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The University of Kent
School of Biosciences

Optimisation of the Expression of Sigma
Non-Opioid Intracellular Receptor 1 (σ 1) in
Membrane Mimetic Systems

By Bradley Hilton

Master's degree (MSc): Biochemistry

Total Word Count: 26,925

Signed _____

Declaration

No part of this thesis has been submitted in support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

Acknowledgments

I would like to say a massive thank you to my master's by research supervisor, Dr Jose Ortega-Roldan at the University of Kent. Throughout my studies he showed a high level of support, guidance and his immense motivation has inspired me to continuously develop as a scientist. Whenever I needed something or was unsure, Dr Ortega-Roldan would personally take time out of his busy schedule to personally sit down with me and explain anything in great detail. I believe this level of individual enthusiasm and care for students can be particularly hard to find in a supervisor, due to their profound commitments in academia. I will always look back on my experience in the Ortega laboratory as a positive experience that I would recommend any undergraduate students to peruse. In addition, I would like to say a massive thank you Alex Hendry, Dr Lorena Varela-Alvarez, Dr Encarnación Medina Carmona, Joe Perkins, Diego Cantoni and Dr Gary Thompson for all of their caring support throughout my studies, even when they were extremely busy with their own research. These individuals taught me so much from their extensive knowledge and I believe they have encouraged me to develop an excellent work ethic, just as they do.

Furthermore, I would like to say an individual thank you to Maria Dodina for all of her patience, support and help throughout this project. Although this PhD student was not a researcher of the Ortega laboratory, she saw potential in me and took time out of her day, all year to motivate me, teach me and give me direction in my studies even when I did not gain the results I wanted. Lastly, I would like to thank my family and my partner, for showing me endless support, love and encouragement throughout my studies. This achievement would have not been possible without them, and I hope that I make them proud.

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Buffer and Media Compositions: Final volume prepared to 1000mL with Milli-Q water

HEPES-buffered saline (HBS): 20mM HEPES, 250mM NaCl, and 20% glycerol (v/v) pH 7.5

Tris-Buffered saline (TBS): 50mM Tris-Cl and 150mM NaCl pH 7.5

Tris-Buffered Saline Tween (TBST): 50mM Tris-Cl, 150mM NaCl, and 0.1% Tween 20 pH 7.5

Transfer buffer (TB): 25mM Tris-Cl, 192mM Glycine, 20% Methanol pH 8.3

Block buffer (BB): 50mM Tris-Cl, 150mM NaCl and 5% skimmed milk pH 7.5

Yeast Peptone Dextrose (YPD): 1% yeast extract, 2% peptone and 2% dextrose

Terrific Broth (TB): Tryptone 1.2%, Yeast extract 2.4% and 0.5% glycerol (No TB salts added)

Abbreviations

- **σ 1, SIGMA-1 and S1R:** Sigma Non-Opioid Intracellular Receptor 1
- **SIGMAR1:** Sigma Non-Opioid Intracellular Receptor 1 Gene
- **IMAC:** Immobilised Metal Affinity Chromatography
- **SEC:** Size-Exclusion Chromatography
- **LB:** Luria Broth Medium
- **LB-AMP:** Luria Broth Ampicillin Medium
- **KDa:** Kilodaltons
- **Da:** Daltons
- **GFP:** Green Fluorescent Protein
- **6XHis:** Polyhistidine tag
- **AmpR:** Ampicillin Resistant
- **NMR:** Nuclear Magnetic Resonance Imaging
- **pH:** Potential Hydrogen
- **RPM:** Rotations Per Minute
- **TB:** Terrific Broth
- ***E. Coli:*** *Escherichia Coli*
- ***S. Cerevisiae:*** *Saccharomyces Cerevisiae*
- **SSRI:** Selective Serotonin Reuptake Inhibitor
- **PK:** Pharmacokinetic
- **PD:** Pharmacodynamic
- **ALS:** Amyotrophic Lateral Sclerosis
- **AD:** Alzheimer's Dementia
- **MW:** Molecular Weight
- **IPTG:** Isopropyl β - d-1-thiogalactopyranoside
- **TBS:** Tris-Buffered Saline
- **TBST:** Tris-Buffered Saline Tween
- **TB:** Transfer Buffer
- **BB:** Blocking buffer
- **MAM:** Mitochondrial Associated Membrane
- **UN:** United Nations
- **SMA:** Styrene maleic acid
- **SMA Imide:** Styrene maleimide
- **NMR:** Nuclear magnetic Resonance
- **CPMG** Carr-Purcell-Meiboom-Gill
- **MBP:** Maltose Binding Protein
- **IP3:** Inositol trisphosphate Receptors
- **AU:** Absorbance Units
- **UV:** Ultraviolet
- **NM:** Nano-meters
- **RFU:** Relative Fluorescence Units
- **PPM:** Parts per million
- **LMNG:** Lauryl Maltose Neopentyl Glycol
- **CHS:** Cholesterol Hemisuccinate
- **YPD:** Yeast peptone dextrose
- **SDS-PAGE:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Abstract

The sigma non-opioid intracellular receptor 1 ($\sigma 1$) is a highly important, yet poorly understood chaperone membrane protein. Although structural studies have come a long way in identifying the crystal structure and its pharmacological characteristics, the proteins relation to disease and illness is yet to be fully established. Currently a world leading biopharmaceutical company called Anavex, is conducting phase 2b and 3 clinical trials on a compound called A2-73 to target the sigma-1 receptor in Alzheimer's disease. This compound has demonstrated a good safety profile and significant therapeutic effects in some patients by slowing Alzheimer's disease progression and even reversing its development if treated within early stage onset.

This research project aimed to develop recombinant protein expression systems for the sigma-1 receptor in C43 *Escherichia coli* and INVSc *Saccharomyces cerevisiae* cell lines. In addition, we set out to compare current detergent solubilization techniques with upcoming nanodisc technology, as previous publications have demonstrated undesirable results when conducting structural studies on this protein. Here we report, the successful isolation of the human sigma-1 receptor in C43 expression systems and effective solubilisation using detergent based methods. Unfortunately, we were unable to solubilise this receptor using nanodisc technology in any of the expression system we used during this study. In addition, we were unable to conclude the definitive isolation of the sigma-1 receptor in the INVSc1 cell line due to potential cloning and expression protocol issues. However, we did report positive results for the use of nuclear magnetic resonance (NMR) spectroscopy in ligand interaction experiments such as CPMG studies. From these results we identified a plausible binding interaction between the sigma-1 receptor and the agonist compound fluoxetine hydrochloride. However, this result was not entirely conclusive due to a possible indication of the agonist binding to micelle formation from the use of detergent. Overall, this study highlighted the challenges and potentials of researching the sigma-1 receptor and provides a strong indication for future research.

1. Introduction

For centuries scientists have attempted to unravel the complexities of the human body. It has only been made possible through scientific research, clinical trials and medical experimentation that proteins are a key biomolecule in the development and progression of disease. Breakthroughs in both chemical sciences and the biosciences, has made it possible to express, purify and characterise proteins on the atomic level, enabling us to understand the underlying pathologies in a range of medical conditions. In the last century, it has only now become possible to identify specific genetic mutations or aberrations in disease enabling us to identify which synthesised proteins are affected. This is a major development, as it enables us to develop investigational medicinal products (IMPs) to target these proteins directly or indirectly to eradicate a variety of diseases. The majority of human proteins can now be studied effectively through expression vectors such as *E. coli* which has enabled scientists to conduct biochemical pharmacology research prior to human experimentation with potentially dangerous pharmacokinetic and pharmacodynamic IMPs, in early phase clinical trials.

Furthermore, the United Nations (UN) have forecasted that the global population is set to rise to 9.8 billion people by 2050, which health experts suggest will put a major strain on health sectors across the world. This increase in population is more than likely to result in an increase in disease rates across the globe. Because of this, it is more important than ever to research potential therapeutic target proteins to develop drugs that will potentially save or improve the quality of life for millions of people. One of these proteins that requires extensive research is the sigma non-opioid intracellular receptor 1 as it has been implicated in a profound number of physiological and psychological condition. Although there have been major breakthroughs in identifying the biochemical structure of this protein, its role in many diseases such as cancer is yet to be established.

1.1 The Sigma Non-Opioid Intracellular Receptor 1 History and Background

In recent years there has been a major increase in the studies of the sigma non-opioid intracellular receptor (σ_1). Over forty years ago in 1976, *Martin et al* hypothesised the existence of multiple opioid intracellular receptors that mediate pharmacological effects of morphine and its direct analogues¹. Following extensive pharmacological investigations *Martin et al* classed these opioid receptors based on specific Greek symbols depending upon the ligands that these receptors mediate, for example mu (μ) opioid receptors bind to morphine to produce analgesia and kappa (κ) opioid receptors cohere to ketocyclazocine to induce dysphoric and anxiety effects¹. Years later, in 1986 *Su et al* identified the true existence of the sigma class of receptors and their pharmacological characteristics. After carrying out numerous selectivity assays it was determined that the sigma class of receptors do not possess a high affinity for naltrexone, which all of the other classes of opioid receptors do bind to. This unique pharmacological characteristic of these receptors is a clear distinction that sets these proteins in a completely different subtype².

Following this discovery researchers were highly motivated to investigate the sigma receptor class of proteins to determine their physiological functions and potential implications in disease. A book released in 2007 called "*Sigma Receptors Chemistry, Cell Biology & Clinical Implications*"³ clearly outlines an overview of the sigma class of receptors pharmacological characteristics and describes their physiological functions in homeostasis & disease. This book contains an excellent collection of chapters from lead researchers across the world, to which provide an in-depth analysis of the sigma class of receptors. In the first chapter Dr Rae Motsumoto from the University of Mississippi describes that the sigma class of receptors consists of two distinct proteins; σ_1 and σ_2 . These receptors can be distinguished from each other based upon their molecular weights, tissue prevalence, and drug selectivity patterns. Both of these proteins are currently the focus of major research as their cellular signalling pathways are yet to be fully established.

1.2 Cellular Functions, Signalling and Chaperone Activity of Sigma-1

The sigma-1 receptor consisted of 223 amino acids and is encoded by the *SIGMAR1* gene, located on chromosome 9 at position 13.3⁴. The sigma-1 receptor is profoundly expressed in neural tissue such as glial cells, oligodendrocytes, microglia and astrocytes. The fully synthesised sigma-1 protein resides anchored in the endoplasmic reticulum (ER) of the mitochondrial associated membrane (MAM). Currently, there has been no identified soluble cytoplasmic state of the sigma-1 protein, and it is believed that its chaperone activities are facilitated in a membrane bound state. Chaperones are proteins that facilitate the correct covalent folding or unfolding of other proteins in order to regulate a specific cellular activity. The sigma-1 receptor is a known chaperone of the inositol-3-phosphate or IP3 receptor, which is a protein also residing in the MAM. The activity of IP3 receptors are regulated via sigma-1 to ensure proper Ca²⁺ signalling between the mitochondria and the ER⁵. Under normal conditions cellular Ca²⁺ signalling is maintained to normal homeostatic levels, however when there is an increase in intracellular stress that reduces ER or Mitochondrial Ca²⁺ signalling, sigma-1 translocates to eradicate apoptotic cellular activities whether that be extrinsic signalling or intrinsic. This activity demonstrates sigma-1 plays an important role in cellular protection and facilitates cell survival under stress.

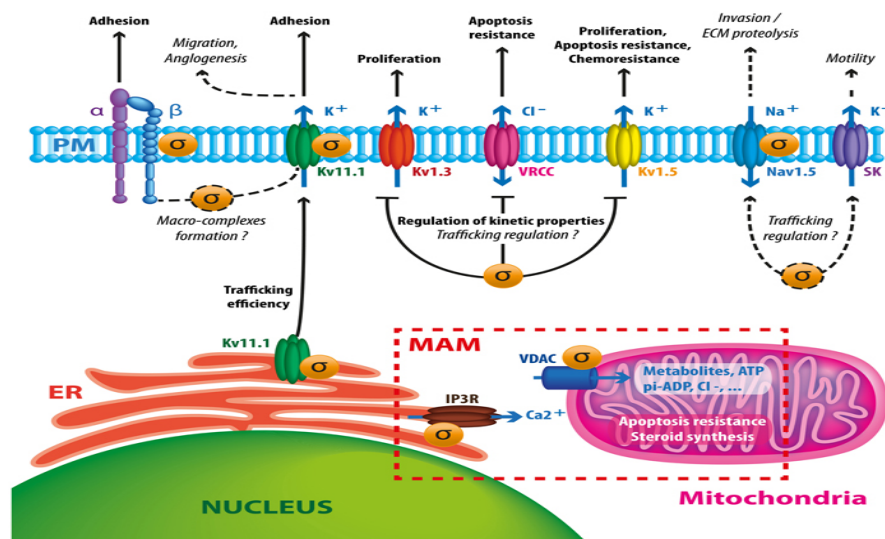


Figure 1: Demonstrates homeostatic Ca²⁺ ER and mitochondrial cell signalling facilitated by sigma-1 chaperone activity on IP3 receptors⁶.
The Sigma-1 Intracellular Ca²⁺ Signalling Model.

1.3 Implications in Disease and Illness

As the sigma-1 receptor functions are crucial for cellular survival under cell stress, abnormalities in its structure and down regulation of its activity have been theorised to play a significant role in various diseases. Some of the most notable physiological and psychological conditions sigma-1 has been linked to include depression, Alzheimer's, Parkinson's, Huntington's disease, neuropathic pain, cancer, addiction and Amyotrophic lateral sclerosis (ALS). As the sigma-1 receptor is highly expressed throughout the central nervous system its links to neurodegenerative disease have been implicated. Specifically, in 2011 *Al-Saif et al* demonstrated that an E102Q autosomal recessive mutation in the *SIGMA1* results in juvenile ALS. This study performed homozygosity mapping and direct sequencing of extracted DNA from peripheral blood samples taken from patients suffering with the condition⁷.

In addition, this study demonstrated that mutations in the *SIGMA1 gene* reduces synthesised sigma-1 distribution throughout the central nervous system and there was a significant reduction in cell viability studies in these patients. This study supports the theory that the sigma-1 receptor plays significant functions in neuroprotection, and potential drug target upregulation of this protein may prove to have a beneficial therapeutic effect in some patients. Another study published in 2017 by *Gueguineo et al* identified that the sigma-1 receptor may play a significant role in the hallmarks of cancer. In this study they identified that activated sigma-1 under cellular stress increased calcium influx by coupling between SK₃, Ca²⁺ activated K⁺ channel (KCNN3) and VI calcium channel Orai1⁸. In the hallmarks of cancer, calcium signalling is a key process hijacked by many carcinogenic cells enabling cell migration in patients with metastatic staged cancers. Furthermore, *Gueguineo et al* also identified that inhibition of the sigma-1 receptor through molecular silencing with a known ligand igmesine, resulted in decreased SK₃ current and Ca²⁺ cellular entry in breast cancer patients. These findings are astounding and demonstrate that the sigma-1 receptor could be a key therapeutic target in a range of disease and illnesses.

1.4 Molecular Structure and Chain Sequence of Sigma-1

The sigma non-opioid intracellular receptor 1 has both a unique molecular structure and sequence identity when comparing to other known proteins. The sigma class of proteins belongs to the ERG2 family of proteins and shares 33% identity and 66% resemblance to the fungal $\Delta 8 \rightarrow \Delta 7$ sterol isomerases⁹. Over 20 years since $\sigma 1$ was first sequence, the crystal structure was finally solved by *Shmidt et al* in 2016 by binding the antagonist compound PD144418 to the protein

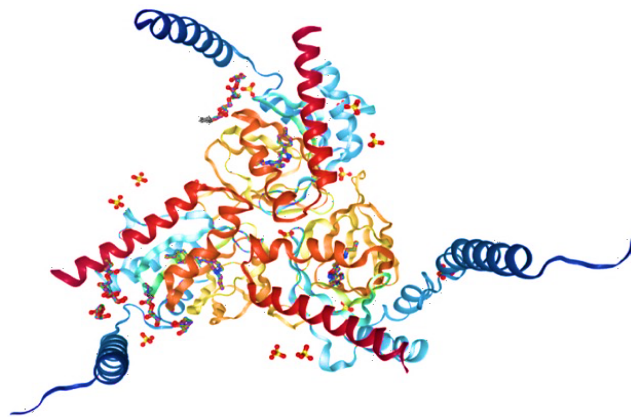


Figure 2: The Crystal Structure of Sigma-1 bound to Compound PD14441 In a Free and Transmembrane State (PDB Database 2019)¹⁰. The Identified Crystal Structure of the Sigma-1 Receptor.

This publication revealed that $\sigma 1$ possess an atypical fold with a single-transmembrane topology. In addition, the crystallization of the protein revealed $\sigma 1$ is folded into a trimer with a transmembrane domain in each corner. In addition, the *Shmidt et al* identified that the crystal structure of the sigma-1 receptor possesses a cupin-like β -barrel with the ligand binding site underneath the centre of the domain. This binding site has been described as highly diverse for ligand recognition even if two different ligands possess different chemical properties. In addition, it has been identified that many ligands for sigma-1 such as PD14441 tend to form a charge to charge interaction with a glutamine at the amino acid position 172 and takes place at high affinity even if the binding pocket is surrounded with highly hydrophobic residues¹¹.

1.5 Introduction to Expression Systems for Sigma-1

The expression and purification of recombinant proteins using bacterial vectors is well documented and is typically the preferred system to isolate desired proteins for research. However, many studies have demonstrated that yeast may prove a more efficient expression system for many proteins i.e. membrane proteins. Although bacterial expression may offer folded and stable recombinant proteins, there may still be a lack of important post-translational protein modifications such as glycosylation or phosphorylation. These systems and biological machinery typically exist in eukaryotic organisms such as yeast, insect, and mammalian cells. Insolubility of proteins expressed in bacterial vectors such as C43 has been extensively publicised, such as in intrinsically disordered proteins which tend to aggregate more than folded proteins. *S. cerevisiae* is a common species of yeast and is one of the most intensively studied eukaryotic model organisms in molecular and cell biology much like *E. coli* cell lines. Currently, there has been no substantial research into the expression of the sigma-1 receptor in *S. cerevisiae* cell lines, even though these systems offer high protein yields and sufficient post-translational modifications. However, a disadvantage of using this expression system can be said for the transformation protocol used to incorporate desired protein DNA fragments into yeast. This process is often timely and costly in comparison to *E. coli* cell lines as the reagents and transformation incubation time differ significantly.

Currently, the most common methods for expressing the sigma-1 receptor is by utilising transformation in insect and mammalian cell lines. Both of these systems if performed correctly, offer highly stable post-translationally modified desired proteins however these expression systems are often prone to contamination human error. Furthermore, successfully expressing recombinant membrane proteins in insect cells can take up to two months, as it is vital to create a high-quality viral titre containing a desired bacmid DNA for sufficient insect cell infection. Therefore, this project aims to find a suitable expression system for the

sigma-1 receptor that can be performed in the least amount of time with the highest protein yield.

E. coli have been utilised by scientific researchers over decades for the purpose of recombinant protein expression. This gram-negative bacterium has become highly popular expression platform for heterologous proteins as they have the capability of high overexpression levels. In order to express the endoplasmic reticulum membrane protein Sigma-1 in *E. coli*, competent cells should be used. *E. coli* bacterium cells have the ability to incorporate foreign plasmid DNA through their cell walls effectively if they have been altered through electroporation or with the addition of specific chemicals. We selected the use of C43 competent cells as they are highly effective at expressing toxic proteins including membrane proteins from many organisms such as; yeast, eubacteria, viruses, plants and mammalian cells. These cells have the necessary molecular machinery to express desired proteins and enhance its expression as a cellular priority. The process by which competent cells take up recombinant DNA for protein expression is known as transformation. Prior to this process it was necessary to ensure that a construct containing our desired protein was cloned and amplified to create stocks for multiple transformation procedures.

1.6 Introduction To Membrane Protein Solubilisation Techniques

Cellular membranes are complex biological structures that consist of various lipids and proteins to ensure cell survival. Many of the proteins that reside on cellular membranes form channels or function in cellular signaling transduction pathways that are vital for almost all cellular activities. One of the most significant challenges scientists have faced when studying these proteins, is being able to successfully extract and solubilize whilst maintaining the membrane bound biochemical structure. A method that revolutionized the extraction of these insoluble proteins is known as detergent extraction. Detergents are soluble amphiphiles (i.e. containing both hydrophobic and hydrophilic groups) and are currently the most adopted method of membrane protein extraction¹². The most

common types of detergents used for this extraction can be divided into three groups; ionically charged (either anionic or cationic), nonionic (uncharged) or zwitterionic (having both polar or nonpolar groups but having a total charge of zero)¹³.

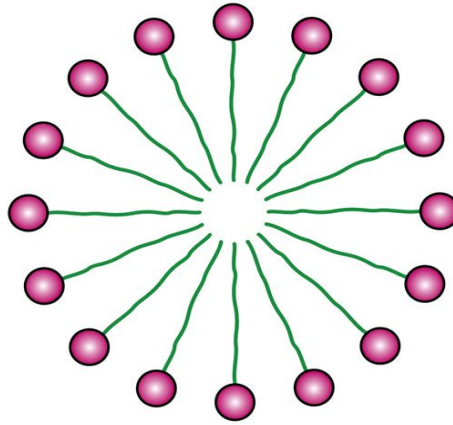


Figure 3: Diagram of the basic structure of a micelle with nonpolar hydrophobic tail groups. An Overview of Detergent Micelle Formations.

In a water-based solution, detergents form soluble micelles which consist of a ring like formation. Although detergents consist of hydrophobic nonpolar tail groups, overall, they have a high level of water solubility enabling efficient extraction of membrane proteins. Their outward pointing polar heads are highly hydrophilic enabling a complete mixture of these micelles with aqueous solutions. For this particular project we will be using a newly developed detergent called lauryl maltose neopentyl glycol (LMNG: chemical formula $C_{47}H_{88}O_{26}$) along with a cholesterol analogue known as cholesterol hemisuccinate (CHS: chemical formula $C_{31}H_{50}O_4$) for sigma-1 extraction. LMNG detergent contains two hydrophobic chains of equal length and two hydrophilic polar maltose head groups. In recent years, multiple publications have shown the potential this detergent has in solubilising difficult membrane proteins and have even demonstrated LMNG to be more effective than dodecyl maltopyranoside (DDM).

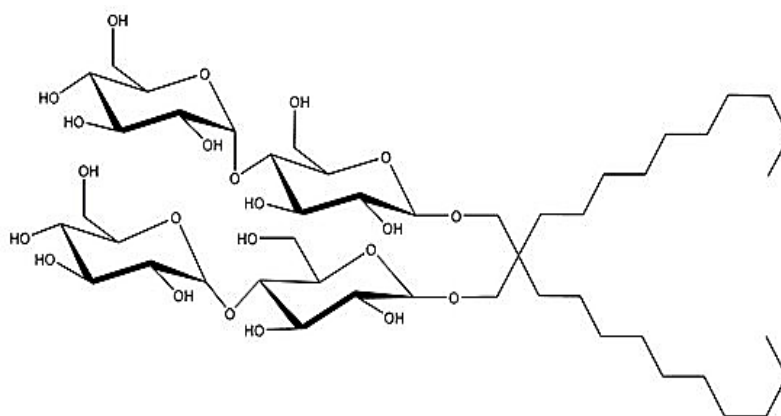


Figure 4: Chemical structure of lauryl maltose neopentyl glycol (LMNG) with two hydrophobic nonpolar tails and two hydrophilic polar maltose heads¹⁵. The Chemical Structure of Lauryl Maltose Neopentyl Glycol (LMNG).

The crystal structure of the sigma-1 receptor was identified by *Shmidt et al* in 2016 using 0.1% (w/v) of this detergent supplemented to their extraction buffer. From their crystallography data they identified that this detergent had little adverse effects to the stability and structure of the protein and managed to successfully bind an antagonist compound. As well as solubilising extracted the sigma-1 receptor with 1% (w/v) LMNG, *Shmidt et al* also used 0.1% CHS (w/v) supplemented to the same extraction buffer. Typically, cholesterol or its analogues are often used for insertion into micelles for increased stability of a desired protein. Many proteins, especially membrane proteins rely on specific lipid interactions to retain correct functions and structure whilst residing in biological membranes. For this reason, we intend to supplement our protein extraction buffers to the same protocol used by *Shmidt et al* and use similar purification procedures with the addition of the appropriate concentration of both LMNG and CHS.

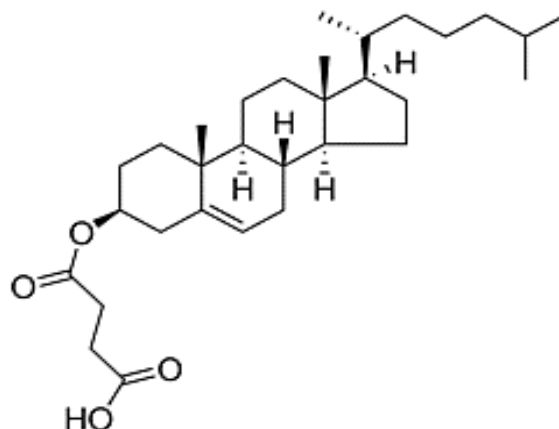


Figure 5: Chemical structure of cholesterol hemisuccinate (CHS), an analogue of cholesterol¹⁶. **The Chemical Structure of Cholesterol Hemisuccinate (CHS)**

In recent years, there has been a major increase in the funding and research into nanodisc technologies in the biotech industry. One of the most notable developments in this field was the synthesis of styrene maleic acid copolymer (SMA) resins for the purpose of membrane protein extraction. Recently in a profound number of publications, it has been demonstrated that solubilised SMAs can dissolve biological membrane bound proteins into the formation nanodiscs. These nanodiscs have shown to natively solubilise spherical sections of biological membranes at varying size and molecular weights. Within these nanodiscs, proteins are still physically embedded into their original patch of lipids to which they would become functionally active. This is facilitated by amphiphilic polymer chains that wrap around the lipids resulting in effective solubilisation.

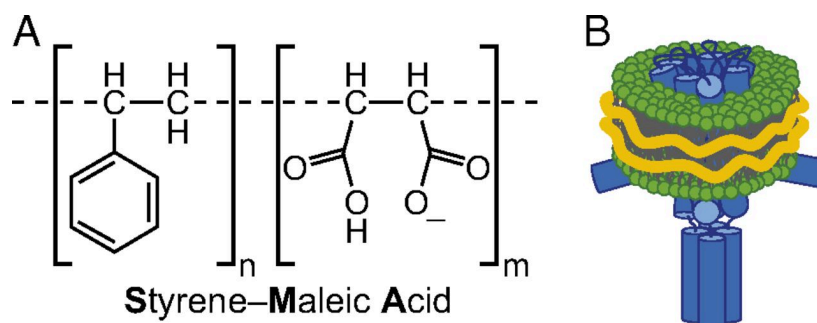


Figure 6: Diagram of the basic chemical structure of a 1:1 styrene maleic acid compound and graphical representation of how SMAs form protein nanodisc complexes¹⁷. **The Formation of SMA Nanodisc Complexes.**

The Styrene-maleic acid chains result in water based solubilization with an increased stability of the extracted proteins. The discovery of SMAs has revolutionized the research of many difficult to solubilize membrane proteins and many researchers are considering adopting this extraction method over detergent based protocols. These SMA nanodiscs can be synthesized in varying ratios of styrene to maleic acid groups, which can be catered to specific protein research needs or through protocol optimization. For example, the most commonly used form of SMAs used for membrane protein structural research, are the SMA 2:1 copolymer as they have been shown to be highly stable and form low molecular weight nanodiscs with a diameter of around 10 to 20nm. For this project we will attempt to solubilize the sigma-1 receptor using 2% (w/v) 2:1 SMA copolymer as there is currently little to no literature available for this research. Furthermore, we will be experimenting solubilization with an SMA copolymer variant known as Styrene Maleimide resins (or SMA imides). These positively charged copolymers consist of alternating styrene and maleimide moieties which also have been demonstrated to effectively extract membrane proteins whilst maintaining a high level of protein stability.

