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KATJA SRPAN

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KATJA SRPAN

IZBOR SPECIFIČNIH scFv FRAGMENTOV ZA E-KADHERIN IZ BAKTERIOFAGNE PREDSTAVITVENE KNJIŽNICE

SELECTION OF E-CADHERIN SPECIFIC scFv FRAGMENTS FROM A PHAGE-DISPLAYED PEPTIDE LIBRARY

DIPLOMSKA NALOGA

Ljubljana, 2013

This Master's Thesis was performed at the French Alternative Energies and Atomic Energy Commission (Commissariat à l'énergie atomique et aux énergies alternatives) in Fontenay aux Roses, and the Faculty of Pharmacy, University of Ljubljana. I worked under mentorship of Prof. Dr. Irena Mlinarič-Raščan, and co-mentorship of Dr. Nicolas Ugolin.

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STATEMENT

I hereby declare that I have performed and written this Master's Thesis solely by myself under the mentorship of Prof. Dr. Irena Mlinarič-Raščan, and co-mentorship of Dr. Nicolas Ugolin.

Ljubljana, July 2013

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ABSTRACT

E-Cadherin is a protein involved in cell-cell adhesion. Its disappearance is known as one of the main molecular events responsible for epithelial-to-mesenchymal transition (EMT), when cancer cells of a primary tumor become malignant and invasive. Cells that undergo EMT detach from the primary site and enter the vascular system. They are known as circulating tumor cells (CTCs). They have the potential of seeding metastases in distant organs.

The transition from epithelial cells to mesenchymal is never complete; therefore newly formed CTCs express beside mesenchymal markers also some epithelial ones, including E-Cadherin. Because normal blood cells do not express E-Cadherin, it can be used for non-invasive detection of those potentially malignant cells in blood.

The main objective of the present work was to develop a method for non-invasive rapid detection of CTCs in blood by phage-display selection of high affinity scFv antibody fragments specific for E-Cadherin.

First we prepared recombinant E-Cadherin by Gateway cloning technique in five different expression clones. In order to achieve best protein expression in bacteria, several *E.coli* strains were tested. Final product was purified with Ni-NTA columns by protein's 6xHis-tag.

Wild type protein was extracted from human breast carcinoma cell line. For better specificity it was enzymatically degraded to smaller particles and sufficiently purified.

Finally scFv antibody fragments were selected by biopanning on recombinant E-Cadherin that was immobilized on magnetic beads. Sixteen randomly selected scFvs were immobilized on adhesive slides and incubated with recombinant and wild type E-Cadherin, labeled with fluorescent dye, in parallel. Affinity of selected fragments was defined by the intensity of fluorescent signal of the fluorescent scanner. Twelve out of sixteen selected fragments showed good affinity for both types of E-Cadherin. There was no big difference between wild type and recombinant protein. According to the results we can conclude that any of those twelve scFvs, positive for E-Cadherin could be used for further development of the method for detection of CTCs.

RAZŠIRJENI POVZETEK

Protein E-kadherin je pomemben sestavni element celičnega stika. Njegova odsotnost je ključna za začetek in ohranjanje epitelno-mezenhimskega prehoda (EMP), v katerem se posamezne celice iz primarnih epitelnih tumorjev, preko številnih spontanih mutacij, spremenijo v maligne, invazivne in metastazne. Le-te se zaradi odsotnosti celičnih stikov odcepijo ter vstopijo v krvo-žilni sistem, preko katerega lahko dostopajo do drugih organov, kjer lahko zasejejo metastaze. Imenujemo jih cirkulirajoče tumorske celice (CTC).

Ker EMP ni nikoli končen proces, imajo nastale CTC ob novo pridobljenih mezenhimskih markerjih še vedno nekaj epitelnih, med drugimi tudi E-kadherin. Normalne krvne celice ne izražajo E-kadherina, zatorej bi ga lahko uporabili za določitev potencialno malignih celic v krvi.

Glavni namen tega diplomskega dela je bil razvoj metode za detekcijo CTC. S pomočjo tehnologije bakteriofagnega prikaza smo želeli iz bakteriofagne knjižnice izbrali scFv fragmente protiteles, specifične za E-kadherin.

Sprva smo z Gateway tehnologijo pripravili rekombinanten E-kadherin v večih vektorjih. Vse smo izrazili v različnih sevih bakterije *E.coli* ter določili pufer, v katerem je izražen protein najbolje topen. Glede na dobljene rezultate smo izrazili večjo količino proteina, ki smo ga prečistili z Ni-NTA kolono.

Nativni protein smo ekstrahirali iz celičnih linij humanega karcinoma dojke. Za boljšo specifičnost smo ga z encimsko razgradnjo razgradili na manjše delce ter ga prečistili z ekstrakcijo iz SDS-PAGE gela in z imunoprecipitacijo.

Rekombinantni protein, imobiliziran na magnetnih kroglicah, smo uporabili za izbor scFv fragmentov iz bakteriofagne predstavitvene knjižnice. Fage, ki antigena niso prepoznali, smo sprali, medtem ko smo tiste, ki so tvorili kompleks s predstavljeno tarčo, eluirali in namnožili v *E.coli*. Izbrane scFv fragmente smo nanesli na stekelca ter jih vzporedno inkubirali z nativnim in rekombinantnim E-kadherinom, označenim s fluorescentnim barvilom. Rekombinantni je služil za kontrolo. Nastale komplekse smo detektirali na fluorescentnem skenerju. Intenziteta signala je odražala afiniteto scFv fragmenta za E-Cadherin.

Po zgoraj opisanem postopku smo uspešno izbrali dvanajst scFv fragmentov, specifičnih za E-kadherin. Vseh dvanajst je pokazalo močno afiniteto za obe tarči, razlike med rekombinantnim in nativnim proteinom pa niso bile opazne. Vsak od njih bi bil lahko uporabljen za nadaljnji razvoj metode za detekcijo CTC.

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APS	Ammonium Persulfate
BSA	Bovine serum albumin
cDNA	Complementary DNA
CTC	Circulating tumor cell
CTM	Circulating tumor microemboli
dH_2O	Purified water
DMP	Dimethyl pimelimidate
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-to-mesenchymal transition
FDA	Food and drug administration
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IPTG	Isopropylthio-β-galactoside
LB	L Broth, also called Luria Bertani medium
MET	Mesenchymal-to-epithelial transition
NP-40	Nonyl phenoxypolyethoxylethanol 40
OD	Optical density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonylfluoride
PVDF	Polyvinylidene difluoride membrane

RPM	Revolutions per minute
RT	Room temperature
scFv	Single chain F variable, recombinant antibody fragment
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis
ssDNA	Single stranded DNA
TBE	Tris/Borate/EDTA buffer
TBS	Tris-buffered saline
TEMED	N,N,N´,N´-tetramethylethylenediamine
TTBS	Tris-buffered saline with Tween20
V_H	Variable heavy chain
V_L	Variable light chain
WB	Western Blot
WT	Wild Type

1. INTRODUCTION

1.1. Circulating tumor cells (CTCs)

The development of metastases starts during the early stages of solid carcinoma progression, when some cells undergo epithelial-to-mesenchymal transition – EMT (described below) and become malignant cancer cells that detach from a primary neoplasm, invade through surrounding tissue and enter the circulation. These cells, called circulating tumor cells (CTCs), are capable of seeding metastasis in distant organs [1][2]. Some of them aggregate together into clusters called circulating tumor microemboli (CTM) [3].

The presence of CTCs in blood is an important indicator of cancer progression. They are commonly present in the blood of patients with malignant cancer, but are rarely found in the blood of healthy population or those with benign disease [4]. As such they could be a strong prognostic marker for patients with metastasis and could provide information about therapeutic efficiency and the tumor profile that could be useful for personalized treatments [5]. It is expected that in future, investigation of CTCs may replace invasive tissue biopsies for defining tumor and metastatic characteristics in much earlier stages than nowadays [2].

However, metastasis is highly inefficient process and CTCs are rare events in vascular system. It is estimated that there are only few CTCs among billions of normal blood cells (Figure 1) [5]. In order to become invasive, cells from the primary tumor have to undergo a significant rate of spontaneous mutations (during EMT) [6]. From model systems it has been estimated that approximately one million of such cells per gram of tumor enter the bloodstream daily (Figure 2). 85% of them die in first few minutes due to anoikis (apoptosis induced by lack of correct cell-ECM attachment), forces of the circulation or immune recognition. Around 2-3% are capable of forming micrometastasis while only 0.01% proliferate into macrometastasis [3], [7].

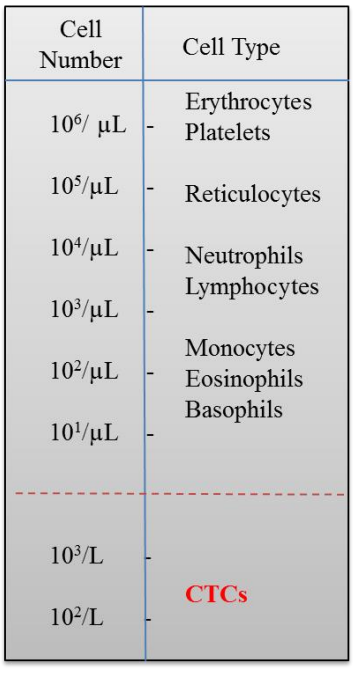

Figure 1: Number of cells in blood. In 10 ml of blood there are only few CTCs hidden among billions of erythrocytes and millions of lymphocytes [3], [7], [66], [67].

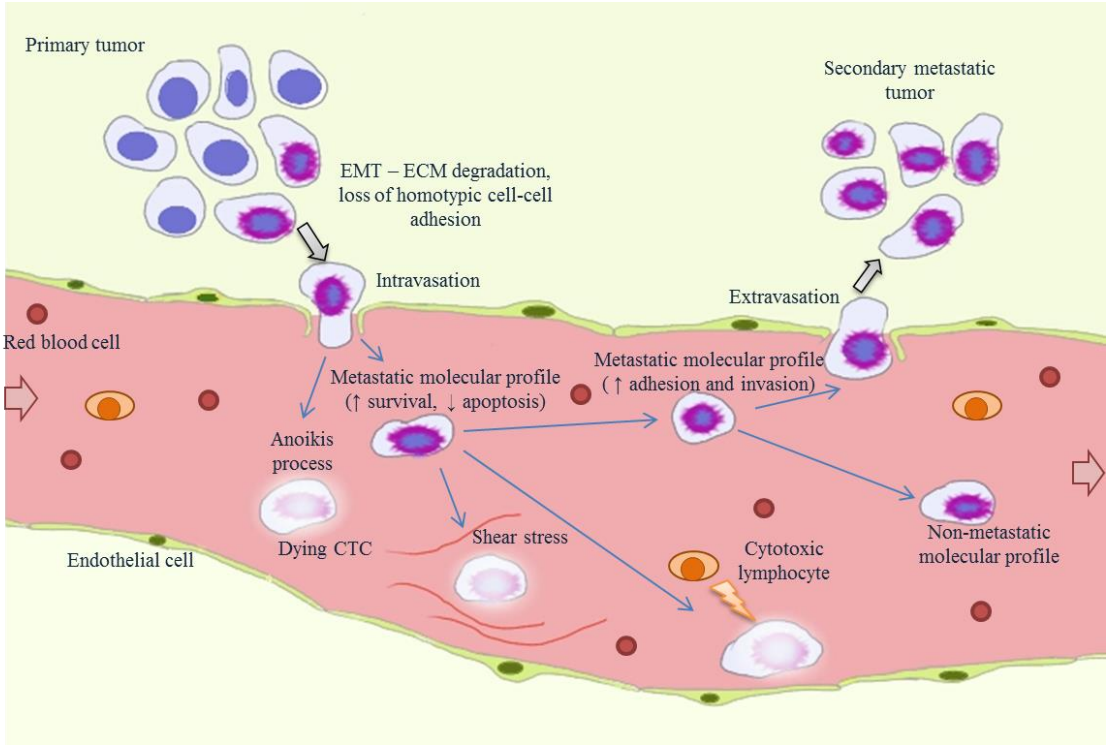

Figure 2: Circulating tumor cells. Cells that undergo epithelial-to-mesenchymal transition (EMT) leave the primary tumor and enter the vascular system as CTCs. These cells have to survive various challenges in a bloodstream, such as anoikis, forces of a blood flow and immune recognition. For that they have to acquire appropriate anti-apoptotic factors. Most of them are gained during EMT. Only few CTCs are able to found metastasis at distant sites. [based on 7].

Because of their limited number, genomic instability and extreme heterogeneity, detection of CTCs presents a great technological challenge. Detection methods should be very specific, sensitive, standardized, and highly reproducible [2]. However, in contrast to invasive procedures like biopsies, CTCs could be easily obtained from peripheral blood. This, far more acceptable procedure for patients and their physicians, called liquid biopsy, could be done safely and frequently [8].

So far, several approaches have been investigated for CTCs detection. Only CellSearch® has been approved by FDA for predicting the prognosis and monitoring the clinical outcome of certain cancers [8].

1.2. Epithelial–to-mesenchymal transition

Epithelial to mesenchymal transition (EMT) describes a reversible multistage cellular process, where a spectrum of distinct genetic and epigenetic inter- and intracellular alterations convert fully differentiated epithelial cells into poorly differentiated mobile mesenchymal cells [9].

Epithelial cells form a monolayer of cells that are tightly connected laterally in a cobblestone-like structure by specialized membrane structures, such as tight junctions, adherens junctions, desmosomes and gap junctions. They play a major structural and functional role in organs. In an intact epithelium they have apical-basolateral polarization through their anchoring to the basal lamina at the basal surface. They are motile in the frames of epithelial layer. On the other hand, spindle-shaped mesenchymal cells have a much more relaxed organization and far lower level of focal cell junctions. They can individually migrate through layers of extracellular matrix (ECM) [3], [10], [11].

During EMT epithelial cells lose their polarity and cell-cell junctions, undergo remarkable cytoskeleton remodeling and acquire mesenchymal characteristics, such as increased cell migration and invasion [12].

This process is important, because it is strongly linked to the life-threatening malignant progression of epithelial cancers [13]. Initiation and early growth of primary carcinomas happen due to a hyper-proliferation of epithelial cells and angiogenesis [14]. But for invasion through adjacent cell layers, carcinoma cells need to break away from the primary tumor. In order to be able to do that, benign tumors cells start transformation to malignant, metastatic and invasive phenotype [13]. Besides inducing cancer cell motility and invasiveness, EMT also enables cancer to avoid apoptosis, anoikis, senescence and general immune defense. It has a crucial role in generation and maintenance of cancer stem cells [9].

When EMT-derived migratory cells that survive the transport through circulation want to invade a distant organ, epithelial-like cells should be re-established from mesenchymal cells via the reverse process, a mesenchymal-to-epithelial transition (MET) in order to regain the ability to proliferate as a secondary tumor (Figure 3) [3].

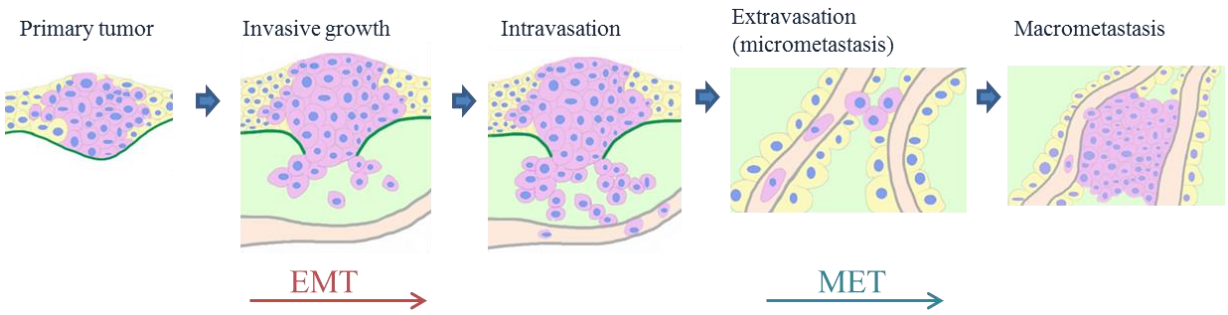

Figure 3: Contribution of epithelial-to–mesenchymal transition (EMT) to cancer progression [15]**.** A series of different sequential stages are involved in tumor metastasis. Epithelial cells first lose their polarity; they detach from basement membrane and proliferate uncontrollably. Epithelial-to-mesenchymal transition (EMT) (red arrow) facilitates tumor malignancy. Cells undergo transmigration through basal membranes, surrounding tissues and blood vessels endothelium. Those that survive the bloodstream can exit vascular system and form micro- and macrometastasis at distant sites (blue arrow) through mesenchymal-to-epithelial transition (MET), a reversion to an epithelial phenotype [based on 3].

Various molecular processes, such as up-regulation of ECM degrading enzymes, production of growth factors, activation of transcription factors, down-regulation or expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs are responsible for initiation and maintenance of EMT (Figure 4) [1], [4].

In many carcinomas tumor itself or tumor-associated stroma up-regulate the proteolitic activity of matrix metalloproteases (MMPs) and produce different growth factors, including transforming growth factor (TGF-β) [16], hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin-like growth factors (IGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), and tumor necrosis factor α (TNFα) [9], [13]. In most cases growth factors signaling promotes the expression of several transcription factors (Snail [17], Slug [18], Sip1 [19], Zeb [20] and Twist [21] to name a few) [14], which could contribute to EMT pleiotropically. They have a potential to down-regulate the expression of genes that are important for the maintenance of polarity and organization of epithelial cells, such as E-Cadherin, claudins, occludins, and ZO-family members [9]. The implementation of EMT is a result of series of intracellular signaling networks involving, among other signal-transducing proteins, ERK, MAPK, PI3K, Akt, Smad, RhoB, Wnt/β-catenin, Notch, lymphoid enhancer binding factor (LEF), Ras, and c-Fos as well as surface proteins such as β4 integrins, α5β1 integrin, and αBβ6 integrin [11], [14].

