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Optimizing the human aryl hydrocarbon receptor (hAHR) expression in Pichia pastoris

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OPTIMIZING THE HUMAN ARYL HYDROCARBON RECEPTOR EXPRESSION IN PICHIA PASTORIS

By

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OPTIMIZING THE HUMAN ARYL HYDROCARBON RECEPTOR EXPRESSION IN PICHIA PASTORIS

Abstract

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The aryl hydrocarbon receptor (AHR) is a transcription factor which heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) to regulate downstream gene transcription. For the purpose of studying the crystal structure of human aryl hydrocarbon receptor (hAHR), it is essential to obtain abundant amount of pure recombinant protein.

Basing on the benefits of using *P. pastoris* system to produce recombinant protein, including appropriate folding, secretion of interest proteins to the external environment of the cell, and easier purification process of protein due to the its limited production of endogenous secretory proteins [Mohsen Karbalaei et al, 2020], our lab chose *P. pastoris* yeast as the host to overexpress human AHR.

My lab has successfully used the protease-deficient *P. pastoris* (ySMD1163) strain to express AHR [Yujuan Zheng et al, 2016], but unfortunately the yield is modest, presumably due to low copy number. My work addressed whether increasing the copy number of hAHR in the yeast genome would increase the expression level of hAHR in *Pichia pastoris*.

Results from my experiments showed that although the copy number correlated with the expression levels of hAHR, the increased expression of the hAHR largely in the pellet, suggesting that the soluble expression of hAHR can’t be enhanced merely by increasing its production.

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LIST OF ABBREVIATIONS

AHR	aryl hydrocarbon receptor
hAHR	human aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
Ab	antibody
CYP	cytochrome P450
qPCR	Quantitative Real-time Polymerase Chain Reaction
gDNA	genomic DNA
GA-hAHR	GeneArt optimized human aryl hydrocarbon receptor
ySMD1163	protease-deficient Pichia strain
BMMY	buffered methanol complex medium
BMGY	buffered glycerol complex medium
HEDG	25mM HEPES, pH7.4,1mM EDTA,1mM DTT, and 10% glycerol
DTT	dithiothreitol
RT	room temperature
HSP90	Heat shock protein 90 (90 comes from the fact that it weighs roughly 90 KiloDalton)
AIP	AHR-interacting protein (also known as XAP2)
PAS	A Per-Arnt-Sim (PAS) domain
	Per – period circadian protein
	Arnt – aryl hydrocarbon receptor nuclear translocator protein
	Sim – single-minded protein
XRE	xenobiotic response elements

DRE	dioxin response element
AP	ammonium persulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
BCA	bicinchoninic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
FICZ	6-formylindolo[3,2-b] carbazole
WT	wild type
NC	negative control
YNB	Yeast Nitrogen Base
YPD	Yeast extract Peptone Dextrose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	immunoglobulin
P. Pastoris	*Pichia Pastoris*
bHLH-PAS	basic helix-loop-helix-PER-ARNT-SIM
kDa	kilodalton

CHAPTER 1: INTRODUCTION

1.1 Aryl Hydrocarbon Receptor

Aryl hydrocarbon receptor (AHR) is a ligand-activated cytoplasmic transcriptional factor known for its contribution for cellular responses against environmental toxins and carcinogens [Hui Zhao et al,2019]. It plays an important role in the human immune system and cancer, making it an interesting target for research as potential treatment options. The aryl hydrocarbon receptor (AHR) is a protein encoded by gene residing on the human chromosome 7, its molecular mass is 104 kDa, this protein is well known as a transcriptional factor which regulates the gene expression. AHR was originally discovered by Poland (1976) as a dioxin-binding protein [Poland A. et al,1976]. It was originally considered as a sensor of xenobiotic chemicals and as the regulator of enzymes such as cytochrome P450s that metabolize these chemicals. AHR, existing in many cells, can be activated by natural and synthetic ligands to connect these environmental chemical stimuli with adaptive response, such as detoxification, cellular homoeostasis, or immune responses [Charlotte Esser,2012].

At the cellular level, AHR is involved in the control of cell proliferation and cell cycle, cell morphology, cell adhesion and cell migration. The new aspect of AHR in biology involves its significance in controlling cell differentiation and its possible involvement in cell pluripotency and stemness. In fact, AHR may help regulate the balance between the differentiation and pluripotency of normal and transformed tumor cells [Sonia Mulero-Navarro & Pedro M. Fernandez-Salguero,2016].

1.2 AHR Signaling Pathway

AHR has several different signaling pathways: (1) XRE-dependent control of gene expression by AHR; (2) Control of gene expression by AHR through non-XRE DNA-responsive elements; (Figure 2a) (3) Additional AHR signaling mechanisms (Figure 2b) [Veit Rothhammer & Francisco J. Quintana,2019]

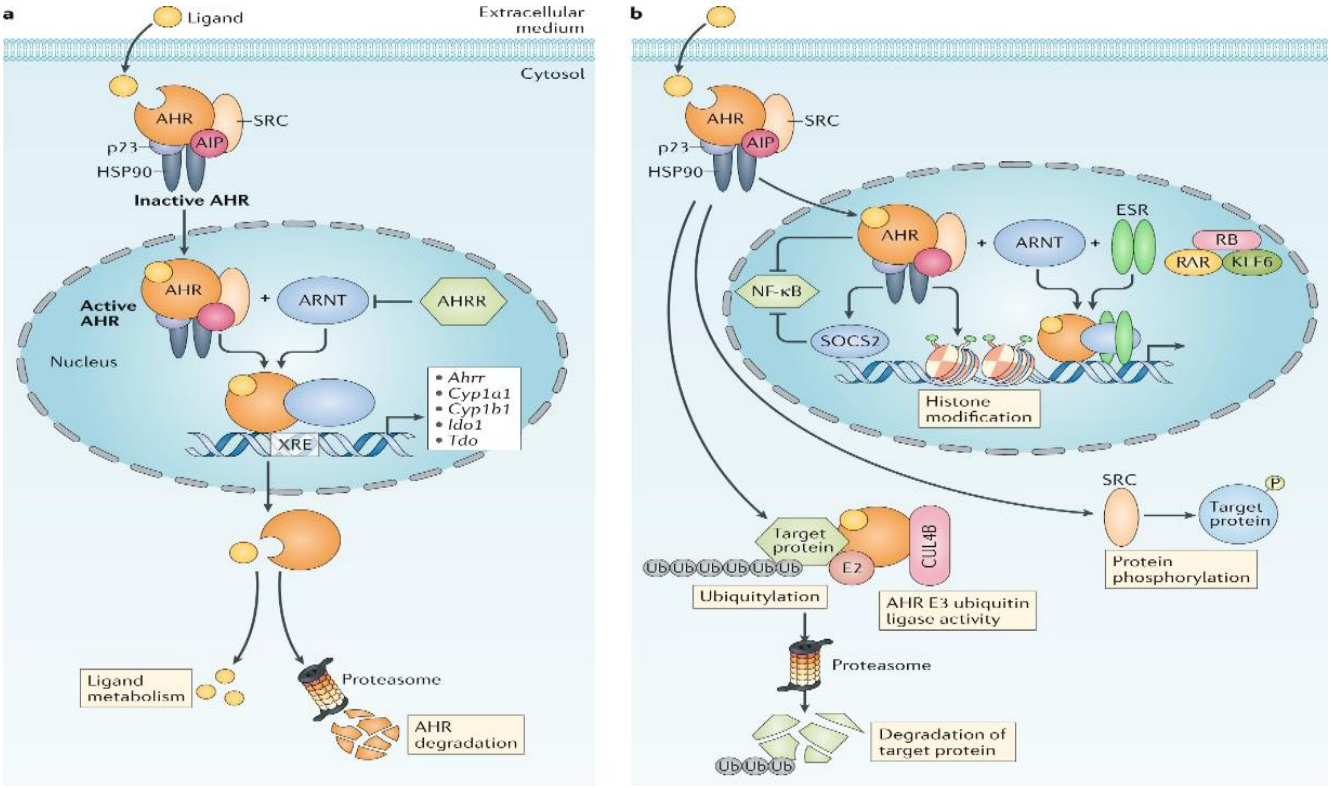

Figure 1. Human AHR signaling pathway. A | Inactive aryl hydrocarbon receptor (AHR) is complexed with 90 kDa heat shock protein (HSP90), AHR-interacting protein (AIP), p23 and SRC. B | AHR can also interact with additional transcription factors to target their specific binding sites and interact with pro-inflammatory nuclear factor-κB (NF-κB) signalling in direct and indirect ways. Moreover, AHR functions as an E3 ubiquitin ligase, promoting the proteasomal degradation of target proteins. In addition, AHR activation triggers phosphorylation cascades driven by SRC upon its release from the AHR chaperone complex. AHRR, AHR repressor; ARNT, AHR nuclear translocator; CUL4B, cullin 4B ubiquitin ligase complex; ESR, oestrogen receptor; KLF6, Krüppel-like factor 6; P, phosphorylation; RAR, retinoic acid receptor; RB, retinoblastoma protein; SOCS2, suppressor of cytokine signalling 2; Ub, ubiquitylation; XRE, xenobiotic response element.

The best known and classic AHR signaling pathway is the XRE-dependent control of gene expression. AHR is a member of the bHLH-PAS family of nuclear transcriptional factors. [Bersten DC et al.,2013] As a ligand-activated transcriptional factor, AHR exists in the inactive state as part of a protein complex, which consists a dimer of HSP90, AHR-interacting protein (AIP; also known as XAP2), the co-chaperone p23 and the protein kinase SRC.

Once the ligand binds to the AHR, the AHR will be activated and undergo a conformational change, then the protein complex of AHR, hsp90, p23 and XAP2 will relocate into the nucleus, and the complex will dissociate. After heterodimerization with ARNT protein in the nucleus, AHR can bind to exogenous response elements (XREs) or dioxin res`ponse elements (DREs) located in the promoter regions of AHR-responsive genes, activating transcription of genes such as the cytochrome P450 genes – cyp1a1, cyp1a2, and cyp1b1[Denison MS et al.,2011]. AHR will then cycle back to the cytosol and be degraded.

