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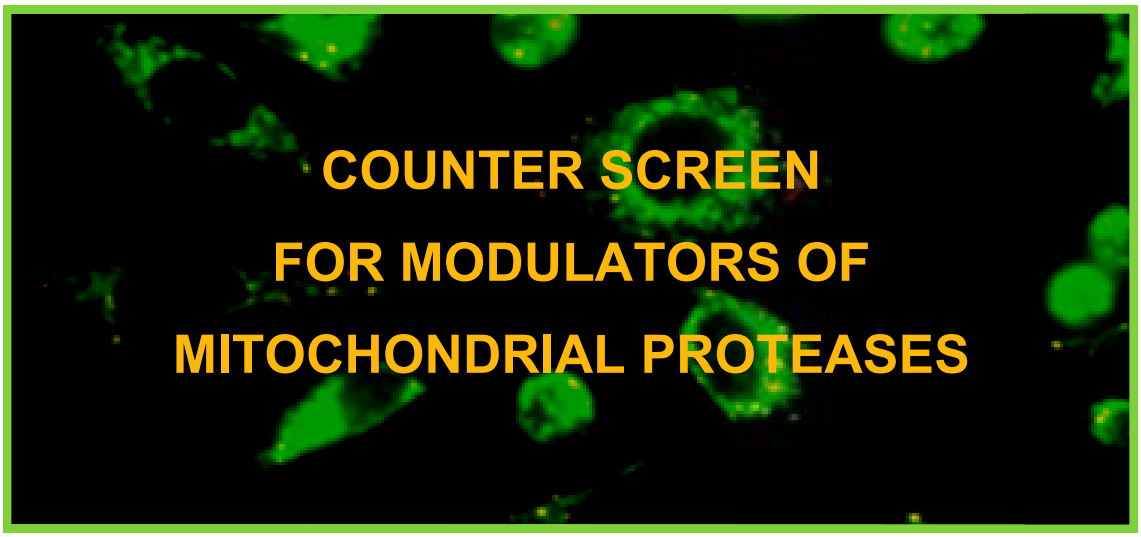

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Innsbruck, 30. August. 2011

Julia E. Mayer

ABSTRACT

The Koehler laboratory at University of California, Los Angeles, has tested 88.000 small molecule modulators for both activation and inhibition of the human Presequence Protease (hPreP), utilizing a high throughput screen (HTS) platform with several *in vitro* assays. In a primary screen it was demonstrated for the first time that the mitochondrial metalloendoprotease hPreP is enhanced by 260 and attenuated by 464 compounds. In the present study a secondary screen was conducted to not only eliminate false-positive hits from the primary screen but also to verify that the small molecules specifically activate and/or inhibit hPreP with Insulin Degrading Enzyme (IDE), which belongs to the same protease family as hPreP and has a similar active site. Therefore, IDE was cloned into low copy expression vector pET28a and recombinant IDE was purified. In order to measure the activity of the purified protein a series of activity assays were performed. The results of the counter screen indicate that IDE activity is not affected by the activators or inhibitors found within the primary screen and these primary modulators are indeed specific for hPreP. Thus, the counterscreening approach confirms that specific modulators can be identified for hPreP. Future studies will develop these modulators for disease studies in human mitochondria, particularly focusing on Alzheimer's disease.

The importance of this study is based on the fact that degradation of the mitochondrial amyloid-beta (Aβ) protein by hPreP may be related to the pathology of Alzheimer 's Disease (AD), which is a neurodegenerative disease, marked by behavioral changes as well as gradual loss of memory and bodily function. Presently, important questions about the development of AD have not been resolved, and it is still not clear whether AD is a cause or a result of mitochondrial dysfunction. However, the intimate correlation between AD and mitochondria (which are important for a range of cellular processes including the production of energy, apoptosis and signaling) has been proven through the aggregation of amyloid-precursor protein (APP) in the mitochondrial translocons and the accumulation of Aβ in brains of AD patients. Aβ is produced by APP, impairs both neuronal and mitochondrial function, and causes oxidative damage in the mitochondrion. Moreover, the idea that the zinc-metalloprotease IDE, which is primarily located in the cytosol, endosomes, mitochondria and the extracellular matrix, is involved in Aβ degradation is prevalently accepted. Due to the fact that hPreP also degrades Aβ after it has been cleaved from APP and that AD patients show mitochondrial dysfunction, there is an obvious connection between hPreP, IDE, mitochondria, and AD that warrants further investigation. The goal of this project is to develop new small molecules that can be used to interrogate the role of hPreP and mitochondria in the development of AD. This study may lead to the development of new strategies to understand and treat neurodegenerative diseases such as AD, since there is neither a definitive diagnosis nor an effective therapy available for the treatment of this most common form of dementia. Herein, the materials, methods and results achieved from this critical part of the study are presented.

KURZFASSUNG

Das Koehler Labor der Universität von Kalifornien, Los Angeles, hat kürzlich 88.000 kleine Molekül-Modulatoren in Bezug auf deren aktivierende und inaktivierende Wirkung auf das Vorläuferpeptid human Presequence Protease (hPreP) getestet. Die Tests wurden unter Zuhilfenahme eines High-Throughput Screen (HTS) mit verschiedenen *in vitro* Assays durchgeführt. Der Primär-Screen hat gezeigt, dass 260 dieser Modulatoren eine verstärkende und 464 eine hemmende Wirkung auf die mitochondriale Metalloendoprotease hPreP haben. Basierend auf diesen Ergebnissen wurde ein sekundärer Screen mit dem Protein Insulin Degrading Enzyme (IDE) - dem funktionellen Analogon zu hPreP - durchgeführt, um einerseits falsch-positive Treffer zu eliminieren und andererseits die Spezifität der aktivierenden und/oder hemmenden Wirkung auf die getesteten Moleküle zu bestätigen. Dafür wurde IDE in den Expressionsvektor pET28a kloniert, das rekombinante Protein aufgereinigt und dessen Konzentration über einen Bicinchoninic-Säure (BCA) Assay gemessen. Des Weiteren wurde die Aktivität des aufgereinigten Proteins über mehrere Aktivität Assays getestet und schlussendlich der Counter Screen durchgeführt. Die Ergebnisse zeigen, dass IDE weder von den 260 Aktivatoren noch von den 464 Inhibitoren des primären Screens beeinträchtigt wird.

Die Wichtigkeit dieser Forschung basiert darauf, dass hPreP für den Abbau von Amyloid-Beta (Aβ) in den Mitochondrien verantwortlich ist, was wiederum in Verbindung mit der Pathologie von Alzheimer steht. Alzheimer ist eine neurodegenerative Gehirnerkrankung, die durch Veränderungen im Verhalten und einem stufenweisen Abbau von Erinnerungen sowie Körperfunktionen gekennzeichnet ist. Wissenschaftler gehen heute davon aus, dass ein enger Zusammenhang zwischen Alzheimer und Mitochondrien, die unerlässlich für eine Vielzahl zellulärer Prozesse sind, besteht. Es wurde gezeigt, dass sich Amyloid-Beta (Aβ), welches vom Amyloid-Vorläuferprotein (APP) abgespalten wird, in den Mitochondrien im Gehirn von Alzheimer Patienten ansammelt, dort neurodegenerative Funktionen beeinträchtigt und so zu schwerwiegenden oxidativen Störungen in den Mitochondrien führt. Zudem ist erwiesen, dass die im Cytosol lokalisierte Zinkmetalloprotease IDE, Aβ abbaut. Auf Grund der Tatsache, dass hPreP im finalen Abbau von mitochondrialen Zielsequenzen mitwirkt und ebenfalls in der Lage ist Aβ abzubauen, liegt die Vermutung nahe, dass ein signifikanter Zusammenhang zwischen hPreP, IDE, Mitochondrien und Alzheimer besteht.

Ein Erfolg in diesem Forschungsbereich könnte wesentlich zur Entwicklung neuer Strategien im Kampf gegen Alzheimer beitragen um diese neurodegenerative Erkrankung besser verstehen und behandeln zu können. Denn bis heute besteht weder die Möglichkeit eine definitive medizinische Diagnose dieser Krankheit zu stellen, noch existieren effektive Therapiemöglichkeiten für Betroffene. In dieser Arbeit werden die verwendeten Materialien, durchgeführten Methoden und erhaltenen Ergebnisse der Forschungsarbeit im Koehler Labor der UCLA präsentiert.

ACKNOWLEDGMENTS

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Of all who have been enormously helpful in the preparation of this thesis, a very special recognition needs to be given to Juwina Wijaya for her guidance and input throughout the process of this research. I am deeply grateful for her continuing encouragement and scientific advice, and especially for her warmth and generosity.

In addition, I would like to explain my deep gratitude to my lovely roommates Mandy Styles, Shima Hafezi, Lee Kyungeun and Sanem Emregul as well as all my friends for their help, consideration and motivation during the time of writing.

I dedicate this thesis to CRL, my father Wolfgang, my mother Marianne, and my siblings Stephanie with Clemo, Lukas, and Raphaela for their unconditional love and support in every way possible.

Thank you.

TABLE OF CONTENTS

1. INTRODUCTION

Mitochondrial dysfunction contributes to various diseases including cancer, diabetes, and neurodegenerative disorders such as Alzheimer's Disease (AD) and Parkinson Disease [1].

Previous studies on the molecular mechanism of the pathology of AD are still contradictory and not clear. Several studies show that the characteristics of AD (Figure 1) are marked by extracellular, progressive accumulation of amyloid plaques mainly composed of amyloid-beta (Aβ). In contrast, other studies show that AD is due to intracellular, neurofibrillary tangles composed of aggregated, microtubule-associated, hyperphosphorylated, protein Tau. Increasing attention has been directed towards mitochondrial dysfunction as an initial cause, or contributing factor, of AD.

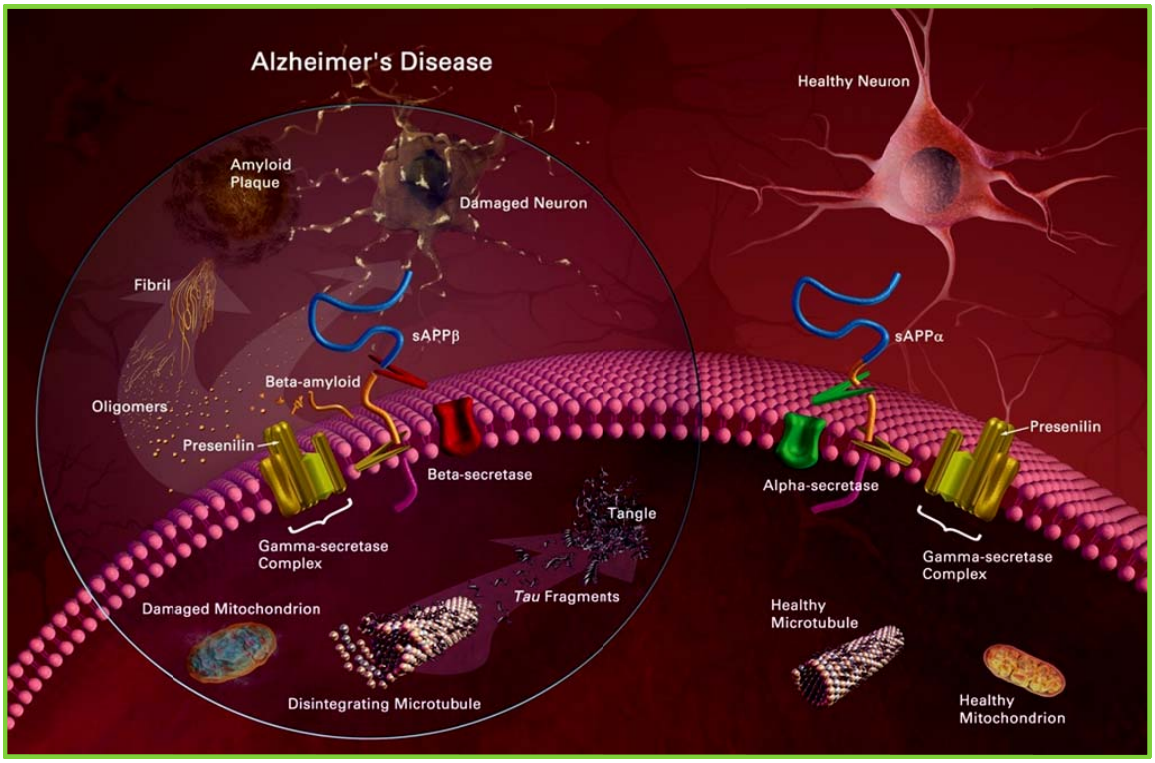

Figure 1: Characteristics of Alzheimer's Disease [2]

The mitochondrion is important for energy metabolism by generating ATP through oxidative phosphorylation and is crucial for many cellular processes including apoptosis, cell differentiation, homeostasis, metabolism, and signaling. Moreover, the mitochondrion is a double-membrane organelle composed of the outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), and the matrix. Previous studies have shown that in the human AD brain, amyloid precursor protein (APP) aggregates in the mitochondrial import channels and that Aβ-toxicity is linked to each of the compartments within mitochondria [3], [4]. Under normal conditions, Aβ is produced by APP and immediately

catabolized, whereas a failure to degrade Aβ leads to pathological deposition [5]. These findings link mitochondrial dysfunction to the pathology of AD.

This study focuses on the role of the Insulin Degrading Enzyme (IDE) and human Presequence Protease (hPreP) in Aβ degradation, because not only IDE but also hPreP have been shown to clave Aβ protein [3]. Both enzymes are metalloendopeptidases belonging to the pitrilysin familiy and are characterized as members of the M16 subfamily of proteases. They contain the zinc-binding domain HXXEH *(H: histidine (zinc ligand), X: any amino-acid, E: catalytic glutamate)*. Characterized in *Arabidopsis thaliana* as *At*PreP, the ATP-independent protease - PreP - is localized in the mitochondrial matrix as well as in the chloroplast stroma. Human PreP is, among other things, responsible for the clearance of damaged, miss-folded, and non-assembled proteins. It degrades the mitochondrial targeting sequence (MTS) of proteins that are localized to the mitochondria. Furthermore, hPreP contains two cysteines (Cys^{90} and Cys^{527}) that form a disulfide-bridge under oxidizing conditions. This disulfide-bridge locks hPreP into an inactive closed conformation. During accumulation of Aβ, the mitochondria becomes more oxidized, which may lead to oxidation and subsequent inactivation of PreP, culminating in an increase in Aβ that leads to mitochondrial dysfunction. Moreover, human IDE, the analogue of hPreP, is believed to be the main culprit in the pathology of AD accumulation in the extracellular matrix [5]. Loss of function of human IDE results in the accumulation of Aβ in the brains of AD patients. Within the cell, IDE is localized predominantly in the cytoplasm, and is also secreted into the extracellular space or associated with the cell surface in different cell types. Interestingly, in CHO (Chinese hamster ovary) cells, a novel, longer isoform of IDE (generated from translation of an alternate intron) containing an N-terminal mitochondrial targeting sequence has been localized to mitochondria and mitochondrial IDE degrades mitochondrially-targeted peptides *in vitro*. Unfortunately, the mechanisms responsible for targeting IDE to subcellular compartments of the nucleus, endosomes, and peroxisomes, as well as its role in AD, remain unclear [5]-[7].

In order to gain a better understanding of the role of mitochondria in AD disease, the Koehler laboratory at the University of California, Los Angeles, strives to find new strategies, as well as better molecular tools, to modulate mitochondrial activity in biological model systems. Juwina Wijaya, a graduate student in the laboratory, developed an *in vitro* assay to measure hPreP activity and implemented a small molecule screen using a high throughput assay [8]-[10]. From this screen, 464 potential inhibitors and 260 potential activators of this protease were identified. To verify that the potential inhibitiors and activators specifically modulate the activity of PreP and were not simply general protease inhibitors, a counter screen was devised with recombinant IDE. IDE was initially cloned into low copy expression vector pET28a. Recombinant IDE was then purified and its concentration quantified using bicinchoninic acid (BCA) assay. Activity assays were used to measure the activity of the purified protein. Finally, the counter screen was performed to determine if the compounds found from the primary HTS

for PreP specifically modulate the proteolytic activity of PreP, eliminating false-positive hits. Herein, the materials, methods and results achieved from this critical part of the study are presented.

1.1 The Mitochondrion

The mitochondrion resides in the cytosol of eukaryotic cells. Mammalian cells have up to 2500 mitochondria, which are typically about 0.5 ± 0.3 micron in diameter and from 0.5 micron to several microns long [11].

