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Overexpression and Purification of Mouse Divalent Metal Transporter 1 (mDMT1) from *Saccharomyces cerevisiae*

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Abbreviations

CHS = Cholesteryl hemisuccinate
CMC = Critical micelle concentration
CV = Column volume
DDM = n-Dodecyl-β-D-Maltopyranoside
DM = n-Decyl- β-D-Maltopyranoside
DMT1 = Human divalent metal transporter 1
DTT = Dithiothreitol
EDTA = Ethylenediaminetetraacetic acid
FC-12 = Fos-Choline-12
FT = Flow-through
GFP = Green fluorescent protein
IEX = Ion-exchange chromatography
IMAC = Immobilized metal ion affinity chromatography
mDMT1 = Mouse divalent metal transporter 1
MRB = Membrane resuspension buffer
MP = Membrane proteins
NG = n-Nonyl- β-D-Glucopyranoside
NTA = Nitrilotriacetic acid
OD_{600} = Optical density measured at 600 nm
OG = n-Octyl- β-D-Glucopyranoside
OGNG = Octyl Glucose Neopentyl Glycol
PAGE = polyacrylamide gel electrophoresis
PMSF = Phenylmethane sulfonyl fluoride
RB = Resuspension buffer
RFU = Relative fluorescent unit
SBDD = Structure based drug design
SDS = Sodium dodecyl sulfate
SEC = Size-exclusion chromatography
SLC11 = Solute carrier 11 protein family
SN = Substantia nigra pars compacta
TM = Transmembrane α-helix domain
URA = Uracil
v/v = volume per volume
w/v = weight per volume
yEGFP = yeast enhanced green fluorescent protein
YSB = Yeast suspension buffer

Abstract

The membrane integrated divalent metal transporter 1 (DMT1) functions as a transporter of mainly iron across cellular membranes within the human body. Studies indicate that dysregulated transport by DMT1 causes accumulation of iron within the midbrain leading to neuronal cell death – a common symptom among patients suffering from Parkinson's disease (PD). Detailed structural information of DMT1 is currently lacking but may aid in drug development for PD. To solve the 3D crystal structure of DMT1, high-purity mono disperse protein must first be obtained. To this end, we expressed mouse DMT1 (mDMT1) in *Saccharomyces cerevisiae* and developed protocols to purify this protein. Based on a detergent screen and our purification trials we find that mDMT1 is soluble in n-dodecyl-β-D-maltpyranoside (DDM) and an addition of cholesteryl hemisuccinate (CHS) increases solubility of the protein in solution. Furthermore, we determine that the highest protein concentration by *S. cerevisiae* is obtained at 23 °C after 43 h. Although none of the performed purification trials was entirely successful at yielding mDMT1 samples suitable for crystallization, we present guidelines as to what areas of the protocol may be optimized to achieve this goal.

Acknowledgement

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1. Introduction

Each cell in our body is enclosed by a hydrophobic lipid bilayer that has an important role in protecting and regulating molecular compounds within the cell [1]. Water and other small uncharged molecules have the ability to cross these lipid bilayers by diffusion. However, larger and hydrophilic molecules cannot pass membranes without assistance from membrane integrated proteins (MP). In addition to their invaluable transporting mechanism, signal transduction and extracellular interaction are other functions representative for MP [1, 2].

Divalent metal-ion transporter 1 (DMT1), also called natural-resistance-associated macrophage protein 2 (Nramp2) or solute carrier family 11 member 2 (SLC11A2), is a membrane integrated protein and a member of the solute carrier 11 (SLC11) family [3]. It is found in many organisms from bacteria to humans [4] where it is mainly expressed in the duodenum, e.g. the microvilli surface and mediates the uptake of divalent cations such as Fe^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+} and Zn^{2+} [5, 6]. These ions are symported with H^+ by the protein where the proton gradient is functioning as a driving force [6, 7]. The presence of Fe, Mn and Zn are important for metabolic and biochemical reactions in all viable cells [6]. However, regulation of the uptake mechanisms is essential for the maintenance of physiological metal levels [3, 6]. It is known that excessive iron uptake can lead to accumulation in the midbrain, e.g. substantia nigra pars compacta (SN), which further causes oxidative stress and α-synuclein fibrillation. The outcome is neuronal cell death, a common symptom among patients suffering from Parkinson's disease (PD) [8, 9]. Moreover, expression of DMT1 in the SN is increased with age, a main risk factor for developing PD and other neurodegenerative disorders. Through this correlation it has now been suggested that DMT1 is one among other elements responsible for neurodegeneration in Parkinson's disease patients [8].

The human and mouse DMT1 both contains 568 amino residues of which 92% are identical meaning that the proteins are highly homologous [23]. According to the predicted topology (Fig 1) of the mouse DMT1 (mDMT1) 12 transmembrane helices (TM) span the membrane and both the C- and N-termini are located in the cytoplasm [4, 5].

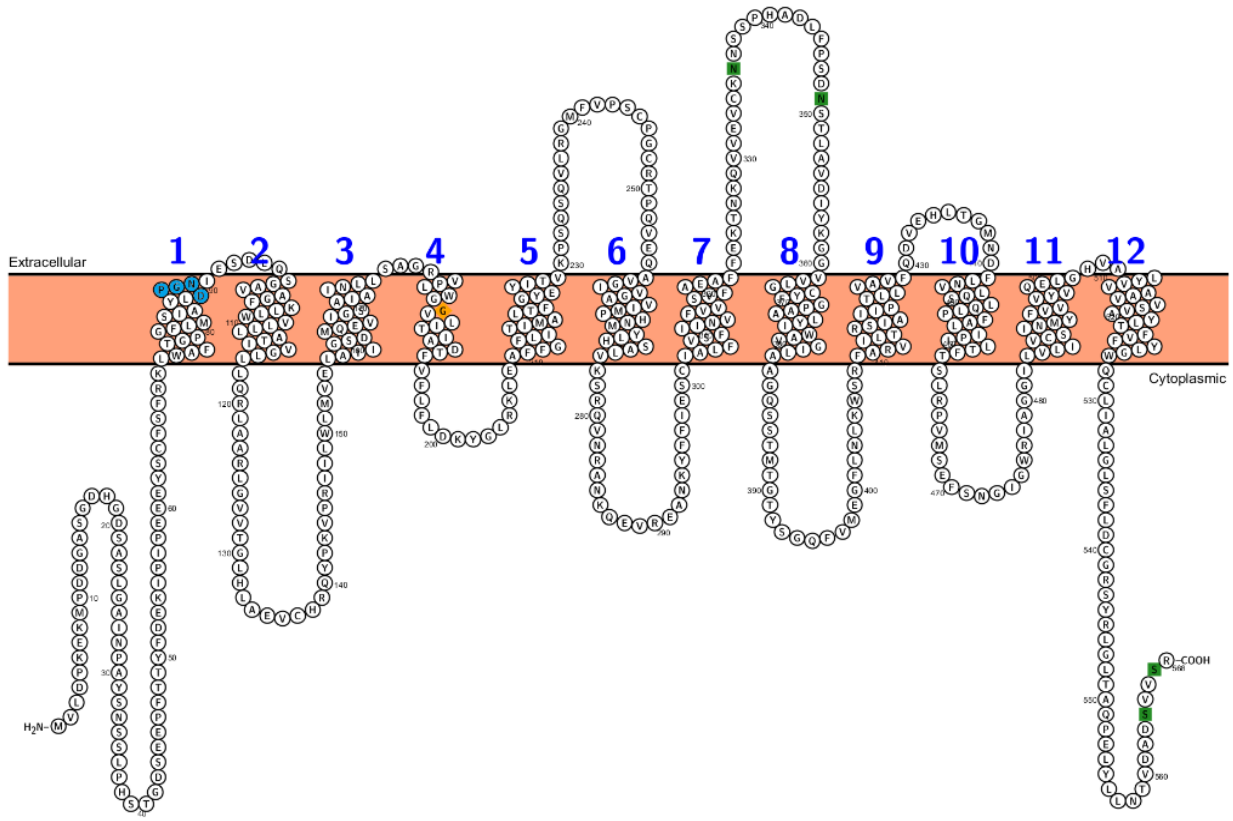


Figure 1. mDMT1 is structurally predicted to contain twelve transmembrane domains with six loops at the extracellular surface and five loops in the cytoplasm [1, 3]. The conserved DPGN motif [5] is highlighted in blue and amino acid G185, which is essential for functional iron transport, is highlighted in orange. Green highlighted amino acids stands for post-translational modification target according to Protter but can be ignored in this thesis Picture retrieved from Protter [20].

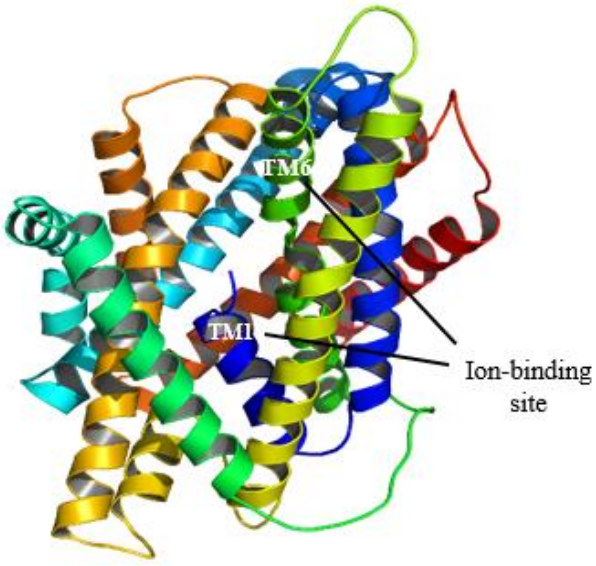


Figure 2. Ribbon representation of the determined DMT structure from *Staphylococcus capitis* [24].

This far, only one crystal structure has been determined from a SLC11 member, namely the divalent metal-ion transporter expressed in *Staphylococcus capitis* (ScaDMT) (Fig 2). Identical amino acids for ScaDMT and human DMT1 is estimated to be 39%, indicating that the basal transport function is similar for the bacteria and eukaryotic counterpart [5].

The SCL11 family's characteristic and highly conserved DPGN motif (highlighted blue in Fig 1) located in TM1 is essential for proper protein function, but has not been shown to be a part of the ion binding site [4, 5] . Nevertheless, TM1 together with TM6 constitute the ion binding site in ScaDMT [5]. Moreover, a single G185R mutation in TM4 (highlighted orange in Fig 1) causes impaired iron transport and contribute to microcytic anemia (any kind of red blood cell anemia) in the *mk* mouse and Belgrade rat, animal models for Parkinson's disease [3, 5, 8]. Due to this, there is a reason to suspect involvement of TM4 in the ion binding site among eukaryotes. Even though prokaryotic DMT and mammalian DMT1 share high homology, there will always be structural and functional differences to some extent between pro- and eukaryotic protein counterparts. Thus, it is of high interest to gain greater knowledge of mammalian DMT1 and especially its 3-dimensional structure in order to support novel drug discoveries in the future [8, 9].

Structure-based drug design (SBDD) is dependent on the structural information of a specific protein as it paves the way for prediction of biochemical and ligand interacting behavior [10]. Crystallization of proteins is a prominent method for acquiring structural data since it may give the advantage of high resolution structures. To gain well diffracting crystals it is of absolute importance that the preparative steps such as purification yields large, as well as pure and monodisperse, quantities of the protein of interest [10, 11]. This is often a troublesome goal to reach when using eukaryotic MP due to numerous reasons. Generally, eukaryotic proteins requires a more complex post-translational modification, such as folding, than prokaryotic proteins and MP furthermore increases the complexity by the means of a correct insertion into the membrane. Endogenous expression of MP is also lower than of soluble proteins and thus a great MP yield is often restricted already from this initial step. The highly hydrophobic feature also make MP sensitive to extraction from their native lipid environment which is an inescapable step when purifying MP. Furthermore, even though extraction succeeds, the tendency of aggregate formation in solution still remains throughout the whole purification process [2, 19]. Thus, it usually requires many optimization steps when expressing, extracting and purifying MP before crystallization of the protein is performed [12].

Aim of project
This study was performed with the aim to express mouse DMT1 in *Saccharomyces cerevisiae* and to find a purification method that yields samples in qualities good enough for crystallization. Success with this may lead closer to the final goal of solving the 3D-structure by X-ray crystallography in the future.

1.1 Theory of methods
Following part of the introduction will give a brief background of methods used to reach the aim of the project.

GFP-tagging
As discussed above, one of the main obstacles to overcome when expressing and purifying eukaryotic MP is to obtain sufficient protein quantities for crystallization [12, 22]. One method that facilitates a faster optimization of expression and purification is the green fluorescent protein (GFP)-based fusion technology. There are several benefits with tagging the sought MP with GFP.

First, GFP fused to the MP C-terminal will fluoresce only if the upstream protein is located within the membrane. Even though a correct subcellular localization does not fully guarantee a proper function of the MP, fluorescence from the GFP-tag indicates that the construct has been expressed. It also simplifies the process of finding good overexpression conditions. Furthermore, by combining fluorescence measurements with other methods such as size-exclusion chromatography and SDS-PAGE, it also speeds up the procedure to obtain pure, monodisperse and soluble samples of the MP. Fluorescence is possible to measure from solutions containing whole cells, crude membranes or purified protein and in-gel fluorescence advantageously identifies the sought protein even at low concentrations and from impure samples [19, 22].

Detergent screen

Since MP are embedded in insoluble membranes, amphiphilic molecules, such as detergents, are commonly used for protein isolation in solution [13]. A critical part is to find a detergent which will disrupt the membranes gently enough to isolate the MP without harming it [13, 14]. In addition to this, it is important that the micelle, created by the detergent, must not be too big since that could completely cover the protein and thus interfere with purification and crystallization. Neither should the micelle be smaller than its ability to maintain a stable form of the protein in solution in order to avoid aggregation and precipitation. To choose which detergent best matches the above criteria, a screen with different detergents could be used to solubilize membranes from a small scale culture. The critical micelle concentration (CMC), defined as the minimum detergent concentration crucial for micelle formation, needs to be taken into account. It is usually suggested to use a detergent concentration ~10 times above CMC when solubilizing. Supplemental methods such as size-exclusion chromatography (SEC) and gel electrophoresis are preferably used for analyzing detergents adequacy [13].

Purification

Ni-Affinity chromatography is one kind of Immobilized Metal Affinity Chromatography (IMAC) common when purifying His-tagged proteins [15]. His-tags bind with high specificity to a variety of divalent metal ions such as Ni and Co and thus is of high importance to separate recombinant protein from any native unwanted protein. Generally imidazole in low concentrations is used to wash away unspecific bound proteins and in higher concentrations to outcompete the more tightly bound protein [15]. Other properties, specific for the protein of interest, could be used when purifying. The isoelectric point (pI) is the pH where a protein carries no net charge, or in other words, where the total amino acid charge equals zero [16]. Ion-Exchange Chromatography (IEX) is another popular purification method used to separate charged molecules after their pI. The stationary phase of the IEX column may be positively charged binding negatively charged ions (anion exchanger) or vice versa (cation exchanger). The choice of buffer pH is crucial for good interaction and a general rule is to use a pH unit 0.5 – 1 above the pI if using an anion exchanger and conversely a pH unit 0.5 – 1 below the pI if using an cation exchanger. Ideally, the buffer should not have a pH so high or low that it may risk any instability of the protein and thus the buffer should be chosen with respect to this. Usually, increasing concentrations of NaCl, e.g. increased ionic strength, are used in order to outcompete the specific bound protein [16].

A supplementary step, to refine purification, commonly used is Size-Exclusion Chromatography (SEC) that separates molecules according to size [17]. Proteins are usually applied into prepacked columns containing a porous matrix, permitting smaller sized compounds to pass through tiny pores and thus end up travelling a longer path. Consequently, larger compounds elute earlier than

smaller. SEC thus make it possible to interpret the quality of the purified protein by analyzing the resolution and numbers of elution peaks as well as what volume it elutes. Furthermore, the void volume reveals information about whether the protein is stable or if it is aggregating under the employed conditions [17].

To analyze purity and quantity of a purified sample, electrophoresis is a fast and easy method. Sodium dodecyl sulfate (SDS) is an anionic molecule which denatures proteins into linear forms and gives them a negative charge [18]. As a result, proteins get a distribution of negative charge per length unit independent from native protein structure. Polyacrylamide gel electrophoresis (PAGE) separates molecules with an electric field across a gel. SDS-PAGE combines the advantage from both properties and separates proteins according to their size after the charge ratio [18].

2. Material & Methods

Methods are inspired from the protocol *GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in Saccharomyces cerevisiae* [19].

All following GFP-fluorescence measurements to be mentioned in the methodology were measured from 200 µl aliquots in 96-Well Clear Bottom Microplates (Corning) in a ClarioStar (BMG Labtech) microplate reader. Emission was detected at 518 nm by excitation at 485 nm for measurements of cells and unpurified membranes. Emission was detected at 512 nm by excitation at 470 nm for samples analyzed after purification. Optical density was measured by a Cary 60 UV-Vis system (Agilent Technologies) at a wavelength of 600 nm (OD_{600}) in semi-micro polystyrene cuvettes (Sarstedt) containing 1-ml aliquots for all samples analyzed.

