An investigation of the interaction between Aquaporin 5 & Ezrin FERM domain

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1 Abstract

The water in our body is one of the most fundamental substances we have. Thanks to the family of water channels called aquaporins, water can be distributed throughout the entire body. The distribution of water helps with vital body functions. Aquaporin-5 is involved in our ability to produce sweat, tears, and saliva. If the water channels were dysfunctional or mutated there is a high chance of complications. Defects in the distribution of aquaporin-5 has been linked to Sjögren's syndrome where the patient has a low production of fluid in the eyes and mouth. The regulation of how membrane proteins are distributed in the cell is called trafficking, and the protein ezrin has been discovered to interact with aquaporins in trafficking processes. In the present study, the interaction between a peptide and a protein were tested, the C-terminus of aquaporin-5 and the FERM domain of ezrin. The peptide and protein were analyzed in SDS PAGE and Western blot after affinity chromatography, and in microscale thermophoresis. The microscale thermophoresis K_d (disassociation constant) value calculated by MO Affinity using a preset equation showed promising result. However, the capillary scan values were above $\pm$10% and the K_d confidence value was high. A lower error of margin would have been desirable, and the MST measurements needs to be repeated and optimized. The results from running a mixture of the C-terminus and the FERM through affinity chromatography, and analyzing with SDS PAGE and Western blot, showed that a complex had been formed between the proteins of interest. Future studies should be done with full length proteins and repeated to give strong evidence of an interaction.

2 Introduction

The average human body consists of more than 50% water. The water in our bodies is vital for our survival (Lote, 2012). Water is essential in several different processes in the body and is retrieved via the food we eat and the liquids we drink. The water is able to be distributed throughout the body and into all cells via membrane protein channels named aquaporins (Yang, 2017). If the water distribution and transport in our bodies were altered through genetic changes, or they somehow worked improperly, serious diseases could occur. Aquaporins have been linked to several human disease states, including respiratory, renal, and neurosurgical diseases (Magouliotis et al., 2020).

Aquaporin-5 (AQP5) is a water channel protein which belongs to the aquaporin family. Aquaporins are membrane-bound proteins that primarily facilitate the diffusion of water across the plasma membrane, but also other small molecules. The aquaporins can be divided into two families. The orthodox aquaporins and the aquaglyceroporins. The orthodox AQPs are only water permeable, and the Aquaglyceroporins are permeable to water and other uncharged molecules (Roche & Tornroth-Horsefield, 2017). The structure of aquaporins is made of four monomers forming a tetramer, where each monomer is a water transporting pore (Fig. 1). Each monomer is made of six transmembrane helices (Kreida & Tornroth-Horsefield, 2015). Aquaporin-5 resides in the apical membrane in acinar cells of salivary, lacrimal, sweat, and other secretory glands. The apical membrane is the last membrane the water needs to cross to create the secretory fluids. These glands are known to produce a significant amount of fluids (Agre, 2004). For example, we secrete water through sweat glands when the body temperature needs to lowered. The distribution of AQP5 to and from the plasma membrane has also been suggested to be linked to Sjögren's syndrome (Agre, 2004). Sjögren's syndrome is a chronic autoimmune disease which leads to dry eyes and dry mouth caused by lymphocytic infiltration and destruction of secretory glands (Chivasso et al., 2021). The importance of AQP5 could be seen in AQP5-knockout mice, where a 60% reduction of secretion from the salivary glands when the AQP5 was not present (Ma et al., 1999). AQP5 affects water permeability via the paracellular pathway and is regulated via trafficking to the plasma membrane when cell stimulation occurs. The trafficking of AQP5 has shown to be impaired in the salivary glands in subjects with Sjögren's syndrome (Lee et al., 2012).

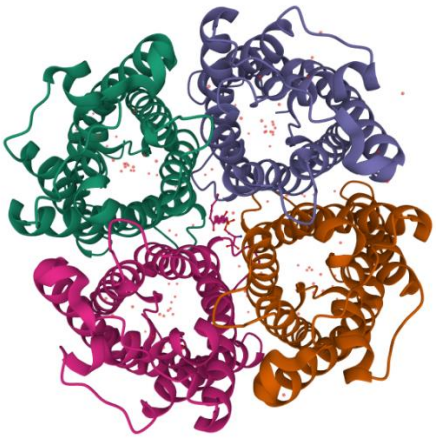

Figure 1: Structure of AQP5 with each color of the structure marking one monomer, together forming a tetramer. [DOI:10.2210/pdb3D9S/pdb]

Trafficking is the transportation of proteins to their designated destination. This is done via transporting protein inside vesicles. Endo- and exocytosis makes it possible to retrieve proteins from the plasma membrane or add proteins to the membrane, in order to up- or down regulate protein expression (Schülein & Rosenthal, 2008).

Ezrin is a protein belonging to the ezrin/radixin/moesin (ERM) proteins. The ERM proteins are a family of proteins that have an important role in endocytosis, phagocytosis, vesicular trafficking, and vesicle maturation. Ezrin is membrane-bound and interacts with actin filaments of the cytoskeleton. The ERM proteins have a conserved domain at the N-terminus called FERM (Fig. 2) and often a cytoskeleton binding domain at the C-terminus. The FERM domain can bind a diversity of proteins, lipids, and molecules. When the C-terminus is unphosphorylated, FERM is viewed as inactive, and when it is phosphorylated FERM is active and prone to bind various partners (Bosanquet et al., 2014).

Previous studies have confirmed the binding between AQP5 and ezrin via immunoprecipitation assays (Chivasso et al., 2021). Computer modelling has also been done to investigate the interacting domains, where the conclusion was AQP5 C-terminus interacts with the sub-domain C β-sandwich of the ezrin FERM domain (Chivasso et al., 2021). Interaction between AQP2 and ezrin has also been found by using anti-ezrin and anti-AQP2 antibodies in a co-immunoprecipitation assay. In the same study the full length-, C-terminus- and N-terminus of ezrin were tested, and the conclusion was AQP2 interacts with full length and N-terminus, which is where the FERM domain is located (Li et al., 2017). Another study presents data on interaction between the C-terminus of AQP0 and ezrin via a co-immunoprecipitation assay and mass spectrometry (Wang & Schey, 2011). It was also discovered that a knockout of the ezrin expression increased the presence of AQP2 in the plasma membrane, and less trafficking via endocytosis of AQP2 occurred (Li et al., 2017). This report aims to study the interaction between the water channel AQP5 and the protein ezrin, to gain more knowledge about the trafficking of aquaporins and its role in disease.

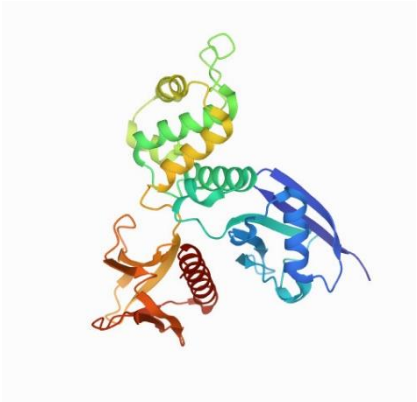

Figure 2: Structure of the active FERM domain of ezrin. [DOI: 10.2210/pdb1NI2/pdb]

3 Methods & Terminology

3.1 Terminology

3.1.1 Induction with IPTG

IPTG is a molecule with structural properties similar to lactose and is used to induce protein expression. IPTG binds to the lac operon repressor and causes it to dissociate. This allows RNA polymerase to start the transcription of the operon where the gene for the protein of interest is located (Martin, n.d).

3.1.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE is a type of gel electrophoresis. The gel is of polyacrylamide and a small amount of N,N'-Methylenebisacrylamide which is a cross-linking agent and helps with the 3D-structure of the gel. The principle is that sodium dodecyl sulfate (SDS) is mixed with a protein solution and disrupts most of the non-covalent bindings. Then beta-Mercaptoethanol or dithiothreitol is added to hinder the amount of disulfide bonds formed. The bound SDS and denatured protein will have a large net negative charge in correlation to the weight of the protein. This formation will be subjected to electrophoresis and move through the porous gel. Small proteins will move faster than the large proteins. The gel must be stained with a dye, such as Coomassie blue, to be able to visualize the protein bands (Berg, 2019).

3.1.3 Western Blot

Western blot is an immunoassay technique which allows for small quantities of protein to be detected. After an SDS PAGE have been performed, a polymer membrane is pressed against the gel, transferring the proteins from the gel to the membrane with the help of an electrical current. An antibody called *primary antibody*, which is specific for the protein of interest is applied to the membrane and binds the protein. Then another antibody called *secondary antibody*, is added to the membrane, and binds to the primary antibody. The secondary antibody is linked with an enzyme that can be detected, often a fluorescent enzyme (Berg, 2019).

3.1.4 Immobilized Metal Affinity Chromatography (IMAC)

IMAC is a type of affinity chromatography which is used for purification of proteins by taking advantage of the unique properties of different amino acids. The protein solution is passed through a column which has an immobilized metal ion that the protein of interest will bind to. The protein is then eluted by adding a buffer which contains molecules with higher affinity to the column than the protein (Berg, 2019).

3.1.5 GST Affinity chromatography

Glutathione S-transferase (GST) is a protein which is commonly used for purification. The GST protein can be fused with a protein of interest and be used in affinity chromatography, where the GST binds to glutathione-conjugated resin in a column. Then a buffer with glutathione is used for elution of the bound protein. If GST is fused with a protein of interest it can be used in a pull-down assay to detect protein-protein interactions (Kim & Hakoshima, 2019). For example, if the GST-protein A binds to a column and is then washed with protein B solution, and protein B interacts with protein A. Then protein B will bind to protein A in the column and will not be eluted until elution buffer is added.

3.1.6 Microscale thermophoresis (MST)

MST is a method for analyzing the binding between different molecules. With the usage of a thermal gradient and fluorescently marked molecules, the binding affinity can be measured. MST is based on the principle that molecule concentration in a specific region correlates to temperature change (Asmari et al., 2018).

3.1.7 Alexa 488

Alexa 488 dye is a fluorescent labeling dye which can be attached to proteins and can be measured at 488nm excitation.

3.1.8 NGC machine/FPLC system

Fast Protein Liquid Chromatography (FPLC) system (Fig. 3) is used for protein separation and purification. The system most often consist of a pump, a UV detector, a conductivity meter, and a fraction collector (Bio-Rad Laboratories Inc., n.d-b). Column liquid chromatography separates molecules based on their physicochemical interactions. Some of these interactions are size exclusion, ion exchange, or affinity chromatography. The UV detector measures light absorption at 280nm, which is the absorption of tryptophan (Bio-Rad Laboratories Inc., n.d-c).