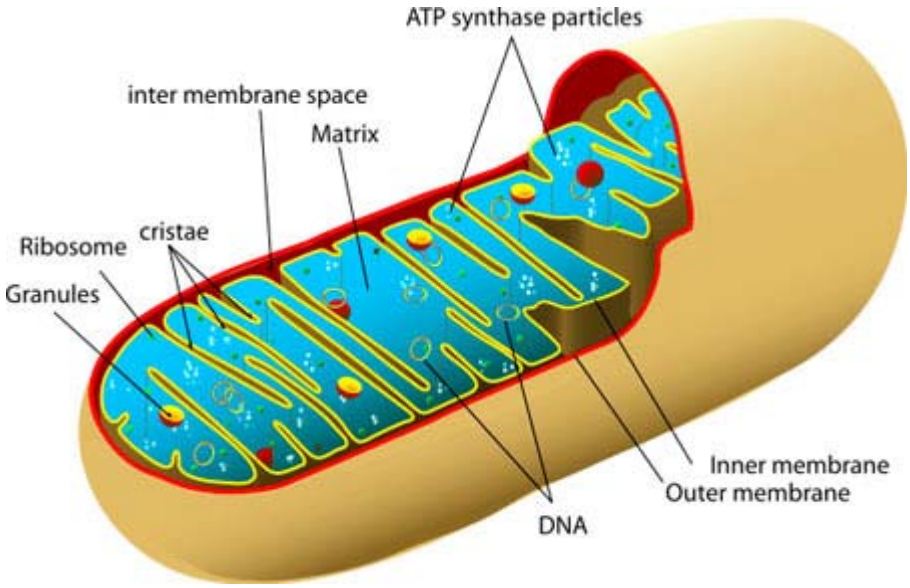

Figure 2: Mitochondrion [12]

The OMM surrounds the whole organelle (Figure 2); and includes many different proteins, such as import machineries and voltage dependent anion channels (VDAC). These channels are responsible for the free passage of substances of small molecular weight (≤ 5000 Da) between the cytoplasm and the IMS. The IMS contains different proteins including cytochrome *c* and several proteins responsible for maintaining the proton electrochemical gradient across the IMM needed for the ATP production. In contrast to the porous, permeable OMM, the more complex IMM provides a virtually impermeable barrier for small molecules. The folded cristae of IMM contain mitochondrial carrier proteins, respiratory enzymes of the electron transport chain (ETC), and the cofactor coenzyme Q. In addition, mitochondrial DNA is located within the matrix [3]. Human mitochondrial DNA is a circular molecule encoding two ribosomal RNA genes, 22 transfer RNA genes, and 13 polypeptides of the respiratory chain for intra-mitochondrial protein synthesis. Due to a lack of protective histone proteins, inefficient DNA repair, and a higher prevalence of reactive oxygen species (ROS) than in the nucleus, mitochondrial DNA has a high mutation rate and is more vulnerable to oxidative damage than nuclear DNA [4], [13].

The generation of energy within mitochondria takes place via two major oxidative metabolic processes that are closely connected to each other: the Krebs cycle (also known as tricarboxylic cycle (TCA) or citric acid cycle) and oxidative phosphorylation.

The Krebs cycle occurs in the mitochondrial matrix (Figure 7), whereas oxidative phosphorylation (Figure 3 and Figure 7) is located in the IMM and is composed of four enzyme complexes (complex I to IV) and the F_1F_0-ATP synthase (complex V).

While complex II is localized to the inner surface of the IMM, complexes I, III and IV are integral proteins of the IMM. Electrons from NADH and $FADH_2$ are transferred to complex I (NADH - dehydrogenase) and complex II (Succinate - dehydrogenase) respectively. The mobile electron carrier coenzyme Q, also known as ubiquinone (UQ), carries electrons from complexes I and II to complex III (Coenzyme Q - cytochrome c reductase). Furthermore, Complex III catalyzes the transfer of electrons from coenzyme Q to the electron carrier cytochrome *c* (Cyt*c*), and finally complex IV (Cytochrome *c* - oxidase) reduces molecular oxygen to water. As electrons are transferred through complexes I, III and IV, protons are pumped from the matrix into the IMS, generating an electrochemical gradient denoted with a unique mitochondrial membrane potential (-150mV to -180mV). This store of redox-energy is used to generate ATP from ADP as protons are reverse transported into the matrix across complex V [3]-[4].

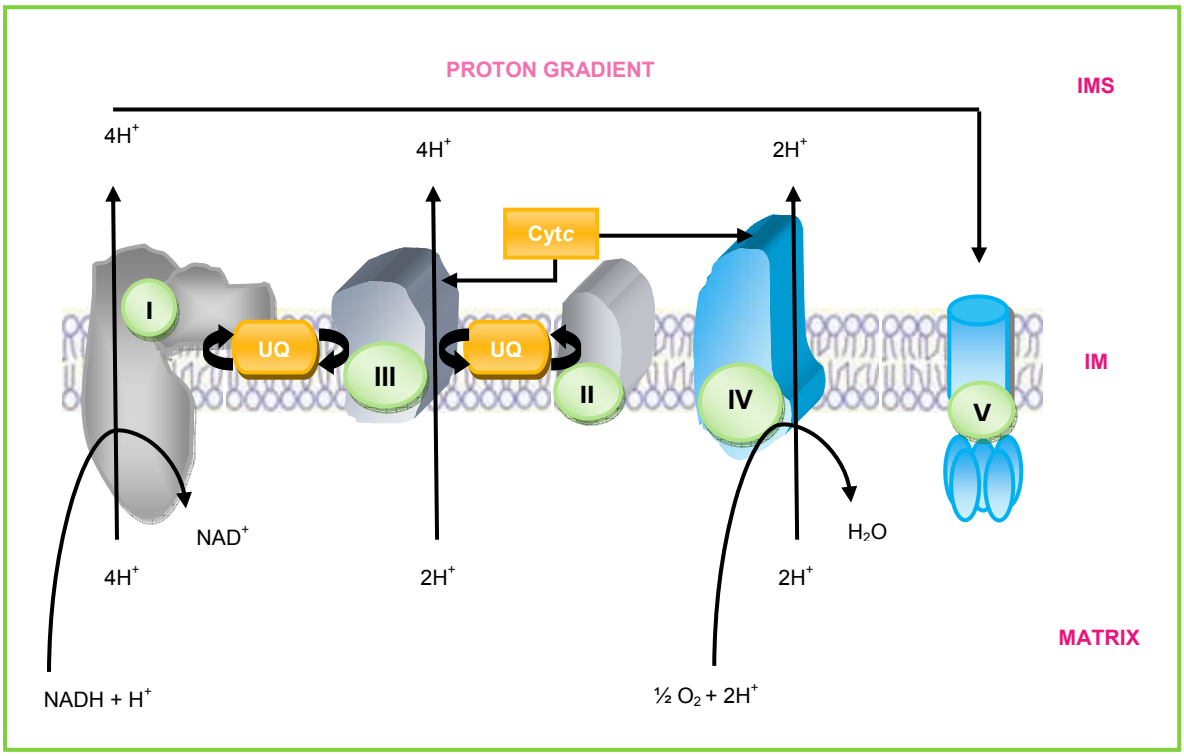

Figure 3: Oxidative Phosphorylation - electron transport and protein translocation

1.2 Pathway for processing targeting peptides in the mitochondrion

The majority of mitochondrial proteins is synthesized on cytosolic ribosomes (Figure 4) as pre-proteins and is imported across the various compartments within the mitochondria. Only 1% of mitochondrial proteins are synthesized inside the organelle on mitochondrial ribosomes [14].

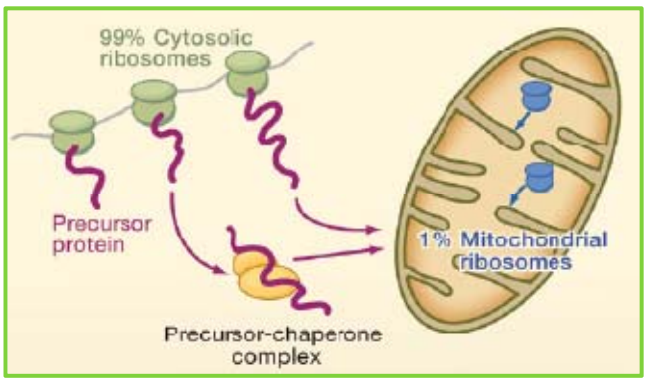

Figure 4: Biogenesis of mitochondrial proteins [14]

As such, the mitochondrion has developed an elaborate system for the proteolysis and the degradation of macromolecular complexes, involving a range of processing peptidases that remove and degrade the pre-proteins (Figure 5).

The mitochondrial pre-proteins contain a N-terminal (20 to 60 amino acids long) mitochondrial targeting signal (MTS) that is recognized by receptors on the OMM. As soon as the MTS is recognized, most of the pre-proteins are imported through the TOM40 (Translocon of Outer Membrane) complex. Depending on its final destination, the imported protein can interact with several translocation systems. Matrix-targeted proteins, as well as most proteins with a single transmembrane (TM) domain, are targeted to the IMM and recognized by TIM23 (Translocon of Inner Membrane). Once the protein reaches its correct compartment, the MTS is cleaved by one or several peptidases. MPP (Matrix Processing Peptidase) conducts the first cleavage of the matrix-targeted pre-protein, while IMP (Inner Membrane Peptidase) cleaves the sorting sequence for IMM- or IMS-localized proteins. Furthermore, MIP (Matrix Intermediate Processing-Peptidase) facilitates an additional cleavage on a small number of pre-proteins. Subsequently, the mitochondrial targeting cleaved pre-sequence is degraded by PreP into smaller oligopeptides. Finally, the oligopeptides are exported from mitochondria by the ABC (ATP Binding Cassette) transporter [8]-[10].

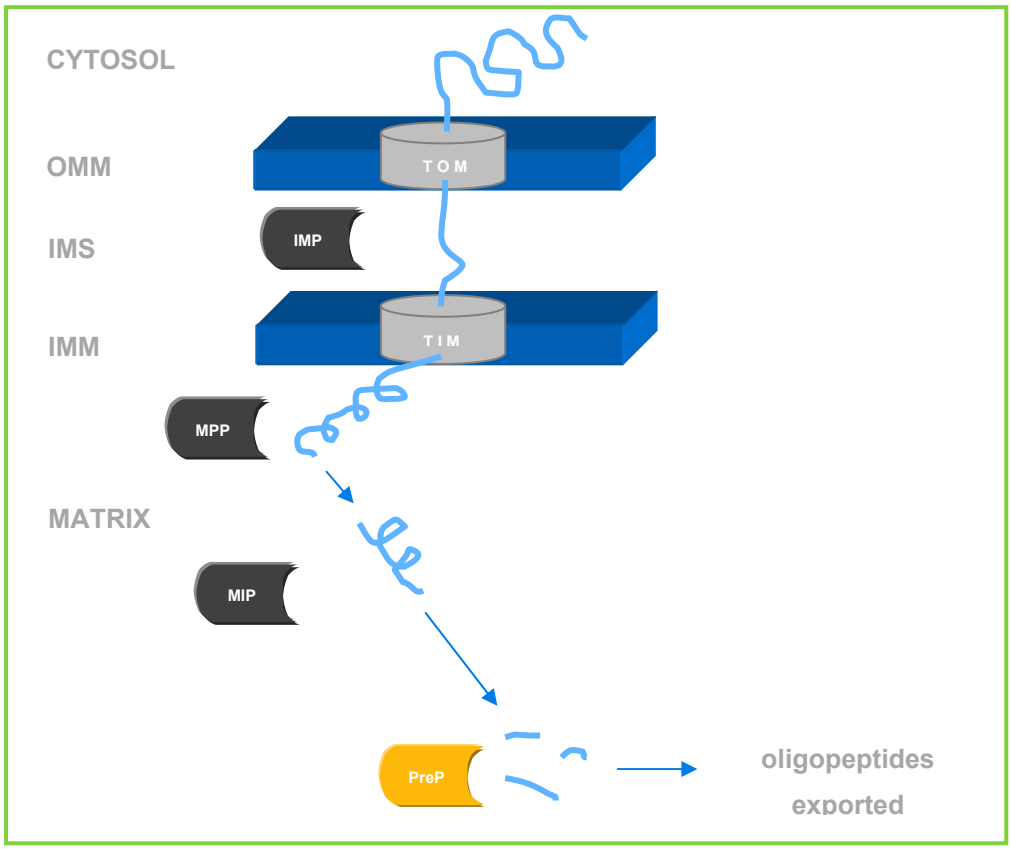

Figure 5: Mitochondrial Proteolytic System

1.3 Amyloid Precursor Protein (APP) and Amyloid-Beta (Aβ)

The aggregation of peptide Aβ, which is normally secreted from healthy neural and non-neural cells and circulates in blood and cerebrospinal fluid, is produced by cleavage of the integral (110-130kDa) glycoprotein APP resulting in the production of 40 or 42 amino-acid sequence called $A\beta_{40}$ and $A\beta_{42}$, respectively. APP is expressed in human tissues and is localized in the plasma membrane and several organelles, including the endoplasmic reticulum (ER), golgi apparatus, and mitochondria.

As Figure 6 illustrates, APP can go through an amyloidogenic and a non-amyloidogenic pathway of cleavage by α- (Alpha-), β- (Beta-), and γ- (Gamma-) secretases.
In the non-amyloidogenic pathway, the proteolytic enzyme α-secretase cleaves APP within the Aβ sequence first, releasing the fragment sAPPα. The second enzyme γ-secretase also cleaves APP in another place. These released fragments are thought to be beneficial for neurons.
In the amyloidogenic pathway, the first cut is made by the action of β-secretase, releasing the fragment sAPPβ. The first cleavage combined with the second cleavage by γ-secretase, results in the release of short Aβ fragments of APP. Normally, Aβ is transported across the blood-brain-barrier by the protein LRP1 (LDL-receptor-related-protein 1), which is able to interact with APP and induces brain clearance. In the brains of AD patients, the Aβ fragments clump together, become toxic, and interfere with the function of neurons. Furthermore, as more fragments are

added to the clumps, these oligomers increase in size, become insoluble, and form Aβ-plaques. As a result, as soon as the clearance is impaired, the aggregation and accumulation of neurotoxic Aβ results in neuronal cell death [3], [13].

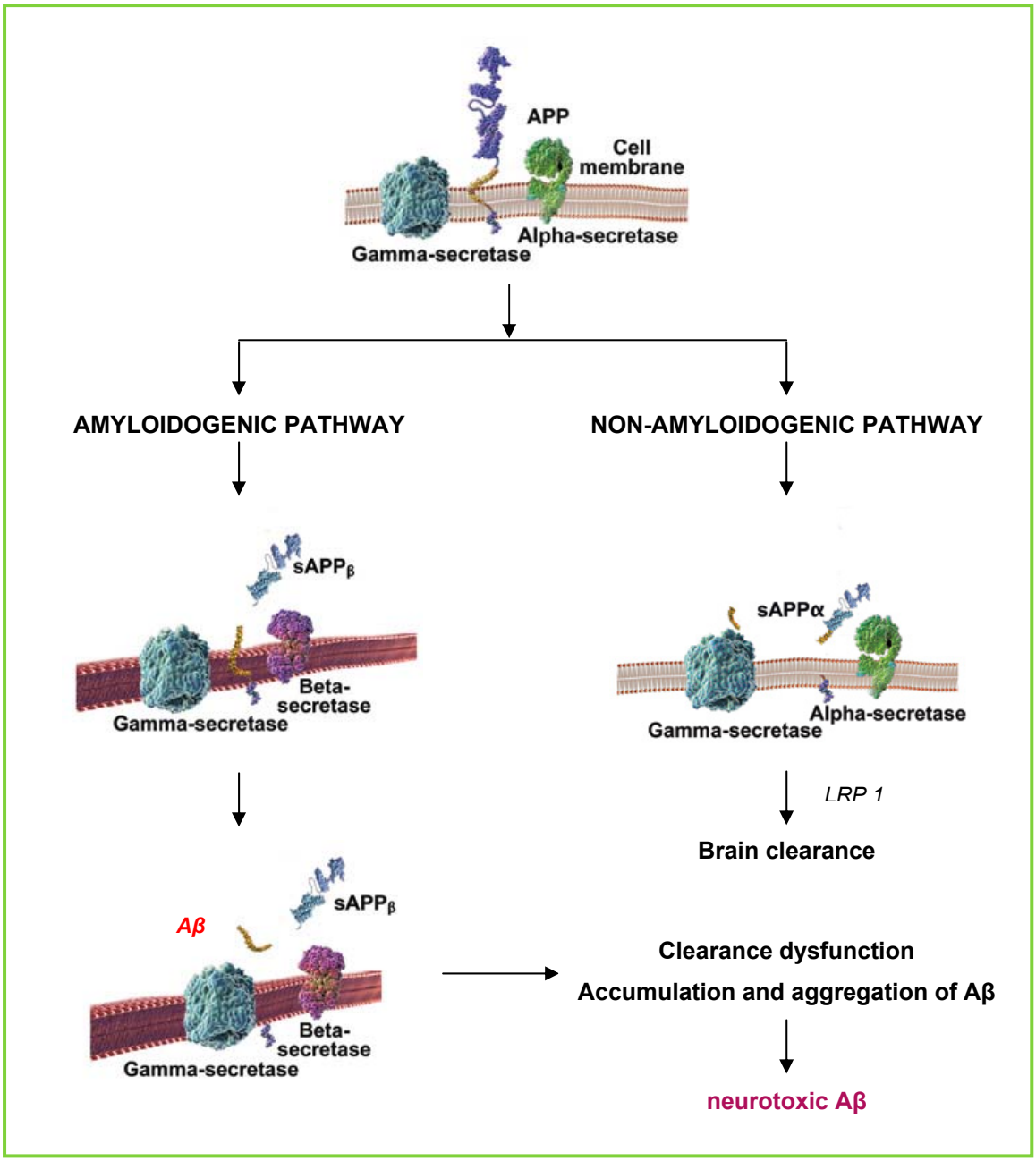

Figure 6: Amyloidogenic Pathway and Non-Amyloidogenic Pathway [2]

Although mitochondrial toxicity induced by Aβ is still not clear, rigorous scientific research has identified numerous mechanisms of Aβ interaction with mitochondria at the OMM, IMS, IMM, and the mitochondrial matrix (Figure 7).

