DNA-BASED ASSEMBLY OF TRANSCRIPTION FACTORS FOR BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) ASSAY

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ABSTRACT

DNA-BASED ASSEMBLY OF TRANSCRIPTION FACTORS FOR BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET) ASSAY

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Bioluminescence Resonance Energy Transfer (BRET) is a promising assay for studying molecular dynamics such as protein-protein interactions especially *in situ* and *in vivo* since the system requires precise distance between the molecules. BRET technique has been used for identification of molecular interactions *in situ*, imaging of deep-tissues in animal models and sensing of organic or inorganic molecules *in vitro* by combining the luciferase derivative that is an obligatory unit for this assay and any fluorescent molecules, such as inorganic fluorescent molecules and fluorescent proteins. Beside the luciferase derivatives must be used as one part of BRET pairs, the overall system would be optimized to adapt desired properties by changing the substrate of the luciferase and fluorescent molecule with suitable excitation and emission wavelength as well as the platform that locates the BRET pairs particularly for sensing studies via BRET technique. As sharing the perspective of Fluorescence Resonance Energy Transfer (FRET) which was described previously and has been used widely in molecular interactions, BRET systems have been established basically on *in situ* studies; however,

BRET could be used in sensing systems more widely thanks to its advantages over FRET technique. Deoxyribonucleic acid (DNA) has been shown as the source of life; nonetheless, it is a remarkable bio-polymer having stable and predictable structure with cheap production of synthetic variants. Basing on these useful properties of DNA, it has been used popularly to develop scaffold to combine molecules with exact distances for any purposes or proof of concept studies using molecular self-assembly principle. In the present study, we aimed to design a BRET assay in which engineered transcription factors are fused to BRET pairs, *Renilla* luciferase (RLuc) and fluorescent protein, mCherry, and signaled on DNA scaffold as a proof of concept study. We fused complementary DNA (cDNA) of CDC DNA-binding protein that is the engineered form of human estrogen receptor α (hERα) to cDNA of RLuc and cDNA of yeast protein, Gal4 DNA-binding domain to cDNA of mCherry with the common protein purification tag of 6-Histidine (6xHis) in a bacterial expression vector by cloning studies. Upon construction of the plasmids that code for related fusion proteins, we tried to over-express, isolate and purify the fusion proteins for next steps of the study and the final process was confirmed by Western Blot (WB) analysis. As an ongoing study, we will try to verify the binding of proteins to DNA scaffold by biochemical methods. Next, we will try to obtain BRET signal and optimize the overall system. This platform would be adapted to *in situ* and regarded to be used to study context-dependent transcription machinery, genome editing and protein binding to DNA.

Keywords: Bioluminescent Resonance Energy Transfer (BRET), DNA scaffold, mCherry

ÖZ

BİYOLÜMİNESANS REZONANS ENERJİ TRANSFERİ (BRET) ANALİZİ İÇİN TRANSKRİPSİYON FAKTÖRLERİNİN DNA TEMELLİ BİR ARAYA GETİRİLMESİ

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Biyolüminesans Rezonans Enerji Transferi (BRET), moleküller arasında kesin bir uzaklık gerektirdiğinden, özellikle *in situ* ve *in vivo* olarak, protein-protein etkileşimi gibi moleküler dinamiklerin çalışılması için umut vaat eden bir analizdir. BRET, tekniğin zorunlu kıldığı lüciferaz enziminin ve inorganik floresan boyalar veya floresan proteinler gibi floresan moleküllerin bir araya getirilmesi ile *in situ* moleküler etkileşimlerin aydınlatılması, hayvan modellerinde derin doku görüntülemesi ve organik ya da inorganik moleküllerin *in vitro* olarak tespiti için kullanılmıştır. Lüsiferaz türevlerinin BRET çiftlerinden biri olması zorunluluğunun yanında, özellikle BRET tekniği üzerinden geliştirilen tespit çalışmalarında, tüm sistem, lüsiferaz sübstratının ya da istenilen uyarım ve emisyon dalga boylarında floresan moleküller kullanılarak optimize edilebilir. BRET'den çok daha önce tanımlanan ve moleküler etkileşim çalışmalarında sıkça tercih edilen Floresan Rezonans Enerji Transferi (FRET) prensibine benzer olarak, BRET sistemleri temel olarak *in situ* çalışmalarda kullanılır

fakat FRET'le kıyaslandığında açığa çıkan avantajları nedeniyle BRET sensör çalışmalarında da daha geniş yer bulabilmelidir. Deoksiribonükleik asit (DNA) yaşamın kaynağı olarak gösterilmekle birlikte durağan ve tahmin edilebilir yapısı ve sentetik varyantlarının ucuza üretilmesi gibi avantajlara sahip olan bir biyo-polimerdir. Bu avantajlar çerçevesinde DNA, moleküler kendiliğinden birleşim prensibine bağlı olarak, herhangi bir amaçla ya da sadece kavram kanıtı için moleküllerin üzerinde belirli uzaklıklarda yerleştirilebileceği iskele çalışmalarında kullanılmıştır. Sunulan çalışmada, tasarlanmış transkripsiyon faktörlerini BRET çifti olan *Renilla* lüsiferaz (RLuc) ve floresan bir protein olan mCherry'ye birleştirildiği ve bu parçaların DNA iskelesi üzerinde bir araya getirildiği bir BRET platformunun dizayn edilmesi amaçlanmıştır. Bu amaçla, RLuc komplementer DNA (cDNA)'sı insan östrojen reseptörü α (hERα)'nın modifiye edilmiş hali olan CDC DNA-bağlanıcı proteinin cDNA'sına ve mCherry cDNA'sı bir maya protein olan Gal4 DNA-bağlanıcı proteinin cDNA'sına, genel bir protein saflaştırma etiketi olan 6-Histidin (6xHis) ile bir bakteriyel ekspresyon vektörü içerisinde birleştirilerek klonlanmıştır. İlgili füzyon proteinlerini kodlayan plasmidlerin oluşturulması sonrasında çalışmanın sonraki aşamalarında kullanılacak füzyon proteinlerinin aşırı-ekspresyonu, izolasyonu ve saflaştırılması denenmiş ve tüm aşamalar Western Blotting Analizi ile doğrulanmaya çalışılmıştır. Henüz devam eden bir çalışma olarak, proteinlerin DNA'ya gerçekten bağlanıp bağlanmadığı biyokimyasal yöntemlerle kontrol edilecek ve BRET sinyali elde edilmeye çalışılacaktır. Dizayn edilmesi planlanan bu platformun, in situ çalışmalara aktarıldığında kompleks transkripsiyon mekanizmasının çalışılmasında, genom mühendisliği ve DNA-protein etkileşimlerinin çalışmalarında kullanılabileceği öngörülmektedir.

Anahtar Kelimeler: Biylüminesans Rezonans Enerji Transferi (BRET), DNA iskelesi, mCherry

To Çiço

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INTRODUCTION

1.1. Transcription Factors (TFs)

Transcription factors are one of the most diverse and large members of the DNA-binding proteins. They regulate gene transcription by binding to specific DNA sequences. As a result of structural studies and sequence comparisons, TFs are grouped into diverse classes, such as helix-turn-helix proteins, helix-loop-helix proteins, homeodomains, zinc finger proteins, leucine zipper proteins and steroid receptors. Each class includes a simple secondary structure which binds to DNA. However, this structure is not enough to bind to DNA. DNA-binding is a total activity of the protein with different interactions between DNA and protein. The orientation of the secondary structures for binding to DNA is protein-specific (Pabo & Sauer, 1992).

Binding of proteins to DNA is carried out via molecular interactions with bases on DNA molecule. The interactions are direct hydrogen bonds between bases and protein side chains, hydrogen bonds between polypeptide chain and bases, hydrogen bonds via water molecule and hydrophobic contacts. Binding occurs most generally to major groove (far side of helical structure of B-DNA) rather than minor groove (close side of B-DNA) since major groove has more convenient orientation to form molecular interactions,

resulting in sequence-specific recognition. Still, there are not obvious recognition codes or interaction patterns for binding of proteins to DNA bases, creating a large complexity how TFs interact with DNA. However, conserved sequences make it possible to generate a docking model for common TFs. In the DNA, sugar-phosphate backbone is used for site-specific recognition by orienting the proteins. Moreover, DNA bending, kinking and flexibility contribute to the binding of proteins to DNA (Harrison, 1991; Pabo & Sauer, 1992).

TFs consist of modular structures, each of which displays different activity. Mainly, TFs are divided into a nucleic acid-binding domain and an effector domain. This modular structure allows combinatorial uses of TFs in protein engineering (Frankel & Kim, 1991).

As an advantage of modular structure, domains of TFs have been modified as chimeric proteins for mainly controlling gene expression (Beerli & Barbas, 2002; Huang et al., 2004) and detection of DNA binding. For instance, TFs labelled with Quantum Dots (QDs) have been used to find out the localization of them on DNA. As exemplified with a proof-of-concept study, QD-labelled T7 RNA polymerase which was detected on the T7 bacteriophage genome (Ebenstein et al., 2009). In another study, modified zinc finger domains (ZFDs) were used to detect the target dsDNA by fusing the ZFDs to split-parts of β-lactamase. Upon bringing into a close proximity of ZFDs, the restoration of β-lactamase activity was shown; this system was suggested to be used in the detection of target dsDNA sequences for diagnostic purpose and further developed as an array system to adjust the detection limit (M.-S. Kim, Stybayeva, Lee, Revzin, & Segal, 2011; Ooi, Stains, Ghosh, & Segal, 2006). A different approach was used to generate an artificial estrogen-inducible Gal4 protein, in which the ligand-binding domain of human estrogen receptor was fused to DNA-binding domain of Gal4. The system was proposed to be used as a new inducible expression system in biotechnology to investigate TF-regulated gene activity in various systems (Braselmann, 1993). The modular structure of

Gal4 protein was also used to detect protein-protein interaction by generating fusion proteins of interest with DNA-binding and transcription activation domains of Gal4, which was called yeast two-hybrid system. The system has been a useful tool for protein interaction studies (Fields & Song, 1989). This system was further modified to be utilized as a mammalian two-hybrid system by changing transcription activation domain from Gal4 to VP16 protein of the herpes simplex virus (Luo, Batalao, Zhou, & Zhu, 1997).

1.1.1. TFs with Zinc-binding Domains

The term of the zinc-binding domain is used for categorizing the DNA-binding domain of TFs. These types of proteins include zinc ion (Zn) as a structural element. In 1991, Harrison reviewed the structurally identified DNA-binding domains and separated zinc-binding domains into three classes, Class 1, 2 and 3. Class 1 is the original zinc-finger proteins with one Zn ion-coordinated 30-residue module; Class 2 includes steroids and related hormone-like receptors; e.g. estrogen receptor, with two Zn ions in loop-helix elements of the DNA binding domain that coordinates four cysteines in a 70-residue module. Proteins including this domain bind to DNA as dimers on a symmetric DNA sequence. Class 3 includes two Zn ions, each coordinate six cysteines and is found in a set of yeast activators; e.g. Gal4 (Harrison, 1991).

1.1.1.1. Estrogen Receptor (ER)

Estrogen receptor (ER) is a TF that is activated by estrogenic hormones. Activated ER regulates variety of physiological and pathophysiological events by up- or down-regulating gene expressions. The gene regulation is controlled by the binding of ER to specific DNA sequences called estrogen response elements (EREs) as a dimer. ER has modular structure with different domains (Figure 1); the N-terminal domain (NTD), DNA-binding domain (DBD) and ligand binding domain (LBD), from N- to C-terminus. The activation function (AF) domain 1 is localized in NTD and the AF2 is localized in LBD, both of which are responsible for gene regulation with hormone-independent (AF1) and hormone-dependent (AF2) manner. Human ER has subtypes encoded by different genes: ERα and ERβ. ERs when co-synthesized form homo- and/or heterodimers (Gronemeyer, 1991; Kumar et al., 2011).

Figure 1. Schematic structure of human estrogen receptor. Human estrogen receptor consists of N-terminus domain (NTD; A/B) in which there is a transcription activation region (AF1); a DNA-binding domain (DBD; C); a hinge domain (D); a ligand binding domain (LBD; E); and the C-terminus domain (F) functioning as the second transcription activation region (AF2).

The hinge region, D domain, includes a nuclear localization signal and provides flexibility for between the amino- and carboxyl-termini. The E/F region displays ligand binding, dimerization and ligan-dependen transactivation activities. The DBD, C domain, is the domain that binds to ERE, idealized with the consensus 5' GGTCAnnnTGACC 3' palindromic sequence (Kumar et al., 2011).

ER domains were used to engineer synthetic transcription factors. For example, generating proteins with different combinations of ER domains, the engineered CDC protein (Figure 2) with two DNA-binding domains (C) and the hinge region (D) was demonstrated to bind to ERE without necessity of dimerization and without a transcription activity (Huang et al., 2004).

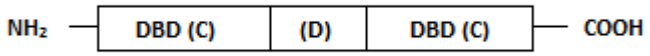

Figure 2. Schematics of the engineered monomeric DNA binding module. DNA-binding domains (DBD; C) of human estrogen receptor were combined with the hinge domain (D) at the middle to promote flexibility and nuclear localization.

1.1.1.2. Gal4

Gal4 is a TF activated by binding to galactose and melibiose in *Saccharomyces cerevisiae* and interacts with 17 base pair (bp) palindromic sequence, Gal4 response element (Gal4RE), as a dimer. It is composed of a DBD which is formed by DNA recognition element and a dimerization domain, and two transcriptional activation domains (Hong et al., 2008). The N-terminus of the intact Gal4 is responsible for DNA-binding and analogous to those of steroid hormone receptors. Amino acids from 1 to 65 bind as a homodimer to CGG half-sites that are separated by 11 bp sequence (Harrison, 1991; Hong et al., 2008; Pan & Coleman, 1989). Zinc and cysteine structures are packed by two helix-turn-strand motifs in DBD distinguishing Gal4 from other zinc finger proteins (Kraulis, Raine, Gadhavi, & Laue, 1992). Gal4 DBD is also responsible for protein thermostability (Hong et al., 2008).