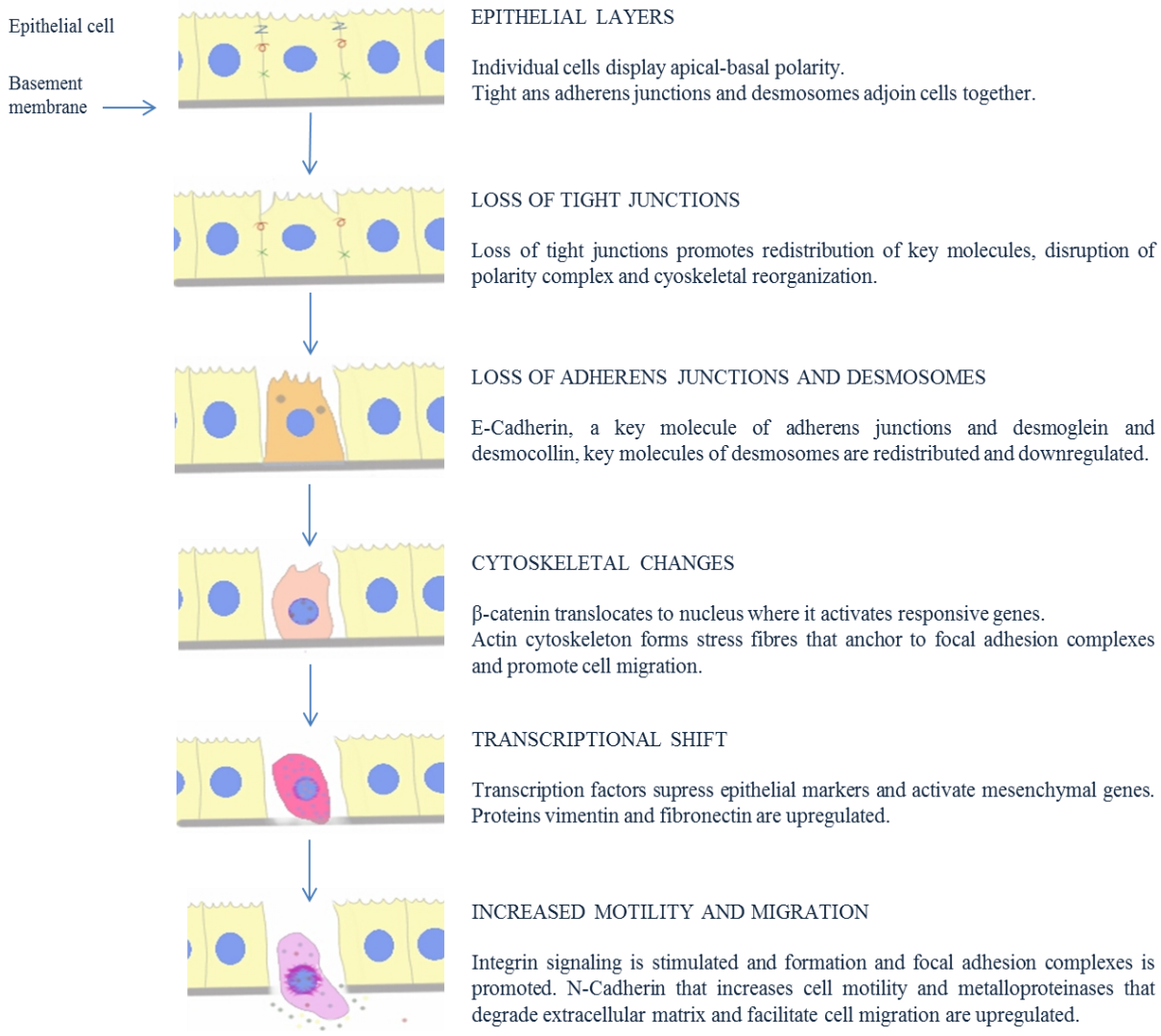

Figure 4: The progressive stages of EMT. After epithelial cells lose their tight and adherent junctions and desmosomes, they go through cytoskeletal changes. Transcription factors suppress epithelial and activate mesenchymal genes. Final up-regulation of N-Cadherin increases cell motility while metalloproteinases degrade extracellular matrix and facilitate cell migration [based on 22].

MicroRNAs are other components that influence the tumorigenesis by acting as oncogenes or tumor-suppressors when being down- or up-regulated respectively. A prime example is miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR429) that decreases the expression of Zeb-1 and Zeb-2 transcription factors resulting in increased expression of E-Cadherin. Conversely, Zeb-1 and Zeb-2 repress the expression of the miR-200 gene clusters. Besides that, many other transcription factors involved in EMT are regulated by different miRNA. MiR-34a represses Snail-1 while miR-192 and miR138 repress Zeb-2 to suppress

EMT. Furthermore, miR-9 represses gene expression of E-cadherin that activates β-catenin signaling and the up-regulation of mesenchymal and angiogenic genes expression. MiR-21 induces TGF-β and therefore influences EMT [9], [14].

Moreover, EMT induction is facilitated by the disruption of cell-cell adherens junctions mediated by cadherins and the cell-ECM adhesions mediated by integrins [11], [23].

Intumoral hypoxic (low oxygen) conditions that appear due to rapid and uncontrolled cell division promote EMT by increased expression of hypoxia-inducible factor 1α (HIF1α). Hypoxia also upregulates expression of Snail while inhibiting Snail's endogenous inhibitor - GSK3β by phosphorylation resulting in reduction of E-Cadherin [24].

To summarize, recent gene expression profiling experiments in several cellular experimental systems claim that gene expression of hundreds of genes changes considerably during EMT [9], [14], [25].

However, it remains a challenge to observe EMT in human carcinomas. Beside the transient and reversible nature of EMT during carcinoma invasion and metastasis, there is only a small minority of carcinoma cells from primary tumors undergoing an EMT. The changed gene expression in such cells can be hidden by the majority of non-malignant cells [21] .

Nevertheless it should be noted that the role of EMT in the progression of carcinomas and their metastatic dissemination is still under discussion. In most carcinomas a full EMT is rarely observed. It is rather manifested by a wide spectrum of phenotypic changes of invasive cells, where certain proteins specific for either epithelial or mesenchymal cells may be expressed at a variable level [3]. Therefore epithelial markers on invasive cells are commonly used for detection and characterization of metastatic epithelial cancers [13]. It also remains unclear if metastatic dissemination of non-epithelial cancer could be explained by the same phenomena and molecular mechanisms [14].

1.2.1. EMT subtypes

Besides above described carcinoma promotion, EMT plays a critical role also in metazoan embryogenesis, organ development and tissue repair in adult [12].

EMT can be classified into three subtypes, depending on the biological and functional setting in which it occurs.

Type 1 is critical during implantation, embryogenesis and organ development. From primitive epithelium (epiblast) EMT generates mesenchymal cells which can undergo mesenchymal to epithelial transition (MET) to form secondary epithelia. It is essential for gastrulation, mobile neural crest, and organ development (Figure 5).

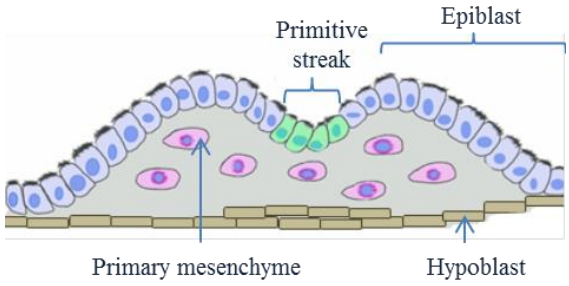

Figure 5: Type 1 EMT. The epiblast (primitive epithelium) generates primary mesenchyme via EMT. A primary mesenchyme could be re-induced by MET to form secondary epithelia. Secondary epithelia may go through further differentiations and undergo subsequent EMT to form other types of connective tissues [based on 14].

Type 2 is associated with the formation of fibroblasts during wound healing, tissue regeneration, inflammation and fibrosis. Organ fibrosis is mediated by inflammatory cells, fibroblasts and ECM components. During inflammation and fibrosis EMT could be reactivated for the extended period of time. If the primary cause of inflammation is not removed, the affected organ could be destroyed (Figure 6).

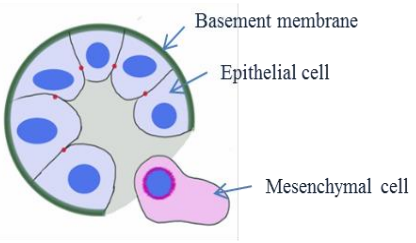

Figure 6: Type 2 EMT. Mesenchymal fibroblasts are formed during EMT from epithelial cells. They are essential for wound healing, tissue regeneration and inflammation process [based on 14].

Type 3 is characterized by the transformation of epithelial tumor cells into invasive metastatic mesenchymal cells that underlie cancer progression. By EMT neoplastic cells become motile and invasive, and leave the primary epithelial tumor site, which may lead to cancer invasion, systemic cancer cell dissemination, and metastasis. A distal mesenchymal-to-epithelial transition - MET event promotes the formation of a secondary tumor and cancer progression in other organs (Figure 7). [9], [14], [26]

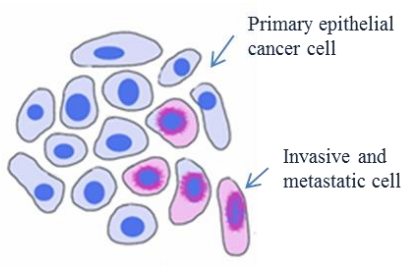

Figure 7: Type 3 EMT. The secondary epithelia can transform into cancer cells that may convert into malignant invasive metastatic cells through EMT [based on 14].

1.3. E-Cadherin

Epithelial (E)-Cadherin protein, known also as uvomorulin, L-CAM, cell-CAM 120/80 or Arc-1 [1], is a fundamental caretaker of epithelial integrity [10] that forms the key functional component of adherens junctions [1].

1.3.1. Role of E-Cadherin

Normal E-Cadherin expression and function are essential for the induction and preservation of polarity and differentiated epithelia during embryonic development [1]. E-Cadherin is also involved in maintenance and homeostasis of a normal adult epithelial tissue [27]. During tumorigenesis it plays an important role in suppressing invasion and metastasis of tumor cells [28].

1.3.2. E-Cadherin gene and protein structure

The human E-Cadherin gene CDH1 is located on chromosome 16q22.1 [29]. It consists of 16 exons separated by 15 introns [27].

CDH1 encodes a 120 kDa single-span calcium-dependent transmembrane glycoprotein with a large extracellular domain, a single transmembrane segment and a short cytoplasmic domain (Figue 8) [23], [27]. The N-terminal extracellular domain contains five tandem repeats of a 100-residue-amino-acid-motif with many adhesive sites that can form adherens junctions through homotypic interactions (by binding only to identical molecules on opposing cell surface) in a process of lateral dimerization [23]. In pockets between tandem repeats are binding sites for calcium ions [27].

The highly conservative cytoplasmic C-terminal of approximately 150 residues is responsible for the regulation of the extracellular domain cell-cell binding function [27], through indirect interactions with actin and microtubule cytoskeleton via three catenins (α, β, and p120) [23], [30]. α-catenin acts like a switch between actin and the E-cadherin–β-catenin complex [28].

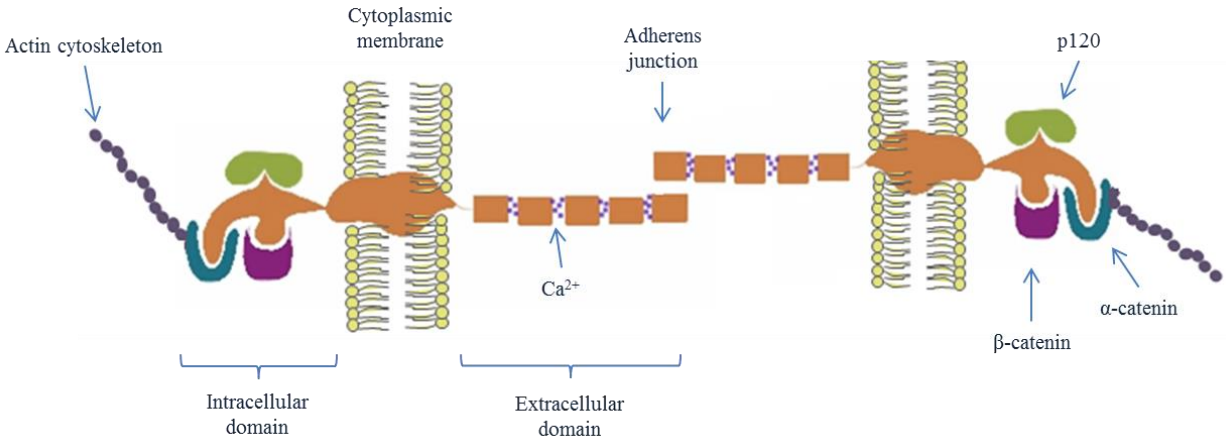

Figure 8: E-Cadherin in adherens junctions. Extracellular domain with adhesive activity consists of 5 tandem repeats with calcium binding pockets. It is responsible for adherens junctions. Intracellular domain, associated with catenins regulates binding activity of extracellular domain through cytoskeleton [based on 27].

1.3.3. Loss of E-Cadherin

In human carcinomas the loss of E-Cadherin has a wide range of transcriptional and functional consequences including abnormal morphogenesis, polarization and organization of epithelial tissues, inhibition of cell-cell contact, unregulated growth and invasion [29]. It promotes metastasis by enabling the first step of cancer cell disaggregation and controlling complex transcriptional network in later stages, by inducing anoikis resistance, and by facilitating

angiogenesis [23], [30]. Tumor stage is inversely correlated to E-Cadherin expression level [12]. It has been shown that in some invasive carcinoma cells E-Cadherin can inhibit their aggressive invasiveness while on the other hand E-Cadherin blocking in non-invasive tumors induces their metastatic ability [31], [32].

Down-regulation of E-Cadherin happens because of somatic mutations, chromosomal deletions, proteolytic cleavage, and silencing of the CDH1 promoter that appears after DNA hypermethylation or through the activation of any of the 19 transcription repressors including Slug, Snail, SIP1, δEF1, E12/E47, and Twist [13], [19], [23]. Some of these transcription factors are regulated by pathways that are known to contribute to tumor progression such as Wnt, TGF-β, FGF, EGF, signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF- κB) signaling [13].

Reduction of E-Cadherin could also happen at the protein level. Phosphorylation of E-Cadherin and catenins could be activated by RTKs, including EGFR, c-Met, IGF-1R, FGF receptors (FGFRs) and the non-RTK c-Src [13] resulting in their ubiquitylation and subsequent endocytosis and degradation [33].

Moreover, secreted proteases such as MMPs can disrupt adherens junction by cleaving E-cadherin [13].

In the absence of E-Cadherin β-catenin is released into cytosol where it can undergo phosphorylation by CK1 and GSK-3β kinases and further ubiquitin-proteasome degradation. Binding of Wnt to its Frizzled receptor at the cell surface induces the β-catenin protection from phosphorylation, resulting in its accumulation in cytosol and eventual translocation into nucleus where it activates T-cell factor/lymphoid enhancer factor (TCF/LEF)-mediated transcriptions (Figure 9) [34], [35]. β-catenin-mediated transcription can induce the expression of Slug and Twist [11] that further repress E-Cadherin expression [9].

Dislocation of p120 catenin due to the ablation of E-Cadherin results in repression of Rho and induction of Cdc42 and Rac1 proteins. Rho therefore stops induction of stress fibers, while Cdc42 activates formation of lamellipodia and Rac1 induces filopodia. These structures are essential for cell migratory behavior [13].

Usually concomitant to the E-Cadherin loss is the gain of mesenchymal cadherins, such as soluble N-Cadherin, which increases the motility and invasiveness of cancer cells. This process is commonly called ″the cadherin switch″ [13].

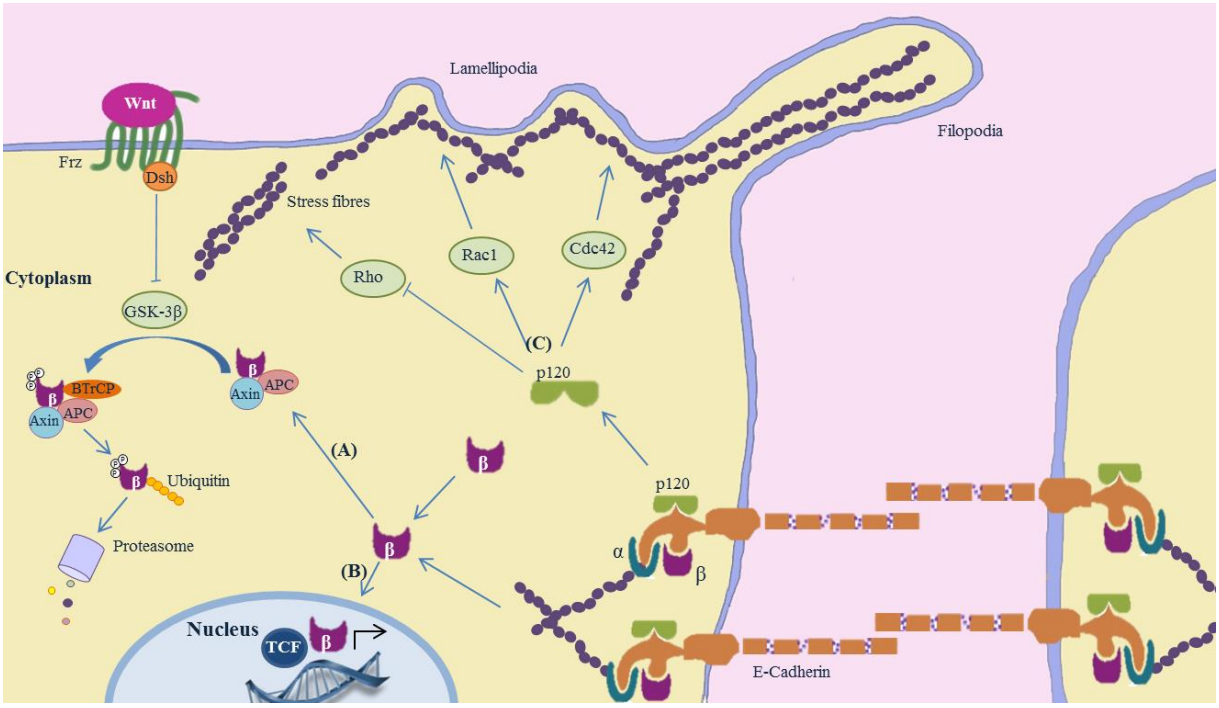

Figure 9: Signaling pathways following the reduction of E-Cadherin. a - When E-Cadherin function is lost, β-catenin is displaced into cytosol, where it forms a complex with adenomatous polyposis coli (APC) -axin that is phosphorylated by GSK-3β, ubiquitylated and finally degraded by proteasome. b – Activated Wnt signaling pathway inhibits GSK-3β, which results in accumulation of β-catenin in cytosol. Consequently β-catenin migrates into nucleus, where it regulates, together with TCF/Lef-1, expression of several transcription factors. c – Released p120 in cytosol represses the Rho and activates Rac-1 and Cdc-42 that together modulate the actin cytoskeleton and the motile properties of tumor cell by promoting the construction of filopodia and lamellipodia [based on 13].