1.3 Recombinant Protein Expression System: *Pichia pastoris* Yeast

Pichia pastoris was originally isolated from exudates of the French chestnut tree, and was named Zygosaccharomyces Pastoris [Zahrl, R. J,2017]. The organism was then classified as a new genus Komagataella or Pichia [Naumov, G. I.et al,2018]. *Pichia pastoris*, an engineered methylotrophic microorganism, using methanol as its sole carbon and energy source [Cereghino, G. P. L.,et al,2002]. Nowadays, there are several existing recombinant protein expression systems: bacteria, yeast, mold, mammals, plants, and insects. Compared with other expression systems, yeast cells have significant advantages such as growth speed, posttranslational modification, secretory expression, and easy genetic manipulation. [Mohsen Karbalaei et al,2020] (Table 1) In addition, linearized exogenous DNA can be efficiently inserted into

chromosomes through the phenomenon of cross-recombination to generate stable cell lines [Rachel Daly, et al.2018].

Table 1.
Basic Characteristics of Different Host Systems for the Expression of Recombinant Proteins

Characteristics	*Escherichia coli*	*Pichia pastoris*	CHO cell
Doubling time	30 min	60–120 min	24 hr
Cost of growth medium	Low	Low	High
Complexity of growth medium	Minimum	Minimum	Complex
Expression level	High	Low to high	Low to moderate
Extracellular expression	Secretion to periplasm	Secretion to medium	Secretion to medium
Protein folding	Refolding usually required	Refolding may be required	Proper folding
N-linked glycosylation	None	High mannose	Complex
O-linked glycosylation	No	Yes	Yes
Phosphorylation & acetylation	No	Yes	Yes
Drawback	Accumulation of LPS	Codon bias	Contamination with animal viruses

Abbreviations: CHO, Chinese hamster ovary; LPS, lipopolysaccharide.

1.4 Expression of Recombinant Gene in *Pichia pastoris*

The expression of any recombinant gene in Pichia pastoris is divided into three stages: (a) cloning the gene of interest into a suitable expression vector, (b) inserting the cloned vector into the Pichia host genome; (c) express the recombinant protein using the Pichia strain [Macauley-Patrick et al,2005].

The inserted gene is integrated into the yeast genome through the phenomenon of cross-recombination (Figure 3), thereby forming a recombinant cell. In most cases, only one crossover occurs in the genome, but in 1-10% of cases, multiple insertions occur. The expression vector in the Pichia pastoris expression system consists of three sequences: a promoter sequence in the 5' region (most commonly AOX1); a transcription termination sequence in the 3' region, which is essential for messenger RNA processing and polyadenylation; and a sequence containing sequences of single or multiple cloning sites, which are necessary for insertion of the gene of interest.

Episomal vectors can replicate autonomously in the cytoplasm or as part of a chromosome. But vectors for Pichia pastoris do not have a stable episomal state; therefore, they should first be linearized with the enzyme and then integrated into the Pichia pastoris chromosome [Li, P.et al,2007].

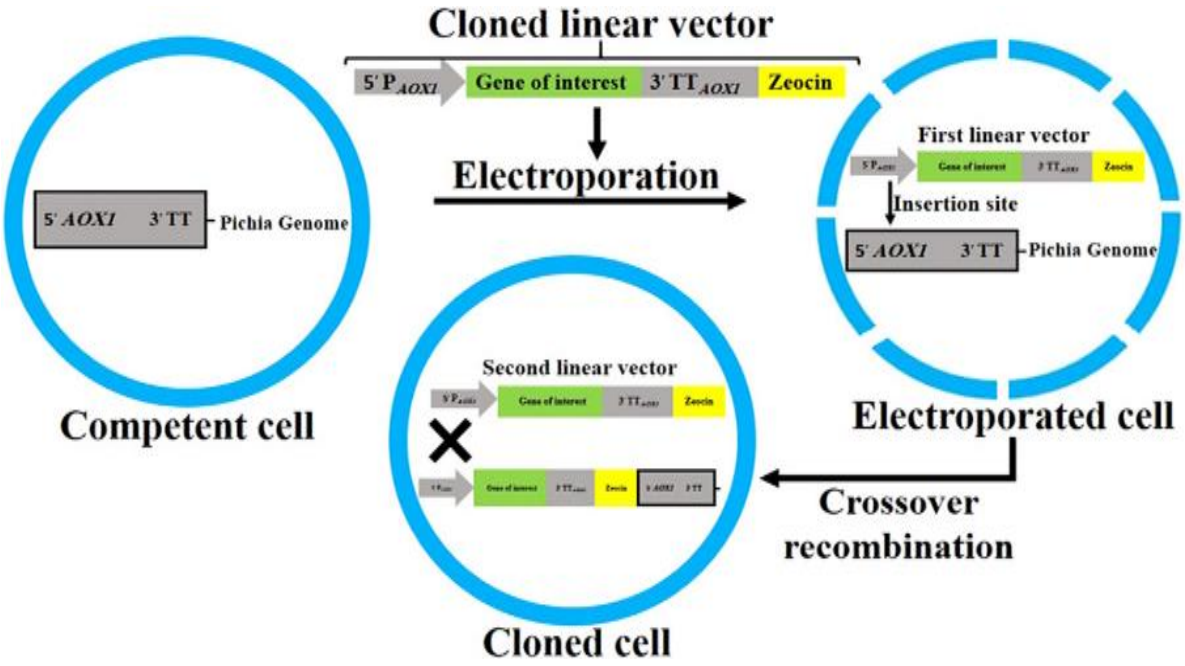

Figure 2. Cross-over recombination phenomenon in the *Pichia pastoris* genome. Following the electroporation process of competent yeast cells, cloned linear vectors are inserted into the electroporated cells. Crossover recombination occurs between 5′ promoter (5′ PAOX1) of vector and AOX1 region of P. pastoris genome. Consequently, cloned cells with a recombinant genome are formed. AOX1, alcohol oxidase 1; TT, transcription termination region

1.5 Gene Electroporation

The basic principle of electroporation is that by applying an electrical pulse of sufficient intensity to the cell, it leads to an increase in the potential difference across the membrane, thereby causing membrane instability. The permeability of the cell membrane increases, followed by the entry of non-permeable molecules into the cell [Kotnik, T et al,2000] [Sweeney DC et al,2018].

Although the mechanism of gene electro transfer is not fully understood, it has been shown that the introduction of DNA occurs only in the cathode portion of the membrane, and that successful transfection requires several steps: electrophoretic migration of DNA into the cell, insertion of DNA into the cell membrane, and ease of transmembrane transfection, translocation, DNA migration to the nucleus, DNA translocation across the nuclear envelope, and finally gene

expression [Satkauskas S et al,2002] (Figure 4). There are many factors that affect the efficiency of gene electro transfer, such as: temperature, electrical pulse parameters, DNA concentration, electroporation buffer used, cell size, and the ability of the cells to express the transfected gene [Gehl J et al,2003]. In vivo gene electro transfer, DNA diffusion through the extracellular matrix, tissue properties, and overall tissue conductivity are also critical [Miklavcic D et al,1998].

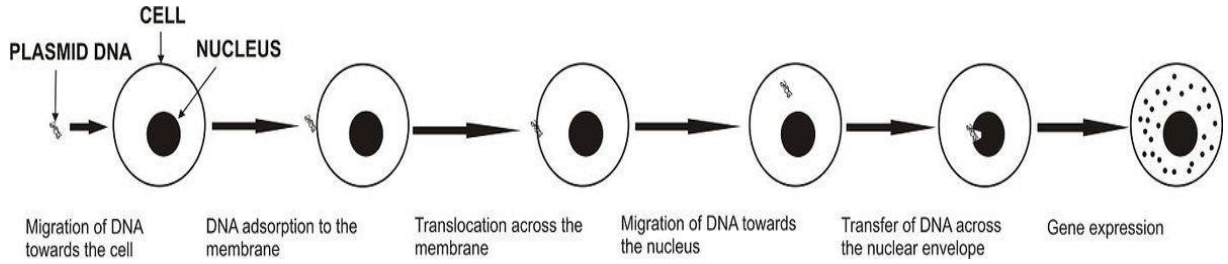

Figure 3. The whole process of gene electroporation

1.6 Purification Using Cobalt

With a polyhistidine (6xHis) tag at the C-terminus of pPICZB, his tags allow researchers to selectively extract proteins of interest from thousands of other proteins found in cells or cell lysates. His-Tag proteins are purified by one-step immobilized metal affinity chromatography (IMAC); a special form of affinity chromatography in which proteins are separated according to their affinity for metal ions immobilized on solid chelating resins. While many divalent metal ions are available for IMAC, nickel (Ni2+) or cobalt (Co2+) are commonly used in chelating resins to coordinate and selectively bind his tags. Cobalt Resin is a tetradentate chelating agarose resin with divalent cobalt (Co2+) used to obtain high purity his-tagged proteins free of metal contamination.

Cobalt resin has several features: (1) high purity (2) specificity (3) low metal leaching (4) versatility (5) cost-effectiveness (6) flexibility

1.7 Why Optimizing the AHR Expression in *Pichia pastoris*

The crystal structural information of full length hAHR is unknown due to the difficulties in obtaining enough purified full-length protein for structural studies. Many researchers are investigating AHR's structure using truncated AHR [Seok SH, Lee W, Jiang L, et al,2017] [Schulte KW, Green E, Wilz A, et al.2017] and homologous modeling coupled with mutagenesis data [Xing Y, Nukaya M, Satyshur KA, et al.2012] [Pandini A, Soshilov AA, Song Y, et al.2009] to provide structural information for AHR. We believe that it will be more straightforward to discover the structural information of AHR by scaling up the production of full length AHR in *Pichia pastoris*. To harvest high yield AHR, there are several studies reported to improve the protein expression in Pichia pastoris, such as different promoter variants, codon optimization or co-expression of helper proteins [Sandra Abad, Kerstin Kitz et al,2010]. Chan's lab has successfully performed electroporation using the codon-optimized hAHR plasmid with protease-deficient *Pichia* strain ySMD1163, and expressed functional full length hAHR which forms the native AHR/Arnt/DRE complex in gel shift assay in vitro [Yujuan Zheng, Jinghang Xie,2016]. Although the yield of AHR expression in Pichia pastoris is comparable to its expression in a baculovirus system, the expression was rather modest at best, making it more difficult to scale up the expression.

1.8 Hypothesis

Chan's lab has used ySMD1163 to express human AHR, but unfortunately only few amounts of colonies grew on the low concentration,100μg/ml Zeocin plate, and its AHR expression yield is very low. Because the AHR gene is carried on the plasmid-pPICZB (Figure1), this plasmid owns a gene fragment which can produce enzyme to degrade Zeocin to help the clone to grown on the Zeocin plate. According to the phenomenon, an assumption crossed our

mind, is that because of the low copy number of the interest gene? As we thought, if the target gene's copy number is very few, that means only few amount pPICZB bound to the yeast genome, so the clone can only produce little enzyme to resist antibiotics, that could be the reason why only few colonies grew on 100μg/ml Zeocin plate. If so, after increasing the copy number of hAHR introduced into yeast genome, it is possible to optimize the AHR expression. Due to the technique problems to perform electroporation with protease-deficient yeast, we try the WT yeast yJC100 to substitute for ySMD1163 for electroporation.

