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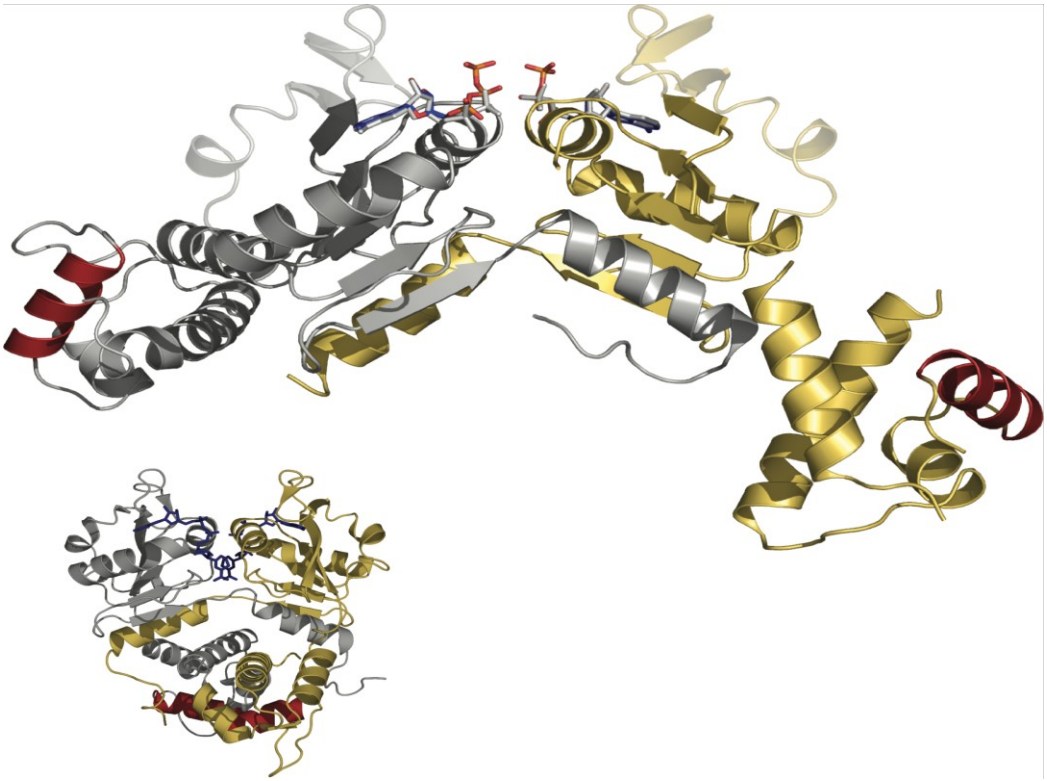
Structure and function of a bacterial redox sensor.



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I would like to convey my gratitude to my mother; you are the greatest in my life, thank you so much for praying for me, for being always there for me, for your support, understanding, and encouragement. Wish you happiness and be always together.

My husband Adel; thank you for all your efforts to keep me happy and smiling. I hope one day I can return some of what you have done for me. I wish us together a great future full of happiness, achievements and success (together for ever).

Abstract

Gram-positive bacteria e.g. *Streptomyces coelicolor* [8], *Bacillus subtilis* [43] and *Staphylococcus aureus* [40] contain a novel sensor for the nicotinamide adenine dinucleotide (NAD) redox balance that functions as a transcriptional repressor called Rex (redox). Rex plays a key role in regulating genes in energy metabolism. It also modulates transcription of important genes for cellular redox homeostasis including genes for alternative metabolic pathways and oxidative stress responses [42]. The current Rex structures have provided novel insights into dinucleotide regulation of gene expression. However, to obtain a deep and complete understanding, additional Rex structural forms are required. In this project wild type and mutant variants of Rex from *Streptococcus agalactiae* were expressed in *Escherichia coli*. The wild type Rex (with or without a His6-tag) was purified, and the affinity to NADH was measured with a novel technique called MicroScale Thermophoresis (MST). To test the effect of mutation on the dissociation constant (K_d) of Rex for NADH binding, the following mutations were investigated; His6-Rex-triple mutant (S158A, E160A and S180T) and two single mutations; His6-Rex-R24S and His6-Rex-D130R. MST analysis revealed that the K_d value for Rex/NADH binding was ~5 µM while, the measured K_d for His6-Rex/NADH binding was ~50 µM. The difference in the measured K_d values might be due to an effect of the His6-tag.

Key words: Rex protein, Microscale thermophoresis (MST), dissociation constant (K_d)

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List of abbreviations

ADP	Adenosine Di Phosphate
AMP	Adenosine Mono Phosphate
ATP	Adenosine Tri Phosphate
Carb	Carbenicillin
dH_2O	deionized H_2O
DNA	Deoxynucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
GF	Gel filtration
His6	6 Histidine residues
His-tag	Histidine-tagged
IEC	Ion Exchange Chromatography
IMAC	Immobilized Metal-Affinity Chromatography
IPTG	Isopropyl ß-D-1-thiogalatopyranoside
Kan	Kanamycin
K_d	Affinity Constant (dissociation constant)
lacI	Lactose repressor
lacO	Lactose operator
lacY	lac permease
mRNA	messenger Ribonucleic Acid
MST	MicroScale Thermophoresis
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
PCR	Polymerase Chain Reaction
(Q)	Quinone pool
Rex	Redox
ROP	Rex-Operator
PMF	Proton motif force
SAR	Structure Activity Relationship
SEC	Size Exclusion Chromatography
SDS-PAGE	Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis

1. Introduction

Bacteria have a fantastic ability to adapt to environmental changes [1]. They can't only synthesize a wide variety of proteins to control all different situations, but also regulate when these proteins are available. One situation where the bacteria are regulating which proteins are made is when they encounter stress. Stress is a chemical or a physical environmental change that can affect important cellular physiological processes or can cause macromolecular cellular damage. So that, the bacterial cell responds by what is called; cellular stress response, to increase the tolerance towards that stress and adapt to the new situation [2]. Pathogenic bacteria are mostly under stress all the time as they are facing the host defense response [3]. By understanding these mechanisms of bacterial stress response, new antimicrobial targets can be discovered and more infectious diseases can be cured.

This study focuses on a transcription regulator called Rex that senses the redox poise in the cytoplasm.

1.1 Cell metabolism and Redox regulator (Rex)

Cell metabolism is a network of chemical reactions carried out by living cell to help extracting energy from the environment to build up macromolecules and perform the biological processes [4]. Cell metabolism includes both catabolism and anabolism, where in catabolic reactions (e.g. Glycolysis), the fuels e.g. carbohydrates, fats and proteins are converted into useful energy of adenosine triphosphate (ATP) which is used later in anabolic reactions (e.g. Gluconeogenesis) to build up cellular macromolecules [5]. Reduction and oxidation (redox) reactions play an essential role in the cell metabolism, as they provide the cell with the most energy supply through the different steps of the metabolic pathways. For example, in the electron transport chain during

cellular respiration, a proton motif force (PMF) is converted into a phosphoryl transfer potential in the form of ATP providing the required free energy for all physiological processes (figure 1) [6].

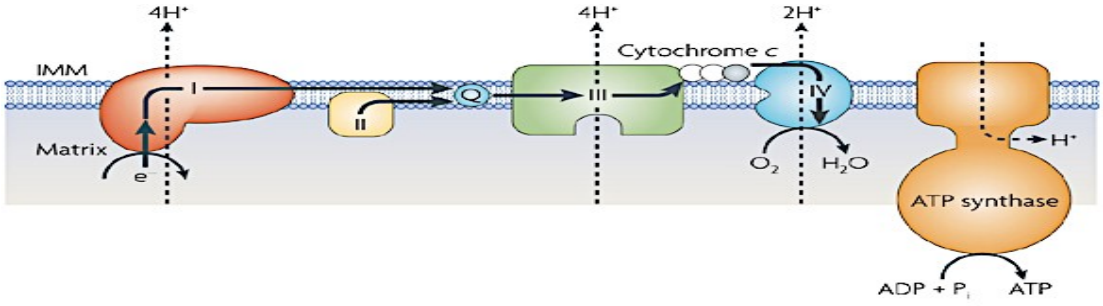


Figure 1: During the electron transport chain, electrons flow through a series of proton (H^+) pumps. The released energy builds a H^+ gradient across the inner membrane. During oxidative phosphorylation; ATP synthase catalyzes ATP synthesis using energy from the H^+ gradient across the membrane. Figure from [6].

It is crucial for the cell to sense the redox balance status and adapt to any changes that affects it. This can be achieved by regulating the required genes, which encode for the proteins in charge of this task. Rex plays a key role in regulating genes in energy metabolism in several bacteria [34]. It senses the redox status by sensing changes in the nicotinamide adenine dinucleotide (NAD) redox poise through direct binding to NADH or NAD^+ [7]. NAD is a ubiquitous redox molecule that is an essential cofactor for many enzymes involved in redox reactions. The oxidized form of NAD carries a positive charge and is donated NAD^+, while the reduced form is NADH. Reductive stress is a result of conditions that enhance the formation of excessive intracellular concentrations of NADH. Changes in ($NADH/NAD^+$) ratio affect a plethora of cellular processes and is associated with longevity and several human diseases [8]. In addition, the activity of many enzymes is regulated by ($NADH/NAD^+$) ratio. Thus, the cellular balance between NAD^+ and NADH acts as a messenger of the global metabolic state of the cell. In facultative aerobic bacteria, catabolism of the fuel molecules is associated with the

reduction of NAD^+ to NADH. During the transition to oxygen-limited growth, NADH is less efficiently reoxidized to NAD^+ and an increased level of NADH builds up as a result of the reduced respiration [9]. These roles of NADH and NAD^+ provide a link between energy homeostasis and gene regulation. In most Gram-positive bacteria a novel sensor of the NAD redox balance is present [10].

1.2 Rex Structure Activity Relationship (SAR)

This thesis shed a light on the effect of cytoplasmic NAD redox balance on the structure and function of Rex for what is known as Structure Activity Relationship (SAR). Rex senses NAD redox balance by functioning as a DNA binding protein that has a transcriptional suppression effect on some respiratory genes under adequate availability of oxygen [11]. Rex was first characterized in *Streptomyces coelicolor* [8], later crystal structures of Rex from different bacterial species have been determined, e.g. Rex from *Thermus aquaticus* [12], *Thermus thermophiles* [13], *Bacillus subtilis* [14] and *Streptococcus agalactiae*. To study SAR of Rex, additional Rex structures need to be determined. Rex is composed of two domains: an N-terminal domain that adopts a winged helix-turn-helix fold that interacts with DNA and a C-terminal NADH binding Rossmann fold domain (figure 2) [14]. Rex is a homodimer, ~24 kDa molecular mass per each monomer, stabilized by swapping of the C-terminal α-helices. In complex with NADH, the two N-terminal domains pack close to each other in a compact dimer that is unable to bind DNA [14].

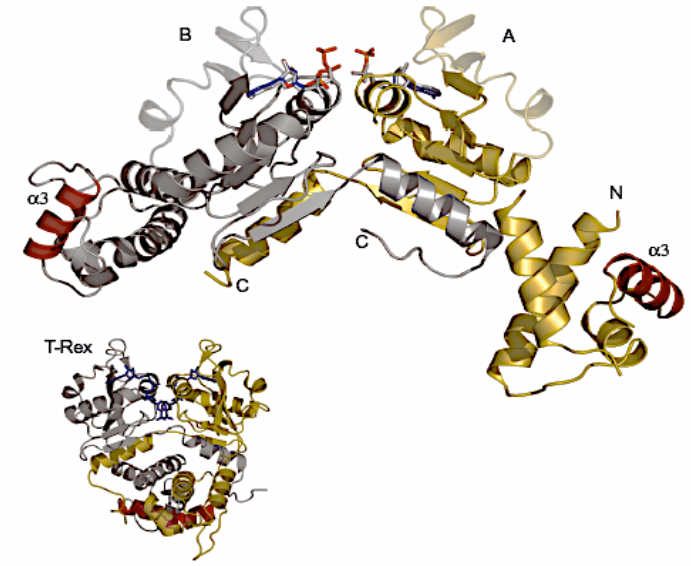


Figure 2: Overall crystal structure of the *B. subtilis* Rex (B-Rex) dimer with ATP bound. The view is perpendicular to the approximate twofold symmetry axis of the C-terminal domains. The A and B monomers are colored gold and grey, respectively. The presumed DNA binding helices, denoted alpha3, are highlighted in red. The bound ATP molecules are shown as stick models. The conformation of the observed ADP moiety (grey stick models) of NADH in crystals of B-Rex soaked with NADH is superimposed on the ATP molecules. Inserted is the structure of the closed form of T-Rex (PDB code 1DT5) with NTDs packed under the CTD dimer core, for comparison, figure from [14].

1.3 Aerobic and anaerobic shift effect on transcription regulation

Under aerobic growth conditions, Rex through its N-terminal domain binds to Rex-Operator (ROP) on the major grooves of the DNA molecule-binding site and functions as a transcriptional repressor of respiratory genes promoters. Under aerobic conditions, NADH is rapidly oxidized to NAD^+ by NADH-dehydrogenase [15,16]. Rex is bound to NAD^+ and can bind to DNA. Under microaerobic conditions, NADH is accumulated and binds to Rex resulting in conformational changes that inhibits its binding to DNA (figure 3).

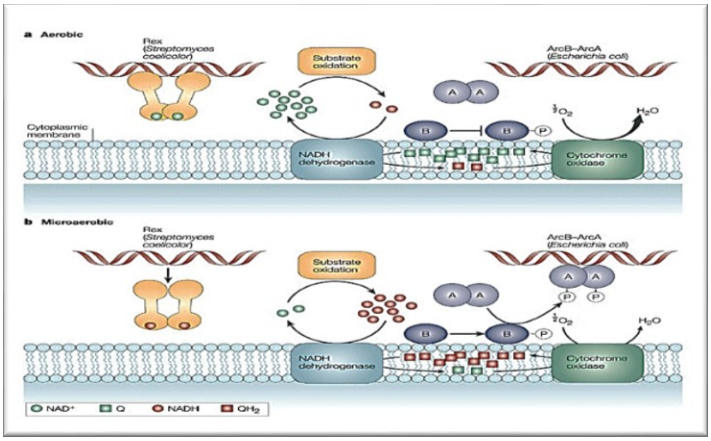


Figure 3: Under aerobic conditions, NADH generated by substrate oxidation is rapidly re-oxidized to NAD^+ by NADH dehydrogenase. The electrons are ultimately passed on to oxygen by way of the quinone pool (Q) and a terminal cytochrome oxidase. Under these conditions, the redox states of the NAD (H) and Q pools are likely to be oxidized. Under microaerobic conditions these respiratory intermediates will accumulate in the reduced state as electrons accumulate in the pathway. Under aerobic conditions, Rex binds to Rex-Operator (ROP) on the major grooves on DNA molecule and functions as a repressor of respiratory genes promoters. Under microaerobic conditions NADH binds to Rex, resulting in conformational changes and Rex can't bind the DNA molecule. Figure from [15].

