Expression and purification of pyruvate transporting human mitochondrial pyruvate carrier (MPC) protein complex for structural studies

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25-05-2022

Master's project in Molecular Biology
MOBY10 30 Credits
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Biochemistry & Structural Biology
Spring 2022

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1 Abstract

Pyruvate is one of the main molecules needed for the citric acid cycle, which produces energy in the form of ATP. The citric acid cycle reaction occurs in the mitochondrial matrix, and for pyruvate to participate in the reaction, it needs to be transported from the cytosol across the mitochondrial membranes into the mitochondrial matrix. A special membrane protein called the mitochondrial pyruvate carrier (MPC) transports pyruvate across the inner membrane of the mitochondria, while pores in the outer mitochondrial membrane allows smaller molecules to diffuse over it. The MPC is made of two proteins, MPC1, and MPC2. The aim of this project is to characterize the structure and functionality of the mitochondrial pyruvate carrier. In this study, the Hu-MPC1 and Hu-MPC2 proteins were expressed separately in *Pichia pastoris*, and then individual and combined protein assays were performed. The assays include SDS-PAGE, Native-PAGE, Western Blot, and Mass spectrometry. To enable chromatography and assays of the proteins, the Hu-MPC1 was tagged with a Strep-tag II at the C-terminal and the Hu-MPC2 was tagged with a His-tag at the C-terminal. The results suggests that a heterodimer complex was formed between Hu-MPC1 and Hu-MPC2. Further research is desired with Hu-MPC1 induction for 24 hours to see if it is possible to produce a larger amount of protein. Further assays are necessary with Hu-MPC1 and Hu-MPC2 and as a complex, to gain more knowledge regarding the complex formation of the two subunits and their dimerization capabilities. Lastly, further studies are essential with purification optimization of Hu-MPC1 and Hu-MPC2, and usage of Cryo-EM or X-ray crystallography to solve the structure of the Hu-MPC complex and subunits.

2 Abbreviations

MPC	– Mitochondrial pyruvate carrier
Hu-MPC	– Human Mitochondrial pyruvate carrier
IMAC	– Immobilized Metal Affinity Chromatography
FPLC	– Fast Protein Liquid Chromatography
AC	– Affinity Chromatography
SDS-PAGE	– Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SDS	– Sodium Dodecyl Sulphate
DTT	– Dithiothreitol
PVDF	– Polyvinylidene Difluoride
Native-PAGE	– Polyacrylamide Gel Electrophoresis
MALDI	– Matrix-Assisted Laser Desorption/Ionization
TOF	– Time of Flight
DDM	– n-Dodecyl-β-D-Maltoside
MW	– Molecular Weight
FD	– Fast Digest

3 Introduction

3.1 Background

Pyruvate, a product from glucose metabolism, is an important molecule in the human energy production. It is converted into Acetyl-Coenzyme A which is the fuel for the citric acid cycle (Fig. 1). The citric acid cycle takes place inside the mitochondria of the cell and generates NADH and $FADH_2$ which is ultimately used in the electron transport chain to produce ATP (Berg, 2019). The mitochondria have an outer membrane and an inner membrane. The outer membrane has porins which allow pyruvate and other small molecules to diffuse over the membrane. To cross the inner membrane, a membrane protein called the mitochondrial pyruvate carrier (MPC) is needed (Gray et al., 2014).

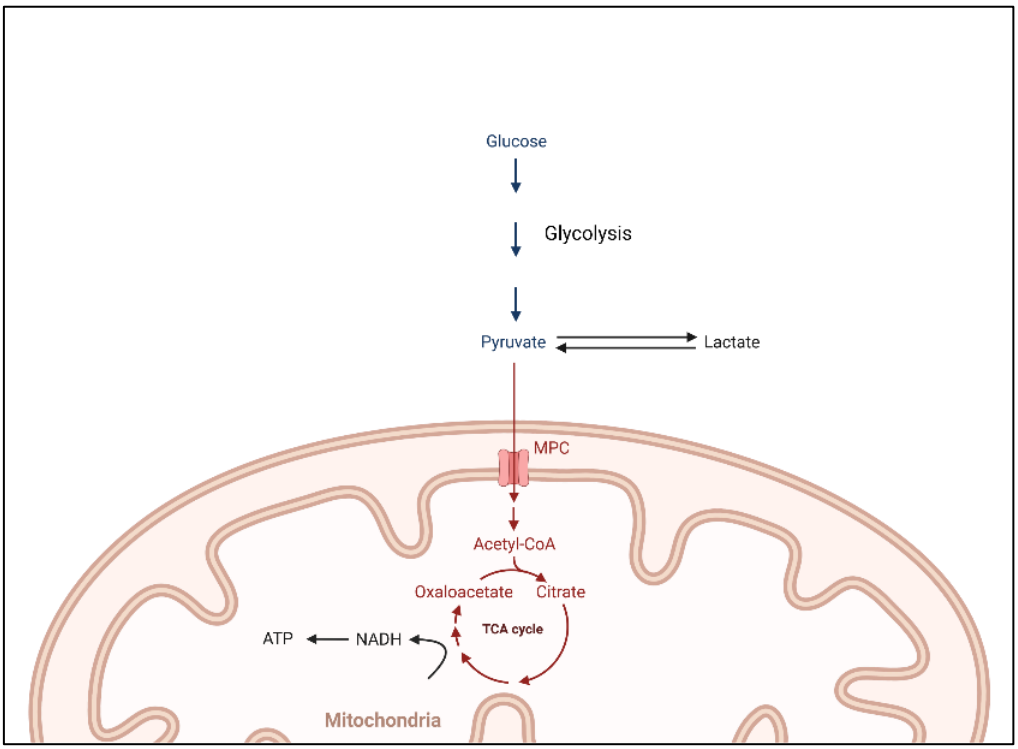

Figure 1: Overview of the metabolic pathway of glucose into pyruvate and the further on transported into the mitochondria. Adapted from "Warburg Effect", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

Human MPC (Hu-MPC) consists of two proteins, MPC1 and MPC2 which theoretically forms a ~150 kDa complex (Bricker et al., 2012; Herzig et al., 2012). The structure of MPC1 (Fig. 2) and MPC2 (Fig. 3) have not been solved, but they are predicted to have two or three transmembrane domains (Xu et al., 2021).

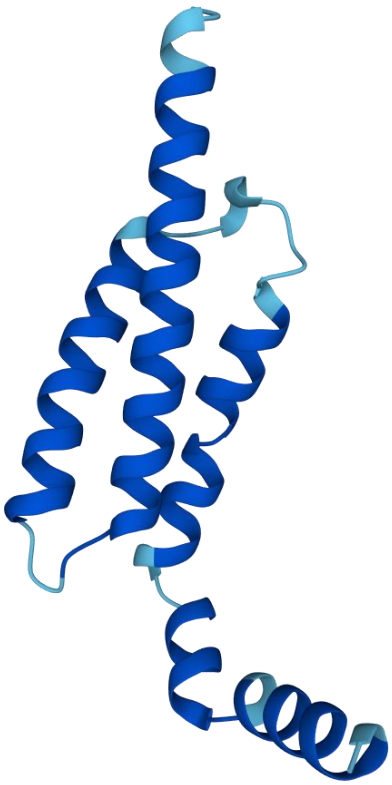

Figure 2: Note. The suggested structure of human MPC1, produced by AlphaFold. Per-residue confidence score (pLDDT) 0-100. Dark blue (pLDDT > 90), light blue (90 > pLDDT > 70). From (UniProt, n.d-a). CC BY 4.0. (https://creativecommons.org/licenses/by/4.0/)

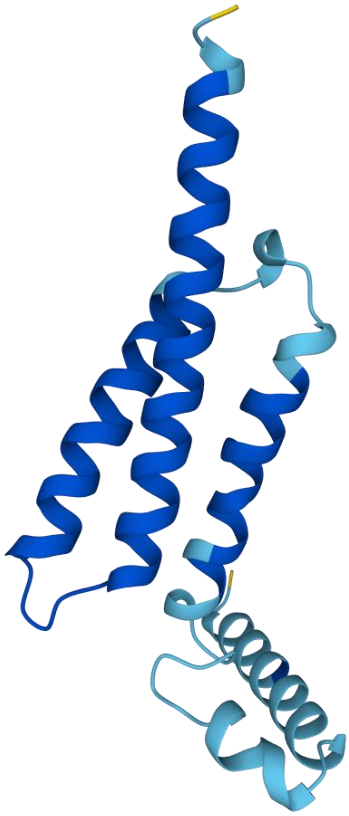

Figure 3: Note. The suggested structure of human MPC2, produced by AlphaFold. Per-residue confidence score (pLDDT) 0-100. Dark blue (pLDDT > 90), light blue (90 > pLDDT > 70). From (UniProt, n.d-b). CC BY 4.0. (https://creativecommons.org/licenses/by/4.0/).

Although the individual protein structures have not been solved, studies on the MPC complex show that MPC1 and MPC2 most likely exists in a heterodimer complex state and not a homodimer complex, as ligand binding affinity results was shown to be higher for the hetero-complex (Lee et al., 2020). The transport of pyruvate is suggested to be coupled with an electrochemical gradient across the inner membrane, and that MPC works as a symport with protons (Papa et al., 1971).

Previous studies suggests that MPC is upregulated when there is glycogen present, and downregulated when insulin is present in the cell (Titheradge, 1976). Previous studies have also shown that MPC can transport other molecules than pyruvate, and that the transport can be inhibited. Some of the inhibiting molecules are α-cyano-4-hydroxi cinnamate, UK-5099, and various thiazolidinediones compounds (Divakaruni et al., 2013; Halestrap, 1975; Halestrap, 1974; Hildyard et al., 2005; Xu et al., 2021). The inhibiting thiazolidinedione compounds are a commonly used as a therapeutic agent for patients with high blood sugar to stop the development of type 2 diabetes. However, thiazolidinedione treatment is problematic since it has shown to have several negative side effects (Divakaruni et al., 2013).

Studies have also linked the dysfunction of MPC to some forms of cancer, where reduced activity of the MPC allows for unchecked proliferation and cell survival (Rauckhorst & Taylor, 2016). The outcome of MPC dysfunction or inhibition could also be dependent on which cells that are affected by the MPC loss. MPC disruption in muscle cells has shown to increase glucose uptake in the muscles and whole-body insulin sensitivity, which could help prevention of type 2 diabetes. Other diseases linked to the MPC are neurodegenerative diseases such as Parkinson's (Ghosh et al., 2016). To summarise, solving the structure of the MPC could prove to be important for future therapeutic agents. This study aims to characterize the structure and functionality of the mitochondrial pyruvate carrier.

3.2 Methodology

3.2.1 Cloning

Figure 4: The plasmid vector pPICZ-B created in SnapGene, containing the AOX1 promoter. Length of 3328bp and restriction enzyme cutting site of EcoRI at 943bp and KpnI at 980bp. SnapGene software (from Insightful Science; available at snapgene.com).

The pPICZ-B (Fig. 4) is a 3328 bp long plasmid which was used as a vector for cloning and contains the following relevant properties; the common and effective *AOX1* promoter which allows for methanol induction, 10 unique restriction enzyme cutting sites which allows for gene insertion, *AOX1* transcription terminator, Zeocin resistance gene, pUC origin which allows for cloning in *Escherichia coli,* and *Sac I* restriction site for linearization and efficient integration into the *Pichia pastoris* genome (Invitrogen, 2010; Vogl & Glieder, 2013).

3.2.2 Strep-Tag II system

The Strep-tag II is an eight amino acid long chain (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) which is used for high-affinity purification and detection of proteins. The Strep-tag II is fused with a protein of interest and allows for protein-protein assays to be performed. The Strep-tag II binds to core streptavidin, which is a shortened version of the bacterial protein streptavidin. The protein of interest with a Strep-tag II binds to a column with streptavidin and then a buffer with a biotin derivative is added to elute the bound protein (Schmidt & Skerra, 2007).