1.4. Phage Display Technology

Phage display is a simple, powerful and widely used method for selecting and engineering polypeptides with novel functions [36]. It can be particularly useful for screening receptor-ligand interactions [37]. Beside that it is used also for many other applications, like isolation and engineering of recombinant antibodies [38], discovery of new drugs [39], development of vaccines [40], diagnostics [41], or biosensing [42].

The technology was initially described in 1985 by George P. Smith [43]. It involves the expression of recombinant (poly)peptides as fusions to capsid proteins on the surface of

bacteriophage, while genetic material resides within the phage particle [39], [43]. Due to this physical linkage between ligand genotype and its corresponding phenotype phages could be enriched by e.g., affinity selection on immobilized target [44]. Non-bound phages are washed out while bound ones can be eluted and easily amplified to amounts required for screening [45], [46].

1.4.1. Biology of filamentous bacteriophage

1.4.1.1. Structure

Bacteriophages that are the most commonly used are nonlytic filamentous phages fd, fl and M13. They are about 1000 nm long flexible rods with 6 nm in diameter (Figure 10). Inside the phage envelope lies a circular single-stranded viral DNA core with 6407-8 nucleotides [47] encoding three proteins required for DNA replication (pII, pV, pX), five different coat proteins (pIII, pVI, pVII, pVIII, pIX) and three proteins required for phage assembly (pI, pIV, pXI) [48], [49].

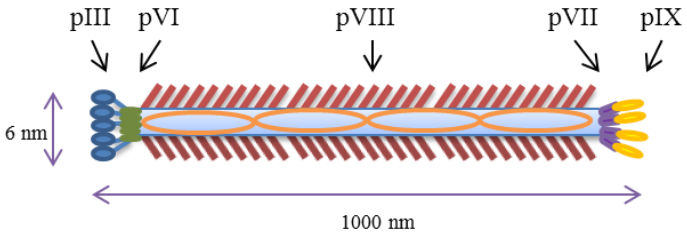

Figure 10: Structure of filamentous bacteriophage. Phage particle is 1000 nm long and has 6 nm in diameter. It consists of five coat proteins, pIII, pVI, pVII, pVIII, and pIX. Inside the capside is a single stranded DNA [based on 66].

The length of a particle is covered by approximately 2700 copies of the major coat protein - pVIII. Ends of the filament are surrounded by five copies of the remaining four minor proteins, pIII and pVI at one site and pVII and pIX at the other. First pair of minor proteins is important for phage stability and infectivity while the second is necessary for efficient initiation of particle assembly and export from bacterial host [49], [50].

1.4.1.2. Life Cycle

There are three stages of phage life cycle: (1) infection, (2) replication of viral genome and (3) assembly of new viral particles and their release from the host (Figure 11). Phage infects male strains of *E.coli* containing the F-conjugative plasmid [51] by the attachment of amino-terminal of pIII minor protein to the tip of the F-pilus receptor resulting in pilus retraction to the cell surface [47], [52]. Coat proteins then dissolve in bacterial surface envelope. Uncoated (+) stranded ssDNA is concomitantly translocated into the cytoplasm. The replication machinery of the host synthesizes a complementary (-) strand and converts DNA into a double stranded replicative form (RF). RF undergoes replication where new ssDNA are formed and it also serves as a template for the expression of phage proteins. Phage progeny particles are assembled by packing the ssDNA into the case of coat proteins acquired from the membrane with the help of proteins for phage assembly and export [53]. Virions are extruded from cell continuously without lysing the cell. The normal life cycle of infected bacteria is slowed down because of the stress of phage production [47].

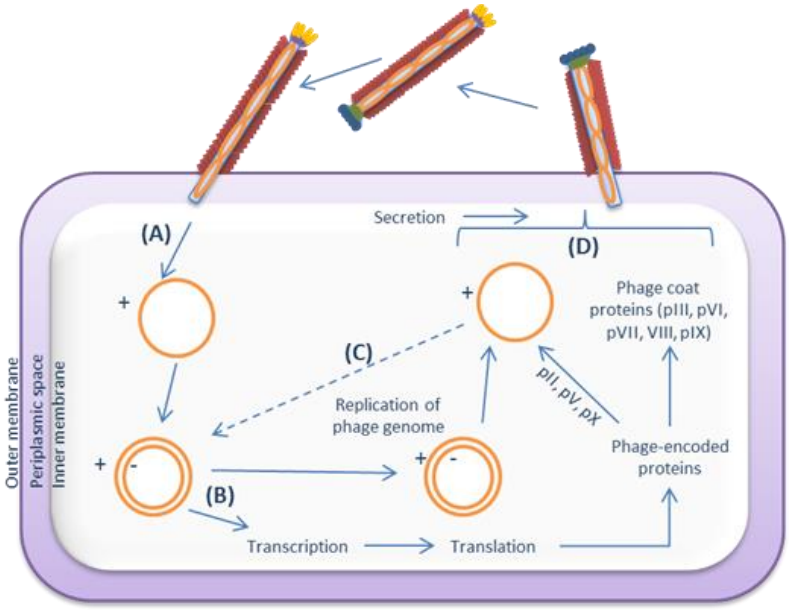

Figure 11: Replication of phage particle in bacteria. After infection of bacterial F-pilus with pIII protein, phage coat proteins dissolve in bacterial membrane. (A) (+) stranded ssDNA enters the cytoplasm where a complementary strand ((−) strand) is synthesized to form dsDNA. (B) Phage-encoded proteins are expressed from dsDNA by host-mediated protein synthesis. (C) The phage genome is replicated using the (+)-strand as a primer and the (−)-strand as a template. (D) Progeny virions are assembled and extruded from the cell [based on 54].

1.4.2. Types of phage display systems

According to Smith [47] phage display systems can be classified according to where genes for foreign protein expression are fused to genes for phage coat proteins [47].

In the simplest system the DNA fragment to be expressed is simply fused to gene of a coat protein (Figure 12; type 3). Foreign proteins will be displayed on every copy of the chosen coat protein. The positive side of such system is that many copies of foreign protein are expressed. But, because there are no original versions of wild type protein present, the endurance and infectious potential of such phage may be decreased.

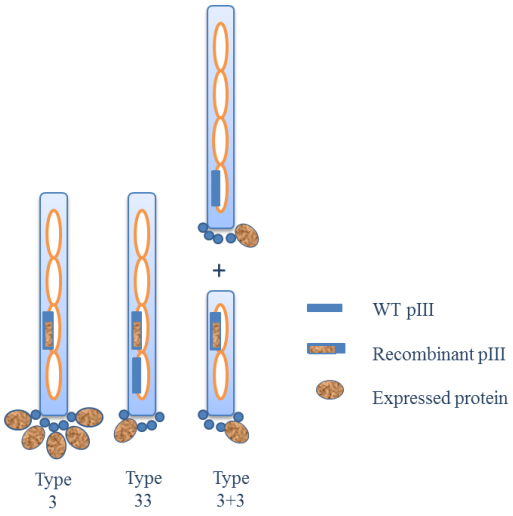

Figure 12: Expression of fusion proteins in different types of phage display system. Fusion to pIII protein is presented. Fusion to other coat proteins acts analogously. In type 3 a desired protein genetic sequence is fused directly to gene of coat protein. All expressed copies on virion surface are fusion proteins. Type 33 genome contains genes for wild type and recombinant protein. Resulting virions are coated with both, wild type and fusion proteins. Type 3+3 presents phagemid system, where genome for wild type proteins is inside helper phage and genes for fusion proteins are in phagemid. Fusion proteins are displayed in minority on all resulting virions [based on 47].

To avoid this disadvantage, a hybrid vector with genome containing genes encoding a wild type and recombinant type coat protein respectfully has been developed (Figure 12; type 3+3). Resulting phage particle has a coat with a mosaic of wild type and fusion proteins. This vector allows the display of larger proteins on the virion surface.

The most widely used is phagemid-based system (Figure 12; type 3+3). There are wild-type genes, required for formation of phage particle, carried by phage (usually called helper phage) and the recombinant sequences are carried by phagemid. Fusion and wild type proteins are displayed as a mosaic on all virions in limited numbers. Use of phagemids has several advantages: smaller phagemid genome can accept a larger nucleotide sequence, expression levels of fusion proteins can be easily controlled and modulated, and they are usually more stable than hybrid phages. However, average number of displayed proteins is lower than expected due to the competition between fusion and wild type proteins for the incorporation into virions [36], [37], [47], [55].

All five coat proteins (pIII, pVI, pVII, pVIII, pIX) could be used for display of foreign peptides, but the most common choices are pIII and pVIII. pVIII is presented in 540 copies while pIII can be seen in only five. That means that proteins fused to pVIII can be expressed in much higher numbers. However, the increase of displayed protein length significantly decreases the number of expressed copies. And generally, pIII can accommodate larger foreign proteins then pVIII [37]. Usually protein pIII accommodates only one copy of foreign protein, which reduces the avidity effect and enables selection of high affinity proteins [44]. On the other hand, pVIII is better to use when a diverse scope of expressed ligands is desired [37].

1.4.3. Tomlinson Human Single Fold scFv Libraries I + J

The Tomlinson libraries are ″naïve″ or ″single pot″ libraries comprised of approximately 100 million of random phagemids derived from B-lymphocytes of non-immunized human donors [56]. Phagemids encode a single chain F variable (scFv) gene fused to the gene gIII. Particles display a single antibody fragment.

The scFv is composed of a single polypeptide with V_H and V_L domains, connected by a flexible glycine-serine linker [57]. They are commonly used in phage display, because of their easier and faster production in recombination form with maintained affinity and specificity [58]. ScFv fragments displayed on filamentous bacteriophage can be expressed at medium to high levels in the periplasm of *E.coli* [44].

2. OBJECTIVES

Circulating tumor cells (CTCs) are strongly correlated with the appearance of metastases in carcinoma patients. Their efficient detection could contribute to an earlier identification of cancer progression and improved therapy resulting in better prognosis. CTCs develop through epithelial to mesenchymal transition (EMT) from primary carcinoma cells. One of the most important characters in this process is E-Cadherin. EMT process is never complete, thus CTCs still express some epithelial markers beside mesenchymal ones, including E-Cadherin. That characteristic could be used to distinguish CTCs from normal blood cells in blood samples.

The main objective of the present work is to select stable E-Cadherin specific scFv antibody fragments from a phage display library.

Firstly recombinant E-Cadherin will be prepared by Gateway cloning technique that enables efficient production of large amounts of desired protein. Various destination vectors providing different tags for protein purification will be used. In order to guarantee the best expression of protein, several bacterial strains will be tested. Wild type E-Cadherin will be extracted from human breast carcinoma cell lines and sufficiently purified by extraction from SDS-PAGE gel and immunoprecipitation. For increased specificity protein will be degraded to smaller particles by enzymatic digestion. Selection of E-Cadherin specific scFv fragments will be performed with recombinant antigen. Finally the specificity and affinity against wild type protein labeled with fluorescent dye will be defined on fluorescent scanner (Figure 13).

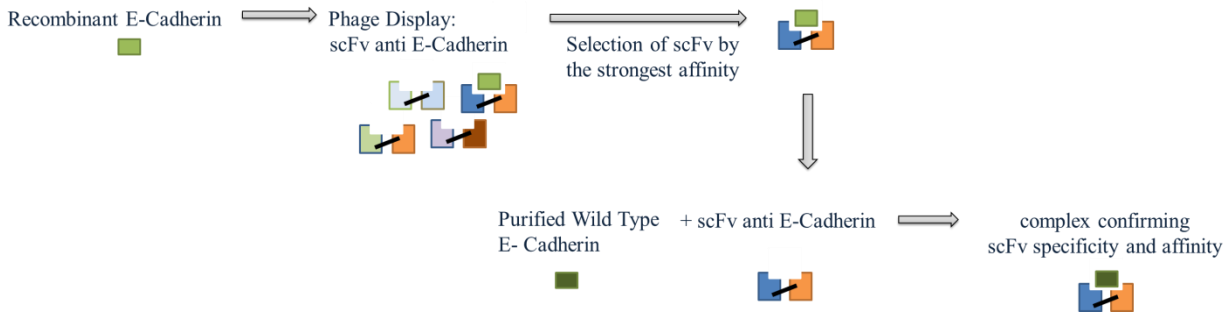

Figure 13: Schematic presentation of the experimental strategy. Recombinant E-Cadherin, produced by Gateway cloning technology, will be used for selection of E-Cadherin specific scFv antibody fragments from phage display library. ScFvs with the strongest affinity will be identified by fluorescent scanner. Affinity of selected scFvs for wild type protein will be defined by sufficiently purified wild type E-Cadherin.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Antibiotics

- Ampicillin Sodium Salt: *Sigma Aldrich, St. Louis, USA*
- Chloramphenicol: *Sigma Aldrich, St. Louis, USA*
- Gentamicin sulphate salt: *Sigma Aldrich, St. Louis, USA*
- Kanamycin Sulphate: *Gibco, Invitrogen, Oslo, Norway*

3.1.2. Antibodies

- Anti-6xHis mouse monoclonal: *Abcam, Cambridge, UK*
- Anti-E-Cadherin mouse monoclonal: *BD Transduction Laboratories, NJ, USA*
- Poly-HRP Secondary Goat-Anti-Mouse Antibodies: *Pierce, Thermo Scientific, Rockford, IL, USA*

3.1.3. Bacteria

- BL21(DE3) competent *E.coli: New England BioLabs, Ipswich, MA, USA*
- BL21(DE3)-pRARE2 competent *E.coli: EMD Millipore, Billerica, MA, USA*
- BL21(DE3)-Star competent *E.coli: New England BioLabs, Ipswich, MA, USA*
- Lemo21(DE3) competent *E.coli: New England BioLabs, Ipswich, MA, USA*

3.1.4. Cell cultures

- Human breast carcinoma cell lines: BT-20, MB-157, MB-231, MB-436, MCF-7, T-47D, ZR-75-1: *American Type Culture Collection, Rockville, MD, USA*

3.1.5. Chemicals and reagents

- 100 bp DNA Ladder TrackIt: *Invitrogen, Oslo, Norway*

- 29:1, 30% Acrylamide Bis Solution: *Bio Rad, Marnes-la-Coquette, France*
- Acetic acid: *Sigma-Aldrich, St. Louis, USA*
- Agarose: *Sigma, St. Louis, MO, USA*
- Ammonium Chloride (NH_4Cl): *Sigma-Aldrich, St. Louis, USA*
- Ammonium Persulfate (APS): *Bio Rad, Marnes-la-Coquette, France*
- Bacto Agar: *BD Biosciences, New Jersey, USA*
- Bacto Tryptone: *BD Biosciences, New Jersey, USA*
- Bacto Yeast Extract: *BD Biosciences, New Jersey, USA*
- Boric Acid: *Sigma, St. Louis, USA*
- Bovine Serum Albuminum (BSA): *Sigma, St. Louis, USA*
- Calcium Chloride Dihydrate ($CaCl_2*2H_2O$): *Merck, Whitehouse Station, NJ, USA*
- Citric Acid: *Sigma, St. Louis, USA*
- Coomassie Brilliant Blue R-250: *Thermo Scientific, Rockford, Il, USA*
- D(+) Glucose monohydrate: *Merck, Whitehouse Station, NJ, USA*
- Dimethyl pemelimidate dihydrochloride (DMP): *Sigma, St. Louis, USA*
- Distilled Water: *Invitrogen, Oslo, Norway*
- DyLight 633 NHS Ester: *Thermo Scientific, Rockford, Il, USA*
- Dynabeads Protein G: *Invitrogen, Oslo, Norway*
- ECL 2 (Enhanced Chemiluminiscence reagent), Western Blotting substrate: *Thermo Scientific, Rockford, IL, USA*
- Ethylenediaminetetraacetic acid (EDTA): *Sigma-Aldrich, St. Louis, USA*
- Ethanol 100%: *VWR, Radnor, PA, USA*
- Ethidium Bromide 10 mg/mL: *Invitrogen, Oslo, Norway*
- Ethylenediaminetetraacetic acid – EDTA: *Sigma, St. Louis, USA*
- Gel Loading Dye Orange 6x: *New England BioLabs, Ipswich, MA, USA*
- Glycine: *Bio Rad, Marnes-la-Coquette, France*
- IPTG (isopropylthio-β-galactoside): *Invitrogen, Oslo, Norway*
- Magnesium chloride hexahydrate ($MgCl_2*6H_2O$): *Merck, Darmstadt, Germany*
- Magnesium sulfate ($MgSO_4$): *Sigma-Aldrich, St. Louis, USA*
- Methanol: *VWR, Radnor, PA, USA*
- Ni-NTA Magnetic Agarose Beads: *Qiagen, Venlo, The Netherlands*

- NP-40 Detergent Solution: *Thermo Scientific, Rockford, Il, USA*
- Phenylmethanesulfonyl fluoride (PMSF): *Sigma-Aldrich, St. Louis, USA*
- Polyethilenglycol – PEG 6000: *Sigma-Aldrich, St. Louis, USA*
- Ponceau Red Soluton: *Sigma, St. Louis, USA*
- Potassium Phosphate Monobasic (KH_2PO_4): *Sigma-Aldrich, St. Louis, USA*
- Protein Ladder 10-250 kDa: *New England BioLabs, Ipswich, MA, USA*
- Sodium dodecyl sulfate (SDS) 20%: *Sigma, St. Louis, USA*
- Sodium Chloride (NaCl): *Sigma-Aldrich, St. Louis, USA*
- Sodium Orthovanadate (Na_3VO_4): *Sigma-Aldrich, St. Louis, USA*
- Sodium phosphate dibasic (Na_2HPO_4): *Sigma-Aldrich, St. Louis, USA*
- Temed (N,N,N´,N´-tetramethylethylenediamine): *Bio Rad, Marnes-la-Coquette, France*
- Thiamine Hydrochloride: *Sigma Aldrich, St. Louis, USA*
- Tris : *Sigma, St. Louis, USA*
- Trizma base: *Sigma, St. Louis, USA*
- Tween20: *Fisher Bioreagents, Hampton, New Hampshire, USA*

3.1.6. Enzymes

- Complete Protease Inhibitor Cocktail Tablets: *Roche, Basel, Switzerland*
- Endoproteinase GluC (*Staphylococcus aureus* Protease V8): *New England BioLabs, Ipswich, MA, USA*
- Trypsin from bovine pancreas: *Sigma, St. Louis, USA*

