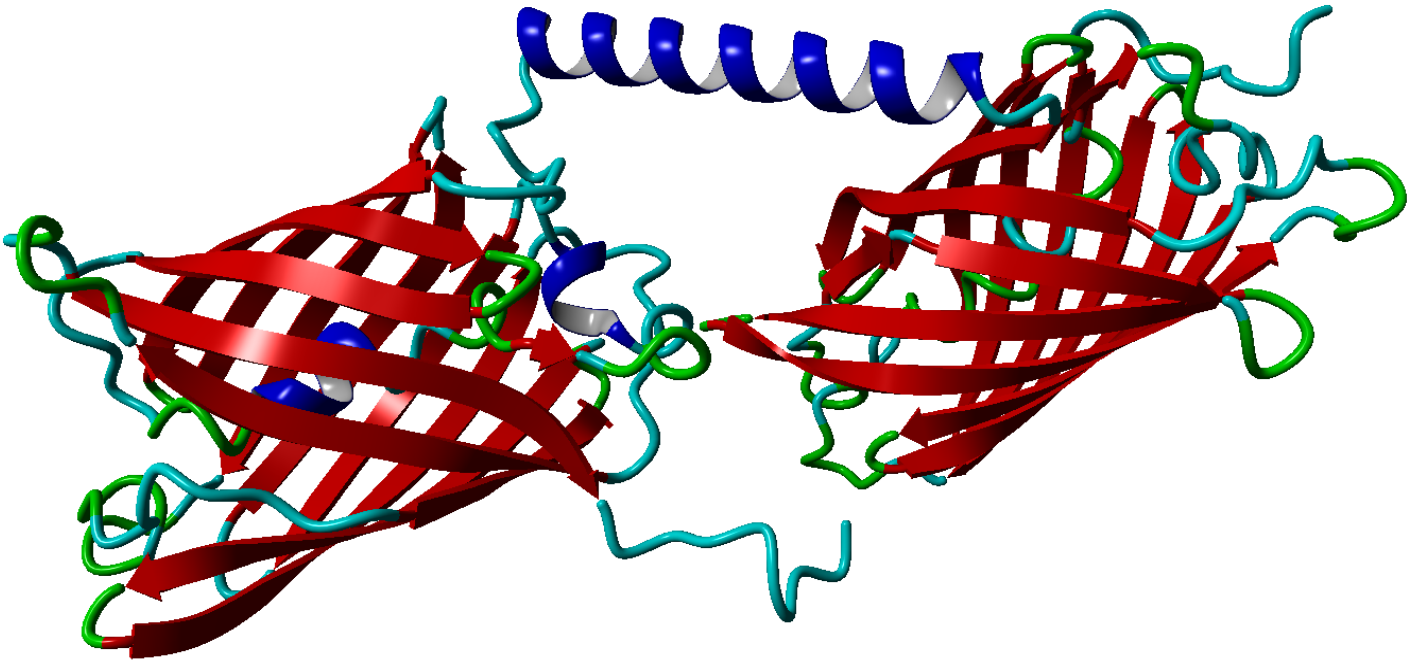
Expression and Investigation of RFP-GFP Fusion Protein



Master Thesis by
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Abstract

In this master's thesis attempts were made at constructing and expressing a TagRFP-T-Linker-GFP S65T fusion protein. These attempts were unsuccessful. Instead, the TagRFP-TagGFP fusion protein gene was transferred from pCasper3-GR to a pET11a vector using PCR, constructing pCasper3. The pCasper3 was expressed in *E. coli* BL21 (DE3). The fusion protein was analyzed using SDS-PAGE, fluorescence spectroscopy and absorption spectroscopy. SDS-PAGE showed expression of a correct size protein. Absorption spectroscopy showed maxima close to those of TagGFP and TagRFP. Fluorescence spectroscopy showed that FRET effects were responsible for the TagRFP fluorescence during TagGFP excitation. Temperature scan fluorescence spectroscopy was used to find the melting temperature of TagGFP which was 78 °C. The melting temperature of TagRFP was found to be at least 78 °C. Homology modelling found that the structure of the fusion protein was two β-barrels connected by an α-helix linker. The distance between the chromophore was found to be 5 nm. Further studies suggested that the linker formed a random coil structure and that the distance between the chromophores was more than 5 nm.

Danish Abstract

I dette kandidat speciale forsøgte vi at konstruere og udtrykke et TagRFP-T-Linker-GFP S65T fusion protein. Dette var forgæves. I stedet blev TagRFP-TagGFP fusion protein genes overført fra pCasper3-GR til en pET11a vector ved hjælp af PCR. pCasper3 blev udtrykt i *E. coli* BL21(DE3). Fusionsproteinet blev analyseret med SDS-PAGE, fluorescens spektroskopi og absorptions spektroskopi. SDS-PAGE viste at et protein med den rigtige størrelse blev udtrykt. Absorptions spektroskopi viste maksima tæt på dem for TagGFP og TagRFP. Fluorescens spektroskopi viste at FRET var ansvarlig for fluorescensen fra TagRFP når TagGFP blev eksiteret. Temperatur skannings fluorescens spektroskopi blev brugt til at finde smelte temperaturen for TagGFP. Den blev målt til at være 78 °C. Smeltetemperaturen for TagRFP blev målt til at være mindst 78 °C. Homologi modellering blev brugt til at finde strukturen af fusionsproteinet. Modelleringen viste at fusionsproteinet bestod af to β-barrels forbundet af en α-helix linker. Afstanden mellem chromoforene blev målt til at være 5 nm. Yderligere studier af linkeren antydede at linkene bestod af en random coil struktur, og at afstanden mellem chromoforene var længere end 5 nm.

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Preface

This Master thesis was written by the authors during the Masterâ€™s degree program Nanobiotechnology at the Department of Physics and Nanotechnology at Aalborg University during the period of September 1, 2014 - September 15, 2015.

Reading Guide

Throughout the report, there will be references to various sources. These will be found on the form [#] where the number in the angular brackets refers to a specific source in the bibliography at the end of the report. In the bibliography the sources will be listed with its title, author, and other relevant information depending on whether the source is a book, article, or web page. The bibliographic references will be listed after the specific section in which they are used; this indicates that the reference applies to all of the above if nothing else is stated.

Tables and figures are listed after the number of the chapter in which they are displayed. Hence the first figure in chapter 4 would be named 'Figure 4.1' whereas the next one would be 'Figure 4.2' et cetera. Since tables are numbered according to the same system both 'Table 4.1' and 'Figure 4.1' are possible in the same chapter. To each figure/table a short descriptive caption will be made together with a bibliographic reference where necessary. All figures can be found on the attached DVD.

Aalborg University, 15/09-2015

Michael Nissen

Rasmus Utoft

Abbreviations

BFP	=	Blue Fluorescent Protein
Casper3	=	Caspase 3 reporter
CFP	=	Cyan Fluorescent Protein
DOT	=	dissolved oxygen tension
DRM	=	Detergent resistant membranes
E. coli	=	*Escherichia coli*
FAK	=	Focal Adhesion Kinase
FbFP	=	Flavin-Binding Fluorescent Protein
FRET	=	Förster resonance energy transfer
GFP	=	Green Fluorescent Protein
IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
PCR	=	Polymerase Chain Reaction
RFP	=	Red Fluorescent Protein
SDS-PAGE	=	Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
YFP	=	Yellow Fluorescent Protein
Wt	=	Wild Type

Contents

Introduction 1

Dynamic interactions between molecules in cells are thought to have a significant role in cellular signal transduction pathways. Interactions between proteins plays a role in the regulation of these. Therefore the interaction between specific proteins in cells are of interest in many areas of biological research. Colocalization using immunofluorescence microscopy has been used to examine protein interactions *in situ*. [1] Here fluorescently labeled antibodies are used to determine the position of proteins. With two antibodies with different fluorescent labeling, it is possible to determine if two specific proteins are in the same area of a cell. However due to the rayleigh criterion the spatial resolution is limited to approximately 200 nm. This only tells us that the two proteins are in the same area of the cell, but it does not guarantee that they are interacting. [2]

1.1 FRET

Förster resonance energy transfer (FRET) is a physical process where energy is transferred from a fluorophore to another molecule (typically another fluorophore). The process was first proposed by Theodor Förster [3]. The process only occurs if the two fluorophores are within 10 nm or less of each other. Therefore, if two labelled proteins produce a signal you can be confident that they are interacting in some way. The Förster theory tells us that the FRET efficiency (E_{FRET}) varies as the sixth power of the distance between the two fluorescent molecules (r)[1]:

$$E_{FRET} = \frac{1}{[1 + (\frac{r}{R_0})^6]} \tag{1.1}$$

Where R_0 is the distance when $E_{FRET} = 50\%$. R_0 varies depending on the fluorophores used and can be calculated. E_{FRET} can be expressed graphically as seen in Figure1.1 with E_{FRET} as a function of the distance r. In the figure the distance is given in units of R_0.

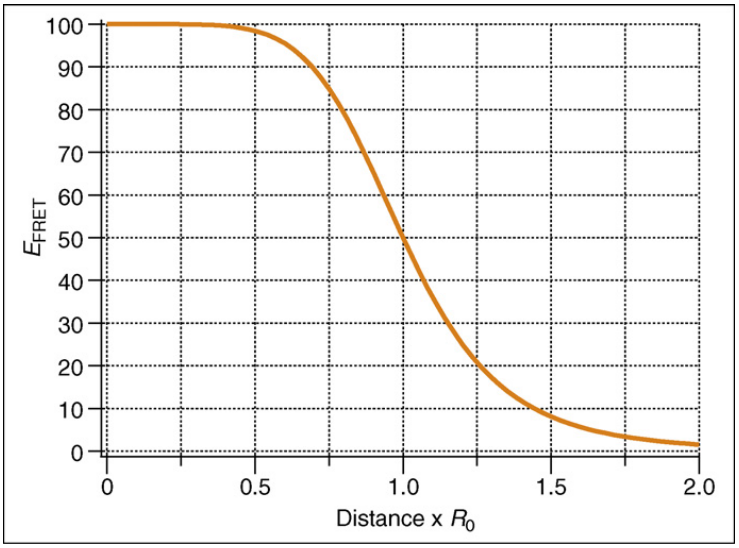

Figure 1.1: *FRET efficiency as a function of distance in units of* R_0.

Because of the $1/r^6$ dependancy there is a sharp slope. For distances less than R_0 E_{FRET} is close to maximal while distances greater than R_0 the efficiency is close to zero. When the distance is close to zero FRET can be used as molecular ruler to determine the distance between two fluorophores. These types of measurement can be used in structural biology but requires precise spectroscopic approaches. For most approaches the signal to noise ratio is often too low, and the distinguish is often between high FRET and low FRET, or FRET and no FRET.

The FRET efficiency can be found experimentally using the following Equation[4]:

$$E = 1 - \frac{F_{DA}}{F_D} \tag{1.2}$$

Where F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor and F_D is the fluorescence intensity of the donor in the absence of the acceptor. Once the FRET efficiency has been calculated the distance can be found using a rearrangement of Equation 1.1:

$$r = R_0 \sqrt[6]{\frac{1}{E} - 1} \tag{1.3}$$

R_0 can be calculated. The following Equation can be used to calculate R_0 is an aqueous solution:

$$R_0 = [2.8 * 10^{17} * \kappa^2 * Q_D * \epsilon_A * J(\lambda)]^{\frac{1}{6}} \tag{1.4}$$

Where κ^2 is the orientation factor, Q_D is the donor quantum yield, ϵ_A is the maximal acceptor extinction coefficient in Mol^{-1} cm^{-1} units and $J(\lambda)$ is the spectral overlap integral between the normalized donor fluorescence $F_D(\lambda)$, and the acceptor excitation spectra $E_A(\lambda)$ gives as:

$$J(\lambda) = \int F_D(\lambda) * E_A(\lambda) * \lambda^4 d\lambda \tag{1.5}$$

The two factors which needs further explanation are κ^2 and J(λ).

The orientation factor is dependent on the angles between the two fluorophores, and simply stated that the FRET transfer will be higher when the fluorophores are aligned parallel compared to when they are aligned perpendicular. The value ranges from 0 to 4 but is often assumed to be $\frac{2}{3}$ which is the average value when integrating over all possible angles.

J(λ) can more simply be describes as the region where the emission of the donor and the absorbance of the acceptor overlaps as seen in Figure 1.2.[1]:

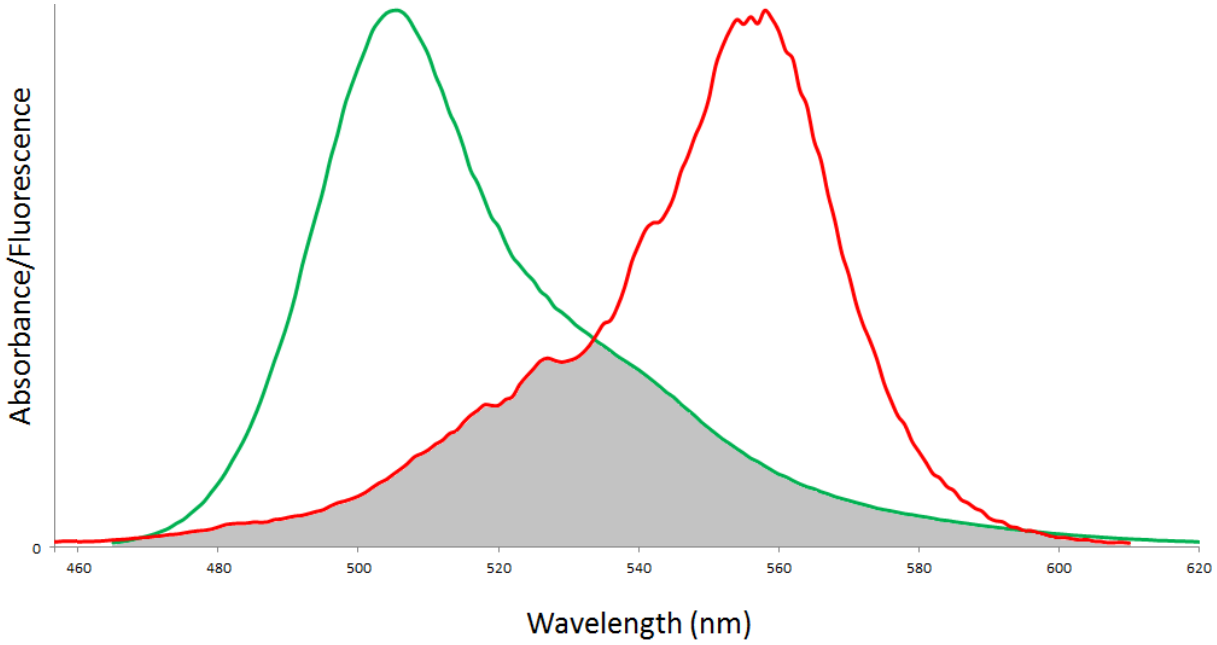

Figure 1.2: *Emission curve of TagGFP (Green) and excitation curve of TagRFP (Red) The gray area is the overlap. Made in Excel using data from Evrogen. [5]*

The other factors which affects the FRET efficiency is the quantum yield of the donor and the extinction coefficient of the acceptor. These two values are therefore an important factor to consider when choosing FRET pairs.

1.1.1 Applications

FRET can be used to analyze proteins interactions or changes in protein structure in different environments. For interaction between two proteins, one is labeled with a donor while the other is labeled with an acceptor. An example of this can be seen in Figure 1.3:

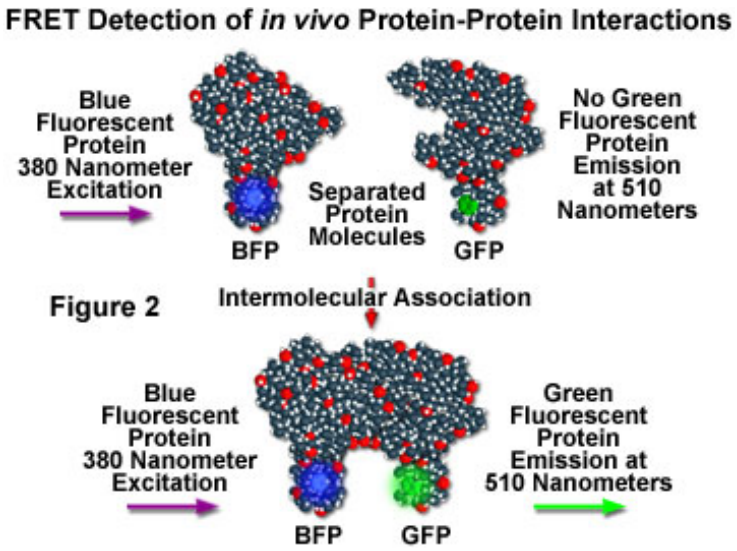

Figure 1.3: *Illustration interaction between two proteins. The interactions can be measured using FRET. [2]*

The blue fluorescent protein is excited and if there is a protein interaction, a green fluorescent signal will be observed. For structural analysis, the two fluorophores are attached to the same protein. When the environment of the protein changes, e.g. due to temperature or pH, a FRET signal is observed.An example of this can be seen in Figure 1.4:

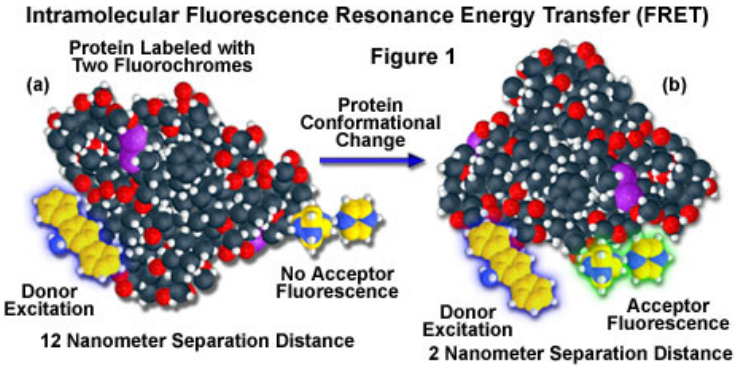

Figure 1.4: *Illustration of conformational change of a protein. The conformational change can be investigated using FRET. [2]*

1.1.2 FRET Pairs

Several FRET pairs has been identified and used. These have often been chemical fluorophores, however lately fluorescent proteins have been used in research. The advantage of these fluorescent proteins is that it is relatively easy to fuse them with other proteins using recombinant DNA technologies. Using these technologies the fluorescent protein can be linked to the N or C termini of the protein. The proteins are also easily expressed in organisms since they do not require additional factors to fold, and the chromophore of the proteins form spontaneously.

The first fluorescent protein to be identified was the Green Fluorescent Protein (GFP) from the jellyfish Aequorea victoria. GFP has primary structure of 238 amino acids with a molecular weight of 26.9 kDa. The tertiary structure is a 11-stranded β-barrel with an α-helix running along its axis. The chromophore consists of the amino acids ser^{65}, Tyr^{66} and Gly^{67}. First, the proteins forms into a near native state. The imidazolinone is formed by nucleophilic attack of the amide of Gly^{67} on the carbonyl of Ser^{65} followed by dehydration reaction. Following this reaction the α-β bond of Tyr^{66} is oxidised by molecular oxygen puttings its aromatic group into conjugation with the imidazolinone. [6] The reaction can be seen in Figure 1.5.

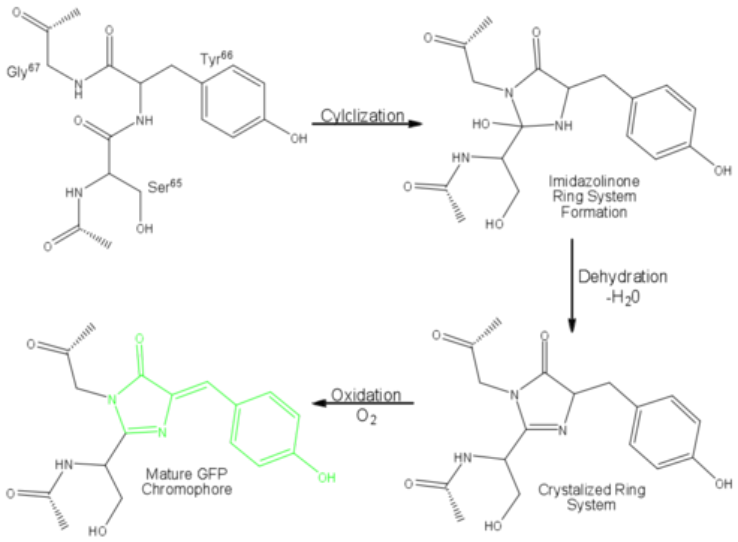

Figure 1.5: *Illustration of the maturation of GFP.[7]*

In this work four different fluorescent proteins has been used. These can be seen in the table below. GFP S65T and TagRFP-T were used for the original work, and TagGFP and TagRFP are part of the Casper3 fusion protein.

Protein	**Excitation maximum (nm)**	**Emmission maximum (nm)**	**Fluorescence quantum yield**	**Extinction coefficient M^{-1} cm^{-1}**	**Chromophore**	**Role in this work**
GFP S65T	489	509 - 511	0.64	52 - 58000	Ser65-Tyr66-Gly67	Donor
TagRFP-T	555	584	0.41	81000	Met67-Tyr68-Gly69	Acceptor
TagGFP	482	505	0.59	58200	Cys311-Tyr312-Gly313 (Position in fusion protein Construct)	Donor
TagRFP	555	584	0.41	100000	Met64-Tyr65-Gly66 (Position in fusion protein construct)	Acceptor

Table 1.1: *List of fluorescent proteins used in this work*

GFP S65T is a mutant of wild type GFP (wtGFP). wtGFP was originally discovered by Osamu Shimomura et al [8]. It was isolated from the jellyfish *Aequorea victoria*. wtGFP has a major excitation

maximum at 395 nm and a minor maximum at 475 nm. These two excitations produced two different emissions with maxima at 508 nm and 503 nm respectively. The S65T mutation greatly suppresses the 395 maximum and shifts the 475 nm excitation maxima to 489 nm and shifts the emission maxima to 509-511 nm. [6]

TagGFP is a mutant of a GFP like protein. The wild type protein was found in *Aequorea macrodactyla* which is another jellyfish [9]. The protein shares 80% of its amino acid sequence with wtGFP, and the chromophore is the same. Evrogen used this protein as a basis for TagGFP. One noticable difference between the GFP like protein and TagGFP is that the chromophore is changed from Ser-Tyr-Gly to Cys-Tyr-Gly.

TagRFP was reported by Ekaterina M Merzlyak et al [10]. The protein is a mutant of eqFP578 from the sea anemone *Entacmaea quadricolor*. The protein is a bright red dimeric fluorescent protein. The mutations resulted in TagRFP being a monomeric protein. TagRFP-T was reported by Nathan C Shaner et all [11]. The protein is a mutant of TagRFP. TagRFP-T has a single mutation at S158T which improved its photostability nine fold. The formation of the TagRFP chromophore is similar to that of GFP, however it includes an additional dehydration reaction of the amino-α-carbon bond of the first amino acid. This reaction is performed before the oxidation of the Tyr α-β bond. Figure 1.6 shows the full chromophore formation of DsRed which in another red fluorescent protein. The formation of the TagRFP chromophore is identical however the chromophore is formed by Met-Tyr-Gly instead of Gln-Tyr-Gly.[12]

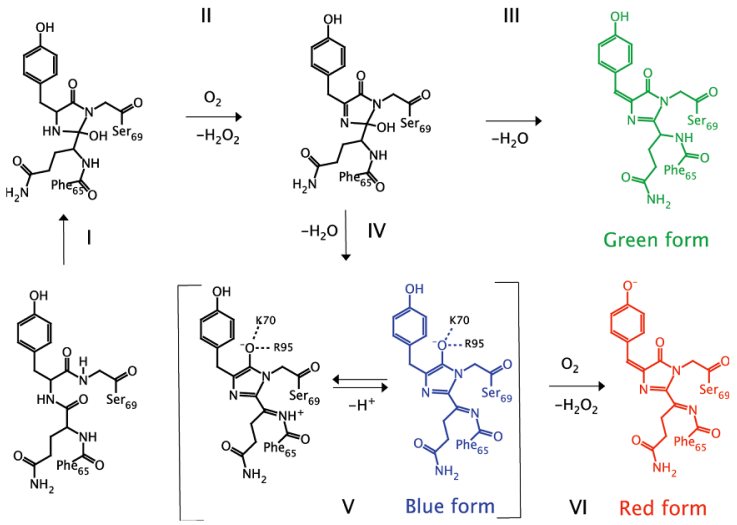

Figure 1.6: *Maturation of the chromophore of DsRed.*

1.2 ProteinConstruct

The desired fusion protein can be split in three sub-components. At the n-terminal of the protein we have RFP, at the C-terminal we have GFP and between these there is a short peptide which consists of seven amino acids. At the start, the GFP S65T is in a pet11a vector (pGFP). The goal is to ligate the linker and RFP gene upstream with respect to the GFP gene. pGFP is digested using the restriction enzymes *Nde*I and *Sma*I . The linker where ordered with these restriction sites and were ready to use for ligation. The linker and pGFP was ligated to produce pLGFP. PCR was used to add restriction sites *Nde*I and *Sal*I to RFP. After the PCR modification the PCR product and pLGFP was digested using the restriction enzymes *Nde*I and *Sal*I, followed by a ligation to produce pRFPLGFP. The process can be seen in Figure 1.7

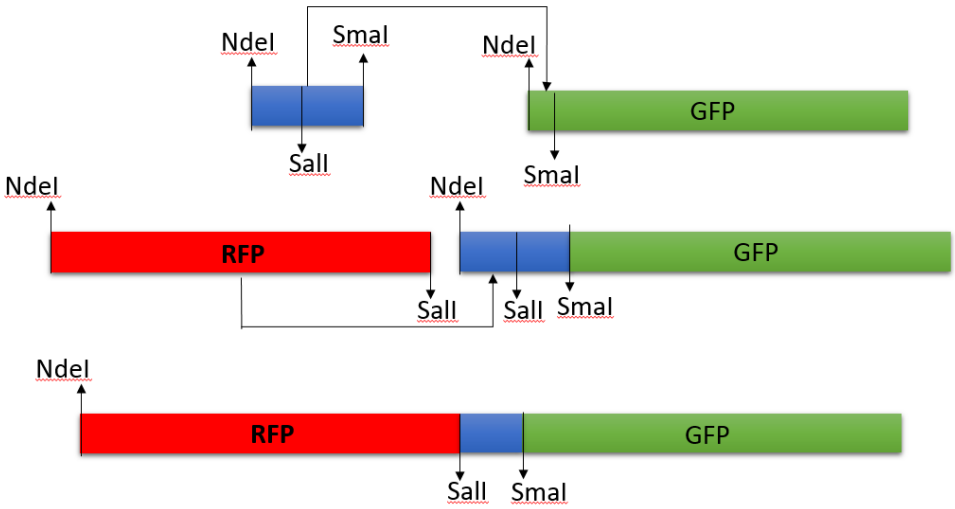

Figure 1.7: *Illustration of the final gene product where the linker and GFP are located downstream of the RFP gene. The green color represents GFP, blue the linker and finally red the RFP.*

1.3 Linker

As a product of recombinant DNA technology, fusion proteins have been developed as a class of novel biomolecules with multi-functional properties. A fusion protein is two or more protein domains connect with a linker. The linker is generally a peptide. The linkers purpose is to ensure a distance between the two protein. A direct fusion of functional domains without a linker may lead to many undesirable outcomes, including misfolding of the fusion proteins, low yield in protein production, or impaired activity. Therefore, construction of a suitable linker is an important part of producing a recombinant fusion protein.

1.3.1 Natural linker

In order to understand linker construction studies of linkers found in natural occurring proteins have been made. Argos[13], George et. al[14] has researched properties of natural linkers including linker length, hydrophobicity, amino acid residues and secondary structures. They found the average length of linkers in natural multi-domain protein were 6,5 residues and 10±5,8 respectively. Additionally they found that the average hydrophobicity of the linker decreased with the increase of length, indicating that longer linkers were more hydrophilic and therefore more exposed in the aqueous solvent than shorter linkers. There were some disagreement in which amino acids are preferable but both suggested glutamine (Gln), proline (Pro) and threonine (Thr).Argos also suggested serine (Ser), glycine (Gly), aspartic acid (Asp), lysine (Lys), asparagine (Asn) and alanine (Ala). Whereas George and Heringa suggested arginine (Arg), phenylalanine (Phe), glutamic acid (Glu). Considering the secondary structure of the linkers, George and Heringa found 38.3% α-helix and 37.6% coil/bend, while Argos found that 59% adopted coil structures. The discrepancy might be explained by the fact that George and Heringa examined on average longer linkers which were able to form α-helices.[15]

1.3.2 Flexible linkers

A flexible linker is used when joining domains that require some distance between them but also a certain degree of movement or interaction. They are generally composed of small, non-polar (eg. Gly) or polar (e.g. Ser or Thr) amino acids. The small size of these amino acids provide flexibility, and allows for mobility of the connecting functional domains. The polar amino acids like Thr and Ser can form hydrogen bonds with the water molecules in aqueous solutions and thereby maintain the stability of the linker. Ser or Thr is in this manner reducing the unfavorable interaction between the linker and the protein moieties. Most of the commonly used flexible linkers are made of Gly and Ser. An example of one of the most flexible linkers has the sequence (Gly-Gly-Gly-Gly-Ser)n, where n is the number of times the sequence is repeated. [15]

In summary, flexible linkers are generally rich in small or polar amino acids such as Gly and Ser to provide good flexibility and solubility. They are a good choice when movements or interactions for fusion protein domains are required. Flexible linkers can serve as a passive linker to keep a distance between functional domains. The length of the linkers can be change to allow a proper folding of the fusion proteins or to achieve optimal biological activity.[15]

1.3.3 Rigid linkers

Although flexible linkers have the advantage to connect the functional domains passively and permitting certain degree of movements, the lack of rigidity can be a problem. There are several examples in the literature where the use of flexible linkers resulted in poor expression yields or loss of biological activity. In these examples the ineffectiveness of flexible linkers were due to an inefficient separation of the protein domains or insufficient reduction of their interference with each other. The solution to this problem is to make a more rigid linker to keep a fixed distance between the domains. A way to make a more rigid linker is to include several prolines in the sequence as it imposes strong conformational constraints. Another method is to create a rigid linker by making a stiff α-helical linker. The α-helical structure is rigid and stable. Studies were made There have been made test on these a-helical linkers to see if they could effectively separate the protein domains. This was done by linkers between blue fluorescent protein (EBFP) and enhanced green fluorescent protein (EGFP) and then measure the (FRET) efficiency between EBFP and EGFP. The FRET efficiency could then be used to measure the distance between the two fluorescent proteins. The study found that the FRET efficiency decreased as the length of helical peptides increased, indicating that helical linkers can control the distance between domains, while flexible linkers of the same length were not nearly as efficient at reducing the FRET signal. In other words, the helical linkers can separate functional domains more effectively.[15]

In conclusion, a rigid linker have relatively stiff structures and can be created as a α-helix structure or by containing multiple Pro residues. Under many circumstances, the rigid linker can separate the functional domains more efficiently than the flexible linkers. Additionally the length of the linkers can easily be adjusted by changing the copy number to achieve an optimal distance between domains.[15][16]

1.3.4 Cleavable linker

The linkers discussed earlier generally consist of stable peptide sequences that will not be preferentially cleaved *in vivo*. These stable linkers covalently join functional domains together to act as one molecule.[15] This give many advantages such as prolonging the plasma half-life (e.g. albumin). However it can also potential have some drawbacks including steric hindrance between functional domains, decreased bioactivity, altered biodistribution and unwanted interference between the domains. If this is the case, cleavable linkers can be made to release free functional domains. These cleavable linkers need to have sequences which are cleaved under specific conditions such as the presence of reducing reagents or proteases which can be seen in Figure 1.8. [15]

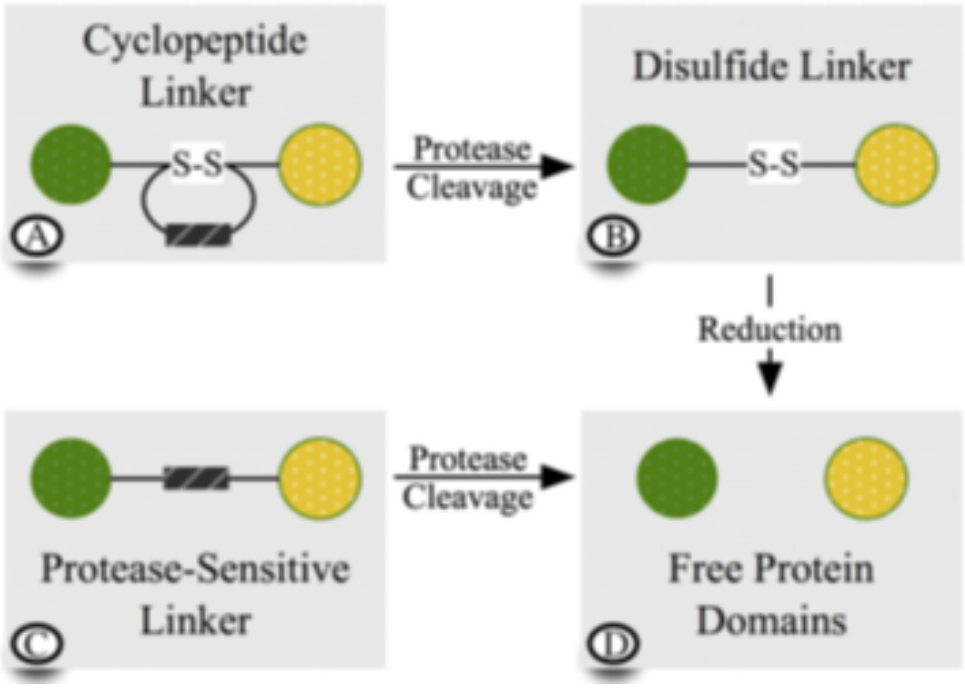

Figure 1.8: *Examples of linkers cleavable by reduction and protease.[15]*

One well-studied *in vivo* process is the reduction of disulfide bonds, which has been applied in drug delivery by chemical conjugation methods. An example of such a disulfide linker is (LEAGCKNFFPR↓SFTSCGSLE), which is based on a dithiocyclopeptide containing an intramolecular disulfide bond formed between two cysteine (Cys) residues in the linker.[15]

The protease cleavable linker can be used to measure the activity of proteases using FRET. A FRET pair linked by a cleavable linker will have a specific FRET efficiency. When the linker is cleaved the signal will change, and the change in signal will be proportional to the activity of the protease.[15]

1.3.5 Engineering of RFP-GFP linker

The linker in this work was designed to be a flexible linker between the RFP and GFP proteins. The requirements for the linker was that it has to relative short(less than 20 amino acids) and it needed to have a number of restriction sites for restriction enzymes that had to be used during the construction of the recombinant fusion protein.