3.2.3 Immobilized Metal Affinity Chromatography (IMAC)

IMAC is an affinity chromatography method which is used for purification of proteins by utilizing the unique properties of different amino acids. A protein solution is passed through a column which has an immobilized metal ion which the protein of interest will bind to, while the rest of the unbound sample passes straight through the column. The bound protein can then be eluted by adding a solution containing molecules with higher binding affinity to the column than the protein of interest. IMAC is used for purification of proteins with a histidine-tag (Berg, 2019).

3.2.4 NGC machine/FPLC system

Fast Protein Liquid Chromatography (FPLC) system is used for affinity chromatography (AC) and size exclusion chromatography. The system commonly consist of a fraction collector, a conductivity meter, a pump, and a UV detector (Bio-Rad Laboratories Inc., n.d-a). Column liquid chromatography separates molecules based on their physicochemical properties and interactions. The UV detector measures light absorption at 280 nm, which is mainly the absorption of tryptophan (Bio-Rad Laboratories Inc., n.d-b).

3.2.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a type of gel electrophoresis. The gel is made of polyacrylamide and N,N'-Methylenebisacrylamide which is a cross-linking agent that creates the 3D-structure of the gel. The principle is, when sodium dodecyl sulphate (SDS) is added in a protein solution, the reagent binds to the proteins and disrupts the non-covalent bindings. The SDS is mixed with either beta-Mercaptoethanol or dithiothreitol (DTT), which acts as a reducing agent to hinder the amount of disulphide bonds formed. The SDS will bind and denature the proteins in the solution and create a net negative charge in correlation to the molecular weight of the protein. This formation will be subjected to electrophoresis and move through the porous gel, where small proteins will advance fast, and large proteins slower. The gel must be stained with a dye, such as Coomassie blue, to be able to visualize the protein bands (Berg, 2019).

3.2.6 Western Blot

Western blot is an immunoassay technique which allows for low concentrations of protein to be detected. After SDS-PAGE, a nitrocellulose- or polyvinylidene difluoride- (PVDF) membrane is pressed against the gel, and the proteins are electroblotted from the gel to the membrane. The membrane is then incubated with a primary antibody, which is specific for the protein of interest. After unbound primary antibody is washed away, the membrane is incubated with a secondary antibody, which binds to the primary antibody. The secondary antibody is conjugated with an enzyme (e.g. horseradish peroxidase), which most commonly has an enzymatic reaction that produces a fluorescent product (Berg, 2019).

3.2.7 Fermenter

A fermenter is used to ferment *P. pastoris* and to induce overexpression of a protein of interest. The fermenter allows certain parameters to be controlled which are crucial for optimal cell growth and to produce a large amount of protein. The oxygen which is needed by the yeast cells to metabolize glycerol and methanol is controlled by agitation (500 – 1500 rpm) and a constant oxygen inlet. The temperature is necessary to be around 30℃ and the pH around 5 for optimal growth conditions, which is controlled by a heating/cooling element and an intake of NH_3 and HCl (ThermoFisher-Scientific, 2002).

3.2.8 Membrane Preparation

Membrane preparation is necessary to perform on cells to isolate the membrane proteins and be able to conduct assays on said proteins. The membrane preparation includes breakage of the cells with buffers and glass beads, and then centrifugation for purification of the desired membrane from soluble proteins and other non-desired debris. The next step is to solubilize the membrane proteins to isolate them from the membrane. This is performed by adding a detergent which creates micelles around the proteins to keep them stable and active (Roy, 2015; Seddon et al., 2004).

3.2.9 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native-PAGE is a gel electrophoresis with a polyacrylamide gel which allow for non-denaturing electrophoresis. The electrophoresis is performed at approximately pH 7.5 and preferably in 4℃ which increases the stability of the gel and proteins. The ability to perform electrophoresis without denaturing the proteins allows for analysis of protein complexes (Life-technologies, 2012).

3.2.10 Mass Spectrometry

Mass spectrometry is an analytical technique where samples are ionized into gaseous charged ions and the mass-to-charge ratio of these are measured, which in our case is proteins. A mass spectrometer consists of three main components: an ion source, a mass analyser, and a detector. In the ion source the sample is ionized, and in the mass analyser the ions are sorted and separated before they are finally detected by the detector and a relative abundance is recorded. The result is presented as a mass spectrum where the intensity, on the y-axis, is plotted against the mass-to-charge ratio (m/z), on the x-axis. In this study we used a mass spectrometer with a matrix-assisted laser desorption/ionization (MALDI) ion source and a time-of-flight/time-of-flight (TOF/TOF) mass analyser. Mass spectrometry can be used to study many different molecules for example proteins, peptides, carbohydrates, lipids, and small molecules. The spectra can among other things, be used to determine the masses of particles and molecules. In this study we used mass spectrometry to determine the intact mass of our protein of interest, the MPC complex (Hofmann & Clokie, 2018; Smith, 2013).

3.3 Strategy

The project was designed to continue the previous work done by Susanna Horsefield's research group on mitochondrial pyruvate carriers. Previously Hu-MPC1 (Tab. 9, Appendix I) and Hu-MPC2 (Tab. 10, Appendix I) had been produced with a His-tag in *P. pastoris.* However, to simplify the protein assays, the tag on Hu-MPC1 was decided to be replaced with a Strep-Tag II. The strategy was to change the existing gene of Hu-MPC1 with His-tag in the vector pPICZ-B with primers for both N- and C-terminus Strep-Tag II (Tab. 11, 12, Appendix I). Then analyse which construct was the most stable and continue with said construct to protein

expression. The Hu-MPC2 had already been produced with a C-terminal His-tag in *P. pastoris* x33 with the membrane prepared and isolated. After protein expression of Hu-MPC1 in *P. pastoris* x33, the two proteins of interest would be solubilized individually and collectively and analysed to the extent of the thesis project's time limit.

4 Methods & Materials

4.1 Method

To test the hypothesis, Hu-MPC1 and Hu-MPC2 was produced separately, and then subjected to different assays individually and collectively. Hu-MPC2 was supplied by Dr. Tamim Al Jubair.

4.1.1 Cloning of Hu-MPC1 gene in pPICZ-B

The gene coding for Hu-MPC1 was multiplicated in a PCR with primers (Tab. 11, 12, Appendix I) to replace current N- and C-terminus for either N- or C-terminus Strep-tag II (Fig. 5).

Figure 5: The two constructs for the human MPC gene. One construct with Strep-tag II at the C-terminal and a thrombin cleavage site in between the Hu-MPC1 gene and the Strep-tag II. One construct with Strep-tag II at the N-terminal and a thrombin cleavage site in between the Hu-MPC1 gene and the Strep-tag II. Created with BioRender.com.

The PCR was run with both constructs with Strep-tag II according to the *Hu-MPC Polymerase Chain Reaction protocol* (Appendix I, 10.1.1). After the PCR of Hu-MPC1 gene, the product was purified with E.Z.N.A. Cycle Pure Kit (Appendix I, 10.1.2) with 65°C MilliQ H_2O as elution buffer. The pPICZ-B vector was digested (Appendix I, 10.1.3) and then run on an 1% agarose gel to purify the digested plasmid. The fragment was extracted according to the *Gel DNA Extraction protocol* (Appendix I, 10.1.4), except elution was with 30 µL 65°C MilliQ H_2O. The digested plasmid fragment was ligated with the Hu-MPC1 gene. Both the N- and C-terminal sequences of Hu-MPC1 were ligated into the pPICZ-B plasmid according to *Vector and Insert Ligation* (Appendix I, 10.1.5).

4.1.2 Transformation into *Escherichia coli* DH5-α

The two pPICZ-B vectors with Hu-MPC1 N- and C-terminal Strep tag II genes were transformed into DH5-α cloning strain of *E. coli* according to the *Transformation into E. coli protocol* (Appendix I, 10.1.6), with Zeocin as a selection marker. Five colonies from both N- and C-Strep tag II Hu-MPC1 construct were chosen for a colony PCR (Appendix I, 10.1.7) to make a crude assessment of the success of the ligation. The colonies with the most promising results were chosen for plasmid extraction according to the *Sigma Aldrich GenElute Plasmid Miniprep Kit Protocol* (Appendix I, 10.1.8) and sent off for sequencing by Eurofins GATC Services.

4.1.3 Transformation into *Pichia pastoris* x33

Once the correct constructs of Hu-MPC1 were confirmed, the plasmids were linearized and transformed into *P. pastoris* (Appendix I, 10.1.9) and plated on *YPD agar* (Tab. 13, Appendix I) plates with 100 µg/mL Zeocin as a selection marker. The colonies of *P. pastoris* was incubated for 48 hours at 30℃ and then re-plated onto a new *YPD agar* plate with 2000 µg/mL Zeocin. The colonies which had the largest area after 48 hours of incubation in 30℃, were chosen for a small-scale expression (Appendix I, 10.1.10). The colonies chosen for the small-scale expression were analysed on a western blot (Appendix I, 10.1.11) with MPC1 rabbit monoclonal antibodies as the primary antibody (1:2000 dilution) and goat anti-rabbit IgG (H+L)-HRP conjugated as the secondary antibody (1:5000 dilution) (Appendix I, 10.1.12).

4.1.4 Fermentation of *Pichia pastoris* x33 & protein induction

The colony which showed the desired and most efficient protein expression was chosen for high-cell density fermentation and 48 hours protein induction (Appendix I, 10.1.13). A small sample of the yeast cells were prepared for a dot blot (Appendix I, 10.1.14) to analyse the induction and overexpression of the Hu-MPC1 protein. After the presence of Hu-MPC1 protein was confirmed, a 100 g sample of the cells were used for membrane preparation (Appendix I, 10.1.15).

4.1.5 Protein purification & assays

The Hu-MPC1 membrane preparation was solubilized in a 1:1 ratio with *Solubilization buffer* (Tab. 15, Appendix I) at 4℃ rotating for 1.5 hours. Insolubilized material was spun down using the Ti70 rotor, 50,000 rpm for 1 hour, and the supernatant was collected. The sample was run through an affinity chromatography with a StrepTrap column (Cytiva, cat#28907547), using *Buffer A1- and B1 Strep* (Tab. 16, 17, Appendix I) for equilibration and elution respectively. The Hu-MPC1 membrane preparation was also solubilized with Hu-MPC2 membrane preparation in a 1:1 volume ratio with *Solubilization buffer* at 4℃ rotating for 1.5 hours. The mixed Hu-MPC1 and Hu-MPC2 solubilization was run through an IMAC with a HisTrap column (Cytiva, cat#17524801), using *Buffer A- and B His* (Tab. 18, 19, Appendix I) for equilibration and elution respectively. The protein solution with Hu-MPC1 and Hu-MPC2 was subsequently run through an affinity chromatography with a StrepTrap column, using *Buffer A2- and B2 Strep* (Tab. 20, 21, Appendix I) for equilibration and elution respectively. Samples from the affinity chromatographs were analysed on SDS-PAGE with both Coomassie blue staining (Appendix I, 10.1.16) and western blot (Appendix I, 10.1.12). The western blots were done with primary antibodies against MPC1 (rabbit MPC1 monoclonal, Cell Signaling Technology, cat#14462), Strep-tag II (rabbit anti-Strep tag II polyclonal, Thermo Scientific, cat#PA5-119772), or His-tag (mouse 6xHis monoclonal, TaKaRa, cat#631212), and with secondary antibodies against either rabbit (goat anti-rabbit IgG (H+L)-HRP conjugated, Bio-Rad, cat#170-6515) or mouse (goat anti-mouse IgG(H+L)-peroxidase labelled, SeraCare, cat#5220-0341). The elution fractions of Hu-MPC1 and Hu-MPC2 from the IMAC with a HisTrap, was run on a Native-PAGE (Appendix I, 10.1.17) and stained according to *Native-PAGE gel Coomassie staining* (Appendix I, 10.1.18).

5 Results

5.1 Hu-MPC1 Production

The Hu-MPC1 gene was inserted into pPICZ-B and the vector with insert was transformed into *E. coli* DH5-α, then extracted and transformed into *P. pastoris* x33. The vector pPICZ-B has resistance against Zeocin, which was used as a selection marker to find the clones with the highest transformation efficiency. To find the *P. pastoris* clone which had the highest number of copies of the Hu-MPC1 gene, 36 N-terminal and 10 C-terminal Strep-tag II transformants were streaked on a *YPD agar* plate with 2000 µg/mL Zeocin (Fig. 6). On the plate, several colonies can be seen with markings for N-terminus or C-terminus Strep-tag (Fig. 6).

