UiT
THE ARCTIC UNIVERSITY OF NORWAY

Department of Pharmacy

Regulation of ERK3/4 Function via Specific Protein-Protein Interactions

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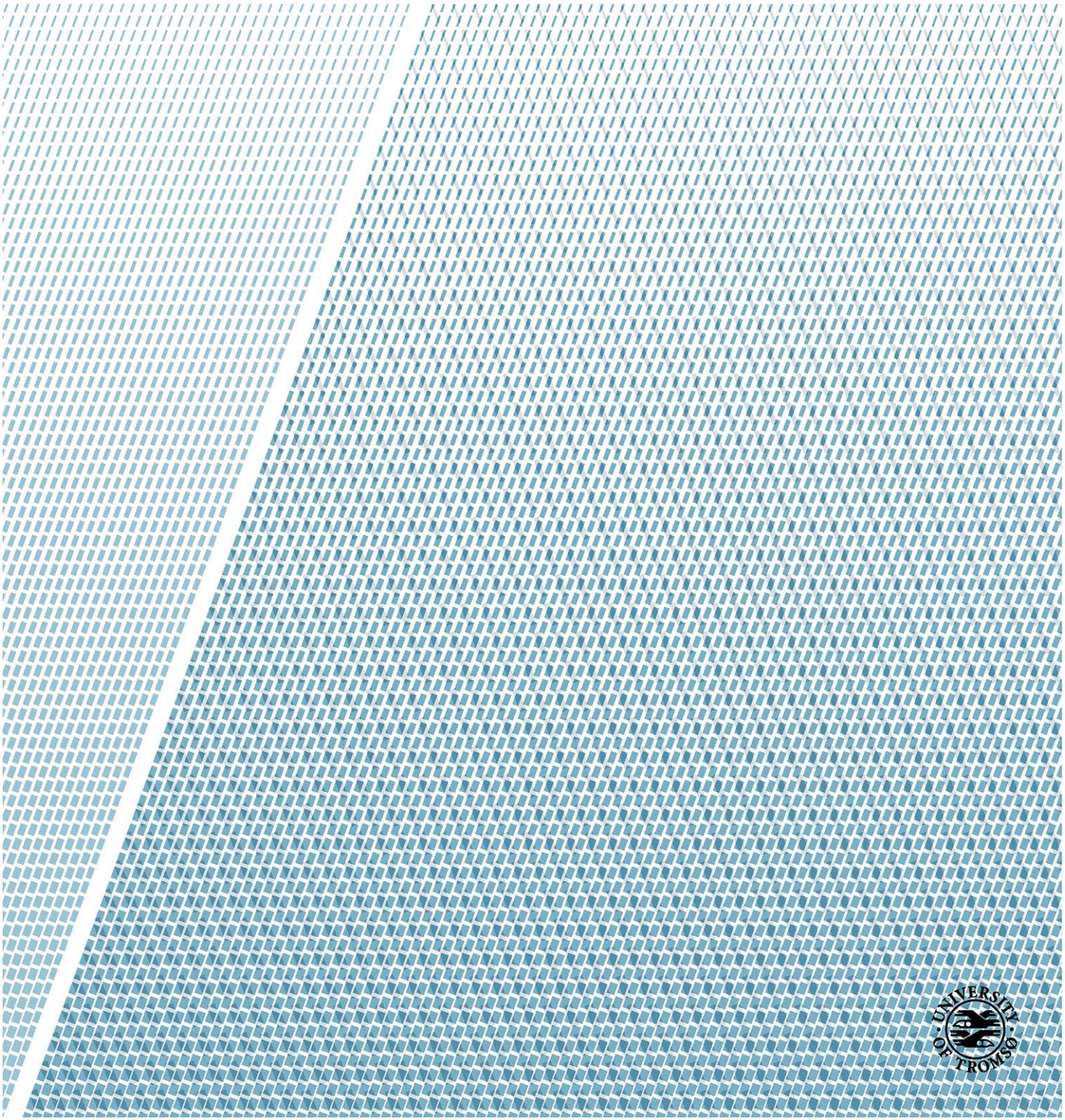
Master thesis in Pharmacy … May 2016



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ABSTRACT

The mitogen-activated protein kinase 5 (MK5) binds to extracellular signal-regulated kinase 3 and 4(ERK3 and ERK4) through a unique FRIEDE motif in ERK3/4. 100 amino acids C-terminal in MK5 is sufficient for this binding. MK5s subcellular localization and activity is regulated by the atypical MAP kinase ERK3 and ERK4. In this project we wanted to express and purify full-length and different domains of MK5 and use it to gain structural information of the kinase to further reveal how it binds to ERK3 and ERK4 at the atomic level. Recombinant technics for generation of these proteins together with functional assays to assess the activity of MK5 have been used. Optimal protocols for expression and purification of the kinase was developed. Finally we used a GST pulldown assay to show that the amino acid domain amino acid domain 372-473 of MK5 are able to bind to ERK4 in extracts from mammalian cells.

ABBREVIATIONS

AMP	Ampicillin
CAM	Chloramphenicol
dNTP	Deoxyribonucleoside triphosphates
GST	Glutathione S-transferase
IMAC	Immobilized metal Ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranosid
LB	Lysogeny Broth
MAPK	Mitogen-activated protein kinase
Ni-NTA	Nickel-nitrilotriacetic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
STM	Streptomycin
TB	Terrific broth
TBS-T	Tris-buffered saline – tween 20

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INTRODUCTION

Cancer is among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012[1]. Norway with a population of more than 5 million people, 31 651 new cases were reported in Kreftregisteret according to Cancer in Norway 2014[2]. Cancer is when abnormal cells divide uncontrollably. They can invade and spread to distant sites of the body. Metastasis is the spread of cancer cells to other organ site. *Hallmarks of Cancer: The Next Generation* describes the six hallmarks of cancer, which are typical for cancer cells[3]. These events are sustained proliferative signaling where cancer cells receive growth-promoting signals, evading growth suppressors, resisting cell death, enabling replicative immortality by exceeding senescence and apoptosis and therefore allowing multiple replication, inducing angiogenesis as blood supply to the cancer tumors, and activating invasion and metastasis. Better understanding of these characteristics will further develop new means to fight cancer[3].

There are more than 100 types of cancer, and types of cancer is usually named after where the cancer occurs in the bodily organs or tissues. For example, carcinoma arises in the epithelium, which is the tissue that covers the outside of the organs and body. Cancer is a genetic disease and three main types of genes contribute to the development of cancer. These are 1) proto-oncogenes, which often encode proteins for function of cell division, and differentiation, and stop cell death. These proto-oncogenes can become tumour-causing genes, the oncogenes, allowing cells to grow uncontrollably and resist cell death. 2) tumor suppressor genes that express proteins for controlling cell growth, and 3) DNA repair genes that repair damaged DNA genes. Mutations in any of these genes may lead to cancer formation[4,5].

Currently cancer can be treated with surgery (removal of malignant tumor), radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy and stem cell transplant. One can only have one type of treatment or combination of treatments[6]. Kinase inhibitors are a subclass of chemotherapy and has been an interesting field among researchers. 30 substances are now in clinical use and more than 130 are in phase 1-3 clinical trials[7].

Signal Transduction

Signal transduction describes how cells receive, process, and respond to information from the environment[8]. Extracellular molecular signals transmit into a cell before it gives a response. How the transmission takes place, is determined by receptors, which are proteins that receive molecular signals outside the cells. Signaling transducing receptors are of four classes; 1)

Enzyme-linked receptors, which penetrate the cell membrane and have intrinsic enzymatic activity or association with intracellular enzyme, 2) G protein-coupled receptors, which are coupled to G proteins inside the cell, 3) nuclear receptors, which are within the cell and alter gene expression and 4) Ligand-gated ion channels that allow passage of ions upon activation.

Upon activation of the receptor, an event of signaling cascade takes place where enzymes act on one another in a specific way. This gives a physiological response that is specific and appropriate. The small molecules, which participate in intracellular signaling, are called second messengers. These include cAMP, cGMP, nitric oxide, lipids and Ca^{2+} ions. Once the receptor is activated, it stimulates the second messenger production, which then activate other enzymes within the cascades[10]. This does not apply for enzyme-linked and nuclear receptors however. Enzyme-linked receptors initiate signaling cascades and hence the intrinsic enzyme activity through multi-subunit transmembrane proteins[9]. Nuclear receptors are a large family of transcription factors that bind to DNA and start transcription and translation of target DNA sequence[11].

Kinases

Kinases are enzymes that phosphorylate other molecules, adding a phosphate group (PO_4^{3-}). This phosphate group is from adenosine trisphosphate (ATP). Kinases are involved in many cellular processes. They are further named after what the target molecule is. For example, kinases that phosphorylate proteins are called protein kinases and kinases that phosphorylate lipids are called lipid kinases[12,13].

Protein kinases attach the phosphate group onto a protein (Figure 1). From there they regulate signal transduction and cellular processes. These include metabolism, transcription, cell cycle progression, cytoskeletal rearrangement and cell movement, apoptosis, and differentiation. In addition, protein kinases are important in intercellular communication during development, in physiological responses and in homeostasis, and in functioning of the nervous and immune systems. Protein kinases phosphorylate serine/threonine (Ser/Thr) or tyrosine(Tyr) on the side chains in proteins[14,15]. There are 518 annotated protein kinases in human, where 478 belong to a single superfamily whose catalytic domains are related in amino acids sequence, which consists of 250-300 amino acids[13,14]. These domains are called eukaryotic protein kinase (ePK) catalytic domain. The remaining 40 protein kinases are what is called atypical protein kinase (aPK)[14,15]. ePKs are further divided in seven major groups (Figure 2) according to their similarity and biochemical functions[16].

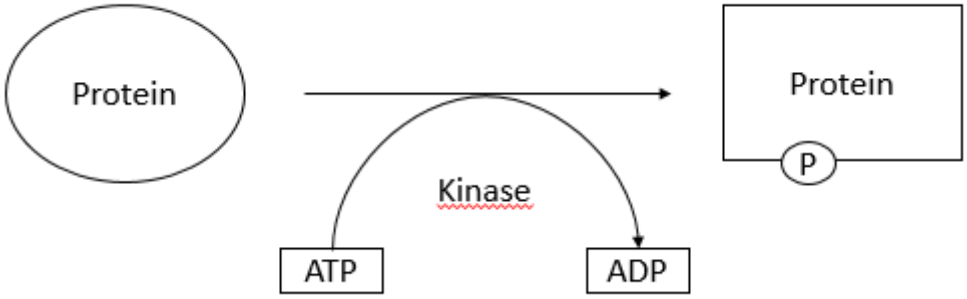

Figure 1 Phosphorylation. The kinase is a protein kinase that adds a phosphate group P from an adenosine triphosphate (ATP) to a protein. ATP becomes adenosine diphosphate (ADP) dues to the loss of a phosphate group. Shape-shifting of the protein occurs due to conformational change.

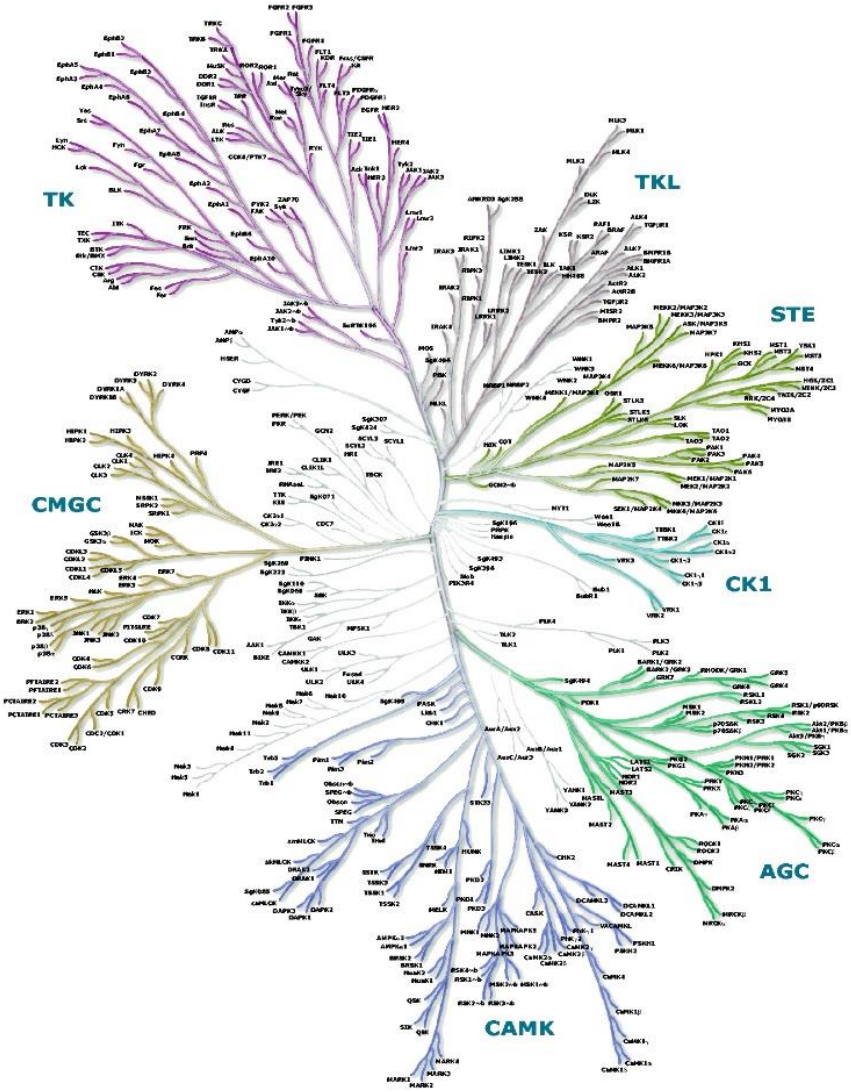

Figure 2 The Human Kinome. The Eukaryotic protein kinases are divided in seven major groups. The ERK3/ERK4 signaling pathway is in the CMGC group (derived from [15]).

Enzyme Inhibition

The activity of an enzyme can be reduced or inhibited by specific molecules. Molecules that inhibit an enzyme are called inhibitors. They play a major role in the regulation mechanism in biological systems. The inhibition is divided into reversible and irreversible. Irreversible inhibitors tightly bind to the target enzyme, either covalently or noncovalently, and dissociate slowly from it. Reversible inhibition is when the inhibition of the enzyme is brief and the inhibitor dissociate fast. Competitive inhibition is when the inhibitor, which is similar to the substrate in structure, binds to the active site of the enzyme. The binding of the enzyme and substrate is thus prevented[17].

Mitogen-activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) family member phosphorylates Ser/Thr residues in the substrate molecules[18]. The MAPK family of protein kinases is further divided into two subfamilies, conventional MAPKs and atypical MAPKs (Figure 4). The conventional family consists of ERK1/2, JNK1-3, p38α, β, γ, δ and ERK 5. The atypical family consists of ERK3/4, NLK and ERK7[19,20]. When a cell receives a stimuli the mitogen-activated protein kinase kinase kinases (MAPKKKs) phosphorylate mitogen-activated protein kinase kinases (MAPKKs), which then phosphorylate mitogen-activated protein kinases (MAPKs)[19]. The MAPKKKs, which themselves are Ser/Thr kinases, are activated by extracellular stimuli like mitogens, cytokines and cellular stresses. In response of these stimuli, MAPKKs become phosphorylated and/or interact with a small G protein of Ras/Rho family[21,22]. This leads to the phosphorylation of MAPKKs, which further activates the individual MAPK through dual phosphorylation on the specific Thr and Tyr residues found in their activation loop[20]. An illustration of the MAPKs demonstrates their general structures (Figure 3).

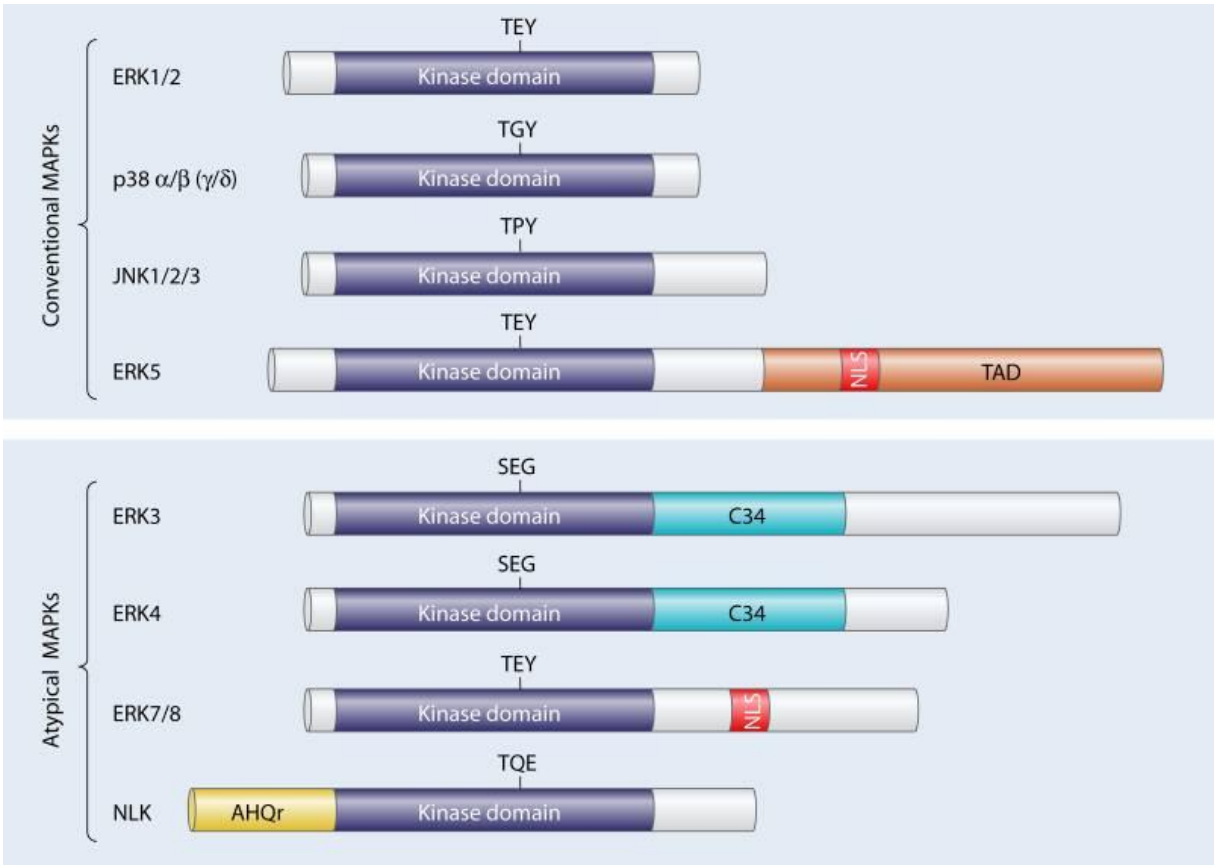

Figure 3 Schematic illustration of the general structures of MAPKs. Different lengths of N- and C-terminal regions flank the kinase domain, which binds to the MAPKs substrate and phosphorylate them at Ser/Thr domains. In some MAPKs, there are additional domains such as transactivation domain (TAD), nuclear localization sequence (NLS), region conserved in ERK3/4 (C34) and domain rich in alanine (Ala), histidine (His), and glutamine (Glu) (AHQr). The three amino acids segment for the phosphorylation of the MAPK above the kinase domain is also presented (Reprinted from [21]).

The atypical MAPKs mechanism is less known due to their divergent characteristic. They do not share the three-tiered kinase cascades. Moreover, a glycine (Gly) or glutamic acid (Glu) represents instead of the Tyr in the activation motif for ERK3/4 and NLK. ERK7 contains the motif Thr-Glu-Tyr motif rather than the Thr-X-Tyr, which presents in conventional MAPK pathway. Phosphorylation of Thr-Glu-Tyr is catalyzed by ERK7 itself instead of by an upstream MAPKK[19].

MAPKs phosphorylate their respective substrates. These include the members of a family of protein kinases called MAPK-activated protein kinases (MAPKAPKs) (Figure 4). This family includes the protein kinases; p90 ribosomal S6 kinases (RSKs), mitogen- and stress-activated kinases (MSKs), MAPK-interacting kinases (MNKs), MAPK-activated protein kinase 2/3 (MK2/3), and MK5[21].

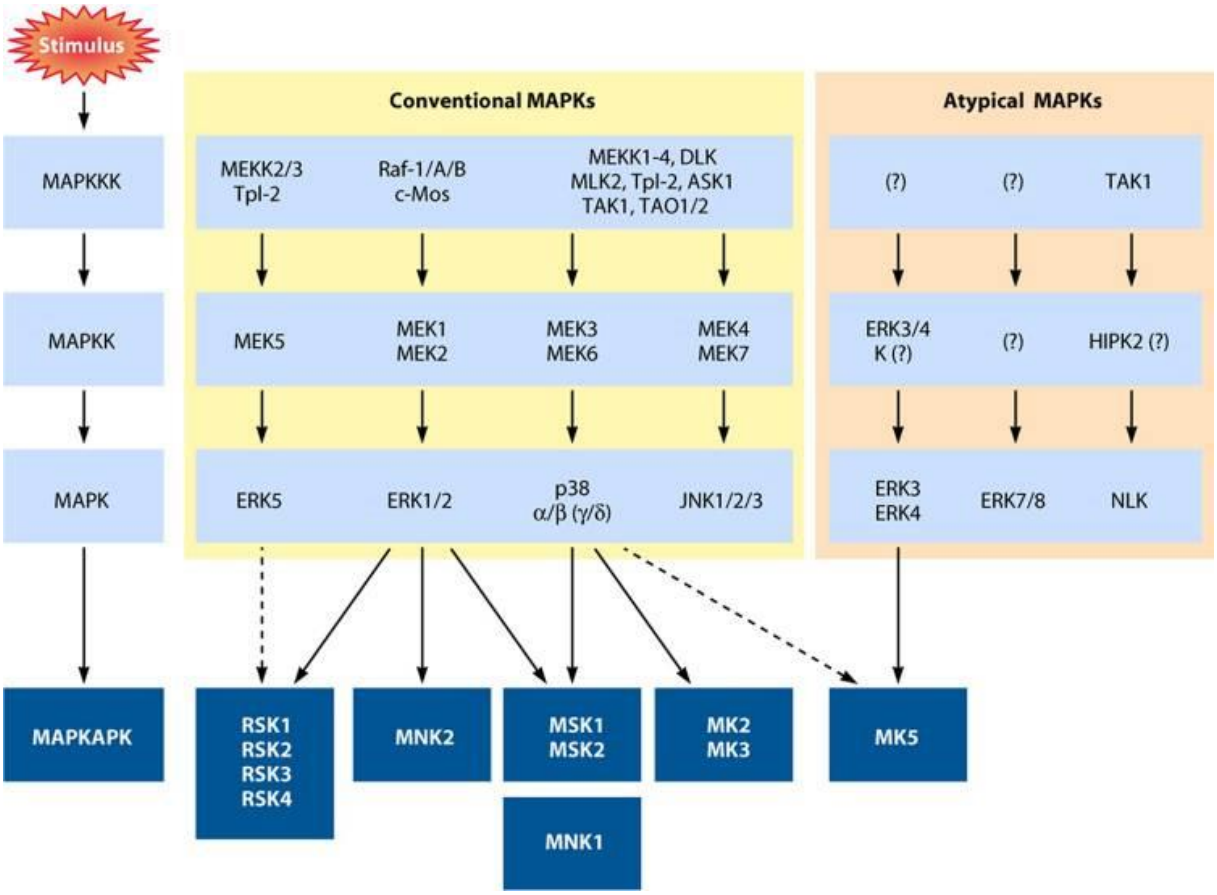

Figure 4 Signalling Patterns of MAPK-Pathway. The conventional MAPKs follow the three-tiered pathway, whereas the atypical MAPKs do not. The dotted lines indicate that it has been reported that RSKs and MK5 have been phosphorylated by their respective MAPKs, but it needs more reports on them (reprinted from [21]).

Structure

Through crystallization of members of ERK1/2, p38 and JNK groups the MAPK domain structure has been understood. The crystallization was done alone or in combination with substrates, scaffolds, inhibitors and derived peptides from substrates or interacting partners[23]. MAPKs are made of two regions, an N-terminal region, which consists of β-sheets and two helices, αC and αL16, and a C-terminal region that is mostly helical, with four short β-strands. Between these two regions lies the catalytic site, where the ATP and two magnesium ions bind. In addition, a flexible linker, which functions as a connecting molecule, maintains the proper orientation of the terminal regions. Proper orientation is important for the enzymatic activity. The MAPK insertion, to be found in the C-terminal region and extend over the whole protein, including the αL16 helix, distinguishes MAPKs from other proteins on the kinase superfamily[24] (Figure 5).