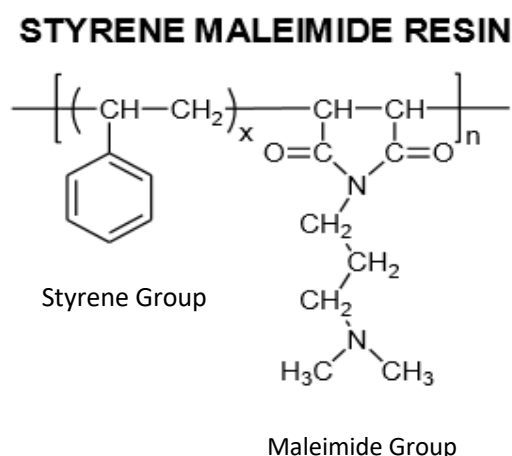


Figure 7: The Two-Dimensional Chemical structure of styrene maleimide (SMA imide) copolymer 1:1 resins¹⁸. An Overview of a Basic SMA Imide Chemical Structure.

Currently there have been no scientific publications demonstrating successful extraction of the sigma-1 receptor using both SMA and SMA imide copolymers.

Although, other studies have demonstrated its effectiveness of extraction such as that of G-coupled receptors in various papers. For this project all SMA and SMA imide resins will be ordered through Cray Valley supplier in a non-water-soluble chemical state. All resins will require a solubilization protocol via protonation in a condensation reaction using an appropriate solvent such as hydrochloric acid (HCl) or sodium hydroxide (NaOH). Post solubilization, all SMA resin solutions will need to be purified and freeze dried to remove any contaminants. For this study, we will be using transmission electron microscopy (TEM) in order to identify the presence of nanodisc formations within our solubilized sigma-1 samples post solubilization and purification.

1.7 NMR Studies for The Identification of The Sigma-1 Receptor

Nuclear magnetic Resonance imaging or NMR has become an increasingly important scientific technique for the screening and characterisation studies of simple and complex biomolecules. In the last 25 years, NMR along with other structural techniques such as crystallography has enabled the identification of three-dimensional molecular protein structures on the atomic level. In addition, not only can NMR provide high resolution structural information, it also has the capability in determining kinetic interactions proteins have in varying environmental conditions i.e. in the presence of agonist or antagonist compounds. The scientific principles surrounding NMR experiments may differ depending upon the study conditions applied and the researcher's analytical objectives.

However, the physical scientific principles that make NMR possible remain the same throughout almost all experiments. The NMR principle is strongly dependent upon atomic particle spin theory and many chemists, biologists and physicists have used this principle to their advantage. Electrons are subatomic particles with the propensity to spin and atomic nuclei also possess this physical property. The nucleus of an atom consists of protons and neutrons, which can also be broken down to subatomic particles such as quarks. Neutrons are comprised of two quarks (down quarks) with a charge of $-e/3$ and one quark (up quark) with

a charge of $+2e/3$, which equates to a net neutron charge equal to 0. In contrast, the proton consists of two up quarks with a charge of $+2e/3$ and one down (down with a charge of $-e/3$, resulting in a net positive proton charge of $+1$. As the neutron number plus the proton number are equal to an odd level of charge it can be said that they have half-integer spin or $\frac{1}{2}$ spin²⁸. When atoms in elements such as H^1 and C^{13} are in $\frac{1}{2}$ spin they are considered to have a low energy state. However, when a magnetic field is applied to these atoms, they will begin to precess on their spinning axis around the direction of the applied field.

If energy is absorbed by the nucleus of these atoms during this low energy state, then they will flip upon the axis opposing the direction of the magnetic field applied. This physical property of these atoms at this stage is considered to be a higher energy state. In terms of NMR imaging, scientists are often interested in the relaxation states of these precessing atomic nuclei to which provides key structural information for biomolecules. This relaxation state is underpinned by the principles of thermodynamics and studies physical changes when atoms transition from a higher energy state to a lower energy state. This shift between energy states of these atomic nuclei can be detected by applying a known frequency of electromagnetic radio wave radiation. Furthermore, this applied radio wave causes an atomic resonance to which can be detected and translated into an NMR peak of specific intensity. In relation to our NMR studies for this project we performed a 1D proton NMR study and performed a Carr-Purcell-Meiboom-Gill (CPMG) experiment.

These studies focussed on identifying the interactions between a known agonist compound fluoxetine hydrochloride and the sigma-1 receptor. The first of our NMR experiments involved the running of CPMG sequences on the individual samples and then creating an overlay for quantitative analysis. This type of NMR experiment is often referred to as relaxation editing, and in recent years this technique has been increasingly used to assess the binding affinities of proteins and their ligands. This experiment depends on the principle that rotational tumbling is proportional to the molecular size of any given protein, i.e. higher

molecular weight proteins have a longer rotational time than that of lower molecular weight proteins. Subsequently, this means smaller proteins will have shorter T_2 relaxation times (resulting in broader peaks) than that of larger proteins. In principle, CPMG experiments measure the T_2 relaxation time of proteins, by monitoring the signal decay as a proportional unit of relaxation delay²⁹. This allows for the analysis of peak intensities, by measuring their width and height for ligand-protein interactions.

In addition, this experiment requires control samples to compare spectra in both the presence and absence of target proteins in solution. Hence, the presence of multiple overlay signals for varying sample conditions in our spectra for this experiment. Once we obtained data for the CPMG experiment it was deemed necessary to determine whether the detergent used to solubilise our target protein was also binding to the agonist ligand. For this we run a normal 1D NMR proton spectrum on the same samples to also assess for the presence of peak shifts and changes in peak intensities³⁰. Although, these experiments do not provide us with K_D values or other pharmacological binding affinity data they serve as an effective method at determining protein interactions. Currently, fluoxetine hydrochloride along with other selective serotonin reuptake inhibitors (SSRI's) have been previously identified in publications, as having affinity for the sigma-1 receptor. Therefore, this final experiment served as a valuable early confirmation technique for the isolation of our target protein by measuring its interactions with a known agonist of the receptor.

1.8 Project Aims and Objectives

The sigma non-opioid intracellular receptor 1 is a notoriously difficult protein to successfully solubilize and express in prokaryotic systems such as *E. coli* cell lines. There have been very few studies that have demonstrated a successful and optimized purification procedure for this chaperone protein in yeast and *E. coli* cell lines. Furthermore, this project aims to produce optimized growth and purification procedures for the sigma-1 receptor in C43 *E. coli* and INVSc1 *S.*

Cerevisiae cell lines, as previous studies have focused on using advanced cell lines such as insect Sf9 cells. The rationale behind this is due to advanced expression systems, such as insect and mammalian cell lines often requiring timely growth procedures and tend to produce low yields of target proteins. Whereas *E. coli* systems, when optimized can produce much higher protein yields. In addition, studies that have successfully solubilized the sigma-1 receptor with the aid of cationic and non-cationic detergents which are very costly and can often affect the structural integrity of the protein during purification.

Furthermore, this project is going to analyze and compare the effectiveness of detergent based methods for membrane solubilization against that of newly developed techniques, such as nanodisc technology in the form of SMAs or SMA imides. Currently, there are no known publications that have studied agonist or antagonist compound interactions with the sigma-1 receptor when natively solubilized in nanodiscs. For this reason, we aim to produce a successful isolated sample of the sigma-1 receptor solubilized by nanodisc technologies or via detergent based methods. Once these sample have been acquired, the ultimate objective is analyzing the interactions a known agonist; fluoxetine has with our desired protein using 1D proton nuclear magnetic resonance (NMR) imaging experiments. These experiments will not focus on the analysis of the proteins structure itself, however we will be analyzing the interacting drugs peak intensities in the presence or absence of the sigma-1 receptor. This project will be comparing the similarities and differences to past publication results such as that of the 2016 *Crystal structure of sigma-1 by Shmidt et al.* Although, this study focused on the expression of sigma-1 receptor in insect expression systems it serves as a valuable reference for this project as we will be using similar purification and characterization methods as the researchers.

Research Project Aims

- Successfully produce a modified pCold-1, transformed C43 cell line containing a DNA fragment for the sigma-1 receptor tagged to green fluorescent protein (GFP)
- Compare transformation procedure in C43 cells with transformed INSVc1 *Saccharomyces cerevisiae* containing the sigma-1 LEU2 construct
- Create bacteriological and yeast growth cultures that are induced at an optimum OD₆₀₀, for sufficient cell culture density of C43 and INSVc1
- Extract the sigma-1 receptor tagged to GFP via solubilisation techniques such as detergent based and native nanodisc technology
- Successfully purify solubilised sigma-1 receptor and isolate via TEV protease cleavage from GFP
- Confirm isolation through antibody screening techniques such as western blot
- Once isolation is deemed successful use NMR studies to assess the sigma-1 receptor interactions with known agonists such as fluoxetine hydrochloride
- Assess retrieved NMR data to draw conclusions between solubilisation techniques for the sigma-1 receptor
- If all aims are reached to the correct deadlines, then we will attempt to express the sigma-1 receptor in Sf9 insect cells to compare and correlate expression results

1. Materials and Methodologies

2.1 Preparation of SMA And SMA Imide Nanodiscs For Solubilization

As both the SMA and Imide nanodiscs from stock supply resided in a water insoluble state it was necessary to solubilize them via a condensation reaction. SMA resins are soluble in water at basic pH so it was necessary to first perform

solubilization via deprotonation using sodium hydroxide (NaOH), whereas SMA Imide resins are soluble in water at acidic pH therefore required protonation using hydrochloric acid. For this study we ordered the stock resins of SMA 2000 (2:1) and SMA imide 1000I (1:1) from the supplier Cray Valley¹⁸. These resins can chemically alter depending upon the ratio of styrene to either maleic acid or maleimide groups (i.e. one styrene group for one maleimide group). The protocol for solubilizing these resins were conveniently provided for the supplier, however we made modifications due to equipment and reagent availability.

Table (1): Copolymer resin chemical properties supplied by Cray Valley¹⁹

Ratio of styrene to maleic acid or maleimide	Copolymer Name	M_n (g/mol)	M_w (g/mol)	Kilodaltons (KDa)
2:1	SMA 2000I	2,700	7,500	7.5
1:1	SMA IMIDE 1000I	2,000	5,500	5.5

In order to solubilize these resins, it was necessary to determine the volume of hydrochloric acid or sodium hydroxide required for this procedure. All of these values were supplied by Cray Valley and it just required equation substitution.

Equation 1: Calculation of NaOH volume for SMA 2:1 (SMA 2000I) Solubilization

$$\text{mL of NaOH} = \frac{(\text{amine or alkali mol. wt.}) (\text{SMA acid no.}) (\text{wt. SMA}) (\text{excess factor})}{56,100 \times (\text{amine or alkali concentration, as a decimal})}$$

In equation 1, we find the method used to determine the volume of NaOH to solubilize our SMA 2:1 (SMA 2000I) resins. The 'amine or alkali mol. wt.' equated to the molecular weight of NaOH (40mw), the 'SMA acid no.' of this calculation was 350, the 'wt. SMA' was the weight of the resin used and the excess volume of NaOH to solubilize the resins equated to the 'excess factor', which is typically 5 to 15% expressed as a decimal i.e. 1.1. This figure from equation 1, was then divided by 56, 100 multiplied by the concentration of NaOH as a decimal. For our

SMA 2:1 solubilization protocol we determine that 1M NaOH would be enough to solubilize our pure copolymer resin. Once we had made this calculation it was then necessary to grind these resins down to a smooth powder base to maximum the surface area to volume ratio of the reaction. Following this, the powdered SMA 2:1 resin was then added to the 1M NaOH solvent within a boiling flask²⁰.

A magnetic stirrer was then added to the boiling flask and the solution was gently stirred under reflux conditions for 2 hours at 60°C, using heated bumping granules. Once the reaction time duration was complete, the solution was removed from the heat source and left to cool down to room temperature. After this, the solution was then precipitated using a 1 M HCl solution to achieve pH.5 for the wash phase. Post precipitation, the precipitate was washed for 5 wash cycles using Milli-Q water. The precipitate suspended in Milli-Q water was then centrifuged at 11,000g using a Beckmann™ J-25 centrifuge (JA-10 rota) for 15 minutes per cycle. Following the wash phase, the precipitated resin was then resuspended in 0.6M NaOH to achieve pH 8.0. Once the desired pH was achieved the precipitate was placed in an orbital shaking incubator set to 25°C and at 180rpm for overnight resin dissolvment. Once the SMA 2:1 resin was assessed for complete resuspension, it was then flash frozen using liquid nitrogen and set up for lyophilises for 1 to 2 days. The freeze dried solubilised SMA 2:1 resin was then weighed out into aliquots and stored at 4°C until required for use²¹.

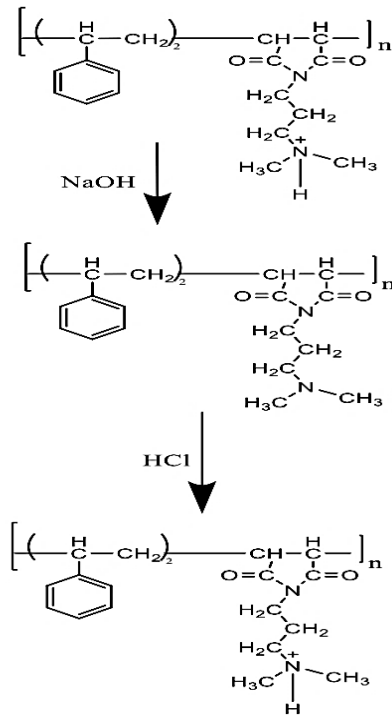


Figure 8: Demonstrates SMA 2000I solubilisation rationale with the aid of NaOH deprotonation and HCl protonation. **Chemical Equation of SMA 2000I Solubilisation.**

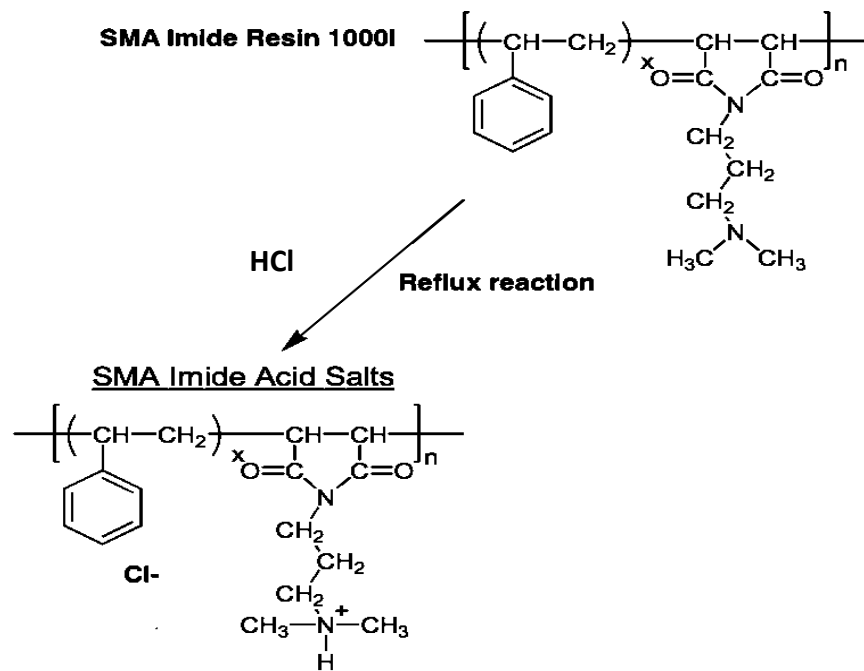


Figure 9: Demonstrates SMA imide 1000I solubilisation rationale with the aid of HCl via a ternary amine protonation. **Chemical Equation of SMA Imide 1000I Solubilisation.**

Equation 2: Calculation of HCl volume for SMA Imide 1:1 (SMA 1000I)

Solubilization

$$\text{mL of HCl} = \frac{(\text{SMI}_{\text{wt}})(\text{SMA}_{\text{index}})(\text{HCl}_{\text{mw}}) (\text{Excess Factor})}{1000 (\text{Acid concentration})}$$

In equation 2, we find the calculation used to determine the volume of hydrochloric acid for solubilising the SMA imide 1:1 resin. From the equation, the 'SMI wt.' equates to the total weight of SMA imide resin used in the reaction, the 'SMI index' for the SMA 1:1 imide resin used in this protocol was 3.13, the 'HCl mw' is the molecular weight of hydrochloric acid i.e. 36.45 mw, the 'excess factor' corresponded to the excess volume of acid used to completely solubilise the resin, and was expressed as a decimal. Finally, this figure was then divided by 1000 times the concentration of acid in the reaction. Just as in the SMA 2:1 solubilisation reflux reaction, the SMA imide 1:1 resin was weighed out for solubilisation using 1M HCl.

The solution was then refluxed for 2 hours at 60°C under reflux conditions until the resin was completely dissolved. Following this, the dissolved resin was then left to cool down to room temperature and then prepared for pH adjustment using 5M NaOH. The dissolved SMA imide 1:1 resin was then adjusted to pH 7.5 and then prepared for flash freezing using liquid nitrogen. Upon the flash freezing of the dissolved resin, it was the lyophilised for 2 to 3 days to ensure complete removal of any moisture from the soluble SMA imide dried reagent. The newly solubilised SMA 1:1 imide was then weighed into 5g aliquots and stored at room temperature until required for use. For our solubilisation techniques these resins were used at 2% (v/w) of the total cell lysate volume. Currently this supplemented concentration of solubilised SMA or SMA imide resins has been recommended by various publications in recent years.

2.2 C43 Expression Protocol for Isolated Sigma-1

In order to insert a suitable construct to transform into C43 cells we firstly ordered the human *SIMGA1* gene incorporated with a N-terminus super folder green fluorescent protein gene sequence. Separating these two proteins was a TEV cleave site enabling for late stage sigma-1 isolation during purification. In addition, the N-terminus was designed to obtain the necessary tags required for purification, including twin Step tag and 6xHis tag. The sigma-1 gene was then inserted into a modified version of the p-Cold1 Vector using a transformation procedure into DH5alpha cells. These cells were then used to create an overnight starter culture in LB medium, at 37°C in a shaking incubator at 180rpm. The overnight starter cultures were then centrifuged at 13,000rpm for 10 minutes to obtain the cellular pellet. The pelleted cells then underwent DNA extraction for isolation of the desired construct in Milli-Q water. The extracted DNA sample was then sent for sequencing to ensure the Sigma-1 gene was correctly inserted into the modified p-Cold1 vector. Once the correct sequence of the final construct was confirmed, it was then possible to move forward to expression of our desired recombinant protein. In Figure 10, It is possible to see the final sequenced construct that was used for transformation into C43 cells.

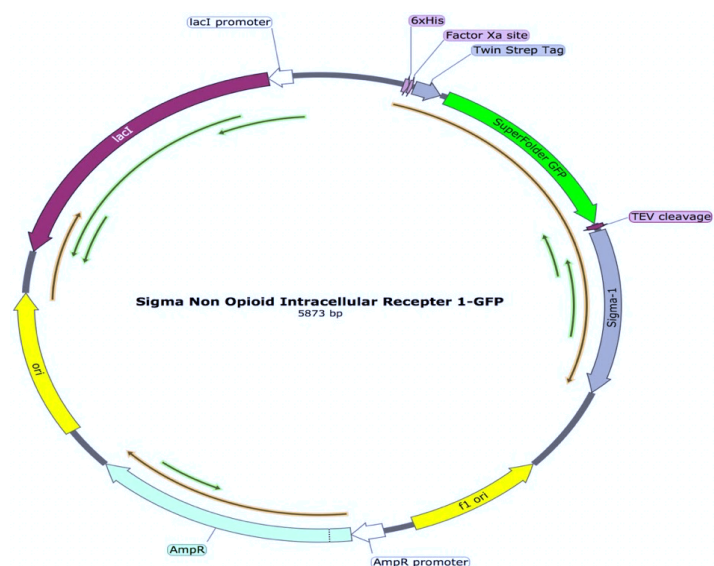


Figure 10: Sigma-1 Final Construct of The Modified p-Cold1 Plasmid (5873 Base Pairs). Image Obtained from SnapGene Software. **Final Sigma-1 Modified p-Cold1 Construct.**

The final construct was designed to be highly suitable for both the expression of Sigma-1 and its characterization. The green fluorescent protein tag for the Sigma-1 was implemented to ensure easier screening for the protein post induction and during purification steps. We decided we could do this by measuring the GFP fluorescence of our samples at different timepoints of the study, whether that be during size-exclusion chromatography (SEC) or via a fluorescent plate reader at any time. On the N-terminus of the GFP, a twin Step tag was implemented for the purpose of one-step purification of the recombinant protein. The twin-step tag (amino acid sequence: SA-WSHPQFEK-(GGGS)₂-GGSA-WSHPQFEK) is a short peptide that has a molecular weight of 6.98KDa. Many publications have demonstrated this tag to be extremely useful as it combines a high specificity and high affinity for Strep-Tactin beads during purification. This makes it possible to even to perform complete affinity chromatography from a cell culture supernatant, reducing the time spent on additional purification steps. The polyhistidine tag adjacent to the twin-step tag also serves as an effective method of purification for Sigma-1. The 6XHis tag is a small peptide consisting of 6 Histidine amino acids (CATCATCATCATCAT) and has a small molecular weight of 840.9 Da.

The purpose of implementing this tag was for having the ability to perform immobilized metal affinity chromatography (IMAC) on Sigma-1 extract samples. The principle of the His tag purification is since histidine residues chelate metal ions such as nickel under specific conditions causing them to bind to one another. This makes it possible to elute desired proteins by using compounds such as imidazole to compete binding with the His tag for the charged metal ion resin thus eluting a specific protein in a single sample. When screening for the target protein it is important to take into consideration the final molecular weight of the expressed complex including all the molecular weights of all tags. We estimated our final Sigma-1 tagged GFP complex to be around 55KDa and we decided to screen samples that contained proteins around this molecular weight. For the expression of Sigma-1 it was necessary to select a cell line that is highly competent, has the ability to express toxic proteins and at high levels. For this

stage we selected the OverExpress™ C43 (DE3) Electrocompetent cell line supplied from Sigma Aldrich. This strain is derived from C41 (DE3) cells by selecting colonies resistant to the expression of toxic proteins. These cells were stored at -80°C until they were required for use during a transformation procedure. In recent years, this cell line has received a high level of recommendation for the expression of membrane proteins throughout various expression system publications.

2.3 Methodology for C43 Electrocompetent Cells Transformation

Once all the pre-study requirements had been reached and the Sigma-1 construct had been successfully sequenced it was then possible continue forward onto the transformation stage. Transformation is a multistep process which includes a heat shock process to increase competent cell wall permeability along with a temperature specific incubation period suitable to the cells being used. However, prior this procedure the electrocompetent C43 cells must undergo a growth process using nutrient rich agar medium with the addition of ampicillin for selective growth. The Sigma-1 modified Pcold1 construct incorporates an ampicillin resistance gene (AmpR) to ensure the required C43 cells will specifically grow without undesired contamination. Prior to the transformation procedure selective agar plates were prepared in order to ensure a level of organization during the process. In order to make these selectivity Agar plates it was necessary to prepare both antibiotic and Luria Broth medium stocks. Ampicillin sodium salt was used to prepare stocks of 100mg/mL in Milli-Q water.

Following the preparation of the antibiotic stocks it was necessary to prepare Luria Broth Agar as it is vital for preparing the selectivity plates. Once this was complete, it was necessary to adjust pH of the LB-Agar to 7.5 as it is the optimum for C43 cells. The medium was then autoclaved for 15 minutes at a temperature of 121°C using a Prestige 2100 Classic autoclave. The sterile autoclaved medium was then cooled down to 50°C using a water bath as the addition of ampicillin to medium at higher temperatures can lead to degradation of the antibiotic. Once the LB-Agar medium had cooled to the optimum temperature, under aseptic conditions we

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dispensed the ampicillin to the medium to create a final concentration of 100ug/mL. Following this the molten LB-Ampicillin Agar was poured into each petri dish and then left at room temperature to solidify. Once the medium had solidified stocks of the LB-Ampicillin Agar plates were completely sealed and then stored at 4°C in a fridge for later use.

After all the necessary preparations were complete, it was then possible to start the transformation procedure using the Sigma-1 modified pCold1 construct and C43. During this procedure, a 1mL stock of electrocompetent C43 cells and a 50µl stock of the Sigma-1 construct (102ng/µl) was removed from the -80°C and thawed on ice for 15 to 20 minutes. Following a single thaw cycle, 2µl of the Sigma-1 construct was gently mixed with competent C43 cells and then placed back on ice for a further 15 minutes. Once the mixture had been incubated on ice, the Sigma-1 construct and C43 cell mixture underwent a heat shock procedure in a water bath at 42°C for 45 seconds. Post to the heat shock procedure the transformation mixture was then incubated back on ice for a further 15 minutes. Following the incubation on ice, the mixture was then supplemented with LB medium without the addition of ampicillin. The transformant suspended in LB medium was then transferred to a shaking incubator with a temperature of 37°C and rotation speed of 250rpm for 1 hour. Following the required incubation time, the transformant mixture was then aseptically plated onto the LB-Amp Agar selectivity plates previously prepared. During this procedure the transformant mixture was suspended onto a single selectivity plate and a sterile loop was used to inoculate the entire surface area of the LB-Amp Agar medium. Furthermore, the newly inoculated plate was then completely sealed, inverted and placed in an incubator overnight at 37°C.

2.4 Transformation Plate Screening and Preparations for Growth

At 12-24 hours following the incubation of the transformation culture, the plate was screened for viable colonies to ensure sufficient growth of the transformed C43 cells had occurred. As these selectivity plates contained a sufficient

concentration of ampicillin, it was decided likely that isolated colonies were probable to have incorporated the Sigma-1 construct containing the AmpR gene. Prior to the preparations of large-scale growth, it was necessary to store the C43 selectivity plates at 4°C post removal of the incubator used overnight. In order to ensure the large-scale growth protocol was followed with efficiency it was vital that all reagents including the growth medium was prepared prior to the protocol. During the large-scale growth protocol, it was decided to be beneficial to use an increasingly nutrient rich medium to maximise cellular density and protein expression. For this we adopted the use of terrific broth (TB) as an effective medium source for our transformed C43 cells. Prior the growth we estimated that 4 litres of terrific broth should be enough to harvest enough cells for screening for our target protein. Typically, terrific broth medium has the addition both mono and dibasic phosphates (such as dipotassium hydrogen phosphate and potassium dihydrogen phosphate).

However, this is usually used to increase the yield of plasmid DNA in suspension of derived C43 cultures, nonetheless for our specific study we deemed this as unnecessary. In order to make a single litre of terrific broth medium. The pH of the terrific broth mixture was then adjusted to 7.5, just as the LB-Amp Agar was previously. The mixture was then autoclaved in a sterilised 1L growth flask for 15 minutes at 121°C. In order to maximise the overexpression of our target protein it was vital that we carried out an induction procedure at a specific point of the C43 cell's exponential growth phase. As described previously, the Sigma-1 modified pCold1 construct holds the *LacI* promoter gene and can be activated with the addition of the reagent IPTG. Prior to the setup of the large-scale growth it was also necessary to prepare stocks of IPTG at the correct concentration required for our induction protocol. We prepared a stock solution of 1M and stored these at -20°C for later use.

