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The production of human G-protein coupled receptors under different *AOX1* promotor variants in *Komagataella phaffii (Pichia pastoris)*

Master thesis

to achieve the University degree of
Diplom-Ingenieurin

Master's degree programme: Biotechnology

submitted to

Graz University of Technology

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Graz, November 2020

Affidavit

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master's thesis.

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Signature Date

Acknowledgement

First of all, I would like to thank my external supervisor Dr. Roland Weis at the company "Validogen" who made this thesis possible and supported me throughout my whole project laboratory and master thesis with an endless effort and persistence despite his lack of time. Next, I want to give a big thank to my internal supervisor at the "Institute of Molecular Biotechnology" Prof. Dr. Harald Pichler who led me through my whole thesis with great advice, provided me with critical discussions and always had time for me.

I want to thank all my colleagues at the company for taking me up so kindly and making an enjoyable and interesting master thesis project possible to me. I want to thank Dr. Iskandar Dib, Dr. Alexander Kern, Dr. Aid Atlic, Hanna Schantl, Heinz Plank and Dr. Thomas Purkharthofer, who cheered me up, gave me advice and critical discussions. Special thanks go to Dipl. Ing. Eva Tacha who supported me with my Western blots and Coomassie stains, was omnipresent in the laboratory and became a best friend to me. I especially want to thank Ulrike Hohensinner and Anita Deutscher who always were there for me with a helping hand and explanations for cloning and detection in the laboratory and for critical discussions. Special thanks also go to my office colleagues Dr. Evelyn Trummer-Gödl and Regina Grach for their emotional support, critical discussions and ordering of labware. I thank Max Pichler for critical discussion and providing me with his fur hat for the cooling chamber. I especially thank Dr. Michael Koncar, the CEO of the "Konvalue Holding GmbH" group for making this thesis possible and employing me for this project.

I thank the "Pichler group" in Petersgasse 14 on the second floor for letting me work in their laboratory, listening to my presentation and for critical discussions. Especially, I thank Dr. Anita Emmerstorfer-Augustin who supported me with ultracentrifuging, precipitating my samples and with critical discussions. Special thanks go to Alina Voit, MSc who`s master thesis gave input for my studies, helped structuring my work and was an inspiration for the cell lysis and protein isolation protocols.

I thank Dr. Tarek Moustafa and his research group at the "Centre for medical research" in Graz for examining my MP-GFP products with fluorescence microscopy and providing me with the fluorescence microscopy images.

A big thank goes to my boyfriend Michael Sommerer who tried to understand my work as best as possible and supported me emotionally. I want to thank my family and friends up in the "Waldviertel" who always cheered me up, showed patience with me and tried to understand my work. I thank also all my friends and study colleagues in Graz for their emotional support and several critical discussions about my work.

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Abstract

G-protein coupled receptors are very important integral membrane proteins that bind many different ligands. They mediate several downstream signalling cascades in cells and are therefore involved in many, diverse physiological functions. As they make up 50 % of all drug targets inside the human body, many drugs against cardiovascular, metabolic and psychiatric diseases bind to them. So it is important to have structural models of them to evaluate binding of novel drugs to them. As they are difficult to isolate naturally, it is tried to produce them recombinantly in different hosts such as *P. pastoris*.

In the thesis, the coding sequences for the human β2-adrenergic receptor, the 5-hydroxytryptamine serotonergic receptor 2c and the 5a subtype with His-tags and/or Strep-tags I were ligated in pPZ plasmids with different methanol-inducible and methanol-free variants of the *AOX1* promoter. The plasmids were used to transform competent *E. coli* DH5-α cells and *P. pastoris* mut^s cells. Clones were cultivated under methanol-induced or methanol-free conditions. The protein lysates were ultracentrifuged and precipitated for clearer results. Lysates were examined for their total protein concentration in BCA assays. They were de-glycosylated before loading them onto SDS-PA gels to obtain sharper distinct bands and reduce background. Anti-His- and anti-Strep-tag I Western blots and Coomassie brilliant blue stains were made to detect recombinant protein production. Optimization of production of the recombinant proteins was assessed by expressing proteins with the α-mating factor (α-MF) signal sequence instead of the native signal peptide (where applicable). It was tried to enhance production by reducing the induction temperature, adding Fos Choline 14 before or after lysis and coupling a green fluorescent protein to the membrane protein (C-terminally).

The β2AR was the best protein expressed with levels of around 1 µg/ml. Receptors were better expressed with their native signal sequence and with methanol-inducible promoters. The 5-HT 5a receptor was expressed despite lacking a signal sequence. Induction at 28 °C was suitable and the addition of the detergent Fos Choline 14 did not succeed in increasing yield or providing a reduction of background on the gels and blots. The 5-HT 2c was expressed well with the native signal sequence, but poorly with the αMF signal sequence. The membrane protein-GFP fusions were expressed at lower levels. From the β2AR-GFP in cells 10 h after induction, very clear fluorescence images with recombinant proteins putatively localized in the endosome were seen.

Zusammenfassung

G-Protein gekoppelte Rezeptoren gehören zu den wichtigsten Membranproteinen. Sie binden die verschiedensten Moleküle, Agonisten und Neurotransmitter. Diese Rezeptoren aktivieren außerdem diverse Signalkaskaden in Zellen und sind darum an vielen physiologischen Prozessen maßgeblich beteiligt. Da sie Ziele von 50 % aller Medikamentenwirkstoffe im menschlichen Körper darstellen, binden die verschiedensten Medikamente gegen Herz-Kreislauf-, Stoffwechselerkrankungen und psychischen Krankheiten an diese Rezeptoren. Daher sind Strukturmodelle der G-protein gekoppelten Rezeptoren sehr wichtig, um das Binden von neuen Medikamenten zu testen. Meist werden die Membranproteine rekombinant in verschiedenen Organismen produziert, weil die Rezeptoren schwierig aus dem menschlichen Körper zu isolieren sind.

In dieser Arbeit wurden die kodierenden Sequenzen von humanen β2-Adrenorezeptoren und 5-Hydroxytryptamin serotonergenen Rezeptoren der Untergruppe 2c und 5a mit His-tags und/oder Strep-tags I in pPZ Plasmide ligiert. Die Membranproteine konnten so später unter Methanol-induzierten und Methanol-freien Varianten des *AOX1* Promoters exprimiert werden. Die Plasmide wurden zur Transformation kompetenter *E. coli* DH5-α und *P. pastoris* mut^s Zellen verwendet. Die Klone wurden unter Methanol-induzierten oder Methanol-freien Bedingungen kultiviert. Die Proteinlysate wurden ultrazentrifugiert und präzipitiert, um reinere Proben zu erhalten. Die Lysate wurden auf ihre Gesamtproteinkonzentration in BCA-Tests untersucht. Die Proben wurden außerdem deglykosyliert bevor sie auf SDS-PA Gele geladen wurden, um stärker differenzierte Banden zu bekommen und um den Hintergrund zu reduzieren. Dann wurden anti-His-tag und anti-Strep-tag I Western blots und „Coomassie brilliant blue“ Färbungen gemacht, um die Produktion der rekombinanten Proteine zu visualisieren. Danach wurde versucht, die Produktion der rekombinanten Membranproteine zu optimieren. Ein Ansatz dabei war, die Proteine mit der Signalsequenz des α-Paarungsfaktors statt mit der natürlicher Signalsequenz zu exprimieren, sofern es möglich war. Außerdem wurde versucht die Temperatur in der Induktionsphase niedriger zu halten, um die Produktionsrate zu erhöhen. Andere Versuche waren, Fos Choline 14 vor oder nach der Zell-Lyse hinzuzufügen oder das Membranprotein mit einem am C-Terminus gekoppelten grünen Fluoreszenzprotein zu detektieren.

Der β2-Adrenorezeptor wurde mit 1 µg/ml Konzentration am besten exprimiert. Die Rezeptoren wurden mit ihrer natürlichen Signalsequenz und mit Methanol-induzierten Promotoren besser exprimiert. Der 5-HT 5a Rezeptor wurde auch ohne erkennbare Signalsequenz exprimiert. Die Induktionsphasen bei 28 °C waren gut geeignet und die Zugabe von Fos Choline 14 zeigte keinerlei Verbesserung in der Erhöhung der Ausbeute und in der Klarheit der Western blots. Der 5-HT 2c Rezeptor wurde mit seiner natürlichen Signalsequenz gut exprimiert, aber eher schlecht mit der Signalsequenz des α-Paarungsfaktors. Die Membranprotein-GFP Fusionen wurden schlechter produziert als die Membranproteine alleine. Sehr gute Fluoreszenzaufnahmen konnten vom β2-Adrenorezeptoren, die sich nach 10 Stunden Induktion vermeintlich in den Endosomen der Zellen befanden, gemacht werden.

List of abbreviations

Table 1: Different words occurring in the thesis and their used abbreviations

Word	**Abbreviation**
5-hydroxytryptamine	5-HT
YPhyD agar plates with the triple amount of Zeocin added	3x Z
Alcohol oxidase 1/2	*AOX1/2*
Alpha mating factor	αMF
Amino terminus	N-terminus
Antibody	AB
Basepairs	bp
β2-adrenergic receptor	β2AR
Bovine serum albumine	BSA
Buffered minimal dextrose medium	BMMD
Buffered minimal methanol medium	BMM2
Buffered minimal methanol medium	BMM10/15
Buffered minimal sorbitol medium	BMS1, BMS15
Bicinchoninic acid	BCA
Carboxyl terminus	C-terminus
*Eco*RI/*Not*I restriction enzyme cut	E/N
Endoglycosidase H	Endo H
Endoplasmatic reticulum	ER
Ethylenediaminetetraacetic acid	EDTA
Fast digest	FD
Gravitational force equivalent = g-force	g
Green fluorescent protein	GFP
G-protein coupled receptors	GPCRs
Guanosine diphosphate	GDP
Guanosine triphosphate	GTP
Hydrochloric acid	HCl
Histidine	His
Horseradish peroxidase	HRP
Human serum albumin	HSA
Lithium dodecyl sulfate	LDS
Lysergic acid diethylamide	LSD
Lysogeny broth	LB
Magnesium chloride	$MgCl_2$
Magnesium sulfate	$MgSO_4$
Membrane protein	MP
Methanol	MeOH
2-(N-morpholino)ethanesulfonic acid buffer	MES

Methanol utilization type plus	mut^+
Methanol utilization type slow	mut^s
New Prom (new promoters)	NP
Optical density at 600 nm	OD_{600}
Phenylmethylsulfonyl fluoride	PMSF
Phosphate buffered saline	PBS
Polyvinylidene difluoride	PVDF
Potassium chloride	KCl
Potassium dihydrogen phosphate	KH_2PO_4
Relative fluorescence units	RFU
Revolutions per minute	rpm
Room temperature	RT
Sodium chloride	NaCl
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	SDS-PAGE
Disodium hydrogenphosphate	Na_2HPO_4
Monosodium dihydrogenphosphate	NaH_2PO_4
Sodium phosphate buffer	NaPi
Streptavidin-binding	Strep
Super optimal broth with catabolite repression	SOC
Tobacco etch virus	TEV
Tris acetate EDTA buffer	TAE
Trichloro acetic acid	TCA
3,3',5,5'-Tetramethylbenzidine	TMB
Tris(hydroxymethyl)aminomethane	Tris
Ultracentrifuge	Ultrac
Yeast nitrogen base	YNB
Yeast extract phyton peptone dextrose media	YPhyD
XhoI/*NotI* restriction enzyme cut	X/N

1 Introduction and scientific background

1.1 Integral membrane proteins

Integral membrane proteins (MPs) are an essential part of biological membranes. In combination with the lipids inside the bilayer, they are responsible for different activities of membranes. These activities include the transport of matter or information, excitation of the membrane and protection of the cell from influences outside. (Guidotti, 1972)

Membrane proteins are of great interest for academia and industry as they are involved in important cellular functions and there are still not many 3D structures available of them. To obtain high amounts of MPs, for example to obtain high resolution structures and benefit from an easier handling, they are produced in recombinant hosts. This enables gaining insights in molecular mechanisms as well. Production processes in hosts are optimized to obtain stable, functional receptors at high yields instead of isolating them from other biological sources. (Hedfalk, 2013)

1.2 G-protein coupled receptors

One of the most abundant group of proteins in humans and other vertebrates are G-protein coupled receptors (GPCRs), membrane proteins. They all share seven times transmembrane spanning α-helix domains. These consist of 25-35 consecutive amino acids going through the plasma membrane. The anti-clockwise α-helixes are conserved and mediate as connectors and also as recognition receptors for ligands that bind on the cell. Apart from their basic structure, they are very different to each other regarding their ligands, interactions with other proteins and physiological functions. The ligands vary from ions, hormones and organic odorants to proteins, lipids and nucleotides. The so-called GRAFS system is used to group the GPCRs according the ligands they bind. The families are glutamate (G), rhodopsin (R), adhesion (A), frizzled/ taste 2 (F) and secretin (S). They can be grouped further into diverse subgroups. (Schiöth, 2005)

1.2.1 Signalling of GPCRs

The GPCRs are in a low-affinity state when no ligand is bound, but turn into a transient high-affinity state upon binding. When the receptor is activated, the GDP molecule on the G-protein coupled to the receptor is exchanged with a GTP. The subunits of the protein bound separate in an α unit and a βγ dimer. These subunits can be divided into further different types. The subunits activate signalling and mediator proteins in the downstream signalling cascade until action of the signal on the cell`s transcription factors. There exist many mechanisms for fine tuning this signalling ways as there are so many different proteins available for the cascade. Rapid dampening of the receptor occurs by regulation through phosphorylation with G-protein specific kinases. (Pierce, 2002)

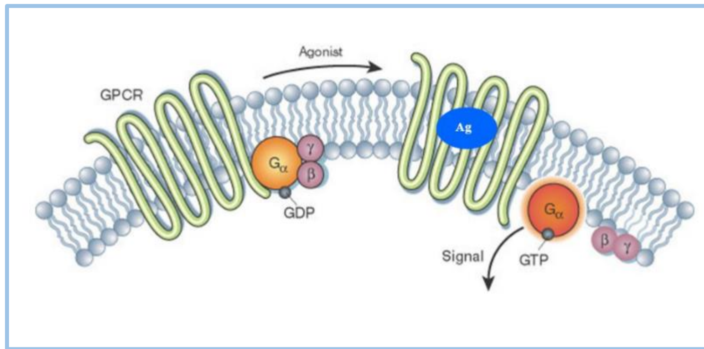


Figure 1: A GPCR and the associated G-protein

Image of the GPCR in a resting state where GDP is bond to the G-protein that is associated with the receptor. The β and γ subunit are associated with the α subunit of the G-protein. On the right side the GPCR with an agonist bond to it is depicted. The GDP is exchanged for a GTP on the α subunit and subsequently the β and γ subunits dissociate. *(Naatz, 2019)*

In addition, some signalling pathways are based on interactions of the GPCRs with adaptor and scaffolding proteins. These enable the receptor to interact with small second messengers in the downstream signalling cascade. Various other receptor-protein complexes have been shown to exist for special signalling pathways. They can deviate from normal mechanisms. (Pierce, 2002)

1.2.2 Signal sequences of G-protein coupled receptors

The proteins produced at the ribosome contain a signal sequence that directs them to their final destination in or outside of the cell. There are N-terminal signal peptides present that are often removed after translocation of the protein. Or there are internal signal sequences that are also called signal anchor sequences that remain in the protein sequence after successful translocation. The anchor sequences are usually in the first transmembrane domains found. Only 5-10 % of all GPCRs contain N-terminal signal sequences, the others only have signal anchor sequences. To determine the N-terminal signal sequences of proteins, prediction programs are used. Yet, signal anchor sequences hardly can be predicted. The signal sequences present in GPCRs are not distributed equally over the families, but are also not concentrated in one type. In the rhodopsin family they appear to be very rare. The GPCR signalling sequences do not only target the receptors to their correct location in the cell membrane, but also control the gating in translocation, receptor densities at the plasma membrane and G-protein coupling selectivity. (Rutz, 2015)

1.2.3 GPCRs in medicine

As GPCRs can bind so many different ligands and participate in such a variety of cellular processes and signal transduction pathways, they are also involved in many cardiovascular, metabolic and also psychiatric diseases and their treatment. This is the reason why they make up 50 % of all current drug targets. For the feasibility studies in pharmaceutical companies to develop new medicinal products it is important to have reasonable structural models of the transmembrane receptors. 3D models and crystallization structures are highly needed to evaluate binding of ligands to them, such as medications. (Lundstrom, 2006) Many proteomic analyses of plasma membrane proteins have been only performed with 2D-gels and mass spectroscopy so far. This is due to the fact that it is very difficult to get clean preparations of natural plasma

membrane proteins. Furthermore, the proteins are big-sized and hydrophobic which leads to difficulties in enrichment of them. (Zhao, 2004) In addition, they are not produced in high amounts naturally. (Andre, 2006)

Therefore, it is tried to produce the membrane proteins and receptors recombinantly in different hosts to have more material available for diligent structural and functional studies. In addition, they show a higher quality and production is optimized more easily. (Andre, 2006)

1.2.4 The β2-adrenergic receptor

β-adrenergic receptors (βAR) are part of the GPCR class. They consist of three subtypes of βAR: β1AR, β2AR and β3AR that are all coupled to a stimulatory Gs-protein subtype of the G-proteins. B1ARs are present largely in the heart to affect cardiac function and in the brain for controlling synaptic plasticity. The β2AR is the predominant type in vascular and bronchia smooth muscles. So it is used to address cardiovascular and pulmonary diseases. B3AR is located in white and brown adipose tissue. The β2AR was chosen to be expressed in the production host *P. pastoris* as it is of high medical relevance, is the best characterized type and was already expressed successfully in this host so far. (Voit, 2017) (Talmont, 2009)

The β2AR consists of 413 amino acid residues and its structure and functionality is of high importance for the development of new medicinal products. (Noguchi, 2006) Signalling pathways induced by the β2AR lead to downregulation of constrictive pathways and activation of dilating pathways in smooth muscles. With synthetic agonists for the receptor it is tried to keep the β2AR in a particular conformation that might be of medical importance. (Mc Graw, 2005)

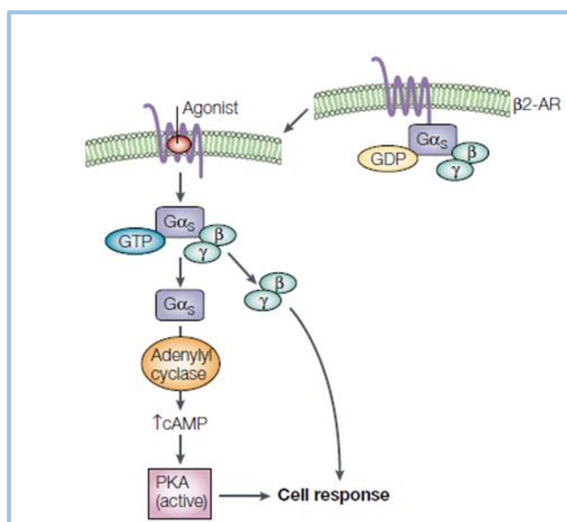


Figure 2: Signalling pathways downstream of GPCRs

Activation of the β2-adrenergic GPCR by binding of a ligand and subsequent downstream signalling by activation of a stimulatory Gαs-protein by exchange of GDP for GTP. The stimulatory G-protein activates further kinases, signalling proteins or secondary messengers. (Pierce, 2002)

1.2.5 The 5-hydroxytryptamine serotonergic receptors

The 5-hydroxytryptamine receptors are a family of GPCRs and are part of different downstream signalling pathways in the central nervous system and in the periphery.

There are different subtypes present that are indicated with numbers, such as the 5-HT 2 subtype. There have been a lot of studies that indicate their presence in several central nervous system disorders such as depression and migraine. So there is interest to produce higher amounts of them recombinantly to be able to form crystallisation structures and 3D-models of them. Furthermore, these studies will help to develop novel pharmaceuticals against these severe psychiatric conditions by binding to these receptors. (Knauer, 2009)

1.2.5.1 The 5-hydroxytryptamine 2c serotonergic receptor

Especially the 5-HT 2c subtype of the serotonergic receptors seems to be appealing as a pharmaceutical target as it has been observed to be involved in the inhibition of transmission and release of dopamine and noradrenaline in the frontal cortex resulting in psychiatric diseases such as schizophrenia and depression. However, it does not inhibit pathways of the other 5-HT receptor subtypes. So by producing this receptor in high amounts and gaining more structural knowledge of it, it will be possible to develop antagonists for this receptor that counteract the inhibition by the 5-HT 2c protein. (Millan, 1998) It is also involved in neuronal activation of the amygdala corticotropin-releasing hormone that is mediating responses in anxiety. So dysfunction of this receptor can also result in chronically elevated anxiety diseases. (Heisler, 2007)

The 5-HT 2c receptor is one of a few GPCRs that has an N-terminal signal sequence. (Jahnsen, 2012)

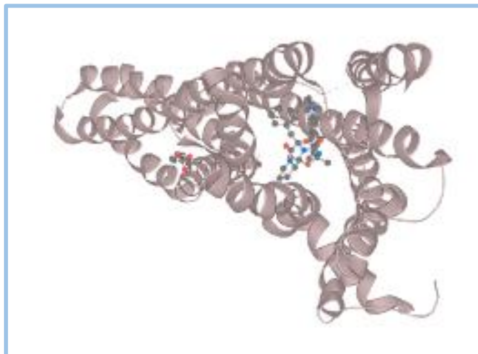


Figure 3: Structure of the 5-HT 2c protein

The 5-HT 2c chain A. A secondary structure in complex with the agonist ergotamine obtained with X-Ray. (Uniprot, 2019)

1.2.5.2 The 5-hydroxytryptamine 5a serotonin receptor

The 5-hydroxytryptamine 5 subgroup activates many different signalling pathways. Especially the 5-HT 5a subtype plays a role in many second messenger cascades. It is found in different parts of the brain regions such as in astrocytes, in the cerebral cortex and in the hippocampus. The receptor takes part in many physiological conditions such as memory, learning, expression of emotions, motor coordination and performance under stressful conditions. The drug LSD seems to mediate its action by binding to this receptor. Mutations in the gene of the 5-HT 5a lead to chronic diseases including schizophrenia and affective disorders. (Noda, 2004) The presence of the 5-HT system is important for a healthy prefrontal cortex. Critical changes of it in the brain have been shown to happen in aging or amnesia. So by characterizing the receptor in

more detail it might be possible to develop medications acting as receptor antagonists against diseases. (Gonzalez, 2013)

1.3 *E. coli* DH5-alpha strain

The *E. coli* DH5-alpha strain was especially designed for cloning approaches and transformations in the laboratory. The strain has a lot of mutations that lead to a high efficiency for cloning. The endA1 mutation for example enables higher plasmid transfer as endonuclease-based degradation is lower. The recA1 mutation lowers homologous recombination and so inserts are more stable. (Microbewiki, 2015)

1.4 *Komagataella phaffii* (*Pichia pastoris*) as expression host

The methylotrophic yeast *Komagataella phaffi* (from now on called *Pichia pastoris* in this thesis) is an important expression host for eukaryotic proteins such as integral membrane proteins for production in high amounts. *P. pastoris* has the cellular machinery capable of performing synthesis, maturation and localization of eukaryotic proteins. (Bornert, 2012) It provides rapid expression at low cost. This expression host is suitable for large scale bioreactor-cultivation where parameters can be easily monitored. Recombinant proteins can be targeted for different cellular compartments or for secretion. (Byrne, 2015)

1.4.1 The methanol-inducible promotor

P. pastoris has a strong methanol-inducible promotor that is associated with the methanol utilization pathway. It is often exploited for the high-level expression of recombinant genes. (Bornert, 2012) The promotor for the *AOX1* gene (P_{AOX1}) is repressed by glucose and induced by methanol. (Byrne, 2015) The gene of the alcohol oxidase is originally expressed to oxidize methanol to formaldehyde and hydrogen peroxide in the peroxisome. The *AOX1* is in contrast to the *AOX2* gene mainly involved in enzyme activity. (Cregg, 2000) The *AOX1* as well as the *AOX2* gene are regulated on transcriptional level by repression, de-repression and induction mechanisms. The two enzymes produced are very closely related in their primary sequences, but their flanking sequences differ. There is always a higher level of *AOX1* transcript due to a difference in the transcription initiation rate of the two genes. So there is an evidence that in nature the *AOX2* enzyme must be conserved for a specific activity that offers a selective advantage over other microorganisms. However, it still remains to be elucidated under which circumstances this advantage occurs. (Cregg J. M., 1989)

1.4.2 The expression vectors

Plasmids that are constructed for recombinant expression in *P. pastoris* need some general elements. Mostly, a yeast/*E. coli* shuttle vector is used. (Bornert, 2012) The foreign gene with a signal sequence that is introduced in the vector is connected to the P_{AOX1} and terminator (T_{AOX1}) or alternative ones. There are many different restriction sites for integration set depending on the vector applied. As a selectable marker a His4 gene for example, is located on the plasmid or an antibiotic resistance gene to provide a growth advantage of clones that have successfully incorporated the plasmid only. Sequences for the replication and maintenance of the vector in bacteria must be also

present if maintenance of the plasmid in a bacterial host is required before going into a eukaryotic host. (Cregg, 2000)

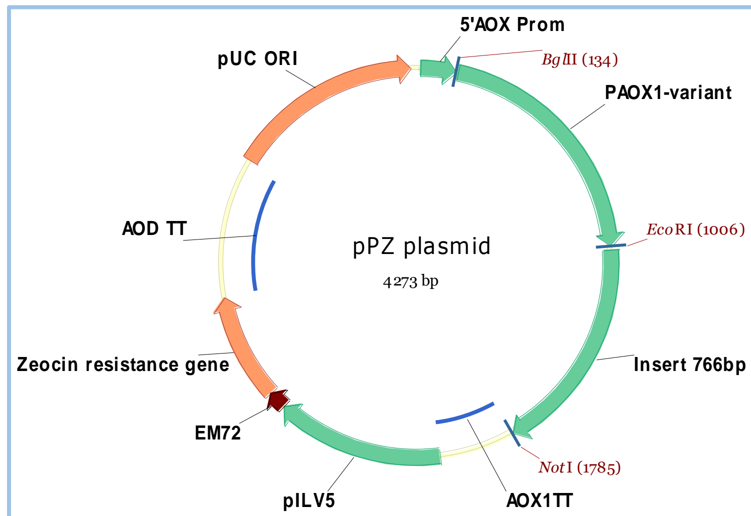


Figure 4: Expression vector for the GPCRs

Expression vector pPZ used for transformation of *P. pastoris* strains. The genes for the membrane proteins are ligated in the multiple cloning site between the *Eco*RI and *Not*I restriction sites. So the gene is under the control of P_{AOX1}. The vector carries a Zeocin resistance cassette controlled by each, one dedicated-promotor for expression in *E. coli* and *P.pastoris*, as well as an origin of replication for maintenance in *E. coli*. The other vectors used in the thesis are displayed in the appendix.

1.4.3 Copy numbers in the genome

The expression vectors integrate into the genome of *P. pastoris* by pairing of a vector region to a corresponding location in the genome. So it is possible to get a stable expression of the gene of interest. The number of vector copies that introduce in the genome cannot be controlled easily. There is a chance to obtain multiple copy integrations. The copy numbers are estimated by colony screening. The concentration of the antibiotic to which the construct carries a resistance gene is increased stepwise in the medium. The higher the concentration of the antibiotic gets, the more copies of the resistance gene are required in the genome. However, the number of copies is not always associated with higher expression of the recombinant protein. So also functional protein expression should be checked with assays. (Byrne, 2015) As the P_{AOX1} produces more enzyme it is tried to get an integration of constructs at its site. The P_{AOX1} initiates the mut pathway and has a very high strength. (Juturu, 2018)

1.4.4 Secretion of recombinant proteins

Heterologous proteins produced in *P. pastoris* can be expressed in the cell or secreted into the cultivation medium. Secretion is a good way as a first purification step for recombinant proteins because native *P. pastoris* proteins are not secreted to significant levels. The recombinant protein needs a signal sequence consisting (at least) of a signal peptide for translocation into the ER and potentially also a “pro”-sequence for guidance to the Golgi-apparatus and eventually secretion to the exterior of the cell. The success of different signal peptides is variable for different protein types. The native signal sequence of the recombinant protein can be used or other prominent

signal sequences as the alpha-mating factor pre-pro signal peptide are usually assessed. (Cregg, 2000)

1.4.5 Methanol free expression

The expression of recombinant proteins with the P_{AOX1} also has some disadvantages. Methanol has to be used for induction that is toxic and highly flammable. One by-product of the metabolic pathway, hydrogen peroxide, poses oxidative stress on the cells. In addition, the P_{AOX1} is very tightly controlled and can overload the transcription machinery of the cells upon induction, potentially resulting in misfolded or mis-targeted proteins. So expression hosts were developed where the P_{AOX1} can be functional without methanol. This is achieved by overexpressing activators and knocking down repressors. Two kinases that are involved in pathways regulating the promotor are knocked out for example. The promotor is then induced with glycerol or dihydroxyacetone and repressed by glucose. (Shen, 2016)

1.4.6 Mut^s strains

In addition to the strong P_{AOX1}, there is a much weaker promotor for the *AOX2* gene (P_{AOX2}) that leads to transcription and translation of an alcohol oxidase 2 that is not as active as the oxidase 1. Mut^s strains stand for methanol utilization type slow. This is due to the fact that the P_{AOX1} is knocked out and only the P_{AOX2} is active. Mut$^+$ strains show higher growth than mut^s strains, but mut^s strains can have higher productivities when certain proteins have to be expressed. In addition, less stress is posed on the cells and there is less production of hydrogen peroxide. They have lower oxygen requirements and lower methanol consumption rates than mut$^+$ strains. (Krainer, 2012) It was decided to use the mut^s strain for expression of membrane proteins to limit the metabolic burden on the cells and assure a correct translation and translocation process.

1.4.7 Fine-tuned expression with methanol-induced and methanol-free promoter variants

A library of P_{AOX1} with different expression characteristics can be formed by combining deletions and duplications of different transcription factor-binding sites of the *AOX1* gene sequence. The cis-acting elements that are involved in protein expression are mutated by random mutagenesis methods such as PCR with a Pfu Ultra polymerase or overlap extension PCR. They are cloned into a vector with a protein, such as GFP, to be expressed and are screened for the expression pattern of cultivated *P. pastoris* clones under different conditions. In case of the GFP it is screened for a fluorescence output. When deleting bases or duplicating regions of transcription factor binding sites different promoter variants are obtained. These variants either up- or downregulate gene transcription. Some show their effect during de-repression after glucose is (almost or completely) depleted or after methanol induction. Some variants increase protein expression whereas others decrease it under methanol induction. The strongest de-repression variants are called d6 and d6*. Deletions in the region called d1 lead to a higher expression under methanol induction. With the results from these mutagenesis studies new, tailor- made promoter variants can be created. This is done by deleting bases in some regions of the promoter sequence and combining this with

duplications of other regions on the sequence. So constructs, such as the d2d6 promoter variant, are generated that combine benefits from both. (Hartner, 2008)

1.4.8 Optimization of recombinant protein production

There are factors that have a strong impact on the expression efficiency of the recombinant protein. Firstly, by optimizing the codons of the gene of interest to the corresponding expression host, less aggregation of the protein occurs. Secondly, N- and C-terminal tags help to verify primary authenticity of the protein and to obtain a highly pure protein by for instance applying affinity chromatography. Most often, FLAG and His tag constructs are used. Thirdly, to increase correct targeting of the recombinant proteins in- or outside of the cell, an alpha-mating factor signal sequence of *S. cerevisiae* is fused at the 5` end of the gene construct. The native signal sequence of the protein can also be used. Fourthly, an optimization of culture conditions such as pH-value, temperature and induction time leads to better expression levels. What is more, the choice of the yeast strain should be done with regard to the protein type of interest and its characteristics. (Byrne, 2015) For the production of membrane proteins higher gene dosages do not always lead to more functional proteins, but rather limit growth and can enforce the production of inclusion bodies. Inducible promoters are favoured over constitutive ones as they retard cell growth. (Freigassner, 2005)

For the 5-HT serotonin receptor and the β2-adrenergic receptor it has been reported that higher copy numbers of the incorporated gene increase the receptor expression levels without overloading the cellular machinery. However, to improve translation and stability of the protein, the N-linked glycosylation and disulphide bridges are removed from the gene construct before. (Hedfalk, 2013)

For some receptor also the addition of 2 % DMSO to the methanol induction medium led to higher expression levels of recombinant proteins. This can be explained by the fact that it slightly permeabilizes the cell membrane at some points. (Shukla, 2007)

Unfortunately, it was observed that upon overexpression, each membrane protein interacts individually in the host and might respond to the measures taken or not. The success rates of expression are closely linked to the specific intrinsic properties of the membrane proteins. (Freigassner, 2009)

1.5 Yeast cell lysis

Host cells are lysed in order to gain proteins of interest such as an integral membrane protein from cellular compartments or the cell membrane. There are different enzymatic, chemical and mechanical techniques possible. The choice of the disruption method has a high impact on the number of steps that remain for subsequent downstream processing, purity of the protein and on the detection of promising results. (Naglak, 1990)

1.5.1 Enzymatic lysis

There are bacteriolytic and yeast-lysing enzymes that can attack the cell walls of the heterologous hosts for release. The cell wall of yeasts consists of glucans and chitin that form a scaffold and give the wall its shape and flexibility. Mannoproteins limit the permeability through the membrane. So a mixture of different enzymes has to be

applied for cell wall degradation. A lytic protease opens the structure, then a glucanase can the inner wall. In vitro, reducing agents such as β-mercaptoethanol are applied to break disulphide bridges. When the wall is broken, the plasma membrane and cell content are released as protoplast. An osmotic support sucrose is added so that the proteins are not degraded. (Salazar, 2007)

1.5.2 Chemical permeabilization

Cells are suspended in treatment buffer, added to guanidine hydrochloride or Triton X-100 solutions as treatment chemicals and shaken. It has been demonstrated that this procedure releases the same amount of protein from cells as other disruption methods. The difference to the other methods is, that it maintains the morphology of the cells and does not actually lyse them. The proteins are released and the cells are easily separated from them. (Naglak, 1990)

1.5.3 Mechanical disruption with glass beads

At small scale, the cells are re-suspended in lysis buffer and glass beads are added. Cells are lysed by using bead beating devices or vortexing. After ultracentrifugation, the lysate is taken up in a Tris-HCl-DTT buffer. (Sarramegna, 2002) At larger scales up to 15 ml, pellets are suspended in breaking buffer and are transferred into a Merckenschlager vessel with glass beads for lysis. (Voit, 2017) This disruption method does not need much specific equipment and is easily handled. Disruption by glass beads is rather used at laboratory scale, but at larger scales with suspended volumes of 500 ml or more, industry bead mills or high pressure homogenizers are required. (Ramanan, 2008)

1.5.4 Solubilisation of membrane proteins

Sometimes it can be difficult to release the hydrophobic domains of membrane proteins from cell membranes. Therefore, it is proposed to use solubilizing agents such as Fos Choline 14. These reagents are amphipathic and form micelles in which they embed membrane proteins and keep them in an aqueous solution. (CubeBiotech, 2013) Solubilisation needs to be optimized to decrease protein denaturation and get higher yields of the target protein. Zwitterionic detergents such as Fos Choline 14 added during cell lysis or afterwards were shown to almost completely solubilize target membrane proteins from the membranes. (Worms, 2019)

1.6 Detection of proteins by their affinity tags

Different affinity tags, such as a His- or a Strep-tag can be added after the signal sequence at the beginning of an amino acid sequence or at the end of the sequence. Various affinity tags are used for the detection and isolation of only the desired protein that has the tag attached. They can be isolated and purified with columns providing special bindings to the tags. (Terpe K. , 2007) Moreover, these primary, or in other cases secondary, antibodies are conjugated to an HRP enzyme that is able to oxidize a benzidene chromogen (TMB) used as substrate to a blue-coloured reaction product. The blue colour is visible only at the protein bands detected by the (primary) antibody before. (Mesulam, 1976)

1.7 Detection of membrane proteins by a GFP tag

Membrane proteins are often poorly expressed and a large number of clones has to be checked. For screening larger amounts of expression clones it is convenient to fuse a GFP to the C-terminus of the recombinant membrane protein and rapidly detect well-expressing clones after cultivation. The GFP is often linked to the gene of the desired protein with a TEV protease cleavage site and a few amino acids as linker-region so that the protein can be released in a later step and further purified. (Brooks, 2013) Linkers have been observed to be very important for stable, bioactive fusion proteins and to avoid mis-fold. As a linker relatively small, polar amino acids such as Glycine and Serine are favourable due to their flexibility. (Chen, 2013) Already a few hours after induction fluorescence intensity can be measured at 395 nm excitation and 509 nm emission with samples in a microplate on a spectrometer. The fluorescence intensity can be given in relative fluorescence units. It is a good estimation on the overexpression levels of the membrane proteins. (Drew, 2006) The fluorescence intensity might decrease with induction time due to overproduction of the recombinant protein and concomitant overload of the translational machinery leading to protein accumulation in the ER. These proteins are then subject to degradation or rescue routes. (Freigassner, 2009)

2 Materials

2.1. Laboratory equipment

For all steps done in the laboratory different equipment was needed.