First a preliminary linker made by designing a DNA sequence with certain restriction sites, this was then translated into an amino acids sequence to investigate if they would resemble a flexible linker. The linker was slightly modified to avoid any problematic amino acids. The final DNA sequence of the linker can be seen in Figure 1.9.

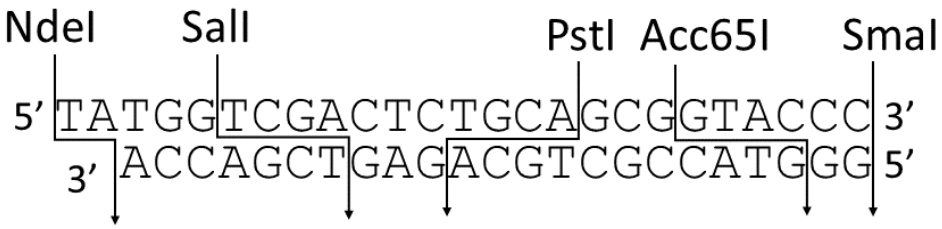

Figure 1.9: *DNA sequence for the linker including restriction sites.*

The linker is mostly made out of small or polar amino acids. When the fusion protein was made the linker has been shortened from nine to seven amino acids, but it still kept two restriction sites. These are important for continuous work, where it is possible to insert a more complicated linker to check for enzymatic activity of a protease for example.

The linker consists of the amino acids (Val - Asp - Ser - Ala - Ala - Val - Pro) and can be seen on Figure 1.10

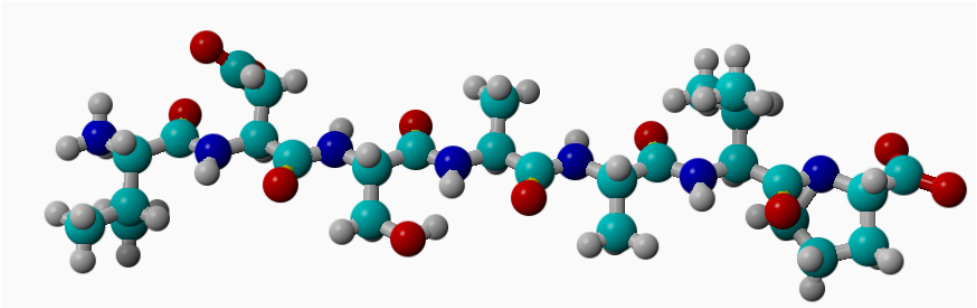

Figure 1.10: *Show the linker used in this project in free form from the N- to the C-terminal. Molecular graphics created with YASARA*

1.3.6 CaspeR3

CaspeR3 (Caspase 3 Reporter) is a fusion protein used for detection of caspase 3. The fusion protein consists of TagRFP linked to TagGFP using a 17 AA peptide linker containing the caspase-3-cleavable motif DEVD. Caspase 3 is a protease and key mediator of apoptosis of mammalian cells. [17] The fusion protein gene is found in pCasper3-GR, which is a mammalian expression vector and was transferred to a pET11a vector using PCR. The PCR introduced a *Nde*I restriction site at the 5' end of the gene and a *Bam*HI restriction site at the 3' end of the gene. The PCR product was ligated with a *Nde*I/*Bam*HI digested pET11a vector constructing pCasper3. The process can be seen in Figure 1.11.

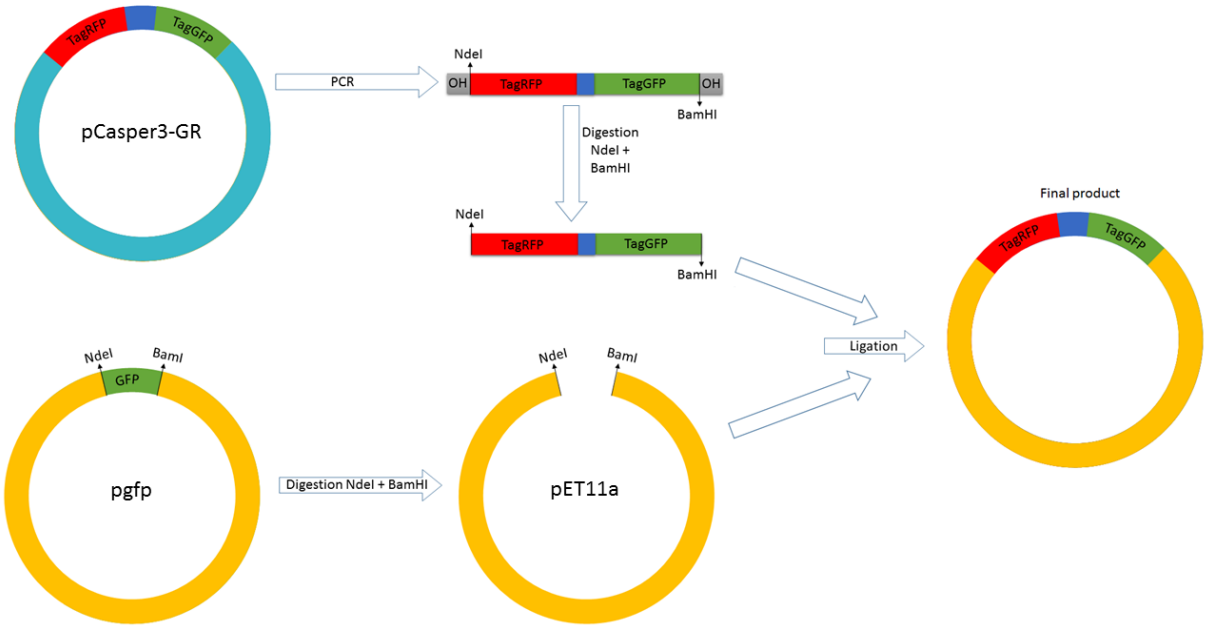

Figure 1.11: *Illustration of construction of pCasper3*

1.4 *E. coli* as an expression system

During expression of recombinant proteins, it is important to consider the various expression systems. In this work, the protein of interest is a combination of GFP, linker and RFP. Neither GFP, the linker or RFP requires post-translational modifications and therefore *E. coli* was chosen as expression system using the expression vector pET11a.

1.4.1 Expression vector

The pET11a vector is one of several pET vectors. It consists of approximately 5700bp [18] and can seen in Figure 1.12.

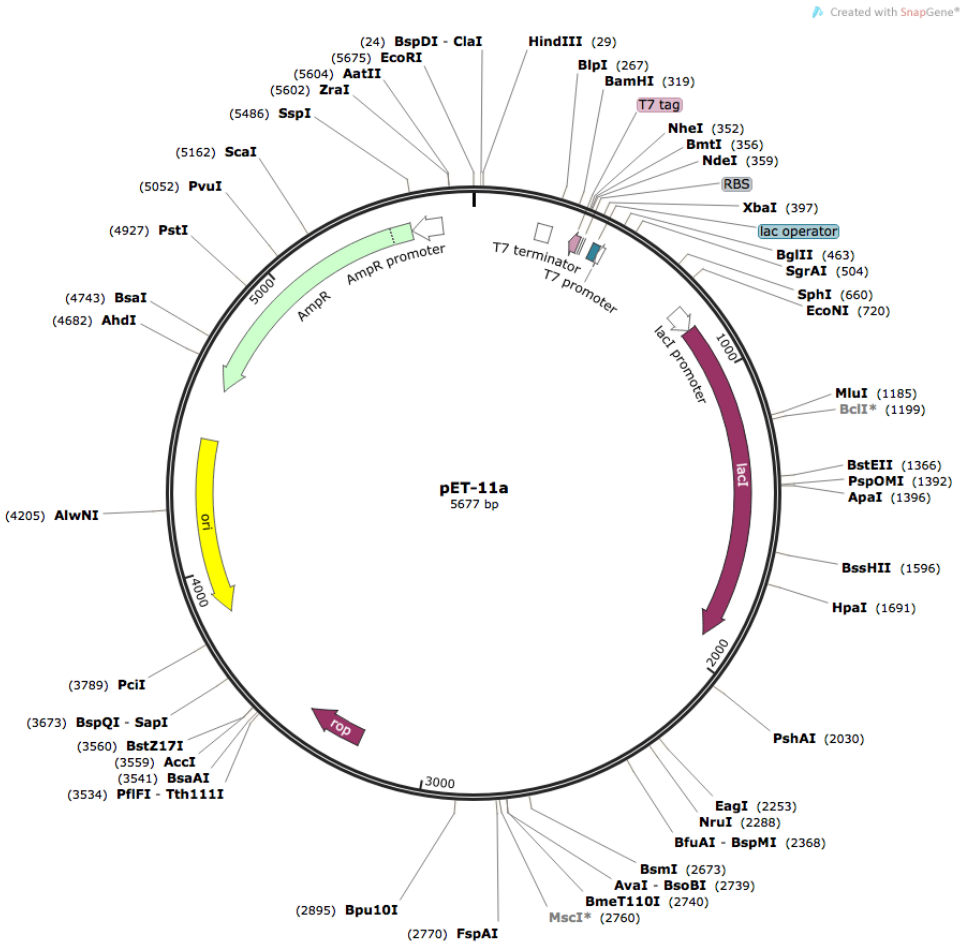

Figure 1.12: *Mapping of pET11a vector [19]*

Expression systems are designed in a way, such that the protein of interest would be expressed in as many copies as possible within the host cell. The vector needs to be inserted into the host cells, and have to include all the genetic coding necessary to yield a protein. This includes a promoter designed for the

host cell and sequences of nucleotide, which are responsible for translation, termination and ribosome binding.

How it works: IPTG in->production of T7 rna polymerase by host cell->T7 binds at promoter->makes mRNA (Transscription)->ribosome makes protein(Translation)!

The pET11a contains the T7 promoter, which comes from bacteriophage T7. In order for the promoter to work, the host cell needs to provide the T7 RNA polymerase. The T7 RNA polymerase is so selective and active that almost all of the hosts resources are used to express the target protein. In fact, up to 50% of the total protein in the cell consists of the target protein within a few hours after induction.[20] The production of T7 RNA polymerase by the host cell is tightly regulated and expression should not be started until induction is performed. Even tho production is regulated some T7 RNA polymerase will be expressed which will inhibit basic functions of the host cell. Therefore cloning is often performed in cloning strains, which does not carry the T7 RNA polymerase gene. [20]

Once the plasmids have been stabile established in the cloning host, they are transferred to an expression host containing a chromosomal copy of the T7 RNA polymerase gene (DE3 lysogen) under T7lac control. The lac promoter, which is responsible for T7 RNA polymerase can be induced by adding either IPTG or lactose.[18] This system can be seen in Figure 1.13.

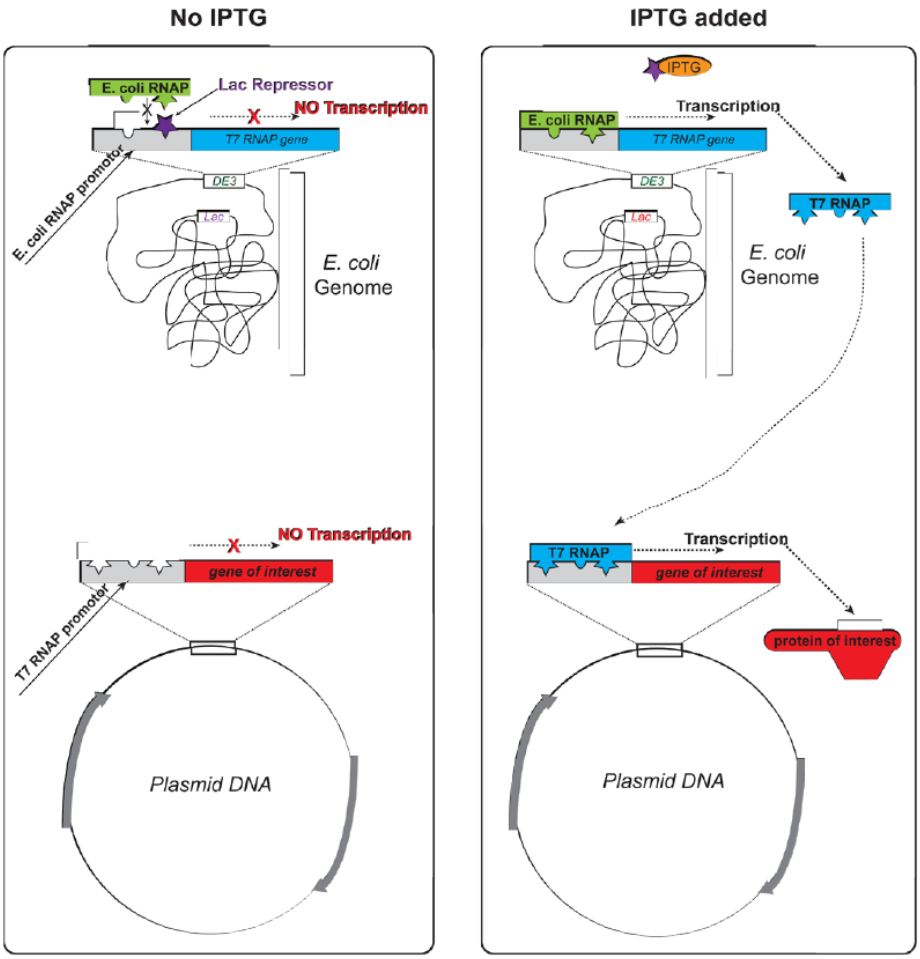

Figure 1.13: *Illustration of T7 lac expression system.[21]*

1.4.2 Cloning strain

For the cloning procedures, competent *E. coli* strain DH5-α was chosen. This strain contains endA1 mutation, which prevents plasmid DNA from being degraded. Furthermore this strain has a high transformation efficiency of 1-3 x 100 colony forming units/μg. The DH5-α is a cloning strain and does not have T7 RNA polymerase gene incorporated into the plasmid.[22]

1.4.3 *E. coli* expression Strain

The strain used in this project is *E. coli* BL21(DE3). The BL21(DE3) contains the T7 RNA polymerase gene and is under control of the lac promoter. This means that in order to produce the protein of interest an inducer (IPTG or lactose) needs to be added. When IPTG is added, the T7 RNA polymerase gene is expressed and thus the gene of interest can be transcribed. [18]

Materials and Methods 2

2.1 Materials

This section contains tables of the different materials used during the experiments. The materials are separated into tables depending on their function: plasmid DNA, Bacterial strains, Enzymes, buffers, Primers, kits, chemicals. The tables contains information about the supplier, the materials and lot/batch numbers.

Buffer	**Description**	**Supplier**
Running buffer TGS	25 mM Tris, 0.2 M glycine, 0.1% SDS	In house
10 x Dream *Taq* Buffer	Lot:00093326	Fermentas
10 x Classic *Taq* Buffer	Lot:PB604-2	PCR Biosystems
10 x Tango Buffer	Lot:00017765	Fermentas
10 x O Buffer	Lot:000024568	Fermentas
Reaction Buffer A	Lot:00024320	Fermentas
10 x Ligation Buffer	Lot:0011101	NEB
1 x TAE Buffer	40 mM Tris acetate, 1 mM EDTA, pH 8.2	In house
TES Buffer pH 7,47	50 mM Tris pH 7.5, 10 mM EDTA, 20% sucrose	In house
Running Buffer	Lot:68879A	Bio Rad
Phosphate buffer	1 m Potassium phosphate buffer pH 6.0	In house

Table 2.1: *A list of the different buffers used in this project*

Chemicals	**Description**	**Supplier**
6x Loading Dye	0071285	Fermentas
ATP	Lot: 077K7012	Sigma Aldrich
Agar	Lot:BCBC2317	Sigma Aldrich
Agarose	Lot:100Mg432V	Sigma Aldrich
Ampicillin	Lot:A9518 -25G	Sigma Aldrich
Nuclease free water	Lot:RNBC3293	Sigma Aldrich
Deoxyribonucleoside triphosphate set PCRGrade		Roche
Elution solution	Lot:024K6600	Sigma Aldrich
Ethanol	Lot:SE10012182	Kemetyl
Ethidium bromide	Lot:SLBF7132V	Sigma Aldrich
Ethylenediaminetetraacetic acid	Lot:056k0078	Sigma Aldrich
GeneRuler 1 kb ladder	Lot:00065513	Fermentas
Isopropanol	Lot:SZBE058BV	Sigma Aldrich
Isopropyl β-D-1-thiogalactopyranoside	Lot:5896F	MP Biomedicals
Lithium chloride	Lot:036K0122	Sigma Aldrich
Lysozyme	Lot:072K7062	Sigma Aldrich
Magnesium chlorid	Lot:00026651	Fermentas
Magnesium chlorid (Classic)	Lot:PB604-2	PCR Biosystems
	0.24g of KH2PO4 in 1 L H_2O	
Sodium acetate 3M pH 5.2	Lot:112K1373	Sigma Aldrich
Sodium chloride		-
Sodium hydroxide		Bie & Berntsen A/S
Sodium dodecyl sulfate	Lot:STBD6276	Sigma Aldrich
Super Optimal Broth	Lot:2751306	NEB
TEMED	Lot: 036K0694	Sigma Aldrich
Glycine Sample Buffer	Lot:310001942	Bio Rad
Tris	Lot: 111M54391V	Sigma Aldrich
Triton X-100	Lot:023K0005	Sigma Aldrich
Tryptone	Lot:2M007007	Applichem
Yeast extract	Lot:VM619326346	Microbiology Fremtech

Table 2.2: *A list of the different chemicals used in this project*

Biologicals	**Description/Genotype**		**Manufacturer**
Bacteria strains			
Competent *E. coli* DH5-α cells			NEB
Competent *E. coli* 5-Alpha cells			NEB
Competent *E. coli* BL21 (DE3) cells			NEB
Plasmids			
pcDNA3-*Tag*RFP-T	Plasmid containing RFP		Dr. Pavel Dráper, Prague
pGFP	pET11a containing GFP		Nanobio, AAU
pLGFP	pET11a containing GFP and linker		This work
pRFPLGFP	pET11a containing RFP, linker and GFP		This work
pCasper3-GR	Plasmid containing Casper3		Evrogen
pCasper3	pET11a containing Casper3		This work
Enzyme			
10 x Dream *Taq* polymerase	Lot:00177587		Fermentas
10 x Classic *Taq* polymerase	Lot:PB604-2		PCR Biosystems
T4 Kinase	Lot:00029977		Fermentas
T4 DNA Ligase	400000 U/ml	Lot:0991012	NEB
*Nde*I	5'...CA TATG...3'	(10 U/μl)	Fermentas
Restriction endonucleases	3'...GTAT AC...5'	Lot:00125955	
*Bam*HI	5'...G GATCC...3'	(10 U/μl)	Fermentas
Restriction endonucleases	3'...CCTAG G...5'	Lot:00125955	
*Sal*I	5'...G TCGAC...3'	(10 U/μl)	Fermentas
Restriction endonucleases	3'...CAGCT G...5'	Lot:00024597	
*Sma*I	5'...CCC GGG...3'	(10 U/μl)	Fermentas
Restriction endonucleases	3'...GGG CCC...5'	Lot:00027513	

Table 2.3: *A list of the bacterial strains, plasmid and enzymes used in this project.*

Primers	Sequence	Supplier	T_M
Linker (+)	5'-TATGGTCGACTCTGCAGCGGTACCC-3'	TAG Copenhagen A/S	67,9 °C
Linker (-)	5'-GGGTACCGCTGCAGAGTCGACCA-3'	TAG Copenhagen A/S	67,8 °C
RFPForward	5'-CTCATATGGTGTCTAAGGGC-3'	TAG Copenhagen A/S	57,3°C
RFPReverse	5'-TAGTCGACCTTGTACAGCTCG-3'	TAG Copenhagen A/S	59,8°C
CasperForward1	5'-TCCATATGGTGAGCGAGCTG-3'	TAG Copenhagen A/S	53,8°C
CasperReverse1	5'-CTGGATCCTCAGCGGTACAG-3'	TAG Copenhagen A/S	55,9°C
CasperForward2	5'-TCGGTACATATGGTGAGCGAGCTGATTAAG-3'	TAG Copenhagen A/S	59,5°C
CasperReverse2	5'-TCATGGATCCTCAGCGGTACAGCTC-3'	TAG Copenhagen A/S	58,8°C
CasperForward3	5'-TCGGTACATATGGTGAGCGAGCTGATTAAG-3'	TAG Copenhagen A/S	61,6°C
CasperReverse3	5'-AGCCGGATCCATGATCTAGAGTCGC-3'	TAG Copenhagen A/S	61°C
Pet11a1	5'-CCTCTTGCGGGATATCCG-3'	Genemed synthesis inc.	57°C
Pet11a2	5'-GATGTCGGCGATATAGGC-3'	Genemed synthesis inc.	55°C

Table 2.4: *A list of the different Primers used in this project*

Kits	Supplier	Lot no:
QIAGENPlasmid Midi kit	QIAGEN	13975934
Centrifugal lter tubes	Millipore	R3MA01181
Purication of PCR product	Mancherey-Nagel	603/001
UltraClean® Standard Mini Plasmid Prep Kit	MO BIO Laboratories, Inc	U14k6

Table 2.5: *A list of the different Kits used in this project*

2.1.1 LB Medium and Agar Plates

For 1 liter of LB medium 10g tryptone (applichem), 5g yeast extract (Microbiology Fremtech) and 10 g NaCl were added to a Blue Cap Bottle. Water was added for a total volume of 1 L. The medium was autoclaved at 121 °C for 30 min, and stored in a fridge. For agar plates 15 g/L agar (Sigma Aldrich) was added before autoclaving. The medium were distributed in petri dishes and the plates were stored in the fridge. For some medium and plates, a selection antibiotic was added. If nothing else is noted the following concentrations of antibiotic is used. Ampicillin was added for a final concentration of 100 μg/ml, and kanamycin was added for a final concentration of 50 μg/ml.

2.2 Methods

In this section, the various methods used in the different experiment will be described. The first section shortly describes general methods used, and some of these are described in detail in appendix A. The second section describes the experiments performed in the laboratory.

2.3 General methods

2.3.1 Chemical transformation of *E. coli*

Transformation of *E. coli* was performed using 20-50 μl of 5-alpha F' I^q Competent (NEB), or BL21 (DE3) (NEB) cells. Before transformation, SOC (NEB) or LB-medium was preheated and the competent cells were thawed on ice. A detailed protocol can be found in appendix A.

2.3.2 Isolation of Plasmid DNA

Plasmid DNA was isolated from *E. coli* 5-alpha (NEB) and *E. coli* Bl21 (DE3) (NEB) using various kits and methods depending on the quantity and quality of DNA needed. For larger plasmid DNA production the QIAGEN plasmid midi kit (QIAGEN) was used, and for smaller production the UltraClean® Standard Mini Plasmid Prep Kit (MO BIO Laboratories, Inc) or the TELT mini preparation mthod was used.

2.3.3 DNA isolation by QIAGEN plasmid midi kit

The plasmid DNA was isolated from 100 ml overnight culture using the QIAGEN plasmid midi kit (QIAGEN) and resuspended in 100 μl nuclease free water. A detailed protocol can be found in appendix A. The DNA concentration was determined using agarose gel electroporation.

2.3.4 DNA Isolation by UltraClean® Standard Mini Plasmid Prep Kit

The plasmid DNA was isolated from 4 ml overnight culture using the UltraClean® Standard Mini Plasmid Prep Kit (MO BIO Laboratories, Inc) and resuspended in 50 μl nuclease free water. A detailed protocol can be found in appendix A. The DNA concentration was determined using agarose gel electroporation.

2.3.5 DNA Isolation by TELT mini-preparation

The plasmid DNA was isolated from *E. coli* 5-alpha (NEB) or E coli BL21(DE3) (NEB) colonies using the TELT mini preparation protocol. The DNA was resuspended in 40 μl nuclease free water (Sigma Aldrich). A detailed protocol can be found in appendix A. The DNA concentration was determined using agarose gel electroporation.

2.3.6 Agarose gel electrophoresis

1% agarose solutions were made by adding 10g/L agarose (Sigma) to 1X TAE buffer (In house). The solution was microwaved until the agarose was completely melted and stored at 60 °C.

DNA size and concentration determination by agarose gel electrophoresis

1 μl EtBr (Sigma) added to 30 ml agarose solution was used for the gel. DNA solution was mixed with an appropriate amount of 6X loading dye (Fermentas) and added to the wells. 5 μl 1k bp Generuler ladder (Fermentas) was used as standard. The gel was run at 90V for 60-90 mins in a Mini-PROTEAN electrophoresis module (BIO-RAD) filled with 1X TAE buffer. The gel was analyzed using UV light (uvitec). The bands were compared to the standard which allowed estimations of size and concentration. The Standard can be seen in appendix A.

DNA extraction from agarose gel

The DNA was localized and cut out of the gel using a scalpel, separated into smaller chunks, packed in parafilm and put in a freezer for 3 hr - overnight. The gel was centrifuged at 5000g for 10 mins in Centrifugal Filter Units (MILLIPORE) and the liquid was collected. The DNA was concentrated using an ethanol precipitation.

2.3.7 Ethanol precipitation

Ethanol precipitation was performed by mixing 1 x DNA solution with 2 x 96% EtOH (Kemetyl) and $\frac{1}{10}$ x 3M NaAc pH 5.2 (Sigma Aldrich). The solution was mixed well and incubated at -20 °C. The solution centrifuged at max RPM for 30 min at at 4°C. The supernatant was discarded. 100 μl 70% EtOH (Kemetyl) was added and the solution was centrifuged at max RPM for 15 min at 4°C. The supernatant was discarded and the DNA pellet was air dried until the smell of ethanol had disappeared. The DNA was suspended in 20-100 μl nuclease free water (Sigma Aldrich).

2.3.8 Polymerase Chain Reaction

The PCR reaction was set up in different ways depending on the experiment, however most of the factors are kept constant unless something else is specified. In the sections where PCR's are described the amount of template (typically in ng), and *Taq* (in units) used will be noted. Additionally the primers used will be noted. The table below shows the general PCR setup. This setup is for 100 μl total volume. For 50 μl total volume the amount (in μl) of 10x Buffer, dNTP's, MgCl, Forward primer, Reverse primer is halved. [23]

Chemical (Concentration)	**Amount (in μl or ng)**	**Final concentration**
10x Buffer	10μl	1x
dNTP's (200 μM each)	1μl	2 μM each
MgCl (50 mM)	6 μl	3 mM
Forward primer (100 μM)	1 μl	1 μM
Reverse primer (100 μM)	1 μl	1 μM
Template	10-20 ng	Variable
Taq (5u/μl)	0.5-1 μl	Variable
H_2O	Until 100 μl	-
Total	100μl	-

Table 2.6: *Chemicals used for the PCR process*

For the PCR experiment the chemicals in the table are mixed. *Taq* is added last and the solution is mixed well. The PCR is run in cycles in a MasterCycler gradiant (eppendorf) and unless anything else is noted the program below is used. Note that the annealing temperature is dependant on the primers used and this temperature will be noted in the PCR sections.

Name:	**Temperature**	**Time**	**Cycles**
Initial Denaturation	95 °C	3 min	1
Denaturation	95 °C	30 sec	35
Anneal	55-65 °C	30 sec	35
Extension	72 °C	1 min	35
Final Extension	72 °C	7 min	1
Hold	4 °C	Till collected	1

Table 2.7: *Program used for PCR process*

2.3.9 PCR cleanup using Macherey-Nagel NucleoTraP®CR

The PCR product was purified using the PCR cleanup kit NucleoTraP®CR from Macherey-Nagel.[37] 200 μl of the PCR product was pipetted into the pre-assembled filter and centrifuged, so the DNA could bind to the filter. The filter was then washed and dried. Finally, the DNA was eluted by adding 50 μl of elution buffer (Sigma Aldrich) and centrifuged for 1 min at 11000 x g. The detailed protocol can be seen in Appendix A.

2.3.10 PCR Cleanup using GenElute^{TM} PCR Clean-Up Kit

The PCR product was isolated from 200 μl PCR solution using the GenElute^{TM} PCR Clean-Up Kit (Sigma) and suspended in 50 μl nuclease free water (Sigma Aldrich). A detailed protocol can be found in appendix A. The DNA concentration was determined using agarose gel electroporation.

2.3.11 SDS-PAGE

The 12% SDS-PAGE was prepared. 1 part protein solutions was mixed with 2 parts glycine sample buffer (BIO-RAD). The samples were heated for 5 min at 95 °C, and added to the wells. The gel was run at 120V for 1 hr. The gel was stained, and then destained. [24] A detailed protocol can be found in appendix A.

2.4 Construction of pRFPLGFP

Flowchart of the process used for construction, screening and expression of pRFPLGFP can be seen in Figure 2.1.