1.2. Bioluminescence Resonance Energy Transfer (BRET)

Resonance energy transfer (RET) described in 1940s is the transfer of energy from a donor to an acceptor. RET is modified to be used as fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer, BRET (Ciruela, 2008). As a specific form of RET, BRET is a natural process that occurs in some marine species. It is the transfer of non-radiative biochemical energy between a light emitting enzyme, such as luciferase, as an energy donor, and an energy acceptor which is mainly a fluorescent protein or an inorganic molecule, such as inorganic dyes and nanoparticles (Bacart, Corbel, Jockers, Bach, & Couturier, 2008; Xia & Rao, 2009; Y. Xu, Piston, & Johnson, 1999). The acceptor molecule absorbs the energy generated by the oxidation of the substrate by luciferase and re-emits energy in an excited state (Xia & Rao, 2009).

The efficiency of BRET method is defined as BRET ratio which is the proportion of acceptor emission to donor emission (Zhang et al., 2006). The ratio is optimized as the formula, [(emission of acceptor)-(emission of donor)xCf]/(emission of donor), where Cf corresponded to (emission of mCherry)/(emission of RLuc) for the 6xHis-CDC-RLuc expressed alone in the same experiments (Angers et al., 2000).

BRET is a powerful tool for monitoring molecular interactions, especially protein-protein interactions via spatial relationship. The signal is detected as a result of functional activity, *in vivo* and *in vitro* by tagging of proteins of interest with RLuc and fluorescent proteins. If the proteins of interest do not interact with each other, only one signal, from oxidation of substrate by RLuc, is obtained. However, if the proteins of interest interact, two signals, enhanced re-emission of fluorescent molecule addition to signal from RLuc, are obtained. BRET also gives clues about the cell-based mapping of signal transduction pathways and receptor-ligand interactions in real time (Boute, Jockers, & Issad, 2002; De, Loening, & Gambhir, 2007; Y. Xu et al., 1999).

1.2.1. Components of BRET Assay

1.2.1.1. Renilla Luciferase (RLuc)

RLuc protein has a rapid turnover rate which is an advantage for kinetic studies (Y. Xu et al., 1999). RLuc is the most commonly used luciferase for BRET assays (Cui et al., 2014). It gives 485nm emission with native substrate, coelenterazine (Figure 3) and the emission changes according to substrate; 400nm with coelenterazine derivative, DeepBlueC (coelenterazine-400a). Native RLuc has a low quantum yield, rate of the ratio of reaction induced by photon absorption to flux of absorbed photons (Sun & Bolton, 1996), resulting in problematic within the cell-based assays. Hence, native RLuc was modified by mutations and named as RLuc8 to increase quantum yield and stability for cell-based assays (De et al., 2007; Hawkins et al., n.d.).

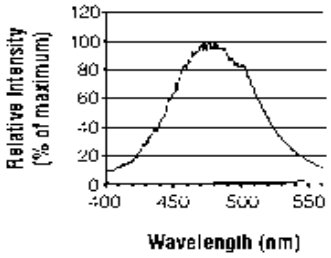

Figure 3. Spectra and luminescence measurements generated by *Renilla* luciferase (Hawkins et al., n.d.).

Signal from RLuc is dependent on substrate type. The signal could be enhanced by ViviRen^{TM} and extended by EnduRen^{TM} substrates (Xie, Soutto, Xu, Zhang, & Johnson, 2011).

1.2.1.2. mCherry

mCherry is a fluorescent protein which was developed by mutations on the monomeric red fluorescent protein (RFP), mRFP1, which was already obtained by molecular evolution and fragmentation of *Discosoma sp.* fluorescent protein, DsRed. Its maximum excitation wavelength is 587nm and maximum emission wavelength is 610nm (Figure 4). It is 231 amino-acid length (Shaner et al., 2004). It was shown that the properties of mCherry are less affected by the fusion to the N- or C- terminus of the acceptor proteins compared to other fluorescent protein. Thanks to these properties, mCherry has been used diverse fluorescence-based studies. Fan et al. generated a split mCherry by dividing mCherry into two parts and show that split protein function is restored only when the parts complemented. This approach become a suitable tool for protein-protein interaction studies (Fan et al., 2008). Its properties to some of other fluorescent proteins were assessed and evaluated for FRET studies (Akrap, Seidel, & Barisas, 2010). For the BRET studies, mCherry was shown to be proper for excitation by the luminescent of *Firefly* luciferase (575nm) even in *in vivo* studies (Iglesias & Costoya, 2009).

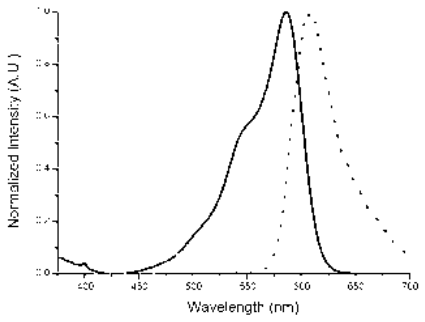

Figure 4. Excitation (line) and emission (dots) wavelength of mCherry (Albertazzi, Arosio, Marchetti, Ricci, & Beltram, 2009).

1.2.2. Advantages and Disadvantages of BRET

Protein-protein interactions, a crucially fundamental concept to identify cellular processes, are figured out by mainly two-hybrid system, reporter assays, surface plasmon resonance (SPR) and fluorescent based methods including protein fragment complementation assay, mass spectrometry, evanescent wave methods, FRET and BRET (Bacart et al., 2008; Cui et al., 2014; Xie et al., 2011; Y. Xu et al., 1999).

The most powerful method to monitor protein-protein interaction is yeast two-hybrid method. However, this method is lack of monitoring dynamic interaction between proteins. Fluorescence-based methods are useful for monitoring dynamic protein-protein interactions (Bacart et al., 2008). BRET is particularly convenient to screen constitutive and intermolecular protein interactions (Perroy, Pontier, Charest, Aubry, & Bouvier, 2004).

A comparable method to BRET, FRET has an uncontrollable excitation. The acceptor of FRET system may also be excited by the external light source even though it is advantageous for single-cell studies. Also, the external light source may damage the cells and could be problematic for photo-responsive cells, autofluorescence, photobleaching, phototoxicity and overlapping signals. Moreover, BRET-based assays are ten-times more sensitive than FRET-based ones and FRET analyses require higher protein amounts. Since there is no need for external excitation, deep tissues in model organisms can be monitored by BRET assay (Bacart et al., 2008; Boute et al., 2002; James, Oliveira, Carmo, Iaboni, & Davis, 2006; X. Xu et al., 2007; Y. Xu et al., 1999).

Fluorescence-based methods (basically FRET and BRET) necessitate appropriate orientation of donor and acceptor, and suitable distance between them (Y. Xu et al., 1999). The distance between donor and acceptor is 10 to 100Å. The BRET efficiency (the ratio of acceptor emission to donor emission) is dependent on the inverse sixth-

power of the distance between the donor and the acceptor. The efficiency is also dependent on the levels of donor or acceptor. This is also valid for strength of interaction between molecules, and density of the molecules (Cui et al., 2014; James et al., 2006).

BRET assay necessitates highly sensitive light-measuring devices since the BRET signal is dim (X. Xu et al., 2007; Y. Xu et al., 1999). Moreover, BRET technique may not be useful for imaging of single cells and small organisms likewise the other protein-protein interaction methods. However, modified BRET pairs are able to overcome these problems (De et al., 2007).

1.2.3. Applications of BRET

By using *Renilla* Luciferase (RLuc) and Enhanced Yellow Fluorescent Protein (EYFP) BRET pair, Xu et al. showed that circadian (daily) clock proteins from cyanobacteria homo-dimerized *in vivo* and *in vitro* (Y. Xu et al., 1999).

The conditional interaction of proteins was monitored by using anti-GST antibody as the conditioner, and *Firefly* Luciferase derivative and DsRed (Red fluorescent protein from *Discosoma* species) as the BRET pair. The system resulted in conditional binding of Glutathione-S-Transferase (GST) to *Streptococcus* protein G IgG binding domain (Arai, Nakagawa, & Kitayama, 2002).

Cui et al. used bacterial luciferase derivative, LuxAB and EYPF to generate a BRET system for bacteria in the light of that previous common BRET couples are not suitable for bacteria because of expression patterns of proteins. They figured out bacterial protein-protein interactions (proteins in the flagellar regulon; FlgM and Flia) in a dynamic manner by using also inducible proteins (Cui et al., 2014).

An *in vitro* insulin detecting system based on BRET was developed. The system uses RLuc and EYFP which are fused to beta subunits of the insulin receptor (IR). Upon autophosphorylation of IR by the tyrosine kinase activity, the subunits get closer. Abnormalities in autophosphorylation are related to insulin resistance, diabetes and obesity. That insulin-like growth factor-1 (IGF1) and anti-insulin receptor antibody (83-14), which activate the IR were shown to affect the BRET signal and epidermal growth factor (EGF) was used as negative control. The system was suggested to correlate with pharmalogical background (Boute, Pernet, & Issad, 2001).

The dynamic nature of ubiquitination, a post–translational modification that is mainly responsible for protein degradation, was studied by BRET. Using the advantages of BRET on real-time *in vivo* monitoring facility, ubiquitination of β-arrestin of which ubiquitination pattern changed from stable to transient according to activated receptor type was studied by BRET using RLuc-β-arrestin and GFP-ubiquitin couple (Perroy et al., 2004).

BRET was used together with site-directed mutagenesis *in vivo* to determine effect of the nuclear exclusion and dimerization of *Arabidopsis* Constitutive photomorphogenesis 1 (COP1). COP1 is the repressor of light transduction as the part of nuclear E3 ubiquitin ligase. Researches showed that one mutation displayed nuclear accumulation with the retained dimerization by BRET pairs, RLuc and YFP. Thus, using BRET assay, an important light-regulator protein, COP1, in plant was characterized by overlapping the nuclear exclusion function and dimerization (Subramanian et al., 2004).

The intermolecular interaction of calcium-sensing receptor (CaR), a protein belonging to family C of G-coupled receptor superfamily, was characterized by using BRET with RLuc and GFP. The approach generated information about the conformational change in the transmembrane proteins of receptor in the absence and the presence of agonist (Jensen, Hansen, Sheikh, & Bräuner-Osborne, 2002).

De et al. modified the BRET pairs to overcome the problem of BRET technique with small living organisms and single cells in which BRET signal is not sensitive. They showed interactions of rapamycin pathway proteins, FKB12 and FRB, in mammalian cells and living mouse model by using GFP and RLuc8 as the BRET pair (De et al., 2007).

A sensor-based usage of BRET assays was designed to detect a specific protease, matrix metalloproteinase-2 (MMP-2) by using RLuc8 and carboxylated QD as the BRET pair. The system worked well for the detection of MMP-2 cleaving the amino acid-based substrate and separating combined BRET pair (Yao, Zhang, Xiao, Xia, & Rao, 2007).

By using advanced imaging systems, BRET assay is also applicable in monitoring subcellular localization of protein-protein interactions. Xu et al. showed the dimerization of CCAAT/enhancer binding protein α (C/EBP α) in the mammalian nucleus with an advanced imaging system (X. Xu et al., 2007).

G-protein coupled receptors (GPCRs) represent the single largest family of transmembrane proteins in cell signaling. They are the most commonly studied receptors by BRET assays. This superfamily of receptors is the key targets for drug developments (Milligan, 2004). Dimerization dynamics of chemokine receptors, CXCR4 and CCR2 were shown by BRET technique. By BRET assay, the binding of ligand to pre-formed CXCR4 and CCR2 dimers changed the conformation, showing that the ligand binding was not responsible for receptor dimerization or dissociation. Moreover, antagonists affecting CCR5-β-arrestin interactions were monitored by BRET. It was shown that some of the antagonist prevented this interaction; giving details for deactivation of GPCRs. A similar study was also carried out for neuropeptide Y family peptides and illustrated that these agonists increased the interaction of GPCRs with β-arrestin (Berglund, Schober, Statnick, McDonald, & Gehlert, 2003; Hamdan, Audet, Garneau, Pelletier, & Bouvier, 2005; Percherancier et al., 2005). In another study, β_2-adregenergic

receptor (β_2AR) homo-dimerization but not hetero-dimerization, and the agonist-dependent increase in the interaction of the homo-dimer structure were demonstrated in cell membrane (Angers et al., 2000). As a newer study, that β1-AR and β2-AR could form homo- and hetero-dimers was demonstrated (Mercier, Salahpour, Angers, Breit, & Bouvier, 2002). McVey et al. showed homo- and hetero-dimerization of δ-opioid receptors in transiently transfected mammalian cells. They also figured out the increase in the agonist-dependent interactions of receptor with other receptors by using BRET approach (McVey et al., 2001). As a further study, the affinity of opioid receptor to form homo- or hetero-dimers was studied by BRET and it was found that there was not any affinity differences to form homo- or hetero-dimers of receptors, the hetero-dimerization of functional μ and κ, and the selectivity of receptors to form oligomers with other receptors (Ramsay, Kellett, Mcvey, Rees, & Milligan, 2002; Wang, Sun, Bohn, & Sadee, 2005).

1.3. Molecular Self-Assembly and DNA as a Scaffold

Molecular self-assembly is to bring about molecular aggregates by the designed and defined interactions of small molecules displaying properties that individual molecules cannot display. DNA is not only an information source but also a stable biopolymer in terms of physicochemistry; and mechanically rigid, a precious self-assembly member via complementation and functionalization properties. DNA is advantageous because of its precise biosynthesis and hybridization, and selective crosslinking. Moreover, generation of synthetic DNA with specific modifications is cheap and easy. Owing to these advantages, DNA has been used in the assembly of supramolecular aggregates in especially nanotechnology (Bandy et al., 2011; Niemeyer, 1997; Tagore, Sprinz, Fletcher, Jayawickramarajah, & Hamilton, 2007).