1.4 Rex three conformational states

Rex from *B. subtilis* exists in three distinct states: Apo form (a flexible state), in complex with DNA and in complex with NADH (figure 4). Upon binding to NAD^+, Rex structure is rigidified and the conformational flexibility is limited, so that it's binding to DNA is stabilized in presence of NAD^+. However, Rex affinity for NADH is 10000 times higher than its affinity for NAD^+, so even at relatively low concentrations of NADH, it binds to Rex, resulting in a conformational changes that abrogates DNA binding [16].

2. Scope and aim of the project

The aim of the current work is to determine the K_d of the wild type as well as different mutants of *S. agalactiae* Rex proteins to NADH and DNA by MST analysis.

The project work plan can be summarized in the following points:

- Development of efficient production and purification procedure for *S. agalactiae* Rex proteins.
- Optimization of MicroScale Thermophoresis (MST) analysis of Rex-NADH interaction.
- Determination of the binding affinities of the purified Rex proteins to NADH.
- Studying the effect of specific Rex mutations on NADH binding.

3. Materials and Methods

MilliQ-water purified with Synergy water-purification system (Millipore, Billerica, MA, USA) was used in all experiments, unless otherwise stated.

3.1 Stock solutions

All stock solutions were sterile.

Table 1: list of stock solutions used in this study

	Item	Conc.
1	Isopropyl ß-D-1-thiogalatopyranoside (IPTG).	1 M
2	Kanamycin (Kan), stored at -20°C.	50 mg/ml
3	Carbenicillin (Carb), stored at -20°C.	100 mg/ml
4	Dithiothreitol (DTT), stored at -20°C.	1 M
5	Di-sodium ethylenediaminetetraacetic acid (Na-EDTA), pH 8.0, stored at room temperature.	0.5 M
6	Ethidium bromide, stored at room temperature.	10 mg /ml

3.2 Culture media

- Luria Bertani broth (LB) (Difco™, Detroit, MI, USA)

The medium was prepared according to the manufacturer's instructions, 25 g LB powder was dissolved in 1 L deionized H_2O (dH_2O), autoclaved at 121°C for 20 min and stored at room temperature. Before usage, Kan or Carb were added to a concentration of 50 µg/ml, and 100 µg/ml, respectively.

- Terrific Broth (TB), Difco™ (Detroit, MI, USA)

The medium was prepared according to the manufacturer instructions, 46.7 g TB powder was dissolved in 1 L dH_2O, 4 ml glycerol was added, the medium was autoclaved at 121°C for 20 min and stored at room temperature. Before usage, Kan 50 µg/ml or Carb 100 µg/ml were added.

- LB agar plates (LA)

For 1L of prepared LB broth, 15 g agar (Bacto™, Mt Pritchard, NSW, Australia) was added prior to autoclaving. After tempering the media to 50°C, Kan 50 µg/ml or Carb 100 µg/ml were added. Plates were sterile poured and stored at 4°C.

3.3 Buffers

The buffers used in this study were listed in table 2, see appendix; all buffers were filtered with 0.45 µm or 47 mm Nylon Membrane Filter, Nylafo™ (Pall Corporation, Port Washington, NY, USA). All used chemicals were lab gradients.

3.4 Bacterial strains used for cloning and expression

E. coli TOP 10 competent cells (Life Technologies, Waltham, MA, USA) were used for cloning and propagation of plasmids, while *E. coli* Tuner(DE3) competent cells (Novagen, Madison, WI, USA) were used for protein expression. *E. coli* Tuner(DE3)

strain contains the gene for T7 polymerase, it also has a mutation in the lac permease (*lacY*) gene, which allows uniform uptake of IPTG in the whole cell population.

3.5 Plasmids constructs

Plasmids; pNIC28_Strep_*rex* and pET101_Strep_*rex* are the expression vectors for *S. agalactiae rex*. pNIC28_Strep_*rex* plasmid is 5995 base pairs (bp), it contains multiple cloning sites, one of which is for EcoRV that divides the plasmid into ~2000 and ~4000 bp. The vectors also code for resistance markers to Kanamycin and Carbenicillin, respectively.

3.6 Primers

Forward and reverse DNA primers for both (R24S) and (D130R) mutations (table 3) were purchased from Invitrogen (Waltham, MA, USA). They bind to template DNA in back to back manner.

Table 2: Forward and reverse DNA primers for (R24S) & (D130R) mutations.

Mutation	Primer	Oligo sequence (5 ´to 3´)
R24S	R24S_mut_F	CTA CCG TAT TTT TAA ATC TTT TAA TAC TGA TGG C
	R24S_rev_R	TAC AGT GAT AAA CGT TTA GCA GTT GCT TTA GG
D130R	D130R_mut_F	GGT AAA ACA ACC GAG CGT GGC ATT CCT GTC
	D130R_rev_R	AAC TAA ATC ATT GCT ATC TAA ATC AAA AGC C

In this work, five different variants of *S. agalactiae rex* have been studied. The first and the second variants were constructed previously while others were constructed during this work. The first variant; pNIC28_Strep_*rex* referring to His6-Rex. The second variant refers to His6-Rex with triple mutations (S158A, E160A and S180T). The third

and the fourth variants represent His6-Rex with single mutations, (R24S) and (D130R), respectively. The fifth variant represents wild type Rex (figure 4).

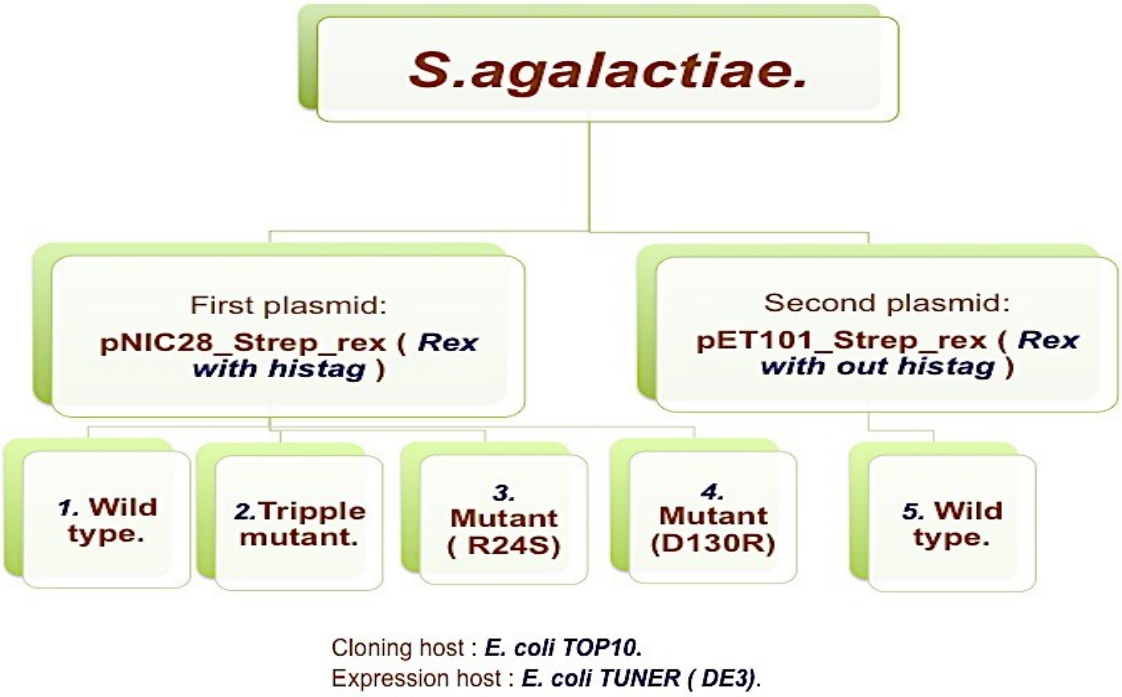


Figure 4: Flow chart represents the five types of *S. agalactiae* Rex proteins used in this study.

3.7 General molecular biology techniques

3.7.1 Plasmid extraction (isolation)

Plasmid extraction was performed according to the manufacturer's instructions (Gene JET Plasmid Midi-prep Kit, protocol B) (Thermo Scientific, Waltham, MA, USA).

3.7.2 Electroporation of *E. coli* Tuner (DE3) cells

40 µl of *E. coli* Tuner (DE3) cells thawed on ice were mixed with 1 µl of plasmid of interest in an Eppendorf tube held on ice. The mixture was transferred to a Gene Pulser Cuvette with 1 mm electrode gap (Bio-Rad, Hercules, CA, USA), avoiding air bubbles and held on ice. Then the cuvette was wiped with a dry Kleenex and placed in the Gene Pulser cuvette holder and pulsed in the Gene Pulser machine (Bio-Rad, Hercules, CA, USA) with the following settings: 1.8 kV, 200 Ω and 25 µFD for 10 sec. Directly after

pulse, the cells were suspended in 1 ml SOC medium (Invitrogen, Waltham, MA, USA) and transferred to a sterile glass tube and incubated at 37°C for 30-60 min. Transformed cells were plated on selective LA plates (100 µl / plate), and incubated upside down overnight at 37°C.

3.7.3 Chemical transformation of *E. coli* TOP 10 cells

50 µl of *E. coli* Top 10 cells thawed on ice were mixed with 5 µl of the ligation mixture in a cold microtube. The microtube was incubated for 30 min on ice, then for 45 sec in a 42°C water bath, then for another 2 min on ice, finally 450 µl of SOC medium were added. After that, the cells were incubated at 37°C, 200 rpm for 1 h. Transformed cells were plated on selective LA plates (100-200 µl/plate), and incubated upside down at 37°C overnight.

3.7.4 Expression trials

E. coli Tuner (DE3) strain harboring the plasmid of interest was grown overnight at 37°C on an LA plates with appropriate antibiotics to prepare the preculture. The colonies were resuspended in 2 ml growth medium with an inoculation loop. The preculture cells suspension was transferred to a glass tube and the OD_{600} nm was measured, growth medium alone was used as a blank. The preculture was used to inoculate 25 ml growth medium supplemented with appropriate antibiotics on 250 ml Erlenmeyer baffled flasks with indentations to OD_{600} 0.1[41]. One flask for induction, and another one for uninduced control were inoculated. Cells were grown at the desired temperatures, 160 rpm with frequent OD_{600} nm monitoring. When the OD_{600} nm reached ~0.6, 0.4 mM IPTG was added, 1 ml samples were taken every 1h. For the uninduced control, 1 ml was taken just before the induction of the induced flask. Samples were directly

centrifuged in a micro centrifuge (>8000 rpm, 5 min at 4°C). Collected cell pellets were resuspended in 0.25 ml of cold Tris buffer and stored at -20°C.

3.7.5 Monitoring the microbial growth

The microbial cell growth was monitored by measuring the turbidity of the bacterial culture with U-1100 Spectrophotometer (Hitachi, Japan). Cell free medium was used as a blank and the absorbance was measured at 600 nm.

3.7.6 Determination of DNA and protein concentrations

The concentration of DNA and proteins in solution were measured with NanoDrop™, ND-1000 Spectrophotometer with ND-1000 software (Thermo Scientific, Waltham, MA, USA). The absorbance of 1 µl sample at 260 nm was used to measure DNA concentration while; the absorbance of 2 µl sample at 280 nm was used to measure protein concentration. The calculated protein molecular weight and the extinction coefficient were specified before measuring.

3.7.7 SDS-PAGE gel electrophoresis

SDS-PAGE was used for separating and detecting proteins. Samples were either prepared from collected cell pellets or collected during protein purification. The frozen culture samples were thawed and sonicated on ice for 3×20 seconds (Vibra cell disrupter, output control 40-60, Pulser 3) (Heinrichstraβe, Berlin, Germany) or until complete cell lysis was achieved. 40 µl of the lysed culture were saved (whole cell fraction W) and the remaining 210 µl were centrifuged (20 000 g, for 45 min at 4°C) to remove cell debris. 40 µl of the supernatant was collected (Soluble fraction S), and stored on ice. 40 µl of each fraction were mixed with 10 µl of 5x SDS sample buffer, heated at 95°C for 10 min, cooled in ice. 10 µl of protein samples and 10 µl of PageRuler™ plus Prestained protein ladder (Thermo Scientific, Waltham, MA, USA)

were loaded in Mini-PROTEAN^{R} TGX^{TM} Gels (Bio-Rad, Hercules, CA, USA) and the electrophoresis started with 1x TGS buffer as a running buffer. Gels were stained with Coomassie blue staining buffer and destined with dH_2O. ChemicDoc^{TM} systems together with image lab software (Bio-Rad, Hercules, CA, USA) were used for gel imaging.

3.7.8 Site-Directed mutagenesis and cloning of (R24S) and (D130R) *S. agalactiae* Rex mutants

Site-Directed Mutagenesis, agarose gel electrophoresis, DNA ligation, cloning of the PCR product into pNIC28_Strep_*rex*, chemical transformation into *E. coli* TOP10, plasmid purification, restriction enzyme analysis for plasmid verification, and electroporation transformation into *E. coli* Tuner (DE3) cells were performed according to the manufacturer instructions "Phusion Site-Directed Mutagenesis Kit for point mutation" (Back to back method) (Thermo Scientific, Waltham, MA, USA).

3.7.9 Expression and purification of His6-Rex protein

The preculture of *E. coli* Tuner (DE3) cells carrying pNIC28_Strep_*rex* plasmid prepared as before was used to inoculate 2 L TB medium with Carb. 100 µg/ml (2×1 L in 5 L baffled E-flasks). Cells were grown at 37^{o}C, 200 rpm until OD 600nm of ~ 0.6, temperature was decreased to 30^{o}C and 15 min later 0.4 mM IPTG was added to induce expression of *rex* gene. The culture was continued at 30^{o}C, 200 rpm for 4 h with frequent sampling. The cell pellet was recovered by centrifugation (5000 rpm, 20 min at 4^{o}C; Avanti J-20XP, Beckman Coulter, Fullerton, USA) and re-suspended in 100 ml ice-cold 50 mM potassium phosphate buffer (pH 7.5)-EDTA free, as EDTA is incompatible with the His-trap column. One tablet of cOmplete EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added. Then another centrifugation (5700 rpm,

20 min at 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA) was done and the pellet was then frozen at -20°C. Later on, the cell pellet was thawed, re-suspended in 35 ml ice-cold 50 mM potassium phosphate buffer (pH 7.5). One tablet of cOmplete EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany) was also added. Then, the cell suspension was lysed (two-three) passes through a French press operated at 18000 psi. The cell suspension was centrifuged at high speed (100 000 g, 60 min, 4°C; Beckman L2-65B ultracentrifuge using an SW 56 rotor; Beckman Coulter, Fullerton, USA) to pellet insoluble cellular debris and get clear supernatant. The supernatant containing Rex protein was then filtered with Acrodisc® 32 mm Syringe Filter with 0.2 µm Super® Membrane (Pall Life Sciences, Port Washington, NY, USA). The filtered Rex solution was purified from the soluble cell fraction using "Äkta Avant preparative chromatography system" (GE Healthcare Bio-science AB, Uppsala, Sweden) with a Ni-NTA column (HisTrap HP 1 ml; GE Healthcare Bio-science AB, Uppsala, Sweden). The soluble fraction was mixed with the wash buffer and loaded to the column, the column was washed 5-10 column volume with the wash buffer to remove any unbound proteins. The purified protein was gradually eluted with the elution buffer. Eluted fractions containing Rex protein as well as the flow through and the wash fractions were collected. To remove imidazole, the protein fractions were dialyzed against 50 mM potassium phosphate buffer (pH 7.5); a fresh 1 L buffer was added after approx. 2 h and the dialysis was continued overnight. Rex protein was concentrated to approximately 5 mg /ml with Centriprep concentrators, 10 kDa cut-off (Millipore, Billerica, MA, USA). Rex protein solution was then sterile filtered with Millex®, Syringe Driven Filter Unit, 0.22 µm sterile (Millipore, Billerica, MA, USA) and stored at 4°C. Pure Rex was used directly for MST analysis.