1.3.1 OMM - IMS - IMM

Several studies show that Aβ crosses mitochondrial membranes via the TOM-TIM import-machinery and may interact with mitochondria, inhibiting protein import directly and indirectly. Due to the fact that Aβ induces an up-regulation of the biosynthesis of membrane components, deposits of Aβ may be enhanced too. In addition, a decline in protein import seems to lead to increased reactive oxygen species (ROS) and decreased mitochondrial membrane potential (MMP, ψ), which may cause gradual disorder of mitochondria. ROS production is closely connected with ψ; the high potential directly correlates with an increase in ROS production. Furthermore, mitochondrial Aβ may play an important role in impaired mitochondrial dynamics, due to a disturbance in the balance of mitochondrial fusion and fission. While fission, mediated by the proteins Mfn1/2 (mitofusion protein) and OPA1 (optic atrophy protein), allows mitochondrial renewal and protein proliferation in synapses; fusion, mediated by mitochondrial Fis1 (fission protein 1) and DLP1 (dynamin-like protein 1), eases mitochondrial movement as well as distribution across axons and synapses. If the balance of these dynamics is disrupted, the mitochondrion becomes either fragmented or elongated. In AD, mitochondrial fission has been shown to be more prevalent than mitochondrial fusion, because there are less mitochondria in brains of AD patients which correlates with an increase in the size of mitochondria [3]-[4], [13].

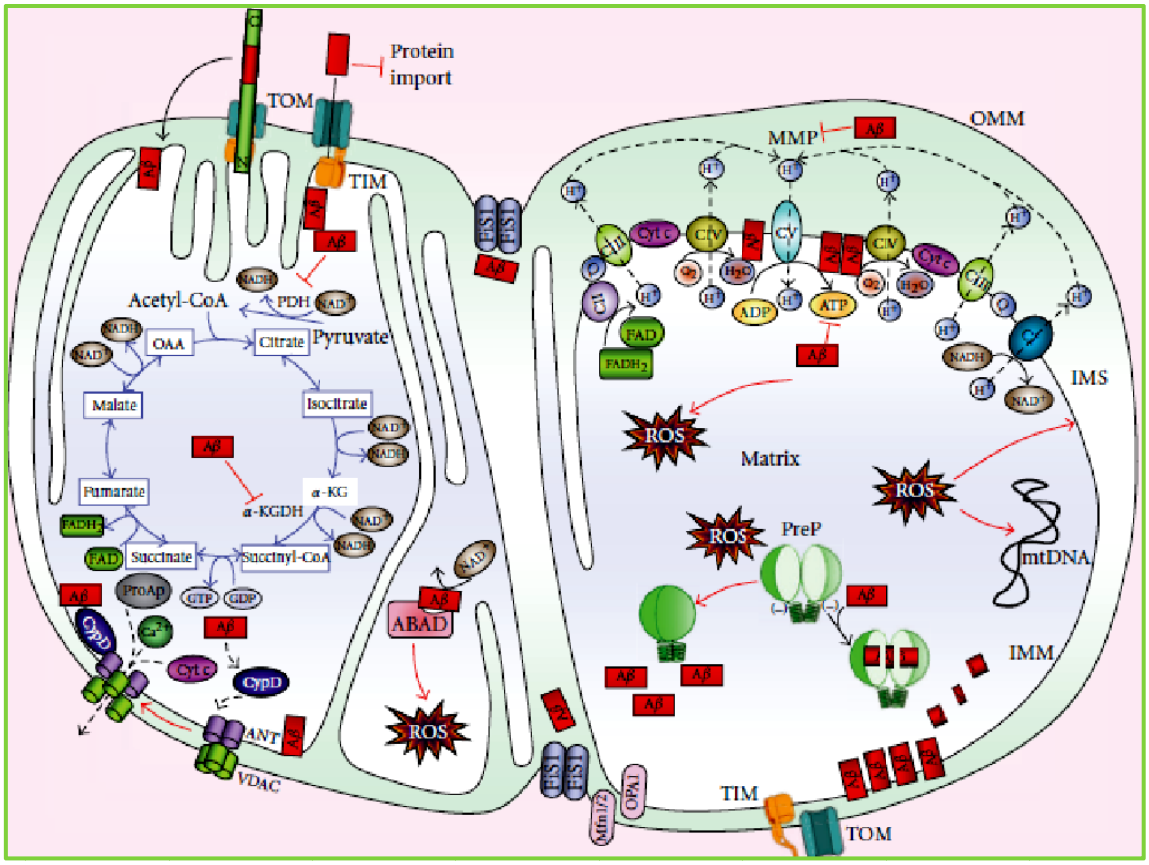

Figure 7: Mitochondrial Targets of Amyloid-Beta [3]

1.3.2 IMM and Matrix

Previous studies have shown that Aβ interacts with components of mitochondria and has the ability to bind to different molecules, such as Aβ binding dehydrogenase (ABAD). It has been shown that in the matrix of brain mitochondria from AD patients ABAD and Aβ interact and inhibit 4-hydroxynonenal (4-HNE) detoxification (a marker for lipid oxidation) leading to the generation of ROS.

Moreover, it is suggested that Aβ interacts with cyclophilin D (CypD) - a mitochondrial protein of the membrane permeability transition pore (mPTP) - which is generally formed by a complex of CypD, VDAC, and adenine nucleotide translocase (ANT). Although earlier studies have shown that the interaction between Aβ and CypD is weaker than the interaction between Aβ and ANT, it has been demonstrated that the translocation of CypD from the matrix to the IMM increases as soon as Aβ and CypD form a complex. This translocation favors the opening of mPTP, impairs calcium (Ca^{2+}) storage and increases apoptosis (through apoptotic factors (ProAp), such as Cyt*c*) as well as necrosis. To date, several hypotheses have been proposed about apoptosis in connection with Aβ and AD, but the role and the exact mechanisms of Aβ in apoptosis are not clarified.

Additional studies have shown that Aβ inhibits mitochondrial respiration. More specifically, it affects several proteins involved in oxidative phosphorylation, such as the α-chain of complex V (CV), Cyt*c*, and complex III (CIII). Furthermore, there is strong evidence that patients with AD have decreased activity of complex IV (CIV).

It has been confirmed that the inefficiency of electron transfer as a result of a decline in mitochondrial respiratory activity leads to a reduction of ATP production, as well as increased ROS generation, inducing severe damage within neuronal cells. Through ROS overproduction in brains of AD patients, there may be an indirect effect of Aβ on mitochondrial DNA and RNA; however, a couple of mitochondrial DNA mutations have associated the respiratory chain with a decline in the activity of the complexes III and IV in AD brain mitochondria.

A link between AD and the Krebs cycle is also plausible as oxidative damage has been implicated to play an important role in AD pathology. With an increase of oxidative damage and decrease in energy metabolism, Aβ has been shown to reduce the activity of pyruvate dehydrogenase (PDH), ATP-citrate lyase, acetoacety-CoA thiolase, and α-ketoglutarate dehydrogenase (α-KGDH), decreasing the NADH reduction [3]-[4], [13].

In summary, it can be stated that these highlighted mitochondrial targets of Aβ need further clarification because the contribution of mitochondrial dysfunction in the pathology of AD is not clear.

1.4 The human Presequence Protease (hPreP) and Insulin Degrading Enzyme (IDE)

Human Presequence Protease (hPreP) was originally identified as human metalloprotease 1 (hMP1) and has been shown to be the mitochondrial neurotoxic-Aβ-degrading protease in the human brain mitochondrial matrix. It contains a 29 amino-acids long mitochondrial targeting presequence. The protease is made up of 1037 amino-acids and has the ability to degrade not only $A\beta_{40}$ but also $A\beta_{42}$. It is encoded by a gene called *PITRM1* (Pitrilysin Metallopeptidase 1) gene located on chromosome 10 [1].

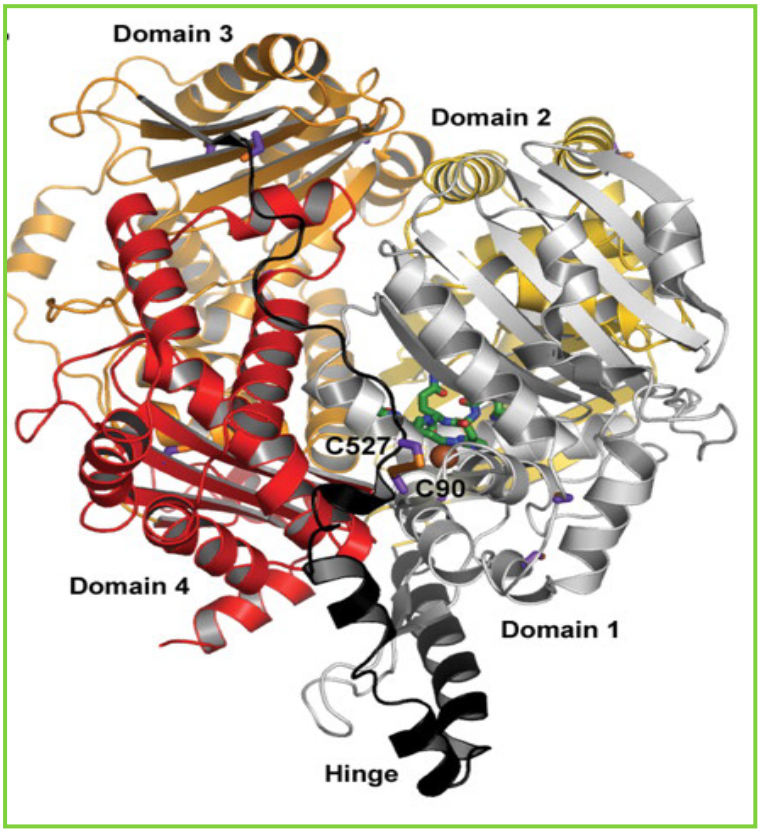

Figure 8: Structural model of hPreP with Aβ (12-17) bound to the active site [7]

The sequence of hPreP shows 48% sequence similarity and 31% identity to *At*PreP, which allows scientists to create a molecular homology structural model of hPreP (Figure 8). It should be noted that this structural model simply illustrates where the substrate is located during proteolysis as hPreP degrades several different substrates at multiple sites (beside the illustrated substrate Aβ (12-17)). Human PreP consists of four topologically similar domains. These domains create two halves that are connected by a hinge region. The two halves come together creating a large proteolytic chamber containing the active site of the protein. The active site contains an inverted zinc-binding motif, as well as a distal glutamate located in the N-terminal domain, and a Tyr^{878} which is located in the C-terminal domain [7].

IDE is also located on chromosome 10. The molecular surface of IDE (Figure 9) consists of two subunits. Each subunit illustrates two halves connected by a flexible linker (Aβ) located inside the proteolytic chamber of the molecule. If IDE is present in its open conformation, it allows substrates to enter and products to leave. In its closed conformation, it just allows substrate entrapment within the proteolytic chamber, which is formed by the two domains, featuring an exo-site that entrenched the N-terminal end of substrates [5].

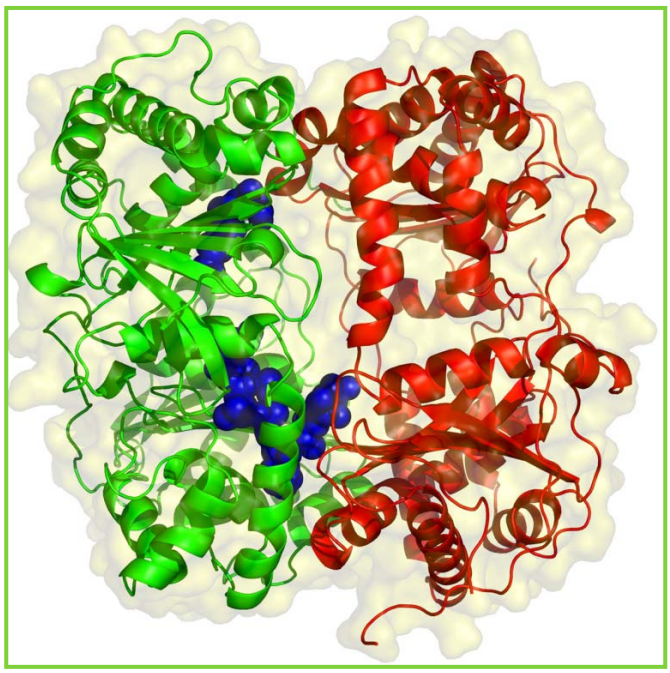

Figure 9: Structure of human IDE in complex with Aβ
[15]

In spite of the fact that both hPreP and IDE belong to the pitrilysin family of proteases and are able to degrade Aβ, it is found that there is only a 28 amino-acid long sequence in IDE, that is closely located to the zinc-binding motif and shows 55% sequence similarity and 38% identity to hPreP. Similarly, both sequence similarity and identity of hPreP and IDE is very low [7].
Human PreP degrades peptides up to 70 amino-acids in length and therefore should be able to degrade insulin, as the size of insulin is appropriate. Interestingly, it has been found that insulin is cleaved by IDE, which also degrades glucagon, amylin, and α-/β-endorphin, but not by hPreP. This may be due to the exo-site in the proteolytic chamber in the structure of the homodimer IDE, which is postulated to unfold small proteins and has not been found in the hPreP structure. Lacking this structure, hPreP might be inactive against small folded proteins [1], [7].

1.5 Principle of TOPO® Cloning

Conventional cloning is time consuming. The method consists of integrating an insert into a vector which mainly consists of both cutting the insert and vector with restriction enzymes and ligating the insert and vector with the ligase.

In some cases the insert-DNA needs to be inserted into the vector without compatible ends. That requires a ligation of an oligonucleotide containing the interfaces for the suitable restriction enzyme into the insert, which is both a time-consuming and elaborate process. In all, cloning consists of multiple steps which are time-consuming.

However, TOPO®-Cloning (Invitrogen) - also known as TA-cloning - is available for high efficient cloning. This one-step cloning strategy allows a direct insertion of blunt-end PCR products into a plasmid vector by exploiting the reaction of Topoisomerase I in order to efficiently clone PCR products. Furthermore, there is neither a ligase, nor PCR primers with specific sequences, and no post-PCR procedures required. Topoisomerase I from *Vaccinia* virus is able to bind to double-stranded DNA .The enzyme cleaves the phosphodiester-backbone after the 5'-CCCTT sequence in one strand. The energy from this cleavage is conserved by formation of a covalent bond between tyrosyl-residue (Tyr-274) of Topoisomerase I and the 3'-phosphate of the cleaved strand, which can subsequently be attacked by the 5'-hydroxyl of the original cleaved strand. This step reverses the reaction and releases the Topoisomerase I [16].

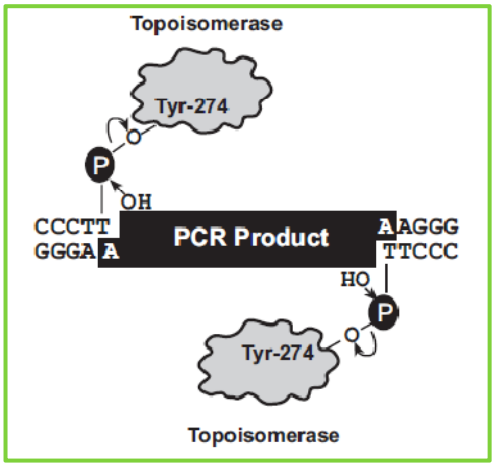

Figure 10: Theoretical Prinicple of TOPO®-Cloning [16]

The linearized plasmid vector (pCR®-Blunt II-TOPO®) contains the Topoisomerase I covalently bound to the 3'-end of each DNA strand and allows direct selection of recombinants through disruption of ccdB, a lethal *E.coli* gene, which is fused to the C-terminus of the *lac*Zα-fragment in the vector. The expression of the *lac*Zα-ccdB gene complex is disrupted by the ligation of a blunt-end PCR product, which permits the growth of only positive recombinants upon transformation. The TOPO®-Cloning reaction can either be transformed into chemically competent cells (our procedure) or electroporated directly into electrocompetent cells [16].

1.6 Expression Vector pET28a

It is important to choose a vector that is suitable for protein expression in certain hosts such as bacteria or yeast.

In this study, the pET28a vector was used for TOPO®-Cloning (see above). The pET28a expression vector was originally constructed by Studier and colleagues and developed at EMD Chemically Inc. Containing the highly efficient ribosome binding site from the phage T7 major capsid protein, pET28a belongs to the translation vectors family and is generally used for the expression of target genes without their own ribosomal binding site. This vector was used primarily for high expression of proteins, which allowed the production of a large amount of active proteins for screening [17].