3.1.7. Kits

- Amersham HyperfilmTM ECL: *GE Healthcare, Buckinghamshire, UK*
- Gateway cloning kit: *Invitrogen, Oslo, Norway*
- Immobilon®Transfer Membrane, Filter type PVDF: *Millipore, Billerica, MA, USA*
- KAPA Taq PCR Kits: *Biosystems, Boston, USA*
- Optitran BA-S 85 Rainforced NC: *GE Healthcare, Little Chalfont, UK*

- QIAprep Spin Miniprep Kit: *Qiagen, Venlo, The Netherlands*
- Qubit Assay Kit: *Invitrogen, Oslo, Norway*

3.1.8. Labware and equipment

- 24 deep-well culture plate
- 96-well microtiter plate
- 96-deep well culture plate
- Autoclave
- Centrifuge: *Beckman, Brea, Ca, USA*
- Centrifuge: *Eppendorf, Le Pecq, France*
- Fluorescent Dye Removal Columns: *Thermo Scientific, Rockford, Il, USA*
- Incubator shaker: *Infors, Basel, Schwitzerland*
- Lab Roller
- Magnetic separator
- Micro centrifuge
- Nanodrop: *Thermo Scientific, Rockford, Il, USA*
- Nexterion®Slide E for protein application: *Schott, Jena Germany*
- Over-and-under turntable
- PCR machine
- Petri Dishes: *Corning Incorporated, Amsterdam, The Netherlands*
- Polyvinylidene Difluoride (PVDF) membrane: *Thermo Scientific, Rockford, Il, USA*
- Qubit: *Invitrogen, Oslo, Norway*
- Scanarray express: *Packard Bioscience, Perkin Elmer, Waltham, MA, USA*
- SDS-PAGE electrophoresis system: *Apelex, Evry Cedex, France*
- SDS-PAGE electrophoresis system: *Bio Rad, Marnes-la-Coquette, France*
- Speed-Vac
- Thermomixer: *Eppendorf, Le Pecq, France*
- Water Bath

3.1.9. Phage Display Libraries

- Tomlinson I + J Libraries: *MRC Laboratory of Molecular Biology, Cambridge, United Kingdom*

3.1.10. Buffers and solutions

- **Agarose gel 2%**

  500 mg agarose, 25 mL TBE, 1.5 µL ethidium bromide
- **Assay Wash Buffer, pH 8.0**

  50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.005% Tween 20
- **Coomassie Blue Destaining solution**

  40% methanol, 10% glacial acetic acid
- **Coomassie Blue Staining solution**

  0.1% Coomassie Brilliant Blue R-250, 50% methanol , 10% glacial acetic acid
- **Elution buffer for protein elution from Ni-NTA beads, pH 8.0**

  50 mM NaH_2PO_4, 30 mM NaCl, 20 mM imidazole, 0.005% Tween 20
- **M9 salts, 5x solution**

  64 g Na_2HPO_4, 15g KH_2PO_4, 5 g NH_4Cl, 2.5 g NaCl, to 1 L dH_2O
- **PEG solution**

  20% PEG, 2.5 M NaCl. Filter through 0.2 µM filter.
- **PBS buffer, pH 7.4**

  2.67 mM KCl, 1.47 KH_2PO_4, 137.93 mM NaCl, 8.06 mM Na_2HPO_4 x 7 H_2O
- **PBST buffer 0.05 – 0.1%**

  1 L PBS 1x solution, 0.05 -0.1% Tween®20
- **Protein binding buffer, pH 8.0**

  50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole
- **RIPA buffer**

  0.15 mL Tris 1 M pH 7.5, 0.45 mL 1 M NaCl, 0.30 mL 10% NP-40, 0.15 mL 10% SDS, 0.006 mL 0.5 M EDTA pH 8.0, 0.10 mL 30 mM Na_3VO_4, 0.03 mL 0.1 M NaF, 0.2 mM PMSF 0.003 mL , 0.06 mL 50 mM Roche mini complete Protease Inhibitor Mixture, 1.75 mL dH_2O

- **Solution for the detection with ECL reagents**

  Solution A: Solution B = 40:1

  0.125mL working solution /cm^2 of membrane
- **TBE buffer 1x solution**

  107.8 g Tris base, 54.4 g boric acid, 4.52 g EDTA, to 1 L dH_2O
- **TBS (tris-buffered saline) buffer 5x solution**

  8.8 g NaCl, 1.21 g Tris base, to 1 L dH_2O
- **TBSC buffer, pH 7.4**

  10 mM Tris pH 7.4, 137 mM NaCl, 1 mM CaCl2
- **TES buffer**

  10 mM Tris pH 8.0, 0.1 mM EDTA, 150 mM NaCl, 20% sucrose
- **TTBS buffer 0,05% - 0,10%**

  1 L TBS 1x solution, 0.05 -0.1% Tween®20
- **Tris 3 M pH 8.9**

  72.66 g Tris base, 200 mL TBS 1x solution
- **Tris 0.5 M pH 6.8**

  12.1 g Tris, 200 mL TBS 1x solution
- **WB Buffer for electrotransfer 10x (PVDF membrane)**

  30.3 g Tris base, 144 g glycine, to 1L dH_2O

  WB Buffer for electrotransfer 1x (PVDF membrane)

  700 mL dH_2O, 100 mL buffer 10x solution, 200 mL 100% Methanol, 125 µL 20% SDS
- **WB Incubation solution (with the primary antibody) for PVDF membrane**

  Anti 6 x His: 1:1000 Primary antibody, 50 mL TBS 1x solution – Tween 0.1%

  Anti-E-Cadherin: 1:5000 Primary antibody, 50 mL TBS 1x solution – Tween 0.1%
- **WB Incubation solution (with the secondary antibody) for PVDF membrane**

  1:30 000 Secondary antibody, 50 mL TBS 1x solution – Tween 0.1%
- **WB Loading buffer, 5x solution**

  2 mL SDS 20%, 1.75 mL Tris 0.5 M pH 6.8, 0.5 mL bromphenol blue 0.25%, 1.25 mL B-mercaptoethanol, 4.5 mL gylcerol

- **WB Migration buffer 10x solution, pH 8.3**

  30.3 g Tris base, 144.2 g glycine, 50 mL 20% SDS, to 1 L dH_2O
- **WB Saturation buffer (5% milk in TBS) for nitrocellulose membrane**

  2.5 g milk powder, 50 mL TBS 1x solution
- **WB Saturation buffer (5% milk in TTBS 0.1%) for PVDF membrane**

  2.5 g milk powder, 50 mL 0.01% TTBS

3.1.11. Bacterial medium

- **L Broth (Luria-Bertani) medium, pH 7.2**

  10 g bacto tryptone, 5 g yeast extract, 10 g NaCl, to 1L dH_2O

  For preparation of Petri dishes add 15 g agar.
- **2x TY, pH 7.2**

  16 g bacto tryptone, 10 g yeast extract, 5 g NaCl, to 1L dH_2O
- **TYE agar plates**

  10 g bacto tryptone, 5 g yeast extract, 8 g NaCl, 15 g agar, 800 mL dH_2O
- **M9 minimal medium glucose plates**

  15 g agar, 800 mL dH_2O, 200 mL M9 salts 5x solution, 10 mL 20% glucose, 1 mL of 1 M $MgSO_4$, 100 µL of 1 M $CaCl_2$, 1 mL of 1 mg/mL Vitamin B1
- **H-TOP Agar**

  10 g bacto tryptone, 8 g NaCl, 7 g agar, to 1L dH_2O

3.2. Methods

3.2.1. Extraction of proteins from eukaryotic cells

$5x10^6$ pelleted cells were extracted with 100 µl of RIPA buffer. When buffer was added to cells, three cycles of freezing in liquid nitrogen and defreezing at 37°C were performed. Tubes were incubated on ice for 30 minutes and vortexed every 5 minutes. Solution was then centrifuged at 1500 x g, 4°C for 15 minutes. Supernatant with extrated proteins was placed into a clean tube.

3.2.2. Western Blot

3.2.2.1. SDS-PAGE

We prepared two polyacrylamide gels with different concentrations as indicated in Table I. The concentration of separation gel depended on the molecular weight of proteins to be analyzed, ranging from 5-12%. The solution of separation gel was pipetted up to 3 cm from the top of the glasses. On top of it a layer of isopropanol was deposited. Isopropanol was removed after gel polymerized, about 1 hour later. Then each glass sandwich was filled with stacking solution and a plastic comb was carefully inserted into it. Approximately 30 minutes later, gels were ready for sample loading.

Table I: Preparation of gels for electrophoresis.

	SEPARATION GEL – 12% (10 ml)	**STACKING GEL – 5% (10 ml)**
dH_2O	3,2 ml	6,8 ml
30% Acrylamide mix	4,0 ml	1,66 ml
1,5 M Tris, pH 8,5	2,6 ml	-
1,5 M Tris, pH 6,5	-	1,26 ml
SDS 10% (w/v)	0,1 ml	0,1 ml
APS 10 % (w/v)	0,1 ml	0,1 ml
Temed*	0,004 ml	0,01 ml

*This reagent was added extemporarily.

Preparation of samples for electrophoresis

Loading buffer 2x solution was mixed with samples in the adequate volume. The mixture was heated at 95°C in Thermoblock for 10 minutes. Samples and the protein standard solution were loaded to gel using a pipette.

Protein migration

Electrophoresis was carried out in the Bio-Rad Mini Protean Cell, in migration buffer. The process was performed at 150 V and 50 mA for first 15 minutes. After samples have entered separation gel the process was slowed down to 60 V and 30 mA for another 1 – 1.5 hours.

Electrotransfer of proteins

Proteins were electrophoretically transferred to PVDF in a cold transfer buffer. Transfer took place at 100 V and 200 mA for 1-2 hours. Finally PVDF membrane was blocked with 5% non-fat dry milk in TTBS 0.1%, both for 30 minutes.

3.2.2.2. Incubation with antibodies

Following 30 minutes incubation with primary antibodies on a lab roller at RT, PVDF membrane was washed 3x for 5 minutes each time with TTBS 0.1% and further incubated with HRP conjugated secondary antibodies for 30 minutes at room temperature and continuous stirring.

3.2.2.3. Identification of proteins

Membrane was exposed to an enhanced chemiluminiscence reagent (ECL). The oxidation of luminol is catalyzed by HRP, and because the HRP is in a complex with the protein of interest on the membrane, the amount and location of light that HRP catalyzes the emission of, is directly correlated with the location and amount of protein on the membrane. Emission was detected by the photographic film.

3.2.2.4. Staining of proteins on electrophoresis gel with Coomassie Blue

The Coomassie stain irreversibly binds to proteins on gel and therefore enables rapid detection of protein bonds directly on gel. To perform it we put gel into staining solution and mixed it

on lab roller for 2 hours. Stain was removed with destaining solution that was replenished several times until background of the gel was fully destained.

3.2.3. Purification of proteins by extraction from SDS-PAGE gel

After separation of proteins according to their size by SDS-PAGE, parts of gel with bonds of interest were cut off with a scalpel and put into a clean tube (Figure 14). A small volume of dH_2O was added, so the gel did not dry out. Gel was crushed into small pieces. To elute proteins from gel, tubes were put to 4°C overnight. Next day, tubes were incubated at 40°C for 2 hours and centrifuged at 5000 x g, 4°C for 10 minutes. The supernatant with eluted proteins was transferred into a clean tube. To concentrate samples we used Speed-Vac machine that evaporated water from them.

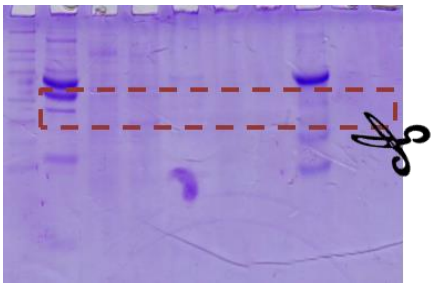

Figure 14: Schematic presentation of purification of proteins from SDS-PAGE gel. Bonds of interest were cut off with a sharp scalpel.

3.2.4. Digestion of proteins with restriction enzymes

For degradation of proteins they were submitted to digestion with restriction enzymes. 5 µL of enzyme Endoproteinase GluC (*Staphylococcus aureus* Protease V8) or Trypsin (100 ng/µL) and 85 µL of reaction buffer were added 10 µL of protein (1 µg/µL). Mixture was incubated at 25°C for 2 hours (for digestion) and at 70°C for 10 minutes (for enzyme denaturation).

3.2.5. Immunoprecipitation with Dynabeads® Protein G

3.2.5.1. Antibody biding

10 µL (0.25 µg/ µL) of anti-E-Cadherin antibodies were added to 1 ml of well mixed Dynabeads®. Mixture was incubated on over-and-under turntable at RT for 10 minutes. Obtained complex was resuspended in 1 ml of PBST 0.1%.

3.2.5.2. Crosslinking

To avoid co-elution of antibody, antibodies were crosslinked to beads with crosslinking reagent – DMP. Antibody–beads complexes were washed in 2 ml of 0.2 M triethanolamine pH 8.2, resuspended in 2 mL of freshly made DMP in 0.2 M triethanolamine pH 8.2 and incubated on over-and-under turntable for 30 minutes at RT. Reaction ended by removing the supernatant and resuspension of beads in 2 ml of 50 mM Tris, pH 7.5. After 15 minutes beads were washed 3x with PBST 0.1%.

3.2.5.3. Immunoprecipitation of target antigen

50 µg of target protein was added to crosslinked complex and incubated at over-and-under turntable at 4°C for 1 hour. Supernatant was removed and antigen was eluted from the complex (Figure 15).

3.2.5.4. Gentle, non-denaturating elution of protein

Antigen was eluted with 200 µL of elution buffer (0.1 M citric acid, pH 2-3) by gentle pipetting and resuspending Ab-Ag-beads complex. Supernatant containing eluate was placed into a clean tube. To adjust the pH of the eluting solution, dialysis with Tris pH 6.8 was performed.

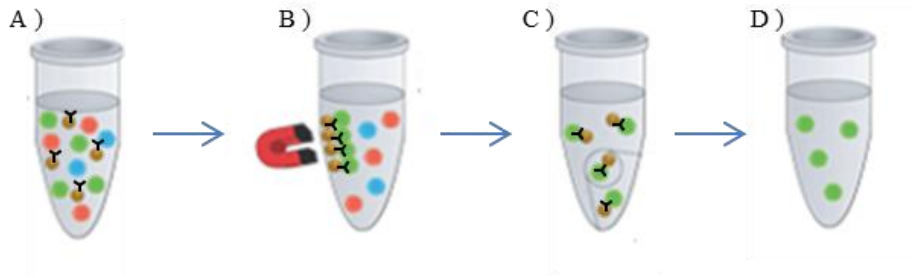

Figure 15: Schematic presentation of immunoprecipitation. A) In the first tube there is a solution of magnetic beads with bound antibodies (golden spheres), target antigen (green spheres) and other proteins (blue and red spheres). B-C) After incubation a complex beads-antibody-antigen creates and it can be easily separated from other proteins with the use of a magnet. D) The elution of target protein results in a solution of a non-bound protein.

3.2.6. Gateway Cloning Technology

Gateway cloning method is based on the site-specific recombination system of bacteriophage lambda [59] which promotes the integration of lambda into *E.coli* genome and the switch between the lytic and lysogenic pathways [60]. It is an extremely rapid and efficient method that can be used for functional analysis and expression in multiple systems while maintaining orientation and reading frame [61]. It allows use and expression from multiple types of DNA sequences (PCR fragments, cDNA, or restriction fragments) (Figure 16).

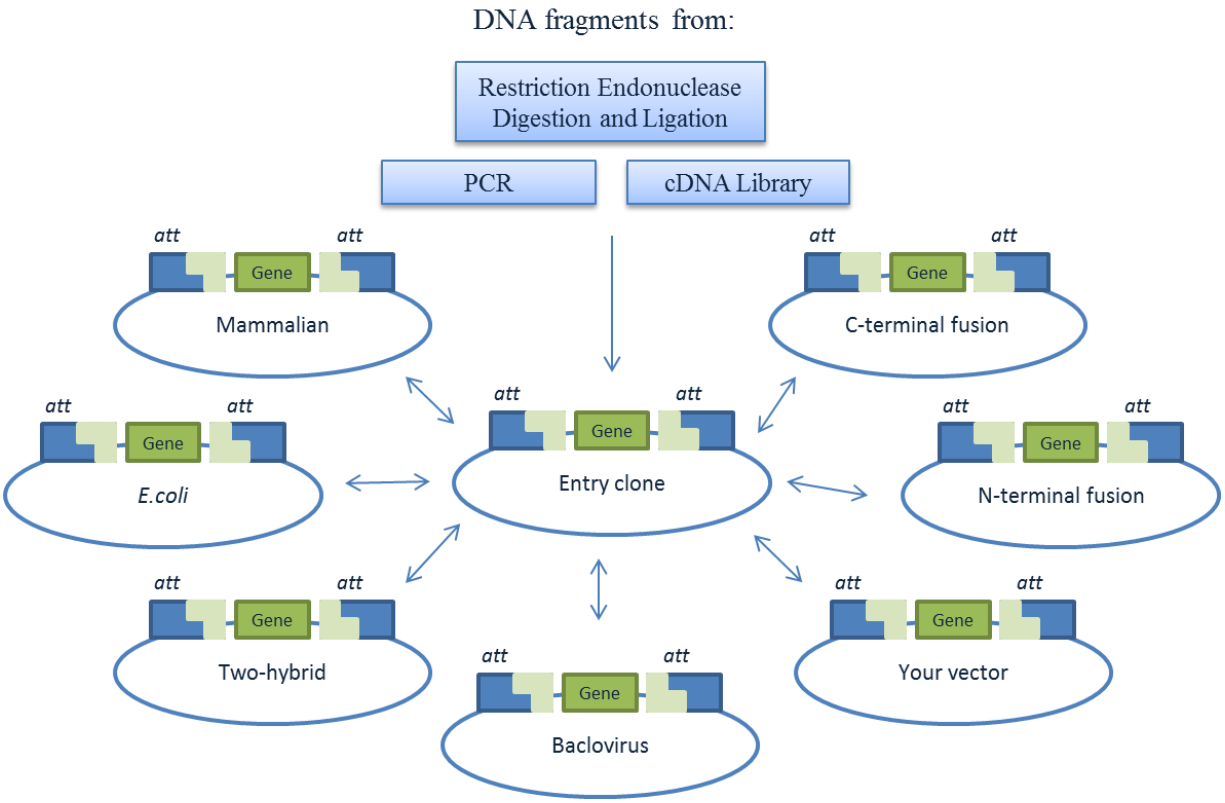

Figure 16: Wide applications of Gateway System. Gene of interest from different possible sources could be inserted into the Gateway system via entry clone. Once the entry clone is formed, it is available to express the gene of interest in a variety of other vectors (e.g. *E.coli*, Mammalian cells…). This reaction is reversible [based on 62].