3.7.10 Expression and purification of His-tag-free wild type Rex protein

The preculture of *E. coli* Tuner (DE3) cells harboring pET101-Strep-*rex* plasmid prepared as described earlier was used to inoculate 2 L TB medium supplemented with 100 µg/ml Carb. (2 × 1 L in 5 L baffled E-flasks) [41]. The culture was incubated at 37°C, 200 rpm until OD600 nm of ~0.6, then 0.4 mM IPTG was added to induce protein expression and the culture was continued at 37°C, 200 rpm for 4 h. At the end of the cultivation, the cells were recovered from the culture supernatant by centrifugation (5000 rpm, 20 min at 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA). The cell pellet was resuspended in 100 ml ice-cold buffer A. One tablet of cOmplete EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added and another centrifugation (5000 rpm, 20 min at 4°C; Avanti J-20XP, Beckman Coulter, Fullerton, USA) was also applied, the resultant pellet was stored at -20°C. Later on, the cell pellet was thawed, resuspended in 35 ml ice-cold buffer A, another tablet of cOmplete EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany) was added. For cell lysis, the cell suspension was passed (two-three) passes through a French press operated at 18000 psi. The cell lysate was centrifuged at high speed (100 000 g, 60 min, 4°C; Beckman L2-65B ultracentrifuge using an SW 56 rotor; Beckman Coulter, Fullerton, USA) to remove the insoluble cellular debris and get clear supernatant. The supernatant containing Rex was then filtered with Acrodisc® 32 mm Syringe Filter with 0.2 µm Super® Membrane (Pall Life Sciences, Port Washington, NY, USA). The filtered solution containing Rex protein was loaded directly to a Q-Sepharose HP column (2.6 ×10 cm) (GE Healthcare Bio-science AB, Uppsala, Sweden) pre-equilibrated with buffer A. The purified protein was gradually eluted over 20 column volumes with elution buffer (buffer A supplemented with 1 M NaCl). The eluted fractions were pooled and concentrated to ~10 ml by ultrafiltration Centriprep concentrators, 10 kDa cut-off (Millipore, Billerica, MA, USA). The concentrated 10 ml

protein sample was further loaded to a gel filtration column packed with Superdex 75 resin (2.6×0.6 m) (GE Healthcare Bio-science AB, Uppsala, Sweden). Purified Rex protein was eluted as a single peak with elution buffer (buffer A supplemented with 0.1 mM EDTA and 150 mM NaCl, pH 7.5), fractions were pooled and diluted with equal volume of 50 mM potassium phosphate buffer (pH 7.5). Due to the high binding affinity of Rex to NADH and the structural similarity between AMP (Adenosine Mono Phosphate) and NADH (8), another purification step was applied. The purified protein was loaded to 5'-AMP -Sepharose 4B column (1.6×6 cm) (GE Healthcare Bio-science AB, Uppsala, Sweden) pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.5). After complete protein loading, the column was extensively washed 10 column volumes with the same buffer to remove any unbound proteins, purified Rex was gradually eluted with elution buffer (buffer A supplemented with 1M NaCl, pH 7.5) over 20 column volumes. Eluted Rex was concentrated by ultrafiltration with Centriprep concentrators, 10 kDa cut-off (Millipore, Billerica, MA, USA) to ~4 ml, then dialyzed against 100 mM potassium phosphate buffer (pH 7.5). The final protein solutions were concentrated as previously described to 3 mg/ml. Pure Rex was then used directly for MicroScale Thermophoresis analysis.

3.7.11 MicroScale Thermophoresis (MST) analysis (NanoTemper Technologies, München, Germany)

As mentioned earlier, MST detects the affinity constant (K_d) of Rex and its ligand (NADH or DNA) complex, through detection of changes in the hydration shell, charge, or molecular size. These changes can be measured through detection of changes in the molecules mobility in microscopic temperature gradients. Rex labeling was performed according manufacturer instructions (Monolith NT™ Protein Labeling Kit RED-NHS; Kit for 1 h labeling of proteins with the RED fluorescent dye NT-647-NHS for use in Microscale Thermophoresis, NanoTemper Technologies, München, Germany).

Protein labelling protocol can be divided in two main steps: A) protein labelling and B) purification of the labelled protein.

A) Protein labelling, the following steps have been followed:

- The protein concentration was adjusted to 20 µM with 100 mM phosphate buffer and use 100 µl of protein solution.
- 30 µl of buffer were added to the solid fluorescent dye to get approx. 435 µM solution.
- The dye was thoroughly mixed and completely dissolved by vortexing.
- The dye was diluted with 100 mM phosphate buffer to a concentration 2-3 fold protein concentration.
- Protein and Dye were mixed in a 1:1 ratio (200 µl final volume).
- The dye-protein mixture was incubated for 30 minutes at room temperature in the dark. In the meantime, prepare Step C

B) Purification, the aim of this part is to purify the labelled protein from any unreactive "free" dye. It includes the following steps:

- Storage solution in column-B was poured off, the bottom cap was removed and placed in 15 ml tube.
- The column-B was extensively washed 3 x 3 ml and equilibrated with 100 mM phosphate buffer.
- 500 µl of labeling reaction was added to the center of Column-B and allowed to penetrate the bed completely, and the flow through was discarded.
- The tube was placed in a new 15 ml collection tube.
- 600 µl buffer were added, the eluate was collected with discarding the first few µl.

- The ratio of protein/dye was measured spectroscopically (absorption at 280 nm for Rex protein and 650 nm for the dye, Molar absorbance: 250.000 M^{-1} cm^{-1}), and protein was stored at 4ºC.

MST experiment, sample loading, use of Monolith NT, 115-instrument and MST data analysis were performed according to the instructions of User Manual for the Monolith NT.115, NANO TEMPER technologies. Two MST experiments were done for His6-Rex, and His-tag-free wild type Rex. The first experiment was done for His-tagged Rex with 42 and 10 000 μM concentrations of Rex and NADH, respectively. The second experiment was done for His-tag free Rex, with 18.8 $\times 10^{-3}$ and 100 μM concentrations of Rex and NADH, respectively.

4 Results

4.1 The first variant; His6-Rex

In previous studies native Rex have been expressed and purified from *E. coli*. To simplify purification a Rex variant with a polyhistidine-tag at the N-terminus was constructed. Transformation of pNIC28_Strep_*rex* into *E. coli* Tuner (DE3) resulted in ~ 400 colonies / plate. Small-scale production of His6-Rex resulted in soluble protein with an increased Rex concentration over the expression time (figure 5).

Large-scale protein expression under the same conditions as small-scale resulted in soluble and nearly constant concentration of Rex all over the expression time (figure 6).

His6-Rex was purified with IMAC, eluted with imidazole and the eluted fractions were pooled together (~ 5.5 ml). The efficiency of the purification procedure was monitored on SDS-PAGE and the protein was purified to homogeneity (figure 7).

For additional purification and aggregates removal, pooled fractions from the IMAC step were subjected to gel filtration with Superdex 75 resin. The purified protein was eluted with DTT-free gel filtration buffer and the purity was checked on SDS-PAGE and showed a homogenous single protein band indicating pure protein (figure 8). The protein concentration in the eluted fractions was measured with NanoDrop and it was ~ 1 mg/ml. The purified Rex protein was dialyzed for desalting and the protein concentration after dialysis measured by NanoDrop was ~1 mg/ml. The purified Rex was used for MST analysis. The measured K_d by MST analysis of purified Rex protein with NADH as a ligand was ~ 50 µM (figure 9).

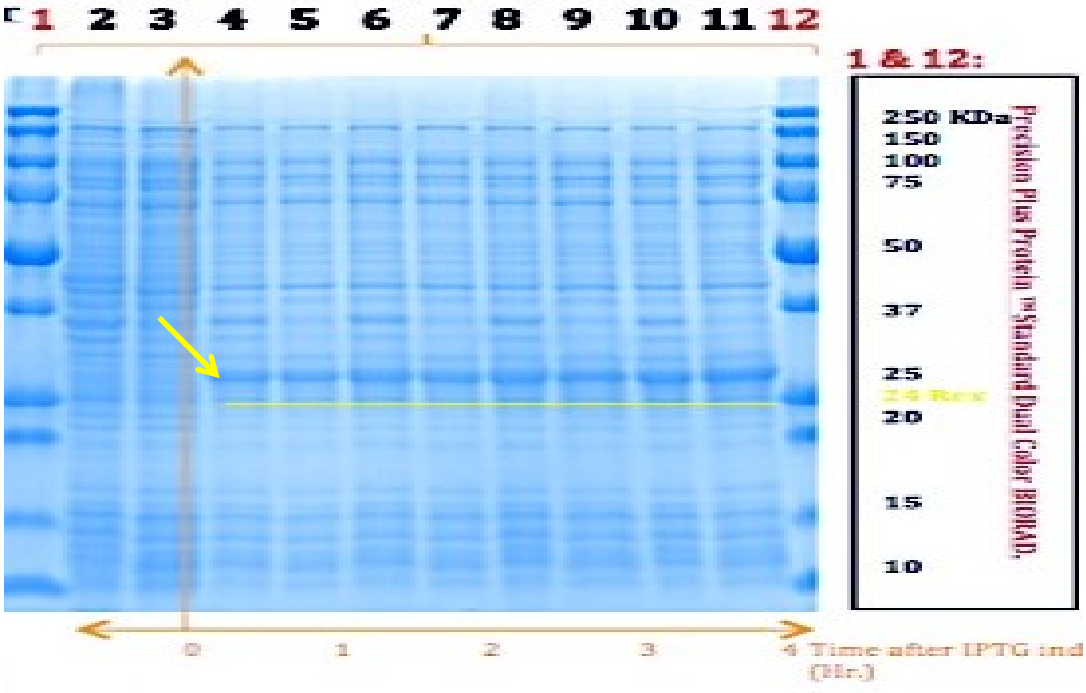


Figure 5: SDS-PAGE of His6-Rex small-scale expression. **L1** & **L12**: Protein marker, **L2**: Whole cell lysate of blank (uninduced) culture, **L3**: Soluble fraction of blank (uninduced) culture, **L4**: Whole cell lysate fraction after 1 h induction, **L5**: Soluble fraction after 1 h induction, **L6**: Whole cell lysate fraction after 2 h induction, **L7**: Soluble fraction after 2 h induction, **L8**: Whole cell lysate fraction after 3 h induction, **L9**: Soluble fraction after 3 h induction, **L10**: Whole cell lysate fraction after 4 h induction, **L11**: Soluble fraction after 4 h induction. Bands at ~24 kDa correspond to Rex protein.

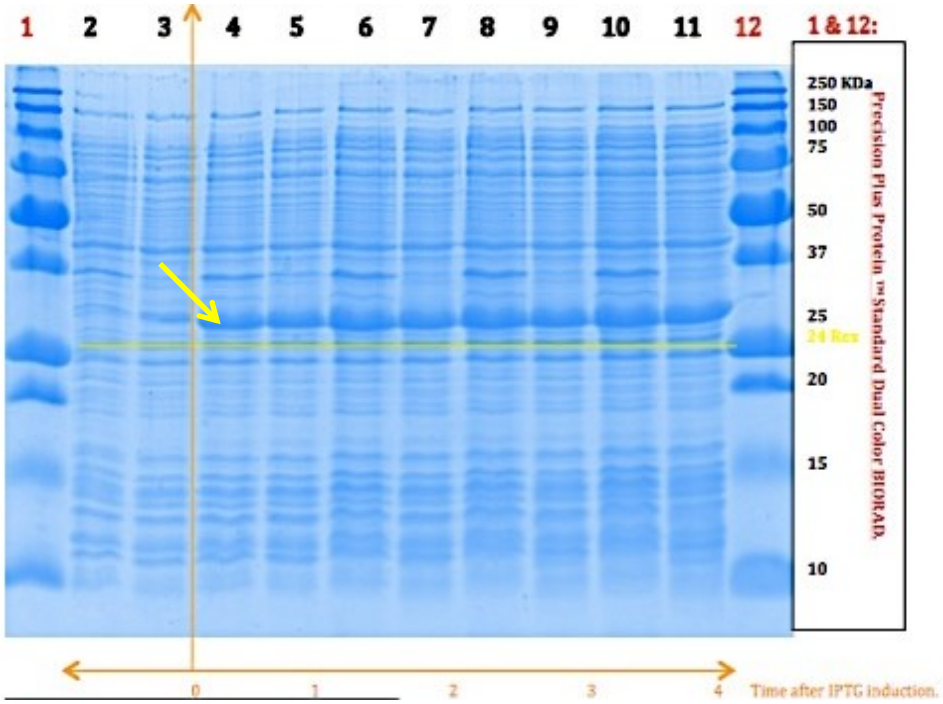


Figure 6: SDS-PAGE of His6-Rex large-scale expression. **L1** & **L12**: Protein marker, **L2**: Whole cell lysate of blank (uninduced) culture, **L3**: Soluble fraction of blank (uninduced) culture, **L4**: Whole cell lysate fraction after 1 h induction, **L5**: Soluble fraction after 1 h induction, **L6**: Whole cell lysate fraction after 2 h induction, **L7**: Soluble fraction after 2 h induction, **L8**: Whole cell lysate fraction after 3 h induction, **L9**: Soluble fraction after 3 h induction, **L10**: Whole cell lysate fraction after 4 h induction, **L11**: Soluble fraction after 4 h induction. Bands at ~24 kDa correspond to Rex protein.

