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# Optimization of phage display selection methods for Affibody<sup>®</sup> molecules

Master's degree project

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## Abbreviations

a. a.	amino acid
ABAS-ELISA	ABD Based Affinity Screening ELISA
ABD	Albumin Binding Domain
AU	Absorbance Units
PBS	Phosphate Buffer Saline
CCD	Charged Coupled Device
Conc	Concentration
Cfu	Colony forming units
CS	Cycle Sequencing buffer
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	<i>Escherichia. coli</i>
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
HRP	Horse Radish Peroxidase
HSA	Human Serum Albumin
IPTG	Isopropyl- $\beta$ -D-Thiogalactopyranoside
LMW	Low Molecular Weight ladder
M	Molar, moles per liter
Min	minutes
MPC	Magnetic Particle Concentrator
mRNA	messenger RNA
MQ	Milli-Q <sup>®</sup> water
NA	Neutravidin
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
Sec	seconds
PBS-T0.05	PBS with 0.05% Tween 20
PBS-T0.1	PBS with 0.1% Tween 20
OD <sub>600</sub>	Optical Density at 600 nm wavelength
ON	Over Night
PAGE	Poly Acrylamide Gel Electrophoresis
Pfu	Plaque-forming units
RNA	Ribonucleic Acid
Rpm	rounds per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
TSB	Tryptic Soy Broth
TYE-plate	Tryptose Yeast Extract agar plates
SA	Streptavidin

# 1 Introduction

In this master thesis work, the aim was to optimize the phage display selection process used at Affibody AB, Bromma, Sweden. Phage display is a commonly used method for screening and selecting proteins with affinity for chosen targets. Affibody uses a selection library comprising Affibody<sup>®</sup> molecules, which are based on *Staphylococcus aureus* protein A, domain B. One problem has been the amount of background, i. e. binders that bind to other molecules than the target in the selection process. Background binders make it difficult and sometimes impossible to find Affibody<sup>®</sup> molecules with the right binding properties. The primary goal of this project was to develop a phage selection method that minimizes the amount of background binders.

A couple of methods have been evaluated:

- Changing the solid support
- Alternating the solid support during selection
- Washing protocols with larger volumes, higher detergent concentrations and longer incubation times
- Pre-clearing

## 2 Background

In this background, a brief overview of the use of selection methods is first described. Because phage display is only one method among many, the basics of the most common selection methods are presented.

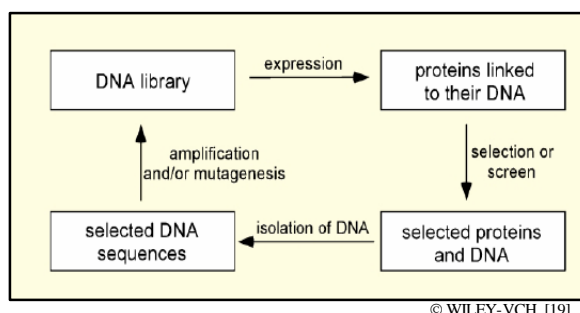
Further, different phage display systems are explained and the problems with unspecific binding. Finally, the methods used in this master thesis work are presented.

### 2.1 Screening and selection methods

Designed molecules can be used in efficient cancer treatment, drugs for other diseases, enzymes, diagnosis tools, protein purifying and blood purifying to mention a few examples. In vitro design of molecules has unfortunately been shown to be a difficult task.

One efficient way to get ligands with eligible properties is to create entire libraries of different molecules and with a selection process find molecules with desired properties. Wide arrays of different molecules are used in screening and selection, i. e. inorganic compounds, DNA and proteins.

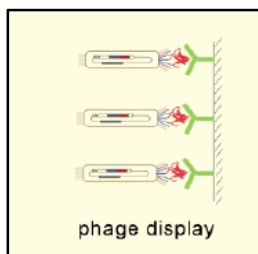
A common type of selection is based on finding molecules with affinity for a given target; an overview can be seen in figure 1. First, the library of different proteins is mixed with the target. The second step is to catch the target-bound proteins, and then wash away all unbound proteins. Last, the bound proteins are amplified and the process is repeated until a mixture of proteins with high affinity to the target is achieved. A protein library can be built by first creating a DNA-library by recombining DNA randomly and then



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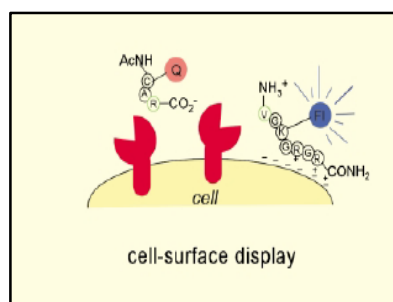
**Figure 1. Overview of a protein selection process Figures 1-7, copyright 2002, reprinted with permission from Wiley-VCH.**

construct the protein library. By coupling proteins to their corresponding DNA the proteins can easily be identified and amplified. There are several ways to achieve the link; the DNA could be linked directly to the protein by a physical bond [19]. It may also be linked by compartmentalization or by spatial separation [19]. Phage display that has been used in this work is based on a physical bond. There are also methods to sequence the protein itself. Mass spectrometry is advancing in this area [19]. Below follows examples of some different selection strategies.

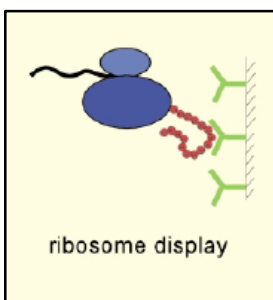


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**Figure 2.** Phage with displayed protein (red) binding to target molecule (green).

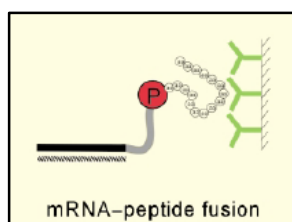


**Figure 3.** Proteins (red) displayed on a cell surface.



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**Figure 4.** mRNA (black) linked to protein (red) by a ribosome (blue).



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**Figure 5.** mRNA (grey & black) connected to protein (grey) by puromycin (red).

### 2.1.1 Physical bond – Surface Display

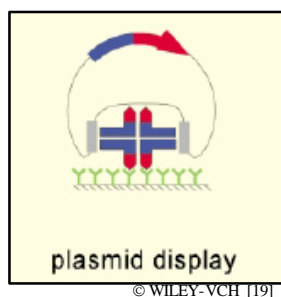
**Phage display** – is a method based on displaying proteins or peptides on bacteriophage (figure 2). The library molecule is genetically fused to one of the phage's natural surface proteins. It is a widely used method but the size of the library is limited by the transformation process of DNA to *Escherichia coli* (*E. coli*) [19]. The method is described in more detail in section 2.2.

**Cell surface display** – is a method based on displaying proteins or peptides on different cells (figure 3). The library molecule is fused to one of the cell's natural surface proteins. Screening is usually made by fluorescence-activated cell sorting (FACS). Cell surface display was not initially developed for screening [16]. The application is rather in the development of live bacteria vaccine delivery systems [19]. Library size is limited due to the low transformation efficiency.

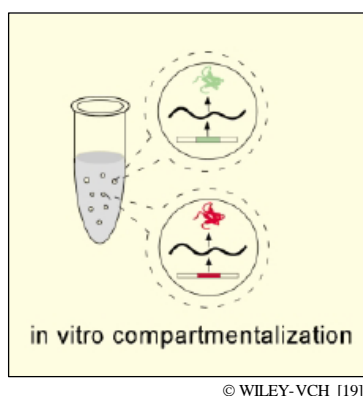
### 2.1.2 Physical bond – Direct linkage

**Ribosomal display** – is a method where the ribosome acts as a bond between the peptide and the mRNA (figure 4). When chloramphenicol is added to the translation reaction of mRNA, translation stops and the bond is created. Selection efficiency is limited due to the fact that the complex between ribosome, mRNA and peptide (or protein) is instable. When a library is built, the yield is decreased with increasing size of the proteins. The method is completely *in vitro*. An *in vitro* method has no restrictions like transformation efficiency but may cause problems with expression of certain proteins [19].

**mRNA display** – mRNA is linked to the protein by puromycin (figure 5) in the translation process. The method is completely *in vitro*. An *in vitro* method has no restrictions like transformation efficiency but may cause problems with expression of certain proteins [19].



**Figure 6.** Plasmid linked to protein (red) by DNA-binding domain (blue).



**Figure 7.** Translation occurs in small water droplets separated by oil.

**Plasmid display** – The displayed proteins are connected to a DNA binding protein that binds to the plasmid with the randomized DNA (figure 6). The binding occurs inside a cell that subsequently is lysed. The library size is limited due to the low transformation efficiency [19].

### 2.1.3 Compartmentalization

**Protein fragment complementation assay** – Binding between target and binder is performed inside a cell that expresses both molecules. The target and the binder are fused to different domains of a protein with a detectable phenotype, for example fluorescence. If binding occurs, the binder can be found by finding the special phenotype [29]. The library size is limited due to the low transformation efficiency.

**Liposome-based assays** – is a method based on small water-in-oil- droplets to link DNA and protein. Each drop contains one gene, transcription and translation factors which produce proteins inside the drop (figure 7). Before dissociation of the drop, a bridge between the DNA and the proteins can be made. Some examples of bridges are: protein-streptavidin-biotin-DNA coupling [17] and protein-antibody-bead-DNA binding [38]. Enzymatic activity selection can also be made by letting the translated protein demethylate the DNA before dissociation of the drop. The demethylation protects the DNA from restriction enzymes added subsequently [18].

## 2.2 Phage display

### 2.2.1 The bacteriophage

A bacteriophage is a virus which infects bacteria and some bacteriophage lyse the host during reproduction (so-called lytic phage). In phage display, lysogenic phage which do not lyse the host are used. These variants are used to display proteins or peptides on the surface and contain the genetic code of the protein inside. By genetically fuse the protein to one of the phage's coat proteins it will be displayed on the surface and be available in selection. After selection, the selected phage can easily be amplified by letting them infect bacteria and reproduce [2]. A number of phage strains are used in phage display, commonly M13, f1 or fd. They are almost identical and are called Ff phage due to their way to bind *E. coli* through the F pilus.

### 2.2.2 The phagemid

A phagemid is a plasmid containing both an ori for *E. coli* and a phage-derived ori. It can be packed inside a phage. To produce new phage containing phagemids, helper phage are used. The helper phage got the genetic information for all phage proteins. By co-infecting a cell with a phagemid and a helper phage, new phage expressing the gene of interest are produced [2].

Phagemids got the advantage over bacteriophage that phage production can be controlled. This is used at Affibody to express proteins from the phagemid vector without producing new phage. By letting the helper phage code for native surface protein it is possible to present only one ligand per phage (i.e. monovalence) [1].

## 2.2.3 Surface presentation on phage

### 2.2.3.1 Poly- and monovalence

By using different display types the number of displayed foreign proteins or peptides differs on the bacteriophage. A system with only one displayed protein is monovalent and if several copies of the foreign protein are displayed the system is polyvalent. A monovalent system shows the real affinity since no cooperative binding from peptide/protein siblings can occur. In a polyvalent system several molecules can bind to target simultaneously. An avidity effect is then achieved. As the experienced affinity often is higher than the real affinity, a polyvalent system is preferred when weaker and more specific binders are desired [14]. A monovalent system can display larger proteins than the polyvalent system [1].

