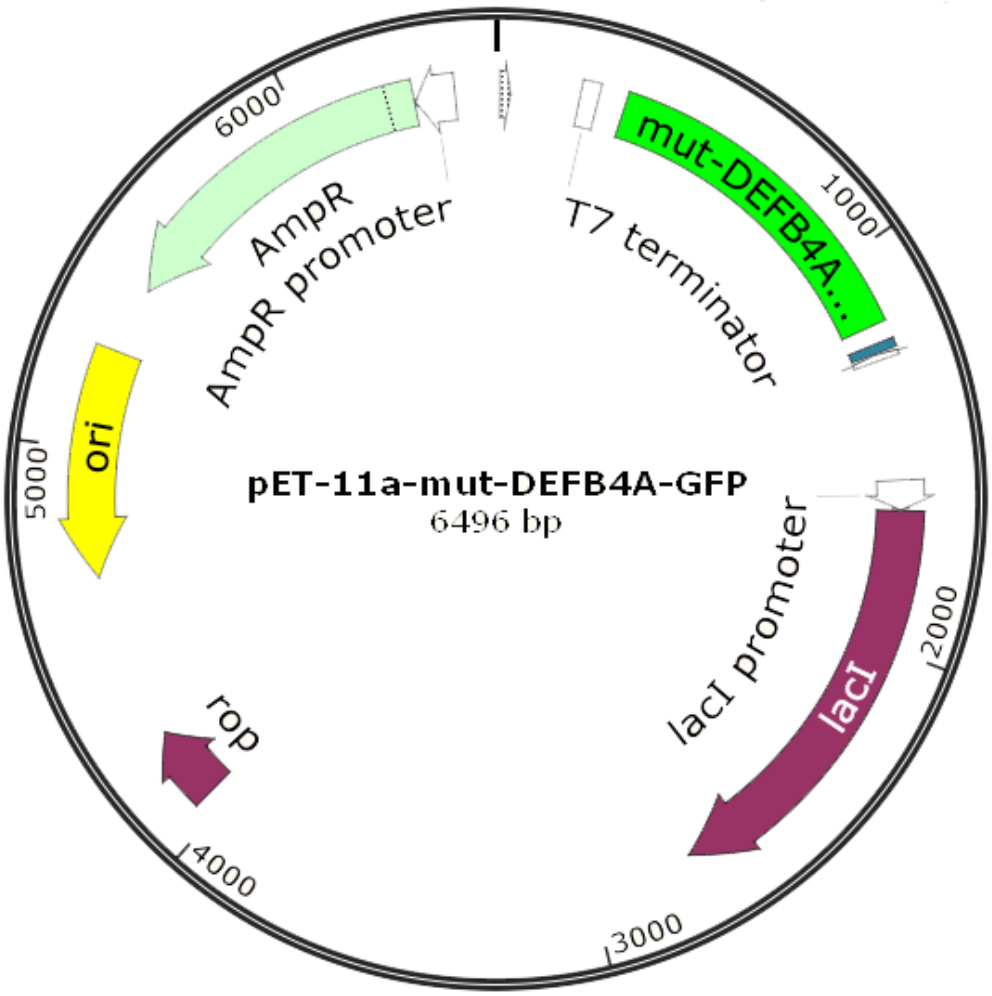
Cloning and Expression of Fusion Protein Human Beta-Defensin 2 Green Fluorescent Protein in *Escherichia coli*



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Master's thesis in Nanobiotechnology

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CLONING AND EXPRESSION OF FUSION PROTEIN HUMAN BETA-DEFENSIN 2 GREEN FLUORESCENT PROTEIN IN *Escherichia coli*

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English abstract

In this master's thesis, soluble HbD-2-turboGFP fusion protein was expressed in *E. coli* Origami 2(DE3). Fluorescence measurements of the fusion protein indicated a correctly folded and active turboGFP fusion-partner. Fluorescence measurements also showed a much higher protein concentration in bacteria grown under IPTG induction, than in the uninduced reference. The fusion protein size was confirmed on an SDS-PAGE gel.

The pET-11a-DEFB4A-GFP vector was constructed by ligation of DEFB4A-GFP and pET-11a, however, normal subcloning strategies were not performed, as the DEFB4A-GFP gene had an on-gene NdeI restriction site. Thus, an NdeI partial digestion of DEFB4A-GFP was first carried out, but this was unsuccessful.

Then a PCR site-directed point mutation was performed to mutate the on-gene NdeI restriction sequence from CATATG to CATTTG. This was successful, and the mutated gene could be digested with NdeI and BamHI restriction enzymes and ligated into digested pET-11a to form pET-11a-mut-DEFB4A-GFP vector.

Sequencing data of the constructed vector showed no mutations other than the point-mutation produced.

Danish abstract

I dette kandidatspeciale blev opløseligt HbD-2-turboGFP fusionprotein udtrykt i *E. coli* Origami 2(DE3). Fluorescensmålinger af fusion proteinet indikerede en korrekt foldet og aktiv turboGFP fusions-partner. Fluorescensmålinger viste også en meget højere proteinkoncentration i bakterier groet under IPTG induktion, end i den ikke-inducerede reference. Fusionproteinets størrelse blev bekræftet på en SDS-PAGE gel.

pET-11a-DEFB4A-GFP vektoren blev konstrueret ved ligation af DEFB4A-GFP og pET-11a, men en normal sub-kloning fremgangsmåde blev ikke benyttet, da DEFB4A-GFP genet har en - på genet - NdeI restriktionssekvens. Således blev en NdeI partiel restriktion af DEFB4A-GFP først udført, men denne var ikke succesfuld.

Derfor blev en PCR punktmutation udført for at mutere den - på genet - NdeI restriktionssekvens fra CATATG til CATTTG. Dette var succesfuldt, og det muterede gen kunne blive klippet med NdeI og BamHI restriktionsenzymer og blive ligeret med klippet pET-11a for at forme pET-11a-mut-DEFB4A-GFP vektor.

Sekventeringsdata af den konstruerede vektor viste ingen mutationer andre end den producerede punktmutation.

Preface

This master's thesis is the outcome of the research carried out by Kasper Pedersen in the period September 2012 - September 2013 at the Department of Physics and Nanotechnology at Aalborg University.

To fully understand this report, a basic understanding of genetechnology and biotechnology in general is required.

The Harvard Referencing System has been used for the literature list, which can be found in Bibliography. This system displays the author's surname or the owner of a website and the year of publication, e.g. [Watson and Crick, 1953]. Chapters, sections, figures, tables, and equations have been enumerated, i.e. figures, tables, and equations have the chapter number and then a sequence number, e.g. the first figure in chapter four is reffered to as: Figure 4.1; the second figure: Figure 4.2; etc. Below figures and above tables an explanatory text is written in which references are also to be found. If no source is present in the figure text, the figure is a product of the author. Abbreviations can be found on page vii. This project contains 6 appendices, which can be found in the back of the rapport.

In the back of the report, a CD-ROM has been attached. This CD-ROM contains sources used in the project, fluorescence measurement data, DNA sequences and sequencing data, and all the figures used in the report. So if a figure needs to be digitally examined or a graphical representation of the sequencing data is needed, this is possible with the CD-ROM.

Kasper Pedersen

Abbreviations

AA	-	Amino acids
AcOH	-	Acetic Acid
AMP	-	Antimicrobial peptide
APS	-	Ammonium Persulfate
CFTR	-	Cystic fibrosis transmembrane regulator
EDTA	-	Ethylenediaminetetraacetic acid disodium salt dihydrate
EtBr	-	Ethidium bromide
EtOH	-	Ethanol
FDA	-	Food and Drug Administration
FRET	-	Fluorescence resonance energy transfer
GFP	-	Green fluorescent protein
Gly	-	Glycine
gor	-	Glutathione reductase
HbD	-	Human beta-defensin
HCl	-	Hydrochloric acid
Ile	-	Isoleucin
IPTG	-	Isopropyl-β-D-thiogalactopyranoside
IND	-	Investigational New Drug
KDS	-	Potassium dodecyl sulfate
lacO	-	lac Operon
LB	-	Lysogeny broth
LMW	-	Low molecular weight
LPS	-	Lipopolysaccharides
MIC	-	Minimum inhibitory concentration
NaCl	-	Sodium chloride
NaOAC	-	Sodium acetate
NaOH	-	Sodium hydroxide
OE-PRC	-	Overlap extension polymerase chain reaction
ORF	-	Open reading frame
ori	-	Origin of replication
PCR	-	Polymerase chain reaction
SDS	-	Sodium dodecyl sulfate
SDS-PAGE	-	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	-	Serine
SOC	-	Super optimal broth with catabolite repression
SUMO	-	Small ubiquitin-related modifier
TAE	-	Tris-acetate-EDTA
TEMED	-	Tetramethylethylenediamine
TES	-	Tris-EDTA-sucrose
tGFP	-	turboGFP
trxB	-	Thioredoxin reductase
Tyr	-	Tyrosine
wtGFP	-	Wild type green fluorescent protein

Contents

Introduction 1

The discovery of penicillin in 1928 by Sir Alexander Fleming was a huge breakthrough and became a shift in paradigm to how infections were treated. Since then many other antiobiotics were developed, e.g. streptomycin against tuberculosis. This meant that the leading cause of death changed from being infections to being non-infectious diseases, i.e. cancer, stroke, etc.
Meanwhile, extended use of antibiotics have led to multi-drug resistance in some pathogenic organisms. Some of the human pathogens that have acquired increased resistance against traditional antibiotics are *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. That has made the treatment of diseases caused by these very difficult. An example of an attempt to overcome this resistance is the creation of linezolid, a new synthetic antibiotic against *S. aureus* infections. However, this effort backfired, and linezolid-resistant *S. aureus* now exist [Aoki *et al.*, 2012] [Hancock and Diamond, 2000] [Yeaman and Yount, 2003].

The search by scientists for novel antimicrobial drugs that are less prone to causing resistance has been diverted back to the antibiotics produced by nature itself. This search had a breakthrough when magainin, the first antimicrobial peptide (AMP), was isolated by Zasloff [1987] from African clawed frog *Xenopus laevis*. This discovery led to further search for more AMPs, and now AMPs are a large group of molecules that are being explored because of their antimicrobial potency against pathogenic organisms. It is believed, that resistance toward AMPs is very unlikely due to the fact that they are still anti-infective after more than 10^8 years [Hancock and Diamond, 2000] [Yeaman and Yount, 2003].

1.1 Antimicrobial peptides

AMPs are an essential part of the innate immune system, hence their description as host defence peptides. The innate immune system evolved 2.6 billion years ago, and is present in all living organisms [Gordon *et al.*, 2005]. It is the front-line defense against infections, and it is very rapid compared to the adaptive immune system. A bacterial infection could occur within 24 hours after contamination, and the adaptive immune system can take up to a week before B-cells and T-cells have been produced to combat this infection. Here, the innate imune system will instead prevent that infection or limit it until the adaptive immune system is ready to take over [Hancock and Diamond, 2000]. The innate immune system is triggered by structures that are found in large groups of microorganisms, and this recognition is very important so that only pathogens and not human cells are killed (Figure 1.1 and 1.2) [Yang *et al.*, 2002].

AMPs are defined as 6-100 aminoacids long anionic antimicrobial molecules. They have shown biocidal activity against Gram-positive and Gram-negative bacteria, fungi, viruses, and tumor cells [Guiliani *et al.*, 2007] [Gordon *et al.*, 2005] [Yang *et al.*, 2002]. The minimum inhibitory concentration (MIC) of some AMPs have been found as low as 0.24-4 μg/ml [Powers and Hancock, 2003]. Defensins and Cathelicidins are the two main families of AMPs found in humans. These exist in epithelial cells, phagocytes, and inflammatory body fluids, and can be found at millimolar concentrations [Ganz, 2003].

Generally, AMPs are divided into four classes based on the secondary structure: α-helical, β-sheets, loop, and extended peptides [Hancock and Lehrer, 1998] (Figure 1.3). A database of all known natural AMPs can be found in The Antimicrobial Peptide Database at *aps.unmc.edu/AP/main.php* and currently there are 2256 AMPs in that database.

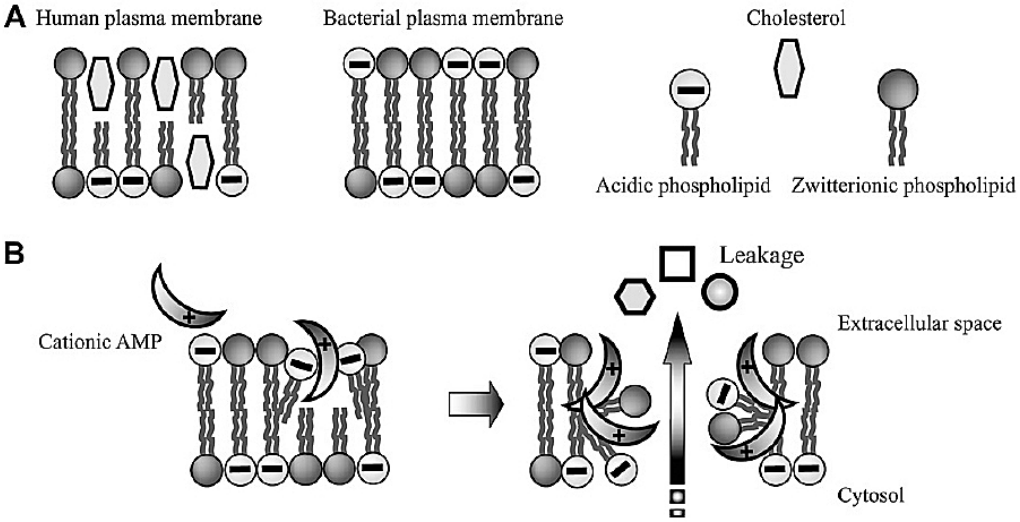

Figure 1.1. Bacterial selectivity and mode of action of AMPs. (A) Composition of human plasma membrane compared to bacterial plasma membrane - bacterial membrane has a higher negative surface charge. (B) Disruption of membrane by cationic AMPs that interact with the membrane because of their affinity for anionic molecules [Aoki *et al.*, 2012].

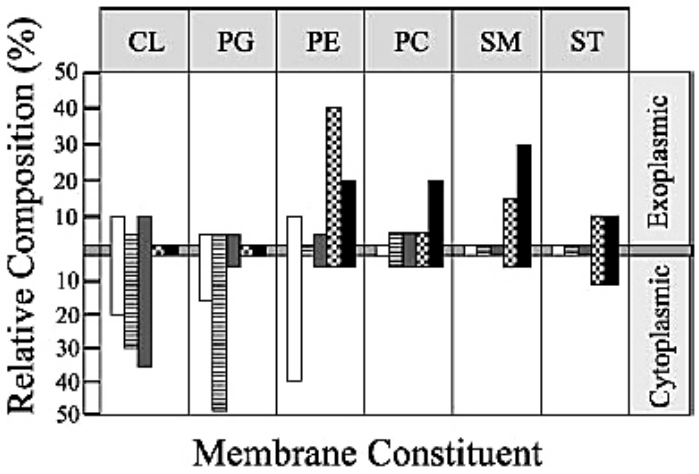

Figure 1.2. Comparison of constituents in microbial and human cytoplasmic membranes. The composition and distribution of membrane constituents between inner and outer membrane leaflets of the cytoplasmic membranes of bacterial (*E. coli*, *S. aureus*, and *B. subtilis*) and fungal (*C. albicans*) pathogens are compared to that of human erythrocytes. The constituents are ranged from anionic (left) to zwitterionic to neutral (right). These are: cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), and sterols (ST) (cholesterol or ergesterol). Key: (white) *E. coli*; (striped) *S. aureus*; (grey) *B. subtilis*; (checkered) *C. albicans*; (black) human erythrocyte [Yeaman and Yount, 2003].

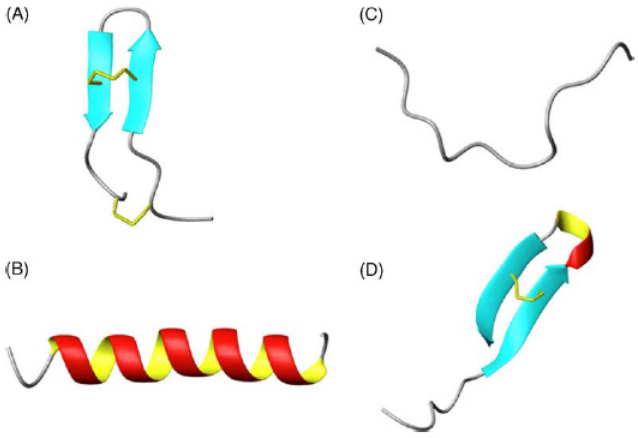

Figure 1.3. Structural classes of AMPs. (A) β-sheet structure (tachyplesin I). (B) α-helical structure (magainin 2). (C) extended structure (indolicidin). (D) loop structure (thanatin) [Powers and Hancock, 2003].

1.1.1 Mechanism of action

The mode of action of antibacterial activity of AMPs have mainly been studied through interactions between AMPs and model membrane systems, but studies have also been conducted using microbial cells with membrane potential sensitive dyes and flurescently labeled peptides. These experiments have made it possible to divide AMPs into two classes of mechanism: membrane disruptive and non-membrane disruptive (through translocation across the membrane and intracellular targeting) [Powers and Hancock, 2003] [Guiliani *et al.*, 2007].

Because the mechanism of action has mainly been studied on Gram-negative bacteria, this is the mechanism that will be presented here. The outside of Gram-negative bacteria consist of an inner cytoplasmic membrane, a peptidoglycan layer, and finally an outer membrane containing lipopolysaccharides (LPS) that trigger the innate immune system as written earlier. A Gram-negative bacteria in its native state has cations such as Mg^{2+} and Ca^{2+} bound to the negative LPS molecules. However, the cationic nature of AMPs give them a higher affinity for LPS, and such, the cations are displaced. This leads to destabilised areas of the membrane, and eventually the outer membrane will disrupt. Now the inner cytoplasmic membrane is accessible, and the AMPs will either disrupt the membrane or translocate into the cytoplasm (Figure 1.4) [Seo *et al.*, 2012a] [Powers and Hancock, 2003] [Hancock and Chapple, 1999].

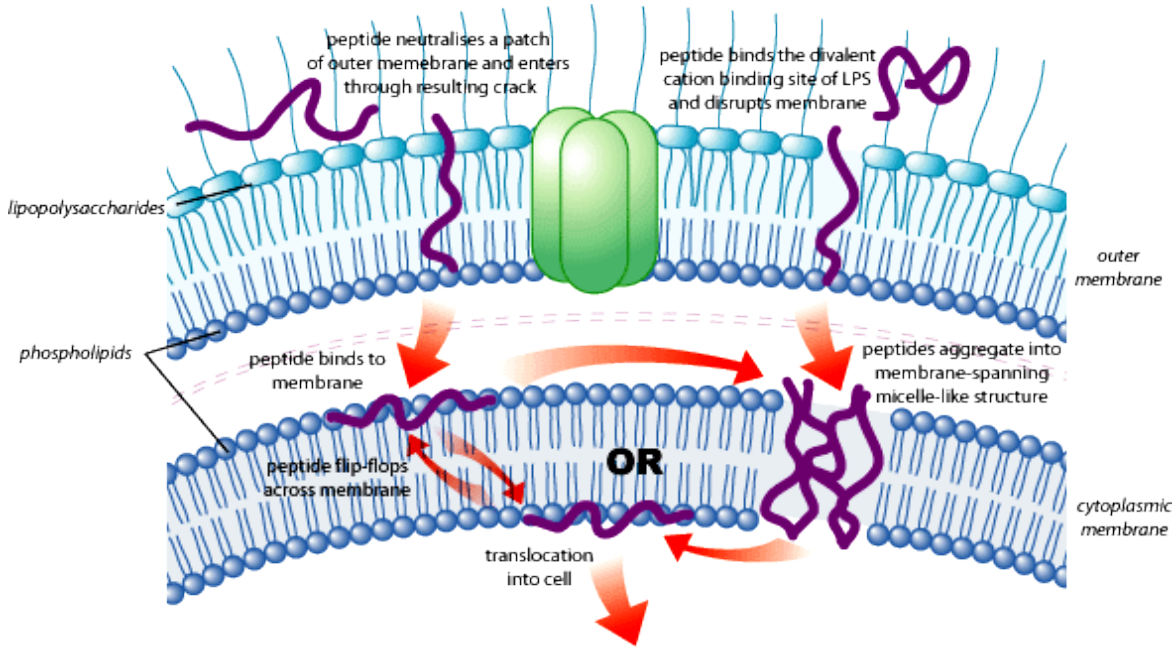

Figure 1.4. AMPs interacting with Gram-negative bacteria. After disrupting the outer membrane, the AMPs will either disrupt the inner cytoplasmic membrane through peptide-induced pore formations, or the AMPs will translocate into the cell and inhibit its growth by intracellular targeting of DNA, RNA, protein synthesis, enzymes, etc. [Wilcox, 2004].

So far, three mechanisms for disruption of the cytoplasmic membrane has been proposed. These are:
The barrel-stave model (Figure 1.5A), the carpet model (Figure1.5B), and the toroidal model (Figure 1.5C).

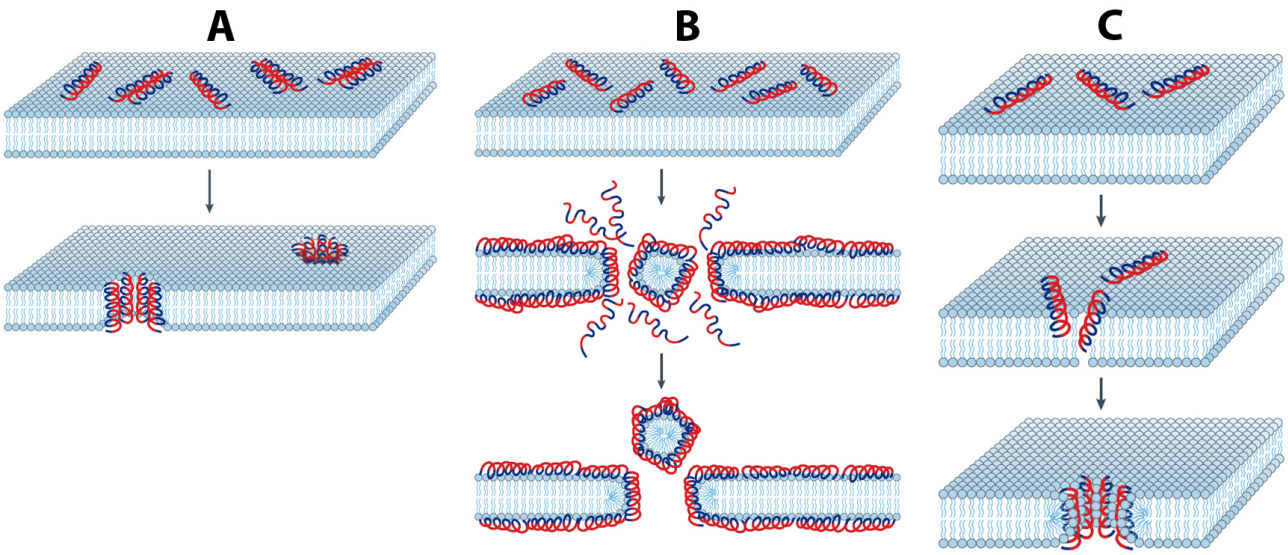

Figure 1.5. (A) The barrel-stave model of AMP-induced killing. The membrane-bound AMPs will aggregate and interact with the membrane bilayer such that a pore is formed. In this pore, the hydrophobic regions of the peptides point outwards toward the hydrophobic lipid tails, and the hydrophilic regions of the peptides point inwards to form a hydrophilic pore. (B) The carpet model of AMP-induced killing. The membrane-bound AMPs will orientate themselves parallel to the surface to form a layer called a carpet. Throught this carpet they will interact with the lipid bilayer, so that the amphiphobic regions of the AMPs reach the amphiphobic lipid tails, and the hydrophilic regions of the AMPs will interact with the hydrophilic lipid headgroups and point outwards from the membrane. This will create regions in the membrane where the peptide can take the place of a lipid and thereby disrupt the membrane. (C) The toroidal model of AMP-induced killing. This model is almost like the barrel-stave model, however, in the toroidal model the lipids will bend through the pore, so that lipid headgroups point toward the pore. Thus, this model could be favored over the barrel-stave model in an energy minimisation consideration. Key: Red regions of AMPs are hydrophilic, blue regions are hydrophobic [Brogden, 2005].

1.1.2 Potential as drugs

The great potential of the use of AMPs as antibiotics has been extensively covered in several studies. But the step from research to actual evaluation of AMPs has happened at a very slow rate, and so far only a few hundred AMPs have been clinically evaluated. Seven years ago Nature published Hancock and Sahl [2006], a review that provided a good insight to what stage the development of commercial drugs was at (Table 1.1). However, so far, no AMPs have been approved by Food and Drug Administration (FDA) for clinical applications. Currently the most promising AMPs are MBI-226 (treatment of catheter-related infections) and MX-594AN (treatment of acne) developed by Migenix (Vancouver, BC, Canada), where MBI-226 (Omiganan) has made it to phase IIIa trails. Furthermore, an AMP developed by Intrabiotics Pharmaceuticals (Mountain View, CA, USA), IB-367 (Iseganan) used for treatment of oral mucositis in cancer patients is going into clinical trials [Teixeira *et al.*, 2012]. In the beginning of 2013, PMX-30063 (Brilacidin), a small-molecule defensin-mimetic antibiotic that is intended for treatment of *Staphylococcus* completed phase II clinical trials [PolyMedix, 2013]. Sadly, PolyMedix, the company developing Brilacidin, filed for bankruptcy only few months later. However, several other AMPs are also under current development. So it seems that even though there are still no FDA approved AMP-based drugs, companies are still optimistic and active in the development of AMPs for therapeutic use.

