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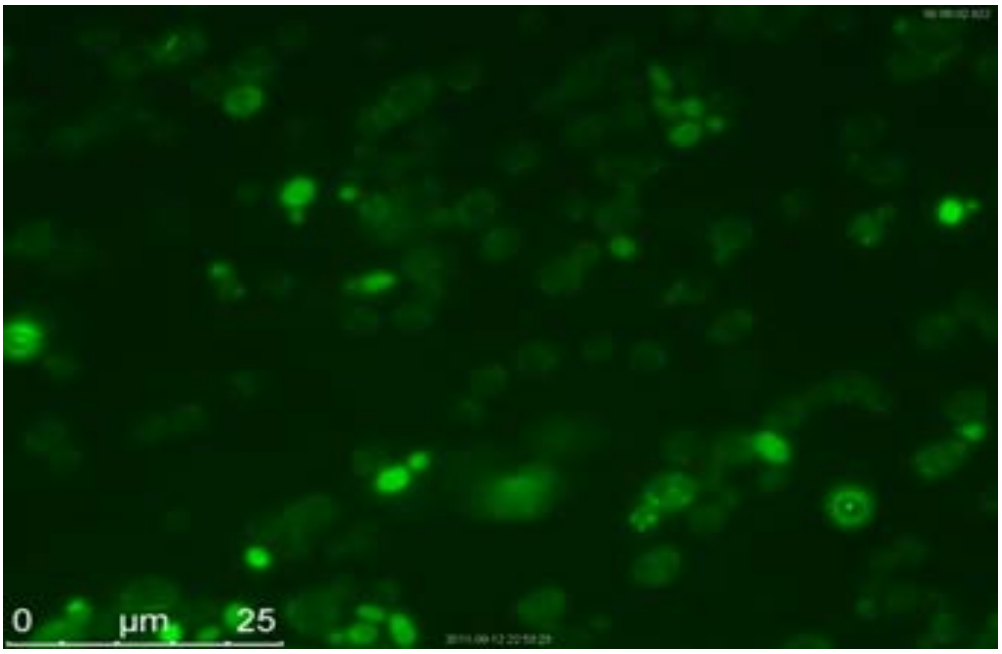

Enlarging the synthetic biology toolbox for *Saccharomyces cerevisiae*

A new synthetic reporter system for transcription dynamics analysis

Master of Science Thesis in the Master Programme of Chemistry and Bioscience

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Göteborg, Sweden, 2012

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SUMMARY
Green fluorescent protein (GFP) has been broadly used as an efficient reporter system, but not only its stability makes it unsuitable for monitoring transcription dynamics but also the oxygen-demanding chromophore formation restricts its application in aerobic systems. An ubiquitin fusion strategy and an N-degron including a destabilizing N-terminal residue and lysine containing Δk linker can tune the decay rate and provide a range of different stabilities, which is theoretically available for any protein. By N-terminally fusing ubiquitin and N-degron with the GFP-like TurboGFP and a novel flavin mononucleotide (FMN) binding fluorescent protein (FbFP), which works both in aerobic and anaerobic conditions, new destabilized reporter proteins were synthesized and evaluated at transcription level and functional translation level.
With methionine, glutamic acid and tyrosine corresponding to relatively strong, middle and weak stability at the conjugate of ubiquitin and Δk linker, TurboGFP and FbFP were integrated into the chromosome of *Saccharomyces cerevisiae* under control of the inducible *GAL1* promoter. The transcription level in each strain was quantified by RT-qPCR and as expected while the functional translation level, i.e. fluorescence intensity was very low. The destabilizing modifications were suspected to affect the fluorescence intensity. Therefore FbFP constructs containing different parts of the modification were developed and proved that the N-terminally fused ubiquitin and linker significantly affected the fluorescence although the mechanisms behind this require further study.

Keywords: reporter gene, green fluorescent protein, flavin mononucleotide (FN) binding fluorescent protein, N-end rule pathway, galactose regulated transcription.

Contents

1. Introduction

1.1 Background

Molecular biological techniques have been broadly utilized in metabolic engineering to modify multi-step biosynthetic pathways for the production of e.g. amino acids, fatty acids and natural products in organisms ranging from *Escherichia coli* to mammalian cells.

As one of the most intensely studied eukaryotic model organisms with comprehensive knowledge of its genetics, biochemistry, physiology, and large-scale fermentation performance, *Saccharomyces cerevisiae* has been extensively applied in metabolic engineering for heterologous substance production like valuable chemicals and proteins.

Among the various kinds of biosynthetic pathway modifications, it is an effective way to modify the key enzymatic steps by replacing the promoters of the corresponding genes. For instance, to increase the lysine yield, the promoters of *dapB*, *lysC*, *pyc* and the *tkt-operon*[a] were replaced by the promoter of *sod* and promoter of *fbp* was replaced by promoter of *eftu* in *Corynebacterium glutamicum*. These replacements on biosynthesis pathway lead to high and effective lysine yield in actinomycete (Becker et al., 2011).

Based on the demand of growing implementing of this strategy, a well characterized library containing promoters with various activity in diverse environments is necessary to be constructed and could facilitate the pathway optimization. In addition, there is also a need to set up an efficient and high-throughput reporter system to evaluate promoter efficiency and monitor gene regulation under various conditions.

The green fluorescent protein (GFP) has been used as a very useful and efficient reporter system for studying regulation of gene transcription. However, because of some drawbacks such as the relatively rigid stability of the GFP proteins, time-consuming protein folding and chromophore formation and the demand of oxygen, GFP is not an ideal candidate to monitor dynamic transcription levels of target genes (Chalfie 1994; Zimmer 2002; Niedenthal et al., 1996; Li et al., 2000). In order to overcome these disadvantages of the GFP reporter system and construct a novel reporter system for *S. cerevisiae* capable of monitoring transcription dynamics of a target gene, a reporter systems based on fluorescent protein with high turnover rate has been designed. TurboGFP and a novel flavin mononucleotide (FMN) binding fluorescent protein (FbFP) were chosen to be modified to achieve a high turnover rate via their capability of fast maturation and through fusion with an N-terminal destabilizing region.

1.2 TurboGFP

In recent years, the derivatives of green fluorescent protein, which was first characterized from *Aequorea victoria* (Prasher et al., 1992), have been extensively

applied in a wide range of organisms as a reporter for gene expression and as fluorescent tag for determination of subcellular localization (Niedenthal et al., 1996). The fluorescence spontaneously generated by GFP without adding any substrates and co-factors shows no disruption in host. These advantages allow scientists to monitor the gene expression *in vivo* with GFP.

However, the slow maturation rate and the high stability (> 420 min in yeast) of GFP restrict further application in monitoring of transient expression (Natarajan et al., 1998). To overcome this problem, a non-aggregating, rapidly maturing variant named TurboGFP (Evdokimov et al.,, 2006) has been developed by optimization of ppluGFP2 which was reported from Copepoda species (Shagin et al.,, 2004).

The TurboGFP emits green fluorescence at a maximum wavelength of 502 nm and is excited at a maximum wavelength of 482 nm. It is proved that TurboGFP is characterized with a fast maturation rate and high brightness (Evdokimov et al.,, 2006). The maturation half-time of TurboGFP is 1468 sec, relatively fast compared with 3915 sec of EGFP *in vitro*. The brightness of TurboGFP is three fold higher than EGFP at the early and two fold higher at the middle gastrula stages in developing *Xenopus laevis* embryos (Evrogen (http://www.evrogen.com/protein-descriptions/TurboGFP-description.pdf)). In addition, TurboGFP shows high stability at a wide range of pH (4-12).

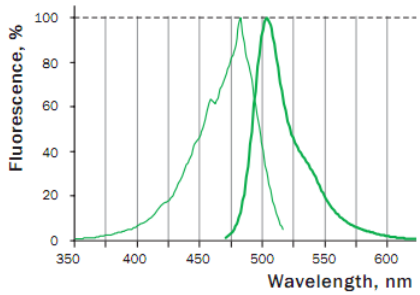


Figure 1 TurboGFP normalized excitation (thin line) and emission (thick line) spectra. The maximum excitation wavelength is 482 nm and the maximum emission wavelength is 502 nm (Evrogen).

Cyclization of the tripeptide serine, tyrosine and glycine (position 65-67) is the initial procedure of GFP chromophore formation with oxidation of the tyrosine residue (Cody et al., 1993; Heim et al., 1994), which requires aerobic conditions for monitoring its expression. The strategy to achieve a proper degradation rate is described in following paragraph.

1.3 Flavin mononucleotide binding fluorescent protein

A new fluorescent protein known as flavin mononucleotide (FMN) binding fluorescent protein (FbFP) was employed as a suitable reporter protein in this project, allowing both fluorescence in aerobic and anaerobic conditions (Drepper et al.,, 2007).

It was derived from the bacterial blue-light photoreceptor YtvA from *Bacillus subtilis* which contains two functional domains: the photoactive N-terminal light oxygen voltage (LOV) domain (amino acid residue 25-126) and the C-terminal sulfate transporter/anti-sigma factor antagonist domain (amino acid residue 147-258). The LOV domain belongs to the structurally conserved PAS (PerArntSim) superfamily. The EcFbFP with highest fluorescence intensity was optimized by replacing the conserved photoactive cysteine residue (Cys62 of YtvA) with an alanine, truncating the gene leaving only the LOV domain (the first 137 N-terminal amino acids) and adjusting the gene sequence to the *E. coli* codon usage bias. This fluorescent protein showed maximal excitation at 450 nm and maximal emission at 495 nm.

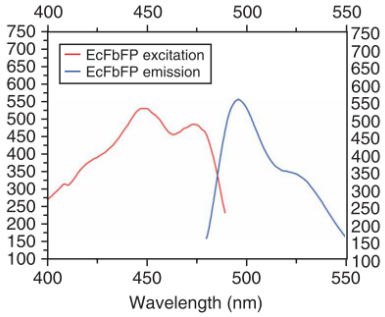


Figure 2 Fluorescence emission spectrum (in blue) of purified EcFbFP recorded at an excitation wavelength of 450 nm. Fluorescence excitation spectrum (shown in red) of the same samples were recorded at an emission wavelength of 495 nm. (Drepper et al.,, 2007)

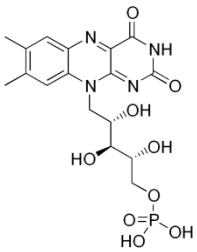


Figure 3. Flavin mononucletide (FMN) functions as prosthetic group of various oxidoreductases as well as cofactor in blue-light photoreceptors.

Unlike the fluorescence of GFP, which is generated by its internal chromophore in areobic condition, FbFP fluorescence occurs via non-covalentlly binding of the FMN chromophore (Christie et al., 1999) without the demand of oxygen (Drepper et al.,, 2007). It was proved that the fluorescence well relfected the change of cell density during the culture because of its short time lag between the fluorescence signal detection and reporter protein production at a certain time point (Drepper et al.,, 2010).

1.4 Destabilizing protein construct

To solve the problem of GFP being unsuitable for monitoring dynamic changes in gene expression caused by its high stability, different modifications have been performed on the fluorescent proteins. For instance, the PEST-rich 178 carboxyl-terminal residues of

G_1 cyclin Cln2 which are thought to target the protein for the ubiqutin-dependent degradation was fused to the C terminus of a GFP variant and led to a remarkly decreased half-life (~30 min) compared to the control (~7 h) (Mateus et al.,, 2000). Ubiquitin is a 76 aa residues regulatory protein that has been found in almost all biological systems and can be convalently conjugated to other proteins and label them for destruction (Hershko et al., 1998).

A more interesting destabilzation stragetgy was based on the N-end rule pathway of degradation which relates the *in vivo* half-life of a protein to the identy of its N-terminal residue. This N-end rule is, in eukaryotes, a part of the ubiquitin system (Varshavsky et al., 1997). When expressing a reporter protein such as *E. coli* *β*-galactosidase (*β*gal) N-terminally fused with ubiquitin in *S. cerevisiae* (Bachmair et al., 1986), the ubiquitin is cleaved off by deubiquitinating enzymes (D'Andrea et al., 1998). As a result of deubiquitination, different N-terminal residues X can be exposed and yield a range of different protein stabilities (Table 1). Stablilizing residues (Met, Gly, Val, Pro, Cys, Ala, Ser, Thr) refer to a long half-life (>20 h) and destabilizing residues (Glu, Gln, Asp, Asn, Ile, Leu, Phe, Trp, Tyr, His, Lys, Arg) refer to half-lives of 2–30 min. A multiubiquitin chain is linked with a lysine residue on the substrate (Chau et al., 1989), which would be processed by the 26S proteasome for degradation (Hoffman et al., 1996).

Table 1. *In vivo* half-life of X-*β*gal in *S. cerevisiae* at 30°C (Bachmair et al., 1989).

Residue X	**Half-life of X-*β*gal in *S. cerevisiae***
Arg	2 min
Lys	3 min
Phe	3 min
Leu	3 min
Trp	3 min
Tyr	10 min
His	3 min
Ile	30 min
Asp	3 min
Glu	30 min
Asn	3 min
Gln	10 min
Cys	> 30 h
Ala	> 30 h
Ser	> 30 h
Thr	> 30h
Gly	> 30h
Val	> 30h
Pro	> 5 h
Met	> 30 h

Methionine, glutamic acid and tyrosine applied in this project are marked in red.

Besides the N-terminal destabilizing residue, the lysine residue of the substrate serving as polyubiquitin binding site also belongs to the N-degron in eukaryotes. Studies with the N-rule indicated that gene engineering of 40 amino acid sequence termed 'ek' linker (extension bearing lysines) showed various efficiency of degradation. The linker is consist of a central KRK motif orginal from *E.coli lac* repressor and located at the N-terminal of *β*gal (Suzuki et al., 1999). The two lysines (K) in the ek linker, either K-15 or K-17 must present to result in an active N-degron. Using only the amino acids to threonine (T) 24 could generate a less efficient N-degron signal (Δk).

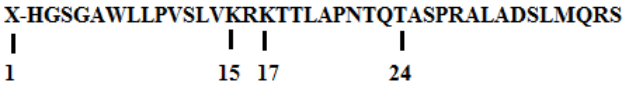


Figure 4. The N-degron sequence comprising the N-terminal destabilizing residue X and the ek linker as polyubiquitin binding site. (Hackett, Esch, Maleri, & Errede, 2006)

The destablization strategy based on ubiquitylation system and N-end rule were performed on cyan fluorescent protein to generate a series of derivatives with a range of stabilities which facilited the research of transient gene expression (Hackett et al., 2006).

These destablizing factors: ubiquitin, first residue at the N-terminal junction and variants of ek sequence are modular, replaceable and programmable which allows design and synthesis of a broad range of protein stability for different purposes and situations.

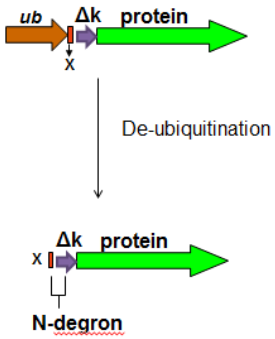


Figure.5 The designed destablized fluorescent protein consists of ubiquitin (brown), first residue at the junction (red), N-degron linker Δk (purple) and fluorescent protein sequence (green). The ubiquitin is cleaved by de-ubiquitination, poly-ubiquitin is bound to the lysine residue in Δk, forms a protein complex and directs it to proteosome.

In this project, methinoine (M), glutamic acid (E) and tyrosine (Y) corresponding to long, middle and short half-life are used as the first residue at the junction as well as the less efficient N-degron linker Δk for both TurboGFP and FbFP.

1.5 Protein construct on chromosome

To control the gene copy number and achieve a stable expression system, the fluorescent protein gene was chosen to be integrated into the yeast chromosome. One integration site named YPRCτ3, a Long Terminal Repeat (no.21) on Chromosome XVI which is situated within 400 bp from the closest antonomously replicating sequence (ARS) was proved to allow high transcritpion acitivity by integrating *lac*Z under the control of *TEF1* and *ACT1* promoters (Flagfeldt et al., 2009).

Generally this reporter system could be separate into two parts: exchangable promoter and integrated destablized fluorescent protein gene with *ADH1* terminator. Firstly the fluorescent protein cassette without promoter i.e. fluorescent protein gene and *ADH1* terminator are integrated into the chromosome of *S. cerevisiae*. Afterwards the *GAL1* promoter is integrated upstream of the fluorescent protein genes to control expression of the variants of TurboGFP and FbFP. After comparing the characteristics of TurboGFP and FbFP, the most suitable one would be selected as the reporter for transcription dynamics and utilized to characterize promoter libraries.

2 Materials and Methods

2.1 Strain construction

2.1.1 Synthesis of TurboGFP and FbFP constructs on plasmid in *E.coli*

The sequences of the fluorescent protein constructs consisting of a ubiquitin encoding sequence, a linker (∆k), the sequence encoding either TurboGFP or FbFP, a FLAG tag (only for FbFP), a stop codon, an *ADH1* terminator, and *Bam*HI restriction sites at both ends were synthesized and the protein encoding sequences codon optimized for expression in *S. cerevisiae* by GenScript (NJ, USA). These two constructs were ligated into the *Bam*HI site of cloning vector pUG57 by the supplying company.

