasmid DNA size, five 10μL restriction digest reactions in a 1.5mL centrifuge tube was set up using three restriction enzymes: EcoRI (1220), HindIII (2539) and BglII (606, 2073) (Roche Diagnostics) (***Figure 5***, ***Table 4***).

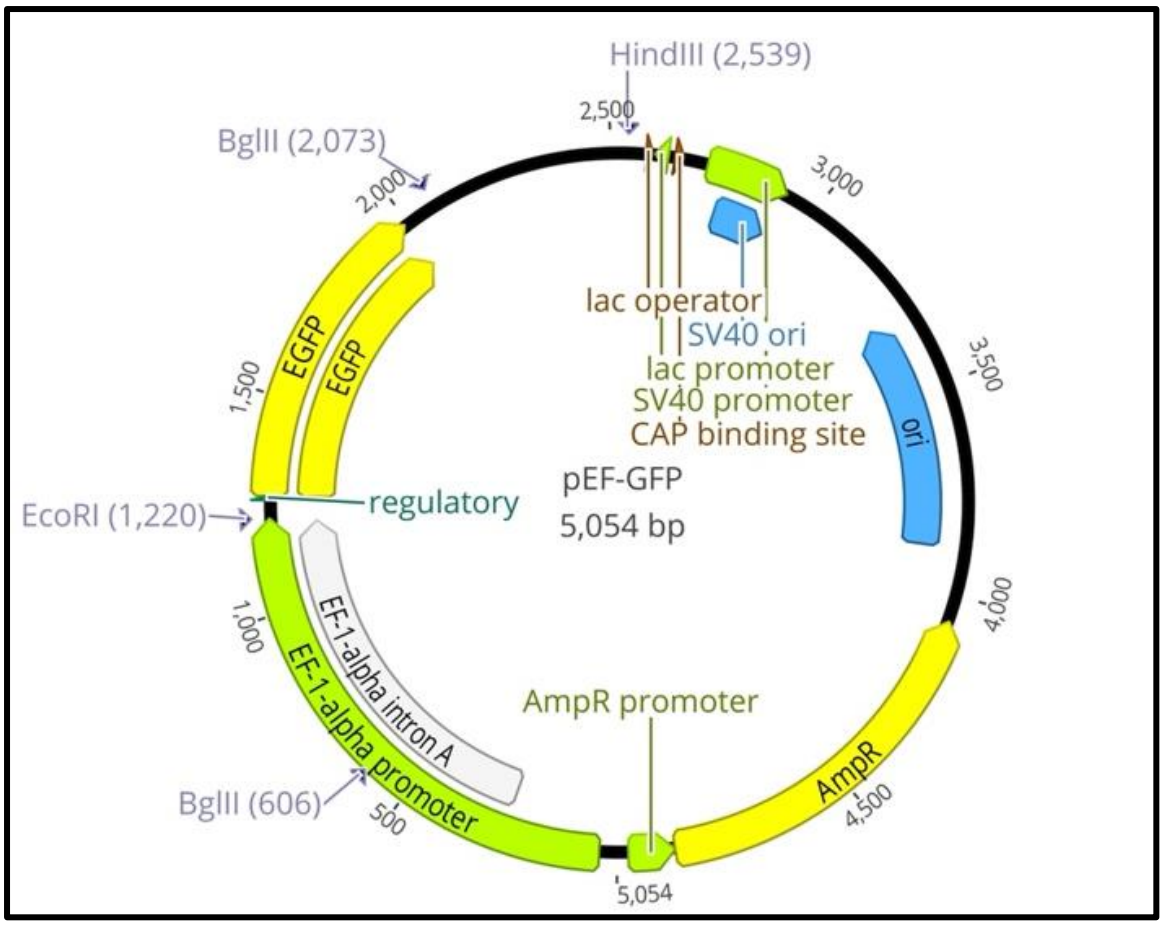

Figure 5 shows the pEF-GFP plasmid with the two genes (GFP and ampicillin resistance) and all the regulatory proteins. This figure also shows the cut sites for the three restriction enzymes that will be used in the restriction digest, EcoRI (1220), HindIII (2539) and BglII (606, 2073). Geneious version 2023.2 created by Biomatters. Available from https://www.geneious.com.

	EcoRI/ HindIII (R1) (1319bp & 3735bp)	**EcoRI (R2) (5054bp)**	**HindIII (R3) (5054bp)**	**BglII (R4) (1467bp & 3587bp)**	**Control (R5)**	**Final Concentration**
Plasmid DNA 200 ng/μL	5μL	5μL	5μL	5μL	5μL	1μg
10X Restriction Enzyme Buffer	1μL	1μL	1μL	1μL	1μL	1X
Restriction Enzyme 1 (10U/μL)	2μL	2μL	2μL	2μL		2U
Restriction Enzyme 2 (10U/μL)	2μL					2U
MQ Water		2μL	2μL	2μL	4μL	
Final Volume	10μL	10μL	10μL	10μL	10μL	

Table 4 The reaction set-up for the Restriction Digest of the pEF-GFP Plasmid using the three restriction enzymes EcoRI, HindIII, and BglII.

The centrifuge tubes were placed in the Eppendorf Thermomixer comfort overnight at 37°C with shaking at 600rpm. The next day, the tubes were spun (10 seconds) to bring all the liquid to the bottom of tube and mixed with 2μL 6X loading dye. A 10μL sample was then loaded onto a 1% Agarose (HydraGene HyAgraose™ LE, Multipurpose Agarose) 1X TAE gel supplemented with 0.5μg/mL thiazole orange (5μL). The gel was run at 100V for 30 min at RT against 10μL of the iNtRON SiZer™ 1000bp plus DNA Marker and was visualised using the iBrightFL1000 Invitrogen (ThermoFisher Scientific).

3.2.12 Generate a stable A549-GFP cell line

Geneticin (G418) (Gibco™, ThermoFisher Scientific, 10131027) is a selective antibiotic that is used to select and maintain cells with a particular characteristic (A549 cells that contain the pEF-GFP plasmid)[106]. Before G418 can be used to help establish a stable A549-GFP cell line after GFP transfection (***Method 3.2.11***), an optimal G418 concentration for A549 cells must be determined using a kill-curve.

Seeding A549 for G418 Kill-Curve

Cells that have been cultured in a T75 flask are used for seeding. The cells were washed, trypsinised, centrifuged, and resuspended in 5mL of A549 cell medium without Penicillin-Streptomycin as this will inhibit the G418. An automated cell counter (***Method 3.2.6***) was used to count the cells. The cells were then seeded into a treated flat-bottom 24-well plate with the appropriate cell number and extra media (to have the wells contain 0.5mL of media. The plate was incubated overnight at 37°C and 5% CO_2. The seeding of the cells is classed as Day 0.

Kill-Curve for determination of optimal G418 concentration

On Day 1 of the kill-curve assay the media was removed from the wells and the cells were then washed with warmed 1X PBS. The cells then had 450µL of fresh warmed media (without Penicillin-Streptomycin). Next, 50µL of G418 with the appropriate dilution (***Table 5***) was then added to the corresponding well. A master-mix was prepared for the each of the G418 dilutions (350µL for each dilution for the seven-day assay) and stored at 4°C.

The media and diluted antibiotic were changed on Day 3 and Day 5. The plate was inspected under the microscope at Days 3, 5, and 7 with photos taken on Day 3 and Day 7 to show the gradual cell death. This assay will determine which concentration will be the optimal dose for killing the cells without the desired plasmid.

0µg	0µg	300µg	300µg	700µg	700µg
50µg	50µg	400µg	400µg	800µg	800µg
100µg	100µg	500µg	500µg	900µg	900µg
200µg	200µg	600µg	600µg	1000µg	1000µg

Table 5 Layout of the antibiotic dilution and well set-up in the 24-well plate G418 kill-curve assay for the determination of the optimal dose needed for cell death of A549 cells that do not contain a plasmid.

3.2.13 Optimisation of the positive pEF-GFP into A549 cells

Two different lipofectamine reagents were investigated for optimal transfection efficiency; Lipofectamine 3000 (positive control[117]) and the new Lipofectamine CRISPRMAX. Based on data from Wightman (2018)[117], Lipofectamine 3000 had worked previously in our lab but unclear for the new Lipofectamine CRISPRMAX. The plasmid DNA from ***Table 14*** (***Results 3.3.4***) was used.

Seeding A549 cells

A549 cells were cultured as described in ***Chapter 3.2.2*** and then the cell confluency was confirmed (30 – 70%). Next, the cells were washed, trypsinised, pelleted (***Method 3.2.4***) and resuspended in 5mL of OPTI-MEM medium. The cells were then counted (***Method 3.2.6***) and seeded into the eight wells of the 24-well plate (***Table 6***) with the appropriate (40,000 – 200,000 cells/0.5mL) cell number and extra media (to have the wells contain 0.5mL of media) was added to each of the experimental wells. The cells were then incubated overnight at 37°C and 5% CO_2.

Lipofectamine 3000	Lipofectamine 3000		Negative Control	Negative Control	
CRISPRMAX 0.3µg	CRISPRMAX 0.3µg		CRISPRMAX 1µg	CRISPRMAX 1µg	

Table 6 The set-up of the 24-well plate for the optimisation of the pEF-GFP transfection.

GFP transfection – Positive Control (Lipofectamine 3000)

Table 7 outlines the kit reagent volumes used for the transfection of 1µg of plasmid using Lipofectamine 3000 (Invitrogen, ThermoFisher Scientific, L3000001).

Briefly, Tube 2 was prepared first followed by Tube 3 and then Tube 1. Next, a 1:1 ratio (25µL: 25µL) of Tube 1 was added to Tubes 2 and 3 with an incubation for 10 minutes at RT. Finally, 50µL of Tube 2 and 3 were added to the seeded cells in addition with the appropriate controls.

	Positive Control – Lipofectamine 3000		
Reagent	**Tube 1 – DNA**	**Tube 2 – Diluted Lipofectamine**	**Tube 3 – Diluted Lipofectamine**
OPTI-MEM	11.8µL	5.9µL	5.9µL
Lipofectamine		0.177µL	0.344µL
Lipofectamine P3000	0.72µL		
GFP (pEF-GFP plasmid) (0.212µg DNA)	2.36µL		

Table 7 Concentration and Volumes used for GFP transfection using Lipofectamine 3000 as a positive control

GFP transfection – Lipofectamine CRISPRMAX and Negative Control

Table 8 outlines the kit reagent volumes used for the transfection of two different concentration of plasmid and a negative control using Lipofectamine CRISPRMAX (Invitrogen, ThermoFisher Scientific, A36496). Tube 1 (0.371µg, 1µg, and the negative control) was prepared first. Tube 1 was then incubated for up to 30 minutes at RT. Tube 2 (0.371µg, 1µg, and the negative control) was then prepared. Tube 2 was incubated for 1 minute at RT. Tube 1 and Tube 2 were then combined and incubated for 10 minutes at RT. Finally, 50µL of the appropriate reagent was added to the appropriate well.

	Lipofectamine CRISPRMAX – 0.371ug DNA		**Lipofectamine CRISPRMAX – 1µg DNA**		**Negative Control**	
Tube	**Tube 1 – Cas9 Plus**	**Tube 2- CRISPRMAX**	**Tube 1 - Cas9 Plus**	**Tube 2- CRISPRMAX**	**Tube 1 - Cas9 Plus**	**Tube 2- CRISPRMAX**
Media	25µL	25µL	22.03µL	25µL	26.75µL	25µL
Lipofectamine Cas9 Plus	2.5µL	-	2.5µL	-	2.5µL	-
Lipofectamine CRISPRMAX	-	1.5µL	-	1.5µL	-	1.5µL
GFP (pEF-GFP plasmid) (0.212µg DNA)	1.75µL	-	4.72µL	-	-	-

Table 8 Concentration and Volumes used for GFP transfection using Lipofectamine CRISPRMAX and the negative control

The 24-well plate containing the two transfection reagents from ***Table 7*** and ***Table 8*** was then incubated at 37°C/5% CO_2. The plate was visualised under a UV light at zero, two, four, eight, 24, 48, and 72 hours.

The next step was to create a stable A549-GFP cell line so that these cells could be used for potential bioprinting. To do this, the transfected cells had to be exposed to the optimal dose of G418 that was determined previously in ***Method 3.2.12***.

Establishment of a stable A549-GFP cell line using G418

Using ***Table 7*** in ***Method 3.2.13*** three individual transfection wells and one negative control were set-up. Following the 72-hour incubation and visualisation of GFP, the media was removed, the cells were washed with warmed (37°C) 1X PBS, and then supplemented with 450µL of fresh warmed media (without Penicillin-Streptomycin). Next, 50µL of G418 with the appropriate dilution (***Method 3.2.12***) was then added to the corresponding well. A master-mix was prepared for the G418 dilutions (750µL in the 700µg/mL for the seven-day assay) and stored at 4°C. This is considered Day 1 of the G418 experiment. The media and diluted antibiotic were changed on Day 3 and Day 5. The plate was inspected for GFP signal under UV light at Days 3, 5, and 7 with photos taken on Day 3 and Day 7 to show the gradual cell death.

Lipofectamine 3000	Lipofectamine 3000	Lipofectamine 3000		Negative Control	

Table 9 The plate set-up for the GFP transfection for the establishment of a stable A549-GFP cell line using volumes reported in Table 6.

Single-cell serial dilution

The transfected A549 cells that were exposed to 700µg/mL of G418 for 7 days were used to prepare a 96-well plate (treated flat-bottom) with the purpose of selecting a single cell with the pEF-GFP plasmid. The media was removed and the cells were then washed and trypsinised. For the serial dilution there needs to be $2x10^4$ cells/mL. The supernatant was removed, and the cells were resuspended in 210µL of cell media, the cells were then counted

(***Method 3.2.6***). The 200µL of cell suspension was added to the first column of each row (GFP1 was added to A1, GFP2 added to B1 and so on). Next, 100µL of complete media was added to all the wells expect those in column 1, then 100µL of the cell suspension was transferred into column 2 and combined by pipetting up and down (being careful not to create bubbles). Then, 100µL of cell suspension from column 2 to column 3 and mixed. This process was continued all the way to column 12 and 100µL of the cell suspension from column 12 was discarded. With all of the well's volume being 100µL, 100µL of cell media was added to the wells so there was a final volume of 200µL. This process was repeated for all the rows of the plate. Incubate at 37°C/5% CO_2, changing the media as needed, maintain until the cells in column 12 are confluent. During cell media change visualise the cells under UV to observe the cells green fluorescing. Continue upsizing the cells, to establish a stable GFP cell line.

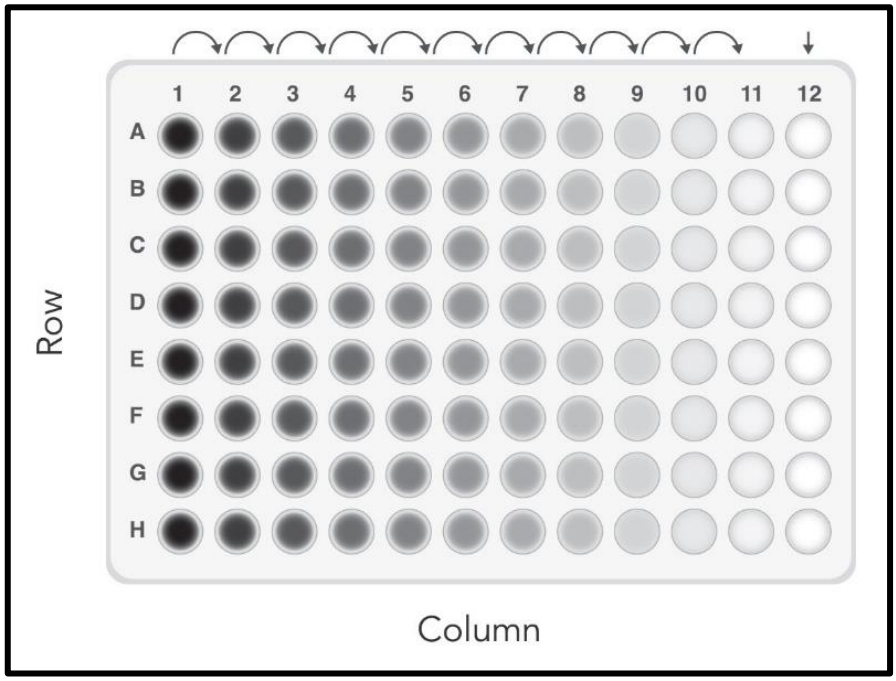

Figure 6 The experimental design of the serial single cell dilution[118]. Column 1 will have the highest number of cells. Every time the cells are added to the next well the number of cells decreases due to the dilution of cells to cell media. As the cells are diluted the final column, Column 12, should be left with only one cells, and that cell should if the G418 was functioning correctly have the pEF-GFP plasmid. The cells will then grow and divide clones that will also contain that plasmid and thus will establish a stable A549-GFP cell line that can be used for other experiments.

3.2.14 Bioprinting A549 cells

The ***Methods 3.2.14, 3.2.15,*** and ***3.2.16*** were sourced and modified from Sawyer (2021)[67], and Hardaker (2021)[29]. The whole cell extraction method (***Method 3.2.14***) was sourced from

G.Y Lee et al (2007)[94], and was modified to better suit the reagents and equipment that was available as well as the bioprinting technique and cell line that was used.

Preparation of the hydrogel

Sodium alginate (Allevi) ($C_6H_9NaO_7$, 0.3g) and Gelatine (Allevi) ($C_{102}H_{151}O_{39}N_{31}$, 0.4g) were weighed into plastic weigh boats and then sterilised under UV light (BLX-254 crosslinker, Life Technologies) at 3000J for 30 minutes. They were then added to 10mL of pre-warmed of complete medium in a 15mL centrifuge tube and gently mixed by hand till the mixture was as homogenous as possible. The centrifuge tube was placed into the rotor (MINI OVEN MK II) for 24 – 48 hours at speed 6 with the temperature remaining at 37°C to further mix the reagents. The solution was mixed once more. A sterile 3mL Pasteur pipette was used to mix and transfer liquids for bioprinting and *in vitro* tissue cultures unless stated otherwise.

Preparing mammalian cells for bioprinting

For cells to be used for bioprinting, the cells first must be ~90% confluent in the T75 cell culture. The cells media was removed, the cells were washed and the trypsinised. Cells were counted using ***Method 3.2.6***. The number of dead and alive cells were counted and recorded in an Excel spreadsheet made by Hardaker, (2021)[29]. The cell viability was calculated (***Equation 2***) to determine the volume of pre-warmed cell media and the cell suspension that is required to mix with 4mL of the hydrogel (made 24 – 48 hours in advance) to make a final bioink volume of 8mL.

$$\text{Cell viability (\%)} = 1 - \left(\frac{Dead\ cell\ count\ (average)}{(Dead\ cell\ count\ (average)) + (Live\ cell\ count\ (average))}\right) \text{x } 100$$

Equation 2 Determination of Cell viability.

Encapsulating A549 cancer cells

Four millilitres of the hydrogel (made in ***Method 3.2.14*)** was transferred to a capped 10mL Leuer slip syringe (HAPOOL MEDICAL TECHNOLOGY). The 4mL cell suspension containing 2 x 10^6 cells/mL was mixed into the hydrogel until the solution was consistent in texture. ***Equations 3 to 7*** were used to make bioink (shown below). To keep the plunger of the syringe sealed during incubation, it was pushed down until no air was left in the syringe without losing bioink liquid and snapped in place to prevent it from moving during

bioprinting. Then, the solution was incubated at 4°C for at least two hours in the upright position to form the bioink for the Allevi 2 Bioprinter. Following the two-hour incubation, the bioink was transferred into the biohood to adjust to RT (20°C). The Leuer slip cap was then replaced with a gauge needle (JG22-0.5HP, 22 Gauge 0.5", ID.016" OD.027"). Before connecting to the bioprinter extruder, the bioink was manually squeezed through to ensure no blockages were present and the flow was consistent.

$$\begin{aligned} &Cell\ suspension\ concentration\ (cells\ per\ mL) \\ &= Average\ live\ cells \times \left(\frac{Initial\ cell\ volume\ (10\mu L) + Trypan\ blue\ volume\ (10\mu L)}{Initial\ cell\ volume\ (10\mu L)}\right) \\ &\times\ 10000 \times Cell\ media\ volume\ resupension\ (2mL) \end{aligned}$$

Equation 3 Cell suspension concentration for desired bioink cell suspension

$$Cell\ suspension\ volume\ (x\ mL) = Bioink\ (8mL) \div \left(\frac{\left(Cell\ suspension\ concentration\ (cells\ per\ mL)\right)}{1{,}000{,}000}\right)$$

Equation 4: Cell suspension volume to make the bioink

$$Cell\ media\ volume\ (x\ mL) = \left(\frac{Bioink\ (8mL)}{2}\right) - Cell\ suspesion\ volume\ (xmL)$$

Equation 5 Cell media volume calculation for bioink

$$\begin{aligned} &Hydrogel\ Volume\ (mL) \\ &= Bioink(8mL) - Cell\ suspension\ volume\ (x\ mL) - Cell\ media\ volume\ (x\ mL) \end{aligned}$$

Equation 6 Hydrogel volume calculation to make the bioink

$$Bioink\ (8mL) = Cell\ Suspension\ (\ x\ mL) + Cell\ media\ (\ x\ mL) + Hydrogel\ (4mL)$$

Equation 7: Calculating the bioink component volumes

Printing using the Allevi 2 3D bioprinter

The Allevi 2 3D bioprinter (Allevi, 3D Systems) was connected to the air compressor (CALIFORNIA AIR TOOLS Ultra Quiet Air Compressor 2010A) with the pressure set to 50psi. The power was switched on followed by activation of the Raspberry Pi, which due to connectivity issues in E.3.13 was connected to an ethernet cable, and Allevi 2 power button. Next, connection of the Allevi 2 to a laptop, that was placed in a location close to the

bioprinter, was achieved by logging in to the Allevi website (https://bioprint.allevi3d.com/login). When the laptop is connected to the printer through the website it showed the printer settings that are adjusted to the printing parameters. The GCODE file was selected, along with the plate type (petri dish), extruder pressure (started at 15psi) and temperature (started at RT (20°C)). The extruder pressure and temperature could be variable during the bioprinting session(s) due to how the bioink flow changed. These adjustments were monitored during the bioprinting session to better analyse the process and that the changes made limited bioink waste. Extruder 2 was calibrated before selecting Extruder 1 and setting it into the appropriate printing position above the selected well of a six-well plate before pressing '*set calibration*'. To print the button '*Print*' was selected and the '*Cancel*' button was pressed to stop the printer once the desired size had been reached. To view the bioprint, the X/Y axis home button was selected (located beneath X (left) and Y (right) axis arrows) and the Z axis (plate holder) was moved down by 20 (see Allevi Bioprinter guide by Hardaker (2021)[29]. Next, the extruder was moved by 40 to the next well. This new X/Y position was set to home. The home button for the Z axis was pressed to return extruder 1 back to printing position to print the next bioprint. It is important to set this new printing position before selecting print or the printer will return to the previous position which can cause damage to the needle. The number of bioprints produced per session varied between 6 to 9. This difference was likely due to cell viability, temperature, bioink viscosity and/or the bioprinter. The changes in the volume of the bioink components may have affected how well the bioink would set during the two-hour incubation step at 4°C.

