Cloning, Expression and Purification of β-Defensins from *S. salar* (Atlantic Salmon)

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Declaration

I (Veronica Gomes), Declare that the work in this thesis is my own. All externally sourced materials and images have been cited and referenced according to the Harvard referencing system.

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Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations

AGD	Amoebic Gill Disease
AMP	Antimicrobial Peptide
AMR	Antimicrobial Resistance
BSA	Bovine Serum Albumin
CRP	C-Reactive Protein
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic
eGFP	Enhanced Green Fluorescent Protein
HIS	Histidine
IHNV	Infectious Hematopoietic Necrosis Virus
IgM	Immunoglobulin M
IPNV	Infectious Pancreatic Necrosis Virus
IPTG	Isopropyl B-D-1-Thiogalactopyranoside
ISA	Infectious Salmon Anaemia
ISAV	Infectious Salmon Anaemia Virus
I-TASSER	Iterative Threading Assembly Refinement
LB	Luria-Bertani
LOMET	Locally Installed Meta-Threading Approach
LPS	Lipopolysaccharide
MBP	Maltose-Binding Protein
MIC	Minimum Inhibitory Concentration
PAMP	Pathogen Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMSF	Phenylmethylsulfonyl Fluoride
PVDF	Polyvinylidene Fluoride
RNA	Ribose Nucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TBST	Tris Buffered Saline (with Tween 20)

Abstract

Introduction: The purpose of this study was to develop a method to recombinantly produce salmon β-defensins in the prokaryotic host Escherichia coli. β-defensins are a group of antimicrobial peptides (AMPs) which display potent and immunomodulatory properties in several species. Three expressed sequence tags (EST) corresponding to salmon β-defensins were previously identified by GenBank accession no: CK892029, CK895920 EG781611). It was hypothesised that these expressed sequence tags from the S. salar genome contain the genes corresponding to 3 β-defensin AMPs that are important mediators of the innate immune system of salmon. Given this information, the aim of this project is to clone these 3 genes into E. coli, followed by induction of overexpression of the gene products and purify these peptides using affinity chromatography.

Methods: β-defensin genes 1, 3 and 4 were genes were commercially synthesised by Integrated DNA Technology and amplified using polymerase chain reaction (PCR). The β-defensin 4 gene was ligated into the pET44a expression plasmid creating a NusA-β-defensin 4 fusion gene. All 3 β-defensin genes were ligated into the pMAL-p4X expression plasmid creating MBP-β-defensin fusion genes. Only the MBP-β-defensin 4 fusion gene were transformed into Rosetta gami and BL21 (DE3) E. coli cells and expression trials were carried out using both IPTG (Isopropyl B-D-1-Thiogalactopyranoside) and auto-induction for HIS tagged-NusA-β-defensin 4 fusion peptide and autoinduction was used to overexpress the MBP-β-defensin 4 fusion peptide. Nickel affinity chromatography using Nickel-NTA Sepharose resin was used to purify the resulting NusA-β-defensin 4 protein and amylose affinity resin was used to purify the MBP-β-defensin 4. Protein concentration and yield was quantified using both the Bradford and BCA assays and purity was assessed using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and Western blotting.

Results: β-defensins 1, 3 and 4 genes were successfully cloned and the sequences of the products were verified by Sanger sequencing. Expression studies were carried out with the β-defensin 4 peptide only. Optimal expression was successful using the pMALp4x expression plasmid in BL21 (DE3) cells with autoinduction for 48 hours at 37°C. A total of 5.2 mg of MBP-β-defensins 4 fusion protein were produced per litre of E. coli expression culture.

Conclusion: The findings from this project have made a significant impact on the development of an in vitro laboratory method to produce recombinant Salmo salar β-defensins in E. coli. Although further work is needed to complete the cleavage of the fusion tag from the mature β-defensin, the majority of the protocol has been optimised and can be applied to β-defensin 1 and 3. The study has investigated important core strategies which central to future investigations of salmon β-defensins such as structural studies, minimum inhibitory concentration (MIC) assays and membrane permeability and amoebicidal assays.

Chapter 1: Introduction

Atlantic salmon are continuously exposed to microorganisms which threaten their health. These include bacteria such as infectious salmon anaemia virus (ISAV), *Neoparamoeba perurans* and sea lice. Salmon aquaculture is a valuable to the Irish economy. Therefore, it is essential that any threats to the salmon industry are eliminated. Antibiotics are used to treat infections and diseases in the salmon industry. One of the biggest problems that humans and animals are facing now is the increase in antibiotic resistance. Antibiotic resistance arises when microorganisms develop mutations allowing them to become resistant to the effect of antibiotics. It is an issue which can have catastrophic consequences. AMPs have been investigated as an alternative to antibiotics. AMPs are useful as an alternative to antibiotics since they are produced naturally and are found in several sites throughout the body. There have been very few cases of resistance to AMPs recorded to date leading researchers to think it is a good alternative (Andersson *et al.*, 2016). This project seeks to develop a strategy whereby significant amounts of these peptides can be produced and purified. It is believed that this will lay the foundation for the development of new antimicrobial treatments for the salmon industry and in turn to human healthcare. β-defensins have been studied for several species however, not much attention has been given to β-defensins in salmon. Three previously identified salmon β-defensins by (Adzhubei *et al.*, 2007) (Bdef1; GenBank accession number: CK892029, Bdef3; GenBank accession number: CK895920 and Bdef4; GenBank accession number: EG781611) are used as the target genes for this project.

It was hypothesised that the 3 previously identified expressed sequence tags from the S. salar genome contain the genes corresponding to 3 β-defensin antimicrobial peptides that play an important role in the innate immune defense system of this teleost. The aim of this project is to isolate and purify 3 β-defensin AMPs from Atlantic salmon. Specifically, the objectives are:

1. Amplify the 3 β-defensin genes using specific primers designed to clone them from Atlantic salmon into *Escherichia coli*.
2. To induce overexpression of the β-defensin genes products.
3. To purify the resulting 3 β-defensin peptides using affinity chromatography and produce large quantities of the β-defensin peptides to be utilised for future studies to investigate the structure, function and mechanism of β-defensins in salmon.

Chapter 2: Development of Theoretical Perspective

2.1. Atlantic Salmon Immunity

Salmon belong to a family of ray-finned fish known as Salmonidae. The Salmonidae family also contain other species such as trout, whitefish and char (ITIS, 2003). Salmon are mostly found in the North Atlantic and the Pacific Ocean. *Salmo salar*, also known as the Atlantic salmon is the only salmon species found in the Atlantic Ocean, whereas many other species are found in the Pacific ocean such as Pink salmon (*Oncorhynchus gorbuscha*), Coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tschawytscha*) (Marine-Institute, 2017). Atlantic salmon are anadromous fish, who migrate from the sea to rivers to spawn (Behnke *et al.*, 2002).

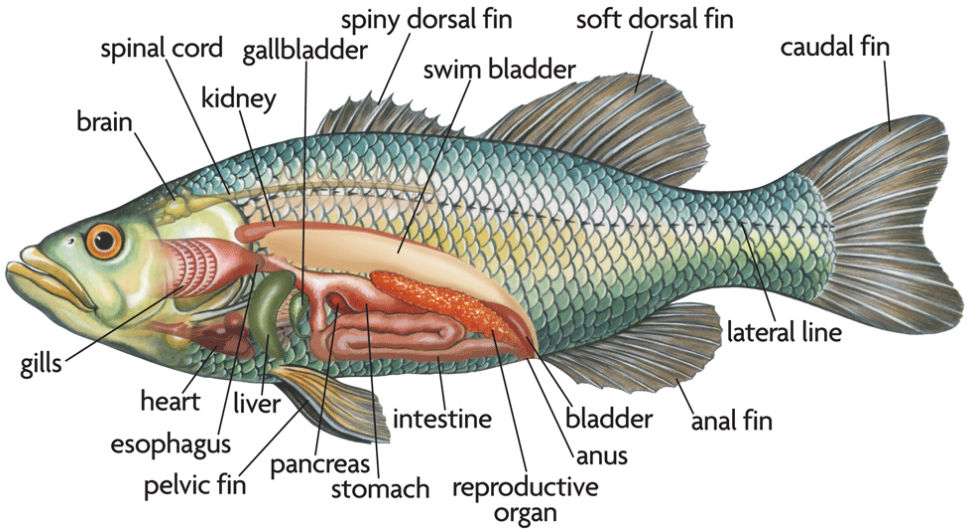


Figure 1: The Internal Anatomy of a Teleost (Animalia-Life, 2017)

Salmon primarily rely on their innate immune system as they are exposed to a range of pathogens from the embryogenesis phase of their life. Their innate immune system consists of many humoral factors, physical barriers and cellular components (refer to Figure 2 for an overview of teleost immune response). There are many similarities between the immunology of teleost and vertebrates. Salmon contain a population of lymphocytes that are comparable to humans. Since salmon are poikilotherms, their lymphocytes are not as efficient as those in humans, hence they have to depend on their innate immune system to fight against pathogens (Whyte, 2007).

Similar to human immunity, immunoglobulins play an important role in the immunity of salmon. Salmon also contain lymphoid organs like spleen and thymus. In salmon, the

thymus and the anterior kidney is completely developed before eggs are hatched (Razquin *et al.*, 1990).

Due to the presence of various pathogens in the oceans, it is crucial for salmon to have robust immune barriers (refer to Figure 1 for the anatomy of salmon). The gills, mucous and secretions act as the first line of defence (Ellis, 2001). The mucous membrane secretions of teleosts contain several important components including complement proteins, lysozyme, antimicrobial peptides and immunoglobulins. These molecules have broad-spectrum antimicrobial functions which prevent the entry of microbes or immediately kill the pathogen upon entry and prevent further spread of infections. Cellular hyperplasia and the thickening of the epidermis in response to different conditions also contributes as a physical barrier. This is also essential to prevent entry of pathogens and maintain homeostasis (Ellis, 2001). The gastrointestinal tract of salmon contains enzymes, acids and bile salts which allow the digestion of many pathogens. Antimicrobial peptides such as defensins and cathelicidins are low molecular weight peptides which have been found in many organs of teleosts (Birkemo *et al.*, 2003). It has been suggested they function in the destruction of bacterial cell (Ellis, 2001).

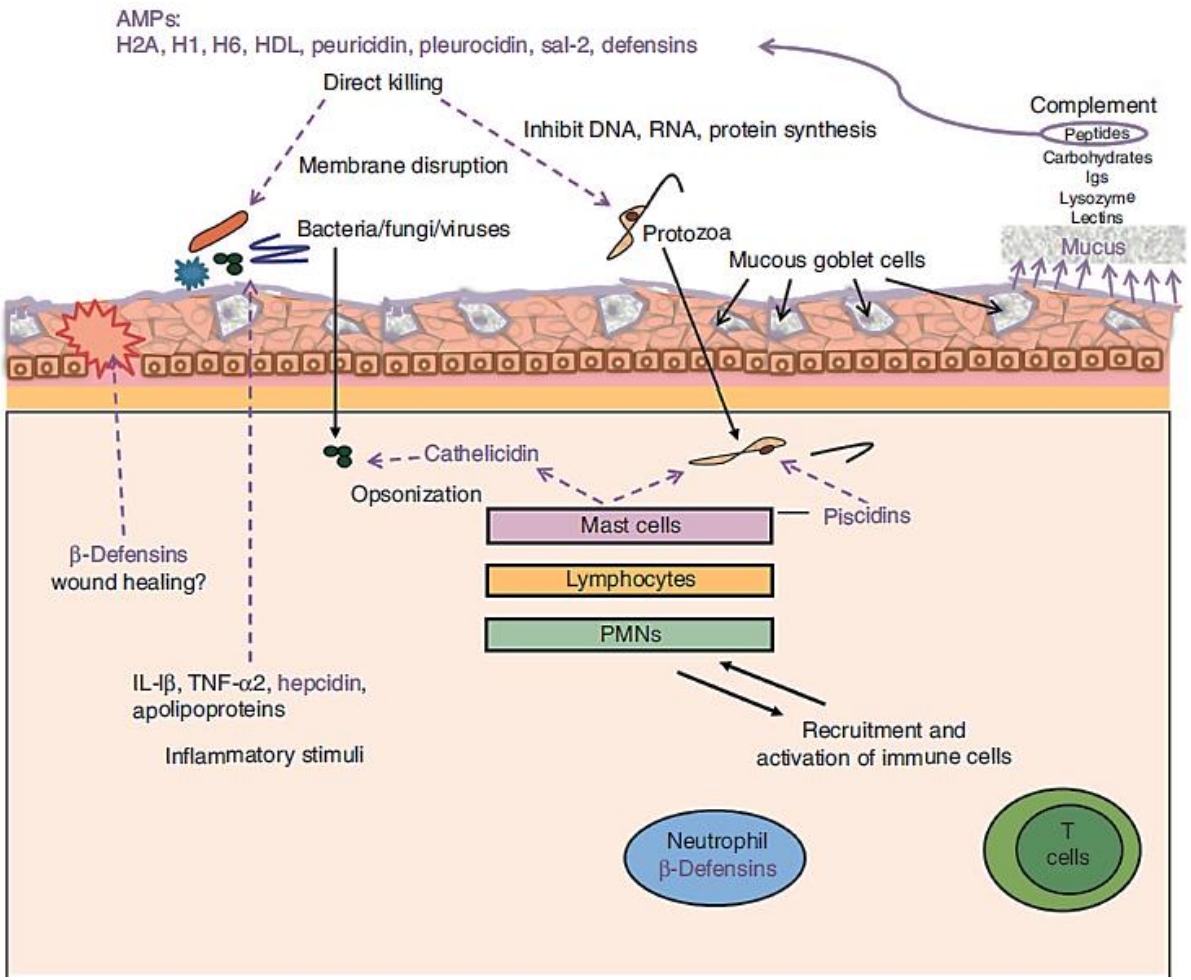
Upon invasion of the teleost by a pathogen, a range of immune reactions take place. Salmon contain receptor proteins such as IL-1 which are capable of recognising pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycans and intracellular components of pathogenic organisms. When these PAMPs are recognised, the complement system is activated. C-reactive protein (CRP) binds to phosphocholine found in pathogens. It has been suggested which have a central role in complement activation. CRP expression has been shown in *S. salar* by (Lund and Olafsen, 1998). Increased levels of expression of CRP have been found as a result of infection and injury. Upon recognition of the gram-negative cell wall of invading pathogens, chemokines are also produced. This allows the attachment of neutrophils and macrophages to the pathogen, resulting in phagocytosis. Complement activation by opsonisation has been found in Salmonids by (Lammens *et al.*, 2000). The complement system can also initiate inflammation and induces the production of antibodies by B cells. These antibodies opsonize the pathogen, also resulting in phagocytosis.

Immunoglobulins in salmon are primarily found in the plasma, skin, gills, intestine and bile (Rombout *et al.*, 1986; Lumsden *et al.*, 1993; Hatten *et al.*, 2001). Salmonid immunoglobulins have neutralised enveloped viruses such as the viral haemorrhagic

septicaemia virus and the infectious hematopoietic necrosis virus (IHNV) previously (Lorenzen and Lapatra, 1999).

Immunoglobulin M (IgM) is the predominant class of antibodies in teleosts (Acton *et al.*, 1971). However, upon comparison of serum IgM levels of salmon and other teleosts, results showed much lower levels in salmon (Uchida *et al.*, 2000). Studies have shown that IgM concentration varies in salmon depending on size, temperature, season and water quality (Olesen and Jørgensen, 1986; Sanchez *et al.*, 1993). A number of bactericidal components are present in the plasma of salmon such as lysozyme. In salmon, lysozyme has been detected in the serum, mucous, secretions and tissues such as intestine and kidney (Grinde *et al.*, 1988). Cytokines also have a role in inflammation in teleosts. Scientists have suggested the IL-1 receptor has a role in the regulation of IL-1β in inflammation (Sangrador-Vegas *et al.*, 2000; Subramaniam *et al.*, 2002). IL-1 receptors have also been cloned from *S. salar*. Constitutive expression was determined for the IL-1 receptor in this species.

During viral infections, interferon is produced by the infected cells, preventing the spread of further infection. IFNα-1 and IFNα-2 have been successfully cloned and characterised from *S. salar* (Kileng *et al.*, 2007; Rokenes *et al.*, 2007). These two interferons have similar characteristics to interferons found in mammals (Rokenes *et al.*, 2007). Studies have found that INFα-1 exhibits a strong antiviral reaction against infectious pancreatic necrosis virus (IPNV). In contrast, it is ineffective against infectious salmon anaemia virus (ISAV) (Kileng *et al.*, 2007).



(Rakers *et al.*, 2013)

Figure 2: Schematic Representation of Immune Responses in Teleosts

2.1.1. Anti-Microbial Peptides

AMPs, also known as host defence peptides are a group of small, unique molecular weight protein molecules (18-46 amino acids). These peptides have a broad antimicrobial function which target bacteria, viruses, fungi and parasites (Katzenback, 2015). They are widely distributed in many species. In teleosts, several classes of AMPs exist including defensins, cathelicidins, hepcidins, histone-derived peptides and piscidins (Masso-Silva and Diamond, 2014). Expression of AMPs is constitutive although they can also be induced during infection. The primary mechanism for AMPs production involves recognition of PAMPs by toll like receptors, which initiates intracellular signalling to produce and activate AMPs (Zasloff, 2002). Upon synthesis of prepropeptides, AMPs are present in an inactive form. Enzymatic cleavage is required to release the activated mature peptide. Many AMPs have been identified to date across various species. A database called The Antimicrobial Peptide Database has been created to organise all the information (Wang,Li, *et al.*, 2016). To date over 110 AMPs have been identified in fish. They can be categorised into five groups; β-defensins, hepcidins, cathelicidins, histone-derived peptides and piscidins (Wang,Li, *et al.*, 2016). The overall structures of these AMPs are remarkably similar, along with their microbicidal and immunomodulation functions.

2.1.1.1. Defensins

Based on the tertiary structure, the defensins are sub-categorised into three families; α-defensins, β-defensins and θ-defensins. Defensins have been found in many fish species to date; Atlantic cod, Chinese loach, common carp, gilthead sea bream, Japanese pufferfish, Mandarin fish, olive flounder, orange-spotted grouper, rainbow trout and many more (Masso-Silva and Diamond, 2014). The θ-defensins have only been found in monocytes and neutrophils of rhesus monkeys (Garcia *et al.*, 2008). Defensin genes are closely related between species, as shown in Figure 3. To generate Figure 3, the FASTA sequence was retrieved from the NCBI Database and Clustal Omega was used to generate a phylogenetic tree to demonstrate the homology of various β-defensins genes amongst different species. Figure 3 illustrates the genetic homology between β-defensins from *S. salar* and other teleosts including *G. morhua, O. mykiss, T. rubripes* and *D. rerio*.

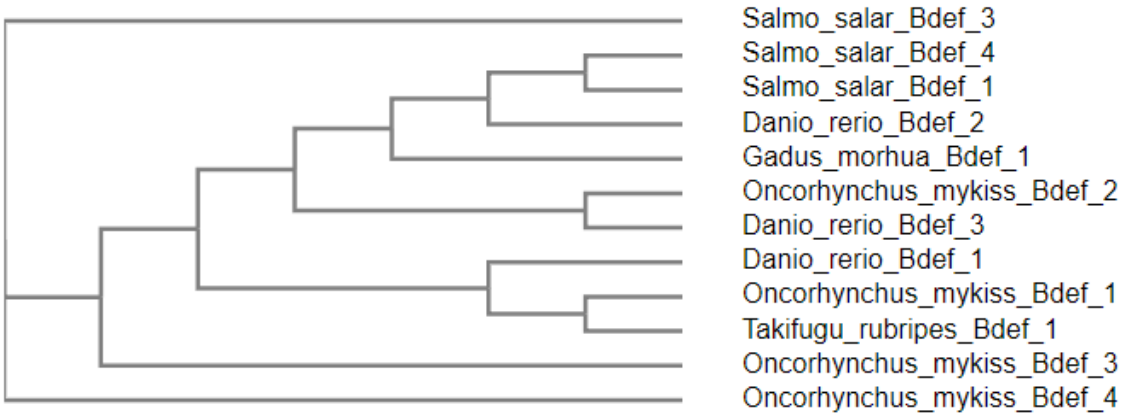


Figure 3: Sequence Alignment and Phylogenetic Tree of β-Defensin between Different Species

The human α-defensin is a small peptide between 3 to 4 kDa, with disulphide bonds between cysteines 1-6, 2-4 and 3-5. In contrast, β-defensins are slightly bigger between 4 to 6 kDa, with disulphide bonds between cysteines 1-5, 2-4 and 3-6. These molecules have a complex tertiary structure (Oppenheim *et al.*, 2003). Θ-defensins are products of translational splicing of 2 α-defensins derived nanopeptides. Constitutive expression has been reported for all types of defensins, however during an inflammatory response, increased levels of expression has been evident (Masso-Silva and Diamond, 2014). Defensins exhibit bactericidal, fungicidal and antiviral activity (Katzenback, 2015). Using Clustal Omega, Figure 4 was generated to show the sequence homology between salmon and human β-defensins. This phylogenetic tree demonstrates the sequence homology between S. salar β-defensins (SsBD1, SsBD3 and SsBD4) and human β-defensins (hBD1-6, hBD26-28, DEFB114, DEFB118, DEFB120 and DEFB130)

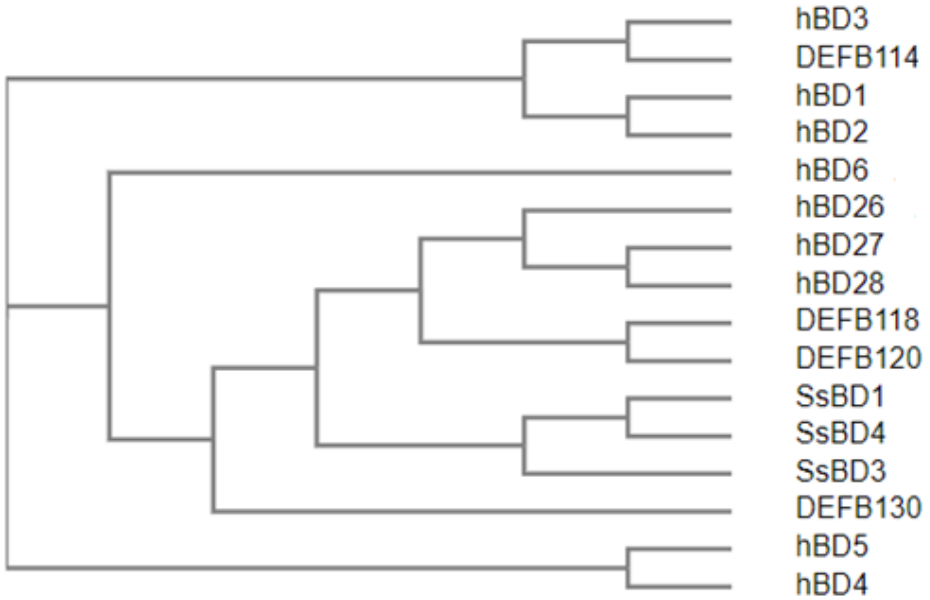


Figure 4: Sequence Alignment and Phylogenetic Tree Illustrating Homology between β-Defensin from S. salar and Humans

2.1.1.2. Cathelicidins

Fish cathelicidins are sub-categorised into two families; cathelicidin 1 and cathelicidin 2 based on the presence or absence of disulphide bonds between the cysteines (Chang *et al.*, 2006). The first cathelicidins in teleosts; HFIAP-1, 2 and 3 was identified and isolated from Atlantic hagfish (Uzzell *et al.*, 2003). Unlike other AMPs, cathelicidins in teleosts do not share similar sequence between different classes, due to presence or absence of disulphide bonds (Masso-Silva and Diamond, 2014). In contrast, there is ~90% sequence homology in cathelicidins from mammals. The cathelicidin polypeptide consists of a signal sequence, a cathelin domain (1-3 exons) and a C-terminal region (4 exons) containing the elastase cleavage site and the unique mature peptide. Cathelicidins are expressed from early stages of life in teleosts. In adult fish expression levels vary and depend on the species, tissue and the cathelicidin gene (Broekman et al., 2011). Comparable to defensins, cathelicidins also have broad-spectrum antimicrobial activity against Gram positive bacteria, Gram negative bacteria and fungi (Masso-Silva and Diamond, 2014).

2.1.1.3. Hepcidins

The first hepcidin to be identified, LEAP-1 was in humans (Krause *et al.*, 2000). However, it has since been identified in many species. Fish hepcidin consist of a three-exon/two-intron genomic structure. However, human hepcidins can be a single gene. Studies for mammals have shown hepcidin expression is predominantly in the liver with the main function being iron homeostasis along with microbicidal activity *in vitro* (Shi and Camus, 2006). Only one copy of the hepcidin gene is present in humans, whereas several copies of the hepcidin gene can be found in teleosts, due to whole genome duplication. Two copies of the hepcidin gene have been found in Atlantic salmon (Douglas *et al.*, 2003).

2.1.1.4. Histone-Derived Peptides

The proteolytic cleavage of histones found in the skin and mucous of fish forms fragments of peptides (~10kda) known as histone-derived peptides (Katzenback, 2015). Studies have shown that these peptides are formed because of damage to the epidermis and in response to LPS. They are bactericidal, parasiticidal, fungicidal and viricidal. It has been demonstrated by (Bustillo *et al.,* 2014) that the mechanism of action of some classes of histone-derived peptides involves binding and permeabilizing the cell membrane of the pathogen (Katzenback, 2015).

2.1.1.5. Piscidins

Piscidins are a family of fish-specific antimicrobial peptides and they consist of piscidins, moronecidin, pleurocidin, misgurin, gaduscidin, epinecidin and dicentracin (Sun *et al.,* 2007; Pan *et al.,* 2008; Browne *et al.,* 2011). The members of the piscidins family differ in gene sequence. Piscidins have been identified in many fish species. Some species only express one class of piscidin while other's can express up to all five classes (Katzenback, 2015). Most of the genes contain four-exon/three-intron gene structure (Sun *et al.,* 2007). Comparable to other AMPs the proteolytic cleavage of the prepropeptide releases the mature peptide (~3kDa). Varying levels of piscidin-gene expression are reported between species. High levels are found in mature fish skin, head kidney, gill and spleen (Katzenback, 2015). Similar to defensins and cathelicidins, piscidins also have broad-spectrum antimicrobial activity against Gram positive bacteria, Gram negative bacteria, parasites and fungi. The mechanisms of microbial actions include pore formation, permeabilization of the membranes, production of oxygen reactive species and initiating apoptosis (Cho and Lee, 2011; Katzenback, 2015).

2.1.2. Beta-Defensins in Teleost

In fish, β-defensins were originally identified in zebrafish (*Danio rerio*) and pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*) (Zou *et al.,* 2007). Generally, β-defensin genes exhibit a three-exon/two-intron structure (Zou *et al.,* 2007). Not all species share the same number of β-defensin genes. Atlantic cod has only one copy of the gene (Ruangsri *et al.,* 2013), while two copies are found in blunt snout bream (Liang *et al.,* 2013), three in carp (Nam *et al.,* 2010; Marel *et al.,* 2012), four gene copies in rainbow trout and five copies in olive flounder (Nam *et al.,* 2010). In teleosts with several β-defensin gene copies, some have been found on the same chromosome, linked to each other while others have been found on different chromosomes. This indicates the presence of multiple loci for β-defensins in teleosts (Zou *et al.,* 2007). β-defensins are produced as prepeptides. Upon synthesis, a signal peptide (18-24 amino acid) and a mature peptide (39-45 amino acid) are produced (Katzenback, 2015). An exception has been found for β-defensins in olive flounder, which also contains a proregion (15 amino acids) (Zou *et al.,* 2007). The mature peptide is small, approximately 4 -6kDa with 6 conserved cysteine residues. The CRRRYK/R motif can be found between cysteines 4 and 5. They have a net charge ranging from +1 to +7 (Zou *et al.,* 2007). Most β-defensin peptides have a 3-β strand secondary structure although some contain an α-helix in the N-terminus region (Ruangsri *et al.,* 2013). Constitutive levels of expression have been found in very early stages of life in teleosts. This indicates that β-defensins play a significant role in the immunity of fish during embryogenesis. Levels of expression vary from defensin gene and species of fish, with primary expression found in mucosal tissue and lymphoid organs (Cuesta *et al.,* 2011). In zebrafish, the expression of the β-defensin zfBD2 gene in ZF4 cells has shown to enhance translocation of NF-κB to the nucleus from the cytoplasm, thus indicating β-defensins play a role in the activation of immune cells through the NF-κB pathway (Zhu and Solomon, 1992). Based on previous work on other teleost species, β-defensins have a broad spectrum of antimicrobial function and some immunomodulatory roles. In zebrafish they have been found to recruit cytotoxic cells to the site of infection (Yang *et al.,* 1999). They have also been suggested to enhance phagocytosis in Atlantic cod (Ganz, 2003).

2.1.3. Beta-Defensins Humans

In humans, seventeen defensin peptides have been identified to date. These include six α-defensins and eleven β-defensins. The first β-defensin in human, hBD-1 was identified in 1995 from the plasma of renal disease patients (Bensch *et al.,* 1995). The β-defensins (n=17) are designated DEFB (Jarczak *et al.,* 2013). DEFB1-3 are structurally similar in terms of the six cystine residues and the presence of disulphide bonds. However, in DEFB5 and DEFB12 the first cysteine residue can be found at the N-terminus Constitutive expression of DEB1 is found in keratinocytes of the skin, whereas DEFB2-4 production is inducible by IL-1, tumour necrosis factor and LPS (Yang *et al.,* 2001). All human β-defensins have been found to inhibit the growth of a wide range of pathogens (refer to Table 1). The levels of antimicrobial activities vary between each type of human β-defensin. DEFB1 and DEFB2 have found significantly effective against Gram-negative bacteria and fungi but not as effective against Gram-positive bacteria (Bals *et al.,* 1998). DEFB3 has been found effective against vancomycin-resistant *Enterococcus faecium* (Garcia *et al.,* 2001). Studies have found DEFB2 and DEFB3 to be more effective against aerobic bacteria in comparison to anaerobic bacteria (Joly *et al.,* 2004).

Table 1: Table Outlining The Growth Inhibition of Pathogens by Human β-defensins

Defensin	Pathogen
hBD-1	*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae Staphylococcus aureus*
hBD-2	E. coli, P. aeruginosa, K. pneumoniae S. aureus, *Streptococcus pneumoniae, Candida albicans, Candida parapsilosis, Candida krusei, Enterococcus faecalis,* HIV-1
hBD-3	*E. coli, P. aeruginosa, K. pneumoniae, S. aureus, Streptococcus pyogenes, Ent. faecium, Strep. pneumoniae, Staphylococcus carnosus, Burkholderia cepacia, Saccharomyces cerevisiae, C. albicans, C. parapsilosis, C. krusei,* HIV-1
hBD-4	*E. coli, S. carnosus, P. aeruginosa, B. cepacia, Strep. pneumoniae, S. aureus, Sacch. cerevisiae,*
hBD-28	*E. coli, P. aeruginosa, K. pneumoniae, Strep. pneumoniae, S. aureus*
hBD-18	*E.coli*
hBD-6 pro-peptide	*E. coli*

Adapted from Pazgier *et al.*, 2006

2.2.Infectious Diseases in Salmon

The biggest threat to salmon health is caused by the infectious salmon anaemia virus (ISAV), *Neoparamoeba perurans* and sea lice. Bacterial infections can also contribute to salmon mortality although they are not as severe.

2.2.1. Infectious Salmon Anaemia

ISAV is the causative agent for infectious salmon anaemia (ISA). This is a systemic disease effecting farmed Atlantic salmon. The first case of ISA was found in Norway, but outbreaks of ISA have been reported since in Chile, Canada, USA and Scotland (Lauscher *et al.,* 2011). Symptoms include pale gills, haemorrhages, exophthalmia and significant decrease in haematocrit. Vaccines have been developed for ISA and an increase in survival of the vaccinated fish have been reported (Rm *et al.,* 1999).

2.2.2. Amoebic Gill Disease

Amoebic gill disease (AGD) is caused by *Neoparamoeba perurans,* which are a species of protozoan amoeba (Vincent *et al.,* 2006). This disease effects salmon by inducing a proliferative reaction in the epithelium of gills, resulting in thickening of the gills. This prevents the diffusion of oxygen through the epithelium, resulting in suffocation. Initial outbreaks were recorded in Australia, but the disease has since spread to Ireland, France, Scotland, Norway, Canada and USA. Symptoms include, mucoid patches, gasping and flared opercula (Vincent *et al.,* 2006). No cures have been discovered to date. Many research groups are currently investigating potential treatments (Nowak *et al.,* 2004).