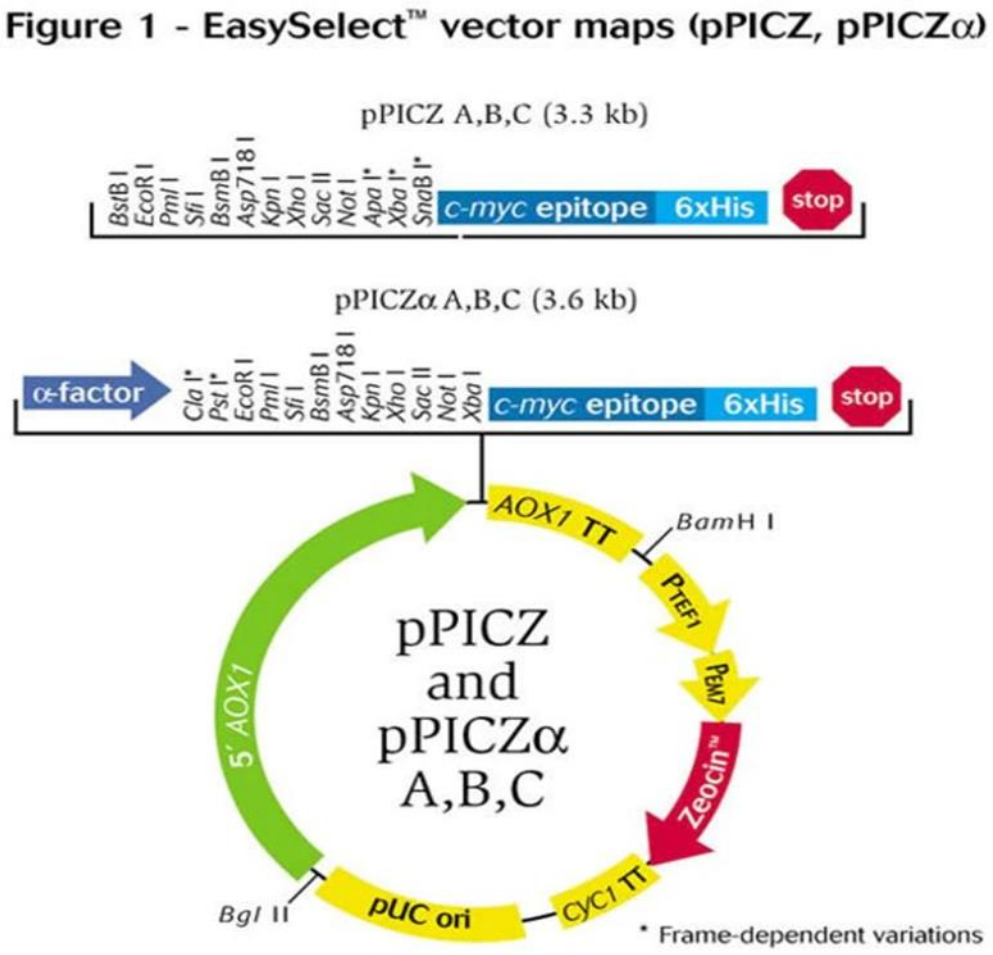

Figure 4. pPICZ vector sequence map

CHAPTER 2: OPTIMIZING THE AHR EXPRESSION IN *PICHIA PASTORIS* BY INCREASING THE COPY NUMBER OF AHR

2.1 Copy Number Section

Gene copy number is one of the most vital factors affecting the efficiency of the recombinant production in the yeast, it should be taken into consideration as an optimization steps of protein production [Roghayeh Shirvania et al,2020]. There are several methods developed in the past decades, including FISH, Southern Blot, and qPCR. Routinely, southern was performed for the copy number determination [Vassileva A, Chugh DA,2001] [Inan M, Aryasomayajula D,2006].

However, recently, real-time PCR has replaced as a popular method to detect gene quantity because it can be performed at high throughput, generates more accurate results, and has a larger dynamic range of detection; reaching to picograms level [Abad S, Kitz K, Hormann A,et al,2010] [Lovatt A.2002].

2.2 Material

All chemicals and ingredients are purchased to prepare bacterial and yeast media (LB, YPD, BMGY, BMMY) were purchased from Thermo Fisher Scientific (Hampton, NH) or Sigma Aldrich (St. Louis, MO). All oligonucleotides, Zeocin, otherwise specified, were purchased from Thermo Fisher Scientific (Hampton, NH). The Nanodrop is from Thermo Fisher Scientific (Hampton, NH). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Zyppy^{TM} Plasmid Miniprep Kit is from Zymo Research corporation. Anti-AHR SA210 rabbit IgG was purchased from Enzo Life Sciences (Farmingdale, NY). IRDye800 donkey secondary IgG was purchased from LI-COR (Lincoln, NE). The Gene Art codon optimized

human AHR sequence (GA-hAHR) were purchased from Life 34Technologies (Thermo Fisher Scientific, Grand Island NY). Cobalt agarose beads were purchased from Gold Biotechnology (St. Louis, MO). The Colony PCR master mix was purchased from Lucigen Corporation (Middleton WI). The pPICZB plasmid and the Pichia strains (ySMD1163 and yJC100) were gifts from Dr. Goeff Lin-Cereghino (University of the Pacific). Western blot analyses were performed using a LI-COR Odyssey imaging system (Lincoln, NE). Electroporation was performed using an Eppendorf 2510 electroporator (Edison, NJ). Cells pellets were homogenized using Bullet Blender Storm 24 (Troy, NY). The iTaq^{TM} SYBR Green Supermix is from BIO-RAD company.

2.3 Methods

2.3.1 Preparation of Plasmid

Our lab used plasmid -pPICZB carrying codon optimized human AHR to perform the electroporation. 23.67% of the nucleotides have been replaced to improve transcription (Figure 5), however, these changes did not alter the final amino acid sequences [Xie, Jinghang,2014].

After recovering the *E. coli* which stored the pPICZB-GA-hAHR from -80℃, we perform the plasmid extraction using the Zyppy^{TM} Plasmid Miniprep Kit, then we use restriction enzyme SacI-HF to linearize pPICZB-GA-hAHR overnight at the cut site: GAGCT/C to increase the efficiency of external DNA integration into the Pichia genome.

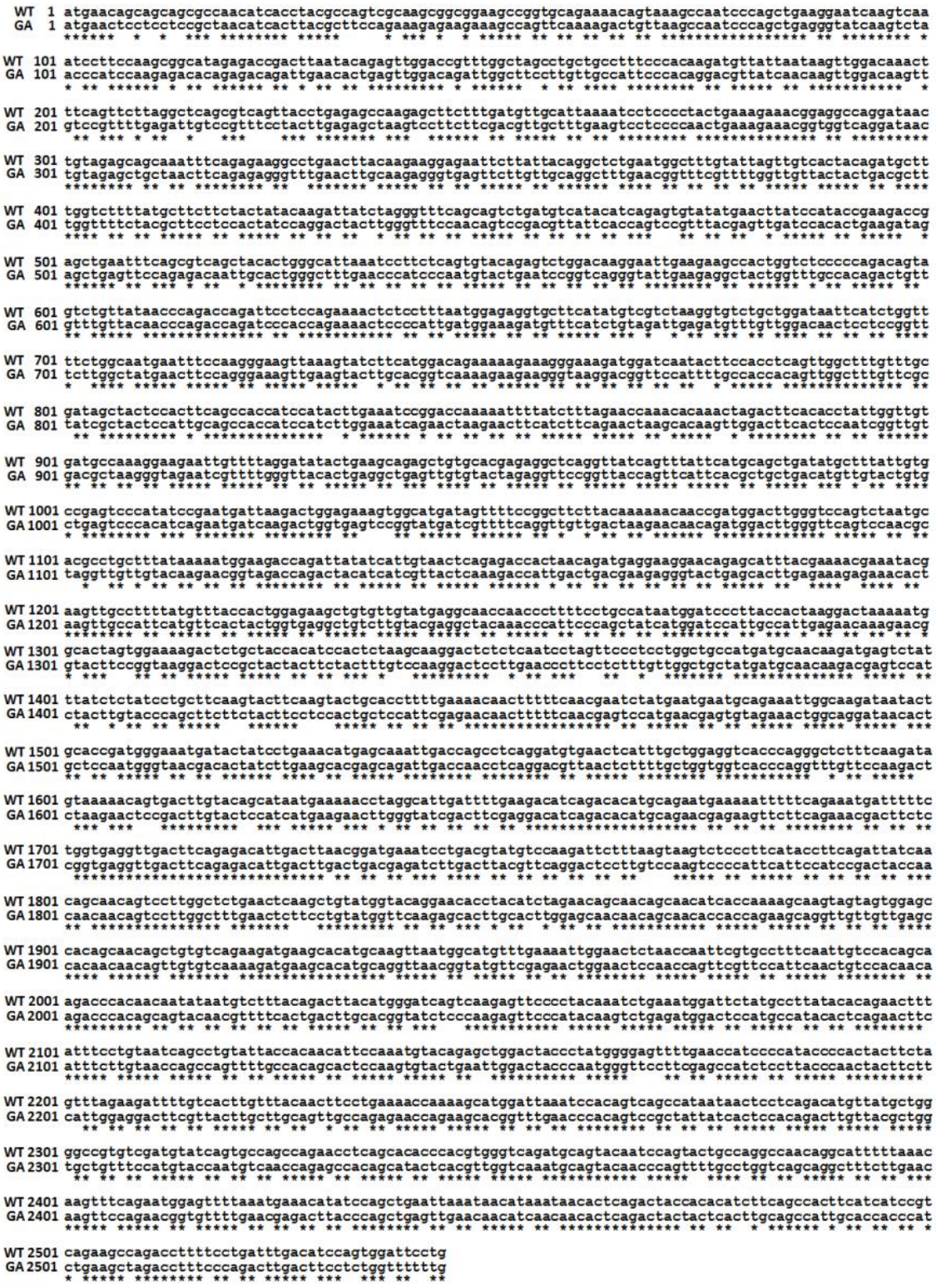

Figure 5. Human AHR wild type (WT) cDNA and gene art (GA) nucleotide sequence. Asterisks (*) represent identical nucleotide sequences between WT and GA