The bioprints were then soaked in 3mL (extra if needed to cover the entire bioprint) of 2% calcium chloride ($CaCl_2$) solution, this is required for the intermolecular cross-linking to take place between the alginate and hydrogel. The $CaCl_2$ solution is toxic to cells so was removed promptly from each well after 2 minutes so bioprints could be soaked with prewarmed 1X PBS. Three millilitres of 1X PBS were used, (plus extra if needed to cover the entire bioprint) for 2 minutes to rinse off any $CaCl_2$ remnants. The bioprints then had the 1X PBS solution removed and 3mL of prewarmed cell culture media was added (depending on the size of the bioprints extra cell media was also added if the bioprints were not fully covered). The bioprints were placed back into the incubator (37°C, 5% CO_2) until required for the selenium exposure assay (3-week incubation) or for bioprint cross-sectioning. Bioprint cell media was replaced every three to four days.

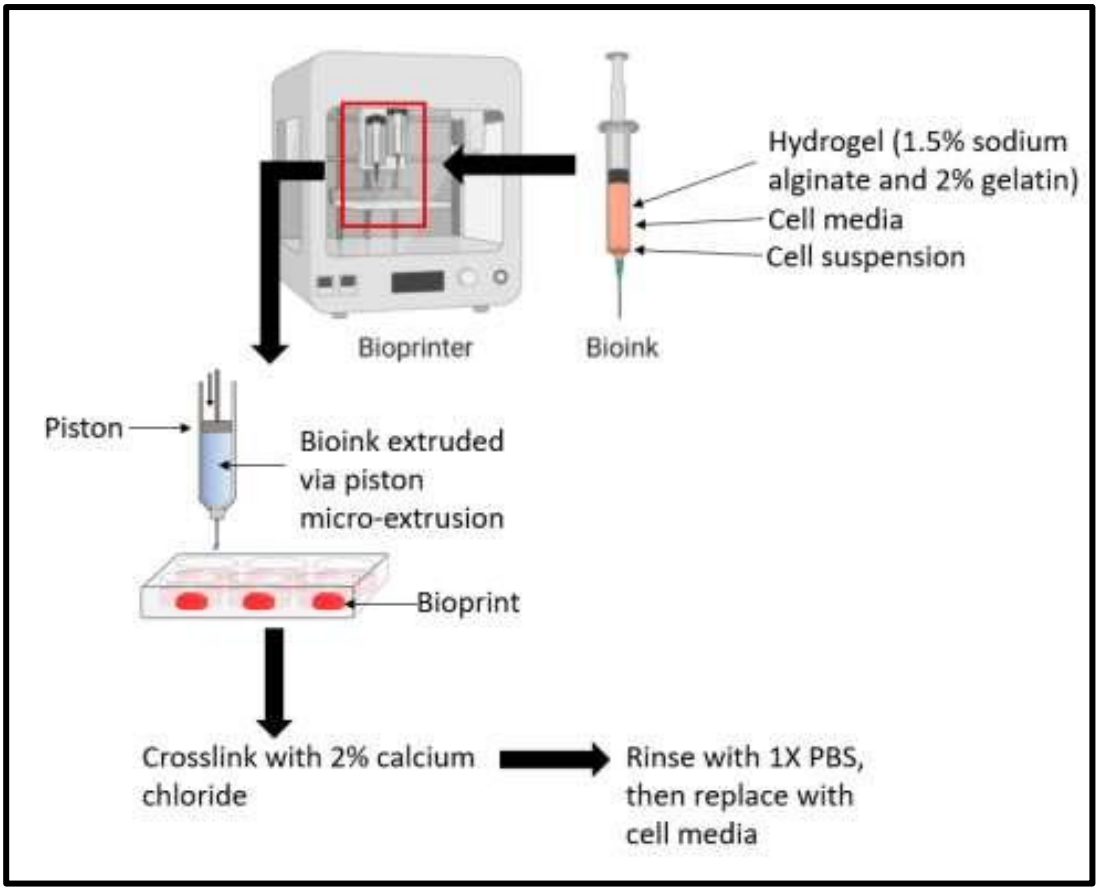

Figure 7 A schematic flow diagram of how the 3D bioprinting method is completed. The diagram was sourced from Sawyer[67].

Sawyer, 2021 RNA extraction from 3D bioprints

For the first bioprinting experiments and MSA drug exposures the focus was on extracting high-quality RNA, to do this the RNA extraction method used by Sawyer (2021)[67], was completed. After 21 days the cell media was removed from wells and the bioprints were placed into a screw cap tube (2mL) containing glass beads; three 2.5mm (BioSpec) and 100mg of 0.1mm (BioSpec) using a scoop cut out of a Pasteur pipette. TRI Reagent® (SIGMA-ALDRICH) was added (0.5mL) into each tube. The samples were then homogenised at 4800rpm for 3 cycles each 20 seconds long using Precellys 24 bead mixer. The samples were then incubated at RT for 5 minutes so that the nucleoprotein complex to dissociate. Samples were either be stored at -20°C until ready to process or processing took place after homogenisation, which saw 200µL of chloroform added to the tubes and shaken robustly (15 seconds) followed by a further mix using a rotating wheel (Labnet LABROLLER II) at RT for a further 3 minutes. Finally, the tubes were centrifuged at RT (12,000g, 15 minutes) to separate the aqueous phase (top) containing the RNA and the organic phase (bottom) containing the protein (The protein sample can be saved and stored at

-20°C). The aqueous phase was transferred to a new sterile tube. In the new tube with the aqueous phase, 125µL of 100% Isopropyl alcohol (IPA) was added to precipitate the RNA. The sample was mixed via hand inversion before adding 125µL of high salt solution (1.2M NaCl, 0.8M NaCit). The mixture was inverted several more times, then incubated at RT (5 minutes). To separate the phases again, the samples were centrifuged (12,000g, 15 minutes). The supernatant was removed, and the remaining RNA pellet was washed with 1mL of 70% EtOH to wash the precipitates followed by a centrifuging step at 12,000g for five minutes at RT. The supernatant was removed, and the RNA pellet was left to air dry in the fume hood. The RNA pellet was then resuspended in 30µL Diethylpyrocarbonate (DEPC) water and mixed by lightly vortexing. Each sample was measured for RNA quality using absorbance (A) ratios of 260/280nm and 260/230nm and concentration using a DeNovix DS-11 FX Spectrophotometer/Fluorometer (DeNovix) by using 1µL of each sample. The samples were stored at -80°C.

Whole Cell Extraction of encapsulated cells in 3D bioprints

This protocol was retrieved and adapted from G.Y Lee et al (2007)[94]. All of the below steps were performed on ice. To extract RNA and/or protein from cells that have been cultured in the 3D bioprints, the cells must be released from the hydrogel that they are encapsulated in by dissolving the hydrogel. Firstly, the cell media was removed from the bioprints and rinsed with 2mL of ice-cold 1X PBS. Secondly, 4mL of ice-cold 1X PBS-EDTA Protease Inhibitor Cocktail Solution (cOmplete™ ULTRA Tablets, *EASYpack* Protease Inhibitor Cocktail, Roche, Sigma Aldrich (05892970001) was added and incubated for 45 minutes at shaking at 70rev/min (Bibby Stuart GYRO-ROCKER STR9). Thirdly, bioprints and PBS supernatant from the 6-well plate were transferred into a 15mL centrifuge tube and further incubated for 30 minutes at 70rev/min. The tube was inspected to ensure that the hydrogel had dissolved (invert the tube gently to see a homogeneous solution of cell colonies), if not more PBS-EDTA was added and shaken for another 15 minutes. Next, the tubes were centrifuged for 5 minutes at 1100g at RT to form a pellet (pellet will be hard to see so take extra care), and remove supernatant. A final wash with PBS-EDTA was completed, the tubes were gently inverted, and centrifuged at 1100g for 5 minutes at RT.

RNA Extraction following whole cell extraction

After whole cell extraction the supernatant was removed, and the cell pellet was resuspended in 1mL of TRI Reagent®. The suspension was pipetted up and down several times to lyse the cells completely. Transfer the suspension into a sterile 1.5mL centrifuge tube and incubate at RT for 3 minutes. After the incubation the RNA samples followed Sawyer (2021)[67], RNA method with the addition of the 200µL of chloroform.

Protein Extraction following whole cell extraction

Following the ***Method 3.2.14 (whole cell extraction),*** the supernatant was removed, and the cell pellet was resuspended in 400µL of 1X cell lysis buffer and 1X protease inhibitor cocktail (cOmplete™ Ultra Tablets mini *EASYpack*, Roche, NZ, 05892970001) (***SA2***). Once the cell pellet had been resuspended, the samples were placed in the -80°C freezer overnight to allow the cells to lyse but prevent the degradation of the protein in the samples. After the incubation had been completed the samples were able to be used for fast Bradford assays, PAGE-gels, and western blotting.

3.2.15. Selenium Drug Exposure Assay

Methylselenic acid (MSA) was prepared using a filtered face mask, safety glasses, two layers of long-cuffed gloves, and a disposable lab coat as personal protective measures for working with a cytotoxic drug. MSA is corrosive reagent, that can cause acute toxicity, it is an environmental and health hazard[110]. MSA was made in a 2mL screw top lid microcentrifuge tubes. The 2mL 1M MSA final volume solution contained 0.41g of elemental Se. Further drug dilutions were (100µM and 10µM) prepared using 1X PBS solution. The solutions were aliquoted into 200µL volume of each dilution into new labelled tubes to avoid multiple freeze- thawing (screw top lid, 2mL). These were stored at -20°C.

Preparation of Se exposure study

Table 10 outlines the reagents and concentrations that were used for the Se exposure assay in the growth media supplemented with and without Se. The final volume of 3.5mL of growth media was used for each bioprint in each well of the six-well plate (the excess of 0.5mL to compensate for any potential pipetting error). For each treatment (2µM, 10µM, and Solvent Control (PBS)) replicates of two or three bioprints (*n*= 2 or 3) were carried out. A measurement of the width, length, and height of each bioprint was collected at the beginning

(*T*=0) and end (*T*=6 hr) of the six-hour exposure of Se. Based on previous MSc research the bioprints were exposed for the six-hours. After the exposure the bioprints then underwent the extraction methods in ***Methods 3.2.14***.

Stock Solutions	**MSA (µM)**		**Solvent Control**	**Final Concentration**
	2	**10**		
100% FBS	350µL	350µL	350µL	10%
100X Pen/Strep	35µL	35µL	35µL	1X
F-12 Media	3010µL	2730µL	2730µL	
100µM MSA	70µL	350µL		2 – 10µM
1X PBS			350µL	20µM
Final Volume	**3500µL**	**3500µL**	**3500µL**	

Table 10 Preparation of the growth media for a single well of the 6-well plate in the MSA (Se) drug exposure assay

3.2.16. Analyse of 3D Bioprints protein extracts

Estimation of protein concentration using the fast Bradford Assay

For a Bradford Assay a dye was prepared by mixing Dye Reagent Concentrate (10mL) with Milli-Q water (40mL) in a 50mL Falcon tube. When not in use it was stored at 4°C. Before use, the dye was inverted and warmed to RT (20°C). Next, the Bovine Serum Albumin (BSA) standard was prepared by first making up 10mg/mL of BSA (SIGMA-ALDRICH, A7906-100G) in 1mL of 1X PBS solution. This reagent was used for an 100µL dilution series (0 – 10mg/mL). The assay was run in a 96-well flat-bottom plate with 100µL of diluted dye reagent added to each well and 1µL of each protein sample/BSA standard. Three replicates were prepared. The plate was incubated at room temperature for five minutes. The protein concentration of each sample was estimated by linking the colour of the sample to the right BSA standard colour. For further accuracy, the plate was run through a plate reader (MultiSkan GO 1510-00337C) with the wavelength set to 595nm. A standard curve was generated using ScanIt software. A coefficient of determination (R^2) values between 0.8 to 1.0 was accepted.

Polyacrylamide gel electrophoresis (PAGE) of protein extracts

Protein samples were made to a final concentration of ~16 mg/mL in 50µL volume in 1.7mL boil proof microcentrifuge tubes (with graduations, VWR®) using 12.5µL of 4X

Laemmili dye and 1X PBS solution (if required). The protein samples were denatured at 95°C using the thermomixer for five minutes, then transferred to ice before loading onto the commercial gel (10% Mini-PROTEAN TGX Stain-Free™, 10-well 30µL precast gels, Bio-Rad, 456-8035).

Commercial gels were removed from the package and rinsed with double distilled water (ddH2O) to remove any air bubbles and the green tape was removed from the base of the gel. Gels were attached to an electrophoresis tank with 1X Tris-Glycine SDS (TGS) Running Buffer (***SA1***) added afterwards. Five microlitres of protein ladder (PAGE MASTER Protein Standard Plus, GenScript, MM1397-500) or 5µL of western blot ladder (WB MASTER Protein Standard, GenScript, M00521) alongside 20µL of denatured protein samples were loaded into the separate gel wells and underwent electrophoresis at 200V, 100mA for an 45 minutes. Gels were removed from the electrophoresis tank, separated from the plastic plates, rinsed with ddH2O, then stained using the Invitrogen Simply Blue™ Safe Stain (ThermoFisher Scientific, LC6060) for a minimum of 1 hour at RT while gently shaking on a plate mixer (ORBITAL SHAKER, BELLCO GLASS, Inc) (If staining was done a new gel was run for western blots ***Method 3.2.16***). After staining the gel was washed with ddH2O on the plate mixer overnight. Images of gels were taken using an iBrightFL1000 Invitrogen (ThermoFisher Scientific) under white light.

Western blot

Once the protein concentration had been determined and visualised on a stained PAGE-Gel, another PAGE-Gel was run (with any optimisations needed). A PVDF membrane (Immobilon-FL, IPFL00010) was cut to the appropriate gel size and was then activated with methanol for 15 seconds, transferred to a clean tray where the membrane was covered in eBlot™ Equilibration Buffer. Using the eBlot™ Protein Transfer apparatus and a packet containing eBlot™ Protein Transfer Pads (anode and cathode pads) these were assembled according to the manufacturer. The protein transfer was set for seven minutes. Protein transfer was visualised by staining the membrane with Ponceau stain for five minutes, followed by rinsing in ddH2O. The membrane was blocked in 10% blocking solution (non-fat milk in 1X TBST) at RT for 1 hour on a plate shaker (ORBITAL SHAKER, BELLCO GLASS, Inc), washed with 1X TBS and antibodies were applied (***Table 11*).**

Antibody	Host Species	Dilution Used	Predicted Molecular Weight	Catalogue Number	Manufacturer
Anti-HSP70	Rabbit	1:1000	70kDa	ab79852	Abcam
Anti-GPX1	Rabbit	1:1000 – 1:5000	22kDa	ab108427	Abcam
Anti-GAPDH	Rabbit	1:2000	36kDa	A92899	Antibodies
Anti-TUBA4A	Rabbit	1:500	55kDa	A17304	Antibodies
Anti-TP53	Rabbit	1:500	53kDa	A167714	Antibodies
Secondary Antibody – Goat anti-Rabbit IgG [HRP]	Goat Anti-Rabbit IgG	1:5000	-	ab97051	Abcam

Table 11: The primary and secondary antibodies that were used throughout the research as well as their host species, dilution used, predicted molecular weight and catalogue number for future uses.

Following exposure of secondary antibody, the membrane was washed twice in 1X TBST for three minutes each and then protein signal was developed using the Supersignal™ West Pico Plus (ThermoFisher Scientific, 34577) and incubated in the dark for 10 minutes. The excess reagent was removed, and the membrane was visualised under the chemi blots setting of the iBright imager. Membranes were stored at 4°C and/or re-used after stripping with either mild stripping buffer.

Mild stripping western blot

The membrane was covered using a mild stripping buffer and left for 10 minutes at RT. Following this, the buffer was removed and replaced with the same mild stripping buffer solution enough to cover the membrane/s for a further 10 minutes. The buffer was removed and replaced with three 10 minute washes in 1X TBS, which were then followed by two 5 minute washes in 1X TBST. The membrane could then be re-blocked, and the western blot protocol can be continued.

3.3 RESULTS

3.3.2 Mini-Prep extraction of plasmid DNA from transformed *E. coli*

Table 12 shows the concentration and quality of plasmid DNA that has been extracted from four samples using the mini-prep plasmid extraction (***Method 3.2.9***). The plasmid DNA concentration ranged from 56ng/μL to 98ng/μL with an average of 70.5ng/μL. These results are lower than the kits expected yield of up to 35μg of high quality plasmid DNA. The 260/230 (a range between 2.0 – 2.2 is generally accepted) and 260/280 (a range between 1.8 – 2.0 is generally accepted) are higher than recommended, except sample 3 which is within those ranges. Thus, the plasmid DNA was re-purified and concentrated (***Method 3.2.10***).

Sample	DNA (ng/μL)	A260	260/230	260/280
1	59.152	1.183	2.430	2.163
2	56.651	1.133	2.318	2.143
3	98.246	1.9649	1.947	2.072
4	69.039	1.3808	2.190	2.062

Table 12 Spectrophotometer results of plasmid DNA extracted using mini-prep kit repeat experiment with optimised condition

Table 13 shows the second plasmid DNA extractions concentration and quality of plasmid DNA that has been extracted from six samples using the mini-prep plasmid extraction (***Method 3.2.9***). The plasmid DNA extraction was repeated to increase the volume of purified plasmid that was in stock for the generation of a stable A549-GFP cell line. The plasmid DNA concentration ranged from 83ng/μL to 94ng/μL with an average of 89ng/μL. This is too low for the future transfection experiments as 1μg of DNA total is recommended. The 260/230 (a range between 2.0 – 2.2 is generally accepted) and 260/280 (a range between 1.8 – 2.0 is generally accepted) are lower than recommended. Thus, the plasmid DNA was re-purified and concentrated (***Method 3.2.10***).

Sample	DNA (ng/μL)	A260	260/230	260/280
1	94.481	1.8896	1.292	1.446
2	89.176	1.7835	1.556	1.435
3	92.796	1.8559	1.616	1.463
4	87.859	1.7572	1.507	1.437
5	83.042	1.6608	1.541	1.427
6	90.594	1.8119	1.577	1.457

Table 13 Spectrophotometer results of plasmid DNA extracted using mini-prep kit repeat experiment with optimised condition

3.3.4 DNA purification and concentration of the extracted plasmid DNA

Table 14 shows the results from the "Zymo Research DNA Clean and Concentrator™ – 5" (Zymo Research, D4013). Three plasmid DNA samples were combined to increase the DNA concentration of the final sample. The plasmid DNA concentration increased to 212ng/μL. The 260/230 from the original extraction seen in ***Table 12*** are close to the 2.0 – 2.2 range and the 260/280 ratio is now within the range with values of 1.8. However, the concentration is still low for transfection which requires a total DNA concentration of around 1μg, due to this the plasmid DNA volume for the transfection will be increased.

Sample	DNA (ng/μL)	A260	260/230	260/280
1 (1+2+4)	212.305	4.2461	1.977	1.858

Table 14 Spectrophotometer results of plasmid DNA that was purified and concentrated from the first extraction

Table 15 shows the results from the "Zymo Research DNA Clean and Concentrator™ – 5" (Zymo Research, D4013). Two plasmid DNA samples were combined to increase the DNA concentration of the final sample. The plasmid DNA concentration increased to a range of 109ng/μL – 124ng/μL. The 260/230 from the original extraction seen in ***Table 13*** are still low but the 260/280 ratio are now within the range with values of 1.8.

Sample	DNA (ng/μL)	A260	260/230	260/280
1 (1+2)	109.471	2.1894	1.809	1.823
2 (2+3)	112.245	2.2449	1.627	1.813
3 (5+6)	124.593	2.4919	1.666	1.821

Table 15 Spectrophotometer results of plasmid DNA that was purified and concentrated

3.3.5 Restriction Digest of the extracted plasmid DNA

Figure 8 shows the gel electrophoresis of four restriction digests of the plasmid DNA compared to a negative control (-ve, no restriction enzyme). Three restriction enzymes EcoRI (E), HindIII (H) and BglII (B). EcoRI and HindIII (EH) were used together and separately to determine that the plasmid was the correct size. The double digest (EH lane) shows in complete digestion with three bands observed at 5054bp, 4000bp and 1500bp instead of two expected band sizes of 1319bp and 3735bp. For the individual EcoRI and HindIII lanes (E and H) a single linearised bright band is visualised at the expected size, around 5000bp. A third restriction enzyme was used, this being BglII. BglII makes two cuts on the plasmid creating two bands at 3587bp and 1467bp which can be seen on the gel. In conclusion, the restriction digest data supports that the correct plasmid has been sourced for the future transfection studies.

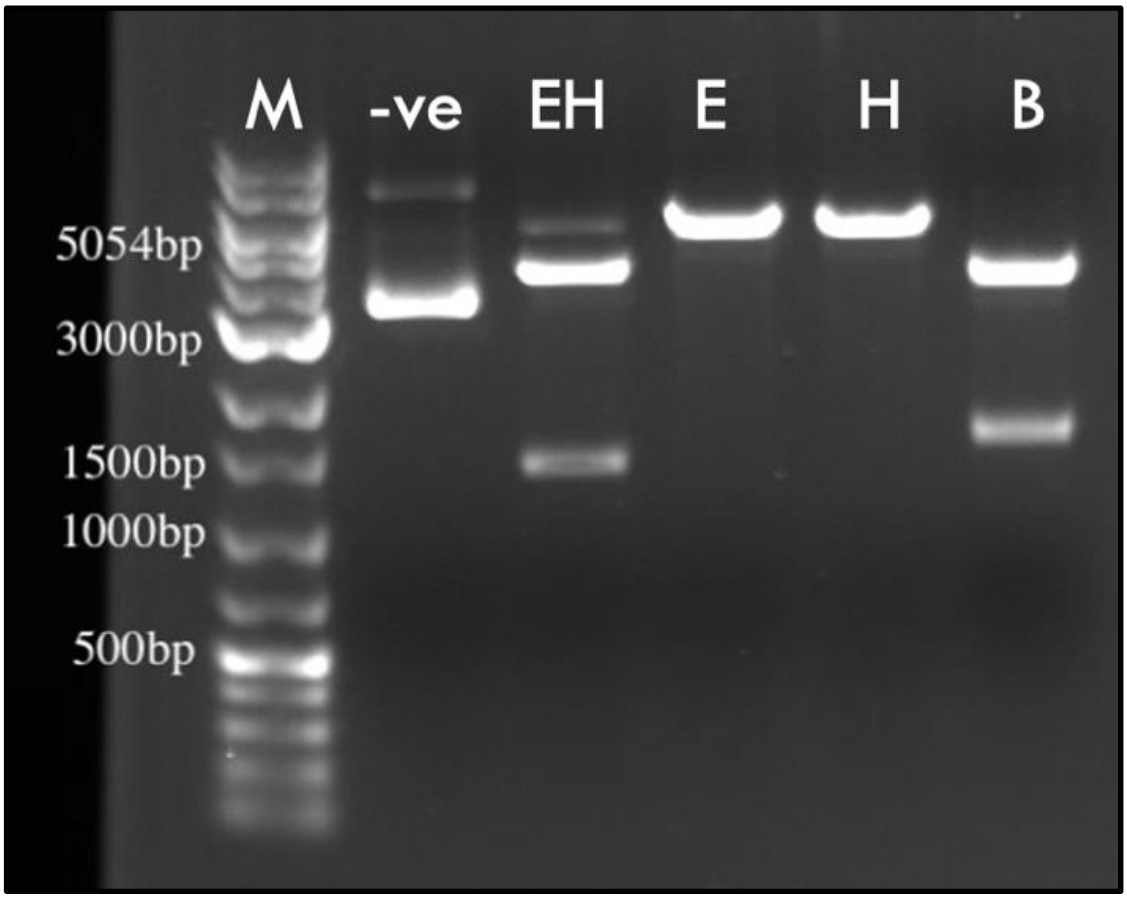

Figure 8 Agarose Gel Electrophoresis of the Restriction Digest of the pEF-GFP plasmid. The lane labelled M is the ladder (iNtRON SiZer™ 1000bp plus DNA Marker (24075)). -ve lane = is the negative control (plasmid DNA with no restriction enzyme). EH lane = plasmid DNA with EcoRI and HindIII restriction enzymes. E lane = plasmid DNA with the EcoRI restriction enzyme. H lane = plasmid DNA with the HindIII restriction enzyme. B lane = plasmid DNA with the BglII restriction enzyme.