2.2.3. Sea Lice

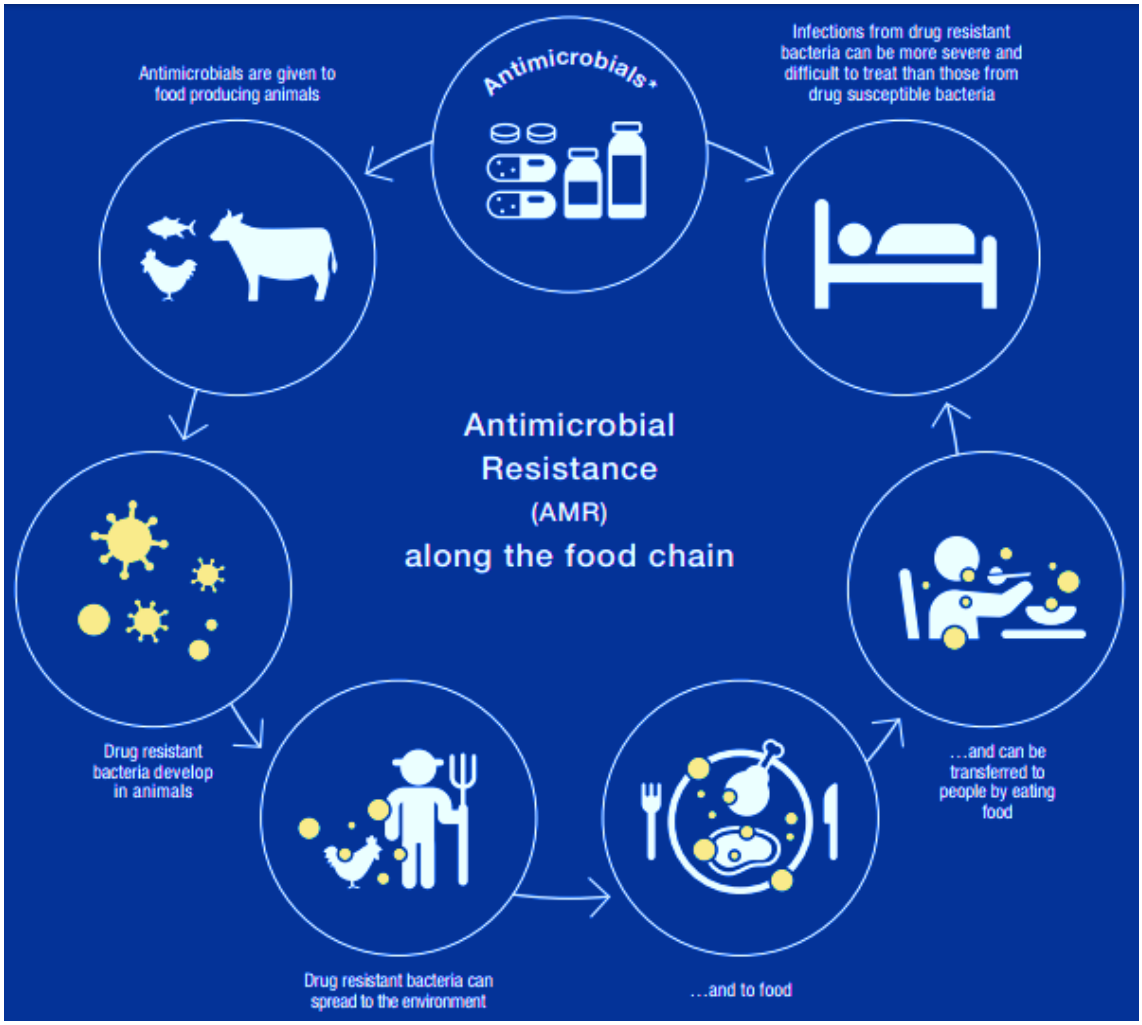
The most common sea lice effecting salmon are *Lepeophtheirus salmonis*. These parasites cause tissue damage. The parasites attach to the exposed fish tissue and feed on the skin, blood and mucous (Valenzuela-Munoz et al., 2016). Medicines are available in forms of feed and bath which can be used to treat sea lice. The use of cleaner fish which feed on sea lice is also an option to remove the sea lice from the infected salmon (Nowak *et al.,* 2010).

2.2.4. Economic and Ecological Effects

Treatments in the form of vaccinations and medicines incorporated into feed and baths are available for some diseases faced by Atlantic salmon. However, treatments for disease such as AGD have yet to be discovered. Salmon aquaculture is of great value to the Irish economy. In 2016, €105 million worth of Salmon were produced (BIM, 2017). Diseases such as AGD, without treatment can result in mortality of up to 10% (Downes *et al.*, 2015). The use of freshwater baths has been found effective, resulting in 86% decrease in amoeba numbers (Clark *et al.*, 2003). However, this method is labour intensive and expensive. Therefore, a new treatment strategy is essential.

2.3.Antimicrobial Resistance and Importance in Human Health

Some microorganisms, also known as superbugs undergo genetic modification when they encounter antimicrobial drugs. They become resistant to that drug and continue to cause damage to the host. This is known as antimicrobial resistance (AMR). AMR is an increasing threat to humans. Resistance have been recorded for many important antimicrobial classes which are used in the treatment of lethal diseases such as malaria and tuberculosis (WHO, 2017a). Resistance can be transferred from food producing animals to humans (refer to Figure 5). Antibiotics are used in aquaculture and agriculture as a preventative measure, treatment and to promote growth. The WHO have established guidelines on using antibiotics with food-producing animals (WHO, 2017a). This document encourages minimum use of antibiotics on food producing animals. Therefore, it is crucial to develop an alternative treatment for AGD.



(WHO, 2017b)

Figure 5: Antimicrobial Resistance Along the Food Chain

2.3.1. Antimicrobial peptides as Alternative Treatment Strategy

The broad spectrum antimicrobial activity of AMPs makes them promising candidates to replace antibiotics. AMPs can be administered to a particular site of infection or alternatively their production in the host can be stimulated.

Nanocarriers have been investigated as an AMP drug-delivery system. Nanocarriers can be produced from biodegradable and biocompatible materials such as lipids and polymers. They allow the encapsulation of the AMPs and in turn prevent degradation of the peptides. Due to the controlled degradation a time controlled release of the AMPs can be administered to ensure the peptides are released and effective at the target site (Zhang *et al.*, 2010; Witting *et al.*, 2015; Sandreschi *et al.*, 2016). For example, hyaluronic acid nanogels have been used to encapsulate LLKKK18, an analogue of LL-37 which is more effective against mycobacteria in comparison to the free peptide, *in vivo* and *in vitro (Silva et al., 2016).* Direct ingestion or drug delivery of AMPs is not the only therapeutic option. β-defensins can be stimulated in the host by certain stimuli, thereby enhancing native immunity. This is a promising alternative treatment avenue. IL-1α and IL-1β have been found to stimulate human β-defensin 2 (Liu et al., 2002). Cyclic adenosine monophosphate and butyrate has been found to induce expression of the β-defensin 9 in chickens (Sunkara *et al.*, 2014). Based on these studies, there is potential to investigate the stimuli which induce and enhance β-defensins production is *S. salar*.

Many AMPs have been investigated in recent years due to their mechanism of action, antimicrobial activity, natural production and low resistance rate (Mahlapuu *et al.*, 2016). Several ongoing clinical trials are investigating these AMPs and potential therapeutic agents for various infections. Pexiganan, which is an analogue of magainin is currently in phase 3 of the trial NCT00563394, for the treatment of infected foot ulcers in diabetics (Lipsky *et al.*, 2008). Novexatin, derived from human defensins is in phase 2 of a clinical trial for the treatment of fungal nail infections (Fox, 2013). LL37, the human cathelicidin is being investigated for the treatment of venous leg ulcers and is currently in phase 2 of the clinical trial (Gronberg *et al.*, 2014).

β-defensins have also been suggested as a treatment alternative to antibiotics (Wang,Zeng, *et al.*, 2016). Some properties of β-defensins make them a desirable option for treatment of infections. They have been previously investigated against various pathogens and have been found effective against Gram-Positive and Gram-Negative

bacteria, fungi, yeast and parasites (Katzenback, 2015). This broad range of antimicrobial activity is a particularly important characteristic of many antimicrobial peptides, signifying their use in treatment of infection caused by a range of pathogens. β-defensins also have a diverse amino acid structure, with variations in sequence and position of the cysteine residues. This property of the β-defensins is important to prevent resistance since they are structurally diverse. Another advantage to using defensins for treatment of infections is the fact that they are naturally occurring. As with other AMPs, their expression can be upregulated through addition of stimuli to the diet. Studies with gilthead seabream have shown increased defensin expression in the head kidney with a microalgae diet (Cerezuela *et al.*, 2012) and in rainbow trout, a peptidoglycan diet exhibited elevated levels of defensin messenger ribose nucleic acid in gills and skin (Maier *et al.*, 2008). These properties of β-defensins suggest they have the potential to be a good alternative to antibiotics for the treatment of infections.

2.4.Recombinant Production of Peptides

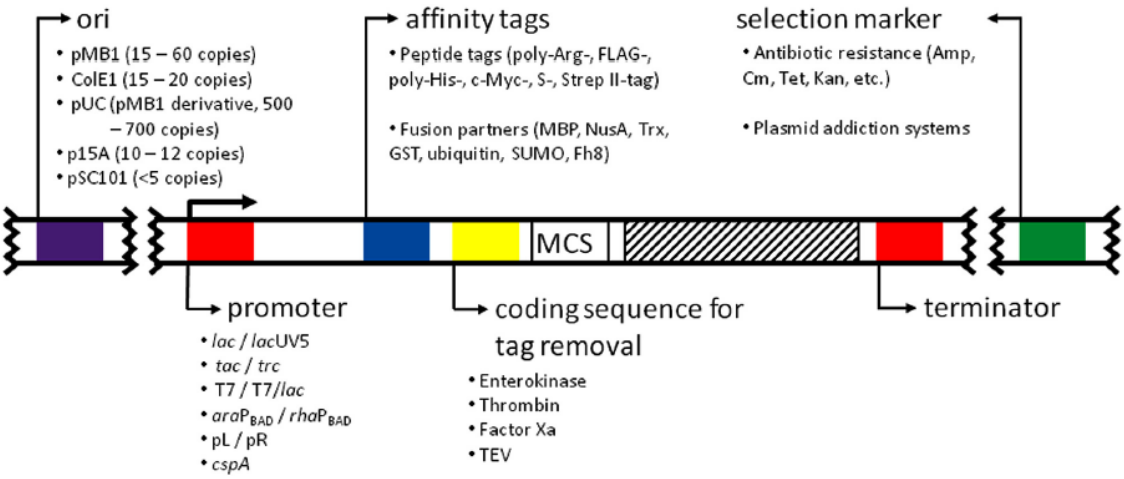
Using microorganisms to produce recombinant proteins have transformed biochemistry in recent years. Traditionally peptide production was very labour intensive, involving kilograms of plant or animal tissues to achieve a low yield (Rosano and Ceccarelli, 2014). However, recombinant technology with the use of microorganisms to produce the peptide of interest in high concentrations has proven invaluable as an alternative technique. Many studies have used recombinant technology to produce small molecular weight peptides like AMPs (Zorko and Jerala, 2010; Li, 2011; Ruangsri et al., 2013). There are several factors to consider before using recombinant technology.

1. **Host Cell:** Bacteria (Ruangsri *et al.*, 2013), fungi (Reichhart *et al.*, 1992) and insect cells (Andersons *et al.*, 1991) have been previously used as host cells for recombinant production of AMPs. *E. coli* is the most common host cell used for recombinant production of AMPs. There are many advantages to using *E. coli*, such as its high growth rate in optimal conditions, allowing culture with high cell densities to be achieved and transformation with target DNA (deoxyribose nucleic acid) is fast and easy (Sezonov *et al.*, 2007).

   Various *E. coli* strains are available for recombinant production. While they all have their advantages and disadvantages, BL21 (DE3) strains have been recommended by researchers (Daegelen *et al.*, 2009). (Studier and Moffatt, 1986) described the BL21 (DE3) strains in 1986. These cell lines have since been used for many recombinant peptide productions because of their beneficial characteristics. BL21 (DE3) lacks the enzyme Lon protease which is responsible for proteolysis of foreign peptides (Rosano and Ceccarelli, 2014). It is also deficient in the gene coding for OmpT, which functions in extracellular proteolysis. The lack of these two enzymes allows the production of the target peptide in the host and reduced the chances of protein degradation (Fathi-Roudsari *et al.*, 2016).

   Another *E. coli* strain commonly used is *R. gami*. This strain is genetically engineered with rare codons to enhance gene expression and disulphide bond formation. The strain contains mutations of the the trxB an gor genes, which aid the formation of disulphide bonds by providing and oxidising cytoplasmic environment (Fathi-Roudsari *et al.*, 2016).

2. **Plasmid:** Due to the advances in recombinant technology over the last number of years, there are a wide selection of expression vectors commercially available. However, several factors are to be considered when choosing the expression vector; number of replicons, promotors, multiple cloning sites, affinity tags and tag removal and selection markers. See Figure 6 below for an outline of the main factors to consider. Each experiment will have specific requirements for these factors. Therefore, it cannot be stated which expression vector is the best as they depend on the experiment itself.



(Rosano and Ceccarelli, 2014)

Figure 6: Main components of an expression vector

3. **Affinity Tags:** Due to the cytotoxic properties of AMPs, it is difficult to use microorganisms as host cells to produce these peptides. This issue has been resolved by using fusion proteins which allows the intrinsic antimicrobial activity of the AMPs to be masked inside the host cell. The fusion proteins also aid the purification process by allowing the protein of interest to be separated from the other non-specific material. The fusion protein can be removed from the target peptide by enzymatic cleavage. There are numerous fusion proteins available; Histidine, Maltose-binding protein, Green fluorescence protein, Glutatione S-transferase, Prochymosin, Ketosteroid isomerase and many more (Zorko and Jerala, 2010). These peptides are encoded by a sequence on the expression vectors. The vector also contains a coding sequence for the removal of the tag such as enterokinase, Factor Xa and TEV. Each of these fusion peptides require a specific chromatography resin for purification.

2.4.1. Recombinant Production of AMPs

In recent years, many research groups have investigated AMPs and their antimicrobial functions. These researchers have used recombinant technology using various combination of host cells, expression systems and affinity tags to ensure successful purification of the target AMP. A database has been created to compile all relevant information regarding the recombinant production of AMPs such as expression host, affinity tags, release method and peptides yields (Li and Chen, 2008).

(Zhong *et al.*, 2006) investigated human β-defensin 2 (hBD2) and their effects against pathogens. They used pET32 as an expression vector and used BL21 (DE3) cells to express the hBD2-pET32 plasmid. As an alternative, they used tandem repeats of the mature hBD2 gene and expressed it as the fusion protein. They successfully purified the protein with 0.76g/l of high purity mature peptide (Zhong *et al.*, 2006).

β-defensins were also produced by (Ruangsri *et al.*, 2013) from Atlantic cod using the pET44a expression vector and BL21 (DE3) cells. The peptides were purified using a histidine tag and Nickel affinity chromatography. The yield of protein recovered is not confirmed. However functional studies and characterisation assays were performed during this study, indicating a large yield of β-defensins were produced.

α-defensins have also been of interest. (Bruhn *et al.*, 2007) have studied α-defensins from horses. Similar to (Zhong *et al.*, 2006) and (Ruangsri *et al.*, 2013), they have also used BL21 (DE3) cells to express the peptide. A pET system was also used, namely pET-30 Xa/LIC and a histidine tag was also used for purification. This study used a similar approach to produce α-defensins as Ruangsri *et al*. Once again, the protein yield was not confirmed, however the characterisation assays indicate a good yield was produced.

Looking at other classes of AMPs, human hepcidins have been of interest for (Zhang *et al.*, 2005). This study used the pGEX-3X expression vector and comparable to previous findings, BL21 (DE3) cells were used to express the AMPs and a histidine tag was used for purification. The purification was successful with a result of 0.97g/l of pure human hepcidin.

ORBK is a cyclic cationic peptide which was produced by recombinant technology using similar purification strategies by (Li *et al.*, 2014). They used the pET28a expression vector and BL21 (DE3) cells to express the peptide. Contrasting to

previously mentioned studies, a maltose binding protein (MBP) tag was used for purification of the peptide with yield of 3mg/l of pure ORBK.

(Krahulec *et al.,* 2010) investigated the human cathelicidin LL-37. This study utilised the pUC19 expression vector and a different strain of *E. coli*, K-12 RV308ai. A histidine tag was used here to purify the LL-37. 2.6mg/l of pure LL-37 was recovered from this purification.

All these studies have successfully purified the peptide of interest even with variations in host cells, expression systems and affinity tags used. *E. coli* was the host strain of choice for all the studies mentioned. This is not a surprise as the advantages of using *E. coli* are incomparable. The pET expression system was the most commonly used system and the histidine tag was the affinity tag of choice for most researchers. However, successful purification was also achieved using the PUC expression system and MBP affinity tag.

2.4.2. Challenges with Recombinant Peptide Production

In theory it is quite simple; the gene of interest in amplified and cloned into the chosen expression vector, the vector is then transformed into the chosen host, the host is induced to express the gene and produce the peptides. The resulting peptide can then be purified and characterised. However, in reality it is not always as simple. There are limitations including difficulty growing the transformed host cells, formation of inclusion bodies, incorrect folding of protein causing inactivity, difficulty during purification and proteolysis (Rosano and Ceccarelli, 2014). The most significant challenges include:

A. Low yield of the protein of interest can be a big issue as milligram/ litre of culture is required for characterisation experiments. Protein toxicity can interfere with the growth and homeostasis of the host microorganism, leading to slow growth, reduced cell numbers, cell death and finally reduced amount of protein (Doherty *et al.*, 1993).
B. Inclusion bodies formation occurs when there is a build-up of protein aggregates. This occurs when the foreign recombinant gene is introduced to the host with a different metabolic environment (pH, cofactors, osmolarity and folding mechanism) in comparison to the original host. Formation of inclusion bodies cause problems during detection, purification and quantification of the peptide (Hartley and Kane, 1988).
C. Disulphide bonds need to be formed correctly to ensure the peptide is biologically active and fully functional. Incorrect formation of disulphide bonds can result in misfolding, inactivity and inclusion body formation. Cysteine oxidation occurs in the periplasm in *E. coli*. In the cytoplasm, disulphide bonds are rare as many enzymes contain cysteine residues in their catalytic sites (Derman *et al.*, 1993). The cytoplasm has a reducing environment sustained by glutaredoxin–glutaredoxin reductase and thioredoxin reductase systems (Stewart *et al.*, 1998). However, protein can still be expressed in the cytoplasm using modified *E. coli* strains with an oxidising cytoplasmic environment to aid disulphide bond formation (Derman *et al.*, 1993).
D. Protein degradation can occur due to the presence of proteases. This results in the breakdown of the peptides in turn resulting in protein inactivity.

E. Affinity tag used for protein purification is an important factor. Many affinity tags are available for recombinant protein purification. However, some tags are better than others. It is important to use a tag which is unique and have a strong affinity for the matrix. The use of an unsuitable tag can result in unsatisfactory purification of the recombinant peptide and low yield (Zhao *et al.*, 2013).

Chapter 3: Research Methodology

3.1. Cloning of β-Defensin 1, 3 and 4

Cloning of the β-Defensin 1, 3 and 4 genes involved PCR amplification of the gene of interest to produce a pure sample of high concentration, enzyme digestion of the target gene to allow for insertion into the expression vector, enzyme digestion of the expression vector to allow for the attachment of the insert, ligation of the two DNA fragments together and transforming the plasmid into the host cell to express the β-Defensin fusion peptide.

3.1.1. Serial Cloner

Serial Cloner is a free molecular biology software, created by a group of developers at Serial Basics. During this project, this software was used to perform *in silico* molecular experiments such as sequence alignment, PCR, insert and vector digestions, ligations and cloning. This software was used to establish the size of the PCR products and digested inserts and vectors, and used as a guide when performing these techniques in real time (Serial-Basics, 2012).

3.1.2. Polymerase Chain Reaction

Primers were designed for β-defensin 1, 3 and 4 based on the ESTs for *Salmo salar with accession numbers;* CK892029, CK895920 and EG781611 from the NCBI database (Adzhubei *et al.*, 2007; Ruangsri *et al.*, 2013). These primers contained overhangs to allow for cloning into a vector. These primers were used to amplify the β-defensin 1, 3 and 4 genes in preparation for cloning. To allow for multiple expression and purification options two sets of gene constructs were prepared for cloning (refer to Table 3 for sequence of all primers used). Primer set one produced construct 1, which added the sequence for the Histidine (HIS) tagged NusA protein on to the C-terminus of the defensin genes creating a HIS-NusA-Defensin fusion using pET-44a plasmid. Primer set 2 produced construct 2, which added the sequence for MBP onto the C-terminus of the defensin gene using pMAL-p4X. See Table 3 on page 25 for the sequence of these primers. Commercial gBlocks® gene fragments corresponding to the sequence of the defensin fusion constructs were synthesised by and purchased from IDT, Iowa (refer to Table 2 for gBlock gene sequences). These were designed to include the restriction sites for cloning. Construct 1 contains enterokinase site and

construct 2 contains factor Xa site for the final cleavage of the peptide. The lyophilised gBlocks®, synthetic gene fragments were prepared according to manufacturer's instructions. 2µl of the gBlocks® template was used for a 25ul PCR reaction using Phusion Polymerase. Phusion polymerase was chosen due to its high fidelity to reduce the risks of error during replication of the β-defensin 4 gene. PCR as carried out using a Biometra® Thermocycler. Refer to Table 4 for the master mix content, Table 5 for PCR conditions used and Table 6 for annealing temperatures of PCR reaction for each gene. Early optimisation of the PCR conditions was performed to determine the optimal conditions for amplification of the template DNA with the primer pair.

Table 2: Sequences of gBlocks used for PCR

Gene	Sequence	Size
Bdef 1 gBlock	GTCATTTCCCTTCTCTTGCCCCACCCTGAGTGGAGTCTGTC GAAAACTTTGCCTGCCAACAGAGATGTTCTTTGGACCACT GGGCTGTGGAAAGGGATTCTTGTGCTGTGTTTCTCATTTCT TATGAGGATCCCG	136bp
Bdef 3 gBlock	GTCTCTACACTTATGTTTCATTAGTGGGGGCGGGTGCAGA AACCTTCGTTTGTGCCTTGCTTCTGGTGGTACTAACATTGG AAAAATGGGATGTACATGGCCGAATGTATGCTGTAAATGA GGATCCCGG	130bp
Bdef 4 gBlock	GTTTCCAATTCCATGGGGATGCTCAAACTACAGTGGGATC TGCCGTGCTGTCTGTCTGTCAGCAGAACTACCATTTGGAC CTTTTGGATGTGCAAAAGGATTTGTATGCTGTGTCGCCCA CGTCTTCTAAGGATC	135bp

Table 3: Primers Used for PCR Amplification of β-Defensin 1, 3 and 4 Gene

Primer		Sequence (5'-3')
Primer set 1	Bdef 1_F_Pet44a	GTCATTTCCCTTCTCTGCCC
	Bdef 1_R_Pet44a	CCGGGATCCTCATAAGAAATG
	Bdef 3_F_Pet44a	GTCTCTACACTTATGTTTC
	Bdef 3_R_Pet44a	CCGGGATCCTCATTTACAG
	Bdef 4_F_Pet44a	GTTTCCAATTCCATGGGGATG
	Bdef 4_R_Pet44a	CCGGGATCCTTAGAAGACG
Primer set 2	Bdef 1_F_PmalP4x	CGCGAATTCTCATTTCCCTTC
	Bdef 1_R_ PmalP4x	CCGGGATCCTCATAAGAAATG
	Bdef 3_F_ PmalP4x	CCGCGAATTCTCTCTACACTTATG
	Bdef 3_R_ PmalP4x	CCGGGATCCTCATTTACAGC
	Bdef 4_F_ PmalP4x	GGCGCGAATTCTTTCCAATTCC
	Bdef 4_R_ PmalP4x	CCGGGATCCTTAGAAGACGTG

Table 4: In-house PCR Master Mixture for β-defensins 1, 3 and 4

Template DNA	2 µl
Reaction buffer	5 µl
Forward Primer	2.5 µl
Reverse Primer	2.5 µl
DNTP's	0.5 µl
Phusion polymerase	0.25 µl
Sterile Water	12.25 µl
Total Volume	25µl

Table 5: PCR Reaction Conditions for β-defensins 1, 3 and 4

PCR Step	Time	Temperature	Cycles
Denaturation	1 minute	98°C	1
Denaturation	15 seconds	98°C	30
Annealing	30 seconds	See Table 6	
Extension	15 seconds	72°C	
Final Extension	5 minutes	72°C	1
Cooling	Infinite	4°C	

Table 6: Annealing Temperatures for Each β-defensins Gene

		Annealing temperature (°C)
Primer set 1	β-defensin 1	47
	β-defensin 3	45
	β-defensin 4	45
Primer set 2	β-defensin 1	56
	β-defensin 3	56
	β-defensin 4	56

3.1.3. Electrophoresis

Following PCR, electrophoresis was performed using 10µl of PCR product stained with GelRed nucleic acid stain, (Biotium, 2017) on 2% agarose gel with 1x TAE running buffer (40 mM Tris (pH 7.6), 20mM acetic acid and 1mM ethylenediaminetetraacetic acid (EDTA) (Bio-Rad, 2017) at 120 volts for 45 minutes to resolve the β-defensin genes. A molecular weight marker, (Bioline, 2017) was used which contains a mixture of gene fragments of known molecular weight. This was used as a standard to determine the size of the β-defensin genes. The expected size of the β-defensin 1, 3 and 4 genes were 136p, 130bp and 139bp respectively for primer set 1 and 144bp, 149bp and 139bp respectively for primer set 2.

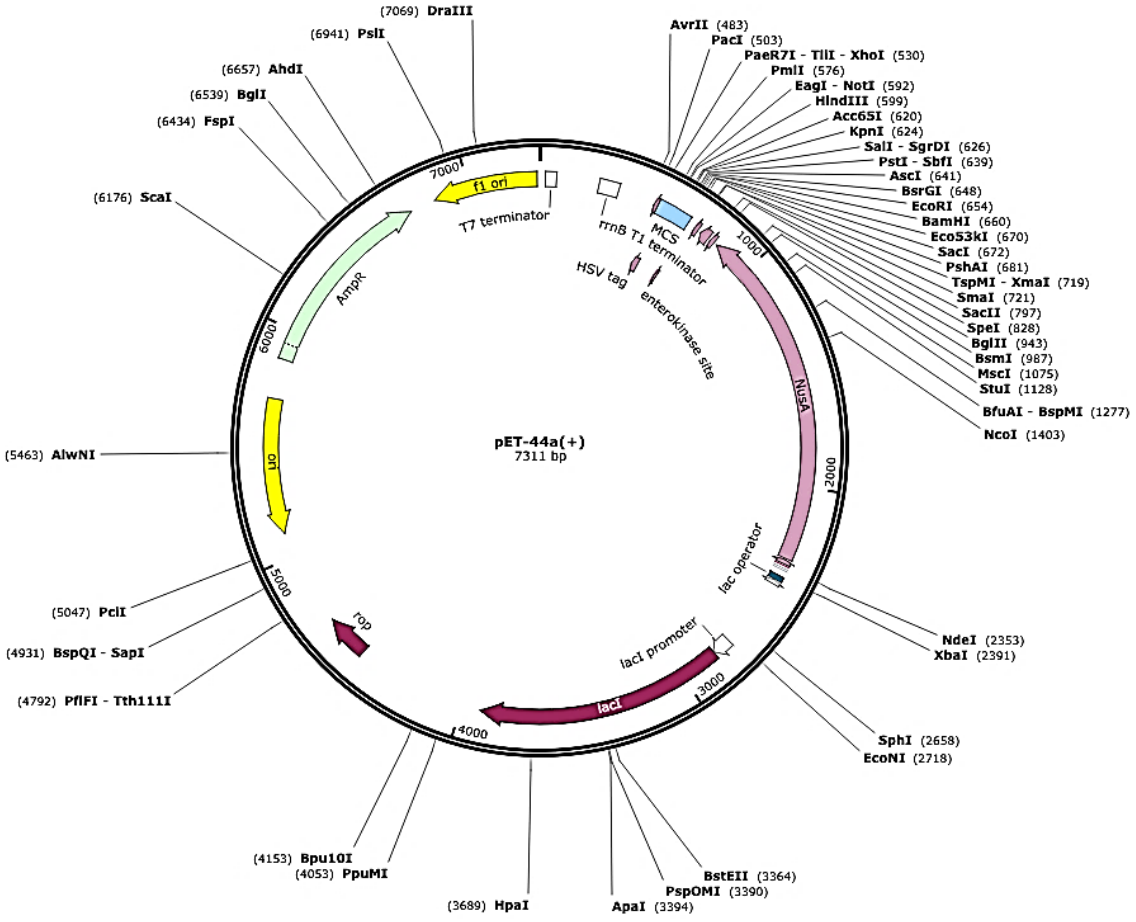
3.1.4. Digestion of Insert

Based on Serial Cloner, positive bands were cut out of the gel and the DNA was extracted from the gel using the QIAGEN® MinElute® Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, 2017). The concentration of the purified DNA was determined using a Nanodrop Spectrophotometer (BioTek, 2017). To digest the DNA insert for pET-44a, the restriction enzyme BamHI (Biolabs, 2017a) was used to digest the template DNA at the 3' end to allow insertion into both cloning vectors. BamHI recognises sequence GGATCC and results in a staggered cleavage of the DNA molecule, leaving the 3' end with sticky ends (Biolabs, 2017a) while the 5' end will be blunt end ligated into the vector. The digestion was performed in a 20µl reaction with 0.5µl BamHI, 10x buffer BamHI and 1µg/µl of DNA at 37°C for 1 hour, as per manufacturer's guidelines. 4µl of 6x loading dye containing ethylenediaminetetraacetic acid EDTA was added to the sample to stop the reaction and the sample was then analysed using agarose gel electrophoresis.

The positive band was cut out and the DNA was extracted using QIAGEN® MinElute® Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, 2017). To digest the DNA for pMAL-p4X, two restriction enzymes were used; BamHI and EcoRI. EcoRI recognises the sequence GAATTC and cuts the DNA fragment leaving sticky ends (Biolabs, 2017b). Similarly, the digestion was performed in a 20µl reaction with 1µl BamHI, 0.5 µl EcoRI, 10x buffer BamHI and 1µg/µl of DNA at 37°C for 2 hours, as per manufacturer's guidelines. Loading dye was added after incubation and electrophoresis was performed. The positive band cut and the DNA was extracted using QIAGEN® MinElute® Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, 2017). The concentrations of the DNA was measured using a Nanodrop Spectrophotometer (BioTek, 2017).

3.1.5. Digestion of Vector

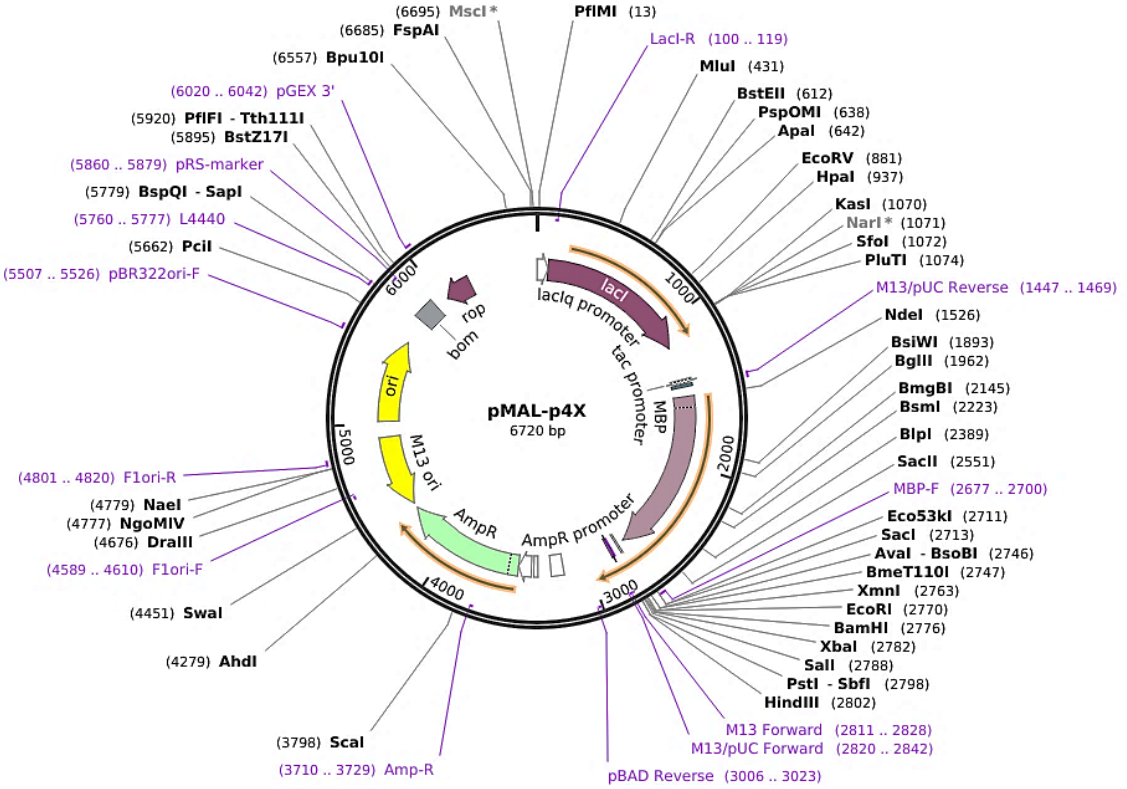
The pET44a cloning vector (Figure 7) was digested using BamHI and PshAI (Biolabs, 2017c). The restriction enzyme PshAI recognises the sequence GACNNNNGTC and cleaves the DNA double stranded molecule in the middle of the sequence, resulting in a blunt end. The vector is left with a sticky end and a blunt end. The digestion was performed in a 20µl reaction with 1µl of PshAI, 0.5µl BamHI, 10x buffer BamHI and 1µg/µl of DNA at 37°C overnight, as per manufacturer's guidelines (Biolabs, 2017a). 4µl of 6x Loading dye was added to the sample to stop the reaction and the sample was analysed using agarose gel electrophoresis. The positive band of 7.3kb was cut out and the DNA was extracted using QIAGEN® MinElute® Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, 2017).