DNA is a biopolymer with changing scales of units in its structure. The conformation of DNA as a result of physical and mechanical studies were determined to be changeable according to parameters, such as handedness, helical repeat, contour length, persistence length and torsional modulus affecting all the properties of molecule in different solutions. DNA is named as letters, such as A, B, L and Z DNA (Feig & Pettitt, 1997; Sheinin, Forth, Marko, & Wang, 2011). As conformation of most natural and synthetic DNAs, B form has been highly studied for DNA behavior in diverse solutions and scales. B form is the right-handed conformation with 10.5bp/turn helical repeat, 0.34 nm/bp contour length, 5.9-6.1Å minor groove width, 11.2-11.3Å major groove width, 4.6-5.7Å minor groove depth and 3.9-4.1Å major groove depth (Sheinin et al., 2011; Stofer & Lavery, 1994; Zimmerman, 1982).

Assembly of metallic nanocrystals provides a convenient environment to study the physical properties of these structures. Biopolymers, particularly DNA on the account of its predictable secondary and tertiary structure, nano-size structure and stability in aqueous solutions, are good candidates to assemble these structures in a spatial-controllable manner. Loweth et al. used chemically conjugated gold nanoparticles (AuNPs) to single strand DNA (ssDNA) and combined the particles on DNA using complementation properties of ssDNA. They studied optical properties of the structures (Loweth, Caldwell, Peng, Alivisatos, & Schultz, 1999). As a different scaffold type for same purpose, a DNA origami scaffold, called DNA slit having six long rectangular spaces, was produced to assembly of AuNPs on DNA with a distinct positioning. Slit cavity was thiolated in a solution including ssDNA and dsDNA to assembly AuNPs on DNA using self-assembly principle on a mica surface (Endo, Yang, Emura, Hidaka, & Sugiyama, 2011).

DNA was used as a template to be able to combine single-wall carbon nanotubes (SWNTs) and metallic wire system to generate a field-effect transistor (FET). The overall system was also included the usage of functionality of homologous

recombination by RecA protein to address the SWNTs on the DNA. Briefly, RecA was polymerized on ssDNA and this complex mixed to double strand DNA (dsDNA) via sequence homology. By the help of a mouse anti-RecA antibody and biotinylated anti-mouse secondary antibody, streptavidin-conjugated SWNTs were placed on the dsDNA. As the same principle, metallic wires and source-drain elements were addressed on the RecA-polymerized dsDNA and whole FET system was constructed. The electronic properties of the system were evaluated (Keren, Berman, Buchstab, Sivan, & Braun, 2003).

Puncher et al. used DNA as a mediator to combine nanoparticles guiding one-by-one assembly by the tip of atomic force microscopy (AFM). They placed biotin molecules on a target plate by taking biotin-coupled DNA with AFM tip on the principle of hybridization and combined them on the plate molecule-by-molecule using again hybridization principle of DNA and regarding geometry and energy facts. They finally used streptavidin-coated QDs to combine QDs on a plate with a certain distance (Puchner, Kufer, Strackharn, Stahl, & Gaub, 2008). Similarly, polycatenated DNA scaffold was used to combine thrombin or AuNPs or fluorescent molecules for precious spatial orientation of these molecules using AFM tip as a director and the principle of hybridization and ligation of DNA molecules. The certain spatial orientations were proved by different microscopy techniques and FRET assay (Weizmann, Braunschweig, Wilner, Cheglakov, & Willner, 2008).

In a well-defined study, DNA was used as a scaffold to combine the enzymes, which were fused to zinc finger DNA binding domains, of multi-enzyme systems to increase the amounts of final products, resveratrol, 1,2-propanediol and mevalonate. Different scaffolds distinguishing in the terms of distance between DNA binding domains were used to examine the most suitable platform for maximal efficiency. BRET system was also used in a different perspective to demonstrate whether DNA binding domains came together (Conrado et al., 2012). As a similar perspective, He and Liu developed a system

in which multistep biosynthesis of small molecules, amine acetylation for this study, was carried out on DNA track in a solution via autonomous change of physical location by the time and RNA-cleaving DNAzyme, which was called DNA walker. They showed the overall system increased the easiness, speed and efficiency of the reaction (He & Liu, 2010).

Roseweig et al. combined more than one C-reactive protein (CRP), a liver-specific protein biomarker produced as the result of infection and inflammation, on a pentameric DNA structure using phosphocholine (PC) head groups to study the properties of the proteins. They assembled CRP on DNA with a high affinity, which was a putative diagnostic tool with the reporter and target binding activity (Rosenzweig & Ross, 2009). Similarly, a stick-like punched DNA origami was used to place streptavidin protein molecule-by-molecule in a programmed manner situation in which the well-positioned streptavidin was stable for microscopic investigations via generated protein nanoarrays (Numajiri, Kimura, Kuzuya, & Komiyama, 2010). Moreover, the mechanisms of microtubules-based motor proteins, dynein and kinesin-1 were investigated using a programmable synthetic cargo and three-dimensional DNA origami (Derr et al., 2012).

DNA scaffold was also used for assembly of porphyrin, a chromophore derivate of which self-assembly is important for generation of photochemical and electrochemical materials. Porphyrin dimer was constructed on a duplex DNA scaffold in a temperature-dependent manner and conformational change of the dimer was investigated (Endo, Fujitsuka, & Majima, 2008; Fendt, Bouamaied, & Thöni, 2007).

1.4. Aim of the Study

The aim of the present study is to perform the recombinant protein studies from cloning to protein purifications so as to construct a novel Bioluminescence Resonance Energy Transfer (BRET) system by using BRET pairs fused to transcription factors and DNA as the template; serving a novel tool to study molecular interactions of proteins and DNA, and is open to development of novel sensor systems. This system is advantageous thanks to exactly adjustable distance between donor and acceptor owing to DNA scaffold.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cloning of constructs

The parts of BRET platform includes bioluminescent (RLuc) and fluorescent (mCherry) proteins fused to CDC, which is an engineered protein from estrogen receptor α and binds to specific estrogen response element (ERE) without any function, and Gal4DBD, which is the DNA-binding domain of Gal4 transcription factor, respectively. Both constructs include 6-Histidine (6xHis) tag at the N-terminus of the proteins for purification. In this section, how cDNAs coding for related fusion proteins were generated will be covered.

2.1.1. Primer designs

In order to obtain cDNAs with appropriate modifications to code related fusion proteins, suitable primers (Figure A1) were designed and ordered from Iontek, Istanbul or Sentegen, Ankara.

2.1.1.1. Primer design for *6xHis-CDC-RLuc*

To generate *6xHis-CDC-RLuc*, the plasmid, pBS-KS-Flag-CDC-VP16 (Figure 5) from Dr. Mesut Muyan's inventory was used as a template plasmid and the primers were ordered in this aspect.

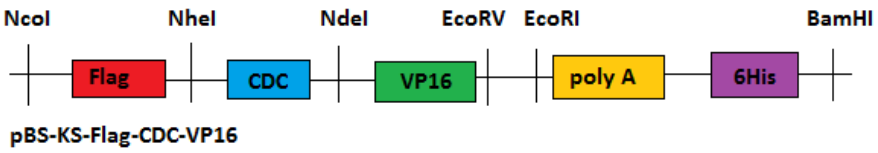

Figure 5. The plasmid used as template for construction of the vectors.

The forward primer includes NdeI restriction enzyme recognition site in addition to specific sequence and the reverse primer contains EcoRI and BamHI restriction enzyme recognition sites and polyA signal sequence in the middle of these sites in addition to specific sequence (Figure A.1). All primers was also added extra non-specific sequences in front of the restriction enzyme recognition sites, located at the ends of the primers to make the restrictions easier.

2.1.1.2. Primer design for *6xHis-Gal4DBD-mCherry*

To obtain *6xHis-Gal4DBD-mCherry* construct, pM-Gal4DBD (Clontech, Inc.), from Dr. Mesut Muyan's inventory, and pcDNA-mCherry, kindly gift from Dr. Çağdas Devrim Son, were used as template plasmids. In order to add *6xHis* tag to 5' end of the fused-cDNA (*Gal4DBD-mcherry*), primers were ordered as NheI recognition site at the beginning of the forward primer and BamHI recognition site at the end of the reverse primer in addition to specific overlapping sequences (Figure A.2).

2.1.2. PCR amplifications

2.1.2.1. PCR amplifications for *6xHis-CDC-RLuc*

In order to obtain *6xHis-CDC-RLuc* cDNA, *RLuc* cDNA was amplified from pRL-SV40 vector (Promega Corporation, USA) with the proper primers using Taq Polymerase (Thermo Fisher Scientific Inc., USA) by PCR. PCR conditions were set to 3 minutes at 95^{o}C as initial denaturation; 30 seconds at 95^{o}C as cyclic denaturation of 30 cycles; 30 seconds at 50^{o}C as annealing; 30 seconds at 72^{o}C as cyclic extension; 5 minutes at 72^{o}C as final extension; and infinite hold at 4^{o}C. 50ng of pRL-SV40 was used to amplify *RLuc*. These primers and conditions were also used for colony PCR to ensure that the vector includes the insert. Colony PCRs were set via bacterial colony or the bacterial suspension (1ul of the re-suspended bacterial pellet obtained from 400ul liquid LB).

2.1.2.2. PCR amplification for *6xHis-Gal4DBD-mCherry*

Using the primers for *Gal4DBD-mCherry*, a product was amplified by using pM-Gal4DBD-mCherry vector as template. This vector was constructed without needing any PCR. However, this vector was problematic and caused frame-shift in the cDNA. Still, it was used as a template to obtain *Gal4DBD* with the restriction enzyme sites NheI at the 5' end and EcoRI at the 3' end by using Gal4DBD-mCherry_FP and mCherry_REP. For PCR conditions were set to conditions given for *6xHis-CDC-RLuc* excepting the annealing temperature 55^{o}C instead of 50^{o}C. As being valid for *6xHis-CDC-RLuc*, these primers and conditions were also used for colony PCRs. Next, using mCherry_FP and mCherry_REP with same conditions given above, *mCherry* with EcoRI restriction site at the 5' end and HindIII restriction site at the 3' end was obtained.

2.1.3. Cloning for *6xHis-CDC-RLuc*

2.1.3.1. Cloning of *RLuc* into pBS-KS (-) vector

All the constructs were created in an intermediary vector, pBS-KS (-) and transferred into expression vectors as a total structure.

The PCR-amplified *RLuc* with the proper restriction enzyme recognition site on the primers was purified using PCR Purification Kit (Qiagen, USA). After purification, *RLuc* PCR product and the vector, pBS-KS-Flag-CDC-VP16, were cut with NdeI and BamHI restriction enzymes. The double digestion of the vector, pBS-KS-Flag-CDC-

VP16, discarded the part, *VP16*, and created a suitable position for the binding of double- digested *RLuc* PCR product. The double digested *RLuc* PCR product and pBS-KS-Flag-CDC-VP16 were loaded into 1% agarose gel and extracted with Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research, USA).

After gel extraction, the concentrations of double-digested products were determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). Depending on the concentrations and the sizes of the products to use same amount vector and insert, the vector and the insert were ligated using Rapid Ligation Kit (Thermo Fisher Scientific Inc., USA) for 15 minutes at room temperature. After 15 minutes, standard transformation protocol was applied. Briefly, the ligation solution was mixed with 90ul of competent bacteria (*Escherichia coli* XL1 Blue), which were generated by standard $RbCl_2$ method, and the mixture was left on ice for 1 hour; heat-shocked for 45 seconds at 42^{o}C; shaken for 1 hour at 200rpm and 37^{o}C; plated onto LB agar with specific antibiotic, ampicillin for this case, by using glass beads; and incubated at 37^{o}C, overnight. Next day, colonies would be seen and were selected by tipping them into 4ml LB plus ampicillin (100ug/ml final concentration) and shaken overnight. Next, 400ul of the cell suspension was used for colony PCR and the colonies including the inserts were used for plasmid isolation. Plasmid isolations were managed with MiniPrep Plasmid Isolation Kit (Qiagen, USA) according to supplier's instructors and the concentrations after isolations were determined by using NanoDrop. The vector was called pBS-KS-Flag-CDC-RLuc. Since the insert was a PCR product and base changes could occur, the isolated plasmids were sequenced by universal T3 forward primer and T7 reverse primer, which were vector-specific, via METU Central Laboratory Molecular Biology and Biotechnology Research and Development Center, Ankara or Refgen, Ankara.

2.1.3.2. Cloning of *CDC-RLuc* into pET28a (+) vector

To obtain *6xHis-CDC-RLuc* in the bacterial expression vector, pET28a (+) with Kanamycin resistance coding sequence, the vector, pBS-KS-Flag-CDC-RLuc and pET28a (+) were cut with NheI and BamHI. The double digested plasmids were loaded to 1% agarose gel and gel extraction was managed. After gel extraction, the concentrations of the parts were determined and the parts were ligated to each other. Standardly, transformation was applied and the plasmids were obtained two-day after transformations. The *6xHis* tag was automatically added at the 5' end of the *CDC-RLuc* as the result of these enzyme restrictions and ligations. The vector was called pET28a-6xHis-CDC-RLuc and it was obtained by using *Escherichia coli* XL1 Blue competent cells in 30ug/ml final concentration of Kanamycin.

2.1.4. Cloning for *6xHis-Gal4DBD-mCherry*

2.1.4.1. Cloning of *Gal4DBD* and *mCherry* into pBS-KS (-) vector as fusion

By using frame-shifted cDNA, NheI and EcoRI-digestible *Gal4DBD* was ligated with pBS-KS-Flag-CDC-VP16 vector double-digested with same enzymes resulting in an empty plasmid backbone with Flag epitope-coding sequence at the 5' end. Thus, a new vector could be named as pBS-KS-Flag-Gal4DBD was obtained. This vector was used as template to form *Gal4DBD-mCherry* construct by cutting the vector and *mCherry* PCR product with EcoRI and HindIII. Hence, the final intermediary vector, called pBS-KS-Flag-Gal4DBD-mCherry was constructed.

2.1.4.2. Cloning of *Gal4DBD-mCherry* into pET28a (+)

The intermediary vector, pBS-KS-Flag-Gal4DBD-mCherry was cut with NheI and HindIII and the product, *Gal4DBD-mCherry* was ligated to pET28a (+) cut with same vector couple, resulting a plasmid backbone with 6xHis epitope-coding region at the 5' end. The vector was named as pET28a-6xHis-Gal4DBD-mCherry.