Once all of the required reagents and medium were correctly prepared it was then possible to continue setting up the large-scale growth. In order to achieve a minimum cellular density of 6.4×10^8 during growth, we needed to inoculate the

medium with a starter culture to reach this level. A starter culture was prepared by inoculating four separate starter cultures per litre of TB medium. Each starter culture contained LB-Amp medium in a sterile falcon tube and was inoculated with a single colony selected from the C43 transformation plate using a sterilised pipette tip. The starter cultures were then transferred to a shaking incubator overnight, set to 37°C at 180rpm. After 12 to 24 hours of growth, the starter cultures could be used to inoculate 4L of terrific broth medium for our large-scale growth phase. Prior to this transfer a 1mL blank sample was taken from the sterilised TB medium for a baseline, for the purpose of tracking the optical density (OD) for each 1L of inoculated TB medium.

Once the TB medium had been inoculated with the starter cultures, they were then transferred to a shaking incubator set to 37°C at 180rpm. The OD of each flask was measured every 30 minutes using a DiluCell 20 device and all data was recorded electronically. During the large-scale growth it was important to induce the culture once the peak of exponential growth had been reached i.e. OD of 0.8 to 1.0 600nm. This was implemented to maximize cell harvesting and to avoid cells entering the death phase of growth. After 180 minutes of culture incubation the average OD equated to 1.12 (8.96×10^8) at 600nm and we decided to induce all 4 liters of growth. As we used the Sigma-1 Modified pCold1 construct it was useful to follow a cold shock induction system for maximizing the overexpression of our target protein. This procedure aims to slow cellular growth during protein induction, reducing the formation of protein aggregation thus minimizing the development of inclusion bodies. Once the sufficient OD had been reached, the cultures were removed from the shaking incubator and placed on ice for 15 minutes. During this time the temperature of the shaking incubator was reduced to 15°C and IPTG 1M stocks were left at room temperature to thaw. Once the cultures were incubated on ice for the specified time, they were then induced using a final IPTG concentration of 1mM. The induced cultures were then placed back in a shaking incubator set to 15°C and 180rpm overnight for 12 to 24 hours. Following the correct culture induction time, the 4L culture was then centrifuged using a Beckman Centrifuge and a T45i rota for 25 minutes at 6,000rpm. The

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supernatants containing the terrific broth medium was discarded and the cellular pellets were placed on ice for resuspension in lysis buffer.

The chosen lysis buffer changed throughout the study due to optimisations in our protocol, we firstly started using 1x tris-buffered saline (TBS) at a pH of 7.5. However, as we had issues with solubilisation of our protein, we decided to use the same sample buffer used by *Schmidt et al* who were the first to confirm the true crystal structure of the Sigma-1 receptor. This buffer consisted of 20mM HEPES, 250mM NaCl, and 20% glycerol (v/v) also at pH 7.5. Depending upon the solubilisation technique used for Sigma-1, the lysis buffer was modified and supplemented with a specific reagent i.e. detergent.

The previously centrifuged cellular pellets incubated on ice were then resuspended using HEPES-buffered saline lysis buffer and then supplemented with Halt™ protease inhibitor single-use cocktail 100X (EDTA free). At this point a reference sample was taken from the cell lysate and stored at 4°C for later screening of our target protein and its tag i.e. GFP fluorescence and SDS-PAGE. Once the cell lysate had been supplemented with a protease inhibitor, it was then possible to continue through to extracting Sigma-1 through a protocol consisting of sonication, ultracentrifugation, homogenisation and nanodisc incubation. The 30mL cell lysate was then placed in a sterile volumetric beaker and then placed back on ice during the sonication procedure. During this protocol the cell lysate was sonicated at 20 kHz using a Soniprep 150 for 15 split cycles at 15 minutes. This technique ensured that the cell lysate was sonicated for 30 seconds at the specified amplitude, and then incubated on ice for a further 30 seconds in one complete cycle.

The sonicated cell lysate was then ultracentrifuged using a Beckman Optima LE-80K centrifuge with a T70ti rotor. The sonicated lysate was centrifuged for 1 hour at 45,000rpm to obtain pelleted cellular membranes and to discard undesirable cellular debris. Following this, the pelleted membranes were then resuspended on ice using the same lysis buffer and homogenised using a micro grinder ensure

complete protein extraction. The sample was then supplemented with 2% (w/v) a SMA or SMI previously prepared reagent and the cell lysate was then mixed overnight at 4°C for solubilised membrane extraction of Sigma-1. After 12-24 hours of solubilisation, the cell lysate was further centrifuged at 45,000rpm using a LE-80K with a T70ti rota to isolate the supernatant. Post centrifugation the supernatant was immediately extracted and placed on ice. In contrast, the pelleted membranes were resuspended in lysis buffer and flash frozen in liquid nitrogen for later screening if Sigma-1 extraction was poor. As previous nanodisc studies had demonstrated that free disc formations in solution can interfere with IMAC purification, we decided to perform a dialysis procedure to remove any free nanodiscs.

2.5 Purification Techniques for Nanodiscs Solubilised Sigma-1 GFP Complexes

The dialysis of solubilised Sigma-1 was set up immediately following extraction of the supernatant. A total of 4 L of HEPES-buffered saline was prepared prior to this procedure and stored at 4°C. The extracted supernatant was then inserted into a sealed 10K MWCO SnakeSkin™ dialysis tubing to be suspended into the 4L of lysis buffer for 12-16 hours overnight. In addition, the dialysing supernatant was slowly spun using a magnetic stirrer at 4°C to prevent aggregation and the precipitation of sigma-1 out of solution. Once the sample was dialysed for the specified time, it was then vital at this stage to take a reference sample that could be used for performing screening techniques such as SDS-PAGE, in gel-fluorescence and GFP fluorescence microplate reading.

2.6 Primary Purification: Immobilised Metal Affinity Chromatography

As the free SMA or SMI nanodiscs had been removed from the solubilised sigma-1 supernatant, it was then possible to purify our desired protein using IMAC. This procedure was performed using the automated AKTA™ start chromatography system in a series of specific protocol phases. Prior to this procedure a 5mL HisTrap™ HP nickel charged column was fitted to the system, so that the sample could be passed directly through its flow path. During the first phase of this purification, double distilled water (ddH₂O) was firstly filtered and degassed to

eradicate contaminants and globules. The ddH₂O was then run through pump A at 0.5mL/minute for three column volumes (15mL) in order to complete the primary wash phase of IMAC procedure.

The confirmation end point of the primary wash phase was determined by analysing the reduction and stability of the UV (AU 280nm) and conductivity (S/m) sensors. Shortly following this wash phase, the AKTA™ system including the 5mL HisTrap™ HP nickel column was equilibrated in buffer A. It was important that Buffer A was identical to that of the sample buffer in order to promote an affinity reaction between the polyhistidine tag and nickel ions of the column. For this reason, five (25mL) column volumes of filtered buffer A (1x HEPES-buffered saline) was passed through the system at a flow rate of 0.5mL/min. Once both the UV and conductivity detectors were stable it was then possible to pass the sigma-1 sample through the system at 0.5 mL/min on ice and collect its flow through from the buffer outlet. At this stage, the analysis of increased UV and conductivity was a priority as it enabled us to determine whether a high concentration of proteins were in present in our sample. In principle Sigma-1-GFP nanodisc complexes should remain bound to the affinity resin via his-tag interactions and undesired cellular components should pass through to the flow through collection on ice. Following the collection of the flow through sample on ice, it was necessary to perform a secondary wash procedure in buffer A to remove any unbound contaminants in the IMAC system. During the secondary wash phase 5 column volumes (25mL) of buffer A were passed through the system and a secondary flow through sample was collected on ice for later analysis.

To ensure that the secondary wash phase was sufficient it was necessary to confirm that both UV and conductivity sensor data decreased to baseline levels, as this would enable easy identification of potential sigma-1 elutions. In order to elute potential desired protein, it was necessary to use a secondary buffer known as buffer B. This buffers composition was identical to buffer A, (1x HEPES- buffered saline) however it was supplemented with a His-tag competing reagent known as imidazole. This buffer was supplemented with 500mM of imidazole and adjusted

to the desired pH of 7.5. Following the correct preparation of buffer B, a concentration gradient of buffer A and B was set up with a final target concentration of buffer B of 100%. A mixture of both buffers with an increasing concentration of buffer B was passed through the column at a flow rate of 0.5 mL/min until the final target concentration was achieved. Over ice, 4mL elutions were collected until UV and conductivity levels decreased to baseline levels. Lastly, a final wash of ddH₂O was applied to the system and its flow through was collected to ensure all possible elutions of sigma-1 were extracted from the system. All samples after collection from IMAC purification were then stored at 4°C until they were used for screening purposes. This was to ensure the stability of our target protein and to minimise the possibility of protease cleavage during storage. Before going further into other phases of our purification protocol it was necessary to screen these elutions for our desired protein.

2.7 SDS-PAGE Sigma-1 Screening Procedure Post IMAC Purification

In order to do so, all elutions were tested on an SDS-PAGE gel (12% acrylamide). To perform this procedure, samples were prepared prior to gel loading in sterile eppendorf tubes. For this, 20µl of each elution was supplemented with 4µl of a 5X sample loading buffer. The SDS-PAGE gel was then placed into an electrophoresis tank and submerged in a 1X Tris-base running buffer at 8.3 pH. Succeeding this, 10µl of PageRuler™ protein ladder (5 to 250 KDa) was loaded into the first well of the SDS gel followed by 10µl of the preprepared samples in ascending order of IMAC elutions. The SDS-PAGE was then run at 200 volts (V) for 30 to 45 minutes until denatured proteins run down the gel 70 % to 90%. After analyzing the SDS-PAGE of eluted fractions we speculated that our desired protein could potentially be present in elutions T7 to T17 based upon the evidence of bands at 50KDa to 70KDa. At this stage we decided it was possible to then move forward into an additional purification procedure in order to separate these proteins by their size and molecular weight. This procedure is known as size-exclusion chromatography (SEC) and required us to concentrate these elutions into a single sample. Furthermore, samples T7 to T17 were spin concentrated using a 10KDa spin column at 4,000rpm, in a Beckman tabletop centrifuge. For our protocol we

concentrated these samples down to 500µl as the injection loop we would later be using has a total volume of 500µl. In addition, prior to this procedure the concentrated sample was centrifuged at 13,000g using a tabletop centrifuge and syringe filtered using a 0.22µm filter into a clean 1.5mL eppendorf tube.

2.8 Size Exclusion Chromatography (SEC) Procedure for Sigma-1 Isolation

This procedure was performed at 4°C in a controlled temperature environment to prevent protein aggregation and cleavage. In addition, this semi-automated procedure would be aided with the activation of UV detectors set to 280nm (AU) and FLD fluorescence detectors set to an excitation of 495nm and emission of 509nm. This setup would enable us to detect sigma-1 GFP tagged nanodisc complexes as it would enable us to prioritise aliquot fractions that have high resolution signals for protein absorbance at 280nm and GFP's specific detectable wavelengths. The first phase of this procedure was performed by introducing a wash phase of the Superdex™ 200 25mL size-exclusion chromatography column. Just as seen in the IMAC procedure using HisTrap™ nickel affinity column, the Superdex™ 200 25mL column was washed for one column volume at 0.5mL/min in degassed and filtered ddH₂O. Following this wash phase, it was necessary to perform a subsequent equilibration phase, using the sample buffer for a total volume of one column volume. Just as in the IMAC purification, 1x HEPES-buffered saline at pH 7.5 was run through the system at 0.3mL/min to ensure protein stability and solubility during this SEC phase. Upon the passing of buffer to reach a total volume of one column volume, the sigma-1 tagged GFP SMI or SMA nanodisc sample was centrifuged at 13,000g using a Beckman tabletop centrifuge for 10 minutes to remove any aggregates or debris contaminant.

The 500µl SEC injection loop was then washed with 500µl of ddH₂O and subsequently equilibrated using 500µl of the equilibration buffer in preparation for sample injection. The previously concentrated 500µl sample was then injected into the SEC injection loop and the systems flow path was set to flow through the

injection loop. During this column phase a flow rate of 0.5mL/min was implemented, and dispensed fractions were set to a total volume of 500µl per sample on the fraction collector. After one column volume of collection, samples were immediately transferred to storage at 4°C to ensure sample integrity. After analysing the SEC systems generated data for UV and Fluorescence detectors, specific samples were nominated for screening to identify whether these samples contained our desired protein. Following the complete run of the SEC purification procedure all data was analysed in order to determine which fractions were most likely to contain our desired protein. As seen in figure 28, we screened all fractions that met the criteria of both high UV 280nm absorbance readings as well those that have a high signal for GFP fluorescence. In addition, the resolution of the peaks was used as a valuable indicator during this procedure as discrete narrow peaks can indicate that a protein at a specific molecular weight and size is eluting through a low sample volume. Furthermore, if it was evident post SDS-PAGE screening that these fractions have no indications for sigma-1 GFP tagged nanodiscs then all the other likely fractions would be exhumed.

Following SDS-PAGE analysis fractions likely contain sigma-1 GFP tagged nanodiscs were spin concentrated into one sample using a 10KDa spin column at 4,000rpm in a Beckman™ tabletop centrifuge. The concentrated sample was then prepared for TEV cleavage in order to isolate sigma-1 from its GFP counterpart. In order to perform this procedure 1mL stocks of TEV protease expressed in C43 cells were thawed on ice for 15-20 minutes before being dispensed into the concentrated sigma-1 GFP tagged sample. In addition, enough TEV stocks were thawed in order to reach a target ratio to protein of 1:100 (w/w) or 10,000 unit (1mg) and this depended upon the total volume of concentrated sample. Typically for a TEV protease cleavage procedure the sample was concentrated to 10mL-20mL and incubated on ice before dialysis. Following the dialysis procedure previously described, 4L of 1x HEPES-buffered saline at pH 7.5 buffer was prepared prior to TEV incubation. Using a 10KDa SnakeSkin™ dialysis membrane the concentrated sigma-1 sample was inserted along with the required volume of TEV protease stocks. Subsequently after the dialysis membrane insertion, the

cleavage procedure was incubated at 4°C and slowly spun using a magnetic stirrer. After 12-16 hours of incubation the potentially cleaved sigma-1 sample was removed from dialysis and immediately placed on ice for further IMAC purification. As we theoretically had cleaved sigma-1 from its GFP tag using the N-terminus TEV cleavage site ^{5'}CTGGAAGTACAGGTTTTC^{3'} this would also equate to the removal of the His-tag from the sigma-1 complex.

Therefore, the procedure for this IMAC purifications protocol would differ in comparison to the previously used method for primary purification. Using the procedure previously described for using the AKTA™ start affinity nickel purification system a primary wash, equilibration, sample loading, secondary wash and elution phase were all implemented accordingly. However, for this protocol, we expected isolate sigma-1 to elute during the sample loading phase as flow through instead of binding to the 5mL HisTrap™ nickel column. In addition, we also anticipated sigma-1 to elute during the secondary wash phase as there could be potentially some remaining inside the system during sample loading phase. In theory both GFP and the TEV protease were expected to remain bound to the nickel column as both these proteins have been designed to have a N-terminus His-tag. As the SDS-PAGE indicated that the flow through sample could potentially contain cleaved sigma-1, free GFP or TEV protease it was necessary to perform an antibody assay (i.e. ELISA) or a western blot to confirm that the protein seen is cleaved sigma-1.

2.9 Detergent Solubilisation Methodology for Sigma-1 Isolation

All procedures for transformation, induction and large-scale growth of C43 cells were followed to the same protocol and standards as described previously. The point at which the protocol for protein extraction and solubilisation alters post the centrifugation of the large-scale growth phase. In order to solubilise sigma-1 with LMNG and CHS it was important to prepare the extraction buffer prior to sonication, homogenisation and ultracentrifugation. Once the entire 4L of C43 cell growth culture had been centrifuged at 6,000rpm for 25 minutes using a

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Beckman™ centrifuge (T45i rota), the supernatants were discarded, and pellets resuspended in extraction buffer. The extraction buffer consisted of the following reagents; 1% LMNG (w/v), 0.1% CHS (w/v), 1x HEPES-buffered saline. Using this buffer, the pelleted culture was resuspended in 50mL of extraction buffer and supplemented with 500µl of Halt™ protease inhibitor single-use cocktail 100X (EDTA free) per 10mL of cell lysate.

Following pellet suspension, the cell lysate was then prepared for sonication just as previously described when using SMI & SMA nanodiscs for solubilisation. Following sufficient cycles of sonication, the cell lysate was homogenised using a tissue grinder and incubated for two to four hours at 4°C whilst being gently mixed. Furthermore, post incubation the cell lysate underwent ultracentrifugation using a Beckmann™ LE-80K with a T70ti rota at 45,000rpm for 1 hour (4°C). The ultracentrifuged cell lysate was then removed from the centrifuge and the supernatant was immediately extracted on ice. In addition, the pelleted cellular membranes were resuspended in extraction buffer and flash frozen in liquid nitrogen for later analysis if necessary. As this protocol is using detergent for solubilisation it was not deemed as necessary to perform a dialysis procedure of the extracted supernatant. Therefore, it was possible to continue forward onto our primary purification phase of IMAC. This purification procedure was followed in the same accordance as described previously, however both the equilibration buffer and elution buffer differed. As we were using detergent for our solubilisation protocol it was crucial that all purification systems must be equilibrated with detergent also to ensure stability and prevent protein precipitation out of solution. Due to pressure constraints and affinity interactions of HisTrap™ nickel columns it was vital that both the LMNG and CHS buffer concentration decreased 10-fold during this procedure. For our detergent IMAC purification 0.1% LMNG (w/v) and 0.01% CHS (w/v) was supplemented to our previously prepared HEPES-buffered saline. In addition, it was crucial that both the LMNG and CHS had completely dissolved into the buffer as this could have compromised the entire procedure.

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Following completion of the IMAC procedure with the addition of LMNG and CHS, 4mL buffer B elutions were immediately collected and transferred for storage at 4°C. Using the same protocol for SEC, used for C43 expressed sigma-1, both the equilibration buffer and elution buffer were prepared to similar standards. The only varying factor was that these buffers were supplemented with a decreased concentration of LMNG (0.01% w/v) and CHS (0.001% w/v) to maintain a stable pressure throughout the procedure. Upon completion of this secondary purification protocol 0.5mL fractions were immediately stored at 4°C whilst data was analysed for the select screening of the peak elutions using SDS-PAGE. Once likely candidates were selected, samples were then spin concentrated using a 10KDa spin column at 4,000rpm for 30minutes using a Beckmann table-top centrifuge.

Furthermore, upon extraction of the concentrated sample a TEV cleavage procedure was set up immediately at 4°C. Due to the expense of LMNG and CHS reagents it was decided that the TEV cleavage procedure would be performed by directly incubating the protease with the sigma-1 sample at pH 7.5. Although these are not the ideal conditions for a TEV cleavage procedure it was deemed as necessary to ensure the stability of sigma-1 and to reduce the overuse of LMNG and CHS. After 12 to 16 hours of TEV protease incubation the sigma-1 detergent solubilised sample was immediately placed on ice for a second IMAC procedure. Using the same protocol previously used for TEV cleaved sigma-1 nanodiscs, all systems and procedures were set up to the same the standards. As our desired protein was still solubilised with detergent it was deemed as necessary to maintain stability of our protein by maintaining the buffer concentration of LMNG at 0.01% (w/v) and CHS at 0.001% (w/v). Following the procedure all wash, flow through and elution samples were stored at 4°C in preparation for SDS-PAGE, in-gel fluorescence and a western blot procedure.

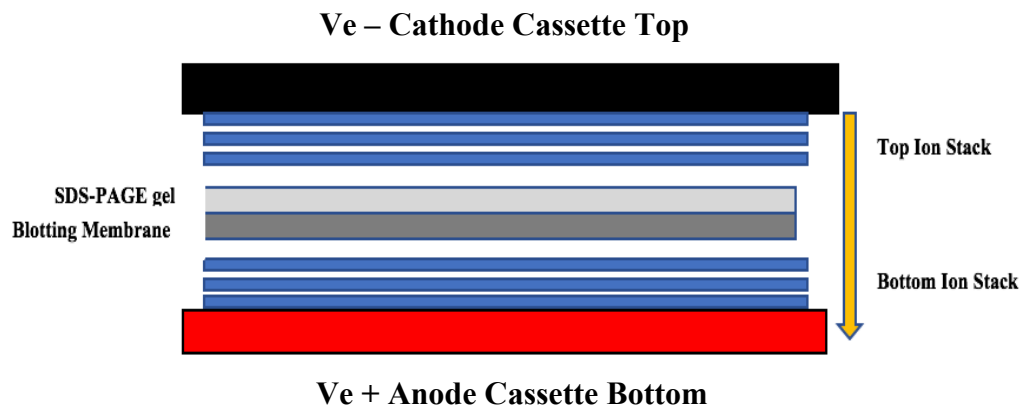
2.10 In-gel fluorescence (IGF) For Green Fluorescent Protein Sample Screening

As an additional screening method for our samples post purification and prior to performing a TEV-cleavage method it was deemed as beneficial to assess whether the protein identified on previous SDS-PAGE gels were in fact free GFP or bound GFP to sigma-1. In order to do so we prepared a unique sample buffer that has been utilised in many studies for the purpose of GFP screening. This buffer also known as IGF-SB (In-gel fluorescence sample buffer) consisted of the following reagents; 50mM Tris-HCl (pH 7.6), 5% glycerol (v/v), 5mM EDTA (pH 8.0) and 0.02% bromophenol blue in stocks of 700 μ l (stored at -20 $^{\circ}$ C). Prior to using the IGF-SB it was necessary to supplement the stocks with 200 μ l of 20% SDS (w/v) and 100 μ l 0.5M DTT. These freshly prepared stocks could be stored at room temperature up to 20 to 30 days or for long term storage at -20 $^{\circ}$ C. Using the IGF-SB, samples were prepared following the same protocol for SDS-PAGE. Furthermore, 20 μ l of concentrated samples were supplemented with 10 μ l of IGF-SB at room temperature and were not heated as this could degrade GFP fluorescence. Following this, all necessary samples were loaded onto a gel along with 5 μ l of Precision Plus Protein Dual Colour Ladder and run at 200V for 30 to 45 minutes. Post SDS-PAGE running, the gel was immediately transferred to a SynGeneTM (CHEMI-XRQ) CCD camera for blue light (EPI source) ultraviolet imaging at 460nm and 515nm cut-off

2.11 Western Blotting for Isolated Sigma-1 Post TEV Cleavage Protocol

Once we had screened our TEV cleaved elutions post IMAC it was then deemed necessary to confirm whether these were isolated sigma-1 samples. This protocol can be divided into five distinct phases, which include; protein electrophoresis, membrane transfer, membrane blocking, antibody incubation and detection. In order to carry out this screening procedures it was necessary to prepare the following buffers; transfer buffer, blocking buffer, Tris-buffered saline (TBS), and Tris-buffered saline-tween (TBST). The primary step of this protocol is to loaded samples following the same method for SDS-PAGE. During this stage proteins will separate according to their molecular weight and do not require post staining for

this procedure. For this protocol 5µl Precision Plus Protein™ Dual Colour pre-stained ladder was used to ensure easy confirmation of successful membrane transfer. Upon running the gel at 200V for 45 minutes, the gel was prepared for polyvinylidene difluoride (PVDF: Bio-Rad Immune-Blot™) membrane transfer using a semi-dry method. In order to carry out this transfer procedure the PVDF membrane was activated by suspension in 1x transfer buffer,



*Figure 11: Diagram showing how western blot was prepared for SDS-PAGE gel transfer to PVDF membrane. **Western Blot Transfer Setup.***

In addition, filter paper stacks (Bio-Rad: Absorbent filter paper) were then equilibrated in 1x Tris-buffered saline. Once both the PVDF membrane and filter stacks had been treated, it was then possible to prepare the cassette for blotting membrane transfer. The transfer cassette was prepared in the following order to ensure that the current was travelling in the correct direction; negatively charged cathode, top filter paper stack, SDS-PAGE gel, blotting membrane, bottom filter paper stack and positively charge anode. Once the final transfer stack had been successfully prepared it was then transferred into Trans-Blot Turbo Transfer system. Upon insertion to the device, the transfer procedure was run for 7 minutes at 20V until complete transfer was achieved. Once the transfer procedure was complete, the PVDF membrane was checked to ensure that the SDS-PAGE gel had been successfully transferred. If the transfer was confirmed, the membrane was then blocked using 10mL of blocking buffer. This buffer consisted of 5% skimmed milk powder (w/v) in 1x Tris-buffered saline-tween. This blocking procedure took place at room temperature using a digital benchtop shaker at

80rpm for 1 hour. Whilst this blocking procedure was taking place, it was necessary to prepare the primary antibody over ice as thawing was timely. For our primary antibody incubation, we ordered the Sigma-1 Receptor Polyclonal Antibody (*product no: 42-3300*) from ThermoFisher™ due to its high specificity for the sigma-1. This unconjugated IgG antibody was derived from rabbit and required incubation with a secondary anti-rabbit IgG antibody for this blotting procedure. In order to prepare the correct working concentration of this antibody (1:1000) a dilution in blocking buffer was used to maintain a final incubation concentration of 2µg/mL.

Once the primary antibody was successfully prepared and the membrane blocking phase was complete, it was the necessary to pour off the blocking buffer from the membrane. Upon removal of the blocking buffer, the membrane was then supplemented with 10mL of primary antibody in blocking buffer and left to incubate at room temperature for 1 hour. Once primary antibody incubation was complete, the membrane was then washed using freshly prepared TBST in 5 individual wash cycles for 5 minutes per cycle. Following this, it was then necessary to prepare the secondary antibody in blocking buffer over ice. For our secondary antibody, we used the Goat Anti-rabbit IgG HRP-Linked Antibody (*product no:65-6120*) supplied from ThermoFisher. This secondary antibody (stock concentration 1mg/mL) was 1/10,000 diluted into blocking buffer and applied to the blotting membrane for 1 hour at room temperature on a digital benchtop shaker at 80rpm. Once the blotting membrane had been incubated for the specified time, it was then necessary to wash the blotting membrane again using 4 TBST wash cycles for 5 minutes and 2 TBS wash cycles at 5 minutes per cycle. Upon completion of the wash cycle it was then necessary to prepare the membrane for imaging using enhanced chemiluminescence reagent (ECL). As our secondary rabbit IgG antibody was linked to the enzyme horseradish peroxidase (HRP) it was possible to detect its activity using photographic imaging. For this procedure we used Peirce™ ECL western blot substrate reagent (*product no. 32106*) due to its compatibility with PDVF blotting membranes.

Once the membrane had been sufficiently washed using both TBST and TBS, 1mL of the ECL reagent was directly applied for imaging. The ECL reagent was prepared in a 1:1 ratio, by mixing peroxide solution with luminol solution in a clean Eppendorf and then wrapped in aluminium foil to ensure chemical stability. The light emission peak for ECL reagent ranges between 5 to 20 minutes so it was crucial that we applied this at the moment of imaging. Once the membrane was exposed to the ECL for 3 minutes in a CCD camera, the blot was imaged at a maximum wavelength of 425nm using a preinstalled method for this procedure. It is considered that most suitable conditions for membrane blocking and antibody incubation, is at 4°C overnight. Typically, when western blotting is performed to this standard there is a stronger detection signal with specificity and sensitivity. Unfortunately, we only blocked our PDVF membrane and incubated our antibodies for 1 hour at room temperature. This may have had an impact on the results obtained for our western blot procedures in both C43 and INVSc1.