Table 1.1. Peptides and peptidomimetics in commercial development in 2006. The table lists known AMPs in development and/or clinical trials in private companies. Peptides that went through clinical trials but were not approved are not included in the table (adapted from Hancock and Sahl [2006]).

Company	Drug	Stage of development	Medical use
AM-Pharma (Bilthoven, The Netherlands)	hLF-1-11 (small peptide derived from human lactoferrin)	Phase 2	Allogeneic bone marrow stem cell transplantation-associated infections
BioLineRx (Jerusalem, Israel/Palestine)	BL2060 (a synthetic compound comprising fatty acid and lysine copolymers)	Lead optimization	Anti-infective
Ceragenix (Denver, CO, USA)	CSA-13 (cationic steroid (ceragenin) that mimics host-defense peptides)	Preclinical	Anti-infective
Helix Biomedix (Bothell, WA, USA)	HB-50 (synthetic natural peptide mimetic of cecropin)	Preclinical	Anti-infective
Helix Biomedix (Bothell, WA, USA)	HB-107 (19-amino-acid fragment of cecropin B)	Preclinical	Wound healing
Inimex (Vancouver, BC, Canada)	IMX942 (5-amino-acid peptide)	Lead optimization	Immunomodulation; treatment of fevers and neutropenia in chemotherapy patients
Lytix Biopharma (Tromso, Norway)	Not available	Discovery	Anti-infective, antitumor
Migenix (Vancouver, BC, Canada)	Omiganan pentahydrocholoride/ CP-226/MX-226/CLS001 (12-mer analog of bactolysin)	Phase 3b/phase 2	Prevention of catheter-related infections; dermatology-related infections
Novacta Biosystems Ltd. (Hatfield, England)	Mersacidin (bacteriocin)	Preclinical	Gram-positive infections
Novobiotics (Cambridge, MA, USA)	Not available	Discovery	Nail fungus; methicillin-resistant
Novozymes A/S (Bagsvaerd, Denmark)	Plectasin (fungal defensin)	Preclinical	Systemic anti-Gram-positive, especially pneumococcal and streptococcal infections
Pacgen (Vancouver, BC, Canada)	PAC113 (based on the active segment of histatin 5 protein found in human saliva)	Investigational New Drug (IND) approval	Oral candidiasis
PepTx (St. Paul, MN, USA)	PTX002 (33-mer peptide) PTX005 (12-mer peptide), PTX006 (N-acylated analog of PTX005) and PTX007 (a nonpeptidic structural analog of PTX005)	Discovery	Broad-spectrum antimicrobial antiendotoxin
Polymedix (Philadelphia, PA, USA)	Peptidomimetics (derived from the arylamide, calixarene, hydrazide and salicylamide series)	Discovery/preclinical	Anti-infectives; antimicrobial polymers and coating materials
Zengen (Woodland Hills, CA, USA)	CZEN-002 (synthetic 8-mer derived from α-melanocyte-stimulating hormone)	Phase 2b	Vulvovaginal candidiasis

1.1.3 Defensins

The AMP family of defensins can be further divided into three classes of α-, β-, and θ-defensins based on the spatial distribution of the cystein residues and disulfide bonds (Figure 1.6 and 1.7) [Chen *et al.*, 2006]. Defensins have the size 3-5 kDa, have six to eight cysteine residues (i.e. three or four disulfide bonds), and exist in plants, vertebrates, and invertebrates. Defensins are widely studied and have been found to show biocidal activity against bacteria, fungi, and some viruses [Guiliani *et al.*, 2007] [Ganz, 2003] [Oppenheim *et al.*, 2003]. Defensins even show activity against tumour cells [Lichtenstein *et al.*, 1986]. This selectivity toward tumour cells compared to normal cells is due to a stronger negative surface charge, which is caused by higher levels of anionic phosphatidylserine [Guiliani *et al.*, 2007] [Schröder-Borma *et al.*, 2005]. And thus, defensins also act as candidates for chemotherapy.

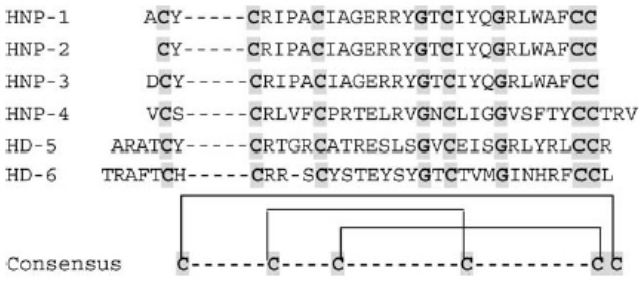

Figure 1.6. Molecular structure of human α-defensin. The cystein residues of six different human α-defensins are marked by grey, and it is shown how the residues are linked in disulfide bonds [Chen *et al.*, 2006].

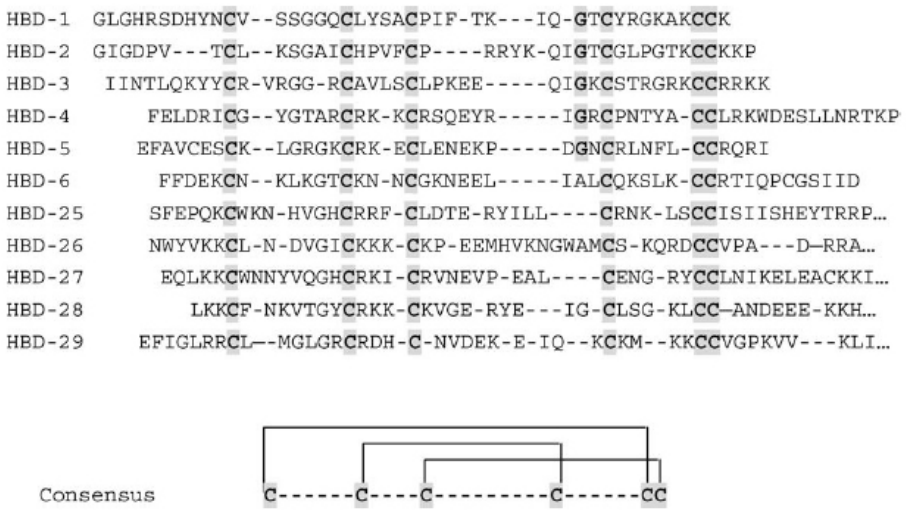

Figure 1.7. Molecular structure of human β-defensin. The cystein residues of 11 different human β-defensins are marked by grey, and it is shown how the residues are linked in disulfide bonds [Chen *et al.*, 2006].

Role in nature

The only AMP family that can be found in both plants, vertebrates, and invertebrates are defensins. Their defense mechanism differ with regards to infection pressure. This means that most plant defensins are only active against fungi, because fungal infections by far are the most common in plants. Only very few plant defensins have shown bactericidal activity, because most bacteria that interact with plants are saprotrophic (uptakes the dead plant matter) and not infective [Thomma *et al.*, 2002].
However, there are still some plant pathogenic bacteria such as *Pseudomonas syringae*. Arenas *et al.* [2006] transformed a defensin-gene from invertebrate Chilean mussel *Mytilus edulis chilensis* into tobacco plants and thereby created plants with resistance toward *P. syringae*. Defensins from invertebrates are classified as anti-fungal vs. anti-bacterial, and defensins in general isolated from marine invertebrates have shown activity against both Gram-negative and Gram-positive bacteria [Otero-González *et al.*, 2010]. Defensins isolated from insects have shown anti-fungal activity, but the main part of the studied insect defensins are anti-bacterial [Bulet *et al.*, 2004].

Human beta-defensin 2

Human beta-defensin 2 (HbD-2) is one of the most studied AMPs; it was first discovered in 1997 by *Harder et al.*. It is found in skin, lungs, trachea epithelia, and uterus and thus is thought to have great importance in the innate immune system. This importance is further grounded by patients suffering from defensin-defiencency; these individuals often get infections from common bacteria [Chen *et al.*, 2006].

HbD-2 (Figure 1.8) consists of 41 aminoacids (AA) and has a molecular weight of 4.3 kDa. The three disulfide bonds are between cystein residues 8-37, 15-30, and 20-38, it is a monomer in solution, has a net charge of +7, and has shown activity against Gram-positive and Gram-negative bacteria, virus (also HIV), fungi. Furthermore, it has shown chemostatic activity (synergy with the adaptive immune system) [AMP-Database].

HbD-2 is a salt-sensitive AMP, and its activity is significantly reduced by concentrations of 100 mM NaCl or KCl [Tomita *et al.*, 2000]. This is a very important feature, that gives way to different theories regarding different illnesses:
The genetic disease cystic fibroses, which is caused by matations in the cystic fibrosis transmembrane regulator (CFTR), causes defective chloride channels to the lungs, which is believed to give an increased concentration of salt. The deactivation of HbD-2 in lungs from this increased salt concentration is then thought to be the reason for a defect in lung mucosal defense and thereby an increase in lung infections [Ganz, 2003]. Furthermore, a higher than normal concentration of HbD-2 is detected in the skin of psoriasis patients, thus, an elevated level of HbD-2 could cause a kind of allergic reaction in the form of psoriasis. Psoriasis is alleviated in salt-water, where HbD-2 should also be inactivated [Harder *et al.*, 2001] [Chen *et al.*, 2006].

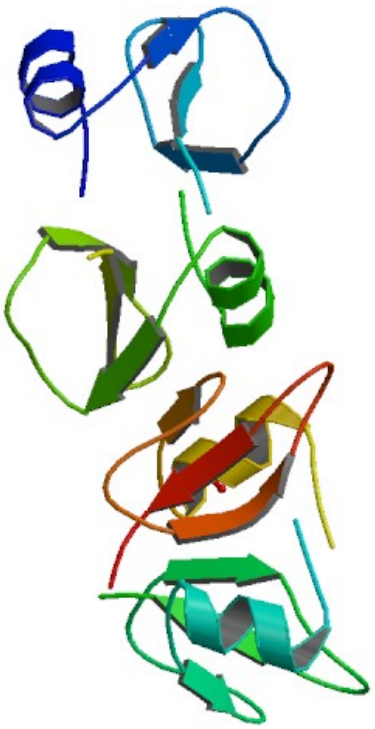

Figure 1.8. 3D structure of human beta-defensin 2 shown from different angles. The secondary structure of HbD-2 is composed of α-helix and β-sheets [PDB, 2011].

1.2 Green fluorescent protein

Green fluorescent protein (GFP) was first discovered by Davenport and Nicol [1955] as they described the green bioluminiscence of jellyfish *Aequorea victoria*. In 1962 a protein, thought to be responsible for the bioluminiscence, named aequorin was isolated by Shimomura et al. However, this protein emitted blue light, so Shimomura et al. isolated another protein that emitted green light, which was to be named wild type green fluorescent protein (wtGFP). It was found that the reason for *A. victoria* to have two fluorophores was fluorescence resonance energy transfer (FRET), so the purpose of aequorin in *A. victoria* is to amplify the fluorescence of wtGFP. Prasher *et al.* [1992] were the first to clone and sequence wtGFP, and only two years later Chalfie *et al.* [1994] had expressed wtGFP in *E. coli*, which meant that all posttranslational information was encoded in the gene. The research in GFP led to Martin Chalfie, Osamu Shimomura, and Roger Tsien recieving the Nobel Prize in Chemistry in 2008 [Shimomura, 2005] [Ehrenberg, 2008] [Tsien, 1998].

wtGFP from *A. victoria* has a length of 238 amino acids, and the fluorescent chromophore is p-hydroxybenzylideneimidazolinone formed by cyclization and oxidation of residues 65-66-67 (Ser-Tyr-Gly) (Figure 1.9) [Reid and Flynn, 1997]. The crystal structure of wtGFP reveals a β-barrel made up by 11 β-strands with an α-helix with chromophore going through the barrel (Figure 1.10) [Ormö *et al.*, 1996]. wtGFP has exitation wavelenghts at 400 nm (major) and 470 nm (minor) and emission at 505 nm (major) and 540 nm (minor).

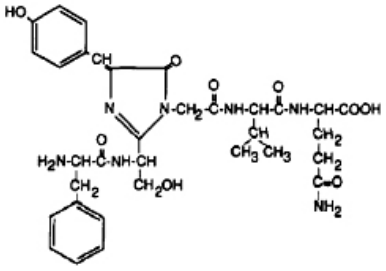

Figure 1.9. p-hydroxybenzylideneimidazolinone, the fluorescent chromophore in GFP, formed by cyclization and oxidation of residues Ser65-Tyr66-Gly67 [Cody *et al.*, 1993].

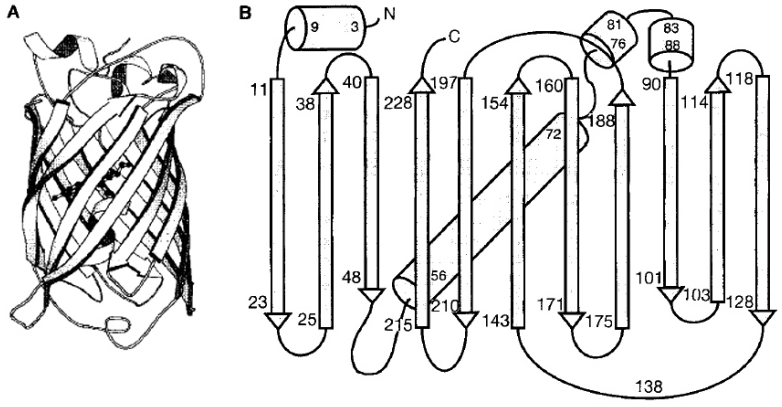

Figure 1.10. (A) Barrel-structure of GFP composed of 11 β-strands, threaded by an α-helix with the Ser65-Tyr66-Gly67 chromophore (shown as ball and stick model). (B) Overall fold of GFP with arrows representing β-strands and cylinders representing α-helices. [Ormö *et al.*, 1996].

1.2.1 Green fluorescent protein as an expression marker

One of the problems when expressing AMPs in *E. coli* is their host-killing activity. If *E. coli* recognises this toxicity, it can stop expression of that gene, and thus no protein will be expressed. Another problem is that the relatively small size of AMPs make them more susceptible to degradation by host cell proteases. These obstacles can be overcome by removing the stop codon of the AMP and adding a protein partner at the C-terminal and thus create a fusion protein. Such fusion partner can alleviate an array of problems that could occur in AMP expression, i.e. prevent degredation of the product, increase solubility, and lower the risc of creating inclusion bodies. Moreover, a fusion protein such as green fluorescent protein (GFP), which will be used in this project, can help detect that expression has occured through its fluorescent nature [Xu *et al.*, 2006] [Piers *et al.*, 1993] [Chalfie *et al.*, 1994].

TurboGFP is a very fast maturing protein with a maturation half-time of 1468 s (24 min), compared to normal GFP which needs several hours to fold. It has a molecular weight of 26 kDa, consists of 232 amino acids, the active structure is a dimer, and it has excitation/emission = 482/502 nm [Evrogen] [Chalfie *et al.*, 1994].

1.3 Aim of project

The aim of this project is to recombinantly clone and express fusion protein HbD-2-turboGFP.

Experimental Design 2

2.1 Expression system

2.1.1 *Escherichia coli* as an expression host

Although coming from the same laboratory, HbD-2 have been reported successfully expressed in *E. coli* in several publications:

- Peng *et al.* [2004] used *E. coli* BL21 (DE3) to express soluble and functional HbD-2 in a fusion protein with thioredoxin.
- Xu *et al.* [2005] used an *E. coli* cell-free system, without cell wall and plasma membrane, to express a soluble fusion protein of HbD-2 with GFP.
- Xu *et al.* [2006] used *E. coli* BL21 (DE3) and expressed HbD-2 in a fusion protein with thioredoxin, again. But this time they removed the prepro-peptide sequence from the HbD-2 gene and achieved both higher solubility and higher concentration of product. After expression, the product was digested with enterokinase to remove thioredoxin and purified. The purified HbD-2 showed antimicrobial activity against *E. coli* K12D31, and the protein also displayed salt-sensitivity as expected (almost totally inactive at a concentration of 150 mM NaCl).

Newer articles, produced by other research teams, also confirm the ability of *E. coli* to express different HbDs in active and soluble form:

- Huang *et al.* [2008] expressed soluble fusion protein HbD-5 and HbD-6 with thioredoxin in *E. coli* BL21 (DE3). The thioredoxin was removed by cleavage with enterokinase, and both HbD-5 and HbD-6 displayed antimicrobial activity against *E. coli* K12, which could be suppressed by a NaCl concentration of 100 mM.
- Li *et al.* [2010] expressed soluble fusion protein HbD-4 with small ubiquitin-related modifier (SUMO) in *E. coli* BL21 (DE3). The fusion-protein was cleaved with a SUMO protease, and the final HbD-4 displayed antimicrobial activity similar to synthetic HbD-4.
- Seo *et al.* [2012b] expressed soluble fusion protein HbD-1 with disulfide bond isomerase in *E. coli* BL21 (DE3). The isomerase was removed by enterokinase cleavage, and the final isolated HbD-1 showed broad antimicrobial activity against Gram-positive and Gram-negative bacteria including *Streptococcus pneumoniae* and *Klebsiella pneumoniae*.

So it seems that using a prokaryotic expression host such as *E. coli* is sufficient for production of HbD-2, and that post-translational modifications such as glycolysation provided in eurkaryotic expression systems are not required for production of active HbD-2.

However, a general obstacle for HbD protein production in *E. coli* is the formation of disulfide bonds. Origami is an *E. coli* strain which have mutations in the thioredoxin reductase (trxB) and glutathione reductase (gor) genes. This greatly improves the disulfide bond formation in the cytoplasm.

Therefore, it was decided to use Origami™ 2(DE3) from Merck (Novagen). This strain has the trxB and gor mutations, and it has a chromosomal copy of the T7 RNA polymerase gene (indicated by DE3 in the name). This gene is regulated by the lacUV5 promoter, which means that a β-galactoside, such as IPTG, can be used as an inducer to activate transcription. The use of IPTG is favorable because it cannot be hydrolysed by β-galactosidase, which means that only a very low concentration of IPTG is needed, and the concentration added remains constant. To make use of this T7 system, the plasmid vector containing the gene of interest must carry a T7 promoter. pET expression vectors all carry the T7 promoter upstream of the polylinker making them ideal for expression in Origami™ 2(DE3). The pET vectors also carry an ampicillin resistance gene, so ampicillin can be used for selection pressure, as the gor gene makes Origami tetracyclin resistant [EMD-Millipore, 2013] [Novagen, 2011].

2.1.2 pET-11a expression vector

In this master's thesis, pET-11a expression vector will be used (Figure 2.1). It is part of the pET system, which is a very good system for production of heterologous proteins in *E. coli*. A very important advantage of working with pET vectors is that they are part of T7 system, which means that transcription of the inserted gene can only happen when the plasmid is inserted in a host carrying the T7 RNA polymerase gene. Thus, initial construction of the plasmid and selection of colonies can occur with no protein produced. This initial cloning will be carried out in *E. coli* NEB5α; a strain that lacks the T7 RNA polymerase gene. When using the pET-11a vector it is favourable to insert the gene between restriction sites NdeI and BamHI, which will put it under influence of the T7 promoter and lac Operon (lacO). Furthermore, the NdeI restriction site is CA'TATG, which means that the start-codon is already there [Novagen, 2011]. This is especially useful when producing heterologous proteins with a signal peptide sequence (that contains the start-codon), which is unwanted for expression in *E. coli*.

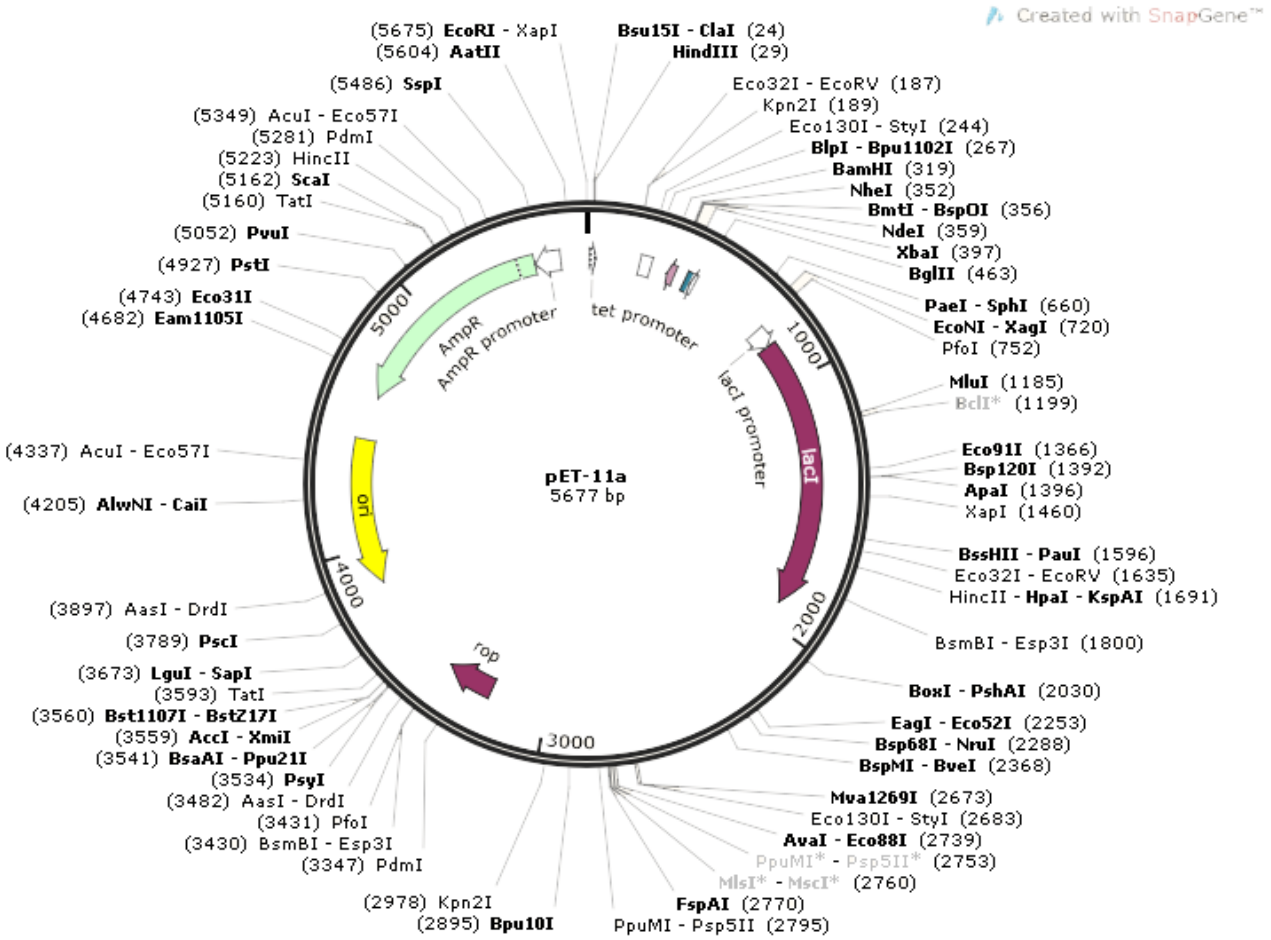

Figure 2.1. Circular map of pET-11a, only Fermentas restriction enzymes are shown. Created with SnapGene software.

2.2 Construction of pET-11a vector with DEFB4A-GFP insert

In this project, it was decided to order a mammalian vector from OriGene containing the cDNA coding for HbD-2 with a C-terminal turboGFP tag. This gene, DEFB4A-GFP, was to be subcloned into pET-11a in order to be able to perform expression in *E. coli*. Normally, the procedure for subcloning (Figure 2.3 on page 15) is to make an overlap extension polymerase chain reaction (OE-PCR) of the gene using primers containing restric-

tion sites, thereby adding these sites to the gene (in this case NdeI and BamHI). Then a digestion with restriction enzymes NdeI (CA'TATG) and BamHI (G'GATCC) is performed on both the destination vector (pET-11a) and the polymerase chain reaction (PCR) product, and finally the open pET-11a vector and the digested DEFB4A-GFP will be ligated in a ligation reaction.

However, in the gene ordered from OriGene there is a second NdeI restriction site from 39-44 bp inside the DEFB4A-GFP gene (Figure 2.2). This means that normal subcloning cannot be performed. First, it was considered whether it was possible to use a different restriction enzyme such as NcoI, which also has ATG in the restriction sequence, but NcoI is also found inside the gene at 173-178 bp. Consequently, either a partial digestion or a point mutation had to be carried out to be able to create the desired pET-11a vector containing DEFB4A-GFP. It was decided to attempt a partial digestion first. The reason being that the creation of a point mutation using PCR would require the purchase of four extra primers, and in theory would take longer time than a partial digestion.