Table 2: The task done with the equipment needed for it and its manufacturer

Working step	**Equipment**	**Manufacturer**
Absorption measurement	Spectra A max Cuvettes	Molecular devices, US Thermofisher Scientific, US
Agarose gel electro-phoresis	GelDoc XR+ Gel chambers, trays, control unit Microwave Balance	Biorad, US Biorad, US Silva Kern & Sohn GmbH, Germany
Autoclaving	Autoclave VX150	Systec GmbH, US
Cell lysis	Glass beads Vortex Ultracentrifuge TLA 100.4	Roth GmbH, Germany Roth GmbH, Germany Beckman Coulter GmbH, US
DNA concentration measurement	NanoVue Plus	Biochrom, US
Electroporation	Micropulser Electroporation cuvettes (2 mm gap)	Biorad, US Biorad, US
Fluorescence screen	Spectra Max M2 Fluorescence plate 96-wells	Molecular devices, US Thermofisher Scientific, US
Glycerolstocks	Pipet for viscous fluids Cryotubes Labelling caps Cryoboxes	Gilson, US Thermofisher Scientific, US Thermofisher Scientific, US VWR, US
Harvest of cells	Tabletop centrifuge 5810 R	Eppendorf, Germany
Heating	Thermomixer	Eppendorf, Germany
Incubation 37°C	Incubator Shaking incubator	Binder GmbH, Germany Labforce
Incubation 28°C	Incubator Shaking incubator	Binder GmbH, Germany Labforce
Microcentrifuge	Centrifuge 5424 R	Eppendorf, Germany
Mixing	Vortex	Heidolph, Germany
PCR	PCR Mastercycler X50s PCR tubes	Eppendorf, Germany
Pipetting	Pipetman P10, P20, P200, P1000 Multichannel pipet	VWR, US Sartorius, Germany VWR, US
SDS-PAGE ,Western blot and Coomassie staining	SDS-PAGE gels 12 % BT Plus 15w Chamber IBlot 2 2 PVDF Regular Stacks	Novex-Thermofisher Scientific, US Thermofisher Scientific, US

	Shaker Printer Bizhub C3350 i Gel doc E Z	Heidolph, Germany Konica Minolta, Germany Biorad, US
Spin down	Microtubes spin down	Biozym, Germany
Sterile filtration	50 ml syringe 10 ml syringe Single use filter units	Henke, Sass Wolf, Germany Braun, Germany Sartorius, Germany
Sterile working	Laminar flow hood Hera safe	Thermo Fisher Scientific, US
Stirring	Magnetic stirring rod Heating plate MR Hei- Mix L	Thermofisher Scientific, US Heidolph, Germany
Weighing	Balance Analytical balance	Kern & Sohn GmbH, Germany

2.2 Reagents

Various reagents were needed for preparing media, antibiotics, supplements and buffers.

Table 3: The reagents needed for the different methods and their suppliers

Reagent	**Supplier**
Acetic acid	Roth GmbH, Germany
Agar-Agar	Roth GmbH, Germany
Agarose	Biozym, Germany
Ampicillin	Roth, GmbH, Germany
Bactor Agar	Becton, Dickinson and Company, US
Bacto yeast extract	Becton, Dickinson and Company, US
Bacto yeast nitrogen base	Becton, Dickinson and Company, US
BCA Protein Assay Kit	Thermofisher Scientific, US
Biotin	Honeywell, US
Buffer Endo H G	New England Biolabs, US
BSA	Thermofisher Scientific, US
Denaturing buffer for de-glycosylation	New England Biolabs, US
D-Glucose (Dextrose)	Roth GmbH, Germany
dNTPs 10mM/2mM	Thermo Fisher Scientific, US
D-Sorbitol	Roth GmbH, Germany
EDTA	Roth GmbH, Germany
Ethanol	Roth GmbH, Germany
Ethidium Bromide	Roth GmbH, Germany
FD Green Buffer	Thermo Fisher Scientific, US
Fos Choline 14	Cube Biotech, US
Gene Miniprep kit	Thermo Fisher Scientific, US
Gene Ruler DNA Ladder Mix	Thermo Fisher Scientific, US
Glucose	Roth GmbH, Germany
Glycerol	Roth GmbH, Germany
HCl	Roth GmbH, Germany
Kanamycin	Roth GmbH, Germany

KCl	Roth GmbH, Germany
KH_2PO_4	Roth GmbH, Germany
LB (Lysogeny Broth)	Roth GmbH, Germany
LDS Sample Buffer 4x Nu PAGE	Thermofisher Scientific, US
Ligase buffer	Thermo Fisher Scientific, US
Loading Dye 6x	Thermo Fisher Scientific, US
MES buffer concentrate	Thermofisher Scientific, US
Methanol	Roth GmbH, Germany
$MgCl_2$	Roth GmbH, Germany
$MgSO_4$	Roth GmbH, Germany
MilliQ water	Merck, Germany
Monosodium phosphate	Roth GmbH, Germany
NaCl	Roth GmbH, Germany
Na_2HPO_4	Roth GmbH, Germany
Nuclease free water	Promega GmbH, US
Phusion buffer HF	Thermo Fisher Scientific, US
Phyton Peptone	Becton, Dickinson and Company, US
pJET PCR Cloning kit	Thermo Fisher Scientific, US
PMSF	Thermo Fisher Scientific, US
Protein Assay Dye Reagent Concentrate	Bio-Rad, US
Reaction buffer 5x HF	Thermo Fisher Scientific, US
Reaction buffer 2x for pJET cloning	Thermo Fisher Scientific, US
Sample Reducing Agent Bolt 10x	Thermofisher Scientific, US
See Blue Plus 2 pre-stained protein standard	Thermofisher Scientific, US
Simply Blue Safe Stain	Thermofisher Scientific, US
Skim milk powder	Roth GmbH, Germany
SOC Outgrowth Medium	New England Biolabs, US
Standard 6x His HSA	Eva Tacha Validogen GmbH, A
TCA	Roth GmbH, Germany
TMB Ultra Blotting solution	Thermofisher Scientific, US
Tris	Roth GmbH, Germany
Trypton	Roth GmbH, Germany
Tween20	Honeywell, US
Wizard SV Gel and PCR Clean- up system	Promega GmbH, US
Yeast extract	Roth GmbH, Germany
Zeocin	Eubio, Andreas Köck, e. U.

2.3 Enzymes

Various enzymes were needed for procedures such as ligation or PCR.

Table 4: The enzymes needed for the different methods

Enzyme	**Supplier**
Endoglycosidase H	New England Biolabs, US
Fast Digest enzymes	Thermo Fisher Scientific, US
Phusion polymerase	Thermo Fisher Scientific, US
Restriction enzymes	Thermo Fisher Scientific, US
T4 DNA ligase	Thermo Fisher Scientific, US

2.4 Antibodies

Some antibodies were needed for the detection of the target proteins on Western blots.

Table 5: The antibodies used, the antibody type and the suppliers of them

Antibody	Type	Supplier
Primary poly-His HRP antibody	Anti His-tag	Sigma Aldrich, US
Streptavidin HRP antibody	Binding to Biotin, anti Strep	Southern Biotech, US
Strep-Tactin HRP antibody	Anti Strep II	IBA-Lifesciences, US

2.5 Liquid media

Different liquid media were needed for cultivation of *E. coli* and *P. pastoris* strains.

2.5.1 YPhyD liquid medium

20 g Phytone peptone and 10 g Bacto yeast extract were weighted in, filled up with 900 ml of distilled water and autoclaved. 100 ml dextrose were added for 1 l and the medium was cooled down to add the desired antibiotic.

2.5.2 SOC medium for glycerol stocks

20 g Trypton, 5 g yeast extract and 0.5 g NaCl were weighed in and filled up to 1 l with distilled water. After autoclaving, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$ and 1 ml of 2 M glucose were added.

2.5.3 BMD1 medium

600 ml distilled H_2O, 200 ml 1 M NaPi, pH 6, 100 ml of 10x YNB, 100 ml of 10x Dextrose and 2 ml of 500x Biotin were mixed to obtain 1 l of cultivation medium.

2.5.4 BMS1 medium

600 ml distilled H_2O, 200 ml 1 M NaPi, pH 6, 100 ml of 10x YNB, 100 ml Sorbitol and 2 ml 500x Biotin were mixed to obtain 1 l of MeOH-free medium.

2.5.5 BMM2 medium

690 ml distilled H_2O, 200 ml 1 M NaPi, pH 6, 100 ml of 10x YNB, 2 ml of Biotin and 10 ml of MeOH were mixed to obtain 1 l of induction medium.

2.5.6 BMM10 medium

650 ml H_2O, 200 ml NaPi, 100 ml YNB, 50 ml of MeOH and 2 ml of Biotin were mixed to obtain 1 l of induction medium.

2.5.7 BMM15 medium

75 ml H_2O, 24 ml NaPi pH 6, 12 ml of 10x YNB, 240 µl of 500x Biotin and 9 ml of MeOH were mixed to obtain a final volume of 120 ml for MeOH induction of shake flask cultures.

2.5.8 BMS15 medium

24 ml distilled H_2O, 8 ml NaPi, pH 6, 4 ml 10x YNB, 80 µl of Biotin and 4 ml of Sorbitol were mixed to obtain 40 ml of medium for MeOH-free induction of shake flask cultures.

2.6 Solid media

Different solid media were prepared to cultivate different *E. coli* and *P. pastoris* clones.

2.6.1 LB agar with Kanamycin

10 g Trypton, 5 g yeast extract, 5 g NaCl and 15 g of agar were weighed in and filled up to 1 L with distilled water. The medium was autoclaved at 121° C, cooled down, 1 ml Kanamycin with a concentration of 40 mg/ml was added per l and plates were poured.

2.6.2 LB plates with Ampicillin

10 g Trypton, 5 g yeast extract, 5 g NaCl and 15 g of agar were weighed in and filled up to 1 L with distilled water. The medium was autoclaved at 121° C, cooled down, 1 ml Ampicillin with a concentration of 100 mg/ml was added per l and plates were poured.

2.6.3 YPhyD plates with Zeocin

20 g Phyton peptone, 10 g Bacto yeast extract and 20 g of Bacto agar were weighed in and filled up with 900 ml of distilled water. The medium was autoclaved at 121 °C, 100 ml of dextrose were added and the medium was cooled down. Zeocin was added for the desired concentration and plates were poured.

2.7 Antibiotics

Various antibiotics were needed for the cultivation of *E. coli* and *P. pastoris* strains.

2.7.1 Kanamycin stock

The Kanamycin stock was prepared to have a concentration of 40 mg/ml. 10 ml of distilled water were mixed with 0.4 g of Kanamycin and the solution was sterile filtered.

2.7.2 Ampicillin stock

A 1000x Ampicillin stock was prepared to have a concentration of 100 mg/ml. 10 ml of distilled water were mixed with 1 g of Ampicillin and the solution was sterile filtered.

2.7.3 Zeocin stock

The Zeocin stock was prepared to have a concentration of 100 mg/ml. 10 ml of distilled water were mixed with 1 g of Zeocin and the solution was sterile filtered. 1 ml of the Zeocin solution was added to 1000 ml YPhyD medium to obtain a final concentration of 100 µg/ml. 3 ml to obtain 300 µg/ml.

2.8 Buffers and reagents

Different reagents and stock solution were needed for media supplementation and buffer ingredients.

2.8.1 Dextrose for the YPhyD medium

20x Dextrose was prepared for the addition to the YPhyD medium. 110 g of Dextrose were filled up with 500 ml of distilled water.

2.8.2 TAE buffer for agarose gel electrophoresis

50x TAE buffer stock was prepared by weighing in 242 g Tris, 57.1 ml acetic acid, 18.6 g EDTA and filling up with 942.9 ml distilled water. The 1x TAE buffer for running the electrophoresis was prepared by mixing 200 ml of 50x TAE buffer with 9.8 l of distilled water.

2.8.3 Glycerol 30 % for glycerol stocks

15 ml of Glycerol were filled up to 50 ml with distilled water in a 50 ml Falcon tube.

2.8.4 YNB medium 10x

67 g Bacto yeast nitrogen base were filled up with other amino acids up to 500 ml.

2.8.5 500x Biotin
10 mg Biotin were filled up to 50 ml with distilled water and were sterile filtered.

2.8.6 1 M NaPi buffer

177.98 g of $NaHPO_4$ and 156.01 g of NaH_2PO_4 were weighed in and filled up with 1 L distilled water. The pH-value was changed by adding 1 M $NaHPO_4$ or 1 M $NaHPO_4$.

2.9 Reagents for cell lysis and protein solubilisation

For cell lysis different buffers and solutions were prepared.

5 % Glycerol were prepared by mixing 23.75 ml of distilled water and 1.25 ml of Glycerol. A 1 M NaPi buffer, pH 7.5, was prepared. 0.25 ml of 1 M NaPi were pipetted to the 25 ml glycerol to obtain a 0.01 M end-concentration of NaPi in the buffer. 9.3 mg of EDTA (with a molecular weight of 372.24 g/mol) were weighed in for an end-concentration of 1 mM in the 25 ml of buffer. The buffer was sterile filtered.

2.9.2 PMSF (protease inhibitor) stock

A 100 mM stock of PMSF was made by weighing in 34 mg of PMSF (molecular weight 174.2 g/mol) and filling it up with 2 ml of MeOH.

2.9.3 10 % Fos Choline 14 stock

1 g of Fos Choline 14 was dissolved in 10 ml of nuclease free water to obtain a 10 % stock solution.

2.10 Reagents for protein concentration and precipitation

Different buffers and solutions were prepared for concentrating and precipitating the isolated proteins.

2.10.1 10 mM Tris-HCl buffer

12.1 g of Tris was weighed in and filled up to 80 ml with distilled H_2O to obtain a 1 M Tris solution. The pH was adjusted to pH 7.4 with 6 to 7 ml of concentrated HCl. The

volume was filled up to 100 ml with distilled H_2O. The solution was sterile filtered. It was diluted 1:100 to obtain a 10 mM Tris-HCl buffer.

2.10.2 TCA 50 %

0.5 g TCA were weighed in and filled up to 10 ml with distilled water to obtain a 50 % TCA solution.

2.11 Buffers for SDS-PAGE and Western blot

For the analytics different buffers and solutions were needed.

2.11.1 MES buffer for SDS-PAGE

A MES buffer concentrate was diluted 1:20 to obtain a MES running buffer for SDS-PAGE.

2.11.2 Blocking buffer for Western blot

5 g BSA or 5 g skim milk powder were weighed in to achieve a final concentration of 5 % in 100 ml of PBS-Tween (reducing unspecific binding) 0.1 % buffer.

2.11.3 Washing buffer for Western blot /PBS-Tween 0.1 % buffer

For 1 L PBS buffer, 8 g NaCl, 0.2 g KCl, 1.78 g Na_2HPO_4 and 0.27 g KH_2PO_4 were weighed in. To obtain a PBS-Tween 0.1 % buffer, 1 g Tween for 1 l was added as well. It was filled up to 1 l with MilliQ water.

2.11.4 AB-diluent primary antibody for anti His or anti Strep Western blot

5 ml of blocking buffer (see above) were filled up with PBS-Tween 0.1 % buffer to 25 ml and 12.5 µl of anti-His or anti-Streptadivin antibody were added to obtain a 1:2000 dilution of the antibody in the buffer. For the Streptactin antibody dilution, 2.5 µl of antibody were added to the same solution to obtain a concentration of 1:10000 of antibody in the buffer.

2.12 Strains and plasmids

E. coli and *P. pastoris* strains were transformed with different plasmid types with genes under various methanol inducible and methanol free promoters that are due to confidentiality reasons only named with letters A to K.

Table 6: The *P. pastoris* and *E. coli* strains used and the plasmids that were put into them

***P. pastoris* and *E. coli* strains**	**Plasmids (with the different promotors)**	**Methanol induced or not**
***E. coli* NEB 5-alpha Competent** fhuA2Δ (argF-lacZ) U169 phoA glnV44 ϕ80Δ (lacZ) M15 gyrA96 recA1 endA1 thi-1 hsd R17 New England Biolabs, US	ATUM pJ 201: 382417 ATUM pJ201: 382418 ATUM pJ201: 382419	
E. coli NEB 5-alpha Competent	pJET plasmid 1.2 blunt-ended (Thermofisher Scientific, US)	
E. coli NEB 5-alpha Competent	pPZ A, B, C, D, E, F, G, H, I, J, K	

E. coli NEB 5-alpha Competent	pPZ αMF E, B, H	
E. coli NEB 5-alpha Competent	pJET for MP-GFP constructs: ß2AR-GFP, 5-HT 2c-GFP, 5-HT 2c aMF-GFP	
E. coli NEB 5-alpha Competent	pPZ ß2AR-GFP, 5-HT 2c-GFP, 5-HT 2c αMF-GFP	
***P. pastoris* mut^s**	pPZ A, B, C, D	MeOH-induced
P. pastoris mut^s	pPZ E	MeOH-induced or free
P. pastoris mut^s	pPZ G, H, I	MeOH-free
P. pastoris mut^s	pPZ F	Weak MeOH promoter
P. pastoris mut^s	pPZ αMF E, B, H	MeOH-induced or free
P. pastoris mut^s	pPZ MP-GFP A, B, E, I or pPZ MP-αMF-GFP A, C, E, I	MeOH-induced or free

2.13 Affinity tags

There were 2 different tags used to detect the membrane proteins. The 5-HT 5a protein only got a Strep-tag I attached as no location for the His-tag could be found as this protein did not contain a signal sequence in the common sense. (Terpe K. , 2007)

Table 7: The different tags bound to the amino acid sequences, their sequence, location at the membrane protein sequence and their size in kDa

	Amino acid sequence	**Location at the protein**	**Approximate size [kDa]**
His-tag	HHHHHHHHH	after signal sequence (N-terminal)	1.4
Strep-tag I	AWRHPQFGG	at end of amino acid sequence (C-terminal)	1.05
GFP	MSKGEELFTGV…	After Strep tag I on protein	26.9

2.14 Primers

Different primers were used for PCR or sending DNA for sequencing.

Table 8: The working step with the PCR done, the sequences of the forward and reverse primer and their melting temperatures in °C

	Primer	**Sequence**	**Melting temperature [°C]**	**Supplier**
Sequencing of the pJ plasmid	Fwd: DNA20_pTF	5`-CTCGAAAATAATAAAGGGAAAATCAG–3`	53	Thermofisher Scientific
	Rev: DNA20_pTR	5`- TGGTAGTGTGGGGACTC – 3`	59	Thermofisher Scientific

Sequencing of the pJET plasmid	Fwd: pJET_for Rev: pJET_rev	5`- CGACTCACTATAGGGAGAGCGGC – 3` 5`- AAGAACATCGATTTTCCATGGCAG – 3´	60 57	Thermofisher Scientific Thermofisher Scientific
Sequencing of the pPZ plasmids	Fwd: $P_{AOX11F1}$ P_{AOX1F2} Rev: TT_aftergene_rev	5`- GTTGCAAACGCAGGACCTC – 3` 5`- GCGACTGGTTCCAATTGAC – 3` 5`- GAAGCCTGCATCTCTCAG – 3`	61 58 56	Invitrogen Invitrogen Invitrogen

3 Methods

3.1 Preparation of protein sequences

The native protein sequences including the signal sequence of the proteins to be expressed were searched on the “Uniprot” database. (Bairoch, 2005) (Uniprotconsortium, 2019) Additionally, the sequence was checked for the signal peptide sequence on the “SignalP” server except for the 5-HT 5a serotonin receptor that has no signal peptide. (http://www.cbs.dtu.dk/services/SignalP/) The native sequences were supplied with a 10x His-tag after the native signal sequence and the Kozak consensus sequence at the N-terminus and with a Strep-tag I after the double stop codons at the C-terminus except for the 5-HT 5a receptor where only a Strep-tag was added at the C-terminus and no His-tag as the receptor was going to be secreted with an αMF-signal sequence. (Terpe, 2007)

3.2 Ordering of the backbone vector with the gene of interest

The protein sequences were commissioned and codon optimized for our host by “ATUM”. (https://www.atum.bio) They were ordered together with the necessary 5´ and 3´ extensions amenable for cloning into the destination plasmids and a list of restriction enzymes not allowed (*Bgl*II / *Eco*RI / *Xho*I / *Not*I / *Pst*I / *Xba*I / *Xma*I / *Eco*RV / *Asc*I) to be used by the company when assembling the backbone vector. The gene of interest was provided on a backbone vector that is used to transform competent *E. coli* cells.

3.3 Preparation of DNA for transformation

The filter with around 4 µg DNA obtained from “ATUM” was placed on a petri dish. 100 µl of nuclease free water were pipetted onto the filter. The filter was transferred in a mini-prep spin column from which the membrane was removed, and placed on top of an Eppendorf tube. The tube was centrifuged for 2 min at full speed and the DNA was collected in a new Eppendorf tube.

3.4 Transformation of competent *E. coli* cells with different plasmids

2 µl of isolated plasmid were mixed with 35 µl of competent NEB *E. coli* cells in an Eppendorf tube on ice. It was incubated for 15 to 20 min on ice. Subsequently, a heat shock at 45 °C was applied for 45 s on a thermocycler followed by incubation on ice

for 5 min. Then, 600 µl of SOC medium were filled into the Eppendorf tube. The cells were shaken at 650 rpm on a thermomixer at 37 °C for 75 min prior to plating on LB agar with the suitable antibiotic Kanamycin, Ampicillin or Zeocin. Plates were incubated at 37 °C overnight.

3.5 Mini-preps of *E. coli* cells

The plasmid was isolated with the "Wizard Purification kit". 250 µl of resuspension buffer were put in an Eppendorf tube. With a toothpick many cells from around a quarter of the plate were scrapped off and immersed in the tube. The cells were well suspended. After adding 250 µl of lysis buffer the tubes were inverted. 350 µl of neutralisation buffer were added, the tubes were inverted and centrifuged at full speed for 10 min. The supernatant was decanted in the filter of a spin column. It was centrifuged for 1 min. 700 µl of washing buffer with EtOH added were pipetted onto the filter. It was centrifuged and the liquid was decanted. The open filters were dried at 37 °C for at least 15 min. DNA was eluted with 50 µl of nuclease free water into fresh Eppendorf tubes.

3.6 Sequencing of plasmids

The isolated plasmids were sent for sequencing to "Biosearch Technologies" (https://www.biosearchtech.com/) in Berlin, Germany. 10 µl of plasmid were mixed with 4 µl of selected reverse and forward primer in separate Eppendorf tubes. DNA and vector sequences were visualized with the "VNTI" software from "Thermofisher Scientific". Sequences were analysed for their correctness with the "Contig Express tool".

3.7 Restriction digest of plasmids

20.5 µl of isolated plasmid were mixed with 2.5 µl of FD Green buffer and cut with 1 µl of suitable FD restriction enzymes for 90 min at 37 °C.

3.8 Phusion PCR

For the PCR protocol an ultra-hot start was chosen for the Phusion polymerase with 35 cycles. The PCR was performed to generate a sufficient amount of plasmid with protein having the αMF-signal sequence attached. The amplification was performed with forward primer "αMF_HIS_MP_for" or "αMF_HIS_fwd" as shown in Table 8 and a reverse primer for the pJ plasmid. Resulting PCR fragments were then cloned in a pJET vector as backbone. The inserts were amplified at their His-tag so that they do not have their signal sequence attached anymore, but are amenable for seamless cloning 3´ of the αMF-pre-pro-signal sequence into blunt-ended destination plasmids.

The PCR programme can be seen in Table 9 and a mastermix was prepared as follows: 35 µl nuclease free water and 10 µl of 5x HF-reaction buffer were mixed with 1 µl of αMF_HIS_MP-for forward primer, 2 µl of DNA20_pTR reverse primer, 1 µl of 10 mM dNTPs, 0.5 µl of Phusion polymerase and 0.6 µl of template (pJ plasmid). After the PCR reaction the samples were mixed with 10 µl of 6x loading dye.

Table 9: The conditions used for every PCR step. Annealing temperature was sometimes decreased to 45 °C

Step	Temperature [°C]	Time
Initial Activation	98	30 s
Activation in each cycle	98	10 s
Annealing	48 or 45	10 s
Extension	72	16 s
		X 35 cycles
	72	5 min
	25	

3.9 Preparative agarose gel

A preparative agarose gel was prepared by weighing in 2.7 g agarose and filling it up with 250 ml of TAE buffer. The mix was heated in microwave for 4 min and cooled down. 2 drops ethidium bromide with a concentration of 50 µg/ml were added and the gel was poured in a middle-sized tray. In the tray a comb with 15 stacks was inserted and the gel was overlaid with TAE buffer. After solidification, all of the restricted plasmid was distributed in two slots and 6 µl DNA ladder were pipetted in a slot for size estimation. The gel was run at 130 V for around 2 h.

3.10 DNA purification and determination of concentration

The gel was visualized on "Geldoc" (Biorad). Gel slices were cut out under UV light with a scalpel on "Geldoc". The DNA was purified with the "Wizard DNA Purification System" exactly as described in the user manual. It was eluted with 30 µl of nuclease free water. From the eluted DNA the concentration was measured by pipetting 1 µl on the "NanoVue" device. If the concentration was too low for subsequent cloning events another round of a mini-prep, digest with suitable FD restriction enzymes, agarose gel and gel slice purification was performed.

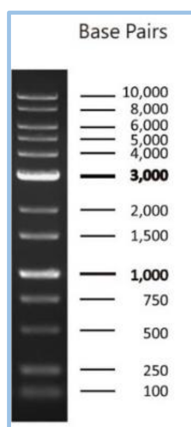


Figure 5: Gene Ruler Ladder DNA mix

Resolution and explanation of the Gene Ruler Ladder DNA mix from Thermofisher Scientific that was used to estimate the size of the bands on the gel and therefore DNA size

3.11 Ligation in pJET plasmid

The purified PCR products of the β2AR and the 5-HT 2c gene were then used for cloning into a pJET 1.2 vector with blunt ends. 5 µl reaction buffer and 1 µl of pJET plasmid from the “pJET Cloning kit” were mixed with 3 µl of PCR eluate and 1 µl of DNA T4 ligase. The mix was shortly spun down at 6000 rpm and let at room temperature for 15 to 20 min to perform the ligation reaction. If PCR products had a very low DNA concentration, 10 µl of 5x HF reaction buffer were mixed with 5 µl of PCR product, 1 µl pJET vector, 1 µl ligase and 3 µl nuclease free water.

3.12 Preparation of pPZ vectors with different promotors

It was decided to clone the genes of the three proteins in different pPZ expression vectors with strong and weak methanol inducible promotors and methanol-free promotors. The pPZ vectors with the different promotors were streaked out from the strain collection, a mini-prep was done, the vectors were cut with Fast Digest enzymes *Eco*RI and *Not*I or with *Xho*I and *Not*I (for the αMF expression) to cut out the original insert and get the vector ready for the desired insert. A preparative agarose gel was run and the gel slices of the vector were cut out and purified with the “Wizard DNA Purification System”. DNA was recovered in 30 µl as described in steps above. The concentration of the vector DNA was measured with “NanoVue”.

3.13 Cloning into the pPZ vectors with the native protein signal sequence

The three DNA inserts β2AR, 5-HT 5a and 5-HT 2c were ligated in pPZ vectors with 5 methanol-induced promotors (A, B, C, D, E), four methanol-free promotors (G, H, I, J) and a weak methanol promotors (F). Therefore, 5 µl of ligase buffer were mixed with 2 µl of DNA insert (previously restricted by FD restriction enzymes) and cut out from a gel as described in step 2.2.10, 2 µl of vector and 1 µl of T4 DNA ligase. The cut was left for 20-30 min at RT. The transformation was done as described in chapter 3.4 and it was plated out on LB agar with Zeocin. From the transformation plates colonies were picked and freshly streaked out on LB for a mini-prep. The following steps were done as described in chapter 3.5 and 3.6. The primers used for sequencing were the P_{AOX1}_F1, that binds to the position 120- 140 of the P_{AOX1}, the P_{AOX1}_F2, binding to the 850- 870 region and the TT_aftergene_rev primer that binds to the 100- 120 region of the T_{AOX1}.

3.14 Preparation of inserts for cloning into pPZ vectors with the αMF signal sequence

The pJET clones carrying the PCR amplified fragments contain an insert with the αMF-signal sequence. They were cloned into three different pPZ vectors (B, E, H). The inserts were removed from the pJET plasmids with the Fast Digest restriction enzyme *BglII* and the inserts were prepared as described previously in chapter 3.12 and 3.13.

3.15 Preparation for transformation of *P. pastoris* with the different pPZ and pPZ_αMF vectors

Positive *E. coli* clones for all approaches were streaked out for one mini-prep of each different insert with each promotor. Additionally, they were streaked out for the strain collection (glycerol stocks). The isolated DNA was cut with 1.5 µl of *Bgl*II and 5.5 µl of enzyme O-buffer for linearization overnight.

Ethanol precipitation of the linearized fragments was performed as follows: 125 µl ice-cold EtOH 100 % were added to the 50 µl of restricted DNA. 19.25 µl of sodium acetate solution were added, tubes were inverted and incubated at -20 °C overnight. The tubes were centrifuged at full speed for 5 min at 4 °C. After decanting, 250 µl ice-cold EtOH 70 % were added, followed by centrifugation for 3 min at full speed at 4 °C. After decanting the supernatant, the same amount ethanol was added and it was centrifuged again. Thereafter, all remaining liquid was sucked out. Samples were left to dry at 37 °C for at least 30 min. As soon as the DNA-pellets were dry, 20 µl of nuclease free water were added to elute the DNA. The eluates were pipetted on “Millipore DNA filter paper” (0.025 µm) that were placed in “MilliQ” water in petri dishes. After 30 min, the remaining liquid was pipetted off the filter back in a new Eppendorf tube and DNA concentration was measured on “NanoVue”.

3.16 Preparation of electrocompetent *P. pastoris* cells

A few colonies of mut^s *P. pastoris* cells were scraped off a cultivation plate and were inoculated in 50 ml of liquid YPhyD in an Erlenmeyer flask with a sterile inoculation loop. They were put in a shaking incubator at 28 °C with 120 rpm overnight. On the next day optical density of the culture was measured at 600 nm with YPhyD as blank. For measurement the culture was diluted 1:10 with YPhyD. The culture was diluted to an OD of 0.2 with YPhyD filled up to 50 ml. It was grown for 4 h to an OD of 0.8 as the generation time was 2 h. The cells were filled in a 50 ml Falcon tube for making them electrocompetent. The cells were harvested in a table top centrifuge in 4 °C for 7 min at 4000 rpm. They were put into a box with ice, tubes were emptied, filled up with 40 ml of MilliQ water and the pellets were resolved by pipetting up and down. The cells were centrifuged in 4 °C for 5 min at 4000 rpm. The supernatant was decanted again, 20 ml of water were added and the pellet was dissolved. It was centrifuged again with the same parameters. Supernatant was decanted, 10 ml of 1 M sorbitol were added and the pellet was dissolved. It was centrifuged with the previously described parameters. The supernatant was decanted and carefully pipetted away from the pellet. The pellet was taken up in 350 µl of sorbitol.

3.17 Transformation of *P. pastoris* cells

Electroporation cuvettes were placed on ice and an amount of DNA eluate was pipetted in that corresponded to 2 µg of DNA. 80 µl of electrocompetent cells were pipetted in the cuvette in a way that they were mixed with the DNA. The mix was left standing for 5 min. On the electropulser the “Fungi” and “Pichia” program was chosen. The electrotransformed cells were taken up in 800 µl of sorbitol mixed 1+1 with YPhyD. The mix was filled in 15 ml Falcon tubes and incubated for 2 h at 400 rpm and 28 °C.

Afterwards, transformants were plated out on YPhyD 3x Z agar. Plates were incubated for 2 days at 28 °C.

3.18 Deep-well plate cultivation

Two clones of transformation plates of each variant were chosen to get further cultivated in deep-well plates. 2 plates were prepared for MeOH-induced and MeOH-free promotors of clones. The plates were filled with 250 µl of BMD1 medium for cultivation. Single colonies of two different clones were picked with toothpicks from and inoculated as described in Figure 6 and 7. The mock strain was a mut^s culture. The plates were incubated at 28 °C and 110 rpm in a shaking incubator.