Lambda-based recombination includes two important components:

- The specific DNA recombination sites (*att* sites) and
- The enzyme clonase mixes that mediate the recombination reaction and are needed for excision and incision of genes.

The main advantage of the system is the conservative recombination between specific attachment (*att*) sites in the interacting DNA molecule. Reaction is catalyzed by a mixture of enzymes that bind to *att* sites, bring together the target sites, cleave them, and covalently attach the DNA. Proteins involved in the reaction differ depending upon whether lambda utilizes the lytic or lysogenic pathway.

Lysogenic Pathway (BP Reaction): *att*B x *att*P $\longrightarrow$ *att*L x *att*R

Catalyzed by: BP Clonase (Int, IHV)

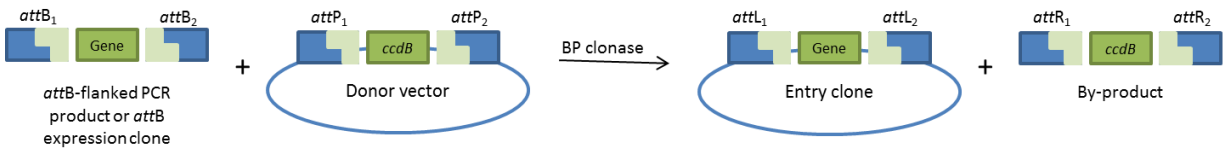

Figure 17: BP reaction. This reaction facilitates recombination of *att*B substrate with an *att*P substrate (donor vector) to create an *att*L-containing entry clone [based on 62].

Lytic Pathway (LR Reaction): *att*L x *att*R $\longrightarrow$ *attB* x *att*P

Catalyzed by: LR Clonase (Int, Xiv, IHF)

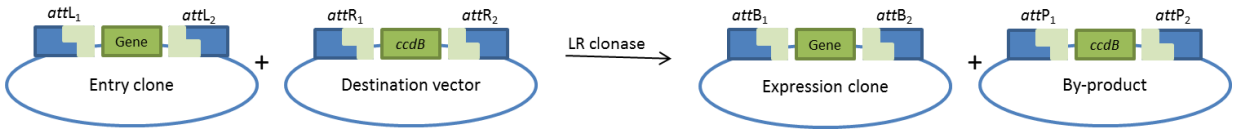

Figure 18: **LR reaction.** The *att*L substrate (entry clone) reacts with an *att*R substrate (destination vector) to create an *att*B-containing expression clone [based on 62].

Reactions BP and LR are directional because *att*B_1 reacts only with *att*P_1, *att*B_2 only with *att*P_2, *att*L_1 with *att*R_1 and *att*L_2 with *att*R_2.

Since the Gateway system is reversible, more entry clones can be formed from one expression clone using BP reaction.

For efficient selection of entry and expression clones, most Gateway vectors contain the *ccd*B gene flanked with two *att* sites and Chloramphenicol resistance gene (Cm^R). The *ccd*B protein interferes with *E.coli* DNA gyrase [63], thereby inhibiting growth of most *E.coli* strains. After recombination and transformation *Ccd*B allows negative selection of the donor and destination (and some entry) vectors in *E.coli*. In BP or LR reaction, *ccd*B and Cm^R containing cassette is replaced by the gene of interest, forming the entry and expression clone respectively. Cells that take up unreacted vectors or by-product with *ccd*B gene will not grow which gives the very high yield of desired clones.

3.2.6.1. BP and LR reaction

Reactions were performed according to the manufacturer's manual (Invitrogen) [62].

Products of BP reaction were transformed into DH5α-T1R competent cells and spread onto Gentamycin selective plate. Positive clones were selected for screening and sequencing with p207S and p207R primers.

For LR reaction, 5 different destination vectors were used (described under section Results and discussion). Reaction products were used for transformation of DH5α-T1R competent cells. Transformed bacteria were spread onto ampicillin selective plate. Plasmids from positive colonies were purified by QIAprep Spin Miniprep Kit according to its manual.

3.2.7. Growing bacterial culture and protein expression

A bacterial colony was scraped from LB-agar and mixed into 10 mL of LB with antibiotic. Tube was incubated at 37°C, 200 RPM overnight. Next day the resulting preculture was poured into 250 mL of LB with antibiotic and incubated at 37°C, 200 RPM until the OD_{600nm} was 0.5. 1 mL of bacteria was centrifuged for the control before protein induction and pellets were mixed with 25 µL of loading buffer 5x solution. IPTG was added to the rest of the culture to the final concentration 0.5 mM. Solution was incubated overnight at 25°C, 200 RPM. Next day cells were centrifuged at 20°C, 4000 x g for 20 minutes. Pellets were stored at -20°C and further used for protein purification.

3.2.8. Native conditions protein purification with His x 6-tag columns

Pellets were thawed on ice and resuspended in 10 mL of native lysis buffer (with lyzosyme and nuclease). After 30 minutes incubation on ice, they were sonicated for one minutes (2 seconds interval). Lysat was centrifuged at 4°C, 14000 x g for 30 minutes. Supernatant was poured into purification column. After Flow Through fraction went through column, it was washed twice with 4 mL of wash buffer. Proteins with 6xHis-tag were finally eluted twice with 1 mL of native elution buffer. All fractions were collected separately. Aliquots of 5 µL of all fractions were mixed with 25 µL of loading buffer 5x and further analyzed on Western Blot.

3.2.9. Phage display

3.2.9.1. Estimation of Phage Titers

a) By spectroscopy

Phage titers were estimated by measuring absorption at 260 nm by nanodrop. Phage was diluted 100-fold in PBS. Titers were calculated according to the empirical formula:

Equation 1:

$$\text{Phage / ml} = OD_{260nm} * 100 * 22.14 * 10^{10}$$

b) By plating infected bacteria

900 µL of TG1 bacteria at OD_{600nm} 0.4 were infected with 100 µL of serially diluted phages in PBS. 10 µL of each culture were plated on TYE plates containing 100 µg/mL ampicillin and 1% glucose and incubated at 37°C overnight. Next day colonies were counted and phage titers estimated by equation 2:

Equation 2:

$$\text{Phage / ml} = \text{Number of colonies} * \text{dilution factor} / 0.01\ \text{mL}$$

3.2.9.2. Selection of phages that show affinity towards antigen

Removing non-specific binders

50 µL of magnetic Ni-NTA beads were used for each Phage Display Library. Buffer was removed with a use of a magnet and beads were washed twice with PBS. They were saturated with 500 µL of 3% BSA in PBS for 1 hour. Non bound BSA was removed from the tube and 100 µL of each library were added separately. Solutions were incubated for 1 hour at RT at the over-and-under turntable. Phages that bound to empty beads were discarded and supernatant was used for the selection.

Selection

200 µL of Ni-NTA beads were used for the selection. Original buffer was replaced with 500 µL of binding buffer. 65 µL of protein were added and well mixed solution was incubated for 1 hour. Supernatant with non-bound protein was discarded and beads were saturated with 3% BSA in PBS for 1 hour. Supernatant was removed and beads were washed 3x with PBS. Libraries, pre-incubated with empty beads, were added to tubes. Incubation took two hours. Non-bound phages were discarded and beads were washed 5x with PBST 0.1% and 3x with PBS to remove detergent. Finally phages were eluted with Trypsin solution or 0.1 M Glycine, pH 2.7 for 1 hour 30 minutes. Glycine eluates were neutralized with 20 µL of Tris 1 M, pH 9. All incubations were performed at RT on over-and-under turntable.

3.2.9.3. Production of soluble fragments

Individual colonies were put separately into 96-well plate containing 200 µl of 2xTY medium supplemented with ampicillin (100 µg/mL) and 4% glucose per well. Clones were grown overnight at 37°C at 200 RPM in a plate with removed lid, laid inside a plastic box. A fresh 96-well plate containing 200 µl of 2xTY medium supplemented with 100 µg/ml of ampicillin and 4% glucose was inoculated with 5 µl of the overnight culture. Glycerol stock of the original 96-well overnight cultures was prepared by adding glycerol to plate (20% final concentration) and stored at -80°C. Freshly inoculated plate was shaken at 37°C, 200 RPM for three hours (in a plastic box as above). Cells were spun down at 3200 x g in a plate centrifuge for 10 minutes. Supernatant was discarded by quickly inverting the plate. Pellets were

resuspended in 200 µl of 2xTY medium supplemented with ampicillin 100 µg/ml and 1 mM IPTG by gentle agitation. Culture was grown overnight at 35°C, 200 RPM in a plastic box for 16 hours. For scFv purification cells were spun down, resuspended in 100 µL of TES buffer and kept on ice for 30 minutes. To obtain periplasmic fraction plate was centrifuged at 1800 x g for 10 min. ScFv containing periplasms were diluted 1:2 in PBST 0.1 %.

3.2.9.4. Labeling proteins with fluorescent dye

50 µL (0,1 mg/mL) of protein were mixed with 50 µl of Dylight 633 NHS-Ester Dye. During 1 hour incubation at room temperature, we prepared dye removal resin for removing the dye that did not bind to protein. We put 100 µl of resin into the column and centrifuged it for 1 minute at 1500 x g. Then we changed the collecting tube, poured protein solution on resin and centrifuged it for 1 minute at 1500 x g.

3.2.9.5. Preparation of slides for fluorescent microarray scanning

1 µL of phages was spoted on Nexterion adhesive slide and left at RT until the slide was completely dry. Slides were saturated with 1% BSA in PBST 0.1% at RT, on a lab roller for 1hour. Saturation solution was replaced with fluorescent-labeled proteins dye and incubated for 1 hour at RT. Finally slides were washed 3x with PBST 0.05% and 2x with PBS and dried at RT. Dry phages were immobilized and disinfected with ethanol. Fluorescence was detected with scanner.

3.2.9.6. Immobilization and disinfection of phages attached to adhesive slides

Before the scanning, slides with attached phages were dipped for few seconds into 10% and then to 30%, 70% and finally 100% ethanol. Procedure immobilized phages to slides and also disinfected them.

4. RESULTS AND DISCUSSION

4.1. Gateway Cloning

Our aim was to produce recombinant C-terminal of E-Cadherin that will serve as antigen for selection of specific antibody fragments by phage display. We decided to produce it by Gateway cloning technique because this is a very rapid and efficient method for production of recombinant proteins.

We focused on carbon-terminal because antibodies that are available on the market attach to it. Therefore we had a tool for confirmation of expressed protein by Western Blot.

First we prepared gene of E-Cadherin flanked with *att*B sites necessary for BP reaction. Resulting product was used in LR reaction with five different destination vectors. Obtained expression clones were purified by Mini Prep. To define best conditions for protein expression and purification Mini expression and solubility test were performed. According to the obtained results, final protein expression was performed.

4.1.1. Primer Design

To generate PCR products suitable for the use as substrates in a Gateway® BP recombination reaction with a donor vector, *att*B sites had to be incorporated into PCR product. We achieved that by designing appropriate forward and reverse primers for the use in PCR reactions.

4.1.1.1. Forward primer

The forward primer for the first PCR reaction encompassed the gene specific sequence and added a NdeI restriction site and the TEV cleavage site encoding sequence.

SP0007 (E-Cadherin Forward primer): 43 mers – Tm = 62 (determined using NEB calculator [64]).

5’ – GAAAACCTGTATTTTCAGGGC**CATATG**CTTCGGAGGAGAGCGG – 3’

The final PCR product was obtained using the Gateway generic forward primer encompassing the *att*B_1 recombination site and the TEV cleavage site encoding sequence.

G1: 5'–GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAAACCTGTATTTTCAGGGC–3'

4.1.1.2. Reverse primer

The reverse primer encompassed the gene specific sequence and added a stop codon and the *att*B_2 recombination site.

SP0008 (E-Cadherin Reverse primer): 53 mers – Tm = 63 (determined using NEB [64]).

5– GGGGACCACTTTGTACAAGAAAGCTGGGTC**GGATCC**TCAGTCGTCCTCGCCGC–3'

4.1.2. PCR product

DNA sequence of E-Cadherin C-terminal was found on NCBI website [65]. DNA template for PCR was prepared by another laboratory.

For the Gateway cloning we first amplified DNA template with SC0007 – E-Cadherin Forward and SC0008 – E-Cadherin Reverse Gateway primers (Figure 19).

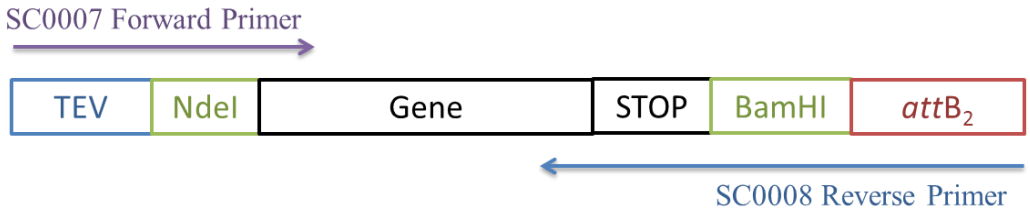

Figure 19: Scheme of first PCR product. DNA template was amplified with SC0007 forward and SC0008 reverse primers. Resulting product encompassed gene of E-Cadherin C-terminal, TEV cleavage site, NdeI and BamHI restriction sites and *att*B_2 recombination site.

Resulting PCR product was amplified with G1 Gateway generic primer and reverse primer from the previous reaction (SC0008 – HsCadhE-R). Final PCR product flanked with *att*B sites was purified with GeneJET Purification Column (Figure 20).

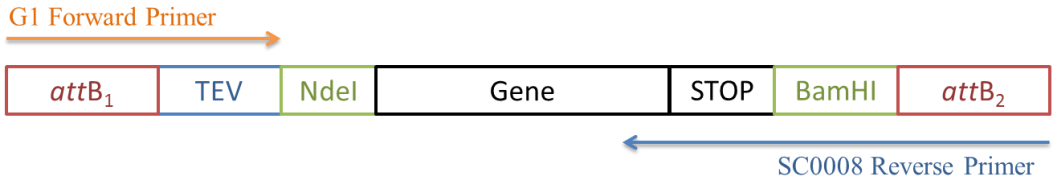

Figure 20: Scheme of the second PCR product. DNA template was amplified with G1 forward and SC0008 reverse primers. Resulting product encompassed gene of E-Cadherin C-terminal, TEV cleavage site, NdeI and BamHI restriction sites and *att*B_1 and *att*B_2 recombination site.

The resulting PCR product contained E-Cadherin DNA sequence with stop codon flanked with TEV cleavage site, *Nde*I and *Bam*HI restriction sites and *att*B_1 and *att*B_2 recombination sites.

4.1.3. BP and LR reactions

The PCR product flanked with *att*B sites was inserted into Donor vector during BP reaction. Resulting Entry clone was sequenced with p207S and p207R primers from the Gateway cloning Kit. Results of sequencing confirmed the expected sequence.

LR reaction was performed with Entry clone resulting from BP reaction and 5 different Destination vectors. Destination vectors contained different fusion tags sequences for protein purification. By using different Destination vectors we enhanced possibilities of efficient protein expression.

Destination vectors:

- pHGWA: 6xHis-tag (→ SC0035),
- pHGGWA: 6xHis and GST-tag (→ SC0036),
- pHMGWA: 6xHis and MBP-tag (→ SC0037),
- pHNGWA: 6xHis and NusA-tag (→ SC0038), and
- pHXGWA: 6xHis and TRX-tag (→ SC0039).

Obtained Expression clones were named SC0033, SC0034, SC0035, SC0036, and SC0037 respectfully. They were used for transformation of DH5α-T1R competent *E.coli* bacteria. All cultures seeded many colonies on ampicillin containing agar plates, thus we concluded that all

transformations were successful. The efficiency of transformation was extremely high, because all bacteria transformed with By-product died due to *cdd*B-protein toxicity and antibiotic sensibility. Expression clones from one colony per each transformation were purified with QIAprep Spin Miniprep Kit.

4.1.4. Mini Expression Test

We wanted to test the expression of prepared constructs in different *E.coli* strains, so we could define which bacteria gives the best results.

First we determined the efficiency of competent cells using pUC19 plasmid. To calculate efficiency in cfu (colony forming units)/µg the following formula was used:

***Equation* 3:**

$$\text{Transformation efficiency (cfu/ µg pUC19 DNA)} = \frac{\text{\# Colonies (colony forming units)}}{\text{pg pUC19 transformed}} \times \frac{10^6 \text{ pg}}{\text{µg}} \times \frac{\text{Final volume (µl) of transformation mix}}{\text{Volume plated (µL)}}$$

Strains tested were:

- BL21(DE3),
- BL21(DE3)-Star,
- BL21(DE3)-pRARE2, and
- Lemo21(DE3).

Table II: The efficiency of chemical competent cells

***E.coli* strains**	**Efficiency (cfu/µg pUC19)**	**Antibiotic**
BL21(DE3)	$5*10^4$	None
BL21(DE3)-Star	$1.5*10^6$	None
BL21(DE3)-pRARE2	$5*10^5$	Chloramphenicol (34µg/ml)
Lemo21(DE3)	$2*10^6$	Chloramphenicol (34µg/ml)

All strains showed sufficient efficiency (Table II) thus all of them were used for transformation of Expression clones. Four bacterial strains and five vectors (SC0033, SC0034, SC0035, SC0036 and SC0037) gave 20 different combinations that were plated on agar plates separately. After an overnight growth at 37°C, colonies were observed for all transformations meaning that all of them worked.

One colony from each transformation was used to prepare preculture that served for preparation of 4 ml of bacterial cultures. When the mean value of OD_{600nm} of cultures reached 0.5 cells were induced with IPTG in order to produce large amounts of required protein. Cultures were incubated at 25°C for 20 hours. Significantly increased OD_{600nm} proved the efficient bacterial growth of all samples.

Obtained cultures were centrifuged. Smaller amounts of all pellets were mixed with 15 µL of WB Loading buffer 5x and detected on SDS-PAGE gel stained with Blue Coomassie (Figure 21).