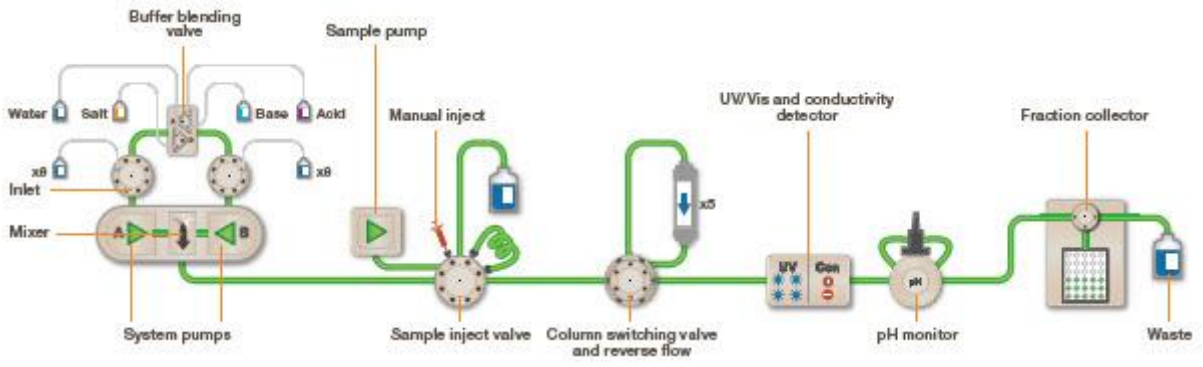

Figure 3: Sample fast protein liquid chromatography flow path. A sample flow path that illustrates the components of the NGC medium-pressure chromatography system. Image is taken from the NGC system's ChromLab™ software (Bio-Rad Laboratories Inc., n.d-b).

3.1.9 Desalting

Desalting is a type of Size exclusion chromatography where a porous column is used to separate proteins from a solution and adding the proteins to another solutions. The proteins are trapped in the column and then eluted with a new solution (Bio-Rad Laboratories Inc., n.d-a). Desalting is commonly used for buffer exchange.

3.2 Method

To test the hypothesis, two different analytical approaches were used. One approach was to mix the AQP5 peptide with ezrin FERM domain and then running the mixture through affinity chromatography, and then on SDS PAGE and Western blot to confirm the protein complex's presence. The other approach was the usage of Microscale thermophoresis (MST).

Protocols can be found in Appendix I. First, the AQP5 peptide-GST was produced by transforming a plasmid with the AQP5 and GST genes (Tab. 1 in Appendix I) into *E. coli* BL21 expression strain. The transformed cells were plated on agar plates with kanamycin to prevent unwanted bacterial growth, where the first plate was a negative control, the second plate was low concentration of cells, and the third plate was high concentration of cells. One colony was taken from the low concentration plate and inoculated overnight. Then a total of 2250ml of LB medium with kanamycin was prepared in 3 flasks, and 3ml of overnight culture was added to each flask. The cells were put in a 37℃-water bath and oxygen was pumped into the culture until the absorbance at 600nm reached OD = 0.58. Samples were collected every hour for analysis of the induction on SDS PAGE. The next step was to induce overexpression of the AQP5 peptide-GST and then harvest the cells after 3 hours. The harvested cells were mixed with *lysis buffer* (Tab. 2 in Appendix I) and then sonicated in order to break the cells. The solution was centrifuged to remove debris and then run through a 0.45 µm filter and degassed to run through affinity chromatography using the GST tag. The *lysis buffer* without PMSF was used as buffer A, and the *elution buffer* (Tab. 3 in Appendix I) was used as buffer B for the affinity chromatography. The GST purification protocol was based on the study (Kim & Hakoshima, 2019). Protein elution can be seen on a chromatogram as peaks of absorbance at 280nm. The black line on the chromatogram shows when buffer B starts, which is the elution phase. The sample application and the elution phase are collected, while the other phases go directly to waste. The collected fractions which suggested that protein had been eluted according to the chromatogram were chosen for SDS PAGE.

The ezrin FERM domain protein solution and the AQP5 peptide-GST protein solution was desalted into *complex forming buffer pH 7.4* (Tab. 4 in Appendix I) and mixed at a 1:1 concentration ratio. The protein mixture was on a shaker in the 4℃ cold room overnight. The mixture was run through a GST-column with *complex forming buffer pH 7.4* as buffer A and *elution buffer* as buffer B. The collected fractions which suggested that protein had been eluted according to the chromatogram was chosen for SDS PAGE and Western blot. The mixing of AQP5 peptide-GST and ezrin FERM domain, affinity chromatography using the GST tag, SDS PAGE, and Western blot had to be redone a second time because the results were not as expected according to previous studies. The second try was done with a new complex forming buffer with pH 8 instead of pH 7.4, *complex forming buffer pH 8* (Tab. 5 in Appendix I). The AQP5 peptide-GST mixed with the ezrin FERM domain was buffer exchanged using a desalting column into *FERM complex forming buffer* (Tab. 6 in Appendix I), then concentrated using a 30kDa cutoff concentrator column, and mixed with Thrombin at a 1:5 molar ratio in the 4℃ cold room overnight. The AQP5 peptide and ezrin FERM mixture was bound to a

IMAC column using the His-Tag on FERM with *FERM complex forming buffer* as buffer A and eluted with *His-Trap elution buffer* (Tab. 7 in Appendix I) as buffer B. The *His-Trap elution buffer* was pH adjusted from 10 to 8 with HCl. The collected fractions which suggested that protein had been eluted according to the chromatogram was chosen for SDS PAGE and Western blot.

For the MST measurement, AQP5 peptide-GST was purified via affinity chromatography using the GST tag on the AQP5 peptide, and the solution was mixed with Thrombin (to cleave off the GST tag) at a 1:5 molar ratio and left in the 4℃ cold room overnight. The AQP5 peptide was purified from GST and Thrombin using a 10kDa cutoff spin concentrator. The AQP5 peptide was buffer exchanged into *FERM complex forming buffer* using a desalting column. Ezrin FERM protein solution was received from a stock and desalted into *FERM complex forming buffer*. The ezrin FERM protein was labeled with Alexa 488 to be used in MST with AQP5 peptide. Microscale thermophoresis was prepared with FERM-Alexa and AQP5 peptide in a 1:1 ratio dilution series with the start concentration of FERM-Alexa at 2.579µM and AQP5 peptide at 67.50µM. The samples were prepared according to the MST protocol in Appendix I. Then 3 runs at 3 different LED power settings, 20%, 40%, and 60%, was performed in triplicates to get a reliable result. The preparations and MST measurements were performed a second time with four different LED power settings, 20%, 40%, 60%, and 80%, and with FERM-Alexa at a lower concentration for a result more according to expectations. The second attempt, FERM-Alexa had the start concentration of 0.2579µM, and AQP5 peptide had a concentration of 67.50µM before the 1:1 dilution series.

A SDS PAGE gel was prepared with four different samples; AQP5 peptide-GST, AQP5 peptide without GST, AQP5 peptide-GST mixed with ezrin FERM, and AQP5 peptide mixed with ezrin FERM domain and GST cleaved off.

3.2.1 MST calculations

The MST machine first inspects all the samples loaded into capillaries by performing a capillary scan and analyzing the comparability of the dilution series. The error of margin should be within 10%. Then the samples are analyzed at 20%, 40%, 60%, and 80% LED power. The binding affinity test at each LED power is tested 3 times. The results are then plotted in a program called MO Affinity by fitting a K_d model and calculated.

K_d model equation:

$$f(x) = \frac{(Bound - Unbound) \times (Ligand + Target + K_d - \sqrt{(Ligand + Target + K_d)^2 - 4 \times Ligand \times Target}}{2 \times Target}$$

(Asmari et al., 2018)

4 Results

The transformation of AQP5 plasmid into *E. coli* BL21 strain gave colonies on both the low and high concentration agar plates but not on the negative control plate (Fig. 4, 5, 6). Protein expression induced with IPTG of the AQP5 peptide showed many protein bands on the SDS PAGE gel (Fig. 7). A specific band can be seen increase in thickness around the 35kDa ladder mark in correlation with how much time that has passed (Fig. 7). A high amount of different proteins are eluted during the sample application phase on the chromatogram (Fig. 8), and then a new peak in protein elution during the elution phase where glutathione binds to the GST column with higher affinity than the GST-protein. The SDS PAGE gel after GST purification shows many different protein bands, the most prominent is around 35kDa (Fig. 9). The chromatogram (Fig. 10) shows elution peaks during the sample application and then a smaller peak during the elution phase. The SDS PAGE gel of the mixed ezrin FERM domain and AQP5 peptide-GST after GST purification shows prominent protein bands around 40kDa from the fractions during sample application, and around 35kDa from the fractions during elution (Fig. 11). Samples on the Western blot membrane with His-Tag antibodies shows protein bands only on the fractions from sample application phase of the GST purification of ezrin FERM domain mixed with AQP5 peptide-GST (Fig. 12). The chromatogram (Fig. 13) starts with a high peak of protein elution during sample application which then reduces until the elution phase where a new peak of eluted protein can be seen. The SDS PAGE gel after GST purification with the second attempted mix of ezrin FERM domain and AQP5 peptide-GST shows prominent protein bands on fraction 26 and 27 at 35kDa and fraction 5 at 40kDa (Fig. 14). Less prominent protein bands at 40kDa can also be seen on fraction 26 and 27 (Fig. 14). The Western blot membrane with His-Tag antibodies shows a reoccurring protein band at 40kDa on fraction 1, 3, 5, 26 and 27, whilst most prominent on fraction 5 (Fig. 15). The SDS PAGE gel of AQP5 peptide-GST, AQP5 without GST, AQP5 peptide-GST-FERM, and AQP5 peptide-FERM without GST, shows prominent protein bands at ~80, 35, <25, and <15kDa for the AQP5 peptide-GST sample, at <10kDa for the AQP5 without GST sample, at 35 and 40kDa for the AQP5 peptide-GST-FERM sample, and at <10, ~30, and ~39kDa for the AQP5 peptide-FERM without GST sample (Fig. 16). The chromatogram shows elution of protein during the elution phase and continuation into the column wash phase (Fig. 17). The SDS PAGE gel of AQP5 peptide-FERM without GST from His-Tag purification shows prominent protein bands on ~30kDa for fractions 31, 32 and 33, and ~38kDa for fraction 33 (Fig. 18).

The MST results from the first run (Fig. 19, 20, 21, 22) shows tendencies for an interaction between the proteins of interest. The MST results from the second run at both 60% and 80% LED power (Fig. 23, 24, 25, 26, 27, 28, 29, 30) shows there is an interaction between the proteins of interest. The dissociation constant K_d confidence, and the response amplitude (Tab. 1) also show an increase in quality of the analytical result with the second run compared to the first run.