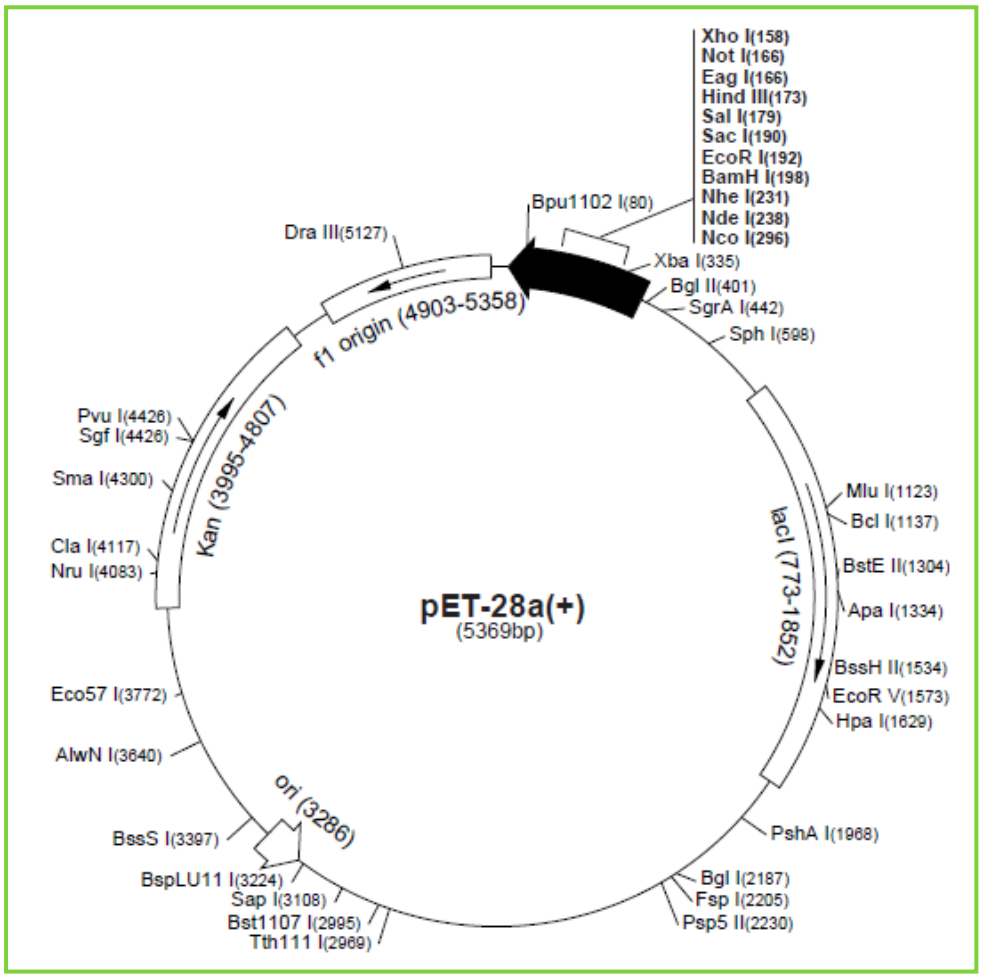

Figure 11: Vector Map pET28a(+) [18]

The pET-28a(+) vector (Figure 11) includes an optional C-terminal His-Tag, an N-terminal His-Tag®/thrombin/T7-Tag® configuration, T7 promoter, T7 transcription start, T7 terminator, pBR322 and f1 origin, as well as cloning sequences for lacI and Kan. Furthermore, the unique multiple cloning sites are shown in the vector map. Note that the T7 expression region is reversed shown (Figure 11), since the sequence is numbered by the pBR322 convention [18].

2. EXPERIMENTAL PROCEDURES

2.1 Cloning IDE into pET28

2.1.1 Preparation of IDE

Insulin degrading enzyme (IDE) cDNA was purchased from Open Biosystems. The primers (IDE2 forward and IDE reverse primer) for Polymerase Chain Reaction (PCR) were designed (Figure 12), ordered from Fisher Science Education, dissolved (100 µmol) in 10x TRIS-Borate-EDTA (TBE) buffer, and diluted (1:10) in water, giving a stock concentration of 10 µM.

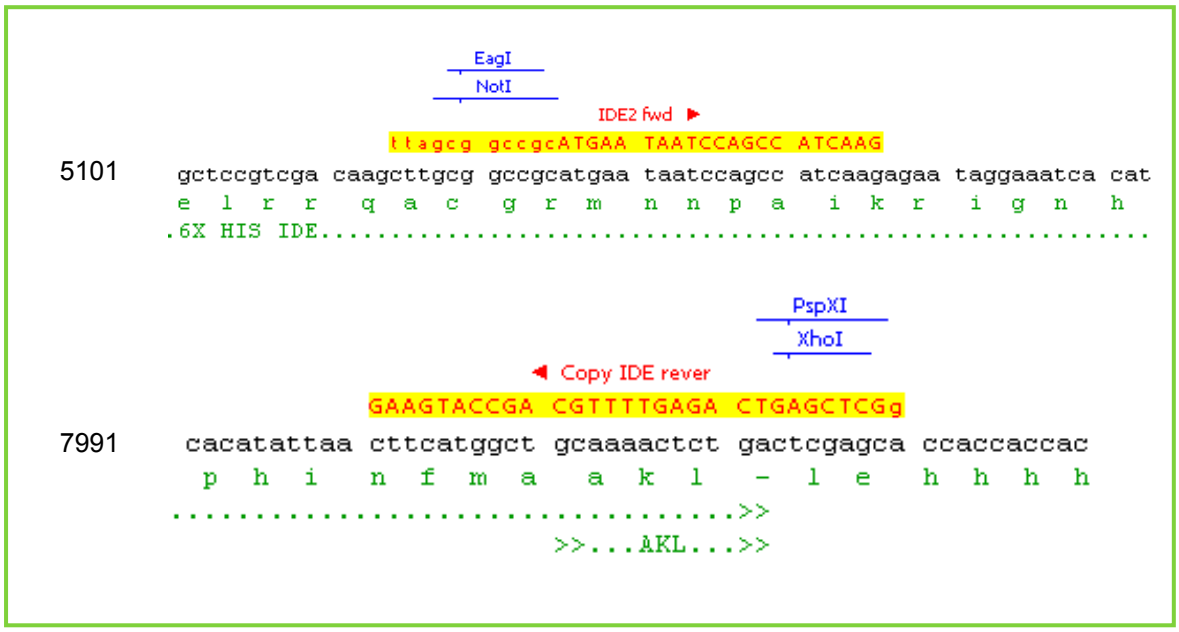

Figure 12: Primer Desing IDE

PCR *(C 1000^{TM} Thermal Cycler – Bio-Rad)* was carried out by following the PCR Protocol (9.1). The final PCR product contains NotI and XhoI restriction sites, which were used to sub-clone the insert DNA into pET28a vector. In addition, the PCR products were purified, as described in the PCR Cleaning Spin Protocol (9.2). All centrifugation steps were carried out with the Centrifuge 5417 C (Eppendorf). PCR products were run in a 0.8% agarose gel electrophoresis *(0.4 g Agarose Low EEO dissolved in 50 mL 1x TBE buffer and 4 µL Ethidiumbromide)* in 1x TBE buffer (120 V, 1 h) and visualized in ultraviolet light to verify the quality and quantity of these products *(Multilimage^{TM} Light Cabinet - Alpha Innotech Corporation)*, (Figure 15).

2.1.2 TOPO® Cloning

The TOPO® Cloning Reaction was performed, following the steps from TOPO® Cloning Protocol (9.3) and transformed into chemically competent *E. coli* cells. The TOPO® cloning reaction was incubated for 30 minutes, and Top10 competent cells were used in lieu of using the proposed One Shot® Competent Cells.

2.1.3 Insert IDE - digestion with NotI and XhoI

Colonies were picked from the TOPO® Cloning plates and overnight cultures were set up, by using 5 mL sterile *(Amso Scientific Series 3023-S Vacamatic)* 2YT (S.O.C.) medium *(ingredients 1L: 1.6% Tryptone (16.00 g), 1% Yeast Extract (10.00 g), 0.5% Sodium Chloride (5.00 g) and 1 M Tris-HCl (10.00 mL) at pH 7.4)* and 5 µL of 1000x kanamycin. The cultures were incubated (37°C) overnight with shaking *(Infors Multiran, ATR)*. The plasmid DNA were isolated out of *E.coli*, using EZgene™ Plasmid Miniprep - Bioland (9.4), and digested with NotI and XhoI restriction enzymes in a 37°C water bath for 90 minutes.

Digest Reaction mix	1(x)	Master Mix (12x)
	[µL]	[µL]
Plasmid	10	-
Buffer 4	5	60
BSA	0.5	6
NotI	0.5	6
XhoI	0.5	6
ddH_2O	33.5	402
Final volume	**50**	**40 aliquot**

Note: Buffer 4, BSA, NotI and XhoI were ordered from New England BioLabs®$_{Inc}$.

To figure out if the TOPO® Cloning was successful, digested DNA were run on a 0.8% agarose gel electrophoresis (120 V, 1 h) and visualized under UV light (Figure 16).

A few days later, colonies were picked from the LB Kan control plates (IDEa, b and d), overnight cultures were set up, incubated (37°C), and the plasmid DNA was isolated out of *E.coli* (9.4). To make sure, the plasmid DNA contains IDE, a 0.8% agarose gel electrophoresis (120 V, 1 h) was run again (Figure 17). Furthermore, the concentration [µg/µL] was measured *(Nano Drop 2000 Spectrophotometer - Thermo Scientific)* to calculate the volume [µL] of insert for the digest (16°C, overnight in PCR), because 3 µg plasmid were needed for the reaction. All centrifugation steps were carried out with the Centrifuge 5417 C (Eppendorf).

Digest Reaction mix		1(x) [µL]	Master Mix (4x) [µL]
	Insert (IDE)	3 ng/µL	-
	Buffer 4	5	20
	BSA	0.5	2
	NotI	1	4
	XhoI	1	4
	ddH_2O	various	various
	Final volume	**50**	**various**

Note: Buffer 4, BSA, NotI and XhoI were ordered from New England BioLabs®Inc.

2.1.4 Expression Vector pET28a - sequential digest with NotI and XhoI

Overnight cultures for pET28a were set up, by using a plate which was plated with pET28a *(Novagen)* from the frozen stock. Plasmid DNA was isolated out of *E.coli* (9.4), a 0.8% agarose gel electrophoresis (140 V, 1 h) was run, visualized, and the DNA concentration was measured as before. All centrifugation steps were carried out with the Centrifuge 5417 C (Eppendorf). The sequential digest was set up, using 8 µg of the vector.

Digest Reaction mix		1(x) [µL]	Master Mix (3x) [µL]
	Buffer 4	20	60
	BSA	2	6
	XhoI	4	-
	pET28a	8 ng/µL	-
	ddH_2O	various	various
	Final volume	**50**	**various**

Note: Buffer 4, BSA, NotI and XhoI were ordered from New England BioLabs®Inc.

The plasmid contained was then digested at 37°C for 3 h before 4 µL of the second restriction enzyme (NotI) was added. Afterwards, the reaction with both enzymes was digested (37°C, overnight in PCR).

2.1.5 IDE and pET28a - Gel Extraction

To extract the digested pET28a and IDE DNA, digested reactions were loaded on 0.8% agarose gel and gel electrophoresis (140 V, 2 h) was carried out, (Figure 18). The bands were then visualized under ultraviolet light *(Foto/Prepl - Fotodyne)*. The vector pET28a and IDE DNA were cut out of the gel then extracted using gel extraction kit (Bioland) and eluted with a final volume of 40 µL, by following the steps from Gel Extraction Spin Protocol (9.5).

2.1.6 IDE in pET28a - Ligation

In order to estimate the concentration of IDE and vector *(by comparing it to the concentration of the 1kb ladder - New England BioLabs®Inc.)* as well as to calculate the volume of DNA needed for ligation reaction, the DNA were run on a 0.8% agarose gel electrophoresis at 120 V for 1 h and visualized under UV light (Figure 19). The Ligation was set up at 16°C, overnight in a PCR machine by using 5 µL and 10 µL of IDE as well as 2 µL and 5 µL pET28a.

Ligation Reaction mix	1(x)	Master Mix (8x)
	[µL]	[µL]
Vector (pET28a)	2/5	-
Insert (IDE)	5/10	-
T4 DNA Ligase Buffer	2	16
ATP	0.5	4
T4 DNA Ligase	1	8
ddH_2O	9.5/1.5	12
Final volume	**20**	**5 aliquot**

Note: T4 DNA Ligase Reaction Buffer and T4 DNA Ligase were ordered from New England BioLabs®Inc.

2.1.7 Transformation into BL 21 Gold Competent Cells

The construct, pJM-IDE-pET28a, was transformed at 37°C into BL 21 Gold Competent Cells *E.coli* strain and plated overnight by following the Transforming Chemically Competent Cells Protocol (9.6), using LB Kan/Tet plates containing yeast extract, tryptone, glycerol, glucose, α-lactose, $MgSO_4$, $(NH_4)_2SO_4$, KH_2PO_4, Na_2HPO_4, and the antibiotics kanamycin and tetracycline.

2.1.8 IDE and pET28a - test digest with EcoRI and HindIII

Colonies were picked from the LB Kan/Tet plate, overnight cultures were set up, by using sterile 5 mL 2YT solution, 5 µL of 1000x kanamycin and 5 µL of 1000x tetracycline, and incubated (37°C) overnight. Plasmid DNA was isolated out of *E.coli* (9.4), LB Kan/Tet control plates were plated, and the digest was set up in the 37°C water bath for 2 hours with restriction enzymes EcoRI and HindIII. All centrifugation steps were carried out with the Centrifuge 5417 C (Eppendorf).

Digest Reaction mix		1(x) [µL]	Master Mix (16x) [µL]
	Plasmid	10	-
	Buffer 2	5	80
	EcoRI	0.5	8
	HindIII	0.5	8
	ddH_2O	34	544
	Final volume	**50**	**40 aliquot**

Note: Buffer 2, BSA, EcoRI and HindIII were ordered from New England BioLabs®Inc.

To figure out if the cloning was successful the DNA was run on a 0.8% agarose gel electrophoresis (Figure 20) at 150 V for 2 hours. Positive clones were further stored frozen at -80°C in 20% glycerol.

2.1.9 Test PCR

To verify that the colonies chosen contained the IDE insert, PCR reactions were set up using the two primers used to amplify the IDE2 insert. First, the concentration [µg/µL] of the digested plasmid was measured in order to figure out the volume [µL] for the PCR reaction. PCR was performed by following the PCR protocol (9.1).

PCR Reaction mix		1(x) [µL]	Master Mix (4x) [µL]
	Pfx RXN MIX Buffer	5	20
	IDE2 fwd. primer	1.5	6
	IDE rev. primer	1.5	6
	Templates	6 ng/µL	-
	cDNA IDE (+control)	6 ng/µL	-
	Pfx Polymerase	0.5	2
	ddH_2O	35.5	142
	Final volume	**50**	**44 aliquot**

Note: Pfx RXN MIX Buffer and Pfx Polymerase were ordered from Invitrogen.

A 0.8% agarose gel electrophoresis (120 V, 1 h) was run to analyze the result of the PCR product (Figure 21).

2.2 Protein Purification

2.2.1 Test Induction

2.2.1.1 Culture Growth

100 mL of 2YT medium, 100 µL of 1000x kanamycin, 10 µL of 1000x tetracycline and one colony (LB Kan/Tet control plate, IDEa2) were inoculated in a 500 mL flask and incubated (37°C, overnight) with shaking. The next day, three new 500 mL flasks were prepared and inoculated with 5 mL of the overnight culture, and incubated (37°C, 0.5 h) with shaking until the OD_{600} *(Ultrospec 10 cell density meter - Amersham Biosciences)* was 0.6. Isopropyl-β-D-Thiogalactopyranoside (IPTG), *(GBT – Gold Bio Technology, Inc.)* was prepared *(0.48 g dissolved in 2 mL ddH_2O, final concentration 1 mM)* and 500 µL of IPTG was added in each flask to induce expression. A 1 mL sample was collected, centrifuged *(Centrifuge 5417 C - Eppendorf)*, and the pellet was stored at -20°C as an uninduced control for SDS-PAGE. In order to figure out the optimal growth conditions for protein expression, the cultures were incubated at three different temperatures (25°C, 30°C and 37°C) with shaking. 1 mL samples were taken, centrifuged, and the pellets were stored at -20°C, after every second hour for a total of 6 hours. After 24 hours, another 1 mL sample was collected, centrifuged, and the pellet was stored at -20°C. The OD_{600} was measured for every sample (Table 3).

2.2.1.2 8% SDS-PAGE

Pellets collected at different time points were resuspended in 60 µL 2x SDS-PAGE Sample Buffer *(1000 µL 5x Sample Buffer and 200 µL 20% β-Mercaptoethanol)*, heated at 95°C for 5 minutes *(Dry Bath Incubator - Fisher Scientific)*, and loaded onto two 8% protein gels *(Seperating Gel: 6.66 mL Acrylamid/BIS (30/0.8%), 6.25 mL Buffer (Tris/Cl 1.5 M, pH 8.8), 12.125 mL ddH_2O, 0.02 mL TEMED, 0.20 mL APS (10%); Stacking Gel: 2.4 mL Acrylamide/BIS (30/0.8%), 4.0 mL Buffer (Tris-Cl 0.5 M, pH 6.8), 9.4 mL ddH2O, 0.02 mL TEMED, 0.20 mL APS (10%)).* Samples were loaded on the gels and ran (Figure 23 and Figure 24) at 70 mV for 5 hours. For SDS-Analysis (Western Blot and Coomassie) standard procedures (9.7) were used. Furthermore, His antibody *(MS mAB to 6x HIS, tag® [HIS H8], 100 µg (1 mg/mL) - ab 18184 abcam)*, and a mouse horseradish peroxidase Hrpsecondary antibody *(1:10,000 goat anti-mouse IgG HRP - Pierce)* were used for Western blot analysis.