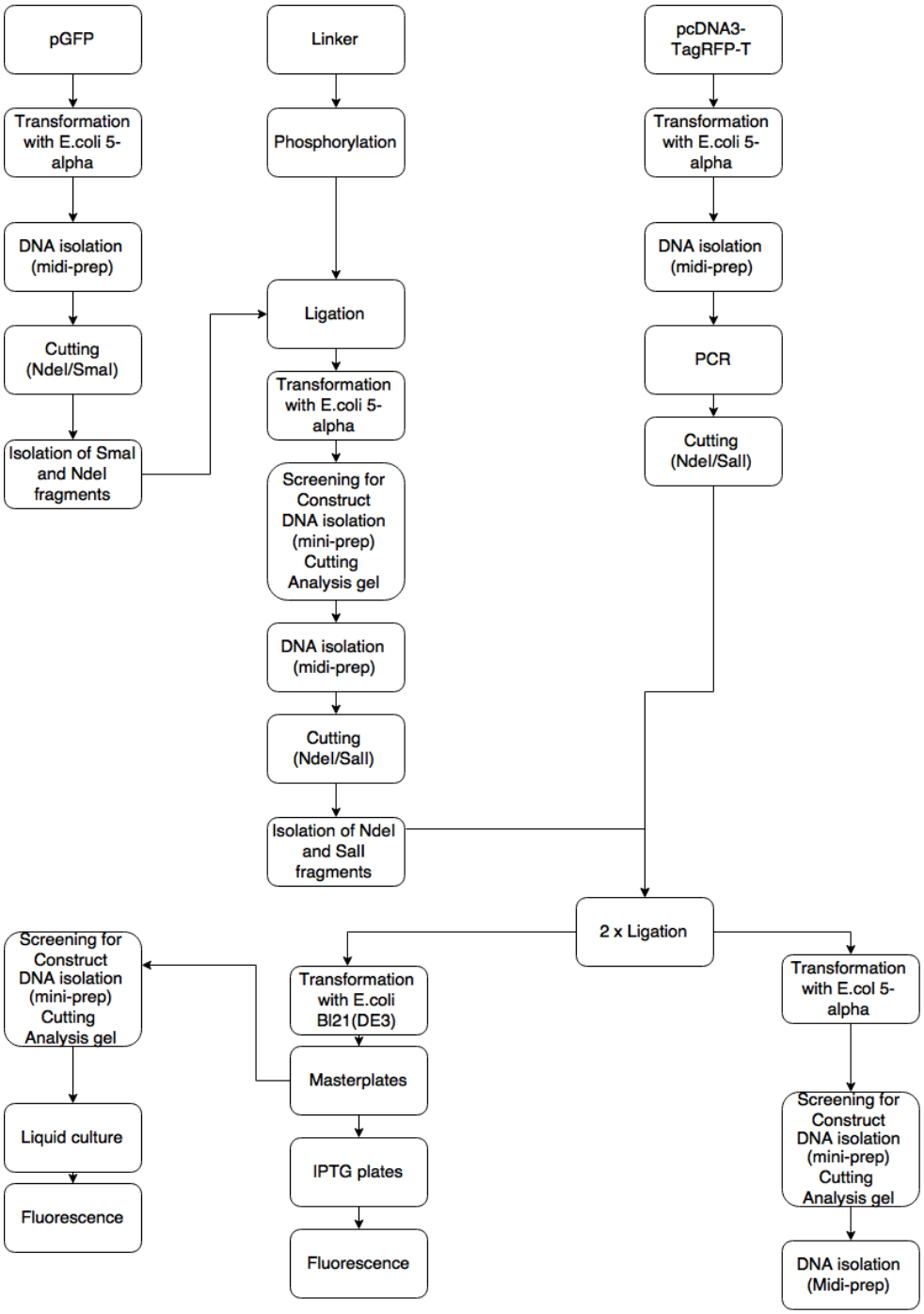

Figure 2.1: *Workflow in construction and expression of pRFPLGFP*

2.4.1 Preparation of pLGFP

The pLGFP was constructed by ligating pGFP with a linker.

2.4.2 Preparation of pGFP

5 μl pGFP was added to 20 μl competent DH5-α *E. coli* and a chemical transformation was performed. The plasmid was isolated using the QIAGEN plasmid midi kit (QIAGEN). Two digestion procedures were set up. 40 μl DNA solution were digested in a total volume of 50 μl. One was digested using 30 U *Sma*I (Fermentas) at 25 °C for 2hrs, and the other using 30 U *Nde*I (Fermentas) at 37 °C for 2 hrs. The enzymes were neutralized using heat shock at 75 °C for 10 min. The digestion was verified using agarose gel electroporation. The two samples were concentrated using EtOH precipitation and cut again with the opposite enzyme and buffers. The two samples were mixed and DNA isolation using agarose gel electroporation was performed to separate the cut DNA from the uncut. An agarose gel electroporation was performed to determine the DNA concentration.

2.4.3 Phosphorylation of linker DNA

Preparation of linker was performed by mixing the two SS DNA linkers (TAG copenhagen) with an appropriate amount of water for a final concentration of 100 pmol/μl. 20 μl of each was mixed and run in a MasterCycler gradient (eppendorf) using the following program:

Steps	**Degree C**	**Time**
Heating	99	3 min
Stabilise	70	5 min
Annealing	65	20 min
Hold	4	Hold

Table 2.8: *Program used for phosphorylation process*

Phosphorlylation

3 μl linker solution was phosphorylated using 50 units of T4 Polynucleotide Kinase (Fermentas) in a total volume of 50 μl in a suitable buffer with added ATP. The solution was incubated at 37 °C for 20 min, and heat shocked for 10 mins at 75 °C. The solution was stored in the fridge.

2.4.4 Ligation of pLGFP

6μl of the phosphorylated linker (TAG Copenhagen A/s) was ligated with 10μl digested pGFP, in a 20μl total volume using 2U T4 ligase (NEB) in a suitable buffer. The solution was incubated at 6 °C overnight, followed by incubation at room temperature for 30 min. The ligase solution was transformed into *E. coli* 5-alpha cells (NEB).

2.4.5 DNA based screening for pLGFP

The verification of linker insertion was done using the digestion enzyme *Sal*I (Fermentas). The screening was done on plasmid DNA which has been collected using the TELT mini-preparation. 3μl pLGFP was

digested using 10 units of *Sal*I (Fermentas) in a total volume of 20 μl in an appropriate buffer. The solution was incubated for two hours at 37°C. The enzyme was neutralized using heat shocking for 10 min at 65 °C. The digested solution was analyzed by an agarose gel. The gel was run with an undiluted uncut reference.

2.4.6 Preparation of pRFPLGFP

The pRFPLGFP was constructed by ligating pLGFP with a PCR product containing the RFP DNA sequence. 30 μl of pLGFP was digested in a total volume of 50 μl. The plasmid DNA was digested using 20U of *Sal*I (Fermentas) and 20U of *Nde*I (Fermentas) in a suitable buffer. The solution was incubated for 2 hr at 37 °C. The enzymes were deactivated using heat shocking at 75 °C for 10 min. The digested plasmid was isolated using agarose gel electroporation. The PCR was performed using 0.5 μl of plasmid DNA containing the RFP sequence as template, the primers RFPForward and RFPReverse , and 2.5u of *Taq* (Fermentas) in a total volume of 100 μl. The annealing temperature used was 55 °C. The PCR was cleaned using the PCR cleanup kit NucleoTraP®CR (Macherey-Nagel). Two digestions were set up. 30 μl of DNA was digested in an appropriate buffer in a total volume of 50 μl. One was digested using 20u of *Nde*I (Fermentas) at 37 °C for 2 hr, and the other was digested using 20u of *Sal*I (Fermentas) at 37 °C for 2 hr. The enzymes were deactivated using heat shocking at 75 °C for 10 min. The solutions were then digested using the opposite enzyme. An ethanol precipitation was used to clean the digested PCR Product.

2.4.7 Ligation of RFP and pLGFP

9μl of the digested PCR product was ligated with 8μl of digested pLGFP in a total volume of 20 μl using 2U of T4 Ligase (NEB) in a suitable buffer. The solution was incubated overnight at 6-8°C. After the incubation, the solution was transformed into competent *E. coli* 5-alpha cells (NEB).

2.4.8 DNA based screening for pRFPLGFP

Screening for pRFPLGFP was performed using digestion and PCR. DNA was isolated from *E. coli* 5-alpha using the QIAGEN Plasmid midi prep kit (QIAGEN). 2 μl of DNA was digested in a total volume of 20 μl. The plasmid DNA was digested using 20U of *Sal*I (Fermentas) in a suitable buffer. The solution was incubated at 37 °C for 2 hr. The Enzyme was deactivated using heat shocking for 10 min at 75 °C. 10U *Nde*I was added and the solution was incubated for 3 hr at 37 °C. The enzymes were deactivated using heat shocking at 75 °C for 10 min. The solution was investigated using an agarose gel.

PCR was performed on the isolated plasmid DNA. The PCR was performed using 0.5 μl plasmid DNA as template, the primers RFPForward and RFPReverse and 2.5U of *Taq* (PCR Biosystems). The annealing temperature was 55 °C. A reference sample were made where the plasmid DNA and the *Taq* was exchanged with nuclease free water. The PCR solutions were investigated using an agarose gel.

2.4.9 Screening for pRFPLGFP in BL21 (DE3)

A second ligation was performed and the pRFPLGFP was transformed into competent *E. coli* BL21 (DE3) cells (NEB) to be used for expression. PCR was used to screening for the insert. Plasmid DNA was isolated using TELT mini preparation. The PCR was performed using 0.5 μl plasmid dna as template, the primers RFPForward and RFPReverse and 2.5 U of *Taq* (PCR Biosystems). A reference was made

with nuclease free water instead of *Taq* enzyme and template DNA. The PCR solutions were investigated using an agarose gel.

2.5 Expression of p*RFPLGFP*

Expression of p*RFPLGFP* was performed on IPTG induced LB-Amp agar plates and in IPTG induced LB-Amp medium. Colonies from the second ligation was transferred to fresh LB-Agar plates and incubated overnight at 37 °C to make master plates. IPTG induced LB-amp Agar plates were made by adding 50 μl of 100 mM IPTG to LB-Amp agar plates. Aliquots of bacteria was taken from the master plates and spread on IPTG induced LB-Amp agar plates. The plates were incubated overnight at 25 °C. In parallel aliquots of bacteria was taken from the master plates and added to 100 ml of LB-amp medium and incubated at 25 °C and 230 rpm. After 8 hr 0.5 ml of 100 mM IPTG was added. The solution was grown for another 8 hr. The solution was centrifuged at 6000 rpm for 6 min using a RC5C centrifuge from Sorvall Instruments with an SLA-1500 rotor. The supernatant was discarded and 5 mL TES-buffer was added and then mixed. The solution was centrifuged at 9000 rpm for 20 min and the TES-fraction was discarded and stored. Then 5 mL of autoclaved water is added and a final centrifugation is done at 9000 rpm for 35 min and the water-fraction was stored as well. All the samples are kept on ice and later analysed by SDS-PAGE

2.5.1 Fluorescence spectroscopy analysis of RFPLGFP

All measurements were conducted with a steady-state spectrophotometer from Photon Technology International. The model used was a PTI Quantamaster QM-6. All motors were adjusted to a slit-width of 3 nm. The spectrophotometer software was FeliX32 Analysis Module. Material from the IPTG plates were added to 720 μl of a 50 mM phosphate buffer at pH 7.2. Fluorescence spectroscopy was conducted where an emission spectrum was measured with an excitation of 489 nm. Finally, an excitation spectrum was done, where the excitation range was 450-500 nm with an emission detection at 509 nm.

Fluorescence spectroscopy was conducted on the liquid cell medium as well as the TES and water fractions. 750 μl were added for each analysis with reference scans having been subtracted. Emission scans had been conducted in the areas 500 nm to 550 nm and 560 nm to 600 nm with excitation of 489 nm and 555 nm respectively to test for GFP and RFP presence. To test for any FRET traces, an emission scan has been done in the range 570 nm to 600 nm with an excitation at 489 nm. Lastly, excitation scans in the range of 470 nm to 500 nm and 540 nm 570 with emission detection at 509 nm and 584 nm respectively.

2.6 Construction of pCasper3

Flowchart of the process used for construction, screening and expression of pCasper3 can be seen in Figure 2.2.

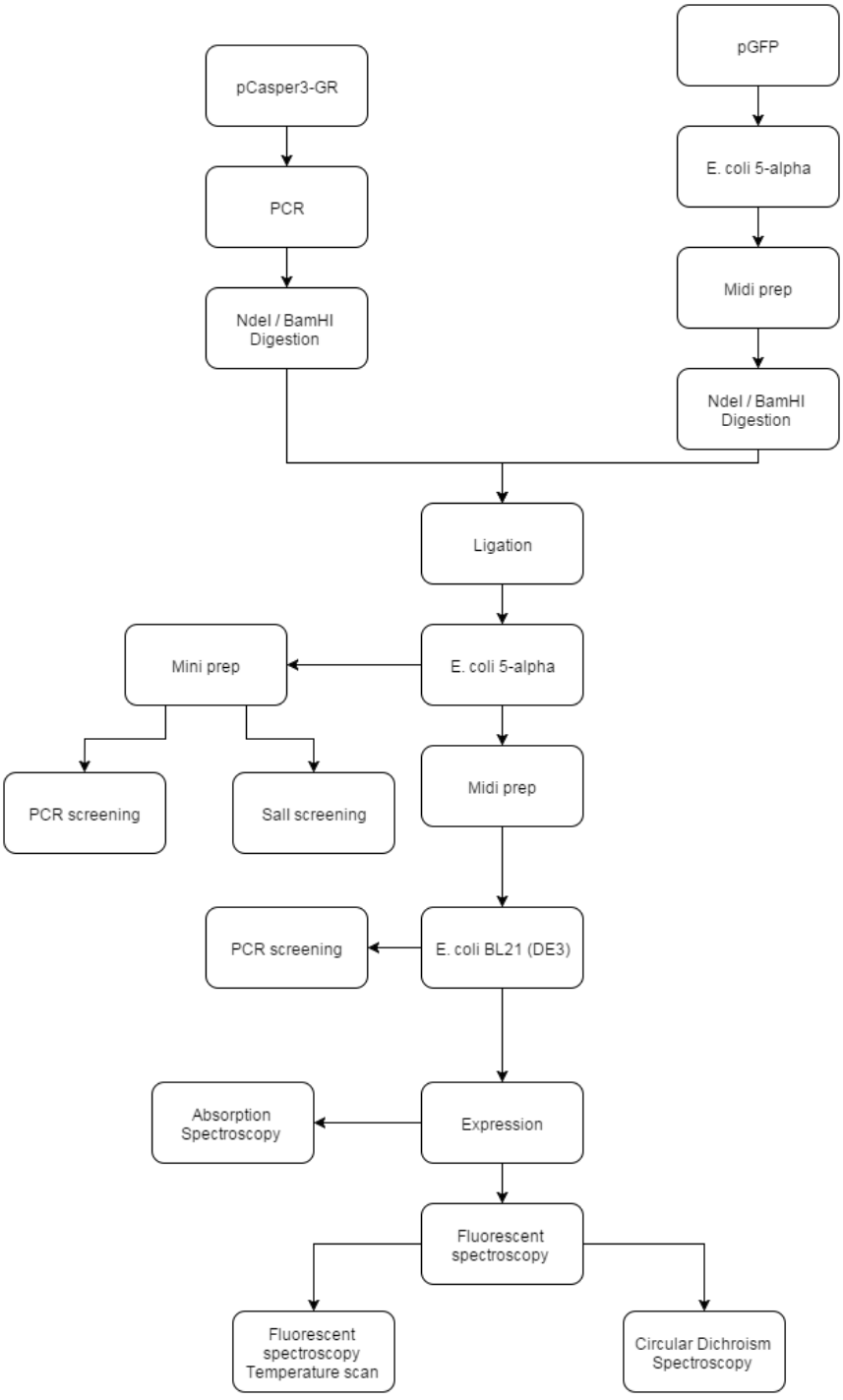

Figure 2.2: *Workflow in construction and expression of pCasper3*

pCasper3 was prepared by inserting a *Nde*I/*Bam*HI fragment containing Casper3 into a pET11a Vector.

2.6.1 Preparation of NBCasper3

The NBCasper3 were made by introduce a *Nde*I site upstream of the start codon of the Casper3 and a *Bam*HI site downstream of the stop codon by using PCR.

The PCR was run using 250 ng of pCasper3-GR as template, the primers CasperForward1 and Casper-Reverse1 and 2.5u of *Taq* (PCR Biosystems) in a total volume of 100 μl with an annealing temperature of 52 °C. Due to problems the PCR was repeated using10 ng DNA and 5u of *Taq*. Due to problems various things were optimized.

- The DNA was transformed into *E. coli* 5-alpha and a PCR was performed on the isolated DNA
- MgCl concentration was varying from 1 mM - 5mM in 1mM interval
- The denaturation temperature was changed from 95 °C to 97 °C
- The pCasper3-GR was linearized by digestion with *Nde*I, before the PCR

New primers were designed and used for the PCR. Two PCR's were run. One with pCasper3-GR, and one with pCasper3-GR from *E. coli* as template. 10 ng of template was used with 5 units of *Taq* in a total volume of 100 μl. The primers used were CasperForward2 and CasperReverse2 and the annealing temperature used was 57 °C. The PCR's were cleaned using a GenElute^{TM} PCR Clean-Up Kit (Sigma-Aldrich).

Digestion of PCR product

1.6 μg of PCR product was digested using 30 units of *Bam*HI for 3 hrs in an appropriate buffer. The enzyme was deactivated by heating in a heating block for 20 mins at 65 °C. 30 Units of *Nde*I was added and the DNA was cut for an additional 3 hrs. The digested PCR product was cleaned using an ethanol precipitation and resuspended in 50 μl of nuclease free water. An agarose gel was used to determine the concentration of DNA.

2.6.2 Preparation of pET11a

100 ng of pGFP was transformed into *E. coli* 5-alpha (NEB) according to the transformation protocol. The DNA was isolated using mini prep. The mini preps was checked and on was used for midi prep. Two digestions were set up. 1.4 μg of DNA was digested in an appropriate buffer in a total volume of 50 μl. One was digested using 20 units of *Nde*I at 37 °C for 3 hrs, and the other was digested using 20 units of *Bam*HI at 37 °C for 3 hrs. The digestion was confirmed using an agarose gel. The solutions were then digested using 20 units of the opposite enzyme. The DNA was extracted from the agarose gel, and then concentrated using an ethanol precipitation.

2.6.3 Ligation of digested pET11a and digested NBCasper3

50 ng digested pET11a was ligated with 40 ng digested NBCasper3 using 10 units of T4 ligase in a total volume of 20μl. A control was set up using the same chemicals except for NBCasper3. The ligation was carried out at 16 °C overnight followed by 1 hr at room temperature. The enzyme was deactivated by heat shocking at 65 °C for 10 mins.

2.6.4 Transformation of ligation product

15 μl ligation product was transformed into 50 μl *E. coli* 5-alpha (NEB) competent cells. 500 μl of SOC medium was used. The bacterial solutions of the ligation and control were spread on three LB-amp plates each with 50μl, 100 μl and the rest respectively. The colonies were incubated at 37 °C overnight. Colonies from the ligation were identified and used to create master plates which were incubated overnight.

2.6.5 Screening for pCasper3

Minipreps were performed on the colonies using the UltraClean® Standard Mini Plasmid Prep Kit (MO BIO) to isolate plasmid DNA from the bacteria. The screening attempted using various methods on the isolated DNA.

- 500 ng of isolated DNA was digested using 30 units of *Sal*I is a total volume of 50 μl.
- A PCR was run using 10 ng of isolated DNA as template, 2.5 units of *Taq* and the CasperForward2 and CasperReverse2 as primers with an annealing temperature of 57 °C
- A PCR was run using 10 ng og isolated DNA as template, 2.5 units of *Taq* and pET11a1 Forward and pET11a2 Reverse as primers with an annealing temperature of 53 °C.

2.6.6 Second attempt on creation of pCasper3

Due to problems the whole process with digestion of pGFP and production of NBCasper3 was repeated. This time all the DNA was cleaned using the GenElute ^{TM} PCR Clean-Up Kit (Sigma-Aldrich) between each step.

1.4 ng of pET11a was digested using 50 units of *Bam*HI in a total volume of 50 μl in an appropriate buffer. 50 units of *Nde*I was added along with buffer to keep the buffer levels at the recommended values. The vector DNA was extracted from an agarose gel and was then concentrated with an ethanol precipitation. PCR was made using pCasper3-GR as template and with CasperForward2 and CasperReverse2 as primers. The DNA was then digested with *Bam*HI and *Nde*I. The DNA was extracted from an agarose gel and then concentrated by an ethanol precipitation. The digested NBCasper3 was ligated with the digested pET11a. The ligated product was transformed into *E. coli* DH5-Alpha (NEB).

2.6.7 Production of NBCasper using new Primers and Preparation of pCasper3

New primers were designed and used for the PCR.The PCR was performed using 10 ng of pCasper3-GR as template, 2.5 units of *Taq* and CasperForward3 and CasperReverse3 as primers with an annealing temperature of 59 °C. The PCR product was cleaned using the GenElute ^{TM} PCR Clean-Up Kit (Sigma-Aldrich).

Two digestions using 1.8 ng of DNA was set up. The DNA was digested for 2 hr at 37 °C using 30U of *Nde*I in a total volume of 50 μl. The DNA was cleaned using the GenElute ^{TM} PCR Clean-Up Kit (Sigma-Aldrich). Two digestions using 1.2 ng of DNA was set up. The DNA was digested for 2 hr at 37 °C using 30U of *Bam*HI in a total volume of 50 μl. The DNA was cleaned using the GenElute ^{TM} PCR Clean-Up Kit (Sigma-Aldrich). A gel isolation was used to further clean the NBCasper3, followed by an ethanol precipitation to concentrate the DNA.

50 ng of digested NBCasper3 was ligated with 30 ng of digested pET11a using 10 units of ligase in a total volume of 20 μl. A control was performed with 30 ng of digested pET11a with 10 units of ligase in a total volume 20 μl. The solutions were incubated in a fridge for three days followed by 30 min at room temperature. The enzymes were inactivated by heating to 65 °C for 10 min.

Transformation of Ligation Product

20 μl of ligation product was transformed into 50 μl of *E. coli* 5- Alpha (NEB). 500 μl of SOC medium was used. The bacterial solutions of the ligation and control were spread on three LB-amp plates with 50 μl, 100 μl, and the rest respectively. The plates were incubated at 37 °C overnight. Colonies were identified and used for master plates.

2.7 Screening for pCasper3

Minipreps were performed on the colonies using the UltraClean® Standard Mini Plasmid Prep Kit (MO BIO) to isolate plasmid DNA from the bacteria. The following methods were used for screening for pCasper3.

- PCR's were run using 10 ng of the plasmid DNA as template, 2.5 units of *Taq* and pET11a1 and pET11a2 as primers with an annealing temperature of 53 °C.
- 700 ng of isolated DNA was digested using 30 units of *Sal*I is a total volume of 50 μl.
- Sall digested DNA was digested using 20 units of *Nde*I.

Samples were identified and used for midi preps using the QIAGEN Plasmid Midi Kit (QIAGEN) and suspended in 100 μl of nuclease free water. A PCR similar to the screening stage was used for screening for pCasper3 and a digestion using *Sal*I was used to determine the concentration of DNA.

2.8 Transformation into *E. coli* BL21 (DE3)

100 ng of pCasper3 was transformed into *E. coli* BL21 (DE3) (NEB). 500 μl of SOC medium was used. The bacterial solution was spread on three LB-amp plates with 50 μl, 100 μl, and the rest respectively. The plates were incubated at 37 °C overnight. Colonies were identified and used to make master plates.

2.8.1 Screening for pCasper3 in BL21 (DE3)

Minipreps were performed on the colonies using the UltraClean® Standard Mini Plasmid Prep Kit (MO BIO) to isolate plasmid DNA from the bacteria. A PCR similar to the screening stage was used to screen for pCasper3.

2.9 Protein Expression

Four colonies was used for the protein expression. Aliquots of bacterial from the master plates were resuspended in 10 ml LB-amp medium to make pre-cultures. The pre-cultures were incubated at 37 °C overnight. The following day the pre-culture were added to 1L Erlenmeyer flask with 200 ml LB-Amp medium and the temperature was reduced to 25 °C. The cultures were incubated until an OD600 of 0.9 was reached. 440μl of 100 mM IPTG stock solution was added for a final concentration of 200 μM IPTG. The bacteria was incubated at 25 °C overnight.

OD600 measurements was done right before harvest in order to determine how much TES buffer was needed. The calculation of the amount needed for opening of cells can be seen below:

Amount of cells	OD600	Amount TES
10 ml	3	0.5 ml
10 ml	4	0.6 ml
10 ml	5	0.8 ml
10 ml	6	1 ml

Table 2.9: *The amount of TES needed according to OD600*

The cells was harvested by centrifugation at around 6000 rpm for 6 min. The medium was saved for later investigation. The pellet was resuspended with chilled TES buffer with a bit of lysozyme and centrifuge again at 7000 rpm for 20 min at 6°C. The supernatant was added to 50 ml greiner tubes and stored at 4 °C for later investigation. The pellet was resuspended with chilled sterile MiliQ water and centrifuge for 40 min. at 12000 rpm at 6°C. The supernatant was added to 50 ml greiner tubes and stored at 4 °C. All the different fraction was later analysed with a SDS-PAGE.

2.10 Spectroscopy Analysis

2.10.1 Fluorescence Spectroscoy

All measurements were done with a steady-state spectrophotometer from Photon Technology International. The model used was a PTI Quantamaster QM-6. All motors were adjusted to a slit-width of 3 nm. The spectrophotometer software was FeliX32 Analysis Module. Analysis of the data was done in Excel 2013. *Tag*GFP excitations were performed using an excitation wavelength of 400nm, 430, 456 and 482 nm. The emission was measured from close to the excitation wavelength to 650 nm. *Tag*RFP excitations were performed using an excitation wavelength of 555 nm. The emission was measured in the range 460-650 nm and 560-650 nm.

Temperature Dependant Fluorescence Spectroscopy

Temperature dependant scans were performed using an excitation wavelength of 482 nm and emission were measured at 502nm and 570 nm, the temperature range 25-85 °C and 85-25 °C.

2.10.2 Absorption Spectroscopy

The measurements were done on a UV-vis steady state absorption spectrometer from VWR INTERNATIONAL. The model was a UV1. The absorption spectrometer software was VISIONLite Scan. Analysis of data was done in Excel 2013. The measurement were performed in the range 422-650 nm.

Expected Absorbance Spectra

The calculation for the expected absorbance spectra were performed in Microsoft Excel 2010. The calculation used data about TagGFP and TagRFP from Evrogen. [25] [26] The data used was the Molar Extinction Coefficient (ϵ) of TagGFP and TagRFP and normalized excitation (λ_{EX}) spectra of TagGFP and TagRFP. The expected absorbance spectra ($\lambda_{ExpectedAbs}$) is given by the formula:

$$\lambda_{ExpectedAbs} = \epsilon_{GFP} * \lambda_{EXGFP} + \epsilon_{RFP} * \lambda_{EXRFP} \tag{2.1}$$

This was then normalized to a maximum value of 100.

2.10.3 Circular Dichroism Spectroscopy

The measurements were done on a J-715 spectrophotometer from Jasco. The software used was Jasco J-715 Hardware Manager. The cuvettes used were Quartzglas SUPRASIL ® 300 PPRÅZISIONS-K*Ü*VETTEN from HELLMA. All measurement were done in the range 260 - 190 nm, with a speed of 50 nm/min, step size of 1 nm and an accumulation of 3 and at room temperature.

2.11 Modeling

The modelling of the fusion protein was performed using YASARA version 15.6.21. A FASTA file was constructed containing the amino acid sequence of the fusion protein. This was used as the target for a homologous modeling using the hm_build macro of YASARA. The workflow and function of the hm_build macro can be seen in Figure 2.3. Additionally an energy minimizations was performed. A Ramachandran plot was constructed using RAMPAGE.[27] The linker was investigated independently using YASARA and PredictProtein.[28] The distance between the chromophores were measured using YASARA.

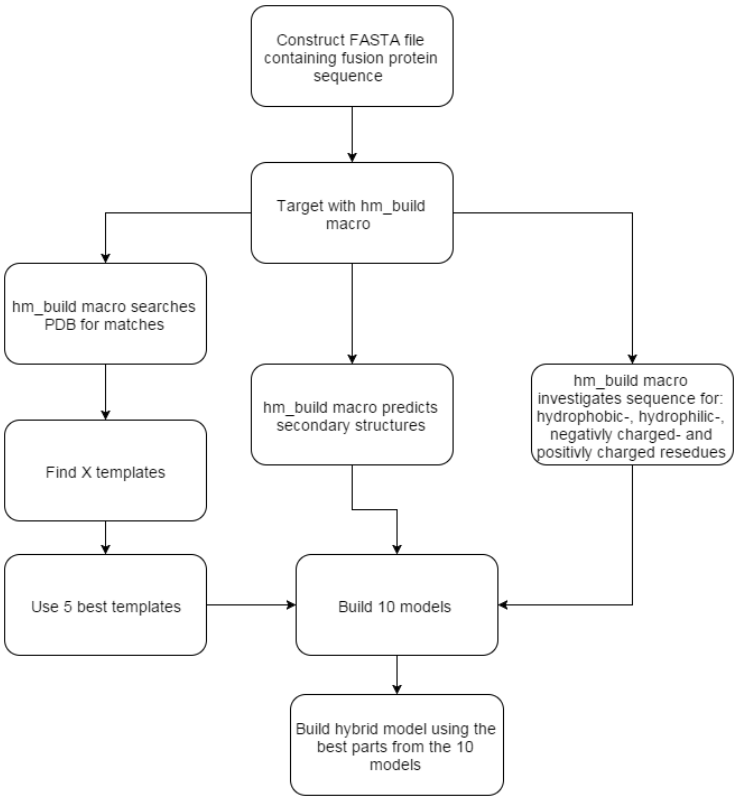

Figure 2.3: *Workflow of homologous modeling and function of the hm_build macro used to model the fusion protein.*

Result 3

3.1 Construction of pRFPLGFP

pRFPLGFP was constructed in two steps: First a linker was inserted into pGFP which is a pET11a vector containing the GFP gene. The linker was inserted up stream of the GFP gene. Secondly, NSRFP was inserted at the 5'-end of the linker. NSRFP is made by introducing a *Nde*I site at the start codon of the RFP gene and a *Sal*I site at the 3'-end of the gene using PCR. An illustration of this process can be seen on Figure 1.7 in the introduction.

3.1.1 Construction of pLGFP

pGFP was isolated and then digested using the restriction enzymes *Sma*I and *Nde*I. The digested pGFP was isolated and ligated with a 21 bp phosphorylated linker. The pGFP with the linker (pLGFP) was transformed into competent *E. coli* DH5-α cells. Plasmid DNA was isolated from 4 colonies and screened for the presence of the linker.

3.1.2 Screening for pLGFP

Plasmid DNA were screened for the presence of the linker by digesting with *Sal*I. The results can be seen in Figure 3.1. The digested DNA samples were compared to the undigested DNA samples.

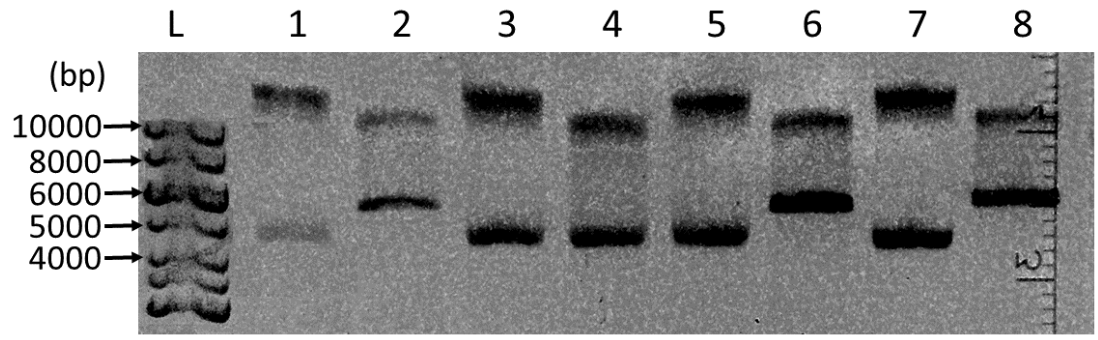

Figure 3.1: *1% agarose gel of SalI digested pLGFP miniprep plasmid DNA.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of Plasmid DNA from colony 1.* ***2)*** *5µl of SalI digested plasmid DNA from colony 1.* ***3)*** *5µl of plasmid DNA from colony 2.* ***4)*** *5µl of SalI digested plasmid DNA from colony 2.* ***5)*** *5µl plasmid DNA from colony 3.* ***6)*** *5µl of SalI digested plasmid DNA from colony 3.* ***7)*** *5µl plasmid DNA from colony 4.* ***8)*** *5µl of SalI digested plasmid DNA from colony 4.*

Digested plasmid DNA from colonies 1, 3 and 4 (in well 2, 6 and 8 in Figure 3.1) show clear differences when compared to the reference samples (in well 1, 5 and 7). At the time of the experiment, it was assumed that the linker had been properly inserted, however it was later concluded that this was not the case.