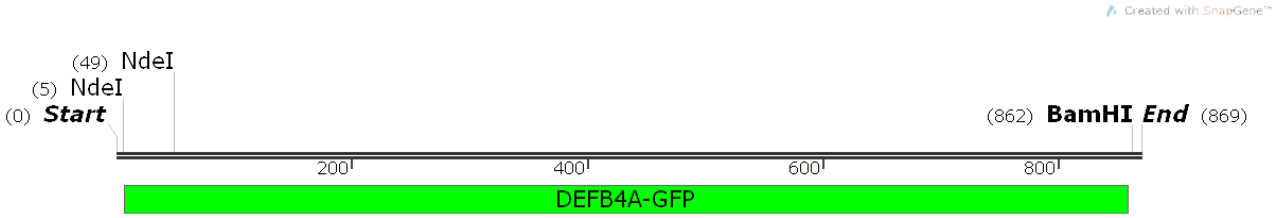

Figure 2.2. A second NdeI restriction sequence is found inside the DEFB4A-GFP gene, which makes a normal subcloning procedure inept. Created with SnapGene software.

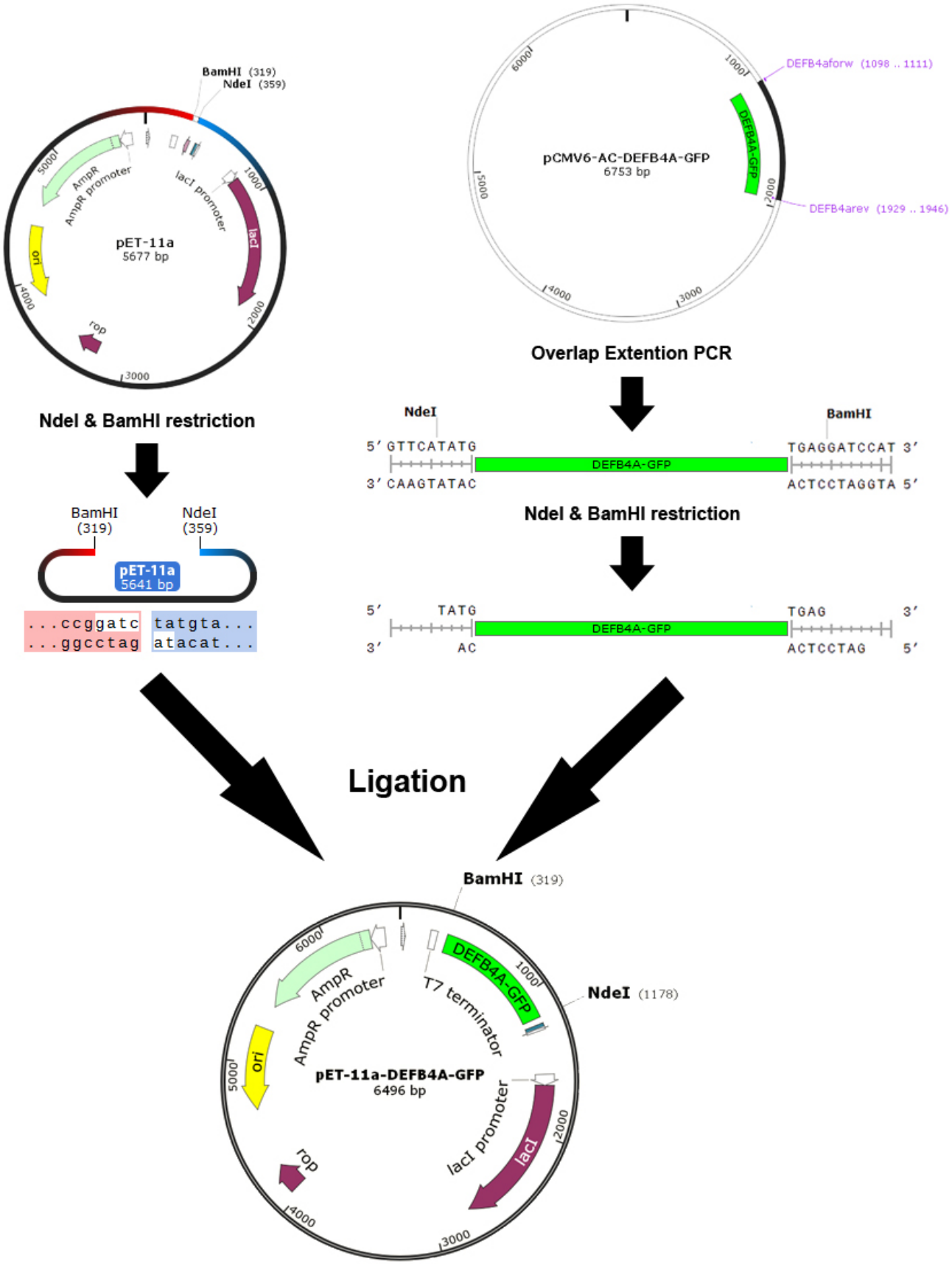

Figure 2.3. Subcloning of a heterologous gene of interest into pET-11a vector. This was how it was originally planned to subclone DEFB4A-GFP into the pET-11a vector. An OE-PCR is performed on the desired gene, GFP-DEFB4A, to add NdeI and BamHI restriction sites. The pET-11a vector and the OE-PCR product are digested with BamHI and NdeI to acquire overhangs (sticky ends) that match. The DEFB4A-GFP gene is inserted into the pET-11a vector by ligation. Parts of the figure was created with SnapGene software.

2.2.1 Partial digestion

The idea of a partial digestion is to first do a complete digestion of the gene using BamHI. This will make all the DEFB4A-GFP DNA strands have a BamHI overhang in the 3' (GFP-end). After this, the point is to make the NdeI restriction with as little enzyme as possible and in a short enough period of time, so that at least some of the DEFB4A-GFP is cut only in the end of the gene. This will create a pool of DEFB4A-GFP DNA strands: Some will remain intact in the NdeI restriction site (Figure 2.4A). Some will be completely digested, so that the first 43 bp of the gene are missing (Figure 2.4B). And some will be correctly digested, so that the gene is intact but with NdeI overhang at the 5' (DEFB4A-end) and BamHI overhang at the 3' (GFP-end) (Figure 2.4C).

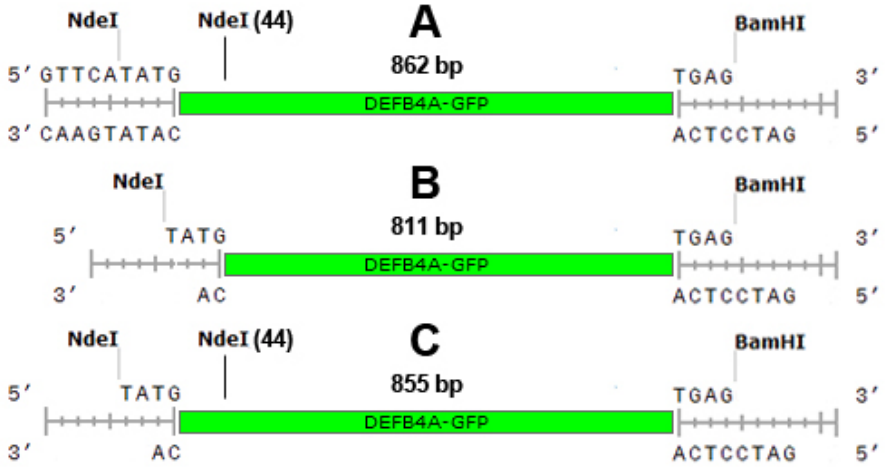

Figure 2.4. Overview of the DEFB4A-GFP pool resulted by partial digestion with NdeI after BamHI digestion. (A) The DEFB4A-GFP gene has not been cut by NdeI - a partial digestion is done in a very short period, so not all DNA strands will be restricted. (B) The DEFB4A-GFP gene has been cut in the on-gene NdeI restriction site, and the DEFB4A part of the fusion gene is no longer intact. This is expected to happen to at least 50 % of the restricted DNA. (C) The DEFB4A-GFP has been restricted correctly in only the NdeI restriction site in the beginning of the gene. This makes the entire gene complete and ready to be inserted into pET-11a. In an ideal partial digestion experiment, 50 % of the restricted DNA will be cut like this. Parts created with SnapGene software.

This pool of DNA strands will be ligated into pET-11a restricted with both NdeI and BamHI. After ligation, the plasmids will be transformed into *E. coli* NEB5α, and as many colonies as possible must be screened to find one that contains the pET-11a vector with a complete DEFB4A-GFP insert.

If the partial digestion is not successful, a point mutation to silence the on-gene NdeI site must instead be carried out by PCR site-directed mutagenesis.

2.2.2 PCR site-directed mutagenesis

To perform the PCR site-directed mutagenesis, six primers are needed (Figure 2.5): Two outer primers (pCMV6forw & pCMV6rev) that anneal 50-100 bp on each side of the gene. Two mutation primers (mutaforv & mutarev) that anneal to the on-gene NdeI site; these have one bp mismatch with the gene where the point mutation will be made (Figure 2.6). Finally, the two original primers (DEFB4Aforw & DEFB4Arev) that add the NdeI restriction site and start codon at the beginning of the gene and the BamHI restriction site at the end of the gene are needed.

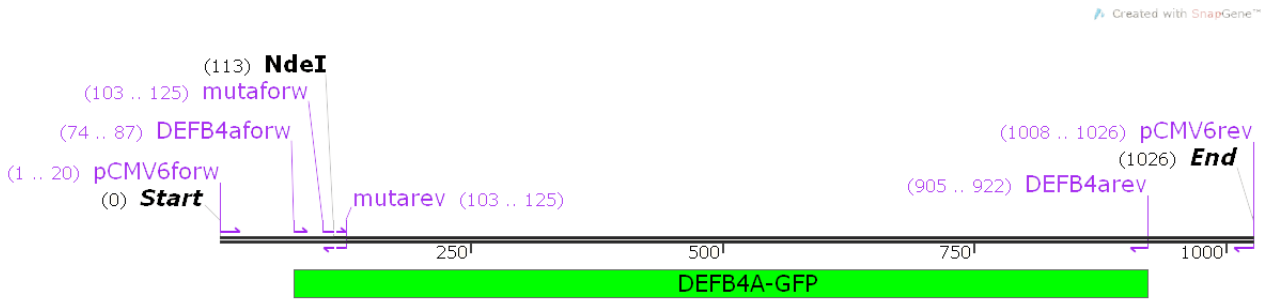

Figure 2.5. Primers that will be used in PCR site-directed mutagenesis of DEFB4A-GFP. pCMV6forw (20 bp) and pCMV6rev (19 bp) primers anneal 50-100 bp from on either side of the gene. mutaforv (23 bp) and mutarev (23 bp) primers anneal to the NdeI restriction sequence, where the mutation is desired. DEFB4Aforw (23 bp, 14 anneals) and DEFB4Arev (29 bp, 18 anneals) primers anneal on each side of the DEFB4A-GFP gene to add NdeI and BamHI restriction sites required for cloning into the pET-11a vector. Created with SnapGene software.

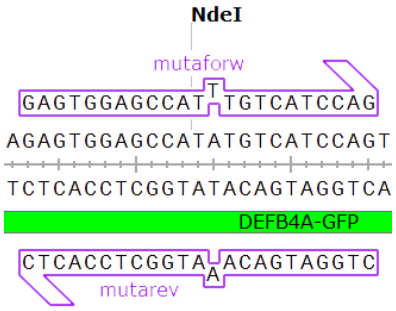

Figure 2.6. The primers used to make the point mutation. The NdeI restriction sequence is mutated from CATATG to CATTTG. The open reading frame (ORF) is such that the codon is mutated from ATA to ATT, both coding for Isoleucin (Ile), but ATA is a rare codon and ATT is not. Thus, the mutation also improves translation. Created with SnapGene software.

In the first round of PCRs, two reactions will be performed. The first will use pCMV6forw and mutarev primers to create a PCR product (mut1) of the first part of the DEFB4A-GFP gene with the mutation (Figure 2.7). The second will use mutaforw and pCMV6rev primers to create a PCR product (mut2) of the second part of the DEFB4A gene with the mutation (Figure 2.8).

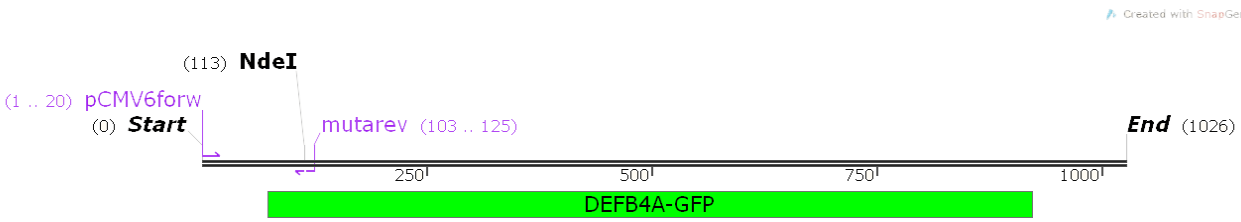

Figure 2.7. In the first PCR, pCMV6forw and mutarev primers are used. This creates a product (mut1) with part of the DEFB4A-GFP gene, now with a mutation on the NdeI restriction site. Created with SnapGene software.

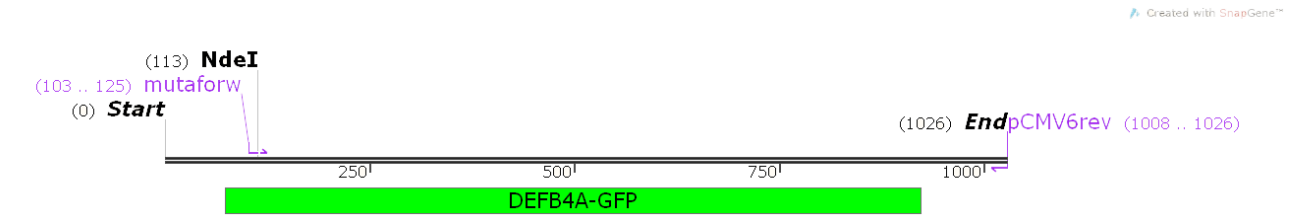

Figure 2.8. In the second PCR, mutaforw and pCMV6rev primers are used. This creates a product (mut2) with the other part of the DEFB4A-GFP gene, now with a mutation on the NdeI restriction site. Created with SnapGene software.

These two PCR products (mut1 and mut2) will be fused in a third PCR where no primers are added. mut1 and mut2 (Figure 2.9) will be added to equimolar concentrations, this PCR solution will be heated causing denaturation, and then cooled down to room temperature. This will make the 5'-3' strand (top strand) of mut1 anneal with the 3'-5' (bottom) strand of mut2 at the mutation site (Figure 2.10). Then, *Taq* Polymerase is added, and a normal PCR program is run.

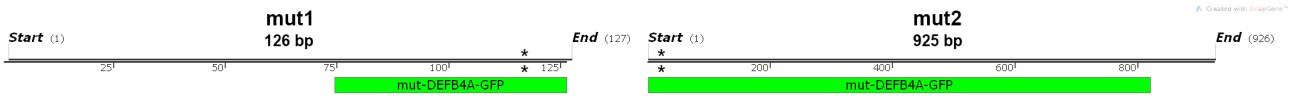

Figure 2.9. mut1 and mut2 products from the mutagenesis PCRs. The mutations are marked by (*). Created with SnapGene software.

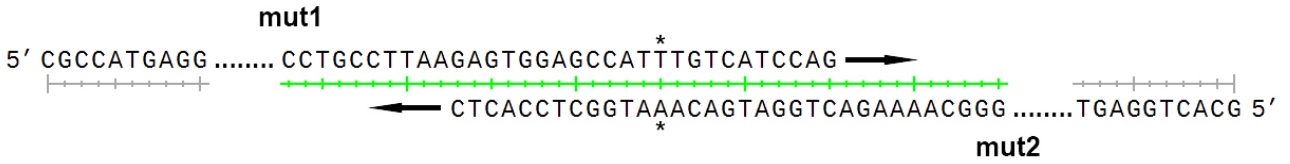

Figure 2.10. In the third PCR, mut1 and mut2 are mixed and denatured, and then cooled so that the mutation sequences anneal to eachother. This is followed by addition of *Taq* Polymerase and a regular PCR program. The mutations are marked by (*). Parts created with SnapGene software.

This will give a PCR product containing DEFB4A-GFP where the NdeI restriction site has been silenced, because of the muatation this gene will now be named mut-DEFB4A-GFP. Now, a final PCR will be run with the DEFB4Aforw and DEFB4arev primers to add the NdeI and BamHI restriction sites to the 5' and 3' ends of the mut-DEFB4A-GFP gene (Figure 2.11). This PCR product can then be cleaned by using a PCR Clean-Up Kit, and is then ready for BamHI and NdeI restriction followed by ligation into the pET-11a vector.

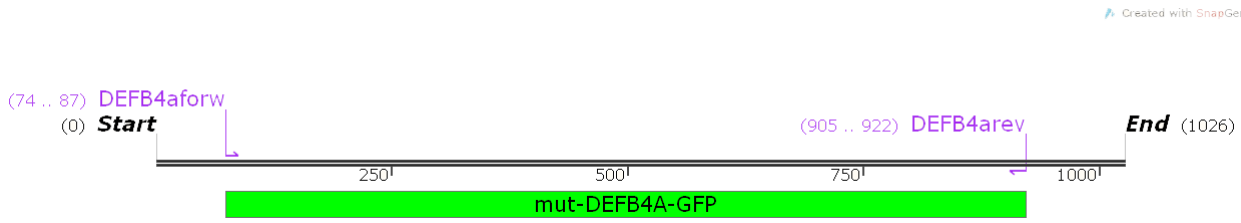

Figure 2.11. mut-DEFB4A-GFP is the product of the third PCR where mut1 and mut2 were fused. In the final step of preparing the gene for NdeI and BamHI digestion, these restriction sites are added by OE-PCR using DEFB4Aforw and DEFB4Arev primers on the mut-DEFB4A-GFP gene. Created with SnapGene software.

Materials and methods 3

3.1 Overview

As explained in section 2.2, a PCR site-directed mutagenesis would be carried out given that a partial digestion was not successful. Unfortunately, this was the case in this master's thesis even though a lot of effort was put into performing this partial digestion. To give an understanding of how the partial digestion was attempted, the method will be described in this chapter (Section 3.5). This will also provide a good base for the discussion.

In general, a lot of laboratory procedures were performed in the experimental work in this master's thesis. A flowchart diagram has been created to provide an overview of the process of these experimental steps (Figure 3.1 on the next page).

Figure 3.1. Flowchart describing the eximental process of this master's thesis. The procedures to the left were used for partial digestion. The procedures to the right were used for PCR site-directed mutagenesis. Key: Green colour indicates work with DEFB4A-GFP DNA. Blue colour indicates work with pET-11a DNA. Red colour indicates work with pET-11a-DEFB4A-GFP DNA. Yellow colour indicates work with HbD-2-turboGFP fusion protein.

3.2 Chemicals & biologicals

Table 3.1. Chemicals

Chemical	Description	Manufacturer	Identifier
2-Propanol	-	Sigma-Aldrich	Lot#8195S
Acetic acid (AcOH)	Glacial (100 %)	VWR	CAS#64-19-7
Acrylamide/Bis Solution	30 %	Bio-Rad	Cat#161-0156
Agar	-	Sigma-Aldrich	Lot#BCBC2317
Agarose	-	Sigma-Aldrich	Lot#100M9432
Ammonium Persulfate (APS)	-	Bio-Rad	Cat#161-0700
Ampicillin sodium salt	100 mg/ml prepared solutions	Sigma-Aldrich	Lot#BCBB6153
Coomassie Brilliant Blue	PhastGel Blue-R350	Amersham Pharmacia	Lot#0289363
Ethanol (EtOH)	96 %	Kemetyl	PR#1680643
Ethidium bromide (EtBr) solution	1 %	Sigma-Aldrich	CAS#7732-18-5
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	-	Sigma-Aldrich	Lot#BCBC5034V
Fermtech Yeast Extract	-	Merck	Cat#1.11926.1000
Glycine	-	Sigma-Aldrich	Lot#109K01072V
Hydrochloric acid (HCl)	-	Sigma-Aldrich	CAS#7647-01-0
Isopropyl-β-D-thiogalactopyranoside (IPTG)	-	VWR	CAS#367-93-1
Super optimal broth with catabolite repression (SOC) Medium	-	New England Biolabs	Lot#2001112
Sodium acetate (NaOAc)	3 M pH 5.2	Sigma-Aldrich	CAS#126-96-5
Sodium chloride (NaCl)	-	Sigma-Aldrich	CAS#7647-14-5
Sodium dodecyl sulfate (SDS)	for molecular biology, approx. 99 %	Sigma-Aldrich	Batch#078K0102
Sodium dodecyl sulfate (SDS) solution	10 %	Sigma-Aldrich	CAS#151-21-3
Sodium hydroxide (NaOH)	-	Sigma-Aldrich	CAS#1310-73-2
Sucrose	99+ %	Sigma-Aldrich	CAS#57-50-1
Tetramethylethylenediamine (TEMED)	for electroporesis, approx. 99 %	Sigma-Aldrich	CAS#110-18-9
Trizma base (tris base)	-	Sigma-Aldrich	Lot#110M54391V
Tryptone BioChemica	Peptone from casein	AppliChem GmbH	Lot#0F007962
Water, Molecular Biology Reagent	DNase, RNase, Protease, free	Sigma-Aldrich	Lot#RNBC3293

Table 3.2. Biologicals

Biological	Description	Manufacturer	Identifier
Buffers			
10x Buffer BamHI	with BSA	Fermentas	Lot#00011792
10x Buffer O	with BSA	Fermentas	Lot#00024568
10x Buffer Tango^{TM}	with BSA	Fermentas	Lot#00033251
10x DreamTaq^{TM} Buffer	Includes 20 mM $MgCl_2$	Fermentas	Lot#00058293
10x Ligation Buffer for T4 DNA Ligase	-	Fermentas	Lot#00023402
DNA & protein standards			
Low molecular weight (LMW) standard	-	Amersham	Cat#17-0446-01
Generuler^{TM} 1 kb DNA Ladder	-	Fermentas	Lot#00065513
Generuler^{TM} 100 bp Plus DNA Ladder	Ready-to-Use	Fermentas	Lot#00073767
Generuler^{TM} 50 bp DNA Ladder	-	Fermentas	Lot#00028112
Enzymes			
BamHI	-	Fermentas	Lot#00071399
DreamTaq^{TM} DNA Polymerase	-	Fermentas	Lot#00082460
NdeI	-	Fermentas	Lot#00092125
T4 DNA Ligase	-	Fermentas	Lot#00029582
Microbial strains			
Escherichia coli NEB 5-alpha F' I^q Competent E. coli	-	New England Biolabs	Cat#C2992H
Escherichia coli Origami^{TM} 2(DE3) Singles^{TM} Competent Cells	-	Merck	Lot#D00126639
Plasmids			
pCMV6-AC-DEFB4A-GFP	Human cDNA ORF Clone	Origene	Cat#RG219487
pET-11a	-	Merck	Cat#69436
Primers			
Pet11a2	5'-GATGTCGGCGATATAGG-3'	Taq Copenhagen	-
GFP8pet	5'-GGGATATCCGGATATAGTTCCTCC-3'	Taq Copenhagen	-
DEFB4aforw	5'-GTT CATATG GGTATAGGCGATCC-3'	Taq Copenhagen	-
DEFB4arev	5'-AT GGATCC TCA TTAAACTCTTTCTTCACC-3'	Taq Copenhagen	-
pCMV6forw	5'-CGCCATGAGGGTCTTGTATC-3'	Taq Copenhagen	-
pCMV6rev	5'-GCACTGGAGTGGCAACTTC-3'	Taq Copenhagen	-
mutation-forw	5'-GAGTGGAGCCATTTGTCATCCAG-3'	Taq Copenhagen	-
mutation-rev	5'-CTGGATGACAAATGGCTCCACTC-3'	Taq Copenhagen	-
Other			
2x Laemmli Sample Buffer	premixed protein sample buffer for SDS-PAGE	Bio-Rad	Cat#161-0737
6x DNA Loading Dye	-	Fermentas	Lot#00071285
dATP	100 mM	Fermentas	Lot#00089642
dCTP	100 mM	Fermentas	Lot#00089634
dGTP	100 mM	Fermentas	Lot#00088329
dTTP	100 mM	Fermentas	Lot#00087995

The pET-11a vector was provided by supervisor Evamaria Petersen with a cutinase insert. It will be referred to as pET-11a-cutinase.

pCMV6-AC-DEFB4A-GFP plasmid map and sequence can be found in Appendix C. pET-11a-cutinase plasmid map and sequence can be found in Appendix D.

3.2.1 Buffers

TAE buffer

50X tris-acetate-EDTA (TAE) buffer was prepared by adding 242 g tris base, 57.1 ml glacial acetic acid, and 18.6 g EDTA to 900 ml Milli-Q water. This solution was mixed with a magnetic stirrer until the chemicals were fully dissolved, and the volume was adjusted to 1000 ml with Milli-Q water for final concentrations of 2 M tris base, 1 M acetic acid, and 50 mM EDTA.