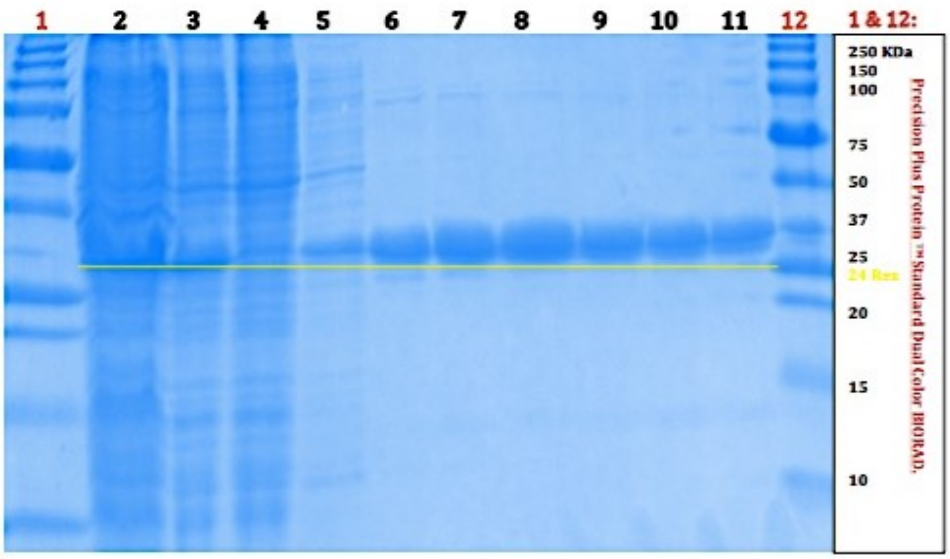


Figure 7: SDS-PAGE of IMAC purification process of His6-Rex. **L1** & **L12**: Protein marker, **L2**: Whole cell lysate before loading, **L3**: Soluble fraction before loading, **L4**: Flow through, **L5**: Column wash, **L6-L11**: Eluted fractions showing a single band of pure protein at ~24 kDa corresponding to Rex protein.

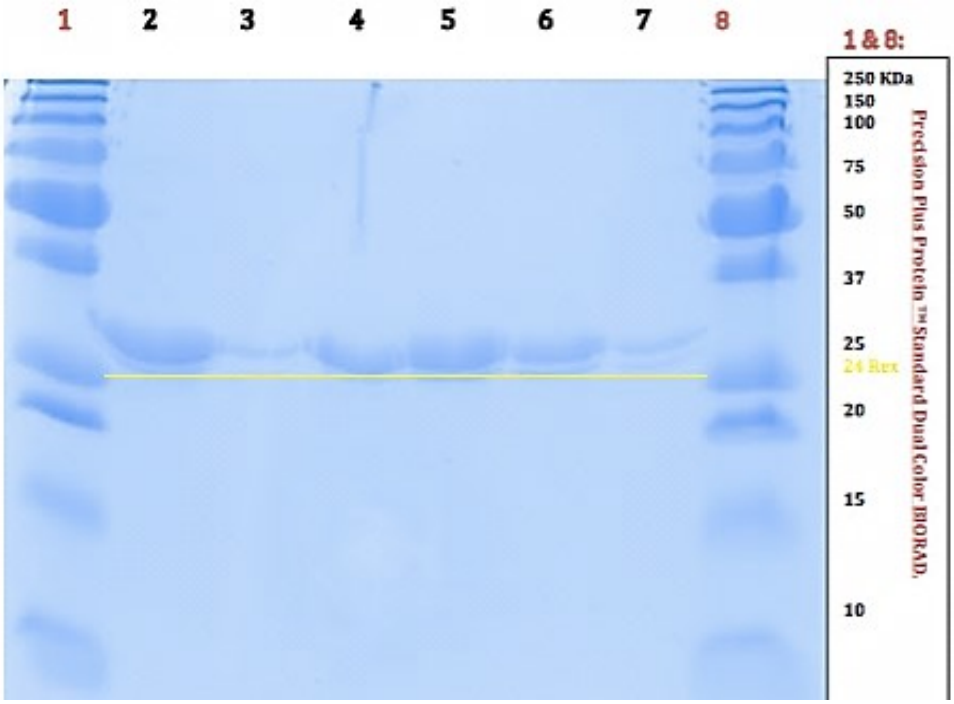


Figure 8: SDS-PAGE of gel filtration step of His6-Rex protein. **L1, L8**: Protein marker, **L2:** Sample before loading to Superdex 75 column, **L3-L7:** Eluted fractions showing single band of pure protein at ~24 kDa corresponding to Rex protein. Fractions 4 & 5 were selected for dialysis step due to their highest purity and concentration.

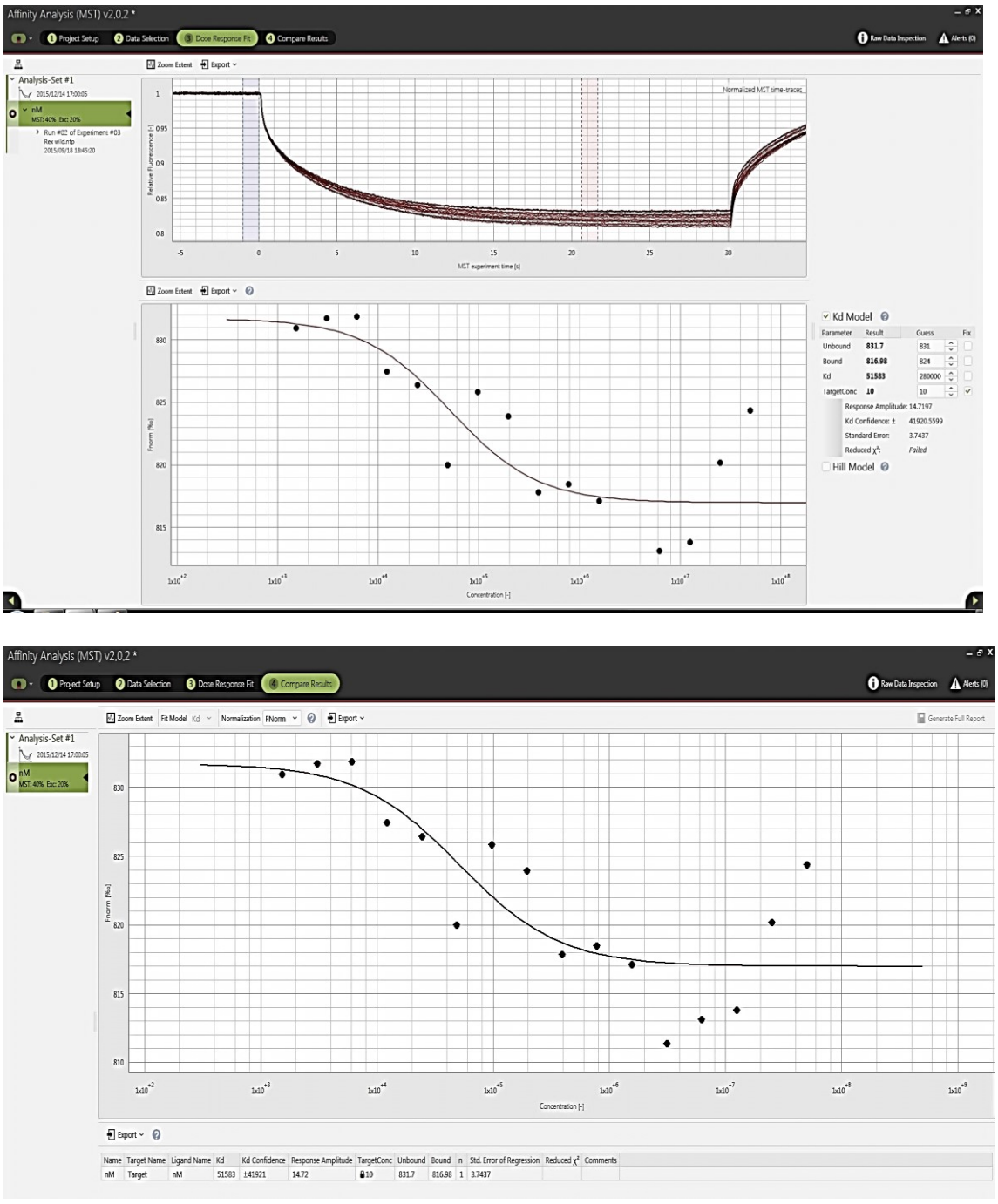


Figure 9: MST analysis of purified His6-Rex protein with NADH as a ligand, the measured K_d was ~ 50 µM. The molar concentration of Rex was 42 µM and for NADH, the starting concentration of the serial dilutions was 10 000 µM.

4.2 The second variant; His6-Rex-triple mutant (S158A, E160A and S180T)

In previous studies (S158A, E160A and S180T) mutations on Rex were predicted to have important effect on Rex binding affinity so the triple mutant with the three mutations was purified so that its binding affinity could be tested. The pNIC28_Strep_*rex*_triple mutant plasmid was extracted and purified from *E. coli* TOP10/ pNIC28_Strep_*rex* strain, the concentration of the purified plasmid measured by NanoDrop was ~67.8 ng/µl. The transformation efficiency of pNIC28_Strep_*rex*_triple mutant into *E. coli* Tuner (DE3) was ~ 200 colonies / plate.

Large-scale expression of His6-Rex triple mutant was performed under the same experimental conditions as the wild type, and the protein was expressed in a soluble form. The culture was harvested, cells were lysed using French press, then ultracentrifugation was done to remove cellular debris.

The protein in the whole cell lysate was purified through IMAC, eluted gradually with imidazole and the eluted fractions were pooled together. The efficiency of the purification process and the purity of the protein were monitored on SDS-PAGE, and the protein was purified to homogeneity (figure 10).

For additional purification and aggregates removal, pooled fractions from IMAC step were subjected to gel filtration with Superdex 75 resin. The purified protein was eluted with DTT-free gel filtration buffer and pure protein was obtained as seen on SDS-PAGE (figure 11).

The eluted fractions were pooled together and stored at 4^{o}C. The purified His6-Rex triple mutant protein was dialyzed for desalting, but the desalted protein was turbid and this might be due to the effect of the triple mutations on the stability of S. Rex protein.

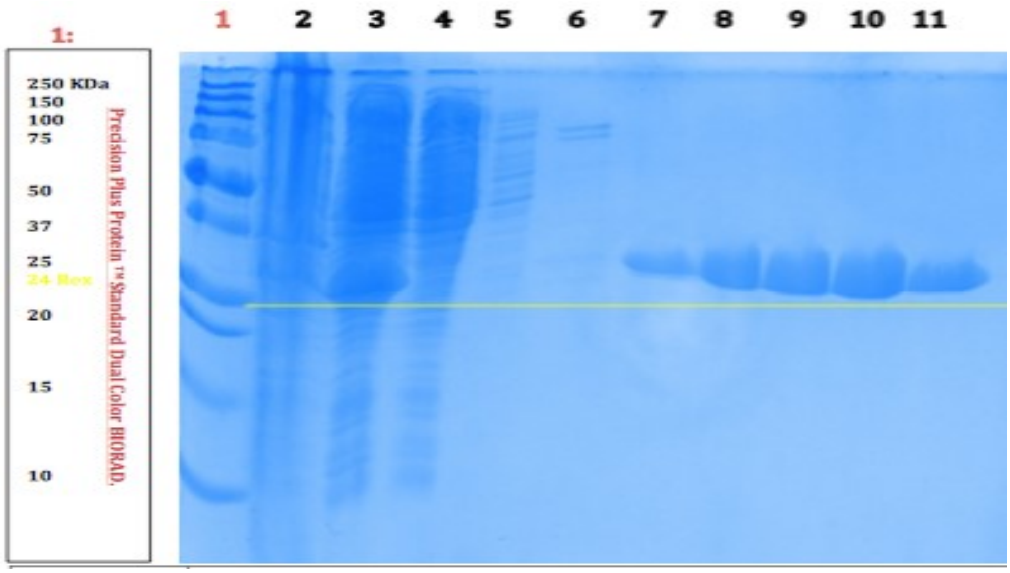


Figure 10: SDS-PAGE of IMAC purification of His6-Rex triple mutant protein. **L1**: Protein marker, **L2**: Whole cell lysate, **L3**: Soluble fraction, **L4**: Flow through, **L5**: Column wash, **L6-L11**: Eluted fractions showing single band of pure protein at ~24 kDa corresponding to Rex protein.

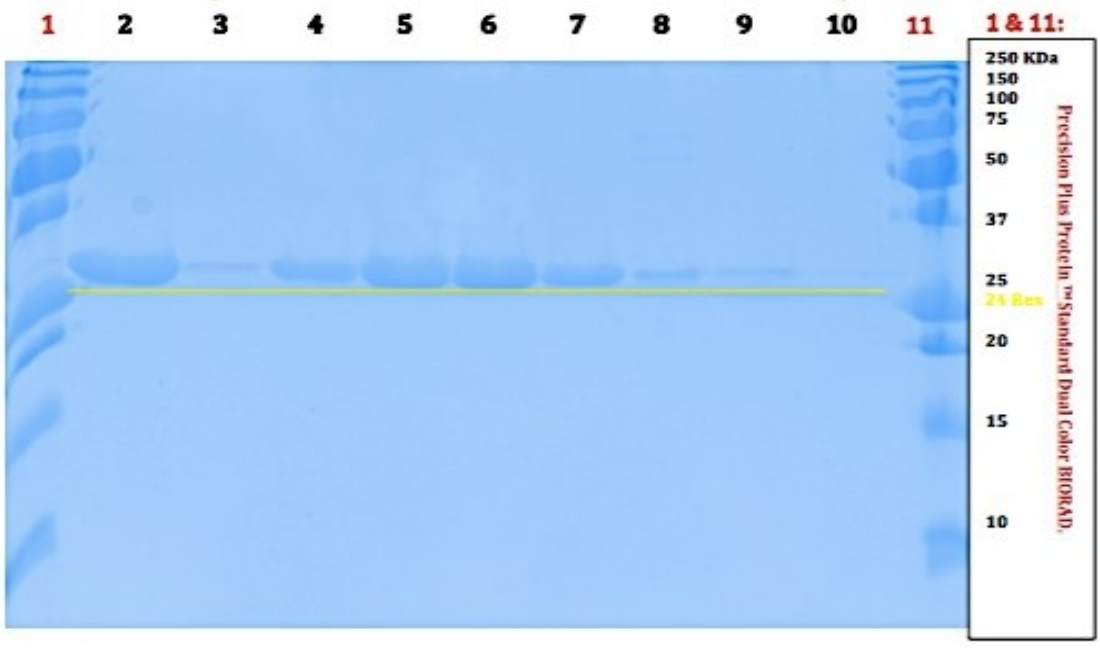


Figure 11: SDS-PAGE of gel filtration step of His6-Rex triple mutant protein. **L1, L11**: Protein marker, **L2:** Sample before loading to Superdex 75 column, **L3-L10:** Eluted fractions showing single band of pure protein at ~24 kDa corresponding to Rex. Fractions 4, 5, 6 & 7 were selected for dialysis.