3.1.3 Preparation of pLGFP

Plasmid DNA from colony 1, 3 and 4 was isolated using midi prep. The plasmid DNA from colony 4 had the highest concentration of DNA and was therefore used for further experiments. The DNA was digested with *Nde*I and *Sal*I. The digested pLGFP was isolated and saved for ligation

3.1.4 Preparation of TagRFP-T Fragment

The 750 bp TagRFP-T fragment was prepared by PCR. pcDNA3-TagRFP-T was used as template. In order to ligate the TagRFP fragment into pLGFP two primers RFPForward and RFPReverse were used to introduce a *Nde*I site at the 5'-end of the fragment and a *Sal*I site at the 3'-end of the fragment. The PCR produced a fragment with a size of 750 bp which can be seen in Figure 3.2. The fragment was digested with *Nde*I and *Sal*I and cleaned as preparation for ligation.

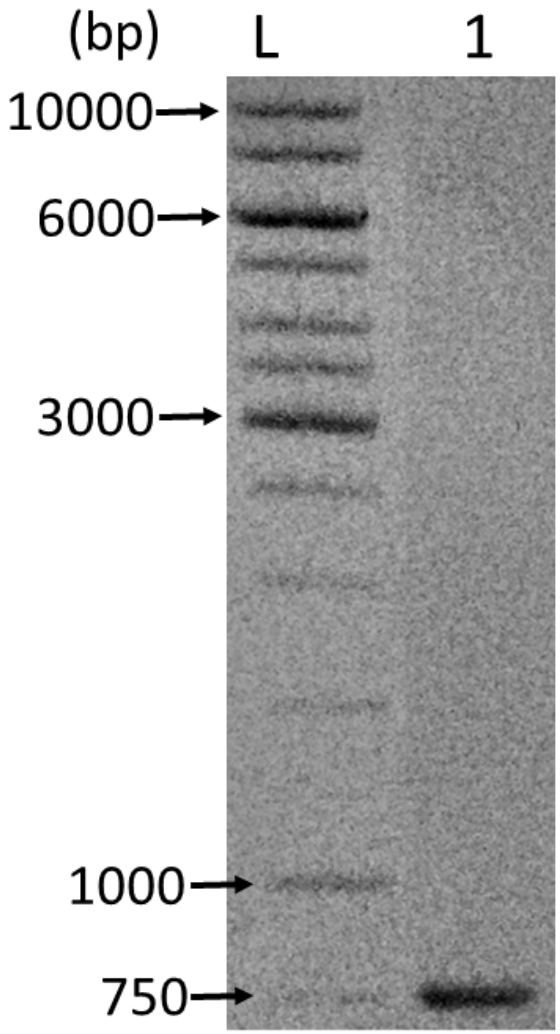

Figure 3.2: *1% agarose gel of PCR product from TagRFP-T using RFPForward and RFPReverse as primers.* ***L)*** *5µl 1 kb ladder (Fermentas).* ***1)*** *5µl PCR product of pcDNA3-TagRFP-T.* ***2)*** *5µl PCR product of pcDNA3-TagRFP-T.*

pRFPLGFP was constructed by ligating the *Nde*I/*Sal*I digested pLGFP with the *Nde*I/*Sal*I digested TagRFP-T PCR product. The ligation was transformed into *E. coli* DH5-α cells. Colonies were grown and 6 were selected for screening.

3.1.5 Screening for pRFPLGFP in *E. coli* DH5-α

The colonies were initially screened by expression and the results can be seen in Figure 3.3.

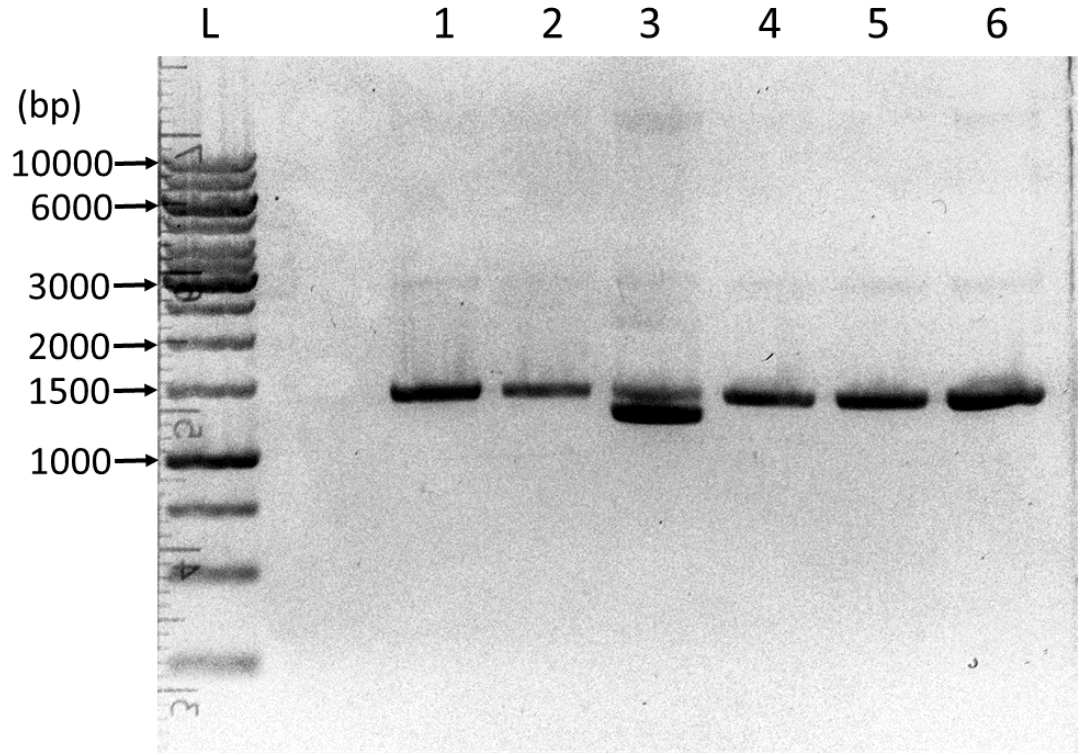

Figure 3.3: *1% agarose gel of isolated plasmid DNA from six randomly selected colonies potentially harboring pRFPLGFP.**L)** 5µl 1 kb ladder (Fermentas). **1)** 5µl pRFPLGFP. **2)** 5µl pRFPLGFP. **3)** 5µl pRFPLGFP. **4)** 5µl pRFPLGFP. **5)** 5µl pRFPLGFP.**6)** 5µl pRFPLGFP.*

The plasmid DNA isolated from the 6 colonies showed a fragments with a size of 1500 bp, around 3000 bp and above 10000 bp. The pattern of the isolated undigested DNA was rather unexpected. The plasmid DNA were digested with *Sal*I and *Nde*I to investigate their size. When digested with *Sal*I a fragment of around 7000 bp (pET11a $\approx$ 5600 bp and TagRFP+TagGFP $\approx$ 750 bp + 750 bp = 1500 bp) should be seen and after digesting with both *Sal*I and *Nde*I a fragment of around 6300 (pET11a + GFP) and a fragment of 750 bp (TagRFP) should be seen. The digesting with *Sal*I can be seen in Figure 3.4 and the digesting with *Sal*I and *Nde*I can be seen in Figure 3.5.

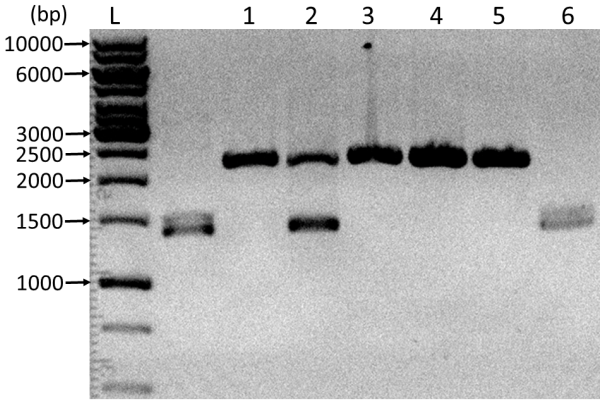

Figure 3.4: *1% agarose gel of SalI digested plasmid DNA from all 6 colonies.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of plasmid DNA from colony 1.* ***2)*** *5µl of SalI digested plasmid DNA from colony 1.* ***3)*** *5µl of SalI digested plasmid DNA from colony 2.* ***4)*** *5µl of SalI digested plasmid DNA from colony 3.* ***5)*** *5µl of SalI digested plasmid DNA from colony 4.* ***6)*** *5µl of SalI digested plasmid DNA from colony 5.* ***7)*** *5µl of plasmid DNA from colony 1 in digestion buffer (without enzyme).*

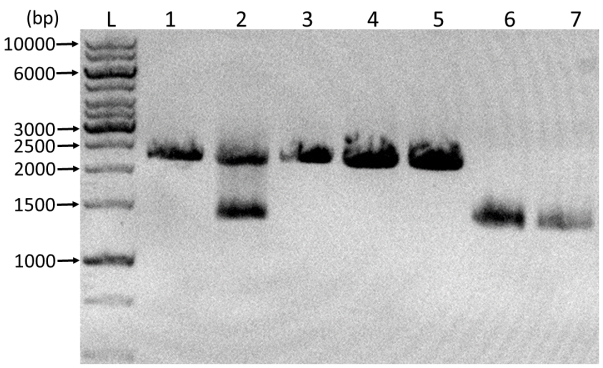

Figure 3.5: *1% agarose gel of SalI/NdeI digested plasmid DNA from all 6 colonies.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of SalI and NdeI digested plasmid DNA from colony 1.* ***2)*** *5µl of SalI and NdeI digested plasmid DNA from colony 2.* ***3)*** *5µl of SalI and NdeI digested plasmid DNA from colony 3.* ***4)*** *5µl of SalI and NdeI digested plasmid DNA from colony 4.* ***5)*** *5µl of SalI and NdeI digested plasmid DNA from colony 5.* ***6)*** *5µl of plasmid DNA from colony 1.* ***7)*** *5µl of plasmid DNA from colony 1 in digestion buffer (without enzyme).*

Undigested plasmid DNA from colony 1 was used as reference. The *Sal*I digested DNA in well 2-6 show fragment with the size of 2500 bp. DNA from colony 2 contains an additional fragment with the size of 1500 bp indicating that some of the DNA is undigested. The following digested with *Nde*I did not change the result as seen in Figure 3.5.

PCR was used to screen for the TagRFP-T fragment using the primers RFPForward and RFPReverse and using the isolated plamid DNA as template. The expected result would be a fragment of the size 750 bp. The PCR products have a size just below 1500 bp which can be seen in Figure 3.6.

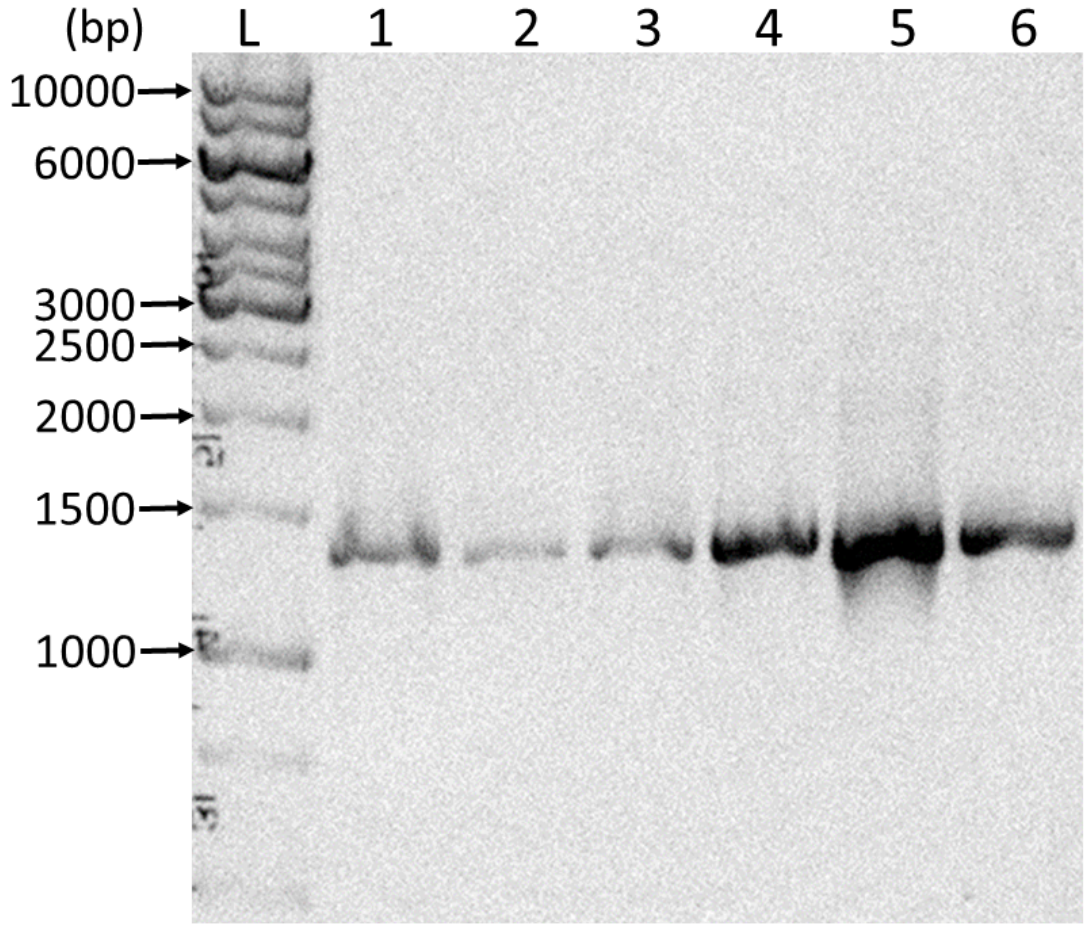

Figure 3.6: *1% agarose gel of PCR products of plasmid DNA from colony 1-6 using RFPforward and RFPbackwards as primers.* ***L)*** *5µl 1 kb ladder (Fermentas).* ***1)*** *5µl PCR product of plasmid DNA from colony 1.* ***2)*** *5µl PCR product of plasmid DNA from colony 2.* ***3)*** *5µl PCR product of plasmid DNA from colony 3.* ***4)*** *5µl PCR product of plasmid DNA from colony 4.* ***5)*** *5µl PCR product of plasmid DNA from colony 5.* ***6)*** *5µl PCR product of plasmid DNA from colony 6.*

It was concluded that the NSRFP had not been correctly inserted, and therefore the ligation was repeated. However this time the ligation product was transformed into *E. coli* BL21 (DE3).

3.1.6 Screening by transformation of pRFPLGFP

After transformation of the ligation product into *E. coli* BL21 (DE3) ten colonies were chosen and their plasmid DNA were isolated. The results of the DNA isolation can be seen in Figure 3.7 and Figure 3.8.

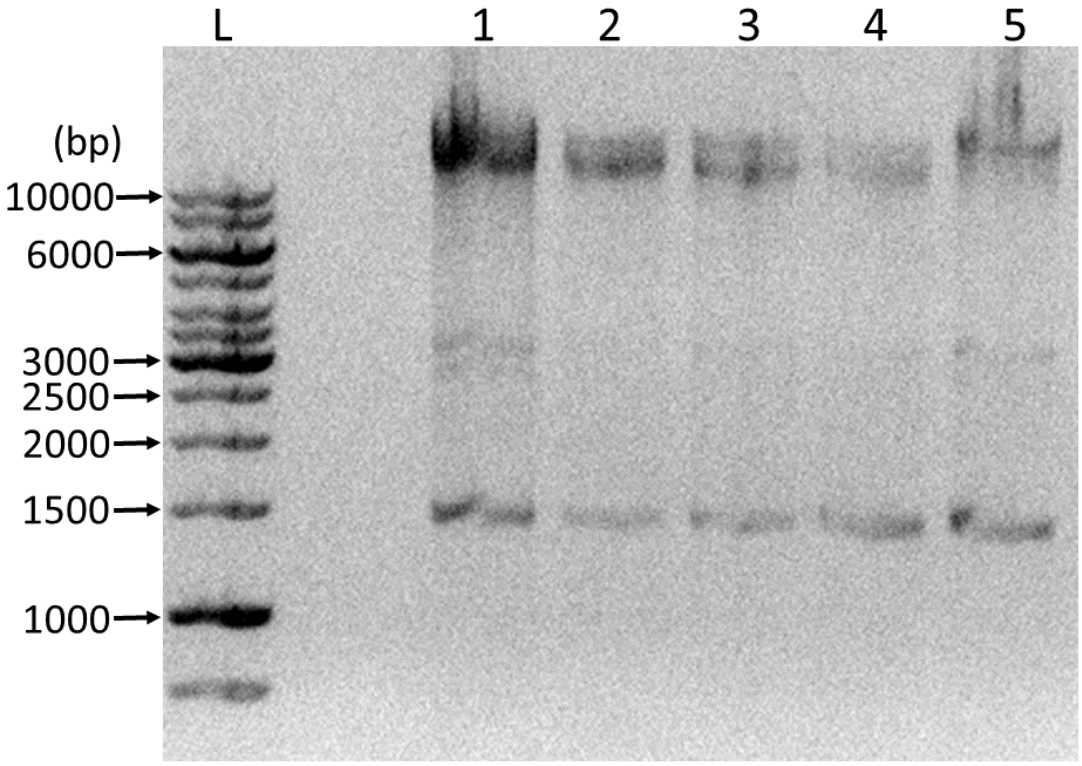

Figure 3.7: *1% agarose gel of minipreps from colony 1-5 .* ***L)*** *5µl 1 kb ladder (Fermentas).* ***1)*** *5µl mini prep. of pRFPLGFP from colony 1.* ***2)*** *5µl mini prep. of pRFPLGFP from colony 2.* ***3)*** *5µl mini prep. of pRFPLGFP from colony 3.* ***4)*** *5µl mini prep. of pRFPLGFP from colony 4.* ***5)*** *5µl mini prep. of pRFPLGFP from colony 5.*

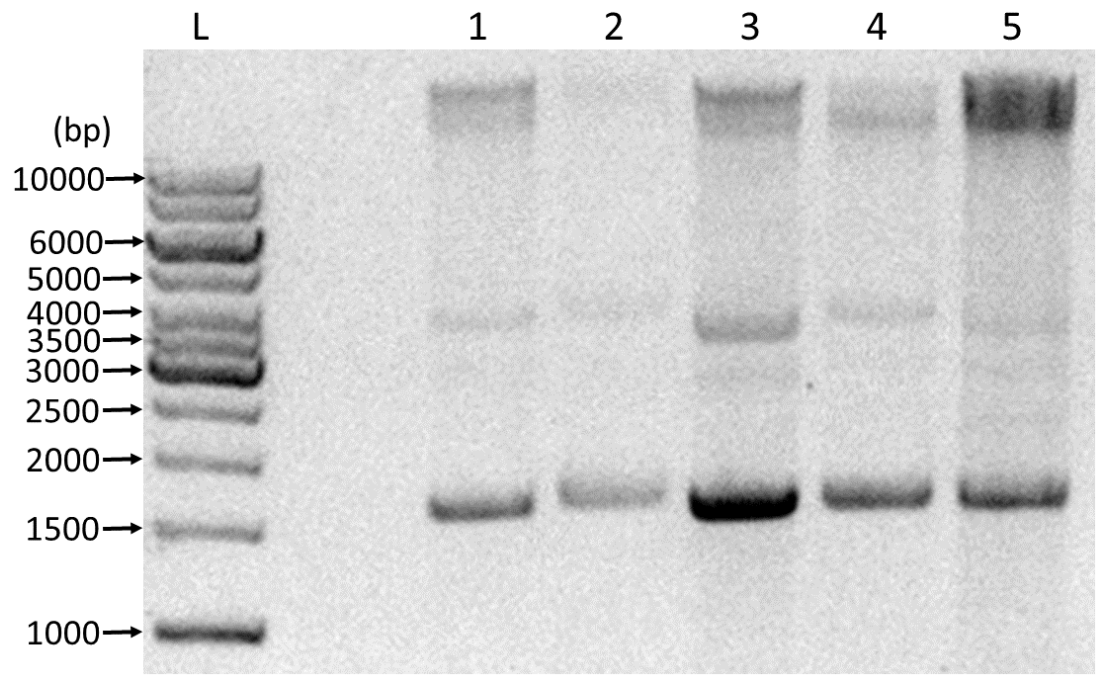

Figure 3.8: *1% agarose gel of minipreps from colony 6-10.* ***L)*** *5µl 1 kb ladder (Fermentas).* ***1)*** *5µl mini prep. of pRFPLGFP from colony 6.* ***2)*** *5µl mini prep. of pRFPLGFP from colony 7.* ***3)*** *5µl mini prep. of pRFPLGFP from colony 8.* ***4)*** *5µl mini prep. of pRFPLGFP from colony 9.* ***5)*** *5µl mini prep. of pRFPLGFP from colony 10.*

DNA from all 10 colonies showed fragments with sizes of 1500 bp, 4000 bp and above 10000 bp.

Plasmid DNA from all colonies were screened by PCR with the primers RFPForward and RFPReverse. The results of the PCR can be seen in Figure 3.9.

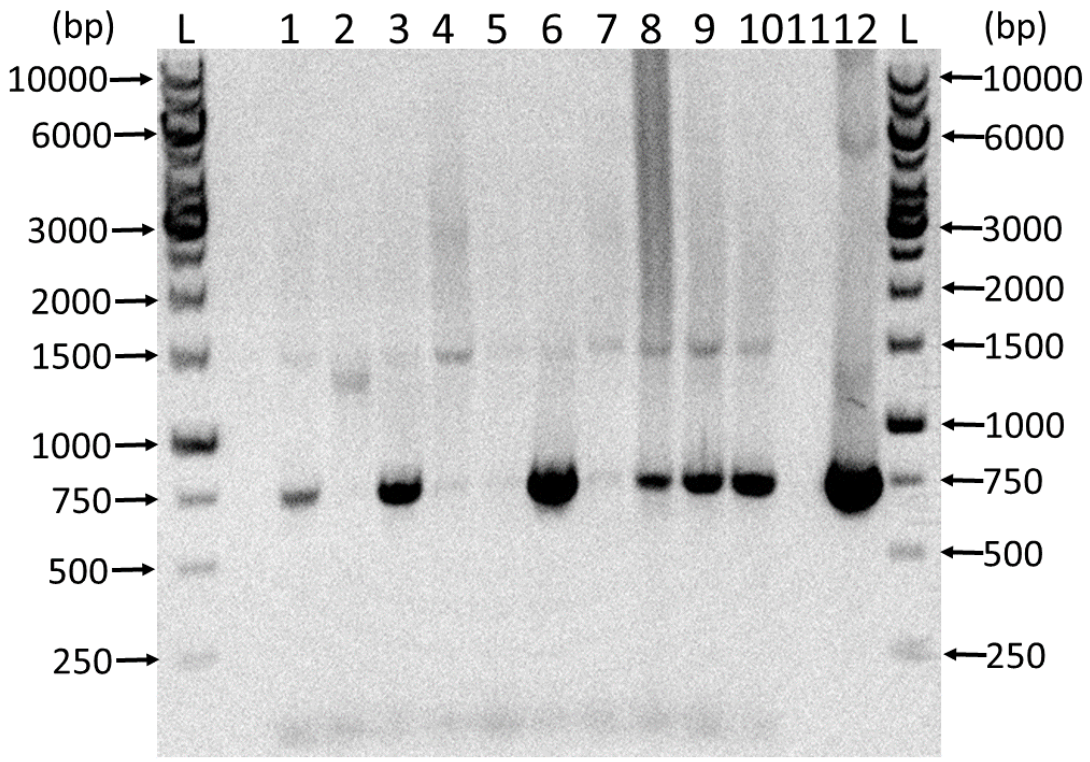

Figure 3.9: *1% agarose gel of PCR products using plasmid DNA from E. coli BL21 (DE3) colonies as template with RFPForward and RFPReverse as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from DNA isolated from colony 1.* ***2)*** *5µl of PCR product from DNA isolated from colony 2.* ***3)*** *5µl of PCR product from DNA isolated from colony 3.* ***4)*** *5µl of PCR product from DNA isolated from colony 4.* ***5)*** *5µl of PCR product from DNA isolated from colony 5.* ***6)*** *5µl of PCR product from DNA isolated from colony 6.* ***7)*** *5µl of PCR product from DNA isolated from colony 7.* ***8)*** *5µl of PCR product from DNA isolated from colony 8.* ***9)*** *5µl of PCR product from DNA isolated from colony 9.* ***10)*** *5µl of PCR product from DNA isolated from colony 10.* ***11)*** *Empty.* ***12)*** *5µl of PCR product from pcDNA3-TagRFP-T using the primers RFPForward and RFPReverse (control).* ***L)*** *6µl 1 kb ladder (Fermentas).*

Well 12 shows a reference where pcDNA3-TagRFP-T was used as template and RFPForward and RFPReverse as primers. In most of the wells fragment of the size of 750 bp, 1500 bp and below 250 bp can be seen. Due to the correct size of the PCR product, some colonies were further investigated. These were colonies 1, 3, 6, 8, 9, 10.

3.2 Expression of plasmid DNA from selected colonies

Colony 1, 3, 6, 8, 9 and 10 were expressed. The bacteria were grown at 37 °C in LB-Amp medium. When the OD reached 0.9 IPTG was added for a final concentration of 200 μM and the temperature was reduced to 25 °C. The cultures were incubated overnight. The cells were opened via an osmotic shock resulting in a water and a TES fraction containing the proteins from the bacterias. The TES and water fractions were analyzed using SDS-PAGE analysis (see Figure 3.10 and Figure 3.11).

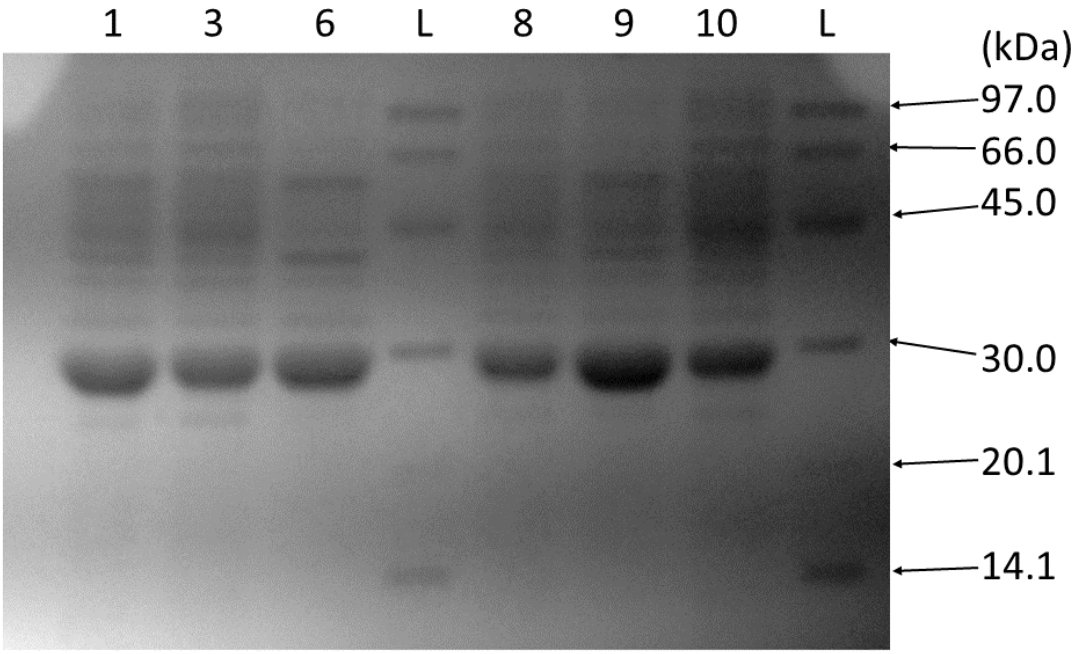

Figure 3.10: *Coomassie stained 12% SDS-PAGE of the TES fraction from expression studies using plasmids from colony 1, 3, 6, 8, 9, 10. All studies were performed in LB amp medium at 25 °C and induced 8 hr with 100 mM IPTG starting at an OD of 0.9).* ***1)*** *4µl TES fraction from colony 1.* ***3)****4µl TES fraction from colony 3.* ***6)****4µl TES fraction from colony 6.* ***L)*** *3µl LMW standard.* ***8)****4µl TES fraction from colony 8.* ***9)****4µl TES fraction from colony 9.* ***10)****4µl TES fraction from colony 10.* ***L)*** *3µl LMW standard*

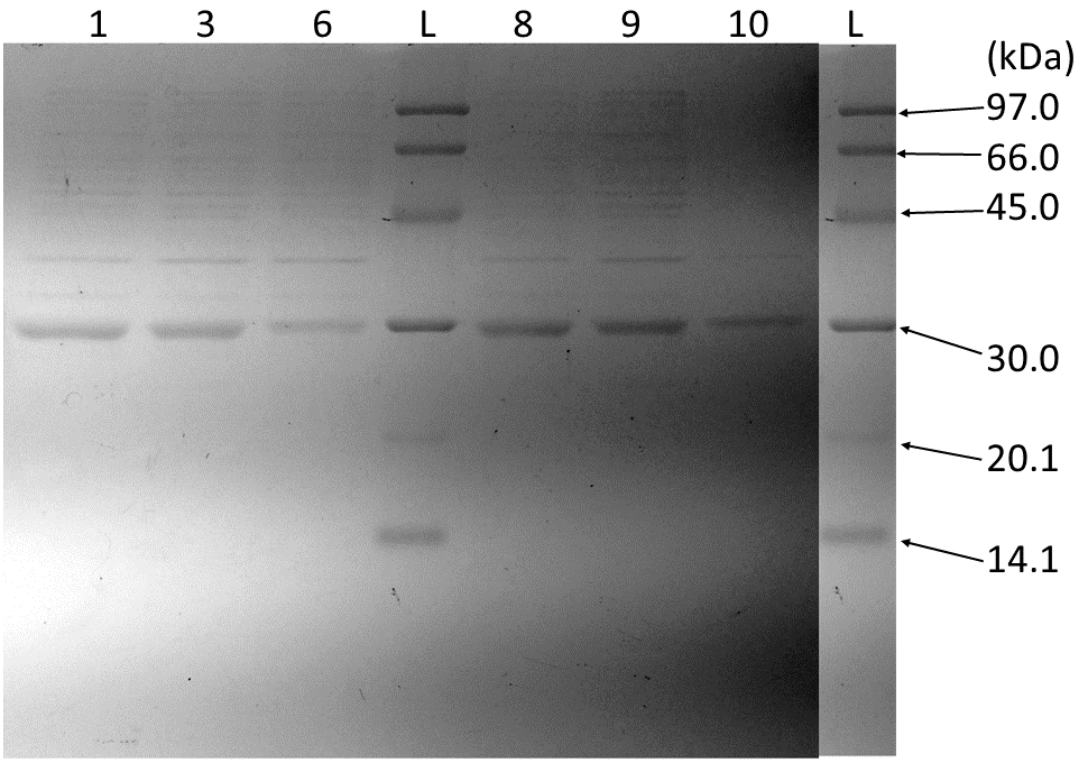

Figure 3.11: *Coomassie stained 12% SDS-PAGE of the water fraction from expression studies using plasmids from colony 1, 3, 6, 8, 9, 10. All studies were preformed in LB amp medium at 25 °C and induced 8 hr with 100 mM IPTG starting at an OD of 0.9.* ***1)*** *4µl water fraction from colony 1.* ***3)****4µl water fraction from colony 3.* ***6)****4µl water fraction from colony 6.* ***L)*** *3µl LMW standard.* ***8)****4µl water fraction from colony 8.* ***9)****4µl water fraction from colony 9.* ***10)****4µl water fraction from colony 10.* ***L)*** *3µl LMW standard.*

The expected size of the protein is around 56 kDa. Both the TES and the water fraction seems to have high amounts of protein of the size of 30 kDa. Both fractions were analyzed using fluorescence spectroscopy however, nothing conclusive was found.

3.3 Construction of pCasper3

pCasper3 was prepared by inserting the Casper3 gene into a pET11a Vector. In order to ligate these the Casper3 gene was modified by introducing a *Nde*I site at the 5'-end of the Casper3 gene and a *Bam*HI site at the 3'-end of the Casper3 gene (NBCasper3). These modifications were done by PCR. There were several attempts at producing the NBCasper3 PCR product.

3.3.1 Initial PCR attempts

The primers CasperForward1 and CasperReverse1 were used, along with the original DNA of pCasper3-GR as template. The expected fragment size is around 1500 (TagGFP + TagRFP). A fragment of this size could not be found but rather a distribution of many different size fragments also known as a smear. The results of this can be seen in Figure 3.12.