(Merck, 2017)

Figure 7: pET-44a Vector Map

The pMAL-p4X cloning vector (Figure 8) was digested using BamHI and EcoRI. The digestion was performed in a 20µl reaction with 1µl BamHI, 0.5 µl EcoRI, 10x buffer BamHI and 1µg/µl of DNA at 37°C overnight, as per manufacturer's guidelines (Biolabs, 2017a). Loading dye was added after incubation and electrophoresis was performed. The positive band of 6.7kb cut and the DNA was extracted using QIAGEN® MinElute® Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, 2017). The concentrations of the DNA was measured using a Nanodrop Spectrophotometer (BioTek, 2017).



(Walker *et al.*, 2010)

Figure 8: pMAL-p4X Vector Map

3.1.6. Ligation

The digested insert and vector were joined together by using T4 DNA ligase to construct a recombinant DNA molecule. The ligation was performed in a 10 µl reaction, with the insert: vector molar ratio of 3:1, 1 µl of T4 DNA ligase, 10x T4 DNA ligase buffer and 1 µl of 50% w/v polyethylene glycol (PEG) 4000. This ligation mix was incubated at 22°C for 1 hour and transformed into *E. coli* Top 10 cells (refer to Section 3.1.8 on page 31 for heat-shock transformation protocol).

3.1.7. Preparation of Chemically Competent Cells

Chemically competent *E. coli* Top10, BL21 (DE3) and *R. gami* cells were prepared in house according to the method of (Mandel and Higa, 1970). An isolated colony from a streak plate was used to inoculate 3ml of Luria-Bertani (LB) broth, which was incubated overnight at 37°C, at 100rpm in a Thermo Electron Corporation shaking incubator. After approximately 20 hours, the overnight culture was diluted 1/100 with fresh LB broth and incubated at 37°C, shaking at 100rpm until the OD_{600} reached 0.5. Cells were placed on ice for 10 minutes, followed by centrifugation at 2990 x g, at 4°C for 5 minutes. The pellet was washed using ice-cold, sterile 0.1M calcium chloride and incubated on ice for 20 minutes (Mandel and Higa, 1970). Cells were centrifuged at 2990 x g, at 4°C for 5 minutes. A second wash was performed using 0.1M calcium chloride, without incubation. The pellet was resuspended gently using 0.1M calcium chloride, 15% glycerol (Mandel and Higa, 1970). 50µl aliquots were transferred to ice-cold eppendorf tubes and stored at -80°C.

3.1.8. Heat-Shock Transformation

The recombinant DNA was transformed into *E. coli* Top10 cells using a heat shock method (Froger and Hall, 2007). *E. coli* Top10 cells were used for transformation of the recombinant DNA as it allows steady replication of high copy number plasmids. This step was essential to ensure a high and pure concentration of the recombinant β-defensins was prepared for the upcoming expression trials (Nakata *et al.*, 1997).

50µl of chemically competent *E. coli* Top10 cells were thawed out on ice. 100ng of recombinant DNA was added to the competent *E. coli* Top10 cells. The sample was incubated on ice for 30 minutes, followed by a heat shock at 42°C for 30 seconds (Froger and Hall, 2007). Samples were cooled on ice for 5 minutes. 1ml of LB broth was added to the sample and incubated at 37°C for an hour. Sample was centrifuged for 30 seconds at 13,604 x g. The pellet was resuspended in 200µl of LB broth, plated on LB agar with ampicillin and incubated at 37°C overnight.

3.1.9. Plasmid Extraction

Ten single isolated colonies were picked after transformation of *E. coli* Top 10 cells with ligation mixture and used to inoculate ten 3ml cultures of LB with 3µl of ampicillin, which was grown at 37°C overnight. The following day cells were pelleted at 2606 x g. The pellet was resuspended and the plasmid was extracted using the QIAGEN QIAprep Spin Miniprep Kit, as per manufacturer's guidelines. (QIAGEN, 2017). The extracted plasmid was quantified using a Nano-spectrophotometer (BioTek, 2017). 3 µl of samples was used to measure the absorbance at 260nm. The purity of the samples was also determined as a ratio of absorbance at 260/280 (Brown and Brown, 2010). All samples used for cloning and expression were of high purity and ranged from 1.7-1.9.

3.1.10. Identification of Plasmids Following Ligation and Extraction

Restriction Digestion

Restriction digestions were used as one of the tools to initially confirm the identity of the putative pET44a- β-defensin plasmids. For pET44a, 100ng of each extracted plasmid was digested using 10x buffer Tango, 0.5µl of BoxI, 0.5µl NcoI, in a 20µl reaction and incubated at 37°C for 2 hours. For pMAL-p4X, 10x buffer Tango, 0.5µl of EcoRI and 1µl HindIII was used instead and incubated at 37°C for 2 hours. An undigested control was used, lacking the restriction enzymes. A negative control was also used which contained 100ng of undigested pET44a plasmid. 4µl of 6x loading buffer containing EDTA was added to each sample to stop digestion. All samples were analysed on a 2% agarose gel.

Polymerase Chain Reaction

Samples were amplified using initial suitable PCR conditions and positive bands were visualised on a 2% agarose gel after electrophoresis.

Sequencing

Positively identified plasmid samples were sent away to GATC, a sequencing centre in Germany to confirm the sequence of the genes using Sanger sequencing.

3.2.Expression Trials of β-Defensin 4 Recombinant Protein

Small-scale expression cultures were prepared to identify the optimal conditions for expression of the fusion proteins.

3.2.1. IPTG Induction

Sequencing confirmed β-defensin 4 gene was heat-shock transformed into BL21 (DE3) cells and grown on LB agar with ampicillin overnight at 37°C as per protocol established by (Froger and Hall, 2007). A single transformant colony was picked and used to inoculate LB broth with ampicillin and grown overnight at 37°C. 200µl of overnight cultures was added to 10ml of LB broth with ampicillin. 5 samples in total were prepared; 3 for checking the growth of cells by measuring $OD_{600,}$ one IPTG induced sample and one uninduced sample. These were incubated at 37°C, shaking at 250rpm and grown until the OD_{600} was 0.5-0.6. The growth check controls were used to check the absorbance at 600nm periodically to ensure growth does not exceed 0.6 at OD_{600}. Sterile 500mM IPTG solution was added to one of the samples. Varying concentrations of IPTG and incubation temperature were tested for IPTG expression trials (refer to Table 7 for conditions). The samples were further incubated for 3 hours, at 37°C, shaking at 250rpm. After incubation, 1ml aliquot of each samples were taken, the absorbance at 600nm was adjusted to 2. The remainder of the sample was harvested by centrifugation at 1914 x g, at 4°C for 20 minutes. The supernatant was removed, and pellet was frozen overnight. The pellet was thawed the following day and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 50 mM NaH_2PO_4).

3.2.2. Autoinduction

Sequencing confirmed β-defensin 4 genes were heat-shock transformed into *R. gami* and BL21 (DE3) cells and grown on LB agar with ampicillin overnight at 37°C. A single transformant colony was picked and used to inoculate LB broth with ampicillin and grown overnight at 37°C. Autoinduction media was prepared using 6g/L Na_2HPO_4, 3g/L KH_2PO_4, 20g/L Tryptone, 5g/L Yeast extract, 5g/L NaCl, 10ml/L, 60% v/v Glycerol, 10% w/v Glucose and 8% w/v Lactose (Studier, 2005). 10ml/L of overnight culture was added to the autoinduction media with ampicillin and incubated at 37°C, shaking at 200rpm, for 24 or 48 hours. The following day cells were harvested by centrifugation at 1914 x g, at 4°C for 20 minutes. The weight of the pellet was determined by subtracting the weight of the flask from the total weight of the pellet. The pellet was resuspended in lysis buffer (3 x weight of pellet). 5µl/ml phenylmethylsulfonyl fluoride (PMSF) was added and the sample was stored at -20°C overnight.

		IPTG Induction					Autoinduction	
Temperature		0.25mM	0.3mM	0.5mM	0.75mM	1.0mM	24 hours	48 hours
	~20	RG	RG	RG	RG	RG	RG	RG
	25	RG	RG	RG	RG	RG	RG	RG
	37	RG	RG	RG	RG	RG	RG BL21 (DE3)	RG BL21 (DE3)

Table 7: Expression Trials Summary Table (RG: *Rosetta gami*)

3.2.3. Cell Lysis

The pellet was thawed at room temperature. 6µl of Lysozyme (50mg/ml stock solution), 0.5µl/ml of DNAse (1mg/ml) = 0.5µg/ml and 2.5 µl/ml of 1M $MgCl_2$ was added to the sample and incubated at room temperature on a platform rocker. The samples were then sonicated for 12 minutes on ice with 10 seconds of 40% power and 40 second to cool down. The sample was centrifuged at 9690 x g for 10 minutes at 4°C. Samples were then analysed using SDS-PAGE.

3.2.4. SDS-PAGE

SDS-PAGE was performed to examine various proteins present in a sample and whether expression of the β-defensin fusion protein was successful. Bio-Rad Laboratories (California) SDS-PAGE analysis system was used. 12% gels were prepared in house using the following recipe. To prepare running gel: 1.05ml of 40% acrylamide, 1.4ml of Tris-HCl (1.875M, pH 8.8), 35µl of 10% SDS, 21µl of 10% ammonium persulfate, 2.1µl of 100% tetramethylethylenediamine and 0.99ml of deionised water was mixed and added to the gel casting chambers. A layer of isopropyl alcohol was added on top of the running gel. When the gel solidified, filter paper was used to remove the excess ethanol from the chambers. To prepare the stacking gel: 0.25ml of 40% acrylamide, 0.32ml of Tris-HCl (0.625M, pH 6.8), 15µl of 10% SDS, 15µl of 10% ammonium persulfate, 1.5µl of 100% tetramethylethylenediamine and 0.90ml of deionised water was mixed and added on top of the running gels. The combs were inserted to allow the formation of sample wells. Gels were allowed to solidify and stored at 4°C overnight. For SDS-PAGE analysis, gels were run at 150 volts for 60 minutes or until the loading dye reached the bottom of the gel. The dual colour Precision Plus Protein™ (Bio-Rad, California) was used as the molecular weight marker for all SDS-PAGE analysis. The gels were then removed from the casting chambers and stained using coomassie blue for 2 hours and destained using 20% methanol and 10% glacial acetic acid, overnight.

3.2.5. Western Blot

To confirm the presence of the HIS-tagged NusA-β-defensin fusion protein and the MBP- β-defensin fusion protein, western blot was used. An SDS-PAGE was performed for 1 hour at 150 volts. 6 filter paper sheets matching the size of the gel was cut out and soaked in the semi-dry transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.04% SDS) (Abcam, 2018). A sheet of polyvinylidene difluoride (PVDF) membrane, the same size as the gel was also cut out and soaked in 20ml of 20% methanol for 3 minutes. The gel was removed and placed in transfer buffer. The membrane was removed from methanol and soaked in transfer buffer for 3 minutes. A gel stack was prepared using three pieces of filter paper, the gel, the membrane and the remaining three pieces of filter paper. The edges were aligned, and the excess filter paper was trimmed using a sharp blade. A roller was used to eliminate air bubbles. The sandwich was placed onto the blotting unit with the membrane under the gel. The blotting was performed at 60 volts, 0.21 amps, for 1.5 hours. After blotting, the membrane was stained with ponceau red stain to ensure transfer was complete, followed by detaining with deionised water and Tris buffered saline with tween 20 (TBST) (Abcam, 2018). 30ml of blocking buffer was added (30ml 1x TBST +4.5g + 45ul of Tween 20) and blocked at room temperature for 1 hour. Membrane was then washed ten times with TBST. 20 ml of primary antibody was prepared according to manufacturer's instruction using 1% bovine serum albumin in TBST. Anti-HIS antibody was used for investigation of the HIS-tagged NusA- β-defensin fusion protein and an anti-MBP antibody was used for the MBP- β-defensin fusion protein. Membrane was incubated with the primary antibody overnight at 4°C. Membrane was then washed three times with TBST. Pierce CN/DAB Substrate Kit (Thermo Scientific, Massachusetts) was used for staining the membrane, according to manufacturer's guidelines. Once the desired intensity was reached, the reaction was stopped by adding deionised water to the membrane.

3.3.Purification β-Defensin 4 Recombinant Protein

During this project, two types of purification systems were used; a peristaltic pump for manual purification and the ÄKTA Purification System for automated purification.

3.3.1. Affinity Chromatography – Peristaltic Pump

The purification of β-defensin 4 was performed using a peristaltic pump and Nickel Sepharose Fast Flow beads (GE-Healthcare, 2017), (refer to Figure 9 for set-up of apparatus). Buffer 1 was used for equilibration and initial washing of the column consist of 20mM Na_2HPO_4, 20mM NaH_2PO_4 and 500mM NaCl. Buffer 2, 3 and 4 were used for washing of the column to remove contaminants. These buffers were identical to buffer 1, with the addition of 50mM, 150mM and 250mM imidazole respectively. Buffer 5 was used for elution of the target peptide and contained 300mM imidazole along with the components of buffer 1. Equilibration was performed using 40 column volumes of buffer and washed using 10 column volumes. These steps were performed using a flow rate of 1ml/min. The elutions were performed using 10ml of elution buffer and 500µl elution fractions were collected at a flow rate of 250µl/ml to be analysed later using SDS-PAGE.

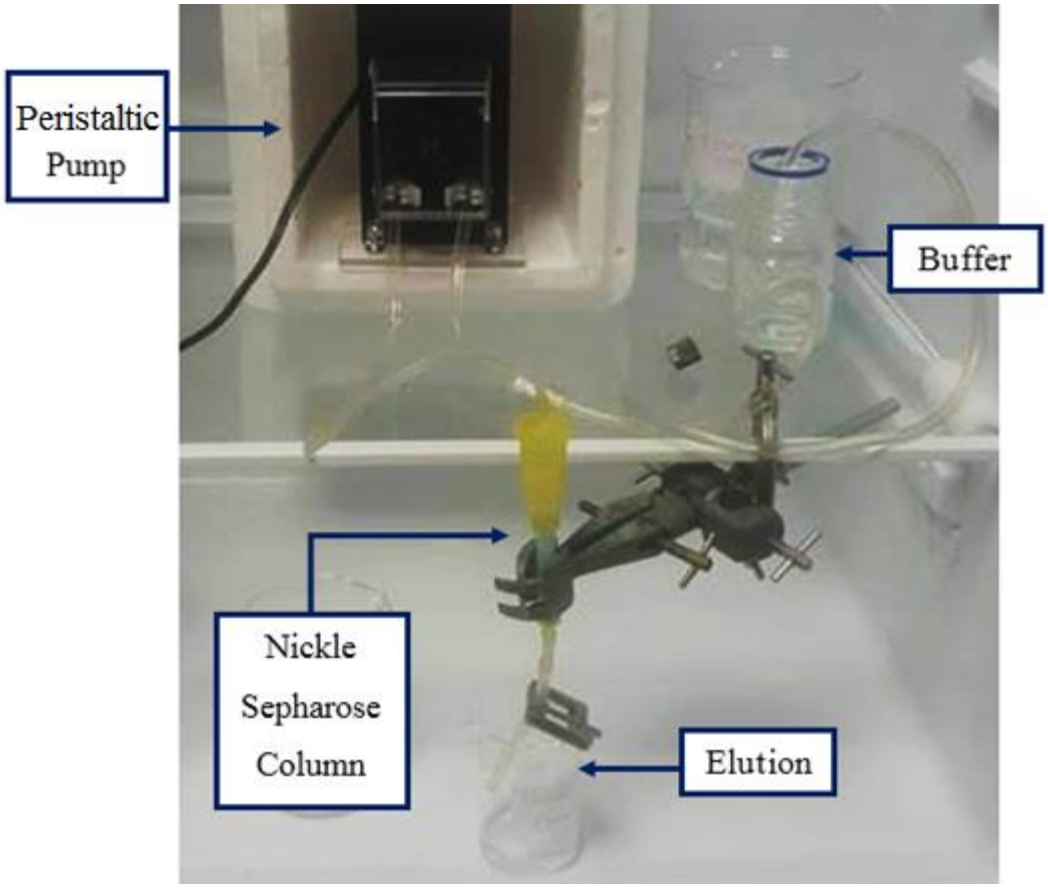


Figure 9: Set Up of Affinity Chromatography Using a Home-made Manual Pump

3.3.2. Affinity chromatography – ÄKTA Purification Systems

An automated purification system was also used for purification of the β-defensin peptides. Using affinity chromatography, the ÄKTA allows purification of peptides and allows the users to set up customised programs for purification. The ÄKTA was used for purification of β-defensins using the MBP tag. Unlike purification with the HIS tag, only 2 buffers were used for MBP tag purification; a binding buffer and an elution buffer. The binding buffer contained 50mM Tris-HCl, pH 8.0, 200 mM NaCl and 1mM EDTA. The elution buffer consisted of 10mM maltose added to the elution buffer. An amylose resin was used with maltose as the competitor for MBP (Duong-Ly and Gabelli, 2015). 3ml of amylose resin was added to fill the column. The column was equilibrated with 10 column volumes of equilibrium buffer. The ÄKTA as set to a flow rate of 1ml/min and maximum pressure of 0.5mPa. 15ml of lysed overexpressed cells were added to the column. The column was washed with 20ml of the equilibrium buffer. Flow rate was changed to 0.5ml/min and 1ml fractions were collected after addition of elution buffer.

3.4.Protein Quantification

The protein concentration was quantified for the crude extract and in each elution fraction from purification using Bradford and BCA methods. Initially the Bradford method was used for protein quantification. However, it was later discovered that imidazole present in the buffers may interfere with the assay. This may have led to unreliable results. Therefore, Bradford assay was replaced with BCA assay.

3.4.1. Bradford Method

1mg/ml bovine serum albumin (BSA) stock solution was used to make 500µl of calibrator samples of 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml and 1000µg/ml, which were diluted using equilibration buffer (previously used for purification). 500 µl of the calibrators and test samples were added to a 96-well microplate and 200 µl of Bradford reagent was added to each well. These samples were incubated for 5 minutes at room temperature. Samples were assayed in duplicates. The absorbance of all samples was measured at 595nm using a plate reader. A calibration curve was plotted using the concentration of BSA (mg/ml) versus absorbance. This curve and the equation of the line of best fit was used to calculate the protein concentration of the unknown samples.

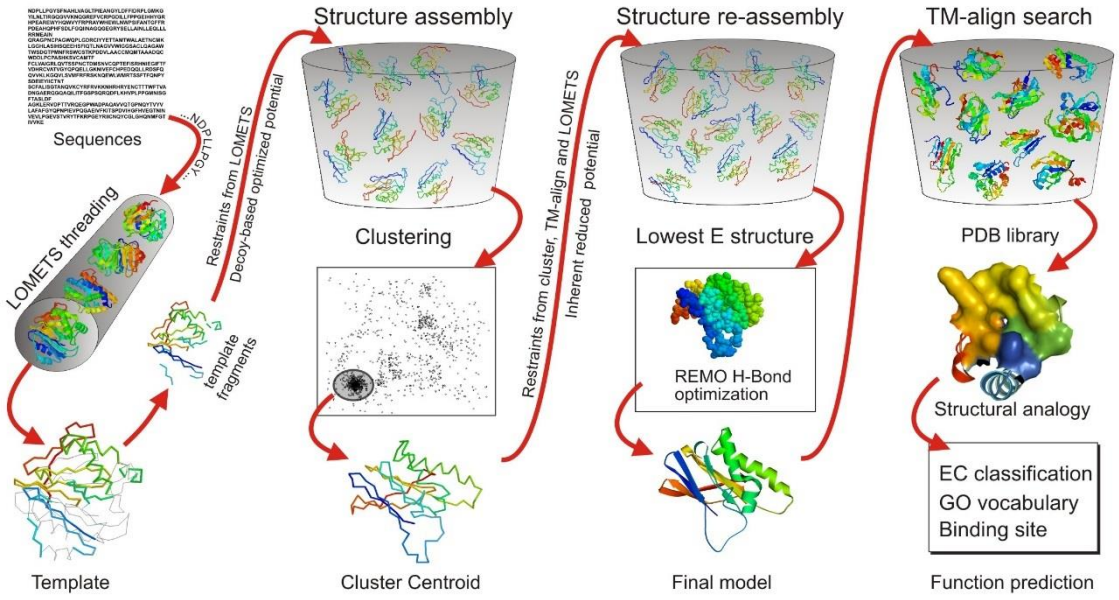
3.4.2. BCA Method

Pierce™ BCA Protein Assay Kit by Thermo Scientific was used for protein quantification. A standard curve was generated using standards provided in the kit, as per manufacturer's guidelines. Assay was performed using elution samples from affinity chromatography following the kit manual and analysed using 96 multi well plates to measure the absorbance at 562nm. The standard curve was used to determine the concentration of the samples.

3.5. Structures Prediction for β-Defensins 1, 3 and 4

Structural models for β-Defensins 1, 3 and 4 were investigated during this project. An online tool called I-TASSER. The FASTA sequence was submitted to I-TASSER which used algorithms and a protein database to provide 5 prediction models of the peptide structure and 10 peptides with highest structural homology.

Iterative Threading ASSEmbly Refinement (I-TASSER) is an online tool which uses algorithms established by (Zhang, 2008; Roy *et al.*, 2010; Yang *et al.*, 2015) to predict the structure and function of the peptide of interest. The amino acid sequence is submitted through an online form and a report is generated which includes 5 3D prediction models of the protein structure. When user submits an amino acid sequence, the server first tries to retrieve template proteins of similar folds (or super-secondary structures) from the PDB library by a locally installed meta-threading approach (LOMETS). See Figure 10 for principle for structure prediction. LOMETs contain several threading programs with each program generating thousands of sequence alignments. A Z-score is then calculated by determining the difference between the average and the raw scores of the standard deviation of each sequence. This Z-score is used to establish sequence of highest significance and only these templates are used by I-TASSER. The template with the highest Z-score is selected from each threading program. For every sequence submitted, a large collection of structural conformations is produced called decoys. The SPICKER program is then used to make the final selection. A C-score is calculated which determines the confidence of each model based on the convergence parameters of the structure assembly simulations and the significance of threading template alignments. C-scores can range from -5 to 2, with a high value proportional to high confidence.



(Zhang, 2008; Roy *et al.*, 2010; Yang *et al.*, 2015)

Figure 10: Principle of I-TASSER Structure Prediction

Chapter 4: Results

4.1. Cloning of β-Defensin 1, 3 and 4 into pET44a using Primer Set 1

Genetic cloning involves many molecular techniques such as PCR, digestion of target gene and expression vector, ligation and transformation. The results for each of these techniques are explained in detailed below. In summary, β-defensin 4 was successfully cloned using primer set 1, while all three β-defensins were successfully cloned using primer set 2.

4.1.1. Polymerase Chain Reaction and Electrophoresis

PCR was used to amplify the template DNA for β-defensins (synthetic gene fragments or gBlocks, supplied by IDT) using the conditions outlined in Section 3.1.1. This was performed using primer set 1 (refer to Table 3). *In silico* PCR using Serial Cloner 2.6 confirmed PCR products for β-defensin 1, 3 and 4 are 136bp, 140bp and 139bp respectively and indeed these were the sizes of the PCR products observed on agarose gels. Hyperladder IV (Bioline) was used for analysis on agarose gel electrophoresis following the method outlined in Section 3.1.3, allowing all amplified bands to be seen between the 100bp and 200bp bands of the molecular weight marker. Figure 11 shows the amplified genes on a 2% agarose gel.

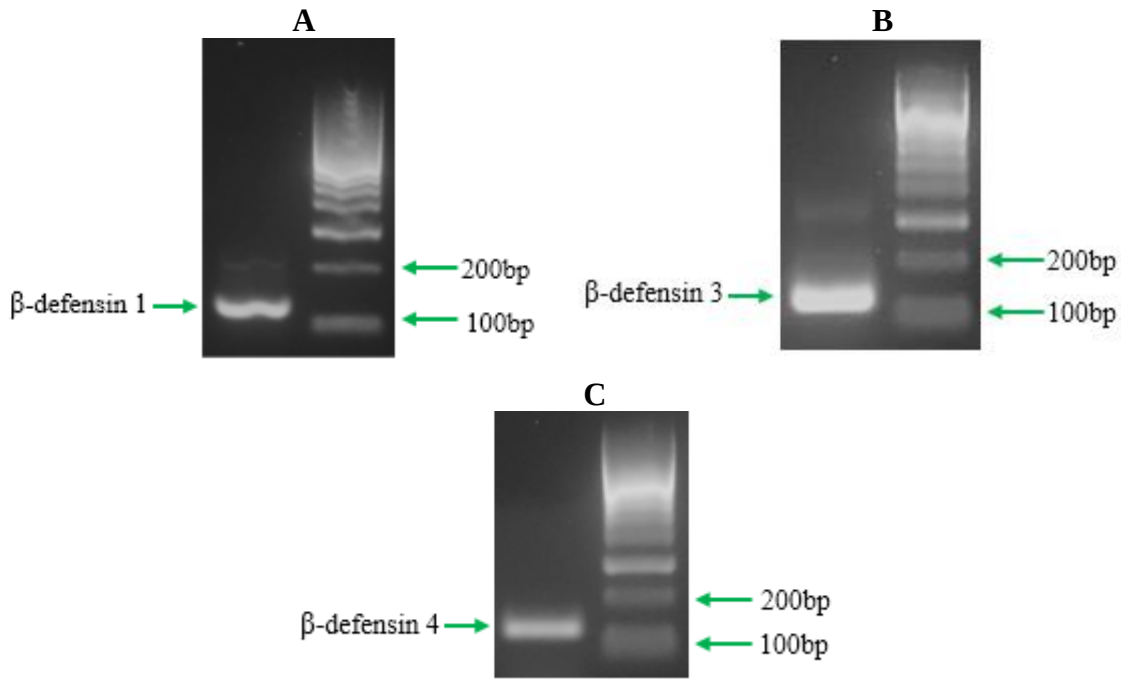


Figure 11: Positive Band for β-Defensin 1, 3 and 4 After PCR

Image A shows the amplified β-defensin 1 PCR product of 136bp, image B shows the β-defensin 3 PCR product of 140bp and image C shows the amplified β-defensin 4 PCR product of 139bp, using primer set 1. Hyperladder IV was used as the molecular weight marker.

4.1.2. Digestion of β-defensin Gene Insert

Insert digestion was carried out as per Section 3.1.4. *In silico* digestion concluded that the digested inserts for β-defensins 1, 3 and 4 respectively were 128bp, 122bp and 131bp respectively and these were the fragment sizes for the genes as observed on the agarose gels. A 100bp ladder also used for β-defensin 1 and 3 while 200bp ladder was used for β-defensin 4 (due to availability) to visualise the digested bands using electrophoresis. Refer to Appendix C for Serial Cloner output illustrating *in silico* digestion. Figure 12 shows the digested gene products on a 2% agarose gel.

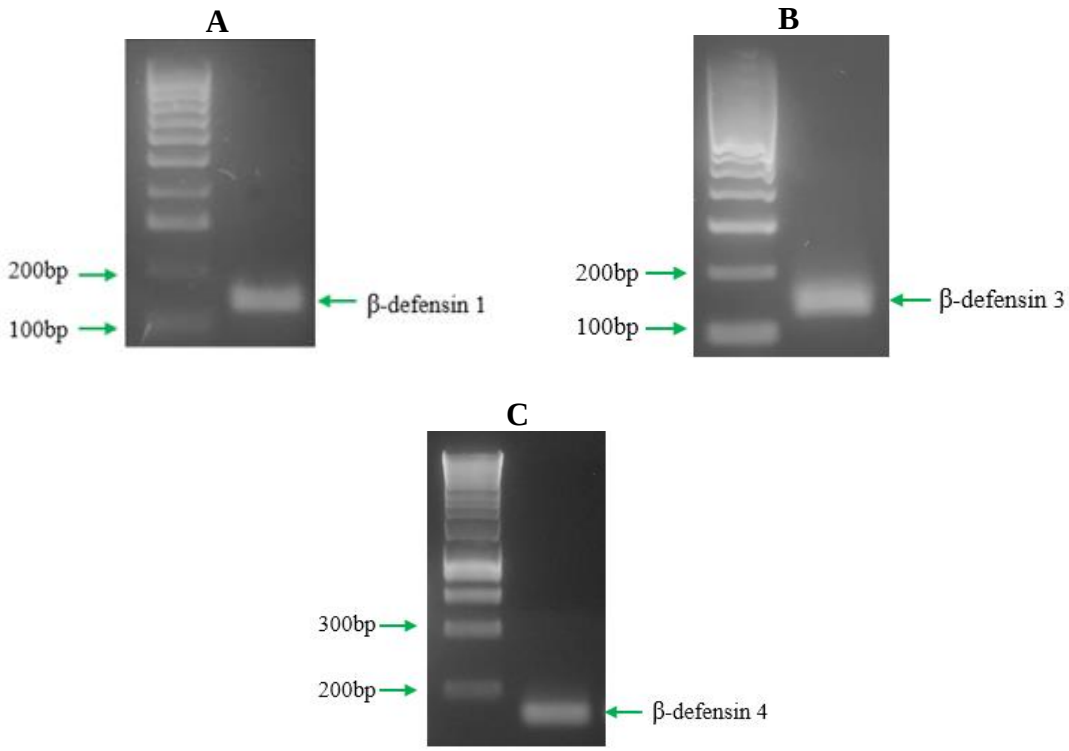


Figure 12: Positive Band for β-Defensins 1, 3 and 4 After Digestion of Insert
Image A shows the digested β-defensin 1 insert of 128bp, image B shows the β-defensin 3 insert of 122bp and image C shows the digested β-defensin 4 insert of 131bp, using BamHI. Hyperladder IV was used as the molecular weight marker for β-defensin 1 and 3 while Hyperladder I was used for β-defensin 4.

4.1.3. Digestion of pET44a Vector

The procedure outlined in Section 3.1.5 was followed to digest the pET44a vector in preparation for cloning. The resulting band after digestion with BamHI and PshAI was 7294bp. Refer to Appendix D for *in silico* output from Serial Cloner. A 10kb ladder (Hyperladder I) was used to allow for digested band to be detected on agarose gel (Figure 13). From the figure below there are clearly contaminating bands below the digested pET44a vector. These may consist of over-digested plasmids. To eliminate these contaminant, the band at 7294bp was cut out of the agarose gel and the plasmid was extracted directly from the gel.

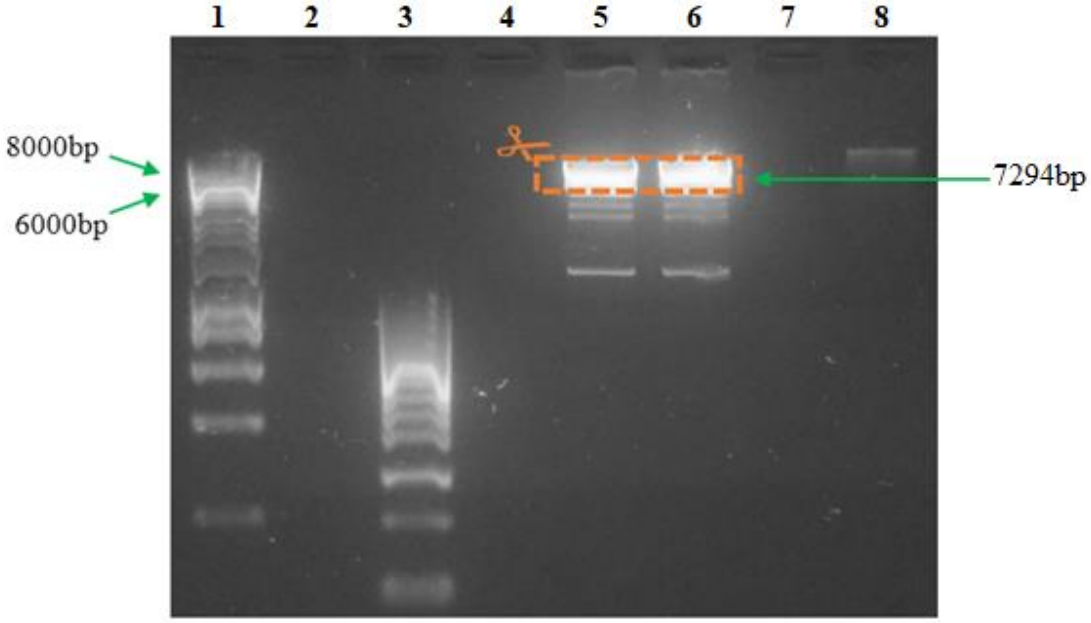


Figure 13: Positive Band for pET44a After Digestion
Image showing the BamHI and PshAI digested pET44a vector in lanes 5 and 6 (7249bp), Hyperladder I in lane 1, Hyperladder IV in lane 3 and a negative control in lane 8.