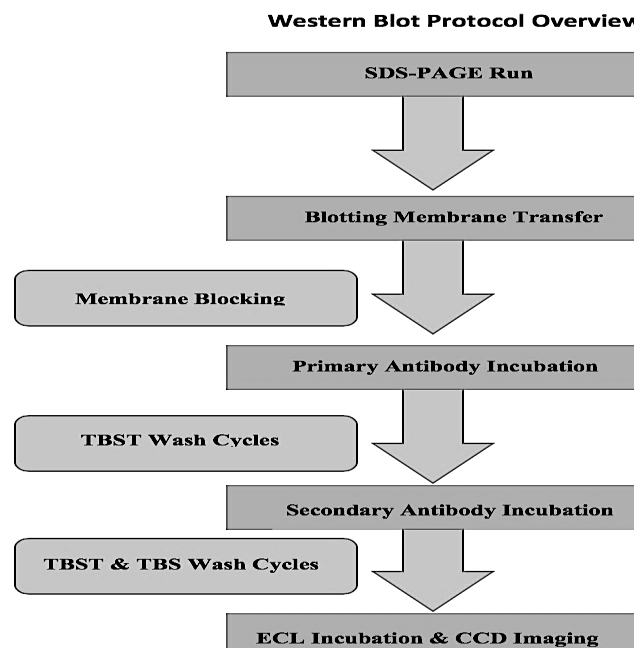


Figure 12: Flow diagram representing key aspects of the western blot protocol implemented for determining sigma-1 isolation in both INVSc1 and C43 expression systems. **Overview of the Implemented Western Blot Protocol.**

2.12 INVSc1 as an Expression System for Sigma-1

In order to create a suitable construct for the transformation of INVSc1 competent cells, a multistep protocol was implemented. This included the following; primer design, fragment/vector PCR, product screening, restriction & ligation,

transformation and sequence screening. The plasmid of choice for the insertion of our sigma-1 fragment was the *LEU2* pop in vector, which has been introduced into both C43 and INVSc1 strains for protein expression in many publications. In order to create this final construct, it was first important to amplify both the sigma-1 fragment and *LEU2* plasmid using specifically designed primers in two PCR's. In principle these reactions would result in both the fragment and vector possessing complementary amino acid overhangs by which they can be incorporated together via pfu polymerase ligation. Using our previously constructed sigma-1 modified pCold-1 vector used for C43 expression it was possible to amplify our protein sequence from this template using two designed primers.

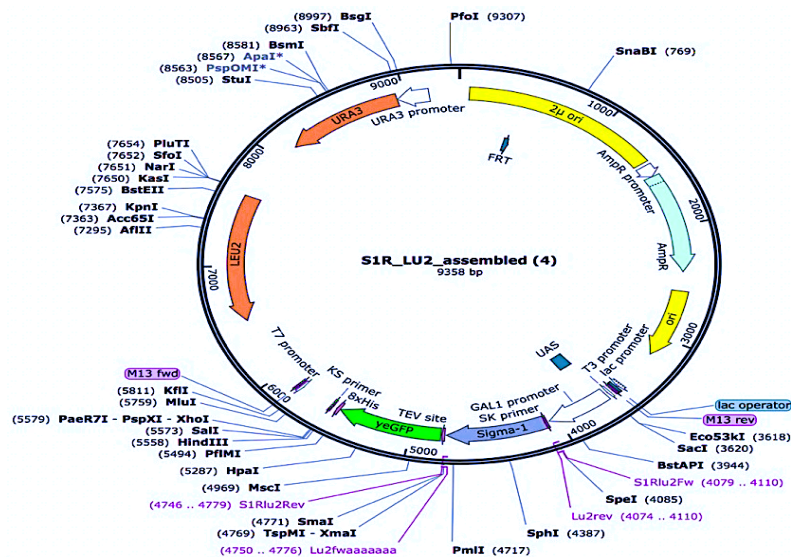


Figure: 13: Image taken of the final S1R-LEU2 construct used for the transformation of *saccharomyces cerevisiae* for sigma-1 expression. **Final Sigma-1 LEU2 Construct for INVSc Transformation.**

In addition to the amplification of the fragment DNA it was also necessary to amplify our vector with its fragment complementary overhangs. These primers were designed to be complementary either upstream and downstream of a SmaI restriction site so that both the fragment and vector can be easily incorporated into a linearized construct. In order to carry out this procedure a standard PCR protocol was followed, however specific phases of the reaction were altered by temperature and time.

2.13 Sample Preparation for Polymerase Chain Reaction (PCR)

For both amplification procedures three reactants were prepared using pfu polymerase to catalyse the reaction. In principle sample 1 and 2 were designed to be our stocks of product whereas sample 3 would be a control without the addition of pfu polymerase. This would enable us to compare sample 3 to the product samples by DNA testing methods such as nanodrop™ and DNA electrophoresis. Once the final volumes of each reagent were supplemented to each sample on ice, they were then transferred to the PCR running device. In order to perform a PCR protocol, it was necessary to determine the overhang T_m 's of both the vector and the fragment primers. This is a crucial step as the annealing phase of PCR is temperature sensitive and may have resulted in unbinding of primers to complementary nucleotides.

Table: (2) Sigma-1 Fragment Primers for Annealing T_m 's in LEU2 Construct.

Primer Name	Sequence	T_m complete primer	T_m part	Annealing T_m
SIGMA1 PLU2lin FOR	TCTAGAACTAGTGGATCATGCAGTGGGCAGTT	65°C	54°C	55°C
SIGMA1 PLU2lin REV	TTTTCCCCGGGGGGCGGGTCCTGACCAAATA AG	72°C	55°C	

Table: (3) LEU2 Designed Plasmid Primer Annealing T_m 's.

Primer Name	Sequence	T_m complete primer	T_m part	Annealing T_m
LEU2FOR	TTTGGTCAGGACCCGCCCCCGGGGA	73°C	55°C	55°C
LEU2REV	AACTGCCCACTGCATGATCCACTAGTTCTAGAA TCCG	67°C	54°C	

Table (4): Polymerase Chain Reaction (PCR) Guidelines for Sample Preparation

Reagent	Sample 1	Sample 2	Sample 3 (Control)
10 x buffer pfu polymerase	5ul	5ul	5ul
DNTP's	1ul	1ul	1ul
Upstream primer	0.5ul	0.5ul	0.5ul
Reverse primer	0.5ul	0.5ul	0.5ul
Vector or Fragment Sample	1ul	1ul	1ul (1/10)
Polymerase	1ul	1ul	0
Milli-Q water	41ul	41ul	42

Both the sigma-1 fragment and *LEU2* vector primers were ordered via Eurofins Scientific and upon obtainment they were stored at -20°C until required for use. Furthermore, once the appropriate melting temperatures of the primers were determined it was then possible to prepare all three reactants including a single control sample. Two of these reactants were to be our stocks of the amplified vector or fragment and the third would be a control sample not containing the enzyme *pfu* polymerase from Promega Express (product no.M7741) with a 10-fold dilution of DNA in Milli-Q water. Following sample preparation over ice, they were then transferred to the PCR reactor system and all parameters were set accordingly. For a standard PCR reaction there are distinct phases; 1) initial denaturation, 2) denaturation, 3) annealing, 4) extension and 5) final extension. The PCR set-up for the plasmid differed to the fragment during at the final extension phase due to the total base pair the size of the vector at 8689bp. Because of this, after optimisation we used 2min/kilobase (kb) for the final extension when amplifying our plasmid and fragment with *pfu* DNA polymerase. Although our sigma-1 fragment size was a total of 669bp, we decided after optimisation to increase the final extension time to a total of 5 minutes to ensure correct amplification.

Table (5): Polymerase Chain Reaction (PCR) Modifiable Protocol for Fragment DNA And Plasmid DNA

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	1 minute	1 Cycle
Denaturation	95°C	45 seconds	1 Cycle
Annealing	55°C	30 seconds	30 cycles
Extension	72°C	2 - 4 minutes	1 Cycle
Final Extension	72°C	5 minutes (Fragment) / 10 minutes (Plasmid)	1 Cycles
Soak (Finish)	4°C	Indefinite	1 Cycles

After a complete run of the PCR procedure both the plasmid and fragment double stranded DNA samples were measured using a nanodrop™ dual device. Using 2ul per sample, all three reactants including the control dsDNA concentrations were

measured at 260nm. For this particular PCR we measured the average fragment concentration at 121.2ng/ μ l, average plasmid concentration at 94.2ng/ μ l and the average control sample at 16ng/ μ l. As the control samples concentration was much less than that of the DNA stock samples it was considered that the PCR procedure had been a success. These samples after analysis were then stored at 4°C until the transformation procedure commenced. Before the transformation procedure took place, it was crucial that the *saccharomyces cerevisiae* cells were treated to develop competency. For this particular protocol it was deemed as efficient to produce chemical competent cells with the addition of lithium acetate (LiAc). This protocol was influenced by a publication in 2008 by *Newstead et al* called "*GFP-based optimisation scheme for the overexpression and purification of eukaryotic membrane proteins in...*". Although the protocol we used for protein in expression in our yeast cells is very similar to *Newstead et al*, there are instances of modified procedure's which were deemed necessary for our desired protein.

2.14 Preparation for Yeast Cell Competency

Prior to the cell competency procedure, a Yeast Peptone Dextrose (YPD) agar plate was inoculated using a sterile loop and incubated overnight at 30°C to create multiple colonies. After 12 to 16 hours of incubation the plate was removed from the incubator and placed on a sterile bench, ready for the next procedure. Under aseptic conditions a sterile loop was used to inoculate YPD medium with the INSVc1 plated cells into a 50mL falcon tube. The starter culture was then transferred into an orbital shaker set to 30°C at 280 rpm for overnight incubation. Following this, the culture was then diluted into 50mL of YPD for an OD₆₀₀ of 0.1 prior to incubation. After assessing the culture, it was then incubated at 30°C in an orbital shaker, set to 280rpm until an OD₆₀₀ of 0.5 was achieved. The culture was then centrifuged at 13,000g for 10 minutes using a Beckmann™ tabletop centrifuge at 4°C. The supernatant was discarded, and the pelleted cells were resuspended in ddH₂O over ice. Following this the resuspended cells were then further centrifuged at 13,000 for 5 minutes using the same centrifuge at 4°C. The supernatant was also discarded; however, the cellular pellet was resuspended in 100mM LiAc over ice. Furthermore, the resuspended cells were further

centrifuged at 13,000g for 30 seconds before the supernatant was discarded and the pellet was resuspended in 500µl of 100 mM LiAc. This cellular competency protocol took a total two days to complete, so it was important that all reagents were prepared prior to performing the next transformation procedure.

In order to prepare the required medium and drop out plates for a selective INSVc1 culture growth, we used a multistep process to do so. In order to prepare the selective drop out medium for -uracil (-URA) and -leucine (-LEU) plates, we used the following reagents; synthetic yeast base without amino acids, dextrose, Bacto-agar and either the -uracil or -leucine drop out medium, which contained all the necessary amino acids for normal yeast cellular growth except those two amino acids. In addition to the preparation of selectivity plates it was also necessary to prepare, the growth medium for the INSVc1 cells post colony selection for the starter culture and larger scale growth. In order to do so we used the same protocol for preparing the selectivity plates, however without the addition of Bacto-agar in the media.

2.15 Transformation Protocol for INSVc1 Expression System

The transformation procedure was more complex and required a high level of time management to perform correctly in comparison to our previous heat shock system in *C43 expression*. Prior to performing this protocol, it was necessary to ensure that the *LEU2* plasmid had been digested by the *SmaI* restriction enzyme to allow insertion of the sigma-1 fragment DNA into the specific site. For this protocol we used the FastDigest *SmaI* restriction enzyme that recognises the 5'CCC-GGG3' sequence and digests with a high level of activity. In addition, this protocol did not require post buffer exchange or further DNA clean up steps and deactivation of the enzyme was possible in a fast procedure. In order to perform this procedure, the following reaction components were added to a 1.5mL Eppendorf tube; Milli-Q water, 1X FastDigest buffer, our plasmid DNA (102ng/µl) and the FastDigest restriction enzyme (total volume of 20µl per reaction). Post preparation, the reactant was gently mixed at room temperature before being incubated in a water thermostat set to 37°C for 5 minutes. After the reaction had

taken place, the *SmaI* was inactivated at 65°C for a further 5 minutes in a different water thermostat. Post to the inactivation protocol the reactant was then stored at 4°C until required for the transformation procedure.

To start the transformation procedure 50% (w/v) PEG 3350 was supplemented to a clean 1.5mL Eppendorf tube on ice. The previously prepared competent INSVc1 cells were also placed on ice and were then transferred to the 50% PEG 3350. The transformant was vortexed for 5s to ensure complete mixture and then placed back on ice. Following this the mixture was then supplemented with 2mg ml⁻¹ salmon sperm (single-stranded carrier DNA) and was then further vortexed for 5 seconds. This transformant was then ready to be supplemented with the addition of a DNA mixture consisting of both *SmaI LEU2* digested plasmid and the sigma-1 PCR fragment. Firstly, Milli-Q water was dispensed into a clean Eppendorf with the addition of 3µl of the digested plasmid (minimum 25ng/µl⁻¹) and 5µl of the sigma-1 PCR fragment (minimum 150ng/µl⁻¹). This 50µl DNA mixture was then added to the transformant and vortexed for a further 5 seconds. The transformant mixture was then immediately transferred to an orbital shaking incubator set to 30°C at 180rpm for 30 minutes. Post Incubation the transformant underwent a heat shock phase in a water thermostat set to 42°C for 25 minutes. Once the heat shock procedure was complete it was then possible to prepare for plating the transformant on a -URA selective agar plate. Furthermore, the heat shocked transformant was then centrifuged at 8,000g for 15 seconds at room temperature. The supernatant was discarded, and the cellular pellet was resuspended in sterile Milli-Q water. This cellular suspension was then plated onto a -URA agar plate under aseptic conditions and left to incubate at 30°C for 2-3 days. Once the -URA selectivity plate had been incubated for the required time, it was then necessary to screen for isolated colonies for further selection. Once isolated colonies had been confirmed a single colony was used to streak a -LEU selectivity plate to aid in the double selection process. This plate was also incubated at 30°C for 2 to 3 days until there were visible colonies present. After -LEU plate incubation it was then stored at 4°C until preparations for growth were achieved

2.16 Sigma-1 Growth Protocol in Transformed INSVc1 Cells

The previously cultured -LEU plate with isolated colonies was used to inoculate the -URA medium supplemented with 2% dextrose (w/v) in a 50mL falcon tube. As yeasts metabolic pathway involves fermentation it was crucial to ensure that the falcon tube cap was loosely sealed to reduce culture toxicity. This starter culture was then transferred to an orbital shaking incubator set to 30°C at 280rpm for overnight incubation. After 12 to 16 hours of growth, the culture was further diluted to an OD₆₀₀ of 0.1 using 10mL of -URA medium supplemented with 0.1% dextrose (w/v). At this stage it was important to decrease glucose concentrations in the growth medium as higher concentrations can suppress the *GAL1* promoter used for induction. Following the dilution stage, the cultures were then transferred to an orbital shaking incubator set to 30°C at 280rpm until an OD₆₀₀ of 0.6 was achieved. Typically, achieving this OD₆₀₀ level would take around 6 to 8 hours as the approximate doubling time for yeast cells is 90 to 120 minutes. Once this OD₆₀₀ level was reached it was then crucial to perform an induction procedure with the addition of galactose. For our induction protocol we prepared a 20% galactose (w/v) stock in -URA medium so that the final culture concentration would equate to 2%. After approximately 22 hours post induction, these cultures were centrifuged at 4,000rpm for 20 minutes using a Beckmann™ tabletop centrifuge. Typically for this procedure we would size up this protocol to gain 500mL of INSVc1 culture per growth in 25 individual 50mL sterile falcon tubes. This would enable us extract and screen for our desired protein even if its overexpression levels are minimum. Post centrifugation, the supernatants of all the cultures were discarded and the INSVc1 cellular pellet was resuspended in the appropriate lysis buffer. Just as in the C43 expression system protocol, the procedure for solubilisation alternated depending upon whether it was a detergent based method or a native extraction using nanodiscs. When extracting sigma-1 GFP tagged complexes with detergent, all pellets were resuspended using 30 to 60mL of 1% LMNG (w/v), 0.1 CHS (w/v) in 1x HEPES-buffered saline.

Just as in the for the C43 expression, this cell lysate would then be supplemented with the appropriate volume of Halt™ protease inhibitor 100X (EDTA free) single-

use cocktail and proceed to sonication. In contract, if the sigma-1 GFP tagged complexes were to be extracted using SMI or SMA nanodisc then the sonicated cell lysate in 1x HEPES-buffered saline, would be supplemented to a final concentration of 2% (w/v) with a pre-prepared nanodiscs reagent. Furthermore, these nanodisc samples would also undergo a dialysis procedure to remove any free unbound nanodiscs. Post extraction the sigma-1 solubilised samples would undergo a primary (IMAC) and secondary (SEC) purification methods along with any necessary screening procedures such as SDS-PAGE, In-gel fluorescence and GFP fluorescence measurements via a plate reader. Once samples had been selected through screening methods as viable then they would undergo a TEV-cleavage procedure to isolate sigma-1 from its counterpart GFP tag. Furthermore, following procedure the sample would undergo a western blot to confirm that we have isolated our desired protein.

2.17 Transmission Electron Microscopy (TEM) Screening Methodology

Following purification of the sigma-1 2% SMA Imide 1:1 complex using size-exclusion chromatography, it was deemed appropriate to screen these samples for presence of these nanodisc complexes. An effective method for this assessment is the use of electron microscopy, which has the ability to visualize particles at a resolution of 0.2 nm (up to 500,000x magnification). After observing UV detector peaks (AU 280nm) generated on the SEC run of the C43 sigma-1 SMA imide (1:1) samples we carried out a negative stain TEM on five fractions that we suspected to be the most likely to contain sigma-1 GFP tagged nanodisc complexes. For this procedure copper grids were set up for negative staining using the carbon floating method. Samples to be tested through this method were not further diluted as we wanted to identify a high concentration of nanodiscs throughout the image. Firstly, samples were dispensed onto carbon-coated mica so that they could be sufficiently absorbed for imaging. Once absorbed, the carbon-coated mica was transferred to be stained with a single drop of 2% uranyl acetate to cause detachment from the carbon grid. Furthermore, a secondary copper grid was placed on top the unmounted carbon grid and the negative stained carbon grids were left to dry before imaging. Once dried, the negative

stained carbon grids were loaded into a FEI Tecnai T12 transmission electron microscope and imaged at 30,000x magnification²². The imaging procedure consisted of scanning across the scanning across the carbon coated grid at a resolution of 20 to 100nm for identification of these nanodisc formations. During the analysis of the samples it was conclude that there may have also been potential contaminants within these samples due to the identification of many unexpected artefacts within the samples. Furthermore, this method proved as highly beneficial for the detection of nanodisc formations within our samples.

2.18 Nuclear Magnetic Resonance (NMR) Sample Preparation

For our NMR studies we prepared samples to a total volume of 550µl with varying experimental factors. All samples were prepared with varying volumes of sample buffer and ddH₂O to achieve the required total volume of 550µl. The NMR experiments included the following samples to be tested on different spectra;

- **Testing sample 1:** 50µM isolated sigma-1 (without drug), sample buffer; 1x HEPES-buffered saline supplemented with 0.1% LMNG and 0.01% CHS and ddH₂O (Total volume of 550µl)
- **Testing sample 2:** 50µM isolated sigma-1, 15.5mM fluoxetine hydrochloride, sample buffer; 1x HEPES-buffered saline supplemented with 0.2% LMNG and 0.01% CHS and ddH₂O (Total volume of 550µl)
- **Drug with detergent sample (no Sigma-1):** 15mM fluoxetine hydrochloride and sample buffer; 1x HEPES-buffered saline supplemented with 0.2% LMNG and 0.01% CHS and ddH₂O (Total volume of 550µl)
- **Drug without detergent sample (no sigma-1):** 15mM fluoxetine hydrochloride and sample buffer; 1x HEPES-buffered saline (Total volume of 550µl)

Once the 550µl samples had been prepared accordingly, they were then dispensed into a green top 7" NMR sample tube carefully to avoid bubble formation. The prepared samples were then individually inserted into a 600 MHz five channel Bruker Avance III spectrometer and the specific experimental system protocol was loaded onto the automated console.

2.19 Study Flow Chart

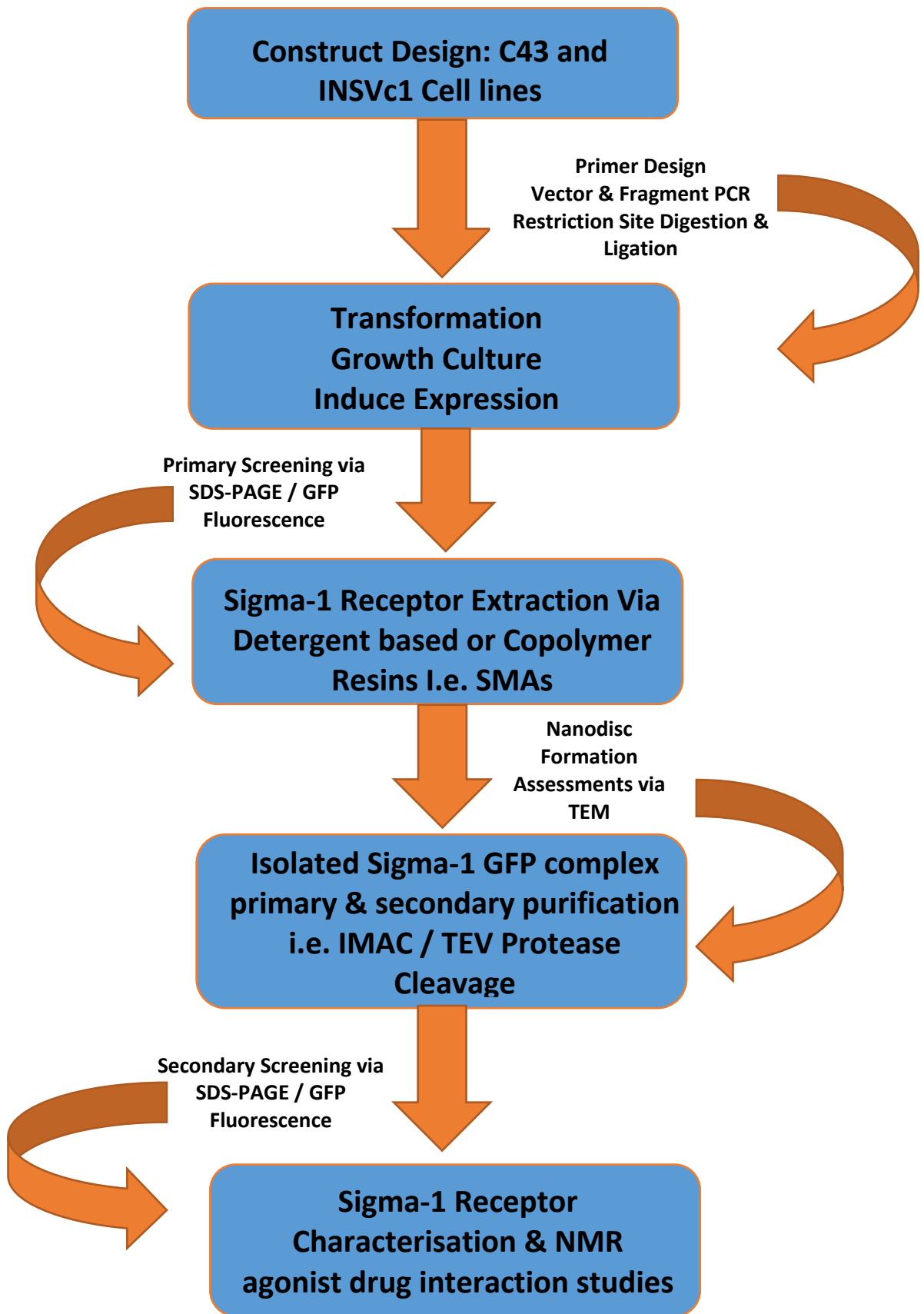


Figure 14: Flow Diagram of basic project phases from construct design to sigma-1 receptor characterisation. Complete Research Project Overview.

3: Results and Discussion

C43 Expression System Results & Discussion

3.1 C43 Cell Growth Data

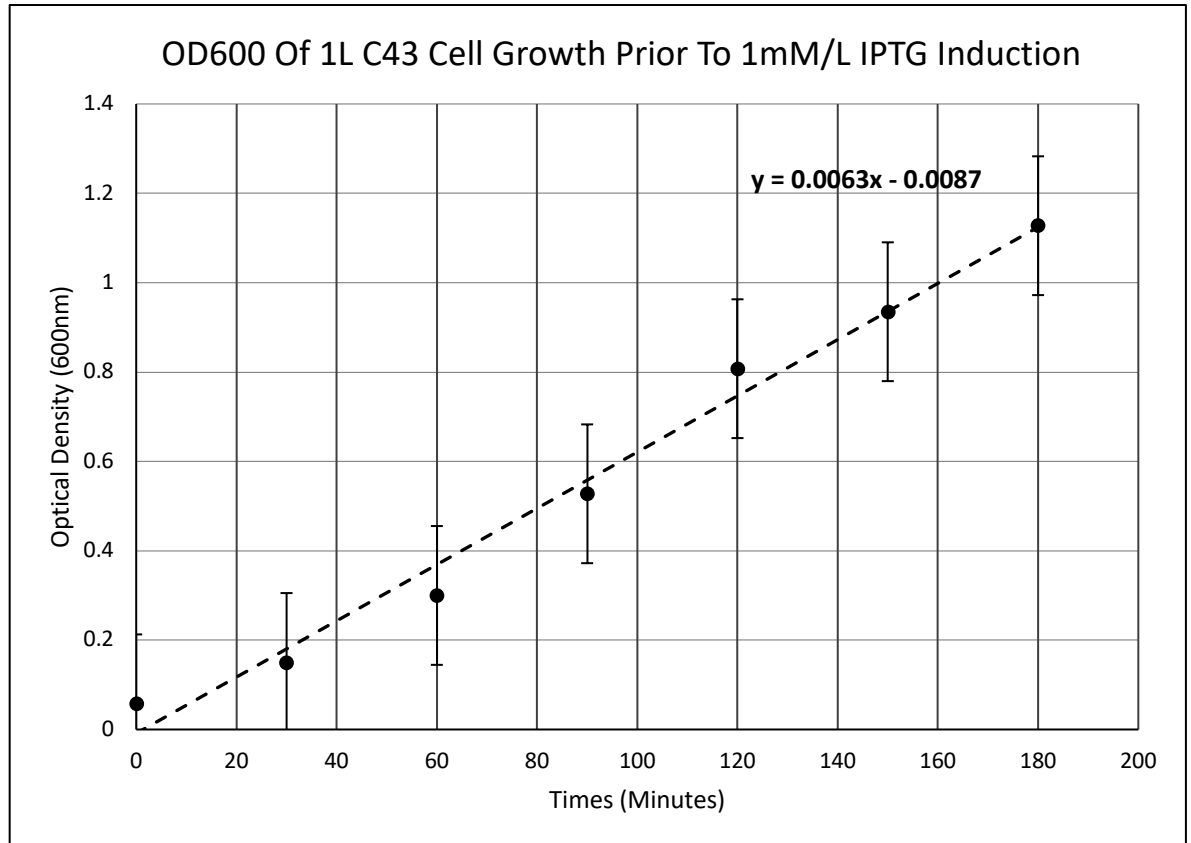


Figure 15: Average Optical Density (OD600) Data of Sigma-1 C43 Cellular Growth in 1L Terrific Broth Medium Prior Induction. **C43 Cell Growth Graph prior to IPTG Induction.**

Prior to inducing our C43 cells with 1mM/L IPTG it was necessary to measure the OD₆₀₀ in order to estimate the cellular concentration in the TB medium. Furthermore, at this stage it was crucial that we reach an OD₆₀₀ that surpassed lag phase of growth and was in exponential growth phase in order to maximise cellular density and viability. The data for figure 15, is comprised of the average OD₆₀₀ measurements of 4L C43 TB growth flasks and the data clearly demonstrates healthy binary fission of the bacteria.