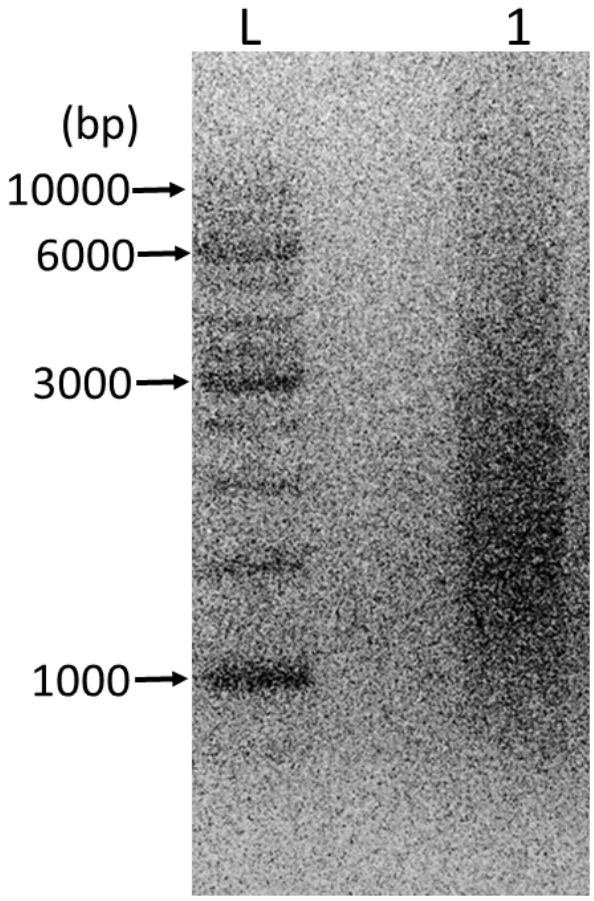

Figure 3.12: *1% agarose gel of the PCR product pCasper3-GR PCR using Casper-Forward1 and CasperReverse1 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of pCasper3-GR PCR product.*

The second PCR was performed using the same procedure, however the template amount was reduced from 250 ng to 10 ng. This did not solve the problem.

For the next PCR attempt freshly isolated DNA was used as template. This did not solve the problem.

3.3.2 Futher investigation of the problem

Since the template was freshly prepared, the problem was believed to be the PCR itself.

Investigation of the Taq enzyme

The PCR procedure was repeated, however this time a control was used. This control was a PCR using pcDNA3-TagRFP-T as template and the primers RFPForward and RFPReverse Controls without Taq were also performed. Figure 3.13 shows that the PCR reaction works when pcDNA3-TagRFP-T (Well1) is used as template indicating that the Taq enzyme was not the problem.

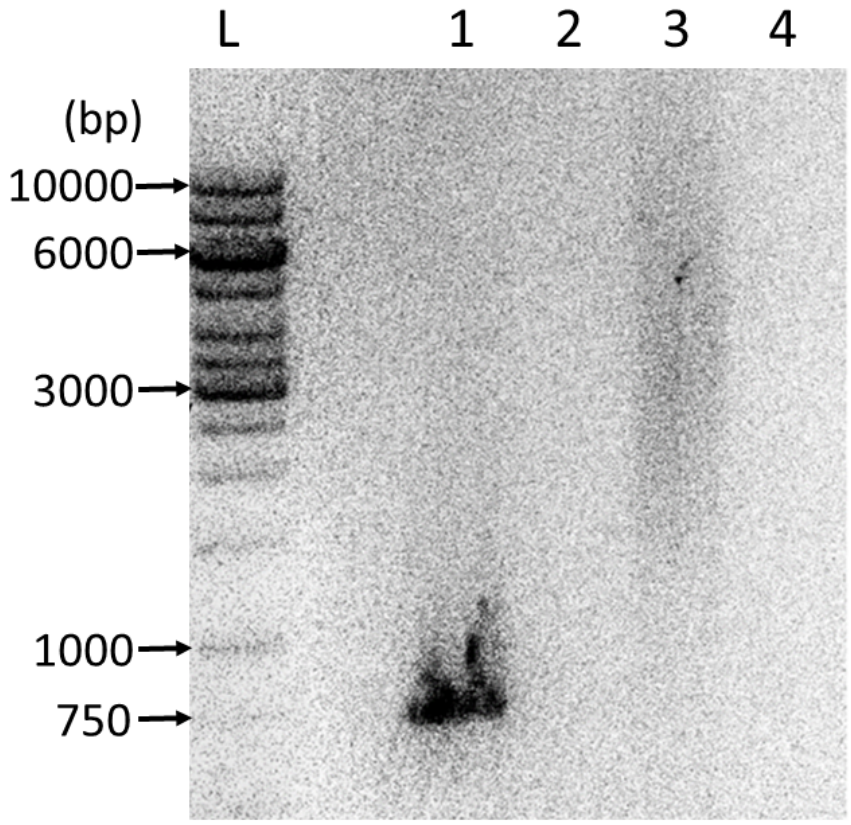

Figure 3.13: *1% agarose gel of PCR products using pCasper3-GR and pcDNA3-TagRFP-T PCR as templates.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of pcDNA3-TagRFP-T PCR product using RFPForward and RFPReverse as primers.* ***2)*** *5µl of pcDNA3-TagRFP-T PCR reaction mixture without Taq.* ***3)*** *5µl of pCasper3-GR PCR product using Casper-Forward1 and CasperReverse1 as primers.* ***4)*** *5µl of pCasper3-GR PCR reaction mixture without Taq.*

Checking template using *Sal*I Digestion

The pCasper3-GR template was investigated by digestion using *Sal*I. pCasper3-GR has two *Sal*I recognition sites at position 639 and 1396. A digestion will therefore give two fragments one with a size of 750 bp and one with a size of 4750 bp (pCasper3-GR vector 5500 bp - 750 bp = 4750 bp). According to Figure 3.14 the template was correct.

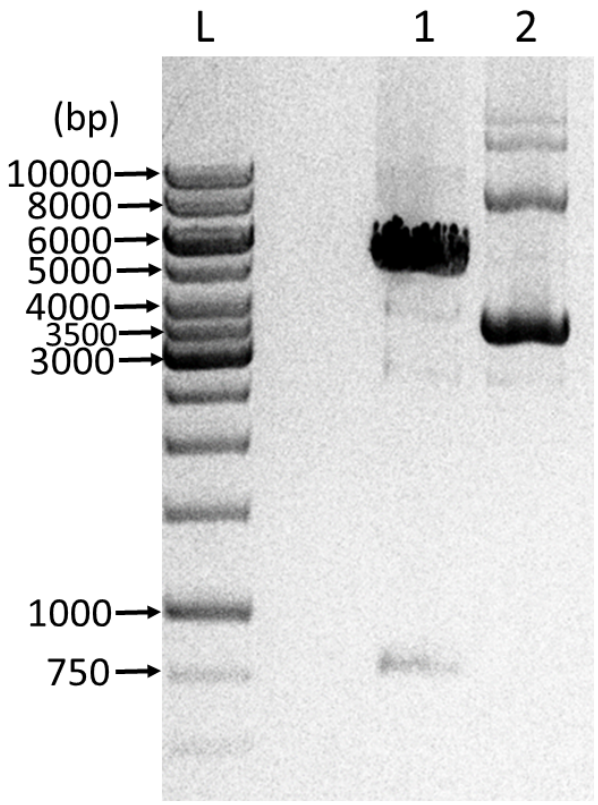

Figure 3.14: *1% agarose gel of SalI digested pCasper3-GR.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of SalI digested pCasper3-GR.* ***2)*** *5µl of pCasper3-GR undigested.*

MgCl optimization

Since the template and the Taq polymerase were not the problem, the PCR reaction was optimized using varying concentrations of MgCl ranging from 1 mM - 5 mM in 1 mM intervals. This did not solve the problems.

PCR with increased denaturation temperature

The PCR process was repeated using a denaturation temperature from 95 °C to 97 °C. This approach had also no positive effect on the PCR reaction.

PCR Using linearized pCasper3-GR

The PCR was performed using linearized pCasper3-GR. The DNA was linearized with *Nde*I. This did not solve the problem.

3.3.3 PCR using new primers

Since none of the previously described attempts improved the result, it was concluded that the primers might not anneal optimally. Therefore, new primers were designed.

PCR reactions were performed using the new primers CasperForward2 and CasperReverse2, and original and freshly isolated pCasper3-GR as template. As a result of this PCR a fragment of the size of 1500 bp was obtained which can be seen in Figure 3.15.

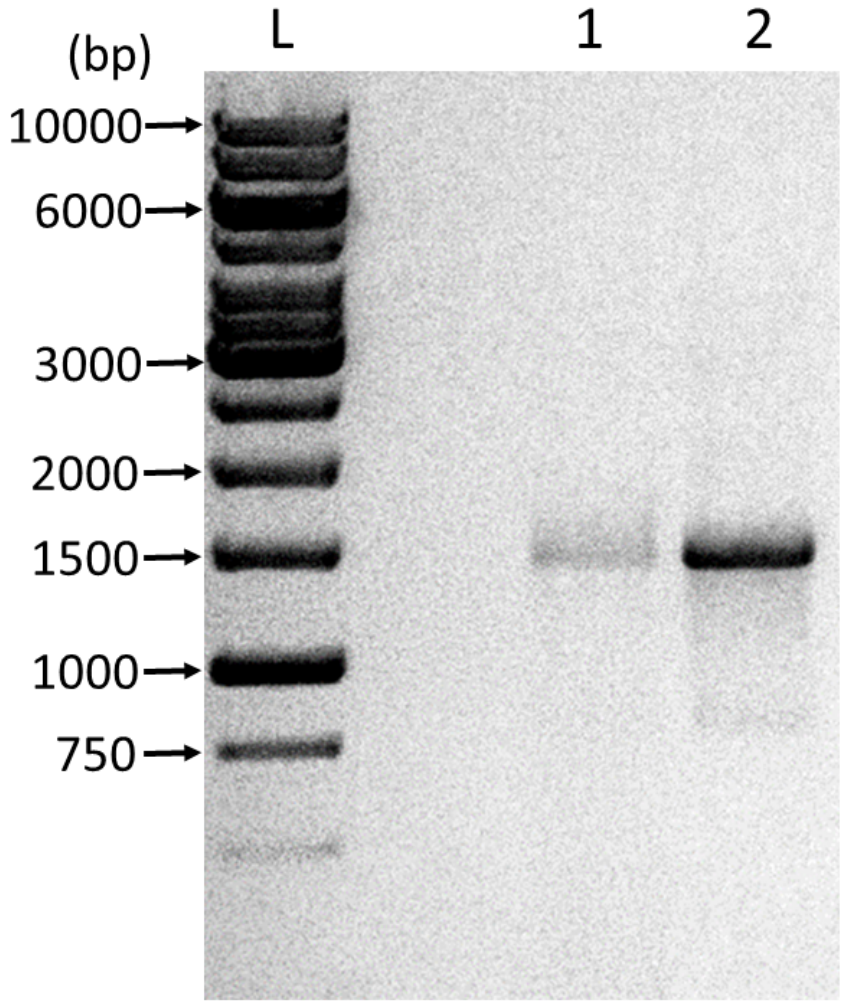

Figure 3.15: *1% agarose gel of pCasper3-GR PCR product using CasperForward2 and CasperReverse2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of original pCasper3-GR PCR product.* ***2)*** *5µl of freshly isolated pCasper3-GR PCR product.*

The PCR product from freshly isolated pCasper3-GR gave the best results and was digested using *Bam*HI and *Nde*I in order to be used for ligation.

3.3.4 Preparation of pET11a vector

pGFP (pEt11a + GFP) was used as source for pET11a in the ligation. The plasmid was digested with *Bam*HI and *Nde*I (see Figure 3.16) to obtain the necessary vector part. The plasmid was digested using an opposite order in the digestion process.

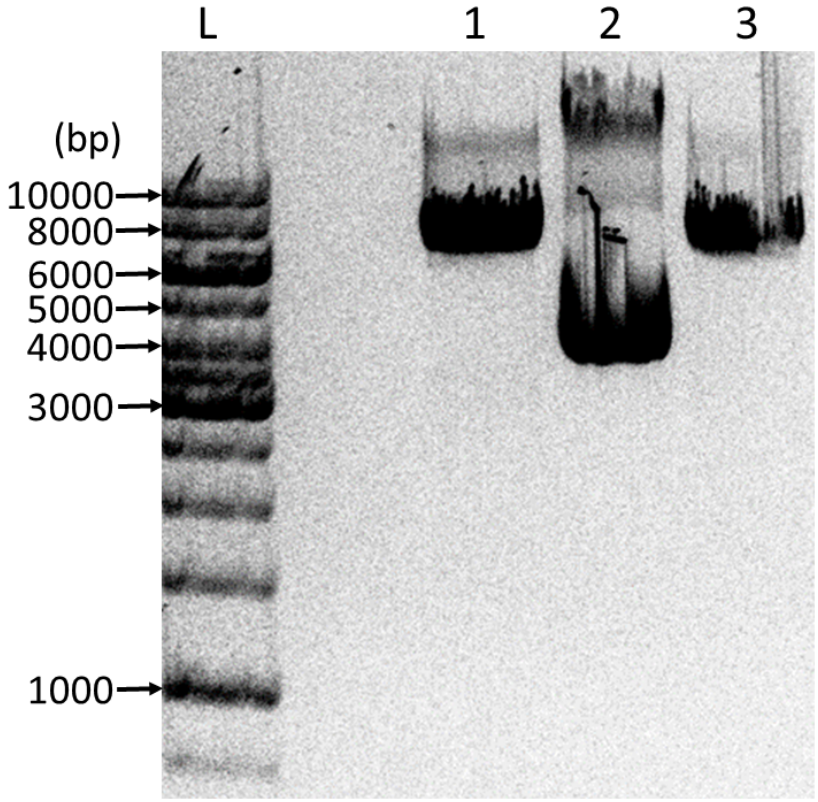

Figure 3.16: *1% agarose gel of NdeI/BamHI digested pGFP.* ***L)*** *5µl 1 kb ladder (Fermentas).* ***1)*** *5µl pGFP digested with BamHI* ***2)*** *5µl pGFP.* ***3)*** *5µl pGFP digested with NdeI.*

3.3.5 Ligation and transformation of pET11a and NBCasper3 fragments

A ligation was made with the digested pet11a vector and the digested NBCasper3 fragment, as well as a control without NBCasper3. Both were transformed into *E. coli* 5-alpha .

12 colonies were chosen for screening with PCR. CasperForward2 and CasperReverse2 were used as primer in the screening. The PCR product of colony 1, 9, 10 and 11 showed smears and the rest showed no product. Figure 3.17 and 3.18 shows the result the PCRs.

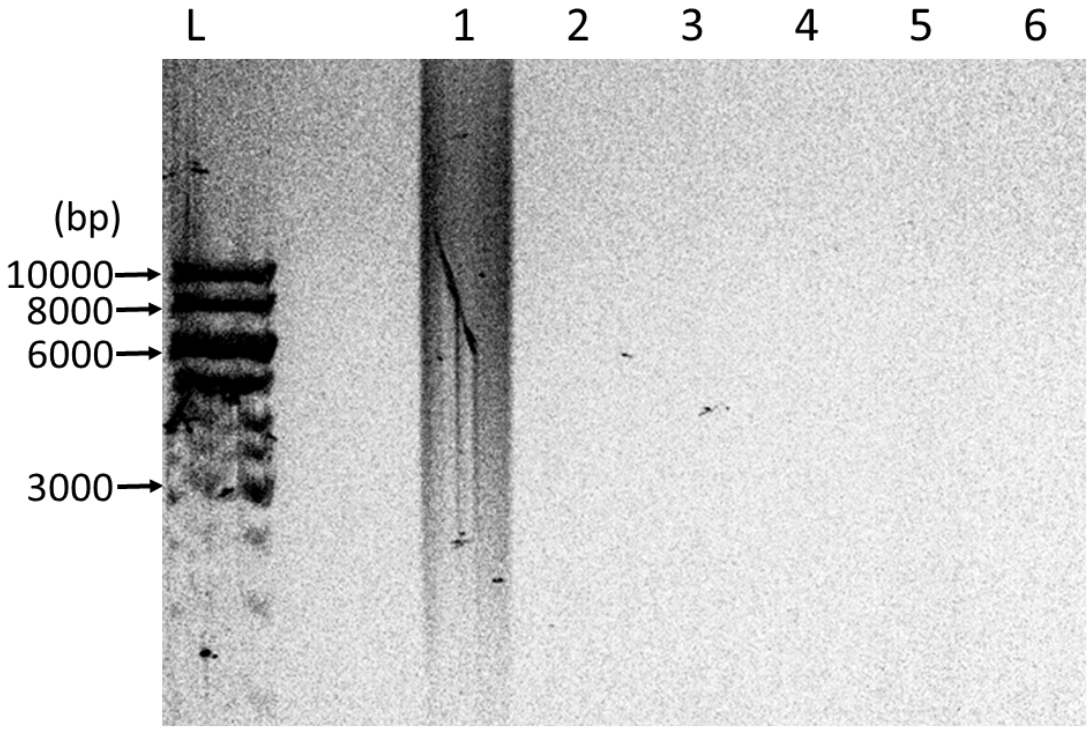

Figure 3.17: *1% agarose gel of PCR products from transformants potentially harboring pCasper3 using CasperForward2 and CasperReverse2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from colony 1.* ***2)*** *5µl of PCR product from colony 2.* ***3)*** *5µl of PCR product from colony 3.* ***4)*** *5µl of PCR product from colony 4.* ***5)*** *5µl of PCR product from colony 5.* ***6)*** *5µl of PCR product from colony 6.*

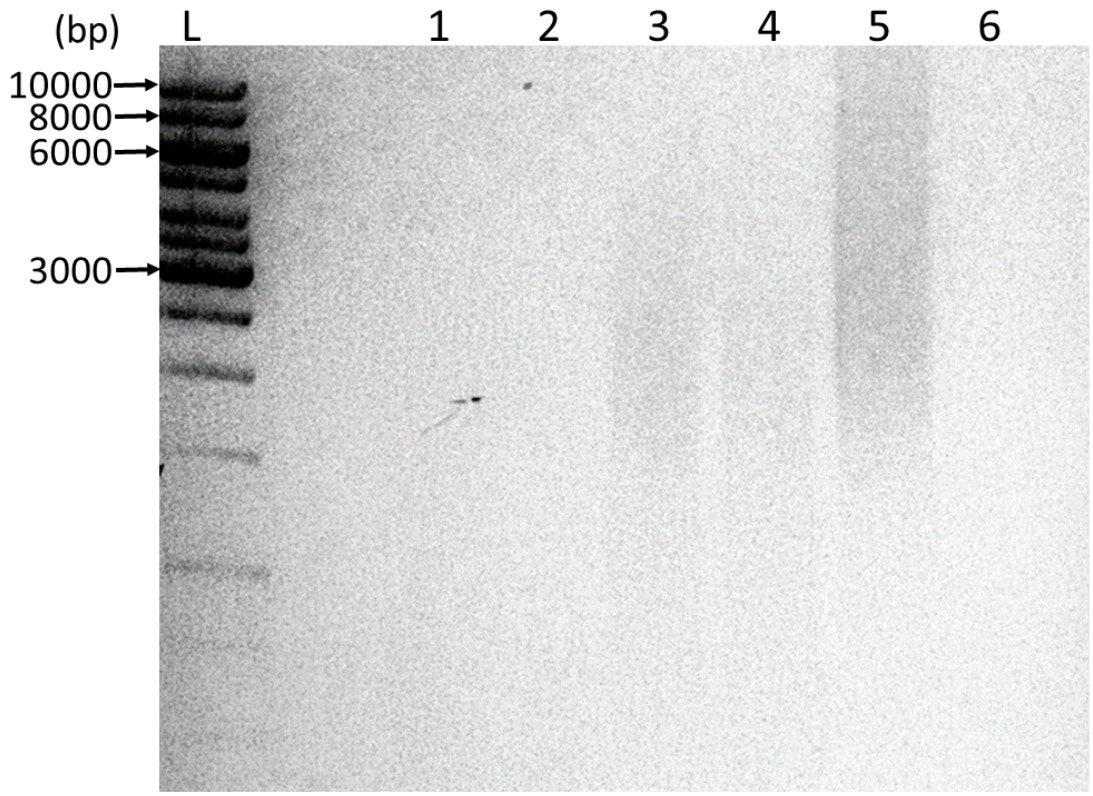

Figure 3.18: *1% agarose gel of PCR products from transformants potentially harboring pCasper3 using CasperForward2 and CasperReverse2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from colony 7.* ***2)*** *5µl of PCR product from colony 8.* ***3)*** *5µl of PCR product from colony 9.* ***4)*** *5µl of PCR product from colony 10.* ***5)*** *5µl of PCR product from colony 11.* ***6)*** *5µl of PCR product from colony 12.*

DNA from colony 1 and 11 were screened by digestion with *Sal*I and the result can be seen in Figure 3.19.

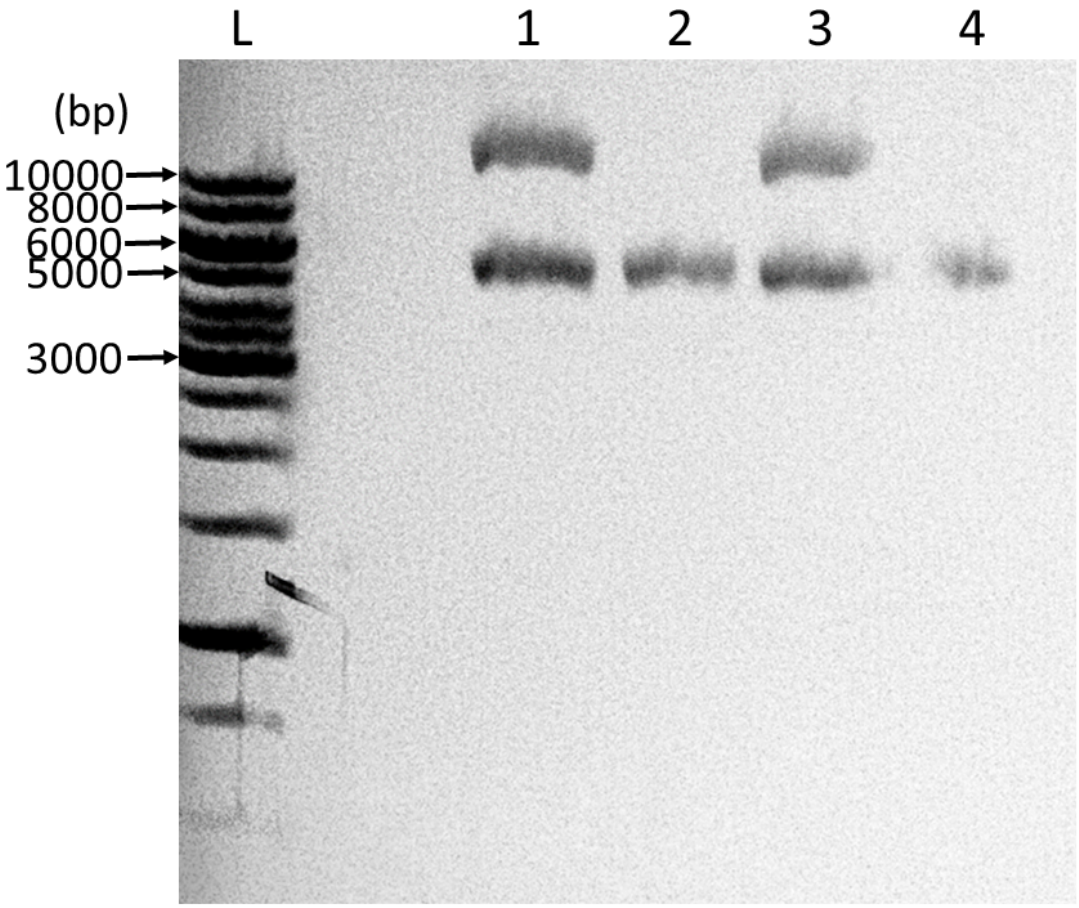

Figure 3.19: *1% agarose gel of SalI digested plasmid DNA from colonies 1 and 11.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of SalI digested plasmid DNA from colony 1.* ***2)*** *5µl of plasmid DNA from colony 1.* ***3)*** *5µl of SalI digested plasmid DNA from colony 11.* ***4)*** *5µl of plasmid DNA from colony 11.*

The *Sal*I restriction site was chosen as it does not exist in pET11a, but is present once in Casper3. The expected result for a successful digestion would be a single fragment around 7000 bp (~ 5500 for pET11a and 1500 for NBCasper3). *Sal*I can have the effect of nicking plasmid DNA and bringing supercoiled DNA into a relaxed state. This can be seen as a change in ratio between supercoiled and relaxed DNA. Well 1 and 3 in Figure 3.19 shows digested DNA while 2 and 4 shows uncut DNA. As a result of this procedure no linearized DNA of the size of 7000bp could be detected but an increase amount of nicked DNA can be seen. Futher colonies were tested but did not reveal any positive results.

An additional screening using the primers pET11a1 and pET11a2 for PCR was performed. These primers were used on selected colonies. The PCR was performed on colonies 2-5, 7-9, 12-14, 16-17 and 19-20. pET11a2 anneals around 170 bp upstream of the start codon of pET11a and pET11a1 anneals around 130 bp downstream of the stop codon. The expected products of the PCR is as follows: If there is no insert a fragment with a size of 300 would appear. If the GFP is still in the vector a fragment will be seen at 1000 bp and if Casper3 is inserted in the vector a fragment at 1800 bp would be obtained. Figure 3.20, 3.21

shows the result of the experiment. The PCR product for most of the colonies screened, gave a fragment around 400 bp.

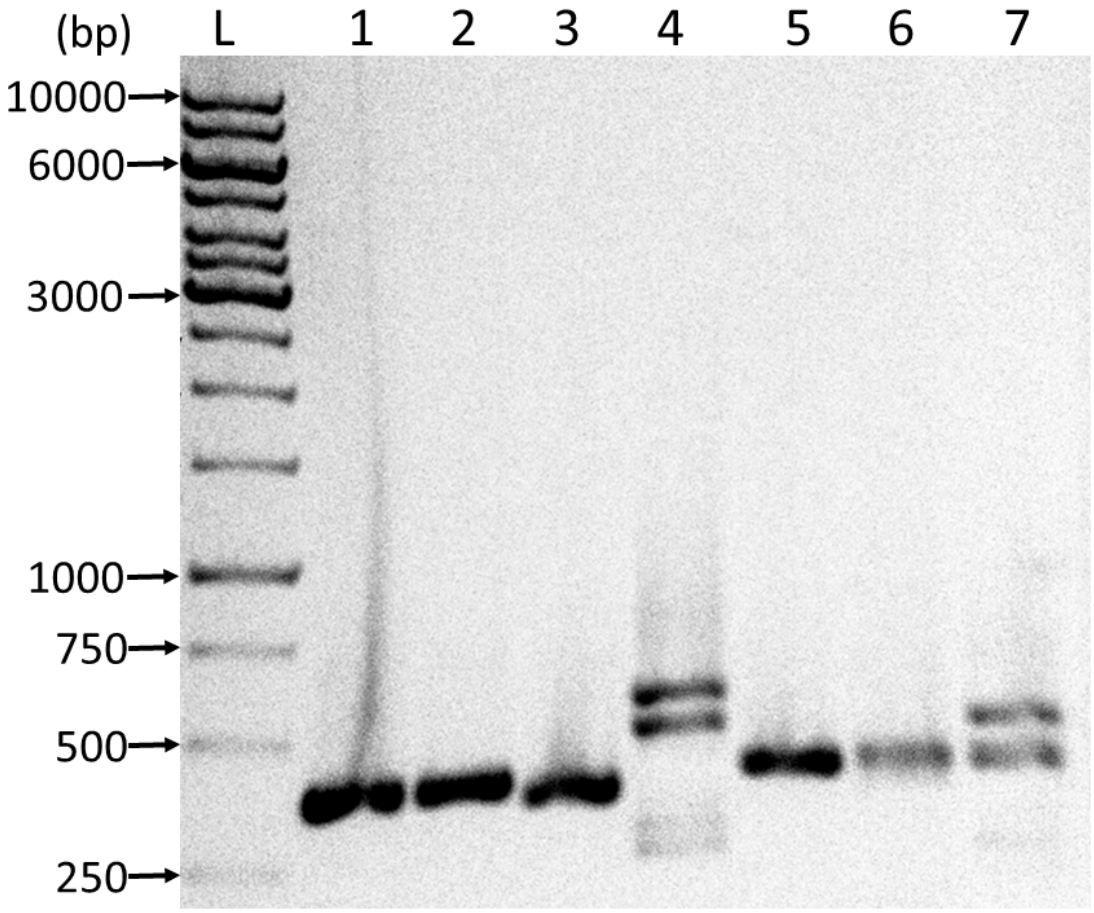

Figure 3.20: *1% agarose gel of PCR products from selected colonies using pET11a1 and pET11a2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from DNA isolated from colony 2.* ***2)*** *5µl of PCR product from DNA isolated from colony 3.* ***3)*** *5µl of PCR product from DNA isolated from colony 4.* ***4)*** *5µl of PCR product from DNA isolated from colony 5.* ***5)*** *5µl of PCR product from DNA isolated from colony 7.* ***6)*** *5µl of PCR product from DNA isolated from colony 8.* ***7)*** *5µl of PCR product from DNA isolated from colony 9.*

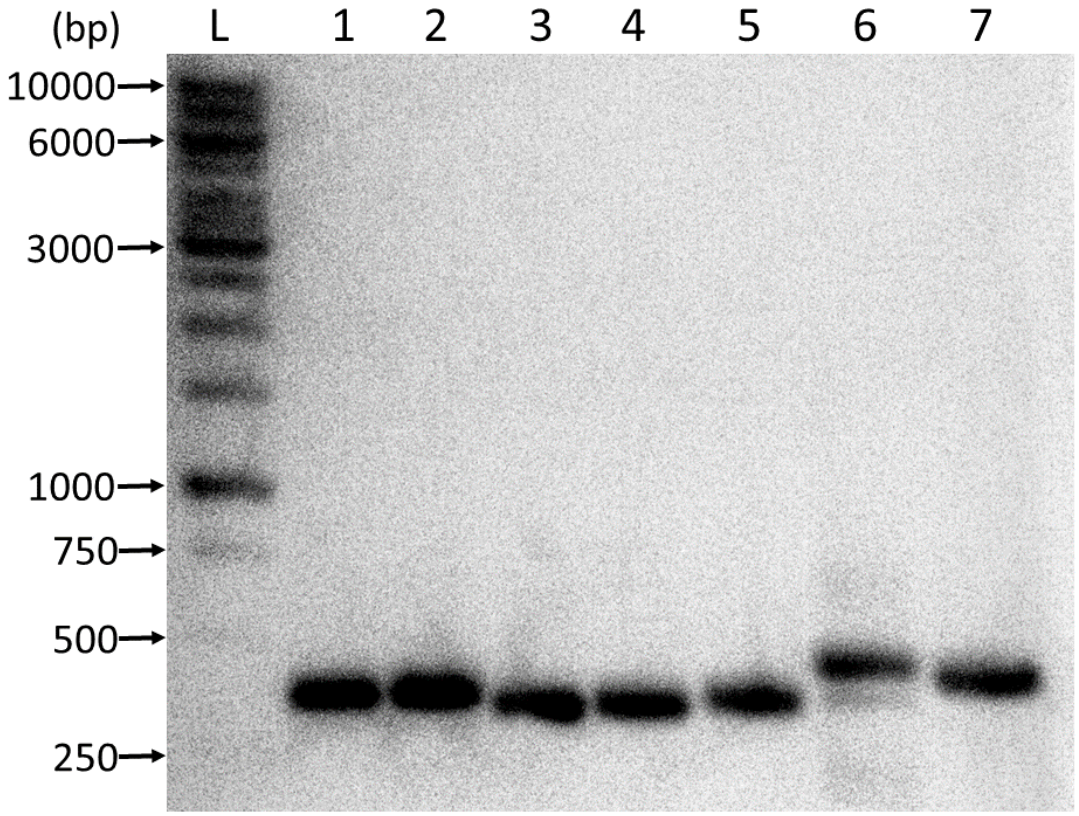

Figure 3.21: *1% agarose gel of PCR products from selected colonies using pET11a1 and pET11a2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from DNA isolated from colony 12.* ***2)*** *5µl of PCR product from DNA isolated from colony 13.* ***3)*** *5µl of PCR product from DNA isolated from colony 14.* ***4)*** *5µl of PCR product from DNA isolated from colony 16.* ***5)*** *5µl of PCR product from DNA isolated from colony 17.* ***6)*** *5µl of PCR product from DNA isolated from colony 19.* ***7)*** *5µl of PCR product from DNA isolated from colony 20.*

According to the result from the PCR none of the investigated DNA from the selected colonies showed an insert of the correct size.

