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# OPTIMIZACIJA KOMBINACIJE METODE SISTEMATIČNEGA RAZVOJA LIGANDOV Z EKSPONENTNO OBOGATITVIJO SELEX IN METODE PRENOSA WESTERN

# OPTIMIZATION OF THE COMBINATION OF SYSTEMATIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT SELEX AND WESTERN BLOT METHODS

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**Povzetek**

Sistematična evolucija ligandov z eksponentno obogatitvijo (SELEX) je *in vitro* metoda, s katero se izberejo nukleinske kisline, ki se z visoko afiniteto in specifičnostjo vežejo na načeloma vsako izbrano tarčo. Izbrani/obogateni oligonukleotidi, ki se imenujejo aptameri, so dobra zamenjava za protitelesa. Kratke enoverižne DNA ali RNA so bolj stabilne in cenejše, prav tako pa tudi ni potrebe po uporabi živali za njihovo proizvodnjo. Njihova selekcija temelji na izmenjavi izbiranja in pomnoževanja zaporedij, ki se vežejo na določeno tarčo in so sestavljena iz dveh regij: naključne in konstantne. Na slednjo se prilegajo oligonukleotidni začetniki, ki so edinstveni za vsako oligonukleotidno knjižnico. Vsaka selekcija se začne z ogromnim številom ($10^{14}$-$10^{15}$) zaporedij, ki se razlikujejo v naključni regiji. Sledi vezava zaporedij na izbrano tarčo, nato odstranitev nevezanih zaporedij in nazadnje prekinitev vezi med tarčo in na njo vezanimi zaporedji. Ta so potem pomnožena z verižno reakcijo s polimerazo (PCR), v kateri nastanejo pomnožene dvoverižne verige DNA, ki se jim v naslednjem koraku odstrani protismerna veriga. Tako nastale enoverižne verige se uporabijo v naslednjem ciklu SELEX-a, ki se začne s ponovno vezavo le-teh na tarčo.

Aptameri se lahko uporabijo v različne namene, npr. v imunoterapiji, za tarčno dostavo zdravil ali v diagnostične namene. Aptameri lahko predstavljajo dobro zamenjavo za protitelesa pri metodi prenosa western. Detekcija proteinov s protitelesi pri metodi prenosa western je običajno zelo drag in dolgotrajen proces, ki velikokrat ne dosega visoke specifičnosti. Protitelesa so že poskušali zamenjati z aptameri, ki so bili izbrani po klasični metodi SELEX in ki so bili konjugirani z molekulo za vizualizacijo. Aptameri, uporabljeni za metodo prenosa western predstavljajo hitro, enostavno in poceni zamenjavo za protitelesa. Vseeno pa bi lahko specifičnost aptamerov za namen detekcije proteinov še izboljšali z direktno selekcijo le-teh na membrani, na katero so prenešeni proteini. Prav tako bi za takšno vrsto selekcije lahko uporabili idejo celičnega SELEX, kjer je kot tarča uporabljena cela celica. Z uporabo tumorskih in netumorskih celic ali dveh različnih tumorskih celic, bi lahko izbrali aptamere, ki se specifično vežejo na neznane biomarkerje, prisotne na določeni tumorski celici. Uporaba pristopa SELEX z dodatno negativno selekcijo odstrani iz selekcije takšne aptamere, ki se vežejo hkrati na obe vrsti celic, kot rezultat pa se obogatijo aptameri, specifični za eno vrsto celic.

Aptameri se na tarče vežejo z vodikovimi vezmi, van der Waalsovimi silami in elektrostatskimi interakcijami. Tarča pri metodi prenosa western so proteini, za katere je znano, da so za vezavo pomembne tudi hidrofobne interakcije, ki pomagajo tvoriti kompleks

protitelesa in proteina. Prav tako je bilo dokazano, da imajo funkcionalne skupine, ki pomagajo pri izbiri najboljših oligonukleotidnih ligandov, po navadi hidrofobni aromatski značaj. Vključitev indola v knjižnico, ki je del stranske verige triptofana in je zmožen tvoriti hidrofobne interakcije s proteini, dokazano izboljša uspešnost selekcije aptamerov. Način, kako lahko to izvedemo, je uporaba knjižnice, kjer so timidini zamenjani s C5-etinil-2'-deoksiuridinom (EdU). Na tega lahko pripnemo s CuAAC klik-reakcijo 3-(2-azidoetil) indol. V naši študiji sta bili uporabljeni dve klik-knjižnici, FT2 in OW1, kot tudi klik-kompetitor, ki sestoji le iz naključne regije zaporedja.

Kot model za našo študijo smo uporabili celični liniji raka prostate: PC3 in LNCaP. Rak prostate je druga najbolj pogosta vrsta raka pri moških. Največ rakov prostate je opredeljenih kot adenokarcinom, ki se izraža z granularno strukturo in izražanjem androgenskega receptorja (AR) in prostatičnega specifičnega antigena (PSA). Večina adenokarcinomov napreduje počasi in so androgen-odvisni. 1 % ostalih epitelijskih obolenj raka prostate predstavlja mali celični (neuroendokrini) karcinom prostate (SCNC). Ti tumorji se pojavljajo redkeje, vendar za razliko od adenokarcinomov ne izražajo AR in PSA. SCNC ni odziven na hormonsko terapijo, zato je ta oblika bolezni zelo agresivna in se po navadi izraža kot lokalno napredovana bolezen ali v obliki oddaljenih metastaz. V večini primerov umrejo bolniki že v nekaj mesecih po diagnozi. Iz študij je razvidno, da kaže celična linija LNCaP podobnosti z adenokarcinomom, medtem ko ima celična linija PC3 podobnosti s SCNC. Zato lahko uporabimo ti celični liniji za iskanje novih biomarkerjev, s katerimi bi lahko razlikovali med tema različno agresivnima tipoma raka prostate.

V ta namen smo obe celični liniji frakcionirali na membranski in citosolni del in proteine obeh celičnih frakcij najprej ločili z elektroforezo SDS-PAGE. Nato smo proteine s pomočjo polsuhega prenosa iz gela prenesli na nitrocelulozno membrano. Membranski del celične linije PC3 smo uporabili kot tarčo za pozitivno selekcijo, medtem ko so nam ostale tri frakcije služile kot negativna selekcija. Tako tarča za pozitivno kot tarče za negativno selekcijo so bile prenešene na isto membrano, ki je služila kot matriks med izbiranjem aptamerov. Prosta mesta na membrani smo najprej blokirali z BSA, nato pa membrano inkubirali z indol-modificirano knjižnico. Nevezana zaporedja smo odstranili z večkratnim spiranjem membrane, nato pa del membrane z membranskimi proteini PC3-celic odrezali in DNA zaporedja eluirali s ChIP ekstrakcijskim pufrom. Pomnožitvi eluiranih zaporedjih je sledila odstranitev protismerne fosforilirane verige, za kar smo uporabili Lambda

eksonukleazo. Tako očiščen vzorec z enoverižno DNA smo modificirali z indolom in uporabili za naslednji cikel SELEX-a. Vse skupaj smo izvedli sedem ciklov selekcije.

Glavna naloga naše študije je bila optimizacija selekcije aptamerov na proteinih, ki so bili z western prenosom prenešeni na nitrocelulozno membrano. Najprej smo minimizirali nespecifično vezavo zaporedij na membrano in proteine z uporabo različnih kompetitorjev. Kombinacija kompetitorjev, ki je nespecifično vezavo zaporedij najbolj znižala, je bila koncentracija 1:1 klik-kompetitorja v 0.01 mg/ml sulfodextrana. Nato smo določili najbolj efektivno raztopino za elucijo zaporedij iz proteinov in očiščenje eluiranega vzorca. Inkubacija membrane s ChIP ekstrakcijskim pufrom pri 99°C in očiščenje vzorca z etanolno precipitacijo je ohranila največ zaporedij. Pred izbiranjem aptamerov smo preverili še čistost membranske in citosolne frakcije z različnimi protitelesi, ki služijo kot markerji za določene frakcije. Žal je bila membranska frakcija še zmeraj kontaminirana s citosolnimi proteini, prav tako pa so bile prisotne tudi sledi jedrnih proteinov v obeh frakcijah PC3-celic.

Celotna študija nam je vzela veliko časa, zato se nismo mogli preveč usmeriti na bolj optimizacijo frakcioniranja celic na celičnomembranski in citoplazemski del. V bodoče predlagamo obširnejšo raziskavo na področju subceličnega frakcioniranja, ki mora biti izvedeno za vsako celico posebej.

Po analizi vezave izbranih aptamerov na različne celične frakcije smo ugotovili, da izbrani aptameri razlikujejo med membransko frakcijo in citosolno frakcijo obeh celic. Vseeno pa še vedno opazimo vezavo aptamerov tako na membranski del PC3 kot tudi celic LNCaP. Verjamemo, da bi lahko z nadaljnjim razmislekom in več poskusi SELEX-a izbrali takšne aptamere, ki bi se specifično vezali le na membranski del celic PC3. Proteine, na katere bi se vezali obogateni aptameri, bi lahko izolirali iz membrane in jih določili z metodo LC-MS/MS. Ti proteini bi predstavljali specifične biomarkerje, ki bi jih lahko uporabili za diagnozo ali prognozo agresivnega tipa raka prostate. Prav tako bi lahko posamezne aptamere uporabili za detekcijo pripadajočih proteinov pri metodi prenosa western. Naša študija kot prva (po našem vedenju) prikazuje sklopitev metode SELEX in metode prenosa western in je zato pomemben temelj za nadaljnje raziskave na tem področju.

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**Ključne besede:** aptameri; click-SELEX; optimizacija protokola; rak prostate; SELEX s prenosom western

**Abstract**

Systematic evolution of ligands by exponential enrichment (SELEX) is an *in vitro* method, by which oligonucleotides with high affinity and specificity can be selected for virtually any target. Due to their many advantages, the selected/enriched oligonucleotides, also known as aptamers, represent a good replacement for antibodies. Short RNA or ssDNA are more stable and cost-effective and animals are not needed for their production.

Protein detection with antibodies by western blot analysis is usually an expensive and lengthy process, which many times suffers from low specificity. To date, researchers have tried to replace antibodies with aptamers, which have been selected through classic-SELEX approach and conjugated with a visualization molecule. Western blot analysis with aptamers has proven to be a fast, simple, and cheap alternative for antibodies. However, with a direct selection of aptamers on the membrane, the specificity of aptamers would be even higher. In addition, it could be used in different ways. For the target, we used two different prostate cancer cell lines, LNCaP and PC3, which are cellular models for adenocarcinoma and small cell (neuroendocrine) carcinoma (SCNC), respectively. Unlike adenocarcinoma, SCNC is a highly aggressive type of cancer and does not respond to hormonal therapy. Therefore, it is important to differentiate between these types of cancer and find specific biomarkers for SCNC.

For this purpose, we fractionated both cells to the membrane and cytosolic fraction and transferred the proteins by western blotting to a nitrocellulose membrane. As we wanted to obtain biomarkers present only on the membrane of PC3 cells, these proteins were used as a positive-selection target, whereas other fractions were used as negative-selection targets. We optimized the background binding signal and found the most efficient recovery solution for bound sequences. We discovered that the combination of 1:1 click-competitor and 0.01 mg/ml of sulfodextran decreases non-specific binding the most. ChIP extraction buffer was confirmed as the most efficient recovery solution with a followed EtOH precipitation of a recovered sample.

The selected aptamers specifically bound to membrane proteins and not to cytosolic proteins. Unfortunately, they could not differentiate between the membrane fraction of the PC3 and LNCaP cell lines as it was our goal.

We believe that the selection and differentiation can be successfully done with further optimization and, most importantly, this study represents the basis for further western blot SELEX studies.

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**Keywords:** aptamers; click-SELEX; protocol optimization; prostate cancer; western blot SELEX

**List of abbreviations**

| Abbreviations | Full name |
|---|---|
| AIPC | Androgen-independent prostate cancer |
| App. | Approximately |
| APS | Ammonium persulfate |
| AR | Androgen receptor |
| Bio | Biotinylated/biotin |
| bp | Base pair |
| BSA | Bovine serum albumin |
| ChIP | Chromatin immunoprecipitation |
| Conc./C | Concentration |
| CuAAC | Copper(I)-catalyzed alkyne-azide cycloaddition |
| CY | Cytosolic fraction |
| $ddH_2O$ | Double-distilled water |
| DNA | Deoxyribonucleic acids |
| dNTPs | Deoxynucleotides |
| dsDNA | Double strand deoxyribonucleic acid |
| ECL | Enhanced chemiluminescence |
| EDTA | Ethylenediaminetetraacetic acid |
| EdU | C5-ethynyl-2'-deoxyuridine |
| ER | Endoplasm reticulum |
| EtOH | Ethanol |
| FCS | Fetal calf serum |
| FDA | Food and Drug Administration |
| fwd | Forward |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| HB | Homogenizing buffer |
| kDa | Kilodalton |
| LC-MS/MS | Liquid chromatography-mass spectrometry |
| LNCaP | Lymph Node Carcinoma of the Prostate |
| NC | Nitrocellulose |
| nt | Nucleotide |
| NTC | No templet control |
| PAGE | Polyacrylamide gel electrophoresis |
| P | Phosphorylated |
| PBS | Phosphate buffered saline |

| | |
|---|---|
| PC3 | Prostate Cancer Cell Line |
| PCR | Polymerase chain reaction |
| PM | Plasma membrane fraction |
| PMSF | Phenylmethylsulfonylfluoride |
| PSA | Prostate-specific antigen |
| PVDF | Polyvinylidene fluoride |
| rev | Reverse |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RT | Room temperature |
| SCNC | Small cell (neuroendocrine) carcinoma |
| SDS | Sodium dodecyl sulphate |
| SELEX | Systematic evolution of ligands by exponential enrichment |
| ssDNA | Single strand deoxyribonucleic acid |
| STR-HRP | Streptavidin-horseradish peroxidase conjugate |
| Sulfodextran | Dextran sulphate sodium salt |
| TEMED | Tetramethylethylenediamine |
| THPTA | Tris(3-hydroxypropyltriazolylmethyl) amine |
| WB | Western blot |

# 1 Introduction

## 1.1 Aptamers

Aptamers are short ssDNA or RNA (usually 20-80 nt with 6-30 kDa molecular weight), that can bind to their target with high affinity and specificity due to their three-dimensional structure [1, 2]. The concept of using nucleic acids for specific binding to proteins began to emerge in the 1980s from a research on adenovirus and HIV. It was discovered that these viruses encode a number of short structured RNAs that bind to cellular or viral proteins with high specificity and affinity [3, 4]. In 1990, Tuerk and Gold isolated two RNA sequences out of a randomized pool of ~65,000 nucleotide sequences that bind to the bacteriophage T4 DNA polymerase with similar affinities [5]. This experiment led to the concept that nucleic acids can be potentially useful in protein recognition and it defined the Systematic evolution of ligands by exponential enrichment (SELEX) process for the first time. In parallel, Ellington and Szostak were investigating whether RNA molecules have the ability to form stable "pockets" for specific interaction with small molecules; consequently, they designed the term aptamer, a combination of the latin "*aptus*" (to fit) and the greek "*mers*" (particle) [6].

### 1.1.1 Aptamers as an alternative to antibodies

Aptamers can be selected *in vitro* for various targets, for example small molecules, ions, proteins, and even cells [7, 8]. Regardless of their functional similarity to antibodies, binding to the target with high affinity and specificity ($K_d$ ~ nmol – pmol range), aptamers have many unique properties that are suitable for industrialization and clinical applications. Aptamers may be better than antibodies in fields such as *in vivo* and *in vitro* diagnoses, precisely controlled drug release, biomarker discovery and targeted therapy [9]. As oligonucleotides, aptamers can withstand repeated rounds of denaturation and renaturation and, therefore, simplify their industrialization. Because of their thermal stability, they can be easily transported and stored. Unlike antibodies, aptamer's production does not require the use of animals, thereby decreasing animal abuse and additional costs [10]. Based on chemical synthesis and modification technologies, a selected aptamer can be produced or modified in large scale, in a short amount of time (even hours), and with minimal batch-to-batch variation [9]. Aptamer synthesis can be automated and, consequently, more cost and time effective [11]. Aptamers can be developed for targets that do not evoke strong immune response, as aptamer selection is not tied to the immune system [12]. Compared to

antibodies, aptamers offer several advantages due to their smaller size and nucleic acid characteristics that can improve their clinical applicability and suitability for industrialization. They can strongly penetrate into tissues and be easily internalized by their target cells, thus improving pharmacokinetics of aptamer-based drugs [13]. With Macugen, an FDA approved drug against age-related macular degeneration (AMD) that is specific for VEGF (vascular endothelial growth factor), one aptamer-based drug is already commercially available and several others are in clinical trials [14, 15]. Additionally, the capability of aptamers to bind cell surface receptors that are then internalized is used for drug delivery systems [16].

## 1.2 SELEX

Aptamers are generated *in vitro* during the SELEX process, which undergoes repeated rounds of selection and amplification [5, 6].