TES buffer

Tris-EDTA-sucrose (TES) buffer (pH 7.5) was prepared by adding tris base, EDTA, and sucrose to Milli-Q water for final concentrations of 50 mM tris, 10 mM EDTA, and 20 % sucrose. This solution was autoclaved at 121 °C for 30 min at 1 bar and stored at 4 °C.

Tris buffer

1.5 M Tris buffer (pH 8.8) was prepared by dissolving 60.6 g tris base in 900 ml Milli-Q water and adjusting the volume to 1000 ml. The pH was adjusted to 8.8 using HCl.

0.5 M Tris buffer (pH 6.8) was prepared by dissolving 181.7 g tris base in 900 ml Milli-Q water and adjusting the volume to 1000 ml. The pH was adjusted to 6.8 using HCl.

SDS-PAGE running buffer

10X sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer was prepared by adding 30.2 g tris base, 144 g glycine, and 10 g SDS to 900 ml Milli-Q water. This solution was mixed with a magnetic stirrer until the chemicals were fully dissolved, and the volume was adjusted to 1000 ml with Milli-Q water for final concentrations of 250 mM tris base, 1.92 M glycine, and 1 % SDS.

3.2.2 LB medium

1000 ml Lysogeny broth (LB) medium was prepared by adding 10 g Tryptone BioChemica, 5 g Fermtech Yeast Extract, and 10 g NaCl to 900 ml Milli-Q water and adjusting the pH to 7.0 using 1 M NaOH. The volume was increased to 1000 ml using Milli-Q water, and the solution was autoclaved at 121 °C for 30 min at 1 bar and stored at 4 °C.

LB Agar Ampicillin-100 plates

Selective agar plates containing 100 μg/ml ampicillin were prepared by adding 15 g/l agar to LB medium prior to autoclavation. This solution was autoclaved at 121 °C for 30 min at 1 bar and left to cool to 50 °C, and ampicillin was added for a final concentration of 100 μg/ml. The solution was gently mixed by swirling and poured into petri dishes. The agar plates were left to solidify at room temperature and stored upside down at 4 °C.

3.3 General procedures

3.3.1 1 % Agarose gel

1 % agarose gels were prepared by adding 1 g/l agarose to 1X TAE buffer and heating in a microwave until all agarose was dissolved. This solution was kept liquid by storage at 60 °C. To cast a gel, 0.5 μl EtBr was added to 30 ml solution, and this was left to solidify at room temperature in a UV-transparent gel tray with a fixed height comb (Bio-Rad, Hercules, CA, USA). The solid gel in tray was submerged into a Mini-SUB Cell GT tank (Bio-Rad, Hercules, CA, USA). An electrical field of 70 V was applied for 90 min using a Power Pac 300 power supply (Bio-Rad, Hercules, CA, USA). The gel was investigated by placing it over a Gel Doc 1000 UV lamp in a camera box (Bio-Rad, Hercules, CA, USA). Generuler™ 1 kb DNA Ladder, Generuler™ 100 bp Plus DNA Ladder, and Generuler™ 50 bp DNA Ladder were used as DNA standards (Appendix A).

3.3.2 Ethanol precipitation

To purify DNA from solution, ethanol precipitations were performed:

To precipitate the DNA, 1/10th the volume 3M NaOAc (pH 5.2) was added and the solution was mixed by gentle inversion. Then, 2.5x the volume 96 % EtOH was added, the solution was mixed by inversion. This was incubated for 40 min at -20 °C, and centrifuged at 20.000 x g for 40 min at 4 °C in a 5417 R centrifuge using an FA45-30-11 rotor (Eppendorf, Hamburg, Germany); this centrifuge and rotor will be used for the rest of this protocol and centrifugations will be carried out in soft mode. The supernatant was decanted, and 300 μl 70 % EtOH was added to wash the DNA pellet. This was centrifuged at 20.000 x g for 15 min at 4 °C, and the supernatant was removed. Finally, the pellet was air-dried for 5-10 min and 20 μl water, molecular biology reagent, was added. The purified DNA was stored at 4 °C.

3.4 Cloning and isolation of pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase plasmids in *Escherichia coli* NEB5α

3.4.1 Transformation of *Escherichia coli* NEB5α with pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase

A tube containing 50 μl competent *E. coli* NEB4α was thawed on ice for 30 min. 1 μl pCMV6-AC-DEFB4A-GFP or pET-11a-cutinase plasmid DNA with a concentration of 100 ng/μl was added to the thawed cells. This was gently mixed by tapping the tube, and incubated on ice for 30 min. The mixture was heat shocked for 45 s at 42 °C in an RM6 Lauda water bath (LAUDA, Lauda-Königshofen, Germany). 500 μl 37 °C LB medium was gently added, and the cells were incubated for 1 hour at 37 °C, 230 RPM. Two aliquots of 10 μl was pipetted and evenly distributed using a drigalski spatula on two selective agar plates containing 100 μg/ml ampicillin preheated to 37 °C. The plates were incubated overnight at 37 °C and stored at 4 °C. To make a plate with a lot of identical clones, one colony was streaked on a new preheated selective agar plate using a plastic inoculation loop and incubated overnight at 37 °C. This plate was stored at 4 °C.

3.4.2 pCMV6-AC-DEFB4A-GFP plasmid isolation using GenElute™ Plasmid Miniprep Kit

pCMV6-AC-DEFB4A-GFP plasmid DNA was isolated from *E. coli* NEB5α using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) [Sigma-Aldrich, 2010]:

Cells from the agar plate with *E. coli* NEB5α containing pCMV6-AC-DEFB4A-GFP plasmid was innoculated in 5 ml LB medium containing 100 μg/ml ampicillin in a 12 ml sterile round bottom PP-tube. This was incubated overnight at 37 °C, 230 RPM. The bacterial growth was transferred to a 15 ml Greiner centrifuge tube, and the cells were harvested by centrifugation at 12,000 x g for 1 min at 4 °C in a 5804 R centrifuge using an F-34-6-38 rotor (Eppendorf, Hamburg, Germany).

The supernatant was removed, and the pellet was resuspended with 200 μl Resuspension Solution by pipetting up and down. The mixture was moved to a 1.5 ml Eppendorf tube. The resuspended cells were lysed by adding 200 μl Lysis Solution and gently inverting the tube until the mixture became clear, followed by incubation at room temperature for 5 min. The cell debris was precipitated by addition of 350 μl Neutralization Solution and gently inverting the tube 5 times. The cell debris, lipids, proteins, SDS, and chromosomal DNA were pelleted by centrifugation at 12,000 x g for 10 min in a MiniSpin plus centrifuge using an F-45-12-11 rotor (Eppendorf, Hamburg, Germany); this centrifuge and rotor will be used for the rest of this protocol. A GenE-

lute Miniprep Binding Column was inserted into a 2 ml microcentrifuge tube, and 500 µl Column Preparation Solution was added. This was centrifuged at 12,000 x g for 1 min, and the flow-through liquid was removed. The cleared lysate from the aforementioned centrifugation was added to the column and centrifuged at 12,000 x g for 1 min, the flow-through liquid was removed. To remove salts and other contaminants from the column, 750 µl Wash Solution was added to the column and centrifuged at 12,000 x g for 1 min. The flow-through liquid was removed, and the tube was centrifuged again at 12,000 x g for 2 min to completely remove the Wash Solution containing ethanol. Finally, the column was moved to a clean 2 ml microcentrifuge tube, and the plasmid DNA was eluted by adding 50 µl water, molecular biology reagent, followed by incubation for 1 min at room temperature and centrifugation at 12,000 x g for 1 min. The purified pCMV6-AC-DEFB4A-GFP plasmid DNA was stored at 4 °C. The concentration of purified pCMV6-AC-DEFB4A-GFP plasmid DNA was determined on a 1 % agarose gel.

3.4.3 pET-11a-cutinase plasmid isolation using QIAGEN Plasmid Midiprep Kit

pET-11a plasmid DNA with cutinase insert was isolated from *E. coli* NEB5α using QIAGEN Plasmid Midi Kit (QIAGEN, Sollentuna, Sweden) [QIAGEN, 2005]:

A starter culture was prepared by innoculating cells from the agar plate with *E. coli* NEB5α containing pET-11a-cutinase plasmid in 5 ml LB medium with 100 µg/ml ampicillin in a 12 ml sterile round bottom PP-tube. This was incubated for 8 hours at 37 °C, 230 RPM. 200 µl of the starter culture was transferred to 100 ml LB medium containing 100 µg/ml ampicillin in a 500 ml conical flask. This was incubated for 16 hours at 37 °C, 230 RPM. The bacterial growth was transferred to a large centrifugation tube, and the cells were harvested by centrifugation at 6010 x g for 15 min at 4 °C in a Sorvall RC 5C Plus Superspeed centrifuge using a Sorvall SLA-1500 rotor (Thermo Scientific, Hvidovre, Denmark); this centrifuge will be used for the rest of the protocol, but with a different rotor where noted.

The supernatant was removed, and the pellet was resuspended in 4 ml Buffer P1 containing LyseBlue particles by pipetting up and down. The resuspended cells were lysed by adding 4 ml Buffer P2 and mixed by gentle inversion until the cell suspension was homogeneously coloured blue. This was incubated at room temperature for 5 min. Genomic DNA, proteins, cell debris, and potassium dodecyl sulfate (KDS) was precipitated by addition of 4 ml chilled Buffer P3 and mixing by gentle inversion until the suspension was colourless. This mixture was incubated on ice for 15 min and then centrifuged at 20,020 x g for 30 min at 4 °C to pellet the precipitated material. The supernatant was decanted into a smaller round bottom centriguation tube and centrifuged at 20,020 x g for 15 min at 4 °C using an SS-34 rotor. A QIAGEN-tip 100 was equilibrated by adding 4 ml Buffer QBT and letting the column empty by gravity flow. The cleared lysate from the aforementioned centrifugation was added to the column and allowed to empty by gravity flow. To remove salts and other contaminants from the column, 2 x 10 ml Buffer QC was added and allowed to empty by gravity flow. The DNA was eluted by addition of 5 ml Buffer QF; this eluate was collected in a 15 ml Greiner centrifuge tube.

Finally, the plasmid DNA was further purified and concentrated by an isopropanol precipitation:
The DNA was precipitated by addition of 3.5 ml isopropanol, mixing, and centrifugation at 15,009 x g for 30 min at 4 °C. The supernatant was carefully decanted, and the DNA pellet was washed with 2 ml 70 % EtOH. This was centrifugated at 15,009 x g for 10 min at 4 °C, and the supernatant was carefully removed. The pellet was air-dried for 5-10 min and redissolved in 100 µl water, molecular biology reagent. The purified pET-11a-cutinase plasmid DNA was stored at 4 °C. The concentration of purified pET-11a-cutinase plasmid DNA was determined on a 1 % agarose gel.

3.5 Construction of pET-11a-DEFB4A-GFP with partial digestion

3.5.1 Polymerase chain reaction

OE-PCR was performed to amplify and add NdeI and BamHI restriction sequences to DEFB4A-GFP. The reagents (Table 3.3) were mixed on ice by pipetting up and down, and PCR program (Table 3.4) was run in a Mastercycler gradient PCR apparatus (Eppendorf, Hamburg, Germany); annealing temperature was set to 5 °C lower than the lowest primer melting temperature. Four PCRs were performed to obtain sufficient DEFB4A-GFP product for further experiments. The PCR product was examined on a 1 % agarose gel.

PCR was also performed with pET-11a-cutinase plasmid DNA isolated with QIAGEN Plasmid Midi Kit for verification that pET-11a-cutinase had been cloned. The reagents (Table 3.3) were mixed on ice by pipetting up and down, and PCR program (Table 3.5) was run in a Mastercycler gradient PCR apparatus. The PCR product was examined on a 1 % agarose gel.

Table 3.3. Polymerase chain reaction recipe.

Reagent	Volume
10X DreamTaq Buffer	10 μL
dNTP Mix, 2 mM each	10 μL
Forward primer (0.1 mM)	1 μL
Reverse primer (0.1 mM)	1 μL
Template DNA ($\sim$10 ng/μL)	1 μL
DreamTaq^{TM} DNA Polymerase	0.5 μL (1.25 u)
Water, nuclease-free (to 100 μL)	76.5 μL
Total	100 μL

Table 3.4. Polymerase chain reaction program for amplification of DEFB4A-GFP gene.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	30 sec	
Annealing	51 °C	30 sec	35
Extension	72 °C	1 min	
Final extension	72 °C	15 min	1
Storage	4 °C	–	1

Table 3.5. Polymerase chain reaction program for amplification of polylinker part of pET-11a vector.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	30 sec	
Annealing	52 °C	30 sec	35
Extension	72 °C	1 min	
Final extension	72 °C	15 min	1
Storage	4 °C	–	1

3.5.2 Clean-up of PCR product

PCR amplification product was purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) [Sigma-Aldrich, 2001]:

A GenElute plasmid mini spin column was inserted into a 2 ml microcentrifuge tube, and 0.5 ml Column Preparation Solution was added. This was centrifuged at 12,000 x g for 1 min using a MiniSpin plus centrifuge with an F-45-12-11 rotor; this centrifuge and rotor will be used for the rest of this protocol. The eluate was removed. 500 μl Binding Solution was added to 100 μl PCR product and mixed by inversion. This mixture was transferred to the column, and centrifuged at 12,000 x g for 1 min. The eluate was discarded, 0.5 ml Wash Solution was added, and the tube was centrifuged at 12,000 x g for 1 min. The eluate was discarded, and another centrifugation was performed at 12,000 x g for 2 min to remove excess Wash Solution. The column was moved to a new 2 ml microcentrifuge tube, and the DNA was eluted by adding 50 μl water, molecular biology reagent, incubation at room temperature for 1 min, and centrifugation at 12,000 x g for 1 min.

3.5.3 Digestion of DEFB4A-GFP with BamHI

DEFB4A-GFP DNA amplified with OE-PCR and purified with GenElute™ PCR Clean-Up Kit was digested with BamHI restriction enzyme to prepare it for partial digestion with NdeI. The reagents (Table 3.6) were mixed on ice by pipetting up and down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 5 hours at 37 °C. After finished digestion, an ethanol precipitation (Section 3.3.2) was performed to purify the BamHI digested DEFB4A-GFP DNA. The concentration of BamHI digested DEFB4A-GFP was measured on a 1 % agarose gel.

Table 3.6. Recipe for BamHI digestion of DEFB4A-GFP.

Reagent	Volume
10x Buffer BamHI	20 μL
DEFB4A-GFP plasmid DNA ($\sim$40 ng/μl)	100 μL
BamHI	4 μL
Water, molecular biology reagent (to 200 μL)	76 μL
Total	200 μL

3.5.4 Partial digestion of DEFB4A-GFP with NdeI

To examine the amount of units NdeI required for the partial digestion, a dilution series with 7 different NdeI concentrations was made. Each reaction tube (A-G) contained 0.1 μl BamHI digested DEFB4A-GFP, 1 μl Buffer O, NdeI (Table 3.7), and water, molecular biology reagent, to 10 μl. All samples were prepared on ice, and the digestion was performed in a Mastercycler gradient PCR apparatus to ensure accurate time and temperature control. The partial digestion was performed for 10 min at 37 °C followed by inactivation of the enzyme for 20 min at 65 °C. A 1 % agarose gel was run to examine what concentration of NdeI that was suitable for partial digestion.

Table 3.7. Units of NdeI in the 7 reaction tubes made to examine the amount of NdeI needed for partial digestion.

Tube	NdeI
A	6.6667 u
B	2.2222 u
C	0.7407 u
D	0.2469 u
E	0.0823 u
F	0.0274 u
G	0.0091 u
Total	10 u

After this test, a scaled up NdeI partial digestion was performed using the most suitable amount of NdeI. The upscaling of the experiment was 30x, which meant that the most suitable concentration of NdeI determined by the dilution row was multiplied by 30, and 3 μl DNA was used instead of 0.1 μl. The reagents (Table 3.8) were prepared on ice and the digestion was performed in a Mastercycler gradient PCR apparatus to ensure accurate time and temperature control. Different concentrations of NdeI (1.5-5 u) were probed. The partial digestion was performed for 10 min at 37 °C followed by inactivation of the enzyme for 20 min at 65 °C. After inactivation, the digested DNA was purified by ethanol precipitation (Section 3.3.2).

Table 3.8.

Reagent	Volume
10x Buffer O	3 μl
DNA	3 μl
NdeI	1.5-5 u
Water, molecular biology reagent (to 30 μl)	24 μl
Total	30 μl

3.5.5 NdeI and BamHI double digestion of pET-11a

pET-11a-cutinase plasmid isolated with QIAGEN Plasmid Midi Kit was double digested with NdeI and BamHI restriction enzymes to remove the cutinase insert, and make NdeI and BamHI overhangs for insertion of DEFB4A-GFP gene. The reagents (Table 3.9) were mixed on ice by pipetting up and down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 4 hours at 37 °C. Two of these restriction reactions were performed to obtain a sufficient amount of digested pET-11a DNA for ligation.

Table 3.9. NdeI and BamHI digestion of pET-11a recipe.

Reagent	Volume
10x Tango Buffer	16 μL (2x)
pET-11a-cutinase plasmid DNA ($\sim$200 ng/μl)	3 μL
NdeI	2 μL
BamHI	2 μL
Water, molecular biology reagent (to 80 μL)	57 μL
Total	80 μL

3.5.6 Isolation of digested pET-11a using Millipore Ultrafree-DA DNA Extraction From Agarose Gels kit

To isolate the digested pET-11a vector, a preparative 1 % agarose gel was made. 16 μl 6x Loading Dye was added to each restriction reaction and mixed by pipetting up and down. Each mix was loaded into a well on the preparative agarose gel, 3 μl 1 kb DNA Ladder was used as a DNA standard, and the gel electrophoresis was run for 90 min. The gel was studied using preparative UV light, wearing a protective UV face shield, and the gel bands corresponding to the size of linearised pET-11a were sliced out using a scalpel. The slices were chopped into smaller pieces, packed in parafilm, and incubated overnight at -20 °C. To extract the DNA from the gel pieces, Millipore Ultrafree-DA DNA Extraction From Agarose Gel kit (Merck Millipore, Billerica, MA, USA) was used [Millipore, 2001]:

The gel pieces were added to the Gel Nebulizer, which acts a funnel to the Ultrafree-MC filter; both are pre-assembled into a 1.5 ml centrifugation tube. A centrifugation at 5000 x g for 10 min in a MiniSpin plus centrifuge with an F-45-12-11 rotor was performed; this centrifuge and rotor will be used for the rest of this protocol. 100 μl water, molecular biology reagent, was added and quickly centrifuged through the funnel for 2 s to soak the gel for 1 min at room temperature. A final centrifugation at 5000 x g for 30 min was performed. After finished protocol, the digested pET-11a was purified from EtBr, salts, and other impurities by an ethanol precipitation (Section 3.3.2). A 1 % agarose gel was run to check the concentration and check the sample for background, i.e. undigested pET-11a-cutinase plasmid.

3.5.7 Ligation of partially digested DEFB4A-GFP and pET-11a

A ligation reaction was performed to ligate the DEFB4A-GFP gene and the pET-11a vector; both carrying NdeI and BamHI sticky-ends:

The reagents (Table 3.10) were mixed by slowly pipetting up and down on ice, and incubated overnight in a fridge door shelf (8-9 °C). The ligation was finished by incubation for 10 min at room temperature.

Table 3.10. Recipe for ligation of partially digested DEFB4A-GFP and pET-11a.

Reagent	Volume
10x T4 DNA Ligase Buffer O	2 μl
DEFB4A-GFP	6 μl
pET-11a	11 μl
T4 DNA Ligase	1 μl
Total	20 μl

3.5.8 Transformation of *Escherichia coli* NEB5α with pET-11a-DEFB4A-GFP

The ligation mix was transformed into *E. coli* NEB5α using an improved transformation protocol:

A tube containing 50 μl competent *E. coli* NEB4α was thawed on ice for 30 min. 20 μl ligation mix was added to the thawed cells. This was gently mixed by tapping the tube, and incubated on ice for 30 min. The mixture was heat shocked for 45 s at 42 °C in a RM6 Lauda water bath, and then put back on ice for 10 min. 500 μl 37 °C SOC medium was gently added, and the cells were incubated for 1 hour at 37 °C, 230 RPM. 50 μl was pipetted and evenly distributed using a drigalski spatula on a selective agar plate containing 100 μg/ml

ampicillin preheated to 37 °C. The remaining 520 μl was distributed on two additional selective agar plates. The plates were incubated overnight at 37 °C and stored at 4 °C.

3.5.9 Verification of construct

To examine if the ligation procedure had been successful, 14 colonies were plated seperately on selective agar plates and incubated overnight at 37 °C. 14 GenElute™ Plasmid Miniprep Kit procedures (Section 3.4.2) were performed on these cells. Subsequently, PCR was performed using the isolated DNA and primers annealing to the NdeI site of the DEFB4A-GFP gene (DEFB4Aforw primer) and pET-11a at the BamHI site (GFP8pet primer). A 15-well 1 % agarose gel was run with these PCR products to determine if any of the isolated DNA was pET-11a with correctly digested DEFB4A-GFP insert.

3.6 Construction of pET-11a-mut-DEFB4A-GFP with PCR site-directed mutagenesis

3.6.1 Creation of point mutation

The nomenclature used to describe the experimental design of the PCR site-directed mutagenesis (Section 2.2.2), will also be used here.

mut1 PCR product was created with pCMV6forw and mutarev primers. The reagents (Table 3.11) were mixed on ice by pipetting up and down, and PCR program (Table 3.12) was run in a Mastercycler gradient PCR apparatus; annealing temperatures were generally set to 5 °C lower than the lowest primer melting temperature.

mut2 PCR product was created with mutaforw and pCMV6rev primers. The reagents (Table 3.11) were mixed on ice by pipetting up and down, and PCR program (Table 3.12) was run in a Mastercycler gradient PCR apparatus.

Table 3.11. Polymerase chain reaction recipe for site-directed mutagenesis - production of mut1 and mut2.

Reagent	Volume
10X DreamTaq Buffer	10 μl
dNTP Mix, 2 mM each	10 μl
Forward primer (0.1 mM)	1 μl
Reverse primer (0.1 mM)	1 μl
pCMV6-AC-DEFB4A-GFP plasmid DNA ($\sim$10 ng/μl)	1 μl
DreamTaq™ DNA Polymerase	0.5 μl (1.25 u)
Water, molecular biology reagent (to 100 μl)	76.5 μl
Total	100 μl

Table 3.12. Polymerase chain reaction protocol for site-directed mutagenesis - production of mut1 and mut2.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	30 sec	
Annealing	53 °C	30 sec	35
Extension	72 °C	1 min	
Final extension	72 °C	15 min	1
Storage	4 °C	–	1

mut1 and mut2 PCR products were examined and their concentrations were determined on a 1 % agarose gel. mut1 and mut2 purified with GenElute™ PCR Clean-Up Kit (Section 3.5.2) were fused in a PCR with no primers, where mut1 and mut2 were added to equimolar concentrations. Three PCRs were run: The first with 3 μl mut1 and 15 μl mut2. The second with 4 μl mut1 and 14 μl mut2. The third with 5 μl mut1 and 13 μl mut2. The reagents (Table 3.13) were mixed on ice by pipetting up and down, and PCR program (Table 3.14) was run in a Mastercycler gradient PCR apparatus. These three fusion PCRs yielded mut-DEFB4-GFP with no BamHI and NdeI restriction sites as a product.

Table 3.13. Polymerase chain reaction recipe for fusion of mut1 and mut2 sequences. Taq polymerase was added after initial denaturation and cooling down to room temperature.

Reagent	Volume
10X DreamTaq Buffer	5 μl
dNTP Mix, 2 mM each	5 μl
mut1	3 - 5 μl
mut2	13 - 15 μl
DreamTaq™ DNA Polymerase	0.5 μl (1.25 u)
Water, molecular biology reagent (to 50 μl)	21.5 μl
Total	50 μl

Table 3.14. Polymerase chain reaction protocol for fusion of mut1 and mut2 sequences. Taq polymerase was added after initial denaturation and cooling to 20 °C.

Step	Temperature	Time	Cycles
Initial denaturation	99 °C	5 min	1
Cooling	20 °C	1 min	1
Addition of 0.5 μl Taq Polymerase	-	-	
Denaturation	96 °C	1 min	
Annealing	50 °C	1 sec	30
Extension	72 °C	3 min	
Final extension	72 °C	10 min	1
Storage	4 °C	–	1

The products of the three mut1 and mut2 fusion PCRs were examined on a 1 % agarose gel. PCRs were run to amplify and add NdeI and BamHI overhangs to the fusion products - all three fusion products were amplified using 0.5, 1, and 10 μl template DNA, resulting in 9 PCRs. The reagents (Table 3.15) were mixed on ice by

pipetting up and down, and PCR program (Table 3.16) was run in a Mastercycler gradient PCR apparatus. The products were examined on 1 % agarose gels. The PCR product with the best mut-DEFB4A-GFP yield, was purified with GenElute™ PCR Clean-Up Kit (Section 3.5.2) and stored at 4 °C. A 1 % agarose was run to examine the concentration and purity of the purified DNA.