4.3 The third (R24S) and the fourth (D130R) variants; His6-Rex with single site directed mutations

Also in previous studies (R24S and D130R) mutations on Rex were predicted to have important effect on Rex binding affinity so the two single directed mutations were done on Rex and the mutants were purified so that their binding affinity could be tested. (R24S) and (D130R) point mutations were successfully introduced into His6-Rex wild type; the PCR was verified with agarose gel electrophoresis (figure 12 A&B). The amount of PCR product was evaluated from the gel by comparing the sample band intensity with the known amount of DNA standard ladder, and it was ~ 75 ng for both R24S and D130R (figure 12 A&B). The plasmids pNIC28_Strep_*rex*_R24S and pNIC28_Strep_*rex*_D130R after ligation were successfully transformed into *E. coli* TOP10 with a transformation efficiency of ~ 300 colonies/ plate, later on the plasmids were extracted, purified and the concentration measured by NanoDrop was ~50 ng/µl for both. Restriction analysis of pNIC28_Strep_*rex*_R24S and pNIC28_Strep_*rex*_D130R by EcoRV fast digest restriction enzyme showed two bands of ~2000 bp and ~4000 bp linear DNA fragments (figure 12 C). The sequencing results confirmed that R24S and D130R mutations have been correctly inserted. The resultant plasmids pNIC28_Strep_*rex*_R24S and pNIC28_Strep_*rex*_D130R were successfully transformed into *E. coli* Tuner(DE3) but with low efficiency ~ 200 colonies/ plate for both.

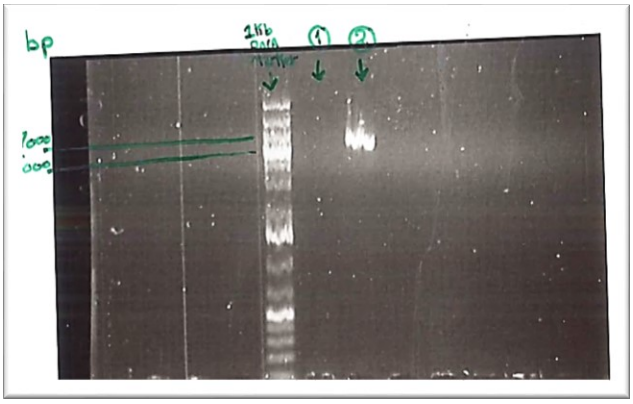
Small-scale expression trials of both R24S and D130R mutants showed acceptable soluble protein expression levels over the expression time, the expression was monitored on SDS-PAGE (figure 13).

Large-scale expression under the same conditions showed good and stable soluble protein expression levels over the expression time with no increase in the expression

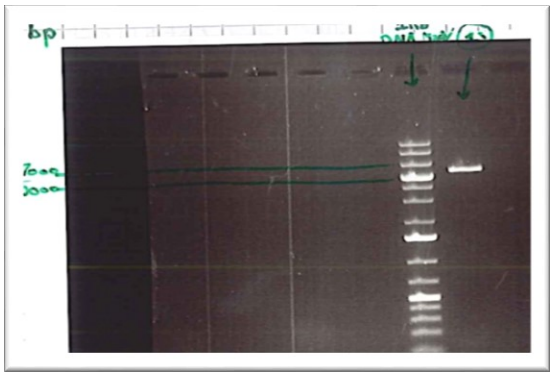
level with time. The cells were successfully lysed with French press then, ultracentrifugation was successfully done to remove cell debris.

IMAC purification of R24S Rex mutant was done and the protein was gradually eluted with imidazole, eluted fractions were pooled together and the efficiency of the purification step was confirmed on SDS-PAGE (figure 14).

A



B



C

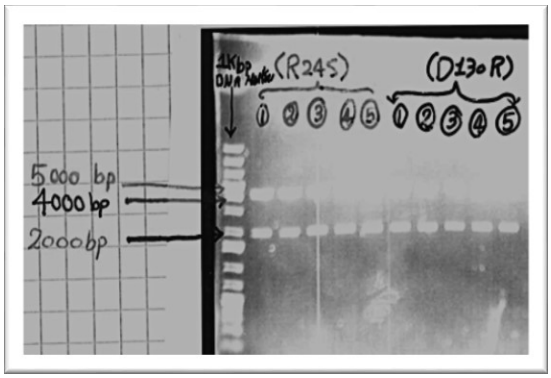


Figure 12: Agarose gel electrophoresis of **A)** PCR products amplification of pNIC28_Strep_*rex*_R24S, **B)** PCR products amplification of pNIC28_Strep_*rex*_D130R and **C)** Restriction enzyme analysis by fast digest *EcoR*V restriction enzyme for pNIC28_Strep_*rex*_R24S and pNIC28_Strep_*rex*_D130R plasmids.

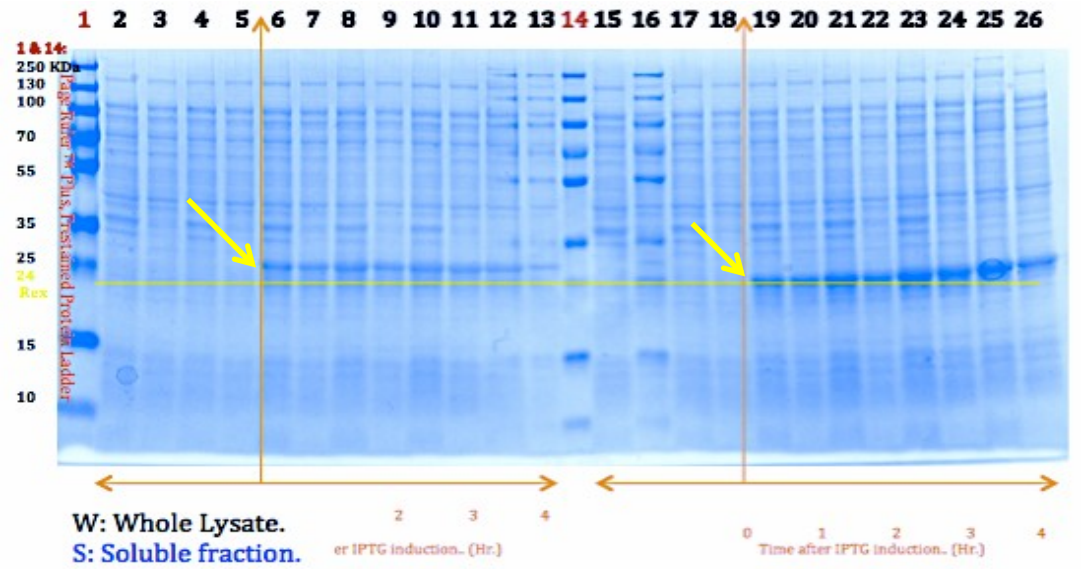


Figure 13: SDS-PAGE of small-scale expression of pNIC28_Strep_*rex* R24S and D130R His6-Rex mutant strains showing acceptable soluble protein expression levels over the expression time. **L1**: Protein marker, **L2**: Whole cell lysate of blank (uninduced) R24S Rex culture, **L3**: Soluble fraction of blank (uninduced) R24S Rex culture, **L4**: Whole cell lysate fraction of R24S Rex culture before induction, **L5**: Soluble fraction of R24S Rex culture before induction, **L6**: Whole cell lysate fraction of R24S Rex culture after 1h induction, **L7**: Soluble fraction of R24S Rex culture after 1h induction, **L8**: Whole cell lysate fraction of R24S Rex culture after 2h induction, **L9**: Soluble fraction of R24S Rex culture after 2h induction, **L10**: Whole cell lysate fraction of R24S Rex culture after 3h induction, **L11**: Soluble fraction of R24S Rex culture after 3h induction. **L12**: Whole cell lysate fraction of R24S Rex culture after 4h induction, **L13**: Soluble fraction of R24S Rex culture after 4h induction. **L14**: Protein marker, **L5**: Whole cell lysate of blank (uninduced) D130R Rex culture, **L16**: Soluble fraction of blank (uninduced) D130R Rex culture, **L17**: Whole cell lysate fraction of D130R culture before induction, **L18**: Soluble fraction of D130R Rex culture before induction, **L19**: Whole cell lysate fraction of D130R Rex culture after 1 h induction, **L20**: Soluble fraction of D130R Rex culture after 1 h induction, **L21**: Whole cell lysate fraction of D130R Rex culture after 2 h induction, **L22**: Soluble fraction of induced D130R Rex culture after 2 h induction, **L23**: Whole cell lysate fraction of D130R Rex culture after 3 h induction, **L24**: Soluble fraction of R24S Rex culture after 3 h induction. **L25**: Whole cell lysate fraction of D130R Rex culture after 4 h induction, **L26**: Soluble fraction of D130R Rex culture after 4 h induction. Protein bands at ~24 kDa corresponds to Rex.

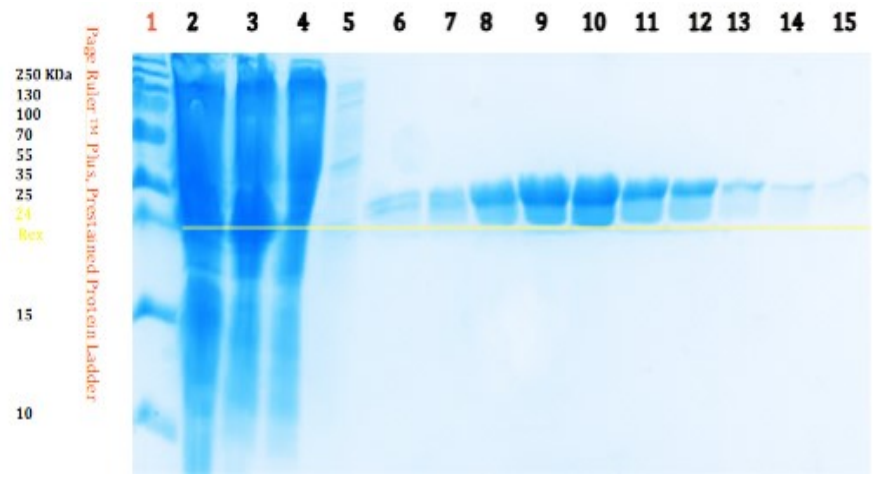


Figure 14: SDS-PAGE of IMAC purification step for His6-Rex R24S mutant. **L1**: Protein marker, **L2**: Whole cell lysate, **L3**: Soluble fraction, **L4**: Flow through, **L5**: Column wash, **L6-L15**: Eluted fractions showing single band of pure protein at ~24 kDa corresponding to Rex.

4.4 The fifth variant; His-tag-free wild type Rex

The measured K_d for His6-Rex done by MST analysis was far from predicted one so a variant of Rex without the his-tag was constructed to test the effect of the his-tag on Rex binding affinity. Large-scale expression of *E. coli* Tuner (DE3) /pET101-Strep-*rex* yielded soluble Rex protein; the expression efficiency was tested on SDS-PAGE, and showed homogenous pure protein (figure 15). The cells were successfully lysed with French press then, ultracentrifugation was successfully done to remove cellular debris. The cell lysate was loaded on to a Q-Sepharose column and the purified protein (122 ml) was eluted with 250-350 mM NaCl concentration. The protein was concentrated to 10 ml with Centriprep concentrators 10 kDa cut-off, and was further loaded on to a Superdex 75 column to remove any aggregates, the purified protein (5 ml) was eluted with solution 1 phosphate buffer. For additional protein purification, the property of high affinity of Rex to AMP was exploited and the 5 ml protein fraction was loaded to 5'-AMP-Sepharose 4B column, the purified protein (150 ml) was eluted at high salt

concentration ~1 M NaCl. After that, the protein was concentrated, buffer exchanged and desalted using Centriprep concentrators 10 kDa cut off to get 7 ml final volume with concentration of 3 mg/ml measured by NanoDrop. SDS-PAGE analysis was performed to follow up the whole purification process, with different purified Rex protein amounts 1, 2 and 4 µg were loaded and it showed clear bands indicating pure protein with no aggregates or any impurities figure 16.

MST analysis of purified His-tag-free Rex protein with NADH as a ligand showed the K_d was ~ 5 µM, the used concentrations were 18.8 $\times 10^{-3}$ µM and 100 µM for Rex and NADH, respectively (figure 17).

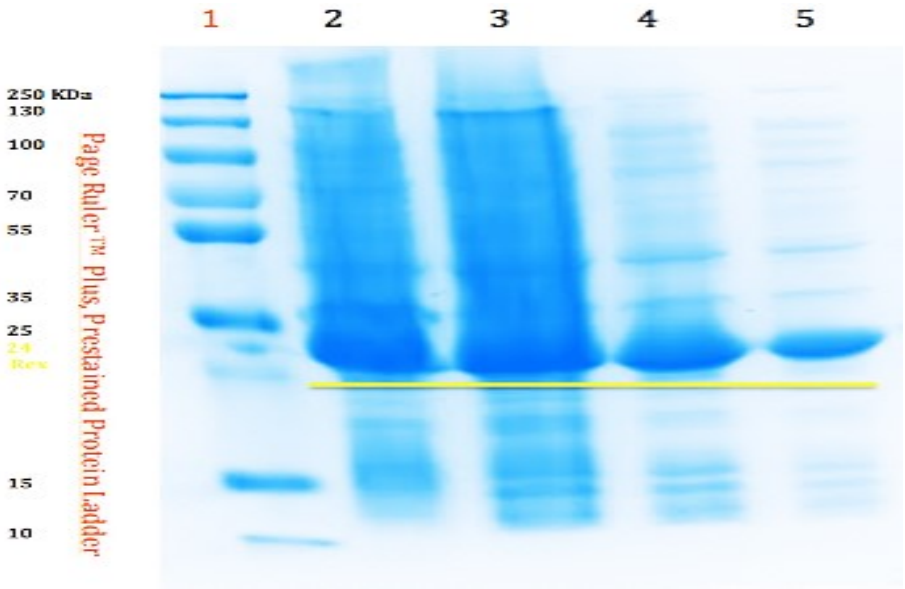


Figure 15: SDS-PAGE of large-scale expression of His-tag-free wild type Rex. **L1**: Protein marker, **L2 & L3**: Whole cell lysate, **L4 & L5**: Soluble fractions showing a band at ~24 kDa corresponding to Rex protein.