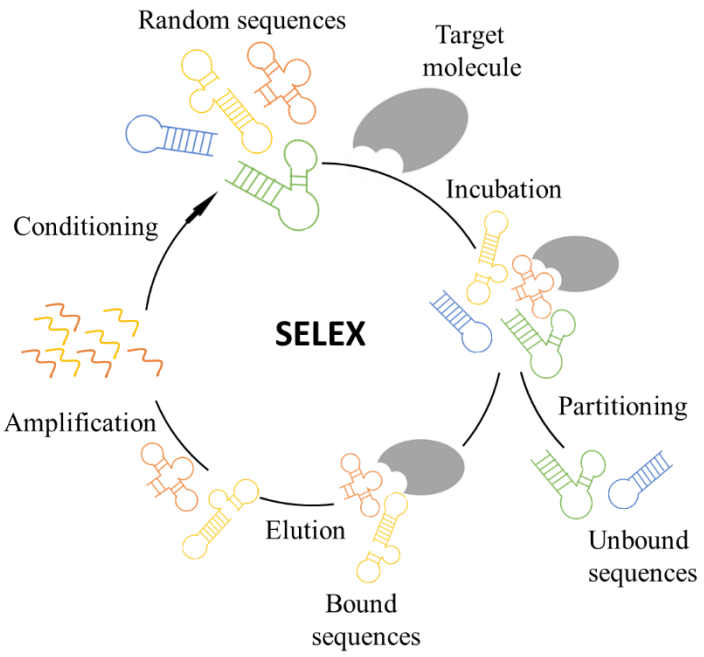


**Figure 1.1: Mayor steps within one SELEX round.**

In *Figure 1.1*, the main steps of SELEX process are shown. Each selection starts with a chemically or enzymatically synthesized oligonucleotide-library which can contain up to $10^{14}$ to $10^{15}$ different sequences. Each sequence is made of a random region and constant region, which is the same for all sequences and where primers anneal (*Figure 1.2*). In the first step, the target of choice is incubated with the library. As mentioned above, almost any molecule can be chosen as a target. The optimum total length of the oligonucleotides needs

to be carefully considered prior to the start of a SELEX. The longer the oligonucleotide sequence, the more nucleotide bases can help to form and be part of three-dimensional structures. However, longer oligonucleotides tend to form base pairs, thus making the SELEX-process less efficient [17]. In the second step, desired oligonucleotides (bound sequences) and undesired oligonucleotides (unbound sequences) must be separated.

**Figure 1.2: Graphic representation of an aptamer.** Depicted in black is the randomized region of an aptamer (20 – 80 bases); primer binding sites are shown in red (18 – 21 bases).

For partitioning, different methods such as capillary electrophoresis, affinity columns, magnetic beads, or membrane filtration can be used [18]. After removing the unbound sequences, the bound oligonucleotides are eluted or recovered from the target for the following steps in the SELEX approach. In most cases, aptamer-target complexes are separated through denaturing methods like heat treatment or an addition of special substances as SDS, EDTA, or urea [19-21]. The separation of bound sequences is possible by elution with competitive binders or by affinity elution with the target [22, 23]. Since the initial oligonucleotide library is highly complex, it is normally expected to recover only few functional sequences. Therefore, these low number molecules are amplified. The settings for PCR performance depends on the length of the designed library and the length of primer-annealing regions, as well as the abundance of guanidine-cytosine base-pairs in primer binding sites. Before going to the next round, amplified sequences need to undergo different steps of preparation for the next SELEX cycle. After the afore-mentioned amplification, the enriched pool is available as dsDNA. A transcription with RNA polymerase has to follow in case of RNA aptamers. The resulting RNA molecules are used as input in the following SELEX round. In case of ssDNA aptamers, single strand displacement has to be carried out. For this purpose, a streptavidin/biotin system can be used in different ways. Biotin molecule can be added to the unwanted strand and the arising size difference in gel electrophoresis can be used to distinguish between both strands [24]. Another possibility is to bind dsDNA (one strand biotinylated) to streptavidin surfaces (plates or beads) and separate strands after DNA denaturation [25]. Furthermore, an asymmetric PCR with either only one or a much higher amount of one primer can be performed [26]. In this study, single strand displacement with Lambda Exonuclease was performed, which is 5'→3' exodeoxyribonuclease. It selectively digests the 5'-phosphorylated strand of dsDNA [27]. For this purpose, a

phosphorylated reverse primer was used, obtaining only forward ssDNA strands after digestion. Before and after Lambda exonuclease digestion, the sample is purified with Nucleospin® Gel and PCR clean-up to remove salts, primers, enzymes, and other impurities. A high amount of chaotropic salts in the binding buffer disrupts hydrogen bonds between water and the silica membrane and between DNA and water molecules. The DNA is attached by hydrogen bonds formation to the silica membrane, whereas all other impurities and small DNA fragments are washed away [28]. Finally, enriched aptamers are prepared for the next round or can be cloned and sequenced with Sanger sequencing or next-generation sequencing.

### 1.2.1 Modifications of aptamers

To date, a lot of aptamer modifications are known, some improving the SELEX process and other improving the final application [29]. For the second one, modification of an aptamer is mostly done post-selectively; for example, introducing 2`-methoxy residues into RNA aptamers during solid phase synthesis or as an addition of a larger polyethylene glycol (PEG) molecule, thus, first, inhibiting enzymatic hydrolysis and second, decreasing renal clearance. Both modifications together help to enhance half-life of an aptamer in the blood stream [30, 31]. However, post-SELEX modification can affect the binding of the aptamer, therefore, some modifications are applied already during the selection. These modifications on a nucleobase level have to be compatible with the enzymatic steps during amplification. The best-characterized position of a nucleobase modification is the C5 position of pyrimidines, as it is the most permissive site for modifications accepted by DNA polymerase [32]. The advantage of nucleobase modification is that the modification is already present during the selection process, thereby assuring that the selected aptamer is functional. Furthermore, nucleobase modification could extend addressed targets with increasing chemical diversity of oligonucleotides [33]. Researchers from SomaLogic reported that with the incorporation of C5 modified nucleotides, the overall success of the selection rises from 30 % of unmodified aptamers to 80 % [34].

### 1.2.2 Click-SELEX

Aptamers can bind to their targets with high specificity and affinity through hydrogen bonding, van der Waals forces, electrostatic interactions, shape complementarity, and stacking of flat moieties [35]. However, it is also known that hydrophobic forces are very important for the formation of antibody-protein interaction. The tyrosine amino acid along

with aromatics tryptophan and phenylalanine are dominating the antibody binding-site [36]. Besides, the examples published by SomaLogic showed that functional groups that produce the best nucleic acid ligands typically have hydrophobic aromatic character [34]. Therefore, by incorporating the indole group, also found in the non-polar side-chain of the tryptophan amino acid, it was shown that the success rate of aptamer selection for proteins strongly increases.

Hydrophobic indole can be included in the library by replacing thymidine with a C5-ethynyl-2'-deoxyuridine (EdU) as shown in *Figure 1.3*. The 3-(2-azidoethyl) indole can be then bound through copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) click-reaction to the EdU [37].

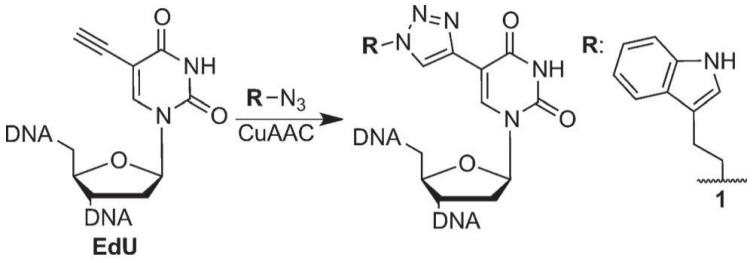


**Figure 1.3: Modification of an EdU with the 3-(2-azidoethyl) indole (R) through CuAAC reaction.**
(scheme taken from [37])

This EdU-modified DNA library is named click-library and can be incorporated with different kinds of azides. In this study, FT2 and OW1 click-libraries (3.1.8) were used, modified only with indole-azide. Apart from the library, click-competitor consisting only of the random region of the FT2 library and modified with indole was used. The FT2 library and both forward primers were linked with a biotin molecule through a C18 spacer (*Figure 1.4*). The spacer creates a distance between an oligonucleotide and a conjugated biotin, thereby preventing the impact of the conjugate on tertiary structure formation [38].

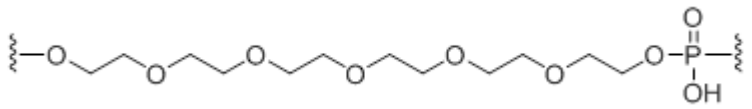


**Figure 1.4: Unit structure of the C18 spacer.**
(scheme taken from [38])

Click-SELEX has the same steps as a classic-SELEX, with an additional click-reaction of the library before incubation with the target, which has to be performed in every SELEX cycle [37].

### 1.2.3 Negative-SELEX

In order to separate unbound and bound sequences, a target is mostly immobilized on a matrix. Unfortunately, many times selected aptamers recognize components present in the selective environment [39]. This behaviour is the reason for the modification of the method to eliminate non-specific interactions, which is called negative-SELEX [40]. This modification excludes those aptamers that bind to the matrix used for immobilization of selection targets, leading to the enrichment of a population of sequences that could form complexes only with the target itself. In this improvement of classic-SELEX procedures, the negative-selection is applied before the positive one. The library is loaded onto the matrix alone and after an incubation period only the washed-off sequences are used for the positive-selection with the target. Instead of matrix, target-similar components can also be used to distinguish between structure related targets. Ellington and Szostak discovered that with this negative-SELEX approach, the affinity of the selected aptamers is approximately 10 times higher [40].

### 1.2.4 Cell-SELEX

Cell-SELEX is a method through which aptamers for the whole cell target are selected. The main advantage of this approach is that generated aptamers are functional with a native conformation of the target molecule on live cells, and thus, cell surface transmembrane proteins would be targets even when their purifications in native conformations are difficult. Additionally, cell-specific aptamers can be obtained without any knowledge about cell surface molecules on the target cells [41]. This approach can be applied to the novel biomarker discovery in the personalized cancer research field. By using specific cancer cells as targets and noncancerous or normal cells as negative-selection target, specific aptamers for unique cancer cells can be obtained, even without prior knowledge of biomarkers present on the tumour cell surface [42, 43].

The use of cell-SELEX approach can also be modified and used not only for the live cell as a target, but also for the cell homogenate and the mixture of proteins inside it. With the following described approach, specific biomarkers can be found. Moreover, hereinafter described type of western blot protein detection has many advantages over conventional method.

### 1.3 Western blot

Western blot analysis, also known as protein immunoblot, is an analytical technique used to detect and quantify specific proteins in a given sample of tissue or cell homogenate [44]. The most common and established procedure for western blot involves SDS–PAGE to separate the proteins according to their size, a transfer to a membrane for immobilization, and the use of primary and secondary antibodies for their detection. This is also the reason why there is an initial requirement for the generation of a specific antibody that can recognize and specifically bind to the protein of interest [45]. However, the production of a specific antibody is time-consuming and not a fool proof procedure. Furthermore, manufactured antibodies do not always achieve requested specificity [46].

The western blot analysis can be improved by replacing antibodies with aptamers. To date, it is only published protein detection with the already selected aptamers through normal SELEX approach [47, 48]. With those studies, it was shown that aptamers are a fast, simple, and cheap alternative to antibodies. Furthermore, the usage of aptamers dramatically reduces the effort and time required for conventional western blot analysis, as there is no need for primary and secondary aptamers, unlike with the detection with antibodies (*Figure 1.5*). It was also shown, that the absence of a blocking step does not increase background signal and can in this way save a lot of time. Last but not least, the *in vitro* selection of aptamers has little to no variation batch-to-batch, compared with the production of antibodies [9].

However, with a direct selection of aptamers on a western blot membrane, the specificity of protein recognition would increase, as the target proteins would already be in the denatured conformation, in comparison with normal selection where they are in the native structure. Besides, it would be possible to select more targets with only one selection, as long as the proteins are of different sizes.

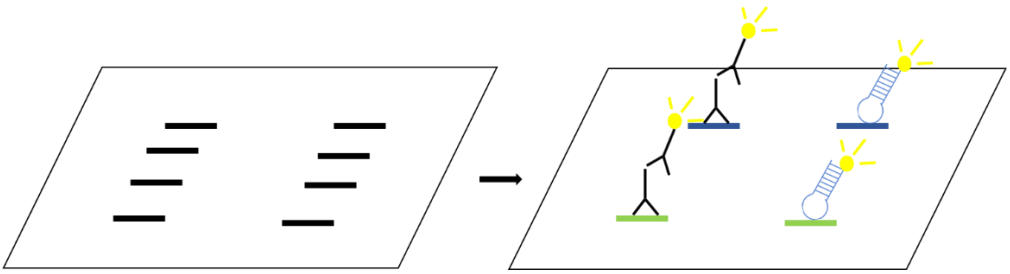

**Figure 1.5: The scheme of immobilized proteins on the membrane and their detection with antibodies and aptamers.**

### 1.4 Prostate cancer

Prostate cancer is the second most common malignancy in men [49]. An estimated 15 % men worldwide were diagnosed with prostate cancer in 2012, with almost 70 % of the cases occurring in more developed regions [50]. Despite the development of novel biomarkers and molecular stratification of many cancers, diagnosis and prognosis of prostate cancer still base on prostate-specific antigen (PSA) levels, local disease state, and histological architecture [51]. PSA levels in blood for men between the ages of 50 and 70 have been shown to powerfully predict a lifelong risk of patients developing prostate cancer metastases and mortality [52]. Although the prolongation of life on a population basis is an important endpoint, it cannot be reached by PSA screening because of insufficient specificity [53, 54]. The researchers identified overdiagnosis as the most relevant harm that limits acceptability of population screening of men at risk [54]. The likelihood of prostate cancer identification with biopsy based on the commonly used PSA threshold of 4 ng/mL is about 21 %. This represents false positive overdiagnosis (30-50 %) of cancers. Therefore, it is important to validate novel biomarkers to complete PSA screening [55]. The initial treatment is usually prostatectomy or radiation in order to remove or destroy the cancerous cells that are still confined within the prostate capsule. However, many patients are either not cured by this therapy and their cancer recurs, or they are diagnosed after cancer has spread. Tumour growth is initially androgen dependent [56]. Androgens are the main regulator of stimulating proliferation and inhibiting apoptosis. So, prostate cancer depends on a crucial level of androgenic stimulation for growth and survival. Androgen ablation causes cancer regression because without androgen, the rate of cell proliferation is lower and the rate of cell death is increased, leading to extinction [57]. Thus, androgen ablation is the mainstay of therapy for progressive prostate cancer. However, many men eventually fail this therapy and die of recurrent androgen-independent prostate cancer (AIPC). AIPC is a lethal form of prostate cancer that progresses and metastasizes. At present, there is no effective therapy for it. There are several pathways by which AIPC can develop; however, androgen receptor (AR) is still present in the tumour cell, only its sensitivity and expression increases [56]. Therefore, it is important to differentiate indolent and aggressive disease as well as find prognostic biomarkers. It is also important to distinguish between different types of prostate cancer according to their histological structure.

### 1.4.1 PC3 and LNCaP cell lines

The majority of prostatic cancers are adenocarcinomas characterized by glandular formation and the expression of luminal differentiation markers AR and PSA. Most adenocarcinomas are indolent and androgen-dependent. The rest of prostatic epithelial malignancies are of various forms including ductal type adenocarcinoma, mucinous (colloid) carcinoma, signet ring cell carcinoma, and small cell (neuroendocrine) carcinoma (SCNC) [58]. SCNCs of the prostate are rare tumours and account for no more than 1 % of all carcinomas of the prostate. Although they may arise *de novo*, such tumours are often seen as recurrent tumours in patients who have a history of conventional prostatic adenocarcinomas and received hormonal therapy [59, 60]. Unlike adenocarcinomas, SCNC does not express AR and PSA, and therefore does not respond to hormonal treatment. In contrast to the majority of prostatic adenocarcinomas that pursue an indolent clinical course, SCNC is highly aggressive which usually presents itself as a locally advanced disease or distant metastasis. Patients in most cases die within months of the diagnosis [61, 62]. It has been shown that the LNCaP cell line is associated with adenocarcinoma and PC3 cell line shares features of SCNC [63]. Therefore, these cell lines could be used to find possible biomarkers by which we could differentiate between these types of prostate cancer.

## 2 Aim of the study

The aim of our study will be to optimize the selection of aptamers with the SELEX method directly on the western blot membrane in order to use selected aptamers for subsequent western blot analysis of potential cancer biomarkers on PC3 cells. In the process of doing so, a thorough background binding of the library will have to be achieved and binding aptamers carefully recovered. We named the combination of these two methods western blot SELEX. The SELEX process will be a combination of different approaches: negative-SELEX, click-SELEX, and cell-SELEX.

For this purpose, we will fractionate PC3 and LNCaP cells to the plasma membrane fraction and the cytosolic fraction. The plasma membrane proteins of PC3 cells were used as positive selection targets (PC3.PM), while the plasma membrane proteins of LNCaP cells (LNCaP.PM) and the cytosolic proteins of PC3 and LNCaP cells (PC3.PM and LNCaP.CY, respectively) were used as a negative selection targets. An indole-modified library will be incubated with all the four proteins mentioned above, the unbound sequences will be washed away, and only the positive selection lane PC3.PM will be used for sequence elution. The recovered sequences will be amplified and used for the next cycle with all the four protein fractions again. To achieve specificity of aptamers, we will keep increasing the selection pressure during the entire SELEX process by decreasing incubation time, increasing washing time and volume, as well as increasing the amount of negative selection proteins. The amount of cytosolic proteins (PC3.CY, LNCaP.CY) will stay the same through the entire selection process and will be equivalent to the amount of positive selection target (PC3.PM), unlike the amount of LNCaP.PM which will increase during the SELEX procedure.