2.2. Recombinant Protein Over-expression and Isolation

2.2.1. Over-expression of fusion proteins

After the vectors, pET28a-6xHis-CDC-RLuc and pET28a-6xHis-Gal4DBD-mCherry, were constructed, it was transformed in to competent bacterial expression host, *E. coli* BL21 DE3 Star by using standard transformation protocol. When the colonies were obtained, one of them was tipped into 4ml LB plus Kanamycin (30ug/ml final concentration) overnight at 200rpm 37^{o}C. Next, 1ml of the bacterial suspension was further incubated in 10ml of LB with Kanamycin until optical density $(OD)_{600}$ reached to 0.6. When OD_{600} reached to 0.6, 2ml of the bacterial suspensions was taken as uninduced control for further studies and the rests were induced with 1mM final concentration of isoproryl β-D-1-thiogalactopyranoside (IPTG; Thermo Fisher Scientific Inc., USA) overnight at 200 rpm shaking at 37^{o}C as a trial. If these conditions were seen not to be suitable for over-expression of the protein(s), the time points and the temperatures were changed from 37^{o}C 5h to 25^{o}C overnight.

2.2.2. Isolations of fusion proteins

After over-expression studies, the bacterial suspensions were spun down at 5000rpm for 5min. At first, the pellets were re-suspended in 50ul of phosphate buffered saline (PBS) and sonicated by probe sonicator, Ultrasonic Processor (Cole-Parmer Instrument Company, Canada) for protein isolations in native conditions. However, since the levels of over-expression were too low in native conditions, denaturing conditions were shifted by using urea. The bacterial pellets were suspended in 50ul of denaturing solubilizing buffer (DSB; 1M NaCl, 50mM NaH_2PO_4 and 8M urea) and sonicated. After sonication, the solutions were spun down at 20000g for 45min and the supernatants were collected as the source of fusion proteins. To see whether the proteins were aggregated in the pellets, the pellets were re-suspended in 50ul of 2X Laemmli buffer (4% (w/v) SDS, 20% glycerol, 120mM Tris-Cl (pH 6.8) and 0.02% (w/v) bromophenol blue) including 10% β-mercaptoethanol. 30 ul of the supernatants were mixed with 6ul of 6X Laemmli and 10% β-mercatoethanol and the samples were incubated at 95^{o}C for 5min. 15ul of the pellets re-suspended with 2X Laemmli were loaded together with uninduced controls to SDS-PAGE (8% separating gel and 5% stacking gel for 6xHis-CDC-RLuc, and 10% separating gel and 5% stacking gel for 6xHis-Gal4-mCherry) by using 3ul of PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific Inc., USA). The runs were voltage-dependent as 100V. SDS-PAGEs were stained with 1X Coomassie Brilliant Blue R250 (Bio-rad Laboratories, Inc., USA) for 30 min. After staining procedure, the gels were destained using destaining solution (40% methanol, 10% glacial acid and dH_20) for 2 hours and then taken into dH_20 overnight to increase visibility of the bands on the gels. Finally, the gels were imaged using a gel imager under the visible light. When the expression levels were satisfactory, the studies were conducted by volume-upping to 1L for 6xHis-CDC-RLuc and 500ml for 6xHis-Gal4-mCherry. 30 ul of these samples were used for approval of the expression, by mixing 1X Laemmli Buffer which

was prepared via 6X Laemmli Buffer as loading dye, by SDS-PAGE with the protocol given above.

When the applied protocol given above was not enough to obtain high level of expressions, a further protocol was applied to increase the level of expressions. For this aim, a web-based protocol (http://structbio.vanderbilt.edu/chazin/wisdom/labpro/inclusion.html) was used. In this protocol, volume-upped samples were spun down at 6000rpm for 20min and the weights of the pellets were determined. The pellets were re-suspended in 3ml Buffer A (50mM tris-HCl, 5mM EDTA and 10mM NaCl, pH 8.0) per each gram of the pellets. The suspensions were transferred into 50 ml falcons and added 1X Complete, EDTA free Protease Inhibitor Cocktail (Roche, Switzerland) and 16ul of 50mg/ml lysozyme per gram of cells. The suspensions were taken into 37^{o}C until it became viscous. The suspensions were then sonicated and spun down at 16000rpm for 30min. The supernatants were stored and the pellets were re-suspended in 3ml of Buffer B (20mM Na_2HPO_4, 20mM NaCl, 5mM EDTA and 25% (w/v) sucrose, pH 7.2) per each gram of the pellets. 1X Protease Inhibitor and 10ul Triton X-100 per ml of solution were added into suspensions and the suspensions were spun down at 16000rpm for 30min. The supernatants were stored and the pellets were fully re-suspended in 8M urea with 50mM final concentration of DTT at 37^{o}C. 30ul of the samples were loaded to SDS-PAGEs with the uninduced control with the protocol given above.

The BRET-control group, 6xHis-RLuc-mCherry was also isolated with Buffer A from inclusion body protocol.

2.2.3. Western Blot (WB) Analysis

To further confirm the over-expression of 6xHis-tagged specific fusion proteins, WB analyses were managed. After the gels were loaded and run as given above, the gels were taken to transfer tank and the proteins were transferred onto PVDF membrane (Roche, Switzerland). The transfer reaction was set to 100V for 1.5h. After transfer, the membrane was blocked by using 5% skim milk in 0.05% Tris Buffered Saline-Tween (TBS-T) for 1h at room temperature. When the blocking finished, the membrane was exposed to primary antibody, rabbit anti-6xHis tag (1:1000; Abcam, USA) in 5% skim milk in 0.05% TBS-T for 1h at room temperature. After this step, membrane was washed with 0.05% TBS-T for 6 times each of which were 5min. Next, the membrane was incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibody, goat anti-rabbit (1:2500; Santa Cruz Biotechnology, USA) in 5% skim milk in 0.05% TBS-T. After that, the membrane was washed with 0.05% TBS-T for 6 times each of which were 5min. The membrane was incubated with 1:3 luminol-enhancer reagent to peroxidase reagent solution from an enhanced chemiluminescence (ECL) kit (Clarity Western ECL Substrate, Bio-rad Laboratories, Inc., USA). The bands on the membrane were imaged by ChemiDoc^{TM} MP System (Bio-rad Laboratories, Inc., USA) and the images were analyzed by Image Lab 5.1 (Bio-rad Laboratories, Inc., USA).

All WB images lack of loading control. However, each steps of SDS-PAGE loading for control of protein isolations were repeated for WB analysis. So, the images for control of protein isolation studies should be also approved for loading control of WB if the extra non-specific bands on the membrane are not shown and/or available.

2.3. Functionality Assays

To assess the functionality of the recombinant proteins, the proteins were analyzed functionally by *Renilla* luciferase assay and/or fluorescence readings after protein isolations and purifications.

2.3.1. *Renilla* Luciferase Assay For 6xHis-CDC-RLuc

To see isolated and purified 6xHis-CDC-RLuc proteins were functional, *Renilla* luciferase assay was performed. Since the fusion protein includes *Renilla* luciferase, the protein moiety could give luciferase signal upon addition of *Renilla* luciferase substrate. For the assay, 25ul of 6xHis-CDC-RLuc isolated and purified proteins were loaded to 96-well white plates (Greiner, Germany) as three replicas in the absence and presence of substrate. As the substrate, the *Renilla* luciferase solutions (Stop and Glo Reagent) from Dual Luciferase Assay Kit (Promega, USA) were used. According to directions of the kit, the substrate (Stop and Glo Substrate) has to be diluted as 1:50 in Stop and Glo Buffer. The final solution was called Stop and Glo Reagent. 25ul of the protein samples from either isolation or purification studies were mixed with 25ul of the Stop and Glo Reagent or 25ul of the Stop and Glo Buffer as negative control. Additionally, only buffer, isolated proteins from uninduced bacterial populations and Buffer A- and Buffer B-isolated proteins were used as controls and comparisons. The luminescence readings were performed by Turner Biosystems Modulus^{R} II Microplate Multimode Reader (Promega, USA) loading Stop and Glo Buffer or Reagent manually. All the results were transferred to GraphPad Prism and the graphs were drawn with GraphPad Prism. The result from the sample, Rluc A (pointing putative 6xHis-CDC-RLuc in Buffer A from

Inclusion Body Protocol) treated with Stop& Glo Substrate was compared to other groups and Dunnett's Multiple Comparison Test was used for statistically showing significance.

2.3.2. Fluorescence Assay For 6xHis-Gal4DBD-mCherry

Since the mCherry protein which was fused to Gal4DBD is a fluorescent protein, it was tested whether the mCherry was functional after protein isolation and purification studies. 50ul of the isolated and purified proteins were loaded into 96-well black plate (Greiner, Germany) as three replicas same as for controls only buffer, isolated proteins from uninduced bacterial populations and isolated proteins by Buffer A and Buffer B. The readings were obtained by SpectraMax Paradigm Multimode Microplate Reader (Molecular Devices, USA) by settings: well-scan (from nine different points of well), 1mm read height and 570nm excitation and 610nm emission wavelengths. The result from the sample, mCherry A (pointing putative 6xHis-Gal4DBD-mCherry in Buffer A from Inclusion Body Protocol) was compared to other groups and Dunnett's Multiple Comparison Test was used for statistically showing significance.

2.4. Purification of fusion proteins

2.4.1. Purification of 6xHis-CDC-RLuc

The isolated 6xHis-CDC-RLuc in Buffer A from Inclusion Body protocol was tried to be purified by using Ni-NTA Spin Kit (Qiagen Spin Kit) according to supplier's instructions. Briefly, the columns were equilibrated with 600ul of lysis buffer (NPI-10: 50mM NaH_2PO_4, 300mM NaCl and 10mM imidazole, pH 8.0) by spinning down at 890g for 2min. 600ul of isolated protein solution was mixed with 10mM imidazole and the solution was loaded to spin columns. The solution spun down at 270g for 5min. Next, the columns were washed for two times with 600ul of wash buffer (NPI-10) by spinning down at 890g for 2min. Finally, the proteins were eluted with 150ul of elution buffer (NPI-500: 50mM NaH_2PO_4, 300mM NaCl and 500mM imidazole, pH 8.0) by spinning down at 890g for 2min. All flow-through was stored and loaded to 8% SDS-PAGE according to volumes of them.

2.4.2. Purification of 6xHis-Gal4DBD-mCherry

Likewise 6xHis-CDC-RLuc, 6xHis-Gal4DBD-mCherry was also tried to be purified by Ni-NTA Spin Kit (Qiagen Spin Kit). All the protocol given for 6xHis-CDC-Rluc; excepting the wash buffer (NPI-30: 50mM NaH_2PO_4, 300mM NaCl and 30mM imidazole, pH 8.0).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning studies

The cDNAs that code for the fusion proteins which are BRET pairs, *CDC-RLuc* and *Gal4DBD-mCherry* were firstly constructed in an intermediary vector, pBS-KS, by using the vectors which were previously designed by Dr. Muyan's laboratory and then sub-cloned into the bacterial expression vector, pET28a (+) with the suitable restriction enzymes which were designed to construct final plasmids step-by-step thanks to designed primers and PCRs. The final cloning of constructed structures into pET28a (+) vector generated an intrinsically coded *6xHis* tag at the 5' end of the structures, which was regarded as to be used in the purification of proteins.

All pET28a (+) vectors including three major fusion protein groups of the project were constructed (Figure 10) and named as pET28a-6xHis-CDC-RLuc and pET28a-6xHis-Gal4DBD-mCherry. During step-by-step construction of the plasmids, all PCR fragments that used in the construction studies were sequenced to check that there were not any base changes comparing to original form of the related sequences. All the plasmids were controlled whether they include the related structures by either colony PCR or digestion by appropriate restriction enzymes.

3.1.1. Cloning for *6xHis-CDC-RLuc*

3.1.1.1. Cloning of *RLuc* into pBS-KS (-) vector

PCR amplified *RLuc* with ~940bp length and suitable restriction enzymes, NdeI and BamHI, was cloned into pBS-KS-Flag-CDC-VP16 (Figure 5) cut with same enzyme couple. The putatively constructed vector, pBS-KS-Flag-CDC-RLuc was controlled whether it included the insert, *RLuc*, by PCR using the primers RLuc_FP and RLuc_Rep after plasmid isolations, and shown that the vector included the *RLuc* (Figure 6). As the positive control, PCR for pRL-SV40, a commercial vector including *RLuc* was performed with a no template control. To further ensure that the sequence of *RLuc* did not change during PCR amplification, the vector was sequenced and confirmed.

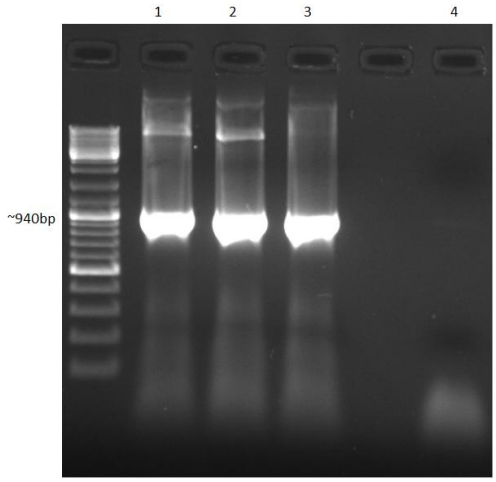

Figure 6. PCR for confirmation of that putative pBS-KS-CDC-RLuc includes *RLuc*. 1. PCR result for isolated plasmid 1; 2. PCR result for isolated plasmid 2; 3. PCR result positive control, pRL-SV40; 4. No template control.

3.1.1.2. Cloning of *CDC-RLuc* into pET28a (+) vector

After the vector, pBS-KS-Flag-CDC-RLuc was obtained, *CDC-RLuc* was cloned into pET28a(+) with the help of NheI and BamHI. The process was confirmed by colony PCR conducted for 10 colonies for putative pET28a(+)-6His-CDC-RLuc by using the primers RLuc_FP and RLuc_REP. The vector, pRL-SV40 was used as positive control with one no template control. As the result, that *CDC-RLuc* was cloned into pET28a(+) was shown (Figure 7).