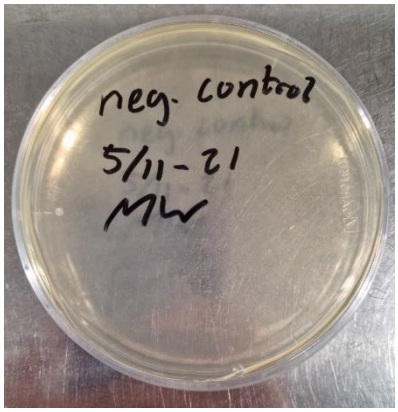

Figure 4: The negative control for the plasmid transformation where water was used instead of plasmid DNA. The figure shows no colonies on the agar plate.

Figure 5: The low concentration cell solution of the plasmid transformation. The figure shows >300 colonies on the agar plate.

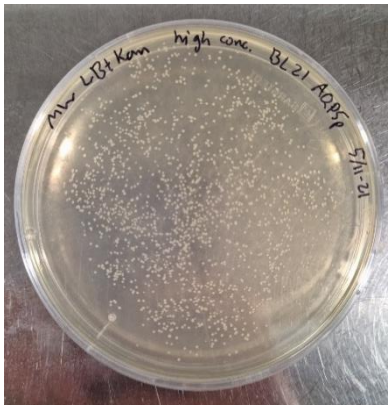

Figure 6: The high concentration cell solution of the plasmid transformation. The figure shows >600 colonies on the agar plate.

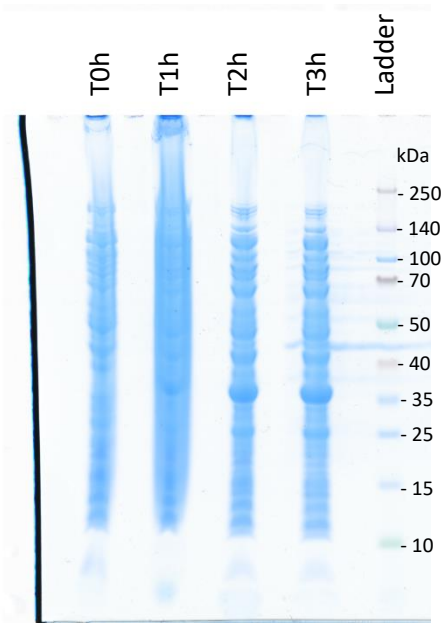

Figure 7: An SDS PAGE gel. The samples in the gel are, T0h (cell culture before induction with IPTG), T1h (cell culture after 1 hour of induction with IPTG), T2h (cell culture after 2 hours of induction with IPTG), T3h (cell culture after 3 hours of induction with IPTG.

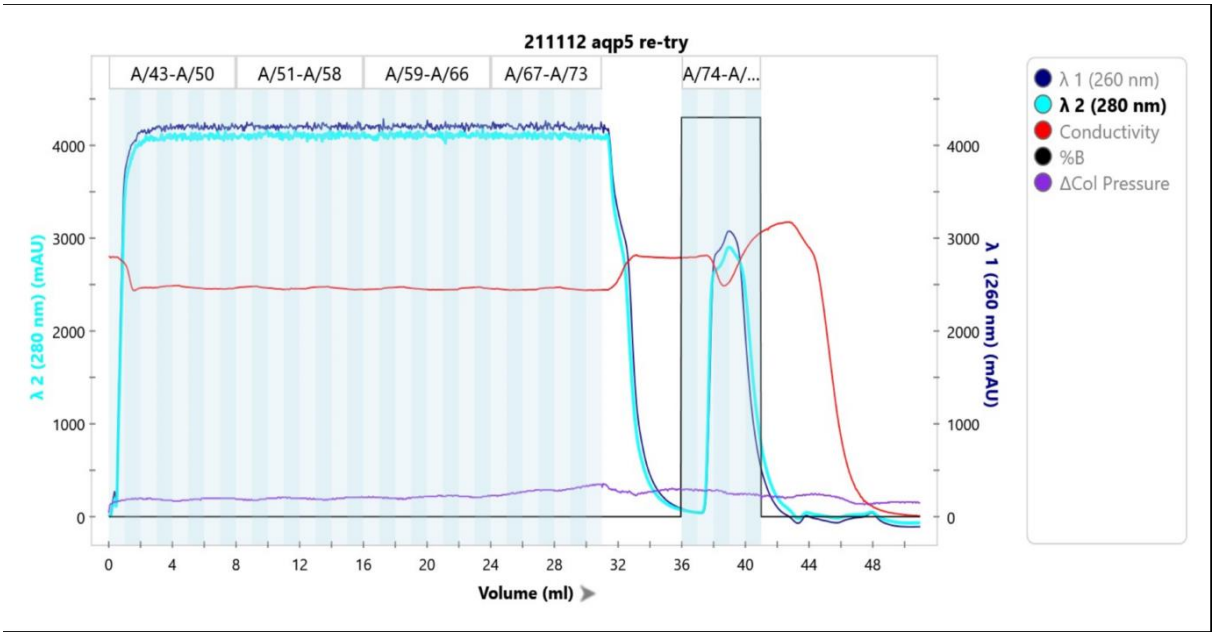

Figure 8: An affinity chromatogram from a GST purification run with the harvested cells. Shown are different phases sample application, elution, and lastly column wash. The black line on the chromatogram shows when buffer B starts, which is the elution phase. The sample application and the elution phase are collected, while the other phases go directly to waste.

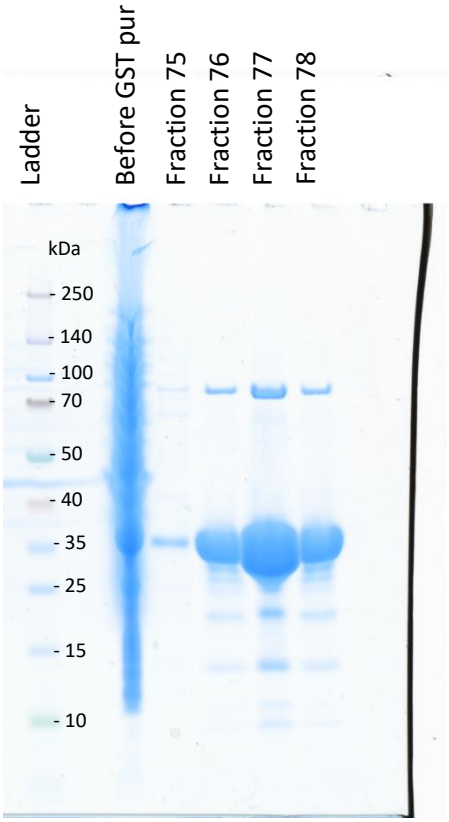

Figure 9: An SDS PAGE gel. The samples in the gel are, the AQP5 peptide-GST sample loaded in the GST purification column, fractions 75, 76, 77, and 78 are from the elution part from the affinity chromatography using a GST tag.

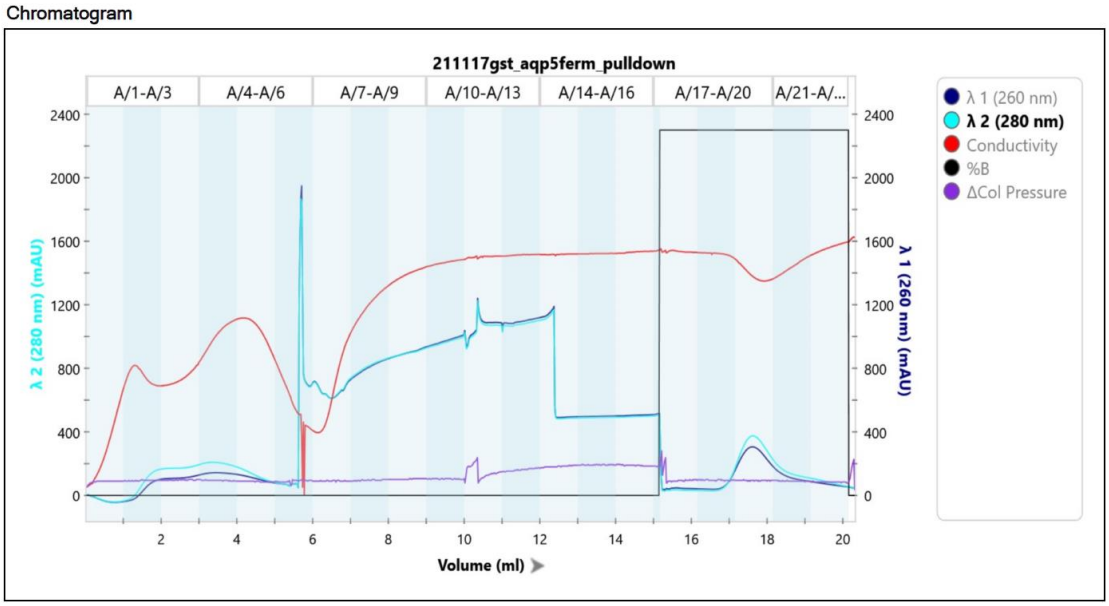

Figure 10: An affinity chromatogram from a GST purification with the AQP5 peptide-GST mixed with ezrin FERM domain. Shown are different phases sample application, elution, and lastly column wash. The black line on the chromatogram shows when buffer B starts, which is the elution phase. The sample application and the elution phase are collected, while the other phases go directly to waste.

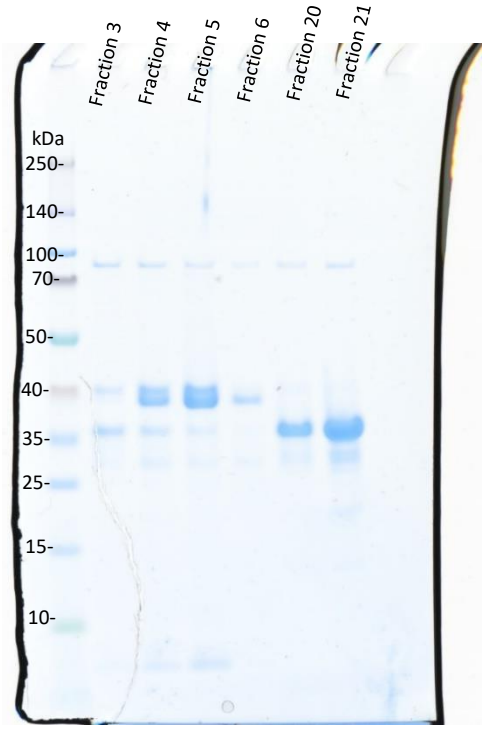

Figure 11: An SDS PAGE gel. The samples in the gel are from an affinity chromatography using a GST tag when attempting to purify AQP5 peptide-GST-FERM complex, the fractions 3, 4, 5, and 6 are from sample application, the fractions 20 and 21 are from the elution phase.