Our hypothesis is that with this approach we can select specific aptamers which will bind only to the proteins present in the PC3.PM fraction and not to the proteins of LNCaP cells.

# 3 Materials and Methods

## 3.1 Materials

All buffers were prepared in $ddH_2O$. The pH was adjusted with HCl or NaOH.

### 3.1.1 Buffers

| | |
|---|---|
| Blocking buffer | 5 % BSA in TBS-T |
| ChIP extraction buffer | 0.1 M $NaHCO_3$ pH 8.0 |
| | 1 % SDS |
| 6x DNA loading buffer | 10 mM Tris pH 7.6 |
| | 60 mM $Na_2EDTA$ |
| | 60 % Glycerol |
| | 0.03 % Xylene cyanole |
| Homogenizing buffer | 5 mM Tris pH 7.4 |
| | 300 mM Sucrose |
| | 0.1 M $Na_2EDTA$ |
| | 1 mM PMSF |
| 4x Laemmli buffer | 78.1 mM Tris pH 6.8 |
| | 12.5 % Glycerol |
| | 6.3 % 2-Mercaptoethanol |
| | 0.003 % Bromophenol Blue |
| | 1 % SDS |
| 10x PBS, pH 7 | 1.37 M NaCl |
| | 27 M KCl |
| | 100 mM $Na_2HPO_4$ |
| | 18 mM $KH_2PO_4$ |
| | 10 mM $CaCl_2$ |
| | 5 mM $MgCl_2$ |
| 10x Running buffer | 250 mM Tris pH 8.9 |
| | 2 M Glycine |
| | 1 % SDS |
| 10x TBE buffer | 0.9 M Tris |
| | 0.9 M Boric acid |
| | 20 mM $Na_2EDTA$ |
| 10x TBS | 200 mM Tris pH 7.6 |
| | 150 mM NaCl |
| TBS-T | 20 mM Tris pH 7.6 |
| | 15 mM NaCl |
| | 0.05 % Tween 20 |

| | |
|---|---|
| | 1 mM $MgCl_2$ |
| | 1 mM $CaCl_2$ |
| 10x Transferring buffer | 25 mM Tris |
| | 20 % Glycine |
| 4x Urea loading buffer | 9 M Urea |
| | 50 mM $Na_2EDTA$ |
| WB mild stripping buffer | 200 mM Glycine pH 2.2 |
| | 1 % Tween 20 |
| | 0.1 % SDS |

### 3.1.2 Chemicals

| Chemicals | Company |
|---|---|
| Acetonitrile | Roth, Germany |
| Agarose | Genaxxon bioscience, Germany |
| Ammonium acetate | Roth, Germany |
| Ammonium persulfate | Roth, Germany |
| 30 % Bis-acrylamide | Roth, Germany |
| Boric acid | AppliChem, Germany |
| Bovine serum albumin | AppliChem, Germany |
| Bromophenol blue Na-salt | Roth, Germany |
| $CaCl_2$ | VWR, USA |
| Chloroform | Roth, Germany |
| Coomassie blue | BioRad, USA |
| $CuSO_4$ | Synthetized in AK Mayer's laboratory (Limes Institute, Bonn, Germany) |
| Dextran sulphate sodium salt | Sigma-Aldrich, USA |
| DMEM Media | Thermo Fisher Scientific, USA |
| Dimethyl sulfoxide | Sigma-Aldrich, USA |
| dNTPs | Larova, Germany |
| DPBS | Thermo Fisher Scientific, USA |
| EdU | Baseclick, Germany |
| FCS | Sigma-Aldrich, USA |
| EtBr | AppliChem, Germany |
| EtOH | VWR, USA |
| Glycerine | Roth, Germany |
| Glycine | Roth, Germany |
| Glycogen | Roche, USA |

| | |
|---|---|
| HCl | VWR, USA |
| Indole-$N_3$ | Synthetized in AK Mayer's laboratory (Limes Institute, Bonn, Germany) |
| Isopropanol | Merck, Germany |
| Lambda exonuclease | Thermo Fisher Scientific, USA |
| Lambda exonuclease buffer | Thermo Fisher Scientific, USA |
| 2-Mercaptoethanol | AppliChem, Germany |
| $MgCl_2$ * 6 $H_2O$ | AppliChem, Germany |
| NaCl | Thermo Fisher Scientific, USA |
| $Na_2EDTA$ | Roth, Germany |
| $NaHCO_3$ | Roth, Germany |
| NaOAc | VWR, USA |
| NaOH | Sigma-Aldrich, USA |
| PageRuler Prestain Protein Ladder | Thermo Fisher Scientific, USA |
| PMSF | Roth, Germany |
| Phenol | Roth, Germany |
| PWO buffer | Genaxxon bioscience, Germany |
| PWO polymerase | Genaxxon bioscience, Germany |
| RPMI Media | Thermo Fisher Scientific, USA |
| Salmon sperm DNA | Invitrogen, USA |
| SDS | Roth, Germany |
| Sodium ascorbate | Sigma-Aldrich, USA |
| Sucrose | Roth, Germany |
| Sulfo-NHS-LC-biotin | Thermo Fisher Scientific, USA |
| STR-HRP | GE Healthcare, UK |
| TEMED | Roth, Germany |
| THPTA | BaseClick, Germany |
| Tris base | Roth, Germany |
| Trypsin-EDTA (0.5 %) | Thermo Fisher Scientific, USA |
| Tween 20 | Merck, Germany |
| Urea | Roth, Germany |
| Ultra-low molecular weight DNA ladder | Thermo Fisher Scientific, USA |

### 3.1.3 Equipment

| Equipment | Company |
|---|---|
| Axiover 25 (inverted microscope) | Carl Zeiss, Germany |
| Bath tube | Grant Instruments, UK |

| | |
|---|---|
| Bath tube (cell culture) | GFL, Germany |
| Balance | Mettler Toledo, USA |
| Hera Cell (cell culture incubator) | Thermo Fisher Scientific, USA |
| Hera Safe (Cell culture hood) | Thermo Fisher Scientific, USA |
| Centrifuge | Eppendorf, Germany |
| Chamber for electrophoresis | BioRad, USA |
| Electrophoresis power supply | Consort, UK |
| Freezer -20°C | Liebherr, USA |
| Freezer -80°C | New Brunswick Scientific, USA |
| Fine balance | Sartorius, Germany |
| GenoPlex (UV-Transilluminator) | VWR, USA |
| Glass/Polytetrafluorothylene (PTFE) potter | Pro Scientific, USA |
| Imaging system Odyssey® | LI-COR Bioscience, Germany |
| Imaging system VersaDoc | BioRad, USA |
| Magnet mixer | Heidolph, Germany |
| Microwave | Bosch, Germany |
| Mini centrifuge | Eppendorf, Germany |
| NanoDrop 2000 (spectrophotometer) | Thermo Fisher Scientific, USA |
| NanoQuant Infinite M200 (spectrophotometer) | Tecan, Switzerland |
| PCR cycler | Biometra, Germany |
| | Thermo Fisher Scientific, USA |
| pH meter | Mettler Toledo, USA |
| Refrigerator | Liebherr, USA |
| Rocker | Stuart, UK |
| Rotator | Heidolph, Germany |
| Semi-dry transfer cell | BioRad, USA |
| Spin device | VWR, USA |
| Thermomixer | Eppendorf, Germany |
| Vortex | VWR, USA |

### 3.1.4 Consumable

| Consumable | Company |
|---|---|
| 0.5 ml vials | Sarstedt, Germany |
| 1.5 ml vials | Sarstedt, Germany |
| 2 ml vials | Sarstedt, Germany |
| 15 ml Falcon tubes | Sarstedt, Germany |

| | |
|---|---|
| 50 ml Falcon tubes | Sarstedt, Germany |
| 96-well plate | Greiner bio-one, Germany |
| Amicon® Ultra 0.5 Centrifugal Filter Devices | Merck, Germany |
| Cell scraper | TPP, Switzerland |
| Glass bottles | DURAN, Germany |
| Nitrocellulose membrane | GE HealthCare, UK |
| Paper for WB | A. Hartenstein |
| Parafilm | Bemis, USA |
| Micro Bio-Spin 6 Chromatography Column | BioRad, USA |
| Pipette for big volumes | Starlab, USA |
| Pipettes for small volumes | Eppendorf, Germany |
| Serological pipettes | Sarstedt, Germany |
| T25 Flask | Sarstedt, Germany |
| T75 Flask | Sarstedt, Germany |
| Tips | Sarstedt, Germany |
| Volumetric flasks | Vitlab, Germany |

### 3.1.5 Kits

| Kits | Company |
|---|---|
| Nucleospin® Gel and PCR clean-up | Macherey Nagel, Germany |
| Pierce® BCA Protein Assay Kit | Thermo Fisher Scientific, USA |
| Pierce® ECL Western Blotting Substrate | Thermo Fisher Scientific, USA |
| Pierce® Reversible Protein Stain Kit | Thermo Fisher Scientific, USA |

### 3.1.6 Antibodies

| Antibody | Company |
|---|---|
| IRDye™ 680 conjugated goat anti-mouse IgG | LI-COR Bioscience, Germany |
| Dylight™ 800 conjugated goat anti-rabbit IgG | Thermo Fisher Scientific, USA |
| Rabbit polyclonal anti-GADPH antibody | Santa Cruz, USA |
| Mouse monoclonal anti-lamin A+C antibody | Santa Cruz, USA |
| Mouse monoclonal anti-CD71 antibody | Santa Cruz, USA |

### 3.1.7 Human cell lines

| Cell line | Company |
|---|---|
| LNCaP | Property of AK Mayer's laboratory (Limes Institute, Bonn, Germany) |
| PC3 | ProQinase, Germany |

### 3.1.8 Nucleic acids

| Click-competitor |
|---|
| N42 (EdU) (N: dA:dG:dC:EdU = 1:1:1:1) |

| FT2 library | |
|---|---|
| Library -N42 (EdU) (N: dA:dG:dC:EdU = 1:1:1:1) | 5'-Biotin- X*CA CGA CGC AAG GGA CCA CAG G-N42-CAG CAC GAC ACC GCA GAG GCA-3' |
| Forward primer | 5'- CAC GAC GCA AGG GAC CAC AGG -3' |
| Forward Bio primer | 5'-Biotin- X*CA CGA CGC AAG GGA CCA CAG G-3' |
| Reverse primer | 5'- TGC CTC TGC GGT GTC GTG CTG -3' |
| Reverse P primer | 5'-Phosphate- TGC CTC TGC GGT GTC GTG CTG -3' |

| OW1 (click_1) library | |
|---|---|
| Library -N44 (EdU) (N: dA:dG:dC:EdU = 1:1:1:1) | 5'- AGC CAC GGA AGA ACC AGA -N44- GCA GAA GCG ACA GCA ACA -3' |
| Forward primer | 5'- AGC CAC GGA AGA ACC AGA -3' |
| Forward Bio primer | 5'-Biotin- X*AG CCA CGG AAG AAC CAG A -3' |
| Reverse primer | 5'- TGT TGC TGT CGC TTC TGC -3' |
| Reverse P primer | 5'-Phosphate- TGT TGC TGT CGC TTC TGC -3' |

*X=C18 Spacer

Both libraries, primers, and click-competitor were purchased from Ella biotech, Germany. The OW1 library was designed by Olga Wolter, AK Mayer (Limes Institute, Bonn, Germany) and FT2 by Fabian Tolle [37].

## 3.2 Methods

### 3.2.1 Cell culture

PC3 and LNCaP cells are prostate cancer cell lines. Both were stored in liquid nitrogen and defrosted at 37°C for 2 min prior culturing. Cells were put into a T25 flask containing prewarmed media. The following day, the media needed to be replaced with fresh one to get rid of DMSO in the storing solution, which inhibits growth of cells. The cells were transferred into a bigger T75 flask after they were 80 % confluent. PC3 cell line was cultured in DMEM media containing 10 % FCS and split every two days with regular medium replacement. The cells were detached from the surface with incubation in 0.05 % Trypsin/0.5 mM EDTA for several minutes. Doubling time of these cells is approximately 33 hours. LNCaP cells are more easily detached, therefore, they were trypsinized only for several seconds and then trypsin was discarded immediately. These cells double in approximately 60 hours [64]. Both cells were washed with DPBS before adding trypsin to decrease inhibitory effect of media on trypsin detachment ability. The cells were cultured at 37°C, 95 % humidity, 5 % CO2, and used for 25 passages.

### 3.2.2 Subcellular fractionation

The protocol used for membrane and cytoplasm isolation is a combination of different steps taken from Pascal Weber's protocol (Limes Institute, Bonn, Germany) and other literature [65-67]. The protocol is based on a series of different centrifugations. Through them proteins are separated according to their molecular weight. At least 2 million cells were used for the subcellular fractionation, cultured in three to four T75 flasks. First, the media was discarded and cells washed with cold DPBS. As the trypsin would change the conformation of some surface proteins, the cells were detached from the surface by adding cold DPBS, pipetting and scraping. The content of all flasks was transferred to a 50-ml tube and cells were counted.

#### *3.2.2.1 Cell counting*

For the determination of total cell number, cells were counted in the Neubauer chamber. The cells were counted in all four quadrants and the final number was calculated with the following equation:

$$total\ number\ of\ cells = \frac{number\ of\ cells\ in\ four\ quadrants}{4} \cdot 10^4 \cdot total\ volume$$

*3.2.2.2 Homogenization and centrifugation*

Detached cells in DPBS were spun down at 1000 x g and RT for 3 min. The supernatant was discarded and pellet was resuspended in 750 µl ice cold and freshly prepared homogenizing buffer containing 0.3 M sucrose to partially dehydrate organelles and keeping them intact. Sucrose also prevents osmotic shock and in this manner, stabilizes proteins. It enables better separation of cell fractions and less contamination between cell fractions. Ethylenediaminetetraacetic acid (EDTA) as a chelating agent and phenylmethylsulfonylfluoride (PMSF) inhibit protein degradation. Cells were homogenized on ice, applying 100 strokes with the homogenizing potter. All the subsequent steps must be performed at 4°C to minimize the activation of proteases. Then, the solution was transferred to a 2-ml vial and centrifuged two times at 800 x g for 5 min at 4°C. Pellet, containing intact cells and nuclei, was discarded and the supernatant was centrifuged at 20,000 x g for 2 hours at 4°C, yielding cytosolic fraction in the supernatant and enriched membrane fraction in the pellet. To dilute cytoplasm proteins in the pellet as much as possible, the pellet was washed with cold DPBS, resuspended in 500 µl homogenizing buffer and centrifuged at 20,000 x g for 1 hour at 4°C, again. The last centrifugation step was repeated and, finally, the pellet was resuspended in 250 µl 5 mM Tris and 1 mM PMSF, pH 7.4.

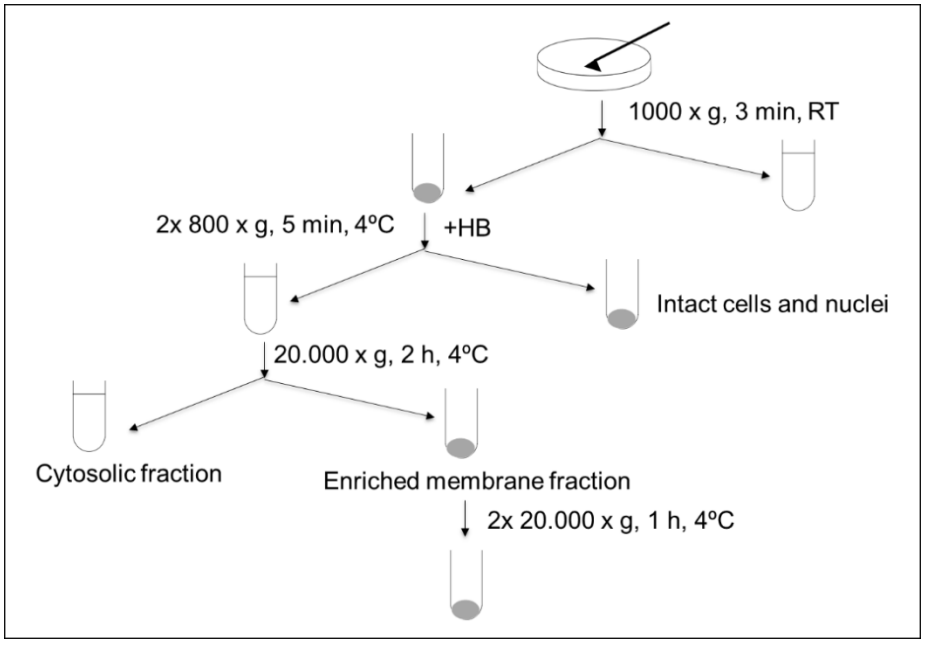


**Figure 3.1: Scheme of subcellular fractionation as described in section 3.2.2.2.**

#### *3.2.2.3 Determination of protein concentration*

The concentration of protein fractions was determined with Pierce® BCA Protein Assay Kit, which is based on bicinchoninic acid assay (BCA). The BCA Protein Assay combines the well-known reduction of $Cu^{2+}$ to $Cu^{1+}$ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection (at 562 nm) of $Cu^{1+}$ by bicinchoninic acid. A standard curve of known BSA concentration was made and out of it, the proteins concentration was calculated [27].

Protein fractions were stored at 4°C and used for approximately 2 weeks. Afterwards, new protein fractions were prepared.