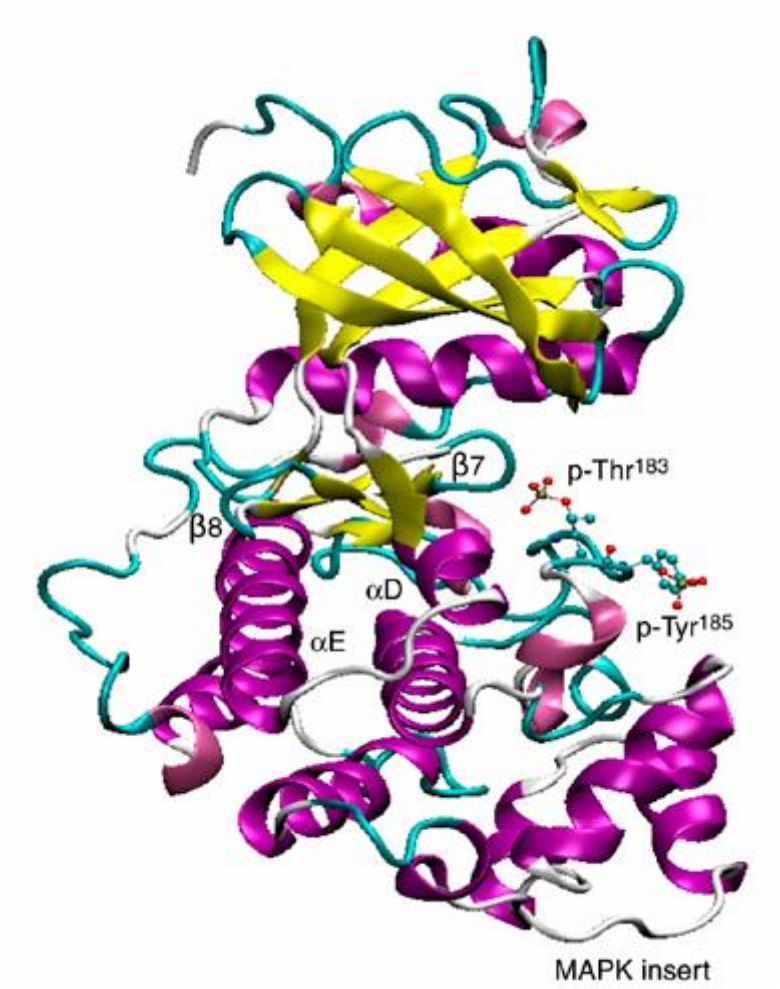

Figure 5 Crystal structure of active ERK2. General structure of MAPKs with the MAPK insertion together with an extension of the C-terminal region distinguishes MAPKs from other proteins of the kinase superfamily. Ribbons colored according to their secondary structure, β-sheet in yellow, α-helices in purple and loops in cyan (Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [MAP kinases and the control of nuclear events][24], copyright 2007).

The Conventional MAPKs

ERK1/2

Extracellular signal-regulated kinase 1 and 2 (ERK1/2) are nearly 85% identical, with 43 and 41 kDa respectively. They are activated through dual phosphorylation of tyrosine and threonine, which are separated by a glutamate residue to yield the motif TEY in the activation loop. Both kinases are vastly expressed in tissues. Stimuli like growth factors, cytokines and certain stresses activate the ERK1/2 signaling pathway[20]. Upstream of this cascade, the MAPKKKs phosphorylate two serine residues or a serine and a threonine to activate the MAPKKs MEK1/2. The Raf isoforms, a-Raf, B-Raf and C-Raf are the main MAPKKKs in ERK1/2 cascade. Mos, Tpl2, and other MAPKKKs are limited to certain cell type signaling[22]. Downstream, ERK1/2

phosphorylate many protein kinases, such as p90 ribosomal S6 kinase (RSK), mitogen and stress activated kinase (MSK), and the transcription factors Elk1, c-Fos, c-Myc, and Ets domain factors.

JNK

c-Jun N-terminal kinases (JNK) has three isoforms; JNK1, JNK2 and JNK3. They are more than 85% identical and exist as 10 or more spliced forms of 46 to 55 kDa. JNK1 and JNK2 are found in all tissues, while JNK3 is mainly in the brain[22]. Stimuli, such as cellular stresses, growth factors and cytokines activate the JNK pathway[25]. Upon activation, JNKs become phosphorylated at tyrosine and threonine residues, which are separated by a proline residue to give a TPY motif in the activation loop[20]. Upstream this cascade, the MAPKKKs MEKKs (MEKK1, -2, -3 and -4), mixed lineage kinases (MLKs), Tpl2, ASKs, TAOs and TAK1 phosphorylate the MAPKKs MKK4 and MKK7. This phosphorylation takes place in the serine and threonine residues of MKK4/7[22]. The transcription factors c-Jun, p53, JunB, c-Myc and ATF2 are among substrates of JNKs[22,26]. Studies indicate that the pro-apoptotic proteins BAX and BAK are substrates of JNKs and an in *vitro study* shows that the anti-apoptotic proteins BCL2, BCL-XL and MCL-1 are phosphorylated by JNKs[27]. The JNK pathway regulates mediation of cell proliferation, differentiation or apoptosis[22].

p38

There are four isomers of the 38 kDa p38; α, β, γ, δ which have 60% identity among themselves. The p38 MAPK cascade is activated by stimuli such as cellular stressors (eg. UV radiation), osmotic shock, hypoxia, pro-inflammatory cytokines and growth factors. Dual phosphorylation and activation of p38 takes place in the TGY motif of the activation loop by the upstream MAPKKs MEK3 and -6. It has been shown that p38 is phosphorylated by MKK4, which is an enzyme in the MAPK JNK pathway in vitro[28]. p38α and –β exist in all tissues, while the γ and δ isomers are more limited to tissues[22]. MEK3/6 are phosphorylated by TAK1, TAO1/2, TPL2, MLK3, MEKK3/4 and ZAK1 in the serine/threonine site of the activation loop. Most of these MAPKKKs stimulate the JNK pathway[28]. Downstream the p38 cascade, p38s phosphorylate

MSK1 and -2, and MK2 and -3. MSK1/2 are important for rapid induction of immediate-early genes, resulting from stress or mitogenic stimuli. MK2/3, in the other hand, play a role in the control of gene expression[28].

ERK5

Extracellular signal-regulated kinase 5 (ERK5) is of 88 kDa and is activated through phosphorylation on threonine and tyrosine residues of the Thr-Glu-Tyr motif in the activation loop. ERK5 is activated upstream in the cascade by MAPKK MEK5, which then becomes phosphorylated by MAPKKKs such as COT, MEKK3 and MEKK2[24]. MEKK2/3 also stimulate the JNK and p38 pathways[29,30]. This pathway is activated by growth factors, oxidative stress and hyperosmolarity and plays a role in cell proliferation and survival. ERK5 phosphorylates MEF2C which belongs to myocyte enhancer factor 2 (MEF2) protein family, Sap1a, SGK, Cx43 and Bad0[31,32]. It has been identified that ERK5 phosphorylates the RSK family of protein kinases[33]

The Atypical MAPKs

The ERK3/4 – MK 5 pathway

Extracellular signal-regulated kinase 3 (ERK3) contains a C-terminal extension of 178 amino acids (aa), giving a protein with a molecular mass of ~100 kDa. Whereas ERK4 is a 578-aa protein with a molecular mass of ~70 kDA. ERK3 and ERK 4 possess 73% amino acid similarity. They are atypical because they lack the phosphoacceptor Tyr residue, which is present in the activation loop of conventional MAPKs[21]. The functions of ERK3/4 are little understood. ERK3 may play a role in the regulation of cell proliferation and differentiation[34].

ERK3/4 contain the sequence S-E-G instead of T-X-Y in their activation loop. The phospho-acceptor in ERK (S189) and ERK4(S186) are phosphorylated in growing cells. Moreover, phosphorylation of these single serine phospho-acceptors are required for their ability to activate MK5 *in vitro* and form stable and active complexes with MK5 in mammalian cells. This phosphorylation in the activation loop of ERK3/4 for binding and activation of MK5 differs from interactions between classical MAPKs and their substrates, such as MK2 and MK3, in that these bind through the so called common docking domain and hence, can occur independently from the activation loop phosphorylation[35].

One known substrate of ERK3/4 is the MAPK-activated protein kinase MK5. There are 2 isoforms, 471 and 473 aa, but the importance of having 2 almost alike molecules remain unclear. When MK5 is overexpressed, its concentration is highest in the nuclei of quiescent cells. Upon cellular stress, the concentration is highest in the cytoplasm [21]. No extracellular stimuli have not been discovered yet for ERK3 and ERK4, thus the connection between and endogenous MK5 and the extracellular stimuli remains questioned[35].

MK5

MAPK-activated protein kinase (MK5) had its first name as the acronym PRAK (p38-regulated/activated protein kinase) because it was described as a serine/threonine protein kinase downstream target of the p38 MAPK-pathway. Upon activation of MK5 through binding with ERK3/4, MK5 translocates from cell nucleus to the cytoplasm. Despite MK5 being activated by both p38 α/β and atypical ERK3/4, only ERK3/4 have been found, when overexpressed in mammalian cells, to form endogenous complexes with MK5[35]. Sun et al. shows that MK5 plays a role in Ras-induced senescene in primary murine fibroblasts in the p38-MK5 signaling pathway. When Ras is activated, the p38-MK5 pathway stimulates the transcription of the tumour suppressor protein p53, which lead to increased level of the p21 protein in the cells. On the other hand, there is strong evidence that the serine 37 within the activation domain of p53 is phosphorylated by MK5.

MK5 and Cancer

Recently, studies of MK5 have been done to define the role of MK5 in the pathology of cancer. Evidences from these studies suggest that MK5 behaves either as a tumour suppressor or a tumour promoting, depending on the cell type or the stage of cancer at which it is activated.

Interactions between ERK3/4 and MK5

MK5 contains a conserved LXTP site in the activation loop where the threonine is the only phospho-acceptor site. It contains a functional nuclear export sequence (NES) and a nuclear localization sequence (NES) within the C-terminal domain, where NLS overlaps with the MAPK docking site. Furthermore, MK5 has a 100 amino acid extension C-terminal to the NLS. This sequence, which cannot be found in MK2 and MK3, contains amino acids that are required for interaction with ERK3/4[36]. The ERK3 and ERK4 interaction domain is termed the FRIEDE-motif after Aberg et al.[37].

Figure 6 Schematic structure of MK5. Phospho-acceptor site at amino acid threonine 182 (T182). The Nuclear Export Signal (NES), Nuclear Localization Signal (NLS) and ERK3 and ERK4 interaction domain (FRIEDE) are shown. (Adapted from [36]).

Aim of Thesis

In this study, we wanted to express and purify full-length and different domains of the mitogen-activated protein kinase 5, a substrate of ERK4 in E. coli.

The purified MK5 will be used to gain structural information of the kinase in experiments to reveal how MK5 physically interacts with ERK4.

MATERIALS

Chemical Reagents

I. Restriction

10x NEBuffer 2	New England Biolabs® inc, USA
10x NEBuffer 2.1	New England Biolabs® inc, USA

II. Gel Electrophoresis

Ethidium Bromide 10 mg/ml	Sigma-Aldrich®, USA
1 Kb plus DNA ladder	Invitrogen, Carlsbad, USA
Seakem® LE Agarose	Lonza Group, Switzerland
Seakem® GTG Agarose	Lonza Group, Switzerland
1xTAE buffer	40 mM Tris-acetate 1 mM Tris-acetate 1 mM EDTA
6x loading buffer	0.03% Bromphenol blue 0.03% Xylene cyanol FF 10 nM Tris HCl pH=8 60% Glycerol

III. Sequencing

5x Phusion® reaction buffer		New England Biolabs® inc, USA
F Primer (MK5)	CCTTTACTTCCAGGGGATGTCGGAGGAGAGCGACATGG	Sigma-Aldrich®, USA
R Primer (MK5)	GCTTGAATTCGGATCCTTATTGGGATTCGTGGGACG	Sigma-Aldrich®, USA
F Primer (Vector)	CGTCCCACGAATCCCAATAAGGATCCGAATTCAAGC	Sigma-Aldrich®, USA
R Primer (Vector)	CCATGTCGCTCTCCTCCGACATCCCCTGGAAGTAAAGG	Sigma-Aldrich®, USA
Deoxynucleotide Mix, 10mM		Sigma-Aldrich®, USA

IV. Reagents for Bacteria and Transformation

Lysogeny Broth (LB)	University hospital in Northern Norway (UNN), Tromsø: 10 g/l Bacto tryptone (DIFCO) 5 g/l Bacto yeast extract (DIFCO) 171 mM NaCl pH adjusted to 7.4 Prepared with the appropriate antibiotic
LB-agar plate	University hospital in Northern Norway (UNN), Tromsø: 10 g/l Bacto tryptone (DIFCO) 5 g/l Bacto yeast extract (DIFCO) 171 mM NaCl pH adjusted to 7.4 10 g/l agar Prepared with the appropriate antibiotic
S.O.C. medium	2% Tryptone 0.5% Yeas extract 10 mM NaCl 2.5 mM KCl Sterile solution of 10 mM $MgCl_2$ and 20 mM Glucose
1 M Isopropyl-β-D-thiogalactopyranosid (IPTG)	University hospital in Northern Norway (UNN), Tromsø:
Terrific Broth (TB) medium	University hospital in Northern Norway (UNN), Tromsø: For 1 l medium: 12 g/l Tryptone 24 g/l Yeast extract 100 ml sterile solution of 0,17 M KH_2PO_4 and 0,72 M K_2HPO_4 Prepared with the appropriate antibiotic

V. Buffers

10x T4 DNA Ligase buffer	New England Biolabs® inc, USA
Odyssey® Blocking Buffer	LI-COR, Lincoln, Nebraska USA
Blotting buffer	29 g Tris base 144 g glycine 1 l methanol Adjusted to 5 l with dH_2O
Buffer A	University of Tromsø, Pharmacology Research group: 50 mM NaH_2PO_4, pH=8 500 mM NaCl
Lysis buffer A	University of Tromsø, Pharmacology Research group: 50 mM NaH_2PO_4, pH=8

	500 mM NaCl 1 tablet of cOmplete, Mini, EDTA-free proteinase inhibitor (Sigma-Aldrich) Lysozyme from chicken egg white (Sigma-Aldrich) 2-4mM β-mercaptoethanol
Elution buffer B	University of Tromsø, Pharmacology Research group: 50 mM NaH_2PO_4, pH=8 300 mM NaCl 500 mM Imidazole 2-4 mM β-mercaptoethanol
Dialysis buffer A	University of Tromsø, Pharmacology Research group: 50 mM Tris pH=7.5 200 mM NaCl 2 mM β-mercaptoethanol
Dialysis buffer B	University of Tromsø, Pharmacology Research group: 50 mM Tris pH=7.5 150 mM NaCl 2 mM β-mercaptoethanol
Gel filtration buffer	University of Tromsø, Pharmacology Research group: 50 mM Tris pH=7.5 150 mM NaCl 4 mM β-mercaptoethanol Filtered
MAPKK lysis buffer	8 ml tris, pH= 7.0 4 ml Triton X-100 8 ml 250 mM NaPPi 40 ml 500 mM NaF 0.8 ml 0.5 M EDTA 0.8 ml 0.5 M EGTA 2 ml 200 mM VO4 (Ortovanadate) 216 ml 0.5 M Sucrose 8 ml 500 mM β-glycerophosphate 112.4 ml MQ water 1 dissolved tablet of cOmplete™, EDTA-free Protease inhibitor cocktail in 10 ml buffer

VI. Antibiotics

Ampicillin	Conc. used: 100 µg/ml, Sigma-Aldrich®, USA
Chloramphenicol	Conc. used: 34 µg/ml, Sigma-Aldrich®, USA

VII. Cell Culture and Transfection Reagents

1x Dulbecco's phosphate buffered saline (PBS)	Sigma-Aldrich®, USA
0.25% Trypsin-EDTA solution	Sigma-Aldrich®, USA
Opti-MEM® 1x, reduced serum medium	Thermo Fisher Scientific, USA
Lipofectamine® 2000 Transfection Reagent	Invitrogen, Carlsbad, USA

VIII. Cell Culture Media and Cells

Cells	**Growth Medium**	**Supplements**
A549	Dulbecco's modified eagle's medium, D5796; Sigma-Aldrich®, USA	10% Fetal bovine; Biochrom GmbH, Berlin, Germany 1% Penicillin-Streptomycin 10,000 U/ml

IX. SDS-PAGE

10x Bolt® Sample reducing agent	Invitrogen, Carlsbad, USA
4x NuPAGE® LDS sample buffer	Invitrogen, Carlsbad, USA
Bolt® 4-12% Bis-Tris Gel 1.0mm x 10/12 wells	Invitrogen, Carlsbad, USA
1x SeeBlue® Plus2 pre-stained protein standard	Invitrogen, Carlsbad, USA
1x NuPAGE® MES SDS Running buffer	Invitrogen, Carlsbad, USA
1x TBS-T	University of Tromsø, Pharmacology Research group: 0.1% Tween 20 10x TBS: 10 g KCL 400 g NaCl 200 ml 1 M TrisCl pH= 7.5 Adjusted to 5 l with dH_2O
MagicMark™ XP Western Protein Standard	Invitrogen, Carlsbad, USA
PageBlue™ Protein Staining Solution	Thermo Fisher Scientific, USA

X. Blotting

10x TBS	10 g KCl 400 g NaCl 200 ml 1 M Tris-HCL, pH=7.5 Adjusted to 5 l with dH_2O
1x TBS-T	1x TBS 0.1% Tween 20
Tween® 20	Sigma-Aldrich®, USA

XI. Protein Purification

β-Mercaptoethanol	AppliChem GmbH, Darmstadt, Germany
Ni-Nta Agarose	QIAGEN Gmbh, Germany

Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane	Merck Millipore, a part of KGaA, Darmstadt, Germany
PBS Dulbecco w/o Ca^{2+}, w/o Mg^{2+} powder	Biochrom GmbH, Berlin, Germany
Glutathione Sepharose 4 Fast Flow	GE Healthcare Life Sciences, Oslo, Norway

XII. GST Pulldown Assays

Pierce™ Gluthatione Agarose	Thermo Fisher Scientific, USA

Enzymes

I. Restriction Enzymes

NdeI	New England Biolabs® inc, USA
XhoI	New England Biolabs® inc, USA
DpnI	New England Biolabs® inc, USA
BamHI	New England Biolabs® inc, USA

II. Ligation Enzymes

T4 DNA Ligase	New England Biolabs® inc, USA

III. Polymerases

Phusion® High-Fidelity DNA polymerase	New England Biolabs® inc, USA

Vectors and Plasmids

I. Expression Vectors

pET-15b	University of Tromsø. Pharmacology Research group
pColdII	University of Tromsø. Pharmacology Research group
Gateway® pDEST™15 Vector	Thermo Fisher Scientific, USA
Gateway® pDEST™17 Vector	Thermo Fisher Scientific, USA

II. Cloning Vectors

Dh5α	University of Tromsø. Pharmacology Research group

III. Plasmids

MK5	University of Tromsø. Pharmacology Research group
MK5 (372-472)	University of Tromsø. Pharmacology Research group
MK5 (383-472)	University of Tromsø. Pharmacology Research group
TEV	University of Tromsø. Pharmacology Research group
pEGFP-MK5 372-473	University of Tromsø. Pharmacology Research group
pEGFP-MK5 383-473	University of Tromsø. Pharmacology Research group
pEGFP-MK5	University of Tromsø. Pharmacology Research group
ERK4	University of Tromsø. Pharmacology Research group
ERK4 S186A	University of Tromsø. Pharmacology Research group
ERK4-FLAG	University of Tromsø. Pharmacology Research group

Kits

QIAquick® Gel Extraction Kit	QIAGENT Gmbh, Germany
QIAgen® Plasmid Plus Midi Kit	QIAGENT Gmbh, Germany
QIAprep® Spin Miniprep Kit	QIAGENT Gmbh, Germany

Bacteria

Dh5-α Competent E. coli	University of Tromsø, Pharmacology Research group (see protocol[38])
BL21(DE3)-CodonPlus-RIL Competent Cells	University of Tromsø. Pharmacology Research group

Antibodies

Primary antibodies

His-ERK4 antibody	Dundee, Scotland
Anti-MK5 P-T182 (3. Bleed 0.21 mg/ml)	Dundee, Scotland
Anti-MK5 P-T182 (1. Bleed 0.12 mg/ml)	Dundee, Scotland
Anti-MK5 P-T182 (3. Bleed 0.13 mg/ml)	Dundee, Scotland
GST antibody (Z-5): Sc-459	Santa Cruz Biotechnology, Texas, USA

Secondary antibodies

Donkey anti-Sheep IgG (H+L) Secondary Antibody, Alexa Fluor® 680 conjugate	Invitrogen, Carlsbad, USA
IRDye® 800CW Donkey anti-rabbit igG (H+L), 0.5 mg	LI-COR, GmbH, Germany

Instruments and Machines

PCR machine	MJ Research PTC-200 Peltier Thermal Cycler DNA Engine, Waltham, USA
Incubator for bacteria plates	Termaks, Bergen, Norway
Shaker for bacteria cultures	Unitron HT InforsAG, Bottmingen, Switzherland
Water bath 42°C	Grant Y6 Instruments, Cambridge, USA
Vortex machine	Labnet VX100 Vortex, MO BIO, Carlsbad, USA
Centrifuge	Thermo scientific PICO 21 Heraeus, Thermo Scientific, Waltham, USA
Nanodrop machine	NanoDrop® ND-1000 Spectrophotometer, Wilmington, USA
Agarose gel chamber	BIO-RAD POWER PAC 3000
UV light	UV Transilluminator UVP Model M-15, CA, USA
Machine for visualization of stained agarose gels	Gel Doc 2000 BIO-RAD, Hercules, USA
Mammalian cells incubator	Thermo Scientific Heracell 150i, CO2 Incubator, 37°C , 5% CO2, Waltham, USA
Shaker	Heidolph Unimax 2010, Schwabach, Germany
Bolt® Mini Gel Tank	Invitrogen, Carlsbad, USA
Centrifuge for larger culture protein	Sorvall RC-3C centrifuge
Sonicator for larger culture protein	SONICS Vibra cell VCX 500
Roller mixer	Analogue tube roller- SRT6
High speed centrifuge (25,000 rpm)	Beckman Coulter Avanti J-25
Magnetic stirrer	Heidolph MR 3001
Machine for infrared detection of Western blots	The Odyssey® Sa Infrared Imaging System
Automatic sonicator	Bioruptor, Diagenode, Seraing, Belgium
Manually sonicator	Microson™ Ultrasonic Cell Disruptor

METHODS

There are many steps involved in expression of protein of interest. For each step in the protocol there are choices to be made regarding the specific protocol. Here the protocols are presented chronologically.

The aim of the project was to modulate the activity of MK5. For that the protein itself had to be made. For this to happen, a cloning procedure had to be done, either by restriction enzyme based subcloning or "fast cloning" using polymerase chain reaction (PCR).

Construction of MAP Kinase 5 (Subcloning)

Subcloning is the process of insertion of a DNA fragment from one to another[39]. The DNA fragment and vector of interest are cut with restriction endonucleases. They are then purified by agarose gel electrophoresis, and treated with DNA ligase to bind the sticky ends of the fragment to the vector. The ligation products are introduced into Escherichia coli cells. The cells are then plated with the appropriate antibiotic before DNA from the colonies are being purified and cut with restriction enzymes. A separation technique, called agarose gel electrophoresis separates the DNA fragments from the cut DNA. Hence, the DNA of interest can be confirmed[40].

Restriction

DNA restriction is a technique used for molecular cloning, either by polymerase chain reaction (PCR) or subcloning. It is also used in diagnostic testing to verify the insert of interest[41]. For this to happen restriction enzymes are required. They are also called restriction endonucleases because of the cutting within the DNA. Each endonuclease recognizes specific DNA sequences (usually palindromic). The recognition sequence range from 4-8 nucleotides. Since each endonuclease is different, there are various conditions to ensure their optimal activity, such as temperature, pH, enzyme cofactor(s), salt composition and ionic strength[42,43].

With DNA restriction method, one can for example with two restriction endonucleases cut and give two different ends for the insert. The insert will then orientate with the compatible ends in the vector due to ligation. Novel constructed plasmids will be made. On the other hand, this method can be used to find out if the new constructed plasmids really have been made. The cut DNAs are to be analyzed visually on agarose gel.

Generally the restriction was performed using these steps:

1 µg DNA

Mix of: 1 µl of restriction enzyme
Appropriate buffer for the endonuclease(s) for optimized cutting
Purified water up to volume 20 µl, depending on volume of DNA to be cut

The sample was mixed carefully and incubated at 37°C for 1-2 hours.

For this study, the plasmids were cut with the restriction enzymes NdeI and XhoI with NEBuffer 2.