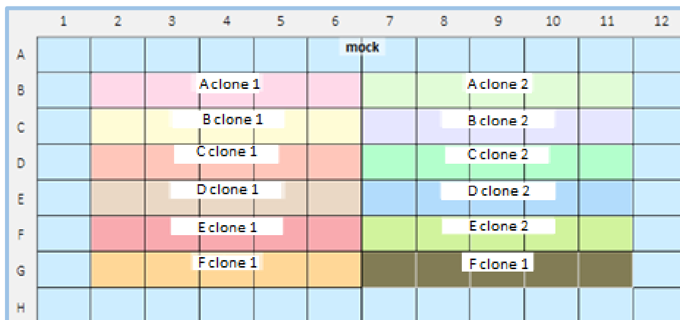


Figure 6: The cultivation scheme for cultivation of the different clones under different promotors that are induced with methanol. The scheme was used for the first cultivation of different β2AR clones.

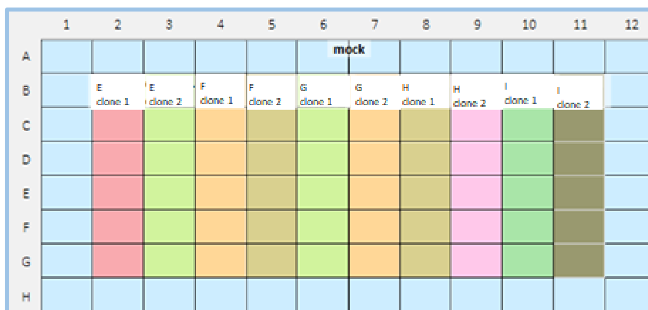


Figure 7: The cultivation scheme for cultivating the clones with different promotors for methanol-free induction. The scheme was used for the first cultivation of different β2AR clones.

After three days of incubation the methanol-induced clones in the plates were induced with 50 µl of BMM2 medium in the morning and BMM10 medium in the evening. The next day and the day after they were induced with 50 µl BMM2 medium in the morning. The methanol-free clones in the plates were induced with 100 µl of BMS1 medium in the morning for three days after the first three days of incubation.

3.19 Shake flask cultivation

For gaining larger volumes of cell lysates and proteins, 250 ml shake flasks were filled with 50 ml of BMD1 or BMS1 medium and inoculated with *P. pastoris* strains. They were incubated over the weekend at 28 °C and 110 rpm. On Monday morning they were induced with 15 ml of BMM15 or BMS15 medium and in the evening with 5 ml of BMM15 medium for the methanol-induced cultures. On Tuesday and Wednesday mornings they were induced with 5 ml of BMM15 or BMS15 medium.

3.20 Harvest of *P. pastoris* clones

After 6 days of incubation had passed, the cells were harvested on the 7th day in the morning. All wells of 12 samples and 1 mock from the methanol plate were emptied with a pipet and all wells of 10 samples and 1 mock of the methanol-free plate were emptied. All liquid inside the wells was sucked out and filled in 15 ml Falcon tubes. The OD_{600} of the samples and the mocks was measured on a photometer with a 1:15 or 1:20 or 1:30 dilution with nuclease free water. The total OD_{600} of the samples in the 15 ml Falcon tubes was calculated.

3.21 Glass bead lysis

The 15 ml Falcon tubes were centrifuged at 4 °C and 4000 rpm for several minutes for harvesting. The supernatant was removed and the pellet was re-suspended in the amount of lysis buffer in µl that corresponded to their OD_{600} value measured (500 µl buffer used for an OD of 100). The same amount of lysis buffer was filled in the form of acid-washed glass beads in a 2 ml Eppendorf tube. The re-suspended pellet was transferred to the tube with the beads. An amount of 100 mM PMSF stock was added to obtain a final concentration of 1 mM in the tube. Cells were lysed in the cooling chamber by 20 cycles of vortexing at maximum speed for 1 min, followed by 1 min chilling on ice. After the final cycle of vortexing and chilling, tubes were centrifuged at 5000 rpm for 30 min at 4 °C. The supernatant was diligently transferred into a pre-chilled tube immediately after centrifugation. (Gurramkonda, 2009)

3.22 Rescreening of well expressing clones

Clones showing a higher expression on Western blots were streaked out again from the glycerol stocks on YPhyD 1xZ plates. They were used to inoculate the respective methanol-induced and methanol-free 96-well plates and 250 ml shake flasks. They were cultivated in 50 ml of BMD1 medium from Friday noon to Monday morning at 110 rpm with a temperature of 28 °C (or 22 °C in induction). On Monday morning they were induced with 15 ml of BMM15 medium or BMS15 medium. On Monday evening 15 ml of BMM15 medium was only given to clones with MeOH-induced promoters only. Tuesday morning and Wednesday morning cultures got induced with 5 ml of BMM15 or BMS15 medium.

On Thursday the cultures were harvested as described in chapter 3.20 and lysed as described in chapter 3.21. One third of the cells harvested were lysed with the addition of 10 % Fos Choline 14 solution to obtain a 1 % final concentration in the broth. After cell lysis and centrifugation, an amount of a 10 % Fos Choline stock was added to another third of the cells harvested for solubilisation. An amount of Fos Choline was added that corresponded to a final concentration of 1 % in the resulting lysate. The remaining third of cells harvested was lysed and centrifuged without the addition of any Fos Choline 14 as a control.

3.23 Cell fractionation and precipitation

From all clones that were chosen to be rescreened, approximately 500 µl of every different third of cell lysate was transferred into sterile Eppendorf tubes, put in an ice

box and brought to the “Department of Molecular Biotechnology” at “Technical University Graz” for ultracentrifugation. It was centrifuged for 1 h at 50,000 rpm in a fixed angle centrifuge with 4 °C. The pellet was re-suspended carefully in 100 µl of 10 mM Tris-HCl buffer at pH 7.4. For precipitation, 350 µl of distilled water and 100 µl of ice-cold 50 % TCA were added. The mix was left to precipitate at 4 °C overnight. (Voit, 2017)

3.24 Determination of total protein concentration with the BCA assay

Protein lysates were diluted 1:10 or 1:20 in sterile Eppendorf tubes. A standard calibration line was generated with 6 dilutions of BSA and water corresponding to 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.05 mg/ml and 0 mg/ml protein according to the BCA protein assay kit from “Thermofisher Scientific”.

25 µl of each dilution were pipetted in a 96-wellplate for optical analysis. For each sample 200 µl of BCA reagent A and 4 µl of BCA reagent B were mixed with each other. 200 µl of this mix were added to a protein sample in the wellplate. The plate was incubated at 37 °C for 30 min. The optical density was measured at 562 nm on a spectrophotometer.

3.25 Precipitation for SDS-PAGE

Samples that were not ultracentrifuged after cell lysis, were exposed to protein precipitation to collect a higher amount of proteins for a subsequent SDS-PAGE. From the BCA assay the amount of µl for 100 µg protein was collected and mixed with 400 µl water. For precipitation 100 µl of 50 % TCA were added. The samples were incubated for 1 h on ice. They were centrifuged at 13,000 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet was re-suspended in 50 µl of 1x SDS sample buffer. 28 µl were mixed with 2 µl of reducing agent. SDS-PAGE was run as described.

3.26 SDS-PAGE

SDS-PAGE gels with 10 or 15 slots were removed from their package and rinsed with water. The combs were removed and the gels were assembled in a tray. The chamber was filled with diluted MES buffer until slots were overlaid completely with buffer. 20 µl of protein extract were pipetted in a well plate, 8 µl of LDS sample buffer and 2 µl of reducing agent were added. The plate was closed with a dark foil, spun down, vortexed and incubated for 10 min at 70 °C. It was again spun down. All of the liquid was pipetted in the slots and 6 µl protein standard was pipetted in one slot. The SDS-PAGE was run for around 50 min at 120 mA and 92 V.

3.27 Western blot

The gel was removed from the tray and its holding frame. The upper and lower ends were cut away and the gel was placed on ready-to-use PVDF stacks as described in the user manual for the I Blot 2 device and the 2 PVDF Regular Stacks manual from “Thermofisher Scientific”. The blotting program was run as described in Table 10. (Invitrogen, 2019)

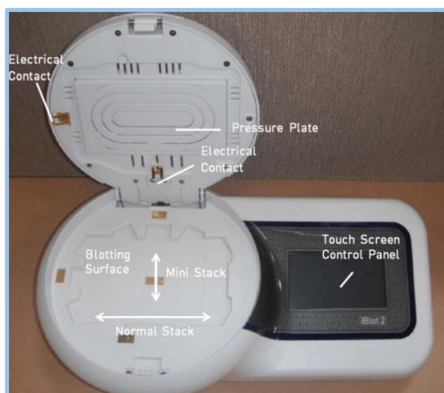


Figure 8: Top view on an open iBlot 2 Gel Transfer Device

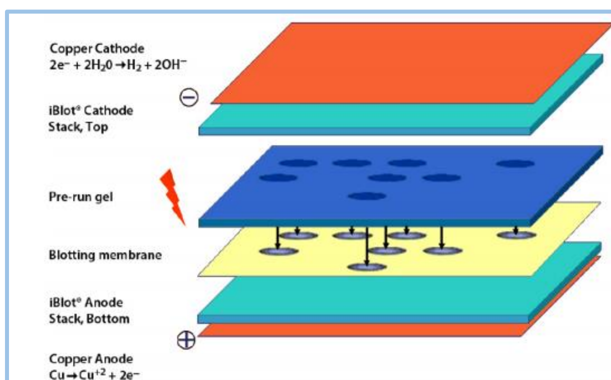
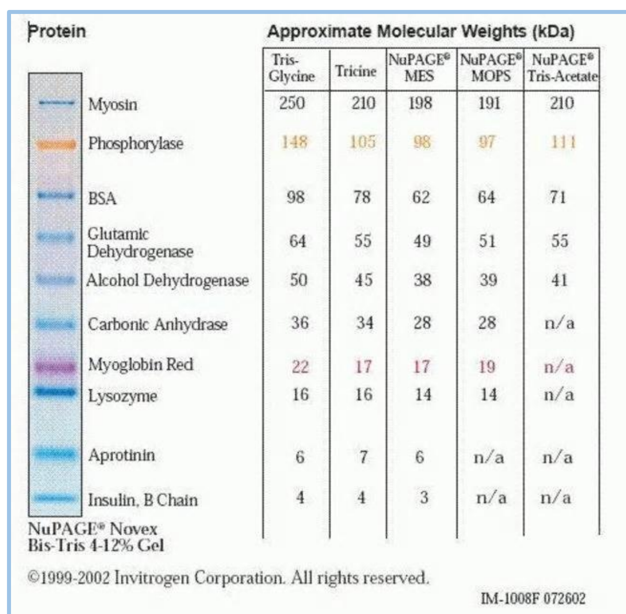


Figure 9: Scheme of iBlot 2 transfer stack assembly

The blot was disassembled and the membrane was put in a plastic box. It was saturated with 25 ml of blocking buffer for 30 min with shaking. The membrane was rinsed once for 5 s with washing buffer and once for 5 min. The primary anti-His-tag or Streptavidin HRP AB were added in the AB-diluent solution to a concentration of 1:2000 or 1:10000. The membrane was incubated with the antibody for 30 min while shaking. The membrane was washed 3 times (1x 5 s, 1x 5 min and 1x 10 min) with PBS 0.1 % and Tween 20 under vigorous agitation. For 1 min it was washed with PBS. 10 ml of TMB Ultra Substrate were added and it was incubated for 10 min. The membrane was washed once with distilled water and incubated with distilled water for 10 min. The membrane was scanned on a printer.

Table 10: The different blotting steps and conditions

Step	Time [min]	Voltage [V]	Ampere [A]
1	1	20	1.4
2	4	23	1.1
3	2	25	1.3



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	198	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex Bis-Tris 4-12% Gel

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IM-1008F 072602

Figure 10: Protein ladder for Western blot membranes. For the samples in the master thesis the NuPAGE MES buffer was used.

The protein ladder used to determine the size of the blotted samples in kDa and an example protein of this size. For the detection of different membrane proteins on the Western blot the size was read from the column “NuPAGE MES”. The β2AR protein for example, shall appear at the fourth band from top at the level of glutamic dehydrogenase of around 49 kDa.

3.28 Coomassie Brilliant Blue staining

After the SDS-PAGE the gels were unpacked, put in an oblong box and rinsed with distilled water. The water was carefully decanted and a “Simply Blue Safe Stain” was poured in the box to overlay the gels. The box was heated by microwave for 30 s and then onto a shaker for 5 to 10 min. The staining solution was decanted and the gels were overlaid with distilled water for de-staining. The box was heated by microwave for 1 min and on the shaker until the water turned blueish. The gels were de-stained three further rounds with the same procedure. They were scanned on a printer or on Gel doc (Biorad).

3.29 De-glycosylation

Some samples that showed two bands above each other at the size of the recombinant protein were de-glycosylated with *Endo* H to see if the two bands come from glycosylated and non-glycosylated recombinant proteins. 10 µl of protein lysate or precipitate were pipetted in a 96-wellplate and 1.1 µl of Denaturing buffer were added. The plate was incubated for 10 min at 80 °C and spun down. To the denatured samples, 1.3 µl of *Endo* H buffer G and 0.5 to 0.7 µl of *Endo* H enzyme were added. The plate was spun down and incubated at 37 °C for 1-2 h. Samples were analysed by SDS-PAGE and a Western blot as usual.

3.30 Screening for multiple integration events

YPhyD agar was prepared as described in chapter 2.6.3 and poured. 5 g of zeocin powder were weighed in 50 ml distilled water to obtain a 1:10 stock solution. 1 ml of stock solution was added to 1000 ml to obtain a final concentration of 100 µg/ml in each plate. The same stock solution was added in different amount to the medium to obtain concentrations of 200 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml. The *P. pastoris* clones and mut^{s} wild type were streaked out on each different plate and incubated for two days at 28 °C. The wild type was additionally streaked out on YPhyD without any antibiotics added as positive control.

3.31 Western blots for quantification with the best expressing clones

A total protein concentration of 20 to 40 µg/ml was loaded on the SDS-PAGE to see clear expression of the recombinant proteins. Therefore, dilutions were made with nuclease free water to obtain the desired concentration in 20 µl. To estimate the concentration of the recombinant protein displayed on the blot by the antibody binding to the His-tag, a standard 6x His-HSA with a concentration of 100 µg/ml was diluted 1:100. The standard was loaded onto the gel with a concentration of 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml. Dilutions were made with a Mock mut^{s} sample, before or after ultracentrifugation and no Fos Choline added, for a negative control.

3. 32 Generation of GFP tagged constructs

Constructs with the native protein attached to a linker to a GFP were generated for the β2AR, 5-HT2c and the 5-HT 2c-αMF gene. The constructs were ordered at “Twist Bioscience” during a 4 months breakup of the thesis. The vectors carrying the desired constructs were used to transform competent DH5-α *E. coli* cells. DNA was extracted, cut with restriction enzymes and sent to sequencing as described previously. DNA was further ligated in pPZ vectors with the different promoters A, B, E and I for the β2AR and the 5-HT 2c and A, C, E, I for the 5-HT 2c-αMF. Plasmids were used to transform competent *E. coli* cells. DNA was isolated, cut with FD restriction enzymes and sent for sequencing. Correct inserts were cut overnight for linearization as previously described. DNA was subject to ethanol precipitation, desalting and concentration measurement, before using it for the transformation of electrocompetent *P. pastoris* mut^{s} cells as described in chapter 3.17.

3.33 Cultivation and screening of clones with MP-GFP constructs

Positive clones were picked from the plates and cultivated in 50 ml BMD1 medium as described in chapter 3.19. They were induced, as previously described, for three days. Before each induction step, a 1 ml sample of cell broth was taken for measuring the OD_{600} and fluorescence emission of the cells. Samples were taken around 0, 8, 24, 48 and 72 h after the first induction. The cell broth was diluted 1:20 or 1:30 in cuvettes and the OD_{600} was measured. Additionally, the cell broth was diluted 1:20 or 1:10 in a fluorescence plate. Samples were excited at 395 nm and their emission was measured at 509 nm on a plate reader. After three days of induction, cells were harvested and lysed according to the procedure described in 3.20 and 3.21. Protein lysates were

analysed with BCA assays, precipitation, SDS-PAGE, Western blots and Coomassie stains as described already before in chapter 3.24 to 3.28.

3.34 Fluorescence microscopy

2 clones of every construct showing the highest fluorescence/OD at a certain time point after induction, were selected and brought in an ice box to Dr. Tarek Moustafa at the "Centre for medical research" in Graz for fluorescence microscopy.

3.35 Generation of glycerol stocks

For the pre-culture of *E. coli* for a glycerol stock, 50 ml SOC medium and the desired antibiotic were mixed. The antibiotic was added to a final concentration of 1:1000 for Kanamycin and Ampicillin and 1:4000 for Zeocin. For *P. pastoris* clones no antibiotics were added to YPhyD medium. 2 ml medium were pipetted in a 15 ml Falcon tube. The different clones were enumerated and a few colonies of them were scraped off the agar with a pipet tip. They were carefully immersed in the medium and grown overnight in a shaker at 37 °C (for *E. coli*) or 28 °C (for *P. pastoris*) at 450 rpm. Cryotubes were enumerated and labelled. The next day, cryotubes were filled with 500 µl of 30% glycerol. In the Falcon tubes the cell pellet was dispersed by pipetting up and down and 500 µl cells were added to the cryotubes. Tubes were filled in a cryobox and stored at -70 °C.

4 Results

4.1 Sizes of the proteins and genes

Expression constructs of membrane proteins with C- and N-terminal fusion tags were designed and sent to ATUM for construction of vectors carrying the desired recombinant genes.

Table 11: The different inserts, their protein sizes and the sizes of their DNA sequences

Insert	**Approximate size (kDa)**	**Approximate size (kB)**
β2AR 10x His with native signal sequence	49	1.35
5-HT 2c 10x His with native signal sequence	54	1.45
5-HT 2c 10x His after αMF	54	1.35
5-HT 5a	41	1.10
β2AR-GFP	76	2.04
5-HT 2c-GFP	81	2.18
5-HT 2c-αMF-GFP	81	2.10

4.2 Sequencing and agarose gel of the introduced genes

The inserts with the β2AR, 5-HT 2c and 5-HT 5a genes in the pJ201 vectors were sequenced with forward and reverse primers. This revealed that the DNA sequences were correct. On the agarose gel 2 correct clones for each of the 5 insert variants were detected at correct sizes. After cut and elution, DNA concentration measured was high enough for subsequent ligations.

4.3 Transformation with the pJET plasmid

The DNA concentrations of the PCR products for pJET cloning were relatively low, but this might be because of some colonies that did not have vectors incorporated.

In Figure 11 it can be seen that not all clones showed an insert corresponding to the correct fragment size. Besides the inevitable re-ligation of the blunt-ended empty pJET vector, this event could have been caused by too low DNA concentration used for the transformation.

Figure 11: Agarose gel of PCR amplified 5-HT 2c-αMF genes

When comparing the size of the insert of the pJET vector to the DNA ladder (Figure 11) on the right side it can be seen that only clones 3, 4 and 7 of the 5- HT 2c constructs amplified by PCR contained an insert. The two inserts amplified by PCR were around 100 bp smaller than the native inserts as their signal sequence is not attached anymore. (Thermofisher Scientific, 2019)

Sequencing of the pJET inserts led to two positive clones (clone numbers 3 and 7) for the 5- HT 2c insert.

4.4 Cloning into the pPZ vectors with different promotors

4.4.1. DNA concentrations of the isolated promotors

All promotors isolated from an agarose gel showed enough DNA concentration after elution for subsequent ligations.

4.4.2 E/N and X/N restriction digests for cloning in the pPZ platform and linearization

In Table 12, the different inserts cut out of the pPZ vectors with different promotors are shown. Sometimes inserts were at the wrong size or sequencing revealed an incorrect DNA sequence. Then, ligation of the insert into the vector was repeated. The vectors with the J and K promoters were completely abandoned as sequencing reactions never resulted in correct sequences. For the 5-HT 5a and the 5-HT 2c genes several ligation reactions had to be repeated.

Table 12: Cloning of the inserts under different promoters

The different DNA inserts with their different promotors introduced in the vector. The promotors are induced by methanol, two are weakly induced by methanol and some are free of a methanol induction. In the table it can be seen which cloning procedures did work with "yes" and which constructs were not able to be followed with "/".

Insert	Promotor	Methanol induction	Positive clones E/N	Positive clones X/N	*BglII* cut
β2AR	A	yes	yes	No αMF ss	yes
	B	yes	yes	No αMF ss	yes
	C	yes	yes	No αMF ss	yes
	D	yes	yes	No αMF ss	yes
	E	yes and free	yes	No αMF ss	yes
	F	yes and free	yes	No αMF ss	yes
	G	free	yes	No αMF ss	yes
	H	free	yes	No αMF ss	yes
	I	free	yes	No αMF ss	yes
	J	free	/	No αMF ss	/
5-HT 5a	A	yes	yes	No αMF ss	yes
	B	yes	yes	No αMF ss	
	C	yes	yes	No αMF ss	yes
	D	yes	yes	No αMF ss	yes
	E	yes and free	yes	No αMF ss	yes
	F	yes and free	/	No αMF ss	/
	G	free	/	No αMF ss	/
	H	free	yes	No αMF ss	/
	I	free	yes	No αMF ss	yes

	J	free	/	No αMF ss	/
5-HT 2c	A	yes	/	/	/
	B	yes	yes	yes	yes
	C	yes	yes	/	yes
	D	yes	yes	/	yes
	E	yes and free	yes	yes	yes
	F	yes and free	/	/	/
	G	free	/	/	yes
	H	free	yes	yes	yes
	I	free	yes	/	yes
	J	free	/	/	/

4.4.3 Agarose gels of the different pPZ vectors and their inserts

As so many different clones were analysed with many different promotors and some cloning and mini-preps had to be repeated, not every agarose gel is shown. The agarose gels shown shall demonstrate examples of some positive clones, the size of their insert and different promotors used (Figure 12).

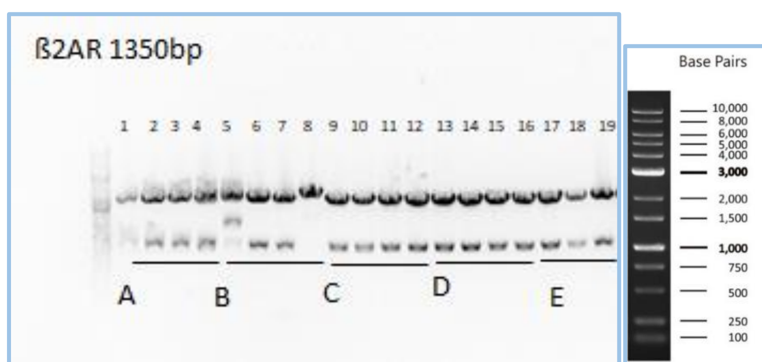

Figure 12: β2AR genes cut out of vectors with different promoters. Four clones were tested for each different promotor, A, B, C, D and E. The inserts of 1350 bp were cut out with Fast Digest restriction enzymes EcoRI and NotI. If all clones of one promotor were positive, one representative was sent for sequencing. (Thermofisher Scientific, 2019)

The DNA concentration measured of the PCR amplified inserts was decisive for the subsequent ligation into the pPZ-αMF vectors with different promotors. DNA concentration of the 5-HT 2c insert was around 20 ng/µl.

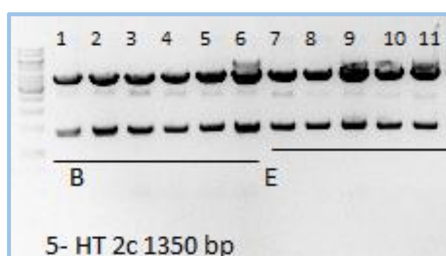

Figure 13: PCR product 5-HT 2c amplified from different pPZ vectors. Clones 3 and 7 of the 5-HT 2c insert amplified by PCR can be seen that were ligated into pPZ vectors with different promotors. The first 6 lanes at around 1300 bp show the insert 5-HT 2c of clone 3 with the B promotor and the following 5 with the E promotor.

4.5 Preparation for transformation of *P. pastoris*

DNA concentration of the *Bgl*II linearized vectors for integration into the genome of electrocompetent *P. pastoris* mut^s cells are depicted in Table 13.

Table 13: DNA concentration measured after desalting of the linearized pPZ vectors. In the table the different genes with their restriction enzymes used and promoter in the plasmid are described in one column. In the other column the concentrations of the different DNA constructs are displayed in µg/ml.

Insert and promotor for it	**DNA concentration measured [µg/ml]**
β2AR E/N A	474
β2AR E/N B	212
β2AR E/N C	331
β2AR E/N D	387
β2AR E/N E	441
β2AR E/N F	368
β2AR E/N G	274
β2AR E/N H	515
β2AR E/N I	186
5-HT 5a E/N A	224
5-HT 5a E/N B	323
5-HT 5a E/N C	218
5-HT 5a E/N D	120
5-HT 5a E/N E	175
5-HT 5a E/N F	495
5-HT 5a E/N G	226
5-HT 5a E/N H	227
5-HT 5a E/N I	111
5-HT 2c E/N B	383
5-HT 2c E/N C	392
5-HT 2c E/N D	298
5-HT 2c E/N E	368
5-HT 2c E/N G	225
5-HT 2c E/N H	309
5-HT 2c E/N I	326
5-HT 2c X/N B	296
5-HT 2c X/N E	285
5-HT 2c X/N H	278

4.6 Transformation of *P. pastoris*

Transformations worked well and transformation plates contained many single colonies as can be seen in Figure 14.

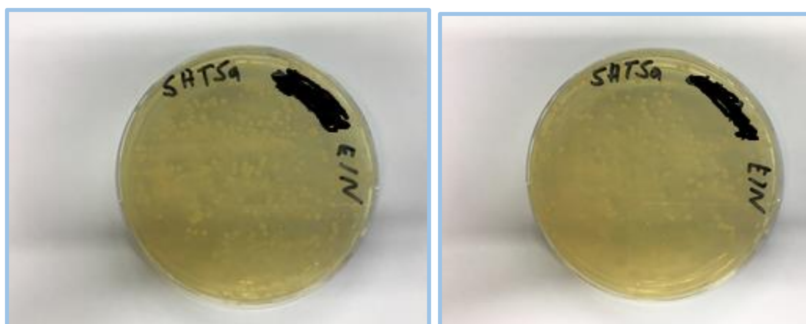

Figure 14: *P. pastoris* transformation plates

Transformation plates with 2 different types of *P. pastoris* clones containing the pPZ vector with the 5-HT 5a insert under promotor B or D.

4.7 Cultivation of *P. pastoris* clones

P. pastoris clones with different pPZ vectors were successfully inoculated as described in chapter 3.18 according to the cultivation schemes in chapter 3.18 and in the appendix.

4.8 Harvest and analysis of *P. pastoris* clones

The entire broth of all *P. pastoris* clones aiming at production of a particular target protein were harvested from the deep-well plates with pipets.

After harvesting, the OD of all clones was measured at 600 nm. Some OD values were consistent whereas others showed more fluctuations. Overall, clones with methanol-free promotors showed higher OD values especially for the 5-HT 5a producing clones. The mocks showed OD values in the higher range. For the 5-HT 5a clones it was shown that measurements are more precise and reliable with 1:30 dilutions. The 5-HT 5a protein producing clones showed generally higher OD values. The optical densities of the different clones and the amount of buffers used are displayed in the appendix in chapter 8.

Before lysis, lysis buffer, glass beads and PMSF were added in amounts calculated in accordance to the OD_{600} values measured. Values for all lyses performed are displayed in the appendix. Cell lysis at 4° with 20 vortexing and cooling steps for 1 min did not destroy the proteins, but lysed the cells efficiently.

In the table below the highest protein concentrations measured of different clones are shown. The remaining sample concentrations can be found in the appendix.

Table 14: Samples with the highest protein concentrations

Samples of different clones with different proteins under different promoters and their absorbance at 562 nm are depicted. It can be seen that there are a few high protein concentrations achieved with some clones when the dilution factor is considered.

Sample	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**
MeOH β2AR A clone 2	0.683	0.725	1:20	14.5

MeOH β2AR C clone 1	0.671	0.929	1:10	9.3
MeOH β2AR C clone 2	1.37	0.475	1:20	9.5
MeOH β2AR D clone 2	0.6	0.63	1:10	6.3
MeOH β2AR E clone 2	0.597	0.619	1:10	6.2
MeOH β2AR F clone 2	0.631	0.76	1:10	7.6
MeOH-free β2AR H clone 2	0.618	0.708	1:10	7.1
MeOH-free 5-HT 5a E clone 2	0.613	0.684	1:10	6.8

The protein concentrations depicted above show especially for one clone very high values. However, the total protein concentrations cannot reveal if there is a high expression of the desired recombinant protein.

4.8.4 Western blots

After lysis and centrifugation, the supernatant was transferred to a new tube. For the β2AR samples, lysate preparations from strains with methanol-induced promotors appeared slimy, but this was not the case for the methanol-free promotors.

4.8.4.1 Western blots of β2AR

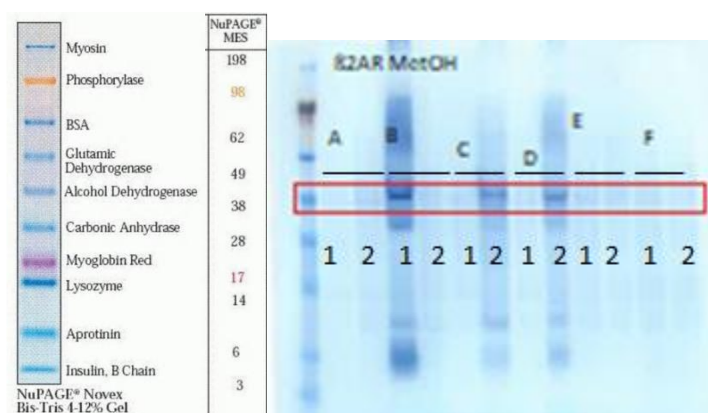

Figure 15: Western blot of the β2AR expressed with methanol induced promoters without ultracentrifugation

As it can be seen in the image of the Western blot of the methanol induced clones in Figure 15, bands at the size of the β2AR protein that is around 49 kDa were found. On the left side a protein standard ladder with protein type and molecular weights is displayed. Clone 1 with the B promotor, clone 2 with the C promotor and clone 2 with the D promotor show thicker and more darkish-blue bands. This indicates that they expressed more target protein than average expressing clones. Especially clone 1 with the B promotor seems to be a promising candidate.

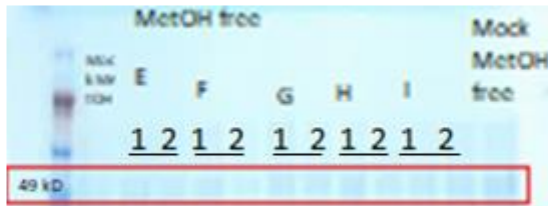

Figure 16: Western blot of the β2AR expressed with methanol-free promoters

On the Western blot of the MeOH-free clones (Figure 16), only weak lanes at the correct size of the β2AR protein are visible. There seemed to be low overall expression of the protein. The methanol-induced mock and the methanol-free mock appear in the same intensity. So the signals might be the recombinant protein at the correct size and the rest of the bands are degradation products from the host. Another possibility is that all bands are unspecific signals from other proteins as the mocks are at the same intensity as the samples.

4.8.4.2 Western blots of 5-HT 5a

For the 5-HT 5a protein, no anti His-tag antibody could be used as no His-tag was added to the N-terminus of the protein. Hence, detection of the Strep-tag I on the C-terminus of the protein with a Streptavidin HRP antibody was assessed. However, as it can be seen on the Western blots (Figure 17), the Streptavidin also unspecifically bound to a lot of other proteins present in the sample. The Streptavidin strongly binds to Biotin and therefore to biotinylated proteins that can be present in the lysate or it binds to different degradation products of expressed protein.

Figure 17: 5-HT 5a protein expressed under different methanol-induced promoters

On the Western blot with methanol-induced clones in Figure 17, there are bands for the 5-HT 5a protein that is around 41 kDa, between the protein standard bands for molecular weights of 38 and 49 kD. Some bands appear very dark blue and bigger, so they maybe contain high-expressing clones, but other proteins can also contribute to the intense colour due to unspecific binding to Streptavidin. On the bottom of the blot it can be seen that there was a lot of unspecific binding of the Streptavidin to other biotinylated proteins present. At the top some unprocessed receptor portions might have led to the background. The mock appears to be a little bit lighter than the other bands. So there might have been an overexpression of proteins in bands that are thicker.

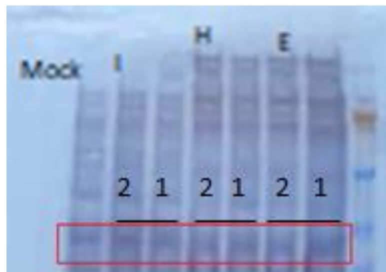

Figure 18: 5-HT 5a protein expressed under different methanol-free promoters

On the Western blot for lysates generated after methanol-free production (Figure 18), there are correct bands for the 5-HT 5a protein. They are not as dark-blue as the methanol-induced samples in Figure 16. On the bottom of the gel there is a lot of unspecific binding to various other biotinylated proteins. At the top, some unprocessed receptor portions might have led to the background. The band for the mock on the left is of similar intensity as those of the clones so it is difficult to prove that expression of the recombinant protein occurred. These bands could originate from unspecific signals. If there is an expression of the recombinant protein by the clones there are also a lot of degradation products visible on the blot.

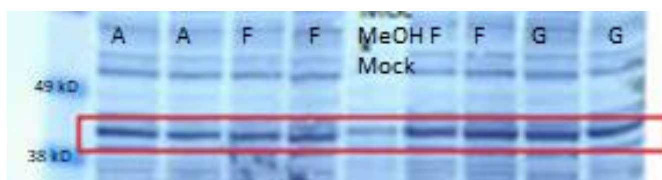

Figure 19: 5-HT 5a protein expressed under methanol-inducible and methanol-free promoters

On the Western blot in Figure 18, the remaining clones with the 5-HT 5a protein expressed under 2 different MeOH-inducible and MeOH-free promotors can be seen at around 41 kDa between the protein standard bands for molecular weights of 49 kDa and 38 kDa. The darker-blueish bands indicate an overexpression. On the bottom of the gel a lot of unspecific binding to the Streptavidin can be seen. At the top some unprocessed receptor portions might have led to additional bands on the Western blot. The mock appears very weak although the same amount of cell lysate was loaded. Apparently, a good expression is shown with the methanol-induced as well as with the methanol-free promoters.

4.8.4.3 Western blots of 5-HT 2c

The Western blot for the 5-HT 2c receptor was performed with an anti His-tag antibody.