### 3.2.3 Protein markers detection by western blot analysis

The key feature of western blotting is the use of immunodetection to identify a specific protein, for example a protein marker for a specific fraction [68]. Thus, the effectiveness of subcellular fractionation and purity of fractions were tested with different antibodies. Once the proteins are immobilized on a nitrocellulose (NC) membrane, they can be probed with a specific primary antibody for the protein of interest. The primary antibody is detected, with a secondary antibody that recognizes the constant domain of immunoglobulin G and is species specific. Furthermore, it carries the tag that allows visualization of the protein.

For the detection with anti-CD71 and anti-GADPH antibodies, 20 µg of plasma membrane (PM) and cytosolic (CY) proteins were loaded on SDS-PAGE gel. For the lamin detection, an aliquot of 10 µl was taken after cell homogenization, first and second nuclei centrifugation, and PM and CY fraction and loaded on the same gel.

#### *3.2.3.1 Primary antibodies*

After membranes were blocked overnight and washed with TBS-T, antibodies were diluted in the blocking buffer. Each blot was incubated in 2.5 ml of antibody solution for 1 hour on a rotator.

Table I: Table of antibody dilutions for belonging protein markers.

| Type of marker | Antibody used | Antibody dilution |
|---|---|---|
| Plasma membrane marker | Anti-CD71 (anti-transferrin receptor) | 1:2,000 |
| Cytosolic marker | Anti-GAPDH | 1:1,000 |
| Nucleus marker | Anti-lamin A+C | 1:1,000 |

#### *3.2.3.2 Secondary antibodies*

After primary antibodies were bound, the membranes were washed three times with 10 ml of TBS-T for 5 min. Secondary antibodies were prepared again in the blocking buffer in a dilution of 1:10,000 and incubated with the membranes for 1 hour.

Anti-rabbit antibodies: Goat anti-rabbit IgG were conjugated with Dylight™ 800 fluorophore and used for detection of rabbit anti-GADPH antibodies.

Anti-mouse antibodies: Goat anti-mouse were conjugated with IRDye™ 680 fluorophore and used for detection of mouse anti-CD71 and anti-lamin A+C antibodies.

#### *3.2.3.3 Visualization with Odyssey® Imaging system*

After the incubation with secondary antibodies, the membranes were washed again. Near-IR light is invisible to the naked eye, but can be visualized with the infrared Odyssey® Imaging system [27]. The emission of Dylight™ 800 was measured at 800 nm and the emission of the IRDye™ 680 at 700 nm.

### 3.2.4 General conditions for western blotting

#### *3.2.4.1 SDS-PAGE gel*

SDS-PAGE is a commonly used technique to separate proteins according to their size. Sodium dodecyl sulphate **(**SDS) is present in the running buffer, in the gel, and, most importantly, in the Laemmli buffer. The last one is heated up with protein samples before electrophoresis in order to make the charge-density of all proteins roughly equal. Heating denatures proteins so that the SDS, an anionic agent, can bind to the uncoiled protein molecule and enables protein separation by size only. In most cases, a reducing agent like 4-mercaptoethanol is added in the Laemmli buffer. It breaks sulfate bridges bonds and guarantees the absence of quaternary or tertiary protein structures [27].

The percentage of bis-acrylamide in the gel defines the size of the proteins, which could be separated (e.g. 12.5 % gels for proteins in the range 10-70 kDa, 10 % gels for proteins in the range 15-100 kDa and 8 % gels for proteins in the range 25-100 kDa) [69]. As this project requires proteins of all sizes, our choice was 10 % gel.

Table II: The recipe for 10 % separating gel and 4 % stacking gel, used for SDS-PAGE electrophoresis.

| Separating gel | | Stacking gel | |
|---|---|---|---|
| **Gel percentage** | **10 %** | **Gel percentage** | **4 %** |
| Water | 2440 µl | Water | 1220 µl |
| 1.5 M Tris pH 8.8 | 1500 µl | 1 M Tris pH 6.8 | 500 µl |
| 10 % SDS | 60 µl | 10 % SDS | 10 µl |
| 30 % Bis-acrylamide | 2000 µl | 30 % Bis-acrylamide | 270 µl |
| TEMED | 6 µl | TEMED | 2.5 µl |
| 10 % APS | 60 µl | 10 % APS | 10 µl |

First, 10 % separating gel was prepared and pipetted in the gel-cassette. Immediately, the gel was overlaid with isopropanol to ensure the straight-line polymerization of the upper layer of the gel. It needed approximately 30 minutes to polymerize, then isopropanol was discarded and 4 % stacking gel was prepared. After adding the stacking gel, a comb with 10 wells and 1 mm width was put in. The gel polymerized in 15 to 30 minutes, and afterwards, it was either used or the glass plates were wrapped with a wet paper towel and stored in a plastic bag at 4°C up to 2 days.

#### *3.2.4.2 SDS-PAGE electrophoresis*

Protein sample was mixed with 4x Laemmli buffer and heated up at 95°C for 5 minutes. In the meantime, electrophoresis chamber was prepared and filled up with the running buffer. Denaturized protein samples and additional PageRuler Prestain Protein Ladder were pipetted into wells. During the electrophoresis, negatively charged proteins were run at 150 V, 150 mA and 10 W, until the lowest protein ladder band came to the end of the chamber.

#### *3.2.4.3 Semi-dry transfer*

Semi-dry blotting is a widespread method to transfer proteins from the gel to a membrane with minimal amount of buffer. The electrodes are placed directly in contact with the gel/membrane sandwich to provide a fast, efficient transfer. Using a platinum-coated titanium plate as the anode and a stainless-steel plate as the cathode, the proteins are transferred in a horizontal position without a buffer chamber or gel cassettes [70].

The transfer buffer used for this experiment did not include methanol, as it shrinks the gel pores when the gel is less than 12 % and thus prevents the transfer of bigger proteins to the

membrane [70]. Methanol is usually added to increase affinity of proteins to the membrane by removing SDS from the proteins [71].

The membrane can be either nitrocellulose or polyvinylidene fluoride (PVDF). As the PVDF needs to be activated with methanol prior blotting, the NC membrane was used instead [69]. The proteins bind to the nitrocellulose membrane through hydrophobic interactions.

After running an SDS-PAGE gel, the gel was equilibrated immediately in the transfer buffer for app. 10 min. Equilibration helped to facilitate the removal of electrophoresis buffer salts and detergents. The NC membrane and four blot papers were soaked in the transfer buffer for 5-10 min. Two of the completely saturated blot papers were placed on the cathode and air bubbles were rolled out with the Falcon tube. Directly on top of it, prewetted NC membrane was placed and air bubbles excluded again. The equilibrated gel was carefully placed on the membrane and the lanes of the proteins were signed with a black marker. Finally, the last two blot papers were put on the top of it and air bubbles rolled out. The semi-dry transfer ran at 20 V and 30 W for 75 min.

By semi-dry transfer, a problem usually arises because bigger proteins are harder to transfer. The problem is mostly solved by applying two different types of transferring buffer. One containing methanol which increases the affinity of proteins to the membrane (anode buffer), whereas the second one containing SDS which aids in the elution of proteins from the gel (cathode buffer) [71]. However, we compared the efficiency of the blotting of a discontinuous system with ours, continuous buffer system, and there were no perceptual differences. In *Figure 3.2*, it is shown that the proteins of all sizes are successfully transferred, even those that are bigger than 170 kDa.

#### *3.2.4.4 Visualization of transferred proteins*

After the transfer, proteins on the NC membrane were visualized using Pierce® Reversible Protein Stain Kit to check the efficiency of blotting. Visualization was not used during the optimization of background binding and western blot SELEX, as the dye could change the binding of sequences to the proteins and the membrane.

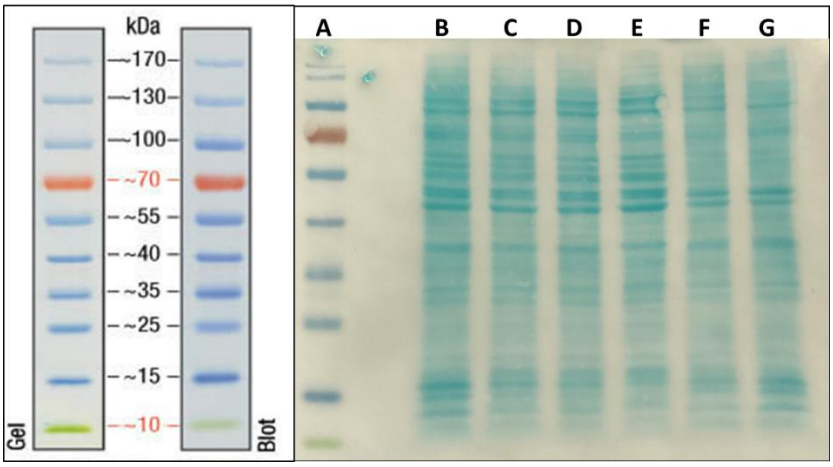


**Figure 3.2: Western blot membrane, blotted with semi-dry transfer.** Proteins of different centrifugation steps after cell homogenization were stained with Pierce® Reversible Protein Stain Kit. With letters A-G following proteins are marked: **A.** PageRuler Prestain Protein Ladder; **B.** homogenized PC3 cells; **C.** supernatant after 1st nuclei centrifugation; **D.** supernatant after 2nd nuclei centrifugation; **E.** CY fraction; **F.** solution after 1st membrane pellet resuspension; **G.** PM fraction. The amount of the proteins by B, C, D, F was 10 µl, as we did not determine proteins concentration during the centrifugation steps, and 10 µg by E and G.

#### *3.2.4.5 Blocking of the membrane*

After blotting, unreacted sites of the membrane needed to be blocked with proteins to reduce non-specific binding of antibodies or oligonucleotides to the empty spaces on the membrane surface. Usually, proteins used for blocking are milk proteins, BSA or casein in TBS-T buffer [68]. This experiment was performed by See Lee Xian, AK Mayer (Limes Institute, Bonn, Germany) where the membrane was blocked with 5 % BSA, 5 % milk or only with TBS-T. The results showed that the best blocking solution was 5 % BSA in TBS-T. The membrane was blocked in 10 ml of blocking solution for 1 hour at room temperature or overnight at 4°C. After blocking the membranes were washed on a rotator 3-times for 5 min: 1x 10 ml, 2x 5 ml of TBS-T on a rotator for 5 min.

After the protein transfer, the membrane was placed into a 50-ml tube. All washing, blocking, and incubation steps were performed at RT on a rotator.

### 3.2.5 Optimization of background binding

#### *3.2.5.1 Click-reaction*

For the click reaction, fresh catalyst-solution needed to be prepared first. $CuSO_4$ is reduced by ascorbate to $Cu^{1+}$. Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) prevents the oxidation of catalytic $Cu^{1+}$ by dissolving oxygen, thus helping to stabilize the $Cu^{1+}$ ion. The catalyst-solution had to rest for 10-15 min at RT prior to be employed for the click-reaction.

Table III: Reagents for catalyst-solution are added in the order from top to the bottom. 100 mM ascorbate-solution needs to be prepared fresh.

| Reagent | Stock conc. | Volume | Final-conc. |
|---|---|---|---|
| Water | | 70 µl | |
| THPTA | 100 mM | 4 µl | 4 mM |
| $CuSO_4$ | 100 mM | 1 µl | 1 mM |
| Ascorbate | 100 mM | 25 µl | 25 mM |

In the meantime, 100 µl of click-reactions were prepared.

Table IV: The components for click-reaction. Alkyne containing sample used for modification can be either click-library or click-competitor (1.2.2). If the initial library or click-competitor was used during the click-reaction, only 5 µl of 100 µM (500 nmol) were added to the final volume 70 µl. During western blot SELEX process, the whole volume of amplified and purified ssDNA was used.

| Reagent | Stock conc. | Volume | Final-conc. |
|---|---|---|---|
| Phosphate buffer (pH 7) | 10x | 10 µl | |
| Sample with alkyne group | | 70 µl | |
| Azide | 10 mM | 10 µl | 1 mM |
| Catalyst-solution | | 10 µl | |

After the reduction of $CuSO_4$ was finalized, catalyst-solution was added to prepared click-reaction solution and incubated for 1 hour at 37°C and 650 rpm in a thermomixer. 5 minutes before the incubation was done, an Amicon® column was pre-rinsed with 400 µl of water for 5 min at 14,000 x g. Water from the collection tube was discarded and 100 µl of click-reaction were pipetted to the column, followed by 400 µl of water. The column was centrifuged again with the same settings. Subsequently, two washing steps followed with 400 µl of water. Finally, the filter was flipped, put into a new collection tube and centrifuged at 1,000 x g for 2 minutes. Approximately 60 µl of recovered solution was collected. More details about Amicon® columns are described in section 3.2.6.4. Finally, the concentration of modified sequences was determined with the NanoDrop 2000.

#### *3.2.5.2 Biotinylation of BSA*

For the purpose of normalization, the BSA was biotinylated (bio-BSA), thus the membranes could be compared to each other. A 10 nmol solution of BSA was mixed with 4 µl of sulfo-NHS-LC-biotin (40 nmol) in a final volume of 100 µl and incubated for 1 hour at RT. After incubation, the remaining NHS-biotin was quenched by adding 20 µl of 200 mM Tris and put on ice for 10 min. In the meantime, P6-column was prepared according to the instructions

of the manufacturer. When quenching was done, the sample was carefully applied onto the gel bed centre in the column. It was centrifuged for 4 min at 1000 x g at RT. Molecules smaller than 6 kDa (NHS) retained on the column, while the biotinylated protein passed through. The bio-BSA was stored at 4°C. Furthermore, SDS-PAGE and dot blot was performed to determine the concentration of the sample and biotinylation efficiency. SDS-PAGE gel was stained with Coomassie blue and the intensity of the band with the unknown bio-BSA concentration was compared to the known BSA concentration in the Aida Software programme.

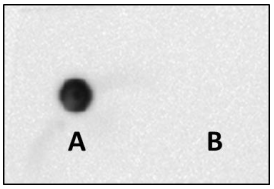


**Figure 3.3: The dot-blot of (A) biotinylated BSA and (B) BSA.**
This part was performed together with See Lee Xian, AK Mayer (Limes Institute, Bonn, Germany).

#### *3.2.5.3 Western blot membrane preparation*

For the SDS-PAGE electrophoresis, proteins samples containing 0.1 µg, 1 µg, 2.5 µg and 5 µg of LNCaP.PM proteins, and 5 ng of bio-BSA were prepared. The required amount of 4x Laemmli buffer was added to the samples, followed by heating them up at 95°C for 5 min. Gel preparation, electrophoresis, blotting, and blocking were performed in the same manner as described in 3.2.4, with blocking overnight at 4°C.

#### *3.2.5.4 Incubation with different competitors*

Usually, different competitors are added to the library during the selection to reduce non-specific binding of sequences to the proteins and to the surface. Most common used competitors are salmon sperm DNA, sulfodextran and, in the case of click-SELEX, click-competitor, which consists of random region of the library only. Since 5 % BSA was used for the blocking of the membrane, it was included also as one of the possible competitors. The amount of FT2 indole-modified library, used for the optimization of background binding, was 20 pmol per membrane. The library and click-competitor were heated up at 95°C for 3 min and cooled down for 5 min prior incubation to break the secondary structure of the sequences.

Table V: Amount or concentration of competitors used for optimization of background binding.

| Competitor | Sulfodextran | | Click-competitor | | Salmon sperm DNA | BSA |
|---|---|---|---|---|---|---|
| Amount | 0.1 mg/ml | 0.01 mg/ml | 20 pmol (1:1) | 200 pmol (1:10) | 200 pmol | 5 % |

Each membrane was incubated in 2.5 ml of TBS-T with 20 pmol of FT2 library and the required amount of a competitor for 30 min at RT. As blank and positive control, the membrane with only TBS-T and TBS-T with the library, respectively, were used. After incubation, all membranes were washed three times with 3 ml of TBS-T for 10 min.

*3.2.5.5 Visualization and quantification with ECL method*

The next step was the visualization of sequences that bound to the proteins on the membrane with the chemiluminescence (ECL) method. As seen in *Figure 3.4*, streptavidin-horseradish peroxidase conjugate (STR-HRP) was incubated with the membrane and, consequently, strong interaction between biotin and streptavidin was formed. For each membrane 2 ml of 1:1000 diluted STR-HRP in TBS-T were used. After the complex ssDNA-biotin and STR-HRP was formed (30 min), unbound STR-HRP was washed away with a 3x 1 ml (2 min), 1x 2 ml (5 min), 1x 3 ml (10 min) and 1x 4 ml (15 min). Finally, the reagent with peroxidase and luminol was added and the reaction catalysed by HRP occurred. The formed light was measured with VersaDoc Imaging system.

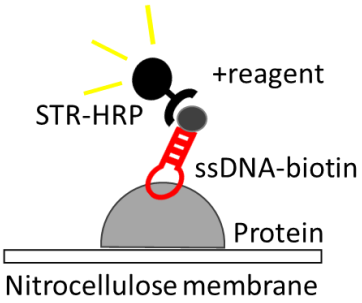


**Figure 3.4: The scheme of ECL visualization.**

The results were quantified with Aida Software where the intensity of the whole lane of each protein was measured. The intensity of protein lanes was normalized to the bio-BSA value that was set to 100. The intensity of the blank was subtracted from the normalized intensity of each lane. Optimization was performed in triplicates and duplicates and the data presented using the Prism software. Triplicates were performed for the first round of the competitor selection and duplicates for the second round of competitor selection, where the best

concentration of selected competitors was determined. With two repetitions of the second experiment, enough reliable results were obtained, therefore, another repetition was not necessary.