### 2.2.3.2 Display types

Five different coating proteins are used for fusing the library molecule to the surface of the phage. pIII and pVIII are the most common but pVI has been used too [1]. pIII and pVI are positioned at one tip of the phage and exists in five copies each [19]. pVIII is a 50-residues peptide that covers the phage in about 2800 copies [2]. An overview of systems based on pIII and pVIII are presented in figure 8. The main characteristics are:

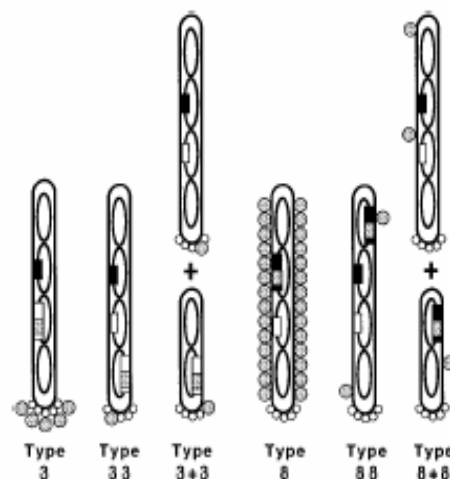
**Type 3** – The phage vector in the phage contains gIII fused to the foreign protein or peptide DNA. The phage displays the protein on all pIIIs. One problem is that if the foreign DNA includes stop-codons, it will stop the translation of the pIII and the phage will not be infective [1].

**Type 33** – The vector codes for two pIII. One of them is fused with the foreign protein and is often slightly suppressed. The phage expresses both pure pIII and pIII fused with the foreign peptide. Thanks to the suppression of the fused vector it is possible to make the phage monovalent, i. e. only one foreign peptide is displayed. A monovalent system can display bigger proteins without losing infectivity [1].

**Type 3+3** – The foreign peptide DNA is fused to specific domains of pIII on a phagemid. Helper phage are used in reproduction.

**Type 6, 66 and 6+6** functions the same way as 3, 33 and 3+3 respectively but with pVI [1].

**Type 8, 88 and 8+8** functions the same way as 3, 33 and 3+3 respectively but with pVIII. There are some differences though. In system 8 only



**Figure 8. Overview of different phage display systems. The long ovals are phage and the shorter ovals are phagemids. The helices inside the ovals are DNA, the black boxes represent gVIII and the white boxes represent gIII. Grey boxes are the library gene and it is fused to one of the coat proteins. The white circles are pIII, pVIII is not displayed, and grey circles represent the library protein or peptide. Reprinted with permission from Elsevier [37]**



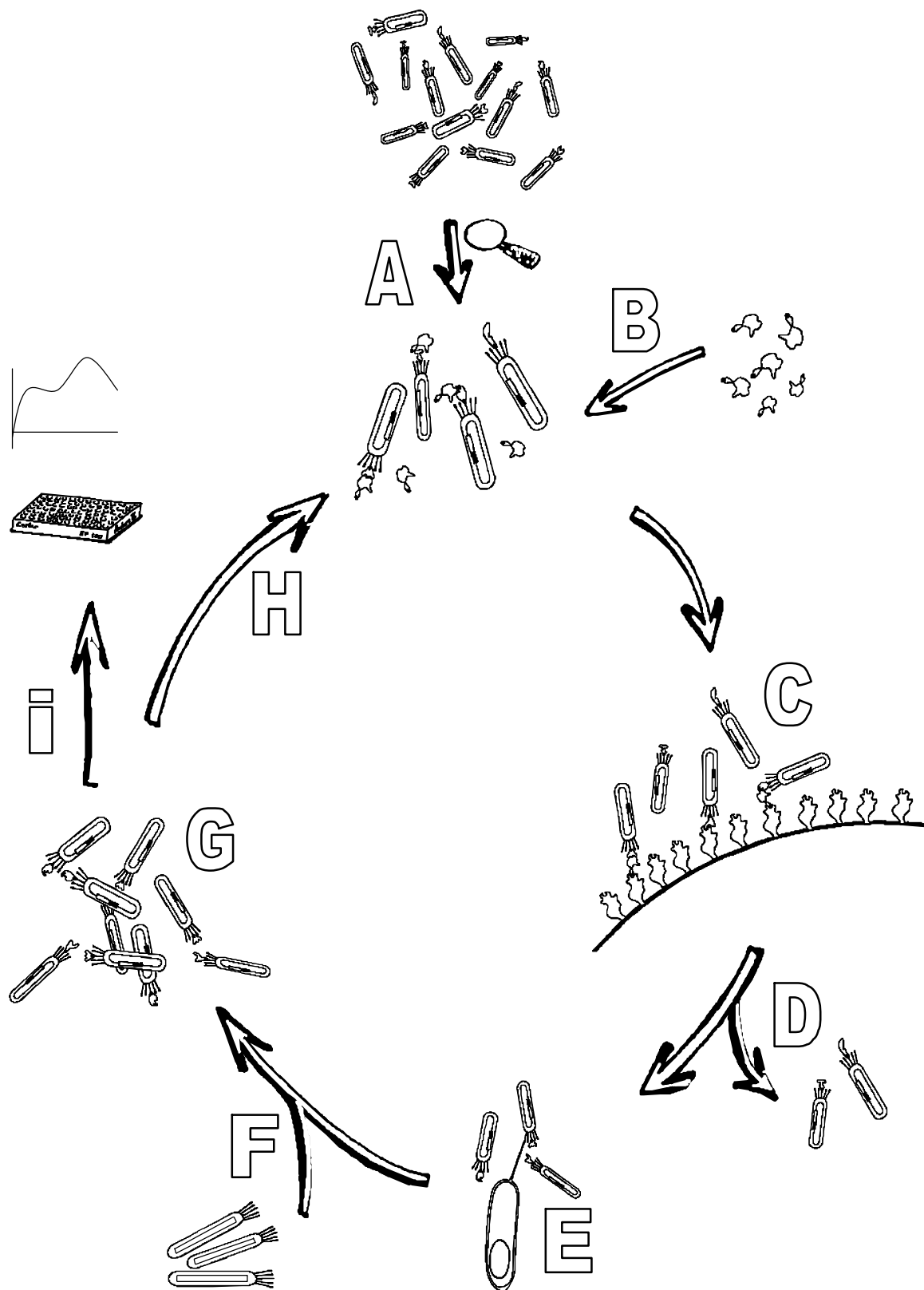
very small peptides can be used because there is not enough space for bigger proteins to coat the phage. The phage also gets reproduction difficulties when displaying larger molecules. An 88 system works better than system 8 but is not monovalent. Instead a mosaic pattern of foreign peptides is displayed over the phage. An 8+8 system displays a mosaic pattern in the same way but is based on phagemids like the 3+3 system [1].

#### 2.2.4 Selection

With a library of different proteins displayed on phage it is possible to screen for and select proteins with affinity to a specific target; in figure 9 an overview of such a selection process is drawn.

By exposure of the target (B) to the library (A), suitable phage are able to bind to the target. The target can either be immobilised on a surface, for example inside wells or at paramagnetic beads (C) (*solid phase selection*), or it can be exposed to phage in a solution (*liquid phase selection*). If the target is in solution, it is necessary to catch it after the phage has bound. Preferably a strong pair of binders is used such as biotin and streptavidin (SA) which have a  $K_d$  of  $10^{-15}$  M [4]. The small biotin molecule is connected to the target and the SA-molecule on a solid surface. If the target is to be selected on a solid phase it can be either immobilised primary, directly on the support matrix, or secondary using a biotin-SA interaction. Depending of the use of the selected peptide, liquid or solid phase is preferred. If high specificity and low affinity is wanted, the solid phase selection allows more multivalent bindings and will be beneficial. Liquid phase is better when selecting for high affinity because affinity discrimination is maximized [14]. Solid phase selection may yield more background binders because the solid phase is exposed for the phage for a longer time [25].

The different selection methods ends with target and phage bound together, connected to a solid phase (C). Still all unbound phage are present in the solution but by washing (D) the surface several times they are more or less removed. The selected phage can be eluted in several ways: pH can be lowered [39] or increased [40] to denature the binding protein. Ethylene glycol, competitive elution with target protein [20], chaotropic agents [3] and enzymatic cleavage [5] are other kinds of elution techniques. Eluted phage are thereafter multiplied by infection of *E. coli* (E) and used for next selection round (H). After two to five selection rounds, phage clones are randomly picked and analysed with ELISA and sequencing (I).



**Figure 9. Overview of a phage selection. The phage library (A) is mixed with biotinylated target (B). Binding occurs and the phage-target-solution is added to SA coated beads (C). Non bound phage are washed away (D). Remaining phage are eluted and allowed to infect *E. coli* (E). Helper phage are added and infects the bacteria too (F). A mix of phage with affinity to the target and background is left (G). Two to five selection rounds are performed (H), and then the phage are screened and analyzed (I).**

### 2.2.5 Definition of background binders

In a library of potential binders different populations of binders exist. In a typical 100 million clones library there are [14]:

- $10^8$  non-binding phage
- $10^4$  phage binding to materials used in the selection. They are often referred to as plastic binders or SA binders. The displayed proteins bind to different parts of the solid support or the blocking agents used
- $10^3$  phage that bind to the target weakly
- 10 phage that bind to the target tightly

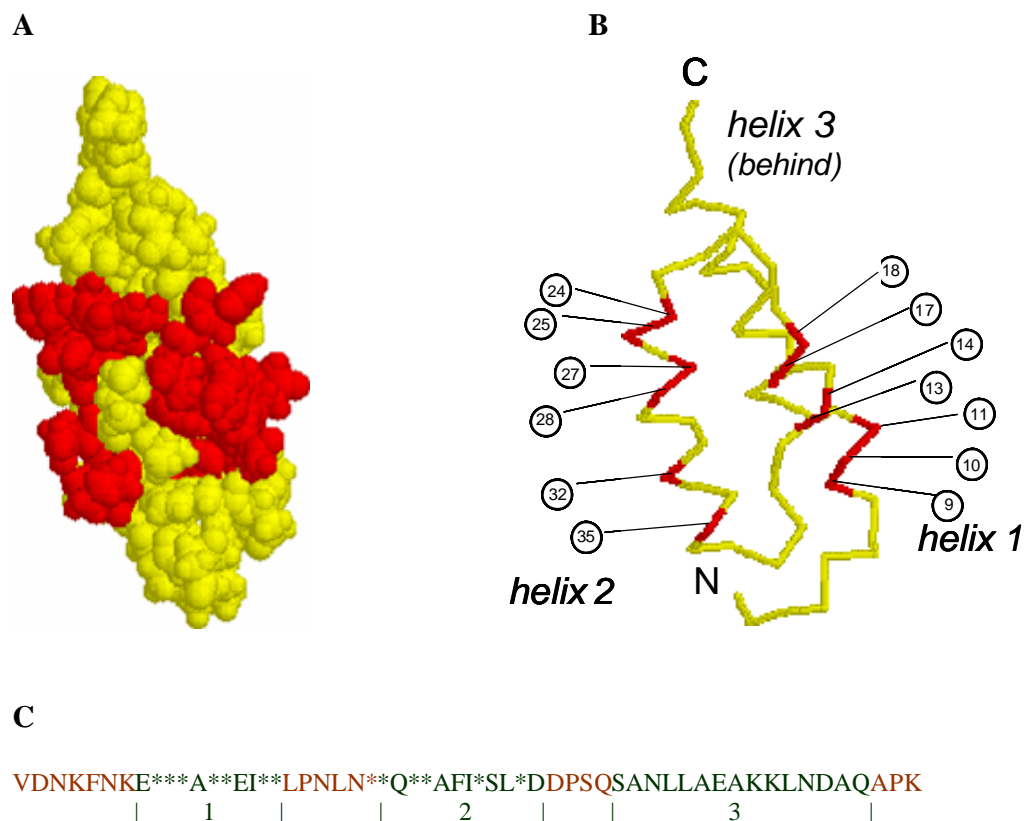
Background binders are binders which do not have affinity to the target. They could either be non-binding phage or material binding phage. If only high affinity binders are wanted the weak binding phage may be called background binders too. Additionally, the phage itself, regardless of displayed protein, can be sticky and survive selections. If background binders succeed to survive a selection round, they may outgrow the target specific binders [14]. Therefore it is of highest importance to use the most appropriate strategy to reduce background binders.