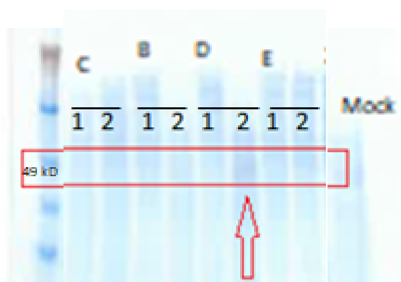

Figure 20: 5-HT 2c protein expressed under methanol-induced promoters

In Figure 20, only for clone 2 with promotor D there was better expression visible on the Western blot, although the lane appeared to be faint. For all other promotors there was hardly any expression found. The mock was weaker than the other bands so there is evidence for an expression of the recombinant protein under promoter D.

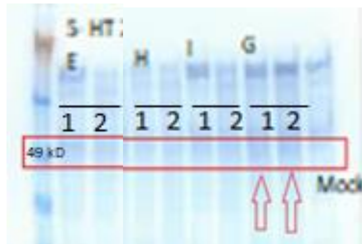

Figure 21: 5-HT 2c protein expressed under methanol-free promoters

In Figure 21, protein expression is confirmed by bands at the size of around 49 kDa on the Western blot. Only for promotor G there was better expression visible. The mock shows the weakest band.

4.8.5 Coomassie Brilliant Blue staining

Coomassie Brilliant Blue stains were performed to confirm the results of Western blots and see if there are any proteins found at the desired size that could be the recombinant protein.

5-HT 5a protein staining

As the Western blots of the 5-HT 5a protein contained a lot of background noise from unspecific antibody-binding, the staining of the gel and determination of protein presence was repeated with a Coomassie Brilliant Blue stain as it can be seen in Figure 22, 23 and 24.

Figure 22: 5-HT 5a protein expressed with methanol-inducible promoters

On the Coomassie stain in Figure 22, clones with the methanol-inducible promotors A, B, C, D and E and a mock can be seen. They should express the 5-HT 5a protein at a size of 41 kDa. On the left and the right side a protein standard ladder was applied. No absolute proof of overexpression of the target protein was given, although bands for mock lysate appeared weaker. As there was not the same protein concentration and only the same volume of every sample loaded, the bands do not show the same colour intensity.

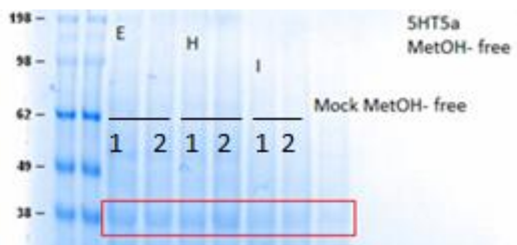

Figure 23: 5-HT 5a protein expressed under different methanol-free promoters

On the gel in Figure 23, clones with methanol-free promotors E, H and I and a mock can be seen. They should express the 5-HT 5a protein at a size of 41 kDa. No overexpression was found as the bands appear to be weakly coloured, but the mock was even fainter. As there was not the same protein concentration and only the same volume of every sample loaded, the bands do not show the same colour intensity.

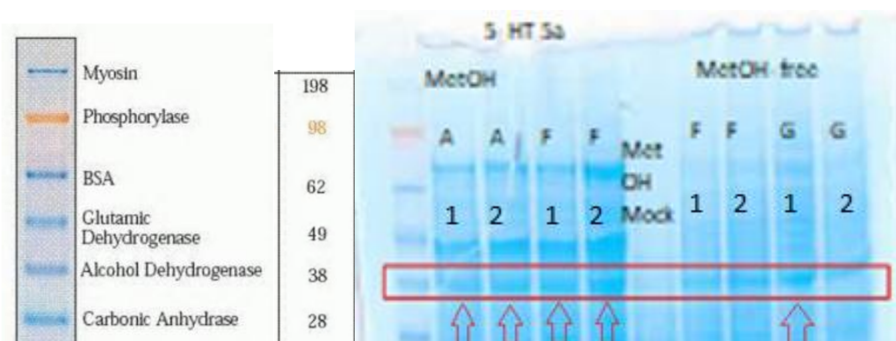

Figure 24: 5-HT 5a protein expressed with different methanol-induced and methanol-free promoters

As there was not the same protein concentration and only the same volume of every sample loaded, the bands do not show the same colour intensity on the gel in Figure 24. Bands can be seen at around 41 kDa slightly above the protein marker for a molecular weight of 38 kDa. The bands for the recombinant protein appear to be at the same size as in the previous Western blots. There seems to be an expression, but no clear overexpression of the protein. The mock appears only slightly coloured.

4.9 Rescreening of well-expressing clones

4.9.1 Selection of rescreened clones

Well expressing clones were indicated by darker blue-coloured bands than the other bands on the Western blots. So some clones with different methanol-inducible or methanol-free promoters were chosen to be rescreened.

Table 15: Well-expressing clones and corresponding methanol-induced or methanol-free promotors

Protein	Induction	Promotor type	Clone number
β2AR	MeOH	B	1
β2AR	MeOH	C	2
β2AR	MeOH	D (later added)	2
5-HT 5a	MeOH	A	2
5-HT 5a	MeOH	B	2
5-HT 5a	MeOH-free	F (later added)	2
5-HT 5a	MeOH-free	G (later added)	1
5-HT 2c	MeOH	D	2

5-HT 2c	MeOH-free	G	1

After cultivation and harvest, one third of each clone was lysed as usual. One third was lysed with Fos Choline 14 added before lysis and one third was treated with Fos Choline 14 after lysis. With the addition of Fos Choline 14 during lysis or after lysis it was expected to obtain more yield. Maybe more overexpression on the Western blots can be seen as the agent was anticipated to release the membrane proteins more efficiently from membrane components. The ODs measured, the amount of buffer and glass beads added and the amount of Fos Choline 14 added are found in the appendix.

Table 16: Rescreened clones with protein inserts under different promoters and Fos Choline added or not

Sample number	**Protein and clone**	**Induction**	**Promoter**	**Fos choline 14 addition**
1	β2AR 1	MeOH	B	None
2	β2AR 2	MeOH	C	None
3	5-HT 5a 2	MeOH	A	None
4	5-HT 5a 2	MeOH	B	None
5	5-HT 2c 2	MeOH	D	None
6	5-HT 2c 1	MeOH-free	G	None
7	Mock	MeOH	/	None
8	Mock	MeOH-free	/	None
9	β2AR 1	MeOH	B	Before lysis
10	β2AR 2	MeOH	C	Before lysis
11	5-HT 5a 2	MeOH	A	Before lysis
12	5-HT 5a 2	MeOH	B	Before lysis
13	5-HT 2c 2	MeOH	D	Before lysis
14	5-HT 2c 1	MeOH-free	G	Before lysis
15	Mock	MeOH	/	Before lysis
16	Mock	MeOH-free	/	Before lysis
17	β2AR 1	MeOH	B	After lysis
18	β2AR 2	MetOH	C	After lysis
19	5-HT 5a 2	MeOH	A	After lysis
20	5-HT 5a 2	MeOH	B	After lysis
21	5-HT 2c 2	MeOH	D	After lysis
22	5-HT 2c 1	MeOH-free	G	After lysis
23	Mock	MeOH	/	After lysis
24	Mock	MeOH-free	/	After lysis
25	β2AR	MeOH	D	None
26	5-HT 5a	MeOH-free	F	None
27	5-HT 5a	MeOH-free	G	None

4.9.2 Total protein concentration after ultracentrifugation

The total protein concentration was measured from all samples after ultracentrifugation and precipitation. In Table 17 the clones with the highest protein concentration are shown. The remaining clones with their concentrations can be found in the appendix.

Table 17: Clones with highest protein concentrations measured

The table shows the different clones under different Fos Choline 14 conditions and their absorbance at 562 nm with dilution factor. The total protein concentration in mg/ml was calculated from the equation of the linear calibration line generated with a serial dilution of BSA.

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**
No Fos β2AR B	0.899	0.632	1:10	6.3
No Fos β2AR C	1.379	0.316	1:20	6.3
No Fos 5-HT 5a B	1.009	0.724	1:10	7.2
No Fos 5-HT 2c D	0.679	0.448	1:10	4.5
Before lysis β2AR C	0.914	0.645	1:10	6.5
Before lysis 5-HT 5a A	0.496	0.294	1:10	2.9
Before lysis 5-HT 2c D	1.246	0.923	1:10	9.2
After lysis β2AR C	0.816	0.562	1:10	5.6
After lysis 5-HT 5a A	1.273	0.945	1:10	9.5

The highest concentrations were found mostly for clones without any Fos Choline added for lysis. However, there were also some clones that showed high overall protein concentrations with the addition of Fos Choline before lysis or afterwards. The highest total protein concentration was found for a clone with the 5-HT 5a protein under the A promoter with Fos Choline added after cell lysis. On the one hand, a total protein concentration can mean that there is also a higher expression of the target protein, but on the other hand the concentration of recombinant protein produced can be low even though high total protein concentrations were measured.

4.9.3 Total protein concentration without ultracentrifugation

The total protein concentration was measured for all rescreened clones after lysis without ultracentrifugation to compare results. In Table 18 the clones with the highest protein concentration are shown. The remaining clones with their concentrations can be found in the appendix.

Table 18: Rescreened clones without ultracentrifugation with highest concentrations

The table shows the different clones under different Fos Choline 14 conditions and their absorbance at 562 nm at a dilution of 1:10. The total protein concentration in mg/ml was calculated from the equation of the linear calibration line generated with a serial dilution of BSA.

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**
No Fos 5-HT 2c D	1.25	0.909	1:10	9.1

No Fos 5-HT 2c G	0.881	0.616	1:10	6.2
Before lysis 5-HT 5a B	1.074	0.769	1:10	7.7

Also without an ultracentrifugation some clones with higher protein concentrations were detected. However, this does not mean that there is also a high expression of the recombinant protein.

4.9.4 Western blots

From all β2AR, 5-HT 2c and 5-HT 5a strains from rescreening under different Fos Choline 14 conditions Western blots were made to compare the yield of the recombinant protein.

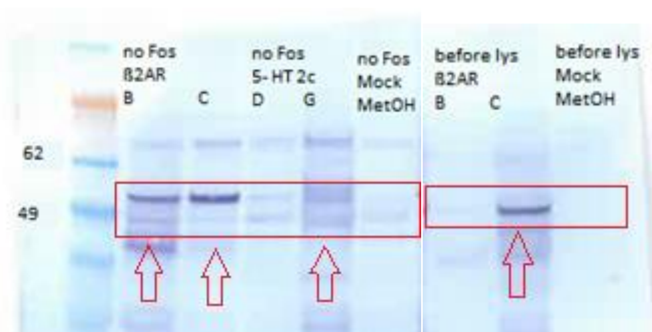

Figure 25: Rescreened clones with β2AR and 5-HT 2c proteins under different promoters and conditions

On the Western blot of protein samples β2AR and 5-HT 2c after ultracentrifugation in Figure 25, only some clones showed a high expression level. Fos Choline 14 addition before the cell lysis seems to have a positive impact only on a few clones. Clones with the β2AR protein under promoter B and C showed a good expression without Fos Choline treatment and only under promoter C with the addition of Fos before lysis. Bands of the recombinant proteins are nearly always stronger than of the mocks. Most intense bands are obtained without the addition of Fos. However, by adding Fos Choline before lysis the degradation products and unspecific bindings on the Western blots generally seem to be reduced.

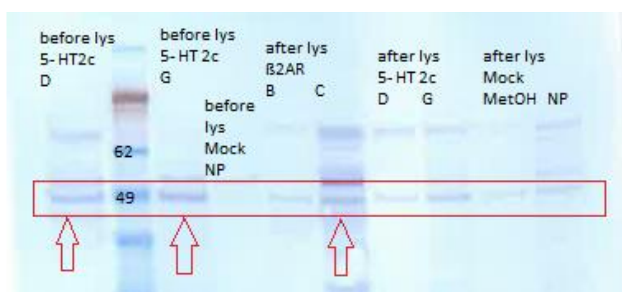

Figure 26: Rescreened clones with β2AR and 5-HT 2c proteins under different promoters and conditions

On the Western blot of protein samples of β2AR and 5-HT 2c after ultracentrifugation in Figure 26, only some clones appear to have a high expression. It can be seen, for example under promoter G, that the addition of Fos Choline 14 before cell lysis is way more beneficial on protein release than after lysis. Clones with the 5-HT 2c protein

under promoter D and G show good expression with the addition of Fos Choline before cell lysis.

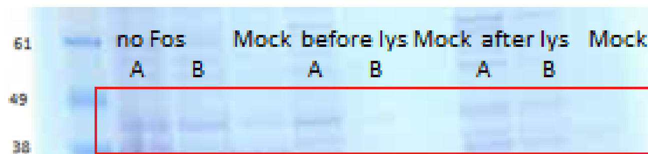

Figure 27: Rescreened clones with 5-HT 5a protein under different promoters and conditions

On the Western blot in Figure 27, a few weak bands at 41 kD can be seen for the 5-HT 5a protein. Bands of the mock appeared weaker than of the recombinant proteins. On the Western blot with the Streptadivin antibody added, some slightly coloured bands can be found at the correct size under promoter A. A slightly positive effect by the addition of Fos Choline 14 can be seen for the reduction of bands from unspecific binding on the Western blots. So sometimes Fos Choline might help to decrease impurities on Western blots. The impurities might largely originate from degradation products from protein transport from the ER to the Golgi apparatus and to the plasma membrane and from protein folding that bind to the Streptadivin and the Strep-Tactin antibody as well.

4.10 Clones induced at lower temperature

Nine different well-expressing clones and two mocks (β2AR B/C/D, 5-HT 5a A/B/F/G, 5-HT 2c D/G, mut^s MeOH-induced and mut^s MeOH-free) were inoculated as previously done in shake flasks, but during application of productive conditions (i.e. with or without methanol) they were incubated at 22 °C instead of 28 ° applied in earlier experiments. This could potentially increase the expression of recombinant membrane proteins as the folding machinery works slower and more diligently. In the appendix, optical densities and the amount of lysis buffer added can be seen.

4.10.1 Total protein concentration

Before and after ultracentrifuging and precipitating, the total protein concentration in the samples was measured.

Table 19: Best expressing clones with proteins under different promoters induced at 22 °C

Sample and Characteristics	Absorbance at 562 nm	Protein concentration [mg/ml]	Dilution factor	Total protein concentration [mg/ml]
β2AR_B vor ulze	0.293	0.137	1:10	1.4
β2AR_D vor ulze	0.339	0.178	1:10	1.8
5-HT 5a_A vor ulze	0.275	0.121	1:10	1.2
5-HT 5a_B vor ulze	0.274	0.12	1:10	1.2
5-HT 2c_D vor ulze	0.416	0.247	1:10	2.5
5-HT 2c_G vor ulze	0.349	0.187	1:10	1.9

5-HT 5a_G nach ulze	0.446	0.275	1:10	2.8
5-HT 2c_D nach ulze	0.126	0.012	1:10	0.1
5-HT 2c_G nach ulze	0.273	0.119	1:10	1.2

In Table 19 it can be seen that the protein concentrations are not as high as in Table 17 and 18. Especially after ultracentrifuging there is only one clone with a rather high protein concentration when looking at other clones of this run, but none was that high as the best expressing clones in the previous studies. This might be due to the lower induction temperature.

4.10.2 Western blots

This time it was tried to apply not only the same volume, but also the same protein concentration of 1 µg/ml on the SDS-PAGE gel after a BCA assay.

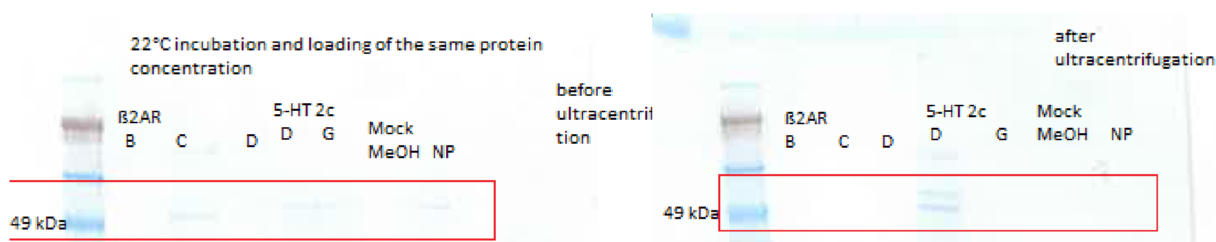

Figure 28: Western blot of β2AR or 5-HT 2c proteins under different promoters induced at 22 °C

Due to the fact that the same protein concentration was loaded for every sample, there is less background visible on the Western blots in Figure 28. On the right Western blot with lysates after ultracentrifugation there are two bands visible for the 5-HT 2c protein expressed under promoter D. The reason for that might be that some of the recombinant proteins were still glycosylated while others were not. In Western blots, only the strongest clones show a band at the correct size. Surprisingly, more proteins seemed to be present, before than after ultracentrifugation and precipitation. Again, promoter C shows expression of the β2AR protein. Promoter D shows expression of the 5-HT 2c protein. However, the bands at the correct size do not appear as strongly coloured as observed previously on the blots of clones cultivated at 28 °C. This might be due to the fact that the same concentration of 10 µg/ml was loaded for every sample on the SDS-PAGE or the more probable reason that there was less protein production at 22 °C which was also suggested by the results of the BCA assay.

Figure 29: β2AR and 5-HT 2c expressed under different methanol-inducible and methanol-free promoters before and after ultracentrifugation

In Figure 29, the promoter D shows a good expression of both membrane protein types. All bands of mocks appear weaker. After ultracentrifugation the background of degradation products and unspecific bindings seems to be lower, but also some bands of the recombinant protein. Bands appear generally stronger than in Figure 29 as 20 µl of each protein were loaded on the SDS-PAGE and not the same concentrations for every sample. However, blots with clones induced at 28 °C were still showing more intense bands overall.

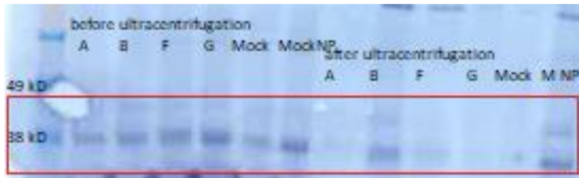

Figure 30: 5-HT 5a protein under different methanol-inducible and methanol-free promoters before and after ultracentrifugation with the same protein concentrations loaded

In Figure 30, a good expression of the 5-HT 5a protein can be seen under methanol-inducible promoters as bands at 41 kD are darker coloured than the methanol-induced mock. So the samples might express the recombinant protein and another protein at the same size that is also present in the mock. However, for methanol-free expression the mock appears with the same intensity as the recombinant proteins. So it is not sure if the bands show an expression of the recombinant protein or just unspecific signals of other proteins present in every sample.

4.11 De-glycosylated samples

Some clones expressing the 5-HT 5a receptor were de-glycosylated before the SDS-PAGE to see if only one of two bands at a similar size remains that originates from non-glycosylated protein.

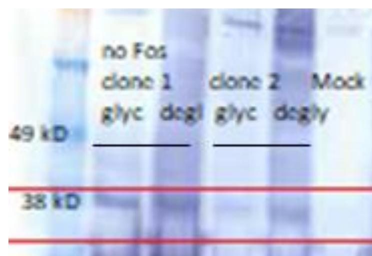

Figure 31: Western blot comparing glycosylated and de-glycosylated 5-HT 5a samples

For the first clone not lysed with Fos Choline, the band of the de-glycosylated recombinant protein at 38 kD is thicker and darker coloured that the band of the glycosylated one. The same is true for clone 2 of lysis without Fos Choline. The mock does not express the recombinant protein. The results indicate that de-glycosylation leads to thicker bands visible at the correct size and so the expression looks stronger than in Figure 33 without de-glycosylation applied before. The de-glycosylated proteins migrate further as they are smaller than glycosylated ones and collect at one size.

4.12 Proteins with the αMF signal sequence attached

3 different clones with the 5-HT 2c sequence without its native signal sequence, but the αMF signal sequence attached at the N-terminus were cultivated in duplicates and harvested as previously done. The optical density of the clones and the amount of buffers used are found in the appendix.

4.12.1 Total protein concentration

In Table 20 the highest total protein concentrations are depicted. With every promoter selected, acceptable total protein concentrations were obtained.

Table 20: Highest total protein concentrations of 5-HT 2c obtained in the BCA assay before and after ultracentrifugation

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration before ultracentrifugation[mg/ml]**	**Total protein concentration after ultracentrifugation [mg/ml]**
5-HT 2c X/N B	0.371	0.604	1:10	6.0	1.1
5-HT 2c X/N H	0.209	0.115	1:10	1.2	0.2
5-HT 2c X/N E NP	0.232	0.185	1:10	1.9	0.2

In Table 20 total protein concentrations significantly decreased after ultracentrifugation and precipitation. However, this does not mean that the concentration of recombinant protein decreased as well.

4.12.2 Western blots

Different Western blots were made to detect the proteins of interest.

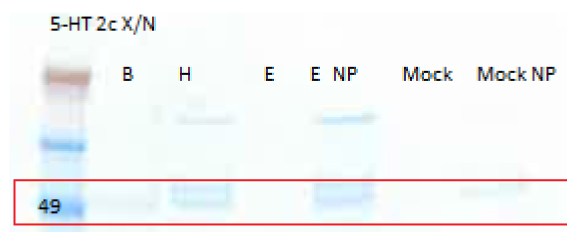

Figure 32: Western blot of 5-HT 2c with αMF signal sequence expressed under different promoters before ultracentrifugation

In Figure 32, there are bands visible for proteins under promoter B and E methanol-free and H. The band of the methanol-free mock is weaker than the band of the methanol-free promoters H and E. 20 µl lysate were loaded to see if every promoter expresses the recombinant protein. For the methanol-free promoters H and E two bands above each other can be seen which might be parts of glycosylated and de-glycosylated protein.

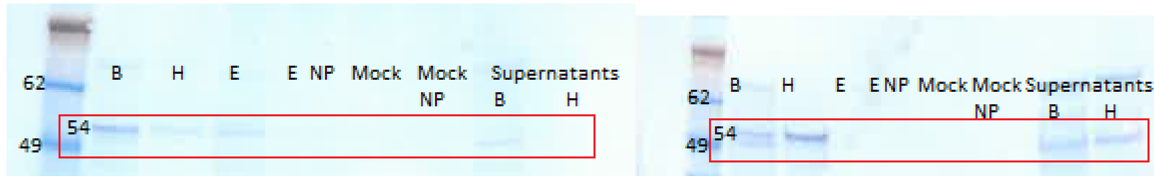

Figure 33: Western blots of 5-HT 2c with αMF signal sequence expressed under different promoters after ultracentrifugation

In Figure 33, on the left blot 7 µg/ml total protein concentration were loaded, therefore bands appear weaker. In comparison to Figure 34, bands appear a bit brighter because lysates were ultracentrifuged additionally after lysis. Especially promoter B in both Western blots in Figure 35 seems to express the recombinant protein very well. Samples of some supernatants after ultracentrifugation were taken. A little part of recombinant protein seems to stay in the supernatant displayed by both Western blots. Both mock samples do not show bands corresponding to the molecular weight range of the recombinant protein. On the right blot in Figure 35 more total protein concentration was loaded and bands appear bigger. The blot shows the same results as the left one. The B and H promoter express the 5-HT 2c with the αMF signal sequence well, but some parts still remain in the supernatant after the treatment.

4.13 Determination of multiple integration events

All clones streaked from the glycerol stocks grew at 100 µg/ml antibiotic concentration and so they were firstly tested on 300 µg/ml Zeocin added to the YPhyD. The concentration was step-wise increased.

Sample	**Growth at 300 µg/ml**	**500 µg/ml**	**1000 µg/ml**	**2000 µg/ml**	**4000 µg/ml**
β2AR A	Yes	Yes	Yes	Yes	Yes
β2AR B	Yes	Yes	Yes	Yes	Yes
β2AR C	Yes	Yes	Yes	Yes	Yes
β2AR D	Yes	Yes	Yes	Yes	Yes
β2AR E	Yes	Yes	Yes	Yes	Yes
β2AR F	Yes	Yes	Yes	Yes	Yes
β2AR G	Yes	Yes	Yes	Yes	Yes
β2AR H	Yes	Yes	Yes	Yes	Yes
β2AR I	Yes	Yes	Yes	Yes	Yes
5-HT 5a A	Yes	Yes	Yes	Yes	Yes
5-HT 5a B	Yes	Yes	Yes	Yes	Yes
5-HT 5a C	Yes	Yes	Yes	Yes	Yes
5-HT 5a D	Yes	Yes	Yes	Yes	Yes
5-HT 5a E	Yes	Yes	Yes	Yes	Yes
5-HT 5a F	Yes	Yes	Yes	Yes	Yes
5-HT 5a G	Yes	Yes	Yes	Yes	Yes
5-HT 5a H	Yes	Yes	Yes	Yes	Yes
5-HT 5a I	Yes	Yes	Yes	Yes	Yes
5-HT 2c B	Yes	Yes	Yes	Yes	Yes
5-HT 2c C	Yes	Yes	Yes	Yes	Yes
5-HT 2c D	Yes	Yes	Yes	Yes	Yes
5-HT 2c E	Yes	Yes	Yes	Yes	Yes

5-HT 2c G	Yes	Yes	Yes	Yes	Yes
5-HT 2c H	Yes	Yes	Yes	Yes	Yes
5-HT 2c I	Yes	Yes	Yes	Yes	Yes
5-HT 2c αMF B	Yes	Yes	Yes	Yes	Yes
5-HT 2c αMF E	Yes	Yes	Yes	Yes	No
5-HT 2c αMF H	Yes	Yes	Yes	Yes	No
β2AR-GFP A	Yes	Yes	Yes	Yes	Yes
β2AR-GFP B	Yes	Yes	Yes	Yes	Yes
β2AR-GFP E	Yes	Yes	Yes	Yes	Yes
β2AR-GFP I	Yes	Yes	Yes	Yes	Yes
5-HT 2c-GFP A	Yes	Yes	Yes	Yes	Yes
5-HT 2c-GFP B	Yes	Yes	Yes	Yes	Yes
5-HT 2c-GFP E	Yes	Yes	Yes	Yes	Yes
5-HT 2c-GFP I	Yes	Yes	Yes	Yes	No
5-HT 2c-αMF-GFP A	Yes	Yes	Yes	Yes	Yes
5-HT 2c-αMF-GFP C	Yes	Yes	Yes	Yes	No
5-HT 2c-αMF-GFP E	Yes	Yes	Yes	Yes	No
5-HT 2c-αMF-GFP I	Yes	Yes	Yes	Yes	No
Mock mut^s	No	No	No	No	No

4.14 Western blots for quantification with the best expressing clones

20 µg total protein concentration of clones before ultracentrifugation and 40 µg total protein concentration after ultracentrifugation that showed the highest concentrations in the BCA assays and darkest bands on the Western blots, were loaded on a SDS-PAGE. Protein expression was detected in Western blots with an anti His-tag AB and a Streptadivin AB. A standard His-HSA protein was loaded additionally at different concentrations for estimation of recombinant protein concentration.

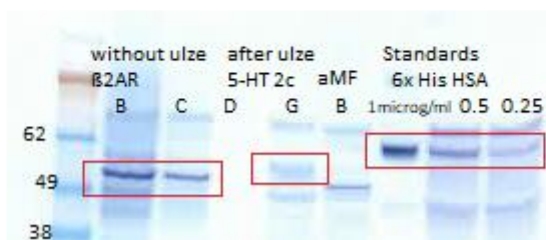

Figure 34: Western blot of the best expressing clones from all samples and HSA standards

In Figure 34, the β2AR clones with promoter B and C without ultracentrifugation show dark bands at the correct size of around 49 kD. For the 5-HT 2c clones the protein under the G promoter appeared higher than the correct size of 54 kD and so might be still glycosylated. For promoter D no band of the recombinant protein was visible. The 5-HT 2c protein with the αMF signal sequence showed a band at the correct size of 54 kD. The standard HSA protein shows a lane at its correct size around 62 kD. From the intensities of the bands it can be estimated that the β2AR protein produced under

promoter B is approximately at the same concentration as the first standard, so 1 µg/ml lysate. The β2AR protein under promoter C seems to have the concentration of the second standard, so 0.5 µg/ml lysate.

After de-staining, the Western blot in Figure 38 was visualized with a Gel doc (Biorad) with the option Silver stain. Samples after ultracentrifugation were chosen as bands appear generally clearer, better separated and with less background on blots.

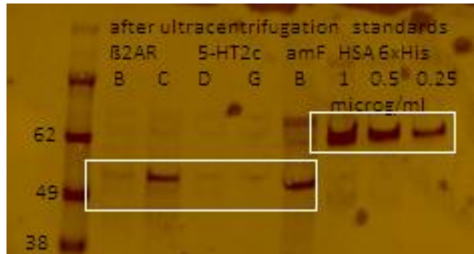

Figure 35: Anti His-tag Western blot with option “silver stain” on Gel doc displaying the β2AR, the 5-HT 2c proteins after ultracentrifugation and a HSA standard protein at three different concentrations. Proteins and standards at their correct size are marked in white.

On the Western blot in Figure 35, an anti His AB was used to detect the β2AR and the 5-HT 2c protein under different promoters and the HSA protein standard at different concentrations. The β2AR is best expressed under promoter C that appears approximately at the same intensity as the first standard, so around 1 µg/ml lysate. As they are displayed a little bigger than 48 kD, they might be glycosylated. The bands of the 5-HT 2c under promoter D and G appear very weak. The 5-HT 2c with the αMF signal sequence appears as intense as the first standard, so at a concentration of 1 µg/ml. Due to the chosen gel imaging stain the standards appear nearly at the same intensity, but are of different concentrations. The 0.5 and 0.25 µg/ml standards were diluted with a MeOH-induced mock.

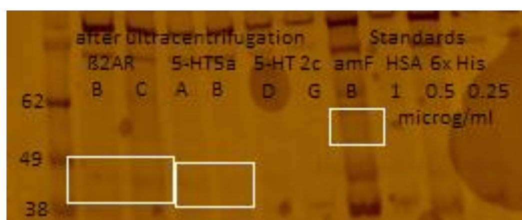

Figure 36: Streptadivin antibody Western blot of the best expressing clones of the β2AR, 5-HT 5a and 5-HT 2c after ultracentrifugation and three HSA standards at different concentrations with “silver stain” option used for photographing the gel. Proteins and corresponding sizes are marked in white.

In Figure 36, some bands of degradation products from the host are visible on the bottom of the blot. Bands of the recombinant proteins appear very weak although 40 µg of total protein concentration were loaded. The band with the highest intensity was visible from the 5-HT 2c protein with the αMF signal sequence under promoter B. Weakness of the bands might relate to inefficient binding of the Streptadivin AB to the target protein.

4.16 β2AR-GFP constructs

Vectors with the β2AR-linker-GFP constructs under different methanol-inducible and methanol-free promoters were used to transform recombinant *E.coli* DH5-α cells and

mut[s] P. pastoris cells. Final clones with plasmids containing the GFP-construct and the promoters A, B, E, E-MeOH-free or I were obtained.

4.16.1 Optical densities and fluorescence measurements

There were always samples taken from the cultivated cells before induction to measure the OD_{600} and fluorescence to see how it changes over time of induction.

Table 21: OD and fluorescence measurements at different time points

Hours after first induction	Clone with promoter	Fluorescence	Total fluorescence	OD_{600}	Total OD_{600}	Fluorescence/ OD
0	A	32,368	647,360	0.856	17.12	37,813.1
0	B	26,086	521,720	0.625	12.5	41,737.6
0	E MeOH	36,902	738,040	0.772	15.44	47,800.5
0	E	22,409	448,180	0.779	15.58	28,766.4
0	I	21,511	430,220	0.764	15.28	28,155.8
8	A	28,679	573,580	0.721	14.42	39,776.70
8	B	105,504	2110,080	0.653	13.06	161,568.15
8	E MeOH	50,132	1002,640	0.705	14.1	71,109.22
24	A	19,521	390,420	0.677	13.54	28,834.6
24	B	101,021	2020,420	0.578	11.56	174,776.8
24	E MeOH	24,392	487,840	0.641	12.82	38,053.0
24	E	21,605	432,100	0.602	12.04	35,888.7
24	I	26,552	531,040	0.89	17.8	29,833.7
48	A	18,562	371,240	0.651	13.02	28,513.1
48	B	55,801	1116,020	0.717	14.34	77,825.7
48	E MeOH	29,943	598,860	0.625	12.5	47,908.8
48	E	30,548	610,960	0.62	18.6	32,847.3
48	I	33,604	672,080	0.693	20.79	32,327.1
72	A	15,566	311,320	0.418	12.54	24,826.2
72	B	49,429	988,580	0.452	13.56	72,904.1
72	E MeOH	29,471	589,420	0.43	12.9	45,691.5
72	E	22,802	456,040	0.716	21.48	21,230.9
72	I	31,491	629,820	0.673	20.19	31,194.7

Samples were taken from all clones with different promoters on their plasmids before the first induction, so at time point 0 (T0). The samples were examined by OD_{600} measurements and fluorescence emission. Eight hours after the first induction only clones with methanol-induced promoters were induced and so there were only samples from methanol-induced clones taken (T1). The next samples were taken 24 h (T2), 48 h (T3) and 72 h (T4) after the first induction. The relative fluorescence units obtained by measuring the emission of the samples is divided by the total optical density obtained. This value should give a first sight how much fluorescence is emitted by the cells and so also a rough estimation on the amount of recombinant protein expressed.

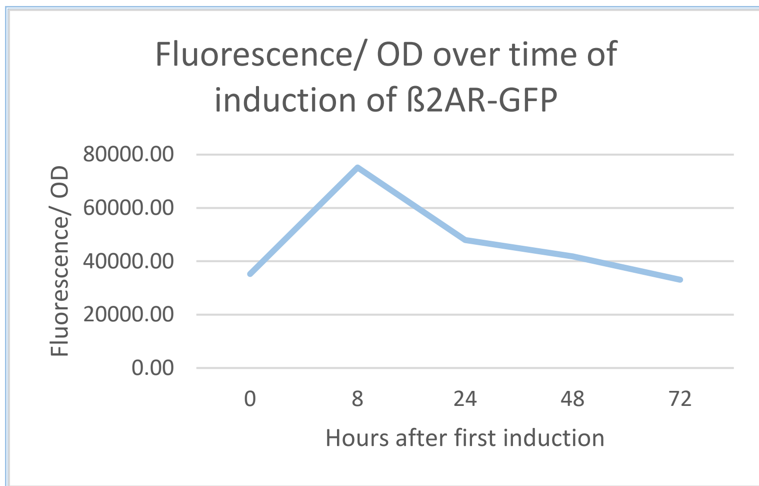

Figure 37: Fluorescence/OD over sampling time points

The fluorescence per optical density at 600 nm was calculated and plotted against the different time points after the first induction. At the beginning of the induction there is an increase of fluorescence, but after subsequent inductions, the fluorescence units decrease.