2.1.2 Cloning pUG66, pUG6 and pUG57-TurboGFP/FbFP in *E. coli*

pUG57 carrying the fluorescent protein constructs, pUG66 harboring the *bleomycin* resistance gene and pUG6 with the *kanamycin* resistance gene were transformed in *E. coli* (DH5α). The chemocompetent *E. coli* cells stored at 80 °C were thaw on ice for 10 min. Plasmid DNA was mixed gently with the competent cells and incubated for 30 min on ice. Then the mixture was heat-shocked for 90 sec at 42 °C followed by incubation on ice for 3 min. 1 ml LB medium was added and the tube was shaken for 1 h at 37 °C afterwards. The cells were spun down to discard the supernatant. The pellet was resuspended in 100 µl water and the cell suspension spread on an LB-Amp plate. Water was used to replace DNA in *E. coli* transformation following the same procedure as negative control. The *E. coli* cells containing the plasmids were selected on ampicillin. Plasmids were extracted with the GeneJet Plasmid Miniprep Kit (Fermentas, Burlington, Canada) for following use. Restriction enzymes *Bam*HI for pUG57, *Hind*III for pUG6, and *Sal*I for pUG66 were used to verify the plasmids.

2.1.3 Replacement of P_{TEF1} in pUG66

The *TEF1* promoter from *S. cerevisiae* was amplified from pSP-GM1by PCR with TEF-f and TEF-r primers and purified using the illustra™ GFX™ PCR DNA and Gel band purification Kit (GE Healthcare, USA). The PCR fragment and pUG66 were restricted with *Xba*I and *Nco*I restriction enzymes (Fermentas). FastAP Thermosensitive Alkaline Phosphatase (Fermentas) was added to the plasmid restriction to prevent potential re-ligation of linearized pUG66. The restricted PCR fragment and linear pUG66 were ligated and amplified in *E. coli*. Linear pUG66 with *TEF1* promoter deletion was transformed in *E. coli* as negative control. pUG66-P_{TEF1} was verified by *Psi*I and sequenced with TEF-f/r primers (Eurofins, Ebersberg, Germany).

2.1.4 Site-direct mutagenesis of pUG57-TurboGFP/FbFP

As the synthesized constructs already contained *ATG* which codes for methionine at the N-terminus of the linker, the other constructs with code for glutamic acid or tyrosine at this position would be synthesized by site-direct mutagenesis. Turbo forward primer, Turbo-E reverse primer, Turbo-Y reverse primer, FbFP-E reverse

primer, FbFP-Y reverse primer (see in appendix) were applied to synthesize TurboGFP/FbFP with a codon of glutamic acid or tyrosine respectively, with the Phusion Site-Directed Mutagenesis Kit (Finnzymes, Vantaa, Finland). Because the designed gene sequences of TurboGFP and FbFP have same Δk linker the Turbo forward primer also can work as FbFP forward primer. The linear PCR products (pUG57-TurboGFP-E, pUG57-TurboGFP-Y, pUG57-FbFP-E pUG57-FbFP-Y) were ligated and transformed in *E. coli*, subsequently verified with *Bam*HI. pUG57-FbFP-M was transformed in *E. coli* as control. f2 and r2 primers were used for sequencing the modified constructs.

2.1.5 DNA fragment preparation for chromosomal integration

The bleomycin resistance gene containing the *TEF1* promoter from *S. cerevisiae* was amplified from pUG66-P_{TEF1} with phleo-f/r primers (see in appendix), the *kanamycin* resistance cassette was amplified from pUG6 with phleo-f/r primers and TurboGFP/FbFP constructs were cut out of pUG57 with *Bam*HI. All products were purified and confirmed by electrophoresis. The up-reverse primer of the marker gene contained a tail which represented the homologous region of the 5' end of the integration site YPRCτ3 while the down-forward primer contained a tail representing the homologous region of 5' end of the reporter gene construct. The 3' end of digested DNA fragment of TurboGFP/FbFP contained homologous region of the 3' end of integration site. After transformation into *S. cerevisiae*, the marker gene and reporter gene constructs were integrated into the chromosome by homologous recombination and verified by colony PCR with f1, r1, f2 and r2 primers.

2.1.6 Chromosomal integration via transformation into *S. cerevisiae*

Yeast transformation was performed according to the Small-Scale LiAc Yeast Transformation Procedure (Clontech Laboratories, Yeast Transformation Procedures, Yeast Protocols Handbook) .Yeast strain CEN.PK113-5D was firstly incubated in 5 ml YPD medium for overnight and this pre-culture used to inoculate 50 ml YPD medium to a starting OD_{600} of 0.2-0.3, which would reach an OD_{600} of 1 after about 4 h of cultivation. The culture was transferred to a 50 ml tube and centrifuged at 1000 g for 5 min at room temperature. The pellet was resuspended in 25 ml sterile 1X TE buffer (see in appendix), followed by another centrifugation at 1000 g for 5 min and removal of the supernatant. The cell pellet was resuspended in 500 µl freshly prepared, sterile 1X TE/1X LiAc. 50 µl of the cell suspension was mixed with a proper amount of DNA fragment (about 200 ng of the largest PCR fragment when destined for integration into the chromosome and a 1:1 molar ratio of the other fragments; 1000 ng of linearized plasmid vector when destined for gap repair cloning and 3 times higher amount of the insert), 50 µl carrier DNA (stored at -20 °C) and 600 µl freshly prepared PEG/LiAc solution by vortexing gently. The mixture was shaken at 30 °C for 30 min, mixed with 70 µl DMSO and incubated in a water bath at 42 °C for 30 min. After spinning down the cells and discarding the supernatant, 1 ml YPD medium was added to the tube and the culture incubated for 2 h to allow expression of the antibiotic resistance genes for selection on corresponding plates. After purification on YPD-phleo/G418 plates, the

positive colonies were verified by colony PCR. 25 µl 20 mM NaOH and acid-treated glass beads were placed in a 1.5 ml eppendorf tube. A small amount of a yeast colony was resuspended in the tube and heated at 100 ℃ for 15 min. Each tube was vortexed for 15 sec and spun down for 1 min with a tabletop centrifuge. 1 µl of supernatant was used as template for a PCR reaction with different combinations of primers f1, f2, r1 and r2 followed by gel electrophoresis.

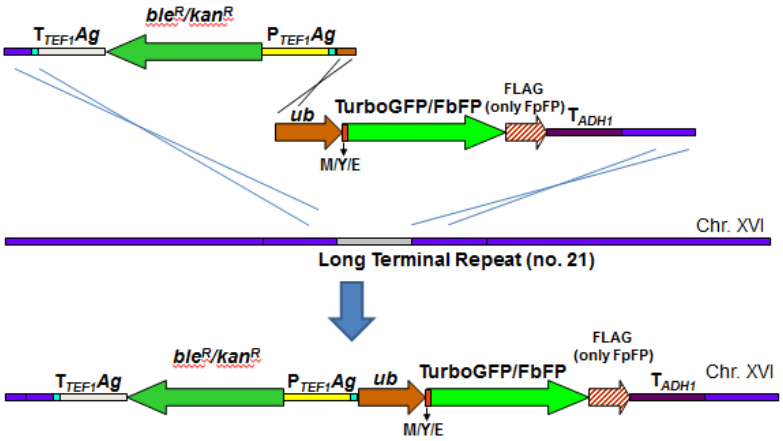


Figure 6. Chromosomal integration constructs of fluorescent protein. Bleomycin and kanamycin resistance marker genes were amplified by phleo-f/r primers from pUG6 and pUG66 separately. The PCR fragment of the marker genes contained the region upstream of LTR21, loxP site, *TEF1* terminator, *ble*/*kan* resistance gene, *TEF1* promoter, a second loxP site and a region homologous to the adjacent ubiquitin gene. After successful transformation, there would be marker gene and fluorescent protein cassette without promoter integrated in chromosome No. 16. of *S. cerevisiae*.

2.1.7 Southern blot analysis

To verify the copy number of chromosomal integration, Southern blot analysis was applied to all strains. The genomic DNA of each strain was extracted with the FastDNA Spin Kit for Soil (MP biomedicals, CA, USA) and digested with *Xba*I for 1 h. After separating the digestion products via gel electrophoresis at 50 V for 45 min, the gel was transferred onto an Amersham Hybond N^+ membrane (GE Healthcare, Little Chalfont, UK) with regents described in Amersham Hybond N^+ protocol. Blotting was performed according to TurboBlotter System Protocol (Whatman, Maidstone, UK). Probes for Southern blot hybridization were amplified with primers Turboprobe-f/r and FbFPprobe-f/r (see in appendix), respectively while labeling with thermostable alkaline phosphatase enzyme and hybridization with target DNA were performed according to the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare). After hybridization, decomposition of a stabilized dioxetane substrate contained in the CDP-Star^{TM} chemiluminescent detection reagents (GE Healthcare) was catalyzed by the probe bound alkaline phosphatase which generated chemiluminescent signals that were detected with help of the ChemiDoc XRS system (BIO-RAD, CA, USA) for 1 h.

2.1.8 Removal of the resistance marker gene

After constructing all chromosomal integration strains, the resistance marker gene was removed via Cre-lox recombination to obtain the loxP TurboGFP-M/E/Y and loxpFbFP-M/E/Y strains. Strains containing plasmid pSH47 (Güldener et al., 1996) containing the *URA3* gene can produce pyrimidine and express Cre recombinase upon induction by galactose. Therefore, after transforming pSH47 into CEN.PK113-5D (*ura3-52*), the resulting transformants can survive on plates not containing uracil. A colony purified from a URA-minus plate was cultured in 5 ml galactose medium (YPG) for around 2 h. Expression of the *cre* gene under the control of *GAL1* promoter was induced and Cre recombinase expressed. The kanamycin or bleomycin resistance cassettes flanked by the two *loxP* sites were subsequently looped out. Afterwards, the cell concentration in the culture was determined by counting chamber and microscope. The sample was diluted and about 300 yeast cells were spread on an YPD plate. The positive colonies would contain two different types of strains, one with marker gene and one without. 10 colonies from each were selected and cultured on two master plates, YPD with or without phleo/G418, to screen for yeast colonies, in which the *ble*/*kan* cassette had been looped out. By comparing the two master plates, the yeast colonies which grew on YPD but not on YPD+phleo/G418 were selected, resuspend in 200 µl water and spread on a 5-fluoroorotic Acid (5-FOA) plate where the 5-FOA would be converted to 5-fluorouracil by Ura3p encoded on pSH47 to kill cells containing the plasmid. A colony growing on the 5-FOA plate therefore represented a strain with neither marker gene nor plasmid pSH47.

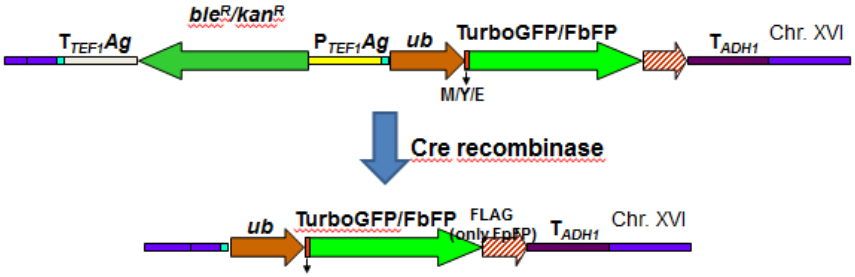


Figure 7. The marker genes were looped out by Cre recombinase expressed by pSH47. There was only the fluorescent protein cassette without promoter remaining in the yeast genome.

2.1.9 *GAL1* promoter integration

The *GAL1* promoter was amplified from pESC-URA with PGal1-f/r primers and subsequently fused with the kanamycin resistance cassette (amplified by phleo-r/kan-r from pUG66) with fusion f/r primers for promoter integration. The 3' end of the *kan* marker gene fragment and 5' end of the *GAL1* promoter fragment had a common overlapping region for fusion PCR. Approximately 1000 ng *kan-GAL1* fused DNA fragment was transformed into the loxpTurboGFP/FbFP strains according to the LiAc method. The strains were verified with f1, f2, r1, r2 primers and purified on YPD-G418 plates.

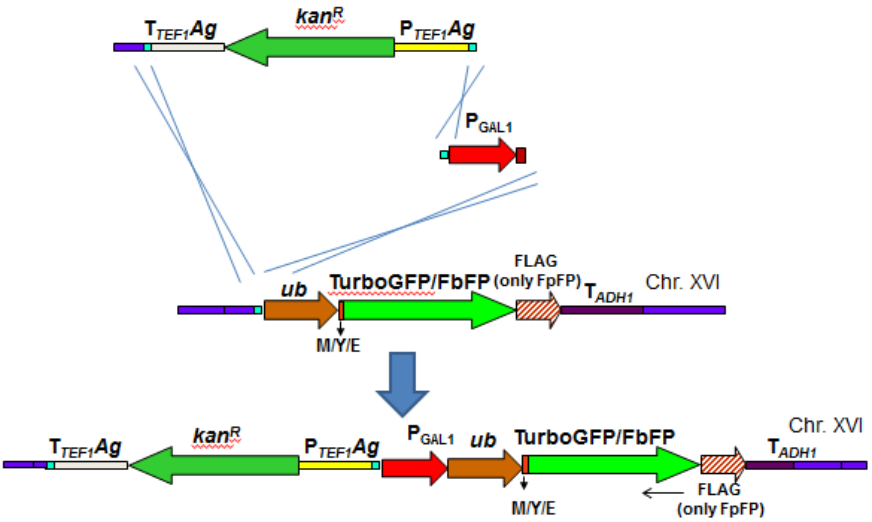


Figure 8. Homologous recombination of GAL1 promoter with the fluorescent protein constructs. The PCR product of the *GAL1* promoter contained ends homologous to the *kan*^{R} marker gene and the ubiquitin gene. *kan*^{R} marker gene and *GAL1* promoter fragment were simultaneously transformed and integrated into the strain containing the incomplete fluorescent protein cassette. In the final strain, the fluorescent protein expression was regulated by the *GAL1* promoter.

2.1.10 FbFP trouble-shooting construct pGFM

Primers of four FbFP trouble-shooting constructs on plasmid were designed to amplify overlapping DNA fragments (Table 2.a). Each of the fragments contained ends overlapping with the adjacent fragment according to the different combinations (Table 2.b). In addition, fragment PGAL1 and the end of ubi-linker-FbFP-FLAG-TADH1, FbFP1-FLAG-TADH1 and TADH1 contained homologous regions with the linearized plasmid pRS416 which was digested with *Bam*HI. Alkaline Phosphatase was applied in pRS416 restriction to prevent potential plasmid re-cyclization. Approximate 1000 ng linear pRS416 and 3 times the molar amount of DNA fragments were transformed and homologous recombined in CEN.PK113-5D yeast competent cell. Empty linear pRS416 was transformed independently as negative control. The plasmids were verified with *GAL1*-fw and ADH-rv primers by colony PCR and isolated on SD-URA plates.

Table 2.a. The DNA fragments for FbFP trouble shooting strain construction were prepared by PCR using corresponding primers and genomic DNA of kGFM as template.

Name of fragment	Template	Primers	Length (bp)
PGAL1	kGFM	*GAL1*-fw/rv	674
ubi-linker-FbFP-FLAG-TADH1	kGFM	ubi-fw/ADH-rv	**952**
ubi	kGFM	ubi-fw/rv	252
FbFP1-FLAG-TADH1	kGFM	FP-fw1/ADH-rv	652
FbFP1	kGFM	FP-fw1/fv	438
TADH1	kGFM	ADH-fw/rv	214
FbFP2	kGFM	FP-fw2/rv	438

Table 2.b. Corresponding fragments were transformed and homologous recombined with linearized pSP416 in CEN.PK113-5D

Strain	Vector	Fragments
pGFM1	pSP416	PGAL1 + ubi-linker-FbFP-FLAG-TADH1
pGFM2	pSP416	PGAL1 + ubi + FbFP1-FLAG-TADH1
pGFM3	pSP416	PGAL1 + ubi + FbFP1 + TADH1
pGFM4	pSP416	PGAL1 + FbFP2 + TADH1

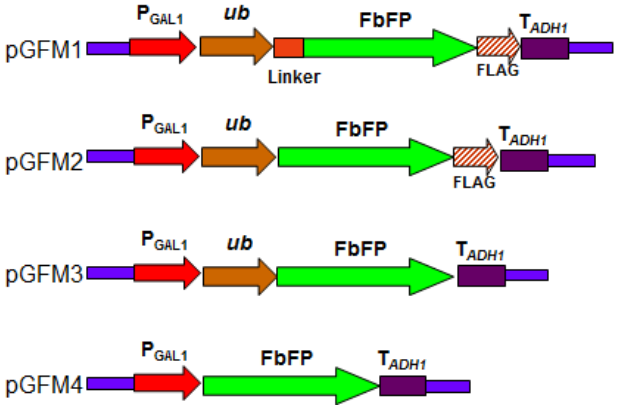


Figure 9. Sketch of FbFP trouble shooting strains pGFM1, 2, 3, 4 corresponding to disparate combination of PCR fragments. pGFM1 contains all the components as the original construct on yeast chromosome while the fluorescent protein in pGFM2 is without linker, in pGFM3 without linker and FLAG-tag and in pGFM4 without ubiquitin, linker and FLAG-tag.