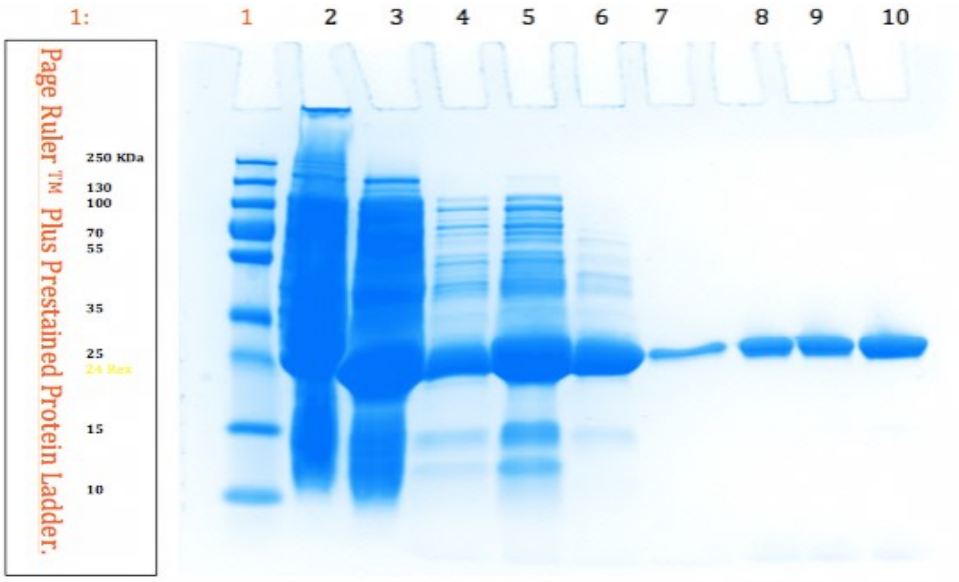


Figure 16: SDS-PAGE of His-tag-free wild type Rex whole purification procedure. **L1**: Protein marker, **L2**: Whole cell lysate, **L3**: Soluble fractions, **L4**: Pooled fractions eluted from Q-Sepharose, **L5**: Fraction collected after ultrafiltration with Centriprep concentrators 10 kDa cut off, **L6**: Pooled fractions eluted from gel filtration Superdex 75 column, **L7**: Pooled fractions eluted from AMP-Sepharose, **L8**: 1 µg loaded of purified protein, **L9**: 2 µg loaded of purified protein, **L10**: 4 µg loaded of purified protein. Protein bands at ~24 kDa correspond to Rex protein.

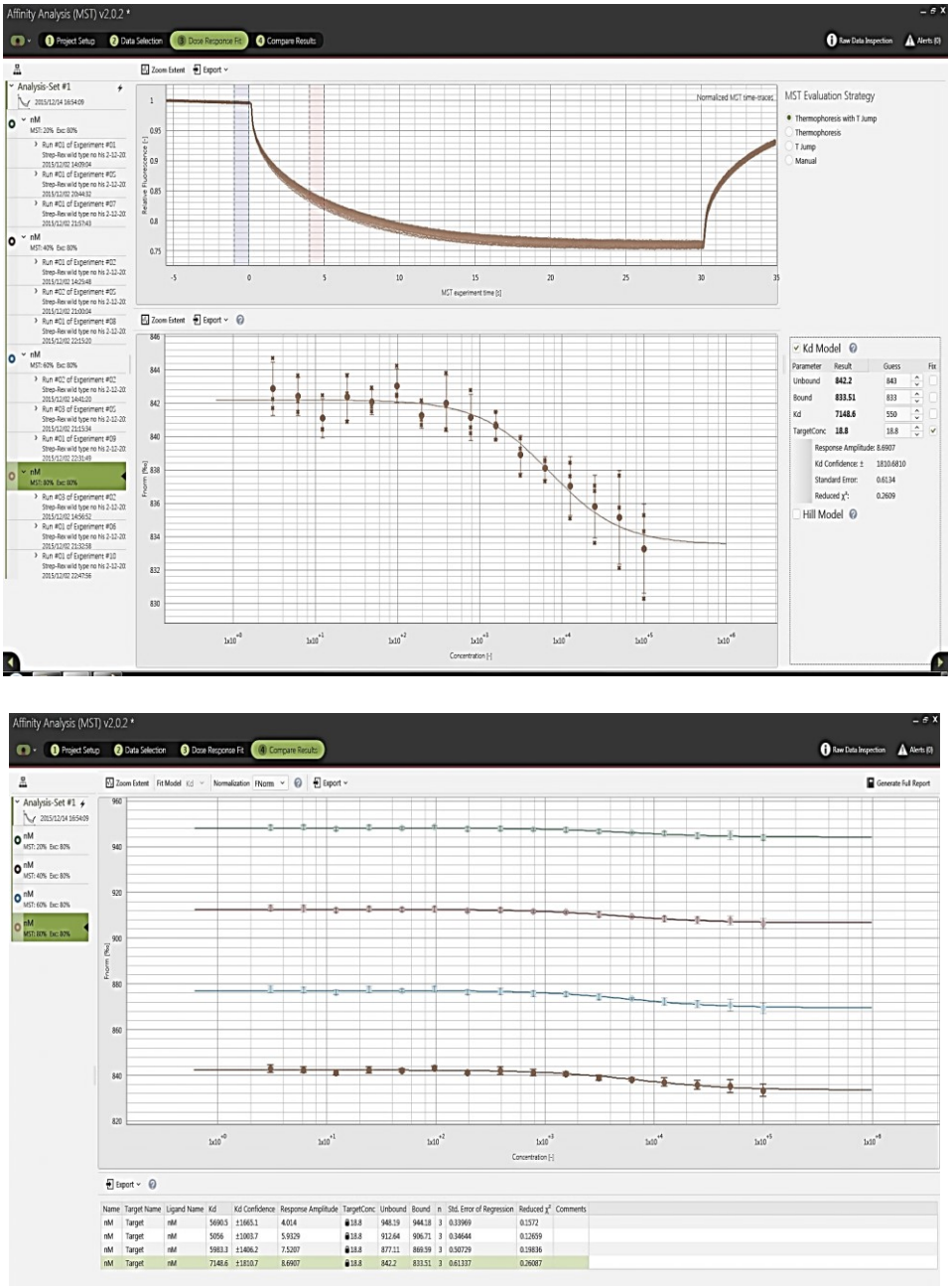


Figure 17: MST analysis of purified His-tag-free wild type Rex protein with NADH as a ligand, the measured K_d was ~5 µM. The molar concentration of Rex was 18.8×10^{-3} µM and for NADH, the starting concentration of the serial dilutions was 100 µM.

5. Discussion

Rex plays a key role in regulating genes in energy metabolism in several bacteria. It senses the redox status through changes in NAD redox poise, through direct binding of NADH or NAD^+ [7]. The aim of this work is to study wild type and mutants of *S. agalactiae* Rex (S. Rex) through development of efficient production and purification procedures, analysis of Rex-NADH interactions by MST. Besides, studying the effect of specific mutations on Rex stability and consequently their effect on Rex-NADH binding.

In this study, production of S. Rex proteins in *E. coli* was tested under different experimental conditions (temperature, growth media, incubation time, IPTG concentration, etc.) to get an optimum and efficient expression procedure. There are many reports for production of different Rex proteins in *E. coli* as an expression host; *B. subtilis* (B-Rex; PDB code 3IKT) [8], *S. coelicolor* (S-Rex; PDB code Q9WX14) [5], *T. aquaticus* (T-Rex; PDB code 1XCB) [32], *T. thermophilus* (Tt-Rex; PDB code 2DT5) [6], and the obligate anaerobe *Thermoanaerobacter ethanolicus* JW200 (RSP-Rex; PDB codes: RSP: 3WG9) [33].

In the current work, Rex proteins have been subjected to different purification steps (IMAC, Gel filtration, affinity chromatography) to get highly purified proteins.

Different techniques have been reported to measure the binding affinity of Rex to either DNA, NADH or NAD^+ for example: McLaughlin et al. [16] used surface plasmon resonance (SPR) to measure T-Rex affinities to DNA of *S. coelicolor* and the apparent K_d was ~12 nM. On the other hand, Ravcheev et al. [1] measured the apparent dissociation constant (K_d) of T-Rex protein interacting with DNA by fluorescence polarization binding assay (PF), while the effect of NADH/NAD^+ on the binding of T-Rex to DNA was measured by electrophoretic mobility shift assay (EMSA) [16]. EMSA was also used by other reserachers to measure the binding of the putative *E. faecalis*

Rex factor; EF2638-His6 to DNA [34]. On the other hand, Isothermal titration calorimetry is another technique that has been used to measure the K_d of *B. subtilis* Rex to NADH and NAD^+ [14].

In this study, we used MST for determination of the K_d of S. Rex to NADH.

MST is a recently introduced tool to characterize protein and small molecule interactions in buffers as well as biological fluids. MST has some more advantages over other K_d measurements tools; it works in free-solution with low sample consumption, it minimizes samples contamination (sample-free contact) as it is an entirely optical method in which sample contact is abolished. Moreover, MST is simple Mix & Read protocol that complex samples preparations are omitted [35]. Many protein-protein interactions, small molecule interactions had been characterized by MST; hIFN-γ and its anti-IFN-γ antibody [36], interaction of the non-NMDA receptor subunits iGluR2 and iGluR6 with different agonists [37], nucleic acid [38], G-protein coupled receptor and its ligand [39].

Rex senses the redox state by either binding to NAD^+ or NADH that enhance or inhibit the binding of Rex to target promoters, respectively [8,40].

Baker et al. [44] showed that Rex binds specifically to the *nox* promoter enhancing *nox* transcription, which is inhibited by NADH. Deletion of *nox* might impair regeneration of NAD^+, and low NAD^+ levels would lead to dissociation of Rex from target promoters. Rex homologs from *S. coelicolor*, *B. subtilis*, and *Thermus aquaticus* bind either NAD^+ or NADH with a strong preference to the later [8, 12, 14, 40]. High NADH levels abolish formation of Rex-DNA operator complex [8, 14, 40]. In contrast, NAD^+ doesn´t inhibit Rex-DNA binding but, competes with NADH for binding to Rex [8]. Indeed, NAD^+ enhanced DNA binding by *B. subtilis* Rex homolog [40].

In the current study, MST analysis showed Rex/NADH affinity constant (K_d) is ~50 μM, which was lower than what had been reported for Rex protein of *Staphylococcus*

aureus that has a K_d of 95 nM for NADH and 150 µM for NAD^+ [40]. Wang et al. [8] showed that Rex from *B. subtilis* has a higher affinity for NADH (24 nM) and lower affinity for NAD^+ (0.49 mM). The difference in K_d values might be attributed to the effect of Histidine tag (His6). To remove the effect of His6, His6-tag free wild type Rex was cloned, produced and purified under the same conditions. MST analysis of His6-tag-free wild type Rex/NADH interaction showed the K_d to be ~ 5 µM which is much lower than His6-Rex and this might be due to the effect of His6 on the binding affinity of Rex to NADH. As MST technique is so sensitive to the dilution effect, the difference between K_d ~5 µM of S. Rex and K_d ~ 0.5 µM of B-Rex might be due to handling and dilution errors. Or might be due to the difference between the techniques used in the measurement; Isothermal titration calorimetry for B-Rex versus MST for S. Rex. The difference in the Rex protein structures between the species or even the genus levels (*B. subtilis* and *S. agalactiae*) might affects the binding of Rex to NADH and contributes to the measured K_d between these two microorganisms.

Expression trials of His6-Rex-triple mutants in *E. coli* Tuner (DE3) resulted in variable and different optimum conditions (incubation time, induction temperature) rather the wild type and this might be the effect of the mutations on the gene transcription and protein production [45]. This effect was clearly observed on the physical properties of the protein; protein stability as the purified protein tended to aggregate directly after purification and this wasn´t noticed before in the wild type. The aggregation obstacle was overcome by centrifugation; the soluble part of the protein was used for protein characterization.

Expression trials of (R24S) and (D130R) His6-Rex mutants in *E. coli* Tuner (DE3) showed that 30ºC was the optimum condition to get soluble protein and this coincide with many reports lowering the induction temperature to avoid inclusion bodies or aggregated insoluble protein forms [46].

6. Conclusions

An efficient production and purification procedure for different variants of *S. agalactiae* Rex was established. Initial procedure of MST analysis of Rex-NADH interaction to determine the K_d value was done. We also could do specific mutations on Rex wild type and could see that they had negative effect on the protein expression, purification and stability. Consequently, these mutations are expected to affect Rex-NADH binding. This work lay the ground for future studies on Rex mutants, as well as the wild type Rex to provide an understanding of Rex structure and function properties.

7 References

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7. Appendix

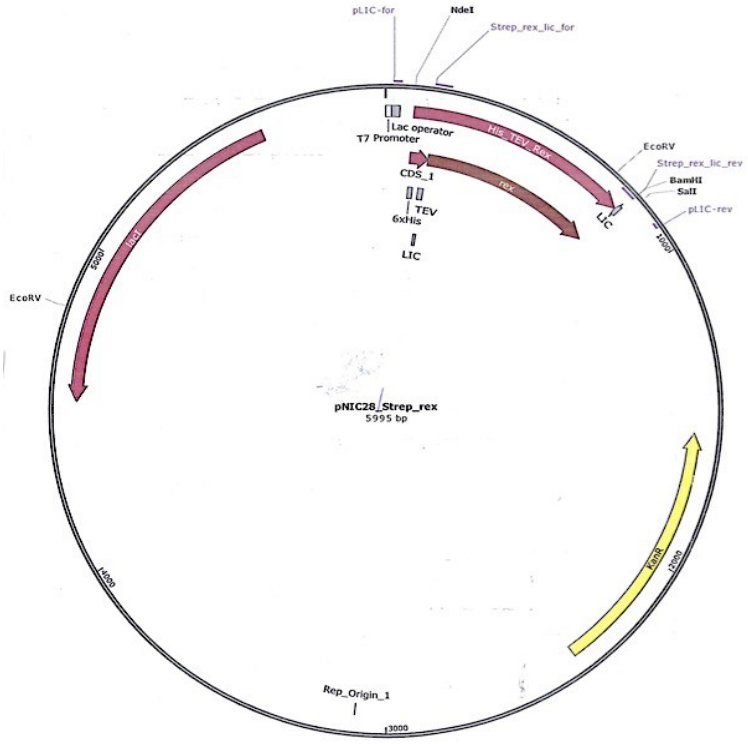


Figure 1. Plasmid map of pNIC28_Strep_*rex*, corresponds to His6-Rex.