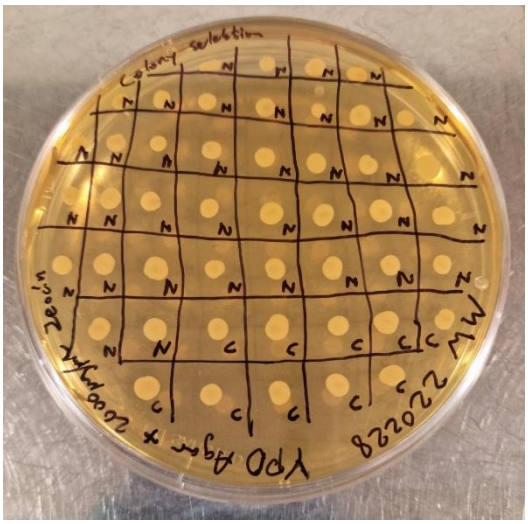

Figure 6: YPD agar plate with 2000 µg/mL Zeocin with Hu-MPC1 C- and N-terminal Strep-tag II transformed into Pichia pastoris.

The transformants which had the largest area after 48 hours of incubation in 30℃ were chosen for a small-scale expression. Samples from the small-scale expression were analysed with western blot after 24 hours and 48 hours of induction with methanol (Fig. 7, 8). The anti-strep antibodies detected a protein band around the 15 kDa protein ladder mark in the positive control (Fig. 7). Protein bands around 15 kDa can also be seen on colonies 1, 2, 3, and 7 with the C-terminal Strep-tag II in the western blot samples after 24 hours of induction (Fig. 7). The anti-strep antibodies detected a protein band around the 15 kDa protein ladder mark in the positive control on the western blot samples after 48 hours of induction (Fig. 8). The western blots (Fig. 7, 8) suggests that there was more protein after 24 hours than 48 hours.

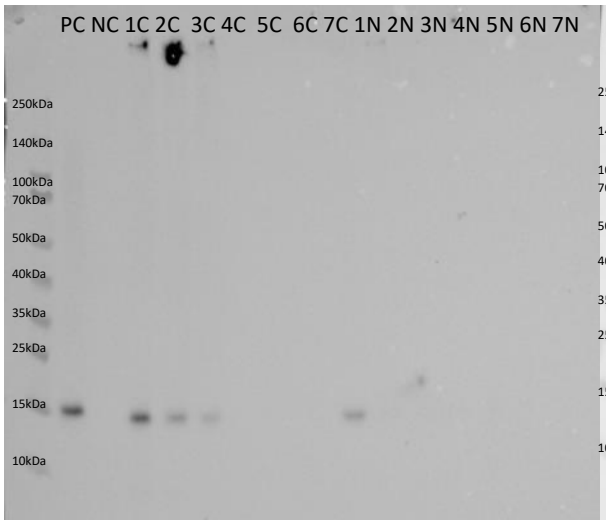

Figure 7: Western blot with anti-strep antibodies on small-scale expression samples after 24 hours of induction. From left to right; positive control (Saccharomyces cerevisiae MPC1), negative control (empty p. pastoris), colony 1-7 with C-terminal Strep-tag II, colony 1-7 with N-terminal Strep-tag II.

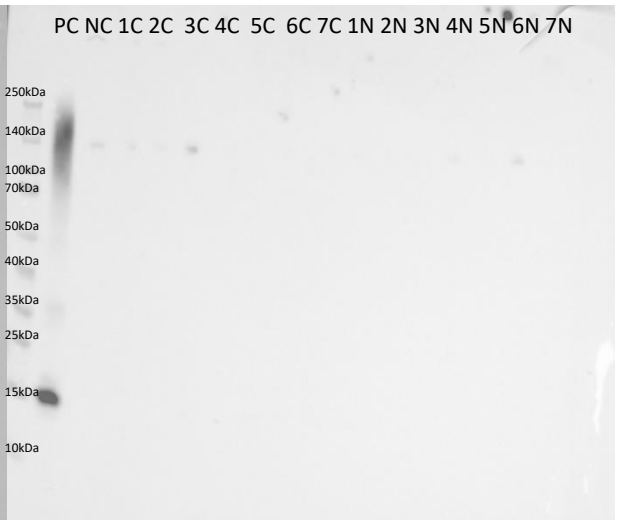

Figure 8: Western blot with anti-strep antibodies on small-scale expression samples after 48 hours of induction. From left to right; positive control (Saccharomyces cerevisiae MPC1), negative control (empty p. pastoris), colony 1-7 with C-terminal Strep-tag II, colony 1-7 with N-terminal Strep-tag II.

5.2 Protein purification and complex formation of Hu-MPC1 and Hu-MPC2

The Hu-MPC1 and Hu-MPC2 was solubilized in n-Dodecyl-β-D-Maltoside (DDM) to replace the membrane with detergent micelles for protein assays. The individual solubilization of Hu-MPC1 was run through an affinity chromatography, and a 280 nm protein absorption peak can be seen during the sample application (Fig. 9). The fact that no protein absorption peak can be seen during the elution phase suggest that the Hu-MPC1 did not bind to the StrepTrap column. The joint solubilization of Hu-MPC1 and Hu-MPC2 was run through an IMAC, and a 280 nm protein absorption peak can be seen during the sample application and the elution phase with imidazole (Fig. 10), which suggest that protein has bound to the HisTrap column and then been eluted with the elution buffer. Fractions 26-29 (Fig. 10), and the positive controls for His-tag, Strep-tag II, MPC1, was analysed on an SDS-PAGE gel with Coomassie blue staining (Fig. 11). The Coomassie blue stained gel (Fig. 11) show several protein bands in the fractions 26-29.

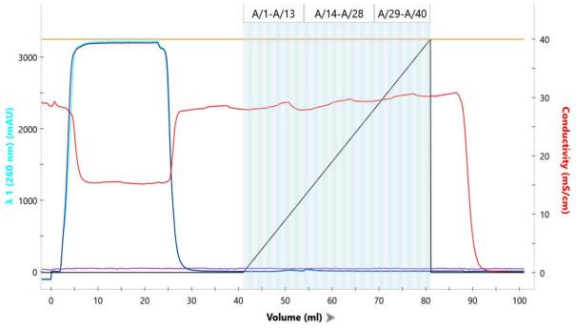

Figure 9: Chromatogram from affinity chromatography StrepTrap with Hu-MPC1 membrane preparation solubilized in 2% DDM. The black line shows elution buffer concentration.

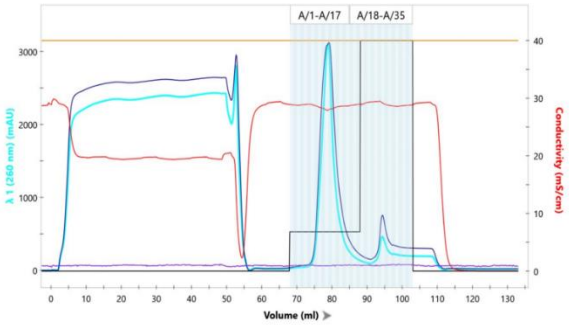

Figure 10: Chromatogram from IMAC HisTrap with Hu-MPC1 membrane preparation mixed with Hu-MPC2 membrane preparation and solubilized in 2% DDM. The black line shows elution buffer concentration.

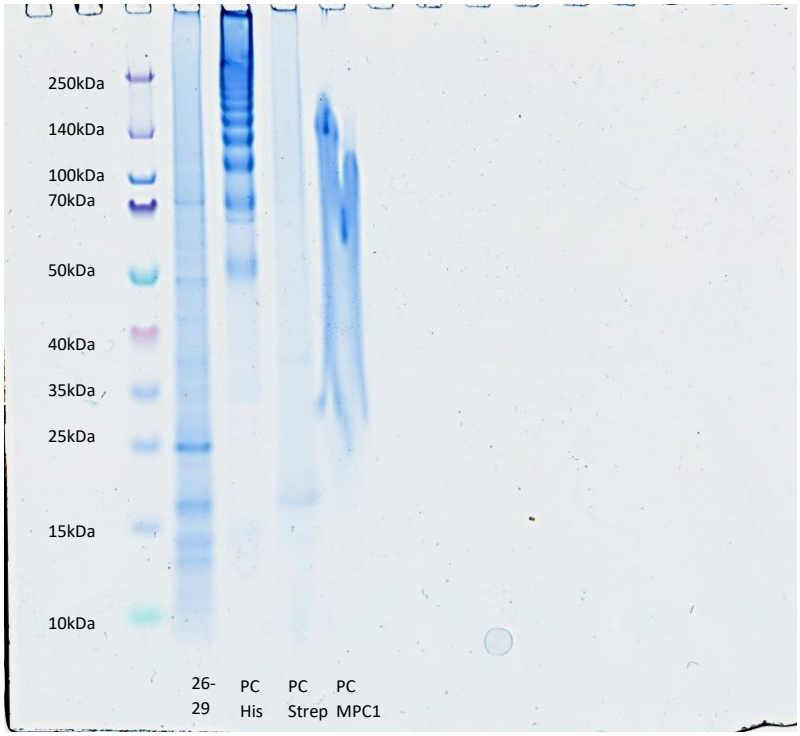

Figure 11: SDS-PAGE with samples from IMAC-His elution and positive controls. From left to right; elution from IMAC-His, positive control for His-tag, Strep-tag, and MPC1.

The fractions 26-29 and the positive controls for MPC1, His-tag, Strep-tag II, was analysed on three separate western blots with antibodies against the different positive controls (Fig. 12, 13, 14). The anti-MPC1 antibodies have bound to the fractions 26-29 (Fig. 12), the anti-his antibodies have bound to the fractions 26-29 and the positive control for His-tag (Fig. 13), and the anti-strep antibodies have bound to the fractions 26-29 and to the positive control for Strep-tag II (Fig. 14). The results from the three western blots (Fig. 12, 13, 14) suggests that both Hu-MPC1 with Strep-tag II and Hu-MPC2 with His-tag are in the fractions 26-29.

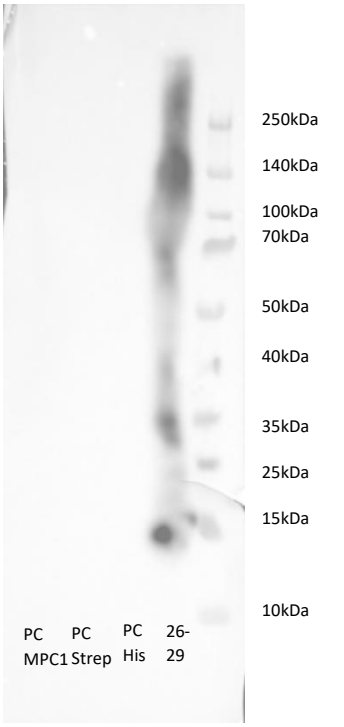

Figure 12: Western blot with anti-MPC1 antibodies on samples from IMAC-His elution and positive controls. From left to right; positive control for MPC1, Strep-tag, His-tag, and elution from IMAC-His.

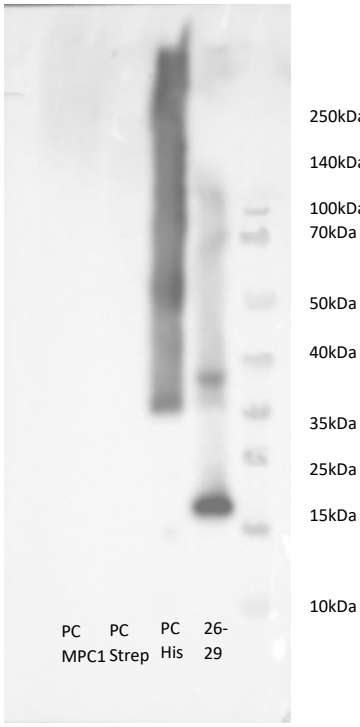

Figure 13: Western blot with anti-His antibodies on samples from IMAC-His elution and positive controls. From left to right; positive control for MPC1, Strep-tag, His-tag, and elution from IMAC-His.