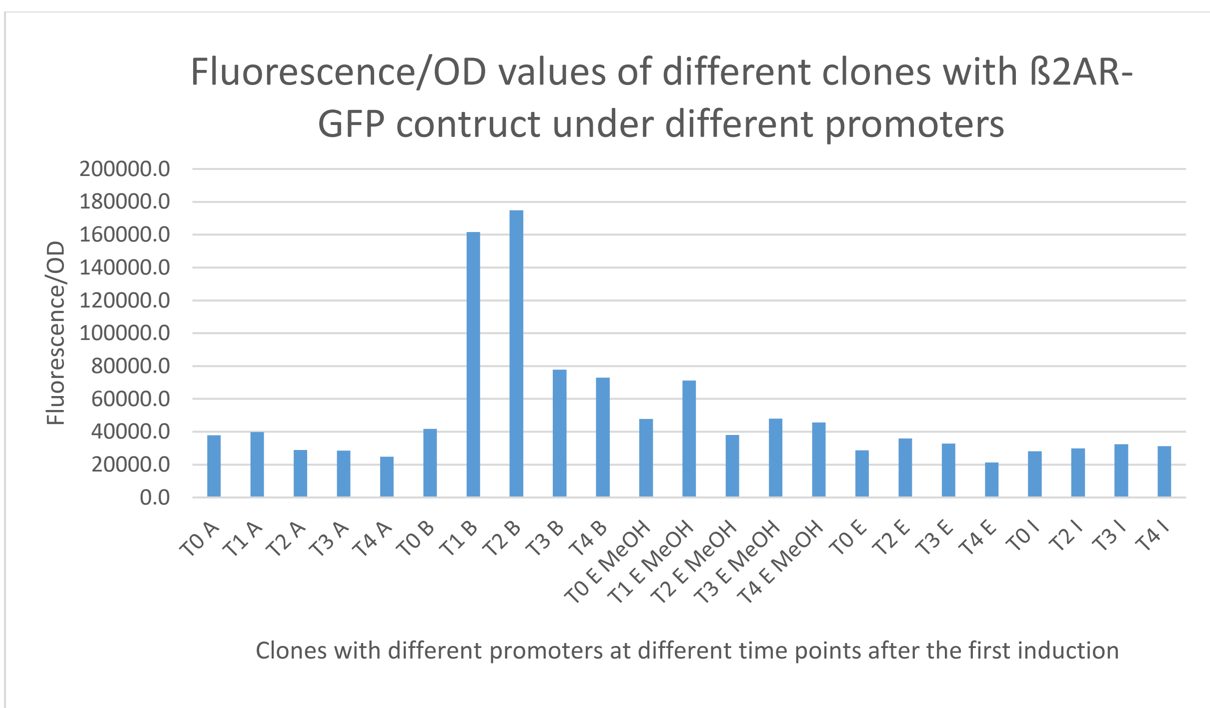

Figure 38: Fluorescence/OD of different promoters at different sampling time points

The fluorescence per OD was calculated for clones with the β2AR-GFP construct under different promoters and measured at different time points after the first induction. T0 was at the time before the first induction, T1 was after 8 h of the first induction, only for the MeOH-induced clones, T2 24 h, T3 48 h and T4 was sampled 72 h after the first induction. The diagram shows an increase in fluorescence after the first induction, but overall a decrease after more induction steps.

The β2AR construct shows the highest fluorescence/OD at 8 h and 24 h after induction under promoter B.

4.16.2 Total protein concentrations

Total protein concentrations of lysates were measured to load the same protein concentration of every sample on the SDS-PAGE to directly compare the expression patterns of the recombinant protein under different promoters.

Table 22: The total protein concentration of clones expressing the β2AR-GFP construct under different promoters, measured in a BCA assay

Sample and Characteristics	Absorbance at 562 nm	Protein concentration [mg/ml]	Dilution factor	Total protein concentration [mg/ml]
β2AR-GFP A	0.452	0.239	1:20	4.8
β2AR-GFP B	0.31	0.105	1:20	2.1
β2AR-GFP E MeOH	0.343	0.136	1:20	2.7
Mock MeOH	0.26	0.057	1:10	0.6
β2AR-GFP E MeOH-free	0.343	0.136	1:10	1.4
β2AR-GFP I	0.349	0.142	1:20	2.8
Mock MeOH-free	0.363	0.155	1:10	1.6

4.16.3 Coomassie blue stain

Coomassie brilliant blue stains were performed to see the diversity of proteins present in the lysates and proteins at the calculated size of the recombinant protein.

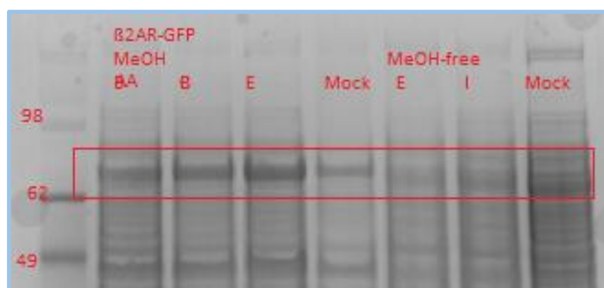

Figure 39: Coomassie blue stain, coloured grey, of β2AR-GFP protein

On the Coomassie blue stained gel in Figure 39, visualized on gel doc and coloured in grey, there are protein bands visible at the correct size of the recombinant protein at around 72 kD under the methanol-induced promoters. There were also bands detected in the MeOH mock sample at similar molecular weights, but none appeared to be exactly matching to the clear bands observed for candidate lysates. Under the methanol-free promoters no recombinant protein was produced at a size of 70 kD.

4.16.4 Western blot

Western blots were performed with anti His-tag antibodies for detection to specifically target the protein of interest and the His-HSA standard.

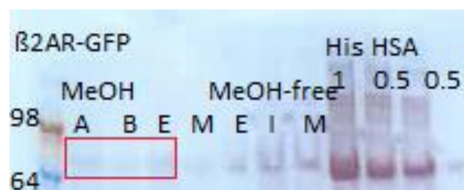

Figure 40: Western blot with the recombinant β2AR-GFP expressed

On the Western blot bands at the size of the recombinant protein are visible. The bands for the expression of the β2AR-GFP construct under methanol-inducible promoters A and E appear strongest. The MeOH-free promoters do not express the recombinant protein. However, another protein approximately present at the same size is expressed as it is also visible in the mock. The other protein bands seen for the methanol-free promoters can originate from HSA that migrated in these wells in the SDS-PAGE. On the right side of the blot a His-HSA standard was applied at different concentrations in µg/ml to estimate the amount of recombinant protein in the other bands. When comparing the MeOH-produced proteins to the bands of the HSA standard, one can immediately see that the recombinant proteins are expressed to very low levels.

4.17 5-HT 2c-GFP constructs

Vectors with the 5-HT2c-linker-GFP constructs under different methanol-inducible and methanol-free promoters were used to transform recombinant *E.coli* DH5-α cells and subsequently mut^{s} *P. pastoris* cells using linearized DNA. Final clones with plasmids containing the GFP-construct and the promoters A, B, E, E MeOH-free or I were obtained.

4.17.1 Optical densities and fluorescence measurements

Table 23: OD_{600} and fluorescence measurements at different time points

Hours after induction	Clone with promoter	OD_{600}	Total OD_{600}	Fluorescence	Total fluorescence	Fluorescence/ OD
0	A	0.835	16.7	17,174	343,480	20,567.7
0	B	0.728	14.56	28,245	564,900	38,798.1
0	E MeOH	0.752	15.04	19,176	383,520	25,500.0
0	E	0.792	15.84	19,632	392,640	24,787.9
0	I	0.762	15.24	18,996	379,920	24,929.1
8	A	0.754	15.08	21,824	436,480	28,944.3
8	B	0.796	15.92	28,053	561,060	35,242.5
8	E MeOH	0.779	15.58	20,486	409,720	26,297.8
24	A	0.663	13.26	21,353	427,060	32,206.6
24	B	0.589	11.78	25,167	503,340	42,728.4
24	E MeOH	0.581	11.62	16,748	334,960	28,826.2
24	E	0.56	11.2	20,584	411,680	36,757.1
24	I	0.65	19.5	18,893	377,860	19,377.4
48	A	0.687	13.74	21,816	436,320	31,755.5
48	B	0.554	11.08	23,166	463,320	41,815.9
48	E MeOH	0.639	12.78	19,037	380,740	29,791.9
48	E	0.726	21.78	21,152	423,040	19,423.3
48	I	0.809	24.27	23,822	476,440	19,630.8

72	A	0.557	16.71	32,675	653,500	39,108.3
72	B	0.35	10.5	24,366	487,320	46,411.4
72	E MeOH	0.356	10.68	21,013	420,260	39,350.2
72	E	0.398	11.94	39,181	783,620	65,629.8
72	I	0.729	21.87	27,998	559,960	25,604.0

Samples were taken from all clones with different promoters on their plasmids before the first induction, so at time point 0 (T_0). The samples were examined by OD_{600} measurements and fluorescence emission. The next samples were taken as described before. The relative fluorescence units obtained by measuring the emission of the samples is divided by the total optical density obtained. This value should give a first evidence how much fluorescence is emitted by the cells and so also a rough estimation on the amount of recombinant protein expressed.

The fluorescence values of the 5-HT 2c-GFP construct expressed under different promoters show a greater variability than the other constructs. So it is difficult to assess if recombinant fusions of membrane protein with GFP were expressed and produced additional fluorescence.

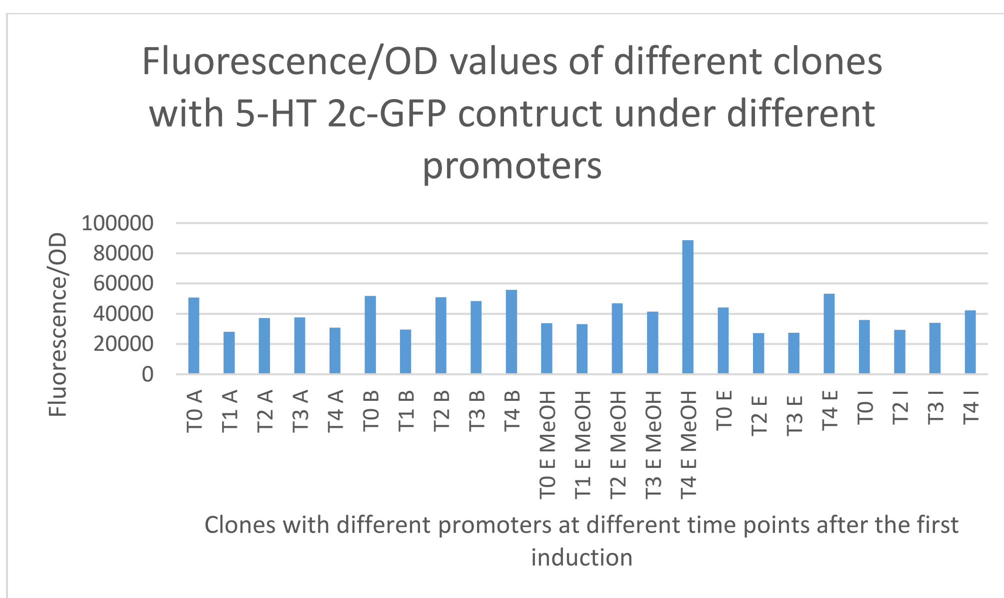

Figure 41: Fluorescence/OD of different promoters at different sampling time points

At the beginning of the induction the fluorescence is not higher than the normal cell fluorescence, but after more than 50 h of induction the fluorescence signal starts to rise especially for the constructs under the E promoter. So the recombinant protein maybe was produced at later stages of induction.

4.17.2 Total protein concentrations

Table 24: The total protein concentration of clones expressing the 5-HT 2c-GFP construct under different promoters measured in a BCA assay

Sample	Absorbance at 562 nm	Total protein concentration before ultracentrifugation[mg/ml]
A	0.687	0.4

B	0.195	0.1
E MeOH	0.206	0.1
E	0.513	0.3
I	0.605	0.4

The total protein concentrations obtained from the samples have overall very low values indicating that the strains had difficulties in producing the recombinant fusion protein. The low values can result from an overloaded translation machinery leading to degradation pathways or a massive protein degradation during cell lysis. This might be due to the fact that the construct is even larger than the β2AR-GFP construct.

4.17.3 Coomassie blue stain

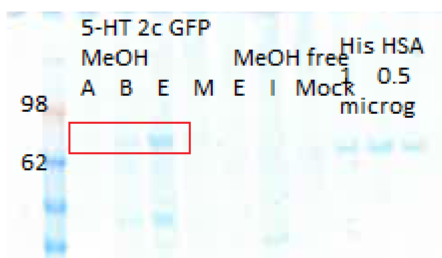

Figure 42: Coomassie blue stain of 5-HT 2c-GFP clone lysates

On the Coomassie blue stained gel there are two protein bands visible produced under the methanol-induced promoters B and E. The bands are at the correct size of the recombinant protein at around 81 kD. The bands do neither appear in the mock samples nor in samples from strains using methanol-free promoters. If the bands solely originate from the recombinant protein, there seem to be 0.5 to 1 µg/ml present when compared to the bands of the His-HSA standards.

4.17.4 Western blot

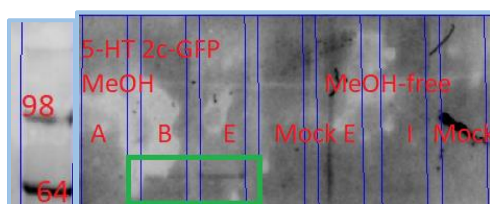

Figure 43: Western blot without HSA as control due to contamination of wells in other Western blots with HSA

Two proteins at 81 kD were produced under the methanol-inducible promoters B and E. For the methanol-free produced proteins no bands are visible as already seen on the Coomassie stain as well. No bands are visible in the mock so the lanes at the correct size originate from the recombinant fusion protein.

4.18 5-HT 2c-αMF-GFP constructs

Vectors with the 5-HT 2c-αMF-linker-GFP constructs under different methanol-inducible and methanol-free promoters were used to transform recombinant *E.coli*

DH5-α cells and mut[s] *P. pastoris* cells. Final clones with plasmids containing the GFP construct and the promoters A, C, E, E MeOH-free or I were obtained.

4.18.1 Optical densities and fluorescence measurements

Table 25: OD_{600} and fluorescence measurements of different sampling time points

Hours after induction	Clone with promoter	OD_{600}	Total OD_{600}	Fluorescence	Total fluorescence	Fluorescence/ OD
0	A	0.495	14.85	21,120	422,400	28,444.4
0	C	0.467	14.01	16,353	327,060	23,344.8
0	E MeOH	0.485	14.55	17,275	345,500	23,745.7
0	E	0.514	15.42	14,925	298,500	23,503.2
0	I	0.513	15.39	18,471	369,420	19,395.7
16	A	0.506	10.12	20,891	417,820	41,286.6
16	C	0.495	9.9	14,752	295,040	29,802.0
16	E MeOH	0.558	11.16	19,411	388,220	34,786.7
24	A	0.307	9.21	15,331	306,620	33,292.1
24	C	0.324	9.72	15,391	307,820	31,668.7
24	E-MeOH	0.331	9.93	17,771	355,420	35,792.5
24	E	0.311	9.33	33,342	666,840	46,420.2
24	I	0.51	15.3	12,029	240,580	43,584.3
48	A	0.345	10.35	23,098	461,960	44,633.8
48	C	0.326	9.78	23,074	461,480	47,186.1
48	E MeOH	0.351	10.53	37,483	749,660	71,396.2
48	E	0.316	9.48	19,556	391,120	38,964.1
48	I	0.581	17.43	24,651	493,020	22,478.2
64	A	0.351	10.53	34,991	699,820	66,459.6
64	C	0.31	9.3	27,649	552,980	59,460.2
64	E MeOH	0.293	8.79	25,777	515,540	58,650.7
64	E	0.342	10.26	24,836	496,720	44,421.1
64	I	0.653	19.59	33,997	679,940	25,355.8

Samples were taken from all clones with different promoters on their plasmids before the first induction, so at time point 0 (T_0). The samples were examined by OD_{600} measurements and fluorescence emission. As induction was started this time in the afternoon, samples from the clones were only taken the next day.16 h after the first induction only clones with methanol-induced promoters were induced and so there were also only samples from methanol-induced clones taken (T_1). The next samples were taken 24 h (T_2), 48 h (T_3) and 64 h (T_4) after the first induction. The relative fluorescence units obtained by measuring the emission of the samples is divided by the total optical density measured. This value should give a first insight in how much fluorescence is emitted by the cells and so also a rough estimation on the amount of recombinant fusion protein expressed.

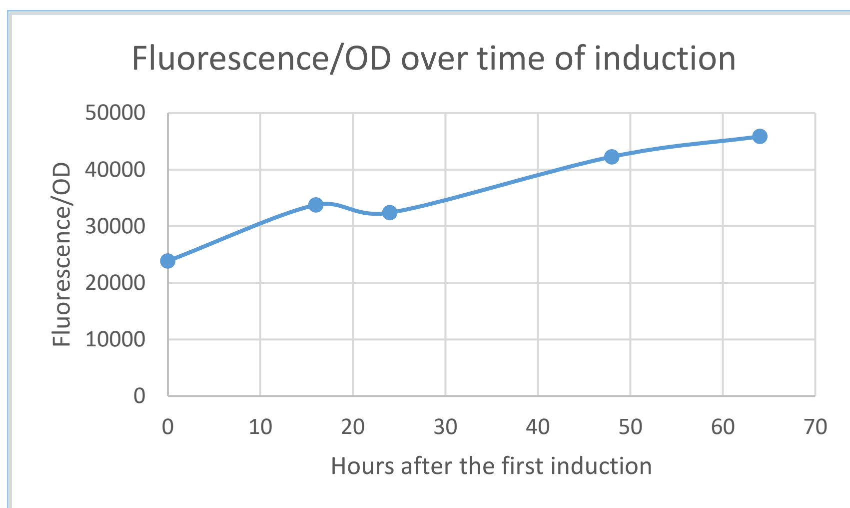

Figure 44: Fluorescence/OD over different sampling time points

The fluorescence per optical density at 600 nm was calculated and plotted against the different time points after the first induction. At the beginning of the first 8 h of induction there was an increase in fluorescence. Then the intensity stayed the same for nearly 20 h. After further inductions, the fluorescence/ OD increased even more. This might occurred due to an adaptation of the cells to the new protein production process and facilitation of protein targeting inside the cell by the αMF signal sequence.

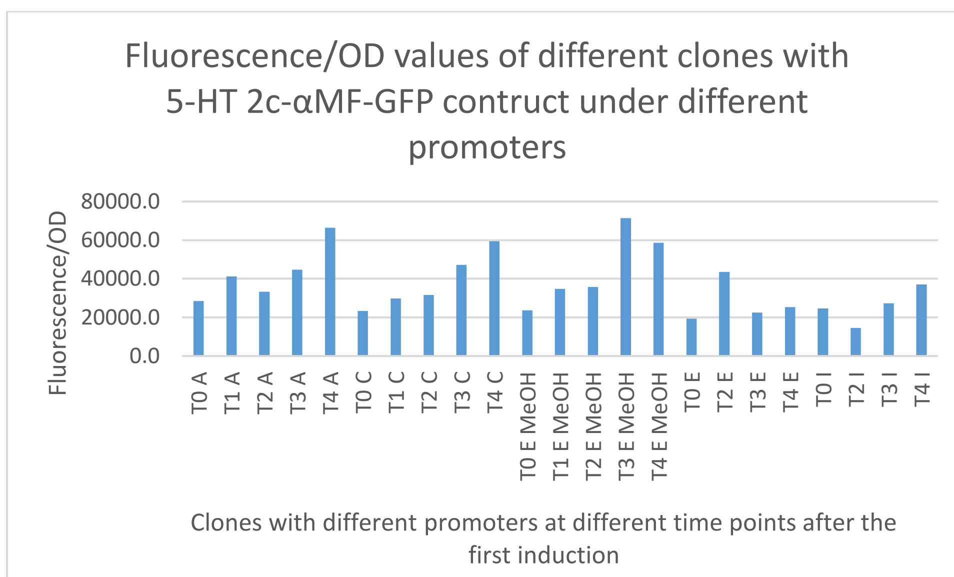

Figure 45: Fluorescence/OD of different promoters at different sampling time points

The fluorescence per OD was calculated for clones with the β2AR-GFP construct under different promoters and measured at different time points after the first induction. T_0 was at the time before the first induction, T_1 was after 16 h of the first induction, only for the MeOH-induced clones, T_2 24 h, T_3 48 h and T_4 was sampled 64 h after the first induction. The graph does not show significant increase in fluorescence with progressing induction, but overall the increase over time was more pronounced compared to the other fusion proteins examined. The fluorescence/OD was generally lower than for the proteins expressed with their native signal sequence.

4.18.2 Total protein concentrations

Table 26: The total protein concentration of clones expressing the 5-HT 2c-αMF-GFP construct under different promoters measured in a BCA assay

Sample	Absorbance at 562 nm	Total protein concentration before ultracentrifugation[mg/ml]
A	0.228	0.1
C	0.248	0.1
E MeOH	0.228	0.1
E	0.26	0.1
I	0.165	0.03

The total protein concentrations obtained from the samples have overall very low values indicating that the strains had difficulties in producing the recombinant fusion protein. The low values can result from an overloaded translation machinery leading to degradation pathways or a massive protein degradation during cell lysis. This might be due to the fact that the construct is even larger than the β2AR-GFP construct and does not have the native signal sequence. The detected values were lower compared to the other two GFP fusion proteins.

4.18.3 Coomassie blue stain

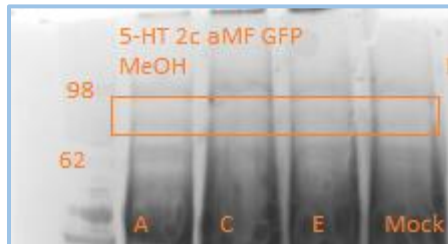

Figure 46: Coomassie blue stain of the 5-HT 2c-αMF-GFP protein lysates

On the Coomassie blue stained gel only faint protein bands were visible, produced under the methanol-induced promoters A and C. The signals were at the correct size of the recombinant protein at around 81 kD. The bands do not appear in the mock sample nor in samples of strains using the methanol-free promoters and are therefore not shown.

4.18.4 Western blots

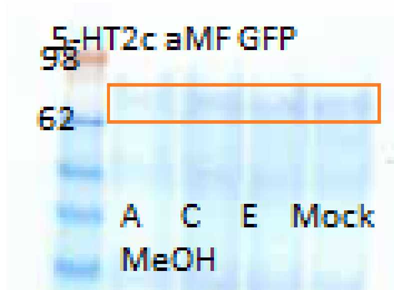

Figure 47: Western blot without HSA as control due to contamination of wells in other Western blots with HSA

Two proteins at around 81 kD were produced under the methanol-inducible promoters C and E. For the methanol-free produced proteins no bands were visible and so not shown. A band at the size of the recombinant protein was also visible in mock sample so that it was unclear if the bands in candidate samples show the recombinant protein. Another reason for a band at the same size in mock sample might be spilling of material from one well into the other.

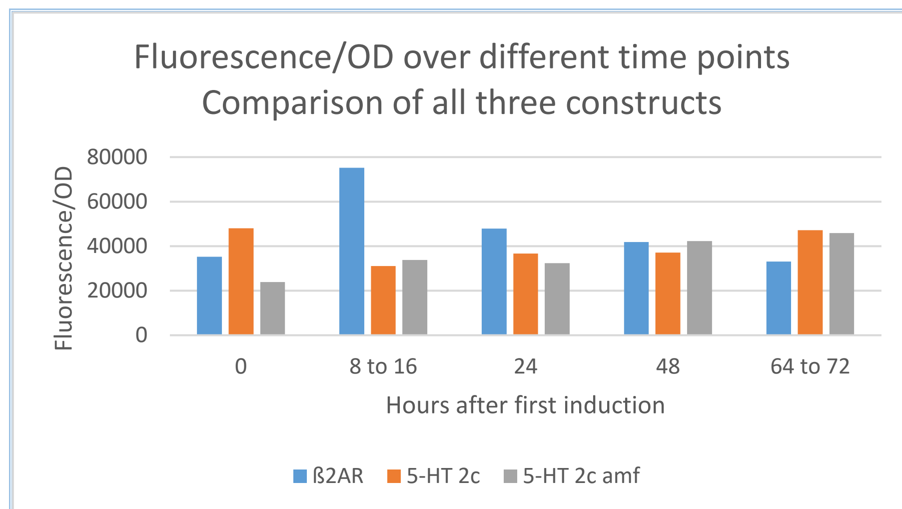

Figure 48: The average fluorescence/OD at every time point measured of the three different membrane protein-GFP fusions

In the diagram above it can be seen that β2AR-GFP showed the highest average fluorescence emission of all proteins. The fluorescence output was strongest 8 to 16 h after induction and decreased after more induction steps which corresponds also to previous studies performed. The 5-HT 2c-GFP did not show marked increase in average fluorescence emission at all, which evidences low or absent production of recombinant fusion protein. The average fluorescence output of the 5-HT 2c-αMF-GFP significantly rised around 50 to 60 h after the first induction.

4.19 Fluorescence microscopy of MP-GFP fusion proteins

4.19.1 Selection of samples for microscopy

From every MP-GFP fusion protein two promoters at a certain time point showing a high fluorescence/OD were chosen to be examined with fluorescence microscopy. As cleavage of the fusion protein in the linking region between fusion partners might release GFP, any fluorescence measured could be caused by free GFP and not from the full-length fusion protein. Hence, microscopy on cellular level shall shed light on localisation of the fluorescence.

Table 27: Samples chosen for fluorescence microscopy

Number	Sample	Time point	Fluorescence/OD
1	β2AR-GFP B	T_1	161,568.2
2	β2AR-GFP E	T_1	71,109.2
3	5-HT 2c-GFP B	T_2	42,728.4
4	5-HT 2c-GFP E	T_2	28,826.2
5	5-HT 2c-αMF-GFP A	T_4	66,459.6

6	5-HT 2c-αMF-GFP E	T_3	71,396.2

4.19.2 Fluorescence microscopy images

For every sample, several images were taken after dilution. The exposure time in milliseconds was applied depending on the strength of the fluorescence signal observed.

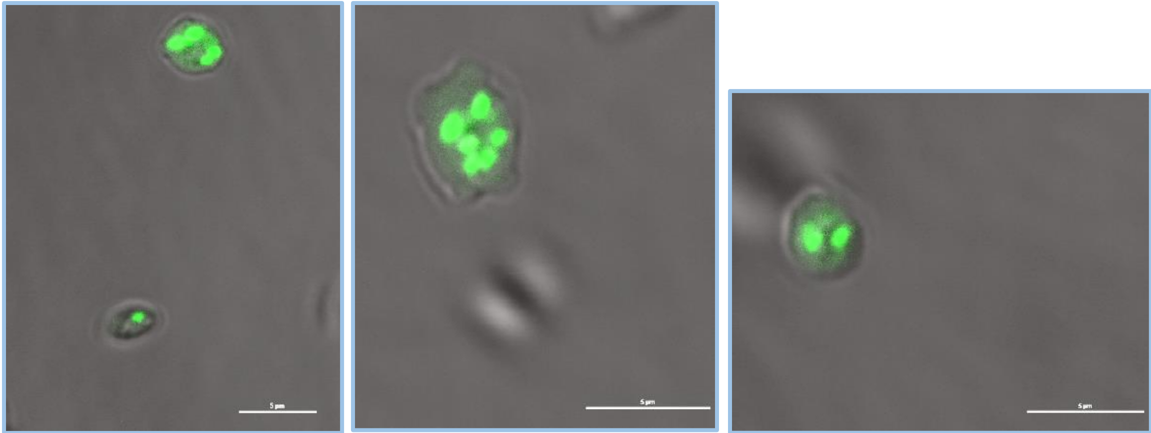

Figure 49: Fluorescence microscopy images of β2AR-GFP produced under promoter B, 8 h after induction

Upon fluorescence microscopy imaging of the β2AR-GFP-fusion protein produced under promoter B, a generally high fluorescence output was visible. This corresponds to the measured fluorescence/OD for this time point and also to specific protein signal visible on the Western blot. On the images the fluorescence is visible in the membranes of several cellular compartments. Most probably the protein is located in endosomes and similar vesicles for sorting of membrane proteins.

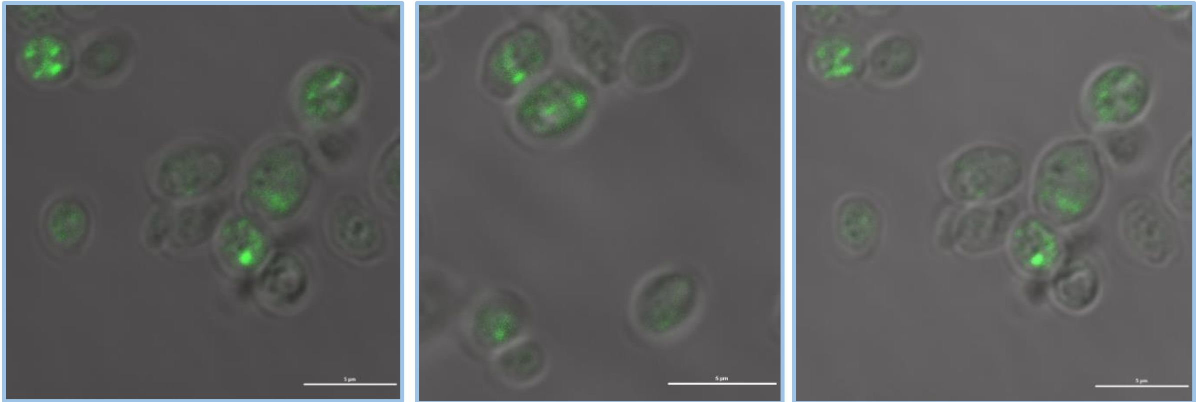

Figure 50: Fluorescence microscopy images of β2AR-GFP produced under promoter E, 8 h after induction

Upon fluorescence microscopy imaging of β2AR-GFP-fusion protein produced under promoter E, a medium to high fluorescence output is visible. This corresponds to the measured fluorescence/OD and the approximate protein bands visible on the Western blots. On the images, the fluorescence was visible in membranes of different cellular compartments. Most probably the proteins are located in endosomes and similar vesicles for sorting.

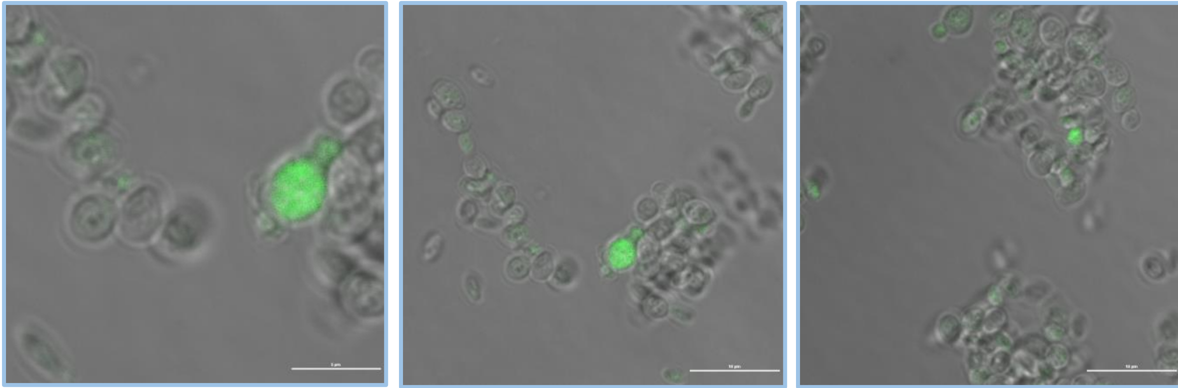

Figure 51: Fluorescence microscopy images of 5-HT 2c-GFP protein produced under promoter B, 24 h after induction

Upon fluorescence microscopy imaging of the 5-HT 2c-GFP-fusion protein produced under promoter B the fluorescence was visible throughout the whole cell. It is not specially localised in cellular compartments or their membranes, but spread throughout the cytosol. The fluorescence produced is rather medium which corresponds to the fluorescence/OD values obtained. This might indicate also cells that are already damaged.

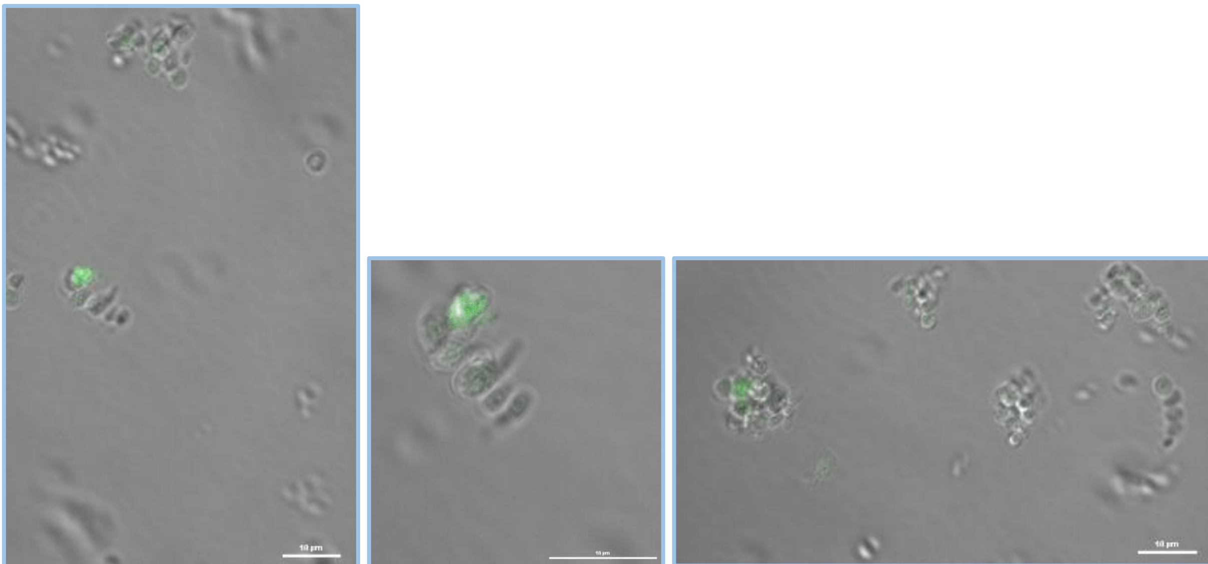

Figure 52: Fluorescence images of 5-HT 2c-GFP produced under promoter E, 24 h after induction

Upon fluorescence microscopy imaging of the 5-HT 2c-GFP-fusion protein produced under promoter E some fluorescence was visible inside cells. The fluorescence is visible throughout the cells and is located mostly in the cytosol and not at the target destination of the membrane protein. The fluorescence output is not very high which corresponds to the measured fluorescence/OD. The measured fluorescence might originate from mitochondrial normal fluorescence and putatively gives false positive fluorescence signals.