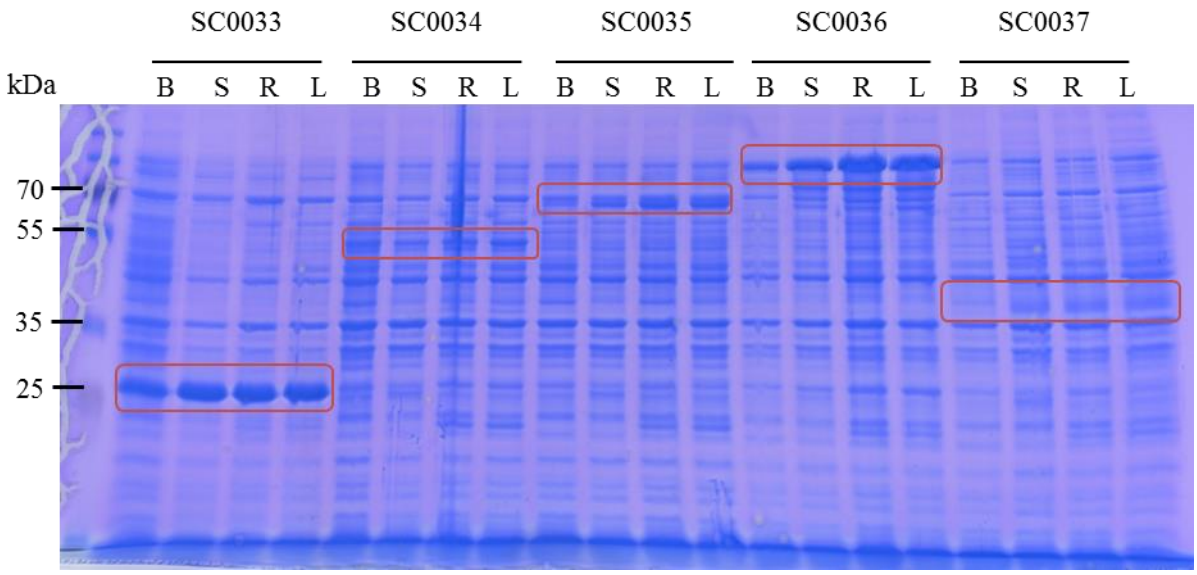

Figure 21: Expression of E-Cadherin C-terminal from all transforming combinations. Bacterial strains (B, S, R, L), transformed with all vectors (SC0033, SC0034, SC0035, SC0036, SC0037) expressed required proteins of different sizes. Results were observed after staining the SDS-PAGE gel with Coomassie blue. Bacterial strains are marked B – BL21(DE3), S – BL21(DE3) – Star, R – BL21(DE3) – pRARE2, L – Lemo21(DE3).

Size of expressed proteins differed due to different tags fused to them:

SC0033: 6xHis-tag E-Cadherin C-terminal: 20.9 kDa

SC0034: 6xHis and GST-tag E-Cadherin C-terminal: 46.6 kDa

SC0035: 6xHis and MBP-tag E-Cadherin C-terminal: 67.9 kDa

SC0036: 6xHis and NusA-tag E-Cadherin C-terminal: 75.8 kDa

SC0037: 6xHis and TRX-tag E-Cadherin C-terminal: 32.8 kDa

From the Figure 21 we could see that the construct SC0033 showed the best expression in all bacterial strains. These proteins were the smallest, due to only one fused tag (6xHis). Smaller proteins are probably easier for bacteria to produce.

But on the other hand, second best expression was shown by the biggest construct (SC0036) especially in BL21(DE3) – pRARE2 and Lemo21(DE3) cells. The lower expression in BL21 (DE3) strain was probably the result of the induction with IPTG at much lower OD_{600nm} than the average value was.

Expressions from SC0034 and SC0037 were the less significant.

4.1.5. Solubility test

In order to get the biggest amounts of expressed proteins from bacteria, we determined protein solubility in different lysis buffers. For the test we used SC0033 construct that showed the best protein expression (section 4.1.5. Mini expression test).

Pellets from all bacterial strains from Mini expression test were suspended in 1.6 mL of LB medium and dispensed 8-times 200 µL in 96-well deep plate. Suspensions were centrifuged at 2500 x g for 15 minutes and the supernatant was discarded.

Pellets were resuspended in different lysis buffers according to the equation 4.

Equation 4:

$$V_L=(OD/2) * V_C * 100$$

V_L = volume of lysis buffer

V_C = volume of culture

Knowing that the mean OD_{600nm} reached was 6.78 and that in each well we had 0.5 mL (4 mL/8) of culture, V_L was 170 µL.

Tested buffers were:

1. 50mM Tris-HCl pH 7 – 10% Glycerol – 150mM KCl – 0.5mg/ml lysozyme
2. 50mM Tris-HCl pH 7 – 10% Glycerol – 400mM KCl – 0.5mg/ml lysozyme
3. 50mM Tris-HCl pH 8 – 10% Glycerol – 150mM KCl – 0.5mg/ml lysozyme
4. 50mM Tris-HCl pH 8 – 10% Glycerol – 400mM KCl – 0.5mg/ml lysozyme
5. 100mM K_2HPO_4/KH_2PO_4 pH 7 – 10% Glycerol – 150mM KCl – 0.5mg/ml lysozyme
6. 100mM K_2HPO_4/KH_2PO_4 pH 7 – 10% Glycerol – 400mM KCl – 0.5mg/ml lysozyme
7. 100mM K_2HPO_4/KH_2PO_4 pH 8 – 10% Glycerol – 150mM KCl – 0.5mg/ml lysozyme
8. 100mM K_2HPO_4/KH_2PO_4 pH 8 – 10% Glycerol – 400mM KCl – 0.5mg/ml lysozyme

Plate containing suspended cells was frozen at -20°C overnight. The day after, cells were thawed for 3 hours at 4°C under shaking. DNAseI (20 ng/µL final) was added for an additional 30 minutes at 4°C under shaking. The plate was centrifuged for 30 minutes at 4000 x g at 4°C.

15 µL of each supernatant containing soluble proteins were mixed with 5 µL of Loading buffer 2x solution and analyzed by staining of SDS-PAGE gel with Coomassie blue. As a control, 25 µL of the total extract obtained for SC0033 in BL21(DE3) were loaded.

Proteins with better solubility showed stronger bond on gel (Figure 22). Conditions 4, 6 and 7 resulted in obtaining partially soluble protein expressed in either BL21(DE3) or in BL21(DE3)-Star while conditions 2, 4 and 6 resulted in obtaining partially soluble protein expressed in either BL21(DE3)-pRARE2 or in Lemo21(DE3).

We assumed that results for expressed proteins of other constructs (SC0034-SC0037) would be similar.

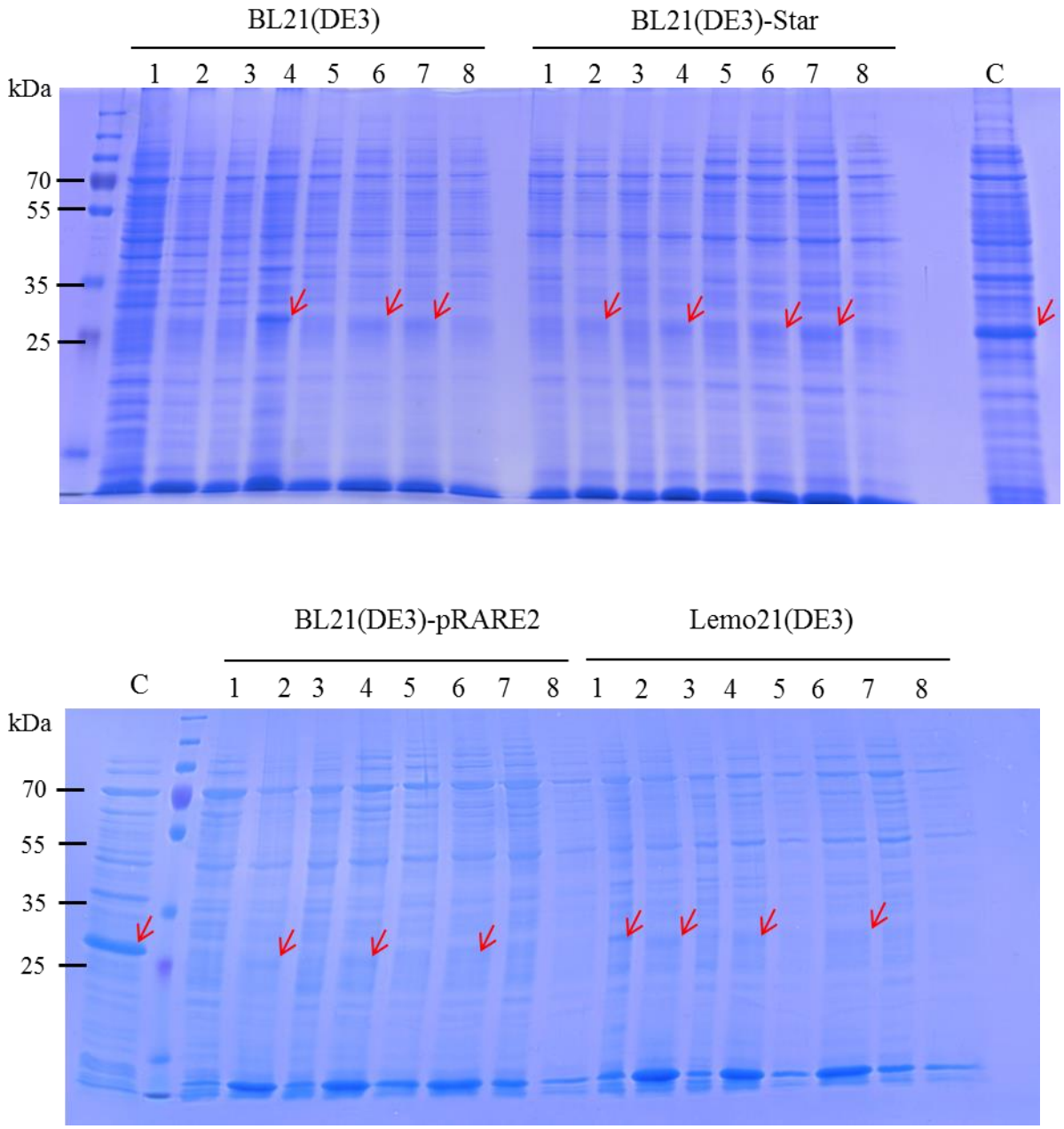

Figure 22: Solubility test. Expressed proteins from different bacterial strains transformed with SC0033 plasmid were extracted with different lysis buffers. SDS-PAGE gel was stained with Coomassie blue. Tested buffers: numbers 1-8. Their composition is described in the text above. Arrows indicate expressed E-Cadherin with better solubility in different buffers. Bacterial strains are marked: B – BL21(DE3), S – BL21(DE3) – Star, R – BL21(DE3) – pRARE2, L – Lemo21(DE3). Control is indicated with letter "C".

4.1.6. Protein expression and purification

According to results from mini expression and solubility test we decided to proceed to larger protein expression using BL21(DE3) *E.coli* transformed with SC0033 and SC0035 respectfully. SC0033 was chosen because it showed the largest protein expression while SC0035 was chosen randomly. BL21(DE3) were used because of the best solubility of expressed proteins (in buffer 4).

Fresh bacteria were used for plasmid transformation. Cells were plated on agar plate. After an overnight growth at 37°C, many colonies were observed. One colony from each transformation was used to prepare pre-culture for 250 mL of bacterial culture. When OD_{600nm} reached 0.4 – 0.5, cells were induced with IPTG (0.5 mM final concentration). Cells were incubated at 25°C overnight shaking at 200 RPM. Next day bacteria were lysed with the lysis buffer 4 from the solubility test (section 4.1.6.) (50 mM Tris-HCl pH 8, 10% Glycerol, 400 mM KCl, 0.5mg/ml lysozyme) and sonicator. Proteins were purified with Ni-NTA purification column. Collected fractions (flow through, wash 1, wash 2, elution 1, and elution 2) were identified by Western Blot. In order to check the loss of proteins in different fractions, all of them were examined. To verify if eluted proteins contained 6xHis-tag, anti-6xHis-tag antibodies were used. Eluates 1 and 2 were also identified with anti-E-Cadherin antibodies in order to guarantee that purified protein is E-Cadherin C-terminal.

4.1.6.1. Expression clone SC0033

Western Blot using anti-6xHis primary antibodies did not confirm the presence of 6xHis-tag on purified proteins. Neither of eluted fractions showed any signal on photographic film. However E-Cadherin was identified in elution fractions by anti-E-Cadherin primary antibodies, with much stronger bond in the second eluate (Figure 23). After an overnight dialysis with Assay Wash Buffer, pH 8.0 concentrations of eluates were measured by nanodrop. Results confirmed the presence of proteins. Concentration of the second eluate was almost 4-times higher than the first one (Table III).

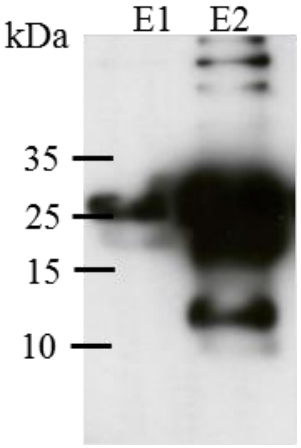

Figure 23: WB using anti-E-Cadherin primary antibodies. E-Cadherin is present in both eluates. Obviously second elution was more successful, because the concentration of E-Cadherin in second eluate is much bigger. Expected size of expressed protein was 20.9 kDa. E1 - first eluate, E2 - second eluate.

Table III: Amounts of purified E-Cadherin C-terminal from SC0033 plasmid

	Eluate 1	**Eluate 2**
c	0.067 mg/ml	0.257 mg/ml
m	17.3 µg	77.0 µg

According to results we assumed that there was the problem with 6xHis-tag on expressed protein. There might have been the problem with tag folding or some other problem that did not allow detection of 6xHis-tag immerged.

To immobilize protein on magnetic Ni-NTA beads for phage display selection of antibody fragments efficient 6xHis-tag was essential, so obtained protein was useless.

4.1.6.2. Expression clone SC0035

On the contrary to the previous sample, the protein expression with clone SC00035 showed expected results. Anti-6xHis primary antibodies in WB confirmed the presence of small amounts of 6xHis-tag in the sample that was taken before purification. Amount increased drastically after IPTG induction. During Flow Through some proteins were lost, but most of them were collected in elution fractions. Anti-E-Cadherin antibodies confirmed that eluted protein was E-Cadherin C-terminal (Figure 24).

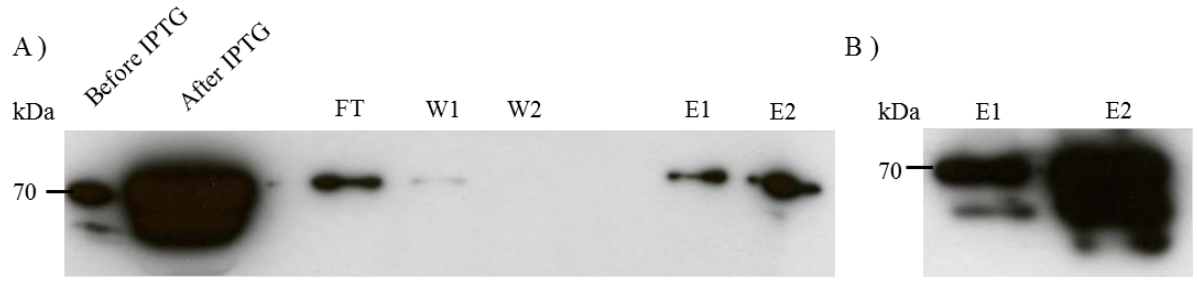

Figure 24: Purification of expressed protein from SC0035 construct. A) For WB anti-6xHis-tag antibodies were used. All purification fractions were analyzed. Expected size of expressed protein was 67.9 kDa. B) Antibodies anti-E-Cadherin confirmed the presence of E-Cadherin C-terminal in both eluates. FT – flow through, W1 - washing 1, W2 – washing 2, E1 – elution 1, E2 – elution 2

After overnight dialysis with Assay Wash Buffer, pH 8.0 protein concentrations were measured spectrophotometrically by nanodrop. The second eluate was approximately twice as concentrated as the first one (Table IV).

Table IV: Amounts of purified E-Cadherin C-terminal from SC0035 plasmid

	Eluate 1	**Eluate 2**
c	0.173 mg/ml	0.310 mg/ml
m	34.6 µg	68.2 µg

Protein expressed from SC0035 construct presented all the properties required for further steps. Because eluate 2 contained more proteins, that fraction was used for phage display selection. Since we used only 10 µg of proteins per selection, the obtained amounts satisfied for 6 selections.

Not very high amount of obtained proteins could be the result of poor protein solubility or of its toxicity to bacteria cells. But if there would be a need to scale up the production of protein, the volume of transformed bacteria producing protein could be easily increased. We could also change the bacterial strain.

Because expressed proteins contain also MBP-tag, purification column that purifies proteins with that tag could be used for achieving better purity. Furthermore purification with HPLC could be used. Because of the limited time we did not proceed all these purification methods, but they would definitely improve the purity of the product. However, a lot of proteins would be lost.

4.2. Extraction and purification of E-Cadherin from eukaryotic cells

Protein extraction was performed from seven different cell lines. Cells with highest concentration of E-Cadherin were used for further procedures. Strongest bonds of E-Cadherin were identified by WB test with anti-E-Cadherin primary antibodies. They were purified by extraction from SDS-PAGE gel and submitted to enzymatic degradation. Again the strongest bonds were identified by WB (with anti-E-Cadherin antibodies) and further purified by extraction from SDS-PAGE gel. Finally the smallest particles, recognized by anti-E-Cadherin antibodies were purified by immunoprecipitation.

4.2.1. Protein extraction from eukaryotic cells

E-Cadherin protein was extracted from seven different types of human breast carcinoma cell lines: MCF-7, T-47D, BT-20, ZR-75-1, MB-436, MB-231, MB-157 with RIPA buffer.

Extracted E-Cadherin was confirmed in first four cell lines by WB, where anti-E-Cadherin antibodies were used as primary antibodies. Apparently last three cell lines did not express any E-Cadherin, because there was no signal for it on photographic film (Figure 25).

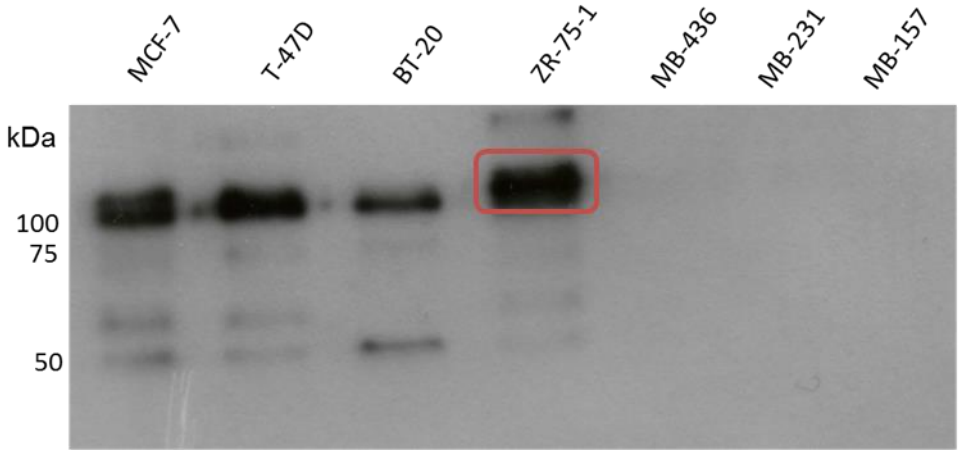

Figure 25: Extracted proteins from human carcinoma cell lines. Presence of E-Cadherin protein was confirmed by WB with anti-E-Cadherin primary antibodies. MCF-7, T-47D, BT-20 and ZR-75-1 cells expressed E-Cadherin while there was no E-Cadherin identified in MB-436, MB-231 and MB-157 cells extracts. ZR-75-1 cells showed the strongest expression of E-Cadherin.