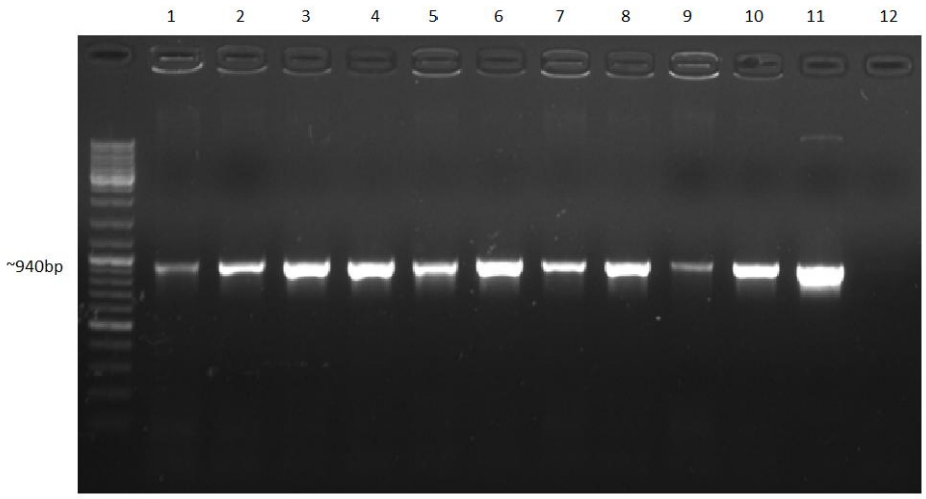

Figure 7. Colony PCR for confirmation of that putative pET28a(+)-6xHis-CDC-RLuc includes *RLuc*. 1-10. Colony PCRs via putative pET28a(+)-6xHis-CDC-RLuc colonies 1-10; 11. PCR for the positive control, pRL-SV40; 12. No template control.

3.1.2. Cloning for *6xHis-Gal4DBD-mCherry*

3.1.2.1. Cloning of *Gal4DBD* and *mCherry* into pBS-KS (-) vector as fusion

By using mCherry_FP and mCherry_REP primers, PCR-amplified *mCherry* with ~450bp length was cloned into pM-Gal4DBD and obtained *Gal4DBD-mCherry* fusion cDNA. However, it was frame-shifted. This vector was used as template to obtain *Gal4DBD* with NheI restriction site at 5' end and EcoRI restriction site at 3' end by PCR using Gal4DBD-mCherry_FP and Gal4DBD-mCherry_REP primers. After the fragment obtained by PCR, it was cloned into pBS-KS-Flag-CDC-VP16 template vector (Figure 5) and pBS-KS-Flag-Gal4DBD plasmid was constructed. To confirm the availability of *Gal4DBD* inside the putative pBS-KS-Flag-Gal4DBD, colony PCR was performed by using Gal4DBD_FP and Gal4DBD_REP primers and that the cloning procedure was successful was shown (Figure 8). Here, pM-Gal4DBD was used as positive control with one no template control. To further ensure that the sequence of *Gal4DBD* did not change during PCR amplification, the vector was sequenced and confirmed.

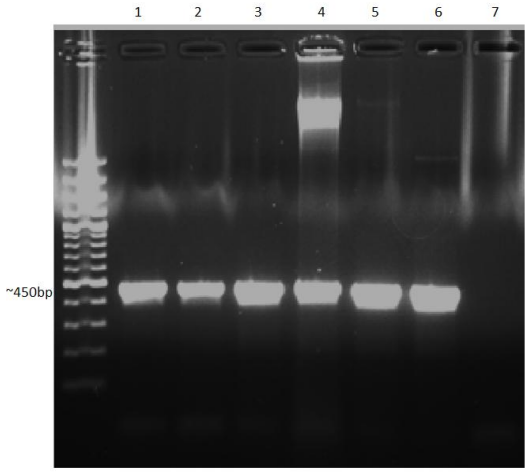

Figure 8. Colony PCR for confirmation of that putative pBS-KS-Flag-Gal4DBD includes *Gal4DBD*. 1-5. Colony PCRs via putative pBS-KS-Flag-Gal4DBD; 6. PCR for positive control, pM-Gal4DBD; 7. No template control.

Next, PCR-amplified *mCherry* with EcoRI restriction site at 5' end and HindIII restriction site at the 3' was cloned into pBS-KS-Flag-Gal4DBD cut with same enzyme couple and the plasmid, pBS-KS-Flag-Gal4DBD-mCherry was constructed. To further ensure that the sequence of *mCherry* did not change during PCR amplification, the vector was sequenced and confirmed.

3.1.2.2. Cloning of *Gal4DBD-mCherry* into pET28a (+)

After the plasmid, pBS-KS-Flag-Gal4DBD-mCherry, was constructed, the vector was double-digested with NheI and HindIII and the cDNA, *Gal4DBD-mCherry* with ~1200bp length, was cloned into pET28a(+), resulting a *6xHis* tag at the 5' end of the fragment. The process was confirmed by digestion of putatively isolated restriction enzymes, NheI and HindIII (Figure 9).

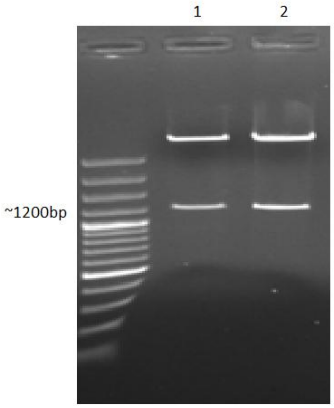

Figure 9. Restriction digestion with NheI and HindIII for confirmation of that putative pET28a(+)-6xHis-Gal4DBD-mCherry includes *Gal4DBD-mCherry*. 1. Restricted plasmid from isolation 1; 2. Restricted plasmid from isolation 2.

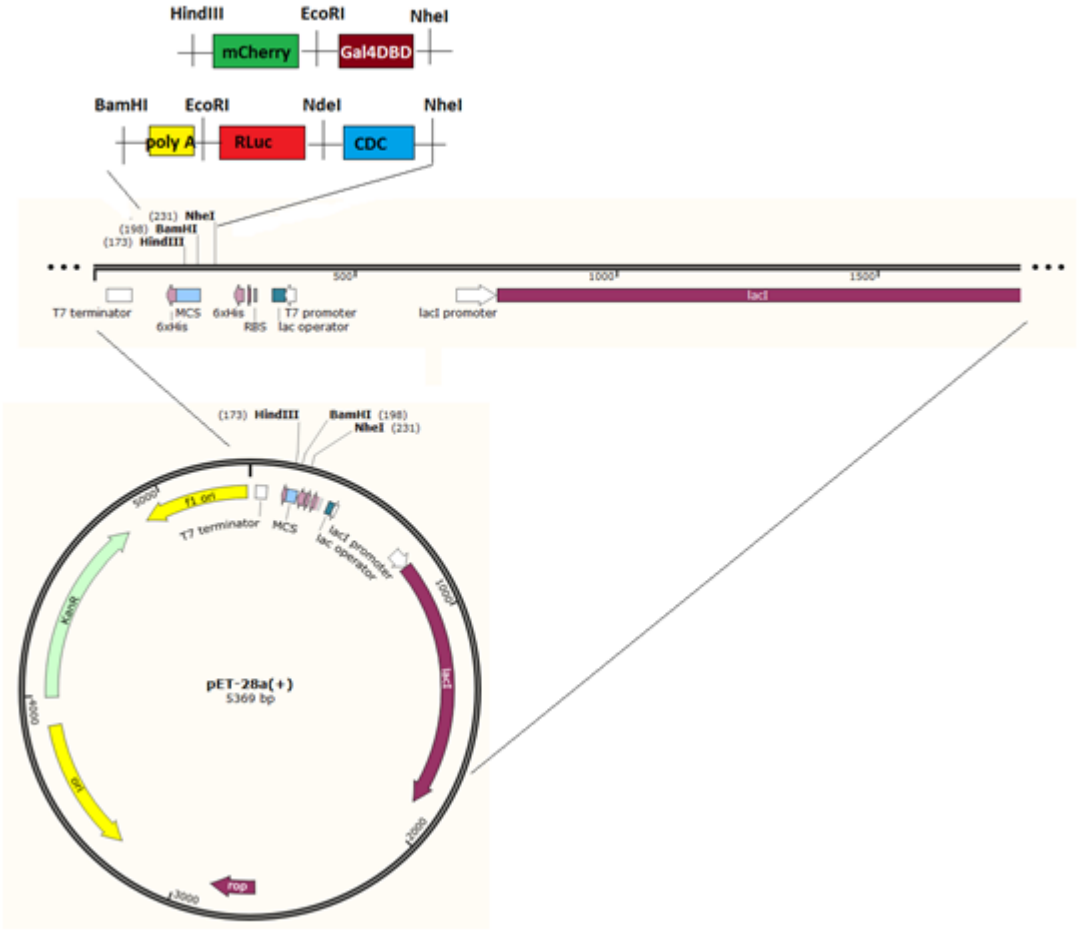

Figure 10. Illustration for finally constructed plasmids in the vector, pET28a (+).

3.2. Protein Over-Expression and Isolation Studies

pET28a(+) vector includes strong bacteriophage T7 transcription and translation signals induced by T7 RNA polymerase of the hosts which do not express T7 RNA polymerase in the uninduced state; thus, the target protein is not expressed in this state. In the uninduced state, the gene coding for T7 RNA polymerase that is regulated by IPTG-

inducible *lacUV5* promoter in the hosts which were specifically generated via bacteriophages for inducible systems and one of which is *Escherichia coli* BL21 DE3 is dominated by *lac* repressor coded by *lac I* gene. Upon induction by IPTG, the host machinery for the production of T7 RNA polymerase initiates. As seen in Figure 11, to minimize basal expression of target protein in the vectors, T7 *lac* operator is localized at the downstream of the T7 promoter to prevent transcription, which is called T7*lac* and also induced by IPTG (Novagen, 2011).

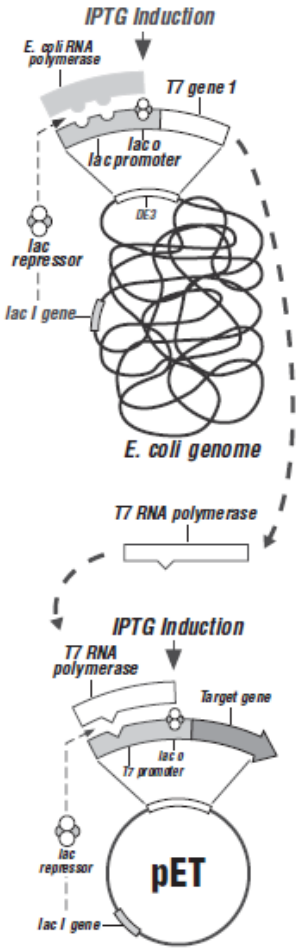

Figure 11. Mechanism of pET systems in the expression hosts. Lac repressors at both host and the vector minimize the expression of target protein coding sequence. Only induction with IPTG initiates the expression of the proteins (Novagen, 2011).

3.2.1. Over-Expression and Isolation of 6xHis-CDC-RLuc

The constructed plasmid for ~58kDa protein, 6xHis-CDC-RLuc which was called pET28a-6xHis-CDC-RLuc was transformed into the bacterial protein expression host, *Escherichia coli* BL21 DE3 Star. After transformation, the cells with small volume were

tried for over-expression studies initially at 37°C for 4h and room temperature overnight using 2X Laemmli Buffer, a SDS-including buffer, to isolate overall proteins of the cells. The expressions were confirmed for both of these conditions even at low levels (Figure 12).

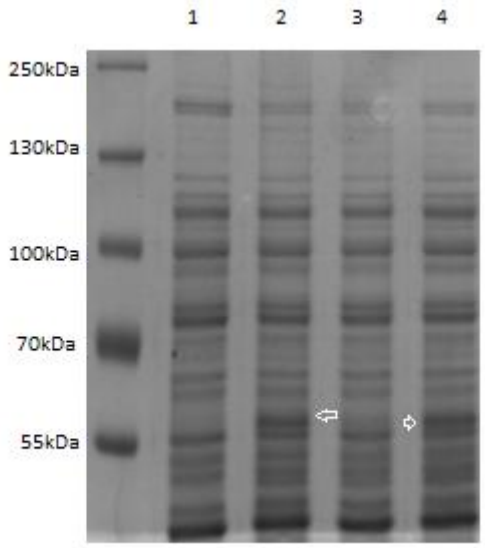

Figure 12. Result of first expression study trial for 6xHis-CDC-RLuc. 1. Uninduced control for 37°C for 4h; 2. Induction for 37°C for 4h; 3. Uninduced control for room temperature overnight; 4. Induction for room temperature overnight. The expression could be seen (marked as white arrows) for induced conditions.

CDC is the engineered form of human estrogen receptor α (hERα) including two zinc binding region with four cysteine residues surrounding each of these regions (Harrison, 1991; Huang et al., 2004). The expression of eukaryotic proteins in bacteria, especially DNA-binding proteins, without any tags or signal peptides that translocate the protein to medium that cells grow is an absolutely challenging phenomena (Sahdev, Khattar, & Saini, 2008). Moreover, expression of some proteins in bacteria may result in toxic effects to the cells, bringing about low expression levels (Novagen, 2011). In the light of

this information, the studies were carried out even the expression was low regarding the optimizations.

Next, the proteins were tried to be isolated under native conditions by using only sonicator as cell disrupter. However, 6xHis-CDC-RLuc could not be shown to be expressed under native conditions at room temperature (Figure 13), the condition that was proved to be used in expression studies (Figure 12).