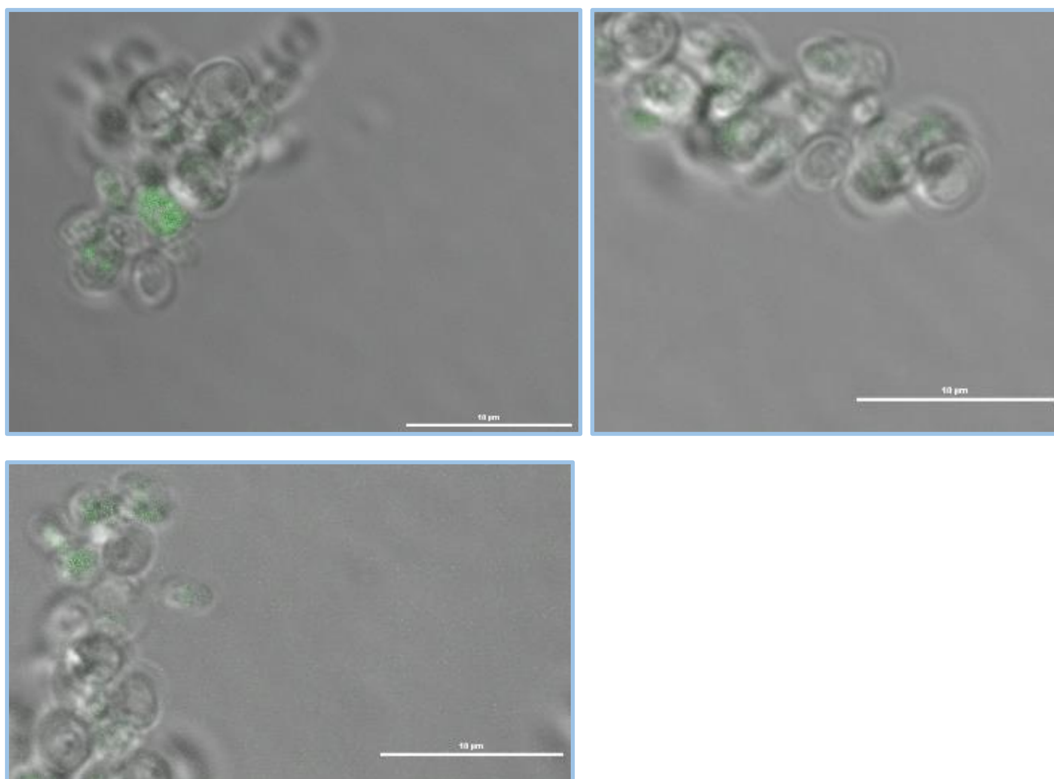

Figure 53: Fluorescence images of the 5-HT 2c-αMF-GFP produced under promoter A, 64 h after the first induction

Upon fluorescence microscopy imaging of the 5-HT 2c-αMF-GFP-fusion protein produced under promoter A, a very weak fluorescence signal was visible in the cells. Upon more careful inspection the fluorescence might originate from normal mitochondrial fluorescence and putatively gave false positive fluorescence signal. The weak signal corresponds to the low fluorescence/OD and the low output on the Western blots.

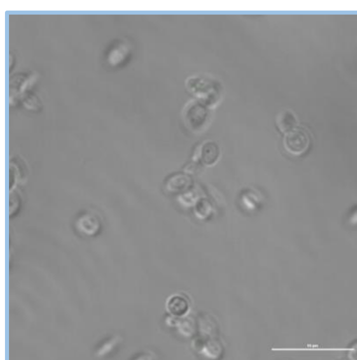

Figure 54: Fluorescence microscopy image of the 5-HT 2c-αMF-GFP fusion protein produced under promoter E, 48 h after the first induction.

Upon fluorescence microscopy imaging of the 5-HT 2c-αMF-GFP-fusion protein under promoter E, no fluorescence output was obtained at all. This does not correspond to the fluorescence/OD measured and the protein bands on the Western blot. However, the measured fluorescence might originate from normal mitochondrial fluorescence and putatively gave a false positive fluorescence signal. On the microscope image, no fluorescence output is seen as cells are maybe already damaged.

5 Discussion

5.1 Different plasmids in *E. coli*

Synthetic genes for the diverse membrane proteins in supplier´s vectors (ATUM, CA, USA) were used to transform competent *E. coli* DH5-alpha cells. Re-cloning of these genes into destination plasmids as well as sub-cloning of amplificates after Phusion PCRs generating protein constructs with the αMF signal sequence for 5-HT 2c and also final cloning into destination plasmids worked well in general. Not for all destination plasmids carrying different promoter types, though, efficient cloning was possible, so that plasmids containing promoters J and K were excluded from all subsequent efforts, but did not compromise the present workflow.

5.2 Transformation and cultivation of *P. pastoris*

Transformation and cultivation of competent *P. pastoris* mut^s cells was performed with several plasmids with different promoters and inserts to source singular colonies for deep-well plate cultivations. The optical density was measured for each clone to verify growth and calculate the amount of lysis buffer needed. In order to obtain more biomass to sustain lysis experiments on a larger scale, cultivation was also performed in shake flasks. It could be confirmed that the OD values obtained per ml cells was roughly the same in well plates and shake flasks, as has been described previously. (Duetz, 2007)

5.3 Expression patterns of *P. pastoris* clones

The total protein concentration was measured using BCA assay to assess protein abundance. For several MeOH-induced promoters expressing β2AR, very high total protein concentrations were obtained which was further substantiated by strong signals on Western blots. For 5-HT 2c, protein concentrations were comparably low, while total protein measurements did not correlate with signal intensities observed on Western blots for 5-HT 5a. Overall, total protein concentrations were not always directly correlated to specific recombinant protein production.

Expression levels were checked by SDS-PAGE followed by Western blot using an anti-His-tag (with conjugated HRP) antibody. Western blot analysis of samples containing β2AR evidenced that production using methanol-inducible promotors performed better than applying methanol-free promotors. This is in line with reports mentioning that the use of methanol-inducible promotors enhances the production rate of membrane proteins. As the anti His antibody successfully bound to the protein`s His-tag, it is assumed that the protein was correctly targeted within the cell. Moreover, it appeared that the signal sequence was cleaved off correctly during protein processing due to the fact that apparent molecular weight of signals on Western blot matched the calculated values. In addition, very low background was observed for lysate preparations on Western blots.

For the detection of 5-HT 5a, a Streptavidin-HRP-conjugate antibody was used, which led to a high background in general. It does not only specifically bind to the Strep-tag

I, but also to Biotin and therefore to all other biotinylated proteins that might be present in the cell lysate. Again, results for MeOH-induced expression seemed to be outperforming the expression with methanol-free promotors. Coomassie Brilliant Blue staining was done additionally for lysates containing 5-HT 5a as Western blot showed high background. Despite the unspecific nature of this staining method, results corresponded to those obtained by Western blots.

The 5-HT 2c receptors expressed under different methanol-inducible and methanol-free promoters did not show high expression rates. A potential cause of this finding might be related to the comparably large molecular weight of the 5-HT 2c receptor (with its native signal peptide) being the largest protein construct of the 5 protein variants assessed in *P. pastoris* in this thesis. The larger the protein, the more difficult it could potentially be to isolate the membrane protein from cellular membranes after lysis and ultracentrifugation.

Around ten selected clones that showed a good expression with a certain promoter on the Western blots were re-cultivated in deep-well plates as well as shake flasks for rescreening.

For the β2AR constructs, MeOH-inducible promoters B and C worked best.

In case of 5-HT 2c production, the MeOH-free promoter G was most efficient.

While more or less every promoter used was equally performing for production of 5-HT 5a, the overall comparability was difficult to judge due to high background on the Western blots.

5.4 Lysis of the rescreening clones with Fos Choline 14

First of all, the total protein concentration of the rescreened clones was measured. Most of the highest protein concentrations were obtained with clones that were not supplemented with Fos Choline 14 for lysis. For target protein β2AR, a correlation of high total protein concentration with high expression of the recombinant protein was observed. For target proteins 5-HT 5a and 5-HT 2c this correlation was not valid. Apparently, a high overall protein concentration does not necessarily translate to high expression of the target protein.

The Western blots for rescreening clones after ultracentrifugation provided clearer signals and better separation of bands. So it is highly recommended to perform an ultracentrifugation after lysis. However, there are still some degradation products found on Western blots. Precipitation with 50 % TCA overnight led to protein bands that were easier to differentiate.

The addition of Fos Choline 14 before or after lysis did not lead to more intense bands. Adding Fos Choline before lysis often displayed the same results as without usage of the reagent. When Fos Choline was added after cell lysis, results were worse than without it. Clones for 5-HT 2c were an exception to this rule as they showed more intense bands upon addition of Fos Choline before lysis. This might be due to the fact that the 5-HT 2c receptor is the largest protein among all tested. Hence, it may be concluded that the addition of Fos Choline 14 before lysis can help to improve the

release of certain large membrane proteins and to remove some background in Western blot analysis.

As most antibodies used to capture protein tags also bind to target-unrelated, non-tagged proteins to some extent, as well as to degradation products of the target protein with similar physiochemical and affinity properties, one has to expect a certain background noise on Western blots (evidenced here for anti His- or anti Strep-tag antibodies). Particularly, the Streptadivin antibody delivered more selective binding to 5-HT 5a protein than the Strep-Tactin antibody that was designed for binding to a Strep-tag II. Overall, it was observed that it is rather difficult to get a significant expression result from recombinant proteins with a Strep-tag for detection as there is a lot of background from degradation products and other unrelated proteins being detected by the antibodies.

The secretion machinery of *P. pastoris* is not always capable of coping with large amounts of recombinant proteins. A high demand for folding or secretion events may induce stress responses that ultimately can lead to degradation of the recombinant target proteins which is manifested by lower molecular weight signals in Western blots. This phenomenon is called “Endoplasmic reticulum-associated protein degradation (ERAD)”. (Zahrl, 2018), (Grisshammer, 2006)

For handling of cultures in the laboratory, it was observed that shake flask cultivation is more convenient and leads to similar results when compared to 96-deep-well plates, especially due to increased volume of the cultures. It was reported that the diameter of the neck of shake flasks did not have an impact on the level of dissolved oxygen inside the flasks, and so impact on cell growth and protein production from different shake flask designs was excluded. (Villatte, 2001)

For lysis using a vortex, 2 ml Eppendorf tubes are recommended rather than e.g. 15 ml Falcon tubes.

It was observed that more conclusive results were obtained when the same concentration of every protein lysate, previously measured by e.g. BCA assay, was loaded onto SDS-PAGEs. Thereby, it was ensured that only very strongly expressing clones were displayed and less background and unspecific signals were detected on the blots.

Rarely, it is possible to establish a direct correlation between total protein concentrations and well-expressed proteins on the Western blots. Only β2AR clones under certain promoter variants showing the highest total protein concentrations were also displaying the highest specific recombinant protein expression on Western blots.

5.5 Lower induction temperature for cultivation

By lowering the induction temperature to 22 °C, no significant increase in expression of the recombinant proteins was observed. Total protein concentrations were lower, accompanied by less intense bands on Western blots. It can be concluded for this survey that 28 °C as standard induction temperature did not overload the translation, folding and secretion machineries when producing the particular membrane proteins assessed. At 22 °C, overall membrane protein production decreased.

5.6 De-glycosylation of lysates

For instances of detecting two bands in close migration proximity on SDS-PAGES and Western blots, the potential reason could be a mixture of the same protein in non-glycosylated and N-glycosylated state. A de-glycosylation step is recommended to verify that the two original signals melt to a single band at the correct size.

Amino acid sequences of β2AR and 5-HT 2c receptor code for an N-linked glycosylation on an asparagine residue close to the signal sequence. Also, the 5-HT 5a receptor with a signal anchor codes for an N-linked glycosylation. (Uniprot, 2020)

From Chapter 4.11 it can be concluded that de-glycosylation can help to increase the thickness and intensity of the single band of the particular recombinant protein. On the Western blots (e.g. Figure 33) it was also demonstrated that no hyper-glycosylation happened with the *P. pastoris* mut^s strain.

5.7 Clones with the αMF secretion signal

Only three different *AOX1* promoter variants in pPZ vectors were used for analysis of production efficiencies using the αMF secretion signal.

5.7.1 Cultivation of *P. pastoris* clones

Cultivation of *P. pastoris* clones expressing 5-HT 2c with the αMF secretion signal was performed in shake flasks at 28 °C. The OD_{600} values were similar to those previously obtained in the other cultivations, but no clone with an extraordinarily high total protein concentration was identified. In general, OD values did not show a large variation between the different clones carrying dedicated promoter variants.

5.7.2 Western blots and Coomassie stains

As only low total protein amount was applied on gels, bands at the correct molecular weight range appeared weak in Western blots and Coomassie stains. Of course, background was also reduced and thin, faint bands for target protein were about the only signal detected. High productivity was especially observed for promoter B controlling expression of 5-HT 2c. Samples with these promoters also had the highest total protein concentrations.

Methanol-free production using promoter E yielded a positive result for the first time, as did promoter H. Western blots after ultracentrifugation produced even clearer signals for the recombinant protein without any background. It seems that the anti His antibody bound to proteins derived from constructs using the αMF secretion signal with higher specificity. Best overall results were obtained with promoters B and H.

It can be concluded that the presence of the αMF secretion signal at the N-terminus of the recombinant protein assessed did not increase yield compared to the native signal sequence, but triggered a lower background on Western blots and Coomassie stains.

5.8 Multiple integration events

Almost all strains carrying constructs coding for the particular membrane proteins and the corresponding GFP-fusions were capable of growing at concentrations up to 4000

µg/ml Zeocin added. Only some strains producing 5-HT 2c αMF and 5-HT 2c-αMF-GFP controlled by certain promoters were not capable of growing at 4000 µg/ml antibiotics, putatively indicating a lower number of integration events.

5.9 Quantification of recombinant proteins with the best expressing clones

Western blots with the proteins produced by the best-expressing clones showed quite good results with the anti His-tag antibody, but not with the Streptadivin antibody. Western blots for Streptadivin showed a very high background from binding of the antibody to degradation products and other *P. pastoris* proteins that are probably more abundant than the recombinant protein.

Best results were detected for production of β2AR (using promoter B and C) and 5-HT 2c (with the αMF secretion signal and promoter B) before as well as after ultracentrifugation.

A calibration using three different concentrations of a 6x His-tagged HSA standard protein was performed to serve as comparison for estimations of band intensities of recombinant proteins. The best results showed a concentration of 1 µg/ml in lysate, but the estimation relied on comparison of intensity and not densitometry. This estimation was only possible for blots targeting the 6x His-tag, though.

For estimating the recombinant protein concentration after ultracentrifugation, more total protein concentration must be loaded on the gel as the total concentration is generally lower after ultracentrifugation. Samples loaded after ultracentrifugation showed clearer bands with less background while mock lysates contained almost no bands.

Best expression was obtained with promoter C for β2AR and with promoter B for 5-HT 2c with the αMF secretion signal after ultracentrifugation. Apparently, for each different GPCR expression and production conditions have to be optimized individually. (Freigassner, 2009)

From the intensities of the bands of the standard only β2AR under promoter C and 5-HT 2c αMF under B appear to show levels of approximately 1 µg/ml lysate recombinant protein produced. However, it seems that several multiple-copy integrations in the genome of *P. pastoris* took place.

It was concluded, that further process optimization needs to be done, including cultivation in fermenters to enable a better control of certain parameters, better cell lysis methods with less protein loss and maybe engineering of the *P. pastoris* strains to make them capable of stabilization of recombinant membrane proteins in their membranes as previously described. (Voit, 2017)

5.10 Cultivation and screening of clones with a GFP tag

Overall, cells expressing the membrane protein-GFP constructs showed higher OD_{600} values than clones expressing only the unfused membrane proteins. The relative fluorescence measured and therefore also the value “fluorescence/OD”, appear very high.

The diagrams for β2AR in chapter 4.16 show that the fluorescence intensity rose in the first hours of induction, but decreased after progressing induction time as already seen in previous studies. (Hirz, 2013) This finding could be explained by a decreasing amount of recombinant protein produced in later induction stages and/or degradation of the recombinant protein. As can be seen in Figure 41, 42 and 44, there was also fluorescence detected before induction start, caused by auto-fluorescence produced by NADPH, flavins, mitochondria and lysosomes when exposed to light. Fluorescence decrease over induction time after more induction steps so also results from degradation of mitochondria and lysosomes. (Monici, 2005) This is also confirmed by the similar fluorescence/OD at T_0 of β2AR-GFP samples and 5-HT 2c-GFP samples before the first induction.

Western blots and Coomassie stains with protein lysates clearly showed production of the membrane protein-GFP fusions. Western blots with the 6x His-HSA standards added at different concentrations clearly emphasized low amounts of target proteins.

The highest fluorescence/OD values were obtained for β2AR-GFP under promoter B which was also proven in the corresponding Western blot. For 5-HT 2c-GFP constructs, the highest fluorescent/OD values were found under methanol-inducible promoter B and E. Upon Western blot analysis, two bands were detected. Interestingly, samples with highest fluorescence intensities showed the lowest total protein concentrations, which could be interpreted in two ways: either a high proportion of the proteins actually represented the fusion protein, or proteolytic cleavage released GFP with degradation of the membrane protein part. Regarding the target protein 5-HT 2c-αMF-GFP, the lowest fluorescence/OD values were detected and also Western blot results confirmed low specific protein detection. This might be due to the fact that fluorescence was highest at T_4 and therefore protein production maybe just started or the produced proteins were degraded immediately before. Another explanation might be that 5-HT 2c is in general more difficult to express without the native signal sequence.

For all GFP fusion proteins methanol-inducible promoters produced higher amounts.

A comparison of the average fluorescence/OD values for all three fusion proteins assessed at different time points revealed that highest fluorescence intensities for β2AR were obtained at 24 h after the first induction. In case of both other fusion proteins it appeared that fluorescence did increase very late in induction.

Again, it needs to be mentioned that a high value of fluorescence does not necessarily correlate to large amounts of intact full-length fusion proteins as potentially the GFP-portion might have been separated from the fusion protein by degradation.

Still, it can be concluded that fusion of β2AR with GFP appeared to be produced at highest levels among all fusion proteins assessed.

5.11 Fluorescence microscopy

In order to prove the intended localization of the various GFP-fused membrane proteins inside the cell, fluorescence microscopy was selected. A localization of the fusion proteins in membrane(s) emphasized by fluorescence of these compartments

ultimately evidences integrity of the fusion protein due to the fact that released GFP by degradation might be expected to be localized in the cytosol.

Cells containing the fusion protein β2AR-GFP (by control of promoters B and E) showed fluorescence emission from membranes of different organelles and cell membranes for sampling point 24 h after first induction. This was a clear indication for integrity of the recombinant β2AR-GFP fusion protein and correct targeting. The fluorescence was mostly visible from endosomes and other types of vesicles that are responsible for the correct targeting of membrane proteins.

Cells producing the fusion protein 5-HT 2c-GFP (by control of promoters B and E) showed lower fluorescence signals compared to target protein β2AR-GFP, and also emission in microscopy was spread over the whole cell, indicating distribution in the cytosol. This might have happened by release of GFP during processing of the entire fusion protein or by mis-localization of the entire fusion protein that was still intact but resided in the cytosol instead of membranes. However, a definite statement can only be made with more fluorescence microscopy images of the candidate samples and mock to better distinguish normal fluorescence from additionally produced one.

Cells using promoter A to express the fusion protein 5-HT 2c-αMF-GFP showed the lowest fluorescence signals overall, but localized in the membranes of different cellular organelles. The fluorescence/OD-values as well as band identification on Western blot were also not very high. Potentially, the larger molecular weight of this target protein imposed severe stress on the cellular machinery, so that only little fusion-protein was produced and correctly targeted to the final destination.

For cells using promoter E to produce the fusion protein 5-HT 2c-αMF-GFP showed no fluorescence signal at all on the microscope images. This did not correspond to the fluorescence/OD-values obtained after 50 h of induction and did also not correlate to protein abundance detected by Western blot. The fluorescence emissions measured for the 5-HT 2c-αMF-GFP samples at early induction might originate only from normal fluorescence of some cellular components such as mitochondria. However, at later time of induction fluorescence rises, especially for the 5-HT 2c αMF-GFP-fusion protein. On the microscope images not much fluorescence is visible as many cells are obviously already damaged at the time of imaging.

Unfortunately, it was not possible to perform a second round of fluorescence microscopy with additional samples and samples of mock to confirm the results or to see if the first samples got damaged in some way. A general problem encountered on fluorescence microscope images are fluorescence particles. When expressing recombinant GFP in high amounts intracellularly in *P. pastoris*, it is possible that fluorescent particles consisting of unfolded proteins and entrapped GFP are generated. In addition, the GFP expressed can be entrapped in different cell organelles, such as the peroxisome where it is kept in insoluble alcohol oxidase clusters. When the GFP is fused to membrane proteins it might happen that fluorescent GFP particles accumulate in the cell membrane and endocytosis is prevented. The fluorescent particles can increase outputs on quantitative determinations in fluorescence assays. This might have happened in the fluorescence images of the β2AR.

Therefore, it is recommended, when whole cells, cytoplasmic or membrane fractions are applied in fluorescence assays, to make sure that the fusion protein is soluble and not in particles when doing fluorescence microscopy for confirmation of recombinant protein production. (Zupan, 2003)

6 Conclusion

It was possible to successfully express the three GPCRs β2AR, 5-HT 2c and 5-HT 5a with their native signal peptide as well as using the αMF secretion signal in case of 5-HT 2c in a *P. pastoris* mut^s strain. The P_{AOX1} variants B and especially C expressed target protein β2AR particularly efficient, while best results were obtained for promoter variants A and B controlling expression of 5-HT 5a, and promoter D and G for target protein 5-HT 2c (with its native signal sequence), respectively. Regarding production of 5-HT 2c using the αMF secretion signal, promoters B and H performed best.

Generally, MeOH-induced promoters showed higher yields of recombinant proteins. It was shown that detection of His-tagged target proteins were much easier to accomplish compared to a Strep-tag I.

A high value for measurement of total protein concentration often, but not always correlated with high amount of specific recombinant protein detected in Western Blot analysis.

Ultracentrifugation led to a much clearer resolution with less background on Western blots in general, despite detection of degradation products and unrelated proteins.

It appeared that larger membrane proteins were more difficult to separate from cellular components, and overall yielded less target protein compared to the smaller membrane proteins assessed. Consequently, addition of Fos Choline 14 for solubilisation of the target protein from membrane fractions only showed a beneficial effect for the large protein 5-HT 2c (when added before cell lysis). In addition, Fos Choline seemingly was able to remove some of the eminent degradation products usually visible on Western blots.

Concerning production conditions, a decrease of induction temperature from 28 °C to 22 °C did not lead to higher expression rates.

De-glycosylation of samples before loading them on SDS-PAGEs sometimes helped to reduce the amount of bands migrating to similar apparent molecular weight regions, but not in every case.

Generation of fusion proteins by adding GFP to the C-terminus of the particular target membrane proteins yielded positive localization results for β2AR-GFP in endosomes and vesicles, while target protein 5-HT 2c-GFP seemed to be distributed cytosolically and no fluorescence was identified for target protein 5-HT 2c-αMF-GFP upon fluorescence microscopy.

7 Future outlook

Additionally, there are many ways to improve the yield of recombinant membrane proteins that are worth to try, but are beyond the scope of this thesis. For instance, with the co-expression of chaperones or proteins that form stable complexes with the membrane proteins, the expression is improved as the GPCRs are more in their native environment. Another approach is to engineer the membrane protein itself by distinct point mutations. A very sophisticated way is the production of membrane proteins with a *P. pastoris* strain that has cholesterol integrated in its membrane as already described by a research group. (Hirz, 2013) So better folding of the human GPCRs is approached. In addition, different locations in the genome of *P. pastoris* might be used for integration. (Freigassner, 2009)

After optimization of the molecular path, the production volume is scaled up for bioreactor cultivation. By scaling up the process to litre scale incredible higher expression rates can be obtained and cells grow to high densities. Induction strategies, growth conditions and temperatures are monitored more easily. (Raina, 2007)

By having achieved the recombinant expression of integral membrane proteins ß2AR, 5-HT 2c and 5-HT 5a with their native signal sequences or the αMF signal sequence in *P. pastoris*, a confirmation of their functionality for example, with a receptor agonist, is necessary. (Mohell, 1989) This activity-based screen, unlike the immunodetection methods, enriches functional and active proteins. (Freigassner, 2009)

After fermentation, diligent purification and characterization steps with HPLC and mass spectroscopy for instance, are applied. After the analytics, it will be possible to perform crystallizations and 3D models as the receptors are available in a high amount then. In addition, feasibility studies of receptor agonist and antagonist binding that might be used as new medicinal products are facilitated. With well characterized and purified receptors produced in sufficient amount new medicinal products against severe diseases will be developed due to better understanding of the structure and binding processes of the transmembrane receptors. (Lundstrom, 2006)

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8 Appendix

8.1 Protein sequences ordered

8.1.1 B2-adrenergic receptor protein sequence with tags and native signal sequence

At the N-terminus there is the signal peptide sequence, followed by a 10x His-tag and the protein sequence. At the C-terminus there is a Strep-tag I attached on the sequence consisting of the eight proteins Tryptophane, Arginine, Histidine, Proline, Glutamine, Phenylalanine and two Glycines. The tag is a Streptavidin-binding-tag and so a Streptavidin antibody or matrix are used for purification or detection of proteins having this tag attached at their N- or C-terminus. (Kosobokova, 2016) Potential N-glycosylation sites on Asparagines on the protein are marked in red.

```
MGQPGNGSAFLLAHHHHHHHHHHGDPNRSHAPDHDVTQQRDEVWVVGMGIVMSLIVLAIVFGNVLV
ITAIAKFERLQTVTNYFITSLACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTASIETL
CVIAVDRYFAITSPFKYQSLLTKNKARVIILMVWIVSGLTSFLPIQMHWYRATHQEAINCYANETCCDFF
TNQAYAIASSIVSFYVPLVIMVFVYSRVFQEAKRQLQKIDKSEGRFHVQNLSQVEQDGRTGHGLRRSS
KFCLKEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQDNLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRI
AFQELLCLRRSSLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNID
SQGRNCSTNDSLLAWRHPQFGG
```

8.1.2 5-HT 2c serotonin receptor protein sequence with tags and native signal sequence

At the N-terminus there is the signal peptide sequence marked in green, followed by a 10x His-tag in yellow and the protein sequence found. At the C-terminus there is a Strep-tag I attached to the sequence marked in yellow. Potential N-glycosylation sites on Asparagines are marked in red.

```
MVNLRNAVHSFLVHLIGLLVWQCDISVSPVAAHHHHHHHHHHIVTDIFNTSDGGRFKFPDGVQNWPA
LSIVIIIIMTIGGNILVIMAVSMEKKLHNATNYFLMSLAIADMLVGLLVMPLSLLAILYDYVWPLPRYLCPV
WISLDVLFSTASIMHLCAISLDRYVAIRNPIEHSRFNSRTKAIMKIAIVWAISIGVSVPIPVIGLRDEEKVFV
NNTTCVLNDPNFVLIGSFVAFFIPLTIMVITYCLTIYVLRRQALMLLHGHTEEPPGLSLDFLKCCKRNTAE
EENSANPNQDQNARRRKKKERRPRGTMQAINNERKASKVLGIVFFVFLIMWCPFFITNILSVLCEKSC
NQKLMEKLLNVFVWIGYVCSGINPLVYTLFNKIYRRAFSNYLRCNYKVEKKPPVRQIPRVAATALSGRE
LNVNIYRHTNEPVIEKASDNEPGIEMQVENLELPVNPSSVVSERISSVAWRHPQFGG
```

8.1.3 5-HT 5a serotonin receptor protein sequence with N-terminal tag

At the N-terminus there is the start codon and the protein sequence with its signal anchor sequence whose exact location was not defined so far. At the C-terminus there is a Strep-tag I attached on the sequence in yellow. The two potential N-glycosylation sites on Asparagines are marked in red.

```
MDLPVNLTSFSLSTPSPLETNHSLGKDDLRPSSPLLSVFGVLILTLLGFLVAATFAWNLLVLATILRVRTF
HRVPHNLVASMAVSDVLVAALVMPLSLVHELSGRRWQLGRRLCQLWIACDVLCCTASIWNVTAIALD
RYWSITRHMEYTLRTRKCVSNVMIALTWALSAVISLAPLLFGWGETYSEGSEECQVSREPSYAVFSTV
GAFYLPLCVVLFVYWKIYKAAKFRVGSRKTNSVSPISEAVEVKDSAKQPQMVFTVRHATVTFQPEGDT
WREQKEQRAALMVGILIGVFVLCWIPFFLTELISPLCSCDIPAIWKSIFLWLGYSNSFFNPLIYTAFNKNY
NSAFKNFFSRQHAWRHPQFGG
```

8.1.4 B2AR protein sequence with N-terminal GFP sequence

At the C-terminus there is the signal sequence of the protein marked in green. On the N-terminus the Strep-tag I is marked in green with a linker in blue. The following GFP is marked in yellow. On the N-terminus three red marked amino acids indicate sites for potential N-glycosylation on an Asparagine.

MGQPGNGSAFLLAGDPNRSHAPDHDVTQQRDEVWVVGMGIVMSLIVLAIVFGNVLVITAIAKFERLQT
VTNYFITSLACADLVMGLAVVPFGAAHILMKMWTFGNFWCEFWTSIDVLCVTASIETLCVIAVDRYFAIT
SPFKYQSLLTKNKARVIILMVWIVSGLTSFLPIQMHWYRATHQEAINCYANETCCDFFTNQAYAIASSIV
SFYVPLVIMVFVYSRVFQEAKRQLQKIDKSEGRFHVQNLSQVEQDGRTGHGLRRSSKFCLKEHKALK
TLGIIMGTFTLCWLPFFIVNIVHVIQDNLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAFQELLCLRRS
SLKAYGNGYSSNGNTGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVPSDNIDSQGRNCSTNDS
LLAWRHPQFGGGSMSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLP
VPWPTLVTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLV
NRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIG
DGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK

8.2 DNA sequences of the inserts

8.2.1 B2-adrenergic receptor DNA sequence with tags and native signal sequence

The signal sequence is marked in green and the His-tag and the Strep-tag attached on the protein in yellow. Base triples marked in red indicate potential N-glycosylation sites on the protein.

GAATTCGAAACGATGGGTCAACCCGGAAACGGATCAGCTTTCCTGCTCGCTCACCACCATCATCA
CCATCACCACCACCACGGTGACCCTAACAGATCACACGCCCCAGACCACGACGTGACCCAACAG
AGAGACGAAGTCTGGGTTGTTGGTATGGGAATTGTTATGTCCCTTATTGTCCTAGCAATTGTCTTC
GGTAACGTTCTGGTTATCACTGCTATTGCCAAGTTTGAGAGATTGCAGACAGTCACTAACTATTTC
ATTACTTCTTTGGCGTGCGCTGATCTAGTTATGGGCCTCGCTGTTGTCCCATTCGGGGCTGCTCA
TATTCTGATGAAGATGTGGACCTTTGGTAATTTCTGGTGTGAGTTTTGGACTTCTATTGATGTTCT
GTGTGTTACTGCATCCATCGAGACTCTTTGCGTCATCGCTGTTGATAGATACTTTGCTATTACTTC
CCCATTCAAGTACCAATCTCTTCTAACCAAAAACAAAGCCAGAGTCATTATTCTAATGGTCTGGAT
CGTGTCTGGTCTCACTTCATTCTTGCCTATTCAAATGCACTGGTATAGGGCTACTCACCAAGAGG
CTATTAACTGTTATGCAAATGAGACGTGCTGTGACTTCTTTACCAACCAAGCCTACGCTATCGCCT
CTTCTATTGTTTCTTTTTACGTTCCTCTCGTGATCATGGTATTCGTTTACAGCAGAGTCTTCCAGGA
GGCTAAGAGACAGCTACAAAAGATTGACAAGAGTGAGGGTAGATTCCACGTTCAAAACCTTTCTC
AAGTAGAACAAGACGGTAGAACTGGTCACGGTCTCCGTAGATCCTCGAAGTTCTGTCTTAAGGAA
CACAAAGCCCTCAAGACTCTAGGTATTATCATGGGAACCTTTACCCTTTGTTGGCTGCCATTCTTC
ATTGTGAACATCGTGCACGTTATCCAAGACAACCTAATCCGTAAGGAAGTCTACATCCTGCTAAAT
TGGATTGGTTACGTTAACTCTGGTTTCAACCCATTAATCTACTGTCGTTCTCCTGACTTTAGAATAG
CATTCCAGGAACTCCTATGCCTCAGACGTTCTTCCCTTAAGGCTTACGGTAACGGATACTCCTCTA
ACGGTAATACAGGTGAGCAATCCGGATACCACGTCGAACAGGAGAAGGAAAACAAACTTCTGTGT
GAGGATTTGCCTGGCACCGAAGATTTCGTGGGACACCAAGGTACCGTTCCATCCGACAACATCG
ATTCTCAGGGTAGAAACTGTTCTACCAATGACTCTCTCCTGGCATGGAGACACCCACAATTCGGT
GGCTAATAGCGGCCGC

8.2.2 5-HT 2c serotonin receptor DNA sequence with tags and native signal sequence

The signal sequence of the protein is marked in green and the His-tag and Strep-tag I attached are marked in yellow.