Table (7): OD₆₀₀ Data Used to Calculate C43 Cellular Density

Optical Density (OD₆₀₀)	C43 Cell Density (Cells/mL)
0.057	4.6 x 10⁷
0.15	1.2 x 10⁸
0.3	2.4 x 10⁸
0.52	4.16 x 10⁸
0.8	6.4 x 10⁸
0.93	7.44 x 10⁸
1.12	8.96 x 10⁸

Using the principle that an OD₆₀₀ of 1.0 is equal to 8 x 10⁸ cells/mL it was possible to calculate each specific OD₆₀₀ corresponding cellular density. Furthermore, it is clear to see in the early stages of growth between timepoint 0 minutes to 120 minutes the cells appear to almost double then the rate of exponential growth decreases at 120 minutes to 180 minutes. This could indicate that these cells were in fact entering stationary phase, meaning that all available growth medium nutrients began to become depleted for further growth. At 180 minutes we achieved an OD₆₀₀ of 1.12 which equates to a cellular density of 8.96 x 10⁸. Ideally, it may have been beneficial and more suitable to induce these cells at a lower OD₆₀₀ as the cell viability may have decreased with increase in cell density. This could have potentially had an impact on our recombinant protein expression in this system, resulting in a slightly decrease sigma-1 expression in total.

3.2 IMAC: AKTA Purification for C43 Growth

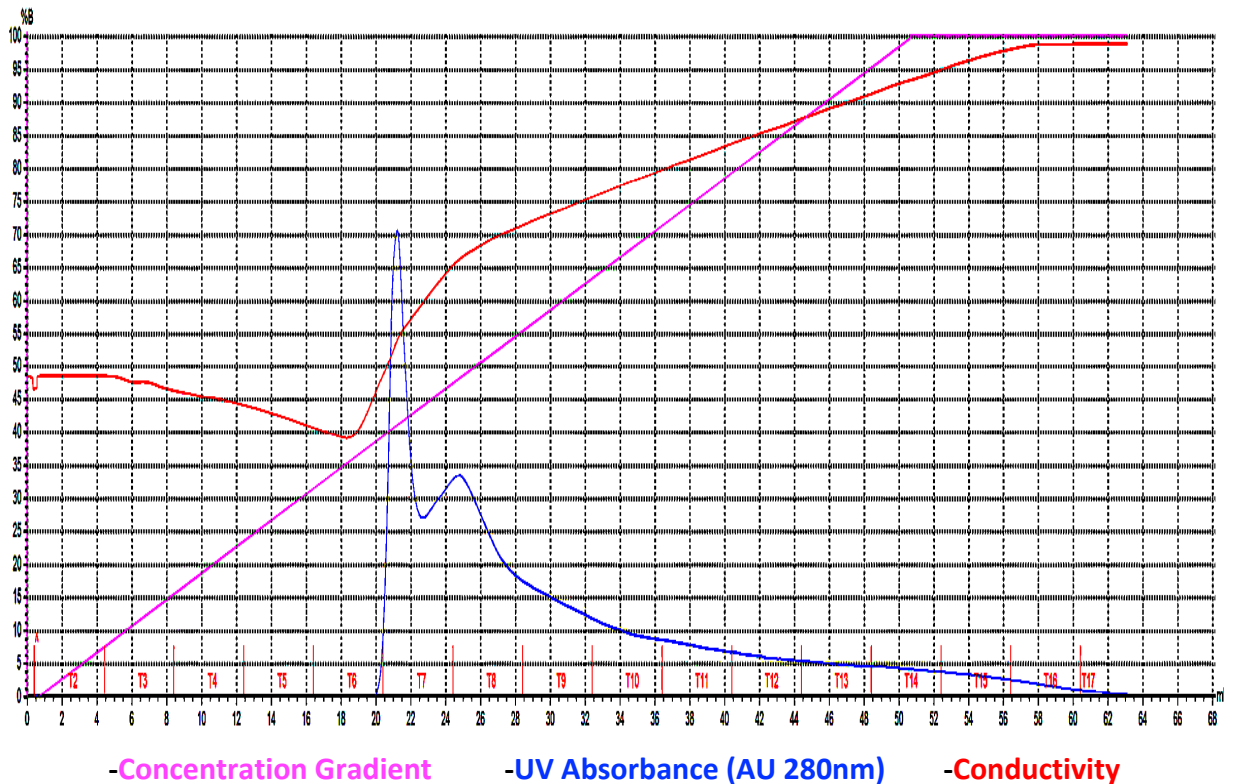


Figure 16: demonstrates graphical representation of the IMAC purification performed on SMI solubilised sigma-1 tagged GFP complexes. At 35% to 100% buffer B concentration we see a sharp increase in UV (AU 280nm) detection followed by an increase in conductivity. Fractions in tubes 6-17 were later screened by SDS-PAGE to determine the likelihood of sigma-1 GFP tagged complexes isolation. **Graph Showing IMAC Run on Solubilised Sigma-1 using SMA Imide 1000I.**

The IMAC AKTA start purification system was deployed as part of our primary purification system for all our expression vectors. In addition, the procedure for running this method remained the same throughout all solubilisation techniques whether that be via nanodiscs or detergent based methods. This procedure was divided into 6 distinct phases; 1) primary column wash, 2) column equilibration, 3) sample run, 4) secondary column wash, 5) elution phase, and 6) sample collection. During all IMAC phases, the key tool for analysis was that of an ultraviolet (UV) detector set to a wavelength of 280nm. Following the sample run procedure, both the UV and conductivity levels sharply increased as a high concentration of proteins passed through the system to the flow through collector.

Once the sample had entirely been passed through the system, and the secondary wash procedure had commenced we saw a steady decrease in the UV (280nm) reading along with conductivity. This was an indication that all unspecific bound proteins had been removed from the system and it was possible to elute our desired protein which should theoretically bound to the 5mL His-Trap™ affinity nickel column. Using a concentration of 500mM imidazole (0% to 100%) any bound proteins to the nickel column were eluted over 17 fractions of 4mL. In figure 16, at 40%-45% imidazole concentration and 24mL to 32mL we see the two peak UV elution of protein in fraction 7 and 8 (T7 & T8). After these peak elutions there is a steady decrease in UV detection until T17 indicating our desired protein may be eluted in high concentration in both fractions 7 & fraction 8, with decreasing concentration in all elutions up to fraction 17.

Although these results provide a strong indication for the presence of sigma-1 GFP tagged nanodisc complexes there are a few factors to take into consideration. Imidazole also has an ultraviolet absorbance at 280nm which could equate to an increase in total UV absorbance throughout the procedure. In addition, the exact concentration gradient readings may be inaccurate to difficulties in equilibrating both pump A and pump B before the elution phase. In order to assess these fractions for our desired protein it was then necessary to screen via SDS-PAGE to ensure eluted proteins were of expected molecular weight. As we solubilised our membrane protein using both nanodiscs and detergents in separate procedures we also obtained AKTA start purification graphs for SMA 2:1 and LMNG. The results of these graphs followed a similar pattern when comparing that to the SMA imide sigma-1 procedure as we detected UV 280nm peak elutions between 40% to 60% imidazole concentration. Unfortunately, due to technical errors in the AKTA purification procedures we were not able to obtain graphs for the IMAC purification of the SMA 2:1 or the 0.1% LMNG sigma-1 samples. However, all analysis at the time of purification matched the same trends as seen in the AKTA purification of SMA imide 1:1 sigma-1 samples.

3.3 IMAC Purification Elution Samples Run On 12% SDS-PAGE Gels

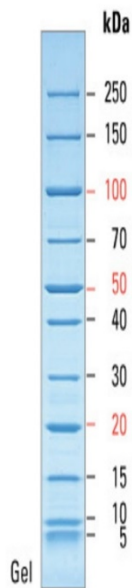


Figure 18: 5-250 kDa PageRuler™ protein ladder²³.
Page Ruler Protein Ladder Reference

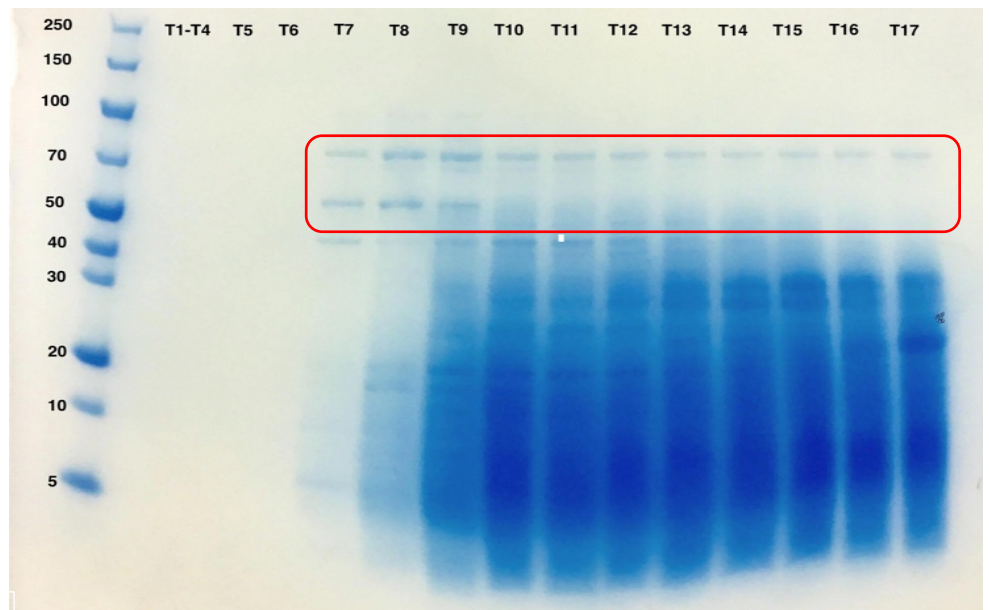


Figure 17: Image taken of SDS-PAGE gel screening for C43 expressed sigma-1-GFP tagged SMA imide nanodisc complexes. Lane 1 contains PageRuler™ protein ladder (5 to 250KDa) and lanes 2 to 15 contain elution samples from previous IMAC purification procedure. Lane 1 contains a spin concentrated sample obtained from elutions 1 to 4 using a centrifuge at 4,000rpm for 30 minutes. In addition, circled is the potential sigma-1 GFP tagged SMI nanodisc complexes. **SDS-PAGE Result Post IMAC Run of Solubilised Sigma-1 Complexes using SMA Imide 1000I.**

After obtaining elutions for the SMA Imide 1:1 AKTA start purification all elutions were run on an SDS-PAGE to screen for sigma-1 GFP tagged SMA imide nanodisc complexes. In figure 17, elutions T1 to T4 were spin concentrated using a 10KDa spin column however we still see no indication for the presence of proteins in these samples. It is only until we analyse elutions T7 to T17 we can see a significant concentration of various proteins in the SDS-PAGE. In samples T7 to T9 we see distinct bands at around 50KDa which proved to be a promising indication for the isolation of sigma-1 GFP tagged SMA imide nanodisc complexes. Furthermore, elutions T7 to T17 we also see distinct bands at around 70KDa which was not to be expected. However, a rational explanation for this could be the fact that previous publications on the analysis of nanodiscs have demonstrated varying disc

formations at increasing molecular weights. In addition, as nanodiscs have the solubilise disc formatio LAD TL6 TL7 TL8 TL9 TL10 T11 T12 T13 T14 FT WH lipids surrounding our protein also vary in size and molecular weight. Furthermore, once we had an indication for the presence of sigma-1 nanodisc complexes these samples were spin concentrated for secondary purification using size-exclusion chromatography (SEC).

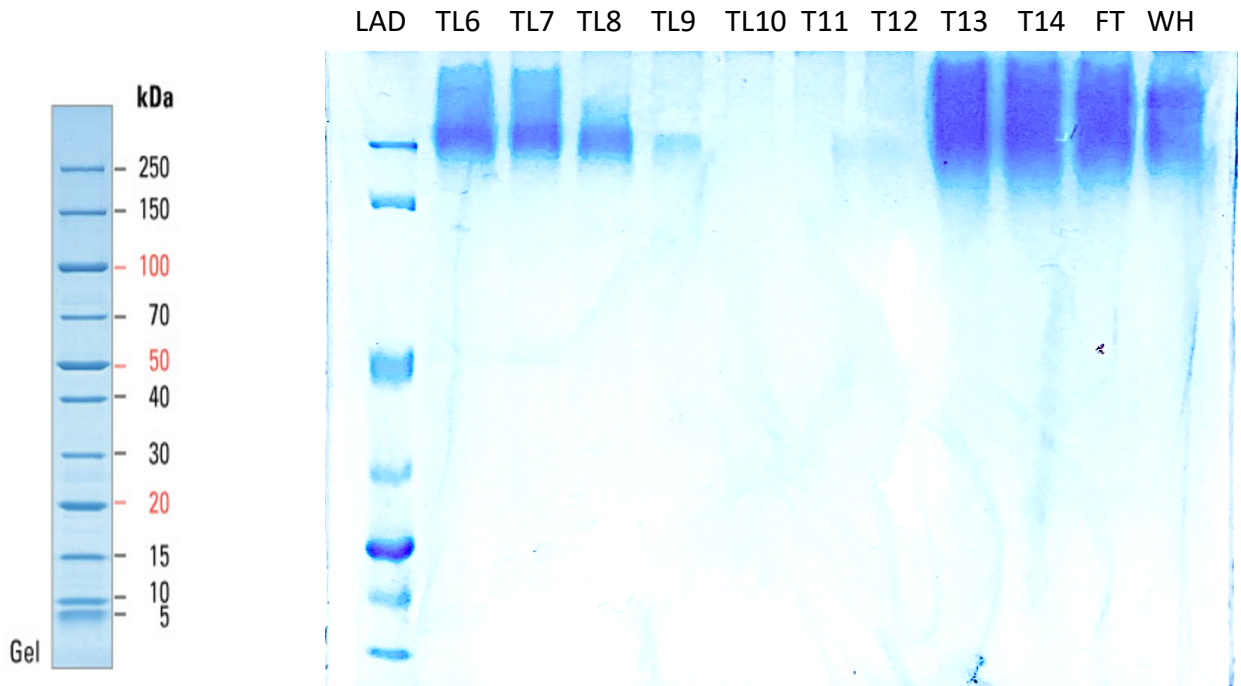


Figure 20: 5-250 kDa PageRuler™ protein ladder ²³.
Page Ruler Protein Ladder Reference

Figure 19: Image taken of SDS-PAGE gel screening for C43 expressed sigma-1 GFP tagged SMA 2:1 nanodisc complexes. Lane 1 contains PageRuler™ protein ladder (5 to 250KDa). lane 2 to lane 10 contained elutions T6 to T14, lane 11 contains flow through sample and lane 12 contains wash sample. **SDS-PAGE Result Post IMAC Run of Solubilised Sigma-1 Complexes using SMA 2000I.**

In addition to the SMA imide 1:1 SDS-PAGE screening it was also necessary to screen the SMA 2:1 elutions post AKTA start purification. The results of this SDS-PAGE surprised us we did not anticipate such abnormal results when running the SMA 2:1 elutions upon an SDS gel. As we can see, figure 19 shows that all elution samples failed to pass through to the separating section of the gel and appear to be majorly aggregated. After multiple attempts to run a successful gel using SMA 2:1 nanodiscs it was deemed unsuccessful and we decided to abandon this method of solubilisation. This gel shows a strong indication that the SDS in the gel did not denature the nanodiscs to produce a net negative charge of the overall

complexes or the SMA nanodiscs resin solubilisation procedure was not performed correctly resulting in an abnormal chemical property. In addition, for the SDS-PAGE procedure we speculated that the acrylamide concentration of 10% was too high and prepared gels at 8% in order to increase gel pore size for heavier molecular weight complexes. This attempt also failed, leading us to stop using SMA 2:1 for the solubilisation of our protein and focus on using SMA imide 1:1 and LMNG detergent.

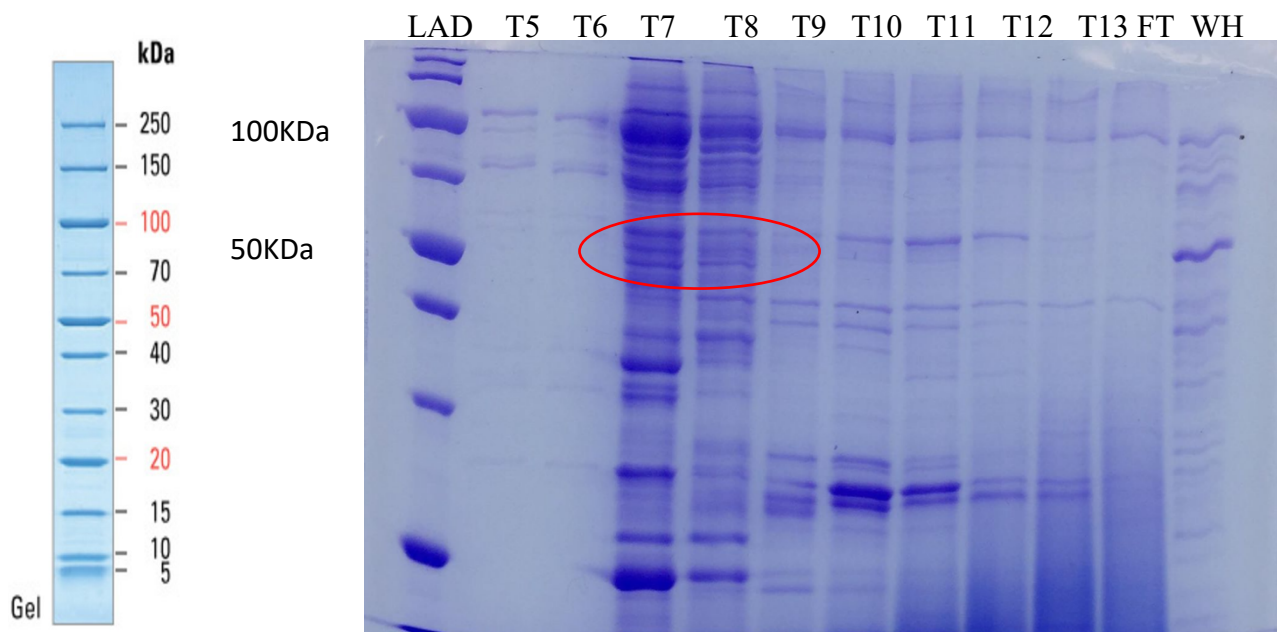


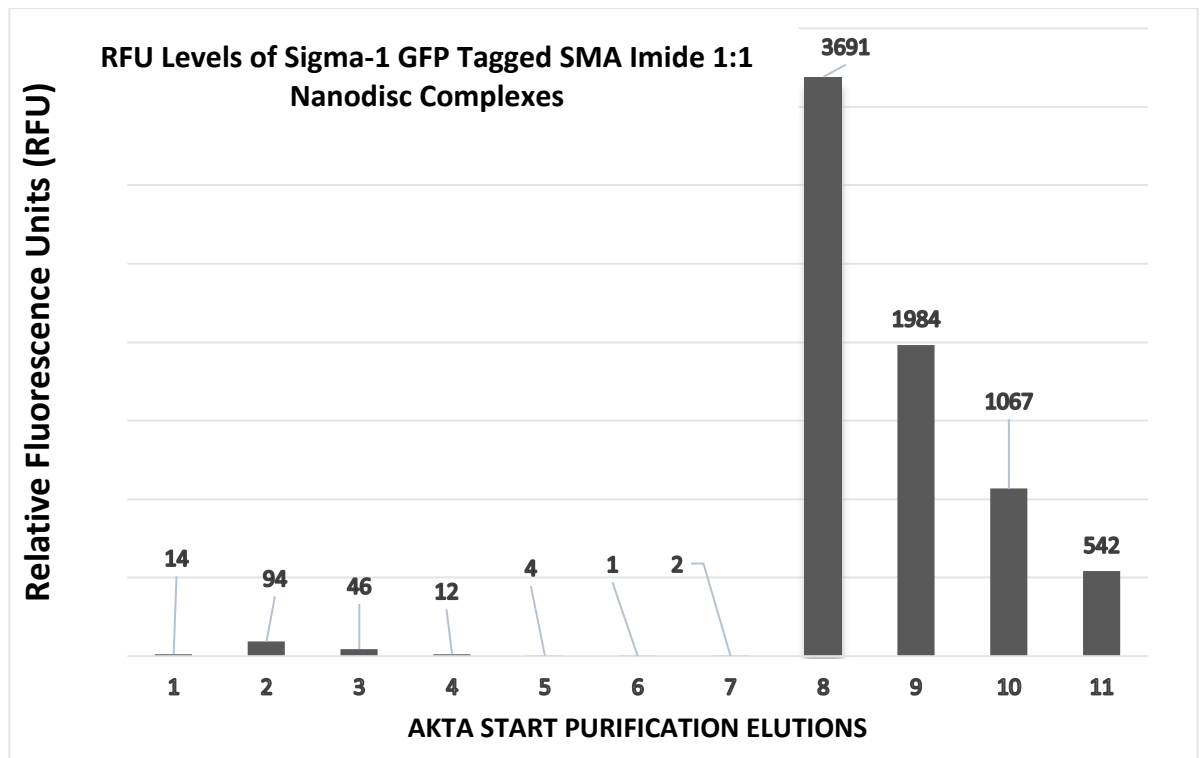
Figure 22: 5-250 kDa PageRuler™ protein ladder ²³.
Page Ruler Protein Ladder Reference

Figure 21: Image taken of SDS-PAGE gel screening for C43 expressed sigma-1 GFP tagged 0.1% LMNG & 0.01% CHS elutions. Lane 1 contains PageRuler™ protein ladder (5 to 250KDa), lane 2 to lane 10 contains elutions T5 to T13, lane 11 contains FT sample, and lane 12 is the wash sample. **SDS-PAGE Result Post IMAC Run of Solubilised Sigma-1 Complexes using LMNG and CHS.**

After analysing figure 21, it is clear to see that there many proteins present at varying molecular weights. Overall, we do not see any bands with particular intensity at any other molecular weights apart from at 100KDa in both elution samples T7 and T8. However, we still do see bands at around 50KDa which still provides some indication of our desired protein being expressed in this system. Ideally, we would have expected these bands at 50KDa to be have much more intensity than any other protein band present on this gel. Furthermore, as the

results of this gel correlates with the SMA imide 1:1 sample elutions, this must be deemed as a significant finding and as these protein bands could potentially be the solubilised sigma-1 receptor.

3.4: GFP Tag Fluorescence Analysis of SMI Imide 1:1 IMAC Elutions



*Figure 23: Shows GFP fluorescence of SMA imide 1:1 elution sample (T1 to T11) following IMAC purification. Samples were loaded into a 96 well plate and GFP fluorescence was measured at excitation of 495nm and an emission of 509nm. **GFP Tag Fluorescence in Solubilised Sigma-1 Complexes using SMI Imide 1000I Post IMAC Run.***

Once there was an indication post AKTA start purification for the presence of sigma-1 GFP tagged complexes, 250µl of elutions T1 to T11 were assessed for GFP fluorescence using a 96 well microplate. As we can see, elutions 1 to 7 contain relatively low levels of GFP fluorescence, however as we get to elution 8 it is clear to see a sharp increase in the RFU of this sample. In the contrary, we begin to see a decrease from elutions 9 to 11 which indicated that elution 8 contained the highest concentration of sigma-1 GFP tagged SMA imide nanodiscs. Although, we see a significant RFU level of GFP in elution 8, this does not necessarily directly indicate that our GFP is directly bound to sigma-1 proteins. Having a high level of

GFP fluorescence, could also indicate there is a high level of free GFP in the sample due to potentially being cleaved by proteases during protein extraction. For this reason, this testing method alone is not suitable for determining the presence of bound GFP to sigma-1 and required further testing methods such as in-gel fluorescence to determine the molecular weight of the fluorescent complexes

3.5 C43 Cell Line In-gel Fluorescence Results

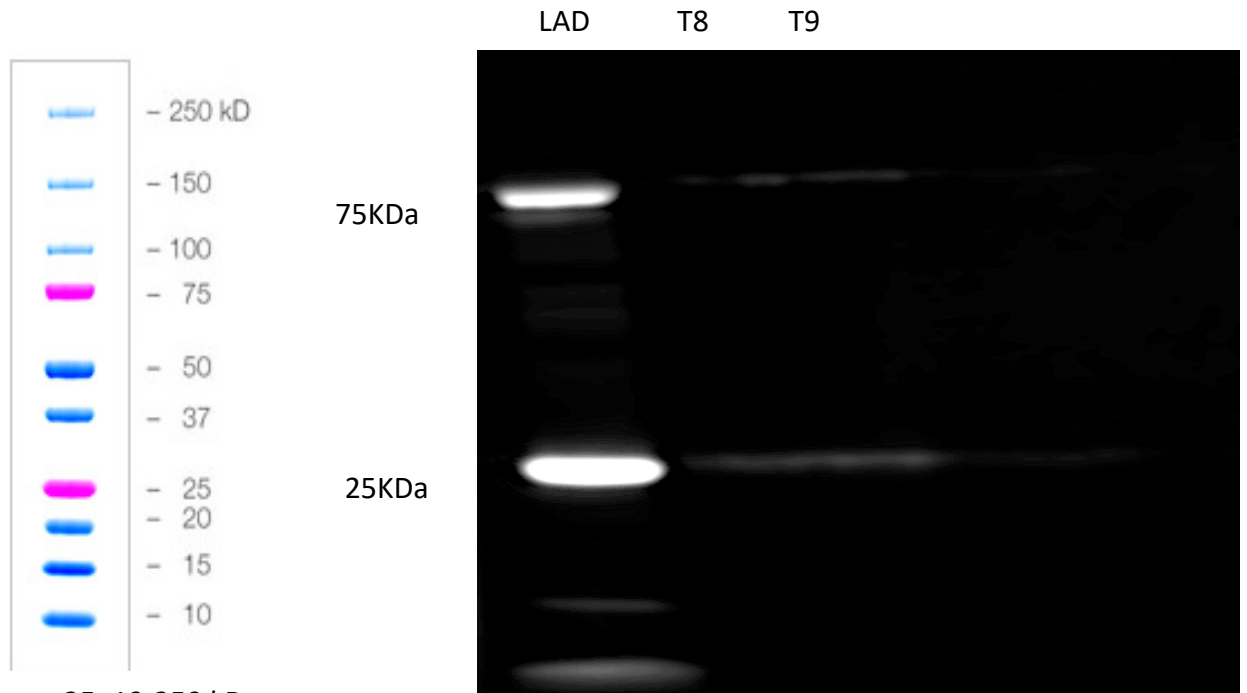


Figure 25: 10-250 kDa Precision Plus Protein™ Dual Colour (pre-stained) ladder². Precision Plus Protein Ladder Reference

Figure 24: In gel-fluorescence using IGFSB as a marker for sigma-1 GFP tagged complexes. Lane 1 contains the Precision Plus Protein™ Dual Colour (pre-stained) ladder, lane 2 contains concentrated elution 8 and lane 3 contains concentrated elution 9 post 0.1% LMNG & 0.01 CHS AKTA Start purification. **In Gel-Fluorescence of LMNG and CHS Sigma-1 GFP tagged Solubilised Complexes.**

Once we had gained peak elutions from the AKTA start purification system and measured the GFP fluorescence via a plate reader it was necessary to determine the molecular weight of the protein emitting this fluorescence. This was done by using the pre-prepared in-gel fluorescence sample buffer and following the previously specified protocol. As we can see in figure 24, In lane 1 the Precision Plus Protein™ Dual Colour ladder emits fluorescence at bands of 75KDa and 25KDa enabling easy tracking of samples in the gel. Using a CCD camera, we could

visualise the elutions fluorescence at 460nm and 515nm cut-off using blue light. In lane 2 for elution 8 we see a strong fluorescence for at 25KDa and 75KDa, which can also be confirmed lane 3 elution 9.