Table 3.15. Polymerase chain reaction recipe for amplification of mut-DEFB4A-GFP gene.

Reagent	Volume
10X DreamTaq Buffer	10 μl
dNTP Mix, 2 mM each	10 μl
Forward primer (0.1 mM)	1 μl
Reverse primer (0.1 mM)	1 μl
Template DNA	0.5 - 10 μl
DreamTaq™ DNA Polymerase	0.5 μl (1.25 u)
Water, molecular biology reagent (to 100 μl)	67.5 - 77 μl
Total	100 μl

Table 3.16. Polymerase chain reaction protocol for amplification of mut-DEFB4A-GFP gene.

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	2 min	1
Denaturation	95 °C	30 sec	
Annealing	51 °C	30 sec	35
Extension	72 °C	1 min	
Final extension	72 °C	15 min	1
Storage	4 °C	–	1

3.6.2 NdeI and BamHI sequential digestion of mut-DEFB4A-GFP

Purified mut-DEFB4A-GFP DNA was sequentially digested with NdeI and BamHI restriction enzymes to make NdeI and BamHI overhangs for ligation into pET-11a. mut-DEFB4A-GFP DNA was first digested with NdeI restriction enzyme: The reagents (Table 3.17) were mixed on ice by pipetting up and down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 5 hours at 37 °C.

Table 3.17. Recipe for NdeI digestion of mut-DEFB4A-GFP.

Reagent	Volume
10x Buffer O	8 μL (2x)
mut-DEFB4A-GFP ($\sim$40 ng/μl)	40 μL
NdeI	4 μL
Water, molecular biology reagent (to 80 μL)	28 μL
Total	80 μL

After ended NdeI digestion, an ethanol precipitation (Section 3.3.2) was performed. This was followed by a digestion with BamHI restriction enzyme: The reagents (Table 3.18) were mixed on ice by pipetting up and

down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 5 hours at 37 °C.

Table 3.18. Recipe for BamHI digestion of mut-DEFB4A-GFP.

Reagent	Volume
10x Buffer BamHI	8 μL (2x)
NdeI digested mut-DEFB4A-GFP	20 μL
BamHI	4 μL
Water, molecular biology reagent (to 80 μL)	48 μL
Total	80 μL

After BamHI digestion, ligation-ready mut-DEFB4A-GFP was isolated by ethanol precipitation (Section 3.3.2). The concentration was determined on a 1 % agarose gel.

3.6.3 NdeI and BamHI sequential digestion of pET-11a

The quantity of pET-11a available for ligation was increased by doing a sequential digestion instead of a double digestion; a sequential digestion allowed a higher concentration of pET-11a to be digested.
pET-11a-cutinase plasmid isolated with QIAGEN Plasmid Midi Kit was first digested with NdeI restriction enzyme. The reagents (Table 3.19) were mixed on ice by pipetting up and down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 5 hours at 37 °C.

Table 3.19. Recipe for NdeI digestion of pET-11a.

Reagent	Volume
10x Buffer O	8 μL
pET-11a-cutinase plasmid DNA ($\sim$200 ng/μl)	15 μL
NdeI	4 μL
Water, molecular biology reagent (to 80 μL)	53 μL
Total	80 μL

After ended NdeI digestion, an ethanol precipitation (Section 3.3.2) was performed. This was followed by a digestion with BamHI restriction enzyme: The reagents (Table 3.20) were mixed on ice by pipetting up and down, and centrifuged for 2 s in a MiniSpin plus centrifuge using an F-45-12-11 rotor to spin down wall-drops. This restriction reaction mix was incubated for 5 hours at 37 °C.

Table 3.20. Recipe for BamHI digestion of pET-11a.

Reagent	Volume
10x Buffer BamHI	8 μL
NdeI digested pET-11a-cutinase DNA	20 μL
BamHI	4 μL
Water, molecular biology reagent (to 80 μL)	48 μL
Total	80 μL

After BamHI digestion, ligation-ready pET-11a was isolated by a 1 % preparative agarose gel and Millipore Ultrafree-DA DNA Extraction From Agarose Gels kit (Section 3.5.6).

3.6.4 Ligation of mut-DEFB4A-GFP and pET-11a

A ligation was performed to ligate mut-DEFB4A-GFP and pET-11a vector; both carrying NdeI and BamHI sticky-ends. The reagents (Table 3.21) were mixed by slowly pipetting up and down on ice, and incubated overnight in a fridge door shelf (8-9 °C). The ligation was finished by incubation for 10 min at room temperature.

Table 3.21. Recipe for ligation of mut-DEFB4A-GFP and pET-11a.

Reagent	Volume
10x T4 DNA Ligase Buffer O	2 μl
mut-DEFB4A-GFP	4 μl
pET-11a	13 μl
T4 DNA Ligase	1 μl
Total	20 μl

The ligation mix was transformed into *E. coli* NEB4α (Section 3.5.8).

3.6.5 Verification of construct

To examine if the ligation procedure had been successful, 6 colonies were plated seperately on selective agar plates and incubated overnight at 37 °C. 6 GenElute™ Plasmid Miniprep Kit procedures (Section 3.4.2) were performed on these cells. Subsequently, PCR was performed using the isolated DNA and primers annealing to the NdeI site of DEFB4A-GFP gene (DEFB4Aforw primer) and pET-11a at the BamHI site (GFP8pet primer). A 1 % agarose gel was run with these PCR products to determine if any of the isolated DNA was pET-11a with mut-DEFB4A-GFP insert.

When a positive clone was found, an NdeI and BamHI digestion of the plasmid DNA was performed to check that the size of the plasmid isolated corresponded to the size of pET-11a-mut-DEFB4A-GFP, and to check that the restriction sites (and start codon) were intact. The reagents (Table 3.22 and 3.23) for the digestions were mixed and incubated for 1 hour at 37 °C. A 1 % agarose gel was run to examine the restriction products.

Table 3.22. Recipe for NdeI digestion of pET-11a-mut-DEFB4A-GFP.

Reagent	Volume
10x Buffer O	0.5 μL
pET-11a-mut-DEFB4A-GFP plasmid DNA	1 μL
NdeI	0.5 μL
Water, molecular biology reagent (to 5 μL)	3 μL
Total	5 μL

Table 3.23. Recipe for BamHI digestion of pET-11a-mut-DEFB4A-GFP.

Reagent	Volume
10x Buffer BamHI	0.5 μL
pET-11a-mut-DEFB4A-GFP DNA	1 μL
BamHI	0.5 μL
Water, molecular biology reagent (to 5 μL)	3 μL
Total	5 μL

3.7 Sequencing of pET-11a-mut-DEFB4A-GFP

A QIAGEN midiprep (Section 3.4.3) was prepared of the positive clone from the verification of insert. The midiprep pellet was resuspended in 200 μl water, molecular biology reagent, to get the right concentration for sequencing (100 ng/μl). A 1 % agarose gel was run to check the concentration of the sample; the concentration was confirmed using Pico200 spectrophotometer (Picodrop Ltd., Hinxton, United Kingdom). Sequencing was performed by Beckman Coulter Genomics (Takeley, United Kingdom).

3.8 Transformation of *Escherichia coli* Origami 2(DE3) with pET-11a-mut-DEFB4A-GFP

A tube containing 50 μl competent *E. coli* Origami 2(DE3) was thawed on ice for 30 min. 1 μl pET-11a-mut-DEFB4A-GFP plasmid DNA with a concentration of 100 ng/μl was added to the thawed cells. This was gently mixed by tapping the tube, and incubated on ice for 30 min. The mixture was heat shocked for 45 s at 42 °C in an RM6 Lauda water bath (LAUDA, Lauda-Königshofen, Germany). 500 μl 37 °C LB medium was gently added, and the cells were incubated for 1 hour at 37 °C, 230 RPM. Two aliquots of 10 μl was pipetted and evenly distributed using a drigalski spatula on two selective agar plates containing 100 μg/ml ampicillin preheated to 37 °C. The plates were incubated overnight at 37 °C and stored at 4 °C. To make a plate with a lot of identical clones, one colony was streaked on a new preheated selective agar plate using a plastic inoculation loop and incubated overnight at 37 °C. This plate was stored at 4 °C.

3.9 Expression of HbD-2-turboGFP fusion protein in *E. coli* Origami 2(DE3)

A starter culture was prepared by innoculating cells from the agar plate with *E. coli* NEB5α containing pET-11a-mut-DEFB4A-GFP in 8 ml LB medium with 100 μg/ml ampicillin in a 50 ml Greiner centrifuge tube. This was incubated for 12 hours at 25 °C, 230 RPM. OD_{600} was measured to 0.555 using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Stockholm, Sweden), and two aliquots of 3 ml of the starter culture was transferred to two 500 ml conical flasks containing 100 ml LB medium with 100 μg/ml ampicillin. This was incubated for 12 hours at 25 °C, 230 RPM. After 6 hours, OD_{600} was measured to 0.275 and 0.250. After 12 hours OD_{600} was measured to 0.990 and 0.975 (calculated from a 1:2 dilution). One of the cultures (OD_{600} = 0.990) was induced with IPTG for a final concentration of 0.1 M IPTG. This was incubated for 11 hours at 25 °C, 230 RPM, and OD_{600} was measured to 1.888 (+IPTG) and 2.352 (÷IPTG) (calculated from a 1:3 dilution).

The cells of the two cultures were harvested by centrifugation at 6000 x g for 15 min at 4 °C in a 5804 R centrifuge using an F-34-6-38 rotor; This centrifuge and rotor will be used for the rest of this protocol. The supernatants were decanted. The two cell pellets were each resuspended in 4 ml TES buffer and incubated for

30 min on ice to open the cells. This was centrifuged at 7000 x g for 15 min at 4 °C. The crude cell lysate supernatant was decanted into a separate tube and stored at 4 °C protected from light. The pellets were each resuspended in 4 ml chilled autoclaved Milli-Q water, and centrigued at 7000 x g for 15 min at 4 °C. The supernatant was decanted into a separate tube and stored at 4 °C protected from light. The pellet was also stored at 4 °C protected from light.

3.10 Fluorescence spectroscopy measurements of expressed HbD-2-turboGFP fusion protein

Fluorescence measurements were performed on the TES buffer and water fractions from the expression experiment (Section 3.9) using a QuantaMaster™ Phosphorescence Spectrofluorometer setup (Photon Technology International, Birmingham, NJ, USA). Samples were loaded in a quartz cuvette with 1 cm path length. Excitation wavelength was set to 480 nm, and emmision was recorded from 485 to 600 nm.

3.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

A 12 % SDS-PAGE gel was cast according to Roche [2011] using SDS-PAGE apparatus (Bio-Rad, Hercules, CA, USA):
The reagents (Table 3.24) for the 12 % resolving gel were mixed (TEMED was added last) and pipetted between the glass plates immobilised in the casting apparatus. Milli-Q water was added to level the resolving gel, and the resolving gel was left to polymerise. After polymerisation, the water was removed with filter paper. A 5 % stacking gel (Table 3.25) was prepared and loaded on top of the polymerised resolving gel. A comb was inserted, and the stacking gel was left to polymerise. The gel was moved to a clamping frame with electrodes, and this was placed in a plastic reservoir. The inner chamber of the clamping frame was filled with 1x SDS-PAGE running buffer and the comb was removed.

Table 3.24. Recipe for 12 % SDS-PAGE resolving gel [Roche, 2011].

Reagent	Volume
Milli-Q water	1.6 μL
30 % acrylamide/bis solution	2.0 μL
1.5 M tris buffer (pH 8.8)	1.3 μL
10 % SDS solution	0.05 μL
10 % ammonium persulfate	0.05 μL
TEMED	0.002 μL
Total	5 μL

Table 3.25. Recipe for 5 % SDS-PAGE stacking gel [Roche, 2011].

Reagent	Volume
Milli-Q water	0.68 μL
30 % acrylamide/bis solution	0.17 μL
0.5 M tris buffer (pH 6.8)	0.13 μL
10 % SDS solution	0.01 μL
10 % ammonium persulfate	0.01 μL
TEMED	0.001 μL
Total	1 μL

The samples were prepared by mixing 1:1 with 2x Laemmli Sample Buffer (the cell pellet was prepared at 1:5), and heated for 3 min at 95 °C. Then incubated on ice until being loaded. 20 μl of each prepared sample was loaded next to 3 μl LMW standard (Appendix B). The tank was filled with 1x SDS-PAGE running buffer, and the power supply was connected. An electrical field of 130 V was applied for 70 min. The gel was developed for 60 min in staining solution (50 % EtOH, 40 % Milli-Q water, 10 % AcOH, and 1 g/l Coomassie Brilliant Blue). The gel was rinsed with Milli-Q water, and destained overnight in destain solution (30 % EtOH, 70 % Milli-Q water, and 10 % AcOH).

Results 4

4.1 Cloning of pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase plasmids

4.1.1 Concentration determination of cloned pET-11a-cutinase and pCMV6-AC-DEFB4A-GFP plasmids

After cloning of pET-11a-cutinase and pCMV6-AC-DEFB4A-GFP plasmids in *E. coli* NEB5α, the plasmids were isolated with QIAGEN Plasmid Midiprep Kit and GenElute™ Plasmid Miniprep Kit, respectively. The concentrations of the isolated plasmids were determined on a 1 % agarose gel (Figure 4.1). The band at 6000 bp in the DNA standard contains 70 ng DNA, thus the concentrations of pET-11a-cutinase and pCMV6-AC-DEFB4A-GFP were both determined to ∼200 ng/μl.

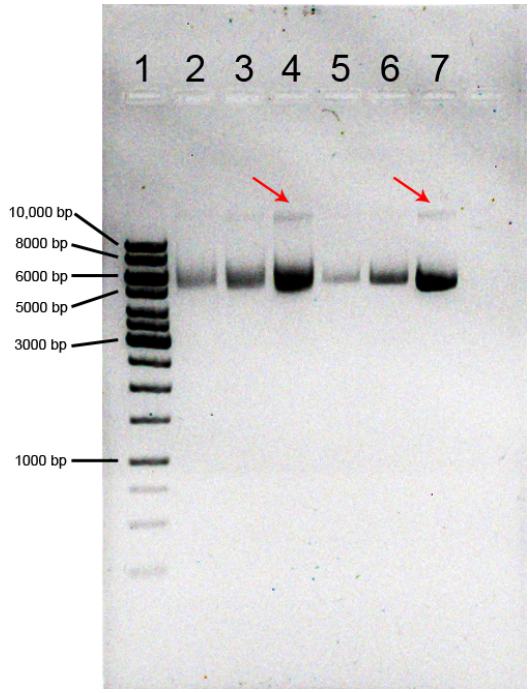

Figure 4.1. 1% agarose gel to determine the concentration of pET-11a-cutinase isolated with QIAGEN Plasmid Midiprep Kit and pCMV6-AC-DEFB4A-GFP plasmid isolated with GenElute Plasmid Miniprep Kit, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler™ 1 kb DNA Ladder. Lane 2 contains 0.1 μl pET-11a-cutinase. Lane 3 contains 0.2 μl pET-11a-cutinase. Lane 4 contains 0.5 μl pET-11a-cutinase. Lane 5 contains 0.1 μl pCMV6-AC-DEFB4A-GFP. Lane 6 contains 0.2 μl pCMV6-AC-DEFB4A-GFP. Lane 7 contains 0.5 μl pCMV6-AC-DEFB4A-GFP. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye. Red arrows are pointing at nicked circular DNA.

4.1.2 pET-11a and DEFB4A-GFP PCR product

To verify that pET-11a-cutinase and pCMV6-AC-DEFB4A-GFP had been cloned, PCR was performed, and the PCR products were analysed on a 1 % agarose gel (Figure 4.2). The expected size of pCMV6-AC-DEFB4A-GFP OE-PCR product was 869 bp. The expected size of pET-11a-cutinase PCR product was 1138 bp. A vague secondary annealing PCR product was observed in the pET-11a-cutinase PCR.

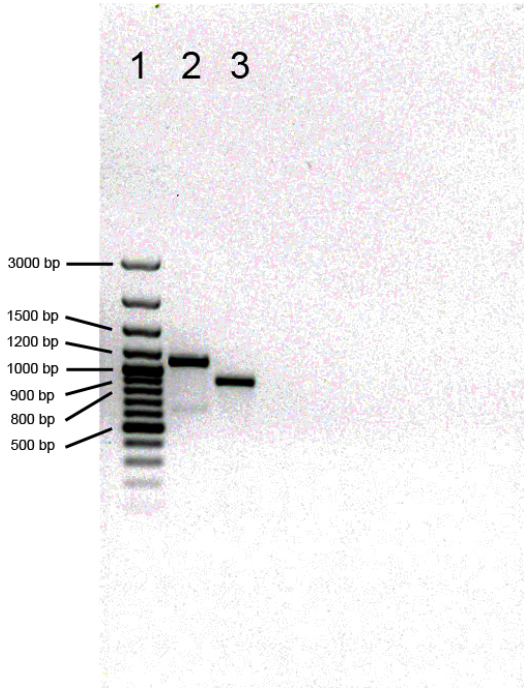

Figure 4.2. 1% agarose gel for examination of PCR product of DEFB4A-GFP using DEFB4aforw and DEFB4arev primers on pCMV6-AC-DEFB4A-GFP plasmid isolated using GenElute Plasmid Miniprep Kit; and PCR product of pET-11a-cutinase using Pet11a2 and GFP8pet primers on pET-11a-cutinase plasmid isolated by QIAGEN Plasmid Midiprep Kit, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl pET-11a-cutinase PCR product. Lane 3 contains 1 μl DEFB4A-GFP PCR product. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

4.2 Construction of pET-11a-DEFB4A-GFP with partial digestion

4.2.1 DEFB4A-GFP PCR clean-up product

After OE-PCR of DEFB4A-GFP with DEFB4Aforw and DEFB4Arev primers, the PCR product was purified with GenElute PCR Clean-Up Kit. During the clean-up, 100 μl PCR product was concentrated, as the clean-up product was eluted with 50 μl.

To check the efficiency of the GenElute PCR Clean-Up Kit and to find the concentration of the PCR clean-up product before cutting with BamHI, a 1 % agarose gel was made (Figure 4.3). The band at 900 bp in the DNA standard contains 27 ng DNA, thus the concentration of DEFB4A-GFP PCR product was determined to $\sim$20 ng/μl, and the concentration of DEFB4A-GFP PCR Clean-up product was determined to $\sim$40 ng/μl.

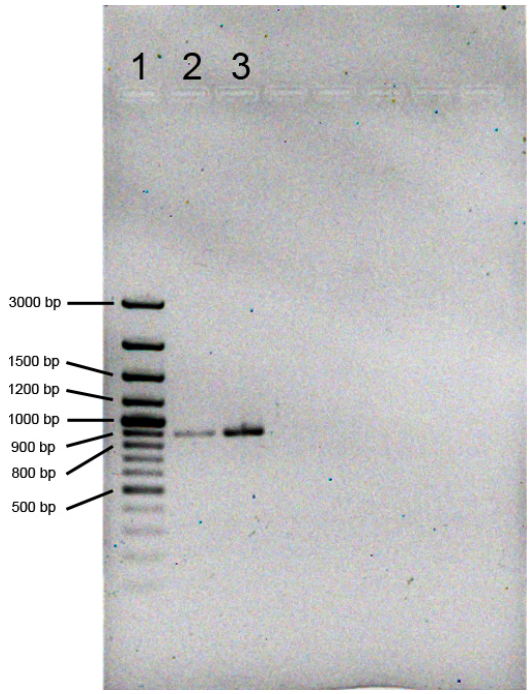

Figure 4.3. 1% agarose gel for examination of PCR product of DEFB4A-GFP after GenElute PCR Clean-up Kit, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 1 μl DEFB4A-GFP OE-PCR product. Lane 3 contains 1 μl DEFB4A-GFP product from PCR Clean-up Kit. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

4.2.2 Digestion of DEFB4A-GFP with BamHI

100 μl DEFB4A-GFP product from PCR clean-up with a concentration of $\sim$40 ng/μl was digested with BamHI for 5 hours at 37 °C. The digested DNA was isolated by an ethanol precipitation, and the concentration was determined on a 1 % agarose gel (Figure 4.4). The band at 1000 bp in the DNA standard contains 60 ng DNA, and the concentration of the loaded BamHI digested DEFB4A-GFP seems well above that. If there was no loss of DNA in the BamHI digestion and ethanol precipitation procedures, the concentration would be $\sim$200 ng/μl (4 μg DNA was digested and redissolved in 20 μl water, molecular biology reagent, after ethanol precipitation).

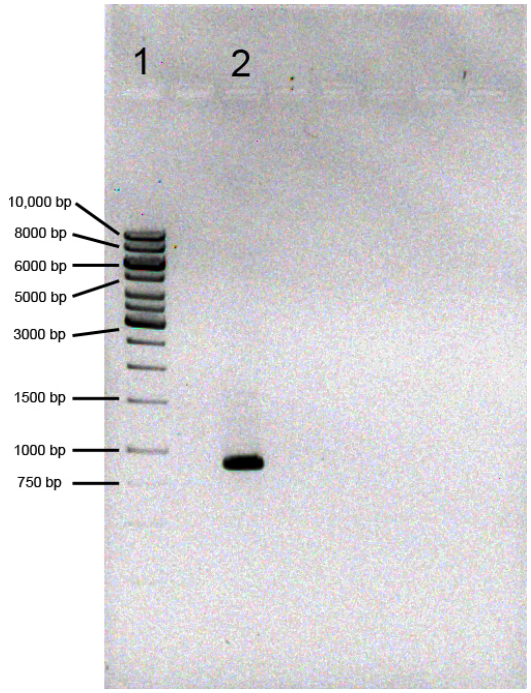

Figure 4.4. 1% agarose gel for examination of DEFB4A-GFP PCR clean-up product digested with BamHI for 5 hours at 37 °C, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 1 μl BamHI digested DEFB4A-GFP. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

4.2.3 Partial digestion of DEFB4A-GFP with NdeI

After ethanol precipitation of BamHI digested DEFB4A-GFP, the ammount of NdeI required to perform a partial digestion was determined by a dilution series experiment. In this experiment, 0.1 μl BamHI digested DEFB4A-GFP was restricted for 10 min with 7 different concentrations of NdeI. The result of this dilution series was examined on a 1 % agarose gel (Figure 4.5). The sample in lane 6, seemed to be both partially digested and overdigested, therefore, this concentration of NdeI (0.0823 u) will be the one chosen for the upscaling of the NdeI partial digestion experiment.

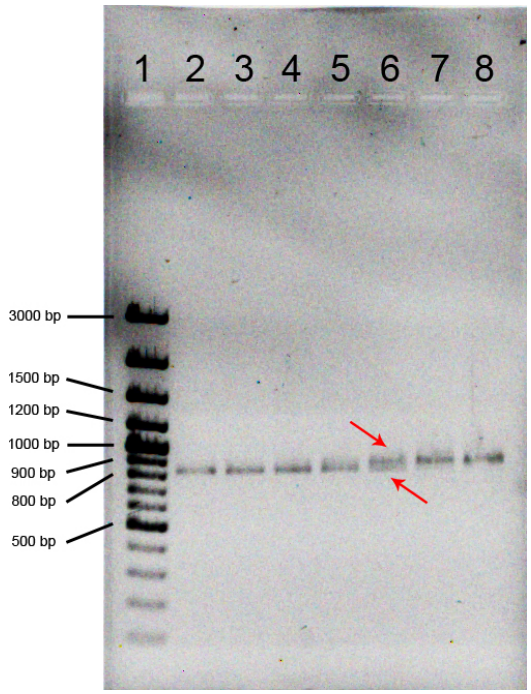

Figure 4.5. 1% agarose gel for examination of NdeI dilution series digestion of BamHI digested DEFB4A-GFP, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 6.6667 u NdeI for 10 min. Lane 3 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 2.2222 u NdeI for 10 min. Lane 4 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 0.7407 u NdeI for 10 min. Lane 5 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 0.2469 u NdeI for 10 min. Lane 6 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 0.0823 u NdeI for 10 min. Lane 7 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 0.0274 u NdeI for 10 min. Lane 8 contains 0.1 μl BamHI digested DEFB4A-GFP, digested with 0.0091 u NdeI for 10 min DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye. The top red arrow is pointing at a band that corresponds to the size of partially digested DEFB4A-GFP. The bottom red arrow is pointing at a band that corresponds to the size of DEFB4A-GFP digested at the on-gene NdeI restriction site.

The upscaled NdeI partial digestion of DEFB4A-GFP was performed with 3 μl DNA in a 30 μl volume. $30 \cdot 0.0823u = 2.47$ u. Thus, the NdeI partial digestion experiment was performed with 1.5 u NdeI, 2.47 u NdeI, and 5 u NdeI. After digestion, the ligation-ready DEFB4A-GFP was purified by an ethanol precipitation.

4.2.4 Ligation and transformation

To prepare pET-11a for ligation, the cutinase insert was removed by NdeI and BamHI double digestion. After digestion, linearised pET-11a was isolated by a preparative 1 % agarose gel, extracted using Millipore Ultrafree-DA DNA Extraction From Agarose Gels kit , and purified by an ethanol precipitation. The concentration of the ligation-ready pET-11a and DEFB4A-GFP was determined on a 1 % agarose gel (Figure 4.6[1]).