2.2.2 Large Scale Protein Purification - 8 Liter

2.2.2.1 Culture Growth

200 mL 2YT medium, 200 µL of 1000x kanamycin, 200 µL of 1000x tetracycline were inoculated with 1 colony (LB Kan/Tet control plate, IDEa2) in a 1000 mL flask and incubated (37°C, overnight) with shaking. 8 L sterile 2YT (S.O.C) medium were prepared in eight 1 L-flasks. The next day, 1000 µL of 1000x kanamycin, 1000 µL of 1000x tetracycline as well as 20 mL overnight culture was inoculated in each flask. The flasks were incubated (37°C) with shaking until the OD_{600} was 0.6. In order to induce expression, IPTG was prepared *(1.19 g dissolved in 5*

mL ddH_2O, final concentration 1 mM) and 500 µL were added in each 1L-flask. A 1 mL sample was collected, OD_{600} was measured (OD_{600} = 0.64), centrifuged, and the pellet was stored at -20°C as an un-induced control for SDS-PAGE. Afterwards, the flasks were grown - at an optimal condition (25°C, 4 h) - with shaking. A second 1 mL sample was collected, OD_{600} was measured (OD_{600} = 0.91, dilution 1:1) centrifuged, and the pellet was stored at -20°C as an induced control for SDS-PAGE. The cells were harvested by centrifugation *(4°C, 1700 rpm; Avanti^{TM} J-20 XP Centrifuge, Rotator ID: JLA - Beckman Coulter)*.

2.2.2.2 Protein Purification under native conditions

To purify the protein, first, the cell pellet (26.15 g) was re-suspended in Lysis Buffer at 2 mL/g pellet (52.3 mL), *(ingredients 1L: 50 mM $NaH_2PO_4 \cdot H_2O$ (6.90 g), 300 mM NaCl (17.54 g), 10 mM imidazole (0.68 g); pH 8.0 - adjusted with NaOH)*. In order to lyse the bacterial cell wall 1 mg/mL lysozyme *(US Biological, lysoyzme - Chicken, RX3 muramidase)* was added, incubated (30 min), and sonicated *(550 Sonic Dismembrator - Fisher Scientific)* on ice. The lysate was centrifuged *(4°C, 30 min., 9000 rpm; Beckman - J2-HC, CJG 99E02)* to pellet the cell-debris. In the meantime, 6.4 mL Ni^{2+} beads *(HIS Pur^{TM} Ni-NTA Resin, 100 mL - Thermo Scientific)* were washed in 1 mL Lysis Buffer three times *(4°C, 3 min., 1200 rpm; Eppendorf Centrifuge 5810R, 15 Amp version)*. Carefully, the supernatant was added to the Ni^{2+} beads, rotated at *4°C* for one hour to allow the His-tagged protein to bind the Ni^{2+} beads and centrifuged (4°C, 3 min., 1200 rpm). Furthermore, a 1 mL sample from the cell-debris pellet as well as a 1 mL sample from the supernatant after binding was collected and stored at -20°C for SDS-PAGE. The Ni^{2+} beads with the bound protein were washed with Wash Buffer *(ingredients 1L: 50 mM $NaH_2PO_4 \cdot H_2O$ (6.90 g), 300 mM NaCl (17.54 g), 20mM imidazole (1.36 g); pH 8.0 - adjusted with NaOH)* for three times (4°C, 3 min/wash, 1200 rpm) to remove non-specific binding proteins. In addition, two 1 mL samples were collected from wash-steps 2, 3 and stored at -20°C for SDS-PAGE. Finally, the protein was eluted with 10 mL (1 mL/tube) Elution Buffer *(ingredients 1L: 50 mM $NaH_2PO_4 \cdot H_2O$ (6.90 g), 300 mM NaCl (17.54 g), 250 mM imidazole (17.00 g); pH to 8.0 - adjusted with NaOH)* at 4°C by using a Ni^{2+} column, and 200 µL sample were collected from the boiled Ni^{2+} beads. The purified protein was stored frozen at -80°C in 20% glycerol at a concentration of 1.22 µg/µL.

2.2.2.3 8% SDS-PAGE

As follows, 2x SDS-PAGE Sample Buffer *(1000 µL 5x Sample Buffer and 200 µL 20% β-Mercaptoethanol)* were added to the samples:

- 40 µL to the uninduced control, induced control and the cell-debris pellet.
- 10 µL to 200 µL supernatant after binding, wash 2 and wash 3.
- 15 µL to 200 µL elution 1 to elution 8 and boiled Ni^{2+} beads.

The samples were heated at 95°C for 5 minutes, and loaded on an 8% SDS-PAGE. The gel was run (8 mV, overnight) and the SDS-Analysis (Coomassie) was performed (Figure 25) according to standard procedures (9.7).

2.3 BCA (Bicin-coninic-acid) Assay

To determine the concentrations of the protein templates (elution 1 to 10) a BCA Protein Assay *(Pierce® BCA Protein Assay Kit, US Patent 4,839,295 - Thermo Scientific)* was performed. As follows, the standards for the assay were prepared, using BSA *(Bovine Serum Albumin - New England BioLabs®Inc)*:

2.00 mg/mL BSA · x = 1.50 mg/mL · 500 µL

x = 375 µL (2.00 mg/mL BSA) filled up to 500 µL with Elution Buffer

1.50 mg/mL BSA · x = 1.00 mg/mL · 300µL

x = 200 µL (1.50 mg/mL BSA) filled up to 300 µL with Elution Buffer

1.00 mg/mL BSA · x = 0.50 mg/mL · 200 µL

x = 100 µL (1.00 mg/mL BSA) filled up to 200 µL with Elution Buffer

0.50 mg/mL BSA · x = 0.2 5 mg/mL · 100 µL

x = 50.0 µL (0.50 mg/mL BSA) filled up to 100 µL with Elution Buffer

Furthermore, the plate (Table 1) for analysis was filled up (25 µL/well) with elution buffer (EB), the standards and the protein samples E1 to E10. 10 mL Pierce® BCA Protein Assay Reagent A and 200 µL Pierce® BCA Protein Assay Reagent B were then mixed and 200 µL of the mixed reagents were pipetted to each well, incubated *(37°C, 30 min.; VWR Scientific Inc.)* and analyzed visually. To measure the protein concentration a spectrophotometer *(µQuant - Bio-Tek Instruments, Inc, VT 05404-0998)* was used (Figure 26).

Table 1: BCA Assay Plate

EB [mg/mL]	2,00 BSA	1,50 BSA	1,00 BSA	0.50 BSA	0.25 BSA
EB [mg/mL]	2,00 BSA	1,50 BSA	1,00 BSA	0.50 BSA	0.25 BSA
EB [mg/mL]	2,00 BSA	1,50 BSA	1,00 BSA	0.50 BSA	0.25 BSA
E1	E2	E3	E4	E5	E6
E7	E8	E9	E10	-	-

2.4 Activity Assay

The following reagents and equipment were used for the activity assay:

- 384-well black plate (Greiner BioOne)
- Flex Station II (Molecular Devices)
- IDE (frozen stock) (cDNA from Open Biosystems)
- Screening Buffer (-)
- Trypsin (-)
- *1,10* O-Phenanthroline (Sigma Aldrich)
- EDTA (Fisher Scientific)
- Peptide (JPT)

Table 2 displays the reactions, which were set up for the activity assay in tubes. All reactions were incubated at room temperature for 1 hour, before the peptide was added. After the peptide was admitted to the reactions the plate was filled and immediately analyzed automatically, by using Flex Station II (Figure 27).

Table 2: Reactions for Activity Assay

REACTIONS	**1**	**2**	**3**	**4**	**5**	**6**
	Trypsin	**10 µL IDE**	**15 µL IDE**	**20 µL IDE**	**Chelated IDE (+EDTA)**	**Peptide alone**
Sceening Buffer	177 µL	177 µL	177 µL	177 µL	177 µL	177 µL
Trypsin	1 µL	-	-	-	-	-
IDE	-	10 µL	15 µL	20 µL	10 µL	-
O-Phenanthroline	-	-	-	-	2 µL	-
EDTA	-	-	-	-	4 µL	-
H_2O	20 µL	11 µL	6 µL	1 µL	5 µL	21 µL
Peptide	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL

2.5 Counter Screen with IDE

To identify specific inhibitor and/or activators of PreP, a counter screen with IDE was performed (Figure 13).

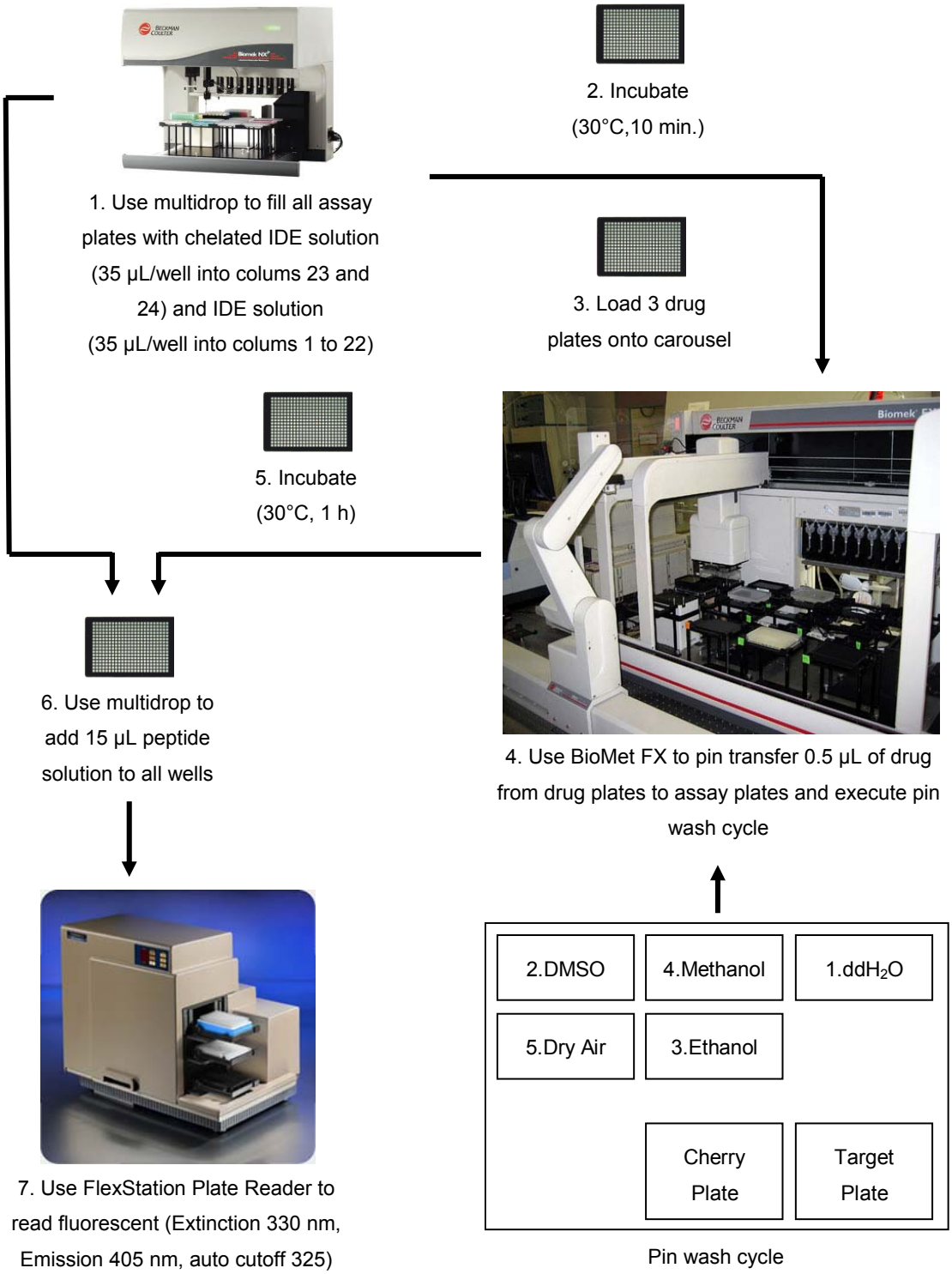

Figure 13: Way of proceeding counter screen with IDE [19], [22]

3. RESULTS

3.1 Assay Development and HTS for modulators of hPreP

The first step in developing the assay has been the cloning and expression of PreP, which was subcloned into pET28a low-copy expression vector. Since the N-terminal targeting sequence is normally processed and can obstruct correct protein folding, the targeting sequence was left out in the generated construct. The recombinant protein was then purified, using a Co^{2+} column and identified by western-blot with a His-antibody. In a preliminary single tube assay, 50 µL reactions were used with a final fluorogenic-peptide concentration of 10 µM by titrating PreP from 125 ng to 550 ng. The peptide used has a fluorescent label at its C-terminus and a quencher at the N-terminus, which quenches the fluorescence. Upon cleavage by the protease the fluorescence increases, which is the readout of the assay.

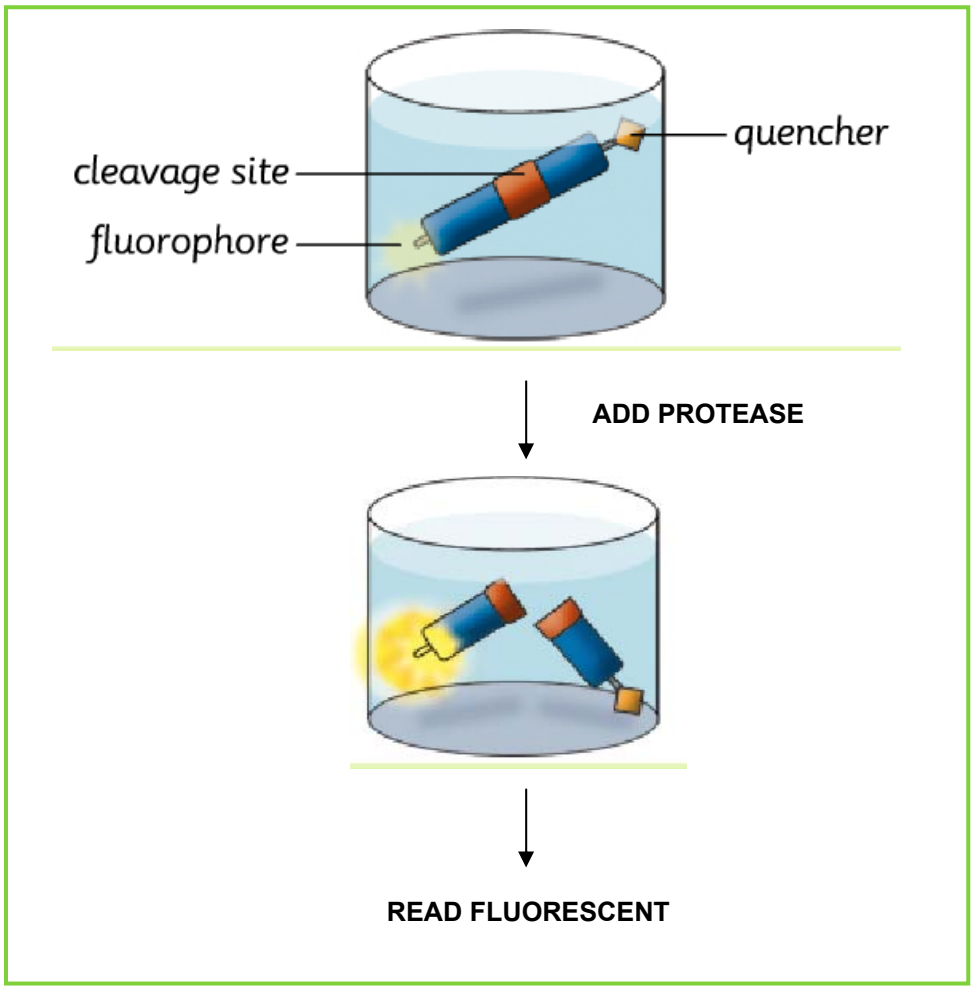

Figure 14: How to see if the protein is active (Image by JPT peptides)

As expected, PreP degraded the peptide in a concentration-dependent manner. To futher optimize the assay for HTS, different parameters were tested. It was found that the PreP activity was inhibited in the presence of 1,10 o-phenanthroline and EDTA. In addition, the protease activity was not affected when the enzyme was frozen and thawed, nor when it has been treated with common HTS buffers like HEPES and phosphate buffer. Moreover, it has been shown that common protease do not significantly inhibit the proteolytic activity of the purified protein. As a result, the identification of specific inhibitors for PreP will be very useful to generate tools to attenuate PreP activity, specifically because small molecule modulators are not available to aid in the understanding how defects in mitochondria contribute to AD.

The second step in assay development was the adaption of the preliminary single tube assay for high throughput screening. In pre-pilot experiments, a Z-prime value of 0.67 was ascertained, which indicates an optimal seperation band between maximum and minimum controls and low standard-deviations, as a Z-prime value ≥ 0.5 stands for good quality.

In a pilot-screen about 600 small molecules were screened for inhibition or activation of PreP. In addition, fluorescent counts were measured before and 20 minutes after the peptide was added because PreP inhibitiors give a fluorescent count that is comparable or lower than the control wells (PreP chelated with 2 mM EDTA and 0.5 mM o-phenanthroline), whereas PreP activators were expected to give a fluorescent count that is higher than the control with 2% DMSO. The hits for both potential inhibitors and activators were analyzed by an algorithm, courtesy of Dr. Samuel Hasson. A Z-prime value ≥0.5 was obtained and the result shows hits for potential inhibitors.
Following the successful pilot-screen, the finding for small molecule modulators of PreP from several libraries continued. The data analysis from the libraries screen revealed 464 potential inhibitors and 260 potential activators of PreP out of 88.000 compounds [8].