Prior to this procedure we expected fluorescence visibility at around 50 to 60KDa for this to be sigma-1 GFP tagged complexes. The presence of GFP fluorescence at around 25KDa was an indication that there were free GFP complexes in the sample unbound to our desired protein. Whereas fluorescence at 75KDa indicated that expression of our desired protein complexes could be abnormal or misfolded in some way. Furthermore, a simpler explanation could be the possibility of contamination from the fluorescent protein ladder in both of the sample's lanes.

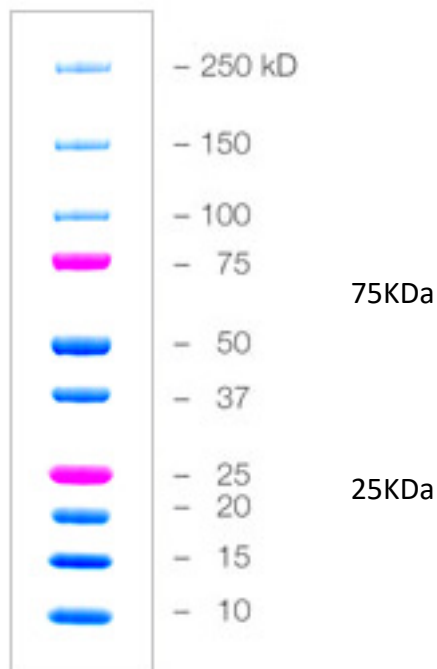


Figure 27: 10-250 kDa Precision Plus Protein™ Dual Colour (pre-stained) ladder².
Precision Plus Protein Ladder

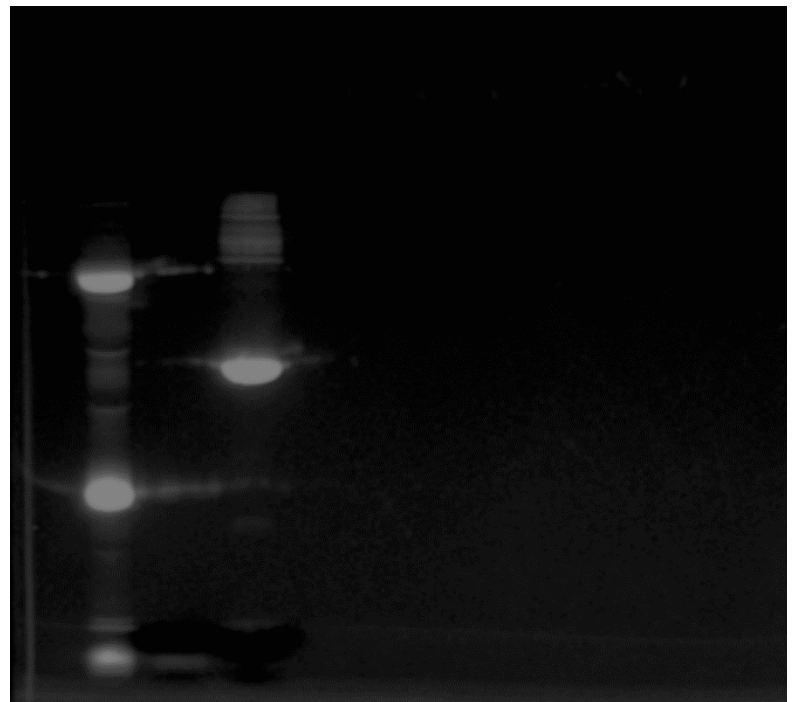


Figure 26: In gel-fluorescence using IG-FSB as a marker for sigma-1 GFP tagged complexes. Lane 1 contains the Precision Plus Protein™ Dual Colour (pre-stained) ladder, lane 2 contains concentrated elution 8 (2% SMA Imide) and lane 3 contains concentrated CLIC1-GFP reference sample. In Gel-Fluorescence of SMA 2000I Sigma-1 GFP tagged Solubilised Complexes Vs. CLIC-GFP Reference

Similarly, to the previously explained in-gel fluorescence result we see the same trend when analysing the concentrated sigma-1 elution solubilised by 2% SMA imide 1:1 nanodiscs. Due to the unexpected results seen in the 0.1% LMNG solubilised sample we decided to compare our sigma-1 GFP tagged sample to a protein that's expression, purification and characterisation protocols had been effectively optimised. The protein we used for this reference was the chloride intracellular receptor 1 (CLIC1), as this protein had been previously analysed in multiple publications in great detail. CLIC1 has a very similar molecular weight to sigma-1 at 27KDa and when tagged with GFP it should also have a molecular weight of around 50 to 60KDa. As we can see in Figure 26, lane 2 containing the sigma-1 GFP tagged SMA imide solubilised elution there is no distinct fluorescent bands at 50KDa. When we compared this our CLIC1 reference sample we can see a clear difference to that of our elution sample.

Furthermore, it is clear to see that the CLIC1 reference sample emits strong GFP fluorescence at around 50KDa whereas our sigma-1 solubilised via SMA imide nanodiscs does not. The only indication that we see in our elution sample is minimal GFP fluorescence at both 25KDa and 75KDa as seen in the 0.1% LMNG sigma-1 gel. This second gel may support the ladder contamination theory previously explained, as both gels appear identical results for the AKTA start purification elution sample. Overall, the data obtained for the in-gel fluorescence of our eluted samples via all solubilisation techniques were unexpected and definitive conclusions were had to come by. Although the results for in-gel fluorescence appeared to be unpromising it was still necessary to continue forward with our purification procedures for sigma-1. Once analysis of in-gel fluorescence took place, samples were run on an Agilent 1100 automated HPLC device.

3.6 SEC Data for Sigma-1 C43 Expressed Samples

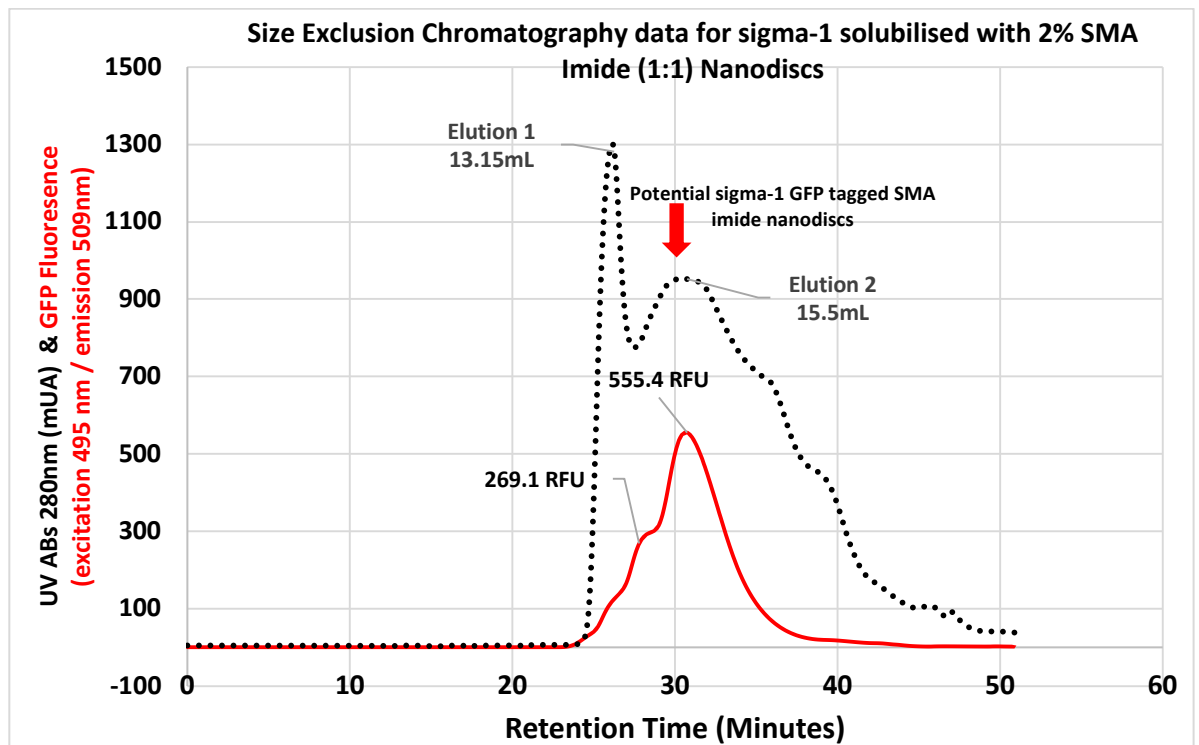


Figure 28: Shows size exclusion chromatography data for sigma-1 solubilised with 2% SMA imide (1:1) Nanodiscs. Here we see detection for UV absorbance at 280nm and GFP fluorescence at excitation 495 nm and emission of 509nm. **Size Exclusion Chromatography of 2% SMA Imide 1000I Solubilised Sigma-1 GFP Complexes.**

Gel filtration chromatography can be divided into two major phases; Stationary Phase and the Mobile Phase. The stationary phase is referred to the point at which the size-exclusion column is void of sample flow or running buffer whereas the mobile phase can be defined as the supply of sample flow or running buffer through the column. Smaller molecules tend to have a greater access to stationary phase enabling them to pass through the sepharos beads at a much slower pace. Larger molecules tend to have an inaccessibility to stationary phase causing them to elute at a much earlier accumulated volume and time during a run. This inaccessibility to stationary phase is referred to as the void volume (V_0) and this is a useful tool that can be used in estimating desired protein peak elutions post SEC. As smaller molecules and low molecular weight proteins readily gain access to stationary phase, they tend to elute before one column volume has passed during a run.

This latent elution of low molecular weight biomolecules is referred to as the total accessible volume (VT), which can be used for column calibration for determining the molecular weights of each peak elution. Unfortunately, for our SEC purification procedure we were unable to successfully or accurately calibrate our column due to a slight compression in the sepharos beads. Furthermore, although we were unable to determine an estimated molecular weight of each peak elution, it was possible to analyse GFP fluorescence of each elution in order to determine which samples were most likely to obtain our desired protein. For this SEC procedures the 500µl 2% SMA Imide concentrated sample obtained post IMAC purification was injected and run at 0.5mL/min. In figure 28 it is clear to see two distinct peaks, one at 13.15mL and the other at 15.5mL of sample running.

However, as we set up GFP fluorescence for this SEC procedure it was deemed necessary to screen UV absorbance peaks simultaneously in order to screen selected fractions via SDS-PAGE. The first UV 280nm peak elution at 13.15mL also corresponds to an increase in GFP fluorescence readings, however it is not until the second peak elution is reached, we see a clear GFP fluorescence corresponding to a specific eluting protein. The total estimated fluorescence peak reached for elution one was determined at 269.19 RFU, whereas elution two was nearly double at 555.4 RFU. Although the fluorescence determined for elution peak two was greater than that of elution peak one, this does not necessarily indicate that a greater fluorescence emitting elution will contain bound GFP to sigma-1. Because of this, it was necessary to screen all elutions for both peak one and peak two to be sure that our desired protein was not situated in an abandoned sample.

3.7 SDS-PAGE Screening Post TEV Protease Cleavage

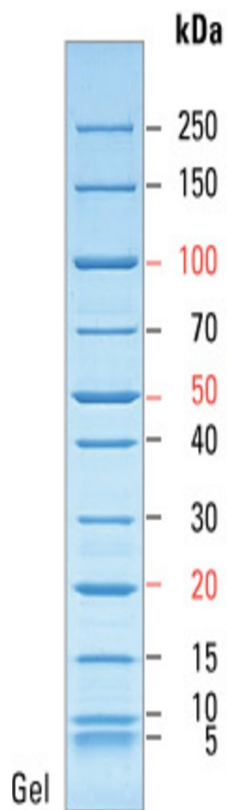


Figure 30: 5-250 kDa PageRuler™ protein ladder²³.
Page Ruler Protein Ladder Reference

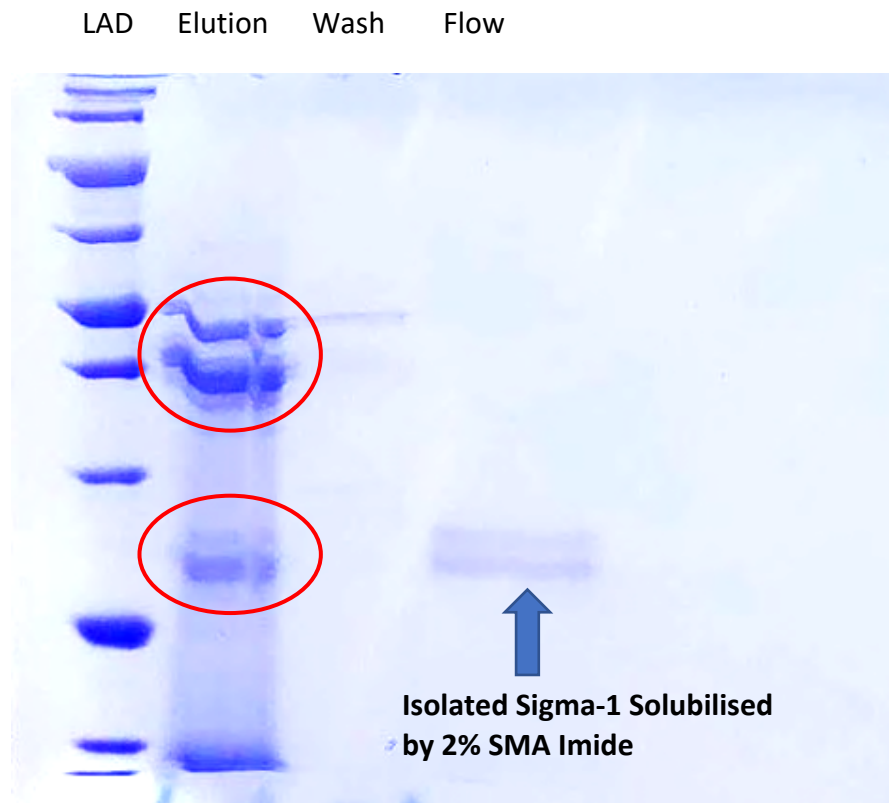


Figure 29: *TeV* cleavage procedure performed in pH 7.5 lysis buffer and at 4°C. Lane one contains the PageRuler™ protein ladder, lane two contains our elution sample, lane three contains a wash sample and lane 4 contains our flow through sample. **SDS-PAGE Post TEV Cleavage Procedure of Solubilised Sigma-1 GFP Complexes in 2% SMA Imide 1000I**

As described previously, our TEV cleavage procedure was not performed in optimum conditions due to issues with the stability and purification of sigma-1. Under optimum conditions such as pH 8.0 and at room temperature, TEV protease exhibits a high level of enzymatic activity with a high level of cleavage for the ENLYFQG amino acid sequence. As our TEV cleavage procedure was performed in pH 7.5 and at 4°C it is unlikely that the activity of our enzyme was at full capability, which is evident when analysing our SDS-PAGE gels for this procedure. In figure 29 we can see this to be true, for this procedure we expected to see no protein bands at around 50kDa following TEV cleavage of GFP from sigma-1. However, in figure 29 we see bands in the elution sample at 50kDa and around 40kDa which indicated the TEV cleavage procedure was not fully effective. In addition, we see a protein band at around 25kDa which corresponds close to that of the TEV

protease or free GFP molecular weight. Furthermore, when analysing the wash sample on the gel we also see a faint protein band at 50KDa which supports the idea the TEV protease activity was hindered by the protocol. A promising result is when we begin to analyse the flow through sample on the gel, and we see two individual protein bands at between 20KDa to 30KDa. As our desired proteins HIS-tag was cleaved during the TEV procedure we would expect to see observe this protein, isolated in a flow through sample non previously bound to the nickel column. It is highly likely that the two bands seen in the flow through sample is isolated sigma-1 and TEV protease that has become unbound to the nickel column.

3.8 TEM For Identification of Nanodisc Complexes

Once there was a strong indication for the solubilisation of the sigma-1 receptor using 2% SMA imide (1:1), we tested our size exclusion chromatography peak elution samples to identify the formation of nanodisc complexes. In figure 31, we can see a profound number of nanodisc like formations at a resolution of 100nm. Upon analysing these nanodiscs formations, we estimated an average diameter of 19nm per disc. When comparing these images to previous publications such as seen in *Swainsbury et al* TEM images on 2:1 SMA nanodisc complexes. It is clear to see distinct similarities between our results and their findings. In their publication, they identified the size of their SMA nanodisc formations to be an estimated 13nm in diameter with the presence of aggregated artefacts (100nm in size) likely to be mass clumps of nanodiscs. As we can see in our sample a profound concentration of nanodiscs throughout, it is likely that this had an impact on the efficiency of our primary AKTA purification for the 2% SMA Imide (1:1) sigma-1 samples. In recent publications, it has been implicated that free nanodisc formations may result in the interference of polyhistidine tags with nickel columns during the sample running phase. This could be a potential factor for why we do

Bradley Hilton

not achieve successful isolation of our target protein when using this method of solubilisation

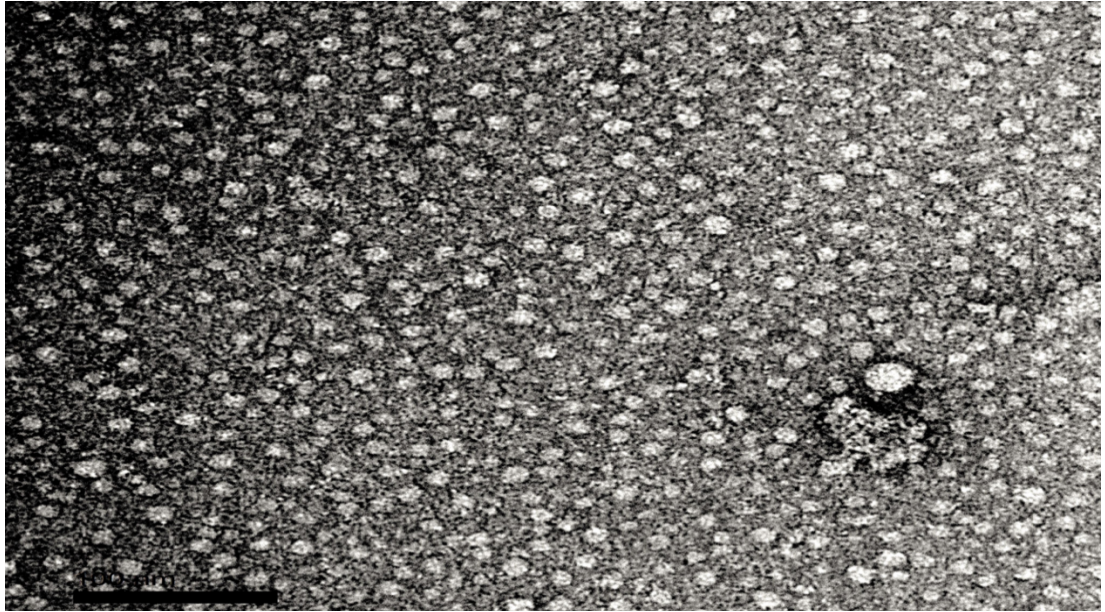


Figure 31: TEM image at 100nm for the identification of 2% SMA imide (1:1) nanodiscs in our SEC purified Sigma-1 GFP Samples. Transmission Electron Microscopy Of 2% SMA Imide (1:1) Nanodiscs at 100nm.

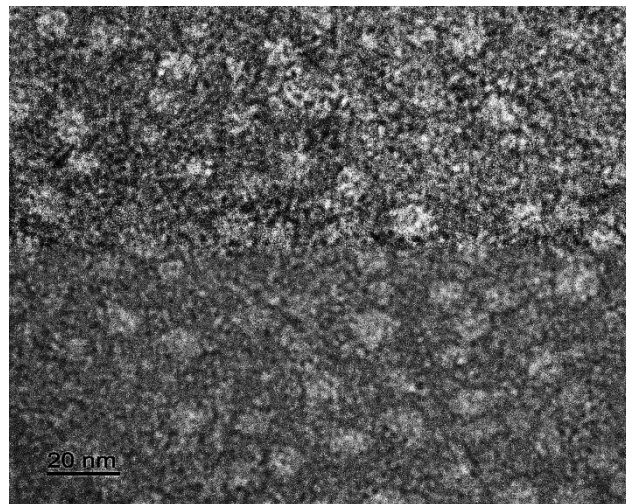


Figure 32: TEM image at 20nm for the identification of 2% SMA imide (1:1) nanodiscs in our SEC purified Sigma-1 GFP Samples. Transmission Electron Microscopy of 2% SMA Imide (1:1) Nanodiscs at 20nm.

3.9 C43 expression results in relation to past literature

The results obtained for the C43 expression protocol of the sigma-1 highlights the challenges of successfully isolating membrane proteins. Currently, there have been very few publications that have demonstrated successful expression of the sigma-1 receptor in C43 being functionally active. A study published in 2013 by Gromek et al, used BL21 and C43 cell lines for the expression of sigma-1 receptor extracted gene from the guinea pig. During their cloning they fused sigma-1 receptor to the maltose binding protein (MBP) as this protein tag has shown to increase recombinant protein yield and solubilisation stability. Similarly, this study concluded sufficient extraction and solubilisation using detergent based methods such as DDM and Triton X-100.

Although, they identified a lower level of sigma-1 functional activity when using these detergents individually rather than used in combination with each other. Overall the the yields obtained of active sigma-1 were not desirable, even after comparing expression levels to other *E. coli* cell lines such as C41 strains. Unfortunately, this study did not provide data for their size-exclusion chromatography and AKTA start purification runs. However, they did conclude these purification methods to be sufficient in isolating the sigma-1 receptor and identified that the addition of supplemented detergent to purification systems increased the functional yield of the protein after assessing via ligand binding studies. In comparison, a previous study published in 2006 by *Ramachandran et al*, also identified similar results when expressing the sigma-1 receptor in *E. coli* expression systems. This study identified that when the sigma-1 receptor is expressed alone without any additional tags such as GFP and MBP, the formation of cell inclusion bodies is evident²⁶.

In contrast even when a GFP tag was present in our study, we also identified the formation of inclusion bodies when induction took place at higher incubation temperatures (>15°C). However, this could also be due to the development of cellular toxicity due to overexpression of both the sigma-1 receptor and GFP at

once. Once the researchers of this study successfully expressed the sigma-1 receptor with its counterpart MBP tag, they also identified a greater level of functional stability in the protein when using a combination of detergent based methods i.e. CHAPS & triton X-100, just as in *Gromek et al* findings²⁵. Just as seen our TEV protease cleavage procedure *Ramachandran et al* identified the isolated sigma-1 receptor at a molecular weight of 25KDa run on a 12% SDS-PAGE gel.

They concluded this isolated sigma-1 protein to also be functionally active using [³H]-(+)-pentazocine for ligand binding assays. Unfortunately, the researchers of this study did not include data obtained for their purification methods (SEC & IMAC) in their publication, therefore we unable to compare our data to their findings. However, the *Shmidt et al* study concluded that the sigma-1 receptor appeared to run as high molecular weight oligomers of high biochemical purity when solubilised with 0.01% LMNG and 0.001% CHS via expression in Sf9 insect cells.

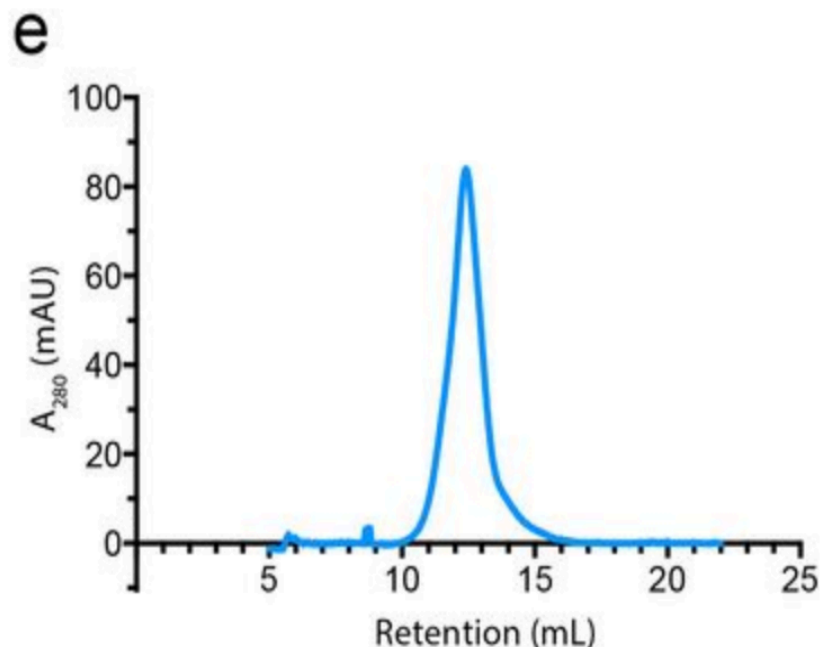


Figure 33: Analytical size exclusion of purified σ_1 receptor in LMNG/CHS detergent buffer on a Superdex 200 column. Image taken from *Shmidt et al* 2016 publication 'the crystal structure of the human sigma-1 receptor'.¹¹ **Schmidt et al LMNG and CHS Size Exclusion Chromatography Data on Isolated Sigma-1 from Sf9 Cells**

As we can see in figure 33, there is a clear distinct high-resolution peak ranging between 10 to 15mL obtained from the *Shmidt et al* SEC purification of the sigma-1 receptor. During this study, they also used a Superdex 200 10/300 GL (25mL) size-exclusion column for their secondary purification and it is clear to see a correlation between their results and ours. Although we do not have data for or sigma-1 0.01% LMNG and 0.001% CHS SEC run, we can see a clear similarity when comparing this to the graph of the sigma-1 2% SMA imide (1:1) SEC purification run. In our study we also identified peak elutions ranging between 10 to 15mL for all of our solubilisation technique SEC runs. Furthermore, although there is not a sufficient amount of available literature to which we can compare our findings directly to, there is still many strong indications that suggest successful expression and isolation of our target protein in the C43 expression system.