The band at 6000 bp in the 1 kb DNA standard contains 70 ng DNA, and the band at 5000 bp contains 30 ng DNA. The concentration of ligation-ready pET-11a was determined to 15 ng/*mu*l.

[1]Even though the partial digestion was performed with 1.5, 2.47 and 5 units NdeI, these all looked the same on the agarose gel; and so this agarose gel only shows the partial digestion performed with 2.47 units NdeI.

The band at 900 bp in the 100 bp DNA standard contains 27 ng DNA, thus the concentration of ligation-ready DEFB4A-GFP was determined to 20 ng/μl.

The ligations were performed using 11 μl (165 ng) ligation-ready pET-11a and 6 μl (120 ng) ligation-ready DEFB4A-GFP. Ligation-ready pET-11a contains 5637 bp, so 165 ng/μl is 0.0451 femtomolμl. Ligation-ready DEFB4A-GFP contains 858 bp, so 120 ng/μl is 0.2155 femtomol/μl. This gave a molar ratio of 4.8:1 (insert:vector); Fermentas recommends 1:1 to 5:1, so the ligation was within that span, and at the high end because not all insert could be digested in the partial digestion.

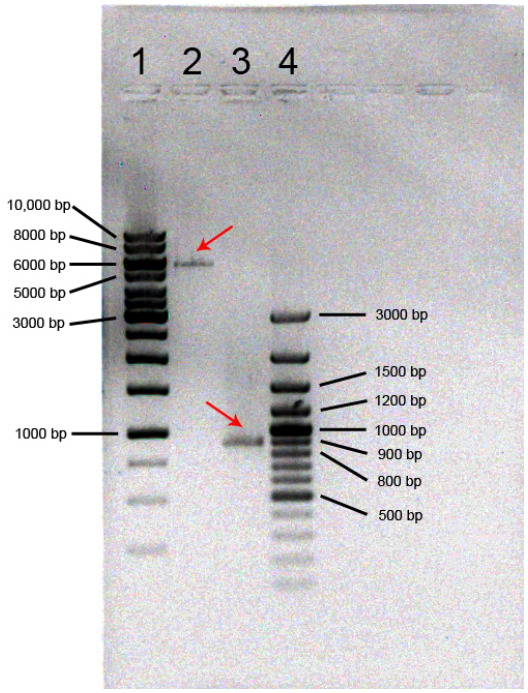

Figure 4.6. 1% agarose gel for concentration determination of ligation-ready pET-11a and DEFB4A-GFP before ligation, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 1 μl ligation-ready pET-11a. Lane 3 contains 1 μl ligation-ready DEFB4A-GFP. Lane 4 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

Ligation mix was incubated overnight in a fridge door shelf, and subsequently transformed into *E. coli* NEB5α. The transformation of the plasmids where DEFB4A-GFP had been partially digested with 1.5 u NdeI yielded only 3 colonies on the selective agar plates. Whereas the two other transformations (partial digestion with 2.47 and 5 units NdeI) yielded more than 40 colonies.

4.2.5 Verification of construct

The two transformations yielding many colonies were used for verification of construct experiment. Plasmids from 14 colonies of each transformation were isolated by GenElute^{TM} Plasmid Miniprep Kit, and PCRs were performed on each miniprep product using DEFB4Aforw and GFP8pet primers. These primers were used because only these two in a combination could tell if a correctly digested DEFB4A-GFP gene had been inserted into pET-11a. The PCR products were analysed on 1 % agarose gels (Figure 4.7 and 4.8). Only a vague band caused by unspecific annealing on one of the agarose gels was observed, and no bands were observed

at the desired PCR product size ∼1000 bp, so the pET-11a-DEFB4A-GFP plasmid had not been successfully constructed.

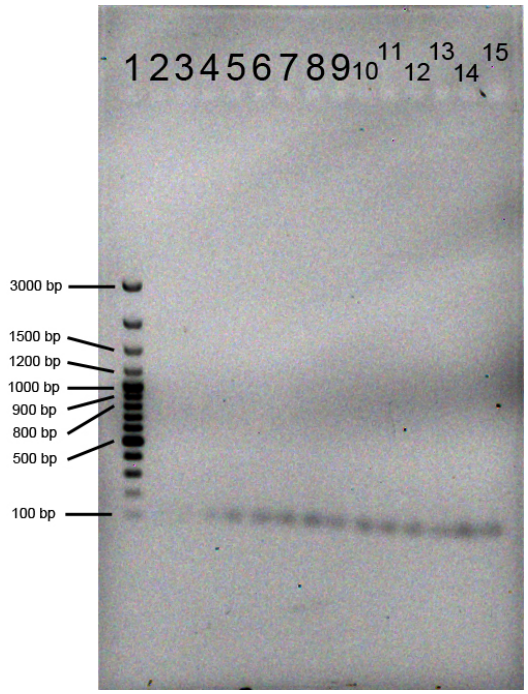

Figure 4.7. 1 % agarose gel to verify if pET-11a-DEFB4A-GFP plasmid had been constructed with partial digestion protocol, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 3 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2-15 contain 2.5 μl PCR product of PCR with DEFB4Aforw and GFP8pet primers and miniprep isolated plasmid DNA from 14 different transformed *E. coli* NEB5α colonies; the partial digestion for construction of these plasmids was performed with 2.47 u NdeI. DNA samples contain 0.5 μl 6x DNA Loading Dye.

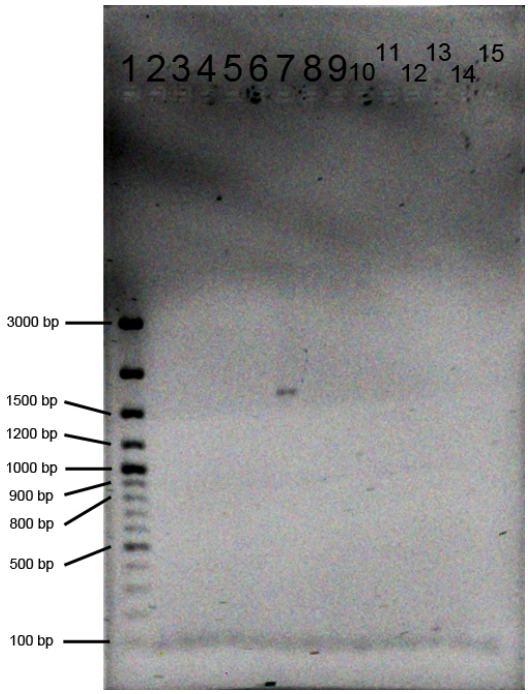

Figure 4.8. 1 % agarose gel to verify if pET-11a-DEFB4A-GFP plasmid had been constructed with partial digestion protocol, run in 1X TAE buffer at 70 V DC electrical field for 120 min. Lane 1 contains 3 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2-15 contain 2.5 μl PCR product of PCR with DEFB4Aforw and GFP8pet primers and miniprep isolated plasmid DNA from 14 different transformed *E. coli* NEB5α colonies; the partial digestion for construction of these plasmids was performed with 5 u NdeI. DNA samples contain 0.5 μl 6x DNA Loading Dye.

To get a better understanding of what plasmid DNA had been isolated with the minipreps, 14 midiprep isolated plasmid samples were examined on a 1 % agarose gel (Figure 4.9). This agarose gel showed plasmid DNA migrating four different distances. six samples had migrated the shortest way through the gel, and another four had only migrated a little further.

To examine what these plasmids consisted of, PCRs of the plasmid DNA loaded in lane 9 and 10 were performed. PCR was performed with primers annealing to pET-11a at the NdeI site and the BamHI site of DEFB4A-GFP (DEFB4Arev and Pet11a2 primers), and with primers annealing to pET-11a (Pet11a2 and GFP8pet primers). The PCR results were examined on a 1 % agarose gel (Figure 4.10).

The PCR of the DNA from lane 9 resulted in a band at ∼1000 bp when using DEFB4Arev and Pet11a2 primers, and a band at ∼1100 bp when using Pet11a2 and GFP8pet primers. This corresponded with the PCR product

sizes expected from pET-11a ligated with an overdigested DEFB4A-GFP gene, i.e. an unsuccessful NdeI partial digestion, where the gene has been digested at the on-gene NdeI restriction site.

The PCR of the DNA from lane 10 resulted in no product when using DEFB4Arev and Pet11a2 primers, but a band at ∼300 bp when using Pet11a2 and GFP8pet primers. This result corresponded with the PCR product size expected from pET-11a ligated with no insert.

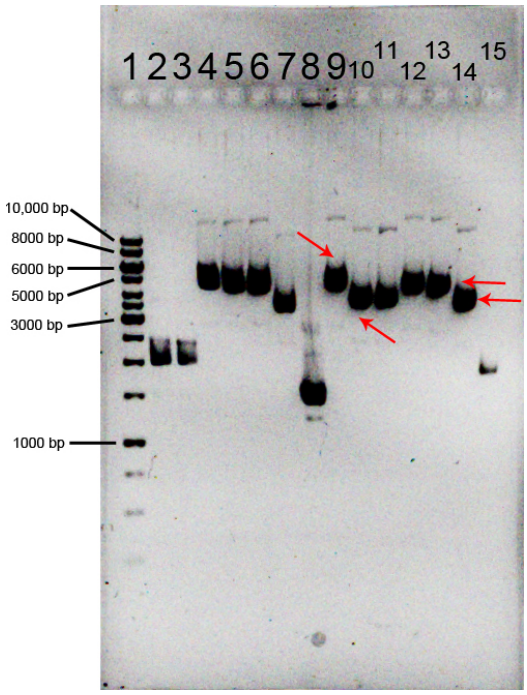

Figure 4.9. 1 % agarose for examination of midiprep isolated DNA from 14 colonies, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 3 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2-15 contain 2.5 μl midiprep isolated plasmid DNA from 14 transformed *E. coli* NEB5α colonies. DNA samples contain 0.5 μl 6x DNA Loading Dye. The red arrows to the right point at the two rows of bands that are most abundant in the agarose gel. The other two red arrows point at the DNA of lane 9 and 10, which was further analysed.

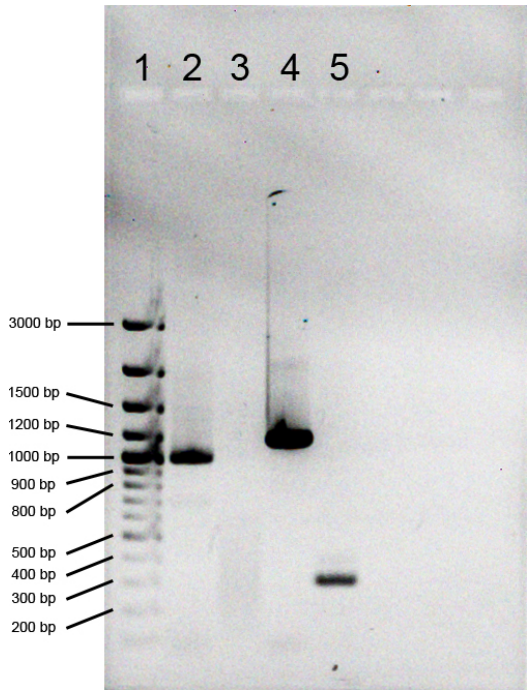

Figure 4.10. 1 % agarose for examination of PCR products for examination of two midiprep isolated plasmid products, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 5 μl PCR product of PCR with "lane 9" plasmid and DEFB4Arev and Pet11a primers. Lane 3 contains 5 μl PCR product of PCR with "lane 10" plasmid and DEFB4Arev and Pet11a primers. Lane 4 contains 5 μl PCR product of PCR with "lane 9" plasmid and Pet11a2 and GFP8pet primers. Lane 5 contains 5 μl PCR product of PCR with "lane 10" plasmid and Pet11a2 and GFP8pet primers. DNA samples contain 1 μl 6x DNA Loading Dye.

4.3 Construction of pET-11a-mut-DEFB4A-GFP with PCR site-directed mutagenesis

Because NdeI partial digestion was unsuccessful, a point-mutation was made to silcence the on-gene NdeI restriction site. The sequence was mutated from CATATG to CATTTG.

4.3.1 Creation of point mutation

The point-mutation was created by a series of PCRs. In the first PCRs two mutated parts, named mut1 and mut2, of the DEFB4A-GFP gene was created. This was done using pCMV6forw and mutarev primers to make mut1 PCR product, and mutaforw and pCMV6rev primers to make mut2 PCR product. The PCR results were examined on a 1 % agarose gel (Figure 4.11). The expected size of mut1 was 125 bp, and 924 bp for mut2. mut1 and mut2 PCR products were purified with a PCR clean-up procedure, and their concentrations were determined on another 1 % agarose gel (Figure 4.12). As the bands at 100 and 200 bp in the DNA standard each contain 30 ng DNA, the concentration of purified mut1 was determined to 40 ng/μl. The band at 1000 bp contain 80 ng DNA, so the concentration of purified mut2 was determined to 80 ng/μl.

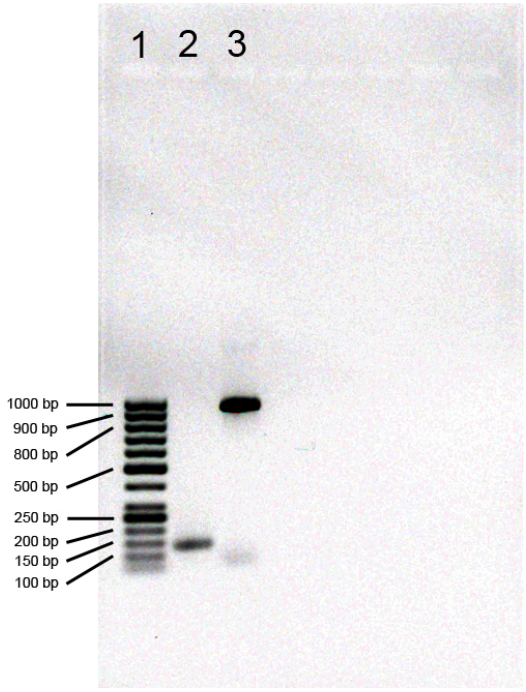

Figure 4.11. 1 % agarose gel for examination of mut1 and mut2 PCR products, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 50 bp Plus DNA Ladder. Lane 2 contains 1 μl mut1 PCR product. Lane 3 contains 1 μl mut2 PCR product. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

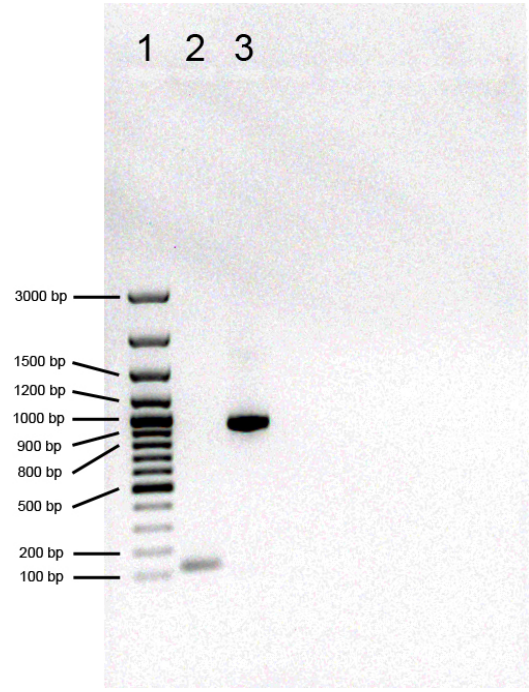

Figure 4.12. 1 % agarose gel for concentration determination of mut1 and mut2 PCR Clean-up products, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl mut1 PCR Clean-up product. Lane 3 contains 1 μl mut2 PCR Clean-up product. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

mut1 and mut2 PCR Clean-up products were fused in a PCR reaction using no primers. mut1 contains 125 bp,

so 40 ng/μl is 0.49 femtomol/μl. mut2 contains 924 bp, so 80 ng/μl is 0.13 femtomol/μl. In an attempt to use equimolar concentrations of mut1 and mut2, three PCRs were made:

PCR1: 3 μl mut1 and 15 μl mut2.
PCR2: 4 μl mut1 and 14 μl mut2.
PCR3: 5 μl mut1 and 13 μl mut2.

The products of these PCRs were examined on a 1 % agarose gel (Figure 4.13). The agarose gel revealed a lot of impurities in the sample but in the lanes where 5 μl sample was loaded, vague bands was observed around 1000 bp. The size of mut1 and mut2 fusion product was expected to 1026 bp.

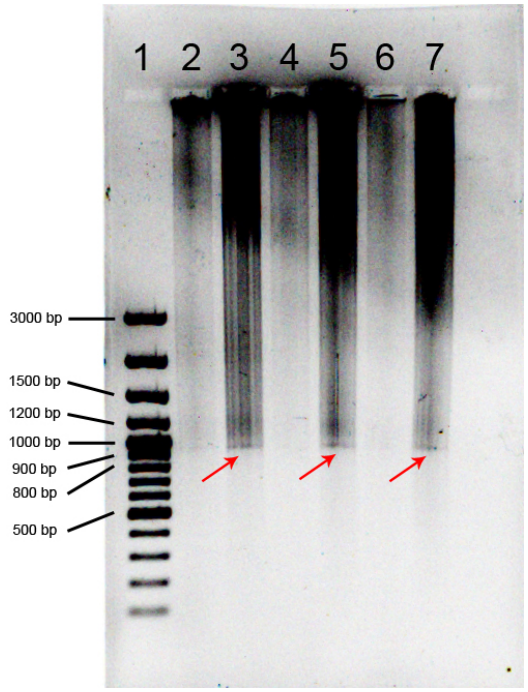

Figure 4.13. 1 % agarose gel for examination of PCR fusion product of mut1 and mut2, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl PCR1 fusion product (3 μl mut1 and 15 μl mut2). Lane 3 contains 5 μl PCR1 fusion product (3 μl mut1 and 15 μl mut2). Lane 4 contains 1 μl PCR2 fusion product (4 μl mut1 and 14 μl mut2). Lane 5 contains 5 μl PCR2 fusion product (4 μl mut1 and 14 μl mut2). Lane 6 contains 1 μl PCR3 fusion product (5 μl mut1 and 13 μl mut2). Lane 6 contains 5 μl PCR3 fusion product (5 μl mut1 and 13 μl mut2). DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye. Red arrows are pointing at the desired size of mut1 and mut2 fusion product.

The fusion products were amplified by PCR using different concentrations of fusion product and DEFB4Aforw and DEFB4Arev primers. This would also add NdeI and BamHI restriction sites to the mutated DEFB4A-GFP gene. The products of these PCRs were mut-DEFB4A-GFP with NdeI and BamHI restriction sites. The concentrations of fusion PCR product used was 0.5 μl, 1 μl, and 10 μl, and PCRs were performed with PCR1, PCR2, and PCR3 - resulting in 9 PCRs in total. The products of these PCRs were analysed on 1 % agarose gels (Figure 4.14). These agarose gels showed that the purest and best amplified PCR product was produced using 0.5 μl fusion PCR product. When using 10 μl, no mut-DEFB4A-GFP was produced, and when using 1 μl mut-DEFB4A-GFP was produced, but with a lot of impurities. PCR with 0.5 μl template yielded strong bands

at the desired size (869 bp), but also two less destinct bands with sizes of ∼2000 and ∼3000 bp.

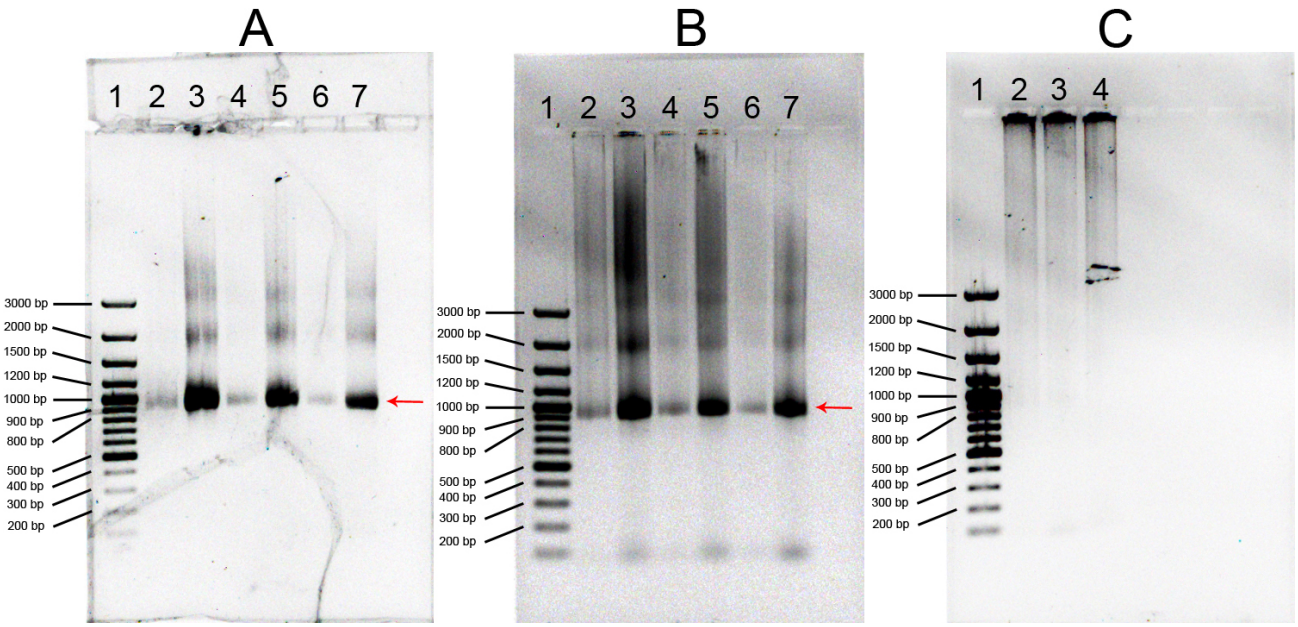

Figure 4.14. 1 % agarose gels for analysis of PCR product with 0.5, 1, and 10 μl PCR1, PCR2, and PCR3 fusion products and DEFB4Aforw and DEFB4Arev primers, run in 1X TAE buffer at 70 V DC electrical field for 90 min. A) and B) contain the same samples, but produced with respectively 0.5 and 1 μl PCR fusion product.**A/B)** Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl PCR product of PCR with 0.5/1 μl PCR1. Lane 3 contains 5 μl PCR product of PCR with 0.5/1 μl PCR1. Lane 4 contains 1 μl PCR product of PCR with 0.5/1 μl PCR2. Lane 5 contains 5 μl PCR product of PCR with 0.5/1 μl PCR2. Lane 6 contains 1 μl PCR product of PCR with 0.5/1 μl PCR3. Lane 7 contains 5 μl PCR product of PCR with 0.5/1 μl PCR3. **C)** 1 % agarose gel for analysis of PCR product with 10 μl PCR1, PCR2, and PCR3 fusion products and DEFB4Aforw and DEFB4Arev primers, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl PCR product of PCR with 10 μl PCR1. Lane 3 contains 5 μl PCR product of PCR with 10 μl PCR1. Lane 4 contains 1 μl PCR product of PCR with 10 μl PCR2. Lane 5 contains 5 μl PCR product of PCR with 10 μl PCR2. Lane 6 contains 1 μl PCR product of PCR with 10 μl PCR3. Lane 7 contains 5 μl PCR product of PCR with 10 μl PCR3. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye. Arrows are pointing at the row of bands that have the size corresponding to mut-DEFB4A-GFP.

It was decided to use mut-DEFB4A-GFP PCR product created using 0.5 μl PCR1 for the following experimental procedures. To prepare the PCR product for NdeI And BamHI digestion, it was purified with a PCR clean-up procedure. The PCR clean-up product was analysed on a 1 % agarose gel (Figure 4.15). The concentration of the band at 800 bp in the DNA standard contains 27 ng DNA, thus the concentration of mut-DEFB4A-GFP was determined to be ∼40 ng/μl. The concentration of the unwanted band at ∼2000 bp was determined to be ∼25 ng/μl.

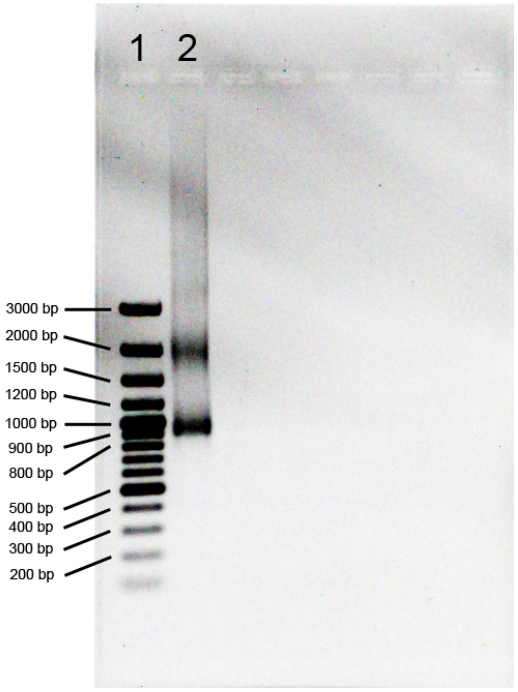

Figure 4.15. 1 % agarose gel for concentration determination of purified mut-DEFB4A-GFP before enzymatic digestion with NdeI and BamHI, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2 contains 1 μl mut-DEFB4A-GFP PCR product purified with PCR Clean-up kit. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

4.3.2 Ligation and transformation

40 μl mut-DEFB4A-GFP and 15 μl pET-11a were sequentially digested with NdeI and BamHI restriction enzymes. And pET-11a was purified by a preparative agarose gel, followed by gel extraction protocol. Before ligation, the concentration of digested and ligation-ready mut-DEFB4A-GFP and pET-11a was determined on a 1 % agarose gel (Figure 4.16). pET-11a was at a very low concentration, even though a partial digestion instead of a double digestion had been performed to increase this concentration. The concentration of ligation-ready pET-11a was determined to $\sim$10 ng/μl. The digested mut-DEFB4A-GFP sample still had impurities at $\sim$2000 bp and a little at $\sim$3000 bp. The concentration of ligation-ready mut-DEFB4A-GFP was determined to 20 ng/μl.