3.3.6 Optimal Geneticin Kill-Curve Assay

Figure 9 shows the gradual cell death on Day 3 and Day 7 of the G418 kill-curve assay on un-transfected A549 cells to determine the optimal dose of G418 of three different concentrations (600, 700, and 800μg/mL) (other concentrations can be seen in ***SA1***). The optimal dose for geneticin is the dose with the lowest antibiotic concentration at which all the cells were dead in the wells of the treated flat-bottom 24-well plate within the 7 days of the assay. From this kill-curve assay it was determined that 700μg/mL is the optimal dose.

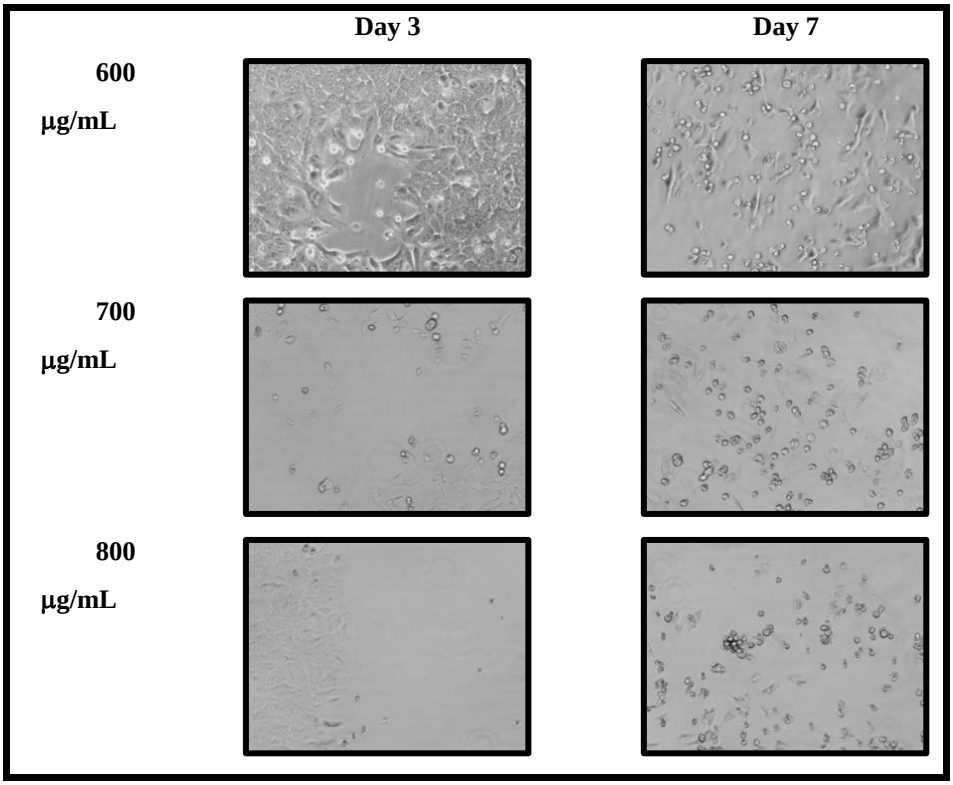

Figure 9 Optimal Kill-curve using geneticin. Photos of geneticin-treated A549 cells after day 3 and day 7 at various antibiotic concentration (0-1000μg/mL) were taken at 10X magnification on the Nikon Coolpix 4500 camera with the Nikon Eclipse TS100 microscope.

3.3.7 Transfection of pEF-GFP plasmid

Figure 10 show the A549 cells that have been transfected with Lipofectamine 3000 (1μg of pEF-GFP plasmid DNA) and CRISPRMAX (0.3μg and 1μg of pEF-GFP plasmid DNA). Photos were taken 72 hours after transfection. The negative control showed no green fluorescence under UV light compared to the GFP samples (data not shown).

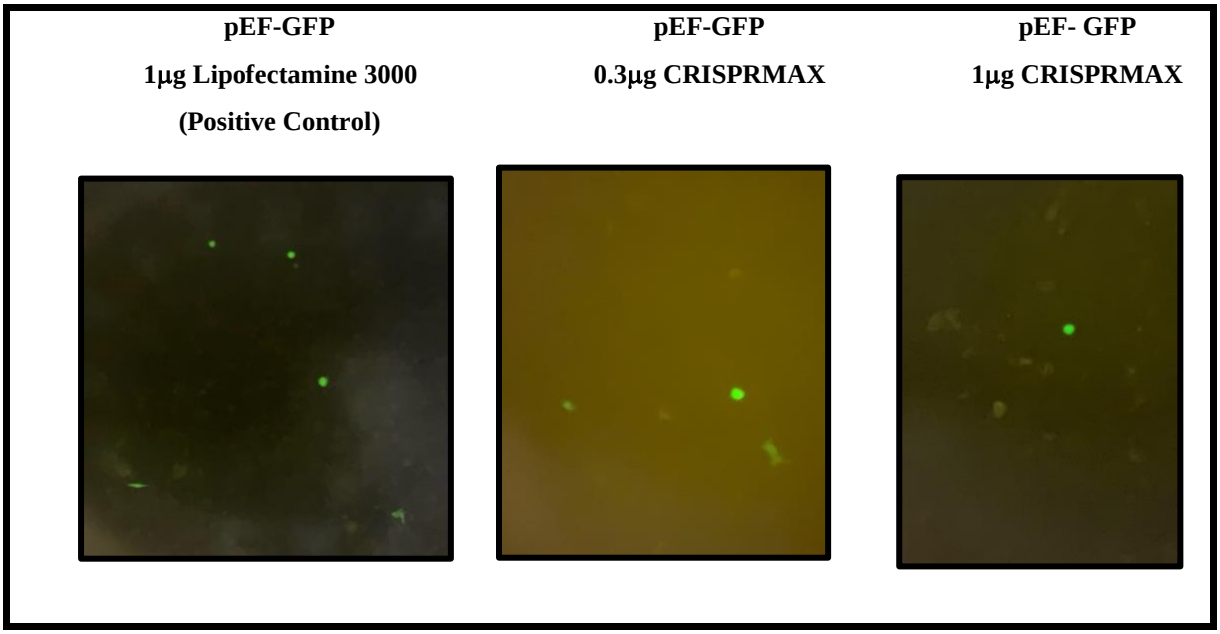

Figure 10 Transfected A549-pEF-GFP cells for both experimental conditions. Visualisation of successful transfection of the pEF-GFP plasmid (0.3µg and 1µg) in to the A549 cell line using the positive control Lipofectamine 3000 and CRISPRMAX after 72 hours of incubation.

3.3.8 Bioprinting

Figure 11 shows the morphology of the A549 cells before and when encapsulated in the hydrogel that was made in ***Method 3.2.14***. A549 bioprints were cultured for a minimum of 21 days before any exposure assays or RNA/protein extractions were undergone. Cells grown in 2D cultures have a stretched epithelial-like morphology compared to the 3D cells that have a pebble-like morphology.

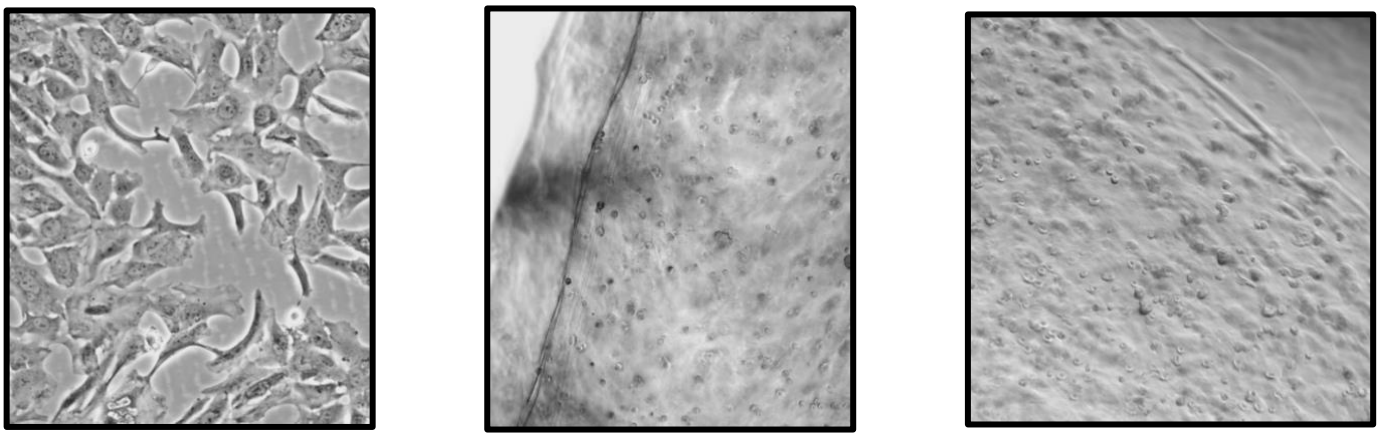

Figure 11 2D vs 3D A549 Cells. Images of three representative A549 bioprints. 2D (left) images taken at 10x magnification and 3D (middle and right) images taken at 4X magnification on the Nikon Coolpix 4500 camera with the Nikon Eclipse TS100 microscope. Left taken before cells were seeded for bioprinting. Middle was taken on Day Zero. Right taken on Day 21.

Protein extraction using whole cell extraction

A new protein extraction protocol was investigated to improve the solubility and folding so that a 70kDa band would be observed on a PAGE gel upon western blot analysis using the HSP70 antibody. This antibody was readily available in our lab for use. The new protein extraction protocol directly removed the cells from the hydrogel and lysed these cells using a cell lysis buffer with protein extraction buffer. Unlike the old method where RNA and protein could be extracted at the same time, only protein or RNA could be extracted at one time.

Figure 12 shows the identification of a 70kDa protein band from four protein samples via a western blot with 1:1000 HSP70 primary antibody. The first two (6mg/mL) protein samples were extracted from A549 cells grown as a monolayer (2D cell culture, non-bioprinted (nBP1 and nBP2)) as a positive control. The next two samples were two 3D Bioprinted cultures (BP1 and BP2). In conclusion, the data suggests that the protein from 3D bioprint is correctly folded for antibody recognition.

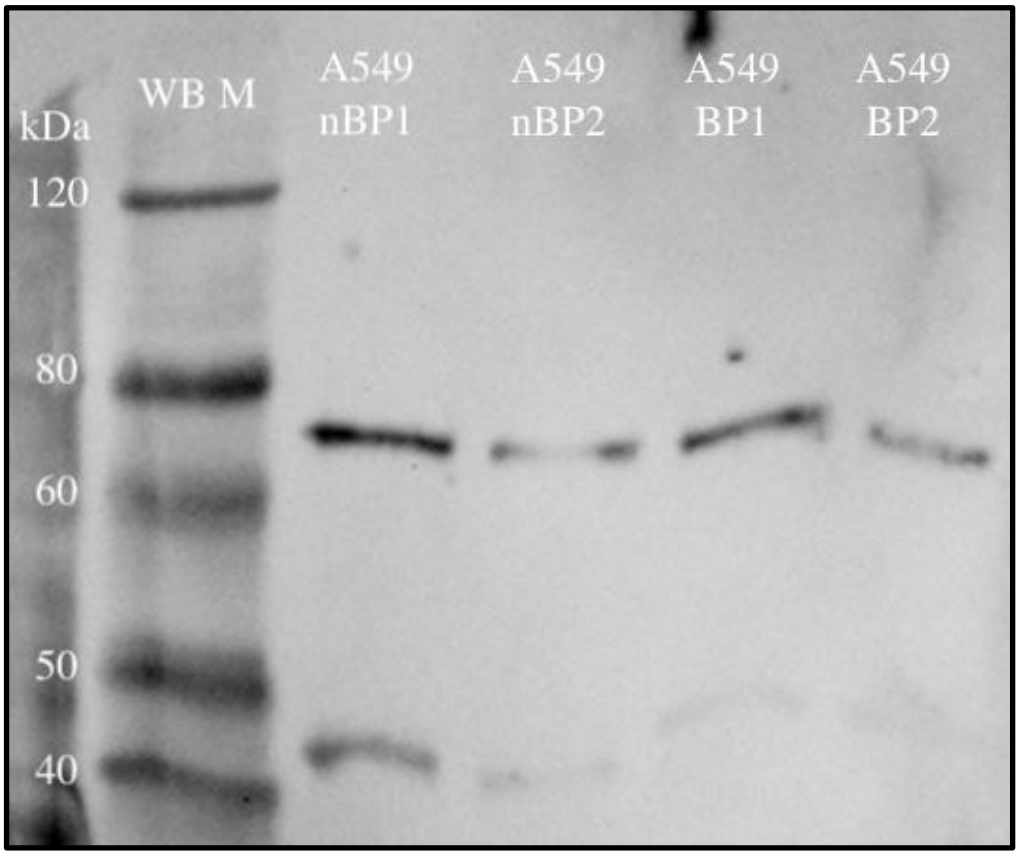

Figure 12 Western Blot using HSP70 1° Antibody. The protein suspension from a 2D or 3D A549 cells was run on the PAGE-Gel for 45 minutes at 200V. The protein concentration was at 6mg/mL.

The next step was to investigate potential primary antibodies that could be used for the future western blots using 3D bioprints that have been exposed to two different MSA concentration and the PBS control. Protein function and RNA expression levels were considered. The potential antibody candidates are shown in ***Table 16***. Using the online Human Protein Atlas database, the A549 cell line RNA expression is measured in nTPM (transcripts per million) and ranged from 100.6 to 931.8 for target genes of interest.

Protein	**Antibody**	**Purpose**	**A459 RNA Expression (nTPM)[120]**	**Selenium Related**	**Hypothesis (Protein Expression – Upregulation, Downregulation)**
Glutathione Peroxidase 1	GPX1	Oxidative Stress	162.0	Yes	Upregulation[66]
Heat shock protein 70	HSP70	Stress response	313.1	No	Upregulation[138]
Tumour Suppressor protein 53	TP53	DNA Damage	100.6	No	Upregulation[136]
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Housekeeping	8134.6	No	No change
Tubulin Alpha 4A	TUBA4A	Housekeeping	254.2	No	No Change

Table 16 Potential primary antibodies for 3D bioprint western blots.

MSA (Selenium) Exposure Assay - whole cell protein extraction method

Bradford Assay protein concentration

Next, 3D bioprints were printed (n=6 or 9) and grown for 21 days. Then, they underwent a six-hour MSA exposure assay with 2 or 10μM MSA or 1X PBS control (n=2 or 3). PBS was used as the control as the MSA powder was dissolved in PBS. There were three independent exposure studies performed on three separate dates: BP1, BP2 and BP3 in duplicate or triplicate. Thus, a total of 27 bioprints were analysed for protein content via the Bradford Assay and expression via a western blot. The Bradford assay showed a concentration range from 0.1mg/mL to 3.42mg/mL (***SA1***).

Figure 13 shows the average total protein concentration (mg/mL) for each experimental replicate. BP1 and BP3 have a trend that shows that the highest average protein concentration is seen in the PBS samples and the lowest concentration is seen in the MSA 10μM samples. BP2 does not follow this trend with the highest being the MSA 2μM samples and the lowest being the PBS samples.

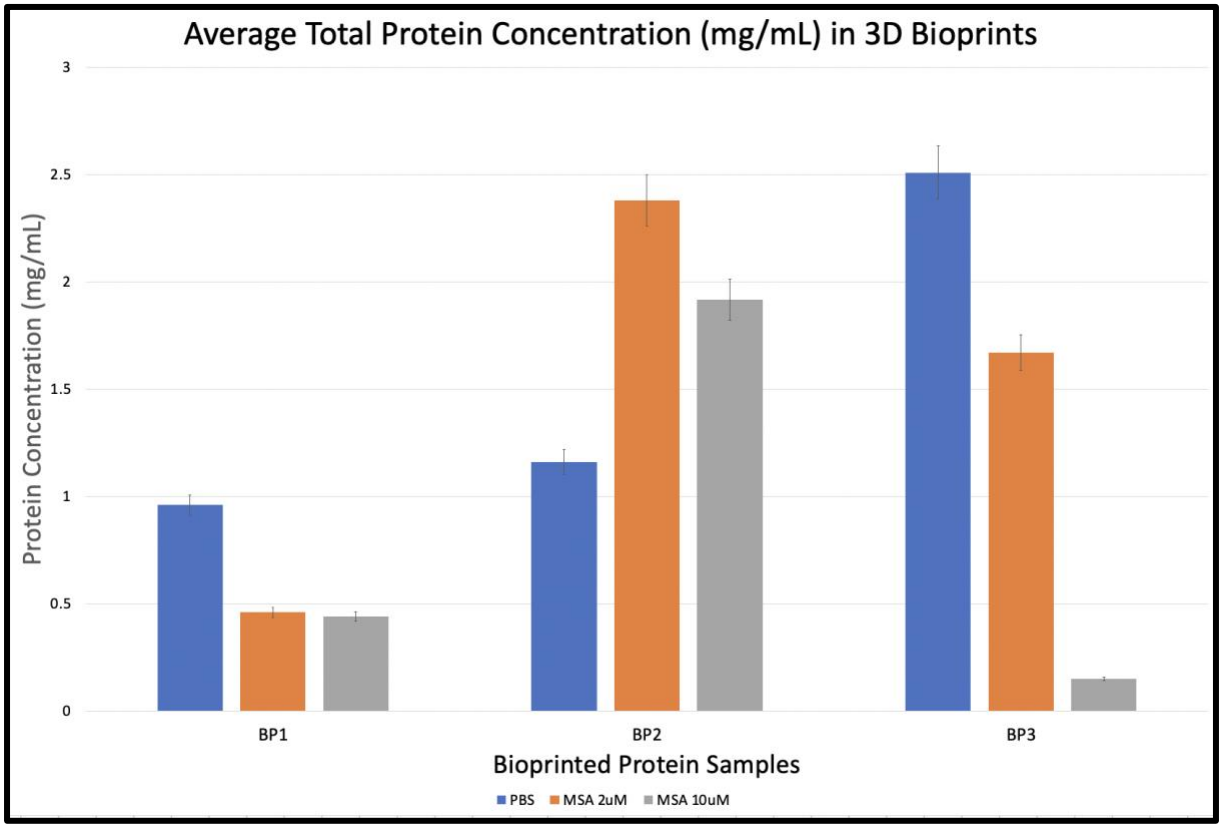

Figure 13 Bradford Assay of the average total protein concentration (mg/mL) extracted from A549 3D Bioprints for each bioprint and experiment condition, PBS (blue), MSA 2μM (orange), and MSA 10μM (grey). BP1 n=2. BP2 and BP3 n=3

PAGE-Gels

Figure 14 shows a representative Coomassie blue staining of eight electrophoresis protein suspensions extracted from the first exposure experiment: Bioprint 1 (BP1). The PAGE gels confirmed a range of proteins were visualised from 120kDa to less than 10kDa. The gel also showed that protein concentration had to be optimised for equal loading for western blot analyses.

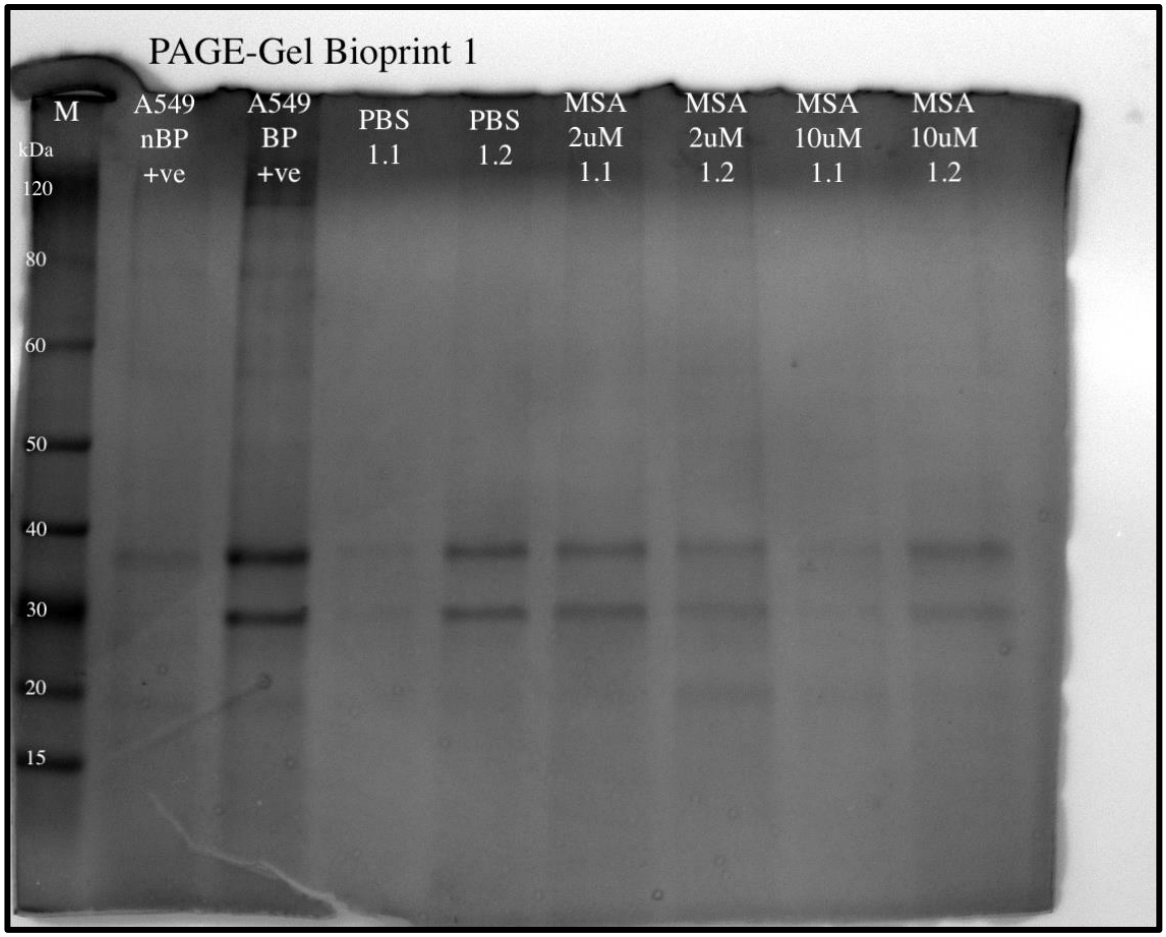

Figure 14 3D Bioprint PAGE-Gel with the samples from Bioprint 1. M = GenScript PAGE-MASTER Protein standard plus (MM1397-500). A549 nBP +ve = 2D A549 protein positive control. A549 BP +ve = 3D A549 protein positive control. PBS 1.1 and 1.2 = PBS control for the MSA drug exposure. MSA 2μM 1.1 and 1.2 = the first MSA concentration used for the drug exposure. MSA 10μM 1.1 and 1.2 = the second MSA concentration used in the drug exposure.