All SDS-PAGE samples were prepared by mixing protein samples with SDS loading dye (see appendix) in a 2:1 ratio. SDS-PAGE samples of 10 µl were applied to a Novex WedgeWell 16% Tris-Glycine Gel or a Novex WedgeWell 4-20% Tris-Glycine Gel (Thermo-Fischer) in 1xTris-Glycine running buffer (see appendix). Proteins were separated at 200V for 45 min by a PowerEase 500 (Invitrogen). Ladders used were 2 – 4 µl of BenchMark Fluorescent Protein Standard (Invitrogen) and 5 µl Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). Fluorescence was detected with 2 minutes exposure from emission at 518 nm by excitation at 485 nm in a Syngene PXi gel imaging system.

2.1 GFP Standard Curve

A GFP standard curve was created so that protein concentrations in crude membrane samples could be estimated. Aliquots from a 6 µg/ml purified free GFP stock were diluted (20 mM Tris-HCl pH 7.5, 300 mM NaCl) to a final volume of 500 µl in a nine sample series as presented in Figure 3. Final concentrations of the purified free GFP are presented in highlighted and bold numbers. GFP-fluorescence was measured for all samples.

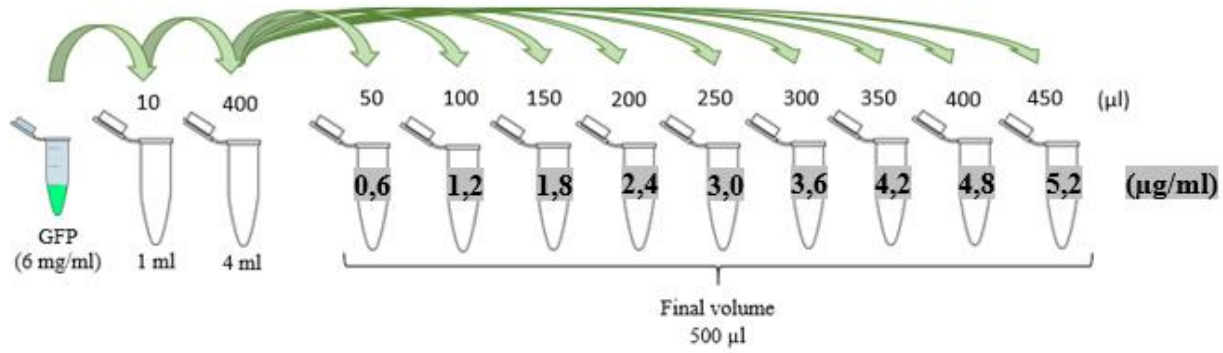


Figure 3. A sketch of the dilution series prepared in order to create a GFP standard curve. The final free GFP concentration is highlighted in grey. Clipart (Eppendorf) retrieved from http://www.clker.com/clipart-eppendorf-tube.html

2.2 Construct

The construct used for protein expression during this project was prepared and provided by Veronika Nesverova (co-supervisor) at the course start. The sequence, bought from GenScript, expresses mouse DMT1 (mDMT1). The expressing vector pDDGFP-2 was transformed into *Saccharomyces cerevisiae* strain FGY217 by homologous recombination. The construct contains yeast-enhanced green fluorescent protein (yEGFP) followed by an 8xHis-tag on the C-terminus. Furthermore, the construct harbors a TEV protease site upstream of the yEGFP-tag, a GAL1 promotor and a URA selection marker. The molecular weight and isoelectric point (pI) of the recombinant protein is predicted to 89 kDa and 5.9 respectively [25].

2.3 Large Scale Cell Culturing and Membrane Preparation

During the practical period of this project large scale culturing was performed numerous times with a small variation in conditions such as culture volume and resuspension buffer. For this part of the methodology culturing of 9 l of cells will be presented.

Following steps were carried out within sterile conditions and all shaker flasks were at least five times bigger than total culture volume to ascertain sufficient aeration. Incubation was always performed at 30 °C on constant shaking at 250 rpm. Cell culturing was prepared by the incubation of yeast cells on agar selection media (see appendix) for ~48 h. 250 ml pre-culture media (see appendix) was inoculated with colonies from the agar plate. Pre-culture was divided in two baffled 250 ml flasks and incubated overnight. OD_{600} was measured and pre-culture was

diluted to 0.12 in 2.5- and 5-l baffled flasks containing 500 ml and 1 l culturing media (see appendix) respectively. Cultures were incubated until OD_{600} reached 0.6 (~6 h). Protein expression was induced with 20% (w/v) galactose (final 2%) in -ura media and the cultures were kept incubating for ~22 h.

Following steps were preceded in as cold conditions as possible. Cells were harvested by centrifugation at 6000 rpm (JLA-8.1000 Beckman) for 25 minutes at 4 °C. Supernatant was discarded, pellet was collected and re-suspended in ~25 ml YSB/original cell culture (see appendix). Protein expression was estimated by measuring GFP-fluorescence of the homogenized cell suspension. Suspended cells were mixed to double the volume with cold Breaking buffer (see appendix) and PMSF (final 1 mM) was added to avoid protein degradation by proteases. Cells were disrupted mechanically with ~200 ml cold glass beads in a Bead-Beater grinder (Biospec Products) kept on ice. Breakage was performed with a 12 time repetition of 30 seconds grinding interspersed with a 30 seconds pause to prevent sample from getting overheated. Lysate was cleared by centrifugation at 9500 rpm (JLA-10.500 Beckman) for 45 minutes at 4 °C. A pellet was discarded and a supernatant collected. GFP-fluorescence from broken cells was measured before and after centrifugation in order to calculate breakage efficiency. Membranes were harvested by ultracentrifugation at 45000 rpm (45Ti Beckman) for 2 h at 4 °C. The pellet was collected and homogenized in MRB or RB (see appendix) with a potter to a final volume of ~50 – 75 ml. A second wash was performed by ultracentrifugation at 45000 rpm (70Ti Beckman) for 1.5 h at 4 °C. Membranes were collected, weighed and re-suspended with MRB or RB to ~6 ml/l original cell culture. Re-suspended membranes were divided into a varying number of aliquots depending on upcoming experiments and stored in –80 °C.

2.4 Detergent Screening

A screen with five different detergents (Anatrace) in three different conditions was performed in order to find a suitable solubilizer for yEGFP-mDMT1. Membranes from 3 l of the cells were prepared and stored in MRB according to previously presented methodology.

Table 1. Solubilizing agents tried out in the detergent screen, their CMC and final concentrations.

Detergent	CMC (% w/v)	Stock concentration (% w/v)	Final concentration (% w/v)
FC-12	0.047	10	1
DDM	0.0087	10	1
DM	0.087	10	1
NG	0.2	20	2
OGNG	0.058	20	2
OG	0.53	30	3

Each detergent was dissolved in 500 µl MRB in Eppendorf's to a final concentration of those presented in Table 1. Three setups of 450 µl crude membranes in MRB were mixed with 50 µl of each detergent stock. A negative control was also prepared out of 450 µl membranes and 50 µl MRB. Setup 1 was incubated at room temperature for 1 h and Setup 2 was incubated overnight at

4 °C. Setup 3 had CHS (Sigma-Aldrich) with a final concentration of 0.2% (w/v) added in each sample and was incubated for 1 h at room temperature. All three setups were kept on mild shaking while solubilizing. The GFP-fluorescence was measured for all the samples before and after (supernatant) centrifugation at 5000 rpm (Beckman, Microfuge 22R) for 1.5 h at 4 °C in order to calculate the solubilization efficiency for each of the detergents.

SEC was performed for the first and second setup. All detergent-solubilized samples were filtered (membrane pore size 0.45 µm) before applied to a Superose 6 10/300 (GE Healthcare) column connected to an automatic chromatography system (Bio-Rad). Superose column was washed with 2 CV equilibration buffer (see appendix). GFP-fluorescence from SEC fractions was measured. A selection of the fractions was analyzed by SDS-PAGE.

2.5 Protein Purification

Different purification methods, such as IMAC and IEX followed by SEC, were tried out with the aim to obtain pure amounts of mDMT1. Conditions throughout these trials were varied in order to optimize the results. Parameters varied were pH, binding time, detergent and buffer composition (Table 2). All purifications performed by the automatic NGC chromatography system (Bio-Rad) was at 4 °C with protein detection by absorbance at λ 260 and 280 nm.

Table 2. Overview of purification trials with varying solubilization and purification conditions. Purification buffers in all trials, except Trial 1, contained 0.01% (w/v) CHS. See appendix for detailed composition of MRB and RB.

		SOLUBILIZATION			PURIFICATION			
Trial	**Type of IMAC**	**Membrane batch (g/ml)**	**Storage buffer**	**Solubilizing detergent**	**Purification detergent**	**pH**	**NaCl (mM)**	**IMAC Binding time**
1	Ni-NTA	0.33	MRB	DDM	DDM	7.5	150	1 h
2	HisTrap	0.33	MRB	DDM	DDM	7.5	150	40 min
3	Ni-NTA	0.33	MRB	DDM	DDM	8.0	150	Overnight
4	Ni-NTA	0.11	RB	DM	DM	8.0	300	Overnight
5	IEX	0.06	RB	DDM	DDM	7.5	15	40 min

Membrane Solubilization
Depending on what buffer the crude membranes were stored in, a solubilization stock was prepared by dissolving selected detergent (final concentration according to Table 1) and a final concentration of 0.2% (w/v) CHS in either MRB or RB (see Table 2). For all trials, membranes were diluted with the detergent stock to a volume of 25 ml. Membrane proteins were solubilized for 1 h at 4 °C at mild shaking and unsolubilized material was pelleted and discarded by ultracentrifugation at 45000 rpm (Beckman 75Ti) for 45 minutes at 4 °C. Supernatant, e.g. protein suspension, was collected and GFP-fluorescence was measured in order to analyze binding efficiency to the IMAC stationary phase. This data is presented as “before binding” in the results.

Immobilized Metal Ion Affinity Chromatography (IMAC)
The 8xHis-tag fused to the protein allowed nickel binding using Ni-NTA Agarose (QIAGEN) or prepacked HisTrap HP 5 ml (GE Healthcare) columns. Several purification trials were performed with varying conditions (Table 2).

IMAC with Ni-NTA
2 ml of Ni-NTA agarose was equilibrated with 5 CV equilibration buffer specific for each trial (see appendix) and incubated with the protein suspension at mild shaking for at least 1 h at 4 °C. Agarose-protein mixture was transferred to a Poly-Prep® Chromatography gravity flow column (Bio-Rad) and was left to settle on ice for ~20 min. Flow-through was collected and GFP-fluorescence was measured in order to analyze binding efficiency to the IMAC stationary phase. This data is presented as "after binding" in the results. The equilibration buffer with increased concentrations of imidazole 25 mM, 40 mM, 50 mM, 150 mM or 300 mM, was used for washing and elution of Ni-bound proteins on bench. Imidazole washes was put together differently in each trial. Fractions of 1 ml was collected and GFP-fluorescence of these was measured.

IMAC with HisTrap
The HisTrap column was connected to the Bio-Rad and equilibrated with 5 CV Buffer A (see appendix). Protein suspension was diluted to a volume of 50 ml with Buffer A (without imidazole) and applied to the HisTrap column through a 50 ml Superloop at a speed of 0.80 ml/min. Buffer A and Buffer B (see appendix) were mixed in a ratio (v/v) that gave a 25 mM imidazole wash (5 CV at 3 ml/min). Buffer B was used to elute protein with 150 mM imidazole in 1-ml fractions (5 CV at 3 ml/min). GFP-fluorescence of the fractions and FT was measured. A selection of the fractions was analyzed by SDS-PAGE.

Ion-Exchange Chromatography (IEX)
A Resource Q (GE Healthcare) 6 ml was connected the automatic chromatography system and equilibrated with 5 CV Buffer A (see appendix). Membranes were diluted to a final volume of 50 ml and the pH had to be adjusted with a few drops of HCl (1 M) to pH 7.5. The sample was applied at 0.80 ml/min to the column with a 50 ml Superloop. Proteins were eluted with 100 mM, 250 mM, 350 mM, 400 mM, 500 mM, 600 mM, 700 mM and 1 M NaCl. 1-ml fractions were collected at 2.5 ml/min. GFP-fluorescence of high 280 nm absorbance peaks was measured and a selection of the fractions were analyzed by SDS-PAGE.

Size-exclusion Chromatography (SEC)
A Superdex 200 10/300 GL (GE Healthcare) with a 10 – 600 kDa fractionation range and a mean bead size of 13 µm was connected to the automatic chromatography system. Calibration of the column was performed by co-workers according to GE Healthcares recommendations before course start. The void volume data was provided as 8 – 9 ml. After IMAC and IEX purification, pooling, concentrating and filtering the protein samples were injected manually to the SEC column equilibrated with a Buffer A. Elutes were collected in 0.5 or 1 ml fractions. According to estimated molecular size (89 kDa) for yEGFP-mDMT1 [25] the protein was predicted to elute around 13 ml [17].

2.6 Optimization of Expression

Six 10-ml small scale cell cultures were prepared by inoculating 25 ml pre-culture media (see appendix) with yeast colonies from agar selection media (see appendix). See the methodology for large scale culturing, until inducing step, to get a full description of growing methodology and conditions. When OD_{600} reached either 0.4, 0.6 or 0.8 two cultures were induced with 20% (w/v) galactose (final 2%) -ura media. Two different incubation temperatures, 23 °C and 30 °C, were tested for 47 h. A culture without induction was kept at 30 °C as a negative control. GFP-fluorescence at different time points were measured for all cultures during the protein expression time.

A second optimization screen setup was prepared as presented above but without glucose in the culturing media. The experiment was cancelled since OD_{600} failed to reach 0.4, 0.6 and 0.8.

3. Results

Following part mainly focuses on the results obtained from the purification performances. Methods may be repeated to some extent in the results for increased clarity.
GFP-fluorescence that was measured per volume throughout this project was mainly functioning as a tool to get an estimated perception of which condition and purification method that seemed to be most suitable for yEGFP-mDMT1. This was done only by the means of comparing RFU between the trials and with less concern about the actual yield. The volume from where the fluorescence was measured is presented for every trial in the result part so that the concentration may be calculated according to guidelines in the protocol *GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in Saccharomyces cerevisiae* [19].

3.1 GFP Standard Curve

The dilution scheme gave a standard curve within the concentration range of 0.6 – 5.4 µg/ml of free GFP (Fig 4). The trend line fitted the plotted data with an R^2 value of 0.992.

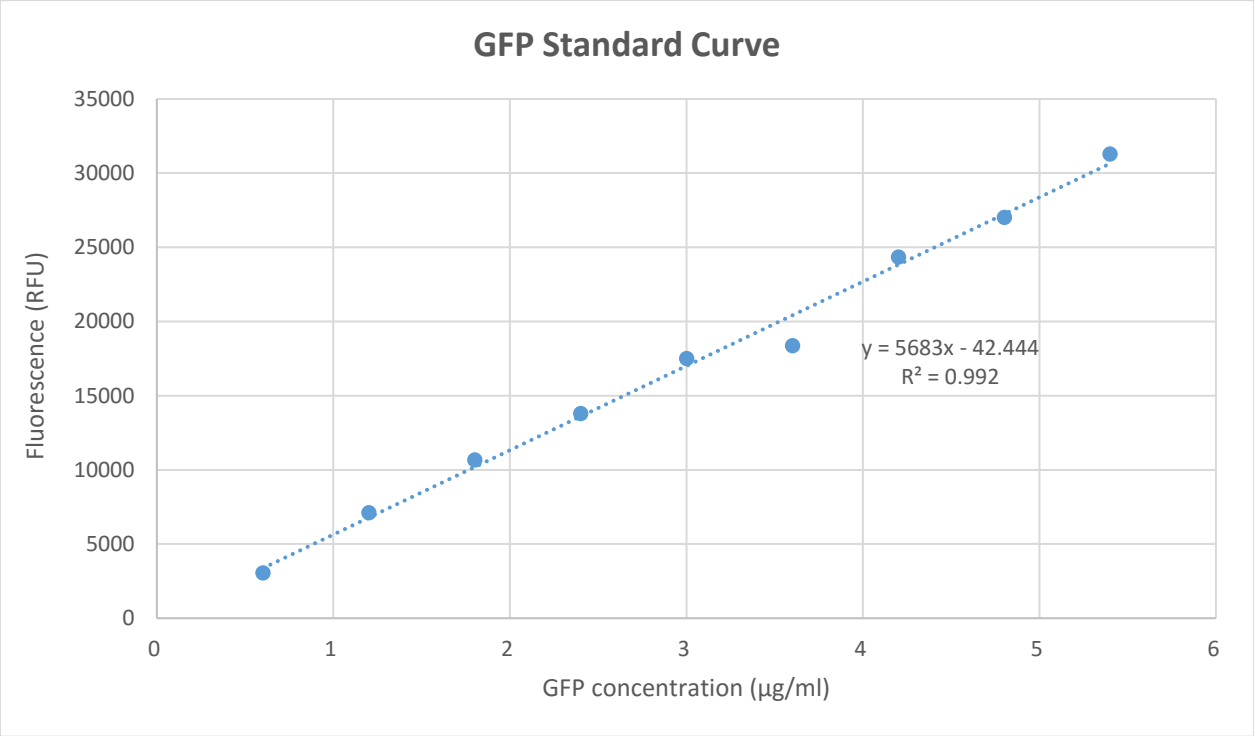


Figure 4. Standard curve created with a dilution scheme from a known start concentration of GFP (µg/ml).