2.3.2 Preparation of Competent Cells

Competent cells are made by growing 500ml cells to desired OD value until it reaches around 0.8-1.2. Then centrifuge the cells for 10 min and add 100ml YPD/0.02M HEPES. Then we add 2.5mL 1.0M dithiothreitol (DTT) dropwise and incubate it for 15 min with shaking at 30℃. Add water up to 500mL and centrifuge cells for 10 min, then resuspend it in 500ml cold water. Centrifuge cells for 10 min and follow by resuspending in 500ml cold water. Centrifuge cells for 10 min. And resuspend in 20 mL 1.0M sorbitol(cold). Pellet the yeast by centrifugation step for 10 min. Finally, the yeast pellet was resuspended in 1mL 1.0 M sorbitol and aliquot into individual 1.5mL tubes. The tubes were placed in -80℃ freezer until needed (Note: the cells were aliquoted, use only once, avoid refreezing. All centrifugation steps were at 4000×g at 4℃) [Joan Lin-Cereghino, William W. Wong et al,2005]

2.3.3 Electroporation

After preparing the linear plasmid and competent cells, thaw the competent cells on ice for approximate 5 minutes. Then combine approximate 4 μl (50-200ng) of linearized plasmid DNA to 40ul of competent cells in a pre-chilled electroporation cuvette. Flick the cell and DNA mixture or tap the cuvette base on the bench top in order to get all components to the bottom of the cuvette with no bubbles present. Incubate the cuvette for 2 minutes on ice. (Transformation efficiency will decline if the cold cells are added to a room temperature cuvette. Also, the presence of bubbles may lead to arcing and cell death.) For my experiment, all electroporation step controls the plasmid concentration as 30ng/μl and the cells volume is 40μl. After wiping the outside of the cuvette to remove any condensation, electroporate samples using Eppendorf electroporator 2510, cuvette gap -2.0mm, charging voltage-1500V, resistance-200Ω, capacitance-25μF.

Immediately after electroporation, for antibiotic resistance markers (i.e Zeocin™ and G418 resistance), resuspend the samples in 0.5 ml 1 M sorbitol and 0.5ml YPD and transfer the mixture to a 1.5ml centrifuge tube. Allow the transformation reaction to recover in 30℃ shaker (225 rpm) for 1 hour and then plate aliquots on YPD medium containing the appropriate of antibiotic. (The hour recovery in YPD/sorbitol is essential to allow the transformed yeast cells to express antibiotic resistance proteins prior to being exposed to the selective agents on the plates. If this step is skipped or shortened, the number of transformants will be greatly decreased. Platting the transformation mixture on increasing concentration of antibiotic (i.e for Zeocin™ 100, 250, 500, or 1000μg/ml) can select for multicopy integrant are more likely to express higher levels of recombinant protein compared to strains containing only single copies. We do not include any sorbitol in the selection plates.) For my project, we use Zeocin as the antibiotics.

Incubate plates at 30°C for 2-3 days until colonies appear. For high Zeocin concentration plate (500 μg/ml,1mg/ml), wait longer until 10 days.

The transformed yeast colonies were selected and grown on another YPD plate containing 100μg/ml Zeocin. Colonies were then validated by colony PCR to confirm the presence of AHR in the genome.

2.3.4 Colony PCR

Pick a nice size amount of colony from your plate using the end of your pipette tip,500 μl of nuclease free water was inoculated with one single colony and vortex well. Heat cell suspension at 95℃ for 5 minutes to burst cells. The homogenized sample was used for PCR amplification in 20μl final volume (5μl cell suspension,10μl of 2×PCR master mix, 1 μl of 50μM OL898, 1 μl of 50μM OL899, 3μl nuclease free water). The reaction was 95 ℃ for 5min at first,

95℃ for 1 min, 53-60℃ for 1 min, 72 ℃ for 1-2 min (35 cycles),72℃ for 10min. The PCR product was analyzed on an agarose gel.

Table 2.
Colony PCR Primer Sets: OL898, OL899

OL898 5'-GAGGGTTTGAACTTGCAAGA-3'
OL899 5'-AGTCTCTGGAACTCAGCTCT-3'

2.3.5 Expression of AHR from *P. Pastoris*: Optimal Methanol-Inducible Time Determination

Inoculate 30 ml BMGY in a 250 ml flask with one yJC100-hAhR-GA transformant single colony, incubate at 29℃, 250rpm overnight. Determine OD_{600} value until the OD_{600} value reach 2-6, when the cells will be in log-phase growth, then pellet and wash culture at 1500g for 5 minutes with $BMM^{(-)}$ Y twice, and then dilute cells into 30 ml of BMMY medium to reach OD=1. After 0, 3, 6, 9, 12, 24h methanol induction, collect 1ml of culture at each time point, then centrifuge at 1,000 g for 5min at 4℃. Wash pellet with cold PBS then freeze pellet in -80 ℃. Add equal volume of ZrOB05 beads onto pellet and 5 times volume of cold lysate buffer (25 mM HEPES, pH7.4,10% glycerol, 0.3 M KCl, 100×Gold protease inhibitor cocktail GB1082). Break cell wall with Bullet Blender at speed 8 for 5 min at 4 ℃, then transfer crude lysate to a new tube then vortex well. Do BCA assay of all samples of crude lysate followed by western blot to determine the optimal induction time. The crude lysate also can be centrifugation at 14,000 g for 30 min at 4℃, followed by centrifugation at 14,000 g for 30 min at 4°C.The resulting supernatant, which has the Pichia expressed hAHR protein, was collected as the starting material for metal-affinity purification using cobalt agarose beads.

2.3.6 Preparation of the gDNA

Inoculate a colony in 3ml BGMY culture at 29℃, 250rpm overnight. Then centrifuge at 1500g for 5-10min at room temperature. Resuspend pellet in 350ul buffer 1, then add to Eppendorf tube for Bullet Blender. Add 20ul 10%SDS, 135ul 3M sodium acetate and 0.2ml ZrOB05 beads. Spin at speed "8" for 5min at 4℃. Spin at 16000g for 30min at 4℃. Transfer 0.4ml of supernatant into new tube. Perform ethanol (~0.8ml) precipitation at -20℃ for 15min, then spin at 16000g for 15min at 4℃. Resuspend pellet in 0.7ml TE buffer, do phenol/chloroform extraction (1:1) and spin the mixture at 16000g for 15min at RT. Take out top layer (~0.45ml) into new tube, add 45ul 3M sodium acetate, do ethanol (~1ml) precipitation at -20℃ for15min, and then spin at 16000g for 15min at 4℃. Finally, air dry the gDNA 30min or more. Resuspend pellet 20ul nuclease-free water for nanodrop to measure the concentration.

During the whole process, do not vortex the gDNA since it is very sensitive. And avoid repeated freezing and thawing, DNA can be aliquoted and stored at -20°C.

2.3.7 Real-time PCR Experiments

Real-time qPCR using SYBR Green, real-time qPCR amplification and analysis were performed using a BIO-RAD CFX Connect ™ Real-Time System instrument with software Bio-Rad CFX Manager version 3.1. The real-time qPCR mixture of 20 μl was prepared using the BIO-RAD iTaq™ SYBR Green Supermix, the reaction system contains: 2ul template DNA (15ng/μl); 2μl forward primer (OL900,400nM); 2μl reverse primer (OL901,400nM), 10μl sybergreen,4μl nuclease-free water.

The thermal cycling protocol was as follows: initial denaturation for 30s at 95 ℃, followed by 40 cycles of PCR at denaturation at 90 ℃ for 10 s, 1 min at 60 ℃ and 5 s at 65 ℃. The fluorescence signal was measured at the end of each extension step at 65 ℃. Each sample

was amplified in triplicate. Specificity of PCR reactions was checked by post-amplification melting curve analysis.

2.3.8 Standard Curve Generation for AHR Copy Number Determination

The standard method to calculate the copy number of the interest gene is building the standard curve using only one copy number target gene (hAHR) template and read off the copies of the sample by Ct value. Unfortunately, we don't have the one copy number human AHR genome, we try to figure out if we can use actin genome to substitute it to build the standard curve. A 10-fold serial dilution series of the yJC100 recombinant cell genome, was used to construct the curve for both actin gene and AHR gene, the concentration of the genome was measured by the nanodrop, and the Ct values in each dilution were measured in duplicate using a real-time qPCR with the hAHR (OL900,901) and actin (OL902,903) primers to generate the curves, respectively. [Table 3]

Table 3.
Human AHR Primers and Actin Primers for Real-Time qPCR Reaction

Human AHR	OL900 5'-CCAGAGACAATTGCACTGGGC-3'
	OL901 5'-GGTGGCTGCAATGGAGTAGCGA-3'
Actin	OL902 5'-CCTGAGGCTTTGTTCCACCCATCT-3'
	OL903 5'-GGAACATAGTAGTACCACCGGACATAACGA-3'

The thermal cycling protocol was as follows: initial denaturation for 30s at 95 ℃, followed by 40 cycles of 10 s at 90 ℃, 1 min at 60 ℃ and 5 s at 65 ℃. The fluorescence signal was measured at the end of each extension step at 65 ℃. The Ct values were plotted against the logarithm of their initial template amount. Each curve was generated by a linear regression of

the plotted points. From the slope of each curve, PCR amplification efficiency I was calculated according to the equation:

$E = 10^{-1/slope} - 1$[Roghayeh Shirvani, Mohammad Barshan-tashnizib et al, 2020]

By comparing two primer set's efficiency, we can compare the similarity and substitutability of the two different curves. To calculate the copy number of the sample's genomic AHR DNA, we can convert the curve of the actin to a standard curve by converting the Y-axis value from Log(amount) to Log(copies), copies of the template is calculated by the equation: DNA (copy)=$6.02x10^{23}$(copy/mol) ×DNA amount(g)/DNA length(dp)×660(g/mol/dp) [Changsoo Lee, Jaai Kim et al,2006]. The DNA length of *P. pastoris* genome is 9.4Mb.

According to the Ct value, the corresponding copies can be read off from the standard curve. The normalized copy number calculated by absolute quantification is given by the following equation:

Copy number $_{\text{Target gene}}$=Copy quantity $_{\text{Target gene}}$/Copy quantity $_{\text{Reference gene}}$ [Sandra Abad, Kerstin Kitz, et al, 2010]

For my project, the reference gene is actin. As a haploid yeast, *P. pastoris* (yJC100 and ySMD1163) only own one copy number of actin.