## 2.3 Phage display method used at Affibody

In the phage selection process at Affibody AB, a 3+3 system is used where the different domains of pIII are encoded partly in the phagemid, pAffi1, and partly in the helper phage, M13K07. When phage are propagated, a mix of phagemids and helper phage is obtained where 6 % are phagemids expressing pIII-fused protein [25]. Paramagnetic beads coated with SA are used as solid phase and the binding between Affibody<sup>®</sup> molecules and target is performed in solution for two hours prior exposure and binding to the solid phase. The beads are washed an increased number of times for each selection round. The bound phage are eluted with acid and then allowed to co-infect *E. coli* together with helper phage to produce new phage for the next panning round. Normally four to five rounds of selection are performed.

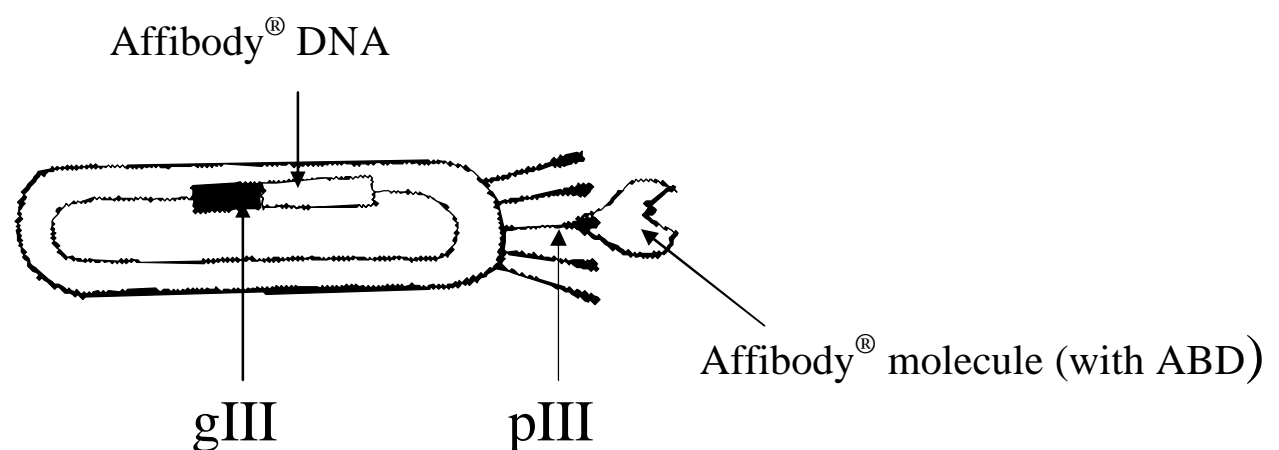
### 2.3.1 Library

The proteins named Affibody<sup>®</sup> molecules that are displayed are 58 amino acids long and based on *Staphylococcal aureus* protein A, domain B [3]. Domain B is engineered at residues 28-29 where asparagine-glycine is exchanged to asparagine-alanine to eliminate a hydroxylamine cleavage site [28]. Furthermore residue 1 is changed from alanine to valine to make a restriction site for *AccI* restriction enzyme, which makes it possible to fuse many domains together at the DNA level [28]. The engineered domain is named Z and has 13 randomized residues [3] which make a library of a theoretical size of  $8.2 \times 10^{16}$  different Affibody<sup>®</sup> molecules. Z is a three-helix structure and the randomised positions are positioned at helix one and two, see figure 10 [26].



**Figure 10.** (A) Z with variable residues marked red, (B) Z with variable residues marked, (C) Affibody<sup>®</sup> sequence, stars indicating variable positions, the green domains are the helices.

The Affibody<sup>®</sup> molecules are fused to a truncated version of pIII (residues 249-406) together with an albumin binding domain (ABD) that is also displayed on the phage [3] (figure 11). The ABD is used as a tag in the analysis of the binders (2.6.2). The library used is called ZLib2002 and has a real size of  $3.3 \times 10^9$  different clones [25]. The library plasmid also contains  $\beta$ -lactamase, an ampicillin resistance vector.



**Figure 11.** Phage with Affibody<sup>®</sup> molecule

## **2.4 Methods to reduce background**

Here is a short presentation of the methods to reduce background used in this project. The list contains both methods which are already in use at Affibody and new methods evaluated in this project.

### **2.4.1 Washing**

After binding of phage to the solid support, the surface of the solid support is washed to remove non-binding phage and weak plastic binders. It has been shown that increasing washing volume, number of washes, detergent concentration and washing time removes more non-binding phage and plastic binders [6]. Studies have been made that compares the efficiency in background reduction of different washing methods [6]. The different washing methods reviewed were:

- 1 10 x 1 ml phosphate buffer saline (PBS) with 0.1% Tween20 (no incubation) + 3 x 1 ml PBS, the relative amount of background was 1.
- 2 3 x 1 ml PBS with 2% Tween20 (10 min incubation) + 1 x 1 ml PBS, the relative amount of background was 0.4.
- 3 3 x 10 ml PBS with 0.5 % Tween20 (19 min incubation) + 1 x 10 ml PBS, the relative amount of background was near 0.2.

The first method is almost identical to the wash procedure used at Affibody today. Each wash, no matter procedure, removes a number of weak binders and tight binders, so a too extensive wash may remove wanted binders. Often weak binders are more specific than strong binders and one method to keep the specific binders is to wash carefully in the first selection round and then increase the washing stringency in the subsequent runs [21]. Three different washing protocols, each similar to the above described protocols were evaluated regarding amount of background binders and the presence of different low and high affinity binders.

### **2.4.2 Pre-clearing of library**

A pre-clearing step is carried out by exposing the whole phage library to naked SA coated beads. The theory is to let the material binders adsorb to the surface and use remaining phage as a new library. The effect of pre-clearing is not very clear and experiments have shown only moderate effects [8]. Still some literature recommends pre-clearing steps [14]. Pre-clearing can also be used to remove phage with special treats like a special binding motif on the target. For that purpose it has been shown to be effective [12, 13]. Pre-clearing is already in use at Affibody at present date but an experiment to test the efficiency of the method was made in this work.

### **2.4.3 Using a more specific biotin binder than streptavidin**

Since many background binders bind to SA in selections made at Affibody and elsewhere [23, 24, 25] a more specific biotin binder can be used. Neutravidin (NA) is a deglycosylated derivative of avidin which has a more neutral pI [30]. This makes NA less attractive for non-specific interactions than avidin and SA. It has been shown that the oligosaccharide side chain is not required for biotin binding [31]. Selections with NA as biotin binding molecules were performed and evaluated in this work.

#### 2.4.4 Blocking of tubes and beads

By adding gelatine to the beads and tubes prior to the selection, non-specific binding sites are blocked by the gelatine. Blocking by gelatine is already in use today at Affibody, the effect of blocking was not evaluated since earlier experiments have shown it effective [25].

#### 2.4.5 Alternating neutravidin and streptavidin

It has been shown that not the same type of binders bind to NA as SA [22]. This can be explained by the differences in structure between SA and NA. By alternating the different biotin binders, the SA and NA binders will have harder to compete with binders to the target. There are experiments that have shown that alternating selection rounds with SA, with rounds without SA present have been an effective way to reduce background binders [8]. Different patterns of alteration was performed and analysed.

##### Comparison between non-glycosylated avidin and SA

- Avidin is an egg white protein [32]
- SA is a protein from bacterium *Streptomyces avidinii* [32]
- Both avidin\* and SA are quaternary  $\beta$ -barrel structures [32]
- The biotin binding pockets of avidin\* and SA are similar but avidin\* has five aromatic groups compared to SA that has four [32]
- The biggest structural differences between avidin\* and SA are the loops connecting the  $\beta$ -sheets [32]
- The sequence identity between avidin\* and SA is 64 % in the homologue segments and 7 % in the other areas. The overall homology is 30 % [32]

\*Experimental data derived on “Lite avidin” a non-glycosylated variant of avidin.

### 2.5 Experimental setup

#### 2.5.1 Target – biotinylated Protein A

Protein A was chosen as a target in this work because several phage display selections have been made using protein A as a target at Affibody AB. It has been easy to find Affibody<sup>®</sup> molecules with affinity for protein A as well as for plastic and SA in these selections. The protein has a mass of 34 kDa [25] and is biotinylated so that it can be caught with a biotin binder in the selection.

#### 2.5.2 Selections performed

##### 2.5.2.1 Test Selection

Initially, with the purpose to test NA coated beads in a small scale selection and examine the possibility of removing SA binders by pre-clearing and altering the biotin binding protein, a selection based on a four member library (Zlib4) was carried out using protein A as target. The library was made of two known protein A binders and two known SA binders originating from Zlib2002. This selection consisted of one panning round and was performed in four different setups:

**TN** – Selection with NA coated beads to examine if selection can be made on this type of beads and to examine if the SA binders are removed.

**TS** – Selection with SA coated beads as a reference to the other selections.

**TNP** – Same as TN but with pre-clearing on NA coated beads prior to selection to examine the effect of pre-clearing.

**TSP** – Same as TS but with pre-clearing on SA coated beads prior to selection to examine the effect of pre-clearing.

#### **2.5.2.2 Optimization selections**

Two separate optimizations with four selections, each using the library Zlib2002, were made to test the effect of NA coated beads compared to SA coated beads and to evaluate alternation between the two types of beads. To see if it was possible to reduce background binders with different washing procedures, three different washing protocols were tested.

##### **Optimization selections 1**

**S** – Selection with SA coated beads. Washing was performed with small volume during minimal time and low concentration of detergent.

**N<sub>1</sub>** – Same conditions as for S but with NA coated beads for comparison of different kind of beads.

**K** – Same as S but with alternating biotin binder, first SA, then NA, SA and NA on the beads. This test was made to test efficiency of alternating the solid support.

**W** – Same as S but with larger washing volumes, longer washing times and more detergent to see if this kind of washing reduces background.

##### **Optimization selections 2**

**N<sub>2</sub>** – Same conditions as for N<sub>1</sub> in optimization selection 1. This selection was made to see if the experiments were reproducible and as a reference between the two selections.