***Saccharomyces Cerevisiae* Expression System Results and Discussion**

3.10 INVS_c1 Pre and Post 2% Galactose Induction Growth Data

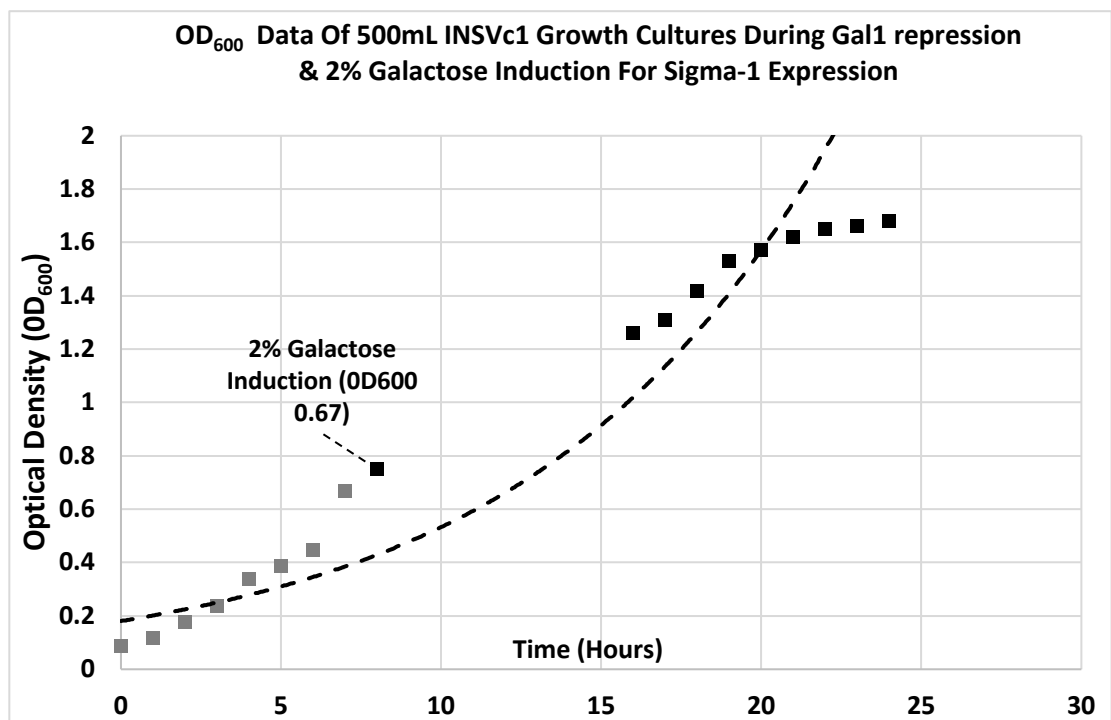


Figure 34: Shows OD₆₀₀ data pre and post induction of the INVS_c1 cell line. Date points in grey are readings taken before induction and data points in black are readings taken post 2% galactose induction. **OD₆₀₀ Growth Data of INVS_c1 cell line prior and post 2% Galactose**

As we can see, figure 34 demonstrates a clear difference between the growth cycles of C43 compared to INVS_c1. In order to achieve an OD of 0.67 from this data, it required 8 hours of incubation at 30°C. When comparing this to the optimum OD₆₀₀ of our C43 *vector*, it is clear to that this expression system required a fraction of that time (140 minutes) to be suitable for induction. However, as we can see the doubling time of this cell line appears to show normal yeast growth as they cells appear to be doubling every 2 hours. Upon induction with a 2% sterile stock of Galactose in -URA medium, we see that the OD₆₀₀ of the growth culture has doubled when hour 16 has been reached. Again, this is to be expected for normal cellular growth and based on the fact galactose increases culture density often resulting in higher OD₆₀₀ results.

After 24 hours has passed, the maximum incubation timepoint has been reached which indicated sufficient protein expression of sigma-1 had been achieved. At hour 24 a final OD₆₀₀ of 1.68 was recorded, which is a normal OD₆₀₀ for a yeast growth culture that has been induced and incubated for a significant time span. We can see from both figures that there is a steady rate of growth up until hour 8, then we see a sharp increase from hour 16 followed by an indication of a stationary phase in the INSV_c1 growth. This is to be expected as the culture reached its maximum timepoint, which signals that the nutrients in the medium had become significantly depleted. As INSV_c1 cells are much larger than that of bacteria, we are unable to accurately calculate the cellular densities of all OD₆₀₀ results at each timepoint.

3.11 In-gel Fluorescence Results Post INSVc1 2% Galactose Induction

LAD Control Induced

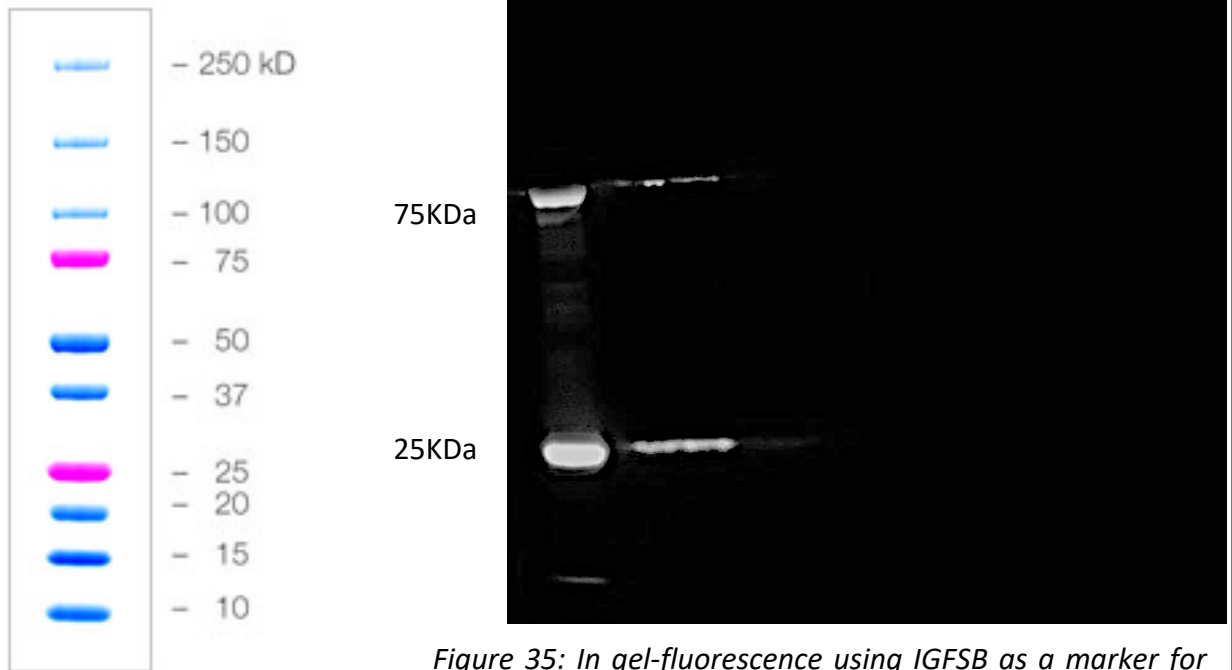


Figure 36: 10-250 kDa Precision Plus Protein™ Dual Colour (pre-stained) ladder²⁴. **Precision Plus Protein Ladder Reference**

*Figure 35: In gel-fluorescence using IGFSB as a marker for sigma-1 GFP tagged complexes. Lane 1 contains the Precision Plus Protein™ Dual Colour (pre-stained) ladder, lane 2 control INSVc1 transformed cells lane 3 contains 2% galactose induced INSVc1 transformed cells. **In Gel Fluorescence of Sigma-1 GFP Tagged Complexes Expressed in INSVc1 Post 2% Galactose Induction.***

In we figure, we can see a comparison in GFP fluorescence between control INSVc1 transformed cells to 2% galactose induced INSVc1 transformed cells. In this gel it is not possible to identify any indications for the presence of sigma-1 GFP tagged complexes in both the control and induced cell. However, as seen before in the in-gel fluorescence for the sigma-1 C43 expressed samples, we see fluorescence at 75KDa and 25KDa. At this stage it is more than likely that these signals are nothing more than false positives due to the high fluorescence of the protein ladder in the adjacent lane 1. It is clear to see this method of GFP fluorescence requires optimisation due to these unexpected results in both the INVSc1 and C43 expression systems.

3.12. Post IMAC 12 % SDS-PAGE Gel of Sigma-1 Expressed in INVSc

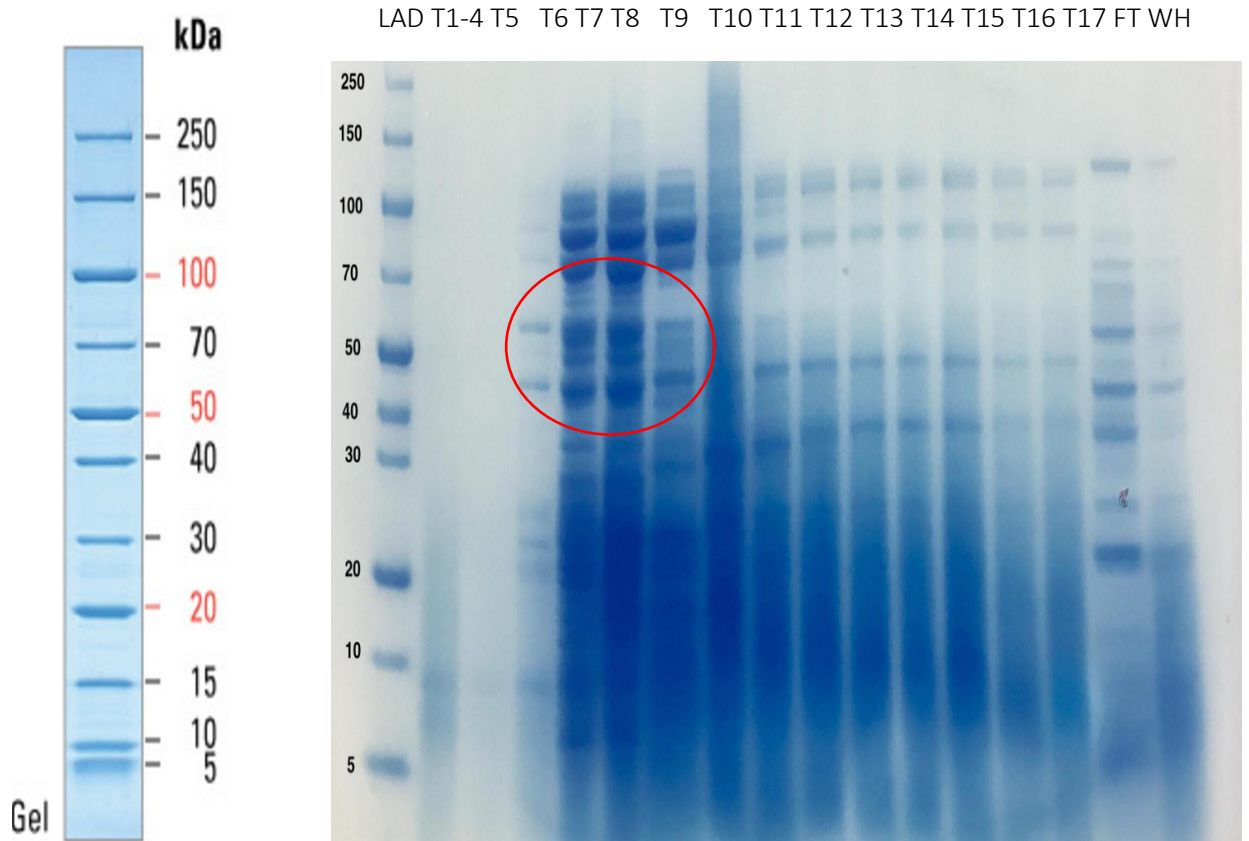


Figure 38: 5-250kDa PageRuler™ protein ladder²³. PageRuler Protein Ladder Reference

*Figure 37: Shows SDS-PAGE gel run for the elutions (T) post IMAC purification using 0.1% LMNG and 0.01% CHS for solubilisation. Lane 1 contains the PageRuler™ protein ladder, lane 2 contains a concentrated sample of elutions T1 to T4, lane 3 to lane 17 contains elutions T5 to T17, lane 18 contains flow through sample and lane 19 contains a wash sample. **SDS-PAGE Result Post IMAC Run on LMNG and CHS Solubilised Sigma-1 GFP Complexes***

By analysing this gel for the elutions obtained post IMAC purification it is clear to see presence of a profound number of proteins within the samples. Theoretically, if the purification was at the highest level of efficiency, we would expect to see fewer proteins within each elution as we expect the only protein to have affinity for the nickel is the sigma-1 receptor based on the presence of a N-terminus GFP His-tag. Although, we do see a significant level of proteins throughout the elutions, flow through, and wash samples we see the highest band intensities in elutions T7 and T8. Specifically, these intensities are at varying molecular weights from 50KDa to 70KDa. This was a strong indication that the INVSc1 were over expressing our desired protein as we had seen similar results in SDS-PAGE gels for

C43. Furthermore, as these gels showed a strong indication for the presence of our target protein it was necessary to investigate further and separate all of these proteins into single fractions via size exclusion chromatography.

3.13: SEC Post IMAC Of Sigma-1 Expressed in INSVc1 Cells

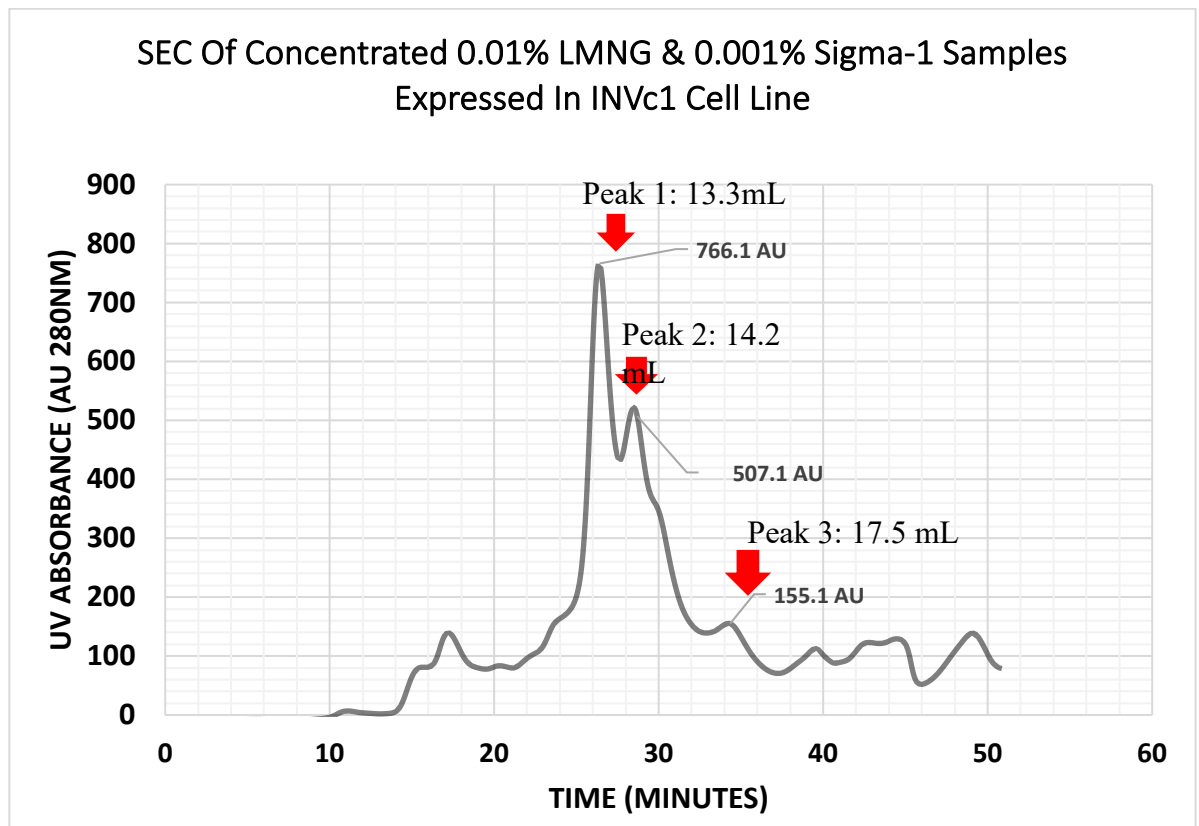


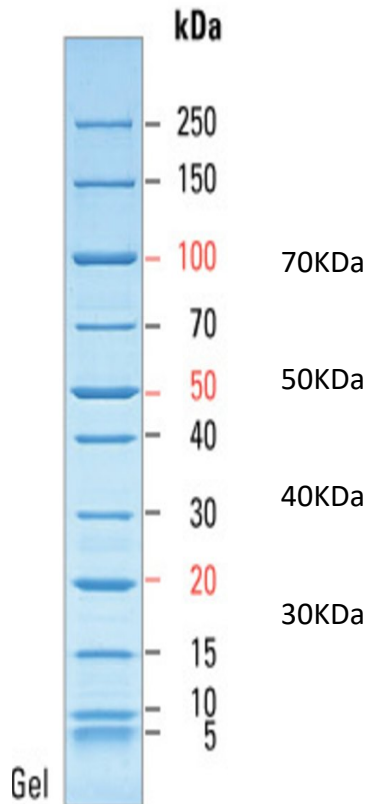
Figure 39: Reports the size-exclusion chromatography data that we obtained for the concentrated elutions obtained from the primary IMAC purification phase in INVSc1. In this graph we see two peaks at 13.3mL and 14.2mL with good resolution peaks. Size Exclusion Chromatography Graph of LMNG and CHS Solubilised Sigma-1 GFP Complexes Expressed in INSVc1 Cells.

The 4mL elutions T5 to T10 run on the SDS-PAGE gel post IMAC purification were spin concentrated down to 500 microlitres to be injected through a 25mL Superdex 2000GL column. Once injected the sample buffer was applied at a flow rate of 0.5mL/min to obtain individual fractions of 0.5mL for the purpose of protein separation. As we can see from the SEC graph in figure 39, we see two distinct peak elutions that significantly correlate to the elution volume previously

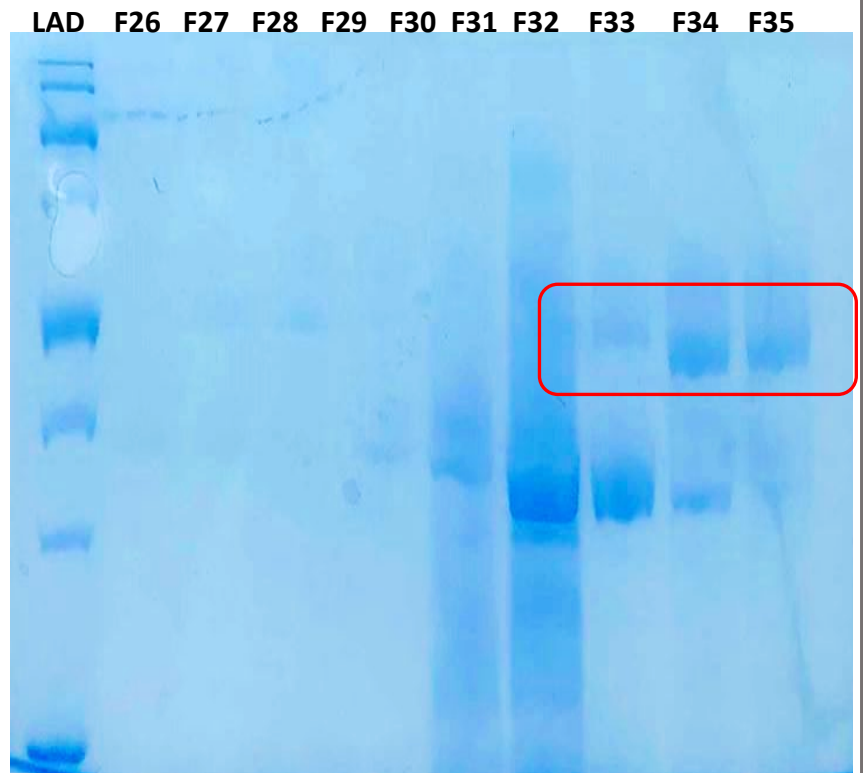
seen in SEC graph for the C43 expression system. In the SMA imide 1:1 sigma-1 purification the first peak elution was also estimated at 13.3mL which can also be in to be the same in this data. Although this SEC purification was not aided by a GFP fluorescence detector, it is safe to say that it is more than likely this fraction is our sigma-1 GFP tagged complex. The second peak elution recorded at 507 AU could also potentially be our desired protein complex or free GFP which could have been cleaved during extraction. In figure 39, we also late peak elutions indicating that something of a smaller molecular weight is eluting. Theoretically this could be the presence of varying sizes of micelles, as this purification was facilitated with a concentration of 0.01% LMNG and 0.001% CHS supplemented to the running buffer. Furthermore, by analysing this data it was necessary to collect fractions from both elutions to screen via SDS-PAGE for protein molecular weights in these samples.

3.14 Gel Filtration Peak Elution SDS-PAGE Screening

The 0.5mL fractions collected post SEC purification, were run on an SDS-PAGE gel to assess both peak elutions for identification of probable molecular weights. As we can see in figure 39, peak 1 (13.2mL: F26 to F27) did not contain any significant concentration of proteins that could be deemed as the presence of the sigma-1 receptor. In addition, peak 2 (14.2mL: F28 to F30) did not contain any proteins at the expected molecular weight of 50KDa except a low intensity band correlating at about 35KDa. However, after including an additional small peak elution (peak elution 3: 17.5mL) on the SDS-PAGE gel, we identified the presence of intense bands at 50KDa. These bands at 50KDa were identified at in fractions 33 to 35, with also the presence of bands at 30KDa to 40KDa.



*Figure 41: 5-250kDa PageRuler™ protein ladder²³. **PageRuler Protein Ladder Reference***



*Figure 40: SDS-PAGE gel post SEC purification procedure on two eluted peak fractions. In lane one we the PageRuler™ protein ladder, lane 2 to 3 we see fractions F26 and 27 (peak 1), lane 4 to 6 we see fractions F28 to F30 (peak 2) and lane 7 to 11 we see fractions F31 to F35 (peak 3). **SDS-PAGE Result of Elution Peaks Post SEC of INSVc1 Expressed Sigma-1 GFP Tagged Complexes.***

The identification of bands at proteins at 50KDa was a positive indication for the presence of the sigma-1 receptor in the INVSc1 cell line. Similarly, to that of the C43 expression vector we also saw intense protein bands at around 30 to 40KDa. This reoccurring trend of lower molecular weight proteins being present as well as those at 50KDa within the samples, is a strong indication that our sigma-1 complexes may be getting cleaved to some structural level. Furthermore, as the results obtained for the INVSc1 and C43 systems demonstrated similar results at this stage of the purification, we deemed it necessary to continue forward into concentrating these fractions for TEV protease cleavage.

3.15 SDS-PAGE post TEV Protease Cleavage Procedure in INSVc1

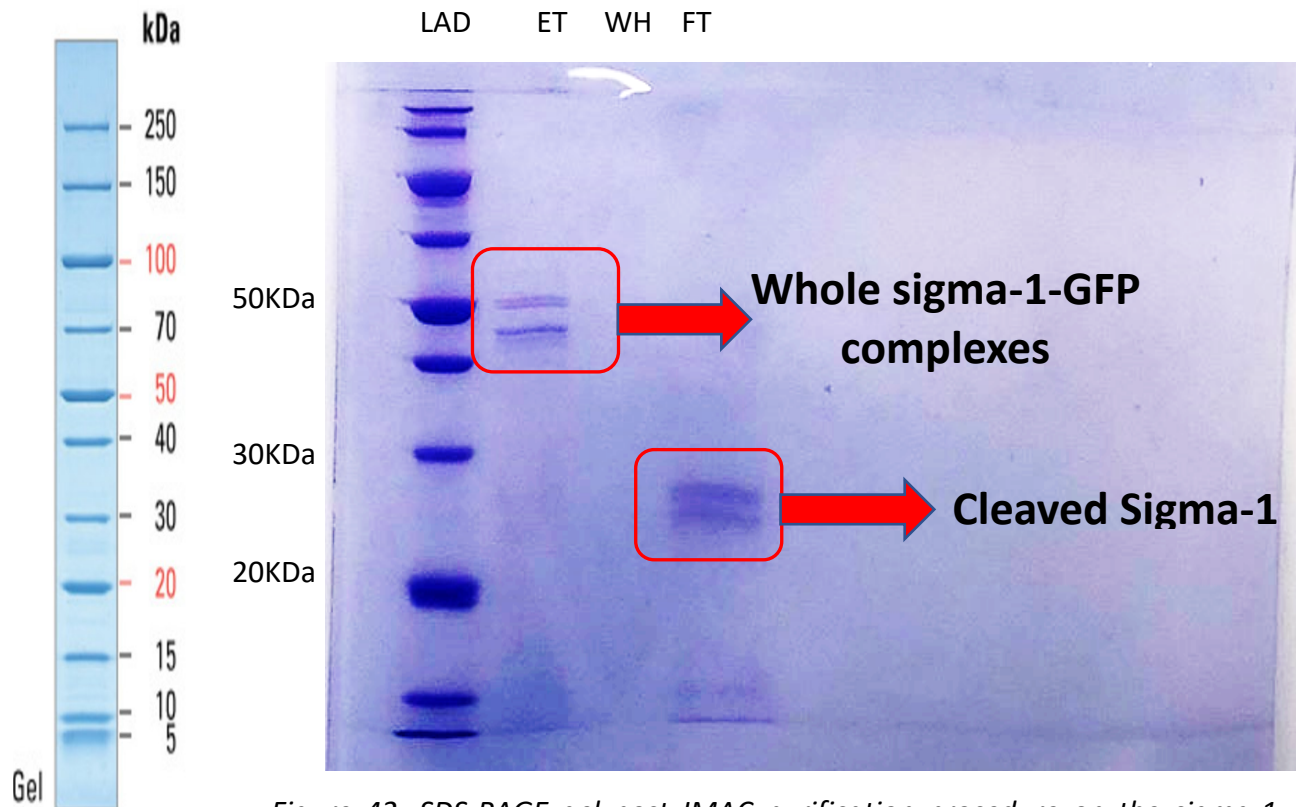


Figure 43: 5-250kDa PageRuler™ protein ladder²³. PageRuler Protein Ladder Reference

Figure 42: SDS-PAGE gel post IMAC purification procedure on the sigma-1 receptor cleaved from GFP via TEV protease cleavage. Lane 1 we see the PageRuler™ ladder, lane 2 the elution sample, lane 3 the wash sample and lane 4 the flow through sample. SDS-PAGE Result post IMAC run on TEV cleaved Sigma-1 Expressed in INSVc1.

Once we had successfully concentrated our SEC fraction samples for TEV cleavage it was necessary to purify potential isolated sigma-1 via IMAC. By analysing figure 42, we can see bands two distinct protein bands present within the imidazole elution sample. One of these protein bands is at 50KDa and the other has a slightly lower molecular weight between 40 to 50KDa. It is likely that the protein band at 50KDa is still sigma-1 tagged to GFP complexes due to poor TEV protease cleavage incubation conditions. The 40 to 50KDa protein band in the imidazole elution sample is likely to be a cleaved construct of the sigma-1 GFP tagged complex, however from this data we are unable to determine the exact structural cleavage location on the protein. In contrast, when analysing this gel, we identified no presence of proteins within the wash sample, which was to be expected. The most

promising result is when we analyse the flow through sample for the presence for isolated sigma-1.