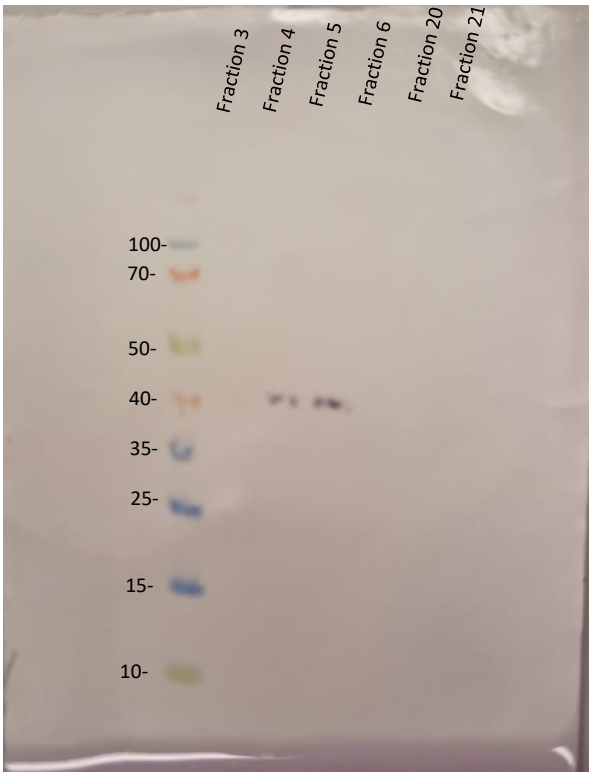

Figure 12: A Western blot membrane. The samples on the membrane are from an affinity chromatography using a GST tag when attempting to purify AQP5 peptide-GST-FERM complex, the fractions 3, 4, 5, and 6 are from sample application, the fractions 20 and 21 are from the elution phase.

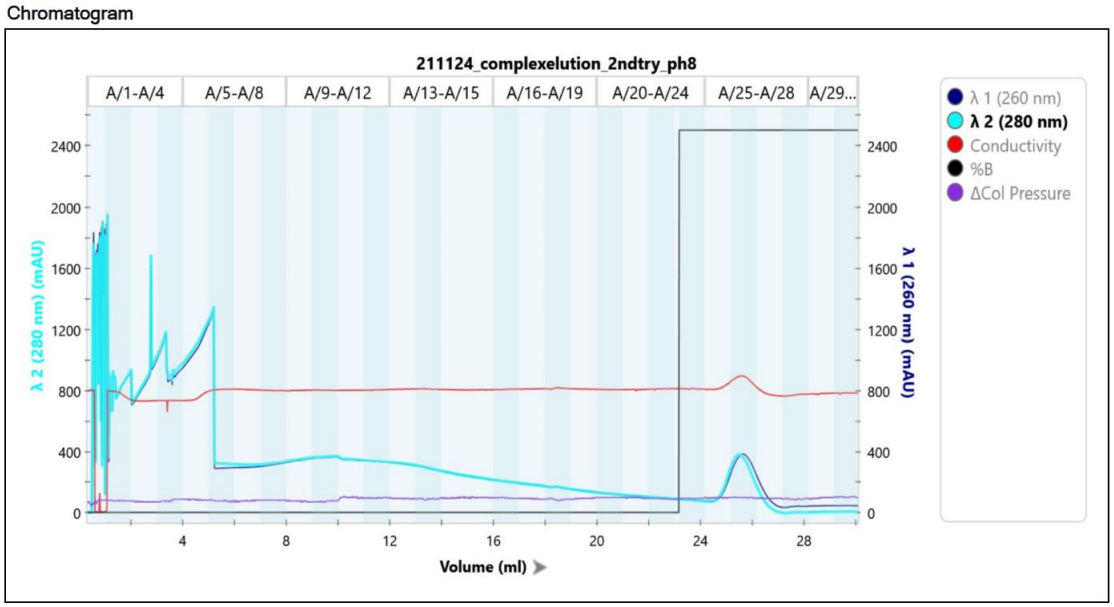

Figure 13: An affinity chromatogram from a GST purification with the AQP5 peptide-GST mixed with ezrin FERM domain. Shown are different phases sample application, elution, and lastly column wash. The black line on the chromatogram shows when buffer B starts, which is the elution phase. The sample application and the elution phase are collected, while the other phases go directly to waste.

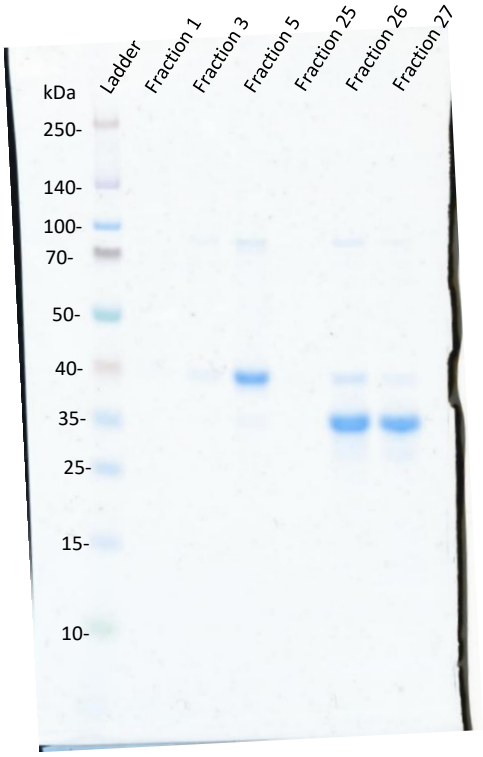

Figure 14: An SDS PAGE gel. The samples in the gel are from an affinity chromatography using a GST tag when attempting to purify AQP5 peptide-GST-FERM complex, the fractions 1, 3, and 5 are from sample application, the fractions 25, 26, and 27 are from the elution phase.

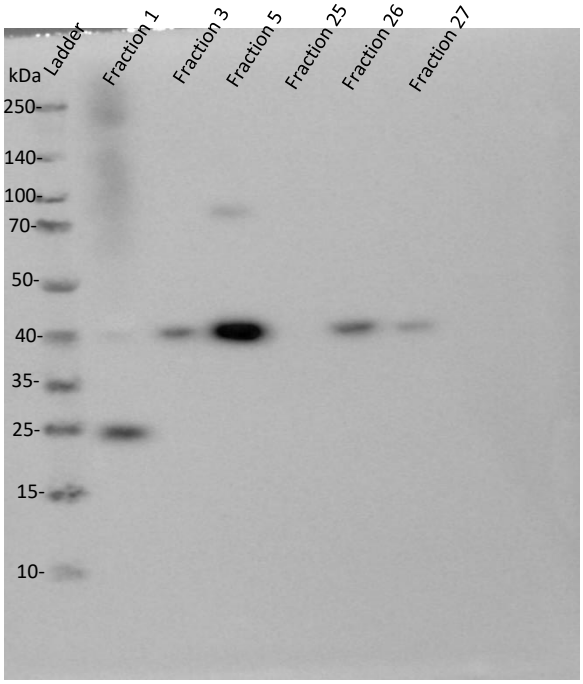

Figure 15: A Western blot membrane. The samples on the membrane are from an affinity chromatography using a GST tag when attempting to purify AQP5 peptide-GST-FERM complex, the fractions 1, 3, and 5 are from sample application, the fractions 25, 26, and 27 are from the elution phase.

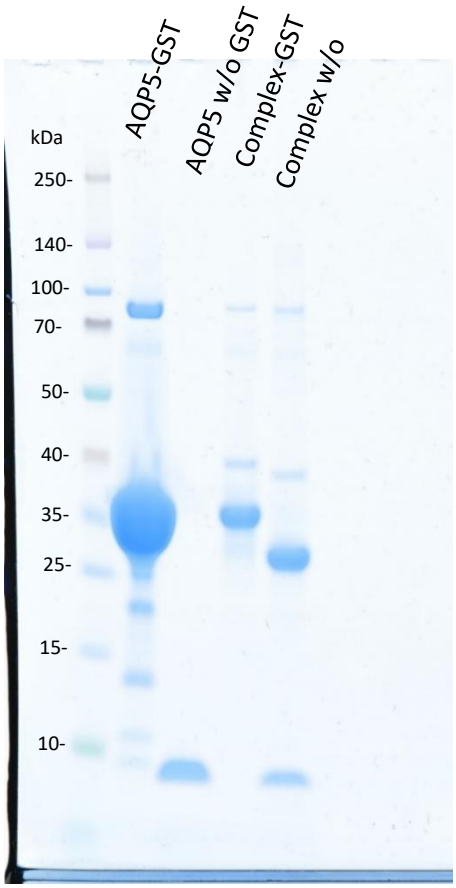

Figure 16: An SDS PAGE gel. The samples in the gel are the AQP5 peptide-GST, AQP5 without GST, AQP5 peptide-GST-FERM complex, AQP5 peptide-FERM complex without GST.

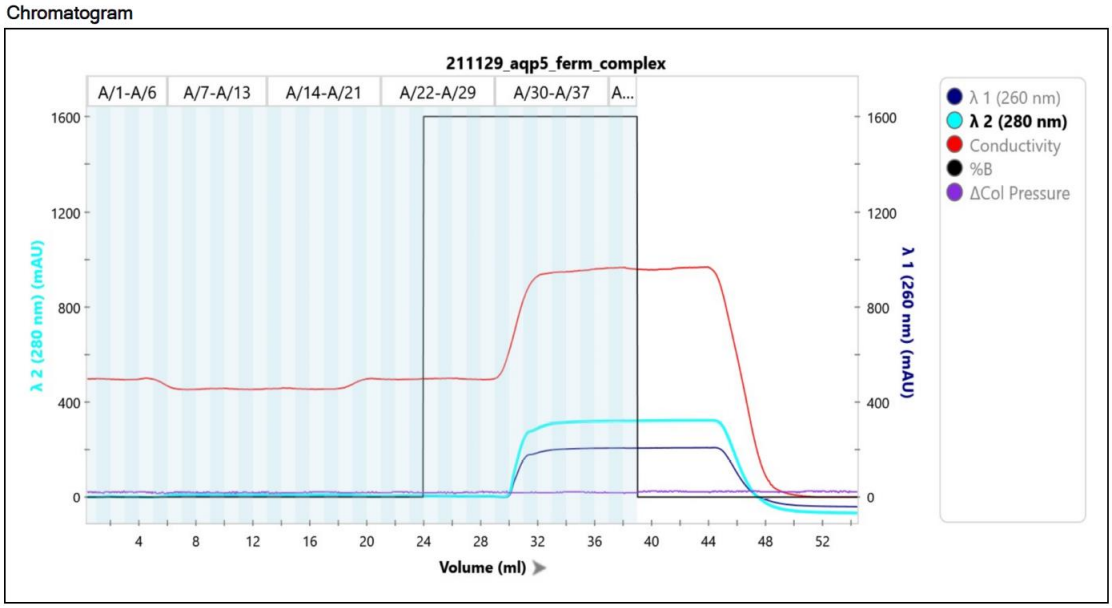

Figure 17: An IMAC chromatogram from a His-Tag purification run with the AQP5 peptide-ezrin FERM complex. Shown are different phases sample application, elution, and lastly column wash. The black line on the chromatogram shows when buffer B starts, which is the elution phase. The sample application and the elution phase are collected, while the other phases go directly to waste.