**A** – Same conditions as for K but starting with NA in the alternations of the biotin binding protein.

**T** – Same conditions as for W but on NA coated beads to see the effect of two background-reducing systems combined.

**E** – Same conditions as for N but with a washing protocol with very high detergent concentration, medium long wash and slightly larger washing volume.

## **2.6 Analysis**

Four different techniques were used to evaluate the selections.

### **2.6.1 SA and NA ELISA**

SA and NA Enzyme-linked immunosorbent assays (ELISA) were used to screen Affibody<sup>®</sup> molecules for affinity to SA or NA. SA or NA coated plates were used to bind SA or NA binding Affibody<sup>®</sup> molecules respectively. The Affibody<sup>®</sup> molecules were detected by two layers of antibodies. The primary antibody layer bind to the Affibody<sup>®</sup> molecules, the secondary antibody bind to the primary antibody and carry a horseradish peroxidase (HRP). HRP catalyses H<sub>2</sub>O<sub>2</sub> oxidation of tetramethylbenzidine. This reaction creates a blue colored product. By adding an acid in the presence of equimolar H<sub>2</sub>O<sub>2</sub> a

stable diimine is formed [27]. The diimine is yellow and the color can be measured with a spectrophotometer.

### **2.6.2 ABD Based Affibody Screening (ABAS) ELISA**

The affinity between Affibody<sup>®</sup> molecules and target is screened by ABAS ELISA. Human serum albumin (HSA) coated plates are used to capture the Affibody<sup>®</sup> molecules through the genetically fused ABD. By adding the biotinylated screening target and then SA-HRP the Affibody<sup>®</sup> molecule-target complex can be detected with oxidation of tetramethylbenzidine ([2.6.1](#)).

### **2.6.3 Sequencing**

Sequencing is used to identify binders after selection. One method to sequence DNA is automated fluorescent DNA sequencing. Differently labelled fluorochrome dideoxy nucleotides (ddNTP) are used to randomly terminate chain extension during a polymerase chain reaction (PCR). This creates sequences with different length and the end is marked with one ddNTP with specific fluorescence for A, G, C and T. Capillary electrophoresis is used to separate the fragments by length. A laser excites the separated fragments and the dyes emit light at different wavelengths. A charged coupled device (CCD) measures the light and the DNA sequence is interpreted from the fragments' emissions [2].

### **2.6.4 Clustering**

By using the program “Clustering of Affibody molecules” Affibody<sup>®</sup> molecule sequences can be analysed for similarities. The program clusters the molecules after their amino acid sequence homology. Since binders with similar sequences often have same binding properties, the cluster tool can be used to investigate if the Affibody<sup>®</sup> molecules are potential SA binders or if they have similarities with other wanted or unwanted binders. In constructing a secondary generation library the information is very useful too. Program calculation formulas are presented in appendix E.



## 3 Materials and methods

### 3.1 Preparations

#### 3.1.1 Neutravidin coating of Dynabeads M280

Tosylactivated Dynabeads<sup>®</sup> M280 (Dyna), 15 mg, were separated from liquid on a magnetic particle concentrator (MPC<sup>®</sup>-S, Dynal) for 2 minutes. The beads were then washed twice with 1 ml 0.1 M H<sub>3</sub>BO<sub>3</sub> pH 9.5 for 2 minutes on a rotor. Subsequently, the beads were coated by removing liquid, adding 1 ml 0.1 M H<sub>3</sub>BO<sub>3</sub> pH 9.5 mixed with 300 µg neutravidin (Pierce) and incubating at 37°C for 24 hours on a rotor. Washing were made twice in PBS (For 1000 ml: 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 8 g NaCl, 11.5 g Na<sub>2</sub>HPO<sub>4</sub>, MQ) pH 7.4 for 5 minutes at 4°C, once in 0.2 M Tris pH 8.5 for 4 hours at 37°C and once in PBS pH 7.4 for 5 min at 4°C, all steps on a rotor. The beads were stored at a final concentration of 10 mg beads/ml in PBS pH 7.4 supplemented with 0.1% BSA (w/v) (CAS 9048-46-8) and 0.02% NaN<sub>3</sub> (Sigma Chemical) at 4°C.

#### 3.1.2 Activity test of neutravidin coated beads

By exposing the NA coated beads to biotinylated protein A (Amersham Biosciences, biotinylated with EZ-link (Pierce)), the beads' binding capabilities were examined. Streptavidin Dynabeads M280 (Dyna) were used as a reference. Beads, 0.5 mg (NA and SA coated beads respectively) were washed in 3 x 1 ml PBS and then mixed with 100 µl PBS 0.05% Tween 20 (Acros. PBS-T0.05) with 24 µg biotin-protein A for 30 min at room temperature (RT) on a rotor (binding I). The supernatant was added to 0.5 mg new, washed, beads and mixed on a rotor for 30 min at RT (binding II). Dry beads from both bindings and supernatant were mixed with 1x or 5x reduction buffer (5x: 100 mM Tris-HCl pH 8.0, 5 mM [Ethylenedinitrilo]tetraacetic acid (EDTA, Merck), 12.5 % sodium dodecylsulfate (SDS, Amresco), 25 % β-merkaptoethanol 14.3 M (Merck), 5 mg bromophenol blue sodium salt (Merck)) respectively to a final volume of 10 µl, warmed to 95°C for 5 min and analysed on a 10-15% SDS-PAGE gel (Amersham Biosciences) with the PhastSystem (Pharmacia) or on a NuPAGE<sup>®</sup> Bis-Tris 4-12 % gel (Invitrogen). A low molecular weight ladder (LMW, Amersham Biosciences) was used as reference on the SDS-gel and the Multimer<sup>®</sup> (Invitrogen) on the NuPAGE-gel. The gels were stained with Coomassie blue (for 400 ml: 1 tablet PhastGel<sup>™</sup> Blue R (Amersham Biosciences), 120 ml 95 % C<sub>2</sub>H<sub>5</sub>OH, 40 ml 17.49 M CH<sub>3</sub>CO<sub>2</sub>H, Milli-Q<sup>®</sup> water (MQ, Millipore)) or with silver staining [11].

### 3.2 Selection process

In table 1, all parameters varying between the different selections and the different rounds are shown.

**Table 1. The varying parameters in each selection round. During rounds II-IV, eluted phage from previous round was used as input material (phage in). Buffer A, PBS with 0.1% Tween20, buffer B, PBS with 0.5% Tween20 and buffer C, PBS with 2% Tween 20.**

Selection round	Name	Library	Phage in (cfu)	Target	Beads		Wash				Analysis	
				Concentration (nM)	Type	Quantity (mg)	Times (x)	Buffer	Volume (ml)	Time (min)	ELISA	Sequencing
I	TN	Zlib4	2,0E+10	10	NA	0,2	3	A	1	1	Yes	No
	TNP	Zlib4	2,0E+10	10	NA	0,2	3	A	1	1	Yes	No
	TS	Zlib4	2,0E+10	10	SA	0,2	3	A	1	1	Yes	No
	TSP	Zlib4	2,0E+10	10	SA	0,2	3	A	1	1	Yes	No
	W	Zlib2002	7,5E+12	50	SA	1	1	B	10	20	No	No
	S	Zlib2002	7,5E+12	50	SA	1	2	A	1	1	No	No
	K	Zlib2002	7,5E+12	50	SA	1	2	A	1	1	No	No
	N <sub>1</sub>	Zlib2002	7,5E+12	50	NA	1	2	A	1	1	No	No
	T	Zlib2002	7,5E+12	50	NA	1	1	B	10	20	No	No
	E	Zlib2002	7,5E+12	50	NA	1	1	C	2	10	No	No
	N <sub>2</sub>	Zlib2002	7,5E+12	50	NA	1	2	A	1	1	No	No
	A	Zlib2002	7,5E+12	50	NA	1	2	A	1	1	No	No
II	W	*		5	SA	0,5	2	B	10	20	No	No
	S	*		5	SA	0,5	3	A	1	1	No	No
	K	*		5	NA	0,5	3	A	1	1	No	No
	N <sub>1</sub>	*		5	NA	0,5	3	A	1	1	No	No
	T	*		5	NA	0,5	2	B	10	20	Yes	Yes
	E	*		5	NA	0,5	2	C	2	10	Yes	Yes
	N <sub>2</sub>	*		5	NA	0,5	3	A	1	1	Yes	Yes
	A	*		5	NA	0,5	3	A	1	1	Yes	Yes
	W	*		5	SA	0,5	3	B	10	20	Yes	Yes
	S	*		5	SA	0,5	4	A	1	1	Yes	Yes
III	K	*		5	SA	0,5	4	A	1	1	Yes	Yes
	N <sub>1</sub>	*		5	NA	0,5	4	A	1	1	Yes	Yes
	T	*		5	NA	0,5	3	B	10	20	Yes	Yes
	E	*		5	NA	0,5	3	C	2	10	Yes	Yes
	N <sub>2</sub>	*		5	NA	0,5	5	A	1	1	Yes	Yes
	A	*		5	NA	0,5	5	A	1	1	Yes	Yes
	W	*		5	SA	0,25	3	B	10	20	Yes	Yes
	S	*		5	SA	0,25	5	A	1	1	Yes	Yes
IV	K	*		5	NA	0,25	5	A	1	1	Yes	Yes
	N	*		5	NA	0,25	5	A	1	1	Yes	Yes

### 3.2.1 Libraries

Zlib2002 comprising  $3.3 \times 10^9$  members [25] or Zlib4 were used in the selections. Zlib4 was made from four different clones from Zlib2002, two known protein A binders and two known SA binders. Each clone was cultivated, harvested, precipitated (3.2.6) and titrated (3.2.4). The clones were mixed together at equal amounts creating a library with  $1.3 \times 10^{12}$  phage/ml. An ABAS ELISA (3.3.2.2) and a SA ELISA (3.3.2.3) were performed to screen the composition of Zlib4.

### 3.2.2 Pre-clearing

In selections TNP and TSP, pre-clearing was performed, by adding  $2 \times 10^{10}$  phage to 0.2 mg beads (that had been washed three times in 1.5 ml PBS-T0.05 for 15 minutes) in 400 µl PBS-T0.1 0.1% gelatine and incubating for 30 minutes on a rotor at RT. The beads were removed and phage were stored at 4°C until selection.

### 3.2.3 A selection round

Prior to the selection rounds, tubes (Eppendorf) were blocked for at least 30 min with PBS 0.1% Tween20 (PBS-T0.1) + 0.1% gelatine at RT. The beads were washed three times in 1.5 ml PBS-T0.05 15 minutes each and blocked for at least 30 min in PBS-T0.1 + 0.1% gelatine on a rotor at RT.

Phage were incubated with biotin-protein A in 1 ml PBS-T0.1 0.1% gelatine for 2 hours on a rotor at RT. The solution was mixed gently with Dynabeads for 15 minutes on a rotor at RT. The beads were washed in PBS and Tween20 according to the washing scheme (table 1). Remaining phage were eluted with 500 µl glycine pH 2.2 at RT for 10 minutes. The supernatant was collected and neutralised with 500 µl 0.1 M Tris HCl pH 7.5. The eluted phages were titrated (3.2.4) and either cultivated (3.2.5) or analysed (3.3).