3.2 Membrane Preparation

From the 9 l of cultured, harvested and lysed cells a yield of 5 g membranes was obtained. Re-suspended membranes fluoresced to 62168 RFU. There were smaller variations in membrane yield for different large scale cultures grown. This result is chosen as a representative example. A numerical estimation of mDMT1 present in the extracted membranes was calculated according to the protocol [19] with complementary data from the GFP standard curve (Fig 4).

Amount of mDMT1 gained from the 9-l of yeast culture: 1.6 mg (see appendix for detailed calculations). The final sample containing 1.6 mg of mDMT1 was divided into three aliquots before further solubilization and purification meaning that the starting protein material was 0.53 mg in every presented trial.

3.3 Detergent Screen

In order to determine what detergent that solubilizes mDMT1 with least aggregation and with most stability a detergent screen with varying conditions was performed once. Results showed that membranes solubilized with DDM and DM in MRB for 1 h in room temperature with the presence of 0.2% (w/v) CHS (Setup 3) gave highest solubilization efficiency after FC-12 that is presented as a positive control (Fig 5). An efficiency of 150% and 110% (for DDM and DM respectively) imply that more protein was detected after centrifugation than before (e.g. after separation of membrane compartments from micelle-incorporated proteins). The solubilization efficiency alone however does not declare what state the protein is in. Possible aggregates will most probably also fluoresce and thus contribute to "false" positive results. With respect to this, SEC was performed for the DDM and DM solubilized samples. The SEC chromatograms (Fig 6 and 7) shows that proteins started to elute at a volume corresponding to the predicted molecular size for yEGFP-mDMT1 (13.5 – 14.5 ml) indicating the protein was present in an non-aggregated state. Fluorescence measured to 2500 RFU from the 1-ml SEC fractions furthermore imply that the GFP-tagged protein was present in them (Fig 8). Aggregated protein, mostly from DDM solubilized membranes, was also observed by fluorescence from samples of the SEC void volume. However, DDM accompanied with CHS gave the most qualifying results, with highest calculated solubilization efficiency and strongest fluorescence from SEC samples, and was thus chosen to be the first detergents to use for large scale solubilization.

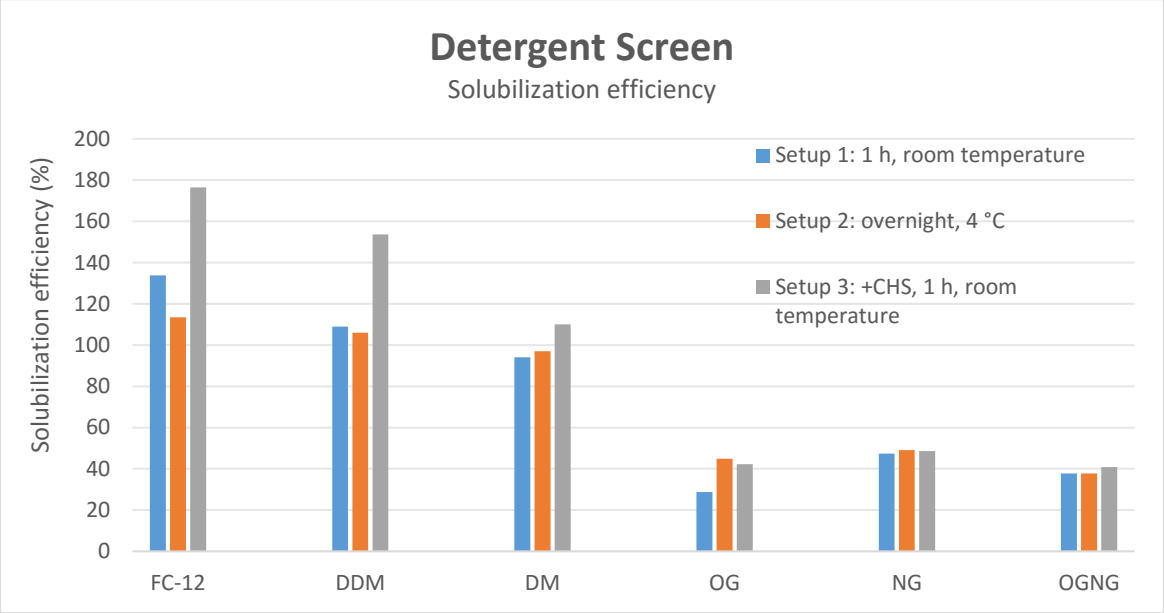


Figure 5. The solubilization efficiency (%) of DMT1 for six different detergents at three different conditions. Percentage calculated from fluorescence measured before and after centrifugation of solubilized membranes.

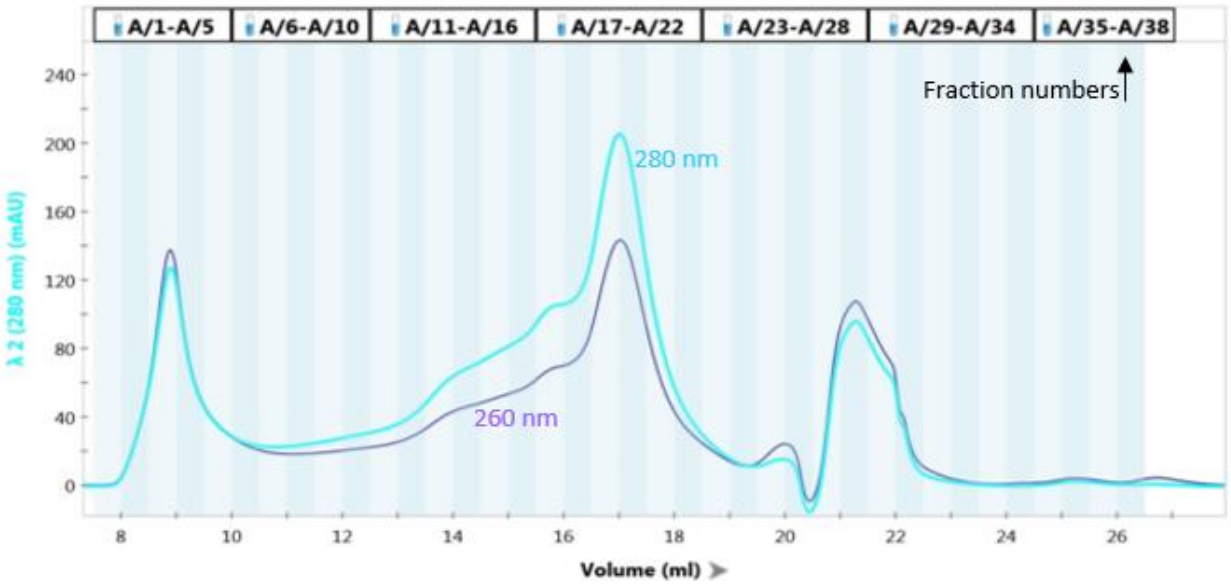


Figure 6. SEC chromatogram from purification of DDM solubilized membranes within conditions according to Setup 3. The volume of each fraction is 0.5 ml. Fraction numbers are labeled in bold above the chromatogram (e.g. fraction 1 equals A/1 and so on).

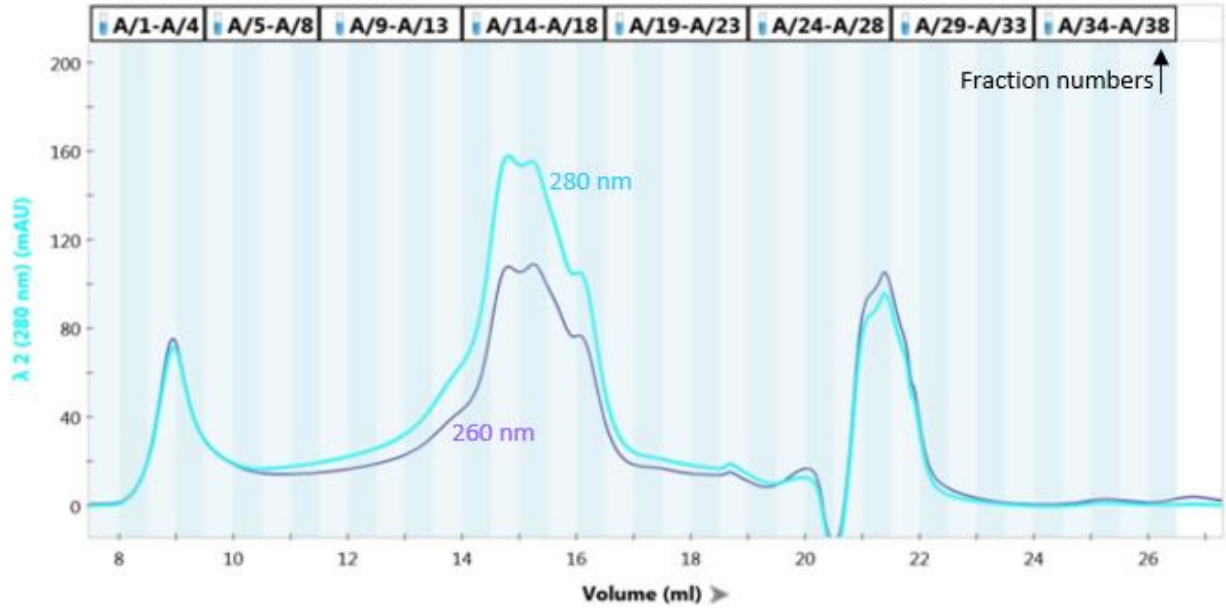


Figure 7. SEC chromatogram from purification of DM solubilized membranes within conditions according to Setup 3. The volume of each fraction is 0.5 ml.

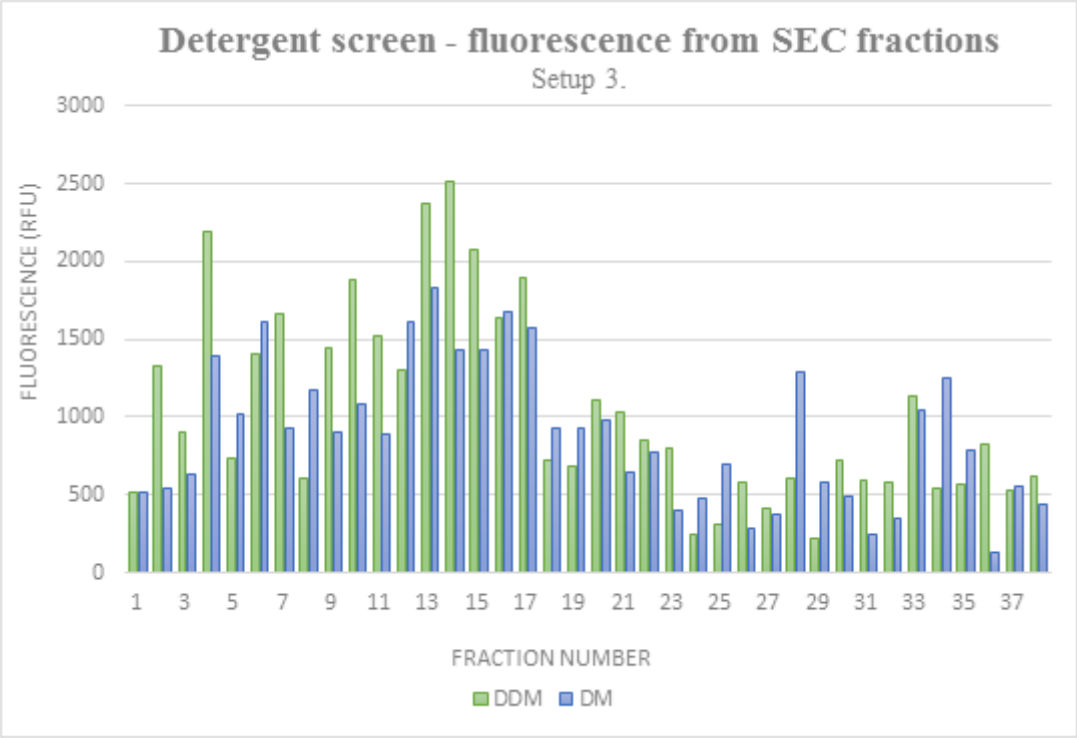


Figure 8. A comparison between fluorescence from SEC fractions (Fig 6 and 7) of DDM and DM solubilized samples within conditions according to Setup 3 (Fig 5). Fluorescence was measured from 0.5 ml SEC fractions and is presented with green and blue columns for proteins solubilized with DDM and DM respectively.

3.4 Purification of Protein

Detailed differences between purification trials are presented in Table 2 and explained in Material & Methods. A short summary of the conditions that varied will be repeated for the trials in the result part for increased clarity.

TRIAL 1.

In Trial 1, DDM and CHS solubilized membranes were binding to Ni resin for 1 h before purification using Ni-NTA. The pH of the MRB was 7.5 and did not contain any CHS. Results show that ~1/4 of the protein bound to the Ni resin (Fig 9) and that most of it eluted with fraction 2, 150 mM imidazole (Fig 10).

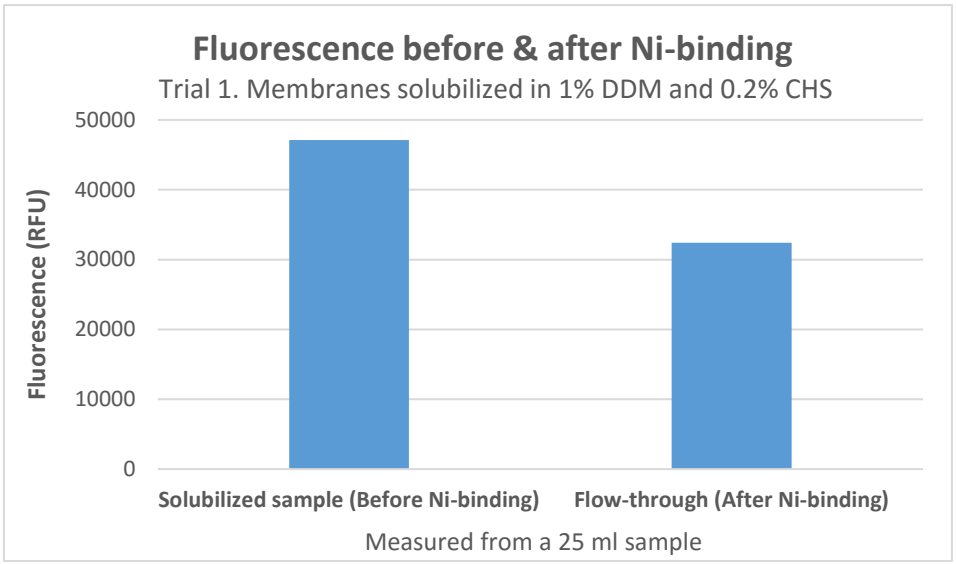


Figure 9. Columns represent GFP-fluorescence from the DDM and CHS solubilized protein sample (25 ml) before interaction with Ni resin (before binding) and from the flow-through after 1 h of Ni resin interaction (after binding).

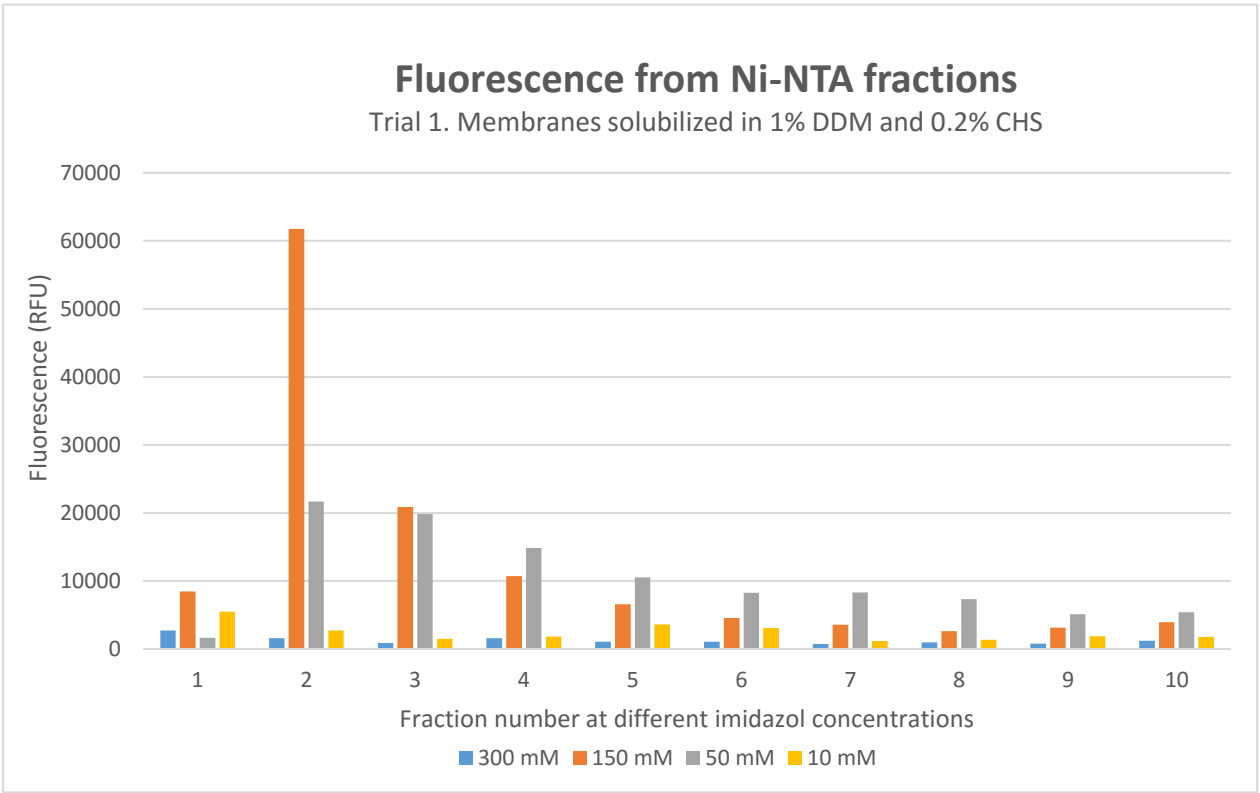


Figure 10. GFP-fluorescence measured from Ni-NTA fractions of 1 ml eluted with different concentrations of imidazole.