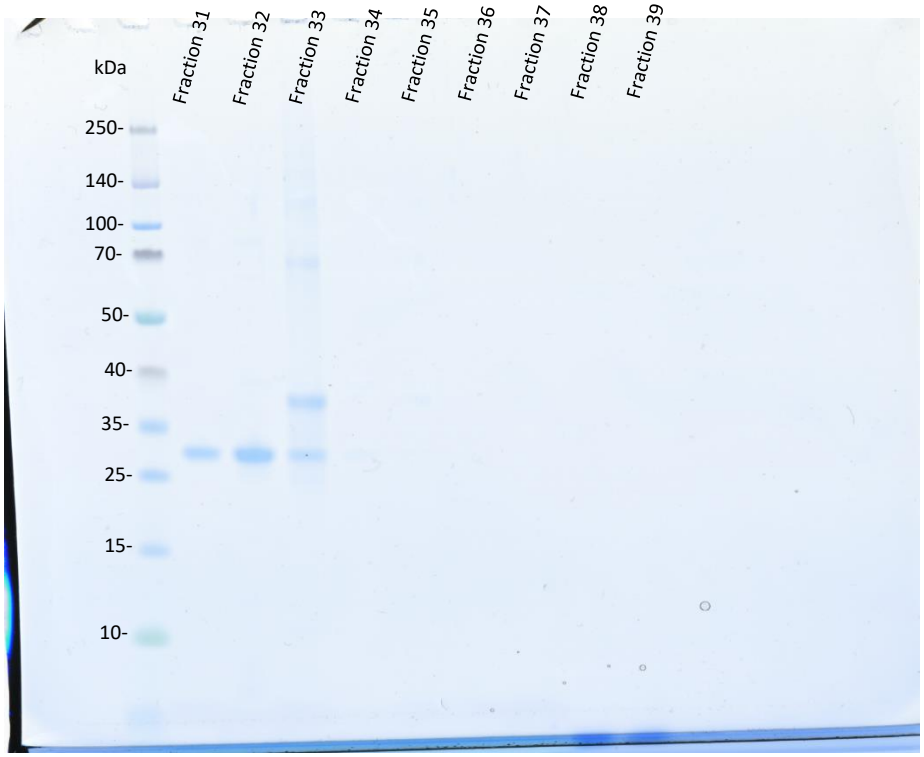

Figure 18: An SDS PAGE gel. The samples in the gel are from a IMAC using His-tag when attempting to purify AQP5+FERM complex without the GST, the fractions 31, 32, 33, 34, 35, 36, 37, 38, and 39, are from the elution phase of the IMAC.

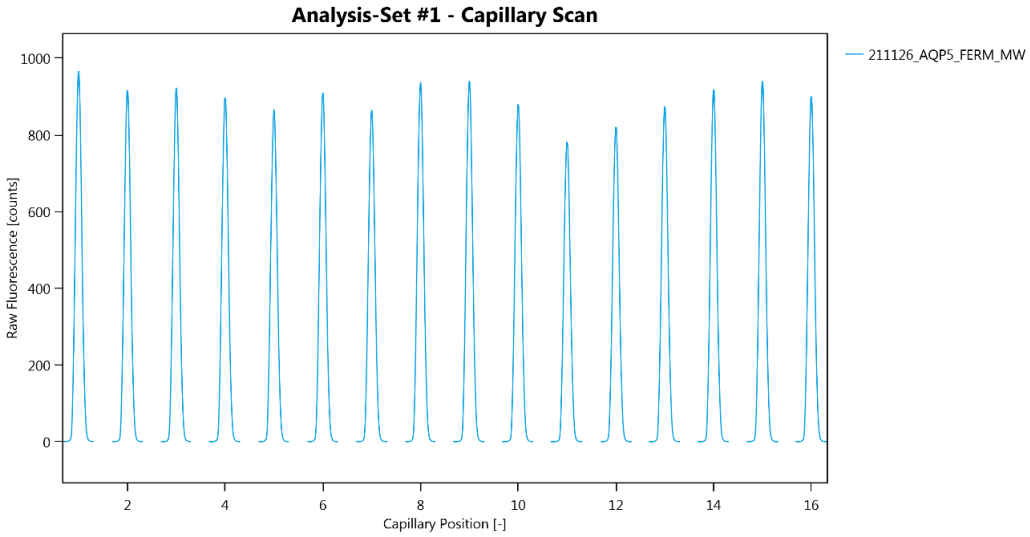

Figure 19: First run of MST. Capillary scan for FERM labeled with Alexa 488 at 60% LED power. The Y-axis shows the raw fluorescence, and the X-axis shows position of the capillary in the MST machine.

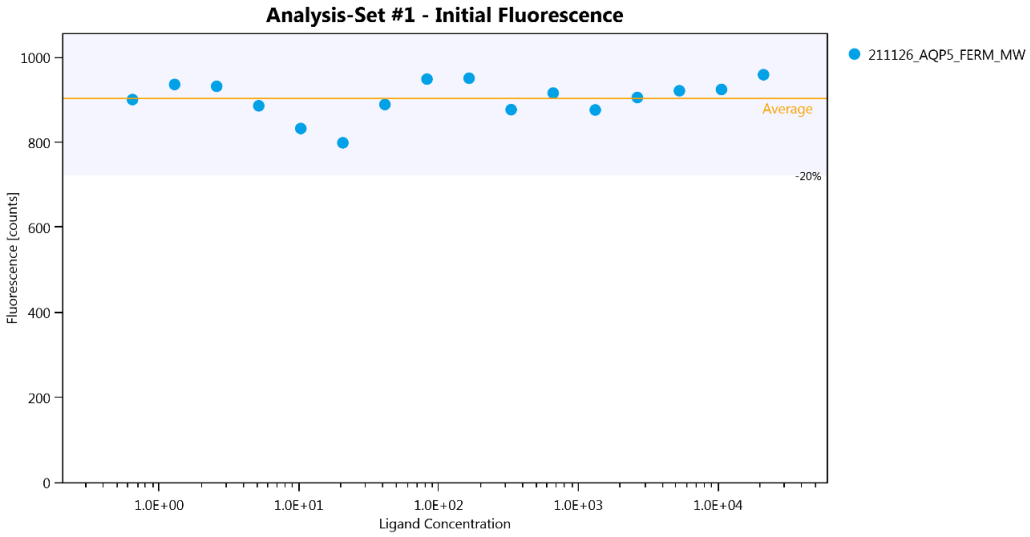

Figure 20: First run of MST. Comparison of the amount of FERM labeled with Alexa 488 to AQP5 peptide at 60% LED power. The Y-axis shows the fluorescence, and the X-axis shows concentration of AQP5 peptide.

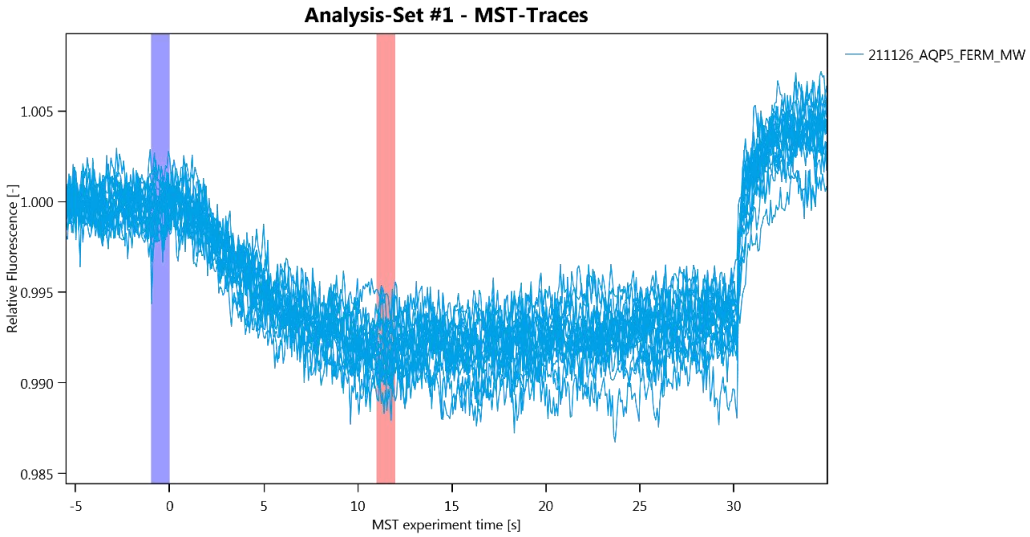

Figure 21: First run of MST. MST trace analysis for FERM labeled with Alexa 488 and AQP5 for 60% LED power. Y-axis shows the relative fluorescence, and the X-axis shows the time passed.

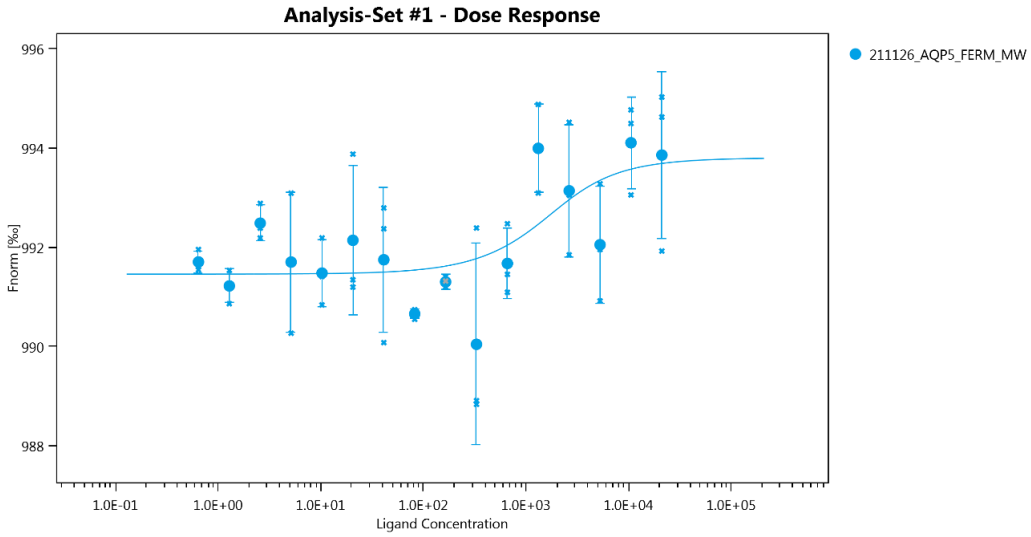

Figure 22: First run of MST, with LED power at 60%. Binding affinity curve for FERM labeled with Alexa 488 and AQP5. Y-axis shows the normalized fluorescence from the MST and the X-axis shows the concentration for the AQP5 peptide.