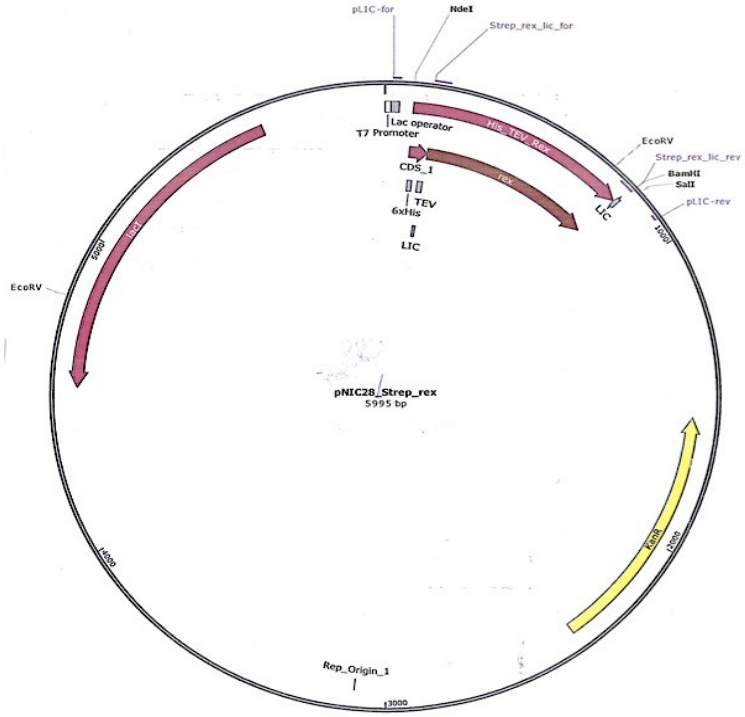


Figure 2. Plasmid map of pNIC28_Strep_*rex* corresponds to His6-Rex-mutated isotype.

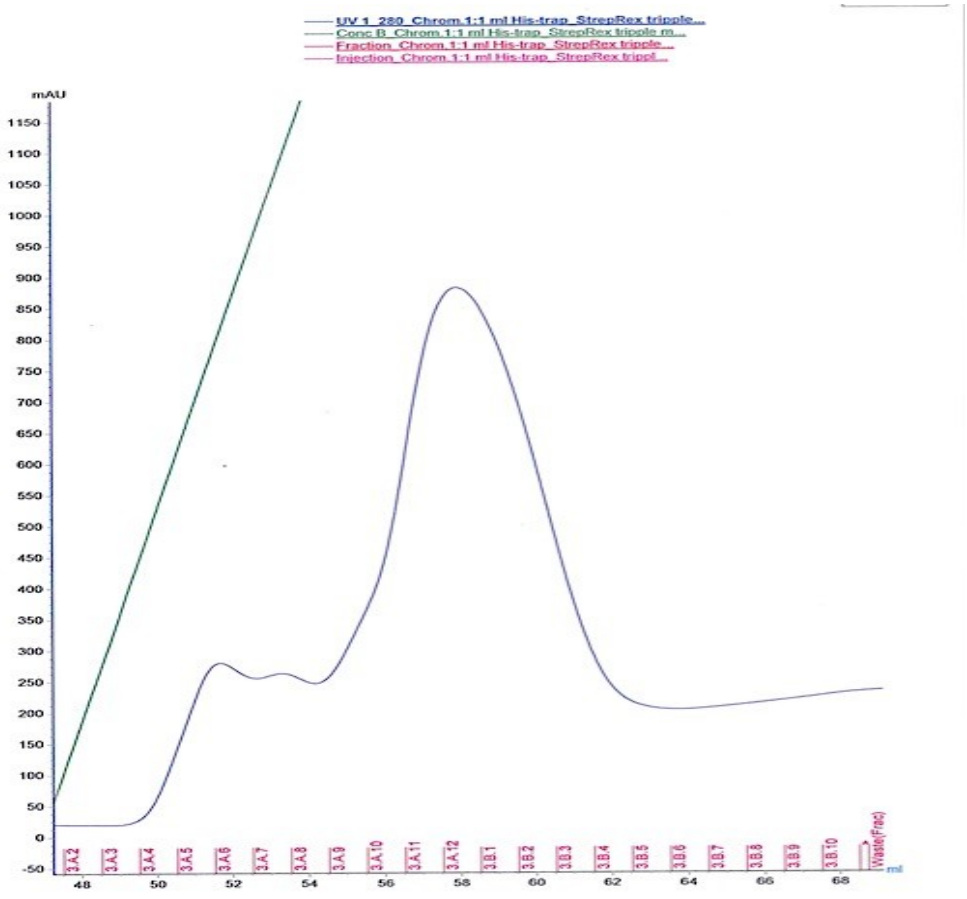


Figure 3. His-trap purification chromatogram of His6-Rex-triple mutant.

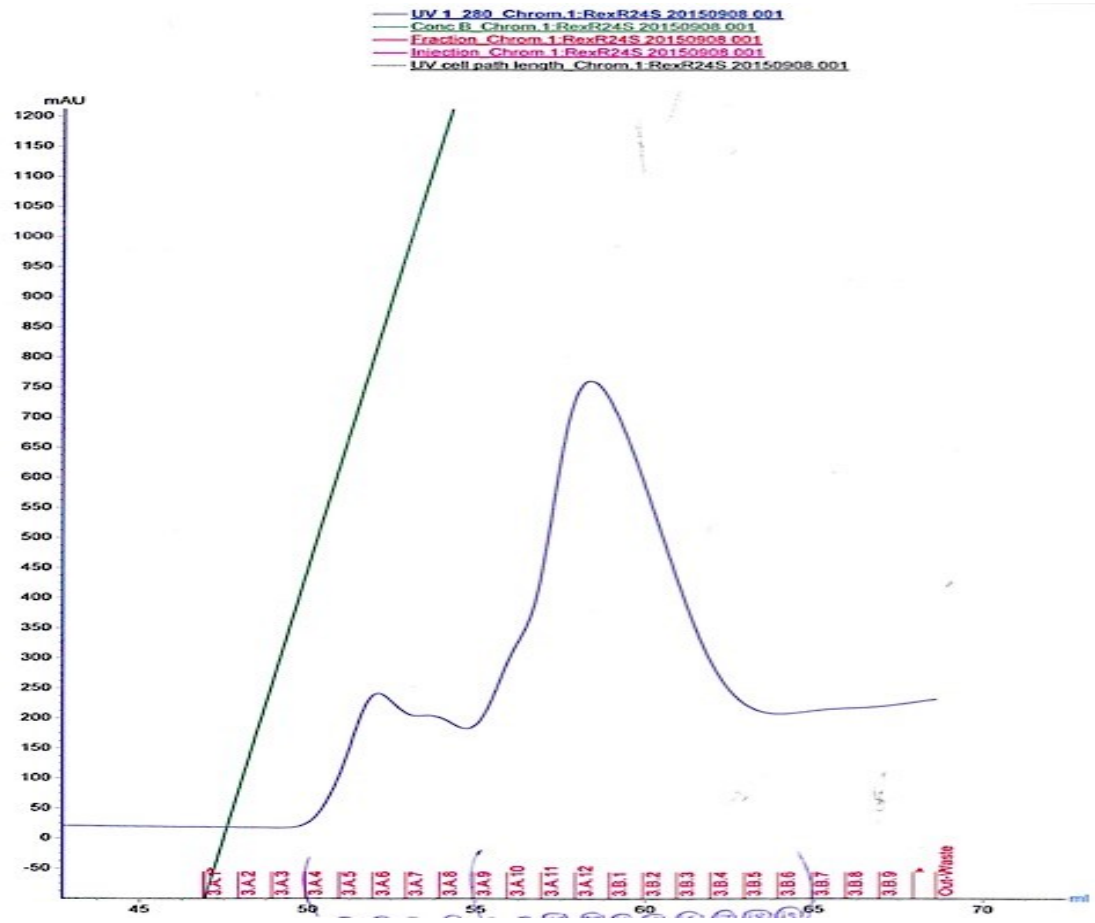


Figure 4. His-trap purification chromatogram of His6-Rex-(R24S) mutant.

Table 2: List of buffers used in this study

Buffer	Composition
50X TAE buffer	242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M Na-EDTA (pH 8) were mixed and stored in room temperature. For 1X buffer, 100 ml 50X TAE buffer were mixed with 4900 ml dH_2O.
5X SDS Gel-loading buffer	250 mM Tris-HCl (pH 6.8), 8% SDS, 0.1% bromophenol blue, 40% v/v glycerol and 100 mM DTT were mixed, distributed into 0.5 ml aliquots and stored at -20°C.
10X TGS Electrophoresis buffer	30.3 g Tris base, 144 g glycine and 10 g SDS were dissolved in dH_2O to a final volume of 1L and stored at room temperature. For 1X buffer, 100 ml 10X TGS was mixed with 900 ml dH_2O.
Tris buffer	(50 mM Tris/HCl (pH 8), 1mM NaCl, 10 mM Na-EDTA); 6.06 g Tris base, 5.84 g NaCl and 3.72 g Na-EDTA were dissolved in approx. 800

	ml of dH_2O. The pH was adjusted to 8; the solution was diluted to 1L, filtered and stored at 4°C.
Gel filtration phosphate buffer	100 mM potassium phosphate buffer (pH 7.5), 150 mM NaCl and 1 mM EDTA.
His-tag wash buffer	1.36 g (20 mM) imidazole or 20 ml of 1M imidazole stock solution, 50 ml of 1M NaH_2PO_4 (50 mM) and 150 ml of 2M NaCl (0.3M) were dissolved in approx. 600 ml dH_2O, pH was adjusted to 8.0, then dH_2O was added to a final volume of 1 L and filtered.
His-tag elution buffer	34.04 g (0.5M) imidazole, 50 ml of 1M sodium phosphate (50 mM) and 150 of ml 2M NaCl (0.3M) were dissolved in 600 ml dH_2O, the pH was adjusted to 8.0, dH_2O was added to a final volume of 1 L and filter.
Solution 1, phosphate buffer	50 mM potassium phosphate buffer (pH 7.5) supplemented with 0.1 mM EDTA and 150 mM NaCl.
Solution 2, phosphate buffer	50 mM potassium phosphate buffer (pH 7.5).
Solution 3, phosphate buffer	50 mM potassium phosphate buffer (pH 7.5), supplemented with 1M NaCl.
Solution 4, phosphate buffer	100 mM potassium phosphate buffer (pH 7.5).
Solution 5, phosphate buffer or **Buffer A**	50 mM potassium phosphate buffer (pH 7.0), supplemented with 1 mM EDTA.

Description of methods used in this project:

Generally, to characterize a protein, it should be expressed in soluble form with adequate amount in suitable expression host, then purified with the optimal efficient purification procedures. In the next section a brief overview of some molecular biology laboratory techniques will be highlighted; Recombinant protein expression in *E. coli,* Site-Directed Mutagenesis using back to back Polymerase Chain Reaction (PCR) amplification method, Immobilized Metal-Affinity Chromatography (IMAC), Ion Exchange Chromatography (IEC), Gel filtration (GF) or Size Exclusion Chromatography (SEC), NanoDrop, Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and MicroScale Thermophoresis (MST).

1.5.1 Expression of recombinant proteins in *E. coli*

It is expression of foreign genes in *E. coli* by inserting the target gene into an expression vector, which is usually a plasmid DNA. This vector should contain certain elements

for expression to be successful: First, a selectable antibiotic resistance marker to ensure the maintenance of the vector in the cell. Second, a controllable transcriptional promoter (e.g. lac) that upon induction with specific inducers can produce large amounts of the mRNA of the targeted cloned gene. Third, translational control sequences, such as an appropriately positioned ribosome-binding site and initiator ATG codon. Finally, a polylinker, or a multiple cloning site to simplify the insertion of the target gene in the correct orientation with in the vector [17].

Production of recombinant proteins in *E. coli* is a very common technique as many researchers have already investigated *E. coli* as an expression host. *E. coli* is considered as the best choice for expression of commercially important proteins as the techniques necessary to express usable amounts of protein in *E. coli* are relatively simple, and the time needed to generate a production strain is short. Moreover, *E. coli* is cheap to grow and 30% of its total proteins can often be obtained as expressed gene product. However, protein expression in *E. coli* does have some disadvantages; eukaryotic proteins are difficult to produce in *E. coli*, due to lack of posttranslational modifications. In additions, proteins expressed in large amounts, tends to precipitate into insoluble aggregates (inclusion bodies) from which they can only be recovered in an active form by solubilization in denaturing agents, followed by careful renaturation [18].

1.5.2 pET expression system

In the T7 expression system the gene of interest is cloned into a pET plasmid expression vector, (a plasmid for expression by T7 RNA polymerase) under the control of a T7 RNA polymerase promoter. To elicit target gene expression, this vector is transferred into an *E. coli* strain (expression strain) that contains a copy of the gene encoding for T7 RNA polymerase under the control of the *lac* promoter. Promoters of both the target gene and T7 gene also contain the *lacO* operator sequence and are therefore inhibited

by the Lac repressor (*lacI*). Both the *E. coli* genome and the pET vector contain the *lacI* gene. Addition of the inducer, isopropyl ß-D-1-thiogalatopyranoside (IPTG) which is an artificial allosteric inducer and can't be metabolized by *E. coli*, will lead to dissociation of the lac repressor and initiates transcription of the T7 gene that codes for T7 RNA polymerase. The T7 RNA polymerase will then bind to the T7 promoter and activate expression of the target gene. As expression of the target gene proceeds and the highly active T7 RNA polymerase will out-come, the host RNA polymerase transcription will proceeds, and after only few hours, the target gene may constitute the majority of the total cellular proteins (figure 5) [19].

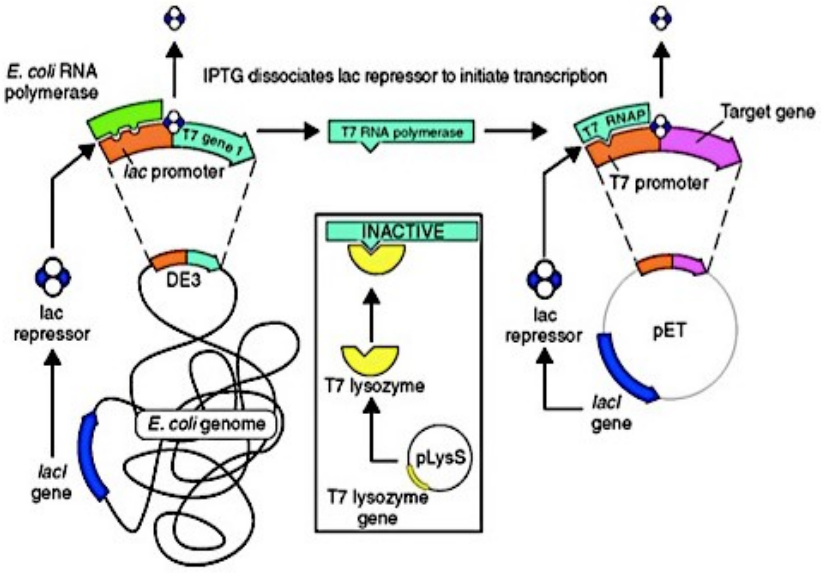


Figure 5: Schematic overview of the T7 RNA polymerase expression system. Figure from [19].