A fluorescent band in the SDS-PAGE, corresponding to the molecular size of GFP-mDMT1, was obtained from a sample of the fraction where most of the protein eluted (Fig 11 a). Even though a fluorescent band, corresponding to yEGFP-mDMT1 molecular size, can be seen by in-gel fluorescence, only a vague band is visible after staining the gel (Fig 11 b). A vague stained band indicates that the protein concentration in this fraction was low. Fluorescence was also detected from the sample corresponding to the Ni-NTA FT confirming that there was unbound protein left after 1 h of Ni-binding in the FT (Fig 11 a). The gel further tells that the protein did not elute significantly with imidazole washes of 10 mM, 50 mM and 300 mM since no strong fluorescing bands are visible. In order to collect as much of the protein as possible, and with less concern about purity, all ten fractions eluted with 150 mM imidazole were pooled and concentrated before SEC purification. No protein was detected eluting however, hence no peaks are visible in the chromatogram (Fig 12).

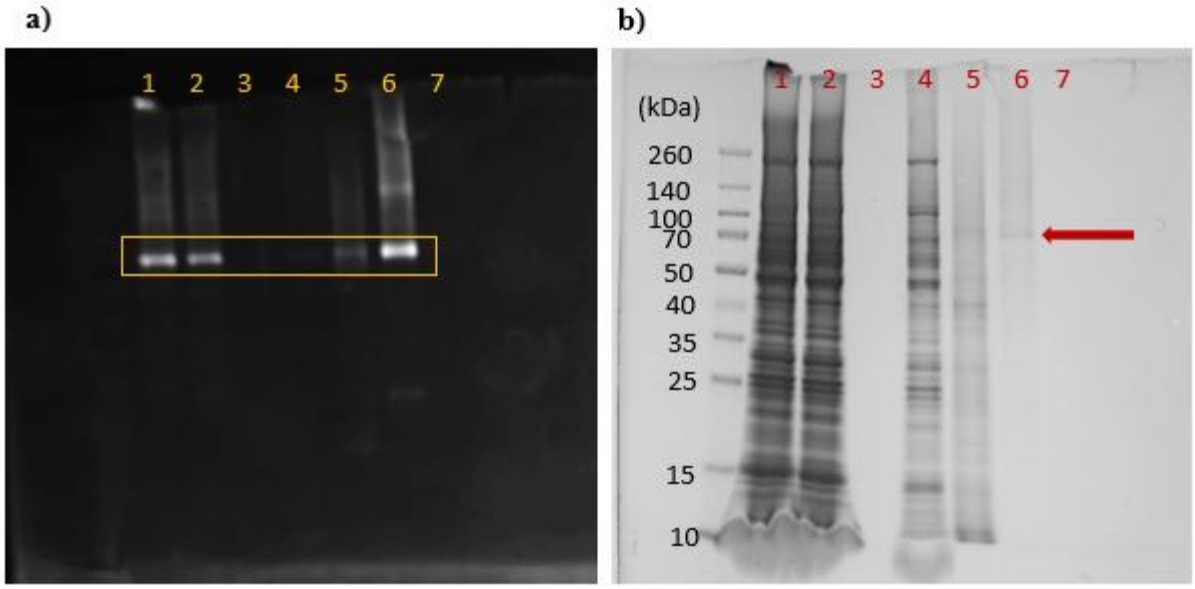


Figure 11. SDS-PAGE from Trial 1 (Table 2). Yellow box and red arrow indicates the correct molecular size for yEGFP-mDMT1. Wells contain: sample before Ni-binding (1), FT after Ni-binding (2), FT after concentration of pooled fractions 1-10 (150 mM imidazole) (3), fraction 1 eluted with 10 mM imidazole (4), fraction 2 eluted with 50 mM imidazole (5), fraction 2 eluted with 150 mM (6) and fraction 1 eluted with 300 mM imidazole (7). **a)** In-gel fluorescence detected with 2 minutes exposure time. Fluorescent ladder is lacking and **b)** stained (SimplyBlue) gel.

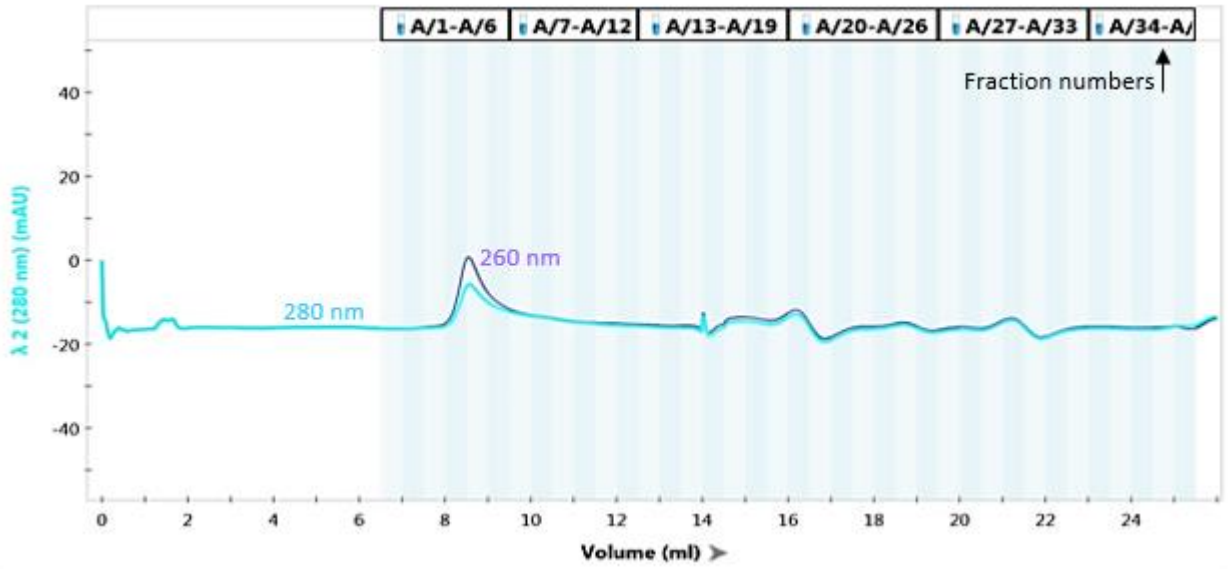


Figure 12. SEC chromatogram from the pooled and concentrated fractions after IMAC purification Trial 1 (Table 2). The volume of each fraction is 0.5 ml.

TRIAL 2

As an attempt to improve binding and avoid possible precipitation, the Ni-NTA column was exchanged to a HisTrap column and CHS was added to the MRB. The DDM and CHS solubilized sample was applied at 0.80 ml/min to the column. High absorbance was detected from non-specifically bound proteins washed out with 25 mM imidazole from the HisTrap in the first nine fractions (Fig 13). Absorbance of ~200 mAU was detected (indicated with a red box in Fig 13) for proteins eluting with the expected eluate concentration, 150 mM imidazole, for mDMT1. Fluorescence up to ~32000 RFU from samples corresponding to those fractions (0.5 ml) confirmed that mDMT1 was present in them (Fig 14). Fluorescence before HisTrap stationary phase binding was measured to 28200 RFU from the solubilized 50 ml protein sample. Data from HisTrap FT is not available.

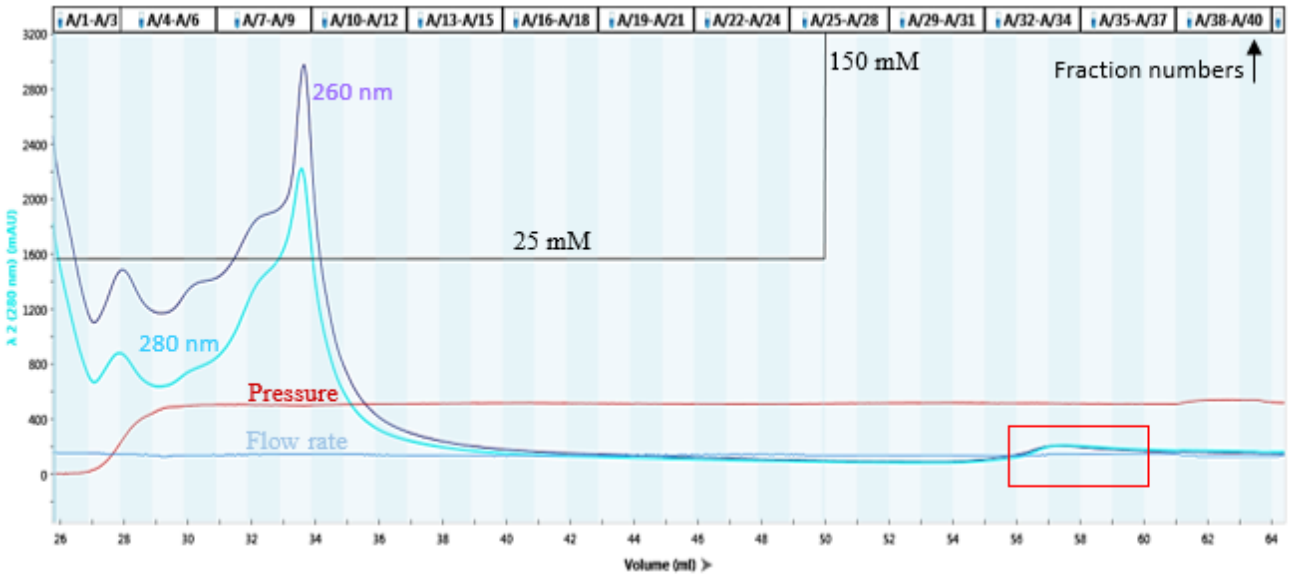


Figure 13. Chromatogram from HisTrap purification Trial 2 (Table 2). The volume of each fraction is 0.5 ml. Fraction 1 – 25 was collected with 25 mM imidazole and fraction 26 – 40 was collected with 150 mM imidazole.

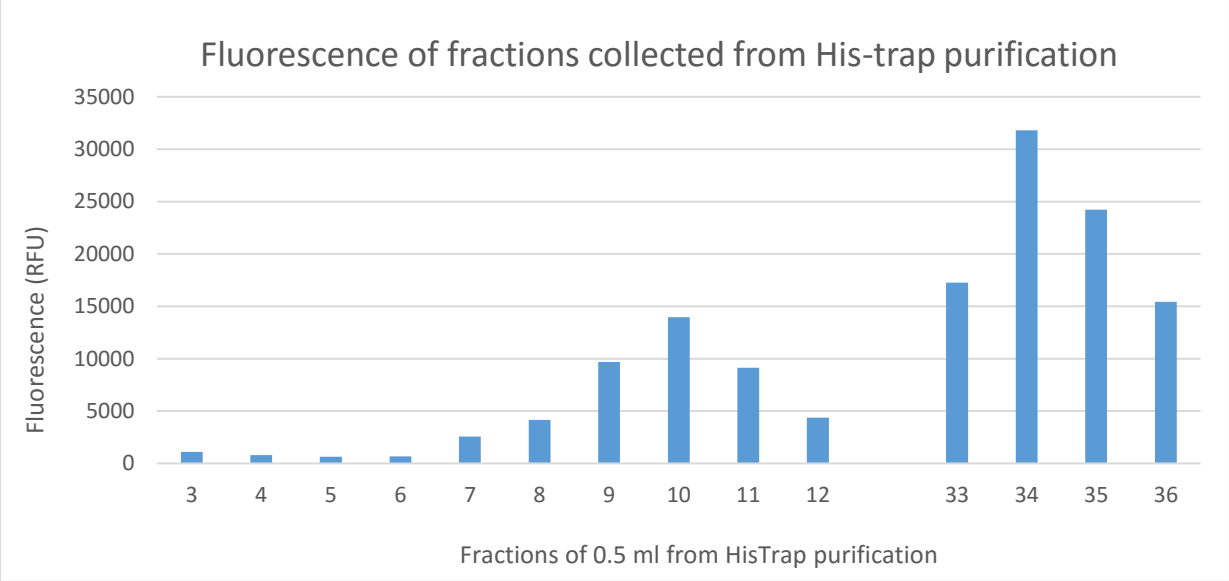
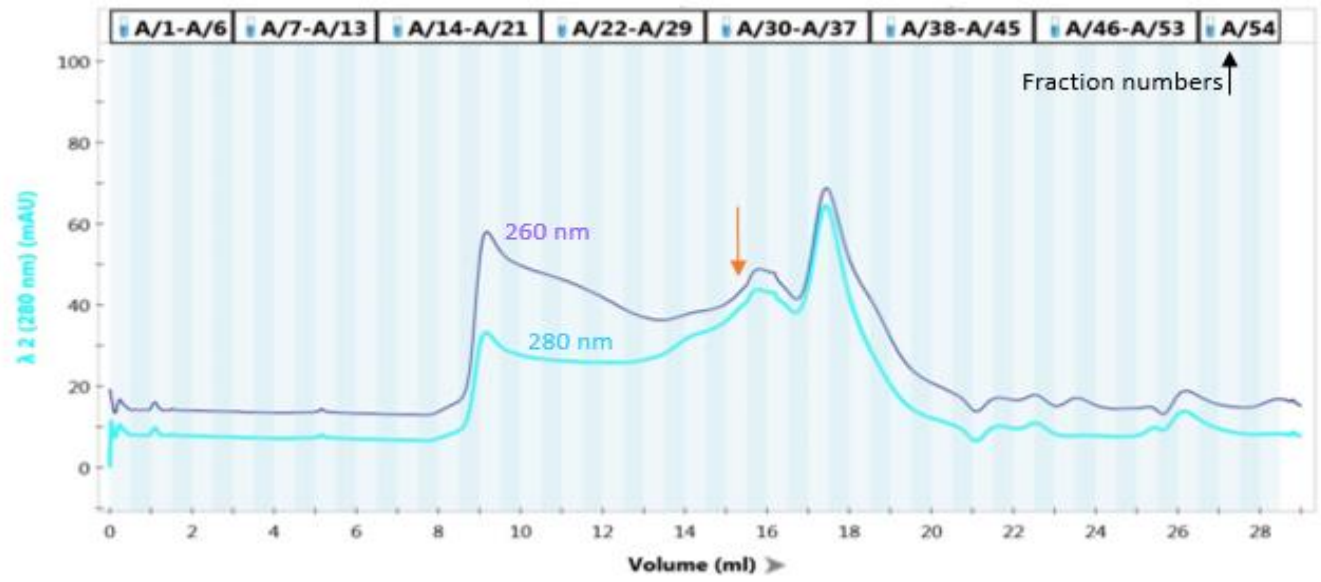


Figure 14. GFP-fluorescence of selected HisTrap-fractions of 0.5 ml from Trial 2 (Table 2). Fractions 3 – 12 are eluted with 25 mM imidazole and 33 – 35 are eluted with 150 mM imidazole.