### 3.2.4 Titration of phage

Phage were serially diluted in autoclaved MQ (sMQ), 100 µl per dilution, to 1/10 to 1/10<sup>12</sup> of the starting concentration in a 96-well microtiter plate (different brands). To each dilution 100 µl RR1? M15-cells [10], cultivated to early log-phase (optical density at 600 nm (OD<sub>600</sub>) = 0.5-0.8 absorbance units (AU)) in tryptic soy broth (TSB. For 1000 ml: 30.0 g TSB (Merck), deionised H<sub>2</sub>O) were added. Infection of the cells was performed for 5 minutes at RT. From each well 5 µl infected cells were applied drop-wise on a tryptose yeast extract agar plate (TYE-plate. For 900 ml: 15 g agar, 3 g NaCl, 10 g Tryptone Water (Merck), 5 g yeast extract, 100 ml 20% glucose, 1 ml ampicillin 100 mg/ml (Saveen & Werner AB), deionised water). The plate was incubated at 37°C over night (ON). Separate colonies were counted and the concentration was calculated.

### 3.2.5 Cultivation of phage

#### 3.2.5.1 Phagemid amplification

From the selection, 500 µl of eluted phage from selection were added to 100 ml (in round 1) or 10 ml (in subsequent rounds) RR1? M15-cells in early log-phase. Infection of the cells was performed for 25 minutes at 37°C. The cells were centrifuged at 3300 g for 15 minutes and the supernatant was discarded. The pellet was resuspended in TSB and spread on TYE-plates, bigger plates for the first round. The plates were incubated ON at 37°C. Grown cells were suspended in TSB. Part of this suspension (approximately 3 ml) was mixed with equal parts of 87 % glycerol and frozen at -80°C.

#### 3.2.5.2 Phage cultivation

Suspended cells, 2x10<sup>10</sup> or 2x10<sup>9</sup> colony forming units (cfu), were added to 400 ml (round 1) or 40 ml (subsequent rounds) tryptose saline buffer with yeast extract (TSB-YE. For 1000 ml: 30.0 g TSB (Merck), 5.0 g yeast extract, deionised H<sub>2</sub>O) with 2% glucose and ampicillin, 100 µg/ml, corresponding a final cell concentration equivalent OD<sub>600</sub> = 0.1 AU (OD<sub>600</sub> = 1 AU equals 5x10<sup>8</sup> cells/ml). The cells containing the phagemids were grown to early log-phase. Helper phage M13K07 (New England Biolabs®), 10<sup>12</sup> plaque-forming units (pfu)/ml, were added to the cells with a 20-fold excess. The amount of cells was estimated from OD<sub>600</sub>-measurements. Infection of helper phage was performed for 25 minutes at 37°C. The cells were then centrifuged at 3300 g for 15 minutes. The supernatant was discarded and the pellet was dissolved in TSB-YE with 100 µg/ml ampicillin, 25 µg/ml kanamycin (Saveen & Werner AB) and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG. Eppendorf) in the original cultivation volume. Phage were grown ON at 30°C, 150 rounds per minute (rpm) in baffled flasks.

### 3.2.6 PEG-Precipitation of phages

Phage from cultivation were harvested through centrifugation at 3300 g for 15 minutes. To the supernatant, 1/5<sup>th</sup> volume 20% polyethylene glycol 6000 (PEG. Merck) 2.5 M NaCl was added. The suspension was mixed thoroughly and then incubated on ice for 2 hours. The precipitate was centrifuged at 10700 g for 30 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in sMQ, to 1/10<sup>th</sup> of the original volume. To the suspension, 1/5<sup>th</sup> the volume 20% PEG 2.5 M NaCl was added. The phage-PEG mix

was incubated on ice for 45 min before centrifugation at 16600 g for 30 minutes. The supernatant was discarded and pellet dissolved in PBS by pipetting. The phage solution was filtered through a Minisart 0.45 µm filter (Sartorius) before titration (3.2.4). Precipitated phage were used in next selection round (3.2.3)

### **3.3 Analysis**

#### **3.3.1 Clone cultivation and Affibody-ABD expression**

Phagemid infected RR1? M15-cells (3.2.5.1) from the selection rounds to be analysed were spread on TYE-plates at different dilutions in order to obtain separate colonies. The plates were incubated at 37°C ON.

From each analysed selection round, 93 colonies were picked with sterile toothpicks and put in autoclaved deep well plates (NUNC), each well containing 1.3 ml TSB-YE, 1mM IPTG and 100 µg/ml ampicillin. The toothpicks were removed and the plate sealed with a gas permeable adhesive seal (Abgene). The plate was incubated at 37°C, 200 rpm for 18-24 hours. Samples from each plate were transferred to a PCR-plate (Abgene) with 50 µl TSB and 50 µl 87% glycerol per well using a 96-well capillary plate. The PCR-plate was frozen at -20°C until sequencing.

#### **3.3.2 ELISAs**

##### **3.3.2.1 Lysating of cells prior to ELISA**

The deep well plate containing cell suspension (3.3.1) was centrifuged at 3000 g for 10 min at RT. The supernatant was discarded and the pellet was dissolved in 450 µl PBS-T0.05 by vortexing. Sealing tape (NUNC) was used to prevent contamination. In order to lyse the cells, the plate was frozen for at least 45 minutes in -80°C and quickly thawed for 30 minutes in lukewarm water. Centrifugation at 3500 g for 30 minutes at 4°C was performed to spin down cell debris. The lysate was immediately used for ELISA or frozen at -20°C.

##### **3.3.2.2 ABAS-ELISA**

A 96 well flat bottom, high binding polystyrene plate (Costar 9018 EIA/RIA plate) was coated with 6 µg/ml HSA (CAS 70024-90-7), 100 µl/well ON at 4°C. The plates were washed with water twice. PBS-T0.05 with 2% (w/v) dry milk (Semper) was used as blocking solution for 1 hour, 200 µl/well at RT using mild shake. Blocking was removed and 100 µl/well cell lysate was added for 1.5 hours at RT allowing Affibody® molecule-ABD to bind HSA. The plate was washed four times with PBS-T0.05. In each well 100 µl biotin-protein A 1µg/ml, diluted in PBS-T0.05 was incubated for 1.5 hours at RT. The plate was washed four times with PBS-T0.05. Streptavidin-horseradish peroxidase (Streptavidin-HRP. Dako Cytomatation), 100 µl/well 0.63 µg/ml diluted in PBS-T0.05 was added and incubated for 1 hour at RT. The plate was washed four times with PBS-T0.05. Immunopure TMB (Pierce), 100 µl/well, was added and incubated dark for 30 minutes at RT. H<sub>2</sub>SO<sub>4</sub>, 2M, 100 µl, was added in each well to stop the reaction. Absorbance at 450 nm was measured with a Tecan Sunrise spectrophotometer.

On each plate the following controls were used:

- Positive - A known insulin binder Z<sub>insulinA</sub>-ABD 1µg/ml was added instead of lysate. It was detected with 100 µl biotinylated insulin 1µg/ml (insulin (Roche), biotinylated with sulfo-NHS- biotin (Pierce)) at Affibody AB).
- Negative - A known insulin binder Z<sub>insulinA</sub>-ABD 1µg/ml was added instead of lysate.

- Blank - PBS-T0.05 was added instead of lysate.

A negative control of all clones in optimization selection 2 was made by not adding biotin-protein A.

### 3.3.2.3 Neutravidin and streptavidin ELISA

For the neutravidin ELISA a 96 well flat bottom, high binding polystyrene plate was coated with 6 µg/ml neutravidin diluted in 50 mM NaHCO<sub>3</sub>, 100 µl/well ON at 4°C.

A streptavidin coated plate (Nunc transparent, c96, 236001) or a neutravidin coated plate was blocked with 200 µl PBS-T0.05 with 2% (w/v) dry milk (Semper) per well for one hour at RT. Blocking was removed, 100 µl cell lysate per well was added and the plate was incubated for 1.5 hours at RT. The plate was washed four times with PBS-T0.05. In each well, 100 µl primary antibody Rabbit IgG (Affibody-ABD binder, 90 µg/ml in PBS-T0.05) was incubated for 1.5 hours at RT using mild shake. The plate was washed four times with PBS-T0.05. Secondary antibody, rabbit immunoglobulin-HRP (P0448 Daco Cytomation, diluted in PBS-T0.05 1:8000), 100 µl/well, was added and incubated for 1 hour at RT. The plate was washed four times with PBS-T0.05. Immunopure TMB, 100 µl/well was added and incubated dark for 30 minutes at RT. H<sub>2</sub>SO<sub>4</sub>, 2M, 100 µl was added in each well to stop the reaction. Absorbance at 450 nm was measured with a Tecan Sunrise spectrophotometer.

On each plate following controls were used:

- Positive - A known SA binder Z-fab L#20-ABD 1µg/ml was added instead of lysate. In the neutravidin ELISA a known NA binder, Z00626, was used in optimization selection 2.
- Negative - A known insulin binder Z<sub>insulinA</sub>-ABD 1µg/ml was added instead of lysate.
- Blank - PBS-T0.05 was added instead of lysate.

## 3.3.3 Sequencing

### 3.3.3.1 PCR reaction prior to sequencing

From the prepared PCR-plates (3.3.1) phagemid-infected RR1? M15-clones were taken for sequencing. Per sequence reaction, 1 µl cell suspension was mixed with 2 µl dNTP (Applied Biosystems), 2 µl 10x PCR-buffer (Applied Biosystems), 0.04 µl AmpliTaq<sup>®</sup> 5 U/µl (Applied Biosystems), 14.16 µl sMQ and primers, 0.4 µl Affi-21 (5'-tgctccggctcgatgtgtgtg) and 0.4 µl Affi-22 (5'-cggaaccagagccaccaccgg) 5 pmol/µl each. The PCR-reaction was performed in PCR-plates or in 0.2 ml PCR-tubes (Abgene AB0266) on a PTC-225 (Peltier Thermal Cycler, MJ Research) with the following program: 94°C 5 min; 30 x (96°C 30 sec; 50°C 30 sec; 72°C 1 min 15 sec); 72°C 7 min. Heated lid was used.

### 3.3.3.2 Sequencing

Diluted PCR-product (1/10), 1 µl, was mixed on ice with 7 µl cycle sequencing buffer (CS. 6.5 mM MgCl<sub>2</sub>, 26 mM Tris-HCl pH 9.0), 1 µl sequencing primer Affi-72 5 pmol/µl (5'-biotin-cggaaccagagccaccaccgg) and 1µl ABI PRISM<sup>®</sup> BigDye<sup>™</sup> Terminator Cycle Sequencing Ready Reaction Kit 3.0 (Applied Biosystems). The mixture was run on a PTC-225 in PCR-plates or in 0.2 ml PCR-tubes. The program run was: 95°C 1 min; 25 x (96°C 30 sec; 50°C 15 sec; 60°C 4 min); 72°C 7 min. The product from the reaction was purified in a Magnatrix 8000 (Magnetic Biosolutions) using program "Magnatrix Sequencing Clean up" with reagents Dynapure<sup>™</sup> Dye terminator removal ver 2 (Dyna),

regeneration buffer (Magnetic Biosolution), 1 x TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA), 70% ethanol, 2x binding/washing buffer (2 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10% PEG). The purified product was transferred to an ABI 3100 plate and sequenced with the ABI 3100 Genetic Analyzer (Applied Biosystems).