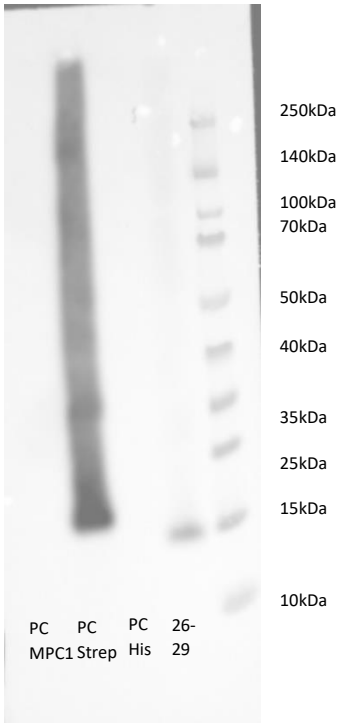

Figure 14: Western blot with anti-Strep antibodies on samples from IMAC-His elution and positive controls. From left to right; positive control for MPC1, Strep-tag, His-tag, and elution from IMAC-His.

The fractions 26-29 was further on analysed with a Native-PAGE to increase the understanding of the interaction between Hu-MPC1 and Hu-MPC2. The Native-PAGE gel was stained with Coomassie R-250. Faint protein bands were visualized around 146 kDa and 600 kDa in the fractions 26-29, and in all three samples a blurry purple discolouring is seen between 20 kDa and 66 kDa (Fig. 15).

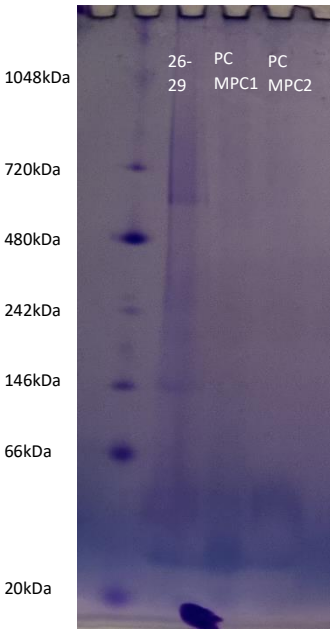

Figure 15: Native-PAGE with samples from IMAC-His elution and positive controls. From left to right; fraction 26-29 elution from IMAC-His, positive control for MPC1, positive control MPC2.

The protein sample containing fraction 26-29 was run through an affinity chromatography with a StrepTrap column, and according to the chromatogram (Fig. 16), two protein elution peaks can be seen. One peak is during the sample application, and the other peak is during the elution phase with desthiobiotin (Fig. 16). The affinity chromatography was run to purify the Hu-MPC1 and Hu-MPC2, and to further investigate the protein-protein interaction. Four samples were analysed on SDS-PAGE with Coomassie blue staining and western blots with antibodies against Strep-tag II and His-tag. The four samples analysed were fractions 26-29 before the AC, and fractions 1, 2, 13 from the AC (Fig. 17, 18, 19). The Coomassie blue staining (Fig. 17) shows no visible protein bands in the fraction 13, and faint protein bands around 25 kDa and 15 kDa in fractions 1 and 2. The western blot with anti-His antibodies show distinct protein bands in the samples pre-AC, fraction 1, and fraction 2 around 15 kDa (Fig. 18). The western blot with anti-Strep antibodies shows distinct protein bands in the samples pre-AC and fraction 13, and faint protein bands in fraction 1 and 2 around 15 kDa (Fig. 19).

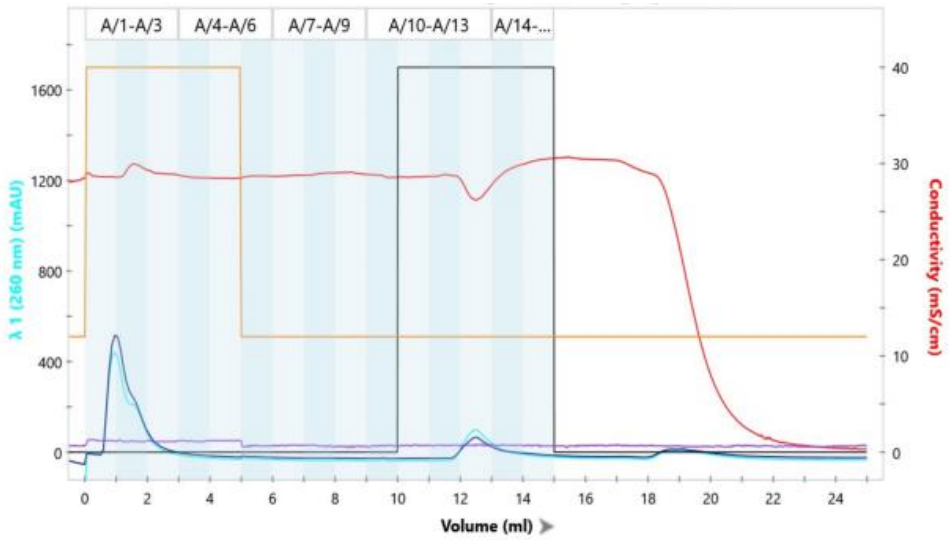

Figure 16: Chromatogram from affinity chromatography StrepTrap with Hu-MPC1 membrane preparation mixed with Hu-MPC2 membrane preparation and solubilized in 2% DDM and run through IMAC-His. The black line shows elution buffer concentration.

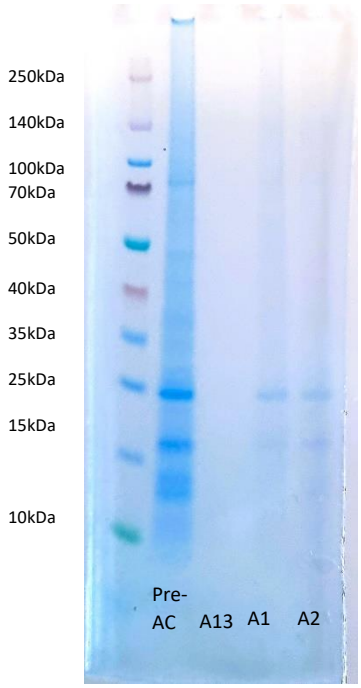

Figure 17: SDS-PAGE with samples from StrepTrap with Hu-MPC1&2. From left to right; Hu-MPC1&2 after IMAC-His, fraction 13 from elution, fraction 1 from flowthrough, fraction 2 from flowthrough.

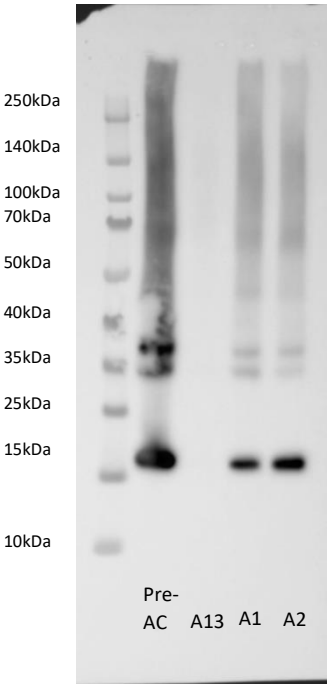

Figure 18: Western blot with anti-His antibodies on samples from IMAC-Strep. From left to right; Hu-MPC1&2 after IMAC-His, fraction 13 from elution, fraction 1 from flowthrough, fraction 2 from flowthrough.

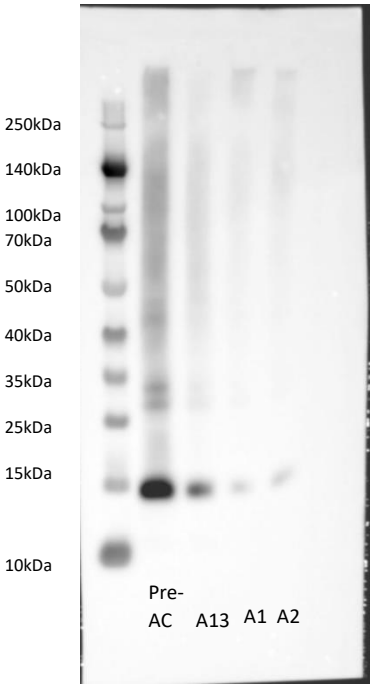

Figure 19: Western blot with anti-Strep antibodies on samples from IMAC-Strep. From left to right; Hu-MPC1&2 after IMAC-His, fraction 13 from elution, fraction 1 from flowthrough, fraction 2 from flowthrough.

The Hu-MPC1 and Hu-MPC2 solubilized sample (fraction 26-29) after IMAC-His purification was sent off for mass spectrometry for further analyses of the complex formation. Mass spectrometry was performed by Dr. Katja Bernfur. The results (Fig. 20) show one relevant intensity peak at 31237 m/z compared to the BSA standards.

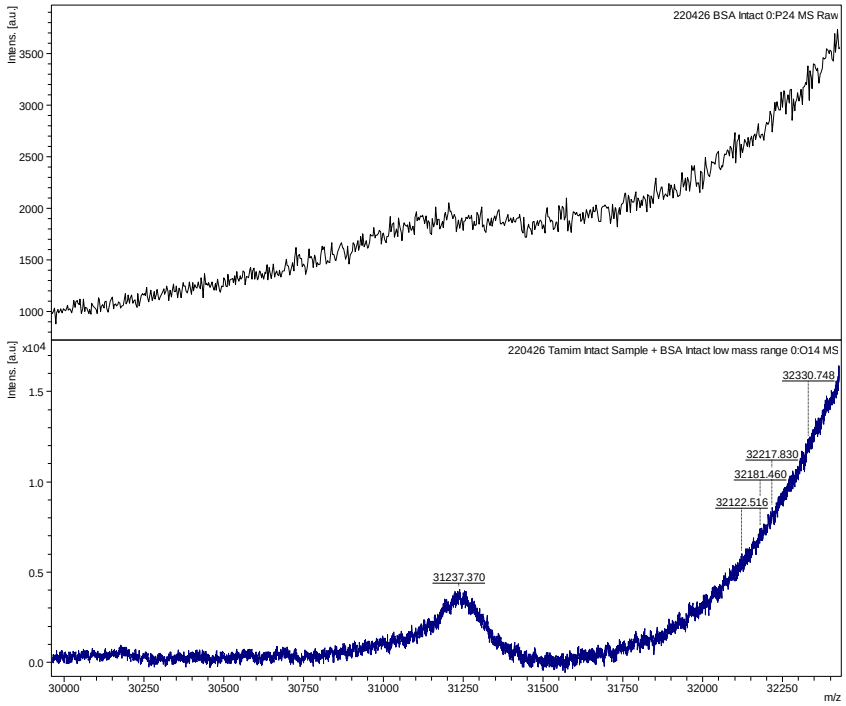

Figure 20: Mass spectrometry results with the BSA standards top graph, and MPC sample bottom graph.10 to 70 kDa.

6 Discussion

6.1 Discussion

The *P. pastoris* transformants plated on 2000 µg/mL Zeocin (Fig. 6) show strong resistance against the selection marker, which suggest that a high number of Hu-MPC1 gene copies was incorporated into the genome of the transformant clones (Hohmann, 1987). In comparison to 2000 µg/mL of Zeocin, a normal amount of Zeocin as selection marker for yeast transformants is between 50-300 µg/mL (Drocourt, 1990; Gatignol, 1987). Therefore the 14 colonies chosen for small-scale expression should be able to overexpress the Hu-MPC1 when induced with methanol, since the pPICZ-B vector contains the AOX1 promotor.