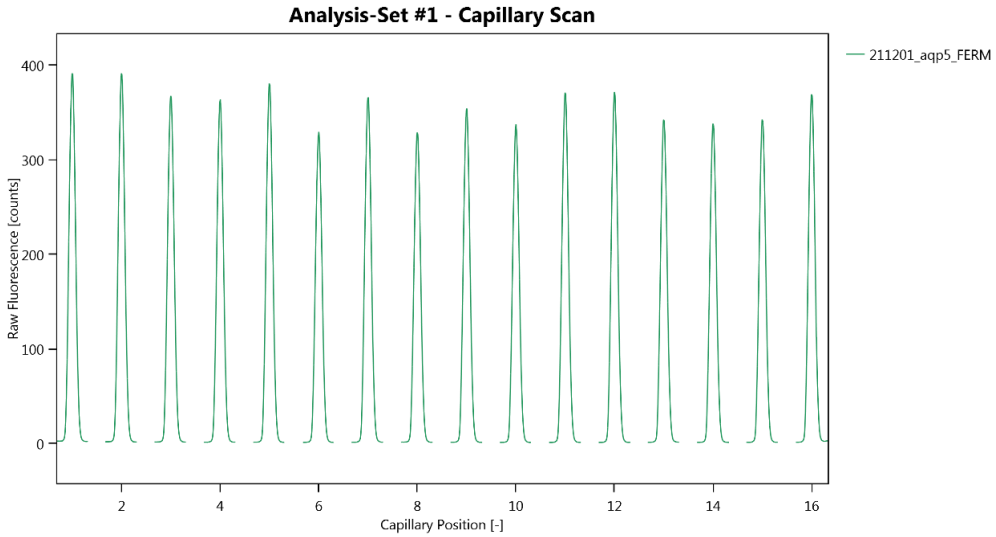

Figure 23: Second run of MST. Capillary scan for FERM labeled with Alexa 488 at 60% LED power. The Y-axis shows the raw fluorescence, and the X-axis shows position of the capillary in the MST machine.

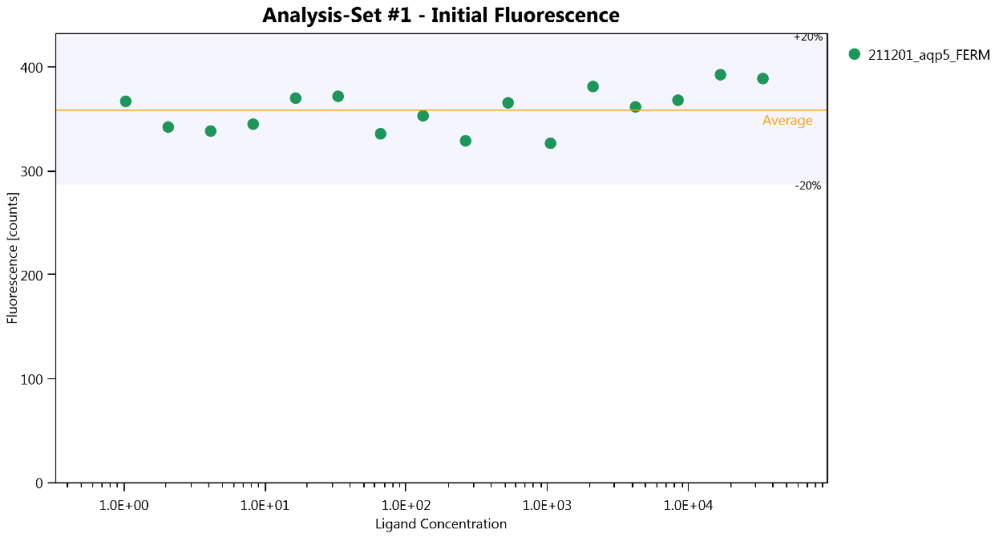

Figure 24: Second run of MST. Comparison of the amount of FERM labeled with Alexa 488 to AQP5 peptide at 60% LED power. The Y-axis shows the fluorescence, and the X-axis shows concentration of AQP5 peptide.

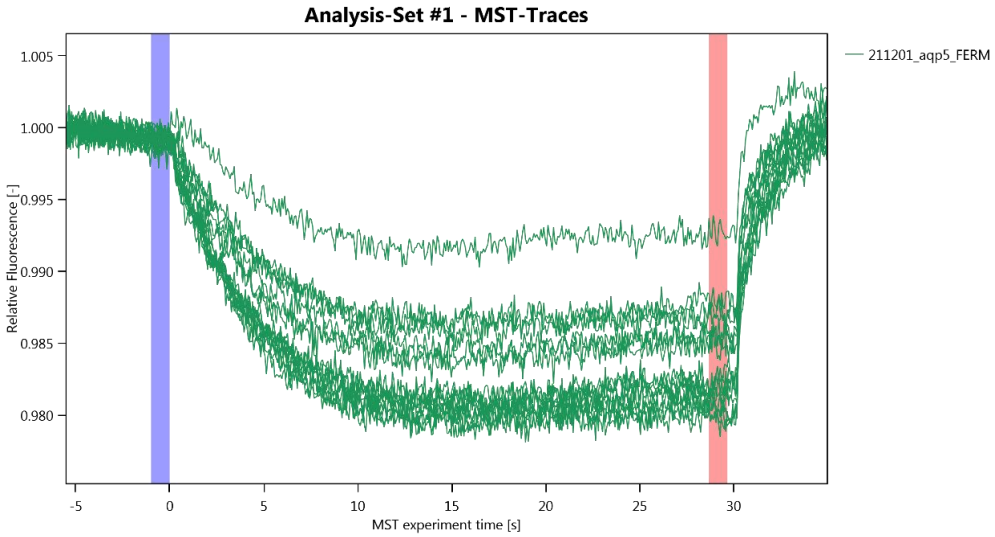

Figure 25: Second run of MST. MST trace analysis for FERM labeled with Alexa 488 and AQP5 for 60% LED power. Y-axis shows the relative fluorescence, and the X-axis shows the time passed.

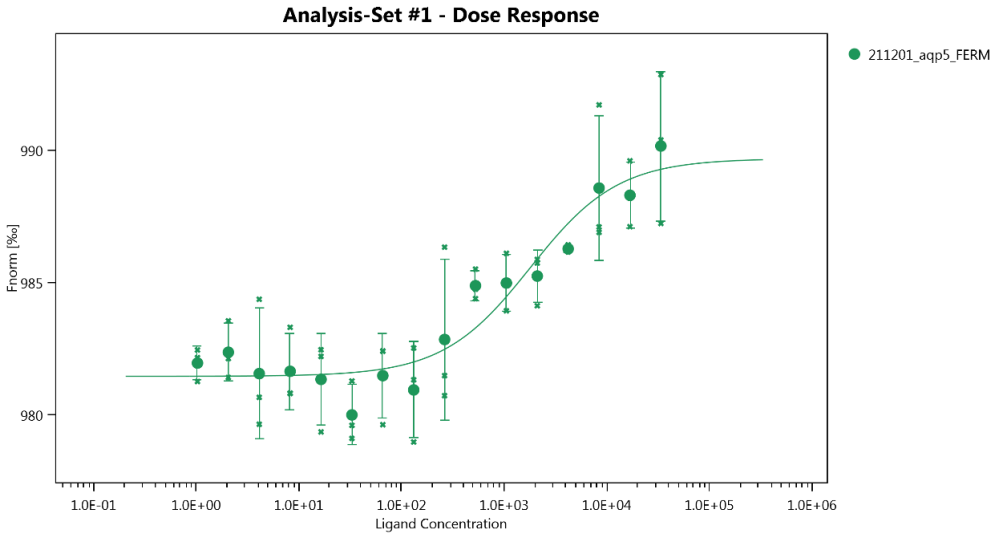

Figure 26: Second run of MST, with LED power at 60%. Binding affinity curve for FERM labeled with Alexa 488 and AQP5. Y-axis shows the normalized fluorescence from the MST and the X-axis shows the concentration for the AQP5 peptide.

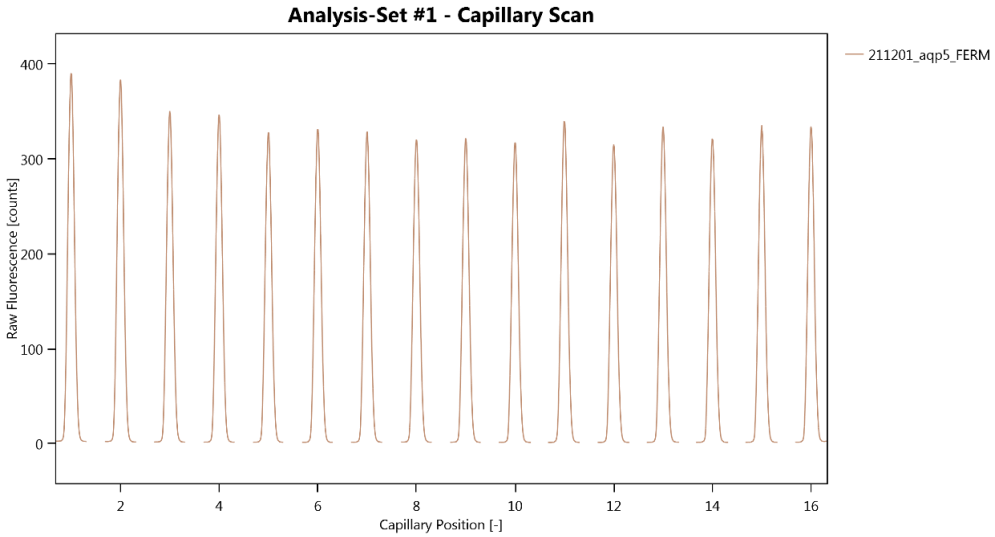

Figure 27: Second run of MST. Capillary scan for FERM labeled with Alexa 488 at 80% LED power. The Y-axis shows the raw fluorescence, and the X-axis shows position of the capillary in the MST machine.