Western blotting

Figure 15 shows the shows the western blot data of three bioprinting experiments; BP1, BP2, and BP3 for each respective antibody. Each 3D bioprint was exposed to 1X PBS, 2μM or 10μM MSA for six-hours. All three western membranes were then exposed to the same primary antibody (HSP70, followed by GAPDH as a loading control). Two positive controls were included. Firstly, protein extracted from 2D monolayer of A549 cells (Lane 1; A549 nBP Positive) and protein previously extracted from a 3D bioprint and confirmed by western blot analysis (Lane 2; A549 BP Positive). The two positive controls show signal at the expected molecular weight but with differing intensities. The GADPH antibody also shows unequal protein loading to visually see if there has been any change in protein expression with respect to MSA treatment.

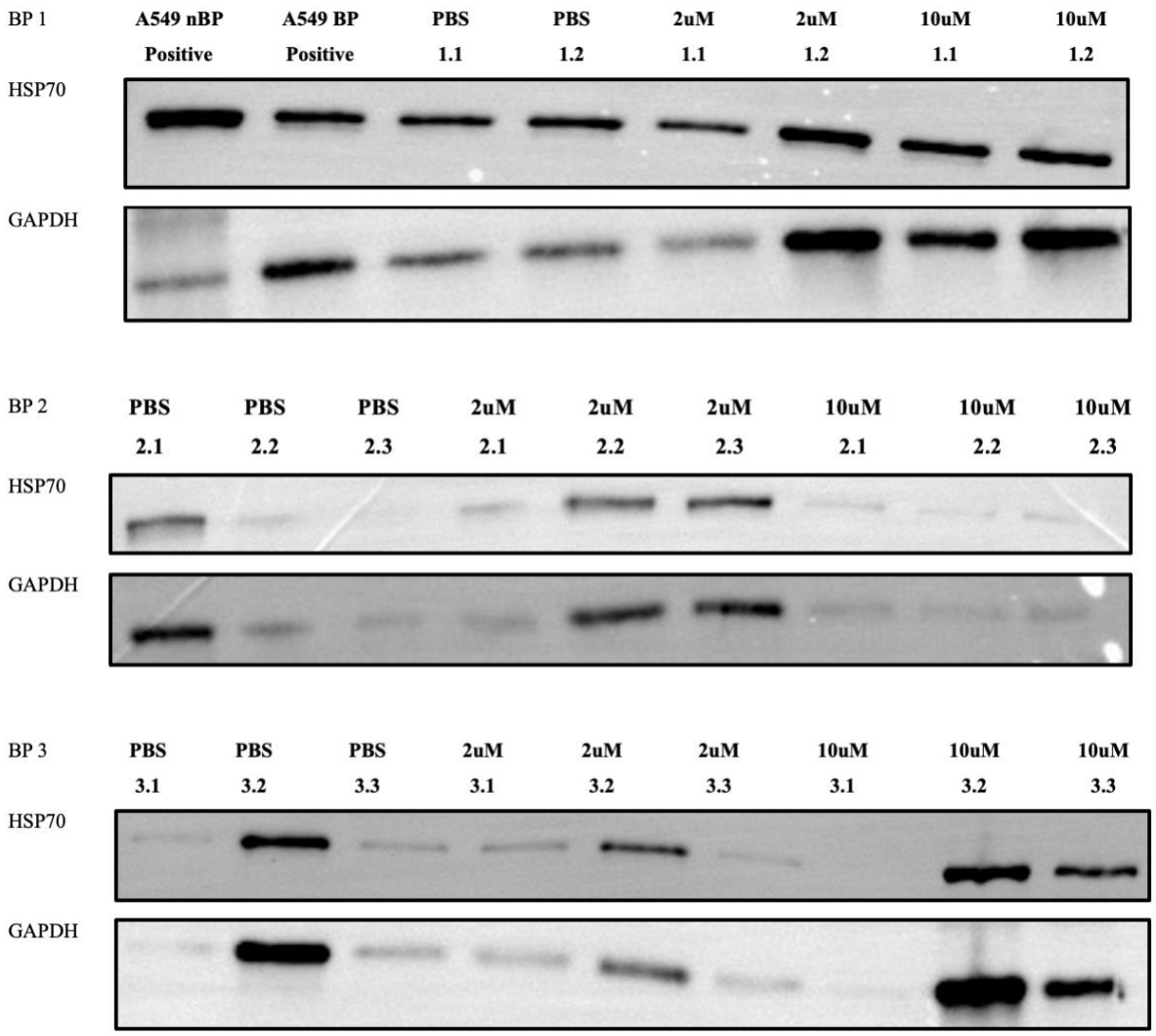

Figure 15 Western blot images of the three PVDF membranes that have been probed with HSP70 or GAPDH primary antibody. BP = Bioprint experiment. Bioprints were exposed to PBS, 2 or 10uM MSA for 6 hrs and then protein was extracted.

Thus, a quantification approach was investigated using the iBrightFL1000 Invitrogen "analysis" feature to determine the pixel intensity of each band.

Figure 16 shows the precentage change in the protein expression of the 3D bioprinted cells. Each protein was normalised againist average PBS GAPDH sample of each bioprint. In the HSP70 protein expression four of the samples had a downregulation in the protein expression when compared to the reference samples (average PBS GAPDH sample of each bioprint). The protein downregulation ranged from -5% – -56%. Two of the samples showed upregulation of the HSP70 protein by 6% and 11%.

Figure 16 Average percentage change in protein expression change of the drug-treated (2μM and 10μM) samples for each bioprint. MSA 2μM (orange), and MSA 10μM (grey). BP1 n=2, BP2 and BP3 n=3.

Figure 17 shows the averaged HSP70 protein expression change (%) of all the experiments conditions from all the bioprints. The average protein expression has been downregulated in both experiment conditions with -22% and -17%.

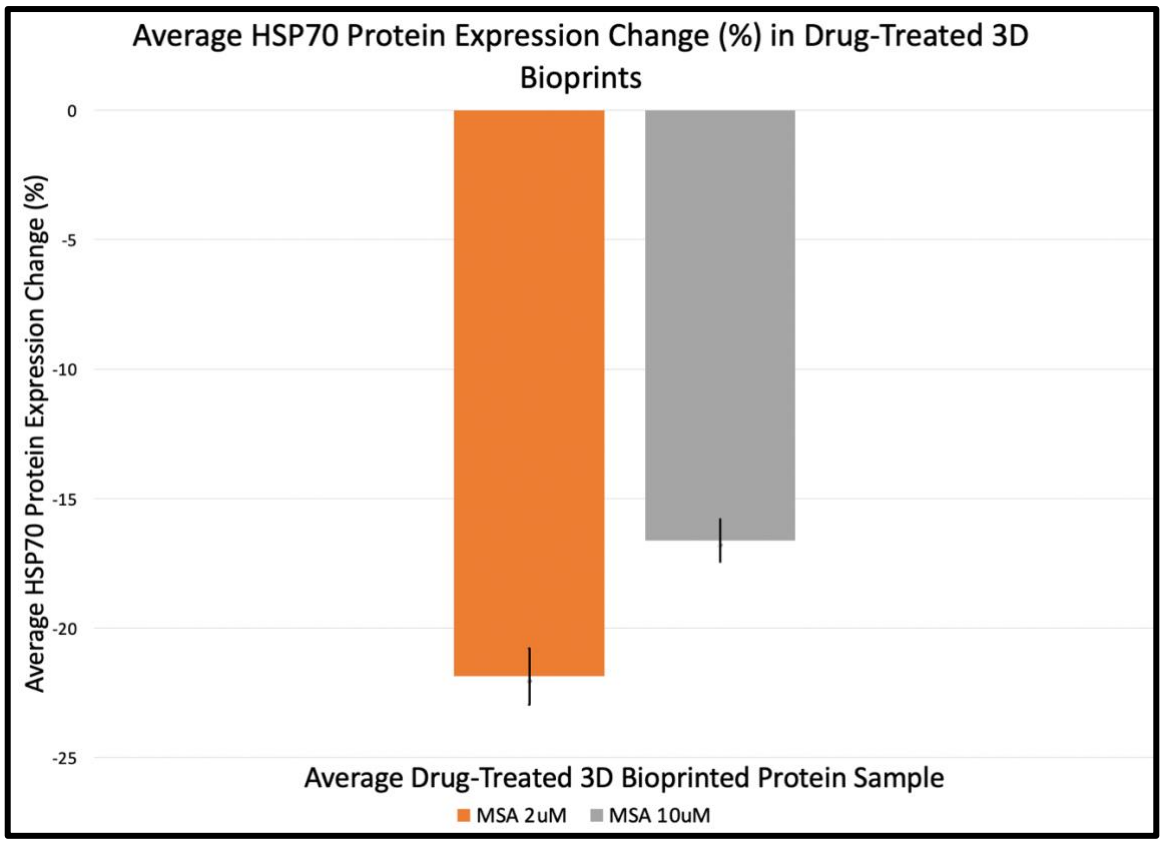

Figure 17 shows the averaged HSP70 Protein Expression Change (%) of all the experiment conditions from all three bioprinting experiments.

RNA Extraction – Whole Cell Extraction Method

Table 17 shows the RNA concentration and quality that was extracted from two individual bioprints using the new whole cell extraction method. The RNA concentration of sample 1 was 61 ng/µL and 94 ng/µL from sample 2. The 260/280 should that the RNA was of high quality with both samples having values at 1.9, falling within the 1.8 – 2.0 guideline.

3D Bioprint Sample	RNA concentration (ng/µL)	A260	260/230	260/280
1	61.147	1.5287	0.394	1.973
2	94.623	2.3656	0.400	1.909

Table 17 The DeNovix results of the RNA that was extracted from two 3D bioprints.

RNA MSA (Selenium) Exposure Assay – Sawyer[67] Method

Figure 18 shows the average total concentration of RNA (µg/µL) each experimental conditions replicate. As RNA was resuspended in different volumes due to different pellet

sizes therefore, the RNA is the total average concentration of the samples. Data shows variability between the bioprinting experiments (BP1 – 3) but less variability within each biological replicate (n=2 for BP1, n=3 for BP2 and BP3). The total RNA concentration for each sample, the RNA concentration (ng/μL), and the quality of the samples was measured well (***SA1***).

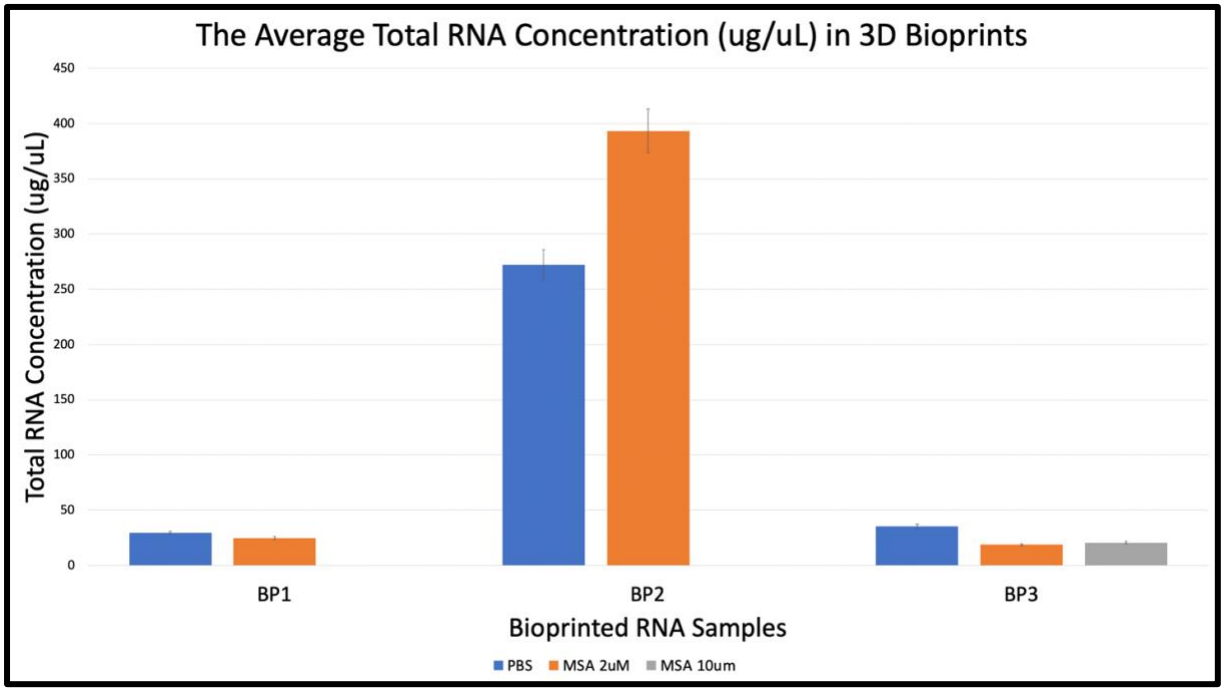

Figure 18 The average total RNA concentration (μg/μL) for each bioprint and experiment condition, PBS (blue), MSA 2μM (orange), and MSA 10μM (grey). BP1, n=2. BP2, PBS n= 2, MSA 2μM n= 3. BP3, n = 3.

3.4 DISCUSSION

3.4.1 Extraction and Confirmation of the pEF-GFP Plasmid from a Bacterial Stock.

Plasmid DNA was extracted from a bacterial stock, but the initial concentration and quality was low but following use of the "Zymo Research DNA Clean and Concentrator™ – 5", the quality and concentration improved. It is recommended in future to use a Maxi-prep kit such as the PureLink™ HiPure Plasmid Maxiprep Kit from ThermoFisher that typically yields 500 – 850µg plasmid DNA from 100 – 200 mL bacterial culture. Also, this kit is yields endotoxin-free plasmid DNA which is better suited for mammalian cell transfections[131].

A restriction digest was used to confirm that the DNA extracted was indeed the correct plasmid. The selection of enzymes demonstrated that the bands were in in agreement with the cuts at 1220bp (EcoRI), 2539bp (HindIII), 606bp and 2073bp (BglII).

3.4.2 Establishment of the Stable A549 pEF-GFP Cell Line

Next, a geneticin (G418) dose kill-curve assay was carried out to determine the lowest antibiotic concentration required to destroy the un-transfected A549 within a seven-day period. G418 is a selective antibiotic that is used to inhibit protein synthesis in prokaryotic and eukaryotic cells, thus killing the cells that do not contain the plasmid DNA[115]. The optimal dose for the A549 cells was found to be as low as 700µg/mL. The gradual killing of cells can be seen in ***Figure 9*** which shows the cells at a particular antibiotic concentration 600, 700, and 800µg/mL (other concentrations can be seen in ***SA1***) at day three and day seven.

Transfection of the pEF-GFP plasmid was then trialled as to be used for the establishment of a stable cell line to aid as visual protein marker in the 3D bioprints but also serve as a transfection positive control when it came time to complete the gene-editing protocol (***Method 4.2.2***). This would make optimisation and troubleshooting simpler if any issues arose in the gene-editing protocol.

Another variable that was used in this protocol was changing the plasmid DNA concentration for the CRISPRMAX cells (0.3µg and 1µg). As the plasmid DNA concentration was less than expected, the plasmid DNA volume was increased to allow for the correct plasmid concentrations. For the Lipofectamine 3000 sample 1µg of DNA was

used, as it was the positive control all the parameters were kept the same to the original experiment that was done.

The cells that had been exposed to the plasmid and transfection reagent were then left to incubate. To observe whether transfection had been successful the cells were visualised under a UV light at seven-time points; zero, two, four, eight, 24, 48, and 72 hours after transfection. It was not until the 72 hours after transfection time point that green fluorescence was visualised in all (except the negative) sample wells. However, a shortfall of the transfection was the low quantity of transfected cells that were visualised under the UV light. There could be several reasons why this low yield is seen, and the most plausible explanations are the quantity of plasmid DNA and/or quality of the transfection reagents used. The efficiency of the transfection reagents could have been affected due to the age of the reagents, the storage of these reagents and the freeze/thaw that these reagents have most likely undergone. Nevertheless, the transfected A549 cells were then exposed to the G418 antibiotic at the optimal dose of 700μg/mL. By Day 7, pEF-GFP plasmid containing-cells were present in the well. Next, these A549 pEF-GFP cells underwent a single-cell serial dilution in hopes of establishing a stable A549-GFP cell line that could be used for bioprinting or other experiments in the future. Unfortunately, these cells in the 96-well plate were exposed to an unknown contaminant which contaminated and killed all the cells which meant that all the transfected cells were lost as they detached from flask surface and were non-viable. Due to a lack of time and reagents it was decided that the generation of a stable cell line was not the focus of this research, and that time and effort should be put instead on bioprinting and optimising a protein extraction protocol and the gene-editing of the A549 cells.

3.4.3 Bioprinting

3D bioprinting has the potential to model the complex tumour microenvironment (TME) and the interactions seen in cancer cells (unlike 2D models). These cellular and non-cellular interactions make the TME promoting cancer progression and influence an individual's response to drug therapies[22].

3D cancer models could become an invaluable tool, better representing the complex TME interactions and properties[22] allowing us to understand cancer and it's progression. Each tumour is with different TME incorporated with various healthy functional cell types.

Several non-printed 3D cancer organoids have been developed over the last 15 years, creating a small representation of the cellular and extracellular interactions seen in tumour tissues and used to maintain the structure[61]. Comparison of the cancer cells grown in 3D vs 2D models show that the 3D organoid models behave differently to the 2D models. These behaviours include differences in growth kinetics, cell morphology, responses to drugs and molecular signatures[62]. However, organoids lack a crucial component, the extracellular matrix (ECM)-like environment. The ECM gives tumours their tissue plasticity and the stromal cell epigenetic modifications seen[22]. This was the reason for developing the 3D bioprinting. To be able to understand the interactions seen in these models it is crucial to determine whether 3D models could be a potential model for drug development and testing. For the determination of the efficacy of this model RNA and protein extraction are required to understand whether there is a different in gene expression when comparing to 2D models.

In this research the 3D bioprints were successfully printed and cultured for 21 days before drug exposure assays with MSA and/or RNA/protein extractions.

The next step for this research was to optimise a protocol for the extraction of protein from the 3D bioprints. A new protocol was modified and optimised from Lee et al (2007)[94]. The goal of the new protocol was to remove the A549 cells from the hydrogel so that only the cells remain for the protein extraction. Unlike the protein protocol used by Sawyer (2021)[67], which required sonification and numerous different reagents, this protocol used basic equipment (shaking plate and centrifuge), PBS, PBS with protease inhibitors and a protein cell lysis buffer. This made to protocol practical and simple to set up and run. The first experiment which used two different bioprints that have only been cultured it was found that not only had protein been extracted but that the protein could be run and seen on a PAGE-Gel and then detected by a 1°Ab (HSP70) with identification of a 70 kDa protein. The protein that was extracted from the 3D bioprints was ~0.5mg/mL. Sawyer (2021),was also able to extract protein with a concentration range of 0.7mg/mL – 3.2mg/mL, respectively. Protein from Sawyer (2021), was also able to run on a 10% PAGE-Gel but was unable to be detected

by the 1°Ab (HSP60). Thus, it was decided to use the new protocol for the protein extraction rather than trying to optimise the Sawyer (2021), protocol.

For the MSA drug-exposure, the duration was decided by following the same protocol as Sawyer (2021). This duration was six-hours for the drug-exposure assay.

In the final three MSA drug exposure, protein was extracted using the new protocol. From the three exposures the protein concentrations ranged from 0.1mg/mL – 3.42mg/mL.

Due to the nature of bioprinting you can control the cell number is the same for each experiment, but you are unable to control the distribution of cells throughout the hydrogel. Because of this lack of control bioprints in the same printing session may have a drastically different number of cells to the next bioprint, affecting the protein concentration of each sample. These differences can explain why variation was observed between the three independent bioprinting experiments.

For western blotting four new antibodies were purchased (five antibodies were probed). The antibodies were tested using a dot blot with the A549 2D protein sample. All samples showed an antibody signal (data not shown).

In the first gel run the total protein concentration was 1.5mg/mL. In all the gels faint bands/smears could be seen in some of the gel lanes from 120kDa – 10kDa.

The gels were transferred, and protein was confirmed to have transferred using TGX gels and Ponceau S staining. The membranes were probed with GPX1 (1:5000). No protein sample bands were seen on the membrane. As neither positive control was detected there were two potential issues. First, the primary 1°Ab is not functioning. Second, the protein concentration is too low.

To solve both the issues two different gels were run. The first gel was a protein concentration gradient using the 2D A549 protein positive control to see if the 1°Ab was functioning correctly and the second gel was using the 3D protein samples with an increased concentration of 3.75mg/mL (the samples volume was increased to 50µL to allow for this

increase in concentration) due to some of the protein samples low concentration this was as high as the protein concentration could be for all samples to have equal loading. The protein was successfully transferred using the same confirmation as the first gel. GPX1 was re-probed, with a higher concentration (1:1000) but again was unsuccessful. The membrane was stripped using a mild stripping buffer (***Method 3.2.16***) and re-probed with the HSP70 1°Ab, which had been shown to be successful (***Results 3.3.8***). On each membrane there was one to two faint bands at 70kDa (data not shown). This showed that the protein was able to be detected by the 1°Ab but that the protein loading was required to increase for all samples to be detected.

In the final PAGE-Gels the total protein concentration was increased to 16mg/mL for all the samples that could (other samples were increased to the highest concentration able within the 50µL volume). The protein transfer was confirmed, and the membranes were probed with the HSP70 1°Ab. All the protein samples in BP1 had strong bands, BP2 and BP3 had all the samples detected but some of the bands were very faint. The membranes were stripped with the mild stripping buffer and re-probed with the GAPDH 1°Ab, yielding very similar results to the HSP70 1°Ab.

The protein expression fold change and expression were measured using the average of each replicate in each bioprinting experiment. The PBS solvent control bioprints were used as the reference since the MSA drug was resuspended in PBS. The GAPDH band intensity was used to calculate the change in the HSP70 protein expression in the two treatment groups (MSA 2µM and MSA 10µM) compared to the PBS group of each bioprint.