The most important expression was observed from ZR-75-1 cells so we decided to proceed with this cell line. Amount of extracted proteins was measured by Qubit fluorometer.

From **$20*10^6$ ZR-75-1 cells 31 mg of proteins** were obtained.

4.2.2. Purification of E-Cadherin from SDS-PAGE gel

To identify the most important bonds of E-Cadherin from the extracted sample from ZR-75-1 cells another Western Blot with anti-E-Cadherin primary antibodies was performed.

The strongest bonds were seen at approximately 35, 60 and above 100 kDa. The whole sample with extracted proteins was applied on SDS-PAGE gel. These bonds were cut off and proteins were extracted from gel. Extracted proteins were identified by WB with anti-E-Cadherin primary antibodies (Figure 26).

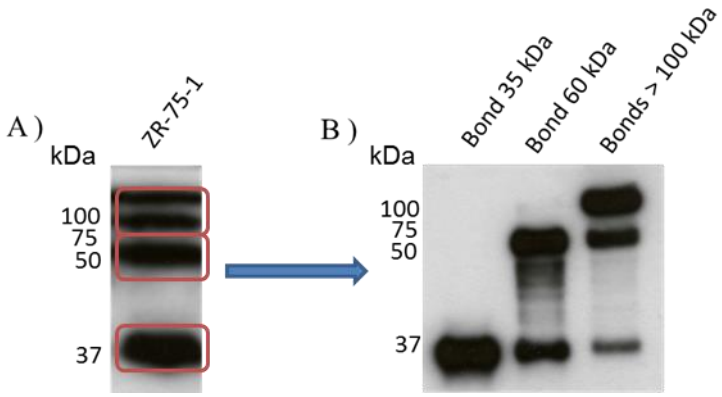

Figure 26: Extracted bonds of E-Cadherin. A) WB with anti-E-Cadherin primary bodies results of extracted proteins from ZR-75-1 cells. Circled bonds are the most important ones. They were purified by extraction from SDS-PAGE gel. B) WB of purified bonds of interest from SDS-PAGE gel. The fact that there is a bond of 36 kDa present in all three samples, and a bond of 60 kDa in the sample that should contain only proteins larger than 100 kDa indicates that bonds of 35 and 60 kDa may be the degradation products of E-Cadherin.

Results of WB confirmed that we purified specific bonds of interest from the SDS-PAGE gel. The bond of 35 kDa was present in all samples and bond of 60 kDa was present in the sample of proteins larger than 100 kDa. That indicates that protein underwent some degradation.

Amounts of purified proteins were measured by Qubit fluorometry (Table V).

Table V: Quantity of bonds 30, 60, >100 kDa, measured by Qubit fluorometer

Sample	Quantity (mg)
Bond 30 kDa	1.15
Bond 60 kDa	1.00
Bonds >100 kDa	1.34

4.2.3. Digestion of proteins with restriction enzymes

For better specificity when confirming antibody fragments selected by phage display, we wanted to prepare the smallest particle possible of E-Cadherin that was still recognized by anti-E-Cadherin antibodies. To achieve that, purified bonds were mixed together and submitted to enzymatic digestion. A small amount of sample was used for testing different enzymes, trypsin and endoproteinase GluC. Digestion took place for 1, 5 and 10 minutes. Results were detected by WB with the anti-E-Cadherin primary antibodies (Figure 27). After trypsin digestion we did not observe any E-Cadherin bonds. There are two possible explanations of this. Firstly, trypsin is quite a strong digestion enzyme so it is possible that our sample was completely degraded. Secondly, digestion products might have not been recognized by E-Cadherin antibodies.

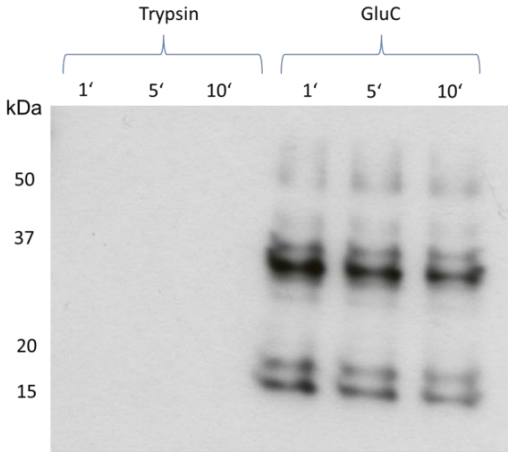

Figure 27: Digestion of E-Cadherin with trypsin and gluC enzymes. Digestion of E-Cadherin bonds (35, 60, above 100 kDa) took 1, 5 and 10 minutes. After WB with anti-E-Cadherin primary antibodies, there was no signal for samples submitted to trypsin digestion. The strongest bonds after gluC digestion were seen at around 35 kDa and 18 kDa. Time of digestion did not have any influence on the final result.

Quite differently digestion with gluC enzyme resulted in many smaller bonds recognized by E-Cadherin antibodies. A strong bond at 35 kDa was still present, but there were also strong smaller bonds (18 kDa) observed. Bonds, larger than 35 kDa were very few and weak indicating that protein degraded completely. There was no signal at the size of the whole protein (120 kDa). There was no big difference between 1, 5 or 10 minutes digestion so obviously time of the digestion did not play an important role. According to results above, the whole sample was submitted to digestion with gluC enzyme for 1 minute.

4.2.4. Purification of degraded E-Cadherin from SDS-PAGE gel

Purification of proteins by extraction from SDS-PAGE gel was performed for smaller bonds that resulted from enzymatic degradation of protein. This time we purified bonds of 18 kDa, 25-50 kDa and >50 kDa. Purification products were confirmed by WB with anti-E-Cadherin antibodies. Only the bond of 18 kDa was observed on WB (Figure 28). Concentrations of other bonds were probably too small to be identified by the test. Because the bond of 18 kDa was the smallest one still recognized by anti-E-Cadherin antibodies and our aim was achieved. There was no need to confirm other bonds.

Concentrations of all bonds were successfully measured by nanodrop (Table VI).

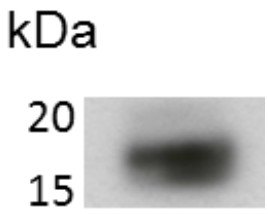

Figure 28: Purified bond of 18 kDa. WB with anti-E-Cadherin primary antibodies confirmed isolated bond of 18 kDa by anti-E-Cadherin antibodies.

Table VI: Quantity of bonds 18, 25-50, >50 kDa, measured by nanodrop

Sample	Quantity (µg)
Bond 18 kDa	835
Bond 25-50 kDa	250
Bonds >50 kDa	232

4.2.5. Immunoprecipitation

As a final purification step immunoprecipitation was used. Dynabeads Protein G® were crosslinked with anti-E-Cadherin antibodies. Usually huge amounts of proteins are lost during immunoprecipitation. Because we did not want to lose too many sample, we immoprecipitated only 100 µg of 18 kDa bonds.

WB with anti-E-Cadherin primary antibodies was performed, but immunoprecipitated amount of proteins was too small for identification. Therefore the identification of immunoprecipitated sample was not possible until the last step of present work, where immunoprecipitated protein was used for targeting phages with anti-E-Cadherin antibody fragments, selected by phage display.

However, the amount of immunoprecipitated proteins was measured by nanodrop (Table VII).

Table VII: Quantity of immunoprecipitated bonds 15-25 kDa, measured by nanodrop

Sample	Quantity (µg)
IP bonds 15-25 kDa	20

4.2.6. Final balance

During the whole procedure a huge amounts of proteins were lost (Figure 29). From $20*10^6$ ZR-75-1 cells 31 mg of proteins were obtained. During purification from SDS-PAGE gel 27.5 mg were lost. Another 2 mg were lost during digestion and second purification from SDS-PAGE gel. So finally 1.3 mg of proteins were obtained, from which we only used 0.8 mg of 18 kDa fraction.

During immunoprecipitation the loss of proteins was 5-fold, meaning that 0.02 mg were eluted from 0.10 mg. If the whole purified bond of 18 kDa was immunoprecipitated, 0.17 mg of proteins would have been collected.

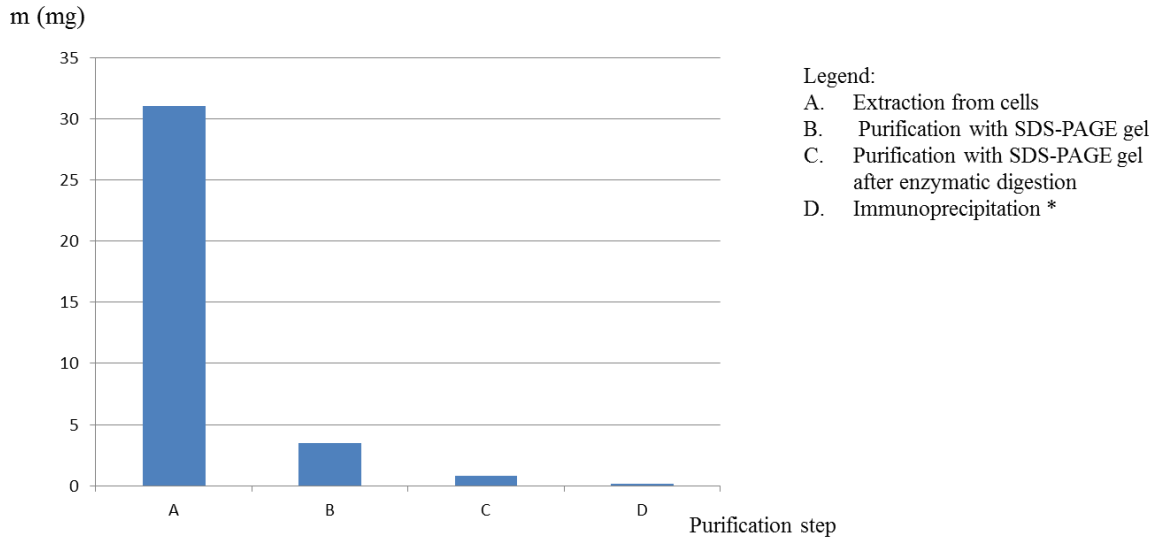

Figure 29: Loss of E-Cadherin during extraction and purification. From $20*10^6$ cells 31 mg of proteins were extracted. After purification from SDS-PAGE gel only 3.5 mg remained. Purification of degraded particles gave 0.8 mg of bond of 18 kDa. Because of commonly significant loss during immunoprecipitation, we decided to immunoprecipitate only 0.1 mg of sample. *From the obtained results the amount was estimated for the immunoprecipitation of the whole sample.

4.3. Phage display

Phage display technology is a powerful method for the selection of monoclonal antibody fragments against an almost unlimited array of biological and non-biological targets. Technology is very simple to use and selection of antibody fragments does not take lot of time.

For the procedure we first thawed the frozen glycerol stock of the repertoire phagemid in TG1 bacteria. Phages were produced in liquid culture after infection with helper phage KM13. They were purified by PEG precipitation and enriched by binding to antigen immobilized to magnetic beads (Figure 30). Non bound phages were washed away. Those that attached to immobilized antigen were eluted with trypsin protease, where a c-myc tag between antibody fragment and the phage gene III protein was cut. Trypsin also removed the background infectivity of trypsin-sensitive helper phage. The eluted phage was used for infection of fresh TG1 bacteria and titers were defined by plated dilution series.

To perform second round of selection, colonies from the first round were scraped from agar plates. Phages were produced in liquid culture and purified by PEG precipitation. The selection procedure by immobilized antigen was repeated.

Finally soluble antibody fragments were extracted from bacteria. Randomly selected clones were immobilized to adhesive slides and incubated with E-Cadherin obtained by Gateway cloning technology, labeled with fluorescent dye. Positive phages were identified by fluorescent scanner and used for further confirmation of their binding properties towards wild type E-Cadherin, prepared as described in section 4.2. (Extraction and purification of E-Cadherin from eukaryotic cells). Because particle, purified by immunoprecipitation needed to be confirmed, a parallel test with sample that did not undergo immunoprecipitation was performed.

Phage display was first performed with anti-ubiquitin as positive control clones and anti-BSA as negative control clones for testing the quality of libraries and defining all parameters of the protocol. For the selection of scFv fragments against E-Cadherin C-terminal libraries Tomlinson I + J were used (section 1.4.3.).

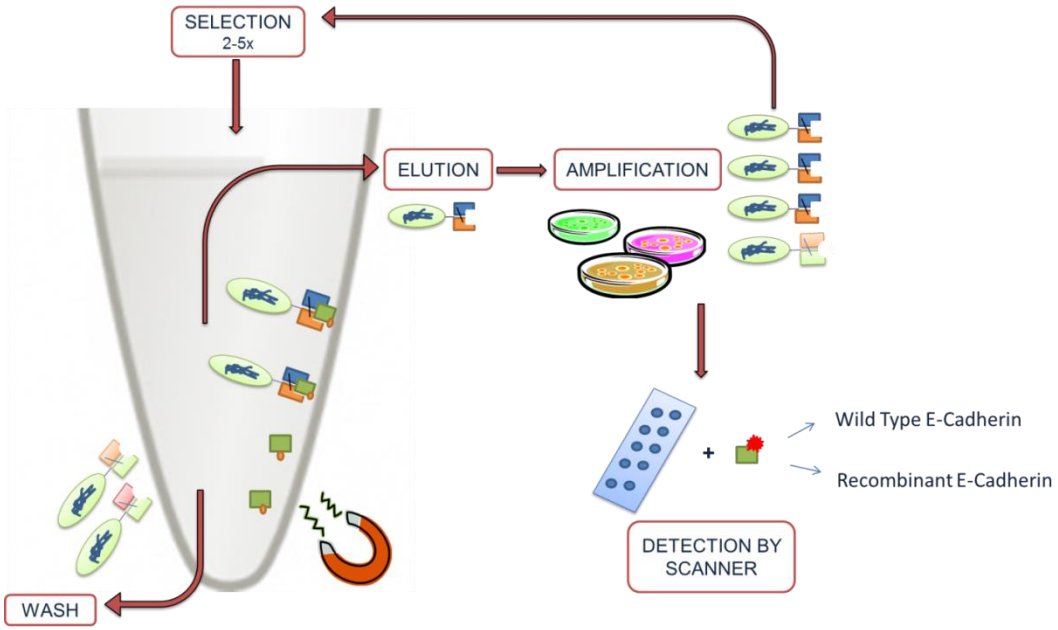

Figure 30: Schematic presentation of phage display. Phage display libraries were incubated with the solution of antigen, immobilized to magnetic beads. Non-bound phages were washed away. Bound phages were eluted and amplified in bacterial culture. For better specificity of selected scFv fragments, selection could be repeated several times. Finally affinity of selected fragments for wild type protein was defined by fluorescent scanner.

4.3.1. Preparation of helper phage KM13

Helper phage KM13 was prepared according to the Tomlinson I + J Libraries protocol. Larger amounts of phage were prepared from one small plaque from agar plate (Figure 31).

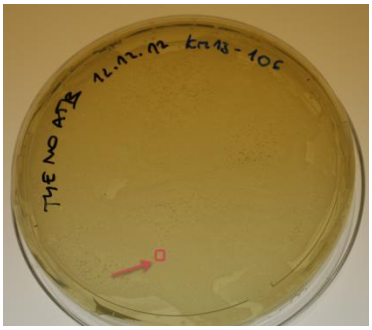

Figure 31: Helper phage plaques on agar plate. Arrow indicates the plaque that was picked for production of larger amounts of KM13.

4.3.1.1. Estimation of Phage Titers

OD_{260nm} was measured by nanodrop.

$OD_{260nm} = 0.107$

Phage / ml = $0.107 * 100 * 22.14 * 10^{10}$ = **$2.369 * 10^{12}$ phages/ml**

4.3.1.2. Test trypsin cleavage

To test the efficiency of purified helper phage, Trypsin cleavage test was performed as described in the protocol. KM13 phage is sensitive to trypsin degradation, thus incubation with this enzyme decreases its efficiency.

Number of colonies obtained from trypsin treated phage was much smaller than for non-treated phage. That confirmed that helper phage was efficient, because cells, that were not infected by helper phage did not gain kanamycin resistance and thus died on agar plate suplemented with kanamycin.

4.3.2. Phage display for anti-ubiquitin control clones

As a positive control anti-ubiquitin scFv in bacterial strain TG1 were used. Anti-BSA scFv in bacterial strain TG1 presented negative control.

4.3.2.1. Growing the cultures with control clones

Control clones were prepared according to the protocol. Phages for positive and negative control were prepared separately.

Estimation of anti-ubiquitin phage titers

OD_{260nm} measured by nanodrop: OD_{260nm}=0.548
Phage / mL = 0.548 * 100 * 22.14 * 10^{10} = **$1.213 * 10^{13}$ phages / ml**

Estimation of anti-BSA phage titers

OD_{260nm} measured by nanodrop: OD_{260nm}=0.337
Phage / mL = 0.337 * 100 * 22.14 * 10^{10} = **$7.461 * 10^{12}$ phages / ml**

4.3.2.2. First round of selection

Protein ubiquitin did not have the 6xHis tag attached; therefore we could have not used Ni NTA magnetic agarose beads for antigen immobilization. Instead Nexterion® slide E for protein application was used.

10 µL of ubiquitin diluted in PBS (0.2 mg/ml) were spotted on slides as showed on Figure 32. When they were completely dry, they were saturated with 1% BSA in PBST 0.05% for 1 hour at RT. Slides were washed 3x with PBST 0.05% and 2x with PBS.

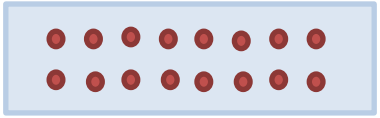

Figure 32: Schematic presentation of adhesive slide with applied proteins.