### 3.2.6 Recovery methods

#### *3.2.6.1 Recovery solutions*

Once the sequences bind to the protein target during the SELEX procedure, it is desirable that most of them are eluted from the target and after amplification transferred to the next round. The most effective recovery solution needs to break bonds between sequences and proteins, or in our case also eventual bonds between proteins and the NC membrane. Usually, high temperature or salts are enough to disrupt hydrogen bounds. All used recovery solutions are summarized in the *Table VI*.

Because of detergents, salts, and possible eluted proteins in the recovery solution, the sample needed to be purified either with phenol/chloroform extraction and EtOH precipitation or only with EtOH precipitation. Both purification approaches were compared to each other and the most effective one was chosen for the purification of recovered sample during the western blot SELEX procedure.

Table VI: Recovery solutions and incubation conditions. For buffer composition see section 3.1.1.

| Recovery solution | Incubation conditions |
|---|---|
| $ddH_2O$ | 99°C, 1400 rpm for 30 min |
| 0.1 M Ammonium acetate | |
| 1 M NaCl | |
| 1x Urea loading buffer | |
| 4x Urea loading buffer | |
| WB stripping buffer | |
| ChIP extraction buffer | |
| Mix of 1x urea + ChIP (1:1) | |
| 40% Acetonitrile in 0.1 M ammonium acetate | 37°C, 1400 rpm for 1 hour |
| Mix of phenol-chloroform (1:1) | Membrane was soaked in the mixture |
| 10 μg of PC3.PM proteins | RT, for 30 min |

#### *3.2.6.2 Phenol/chloroform extraction*

Phenol/chloroform extraction is a widespread method for purifying DNA from proteins, e.g. eluted proteins off the membrane. Proteins accumulate at the border between water and hydrophobic phenol, thus allowing easy removal of the aqueous upper part containing DNA

while the proteins remain in the border solution. Potential traces of phenol within the aqueous upper phase are subsequently removed by adding chloroform. For the extraction procedure, equal volumes of phenol were added to the hydrophilic nucleic acid samples. Following vigorous mixing, samples were centrifuged at 20817 x g for 10 min at RT. The aqueous upper phase was transferred to a new tube and two volumes of chloroform were added. After another round of mixing and centrifugation (20817 x g, for five min, RT), the aqueous upper phase was again collected to continue with the precipitation of DNA.

#### *3.2.6.3 Ethanol precipitation*

Nucleic acids can easily be precipitated using a mixture of salts and ethanol. For this purpose, DNA samples were mixed with 1/10 volume of 3 M NaOAc solution (pH 5.4) and 3 volumes of absolute EtOH. After gently mixing the samples, they were incubated at -80°C for 20 minutes. Subsequently, samples were centrifuged at 20817 x g for 30 min at 4°C. The supernatant was carefully removed and the DNA pellet was washed with 100 µl of 70 % EtOH. After an additional centrifugation, at 20817 x g and 4°C, for 5 min, the supernatant was removed. Before the pellet was resuspended in 10 to 20 µl of $ddH_2O$, it was air dried under the hood for 10 min.

#### *3.2.6.4 Purification with Amicon® columns*

Amicon® Ultra 10K device can be used for desalting and concentration of biological samples such as ssDNA. A special filter device retains small oligonucleotides, while salts and detergents pass through the column. First, the column needed to be pre-rinsed with 400 µl of water at 14,000 x g for 5 min. Furthermore, 100 µl of the sample was added and filled up with water to the top and centrifuged again. In the next two steps, the column was washed with 500 µl of water. Finally, the filter was flipped and the content spun down at 1,000 x g for 2 min to a new collection tube. For the final application, the whole amount of recovered sample (1 ml) was transferred to the column, followed by 5x washing.

#### *3.2.6.5 Polymerase chain reaction*

PCR is a commonly used method to amplify parts of DNA with specific primers and thermostable polymerase. All sequence oligonucleotides of the library are made of a random region in the middle and two constant regions at both ends, where specific primers anneal. PCR is performed in more rounds, where each consists of different steps at different temperatures and timings (*Table VII*).

Table VII: Settings used in the PCR reaction.

| Step no | Step | Temperature | Time |
|---|---|---|---|
| 1 | Initial denaturation | 95°C | 2 min |
| 2 | Denaturation | 95°C | 30 s |
| 3 | Annealing | 62°C | 30 s |
| 4 | Elongation | 72°C | 1 min |
| 5 | Repetition of step 2-4 | | |
| 6 | Final elongation | 72°C | 2 min |
| 7 | Cooling down | 4°C | ∞ |

In this part of the project, PCR was used to discover which recovery solution elutes the most of the sequences and which one is the most compatible with the PCR. First, FT2 library was planned to be used during the selection. Unfortunately, FT2 library was forming non-specific bands very often, therefore, the whole recovery process and PCR with another click-library, OW1, was performed. PCR was performed with samples that were recovered with different recovery solutions (*Table VI*) and that went through different purification processes. Thus, the most efficient recovery solution and purification steps were chosen.

Table VIII: The table of PCR reaction ingredients for every 100 µl of reaction mix. Some concentrations vary between FT2 and OW1 library.

| Reagent | FT2 library | | | OW1 library | | |
|---|---|---|---|---|---|---|
| | Stock C | Final C | 1x | Stock C | Final C | 1x |
| 10x PWO buffer | 10x | 1x | 10 µl | 10x | 1x | 10 µl |
| fwd primer | 100 nM | 0.5 µM | 0.5 µl | 100 nM | 1 µM | 1 µl |
| rev primer | 82.2 nM | 0.5 µM | 0.6 µl | 100 nM | 1 µM | 1 µl |
| dNTPs (each) | 25 mM | 1.25 µM | 1 µl | 25 mM | 1.25 µM | 1 µl |
| PWO Polymerase | 2.5 U/µl | 0.025 U/µl | 1 µl | 2.5 U/µl | 0.025 U/µl | 1 µl |
| dd$H_2O$ | | | 76.82 µl | | | 76 µl |
| Sample | | | 10 µl | | | 10 µl |

*3.2.6.6 Agarose gel electrophoresis*

The effectiveness of recovery and compatibility of recovery solution with the PCR was checked with agarose gel electrophoresis. 5 µL of each PCR product were mixed with 1 µL of 6x DNA loading buffer and loaded onto a 4 % agarose gel (4 % agarose in TBE buffer with 4 µl/50 ml of ethidium bromide). 2.5 µl of Ultra-low molecular weight DNA ladder

was added to the first well. Gel electrophoresis was performed at 150 mA, 150 V, and 30 W. Gels were visualized using a UV camera and the GenoPlex software.

### 3.2.7 Western blot SELEX procedure

The buffer used during the selection was TBS-T buffer, with additional 1 mM of $Ca^{2+}$ and $Mg^{2+}$ ions. Magnesium and calcium ions help oligonucleotide sequences to form three-dimensional structures. All steps after blotting were performed in a 50-ml tube at RT and on a rotator.

For this selection, 1 nmol of the indole-modified OW1 library and 1 nmol of indole-modified click-competitor was used. For further rounds, the whole amount of amplified and purified ssDNA was chemically modified with indole-azide through click-reaction and used for the next round. More details about click-reaction are described in section 3.2.5.1.

Table IX: Table of the western blot SELEX process.

<table>
<tr><th>Round</th><th>Amount of target</th><th>Selection</th><th>Amount of library</th><th>Competitor</th><th>Incubation</th><th>Washing</th></tr>
<tr><td>1</td><td rowspan="3">PC3.PM:<br>1 µg<br>PC3.CY:<br>1 µg<br>LNCaP.CY:<br>1 µg</td><td rowspan="3"><u>Positive:</u><br>PC3.PM<br><u>Negative:</u><br>PC3.CY<br>LNCaP.CY</td><td>1 nmol</td><td rowspan="7">1:1 click-competitor<br>&<br>0.01 mg/ml sulfodextran</td><td rowspan="2">3 hours</td><td rowspan="2">3x5 min,<br>3 ml</td></tr>
<tr><td>2</td><td rowspan="5">↓</td></tr>
<tr><td>3</td><td rowspan="2">2 hours</td><td rowspan="2">3x10 min,<br>5 ml</td></tr>
<tr><td>4</td><td rowspan="3">LNCaP.PM:<br>2 µg</td><td rowspan="4"><u>Additional negative:</u><br>LNCaP.PM</td></tr>
<tr><td>5</td><td rowspan="3">1 hour</td><td rowspan="3">3x15 min,<br>7 ml</td></tr>
<tr><td>6</td><td>10 pmol</td></tr>
<tr><td>7</td><td>LNCaP.PM:<br>10 µg</td><td>10 pmol</td></tr>
</table>

SDS-PAGE gel was prepared in the same way as described in section 3.2.4.1. Meanwhile, protein samples were prepared as described in *Table IX* (column *Amount of target*). For the first three rounds, only 1 µg of PC3.PM, PC3.CY and LNCaP.CY were used, and additional 2 µg of LNCaP.PM in the last three rounds. As the specific aptamers were not selected after

six rounds of selection (the firstly planned selection), an additional seventh round was performed.

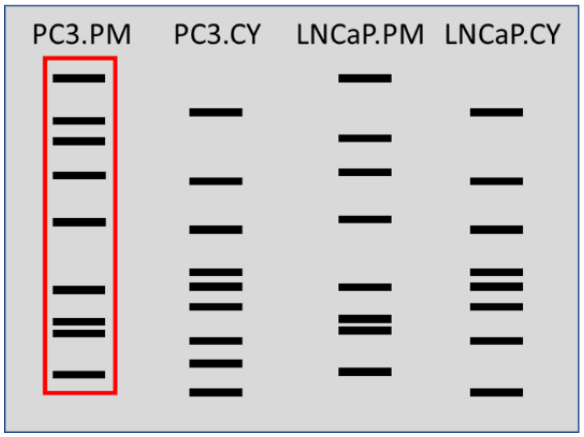


**Figure 3.5: The scheme of a blot with different protein fractions.** The proteins in the red frame present positive-selection target.

Semi-dry transfer was performed in the same way as described in section 3.2.4.3. After blotting, the membrane was blocked for 1 hour in 10 ml of the blocking buffer. Afterwards, the membrane was washed three times with 10 ml and the next two times with 5 ml of TBS-T for 5 min.

The blocked membrane was incubated in 2.5 ml of TBS-T and with the library and competitors, as defined in *Table IX* (column *Amount of library* and column *Competitor*). In rounds 2-5, the whole amount of amplified and purified ssDNA was chemically modified with indole-azide through click-reaction and used for the next round. Incubation time decreased through the selection from 3 hours to 1 hour.

After incubation, the membrane was washed with TBS-T as defined in *Table IX* (column *Washing*).

The PC3.PM protein fraction lane of the membrane was cut away and coiled into a 2-ml vial (*Figure 3.5*). 1 ml of ChIP extraction buffer was added to the vial, so that whole lane was submerged. The vial was incubated at 99°C for 10 min and 1,400 rpm in a thermomixer. After incubation, the membrane lane was discarded. The recovered sample was split into four 1.5-ml vials, each containing app. 200 µl of the sample and EtOH precipitation was performed as described in section 3.2.6.3. Each dry DNA pellet was resuspended in 20-25 µl of dd$H_2O$.

Recovered and purified sequences were amplified before going to the next round. PCR reaction volume was 800 µl for the first five rounds, and for the sixth and seventh round

1000 µl. Additionally, a no template control (only ddH2O instead of the sample) and a positive control (6 pmol of initial library was mixed with Chip extraction buffer, purified with EtOH precipitation and the pellet resuspended in 60 µl of $ddH_2O$) were amplified with the PCR reaction. The PCR master mix was prepared on ice using freshly prepared $ddH_2O$. During the selection, thymidine in the dNTPs mixture was replaced with EdU and instead of usual primers, a biotinylated forward and phosphorylated reverse primers were used. 100 µl of the reaction mixture were aliquoted into 0.5 ml vials, and transferred from ice directly to a preheated PCR cycler in order to avoid formation of primer-dimer structures. The PCR product was checked between the PCR cycles with agarose electrophoresis to avoid over-amplification.

To get rid of salts, primers, enzymes, and other impurities, Nucleo Spin Gel and PCR clean-up Kit was used to purify DNA sequences. 200 µL of Binding buffer NTI was added to each of the PCR tubes containing approximately 100 µL of PCR product. All PCR tubes were put on a silica membrane column and centrifuged at 11,000 x g for 1 min. In the next step, the column was washed with 680 µL of NT3 washing buffer, which contains mostly ethanol. The intermediate step before elution was column drying, now by centrifuging for 2 min. The final step was the elution of the DNA in a new tube with 25 µL ddH2O, again by centrifuging for only 1 min and 1-min incubation before centrifuging. The latter process was repeated 4 times. The final volume of pure dsDNA in water was app. 100 µL. 10 % of purified PCR product (app. 10 µl) was stored at -20°C for binding studies.

After purification, 10 µL of Lambda Exonuclease buffer and 1 µL of Lambda Exonuclease were added to 90 µL dsDNA. The mixture was incubated at 37°C for 15 min and 1,000 x g in a thermomixer. The completeness of digestion was checked with the agarose gel electrophoresis, where the band at 80 bp should not be visible anymore, but only the band of ssDNA at ~50 bp, as shown in *Figure 3.6*.

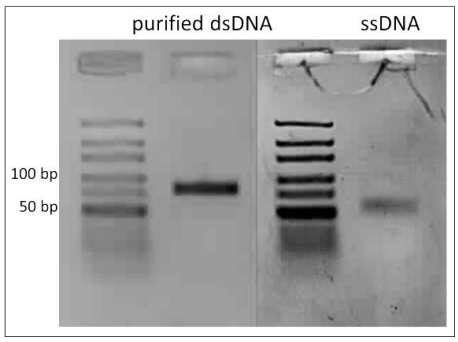


**Figure 3.6: Agarose gel with purified dsDNA at ~80 bp and ssDNA at ~50 bp.**

After digesting one strand of DNA, the solution needed to be purified of salts, enzymes, and small digested DNA fragments again. The procedure was similar to the dsDNA purification, with the exception that instead of using NTI binding buffer, 400 µL of NTC binding buffer was used. The washing and drying steps remained the same. The final elution was performed 4x 15 µL of ddH2O. The final volume of pure ssDNA was app. 60 µL.

### 3.2.8 Binding studies

The final step in this study needs to show whether the selection of specific aptamers that bind with high affinity and specificity to the target succeeded. For this purpose, the binding of selected sequences to specific proteins on the membrane was tested. Binding studies are usually made with selected aptamers after the final round and with aptamers selected in optional rounds during the selection. Thus, the development of enriched library is observed. Binding studies were made in duplicates.

#### *3.2.8.1 PCR and click-reaction*

As the initial OW1 library was not biotinylated (3.1.8), a PCR reaction with biotin conjugated forward primers needed to be performed first. Sequences, obtained after the second, fourth and sixth round were amplified in 800-1000 µl of PCR reaction mix, for which approximately 0.1 pmol of selected sequences per 100 µl were used. As the results showed that the selection was not yet satisfactory, the seventh round was performed. PCR products were purified and single strand displaced. The final step in selected sequences preparation was click-reaction with indole-azide. 20 pmol of amplified ssDNA selected after the sixth round were left unmodified in order to compare the effectiveness of binding studies of modified selected sequences with unmodified ones.

#### *3.2.8.2 Incubation*

Western blot membrane with 1 µg of all protein fractions (PC3.PM, PC3.CY, LNCaP.PM, LNCaP.CY) and 0.001 µg of biotinylated BSA was prepared. Biotinylated BSA was used for normalization and final quantification of the data. The membrane was blocked in a 50-ml Falcon tubes with the blocking buffer for one hour at RT. Then, the membrane was washed three times, and each membrane was incubated in 2.5 ml of TBS-T with 10 pmol of selected ssDNA sequences, 1:1 click-competitor and 0.01 mg/ml sulfodextran. Subsequently, tubes with the membranes were rotated for 1 hour at RT.

#### *3.2.8.3 Washing*

After incubation, the membranes were washed three times with 5 ml of TBS-T for 10 minutes.

#### *3.2.8.4 ECL method and quantification*

Finally, the membranes were visualized with the ECL method, the same way as described in section 3.2.5.5.

# 4 Results and discussion

This chapter provides results and discussion about subcellular fractionation and protein markers detection by western blot analysis, optimization of background binding with various competitors, recovery and purification methods, western blot SELEX and binding studies.

## 4.1 Subcellular fractionation and protein markers detection by western blot analysis

Fractionation of a cell lysates to pure PM and CY fractions is a lengthy procedure, which requires ultracentrifugation, a special kit for subcellular fractionation or final centrifugation on a sucrose gradient [27, 72, 66]. As the kit and ultra-centrifuge were not available in our laboratory, and the centrifugation steps require two full days of work [66], a shorter process was applied instead. The proteins of the nucleus had to be avoided in the fractions, due to their ability to non-specifically bind nucleic acids with high affinity. The PM fraction contained proteins of cell membranes (mitochondria, ER, etc.), along with the PM proteins while, the CY fraction contained the cytosolic proteins. We supposed that, the cell membranes' proteins in the PM fraction (except the PM proteins) of PC3 and LNCaP cells are very similar and, therefore, the binders to cell membrane proteins will be eliminated through negative selection on the LNCaP.PM fraction. To prove the purity of PM and CY fractions, we checked for the absence of PM proteins in the CY fraction and for absence of CY proteins in the PM fraction. At different stages during the subcellular fractionation process, possible nucleic markers were checked in both fractions. It is practically impossible to obtain completely pure fractions with different centrifugation steps only, therefore, small impurities were acceptable. The presence of markers of cytosolic proteins, plasma membrane proteins and nuclei proteins in PM and CY fractions of PC3 and LNCaP cells is given in *Figure 4.1*.