2.1.11 Strain cultivation and sampling

Strains kGTM, kGTE, kGTY, kGFM, kGFE, kGFY with chromosomal integrations were mitotically stable and therefore selection pressure was not essential for strain cultivation. SD-2% galactose+0.2% glucose medium was used to induce fluorescent protein expression while cultivation in SD-2% glucose served as negative control. On the other hand, SD minus URA -2% galactose+0.2% glucose medium was used to cultivate strains pGFM1, 2, 3, 4 while cultivation in SD minus URA-2% glucose served as negative control. Each strain was picked from an isolated colony on a plate and incubated in 5 ml corresponding medium for a certain time (over 1 or 2 nights) to achieve an OD_{600} of about 2 or 3. The seed culture was subsequently used to inoculate in triplicate 10 ml main culture to an OD_{600} of 0.1 using the same medium. Triplicates of 1 ml sample of each culture were taken at around OD_{600}=1 and spun down to discard the medium and then frozen in liquid nitrogen for mRNA extraction and western blot analysis. 1 ml sample of each culture was taken and spun down to discard the medium, then resuspended in 1 ml 0.7% NaCl solution. 100 µl of each NaCl solution was used to measure the fluorescence intensity at the corresponding wavelength.

2.2 Transcription level quantification

To quantify the transcription level of each engineered gene, mRNA of each strain was extracted and reverse transcribed into cDNA used as template for RT-qPCR. Primers

were designed for amplification of target genes *TurboGFP/FbFP* and three reference genes: *ALG9*, *TAF10* and *TFC1*.

2.2.1 RT-qPCR primer analysis

Reference genes *ALG9*, *TAF10*, *TFC1* were selected to normalize the RT-qPCR data. Primers for *ALG9*, *TAF10*, *TFC1*, TurboGFP, FbFP were designed with Primer 3 (http://frodo.wi.mit.edu/, for primer design conditions and primer sequences see in appendix). To analyze the suitability of the primers, genomic DNA of kGFM was extracted as template and diluted to 10 ng, 1 ng, 100 pg, 10 pg, 1 pg per µl. Genomic DNA of CEN.PK113-5D was extracted and diluted to 10 ng/µl as no-template-control (NTC) for FbFP primers and templates for reference genes. The RT-qPCR reactions were performed in triplicates using the Brilliant II qPCR Kit (Agilent, Santa Clara, CA, USA) and the Mx3005P qPCR system (Agilent). Standard curves, PCR efficiency, application curve, C_q and melting curves were analyzed based on the experimental data.

2.2.2 mRNA extraction

The yeast cells were lysed by agitation in a Lysing Matrix tube (MP biomedicals) containing acid-washed glass beads at 4.0 meter/sec for 20 sec in a FastPrep Homogenization System (MP biomedicals) followed by cooling on ice for 5 min and repeated agitation. After lysis, mRNA was extracted with help of the QIAGEN RNeasy Mini Kit (Qiagen, Hilden, Germany), eluted with 30 µl RNase-free water, and stored at -20 °C. The RNA concentration and integrity were measured using an RNA 6000 nano chip (Agilent) and the Agilent Bioanalyzer (Agilent).

2.2.3 Reverse transcription and qPCR

1µl mRNA was reverse transcribed into cDNA in a 20 µl reaction using the Maxima First Strand cDNA synthesis Kit for RT-qPCR (Fermentas) and stored at 20 °C for up to a week. 1 µl cDNA was used in a 20 µl RT-qPCR reaction using the Brilliant II qPCR kit and an Mx3005P qPCR system. Application curve, C_q and melting curve were calculated based on experimental data.

2.2.4 RT-qPCR data analysis

As the expression of the target genes encoding TurboGFP and FbFP was induced by galactose, the transcription level of each gene was calculated according to a relative quantification method to relate the transcription level in galactose condition to that in glucose condition.

$$\text{Relative transcription level} = (1 + \text{PCR efficiency})^{-\Delta\Delta C_q}$$

Formula 1. ΔC_q = C_q of target gene - C_q of reference gene, $\Delta\Delta C_q$ =ΔC_q in galactose - ΔC_q in glucose. In this project, the PCR efficiency was assumed to be 100% for all genes. The target genes are TurboGFP and FbFP and the reference genes indicate *ALG9*, *TAF10* and *TFC1*.

2.3 Translation level quantification

2.3.1 Western blot

1 ml sample of yeast culture at an OD_{600} of 1 was spun down and frozen with liquid nitrogen. The total protein was extracted with help of the Y-PER Yeast protein extraction reagent (Thermo Scientific, Waltham, USA). Concentration of total protein was measured with help of the Pierce BCA protein assay kit (Thermo Scientific). After separating the proteins on a Novex pre-cast Tris-glycine gel (Invitrogen, USA), the proteins were transferred onto a nitrocellulose membrane (VWR, Radnor, PA, USA) using a TE70X Semi-dry blotter (Hoefer, Holliston, USA). The ladder used for SDS-PAGE was the Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). After washing the membrane with blocking reagent (Sigma-Aldrich, St. Louis, MO, USA), anti-tGFP monoclonal antibody (amsbio, CA, US) was used as first antibody to bind with TurboGFP, while anti-DDK monoclonal antibody (amsbio) was used for FbFP. Goat Anti-Mouse IgG F(ab')2 (amsbio) secondary antibody which has been conjugated to alkaline phosphatase (AP) was subsequently applied for both GFP and FbFP. The powerful substrate and enhancer of Immun-Star chemiluminescent protein detection systems (BIO-RAD) was activated by the alkaline phosphatase enzyme conjugate of the secondary antibody after incubating with the membrane and generated chemiluminescent signals.

2.3.2 Fluorescence intensity measurement

After the cultures had grown to an OD_{600} of around 1, a 1 ml sample was spun down and resuspended in 1 ml 0.7 % NaCl solution. 100 µl of the cell suspension was transferred to black FluoroNunc 96 well plates (VWR) to measure the fluorescence intensity by using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). The excitation filter was 485 nm for GFP and the excitation filter was 400-480 nm for FbFP. The emission filter was 520 nm for both fluorescent proteins.

Following concentrating 100 µl samples to 5 µl, 2 µl of each sample were examined using a Leica DMI 4000B fluorescence microscope in conjunction to a Leica EL 6000 compact light source, Leica CTR 4000 electronic box and Leica Application Suite software (Leica Microsystems, Wetzlar, Germany). Pictures of DIC (differential interference contrast) and fluorescence images using the filter for green fluorescent protein were taken at 437 nm excitation and 520 nm emission.

3. Results

3.1 Strain construction

The gene sequences of TurboGFP and FbFP were synthesized by GenScript (NJ, USA) and cloned into plasmid pUG57, separately. To generate fluorescent proteins with high turnover rate, TurboGFP and FbFP were N-terminally fused with ubiquitin, methionine at the linker which affect the protein stability according to the N-end rule. Transcription of both TurboGFP and FbFP was regulated by the downstream *ADH1* terminator. The gene sequences were codon optimized for expression in *S. cerevisiae*.

To modulate the stability of the fluorescent proteins, site-directed mutagenesis was applied to generate pUG57-TurboGFP/FbFP constructs with glutamic acid or tyrosine instead of methionine at the N-terminus with Turbo forward primer, Turbo-E reverse primer, Turbo-Y reverse primer, FbFP-E reverse primer, and FbFP-Y reverse primer. *Bleomycin* and *kanamycin* marker genes were separately amplified from pUG6 and pUG66 with phleo-f/r primers.

The TurboGFP/FbFP constructs were cut by *Bam*HI and simultaneously transformed with the *ble*/*kan* marker cassette into *S. cerevisiae* strain CEN.PK113-5D. Strains containing marker gene and the fluorescent protein construct were named as *ble*-TurboGFP-M for instance. The correct integration of the fragments was verified by colony PCR with primers f1, r1, f2, r2 (Table 3, Figure 8).

Table 3. Expected colony PCR result of *ble*-TurboGFP-M, *ble*-TurboGFP-Y, *ble*-FbFP-M, *ble*-FbFP-E, *kan*-TurboGFP-E, *kan*-TurboGFP-Y, *kan*-FbFP-E, *kan*-FbFP-Y with primers f1/r1 and f2/r2.

Primers	Template	Expected length (bp)
f1/r1	*ble*-TurboGFP-M,	1476
	ble-TurboGFP-Y	
	ble-FbFP-M	
	ble-FbFP-E	
	kan-TurboGFP-E	1871
	kan-TurboGFP-Y	
	kan-FbFP-E	
	kan-FbFP-Y	
f2/r2	*ble*-TurboGFP-M,	1078
	ble-TurboGFP-Y	
	ble-FbFP-M	1339
	ble-FbFP-E	
	kan-TurboGFP-E	1078
	kan-TurboGFP-Y	
	kan-FbFP-E	1339
	kan-FbFP-Y	

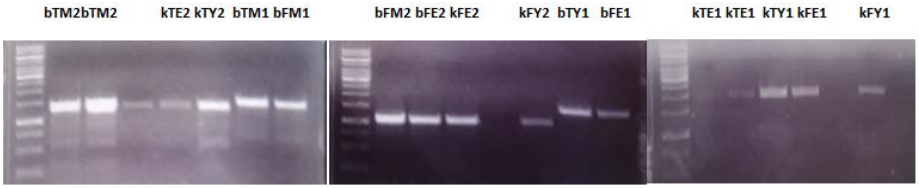


Figure 10. Electrophoresis result of verification of *ble*-TurboGFP-M, *ble*-TurboGFP-Y, *ble*-FbFP-M, *ble*-FbFP-E, *kan*-TurboGFP-E, *kan*-TurboGFP-Y, *kan*-FbFP-E, *kan*-FbFP-Y by colony PCR. The DNA ladder used was the GeneRuler 1 kb DNA Ladder (Fermentas). *ble*-TurboGFP-M is abbreviated as bTM, the other strains accordingly. Digit 1 indicates PCR with primers f1/r1 while digit 2 indicates primers f2/r2.

By comparing the gel photo and the predicted colony PCR results, it can be concluded that strains *ble*-TurboGFP-M, *ble*-TurboGFP-Y, *ble*-FbFP-M, *ble*-FbFP-E, *kan*-TurboGFP-E, *kan*-TurboGFP-Y, *kan*-FbFP-E, *kan*-FbFP-Y showed a PCR fragment of the expected size and therefore were successfully constructed. *ble*-TurboGFP-M, *ble*-FbFP-M, *kan*-TurboGFP-E, *kan*-TurboGFP-Y, *kan*-FbFP-E, *kan*-FbFP-Y were selected for subsequent experiments.

After chromosomal integration, it was essential to ensure that only one copy of the fluorescent protein construct had integrated into the genomic DNA so that unexpected influence on protein expression could be prevented. The genomic DNA of all the six strains was restricted by *Xba*I, separated on an agarose gel and transferred to a nylon membrane. Among the digested genomic DNA fragments, the one containing the TurboGFP construct was predicted to be 2743 bp while the FbFP construct was supposed to lie on a fragment of 2482 bp. A probe of 491 bp designed for TurboGFP and a probe of 481 bp designed for FbFP were labeled with thermostable alkaline phosphatase enzyme and hybridized to the target fragments containing TurboGFP and FbFP. A stabilized dioxetane substrate added was decomposed by alkaline phosphatase and generated a chemiluminescence signal. According to the chemiluminescence photo, the bands were located between 2.5 kb and 3 kb, which that corresponds to the predicted results (Figure 9). There was only one band for each strain, which proved that each strain only contained one copy of the integrated gene.

Figure 11. Chemiluminescence signals of Southern blot of *ble*-TurboGFP-M, *kan*-TurboGFP-E, *kan*-TurboGFP-Y, *ble*-FbFP-M, *kan*-FbFP-E, *kan*-FbFP-Y. Each strain only showed one band which between 2.5 kb and 3 kb. Band sizes were determined by comparison with GeneRuler 1 kb DNA Ladder on the corresponding agarose gel (not shown).

To remove the resistance marker cassettes pSH47 was transformed in each strain and Cre recombinase was expressed by incubation of the strains in YPG galactose medium.

The Cre recombinase looped out *bleomycin* and *kanamycin* marker genes located between two loxP sites. The strains without the marker gene were verified by colony PCR with f1, r2 primers and named loxpTurboGFP-M, loxpTurboGFP-E, loxpTurboGFP-Y, loxpFbFP-M, loxpFbFP-E, loxpFbFP-Y (TM, TE, TY, FM, FE, FY in Fig. 12). The PCR product of the TurboGFP strains was predicted to be 1617 bp while the fragment resulting from strains containing FbFP was expected to be 1355 bp long. All the six strains showed the expected PCR fragments (Figure 10).

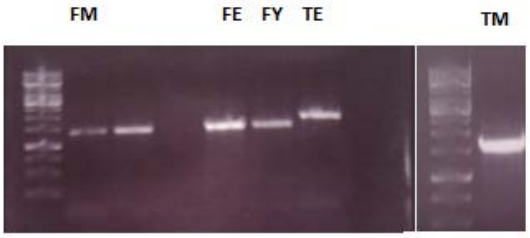


Figure 12. Electrophoresis result of verification of loxpTurboGFP-M, loxpTurboGFP-E, loxpFbFP-M, loxpFbFP-E, loxpFbFP-Y by colony PCR with f1/r2 primers. The DNA ladder used was the GeneRuler 1 kb DNA Ladder. Result of loxpTurboGFP-Y is not shown.

PGal1-f/r primers were used to amplify the *GAL1* promoter from pESC-URA and phleo-f/kan-r primers were used to amplify the kanamycin resistance gene from pUG66. These two fragments were fused by fusion-f/r primers for *GAL1* promoter integration. The fused DNA fragment was transformed into the loxpTurboGFP/FbFP strains. The new synthesized strains named *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E, *kan-GAL1*-TurboGFP-Y, *kan-GAL1*-FbFP-M, *kan-GAL1*-FbFP-E, *kan-GAL1*-FbFP-Y (kGTM, kGTE, kGTY, kGFM, kGFE, kGFY in short) were verified with f1, f2, r1, r2 primers and purified on YPD-G418 plates. The electrophoresis band of *kan-GAL1*-TurboGFP-M/E/Y and *kan-GAL1*-FbFP-M/E/Y with primers f1/r1 located at 2.5 kb, close to the predicted 2572 bp. The electrophoresis band of *kan-GAL1*-TurboGFP-M/E/Y with f1/r2 located at about 4 kb, close to the predicted 3824 bp. The electrophoresis band of *kan-GAL1*-FbFP-M/E/Y with f1/r2 located at around 3.5 kb, close to predicted 3563 bp. All the strains with the *GAL1* promoter integrated have been confirmed.

Table 4. Expected colony PCR result of *kan-GAL1*-TurboGFP-M/E/Y and *kan-GAL1*-FbFP-M/E/Y with primers f1/r1 and f1/r2.

Primers	**Template**	**Expected length (bp)**
f1/r1	*kan-GAL1*-TurboGFP-M/E/Y	2572
f1/r2	*kan-GAL1*-TurboGFP-M/E/Y	3824
f1/r1	*kan-GAL1*-FbFP-M/E/Y	2572
f1/r2	*kan-GAL1*-FbFP-M/E/Y	3563

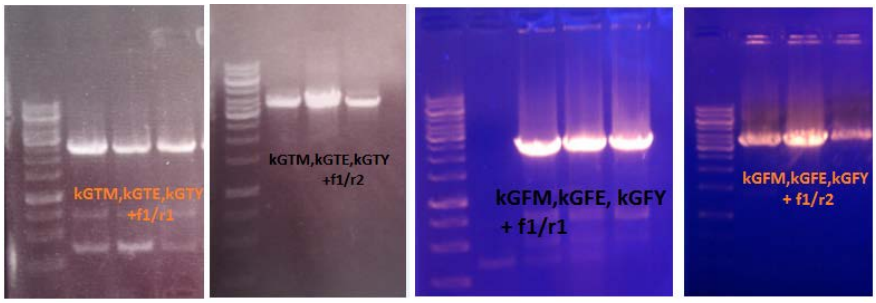


Figure 13. Electrophoresis result of verification of *kan-GAL1*-TurboGFP-M/E/Y and *kan-GAL1*-FbFP-M/E/Y with primers f1/r1 and f1/r2. The DNA ladder used was the GeneRuler 1 kb DNA Ladder.