Second ligation and transformation of pCasper3

Due to no positive clone a new attempt was performed to generate pCasper3. This includes a new digestion of pGFP and a new production of NBCasper3 using PCR. As a variation from the last approach the PCR product was cleaned after each digestion. This was done to maximize purity, however it did reduce the concentration of DNA greatly. The digested NBCasper3 and digested pET11a were ligated and transformed into *E. coli* 5-alpha, However, no growth of transformants was detected.

3.3.6 PCR using new primers

Since the Casper3 was not inserted correctly, there are 3 possible causes: Problems with the Vector, problem with the ligase or problem with the PCR product. Since it has been observed that the ligase successfully recircularized digested pET11a, it was concluded that the PCR product was the problem. Digestion of DNA close to the ends of the DNA can be a problem. If the overhang of nucleotides at the recognition site are not high enough the cleavage can be incompleted. New primers were designed with a *Nde*I overhang of 6, and a *Bam*HI overhang of 4. These were called CasperForward3 and CasperReverse3.

The primers were used to generate NBCasper3. The digested NBCasper3 and digested pET11a were ligated and transformed into *E. coli* 5-alpha.Transformants could be detected. Growth was also seen on control plates.

3.3.7 Screening for pCasper3

12 Transformants were screened. Primers pET11a1 and pET11a2 were used for screening by PCR. Figure 3.22 and Figure 3.23 show the results of this screening.

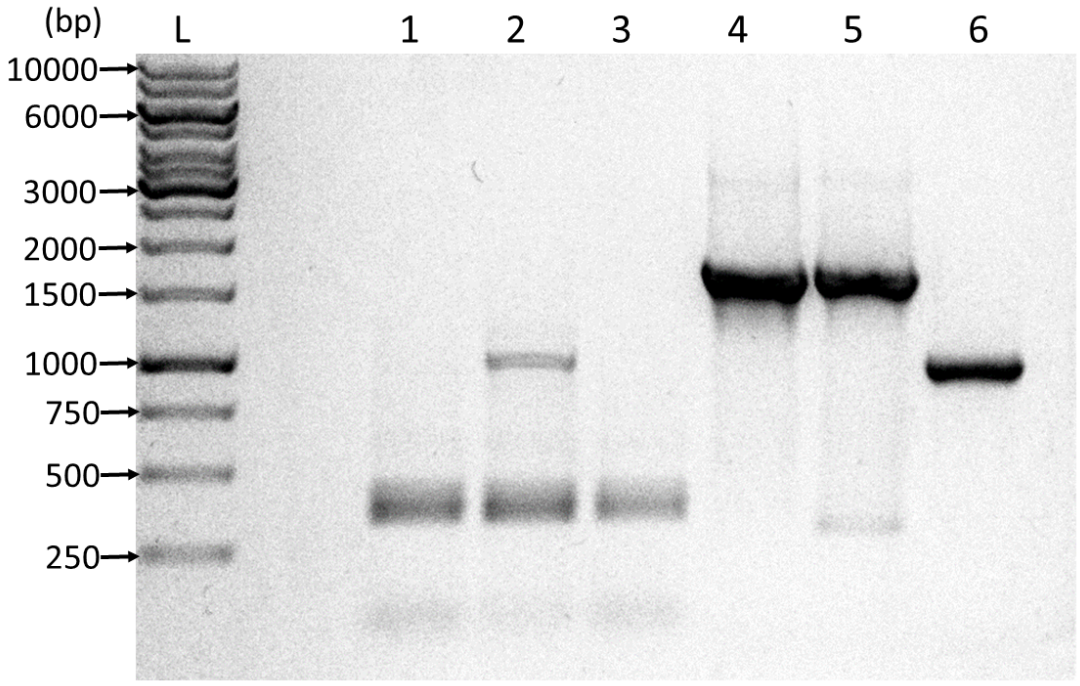

Figure 3.22: *1% agarose gel of PCR products from transformants using pET11a1 and pET11a2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from colony 1.* ***2)*** *5µl of PCR product from colony 2.* ***3)*** *5µl of PCR product from colony 3.* ***4)*** *5µl of PCR product from colony 4.* ***5)*** *5µl of PCR product from colony 5.* ***6)*** *5µl of PCR product from colony 6.*

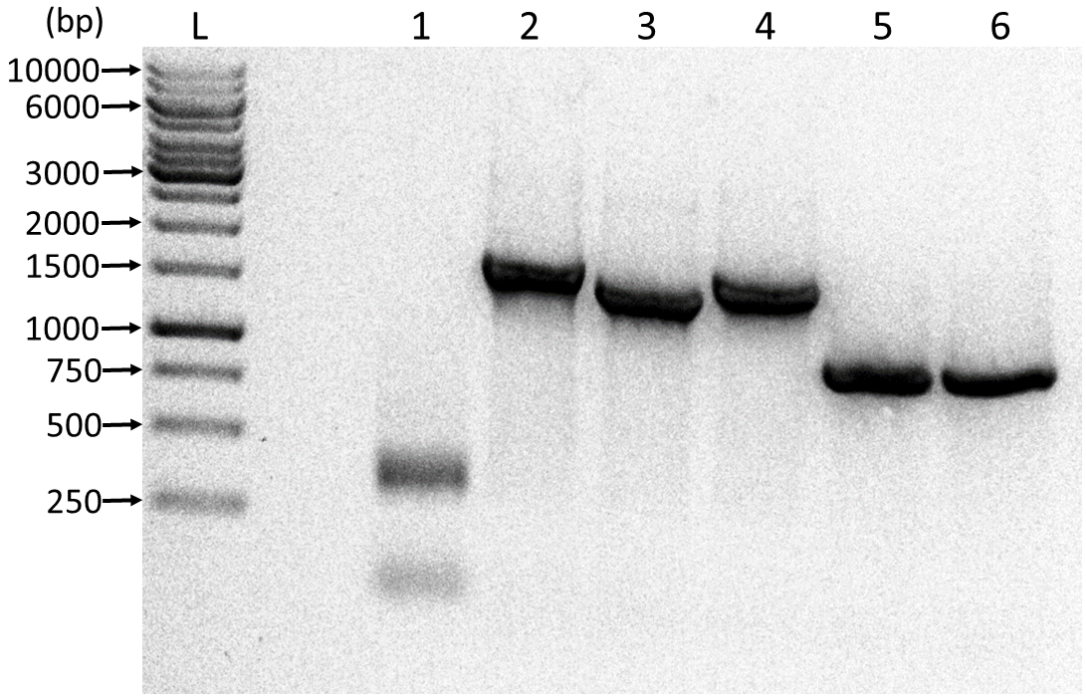

Figure 3.23: *1% agarose gel of PCR products from transformants using pET11a1 and pET11a2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from colony 7.* ***2)*** *5µl of PCR product from colony 8.* ***3)*** *5µl of PCR product from colony 9.* ***4)*** *5µl of PCR product from colony 10.* ***5)*** *5µl of PCR product from colony 11.* ***6)*** *5µl of PCR product from colony 12.*

Transformants 4 and 5 (Figure 3.22) and Transformants 8+9+10 (Figure 3.23) yield PCR products of the size of 1800 bp which indicate that the insert of these transformants is correct.

Transformants 4,5,8,9,10 were further screened using *Sal*I digestion (Figure 3.24) Transformant 11, which has an insert of 750 bp (size of GFP, see Figure 3.23), was used as control, since it does not have a *Sal*I site.

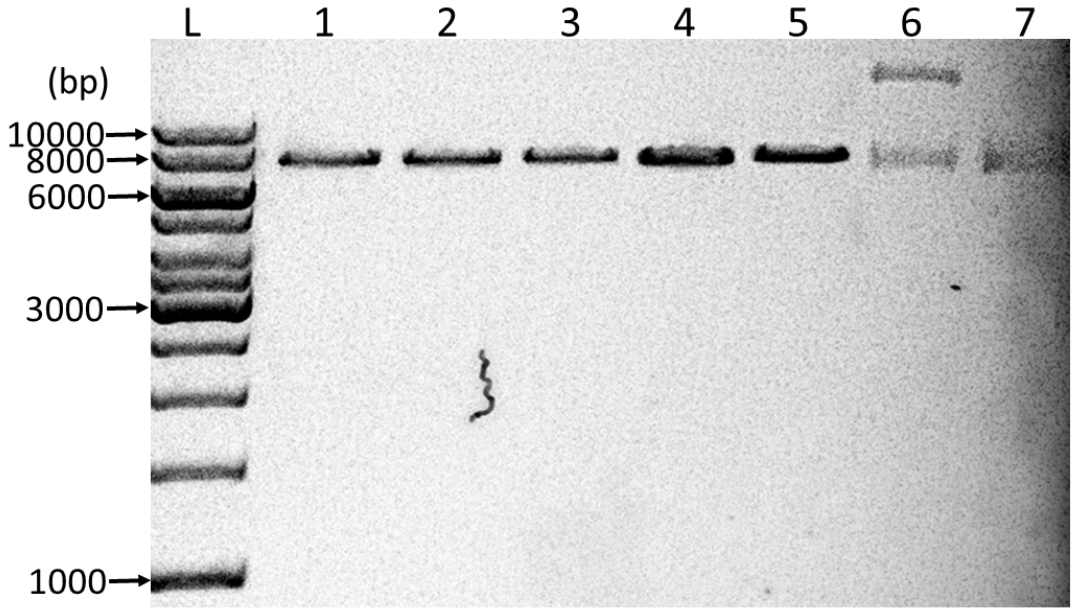

Figure 3.24: *1% agarose gel of SalI digested transformants harboring pCasper3.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of SalI digested plasmid DNA from colony 4.* ***2)*** *5µl of SalI digested plasmid DNA from colony 5.* ***3)*** *5µl of SalI digested plasmid DNA from colony 8.* ***4)*** *5µl of SalI digested plasmid DNA from colony 9.* ***5)*** *5µl of SalI digested plasmid DNA from colony 10.* ***6)*** *5µl of SalI digested plasmid DNA from colony 11 (Control).* ***7)*** *5µl of plasmid DNA from E. coli colony 11 in digestion mixture without SalI (Control).*

Figure 3.24 shows that all selected transformants can be linearized with *Sal*I, which results in fragments of about 8000 bp. The size of the linear fragments suggests that all selected transformant are correct.

Transformants 4, 5, 8 and 9 were further screened by using *Nde*I/*Sal*I. The *Nde*I digestion site is located at the start codon while the *Sal*I digestion site is located in the linker between the TagRFP and TagGFP sequence in Casper3. The result of this digestion should be DNA fragments with a size of about 750 bp + 7000 bp.

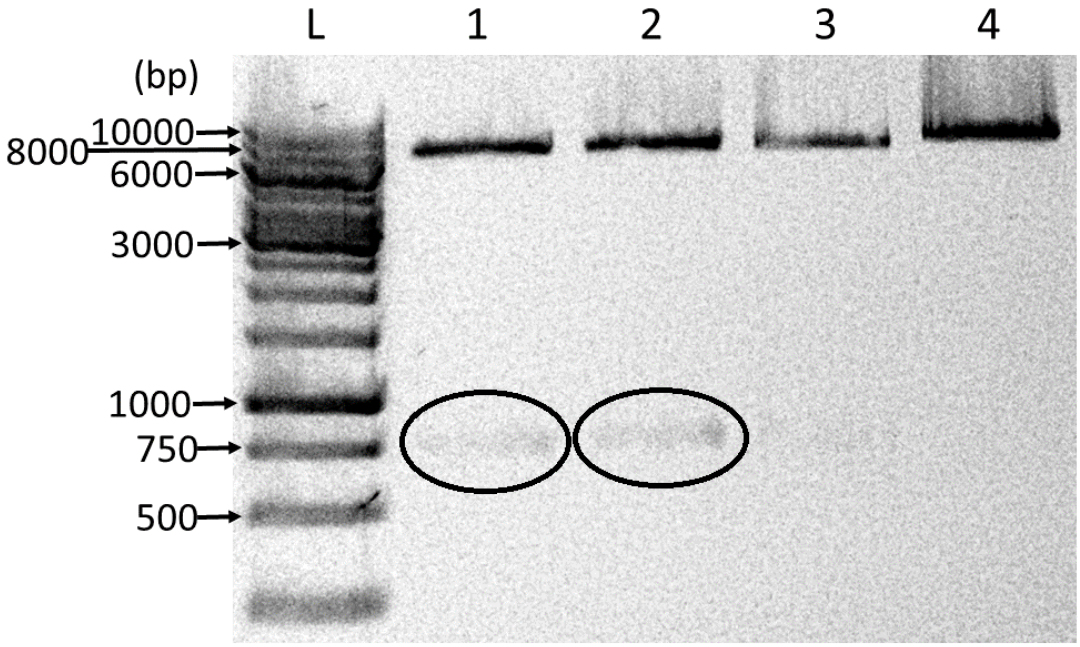

Figure 3.25: *1% agarose gel of SalI/NdeI digested Transformants potentially harboring pCasper3.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of SalI and NdeI digested plasmid DNA from colony 4.* ***2)*** *5µl of SalI and NdeI digested plasmid DNA from colony 5.* ***3)*** *5µl of SalI and NdeI digested plasmid DNA from colony 8.* ***4)*** *5µl of SalI and NdeI digested plasmid DNA from colony 9.*

According to Figure 3.25 transformants 4+5 revealed fragments of the size of 750 bp and around 7000 bp. Plasmids from transformant 5 were used for expression studies.

3.4 Transformation in *E. coli* Bl21 (DE3)

Plasmid DNA from colony 5 were used for transformation into *E. coli* BL21 (DE3). 8 colonies were selected and screened using PCR with pET11a1 and pET11a2 as primers. The result of this can be seen in Figure 3.26.

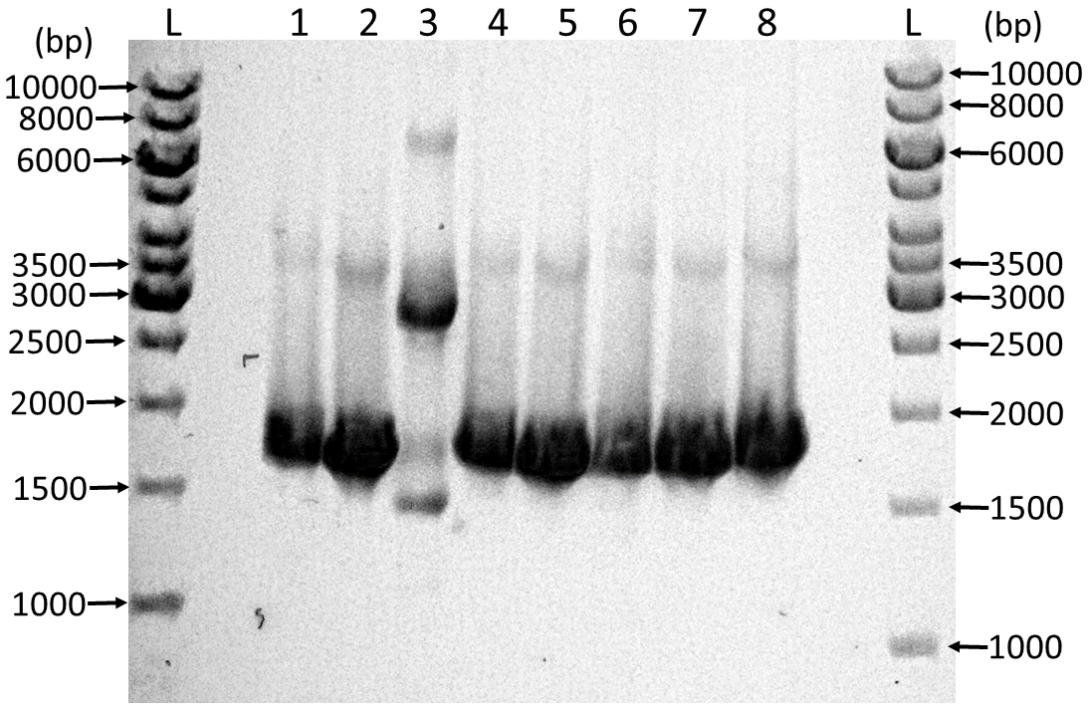

Figure 3.26: *1% agarose gel of products from PCR with plasmid DNA from E. coli BL21 (DE3) colonies from transformation potentially harboring pCasper3 as template using pEt11a1 and pET11a2 as primers.* ***L)*** *6µl 1 kb ladder (Fermentas).* ***1)*** *5µl of PCR product from colony 1.* ***2)*** *5µl of PCR product from colony 2.* ***3)*** *5µl of PCR product from colony 3.* ***4)*** *5µl of PCR product from colony 4.* ***5)*** *5µl of PCR product from colony 5.* ***6)*** *5µl of PCR product from colony 6.* ***7)*** *5µl of PCR product from colony 7.* ***8)*** *5µl of PCR product from colony 8.* ***L)*** *6µl 1 kb ladder (Fermentas).*

According to Figure 3.26 all transformants, except transformant 3 revealed the expected fragments of around 1800 bp. Transformants 1, 2, 4 and 5 were used for expression.

3.5 Expression of pCasper3 in *E. coli* Bl21 (DE3)

Transformants 1, 2, 4 and 5 were grown in LB-Amp medium at 37 °C until OD reached 0.9. IPTG was added for a final concentration of 200 μM and the temperature was reduced to 25 °C. The cultures were further incubated for 10 hours. The final OD for the different cultures can be seen in table 3.1.

Sample	1	2	4	5
OD600	6.22	6.2	5.75	8

Table 3.1: *OD600 measured before harvesting of cells.*

The cells were harvested and opened by an osmotic shock using TES buffer containing 20 % w/v sucrose.

3.6 Analysis of fusion protein

3.6.1 SDS-PAGE Analysis

Two 12 % SDS-PAGE were used to investigate the medium fraction, TES fraction, water fractions and the membrane fraction. All fractions were analysed for fusion protein. The expected size of the fusion protein is 54 kDa. Proteins with the size of 30 kDa and between 45 and 66 kDA can be seen in the TES fraction (see Figure 3.27), in the water fraction and in the membrane fraction (see Figure 3.28)

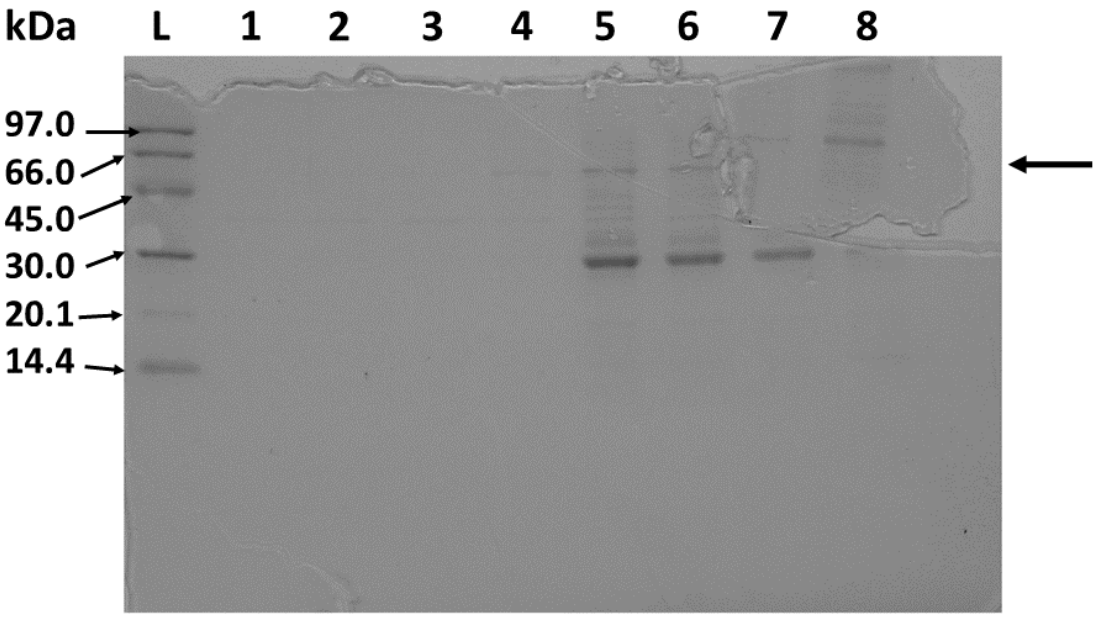

Figure 3.27: *Coomassie stained 12% SDS-PAGE of LB-medium and TES fraction supernatant from the recombinant expression of Casper3 in E. coli BL21 (DE3).* ***L)*** *3µl LMW standard* ***1)*** *4µl LB-medium supernatant of pCasper3 expressed in E. coli from culture 1.* ***2)*** *4µl LB-medium supernatant of pCasper3 expressed in E. coli from culture 2.* ***3)*** *4µl LB-medium supernatant of pCasper3 expressed in E. coli from culture 4.* ***4)*** *4µl LB-medium supernatant of pCasper3 expressed in E. coli from culture 5.* ***5)*** *4µl TES fraction supernatant of pCasper3 expressed in E. coli from culture 1.* ***6)*** *4µl TES fraction supernatant of pCasper3 expressed in E. coli from culture 2.* ***7)*** *4µl TES fraction supernatant of pCasper3 expressed in E. coli from culture 4.* ***8)*** *4µl TES fraction supernatant of pCasper3 expressed in E. coli from culture 5.*

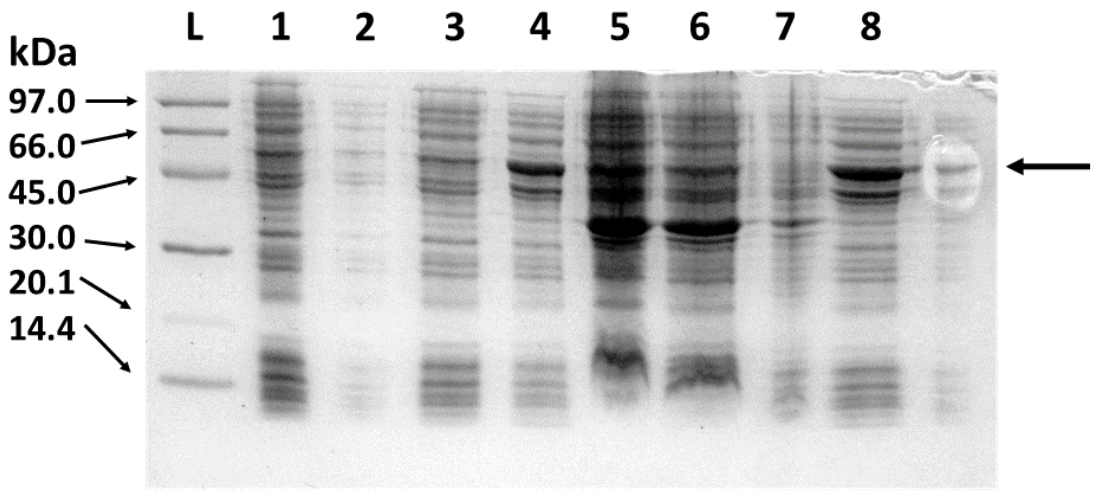

Figure 3.28: *Coomassie stained 12% SDS-PAGE of LB-medium and TES fraction supernatant from the recombinant expression of Casper3 in E. coli BL21 (DE3).* ***L)*** *3µl LMW standard* ***1)*** *4µl Water supernatant of pCasper3 expressed in E. coli from culture 1.* ***2)*** *4µl Water supernatant of pCasper3 expressed in E. coli from culture 2.* ***3)*** *4µl Water supernatant of pCasper3 expressed in E. coli from culture 4.* ***4)*** *4µl Water supernatant of pCasper3 expressed in E. coli from culture 5.* ***5)*** *4µl membrane fraction of pCasper3 expressed in E. coli from culture 1.* ***6)*** *4µl membrane fraction of pCasper3 expressed in E. coli from culture 2.* ***7)*** *4µl membrane fraction of pCasper3 expressed in E. coli from culture 4.* ***8)*** *4µl membrane fraction of pCasper3 expressed in E. coli from culture 5.*

3.6.2 Fluorescence Spectroscopy

The medium fraction, TES fraction and water fraction of colony 1, 2, 4, and 5 were analysed using fluorescent spectroscopy. Evrogen have found that the emission maximum of TagGFP should be located at 505 nm[25], while TagRFP's emission maximum should be located at 584 nm[26].

Some results are normalized to a maximum value of 100 arbitrary units (a.u.). The normalization is towards the data point with the highest value in that specific data set. For the initial scans all fractions were excited at 482 nm and the emission was measured in the range 485 - 650 nm (see Figure 3.29).

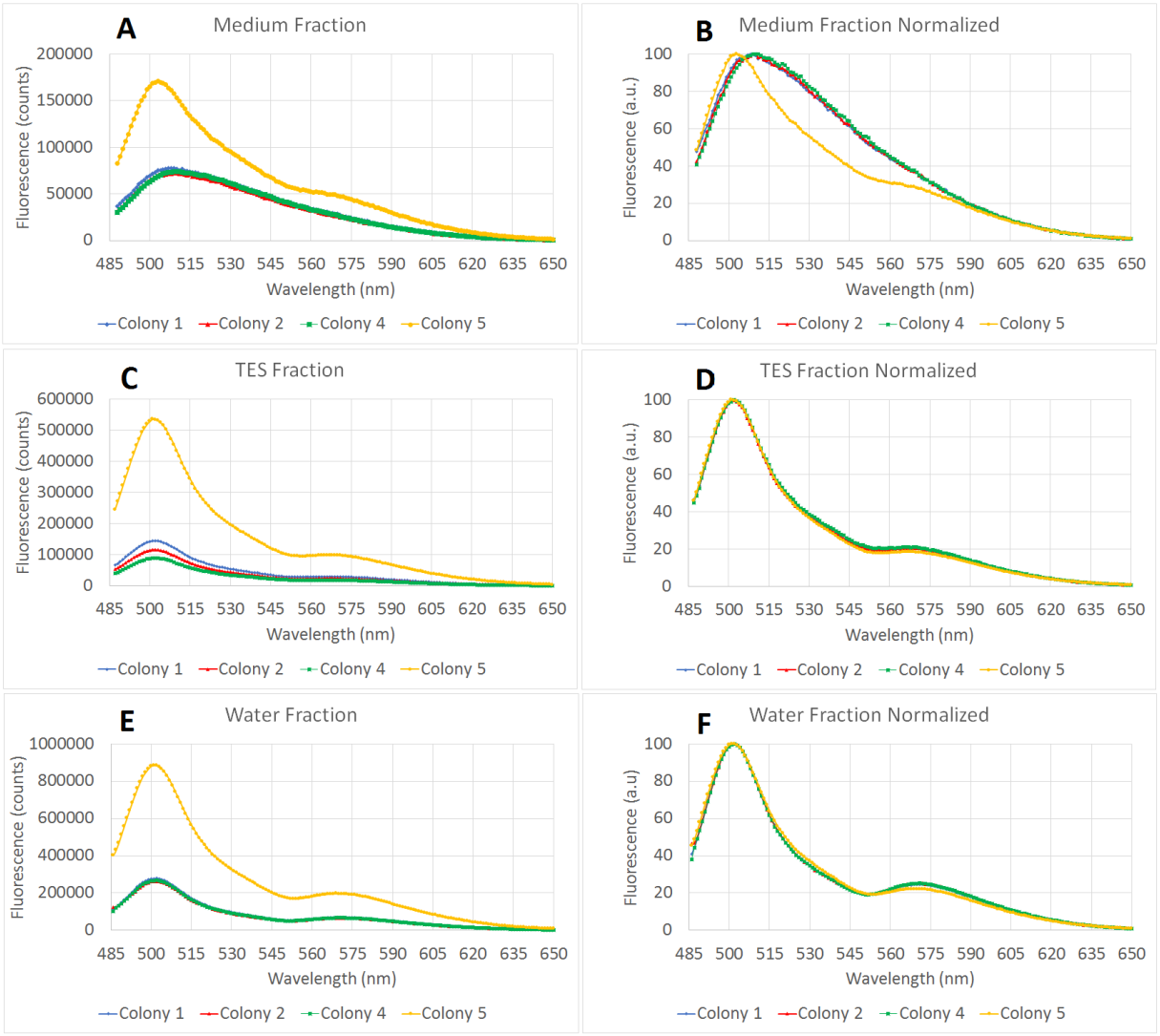

Figure 3.29: *Fluorescence measurements of the different fraction for all 4 colonies originating from expression of the fusion protein. Fluorescence was measured in the range 485-650 nm using an excitation wavelength of 482 nm. (A): Medium fraction, LB medium was used as baseline. (B): Medium fraction normalized. (C): TES fraction, TES buffer was used as baseline. (D): TES fraction normalized. (E): Water fraction, milliQ water was used as baseline. (F): Water fraction normalized.*

One result, common for all measurements is that the fractions from colony 5 produce a much higher signal intensity compared to fractions from the other colonies. The protein from the water fractions produced a much higher signal that the protein from the medium and TES fractions and was therefore diluted 1:20 to reduce the fluorescence intensity to reasonable levels.

In the medium fraction(see Figure 3.29 A) the proteins from colony 5 produced an emission maximum at 503 nm and a local emission maximum around 570 nm. The protein from medium fraction 1, 2 and 4 produced emission maxima at 510 nm.

The normalized medium faction data show that the emission maximum of protein from colony 5 is slightly blue shifted compared to the other samples. Additionally there is an emission peak around 570 nm which could indicate that medium fraction from colony 5 contains the fusion protein (see Figure 3.29 B).

The protein in the TES and water fractions from all colonies produce an emission maxima at 503 nm and local emission maxima at 570 nm (See Figure 3.29 D and F) indicating that all of the TES and water fractions contains the fusion protein. The water fraction from colony 5 produced the highest signal and was therefore chosen for further experiments.

The excitation wavelength for the previous experiments were kept at 482 nm. At this wavelength TagGFP has as excitation efficiency of 100% while TagRFP has an excitation efficiency of 7%. [5] In order to be sure that the emission maximum observed from TagRFP at 570 nm was solely produced by FRET the excitation wavelength was lowered. The excitation wavelength was lowered in the following steps: 475 nm, 460 nm and 400 nm. The table below gives the absorption efficiency of TagGFP and TagRFP at these wavelength. The absorption efficiency (Abs_E) is given by the formula: $Abs_E = \epsilon * \lambda_\lambda$. Where ϵ is the Extinction coefficient of the protein and λ_λ is the absorption intensity of the protein at a specific wavelength λ. The absorption efficiencies were normalized to a maximum value of 100.

Excitation wavelength	GFP	RFP	Ratio G/R
400	8,608641	0,445436	19,32634
460	77,71287	1,704187	45,60114
475	91,73569	4,540039	20,20593
482	100	7,541962	13,25915

Table 3.2: *Table of absorption efficiency of TagGFP and TagRFP at various wavelengths. The values are relative to the absorption efficiency of TagGFP being excited at 482 nm.*

Emission scans at different excitation wavelength were performed (see Figure 3.30 A) and the data was normalized to the maximum value (see Figure 3.30 B).

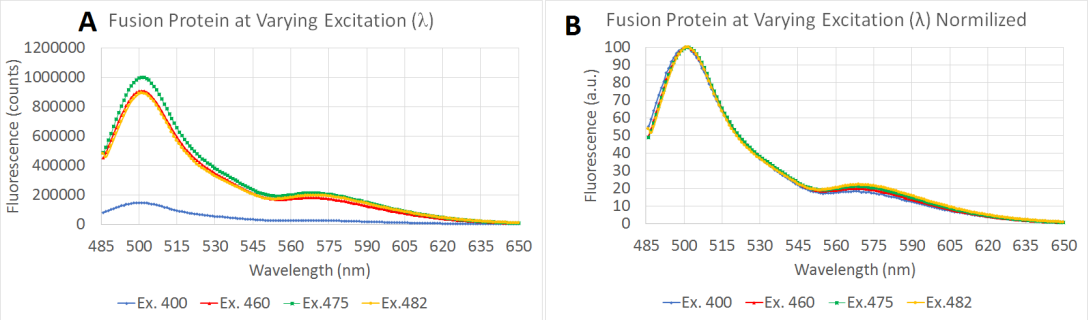

Figure 3.30: *Emission scans performed on water fraction from colony 5 at varying excitation wavelengths (A). Normalization of A to compare emission scans (B). MilliQ water was used as baseline.*

The fluorescence intensity increased when an excitation wavelength of 475 nm was used which was unexpected. The shape of the curve representing the fluorescence intensity distribution over the whole scan seems to be independent of the used excitation wavelength. This result indicate that the signal at 570 (TagRFP) is produced due to FRET.

