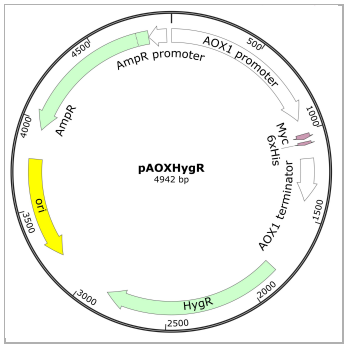

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Assembly of pAOXHygR vector for protein expression in the yeast Pichia pastoris

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ABSTRACT

The protocol describes assembly of an *Pichia pastoris* expression vector composed of the following building blocks: alcohol oxidase 1 (AOX1) promoter, multiple cloning site, *myc* epitope, 6xHis tag, AOX1 terminator, hygromycin B resistance marker, origin of replication and ampicillin resistance marker. The pAOX1HygR expression vector can be used for integrating the gene of interest into the AOX1 locus of the genome of *P. pastoris* and the subsequent methanol induced intracellular expression of the recombinant protein. Ampicillin resistance marker is employed for selecting the transformed *E. coli* cells and hygromycin B resistance marker for selecting the transformed *P. pastoris* cells. The protocol is part of the article "Identification, characterisation and recombinant expression of flavonoid 3',5'-hydroxylases and cytochrome P450 reductases from *Vaccinium* species". The protocol was created during implementing the project no 1.1.1.2/VIAA/2/18/286 "Optimization of novel plant derived enzyme expression in microorganisms for biotechnological application" funded by European Regional Development Fund.

IMAGE ATTRIBUTION

The image was created with SnapGene Viewer 1.6.2.

MATERIALS

- GeneJET Gel Extraction and DNA Cleanup Micro Kit **Thermo Fisher Catalog #K0832**
- pPICZ A, B, & C Pichia Vectors **Thermo Fisher Catalog #V19020**
- CloneJET PCR Cloning Kit **Thermo Fisher Catalog #K1231**
- pUDP082 plasmid **addgene Catalog #103875**
- Phusion High-Fidelity DNA Polymerase (2 U/µL) **Thermo Fisher Catalog #F530L**
- T4 DNA Ligase (5 U/µL) **Thermo Fisher Catalog #EL0011**
- Eco31I (BsaI) (10 U/µL) **Thermo Fisher Catalog #ER0291**
- BglII (10 U/µL) **Thermo Fisher Catalog #ER0081**

1 The sequences of the vectors pPICZ A (Thermo Fisher), pUDP082 (Addgene, plasmid #103875) and pJET1.2 (CloneJET PCR Cloning Kit,Thermo Fisher) were stored in the Benchling R&D cloud. The pPICZ A vector was used as a source for the sequence encoding AOX1 promoter followed by multiple cloning site (MCS), *myc* epitope, 6xHis tag and AOX1 terminator. pUDP086 was used as a template for amplifying

hygromycin B resistance marker and pJET1.2 for amplifying ampicillin resistance marker and origin of replication.

2 The assembly wizard of the Benchling workspace was used to design primers for amplifying DNA fragments with proper overhangs for running Golden Gate assembly. The primer sequences were manually edited (longer) so that the annealing temperatures were at least 63°C. The primer sequences used for obtaining the first two building blocks are presented in the table below (Table 1), whereas the primer prAOX1MCSHis6TT REV eliminated the unwanted Eco31I (BsaI) site from the 3' end of AOX1 transcription terminator. Phusion High-Fidelity DNA polymerase (Thermo Fisher) was used for running PCR according to manufacturer's instructions. The PCR products with the expected size (1420 and 1715 bp) were purified from agarose gel using GeneJet Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific). DNA concentration was measured with Mettler Toledo UV5nano spectrophotometer.

A	B
Primer name	Primer sequence
prAOX1MCSHis6TT FWD	TGGACTGGTCTCGTGAGATCTAACATCCAAAGACGAaagg
prAOX1MCSHis6TT REV	TGGACTGGTCTCGgtGCACAAACGAActTCTCACTTAATCTTCTGTA Ctctgaag
pHygRt FWD	TGGACTGGTCTCCGCactatatgtgaaggcatggctatg
pHygRt REV	TGGACTGGTCTCCACCGttgttgaacattcttaggctg

Table 1. Primer names and sequences used for amplifying the sequence from AOX1 promoter to transcription terminator from pPICZ A (prAOX1MCSHis6TT FWD vs prAOX1MCSHis6TT REV) and the hygromycin B resistance marker from pUDP082 vector (pHygRt FWD vs pHygRt REV).

3 The sequence encoding ampicillin resistance in pJET1.2 contains an unwanted Eco31I (BsaI) site that was removed before running the Golden Gate assembly. Silent mutation was introduced (GGG to GGA) through running PCR in two rounds. In the first round the primer pairs below (Table 2) and Phusion High-Fidelity DNA polymerase were used. The PCR products with the expected size (990 and 880 bp) were purified from agarose gel using GeneJet Gel Extraction and DNA Cleanup Micro Kit.

A	B	C	D	E
Fragment I	oriAmpR FWD	TGGACTGGTCTCCCGGT GAGCAAAAGGCCAGCA	AmpRwoBsaI REV	AGCCGGTGAGCGTGGATCTCGCGGTATCAT T
Fragment II	AmpRwoBs aI FWD	AATGATACCGCGAGAT CCACGCTCACCGGCT	oriAmpR REV	TGGACTGGTCTCCCTCAGGTGGCACTTTTC GGG

Table 2. Primers used for amplifying ampicillin resistance marker and origin of replication from pJET1.2 vector.

In the second round of PCR the fragments were joined by carrying out a two-step PCR by combining one microliter of both purified DNA fragments per 50 µl reaction mix, the primers oriAmpR FWD and oriAmpR REV, and dNTPs, HF buffer and Phusion High-Fidelity DNA polymerase, as recommended by the manufacturer. As before, the PCR product with the correct size was purified from agarose gel.

4 Golden Gate assembly was carried out whereas the molar ratio of the oriAmpR fragment ("backbone") to the inserts - prAOX1MCSHis6TT and HygR was 1:2. 20 µl of the assembly mix contained approximatelly 75 ng of the backbone, 150 ng of both inserts, 2 µl of T4 DNA ligase buffer, 1 µl of Eco31I (BsaI) (Thermo Fisher) and 1 µl of T4 DNA ligase (Thermo Fisher). The assembly reaction was carried out in 250 µl PCR tubes in a PCR machine with the lid heating turned on (105 °C). The programme was as follows: 30 cycles of 2 min at 37 °C followed by 2 min at 16 °C, one cycle of 5 min at 55 °C and one cycle of 5 min of 80 °C.

5 10 µl of the assembly mix was used for transforming 100 µl of competent *E. coli* DH5α cells. The transformation mix was plated on LB agar plates (Luria-Bertani, 1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 75 µg/ml ampicillin. Transformation yielded a lot of colonies confirming that the ampicillin resistance gene was functional. Sequencing with 3'AOX1 primer (GCAAATGGCATTCTGACATCC) was carried out to confirm that MCS was free of errors. *P. pastoris* GS115H (GS115 with HIS4 phenotype, obtained by transforming GS115 with pPIC3.5 linearised with StuI) was transformed with the empty pAOXHygR vector (linearised with BglII) and plated on YPD agar plates (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing 100 µg/ml hygromycin B. Many colonies were obtained, demonstrating that the hygromycin B resistance marker was functional. The vector sequence can be found in the Supplementary files of the manuscript.