1.5.3 Site-Directed Mutagenesis using back-to-back PCR amplification method

Site-Directed mutagenesis is a widely used method to study gene and protein functions. In this method, certain mutations; point mutations, insertions or deletions can be introduced into the plasmid [20]. The mutagenesis process is composed of three steps: First, PCR amplification of the target plasmid with two phosphorylated primers. Either one or both primers with desired mutation(s) are designed to anneal back to back to the

plasmid DNA. Second, circularization of the mutated PCR products with T4 DNA Ligase. Finally, transformation of the ligation mixture into selected *E. coli* strain (figure 6).

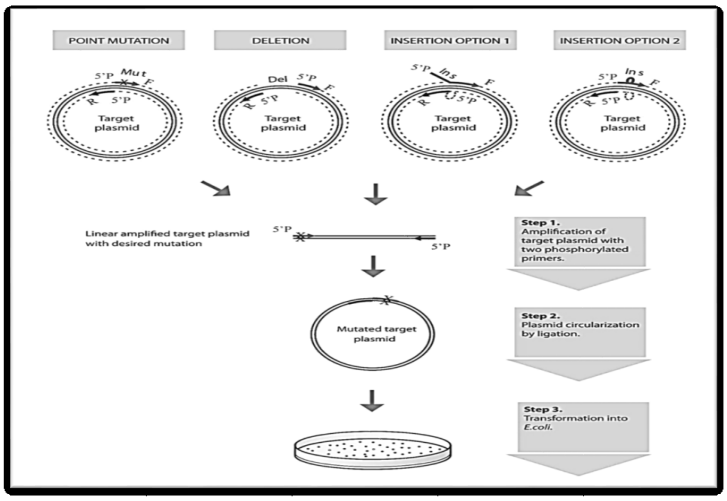


Figure 6: Flow chart of the Phusion Site-Directed Mutagenesis Kit protocol, F = 5' phosphorylated forward primer, R = 5' phosphorylated reverse primer. Figure from [20].

1.5.4 Immobilized Metal-Affinity Chromatography (IMAC)

IMAC is an efficient purification method of Histidine-tagged (His-tag) proteins, where a strong complex is formed between Histidine residues on the target protein and the metal ions on the carrier beads. The bound protein can be eluted by imidazole; the functional group on Histidine, or by lowering the pH [21]. To introduce His-tag to the target protein, N, C-terminal or both of the target protein is engineered with 6-10 consecutive His_{6-10} residues during primers or vector plasmid design [22].

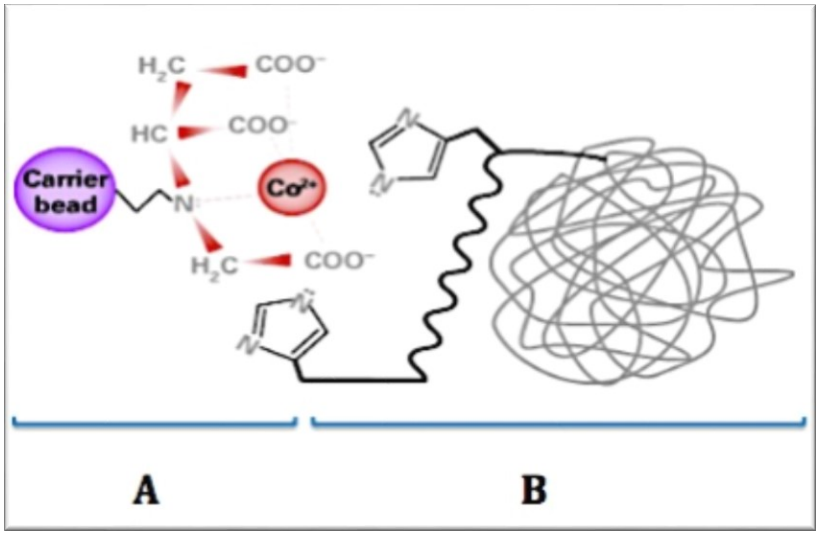


Figure 7: Schematic diagram of IMAC system **A**) Carrier beads bearing the Co^{2+} metal ion, **B**) The Histidine-tagged protein binds to the resin. Figure from [23].

1.5.5 Ion Exchange Chromatography (IEC) and Gel Filtration (GF) or Size Exclusion Chromatography (SEC)

Classical and still widely used purification techniques for proteins and other molecules as well (figure 8).

IEC, separation of proteins in a mixture depends on the charge on the surface of the separated proteins. IEC includes both Cation and Anion exchange chromatography. In cationic type, the protein of interest is positively charged, and attracted by ionic interactions to a negatively charged support, the more positively charged the protein is, the stronger ionic interaction will be formed with the negative support. On the other hand, in anionic type, the protein of interest is negatively charged, and attracted by ionic interaction to a positively charged support, the more negatively charged the protein is, the stronger ionic interaction will be formed with the positively charged support. To optimize the binding of the charged proteins to the oppositely charged support, the mobile phase containing the proteins mixture should be of low to medium conductivity (low salt content). For elution, gradient salt (NaCl) concentration is used; molecules

with the weakest ionic interactions will be eluted firstly, then molecules with stronger ionic interactions that require higher salt concentration will be eluted later in the gradient [24].

Gel filtration or SEC separates proteins mixture according to their size. Protein molecules move through a bed of porous beads, diffusing into the beads to greater, or lesser degrees. Smaller molecules diffuse further into the pores of the beads and therefore move through the bed more slowly, while larger molecules enter lesser or not at all and thus move through the bed more quickly. Both molecular weight and three-dimensional shape of the molecules contribute to the degree of retention. Gel filtration chromatography can be used for molecular size analysis, separation of compartments in a mixture, or salt removal and buffer exchange [25].

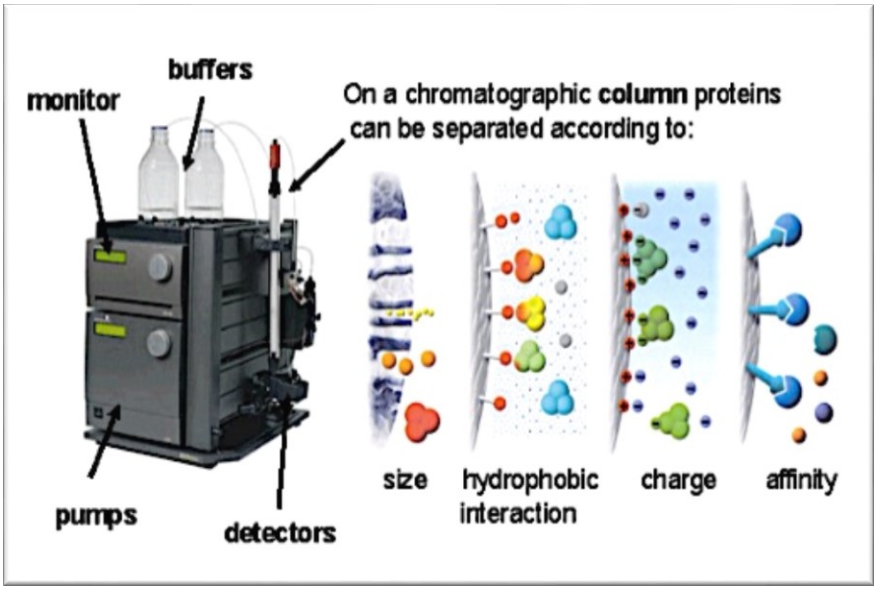


Figure 8: A schematic diagram of IEC and SEC purification methods, illustrating the mechanism of proteins separation in a mixture on different matrices. Figure from [26].

1.5.6 NanoDrop for measurement of protein concentration

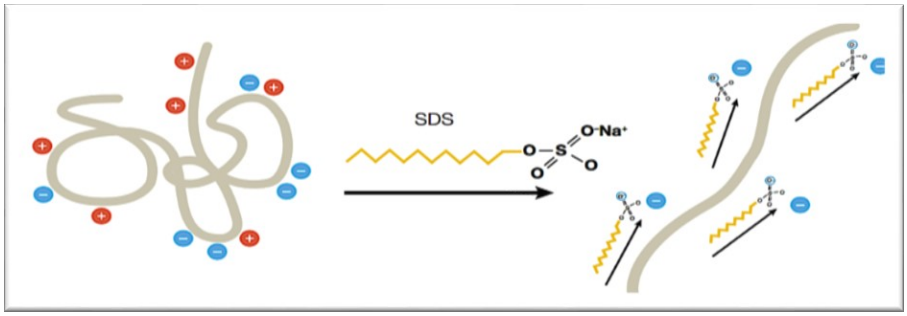
A method used to measure the concentration of purified proteins in (1-2 µl) volumes at 280 nm. Proteins usually contain amino acids such as tryptophan, tyrosine, or cysteine-

cysteine disulfide bonds, which exhibit absorbance at 280 nm. The Beer's-Lambert law (**A** = **E** × **b** × **c**) is used for all protein calculations to correlate absorbance with concentration. Where, **A** is the absorbance value, **E** is the extension coefficient Liter/Mol^{-1}.cm, **b** is the path length in centimeters (usually equal 1cm), **c** is the molar concentration of the protein [27].

1.5.7 Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Widely used technique for separation of proteins according to their size. SDS is a denaturing agent; it denatures proteins giving their negative charges, so that they migrate through the gel from the negative (anode) to the positive (cathode) according to their molecular size. By using standard proteins marker, the molecular mass per Daltons (Da) of each protein band can be determined, (figure 9) [28].

A



B

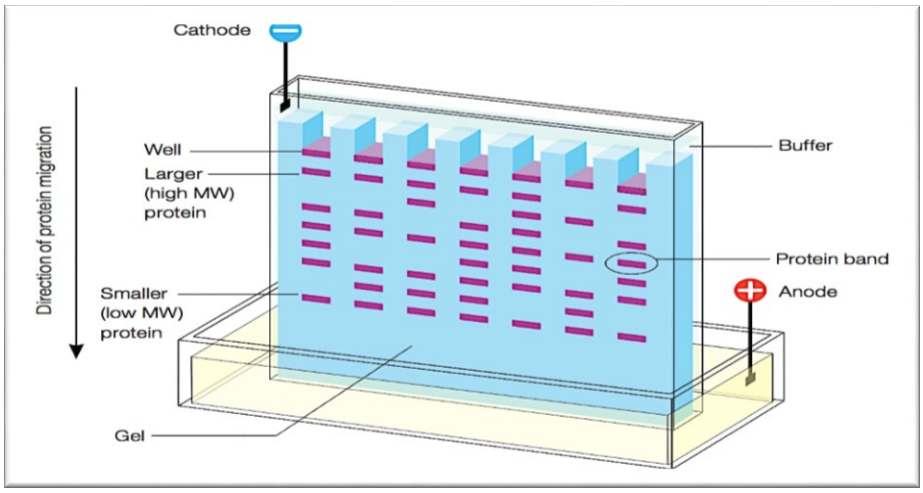


Figure 9: A) the effect of SDS on the conformation and charge of proteins. B) Schematic diagram of electrophoretic protein separation in a polyacrylamide gel, MW: Molecular Weight. Figure from [29].

1.5.8 MicroScale Thermophoresis (MST)

A technique used for analysis and quantification of biomolecular interactions [30]. It measures the dissociation constant or affinity constant (K_d) of the protein-ligand complex by detecting changes in the hydration shell, charge, or size of the molecules, through measuring changes of the molecules mobility in microscopic temperature gradients, (figure 10) [31]. The most important feature of MST is working on the proteins under their native or close to native conditions. However, certain precautions should be taken into account on sample handling:

- Concentration of the fluorescent molecule should be in the same range or lower than the expected K_d.
- Ligand concentration should be 20 fold above the expected K_d.
- Final sample volume per titration point should be ~ 20 µl.
- Small tubes should be used for serial dilutions (e.g. PCR tubes).
- The composition and concentration of the dilution buffer should be held constant all over the serial dilution series.
- Accurate pipetting is essential.
- Pipetting instead of vortexing should do mixing.
- Touching the capillaries in the center should be avoided.

The K_d can be calculated as follows:

*If Protein + Ligand ↔ Protein Ligand complex

*Then, K_d = [P] × [L] ÷ [PL]$[P] \times [L] \div [PL]$

Where [P], [L] and [PL] [P], [L], and[PL]are the molar concentrations of protein, ligand and protein-ligand complex, respectively.

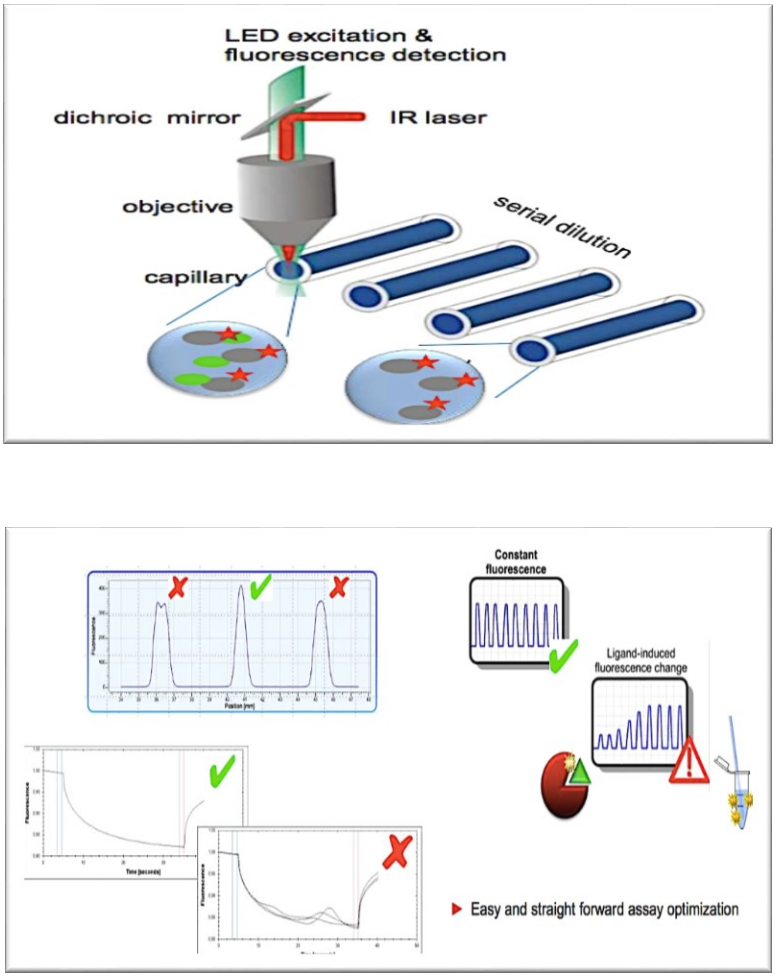


Figure 10: MST assay mechanism and optimization summary, where the random fluorescence changes shouldn´t be greater than 10 %, aggregation should avoided. Figure from [31].