Gel Electrophoresis

The DNA fragments, which are cut by restriction enzymes are separated by means of agarose gel electrophoresis. The gel act like retentive filter for negative charged fragments which could be resolved by range from ~0.5 to 25 kb for standard agarose. The fragments start near negative pole and migrate to positive one. Hence the smaller fragments will migrate faster and be nearer to the positive pole[44,45].

- 1% agarose gel was prepared by dissolving 0.5 or 1 g Seakem® GTG Agarose, depending on how many samples that were run, in 50 or 100 ml 1x TAE buffer under heating.
- 0.2-0.5 µg/ml of ethidium bromide 10mg/ml solution was then added. Ethidium is a DNA intercalating agent, and fluoresces under UV light.
- The gels was poured into the chamber with a comb on top of it. The comb makes small pockets in the gel, so samples can be loaded in them. 3 µl of 6x loading buffer was mixed to each sample and put in each comb.
- 6x loading buffer contains dye and glycerol, this makes the sample visible and heavier than water so that the sample can be followed.
- The gel ran for 90V in approximately 45 minutes with in 1x TAE buffer. 8 µl 1 kb plus ladder serves as a reference because of the known molecular weight for each bands it presents[46].
- After the gel was run, the DNA fragment was excised with a scalpel under low doses of UV-light to minimize the damage of DNA.
- The gel fragment was then purified with QIAquick® Gel Extraction Kit.

Gel electrophoresis can also be used to separate DNA for qualitative analysis. Here Seakem® LE Agarose was used under the same conditions. The result was analyzed in Gel Doc 2000 UV transilluminator (Bio-rad®, Norway) with the Quantity One 4.30 software (Bio-rad®, Norway), using UV-light.

- 0.5g Seakem GTG Agarose was dissolved in 50ml TAE buffer and 20 µl ethidium bromide 1 mg/ml was added.
- The samples with vector pEt-15b and insert GAD-MK5 were added with 3 µl Gel Loading Dye (6X). 10 µl 1 Kb Plus DNA Ladder was used.
- Gel was run for approximately 40 minutes.

Bands of 5.5 kb for pET-15b and 1.5 kb for GAD-MK5 were extracted and purified using QIAquick® Gel Extraction Kit[47].

Ligation

Ligation enzymes exist to join Okazaki fragments during DNA replication and to repair stranded nick in DNA[48]. The enzymes join the 3'-hydroxyl end of one nucleotide to the 5'-phosphate end of another one. This property can be used to form new plasmids. T4 ligase was used in this study and uses ATP as a cofactor[49].

Mix of: 2 µl purified cut vector
6 µl DNA template
1 µl 10x T4 ligase buffer
1 µl ligase

Incubated at 24°C for two hours. Vector alone + ligase control was done for every ligase reaction, to verify the if the vector and DNA insert were cut during the restriction step.

Control: 2 µl purified cut vector
6 µl purified water
1 µl 10x T4 ligase buffer
1 µl ligase

Incubated for 2 hours at 24°C.

Transformation into DH5α cells

Transformation is a process of inserting DNA into bacteria cell. The first transformation was carried out by Avery[50]. The DNA can only be replicated if it contains an origin of replication, OriC, recognized by the polymerases of the bacteria cells. A DNA sequence encoding for antibiotic resistant allows the cloning and isolation of plasmid DNA in the bacterial cells when plated[51].

In order for the competent cells to uptake DNA, they have to be heat shocked at 42°C.

<u>Day 1</u>

- 50 µl of DH5α cells was thawed in ice for ~20 minutes[52].

- Then 1 µl plasmid was added and incubated for ~20 minutes in ice.
- The mix heat shocked for 90 seconds and was followed by 2 minutes in ice.
- 50 µl of the transformation mix was plated out on LB plates with appropriate antibodies.
- The plates incubated overnight in 37°C.

Day 2

- One grown colony was inoculated in 3 ml LB medium with selection antibiotic and incubated at 37°C with shaking.

Day 3

- Bacteria cells were harvested by centrifugation.
- To isolate and purify the DNA plasmids, QIAprep® Spin Miniprep Kit was used[53]. Strong alkaline with SDS solution was used to lysis and denature the bacterial membrane and DNA.
- The DNA was bound to the column.
- It was then washed with several steps before the DNA was eluted.
- For control if the DNA was successful generated, the plasmid was cut with the same enzymes which yield matching ends during ligation.
- An analytical agarose gel was run to confirm the outcome.

Construction of MAP Kinase 5 (FastCloning)

FastCloning is a cloning method that is generated by Li et al[54]. It excludes the need of joining the insert and vector by T4 DNA ligase after restriction, which has multiple steps and is time consuming. Therefore, this technique makes it easy to make constructs for fusion proteins and chimeras, along with making short insertions and mutation up to 120bp in cDNA. FastCloning uses Polymerase Chain Reaction as a foundation before the products are digested with DpnI restrion enzyme. Eventually, the mix of vector and insert are transformed in competent cells for protein expression.

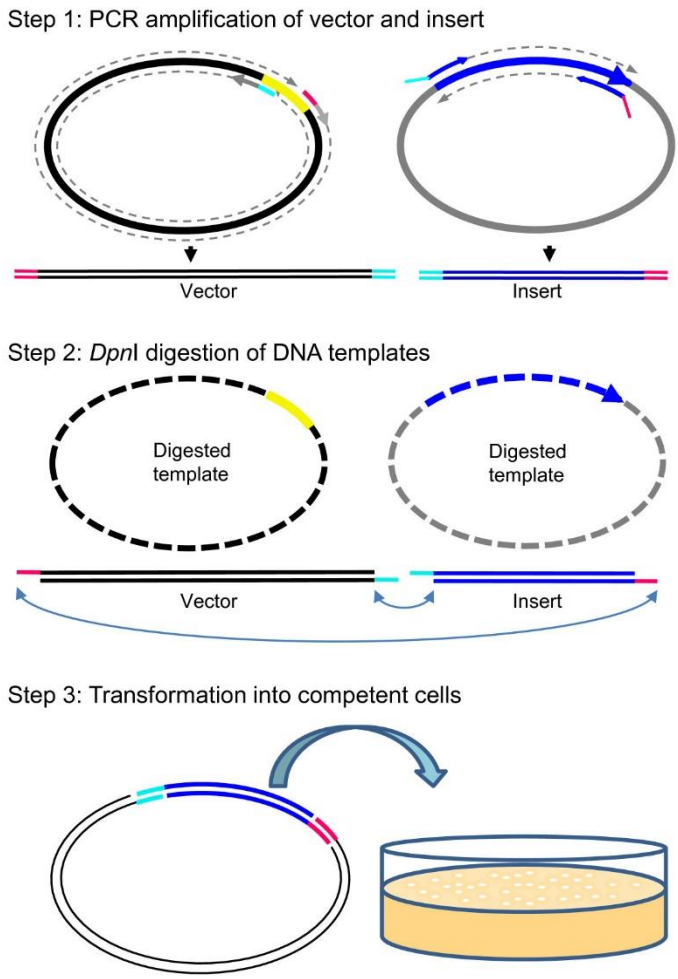

Figure 7 Illustrated overview of FastCloning. Step 1: PCR amplification of vector and insert. Step 2: DpnI digestion. Step 3: Transformation into competent cells (reprinted from [54])

The Polymerase Chain Reaction

The polymerase chain reaction was developed by Kary Mullis in 1983[55]. It is a method for cloning DNA segments of interest without restriction digestion. With the use of primers, which are short single-stranded DNAs with any kind of sequences combination, one can determine which DNA segments in the genome to be copied. Through complementary base pairing, one primer attaches to the start on one end of the DNA strand. The other primer, reverse primer, attaches to the end on the other end of the DNA strand. Primers are starting point for DNA polymerases to start copying. When a DNA polymerase combines with a base pair complex of the primer and DNA strand, it starts adding nucleotides homologous to the DNA sequences. Nucleotides are the building blocks in the PCR reaction. A mixture of four types of nucleotides exist in the liquid together with the primers and polymerase – A's, C's, G's and T's[56], also known as deoxynucleoside triphosphates (dNTPs)[56].

For synthesis of new DNA sequences, the origin DNA template has to denature into two single-stranded DNA (ssDNA)[57]. This is done by heating the samples. Primers can then anneal to the each of the ssDNA complementary to their 5' → 3' ends. This is where polymerases can start extending new DNA strands d. The process of denaturation, annealing and extension go in cycles of 20-30. For every cycle, new strands of DNA are synthesized exponentially. Eventually, 30 cycles will yield 2^{28}-fold amplification of the DNA segments of interest (Figure 8).

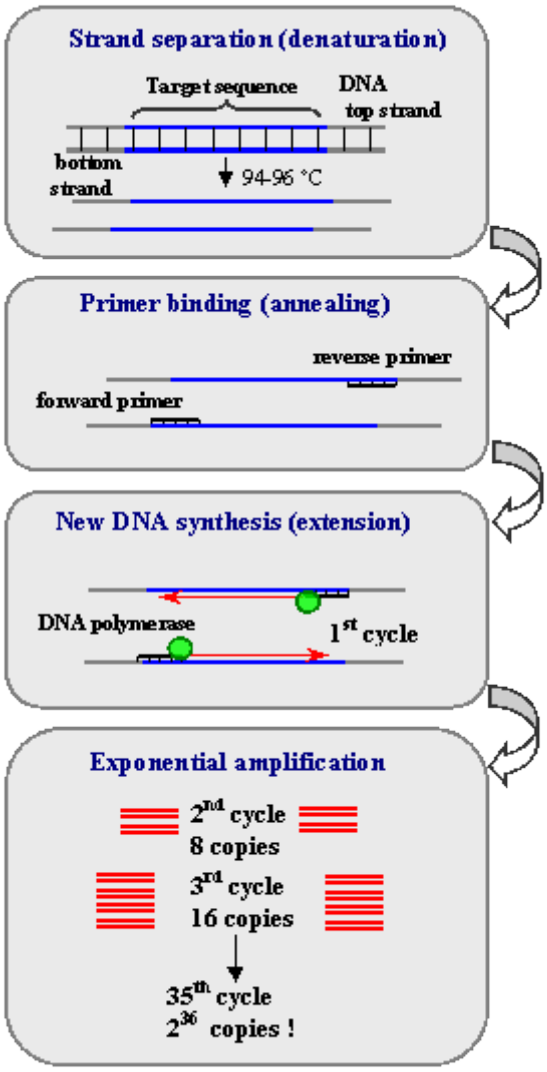

Figure 8 Polymerase chain reaction overview. Template DNA with the target sequence denatures at 94-96°C before primers bind to sense and antisense of the target sequence. Annealing takes place and polymerase of interest synthesize new nucleotides complementary to the ssDNA. After 2nd cycle, two discrete DNA segments generates (reprinted from [58]).

For both MK5 insert and vector separately:

Component	50 µl reaction	Final concentration
5x Phusion GC Buffer	10 µl	1x

10 µM dNTPs	1-2 µl	200-400 µM
10 µM Forward Primer	1 µl	0,5 µM
10 µM Reverse Primer	1 µl	0,5 µM
Template DNA	20-150 ng	Variable |<250 ng
Phusion Polymerase	0,5-1 µl	1,0-2,0 units/50 µl PCR
Nuclease free water	To 50 µl	

PCR program:

1 cycle	3 min at 98°C	Initial denaturation
18-20 cycles	15 sec at 98°C 30 sec at 55°C 30 sec/kb at 72°C	Denaturation, annealing and extension
1 cycle	10 min at 72°C	Final extension

- 5 µl of each PCR product was for routine examination with 1% agarose gel electrophoresis.

Digestion with DpnI and Transformation

- 1 µl DpnI was added into the remaining 45 µl for insert and vector separately.
- The vector and insert were then mixed with 1:1 ratio, and digested at 37°C for 1 hour.
- 3 µl vector-insert mixture were then added to 50 µl of competent DH5α E.coli cells.
- The mixture was then incubated for 30 min on ice.
- After heat shock at 42°C for 45 sec, 300 µl of SOC medium was added to the mixture.
- The mixture incubated at 37°C for 1 hour with shaking at 225 rpm.
- The entire content was plated onto LB agar plate containing 100 µg/ml ampicillin.
- The plate was then incubated at 37°C overnight.
- Next day, colonies were picked for verifying with restriction digestion with BamHI:

Construction of MAPK Kinase 5 Domains

By the means of "Gateway" recombination system, the amino acids 372-473 and 383-473 of MK5 was generated. "Gateway" recombination cloning system is a one-hour recombination reaction with several benefits. It does not use restriction enzymes, ligase and screening of colonies. The method involves two steps. These are: 1) Determination of the Entry clone that contains the DNA insert flanked by attL sequences, which could be recombined with attR sequences in the expression vector; 2) Mediating the reaction with LR Clonase enzymes, which catalyze the reaction between attL and attR sequences so that the DNA insert is integrated in the destination vector, the expression vector. Once the Entry clone is determined, i.e. the DNA

insert in a "Gateway" vector, the gene of interest can be shuttled between any other vector of interest according to which study to conduct[59]. Here our genes of interest were recombined in vectors for protein expression in E. coli.

- pDEST15 and pDEST17 were used as destination/expression vectors.
- The vectors were transformed into DH5α cells.

Transformation with BL21(DE3)-CodonPlus-RIL Cells

Competent BL21(DE3)-CodonPlus-RIL cells are different than competent DH5α cells, in that they are suitable for protein expression[60]. They contain plasmids that encode arginine, isoleucine and leucine tRNAs which are rare in E.coli, but common in human proteins. Moreover, BL21 strains do not express the proteases ompT and lon, which degenerate recombinant protein expression effencieny[61].

Transformation of MK5-HIS, MK5-HIS 372-473, MK5-GST 383-472 and TEV was done into BL21(DE3)-CodonPlus-RIL competent cells, which have resistant genes for chloramphenicol. This yields a more specific isolation of competent cells of interest[62].

- One positive sample of 2 µl was transformed into 50 µl BL21(DE3)-CodonPlus-RIL cells.
- The BL21(DE3)-CodonPlus-RIL cells were thawed in ice for 20 minutes before it was mixed with the DNA.
- The mix was placed on ice for 20 minutes, heat shocked for 30 seconds and put back on ice for 2 minutes.
- 300 µl SOC medium was then added to the mix and grew in 37°C shaking incubator, 225 rpm for 45 min.
- The mix was plated on LB plate with 100 µg/ml ampicillin and incubated at 37°C overnight.

Quantification of DNA and Protein

In order to determine the concentration of DNA from Miniprep or protein from purification, Nanodrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) was used. For DNA the spectrophotometer measures absorbance at the wavelengths 230, 260, which is the nucleic acids' wavelength, and 280 nm. The purity was assessed with the ratio between these wavelengths. For proteins the samples are absorbed at 260 and 280 nm. The wavelength for proteins is 280 nm. 2 µl was used each time for measuring[63].

Full-length MK5, MK5 fragments and TEV Protease Small-Scale Expression

During this step, many experiments were conducted with different conditions for best protein expression in order to get the most soluble proteins for further studies.

Overnight culture and Protein Expression

Table 1 Conditions of MK5-HIS5 (expression optimization)

Trials of MK5-HIS	1	2
Dilution	2:100	2:100
Temp during protein expression	28°C	24°C
Sonication	3 x 10 sec (Microson™ Ultrasonic Cell Disruptor)	5 min, 30 sec on and off (Bioruptor) with 1,5ml microcentrifuge tubes

Table 2 Conditions of TEV protease (expression optimization)

Trials of TEV protease	1	2	3	4	5	6
Dilution	2:100	2:100	2:100	2:100	2:100	2:100
Temp during protein expression	37°C	37°C	37°C	24°C	24°C	24°C
Sonication	5 min, 30 sec on and off (Bioruptor) with 1,5 microcentrifuge tubes	5 min, 30 sec on and off (Bioruptor) with 1,5 microcentrifuge tubes	5 min, 30 on and off (Bioruptor) with 1,5 microcentrifuge tubes	5 min, 30 sec on and off (Bioruptor) with 1,5 microcentrifuge tubes	5 min, 30 sec on and off (Bioruptor) with 1,5 microcentrifuge tubes	5 min, 30 sec on and off (Bioruptor) with 1,5 microcentrifuge tubes

Table 3 Conditions of MK5-HIS 372-473 (expression optimization)

Trials of MK5-HIS 372-473	1	2	3	4
Dilution	2:100	2:100	2:50	2:50
Temp during protein expression	37°C	24°C	24°C	37°C
Sonication	3 x 10 sec (Microson™ Ultrasonic Cell Disruptor)	6 x 10 sec (Microson™ Ultrasonic Cell Disruptor)	6 x 10 sec (Microson™ Ultrasonic Cell Disruptor)	6 x 10 sec (Microson™ Ultrasonic Cell Disruptor)

Table 4 Conditions of MK5-HIS 383-473 (expression optimization)

Trials of MK5-HIS 383-473	1
Dilution	2:100
Temp during protein expression	37°C
Sonication	3 x 10 sec (Microson™ Ultrasonic Cell Disruptor)

Table 5 Conditions of MK5-GST 372-473 (expression optimization)

Trials of MK5-GST 372-473	1
Dilution	2:100
Temp during protein expression	37°C
Sonication	6 x 10 sec (Microson™ Ultrasonic Cell Disruptor)

Table 6 Conditions of MK5-GST 383-473 (expression optimization)

Trials of MK5-GST 383-473	1
Dilution	2:100
Temp during protein expression	37°C
Sonication	6 x 10 sec (Microson™ Ultrasonic Cell Disruptor)

- One colony was inoculated in LB medium with 100 µg/ml ampicillin for MK5-HIS. For MK5 fragments and TEV protease, colonies were inoculated in LB medium with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol.
- Next day the cultures were diluted (see tables above) in their respective medium.
- The cultures were let growth to OD_{600} of 0,6-1 at 37°C with shaking at 225 RPM.

- One sample was taken frequently to check the absorbance.
- IPTG was then added 1:1000 to the culture was shaken at 225 rpm at designated temperature (see above).
- 5 ml was sampled every hour up to 5 hours and overnight, total of 6 samples. Total lysates were sampled before IPTG induction and after overnight.
- The samples except the total lysates were spinned down 20 min at 4000 RPM, 4°C and the supernatants were removed.
- The pellets were resuspended in 500 µl TBS-T and sonicated (see above conditions).
- The samples were then centrifuged at 4°C 10 min, 10.000 RPM.
- The supernatants were transferred to new Eppendorf tubes, and both the pellets and supernatants were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a method for separating proteins by mass and their purity. The proteins wander from negative cathode to positive anode via pores in a gel matrix. The pores size decrease with the wandering towards the anode because of higher acrylamide concentrations. The results of gel pore size and protein size, charge, and shape determines the wandering rate of the protein. SDS is used to denature the protein prior to gel electrophoresis[64].

- For each supernatant samples, 39 µl supernatant was mixed with 15 µl 4x LDS buffer and 6 µl 10x reducing agent.
- 50 µl 4x LDS buffer, 40 µl H_2O and 10 µl 10x reducing agent were added into each pellet instead.
- The supernatant samples were heated 70°C for 10 minutes. Pellet samples were heated 90°C for 10 minutes. This is to shake up the hydrophobic interactions between the molecules within the proteins.
- The samples were run with 1x MES buffer on Bolt™ 4-12% Bis-Tris Plus Gel, 10 well. SeeBlue® Plus2 Pre-Stained Standard was used to visualize the protein molecular weight ranges during electrophoresis.
- The gels were then analyzed Gel Doc 2000 UV transilluminator (Bio-rad®, Norway) with the Quantity One 4.30 software (Bio-rad®, Norway), using UV-light.

MK5 and MK5 Fragments Protein Expression

Conditions	Medium	Resuspension solution	Induction temperature
MK5-HIS	TB + 100 µg/ml AMP + 34 µg/ml CAM	15 ml Lysis buffer A	24°C
MK5-HIS 372-473	LB + 100 µg/ml AMP + 34 µg/ml CAM	15ml buffer A + 1 dissolved tablet of cOmplete™, EDTA-free Protease inhibitor cocktail + 1:1000 β-mercaptoethanol	24°C
MK5-GST 383-473	LB + 100 µg/ml AMP + 34 µg/ml CAM	40 ml PBS solution + 1:1000 β-mercaptoethanol + 1 dissolved tablet of cOmplete™, EDTA-free Protease inhibitor cocktail	37°C

- One colony was picked for 51 ml starter culture in LB medium with 100 µg/ml ampicillin and 34 µg/ml.
- Next day the starter culture was diluted 1:20 in 2 2L erlenmeyer flasks á 500 ml TB medium with 100 µg/ml ampicillin and 34 µg/ml.
- Both flasks were then incubated till OD_{600} of ~1.00 with shaking in 37°C, samples was taken frequently to check the absorbance.
- 1mM IPTG was added and induced for 3-5 hours according to the optimization conditions. The protein solutions were then harvested.
- The protein solutions were then centrifuged for 30 min 10°C at 4.500 RPM using Sorvall RC-3C centrifuge, the supernatants trashed and flasks with pellets were put in -20°C freezer.
- Next day the pellets were resuspended in their appropriate medium (see table above).
- The solutions were then sonicated on ice with Sonics Vibra cell VCX 500 with following settings:

Duration	20 min
Temperature	12°C
Pulse	5 sec
Break	5 sec
Amplitude	50%

- After sonication the solutions were centrifuged for 30 minutes, 25.000 RPM (rotor JA 25.50) at 4°C with Beckman Coulter Avanti J-25.
- The lysates with dissolved proteins were collected for purification.

Protein Purification

In order for further assays of MK5-proteins, they have to be as pure as possible so the impurities do not interfere with the actual results. For this purpose different purification methods were carried out.

Immobilized-Metal Affinity Chromatography

Nickel-nitriloacetic acid (Ni-NTA) agaroses are used for affinity binding to the polyhistidine tag. The recombinant protein with this fusion protein will bind to the immobilized nickel-nitriloacetic acid on the agarose beads. The beads are then washed for unwanted substances, and eventually elution takes place for the purified recombinant protein. Because 6xHis tag has a pK_a of 6.0, raised molar concentration of imidazole during elution will cause reduced pH and the histidine residue will be protonated. This causes the 6xHis tag to dissolve from the Ni-NTA beads[65]. For full length and His-tagged MK5:

- 2 ml slurry of NI-NTA beads were equilibrated by centrifugation twice with 45ml ddH_2O at 2,000g, 5 min, 4°C.
- The water was then removed and beads retained.
- The beads were then washed twice with 45 ml Lysis buffer A and centrifuged at 2,000 x g, 5min at 4°C.
- The supernatant was removed and the beads were mixed with the cell lysate for 2 hours on rotor (Analogue tube roller- SRT6) at 4°C.
- The cell lysate was then centrifuged for 5 minutes at 2000 x g, 4°C and the supernatant was removed.
- The beads with the bound proteins were then washed twice with Lysis buffer A, centrifuged for 5 min, 2000 x g at 4°C, and supernatant removed.
- The beads were further washed with Lysis Buffer A + 25 mM imidazole, centrifuged at 2000 x g, 5 min at 4°C, and the supernatant removed.
- The slurry was then transferred to a gravity column in 4°C. For elution 20 ml of Elution buffer B was used and 9x1 ml elution fractions were taken with a gravity column for further evaluation by SDS-PAGE.
- Protein concentration was measured using Nanodrop® ND-1000 spectrophotometer.

For MK5-His 372-473:

- 4 ml slurry of NI-NTA beads were equilibrated by centrifugation twice with 45ml ddH_2O at 2,000g, 5 min, 4°C.
- The water was then removed and beads retained.
- The beads were then washed once with 20 ml Buffer A and one more time with Buffer A + 5% imidazole 0,5 M
- Centrifuged at 2,000g for 5 minutes at 4°C.
- The supernatant was removed and the beads were mixed with the cell lysate overnight on rotor (Analogue tube roller- SRT6) at 4°C.
- The cell lysate was then centrifuged for 5 minutes at 2000 x g, 4°C and the supernatant was removed.
- The beads with the bound proteins were then washed twice with Buffer A + β-mercaptoethanol + protease inhibitor, centrifuged for 5 min, 2000 x g at 4°C, and supernatant removed.
- The beads were further washed with Buffer A + β-mercaptoethanol + protease inhibitor + 25 mM imidazole, centrifuged at 2000 x g, 5 min at 4°C, and the supernatant removed.
- It was then eluted with Elution buffer B. 2 x 10 ml elutions were taken. The elutions were analyzed with SDS-PAGE.
- Protein concentration was measured using Nanodrop® ND-1000 spectrophotometer.