Fraction 31 – 37, that presented highest fluorescence, was pooled, concentrated and separated with SEC. This resulted in fluctuating absorbance around 40 – 70 mAU (Fig 15 a). No clear peaks can be observed in the SEC chromatogram and thus protein elution was not well-separated into specific fractions. Fluorescence, measured from samples corresponding to all the SEC fractions, shows that no aggregate of the protein was present and that it eluted with fractions in-between 14.5 – 16.5 ml (Fig 15 b).

a)



b)

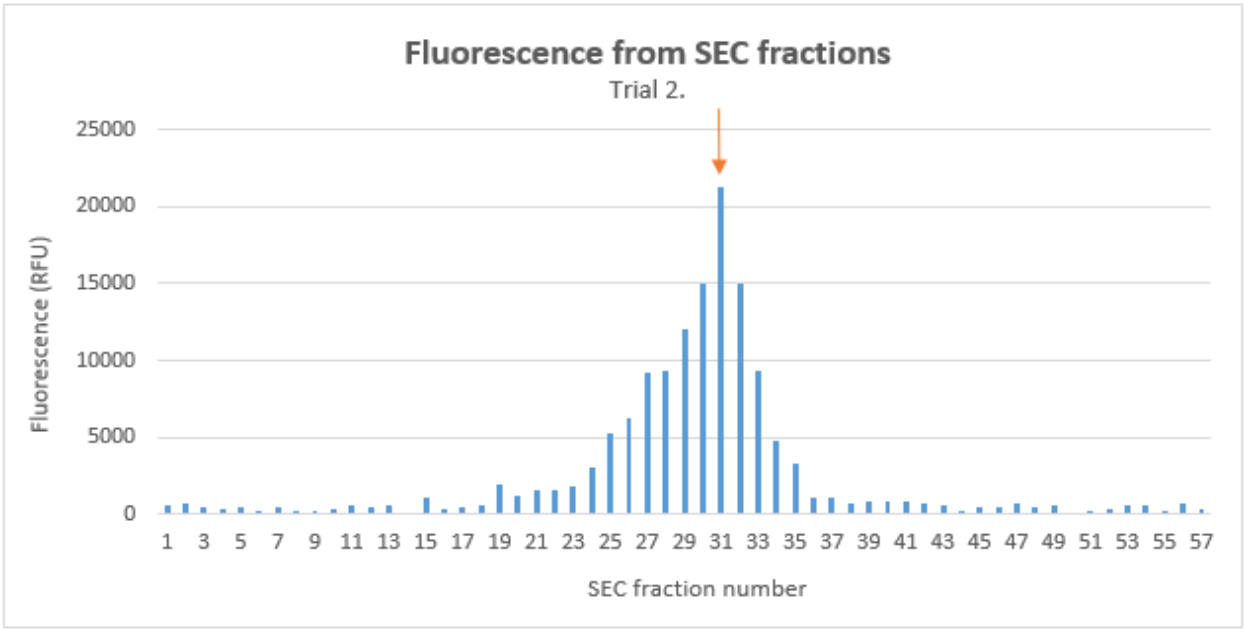


Figure 15. a) SEC chromatogram from pooled and concentrated fractions after HisTrap purification, Trial 2 (Table 2). The volume of each fraction is 0.5 ml **b)** GFP-fluorescence of 0.5 ml fractions from the SEC gel-filtration presented in a). Orange arrows clarifies which SEC fraction that corresponds to highest measured fluorescence.

Fluorescing bands from the SDS-PAGE (Fig 16 a) confirm that the protein was present in the latter mentioned fractions. However, no corresponding bands are detectable in the stained gel (Fig 16 b) meaning that the protein yield was low.

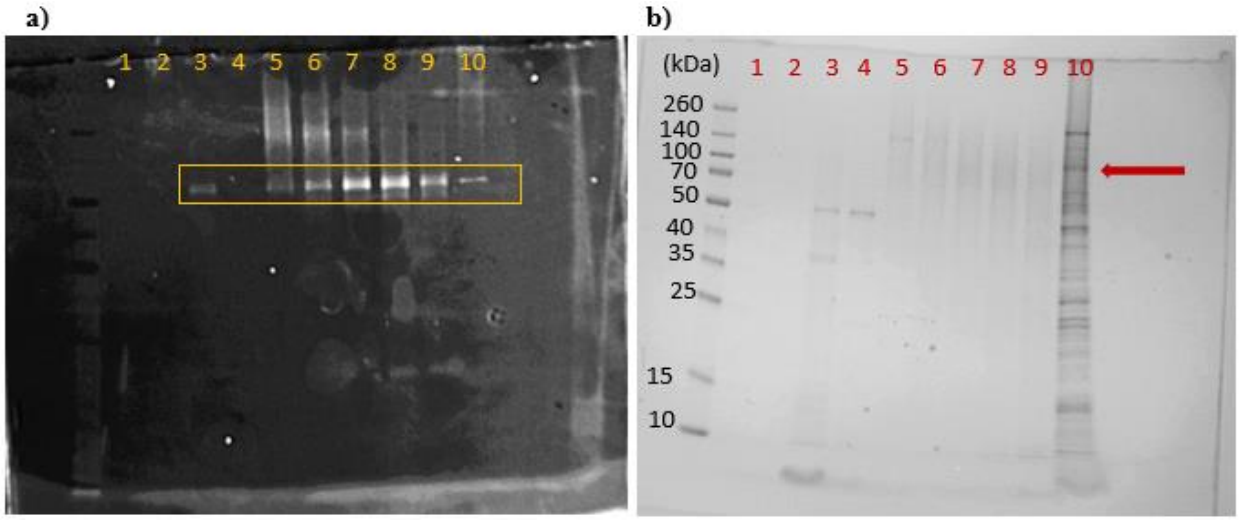


Figure 16. SDS-PAGE of SEC fractions Trial 2 (Table 2). Yellow box and red arrow indicates the correct molecular size for yEGFP-mDMT1. Well 1 – 4 contained SEC fractions 19, 23, 35 and 38 respectively. Well 5 – 9 contained SEC fractions 29 – 33 respectively. Well 10 contained FT from the HisTrap purification. **a)** In-gel fluorescence detected with 2 minutes exposure time. Fluorescent ladder is lacking (compare with Fig 20) **b)** stained (SimplyBlue) gel.

TRIAL 3

Trial 3 was again executed with Ni-NTA and the protein-resin interaction time was increased from 1 h to overnight as an attempt to allow more protein binding. Also, the pH of the buffer was increased to 8 in order to fully deprotonate the 8 histidines on the His-tag. Results show that fluorescence of the solubilized 25 ml sample was almost 50% lower after binding to Ni resin (Fig 17) implying that half of the protein amount bound to the stationary phase. Fluorescence measured from the 1 ml Ni-NTA fractions moreover indicate that highest concentration of yEGFP-mDMT1 was eluted with 150 mM imidazole in fraction 2 (Fig 18).

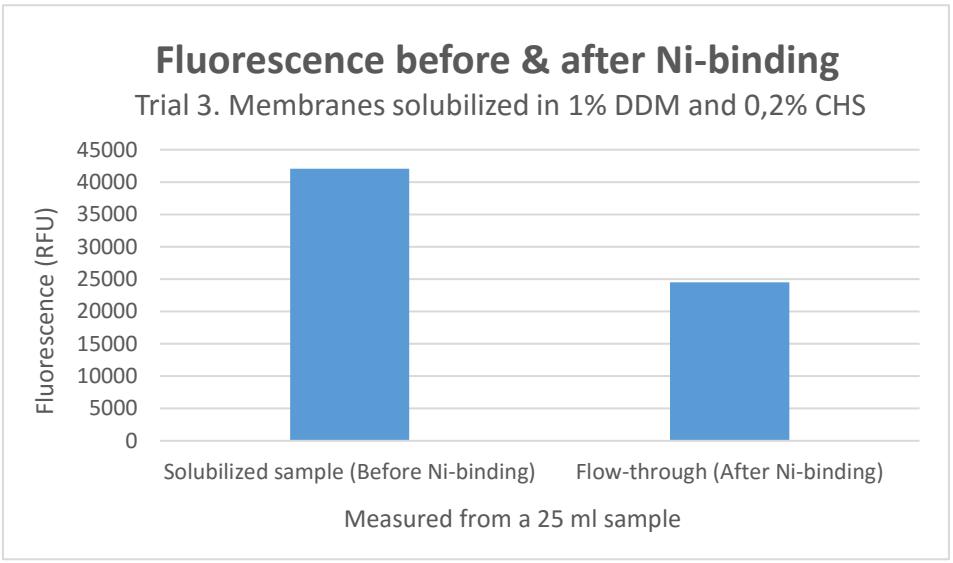


Fig 17. Columns represent GFP-fluorescence from the DDM and CHS solubilized protein sample (25 ml) before interaction with Ni resin (before binding) and from the flow-through after 1 h of Ni resin interaction (after binding).

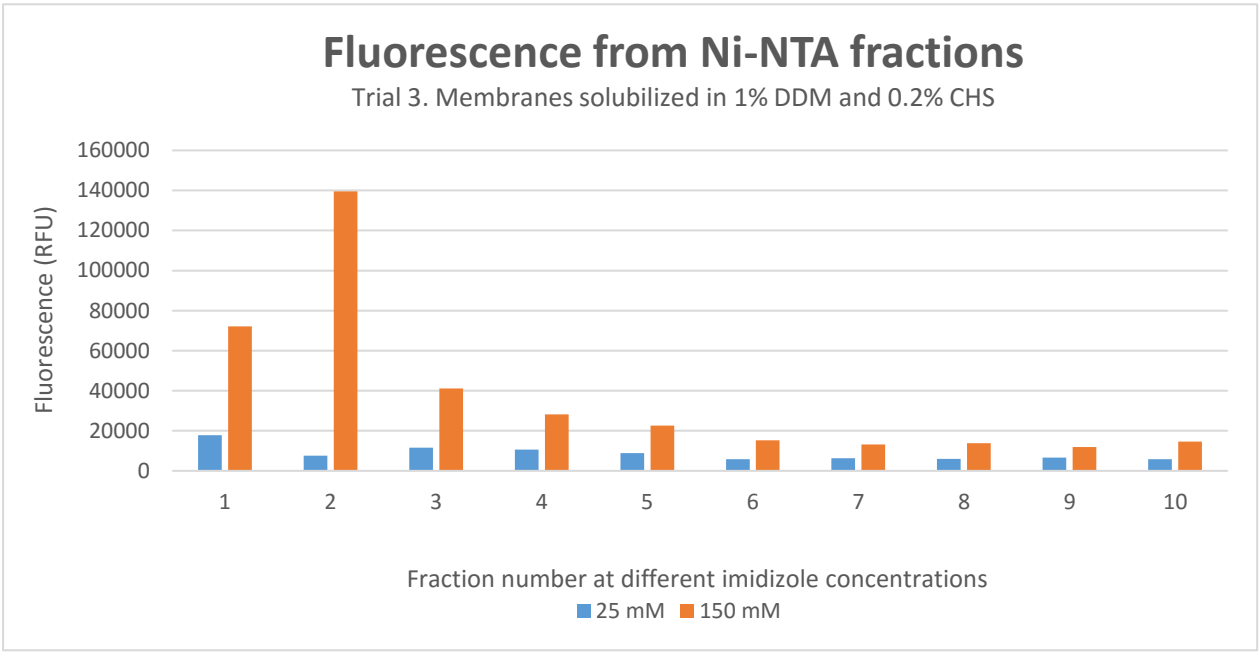


Figure 18. GFP-fluorescence measured from Ni-NTA fractions of 1 ml eluted with different concentrations of imidazole.

Fraction 1 – 5, that presented highest fluorescence from the 150 mM imidazole elution, was pooled, concentrated and further purified by SEC. The SEC chromatogram (Fig 19 a) presents a clear absorbance peak in the void volume fused with a "shoulder" peak at 11 ml. Moreover, all proteins that eluted were within a fraction range of 2 ml indicating imprecise separation of the sought and unwanted proteins. Fluorescence ~24000 RFU was detected for samples corresponding to fractions eluted at 11.5 ml implying that this was where mDMT1 eluted (Fig 19 b). Samples corresponding to the void volume fluoresced approximately 75% less than later eluted sample and thus less amount of aggregated protein than non-aggregated protein was present (Fig 19 b).

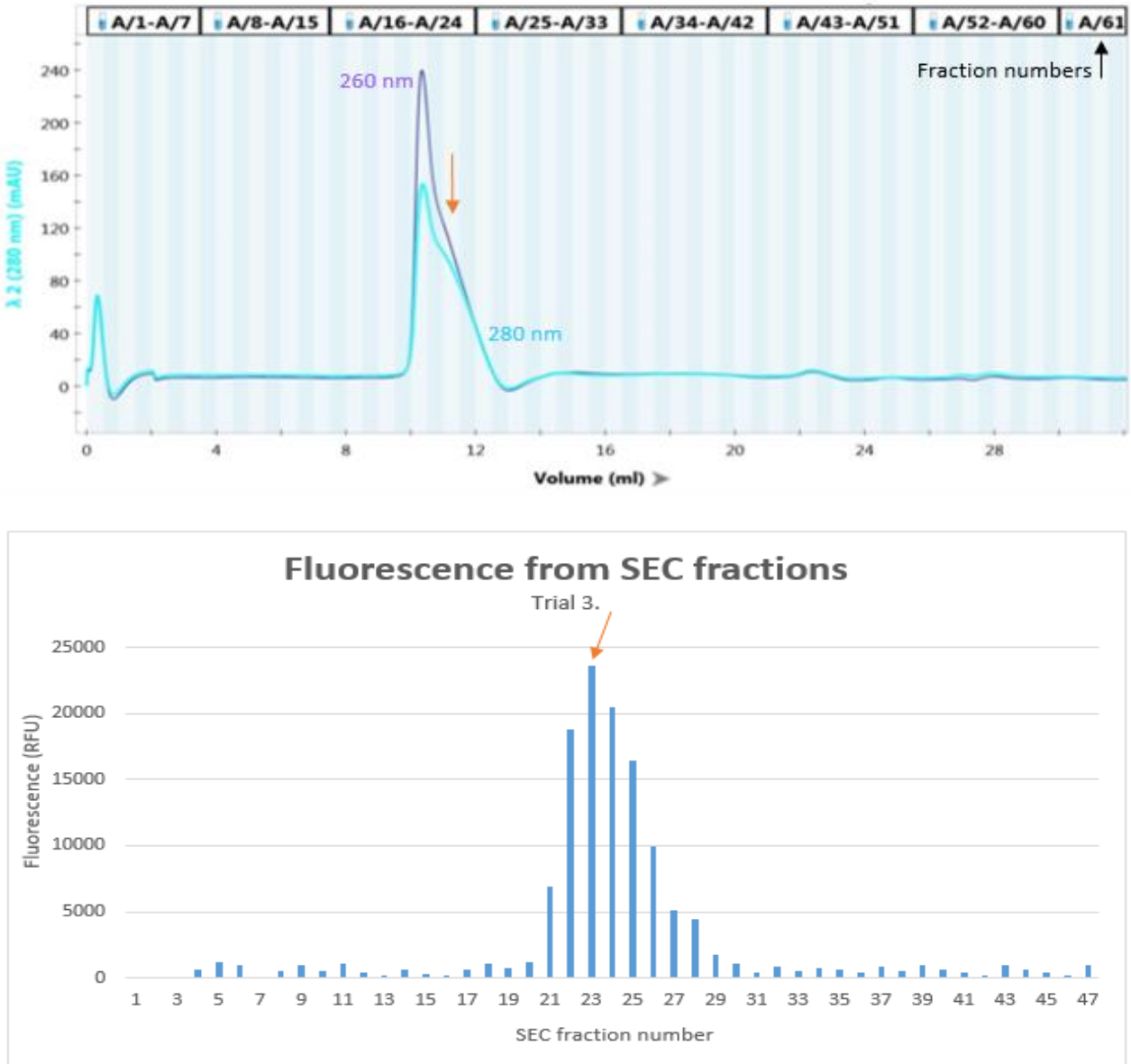


Figure 19 a) SEC chromatogram from pooled and concentrated fractions after IMAC purification Trial 3 (Table 2). The volume of each fraction is 0.5 ml. **b)** GFP-fluorescence of fractions from the SEC gel-filtration presented in a). Orange arrows clarifies which SEC fraction that corresponds to highest measured fluorescence.

It is apparent that there was unbound protein left in the FT since the corresponding sample show a fluorescing band in the SDS-PAGE (Fig 20 a). Moreover, results from the same gel show that protein eluted with the 25 mM imidazole wash. Fluorescence from samples corresponding to fractions which contained most of the eluted protein (fraction 1 and 2, 150 mM imidazole), is smeared along the well thus indicating that the protein aggregated with this wash (Fig 20 a). Weak bands was observed in the stained gel from samples of the SEC fractions implying that low amounts of the protein eluted in that purification step (Fig 20 b)

Last well (nr 11) contained a sample from a SEC fraction from Trial 2. This was simply to enable the possibility of protein size comparison since the gel in that trial lacked a fluorescent ladder.