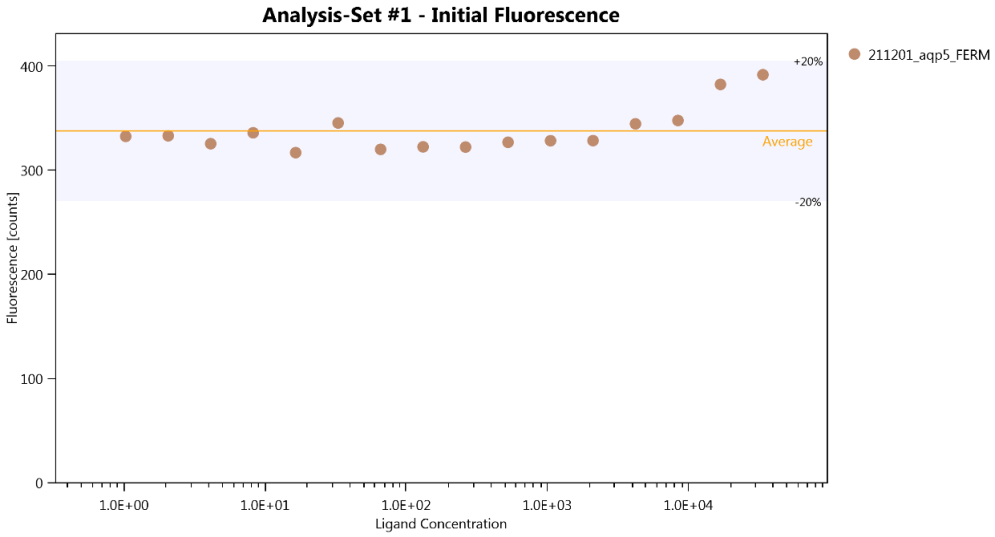

Figure 28: Second run of MST. Comparison of the amount of FERM labeled with Alexa 488 to AQP5 peptide at 80% LED power. The Y-axis shows the fluorescence, and the X-axis shows concentration of AQP5 peptide.

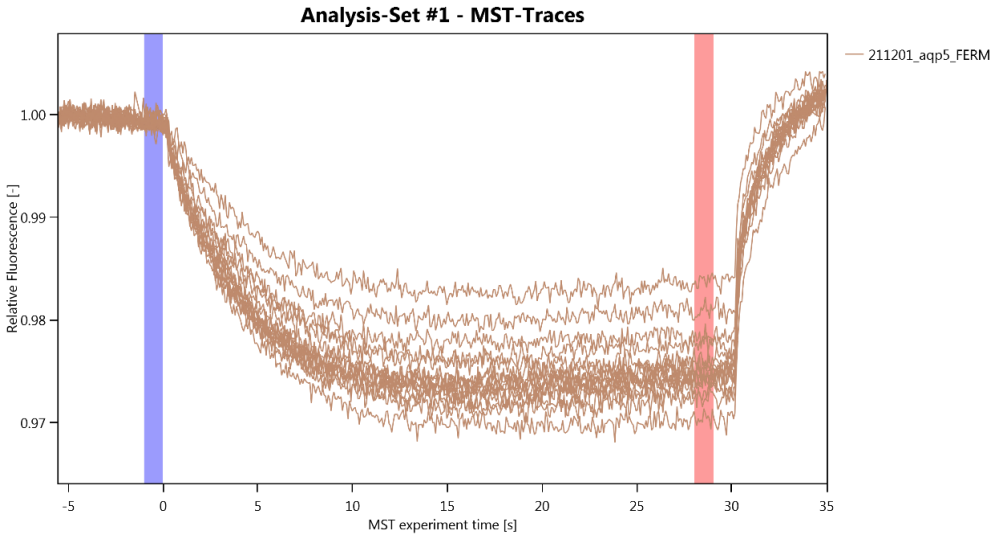

Figure 29: Second run of MST. MST trace analysis for FERM labeled with Alexa 488 and AQP5 for 80% LED power. Y-axis shows the relative fluorescence, and the X-axis shows the time passed.

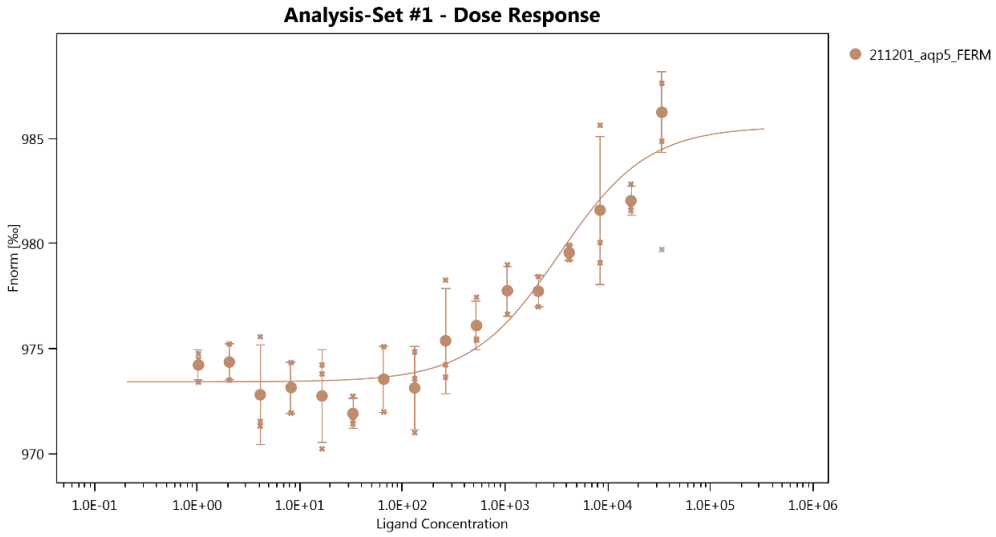

Figure 30: Second run of MST, with LED power at 80%. Binding affinity curve for FERM labeled with Alexa 488 and AQP5. Y-axis shows the normalized fluorescence from the MST and the X-axis shows the concentration for the AQP5 peptide.

Table 1: K_d value, K_d confidence value, and response amplitude, calculated by the MST machine from 3 different runs.

MST run	K_d	K_d Confidence $\pm$	Response Amplitude
1st run 60%	1019.3nM	1987.2nM	2.3437
2nd run 60%	3318.1nM	1195.3nM	12.1629
2nd run 80%	1744.8nM	667.32nM	8.2310

5 Discussion

5.1 GST purification & MST Results

The transformation of the AQP5 peptide-GST gene which was in a plasmid, showed to be successful (Fig. 4, 5, 6). The plates contained kanamycin which is used as a selection marker. The plasmid with AQP5 peptide-GST also contained a gene for kanamycin resistance. It can be concluded that the transformation was successful when our cells with transformed plasmid was able to grow, and the negative control cells did not show growth (Fig. 4, 5, 6).

The protein expression of the AQP5 peptide-GST can be seen around 35kDa (Fig. 7). The samples from the cell culture contain a lot of debris and uninteresting proteins, but at 35kDa a protein band is growing from 0 hours until 3 hours after induction. The AQP5 peptide which is 5.2kDa (Tab. 8 in Appendix I) tagged with GST which is 26kDa (Tab. 9 in Appendix I) should in theory form a 31.2kDa protein. The 35kDa protein band that we see is assumed to be the AQP5 peptide-GST since we induced protein expression with IPTG. 31.2kDa is not equal to 35kDa but SDS PAGE have an error of margin around 10%, it can be difficult to distinguish protein bands on the gel, and other factors such as the SDS to protein ratio can affect the result and could explain the different values (Bio-Rad Laboratories Inc., n.d-d).

On the affinity chromatogram of the GST purification after protein expression shows that elution begins during the sample application (Fig. 8). This shown elution is all the protein and debris that do not have a GST tag and do not have affinity for the GST column. The amount of protein and debris shown on the chromatogram (Fig. 9) correlates to SDS PAGE gel (Fig. 6). During the elution phase a peak at 280nm can be seen (Fig. 7), which should be the AQP5 peptide-GST being eluted with buffer B since absorption at 280nm is the amino acid tryptophan in proteins (Bio-Rad Laboratories Inc., n.d-c).

On the SDS PAGE gel after GST purification the most prominent protein bands are around 35kDa (Fig. 9), which correlates with the increasing protein band on the protein expression SDS PAGE gel (Fig. 7). Protein bands can be seen at other kDa locations, this is probably proteins that have bound unspecifically to the protein of interest or to the GST column. The bands could also be multimers of the AQP5 peptide-GST, but this is unlikely for AQP5 peptide since it is only a small peptide and not a full-length protein. The full length AQP5 is normally in a tetrameric structure. However, GST could form a multimer and be the unknown protein band on the SDS PAGE gel (Fig. 9).

The chromatogram of the GST purification for AQP5 peptide-GST with ezrin FERM domain shows a high amount of protein being eluted during sample application and only a small amount of protein during the elution phase (Fig. 10). If a complex had been formed between our proteins of interest, the chromatogram (Fig. 10) should have had most of the protein eluted during the elution phase. The protein should have been eluted during the elution phase because the AQP5 peptide-GST would bind to the GST column and the ezrin FERM domain would bind to the AQP5 peptide. Based on the results from the chromatogram (Fig. 10), it was decided to test fractions from both the sample application and the elution phase on SDS PAGE and Western blot to locate the proteins of interest.

The SDS PAGE gel following GST purification (Fig. 11) of the wanted complex (AQP5 peptide-GST with ezrin FERM domain) showed that the fractions from the sample application primarily contain a protein around 40kDa, and the fractions from the elution phase primarily

contain a protein around 35kDa. This suggests that AQP5 peptide-GST is the protein band around 35kDa and ezrin FERM domain is the protein band around 40kDa. This conclusion is drawn from the previous result (Fig. 9), where only the AQP5 peptide-GST showed a 35kDa protein band. The ezrin FERM domain protein is suggested to be 35kDa, but in the gel it seems to be 40kDa. Both proteins appear to have shifted 5kDa on the gel (Fig. 11) compared to their theoretical molecular weight. These conclusions are supported by the western blot done with the same samples, where protein bands are only seen in fractions 5 and 6 (Fig. 12). The protein bands seen on the Western blot (Fig. 12), are proteins which have a His-Tag, and only the ezrin FERM domain has a His-Tag.

The second attempt to form a complex was done with a higher pH, because the first attempt did not work. The pH goes up when the protein mixture is in the 4°C cold room. The first attempt sample mixture was pH 7.4 before the cold room, and in the cold room the mix will be around pH 8. The second attempt sample mix was pH 8 before the cold room and will be around pH 8.6 in the cold room (Castells et al., 2003). This might be the reason why we did not manage to form a complex between our AQP5 peptide and the ezrin FERM peptide the first attempt.