The glutathione S-transferase (GST) is a 26 kDa protein, whereas the His tag is 1 kDa. The elution condition is milder than of the His tag. GST binds to immobilized glutathione on the agarose beads, washed and eluted with excess of glutathione. Because the affinity to the glutathione in elution buffer is higher than of the beads, the GST-tag with the recombinant protein dissolves from the beads[66]. A batch purification was done in this study[67]. For GST-tagged MK5 383-473 domain:

- 5 ml slurry of Glutathione Sepharose® 4 Fast Flow was equilibrated by washing with 20 ml dH_2O, centrifuged at 4°C, 3000 RPM for 5 min.
- The beads were then washed with 45 ml PBS solution, centrifuged at 4°C, 3000 RPM for 5 min.
- The lysate was mixed with the beads and incubated with rotation for 30 min at 4°C.
- Medium was sedimented by centrifugation at 500 x g, 4°C for 5 min, and the supernatant was discarded.

- The beads with the bound protein was then washed three times with 20 ml PBS solution. Centrifuged 500 RPM at 4°C for 5 min after each wash.
- 3x5 ml elutions were taken by adding 50 mM Tris, 10 mM reduced glutathione and 1:1000 β-mercaptoethanol, pH=8.0.
- The proteins were analyzed with SDS-PAGE, aliquoted and stored at -20°C.

Gel-Filtration Chromatography

Gel-filtration chromatography separates proteins by the means of different molecular sizes. The gel matrices may differ in porosity, hydrophobicity and charge. Porosity leads to selection of smaller proteins, in which larger proteins may be excluded and swept through the column. Hydrophobicity and charge give chemical properties to the matrices where they can interact with the corresponding properties of the protein molecules. The matrix is packed in a chromatography column, sample mixture applied and an appropriate solution (mobile phase) is running through the column. The solution is eluted in decreasing order of size into fractions. An UV detector analyzes the samples[68,69].

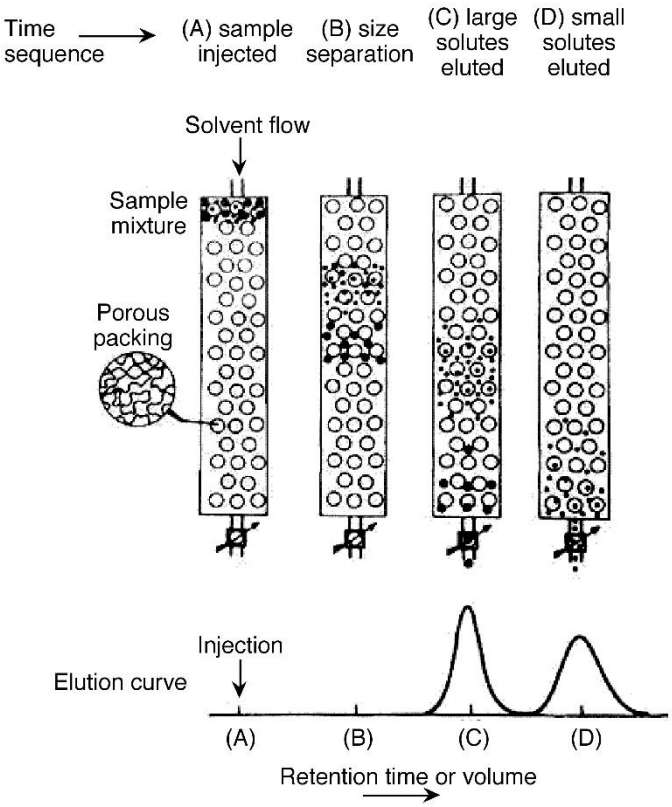

Figure 9 Schematic presentation of the principle of gel-filtration chromatography. Bigger protein molecules that can not penetrate the porous matrix, are eluted first. The small proteins retain in the matrix is eluted later (adapted from [70]).

- Gel-filtration chromatography was done with following run parameters:

Flow 1ml/min
Pressure 0.5 MPa
Elution length 1.5 CV
Eluted fractions: 2ml

Dialysis

By dialysis, small molecular weight substances which are unwanted and may interfere subsequent assays are being eliminated. The sample proteins are placed in a semi-permeable membrane with a fixed pore size. Protein molecules which are bigger than that size, are being retained in the membrane. Smaller molecules and buffer salts pass through the membrane by means of passive diffusion, in which reduce their concentration in the sample. The membrane is embraced with a buffer solution, also called dialysate, which is usually 200-500 times the volume of the sample[71]. For MK5-His:

- All the eluted fractions were collected together and were set to dialyze overnight at 4°C with 2L of the Dialysis buffer A. 4mg of recombinant TEV protease (Norstruct) was added to the buffer to cut off the His-tag on the MK5 proteins.
- The collected elution was transferred to Snake Skin Dialysis tubing 3500 MWCO, which cut off molecules more than 3,5 kDa, lower size molecules will be able to pass through the membrane. His-tag is of 1 kDa and will pass through.
- The membrane was then sealed and put in the buffer with stirring.
- The solutions were aliquoted with glycerol and freezed at -20°C.

For MK5-His 372-473:

- The protein dialyzed in Dialysis buffer B with 4mg of recombinant TEV protease (Norstruct).
- Snake Skin Dialysis tubing 3500 MWCO membrane was used.
- The solutions were aliquoted with glycerol and freezed at -20°C.

Concentration of the Protein

Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane was used for protein concentration. It has nominal molecular weight at 3 kDa, process volume of 15ml and is non-sterile. With the nominal molecular weight of 3 kDa means molecules with 3 kDa and above will get filtrated out during centrifugation[72].

- Added up to 15ml of sample to the Amicon® Ultra filter device.
- Centrifuged for 15 min at 4,000xg.

- The concentrated solute was recovered with pipettor inserting into the bottom edge of the filter device.
- The solutions were aliquoted with glycerol and freezed at -20°C.

Mammalian Cell Culture Techniques

Cell culture

The A549 cells, which were derived from lung cancer cells from human, were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cell were incubated in Thermo-scientific nunc® EasyFlasks 175cm^2 inside a HERAcell® 150i CO_2 incubator at 37°C, 5% CO_2 and 80-95% relative humidity.

Cell splitting

The cell culture was split 1-3 times a week, depending on cell confluence and planning of experiments. Before the splitting took place, medium and trypsin were warmed in 37°C heat incubator, PBS was kept at room temperature.

- Old medium was discarded.
- Washed Cells with 3-5ml PBS and removed.
- Added 3ml 0.25% trypsin-EDTA and put in the heat incubator for few minutes for the trypsin to work. Made sure that the cells were not adherent to the surface by tapping the flask after heating.
- 7ml medium with supplements were added and diluted 1:10. Fresh medium was filled up to 25ml.

Counting Cells

Bürker hemocytometer was used for cell counting. Cell counting is necessary prior to transfection for the calculation of 50-80% confluency of cells on the transfection day[73,74].

- The coverslip was affixed properly on the chamber.
- After resuspension of cells with fresh medium, some cell culture was transferred under the affixed coverslip.
- Cells were counted in at least 3 of the 9 A-squares under Zeiss AxioVert S100 inverted microscope.
- Total cells/ml were obtained with the following formula:

$$\frac{\text{Cells}}{\text{ml}} = \frac{\text{Total cells counted x dilution factor x } 10^4}{\text{number of squares}}$$

Seeding of Cells

After cell concentration estimation, approximately 500 cells/well were seeded in 60mm dishes. Cells were always seeded day before the planned transfection assay. The cells were seeded corresponding to "useful numbers for cell culture" by invitrogen™.

- Total amount of cell suspension needed in mL was calculated with this formula:

$$\text{Cell suspension} = \frac{\text{number of dishes x desired cell number in each dish}}{\text{Cell concentration } \left(\frac{\text{cells}}{\text{ml}}\right)}$$

- The calculated cell suspension and 2 ml of medium were added to each dish.
- The dishes were incubated overnight in the humidified incubator at 37°C and 5% CO_2 for further assay.

Transfection

Transfection is the introduction of genes into mammalian cells. The method can be used to check for gene expression on cell growth, genetic elements for regulation of expression, mutational analyses and to produce of specific protein[73,75]. There are various techniques for transfection. Calcium phosphate transfection and DEAE-dextran transfection attach the DNA on the cell surface before being endocytosed into the cells. Electroporation uses electrical field to open the pores of the cell so DNA can diffuse. The mechanism behind liposome-mediated transfection is not completely understood, negative charge of the phosphate group in DNA binds to the positively charged liposome. The positive surface of the liposome mediates the interaction between the DNA and the cell surface and the DNA enters the cell[75].

Transfection into A549 cells was done with Lipofectamine® LTX & PLUS™ Reagent[76]. 6-8 60mm dishes were used each experiment. One dish was used for one transfection.

- 1-3µg DNA was mixed with 500µl Opti-Mem® Medium and 1,5µl PLUS™ Reagent and incubated 5 min in room temperature.
- 3 µl Lipofectamine® LTX Reagent was added to the mix and incubated for 30 min.
- The whole content was transferred drop by drop into the dish with A549 cells.
- Incubated overnight at 37°C in heat incubator.

Harvesting

The cells are usually harvested 24-72 hours after transfection[77].

- The medium from the 6mm dishes after transfection was discarded.

- Dishes were washed with 250µl PBS.
- 200µl MAPKK lysis buffer + protease inhibitor was added to each dish.
- The adherent cells were scraped off with a cell scraper.
- Cells lysate was transferred to 1.5 ml pre-cooled microcentrifuge tube.
- The tubes were put on ice for 15 min.
- Tubes were then centrifuged for 10 min at 13.000 RPM in 4°C.
- Supernatant was transferred to new tubes.
- 39µl of sample was taken out for SDS-PAGE assay.
- Lysate was stored in -70°C freezer with nitrogen pre-cooling first.

Western Blot

Western blot is a technique for separating and identifying proteins. The proteins are first separated by molecular weight by electrophoresis resulting in bands, which are transferred to a membrane. The membrane is then exposed for proper primary and secondary antibodies. Eventually, the antibodies bound to the bands can be visualize[78].

SDS-PAGE

- SDS-PAGE was run with 2 standards: SeeBlue® Plus2 Pre-stained Protein Standard and MagicMark™ XP Western Protein Standard.

Blotting

- Sponge pads, filter papers and membrane were prior soaked in 1x blotting buffer until saturation before use.
- The pads, filter papers and membrane were assembled like a sandwich. Figure 10 illustrates that.

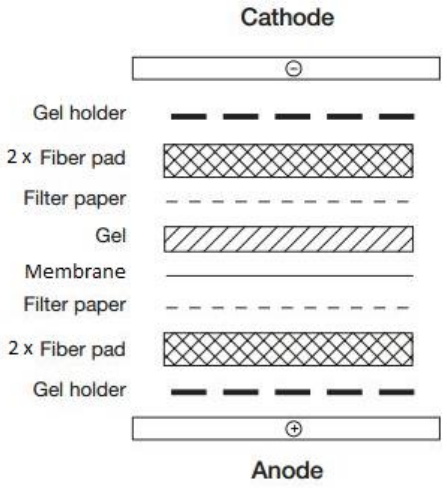

Figure 10 Blotting sandwich. The signals from the gel are transferred to the membrane (adapted from [79]).

- The blot was run at 25V for 1-2 hours.

Blocking

- After blotting was completed, the membrane was removed from blotting sandwich.
- Membrane was soaked in 15 ml Odyssey® Blocking Buffer for 1 hour with gentle rocking.

Antibodies Staining

- 5 ml blocking buffer and 5 µl Tween 20 (1:1000 dilution) was mixed in new 50 ml tube by vortex.
- Primary antibodies were the diluted in the solution and mixed.
- Membrane was rolled with bands towards the inside in the new tube.
- The membrane incubated overnight at 4°C on roller mixer.
- Membrane was washed 4 x 5 minutes in TBS-T solution.
- After washing, the membrane was incubated in 5 ml TBST-T solution with appropriate and diluted secondary antibody for 1 hour at 4°C.
- Membrane was the once again washed with 4 x 5 minutes in TBST-T solution.
- The membrane was analyzed with The Odyssey® Sa Infrared Imaging System.

In Vitro Kinase Assay

- Following were prepared on ice and incubated for 45 min at 30°C:

Phosphorylated MK5	**Unphosphorylated MK5**
5 µg MK5-HIS	5 µg MK5-HIS
1 µl PET38	100 µM ATP
100 µM ATP	5 µl 10x kinase buffer
5 µl 10x kinase buffer	X dH_2O

X dH_2O	
Total of 50 µl	Total of 50 µl

- 2x LDS sample buffer was added and heated for 10 min at 70°C.
- The mixes were then diluted to 0,1 ng/µl, 1 ng/µl and 10 ng/µl.
- 1x LDS sample buffer were added to the diluted solutions.
- SDS-PAGE was then run.

GST Pulldown Assays

Pulldown assays are used to determine interaction between two proteins. The "bait" protein, which is tagged , can be an interacting protein for other proteins. With the bait protein bound to this other protein, they can be isolated by which the tag on the bait protein bind to immobilized affinity ligands[80].

- After transfection, the extract was thawed on ice.
- 1µg GST and 1µg GST-MK5 382-473 was added to each extracts and incubated for 1 hour at 4°C.
- 10 µl washed Glutathion agarose (50% slurry) was added and incubated for further 30 min at 4°C with rotation.
- The samples were transferred to SigmaPrep™ spin column and washed 3 x 400µl MKK lysis buffer + protease inhibitor.
- Eluted by adding 40µl 2x LDS sample buffer, heating at 70°C for 10 min, and spun out.
- 20 µl of each sample was taken out for Western Blot assay with appropriate antibody.

RESULTS

Generation of Vectors for Bacterial Protein Expression

The aim was to insert the MK5 gene into a vector for protein expression in E. coli cells. For that the expression vector needs 1) a DNA sequence encoding a selectable marker that confers a trait for selection. This could be an antibiotic resistant gene, 2) a transcriptional promotor (e.g lac) that is controllable and when induced, can transcribe large amounts of mRNA from cloned gene for translation, 3) translational control sequences, such as a ribosomal-binding site (eg. Shine-Dalgarno, SD) and the start codone, ATG, for initiating expression, 4) a multiple cloning site for inserting the gene in a correct way in the vector[81]. MK5 protein should also have a fusion protein such as the His-tag, so that it could be purified later on. This can be achieved by inserting the MK5 gene into an expression vector.

In addition to the expression vector, the DNA has to be introduced into an E. coli strain for protein expression. The BL21 (DE3) strain contains the rare codons encoding the amino acids arginine, isoleucine, and leucine that are rare in ordinary E. coli cells. It is also deficient in Ion and ompT protease, which degenerate the target protein *in vivo*[61]. The strain uses a T7 polymerase system under the control of the lac promotor. Under conditions without IPTG, an inducer for protein expression, the lac promotor is repressed. Once IPTG is introduced, the repressor falls off and the T7 RNA polymerase can be expressed. When the T7 polymerase is expressed, it can bind to the T7 promotor on the expression vector and generate our gene of interest. The T7 RNA polymerase has many benefits such as: 1) faster RNA synthesizing than E. coli RNA polymerases, and less transcription terminating frequency, 2) selective for its initiation at its own promotor sequence, it does initiate for any other sequences in E. coli DNA, and 3) resistant to antibiotics that inhibit E. coli polymerases[82].

Construction of MK5 Expression Plasmid by Subcloning

Experiments of cloning His-tagged MK5 with restriction enzyme digestion and ligation were conducted. Several clones were analyzed using the same restriction enzymes used in the cloning. One representative gel from a restriction enzyme analysis of 10 clones is shown in Figure 11. Cutting of a correct cloned expression vector should result in two fragments, one of 1419 bp representing the MK5 gene and 5701 bp representing the pET-15b vector. The result depicted in Figure 11 show only one single fragment of around 5000 bp representing the empty vector from clone 1-2 and 4-10. In lane 3 we observe a major band at 3000 bp and two minor

bands at 6000 and above 7000 bp respectively. This represents uncut vector. We repeated these experiments several times with similar results.

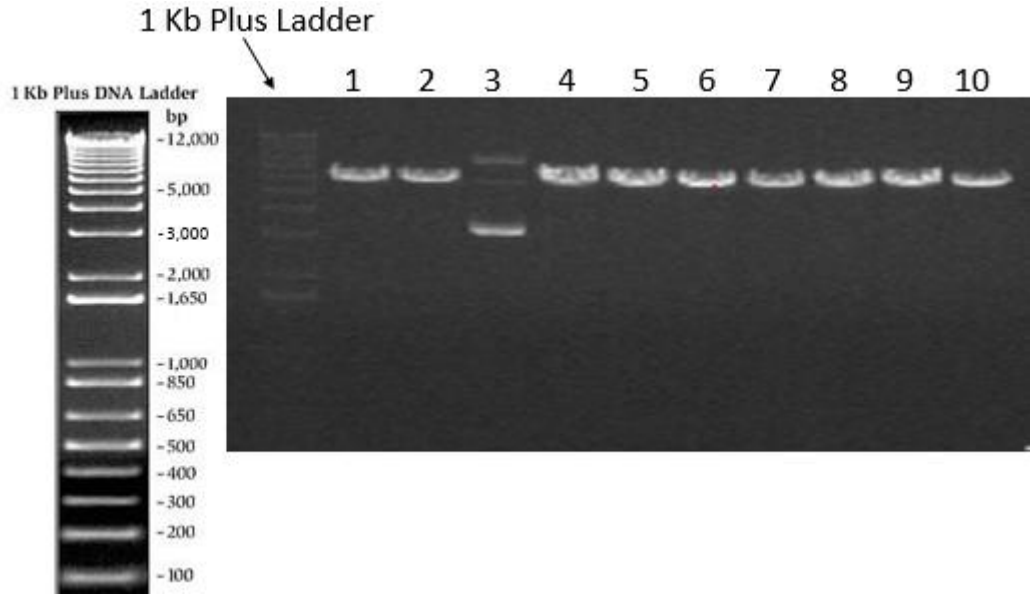

Figure 11: Restriction Digestion of MK5. Purified plasmid DNA from 10 individual clones (1-10) digested with XhoI and NdeI, and run on a 1% agarose for 45 min at 90v. 1 Kb Plus DNA Ladder was used to visualize the molecular weight of linear double-stranded DNA fragments in UV-light.

Construction of MK5 Expression Plasmid by "FastCloning"

As we were unable to clone the His-tagged MK5 in a prokaryotic expression vector by standard restriction enzyme based cloning we need to use an alternative method. We choose to use the method called "FastCloning". With "FastCloning", the DNA insert and expression vector are PCR amplified before they are cut with the restriction enzyme DpnI and transformed into competent cells. Restriction with DpnI will cut methylated DNA sequences, reducing the background DNA templates. After PCR amplification of MK5 DNA insert and pCold II vector, the generation of PCR products were verified with agarose gel electrophoresis. Results depicted in Figure 12 shows that we were able to generate PCR fragments from the MK5 gene (1413 bp) and from the vector (4392 bp).

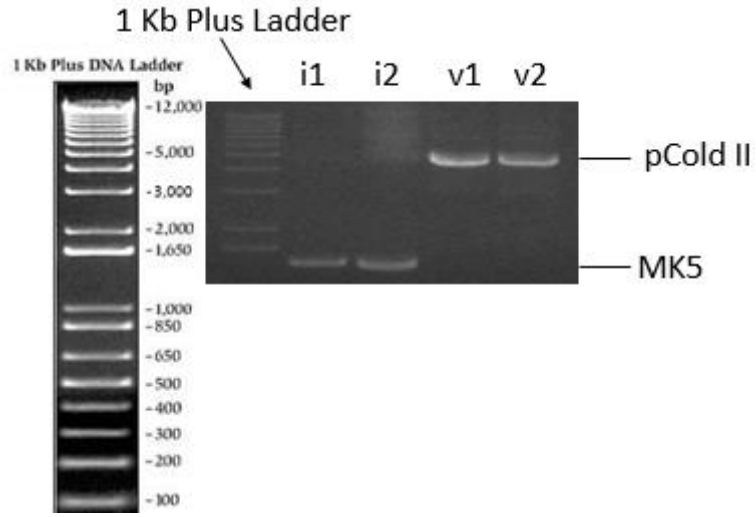

Figure 12: Post-PCR amplication of MK5 and pCold II. 1% agarose gel stained with ethidiume bromide and run for 45 min at 90v. Two samples of MK5 DNA (i1 & i2) and pCold II DNA (v1 & v2) were analyzed after PCR amplication. The gel shows MK5 with bands of 1413 bp and pCold II bands of 4392 bp. 1 Kb Plus DNA Ladder was used to visualize the molecular weight of linear double-stranded DNA fragments in UV-light.

After DpnI digestion, the inserts and vectors were mixed 1:1, and transformed into Dhα cells. Plasmid DNA was isolated from 4 individual colonies and analyzed by restriction enzyme digestion with BamHI. Restriction enzyme analysis with BamHI should result in two separated fragments of 1413 and 4392 bp if the cloning was successful. As shown in the picture of the ethidium-bromide stained agarose-gel from this analysis, 3 of 4 clones were correct (Figure 13).

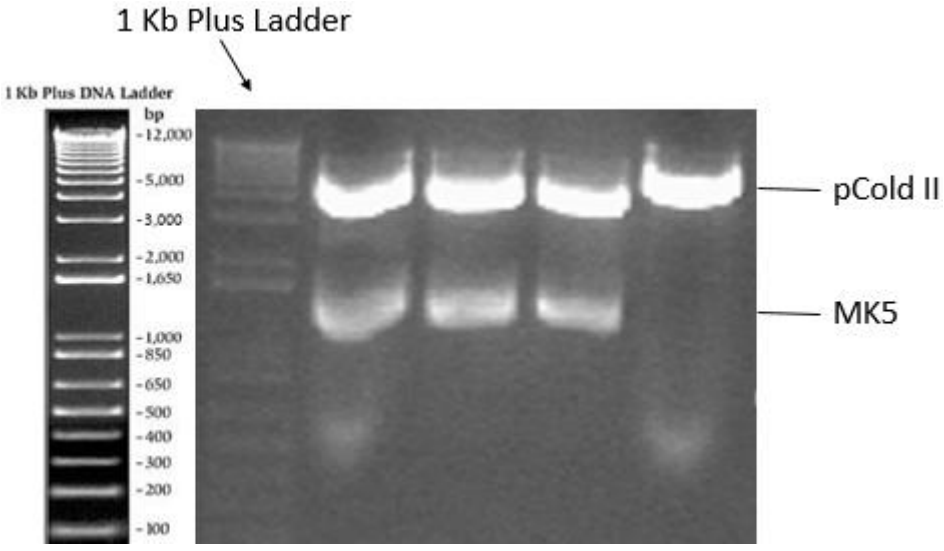

Figure 13: Cut with BamHI. DNA from 4 colonies were cut prior to 1% agarose gel electrophoresis (run for 45 min at 90v). 3 of the 4 lanes were successfully cut, which gave two bands, one at 1413 bp (MK5) and 4000 bp (pCold II). One lane had one band of 5000 bp. 1 Kb Plus DNA Ladder was used to visualize the molecular weight of linear double-stranded DNA fragments in UV-light.

Construction of Expression Plasmids for MK5 Domains

We also wanted to express and purify the unique C-terminal domain of MK5. In order to do this we generated separate expression plasmid constructs with help of the "Gateway" recombination system as described in the methods sections. We cloned two different fragments representing amino acid 372-473 and 383-473 of MK5 respectively. These two domains of MK5 were cloned into two different vector, one with an N-terminal His-tag (pDEST17) and one with a N-terminal GST-tag (pDEST15) respectively. Subsequently, the purified DNAs from 8 individual colonies were cut with BsrGI before they were verified with 1% agarose gel electrophoresis. Successful cutting of GST-tagged MK5 domains should result in two fragments, around 400 and 5328 bp. For His-tagged MK5 domains, the two fragments are expected to be at around 350 and 4669 bp respectively. As depicted in Figure 14, all the clones were correct cut.