4.1.4. Ligation and transformation

Ligation of the β-defensin inserts to the pET44a cloning vector was carried out as per Section 3.1.6. A schematic representation of this step is illustrated below in Figure 14. The figure highlights the position in the vector where the insert will ligate. The figure highlights the position in the vector where the insert will ligate.

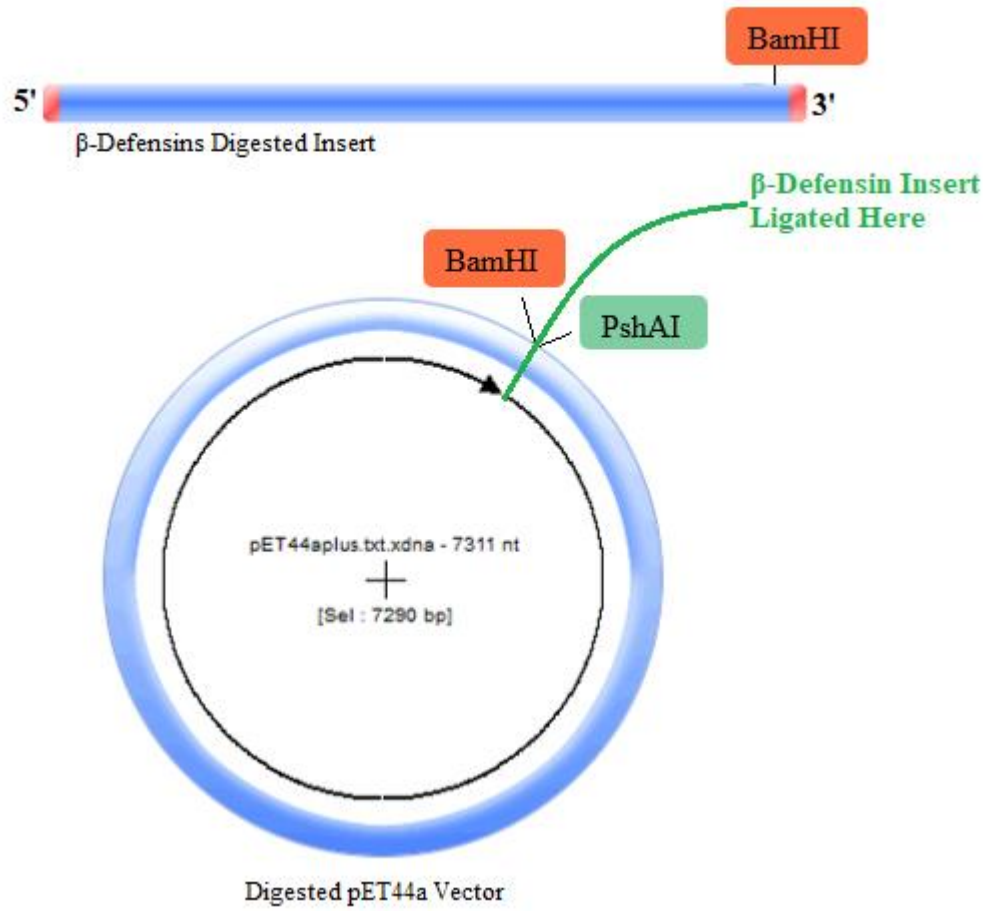


Figure 14: *In Silico* Ligation of β-Defensin 4 Insert Ligation to pET44a

4.1.5. Identification of Putative pET44a-β-Defensin Plasmids

Restriction Digestion

Restriction digestion with BoxI and NcoI was used to identify putative pET44a-β-defensin plasmids, as outlined in Section 3.1.4. *In silico* restriction digestions were performed using Serial Cloner to determine the expected bands for ligated and unligated pET44a plasmids. Refer to Appendix E for Serial Cloner output. Figure 18, Figure 19 and Figure 20 displays the results of restriction digestion for β-defensin 1, 3 and 4 respectively. pET44a-β-defensin plasmids are highlighted in green circles while empty pET44a plasmids are highlighted in red circles.

In Figure 18, pET44a-β-defensin 1 plasmids are expected to show a single band at 7422bp and empty pET44a plasmids are expected to have 2 bands, 6585bp and 726bp. The remaining samples considered to be undigested. Hyperladder I was used as the molecular weight marker. 3 potential pET44a-β-defensin 1 plasmids were evident on the agarose gel.

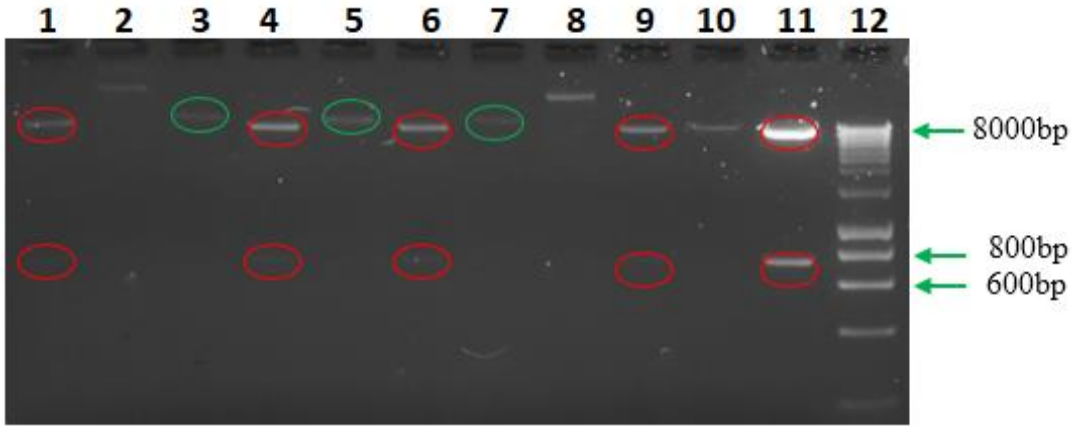


Figure 15: Restriction Digestion of Putative pET44a-β-defensin 1 with BoxI and NcoI
= pET44a-β-defensin plasmid, with 1 band at 7422bp
= Empty pET44a plasmid, with 2 bands at 6585bp and 726bp
Samples in lanes 2, 8 and 10 are undigested and Hyperladder I in lane 12 as molecular weight marker.

In Figure 16, pET44a-β-defensin 3 positive clones are expected to show a single band at 7416bp and empty pET44a are expected to have 2 bands, 6585bp and 743bp. Many samples showed more than 2 bands which were concluded as undigested. Hyperladder I was used as the molecular weight marker. 5 potential positive clones were evident on the agarose gel.

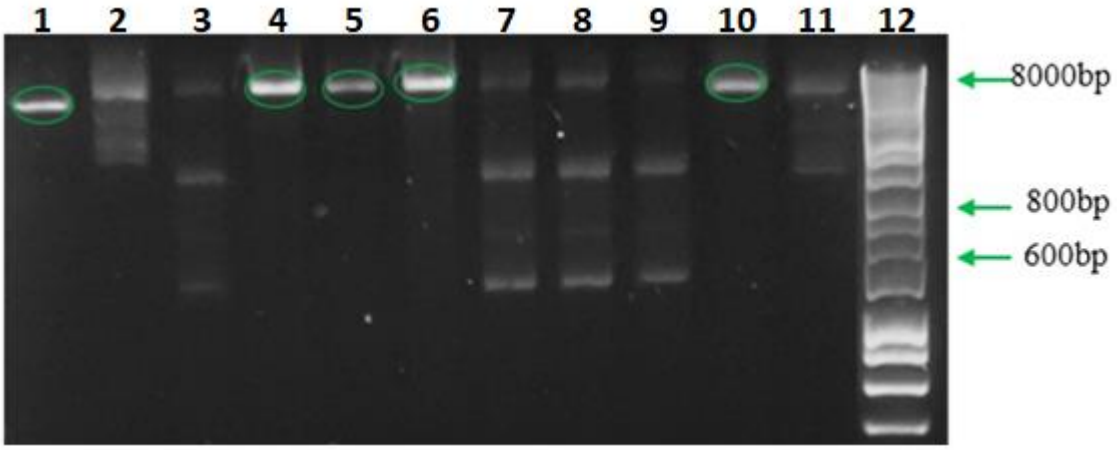


Figure 16: Restriction Digestion of Putative pET44a-β-defensin 3 with BoxI and NcoI
= pET44a-β-defensin 3 plasmids, with 1 band at 7416bp
Samples in lanes 2, 3, 7, 8, 9 and 11 assumed undigested and Hyperladder I in lane 12 as molecular weight marker.

In Figure 17, pET44a-β-defensin 4 plasmids are expected to show 3 bands at 6568bp, 737bp, 120bp and empty pET44a plasmids are expected to have 2 bands, 6585bp and 726bp. Hyperladder I was used as the molecular weight marker. 2 potential pET44a-β-defensin 4 plasmids were seen on the agarose gel.

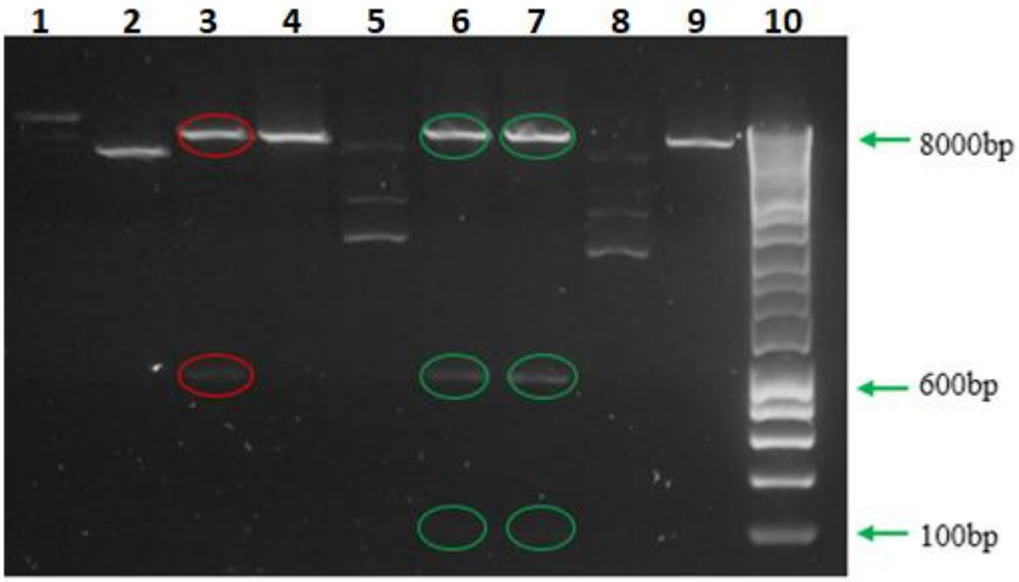


Figure 17: Restriction Digestion of Putative pET44a-β-defensin 4 with BoxI and NcoI
= pET44a-β-defensin 4 plasmids, with 3 bands at 6568bp, 737bp and 120bp
= empty pET44a plasmids, with 2 bands at 6585bp and 726bp
Samples in lanes 1, 2, 4, 5, 8, and 9 assumed undigested and Hyperladder I in lane 10 as molecular weight marker.

Polymerase Chain Reaction

PCR was used to confirm the identity of the pET44a-β-defensin plasmids deemed to be positive by restriction digestion. As stated previously in Section 4.1.1, *in silico* PCR of β-defensin 1, 3 and 4 resulted in PCR products of 136bp, 140bp and 139bp respectively. Using this information, the identity of the pET44a-β-defensin plasmids was confirmed.

Figure 18 shows PCR amplification of putative pET44a-β-defensin 1 plasmids from Figure 15 in lanes 1-3, negative control at lane 4 and molecular weight marker in lane 5 (Hyperladder IV). Lack of bands at 136bp indicates that none of the samples were pET44a-β-defensin 1 plasmids.

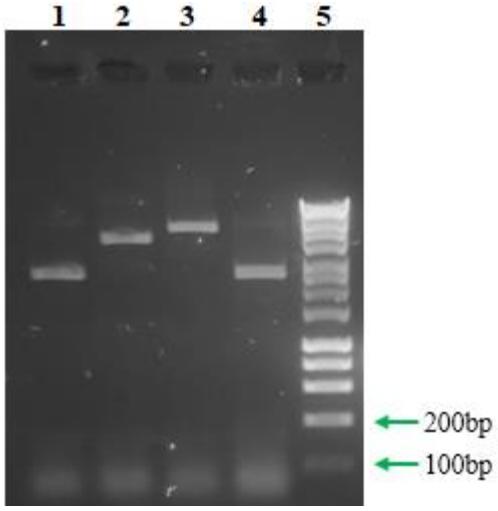


Figure 18: PCR Amplification of Potential pET44a-β-defensins 1 Plasmids
2% agarose gel image showing that amplification of β-defensins 1 gene (136bp) did not occur in lanes 1-3, indicating the plasmids did not contain the gene.

Figure 19 shows PCR amplification of pET44a-β-defensin 3 plasmids from Figure 16 in lanes 1-3, negative control at lane 4 and molecular weight marker in lane 5 (Hyperladder IV). Bands were seen in lanes 1-3 between 100bp and 200bp, demonstrating that all the samples were potentially pET44a-β-defensin 3 plasmids. The negative control in lane 4 did not show any amplification, as expected.

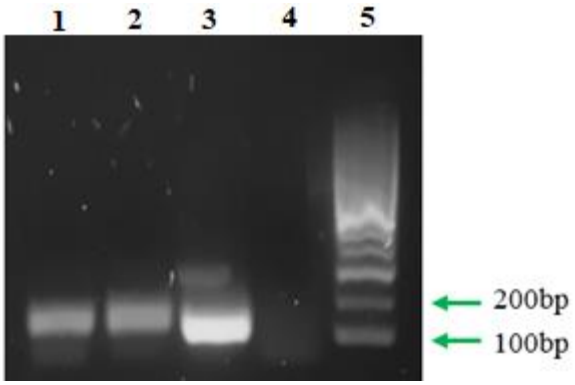


Figure 19: PCR Amplification of Potential pET44a-β-defensin 3 Plasmids
2% agarose gel image showing the amplification of β-defensins 3 gene (140bp) occurred in lanes 1-3, indicating the plasmid contained the β-Defensins 3 insert.

Figure 20 shows PCR amplification of pET44a-β-defensin 4 plasmids from Figure 17;

Lane 1 contains sample 3 from the gel in Figure 17, lane 2 contains sample 4, lane 3 contains sample 7, lane 4 contains sample 8, lane 5 contains sample 9, lane 6 and 10 contains the molecular weight marker, undigested control in lane 8 and negative control in lane 9.

Strong bands were seen in lanes 1-4 between 100bp and 200bp, indicating that all the plasmids may be pET44a-β-defensin 4. Lane 1 contained sample 3 from Figure 20, which was assumed to be an empty pET44a plasmid. A band was also seen for sample 9 in lane 5 which was initially assumed to be undigested. The negative control in lane 9 did not show any amplification at the expected 139bp, as expected. Samples were sent for Sanger Sequencing for confirmation.

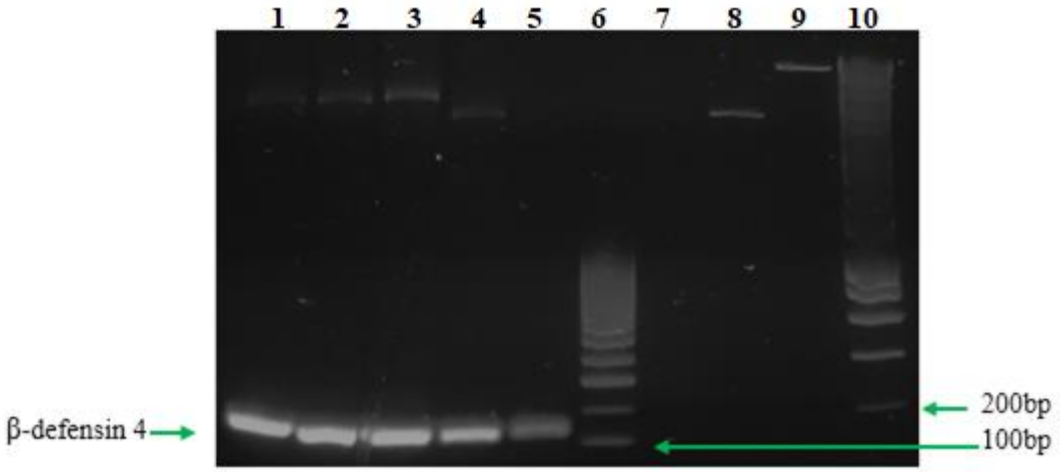


Figure 20: PCR Amplification of Potential pET44a-β-Defensins 4 Plasmids
2% agarose gel image showing the amplification of β-defensins 4 gene (139bp) occurred in lanes 1-5, indicating the plasmids contained the β-Defensins 4 insert.

Sanger Sequencing

According to the Sanger Sequencing carried out by GATC (Germany) putative pET44a-β-defensin 4 plasmid contained the correct sequence and this was used to transform the *E. coli* expression cells in the next stage of the project. The chromatogram in Figure 21 highlights the nucleic acid sequence corresponding to the mature β-defensin 4 peptide sequence in orange. Refer to Appendix F and Appendix G for sequences chromatograms of pET44a-β-defensin 1 and 3 plasmids which did not contain the correct insert.

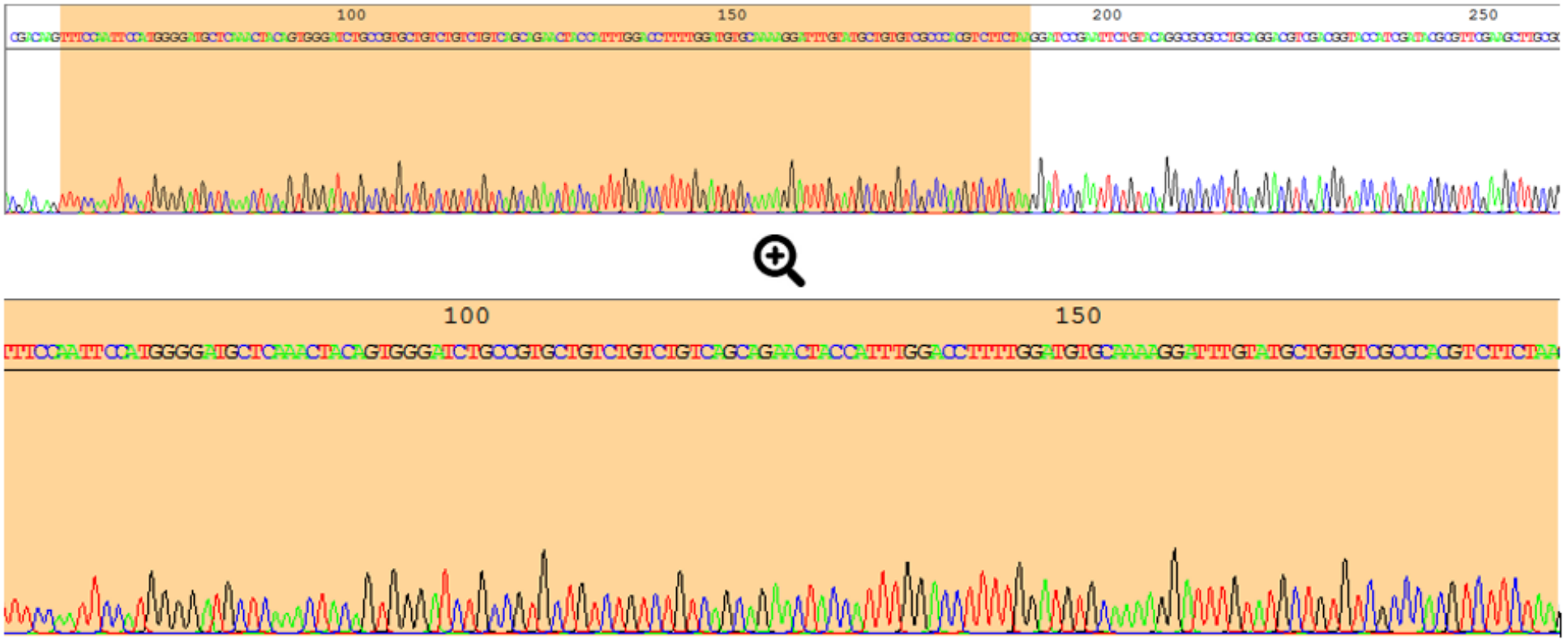


Figure 21: Sanger Sequencing Result for β-Defensin 4, generated using the GATC Viewer (Eurofins Scientific)

4.2.Expression of Recombinant NusA-β-defensin 4 Fusion Protein in *R. gami* and BL21 (DE3) cells

To express the Recombinant NusA-β-defensin 4 fusion protein, both IPTG induction and autoinduction was used for test expression trials to determine the optimal conditions for highest expression.

4.2.1. IPTG Induction

Expression levels of recombinant NusA-β-defensin 4 fusion protein were investigated by analysis of induced whole cell extracts using SDS-PAGE. IPTG induction at a concentration range of 0.25 to 1 mM, at temperatures ranging from 20 to 37 °C, was carried out on *R. gami* cells transformed with the pET44a-β-defensin 4 plasmid, using the protocol outlined in Section 3.2.1. The expected band size for the resulting HIS-tagged NusA-β-defensin fusion protein was approximately 60kDa. Unfortunately, IPTG failed to induce the expression of the fusion protein. As a result, other induction methods and host cells were considered for the expression of the protein.

4.2.2. Autoinduction

Alternatively, autoinduction-induced overexpression was performed as per Section 3.2 using both *R. gami* and BL21 (DE3) cells. Figure 22 shows an SDS-PAGE of the whole cell extracts after autoinduction. Lane 1 contains a protein standard (Precision Plus Protein™ Dual Colour), lane 2 shows autoinduction using BL21 (DE3) for 24 hours, lane 3 shows autoinduction using BL21 (DE3) for 48 hours, lane 4 shows autoinduction using *R. gami* for 24 hours and lane 5 shows autoinduction using *R. gami* for 48 hours. Significant expression bands are clear in lanes 2 and 3 with the target band below 75kDa, indicating overexpression of HIS-tagged NusA-β-defensin 4 fusion protein. The expected expression band should be at approximately 60kDa. For *R. gami* cells, no significant expression bands were seen, indicating that while some expression may have occurred, overexpression did not occur. Therefore, this gel showed that BL21 (DE3) cells were successful hosts for the overexpression of the NusA-β-defensin 4 fusion protein. This host and expression protocol were adopted for use at this stage.

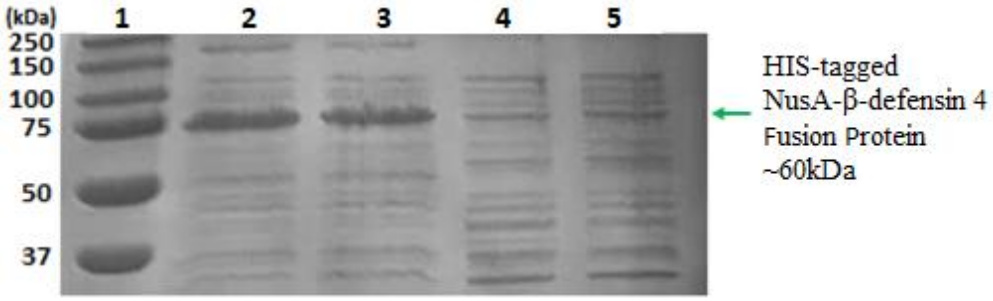


Figure 22: SDS Gel Illustrating Expression Bands in BL21 (DE3) and *R. gami* Cells
Lane 1: protein standard (Precision Plus Protein™ Dual Colour), lane 2: whole cell extract after autoinduction with BL21 (DE3) at 24 hours, lane 3: whole cell extract after autoinduction using BL21 (DE3) at 48 hours, lane 4: whole cell extract after autoinduction using *R. gami* for 24 hours and lane 5: whole cell extract after autoinduction using *R. gami* for 48 hours. The expected band for HIS-tagged NusA-β-defensin 4 fusion protein is ~60kDa.

4.2.3. Western Blot

Western blot was performed to confirm expression of the HIS-tagged NusA-β-defensin fusion protein as seen in Figure 23. The anti-HIS antibody was used (refer to Section 3.2.5. for protocol). Lane 1 contains a protein standard (Precision Plus Protein™ Dual Colour), lane 2 contains β-defensin 4 transformed BL21 (DE3) cells after autoinduction and a positive control, HIS-tagged enhanced green fluorescent protein (eGFP) in lane 7. A sharp band is seen in lane 2 between 50kDa and 75kDa, confirming expression of NusA-β-defensin 4 fusion protein in BL21 (DE3) cells by autoinduction as the expected band is approximately 60kDa (highlighted with ⬭). The eGFP positive control also showed a large band below 37kDa (highlighted with ⬭). The expected size of eGFP is 27kDa (Arpino *et al.*, 2012).

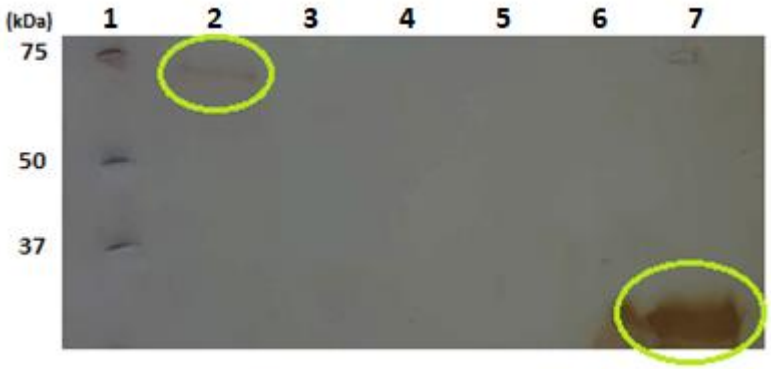


Figure 23: Western Blot Confirming Expression in BL21 (DE3) Cells
⬭ = Positive band using anti-HIS antibody
Lane 1: protein standard (Precision Plus Protein™ Dual Colour), lane 2: whole cell extract after autoinduction with BL21 (DE3), lane 7: HIS-tagged enhanced green fluorescent protein (eGFP) used as a positive control. The expected band for HIS-tagged NusA-β-defensin 4 fusion protein is evident in lane 2 at ~60kDa with the positive control at ~27kDa in lane 7 as expected.

4.3.Purification HIS-tagged NusA-β-defensin 4 Fusion Protein Using Nickel Affinity Chromatography

The following section outlines the results of the initial purification of the NusA-β-defensin 4 fusion protein and the optimization that was carried out to try and improve the purification protocol.

4.3.1. Nickel Affinity Chromatography with Tris buffer

Purification was performed using a peristaltic pump, "Ni Sepharose Fast Flow" beads and tris buffer. Figure 24 shows various fractions from the affinity chromatography purification (method outlined in Section 3.3) for NusA-β-defensin 4 fusion protein from 1 litre of BL21 (DE3) cells. β-defensin 4 transformed BL21 (DE3) whole cells in lane 1, supernatant after cell lysis is in lane 2, flow through from Nickel Sepharose column in lane 3, the elution from the wash steps are in lanes 4 to 6 and the final elution fractions containing the NusA-β-defensin 4 fusion are in lanes 8 to 15. Lanes 7 and 16 contain the Precision Plus Protein™ Dual Colour standard. A single band at approximately 60kDa was expected for the pure NusA-β-defensin 4 fusion protein however it is clear that there is an additional major contaminant band at approximately 50 kDa in all of the elution fractions. There is also some large aggregated protein at higher molecular weights in some of the fractions e.g. 10 and 11. This result indicated that the NusA-β-defensin 4 fusion protein was not isolated in a pure state. It was proposed that the 50 kDa contaminant band may be as a result of degradation of the fusion protein, therefore further optimization of the purification protocol was necessary.

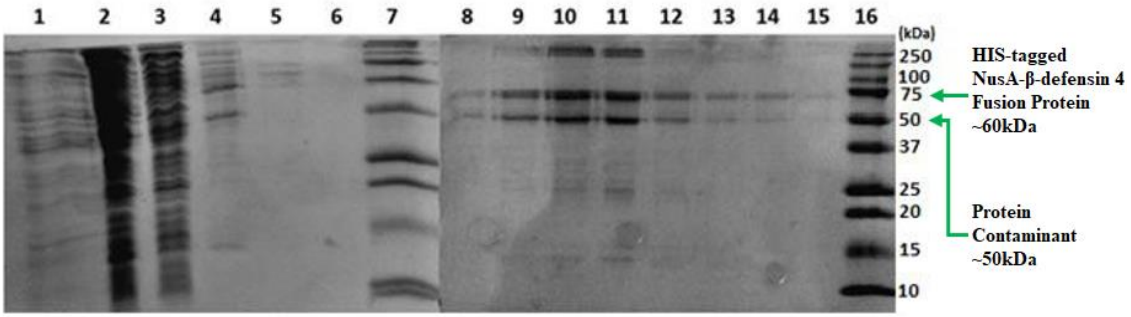


Figure 24: Affinity Chromatography Using Tris Buffer without PMSF

Lane 1: β-defensin 4 transformed BL21 (DE3) whole cells, lane 2: supernatant after cell lysis, lane 3: flow through from Nickel Sepharose column, lane 4: elution from first wash, lane 5: elution from second wash, lane 6: elution from third wash, lane 8: elution fraction 2, lane 9: elution fraction 3, lane 10: elution fraction 4, lane 11: elution fraction 5, lane 12: elution fraction 6, lane 13: elution fraction 7, lane 14: elution fraction 8, lane 15: final elution (300mM imidazole), lane 16 and Precision Plus Protein™ Dual Colour standard in lanes 7 and 16. Single band expected at ~60kDa for NusA-β-defensin 4 fusion protein in elution fractions.

4.3.2. Optimisation Summary of NusA-β-defensin 4 Fusion Protein Purification

In attempts to optimize the purification of the NusA-β-defensin 4 fusion, the following strategies were proposed and followed:

a) Addition of PMSF to the cells before sonication. PMSF is a serine-protease inhibitor and addition of this chemical was proposed to prevent degradation of the fusion protein that was suspected to be causing the presence of the contaminant band at 50 kDa as seen in Figure 24. Refer to Figure 44 in Appendix H for SDS-PAGE image.

b) Adjustment of the purification buffers by changing from Tris-based to Na-Phosphate based, in an attempt to reduce aggregation and potential degradation. Refer to Figure 45 in Appendix I for SDS-PAGE image.

c) Verification of presence of non-specific binding of *E. coli* contaminant proteins to the Nickel affinity column. It was thought that HIS-containing *E. coli* proteins in the cell lysate may be competing with the binding of the tagged NusA-β-defensin 4 fusion, therefore preventing it from being successfully isolated during the purification. Refer to Figure 46 and Figure 47 in Appendix J for SDS-PAGE image.

Unfortunately, despite many purification attempts, none of these optimization methods improved the purification. In fact, the protein, although expressed, appeared not to bind to the affinity column during subsequent purifications and no NusA-β-defensin 4 fusion was recovered.

At this stage, it was suspected that the fusion protein was not being folded properly within the host cells and that the HIS-tag may not be accessible for binding to the Nickel affinity column. As an alternative approach, it was decided to try a different affinity tag/fusion protein, namely MBP.

4.4.Affinity Chromatography Yield

The Bradford method was used to quantify the amount of protein present in all fractions loaded onto and eluted from the Nickel affinity column.

4.4.1. Bradford Method

Each elution fraction of the affinity chromatography purification from Figure 24 was analysed using SDS-PAGE (refer to Section 3.2.4 for protocol) and the protein concentration was determined using Bradford assay (Section 3.4.1). Refer to Appendix K for Bradford assay standard curve. Table 8 summarises the results of the quantification. As can be seen from the table a total of 0.97mg of protein was recovered from 1 L of BL21 (DE3) cells.