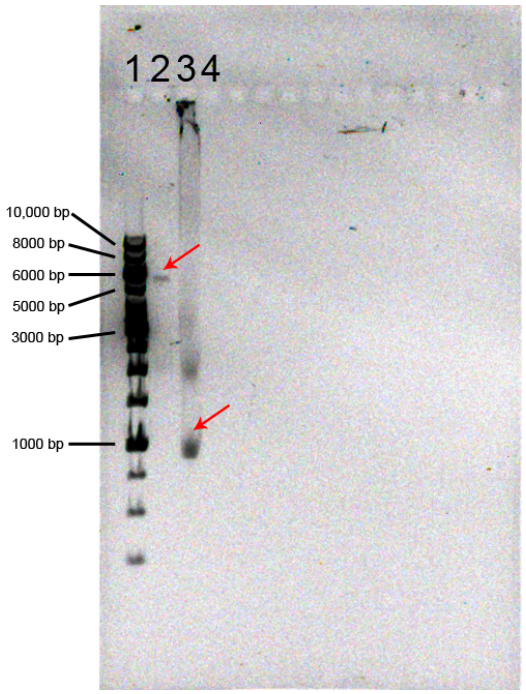

Figure 4.16. 1 % agarose gel for concentration determination of ligation-ready pET-11a and mut-DEFB4A-GFP, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 3 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 1 μl ligation-ready pET-11a. Lane 3 contains 1 μl ligation-ready mut-DEFB4A-GFP. DNA samples contain water, molecular biology reagent, to 2.5 μ and 0.5 μl 6x DNA Loading Dye.

The ligation mix was incubated overnight in a fridge door shelf, and subsequently transformed into *E. coli* NEB5α. The transformed cells were plated on selective agar plates, and incubated overnight at 37 °C. This yielded more than 30 colonies.

4.3.3 Verification of construct and sequencing

Plasmid DNA from 6 colonies was isolated by GenElute^{TM} Plasmid Miniprep Kit, and 6 PCRs were performed on each miniprep product using DEFB4Aforw and GFP8pet primers. The PCR products were examined on a 1 % agarose gel (Figure 4.17). This gel showed no PCR products with the desired size of ∼1000 bp, but there was a band at ∼3000 bp in lane 5, which could indicate that one of the impurities had been inserted into pET-11a. Lane 8-13 contain plasmid DNA BamHI digested for 20 min - since this was not a long enough period of time, nothing can be eluded from these results.

It was decided to pick 6 more colonies, isolate the plasmid DNA, and perform PCRs. Again, the PCR products were examined on a 1 % agarose gel (Figure 4.18). This time there was a positive result, as lane 5 contains a PCR product with the size of ∼1000 bp.

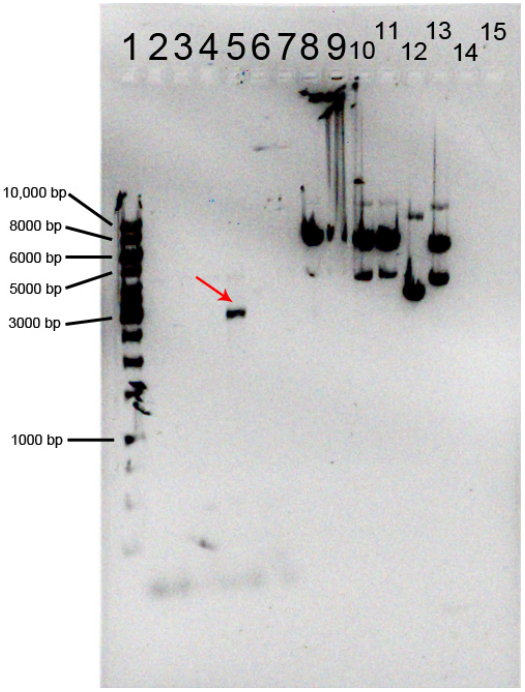

Figure 4.17. 1 % agarose gel to verify if pET-11a-mut-DEFB4A-GFP plasmid had been constructed, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 3 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2-7 contain 2.5 μl PCR product of PCR with DEFB4Aforw and GFP8pet primers and miniprep isolated plasmid DNA from 6 different transformed *E. coli* NEB5α colonies. Lane 8-13 contain 2.5 μl plasmid DNA unsuccessfully digested with BamHI. DNA samples contain 0.5 μl 6x DNA Loading Dye.

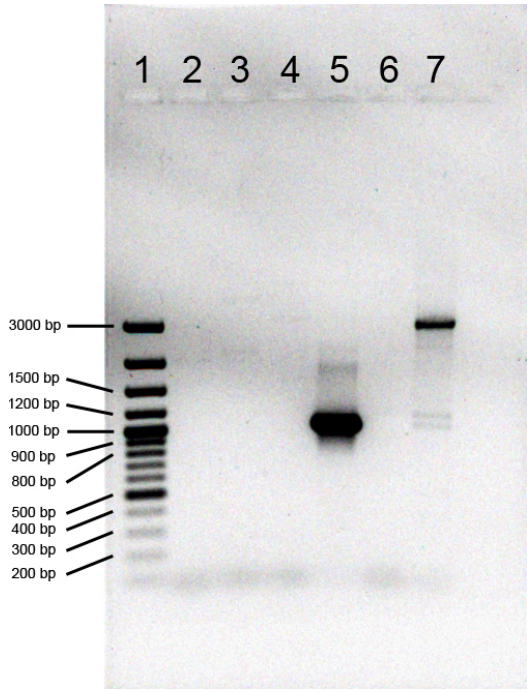

Figure 4.18. 1 % agarose gel to verify if pET-11a-mut-DEFB4A-GFP plasmid had been constructed, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 100 bp Plus DNA Ladder. Lane 2-7 contain 5 μl PCR product of PCR with DEFB4Aforw and GFP8pet primers and miniprep isolated plasmid DNA from 6 different transformed *E. coli* NEB5α colonies. DNA samples contain 1 μl 6x DNA Loading Dye.

To further verify the construct, NdeI and BamHI digestions of the plasmid DNA were performed. This would confirm if the size of the plasmid isolated corresponded to the size of pET-11a-mut-DEFB4A-GFP, and that the restriction sites (and start codon) were intact. The digestion products were studied on 1 % agarose gels (Figure 4.19 and 4.20). The size of the NdeI and BamHI digested pET-11a-mut-DEFB4A-GFP corresponded to the theoretical size of pET-11a-mut-DEFB4A-GFP: 6496 bp (Appendix E). Furthermore, it was observed that supercoiled DNA had migrated further down the gel than the digested DNA, which meant that digestion was successful, and that NdeI and BamHI restriction sites were intact.

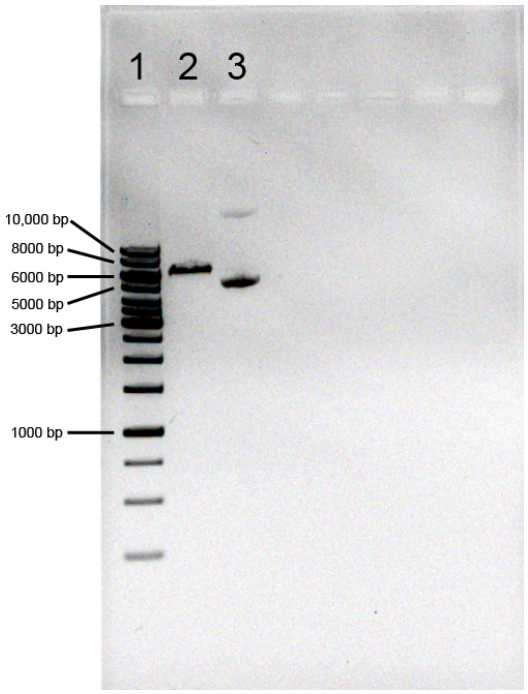

Figure 4.19. 1 % agarose for examination of NdeI digested pET-11a-mut-DEFB4A-GFP, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 1 μl pET-11a-mut-DEFB4A-GFP digested with 5 u NdeI for 1 hour at 37 °C. Lane 3 contains 1 μl pET-11a-mut-DEFB4A-GFP uncut plasmid. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

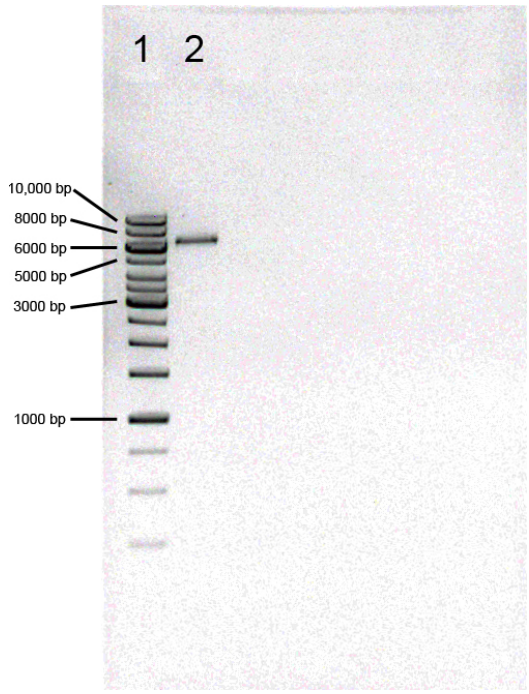

Figure 4.20. 1 % agarose for examination of BamHI digested pET-11a-mut-DEFB4A-GFP, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 1 μl pET-11a-mut-DEFB4A-GFP digested with 5 u BamHI for 1 hour at 37 °C. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

After verifying that pET-11a-mut-DEFB4A-GFP had been cloned, and that both restriction sequences were intact, a QIAGEN midiprep protocol was performed to obtain a high concentration and purity of plasmid DNA for sequencing. To check the concentration of the midiprep product, a 1 % agarose gel was made (Figure 4.21). The concentration was determined to $\sim$100 ng/μl. Sequencing was performed by Beckman Coulter Genomics (Takeley, United Kingdom). The sequencing data verified that mut-DEFB4A-GFP gene had been inserted into pET-11a vector (appendix F). Furthermore, the sequencing data showed that the PCR point-mutation was successful, and that there was no other mutations of the gene.

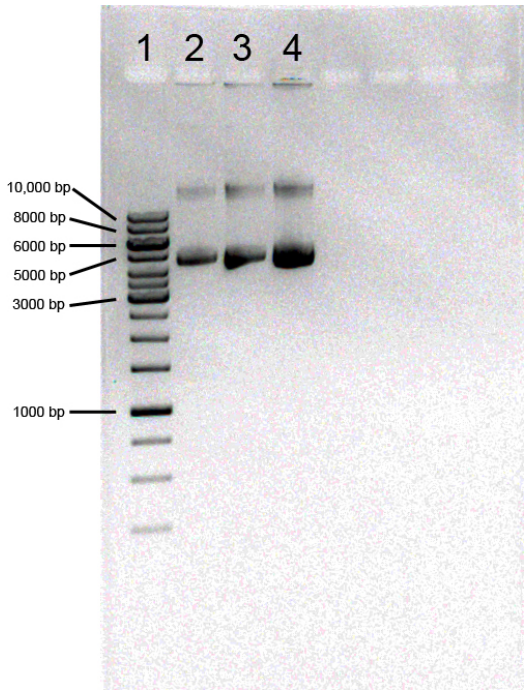

Figure 4.21. 1 % agarose gel for concentration determination of midiprep isolated pET-11a-mut-DEFB4A-GFP, run in 1X TAE buffer at 70 V DC electrical field for 90 min. Lane 1 contains 6 μl Generuler^{TM} 1 kb DNA Ladder. Lane 2 contains 0.5 μl midiprep isolated pET-11a-mut-DEFB4A-GFP. Lane 3 contains 1 μl midiprep isolated pET-11a-mut-DEFB4A-GFP. Lane 4 contains 2 μl midiprep isolated pET-11a-mut-DEFB4A-GFP. DNA samples contain water, molecular biology reagent, to 5 μl and 1 μl 6x DNA Loading Dye.

4.4 Expression of HbD-2-turboGFP fusion protein in *E. coli* Origami 2(DE3)

After verification of construct, *E. coli* Origami 2(DE3) cells were transformed with the isolated pET-11a-mut-DEFB4A-GFP plasmid. An 8 ml selective LB medium starter culture was grown for 12 hours at 25 °C, 230 RPM, and OD_{600} was measured to 0.555. 2x3 ml starter culture was transferred to 2x100 ml selective LB medium and grown for 12 hours at 25 °C, 230 RPM; OD_{600} was measured to 0.990 and 0.975 (after 6 hours OD_{600} was measured to 0.275 and 0.250). One of the cultures (OD_{600} = 0.990) was induced with 0.1 M IPTG, and the cultures were incubated for 11 hours at 25 °C, 230 RPM; OD_{600} was measured to 1.888 (+IPTG) and 2.352 (÷IPTG).

Expression was terminated by centrifugation. The cells were harvested in the first centrifugation, and it was visually noted that the IPTG induced cells had a greener colour than the uninduced. This was applicable after each centrifugation step - the pellet of the induced sample remained greener than the one of the uninduced. The harvested cells were resuspended in TES buffer, incubated for 30 min on ice to lyse the cells, and centrifuged. The crude cell lysate supernatants were saved for further experiments, the pellets were redissolved in Milli-Q water, and the samples were centrifuged. The supernatants and the pellets were saved separately.

4.5 Fluorescence spectroscopy

To examine if the fusion partner turboGFP had been folded correctly and retained its fluorescent function, fluorescence spectroscopy measurements were performed on the supernatant fractions collected by centrifugations in the expression experiment (Figure 4.22). turboGFP has excitation/emission max at 482/502 nm. Emission

was performed at 480 nm, and excitation was collected from 485 nm to 600 nm. The induced samples showed a much higher fluorescence at 502 nm than the uninduced, and the water fraction had a higher fluoresence intensity than the TES buffer fraction.

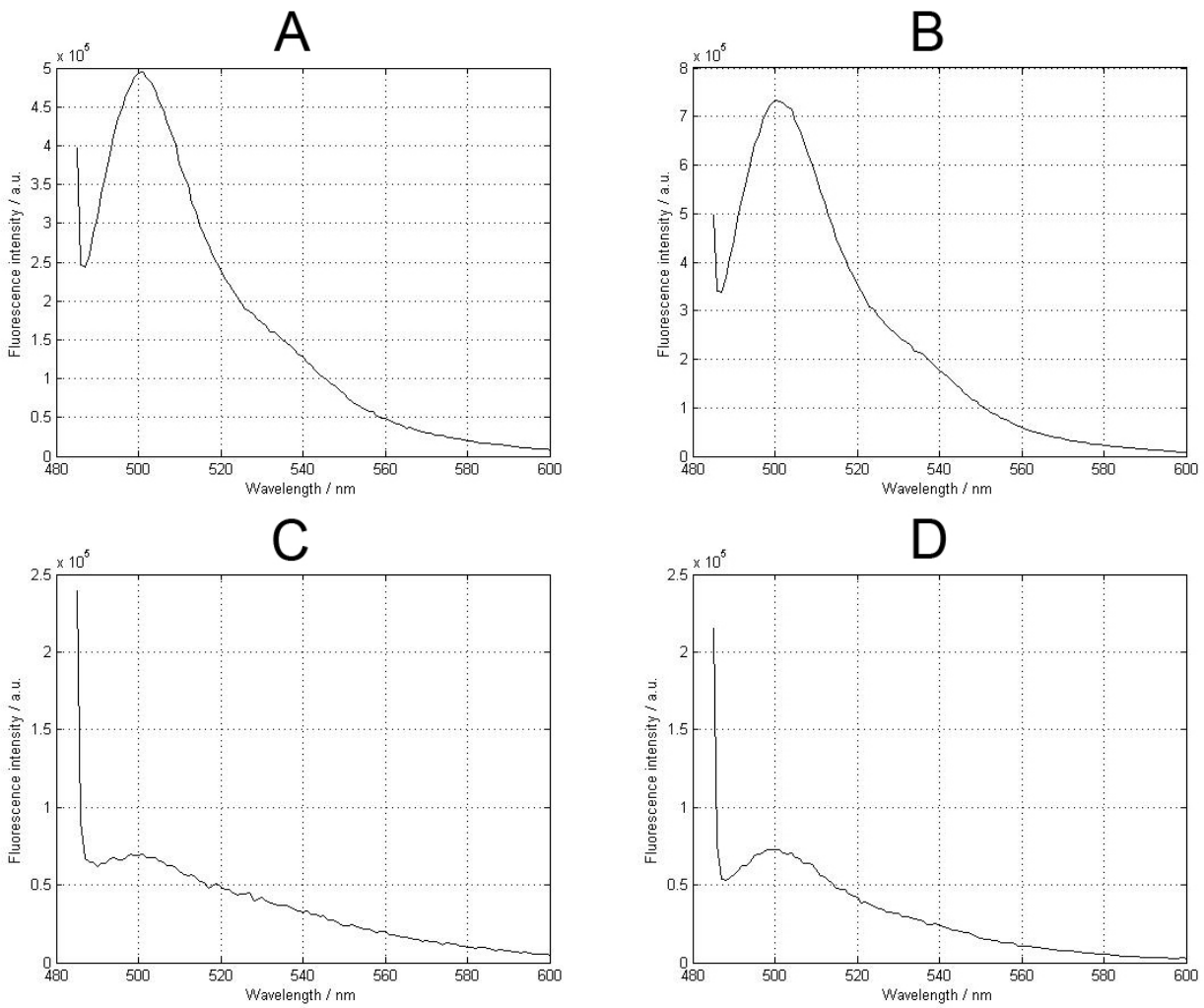

Figure 4.22. Fluorescence measurements of HbD-2-turboGFP fusion protein expressed in *E. coli* Origami 2(DE3) cells at 25 °C, 230 RPM. A) Induced with 0.1 M IPTG; TES fraction of protein collection protocol. B) Induced with 0.1 M IPTG; water fraction of protein collection protocol. C) Uninduced; TES fraction of protein collection protocol. D) Uninduced; water fraction of protein collection protocol.

The measured values at excitation wavelength of 502 nm was collected in a barplot (Figure 4.23). This indicated that the induced water fraction contained ∼50 % more folded turboGFP than the induced TES buffer fraction.

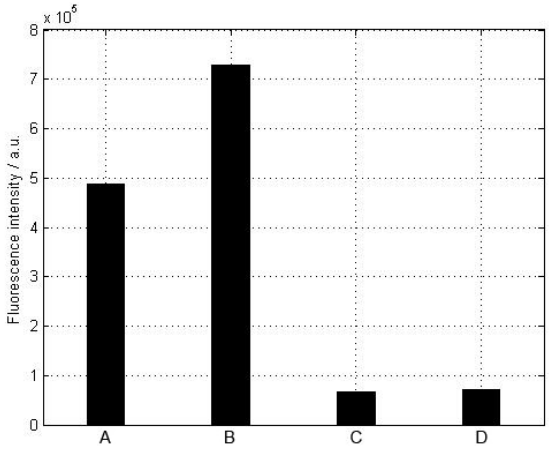

Figure 4.23. Barplot for illustration of fluorescence intensities measured at excitation wavelength of turboGFP: 502 nm. A) Induced with 0.1 M IPTG; TES fraction of protein collection protocol. B) Induced with 0.1 M IPTG; water fraction of protein collection protocol. C) Uninduced; TES fraction of protein collection protocol. D) Uninduced; water fraction of protein collection protocol.

4.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

To further analyse the expressed HbD-2-turboGFP protein, a 12 % SDS-PAGE was made in which the TES buffer and water fractions were loaded, but also the pellets (Figure 4.24). turboGFP has a molecular weight of 26 kDa, and HbD-2 has a molecular weight of 4.3 kDa. This gives a fusion protein with a molecular weight of 30.3 kDa. β-lactamase, the protein responsible for ampicillin resistance, has a molecular weight of 29 kDa. This made analysation of the gel more difficult, and as the OD_{600} of the harvested cells was almost 0.5 higher for the uninduced cells than the induced cells, hence the uninduced cells contained more β-lactamase. However, it was possible to identify a very strong band in the induced water fraction at 30 kDa. And there was also a pattern that the bands at 30 kDA in the water fractions were stronger than the ones in the TES buffer fractions. It was difficult to deduct anything from the pellet samples, as too much material was loaded.

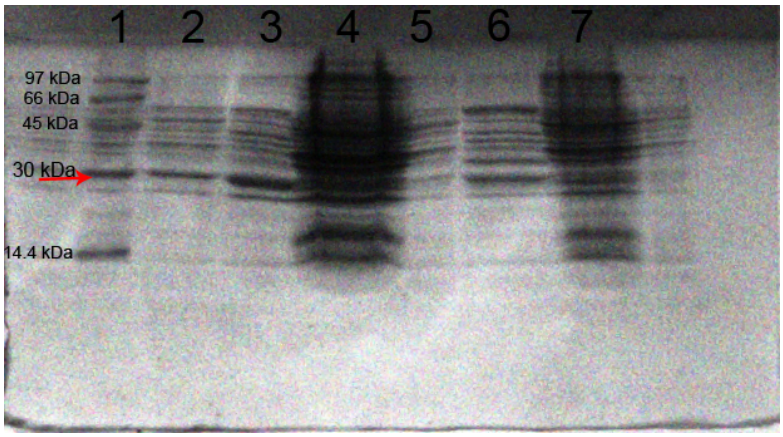

Figure 4.24. 12 % SDS-PAGE for analysis of cell fractions collected in HbD-2-turboGFP expression experiment. Lane 1 3 μl LMW standard. Lane 2 contains 10 μl induced TES fraction. Lane 3 contains 10 μl induced water fraction. Lane 4 contains 3 μl induced pellet sample. Lane 5 contains 10 μl uninduced TES fraction. Lane 6 contains 10 μl uninduced water fraction. Lane 7 contains 3 μl uninduced pellet sample. Samples were prepared by mixing 1:1 with 2x Laemmli sample buffer; pellets were prepared by mixing 1:5 with 2x Laemmli sample buffer. The red arrow is pointing at the row of gel bands at 30 kDa, which corresponds to the size of HbD-2-turboGFP.

5 Discussion

5.1 Cloning of pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase plasmids

Transformation of *E. coli* NEB5α with pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase plasmids yielded, as expected, hundreds of colonies on selective agar plates. 50 μl pCMV6-AC-DEFB4A-GFP plasmid DNA was isolated using GenElute™ Plasmid Miniprep Kit, with a total yield of 10 μg plasmid DNA. According to [Sigma-Aldrich, 2010], the miniprep kit can recover up to 15 μg high copy plasmid DNA. According to Origene [2009], the pCMV6-AC vector carries ColE1 origin of replication (ori) which makes it a low-copy plasmid, so a 10 μg yield is very high.

100 μl pET-11a-cutinase plasmid DNA was isolated using QIAGEN Plasmid Midiprep Kit, with a total yield of 20 μg plasmid DNA. According to [QIAGEN, 2005], the midiprep kit can recover up to 100 μg plasmid DNA, and according to Novagen [2011], the pET-11a vector carries pBR322 ori which makes it a low-copy plasmid as pBR322 has a copy number of 15-20 [Lee *et al.*, 2006]. Thus, in this case a yield of 20 μg plasmid DNA seems realistic due to the use of a low-copy plasmid.

A PCR was run to verify that pCMV6-AC-DEFB4A-GFP and pET-11a-cutinase plasmids had been isolated. The 1 % agarose gel of the PCR products (Figure 4.2) showed bands corresponding to the sizes expected (869 bp for pCMV6-AC-DEFB4A-GFP PCR product, and 1138 bp for pET-11a-cutinase PCR product). Thus, the cloning procedure proved successful. There was a vague secondary annealing product in the pET-11a-cutinase PCR; this could probably have been avoided by a higher annealing temperature, but as the PCR product was not used in further experiments, the secondary annealing was not of importance.

5.2 Construction of pET-11a-DEFB4A-GFP with partial digestion

OE-PCR product of DEFB4A-GFP was purified using a GenElute™ PCR Clean-Up Kit. The efficiency of this protocol was examined on a 1 % agarose gel (Figure 4.3). It seemed that almost 100 % of the PCR product was recovered, and this was justified as the clean-up kit should be able to recover up to 95 % of the DNA according to Sigma-Aldrich [2001].