To investigate the relationship between fluorescence intensity and the different modifications of the FbFP protein, 4 variants of FbFP were designed to be constructed on plasmid pRS416 representing successive deletions of ∆k linker, FLAG tag and ubiquitin (Figure 7). The linker was designed to contain an exposed lysine residue as potential ubiquitin binding site therefore the removal of the linker could inhibit the poly-ubiquitination of TurboGFP but possibly not FbFP, since FbFP still contains other lysine residues close to the N-terminus for potential ubiquitin binding. DNA fragments for homologous recombination with linearized pRS416 in CEN.PK113-5D were amplified and transformed into CEN.PK 113-5D (Table 1.a).. The positive colonies were verified with GAL1-fw and ADH-rv primers by colony PCR and purified on SD-URA plates (Table 5, Figure 12). The electrophoresis band of pRS416-*GAL1*-FbFP1 located at 1.5 kb, close to the predicted 1602 bp. The electrophoresis band pRS416-*GAL1*-FbFP2 located at about 1.5 kb, close to predicted 1530 bp. The electrophoresis band of pRS416-*GAL1*-FbFP3 located at around 1.5 kb, close to predicted 1506 bp. The electrophoresis band of pRS416-*GAL1*-FbFP4 located between 1 kb and 1.5 kb, close to predicted 1278 bp. Therefore all the four strains have been confirmed.

Table 5. Expected colony PCR result of pRS416-*GAL1*-FbFP1, 2, 3, 4 with Gal1-fw/ADH1-rv primers.

Primers	Template	Expected length (bp)
Gal1-fw /ADH1-rv	pRS416-*GAL1*-FbFP1	1602
	pRS416-*GAL1*-FbFP2	1530
	pRS416-*GAL1*-FbFP3	1506
	pRS416-*GAL1*-FbFP4	1278

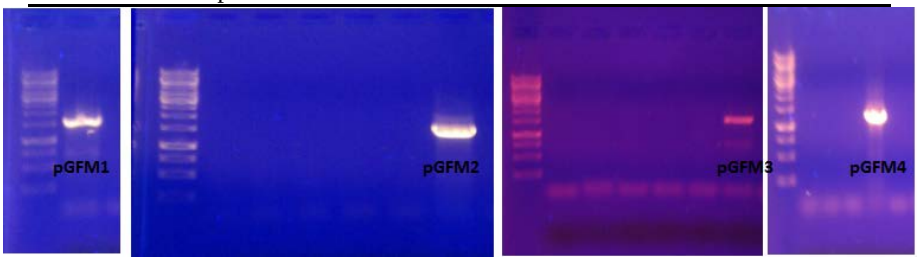


Figure 14. Electrophoresis result of colony PCR verification of pRS416-*GAL1*-FbFP1, 2, 3, 4 with Gal1-fw/ADH1-rv primers. The DNA ladder used in electrophoresis was GeneRuler 1 kb DNA Ladder.

3.2 Transcription level quantification

After finishing strain construction, transcription level quantification was essential to evaluate the performance of the *GAL1* promoter and see whether the mRNA level correlated with the protein level to achieve a sensitive reporter system afterwards.

3.2.1 RT-qPCR primer analysis

One requirement for a suitable qPCR primer pair is that it should bind to the target DNA specifically to avoid any possible additional amplification products. Melting curves of qPCR reactions were analyzed to reveal the possible unspecific binding during the reaction. Another requirement is reasonable PCR efficiency which would be used in transcription level calculations. Primers for reference genes *ALG9, TAF10, TFC1* and target genes *TurboGFP/FbFP* were designed via Primer 3 (http://frodo.wi.mit.edu/, see in appendix).

The binding specificity and PCR efficiency were measured by qPCR reactions with genomic DNA of wild-type and serial dilutions of genomic DNA of *kan-GAL1*-FbFP-M (1x, 10^{-1}x, 10^{-2}x, 10^{-3}x, 10^{-4}x). Concentrations were 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl. Self-interaction of primers was tested by no-template-control reactions. The qPCR reactions were performed according to the Three-Step Cycling Program (Table 6. Agilent).

Table 6. Parameters of Agilent Three-Step qPCR cycling program

Cycles	Process	Duration	Temperature °C
1	Initial incubation	10 min	95
40	Denaturation	30 sec	95
	Annealing	1.0 min	55 for TurboGFP, 60 for FbFP
	Extension	30 sec	72

Table 7. qPCR primer evaluation. Triplicate reactions with serial dilution of kan-GAL1-FbFP-M (1x, 10^{-1}x, 10^{-2}x, 10^{-3}x, 10^{-4}x), wild-type and no-template-control (NTC).

ALG9			TAF10			TFC1			FbFP		
1x	1x	1x	1x	1x	1x	1x	1x	1x	1x	1x	1x
10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x	10^{-1}x
10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,	10^{-2}x,
10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x	10^{-3}x
10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x	10^{-4}x
WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC	NTC

Standard curves were plotted with C_q values on the y axis and exponent of the serial dilutions on the x axis. The PCR efficiency was calculated by linear regression of the standard curve. The PCR efficiencies of *ALG9, TAF10, TFC1* and *FbFP* were 92.1%, 88.4%, 92.9% and 94.5% respectively.

The amplification curve of *ALG9, TAF10, TFC1* primers with genomic DNA of wild-type and 10 ng/µl *kan-GAL1*-FbFP-M showed similar results as expected and only one peak on the first derivative of the dissociation curve which indicated specific binding of the primers. Test with TurboGFP was not performed in this time. No amplification

curve was generated in the NTC reaction indicating that primer self-interaction like dimer formation did not happen. No amplification curve generated between FbFP primers and wild-type DNA indicated there was no unspecific binding between FbFP primers and yeast genomic DNA.

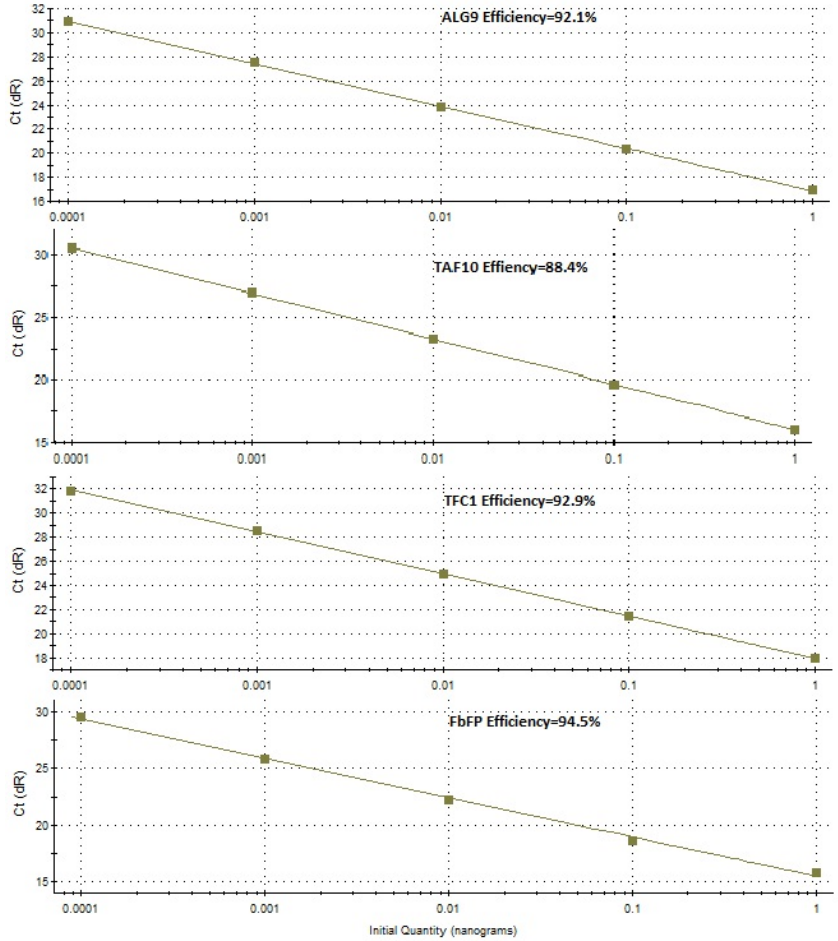


Figure 15. Standard curves of each primer pair: *ALG9, TAF10, TFC1* and *FbFP* (Standard curve of *TurboGFP* was not measured). x axis represented component of serial concentration of template DNA and y axis represented average C_q of triplicate reactions. PCR efficiencies were calculated by linear regression of standard curve.

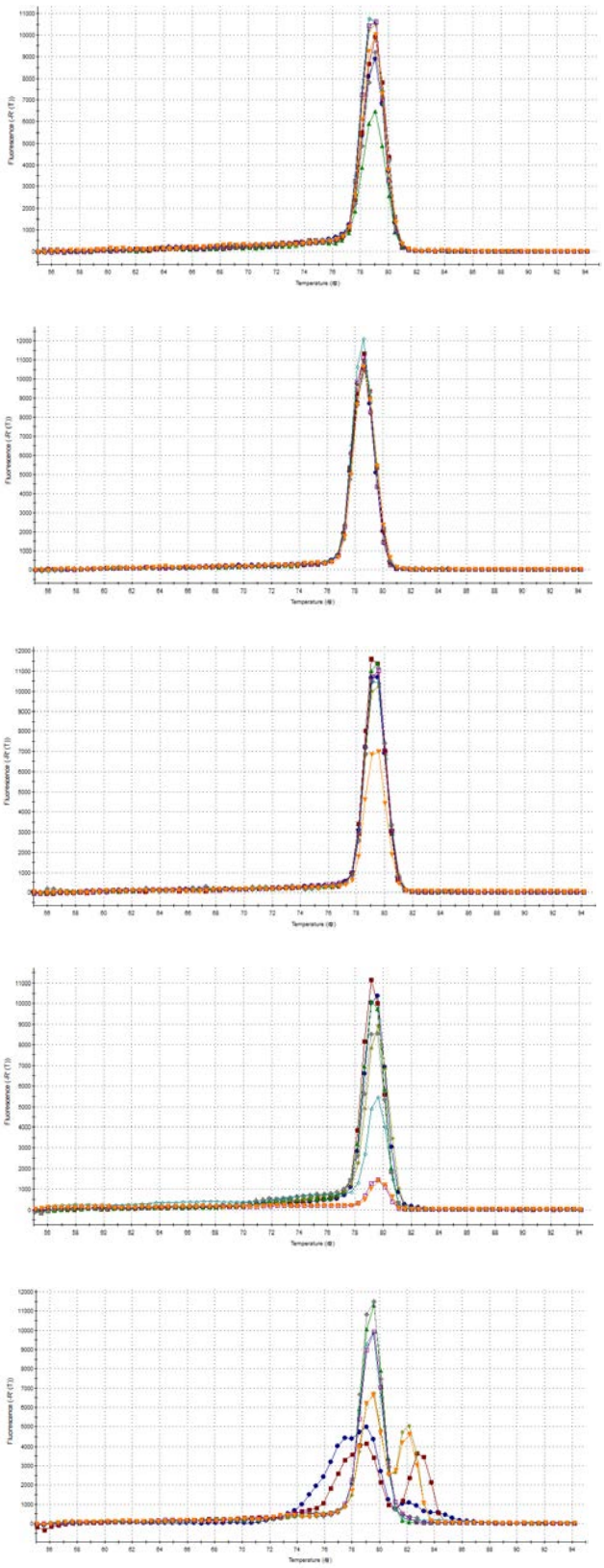


Figure 16. First derivative of dissociation curves of qPCR products generated with primers of *ALG9*, *TAF10*, *TFC1*, *FbFP* and *TurboGFP*.

Because the fluorescence signals generated between primers and wild-type DNA and NTC were all below the threshold, there should be no signal either in the qPCR reactions with the control strains loxpTurboGFP-M, loxpTurboGF-E and loxpTurboGFP-M since theoretically the *TurboGFP* and *FbFP* genes would not be transcribed without promoter. However, the experimental results were not in agreement with the hypothesis. The C_q values of *TurboGFP* in loxpTurboGFP-M and loxpTurboGFP-E were 22.24 and 21.78 in galactose conditions similar with 22.55 and 22.04 in glucose conditions. Since the relative transcription levels of *TurboGFP* in the two control strains calculated via formula 1 were close to zero which indicated no significant variation between galactose and glucose condition, this possible transcription might be caused by a putative promoter region upstream of the target gene. The same thing was observed in loxpFbFP-M, where the C_q value was 19.65 and might have been caused by the same reason since the *TurboGFP* and *FbFP* genes were integrated in the same chromosomal position.

3.2.2 RT-qPCR results

All strains were firstly seed cultured in 5 ml SD-2% glucose and SD-2% glactose+0.2% glucose separately and transferred into triplicate 10 ml corresponding main cultures. The shake flask cultures were grown until OD_{600}=1 with 200 rpm shaking at 30 °C. Cell pellets concentrated from 1 ml culture of each sample were frozen in liquid nitrogen after being spun down for 2 min at 3000 g. mRNA of each strain was extracted and the concentrations were measured by Agilent Bioanalyzer. 1µl mRNA of each strain was reverse transcribed into cDNA for following qPCR reactions.

3.2.2.1 Relative transcription levels of chromosomally integrated TurboGFP

Since the transcription of the TurboGFP gene was induced by the *GAL1* promoter in galactose conditions, the relative transcription level was calculated by normalizing 1) the transcription level of the target gene to three different reference genes, 2) the transcription level of the target gene in galactose to transcription in glucose. Two strains loxpTurboGFM-M and loxpTurboGFP-E without *GAL1* promoter were used as negative control. Relative transcription levels of *kan-GAL1*-TurboGFP-M and *kan-GAL1*-TurboGFP-E were expected to be similar since there was only one codon at the junction of ubiquitin and linker different between the two strains.

After normalization with formula 1, the relative transcription levels of the two control strains were close to zero. *kan-GAL1*-TurboGFP-M showed a 11, 7 and 6 fold higher transcription level in galactose than in glucose, which was very low in contrast to the 478, 410 and 254 fold relative transcription level of *kan-GAL1*-TurboGFP-E in galactose over glucose. The three transcription levels of each strain were calculated depending on the different reference genes used for normalization (Fig. 17). The relative transcription level of *kan-GAL1*-TurboGFP-M was supposed to be an experimental error considering the significant difference with *kan-GAL1*-TurboGFP-E.

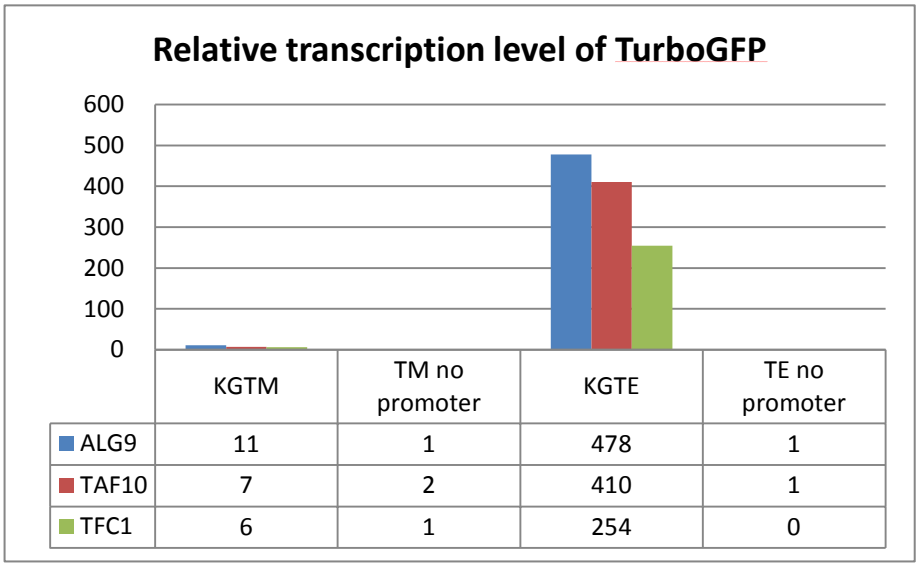


Figure 17. Relative transcription levels of *TurboGFP* in *kan-GAL1*-TurboGFP-M, loxpTurboGFM-M, *kan-GAL1*-TurboGFP-E and loxpTurboGFM-M on galactose and glucose. The blue column indicates the transcription level normalized to *ALG9* expression while the red one refers to *TAF10* and green one refers to *TFC1*.

3.2.2.2Relative transcription levels of chromosomally integrated FbFP

Two strains, *kan-GAL1*-FbFP-M and *kan-GAL1*-FbFP-E, were grown in galactose and glucose conditions. Transcription of the *FbFP* gene was induced by the *GAL1* promoter in SD-2% galactose + 0.2% glucose medium. The transcription level was normalized with *ALG9*, *TAF10* and *TFC1* via formula 1 and expected to be similar since the gene sequence of the two strains were the same except of one codon at the junction of ubiquitin and linker sequence.

The relative *FbFP* transcription level of *kan-GAL1*-FbFP-M was 1033, 1333 and 1574 depending on the three reference genes and the relative transcription level of *kan-GAL1*-FbFP-E was 836, 849 and 891 (Fig. 18). Two strains presented similar results as predicted.