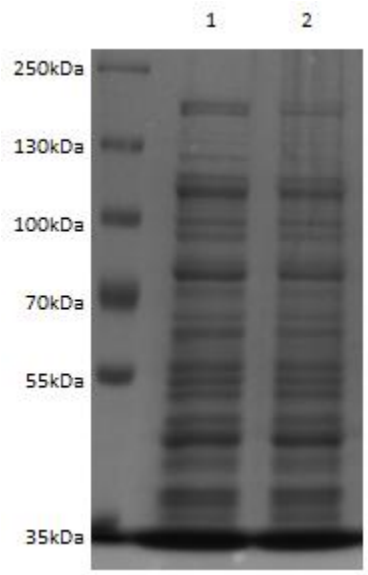

Figure 13. Trial for isolation of 6xHis-CDC-RLuc under native conditions. 1. Protein expression under native condition for uninduced control; 2. Protein expression under native condition for induced situation. There were not differences in terms of expression for induced condition comparing to uninduced control.

Eukaryotic proteins are generally insoluble in bacterial expression because of non-specific disulfide-bond formation, solving of which requires reducing agents (Sahdev et al., 2008). Thus, denatured conditions for isolations of these proteins are more proper. Here, 2X Laemmli Buffer is more convenient to isolate such proteins owing to including mainly SDS by disrupting all cellular components in a mixture; however, since the

isolation of proteins with SDS is difficult (Kurucz, Titus, Jost, & Segal, 1995), the studies later on were carried out by under denaturing conditions, using urea in Denaturing Solubilizing Buffer (DSB).

The expression level was tried to increase by changing the growth conditions of the bacterial populations. When the temperature decreases, folding and solubility of recombinant proteins in bacterial cells increases (Khow & Suntrarachun, 2012). Thus, the growth temperature was decreased from 37^{o}C up to 15^{o}C.

The expression was tried at 37^{o}C for 4h, 37^{o}C overnight, room temperature overnight and 15^{o}C overnight with DSB and 2X Laemmli Buffer as the control. Only the condition, 15^{o}C overnight gave slightly positive result with DSB (Figure 14).

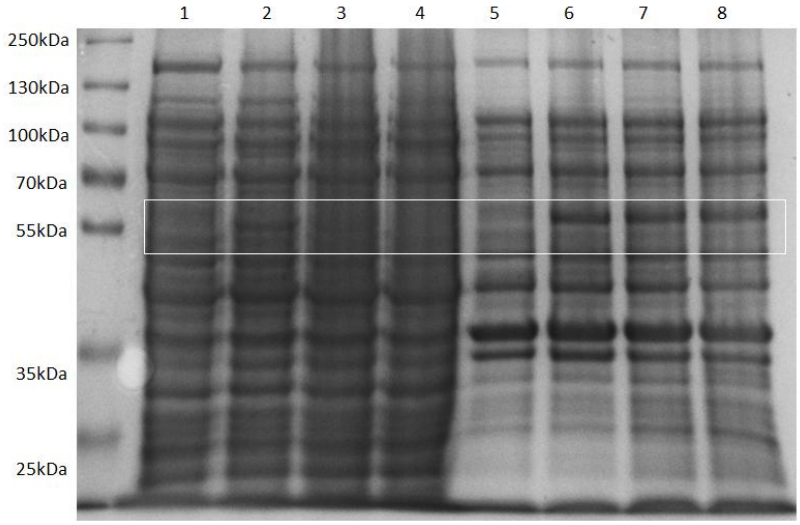

Figure 14. Isolation trial for 6xHis-CDC-RLuc under denaturing conditions. 1. Uninduced control isolated with DSB; 2. Isolated proteins with DSB after induction at 15^{o}C overnight; 3. Isolated proteins with DSB after induction at 28^{o}C overnight; 4. Isolated proteins with DSB after induction at 37^{o}C for 5h; 5. Uninduced control isolated with Laemmli; 6. Isolated proteins with Laemmli after induction at 15^{o}C overnight; 7. Isolated proteins with Laemmli after induction at 28^{o}C overnight; 8. Isolated proteins with Laemmli after induction at 37^{o}C for 5h. Only 15^{o}C

overnight induction conditions gave relatively positive result which could be used in further studies.

Even it was low level, the expression of 6xHis-CDC-RLuc was tried to confirm by Western Blot (WB). The specific band for the protein was demonstrated by WB but again with very low levels (Figure 15).

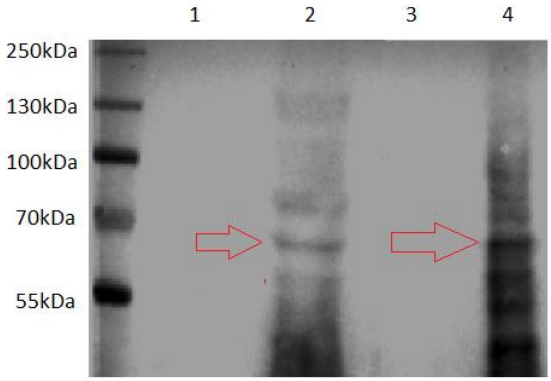

Figure 15. Western Blotting result for isolated 6xHis-CDC-RLuc. 1. Uninduced control isolated with DSB; 2. Isolate proteins with DSB; 3. Uninduced control isolated with Laemmli; 4. Isolate proteins with Laemmli. A low level expression could be seen in the lanes for isolated proteins after induction at 15^{o}C overnight (Lane 2 and 4).

The expression levels of the 6xHis-CDC-RLuc could not be increased. In fact, it was a strategy problem to solve which solubilizing tags should be used at the first step of the studies. For not going back, a web-based protocol to dissolve inclusion bodies to obtain insoluble proteins was applied. As a result of this protocol, a high level expression was shown for isolation of proteins from inclusion body comparing to DSB (Figure 16 and 17). Interestingly, putative soluble form of proteins which was obtained during the intermediary step of inclusion body protocol was seen with remarkable band intensity. This isolated putative protein mixture under native condition was dissolved in only salt

solution including metal chelating EDTA (Buffer A; 50mM tris-HCl, 5mM EDTA and 10mM NaCl, pH 8.0).

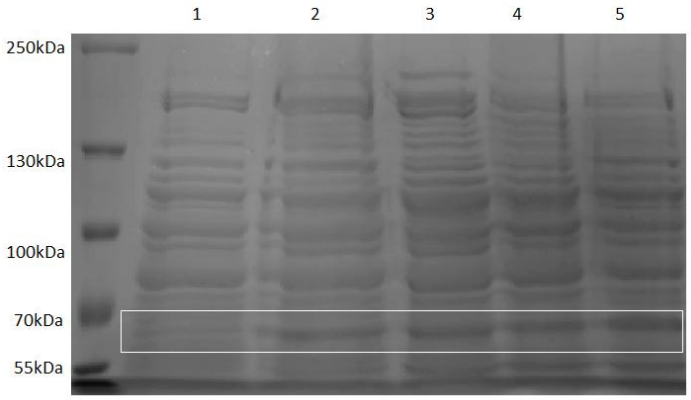

Figure 16. Isolation study of 6xHis-CDC-RLuc after inclusion body protocol. 1. Uninduced isolated with DSB; 2. Isolated proteins with DSB; 3. Isolated proteins after Buffer A resuspension, a step in inclusion body protocol; 4. Isolated proteins after Buffer B resuspension, a step in inclusion body protocol; 5. Isolated proteins after resuspension of inclusion body. All sets of protein isolation steps include the band for 6xHis-CDC-RLuc.

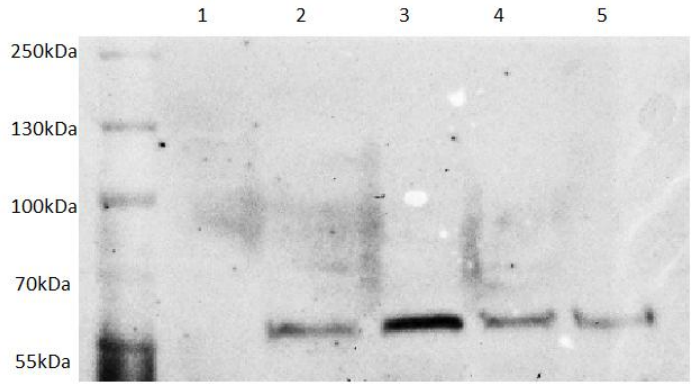

Figure 17. Western Blot result for 6xHis-CDC-RLuc after inclusion body protocol. 1. Uninduced control; 2. DSB-isolated proteins; 3. Isolated proteins after Buffer A resuspension; 4. Isolated proteins after Buffer B resuspension; 5. Isolated proteins after resuspension of inclusion body. All isolated proteins with each isolation method include 6xHis-CDC-RLuc which was confirmed by WB.

The native conditions for isolation of 6xHis-CDC-RLuc has been applied and could not be seen any isolation of related protein. However, the applied protocol was the only disruption of cells resuspended in PBS with sonicator. The drawback with this condition would be the lack of salts. Salts such as NaCl and Na_2SO_4 hydrate the proteins and change the free-energy level of proteins, resulting in increased solubility of proteins (Arakawa & Timasheff, 1982; Golovanov, Hautbergue, Wilson, & Lian, 2004). Hence, the isolation of proteins with only sonication which was considered as isolation of proteins under native conditions would fail because of lack of salts. The salt condition was promoted in the steps of inclusion body protocol and obtained 6xHis-CDC-RLuc under native conditions.

The isolation of 6xHis-CDC-RLuc under native conditions make the purification of the protein easy since it puts away the extra obligatory step, dialysis to remove excessive urea concentration, which is a promising advantage for the next studies.

3.2.2. Over-Expression and Isolation Studies of 6xHis-Gal4DBD-mCherry

The over-expression studies for the second part of BRET pair, mCherry which was fused to Gal4DBD and 6xHis at the N-terminus of the protein as purification tag was carried out after the studies for 6xHis-CDC-RLuc. So, the obstacles faced during the over-expression and isolation studies of 6xHis-CDC-RLuc were skipped and the relatively optimized conditions were directly applied for studies for 6xHis-Gal4DBD-mCherry as Gal4DBD is structurally similar to CDC.

The over-expression studies for 6xHis-Gal4DBD-mCherry with ~48kDa weight with the extension sequences from the vector, pET28a (+) were tried as induction at 15^oC overnight, which was the condition in which 6xHis-CDC-RLuc was over-expressed

maximally. Under this condition, 6xHis-Gal4DBD-mCherry was over-expressed, which was shown for both isolation with DSB and Laemmli Buffer (Figure 18).

That the bands in the gels were faint was the common problem for both SDS-PAGE and Western Blot analysis. The reason could be excess concentration of salt and/or urea. Figure 18 had also this problem; however, that there was expression would be commented.

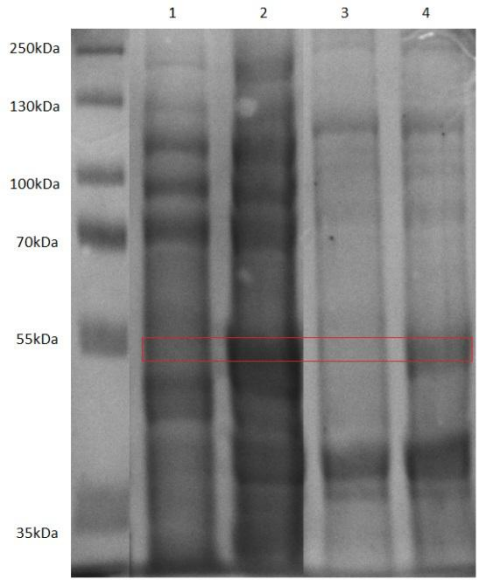

Figure 18. Over-expression and isolation study for 6xHis-Gal4DBD-mCherry. 1. Uninduced control isolated with DSB; 2. Isolated proteins with DSB after induction at 15^{o}C overnight; 3. Uninduced control isolated with Laemmli; 4. Isolated proteins with Laemmli after induction at 15^{o}C overnight. The white frame shows the bands related to weight of 6xHis-CDC-RLuc (~48kDa).

As done for 6xHis-CDC-RLuc, the expression of 6xHis-Gal4DBD-mCherry was tried to confirm by Western Blot analysis using anti-6xHis tag antibody. The over-expression was successfully shown for 6xHis-Gal4DBD-mCherry (Figure 19).

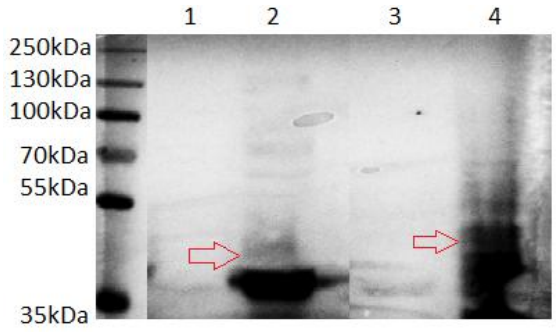

Figure 19. Western Blot for control of over-expression of 6xHis-Gal4DBD-mCherry. 1. Uninduced control isolated with DSB; 2. Proteins isolated with DSB after induction at 15^{o}C overnight. 3. Uninduced control isolated with Laemmli; 4. Isolate proteins with Laemmli. A low level expression could be seen in the lanes for isolated proteins after induction at 15^{o}C overnight (Lane 2 and 4).

The expression level for 6xHis-Gal4DBD-mCherry was better than those of 6xHis-CDC-RLuc under denaturing conditions (Figure 14). Nonetheless, since the over-expression of 6xHis-CDC-RLuc was also shown for putatively under native conditions using Buffer A from inclusion body protocol, 6xHis-Gal4DBD-mCherry was also applied for inclusion body protocol and the over-expressions were shown for both under native and denaturing conditions (Figure 20). The expressions were confirmed by Western Blot analysis (Figure 21). The upper bands in SDS-PAGE and WB image were probably the effect of excessive salt and/or urea concentration.