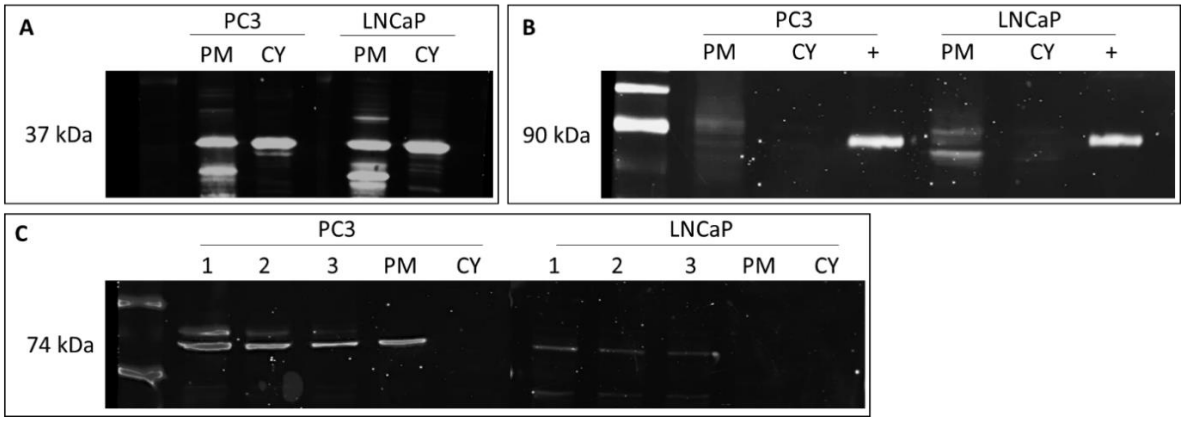


**Figure 4.1: Immunodetection of markers of cytoplasmic proteins, plasma membrane proteins and nuclei proteins.** Antibodies adopted**: A.** anti-GAPDH; **B.** anti-CD71; **C.** anti-lamin A+C. **PM =** plasma membrane fraction; **CY** = cytosolic fraction. **+ =** positive control, (CD71 protein) **1** = homogenized cell; **2** = supernatant after 1st nuclei centrifugation; **3** = supernatant after 2nd nuclei centrifugation.

CD71 (transferrin receptor) is a cell surface receptor necessary for cellular iron uptake by receptor-mediated endocytosis. It is present on the plasma membrane of most human cells (including the prostate cells) and thus suitable as a plasma membrane marker [74]. In both CY fractions, there was no visible band at ~90 kDa, which indicates the absence of plasma membrane proteins in the CY fraction (see *Figure 4.1B*). On the other hand, there was a weak band at ~90 kDa detected in both PM fractions, which is due to a weak expression of CD71 in prostate cells. In the LNCaP.PM fraction there were two CD71 bands which could represent two isoforms of CD71 [75].

The nuclear lamins are a type of V intermediate filament proteins that are critically important for the structural properties of the nucleus [76]. They are present only in the nuclear envelope and can be therefore used as markers of nuclear proteins. A nuclear lamin band was detected in the whole homogenate (*Figure 4.1C*, lane 1) of both PC3 and LNCaP cells. The band in the supernatant after the first and second nuclei centrifugation (lanes 2 and 3) should be much weaker compared to the whole homogenate, but its intensity remained the same. The reason for that could be a too harsh homogenization step that damaged the nuclei that released nuclear proteins into the membrane fraction (band visible in the PC3.PM fraction). Because of different fragility of the cells, fractionation of cells should be optimized individually. Due to lower expression in LNCaP cells, the PM and CY fractions of LNCaP cells did not contain detectable amounts of nuclear lamins. Besides that, another reason might be their resuspension in the last two centrifugation steps without being precipitated again [76]. The number of cells required for fractionation was initially 9 million, which was

very difficult to reach, as it would require at least eight flasks of cultured cells for one fractionation. With a doubling time of 60 hours of LNCaP cells, this number of cells would be hard to obtain [64]. With four culture flasks we managed to get approximately 2-7 million cells per fractionation which consequently meant less total protein extracted.

## 4.2 Optimization of background binding

Optimization of background binding is necessary to decrease non-specific binding of ssDNA sequences to the proteins. Without any competitor, the sequences bind in abundance to the proteins and result in misleading aptamer selection (*Figure 4.2*). This binding is non-specific and has to be decreased. Therefore, different competitors were tested to find which decreased non-specific binding the most.

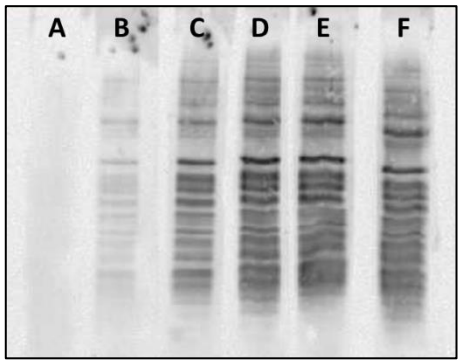


**Figure 4.2: Western blot membrane with a different amount of PC3.PM proteins incubated with bio-FT2 library. A.-G.** 0.1 µg, 1 µg, 2.5 µg, 5 µg, 7.5 µg and 10 µg of total plasma membrane proteins, respectively.

The background optimization comprised western blot membrane preparation, incubation with different competitors and final protein quantification.

### 4.2.1 Western blot membrane preparation

Different amounts of LNCaP.PM proteins and 0.5 ng of bio-BSA were transferred from SDS-PAGE gel to NC membrane by western blotting. To check the effectiveness of transfer, one membrane was stained with Pierce® Reversible Protein Stain Kit (*Figure 4.3*). Proteins of all sizes were transferred adequately.

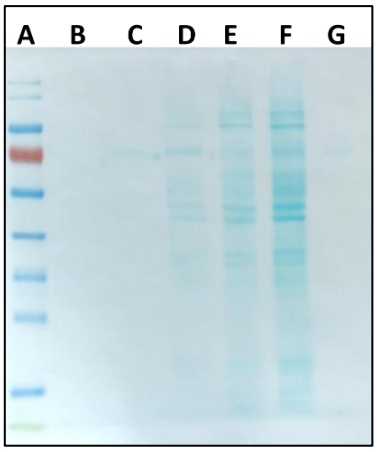


**Figure 4.3: Transfer efficiency for LNCaP.PM proteins. A.** PageRuler Prestain Protein Ladder, **B.-F.** 0 µg, 0.1 µg, 1 µg, 2.5 µg and 5 µg of LNCaP.PM proteins **G.** 0.5 ng of bio-BSA.

### 4.2.2 Incubation with different competitors and ECL visualization

Competitors present during selection prevent non-specific interactions (e.g. ionic interactions of phosphate groups) between the library and the target proteins. The commonly used competitor for SELEX approach is sulfodextran, which competes with its negatively charged groups with the phosphate group of oligonucleotides for the binding on positively charged protein residues [34]. Another frequent competitor is salmon sperm DNA that prevents the binding of short sequences from the library. For click-libraries the addition of a click-competitor is recommended at the ratio 1:1 [77]. The click-competitor should have the same modifications as the library itself, i.e. the azide groups of the competitor compete with the same groups on the library and the stringency of the selection is increased. To eliminate non-specific binding of antibodies to the western blot membrane, 5 % BSA was used as the blocking agent; hence, 5 % BSA was also used as a competitor during SELEX.

Taking into account the narrow linear dynamic range of ECL visualization we chose to work with total protein amounts in the range 0.1 - 5 µg, while smaller protein intensities would be difficult to differentiate [78]. In order to examine possible binding of the sequences to the blocking BSA, the first lane on each membrane contained no cellular proteins loaded (*Figure 4.4*).

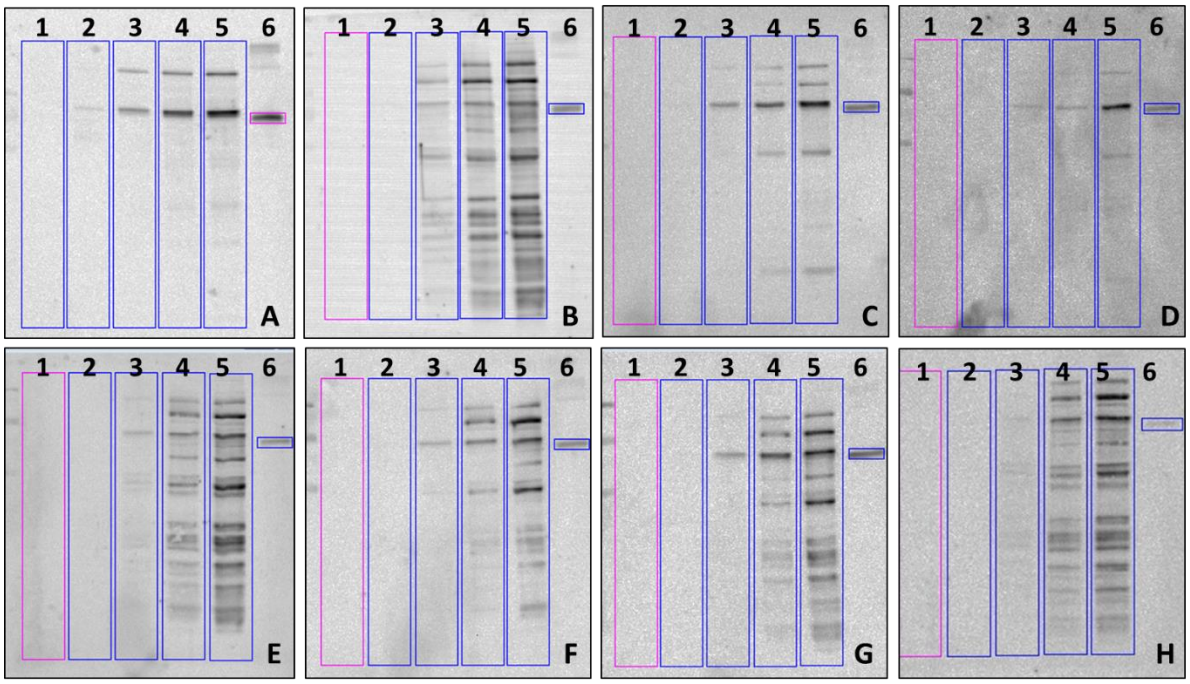


**Figure 4.4: Visualized membranes, incubated with modified FT2 library and different types of competitors.** Bound sequences are visualized with ECL method. **A.** no library and no competitor; **B.** 20 pmol FT2 library, no competitors; **C.-H.** 20 pmol FT2 library and competitors. The competitors were 0.01 mg/ml sulfodextran (**C**), 0.1 mg/ml sulfodextran (**D**), 5 % BSA (**E**), 20 pmol click-competitor (**F**), 200 pmol click-competitor (**G**), and 200 pmol salmon sperm DNA (**H**). Amount of total LNCaP.PM proteins loaded in lanes: **1** no proteins; **2-5** 0.1 µg, 1 µg, 2.5 µg, and 5 µg, respectively; **6** bio-BSA.

To check if the STR-HRP conjugate recognizes any proteins in the fractions, we incubated the membrane with total proteins alone (with no aptamer sequences bound) (*Figure 4.4A*) Streptavidin binds non-specifically to proteins sized ~70 kDa and ~130 kDa, which possibly indicates a naturally biotinylated protein. In mammalian cells, four carboxylase enzymes contain biotin, so the bands are probably their biotinylated subunits, ranging between 70 to 220 kDa. The band at ~70 kDa is most likely a subunit of propionyl CoA carboxylase, which is involved in the metabolism of valine, isoleucine, and methionine. Pyruvate carboxylase involved in the formation of oxaloacetate is a single-chain, a multi-domain enzyme, with the size of ~130 kDa which corresponds to the second band [79, 80].

Surprisingly, there is no much background binding to blocking BSA (lane 1 on each membrane **A**-**H**). We can also conclude that 5 % BSA (membrane **E**) and 200 pmol salmon sperm DNA (membrane **H**) do not represent good competitors, as there is still non-specific binding of sequences to a variety of proteins. On the other hand, click-competitor (membranes **F** and **G**) and especially sulfodextran (membranes **C** and **D**) showed less binding of the library to the proteins. The reason for such strong effectiveness of sulfodextran

is probably due to its high concentration in comparison with other competitors. However, for more accurate results the quantification was done.

### 4.2.3 Quantification

The intensity of each lane on every membrane on *Figure 4.4* was measured with the Aida Software in triplicates. The inverted intensity was given; therefore, the intensity was calculated as $1/intensity$. The normalized intensity value of each lane was calculated by setting the intensity of the BSA lane on each membrane to 100. The following equation was used:

$$normalized\ intensity = \frac{BSA\ intensity \cdot 100}{intensity}$$

Normalized intensity of the blank was subtracted from the normalized intensity of each lane. The mean and standard deviation of normalized intensities were calculated with the Prism software (*Figure 4.5*).

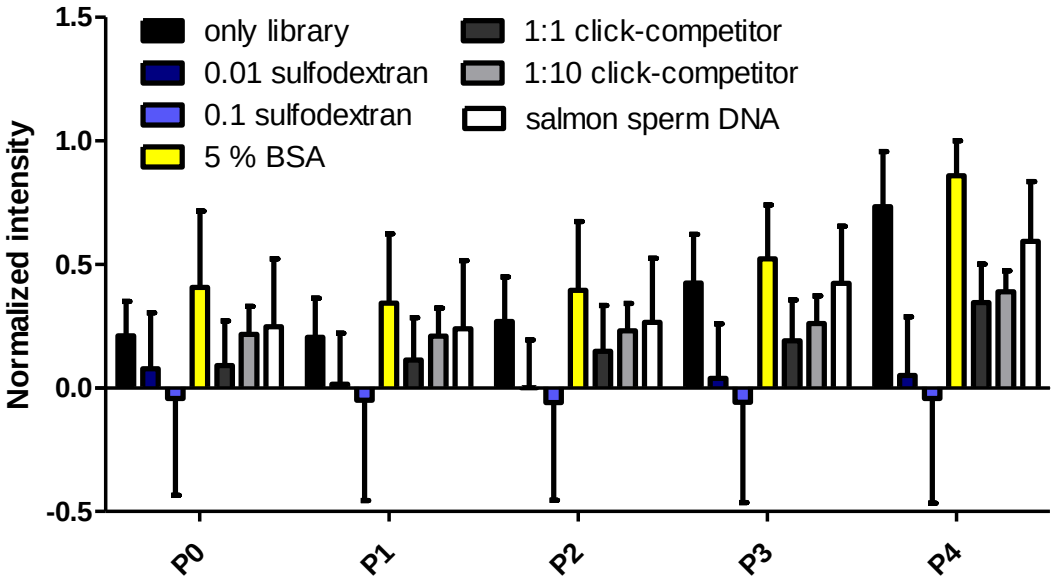


**Figure 4.5: Background binding analysis of various amounts of proteins with addition of different competitors by ECL visualization.** Normalized intensities of sequences bound to LNCaP.PM proteins in the presence of different competitors; **P0.** no blotted proteins; **P1.** 0.1 µg of proteins; **P2.** 1 µg of proteins; **P3.** 2.5 µg of proteins; **P4.** 5 µg of proteins.

The most effective competitor, the lower the intensity expected. The intensity of lanes with 0.1 mg/ml sulfodextran even reached a negative value, meaning that the high concentration of sulfodextran inhibited the binding of streptavidin to biotin.

The highest intensity was expected with the library with no competitors. With all protein quantities used, the intensity with 5 % BSA was even higher than the intensity of the library

with no competitors. In most protein quantities, also salmon sperm DNA produced higher intensities. This means that the sequences are binding even more in the presence of 5 % BSA and salmon sperm DNA.

Data from *Figure 4.4* and *Figure 4.4* apparently contradict each other. We suppose that ECL visualization may not be the best choice for quantification. For the purpose of quantification, IR fluorometry or colorimetry would be better choices, as IR fluorescence has a wider linear range [81]. Unfortunately, the library we employed was not available as fluorophore or alkaline phosphatase conjugates [78]. Furthermore, correct quantification depends also on protein fractionation, blotting, enzymatic reaction, and variations of intensity of bio-BSA.

Data from *Figure 4.4* and *Figure 4.4* agree in the conclusion that 5 % BSA and salmon sperm DNA are not effective competitors, while sulfodextran and click-competitor represent the most efficient competitors as they decreased non-specific binding the most.

For even better results, a combination of sulfodextran and click-competitor was also tested. Instead of the too harsh sulfodextran concentration of 0.1 mg/ml; concentrations of 0.01 mg/ml and 0.001 mg/ml were used instead (*Figure 4.6*).