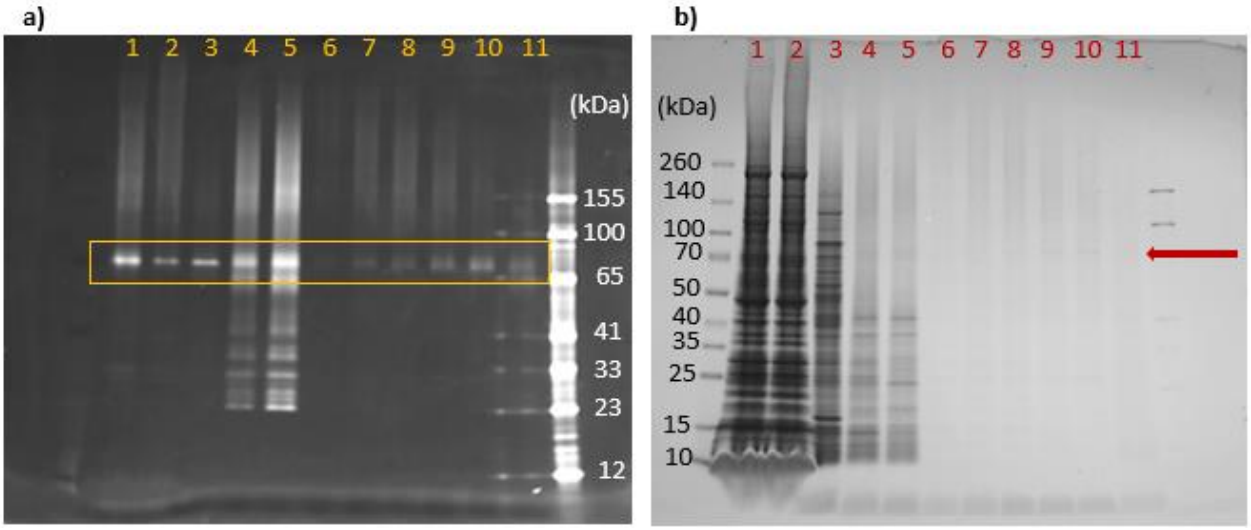


Figure 20. SDS-PAGE of samples from IMAC and SEC fractions Trial 3 (Table 2). Yellow box and red arrow indicates the correct molecular size for yEGFP-mDMT1 Before Ni-binding (1), FT after Ni-binding (2), Ni-NTA fraction 3 eluted with 25 mM imidazole (3), Ni-NTA fraction 1 eluted with 150 mM imidazole (4), Ni-NTA fraction 2 eluted with 150 mM imidazole (5), SEC fraction 21 – 25 (6 – 10 respectively) and SEC fraction 32 from Trial 2 (11). **a)** In-gel fluorescence detected with 2 minutes exposure time and **b)** stained (SimplyBlue) gel.

TRIAL 4

As an attempt to achieve more protein-resin binding and gain higher concentrations of purified protein, DDM was substituted with DM in both the solubilizing and purifying steps. Moreover, MRB was substituted with RB containing 500 mM NaCl. . Results show that ~2/3 of the protein bound to the Ni resin (Fig 21). Most of the bound protein eluted with 150 mM imidazole and, to a lower degree, with 25 and 50 mM imidazole as well (Fig 22).

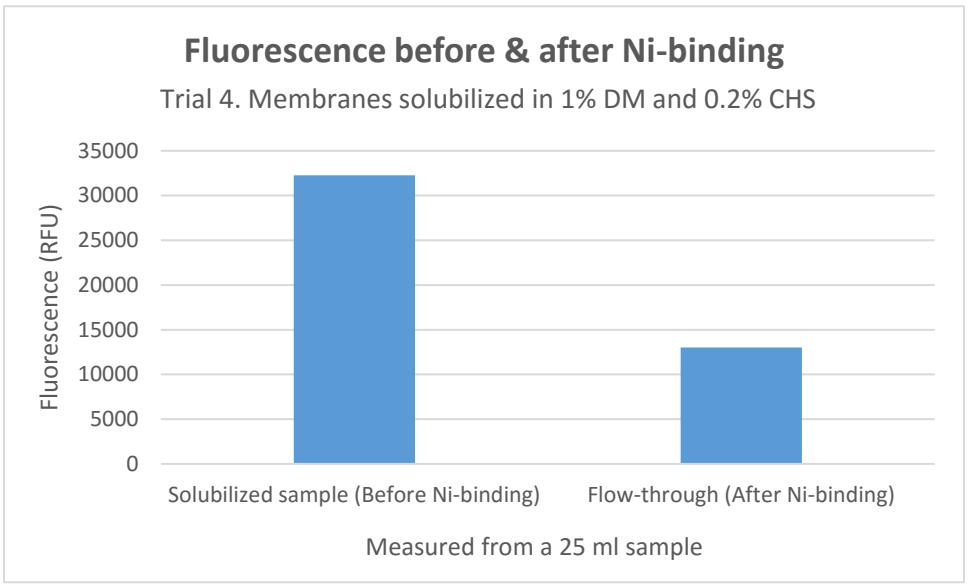


Fig 21. Columns represent GFP-fluorescence from the DM and CHS solubilized protein sample (25 ml) before interaction with Ni resin (before binding) and from the flow-through after 1 h of Ni resin interaction (after binding).

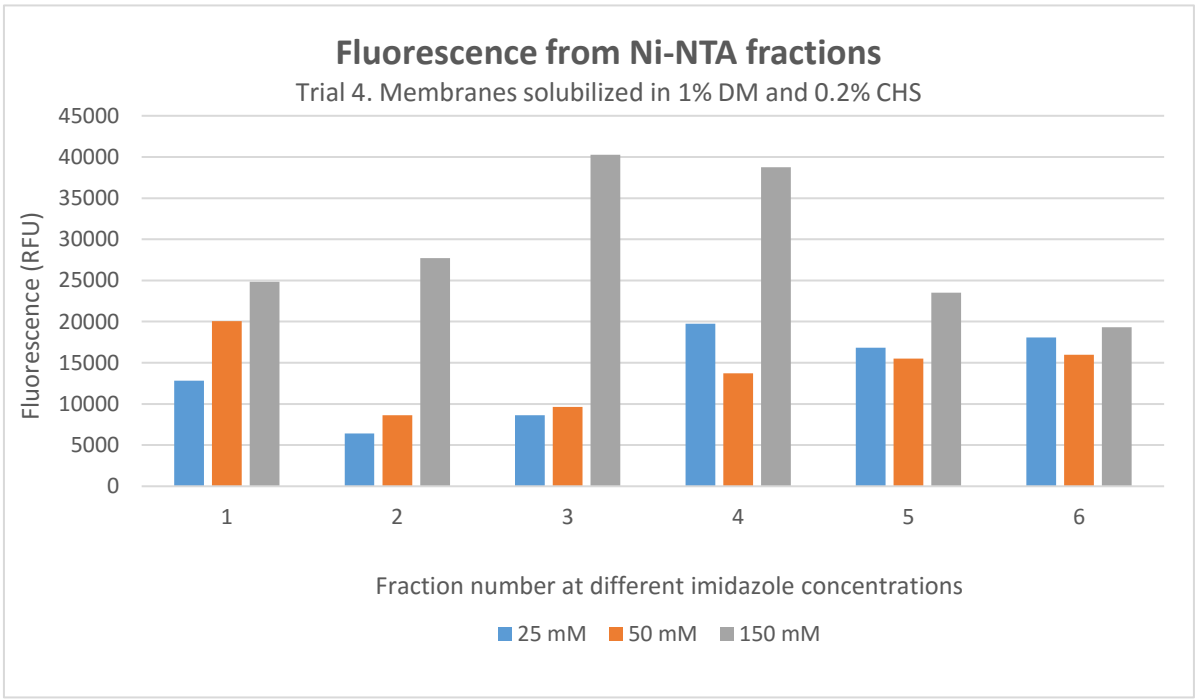
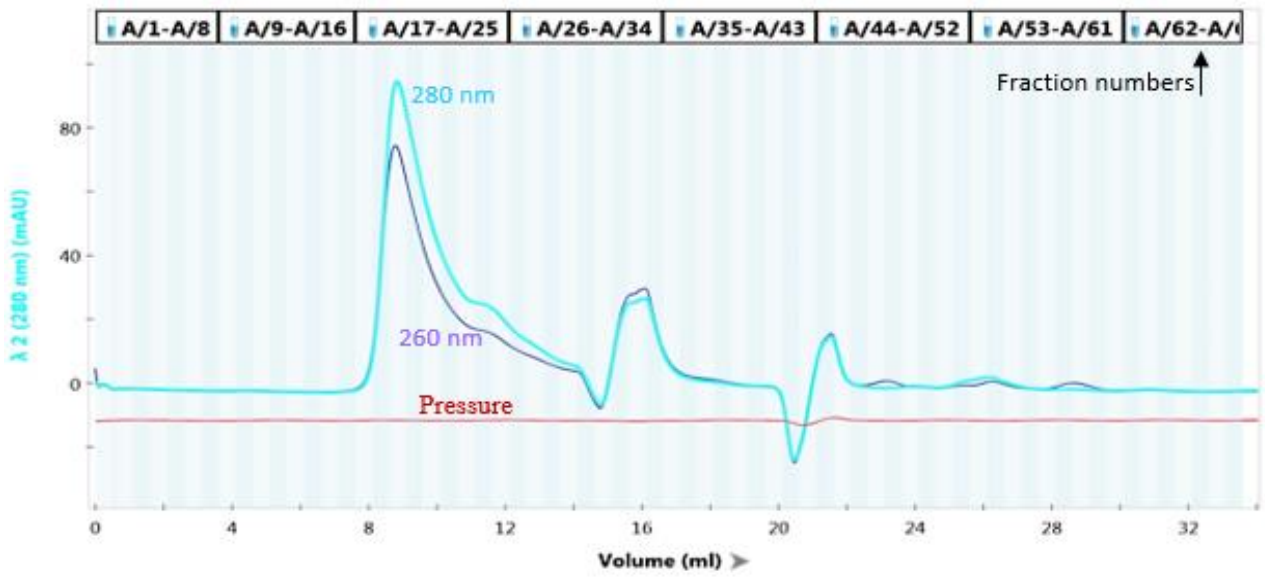


Figure 22. GFP-fluorescence measured from Ni-NTA fractions of 1 ml eluted with different concentrations of imidazole.

Pooled and concentrated Ni-NTA fraction 1 – 5, that presented highest fluorescence, from the 150 mM imidazole wash was purified by SEC. Results show a higher void volume peak followed by two lower ~20 mAU peaks (Fig 23 a). Fluorescence from samples of the SEC fractions was detected mainly for those in the void volume, e.g. 10 – 10.5 ml (Fig 23 b) indicating that the protein had aggregated. Since no significant fluorescence was obtained from samples of other peaks in the SEC chromatogram it is apparent that the protein did not elute with any other fraction.

a)



b)

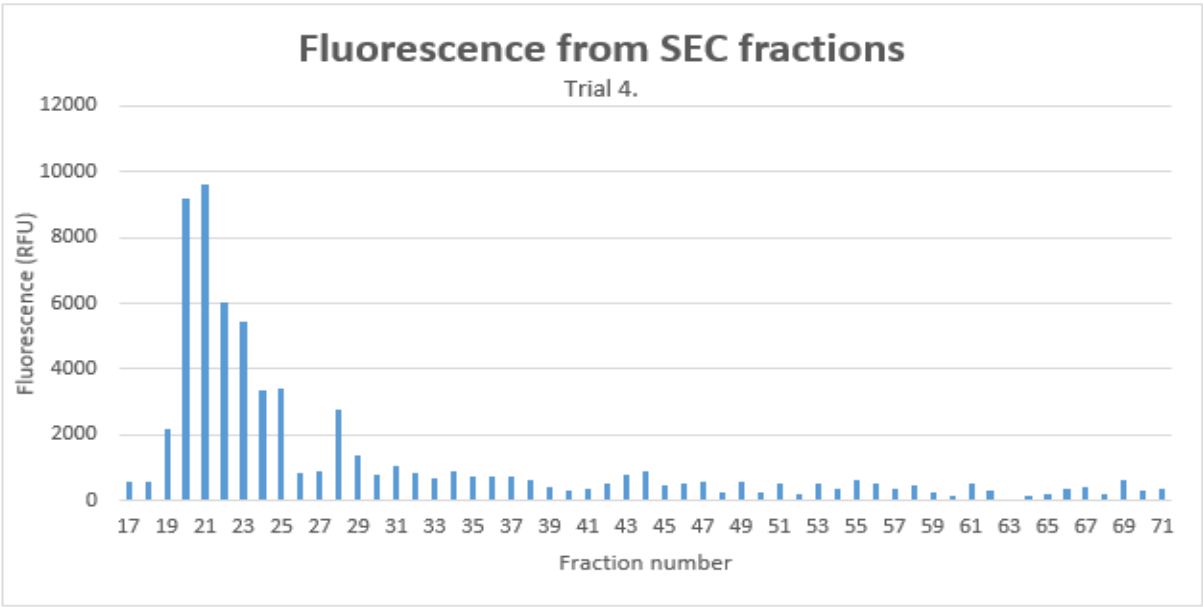


Figure 23 a) SEC chromatogram from the pooled and concentrated fractions after IMAC purification Trial 4 (Table 2). The volume of each fraction is 0.5 ml. **b)** GFP-fluorescence of fractions from the SEC gel-filtration presented in a).

Fluorescing bands were detected in the SDS-PAGE from the solubilized sample before binding to Ni resin (Fig 27 a) confirming presence of the protein before any purification steps. Further, close to no fluorescing band was detected from the sample corresponding to the IMAC fraction with highest fluorescence in solution (fraction 2 eluted, 150 mM imidazole). This implies that low amounts mDMT1 eluted with the fraction. It appears that the protein is almost fully lost after purifying, pooling and concentrating since no clear fluorescing band can be observed from that sample in the gel (Fig 27 a). Numerous bands through the whole lane are visible for all samples mentioned (Fig 27 b) implying presence of other proteins.

TRIAL 5

In Trial 5, detergents for solubilization were changed back to DDM and CHS. A new purification method, IEX, was performed by using an anion exchanger. The sample was applied at 0.80 ml/min. The RB had pH 7.5 and the NaCl concentration was decreased to 15 mM in order to allow protein-matrix binding.

Fluorescence was measured before and after the sample was applied to the column and results show that ~5/6 of the protein bound to the IEX matrix (Fig 24). Further, the IEX chromatogram (Fig 25 a) presents protein elution as three high and clear-cut peaks in the 250 mM, 350 mM and 600 mM NaCl wash. mDMT1 presence was observed by fluorescence measured from samples of the 250 mM NaCl wash (fraction 32 – 37) and the 350 mM NaCl wash (fraction 44 – 46) (Fig 25 b). No significant fluorescence was detected from samples of the 600 mM NaCl wash.

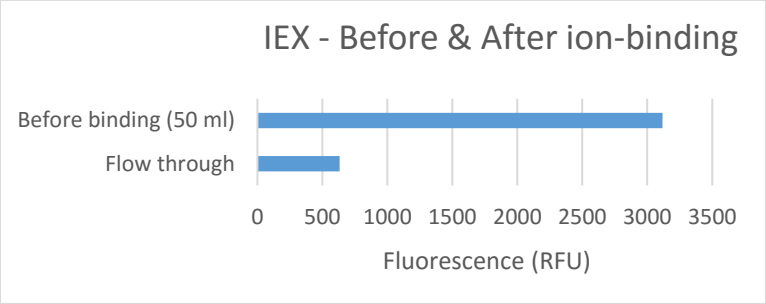
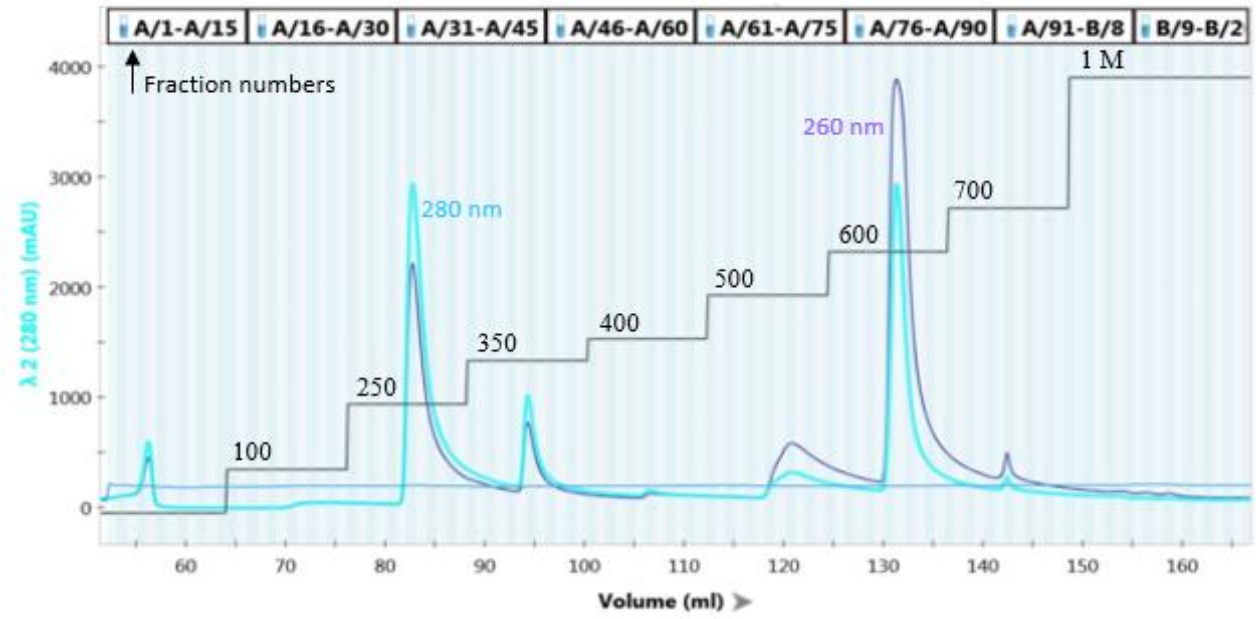