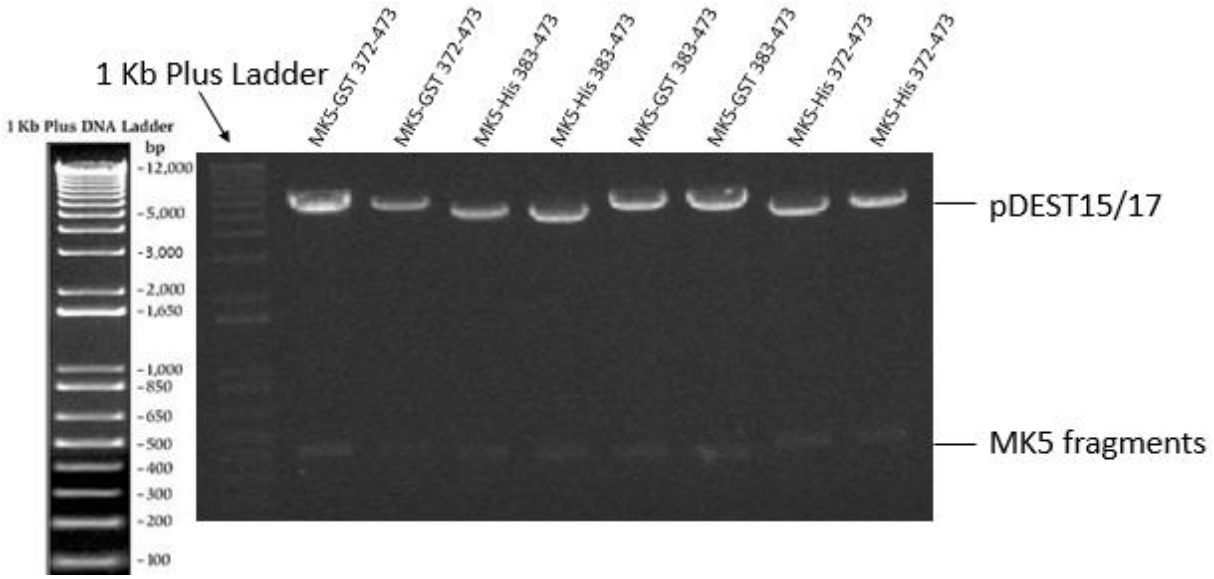

Figure 14 MK5 fragments. Purified DNA from 8 individual colonies were cut with BsrGI prior to 1% agarose gel electrophoresis. Bands of around 6000 bp represent pDEST15 or pDEST17 vector. Bands of 400 represents the MK5 fragments. Analyzed with UV-light.

Protein Expression

Our goal was to express full-length MK5 and MK5 fragments for use in different assays. We wanted to produce large amounts of pure and soluble proteins in the shortest time as possible. The proteins should be in soluble form for easier purification with subsequently purification methods. Moreover, our aim was to gain structural information of the proteins. For that, the proteins should be refolded precisely to its native form in solutions. Purification with

denaturation and renaturation of the insoluble form of the protein do not assure correct refolding[81].

The solubility of proteins can be improved by changing expression conditions. These include the temperature, concentration of the inducer, media, sonication and OD_{600} prior to induction. Some fusion proteins enhance the solubility such as the maltose-binding protein (MBP), but in this study we used GST- and the His-tag. These tags do not enhance solubility[61]. We wanted to focus on OD_{600}, temperature and sonication because these variables were critical for the proteins to be soluble. When the protein gels show significant and distinct bands of our protein generated via small-scale expression, large-scale expression can be proceeded.

The optimization was carried out prior to the actual large scale protein expression and purification. This was to save time and resources in case the actual protein expression did not take place. When the protein gels show significant and distinct bands of the molecular weight of our protein of interest, we continued on with large scale expression.

Optimization of MK5-His

At first we wanted to conduct a small scale expression experiment of His-tagged MK5 to decide which conditions are best for protein expression. As shown in Figure 15 A, we did the first optimization experiment to set a reference. Right before induction, one sample 0 was taken. During induction with 1 mM isopropyl β-D-1-thiogalactopyranoside, one sample was harvested after 1,2,3,4, 5 hours and overnight. OD_{600} was at 0.650 prior to induction and temperature. during induction time was at 28°C.

We wanted to test different OD_{600} and induction temperature. Higher OD_{600} and lower induction temperature were tested and this should result in stronger bands of 52 kDa (MK5-His) in the supernatant gel representing more soluble protein because of the lower temperature. As shown in Figure 15 B, this was true. The supernatant gel showed stronger bands of MK5-His in comparison to the background compared with condition A. Therefore, condition B was carried out for large scale protein expression.

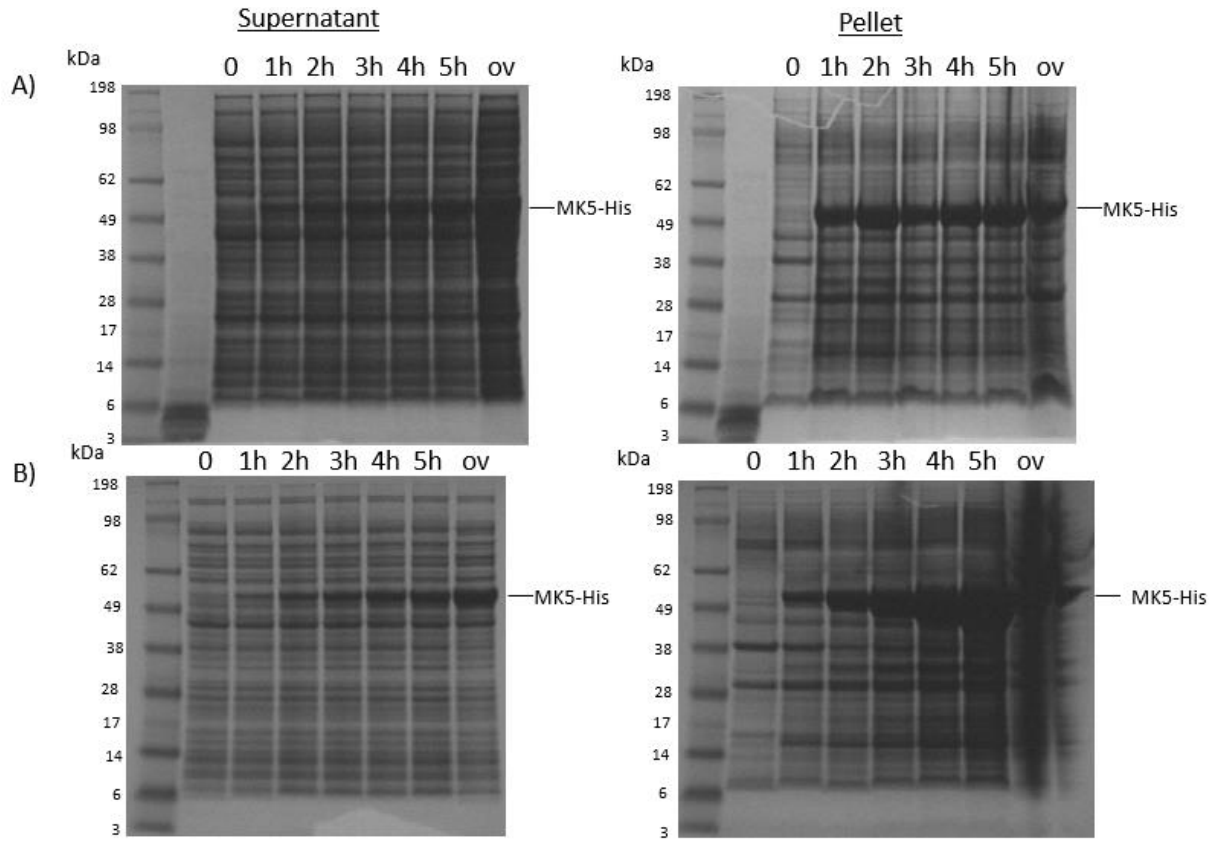

Figure 15: Supernatant and pellet of MK5-His. SDS-PAGE run with MES running buffer. Bands of ~52 kDa revealed the MK5-His protein. **A**: Protein gels of supernatant and pellet before inducing with IPTG (0) and during 1mM IPTG induction after 1, 2, 3, 4, 5 hours and overnight (ov). The protein was harvested after overnight (ov) and analyzed as well. Induced at 28°C during protein expression and sonicated 3 x 10s with Microson™ Ultrasonic Cell Disruptor. OD_{600}=0.650 before induction. Stained with Coomassie Brilliant Blue. **B**: The protein was harvested with the same time interval as A), but temp. during protein expression the temp. was at 24°C and the protein sonicated for 5 min, 30 sec on and off with Bioruptor instead. OD_{600}=0.860 before induction.

Optimization of TEV

Tobacco Etch Virus nuclear-inclusion-a endopeptidase (TEV) protease is used in cleaving off the His-tag. The TEV protease is a 27 Kda large catalytic domain from tobacco etch virus. The native form of TEV cleaves itself so that it becomes truncated yielding diminished enzyme activity. Therefore, a more stable construct of TEV, the mutated TEVsh, was expressed in this study. The mutated form is more soluble and stable. The expression vector of TEVsh is pTH24, which is a derivate of the gateway vector pET-DEST42. It expresses a C-terminal V5 epitope and His-tag and the ampicillin resistance gene and the LacI gene. TEVsh protease is 33 kDa due to its extra amino acids sequences from the vector[83].

We carried out a total of six optimization experiments for the His-tagged TEVsh. We wanted to test different OD_{600} prior to induction and induction temperature to see what the best condition is. One sample, 0, was taken before induction with 1 mM IPTG. The samples were

also harvested after 1, 2, 3, 4, 5 hours and overnight and centrifuged the next day to separate the supernatant and pellet. The supernatant and pellet samples were analyzed with run with SDS-PAGE and analyzed as shown in Figure 16 and Figure 17.

Figure 16 shows three experiments conducted with OD_{600}=1.350, 0.573 and 0.992 under the induction temperature of 37°C. Samples taken overnight (ov) for supernatant in 37°C did not show bands of TEV, while after 1 hour of induction we could already see the TEV of 33 kDa was expressed for experiments A-C. Gel B (supernatant) seems to have the strongest and purest band after 2 hours of induction with OD_{600}=0.573 out of the three supernatant gels.

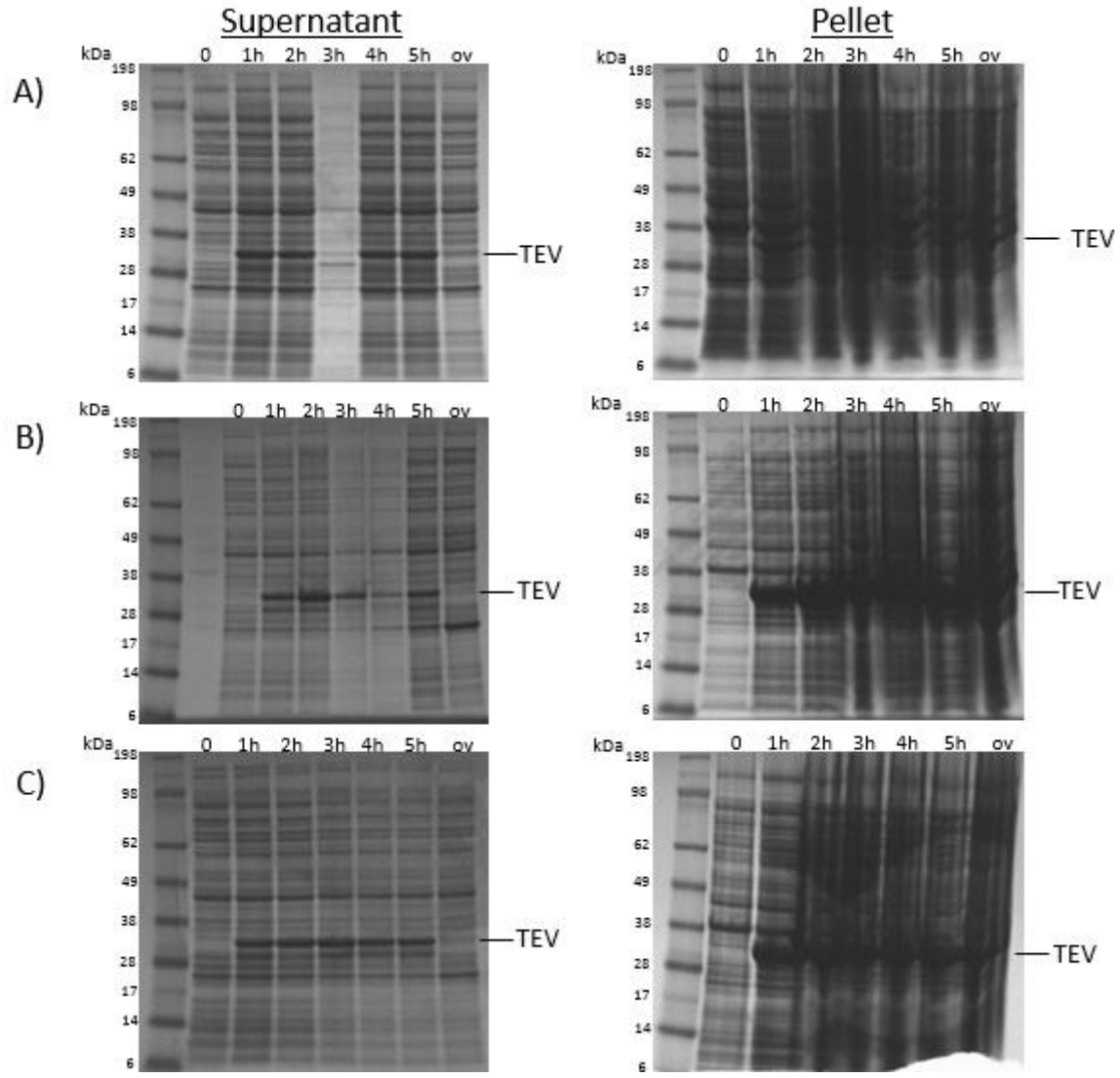

Figure 16: Supernatant and pellet of TEV, induced at 37°C. Three optimization experiments, A), B) and C), of TEV protease under the induction temperature of 37°C and run on SDS-PAGE with MES running buffer. Lane 0 indicates before IPTG induction. Samples were harvested after 1, 2, 3, 4, 5 hours and after overnight (ov). Sonicated for 5 min, 30 sec on and off with Bioruptor. The 33 kDa bands revealed the TEV protein. Stained with Coomassie Brilliant Blue. **A**: OD_{600}=1.350 prior to induction. **B**: OD_{600}=0.573. **C**: OD_{600}=0.992.

We also tested with induction temperature at 24°C of OD_{600}=1,280, 0.865 and 0.473 as depicted in Figure 17. Total lysate, t0 and t1, were also taken, before induction and after overnight, respectively. Total lysates are solutions before separation to supernatant and pellet. The total lysates before induction should result in no distinct band of 33 kDa representing TEV. Visible bands of TEV could first be seen after 2 hours of induction for all experiments depicted in Figure 17 D-F. Out of all the experiments, A-F, experiment B (Figure 16) with OD_{600}=0.573 and induction temperature at 37°C, seems to yield the strongest distinct band from the background after 2 hours of induction. We did not proceed with large-scale expression and purification of TEV, because we got some stored TEV protease left for using in the purification step of His-tagged MK4 and its segments. For the TEV protease protocol for expression and purification, please refer to Carlsen et al.'s protocol[83].

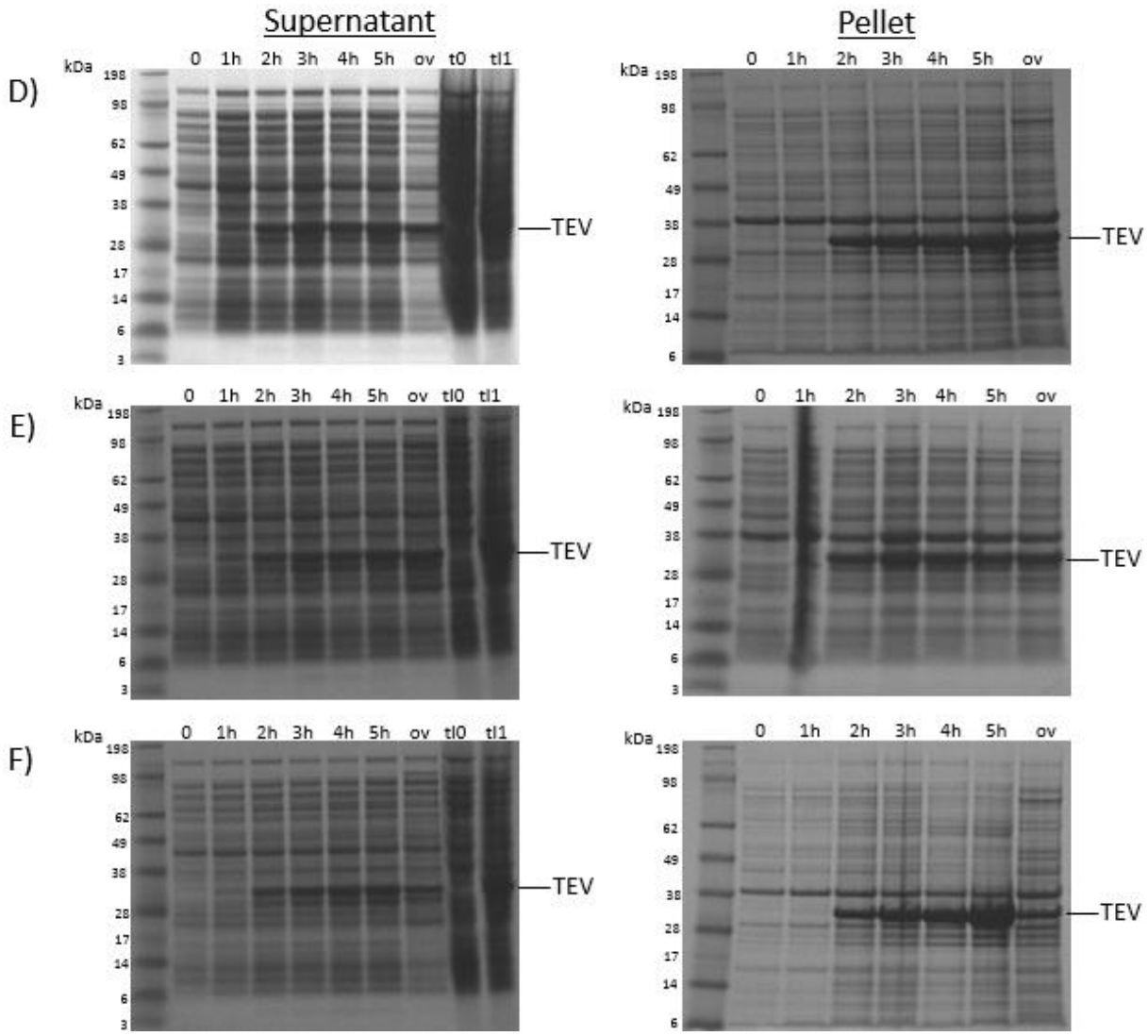

Figure 17 Supernatant and pellet of TEV, induced at 24°C. Samples were harvested before induction (0) and after 1, 2, 3, 4, 5 hours and after overnight (ov). tl0=total lysate (both supernatant and pellet) before induction, tl1=total lysate after overnight. SDS-PAGE with MES running buffer. Sonicated for 5 min, 30 sec on and off with Bioruptor. Bands of 33 kDa revealed the TEV protein. Stained with Coomassie Brilliant Blue. **D**: OD_{600}=1.280 prior to induction. **E**: OD_{600}=0.865. **F**: OD_{600}=0.473.

Optimization of MK5 Segments

The MK5 segments of 372-473 and 383-473 are of important. In order for expression of these proteins, a new series of optimization experiments were carried out. Since each protein has its own biophysical characteristics, it is often required to change the expression protocol to yield successful soluble proteins. Therefore, different OD_{600} and temperature were tested with the MK5 fragments. We used temperature of 37°C to start off with, because this would give shorter induction time before proteins are expressed.

MK5-His fragments

The His-tagged amino acids 383-473 and 372-473 of MK5 were tested with induction temperature of 37° as shown in Figure 18. We expected to get bands of 15 kDa representing our protein. A before induction sample (0) was taken. After induction with 1 mM IPTG, samples were taken after 1.75, 4.258 hours and overnight (ov) for the 383-473 domain. Samples were taken after 2.5, 5 hours and overnight (ov) for the 372-473 domain. The supernatant gel of experiment B showed major bands of 15 kDa than in experiment A. Moreover, there are bigger 15 kDa bands in the pellet gels than in the supernatants.

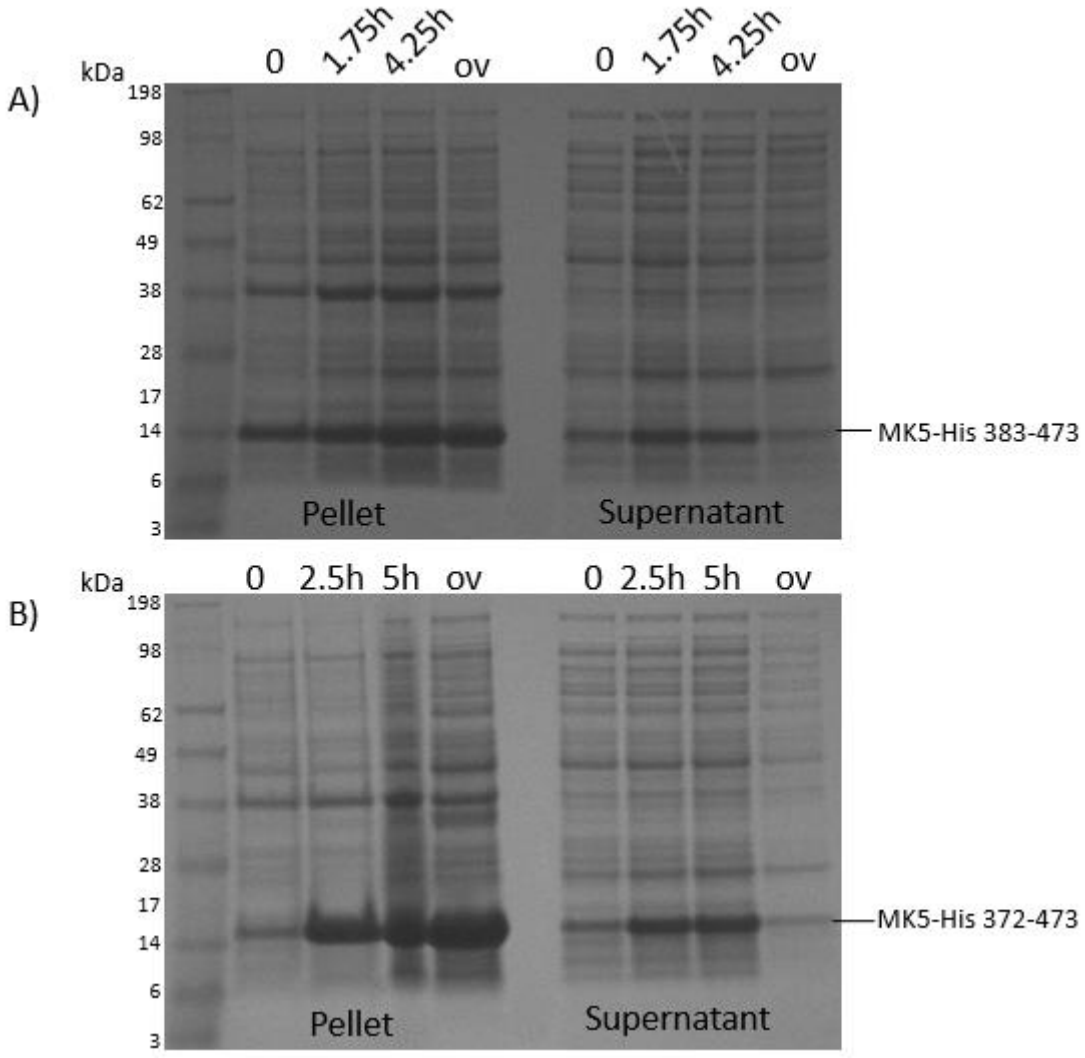

Figure 18: MK5-His 383-473 and MK5-His 372-473. SDS-PAGE with MES running buffer of MK5-His fragments A and B for both pellet and supernatant. The separation to pellet and supernatant was due to centrifugation of the samples taken after the specified time (A: 0, 1.75, 4.25 hours and overnight. B: 0, 2.5, 5 hours and overnight). Induced at 37°C and sonicated 3 x 10 sec (Microson™ Ultrasonic Cell Disruptor). 0=prior to 1 mM IPTG induction. Bands of ~14-15 kDa reveal the MK5-His fragments. Stained with Coomassie Brilliant Blue. **A**: OD_{600}=0.710. Samples were harvested and run on gel after 1.75, 4.2 hours and overnight (ov). **B**: OD_{600}=0.872. Harvested after 2.5, 5 hours and overnight (ov).

MK5-His 372-473

As the supernatant gel for MK5-His 372-473 showed strongest bands out of the two experiments of MK5-His domains (372-473 and 382-473), we chose the His-tagged MK5 372-473 to proceed with. We changed the induction temperature to 24°C, increased the OD_{600} to 1.640 and extended the sonication (6 x 10 sec). The extended sonication was due to the stronger bands in the pellet gel than in the supernatant (Figure 18). We expected not to get any strong bands at around 15 kDa representing the MK5-His 372-473, because the OD_{600} was much higher than the mid-log phase (OD_{600} of ~0.6 to 0.9). The mid-log phase is when bacterial cells is growing exponentially, this assures new and fresh cells for expression. As depicted in Figure 19 A, the results were true, the bands representing the domain were weak.

We wanted to do the experiment of the His-tagged 372-473 domain again, but this time with lower OD_{600}. Two experiments were conducted with induction temperatures 24°C and 37°C, respectively. Lower OD_{600} should result in stronger bands of ~15 kDa. It was as expected as shown in Figure 19 B and C. The supernatant gel in B showed strongest bands out of the three gels. Condition B was therefore proceeded for large scale expression.