Table 8: Protein Quantification results of each purification fraction from Figure 24, using Bradford Method

Sample	Abs. at 595nm	Conc. (mg/ml)	Volume available (ml)	Total Conc. (mg/ml)	
Whole Cells	0.623	0.12			
Sonicated supernatant	1.614	0.45			
Flow through	1.532	0.42			
Wash 1	0.651	0.13			
Wash 2	0.494	0.08			
Wash 3	0.462	0.07			
Elution 1	0.462	0.07	1	0.07	Total = **0.97mg** of HIS-tagged NusA-β-defensin 4 fusion protein in **10 ml**
Elution 2	0.503	0.08	1	0.08	
Elution 3	0.578	0.11	1	0.11	
Elution 4	0.735	0.16	1	0.16	
Elution 5	0.675	0.14	1	0.14	
Elution 6	0.551	0.10	1	0.10	
Elution 7	0.502	0.08	1	0.08	
Elution 8	0.487	0.08	1	0.08	
Elution 9	0.491	0.08	1	0.08	
Elution 10	0.470	0.07	1	0.07	

4.5. Cloning of β-Defensin 1, 3 and 4 into pMAL-p4X using Primer Set 2

To begin production of the new MBP-β-defensin fusion proteins, it was first necessary to re-clone the β-defensin genes into the new expression vector, namely pMAL-p4X. Primer set 2 (described in Table 3) were used for the amplification containing the restriction sites BamHI and EcoRI to allow for cloning of the genes into the multiple cloning site of pMAL-p4X.

4.5.1. Polymerase chain reaction and Electrophoresis

Amplification of all 3 β-defensin gBlocks using primer set 2 was performed by PCR using the protocol outlined in Section 3.1.1. Refer to Table 3 for primer sequences. *In silico* PCR using Serial Cloner confirmed that the PCR products for β-defensin 1, 3 and 4 are 144bp, 149bp and 139bp respectively and indeed these were the sizes of the PCR products observed on agarose gels. A 50bp ladder (Hyperladder II) was used for analysis on a 2% agarose gel following the method outlined in Section 3.1.3, allowing all amplified bands to be seen between 100bp and 200bp. Figure 25 shows the amplified genes on a 2% agarose gel.

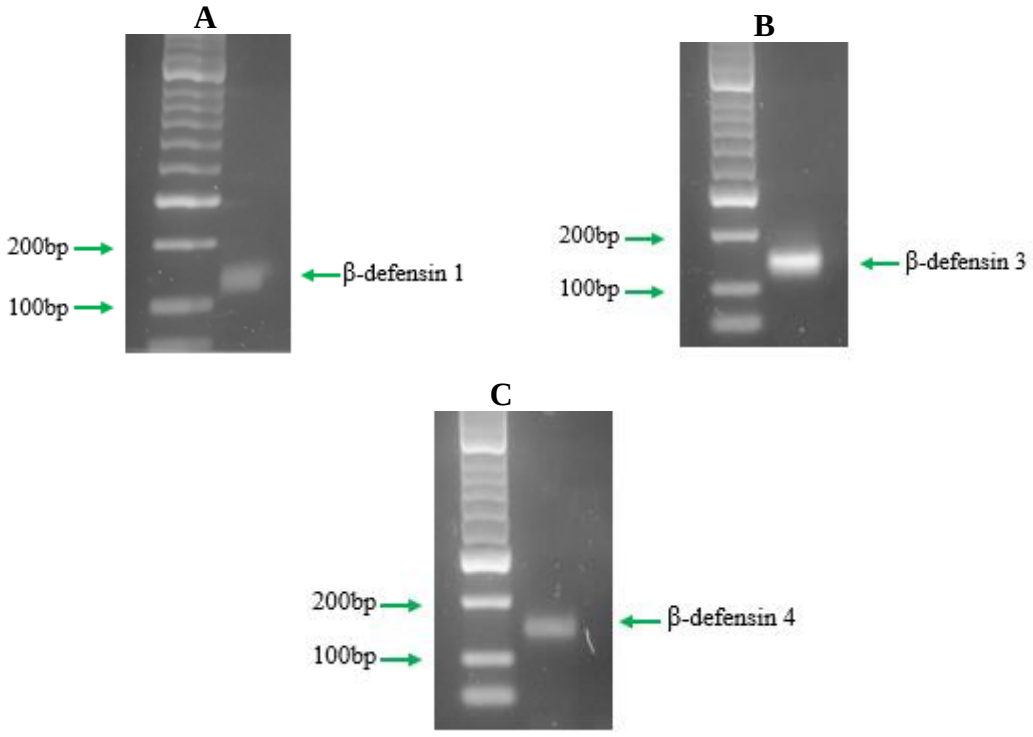


Figure 25: Positive Band for β-Defensin 1, 3 and 4 after PCR
Image A shows the amplified β-defensin 1 PCR product of 144bp, Image B shows the β-defensin 3 PCR product of 149bp and Image C shows the amplified β-defensin 4 PCR product of 139bp, using primer set 1. Hyperladder II was used as the molecular weight marker.

4.5.2. Digestion of Insert

Insert digestion was carried out as per Section 3.1.4. *In silico* digestion concluded the digested inserts for β-defensins 1, 3 and 4 were 132bp, 126bp and 135bp respectively. These were the fragment sizes for the genes as observed on agarose gels. A 50bp ladder (Hyperladder II) was used for β-defensin 1, 3 and 4 to visualise the digested bands using electrophoresis on a 2% agarose gel. Refer to Appendix L for Serial Cloner output illustrating *in silico* digestion. Figure 26 shows the digested gene products on a 2% agarose gel.

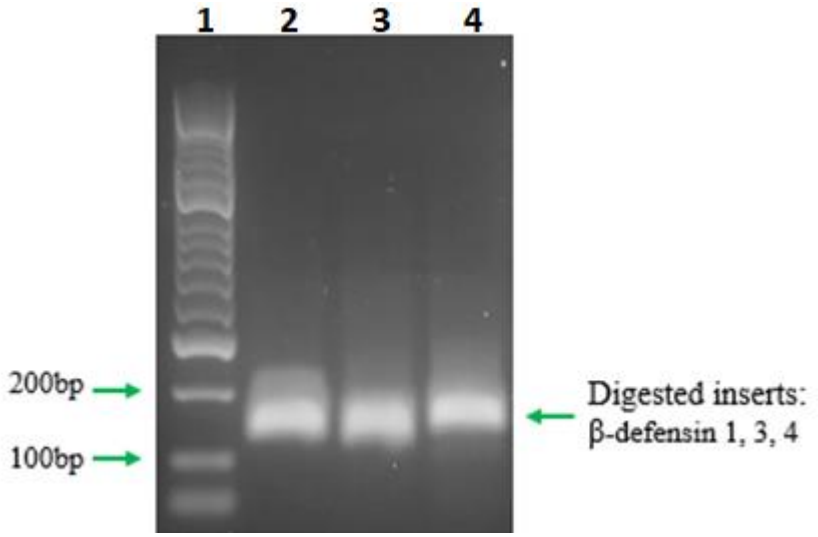


Figure 26: Positive Band for β-Defensins 1, 3 and 4 after Digestion of Insert
Image shows the digested β-defensin 1 insert of 132bp in lane 2, β-defensin 3 digested insert of 126bp in lane 3 and digested β-defensin 4 insert of 135bp in lane 4, using BamHI and EcoRI. Hyperladder II was used as the molecular weight marker.

4.5.3. Digestion of pMAL-p4X Vector

The procedure outlined in Section 3.1.5 was followed to digest the pMAL-p4X vector in preparation for cloning. The resulting band after digestion with BamHI and EcoRI was 6714bp. Refer to Appendix M for *in silico* output from Serial Cloner. A 10kb ladder (Hyperladder I) was used to allow for digested band to be detected on a 2% agarose gel (Figure 27).

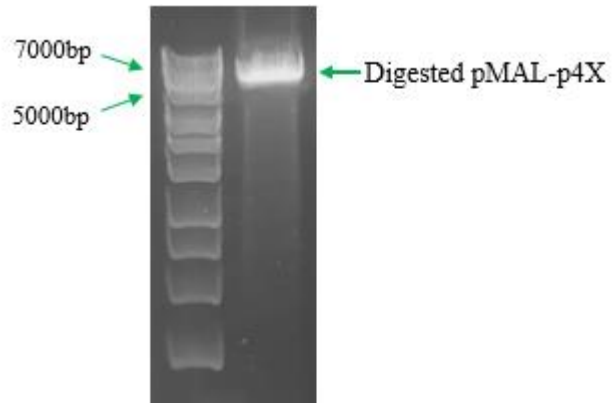


Figure 27: Positive Band for pMAL-p4X after Digestion
Image showing BamHI and EcoRI digested pMAL-p4X vector at ~6714bp. Hyperladder I was used as the molecular weight marker.

4.5.4. Ligation and transformation

Ligation of the β-defensin inserts to the pMAL-p4X cloning vector was carried out as per Section 3.1.6. A schematic representation of this step is illustrated below in Figure 28. The figure highlights the position in the vector where the insert will ligate.

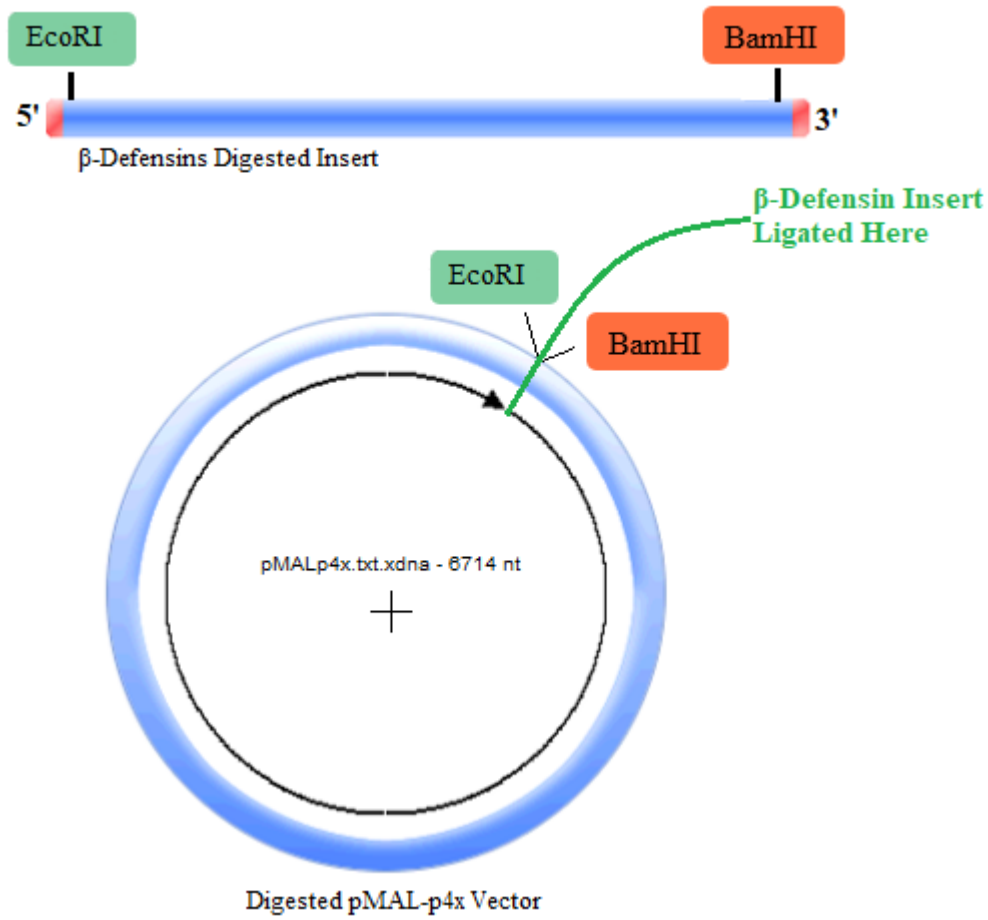


Figure 28: *In Silico* Ligation of β-Defensin 1 Insert to pMAL-p4X Vector

4.5.5. Identification of Putative pMAL-p4X-β-defensin plasmids

Restriction Digestion

Restriction digestion with EcoRI and HindIII was used to identify putative pMAL-p4X-β-defensin plasmids was performed as outlined in Section 3.1.10. *In silico* pMAL-p4X restriction digestions were performed using Serial Cloner to determine the size of the expected bands for ligated and unligated pMAL-p4X plasmids. Refer to Appendix N for Serial Cloner output. Figure 29, Figure 30 and Figure 31 displays the results of restriction digestion for β-defensin 1, 3 and 4 respectively. pMAL-p4X-β-defensin plasmids are highlighted in green circles.

In Figure 29, pMAL-p4X-β-defensin plasmids are expected to show two bands at 158bp and 6688bp while the empty pMAL-p4X plasmids are expected to have 2 bands at 32bp, 6688bp. 6 potential positive clones were evident on the agarose gel.

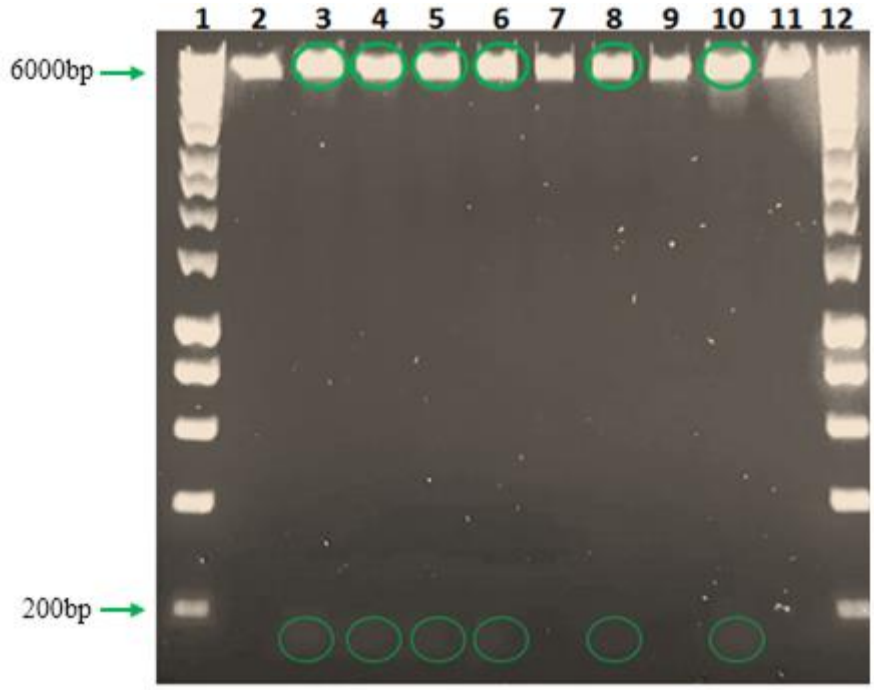


Figure 29: Restriction Digestion of Putative pMal-p4X-β-defensin 1 using EcoRI and HindIII

= β-defensin 1 positive clones, with 2 band at 158bp and 6688bp

Samples in lanes 2, 7, 9 and 11 were undigested and Hyperladder I in lanes 1 and 12 was the molecular weight marker.

In Figure 30, pMAL-p4X-β-defensin 3 plasmids are expected to show two bands at 152bp and 6688bp while the empty pMALp4x plasmids are expected to have bands at 32bp and 6688bp. 6 potential pMAL-p4X-β-defensin 3 plasmids were also evident on the agarose gel.

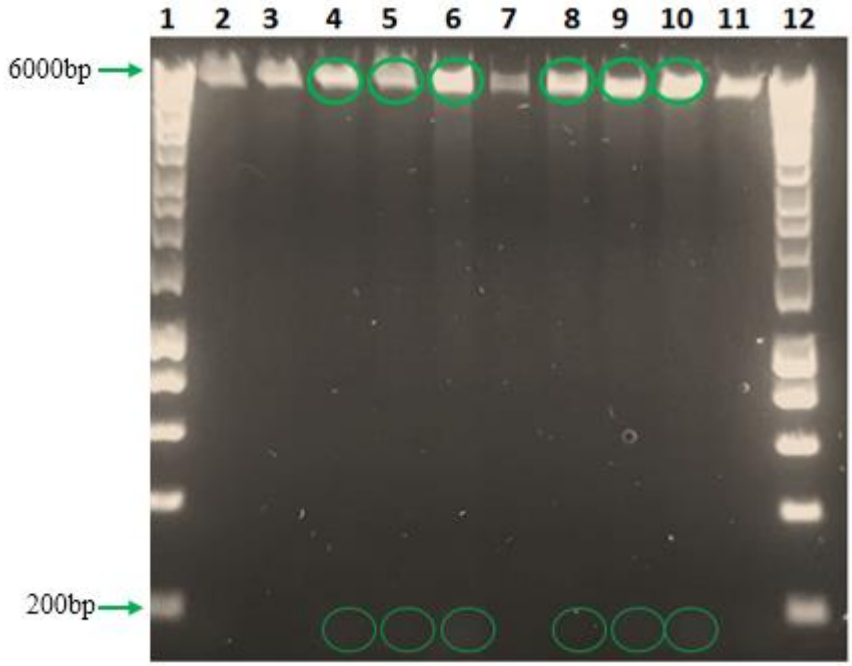


Figure 30: Restriction Digestion of Putative pMal-p4X-β-defensin 3 using EcoRI and HindIII

= pMal-p4X-β-defensin 3 plasmids, with 2 band at 152bp and 6688bp

Samples in lanes 2, 3,7 and 11 were undigested and Hyperladder I in lanes 1 and 12 was the molecular weight marker.

In Figure 31, pMAL-p4X-β-defensin 4 plasmids are expected to show two bands at 161bp and 6688bp while the empty pMAL-p4X plasmids are expected to have two bands at 32bp and 6688bp. One potential pMAL-p4X-β-defensin 4 plasmid was seen on the agarose gel.

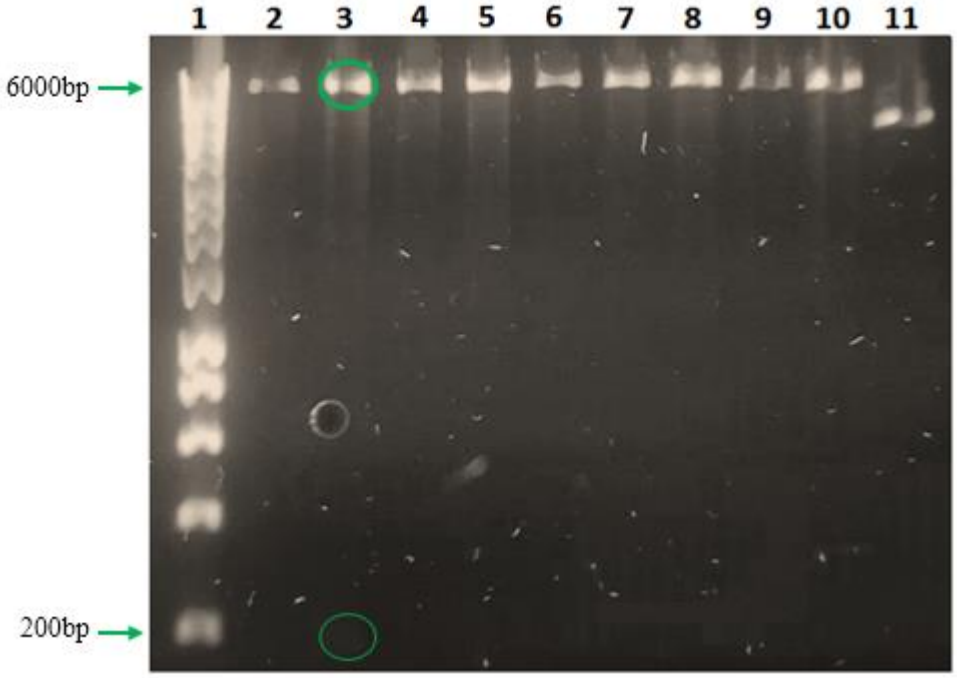


Figure 31: Restriction Digestion of Putative pMal-p4X-β-defensin 4 using EcoRI and HindIII

= pMAL-p4X-β-defensin 4 plasmids, with 2 band at 161bp and 6688bp

Samples in lanes 2, and 4-11 were undigested or negative. Hyperladder I in lane 1 was used as the molecular weight marker.

Sanger Sequencing

One putative pMAL-p4X-β-defensin plasmids for each β-defensin gene was sent for sequence verification by Sanger sequencing to GATC (Germany). The sequencing results confirmed that all 3 plasmids contained the correct sequence. The chromatograms below in Figure 32, Figure 33 and Figure 34 highlights the nucleic acid sequence corresponding to the mature β-defensin 1, 3 and 4 peptides in orange.

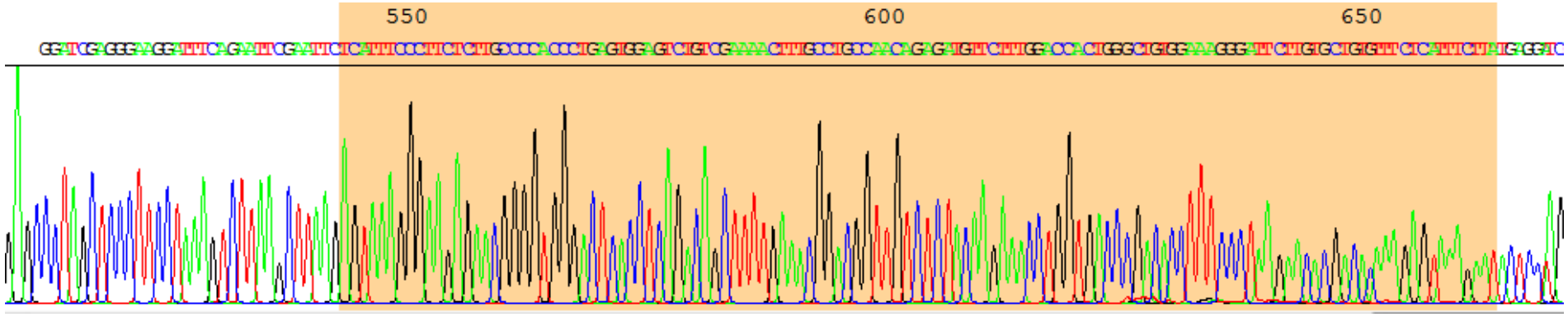


Figure 32: Confirmation of B-Defensin 1 Sequence by Sanger Sequencing, generated using the GATC Viewer (Eurofins Scientific)

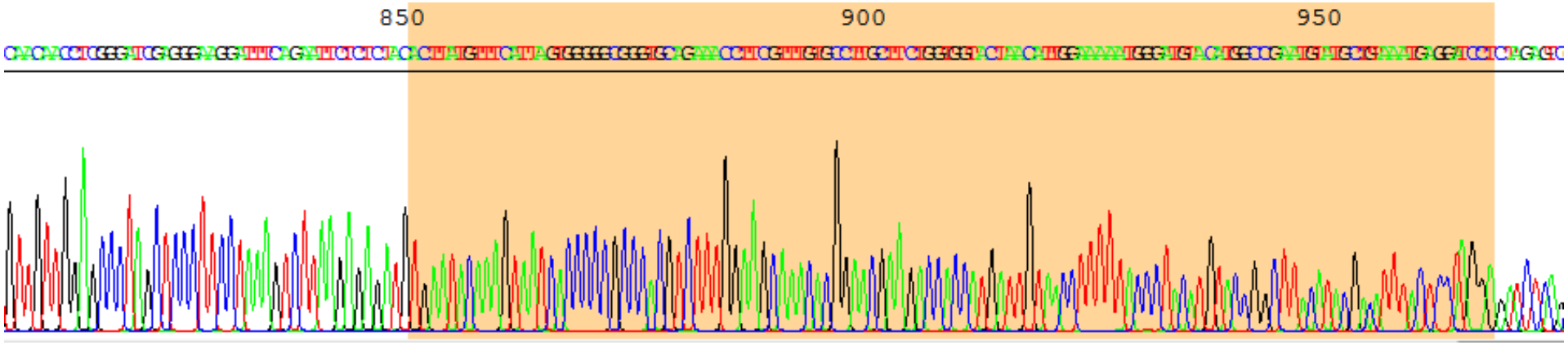


Figure 33: Confirmation of B-Defensin 3 Sequence by Sanger Sequencing, generated using the GATC Viewer (Eurofins Scientific)

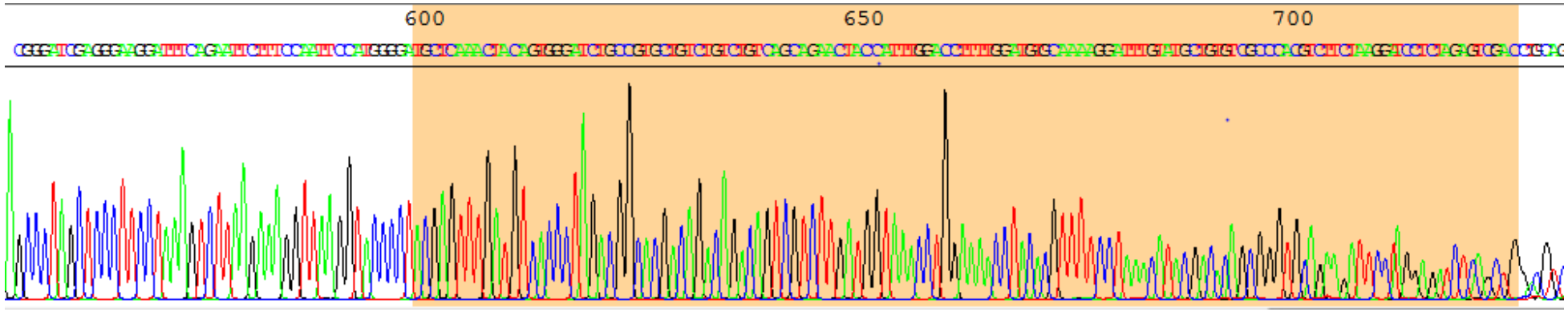


Figure 34: Confirmation of B-Defensin 4 Sequence by Sanger Sequencing, generated using the GATC Viewer (Eurofins Scientific)

4.6.Expression of MBP-β-defensin 4 Fusion Protein in *R. gami* cells and BL21 (DE3) cells

The MBP-β-defensin 4 fusion protein was expressed and purified, with aims to repeat expression and purification using MBP-β-defensin 1 and 3 fusion proteins upon successful purification of MBP-β-defensin 4 fusion protein. Both IPTG induction and autoinduction was performed as per Sections 3.2.1 and 3.2.2 using *R. gami* and BL21 (DE3) cells. Figure 35 demonstrates an SDS-PAGE of the whole cell extracts after overexpression of MBP β-defensin 4 fusion protein using both methods. Lane 1 contains a protein standard (Precision Plus Protein™ Dual Colour), lane 2 shows autoinduction using BL21 (DE3) for 48 hours, lane 3 shows autoinduction using *R. gami* cells for 48 hours and lanes 4 and 5 show IPTG induction using BL21 (DE3) and *R. gami* for at 37ºC, induced for 3 hours. Expression bands are clear in lanes 2 and 3 with the target band approximately 49kDa. BL21 (DE3) shows a larger expression band in comparison to *R. gami*. Similarly, for IPTG induction, small expression band can be seen in lane 4 however, lane 5 does not show similar results.

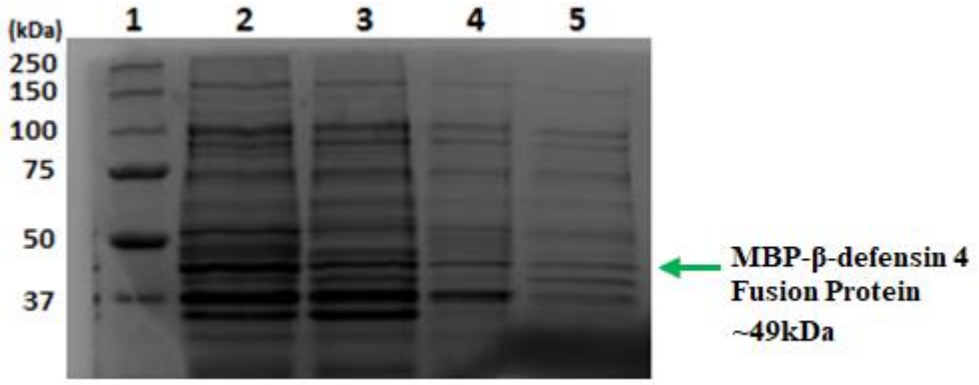


Figure 35: Expression Bands from Autoinduction of BL21 (DE3) and *R. gami*
Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: autoinduction using BL21 (DE3) for 48 hours, lane 3: autoinduction using *R. gami* cells for 48 hours, lane 4 IPTG induction using BL21 (DE3) for at 37ºC, induced for 3 hours and lane 5: IPTG induction using *R. gami* for at 37ºC, induced for 3 hours. The expected size of MBP-β-defensin 4 fusion protein is ~49kDa.

4.7.Purification using Maltose Affinity Chromatography

To verify the expression of the MBP β-defensin 4 fusion protein in both *R. gami* and BL21 (DE3) cells a test expression (1L of cell culture), followed by purification was carried out to determine if the protein could be isolated. Purification was performed using the ÄKTA purification system and an amylose affinity resin using the protocol outlined in Section 3.3.2. Purification trials were performed using BL21 (DE3) and *R. gami* cells and to compare which strain yielded the highest expression. Figure 36 shows elution fractions from affinity chromatography using *R. gami* cells. Lane 1 shows the protein standard, lane 2 shows β-defensin 4 transformed *R. gami* whole cells after autoinduction, lane 3 contains the crude extract after cell lysis, lane 4 contains flow through from column, lanes 5 to 8 contain elution fractions that were expected to contain a single band at approximately 49kDA corresponding to the MBP-β-defensin 4 fusion protein, however multiple bands are visible on the gel for these fractions. Therefore, it can be concluded that insufficient MBP β-defensin 4 fusion protein was expressed in these cells as it could not successfully be isolated using the affinity column.

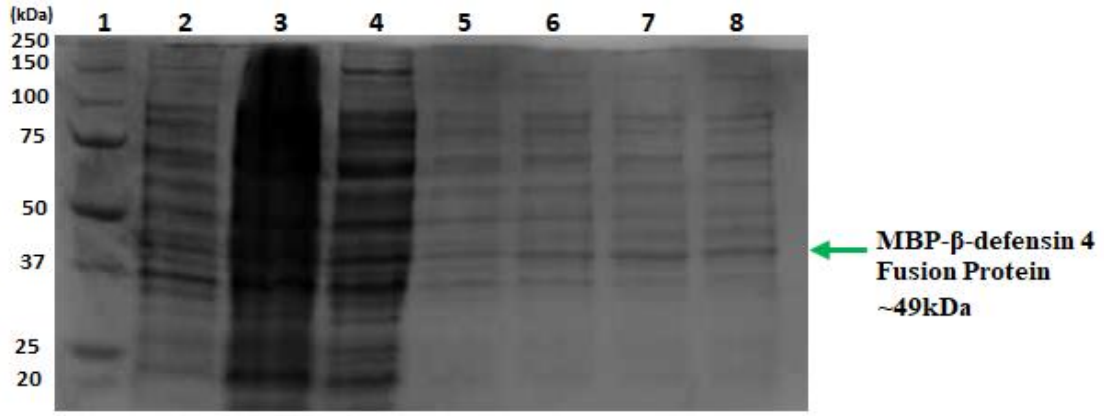


Figure 36: Purification Fractions of β-Defensin 4 Expressed in *R. gami*
Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2 β-defensin 4 transformed *R. gami* whole cells after autoinduction, lane 3: crude extract after cell lysis, lane 4: flow through from column, lane 5: elution fraction 1, lane 6: elution fraction 2, lane 7: elution fraction 3 and lane 8: elution fraction 4. Single band is expected at ~49kDa for MBP-β-defensin 4 fusion protein.

Figure 37 shows elution fractions from affinity chromatography using BL21 (DE3) cells. Lane 1 shows the protein standard, lane 2 shows β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3 contains the pellet after cell lysis, lane 4 contains the crude extract after cell lysis, lane 5 contains the first flow through from column, lane 6 contains second flow through from column, lane 7 contains first wash from the column, lanes 8 to 10 contain elution fractions. A large band is visible on gel for the elution fractions and this correlated with the expected at band for the MBP β-defensin 4 fusion protein at 49kDa. Therefore, it can be concluded that the MBP β-defensin 4 fusion protein was not only successfully expressed in the BL21 (DE3) cells but also that the protein could be successfully isolated.

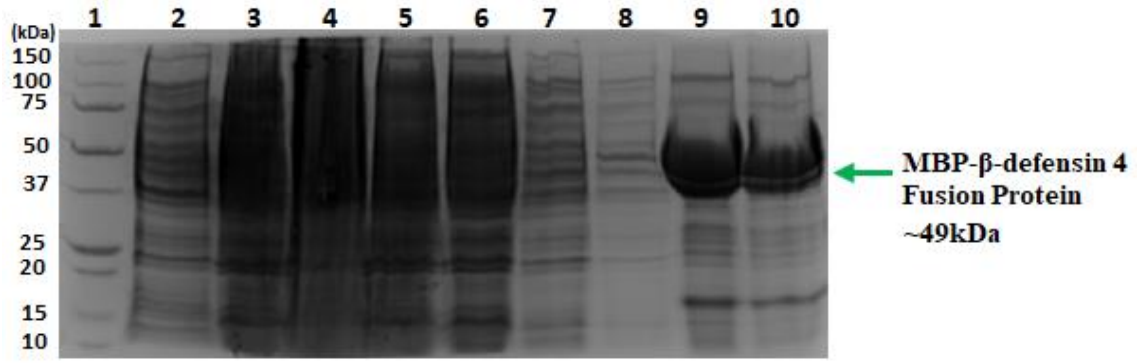


Figure 37: Purification Fractions of β-Defensin 4 Expressed in BL21 (DE3)
Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3: pellet after cell lysis, lane 4: crude extract after cell lysis, lane 5: first flow through from column, lane 6: second flow through from column, lane 7: first wash from the column, lane 8: elution fraction 17, lane 9: pooled elution fraction 18 and 19 and lane 10: elution fraction 20. Single band seen as expected at ~49kDa in lanes 9 and 10, indicating successful purification of MBP-β-defensin 4 fusion protein.