2.3.9 Purification of hAHR Protein in *P. pastoris*

To purify AHR proteins, after 1 mL of cobalt resin column was preconditioned by water and lysis buffer, 3 ml Pichia cell lysate supernatant, were poured onto the 1 mL cobalt resin column at 4°C. The Pichia cell lysates were passed through the column 10 times.

The column was washed with 5 mL lysis buffer (25 mM HEPES, pH 7.4, 10% glycerol, 0.3M KCl) for hAHR purifications and the first wash was collected as W1. To elute the protein that was bound to the resin, 1.5 mL of lysis buffer containing 500 mM imidazole (Elution

buffer:25 mM HEPES, pH 7.4, 10% glycerol, 0.3M KCl,0.5M imidazole) was applied onto the column and the eluate was collected as E1, this process was repeat three times and we collect E1, E2, E3. The majority of was captured in the first 1.5 mL of the eluates and went through dialysis to obtain *Pichia pastoris* hAHR protein exists in the elution buffer. *Coomassie* Blue staining and Western blot analysis of 12% SDS-PAGE were used to determine the presence and purity of the purified proteins.

2.3.10 Western Blot Analysis

The general Western blotting protocol using a near-infrared detection method was described previously with slight modification [38,39]. Briefly, samples were mixed with SDS-PAGE electrophoresis sample treatment buffer, loaded, and ran on a 12% SDS-PAGE gel to separate the proteins based on size. Proteins on the gel were transferred to a nitrocellulose membrane at 300mA for 120 mins at 4℃, followed by a blocking step incubation in a blocking buffer containing 5% BSA, 0.5% NaN3 and 0.1% Tween 20 at room temperature for 1 h. Subsequently the membranes were treated with primary and secondary antibodies. Anti-AHR SA210 (1:5,000) was used as primary antibodies diluted in blocking buffer and incubated overnight at 4°C. Secondary antibody incubation (LI-COR IRDye 680 donkey anti-rabbit IgG, 1:10000) was performed in the blocking buffer for 2 h at room temperature. A LI-COR Odyssey imaging system was used for detection.

2.4 Results

2.4.1Electroporation

Below, I list the timeline of the whole electroporation experiment (Figure 6), the electroporation was performed from spreading the culture on the lower Zeocin concentration 100μg/ml to the highest concentration 1mg/ml YPD plate, to avoide wasting Zeocin reagent.

The positive control (yJC100-pPICZB) and samples (yJC100-pPICZB-hAHR-GA) electroporation were performed at the same time, the reason why I list this timeline is because the competent cells might be sensitive to times stored in -80℃.

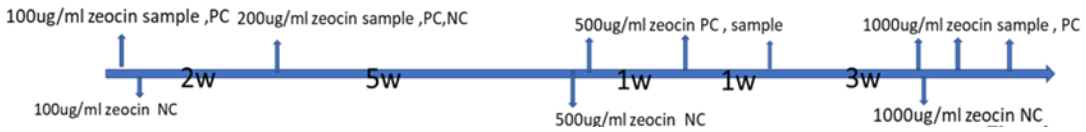

Figure 6. Electroporation experiment timeline
NC: negative control; PC: positive control; w: week

Obviously, there is nothing shows in the negative control (Table 4), which matches the expectation. For the samples, the colonies' amount will dramatically decrease comparing with the increasement of the concentration of Zeocin. For 500μg/ml Zeocin plate, because the clones are very hard to exist, so we repeat the electroporation for several times to get enough clones. The situation is that, sometimes, nothing appears, sometimes, one or two clones appear on the plate, after several times, we got total three clones. For the highest Zeocin concentration, 1mg/ml Zeocin plate, there was nothing appear.

Comparing the positive control and the sample, the clones' amount of the sample is less than the positive control at each different Zeocin concentration. Which suggests that the human AHR gene may inhibit the growth of yeast on the Zeocin plate.

Table 4.
Electroporation Result: Quantity of the Positive Control, Negative Control and Samples on 100μg/ml,200μg/ml,500μg/ml,1mg/ml Zeocin YPD Plate

Zeocin concentration	100ug/ml	200ug/ml	500ug/ml	1000ug/ml
Positive control: pPICZB in yJC100	+++(168)	++(32)	0-2	0
Negative control: No plasmid in yJC100	0	0	0	0
Sample: pPICZB-GA-hAHR in yJC100	++(51)	+(28)	0-3	0

2.4.2 Optimal Methanol-Inducible Time Determination

Through Western blotting result (Figure 7), we find about 9h induction, the normalized protein expression level is the highest. So, we choose 9h as the optimal induction time point for the downstream experiment. This experiment was repeated three times and has same result.

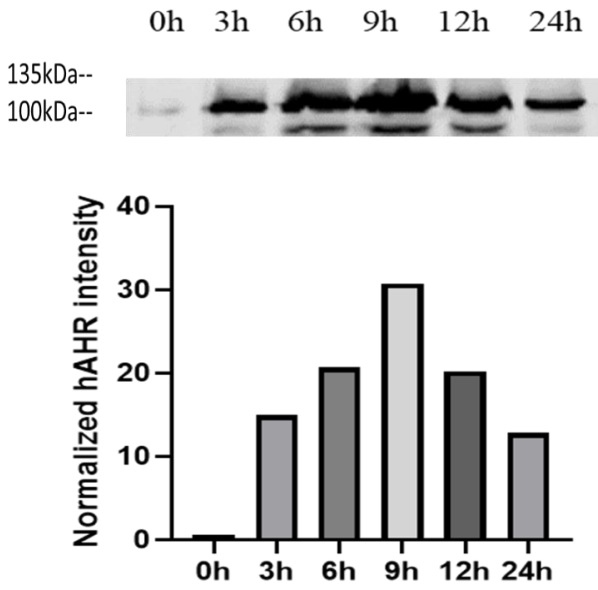

Figure 7. Time dependent expression determination. Western blot (Top panel) showing the time dependent expression of huAhR-GA. 50 µg of huAhR-GA crude lysate at each time point was loaded. Lower panel is the representative of western blot.

2.4.3 Copy Number Calculation

For the below two curves (Figure 8), I did 10 times genome dilution to build the curve. These two curves are built to compare the efficiency of the two sets of primers for actin and hAHR. By comparing the two different sets efficiencies, we can define how similar they are. Actin's efficiency is 65.28%, hAHR is 77.63%, since they are quite similar, we can use the actin curve to substitute.

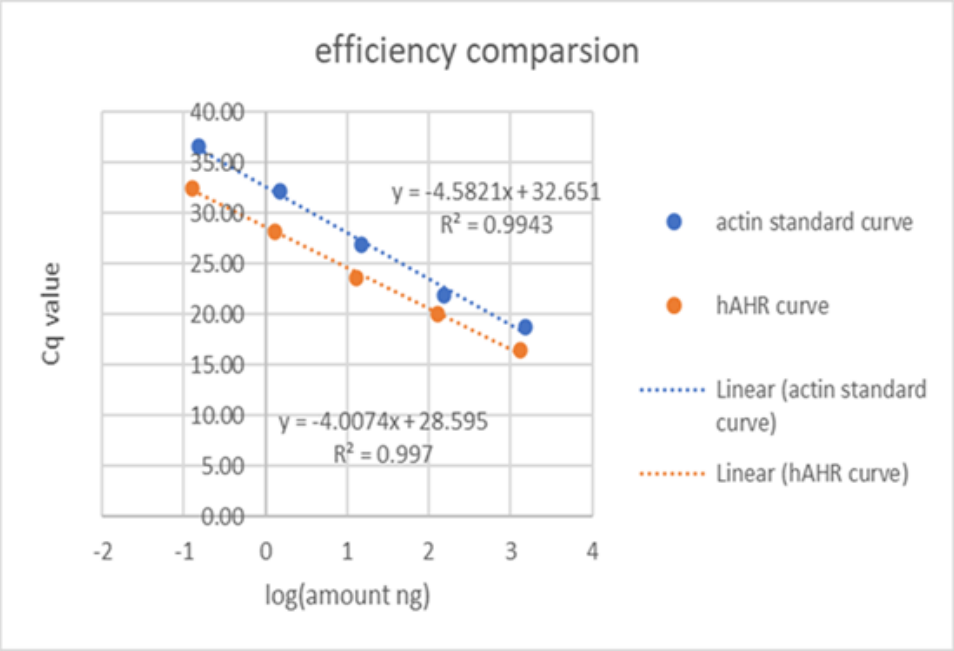

Figure 8. Human AHR and actin curves' efficiency comparison, the Y-axis is Cq value, the X axis is Log (amount of genome); e.g., 15ng yeast genome DNA, actin, x axis: log(15ng) =1.176, y axis:(26.39+27.01+27.22)/3=26.87; each point is repeated three times. For actin efficiency calculation, E(actin)=10^ (1/4.5821)-1=65.28%

Then we plot the standard curve of actin by changing the x axis from DNA amount to copies using the equation we introduced before (Figure 9). For example, for 15ng genome, the 15ng genome log(copies) value is 6.16. For 9 different clones I picked from different Zeocin concentrations, according to the Cq value of each sample, we can read off the log(copies) from the standard curve, since each sample genomic amount we applied to qPCR is 30ng, we can calculate the copy number of them by dividing the copies of hAHR by copies of actin. I list the copy number of 9 different samples at the below table (Table 5). The highest copy number's clone is 200(2), its copy number is 15.68. As we previously assumed, the highest copy number will appear on the highest concentration Zeocin 500μg/ml plate, but it was on the 200μg/ml Zeocin plate.

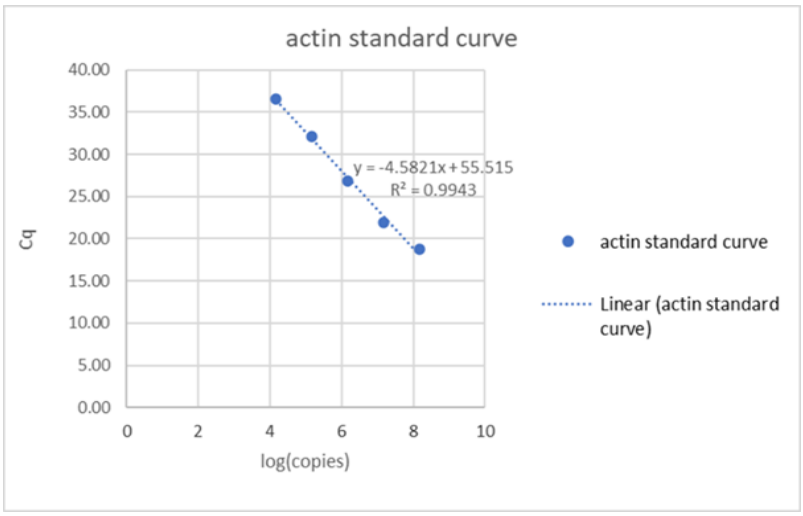

Figure 9. Actin standard curve, Y axis is Cq value, X axis is Log (copies);
e.g. (1) standard curve:15ng yeast genome converts to copies: log (copies of actin) =log(amount*6.022*10^23/length*10^9*660) =log (15*6.022*10^23/9.4*10^6*10^9*660) =6.16; (2) sample:100(1) ,30ng genome are applied to qPCR experiment, Cq value=23.8 Log (Copy number) =log (copy quantity of target gene/copy quantity of reference gene) =log (copies of Cq=23.8)-log (copies of 30ng actin) = (55.515-23.8)/4.5821-6.4708 =0.449; Copy number=2.82

Table 5.
Cq Value and Copy Number of 9 Different Clones.