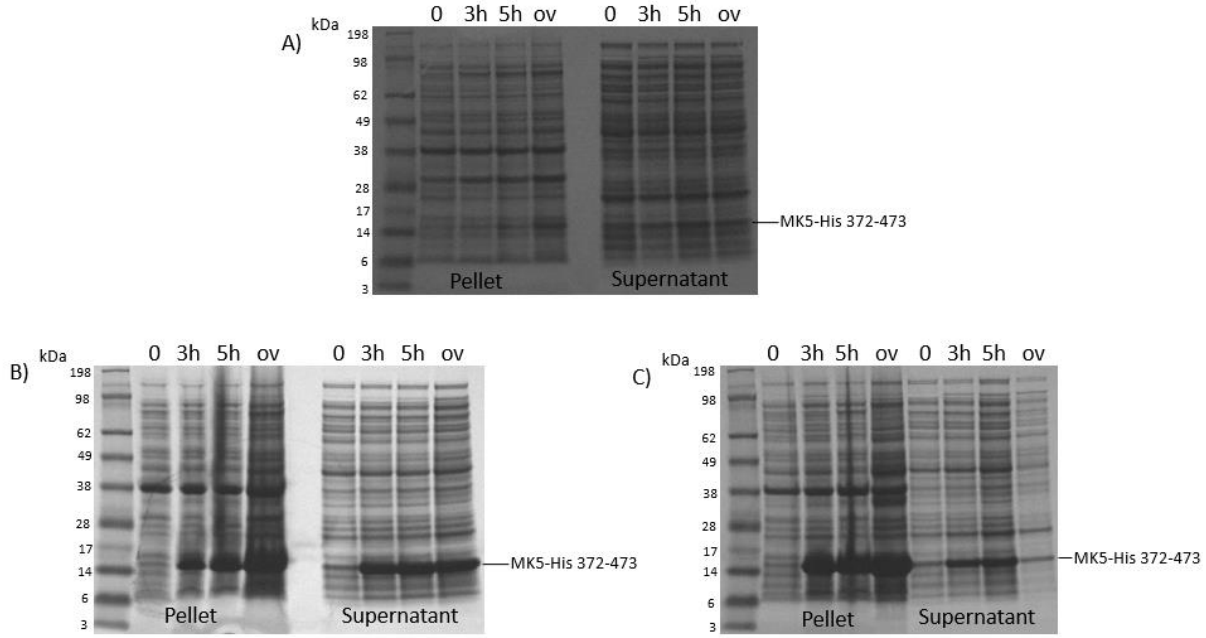

Figure 19: MK5-His 372-473 fragments. SDS-PAGE with MES running buffer of MK5-His fragments in various conditions. 0=prior to 1 mM IPTG induction. The protein was harvested after 3 and 5 hours and overnight (ov). The samples were sonicated 6 x 10 sec (Microson™ Ultrasonic Cell Disruptor) prior to separation to pellet and supernatant with centrifugation, and run on SDS-PAGE. Bands of ~15 kDa indicate the MK5-His 372-473. Stained with Coomassie Brilliant Blue. **A**: OD_{600}=1.640 prior to induction. Induction temp.=24°C. **B**: OD_{600}=0.664. Induction temp.: 24°C. **C**: OD_{600}=0.644. Induction temp.: 37°C.

MK5-GST fragments

We wanted to proceed with MK domains that are GST-tagged too, so one experiment of amino acids 372-473 and 382-473 were carried out. The induction temperature was set to 37°C. The protein gels should result in bands of 39 kDa representing the MK5 domains. As shown in Figure 20, this was true. The supernatant gel in B showed strongest bands of 39 kDa after 3 and 5 hours. Therefore, condition B was proceeded for large scale expression.

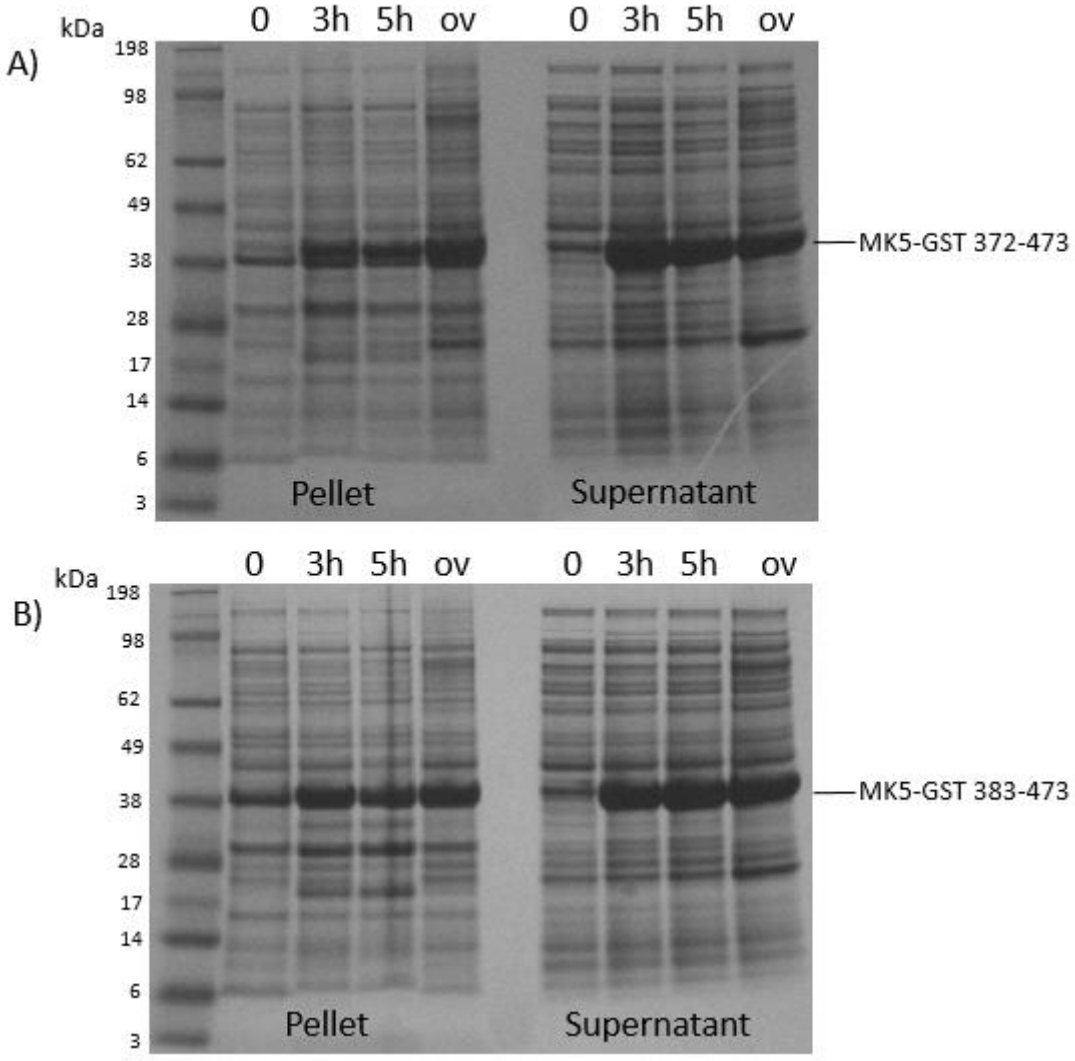

Figure 20 MK5-GST fragments. Protein gel of MK5-GST 372-473 (A) and MK5-GST 383-473 (B) of both pellet and supernatant. Samples were taken prior to 1 mM IPTG induction (0), after 3, 5 hours and overnight, and separated to pellet and supernatant with centrifugation. The samples were then analyzed with SDS-PAGE with MES running buffer. Bands of ~39 kDa indicate the MK5-GST fragments. Sonicated 6 x 10 sec (Microson™ Ultrasonic Cell Disruptor). Induction temp.: 37°C. Stained with Coomassie Brilliant Blue. **A**: OD_{600}=0.804. **B**: OD_{600}=0.960.

Protein Purification

We wanted our proteins as pure as possible. Therefore, many purification techniques were carried out.

IMAC of MK5-His

The goal for the method immobilized affinity chromatography is to bind the 6 consequently histidine that are coupled with MK5 to NiNTA agarose beads as described in the methods section. The bound protein was then washed with with Lysis buffer A and 25 mM imidazole. The lysis buffer contains appropriate salts at pH=8, lysozyme for breaking down bacterial cell walls, β-mercaptoethanol for reducing disulfide bonds between proteins and protease inhibitor for reduction of the degeneration of protein. The bound protein was then eluted with Elution buffer B. In this way, our protein becomes isolated from impurities. 9 eluates were taken, with the 1st eluate from left to the 9th eluate to the right as depicted in Figure 21. If the purification was successful, the SDS-PAGE gel after elution would result in bands of 52 kDa representing MK5-His. Moreover, the background bands should be of lesser because of the washing step. The gel shows that was true. Eluates 1 and 3-9 show little background and lane 2 show strongest band representing MK5. Since the eluates were generally pure and all lanes contained our protein, they were collected together with 6 more eluates of the protein. The last 6 eluates were not analyzed with SDS-PAGE.

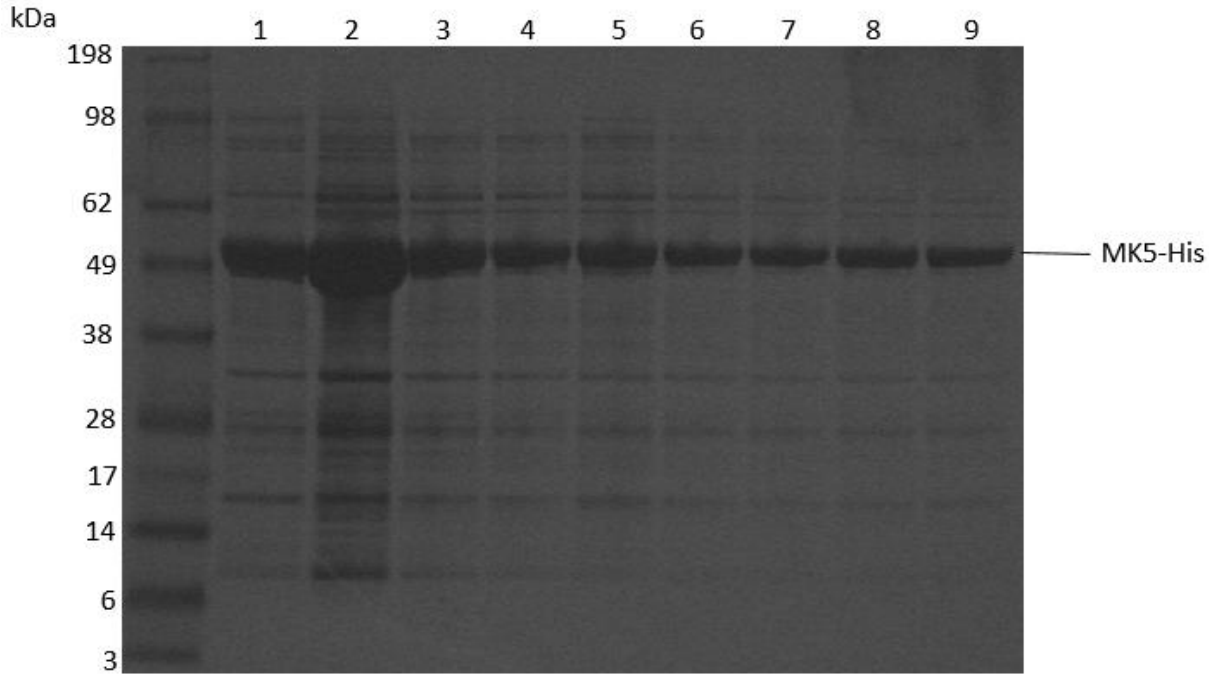

Figure 21 SDS-PAGE of 9 eluates of MK5-His after IMAC. 9 eluates separated on SDS-PAGE run with MES running buffer. Stained with Coomassie Brilliant Blue. Bands of ~52 kDa indicate the MK5-His protein. Lane 1 with the first eluate, lane 2 with the second eluate and so on of totally 9 eluates. Eluate 2 shows biggest band representing the His-tagged MK5. The protein solution was harvested after 5 hours of 1 mM IPTG induction with induction temp. of 24°.

Gel-Filtration Chromatography of MK5-His

With gel-filtration chromatography, we wanted to isolate the collected MK5-His eluates. Gel-filtration chromatography separates the protein molecules by size with the biggest size being eluted out first. Gel-filtration chromatography has many advantages. It can be tailored to your study of interest; matrix, column and buffer can be varied leading to different resolutions on the chromatogram. The stationary phase is inert, which will not react with the samples. It has some disadvantages too. When the compression of the bed is not properly set, it can decrease the flow rate, air bubble in the column and tailing arises. Tailing is when a peak is leaving a tail on the chromatogram[69]. Eluates were collected in 2 ml tubes.

We expected to have a peak for void volume, where protein molecules bigger than 600 kDa are excluded from the column, i.e. not passing through the pores in the matrix. The highest peak representing the His-tagged Mk5 and a peak at 33 kDa are also expected. During optimization step, we investigated that two strong bands at 52 and 33 kDa on the supernatant gel after 5 hours of induction (Figure 15 B). Figure 22 shows the void volume, the highest peak in eluate D9 and two overlapping minor peaks right to the highest one.

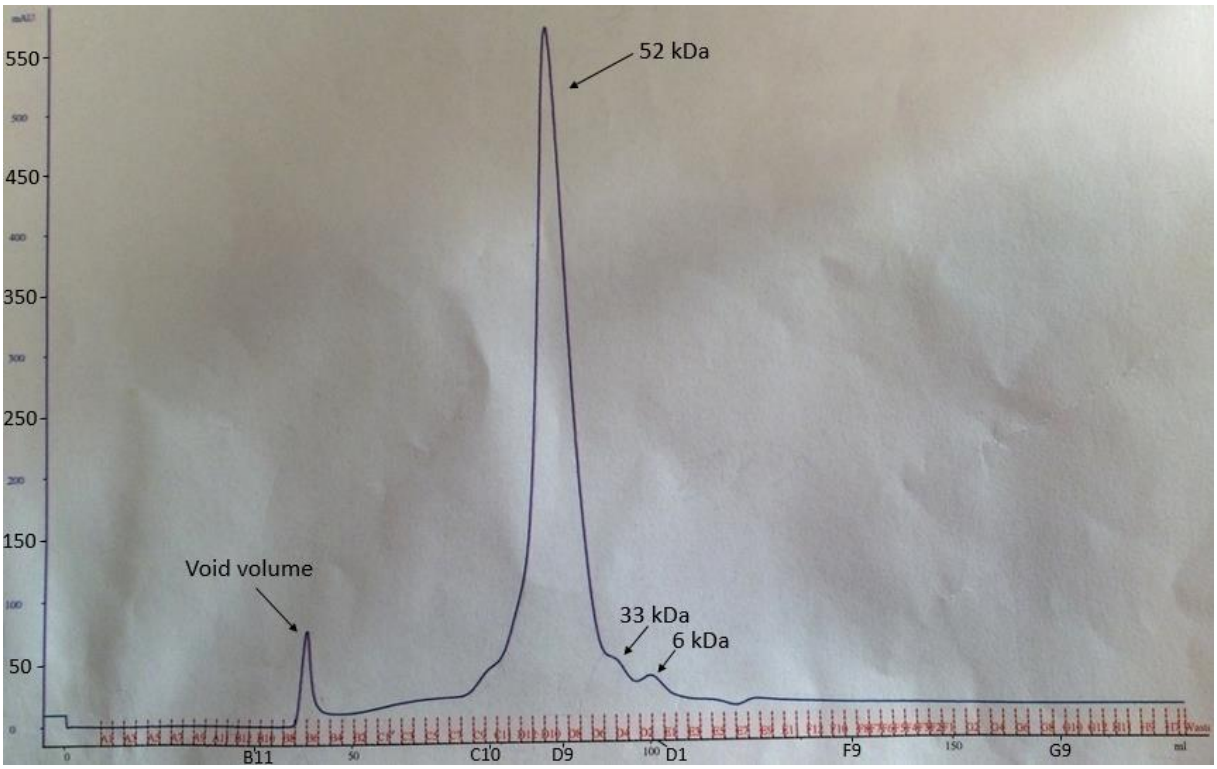

Figure 22 Gel-filtration of MK5-His. Gel-filtration chromatogram of MK5-His. X-axis: mAU (milli absorbance units) at OD_{280}. Y-axis: Elution volume (ml). Exclusion limit=600 kDa. After IMAC purification, the eluates were collected and run on gel-filtration chromatography. Analyzed on UV with OD_{280}.

As the gel-filtration chromatogram only showed the separation of different protein molecules by weight, we wanted to investigate what molecular weight the different peaks represent. In order to do that, SDS-PAGE was done for eluates C10, C12, D12, D11, D9, D8, D7, D6, D5,, D4, D2 and D1 as shown in Figure 23. The eluates D9, D6 and D1 should result in three distinct bands of different weights respectively. This was correct, D9 shows strongest band of 52 kDa representing MK5-His, D6 has two strong bands of 52 and 33 kDa, D1 show a band of 6 kDa. Therefore, these molecular weights represent the 3 overlapping peaks in the chromatogram (Figure 22). Eluates D11-7 contained our protein, so they were collected together for dialyses and concentration steps as described in the methods section.

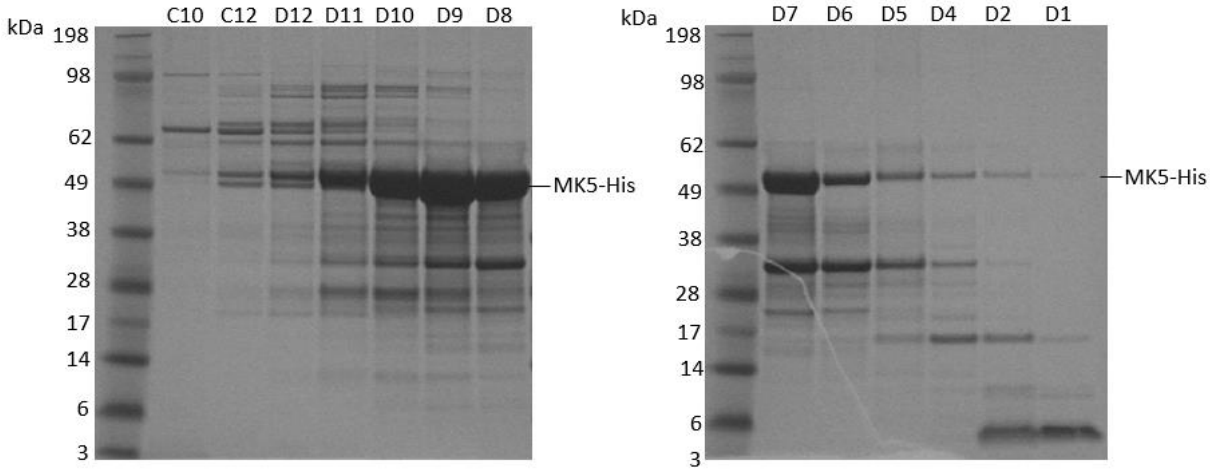

Figure 23 SDS-PAGE of gel-filtration eluates. 11 eluates from the gel-filtration chromatography were verified on SDS-PAGE stained with Coomassie Brilliant Blue. Eluate D9 shows strongest band of MK5-His.

IMAC of MK5-His 372-473

The goal for this experiment was to isolate and purify the His-tagged MK5 with the domain 372-473. The His-tagged MK5 372-473 was washed with Buffer A + 1:1000 β-mercaptoethanol + protease inhibitor + 25 mM imidazole instead of the Lysis buffer A, and eluted with Elution buffer B. The washing buffer used in this purification step did not contain lysozyme, which breaks down the cell walls of bacterial cell to yield more soluble proteins. As seen in the optimization step for MK5-His 372-472 (Figure 19 B), the supernatant gel showed strong and distinct bands representing the MK5 domain. Therefore, it was not necessary to include the lysozyme in the washing buffer. 2 x 10 ml eluates were taken and analyzed with SDS-PAGE. The gel should result in bands of 15 kDa representing our protein. This was true as depicted in Figure 24. Eluates 1 and 2 show our protein of around 15 kDa The eluates were therefore collected together for dialysis and storage as described in the methods section.

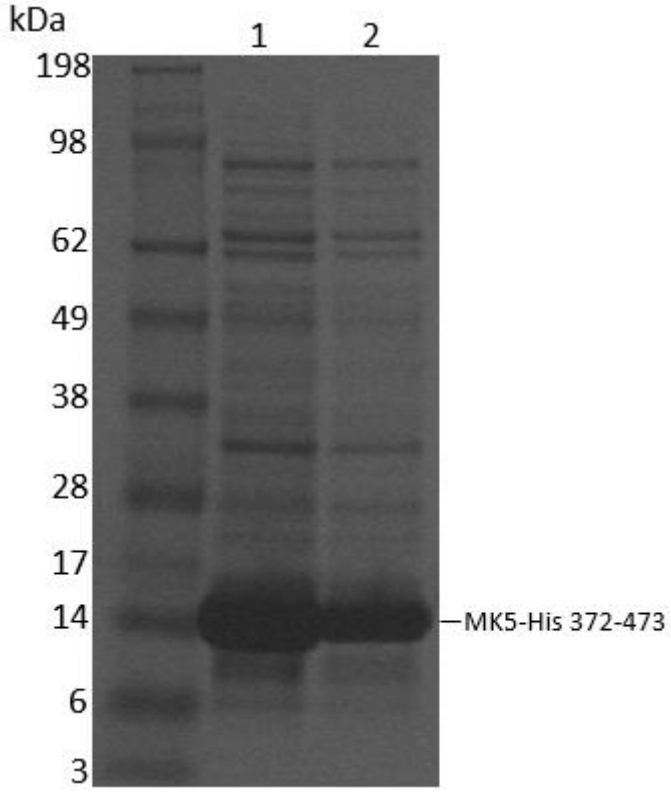

Figure 24 Eluates of MK5-His 372-473. SDS-PAGE of 2 eluates after IMAC, stained with Coomassie Brilliant Blue. Eluate 1 has stronger band of 15 kDa representing MK5-His 372-473 than in eluate 2. Induced at 24°C with 1mM IPTG and harvested after 3 hours prior to SDS-PAGE analysis.

IMAC of MK5-GST 383-473

We wanted purify and isolate the GST-tagged MK5 383-473 fragment. The GST-tag is a 26 kDa fusion protein, while the His-tag is of 1 kDa. The GST-tag is easily detected in enzyme assay and immunoassay. It has mild elution condition, minimizing the risk of degenerating the recombinant protein and because of its size, it will form dimers. The His-tag is only easily detected on immunoassays. Its elution condition is not mild as for GSTs, imidazole may cause precipitation. His-tag is small and will less likely interfere with the structure and function of the recombinant protein[66].

In order to purify the GST-tagged MK5 383.473, the protein was first bound two glutathione agarose beads and washed with PBS solution. 3x5 ml eluates were then taken by adding 50 mM Tris, 10 mM reduced glutathione and 1:1000 β-mercaptoethanol, pH=8.0. The proteins were then analyzed with SDS-PAGE. The protein gel should result in bands of 39 kDa representing MK5-GST 383-473. Figure 25 shows 3 eluates having the bands of 39 kDa. Thus, all the eluates consisted of our protein. They were then collected together and aliquoted.

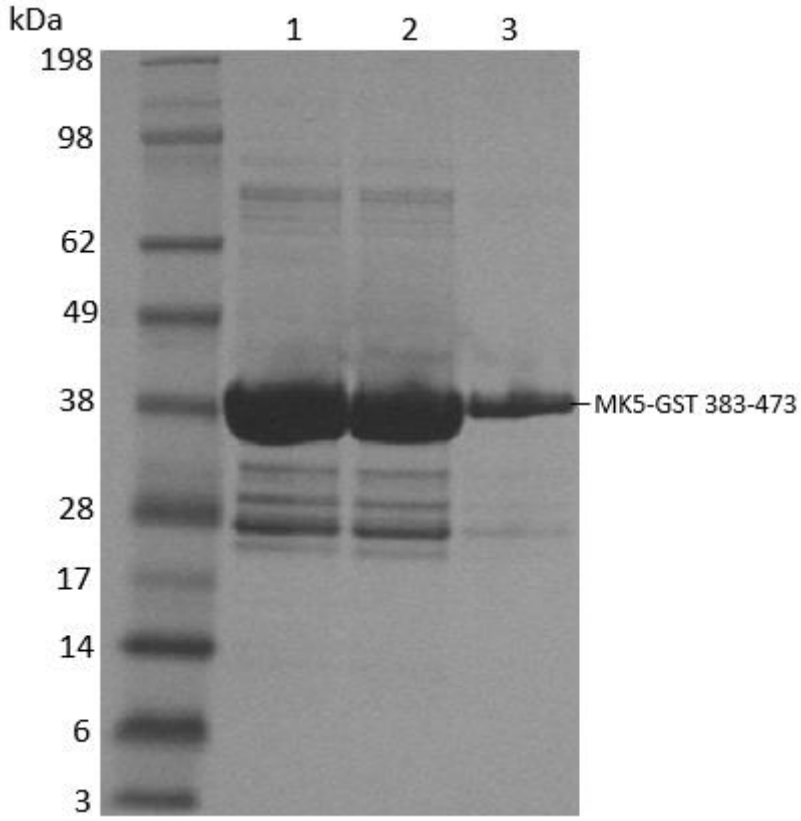

Figure 25 Eluates of MK5-GST 383-473 after IMAC. Three Eluates of MK5-GST 383-473 with bands of 39 kDa were revealed on SDS-PAGE stained with Coomassie Brilliant Blue. Induced with 1 mM under the temp. of 37°C and harvested after 5 hours.