3.2 Cloning IDE into pET28a

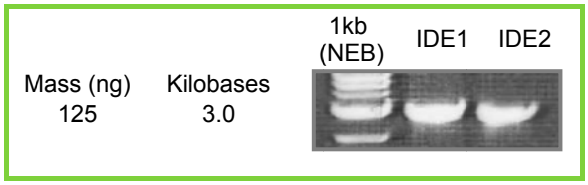

Figure 15: PCR Products of IDE

Figure 15 shows two single and discrete bands of the PCR products (IDE1 [0.23 µg/µL] and IDE2 [0.18 µg/µL]) of the correct size (2.96 kb). Due to the fact that the IDE sequence has two start-codons (ATG), PCR was performed with two IDE forward primers (one for IDE1 and one for IDE2) and one IDE reverse primer. The IDE1 PCR product includes the presequence, which may prevent protein folding and therefore cause the protein to be non-functional. Usually the presequence is cleaved before it folds and becomes mature and functional; however, bacteria - like *E.coli* - might not have the ability to cleave the presequence. That is why when presequence is present, protein might not fold properly and therefore becomes non-functional. Subsequently, we did not use the IDE1 PCR product.

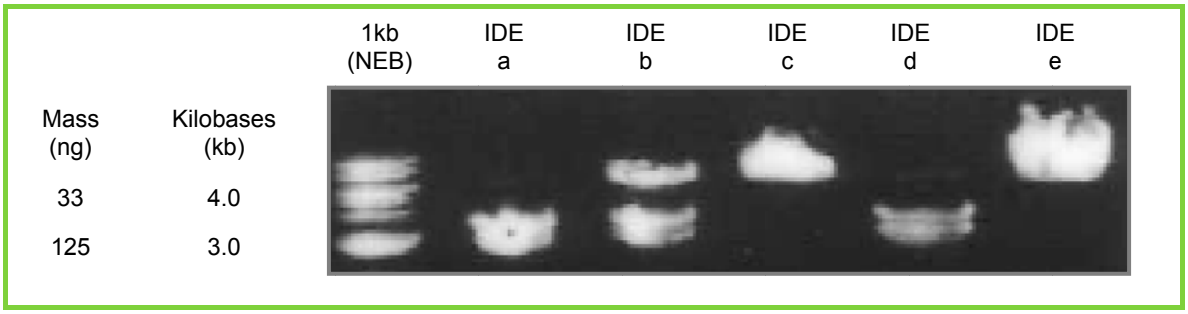

Figure 16: Zero Blunt® TOPO® Cloning result (IDE a-e and TA-vector)

Zero Blunt® TOPO® Cloning was chosen because this one-step cloning strategy requires no ligase and PCR primers containing specific sequences. Unfortunately, the digest with the restriction enzymes NotI and XhoI supplied no explicit results (Figure 16). Commonly, there should be two clear bands detectable. One for IDE at 2.96 kb and another for pCR®-Blunt II-TOPO® vector (also known as TA-vector) at 3.90 kb. The results for IDE (c) and (e) show only one strong band. This could be the result of a huge amount of DNA in the template; which could cause the restriction enzymes to not cut efficiently. The sample IDE (b) offers not only the same band, less strongly, but also two further bands at the expected sizes for IDE and the TA-vector. Furthermore, these two expected bands are discernible at the results for IDE (a) and (d). To be on the safe side with this suggestion, the samples were sent to Fischer Oligo for sequencing.
The sequencing results confirm that the Zero Blunt® TOPO Cloning was successful for the samples IDE (a), (b) and (d), as no mutation was found.

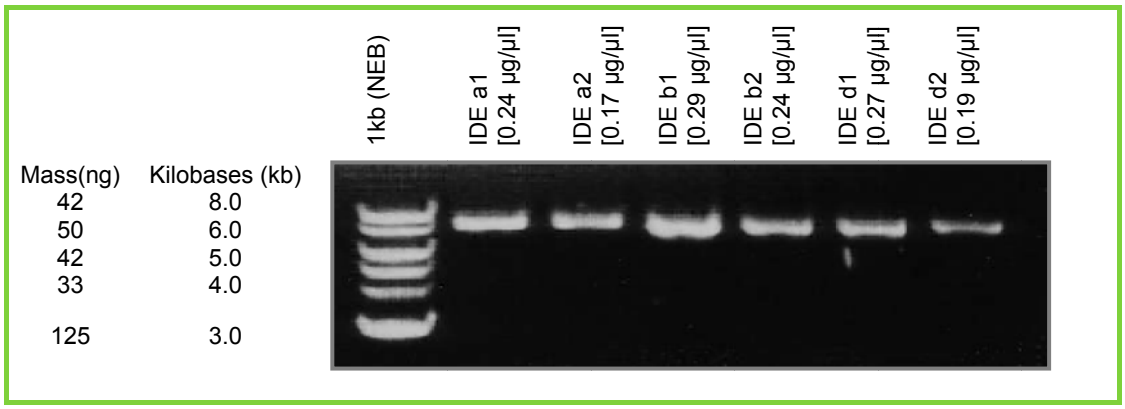

Figure 17: Isolated Plasmid DNA

Figure 17 confirms that the plasmid DNA was successfully isolated out of *E.coli*. As expected, the six bands for IDE ((a), (b) and (d)) are discernible at 6.86 kb (2.96 kb for IDE plus 3.90 kb for pCR®-Blunt II-TOPO® vector).

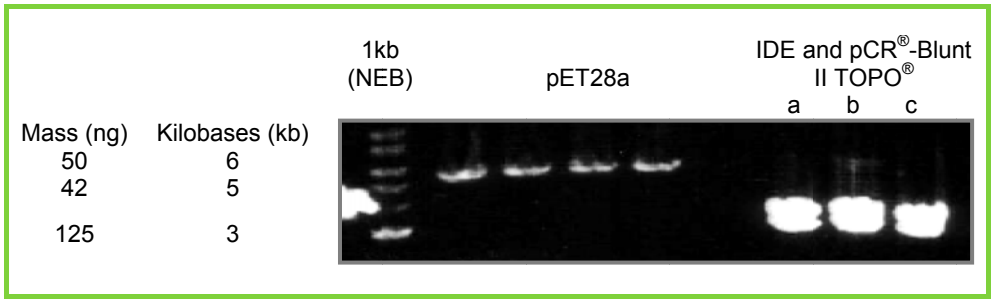

Figure 18: 0.8% agarose gel for gel extraction of pET28a and IDE after digest

Figure 18 validates that not only the expression vector pET28a, but also the insert IDE were well-prepared for ligation, as they are conspicuous at their correct sizes. The size of the vector pET28a was expected to be at 5.37 kb and the size for IDE at 2.97 kb. To get better fractionation for IDE and pCR®-Blunt II-TOPO® and make it easier to cut the band for IDE out of the gel, it might be better to run the 0.8% agarose gel longer.

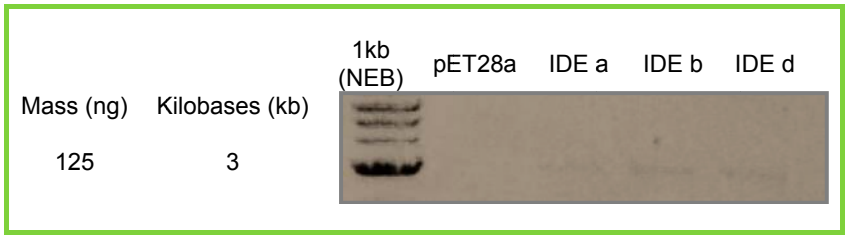

Figure 19: 0.8% agarose gel to estimate the size of the insert and the vector

Unfortunately, the bands in Figure 19 are barely visible. However, this gel was needed to estimate the concentration of insert and vector as well as to calculate the volume needed for the ligation reaction.

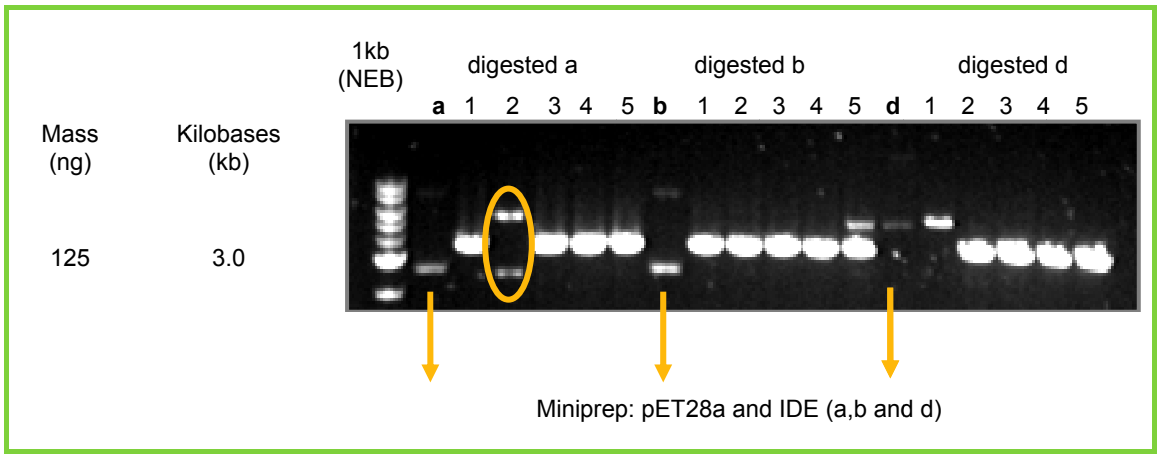

Figure 20: Test digestion (IDE in pET28a)

The test digest of the cloning was conducted with the restriction enzymes EcoRI and HindIII, because HindIII cuts within the insert. The expected sizes are 2.90 kb and 5.43 kb. Figure 20 shows that only one out of fifteen trials to clone IDE into the low copy expression vector pET28a was successful (orange circle), because the two expected bands are discernible. The concentration for the positive clone totals 0.14 µg/µL.

Unfortunately, the resolution of the gel and the 1kb Ladder (NEB) disallows us to take a definite stand on the result. Given the fact that supercoiled DNA runs faster on the agarose gel than linear DNA, the uncut isolated plasmid DNA (Miniprep: pET28a and IDE (a) and (b)) could be supercoiled. The stronger bands are visible at around 3.0 kb, although they should be at around 8.33 kb (2.96 kb for IDE plus 5.37 kb for pET28a), like the lighter ones.

The three bands - visible at around 3.0 kb (Miniprep: pET28a and IDE (d), sample (b5) and (d1) - could be empty vector without insert. For all remaining results there are two suggestions. One option is that restriction enzymes were not able to cut because the samples contain a huge amount of DNA. The other option is that these samples are just empty vector without insert and the DNA is supercoiled.

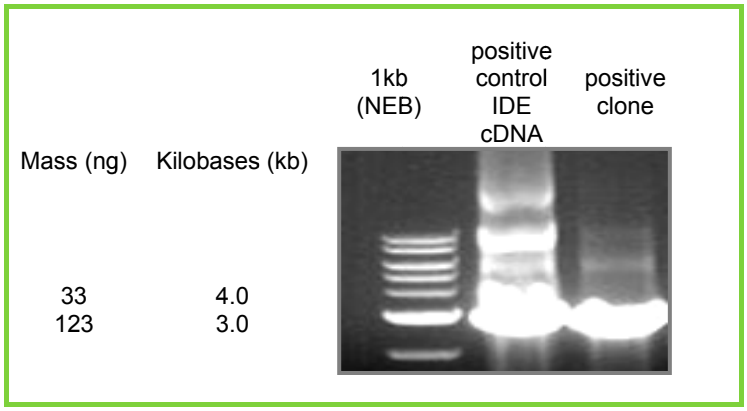

Figure 21: Test PCR

To confirm the positive clone a test PCR was implemented, as shown in Figure 20. The IDE cDNA was loaded on the gel as a positive control. With reference to the test digest (Figure 21) this result verifies our suggestions. The estimate size for the positive control (IDE cDNA) is at around 3.24 kb whereas the size for the IDE is at around 2.96 bp. The smears at the positive control (cDNA) can be pronounced due to the fact that the ordered IDE cDNA was overloaded on the gel and may not have been digested to completion.

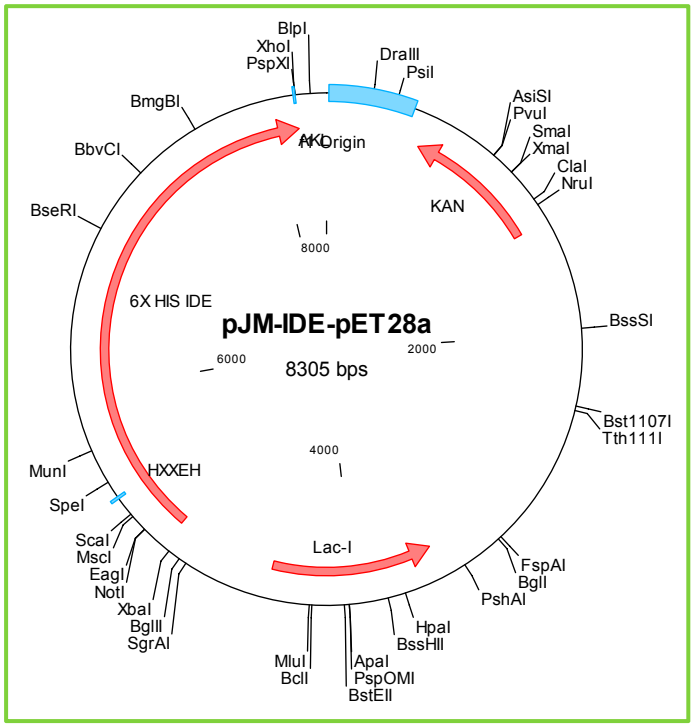

Figure 22: Plasmid map of IDE subcloned into pET28a expression vector

Figure 22 shows the plasmid map of the resulting clone. The sequence was confirmed by sequencing (Fischer Oligo).

3.3 Protein Purification

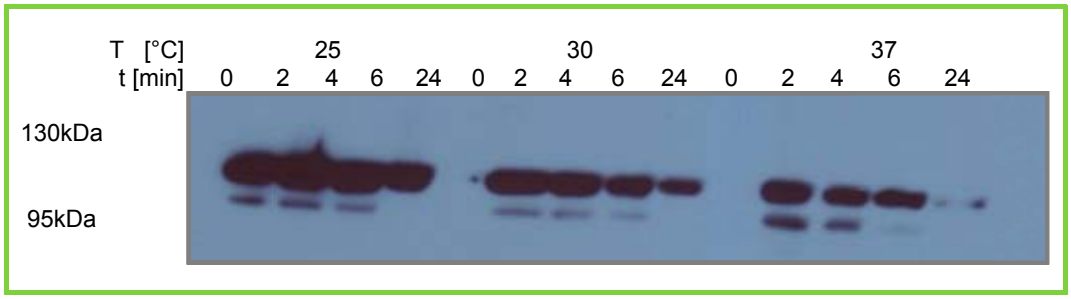

Figure 23: Test Induction – Western-blot IDE and pET28a (0.5 mM IPTG)

The result, showed in Figure 23 is consistent with the expected size for IDE at 118 kDa. It was found that incubation at 25°C for 4 hours provides the highest amount of IDE, and therefore these are the optimal growth conditions for IDE. The smaller bottom band could be a specific degradation product of the protein.

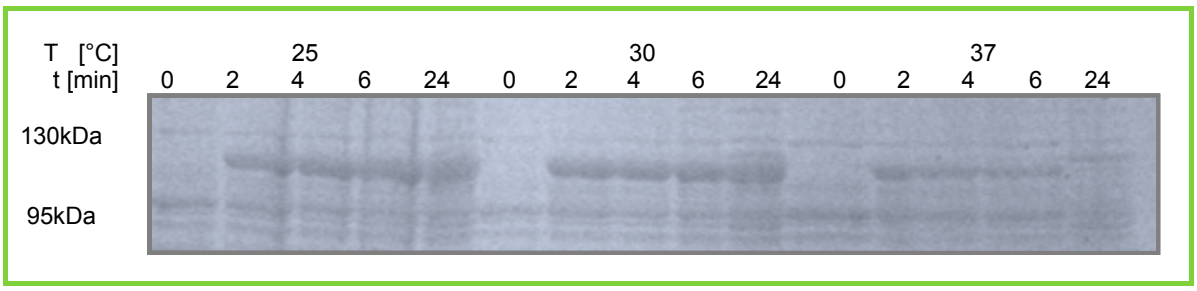

Figure 24: Test Induction - Coomassie-stained gel IDE and pET28a (0.5 mM IPTG)

Figure 24 also confirms that incubation at 25°C for 4 hours is the optimal growth conditions for IDE. As expected there is no protein detectable at time zero (t=0).