The small-scale expression (Fig. 7, 8) suggests that the colonies 1, 2, 3, and 7 with the C-terminus Strep-tag II produced the Hu-MPC1, since prominent protein bands can be seen around the 15 kDa ladder mark, and the theoretical molecular weight of Hu-MPC1 is 14.8 kDa (Walker, 2005) with a linker sequence (DYDIPTT), thrombin cleavage site sequence (LVPRGS), and Strep-tag II sequence (WSHPQFEK). The small-scale expression also suggests that 24 hours of induction produces more protein than 48 hours of induction, since no protein bands can be seen on the western blot with samples taken from the colonies after 48 hours (Fig. 7, 8). The reason could be instability and degradation of the Hu-MPC1. Other reasons might be toxicity to the host, or allocation limitations in the mitochondrial membrane if the protein is overexpressed and disproportionate to the need of the cell host. In *E. coli*, previous studies have shown that an overexpression of membrane protein is toxic for the cell due to the limiting capacity of the Sec translocon (Wagner et al., 2007). The results from the small-scale expression also suggest that the C-terminus Strep-tag II is more stable than the N-terminus, since we can see anti-strep antibodies bound to the C-terminus colonies and no detection of Strep-tag II in the N-terminus colonies. Previous studies with Hu-MPC1 have encountered similar difficulties to express N-terminal with 10x His-tag and FLAG tag (Lee et al., 2020).

The AC of Hu-MPC1 solubilized in DDM suggests that the Strep-tag II somehow is not binding to the StrepTrap, since no elution can be seen during the elution phase with 2.5 mM desthiobiotin (Fig. 9). Instead, all the protein in the solubilized sample is eluted during the sample application. Reasons for the non-binding of Strep-tag II to the column might be that the tag is not accessible. The tag could be concealed in the detergent micelle, or remains of biotin or biotinylated proteins could cause competitive binding with the Strep-tag II to the column (Schmidt et al., 2013; Schmidt & Skerra, 2007).

However, when the Hu-MPC1 was solubilized with Hu-MPC2 in DDM and run through an IMAC with HisTrap, protein elution can be seen during the sample application, wash phase, and elution phase (Fig. 10). During the wash phase the column was washed with 51 mM imidazole, and during the elution phase with 300 mM imidazole. The protein eluted during the sample application is most likely some of the Hu-MPC1 and other protein which do not have affinity for HisTrap column. The protein eluted during the wash phase is protein with a weak binding affinity to the HisTrap column. The assays with the elution fractions 26-29 (Fig. 11, 12, 13, 14) suggests that both Hu-MPC1 and Hu-MPC2 are present in the sample. The antibodies against His-tag and Strep-tag II strongly indicate the presence of His-tag and Strep-tag II in both the positive controls and the fractions 26-29 at the 15 kDa protein ladder mark. The theoretical molecular weight of Hu-MPC2 with 8 histidine is 15.4 kDa (Walker, 2005),

which is about 600Da more than Hu-MPC1. The weight difference can be seen when comparing the protein band location of anti-strep and anti-his antibodies on the western blots (Fig. 13, 14), where the His-tag attached to the Hu-MPC2 is slightly above the 15 kDa protein ladder mark and Strep-tag II attached to the Hu-MPC1 is slightly below. The western blot with anti-MPC1 antibodies shows a strong signal of MPC1 presence in the fractions 26-29, however, the positive control is not detected (Fig. 12). The absence of MPC1 positive control could be explained by the Coomassie stained gel (Fig. 11), where no protein can be seen in the positive control.

The protein samples loaded in the Native-PAGE are difficult to see, although faint protein bands can be seen (Fig. 15), they are not conclusive results. The protein band seen at 146 kDa protein ladder mark (Fig. 15) could be the complex of Hu-MPC1 and Hu-MPC2, as the theoretical molecular weight of the complex is 150 kDa. The poor visualization of the protein samples on the Native-PAGE gel (Fig. 15) could be due to a low concentration of proteins. No distinct protein band can be seen in the MPC1 and MPC2 positive controls (Fig. 15), this could be explained by the discolouring covering the lower part of the gel. However, the blurry purple discolouring between 20 kDa and 66 kDa might also be homodimers of the MPC proteins.

After the fractions 26-29 had passed through IMAC purification, impurities could still be seen in the sample (Fig. 11). Therefore, the complex of Hu-MPC1 and Hu-MPC2 went through another attempt to purify and isolate the complex via AC with Strep-tag II. If there was an interaction between the two proteins of interest, the Hu-MPC1 should be able to be caught by the HisTrap via Hu-MPC2, and vice versa with the StrepTrap. The AC with StrepTrap show a high amount of protein elution during the sample application phase compared to the elution phase with 2.5 mM desthiobiotin (Fig. 16). The assays with the eluted fractions 1, 2, and 13, (Fig. 16) with SDS-PAGE Coomassie staining (Fig. 17) and western blot (Fig. 18, 19) suggest the Hu-MPC1 had difficulties to bind the StrepTrap, similar to the AC with only Hu-MPC1. Since Strep-tag II can be seen in the sample application fractions 1 and 2, and elution phase fraction 13 (Fig. 19), and His-tag can only be seen in fraction 1 and 2 (Fig. 18), it is suggested that the interaction between Hu-MPC1 and Hu-MPC2 was not strong enough to maintain as a complex. Another reason for the lack of presence of His-tag in fraction 13 might be that there is a very low concentration of Hu-MPC2, since no protein can be visualized on the Coomassie staining (Fig. 17), or due to fluorescent overshadowing exposure. The antibody detection is possible by the secondary antibody which is conjugated with Horseradish Peroxidase (HRP) enzyme which reacts with a fluorescent dye (Alegria-Schaffer et al., 2009). However, when detecting the fluorescence, the camera exposure can create difficulties to visualize weak signals that are overshadowed by strong fluorescent signals.

The results from the mass spectrometry (Fig. 20) are inconclusive and need further investigation. However, the intensity peak at 31237 m/z from the mass spectrometry (Fig. 20) could be the homodimer of Hu-MPC2. The m/z does not correlate perfectly with the 2x MW of Hu-MPC2, or the MW of Hu-MPC1 and Hu-MPC2. Reasons for the m/z to not be exactly correlating with the MW of the hetero- or homodimer might be explained by ion-suppression or other sources of error (Dietrich A. Volmer, 2006).

7 Conclusion & Future studies

7.1 Conclusion

The presence of Hu-MPC1 and Hu-MPC2 after IMAC with HisTrap in a combined sample suggest that there is a heterodimer complex. The purpose of this project was to study the structure of Hu-MPC, however, no purified functional formation of the MPC complex could be obtained.

7.2 Future studies

Further research is desired with Hu-MPC1 induction for 24 hours to see if it is possible to produce a larger amount of protein. Further assays are necessary with Hu-MPC1 and Hu-MPC2 and as a complex, to gain more knowledge regarding the complex formation of the two subunits and their dimerization capabilities. Lastly, further studies are essential with purification optimization of Hu-MPC1 and Hu-MPC2, and usage of Cryo-EM or X-ray crystallography to solve the structure of the Hu-MPC complex and subunits.

8 Acknowledgements

I would like to thank my co-supervisor Dr. Tamim Al Jubair for all the guidance and help in the lab. I would like to thank my supervisor Prof. Susanna Horsefield for all the input and planning of the project. I want to thank Dr. Katja Bernfur for assisting with the mass spectrometry. Lastly, I would like to thank (soon to be Dr.) Carl Johan Hagströmer for all the good and bad life advice.

9 References

Alegria-Schaffer, A., Lodge, A., & Vattem, K. (2009). Chapter 33 Performing and Optimizing Western Blots with an Emphasis on Chemiluminescent Detection. In *Guide to Protein Purification, 2nd Edition* (pp. 573-599). https://doi.org/10.1016/s0076-6879(09)63033-0

Berg, J. M. (2019). *Biochemistry* (9th ed.). W.H. Freeman and Company.

Bio-Rad Laboratories Inc. (n.d-a). *Fast Protein Liquid Chromatography*. Bio-Rad Laboratories. Retrieved 29/12 from https://www.bio-rad.com/en-se/applications-technologies/fast-protein-liquid-chromatography?ID=MWHBF4CZF

Bio-Rad Laboratories Inc. (n.d-b). *Liquid Chromatography Principles*. Bio-Rad Laboratories. Retrieved 2021-12-29 from https://www.bio-rad.com/en-se/applications-technologies/liquid-chromatography-principles?ID=MWHAS7E8Z

Bricker, D. K., Taylor, E. B., Schell, J. C., Orsak, T., Boutron, A., Chen, Y.-C., Cox, J. E., Cardon, C. M., Vranken, J. G. V., Dephoure, N., Redin, C., Boudina, S., Gygi, S. P., Brivet, M., Thummel, C. S., & Rutter, J. (2012). A Mitochondrial Pyruvate Carrier Required for Pyruvate Uptake in Yeast, Drosophila, and Humans. *Science*, *337*(6090), 96-100. https://doi.org/doi:10.1126/science.1218099

Dietrich A. Volmer, L. L. J. (2006). Ion Suppression: A Major Concern in Mass Spectrometry. *LCGC North America*, *24*(5), 498-510. https://www.chromatographyonline.com/view/ion-suppression-major-concern-mass-spectrometry

Divakaruni, A. S., Wiley, S. E., Rogers, G. W., Andreyev, A. Y., Petrosyan, S., Loviscach, M., Wall, E. A., Yadava, N., Heuck, A. P., Ferrick, D. A., Henry, R. R., McDonald, W. G., Colca, J. R., Simon, M. I., Ciaraldi, T. P., & Murphy, A. N. (2013). Thiazolidinediones are acute, specific inhibitors of the mitochondrial pyruvate carrier. *Proceedings of the National Academy of Sciences U S A*, *110*(14), 5422-5427. https://doi.org/10.1073/pnas.1303360110

Drocourt, D., Calmels, T., Reynes, J. P., Baron, M., & Tiraby, G. (1990). Cassettes of the Streptoalloteichus hindustanus ble gene for transformation of lower and higher eukaryotes to phleomycin resistance. *Nucleic acids research*, *18(13)*, 4009. https://doi.org/https://doi.org/10.1093/nar/18.13.4009

Gatignol, A., Baron, M. & Tiraby, G. (1987). Phleomycin resistance encoded by the ble gene from transposon Tn 5 as a dominant selectable marker in Saccharomyces cerevisiae. *Molecular and General Genetics*, *207*, 342-348. https://doi.org/https://doi.org/10.1007/BF00331599

Ghosh, A., Tyson, T., George, S., Hildebrandt, E. N., Steiner, J. A., Madaj, Z., Schulz, E., Machiela, E., McDonald, W. G., Galvis, M. L. E., Kordower, J. H., Raamsdonk, J. M. V., Colca, J. R., & Brundin, P. (2016). Mitochondrial pyruvate carrier regulates autophagy, inflammation, and neurodegeneration in experimental models of Parkinson's disease. *Science Translational Medicine*, *8*(368), 368ra174-368ra174. https://doi.org/doi:10.1126/scitranslmed.aag2210

Gray, L. R., Tompkins, S. C., & Taylor, E. B. (2014). Regulation of pyruvate metabolism and human disease. *Cellular and Molecular Life Sciences*, *71*(14), 2577-2604. https://doi.org/10.1007/s00018-013-1539-2

Halestrap, A. P. (1975). The mitochondrial pyruvate carrier. Kinetics and specificty for substrates and inhibitors. *The Biochemical journal*, *148(1)*, 85–96. https://doi.org/https://doi.org/10.1042/bj1480085

Halestrap, A. P., & Denton, R. M. (1974). Specific inhibition of pyruvate transport in rat liver mitochondria and human erythrocytes by alpha-cyano-4-hydroxycinnamate. *The*