Figure 24. Fluorescence from solubilized and diluted membranes before and after application to the IEX column (50 ml).

a)



b)

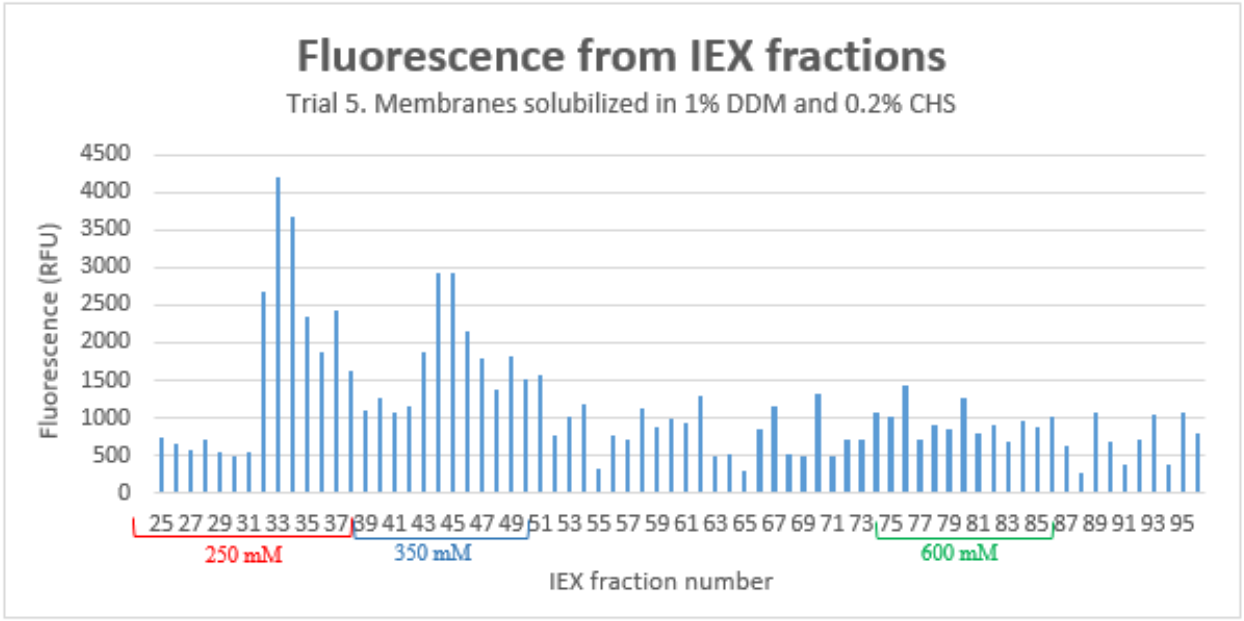
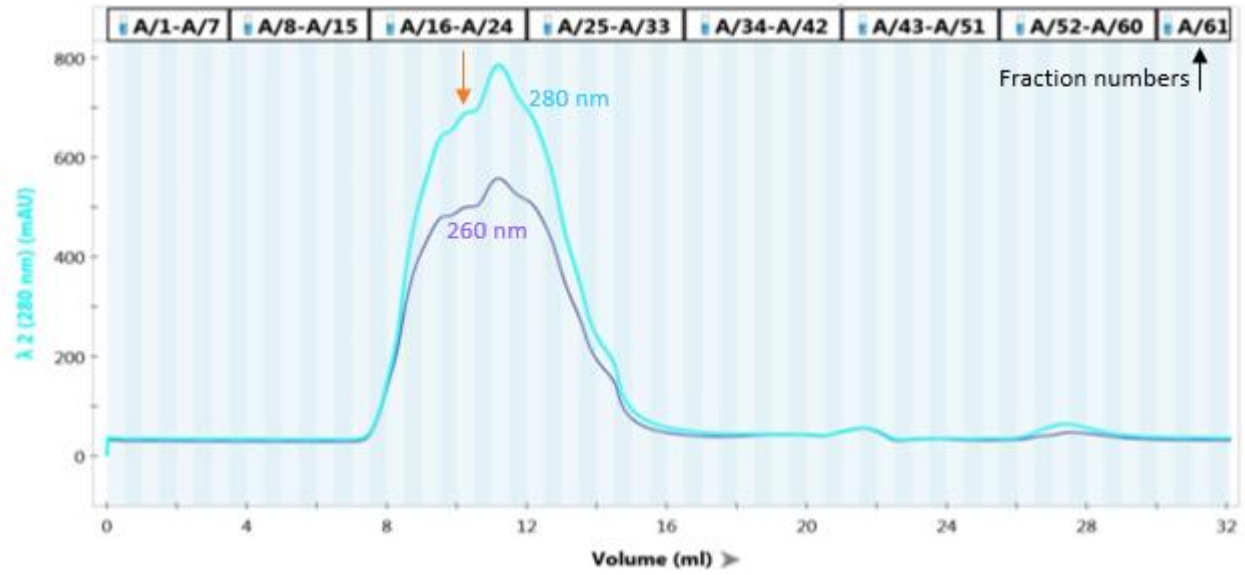


Figure 25 a) IEX chromatogram showing fractions eluting with a stepwise increased concentration of NaCl (mM). The volume of each fraction is 1 ml. **b)** Fluorescence from IEX fractions presented in a).

The IEX fractions that presented highest fluorescence were pooled and concentrated (fraction 32 – 38, 250 mM NaCl and 43 – 46, 350 mM NaCl) and further separated by SEC. This resulted in an 800 mAU "blunt peak" starting from the void volume and 8 ml ahead (Fig 26 a) implying presence of protein aggregation. Results also indicate that there may have be non-aggregated

protein present in a later eluted fraction, at 11.5 ml, which in that case was not well separated from the aggregates. Fluorescence, that was lower than 35000 RFU, confirmed that low amounts of the protein was present both in the void volume (aggregates) and in fractions eluting at 11.5 ml (Fig 26 b).

a)



b)

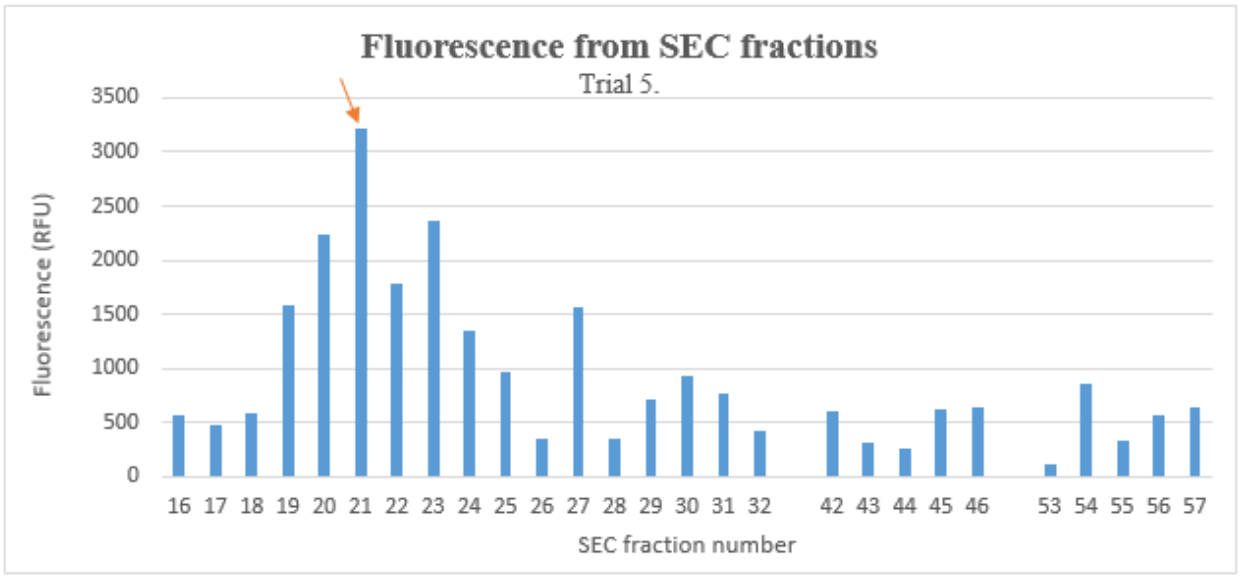


Figure 26 a) SEC chromatogram from the pooled and concentrated fractions after IEX purification Trial 5 (see Table 2). Fractions collected from a Superdex 200 column. The volume of each fraction is 0.5 ml. **b)** Fluorescence from selected SEC fractions presented in a). Orange arrows clarifies which SEC fraction that corresponds to highest measured fluorescence.

The SDS-PAGE (Fig 27 a) shows a non-migrated fluorescence from top of the well that contained a sample from the highest fluorescing IEX fraction (nr 33, 250 mM NaCl). The second IEX fraction which also gave high fluorescence (nr 44, 350 mM NaCl) did not show a strong fluorescing band in the gel (Fig 27 a). Further, low mDMT1 amounts were confirmed by the stained gel as no visible band at 89 kDa was detected (Fig 24 b). The same results were obtained for the pooled and concentrated sample which show a smeared result in the stained gel and an almost invisible band in the fluorescing gel (Fig 27).

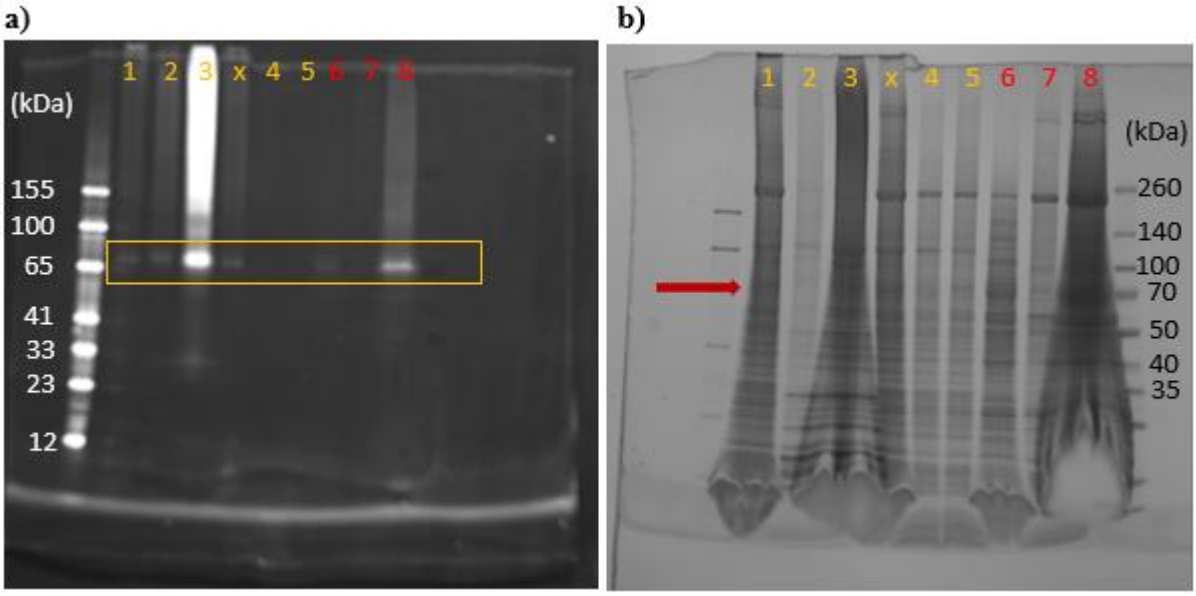


Figure 27. SDS-PAGE from IEX fractions Trial 5 (indicated with yellow numbers) and IMAC fractions Trial 4 (indicated with red numbers). Yellow box and red arrow indicates the correct molecular size for yEGFP-mDMT1. Trial 5: Pooled and concentrated fractions (1), fraction 44 (2), fraction 33 (3), diluted sample before ion binding (4), solubilized sample before dilution (5). Trial 4: pooled and concentrated fractions (6), fraction 3, 150 mM imidazole wash (7), before binding to Ni resin (8) **a)** In-gel fluorescence detected with 2 minutes exposure time and **b)** stained (SimplyBlue) gel.

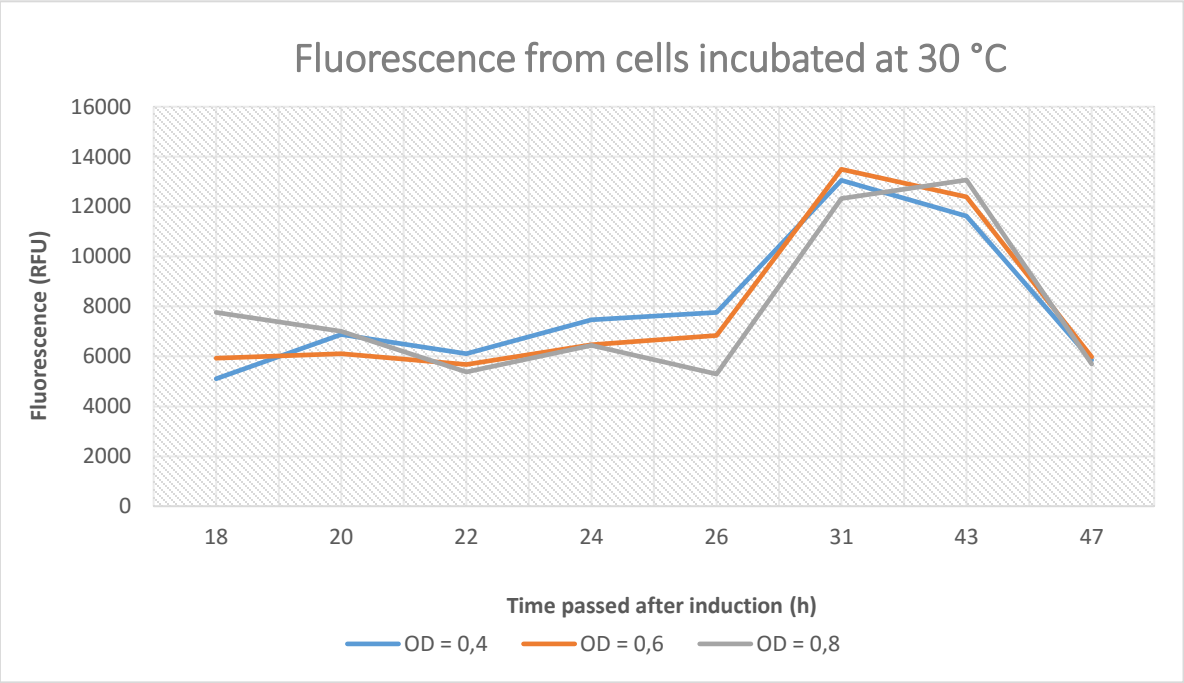
3.5 Optimization of Expression

As expected, one result was consistent throughout the presented trials, namely a low concentration of purified mDMT1. It was expected since the estimated mDMT1 yield was low (1.6 mg per 9-l of cell culture) already before purification. In order to optimize the expression of mDMT1, yeast cells induced at three different OD_{600} units (0.4, 0.6 and 0.8) were incubated at two different temperatures (23°C and 30°C).

Results (Fig 28 a) shows that the 0.4-culture expressed more protein 22 – 26 h after induction when growing at 30 °C compared with the other two cultures. The culture that showed most expression at the recommended time for harvesting (22 h after induction) was the 0.6-culture incubated at 23 °C (Fig 28 b). Further, for cultures incubated at 30 °C, fluorescence was more than two times higher 31 h after induction when comparing with the time point 9 h earlier (22 h after induction). Highest measured fluorescence was however obtained for cultures incubated at

23 °C by showing a fluorescence almost 2.5 times higher 43 h after induction when comparing with the fluorescence measured 22 h after induction. Results for 0.8-cultures did overall not show to express more protein than the other cultures at the time points measured.

a)



b)

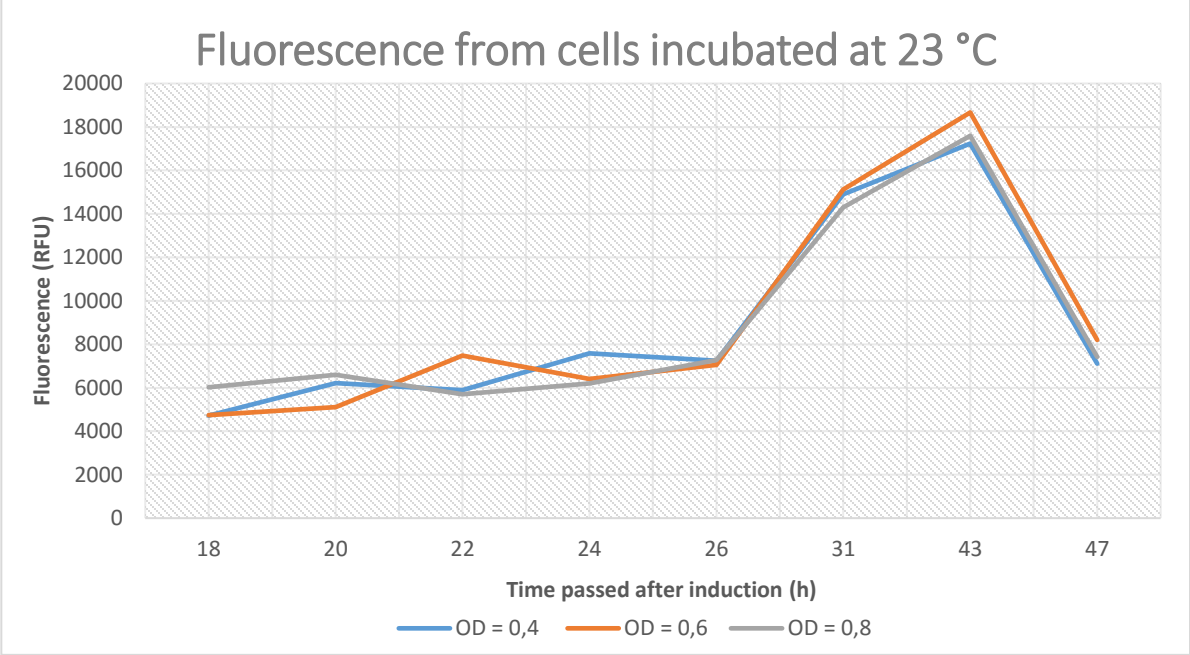


Figure 28. Fluorescence per yeast cell number, induced at different OD_{600}, measured from 10 ml in a time point series. **a)** cells incubated at 30 °C and **b)** cells incubated at 23 °C.