Control phages, diluted 1:100 in PBS were first incubated with empty slides that were saturated with 1% BSA in PBS and shook on lab roller for 1 hour at RT. Non-bound phages were used for selection on ubiquitin slides. After 2 hours incubation on lab roller at RT, they were washed 5x with PBS-Tween 0.1% and 3x with PBS. Finally phages were eluted with 4 mL of trypsin solution for 1 hour at RT.

Estimation of anti-ubiquitin phage titers

OD_{260nm} measured by nanodrop: OD_{260nm}=0.004
Phage / mL = 0.004 * 100 * 22.14 * 10^{10} = **$8.856 * 10^{10}$ phages / ml**

Estimation of anti-BSA phage titers

OD_{260nm} measured by nanodrop: OD_{260nm}=0.007
Phage / mL = 0.007 * 100 * 22.14 * 10^{10} = **$1.550 * 10^{11}$ phages / ml**

4.3.2.3. Amplification

For amplification of phages three different methods were used, because we wanted to define the one that gives best results. For first two options dilution series of phages in PBS (10^{10} to 10^{1}) were prepared.

1) 10 µL of each dilution series was used to infect 200 µl of TG1 bacteria at OD_{600nm} 0.5 in water bath at 37°C for 30 minutes. 3 mL of H-top agar were added and cultures were poured on pre-warmed TYE plates. They were incubated overnight at 37°C.
2) Bacteria were grown on the whole surface of TYE plate. Phages from dilution series were sprayed to plates from a 10 cm distance with a perfume-tester spray of 5 mL. Plates were incubated overnight at 37°C.
3) To 1.75 ml of TG1 bacteria at OD_{600nm}=0.5 250 µL of eluted phage were added. Cultures were incubated in water bath at 37°C for 30 minutes. 10 µL of the culture and 10 µL of 1/1, $1/10^{2}$, $1/10^{4}$ dilutions in 2xTY were spotted on TYE plates containing 100 µg/mL ampicillin and 1% glucose. Bacteria grew overnight at 37°C.

Phages from method 1 and 2 formed small plaques of lysed bacteria overnight. They were picked from plates and directly detected by scanner. For the detection they were directly

picked from the plate with a sterile toothpick. Obtained colonies from the third method were used for the production of soluble fragments.

Because third method allowed direct production of soluble fragments, we decided to use it also for the selection of anti-E-Cadherin scFvs.

4.3.2.4. Detection on the fluorescent scanner

For the detection of phages from methods 1 and 2, phages scraped off plaques from TYE plates were directly applied to Nexterion adhesive slide. Soluble fragments from method 3 were applied in 1 µL drops. After saturation of the slide with 1% BSA in PBST 0.05%, ubiquitin (0.2 mg/ml) labeled with fluorescent dye Dylight 633 was added to target positive clones.

Because we did not want to contaminate scanner with phages, phages were disinfected by ethanol before the detection.

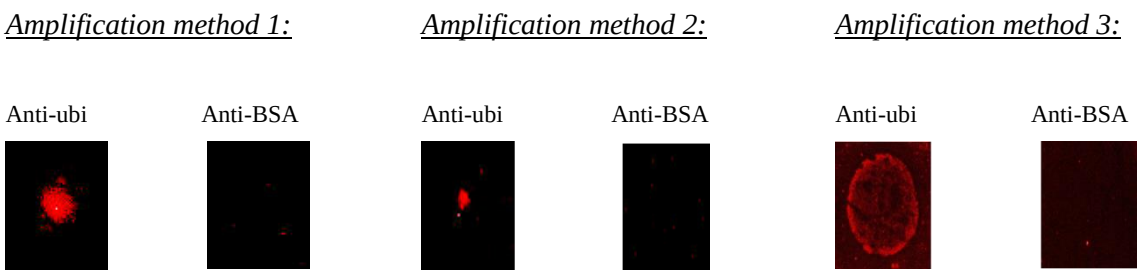

Figure 33: Selected positive phages against ubiquitin target. Ubiquitin, labeled with fluorescent dye Dylight 633 attached to immobilized scFv fragments. Fluorescence detected by scanner corresponded to the quantity scFv-ubiquitin complex. Fragments with stronger affinity towards antigen attached more protein which resulted in stronger signal. If scFv did not show any affinity to ubiquitin, no signal was observed on scanned image. Generally, a positive signal was observed where most of the positive clones were applied, while the negative controls gave no fluorescent signal. There was no significant difference between different amplification methods.

Immobilized anti-ubiquitin scFv fragments captured antigen. Scanner detected the fluorescent signal of labeled ubiquitin (Figure 33). The stronger the affinity of antibody fragments was, more proteins were attached, thus stronger the signal on the scanner was. From positive anti-ubiquitin control most of phages proved to be positive, while phages selected from negative anti-BSA control showed no positive signal against ubiquitin. From these results we concluded that protocol is suitable for further use in selection of anti-E-Cadherin scFvs.

4.3.3. Libraries Tomlinson I + J

4.3.3.1. Growing the libraries

Libraries were grown according to the Tomlinson protocol. We prepared also the secondary stock and stored it at -80°C.

Estimation of Tomlinson I phage titers

OD_{260nm}, measured by nanodrop: OD_{260nm}=0.020

Phage / mL = 0.020 * 100 * 22.14 * 10^{10} = **4.428 * 10^{11} phages / ml**

Estimation of Tomlinson J phage titers

OD_{260nm}, measured by nanodrop: OD_{260nm}=0.036

Phage / mL = 3.600 * 100 * 22.14 * 10^{10} = **7.970 * 10^{10} phages / ml**

Phage titers were also estimated by plating infected bacteria on TYE plates.

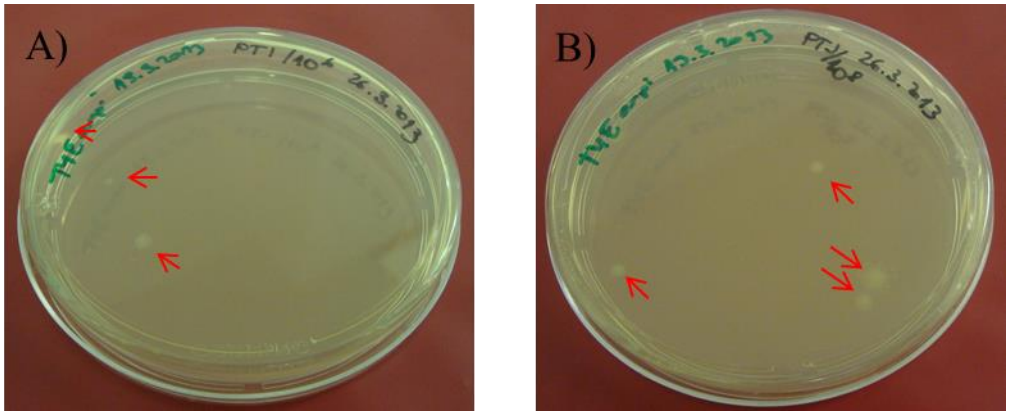

Figure 34: Phage titer for Tomlinson I and J libraries. A) From Library I last dilution series that resulted in colonies was $1/10^6$ with three colonies. B) Library J grew four colonies in dilution $1/10^8$. According to these results, there are $3*10^8$ phages/mL in Library I and $4*10^{10}$ phages/mL in Library J.

Phages from libraries infected bacteria and gave them ampicillin resistance, so that they were able to grow colonies on TYE plates supplemented with ampicillin. The least concentrated dilution from dilution series of phages that still grew colonies on TYE plates from Tomlinson I Library was $1/10^6$, which resulted in three colonies. Dilution of still efficient phage from Library J was $1/10^8$ and grew 4 colonies (Figure 34).

Tomlinson I phage titers:

Phage / ml = $3 * 10^6$ / 0.01 mL = **$3 * 10^8$ phages/mL**

Tomlinson J phage titers:

Phage / ml = $4 * 10^8$ / 0.01 mL = **$3 * 10^{10}$ phages/mL**

Titers from plated phages are slightly smaller than those from spectrophotometry. This may be the consequence of inaccuracy during work. The infected bacteria might have not been mixed or spread on plates homogenously. There is also a possibility that spectrophotometer did not measure OD accurately. However, the exact number is not that important for this work. By tittering the phage we proved that there were many phages in Libraries, providing a wide spectrum of different scFv fragments.

4.3.3.2. First round of selection

For the selection antigen (recombinant E-Cadherin C-terminal, obtained by Gateway cloning, section 4.1.) was immobilized to Ni-NTA magnetic agarose beads by 6xHis-tag of protein. The selection was performed with both libraries in parallel. To remove non-specific binders, libraries were first incubated with empty beads, saturated with 1% BSA in PBS. Only non-bound phages entered the selection by immobilized antigen.

One half of phages in the first round of selection were eluted with trypsin solution while 0.1 M glycine, pH 2.7 was used for the second part. Phages eluted with glycine were further neutralized with 1 M Tris, pH 9.

250 µL of eluted phage were used for infection of 1.75 mL of TG1 at an OD_{600nm} 0.4. Dilution series (1/1, $1/10^2$, $1/10^4$) were prepared for tittering the phage. Remaining TG1 culture was centrifuged at 11 600 x g in a micro centrifuge for 5 minutes. Pelleted bacteria were resuspended in 50 µL of 2xTY and plated on a regular TYE plate containing 100 µg/mL ampicillin and 1 % glucose. Plates were grown at 37°C overnight.

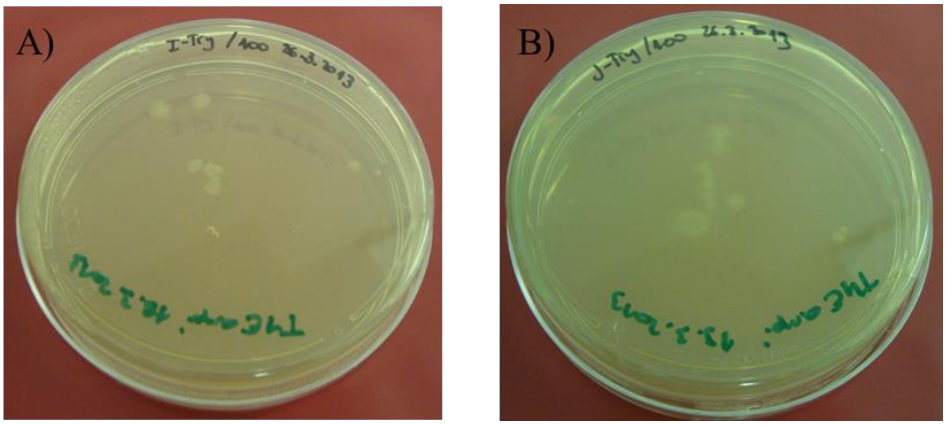

Figure 35: **Phage titer for trypsin eluates from libraries I and J after first round of selection.** A) $1/10^2$ dilution of Library I resulted in 10 colonies, indicating that there are $1*10^5$ phages/mL. B) $1/10^2$ dilution of Library J resulted in 14 colonies, indicating that there are $1.4*10^5$ phages/mL.

Bacteria infected with phages eluted with trypsin resulted in many colonies, while those infected with phages, eluted with glycine, did not grow on ampicillin containing TYE. From these results we concluded, that glycine did not elute any phages. Bacteria infected with these eluates did not gain any ampicillin resistance and therefore did not survive on plates.

From the phage titer we calculated the number of phages per ml (Figure 35).

Tomlinson I phage titers:

Phage / ml = $10 * 10^2 / 0.01$ mL = **$1 * 10^5$ phages/mL**

Tomlinson J phage titers:

Phage / ml = $14 * 10^2 / 0.01$ mL = **$1.4 * 10^5$ phages/mL**

Number is significantly smaller than before the selection, meaning that many phages with no binding affinity towards given antigen were already lost.

4.3.3.3. Further rounds of selection

Second round of selection was performed as proposed in the protocol of the Libraries. This time only trypsin solution was used for elution process. Phage titers were defined by plating infected bacteria on TYE plates (Figure 36).

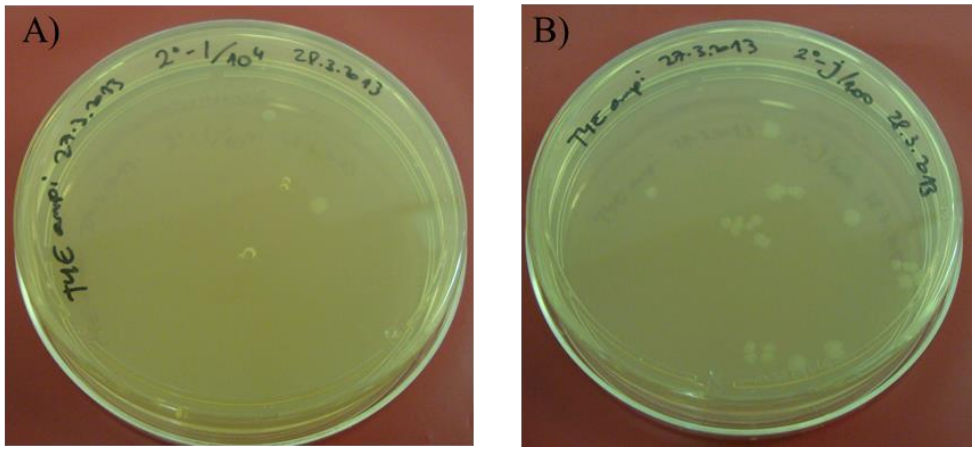

Figure 36: Phage titer for trypsin eluates from libraries I and J after second round of selection. A) $1/10^4$ dilution of Library I resulted in 2 colonies, indicating that there are $2*10^6$ phages/mL. B) $1/10^2$ dilution of Library J resulted in 23 colonies, indicating that there are $2.3*10^5$ phages/mL.

Tomlinson I phage titers:

Phage / ml = $2 * 10^4$ / 0.01 mL = **$2 * 10^6$ phages/mL**

Tomlinson J phage titers:

Phage / ml = $23 * 10^2$ / 0.01 mL = **$2.3 * 10^5$ phages/mL**

Normally number of phage titers should be smaller after every round of selection, because all phages that do not bind the antigen are washed away. In this case, number of phages after second selection is a bit higher than after the first selection. This could be the result of some smaller inaccuracies during the process.

Individual colonies that grew on TYE ampicillin plates after infection of fresh TG1 bacteria with phages from the second round of selection were used for the production of soluble fragments.

4.3.3.4. Confirmation of affinity of obtained soluble scFv fragments

To confirm the affinity of obtained soluble fragments for E-Cadherin, eight randomly selected phages from each library were identically immobilized to three Nexterion® slides. They were targeted with three types of protein, marked with Dylight 633 NHS-Ester Dye.

Proteins used as antigens were:

A) Wild Type E-Cadherin extracted and purified from ZR751 cells before immunoprecipitation
B) Wild Type E-Cadherin extracted and purified from ZR751 cells after immunoprecipitation
C) Recombinant E-Cadherin – C-terminal, produced by Gateway technology

Fluorescence was detected by fluorescent scanner. On the Figure 37 results of two different phages are presented.

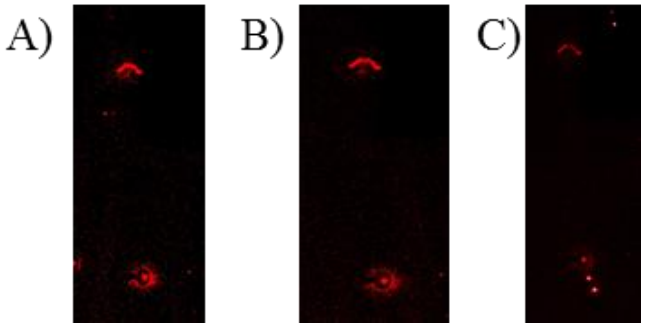

Figure 37: Results on fluorescent scanner of two different phages. Immobilized phages expressing scFv fragments captured antigen, labeled with fluorescent dye. Non bound antigens were washed away. Red spots detected by fluorescent scanner proved the presence of scFv fragments with binding affinity for E-Cadherrin particles. A) Phages were incubated with wild type E-Cadherin, non-imunoprecipitated. B) Slide was incubated with wild type E-Cadherin particles, purified by immunoprecipitation. C) Phages were incubated with recombinant E-Cadherin C-terminal. A,B,C) Both phages proved to be efficient with all three antigens.

Amongst all sixteen immobilized phages twelve of them resulted in antibody fragment-antigen complex, which fluorescence was detected by scanner. Four of them did not show sufficient binding properties towards given antigen. All twelve positive phages were equally efficient against all three differently prepared antigens. Therefore we could conclude that selected phages show appropriate affinity against recombinant and wild type E-Cadherin C-terminal. The signal was very similarly strong for all phages, which indicates that affinity of all of them was very equal. Binding of immunoprecipitated antigen also proved the efficiency of immunoprecipitation that could have not been determined by WB (section 4.2.5.). Any of these phages could be selected for further investigations for development of E-Cadherin detection method.

5. CONCLUSIONS

In the present work we managed to successfully produce recombinant E-Cadherin C-terminal by Gateway cloning technology. This method proved to be efficient for protein expression in bacteria. Gene of interest was successfully inserted into five different destination vectors that enabled the expression of E-Cadherin with different tags for purification. By expression test we defined the best strain of *E.coli* for protein expression. The most appropriate buffer was chosen by the solubility test. According to results we expressed bigger amount of E-Cadherin in bacteria. The obtained amount was sufficient for two selections by phage display. Amount of expressed proteins could be easily increased by using bigger volume of bacterial culture.

During the second stage of the research work we successfully extracted wild type E-Cadherin from human breast carcinoma cell line. To confirm high specificity of scFv antibody fragments selected by phage display, protein was degraded to smaller particles that were still recognized by anti-E-Cadherin antibodies and purified by immunoprecipitation. During the long procedure a large amount of protein was lost. The most critical parts were purification by extraction of specific bonds of interest from SDS-PAGE gel and immunoprecipitation.

Finally we performed two rounds of phage display selection of phages from libraries expressing scFv antibody fragments with binding affinity for recombinant E-Cadherin. Randomly selected phages were immobilized to adhesive slides and incubated with three types of same antigen, labeled with fluorescent dye: recombinant E-Cadherin from Gateway cloning, purified wild type E-Cadherin particle before immunoprecipitation, and wild type E-Cadherin particle purified by immunoprecipitation. Affinity of fragments was detected by fluorescent scanner. The signal was stronger there, where more protein attached to immobilized scFv fragment. The majority of immobilized phages showed strong affinity for all three antigens, proving that the selection procedure was efficient. Any of these positive phages could be used for the further development of a method for efficient detection of wild type E-Cadherin and thus circulating tumor cells.

6. REFERENCES

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