In order to investigate the results of correctly folded TagRFP, the fusion protein was excited at 555 nm. Since TagGFP is not excited at this wavelength, the emission obtained from this experiment should only originate from TagRFP. An emission scan was performed in the range of 560-650 nm (See Figure 3.31). The scan reveals a maximum in intensity at around 573 nm which is close to the emission maximum for TagRFP at 584 nm.

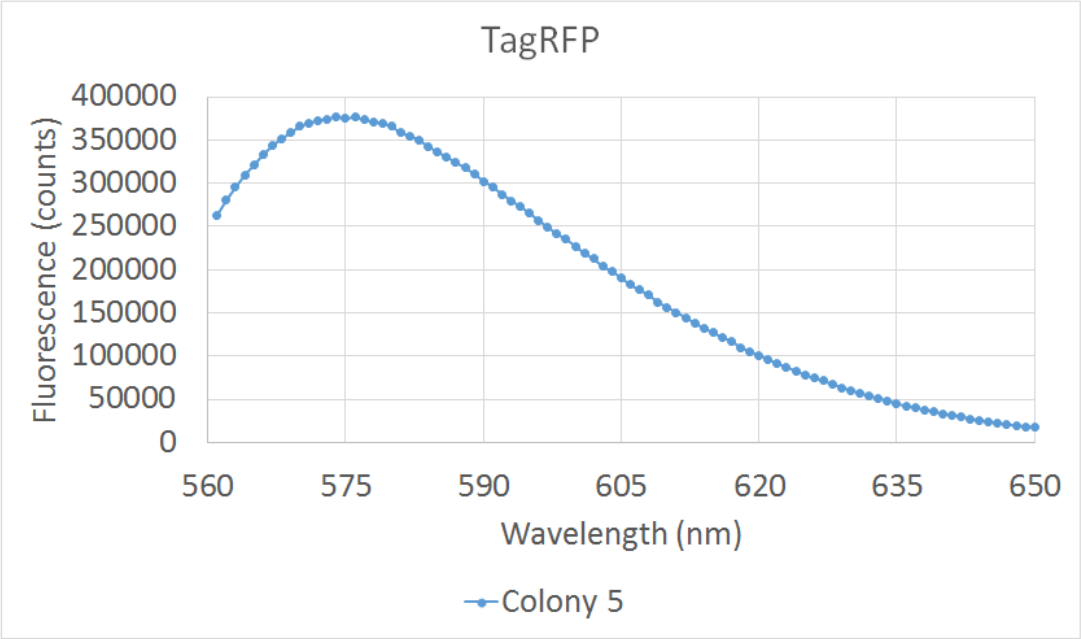

Figure 3.31: *Fluorescence measurements on water fraction on fusion protein from sample 5. Fluorescence was measured in the range 560-650 nm using an excitation wavelength of 555 nm with MilliQ water as baseline.*

3.6.3 Absorption spectroscopy

An absorption scan was performed on all fraction from expression in order to estimate the concentration of fusion protein in the different fractions, and to verify the presence of the fusion protein. The expected result was calculated using the extinction coefficients and excitation spectra of TagGFP and TagRFP. [25][26] The expected result show an absorption maximum for TagGFP at 482 nm and a local absorption maximum for TagGFP at 558 nm. Additionally intensity of the signal for tagGFP is expected to be 40 % lower than that for TagRFP (see Figure 3.32).

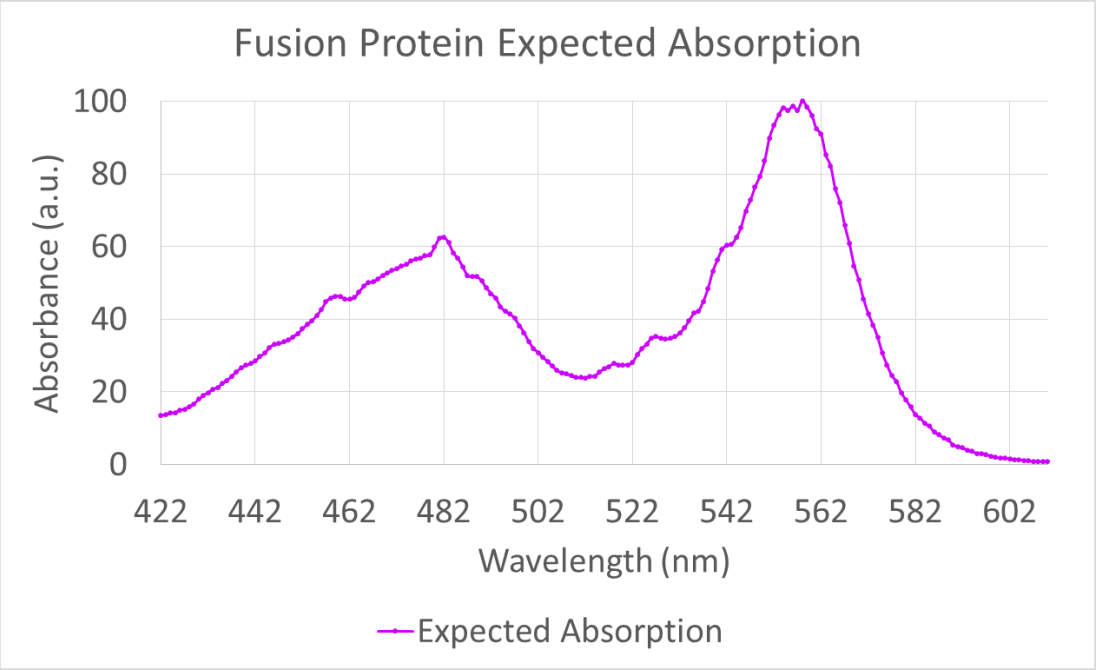

Figure 3.32: *Expected absorption spectra of the fusion protein in the range 422-602 nm normalized*

Absorption measurement on samples from the medium fractions and the TES fractions showed no significant deviation from the baseline and were therefore not included. All of the samples (in Figure 3.33 A) show maxima absorption at 475 nm and 555 nm. The signal intensity of sample 5 was much higher than the rest. The signal intensity at 482 nm and 555 nm were used to calculate an approximate concentration of the samples using Lambert-beers law. The calculated concentrations can be seen in table 3.3. The calculated concentrations shows a GFP/RFP chromophore ratio of around 1.5.

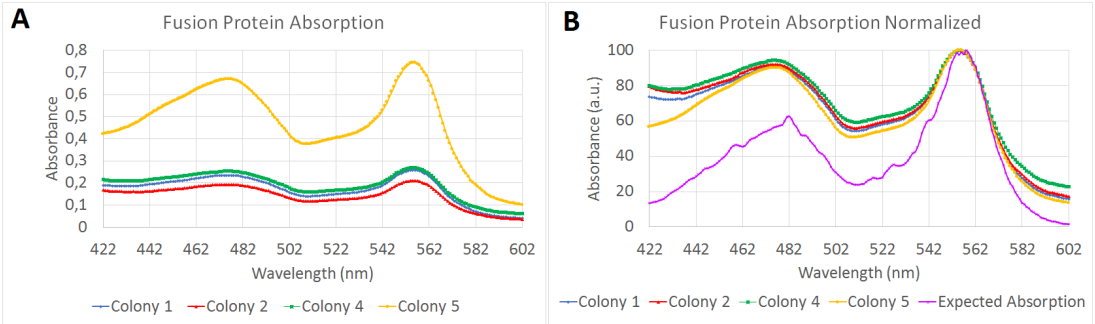

Figure 3.33: *Absorption measurement on samples from the water fraction originating from production of the fusion protein (A). Absorption was measured in the range 422-602 nm with MilliQ water as baseline. B: Absorption measurements and expected absorption normalized.*

The data was normalized and plotted against the expected absorption spectra (see Figure 3.33 B). It can be

seen that the measured signal intensity for TagGFP is higher than the expected. The absorptions maxima are almost the same for the measured and the expected.

	Sample 1	**Sample 2**	**Sample 4**	**Sample 5**
RFP concentration	0,2589 μM	0,2108 μM	0,2689 μM	0,7437 μM
GFP concentration	0,3935 μM	0,3249 μM	0,4244 μM	1,1155 μM
GFP/RFP ratio	1,52	1,54	1,58	1,50

Table 3.3: *Calculated TagGFP and TagRFP concentrations of water fraction samples from protein extraction.*

3.6.4 Temperature Scan using Fluorescence Spectroscopy

Three sets of experiments using Temperature Scan Fluorescence Spectroscopy on the water fraction from colony 5 were performed. The first set of measurements was performed using an excitation wavelength of 482 nm, in the temperature range 25-85 °C. The emission was measured at 502 nm and 570 nm for TagGFP and TagRFP respectively. The spectra was differentiated in order to find the melting temperature of TagGFP and TagRFP. The second set of measurements was performed using an excitation wavelength of 482 nm, in the temperature range 85-25 °C. The emission was measured at 502 nm and 570 nm for TagGFP and TagRFP respectively. The third set measured emission in the ranges 485-650 and 560-650 using excitation wavelength of 482 nm and 555 nm respectively. The measurement were performed on three structures of the fusion protein: Native structure at 25 °C, unfolded structure at 85 °C and a refolded structure at 25 °C.

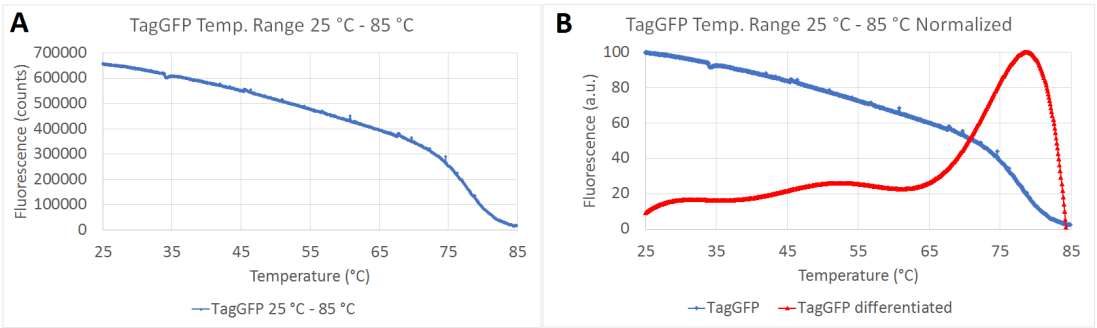

Figure 3.34: *Temperature scans on water fraction from colony 5 originating from expression of fusion protein from colony 5. Emission was measured at 502 nm, using an excitation wavelength of 482 nm in the temperature range 25-85 °C using MilliQ water as baseline. The data was normalized (B). The first derivative of the data set was used to find the melting temperature of TagGFP.*

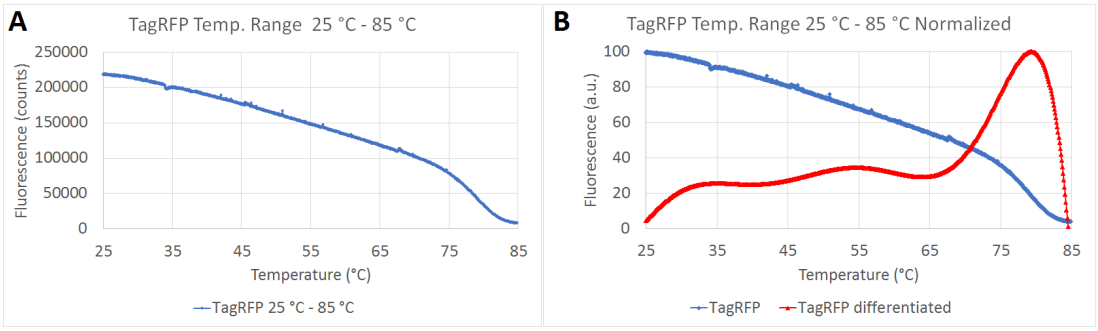

Figure 3.35: *Temperature scans on water fraction from sample 5 originating from expression of fusion protein from colony 5. Emission was measured at 570 nm, using an excitation wavelength of 482 nm in the temperature range 25-85 °C using MilliQ water as baseline. The data was normalized (B). The first derivative of the data was found in order to figure out the melting temperature of TagRFP.*

The fluorescence intensity of TagGFP decreased steadily as the temperature increased. Around 70 °C the slope increased and at 80 °C the curve smoothed which can be seen in Figure 3.34 A. The first derivative of the curve is shown in Figure 3.34 B). The derivative was normalized used to find the melting temperature of TagGFP. The point where the derivative is at its maximum is defined as the melting temperature. The melting temperature was found to be around 78 °C. The differentiation of the data obtained from the temperature scan was performed in Excel and the data point were plotted. The curve was approximated using a polynomial approximation which explains the sharp slope around 85 °C. The fluorescence intensity of TagRFP changes in way similar to TagGFP. The melting temperature was found to be 79 °C which can be seen in Figure 3.35 B.

The change in fluorescence intensity in relation to cooling of TagGFP and TagRFP was measured in the temperature range 85-25 °C (see Figure 3.36).The fluorescence intensity does not change for TagGFP however it increases by about 100% for TagRFP indicating some refolding.

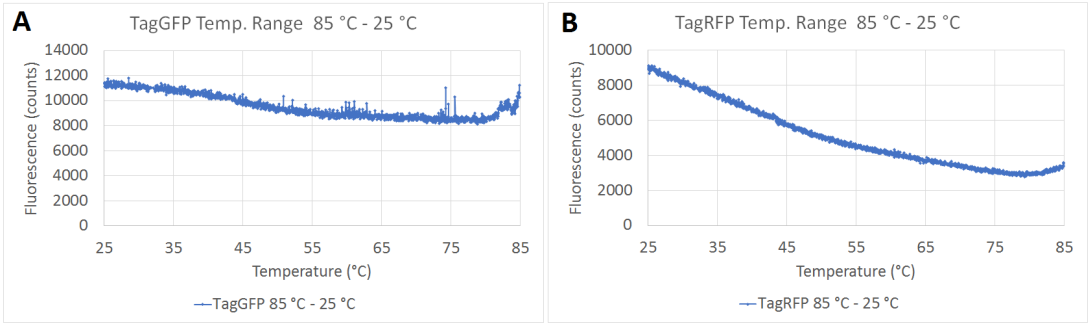

Figure 3.36: *Temperature scans on water fraction from sample 5 originating from expression of fusion protein from colony 5. Emission was measured at 502 nm(GFP) (A) and at 570 nm(RFP) (B), using an excitation wavelength of 482 nm. The temperature range is 85 - 25 °C using MilliQ water as baseline.*

Emission scans have been performed at different temperatures to investigate the thermostability of the

fusion protein (see Figure 3.37 A). Due to the high intensity of the native TagGFP a zoom in was made (see Figure 3.37 B). Both curves have an exponential decrease in intensity, however the data obtained after cooling shows a small maximum around 570 nm indicating a small percentage fluorescence from TagRFP.

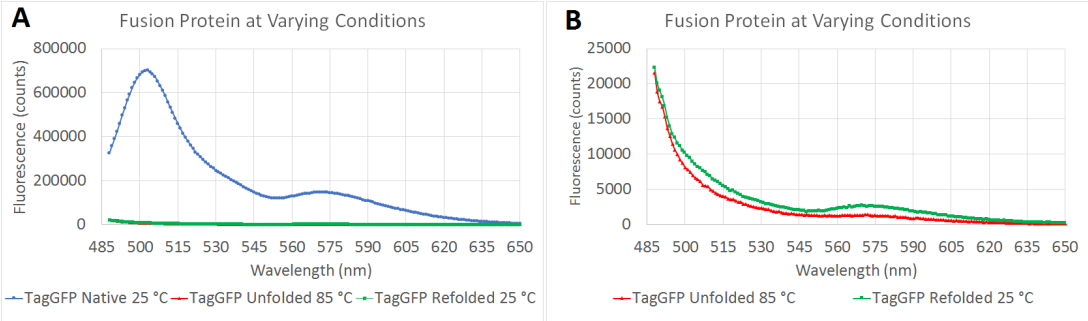

Figure 3.37: *Emission scans performed on water fraction from colony 5 at varying condition. 482 nm was used as excitation wavelength and emission was measured in the range of 485 nm - 650 nm. A: emission scan of fusion protein at, its native from (25 °C), its unfolded form (85 °C) and at its refolded form (25 °C. B: Zoom in on unfolded form (85 °C) and refolded form (25 °C)*

Emission scans have been performed at different temperatures to investigate the thermostability of the fusion protein (see Figure 3.38 A). Due to the high intensity of the native TagRFP a zoom in was made (see Figure 3.38 B). Both graphs have a emission maximum around 573 indicating that a small amount of TagRFP is stable at 85 °C. The fluorescence increases as it refolds. The normalized data (see Figure 3.38 C) suggest that the fluorescence is from TagRFP.

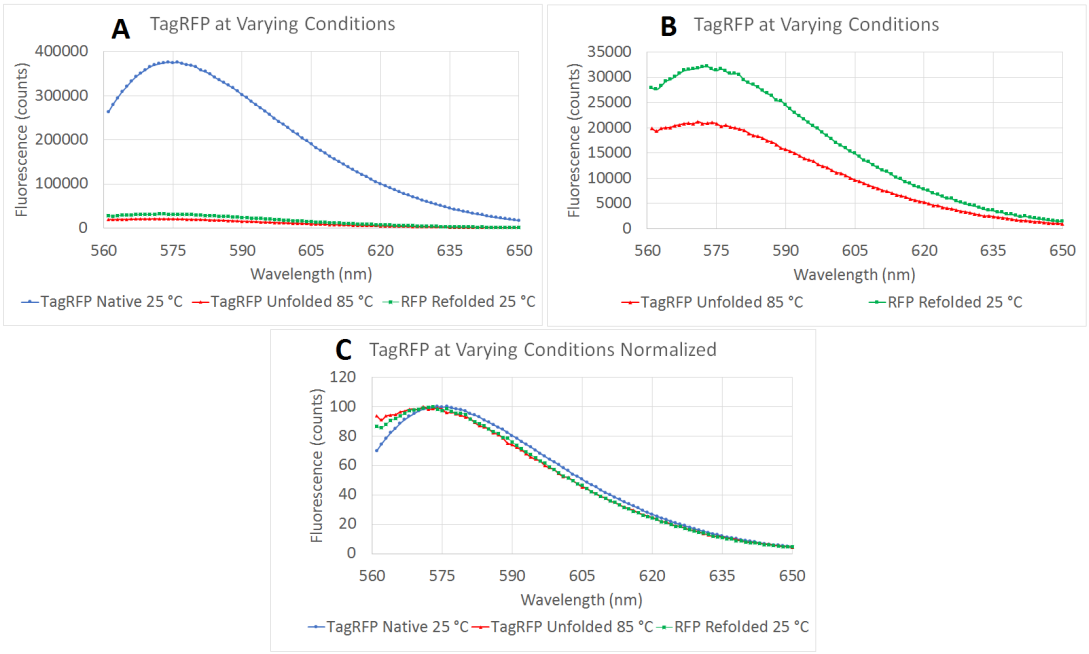

Figure 3.38: *Emission scans performed on water fraction from colony 5 at varying condition. 555 nm was used as excitation wavelength and emission was measured in the range of 560 nm - 650 nm. A: emission scan of fusion protein at, its native from (25 °C), its unfolded form (85 °C) and at its refolded form (25 °C. B: Zoom in on unfolded form (85 °C) and refolded form (25 °C)*

3.7 Modelling of Fusion Protein

The modelling of the fusion protein was performed in YASARA. A FASTA file containing the sequence was constructed and loaded into YASARA. The file was set as target, and a homologous modeling was performed using the hm_build macro. The workflow of the hm_build macro can be seen in Figure 2.3. The templates identified by the hm_build macro can be seen in table 3.4

Templates for Casper3	Cover	ID	Header
1	84%	4KF5 (PDB)	Crystal Structure of Split GFP complexed with engineered sfCherry with an insertion of GFP fragment
2	89%	4ANJ (PDB)	Myosin Vi (Mdinsert2-Gfp Fusion) Pre-Powerstroke State (Mg.Adp.Alf4)
3	90%	4PA0 (PDB)	Omecamtiv Mercarbil Binding Site On The Human Beta-cardiac Myosin Motor Domain
4	56%	4NDJ (PDB)	Crystal Structure Of A Computational Designed Engraile Homeodomain Variant Fused With Yfp
5	47%	3SVS (PDB)	Crystal structure of mkate mutant S158A/S143C at pH 4.0

Table 3.4: *List of templates used for the homology modelling of the fusion protein in YASARA.*

This hybrid model was saved as a pdb file and loaded in YASARA. Figure 3.39 shows the fusion protein structure in YASARA showing the two β-barrels with α-helixes running along its axis. This was expected as it is the prevalent structure in fluorescent proteins.

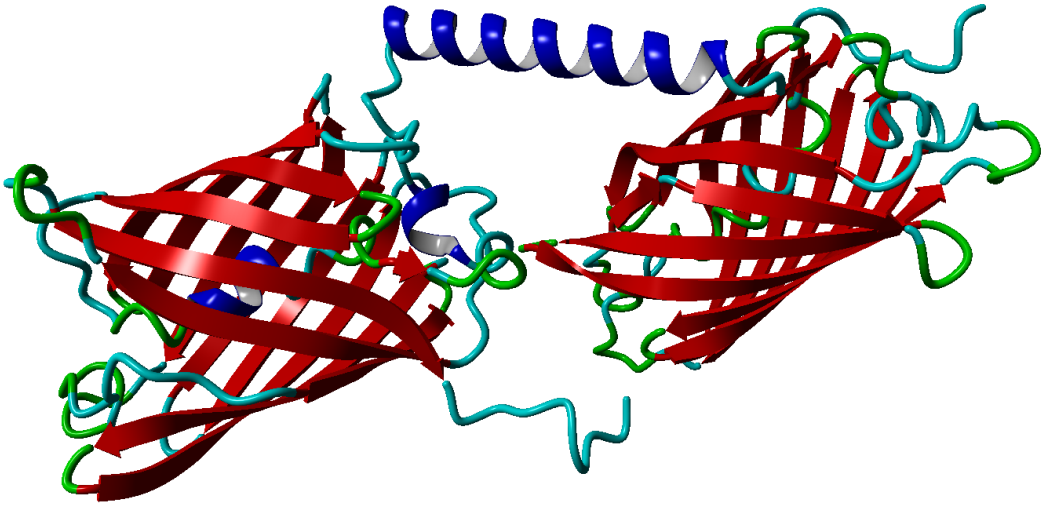

Figure 3.39: *Illustration of Casper3 made in YASARA.*

The linker is shown to consist of random coils and an α-helix. The amino acids used to form the α-helix is Cys^{223}- Thr^{247} (CDLPSKLGHRGGNSGDEVDGTSVAT).

The full linker including random coil between the two β-barrels consists of the amino acids Arg^{221}-Ile^{257} (RYCDLPSKLGHRGGNSGDEVDGTSVATGSGAELFAGI). This sequence was called the long linker. The linker inserted by Dmitry Shcherbo et al [17] to link TagRFP and TagGFP consists of Gly^{233}-Ser^{249} (GGNSGDEVDGTSVATGS). The sequences of the long linker and the short linker were analysed using PredictProtein. [28] The analysis predicted the short linker to consist of 100 % random coil, and the long linker to consist of 23 % strand and 77 % random coil.

The linkers were modelled using the hm_build macro. Table 3.5 shows the templates used for the short linker, and Table 3.6 shows the templates used for the long linker.

Templates for short linker	**Cover**	**ID**	**Header**
1	100%	4NLE (PDB)	Crystal Structure Of Apo Adenylosuccinate Lyase From Mycobacterium Smegmatis

Table 3.5: *List of templates used for the homology modelling of the short linker in YASARA.*

Templates for long linker	Cover	ID	Header
1	69%	3RWA	Crystal Structure Of Circular-Permutated Mkate
2	69%	3RWT	Crystal Structure Of Circular Permutated Red Fluorescent Protein Mkate
3	31%	3PIB	Crystal Structure Of Red Fluorescent Protein Eqfp578 Crystallized At Ph 5.5

Table 3.6: *List of templates used for the homology modelling of the long linker in YASARA.*

Figure 3.40 shows the results of the short linker modelling. The short linker consists solely of random coil structures.

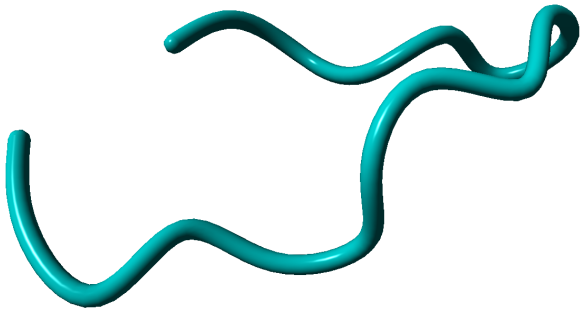

Figure 3.40: *Illustration of the short linker made in YASARA.*

Figure 3.41 shows the results of the long linker modelling. The long linker consists of two β-strands and some random coil.

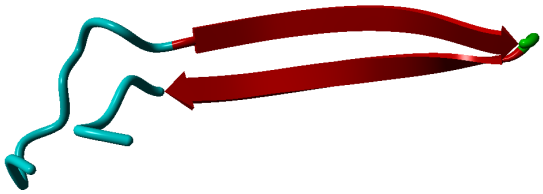

Figure 3.41: *Illustration of the long linker made in YASARA.*

3.7.1 Chromophore distance

The distance between the chromophores was measured in YASARA. The distance was approximated by averaging over the distances between the amino acids of the two chromophores. The distances can be seen in table 3.7.

	Cys^{311}	TYR ^{312}	Gly^{313}
Met^{64}	50.3 Å	48.7 Å	47.1 Å
TYR^{65}	49.8 Å	48.1 Å	46.8 Å
Gly^{66}	53.5 Å	51.7 Å	50.4 Å

Table 3.7: *Distances between amino acids in the chromophores of TagGFP and TagRFP. Distances were measured in YASARA on the modelled fusion protein.*

The average distance has been calculated to be 49.6 Å. This value was used to calculate the FRET efficiency of the modelled fusion protein. The FRET efficiency was calculated to be 70%.

3.7.2 Ramachandran Plot

The modelled fusion protein was analysed using RAMPAGE [27] in order to construct a ramachandran plot. The ramachandran plot was used to validate the homology modeling. Figure 3.42 shows the ramachandran plot of the fusion protein. The analysis shows that 97.1 % (470 residues) are in favoured regions, 2,1 % (10 residues) are in allowed region and 0.2 % (1 residue) is on the border of the generally allow region and the disallowed region. Since this residue turned out to be glycine^{248} in the generally disallowed region this structure was possible.

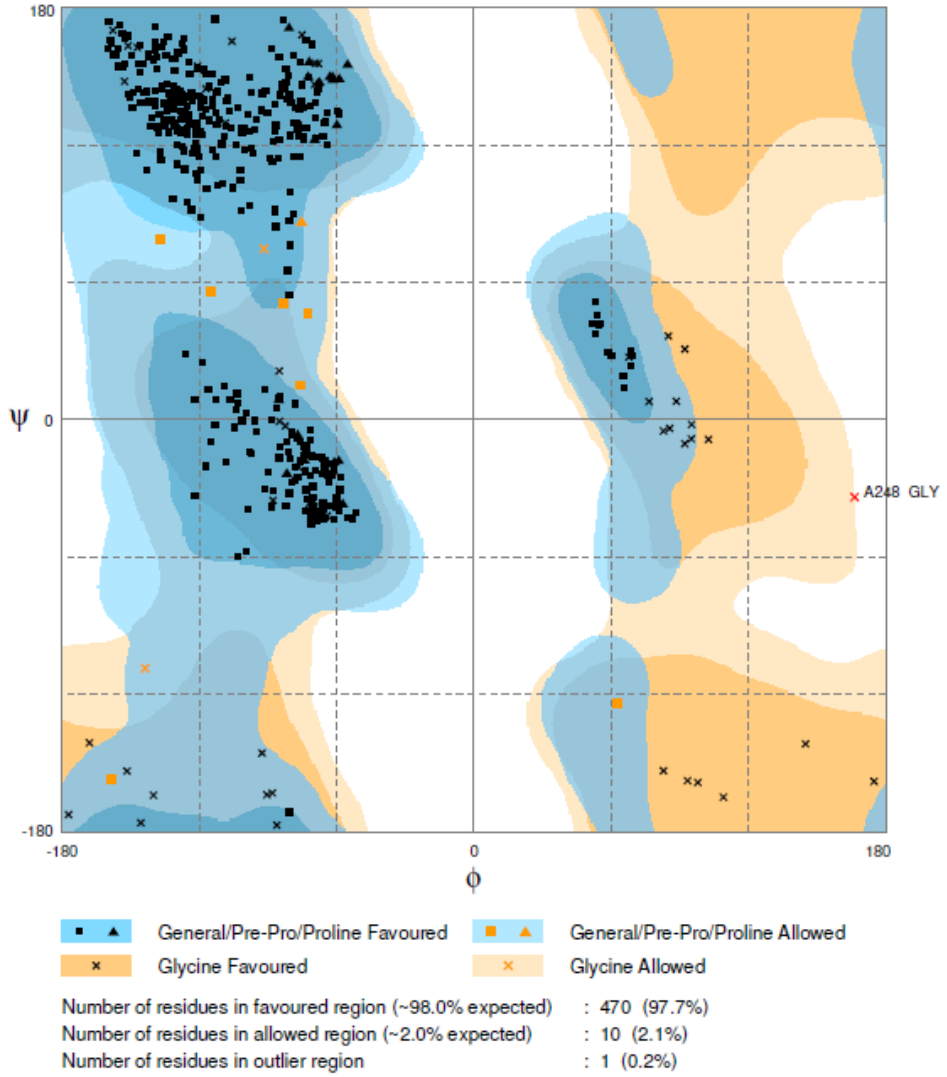

Figure 3.42: *General ramachandran plot of Casper3. The plot was made using RAMPAGE.[27]*

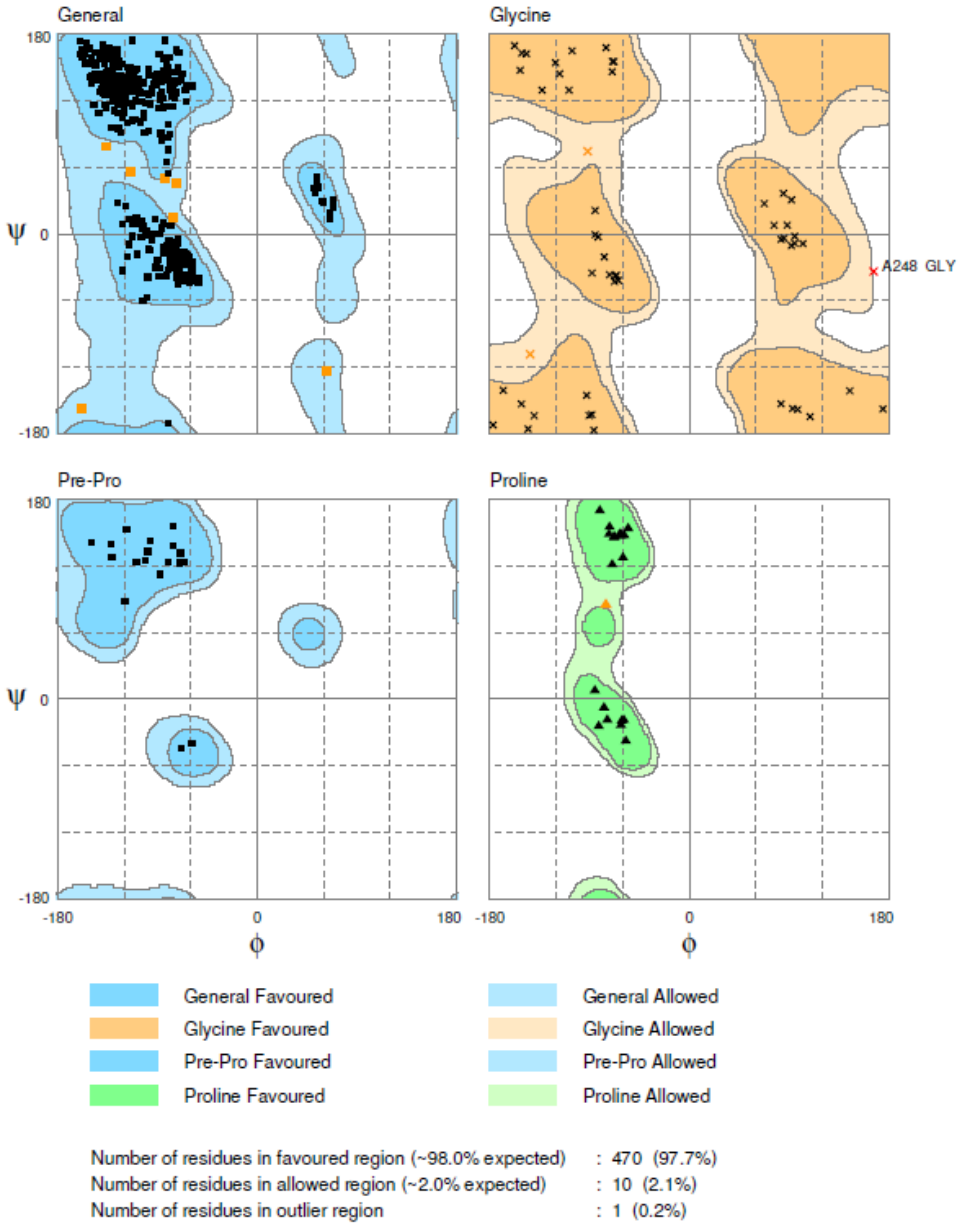

Figure 3.43: *General-, Glycine-, Pre-Proline and Proline ramachandran plots of Casper3. The plot was made using RAMPAGE.[27]*

Discussion 4

The construction of pRFPLGFP was not successful. During the screening of pLGFP which can be seen in Figure 3.1 we found that the linker had not been correctly inserted. The experiment should have been repeated, however at the time of this experiment the result was interpreted as a successful insertion. Since the initial construction of pRFPLGFP was unsuccessful, pCasper3-GR was purchased and used for construction of pCasper3.