The western blot data for each treatment group replicate shows upregulation in the protein expression of HSP70 for BP2 MSA2µM and BP3 MSA10µM, this was 6% and 11%, respectively. HSP70 is a chaperone protein that is used to help with protein folding when the cell is under stress. The upregulation of the HSP70 protein suggests that the cells in these samples are under stress. This stress could be due to the drug-treatment or due to the bioprinting process. The other four samples were all downregulated with a percentage range of -5% – -56%. This downregulation shows that the cells of this sample are not under stress unlike the other two. There was no clear trend with these BP1 – 3 experiments and thus a

statistically testing was not completed. All BP1 samples (including the PBS samples) were downregulated but for BP2 and BP3 there is difference in which sample is up/downregulated.

There is a large variability of the in the protein concentration, this is likely contributed to the lack of control in the number and distribution in the cells in each bioprinted sample. The number of cells that are used in the bioink remains consistent for all the samples. A cell count was not conducted during any of the protein or RNA extractions and so the number of cells compared to the concentration of protein/RNA cannot be determined, this means that an assumption is made that the cells with the lowest concentration had the lowest number of cells. In future experiments methods should be investigated to incorporate a cell count into the extraction protocols. The distribution of the cells could also impact the gene expression in each sample. The belief with the MSA drug exposure is that in the six-hours the drug is able to be absorbed all the was into the centre of bioprint, however this theory has not yet been proven. If the drug is unable to make it to the entireity of the bioprint. The distribution of cells would then have a dramatic impact of the gene expression results. Bioprints were cells have a large majority of cells on the outer layer of the bioprint will see more impacts of the drug then bioprints with the majority of cells in the centre. For future studies investigations on the absorption of drugs into the bioink should be undertaken to better understand the uptake of drugs when using 3D bioprints, potentially using a colour-changing drugs or indicators could give a clearer picture of the drug absorption. If the experiments could be repeated establishing and printing a GFP cell line would also help with visually observing the cell number and distribution in the bioprints.

Finally, equal loading of the proteins on the gel needs to be improved. Due to the low concentrations of some of the samples it was not possible. Future research should investigate methods to increase protein concentration such as using a Amicon® Ultra – 0.5 centrifugal filter devices.

Three antibodies (GPX1, TUBA4A, and TP53) were evaluated by western blotting with no success. Different optimisations including length of antibody incubation and temperature, and different blocking buffer concentration (3%) were made but with no success. The same membrane was positive with the GAPDH or HSP70 detection. Thus, it is possible, that the antibody does not work, epitope was not present in the folded protein, or unable to detect the low concentration of the desired protein. If more time was available,

aqueous size-exclusion chromatography (SEC) kit could be purchased to characterise and extract the desired protein by separating them based on their molecular weight[132].

In ***Chapter 5*** more future recommendations are discussed in detail on ways that the protein expression could be measured and analysed.

In the MSA drug exposure of the first three bioprints, extraction of RNA using the original protocol used by Sawyer (2021)[67], was followed. The first two bioprints had four and five bioprints printed, this meant that only two sample conditions were used, the PBS control and 2μM MSA. The final bioprint had nine prints made and all three sample conditions were used, the PBS control, 2μM MSA, and 10μM MSA. The RNA that was extracted from these samples was resuspended in different volumes of DEPC H_2O due to the different pellet sizes. When looking at the had a concentration range of 1μL of each sample, there was a range from ~100 – 7479ng/μL. The total RNA concentration ranged from 9μg/μL – 671μg/μL.

The purity of each sample (determined by the 260/280 – RNA is consider "pure" when the value is at ~2.0) had a range of 260/280 value from 1.4 – 2.0. These samples have not been cleaned or had DNase added to the samples therefore, the low 260/280 values could be due to other contaminants in the sample like proteins or phenols.

The RNA protocol used for this research utilised Tri-Reagent® to lyse the cells. Tri-Reagent® is considered a contaminant when looking at the 260/230 values and so the Tri-Reagent® residue in the RNA samples lowers the 260/280 values drastically, instead of having the recommended guidelines of 2.0 – 2.2, the values seen in the samples ranges from 0.4 – 1.1.

Sawyer (2021)[67], showed in her research that high-quality RNA could be extracted from bioprints so when looking at her RNA data compared to the samples achieved in this research, the concentration of the RNA extracted was higher compared to Sawyer (RNA concentration range of ~4 – 250ng/μL with a mean concentration of 66ng/μL). However, the 260/280 values have is decreased in the 260/280 range compared to Sawyer's RNA 260/280 value (~1.7 – 2.0). Of all of Sawyer's samples only three falls outside the recommended range of 1.8 – 2.0, where only six of the samples from this research fall in that range.

Sawyer's 260/230 values have also been affected by the Tri-Reagent®. The RNA samples that were obtained from the protocol were stored at -80ºC. Due to the high-quality RNA samples and the lack of time to process them, the best nine (3 from each experiment condition) samples were quickly run on an 1% 1X TAE Agarose gel to determine whether the samples had degraded. If not, the samples would then be sent to China for sequencing. There are two likely possibilities, the first is that the nucleic acid detected by the spectrophotometer is gDNA and if so the gDNA is too large to run on a gel. The second is that the results given by the spectrophotometer are incorrect and an over-estimation of the RNA that is present in the samples. A future recommendation would be to use the university's newly acquired tape station to run the RNA samples. The tape station will give a far more accurate picture of the quality and quantity of RNA present in the samples by providing an RNA integrity number. The aim was to send the RNA samples to China for RNA sequencing.

CHAPTER FOUR - Developing a Gene-Edited 3D Cancer Cell Model

4.1 INTRODUCTION

Fraser-Jones (2022)[112], used the A549 cell line as a positive control for her CRISPR experiments but the T7 Endonuclease Assay showed a low editing efficacy of 16.875%, with the manufacturer recommended editing efficiency of ~90%[116]. Thus, new reagents were purchased. This research followed on with the aim of improving the transfection efficiency of the positive control and developing a gene edited *CDK4* cell line as proof of concept for a 3D model.

4.2 METHODS

4.2.1 Seeding of A549 cells for CRISPR experiments

Lipofectamine CRISPRMAX (Invitrogen, ThermoFisher Scientific, A36496) was used with for the gene-editing of the A549 cell line. ***Method 3.2.13*** was followed ***Table 18*** shows the experimental set up of the 24-well plate.

Negative	NT1	NT2		Negative	GFP (positive)
GE1	GE2	GE3	GE4	GE5	GE6

Table 18 The set-up of 24-well plate for gene-editing of the A549 using the CDK4 sgRNA (GE =gene-edited), Non-targeted (NT) sgRNA, negative control and the positive control which is using GFP transfection.

4.2.2 Gene-Editing using CRISPRMAX

Table 19 outlines the kit reagent volumes used for the gene-editing of the A549 cells using *CDK4* sgRNA, NT sgRNA and a negative control using Lipofectamine CRISPRMAX (Invitrogen, ThermoFisher Scientific, A36496). Tube 1 (*CDK4*, NT, and negative) was prepared first. Tube 1 was left to be incubated for up to 30 minutes at RT. Tube 2 (*CDK4*, NT, and negative) was then prepared. Tube 2 was incubated for 1 minute at RT. Tube 1 and Tube 2 were then combined and incubated for 10-15 minutes at RT. Finally, 50µL of the appropriate reagent was added to the appropriate well. A positive GFP control was also used using CRISPRMAX and the ***Method 3.2.13*** (0.371µg of pEF-GFP plasmid)

	Gene-Edited *CDK4*		Non-targeted sgRNA		Wild-Type (negative control)	
Tubes	**Tube 1 – Cas9 Plus**	**Tube 2 – CRISPRMAX**	**Tube 1 – Cas9 Plus**	**Tube 2 – CRISPRMAX**	**Tube 1 – Cas9 Plus**	**Tube 2 - CRISPRMAX**
OPTI-MEM	25 µL	25 µL	25 µL	25 µL	25 µL	25 µL
Cas9	0.25 µL		0.25 µL		0.25 µL	
CDK4 sgRNA (5'-CACUCUUGAGGGCCACAAAG-3')[116]	1.5 µL					
NT sgRNA (5'-AAAUGUGAGAUCAGAGUAAU-3')[116]			1.5 µL			
Lipofectamine Cas9 Plus	2.5 µL		2.5 µL		2.5µL	
Lipofectamine CRISPRMAX		1.5 µL		1.5 µL		1.5 µL

Table 19 Concentration and Volumes used for CDK4 gene-editing using Lipofectamine CRISPRMAX with the sgRNA (CDK4), the non-targeted sgRNA and the wild-type (negative control).

4.2.3 Single-cell serial dilution

The gene-edited cells were used to prepare a 96-well plate (treated flat-bottom) with the purpose of selecting a single cell with the *CDK4* gene-edit. The media was the cells were then washed and trypsinised. For the serial dilution there needs to be $2x10^4$ cells/mL. The supernatant was removed, and the cells were resuspended in 210µL of cell media, the cells were then counted (***Method 3.2.6***). The 200µL of cell suspension was added to the first column of each row (GE1 was added to A1, GE2 was added to B1 and so on). Next, 100µL of complete media was added to all the wells expect those in column 1, then 100µL of the cell suspension was transferred into column 2 and combined by pipetting up and down (being careful not to create bubbles). Then, 100µL of cell suspension from column 2 to column 3 and mixed. This process was continued all the way to column 12 and 100µL of the cell

suspension from column 12 was discarded. With all the well's volume being 100µL, 100µL of cell media was added to the wells so there was a final volume of 200µL. This process was repeated for all the rows of the plate. The plate was incubated at 37°C/5% CO_2, changing the media as needed, maintain until the cells in column 12 are confluent. The cells continued to be upsized to establish a stable gene-edited cell line.

4.2.4 DNA Extraction

Half of the cell pellets from the gene-editing method had been kept aside. The cells were recentrifuged, supernatant removed, and the pellets were resuspended in 190µL of digestion buffer (***SA2***) and 10µL of freshly thawed Proteinase K (10mg/mL). The samples were digested overnight at 65°C in a thermomixer (Eppendorf). Samples were cooled to RT, vortexed for 20 seconds before 200µL of chloroform was added to remove contaminating proteins. Samples were vortexed again then mixed using a rotating wheel for 5 minutes at RT. Samples were centrifuged for 5 minutes at RT at 10,000g. The top aqueous layer was transferred to a new 1.5mL tube and twice the volume of ice-cold 70% EtOH was added. Samples were incubated at -20°C for at least an hour to precipitate the DNA. TE buffer (pH 8) was heated at 65°C while the samples were incubating. After the incubation, samples were centrifuged (16,000g, 15 minutes at RT). The supernatant was removed, and the pellet was washed with 70% EtOH. A final centrifuge was completed (16,000g for 5 minutes). The supernatant was removed making sure to remove all the EtOH residue. Samples were left to airdry for 5 minutes. The DNA pellet was resuspended in 20µL of TE buffer. The DNA quality was measured using A ratios of 260/280nm and 260/230nm and concentration using a DeNovix DS-11 FX Spectrophotometer/Fluorometer (DeNovix). If the 260/280nm quality of the DNA was less than 1.8 the DNA was cleaned using the "Zymo Research DNA Clean and Concentrator^{TM} – 5" (Zymo Research, D4013). Each DNA sample was diluted in TE buffer in a new aliquot to ensure that all the samples were at the same concentration. The original samples were stored at -20°C and diluted as needed.

4.2.5 T7 Endonuclease Assay for verification of editing

The DNA that was extracted from the gene-edited cells need to be verified for gene editing as well as the efficiency of editing using the T7 endonuclease assay. This T7 enzyme is used to recognize and cleave the strand of DNA that contain the mismatch in the DNA sequence and is used as a key tool for genome editing by detecting the mutation formed in the

sequence[106,107]. The mismatches in the DNA form a heteroduplexes in the double strand. The T7 endonuclease cuts the strands of DNA at the site of the mutation and digests it into a smaller piece that appear as lower density strands on an agarose gel.

A PCR reaction was conducted before doing the T7 endonuclease assay to increase the number of copies of DNA with the gene of interest *CDK4*. A 50µL PCR reaction of *CDK4* with controls was set up in a 200µL PCR tube on ice. ***Table 21*** and ***Table 22*** shows the reagents, volumes and concentrations required for the *CDK4* PCR reaction. ***Table 20*** shows the *CDK4* PCR primers used for the reaction, supplied by the GeneArt™ Genomic Cleavage Detection Kit, Invitrogen, ThermoFisher Scientific, (A24372).

Primer	**Sequence (5' – 3')**	**T_M (°C)**	**GC%**	**Product Size**
CDK4 Forward	GCACAGACGTCCATCAGCC	61.1	63.2	556bp
CDK4 Reverse	GCCGGCCCCAAGGAAGACTGGGAG	71.0	70.8	

Table 20 The PCR primers that were used and designed for the amplification of the CDK4 gene sequence that should contain the gene-edit. The amplification of this gene sequence will be used for the verification of the gene-edit using the T7 Endonuclease and Massey Genome Sequencing.

Components	**Individual Volume**	**Final Volume**	**Final Concentration**
5U SOLIS BIODYNE FIREPol® DNA Polymerase (01-01-0500)	0.5µL	This is dependent on how many samples there are.	2.5U
10X B1 PCR Buffer	5µL		1X
25mM $MgCl_2$	5µL		2.5mM
20mM dNTPs	0.5µL		200µM
Sterile MQ H_2O	36.0µL		
Final Volume	**47.0µL**		

Table 21 Master Mix of PCR components for the CDK4 PCR reaction for the amplification of the CDK4 gene sequence that will have the potential gene-edit and that will be verified using the T7 Endonuclease Assay.

Component	CRISPR Samples	Positive Control	Negative Control	Final concentration
Purified A549 DNA < 25 ng/µL	2.0µL			Up to 50ng
5µM F/R CDK4 primers	1.0µL		1.0µL	0.1µM
Control Template and Primers (A24372)		1.0µL		
Master Mix	47.0µL	47.0µL	47.0µL	
Sterile MQ H_20		2.0µL	2.0µL	
Final Volume	**50.0µL**	**50.0µL**	**50.0µL**	

Table 22 PCR sample set-up for the CDK4 PCR reaction for the amplification of the CDK4 gene sequence that will have the potential gene-edit and that will be verified using the T7 Endonuclease Assay.

The PCR cycling conditions were as shown below in ***Table 23***.

Temperature	Time	Cycle Number
95°C	15 minutes	
95°C	30 seconds	X 35 Cycles
58°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	
12°C	∞ Hold	

Table 23 PCR cycling conditions for the CDK4 PCR reaction amplification of the CDK4 gene sequence that will have the potential gene-edit and that will be verified using the T7 Endonuclease Assay.

After the PCR was complete, 5µL of PCR sample (5 µL of PCR amplicon mixed with 2µL 6X Loading Dye) was loaded onto a 2% 1X TAE agarose gel with 1X Thiazole Orange to confirm that the PCR amplification was successful. The gel was run at 90V for 30 minutes. To ensure that only the amplified *CDK4* DNA is used for the T7 endonuclease assay, the remaining 45µL of PCR sample (only the gene-edited samples) was added to a 2% low-melting point 1X TAE agarose gel (MetaPhor® agarose, Lonza Bioscience, 50180) with 1X Thiazole Orange was loaded and run at 90V for 30 minutes. The DNA fragment was extracted using a "ZymoClean^{TM} Gel DNA Recovery Kit" (D4001, Zymo Research. The

PCR DNA quality was measured using absorbance (A) ratios of 260/280nm and 260/230nm and concentration using a DeNovix DS-11 FX Spectrophotometer/Fluorometer (DeNovix).

4.2.5.1 T7 Endonuclease Assay

For the T7 Endonuclease Assay only gene-edited amplified samples are needed. ***Table 24*** shows the setup of the T7 Endonuclease Assay which requires two reactions per gene-edited PCR sample a positive control (containing the T7 Endonuclease enzyme) and a negative control (does not contain the T7 Endonuclease enzyme). ***Table 25*** shows the cycling conditions of the assay.

Component	**CDK4 Positive Control (Tube 1)**	**T7 Negative Control (Tube 2)**	**Final Concentration**
PCR reaction	Up to 8.5µL	Up to 8.5µL	200ng
10x T7 Endonuclease Reaction Buffer	1.0µL	1.0µL	1X
Sterile MQ H_2O		+ 0.5µL*	
T7 Endonuclease Enzyme *(Leave room to add enzyme once you have completed the re-annealing step)*	0.5µL*		0.5U
Final Volume	**10µL**	**10µL**	

*Table 24 T7 Endonuclease Assay sample set up. * Indicates that these reagents will be added after the re-annealing step.*

Temperature	**Time**
95°C	5 minutes
95-85°C	-2°C/seconds
85-25°C	-0.1°C/seconds
4°C	15 minutes (minimum) (up to an hour)

Table 25 T7 Endonuclease Re-annealing conditions

After the re-annealing step, 0.5µL T7 endonuclease enzyme (10,000 units/mL) (GeneArt™ Genomic Cleavage Detection Kit, Invitrogen, ThermoFisher Scientific, A24372) was added to the PCR reaction tube (Tube 1) and mixed thoroughly. In the control tube 0.5µL of sterile MQ H_2O was added. The samples were then incubated at 37°C for 15

minutes in a BioRad T100 thermal cycler, the samples were briefly vortexed and then spun down. Next, 0.75µL of 0.5M EDTA was added to each sample to stop the reaction. Each sample had 2µL of 30% Glycerol (used as a loading dye for electrophoresis). All the samples onto a 2% 1xTAE agarose gel with 1x Thiazole Orange and run for 30 minutes at 90V. Once the gel had run the iBright™ analysis report was used to compare band concentrations of the expected and digested sizes. The cleavage efficiency determines how effective the CRISPR editing was. ***Equation 8 and 9*** are the equations used to determine the efficiency of the CRISPR editing.

$$FC = \frac{(Band\ 2 + Band\ 3)}{Band\ 1 + \ Band\ 2 + Band\ 3}$$

Equation 8 Fraction Cleaved equation.

$$Cleavage\ Efficiency\ = 1 - ((1 - FC)^{\frac{1}{2}})$$

Equation 9 Cleavage efficiency equation for determining gene-editing.

4.2.6 Bioinformatics

Bioinformatics analyses were undertaken throughout this research to understand where the gene-editing took place in the *CDK4* gene, to verify qPCR primers for genes, protein expression and other areas of interest. ***Table 26*** shows the websites used and their links.

Website	Link
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/
NCBI	https://www.ncbi.nlm.nih.gov/
Geneious Prime	https://www.geneious.com/
UniProt	https://www.uniprot.org/
Gene Cards	https://www.genecards.org/
The Human Protein Atlas	https://www.proteinatlas.org/

Table 26 Bioinformatic websites used throughout this research.

4.2.6.1 CDK4 gene-editing analysis

To determine whether the CRISPR experiment successfully introduced a gene edit in the cell line, 25ng/µL of gel purified CRISPR PCR samples with 4pmol/µL of primers were

then sent to Massey University for genome sequencing. Once the samples were sequenced the data was analysed using Geneious Prime.

4.3 RESULTS

The CRISPR experiment has been set up with three experiments the *CDK4* (Gene-Edited, GE) sgRNA, non-targeted (NT) sgRNA and the negative control (Wild-type, WT). All the replicates were performed in the same 24-well plate (***Table 17, Method 4.2.1***). After the gene-editing experiment had been incubated for 48 hours, half of the each of GE samples cells were taken for DNA extraction and the other half of those samples were used for single-cell serial dilution to establish a gene-edited A549 cell line. All the cells from one of the negative controls and both NT samples were taken for DNA extraction.

4.3.1 DNA Extraction

The genomic DNA from the potentially six gene edited cell samples was extracted using ***Method 3.2.4***. ***Table 27*** shows the DNA concentration and quality for the six gene-edited samples and three controls (WT and NT) after the DNA extraction protocol. The concentration range (just gene-edited) 6ng/μL – 160ng/μL with five samples exhibiting high quality.

Sample	Concentration (ng/μL)	A260	260/230	260/280
Wild-Type	196.985	3.9397	0.874	1.872
Non-Targeted 1	174.863	3.4973	0.839	1.892
Non-Targeted 2	234.568	4.6914	1.278	1.992
Gene-Edited 1	17.431	0.3486	0.254	1.629
Gene-Edited 2	6.545	0.1309	0.133	2.058
Gene-Edited 3	160.766	3.2153	0.903	1.887
Gene-Edited 4	48.196	0.9639	0.561	2.014
Gene-Edited 5	78.125	1.5625	0.9657	2.025
Gene-Edited 6	37.400	0.7480	0.609	2.128

Table 27 The genomic DNA concentration and quality of the DNA that was extracted from the cells that underwent CRISPR/Cas9 gene-editing. The red boxes are the samples where 1000μL of cells had genomic DNA extracted. The green boxes are the sample where 500μL of cells had genomic DNA extracted.

4.3.2 PCR Amplification

PCR amplification was completed to amplify the *CDK4* edited gene sequence that will potentially contain the gene-edit. ***Figure 19*** shows that there is in the negative lane no bands are presents except for the primer dimer at 100bp. The six gene-edited (GE) samples have all been amplified which can be shown with the band present at ~550 bp.

In the negative lane no band can be seen except for a primer dimer (100bp). All six samples have been shown to be successfully amplified (GE1 – 6) with T_a = 58°C and 35 number of cycles. Primer-dimers were still present at the 100bp mark even with multiple attempts of optimisation with PCR conditions. Thus, PCR gel fragment extraction was used to isolate and purify the amplified *CDK4* samples.

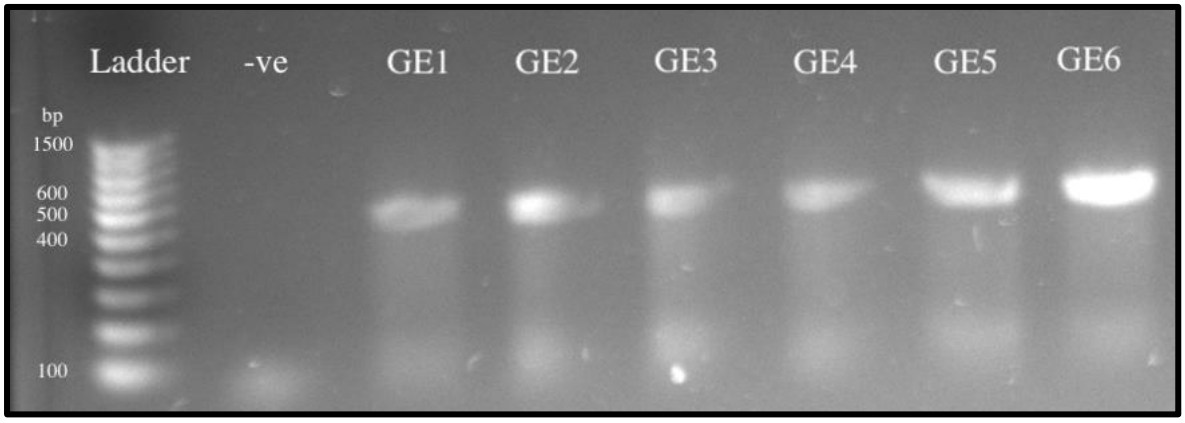

Figure 19 The DNA (20ng) was used for the PCR Amplification of the CDK4 gene sequence. The PCR products were run on a 2% 1X TAE Agarose gel at 90V for 30min. The GenScript 100bp DNA Ladder (M102R) was used. -ve Negative control; GE = Gene edited.