Figure 38 shows the output from the ÄKTA purification system software with the elution fraction number on the x-axis and UV absorbance (mAu) on the y-axis. The large peak at elution 3 shows the elution of non-specific proteins from the column. At fraction 18 a second peak is evident after the addition of elution buffer, detecting the β-defensin 4 peptide fused MBP tag being eluted from the column. The peak eventually plateaus as all the protein is eluted from the column.

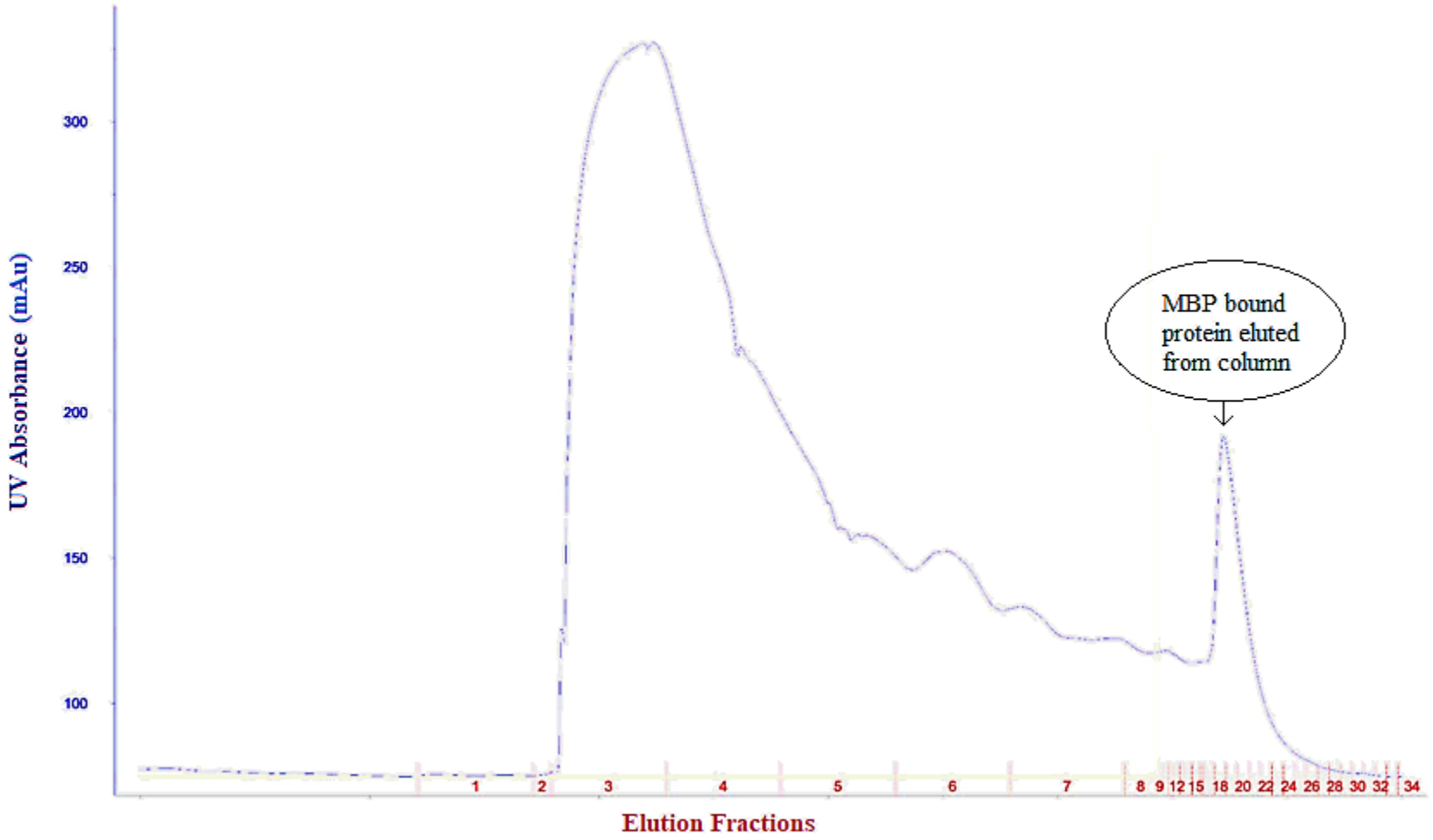


Figure 38: UV Absorbance of Elution Factions during Purification Using AKTA

4.7.1. Confirmation of Expression and Purification by Western Blot

Western blot was performed to confirm expression of the β-defensin protein with MBP tag. Figure 39 shows 2 duplicate SDS-PAGE gels; one stained with Coomassie blue (left) and the other used for western blot (right). Lane 1 contains a protein standard and lanes 2-5 contains serial dilutions (x100) of the elution fraction 18 from purification (Figure 37). Significant bands are seen on both images between 37kDa and 50kDa which correlates to the expected MBP fused β-defensin peptide of ~49kDa. As the samples are diluted in lanes 4 and 5, a sharper band can be seen. A faint band on the Coomassie stained gel correlates to a classic positive western blot band on the PVDF membrane.

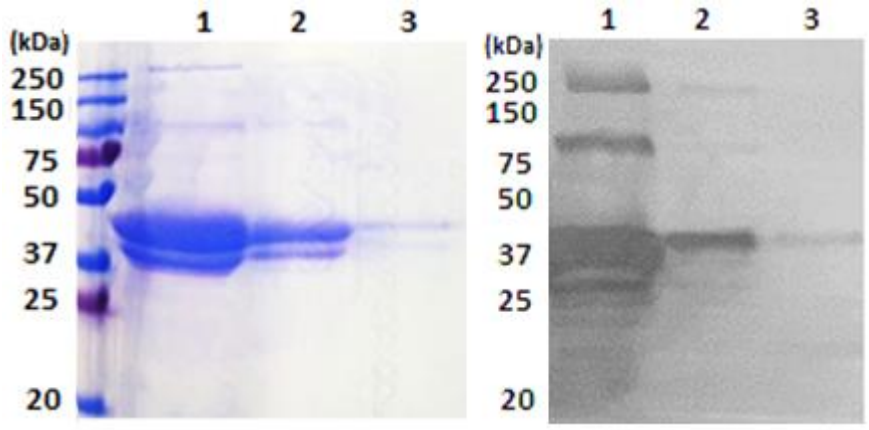


Figure 39: Western Blot Confirming Expression and Purification of β-Defensin 4
Image shows duplicate SDS-PAGE gels. Left gel stained with Coomassie blue and right gel unstained and blotted instead with anti-MBP antibody. Lane 1 contains a protein standard and lanes 2-5 contains serial dilutions (x100) of the elution fraction 18 from purification (Figure 37). The expected band for MBP fused β-defensin peptide is ~49kDa.

4.8.Protein Quantification

Each elution fraction of affinity chromatography purification from Figure 37 was analysed using SDS-PAGE and the protein concentration was determined using BCA assay. Table 9 shows the sample identification and raw date for BCA quantification. Refer to Appendix O for BCA assay standard curve. From Table 9, the yield of MBP-β-defensin 4 fusion protein was 5.24 mg from 1L of BL21 (DE3) cell culture.

Table 9: Protein Quantification results of each purification fraction from Figure 37, using BCA Method

Sample	Abs. at 562nm	Conc. (mg/ml)	Volume available (ml)	Total Conc. (mg/ml)	
Whole Cells	2.2255	3.87			
Sonicated Pellet	3.7755	6.69			
Crude Extract	2.395	4.18			
Flow Through 1	2.4485	4.27			
Flow Through 2	1.177	1.96			
Wash 1	0.354	0.46			
Wash 2	0.1415	0.08			
Elution 17	0.256	0.29	1	0.29	Total = **5.24mg** of MBP-β-defensin 4 fusion protein in **5 ml**
Elution 18 + 19	0.976	1.60	2	3.20	
Elution 20	0.714	1.12	1	1.12	
Elution 21	0.4455	0.63	1	0.63	

4.9.Summary of Results and Optimised Protocol

In summary:

- Cloning of β-defensin 4 was successful using primer set 1 and pET44a vector producing a HIS-tagged recombinant β-defensin 4
- Expression trials were performed using this HIS-tagged recombinant β-defensin 4 using IPTG induction (various [IPTG] and temperature) and autoinduction (Various temperature). However the expression levels were unsatisfactory.
- Purification trials were performed using a peristaltic pump and various buffers to purify the expressed HIS-tagged β-defensin 4 fusion peptide. However purification was not successful.
- Decision was made to change strategy.
- Cloning of β-defensins 1, 3 and 4 were successful using newly designed primer set 2 and pMAL-p4X vector, producing an MBP-tagged recombinant β-defensin 1, 3 and 4.
- Autoinduction was used to express the MBP-tagged β-defensin 4 fusion peptide.
- The ÄKTA was used to purify the MBP-tagged β-defensin 4 fusion peptide.

Figure 40 below summarises the optimised cloning, expression and purification methods and results from this project in chronological order.

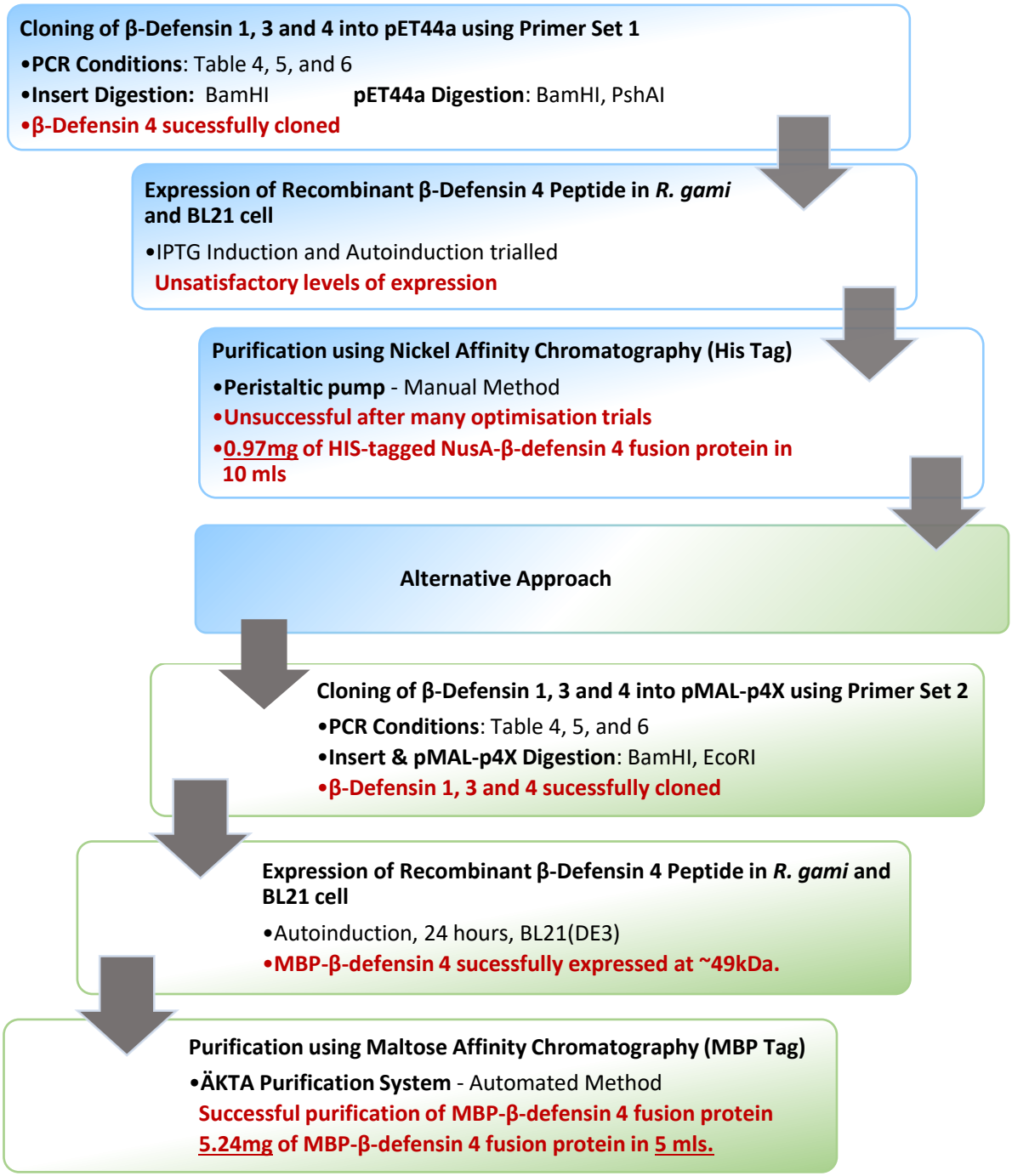


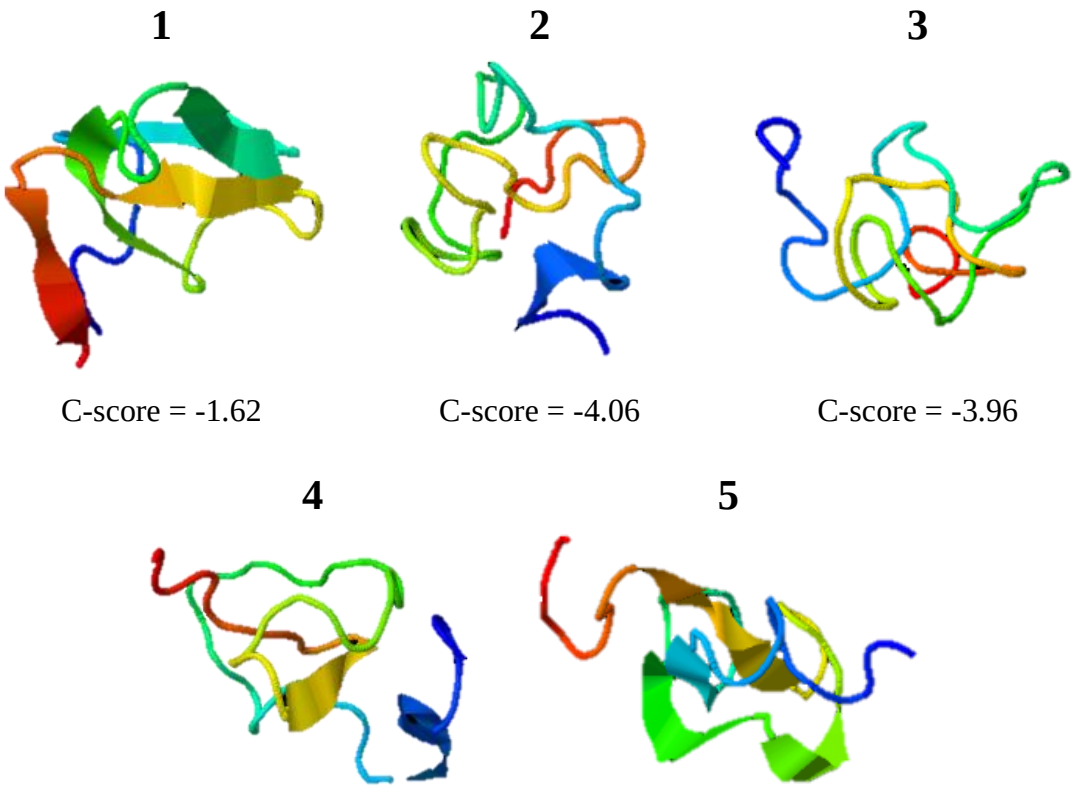
Figure 40: Summary of Cloning, Expression and Purification Methods and Results

4.10. Structure Prediction of β- Defensins

Structural models were investigated during this research project. An online tool called I-TASSER was used which used algorithms and a protein database to provide 5 prediction models of the peptide structure and 10 peptides with highest structural homology. Refer to Section 3.5 for the principle of this prediction. A C-score is calculated which determines the confidence of each model based on the convergence parameters of the structure assembly simulations and the significance of threading template alignments. C-scores can range from -5 to 2, with a high value proportional to high confidence.

4.10.1. β-Defensin 1

Figure 41 provides the best 5 structural models predicted by I-TASSER for the β-defensin 1 peptide. Based on the C-score, prediction 1 has the highest score, indicating this is the best prediction of β-defensin 1.

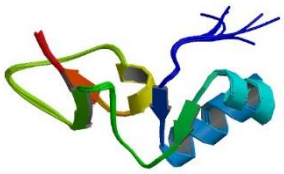
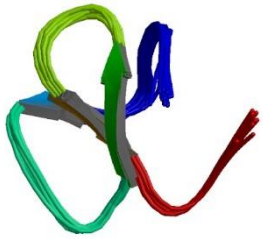


(Yang *et al.*, 2015)

Figure 41: Top 5 Prediction Models of β-Defensin 1 Generated by I-TASSER

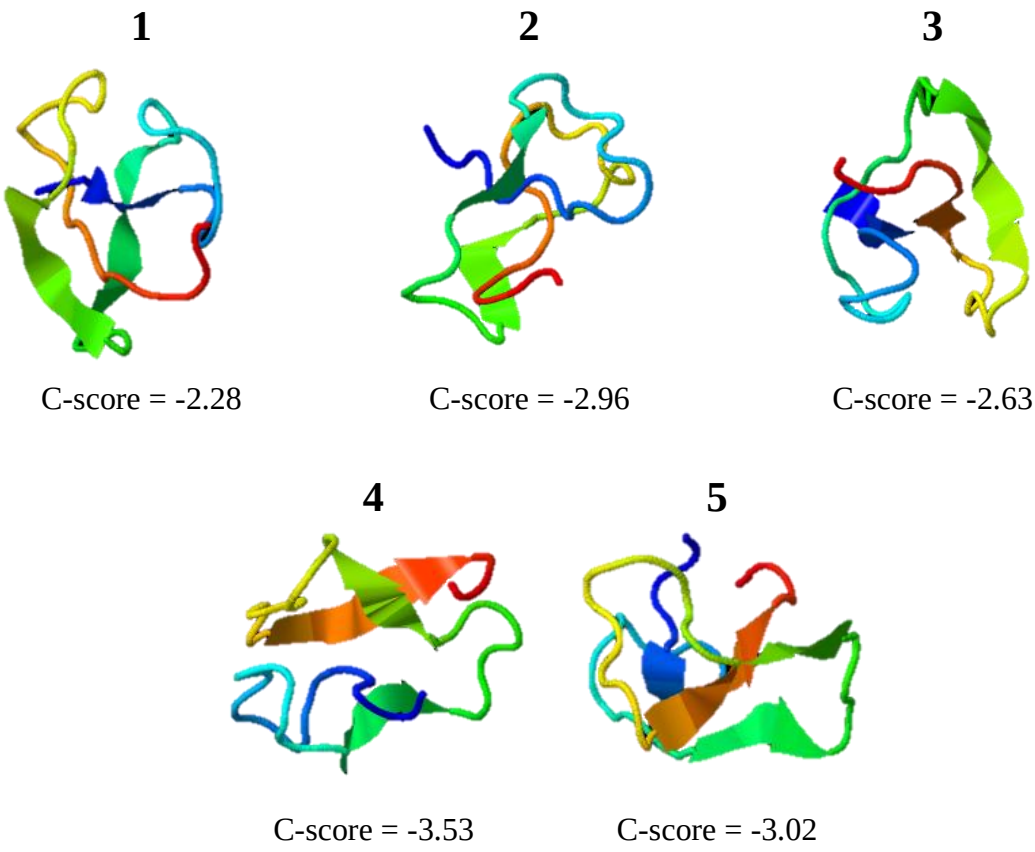
Table 10 below shows the 3 most structurally similar peptides to β-defensin 1, their classification and organism. From the table it is clear that β-defensin 1 shares homology with the human β-defensin 2 and defensins from *Tachypleus tridentatus* and *Gallus gallus.*

Table 10: Proteins Structurally Similar to β-Defensin 1

Peptide	Description	Organism	Classification	Reference
 **1FD3**	Human β-defensin 2	*Homo sapiens*	Antimicrobial Protein	(Hoover *et al.*, 2000)
 **2RNG**	Solution structure of big defensin	*Tachypleus tridentatus*	Antimicrobial Protein	(Kouno *et al.*, 2008)
 **2MJK**	Nmr structure of hen egg β-defensin gallin (chicken ovo-defensin)	*Gallus gallus*	Antimicrobial Protein	(Herve *et al.*, 2014)

4.10.2. β-Defensin 3

Figure 42 provides the best 5 structural models predicted by I-TASSER for the β-defensin 3 peptide. Based on the C-score, prediction 1 has the highest score, indicating this is the best prediction of β-defensin 3.

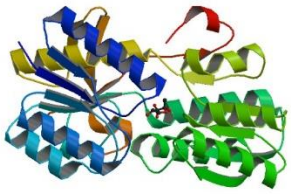
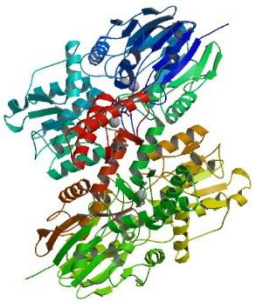
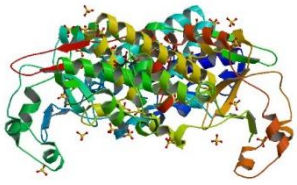


(Yang *et al.*, 2015)

Figure 42: Top 5 Prediction Models of β-Defensin 3 Generated by I-TASSER

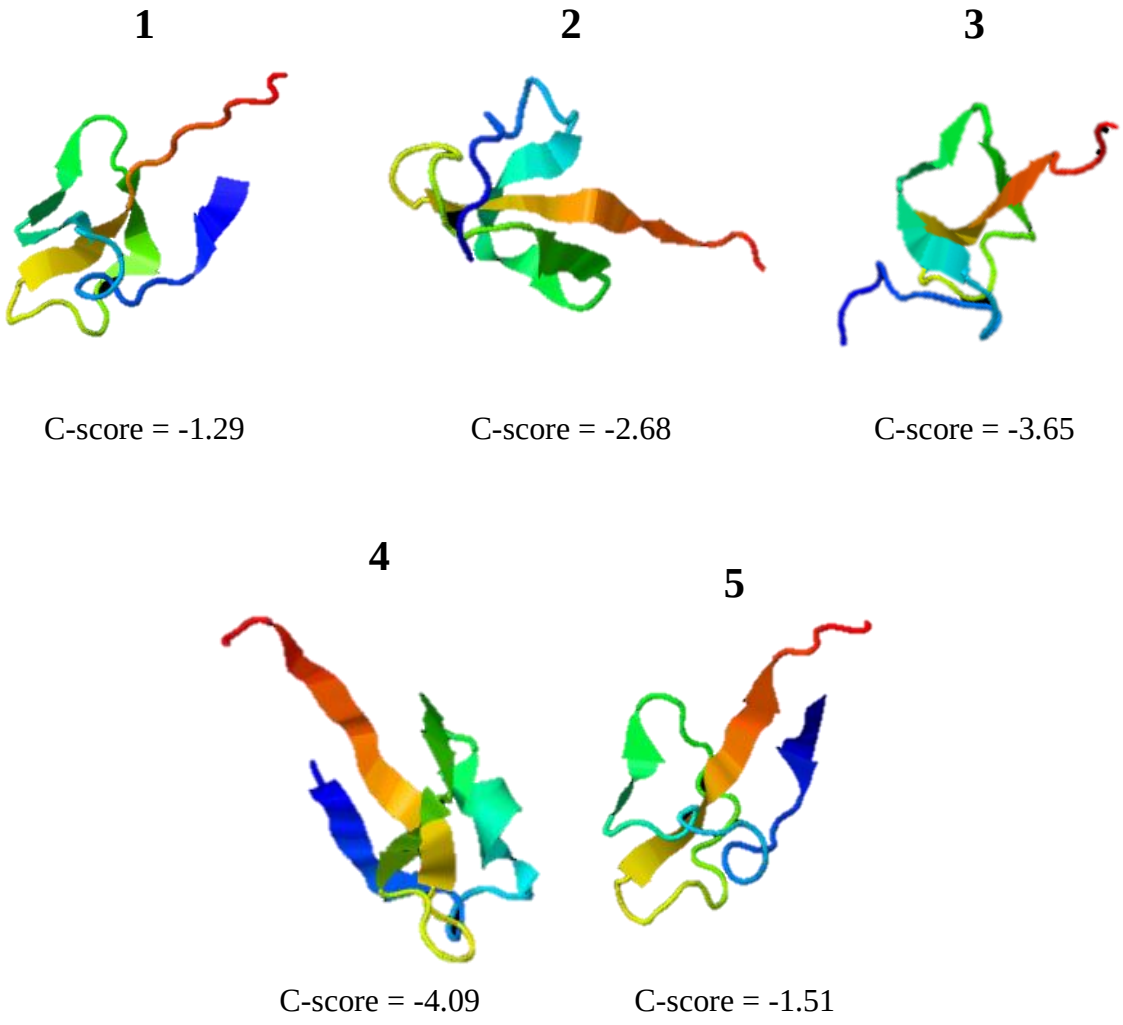
Table 11 shows the 3 most structurally similar peptides to β-defensin 3, their classification and organism. From the table it is clear that β-defensin 3 does not share homology with β-defensins from other species. This was expected as the sequence of the *Salmo salar* β-defensin 3 gene is unique in comparison to *Salmo salar* β-defensin 1 and 4.

Table 11: Proteins Structurally Similar to β-Defensin 3

Peptide	Description	Organism	Classification	Reference
 **3EJW**	Crystal Structure of the *Sinorhizobium meliloti* AI-2 receptor, SmLsrB	*Rhizobium meliloti* (strain 1021)	Signalling Protein	(Pereira *et al.*, 2008)
 **4XWT**	Crystal structure of RNase J complexed with UMP	*Deinococcus radiodurans*	Ribose Nucleic Acid (RNA) Binding Protein	(Zhao *et al.*, 2015)
 **4087**	Crystal structure of a N-tagged Nuclease	*Millerozyma acaciae*	Hydrolase	(Chakravarty *et al.*, 2014)

4.10.3. β-Defensin 4

Figure 43 provides the best 5 structural models predicted by I-TASSER for the β-defensin 4 peptide. Based on the C-score, prediction 1 has the highest score, indicating this is the best prediction of β-defensin 4.

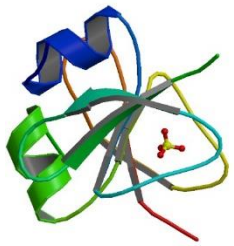
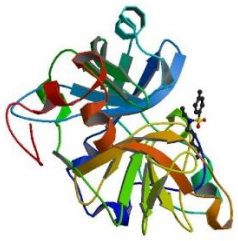
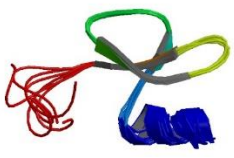


(Yang *et al.*, 2015)

Figure 43: Top 5 Prediction Models of β-Defensin 4 Generated by I-TASSER

Table 12 below shows the 3 most structurally similar peptides to β-defensin 4, their classification and organism. From the table it is clear that β-defensin 4 shares homology with human β-defensin 2 and 6 and Protease I from *Achromobacter lyticus*.

Table 12: Proteins Structurally Similar to β-Defensin 4

Peptide	Description	Organism	Classification	Reference
 **1FD4**	Human β-defensins 2	*Homo sapiens*	Antimicrobial Peptide	(Hoover *et al.*, 2000)
 **1ARC**	The primary structure and structural characteristics of *Achromobacter Lyticus* Protease I, a Lysine-specific Serine Protease	*Achromobacter lyticus*	Hydrolase/hydrolase Inhibitor	(Tsunasawa *et al.*, 1989)
 **2LWL**	Structural basis for the interaction of human β-defensin 6 and its putative chemokine receptor ccr2 and breast cancer microvesicles	*Homo sapiens*	Antimicrobial Protein	(De Paula *et al.*, 2013)

Chapter 5: Discussion

Antimicrobial resistance is a growing economic issue for humans and animals. AMPs are potentially the answer to this problem due their natural production by the host, diverse structures and board spectrum antimicrobial activity (Katzenback, 2015). Salmon aquaculture is a big part of the Irish economy. The natural habitat for this fish are inoculated with pathogens causing salmon mortality, which has significantly risen over the years due to diseases like ISA and AGD (Clark and Nowak, 2001). Although vaccines have been developed in the last number of years for treating infections in salmon (Valdenegro-Vega *et al.*, 2015), antibiotics such as fluoroquinolones and oxytetracycline are still in use in some fish farms. Antimicrobial resistance to some of these antibiotics have been recorded to date (Buschmann *et al.*, 2012). Horizontal gene transfer makes this a treat to not only salmon, but humans who consume the effected fish. Therefore, treatment of salmon with antibiotics will only contribute to the worldwide issue of antibiotic resistance. AMPs have been investigated in many fish species but not much research has been done on salmon. This project aimed to establish a protocol to successfully clone, express and produce β-defensins in *E. coli.* Once a protocol has been established to produce β-defensin in salmon, the techniques can be used for other species. Therefore, eliminating the use of antibiotics and in turn reducing antibiotic resistance.

5.1. PCR Amplification of β-defensin Genes for Cloning

Before cloning, PCR amplification of the β-defensin genes (1, 3 and 4) were performed using commercially available synthesised genes fragments (gBlocks from IDT) as DNA templates. This provided pure concentrated DNA for amplification. Initial troubleshooting was carried out for each set of primers using touchdown PCR, where a gradient of annealing temperatures was used from 40ºC to 60ºC in the same reaction, to ensure the target DNA was being amplified. PCR products of the correct predicted size were produced for each β-defensin gene with each primer set.

Of the 3 β-defensin genes, β-defensin 4 was successfully cloned into the expression vector pET44a. All 3 β-defensin genes were successfully cloned into the expression vector pMAL-p4X.

5.2.Expression System: Vector and Expression Host

Critical to the success of producing recombinant β-defensin AMPs is the choice of expression system i.e. the choice of expression vector and expression host. *E. coli* was chosen as the expression host for the AMPs given the previous success in production of recombinant AMPs (Daegelen *et al.,* 2009) as well as ease of culture and fast growth times (Sezonov *et al.,* 2007).Two strains of *E. coli* were used during the course of this project, namely *R. gami* and BL21 (DE3). The *R. gami* strain contains mutations of the the trxB an gor genes, which aid the formation of disulphide bonds by providing and oxidising cytoplasmic environment and the the lack of the Lon protease and the OmpT enzymes in BL21 (DE3) strains allows the production of the target peptide in the host and reduces the chances of protein degradation (Fathi-Roudsari *et al.,* 2016). Suitable expression plasmids for *E. coli* were chosen for use (pET44a and pMALp4x), both containing genes for soluble proteins that would be used as fusion proteins for the recombinant AMPs.

5.2.1. Expression Vectors Chosen

Initially the pET-44a vector was chosen as the cloning vector based on the protocol as outlined in Ruangsri *et al.,* (2013) who successfully cloned and purified β-defensins from Atlantic cod. The pET-44a vector is capable of producing high levels of expression of the target peptide fused with a NusA tag. This is crucial as the natural properties of the β-defensins to kill bacteria would interfere with the expression since bacterial cells were used for overexpressing the peptides. Fusion of the peptide to the NusA protein also aids in folding of the peptide in the cytoplasm. Studies have found that NusA has the ability to self-assemble into oligomers along with exhibiting chaperone activity and prevention of protein aggregation (Li *et al.,* 2013). The NusA tag brings the fused target peptide into a chaperone-mediated folding pathway and has been reported to interact with the GroEL chaperonin system in *E. coli* (Costa *et al.,* 2014). The vector utilises a HIS tag which allows for affinity purification using a Nickel Sepharose column. The pET expression system is widely used for successful cloning and expression of genes in *E. coli* (Mierendorf *et al.,* 1998; Novo *et al.,* 2006). A pET44a expression vector was used by (Witek *et al.,* 2017) to produce recombinant TlyA from *Mycobacterium tuberculosis* and the pET32 expression vector was used to produce recombinant human β-defensin 2 by Zhong *et al.,* (2006). However, as will be discussed later, difficulties arose in this project when using this expression vector with

the BL21 (DE3) cells for production of the NusA-β-defensin 4 fusion. It was suspected that difficulties arose with folding of the protein in the expression host. Therefore, in the latter stages of the project, the pMAL-p4X vector was utilized.