Clone	100(1)	100(2)	100(3)	200(1)	200(2)	200(3)	500(1)	500(2)	500(3)
Cq value	23.80	22.25	23.5	20.63	20.38	22.94	20.45	23.12	24.09
Copy number	2.82	6.14	3.25	13.85	15.68	4.34	15.21	3.97	2.43

The qPCR of each sample repeated 3 times and the Cq value is the average value.100 μg/ml Zeocin YPD plate grown colonies:100(1),100(2),100(3); 200μg/ml Zeocin YPD plate grown colonies:200(1),200(2),200(3); 500μg/ml Zeocin YPD plate grown colonies:500(1),500(2),500(3).

Then we express total 9 clones and generate the culture of them after 9h induction. By performing Western blotting using the crude lysate, we can compare the protein expression difference from each clone. The below figure, we relate the copy number and the protein

expression of each clone (Figure 10), the result shows the 200(2) will have the highest protein expression and it also is the one has the highest copy number. There is a trend the expression level will increase with the increasement of the copy number, this conclusion basically verified our hypothesis. All this experiment were repeated three times.

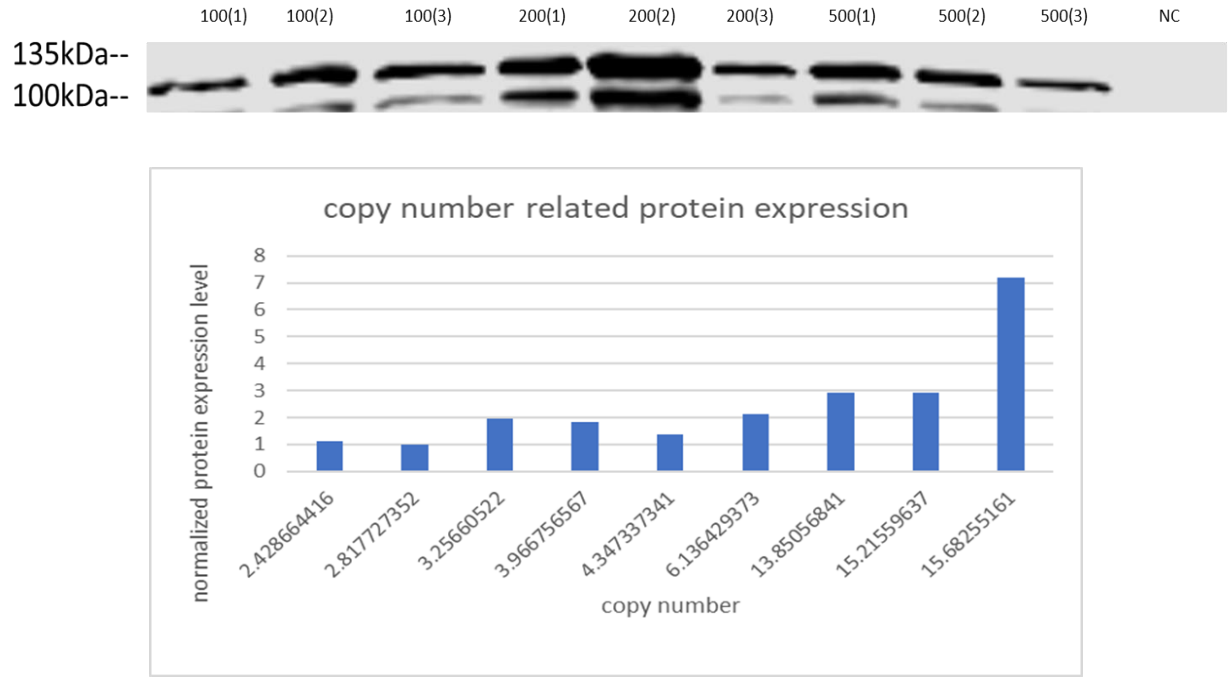

Figure 10. Nine clones AHR protein expression comparison. Above western blotting result, each lane we load 30μg, shows 9 clones AHR expression difference after 9h induction by methanol.NC: negative control, pPICZB in yJC100; Below figure shows the copy number related AHR expression level.

2.4.4 ySMD1163 Copy Number Calculation and AHR Expression Comparison

We use the same method introduced before to calculate the copy number of the pPICZB-GA-hAHR-ySMD1163.According to the Cq value=23.67 (repeated three times, mean value), the copy number of ySMD1163 should be 3. The low copy number might be the reason why the ySMD1163 can't grow on higher Zeocin plate, which match my hypothesis. The culture of

ySMD1163 was generated after 9h methanol induction [Yujuan Zheng, Jinghang Xie et al,2016], followed by Western blotting to compare the difference between the ySMD1163 and yJC100 200(2) clone (Figure 11), the result shows 200(2) expression level is about double amount of the ySMD1163. Since yJC100 200(2) have higher hAHR expression, we can try to figure out if we can purify more hAHR from this clone.

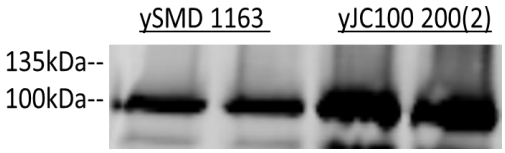

Figure 11.GA-hAHR-ySMD1163 and yJC100 200(2) AHR expression comparison. Each lane we load 30μg crude lysate. ySMD1163:yJC100=1: 2.28

2.4.5 Copy Number =15.21 yJC100 AHR Protein Purification

The whole Western blot experiments before this step, we load the crude lysis of the yeast, the lysate contains both the pellet and supernatant, however, what is useful is hAHR from the supernatant since only the correctly folded AHR are functional, which is dissolved in the eluent. And for my project, what I try to harvest is the purified hAHR since the supernatant also contains a lot of other proteins. The Pichia expressed GA-hAHR protein was detectable by Western blot but not by Coomassie blue staining (Figure 12)

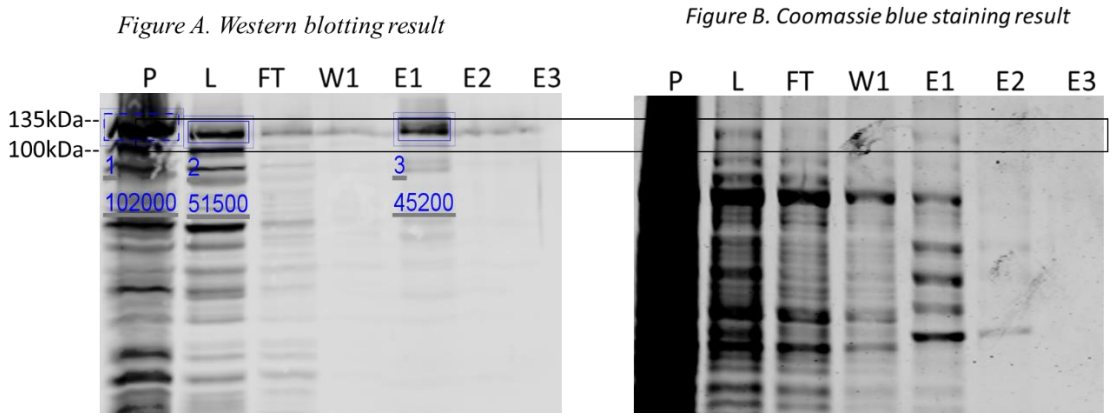

Figure 12. Characterization of human AHR expression in copy number =15.21 yJC100 by Western blot analysis and Coomassie staining. Analysis of Pichia fractions from metal-affinity purification. Arrows indicate GA-hAHR. Left side is the image from Western blot, 15µL samples were loaded per lane. Right side is the image from *Coomassie* staining, 15µL samples were loaded per lane. L, loading sample; FT, flow through sample; W, wash; E1-3, eluted fractions 1-3. P: L: E1 =1.98: 1.14: 1. This experiment was repeated one time with similar result.

2.4.6 Copy Number =15.68 yJC100 AHR Protein Purification

For the copy number=15.68 yJC100 AHR purification, it shows similar result, AHR was successfully purified according to western blot result, but it is not enough to be detected by *Coomassie* blue staining.

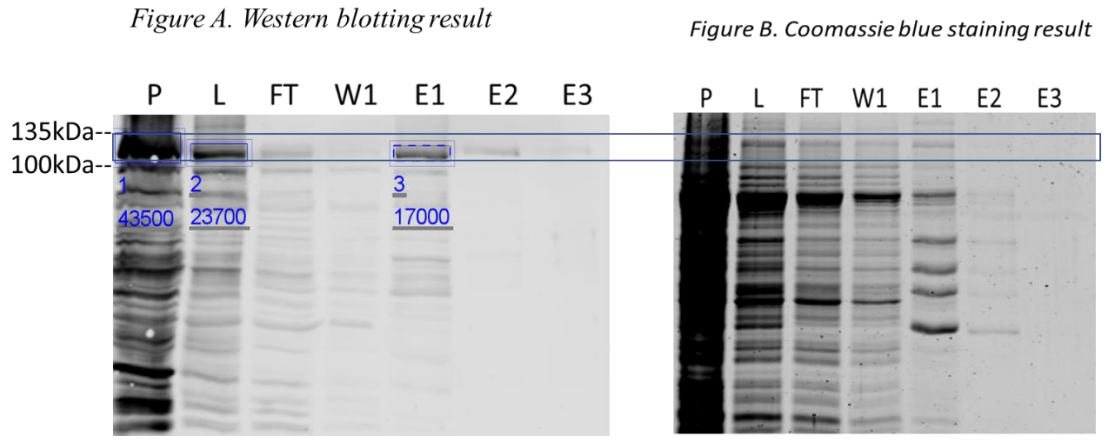

Figure 13. Characterization of human AHR expression in copy number =15.68 yJC100 by Western blot analysis and Coomassie staining. Left side is the image from Western blot, 15µL

(Figure 13 Continued)
samples were loaded per lane. Right side is the image from Coomassie staining, 15µL samples were loaded per lane. L, loading sample; FT, flow through sample; W, wash; E1-3, eluted fractions 1-3. P: L: E1 =2.5 :1.39 :1. This experiment was repeated one time with similar result.