4.3.3 PCR Gel Fragment Extraction

After the amplified PCR samples were run on the 2% 1X TAE Low-Melting Point Agarose Gel, the gel fragments at the desired bp size (~550bp) were extracted using the "ZymoClean™ Gel DNA recovery kit" (D4001) (data not shown). The amplified DNA concentration and quality was extremely low, so the DNA samples were cleaned using the "Zymo Research DNA Clean and Concentrator™ – 5" (D4013). ***Table 28*** below shows the cleaned DNA's concentration and quality. The amplified DNA had a concentration range of 82 ng/µL – 92ng/µL. The 260/280 values are higher than the recommended range of 1.8 – 2.0, with the samples ranging between 2.2 – 2.35.

Sample	Concentration (ng/μL)	A260	260/230	260/280
Gene-Edited 1	90.891	1.8178	2.955	2.300
Gene-Edited 2	83.989	1.6780	2.614	2.234
Gene-Edited 3	82.035	1.6407	2.766	2.314
Gene-Edited 4	83.619	1.6724	2.797	2.296
Gene-Edited 5	92.297	1.8459	2.646	2.335

Table 28 Clean and Concentrated amplified DNA concentration and quality. Gene-edited 6 sample was not processed as the original cells dying.

4.3.4 T7 Endonuclease Assay

To confirm a gene edit had been made, a T7 Endonuclease Assay was applied to the purified amplified CDK4 DNA using five of the GE samples (1 – 5) with (+) and without (-) T7 Endonuclease enzyme. According to the manufacturer, the expected band size is around ~550bp for the non-digested samples (-) and three bands (~550bp, ~300bp and ~200bp) for the digested samples (+). ***Figure 20*** shows detection of a 550bp band but no evidence of gene editing with bands of 300bp and 200bp.

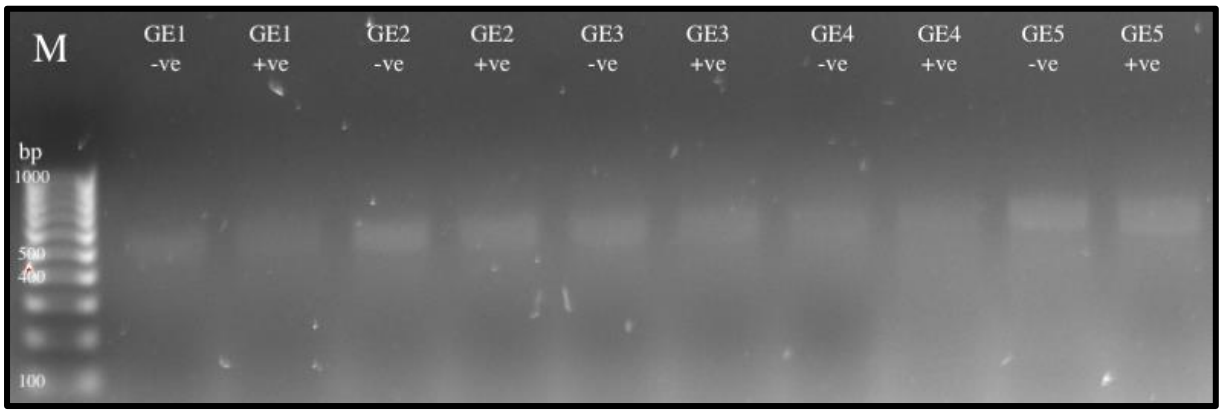

Figure 20 Gene-Edited A549 DNA samples that underwent a T7 Endonuclease Assay. The samples were run on a 2% 1X TAE Agarose Gel at 90V for 30 minutes. M is ladder what are -ve and +ve GE = gene-edited

4.3.5 Massey Sequencing

Five gene-edited samples (GE1 – 5) were sent to Massey for sequencing with the *CDK4* forward and reverse primers. Of the samples sent only one gene-edited sample had high-quality sequencing with well-defined peak and uniform peak spacing.

Figure 21 shows the four-color chromatogram of the GE2 (gene-edited 2) sample forward and reverse sequence. In the GE2 R sample there are two potential SNPs which fall close to the region of the sgRNA (substitutions, 2112T>G; 2115C>T). However, they are not supported by the GE2 F sequence which shows high quality sequencing and no SNPs. Thus, no gene editing is present. This sample could be used for future T7 Endonuclease Assay as a negative control (i.e. no-gene editing).

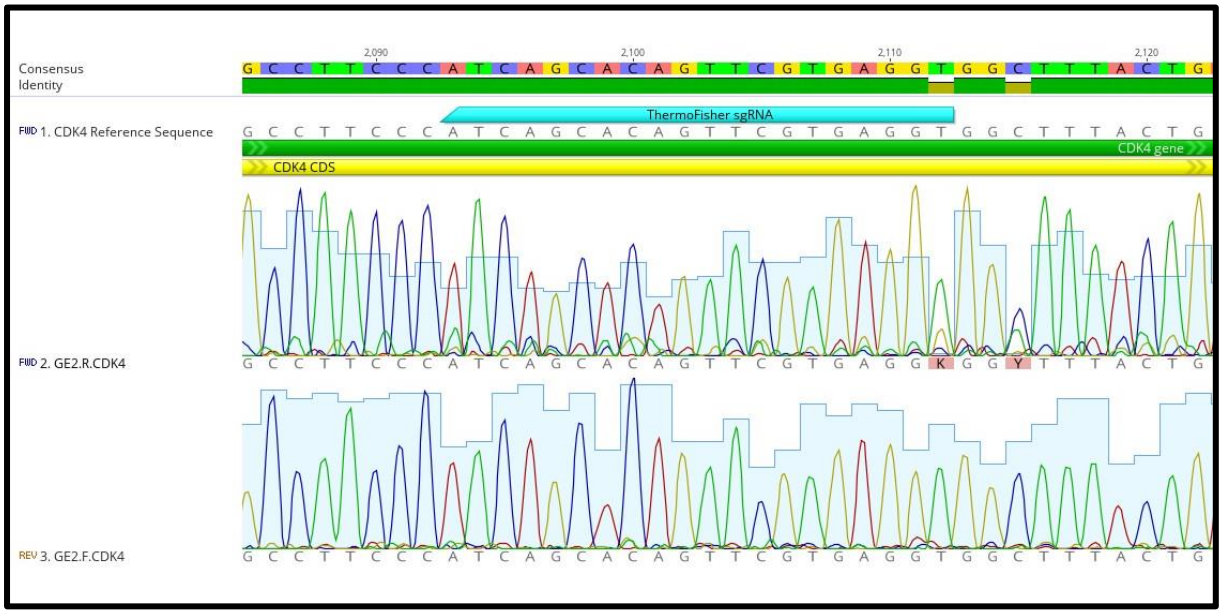

Figure 21 shows the nucleotide alignment of the two chromatograms (.GE2 R and GE2 F) against the .CDK4 Reference Sequence (1019). The CDK4 sgRNA is depicted in aqua.

4.4 DISCUSSION

In ***Results 3.3.8***, the transfection of the GFP plasmid using both the positive control (Lipofectamine3000) and CRISPRMAX had a successful outcome giving some confidence that the reagents (excluding the sgRNA and Cas9) were functioning and that proceeding with the CRISPR/Cas9 gene-editing of the A549 cell using GFP as a positive (visual) control with all the reagents was appropriate. The GFP control had similar results to ***Results 3.3.8***, with the yield of transfected cells was low which could be due to the concentration of the plasmid DNA. The gene-edited cells were split, half for the T7 Endonuclease Assay and half for single cell serial dilution in hopes of isolating and replicating a single gene-edited clone. All the cells that were grown in Column 12 of the serial dilution were upsized until they reached a 6-well plate, they were then frozen at -80°C until the gene-edit could be confirmed.

4.4.1 DNA Extraction and PCR optimisations

The other half of the cells that were used for T7 Endonuclease Assay, had their DNA extracted and underwent PCR amplification to increase the concentration of the gene sequence with the potential gene-edit. Optimisations were made for the succeeding PCR reactions to reduce primer-dimers and to increase the yield of amplified DNA fragments so that easily DNA could be visualised on the T7 Endonuclease Assay agarose gel.

The first optimisation investigated the PCR annealing temperature[119]. The starting annealing temperature was 55°C, this was the recommendation given by Invitrogen for the *CDK4* primers. First all the PCR reactions had their annealing temperatures increased to 58°C, while there was an improvement in the formation of primer-dimers there was still room for a reduction in the formation of the primer-dimers. Higher annealing temperatures (58, 60 and 62°C) were then tested using a gradient PCR to see if the primer-dimers could be reduced. It was observed that in the 62°C reaction the DNA yield had decreased compared to the other two temperatures, while 58°C and 60°C had practically no difference between the two when looking at DNA yield and the formation of primer-dimers. Because of the lack of significant difference between the two and the forward primer having a T_M of 61.1°C it was decided that the annealing temperature would remain at 58°C (data not shown).

The second optimisation investigated the cycling number of the PCR reaction. Following the products recommendations, the first PCR reaction used 40 cycles for the

denaturation, annealing and elongation of the DNA, this was optimised to 35 cycles and 30 cycles. In the 30 cycles the primer-dimers formed were the most reduced compared to 35, and 40 cycles but the amplification yield had also decreased. The decrease in the DNA was not ideal as this amplified DNA is needed for the T7 Endonuclease Assay (which requires a total DNA concentration of 200ng in a 10μL reaction, 24ng/μL). Because the DNA yield is crucial for the next steps it was decided that the number of PCR cycles would be 35 cycles and that other optimisations would be made to increase the amplification of the desired gene sequence and to reduce the primer-dimers.

Thirdly, the primer concentration was adjusted to 0.05μM but none of these reactions were successful and so 0.1μM was used for the remaining PCR reactions.

As the primer-dimers were able to be reduce but not eliminated it was decided that the best way to proceed with the T7 Endonuclease Assay was to upsize the PCR reaction to a 50μL and to then run those samples on a low-melting temperature agarose gel so that the desired DNA band fragment could then be exercised from the gel and then purified and concentrated into a smaller volume. This shifted the focus of the PCR optimisation to examine opportunities to increase the yield of PCR products rather than removing the primer-dimers entirely. To increase the DNA yield of the PCR reaction for the T7 Endonuclease Assay, the concentration of $MgCl_2$ and DNA template quantity was also optimised[119].

In the first few reactions the $MgCl_2$ concentration was 1.5mM, this concentration was increased to 2.5mM. $MgCl_2$ is a co-factor for the Taq polymerase that is used in the elongation of the PCR template[119]. $MgCl_2$ not only interacts with the Taq but with all the PCR components and so increasing the concentration of $MgCl_2$ can increase the yield of DNA. When comparing the PCR reactions of the different concentrations there was no obvious difference and as there was no difference it was decided to keep the higher concentration of $MgCl_2$ rather than the lower (data not shown).

The final PCR optimisation made was to increase the DNA quantity from 3ng of template genomic DNA to 20ng.

Following PCR optimisation and gel purification, the spectrophotometer data shows a DNA concentration range of ~82 – 92ng/μL. This was enough DNA for both the T7 Endonuclease Assay and Massey Sequencing.

4.4.2 Gel Fragment Extraction, Massey Sequencing and T7 Endonuclease Assay

Before the samples were sent to Massey for sequencing the T7 Endonuclease Assay was done determine if any gene-editing had been accomplished in the samples. In the first assay the initial the first 95°C was only for 1 second before the other cycling conditions started, this was a recommendation from the manufacturer but after further investigation and suggestion from others this steps timing was increased to 5 minutes to allow better denaturation of the DNA strands. The final hold step was also adjusted from its original time of 5 minutes to the new timing of 15 minutes to 1 hour. There were different recommendations on this, but the consensus was that the longer the better for the final hold.

Despite the optimisations that were made but no change in the results were seen to indicate gene editing had worked. When examining the ***Results 4.3.4***, three bands should be seen in the positive sample lanes. Depending on how well the gene-editing efficiency was would depend on the brightness of the band, however generally speaking, the brightest band should be the band at 500bp as those are the not gene-edited cells. The other two bands expected at ~300bp and ~200bp are normally fainter due to the lower number of gene-edited cells. The goal of gene-editing is to have a high efficiency in which during the single-cell serial dilution a gene-edit will be isolated and grown into a stable gene-edited cell line, the lower the efficiency the lower the chances of the stable cell line.

There are several possibilities as to why multiple bands are not observed on the T7 endonuclease agarose gel. The first and most obvious answer is that there was no gene-editing in my samples. Thus, it is recommended to import/purchase and use a *CDK4* gene-edited cell-line to serve as positive control DNA for the T7 Endonuclease assay so the three bands can be observed to demonstrate that thee T7 endonuclease is working.

Even though the positive GFP transfection controls showed detection of green fluorescence, the yield of transfected cells with the GFP plasmid was low. Thus, this could raise the question of "was the Cas9 or sgRNA working correctly?". Testing these is harder as

there was no visualisation confirmation like with the other reagents and the GFP transfection. Because of this there is no way to no whether it was the Cas9 enzyme that was not functioning correctly or if there was an issue in the sgRNA. Another issue is that as the A549 cell-line that is being used for these experiments is a cancer line, new mutations can occur at random and could affect how the sgRNA binds to the DNA.

When working with enzymes and DNA, the DNA samples should be free of contaminants like organic solvents, salts, and nucleases. Considering the purified amplified DNA results in ***Results 4.3.3,*** the quality has been impacted by contaminants based on the A260/280 and A260/230 reading being greater than 2.0. These contaminants could most likely impact how well the T7 Endonuclease enzyme is supposed to function and therefore it is possible T7 enzyme was unable to function and detect these edits. Purchase of a new gel extraction kit and higher accuracy for validating DNA such as the Qubit Fluorometer are recommended for future studies.

The final explanation is that the despite the poor DNA quality and if the Cas9/sgRNA were functioning, the root cause for the lack of results is that the T7 enzyme is not working at all. Thus, having positive control gene-edited DNA would be very helpful to address this. All the reagents that were used for this section of the research were stock from two previous projects and so it is likely that the quality of the reagents used had been compromised from use overtime, freeze-thaw cycles, and long-term storage. Therefore, it is highly likely that the T7 enzyme had lost its activity all together and so no matter the optimisation made the assay would have never been successful.

Due to the uncertainty of the T7 Endonuclease Assay, it was decided that the gene-edited samples should be sent to Massey for genome sequencing to determine whether a gene-edit had been made in any of the samples and investigate the mutation status of CDK4 in the A549 cells. As mentioned above the sequencing was on successful with a high-quality in one of the ten samples, and two samples were sequenced with warnings. Using Geneious Prime[108] the sequences were analysed and aligned to form a consensus sequence (447bp and 478bp). This consensus sequence showed a high identity and that there could be a potential gene-edit in the binding region of the sgRNA. However, because only one of these sequences showed this potential edit, it is likely that these are random SNPs and not a gene-edit. The sequence without the edit shows a higher quality sequence then the sequence with them.

Another reason why it is not for certain if this was an edit or just a random SNP is due to the fact the alignment was compared to the reference sequence and the cell-line that is being used for the gene-editing is a cancer cell-line and so it is highly likely that there are an accumulation of SNPs that will differ from the reference sequence and so what may appear to be a gene-edit could be a SNP that was already present in the wild-type DNA. Without the non-edited sequence and the presence of high-quality sequencing in all or most of the samples the results from the Massey sequencing cannot be interpreted with high confidence.

This chapter focussed on the aim of establishing a stable gene edited A549 cell line. Gene-editing methods were attempted for this research but were unable to be prove whether successful editing had occurred. Once the gene-editing method is optimised and a stable gene-edited cell line is established, gene-edited 3D models are the next step for future research. Being able to look at the gene-expression at gene-edited cells will give researchers a better idea of how particular mutations effect the efficacy of different drug therapies.

Having two solid methods for the extraction of RNA and protein is important for the use of 3D cancer models and drug research in being able to understand the cellular responses in an *in-vivo*-like model of the TME. With further research and optimisation this study could provide insight into the gene expression of 3D gene-edited bioprinted cells.

CHAPTER FIVE – Future Recommendations

The overall aims of this research were to investigate the gene expression profiles of 3D gene-edited bioprinted cell that have been exposed to Se compound MSA. There were five objectives that were successfully accomplished.

This chapter will delve deeper into five future optimisations and recommendations that could be taken if this research was wanted to progress further to achieve research objectives 4, 5 and 6.

5.1 Improve the Level of Detection of Protein Expression using Different Primary Antibodies

Order New Antibodies

In this research five 1° Ab's were investigated (***Table 11***). Of those five, only two 1°Ab's were able to bind and detect the protein on the membrane: HSP70 and GAPDH. GPX1, TUBA4A, and TP53 had relatively good expression in A549 cells (162.0, 254.2, and 100.6 nTPM) and should be seen even if faintly with the protein concentration used on the final membranes and protein extracted from 2D cells. Multiple different optimisations were made in attempts to make these 1°Ab's work. Some of these optimisations were increasing the antibody dilution, increasing the incubation period, and changing the blocking buffers concentration and the buffers that the antibodies were diluted in from 10% blocking buffer to 3% blocking buffer. It is recommended to reorder the antibodies that did not work.

Use an Alternative Protein Expression Detection Method

Enzyme-linked immunosorbent assay (ELISA) is a quantitative analyse that show antigen-antibody recognition through fluorescence or colour-change[127]. ELISA utilises enzyme-linked conjugate and substrates to identify the presence and concentration of the desired protein. ELISA was developed by two different researchers in 1971 by modifying another method. ELISA's are a simple technique that is sensitive, versatile and can process many samples quickly for protein expression in a 96-well plate[122]. One advantage of ELISA over a western blot is that the level of protein that can be detected, as low as 100pg. This, this is ideal for the bioprinting samples. ELISA's do not require special equipment or radioactive

labels like previous methods[127]. There are four different ELISA methods: direct, indirect, sandwich, and competitive. Direct is the fastest but has the lowest sensitivity. Indirect and sandwich can quantify the amount of antibody/antigen present in the sample with a high sensitivity. The competitive ELISA method can detect the compositional differences in the complex antibody/antigen mixtures (more than one). A downfall is that ELISA kits are expensive so before an ELISA protocol can be carried out the protein samples should be verified on a WB, this still means that having quality 1° and 2° Ab's is important.

Most ELISA kits set up as a 96-well plate and so all the protein samples can be loaded with replicates and the protein expression analysed and interpreted accurately. This is important when studying how a particular drug therapy effects the cells.

Even though ELISA's have many positives, there is some limitations. ELISA can only be used once and only have one 1°Ab that recognises one protein (except competitive ELISA's). That means that multiple different ELISA kits would have to be ordered to look at each different protein of interest. This is where ELISA kits become expensive and impractical. A more practical approach would be to get the proteome of each protein sample sequenced for all the proteins.

5.2 Investigate the Transcriptome, Epigenome, and Proteomic Profile of each 3D Bioprint

Proteomics has become a useful tool in not only cancer research but many biological research fields due to its ability to process and understand the biochemical changes[123]. Using the current generation of mass spectrometry which allows for quicker and more sensitive analysis and the new sample fractionation, labelling and processing strategies using proteomics to help understand and interpret gene expression has become far more accessible for researchers. Using proteomics for each of the 3D bioprinted samples with the use of 2D protein will give a comprehensive understanding of how the proteome has been affected by first the bioprinting process and how the different concentrations of MSA affect the cells gene expression.

Sequencing the transcriptome using the RNA samples is another recommendation. RNA-Seq is a precise measure of the levels of transcripts and isoforms present in the cell

samples[136]. RNA-Seq uses deep-sequencing technologies to catalogue all the species of transcripts (mRNA, non-coding RNA, and much more).

The epigenome could also be sequenced to investigate the methylation modifications within the DNA[134]. These modifications change the regulation of gene activity that is independent of the DNA sequence. Using Transposase-Accessible Chromatin Sequencing (ATAC-seq) which is a simple and scalable method used for detecting these modifications[135]. ATAC-seq requires a small number of input cells which is ideal for bioprinting and does not require and prior knowledge on the epigenetics of the samples.

Using all three of these methods will give a clear understanding on the effects of bioprinting and Se on the cells gene expression and the changes that are seen within each experimental condition.

5.3 Including a Positive CRISPR Control for the T7 Endonuclease Assay

As discussed in ***Chapter 4.4***, a problem that was major problem that was faced in the gene-editing protocol was the inability to confirm without a doubt whether gene-editing had occurred in the GE samples. Use of a positive gene-edited DNA control samples that will be detected and cleaved by the T7 endonuclease.

5.4 Improving the Gene-Editing Transfection Efficiency with Electroporation

While lipofectamine is cost effective and can provide high throughput its transfection efficiency is too low[124]. Thus, an alternative is electroporation which uses an electric pulse that forms pores in the cells membrane to allow for the entry of Cas9 and sgRNA to enter the cytoplasm [124]. This method is easy and fast, with a high editing efficiency, with one study showing a transfection efficiency of >60%[128]. A second option is Nucleofection, like electroporation in that it uses electric pulses, but uses a Nucleofector to create special pulses that have been pre-optimised for the specific cell type for nuclear delivery[124].

5.5 Using Gene-Editing Reporters to Aid Visualisation of Gene-Edits

Finally, the implementing the use of fluorescent reporters like SRIRACCHA and GEmCherry2 into the CRISPR detail will provide a colour readout when editing has

occurred[125]. SRIRACCHA was created in 2017 and can integrate a reporter gene (containing a puromycin resistance gene) that is followed by the target sequence and then an out-of-frame histone (H2B)-GFP reporter. If gene-editing is successful, the cells will express both the puromycin resistance gene and the GFP. One research used SRIRACCHA and deep sequencing assays[129] to predict which gRNA had the highest efficiency using wild-type and SRIRACCHA Cas9. The SRIRACCHA Cas9 assay showed a higher sensitivity compared to the wild-type Cas9. From the results shown in this research it was suggested that SRIRACCHA could be an advantageous reporter for HDR and valuable for showing precise localisation of the nuclease cleavage.

GEmCherry2 is a modified monomeric red fluorescent protein (RFP). It was developed to rapidly evaluate the efficiency of the Cas9 activity[125]. GEmCherry2 has an out-of-frame genomic CRISPR target region that has been incorporated into the mCherry N-terminus. The genomic region that has been inserted can be targeted by the sgRNA guided Cas9, thus causing a DSB. This DSB is repaired using NHEJ causing an in-frameshift of mCherry resulting in expression. The GEmCherry2 assay was compared with the TIDE assay and showed similar ranking of sequences[130]. GEmCherry2 systems are also able to be used to assess the efficiencies of ZFN, TALENS, and other DNA systems. The expression of mCherry confirm the efficiency of both the sgRNA and Cas9, which are the biggest unknowns of this research. One research looking to establish a beta-2-microglobulin deficient human iPS cells, co-transfecting a sgRNA with the mCherry plasmid[131]. The cells that had successful HDR expressed the EGFP and could be analysed using a flow cytometer. The data showed the 12 - 23% of the cells expressed the mCherry region. A significant number of cells that were mCherry positive also expressed the EGFP. Parrish et al (2021)[133], conducted a successful CRISPR using mCherry EGFP to create a knock-out (KO) of the *CDK4.*

Using the fluorescent reporters like SRIRACCHA and GEmCherry2 will provide a rapid, efficient, and real-time information on the efficiency of the Cas9 and sgRNA. By using these tools at the start of editing which can prevent the investment of time into experiments that are not successful.