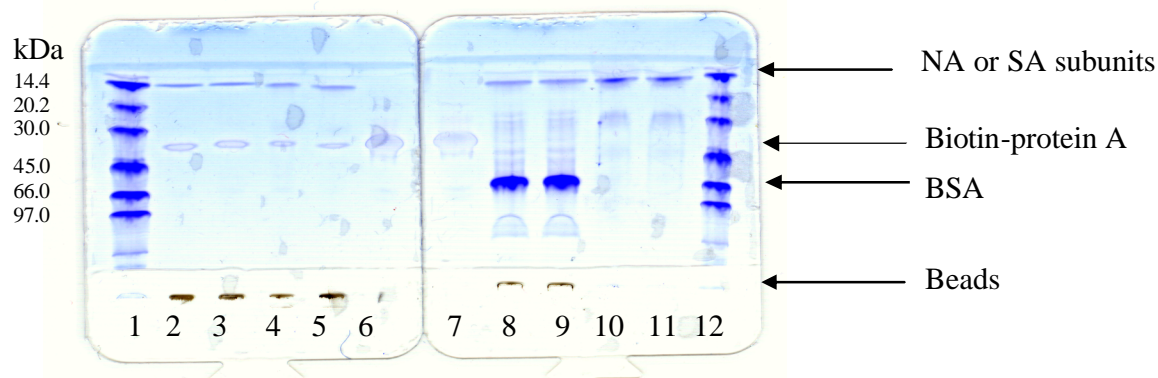
#### **3.3.4 Clustering**

The Affibody<sup>®</sup> molecules with affinity to protein A confirmed in ABAS-ELISA were compared and clustered according to the a.a. sequences. Affibody<sup>®</sup> molecules with protein A affinity found earlier at Affibody AB were included in the analyzes too. The clustering was made using the program “Clustering of Affibody molecules”.

## 4 Results

### 4.1 Activity test of neutravidin coated beads

A test to examine the ability of NA coated beads to bind biotin-protein A was performed. Analysis was made on SDS-PAGE and SA coated beads was used as a reference. By comparing the strength of the band from the pure biotin-protein A (lane 6 and 7) with the bound materials (lane 2-5) it was determined that the SA coated beads bound approximately 2-3  $\mu\text{g}$  and the NA coated beads approximately 1.5  $\mu\text{g}$  biotin-protein A (figure 12).



**Figure 12.** From left to right: (1) LMW, (2) 0.5 mg SA coated beads with bound protein A [binding I], (3) 0.5 mg SA coated beads with bound protein A [binding II], (4) 0.5 mg NA coated beads with bound protein A [binding I], (5) 0.5 mg NA coated beads with bound protein A [binding II], (6) 7.2  $\mu\text{g}$  biotin-protein A, (7) 7.2  $\mu\text{g}$  biotin-protein A, (8) and (9) Samples not from study, (10) Supernatant from SA coated beads, (11) Supernatant from NA coated beads, (12) LMW

### 4.2 Definition of binders

A clone was defined a binder if its absorbance in ELISA where at least twice of the highest absorbance of the blank or the negative control on the same plate. A binder was defined a high affinity binder if the measured absorbance in ELISA was at least 0.7 AU. Below 0.7 AU the binders were defined low affinity binders.

### 4.3 Test selection

In the test selection a small library, Zlib4, consisting of two known protein A binders and two known SA binders was used. Zlib4 was screened with an ABAS-ELISA and a SA ELISA, giving a library composition of 29% protein A binders and 71% SA binders (compare with theoretical 50% / 50% [3.2.1]). A single selection round was performed to examine the discrimination of SA binders on NA coated beads compared to SA coated beads. The effect of pre-clearing was also tested. Evaluation of the selections was made with ELISA. The amount of protein A binders and SA binders was compared between each kind of selection and the original library. During the test selection, no greater differences between selection with SA and NA coated beads appeared. A two- to threefold enrichment of the protein A binders was seen for both bead variants and no effect of the pre-clearing. The selection with NA coated beads reduced the number of SA binders to 23% compared to the selection with SA coated beads that ended up with 15% SA binders after one cycle. The selections with pre-cleared library resulted in an amount of SA binders of 23% for NA coated beads and 26% for SA coated beads.

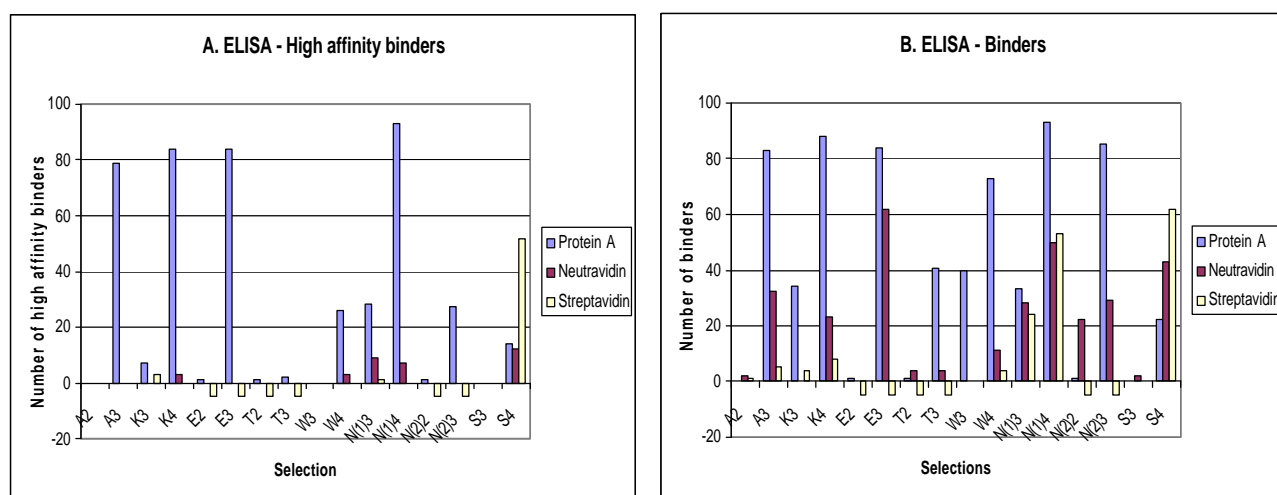
## 4.4 Optimization selections

The optimization selections were made in two sets with four parallel selections in each set. In the first optimization, a selection with SA coated beads (S) was compared with a selection with NA coated beads ( $N_1$ ), an alternating bead selection starting with SA coated beads (K) and a SA coated bead selection using an extended wash strategy, i. e. longer washes, more Tween and larger volumes (W). In the second set,  $N_1$  was repeated ( $N_2$ ) and W was repeated too but on NA coated beads (T). Another washing procedure with even higher Tween concentrations on NA coated beads (E) was also made and an alternating bead selection starting with NA coated beads (A). In the second set only three selection rounds were performed. The selections that generated the best results in terms of number of high affinity protein A binders, few background binders and few number of selection cycles were selection A and E. Selection S was the selection that showed most background (binders without protein A affinity) in each cycle.

### 4.4.1 Background analysis

#### 4.4.1.1 ELISA analysis of background

To examine the composition of binders with different affinities after a selection round, affinity screening for protein A, NA and SA were done using ELISA assays. The binders where no protein A affinity was found were defined background binders. The number of background binders was lowered with an increasing number of selection rounds in all analysed selections steps as it should. In selection rounds A3, K4, E3, W4,  $N_1$ 4 and  $N_2$ 3 (the letter is the selection name and the number is the analysed selection round) the background was very low as over 70 of 93 ELISA analysed colonies were protein A binders (figure 13). In figure 14 the ELISA results are shown as bar diagrams to illustrate the big differences between some selection rounds in another way.

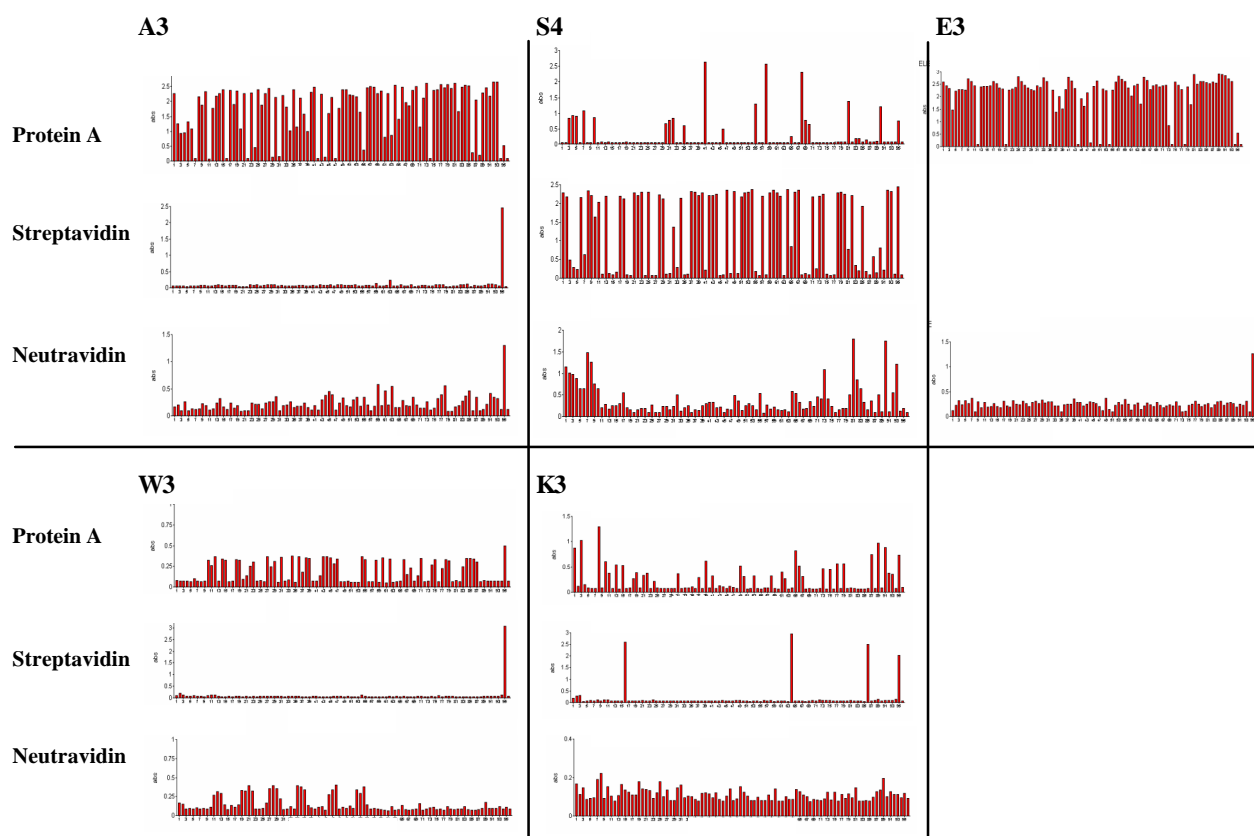


**Figure 13: Results from ELISA analysis, 93 clones were examined in ABAS-ELISA, NA ELISA and SA ELISA in most selections. A negative bar represents if no ELISA was made. (A) The total number of clones with an absorbance of at least 0.7 AU are presented regardless if the clones had high affinity in more than one ELISA. (B) The total number of clones with an absorbance of at least twice the highest of the negative control and the blank are presented regardless if the clones had such affinity in more than one ELISA.**

In S4, over 50% of the ELISA analysed colonies were shown to be high affinity SA binders. Neither after round 3 or 4 high affinity SA binders were found in the other selections. Low affinity to NA however, were found among many of the protein A binders generated in all selections, even in selections with only SA coated beads (figure 14 and Appendix A). Looking at the different NA affinities of the protein A binders no significant differences could be found between the different selection types. Because of



the double affinity of many of the protein A binders, negative controls was performed on whole 96-well plates to check that the absorbances in ABAS-ELISA really were the result of protein A affinity and not streptavidin-HRP affinity. These negative controls did not give any results indicating that it was not protein A affinity that was shown. The NA affinity of the protein A binders was not further investigated. The high number of NA binders in S4 (figure 13) can be explained by the fact that the SA binders also have NA affinity, weaker (except one clone) than the SA affinity but in some cases strong enough to be regarded as a high affinity NA binder. Overall, there were very few binders with affinity only to NA.