The DEFB4A-GFP clean-up product was digested with BamHI to prepare it for NdeI partial digestion. The concentration after BamHI digestion was determined to 200 ng/μl. And in the partial digestion dilution series 0.1 μl (20 ng) was used. In the 1 % agarose gel with the partial digestion dilution series, two bands on top of each other were observed in the sample that had been digested with 0.0823 u NdeI for 10 min at 37 °C (Figure 4.5). This indicated that not all of the DNA had been digested. According to ThermoScientific [2012a], 1 u NdeI can digest 1 μg DNA in 50 μl reaction volume in 60 min at 37 °C. A downscaling of this unit definition gives: 0.0823 u NdeI can digest 13.7 ng DNA in 50 μl reaction volume in 10 min at 37 °C. So the theory that not all 20 ng DEFB4A-GFP was digested using 0.0823 u NdeI seems reasonable. And when not all DEFB4A-GFP

was digested, there was a probability that some of the digested DNA had only been partially digested.

However, after partial digestion with three different NdeI concentrations, ligation, transformation, and verification of construct experiment, it was concluded that partial digestion was not successful. And it was found that the plasmids created either were pET-11a with no insert or pET-11a with an overdigested DEFB4A-GFP insert (Figure 4.10).

A reason for the unsuccessful partial digestion could be attributed to a theory that NdeI has a much higher affinity for restriction of the on-gene NdeI restriction site. The OE-PCR product of DEFB4A-GFP used in these experiments only has 3 bp from the end of the DNA strand to the NdeI restriction sequence. According to ThermoScientific [2012a], NdeI restriction enzyme from Fermentas - which was used in the experiments - needs only 3 bp from the end of the DNA strand to the NdeI restriction sequence to perform restriction with 50-100 % cleavage efficiency. And so 3 bp seems sufficient, however, according to NEB [2000], NdeI restriction enzyme from New England BioLabs needs at least 7 bp from the end of the DNA strand to the NdeI restriction sequence to be able to perform restriction.

In later digestion experiments of mut-DEFB4A-GFP, NdeI displayed successful restriction with only 3 bp to the end of the DNA. So digestion close to the terminus is possible, but a preference for the on-gene NdeI restriction site could be the reason why partial digestion was unsuccessful.

5.3 Construction of pET-11a-mut-DEFB4A-GFP with PCR site-directed mutagenesis

mut1 and mut2 PCR products were successfully produced and a PCR Clean-up kit protocol was performed. mut1 and mut2 were successfully fused using three different concentrations. However, a lot of long-length DNA impurities was also produced in the fusion process (Figure 4.13). This was expected, as the DNA molecules in theory could anneal almost indefinitely, with reaction time being the only limiting factor.

Because of all this contamination, it was expected that using the least DNA for production of mut-DEFB4A-GFP would yield the best result. This expectation was met (Figure 4.14) as the PCR run with 0.5 μl fusion product by far yielded the purest PCR product, and the PCR run with 10 μl fusion product did not give any PCR product at all. The PCR protocol could maybe have been more optimised by PCR with only 0.1 μl fusion product; this could have influenced how many colonies that had to be checked for a positive construct in the verification experiment.

In the protocol where partial digestion was used, pET-11a was double digested with NdeI and BamHI. This double digestion was very inefficient, and only 600 ng vector could be digested for 5 hours at 37 °C. When more plasmid was digested, the sample contained background, i.e. undigested pET-11a after 5 hours. This low efficiency could be due to NdeI inhibiting BamHI activity, or vice versa, or because of low activity in 2x Tango buffer. More likely, it could have been because of isopropanol not being fully evaporated from the pET-11a plasmid which was purified by a propanol precipitation at the end of the QIAGEN midiprep protocol. Residual isopropanol in the sample would definitely reduce restriction activity and enzyme stability.

To try to overcome this, 15 μl pET-11a was instead sequentially digested, and in the preparative gel no background was observed. A large amount of pET-11a was lost during gel extraction from the preparative gel. According to [Merck, 2001], only $\sim$35 % of the DNA can be recovered because of its large size; up to 78 % can

be recovered if the size was 100 bp. This low activity of NdeI and BamHI toward pET-11a combined with low recovery from preparative gel, proved to be one of the bigger obstacles in the laboratory procedures. An obstacle because a high amount of pET-11a had to be digested to get anything for ligation, but when too much was digested, background was observed. Background could also be the reason why 12 colonies had to be screened before one with pET-11a-mut-DEFB4A-GFP plasmid was found. The rest of the isolated plasmids (Figure 4.17 and 4.18) could either have a DNA impurity inserted, could be pET-11a ligated with no insert (Figure 4.10), or could be undigested pET-11a-cutinase background.

After verification of construct, the construct was digested with both NdeI and BamHI enzymes to check that the size of the plasmid was as expected 6496 bp, and that digestion was possible - which would mean that NdeI and BamHI restriction sequences were intact, as especially the NdeI site can ligate in a way where restriction site, and thereby start codon is lost [ThermoScientific, 2012a]. The digestions were successful, as both NdeI and BamHI could linearise the plasmid, and the size of the linearised DNA was $\sim$6500 bp (Figures 4.19 and 4.20).

5.4 Sequencing of pET-11a-mut-DEFB4A-GFP

For sequencing of the isolated pET-11a-mut-DEFB4A-GFP plasmid, a QIAGEN midiprep was carried out to get the required concentration and maximal purity. The concentration was measured on a 1 % agarose gel, and again 20 μg plasmid DNA had been isolated, meaning that the yield of QIAGEN midiprep protocol was reproducable.

The sequencing was carried out by Beckman Coulter Genomics, and the sequencing data showed no mutations other than the one produced by PCR mutagenesis. Mutations could have occurred, because many PCRs were performed to make the final mut-DEFB4A-GFP, some carried out with 35 cycles, and Taq polymerase has an error rate of $2.2 \cdot 10^{-5}$ errors per nt per cycle [ThermoScientific, 2012b].

5.5 Expression of HbD-2-turboGFP fusion protein in *E. coli* Origami 2(DE3)

Expression of fusion protein HbD-2-turboGFP was carried out in *E. coli* Origami 2(DE3), which has a chromosomal copy of T7 RNA polymerase which makes expression controlable. The strain also has trxB and gor mutations, which greatly improves its ability to form disulfide bonds. As HbD-2 contains three disulfide bonds, this was an optimal strain for expression purposes. However, the DEFB4A-GFP gene does contain some rare codons: ATA, AGA, AGG, CCC. The percentage of rare codons was calculated to 4 % using GenScript Rare Codon Analysis Tool. One of the rare codons was removed by the point-mutation in which ATA was mutated to the regular ATT codon.

The obstacle of these rare codons could have been overcome by using an *E. coli* strain which co-expresses the tRNAs for these codons. An example of one of these strains is BL21 (DE3), which has already been used by many laboratories to express defensins in fusion proteins (Section 2.1). Another strain is the Rosetta 2 strain, which co-expresses tRNAs for seven different rare codons. The best strain however would be the Rosetta-gami B strain, which is a combination of the Rosetta strains and the Origami 2 strain. This strain has co-expresses the tRNAs for rare codons and has trxB and gor mutation for disulfide bond formation [Merck] [Novy *et al.*, 2001].

Expression was carried out at 25 °C to enhance protein folding and disulfide bond formation. The cells were induced with IPTG, and just before harvesting the cells by centrifugation OD_{600} was measured to 1.888 (+IPTG) and 2.352 ($\div$IPTG). This difference in growth rate was due to IPTG induction, as the cells were stressed to

expressing protein instead of growing. After harvesting the cells by centrifugation, it was noted that the induced cell pellet was more green coloured than the uninduced cell pellet. This meant that turboGFP had already folded and was fluorescently active.

5.6 Characterisation of HbD-2-turboGFP fusion protein

The crude cell lysates from TES buffer and water fractions were examined using fluorescent spectroscopy to measure the fluorescence of turboGFP. These measurements showed high fluorescence intensities in the protein samples isolated from the induced cells, and low intensities in those not induced with IPTG. The unindiced did however show a little fluorescence, indicating that the promoter was not 100 % tight, and that a little protein was expressed without induction. The fluorescence of the samples meant that turboGFP was correctly folded and active, but did not reveal anything about the folding and activity of HbD-2. However, the other research groups that expressed defensin fusion proteins removed the fusion partner by protease cleavage before measuring the antimicrobial activity (Section 2.1), so it seems that defensins are not active in fusion protein form.

The HbD-2-turboGFP fusion protein was further analysed on a 12 % SDS-PAGE gel (Figure 4.24). In this gel the TES buffer, water, and pellet fractions were loaded to investigate if a protein with the MW of HbD-2-turboGFP was present (30.4 kDa). The pellet sample would also reveal if inclusion bodies had been formed. A hurdle when analysing the gel was that β-lactamase, responsible for ampicillin resistance, has a MW of 29 kDA, so it would show in the same band as HbD-2-turboGFP. When the cells were harvested OD_{600} was measured to 1.888 (+IPTG) and 2.352 (÷IPTG), so the uninduced cells had an optical density that was 0.5 higher than the induced. This would further complicate the analysation, as the uninduced cells would contain an increased β-lactamase concentration compared to the induced cells. Had the cells been grown to the same OD_{600}, the amount of β-lactamase would have been the same in both samples, and could be subtracted in the SDS-PAGE gel analysation. Furthermore, too much pellet sample was loaded, so it was difficult to analyse what was in that sample. It does seem that the induced TES fraction contains the most protein, which correlates well with the result of fluorescence spectroscopy, in which the induced TES fraction had a fluorescence intensity at 502 nm that was almost 50 % higher than that of the induced water fraction.

6 Conclusion

As the DEFB4A-GFP gene had an on-gene NdeI restriction sequence, two approaches were conducted in an attempt to construct pET-11a-DEFB4A-GFP plasmid; the two approaches were an NdeI partial digestion and PCR site-directed mutagenesis.

Construction of pET-11a-DEFB4A-GFP plasmid was not succeeded by NdeI partial digestion of the gene. This approach only yielded pET-11a with no insert and pET-11a with an overdigested DEFB4A-GFP insert (digested on-gene). The partial digestion of 600 ng BamHI digested DEFB4A-GFP was tested with 1.5, 2.47, and 5 units of NdeI, and none of these concentrations worked as intended.

Construction of pET-11a-mut-DEFB4A-GFP plasmid was succeeded by PCR site-directed mutagenesis. Here, the on-gene NdeI restriction sequence was mutated from CATATG to CATTTG, and was no longer susceptible to NdeI restriction. This enabled the possibility of BamHI and NdeI overdigestion assays, followed by insertion into digested pET-11a vector.

The constructed pET-11a-mut-DEFB4A-GFP plasmid was sequenced by Beckman Coulter Genomics, and the sequencing data showed no mutations of the gene except for the induced point-mutation. The sequencing data also showed intact NdeI and BamHI restriction sequences.

Soluble fusion protein HbD-2-turboGFP was expressed in *E. coli* Origami 2(DE3) cells at 25 °C, induced with 0.1 M IPTG. It was confirmed that turboGFP was correctly folded and active by fluorescence spectroscopy measurements. These measurements also showed a much higher concentration of HbD-2-turboGFP in the IPTG induced samples than in the uninduced reference. Finally, the protein samples were loaded on an SDS-PAGE gel, the results were a little inconclusive, but the induced water fraction, which had the highest fluorescence intensity, also had the strongest band at 30.3 kDa, which is the size of HbD-2-turboGFP.

Perspective 7

After analysing, discussing and concluding on the experinental procedures and results thereof, it is now possible to put forward some ideas to what experiments that could succeed the experiments performed in this master's thesis.

First it would be interesting to further analyse the expressed HbD-2-turboGFP fusion protein. A new SDS-PAGE should be made, where the results are more clear. A bacterial growth experiment could be carried out, to analyse the bactericidal effect of the fusion protein, and to analyse the inhibitory effect of salt.

As all of the research articles found have expressed human defensins in fusion proteins and afterwards removed the fusion partner by proteolytic cleavage, it would be interesting to try a similar approach. In this, the fusion protein expression could be optimised by expression in *E. coli* Rosetta-gami B strain. But first an enterokinase protease sequence must be added between the DEFB4A and GFP genes. This would make it possible to express the protein, remove the turboGFP fusion partner, and test the proteolytic activity of HbD-2. HbD-2 could also be analysed with circular dichronism, to make sure it was correctly folded.

Fermentas GeneRuler™ DNA Ladders

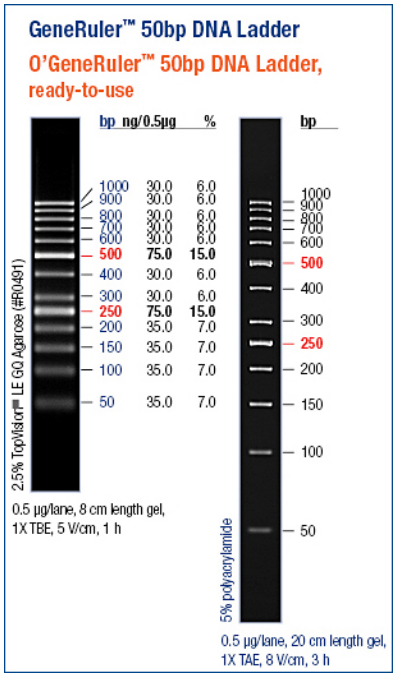

Figure A.1. GeneRuler™ 50 bp DNA Ladder.

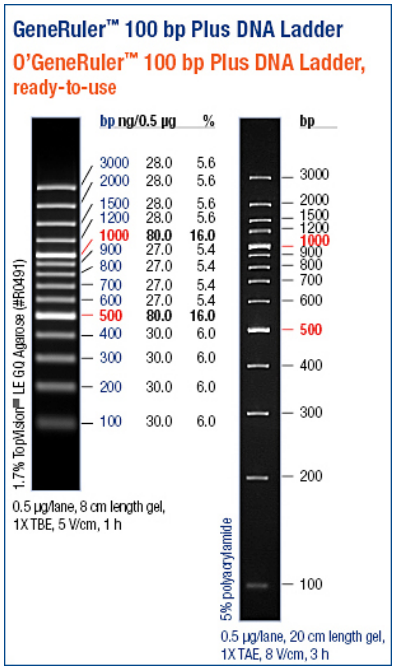

Figure A.2. GeneRuler™ 100 bp Plus DNA Ladder.

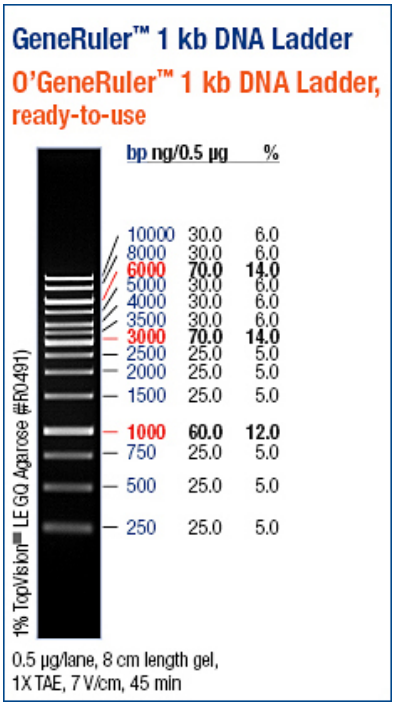

Figure A.3. GeneRuler™ 1 kb DNA Ladder.

Amersham LMW standard B

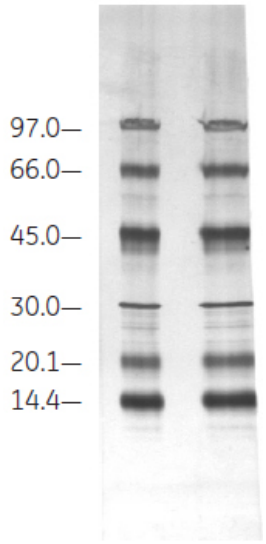

Figure B.1. Amersham Low Molecular Weight Standard for SDS-PAGE.

pCMV6-AC-DEFB4A-GFP plasmid map and sequence

C

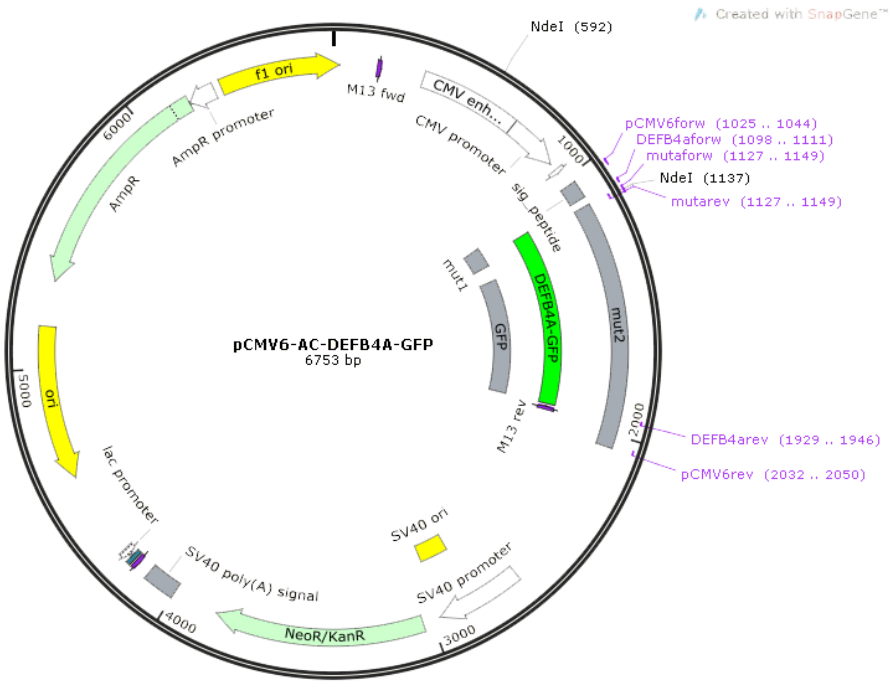

Figure C.1. pCMV6-AC-DEFB4A-GFP plasmid map. Created with SnapGene software.

pCMV6-AC-DEFB4A-GFP sequence

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A more graphical representation of the sequence can be found on the provided CD-ROM.

pET-11a-cutinase plasmid map and sequence

D

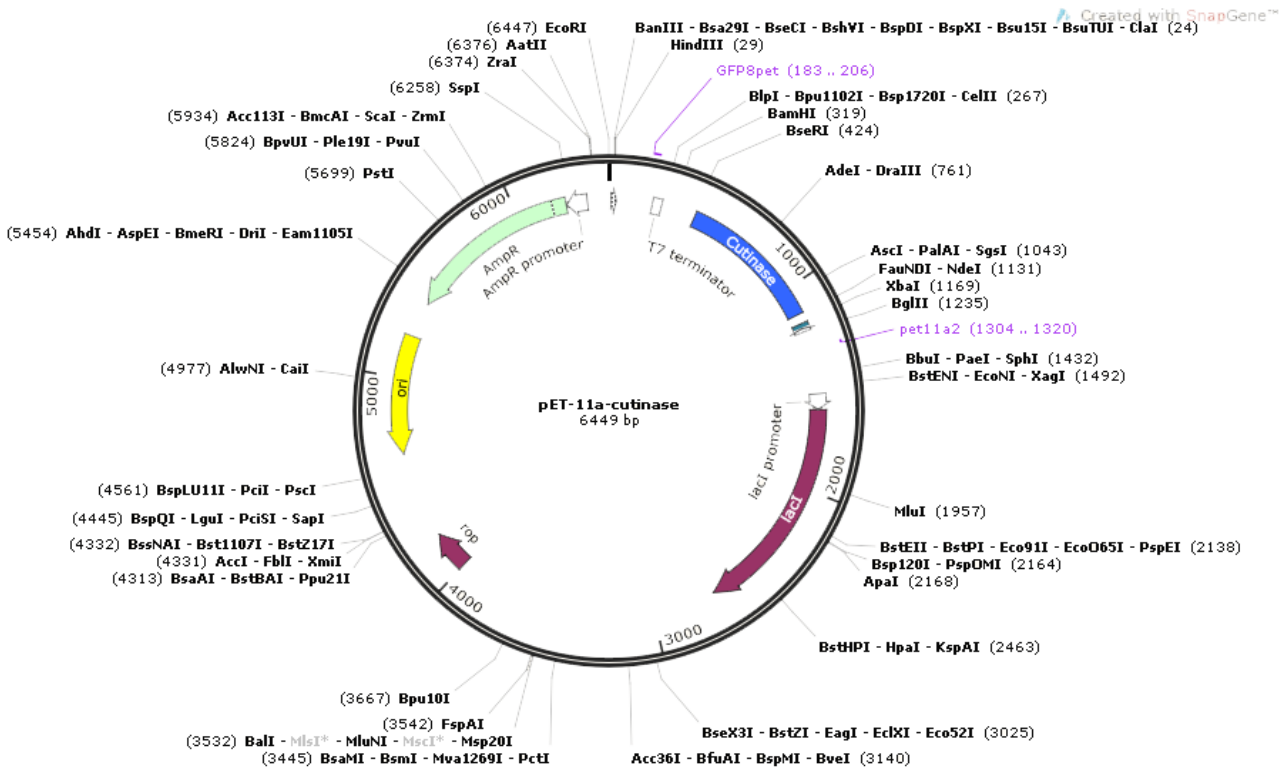

Figure D.1. pET-11a-cutinase plasmid map. Created with SnapGene software.

pET-11a-cutinase sequence

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CCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAA

A more graphical representation of the sequence can be found on the provided CD-ROM.

pET-11a-mut-DEFB4A-GFP plasmid map and sequence

E

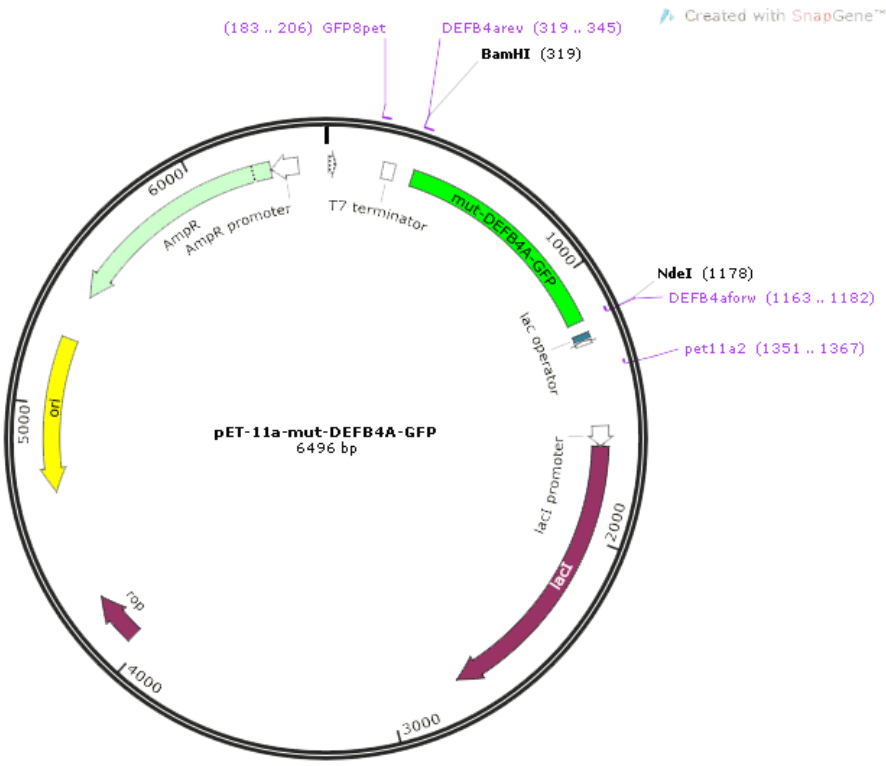

Figure E.1. pET-11a-mut-DEFB4A-GFP plasmid map. Created with SnapGene software.

pET-11a-mut-DEFB4A-GFP sequence

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AAGAA

A more graphical representation of the sequence can be found on the provided CD-ROM.

Sequencing data of pET-11a-mut-DEFB4A-GFP

F

Sequencing of pET-11a-mut-DEFB4A-GFP was performed by Beckman Coulter Genomics using Pet11a2 and GFP8pet primers.

Sequencing files can be found on the provided CD-ROM; these can also be viewed graphically.

Sequencing data using Pet11a2 primer

--TTGTGGC-TG----GCGCC-GGAGCCGGCCCGA--CGTCCGGCGTAGAGGATCGAGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGGAATTGTGAG
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ACGG---CCA--GAT-ACAATAA-CGCATTGTTAA-TTT------CGGG-CCGGATGGC-TT---ATTTAC---GGAAA-CA-CC--TTAA---TA-C---G-AAA-----CAAC----A--T---
TG-AAA--AAGG-CCT---A--CC---TTT-------AA--------AAAAAG------------GGG----TTTTTGGGAAA-G--------CCC---T-T--TTTTT--A--------AA---CCC------AAC--C---
-A----------TAAA---A-----AA--TAAT--------C-------------GG---C---TCT----T----------C-AAA------AAA--A----A---------------------------------C-----

Sequencing data using GFP8pet primer

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--A----CCC-A----TCC---GG---AAA-CTTT-CCG----GGGTAA--ACCCC--AAAAA--C---T--TGGGG-G----GAA----AA---TT---AA------AAAAA---GG-----T-----A---T------
----G---------CC---------TA-A----AAA--A-A-A---A---GG-------------------------A--------A----------

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