Initial attempts at isolating the fusion protein gene from pCasper3-GR using PCR did not work and instead produced a distribution of many different size fragments which can be seen in Figure 3.12. Several attempts were made trying to solve the problem, and in the end, the construction of new primers solved the problem. The new primers solved the smearing problem however the ligation of the PCR product and pET11a vector was not successful which can be seen in Figure 3.20 and 3.21. The problem was caused by not having enough overhang at the 5' end of the restriction site on the primers. [29] New primers solved the this problem which resulted in successful ligation which can be seen in Figure 3.25 .

Protein of the correct size was expressed in *E. coli* Bl21 (DE3) which was seen on the SDS-PAGE in Figure 3.27 and 3.28. Absorption spectroscopy in Figure 3.33 A showed absorption maxima matching those of TagGFP[25] and TagRFP[26] indicating that the fusion protein had been successfully expressed. The absorption spectroscopy indicated that the concentration of fusion protein was around 3 times higher in the water fraction from colony 5 which can be seen in Figure 3.33 B. During expression it was observed that the plug on the erlenmeyer flask had fallen off which increased the oxygen availability which increased the growth which can be seen in table 3.1. The increased oxygen should not affect the expression however; it could affect the maturation of the TagGFP and TagRFP chromophores, which was used to calculate the concentration of fusion protein. Absorption spectroscopy should be repeated to include the absorption of Phe, Tyr and Trp. This experiment would show if the concentration in water fraction 5 was actually higher.

The fusion protein was shown to produce a FRET signal which can be seen in Figure 3.29. The source of the TagRFP fluorescence was concluded to be from FRET which can be seen in Figure 3.30 B. The results are similar to those reported by Dmitry Shcherbo et al. [17] who constructed the fusion protein originally. We did not measure a FRET efficiency which could determine the distance between the chromophores, however Dmitry Shcherbo et al. reported a FRET efficiency of 50 % for the fusion protein upon cleavage using recombinant caspase 3 *in vitro*, which would result in a distance between the chromophores of 5.7 nm. The homology modelling performed suggests a distance of 5 nm and a 25 AA α-helix connecting

the two β-barrels.

Ryoichi Arai et al [30] investigated the effects of α-helixes in fusion protein. The idea was to use α-helices to separate the two proteins in case they showed dimerization tendencies. The group used helical linkers with the sequence A(EAAAK)$_n$A (n=2-5), and the two GFP variants EBFP (Blue Fluorescent Protein) and EGFP. The group used flexible linkers as references. The group calculated the distance using the förster distance of the FRET pair (4.2 nm) and experimentally measured FRET efficiency. The group found that the distance changed slightly when flexible linkers of 19 AA (-0.2Å) and 25 AA (+0.5Å) were used. The group found that the distance changed more when helical linkers of 15 AA (+1.2Å), 20 AA (+3.5Å), 25 AA (+7.1Å) and 30 AA (+11.8Å) were used. All distance are compared to EBFP and EGFP linked by a 4 AA linker (LAAA). The linkers were investigated using circular dichroism and the group found that α-helix content increased in proportion to the number of linker residues.

The finding of Ryoichi Arai et al and the fluorescence spectroscopy results contradicts the results of the modelling. The α-helix sequence also includes 1 proline (residue 4) and 5 glycines (residues 8, 11, 12, 15 and 20) which have poor α-helix forming properties. [31] These results indicate that the linker does not form an α-helix, and more likely form a random coil. The reason for the difference in distance between the EBFP/EGFP and TagGFP/TagRFP is most likely because EBFP and EGFP forms a weak dimer while TagGFP and TagRFP does not.

The melting temperature of TagGFP was found to be 78 °C which can be seen in Figure 3.34 B. Attila Nagy et al [32] found the melting temperature of EGFP, which is a GFP derivative with two mutations, to have a melting temperature of 82,8 ± 0.3 °C. TagGFP shares 80% amino acid sequence identity with EGFP which could explain the difference in melting temperature. The melting temperature of TagRFP was not found as the fluorescence signal is proportional to that of TagGFP, however it was found to be at least 78 °C. Mengyang Xu et al found the temperature of TagRFP-T to be 72 °C using circular dichroism.[33] The difference in melting temperature could be due to TagRFP being in a fusion protein. The melting temperature experiment could be repeated using an excitation wavelength of 555 nm to determine the melting temperature of TagRFP.

Refolding studies showed that TagGFP lost all fluorescence after unfolding, and did not regain any upon refolding which can be seen in Figure 3.36 A. Klaithem M. Alkaabi et al [34] investigated the denaturation and refolding of GFP. The group found that GFP lost all fluorescence when heated to 70 °C at pH 6.5 for 30 min, while only losing 50% at pH 7.5. The group suggested that GFP undergoes a conformational shift between pH 7.5 and 6.5, and that the protein is susceptible to chemical and thermal denaturation. The TagGFP in our study was exposed to 85 °C for at least 30 minutes causing it to lose all fluorescence.

TagRFP retained 5 % fluorescence at 85 °C and had 10 % fluorescence after refolding which can be seen in Figure 3.38 B. Our studies and the studies of Mengyang Xu et al suggests that TagRFP should be unfolded at this temperature exposing the chromophore which is unstable in solution [35]. This suggests that there might be some impurities in the solution. The fusion protein could be purified by adding a His-tag and purified using nickel-nitrilotriacetic acid chromatography.

FRET has been widely used for *in vivo* biosensors lately. One of its uses is in investigation of protein phosphorylation by intracellular kinases. These kinases play an important role in the signaling pathways within cells. Kinases are responsible for transferring phosphate groups from e.g. ATP to a protein. The phosphate is negatively charged resulting in a conformational change, which activates the protein. One

of these kinases is the insulin receptor. Insulin receptors are transmembrane tyrosine kinase receptors activated by insulin. Moritoshi Sato et al [36] used insulin receptors as a proof of concept for FRET biosensors in signaling pathway investigation. The group made a ratiometric FRET biosensor. The setup for their first biosensor can be seen in figure 4.1.

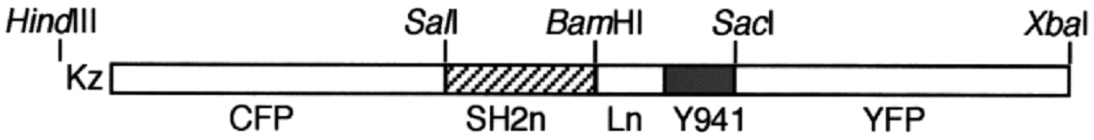

Figure 4.1: *Schematic for insulin receptor FRET biosensor by Moritoshi Sato et al [36]*

CFP and YFP are cyan fluorescent protein and yellow fluorescent protein respectively. Ln is a flexible linker. Y941 is a substrate domain containing tyrosine derived from Insulin Receptor Substrate I. SH2n is the Scr homology 2 domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase. Both Insulin Receptor Substrate I and phosphatidylinositol 3-kinase play important roles in the insulin signaling pathway. Upon activation of the insulin receptors, the tyrosine in the substrate domain is phosphorylated. The phosphorylated tyrosine is recognised and bound by SH2n reducing the distance between CFP and YFP resulting in increased FRET. The reaction can be seen in figure 4.2.

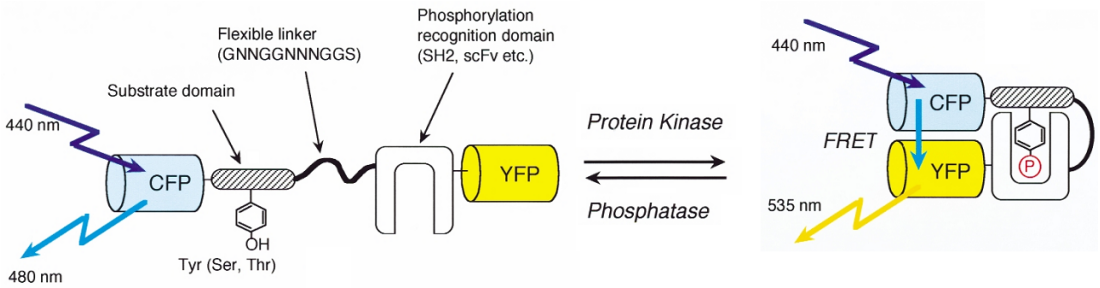

Figure 4.2: *Illustration of Insulin Receptor FRET biosensor by Moritoshi Sato et al. Upon induction on insulin the Insulin Receptor phosphorylates the tyrosine in substrate region. The phosphorylated tyrosine is then recognized and bound by the SH2 domain. This conformational change brings the fluorescent proteins CFP and YFP closer together increasing the FRET efficiency.[36]*

The biosensor was expressed in Chinese hamster ovary cells overexpressing human insulin receptor. The sensor was excited using a wavelength of 440 nm. Emission was measured at 480 nm and 535 nm for CFP and YFP respectively. The signal was measured as the ratio 480 nm/535 nm. The ratio decreased as insulin was added to the cells showing that the insulin receptor were activated. The sensor was shown to be fully reversible by washing the cells. Phosphatases in the cells dephosphorylates the tyrosine inactivating the sensor which increases the ratio.

Jihye seong et al [37] used a similar setup for the detection and investigation of Focal Adhesion Kinase (FAK). FAK play an important role in cell adhesion, cell migration and mechanotransduction. It also plays a role in cell proliferation in response to growth factors. The group wanted to monitor FAK activity at different membrane microdomains. The membrane microdomains of interest were detergent-resistant

membranes (DRM). The group designed a biosensor similar to the one by Moritoshi Sato et al.[36] They used ECFP and Ypet as FRET pair, along with a substrate domain which could be phosphorylated by FAK, and a SH2 recognition domain. The difference between the two sensors were that upon activation the sensor by Jihye seong et al increased the distance between the fluorescent protein resulting in a reduction in FRET. The sensor was tagged with sequences which either bound it to DRM or non-DRM regions in the cell membrane. The sensor was expressed in Mouse Embryonic Fibroblast. The measurements were performed using an excitation filter for ECFP at 420 ± 20 nm and the emission filters 475DF40 and 535DF25 for ECFP and YPet respectively. The results showed that FAK activity was significantly higher at DRM regions upon stimulation by e.g. platelet-derived growth factor. The sensor was also shown to be fully reversible by adding a FAK inhibitor.

These two systems were examples where FRET ratiometric biosensors were used to investigate the activity of specific enzymes *in vivo*. FRET biosensors can also be used to measure the concentration of specific chemicals *in vivo*. One of these is ATP. ATP is considered the energy currency of cells as it is used in various processes. Hiromi Imamuraa et al. [38] constructed a ratiometric FRET biosensor for detection of ATP levels inside cells. The sensor was constructed by linking the FRET pair mseCFP and mVenus to the N and C termini of the ϵ domain of bacterial F_0F_1 ATP synthase. mseCFP is a variant of cyan fluorescent protein and mVenus is a monomeric variant of yellow fluorescent protein. The ϵ domain consists of 1 N-terminus β-barrel and 2 C-terminus α-Helices. The domain binds ATP without hydrolysing it resulting in a conformational change form a relaxed form to a folded form. This conformational change brings the N and C terminus closer together. Additionally the domain binds with high specificity compared to GTP, TTP and CTP.

The sensor was expressed in HeLa cells to investigate ATP levels in different parts of the cell. Targeting sequences were used for measurements in the mitochondria. The sensor was excited using an excitation wavelength of 435 nm. The ATP levels were measured as the ratio YFP/CFP, therefore a higher ratio indicated a higher ATP concentration. The analysis showed that the highest levels of ATP was found in the cytoplasm, followed by the nucleus and mitochondria.

GFP like fluorescent proteins are not the only fluorescent protein which can be used for FRET. Thomas Drepper et al [39] used the blue-light photoreceptors of *Bacillus subtilis* and *Pseudomonas putida* as a starting point and engineered a flavin-binding fluorescent protein (FbFP). This protein uses flavin mononucleotide as chromophore and therefore does not require oxygen to enable fluorescence like GFP like proteins. One of these FbFPs had properties similar to those of CFP with an excitation maximum at 450 nm and emission maximum at 495 nm making it an excellent candidate for a FRET biosensor with YFP.

Janko Potzkei et al.[40] constructed such a biosensor for the detection of molecular oxygen *in vivo*. The fluorescent protein were linked by a linker containing a thrombin protease digestion site. The biosensor was expressed in *E. coli* Bl21 (DE3). The bacteria was grown in TB buffer in a microbioreactor system. The primary carbon source of TB buffer is glycerol. An excitation wavelength of 380 nm was used and the fluorescence of YFP and FbFP were measured at 532 nm and 492 nm respectively. The fluorescence was used to measure the ratio of YFP/FbPF. Additionally the cell density and dissolved oxygen tension (DOT) was measured.

The analysis showed the expected growth curve for *E. coli*. The DOT decreased rapidly and reached 0 after about 5 hr. After induction, using IPTG after 3 hr signals from both the donor and acceptor was

detected. Both increased steadily. The YFP/FbFP ratio increased steadily as well. After 13 hr of cell cultivation the change of carbon source from glycerol to acetate led to a reduced oxygen consumption by the *E. coli*. This diauxic growth temporarily increased the intracellular oxygen concentration which could be detected as an increase in YFP fluorescence and a decrease in FbFP fluorescence. After 16 hr the *E. coli* entered the stationary phase resulting in increased DOT, increased YFP fluorescence and decreased FbFP fluorescence. The group found that d(fluorescence ratio (YFP/FbFP)/dt was proportional to DOT which allowed for intracellular oxygen concentration measurements.

The main difference between these 4 sensors and Casper3 is the FRET pair used. The Casper3 uses a Green/Red instead of Cyan/Yellow. The Casper3 has a higher Förster distance which is advantageous for some applications. Another advantage of the Green/Red pair is that the photons used for excitation have a lower energy and is therefore less damaging towards the cells. The sensors shows that FRET pairs can be used for *in vivo* measurements for just about everything.

Conclusion 5

Attempts were made at constructing and expressing a TagRFP-T-Linker-GFP S65T fusion protein. These attempts were unsuccessful. Instead, a TagRFP-TagGFP fusion protein from evrogen was expressed. The fusion protein gene was transferred from pCasper3-GR to a pET11a vector using PCR. The fusion protein was expressed in *E. coli* BE21 (DE3), and analyzed using SDS-PAGE, fluorescence spectroscopy and absorption spectroscopy.

SDS-PAGE showed expression of a correct size protein, and absorption spectroscopy showed maxima matching those of TagGFP and TagRFP. The fluorescence spectroscopy showed that FRET effects were responsible for the fluorescence of TagRFP during excitation of TagGFP. Temperature Scan Fluorescence Spectroscopy was used to find the melting temperature of TagGFP which was found to be 78 °C. The melting temperature of TagRFP was found to be at least 78 °C.

Homology modelling was performed on the fusion protein to investigate the structure and estimate the distance between the chromophores of TagGFP and TagRFP. The modelling found that the fusion protein structure consisted of two β-barrels connected by an α-helix linker. The distance between the chromophore was found to be 5 nm. Further investigation suggested that the linker structure was random coil and that the distance between the chromophore was longer.

The idea for the fusion protein was to use it for investigation of individual proteins, interaction between two different proteins or as a biosensor. Different FRET biosensor designs were investigated and compared to the TagRFP-TagGFP fusion protein.

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Appendix A

The following section will describe some of the methods used in more detail, than the one found in the materials and methods section.

A.1 Chemical Transformation of 5-alpha F' I^q Competent *E. coli* (NEB)

- Thaw a tube of 5-alpha Competent *E. coli* on ice for 10 mins.
- Add 1-5 μl DNA solution containing 10-100 ng of plasmid DNA. Carefully flick the tube 4-5 times and incubate on ice for 30 mins. For transformation of ligated DNA 15-20 μl was used.
- Heat shock at exactly 42 °C for exactly 30 sec, and incubate on ice for 5 mins.
- Pipette 500 μl SOC medium into the mixture.
- Incubate the solution at 37 °C for 1hr while shaking vigorously (230-250 RPM).
- Warm three selection plates to 37 °C
- Mix the cells thoroughly by flicking, and inverting the solution.
- Add solution to the three plates in the following amounts: 50 μl, 100μl and rest.
- Incubate at 37 °C overnight.
- Optional: Use colonies to make master plates.

A.2 Chemical Transformation of *E. coli* BL21 (DE3) (NEB)

- Thaw a tube of BL21 (DE3) Competent *E. coli* on ice for 10 mins.
- Add 1-5 μl DNA solution containing 10-100 ng of plasmid DNA. Carefully flick the tube 4-5 times and incubate on ice for 30 mins.
- Heat shock at exactly 42 °C for exactly 10 sec, and incubate on ice for 5 mins.

- Pipette 500 μl SOC medium into the mixture.
- Incubate the solution at 37 °C for 60 mins while shaking vigorously (230-250 RPM).
- Warm three selection plates to 37 °C.
- Mix the cells thoroughly by flicking, and inverting the solution.
- Add solution to the three plates in the following amounts: 50 μl, 100μl and rest.
- Incubate at 37 °C overnight.
- Optional: Use colonies to make master plates.

A.3 Midi prep using the Qiagen Plasmid Midi prep kit

- Transfer an aliquot of bacteria from a selective plate (master plate) to a 500 ml erlenmeyer flask containing 100 ml selective medium. Incubate overnight at 37 °C with vigorous shaking (250 RPM)
- Transfer the medium to sterile centrifuge tubes and harvest the cells by centrifugation at 6000 x g for 15 mins at 4 °C. Discard the supernatant.
- Resuspend the bacterial pellet in 4 ml of Buffer P1.
- Add 4 ml of Buffer P2. Invert the centrifuge tubes 4-6 times and incubate at room temperature for 5 min.
- Add 4 ml of buffer P3. Immediately invert the centrifuge tubes 4-6 times and incubate on ice for 15 min.
- Centrifuge at ≥ 20000 x g for 30 min at 4 °C. Remove the supernatant immediately.
- (Optional) Transfer the supernatant to sterile centrifuge tubes and and centrifuge at ≥ 20000 x g for 15. Remove the supernatant immediately.
- Equilibrate a QIAGEN-tip 100 by applying 4 ml of Buffer QBT. Allow the column to empty by gravity flow.
- Add the supernatant to the QIAGEN-tip and allow the column to empty by gravity flow.
- Wash the QIAGEN-tip with 2 times 10 ml of Buffer QC.
- Elute the DNA using 5 ml of Buffer QF
- Precipitate the DNA by adding 3.5 ml room temperature isopropanol, mix and centrifuge immidiately at ≥ 15000 x g for 30 min at 4°C. Carefully decant the supernatant.
- Wash the pellet with 2 ml 70% EtOH and centrifuge at ≥ 15000 x g for 10 min.
- Air dry the pellet untill the smell of ethanol has disappeared and redissolve in a suitable volume of buffer (typically 100 μl of Nuclease free water or elution solution)

A.4 Miniprep using the Telt method

A TELT buffer was made using the following recipe:

TELT	
50	mM TRIS
62,5	mM EDTA (Sigma Aldrich)
2,5	M LiCl (Sigma Aldrich)
0,4 %	Triton X-100 (Sigma Aldrich)

- Suspend cells from a selection plate in 400 μl TELT Buffer
- Add a bit of Lysozyme and vortex well
- Heat the samples 95 °C for 90 sec
- Transfer to ice immediately and incubate for 10 min
- Centrifuge at 11000 x g for 20 min at 4 °C.
- Remove the pellet using a sterile tooth pick
- Add 240 μl isopropanol, mix and incubate on ice for 10 min
- Centrifuge at 11000 x g for 30 min at 4 °C. Discard the supernatant
- Wash the pellet with 300 μl 70% EtOH
- Discard the supernatant
- Air dry the pellet untill the smell of ethanol has disappeared and redissolve in a suitable volume of buffer (typically 30-50 μl of nuclease free water or elution solution)

A.5 Miniprep using the Ultraclean® plasmid Mini prep kit

- Transfer an aliquot of bacteria from a selective plate (master plate) to a 5 ml Greiner culture tube containing 4 ml a suitable selection medium. Grow the cultures overnight.
- Transfer 2 ml to the provided tube and centrifuge at 13000 x g for 1 min, and discard the supernatant. Repeat this process until all the overnight culture has been used.
- Resuspend the pellet using 100 μl of Solution 1
- Add 200 μl of Solution 2 and invert 8 times
- Add 400 μl of Solution 3 and invert 8 times
- Centrifuge at 13000 x g for 3 min and transfer the supernatant to the provided spin filter unit.

- Centrifuge at 10000 x g for 60 sec. DIscard the flow-through
- Add 600 μl of Solution 4 to the spin filter unit and centrifuge at 13000 g for 60 sec. Discard the supernatant.
- Centrifuge at 13000 x g for an additional 60 sec.
- Transfer the spin filter unit to a 2ml collection tube
- Add 50 μl of Solution 5 to the center of the spin filter membrane. Leave at room temperature for 60 sec, then centrifuge at 10000 x g for 60 sec
- Remove the spin filter unit

A.6 PCR cleanup using the GenElute^{TM} PCR Clean-Up Kit

- Insert a GenElute plasmid mini spin column into a provided collection tube. Add 0,5 ml Column Preparation Solution the mini spin column and centrifuge at 12000 x g for 60 sec. Discard the eluate.
- Mix 1 volume of PCR solution with 5 volumes of Binding Solution. Transfer the solution to the binding column and centrifuge at 14100 x g for 60 sec. Discard the eluate
- Add 0.5 ml of diluted Wash Solution to the column and centrifuge at 14100 x g for 60 sec. Discard the eluate
- Centrifuge at 14100 x g for an additional 2 min. discard the eluate
- Transfer the mini spin column into a fresh collection tube. Apply 50 μl of Elution solution or nuclease free water to the center of the mini spin column. Incubate at room temperature for 60 sec.
- Centrifuge the collection tube at 14100 x g for 60 sec

A.7 SDS-PAGE

For an SDS page a stacking gel and a separation gel is needed. For a 12% SDS-PAGE these are made in the following way:

5 ml Separation gel:

Amount	**Chemical**
1,68 ml	MilliQ water
1,25 ml	1,5 M Tris-HCl, pH 8,8
50 μl	10 % SDS
2,0 ml	30 % Acrylamide/Bis
30 μl	10 % APS
10 μl	TEMED

5 ml Stacking gel:

Amount	Chemical
3,05 ml	MilliQ water
1,25 ml	0,5 MTris-HCl, pH 6,8
50 μl	10% SDS
0,65 ml	30% Acrylamide/Bis
30 μl	10% APS
10 μl	TEMED

The making on the 12% SDS page is as follows:

- Set the casting frames on the casting stands
- Prepare the separation gel and swirl the solution
- Pipet an appropriate amount of separation solution into the gap between the glass plates
- Fill the remaining space with water to make the top of the separation gel horizontal
- Wait 20-30 min for the separation gel to gelate.
- Discard the water
- Prepare the stacking gel
- Fill the remaining space with stacking gel
- Insert the well-forming comb without trapping air
- Wait 20-30 min for the stacking gel to gelate
- Remove the well-forming comb carefully
- Remove the casting frames from the casting stand and move them to the cell buffer dam
- Pour running buffer into the inner chamber, let it overflow and keep pouring until the running buffer reaches the required level in the outer chamber
- Mix 1 part protein sample with 2 parts glycine sample buffer (BIO-RAD)
- Heat the samples for 5 min at 90-95 °C
- Add the samples to the wells
- Run the SDS-PAGE at 120V for 1 hr
- Remove the SDS-PAGE from the casting frames, rinse it in milliQ water and move it to a container.
- Add enough Coomassie Stain to cover the SDS-PAGE

- Microwave the container for 40-60 sec on high power until the Coomassie Stain boils
- Incubate on a rocking table for 10 min
- Remove the Coomassie Stain solution.
- Rinse the SDS-PAGE in milliQ water twice to remove the Coomassie Stain
- Add enough fresh Destaining Solution to cover the gel and incubate on a rocking table overnight
- Remove the Destaining Solution, rinse in milliQ water and keep the gel in milliQ water

A.8 Protocol for direct purification of PCR product

- Mix x volumen of sample with 2x volumes of buffer NT
- **Binding of DNA:** Place a Nucleo spin extract II column into a 2 ml collecting tube and load the mixed sample. Centrifuge it for 1 min at 11000 x g and discard the flow-through.
- **Wash silica membrane:** Add 600μl of buffer NT3. Centrifuge for 1 min at 11000 x g and discard the flow-through.
- **Dry silica membrane:** Centrifuge for 2 min at 11000 x g to remove the remaining NT3 buffer.
- **Elute DNA:** Place the Nucleo spin extract II column into a clean tube and add 15 -50μl elution buffer NE. Incubate the solution at room temperature for 1 min and centrifuge for 1 min at 11000 x g.

Appendix B

B.1 GeneRuler 1kb DNA ladder

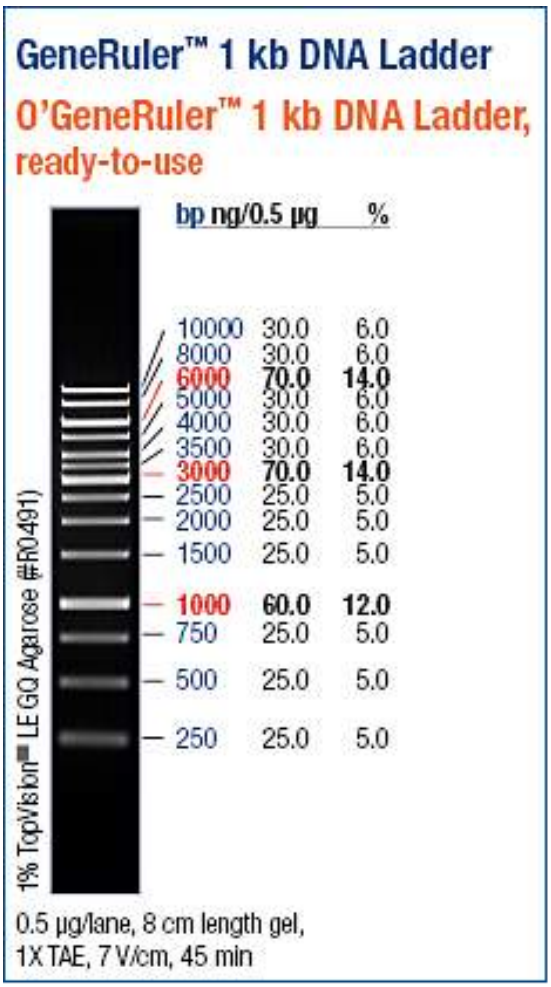

Figure B.1: *The GeneRuler 1kb DNA ladder to approximate the concentration of the DNA on the agarose gels.[41]*

B.2 Amino Acids

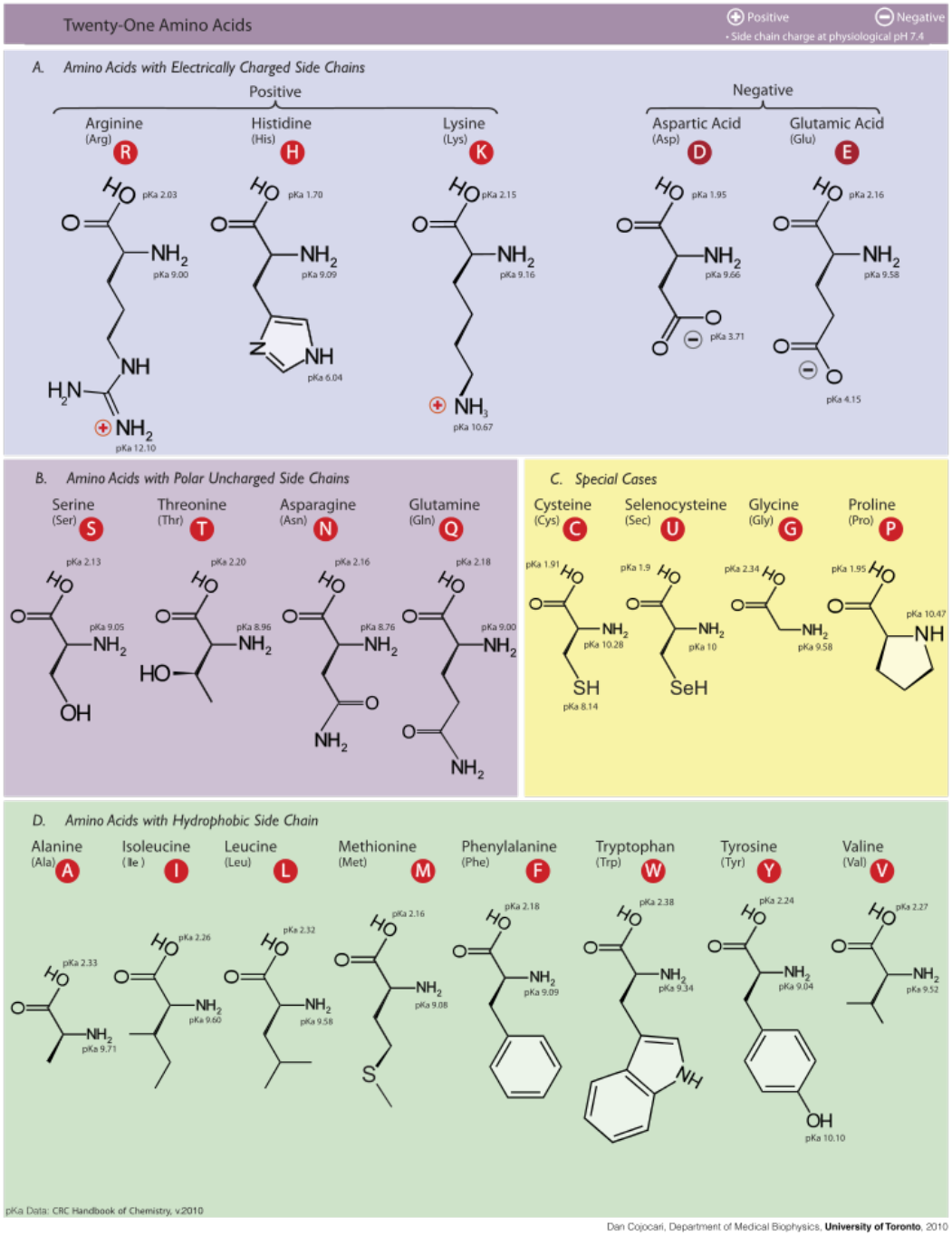

Figure B.2: *Figure of the 20 naturally occurring amino acids.[42]*