Table 3: Test Induction OD_{600} Measurement

	t=0 [min]	t=2 [min]	t=4 [min]	t=6 [min]	t=24 [min]
T=25 [°C]	**0.58**	**0.77**	**0.23**	**0.25**	**1.00**
T=30 [°C]	**0.61**	**0.82**	**0.21**	**0.21**	**1.04**
T=37 [°C]	**0.63**	**0.83**	**0.17**	**0.17**	**1.04**
dilution (2YT)	/	1:1	1:10	1:10	1:10

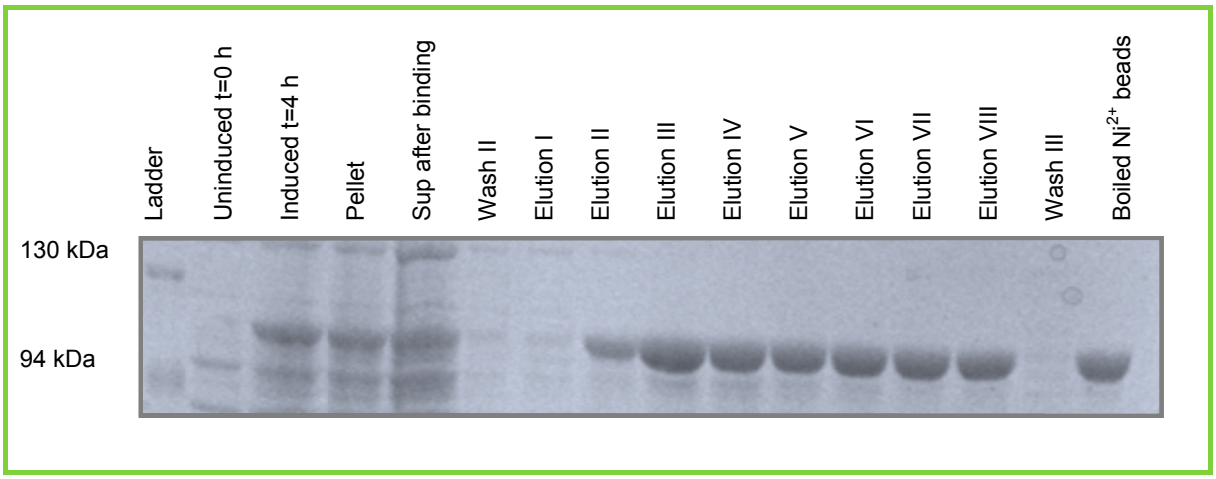

Figure 25: Large Scale Protein Purification - Coomassie-stained gel result (8 Liter)

E.coli overexpression strain, BL21 Gold Competent Cells, were transformed with the construct pJM-IDE-pET28a and expressed for 4 hours at 25°C as optimized by previous results. The lysate was collected and IDE was purified using Ni^{2+} column. 1 mL fractions were eluted and analyzed by SDS-PAGE. The result (Figure 25) indicates that the 118 kDa protein (IDE) was successfully over-expressed and purified. The induced sample, the pellet, the supernatant after binding, the boiled Ni^{2+} beads as well as the elutions II to VIII contain the protein. There is no protein visible in elution I because there might still have been huge amount of Wash Buffer in the column.

We could elute a high amount of IDE with our large scale protein purification as confirmed by elutions II to VIII.

3.4 BCA Assay

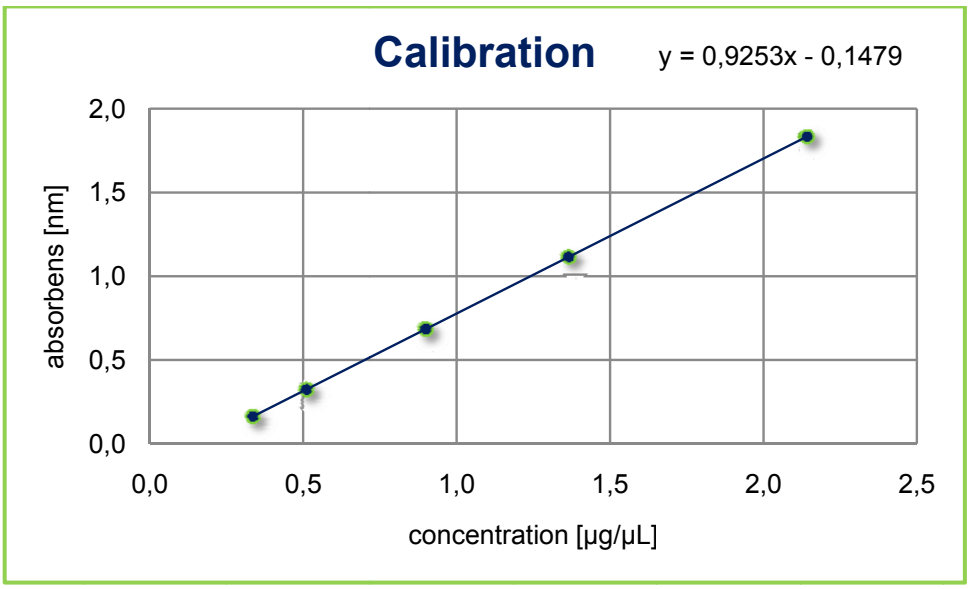

Figure 26: BCA Protein Assay - calibration with BSA

Table 4: Measurement of concentration and absorbance of BSA

BSA [mg/mL]	2.00	1.50	1.00	0.50	0.25
- concentration [µg/µL]	2.14	1.36	0.90	0.51	0.34
- absorbance [nm]	1.83	1.11	0.68	0.33	0.16

The calibration for the BCA protein assay was performed with protein concentration reference standards BSA (bovine serum albumin) solutions (Table 4) because BSA protein is universally accepted as a reference protein for total protein quantification. To estimate the total IDE concentration (Table 5) the BCA assay values for the IDE samples were compared to the known concentration values BSA standard (Figure 26).

Table 5: Measurement of concentration and absorbance of IDE

Elution - IDE	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Concentration [µg/µL]	1.37	1.58	2.33	1.79	1.20	1.03	0.85	0.72	0.72	0.67
Absorbance [nm]	1.12	1.31	2.01	1.51	0.97	0.80	0.64	0.52	0.52	0.47

3.5 Activity Assay

15 µM of the same fluorogenic peptide used for HTS was incubated with about 0.85 nM protein IDE/well, which yielded a good linear range that follows classical kinetic activity profile (Figure 27).

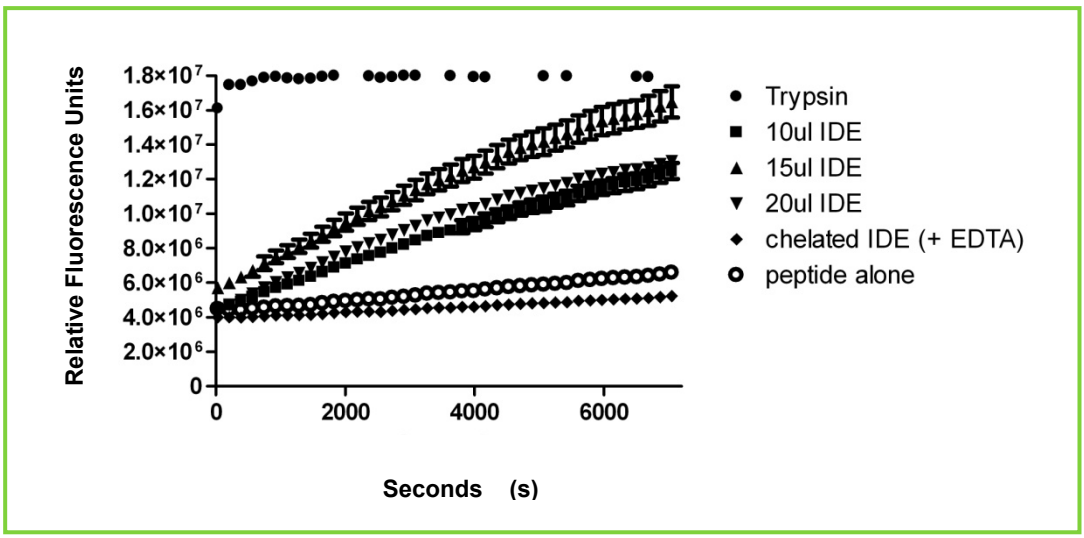

Figure 27: IDE cleaves the fluorogenic peptide substrate

In principal, the fluorescence of the peptide is quenched by the *JPT* quencher group if it is intact, with just a basal level of fluorescence is detected. As soon as IDE cleaves the peptide, the fluorescence increases because the fluorophore is no longer quenched. More precisely, if the concentration of IDE increases, the activity increases as well. Based on the findings of the HTS, Trypsin and peptide alone samples were used to measure the maximum and minimum fluorescence. As a further control, IDE was treated with 2 mM EDTA and 0.5 mM o-Phenanthroline to chelate the metals in the active site. The activity was decreased to a level similar to the peptide alone control.

3.6 Counter Screen

The counter screen was performed with the 260 enhancers and 464 attenuators (3 plates) found in primary HTS. The data were processed by using the same algorithm courtesy of Dr. Samuel Hasson as the data for HTS were processed. First, we average the values obtained of positive and negative controls IDE and DMSO (column 1 and 2), and chelated IDE and DMSO (column 23 and 24), then we set the positive control (column 1 and 2) as 100% activity and the negative control (column 23 and 24) as 0%.

For instance, if the value of column 1 set at 9,000,000 is 100% and the column 23 set at 2,000,000 is 0% and the wells treated with the drugs (260 enhancers and 464 attenuators) give a reading of 2,000,000, it means that it is 0% activation and 100% inhibition.

Furthermore, the Z'-prime values for the 3 plates are more than 0.5, which indicates that the separation between maximum and minimum controls as well as a low standard-deviation were achieved with the counter screen.

The result of the processed data show no hit from the counter screen. This indicates that there is no molecule on the 3 plates that reduces the activity of IDE by 70% or increases the activity of IDE by 50%.

4. DISCUSSION AND SIGNIFICANCE OF THIS RESEARCH STRATEGY

It is estimated that about one in four thousand children in the United States will develop mitochondrial disease before 10 years of age and that one thousand to four thousand children per year are born with a mitochondrial dysfunction. In addition mitochondrial defects contribute to diseases of aging including not only AD, but also cancer, cardiovascular diseases, diabetes, Parkinson disease, and Huntington disease, which primarily affect adults [23]. Thus the development of small molecules modulators will have an enormous impact upon a broad population and will be helpful because we can extend not only our knowledge but also the understanding of the mitochondrion's contribution to these diseases, especially AD.

The objective of Koehler laboratory at UCLA is to identify small molecules modulators that selectively attenuate hPreP activity in mammalian mitochondria, test them in biological model systems such as yeast, worms, fish, mice and cultured cells, and make them available to other research communities that work on diseases that have been suggested to have a mitochondrial component. Utilizing a HTS platform with several *in vitro* assays, they demonstrate and also confirm for the first time that the mitochondrial metalloendopeptidase hPreP is attenuated by 464 and enhanced by 260 compounds out of 88.000 compounds tested.

A key question that this study presents is, if the compounds truly activate or rather inhibit the activity of hPreP or if they are just false-positive hits. To assess the above we performed a counter (secondary) screen with recombinant IDE (a functional analogue of hPreP) against the hits from HT-primary screen in order to mark the hits that specifically affect the proteolytic

activity of hPreP. Our results show that IDE is not affected by any compounds found within the primary screen. Due to the fact that some drugs (3 plates) may fluoresce under the same excitation and emission wavelenght and therefore may give false positive-hits, we took a time zero reading so as to identify compounds with instrinsic fluorescence.

A second interesting question opened by the current discussion and results is, if hPreP's activity is peptide specific or not. On that account, the Koehler laboratory is currently working on another screen, using recombinant Matrix Processing Peptidase (MPP) and a different fluorescent peptide from the peptide we used. As MPP belongs to the same protease family as PreP and IDE; this screen is designed similarly to that for hPreP because again, our interest lies in testing small molecules that specifically alter hPreP activity.

One important point to note is that a lot more protein was used for the secondary screen with IDE than protein for the primary screen with hPreP, because of the Z' prime value. Therefore, the performance of an additional counter screen with MPP is also important not only to eliminate false-positive hits of the primary screen but also because the concentration of the inhibitors or enhancers might not be enough to inbibit or enhance IDE activity.

As a future step, additional experiments need to be performed to characterize the hits. The Koehler laboratory will take advantage of an in-gel activity assay to determine the effect of modulators on Aβ-degradation. Genarally speaking, the modulators are expected to either inhibit (attenuate the cleavage of MTS) or activate (enhance the cleavage of MTS) the proteolytic activity of hPreP. If the modulator inhibits hPreP's proteolytic activity, then they will determine its effect on degradation of Aβ as well as mitochondrial function. More precisely, the degradation of the peptide will be monitored over a time period using 16% (high-percentage) Tris-Tricine gels or mass spectrometry. Furthermore, they will perform an *in vitro* import assay with radio-labeled preprotein into yeast or mamalian mitochondria to visualize the cleavage of the MTS on 16% Tris-Tricine gel by autoradiography, because they expect, that the presequence will not be degraded after cleavage if the modulator inhibits the activity of hPreP. To learn how vertebrate development is affected, the modulators will then be applied to specific model systems such as zebrafish, and cell lines derived from patients. As the Koehler laboratory has already established zebrafish as a model for mitochondrial diseases, they further can apply the modulators (found in the previous screens) to zebrafish embryos to determine how abrogation of hPreP contributes to developmental defects. The study of small molecules that specifically attenuate hPreP will provide new tools for understanding the role of hPreP in mtiochondrial function in general and potentially the molecular events leading to AD. This research is also relevant to public health because to date no treatment has been established to stop or reverse AD. Therefore, small molecules modulators will hopefully provide a steping stool for developing new therapeutics and will also enhance the research of those researchers elucidating mechanisms of AD.

Although this study is still in its early stages, the Koehler laboratory endeavors to find specific modulators of hPreP.

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6. LIST OF FIGURES

7. LIST OF TABLES

8. LIST OF ABBREVIATIONS

A

A	Adenine
α	Alpha
ABAD	Amyloid-Beta Binding Alcohol Dehydrogenase
ABC	ATP Binding Cassette
Aβ	Amyloid Beta Peptide
AD	Alzheimer's Disease
ADP	Adenosine-Di-Phosphate
ANT	Adenine Nucleotid Translocase
APP	Amyloid Precursor Protein
APS	Adenosine Phosphosulfate
ATP	Adenosine-Tri-Phosphate
*At*PreP	*Arabidopsis thaliana* Presequence Protease

B

β	Beta
BCA	Bicin-Choninic-Acid
BSA	Bovine Serum Albumin

C

cDNA	circular Desoxyribo-Nuclein-Acid
C	Carboxyl; Cytosine
Ca^{2+}	Calcium
ccdB	control of cell death B
CHO	Chinese Hamster Ovary
Co^{2+}	Cobalt
CypD	Cyclophilin D
Cys	Cystein
Cyt*c*	Cytochrome *c*

D

Da	Dalton
Cl	Chloride
DLP1	Dynamin-Like Protein 1
DMSO	Di-Methyl-Sulf-Oxide
DNA	Desoxyribo-Nuclein-Acid

E

E	Catalytic Glutamate; Elution
EB	Elution Buffer
EDTA	Ethylene-Diamine-Tetraacetic-Acid
ER	Endoplasmatic Reticulum
ETC	Electron Transport Chain

F

$FADH_2$	reduced form from Flavin-Adenine-Dinucleotid (FAD)
Fis	Fission Protein
fwd.	forward

G

-	-

H

H (HIS)	Histidine
HCl	Hydrogen-Chloride
HEPES	4-(2-Hydroxy-Ethyl)-1-Piperazine-Ethane-Sulfonic acid
hMP1	Human Metallo-Protease 1
hPreP	Human Presequence Protease
HTS	High-Throughput Screen
4-HNE	4-Hydroxynoneal

I

IAA	Iod-Acet-Amide
IDE	Insulin Degrading Enzyme
IgG	Immuno-globulin G
IMM	Inner Mitochondrial Membrane
IMP	Inner Membrane Peptidase
IMS	Inter Membrane Space
IPTG	Iso-Propyl-β-D-Thio-Galactopyranoside

J

JM	Julia Mayer
JW	Juwina Wijaya

K

Kan	Kanamycin
Kb	Kilo Bases
kDa	Kilo-Dalton
KGDH	Keto-Gluterate De-Hydrogenase
KH_2PO_4	Potassiumdihydrogenphosphate

L

LB	Lysogeny Broth
LDL	Low Density Lipoprotein
LRP1	LDL Receptor related Protein 1

M

M	Metalloprotease
mM	mill-Molar
MCA	7-Methoxy-Coumarinyl-4-Acetyl
MCI	Management Center Innsbruck
Mfn	Mito-fusion Protein
$MgSO_4$	Magnesium Sulfate
MIP	Matrix Intermediate Processing-Peptidase
MMP	Mitochondrial Membrane Protein
MP1	Metallo-Peptidase 1
MPP	Matrix Processing Peptidase
mPTP	Membrane Permeability Transition Pore
MSSR	Molecular Screening Shared Resource
MTS	Mitochondrial Targeting Sequence
mV	mill Volt

N

N	Amino
NaCl	Sodium Chloride
NADH	Nicotinamid-Adenine-Dinucleotide
Na_2HPO_4	Sodium Hydrogen Carbonate
NaH_2PO_4	Sodium Dihydrogen Phosphate
NaOH	Sodium Hydroxide
NEB	New England Biolabs
$(NH_4)_2SO_4$	Ammonium Sulfate
Ni^{2+}	Nickel

O

OD	Optical Density
OMM	Outer Mitochondrial Membrane
OPA	Optic Autrophy Protein

P

PCR	Polymerase Chain Reaction
PDH	Pyruvate Dehydrogenase
pH	potential Hydrogenii
PITRM1	Pitrilysin Metallopeptidase 1
PMSF	Phenyl-Methane-Sulfonyl-Fluoride
PreP	Presequence-Peptidase
ProAp	Apoptotic Factors

Q

-	-

R

rev.	reverse
RFU	Relative Fluorescence Units
RNA	Ribo-Nuclein-Acid
ROS	Reactive Oxygen Species

S

SDS-PAGE	Sodium-Dodecyl-Sulfate - Poly-Acrylamide-Gel-Electrophoresis

T

T	Threonine
TBE	TRIS Borate EDTA
TCA	Tri-Carboxylic-Acid
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TIM	Translocon of Inner Membrane
TM	Transmembrane
TOM	Translocon of Outer Membrane
TRIS (Tris)	Hydroxymethylaminoethane
Tyr	Tyrosine

U

UCLA	University of California, Los Angeles
UQ	Ubiquinone

V

V	Volt
VDAC	Voltage-Dependent-Anion-Channel

W

-	-

X

X	any amino-acid

Y

γ	Gamma
YT	Yeast-Tryptone (also abbreviated as S.O.C)

Z

-	-

9. APPENDIX

9.1 PCR Protocol

The following general procedure is suggested as a starting point when using AccuPrime™ *Pfx* DNA Polymerase in any PCR amplification.