After analysing the flow through sample, we identified two distinct bands in close proximity to one another. Here, we estimate the lower molecular weight protein at 25KDa and the higher molecular weight protein at around 27KDa. The protein band identified at 25KDa is more than likely to be isolated sigma-1 as our expressed DNA fragment sequence equates to this molecular weight i.e. 669 bp equates to 25.1 KDa. Whereas, the protein identified at 27KDa within the flow through sample is likely to be TEV protease that has not successfully remained bound to the nickel column during the loading phase of IMAC. Theoretically, we would expect to see the TEV protease within the elution sample due its possession of a His-tag in its expressed structure however, the presence of this may indicate adverse characteristics of our purification procedure. Due to the presence of TEV protease within the flow through sample, it is difficult to make a definitive conclusion that the protein seen at 25KDa is in fact the sigma-1 receptor. In addition, this draws the possibility of this band potentially being free GFP as this protein also has a similar molecular weight to both TEV protease and sigma-1.

3. 16 S. *Cerevisiae* Sigma-1 Expression in Relation to Past Literature

Currently there have been little research publications regarding the expression of the sigma-1 receptor in yeast cell lines, of which is unexpected as the sigma-1 receptor is shares close homology to the yeast C8-C7 sterol isomerase expressed by the *ERG2* gene. Interestingly, this yeast protein also shares a similar pharmacological profile to the sigma-1 receptor and is also able to bind to its ligands with high affinity. Furthermore, we are unable to compare our results to past literature regarding the expression of the sigma-1 receptor in yeast, however it is possible to compare our findings to the *Newstead et al* publication²⁷. Although, this publication aimed at providing an optimised membrane protein expression protocol, it does allow the researcher to identify key characteristics of successful growth in yeast. In the *Newstead et al membrane protein expression*

publication they identified a sufficient induction, OD_{600} at 0.6 for maximum protein expression. When comparing our INSVc1 growth data pre and post induction we can confirmed a normal growth curve and doubling time of our INSVc1 cells. However, when comparing our in gel-fluorescence data are unable to successfully identify sigma-1 tagged GFP complexes at 50KDa. At this stage, it was a strong indication that there may have been cloning issues with our *LEU2* construct. It is possible that the sigma-1 fragment was not successfully incorporated to the LEU2 vector containing the gene for GFP. Unfortunately, the final cloned LEU2 sigma-1 construct was not sent for amino acid sequencing which may have resulted in false positive results. In addition, it may have been beneficial to run DNA electrophoresis gels on all of our amplified PCR fragments before and after transformation to confirm correct DNA fragment LEU2 construct insertion.

Although we finally identified an isolated protein with a molecular weight of 25KDa via SDS-PAGE, it did not test positive for the sigma-1 receptor through western blot screening in any of the INVSc1 samples. In the *Newstead et al* protocol, the researchers also estimated the total membrane overexpression levels using a microplate spectrofluorometer post culture induction with 2% galactose. If we had done this during this study, this may have provided us with an indication of whether our target protein had been successfully expressed or not. Another potential factor that may have resulted into undesirable sigma-1 expression in our INSVc1 cell line, may be due to the measuring of OD_{600} post induction of our growth culture. This was not followed to the same protocol as seen in the *Newstead et al study*, as they advised removing the culture from incubation post induction procedure. This could have potentially resulted in the inhibition of sigma-1 expression and lowered cell culture density overall during this incubation period. Overall, although we did not successfully isolate the sigma-1 receptor in INSVc1 cells, it is clear that this expression system requires major optimisation. In principle, the use of yeast cell lines for the expression of recombinant proteins has been demonstrated to be highly desirable for membrane stability and biochemical functionality.

Final Results and NMR Studies

3.17. Confirmed Isolation of The Sigma-1 Receptor Via Western Blotting

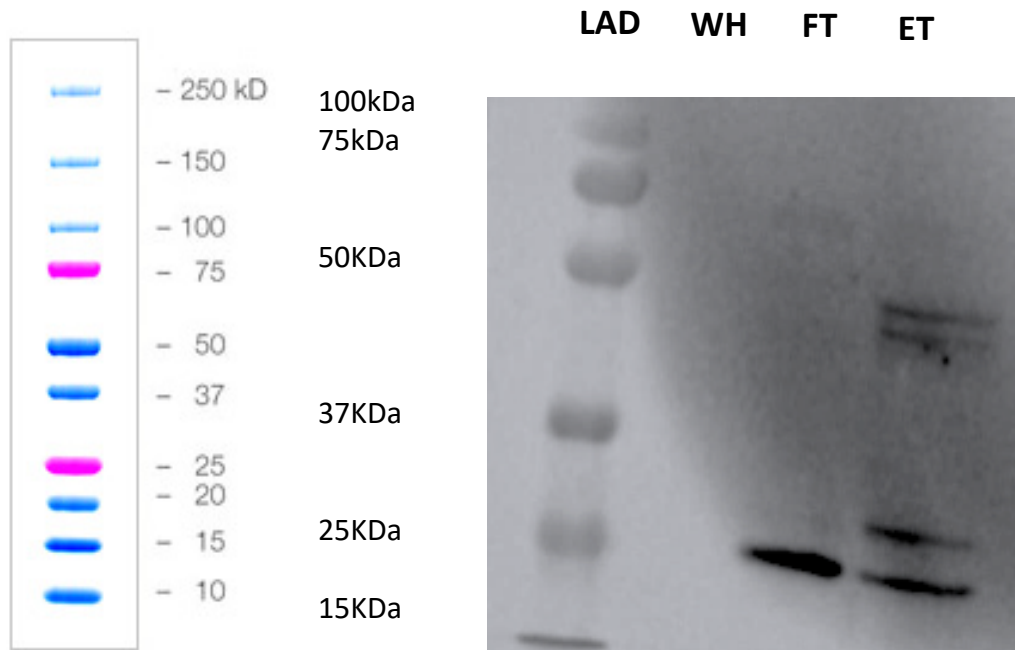


Figure 44: 5-250kDa Precision Plus Protein™ Dual Colour (pre-stained) ladder²⁴.
Precision Plus Protein Ladder Reference

LAD WH FT ET

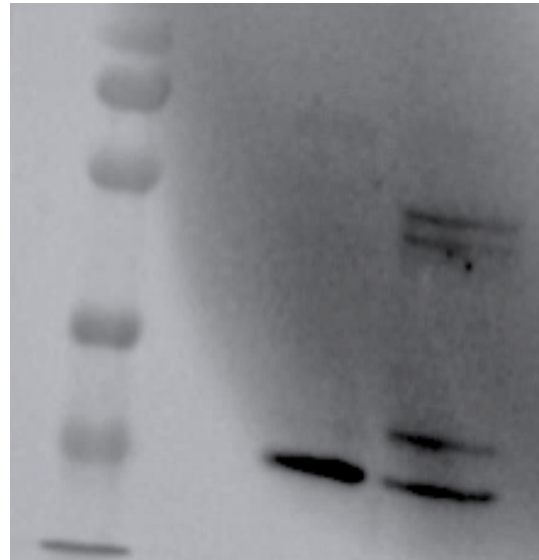


Figure 43: Western blot post TEV cleavage procedure in C43 expressed sigma 1. The sigma-1 receptor was finally solubilised in 0.01% LMNG and 0.001% CHS. Lane 1 is the ladder, lane 2 is the elution, lane 3 is the flow through and lane 4 is the wash sample. **Western Blot Confirmation for Isolated Sigma-1 Expressed in C43 Cells.**

As we can see in figure 43, the confirmed isolation of the sigma-1 receptor was performed in the C43 expression system. After endless attempts to successfully isolate the protein using nanodiscs and LMNG detergent, it would be finally isolated using the same methods as seen by the *Shmidt et al* study. In figure 43, we see no antibody detection of our desired receptor present within the wash sample, which was to be expected due to a previously run SDS-PAGE result. Within the flow through sample we see an intense reaction at 25kDa mediated via the addition of ECL onto a secondary conjugated HRP antibody. This identification concludes the presence of the isolated sigma-1 receptor expressed in C43 cells and solubilised by detergent based methods. Interestingly, when we analyse the elution sample, we also see the presence of secondary antibody reactions at 50kDa and 25kDa. The two bands positive for our desired protein at 50kDa are

likely to be uncleaved sigma-1 GFP tagged complexes due to issues with the TEV cleavage procedure. In addition, the second protein detection band just below 50KDa suggests protein cleavage during our expression protocol. In addition, when we analyse the elution sample at 25KDa we also can identify two distinct positive results. The first band located at exactly 25KDa corresponds a similar result to the flow through sample, indicating this is also isolated sigma-1. However, the protein band just above 25KDa (estimated at 30KDa) is likely to be a combination of unexpected cleaved sigma-1 and GFP complexes. In order to obtain this result via western blot, this project failed multiple times to successfully isolate this receptor. When the nanodiscs solubilised sigma-1 samples were tested via western blot there was no indication of its presence. This could be due to a profound number of protocols, purification, and western blot factors resulting in a false negative result. In addition, when the sigma-1 solubilised 0.01% LMNG samples expressed in INSVc1 were tested via western blot they also showed no indication for the presence of sigma-1. However, this is more than likely due cloning issues that lead to false indications throughout the screening procedures

3.18 NMR Studies for the Identification of the Sigma-1 Receptor

Testing Sample 1: S1R in 0.1% LMNG (Control Without Drug)

Testing Sample 2: S1R (0.2% LMNG) & 15mM Fluoxetine Hydrochloride

Sample 3: Fluoxetine Hydrochloride (0.2% LMNG & No Sigma-1)

Sample 4: 15mM Fluoxetine Hydrochloride (No LMNG & Sigma-1)

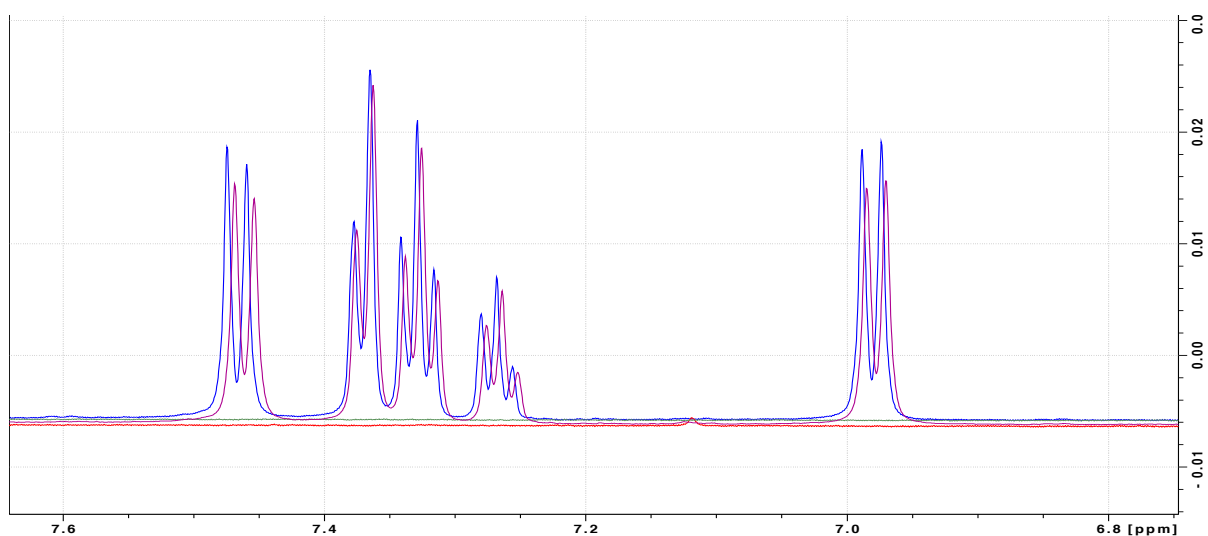


Figure 45: NMR CMPG (15.5mM fluoxetine hydrochloride) spectra overlay, of all varying sample conditions for the experiment. **Overview of Complete NMR CMPG Spectra Overlay.**

Testing Sample 1: S1R in 0.1% LMNG (Control Without Drug)

Testing Sample 2: S1R (0.2% LMNG) & 15mM Fluoxetine Hydrochloride

Sample 3: Fluoxetine Hydrochloride (0.2% LMNG & No Sigma-1)

Sample 4: 15mM Fluoxetine Hydrochloride (No LMNG & Sigma-1)

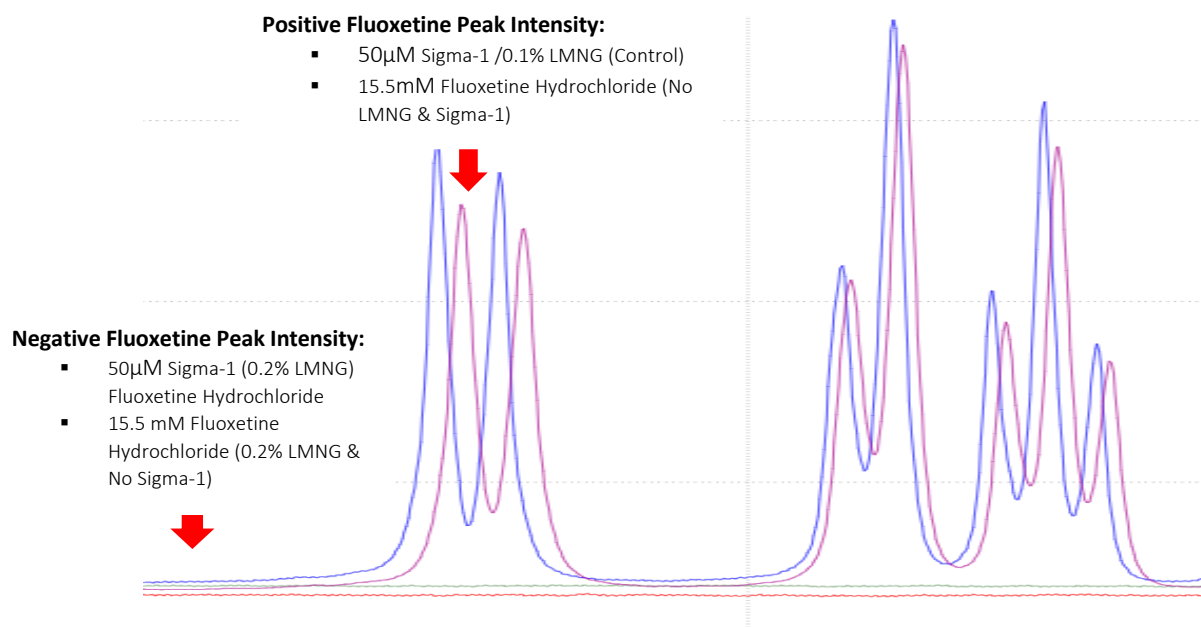


Figure 46: Magnified image of Peak Intensities: NMR CPMG (15.5mM fluoxetine hydrochloride) spectra overlay, of all varying sample conditions for the experiment.

Magnified Peak Intensities for the NMR CPMG Spectra Overlay.

In figure 45, we identified the CPMG spectra of varying peak intensities for 15.5mM fluoxetine hydrochloride spectra. This is the spectra overlay consisting of individual signals detected for the manipulated sample conditions in the presence or absence of the sigma-1 receptor, and varying LMNG concentrations. As we can see the 15.5mM fluoxetine hydrochloride sample (without detergent and target protein blue peak) correlates almost perfectly to that of the 15.5mM fluoxetine hydrochloride/50 μ M sigma-1 sample supplemented with 0.1% LMNG (magenta peak). This allows us to use the 50 μ M sigma-1 and 0.1% LMNG supplemented sample as a control and the 15.5mM fluoxetine without sigma-1 and LMNG as a reference for this spectrum. As the concentration of LMNG increased to 0.2%, the 50 μ M sigma-1/15.5mM fluoxetine hydrochloride sample (red line), and the 15.5mM fluoxetine hydrochloride sample without 50 μ M sigma-1 (green line) resulted in a total net loss of peak intensity in both samples.

This is a strong indication that the sigma-1 receptor was binding to the agonist compound fluoxetine hydrochloride. This can be concluded as we saw no loss of peak intensity in both the 50 μ M sigma-1 in 0.2% LMNG control sample (without drug) and the 15.5mM fluoxetine hydrochloride in 0.2% LMNG sample (without sigma-1). This was an extremely positive result, however there was an important consideration when making final conclusions on this data. As we also saw a total net loss of intensity for the 15.5mM fluoxetine hydrochloride in 0.2% LMNG sample without the presence of the sigma-1 receptor, this provides an indication that the drug may be interacting with detergent micelle formation. In recent years, many publications have demonstrated that certain drugs can bind to micelles in various types of detergents. One possible explanation for this may be due to interactions of fluoxetine hydrochloride structural aromatics with LMNG's maltose groups. These interactions are considered as undesirable for pharmacological studies of specific proteins and for structural characterisation. Which demonstrates the adverse responses when solubilising membrane proteins with detergent based methods. Furthermore, this hypothesis requires more investigation through other experimental conditions and characterisation techniques.

Testing Sample 1: 50 μ M S1R in 0.1% LMNG (Control Without Drug)

Testing Sample 2: 50 μ M S1R (0.2% LMNG) & 15mM Fluoxetine Hydrochloride

Sample 3: 15mM Fluoxetine Hydrochloride (0.2% LMNG & No Sigma-1) Control

Sample 4: 15mM Fluoxetine Hydrochloride (No LMNG & Sigma-1)

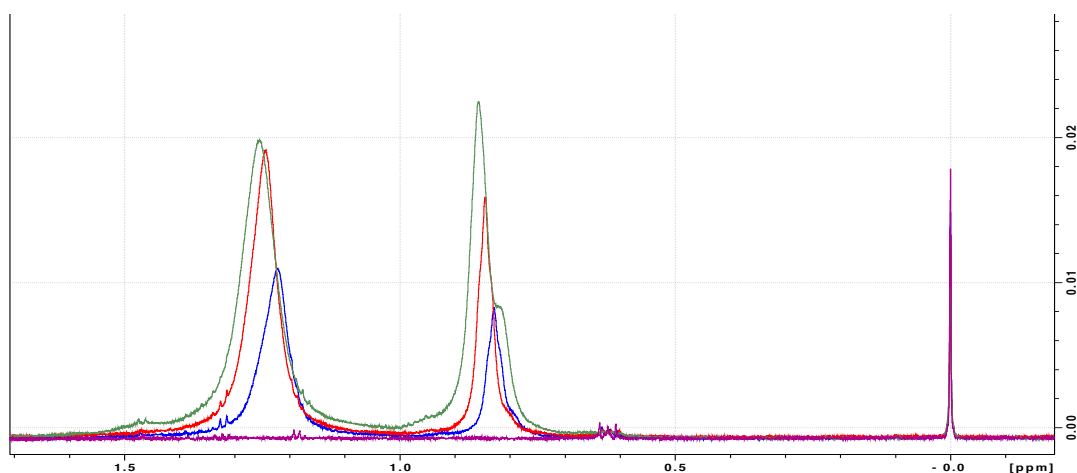


Figure 47: ID proton NMR (15.5mM fluoxetine hydrochloride) spectra overlay, of all varying sample conditions for the experiment. **Overview of ID Proton NMR Spectra Overlay.**

After we had successfully obtained data for the CPMG sequence experiment, it was decided to run these samples on a standard 1D proton NMR spectrum to provide credibility to our hypothesis. In figure 47, we identify a signal overlay corresponding to peaks for the identification of our LMNG detergent in the sample buffer. The data from this spectrum strongly supports the previous hypothesis regarding our agonist's interactions with the sample buffer detergent. When we analyse the 15.5mM fluoxetine hydrochloride (magenta line: without LMNG and sigma-1) we see a complete disappearance of the peak, which is to be expected for this reference sample. However, when we analyse the other samples, we see distinct peaks at varying intensity for the concentration of LMNG in the samples. Firstly, when we see compare the LMNG concentration for the 50µM sigma-1, in 0.2% LMNG 15mM fluoxetine hydrochloride (green peak) against the 15mM fluoxetine hydrochloride (red peak: control sample) in 0.2% LMNG with no sigma-1, it is clear to see a difference in LMNG concentration. The 50µM sigma-1, in 0.2% LMNG 15mM fluoxetine hydrochloride appears to have a greater LMNG concentration than that of the control sample.

Testing Sample 1: 50µM S1R in 0.1% LMNG (Control Without Drug)

Testing Sample 2: 50µM S1R (0.2% LMNG) & 15mM Fluoxetine Hydrochloride

Sample 3: 15mM Fluoxetine Hydrochloride (0.2% LMNG & No Sigma-1) Control

Sample 4: 15mM Fluoxetine Hydrochloride (No LMNG & Sigma-1)

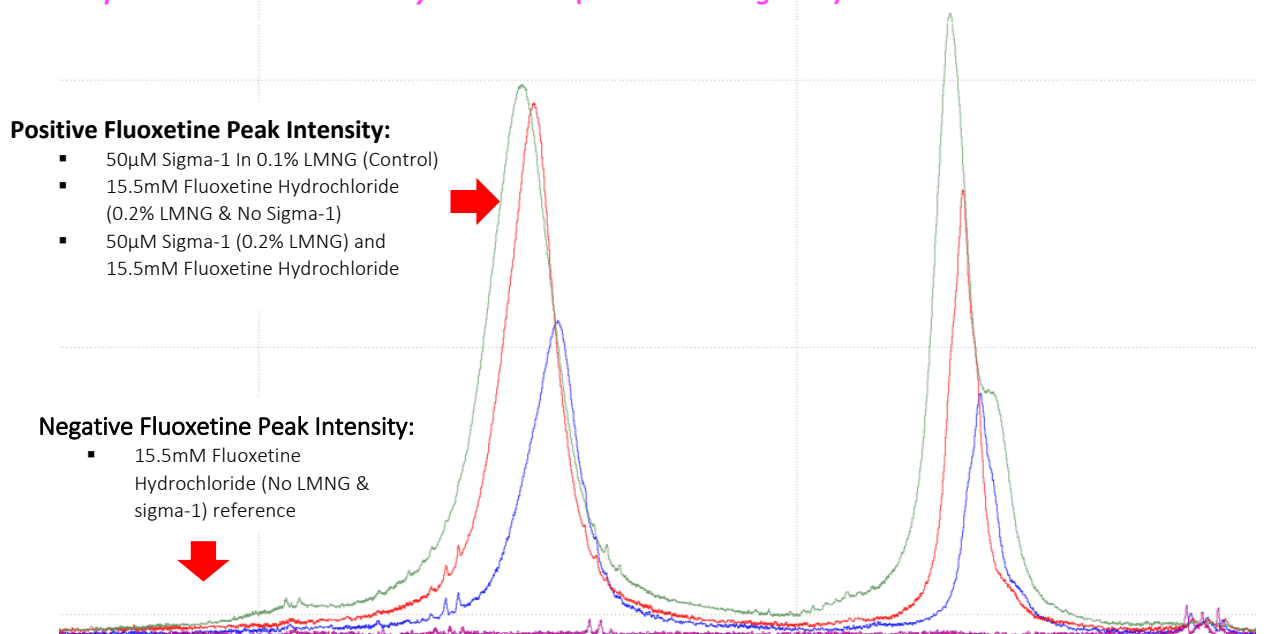


Figure 48: Magnified image of Peak Intensities: 1D proton NMR (15.5mM fluoxetine hydrochloride) spectra overlay, of all varying sample conditions for the experiment.
Magnified Peak intensities of 1D Proton NMR Spectra Overlay

This makes it difficult to determine whether the sigma-1 receptor is directly binding to the agonist or if the LMNG detergent is binding to the drug. Although we could not conclude whether the drug was binding to micelles or the sigma-1 receptor, the data retrieved from the experiments highlights the difficulties in detergent based methods. Unfortunately, as we were unable to successfully solubilise the sigma-1 receptor in nanodiscs we are unable to compare this to the data obtained for our detergent based protocol. Furthermore, these NMR experiments demonstrate the potential for further NMR research into using C43 cell lines as an expression system for the sigma-1 receptor. In addition, it would be advisable in future structural research to determine whether different expression systems result in biochemical changes to that of expressed protein. As current expression systems in eukaryotic and prokaryotic organisms offer varying post translational modifications to the synthesised protein depending on the cell line of choice.

3.19 Protocol Optimisation Considerations for The Expression of The Sigma-1 Receptor

The successful expression and purification of the sigma-1 receptor has proved to be a challenging objective given the results obtained during his project. Although we were able to express the sigma-1 receptor using the C43 cell line, we were still unable to effectively solubilise the receptor using nanodisc technology and in INSVc1. This was more than likely due protocol issues regarding expression and solubilisation of this receptor due to some of the unexpected results obtained. One of the most important optimisation considerations for this project should be an increased analysis when performing cloning for a designed construct. As we did not implement DNA electrophoresis, nor send our *LEU2* final construct for sequencing it is likely that the sigma-1 fragment was not correctly inserted to this vector. This should be a crucial optimisation if further research is to be conducted, as this will avoid potential false positive results and reduce time spent in the expression of the receptor in this system.

Furthermore, it is also vital that the solubilisation of the sigma-1 receptor is optimised to identify the best suited nanodisc technology for its extraction. In this project we predominantly used SMA 2:1 and SMA imide 1:1 nanodisc solubilised copolymers for this extraction. It may be necessary to try the formulations of copolymer resins such as the SMA imide 2:1 or the SMA 3:1, to identify whether these produce better results. In addition, it may be advisable to assess these nanodiscs further using methods such as dynamic light scattering (DLS) and Zita-potential to assess the structural size, molecular weight and stability of these disc formations, once the sigma-1 receptor has been extracted. This would enable us to assess whether these nanodiscs are degrading, as this would result in the insolubility of the receptor and therefore result in protein precipitation in solution. Currently, there is little research that has optimised the use of these nanodiscs for membrane protein extraction. Therefore, factors such as pH and storage temperature may be necessary to determine in an optimised protocol.

4. Conclusion

The sigma non-opioid intracellular receptor 1 is a highly important yet poorly understood membrane chaperone protein. Previous publications have also concluded the challenges they have encountered when trying to express and solubilise this protein in almost all expression systems. Studies such as that of the 2016 *Shmidt et al* study, have highlighted the usefulness of using expression systems such as Sf9 insect cells in producing high yield and stable sigma-1. Our findings also clearly correlate with the challenges of previous publications into the expression of this protein. After a profound number of attempts to solubilise the receptor in C43 using SMA 2:1 and SMA imide 1:1 nanodiscs, we failed in doing so. This can also be said for the expression and all solubilisation techniques used in the INVSc1 cell line.

However, a positive outcome of this project was that we were able to successfully extract and solubilise the receptor in C43 using varying concentrations of LMNG and CHS. In a last attempt before attempting expression in Sf9 insect cells, we

confirmed the isolation of the receptor via western blot. This was a significant achievement as very few publications have been able to successfully express this receptor in prokaryotic cell lines. The successful expression of the sigma-1 receptor in C43 with detergent based techniques may reduce the time and resources for its research. It is well publicised that both mammalian and insect cell lines require a significant level of time to transfect and expressed desired proteins. Therefore, it may be necessary for future research to optimise the C43 expression system for the sigma-1 receptor and analyse its biochemical functionality when solubilised. Overall, the data obtained in this research project whether that be positive or negative results, allows future researchers of this protein to optimise its expression to higher standards. In addition, for future research it be interesting to determine whether the sigma-1 receptor can be successfully expressed in yeast cell lines and if upcoming nanodisc technology offers a better level of solubility then current detergent-based methods.

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