4. Discussion

In this part, results from the different trials will be discussed with an approach of how well purification of mDMT1 succeeded. Since the estimated amount of expressed mDMT1 per large scale cell culturing was 1.6 mg, it was not expected to obtain purified samples in quantities sufficient for crystallization. Instead, the results should be perceived as a guideline for what methods that seems promising to optimize for future purification trials.

4.1 Detergent screen

The presence of CHS, complementary with DDM, has a positive effect on the solubilization efficiency of mDMT1. An explanation for this could be its similarity to natural cholesterol that often is present within mammalian cell membranes [21]. Ideally, a single peak around 13 ml should be seen when purifying the recombinant protein with the columns used in SEC since that is the volume corresponding to yEGFP-mDMT1 molecular size [17]. For the detergent screen though, it was not expected to see a protein separation with high resolution since a detergent screen is more of a rough analytic step without any preparative purification (such as IMAC). Overall, the results indicated, by the means of fluorescence, that DDM is the most suitable detergent to use for solubilizing mDMT1.

4.2 Purification

Trial 1. The binding of protein to Ni resin was poor in Trial 1. As a first trial however, elution looked promising considering there was a specific fraction (nr 2, 150 mM) where the protein eluted to a larger extent (Fig 10). Even though results revealed that the protein concentration in this fraction was low it looks like it did not contain a lot of impurities. This allow the conclusion that a 10 mM and 50 mM imidazole wash, followed by a 150 mM eluate, is a suitable wash scheme to use for Ni-NTA purification of mDMT1. There is reason to suspect precipitation of protein in the column and one possible reason for this is that no CHS was present in the buffer. Precipitation would explain the absence of absorbance at 280 nm from pooled and concentrated IMAC fractions (Fig 12).

Trial 2. It seems, when comparing fluorescence from IMAC fractions, that the new conditions in Trial 2 eluted only half as much protein as in Trial 1 (Fig 10 and 14). However, since the sample was diluted to 50 ml before it was applied to the HisTrap at 0.8 ml/min, the actual binding time was decreased from 1 hour (Trial 1) to 40 minutes. The decreased binding time was could be a reason for less bound protein and thus less detected fluorescence. A slower sample application should preferably be used if a second trial were to be performed. However, better success in the SEC purification step was obtained since no fluorescence was detected for samples corresponding to the SEC void volume (Fig 15 b). This means that aggregation of the protein was avoided and indicates that addition of 0.01 % (w/v) CHS to the purification buffer improves the solubility (e.g. avoids precipitation) of mDMT1 in solution. The SEC chromatogram (Fig 15 a) moreover show that eluted proteins was not well separated meaning that the samples were not monodisperse [17, 19].

It is hard to interpret how well the protein bound to the HisTrap stationary phase since data from FT fluorescence in Trial 2 is unavailable. Fluorescing bands in SDS-PAGE gels from Trial 1 and 2, however, imply that there was a somehow better binding in Trial 2 (Fig 16 a). Again, it must be kept in mind that the FT from Trial 2 is a $\sim \times 2$ diluted sample compared to that in Trial 1. These bands, in other words, are not very comparable. On the whole, Ni-NTA seemed to give more mDMT1 concentrated fractions than HisTrap and the presence of CHS in buffers is important to avoid precipitation of the protein. Further, SEC purification did not manage to elute the protein with high resolution (Fig 15 a) concluding that purification conditions must be optimized.

Trial 3. It seems that the increased interaction time of protein to Ni resin in Trial 3 increased the binding compared to Trial 1. More protein also seemed to elute with fraction 2, 150 mM imidazole, only by comparing RFU from trial 1 and 3 measured from equal volumes (Fig 10 and 18).This is most probably as a consequence of the improved binding, but could also be a consequence of having less imidazole washes before elution. Further, the more distinct absorbance peak in the SEC chromatogram (Fig 19 a) imply an improved resolution of protein separation [17] when comparing with Trial 2. That peak, however, is detected from unwanted proteins eluting within the same fraction range as mDMT1 probably resulting in impure samples, i.e. non monodisperse samples, unsuitable for crystallization. Preferably, in a second try of this trial, a longer SEC column should be used in order to optimize the separation of mDMT1 from unwanted proteins [17].

It is hard to elucidate why there was aggregation of mDMT1 in the 150 mM imidazole wash for this trial. One reason could be the increased binding time that allows proteases to degrade the protein during a longer period. Degradation exposes hydrophobic residues of the protein and thus leads to more aggregation [13].

Trial 4. The amounts of protein binding to Ni resin in Trial 4 increased with approximately 20% when comparing with Trial 3. This is most probably a consequence of using DM for solubilizing proteins instead of DDM. In theory, it could have been that DDM is a detergent big enough to cover the protein His-tag hence interfering with Ni-binding. Since DM creates smaller micelles it may also have allowed better exposure of the His-tag [12, 13]. Elution was not as specific in this trial compared with previous ones. It also seemed like a big part of the bound protein did not elute at all. If comparing bands from the solubilized sample before Ni binding with the concentrated sample in the SDS-PAGE gel (Fig 27 a), fluorescence intensity is lower after concentrating the sample. This indicates that there was a loss of protein during the purification step. Since the protein had better binding in this trial, it is reasonable to suppose that it also bound harder and hence requires higher imidazole concentrations in order to elute. This trial should be performed again, preferably with a $\sim$15 mM imidazole wash directly followed by a second one with $\sim$200 - 300 mM imidazole as an attempt to elute more protein. Conclusions regarding the purifying suitability of DM should not be drawn before this.

There were some alarming results when analyzing the SDS-PAGE further though. It seemed like the protein could have aggregated or precipitated already after the solubilizing step since smeary fluorescing bands were detected from the sample that was collected before any Ni-binding (Fig 27 a). This suggests that DM is not a suitable detergent for solubilization of mDMT1 after all.

SEC and fluorescence measurements, which did not show significant presence of the protein, supports this theory. However, as discussed for Trial 3 already, the increased binding time could also have a negative impact on the protein stability, i.e. aggregation formation, due to degradation by proteases [12, 13].

There may also be a reason to think that the change of buffer from MRB to RB (see appendix) had a negative impact on stability of the membranes and/or protein. It could be of interest to redo this trial from a membrane batch stored in MRB and likewise also use a solubilization stock prepared with MRB.

Trial 5. IEX seems to be a promising method for mDMT1 purification since it showed off to bind more protein than any other method tried during the project. However, the binding is not as specific in IEX as it is when using affinity-tags in IMAC. Unwanted proteins, with similar pI, will also bind and further elute with a certain NaCl concentration yielding a mixture of different proteins in the fractions [16]. It is apparent that this was the case for this trial since mDMT1 eluted with fractions detected as a ~3000 mAU peak in the IEX chromatogram (Fig 25 a). An option in order to circumvent unspecific elution is to use complementary IEX purification steps within different conditions. For this trial, a suggestion could be to pool all the fractions that present significant fluorescence and perform a second anion exchange chromatography where the sample and the buffer A has an increased pH to 8.0. In theory, this increases the negative net charge of the protein and thus the affinity to the positively charged matrix. Higher affinity allow the prediction that elution will occur with higher NaCl concentrations [16]. A second option is to combine IEX with IMAC as the second purification step. Preferably within conditions according to Trial 5 and Trial 3 (Table 2) respectively.

Protein was also eluting later, in the 350 mM NaCl wash but with lower absorbance. Fluorescence from samples and from SDS-PAGE bands corresponding to this wash indicate that the protein yield was low. It should be kept in mind though, that the batch used for this trial also had a lower concentration of membranes compared with other trials (Table 2). The SEC chromatogram show a big and “bulky” void volume peak spread over numerous fractions (Fig 26 a) meaning that separation of aggregated and non-aggregated protein failed [17]. Pooling and concentration of fractions that showed highest fluorescence after IEX was wrongfully performed before analysis of the in-gel fluorescence (Fig 27 a) that revealed presence of aggregated protein. Staining of the gel (Fig 27 b) further confirmed presence of aggregate and other proteins in the IEX fractions. This is most probably also the reason for why the separation by SEC was unsuccessful.

4.3 Optimization of expression

Expression in between 31 – 43 h after induction gives significantly more protein than after 22 h of expression which is the recommended time point for cell harvesting [19]. Interestingly, results also imply that there is a higher yield of protein when incubating cultures at 23 °C. One explanation for this could be that since cells in general work slower at low temperatures, more time for correct translation and folding is given. As a result, increased amounts of functional recombinant protein, and by this more fluorescence, is obtained. Conclusively, results suggest that more than twice as much protein may be isolated if cells, induced at OD_{600} = 0.6 and

incubated at 23 °C, were harvested 43 h instead of 22 h after induction (Fig 28 b). These parameters and conditions should thus preferably be used in future in order to obtain as much expression of mDMT1 from *S. cerevisiae* as possible.

There are some aspects to consider though. Since this experiment only relied on fluorescence data, there is no guarantee that the expressed protein after 31 h is in good state, e.g. correctly folded. It could be that too high concentrations of the protein within the membrane, e.g. restricted space, causes aggregate formations. One option in order to elucidate this is by harvesting and disruption of cells followed by purification with SEC or by separation by centrifugation.

5. Conclusions & Future Aspects

Eukaryotic MP are difficult targets to overexpress, extract and purify. Here, by trying to optimize the expression, screening for a suitable detergent and test different purification techniques we establish a guideline for what conditions and methods that are worth to further optimize in order to yield pure samples of mDMT1 in high concentrations from *S. cerevisiae*. Taken together, none of the tested purification methods with the specific conditions fully succeeded to reach the aim of this project which was to obtain mDMT1 samples in qualities and quantities good enough for crystallization. However, results from this project unravel promising optimization options that may lead closer to the final goal. Results suggests that;

- mDMT1 is soluble with 1% (w/v) DDM and 0.2% (w/v) CHS
- presence of 0.01% (w/v) CHS during purification increases solubility of mDMT1 in solution
- 1% (w/v) DDM solubilized mDMT1 elutes with 150 mM imidazole when purifying by Ni-NTA
- Ni-NTA give fractions containing the highest concentration of mDMT1 among the purification methods tested
- preliminary data suggests that Ni resin interaction with mDMT1 overnight results in ∼20% greater binding than interaction for 1 h
- preliminary data suggests that, at pH 7.5, up to 35% more mDMT1 binds to the anion exchanger matrix than to Ni resin
- preliminary data suggests that the maximum whole cell fluorescence is obtained from *S. cerevisiae* induced at an OD_{600} of 0.6 and incubated at 23 °C for 43 h

Suggestions for fast optimization steps by the means of;

- Expression: Grow large scale yeast cell cultures according to presented “optimal” conditions in order to obtain a greater yield of mDMT1.
- Solubilization: Solubilize mDMT1 with DDM and CHS as performed in the most succeeded trials in this protocol. For further detergent screens, try detergents with similar micelle formation size as DDM.
- Purification: Conditions and methods according to Trial 3 (Table 2) did overall give the most successful results. Use a longer SEC column to further improve the outcome from this trial in order to obtain a better separation of mDMT1 from contaminants.
- Purification: The anion exchange matrix within conditions according to Trial 5 (Table 2) bound highest amounts of mDMT1. Optimize by using complementary IEX purifications with different conditions such as increased pH of sample and a buffer A or by combining IEX with IMAC purification.

5.1 Future Aspects

Success in fulfilling the goal of this project within the near future looks promising. Thus, in a longer prospective, it is not unreasonable to expect that the 3D-structure of mDMT1 will be determined. Two important results made progress for the project – the finding of optimal conditions for increased mDMT1 expression and a detergent combination of DDM and CHS that prevents aggregation in solution. With further optimization of this protocol it is highly likely that future experiments will succeed in yielding samples qualitative for structural work.

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Appendix

Cell Culturing & Membrane Preparation

Agar selection media

6.7 g/l YNB
2 g/l -URA amino acid mix
2% (w/v) agar
2% (w/v) glucose

Pre-culture media

6.7 g/l YNB
2 g/l -URA amino acid mix
2% (w/v) glucose

Culturing media

6.7 g/l YNB
2 g/l -URA amino acid mix
2% (w/v) agar
0.1% (w/v) glucose

YSB

50 mM Tris-HCl pH 8.0
5 mM EDTA
10% (v/v) glycerol
1 tablet/50 ml YSB of cOmplete™ Protease Inhibitor Cocktail (Sigma-Aldrich) without EDTA

Breaking buffer

50 mM Tris-HCl pH 8.0
2 mM EDTA
5% (v/v) glycerol

MRB

20 mM Tris-HCl pH 8.0 (pH 7.5 in detergent screen and Trial 1)
0.1 mM $CaCl_2$
300 mM sucrose

RB

20 mM Tris-HCl pH 8.0
500 mM NaCl
10% (v/v) glycerol

Detergent Screen

MRB
See Cell Culturing & Membrane Preparation in Appendix

Equilibration buffer
20 mM Tris-HCl pH 7.5
150 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM

Purification of Protein

IMAC

Equilibration buffer – Trial 1
20 mM Tris-HCl pH 7.5
150 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM

Equilibration buffer/Buffer A – Trial 2 (HisTrap)
20 mM Tris-HCl pH 7.5
150 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM
0.01% (w/v) CHS

Buffer B – Trial 2 (HisTrap)
20 mM Tris-HCl pH 7.5
150 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM
0.01% (w/v) CHS
150 mM imidazole

Equilibration buffer – Trial 3
20 mM Tris-HCl pH 8.0
150 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM
0.01% (w/v) CHS
10 mM imidazole

Equilibration buffer – Trial 4
20 mM Tris-HCl pH 8.0
300 mM NaCl
5% (v/v) glycerol
0.03% (w/v) DDM
0.01% (w/v) CHS
10 mM imidazole

IEX

Equilibration buffer/Buffer A – Trial 5
20 mM Tris-HCl pH 7.5
15 mM NaCl
0.03% (w/v) DDM
0.01% (w/v) CHS

Buffer B – Trial 5 (IEX)
20 mM Tris-HCl pH 7.5
1 M NaCl
0.03% (w/v) DDM
0.01% (w/v) CHS

SEC

Buffer A
Prepared according to each trials specific equilibration buffer/buffer A. See above.

SDS-PAGE

5X SDS loading dye

10% SDS
250 mM Tris-HCl pH 6.8
0.02% Bromophenol blue
30 % Glycerol
5% β-mercaptoethanol
3 mM DTT

Tris-Glycine Buffer

250 mM Tris
1.9 M Glycine
1% (v/v) SDS

Estimation of mDMT1 amounts expressed from 9 l of cells calculated according to the protocol [19].

Membrane preparation yielded 5 g of membranes from 9 l of cells. Membranes were re-suspended in 45 ml RB (0.11 mg/ml membranes). Fluorescence from corresponding sample was 62167.5 RFU.

$$\frac{(RFU\ in\ solution)}{(RFU\ of\ free\ yEGFP)} \times (conc.yEGFP) = Conc.GFP \quad (1)$$

Equation from standard curve (Fig 4): y = 5683x – 42.444 where x = 3 µg/ml (2)

y = (RFU of free yEGFP) = 5683 × 3 – 42.444 = 17006.56 RFU

Put y-value from eq. 2 into eq. 1

$$\frac{62167.5\ RFU}{17006.5\ RFU} \times \left(3\ \frac{\mu g}{ml}\right) = 10.97 \frac{\mu g}{ml}\ of\ GFP$$

With respect to dilution to 45 ml:

Amount of GFP-mDMT1 fusion = 10.97 µg/ml × 45 ml = 493.7 µg GFP-mDMT1

Amount of mDMT1:

$$\frac{GFP\ fused\ mDMT1\ (89\ kDa)}{GFP\ (28\ kDa)} \times\ 493.7\ \mu g\ = 1596\ \mu g\ \approx \mathbf{1.6\ mg\ mDMT1}$$