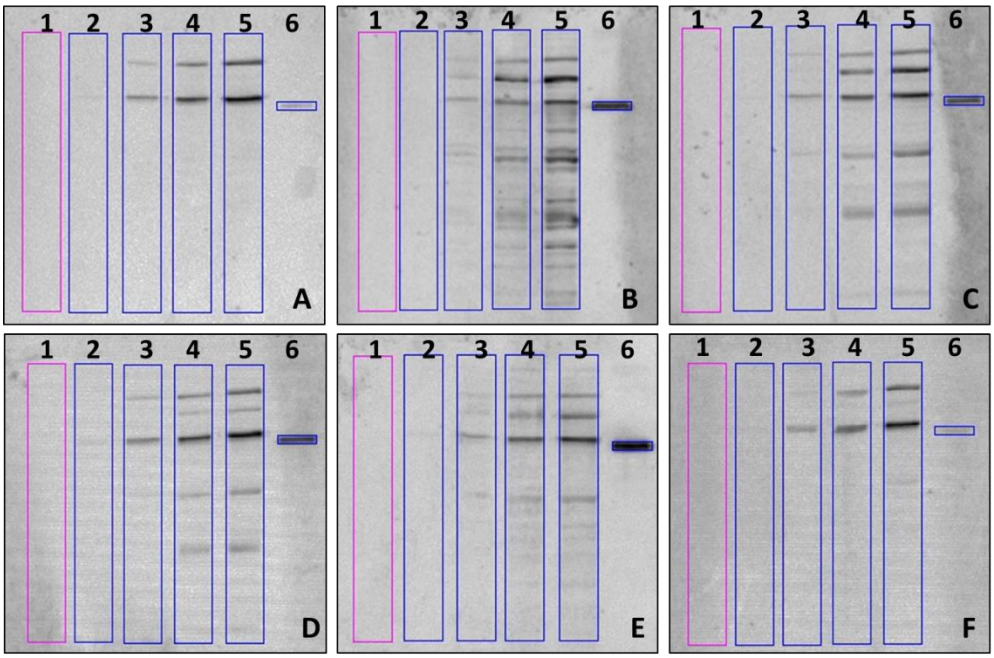


**Figure 4.6: Membranes, incubated with the modified FT2 library and combination of different concentrations of competitors. A.** no library and no competitor; **B.** 20 pmol of FT2 library with no competitor; **C.-F.** 20 pmol of FT2 library with competitors: 20 pmol click-competitor and 0.001 mg/ml sulfodextran (**C**); 20 pmol click-competitor and 0.01 mg/ml sulfodextran (**D**); 200 pmol click-competitor and 0.001 mg/ml sulfodextran (**E**); 200 pmol click-competitor and 0.01 mg/ml sulfodextran (**F**). Amount of total LNCaP.PM proteins loaded in lanes: **1** no proteins; **2-5** 0.1 µg, 1 µg, 2.5 µg, and 5 µg, respectively; **6** bio-BSA.

The membrane with the combination of 200 pmol click-competitor and 0.01 mg/ml sulfodextran (*Figure Figure **4.6**F*) looks very similar to the blank membrane with no library incubation (*Figure **4.6**A*). This means that the 10-times higher click-competitor (200 pmol) amount compared to the library amount (20 pmol) together with 0.01 mg/ml sulfodextran represent a too harsh combination that prevents the binding of almost all sequences. On the other hand, the 1:1 click-competitor and 0.001 mg/ml sulfodextran combination (*Figure **4.6**C*) still allow quite some non-specific binding. The optimal selection results were obtained with the combination of 1:1 click-competitor with 0.01 mg/ml sulfodextran (*Figure **4.6**D*) and 1:10 click-competitor with 0.001 mg/ml sulfodextran (*Figure **4.6**E*). As the click-competitor is much more expensive than sulfodextran, we decided to use the combination of 1:1 click-competitor and 0.01 mg/ml of sulfodextran. We decided to use 1µg of proteins for the selection, as it represents the lowest amount of proteins with still visible bands.

### 4.3 Recovery methods

After the sequences bind to the proteins, in the next step they need to be eluted (recovered) from the target by breaking the interactions between the protein and ssDNA. Recovery of sequences is sometimes poor, therefore, we tried different detergents, salts, and solvents e.g. ammonium acetate, NaCl, Urea, WB buffer, phenol-chloroform, ChIP extraction buffer (*Table VI*).

1 M NaCl can disrupt intermolecular electrostatic interactions and destabilize the folding of the oligonucleotides by hindering intramolecular interaction between nucleobases [82]. The ChIP extraction buffer is commonly used to break covalent bonds between DNA and protein in Chromatin Immunoprecipitation assay, while SDS present in the extraction buffer disrupts non-covalent bonds [83]. 1x Urea is used to break secondary structures of nucleic acids. The WB stripping buffer is employed in western blot assays to remove antibodies bound to proteins. The addition of Tween-20 to the buffer is also known to break apart non-covalent bonds [84].

A mixture of ChIP extraction buffer and 1x Urea buffer can be used for recovery, combining the effect of disrupting secondary structure and breaking non-covalent bonds. For stripping proteins from the NC membrane 40 % acetonitrile in 0.1 M ammonium acetate is also very effective [85]. Lastly, a 10x higher protein amount (compared to the amount of the amount of blotted proteins) was used as a recovery solution to compete for bound DNA sequences [86].

### 4.3.1 Estimation of remained non-eluted sequences

To narrow the choice of different recovery solution, the lanes of the membrane were first cut apart and each lane was eluted with a different recovery solution (*Table VI*) and visualized with ECL method (*Figure 4.7*). The membrane incubated with 40 % acetonitrile in 0.1 M of ammonium acetate shrank and the visualization was not possible.

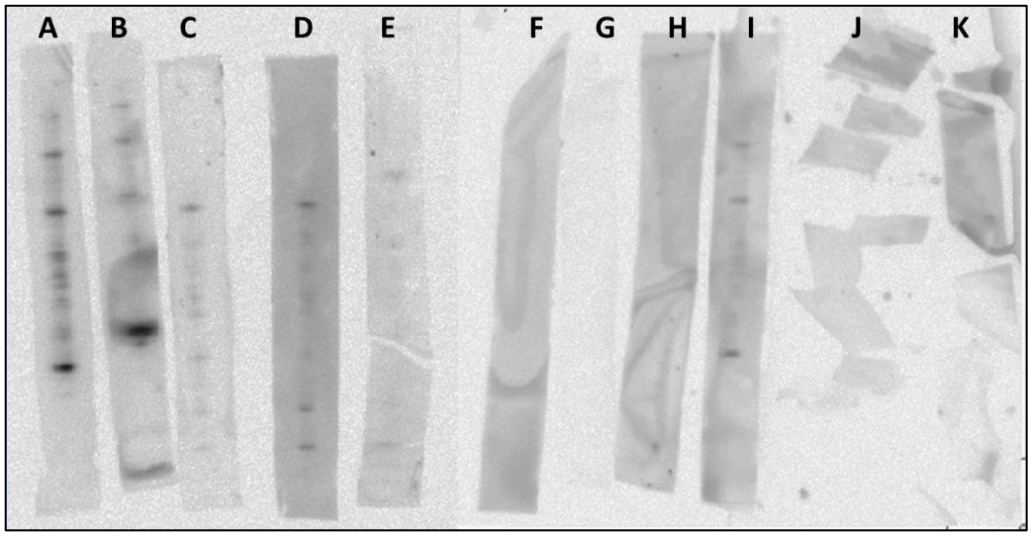


**Figure 4.7: Membranes incubated with different recovery solutions.** 1 µg of PC3.PM proteins were blotted and three lanes were incubated with 20 pmol of modified FT2 library with click-competitor in 0.01 mg/ml sulfodextran. **A.** control with no recovery; **B.** ddH2O; **C.** ammonium acetate; **D.** 1M NaCl; **E.** 1x Urea; **F.** WB buffer; **G.** phenol-chloroform; **H.** 1xUrea+ChIP; **I.** 10 x protein amount in solution; **J.** 4x Urea; **K.** ChIP buffer.

From this experiment, we can conclude that water (*Figure **4.7**B*), ammonium acetate (*Figure **4.7**C*), 1 M NaCl (*Figure **4.7**D*), and competitive proteins (*Figure **4.7**I*) are not effective recovery solutions. On the other hand, the intensity of lanes *E, F, G, H, J* and *K* decreases in comparison with lane *A* (non-eluted proteins). Hence, only the recovery solutions 1x Urea, WB buffer, Phenol-Chloroform, 1xUrea+ChIP, 4x Urea, ChIP buffer and 40 % acetonitrile in 0.1 M ammonium acetate, were used in further studies.

### 4.3.2 Comparison between phenol/chloroform extraction and EtOH precipitation

We also tested whether salts, detergents, and eventually eluted proteins have an impact on the ability of PCR amplification. Each recovery solution was first purified with phenol/chloroform extraction and EtOH precipitation or EtOH precipitation alone. Purified recovered samples were amplified with PCR reaction and the amount of PCR product was estimated with the agarose gel electrophoresis. The recovery solution in combination with the purification method that resulted in the most intensive bands are the most effective one.

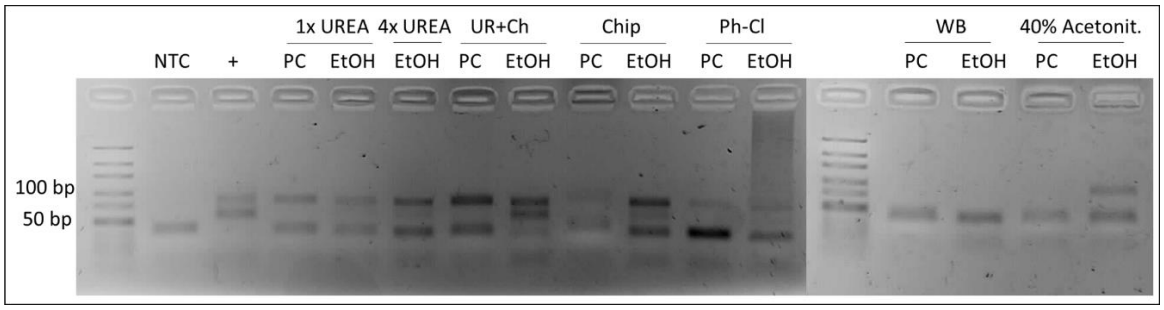


**Figure 4.8: Agarose gel with PCR product after 24 amplification cycles with purified recovered samples.** 20 pmol of modified FT2 library was used per three lanes. Samples were purified with phenol/chloroform extraction (PC) and EtOH precipitation (EtOH). NTC = no template control.

From the results presented in *Figure 4.8*, we can narrow down the choice of recovery solution to: 4x Urea loading buffer, 1x Urea + ChIP mixture, and ChIP extraction buffer. Concerning the purification method, we can apprehend that precipitation with EtOH alone is as effective or even better than phenol/chloroform extraction. In all samples, even in the no template control (NTC), non-specific bands were visible. The reason for that might be the FT2 library, which is more likely to amplify non-specific bands. For this reason, another click-library OW1 was used in further investigation in addition to the FT2 library [87].

### 4.3.3 Comparison between EtOH precipitation and Amicon® column purification, and FT2 and OW1 library

We also tried to purify recovered samples with the Amicon® columns. We used 0.1 pmol and 0.5 pmol, of FT2 or OW1 library, respectively, and recovered the sequences with 100 µl of 4x Urea loading buffer, ChIP extraction buffer or mixture of 1x Urea + ChIP. Samples were purified with EtOH precipitation or Amicon® column and amplified. After the purification with Amicon® column, only 1/5 (out of initial 0.5 pmol) was taken for further amplification. With this experiment, the efficiency of the purification methods and the impact of the remaining impurities on the PCR were investigated, while the elution ability of the recovery solutions was not the primary goal.

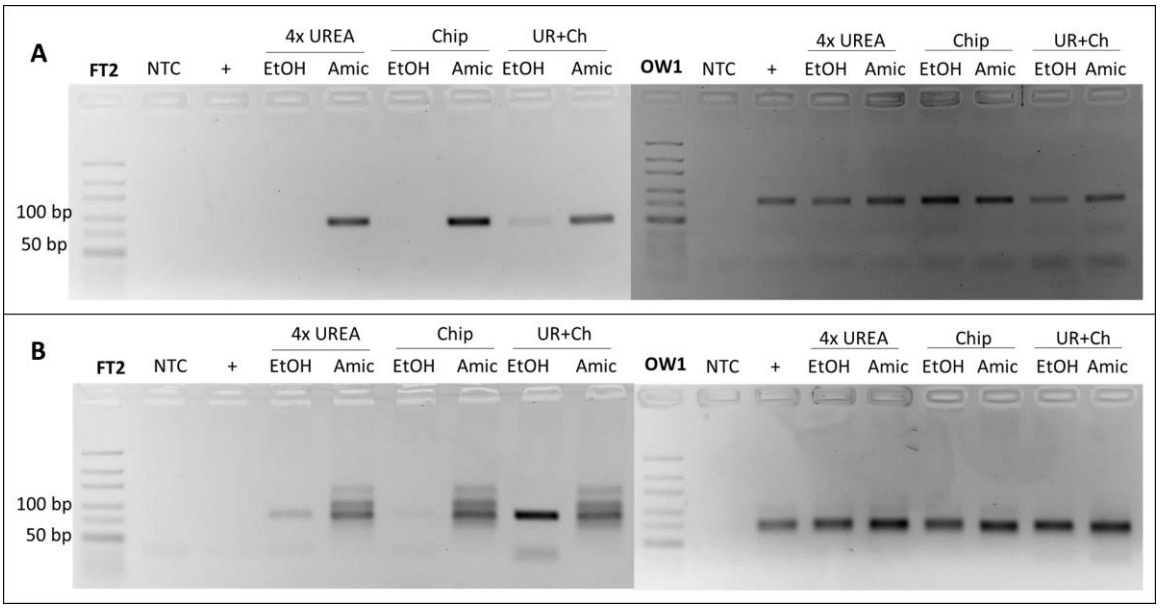


**Figure 4.9: Agarose gel with PCR products amplified after being recovered with different recovery solutions and purified with EtOH precipitation or Amicon® column. A.** PCR products after 14 cycles; **B.** PCR products after 18 cycles.

From the results, we can conclude that purification with Amicon® columns is more effective, especially when the FT2 library is used for selection (*Figure 4.9*). The most intense band is visible after recovery with ChIP extraction buffer, which means that this recovery solution is the most compatible with PCR reaction. However, elution ability of the recovery solutions needed to be further investigated.

During the PCR studies, it happened a couple of times that positive control was negative or the band came out in later PCR cycles, as seen in the case of FT2 library. The reason was probably a too low initial amount of the library (0.1 pmol).

During this experiment, we were able to confirm that FT2 library is more likely to form non-specific bands. For this reason, the OW1 library was preferably used for further studies and for the western blot SELEX approach.

Finally, both, the elution ability and compatibility with PCR of the recovery solution were combined and tested. Sequences were recovered from the membrane, purified, and amplified.

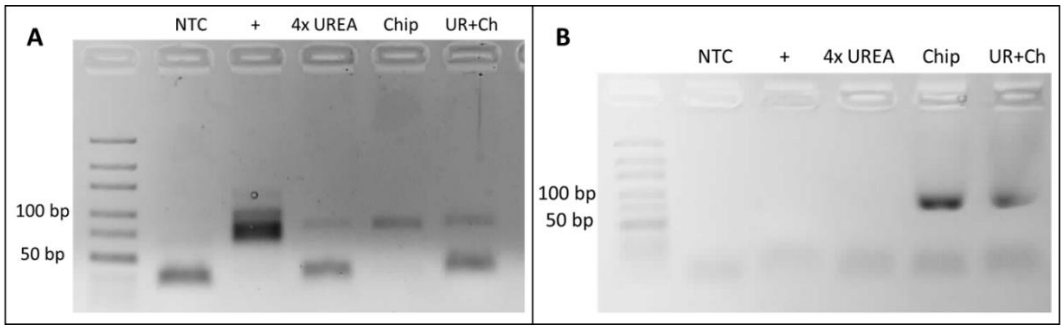


**Figure 4.10: Agarose gel with OW1 PCR product, recovered from the membrane and purified with Amicon® column. A.** PCR product after 22 cycles (1/10 of the eluate); **B.** PCR product after 14 cycles (1/8 of the eluate).

Based on the results in *Figure 4.10*, ChIP extraction buffer has proved to be the most effective recovery solution. Unfortunately, the use of OW1 library resulted in nonspecific bands and primer-dimers. We solved this problem by replacing unmodified primers with phosphorylated and biotinylated ones and with the preparation of PCR master mix on ice and placing the samples directly onto a preheated PCR cycler. By doing this, we successfully amplified only specific, 80 bp-long band and not the non-specific primer-primer structures (~50 bp). We believe that this is because the library does not have any thymidine/EdU in the primer-binding sites. The library is designed this way because the removal of synthesized EdU-containing DNA library from solid phase under alkaline conditions results in the partial oxidation of the ethynyl-moiety, obtaining a ketone containing C5-acetyl residue (KdU) [88]. The KdU is no longer available for functionalization by click-chemistry, thereby leading to an inhomogeneous product. Homogenous product can be obtained by PCR; however, it is difficult to synthetize completely functional DNA in a large scale [89]. A possible way to yield totally functional DNA containing EdU by solid phase synthesis is to perform the click-reaction first, by which alkyne on EdU is protected, and can be removed from the solid phase under alkaline conditions [90]. However, in this way there is no possibility to optionally modify the library after the synthesis. Another way to obtain totally functional product is by using "protection tips", through which alkyne is protected (Ella Biotech). Unfortunately, this kind of synthesis is very expensive, therefore, our library was preferably synthesized without any EdU in primer-binding sites. In this way, we saved a lot of unnecessary costs for the synthesis of the forward primer [37, 87]. The synthetized initial library still contained some KdU in the random region; however, we believed, it was not affecting the selection of aptamers, since we used the synthetized library only in the first round of the selection.