Step 1: The following components were added to an autoclaved micro centrifuge tube on ice:

Component	**Volume [µL]**	**Final Concentration**
10x Accu Prime™ *Pfx* Reaction mix	5	1x
Primer mix (10 µM each)*	1.5	0.3 µM each
Template DNA (10 pg – 200 ng)	1	As required
AccuPrime™ *Pfx* DNA Polymerase**	0.5	1.5 units
Autoclaved, distilled water	To 50	

* AccuPrime™ *Pfx* DNA Polymerase will not function in reactions that contain dUTP either in the primers or in the dNTP mix.

** For most targets, 1 unit is optimal. Higher concentrations may be inhibitory. More enzyme may be required for longer targets (>3 kb).

Step 2: The tubes were caped and centrifuged briefly to collect the contents.

Step 3: The templates were denatured for 2 minutes at 95°C. 25-35 cycles of PCR amplification were performed as follows:

Three-step cycling	Two-step cycling
Denature: 95°C for 15 seconds	Denature: 95°C for 15 seconds
Anneal: 55-64°C for 30 seconds	Extend: 68°C for 1 minute per kb
Extend: 68°C for 1 minute per kb	

Note: Two-step cycling can be used for long primers with high T_M

Step 4: The reaction was maintained at 4°C after cycling. The samples were stored at -20°C until use.

Step 5: The products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Note: This is just a cut-out of the whole PCR Protocol [23]

9.2 PCR Cleaning Spin Protocol

Step 1: 2 volumes Buffer GC were added to the PCR reaction and mixed completely by vortexing. The tubes were spinned briefly to collect any drops from the inside wall and tube lid.

Step 2: Up to 700 µL DNA/Buffer GC mixture were transferred to a spin column with a collection tube and centrifuged at 13,000 x g for 1 minute at room temperature. The flowthrough was discarded and the column was put back to the collection tube. This step was repeated (to process the remaining solution).

Step 3: 500 µL DNA Wash Buffer were added to the column and centrifuged at 13,000 x g for 1 minute at room temperature. The flowthrough was discarded. Ethanol has been added to DNA Wash Buffer as instructed before use.

Step 4: Step 3 was repeated.

Step 5: The empty column was centrifuged at top speed for 1 minute because the removal of ethanol is critical for DNA elution. (Optional: Spin the empty column in the collection for 1 minutes at top speed).

Step 6: The column was placed into a clean 1.5 mL micro centrifuge tube and 30-50 µL Elution Buffer were added to the column. An incubation at room temperature for 1 minute followed. To elute the DNA a further centrifugation step at 13,000 x g for 1 minute was performed [25].

9.3 TOPO® Cloning Protocol

PERFORMING THE TOPO® CLONING REACTION

The procedure below was used to perform the TOPO® Cloning reaction. The TOPO® Cloning reaction was set up using the reagents in the order shown.

Note: The blue color of the pCR®II-Blunt-TOPO® vector solution is normal and is used to visualize the solution.

Reagent*	**Chemically Competent *E.coli***
Fresh PCR product	0.5 to 4 µL
Salt Solution	1 µL
Water	add to a final volume of 5 µL
pCR®II-Blunt-TOPO®	1 µL
Final Volume	**6 µL**

*All reagents were stored at -20°C. Salt solutions and water were stored at room temperature.

1. The reaction was mixed gently and incubated for 5 minutes at room temperature (22-23°C)

   Note: For most applications, 5 minutes will yield plenty of colonies for analysis. Depending on your needs, the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (>1kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time will yield more colonies.
2. The reaction was placed on ice and prepared to Transforming One Shot® Competent Cells.

   Note: You may store the TOPO® Cloning reaction at -20°C overnight.

TRANSFORMING ONE SHOT® COMPETENT CELLS

Introduction

After the TOPO® Cloning reaction was performed, the pCR®-Blunt II-TOPO® construct was transformed into competent *E.coli* provided with the kit.

Materials Supplied by the User

In addition to general microbiological supplies (e.g. plates, spreaders), the following reagents and equipment were used:

- TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, Step 2 (Performing the TOPO® Cloning Reaction).
- S.O.C. medium (included with the kit)
- 42°C water bath or electroporator with 0.1 cm cuvettes
- 15 mL snap-cap plastic tubes (sterile) (electroporation only)
- LB plates containing 50 µg/mL kanamycin or Low Salt LB plates containing 25 µg/mL Zeocin™ (use two plates per transformation)
- 37°C shaking and non-shaking incubator

Preparing for Transformation

For each transformation one vial of competent cells and two selective plates were used.

- The water bath was equilibrated to 42°C.
- The vial of S.O.C. medium was brought to room temperature.
- LB plates containing 50 µg/mL kanamycin or 25 µg/mL Zeocin™ were warmed at 37°C for 30 minutes
- 1 vial of One Shot® cells was thawed on ice for each transformation.

One Shot® Chemical Transformation

1. 2 µL of the TOPO® Cloning reaction were added (Step 2, Performing the TOPO® Cloning Reaction) into a vial of One Shot® Chemically Competent *E. coli* and mixed. **Do not mix by pipetting up and down.**
2. The reaction was incubated on ice for 5 to 30 minutes.
   Note: Longer incubations on ice do not seem to have any effect on transformation efficiency. The length of the incubation is at the user's discretion.
3. The cells were heat-shocked for 30 seconds at 42°C without shaking.
4. The tubes were transferred to ice (immediately).
5. 250 µL of room temperature S.O.C. medium were added.
6. The tube was caped tightly and shake horizontally (200rpm) at 37°C for 1 hour.
7. 10-50 µL from each transformation were spread on a pre-warmed selective plate and incubated overnight at 37°C. To ensure even spreading of small volumes, 20 µL of S.O.C. medium was added. Two different volumes were plated to ensure that at least one plate will have well-spaced colonies. The plates were incubated over night at 37°C.
8. 10 colonies were picked for analysis.

Note: This is just a cut-out of the whole TOPO® Cloning Protocol [16].

9.4 EZgene™ Miniprep Protocol

Step 1: 1-3 mL (high-copy-number plasmid) of overnight culture were harvested by centrifugation for 1 minute at 10,000 x g in a tabletop centrifuge. The supernatant was poured off and the inverted tube was blotted on a paper towel to remove excess media.

Step2: 250 µL Buffer A1 were added and the bacterial pellet was completely resuspended by pipetting or vortexing (complete resuspension is critical for optimal yields).

Step 3: 250 µL Buffer B1 were added, mixed gently but thoroughly by inverting 5-8 times (without vortexing), and incubated for approximately 5 minutes until the solution became slightly clear (don't incubate longer than 5 minutes because over-incubating causes genomic DNA contamination and plasmid damage). **Note:** Precipitation forms in Buffer B1 below room temperature. Warm the bottle at 37-55°C to dissolve.

Step 4: 300 µL Buffer N1 were added, mixed immediately by inverting 5 times and vortexed for 2 seconds (It is critical to mix the solution well, if it appears conglobated or viscous, more mix is required).

Step 5: The samples were centrifuged at 13,000 rpm (14,000-18000 g) for 10 minutes at room temperature and the clear supernatant was carefully transferred into a DNA column with the collection tube (do not bring any floating material into the tube and if the lysate doesn't appear clear, reverse the tube angle, centrifuge for 5 minutes and then transfer the lysate to the DNA column).

Step 6: The samples were centrifuge at 13,000 rpm (14,000-18,000 g) for 1 minute at room temperature and the flowthrough liquid in the collection tube was discarded.

Step 7: 500 µL DNA Wash Buffer were added into the spin column and centrifuged at 13,000 rpm (14,000-18,000 g) for 1 minute at room temperature. The flowthrough was discarded. Absolute ethanol (96-100%) has been added to DNA Wash Buffer before use. (Optional: Add 500 µL DNA Wash Buffer into the spin column, centrifuge at 13,000 rpm (14,000-18,000 g) for 20 seconds at room temperature. Discard the flowthrough).

Step 8: The spin column was put into the collection tube and centrifuged for 1 minute at 13,000 rpm (14,000-18,000 g). The flowthrough was discarded.

Step 9: The column was centrifuged for another minute (It is critical to remove residual ethanol for optimal elution in the following step).

Step 10: The spin column was moved into a clean sterile 1.5 mL micro centrifuge tube, 50µL Elution buffer were added into the column and let stand for 1 minute. The DNA was eluted by centrifugation at 13,000 rpm (14,000-18,000 g) for 1 minute [26].

9.5 Gel Extraction Spin Protocol

Step 1: The DNA fragment was excised from the agarose gel and weight in a 1.5 mL micro tube (A gel slice of 100 mg approximately equals to 100 µL). 1 volume Buffer GC was added to the 1.5 mL microtube and the mixture was incubated at 55-60°C for 8 minutes with mixing the tube by tapping the bottom of the tube every 2 minutes till the gel has melted completely. The tube was cooled to room temperature.

Step 2: Up to 700 µL DNA/Buffer GC mixture were added to a spin column with a collection tube and centrifuged at 13,000 x g for 1 minute at room temperature. The flowthrough was discarded and the column was put back to the collection tube. This step was repeated to process the remaining solution.

Step 3: 500 µL DNA Wash Buffer were added to the column and centrifuged at 13,000 x g for 1 minute at room temperature. The flow through was discarded. Ethanol has been added to DNA Wash Buffer as instructed before use.

Step 4: Step 3 was repeated.

Step 5: The empty column was centrifuged with the lid open at top speed for 1 minute to remove the residual ethanol in the column since the removal of residual ethanol is critical for DNA elution. (Optional: Spin the empty column in the collection for 1 minute at top speed).

Step 6: The column was placed into a clean 1.5 mL micro centrifuge tube and 30-50 µL Elution Buffer were added to the column. The samples were incubated at room temperature for 1 minute and centrifuged at 13,000 x g for 1 minute to elute the DNA. (Optional: Re-apply the eluate to the column, elute as Step 7. This will increase DNA yield) [27].

9.6 Transforming Chemically Competent Cells Protocol

Chemical Transformation Procedure

The instructions provided below are for general use. Specific instructions for particular applications such as Zero Blunt® PCR Cloning are provided in the manual for that kit.

Step1: The vials containing the ligation reactions were centrifuged and place on ice.

Step2: One 50 µL vial of OneShot® cells was thaw on ice for each ligation/transformation.

Step 3: 1 to 5 µL of each ligation reaction were pipetted directly into the vial of competent cells and mixed by tapping gently. **Do not mix by pipetting up and down**. The remaining ligation mixtures were stored at -20°C.

Step 4: The vials were incubated on ice for 30 minutes.

Step 5: An incubation for exactly 30 seconds in the 42°C water bath followed without mixing or sharking.

Step 6: The vials were removed from the 42°C bath and placed on ice.

Step 7: 250 µL of pre-warmed S.O.C medium were added to each vial. (S.O.C is a rich medium; therefore sterile technique must be practiced to avoid contamination).

Step 8: The vials were placed in a micro centrifuge rack on its side and secured with tape to avoid loss of the vials. The vials were shaken at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.

Step 9: 20 µL to 200 µL from each transformation vial were spread on separate, labeled LB agar plates.

Step 10: The plates were inverted and incubated at 37°C overnight.

Step 11: Colonies were selected and analyzed by PCR, and sequencing [28].

9.7 SDS-PAGE, Western Blot and Coomassie

Preparing Protein Gel

1. Gel equipment was assembled.
2. The separating gel (*8% gel: 6.66 mL Acrylamid/BIS (30/0.8%), 6.25ml Buffer (Tris/Cl 1.5 M, pH 8.8), 12.125 mL ddH_2O, 0.02 mL TEMED, 0.20 mL APS (10%))* and the stacking gel *(2.4 mL Acrylamid/BIS (30/0.8%), 4.0 mL Buffer (Tris-Cl 0.5 M, pH 6.8), 9.4 mL ddH2O, 0.02 mL TEMED, 0.20 mL APS (10%)*) were prepared according to the size of our protein. **Note:** Proceed quickly after adding TEMED and APS. Do not add these ingredients before pouring the gel.
3. The separating gel was poured between the two glass-plates and coated with a 0.5 cm layer of ethanol for 30 minutes.
4. The ethanol layer was removed and the separating gel was rinsed with water.
5. The stacking gel was poured above the separating gel. The comb was pressed into the gel (avoid bubbles).
6. The comb was removed after 30 minutes and the glass-plates were put with the gel into the gel cassette.
7. The gel cassette was filled with Running Buffer (*ingredients: 25 mM Tris, 190 mM Glycine and 0.1% SDS*).
8. The gel was loaded with the templates (avoid bubbles).
9. The gel was run.

Coomassie

1. The glass-plates with the gel were taken out of the gel cassette.
2. The top glass-plate was removed carefully and the separating gel was peeled away.
3. The stacking gel was put in a tray and rinsed with water for 1 minute.
4. The water was discarded, the gel as covered with Coomassie Blue stain and shaken for 30 minutes.
5. The Coomassie Blue stain was discarded
6. The gel was covered with Destain solution and the tray was replaced on the shaker for 12 hours.
7. The Destain solution was changed for three times after the first 3 hours.
8. The gel was visualized and dried.

Western Blot

1. See Coomassie steps 1 and 2.
2. The stacking gel was put in a tray and equilibrated in Blotting Buffer (*ingredients: 25 mM Tris, 190 mM Glycine and 20% v/v Methanol*) for a couple of minutes.
3. Blotting sandwich: A container was filled with blotting buffer and the plastic gel holder cassette was put on the buttom with the black side in it. The plastic gel holder cassette was opened and a wet fiber-pat was laid on the black side. The wet blotting paper was

placed on the fiber-pat (role out air and avoid bubbles). The gel was carefully put on the blotting-paper and covered with the nitrocellulose membrane (move the membrane as little as possible). A second wet blotting-paper was placed on the membrane as well as a wet fiber-pat on the blotting-paper. The sandwich was closed by putting the white side of the plastic gel holder cassette down (Make sure that the white clip of the cassette is closed the right way).

4. The blotting sandwich was put into the blotting apparatus (black to black and white to white) and the tank was filled with blotting buffer.
5. The blotting apparatus was attached to power supply and the blot was run (1 A, 1 h).
6. The cassette was removed out of the blotting apparatus, the membrane was taken out of the sandwich by using a pincer and put into a staining tray.
7. The membrane was covered with Ponseau solution and the tray was put on a shaker for a couple of minutes.
8. All used apparatuses were washed.
9. The Ponseau solution was discarded and the colored bands (PreStained *RecProtein Ladder EZ-RUN™ Fischer Bio Reagents*) were marked with a pen.
10. The membrane was covered with Blocking Buffer (*ingredients: 5% dry milk, 0.05% Tween-20 in PBS (Phosphate buffered saline) or TBS (Tris buffered saline)*) and rocked at room temperature for 2 hours.
11. The Primary Antibody solution was prepared (*14 mL PBST (1x PBS and 0.2% Tween-20 and 7 µL of MS mAB to 6xHIS, tag® 100 µg, (1 mg/mL) His-Antibody*).
12. The Blocking Buffer was discarded; the membrane was covered with the Primary Antibody solution and rocked for 2 hours.
13. The membrane was washed with PBST three times for 10 minutes/wash.
14. The Secondary Antibody solution (*14 mL of a 1:1 mixture of Blocking Buffer and PBST and 1.4 µL Goat anti-mouse IgG HRP from Pierce for mouse IgG antibodies*) was prepared.
15. The membrane was covered with Secondary Antibody Solution and shaken at room temperature for 45 minutes.
16. Step 13 was repeated.
17. The membrane was washed with PBS two times for 10 minutes/wash.
18. ECL was performed: A 1:1 solution from Super Signal® West Pico stable Peroxide solution and Super Signal® West Pico Luminol/Enhancer solution was prepared. The solution was placed on the semi-dry membrane (allow the reaction to proceed for at least 3 minutes). Afterwards the membrane was wrapped in the sheet protector.
19. The membrane was exposed to a film.