GAATTCGAAACGATGGTCAATCTTAGAAACGCAGTCCACTCTTTCTTGGTGCATCTCATTGGACTA
CTTGTCTGGCAATGTGACATTTCCGTCTCCCCAGTTGCTGCCCACCACCATCATCACCATCACCA
CCACCACATTGTTACTGACATCTTTAACACCTCTGACGGTGGAAGATTCAAGTTCCCAGATGGAG

TTCAAAACTGGCCTGCACTATCCATCGTTATCATCATTATCATGACAATCGGTGGTAACATTCTAG
TTATCATGGCTGTTTCTATGGAAAAAAAGCTTCACAACGCCACTAACTATTTTCTCATGTCACTCG
CTATTGCCGACATGCTCGTTGGACTGCTCGTAATGCCTCTCTCCCTTCTCGCTATTCTTTACGACT
ACGTCTGGCCTCTACCAAGGTATCTCTGTCCAGTTTGGATTTCACTTGACGTTTTGTTTTCTACAG
CCTCCATTATGCACCTGTGTGCCATCTCACTTGACAGATACGTTGCTATCCGTAATCCAATTGAGC
ACTCTCGTTTCAACTCTCGTACTAAGGCTATAATGAAGATCGCTATTGTTTGGGCAATCTCGATTG
GTGTCTCTGTTCCTATTCCTGTGATTGGTCTGAGAGACGAGGAAAAGGTTTTTGTCAACAATACCA
CTTGCGTCTTGAATGATCCTAACTTTGTACTTATCGGTTCCTTCGTTGCTTTCTTCATTCCACTGAC
TATCATGGTGATCACTTATTGCCTCACTATCTACGTTTTGAGAAGACAGGCTCTGATGCTACTCCA
CGGACACACGGAGGAGCCACCAGGTCTTAGCCTGGACTTTCTGAAGTGCTGTAAGAGGAACACC
GCTGAAGAGGAAAACTCTGCAAACCCTAACCAAGATCAAAATGCTAGACGTAGAAAAAAAAAGGA
GAGAAGACCAAGAGGTACTATGCAGGCTATTAACAACGAGAGAAAGGCATCTAAGGTGCTCGGT
ATTGTATTCTTCGTTTTCCTGATTATGTGGTGCCCATTCTTCATTACCAACATTCTTTCCGTCCTGT
GTGAAAAGTCCTGTAACCAGAAGCTAATGGAGAAACTGCTAAACGTTTTTGTTTGGATCGGTTAC
GTTTGTTCTGGTATAAACCCTCTTGTCTACACCCTATTCAACAAGATTTACCGTAGAGCTTTCTCAA
ACTACCTAAGATGTAACTACAAGGTGGAGAAAAAGCCACCAGTCAGACAAATTCCTAGAGTCGCA
GCTACCGCGCTGTCTGGCAGAGAACTAAACGTCAATATCTACAGACACACCAATGAACCTGTTAT
TGAGAAGGCCTCTGATAACGAGCCAGGCATCGAGATGCAAGTCGAAAACCTAGAATTACCCGTG
AACCCTTCTTCCGTGGTTTCTGAACGTATCAGTTCTGTTGCTTGGAGACACCCACAATTCGGTGG
TTAATAGCGGCCGC

8.2.3 5-HT 5a serotonin receptor DNA sequence with N-terminal tag

The Strep-tag on the protein is marked in yellow. The protein only has signal anchor sequence that was not defined yet. Potential N-glycosylation sites are marked in red.

GAATTCGAAACGATGGATTTGCCTGTTAACCTGACATCTTTCTCACTATCTACCCCATCCCCATTG
GAGACTAACCATTCTCTAGGAAAGGACGACCTCCGTCCATCGTCGCCACTTCTTTCCGTTTTTGG
TGTTCTGATTCTTACCCTTCTGGGTTTCCTGGTTGCTGCTACTTTTGCTTGGAACCTCCTAGTCCT
AGCCACCATCCTAAGAGTCAGAACTTTCCACAGAGTGCCTCACAATCTCGTCGCATCTATGGCCG
TTTCCGATGTTCTGGTTGCCGCGCTTGTCATGCCTCTGTCTCTAGTCCACGAACTGTCTGGTAGA
CGTTGGCAGCTTGGTAGAAGACTCTGTCAACTCTGGATTGCTTGCGACGTTCTATGCTGTACTGC
TTCCATTTGGAACGTTACTGCTATCGCTTTGGACAGATATTGGAGCATCACTCGTCACATGGAATA
CACTCTGAGAACCAGAAAGTGTGTTTCTAACGTTATGATCGCATTGACATGGGCTCTTTCTGCCGT
CATTTCTCTTGCTCCTTTACTATTCGGATGGGGAGAAACTTACTCTGAGGGTTCTGAGGAGTGTCA
AGTTAGTAGAGAACCATCCTACGCTGTGTTTTCTACCGTTGGAGCTTTCTATCTACCACTCTGTGT
GGTTCTCTTCGTGTACTGGAAGATTTACAAGGCAGCAAAGTTCAGAGTAGGTAGTCGTAAAACTA
ATTCCGTTTCACCTATTTCTGAGGCTGTCGAGGTGAAGGACTCTGCTAAGCAACCTCAAATGGTC
TTCACGGTTAGACACGCTACCGTCACCTTTCAACCTGAAGGTGATACCTGGAGGGAGCAGAAGG
AACAAAGAGCTGCACTCATGGTTGGTATCCTTATCGGCGTATTTGTCCTGTGTTGGATTCCATTCT
TCCTGACTGAGCTAATTTCTCCACTCTGTTCCTGCGACATTCCTGCCATCTGGAAGTCAATCTTCC
TTTGGCTCGGTTACTCTAACTCCTTCTTCAACCCCCTCATATACACCGCCTTCAACAAAAACTACA
ATTCCGCATTCAAAAACTTCTTTTCAAGACAGCACGCCTGGAGACATCCACAATTTGGTGGTTAAT
AGCGGCCGC

8.2.5 B2AR protein with a GFP tag

The native signal sequence at the N-terminus is displayed in yellow. The 10x His-tag at the N-terminus after the signal sequence and the Strep-tag I, with parts at the C-terminus of the gene and other parts after the GFP, are displayed in green. The linker sequence is highlighted in red and the GFP in blue. From the GFP sequence the start codon ATG is removed.

GAATTCGAAACGATGGGTCAACCCGGAAACGGATCAGCTTTCCTGCTCGCTCACCACCATCATCA
CCATCACCACCACCACGGTGACCCTAACAGATCACACGCCCCAGACCACGACGTGACCCAACAG
AGAGACGAAGTCTGGGTTGTTGGTATGGGAATTGTTATGTCCCTTATTGTCCTAGCAATTGTCTTC

GGTAACGTTCTGGTTATCACTGCTATTGCCAAGTTTGAGAGATTGCAGACAGTCACTAACTATTTC
ATTACTTCTTTGGCGTGCGCTGATCTAGTTATGGGCCTCGCTGTTGTCCCATTCGGGGCTGCTCA
TATTCTGATGAAGATGTGGACCTTTGGTAATTTCTGGTGTGAGTTTTGGACTTCTATTGATGTTCT
GTGTGTTACTGCATCCATCGAGACTCTTTGCGTCATCGCTGTTGATAGATACTTTGCTATTACTTC
CCCATTCAAGTACCAATCTCTTCTAACCAAAAACAAAGCCAGAGTCATTATTCTAATGGTCTGGAT
CGTGTCTGGTCTCACTTCATTCTTGCCTATTCAAATGCACTGGTATAGGGCTACTCACCAAGAGG
CTATTAACTGTTATGCAAATGAGACGTGCTGTGACTTCTTTACCAACCAAGCCTACGCTATCGCCT
CTTCTATTGTTTCTTTTTACGTTCCTCTCGTGATCATGGTATTCGTTTACAGCAGAGTCTTCCAGGA
GGCTAAGAGACAGCTACAAAAGATTGACAAGAGTGAGGGTAGATTCCACGTTCAAAACCTTTCTC
AAGTAGAACAAGACGGTAGAACTGGTCACGGTCTCCGTAGATCCTCGAAGTTCTGTCTTAAGGAA
CACAAAGCCCTCAAGACTCTAGGTATTATCATGGGAACCTTTACCCTTTGTTGGCTGCCATTCTTC
ATTGTGAACATCGTGCACGTTATCCAAGACAACCTAATCCGTAAGGAAGTCTACATCCTGCTAAAT
TGGATTGGTTACGTTAACTCTGGTTTCAACCCATTAATCTACTGTCGTTCTCCTGACTTTAGAATAG
CATTCCAGGAACTCCTATGCCTCAGACGTTCTTCCCTTAAGGCTTACGGTAACGGATACTCCTCTA
ACGGTAATACAGGTGAGCAATCCGGATACCACGTCGAACAGGAGAAGGAAAACAAACTTCTGTGT
GAGGATTTGCCTGGCACCGAAGATTTCGTGGGACACCAAGGTACCGTTCCATCCGACAACATCG
ATTCTCAGGGTAGAAACTGTTCTACCAATGACTCTCTCCTGGCATGGAGACACCCACAATTCGGT
GGCGGATCTGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGA
TGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAA
AGCTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTA
CTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAA
GAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACA
AGACGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATT
GATTTTAAAGAAGATGGAAACATTCTCGGACACAAACTTGAGTACAACTATAACTCACACAATGTA
TACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGTCACAACATTGAA
GATGGTTCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTT
TTACCAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGA
CCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAATTGTACAA
GTAATAGCGGCCGC

8.2.6 The 5-HT 2c protein with a GFP tag

The native signal sequence at the N-terminus is displayed in yellow. The His-tag at the N-terminus after the signal sequence and the Strep-tag I, with parts at the C-terminus of the gene and other parts after the GFP, are displayed in green. The linker sequence is highlighted in red and the GFP in blue. From the GFP sequence the start codon ATG is removed.

GAATTCGAAACGATGGTCAATCTTAGAAACGCAGTCCACTCTTTCTTGGTGCATCTCATTGGACTA
CTTGTCTGGCAATGTGACATTTCCGTCTCCCCAGTTGCTGCCCACCACCATCATCACCATCACCA
CCACCACATTGTTACTGACATCTTTAACACCTCTGACGGTGGAAGATTCAAGTTCCCAGATGGAG
TTCAAAACTGGCCTGCACTATCCATCGTTATCATCATTATCATGACAATCGGTGGTAACATTCTAG
TTATCATGGCTGTTTCTATGGAAAAAAAGCTTCACAACGCCACTAACTATTTTCTCATGTCACTCG
CTATTGCCGACATGCTCGTTGGACTGCTCGTAATGCCTCTCTCCCTTCTCGCTATTCTTTACGACT
ACGTCTGGCCTCTACCAAGGTATCTCTGTCCAGTTTGGATTTCACTTGACGTTTTGTTTTCTACAG
CCTCCATTATGCACCTGTGTGCCATCTCACTTGACAGATACGTTGCTATCCGTAATCCAATTGAGC
ACTCTCGTTTCAACTCTCGTACTAAGGCTATAATGAAGATCGCTATTGTTTGGGCAATCTCGATTG
GTGTCTCTGTTCCTATTCCTGTGATTGGTCTGAGAGACGAGGAAAAGGTTTTTGTCAACAATACCA
CTTGCGTCTTGAATGATCCTAACTTTGTACTTATCGGTTCCTTCGTTGCTTTCTTCATTCCACTGAC
TATCATGGTGATCACTTATTGCCTCACTATCTACGTTTTGAGAAGACAGGCTCTGATGCTACTCCA
CGGACACACGGAGGAGCCACCAGGTCTTAGCCTGGACTTTCTGAAGTGCTGTAAGAGGAACACC
GCTGAAGAGGAAAACTCTGCAAACCCTAACCAAGATCAAAATGCTAGACGTAGAAAAAAAAAGGA
GAGAAGACCAAGAGGTACTATGCAGGCTATTAACAACGAGAGAAAGGCATCTAAGGTGCTCGGT
ATTGTATTCTTCGTTTTCCTGATTATGTGGTGCCCATTCTTCATTACCAACATTCTTTCCGTCCTGT
GTGAAAAGTCCTGTAACCAGAAGCTAATGGAGAAACTGCTAAACGTTTTTGTTTGGATCGGTTAC
GTTTGTTCTGGTATAAACCCTCTTGTCTACACCCTATTCAACAAGATTTACCGTAGAGCTTTCTCAA

ACTACCTAAGATGTAACTACAAGGTGGAGAAAAAGCCACCAGTCAGACAAATTCCTAGAGTCGCA
GCTACCGCGCTGTCTGGCAGAGAACTAAACGTCAATATCTACAGACACACCAATGAACCTGTTAT
TGAGAAGGCCTCTGATAACGAGCCAGGCATCGAGATGCAAGTCGAAAACCTAGAATTACCCGTG
AACCCTTCTTCCGTGGTTTCTGAACGTATCAGTTCTGTTGCTTGGAGACACCCACAATTCGGTGG
TGGATCTGCTAGCAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGG
TGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCTACATACGGAAAGC
TTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTT
CTCTTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTCAAGAG
TGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAAAGATGACGGGAACTACAAGA
CGCGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATT
TTAAAGAAGATGGAAACATTCTCGGACACAAACTTGAGTACAACTATAACTCACACAATGTATACA
TCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTCGTCACAACATTGAAGATG
GTTCCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTAC
CAGACAACCATTACCTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCAC
ATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGGATGAATTGTACAAGTAA
TAGCGGCCGC

8.2.7 The 5-HT 2c αMF protein with a GFP tag

The pro-αMF signal sequence at the N-terminus is displayed in yellow. The His-tag at the N-terminus after the signal sequence and the Strep-tag I, with parts at the C-terminus of the gene and other parts after the GFP, are displayed in green. The linker sequence is highlighted in red and the GFP in blue. From the GFP sequence the start codon ATG is removed.

CTCGAGAAGAGACACCACCATCATCACCATCACCACCACCACATTGTTACTGACATCTTTAACACC
TCTGACGGTGGAAGATTCAAGTTCCCAGATGGAGTTCAAAACTGGCCTGCACTATCCATCGTTAT
CATCATTATCATGACAATCGGTGGTAACATTCTAGTTATCATGGCTGTTTCTATGGAAAAAAAGCTT
CACAACGCCACTAACTATTTTCTCATGTCACTCGCTATTGCCGACATGCTCGTTGGACTGCTCGTA
ATGCCTCTCTCCCTTCTCGCTATTCTTTACGACTACGTCTGGCCTCTACCAAGGTATCTCTGTCCA
GTTTGGATTTCACTTGACGTTTTGTTTTCTACAGCCTCCATTATGCACCTGTGTGCCATCTCACTT
GACAGATACGTTGCTATCCGTAATCCAATTGAGCACTCTCGTTTCAACTCTCGTACTAAGGCTATA
ATGAAGATCGCTATTGTTTGGGCAATCTCGATTGGTGTCTCTGTTCCTATTCCTGTGATTGGTCTG
AGAGACGAGGAAAAGGTTTTTGTCAACAATACCACTTGCGTCTTGAATGATCCTAACTTTGTACTT
ATCGGTTCCTTCGTTGCTTTCTTCATTCCACTGACTATCATGGTGATCACTTATTGCCTCACTATCT
ACGTTTTGAGAAGACAGGCTCTGATGCTACTCCACGGACACACGGAGGAGCCACCAGGTCTTAG
CCTGGACTTTCTGAAGTGCTGTAAGAGGAACACCGCTGAAGAGGAAAACTCTGCAAACCCTAACC
AAGATCAAAATGCTAGACGTAGAAAAAAAAAGGAGAGAAGACCAAGAGGTACTATGCAGGCTATT
AACAACGAGAGAAAGGCATCTAAGGTGCTCGGTATTGTATTCTTCGTTTTCCTGATTATGTGGTGC
CCATTCTTCATTACCAACATTCTTTCCGTCCTGTGTGAAAAGTCCTGTAACCAGAAGCTAATGGAG
AAACTGCTAAACGTTTTTGTTTGGATCGGTTACGTTTGTTCTGGTATAAACCCTCTTGTCTACACC
CTATTCAACAAGATTTACCGTAGAGCTTTCTCAAACTACCTAAGATGTAACTACAAGGTGGAGAAA
AAGCCACCAGTCAGACAAATTCCTAGAGTCGCAGCTACCGCGCTGTCTGGCAGAGAACTAAACG
TCAATATCTACAGACACACCAATGAACCTGTTATTGAGAAGGCCTCTGATAACGAGCCAGGCATC
GAGATGCAAGTCGAAAACCTAGAATTACCCGTGAACCCTTCTTCCGTGGTTTCTGAACGTATCAG
TTCTGTTGCTTGGAGACACCCACAATTCGGTGGTGGATCTGCTAGCAAAGGAGAAGAACTTTTCA
CTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTG
GAGAGGGTGAAGGTGATGCTACATACGGAAAGCTTACCCTTAAATTTATTTGCACTACTGGAAAA
CTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGTGTTCAATGCTTTTCCCGTTATC
CGGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGC
ACTATATCTTTCAAAGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGATAC
CCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTCGGACACAA
ACTTGAGTACAACTATAACTCACACAATGTATACATCACGGCAGACAAACAAAAGAATGGAATCAA
AGCTAACTTCAAAATTCGTCACAACATTGAAGATGGTTCCGTTCAACTAGCAGACCATTATCAACA
AAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCGACACAATCTGC

CCTTTCGAAAGATCCCAACGAAAAGCGTGACCACATGGTCCTTCTTGAGTTTGTAACTGCTGCTG
GGATTACACATGGCATGGATGAATTGTACAAGTAATAGCGGCCGC

8.3 Different plasmids

8.3.1 Backbone carrier plasmids obtained from ATUM for expression in *E. coli* strains.

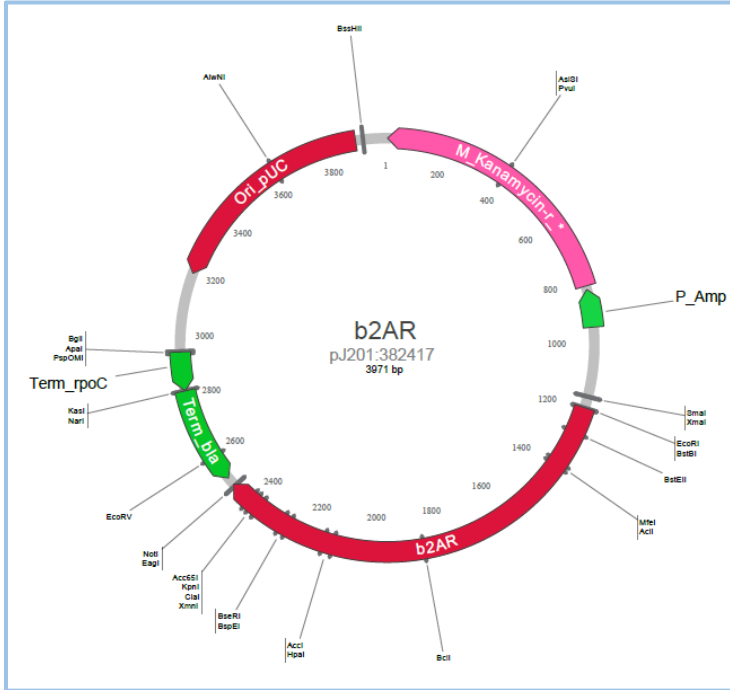

Figure 55: The plasmid vector obtained by ATUM with the β2AR gene incorporated

The plasmid carries an antibiotic resistance gene for Kanamycin and a pUC origin of replication. The restriction enzymes sites for *Eco*RI and *Not*I for isolating the insert can be seen. There is no promoter present in front of the gene of interest as the protein is only going to be expressed in pPZ vectors under various different promoters in *P. pastoris*. (Atum, 2019)

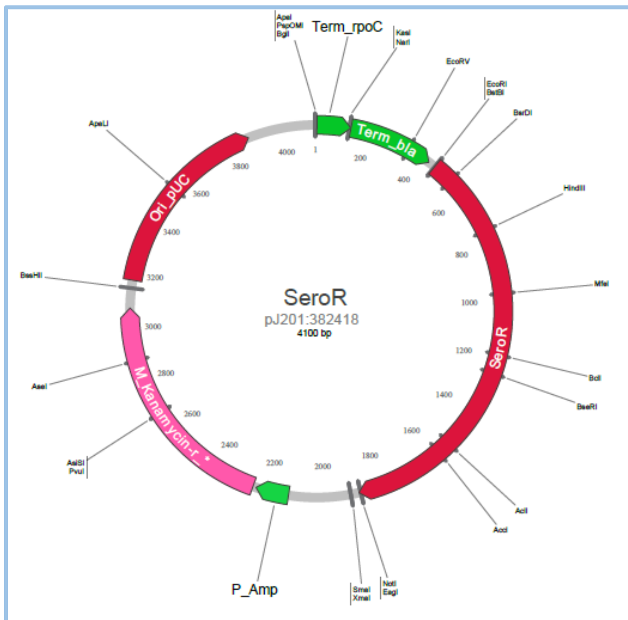

Figure 56: The plasmid vector obtained by "ATUM" with the 5-HT 2c gene incorporated. The plasmid carries an antibiotic resistance gene for Kanamycin and a pUC origin of replication. (Atum, 2019)

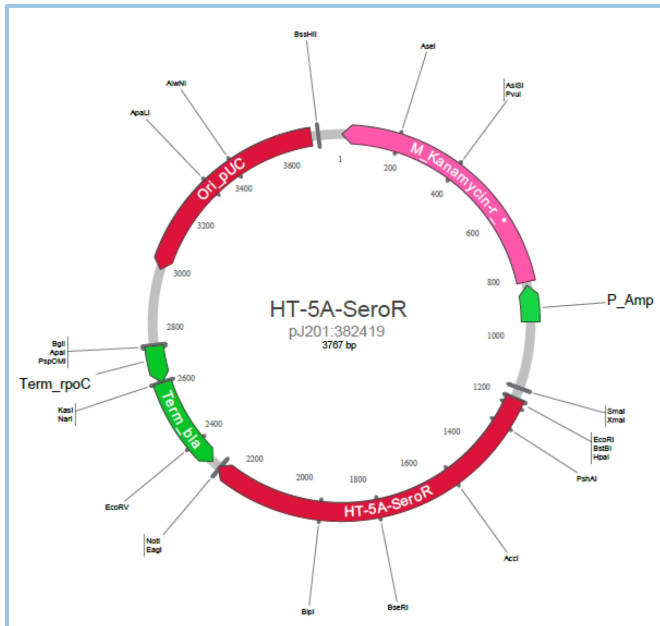

Figure 57: The plasmid vector obtained by "ATUM" with the 5-HT 5a gene incorporated. The plasmid carries an antibiotic resistance gene for Kanamycin and a pUC origin of replication. (Atum, 2019)

8.3.2 pPZ plasmids for recombinant expression in *P. pastoris*

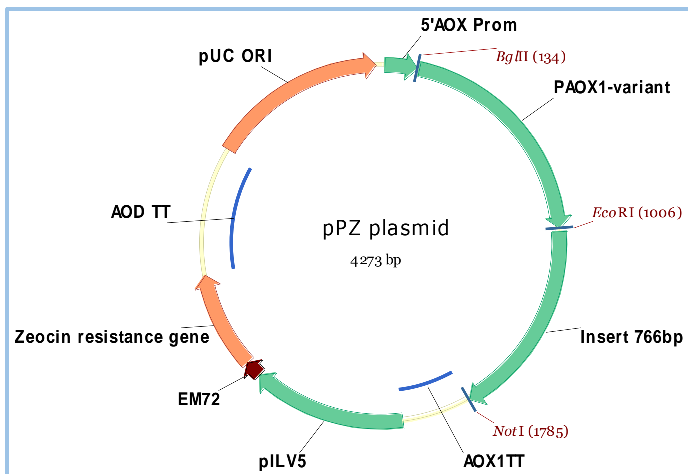

Figure 58: The pPZ plasmid for recombinant protein expression in *P. pastoris*.

It is an *E. coli*/yeast shuttle vector with the P_{AOX1} and T_{AOX1} for gene expression and a zeocin resistance cassette. The insert can be cut from the plasmid with the restriction enzymes *Eco*RI and *Not*I. A pUC origin of replication is for the maintenance of the plasmid in the *E. coli* host. This plasmid type does not contain a secretion signal before the gene of interest, but uses the native signal sequences of the genes of the proteins to be expressed. The proteins are expressed under different variants of the P_{AOX1} named A, B, C, D, E, F, G, H and I.

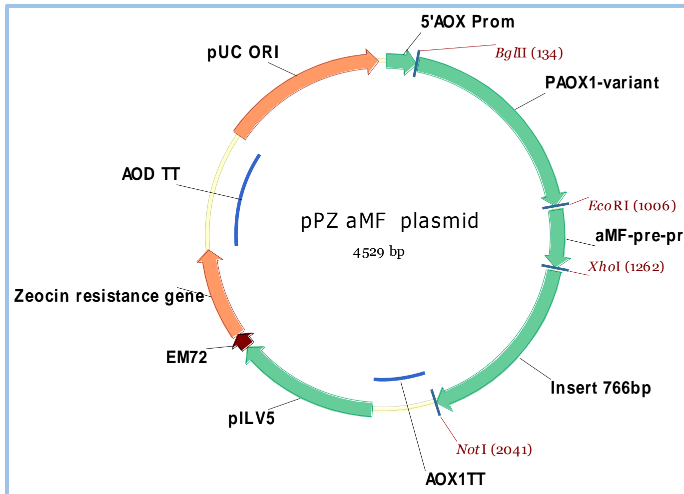

Figure 59: The pPZ_αMF plasmid for recombinant protein expression in *P. pastoris*

The pPZ_αMF plasmid has the same setup as the pPZ plasmid, but it also encodes signal sequence of the alpha-mating factor from *S. cerevisiae*. So it is suited for genes of proteins that have no native signal sequencing targeting to their final destination or for secretion such as the 5-HT 5a gene.

8.3.3 Constructs with the pPZ plasmid and the protein designed for the expression with a GFP tag

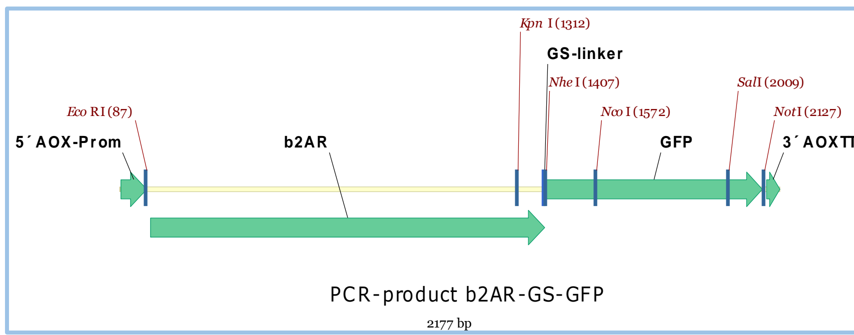

Figure 60: The construct was ordered at “Twist”. In the figure the different restriction enzyme sites for verifying a correct integration of the fragments in the expression vectors. The construct is around 2170 bp long and after linearization it integrates into the genome of *P. pastoris*.

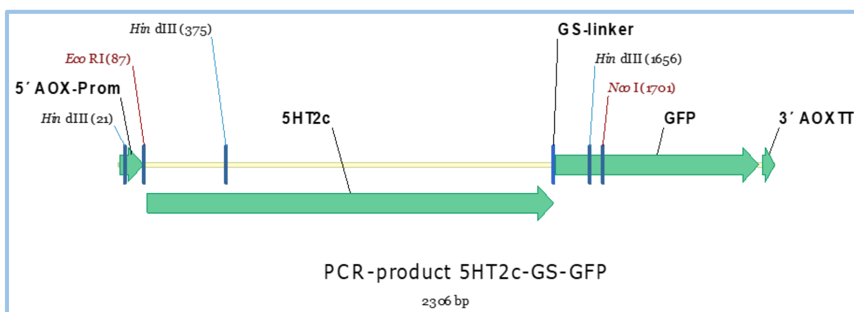

Figure 61: The construct was ordered at “Twist”. It is around 2300 bp in size.

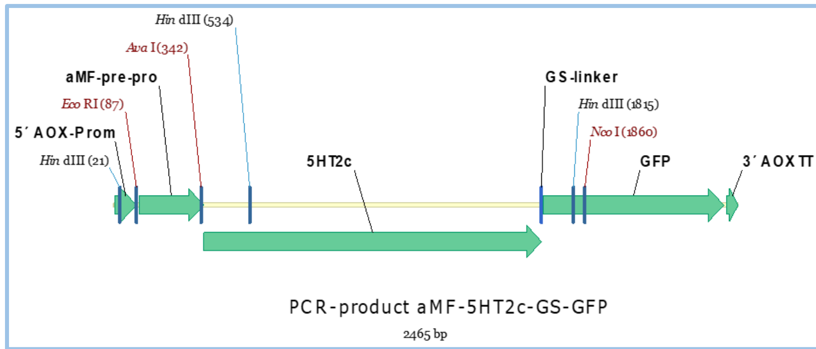

Figure 62: The construct with the αMF signal sequence was ordered at “Twist”. It is around 2460 bp in size.

8.4 Cultivation schemes for *P. pastoris* clones

8.4.1 Cultivation scheme for 5-HT 5a inserts

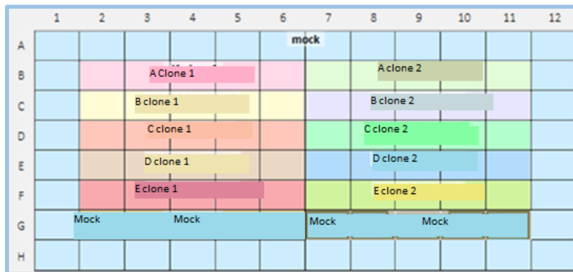

Figure 63: Cultivation scheme for 2 different clones with pPZ vectors carrying the 5-HT 5a insert and the different methanol inducible promoters A, B, C, D and E.

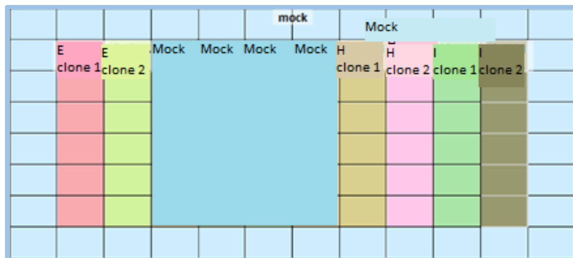

Figure 64: Cultivation scheme for 2 different clones with pPZ vectors carrying the 5-HT 5a insert and the different methanol free promoters E, H and I.

8.4.2 Cultivation schemes for 5-HT 2c inserts and remaining 5-HT 5a clones

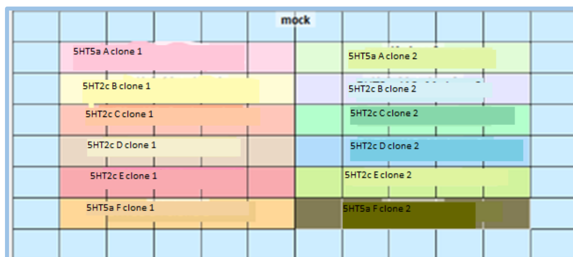

Figure 65: Cultivation scheme for 2 different clones with pPZ vectors carrying the 5-HT5a or 5-HT 2c insert and the different methanol inducible promoters A, B, C, D, E and F.

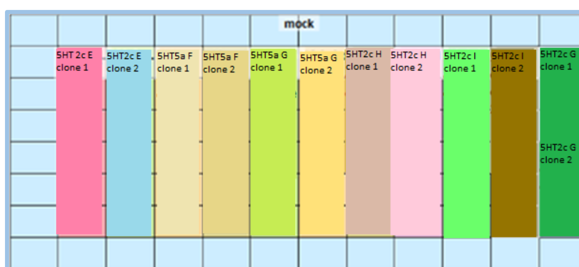

Figure 66: Cultivation scheme for 2 different clones with pPZ vectors carrying the 5-HT 5a or 5-HT 2c insert and the different methanol free promoters E, F, G, H and I.

8.5 OD_{600} measurement of all clones

Table 28: The OD_{600} measured for every clone. From the total ODs the amount of buffers needed was calculated.

Sample	**OD_{600} and dilution factor**	**Average OD_{600} (rounded)**	**Total OD_{600} available (rounded)**	**Amount of lysis buffer [µl] and glass beads [mg] to be used**	**Amount of 100 mM PMSF stock to be used [µl]**
MeOH Mock mut^s	0.519 1:20 0.775 1:15	11.00	28	138	1.4
MeOH β2AR A clone 1	0.324 1:15 0.765 1:20	8.98	23	113	1.1
MeOH β2AR A clone 2	0.675 1:20	13.50	34	169	1.7
MeOH β2AR B clone 1	0.182 1:20	3.64	12	58	0.6
MeOH β2AR B clone 2	0.608 1:20 0.769 1:15	11.85	30	150	1.5
MeOH β2AR C clone 1	0.716 1:20	14.32	43	215	2.2
MeOH β2AR C clone 2	0.280 1:20 0.505 1:15	6.56	18	89	0.9
MeOH β2AR D clone 1	0.546 1:20	10.92	33	165	1.7
MeOH β2AR D clone 2	0.344 1:20 0.497 1:15	7.17	14	72	0.7
MeOH β2AR E clone 1	0.493 1:20 0.585 1:15	9.32	26	130	1.3
MeOH β2AR E clone 2	0.597 1:20	11.94	36	179	1.8
MeOH β2AR F clone 1	0.636 1:20 0.680 1:15	11.46	29	143	1.4
MeOH β2AR F clone 2	0.583 1:20	11.66	33	163	1.6
MeOH-free β2AR E clone 1	0.686 1:20	13.72	34	172	1.7

MeOH-free β2AR E clone 2	0.559 1:20	11.18	34	168	1.7
MeOH-free β2AR F clone 1	0.722 1:20	14.44	43	217	2.2
MeOH-free β2AR F clone 2	0.576 1:20	11.52	35	173	1.7
MeOH-free β2AR G clone 1	0.712 1:20	14.24	43	214	2.1
MeOH-free β2AR G clone 2	0.692 1:20	13.84	42	208	2.1
MeOH-free β2AR H clone 1	0.692 1:20	13.84	42	208	2.1
MeOH-free β2AR H clone 2	0.560 1:20	11.2	34	168	1.7
MeOH-free β2AR I clone 1	0.708 1:20	14.16	35	177	1.8
MeOH-free β2AR I clone 2	0.740 1:20	14.8	44	222	2.2
MeOH-free mock	0.273 1:30	13.65	34	171	1.7
MeOH 5-HT 5a A clone 1	0.339 1:30	10.17	31	150	1.5
MeOH 5-HT 5a A clone 2	0.38 1:30	11.41	34	175	1.8
MeOH 5-HT 5a B clone 1	0.43 1:30	12.90	39	200	2.0
MeOH 5-HT 5a B clone 2	0.466 1:30	13.98	42	200	2.0
MeOH 5-HT 5a C clone 1	0.466 1:30	13.98	42	200	2.0
MeOH 5-HT 5a C clone 2	0.426 1:30	12.78	38	200	2.0
MeOH 5-HT 5a D clone 1	0.468 1:30	14.04	42	200	2.0
MeOH 5-HT 5a D clone 2	0.413 1:30	12.39	37	200	2.0
MeOH 5-HT 5a E clone 1	0.447 1:30	13.41	40	200	2.0
MeOH 5-HT 5a E clone 2	0.490 1:30	14.7	44	250	2.5
MeOH Mock	0.365 1:30	10.95	33	175	1.8
MeOH-free 5-HT 5a E clone 1	0.729 1:30	21.87	66	325	3.3

MeOH-free 5-HT 5a E clone 2	0.531 1:30	15.93	48	250	2.5
MeOH-free 5-HT 5a H clone 1	0.684 1:30	20.52	62	300	3.0
MeOH-free 5-HT 5a H clone 2	0.710 1:30	21.3	64	325	3.3
MeOH-free 5-HT 5a I clone 1	0.537 1:30	16.11	48	250	2.5
MeOH-free 5-HT 5a I clone 2	0.687 1:30	20.61	62	300	3.0
MeOH-free mock	0.640 1:30	19.2	48	250	2.5
MeOH 5-HT 5a A clone 1	0.417 1:30	12.51	38	190	1.9
MeOH 5-HT 5a A clone 2	0.409 1:30	12.27	37	185	1.9
MeOH 5-HT 2c B clone 1	0.457 1:30	13.71	41	205	2.0
MeOH 5-HT 2c B clone 2	0.337 1:30	10.11	30	150	1.5
MeOH 5-HT 2c C clone 1	0.475 1:30	14.25	43	215	2.2
MeOH 5-HT 2c C clone 2	0.342 1:30	10.26	31	155	1.6
MeOH 5-HT 2c D clone 1	0.362 1:30	10.86	33	165	1.7
MeOH 5-HT 2c D clone 2	0.365 1:30	10.95	33	165	1.7
MeOH 5-HT 2c E clone 1	0.499 1:30	14.97	45	225	2.3
MeOH 5-HT 2c E clone 2	0.450	13.5	41	205	2.1
MeOH 5-HT 5a F clone 1	0.542	16.26	49	245	2.5
MeOH 5-HT 5a F clone 2	0.475	14.25	43	215	2.2
MeOH Mock	0.381	11.43	34	170	1.7
MeOH-free 5-HT 2c E clone 1	0.711	21.33	64	320	3.2
MeOH-free 5-HT 2c E clone 2	0.551	16.53	50	250	2.5
MeOH-free 5-HT 5a F clone 1	0.491	14.73	44	220	2.2

MeOH-free 5-HT 5a F clone 2	0.615	18.45	55	275	2.8
MetOH-free 5-HT 5a G clone 1	0.597	17.91	54	270	2.7
MeOH-free 5-HT 5a G clone 2	0.571	17.13	51	255	2.6
MeOH-free 5-HT 2c H clone 1	0.576	17.28	52	260	2.6
MeOH-free 5-HT 2c H clone 2	0.601	18.03	54	270	2.7
MeOH-free 5-HT 2c I clone 1	0.710	21.31	64	320	3.2
MeOH-free 5-HT 2c I clone 2	0.608	18.24	55	275	2.8
MeOH-free 5-HT 2c G clone 1	0.806	24.18	24	120	1.2
MeOH-free 5-HT 2c G clone 2	0.649	19.47	19	95	1.0
MeOH-free Mock	0.711	21.33	53	265	2.7

8.6 OD_{600} measurement of the rescreened clones for ultracentrifugation

Table 29: Clones for ultracentrifugation were harvested and their OD at 600 nm was measured with a dilution of 1:30. The overall OD, the amount of lysis buffer, glass beads, PMSF and Fos Choline was calculated from the measurement.