Biochemical journal, 138(2), 313–316. https://doi.org/https://doi.org/10.1042/bj1380313
Herzig, S., Raemy, E., Montessuit, S., Veuthey, J.-L., Zamboni, N., Westermann, B., Kunji, E. R. S., & Martinou, J.-C. (2012). Identification and Functional Expression of the Mitochondrial Pyruvate Carrier. *Science, 337*(6090), 93-96. https://doi.org/doi:10.1126/science.1218530
Hildyard, J. C., Ammala, C., Dukes, I. D., Thomson, S. A., & Halestrap, A. P. (2005). Identification and characterisation of a new class of highly specific and potent inhibitors of the mitochondrial pyruvate carrier. *Biochimica et Biophysica Acta, 1707*(2-3), 221-230. https://doi.org/10.1016/j.bbabio.2004.12.005
Hofmann, A., & Clokie, S. (Eds.). (2018). *Wilson and Walker's Principles and Techniques of Biochemistry and Molecular Biology* (8 ed.). Cambridge University Press. https://doi.org/10.1017/9781316677056.
Hohmann, S. (1987). A region in the yeast genome which favours multiple integration of DNA via homologous recombination. *Current Genetics, 12*, 519-256. https://doi.org/https://doi.org/10.1007/BF00419561
Invitrogen. (2010). *EasySelect™ Pichia Expression Kit: For expression of recombinant proteins using pPICZ and pPICZα in Pichia pastoris: User manual (MAN0000042)*. https://tools.thermofisher.com/content/sfs/manuals/easyselect_man.pdf
Lee, J., Jin, Z., Lee, D., Yun, J. H., & Lee, W. (2020). Characteristic Analysis of Homo- and Heterodimeric Complexes of Human Mitochondrial Pyruvate Carrier Related to Metabolic Diseases. *International journal of molecular sciences, 21*(9). https://doi.org/10.3390/ijms21093403
Life-technologies. (2012). *NativePAGE™ Novex® Bis-Tris Gel System: A system for native gel electrophoresis*.
Omega Bio-tek. (2019). *E.Z.N.A. Cycle Pure Kit*.
Papa, S., Francavilla, A., Paradies, G., & Meduri, B. (1971). The transport of pyruvate in rat liver mitochondria. *FEBS letters, 12*(5), 285-288. https://doi.org/https://doi.org/10.1016/0014-5793(71)80200-4
Rauckhorst, A. J., & Taylor, E. B. (2016). Mitochondrial pyruvate carrier function and cancer metabolism. *Current Opinion in Genetics & Development, 38*, 102-109. https://doi.org/10.1016/j.gde.2016.05.003
Roy, A. (2015). Membrane preparation and solubilization. *Methods in Enzymology, 557*, 45-56. https://doi.org/10.1016/bs.mie.2014.11.044
Schmidt, T. G., Batz, L., Bonet, L., Carl, U., Holzapfel, G., Kiem, K., Matulewicz, K., Niermeier, D., Schuchardt, I., & Stanar, K. (2013). Development of the Twin-Strep-tag(R) and its application for purification of recombinant proteins from cell culture supernatants. *Protein Exprssion and Purification, 92*(1), 54-61. https://doi.org/10.1016/j.pep.2013.08.021
Schmidt, T. G., & Skerra, A. (2007). The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nature Protocols, 2*(6), 1528-1535. https://doi.org/10.1038/nprot.2007.209
Seddon, A. M., Curnow, P., & Booth, P. J. (2004). Membrane proteins, lipids and detergents: not just a soap opera. *Biochimica et Biophysica Acta, 1666*(1-2), 105-117. https://doi.org/10.1016/j.bbamem.2004.04.011
Sigma-Aldrich. (2014). *GenElute Plasmid Miniprep Kit*.
Smith, R. W. (2013). Mass Spectrometry. In *Encyclopedia of Forensic Sciences* (pp. 603-608). https://doi.org/10.1016/b978-0-12-382165-2.00250-6
ThermoFisher-Scientific. (2002). *Pichia Fermentation Process Guidelines*.

ThermoFisher-Scientific. (2015). *Thermo Scientific GeneJET Gel Extraction Kit #K0691, #K0692*.
ThermoFisher-Scientific. (2021). *Phusion™ High–Fidelity DNA Polymerase*.
Titheradge, M. A., & Coore, H. G. (1976). Hormonal regulation of liver mitochondrial pyruvate carrier in relation to gluconeogenesis and lipogenesis. *FEBS letters*, *72(1)*, 73–78. https://doi.org/https://doi.org/10.1016/0014-5793(76)80901-5
UniProt. (n.d-a). *UniProtKB - Q9Y5U8 (MPC1_HUMAN)*. Retrieved May 9 2022 from https://www.uniprot.org/uniprot/Q9Y5U8
UniProt. (n.d-b). *UniProtKB - Q95563 (MPC2_HUMAN)*. Retrieved May 9 2022 from https://www.uniprot.org/uniprot/O95563
Vogl, T., & Glieder, A. (2013). Regulation of Pichia pastoris promoters and its consequences for protein production. *New Biotechnology*, *30*(4), 385-404. https://doi.org/10.1016/j.nbt.2012.11.010
Wagner, S., Baars, L., Ytterberg, A. J., Klussmeier, A., Wagner, C. S., Nord, O., Nygren, P. A., van Wijk, K. J., & de Gier, J. W. (2007). Consequences of membrane protein overexpression in Escherichia coli. *Mollecular & Cellular Proteomics*, *6*(9), 1527-1550. https://doi.org/10.1074/mcp.M600431-MCP200
Walker, J. M. (Ed.). (2005). *The Proteomics Protocols Handbook*. Humana Press.
Xu, L., Phelix, C. F., & Chen, L. Y. (2021). Structural Insights into the Human Mitochondrial Pyruvate Carrier Complexes. *Journal of Chemical Information and Modeling*, *61*(11), 5614-5625. https://doi.org/10.1021/acs.jcim.1c00879

10 Appendix I

10.1 Protocols

10.1.1 Hu-MPC Polymerase Chain Reaction (PCR)

Prepare PCR reactions. Add the following components in the order listed in the following table, Tab. 1, and run the PCR according to the settings in Tab. 2, Appendix I:

Table 1: The components and amounts of the PCR reaction for Hu-MPC1 gene.

Component	50µL	Final concentration
H_2O	Add to 50 µL	
5X Phusion™ HF Buffer	10 µL	1X
10 mM dNTPs	1 µL	200 µL each
10 µM Forward primer	2.5 µL	0.5 µL
10 µM Reverse primer	2.5 µL	0.5 µL
Template DNA	1 µL	10 ng
Phusion™ High–Fidelity DNA Polymerase	0.5 µL	0.02 U/µL

Table 2: The settings for the PCR of the Hu-MPC1 gene with primers for N- and C-terminal Strep-tag II.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98°C	1 min	1
Denaturation	98°C	5 s	25-35
Annealing	65°C	10 s	
Extension	72°C	15 s	
Final Extension	72°C	5 min	1
Hold	4°C	Hold	Hold

(ThermoFisher-Scientific, 2021).

10.1.2 E.Z.N.A. Cycle Pure Kit

Perform agarose gel electrophoresis to analyse PCR product. Determine the volume of your PCR reaction. Transfer the sample into a clean 1.5 mL microcentrifuge tube (not provided). Add 4-5 volumes CP Buffer. For PCR products smaller than 200 bp, add 5 volumes CP Buffer and 0.4 volumes 100% isopropanol. Note: Volume refers to the size of your PCR reaction. Vortex to mix thoroughly. Briefly centrifuge to collect any drops from the inside of the lid. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube (provided). Add the sample from Step 5 to the HiBind® DNA Mini Column. Centrifuge at maximum speed (≥13,000 x g) for 1 minute at room temperature. Discard the filtrate and reuse collection tube. Add 700 µL DNA Wash Buffer. Note: DNA Wash Buffer must be diluted with 100% ethanol before use. Centrifuge at maximum speed for 1 minute. Discard the filtrate and reuse collection tube. Repeat wash steps for a second DNA Wash Buffer wash. Centrifuge the empty HiBind® DNA Mini Column at maximum speed for 2 minutes to dry the column. Note: This step is critical for removal of trace ethanol that may interfere with downstream applications. Transfer the HiBind® DNA Mini Column into a clean 1.5 mL microcentrifuge tube (not provided). Add 30-50 µL Elution Buffer, TE Buffer, or sterile deionized water directly to the centre of column matrix. Let sit at room temperature for 2 minutes. Centrifuge at maximum speed for 1 minute. Note: This represents approximately 80-90% of bound DNA. An optional second elution will

yield any residual DNA, though at a lower concentration. Store DNA at -20°C (Omega Bio-tek, 2019). The PCR clean-up kit used was Omega Bio-tek, cat#D6492-01.

10.1.3 pPICZ-B Plasmid Digestion

Table 3: The components and concentrations for the digestion reaction of the plasmid pPICZ-B.

Component	20 µl Reaction	Final concentration
Nuclease-free water	0 µL	Up to 20 µL
pPICZB (5 ng/µl)	16 µL	Up to 1 µg
10X Green FD Buffer	2 µL	1X
EcoRI FD	1 µL	Up to RE 1/10 of total
KpnI FD	1 µL	Up to RE 1/10 of total

Mix the reagents (Tab. 3, Appendix I) and incubate at 37 °C for 5-15 min.

10.1.4 Gel DNA Extraction

All the centrifuge steps are to be done >12000 x g.

Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 ml tube and weigh. Record the weight of the gel slice. Add 1:1 volume of Binding Buffer to the gel slice (volume:weight). Incubate the gel mixture at 50-60°C for 10 min or until the gel slice is completely dissolved. Mix the tube by inversion every few minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Transfer up to 800 µL of the solubilized gel solution (from step 3) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. Add 700 µL of Wash Buffer (diluted with ethanol prior to first use) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube. Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube. Add 50 µL of Elution Buffer to the centre of the purification column membrane. Centrifuge for 1 min (ThermoFisher-Scientific, 2015). The gel extraction kit used was Thermo Scientific, cat#K0691.

10.1.5 Ligation of Vector and insert

Mix the reagents (Tab. 4, Appendix I) in PCR tubes.

Table 4: The components for the ligation of pPICZ-B with N- and C-terminal Strep-tag II Hu-MPC1 gene.

Ratio vector:insert	1:0 (negative control)	1:3 (N-terminal)	1:3 (C-terminal)
Vector (pPICZ-B)	5 µL (6 ng/µL)	5 µL (6 ng/µL)	4 µL (3.8 ng/µL)
Cut insert	0 µL	1 µL (89.7 ng/µL)	1 µL (50.2 ng/µL)
10 X T4 DNA ligase buffer	2 µL	2 µL	2 µL
T4 DNA ligase	1 µL	1 µL	1 µL
Nuclease-free water (up to 20 µl)	12 µL	11 µL	12 µL

Incubation 1h at 22°C or 16℃ o/n, then transformation of 3 µL the ligation product in 50 µL *E. coli*. Keep the rest at -20℃.

10.1.6 Transformation into *E. coli*

Thaw competent cells on ice. Chill approximately 5 µL of the ligation mixture in a 1.5 mL micro centrifuge tube. Add 30 µL of competent cells to the DNA. Mix gently by pipetting up and down or flicking the tube 4-5 times to mix the cells and DNA. Do not vortex. Place the mixture on ice for 30 minutes. Do not mix. Heat shock at 42℃ for 45 seconds. Do not mix. Add 965 µL of room temperature low salt LB medium. Do not mix. Place tube at 37℃ for 60 minutes. Shake vigorously (250) rpm or rotate. Warm low salt agar plates to 37℃. Spread 100 µL of the cells and ligation mixture onto the plates, centrifuge the remaining sample at 5000 rpm for 5 min, then remove 800 µL of supernatant and resuspend the pellet in 100 µL and spread onto the plates. Incubate overnight at 37℃.

10.1.7 Colony Polymerase Chain Reaction (PCR)

Prepare PCR reactions. Add the following components in the order listed in the following table, Tab. 5, and run PCR according to the settings in Tab. 6, Appendix I:

Table 5: The components for the PCR reaction for the colony PCR of the Hu-MPC1 gene with primers for N- and C-terminal Strep-tag II.

Component	20µL	Final concentration
H_2O	Add to 20 µL	
5X Phusion™ HF Buffer	4 µL	1X
10 mM dNTPs	0.4 µL	200 µL each
10 µM Forward primer	0.2 µL	0.5 µL
10 µM Reverse primer	0.2 µL	0.5 µL
Template DNA	2 µL	10 ng
Phusion™ High–Fidelity DNA Polymerase	0.2 µL	0.02 U/µL

Table 6: The settings for the colony PCR of the Hu-MPC1 gene with primers for N- and C-terminal Strep-tag II.

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	31
Annealing	48°C	10 s	
Extension	72°C	15 s	
Final Extension	72°C	10 min	1
Hold	4°C	Hold	Hold

(ThermoFisher-Scientific, 2021).