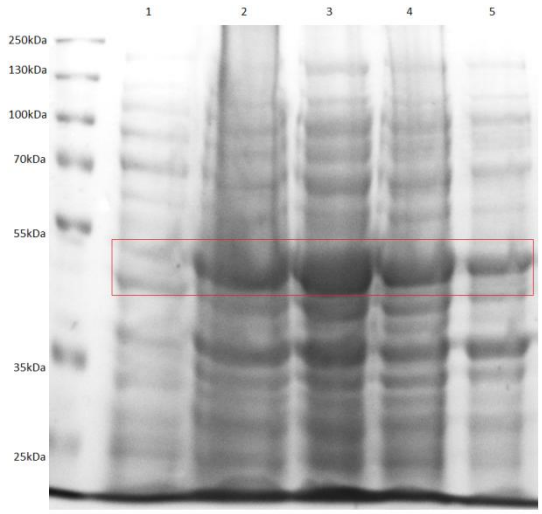

Figure 20. Isolation study of 6xHis-Gal4DBD-mCherry after inclusion body protocol. 1. Uninduced isolated with DSB; 2. Isolated proteins with DSB; 3. Isolated proteins after Buffer A resuspension, a step in inclusion body protocol; 4. Isolated proteins after Buffer B resuspension, a step in inclusion body protocol; 5. Isolated proteins after resuspension of inclusion body. All sets of protein isolation steps include the band for 6xHis-Gal4DBD-mCherry shown in red frame.

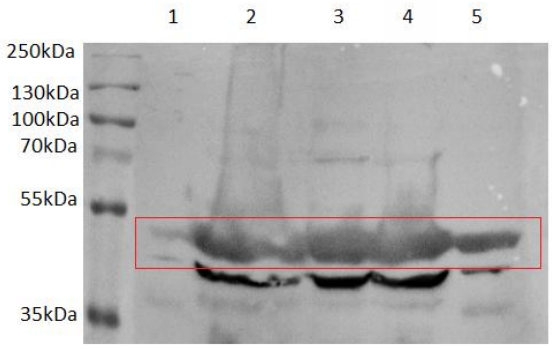

Figure 21. Western Blot result for 6xHis-Gal4DBD-mCherry after inclusion body protocol. 1. Uninduced control; 2. DSB-isolated proteins; 3. Isolated proteins after Buffer A resuspension; 4. Isolated proteins after Buffer B resuspension; 5. Isolated proteins after resuspension of inclusion body. All isolated proteins with each isolation method include 6xHis-Gal4DBD-mCherry shown is red frame. The bands just below the red frame were probably non-specific response to anti-6xHis tag antibody.

These isolation results were also promising for next steps of the overall study by removing the step, refolding of proteins by dialysis thanks to giving band for isolation with Buffer A.

3.3. Functionality Assays

The isolated 6xHis-CDC-Rluc and 6xHis-Gal4DBD-mCherry were in a buffer, Buffer A, including only salt and EDTA (50mM tris-HCl, 5mM EDTA and 10mM NaCl, pH 8.0). Thus, the proteins would be functional. The functionalities of proteins were assessed via *Renilla* luciferase for 6xHis-CDC-RLuc and mCherry for 6xHis-Gal4DBD-mCherry.

3.3.1. *Renilla* Luciferase Assay For 6xHis-CDC-RLuc

To see whether 6xHis-CDC-RLuc in Buffer A was functional, *Renilla* luciferase assay was performed. Isolated proteins were treated with the substrate of *Renilla* luciferase, called Stop& Glo Reagent consisting of Stop& Glo Buffer and Stop& Glo Substrate, from Dual Luciferase Assay Kit (Promega, USA). Buffer A- and Buffer B-isolated proteins were scanned for activity in the absence and presence of the substrate. All the results were at GraphPad Prism (Figure 22).

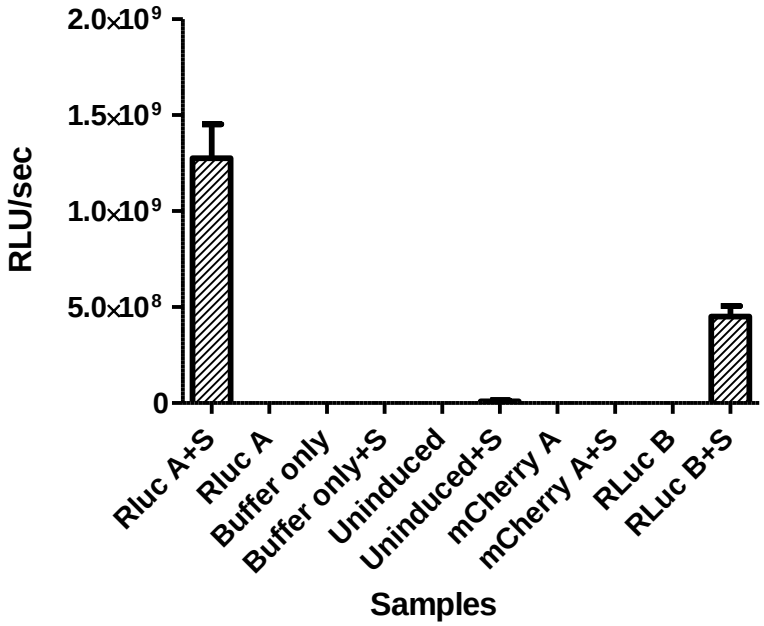

Figure 22. Renilla luciferase assay for 6xHis-CDC-RLuc. All the samples, except buffer only were isolated proteins. Only Buffer refers to Buffer A; Rluc to 6xHis-CDC-RLuc; mCherry to 6xHis-Gal4DBD-mCherry; S: Substrate-treated; A: Proteins isolated with Buffer A; B: Proteins isolated with Buffer B. All readings were performed for single time for triplicate samples. According to Dunnet's Multiple Comparison Test, Buffer A-isolated and substrate-treated 6xHis-CDC-RLuc was statistically significant over the all other samples ($p < 0.0001$).

The assay results were analyzed statistically using Dunnett's Multiple Comparison Test. In this test, result for 6xHis-CDC-RLuc isolated with Buffer A (named as Rluc A in the Figure 22) was compared to all other samples. According to result being able to seen in Figure 22, Stop& Glo Reagent (S)-treated 6xHis-CDC-RLuc isolated by Buffer A is significant to all samples ($P<0.0001$). The activity of *Renilla* luciferase requires a complete protein with proper confirmation (S. B. Kim, Ozawa, Watanabe, & Umezawa, 2004). So, the Buffer A- and Buffer B-isolated 6xHis-CDC-RLuc would be commented as intact, functional and with accurate confirmation. The sucrose in the Buffer B would

affect the result of assay. The activity for Buffer B-isolated proteins could be seen but it is not as much as isolation with Buffer A.

In the further studies of the project, 6xHis-CDC-RLuc will be mixed by 6xHis-Gal4DBD-mCherry in the absence and presence of DNA scaffold. To show that the luminescence signal will be detected only from isolated (or purified) 6xHis-CDC-RLuc, 6xHis-Gal4DBD-mCherry isolated by Buffer A was also assessed during *Renilla* luciferase assay (Figure 22; mCherry A). As seen in Figure 22, the signal detected from 6xHis-CDC-RLuc in the presence of substrate is significant over the 6xHis-Gal4DBD-mCherry treated with luciferase substrate. This is a fundamental result shown for the further studies.

3.3.2. Fluorescence Assay For 6xHis-Gal4DBD-mCherry

The functionality of 6xHis-Gal4DBD-mCherry would be determined via mCherry by fluorescent assay. mCherry is a red fluorescent protein with maximal excitation wavelength at 575nm and maximal emission wavelength at 610nm (Shaner et al., 2004). 50ul of the isolated proteins with Buffer A and Buffer B with control groups were excited at 575nm and the emission at 610nm was detected. All the results were graphed at GraphPad Prism (Figure 23).

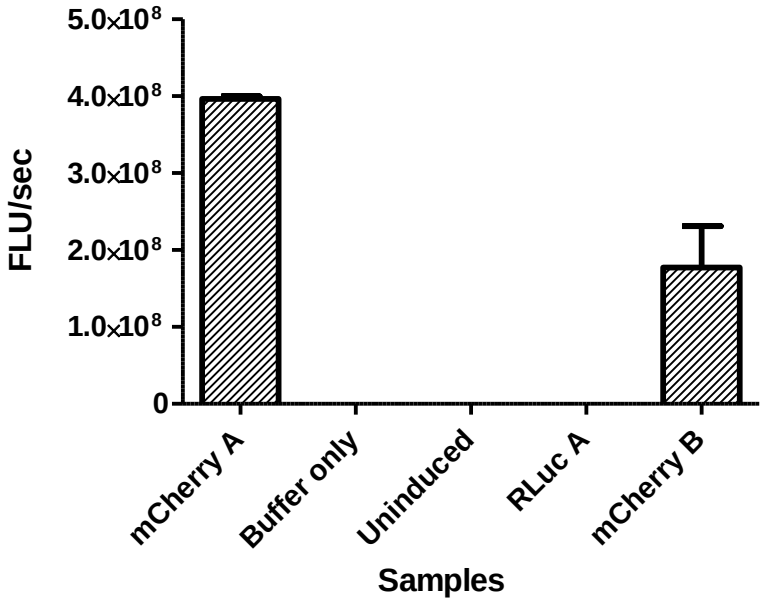

Figure 23. Fluorescence assay for 6xHis-Gal4DBD-mCherry. Buffer only refers to Buffer A; mCherry to 6xHis-Gal4DBD-mCherry; RLuc 6xHis-CDC-RLuc. A: Proteins isolated with Buffer A; B: Proteins isolated with Buffer B. All the readings were performed for only one time point for triplicate samples. According to Dunnett's Multiple Comparison Test, Buffer A-isolated mCherry was statistically significant over the all other samples ($p<0.0001$)

The assay results were analyzed statistically using Dunnett's Multiple Comparison Test. In this test, result for 6xHis-Gal4DBD-mCherry isolated with Buffer A was compared to all other samples. As seen in Figure 23, the fluorescence detected at 610nm by Buffer A-isolated 6xHis-Gal4DBD-mCherry is significant ($P<0.0001$) over the all other samples. It was expected that there was not mCherry protein in buffer only, Buffer A-isolated uninduced control and Buffer A-isolated 6xHis-CDC-RLuc. Buffer A-isolated 6xHis-Gal4DBD-mCherry is also significant over Buffer B-isolated 6xHis-Gal4DBD-mCherry. The reason would be the effect of sucrose which is in Buffer B. Sucrose changes refractive index (medium effect on light), resulting in decreased light scattering in the medium (Ardhammar, Lincoln, & Nordén, 2002).

To be able to be functional, mCherry has to be complete and at proper conformation (Fan et al., 2008). As the result of fluorescence assay, it would be stated that the Buffer A-isolated proteins are intact and functional.

In the further studies of the project, 6xHis-CDC-RLuc will be mixed by 6xHis-Gal4DBD-mCherry in the absence and presence of DNA scaffold. To show that the fluorescence signal will be detected only from isolated (or purified) 6xHis-Gal4DBD-mCherry, Buffer A-isolated 6xHis-CDC-RLuc was also assessed during fluorescence assay (Figure 23; RLuc A). As seen in Figure 23, the signal detected from Buffer A-isolated 6xHis-Gal4DBD-mCherry is significant over 6xHis-CDC-RLuc. This is also an important result shown for the further studies.

3.4. Purification of fusion proteins

The Buffer A-isolated 6xHis-CDC-RLuc and 6xHis-Gal4DBD-mCherry were tried to be purified by the means of 6xHis tag.

3.4.1. Purification of 6xHis-CDC-RLuc

Buffer A-isolated 6xHis-CDC-RLuc was loaded to Ni-NTA Spin Columns (Qiagen, USA) and tried to be purified. The flow-through from each step was loaded to SDS-PAGE with 8% separating gel according to volumes (Figure 24).

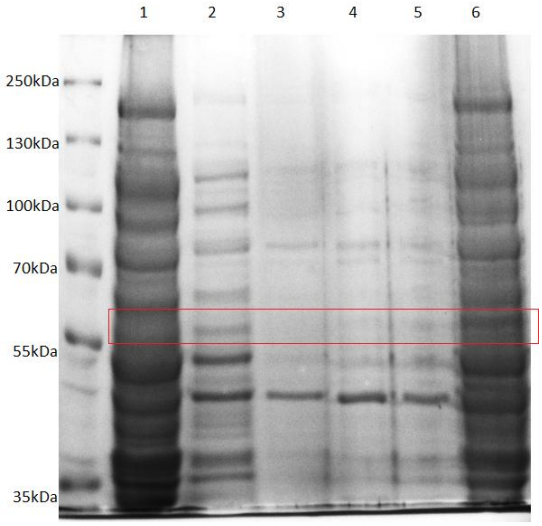

Figure 24. Purification study for 6xHis-CDC-RLuc. 1. After binding; 2. Wash 1; 3. Wash 2; 4. Elution 1; 5. Elution 2; 6. Buffer A-isolated 6xHis-CDC-RLuc as control.

As seen at Figure 24, there was not any problem with the binding of the proteins to the column. However, the proteins were lost in wash step. The imidazole amount for wash steps was nearly minimal (10mM) and the studies that the imidazole was not used for wash steps have given a seriously problematic gel image. Still, there are faint bands for related size, promising for further purification studies. The overall problem is related to low level expressions but since CDC is a DNA-binding protein with eight cysteines, the over-expression of protein is definitely difficult. Even so, proteins with low level of purification or only isolated proteins would be used for the further studies of the project.

3.4.2. Purification of 6xHis-Gal4DBD-mCherry

Like 6xHis-CDC-RLuc, 6xHis-Gal4DBD-mCherry was also tried to be purified by same kit and changing washing buffer from 10mM imidazole to 30mM imidazole. The flow-through from each step was loaded to SDS-PAGE with 8% separating gel according to volumes (Figure 25).