Protein-protein Interactions Studies

In order for testing interactions between MK5 and its fragments with ERK4, the ERK4 protein have to be generated. It is generated by introducing the gene, encoding the ERK4 in a eukaryotic expression vector, into mammalian cells. The eukaryotic expression vectors should at least contain; 1) eukaryotic transcription regulatory elements such enhancer and promoter, 2) prokaryotic replication origin, for replicating in E. coli and 3) prokaryotic selective marker[84].

Overexpression of ERK4 Protein

The goal was to express proteins in eukaryotic cells. By mammalian expression the recombinant proteins expressed are usually properly modified where their structure is generated correctly and their activity is fully mained[85,86].

We wanted to produce cell extracts with overexpression of ERK4 proteins, both wild-type and several mutants. In order to do this, eukaryotic expression plasmids encoding myc-tagged ERK4 (wt), myc-tagged ERK4 where the serine 186 in the activation-loop is changed to the unphosphorylable alanine (ERK4 S186A) or FLAG-tagged ERK4 were transfected into A459 mammalian cells and lysates were harvested for Western-Blot assay. The membrane was

probed with anti-ERK4 primary antibodies and anti-sheep thereafter. Anti-sheep secondary antibodies were used for visualization. We expected the ERK4 proteins to be around 70 kDa. As shown in Figure 26, the results from the ERK4 antibody revealed us that we succeeded in transfection of myc-tagged ERK4 and S186A. Bands of ERK4-FLAG were weaker than the two other ERK4 protein variants. Myc-tagged ERK4 and ERK4-S186A were used as cell extracts in the GST-pulldown assay.

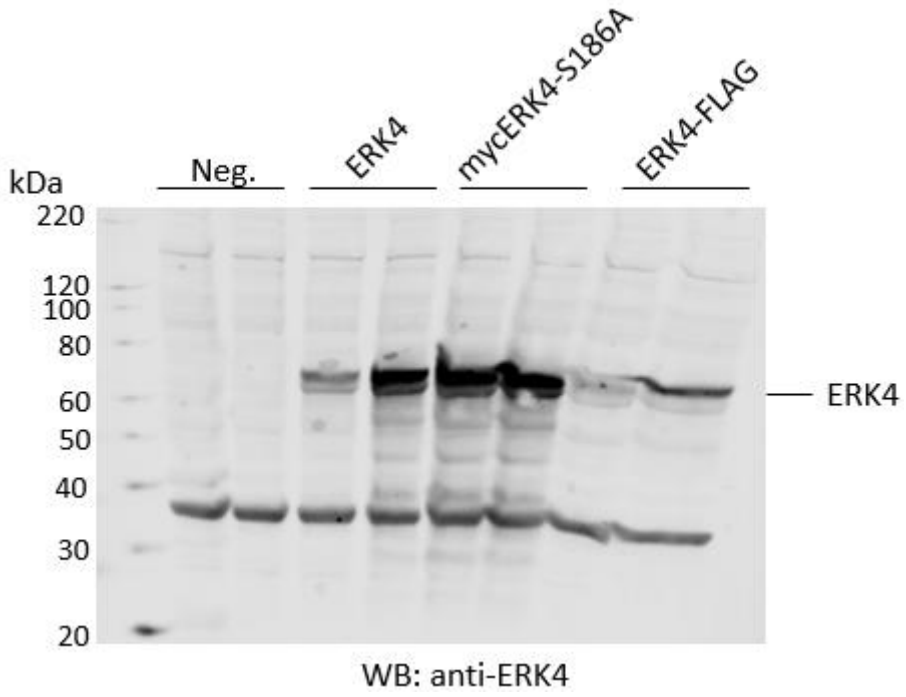

Figure 26 Western-blot of ERK4. Membrane of 2 samples of negative control (Neg.) myc-tagged ERK4 (wt), myc-tagged ERK4-S186A and FLAG-tagged ERK4. The analysis revealed bands of 70 kDa representing ERK4 (wt), myc-tagged ERK4-S186a and FLAG-tagged ERK4. Bands of ~38 is also revealed. Probed with primary antibody: anti-ERK4 and secondary antibody: anti-mouse (IR 700).

GST-pulldown of ERK4

MK5 acts as a substrate for ERK4. In this experiment we wanted to test if myc-tagged ERK4 protein generated from A549 cells could interact with GST-MK5 383-473. In order to achieve this, extracts containing myc-tagged ERK4 (wt) and ERK4 S186A were incubated with the GST-tagged MK5 383-473, and with the help of glutathione-agarose we isolated the GST-MK5 together with ERK4. After GST-pulldown assay as described in the methods section, the samples underwent western-blot and probed first with anti-ERK4 and subsequently with anti-GST to verify if the fusion protein GST was present in the pulldowns. Successful pulldown of ERK4 proteins should result in bands of 70 kDa representing the ERK4 proteins in lanes representing the MK5-GST 383-473 and the ERK4 proteins. In the control lanes (GST + ERK4 proteins), no bands of 70 kDa should be present. As depicted in Figure 27 A, a weak band of

around 62 kDa may indicate the pulled down of ERK4 S186A by the means of MK5-GST 383-473. When re-probing with anti-GST, we should get bands of 26 kDa representing the GST protein for the GST + ERK4 control, and bands of 39 kDa representing the GST-tagged MK5 383-473. Figure 27 B shows 3 bands of 26, 38 and 80 k Da for the GST protein incubated with the ERK4 proteins (GST + ERK4 and GST + ERK4 S186A). For MK5-GST 383-473 + ERK4 proteins, 3 bands of 16, 27 and 51 kDa were reaveled. The ones at 16 kDa being weaker than other two bands. As expected in the GST + ERK4 controls, GST bands of 26 were present. However, the other two bands may implicate impurities. For MK5-GST + ERK4 proteins, the band of 39 kDa representing the GST-tagged Mk5 383-473 did not appear as expected, but instead three other bands, which could represent impurities, were present.

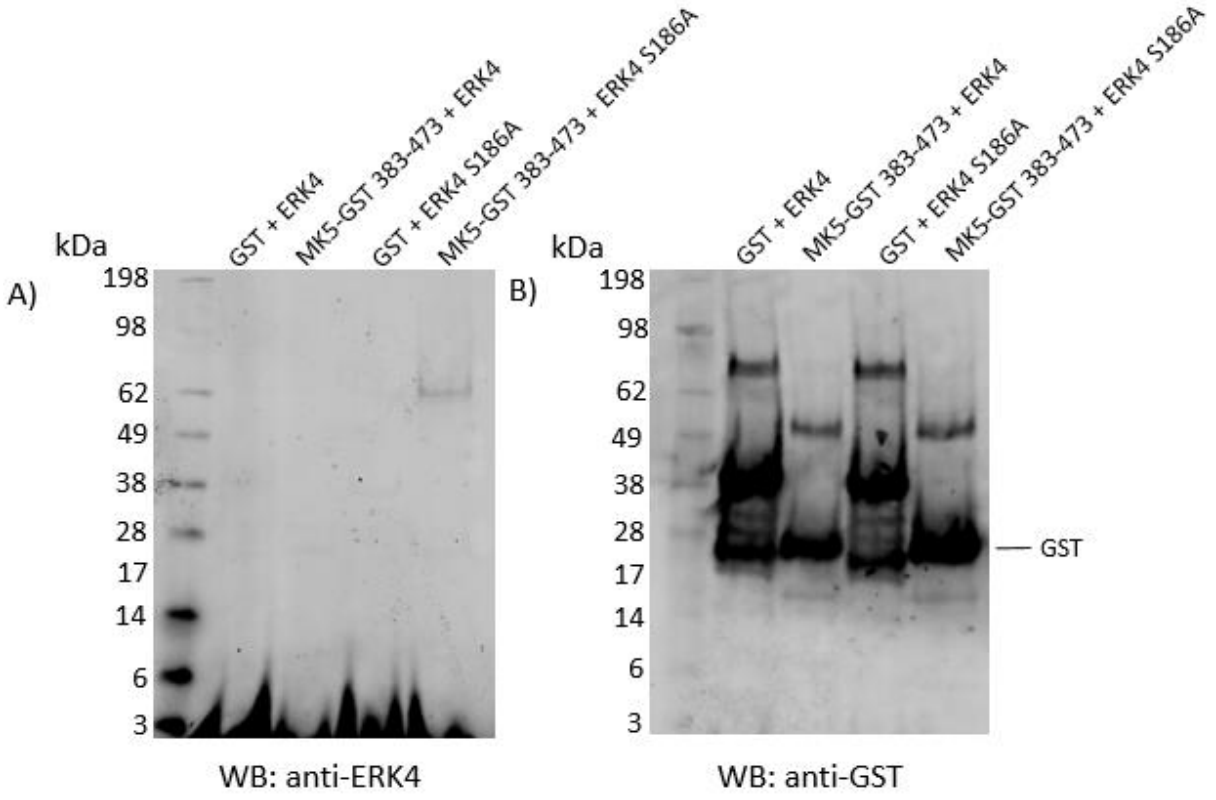

Figure 27 GST-pulldown of ERK4. Western-Blot after GST-pulldown of the myc-tagged ERK4 (wt) and myc-tagged ERK4 S186A by the means of MK5-GST 383-473. GST protein + ERK 4 proteins serve as a control. **A:** Probed first with the primary antibody anti-ERK4. Visualized by probing with the secondary antibody anti-sheep under IR700. A band of ~70 kDa may indicate ERK4. **B:** Reprobed with anti-GST and anti-rabbit IR800.

Co-Transfection

In this study we wanted to test if myc-tagged ERK4 co-expressed with GST-MK5 or GST-MK5 372-473 in the lysate of A549 cells could interact with each other. In order to achieve this, expression vectors encoding myc-tagged ERK4 (wt) and ERK4 S186A were co-transfected with expression vectors encoding GST-tagged fusion protein with the full length MK5 or the

amino acid 383-473 of MK5, and with the help of glutathione-agarose isolate the GST-MK5 proteins together with ERK4 proteins. After transfection and the cell lysates were harvested, they were analyzed on Western-blot and probed first with anti-GST to verify the presents of GST fusion proteins bound to MK5 and MK5 372-473. This would result in bands of 80 and 40 kDa, representing MK5-GST and MK5-GST 372-483 respectively if the transfection was successful. Shown in the left picture in Figure 28, are the bands for MK5-GST and MK5-GST 372-483, which means successful transfection of MK5 proteins.

To test if the ERK4 proteins were successfully transfected, the membrane was reprobed with anti-ERK4 and it should result in bands of 70 kDa representing the ERK4 protein where the ERK4 proteins are presented. As depicted in the right picture in Figure 28, 4 last lanes representing the GST-MK5 and ERK4 proteins showed positive results.

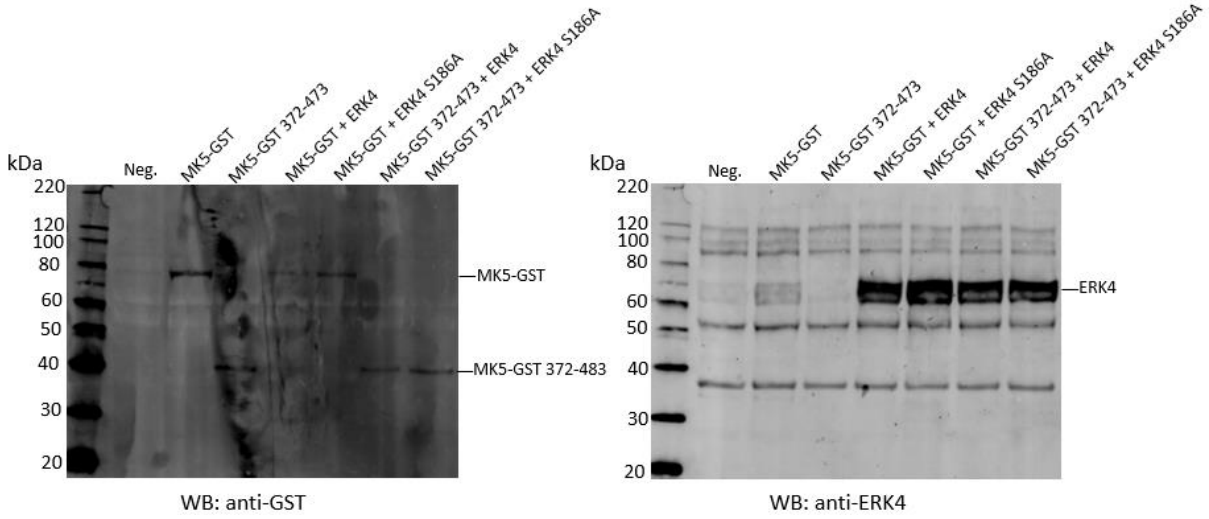

Figure 28 Pre-GST pulldown assay. Western-Blot gel after co-transfection of MK5 + ERK4, MK5 + ERK 4 S186A, MK5 372-473 + ERK4 and MK5 372-473 + ERK4 S186A. Negative control (Neg.) lane has none of the MK5 or ERK proteins transfected. Probed with anti-GST (left) and anti-rabbit IR800. Reprobed with anti-ERK4 and anti-sheep IR700.

As it was shown successful transfection of MK5 and ERK4 proteins, the samples were bound to glutathione agarose, washed and eluted as described in the GST-pulldown assay method section. The eluates were analyzed with Western-blot probing with anti-ERK4 first. This should result in bands of 70 kDa representing ERK4 except for the lane containing just MK5-GST. The left picture in Figure 29 shows that it was correct, the 4 last lanes containing the ERK4 proteins had bands of 70 kDA. In order to check if the GST-tagged MK5 proteins were successfully pulled down with glutathione and to verify that ERK4 proteins were bound to the GST-MK5s, the membrane was reprobed with anti-GST. Bands of 80 and 40 kDa representing

the MK5-GST and MK5-GST 372-483 respectively, were present in the right picture in Figure 29. This shows that the ERK 4 proteins bind to MK5 proteins during GST-pulldown assay.

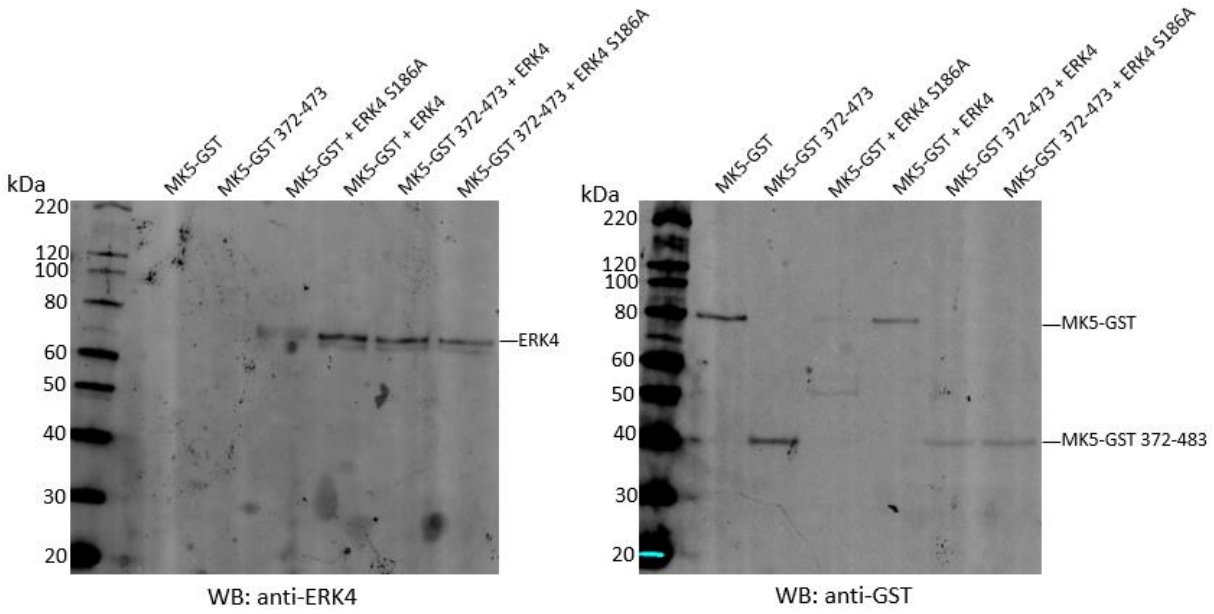

Figure 29 Post-GST pulldown. Membrane probed with anti-ERK4 (anti-sheep IR700) and reprobed with anti-GST antibodies (anti-rabbit IR800). MK5-GST and MK5-GST 372-473 co-transfected with ERK4 (wt) and its mutation, ERK4 S186A. MK5-GST serves as a control. MK5-GST + ERK4 in the left picture has the weakest band of the 4 bands. In the right picture, the same lane showed in addition to a weak band of MK5-GST, a weak band of 50 kDa.

Protein Kinase Activity Assays

MK5

We wanted to test if 3 different MK5 antibodies bind to the phosphorylated His-tagged MK5 of 3 different concentrations (0.1, 1 and 10 ng/µl). The MK5 antibodies only bind to the phosphorylated amino acid threonine 182 in the activation loop of MK5 proteins. The antibodies to be tested were anti-MK5 P-T182 (3. Bleed 0.21 mg/ml), anti-MK5 P-T182 (1. Bleed 0.12 mg/ml) and anti-MK5 P-T182 (3. Bleed 0.13 mg/ml). In order to do this, the *in vitro* kinase assay was carried out as described in the methods section. The Western-blot membrane should result in bands of 52 kDa representing the His-tagged MK5 protein if antibodies were able to bind. The 10 ng/µl lane should yield the strongest band because of higher MK5-His concentration. As shown in Figure 30, both anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) and anti-MK5 P-T182 (3. Bleed 0.13 mg/ml) were able to bind to the phosphorylated amino acid threonine 182 in the activation loop of MK5. Moreover, the testing with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) yielded the strongest band in lane 10 ng/µl.

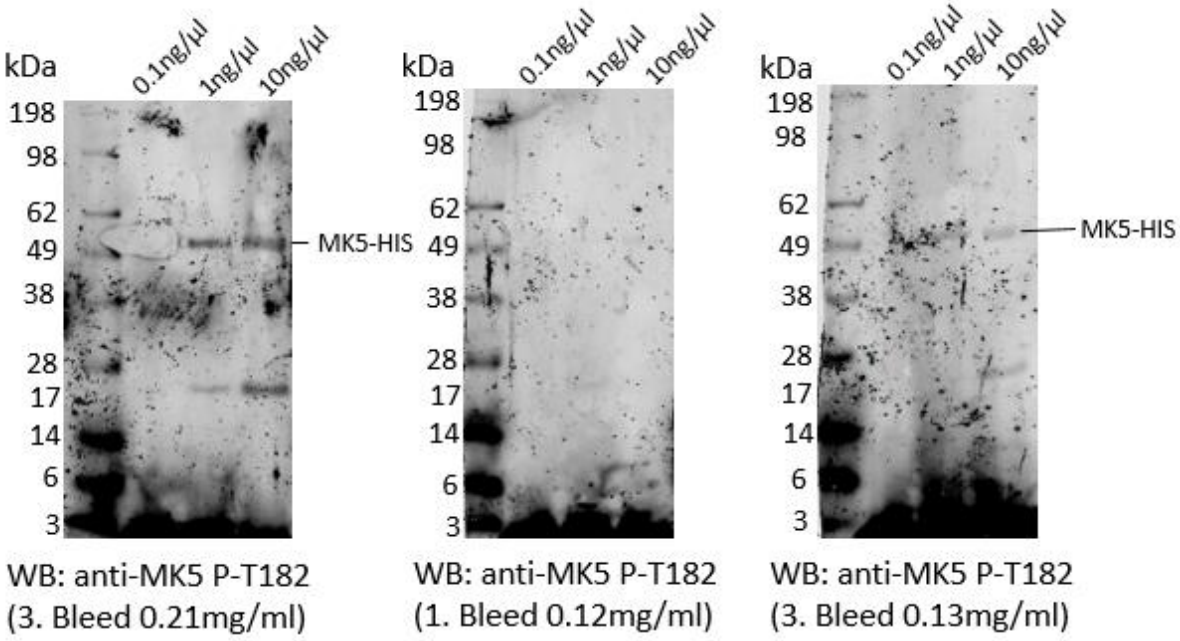

Figure 30 Different primary antibodies for phosphorylated MK5-His. Probed with 1:1000 dilution of anti-MK5 P-T182 (3. Bleed 0.21 mg/ml), anti-MK5 P-T182 (1. Bleed 0.12 mg/ml) and anti-MK5 P-T182 (3. Bleed 0.13 mg/ml) (anti-sheep IR700). 3 lanes of dilutions of MK5-His, 0.1, 1 and 10 ng/µl. Strongest bands of MK5-His appeared with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml). In addition to this one band, bands of 20 kDa were revealed in membranes probed with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) and anti-MK5 P-T182 (3. Bleed 0.13 mg/ml).

As the anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) gave strongest bands in 1:1000 solution, we wanted to investigate again this time in 1:200, 1:500 and 1:1000 dilution of anti-MK5 P-T182 (3. Bleed 0.21 mg/ml). In order to do this, three new membranes with the same concentration of MK5-His (0.1, 1 and 10 ng/µl) were probed with different anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) concentrations. It was to get the strongest band of 52 kDa representing the MK-His in lane 10 ng/µl when probed with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) in 1:200 dilutions. This was true as shown in Figure 31. All three membrane showed bands of 20 kDa as well. The bands of MK5-His in the 1:200 and 1:500 dilutions were similar, so we proceeded with the 1:500 dilutions of anti-MK5 P-T182 (3. Bleed 0.21 mg/ml).

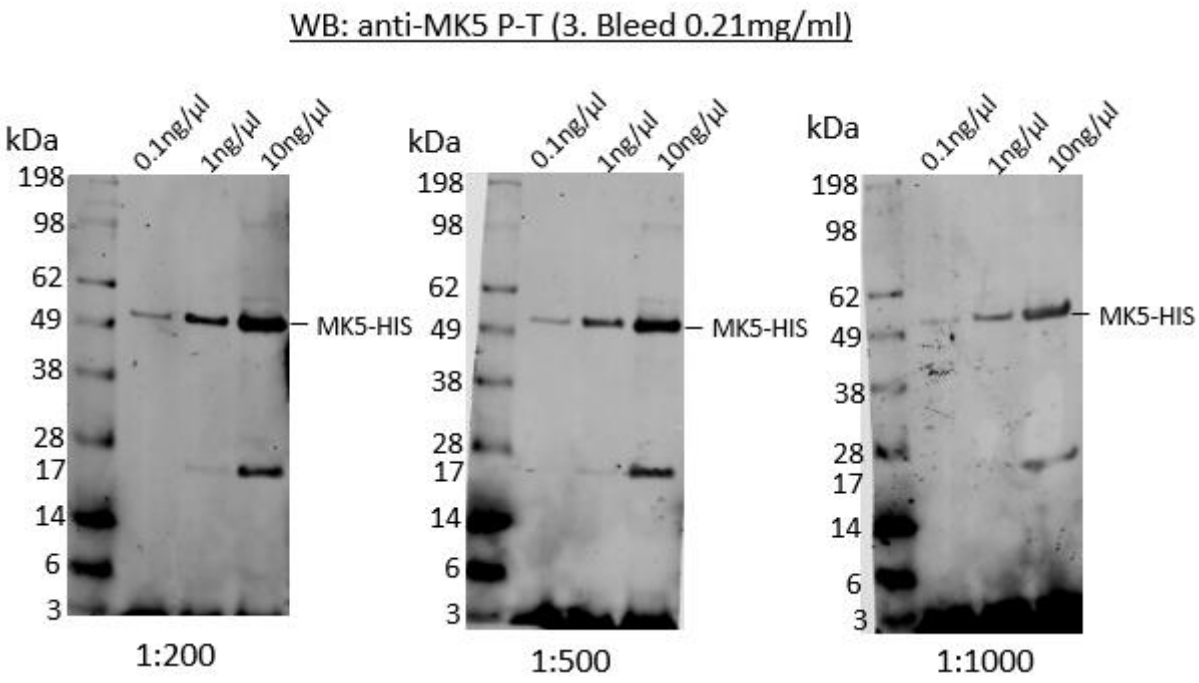

Figure 31 MK5-His with different anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) concentrations. Membranes of phosphorylated MK5 in 3 different concentrations (0.1, 1 and 10 ng/µl) were probed with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) (in 1:200, 1:500 and 1:1000 dilutions). Visualize with anti-sheep IR700. Bands of 52 and 20 kDa were detected.