**Figure 14. Examples of ELISAs.** In each selection (except E3) three ELISAs were made after each analyzed round, ABAS-ELISA (protein A), SA ELISA and NA ELISA. All 93 clones in each selection were analyzed in the same order in the three different ELISAs. The controls are in the rightmost columns in following order: positive (in S4, W3 and K4 Z-fab L#20-ABD was used without positive result in NA ELISA), negative and blank).

#### 4.4.1.2 Sequence analysis of background

After round four in the first optimization selection set, all picked colonies that were analysed with ELISA were sequenced to analyze if the background binders converged to a few unique background binders. In table 2 the number of unique binders with affinity to each of the proteins protein A, SA and NA are presented. If a clone had affinity to more than one target, the target with highest affinity was chosen. For the standard selection S, five SA binders were found compared to no SA binders for the optimization selections W, K and N. The NA binder in selection W has also SA affinity.

**Table 2. The number of sequenced clones with an affinity for the different screening proteins protein A, SA and NA. If the clones had affinity for several targets, the target which achieved the highest affinity was chosen. ‘?’ indicates clone with unknown affinity.**

Number of different clones binding to each protein			Round 4
<b>W</b>	Protein A		2
	Streptavidin		0
	Neutravidin		1
	?		5
<b>S</b>	Protein A		3
	Streptavidin		5
	Neutravidin		0
	?		10
<b>K</b>	Protein A		5
	Streptavidin		0
	Neutravidin		0
	?		2
<b>N</b>	Protein A		2
	Streptavidin		0
	Neutravidin		0
	?		0

#### 4.4.2 Protein A binder comparisons

To analyze the found protein A binding clones, a number of protein A binding clones was chosen (based on the results from ABAS-ELISA) for sequencing. The sequencing results (table 3) show that the extended wash protocol, round 3, (W3) generated the highest number of unique protein A binders (16 out of 17 sequenced). Only one of these binders was however a high affinity binder. A3, one of the alternating bead selections did yield less unique protein A binders than W but has most high affinity protein A binders of all selections. Another interesting result was that selection E, which had the highest Tween concentration and medium long washes, generated only high affinity binders after round 3. The total number of unique binders were however somewhat lower than in selection A.

The standard selection S was the only selection that did not show any binders at all after the third round. After 4 rounds of selection the distinction between the numbers of unique clones found in the different selections was not as obvious as after round 3. S, round 4 (S4) shows close to equivalent number of different protein A binders as the other selections' round 4. The protein A binder density in S4 was however lower since the number of SA binders was abundant. In the selections where four rounds were made, the diversity ([number of unique protein A binders] / [number of sequenced clones]) are lowered in all selections except for S (which did not show any binders at all after round 3). This is because the binders are converging to a few strong protein A binders due to over selection, i. e. more selection than necessary to find wanted binders.

**Table 3. Summary of optimization selections sequencing results. Table is based on appendix A. Results from round 2 are not displayed.**

Selection	A3	K3	K4	E3	T3	W3	W4	N <sub>1</sub> 3	N <sub>1</sub> 4	N <sub>2</sub> 3	S3	S4
Total # of sequenced protein A-binders	23	30	87	24	6	17	72	33	89	22	0	23
# of unique binders with protein A affinity	6	4	6	3	2	16	2	3	2	4	0	3
# of unique binders with high protein A-affinity	4	2	3	3	1	1	1	2	2	2	0	2
Diversity of binders (# of unique binders / # total binders)	26%	13%	7%	13%	33%	94%	3%	9%	2%	18%	0%	13%
Diversity of high affinity binders (# of high affinity binders / # total binders)	17%	7%	3%	13%	17%	6%	1%	6%	2%	9%	0%	9%

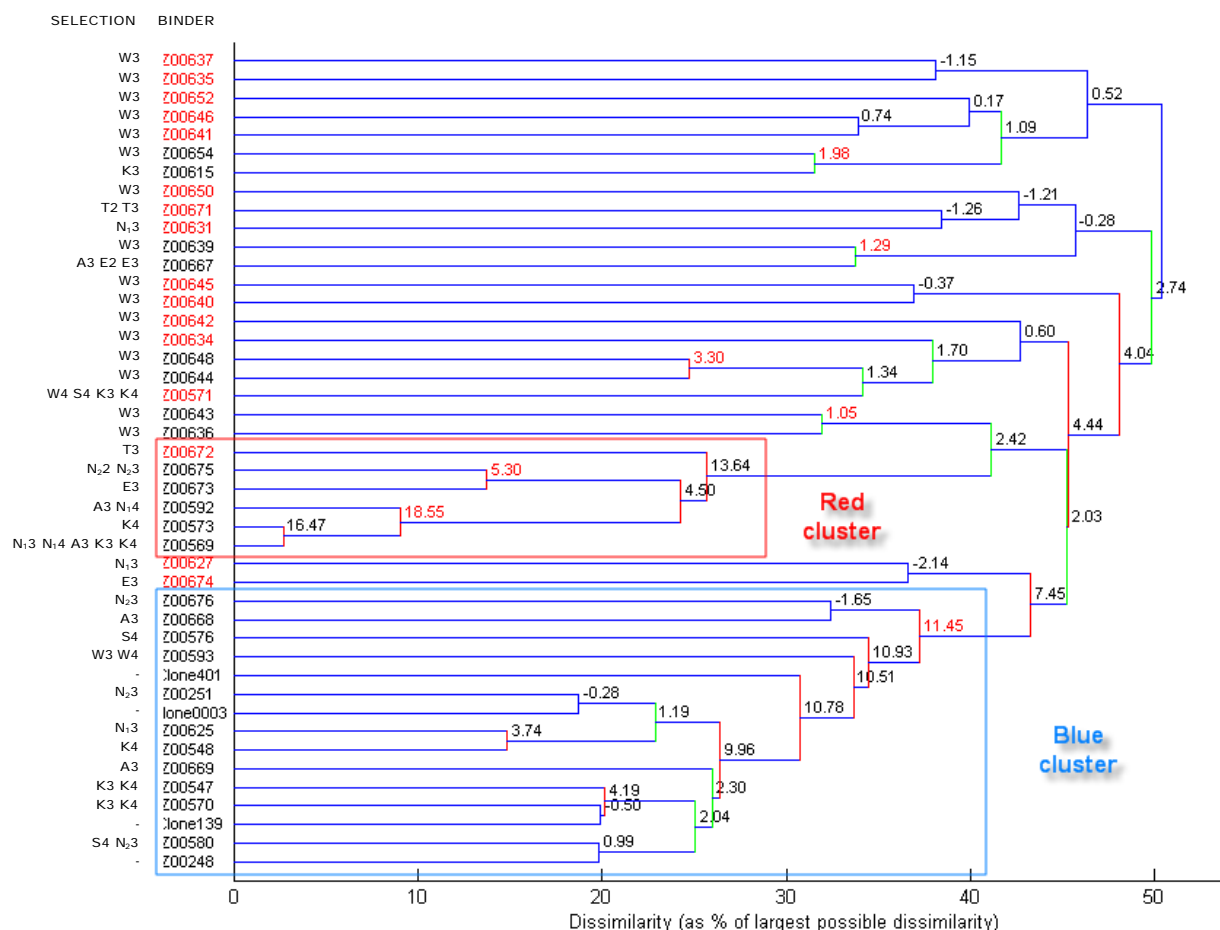
In selections A, E, T and N<sub>2</sub> analyses were made after two rounds too. One protein A binder was found in E, T and N<sub>2</sub> each after the second round, no binder was found in A.

### 4.4.3 Clustering results

To evaluate if the clones in the different selections tended to draw towards a group of similar binders, all sequenced protein A binding Affibody<sup>®</sup> molecules were clustered (figure 15). The sequences used to build the dendrogram are presented in appendix B. Two groups of protein A binders seemed to cluster more tightly than the other binders. The two clusters were called “red cluster” and “blue cluster” respectively. More information of about the different clusters is given in appendix C. For each selection, the number of Affibody<sup>®</sup> molecules belonging to each cluster is represented as a measure of the diversity of the selection (table 4). Looking at the different selection types, it can be seen that both selections with SA or NA coated beads have binders in both clusters when including previous selections performed at Affibody AB (appendix A). Several binders found in these previously done selections have also been found in selections performed with NA coated beads and the extended wash protocols (figure 15 and appendix A). The alternative washes seem to have affected the type of binders though. In the clusters, only one binder from each of the W and T selections can be found and the binders from W are overrepresented outside the clusters. The affinities of the binders from W and T which belong to the clusters have the highest protein A affinities of all binders found in W and T. All other binders in these two selections have poor affinities compared to most binders in the other selections.

**Table 4. Number of different clones belonging to the red or blue cluster in each analysed selection round. The results in this table are presented in appendix A. Results from round 2 are not displayed.**

Selection	A3	K3	K4	E3	T3	W3	W4	N,3	N,4	N,3	S3	S4
Clones in red cluster	2	1	2	1	1			1	2	1		
Clones in blue cluster	2	2	3			1		1		3		1

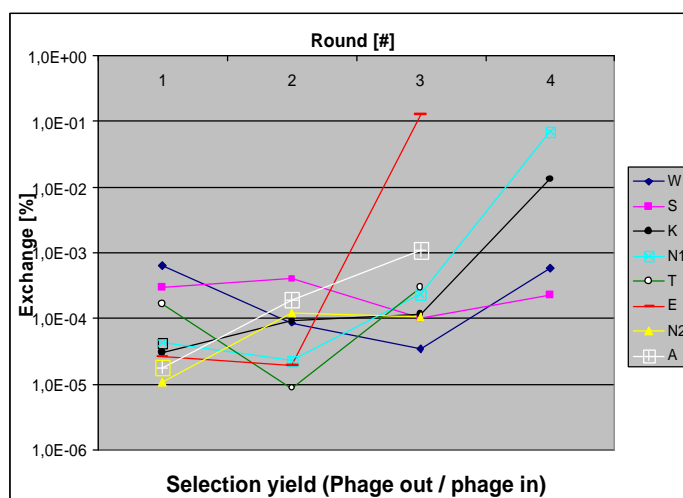


**Figure 15. Result of clustering.** All protein A binders found in the optimization selections and selections previously performed at Affibody AB have been clustered together. Two different clusters have been chosen from the dendrogram and named “Blue cluster” and “Red cluster”. Protein A binders that only has been found in previous selections are marked ‘-’. The numbers in the figure are cluster quality numbers, the higher the better cluster integrity.