The second attempt used buffers with a pH of 8, instead of 7.4. The mixed GST-AQP5 peptide with FERM was again run through a GST purification. The affinity chromatogram (Fig. 13) from the GST purification shows sample being eluted during the sample application and elution phase. The SDS PAGE (Fig. 14) shows fraction 5 from the sample application has a protein band at 40kDa which should be unbound ezrin FERM domain. Fractions 26 and 27 shows protein bands at 40kDa and 35kDa (Fig. 14). This suggests that both the ezrin FERM domain and AQP5 peptide-GST were eluted during the elution phase. This means that FERM bound to AQP5 peptide-GST, since ezrin FERM domain does not have affinity for the GST column. The protein bands shown on the Western blot with the same sample (Fig. 15) confirms it is ezrin FERM domain which is the protein band at 40kDa. The conclusion that a complex has successfully been form is drawn from the fact that only the AQP5 peptide-GST should be eluted from GST purification, and only ezrin FERM domain has His-Tag which can be shown on Western blot.

A SDS PAGE was made of AQP5 peptide-GST, AQP5 peptide with GST cleaved off, AQP5 peptide-GST mixed with ezrin FERM, and AQP5 peptide mixed with ezrin FERM and GST cleaved off (Fig. 16). This gel is good to use as reference point. The AQP5 peptide-GST is clearly seen around 39kDa (Fig. 16). AQP5 peptide with GST cleaved of with Thrombin shows a clear and single protein strand below 10kDa (Fig. 16), which means the cleaving and the purification of the AQP5 peptide was successful.

The chromatogram from the His-Trap purification of the complex (Fig. 17) shows only protein being eluted during the elution phase. The elution continues after the elution phase and into the column wash phase (Fig. 17). This shown elution is most likely imidazole which measures at the same 280nm wavelength as the protein. All the fractions from the elution phase which showed that protein was eluted on the chromatogram, were run on SDS PAGE gel (Fig. 18). The gel (Fig. 18) only shows the three first fractions having protein in them, and the protein band is around 30kDa. The 30kDa protein band might be the ezrin FERM, but this conclusion does not align with previous results. The AQP5 is also missing or very hard to see on the gel (Fig. 18), which might be due to a low concentration of protein in the sample.

The MST measurements capillary scan (Fig. 19, 23, 27) show that there was no absorption of the sample to the capillary wall (NanoTemper Technologies, 2013). The error margin between the capillaries is quite high (Fig. 20, 24, 28), which means the pipetting was done poorly and the results are less reliable. Preferably the error of margin should be within $\pm$10%. The MST results (Fig. 25, 29) (Tab. 1) for the second MST run still shows tendencies for interaction between the AQP5 peptide and ezrin FERM domain. The dissociation constant K_d is higher than other known protein-protein interactions, which suggests that there is an interaction (Asmari et al., 2018). The response amplitude (Fig. 22, 26, 30) should be as high as possible but a value above 3.5 is classified as a binding between two substances (NanoTemper Technologies, n.d).

5.2 Previous studies

The hypothesis in this report is based on the previous findings regarding aquaporins and ezrin. It was confirmed in this study that the C-terminus of AQP5 interacts with the FERM domain of ezrin. The previous studies with aquaporins and ezrin have used immunoprecipitation assays to isolate the proteins of interest while this study used affinity chromatography. This study also confirmed the proteins' possibility to form a complex with usage of MST measurements on AQP5 and ezrin. While the previous studies commonly use mass spectrometry as a second assay. The execution of this study and previous studies differ and are therefore less comparable.

5.3 Conclusion & Prospects

A complex was formed between AQP5 peptide and the FERM domain of ezrin which confirms there is an interaction. Future studies should be done with full length AQP5 and ezrin, and repeated >3 times to give strong evidence and statistical significance of an interaction. The mixture of AQP5 peptide-GST and ezrin FERM domain should be repeated at various buffer pH, temperature, and mixing duration, to find the optimal conditions for complex formation. The MST measurements should be repeated with a lower margin of error in the capillary scan.

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7 Appendix I

7.1 Protocols

7.1.1 Transformation

Thaw Calcium competent *E. coli* cells on ice. Mix carefully by pipetting slowly up and down: 2µl plasmid DNA/water (for control) with 50µl of cells in a sterile Eppendorf tube. Incubate for 30 min – 1 hour on ice. Apply heat shock: 45 seconds in a 42℃-water bath, put immediately on ice. Cool down cells for 2-5 min on ice. Add 500µl of LB medium to the cells and incubate for 1 hour at 37℃, shaking. Plate the whole sample onto an LB-agar plate 1µl kanamycin per 1ml agar. Leave the agar plates to dry, then turn them upside down and put them into a 37℃ incubator overnight.

7.1.2 Protein purification

Inoculate 2250ml LB in 3 750ml flasks with 3ml of O/N-culture AQP5 peptide in BL21 *E. coli*. Leave flasks in the incubator at 37℃ until OD600 = 0.4-0.6, and then add 0.5mM IPTG to each flask. Induce for 3 hours and then harvest the cells by centrifugation at 10000xg for 15 min. Resuspend the cells in Lysis buffer (Tab. 2 in Appendix I) and sonicate for 5x1 min.

7.1.3 Cleaving

Desalt your sample into Thrombin cleaving buffer (Tab. 10 in Appendix I). Add Thrombin at a 5:1 ratio to your sample. Let it mix overnight in on a shaker in the 4℃ cold room.

7.1.4 SDS PAGE & Western blot

Mix the sample with 4x SDS loading dye at a 4:1 ratio and heat it for 5 min at 95℃. Load 15µl of each sample twice onto a NuPage Bis-Tris gel. Run the gel in NuPAGE-running buffer (Tab. 11 in Appendix I) for 35 min at 200 V and approximately 120 mA. After the SDS run, split the gel, and continue with Coomassie blue staining for one half and Western blot for the other half.

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. Destain the gel by adding water and shaking for 15 min.

Western blot: Soak cotton in NuPAGE-transfer buffer (Tab. 12 in Appendix I) and compress the gel between two filter papers and NC-membrane, (with cotton being the filler which creates pressure) and run for 1 h. Soak the blotted membrane in 20ml 5% milk solution with TBS-T (Tab. 13 in Appendix I) for 15 min on the shaker. Incubate with 4µl of 6xHis monoclonal antibody in 20 ml of 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, do this 3 times. Soak the blotted membrane in 20ml 5% milk solution with TBS-T for 15 min on the shaker. Incubate with 4µl of goat anti-mouse IgG (H+L) secondary 14 antibody in 20 ml of 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, do this 3 times.

7.1.5 MST

Prepare 16 PCR tubes and label them 1-16. Add 5µl FERM complex forming buffer (Tab. 6 in Appendix I) to tube 2-16. Add 10µl of AQP5 peptide without GST to tube 1. Do a 1:1 dilution series, take 5µl from tube 1 and add to tube 2, then 5µl from tube 2 and add to tube 3, and so on until tube 16. Take 5µl from tube 16 and discard it. Then add 5µl of FERM labeled with Alexa 488 into each tube. Mix carefully by pipetting up and down at each step.

7.2 Buffers & Sequences

Table 1: The DNA sequence for the AQP5 peptide.

AQP5 DNA sequence; 147bp
ACTAGT TTAGTACCCAGGGGAAGTAATAGCCTATCATTGAGCGAACGTGTTGCGATTATCAAGGGTA CGTACGAGCCGGATGAGGACTGGGAAGAGCAGCGTGAAGAGCGCAAAAAGACCATGGAACT GACCACCCGTTAA CCTAGG

Table 2: The solutions and concentrations to make Lysis buffer.

Lysis buffer
20mM Tris pH 7.4
250mM NaCl
5% Glycerol
1mM DTT
1mM PMSF

Table 3: The solutions and concentrations to make Elution buffer.

Elution buffer
50mM Tris pH 8
20mM Glutathione
1mM DTT
250mM NaCl

Table 4: The solutions and concentrations to make Complex forming buffer.

Complex forming buffer pH 7.4
20mM Tris pH 7.4
100mM NaCl
5% Glycerol
1mM DTT

Table 5: The solutions and concentrations to make Complex forming buffer.

Complex forming buffer pH 8
20mM Tris pH 8 100mM NaCl 5% Glycerol 1mM DTT

Table 6: The solutions and concentrations to make FERM complex forming buffer.

FERM complex forming buffer
20mM Tris pH 8 100mM NaCl 0.5mM DTT

Table 7: The solutions and concentrations to make His-Trap buffer.

His-Trap buffer
300mM Imidazole 20mM Tris pH 8 100mM NaCl 0.5mM DTT

Table 8: The amino acid sequence for the AQP5 peptide.

AQP5 amino acid sequence; 45AA (5.2kDa)
LVPRGSNSLSLSERVAIIKGTYEPDEDWEEQREERKKTMELTTR*

Table 9: The amino acid sequence for Glutathione S-transferase.

Glutathione S-transferase (GST); 218AA (26kDa)
MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDG DVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDF LSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRI EAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPK

Table 10: The solutions and concentrations to make Thrombin cleaving buffer.

Thrombin cleaving buffer
2.5mM CaCl 20mM Tris pH 8 100mM NaCl 1mM DTT

Table 11: The solutions and concentrations to make NuPAGE-running buffer.

NuPAGE-running buffer
50mM MES
50mM Tris base
0.1% SDS
1mM EDTA

Table 12: The solutions and concentrations to make NuPAGE-transfer buffer.

NuPAGE-transfer buffer
25mM Bicine
25mM Bis-Tris
1mM EDTA
pH 7.2

Table 13: The solutions and concentrations to make TBS-T buffer.

TBS-T buffer
0.2% Tween-20 in TBS

Table 14: The solutions and concentrations to make TBS buffer.

TBS buffer
20mM Tris-HCl pH 7.4
150mM NaCl

Table 15: The amino acid sequence for ezrin FERM domain.

Ezrin FERM domain amino acid sequence; 296AA (35kDa)
MPKPINVRVTTMDAELEFAIQPNTTGKQLFDQVVKTIGLREVWYFGLHYVDNKGFPTWLKL DKKVSAQEVRKENPLQFKFRAKFYPEDVAEELIQDITQKLFFLQVKEGILSDEIYCPPETA VLLGSYAVQAKFGDYNKEVHKSGYLSSERLIPQRVMDQHKLTRDQWEDRIQVWHAEHRGML KDNAMLEYLKIAQDLEMYGINYFEIKNKKGTDLWLGVDALGLNIYEKDDKLTPKIGFPWSE IRNISFNDKKFVIKPIDKKAPDFVFYAPRLRINKRILQLCMGNHELYMRRRK