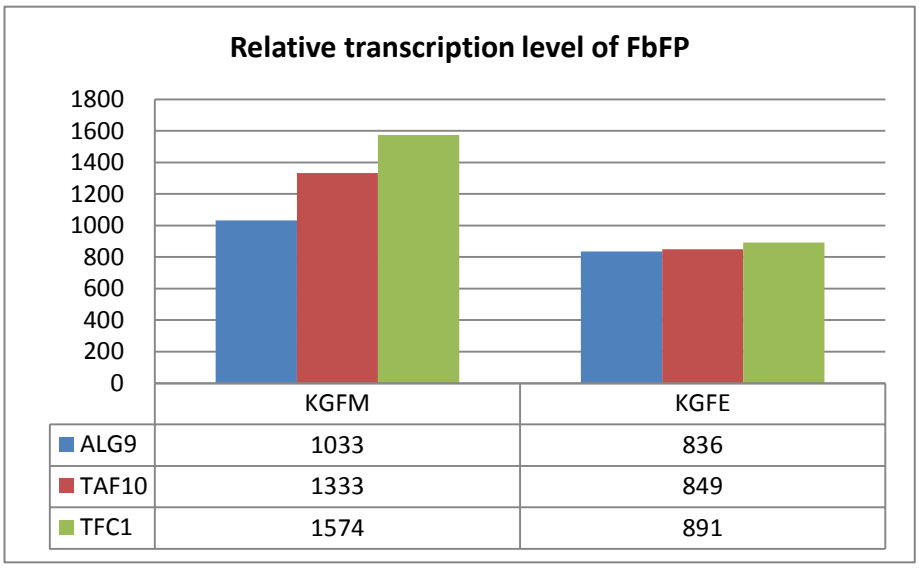


Figure 18. Relative transcription level of *FbFP* in galactose vs. glucose in *kan-GAL1*-FbFP-M, and *kan-GAL1*-FbFP-E. The blue column indicates transcription level normalized to *ALG9* expression while the red one refers to *TAF10* and the green one refers to *TFC1*.

3.2.2.3 Relative transcription level of FbFP in pGFM strains

The experimental results showed that depending on the different plasmid constructs, the relative transcription level of *FbFP* changed. The strain containing pRS416-*GAL1*-FbFP-M 1 with complete the FbFP construct showed the highest relative transcription level of the *FbFP* gene (1820, 803 and 1370 folds in galactose over glucose). The strain containing pRS416-*GAL1*-FbFP-M 2 without linker showed the lowest relative transcription level (251, 101 and 141 folds). The strain containing pRS416-*GAL1*-FbFP-M 3 without linker and FLAG-tag had the second highest relative transcription level (626, 422 and 523 folds) and the strain harboring pRS416-*GAL1*-FbFP-M 4 without linker, FLAG and ubiquitin showed the third highest relative transcription level (375, 234 and 324 folds).

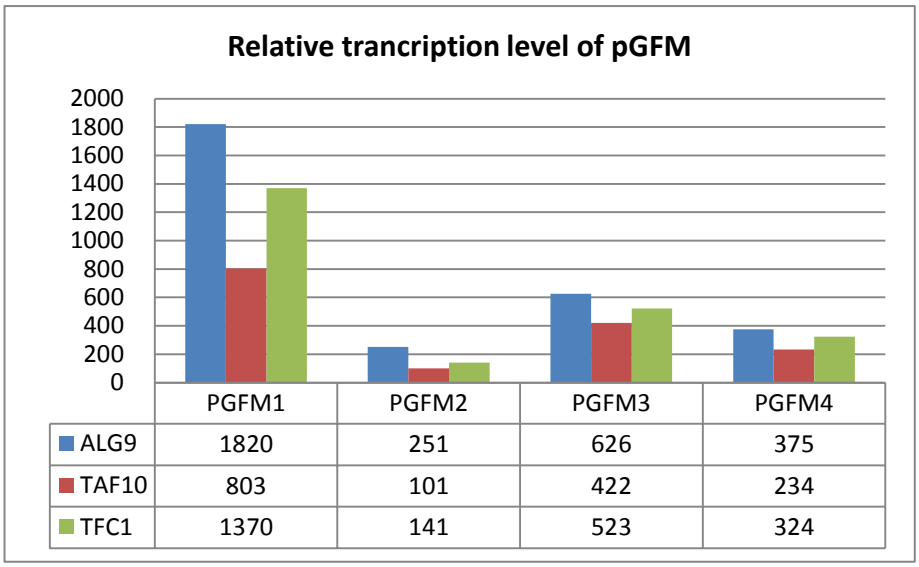


Figure 19. Relative transcription level of *FbFP* in pRS416-*GAL1*-FbFP-M 1, 2, 3 and 4 on galactose vs. glucose. The blue column indicates the transcription level normalized to *ALG9* expression while the red one refers to *TAF10* and the green one refers to *TFC1*.

3.3 Translation level determination

3.3.1 Western blot analysis

Total protein from 1 ml sample of yeast culture at OD_{600}=1 was extracted and the concentration was measured. After separating the proteins by SDS-PAGE with spectra Multicolor Broad Range Protein Ladder as a size marker and transfer of the proteins to a membrane, anti-tGFP monoclonal antibody and anti-DDK monoclonal antibody were used to bind to their respective targets, TurboGFP and FbFP-FLAG, followed with further binding with Goat Anti-Mouse IgG F(ab')2 secondary antibody which had been conjugated to alkaline phosphatase. A chemiluminescence signal was generated and detected after incubating the membrane into a mixture of substrate and enhancer.

TurboGFP consists of 256 amino acids and its molecular weight was calculated to be about 28 kD. Strains loxpTurboGFP-M and loxpTurboGFP-E were used as negative controls. According to the western blot result (Fig. 20), strong bands for kan-GAL1-TurboGFM-E were observed whereas no significant signal of kGTM appeared. Molecular weights of each pGFM variant were calculated to be about 23.3 kD, 20 kD, 19 kD and 19 kD. However, no significant signals were observed on the western blot.

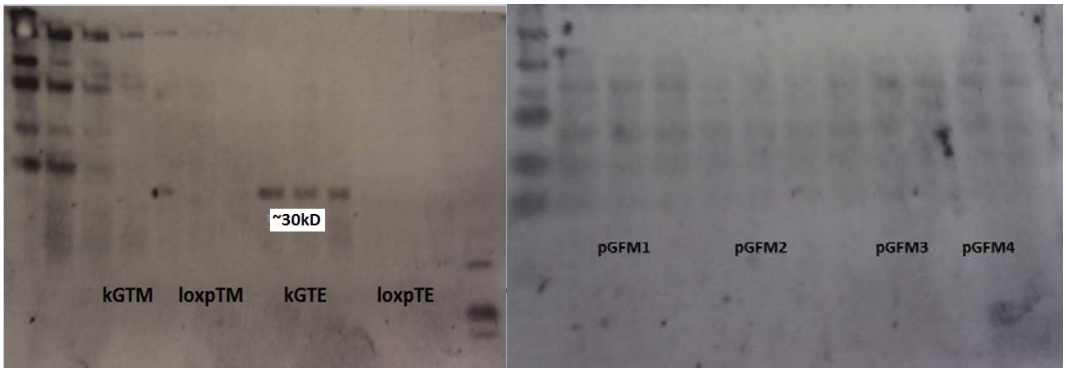
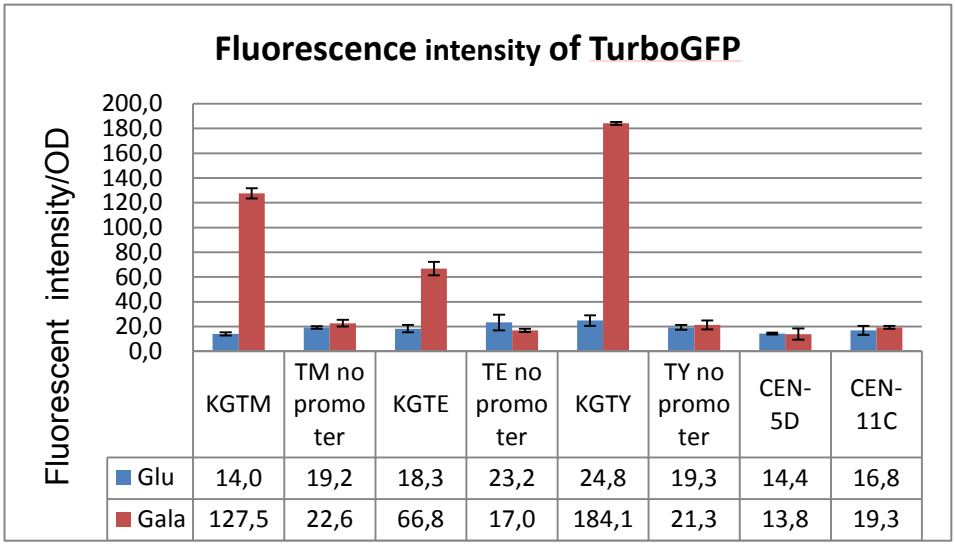


Figure 20. Western blots. The extracted total proteins were separated in a Novex pre-cast Tris-glycine gel by SDS-PAGE together with a Spectra Multicolor Broad Range Protein Ladder. anti-tGFP monoclonal antibody and anti-DDK monoclonal antibody were used as first antibody to bind TurboGFP and FbFP separately while Goat Anti-Mouse IgG F(ab')2 antibody conjugated to alkaline phosphatase was used as secondary antibody and to catalyze the substrate conversion for chemiluminescence signal detection. The left picture represents the western blot result of *kan-GAL1*-TurboGFP-M, loxpTurboGFP-M, *kan-GAL1*-TurboGFP-E and loxpTurboGFP-E. The right picture represents the western blot result of pRS416-*GAL1*-FbFP-M with complete construct, without linker, without linker and FLAG-tag, and without linker, FLAG-tag and ubiquitin.

3.4 Fluorescence intensity quantification and microscopy observation

3.4.1 Fluorescence of TurboGFP strains

The strains were cultured in SD-2 % galactose+0.2 % glucose and SD-2 % glucose from OD 0.1 to 1. Yeast cells were resuspended in 0.7 % NaCl solution and fluorescence was measured at 485 nm excitation wavelength and 520 nm emission wavelength. Three TurboGFP strains, loxpTurboGFP-M, loxpTurboGFP-E and loxpTurboGFP-Y without the *GAL1* promoter and two wild-type strains CEN.PK113-5D, CEN.PK113-11C in galactose and glucose conditions have been considered as negative controls. The fluorescence intensity of those five negative control strains could be considered as background fluorescence intensity which was about 19 in this measurement. The fluorescence intensities of *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E and *kan-GAL1*-TurboGFP-Y in glucose conditions showed similar levels compared with the background intensity which indicated that no functional fluorescent protein was expressed in glucose. The intensities of *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E and *kan-GAL1*-TurboGFP-Y in galactose condition were 127, 66 and 184, respectively, which were about 9.2 fold, 4.8 fold and 13.3 fold higher than the negative control. Considering the strong activity of *GAL1* promoter, these fluorescence intensities were not high. On the other hand, the strains with the chromosomally integrated FbFP, *kan-GAL1*-FbFP-M and *kan-GAL1*-FbFP-E, showed basically no fluorescence signal which led to the trouble shooting approach using different constructs of FbFP destabilized protein.



	KGTM	TM no promoter	KGTE	TE no promoter	KGTY	TY no promoter	CEN-5D	CEN-11C
Glu	14,0	19,2	18,3	23,2	24,8	19,3	14,4	16,8
Gala	127,5	22,6	66,8	17,0	184,1	21,3	13,8	19,3

Figure 21. Fluorescence intensities of *kan-GAL1*-TurboGFP-M/E/Y, loxpTurboGFP-M/E/Y, CEN.PK113-5D and CEN.PK113-11C were measured at 485/520 nm wavelengths in both galactose and glucose conditions. The red columns refer to the intensities in galactose condition and the blue ones refer to the intensities in glucose conditions.

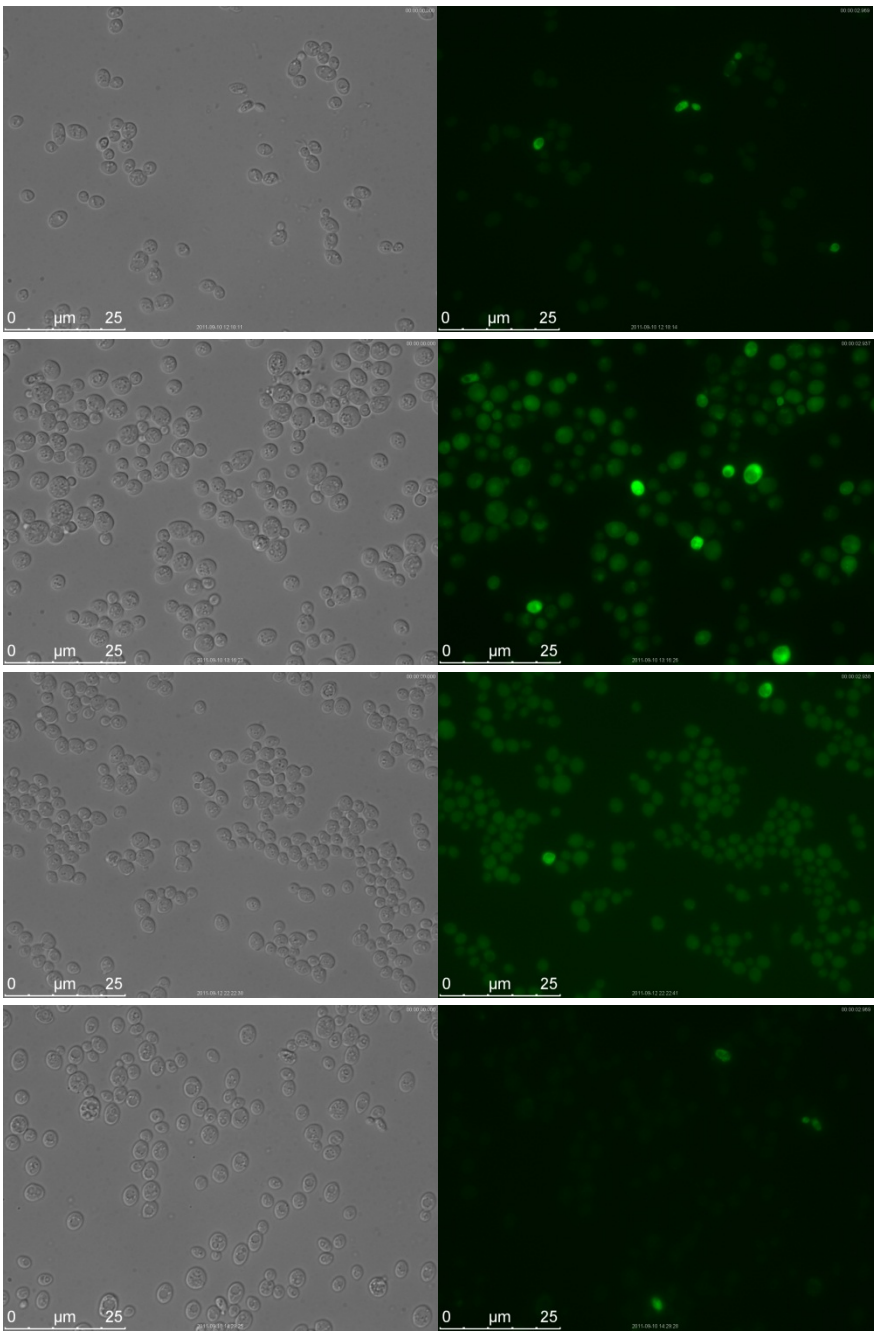
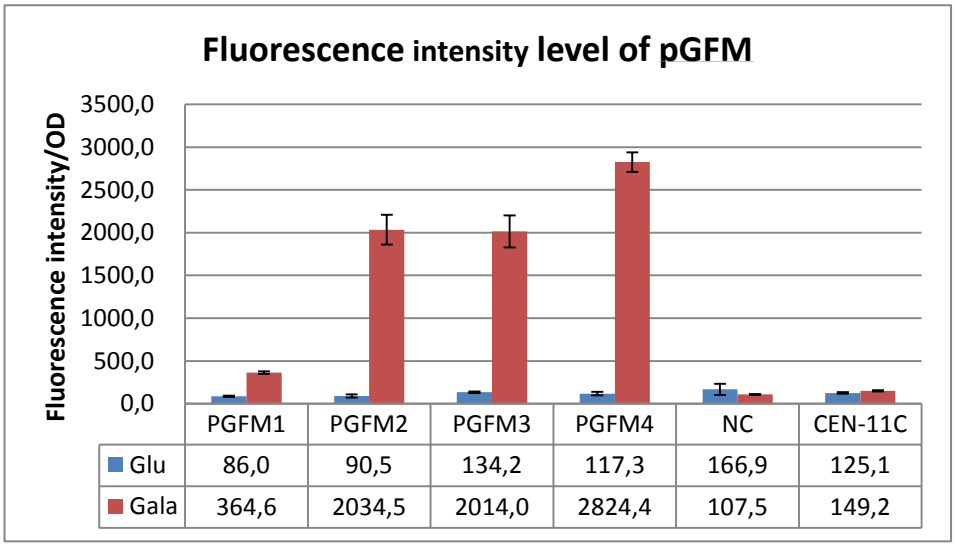


Figure 23: *kan-GAL1*-TurboGFP-M (row 1), *kan-GAL1*-TurboGFP-E (row 2), *kan-GAL1*-TurboGFP-Y (row 3) and wild-type CEN.PK113-5D (row 4) were observed using a Leica DMI 4000B fluorescence microscope with DIC (differential interference contrast) and GFP filter modes separately. The DIC images are on the left column and the fluorescent images on the right.