The pMAL-p4X expression vector contains the gene for MBP, a carbohydrate binding protein from *E. coli* (Riggs, 2000) that is used to aid folding of fused proteins but also for affinity purification via highly specific binding to an amylose affinity column. Additionally, this vector contains the native signal sequence for MBP which directs the protein and anything to which it is fused, to the periplasm (Riggs, 2000). This was considered to be an advantage as it would allow for folding of the β-defensin peptides in the non-reducing environment of the periplasm, aiding the correct formation of the disulphide bonds in the structure. Other AMPs have been successfully purified using the MBP tag. Epilancin 15X is a genetically engineered AMP which were successfully purified using the MBP tag (Velásquez *et al.*, 2011). ORBK is a cyclic cationic peptide which was also purified using the MBP tag (Li *et al.*, 2014). New primers (primer set 2) were designed to facilitate cloning of the β-defensin genes into the vector. All 3 β-defensin genes were successfully cloned in comparison to using pET44a where only β-defensin 4 was successfully cloned. Further optimisation of the cloning procedure using the pET44a vector is warranted for this study.

5.2.2. Expression Hosts

Of the two host strains used in the expression studies, BL21 (DE3) cells produced higher levels of β-defensin 4 fusion protein compared to *R gami* cells, as evidenced from the SDS-PAGE and western blot analysis. Bands for both β-defensin 4 fusion proteins were clearly evident at the correct size when using BL21 (DE3) cells (refer to Figure 22 and Figure 35).

R. gami cells were initially chosen for expression of the β-defensin genes as they can produce peptides with enhanced disulphide bond formation in the cytoplasm (Fathi-Roudsari *et al.*, 2016). However, difficulties were encountered when using *R. gami* for expression of the peptides in this study. *R. gami* cells grew very slowly in comparison to other strains such as BL21 (DE3) cells and indeed this was observed during the course of this project. Additionally, the *R gami* strain was consistently shown to express lower levels of β-defensin 4 fusion proteins than the comparator strain BL21 (DE3) The latter strain is not known for its ability to form disulphide bonds in proteins expressed in the cytoplasm (Daegelen *et al.*, 2009). Normally, proteins must be exported to the periplasm for this to take place. However, as the expression levels of the NusA-β-defensin 4 protein were higher in the BL21 (DE3) strain compared with the *R. gami* strain it was decided to pursue this option. BL21 (DE3) cells have been used for the recombinant production of AMPs such as; human β-defensin 2 (Zhong *et al.*, 2006), β-defensins from Atlantic cod (Ruangsri *et al.*, 2013), α-defensins from horses (Bruhn *et al.*, 2007) and human hepcidins (Zhang *et al.*, 2005).

Following some initial success in isolating the NusA-β-defensin 4 fusion protein from the BL21 (DE3) cells, later purifications were unsuccessful and difficulties were encountered with protein binding to the Nickel affinity column. Therefore, it was concluded that the protein may indeed not be folding properly resulting in lack of exposure of the tag which may be interfering with binding to the column. The limited ability of the BL21 (DE3) cells to form the disulphide bonds in the peptide may have resulted in poorly folded protein being produced, despite the assistance of the highly foldable NusA fusion protein (Li *et al.*, 2013).

Although further optimization experiments were performed in order to address some of the problems which arose using the NusA-β-defensins 4 fusion protein expression in BL21 (DE3) cells, which included evaluating the inclusion of PMSF, a change from

Tris to phosphate buffer and investigating non-specific binding to the column, none of these changes yielded any significant purified protein. Therefore, it was then decided to discontinue using this expression system and the further experiments were performed using pMAL-p4X expression plasmid.

Greater success in terms of fusion protein expression was also seen when using the combination of BL21 (DE3) cells and the pMAL-p4X expression plasmid compared with the use of *R. gami* with this plasmid and ultimately this was the final combination that was used to produce significant quantities of MBP-β-defensins 4 protein. As previously mentioned, the issue of lack of disulphide bond formation that may have contributed to poor production of the NusA-β-defensins 4 protein was overcome here by utilizing the signal sequence of the MBP to export the protein to the non-reducing periplasmic space in the BL21 (DE3) cells where disulphide bonds could form in the β-defensin peptide.

5.2.3. Induction Methods

IPTG induction and autoinduction were both used for the expression of the NusA-β-defensin 4 and MBP-β-defensin 4 fusion proteins in this project (Sections 4.2 and 4.6). Both the *R. gami* and BL21 (DE3) strains of *E. coli* contain the λDE3 lysogen carrying the T7 polymerase gene under control of the lac operon, and therefore allowing induction either through addition of IPTG (Rosano and Ceccarelli, 2014) or via controlled metabolism of glucose of lactose using the autoinduction method (Studier, 2005) Autoinduction is a better method for overexpression of the target peptide because it is quicker, less labour-intensive and cheaper (Fox and Blommel, 2009). Both induction methods were trialled in this project.

Originally induction using IPTG was used to express the NusA-β-defensin 4 genes in *R. gami* cells. A range of temperatures from 20ºC to 37ºC and varying concentrations of IPTG from 0.25mM to 1.0mM were used in small-scale expression trials (refer to Table 7 for a summary of conditions used for expression). In these experiments, low levels of expression were found at all concentrations of IPTG. Expression trials using autoinduction were next performed, where BL21 (DE3) and *R. gami* cells were tested for β-defensin expression using autoinduction at 24 and 48 hours. The results showed significantly stronger bands for the NusA-β-defensins 4 fusion protein at ~60 kDA in BL21 (DE3) when analysed with SDS-PAGE as shown in Figure 22 of the results section.

When MBP-β-defensins fusion protein was used in expression trials, expression was seen in both BL21 (DE3) and *R. gami* cells with autoinduction at 24 hours. The BL21 (DE3) expression host with autoinduction media was chosen as the optimal expression conditions for production of the MBP-β-defensin 4 fusion protein. Indeed, the MBP-β-defensins 4 protein was successfully produced using this system as reflected in the chromatogram in Figure 38 and the SDS-PAGE image in Figure 37. The final yield of the pure protein was 5.2 mg from 1L of cells (Table 9).

5.2.4. Summary of Optimised Protocol for Cloning, Expression and purification of β-defensin Genes

This study resulted in the development of an optimised protocol for the cloning, expression and purification of salmon β-defensin peptides in *E. coli* which can be summarised as follows:

- All three β-defensin genes were cloned using the pMAL-p4X expression vector (Figure 32, Figure 33 and Figure 34).
- Autoinduction was used to overexpress the MBP-β-Defensin 4 fusion peptide at 37ºC, for 24 hours, using BL21 (DE3) cells. This was visualised by SDS-PAGE (Figure 35) and confirmed by western blot using anti-MBP antibodies (Figure 39).
- Purification was successful using the ÄKTA Purification System (Figure 38) with 5.24mg of MBP-β-Defensin 4 fusion peptide from 1L of BL21 (DE3) expression culture (Table 9).

5.3.Structural Modelling using I-Tasser

An online tool called I-TASSER was used which utilises algorithms and a protein database to provide 5 prediction models of the peptide structure and 10 peptides with highest structural homology. Figure 41, Figure 42 and Figure 43 illustrate the top 5 peptide structures predicted by I-TASSER for β-defensins 1, 3 and 4 respectively. The structure of β-defensins are characterised by a 3 β-sheet structure and with the presence of an α-helix in the N-terminus for some β-defensins. From Figure 41 it can be concluded that prediction 1 and 5 contain the expected β-sheet structure. Since some β-defensins may or may not contain an α-helix in the N-terminus, it is difficult to conclude whether the predictions are correct based on the presence of α- helix in the N-terminus. Based on the C-score, prediction 1 has the highest score, indicating this is the best prediction of β-defensin 1. Figure 42 shows prediction 1, 3, 4 and 5 with the expected β-sheet structure for β-defensin 3. Based on the C-score value, prediction 1, with the highest C-score value seems to be the best prediction for β-defensin 3. Figure 43 all of the 5 predictions contain the expected β-sheet structure for β-defensin 4. I- TASSER also established 10 peptides that are structurally similar to the target peptide. 3 peptides of highest structural homology were chosen for comparison to the target peptides. Table 10, Table 11 and Table 12 illustrates a summary table of the top 3 structurally similar peptides to β-defensin 1, 3 and 4 respectively. For β-defensin, all 3 homologous peptides are antimicrobial peptides, with the most structurally similar peptide being from humans. This further supports the hypothesis that β-defensins are conserved among species. The β-defensin 3 structure is unique and it is not like the other two β-defensins. This is supported by the phylogenetic tree and the sequence alignment demonstrated in Figure 3. This also explains why none of the predicted homologous structures are antimicrobial peptides for β-defensin 3. The top 3 homology predictions included a signalling protein, an RNA binding protein and a hydrolase. These peptides do not share functional homology with AMPs. However, for β-defensin 4, Table 12 illustrated that 2 out of 3 peptides of structural homology are AMPs from humans. This supports the earlier statement that β-defensins are closely related between species and that β-defensin 1 and 4 are more closely related than β-defensin 3.

5.4.Need for Recombinant AMPs Production

Recombinant technology has previously been used to generate AMPs (Zhong *et al.*, 2006; Bruhn *et al.*, 2007; Ruangsri *et al.*, 2013) and this study has focused on the development of a protocol which allows for generation of purified β-defensins from salmon. The alternative strategy would be to synthesise the peptide but this has limitations as regards its cost effectiveness and structural integrity of the synthesised peptide.

Various companies such as Abcam and Aurore FIESCHI were contacted over the duration of this project, and estimates were received for the synthetic β-defensins. Refer to Appendix P for quotation of β-defensins to be synthesised, where each peptide would cost €3300 for 2-5 mg of synthesised peptide. This would bring the total to €9900 for all 3 β-defensins. Keeping in mind this is only for 2-5mg and given functional studies require a large amount of purified peptide, more than likely 5 mg would not be enough to complete functional and structural investigations.

The second issue with synthetic peptides is the purity, which due to the length and the sequence the β-defensins peptides, some solubility and purification problems might be expected, and hence the final purity of 95% cannot be guaranteed.

Another challenge was the correct formation of disulphide bonds. Companies could not guarantee that all 3 disulphide bonds would be correctly formed during synthesis. Without this guarantee, synthesis was not an option as the peptides were required in their fully functioning, native state for characterisation assays. Due to all these reasons, the decision was made to produce the peptides by recombinant production.

5.4.1. Future Studies

During this project the MBP-β-defensin 4 fusion peptide was successfully purified. This optimised protocol can now be utilised to express and purify all the *Salmo salar* β-defensins. The next step in the protocol would be to cleave the MBP tag to obtain the pure β-defensin peptide. This can be done during purification with the addition of Factor Xa to cleave the β-defensin peptide from the MBP tag, allowing it to elute from the column with the MBP tag still bound to the column, followed by drying and concentration of the peptide.

Once the pure β-defensin peptide is obtained, there are multiple experiments and future studies that can be performed. The structure of each of these peptides can be investigated and crystallization trials can be performed using the purified peptides to obtain protein crystals for X-ray diffraction analysis (Ilari and Savino, 2008). The *in-meso* method has successfully been used for the crystallization of other peptide molecules including human Glycophorin A (Trenker *et al.*, 2015) and the antimicrobial peptide Gramicidin from *Bacillus brevis* (Höfer *et al.*, 2011). These studies will provide insight into how the structure of the β-defensins influence their molecular mechanism of action.

Future studies can also focus on functional and characterisation assays to analyse all three β-defensin peptides *in vitro*. MIC assays can be performed to determine the effect of the β-defensins on salmon pathogens such as *Neoparamoeba perurans, Yersinia ruckeri, Aeromonas salmonicida* and *Vibrio anguillarum*, along with a range of Gram-positive and Gram-negative bacteria. The antibacterial activity will be measured by a liquid growth inhibition assay in a 96-well microtiter plate as previously reported (Fernandes *et al*., 2004)

The membrane permeabilisation effect of the β-defensins can also be investigated to determine their mechanism of action. The effect of β-defensins on the inner and the outer membrane of pathogens such can be investigated. The inner membrane permeabilisation assays will be investigated as previously described by (Skerlavaj *et al.*, 1990) and the outer membrane permeabilisation assays will be examined as per (Loh *et al.*, 1984).

If it is determined that β-defensins are effective at killing salmon pathogens such as ISAV, *Neoparamoeba perurans* and sea lice, future studies can also focus on drug

delivery to allow the administration of the recombinant AMPs and alternatively how to stimulate the β-defensins in the host.

Local administration of AMPs to a particular tissue has been previously investigated. Nanocarries have shown promising results as the drug delivery system. This can be investigated once the pure β-defensin has been achieved. Hyaluronic acid nanogels have been used to encapsulate LLKKK18, an analogue of LL-37 which is more effective against mycobacteria in comparison to the free peptide, *in vivo* and *in vitro (Silva et al., 2016)*. Cubic phase drug delivery systems can also be used for the administration of AMPs (Trenker *et al.*, 2015). The AMPs AP114, DPK-060 and LL-37 have been evaluated using cubic liquid crystalline gel i.e. cubosomes (Boge *et al.*, 2017). Phytantriol based smart nano-carriers are also valuable as they are chemically stable, non-toxic and biocompatible (Akbar *et al.*, 2017).

Alternatively, β-defensins can be stimulated in the host by certain stimuli. Future studies can also focus on the discovery of the stimuli which induces and enhances β-defensins production is *S. salar*. IL-1α and IL-1β have been found to stimulate human β-defensin 2 (Liu *et al.*, 2002). Cyclic adenosine monophosphate and butyrate has been found to induce expression of the β-defensin 9 in chickens (Sunkara *et al.*, 2014).

The wealth of information that can be gathered in relation to β-defensin peptides as outlined in this section, is dependent on a robust production system, the foundation for which has been laid in this project.

Chapter 6: Conclusion

Over 200 AMPs have been described to function as a first line of defense against pathogens (Li and Chen, 2008; Wang,Li, *et al.*, 2016). Development of a robust expression system using *E coli* cells for production of purified β-defensins is described here. This study provides a platform and methodology which could be adapted for other species.

The findings from this project have made a significant impact on the development of an *in vitro* laboratory method to produce recombinant *Salmo salar* β-defensins. Although further work is needed to complete the cleavage of the fusion tag from the mature β-defensin, the majority of the protocol has been optimised. The study has investigated important core strategies which central to future investigations.

All 3 chosen β-defensin genes from salmon were successfully cloned into the pMAL-p4X cloning vector and the sequence was verified by Sanger sequencing. The best expression was evident using BL21 (DE3) cells and autoinduction for 48 hours at 37°C, shaking at 200rpm. The expression of the 49 kDa MBP-β-defensin 4 fusion protein was confirmed by western blotting. Optimal purification was carried out using the Akta purification system and amylose affinity chromatography, yielding 5.24 mg of MBP fused β-defensin 4 from 1 litre of cell culture.

The next step in this protocol will be to cleave of the MBP tag from the β-defensin peptide using factor Xa. After cleavage the peptide is available in a pure state and characterisation of the AMPs can be done such as MICs, membrane permeability and amoebicidal assays to investigate the function and x-ray crystallisation to confirm the structure.

AMPs are naturally occurring molecules which are likely to serve as a new class of drugs for bacteria and other pathogens, as they are becoming increasingly more difficult to treat by existing antibiotics and standard commercial drugs. β-defensins are one of the most important AMPs in multiple species and description of low cost methods which allow for in vitro production β-defensins is essential to enable future investigations and development of alternative treatments. This study is a starting point for a large area of research in which has the potential to reduce or even eliminate the use of antibiotics in aquaculture, agriculture and healthcare.

Chapter 7: References

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Chapter 8: Appendix

Appendix A: Risk Assessment

Biological Hazards
Hazard: Infection of staff handling specimens • Infectious samples: Infected fish • Bacteria: *Escherichia coli, Rosetta-gami* • Controls and reagents: may contain traces of biological material. • Biological waste.
Category: Medium
Current control Measures: • All fish specimens are dealt with in the class 1 safety cabinet, to prevent contamination and the production of potentially hazardous aerosols. • Biological specimens are disposed of in biohazard bins which are then autoclaved before disposal. • Aseptic technique is used when working with bacterial cultures. Separate incubator is used for microbiology plates. • Personal protective equipment i.e. white Howie laboratory coat are available and worn at all times inside the laboratory. Open toed shoes are not allowed to be worn inside the laboratory. Gloves must be worn when handling specimens/ reagents. • Hands must be washed using appropriate handwashing techniques after removing gloves and before leaving a section. • First aid kits & eye wash station is available in the department in the event of an accident. • Health & safety training is provided to all members of staff and a documented evidence is kept.
Options for improved controls: All appropriate control are in place.

Chemical Hazards
Hazard: Chemical Burns • Sodium Azide, Ethanol, Tris-acetate-EDTA (TAE) buffer, SyberSafe gel stain, Gel Red, HCl, Tetramethylethylenediamine, Isopropynol
Category: Medium
Current control Measures: • Personal protective equipment i.e. white Howie laboratory coat are available and worn at all times inside the laboratory. Open toed shoes are not allowed to be worn inside the laboratory. • Gloves must be worn when handling reagents. In case of contact with skin/ mucous membrane, effected area must be washed with water thoroughly and medical attention should be sought. • Hands must be washed using appropriate handwashing techniques after removing gloves and before leaving a section. • Any spills are wiped and the surface is disinfected immediately. Spill kits are available in the department in the event of a large spill. • First aid kits & eye wash station are available in the department in the event of an accident. • Sodium Azide is capable of reacting with copper plumbing and form explosive metal Azide. To avoid this, large volumes of water should be flushed with any reagent containing Sodium Azide.

Chemical Hazards
Hazard: Chemical Burns, hazardous upon contact with skin and ingestion, risk of harm to unborn child, damage to mucous membrane. • Tris-borate-EDTA (TBE) buffer, Chloramphenicol, Imidazole, Phenylmethylsulfonyl fluoride, Methanol, Acrylamide
Category: High
Current control Measures: • Personal protective equipment i.e. white Howie laboratory coat are available and worn at all times inside the laboratory. Open toed shoes are not allowed to be worn inside the laboratory. • Gloves must be worn when handling reagents. In case of contact with skin/ mucous membrane, effected area must be washed with water thoroughly and medical attention should be sought. • Hands must be washed using appropriate handwashing techniques after removing gloves and before leaving a section. • Any spills are wiped and the surface is disinfected immediately. Spill kits are available in the department in the event of a large spill. • First aid kits & eye wash station are available in the department in the event of an accident. • If ingested, do not induce vomiting unless instructed by medical professional. Contact medical aid team and poison control.

Physical Hazards
Hazard: Sharps (broken glass), moving parts in centrifuge, heavy objects
Category: Low
Current control Measures: • Safety features on equipment; e.g. covers over moving parts, caution stickers on instruments with moving parts, auto shutdown when covers open, alarms, etc. • Training on manual handling is provided to all staff members. Records of all training is documented. Trolleys are available for transportation of heavy objects e.g. equipment and high-volume reagent/waste containers. • Separate disposal of sharps in the "Sharps bin". All waste is labelled, dated and signed before removal from the laboratory. • First aid kits are available in the department in the event of an accident.

Electrical Hazards
Hazard: Electrical Shock Computers, Centrifuge, Fridge, Vortex, Thermocycler, Oven
Category: Low
Current control Measures: • Electrical equipment in used should be designed and manufactured to comply with the appropriate safety requirements, which is checked at installation. • Electrical wires are organised and stored neatly, out of the operators' way. • When possible, instruments are switched off when not in use. • Any spills are wiped and the surface is disinfected immediately. Spill kits are available in the department in the event of a large spill. • Appropriate health and safety training is provided to members of staff prior to the use of an analyser/instrument.

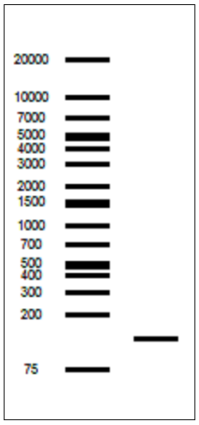
Appendix B: Material List

Product	Manufacturer	Lot no./ Serial no.	Expiry date
50% PEG 4000 solution	Sigma	25322-68-3	N/A
50ml Falcon Tubes	Lennox	280712	N/A
Acrylamide	Fluka	BCBC6548	N/A
Agarose	Sigma	A6236	N/A
Ampicillin	Roche	21442020	03/2019
BamHI	Biolabs	R0136S	N/A
BamHI buffer (10X)	Biolabs	R0136S	N/A
BCA Protein Assay Kit	Thermo Fisher	RG235628	N/A
Boric Acid	Promega	219795	N/A
BoxI (PshAI)	Thermo Fisher	ER1431	N/A
Buffer Tango	Thermo Fisher	BY5	N/A
Centrifuge	Eppendorf	5418FM724486	N/A
Chloramphenicol	Sigma	72F0450	N/A
DAB Substrate and Buffer	Thermo Fisher	RA226479	N/A
DNAse	Sigma	9003989	N/A
DNTP's (10mM)	Thermo Fisher	00346421	07/2018
Dried Milk	Marvel	3023036	09/2018
DTT	Sigma	MKBD8606	N/A
EcoRI	Thermo Fisher	FR0271	N/A
EDTA	Promega	233219	N/A
Electrophoresis Power Pack	BioRad	041BR	N/A
Ethanol	Lennox	R160823ET2	N/A
Filter Paper	GE Healthcare	G9958990	N/A
gBlock	Integrated DNA technologies	72619741 72999964 72999965	N/A
Gel Red	Biotium	41001	N/A
Glucose	Sigma	50997	N/A
Glycerol	AppliChem	4R010080	N/A
Glycine	Thermo Fisher	0927652	N/A
Glycine	Thermo Fisher	0927652	N/A
HCL	Fixanal	73400	N/A
Heating Block	Labnet	52602038	N/A
Hotplate/ Stirrer	Stuart	R000108825	N/A
Hyperladder	Bioline	BIO-33054	N/A
Hyperladder Loading Dye (6x)	Promega	0000043043	28/06/2018
Imidazole	VWR lifesciences	0856C380	N/A
Lactose	Sigma	63423	N/A
LB agar	Sigma	L2897	N/A
Lb Broth	Sigma	BCBN9038V	N/A
Lysozyme	Sigma	0001356468	N/A
Magnesium Chloride	Sigma	7791186	N/A
Methanol	Fluka	9222S	N/A
$MgCl_2$ (25mM) -	Sigma Aldrich	SLBK3871V	N/A

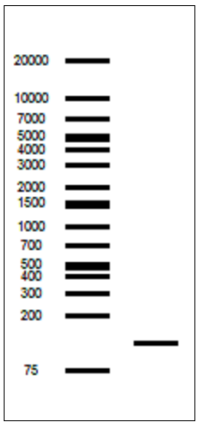
Molecular Grade			
NcoI	Thermo Fisher	ER0571	N/A
Nuclease free water	Invitrogen	1838065	N/A
Nuclease Free water	Invitrogen	1838065	11/2018
PFU	Promega	M7741	N/A
PFU buffer with $MgSo_4$ (10X)	Promega	M7741	N/A
Phusion	Thermo Fisher	F530L	N/A
Phusion GC buffer (5X)	Thermo Fisher	F530L	N/A
PMSF	Sigma	BCBQ7707V	N/A
Ponceau S	Sigma	MKBV6870V	N/A
Potassium Dihydrogen Phosphate	Scientific Chemical Supplies Ltd.	7778770	24/02/20
Rocker	Stuart	R00010507	N/A
SDS	BDH chemicals	S373341030	N/A
Shaking Incubator	Thermo Electron Corporation	14650	N/A
Sodium chloride	BDH laboratory reagent	30104	N/A
Sodium phosphate dibasic	Sigma	075K0167	N/A
Sonicator	Thermo Fisher	FB120	N/A
Spectrophotometer			N/A
T4 DNA ligase	Invitrogen	15224025	N/A
T4 DNA ligase buffer	Invitrogen	15224025	N/A
Taq polymerase	Sigma Aldrich	SLBK5076V	N/A
Taq Buffer without $MgCl_2$ (10X)	Sigma Aldrich	SLBM5556V	N/A
TEMED	Sigma	058K1152	N/A
Thermocycler	Biometra	9069329	N/A
Transilluminator	UVP	050707001	N/A
Tris HCL	Promega	182602	N/A
Tris Base	Sigma	129K5430	N/A
Tryptone	Fluka	134968854507097	N/A
Tween 20	Sigma	9005645	N/A
Urea	Sigma	BCBF5177V	N/A
Vortex	Labnet	52602038	N/A
Water Bath	Genlab	94M132	N/A
Weighing Scale	Pioneer	8730052966	N/A

Appendix C: In silico β-Defensin Insert Digestion with BamHI

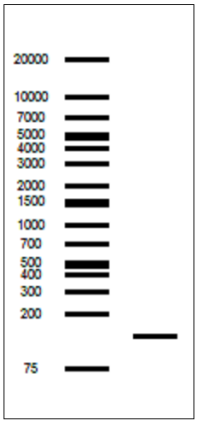
```
<Serial Cloner V2.5> -- <16 Aug 2017   14:58>

Restriction analysis of PCR(gBlock_bdef1-CP).txt.xdna [Linear]
Incubated with BamHI

2 fragments generated.

   1:      128 bp  -  From ---           To  BamHI[128]

   2:        8 bp  -  From BamHI[128]    To  ---
```




```
<Serial Cloner V2.5> -- <16 Aug 2017   15:04>

Restriction analysis of PCR(gBlock_bdef3-CP).txt.xdna [Linear]
Incubated with BamHI

2 fragments generated.

   1:      122 bp  -  From ---           To  BamHI[122]

   2:        8 bp  -  From BamHI[122]    To  ---
```

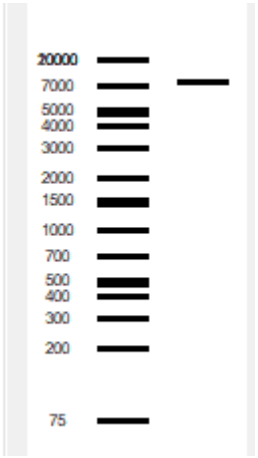



```
<Serial Cloner V2.5> -- <16 Aug 2017   15:07>

Restriction analysis of PCR(gBlock_bdef4-CP).txt.xdna [Linear]
Incubated with BamHI

2 fragments generated.

   1:      131 bp  -  From ---           To  BamHI[131]

   2:        8 bp  -  From BamHI[131]    To  ---
```



Appendix D: *In silico* Digestion of pET44a

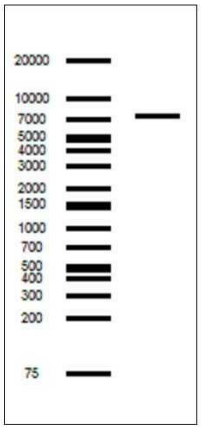
```
Restriction analysis of pET44aplus.xdna [Circular]
Incubated with BamHI + PshAI

2 fragments generated.

   1:    7,294 bp  -  From PshAI[677]    To  BamHI[660]

   2:       17 bp  -  From BamHI[660]    To  PshAI[677]
```



Appendix E: *In-Silico* Restriction Digestion of β-defensin Gene Inserts in Preparation for Cloning β-Defensin Genes using pET44a Cloning Vector

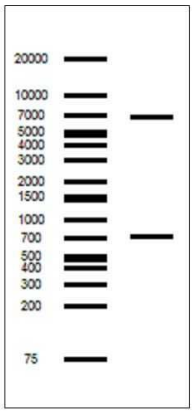
β-defensin 1 Positive

```
<Serial Cloner V2.5> -- <15 Jun 2017   16:21>

Restriction analysis of pET44a_Bdef1.xdna [Circular]
Incubated with NcoI + PshAI

1 fragment generated.

   1:    7,422 bp  -  Linearized by NcoI[5904]
```



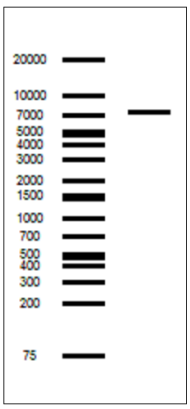
β-defensin 1 Negative

```
<Serial Cloner V2.5> -- <15 Jun 2017   16:24>

Restriction analysis of pET44aplus.txt.xdna [Circular]
Incubated with NcoI + PshAI

2 fragments generated.

   1:    6,585 bp  -  From NcoI[1403]    To  PshAI[677]

   2:      726 bp  -  From PshAI[677]    To  NcoI[1403]
```



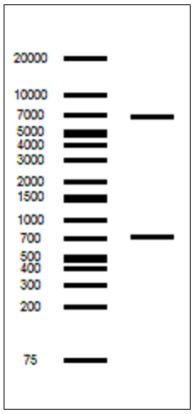
β-defensin 3 Positive

```
<Serial Cloner V2.5> -- <24 Feb 2017   13:41>

Restriction analysis of pET44a_Bdef3.txt.xdna [Circular]
Incubated with NcoI + PshAI

1 fragment generated.

   1:    7,416 bp  -  Linearized by NcoI[5904]
```



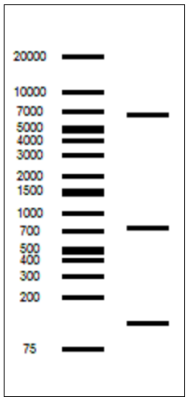
β-defensin 3 Negative

```
<Serial Cloner V2.5> -- <24 Feb 2017   13:40>

Restriction analysis of pET44aplus.txt.xdna [Circular]
Incubated with NcoI + PshAI

2 fragments generated.

   1:    6,585 bp  -  From NcoI[1403]    To  PshAI[677]

   2:      726 bp  -  From PshAI[677]    To  NcoI[1403]
```



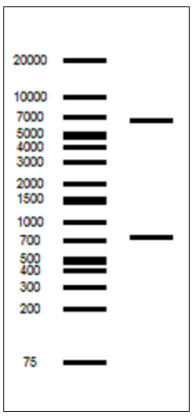
β-defensin 4 Positive

```
<Serial Cloner V2.5> -- <24 Feb 2017   13:39>

Restriction analysis of pET44aBdef4.txt.xdna [Circular]
Incubated with BamHI + NcoI

3 fragments generated.

   1:    6,568 bp  -  From BamHI[6761]   To  NcoI[5904]

   2:      737 bp  -  From NcoI[5904]    To  NcoI[6641]

   3:      120 bp  -  From NcoI[6641]    To  BamHI[6761]
```