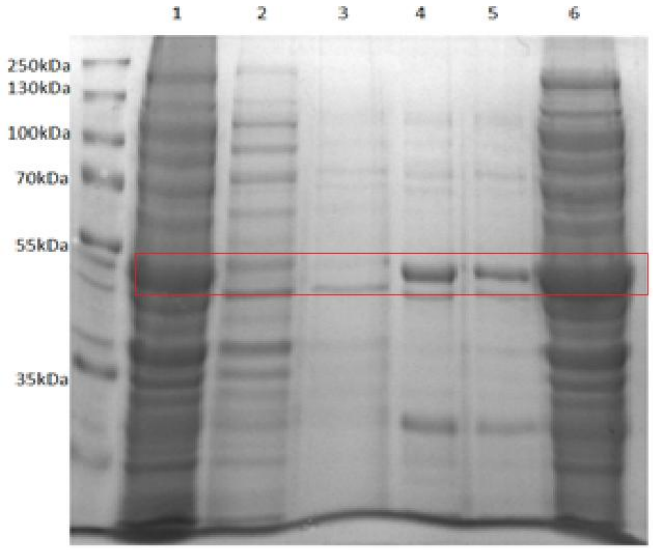

Figure 25. Purification study for 6xHis-Gal4DBD-mCherry. 1. After binding; 2. Wash 1; 3. Wash 2; 4. Elution 1; 5. Elution 2; 6. Buffer A-isolated 6xHis-Gal4DBD-mCherry as control.

Contrary to 6xHis-CDC-RLuc, 6xHis-Gal4DBD-mCherry was purified more successfully. This would be because the expression level of 6xHis-Gal4DBD-mCherry. Still, the purification would be optimized since there were protein losses during the

washing steps. However, the purified level of the 6xHis-Gal4DBD-mCherry would be used for the next steps of the project.

CHAPTER 4

CONCLUSION AND FUTURE PERSPECTIVE

In this study, the construction of the parts of a novel Bioluminescence Resonance Energy Transfer (BRET) system was aimed. This system includes transcription factor fused to protein of BRET pair, *Renilla* luciferase (RLuc) and mCherry red fluorescent protein. The usage of these proteins in a BRET study is novel. *Firefly* luciferase (FLuc) has been used as the BRET pair to mCherry protein (Iglesias & Costoya, 2009); however, the bioluminescence wavelength of FLuc (575nm maximal) overlaps with the excitation wavelength of mCherry (Figure 4). Thus, to overcome such a problem, RLuc was chosen as the BRET pair to mCherry.

The cloning studies to synthesize recombinant fusion proteins, protein over-expression and isolations, and functionality assays were managed successfully. Moreover, possibility of the proteins, RLuc and mCherry to be BRET pairs was proved. Purifications of the proteins were tried and shown to be possible.

As another novelty, the BRET system was regarded to establish on a DNA scaffold. DNA as scaffold has been a popular issue from 1990s owing to advantages of DNA as biopolymer (Niemeyer, 1997). This system combines DNA scaffold approach with

BRET principle, as proof-of-concept, which would be pre-step of development of novel biosensors or be used in bionanotechnology studies.

The system was designed *in vitro* by bacterial protein purifications. In the present study, the prior studies, cloning and protein isolations, were managed and the samples were prepared for further studies, DNA-binding studies by electro mobility shift assay (EMSA) and BRET signal detection.

Even the study was designed *in vitro*, the system would be adapted to *in situ* studies with some major modifications, such as adjustment of transfections and transfection efficiencies, optimization of uptake of RLuc substrate and control of timing of detection of BRET signal. From the perspective of *in situ* studies, this system could be used in context-dependent transcription studies (Hwang, Kim, Shin, & Lee, 2011), genome editing (Hsu, Lander, & Zhang, 2014), studies on parameters for DNA-protein interaction, evaluation of proteins from zinc-finger protein engineering studies (Porteus, 2006) and *in situ* sensing and imaging systems.

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APPENDICES

APPENDIX A. FIGURES FOR PRIMERS

Rluc_FP

5’ CGCAT CATATG ACTTCGAAAGTTTATGATCC 3’
NdeI

RLuc_REP

5’ CGCAT GGATCC TTTATTA GAATTC TTGTTCATTTTTGAGAACTCG 3’
BamHI polyA EcoRI

Figure A. 1. Primers used for amplification of *RLuc*.

Gal4DBD-mCherry_FP

5’ CGCAT GCTAGC ATGAAGCTACTGTCTTCTATC 3’

NheI

mCherry_FP

5’ CGCAT GAATTC GTGAGCAAGGGCGAGGAGGAT

EcoRI

mCherry_REP

5’ CGCAT AAGCCT CTTGTACAGCTCGTCCATGCC

HindIII

Gal4DBD_FP

CGCAT AGATCT GGAGGCAGT aagctactgtcttctatcgaac

BglII Linker

Gal4DBD_REP

CGCAT GAATTC cgatacagtcaactgtctttg

EcoRI

Figure A. 2. Primers designed for amplification of *Gal4DBD-mCherry*. Here, Gal4DBD_FP and Gal4DBD_REP were used only for colony PCRs.

APPENDIX B. MAPS OF VECTORS

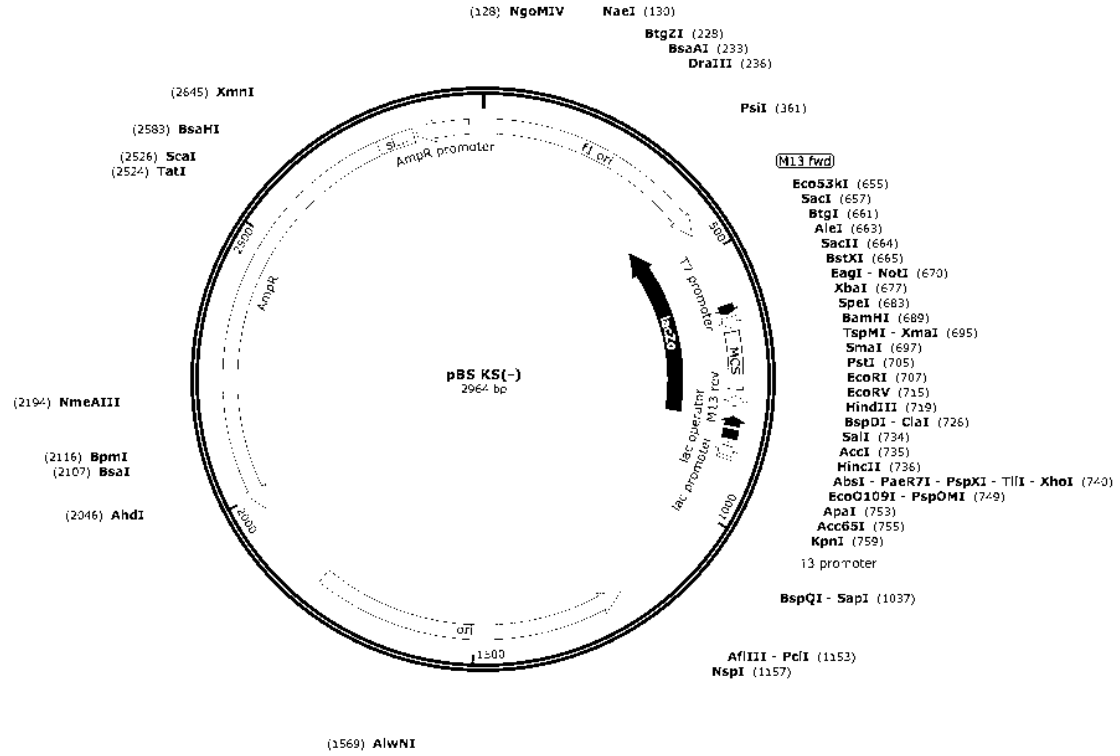

Figure B. 1. Map of the intermediary vector, pBS-KS (-).

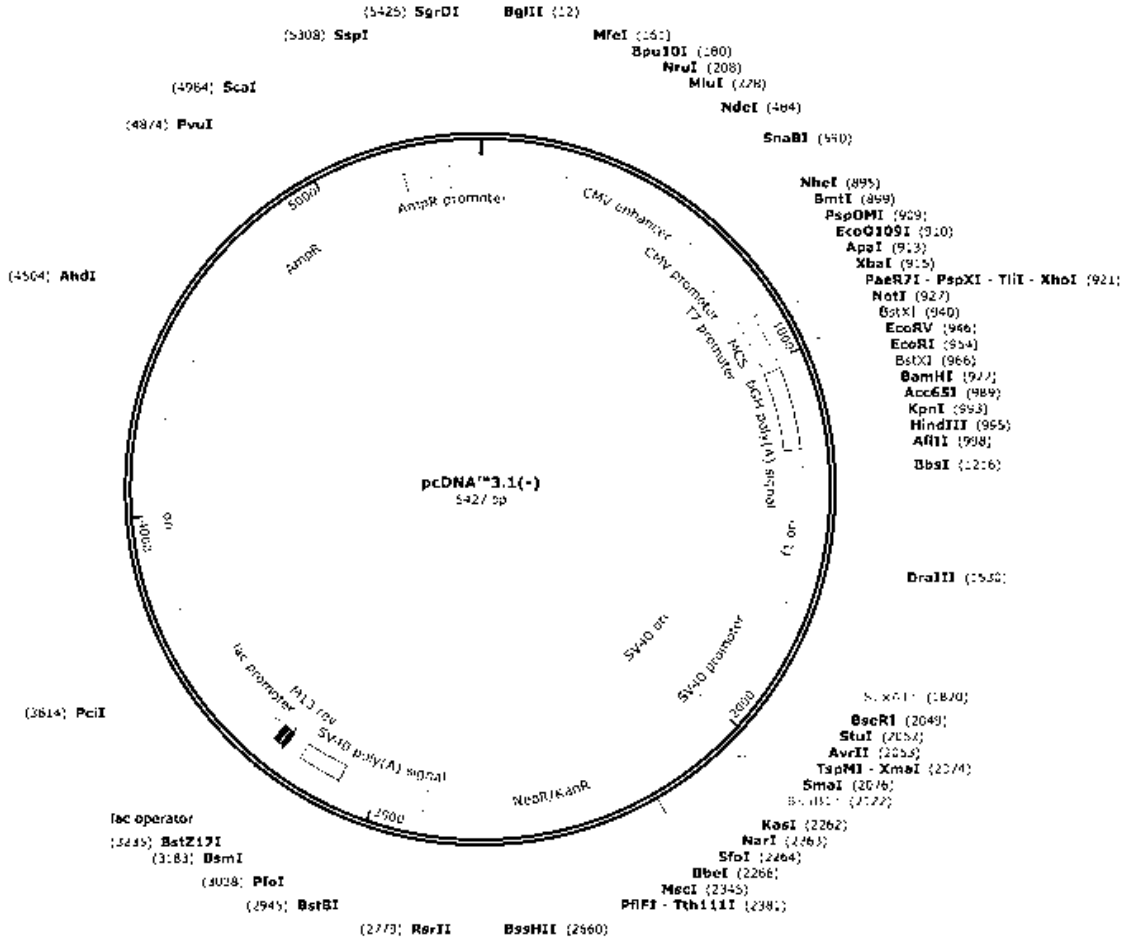

Figure B. 2. Map of pcDNA3.1(-).

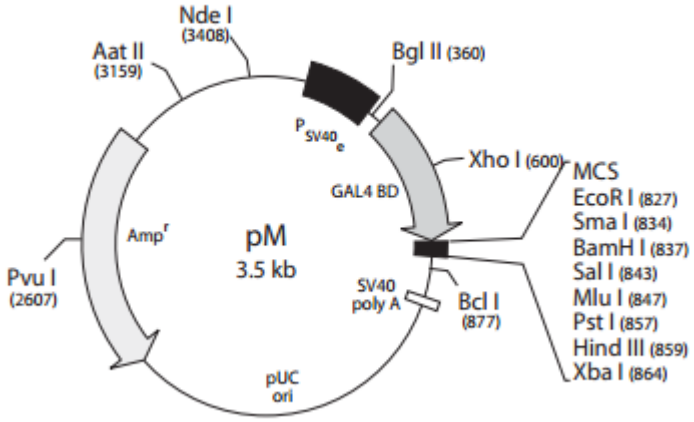

Figure B. 3. Map of pM-Gal4DBD (Clontech, Inc.).

APPENDIX C. STATISTICAL TEST FOR FUNCTIONALITY ASSAYS

	1way ANOVA					
1	Table Analyzed	Data 1				
2						
3	One-way analysis of variance					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	10				
8	F	50.03				
9	R square	0.9575				
10						
11	ANOVA Table	SS	df	MS		
12	Treatment (between columns)	4.592e+018	9	5.102e+017		
13	Residual (within columns)	2.040e+017	20	1.020e+016		
14	Total	4.796e+018	29			
15						
16	Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	RLuc A+S vs RLuc A	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
18	RLuc A+S vs Buffer only	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
19	RLuc A+S vs Buffer only+S	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
20	RLuc A+S vs Uninduced	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
21	RLuc A+S vs Uninduced+S	1.267e+009	15.37	Yes	***	1.024e+009 to 1.510e+009
22	RLuc A+S vs mCherry A	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
23	RLuc A+S vs mCherry A+S	1.276e+009	15.47	Yes	***	1.033e+009 to 1.519e+009
24	RLuc A+S vs RLuc B	1.277e+009	15.48	Yes	***	1.034e+009 to 1.520e+009
25	RLuc A+S vs RLuc B+S	8.267e+008	10.03	Yes	***	5.837e+008 to 1.070e+009

Figure C. 1. Statistical test for *Renilla* Luciferase Assay.

	1way ANOVA					
4	P value	< 0.0001				
5	P value summary	***				
6	Are means signif. different? (P < 0.05)	Yes				
7	Number of groups	5				
8	F	52.96				
9	R square	0.9549				
10						
11	ANOVA Table	SS	df	MS		
12	Treatment (between columns)	3.681e+017	4	9.202e+016		
13	Residual (within columns)	1.738e+016	10	1.738e+015		
14	Total	3.854e+017	14			
15						
16	Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	mCherry A vs Buffer only	3.964e+008	11.65	Yes	***	2.980e+008 to 4.948e+008
18	mCherry A vs Uninduced	3.962e+008	11.64	Yes	***	2.979e+008 to 4.946e+008
19	mCherry A vs RLuc A	3.962e+008	11.64	Yes	***	2.979e+008 to 4.946e+008
20	mCherry A vs mCherry B	2.189e+008	6.432	Yes	***	1.205e+008 to 3.173e+008

Figure C. 2. Statistical test for fluorescence assay.