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APPENDIX ONE

EPA - GMO Approval

Environmental
Protection Authority
Te Mana Rauhī Taiao

DECISION

Amended under s67A on 25 January 2013, on 17 March 2015, on 14 July 2015, 11 March 2016, 21 May 2018, and 19 March 2020

Date	15 December 2011
Application code	APP201152
Application type	To develop in containment genetically modified organisms under sections 40(1) and 42A of the Hazardous Substances and New Organisms Act 1996
Applicant	University of Waikato
Date application received	15 December 2011
Consideration date	15 December 2011
Considered by	Chief Executive, Environmental Protection Authority (EPA)
Purpose of the application	To develop a range of genetically modified non-pathogenic microorganisms, cell lines and zebrafish carrying genes coding for proteins involved in causation of disease, in the evolution of protein stability and cellular functions

1. Summary of decision

1.1 Application APP201152 to develop, as a project, genetically modified organisms (as described in Table 1 of this decision) in containment is **approved, with controls**.

1.2 The EPA considered that it had sufficient information to assess the application. The application was considered in accordance with section 42A of the Hazardous Substances and New Organisms (HSNO) Act 1996 ("the Act"), the Hazardous Substances and New Organisms (Low-Risk Genetic Modification) Regulations 2003 ("the Regulations"), and the Hazardous Substances and New Organisms (Methodology) Order 1998 ("the Methodology").

2. The approved genetically modified organisms and the controls imposed

Purpose of the project

2.1 The purpose of this application is to develop a range of genetically modified non-pathogenic microorganisms, cell lines and zebrafish that carry genes coding for proteins involved in causation of disease, in the evolution of enzyme stability and cellular functions. Proteins are biochemical compounds that facilitate biological function and participate in virtually every process within cells (eg, enzymatic, structural, mechanical, cell signalling, ligand binding, immune responses, cell adhesion and the cell cycle). The aims of the research are to express and purify these proteins to study structure, biochemical and cellular functions, and evolution of enzyme stability (through the reconstruction of ancestral enzymes).

2.2 The EPA considers that this application describes a project for the development of genetically modified organisms (GMOs). This project represents a particular line of scientific inquiry and has clearly defined objectives regarding the development of non-pathogenic microorganisms, cell lines and zebrafish within a containment structure for research into the role of proteins in the causation of disease, in the evolution of enzyme stability and cellular functions. The EPA considers that the organism description in this application falls within the bounds of a project for the development of GMOs as described below

2.3 The EPA has determined that this application is for a valid purpose being the development of any new organism as provided for in section 39(1)(a) of the Act.

Description of the organisms to be developed

2.4 As per section 42A(2) of the Act, the EPA is satisfied that the host organisms and the proposed genetic modifications conform to the requirements of a Category 1 or 2 host organism (as per clause 7 of the Regulations) and Category A or B genetic modifications (as per clause 5 of the Regulations) (as described in Table 1).

Table 1: Description of approved organisms

Host organisms	**Bacterial species:** *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 non-pathogenic laboratory strains *Pseudomonas fluorescens* (Migula 1895) *Sulfolobus solfataricus* Zillig et al. 1980 *Acidovorax temperans* (Willems et al 1990) *Brevibacillus choshinensis* (Takagi et al. 1993), Shida et al. 1996 *Synechocystis* sp. PCC8303 (Stanier et al. 1971) **Viruses:** *Autographa californica multiple nucleopolyhedrosis virus* (AcMNPV) (Chapman and Glasier 1915, Allen 1921) disarmed laboratory strains Bacteriophage Lambda (ICTV approved name is *Enterobacteria phage λ*) **Yeasts:** *Saccharomyces cerevisiae* Meyen ex EC Hansen (1883) non-pathogenic laboratory strains *Pichia pastoris* (Guillierm.) Phaff 1956 non-pathogenic laboratory strains *Pichia angusta* (Teun., H.H. Hall & Wick.) Kurtzman 1984 non-pathogenic laboratory strains *Schizosaccharomyces pombe* Lindner 1893 non-pathogenic laboratory adapted, non-sporulating strains **Cell lines (including primary cell lines)** *Homo sapiens* Linneaus, 1758 (Excluding human embryonic stem cell lines and cell lines derived from people of known Māori origin). *Mus musculus* Linneaus, 1758 *Mus spretus* Lataste, 1883 *Rattus norvegicus* (Berkenhout 1769) *Rattus rattus* (Linnaeus, 1758) *Mesocricetus auratus* (Waterhouse, 1839) *Chlorocebus aethiops* (Linnaeus, 1758) *Chlorocebus pygerythrus* (Cuvier, 1821) *Bos taurus* Linnaeus, 1758 *Bos indicus* Linnaeus, 1758 *Ovis aries* Linnaeus, 1758 *Cricetus cricetus* (Linnaeus, 1758) *Cricetulus griseus* Milne-Edwards, 1867

	Canis familiaris Linnaeus, 1758 *Cavia porcellus* (Linnaeus, 1758) *Gallus gallus* (Linnaeus, 1758) *Drosophila melanogaster* Meigan 1830 *Trichoplusia ni* (Hübner 1803) *Spodoptera frugiperda* (Smith 1797) *Danio rerio* (Hamilton 1822) *Oncorhynchus mykiss* (Walbaum 1792) *Salmo salar* (Linnaeus, 1758)
Category of host organisms	The bacterial species, yeasts, viruses and human and animal cell lines are Category 1 host organisms because: • they are clearly identifiable and classifiable; • they are characterised to the extent that their main biological characteristics are known; • they are not normally able to (or contain infectious agents normally able to) cause disease in humans, animals, plants or fungi; • they do not normally infect, colonise or establish in humans; and • they do not produce desiccation-resistant structures such as spores or cysts that can be normally disseminated in the air.
Modification	Standard molecular biology and genome-editing techniques may be used including, but not limited to, Zinc Finger Nucleases, CRISPR-Cas9 and Talens, which involve vectors or oligonucleotides for targeted gene knockdown, mutation introduction, the introduction of fluorescent proteins or the localisation of proteins or RNAs to study gene causation of disease, structural mechanical, enzymatic, cell signalling, ligand binding, immune responses, cell adhesion and/or cell functions. **Vectors** Non-conjugative and conjugative (but non-transmissible) *E. coli* cloning and expression plasmid vectors, bacteriophage plasmid vectors (including commercially available helper phage vectors, yeast cloning and expression vectors, mammalian expression plasmid vectors (excluding retroviral vector systems); and baculoviral vectors (including transfer vectors, bacmids). Vectors will consist of promoters and gene regulatory elements, reporter and selectable marker genes, secretory and targeting signals, recombination sites, flanking sequences and sequences that facilitate recombination, solubility enhancement tags, protein purification and affinity tags including epitope tags, and origins of replication. Donor genetic material is sourced from humans, animals (including mammals, birds, fish and insects), plants, fungi, bacteria, archaebacteria, viruses and non-pathogenic protozoa, with the exception of donor genetic material sourced from *Plasmodium falciparum* and *Neisseria gonorrhoeae* for expression in *Escherichia coli*, which may be in the form of artificially synthesised DNA that is codon-optimized for expression in *E. coli* as the host organism. The donor genetic material may contain genomic or complementary DNA, antisense sequences or other RNA interference-inducing sequences that encode genes and their associated regulatory elements that code for proteins that are involved in causation of disease, involved in enzymatic, structural, mechanical, cell signalling, ligand binding,

	immune responses, cell adhesion and cell cycle functions. The modifications will exclude: • Genetic material from persons of Māori descent. • Genetic material from native flora or fauna • Genetic material that increases the pathogenicity, virulence, or infectivity of the host organism. • Uncharacterised sequences from pathogenic microorganisms. • Genes that encode for vertebrate toxins with an LD_{50} < 100 µg/kg. • Those that result in the GMO having a greater ability to escape from containment than the unmodified host organism.
Category of modification	The modifications are Category A because these modifications are carried out under a minimum of PC1 containment as defined in the Regulations and do not increase the pathogenicity, virulence or infectivity of the host organism to laboratory personnel, the community or the environment and do not result in the GMO having a greater ability to escape from containment than the unmodified host organism.
Minimum containment level required	PC1

Host organisms	**Bacterial species:** *Bacillus subtilis* (Ehrenberg 1835) Cohn 1872 laboratory strains *Bacillus stearothermophilus* (Donk, 1920) *Neisseria gonorrhoeae* (Zopf 1885) Trevisan 1885 **Animal species:** *Danio rerio* (Hamilton 1822) whole fish
Category of host organism	*Bacillus subtilis, Bacillus stearothermophilus* and *Neisseria gonorrhoeae* are category 2 host organisms because: • they are clearly identifiable and classifiable according to genus, species, strain or other subspecific category and • they are Risk Group 1 organism as defined in the Regulations that produces desiccation-resistant structures such as spores or cysts that can be normally disseminated in the air. *Danio rerio* (whole fish) is a category 2 host organism because: • it is clearly identifiable and classifiable according to genus, species, strain or other subspecific category and • it is a whole animal, vertebrate or invertebrate including oocytes, zygotes, early embryos and other cells able to grow without human intervention into a whole animal.
Modification	Standard molecular biology and genome-editing techniques may be used including, but not limited to, Zinc Finger Nucleases, CRISPR-Cas9 and Talens, which involve vectors or oligonucleotides for targeted gene knockdown, mutation introduction, the introduction of fluorescent proteins or the localisation of proteins or RNAs to study gene causation of disease, structural mechanical, enzymatic, cell signalling, ligand binding, immune responses, cell adhesion and/or cell functions.

	Vectors Non-conjugative and conjugative (but non-transmissible) *E. coli* cloning and expression plasmid vectors, mammalian expression plasmid vectors (excluding retroviral vector systems); and baculoviral vectors (including transfer vectors, bacmids). Vectors will consist of promoters and gene regulatory elements, reporter and selectable marker genes, secretory and targeting signals, recombination sites, flanking sequences and sequences that facilitate recombination, solubility enhancement tags, protein purification and affinity tags including epitope tags, and origins of replication. Donor genetic material is sourced from humans, animals (including mammals, birds, fish and insects), plants, fungi, bacteria, archaebacteria, viruses and non-pathogenic protozoa. The donor genetic material may contain genomic or complementary DNA, antisense sequences or other RNA interference-inducing sequences that encode genes and their associated regulatory elements that code for proteins that are involved in causation of disease, involved in enzymatic, structural, mechanical, cell signalling, ligand binding, immune responses, cell adhesion and cell cycle functions. The modifications will exclude: • Genetic material from persons of Māori descent. • Genetic material from native flora or fauna • Genetic material that increases the pathogenicity, virulence, or infectivity of the host organism. • Uncharacterised sequences from pathogenic microorganisms. • Genes that encode for vertebrate toxins with an LD_{50} < 100 µg/kg. • Those that result in the GMO having a greater ability to escape from containment than the unmodified host organism.
Category of modification	The modifications are Category B because these modifications are carried out under a minimum of PC2 containment as defined in the Regulations and do not increase the pathogenicity, virulence or infectivity of the host organism to laboratory personnel, the community or the environment and do not result in the GMO having a greater ability to escape from containment than the unmodified host organism.
Minimum containment level required	PC2

Controls imposed

2.5 The EPA notes that the approval holder must ensure that the location and nature of the development, and the disposal of the approved GMOs are in accordance with the application unless otherwise required by the controls the EPA imposes below.

2.6 As per section 42A(3)(b) of the Act, the EPA imposes the controls detailed in Table 2 to provide for the matters detailed in Part I of the Third Schedule of the Act and other matters to give effect to the purpose of the Act.

2.7 The EPA notes that the use of cells lines derived directly from humans or animals will be overseen by the appropriate Human Ethics or Animal Ethics committees.

2.8 The EPA is not imposing a control directing the applicant to provide progress reports as this current application does not raise any novel issues.

Table 2: Controls

Any reference to MAF/ERMA New Zealand or AS/NZS Standards in these controls refers to any subsequent version approved or endorsed by the EPA	
1)	The approval holder, University of Waikato, must ensure compliance with the following controls.
2)	This approval is limited to the development of the GMOs described in Table 1 ("approved organisms"), to develop a range of genetically modified non-pathogenic microorganisms, cell lines and zebrafish carrying genes coding for proteins involved in causation of disease, in the evolution of protein stability and cellular functions.
3)	The approved organisms must be developed and maintained within a containment facility in accordance with: • MAF/ERMA New Zealand Standard: Facilities for Microorganisms and Cell Cultures: 2007a; and • Australian/New Zealand Standard AS/NZS 2243.3:2002 Safety in laboratories: Part 3: Microbiological aspects and containment facilities; and • Physical Containment level 1 (PC1) requirements of the above Standards (at minimum) for developments involving *Escherichia coli*, *Pseudomonas fluorescens*, *Sulfolobus solfataricus*, *Acidovorax temperans*, *Brevibacillus choshinensis*, AcMNPV, Bacteriophage Lambda, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Pichia angusta*, *Schizosaccharomyces pombe* and cell lines; and • Physical Containment level 2 (PC2) requirements of the above Standards (at minimum) for developments involving *Bacillus subtilis* and *Bacillus stearothermophilus*. • MAF/ERMA New Zealand Standard Containment Facilities for Vertebrate Laboratory Animals; and • Australian/New Zealand Standard AS/NZS 2243.3:2002 Safety in laboratories: Part 3: Microbiological aspects and containment facilities; and • Physical Containment level 2 (PC2) requirements of the above Standards (at minimum) for developments involving *Danio rerio* (whole zebrafish).
4)	The approval holder must ensure that within 24 hours of the discovery of any breach of containment (includes the escape of an organism(s), unauthorised entry to the containment facility, or a failure in the structural integrity of physical containment mechanisms), the MAF Inspector responsible for supervision of the facility, has received notification of the breach, and the details of any action taken by the facility since the

breach occurred.

3. The decision

3.1 The EPA considers it has sufficient information to assess the application. The EPA waives any legislative information requirements (i.e. concerning the information that shall be supplied in an application) that the application may not meet.

3.2 The EPA considers that the information provided by the applicant is relevant and appropriate to the scale and significance of the risks, costs, and benefits associated with the application (as required by clause 8 of the Methodology). This assessment of risks and costs has been conducted in accordance with the Methodology, particularly clauses 9, 10, 12 and 13 (which incorporate sections 5, 6, and 8 of the Act).

3.3 Given the biological characteristics of the GMOs (as detailed in Table 1 of this decision), the containment system and the controls imposed (Table 2 of this decision), there is no evidence for, nor any reason to expect any non-negligible risks (adverse effects) or costs of the proposed GMOs on humans, animals, plants, other organisms, the environment, the economy, society or community, or Māori culture.

3.4 The EPA considers that a benefit of this application will be the gains in knowledge that will result from conducting this research.

3.5 The EPA considered the other matters required under the Act, the Regulations, and the Methodology, and were satisfied that none or those matters would be affected by the development of the GMOs in containment.

3.6 Therefore application APP201152, to develop in containment GMOs (as described in Table 1 of this decision), **is approved, with controls under section 42A(3)** (as described in Table 2 of this decision). This decision was based on the information supplied by the applicant and was considered in accordance with section 42A of the Act, the Regulations, and the Methodology.

Mr Rob Forlong — Date: 15 December 2011

Chief Executive under delegated authority from the EPA

Approval codes: **GMD101146 – GMD101179, GMD101355, GMD101748**

First Amendment: January 2013

To add the non-pathogenic organism *Bacillus stearothermophilus* to the list of approved host species, under appropriate containment.

Mr Rob Forlong
Chief Executive under delegated authority from the EPA

Date: 25 January 2013

Second Amendment: March 2015

The list of approved host organisms was expanded to include the cyanobacterium *Synechocystis* sp. PCC3803.

Mr Rob Forlong
Chief Executive under delegated authority from the EPA

Date: 17 March 2015

Third Amendment: July 2015

The donor genetic material for Category A modifications was amended to allow the use of gene sequences from *Plasmodium falciparum* that are codon-optimized for expression in *Escherichia coli*.

Mr James Palmer
Acting Chief Executive under delegated authority from the EPA

Date: 14 July 2015

Fourth Amendment: March 2016

The donor genetic material for Category A modifications was amended to allow the use of gene sequences from *Neisseria gonorrhoeae* (which may be codon-optimised) for expression in *Escherichia coli*.

Dr Allan L. Freeth
Chief Executive under delegated authority from the EPA

Date: 11 March 2016

Fifth Amendment: May 2018

The list of approved host organisms was expanded to include *Neisseria gonorrhoeae* (Zopf 1885) Trevisan 1885.

Dr Fiona Thomson-Carter
General Manager Hazardous Substances and New Organisms under delegated authority from the EPA

Date: 21 May 2018

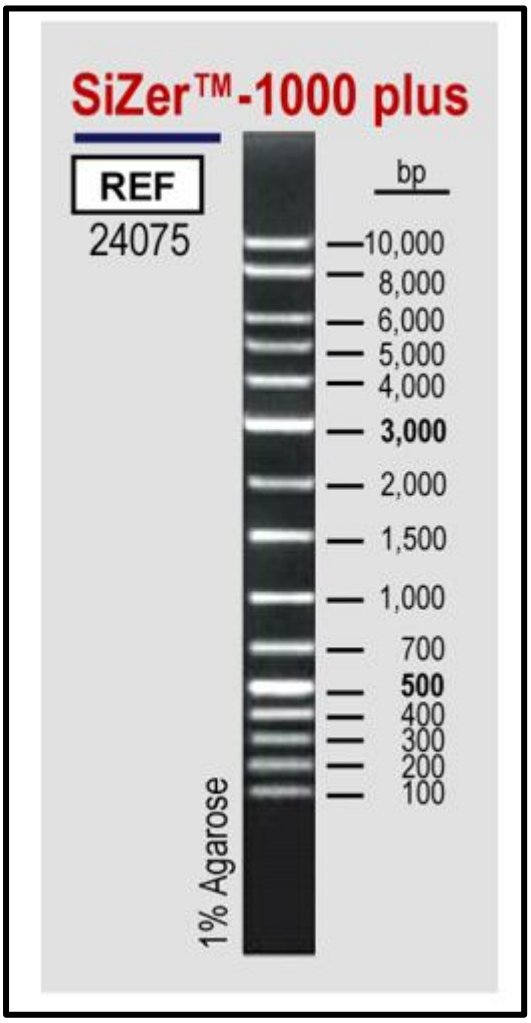
Sixth Amendment: March 2020

To include genome-editing techniques such as Zinc Finger Nucleases, CRISPR-Cas9 and Talens.

Doug Jones Date: 19 March 2020

Acting General Manager Hazardous Substances and New Organisms under delegated authority from the EPA

Ladders



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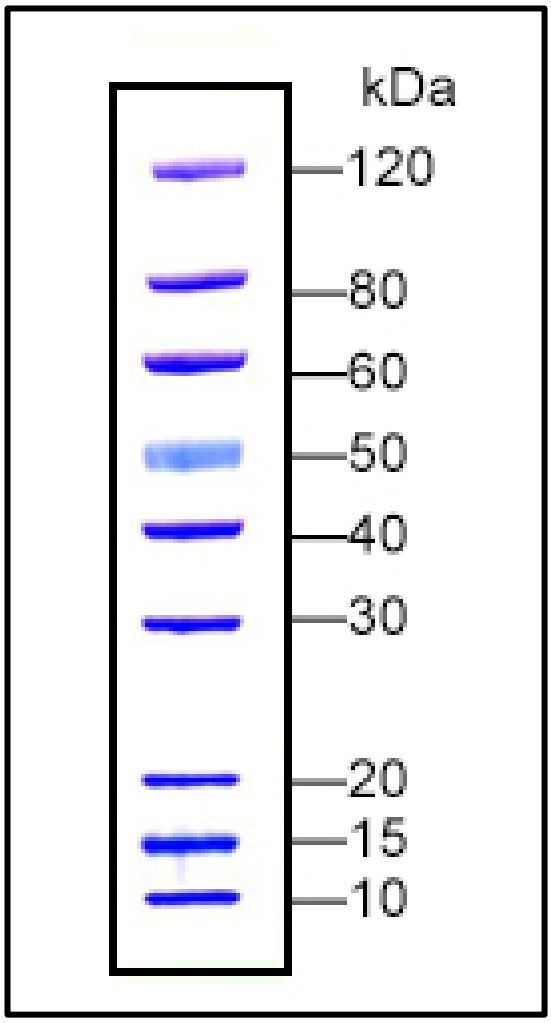

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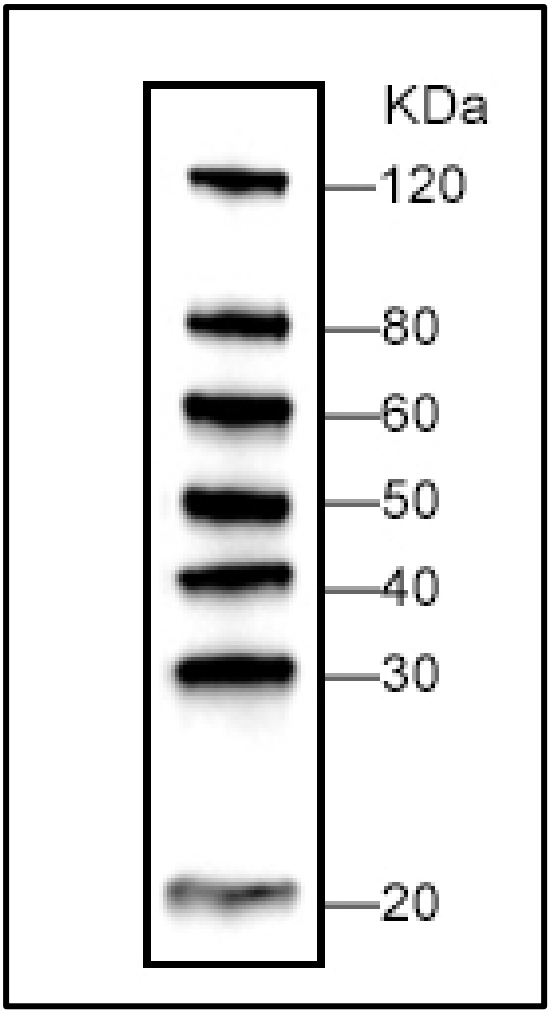