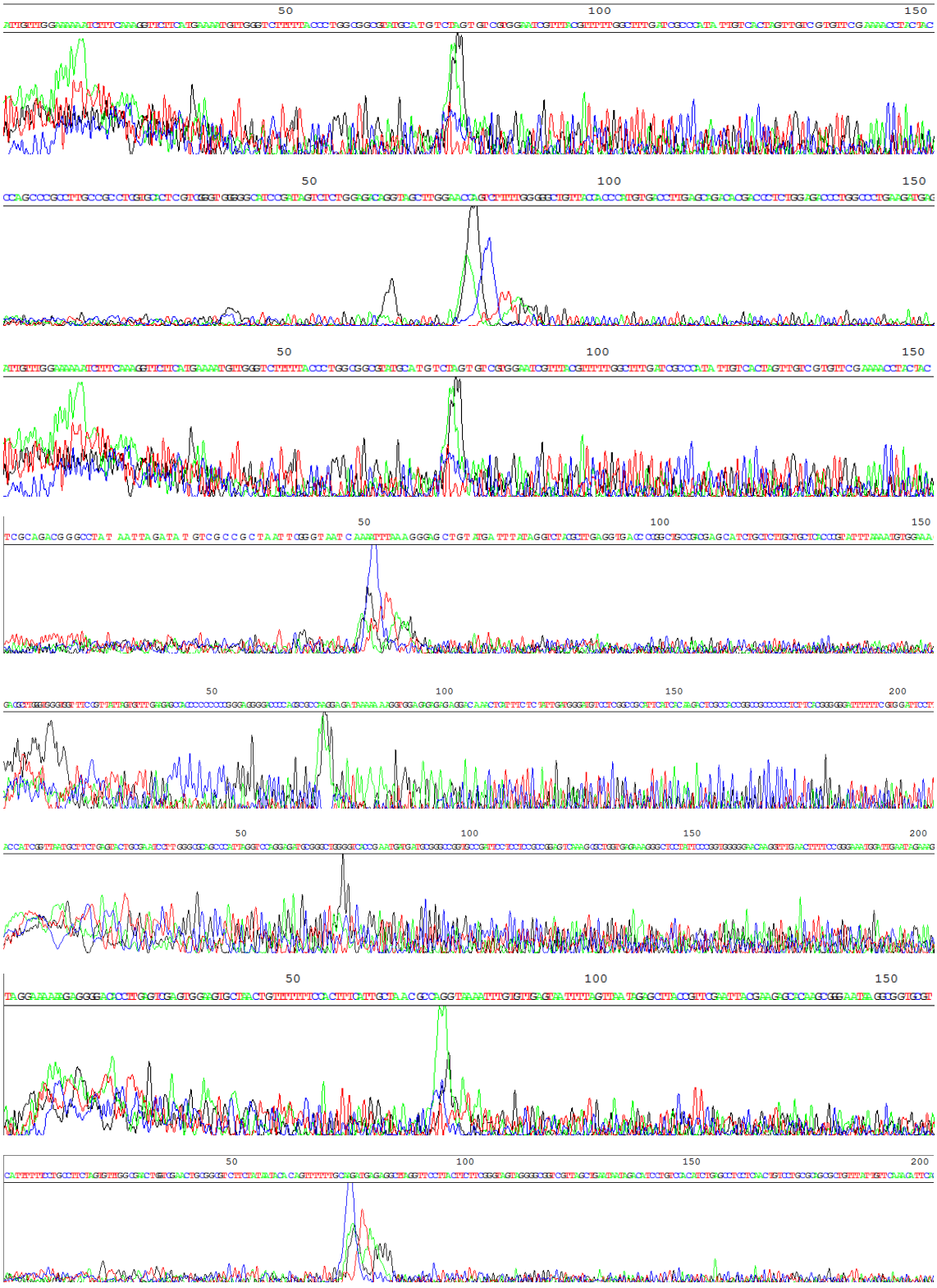


β-defensin 4 Negative

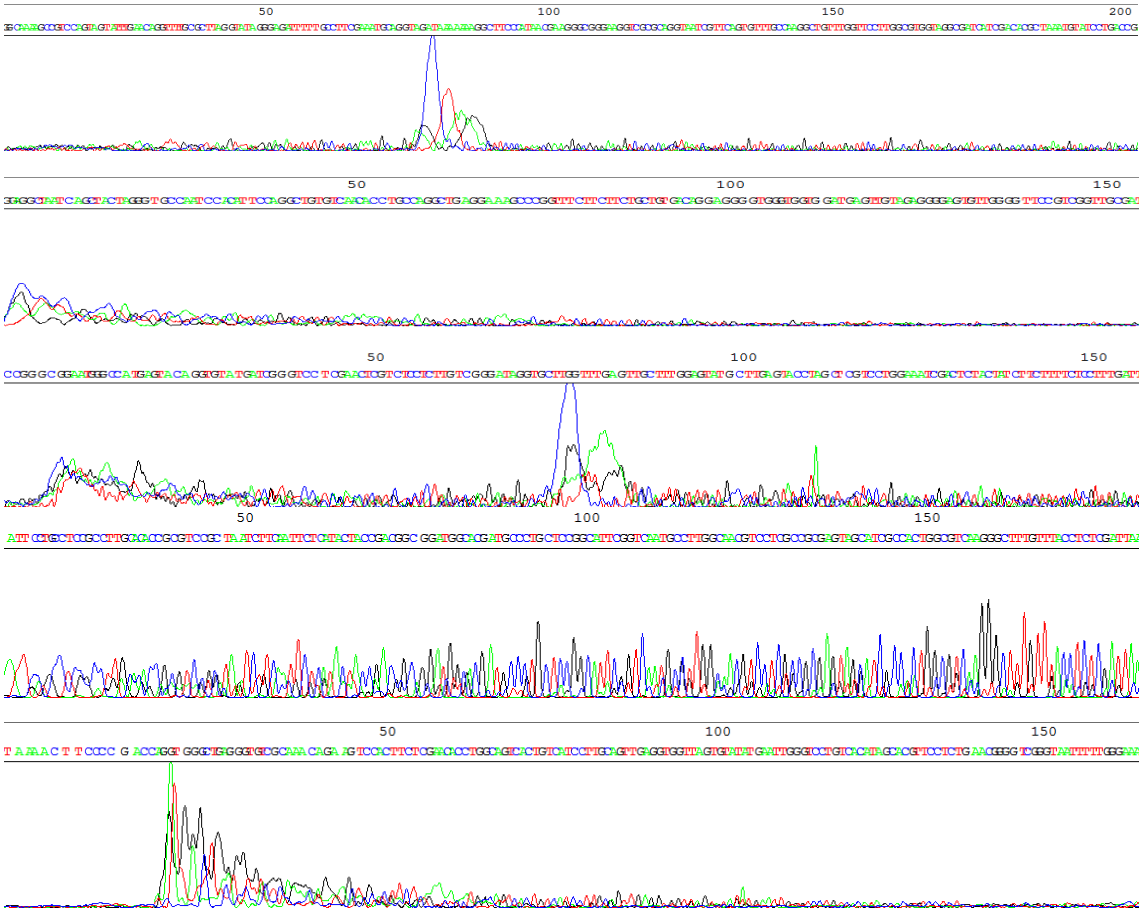
```
<Serial Cloner V2.5> -- <24 Feb 2017   13:40>

Restriction analysis of pET44aplus.txt.xdna [Circular]
Incubated with BamHI + NcoI

2 fragments generated.

   1:    6,568 bp  -  From NcoI[1403]    To  BamHI[660]

   2:      743 bp  -  From BamHI[660]    To  NcoI[1403]
```



Appendix F: β-defensin 1 Cloning



Appendix G: β-defensin 3 Cloning



Appendix H: Nickel Affinity Chromatography with Tris buffer and PMSF

Affinity chromatography purification was repeated with the addition of PMSF to the cell cultures after harvest, which is a protease inhibitor. Figure 44 shows various fractions from the affinity chromatography purification for β-defensin 4 using BL21 (DE3) cells. Lane 1 contains protein standard (Precision Plus Protein™ Dual Colour), lane 2 shows β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3 contains the crude extract after cell lysis, lane 4 contains the flow through from the column, lane 5 shows elution after first wash, lane 6 shows elution after second wash, lane 7 shows elution after third wash, lane 8 contains elution fraction 1, lane 9 contains elution fraction 2 and lane 10 contains elution fraction 3. No bands were detected on the gel for elution fractions. Crude extract and flow through fractions in lanes 3 and 4 also showed identical patterns, may indicate issues with binding of fusion protein to column. A single band is expected at approximately 60kDa for NusA-β-defensin 4 fusion protein.

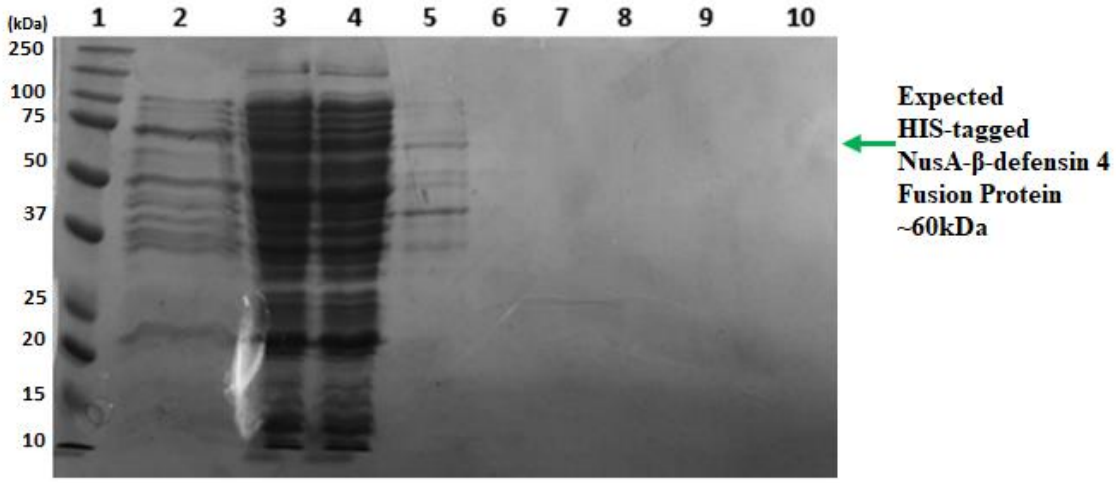


Figure 44: Affinity Chromatography Using Tris Buffer With PMSF

Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3: crude extract after cell lysis, lane 4: flow through from column, lane 5: elution after first wash, lane 6: elution after second wash, lane 7: elution after third wash, lane 8: elution fraction 1, lane 9: elution fraction 2 and lane 10: elution fraction 3. Single band expected at ~60kDa for NusA-β-defensin 4 fusion protein in elution fractions.

Appendix I: Nickel Affinity Chromatography with Phosphate buffer

As an alternative to tris buffer, phosphate buffer was used in a trial purification, SDS-PAGE results shown in Figure 45. Lane 1 contains protein standard (Precision Plus Protein™ Dual Colour), lane 2 shows β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3 contains the crude extract after cell lysis, lane 4 contains the flow through from the column, lane 5 shows elution after first wash, lane 6 shows elution after second wash, lane 7 shows elution after third wash, lane 8 contains elution fraction 1, lane 9 contains elution fraction 2 and lane 10 contains elution fraction 3. Similarly, no bands were detected on the gel for elution fractions. Crude extract and flow through fractions in lanes 3 and 4 also gave identical patterns. A single band at approximately 60kDa is expected for elution fractions to indicate successful purification.

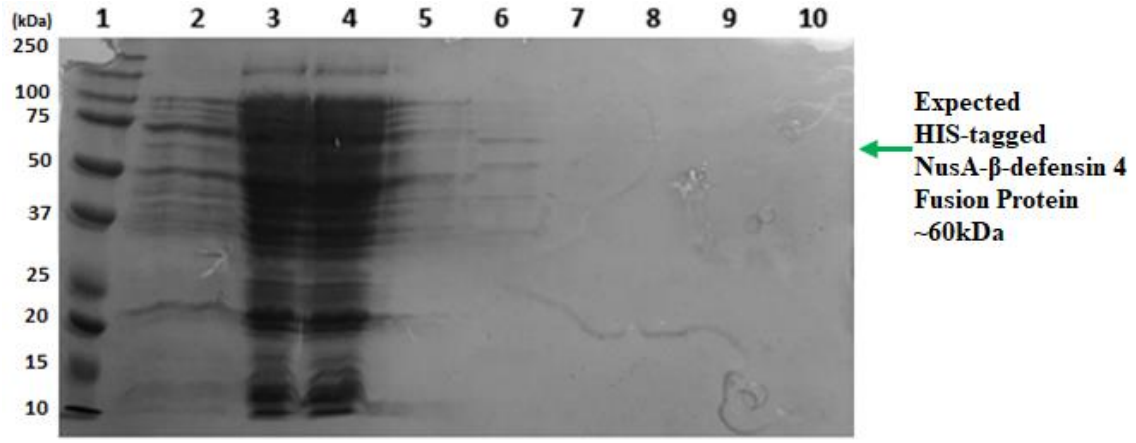


Figure 45: Affinity Chromatography Using Phosphate Buffer with PMSF

Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: β-defensin 4 transformed BL21 (DE3) whole cells after autoinduction, lane 3: crude extract after cell lysis, lane 4: flow through from column, lane 5: elution after first wash, lane 6: elution after second wash, lane 7: elution after third wash, lane 8: elution fraction 1, lane 9: elution fraction 2 and lane 10: elution fraction 3. Single band expected at ~60kDa for NusA-β-defensin 4 fusion protein in elution fractions.

Appendix J: Control Experiment

To investigate issues with binding of cellular material to Nickel Sepharose beads a control experiment was performed. An affinity chromatography purification was repeated with fresh Nickel Sepharose beads. An untransformed *BL21 (DE3)* crude extracts was used as a control. 10mM Imidazole was also added to the equilibration buffer to reduce non-specific binding.

Figure 46 shows protein standard in lane 1, flow through of β-defensin 4 transformed BL21 (DE3) from the column in lane 2, elution from first wash in lane 3, elution from second wash in lane 4, elution from third wash in lane 5, elution fraction 1 in lane 6, elution fraction 2 in lane 7, elution fraction 3 in lane 8, elution fraction 4 in lane 9, and the final elution with the addition of buffer containing 300mM imidazole in lane 10.

Figure 47 follow the exact same layouts of samples as Figure 46 respectively, except the BL21 (DE3) cell for this trial was not transformed with the recombinant peptide.

No bands seen in elution fractions on either gel. Both gels show similar banding patterns. A single band is expected at approximately 60kDa for NusA-β-defensin 4 fusion protein.

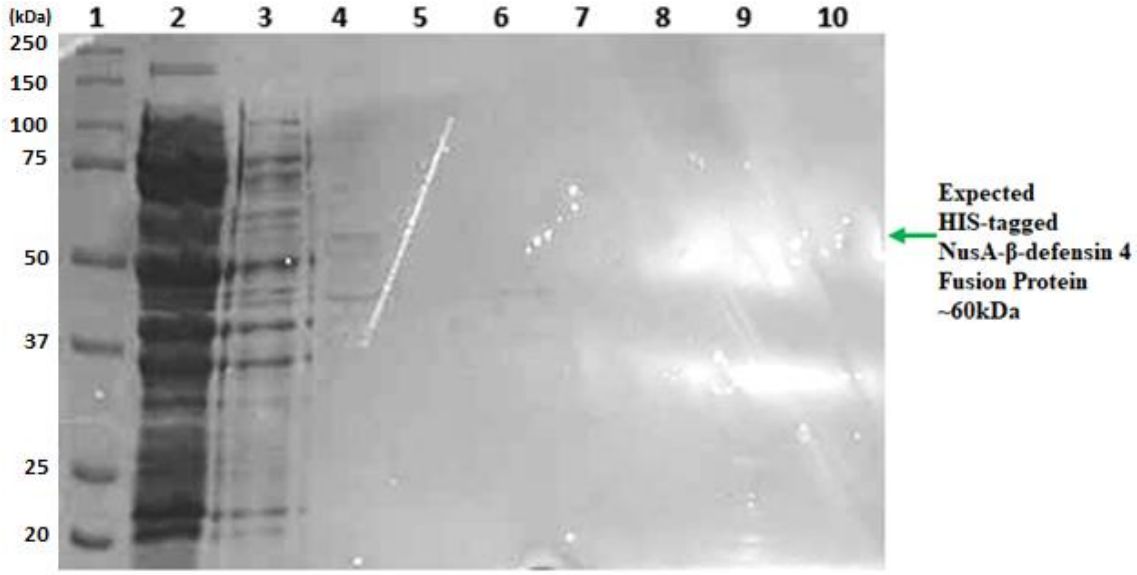


Figure 46: Purification of BL21 (DE3) Cells Transformed with Recombinant β-defensin 4

Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: flow through of β-defensin 4 transformed BL21 (DE3) from the column, lane 3: wash 1, lane 4: wash 2, lane 5: wash 3, lane 6: elution fraction 1, lane 7 elution fraction 2, lane 8: elution fraction 3, lane 9: elution fraction 4 and lane 10: final elution (300mM imidazole). Single band expected at ~60kDa for NusA-β-defensin 4 fusion protein in elution fractions.

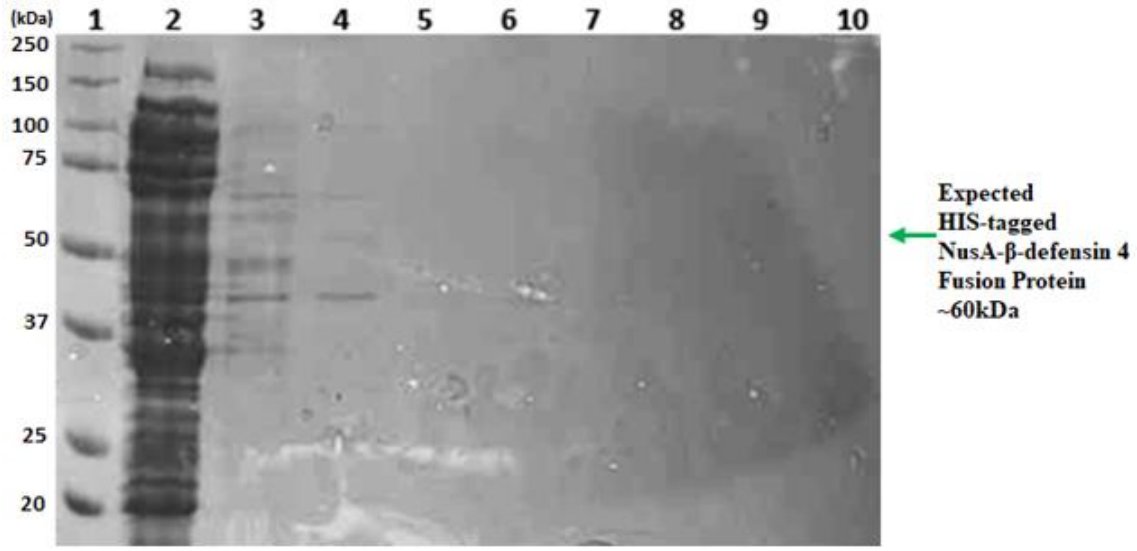


Figure 47: Purification of untransformed *BL21 (DE3)* Cells

Lane 1: Precision Plus Protein™ Dual Colour standard, lane 2: flow through of untransformed BL21 (DE3) from the column, lane 3: wash 1, lane 4: wash 2, lane 5: wash 3, lane 6: elution fraction 1, lane 7 elution fraction 2, lane 8: elution fraction 3, lane 9: elution fraction 4 and lane 10: final elution (300mM imidazole). No bands expected at ~60kDa for as BL21 (DE3) cells were not transformed with β-defensin 4.

Appendix K: Standard Curve Generation for Bradford Assay

Table 13: Concentration (µl/ml) of Bradford Assay Standards at 595nm

Calibrator Concentration (µl/ml)	Absorbance at 595nm
0	0
100	0.721
250	1.24
400	1.484
500	1.565

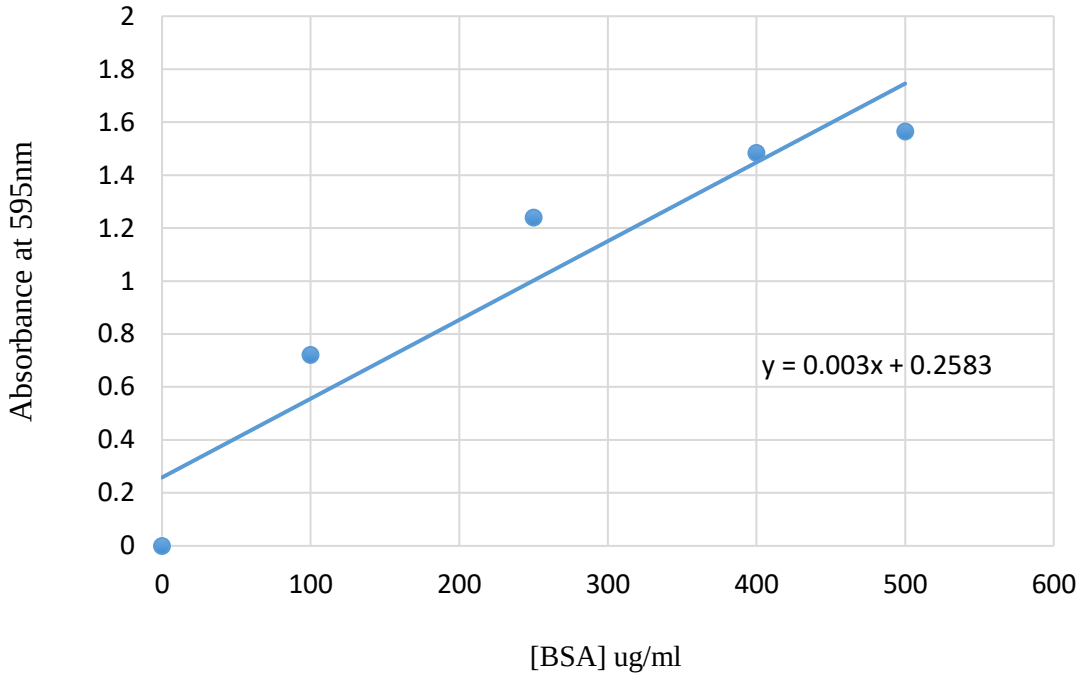
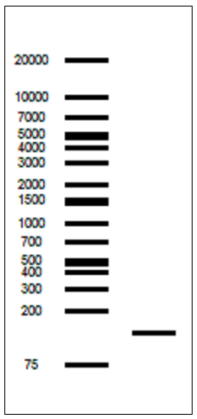


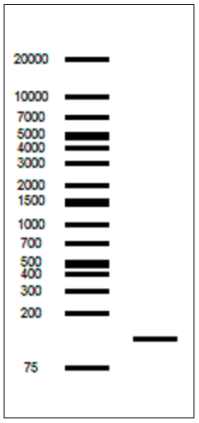
Figure 48: Bradford Assay Standard Curve

Appendix L: *In silico* β-defensin Insert Digestion with BamHI and EcoRI

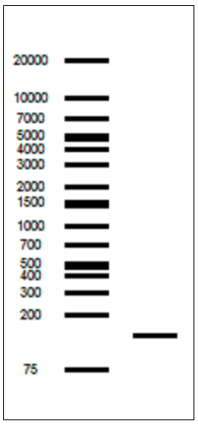
```
<Serial Cloner V2.5> -- <26 Sep 2017   14:33>

Restriction analysis of PCR(Bdef 1MBP) (1).xdna [Linear]
Incubated with BamHI + EcoRI

3 fragments generated.

   1:      132 bp  -  From EcoRI[4]      To  BamHI[136]

   2:        8 bp  -  From BamHI[136]    To  ---

   3:        4 bp  -  From ---           To  EcoRI[4]
```




```
<Serial Cloner V2.5> -- <26 Sep 2017   14:34>

Restriction analysis of Sequence Window #2 [Linear]
Incubated with BamHI + EcoRI

3 fragments generated.

   1:      126 bp  -  From EcoRI[5]      To  BamHI[131]

   2:        8 bp  -  From BamHI[131]    To  ---

   3:        5 bp  -  From ---           To  EcoRI[5]
```

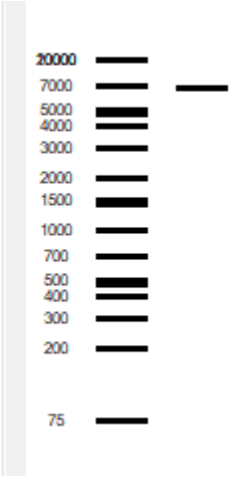



```
<Serial Cloner V2.5> -- <26 Sep 2017   14:35>

Restriction analysis of Sequence Window #2 [Linear]
Incubated with BamHI + EcoRI

3 fragments generated.

   1:      135 bp  -  From EcoRI[6]      To  BamHI[141]

   2:        8 bp  -  From BamHI[141]    To  ---

   3:        6 bp  -  From ---           To  EcoRI[6]
```



Appendix M: *In silico* Digestion of pMAL-p4X

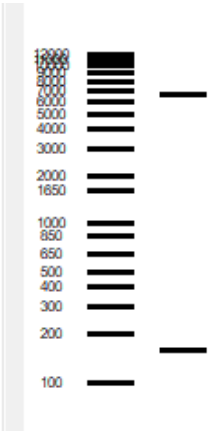
```
Restriction analysis of pMALp4x.txt.xdna [Circular]
Incubated with BamHI + EcoRI

2 fragments generated.

   1:    6,714 bp  -  From BamHI[2776]   To  EcoRI[2770]

   2:        6 bp  -  From EcoRI[2770]   To  BamHI[2776]
```



Appendix N: *In-Silico* Restriction Digestion of β-defensin Gene Inserts in Preparation for Cloning β-Defensin Genes using pMAL-p4X Cloning vector

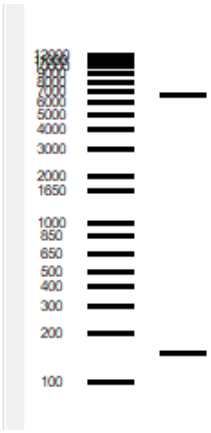
β-defensin 1 Positive

```
Restriction analysis of pMALp4x_bdef1.xdna [Circular]
Incubated with EcoRI + HindIII

2 fragments generated.

   1:    6,688 bp  -  From HindIII[2928] To  EcoRI[2770]

   2:      158 bp  -  From EcoRI[2770]   To  HindIII[2928]
```



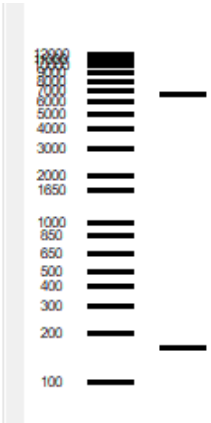
β-defensin 3 Positive

```
Restriction analysis of pMALp4x_bdef3.xdna [Circular]
Incubated with EcoRI + HindIII

2 fragments generated.

   1:    6,688 bp  -  From HindIII[2922] To  EcoRI[2770]

   2:      152 bp  -  From EcoRI[2770]   To  HindIII[2922]
```



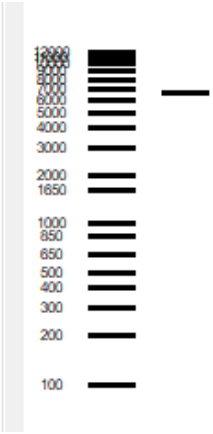
β-defensin 4 Positive

```
Restriction analysis of pMALp4x_bdef4.xdna [Circular]
Incubated with EcoRI + HindIII

2 fragments generated.

   1:    6,688 bp  -  From HindIII[2931] To  EcoRI[2770]

   2:      161 bp  -  From EcoRI[2770]   To  HindIII[2931]
```



β-defensin 1, 3, 4 Negative

```
Restriction analysis of pMALp4x.txt.xdna [Circular]
Incubated with EcoRI + HindIII

2 fragments generated.

   1:    6,688 bp  -  From HindIII[2802] To  EcoRI[2770]

   2:       32 bp  -  From EcoRI[2770]   To  HindIII[2802]
```



Appendix O: Standard Curve Generation for BCA Assay

Table 14: UV Absorbance at 595nm for Protein Standards

Calibrators (µg/ml)	Absorbance at 562nm
0	0
5	0.088
50	0.088
125	0.201
250	0.453
500	0.759
750	1.046
1000	1.294
1500	1.7495
2000	2.2985

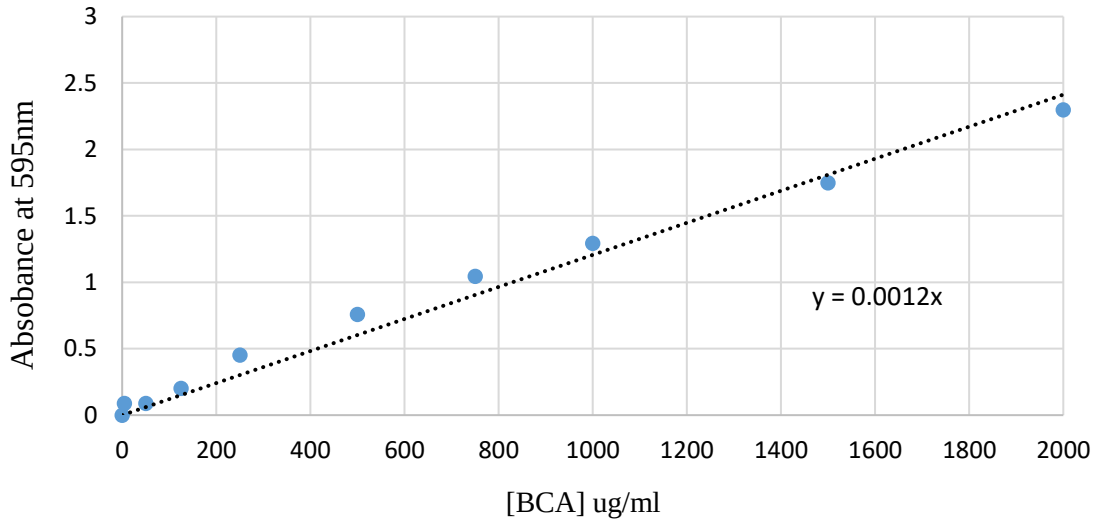


Figure 49: Standard Curve for BCA Assay

Submitted Sequence in FASTA format

```
>protein
SFPFSCPTLSGVCRKLCLPTEMETFFGPLGCGKGFLCCVSHFLSTP
```

Predicted Secondary Structure

```
                    20                  40
                     |                   |
Sequence    SFPFSCPTLSGVCRKLCLPTEMETFFGPLGCGKGFLCCVSHFLSTP
Prediction  CCCCCCCCHHHHHHHHCCHHHHHHCCCCCCCCCCHHHHHHHHCCC
Conf.Score  98765740650487762605568755686567641346767614 88
            H:Helix; S:Strand; C:Coil
```

Predicted Solvent Accessibility

```
                    20                  40
                     |                   |
Sequence    SFPFSCPTLSGVCRKLCLPTEMETFFGPLGCGKGFLCCVSHFLSTP
Prediction  83434244144204412133514432222223431200033237 48
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

Figure 50: Data Generated by I-TASSER for β-defensin 1 Mature Peptide

Submitted Sequence in FASTA format

```
>protein
SLHLCFISGGGCRNLRLCLASGGTNIGKMETGCTWPNVCCKSTP
```

Predicted Secondary Structure

```
                    20                  40
                     |                   |
Sequence    SLHLCFISGGGCRNLRLCLASGGTNIGKMETGCTWPNVCCKSTP
Prediction  CSSSSSSCCCCCCCSSSSSCCCCCCCSSCCCCCCCCSSSCCCC
Conf.Score  907999728992231688826886410002588678603335 88
            H:Helix; S:Strand; C:Coil
```

Predicted Solvent Accessibility

```
                    20                  40
                     |                   |
Sequence    SLHLCFISGGGCRNLRLCLASGGTNIGKMETGCTWPNVCCKSTP
Prediction  72300103344144131011344342342454141332126648
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

Figure 51: Data Generated by I-TASSER for β-defensin 3 Mature Peptide

Submitted Sequence in FASTA format

```
>protein
FPIPWGCSNYSGICRAVCLSAELPFGPFGCAKGFVCCVAHVFSTP
```

Predicted Secondary Structure

```
                    20                  40
                    |                   |
Sequence    FPIPWGCSNYSGICRAVCLSAELPFGPFGCAKGFVCCVAHVFSTP
Prediction  CCCCCCCCCHHHHHHHHCCCCCCCCCCCCCCCCCSSSSSSSSCCC
Conf.Score  985644752540066651443122256655775457777642068
            H:Helix; S:Strand; C:Coil
```

Predicted Solvent Accessibility

```
                    20                  40
                    |                   |
Sequence    FPIPWGCSNYSGICRAVCLSAELPFGPFGCAKGFVCCVAHVFSTP
Prediction  653324244144203312233523323223431200033337 48
            Values range from 0 (buried residue) to 9 (highly exposed residue)
```

Figure 52: Data Generated by I-TASSER for β-defensin 4 Mature Peptide

Appendix P: Quotation for Synthesis of β-Defensins

ESTIMATE N° 92001

Dear Dr SLATTERY,

Thank you very much for your e-mail .
As requested we are pleased to send you our price quotation as follows :

In order to secure the process and for correctness , please check the sequence(s) mentionned .
*Considering the length and the sequence this peptide, some solubility and purification problems are expected, this is why the final purity of 95% cannot be guaranted at this stage.
Of course make sure we will do our best to reach the highest purity possible.
On the other hand , we cannot garantuee the specific disulphide bridge but we propose you a natural folding .

This peptide will be delivered as lyophilized powder, as Gross Weight, as ACETATE salt and with a Certificate of Analysis including :

>HPLC profile (method used at PolyPeptide France)
>Mass Spectromety Results (Monoisotopic or Average Mass)

>Addional Analysis can be proposed upon request .

Designation Sequence	Qty	Purity	Total H (EUR)
Synthesis of peptide S-41-C-NH2,Bdef1 **Ser-Phe-Pro-Phe-Ser-Cys-Pro-Thr-Leu-Ser-** **Gly-Val-Cys-Arg-Lys-Leu-Cys-Leu-Pro-Cys-Val-** **Ser-His-Phe-Leu-Thr-Glu-Met-Phe-Phe-Gly-Pro-** **Leu-Gly-Cys-Gly-Lys-Gly-Phe-Leu-Cys-NH2** **Cyclic Cys6,Cys35** **Cyclic Cys13,Cys29** **Cyclic Cys17,Cys36** delivered in gross weight The peptide will be delivered as an ACETATE salt We would like to draw to your attention to the fact that M(O) of the peptide could be an impurity present in the peptide. The final purity can not be guaranted as a monomer	2 to 5 mg	90-95%	3300.00
Synthesis of peptide S-39-K ,Bdef3 **Ser-Leu-His-Leu-Cys-Phe-Ile-Ser-Gly-Gly-Gly-** **Cys-Arg-Asn-Leu-Arg-Leu-Cys-Leu-Ala-Ser-** **Gly-Gly-Thr-Asn-Ile-Gly-Lys-Met-Gly-Cys-Thr-** **Trp-Pro-Asn-Val-Cys-Cys-Lys** **Cyclic Cys5,Cys37** **Cyclic Cys12,Cys31** **Cyclic Cys18,Cys38** delivered in gross weight The peptide will be delivered as an ACETATE salt We would like to draw to your attention to the fact that M(O) of the peptide could be an impurity present in the peptide. The final purity can not be guaranted as a monomer	2 to 5 mg	90-95%	3300.00
Synthesis of peptide F-42-F,Bdef4 **Phe-Pro-Ile-Pro-Trp-Gly-Cys-Ser-Asn-Tyr-Ser-** **Gly-Ile-Cys-Arg-Ala-Val-Cys-Leu-Ser-Ala-Glu-** **Leu-Pro-Phe-Gly-Pro-Phe-Gly-Cys-Ala-Lys-Gly-** **Phe-Val-Cys-Cys-Val-Ala-His-Val-Phe** **Cyclic Cys7,Cys36** **Cyclic Cys14,Cys30** **Cyclic Cys18,Cys37** delivered in gross weight The peptide will be delivered as an ACETATE salt	2 to 5 mg	90-95%	3300.00