### 4.4 Western blot SELEX

Initially we wanted to perform western blot SELEX with negative selection of the LNCaP.PM already in the first round. Unfortunately, LNCaP cells were contaminated and needed to be discarded. As these cells grow really slowly (2-3 weeks) and we were short on time, the selection needed to start without LNCaP.PM proteins. An aliquot of LNCaP.CY proteins was still available from the previous cell fractionation and could be included in the selection from the beginning.

We considered setting the incubation time during the selection to 1 hour, correspondingly to the incubation time with primary antibodies during western blot analysis. In most cases, incubation time during selection decreases with every additional round, thus increasing the stringency of the process. As we also wanted to select aptamers with slow on and off binding rate, the starting incubation time was set on 3 hours. The idea behind such a long incubation time was that there could be potential strong and specific binders among slow binding aptamers that have less chance to bind in a short period of time than aptamers with a faster on and off rate.

When everything was optimized, we could start with the first round of selection. Unfortunately, a problem arose during amplification, as the PCR product of all eight reaction tubes had unusual smear and was not the right size (*Figure 4.11*). Similar bands also occurred during the recovery experiments (*Figure 4.10B*), however, the reason for that was probably the oversaturation of Amicon® column with salts and detergents, as the whole 1 ml eluate was poured on the column. The salts and detergents in the sample could have caused the smear and different traveling of the PCR product. We used a PCR clean-up Kit to remove the impurities from the samples. Unfortunately, the silica gel column of the clean-up kit did not bind the DNA sequences, instead washed through the column most likely due to the SDS present in the ChIP extraction buffer.

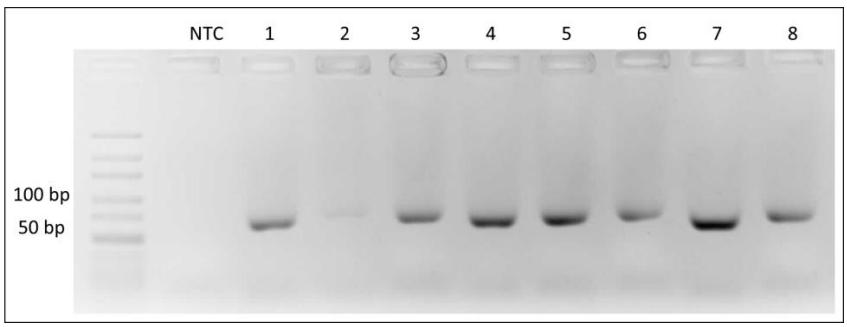


**Figure 4.11: Agarose gel of first round PCR product.** Recovered sample was purified with Amicon® column. 1-8 are numbers of PCR reaction tubes.

The first round of selection needed to be repeated, this time using EtOH precipitation for purification instead of Amicon® columns. Purification of sequences of OW1 library was as efficient with EtOH precipitation as with Amicon® columns (*Figure 4.9*). The PCR product after performing EtOH precipitation was the right size and without any smear (*Figure 4.12*).

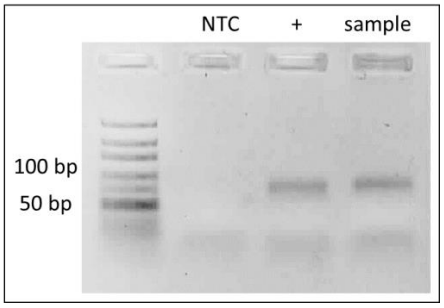


**Figure 4.12: Agarose gel of first round PCR product (second try).** The recovered sample was purified with EtOH precipitation.

For further rounds, the whole amount of recovered and amplified products (in 800 µl of reaction mix) was used during incubation. Interestingly, only 15 pmol of sequences were obtained in the second round. Using only 15 pmol was quite risky, but as only 20 pmol per three lanes of proteins was used during optimization of recovery methods (*Figure 4.9*), we assumed that 15 pmol would still be enough. The assumption was correct, as we managed to recover sequences in the second round. The complete information about selection parameters is collected in *Table X*.

In the sixth and seventh round, only 10 pmol were used for incubation, as it was also the same amount used for further binding studies. Furthermore, recovered samples were amplified in 1000 µl of PCR reaction mix, as the remaining product was intended to be used directly for binding studies.

| Round | Target | | Library | | Incubation | Washing | | | PCR | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Selection | µg | pmol (OW1) | Competitor | Time | N | Time | Volume | Number of cycles | Volume |
| 1 | POSITIVE: PC3.PM<br>NEGATIVE: PC3.CY LNCaP.CY | PC3.PM: 1<br>PC3.CY: 1<br>LNCaP.CY: 1 | 1,000 | 1:1 click-competitor & 0.01 mg/ml sulfodextran | 3 hours | 3x | 5 min | 3 ml | 18 | 800 µl |
| 2 | | | 15 | | | | | | 16 | 800 µl |
| 3 | | | 14 | | 2 hours | | 10 min | 5 ml | 16 | 800 µl |
| 4 | +NEGATIVE: LNCaP.PM | + LNCaP.PM: 2 | 28 | | | | | | 16 | 800 µl |
| 5 | | | 24 | | 1 hour | | 15 min | 7 ml | 16 | 800 µl |
| 6 | | | 10 | | | | | | 18 | 1000 µl |
| 7 | | + LNCaP.PM: 10 | 10 | | | | | | 16 | 1000 µl |

*Table X: Complete parameters used for western blot SELEX.*

### 4.5 Binding studies

In the final part of the study, specificity of selected sequences was tested. We expected that after six rounds of selection, sequences will bind exclusively to proteins of the PC3.PM fraction. Unfortunately, after performing the first binding study, we saw that the selection was not done yet, as there was also binding to proteins of the LNCaP.PM fraction (*Figure 4.13*), therefore, another, seventh round was performed. In the seventh round, we wanted to increase stringency as much as possible by increasing the amount of LNCaP.PM proteins to 10 μg (instead of 1 μg or 2 μg), while other parameters stayed the same as in the sixth round.

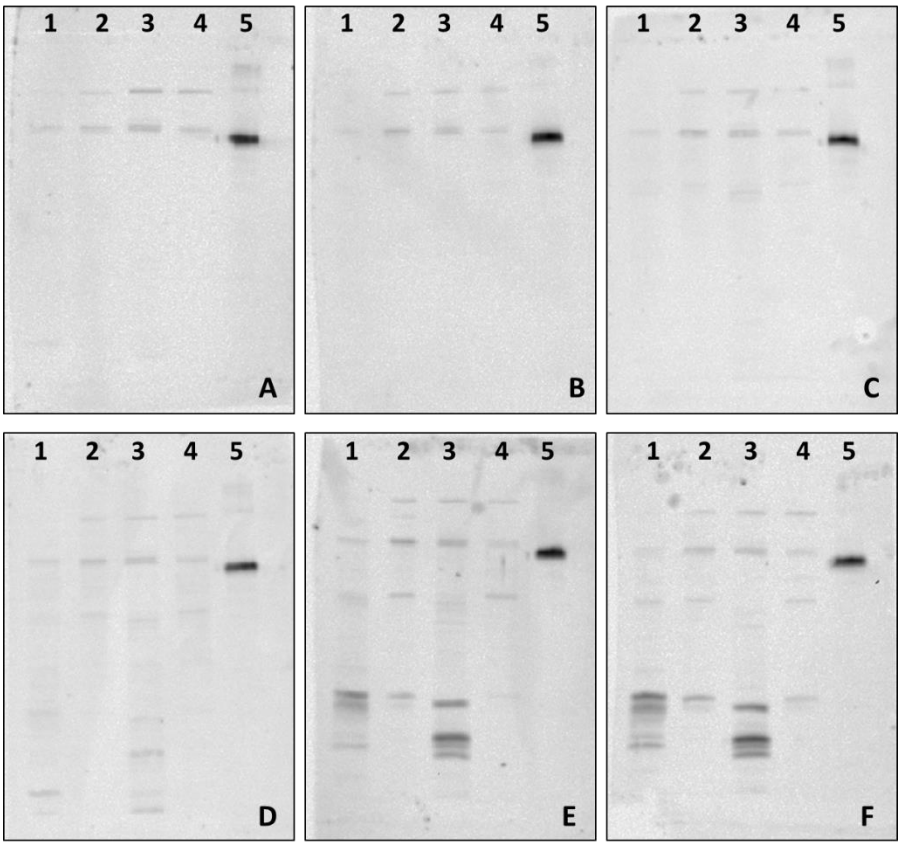


**Figure 4.13: Membranes used for binding studies.** Each membrane was incubated in TBS-T buffer with 10 pmol of click-competitor, 0.01 mg/ml of sulfodextran and **A.** no library; **B.** 10 pmol of indole-modified OW1 library; **C.** 10 pmol of sequences obtained after 2. round; **D.** 10 pmol of sequences obtained after 4. round; **E.** 10 pmol of sequences obtained after 6. round; and **F.** 10 pmol of sequences obtained after 7. round. **1.-4.** proteins form fractions PC3.PM, PC3.CY, LNCaP.PM and LNCaP.CY, respectively; **5.** bio-BSA.

The LNCaP.PM lane of the seventh round looks slightly less intense than the one after the sixth round, while some of the bands of PC3.PM lane are getting slightly stronger. Unfortunately, we still could not get rid of all LNCaP.PM binders, but maybe with another one or two rounds, where the stringency would be even higher, this could have been

achieved. However, the different binding of sequences to proteins of PM fractions and CY protein lanes is already successful. Already after the second round, stronger binding to PM fraction could be observed. In our opinion, the difference of binding to PC3.PM and LNCaP.PM would be stronger, if the negative selection, LNCaP.PM, would be included from the first round, as it was planned in the first place. It can be observed that sequences binding to lower-sized proteins are enriched more efficiently. The reason could be the sticking of the coiled NC lane during the recovery phase, that prevents sequences to be removed from the membrane. For the confirmation of this hypothesis, further investigation should be done, where lanes would be cut in pieces and each piece would be put into different vial with smaller volume of recovery solution.

We wanted to find out if the indole on the EdU plays a role in the interaction of sequences with proteins. Therefore, indole-modified sequences obtained after the sixth round were compared with the same unmodified ones (*Figure 4.14*). Surprisingly, there is almost no binding of the unmodified sequences.

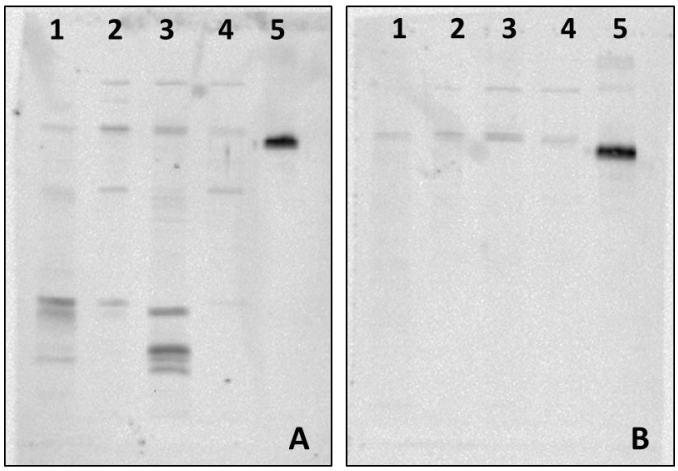


**Figure 4.14: The comparison between the binding of modified sequences (A) and sequences without modification (B). 1.-4.** proteins from fractions PC3.PM, PC3.CY, LNCaP.PM and LNCaP.CY, respectively; **5** bio-BSA.

The intensity of all protein lanes was determined, and normalized to the intensity of bio-BSA band on each membrane. Quantification did not give us meaningful information in comparison with the pictures of visualized membranes (*Figure 4.15*). By comparing the normalized intensities after round zero (R0, initial library) with the intensities after round seven (R7), we can conclude that the sequences were in fact enriched. The enriched library of R7 showed a significant increase in binding to the PC3.PM and LNCaP.PM proteins in comparison to the binding of R0. However, the binding to PC3.CY and LNCaP.CY at R7

was too high to be credible. Moreover, binding of R2, R4 and R6 library showed lower binding than R0. Comparison of R6 library with R6-1 (= R6 library without indole-modification) does not confirm results shown in *Figure 4.14*. In summary, the quantified intensities presented in *Figure 4.15* are not reliable and will not be taken into account in the final conclusions. IR fluorometry and colorimetry would be better visualization methods, as they enable much better quantification than ECL method. The conclusions will be based only on data represented in *Figure 4.13* and *Figure 4.14*.

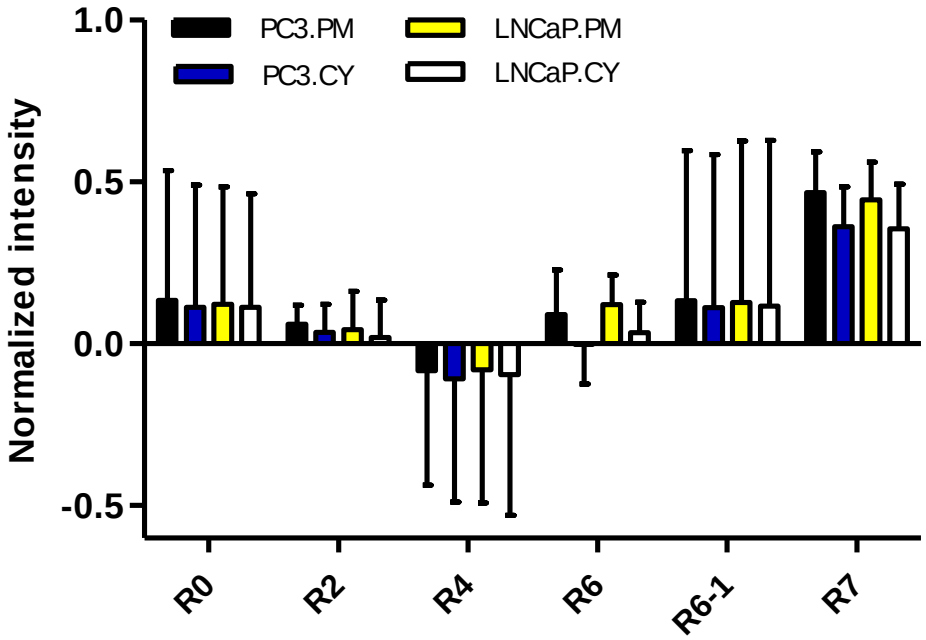


**Figure 4.15: Binding analysis of the 2nd, 4th, 6th and 7th round of SELEX library by ECL method. R0** = initial library, **R2-R7** = selection rounds; **R6-1**=library without indole-modification.

# 5 Conclusions

Protein detection with antibodies by western blot analysis can be an expensive and lengthy procedure, many times even with low specificity. By replacing antibodies with aptamers by direct selection on western blot membranes, the specificity would increase; moreover, the approach can be used for discovery of novel biomarkers. However, to achieve these goals, the study would require much more time than expected.

The optimization and selection on the western blot membrane with SELEX was done for the first time (to our knowledge) and it represents an important basis for further studies. To establish the new SELEX approach, we had to minimize background binding first. For this purpose, we used different competitors and tested their ability to decrease non-specific binding of the library. We discovered that the combination of 1:1 click-competitor and 0.01 mg/ml sulfodextran is the most efficient in doing so. This combination of competitors could be used for selection of other proteins as well. In the next step, we selected the most effective recovery solution, which elutes the most of the bound sequences from the target. We showed that incubation of the membrane with bound complexes target-sequences in ChIP extraction buffer at 99°C and followed EtOH precipitation recovered the highest amount of the sequences.

We fractionized PC3 and LNCaP prostate cancer cell lines on enriched membrane fraction and cytosolic fraction, in order to find aptamers that could bind specifically to the membrane of PC3 cells only. For this purpose, we used different centrifugation steps, and afterwards, we tested the purity of the fractions with different antibodies detecting specific protein markers. Unfortunately, the membrane fractions were still contaminated with cytosolic proteins, as well as there were nucleus proteins in the both fractions of PC3 cells. The whole process of western blot SELEX optimization was time consuming; therefore, we spent too little time for cell fractionation optimization that would need to be done for each cell individually. Moreover, further fractionation to pure PM fractions should be done as well.

We did not completely succeed with the selection of specific binders for PC3.PM, however, it was already a success for us to see differences in sequences binding to proteins of PM and CY fractions. This is a proof that our optimization was successful and with further experimenting, we believe that it would be possible to select aptamers for proteins that are expressed in individual cell lines.

If aptamers are going to be used as an alternative for western blot instead of antibodies, the biotin on the aptamers would need to be replaced by an IR fluorophore as we believe that this will save time and enable quantification, retaining the detection sensitivity. Furthermore, proteins of the size ~70 kDa and ~130 kDa in the cell lysate, which are also the sizes of subunits of naturally biotinylated proteins, could be detected, too, as there are no natural proteins in the cell with conjugated fluorophore.

The final step after the successful selection of aptamers in our study would represent the elution and LC-MS/MS characterization of proteins recognized by aptamers as cell specific biomarkers that could represent new diagnostic, prognostic or drug-targeting molecules.

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