We wanted to verify if the phosphorylated membranes were properly phosphorylated. In order to do this, a membrane of unphosphorylated MK5-His was probed with anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) in 1:500 dilutions. This should not result in bands of 52 kDA representing the unphosphorylated His-tagged MK5. Figure 32 shows that it was true. The anti-MK5 P-T182 (3. Bleed 0.21 mg/ml) antibody only visualize phosphorylated MK5-His.

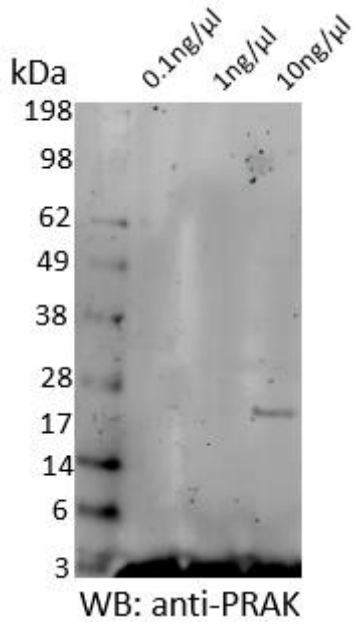

Figure 32 Nonphosphorylated MK5-His with anti-PRAK antibody. Unphosphorylated membrane of MK5-His in three concentrations (0.1, 1 and 10 ng/µl) probed with 1:500 anti-PRAK (anti-sheep IR700). A band of 20 kDa was shown in the last lane.

DISCUSSION

The electrophoresis of the 10 clones resulted in a lane with a major band at 3000 bp and two minor bands at 6000 and above 7000 bp respectively. This raised our concern what this would be. Looking at the MK5 DNA insert and the pET-15b expression vector, they contain 1419 bp and 5708 bp respectively. Cut with NdeI and XhoI of MK5 DNA would give many 1419 bp DNA fragments with compatible sticky ends. During ligation, two of them could have bound to each other through compatible ends where NdeI or XhoI cuts, yielding the 2838 bp long fragment representing 2 MK5 DNA inserts. Consistently, this could successful cut of the MK5 DNA inserts and not the cut of vector. Moreover, with this basic idea more than 2 of the MK5 DNA insert could bind each other, yielding the 4257 bp, 5676 bp and 7095 bp for 3, 4 and 5 DNA inserts bound together respectively. Thus, the 6000 bp and 7000 bp band could indicate the 4 and 5 ligated MK5 inserts. However, one can question why the vector was not cut and ligated with the MK5 insert instead. This possibility could be ruled out because the vector was uncut. Furthermore, why 3 insert strands did not ligate each other remain questionable.

From optimization of protein expressions show that extended sonication, lower induction temperature and OD_{600} in mid-log phase yield good amount of soluble proteins. However, the pellet gels representing our proteins in inclusion bodies indicate much more of proteins that could be soluble. According to Francis et al.'s Strategies to Optimize Protein Expression in E.coli[61], reducing the temperature would give a slower transcription and translation rate, thus reducing the aggregation of the proteins in inclusion bodies. We carried out the expression at 24°C at the lowest, and therefore, lowering the induction temperature may give more soluble proteins. However, longer induction time is required then. Another parameter is the IPTG that we used 1 mM of in all our expression experiments. Lowering this inducer will give slower transcription rate resulting in more soluble proteins. Hence, it is possible to get more soluble proteins by changing these parameters.

It is known that MK5 interacts with ERK3/4 through the phosphorylation-dependent FRIEDE-motif located C-terminal to the common docking domain sequence in ERK3/4[35]. The sequence that the MK5 protein interacts ERK3/4 with is in the C-terminal and it is of 100 amino acids[36]. The results from the GST-pulldown after co-expression of amino acid 372-473 of MK5 and ERK4 showed binding of the segment to ERK. According to Aberg et al., they describe a 383-473 residues of MK5 in the C-terminal binds to the FRIEDE site when co-transfected into HeLa cells. Consistently, the result picture shows that the 372-473 C-terminal motif of MK5 binds to ERK3/4. The amino acid 383-473 of MK5, which is 12 amino acids shorter in the C-terminal

of FRIEDE motif, indicate that the binding site required to interact with ERK3/4 is of shorter sequence.

Another finding of ours, is that the 383-473 residues of MK5 protein bind to ERK4 where the phosphor-acceptor serine 186 is replaced with alanine, *in vitro* with GST-pulldown. This was in consistent with the fact that MK5 is partially phosphorylated and thus activated by this mutant[87]. However, the same binding to ERK4 wildtype did not occur despite that MK5 is fully bound and activated by ERK3/4 when the phospho-acceptor sites in the activation loop are phosphorylated *in vivo* and *in vitro*.

CONCLUSION

- Mid-log OD_{600}, lower induction temperature and extended sonication time are important for expression of soluble proteins.
- Amino acid domain 372-473 binds to ERK4.

Future Perspectives

- The found protein expression conditions for TEV, MK5 and its fragments can be used in future studies to save time spending on optimization of protein expression in E. coli.
- More work is required to carry out the crystallization of MK5.

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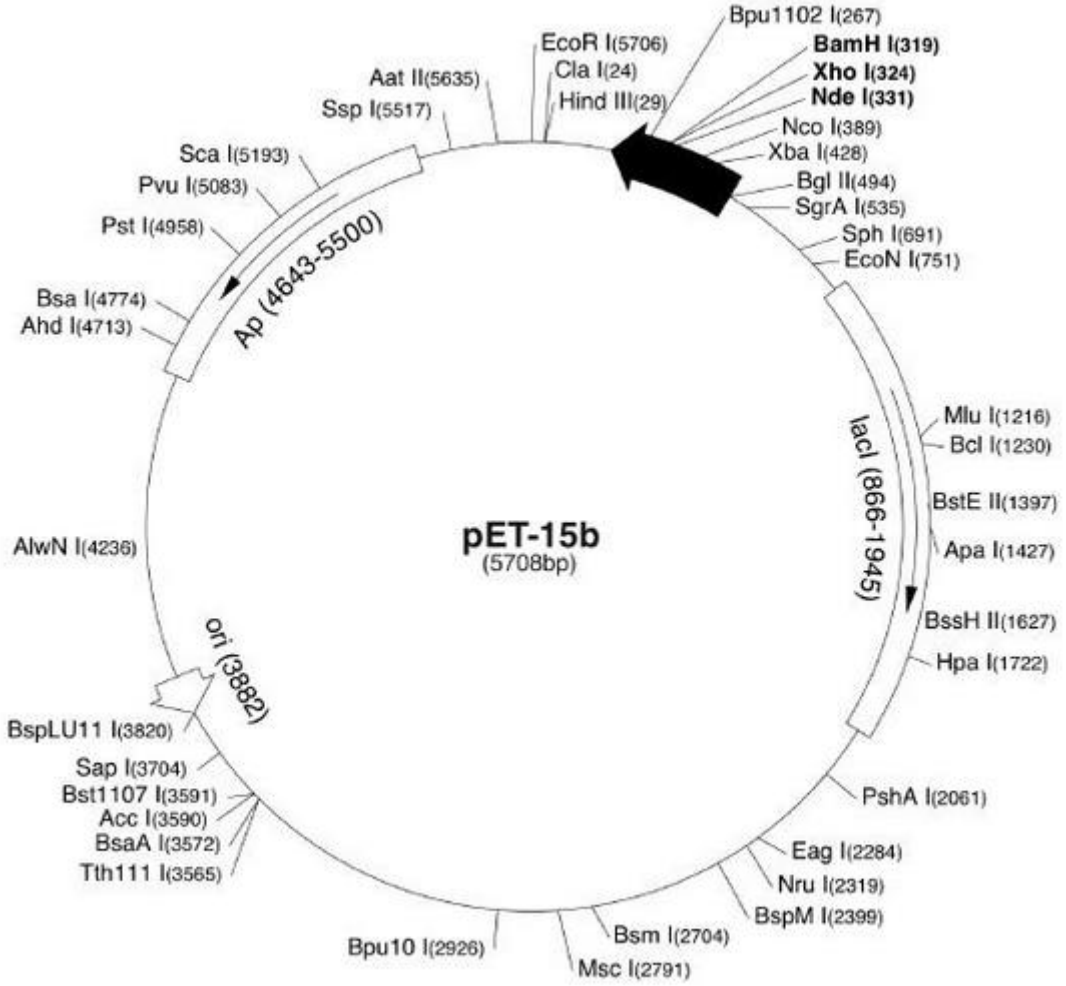
APPENDICES

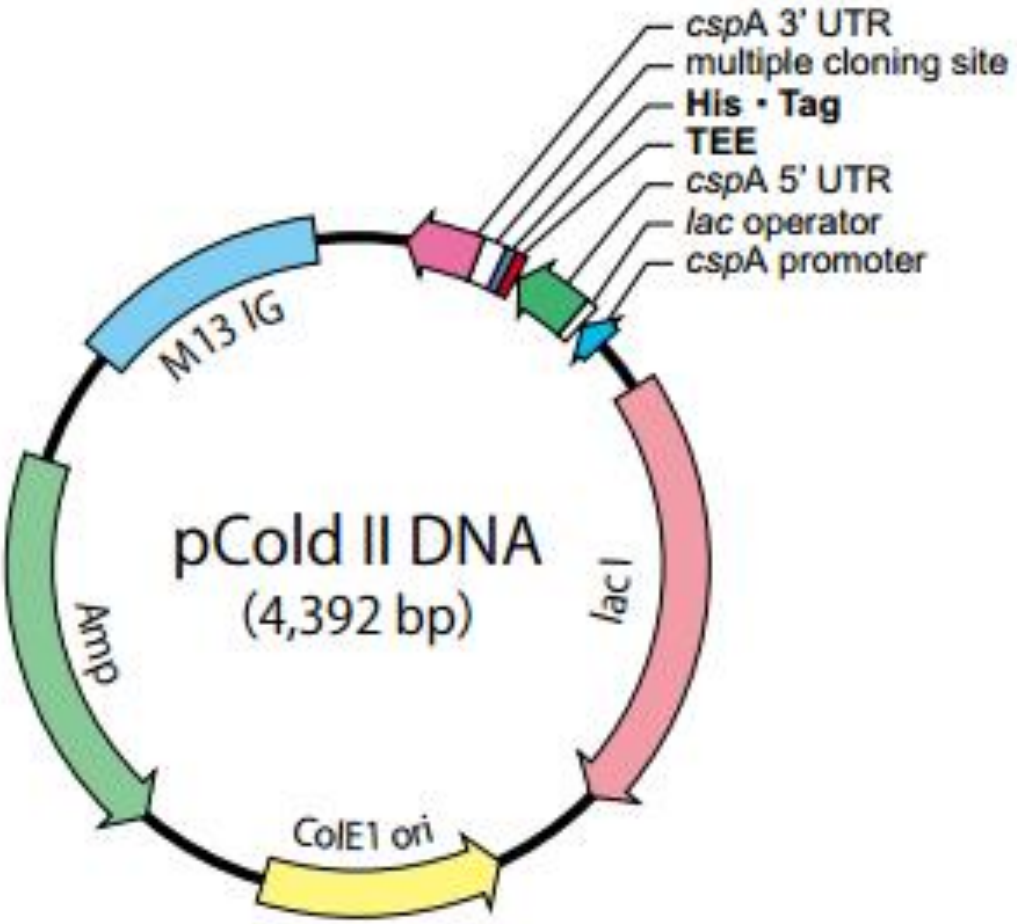
Appendix A: Map of pET-15b

Novagen



Appendix B: Map of pCold II

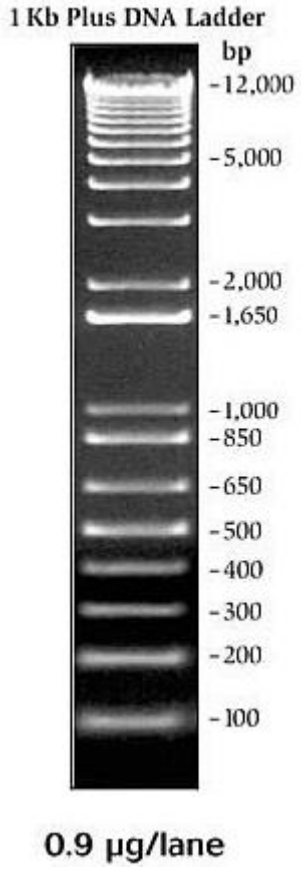
Takara



Appendix C: 1 Kb Plus DNA Ladder

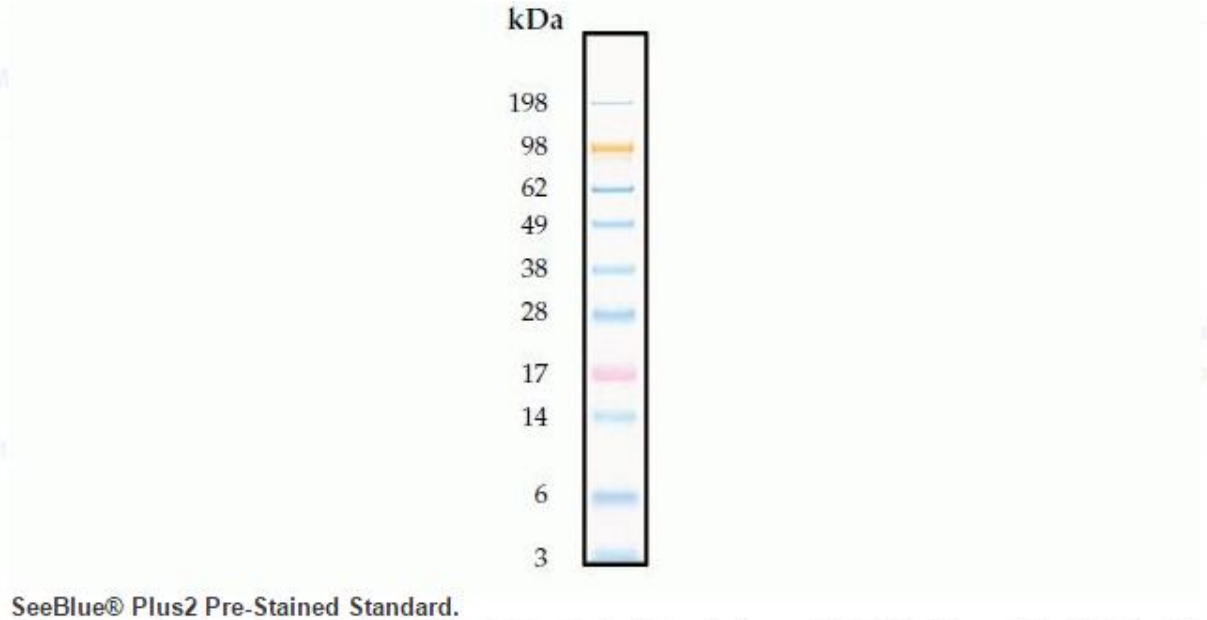
Thermo Fischer Scientific

0.9% agarose gel stained with ethidium bromide



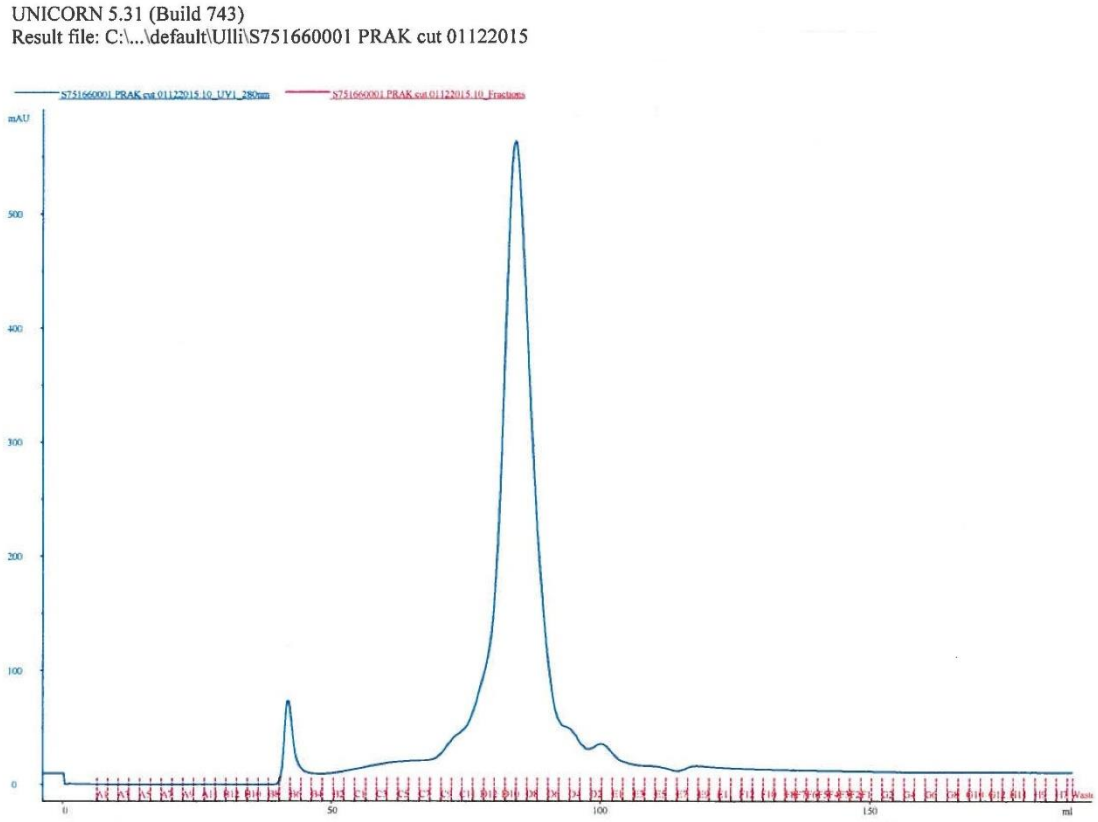
Appendix D: SeeBlue® Plus2 Pre-Stained Standard with MES

Thermo Fisher Scientific



SeeBlue® Plus2 Pre-Stained Standard.
Apparent molecular weights of the SeeBlue® Pre-Stained Standard on a NuPAGE® Novex® 4-12% Bis-Tris Gel with MES.

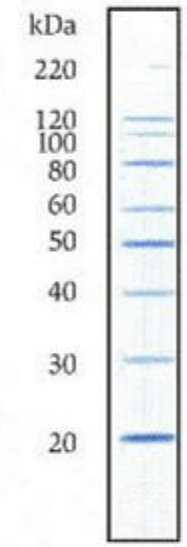
Appendix E: Gel-Filtration Chromatogram of MK5-His



Appendix F: MagicMark™ XP Western Protein Standard

Thermo Fisher Scientific

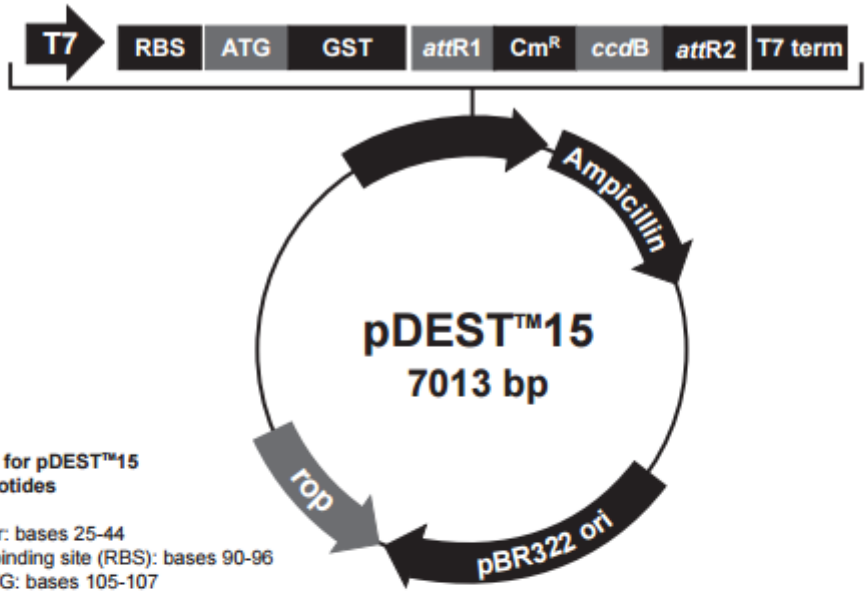
Figure 2 - MagicMark™ XP Standard visualized with Coomassie® stain on an SDS-PAGE gel



NuPAGE® 4-12% Bis-Tris Gel with MES SDS Running Buffer, stained with SimplyBlue™ SafeStain.

Clear bands visualized with Coomassie® stain on SDS-PAGE gel.

Appendix G: Map of pDEST15



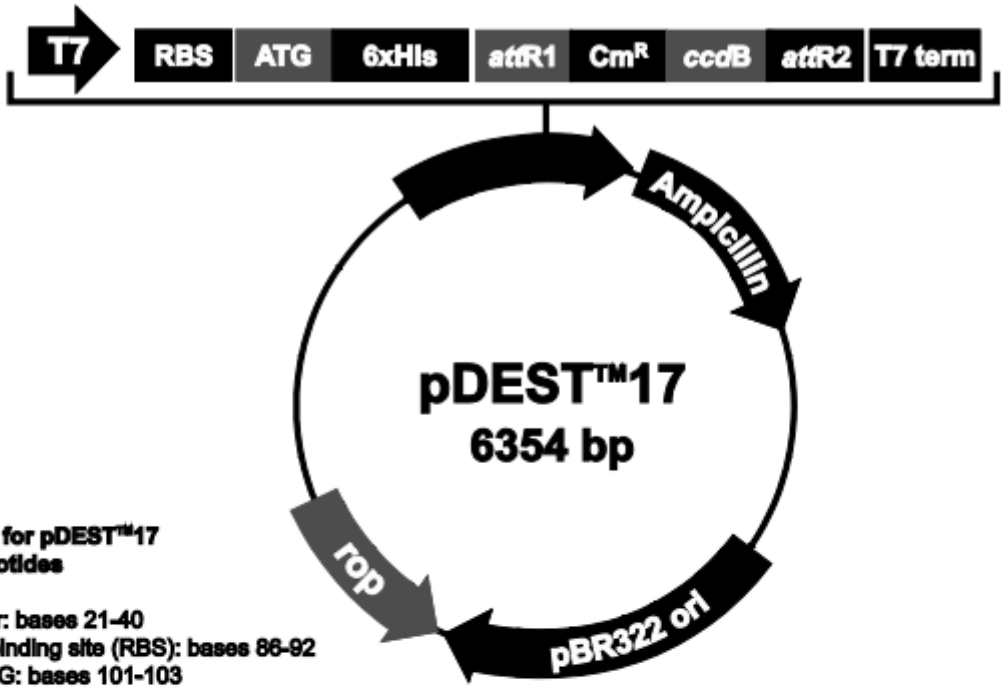
Comments for pDEST™15
7013 nucleotides

T7 promoter: bases 25-44
Ribosome binding site (RBS): bases 90-96
Initiation ATG: bases 105-107
GST tag: bases 108-776
*att*R1: bases 792-916
Chloramphenicol resistance gene (Cm^R): bases 1025-1684
*ccd*B gene: bases 2026-2331
*att*R2: bases 2372-2496
T7 transcription termination region: bases 2518-2646
bla promoter: bases 3134-3232
Ampicillin (*bla*) resistance gene: bases 3233-4093
pBR322 origin: bases 4238-4911
ROP ORF: bases 5282-5473 (C)

C=complementary strand

Invitrogen™
life technologies

Appendix H: Map of pDEST17



Comments for pDEST™17
6354 nucleotides

T7 promoter: bases 21-40
Ribosome binding site (RBS): bases 86-92
Initiation ATG: bases 101-103
6xHis tag: bases 113-130
***att*R1: bases 140-264**
Chloramphenicol resistance gene (Cm^R): bases 373-1032
***ccd*B gene: bases 1374-1679**
***att*R2: bases 1720-1844**
T7 transcription termination region: bases 1855-1983
***bla* promoter: bases 2471-2569**
Ampicillin (*bla*) resistance gene: bases 2570-3430
pBR322 origin: bases 3575-4248
***ROP* ORF: bases 4619-4810 (C)**

C=complementary strand

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by Thermo Fisher Scientific