2.4.7 Copy Number=3 ySMD1163 AHR Protein Purification

For the copy number=3 ySMD1163 AHR purification, it shows similar result, AHR was successfully purified according to western blot result, but it is not enough to be detected by Coomassie blue staining.

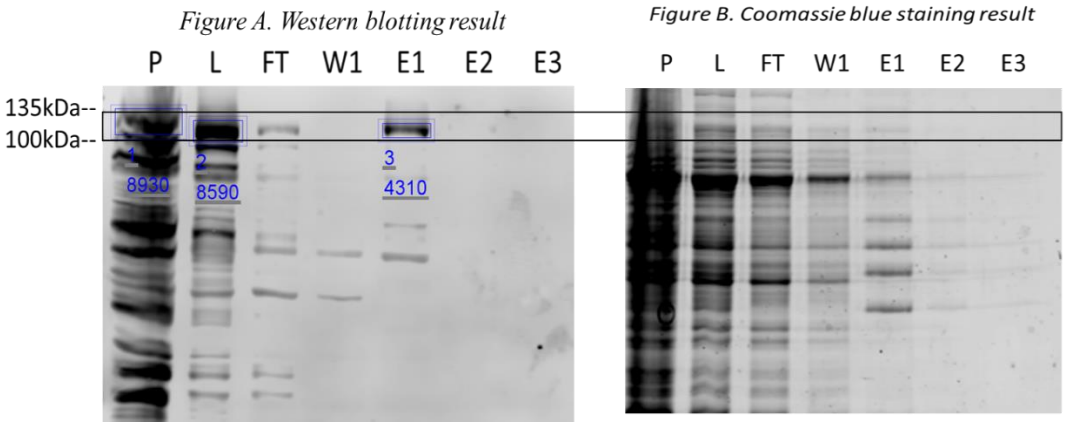

Figure 14. Characterization of human AHR expression in copy number =3 ySMD1163 by Western blot analysis and Coomassie staining. Left side is the image from Western blot, 15µL samples were loaded per lane. Right side is the image from Coomassie staining, 15µL samples were loaded per lane. L, loading sample; FT, flow through sample; W, wash; E1-3, eluted fractions 1-3. P: L: E1 = 2.07:1.99:1. This experiment was repeated one time with similar result.

2.4.8 Eluent 1 Comparison

According to previous AHR crude lysate Western blot comparison result, copy number= 15.68 yJC100 AHR expression should be about double amount than the copy number=15.21yJC100 and the copy number=3 ySMD1163.Since eluent 1 is the final product for downstream research, we try to compare them to see if the ratio between three yeast strain keeps

similar. The western blot result shows even the copy number=15.68 yJC100 still has the highest AHR expression, but it is not very different from the other two yeasts. (Figure 15) Compare with the crude lysate result, the obvious difference is that the crude lysate lane contains more miscellaneous bands, perhaps due to the nonspecific binding of primary antibody.

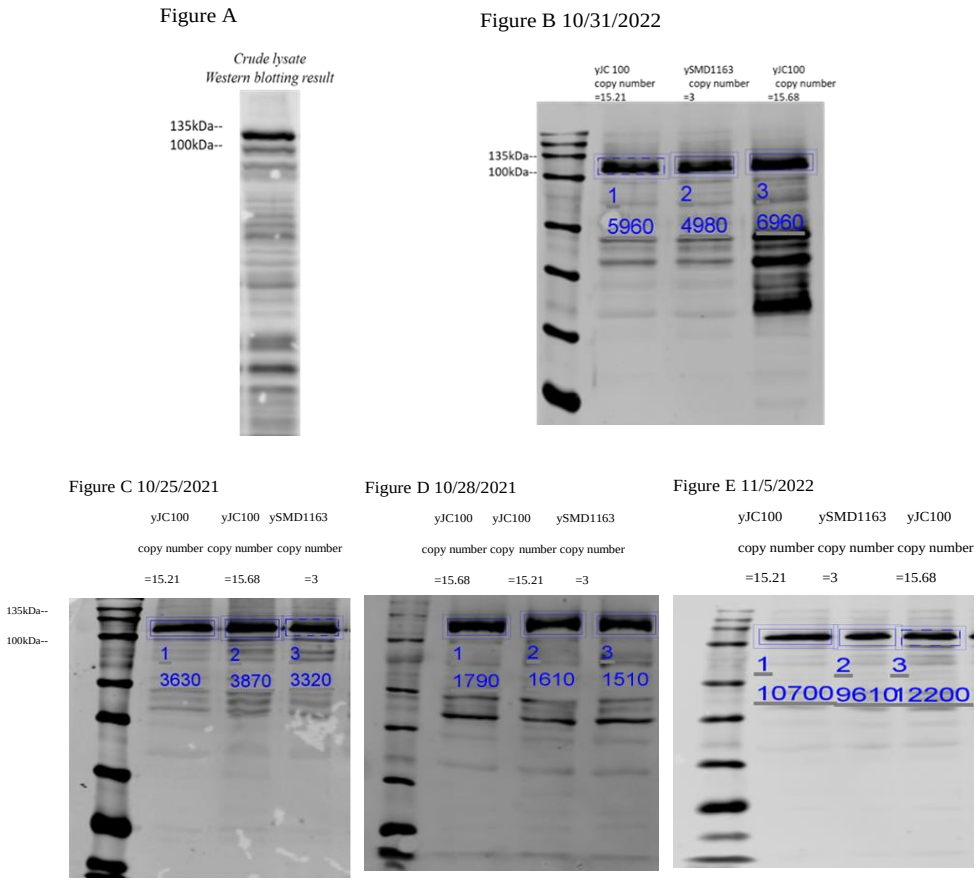

Figure 15. Eluent1 AHR expression comparison through western blot.
Figure A shows the crude lysate western blotting result, Figure B-E is the E1 comparison by western blotting.

2.4.9 Loading Comparison

Since the E1 comparison result isn't ideal, to figure out what happened, we try to compare three different loading samples (same thing as supernatant).

Western blot result shows three yeast strain supernatants' AHR expression signals are quite similar too.

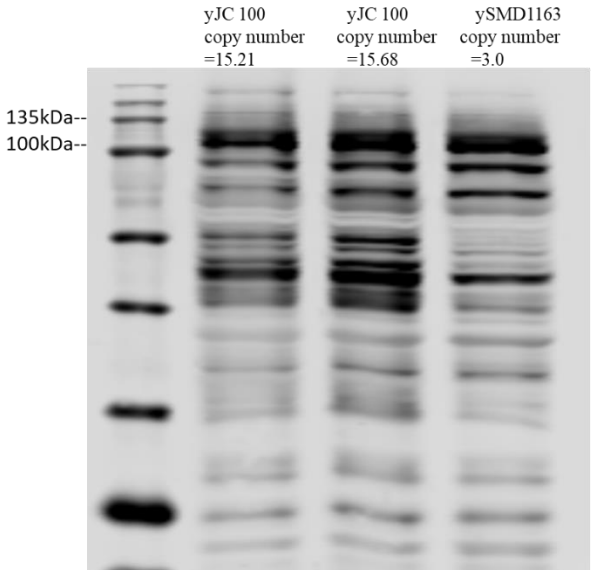

Figure 16. Supernatant AHR expression comparison through western blot. The signaling level between three different strains is yJC100 Copy number=15.21: yJC100 copy number=15.68: ySMD1163 copy number=3=1: 1.21: 1.28.

CHAPTER 3: DISCUSSION AND CONCLUSION

3.1 Discussion

Since AHR is not well solubilized in the supernatant, even with more copy numbers, no more AHR was produced in the supernatant from P. pastoris. This means that most of the AHR is insoluble in the buffer and exists in the pellet. In order to better verify this conjecture, we can compare the three yeast pellets (yJC100 Copy number=15.21: ySMD1163 copy number=3: yJC100 copy number=15.68) by Western blot.

For the E1 comparison Figure B (Figure 15), there are obvious miscellaneous bands under the AHR band in the copy number=15.68 yJC100 E1 lane, at first, we thought it may be the degradation of AHR from yJC100, but since we purified AHR from copy number =15.21 yJC100 strain at the same time, in other word, it should be degraded as well. However, the results conflicted. The experiment was repeated many times and the reason why the miscellaneous bands appeared is still unclear.

In Figure 10, for the copy number=15.21and 15.68 yJC100 strain, even their copy number are very similar, but AHR expression level differ a lot, copy number=15.68 yeast express about double amount of AHR in the crude lysate than copy number=15.21 strain. As expected, they should be same, the reason may due to the inaccuracy of calculation method using actin as template to establish standard curve.

3.2 Conclusion

For all the experiments, we draw two conclusions:

1.hAHR can express more in the crude lysate when the copy number increases. Besides, low copy number may result in low resistance to antibiotic (e.g ySMD1163).

2.Even AHR can express more in the crude lysate, it is not better soluble in the supernatant and eluent than others. Most of AHR exists in the pellet, making it difficult to scale up the production.

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APPENDIX A: RECIPES

1.YPD (Yeast extract Peptone Dextrose) plate: 1% yeast extract, 2% peptone, 2% dextrose, 2% Agar.
2.YPD liquid media: 1% yeast extract, 2% peptone, 2% dextrose
3.BMGY (Buffered Glycerol complex Medium): 1% yeast extract, 2% peptone, 10% 1 M potassium phosphate buffer at pH6.0, 1.34% YNB (Yeast Nitrogen Base), 4×10-5 % biotin, 1% glycerol.
4.BMMY is the same as BMGY except the replacement of 1% glycerol with 0.5% methanol
5.Lower Tris Buffer (1.5 M Tris):181.7 g Tris in 800 mL H2O. Adjust pH to 8.8 and qs 1000 mL.
6.Upper Tris Buffer (0.5 M Tris):30.3 g Tris base in 400 mL H2O. Adjust pH to 6.8 and qs 500 mL.
7.pH 6.8 Bis-Tris buffer (1.4 mol/L Bis-Tris): Bis-Tris base 29.9 g in 50 mL water. Then add 5 mL concentrated HCl (12M) and q.s. to 100 mL (no need to adjust pH). Keep at 4 ℃ in the dark.
8.10 mM ZnCl2 Solution:14 mg in 10 mL water. Filter to remove impurities. Prepare just before use.
9.10% AP:1 g ammonium persulfate in 10 mL dH2O. Aliquot to 0.5 mL fractions and stored at -20 ℃. After taken out, do not put back (avoid freeze-thaw). Use within two weeks.
10.Acrylamide/Bisacrylamide 30/0.8 (30% acrylamide/ 0.8% Bisacrylamide):
120 g acrylamide + 3.2 g Bisacrylamide, qs 400 mL (dissolve in ~200 mL H2O then qs 400 mL)
Caution: only use special spatula (only for acrylamide and Bis); weight in hood; wear gloves, mask, and lab coat.
11.HEDG Buffer:25 mM HEPES (5.96 g) + 1 mM EDTA (2 mL of 0.5 M EDTA) + 1 mM DTT (154.25 mg) + 10% glycerol (100 mL), qs with water to 1 L. Adjust pH to 7.4.