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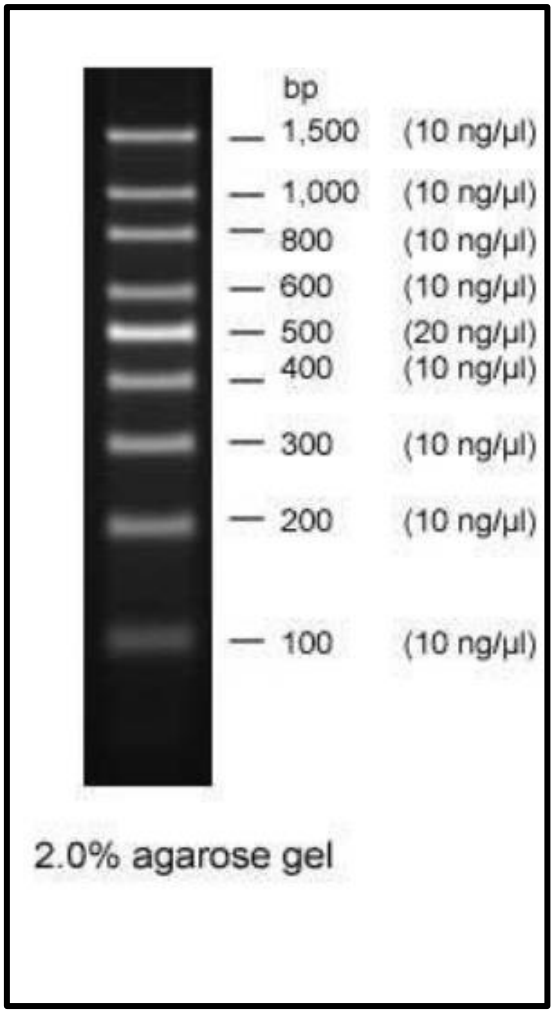

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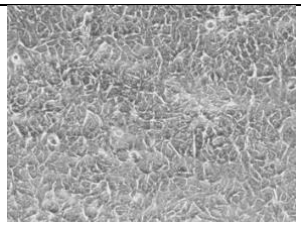
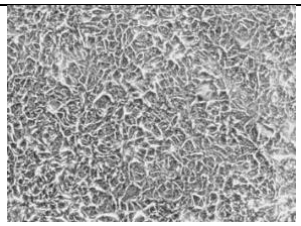
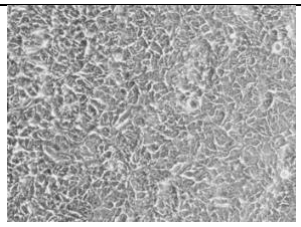
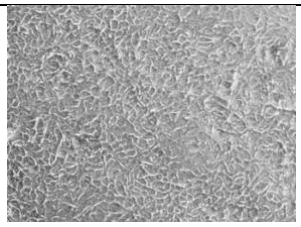
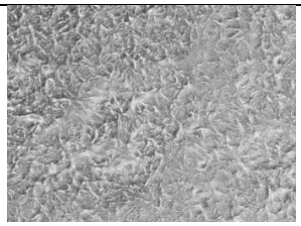
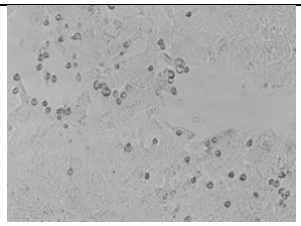
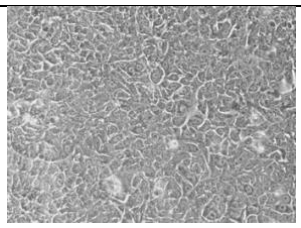
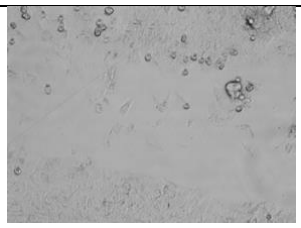
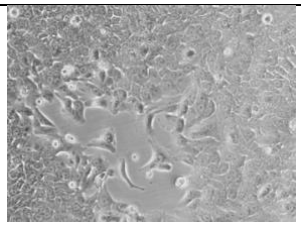
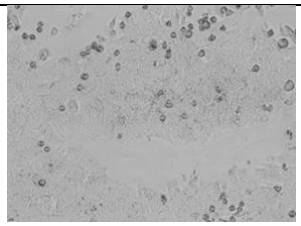
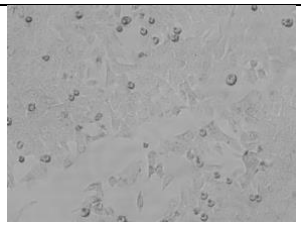
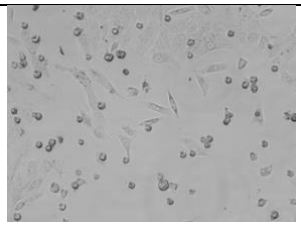
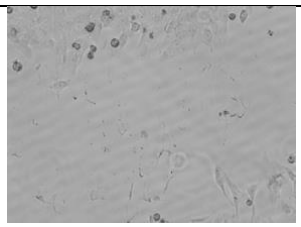
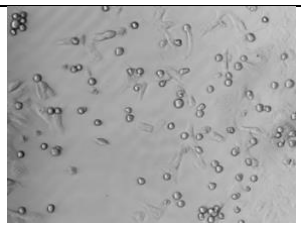
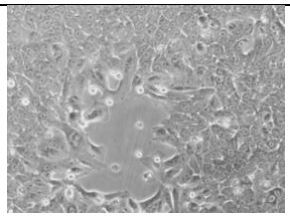
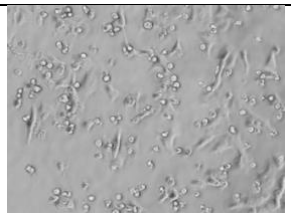
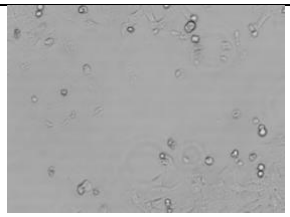
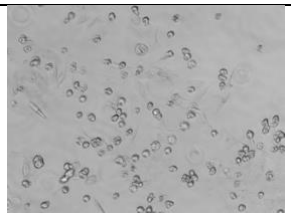
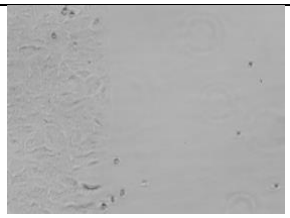
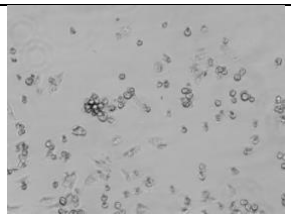
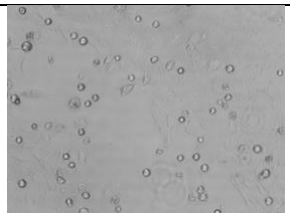
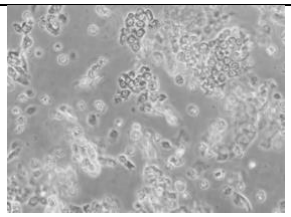
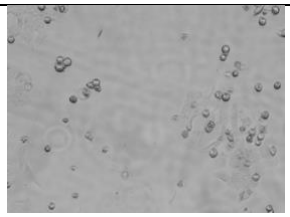
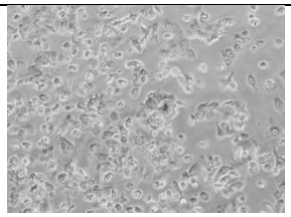
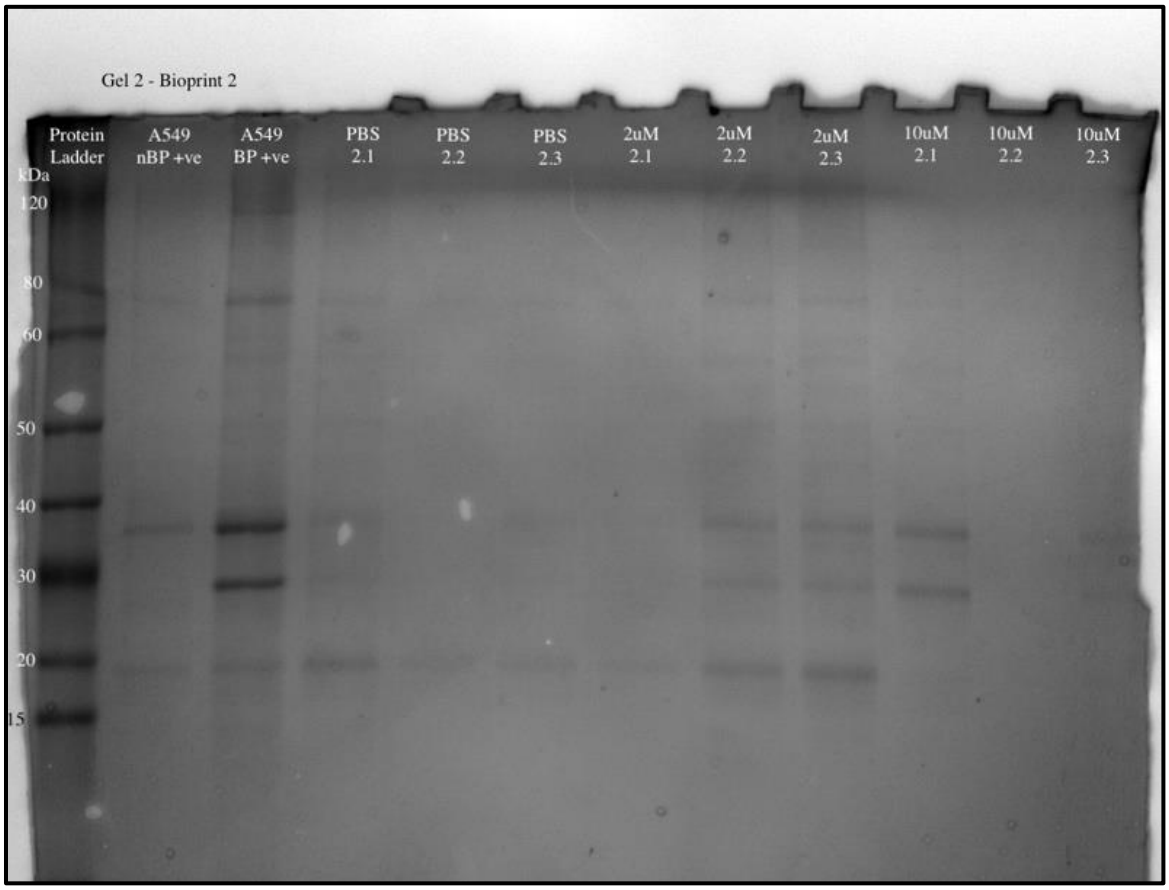
0 μg/mL		
50 μg/mL		
100 μg/mL		
200 μg/mL		
300 μg/mL		
400 μg/mL		
500 μg/mL		

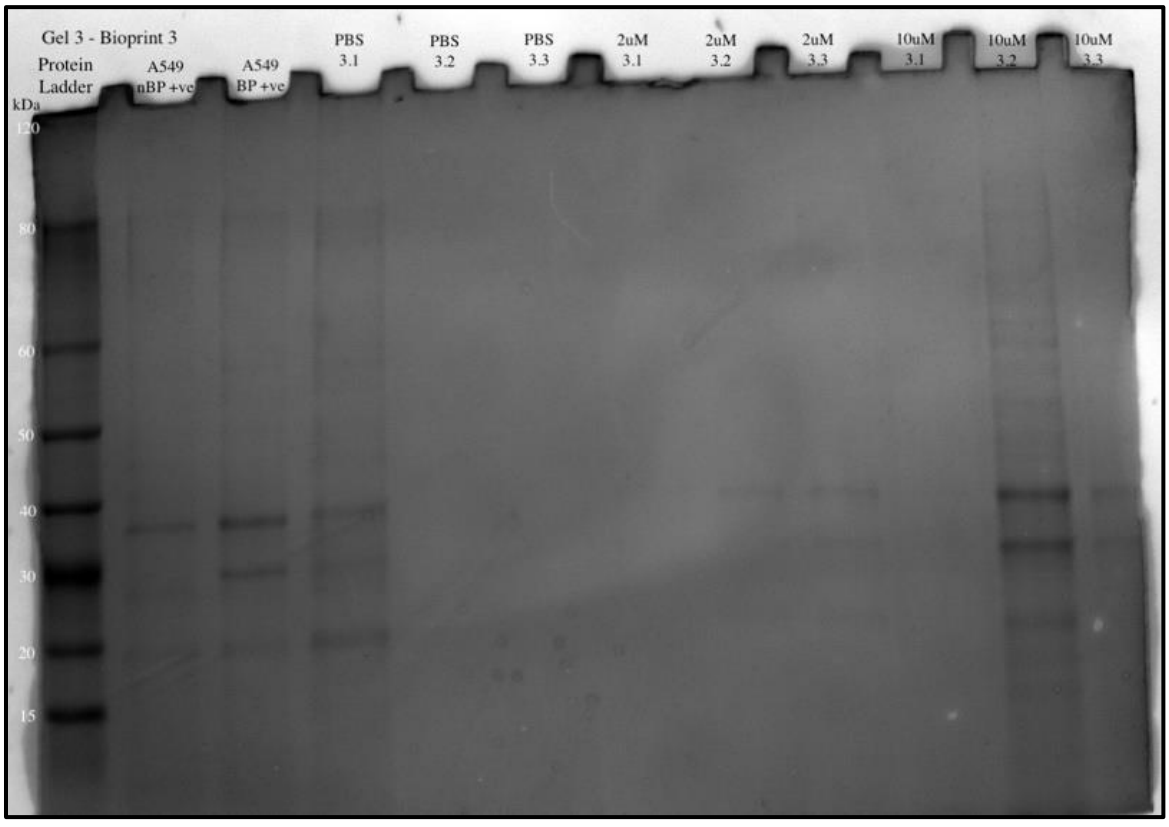
600 µg/mL		
700 µg/mL		
800 µg/mL		
900 µg/mL		
1000 µg/mL		

Bioprinting Protein Concentration and Quality

Sample	Concentration (mg/mL)
BP1 (28/8/23)	
PBS1.1	0.50
PBS1.2	1.42
2μM 1.1	0.511
2μM 1.2	0.407
10μM 1.1	0.714
10μM 1.2	0.173
BP2 (14/9/23)	
PBS2.1	0.994
PBS2.2	1.8
PBS2.3	0.696
2μM 2.1	2.19
2μM 2.2	2.97
2μM 2.3	1.99
10μM 2.1	0.821
10μM 2.2	2.77
10μM 2.3	2.16
BP 3 (25/9/23)	
PBS3.1	0.856
PBS3.2	3.24
PBS3.3	3.42
2μM 3.1	0.445
2μM 3.2	1.12
2μM 3.3	0.1
10μM 3.1	0.1
10μM 3.2	0.25
10μM 3.3	0.1

Bioprinting PAGE-Gels





Bioprinting RNA Concentration and Quality

Sample	Concentration (ng/μL)	Total Concentration (μg/μL)	A260	260/230	260/280
BP1 (30μL)					
PBS 1.1	1642.231	49.26	41.0858	0.696	1.671
PBS 1.2	318.935	9.54	7.9734	0.518	1.585
MSA 2μM 1.1	1189.009	35.67	29.7252	0.923	2.025
MSA 2μM 1.2	467.417	14.01	11.6854	0.588	1.487
BP2 (90μL)					
PBS 2.1	303.539	27.27	7.5885	0.775	1.982
PBS 2.2	5750.260	517.5	143.7565	1.015	1.906
MSA 2μM 2.1	479.435	43.11	11.9859	0.504	1.734
MSA 2μM 2.2	5155.055	463.95	128.8764	1.107	1.874
MSA 2μM 2.3	7479.594	673.11	186.8649	1.028	1.979
BP3 (90μL)					
PBS 3.1	140.544	12.6	3.5136	0.597	1.700
PBS 3.2	913.813	82.17	23.2953	0.567	1.880
PBS 3.3	122.514	10.98	3.0628	0.736	1.709
MSA 2μM 3.1	380.627	34.2	9.5157	0.452	1.596
MSA 2μM 3.2	133.413	11.97	3.3353	0.459	1.641
MSA 2μM 3.3	109.069	9.81	2.7267	0.409	1.655
MSA 10μM 3.1	255.187	22.95	6.3797	0.524	1.578
MSA 10μM 3.2	270.782	24.3	6.7695	0.603	1.516
MSA 10μM 3.3	164.415	14.76	4.1104	0.582	1.480

APPENDIX TWO

Recipes:

0.5M EDTA:

18.61g EDTA

Dissolve in 80mL of ddH_2O. pH to 8 with sodium hydroxide.

Autoclave.

Store at RT.

10% Blocking Solution

In a 50mL falcon tube mix:

2.5g Anchor Non-fat Dry Milk

Make up to 25mL with 1X TBST.

Store for up to one week at 4 °C.

10mg/mL Proteinase K

10mg Proteinase K

10mM 1M Tris

1mM 0.5M EDTA

Make up to 1mL with sterile MQ water.

Store at -20°C.

10X PBS

40g sodium chloride

1g potassium chloride

7.2g disodium phosphate

1.2g monopotassium phosphate

Dissolve in 800mL ddH_2O.

Make up to 1L with ddH_2O.

Store at RT.

10X TBS

24.2g Tris base

80g NaCl

Mix in 800mL sterile MQ H2O

Adjust pH to 7.6 with HCl

Make up to 1000mL with sterile MQ water.

Autoclave.

Store at RT.

1X TBST

100mL 10X TBS

1mL of Tween20 (Final concentration – 0.1%)

Make up to 1000mL with sterile MQ water.

Store at RT.

10X Tris-Glycine SDS Buffer

30.2g Tris

188g Glycine

10g SDS

Fill to 800mL ddH_2O, add all the reagents and mix.

Top up to 1000mL ddH_2O.

Store at RT.

1M Tris (Tris-HCl):

12.11g Tris

Dissolve into 80mL of ddH_2O. Adjust to pH7 with hydrogen chloride.

Allow to cool to room temperature before making final adjustments.

Make up to 100mL.

If solution turns yellow, discard and start again.

Store at RT.

1X Ponceau Stain (0.1% Ponceau-S, 1% Acetic Acid)

0.5g Ponceau-S

5mL Acetic acid

Up to 500mL sterile MQ water.

Store at RT.

2X Protease Inhibitor Cocktail Solution

cOmplete™ Ultra Tablets mini *EASYpack*, Roche, NZ, 05892970001

1 Tablet

5mL ddH_2O

Vortex for 3 minutes until the tablet has completely dissolved.

Aliquot into 200µL.

Store at -20°C.

3M Sodium Acetate

40.8g sodium acetate

Dissolve in 70mL of DDI. pH to 5.2 with glacial acetic acid.

Adjust to 100mL with DDI.

Autoclave.

Store at RT.

4M Sodium chloride

23.38g sodium chloride

Dissolve in 80mL of DDI.

Adjust to 100mL with MQ H_2O.

Autoclave.

Store at RT.

4X Laemmili dye with βME

900µL 4X Laemmili Dye

100µL β mercaptoethanol (βME)

Make in 5mL tube.

Store at RT.

50X TAE Buffer

242g Tris base

57.1mL Glacial acetic acid

100mL EDTA

Dissolve into 1000mL MQ H_2O.

Dilute 40mL 50X TAE into 2L of water for 1x TAE buffer.

Store at RT.

5M Sodium chloride

29.2g sodium chloride

Dissolve in 80mL of DDI.

Adjust to 100mL with MQ H_2O.

Autoclave.

Store at RT.

70% (v/v) EtOH

70mL of absolute EtOH (100%)

30mL of sterile DEPC water.

Store at RT.

A549 Cell Media:

10% FBS

1X Pen/Strep

F.12 Medium

Store at 4°C.

A549 Freezing Media:

10% FBS

1X Pen/Strep

5% DSMO

F.12 Medium

Store at 4°C.

Ampicillin 50mg/mL

50mg/mL Ampicillin

1mL ddH_2O

Sterilise using 0.22μM filtration.

Store at -20°C.

DEPC Water (0.1%)

1mL DEPC

Fill to 1L using ddH_2O

Mix overnight in fume hood using a magnetic stirrer.

Autoclave.

Store at RT.

Digestion Buffer

0.5mL 1M Tris

1mL 0.5M EDTA

1mL 5M sodium chloride

2.5mL 10% SDS

Make up to 50mL with ddH_2O.

Do not autoclave.

Store at RT.

Digestion Buffer with Proteinase K

10μL 10mg/mL Proteinase K

190μL Digestion Buffer

Make fresh and use immediately.

LB Agar Plate

32g LB Agar powder

1000mL ddH_2O

Add half of the ddH_2O first then the powder, mix with a magnetic spinner.

Add the other half of the ddH_2O.

Autoclave.

Store at 4°C.

LB Broth

25g LB Powder

1000mL ddH_2O

Add half of the ddH_2O first then the powder, mix with a magnetic spinner.

Add the other half of the ddH_2O.

Autoclave.

Make fresh and use immediately.

Lysis Buffer

2.5mL 1M Tris

1.9mL 4M NaCl

200µL 0.5M EDTA

250µL 100% Triton X-100

Prepare in a 50mL Falcon Tube.

Make up to 25mL with MQ water.

Store at RT.

When protease inhibitor is added the lysis buffer will be 1X dilution.

Lysis Buffer with Protease Inhibitors (1:1)

200µL Lysis Buffer

200µL 2X Protease Inhibitor Cocktail Solution

Make fresh and use immediately.

Methylselenic Acid (MSA)

CH4O2Se molecular weight = 127g/mol

127g into 1000mL = 1M

0.127g into 1mL = 1M

To make to 2mL of **1M MSA**: 0.127g x 2 = 0.254g of MSA

Weigh 0.254g of MSA in a screw top 2mL tube and fill to 2mL with 1X PBS. Vortex.

#Note: there is 0.41g of elemental Se in 2mL of MSA solution (1M): Se ÷ CH4O2Se = x

78.96u ÷ 127g = 62%

0.254g ÷ 62% = 0.4096g

= 0.41g of Se in 2mL MSA (1M)

To make **100µM**: 100µL of 1M MSA solution was pipetted into a new tube (screw top, 2mL) and made up to 2mL with 1X PBS. Solution was vortexed.

To make **10µM**: 100µL of 100µM MSA solution was pipetted into a new tube (screw top, 2mL) and made up to 2mL with 1X PBS. Solution was vortexed.

200µL of each 1M, 100µM and 10µM solutions were aliquoted out into separate tubes.

Mild Stripping buffer

15g Glycine

1g SDS

10mL Tween20

Adjust pH to 2.2

Bring volume up to 1L with MQ water.

Store at RT.

Ponceau Destain (1% acetic acid)

5mL Acetic acid

Fill up to 500mL with sterile MQ water.

Store at RT.

Sodium Chloride (1.2 M), Sodium Citrate (0.8 M) 50 mL Solution

Weighed 3.5g NaCl (58.44g/mol)

Weighed 10.33g NaCit (258.1g/mol)

Transfer NaCit and NaCl to a Schott bottle (100mL).

Make up to 45mL using ddH2O.

Adjust pH to 7.0 using HCl and NaOH.

Adjust volume to 50mL using ddH2O.

Autoclave.

Store at RT.

TE Buffer (pH 8.0)

0.2mL 0.5M EDTA

1mL 1M Tris

Make up to 100mL with sterile ddH_2O.

Store at RT.