3.4.2 Fluorescence of pGFM strains

The strains were cultured in SD minus URA-2% galactose+0.2% glucose and SD minus URA-2% glucose from OD 0.1 to 1. Yeast cells were resuspended in 0.7% NaCl solution. Fluorescence intensity was measured at 400-480 nm excitation and 520 nm emission wavelength. The stain CEN.PK113-11C with empty pRS416 was used as negative control as well as wild-type CEN.PK113-11C in both glucose and galactose conditions. According to the measurement of the two negative control strains, the background fluorescence intensity was about 122. The intensities of pGFM1, pGFM2, pGFM3 and pGFM4 in galactose condition were 365, 2035, 2014 and 2824, respectively, which was 3 folds, 16.7 folds, 16.5 folds and 23.1 folds higher than the background intensity.

The destabilized fluorescent protein construct of in the pGFM1 strain was same as the chromosomally integrated *kan-GAL1*-FbFP-M, including ubiquitin, Δk linker, fluorescent protein, FLAG-tag and *ADH1* terminator. It also showed low fluorescence intensity just as unapparent as *kan-GAL1*-FbFP-M. After removal of the Δk linker, i.e. in strain pGFM2, the fluorescence intensity increased sharply from 3.4 fold above background to 18.9 folds. The FLAG tag was subsequently removed, but this did not show any significant effect on the fluorescence intensity of pGFM3 (18.7 folds to 18.3 folds). The modifications including ubiquitin, Δk linker and FLAG tag have been entirely deleted in GFM4, which led to a slightly increased fluorescence intensity from 18.7 times the background level to 26.3 folds. Generally speaking, the Δk linker mostly affected the fluorescence of FbFP while ubiquitin slightly affected it and the FLAG tag showed no effect.



	PGFM1	PGFM2	PGFM3	PGFM4	NC	CEN-11C
Glu	86,0	90,5	134,2	117,3	166,9	125,1
Gala	364,6	2034,5	2014,0	2824,4	107,5	149,2

Figure 22. Fluorescence intensities of pGFM1, 2, 3, 4 were measured at 400-80/520 nm wavelengths in both galactose and glucose conditions. The red columns refer to the intensities in galactose condition and the blue ones refer to the intensities in glucose conditions.

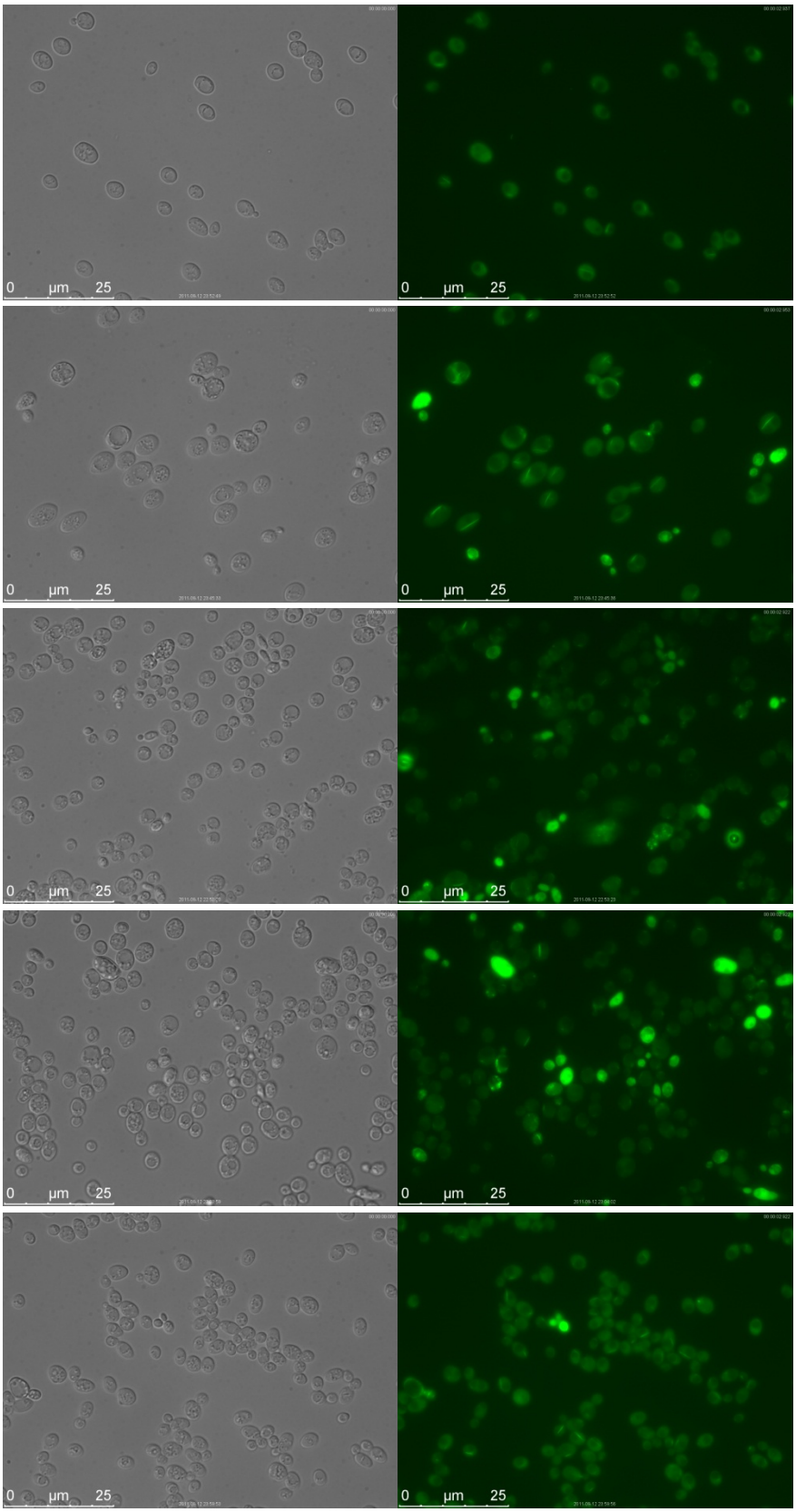


Figure 24: pRS416-*GAL1*-FbFP-M1 (row 1), pRS416-*GAL1*-FbFP-M2 (row 2), pRS416-*GAL1*-FbFP-M3 (row 3), pRS416-*GAL1*-FbFP-M4 (row 4) and CEN.PK113.5D with empty pRS416 as negative control (row 5) were observed using

a Leica DMI 4000B fluorescence microscope with DIC (differential interference contrast) and GFP filter modes separately. The DIC images are on the left column and the fluorescent images on the right.

4. Discussion

Reporter proteins destined for monitoring transcription dynamics and evaluating transient promoter activity are required to have a fast maturation rate and relatively short half-life to sensitively respond to the temporal changes. The N-end rule pathway involving ubiquitin and N-degron signal was previously used to promote the degradation rate of cyan fluorescent protein in *S. cerevisiae* (Hackett et al.,, 2006). In order to construct a novel reporter system of *S. cerevisiae* capable of monitoring transcription dynamics of target genes, a reporter system based on two destabilized fluorescent proteins, Turbo green fluorescent protein (TurboGFP) and flavin mononucleotide (FMN) binding fluorescent protein (FbFP), was designed and synthesized in this project. The reporter proteins were constructed to be capable of fast maturation and relatively fast degradation in order to sensitively response to transient transcription. To achieve a proper degradation rate, 76 residues of ubiquitin and 24 residues of the Δk linker with three different N-terminal residues, methionine, glutamic acid and tyrosine corresponding to long, middle and short half-lives (Bachmair et al., 1986) were N-terminally fused with the fluorescent protein. Six chromosomal integration strains with the *GAL1* promoter: *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E, *kan-GAL1*-TurboGFP-Y, *kan-GAL1*-FbFP-M, *kan-GAL1*-FbFP-E, *kan-GAL1*-FbFP-Y were successfully synthesized to evaluate the performance of *GAL1* induced transcription and destabilizing fluorescent proteins.

The relative transcription level of chromosomally integrated TurboGFP and FbFP on glucose and galactose was quantified by RT-qPCR. Clear transcription induction on galactose mediated by the *GAL1* promoter was observed for most of the strains as expected. However the strains without promoter also showed some kind of transcription which might be caused by putative promoter region upstream of the integration site or result from amplification of contaminating DNA in these samples since there was not any fluorescence being observed.

Comparing the relative transcription level of *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E with their expression determined by western blot, it reflected a rough congruent relationship between transcription and translation that high transcription consistent with clear western blot band while low transcription with nearly no band at all.

Functional translation level, i.e. fluorescence intensity measured by plate reader revealed that *kan-GAL1*-TurboGFP-M, *kan-GAL1*-TurboGFP-E and *kan-GAL1*-TurboGFP-Y showed 9.2, 4.8 and 13.3 higher intensity than the wild-type control. The overall intensity was however very low. On the other hand, the strains of *kan-GAL1*-FbFP-M, *kan-GAL1*-FbFP-E and *kan-GAL1*-FbFP-Y showed nearly no signal. According to the N-end rule, methinoine (M), glutamic acid (E) and tyrosine (Y) combined with the Δk linker should present a decrease in protein stability in this order (Bachmair and Varshavsky 1989). In a previous study of destabilized CFP, it was proved that the fluorescence intensity of destablized ubi-M-Δk-CFP, ubi-E-Δk-CFP and ubi-Y-Δk-CFP showed decrease from 76 to 51 and 5 a.u. If other fluorescence factors were assumed to be constant between the strain variants and the accumulation of

fluorescent protein reached the equilibrium i.e. the protein expression rate was equal to the degradation rate when the intensity was measured, the intensity of the strains with methionine, glutamic acid and tyrosine at the N-terminus of the protein in this project should also show the same downtrend. However the *kan-GAL1*-TurboGFP-Y showed a surprising increase compared with the other two which did not match the hypothesis.
In consideration of the small size of TurboGFP/FbFP compared to ubiquitin and linker, it was suspected that the destabilizing components might have effects on the protein structure which may lead to incorrect folding and chromophore maturation (TurboGFP) or binding of the chromophore (FbFP). If the ubiquitin is cleaved just after synthesis on the ribosome, it might not affect the folding significantly but if it is only cleaved after synthesis of the entire fusion protein, the subsequent folding and maturation might be affected.

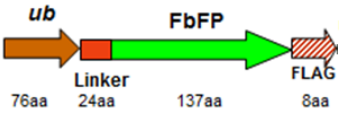


Figure 25: Sketch of complete construct of FbFP. FbFP consists of 137 residues while ubiquitin 76 residues, linker 24 residues and FLAG 8 residues.

To figure out whether the protein extensions affect the fluorescence, a centromeric plasmid approach was applied. Plasmid constructs with different combinations of FbFP modifications were successfully synthesized. The variations in relative transcription level of pRS416-*GAL1*-FbFP in galactose over glucose condition ranging from 101 to 1820 folds may reflect differences in the stability of FbFP mRNA in the different constructs. Transcription level differences had also been observed when the *FUS1* promoter was used to regulate expression of ubi-Y-ΔkCFP (Hackett et al.,, 2006). The significant increase of fluorescence intensity between pRS416-*GAL1*-FbFP 1 (3.4 fold increase over negative control) and pRS416-*GAL1*-FbFP 2 (18.9 fold increase over negative control) indicates that the Δk linker prominently influenced the fluorescence. The deletion of FLAG epitope tag did not clearly affect the fluorescence (18.7 fold increase over negative control) while the removal of ubiquitin again increased the fluorescence (26.3 fold increase over negative control). But unfortunately, the final overall intensity was still not very high.

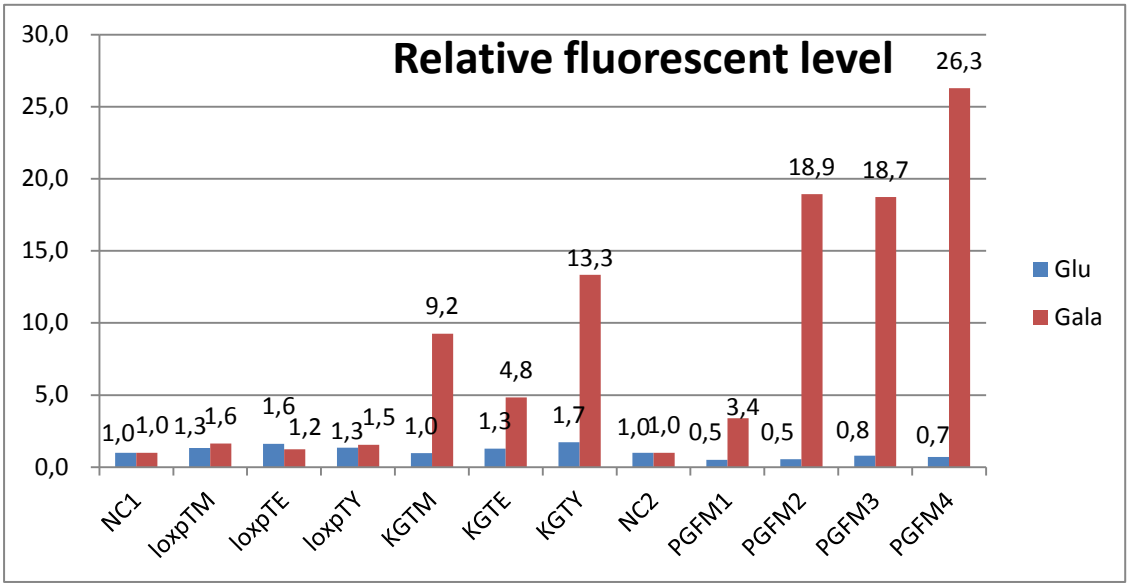


Figure 26: The relative fluorescence level was calculated by dividing fluorescence intensity of TurboGFP and FbFP strains with negative control.

The reason why Δk linker and ubiquitin affected the fluorescence has not been determined, but two aspects might explain this observation. 1) An increased stability. The Δk linker including its KRK motif here worked as ubiquitin binding site which initiates the formation of the ubiquitin-protein complex for degradation by the 26S proteasome. Although the FbFP protein itself contains lysine residues close to its N-terminus they might not work as efficiently as in the context of the Δk linker. Because of the deletion of this component, the protein stability might have increased resulting in the increase of fluorescence intensity. The deletion of the FLAG tag did not affect the protein stability therefore the intensity did not change. This deduction could be confirmed by measuring the half-life of each FbFP protein construct to see whether there is clear relationship between instability and fluorescence intensity. 2) A structural change. Considering the size of ubiquitin, Δk linker and fluorescent protein, it was possible that the N-terminal modifications affected the ultimate steric configuration which was relevant for the capability of fluorescence. Incorrect configuration might either influence the chromophore maturation of TurboGFP or the FbFP binding with FMN. Whether the subsequent folding and maturation after translation could be disturbed by ubiquitin was not clear since the time point of its cleavage, i.e. whether it happened before or after folding of the fluorescent protein, was not confirmed.

Besides the intrinsic fluorescence ability, the low overall fluorescence intensity might be influent by the measuring method. With regard to the FbFP fluorescence spectrum, the maximum excitation and emission wavelength are about 445 nm and 495 nm, respectively, and the fluorescence intensity decreased remarkably after 495 nm. Therefore the low intensities of FbFP might due to the emission filter of the fluorescence plate reader at 520 nm.

In summary, six strains with chromosomal integration of TurboGFP and FbFP variants and four strains with different FbFP plasmid constructs were synthesized and analyzed.

Transcription and functional translation level were quantified to evaluate the performance of destabilized fluorescent protein. The FbFP construct was modified to achieve satisfactory fluorescence intensity, which demonstrated that the low fluorescence level was – at least partially – caused by the N-terminally fused ubiquitin and Δk linker. Further work following the same strategy of design and synthesis is expected to generate improved destabilized fluorescent proteins with stronger intensity and better sensitivity as reporters of transcription dynamics.