APPENDIX B: PROTOCOLS

1.SDS-PAGE Gel

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most used protein expression analysis techniques in polyacrylamide gel electrophoresis. The principle of this technique is to separate the protein in the electrophoretic gel according to the different molecular weight sizes of the proteins in the specimen. The percentage of gels depends on the size of target proteins. Usually, smaller size proteins run higher percentage gels. Below are lists of stacking and separating gels used in our lab. (Table 6)

Table 6.
SDS-PAGE Gel Recipe for 2 Gels

Separating Gel				Stacking Gel	
acrylamide percentage	10%	12%	15%		
Water	8.2 ml	6.9ml	4.8ml	Water	3.4ml
acrylamide/bis-acrylamide (30/0.8)	6.7 ml	8.0ml	10.1 ml	acrylamide/bis-acrylamide (30/0.8)	0.575ml
1.5 M Tris Buffer pH 8.8	5 ml	5 ml	5 ml	0.5 M Tris Buffer pH 6.8	1.25ml
10 % ammonium persulfate (AP)	100ul	100ul	100ul	10 % ammonium persulfate (AP)	50ul
TEMED	10ul	10ul	10ul	TEMED	5ul

2.Bicinchoninic Acid Protein Assay (BCA)

Use 96-well plates for BSA standards and samples. Do duplicates for each standard or sample. The first two column are for standards with concentration ranging from 0 to 1.0 mg/mL BSA as shown in Table 7. The recipe for each concentration is shown in Row1 well content of the table. The volume for each standard or sample is 10 μL. For samples wells, try to dilute the samples to make a final concentration in the range of 0.4-0.7 mg/mL. I usually do 1:10 dilution. After adding all the standards and samples, add 190 μL BCA reagent mix to each well using multichannel pipette and mix well, making a total volume of 200 μL. BCA reagent mix is composed of 98% BCA reagent A and 2% BCA reagent B (by volume). Incubate the plate at 37 ℃ for 30min and determine the absorbance at 562 nm using Epoch plate reader. Calculate protein concentration based on standard curve and absorbance.

Table 7.
BCA Assay 96-Well Plate Standards Layout.

Concentration (mg/mL)	Column	Row 1 well content		
		BSA dilution (μL)	HEDG buffer (μL)	
0	A	0	10	1:100 BSA dilution
0.05	B	5	5	
0.1	C	10	0	
0.2	D	2	8	1:10 BSA dilution
0.3	E	3	7	
0.5	F	5	5	
0.7	G	7	3	
1	H	10	0	

Note. 1:10 BSA dilution=1:10 dilution of 10mg/mL BSA stock solution. 1:100 BSA dilution=1:10 dilution of 1:10 BSA dilution.

3.SDS-PAGE Gel Electrophoresis

General Procedure:

(1) Prepare running buffer.
- a. Running buffer = TGS buffer (contains Tris pH 8.3, Glycine, and SDS)
- b. In Chan's lab, the running buffer is prepared by diluting TGS buffer to 1: 10 (In a total volume of 350 ul running buffer, dilute 35 ul of TGS buffer into 315 ul of dH2O).

(2) Make all the lysates to the same protein loading amounts (20 ug – 50 ug).
- a. Since you already obtained the protein conc. In each lysate, the volume of each lysate = the amount of protein you want to load / the protein conc. Of that lysate.
- b. For example: If you are going to load 20 ug of the protein to SDS-PAGE gel, the volume of lysate you need for running SDS-PAGE is 20 ug/actual conc (ug/ul) of the lysate.

(3) Prepare loading (treatment) buffer.
- a. Loading buffer should contain SDS (detergent), glycerol (density), Bromophenol blue (tracking dye), Beta-Mercaptoethanol (BME) (reducing agent), and Tris-HCl (salt, prevent unnecessary ionic interaction between proteins) pH 6.8.
- b. In Chan's Lab, the loading buffer is prepared by adding 950 ul of 1xTreatment buffer to 50 ul of BME.

(4) Add loading buffer to each lysate.
- a. Volume of loading buffer added to a lysate = Volume of that lysate (1:1 ratio)

(5) Prepare the marker (two ways).
- a. Dilute marker in the loading buffer (2 ul of the marker + a median number of the volumes of loading buffer to the lysates)
- b. Or, directly prepare a 3.5 – 5 ul marker without any dilution

(6) Heat the lysates at 95 deg. Celsius for 1.5 mins to denature the proteins.

(7) Cool the lysates and centrifuge to remove the steam.

(8) Place a gel into an electrophoresis unit and fill the low and upper tanks of the unit with running buffer.

(9) Remove the comb and add a spacer on top of the gel.
(10) Using fine tips, load the marker into the wells in the gel. Then, load the lysates into the wells in the gel.
(11) Remove the spacer and close the lid. Connect the electrophoresis unit to a power source.
(12) Apply appropriate voltages.
a.100 volts in stacking gel (100 volts 15 mins)
b.200 volts in running gel (200 volts ~30mins)
c. Bubbles appearing indicate the current is flowing.
(13) Turn the power off once the dye front reached the bottom of the gel (30 – 60 mins).
(14) Remove the gel cassette from the electrophoresis unit, then separate the gel from the cassette.
(15) Gel is ready for western blot transfer.

4.Gel Transfer for Western Blotting

General Procedure:
(1) Prepare transfer buffer (contains Tris pH 8.3, glycine, and 20% methanol).
(2) Prewet the filter paper and nitrocellulose membrane in a container with transfer buffer.
(3) Cover the container with a plastic wrap to prevent the vaporization of methanol.
(4) Assemble the transfer "sandwich" in the transfer buffer.
a. Order of the sandwich: black cassette, sponge, 2 layers of filter paper, gel, nitrocellulose membrane, 2 layers of filter paper, sponge, clear cassette.
b. Remove the bubbles between each layer.
(5) Insert the "sandwich" into the electrode module.
a. Make sure to place the black side of the cassette facing the black side of the module, and the clamp of the cassette is facing up.
6. Place the electrode module into the electrophoresis chamber. Add a frozen cooling unit and fill the chamber with transfer buffer until the sandwich is submerged.
(7) Connect the electrical leads to the power source.
a.300 mA for 120 mins at 4 deg. Celsius (in cold room).
8.Turn the power off and disassemble the sandwich to remove the membrane.
(9) Cut any unnecessary edges of the membrane.
(10) Place the membrane in 20 ml of blocking buffer for 1 hour with shaking at room temp.
a. Blocking buffer contains PBS solution, Sodium azide, Bovine serum albumin (BSA)
(11) Dilute primary and secondary antibody in blocking buffer if the antibodies are not prepared in the fridge.
a. Dilute primary antibody to 1:2000 or 1:4000, depending on the type of primary antibody you use (eg. To make a 1:2000 dilution, add 5 ul of primary antibody to 10 ml of blocking buffer).
(12) Incubate the membrane with primary antibody overnight with gentle shaking at 4 deg. Celsius in the cold room.
(13) Wash the membrane in PBST x 5, with 5 mins shaking each time.
a. PBST = 100 ml 10xPBS + 890 ml dH2O + 10 ml Tween-20
(14) Incubate the membrane with secondary antibody at room temp. for 2 hours.
(15) Wash the membrane in PBST x 5 with 5 mins shaking each time.
(16) Wash the membrane in 1xPBS with 5 mins shaking. (To reduce the background intensity, to remove Tween20)

(17) Dry the membrane in folded paper towels for 30 mins. (Dry membrane will give a stronger signal for visualization)
(18) Visualize the protein signals on the membrane using LI-COR x Odyssey system.
(19) Use foils to seal the membrane for storage.

5.Agrose Gel Preparation

Agarose gels are usually prepared using a weight/volume solution in the 0.5-2% range, which should be optimized for the size of DNA fragments being analyzed. Optimal percentage of agarose gels will result in best separation and resolution of bands (DNA fragments). (Figure 8)

(7) Weigh out the agarose and add it to the flask/beaker containing the TBE buffer. For example, for a 1% agarose gel, add 1 g agarose to 100 ml buffer. Allow the agarose to sit in solution for a few minutes before swirling the flask/beaker and suspending it in the solution. Higher percentage gels (> 1.5%) should hydrate for longer than lower percentage gels. Use a stir bar and stirring plate to rapidly mix the solution.

Note: Remember to remove the stir bar before microwaving.
(2) Cover the mouth of the flask/beaker with plastic wrap and make a small hole in the top to allow the solution to vent.
(3) Heat the flask/beaker in the microwave. After 30 seconds, remove and swirl to mix well. Repeat every 30 seconds until all the agarose has dissolved.
Note: If solid agarose or gel pieces remain, return the flask to the microwave, and continue heating in 30-second intervals until all product is in solution. This may take a few minutes, depending on the gel concentration you are making and the power of the microwave.
(4) Remove the flask/beaker from the microwave and very gently swirl.
WARNING: The microwaved solution can become superheated and foam over quickly when agitated. Wear appropriate protection and use caution.
(5) Ethidium bromide (EtBr) is used to visualize DNA. Add 0.5 µg/ml EtBr to the melted agarose and swirl to mix well.
Note: EtBr is a potential carcinogen and should be handled appropriately.
(6) Cool the solution to 55-60°C.
(7) Cast the gel following the instructions provided for your casting apparatus.

Table 8.
Optimal Buffer Concentrations for Best Nucleic Acid Fragment Separation

TBE	DNA Size Resolution (bp)
0.5	1,000 – 25,000
0.75	800 – 12,000
1	500 – 10,000
1.2	400 – 7,500
1.5	200 – 3,000
2.0	50 – 1,500