Sample	**OD_{600} with dilution 1:30**	**OD_{600} with dilution factor calculated**	**Total OD_{600} (rounded)**	**Lysis buffer [µl] and glass beads [mg]**	**100 mM PMSF stock [µl]**	**10 % Fos Choline 14 added before lysis [µl]**	**Harvest after lysis [µl]**	**Fos Choline 14 added after lysis [µ]**
1	0.256	7.68	77	384	4	38	400	40
2	0.333	9.99	100	500	5	50	500	50
3	0.412	12.4	124	600	6	60	600	60
4	0.330	9.9	100	500	5	50	500	50
5	0.201	6.3	60	302	3	30	300	30
6	0.526	15.8	158	600	6	60	500	50
7	0.224	67.2	67	335	3	36	500	50
8	0.517	155.1	155	600	6	60	500	50

8.7 Example for a calibration curve in the BCA assay

To estimate the concentration of total proteins in an unknown sample, a calibration curve with measured absorbance at 562 nm and the protein concentration in mg/ml of six BSA dilutions was created.

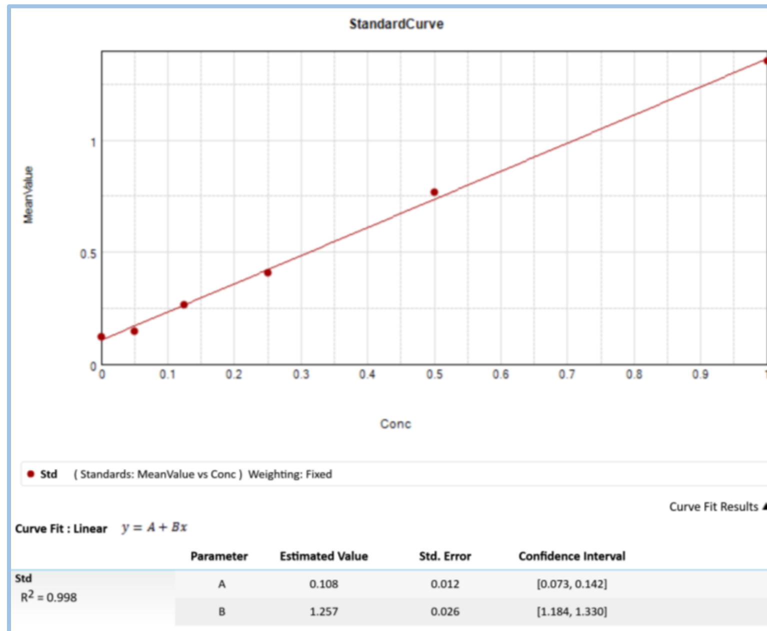

Figure 67: Example of a standard curve for the BCA assay generated with serial dilutions of BSA at known concentrations. With the equation of the line the unknown protein concentration x can be calculated with the rest of the parameters measured.

The absorbance at 562 nm of six standard protein dilutions was measured and the real concentration was calculated from the calibration curve generated with the anticipated concentrations of BSA.

Table 30: Concentrations of the BSA dilutions generated, their real concentrations measured and their absorbance.

Concentration anticipated	Concentration calculated	Well	Absorbance at 562 nm
1	0.991	D1	1.354
0.5	0.524	D2	0.766
0.25	0.24	D3	0.409
0.125	0.126	D4	0.265
0.05	0.032	D5	0.148
0	0.012	D6	0.123

8.8 Total protein concentrations

8.8.1 Total protein concentrations of all different clones with different promoters

Table 31: Total protein concentrations of all clones with their different genes, promoters and inductions. The highest total protein concentrations obtained are depicted in the "Results" section as well.

Sample	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**
MeOH β2AR A clone 1	0.524	0.315	1:10	3.15
MeOH β2AR A clone 2	0.683	0.725	1:20	14.5
MeOH β2AR B clone 1	0.519	0.296	1:10	2.96
MeOH β2AR B clone 2	0.507	0.246	1:10	2.46
MeOH β2AR C clone 1	0.671	0.929	1:10	9.29
MeOH β2AR C clone 2	1.37	0.475	1:20	9.5
MeOH β2AR D clone 1	0.515	0.277	1:10	2.77
MeOH β2AR D clone 2	0.6	0.63	1:10	6.3
MeOH β2AR E clone 1	0.571	0.512	1:10	5.12
MeOH β2AR E clone 2	0.597	0.619	1:10	6.19
MeOH β2AR F clone 1	0.568	0.499	1:10	4.99
MeOH β2AR F clone 2	0.631	0.76	1:10	7.6
MeOH-free β2AR E clone 1	0.525	0.318	1:10	3.18
MeOH-free β2AR E clone 2	0.579	0.544	1:10	5.44
MeOH-free β2AR F clone 1	0.575	0.528	1:10	5.28
MeOH-free β2AR F clone 2	0.567	0.494	1:10	4.94
MeOH-free β2AR G clone 1	0.569	0.501	1:10	5.01
MeOH-free β2AR G clone 2	0.585	0.568	1:10	5.68
MeOH-free β2AR H clone 1	0.582	0.557	1:10	5.57
MeOH-free β2AR H clone 2	0.618	0.708	1:10	7.08
MeOH-free β2AR I clone 1	0.583	0.561	1:10	5.61

MeOH-free β2AR I clone 2	0.588	0.583	1:10	5.83
MeOH mock	0.58	0.549	1:10	5.49
MeOH-free mock	0.679	0.961	1:10	9.61
MeOH 5-HT 5a A clone 1	0.613	0.684	1:10	6.84
MeOH 5-HT 5a A clone 2	0.566	0.489	1:10	4.89
MeOH 5-HT 5a B clone 1	0.552	0.433	1:10	4.33
MeOH 5-HT 5a B clone 2	0.554	0.438	1:10	4.38
MeOH 5-HT 5a C clone 1	0.549	0.419	1:10	4.19
MeOH 5-HT 5a C clone 2	0.548	0.416	1:10	4.16
MeOH 5-HT 5a D clone 1	0.541	0.386	1:10	3.86
MeOH 5-HT 5a D clone 2	0.56	0.464	1:10	4.64
MeOH 5-HT 5a E clone 1	0.552	0.433	1:10	4.33
MeOH 5-HT 5a E clone 2	0.526	0.322	1:10	3.22
MeOH-free 5-HT 5a E clone 1	0.552	0.433	1:10	4.33
MeOH-free 5-HT 5a E clone 2	0.613	0.684	1:10	6.84
MeOH-free 5-HT 5a H clone 1	0.57	0.506	1:10	5.06
MeOH-free 5-HT 5a H clone 2	0.57	0.507	1:10	5.07
MeOH-free 5-HT 5a I clone 1	0.581	0.55	1:10	5.5
MeOH-free 5-HT 5a I clone 2	0.573	0.52	1:10	5.2
MeOH mock	0.538	0.375	1:10	3.8
MeOH-free mock	0.54	0.381	1:10	3.8
MeOH 5-HT 5a A clone 1	0.512	0.265	1:10	2.68
MeOH 5-HT 5a A clone 2	0.513	0.268	1:10	2.68
MeOH 5-HT 2c B clone 1	0.529	0.336	1:10	3.36
MeOH 5-HT 2c B clone 2	0.524	0.316	1:10	3.16

MeOH 5-HT 2c C clone 1	0.517	0.284	1:10	2.84
MeOH 5-HT 2c C clone 2	0.532	0.346	1:10	3.46
MeOH 5-HT 2c D clone 1	0.52	0.297	1:10	2.97
MeOH 5-HT 2c D clone 2	0.503	0.227	1:10	2.27
MeOH 5-HT 2c E clone 1	0.493	0.185	1:10	1.85
MeOH 5-HT 2c E clone 2	0.476	0.114	1:10	1.14
MeOH 5-HT 5a F clone 1	0.592	0.6	1:10	6
MeOH 5-HT 5a F clone 2	0.578	0.539	1:10	5.39
MeOH-free 5-HT 2c E clone 1	0.527	0.327	1:10	3.27
MeOH-free 5-HT 2c E clone 2	0.572	0.514	1:10	5.14
MeOH-free 5-HT 5a F clone 1	0.553	0.436	1:10	4.36
MeOH-free 5-HT 5a F clone 2	0.527	0.327	1:10	3.27
MeOH-free 5-HT 5a G clone 1	0.577	0.537	1:10	5.37
MeOH-free 5-HT 5a G clone 2	0.505	0.236	1:10	2.36
MeOH-free 5-HT 2c H clone 1	0.524	0.315	1:10	3.15
MeOH-free 5-HT 2c H clone 2	0.541	0.385	1:10	3.85
MeOH-free 5-HT 2c I clone 1	0.532	0.347	1:10	3.47
MeOH-free 5-HT 2c I clone 2	0.559	0.461	1:10	4.61
MeOH-free 5-HT 2c G clone 1	0.575	0.528	1:10	5.28
MeOH-free 5-HT 2c G clone 2	0.594	0.605	1:10	6.05
MeOH mock	0.542	0.389	1:10	3.89
MeOH-free mock	0.551	0.429	1:10	4.29

8.8.2 Total protein concentrations of rescreened clones after ultracentrifugation

Table 32: The total protein concentrations of all clones with different promoters, genes, Fos Choline conditions and inductions, after ultracentrifugation, are depicted. Clones with very high protein concentrations are displayed also in the “Results” section.

Sample	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**
No Fos β2AR B	0.899	0.632	1:10	6.32
No Fos β2AR C	1.379	0.316	1:20	3.16
No Fos 5-HT 5a A	0.168	0.019	1:10	0
No Fos 5-HT 5a B	1.009	0.724	1:10	7.24
No Fos 5-HT 2c D	0.679	0.448	1:10	4.48
No Fos 5-HT 2c G	0.36	0.18	1:10	1.8
No Fos Mock MeOH	0.225	0.067	1:10	0.67
No Fos Mock NP	0.606	0.387	1:10	3.87
Before lysis β2AR B	0.15	0.004	1:10	0.04
Before lysis β2AR C	0.914	0.645	1:10	6.45
Before lysis 5-HT A	0.496	0.294	1:10	2.94
Before lysis 5-HT 5a B	0.408	0.221	1:10	2.21
Before lysis 5-HT 2c D	1.246	0.923	1:10	9.23
Before lysis 5-HT 2c G	0.139	-0.005	1:10	-0.05
Before lysis Mock MeOH	0.164	0.016	1:10	0.16
Before lysis Mock NP	0.178	0.028	1:10	0.28
After lysis β2AR B	0.316	0.143	1:10	1.43
After lysis β2AR C	0.816	0.562	1:10	5.62
After lysis 5-HT 5a A	1.273	0.945	1:10	9.45
After lysis 5-HT 5a B	0.175	0.025	1:10	0.25
After lysis 5-HT 2c D	0.176	0.026	1:10	0.26
After lysis 5-HT 2c G	0.189	0.037	1:10	0.37
After lysis Mock MeOH	0.221	0.064	1:10	0.64
After lysis Mock NP	0.537	0.329	1:10	3.29

8.8.3 Total protein concentration of rescreened clones without ultracentrifugation

Table 33: The total protein concentrations of all clones with different promoters, genes, Fos Choline conditions and inductions, without ultracentrifugation after cell lysis, are depicted. Clones with very high protein concentrations are displayed also in the “Results” section.

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**

No Fos β2AR B	0.479	0.296	1:10	2.96
No Fos β2AR C	0.583	0.378	1:10	3.78
No Fos 5-HT 5a A	0.35	0.193	1:10	1.93
No Fos 5-HT 5a B	0.58	0.376	1:10	3.76
No Fos 5-HT 2c D	1.25	0.909	1:10	9.09
No Fos 5-HT 2c G	0.881	0.616	1:10	6.16
No Fos Mock MeOH	0.403	0.235	1:10	2.35
No Fos Mock NP	0.465	0.284	1:10	2.84
Before lysis β2AR B	0.518	0.327	1:10	3.27
Before lysis β2AR C	0.639	0.423	1:10	4.23
Before lysis 5-HT 5a A	0.626	0.413	1:10	4.13
Before lysis 5-HT 5a B	1.074	0.769	1:10	7.69
Before lysis 5-HT 2c D	0.676	0.452	1:10	4.52
Before lysis 5-HT 2c G	0.387	0.222	1:10	2.22
Before lysis Mock MeOH	0.842	0.584	1:10	5.84
Before lysis Mock NP	0.446	0.269	1:10	2.69
After lysis β2AR B	0.311	0.162	1:10	1.62
After lysis β2AR C	0.595	0.387	1:10	3.87
After lysis 5-HT 5a A	0.362	0.202	1:10	2.02
After lysis 5-HT 5a B	0.614	0.403	1:10	4.03
After lysis 5-HT 2c D	0.376	0.213	1:10	2.13
After lysis 5-HT 2c G	0.198	0.072	1:10	0.72
After lysis Mock MeOH	0.334	0.18	1:10	1.8
After lysis Mock NP	0.332	0.178	1:10	1.78

8.9 The rescreened clones induced at a lower temperature

8.9.1 Harvest of the rescreened clones

Table 34: Samples induced at 22 °C were harvested as usual, their optical density was measured at 600 nm. The overall OD, the amount of lysis buffer, glass beads, PMSF and Fos Choline needed were calculated.

Sample	**OD_{600} with dilution 1:30**	**OD_{600} with dilution factor calculated**	**Total OD_{600} (rounded)**	**Lysis buffer [µl] and glass beads [mg]**	**100 mM PMSF stock [µl]**	**10 % Fos Choline 14 added before lysis [µl]**	**Lysate obtained [ml]**
β2AR B	0.28	8.4	126	630	6.3	/	0.6
β2AR C	0.46	13.9	208	1040	10.4	/	1
β2AR D	0.52	15.5	232	1160	11.6	/	1.1

5-HT 5a A	0.56	16.7	250	1250	12.5	/	1.3
5-HT 5a B	0.67	20.0	300	1500	15	/	1.3
5-HT 5a F	0.44	13.3	200	997	10	/	1
5-HT 5a G	0.48	14.5	217	1086	10.9	/	1
5-HT 2c D	0.55	16.6	248	1242	12.4	124	1.6
5- HT 2c G	0.50	15	225	1125	11.3	113	1.5
Mock MeOH	0.60	18.1	271	1357	13.6	/	1.3
Mock MeOH-free	0.49	14.6	219	1100	11	/	1.1

8.9.2 Total protein concentration of the rescreened clones

Table 35: Total protein concentrations of rescreened clones induced at 22 °C with samples before and after ultracentrifugation

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration [mg/ml]**	**Total protein concentration [µg/ml]**
β2AR B before ultrac	0.293	0.137	1:10	1.37	1370
β2AR C before ultrac	0.104	0.032	1:10	0.32	320
β2AR D before ultrac	0.339	0.178	1:10	1.78	1780
5-HT 5a A before ultrac	0.275	0.121	1:10	1.21	1210
5-HT 5a B before ultrac	0.274	0.12	1:10	1.2	1200
5-HT 5a F before ultrac	0.211	0.064	1:10	0.64	640
5-HT 5a G before ultrac	0.27	0.117	1:10	1.17	1170
5-HT 2c D before ultrac	0.416	0.247	1:10	2.47	2470
5-HT 2c G before ultrac	0.349	0.187	1:10	1.87	1870
Mock before ultrac	0.233	0.083	1:10	0.83	830
Mock NP before ultrac	0.271	0.118	1:10	1.18	1180
β2AR B after ultrac	0.244	0.094	1:10	0.94	940
β2AR C after ultrac	0.173	0.03	1:10	0.3	300

β2AR D after ultrac	0.22	0.072	1:10	0.72	720
5-HT 5a A after ultrac	0.149	0.008	1:10	0.08	80
5-HT 5a B after ultrac	0.161	0.019	1:10	0.19	190
5-HT 5a F after ultrac	0.126	0.012	1:10	0.12	120
5-HT 5a G after ultrac	0.446	0.275	1:10	2.75	2750
5-HT 2c D after ultrac	0.126	0.012	1:10	0.12	120
5-HT 2c G after ultrac	0.273	0.119	1:10	1.19	1190
Mock after ultrac	0.26	0.107	1:10	1.07	1070
Mock NP after ultrac	0.183	0.039	1:10	0.39	390

8.10 Proteins expressed with the αMF signal sequence

8.10.1 OD_{600} measurements

Table 36: Optical densities at 600 nm of clones expressing the 5-HT 2c protein with the αMF signal sequence under different promoters. From the OD the amount of buffer needed was calculated.

Sample	**OD_{600} with dilution 1:30**	**OD_{600} with dilution factor calculated**	**Total OD_{600} in 15 ml(rounded)**	**Lysis buffer [µl] and glass beads [mg]**	**100 mM PMSF stock [µl]**	**Lysate obtained [ml]**
5-HT 2c X/N B	0.43	13	208	1000	10	2
5-HT 2c X/N H	0.40	12	180	900	9	1.5
5-HT 2c X/N E MeOH	0.44	13	208	1000	10	2
5-HT 2c X/N E NP	0.36	11	165	825	8.3	1.6
Mock MeOH	0.48	14	210	1050	10.5	1.9
Mock NP	0.51	15	225	1125	11.3	2

8.10.2 Total protein concentrations

Table 37: Total protein concentrations of clones expressing the 5-HT 2c protein with the αMF signal sequence under different promoters.

Sample and Characteristics	**Absorbance at 562 nm**	**Protein concentration [mg/ml]**	**Dilution factor**	**Total protein concentration before ultracentrifugation[mg/ml]**	**Total protein concentration after ultracentrifugation [mg/ml]**
5-HT 2c X/N B	0.371	0.604	1:10	6.04	1.14
5-HT 2c X/N H	0.209	0.115	1:10	1.15	0.19
5-HT 2c X/N E MeOH	0.185	0.042	1:10	0.42	0.22
5-HT 2c X/N E NP	0.232	0.185	1:10	1.85	0.19
Mock MeOH	0.196	0.076	1:10	0.76	1.51
Mock NP	0.206	0.105	1:10	1.05	0.21

8.11 Determination of possible multiple integration events

For the determination of possible multiple integration events in the *P. pastoris* genome clones for every protein were streaked out on YPhyD agar with increasing concentrations of zeocin added up to 4000 µg/ml.

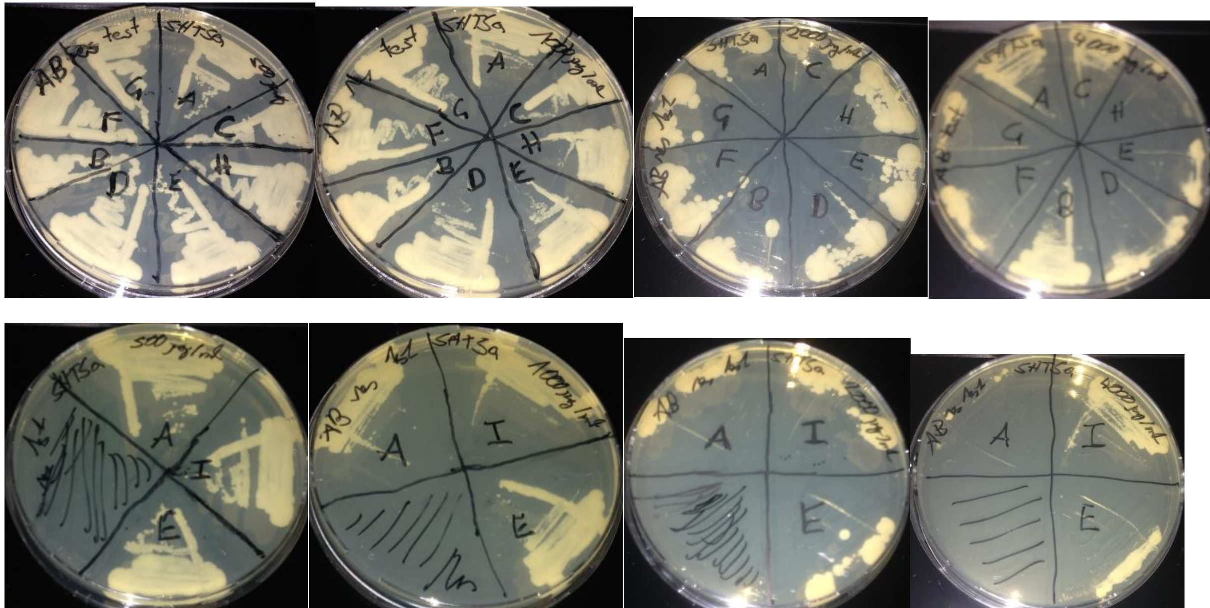

Figure 68: *P. pastoris* mut[s] with the 5-HT 5a construct under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml of zeocin added.

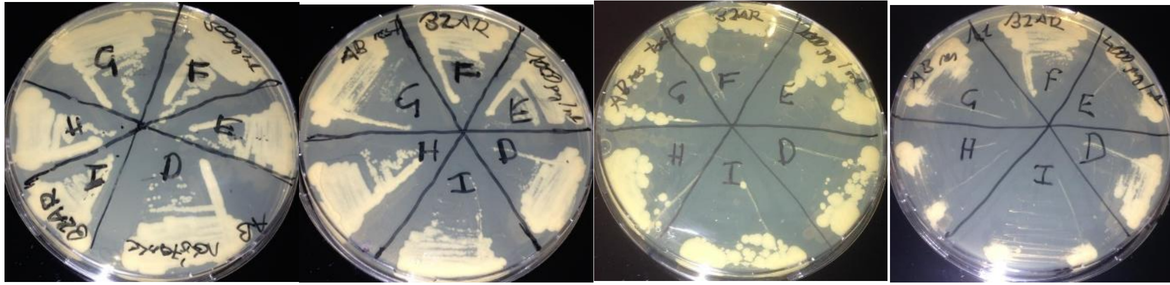

Figure 69: *P. pastoris* mut^s with the β2AR construct under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml of zeocin added.

Figure 70: *P. pastoris* mut^s with the 5-HT 2c construct under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml of zeocin added.

Figure 71: *P. pastoris* mut^s with the 5-HT 2c αMF construct under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml of zeocin added.

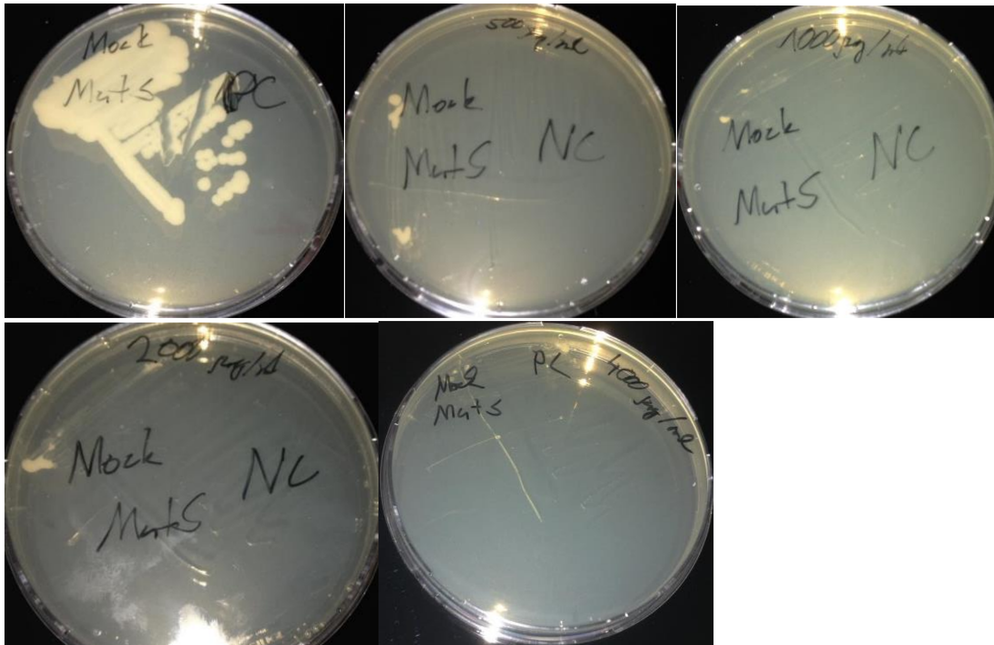

Figure 72: *P. pastoris* mut^{s} growing on YPhyD agar without zeocin, but not under 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml zeocin.

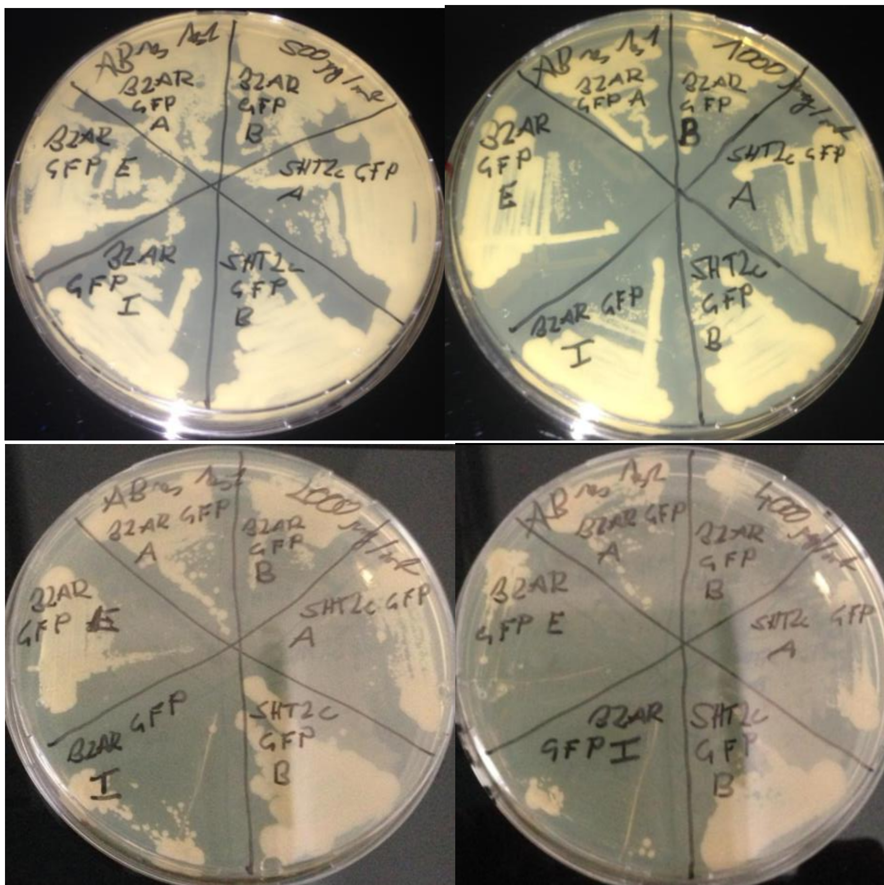

Figure 73: *P. pastoris* mut^{s} with the β2AR-GFP and the 5-HT 2c-GFP constructs under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml zeocin added.

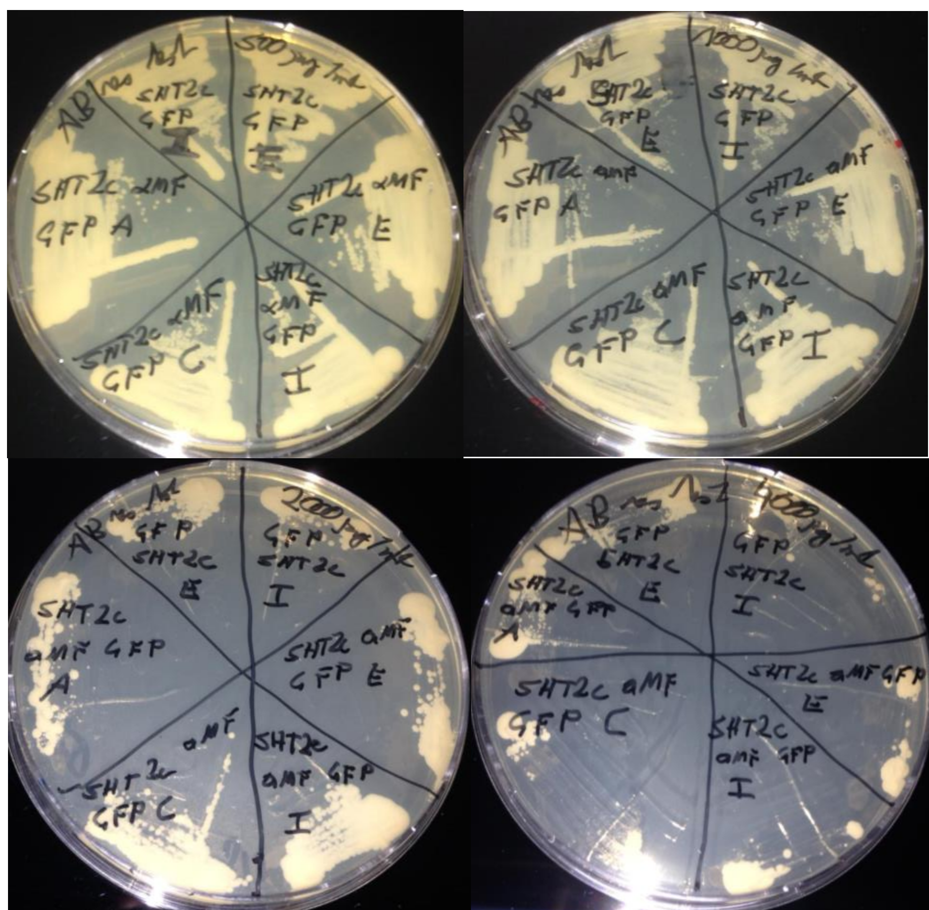

Figure 74: *P. pastoris* mut^s with the 5-HT 2c-GFP and the 5-HT 2c-αMF-GFP constructs under different promoters growing on YPhyD agar with 500 µg/ml, 1000 µg/ml, 2000 µg/ml and 4000 µg/ml zeocin added.

8.12 Proteins with a GFP tag

8.12.1 Harvest of MP-GFP constructs

Table 38: MP-GFP constructs, their OD before lysis and the amount of buffer, glass beads and PMSF added

Sample	OD_{600} at dilution factor 1:30	Average OD_{600} (rounded)	Total OD_{600} available in 50 ml (rounded)	Amount of lysis buffer [µl]	Amount of 100 mM PMSF stock to be used [µl]
β2AR-GFP A	0.418	12.54	627	3135	31
β2AR-GFP B	0.452	13.56	678	3390	34
β2AR-GFP E MeOH	0.430	12.99	650	3248	32
Mock MeOH	0.458	13.74	687	3435	34
β2AR-GFP E MeOH-free	0.716	21.48	1074	5370	54
β2AR-GFP I	0.673	20.19	1010	5048	50
Mock MeOH-free	0.652	19.56	978	4890	50

5-HT 2c-GFP A	0.557	16.71	753	3600	36
5-HT 2c-GFP B	0.350	10.5	474	2400	24
5-HT 2c-GFP E	0.356	10.7	483	2400	24
Mock MeOH	0.398	11.9	537	2700	27
5-HT 2c-GFP E MeOH-free	0.729	21.9	990	5100	51
5-HT 2c-GFP I	0.735	22	990	5100	51
Mock MeOH-free	0.792	24	1080	5400	54
5-HT 2c-αMF-GFP A	0.351	16.5	480	2500	25
5-HT 2c-αMF-GFP B	0.310	9.3	450	2250	23
5-HT 2c-αMF-GFP E	0.293	8.8	400	2000	20
Mock MeOH	0.342	10.3	480	2500	25
5-HT 2c-αMF-GFP E MeOH-free	0.653	19.6	900	4500	45
5-HT 2c-αMF-GFP I	0.610	18.3	840	4200	42
Mock MeOH-free	0.665	20	920	4500	45