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6 Appendix

6.1 Primer sequences

Name	Sequence	Length (bp)	Melting temperature ℃
TEF-f (XbaI)	GAAGAATCTAGAGCACACACATAGCTTCAAA	31	54.3
TEF-r (NcoI)	GAAGTTCATGGTTGTAATTAAAACTTAGATTAGATTGC	37	54.8
Turbo-F	GTGCATGGTTGTTACCAGTCTCAT	24	66.3
Turbo-RE	CAGAACCGTGTTCACCACCTCTCA	24	68.0
Turbo-RY	CAGAACCGTGATAACCACCTCTCA	24	68.0
FbFP-RE	CGGAACCGTGTTCACCACCTCTC	23	69.6
FbFP-RY	CGGAACCGTGATAACCACCTCTC	23	69.6
f1	GTTGCTGAGTAATCTTCATTGC	22	60.8
r1	TCTTGGATCTTAGACTTGACG	21	59.1
f2	CTTCGTTAAGACCTTGACTGG	21	60.8
r2	CGCCTACATACAACTTAAACTCG	23	61.8
phleo-f	CAAGGTCTTAACGAAGATTTGCATCGTACGCTGCAGGTCGACAACC	46	73.0
phleo-r	ATCGTCCTTGTATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAAACTATAGGGAGACCGGCAGATCCG	74	71.0
Turboprobe-f	AATTAGTAGGTGGTGGTG	18	52.6
Turboprobe-r	ACTTGGATGTATTGCGGA	18	59.6
FbFPprobe-f	TGGTTAAGAGAAAGACTACATTGG	24	60.5
FbFPprobe-r	AAATTCGCTTACTTGTCATCG	21	61.1
fusion-f	ATCGTCCTTGTATGGAAGTATCAAAGG	27	63.7
fusion-r	ACTTCCAAGGTGATTGTTTTACCAGTC	27	63.6
kan-r	CGTACGCTGCAGGTCGACAACC	22	72.9
PGal1-f	AAGGGTTGTCGACCTGCAGCGTACGTTTCAAAAATTCTTACTTTTTTTTTGG	52	62.5
PGal1-r	ACTTCCAAGGTGATTGTTTTACCAGTCAAGGTCTTAACGAAGATTTGCATGGGTTTTTTCTCCTTGACGTTA	72	61.5
Construct-f	GTACGATGCAAATCTTCGTTAAGACCTTG	29	69.6
Construct-r	GGCTTATAATGTCAGTATGCATTGTTACGCTTAG	34	70.0
GAL1-fw	GATATCGAATTCCTGCAGCCCGGGTTTTTTTTGGATGGACGCAAAGAAG	49	69.4
GAL1-rv	GGGTTTTTTCTCCTTGACGTTAAAGTA	27	66.3
Ubi-fw	TTTAACGTCAAGGAGAAAAAACCCATGCAAATCTTCGTTAAGACCTTGACT	51	66.8
Ubi-rv	ACCACCTCTCAATCTCAAAACCAAAT	26	67.6
FP-fw1	TTGGTTTTGAGATTGAGAGGTGGTATGGCATCCTTTCAAAGTTTCGG	47	69.0
FP-fw2	TTTAACGTCAAGGAGAAAAAACCCATGGCATCCTTTCAAAGTTTCGG	47	69.0
FP-rv	TTATTCTAACAACTTTTCGTATTCCTTTTGC	31	66.5

ADH-fw	GAATACGAAAAGTTGTTAGAATAAGCGAATTTCTTATGATTTATGATTTTTATTATTA	58	63.8
ADH-rv	GGTGGCGGCCGCTCTAGAACTAGTGAGCGACCTCATGCTATACCTGAG	48	67.3
ALG9-Yuan-Fw	GCCATGTTGTTGTATGTAGGTG	22	57.96
ALG9-Yuan-RV	TATACTAGCCAGGAAATTGTACG	23	55.49
TAF10-Yuan-Fw	CAGATGTACGAGTGAAACGACTT	23	58.46
TAF10-Yuan-Rv	GACTGTTGTTAGCATTAGATACCG	24	57.17
TFC1-Yuan-Fw	AACAAAAGTCTATCCAGGCACT	22	57.06
TFC-Yuan-Rv	GTGTATCTTCTTCGGGACAATC	22	57.67
Turbo-Yuan-Fw	CTATGGGTGACAACGACTTAGAT	23	57.33
Turbo-Yuan-Rv	ACTTGGATGTATTGCGGACTTA	22	58.65
FbFP-Adnan-F	TCCAAAAGGAATCAACATTGC	21	64.0
FbFP-Adnan-R	AAGGATGCCATGGTGTTAGG	20	64.0

6.2 Strain list

No.	Strain name	Marker gene
	***E. coli* (DH5α)**	
1	DH5α-pUG57-TurboGFP-M	*amp*^{R}
2	DH5α-pUG57-TurboGFP-E	
3	DH5α-pUG57-TurboGFP-Y	
4	DH5α-pUG57-FbFP-M	
5	DH5α-pUG57-FbFP-E	
6	DH5α-pUG57-FbFP-Y	
	***S. cerevisiae* (CEN.PK113-5D)**	
13	loxpTurboGFP-M	No marker
14	loxpTurboGFP-E	
15	loxpTurboGFP-Y	
16	loxpFbFP-M	
17	loxpFbFP-E	
18	loxpFbFP-Y	
19	*kan-GAL1*-TurboGFP-M	*kan*^{R}
20	*kan-GAL1*-TurboGFP-E	
21	*kan-GAL1*-TurboGFP-Y	
22	*kan-GAL1*-FbFP-M	
23	*kan-GAL1*-FbFP-E	
24	*kan-GAL1*-FbFP-Y	
25	pRS416-*GAL1*-FbFP-M 1	*URA3*
26	pRS416-*GAL1*-FbFP-M 2	
27	pRS416-*GAL1*-FbFP-M 3	
28	pRS416-*GAL1*-FbFP-M 4	
29	*ble*-TurboGFP-M	*ble*^{R}
30	*ble*-TurboGFP-Y	
31	*ble*-FbFP-M	
32	*ble*-FbFP-E	
33	*kan*-TurboGFP-E	*kan*^{R}
34	*kan*-TurboGFP-Y	
35	*kan*-FbFP-E	
36	*kan*-FbFP-Y	

6.3 Medium and Buffer recipes

LB medium

Component	LB medium	LB Agar plates
peptone/tryptone	10 g	10 g
NaCl	10 g	10 g
yeast extract	5 g	5 g
agar	-	16 g
Water	1 L	1 L

* Ampicillin: 80 mg/ml (1000X stock) dissolved in 50% EtOH/H_2O and sterile filtered (stored at -20^oC).

Final concentration to use 80 mg/L (= 1ml/1L) for both media and plates. Add after autoclavation.

YPD medium and YPD Agar plates

Component	YPD medium	YPD Agar plate
yeast extract	10 g	10 g
peptone	20 g	20 g
glucose	20 g	20 g
agar	-	20 g
Water	1 L	1 L

* G418 working conc. = 200 mg/L, stock conc. = 50 mg/ml, dilute 4 ml stock solution in 1 L medium. Add after autoclavation.

SD 2% glucose and SD 2% galactose + 0.2% glucose

Component	SD 2% glucose	SD 2% galactose + 0.2% glucose
yeast nitrogen base without amino acid (YNB)	6.7 g	6.7 g
complete supplement mixture (CSM)	0.77 g	0.77 g
galactose	20 g	20 g
glucose	-	2 g
Water	1 L	1 L

* SD-URA-2% glucose and SD-URA-2% galactose + 0.2% glucose: replace CSM with CSM-URA

5-FOA plate

Component	600 ml
Solution 1	
Agar (final conc. 2%)	12 g
H_2O	255 ml
Solution 2	
yeast nitrogen base without amino acid (YNB)	4.14 g
complete supplement mixture without Ura (CSM-URA)	0.462 g
Uracil	30 mg
5-FOA	450 mg
Glucose	12 g
MQ water	345 ml

* Solution 1 autoclave and keep at 60^{o}C.

* Solution 2 gently heat in microwave to about 60^{o}C. Stir until everything is dissolved. Filter sterilization. Add to the autoclaved agar. Make sure both solutions are still warm. Mix well without adjusting pH.

LTE Buffer and Transformation Mix

Component	LTE Buffer 50ml	Transformation Mix 10ml
0.1 M LiOAc	0.51 g	0.102 g
200 mM Tris-HCl (pH=7.5)	2.5 ml	0.5 ml
500 mM EDTA	0.1 ml	0.02 ml
PEG 3350	-	4 g
Water	50 ml	10 ml

* Mix LiOAc, Tris-HCl and EDTA, adjust to 50 ml, shake until LiOAc is dissolved, and filter sterilize

*Mix LiOAc, Tris-HCl, EDTA and PEG 3350, adjust to 9 ml, shake until all is dissolved, adjust Transformation Mix to 10 ml, and filter sterilize

6.4 qPCR primer design conditions with Primer 3

1. **Primer 3 design**
    1. Primer length: 18-30bp
    2. GC% content: 40-60%
    3. Tm: 55-60℃, ∆Tm≤4℃
    4. avoid 3’ end of runs of identical nucleotide, especially 3 or more Gs/Cs
    5. avoid 3’ end T
    6. check 3’ stability
    7. avoid self-dimers and 3’ dimer
    8. product length: 80-150bp
    9. product GC% content: 40-60%
    10. avoid secondary structure in the amplicon
    11. Check with NetPrimer
        i. Hairpin: ∆G of 3’ end hairpin ≤-2kcal/mol, ∆G of internal hairpin ≤-3kcal/mol
        ii. Self-Dimer: ∆G of 3’ end dimer ≤-5kcal/mol, ∆G of internal dimer ≤-6kcal/mol
    12. The pair of primers should be located near the 3’ end of the target gene since cDNA synthesis with poly dT primer starts from the 3’ end and the amplicon nearby the 3’ end can avoid influence of uncompleted cDNA synthesis.
2. **BLAST the PCR product to *Saccharomyces cerevisiae* sequences**
   Only one BLAST hit
3. **BLAST the primer with cutoff score 30**
   No unspecific primer binding site
4. **Check the potential amplicon target with the primer in genome by in-silico PCR** (http://insilico.ehu.es/PCR/)

Only one potential amplicon target for *ALG9, TAF10, TFC1*, no potential amplicon target for *TurboGFP* and *FbFP*

6.5 Sequence of *kan-GAL1*-TurboGFP on chromosome

5’ yeast homologous recombination part (Ty3 LTR – ARS1630) – ***phleo reverse primer*** – loxP– ***phleo forward primer*** – ubiquitin – M/Y/E mutation site - TurboGFP – stop codon – Terminator ADH1 – yeast homologous recombination part 3’

5’CAGCACCTTGAATTTCATGTTGCCTTGTATGTACAAACTAAACCCTTACTCAGTAATAAAAACTAAAATGTGTCTTCT
AGTTATGAATGCTCTTAGTATAACTCTCATGAAGCAGTCAAGTCACTTTCATGTATTTTATAATACGAGGCGAATGTCTA
GGCTAAGTTAAAAAACTAGATATAGAAACCATTCCCGAAATATCATATAATTATAATATCCTGGACACTTTACTTATCTAG
CGTATGTTATTACTCGATAAGTGCTAAAGGAGGTGCACGCATTATGGAGACCACTACGATACGATAGCTGCGTTGTTGT
TGAAGGGGTTTCTTAAGGTTGTTTTCGTTGAAGGTAAATATTGGTCGTTTTTGTGCAGCATATTGTCCTCTAGATGCAA
ACTCTGCAGGTCCATTTGCAGTAAAGTGAGTTGCCTCTCGAAGAATCATTAATTTCGTATAACCGTCACTATTAAAGTC
AGAAAATAAATTCTGTCGTAGACAATGTTACCATAATGTTCTTGTCCATTTTGCATACACTTTAAATATTCATTTGATTTC
TCAGGGTTCATGATCATAATAAATTGCGCATTCGCAAGGCGGTAGTATTATAATGGGGTCCATCATTCTGTAGCAAGAA
GTTACAGTACGCTGTTCAAGCGTTAAACAAGATAAGTAATCTCGAATGAAACATTCATATTTCGCATGAGCCAACATAC
AGTTGCTGAGTAATCTTCATTGCGCTTATTTATCGGCATTGAGATTGTAAAGGAAGTAAAACGCATTTTTGCAGATCTG
TTCTCTTATGTATTTTTA*ATCGTCCTTGTATGGAAGTATCAAAGG*GGACGTTCTTCACCTCCTTGGAA***ACTATAGGGAGACCG***
GCAGATCCGCGGCCGCATAGGCCACTAGTGGATCTGATATCACCTAATAACTTCGTATAGCATACATTATACGAAGTTAT
ATTAAGGGTTCTCGAGAGCTCGTTTTCGACACTGGATGGCGGCGTTAGTATCGAATCGACAGCAGTATAGCGACCAGC
ATTCACATACGATTGACGCATGATATTACTTTCTGCGCACTTAACTTCGCATCTGGGCAGATGATGTCGAGGCGAAAAA
AAATATAAATCACGCTAACATTTGATTAAAATAGAACAACTACAATATAAAAAAACTATACAAATGACAAGTTCTTGAA
AACAAGAATCTTTTTATTGTCAGTACTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAG
GATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGG
CAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAG
GTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGAC
TTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCC
TGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACA
CTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTGCCGGGGATCGC
AGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCA
GTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCG
GGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGC
ATCCATGTTGGAATTTAATCGCGGCCTCGAAACGTGAGTCTTTTCCTTACCCATGGTTGTAATTAAAACTTAGATTAGAT
TGCTATGCTTTCTTTCTAATGAGCAAGAAGTAAAAAAAGTTGTAATAGAACAAGAAAAATGAAACTGAAACTTGAGA
AATTGAAGACCGTTTATTAACTTAAATATCAATGGGAGGTCATCGAAAGAGAAAAAAATCAAAAAAAAAAATTTTCA
AGAAAAAGAAACGTGATAAAAATTTTTATTGCCTTTTTCGACGAAGAAAAAGAAACGAGGCGGTCTCTTTTTTCTTTT
CCAAACCTTTAGTACGGGTAATTAACGACACCCTAGAGGAAGAAAGAGGGGAAATTTAGTATGCTGTGCTTGGGTGT
TTTGAAGTGGTACGGCGATGCGCGGAGTCCGAGAAAATCTGGAAGAGTAAAAAAGGAGTAGAAACATTTTGAAGCT
ATGGTGTGTGCTCTAGACCTAATAACTTCGTATAGCATACATTATACGAAGTTATATTAAGGG***TTGTCGACCTGCAGCGT***
ACG***TTTCAAAAATTCTTACTTTT****TTTTTGG*ATGGACGCAAAGAAGTTTAATAATCATATTACATGGCATTACCACCATATA
CATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCTAAAAAA
ACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCCGAGCGG
GTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGATGTGC
CTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTGGCAGTA
ACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTAGCCTTA
TTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAACCACTT

TAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTGTTAATAT

ACCTCTATACTT***TAACGTCAAGGAGAAAAAACCCATGCAAATCTTCGTTAAGACCTTG***ACTGGTAAAACAATCACCTT

GGAAGTAGAATCTTCAGACACTATCGATAACGTCAAGTCTAAGATCCAAGACAAGGAAGGTATACCACCTGAT

CAACAAAGATTGATATTCGCTGGTAAACAATTGGAAGACGGTAGAACTTTGTCCGATTACAACATCCAAAAAG

AAAGTACATTGCATTTGGTTTTGAGATTGAGAGGTGGTATTCACGGTTCTGGTGCATGGTTGTTACCAGTCTCATT

GGTTAAAAGAAAGACTACATTAGCCCCAAATACAATGGAATCCGATGAAAGTGGTTTGCCTGCTATGGAAATCGAATG

TAGAATTACTGGTACATTGAACGGTGTTGAATTTGAATTAGTAGGTGGTGGTGAAGGTACTCCAGAACAAGGTAGAAT

GACAAACAAAATGAAGTCTACTAAGGGTGCCTTAACATTCTCTCCTTACTTGTTATCACATGTTATGGGTTATGGTTTTT

ACCACTTCGGTACTTATCCATCAGGTTACGAAAATCCATTTTTGCATGCTATCAATAACGGTGGTTACACCAACACTAG

AATCGAAAAGTACGAAGATGGTGGTGTATTGCATGTCTCTTTCTCATACAGATACGAAGCAGGTAGAGTCATAGGTGA

CTTTAAGGTTATGGGTACAGGTTTCCCAGAAGACTCCGTTATTTTTACAGATAAGATAATCAGAAGTAATGCCACCGTT

GAACATTTGCACCCTATGGGTGACAACGACTTAGATGGTTCTTTTACAAGAACCTTCTCATTAAGAGACGGTGGTTATT

ACTCCAGTGTTGTAGATTCCCACATGCACTTTAAGTCCGCAATACATCCAAGTATCTTGCAAAATGGTGGTCCTATGTT

TGCCTTCAGAAGAGTTGAAGAAGATCATTCTAACACCGAATTAGGTATTGTAGAATATCAACACGCTTTCAAAACACC

AGACGCTGATGCAGGTGAAGAA**TAA**GCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAA

GTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAG

GTTGCTTTCTCAGGTATAGCATGAGGTCGCTCGATGGGACGTCAGCACTGTACTTGTTTTTGCGACTAGATTGTAAATC

ATTCTTTATTTAATCTCTTTCTTTAACTACTGCTTAAAGTATAATTTGGTCCGTAGTTTAATAACTATACTAAGCGTAACA

ATGCATACTGACATTATAAGCCTGAACATTACGAGTTTAAGTTGTATGTAGGCGTTCTGTAAGAGGTTACTGCGTAAATT

ATCAACGAATGCATTGGTGTATTTGCGAAAGCTACTTCTTTTAACAAGTATTTACATAAGAATAATGGTGATCTGCTCA

ACTGATTTGGTGATAACTCTAACTTTTTTAGCAACAATTTAAAAGATAATTCGAACATATATAACAGTAGGAAGAATTT

GTGTACGTCAAATTAAGATAATTTAGCATTACCAAAGTTATTAACCTAAACATAAAATATATATGAGACACATGTGGAAA

TCGTATGAAACAACTGTTATGAAACTGACAAGAATGAATATATAGAGTAAGCTCCGCTTGTAAAGAGGAATCACTTAA

GTGTATAAATGTCTCGACGATTACTTTAGATCCAAGATTGATGATTGATATTACTCTGTAATACTTAAGCTCTTTTAATAG

CTCACTGTTGTATTACGGGCTCGAGTAATACCGGAGTGTCTTGACAATCCTAATATAAACAGT

6.6 Sequence of *kan*-GAL1-FbFP on chromosome

5’ yeast homologous recombination part (Ty3 LTR – ARS1630) – ***phleo reverse primer*** – loxP– ***phleo forward primer*** – ubiquitin – M/Y/E mutation site - FbFP – stop codon – Terminator ADH1 – yeast homologous recombination part 3’

5’CAGCACCTTGAATTTCATGTTGCCTTGTATGTACAAACTAAACCCTTACTCAGTAATAAAAACTAAAATGTGTCTTCT
AGTTATGAATGCTCTTAGTATAACTCTCATGAAGCAGTCAAGTCACTTTCATGTATTTTATAATACGAGGCGAATGTCTA
GGCTAAGTTAAAAAACTAGATATAGAAACCATTCCCGAAATATCATATAATTATAATATCCTGGACACTTTACTTATCTAG
CGTATGTTATTACTCGATAAGTGCTAAAGGAGGTGCACGCATTATGGAGACCACTACGATACGATAGCTGCGTTGTTGT
TGAAGGGGTTTCTTAAGGTTGTTTTCGTTGAAGGTAAATATTGGTCGTTTTTGTGCAGCATATTGTCCTCTAGATGCAA
ACTCTGCAGGTCCATTTGCAGTAAAGTGAGTTGCCTCTCGAAGAATCATTAATTTCGTATAACCGTCACTATTAAAGTC
AGAAAATAAATTCTGTCGTAGACAATGTTACCATAATGTTCTTGTCCATTTTGCATACACTTTAAATATTCATTTGATTTC
TCAGGGTTCATGATCATAATAAATTGCGCATTCGCAAGGCGGTAGTATTATAATGGGGTCCATCATTCTGTAGCAAGAA
GTTACAGTACGCTGTTCAAGCGTTAAACAAGATAAGTAATCTCGAATGAAACATTCATATTTCGCATGAGCCAACATAC
AGTTGCTGAGTAATCTTCATTGCGCTTATTTATCGGCATTGAGATTGTAAAGGAAGTAAAACGCATTTTTGCAGATCTG
TTCTCTTATGTATTTTTA*ATCGTCCTTGTATGGAAGTATCAAAGGGGACGTTCTTCACCTCCTTGGAA**ACTATAGGGAGACCG*
GCAGATCCGCGGCCGCATAGGCCACTAGTGGATCTGATATCACCTAATAACTTCGTATAGCATACATTATACGAAGTTAT
ATTAAGGGTTCTCGAGAGCTCGTTTTCGACACTGGATGGCGGCGTTAGTATCGAATCGACAGCAGTATAGCGACCAGC
ATTCACATACGATTGACGCATGATATTACTTTCTGCGCACTTAACTTCGCATCTGGGCAGATGATGTCGAGGCGAAAAA
AAATATAAATCACGCTAACATTTGATTAAAATAGAACAACTACAATATAAAAAAACTATACAAATGACAAGTTCTTGAA
AACAAGAATCTTTTTATTGTCAGTACTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAG
GATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGG
CAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAG
GTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTCCAGAC
TTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCC
TGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACA
CTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTGCCGGGGATCGC
AGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCA
GTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCG
GGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGC
ATCCATGTTGGAATTTAATCGCGGCCTCGAAACGTGAGTCTTTTCCTTACCCATGGTTGTAATTAAAACTTAGATTAGAT
TGCTATGCTTTCTTTCTAATGAGCAAGAAGTAAAAAAAGTTGTAATAGAACAAGAAAAATGAAACTGAAACTTGAGA
AATTGAAGACCGTTTATTAACTTAAATATCAATGGGAGGTCATCGAAAGAGAAAAAAATCAAAAAAAAAAATTTTCA
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*CGTTTCAAAAATTCTTACT**TTTTTTTTGG**ATGGACGCAAAGAAG*TTTAATAATCATATTACATGGCATTACCACCATATAC
ATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCTAAAAAAA
CCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCCGAGCGGG
TGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAGATGTGCC
TCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTGGCAGTAA
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AACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTGTTAATATA
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ACAAAGATTGATTTTCGCTGGTAAACAATTGGAAGATGGTAGAACTTTGTCTGACTACAACATCCAAAAGGAAT
CAACATTGCATTTGGTTTTGAGATTGAGAGGTGGTATGCACGGTTCCGGTGCATGGTTGTTACCAGTCTCATTGGT
TAAGAGAAAGACTACATTGGCTCCTAACACCATGGCATCCTTTCAAAGTTTCGGTATTCCAGGTCAATTGGAAGTTATT
AAGAAAGCATTGGATCATGTCAGAGTTGGTGTTGTAATCACAGATCCAGCCTTAGAAGACAATCCTATTGTATATGTCA
ACCAAGGTTTTGTTCAAATGACTGGTTACGAAACAGAAGAAATCTTGGGTAAAAATGCTAGATTCTTACAAGGTAAA
CACACTGATCCTGCCGAAGTAGACAATATAAGAACCGCTTTGCAAAACAAAGAACCAGTTACTGTACAAATACAAAA
CTACAAAAAGGATGGTACAATGTTCTGGAACGAATTGAACATCGATCCTATGGAAATAGAAGACAAGACATACTTCGT
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CTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGCATGA
GGTCGCTCGATGGGACGTCAGCACTGTACTTGTTTTTGCGACTAGATTGTAAATCATTCTTTATTTAATCTCTTTCTTTA
ACTACTGCTTAAAGTATAATTTGGTCCGTAGTTTAATAACTATACTAAGCGTAACAATGCATACTGACATTATAAGCCTG
AACATTACGAGTTTAAGTTGTATGTAGGCGTTCTGTAAGAGGTTACTGCGTAAATTATCAACGAATGCATTGGTGTATT
TGCGAAAGCTACTTCTTTTAACAAGTATTTACATAAGAATAATGGTGATCTGCTCAACTGATTTGGTGATAACTCTAACT
TTTTTAGCAACAATTTAAAAGATAATTCGAACATATATAACAGTAGGAAGAATTTGTGTACGTCAAATTAAGATAATTTA
GCATTACCAAAGTTATTAACCTAAACATAAAATATATATGAGACACATGTGGAAATCGTATGAAACAACTGTTATGAAA
CTGACAAGAATGAATATATAGAGTAAGCTCCGCTTGTAAAGAGGAATCACTTAAGTGTATAAATGTCTCGACGATTACT
TTAGATC

6.7 Sequences of pRS416-GAL1-FbFP

6.7.1 pRS416-GAL1-FbFP1 with complete construct

homologous region-GAL1-**ubiquitin**-linker-FbFP-FLAG-ADH1-homologous region, 1602 bp

GATATCGAATTCCTGCAGCCCGGG**TTTTTTTTGGATGGACGCAAAGAAG**TTTAATAATCATATTACATGGCATTACCA
CCATATACATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCT
AAAAAAACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCC
GAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAG
ATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTG
GCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTA
GCCTTATTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAA
CCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTG
TTAATATACCTCTATACT***TTAACGTCAAGGAGAAAAAACCC***ATGCAAATCTTCGTTAAGACCTTGACTGGTAAAACAAT
**CACCTTGGAAGTAGAATCTTCAGATACTATCGACAACGTCAAGTCTAAGATCCAAGATAAGGAAGGTATCCCAC
CTGATCAACAAAGATTGATTTTCGCTGGTAAACAATTGGAAGATGGTAGAACTTTGTCTGACTACAACATCCAA
AAGGAATCAACATTGCATTTGGTTTTGAGATTGAGAGGTGGTATCCACGGTTCCGGTGCATGGTTGTTACCAGTC**
TCATTGGTTAAGAGAAAGACTACATTGGCTCCTAACACCATGGCATCCTTTCAAAGTTTCGGTATTCCAGGTCAATTGG
AAGTTATTAAGAAAGCATTGGATCATGTCAGAGTTGGTGTTGTAATCACAGATCCAGCCTTAGAAGACAATCCTATTGT
ATATGTCAACCAAGGTTTTGTTCAAATGACTGGTTACGAAACAGAAGAAATCTTGGGTAAAAATGCTAGATTCTTACA
AGGTAAACACACTGATCCTGCCGAAGTAGACAATATAAGAACCGCTTTGCAAAACAAAGAACCAGTTACTGTACAAA
TACAAAACTACAAAAAGGATGGTACAATGTTCTGGAACGAATTGAACATCGATCCTATGGAAATAGAAGACAAGACAT
ACTTCGTTGGTATTCAAAACGATATCACCAAGCAAAAGGAATACGAAAAGTTGTTAGAAGATTACAAAGATGACGAT
GACAAGTAAGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGTATACAAATTTTAA
AGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTAT
AGCATGAGGTCGCTCACTAGTTCTAGAGCGGCCGCCACC

6.7.2 pRS416-GAL1-FbFP2 with linker deletion

homologous region-GAL1-**ubiquitin**-FbFP-FLAG-ADH1-homologous region, 1530 bp

GATATCGAATTCCTGCAGCCCGGG**TTTTTTTTGGATGGACGCAAAGAAG**TTTAATAATCATATTACATGGCATTACCA
CCATATACATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCT
AAAAAAACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCC
GAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAG
ATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTG
GCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTA
GCCTTATTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAA
CCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTG
TTAATATACCTCTATACTT***TTAACGTCAAGGAGAAAAAACCC***ATGCAAATCTTCGTTAAGACCTTGACTGGTAAAACAAT
**CACCTTGGAAGTAGAATCTTCAGATACTATCGACAACGTCAAGTCTAAGATCCAAGATAAGGAAGGTATCCCAC
CTGATCAACAAAGATTGATTTTCGCTGGTAAACAATTGGAAGATGGTAGAACTTTGTCTGACTACAACATCCAA
AAGGAATCAACATTGCATTTGGTTTTGAGATTGAGAGGTGGTATGGCATCCTTTCAAAGTTTCGGTATTCCAGGTC**
AATTGGAAGTTATTAAGAAAGCATTGGATCATGTCAGAGTTGGTGTTGTAATCACAGATCCAGCCTTAGAAGACAATC
CTATTGTATATGTCAACCAAGGTTTTGTTCAAATGACTGGTTACGAAACAGAAGAAATCTTGGGTAAAAATGCTAGATT
CTTACAAGGTAAACACACTGATCCTGCCGAAGTAGACAATATAAGAACCGCTTTGCAAAACAAAGAACCAGTTACTG

TACAAATACAAAACTACAAAAAGGATGGTACAATGTTCTGGAACGAATTGAACATCGATCCTATGGAAATAGAAGACA
AGACATACTTCGTTGGTATTCAAAACGATATCACCAAGCAAAAGGAATACGAAAAGTTGTTAGAAGATTACAAAGATG
ACGATGACAAGTAAGCGAATTTCTTATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGTATACAAAT
TTTAAAGTGACTCTTAGGTTTTAAAACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCA
GGTATAGCATGAGGTCGCTCACTAGTTCTAGAGCGGCCGCCACC

6.7.3 pRS416-GAL1-FbFP3 with linker and FLAG-tag deletion

homologous region-GAL1-**ubiquitin**-FbFP-ADH1-homologous region, 1506 bp

GATATCGAATTCCTGCAGCCCGGG**TTTTTTTTGGATGGACGCAAAGAAG**TTTAATAATCATATTACATGGCATTACCA
CCATATACATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCT
AAAAAAACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCC
GAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAG
ATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTG
GCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTA
GCCTTATTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAA
CCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTG
TTAATATACCTCTATACTT***TAACGTCAAGGAGAAAAAACCC***ATGCAAATCTTCGTTAAGACCTTGACTGGTAAAACAAT
**CACCTTGGAAGTAGAATCTTCAGATACTATCGACAACGTCAAGTCTAAGATCCAAGATAAGGAAGGTATCCCAC
CTGATCAACAAAGATTGATTTTCGCTGGTAAACAATTGGAAGATGGTAGAACTTTGTCTGACTACAACATCCAA
AAGGAATCAACATTGCATTTGGTTTTGAGATTGAGAGGTGGTATGGCATCCTTTCAAAGTTTCGGTATTCCAGGTC**
AATTGGAAGTTATTAAGAAAGCATTGGATCATGTCAGAGTTGGTGTTGTAATCACAGATCCAGCCTTAGAAGACAATC
CTATTGTATATGTCAACCAAGGTTTTGTTCAAATGACTGGTTACGAAACAGAAGAAATCTTGGGTAAAAATGCTAGATT
CTTACAAGGTAAACACACTGATCCTGCCGAAGTAGACAATATAAGAACCGCTTTGCAAAACAAAGAACCAGTTACTG
TACAAATACAAAACTACAAAAAGGATGGTACAATGTTCTGGAACGAATTGAACATCGATCCTATGGAAATAGAAGACA
AGACATACTTCGTTGGTATTCAAAACGATATCACCAAGCAAAAGGAATACGAAAAGTTGTTAGAATAAGCGAATTTCT
TATGATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAA
AACGAAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGCATGAGGTCGCTCACTA
GTTCTAGAGCGGCCGCCACC

6.7.4 pRS416-GAL1-FbFP4 with ubiquitin, linker and FLAG-tag deletion

homologous region-GAL1-FbFP-ADH1-homologous region, 1278bp

GATATCGAATTCCTGCAGCCCGGG**TTTTTTTTGGATGGACGCAAAGAAG**TTTAATAATCATATTACATGGCATTACCA
CCATATACATATCCATATACATATCCATATCTAATCTTACTTATATGTTGTGGAAATGTAAAGAGCCCCATTATCTTAGCCT
AAAAAAACCTTCTCTTTGGAACTTTCAGTAATACGCTTAACTGCTCATTGCTATATTGAAGTACGGATTAGAAGCCGCC
GAGCGGGTGACAGCCCTCCGAAGGAAGACTCTCCTCCGTGCGTCCTCGTCTTCACCGGTCGCGTTCCTGAAACGCAG
ATGTGCCTCGCGCCGCACTGCTCCGAACAATAAAGATTCTACAATACTAGCTTTTATGGTTATGAAGAGGAAAAATTG
GCAGTAACCTGGCCCCACAAACCTTCAAATGAACGAATCAAATTAACAACCATAGGATGATAATGCGATTAGTTTTTTA
GCCTTATTTCTGGGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAATGCAAAAACTGCATAA
CCACTTTAACTAATACTTTCAACATTTTCGGTTTGTATTACTTCTTATTCAAATGTAATAAAAGTATCAACAAAAAATTG
TTAATATACCTCTATACTT***TAACGTCAAGGAGAAAAAACCC***ATGGCATCCTTTCAAAGTTTCGGTATTCCAGGTCAATTG
GAAGTTATTAAGAAAGCATTGGATCATGTCAGAGTTGGTGTTGTAATCACAGATCCAGCCTTAGAAGACAATCCTATT
GTATATGTCAACCAAGGTTTTGTTCAAATGACTGGTTACGAAACAGAAGAAATCTTGGGTAAAAATGCTAGATTCTTA
CAAGGTAAACACACTGATCCTGCCGAAGTAGACAATATAAGAACCGCTTTGCAAAACAAAGAACCAGTTACTGTACA
AATACAAAACTACAAAAAGGATGGTACAATGTTCTGGAACGAATTGAACATCGATCCTATGGAAATAGAAGACAAGA

CATACTTCGTTGGTATTCAAAACGATATCACCAAGCAAAAGGAATACGAAAAGTTGTTAGAATAAGCGAATTTCTTATG
ATTTATGATTTTTATTATTAAATAAGTTATAAAAAAAATAAGTGTATACAAATTTTAAAGTGACTCTTAGGTTTTAAAACG
AAAATTCTTATTCTTGAGTAACTCTTTCCTGTAGGTCAGGTTGCTTTCTCAGGTATAGCATGAGGTCGCTCACTAGTTCT
AGAGCGGCCGCCACC

6.7.5 Integration site on pRS416

pRS416 4898bp, blue sequence indicates the homologous region recombined with insert DNA

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CCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTA
AAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGAC
GGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATATCGAATTCCTGCAGCCCGGG - inserted DNA -
ACTAGTTCTAGAGCGGCCGCCACCGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCC
CTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAA
TTCCACACAACATAGGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG

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Master Thesis in Master Programme of Chemistry and Bioscience

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