10.1.8 Sigma Aldrich GenElute Plasmid Miniprep Kit Protocol

All the centrifuge steps are to be done >12000 x g.

Harvest cells:

Pellet 1–5 ml of an overnight recombinant E. coli culture by centrifugation. The optimal volume of culture to use depends upon the plasmid and culture density. For best yields, follow the instructions in the note below. Transfer the appropriate volume of the recombinant E. coli culture to a microcentrifuge tube and pellet cells at ≥12,000 x g for 1 minute. Discard the supernatant.

Resuspend cells:

Completely resuspend the bacterial pellet with 200 µL of the Resuspension Solution. Vortex or pipette up and down to thoroughly resuspend the cells until homogeneous. Incomplete resuspension will result in poor recovery.

Lyse cells:

Lyse the resuspended cells by adding 200 µL of the Lysis Solution. Immediately mix the contents by gentle inversion (6–8 times) until the mixture becomes clear and viscous. Do not vortex. Harsh mixing will shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA. Do not allow the lysis reaction to exceed 5 minutes. Prolonged alkaline lysis may permanently denature supercoiled plasmid DNA that may render it unsuitable for most downstream applications.

Neutralize:

Precipitate the cell debris by adding 350 µL of the Neutralization/Binding Solution. Gently invert the tube 4–6 times. Pellet the cell debris by centrifuging at ≥12,000 x g or maximum speed for 10 minutes. Cell debris, proteins, lipids, SDS, and chromosomal DNA should fall out of solution as a cloudy, viscous precipitate.

Prepare column:

Insert a GenElute Miniprep Binding Column into a provided microcentrifuge tube, if not already assembled. Add 500 µL of the Column Preparation Solution to each miniprep column and centrifuge at ≥12,000 x g for 30 seconds to 1 minute. Discard the flow-through liquid.

Load cleared lysate:

Transfer the cleared lysate from step 3 to the column prepared in step 4 and centrifuge at ≥12,000 x g for 30 seconds to 1 minute. Discard the flow-through liquid.

Wash column:

Add 750 µL of the diluted Wash Solution to the column. Centrifuge at ≥12,000 x g for 30 seconds to 1 minute. The column wash step removes residual salt and other contaminants introduced during the column load. Discard the flow-through liquid and centrifuge again at maximum speed for 1 to 2 minutes without any additional Wash Solution to remove excess ethanol.

Elute DNA:

Transfer the column to a fresh collection tube. Add 100 µL of Elution Solution or molecular biology reagent water to the column. For DNA sequencing and other enzymatic applications, use water or 5 mM Tris-HCl, pH 8.0, as an eluant. Centrifuge at ≥12,000 x g for 1 minute. The DNA is now present in the eluate and is ready for immediate use or storage at –20 °C (Sigma-Aldrich, 2014). The plasmid extraction kit used was Sigma Aldrich, cat#PLN70-1KT.

10.1.9 Transformation into *Pichia pastoris* x33

Linearization of DNA:

Cut 5-10 ug of DNA (58 µL) with 5 µL of Fast digest (FD) SacI in 7 µL FD buffer, incubate at 37℃ for 1h. Run agarose gel to verify the enzyme cut the plasmid. Purify with PCR clean up kit (E.Z.N.A. Cycle Pure Kit, Appendix I), elute with 50 µL 65℃ MQ water. Concentrate DNA so that concentration is >250 ng/µL using a speed vac.

Preparation of Competent Cells:

Prepare 5 mL *P. pastoris* x33 overnight culture in *YPD medium*. Inoculate 100 mL *YPD* medium, grow until OD_{600} = 1.3. Transfer cells into two 50 mL Falcon tubes. Put on ice and work on ice from now on. Spin down cells at 478 x g (5 min) 4℃. Remove supernatant and wash cells with 100 mL autoclaved ice cold MilliQ water. Spin down cells at 478 x g (5 min) 4℃. Remove supernatant and wash cells with 50 mL Autoclaved ice cold MilliQ water. Spin down cells at 478 x g (5 min) 4℃. Remove supernatant and wash cells with 5 mL Autoclaved ice cold 1M Sorbitol. Resuspend cells in 350 µL 1M ice cold Sorbitol. Put on ice for now.

Electroporation:

Mix 20 µL SacI-digested DNA (total amount should be >5µg) with 80 µL cold cells in a sterile Eppendorf tube. Transfer to electroporation cuvette. Electroporate according to the pre-installed *Pichia* settings on the machine. Add 1 mL ice cold 1M Sorbitol immediately after electroporation. Transfer sample to a sterile 12 mL culture tube, incubate at 30℃ for 1h (no shaking). Add 1 mL *YPD medium*, incubate at 30℃ for 30 min (shaking). Plate on *YPD agar* plates containing 100 µg/mL Zeocin. For each construct make three plates, add 25 µL electroporated cells to one and 250 µL to the other, then spin down the cells at 3000 rpm for 5 min and remove supernatant, resuspend in 50 µL *YPD medium* and plate. Incubate the plates at 30℃ for 2 days.

Colony selection:

Prepare 2000 µg/mL Zeocin plate. Draw a numbered grid on the petri dish. Using a 10 µL pipette tip, spot colonies from the transformation plates onto the high-Zeocin plate.

10.1.10 Small scale expression in *Pichia pastoris*

One colony in 5 mL *Buffered Glycerol-complex medium* (Tab. 24, Appendix I) in 50 mL falcon tube overnight at 250 rpm rotation at 30℃. Check the OD_{600} and dilute the OD_{600} to 1.0 in 5 mL *Buffered Methanol-complex medium* (Tab. 25, Appendix I) and grow for 24 hours. After 24 hours of growth take out 1 mL from each sample to use for SDS-PAGE and then add 20 µL of 100% methanol to the original culture and grow for 24 hours. Take 1 mL of the OD_{600} = 2 culture in 1.5 mL Eppendorf tube. Centrifuge at 3000 rpm for 5 min. Remove the supernatant and freeze the pellet for storage.

10.1.11 Yeast sample preparation for SDS PAGE followed by Western blot

Thaw cell pellets quickly and place on ice. For each sample, add 100 µL *Breaking buffer 1* (Tab. 26, Appendix I) to the cell pellet and resuspend. Add an equal volume of acid-washed glass beads (size 0.5mm). Estimate equal volume by displacement. Vortex 30 seconds, then incubate on ice for 30 seconds. Repeat for a total of 8 cycles. Remove the glass beads and centrifuge at max (10,000 x g) speed for 10 min at room temperature. Transfer the clear supernatant to a fresh microcentrifuge tube. To pellet crude membranes, centrifuge the supernatant at 20,000 x g for 1 h in a desktop centrifuge. Remove the supernatant. Resuspend crude membrane pellet in 30 µL of 2X SDS loading buffer with DTT.

10.1.12 Western Blot

Western blot: Soak cotton pads, filter paper, and the Nitrocellulose-membrane in *NuPAGE transfer buffer* (Tab. 27, Appendix I). Compress the gel between two filter papers and Nitrocellulose-membrane, (with cotton pads being the filler which creates pressure) and run for 1 h at 25V, 160mA. Incubate the blotted membrane in 5% milk solution with *TBS-T* (Tab. 28, Appendix I) for 30 min on the shaker. Add the primary antibody to the 5% milk solution with *TBS-T* and incubate on the shaker for 1 hour in room temperature or 4℃ overnight. Wash the membrane with *TBS-T* on the shaker for 5 min, repeat 3 times. Incubate with the secondary antibody in a 5% milk solution with *TBS-T* on the shaker for 1 hour in room temperature. Wash the membrane with *TBS-T* on the shaker for 5 min, repeat 3 times.

The same procedure is followed for Native-PAGE western blot except with PVDF-membrane instead of Nitrocellulose-membrane, and the PVDF-membrane should be soaked in ethanol for 30 seconds prior to the blot.

10.1.13 *Pichia pastoris* Fermentation in Fermenter

Day 1:

Prepare a *YPD agar* plate without antibiotics, and 100 mL *YPD medium* in a 250 mL shaking flask. Streak yeast on the *YPD agar* plate once cool and grow at 30℃ for 48 hours.

Day 4:

Swab cells from plate and resuspend a decent lump of cells into 100 mL *YPD medium* in a 250 mL flask. Grow at 30℃ overnight. OD600 = 25 is required on day 5. Assemble the fermenter and pour the *Basal salt medium* (BSM) (Tab. 29, Appendix I) into it.

Autoclave the following overnight:

- Fermenter, containing *BSM*
- Glycerol tubing and bottle lid (which has a filter on it), both in aluminium foil
- 50% glycerol (200 mL)
- An empty 500 mL bottle

Do not autoclave the following:

- Beige HCl tubing
- Beige ammonia tubing
- pH electrode

Day 5:

Aim to inoculate the fermenter at 16:00, as the glycerol will then be depleted by 7-8:00 the following day. Assemble the fermenter, connect all tubing, add oil before inserting the thermometer, put on the heating sleeve, and switch on the gas and a gentle flow of water. Calibrate the pH electrode through two-point calibration. Once calibrated, store and set as default. Wash the pH electrode with 70% ethanol, air dry for a few seconds, and insert it carefully into the port you reserved for it. Calibrate the oxygen detector through two-point calibration. Insert needles (base, acid, and 2.5 mL syringe with 1.5 mL anti-foam) into membranes. Adjust the pH to 5 with the base.

Add the following:

- *PTM* 6.5 mL (Tab. 30, Appendix I)
- Anti-foam 3 drops
- Culture 100 mL

Run overnight with following settings:

- Temp = 30℃
- pH = 5.0
- D0 = 25
- Gas = 1
- PU = 0
- PU = 0
- Stir = 800 – 1500

Day 6:

Around 7-8:00, the glycerol will have been consumed, generating a spike in dissolved oxygen (DO). Set PU4 to 10 and switch it on. Throughout the day, slowly increase the feed on PU4 from 10 to 27. To see if it is time to increase the feed, switch off PU4, and see if DO spikes within a minute. If it does, increase the feed. Do not increase the feed too much; you want to keep the DO around 20. Once the bottle of glycerol is depleted, switch it with the methanol and *PTM*. Set PU4 to 2.

Day 7:

Throughout the day, gradually increase the feed of PU4 to 7. Check the DO spike. It should be below 30%. Once stable, you can leave it overnight. The gas and stirring will be quite high. Make sure there is enough methanol left so that the bottle will not be depleted during the night.

Day 8:

In the afternoon, turn everything off but the stirrer and gas (leave at 500 rpm and 1, respectively). Take the tube from the sample collector and aim it into a centrifuge bottle. Place a clamp on the tube going from the condenser to the 5 L flask. Cells will now flow. Fill the centrifuge bottles to 75% of the total volume, balance them, and centrifuge at 6000 rpm for 20-30 minutes. Once pelleted, discard the supernatant, and spoon the cells into a large plastic Ziploc bag. Scrape out the last bits with a spatula. Flatten the contents and put it in the freezer.

10.1.14 Dot Blot

Thaw cell pellet quickly and place on ice. Add 400 µL *Breaking buffer 2* (Tab. 31, Appendix I) to the cell pellet and resuspend. Add an equal amount of acid-washed glass beads. Vortex 30 seconds, then incubate on ice for 30 seconds, repeat for a total of 8 cycles. Centrifuge at 10 000 x g for 10 minutes. Transfer the clear supernatant to a fresh microcentrifuge tube. Use a strip of Nitrocellulose-membrane. Blot 5 µL of 3 different concentrations (1:1, 1:2, 1:4) of positive control protein onto the membrane. Blot 5 µL of 3 different concentrations (1:1, 1:2, 1:4) of cell lysate onto the membrane. Blot 5 µL of 100 µg/mL of primary antibody (anti-Strep Tag II polyclonal antibody) onto the membrane. Incubate the membrane for 1 hour at room temperature. Ensure that the blots are dry before going to the next step. Soak the blotted membrane in 5% milk solution with *TBS-T* for 30 min on the shaker. Incubate with primary antibody (rabbit anti-Strep tag II polyclonal, 1:2000 dilution) in a 5% milk solution with *TBS-T* on the shaker for 1 hour in room temperature. Wash the membrane with *TBS-T* on the shaker for 5 min, repeat 3 times. Incubate with secondary antibody (goat anti-rabbit IgG (H+L)-HRP conjugated, 1:2000 dilution) in a 5% milk solution with *TBS-T* on the shaker for 1 hour in room temperature. Wash the membrane with *TBS-T* on the shaker for 5 min, repeat 3 times.

10.1.15 Membrane preparation

Cell breaking and membrane preparation:

Thaw 100 g *P. pastoris* cells while stirring in 200 mL *Breaking buffer 2*. Bead beating: Use 200 mL acid-washed glass beads (0.5 mm). Add the yeast cell suspension and fill up with *breaking buffer 2* and add 1 mM PMSF to inhibit proteases. Run the bead beating for 12 x 30 seconds with 30 seconds pause between each run. Centrifuge: Decant into medium-size flasks. Wash beads with *breaking buffer 2* and collect this as well. Using a JLA-10,500 rotor, run at 9,500 rpm at 4°C for 40 min. Ultracentrifuge: Transfer supernatant to Ti45 tubes. Using a Ti45 rotor, centrifuge at 40,000 rpm at 4°C for 2 hours. Discard the supernatant and weigh the pellets. Homogenize pellets using a potter homogenizer with *Urea buffer* (Tab. 33, Appendix I). If you have less than 8 g of pellets, use one tube, if more; two tubes. Ultracentrifuge: with Ti45 rotor at 40,000 rpm at 4°C for 2 hours. Carefully remove the supernatant. Homogenize the pellets in *Membrane buffer* (Tab. 33, Appendix I) + 1 mM PMSF + 2 mM EDTA. Ultracentrifuge: with Ti45 rotor at 40,000 rpm at 4°C for 1.5 hours. Remove supernatant, weigh pellet, and homogenize pellet in 2 mL Membrane buffer / gram membrane. Flash freeze membranes in liquid nitrogen and save at -80°C.

10.1.16 Coomassie blue

Coomassie blue staining: Put the gel in a plastic container and fill it with water. Microwave the container for 1 min at 700W, and then shake it for 1 min. Repeat these 3 steps 3 times. Cover the gel with Coomassie blue, microwave for 30 seconds and then shake for 15 min. De-stain the gel by adding deionized water and shaking for 15 min. The Coomassie blue dye used was Novex, cat#LC6065.

10.1.17 Native-PAGE

Prepare samples according to Tab. 8, Appendix I:

Table 8: The components for sample preparation for Native-PAGE with samples in detergent.

Components	Sample with Detergent
Sample	10 µL
Native-PAGE Sample Buffer (4X) (Tab. 34, Appendix I)	2.5 µL
Native-PAGE 5% G-250 Sample Additive	0.25-1 µL *
Deionized Water	To 10 µL
*Ensure that the final G-250 concentration is 1/4th the detergents concentration. Do not heat samples for native gel electrophoresis. Prepare 1X Sample Buffer for dilutions if needed.	

Remove the comb and rinse the gel wells three times with 1X Native-PAGE Dark Blue Cathode Buffer (95% H_2O, 5% *Native-PAGE Running buffer* (Tab. 35, Appendix I), 5% *Native-PAGE Cathode Additive* (Tab. 36, Appendix I)). Remove the white tape near the bottom of the gel cassettes. Place the gels in the XCell SureLock Mini-Cell gel running tank. Fill the gel wells with 1X Native-PAGE Dark Blue Cathode Buffer. Load samples into sample wells filled with 1X Native-PAGE Dark Blue Cathode Buffer prior to filling the cathode chamber to better visualize the sample wells. Load an appropriate volume and protein mass of samples on the gel. Then load your standards. Fill the Upper Cathode Buffer Chamber with 200 mL 1X Native-PAGE Dark Blue Cathode Buffer and fill the Lower Anode Buffer Chamber with 550 mL Native-PAGE Anode Buffer (5% *Native-PAGE Running buffer*, 95% H_2O). Run the 4-16% Bis-Tris gel (Invitrogen, cat#BN1004BOX) in 4°C with pre-chilled buffers, 150V constant for 60 minutes and then 250V for 40 minutes. After the sample has progressed 1/3 of the gel, replace the Dark Blue Cathode Buffer with Light Blue Cathode Buffer (95% H_2O, 5% *Native-PAGE Running buffer*, 0.5% *Native-PAGE Cathode Additive*) (Life-technologies, 2012).

10.1.18 Native-PAGE gel Coomassie Staining

Place gel in 100 mL of Fix Solution (40% methanol, 10% acetic acid) and microwave on high (950-1100) watts for 45 seconds. Shake the gel on an orbital shaker for 15-30 minutes at room temperature. Decant Fix Solution. Repeat step 1 and 2 once. Add 100 mL Coomassie R-250 stain (0.02% Coomassie R-250 in 30% methanol and 10% acetic acid) and microwave on high (950-1100 watts) for 45 seconds. Shake the gel on an orbital shaker for 15-30 minutes. Decant the stain. Add 100 mL De-stain Solution (8% acetic acid) and microwave on high (950-1100 watts) for 45 seconds. Shake the gel on an orbital shaker at room until the desired background is obtained (Life-technologies, 2012).

10.2 Buffers & Sequences

Table 9: The amino acid sequence of the human mitochondrial pyruvate carrier 1.

Amino acid sequence of Human mitochondrial pyruvate carrier 1; 109AA
MAGALVRKAADYVRSKDFRDYLMSTHFWGPVANWGLPIAAINDMKKSPEIISGRMTFALC CYSLTFMRFAYKVQPRNWLLFACHATNEVAQLIQGGRLIKHEMTKTASA

Table 10: The amino acid sequence of the human mitochondrial pyruvate carrier 2.

Amino acid sequence of Human mitochondrial pyruvate carrier 2; 127AA
MSAAGARGLRATYHRLLDKVELMLPEKLRPLYNHPAGPRTVFFWAPIMKWGLVCAGLADM ARPAEKLSTAQSAVLMATGFIWSRYSLVIIPKNWSLFAVNFFVGAAGASQLFRIWRYNQE LKAKAHK

Table 11: The forward and reverse primer for N-terminal Strep-tag II, which was used to replace the current His-tag on the Hu-MPC1 gene.

Forward and Reverse primer for N-terminal Strep-tag II
>Forward GGCGAATTCACGATGTGGAGCCACCCCCAGTTCGAGAAGGATTACGATATCCCAACTACCC TGGTGCCCAGGGGCAGCGCTGGTGCATTGGTTAGAA >Reverse CTTGGTACCTCATGCTGAAGCTGTCTTAGTCATTTC

Table 12: The forward and reverse primer for C-terminal Strep-tag II, which was used to replace the current His-tag on the Hu-MPC1 gene.

Forward and Reverse primer for C-terminal Strep-tag II
>Forward GGCGAATTCACGATGGCTGGTGCATTGGTTAGAA >Reverse CTTGGTACCTCACTTCTCGAACTGGGGGTGGCTCCAGCTGCCCCTGGGCACCAGTGTAGTT GGGATATCGTAATC

Table 13: The reagents and concentrations to make yeast extract peptone dextrose agar.

Yeast Extract Peptone Dextrose (YPD) Agar
1% Yeast extract 2% Peptone 2% Dextrose 2% Agar

Table 14: The reagents and concentrations to make yeast extract peptone dextrose medium.

Yeast Extract Peptone Dextrose (YPD) Medium
1% Yeast extract 2% Peptone 2% Dextrose

Table 15: The reagents and concentrations to make Solubilization buffer.

Solubilization buffer
20 mM Tris-HCl pH 8
300 mM NaCl
4% DDM
1 mM PMSF

Table 16: The reagents and concentrations to make StrepTrap buffer A.

Buffer A1 Strep
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM
1 mM EDTA

Table 17: The reagents and concentrations to make StrepTrap buffer B.

Buffer B1 Strep
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM
1 mM EDTA
2.5 mM Desthiobiotin

Table 18: The reagents and concentrations to make HisTrap buffer A.

Buffer A His
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM

Table 19: The reagents and concentrations to make HisTrap buffer B.

Buffer B His
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM
300 mM Imidazole

Table 20: The reagents and concentrations to make StrepTrap buffer A.

Buffer A2 Strep
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM

Table 21: The reagents and concentrations to make StrepTrap buffer B.

Buffer B2 Strep
20 mM Tris-HCl pH 8
300 mM NaCl
5% Glycerol
0.05% DDM
2.5 mM Desthionbiotin

Table 22: The reagents and concentrations to make Low salt LB agar.

Low salt LB Agar
1% Tryptone
0.5% NaCl
0.5% Yeast extract
15g/L Bacto Agar

Table 23: The reagents and concentrations to make Low salt LB medium.

Low salt LB medium
1% Tryptone
0.5% NaCl
0.5% Yeast extract

Table 24: The reagents and concentrations to make Buffered Glycerol-complex medium.

Buffered Glycerol-complex Medium (BMGY)
2% Peptone
1% Yeast extract
100 mM Potassium Phosphate pH 6.0
1.34% Yeast Nitrogen Base with Ammonium Sulphate without amino acids
4 x 10^{-5}% Biotin
1% Glycerol

Table 25: The reagents and concentrations to make Buffered Methanol-complex medium.

Buffered Methanol-complex Medium (BMMY)
2% Peptone
1% Yeast extract
100 mM Potassium Phosphate pH 6.0
1.34% Yeast Nitrogen Base with Ammonium Sulphate without amino acids
4 x 10^{-5}% Biotin
0.5% Methanol

Table 26: The reagents and concentrations to make Breaking buffer 1.

Breaking buffer 1
50 mM Tris-HCl pH 6
5 mM EDTA
10% Glycerol
1X Complete protease inhibitor cocktail tablets

Table 27: The reagents and concentrations to make NuPAGE Transfer buffer.

NuPAGE Transfer buffer
25mM Bicine
25mM Bis-Tris
1mM EDTA
pH 7.2
10% Methanol

Table 28: The reagents and concentrations to make TBS-T buffer.

TBS-T buffer
0.2% Tween-20
20mM Tris-HCl pH 7.4
150mM NaCl

Table 29: The reagents and concentrations to make Basal salt medium.

Basal Salt Medium (BSM)
0.600 g Calcium sulphate
27.30 g Potassium sulphate
22.35 g Magnesium sulphate heptahydrate
6.195 g Potassium hydroxide
60.30 mL Glycerol 99%
40.05 mL Phosphoric acid
-> 1.5 L H_2O

Table 30: The reagents and concentrations to make PTM trace salts.

PTM Trace Salts
0.6 g Cupric sulphate
8 mg Sodium iodide
0.3 g Manganese sulphate x H_2O
0.02 g Sodium molybdate x2 H_2O
2 mg Boric acid
0.05 g Cobalt chloride
2 g Zink chloride
6.5 g Ferrous sulphate x7 H_2O
0.02 g Biotin
500 µL Sulphuric acid
-> 100 mL H_2O

Table 31: The reagents and concentrations to make Breaking buffer 2.

Breaking buffer 2
50 mM Tris-HCl pH 7.5
2 mM EDTA
5% Glycerol

Table 32: The reagents and concentrations to make Urea buffer.

Urea buffer
50 mM Tris-HCl pH 9.5 4 M Urea 2 mM EDTA

Table 33: The reagents and concentrations to make Membrane buffer.

Membrane buffer
20 mM Tris-HCl pH 8 20 mM NaCl 10% Glycerol

Table 34: The reagents and concentrations to make 10 mL Native-PAGE sample buffer 4X.

Native-PAGE Sample Buffer 4X
0.418 g BisTris 0.0535 mL 37% HCl 4 g Glycerol 0.117 g NaCl 0.4 mg Ponceau S 10 mL H_2O

Table 35: The reagents and concentrations to make 500 mL Native-PAGE running buffer 20X.

Native-PAGE Running Buffer 20X
104.6 g BisTris 89.6 g Tricine 500 mL H_2O

Table 36: The reagents and concentrations to make 100 mL Native-PAGE cathode buffer additive 20X.

Native-PAGE Cathode Buffer Additive 20X
0.4 g Coomassie G-250 100 mL H_2O