#### 4.4.4 Yield

In figure 16 the yield in each selection round is presented. The yield is based on the ratio of phage in and phage out in each selection round (appendix D). When the number of phage binding to the target (or to other components in the selection) increases, the graphs peak. This was used as an indication of the number of protein A binders expected to be found in screening after a specific round. For example, the selection rounds E3, N<sub>1</sub>4, K4 and A3 have the highest yields and also the best protein A binder to background ratios.

Note that selection A that generated the highest number of high affinity binders and few background binders, has a curve, which is increasing almost exponentially.



**Figure 16. Exchange ratios of the number of phage in into each selection round relative phage out of the same round.**

## 5 Discussion

In this work a number of methods to reduce unwanted binders in a phage selection process were tested. The effect of pre-clearing, longer washing times, larger washing volumes, increased Tween 20 concentration during wash, different solid supports and alternating solid supports have been evaluated. The effect of these different approaches has been tested on phage display selection (3+3 display system) against biotinylated protein A. The main conclusions from the experiments were that alternating the biotin binding molecule on the solid support has reduced the amount of background binders from near 100% to 11% in panning round 3 (figure 13) and given a more diverse array of protein A binders (table 3). Increasing the Tween concentration from 0.1% to 2% and changing the biotin binder from streptavidin to neutravidin has reduced the amount of background binders from near 100% to 10% in round 3 (from figure 13) and has also resulted in more high affinity binders (table 3 and 4). With both of these changes it has been possible to reduce the number of panning rounds required to find wanted binders from four to three.

### 5.1 Pre-clearing

The result from the test selection shows that pre-clearing has no positive effect on the number of SA binders. The pre-clearing was however made on a four member library and only one selection round was performed. It is possible that the pre-clearing could be more effective when repeating pre-clearing in each panning during a selection or with another library. Higher concentration of beads may also give better results. No ELISA analyses to screen the binder composition were made directly after the pre-clearing step. Instead the ELISA analyses were made after the subsequent selection. A screen immediately after the pre-clearing step should have given clearer results.

The results show that the effects of pre-clearing are, if any, so small that the method is unnecessary when using other more powerful methods to reduce background.

### 5.2 Efficiency and diversity

In all selections, except the standard selection with SA coated beads, it was possible to find protein A binders already after three selections. It is not possible to say, however, if it is enough to do only three rounds with all kinds of targets. If it is possible, this will make selection less time consuming and cheaper. In two ( $N_1$  and W) of the four selections analyzed after four rounds, the results were actually better after three rounds in the aspect of number of unique binders (table 3), although there was a higher background after three rounds in these selections. After four rounds, mainly one clone was representing all the binders left in each of the two selections. In  $N_1$ , 99% of the colonies analysed after round four were the same clone and in W, 60% of the analysed colonies were identical (Appendix A). When the diversity falls so much in a round that the total number of different wanted clones are reduced to one or a few, the selection may have gone too far (depending on the goal of the selection). If only one high affinity binder and as little screening as possible are wanted, a selection that has gone this far would be good. Several binders are often wanted for analysis though.

### 5.3 Conclusions of streptavidin versus neutravidin

There were protein A binders from the same clusters in both SA and NA coated bead selections indicating that the different avidins do not select different types of binders with target affinity. Even the same clones reappeared in the different selection types. The selections with only NA coated beads did not have as many background binders as the

selection with only SA coated beads. This point towards that NA is a more specific biotin binder than SA, which may be a result from the more neutral pI [30, 41]. Interestingly the best results have been with alternating NA and SA coated beads in the different selection rounds. The orders NA-NA-NA, SA-NA-SA, NA-SA-NA and SA-SA-SA (where NA is a round with NA coated beads and SA is a round with SA coated beads) have been tested and the system which gave most diverse types of binders and the least amount of background was NA-SA-NA. Apparently, there are binders which have affinity to NA but not to SA and the other way around. These binders were more or less removed in the round with alternate biotin binding protein. The conclusion would be that a number of Affibody<sup>®</sup> molecules bind to different domains of the different avidins. Still NA seems to be more specific than SA, yielding a better selection when alternating beads and starting with NA coated beads. It is possible that a selection with beads in the order NA-NA-SA would give even better results since the NA-NA combination generated one binder after two rounds and the NA-SA selection did not generate any binders. This result was not statistically evaluated, though. Besides, results originating from the order NA-SA-NA were so good that possible improvements would probably not be of any greater importance.

In this work, no draw-backs of NA coated beads as compared with SA coated beads could be found. The NA coated beads have given excellent results in reducing background compared to the SA coated beads. There is however one practical drawback with the NA coated beads; they have to be coated and it is hard to get a value of the biotin binding capacity.

#### **5.4 Evaluation of the different washing methods**

Washing with higher Tween concentration, larger washing volume and longer washing time was efficient to eliminate background binders. In all selections with these alterations however, the type of protein A binders, regarding sequence similarities and affinity, were affected too. For example, the washing protocols with a washing volume of 10 ml, 0.5% Tween 20 and 20 minutes washing steps, W and T, got no common kind of binders and both of the selections differ widely from the selections with the standard washing protocols concerning both amount of binders and type of binders (table 3 and 4, figure 15). Actually, W generated almost only low affinity binders. This may be explained by an accidental removal of high affinity binders due to too stringent washing in early rounds. Selection T which had longer washing and larger washing volumes probably washed several binders away, explaining the poor result from that selection. The results from the protocols with 20 minutes washing steps are not very conclusive but the longer washes definitely changed the selection results. With 10 minutes washing steps and 2% Tween (E), three high affinity binders completely takes over the selection (table 3). The washing procedure in E seems to effectively reduce low affinity binders pushing the stronger binders forward at a more rapid rate without being too harsh against them. In a case where specificity is a desired property rather than affinity this type of wash may not be appropriate. The wash protocol with many short washes and small volumes that is used at Affibody today seems from the results in this thesis to allow more background binders per protein A binder than the new types of washes. In selections against new targets, different wash setups should be used in each selection since the binding properties between potential binders and target are unknown [14]. More experiments should be made to compare the efficiency of reducing background binders, without losing wanted binders.

## 5.5 Yield considerations

By looking at the yield in each selection (figure 16) it was possible to get a hint of the effect of the stringency in each round and the possible outcome of a screening. It can be seen that E3, N<sub>1</sub>4, K4 and A3 which were the selections rounds with least background (figure 13) also had the highest yields. Selection A which generated the highest number of high affinity binders, the second most protein A binders and a low degree of background binders had a yield which is increasing almost exponentially between the selection rounds. It may be a sign of optimized stringency.

## 5.6 Future work

Some work that may be done for further optimization studies is presented with comments and a short description.

### *Selections with other targets.*

Repeating the best selection methods with other targets than protein A would answer if the methods with alternating beads and wash protocols with more Tween, longer washes and larger volumes are generally better than selecting with SA coated beads and the less extreme washes which are used today.

### *Increasing stringency*

By increasing the Tween concentration, washing volume and washing time first in later rounds it may be possible to reduce the number of background binders without losing wanted binders [21].

### *Competitive elution*

By eluting phage with non-biotinylated target it is possible to release bound-up target without releasing background binders from the beads [20]. The drawbacks are that it is possible that background binders will flush loose and show up in the eluted material anyway. Further, the competitive elution requires protein that is much more expensive than an acidic elution buffer.

### *Alternating hydrophobic and hydrophilic beads.*

By alternating hydrophobic and hydrophilic beads (the SA coated beads and NA coated beads used in this work are hydrophobic [6, 7]), it may be possible to avoid background binders that have an affinity to either kind of surface. The choice of different beads may alter the biotin binding molecule slightly as it may show its hydrophobic side towards the liquid support when using hydrophilic beads and the opposite with hydrophobic beads [6]. In the case of neutravidin and streptavidin however, it is questionable if exposing another side will do any difference since they have tetramer structures [32].

### *Usage of Tween and salt.*

Higher salt concentrations has previously been shown to reduce hydrophilic background binding [14], i. e. binders with affinity to hydrophilic beads. When using hydrophobic beads on the other hand, it is possible that a higher salt concentration would increase the background binding [36]. Tween 20 inhibits unspecific hydrophobic binding due to its emulsifying properties [7]. A test on using high salt concentration in combination with hydrophilic beads (in a selection with alternate hydrophobic and hydrophilic beads for example) could be made to try to reduce background binding.

### *Other alternate supports*

By immobilizing target by other methods than with a biotin binding molecule and alternate with SA-biotin immobilization it has been possible to minimize the amount of background binders [8].

#### *Alternating blocking*

By alternating BSA and gelatine as blocking agents in the different selection rounds, the amount of possible blocking binders may be reduced.

#### *Basic elution when using Captavidin*

Captavidin (Molecular Probes) is a derivative of avidin in which the tyrosine in the biotin binding site is nitrated [35]. Captavidin binds to biotin with a best  $K_a$  of  $\sim 10^9 \text{ M}^{-1}$  at pH 4 but dissociates completely at pH 10 [34]. By using Captavidin it would be possible to release the phage at pH 10. The release would be between the biotin and the Captavidin and would not be dependent of the affinity between the phage and its target. This could enable release of the phage at such mild conditions that the remaining background binders will not release so easily. Still milder acidic washing would be possible thanks to the high  $K_a$  value at lower pH. The strategy when using Captavidin as a biotin binder would preferably be as one of a couple of different biotin binders in a selection process with alternating kind of solid support. The main drawback of Captavidin is the low affinity to biotin, the  $K_a$  of  $\sim 10^9 \text{ M}^{-1}$  [34] compared to streptavidin which got a  $K_a$  of  $10^{15} \text{ M}^{-1}$  with biotin [2].

#### *pH gradient elution*

Making several elution steps while lowering the pH has been showed as an effective method to reduce background binders [15].

#### *Biotin blocking*

After the phage-target complex has bound to the solid phase, biotin could be added to block remaining SA/NA molecules. The biotin will also compete with Affibody<sup>®</sup> molecules that have bound directly to the biotin-binding site of SA/NA and make them release. Since the biotin-SA and the biotin-NA bindings are very strong the phage-target-biotin-SA/NA complex will remain intact during this action [14].

#### *Optimization of binding time to beads*

$K_a$  between biotin and SA is  $10^{15} \text{ M}^{-1}$  [2] and biotin will most probably associate faster than the background binders to the coated beads. Shortening the binding time to the solid phase will reduce the time for phage binding directly to the NA or SA coated beads. The shorter time will hopefully not affect the phage-target complex' binding to the coated beads as much as the background binding phage's binding.

## **5.7 Conclusions**

By alternating NA and SA on the solid support it has been possible to make a major reduction of background binders and thus allowing more protein A binders to be found. The alternating bead selection, when started with NA, was so effective that further optimizations to reduce the amount of background binders may be unnecessary for the moment. Different alternations of the washing procedure have been tested with good results in reducing background binders; unfortunately the washes also affected the properties of the found protein A binders. However, the tested washing protocol with 2 % Tween 20 and 10 min of washing was so effective in reducing background binders that the results should be considered when setting up new selections. More data is required from selections with other targets than protein A before drawing any guidelines regarding the wash protocols in future selections.



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## **Appendices**

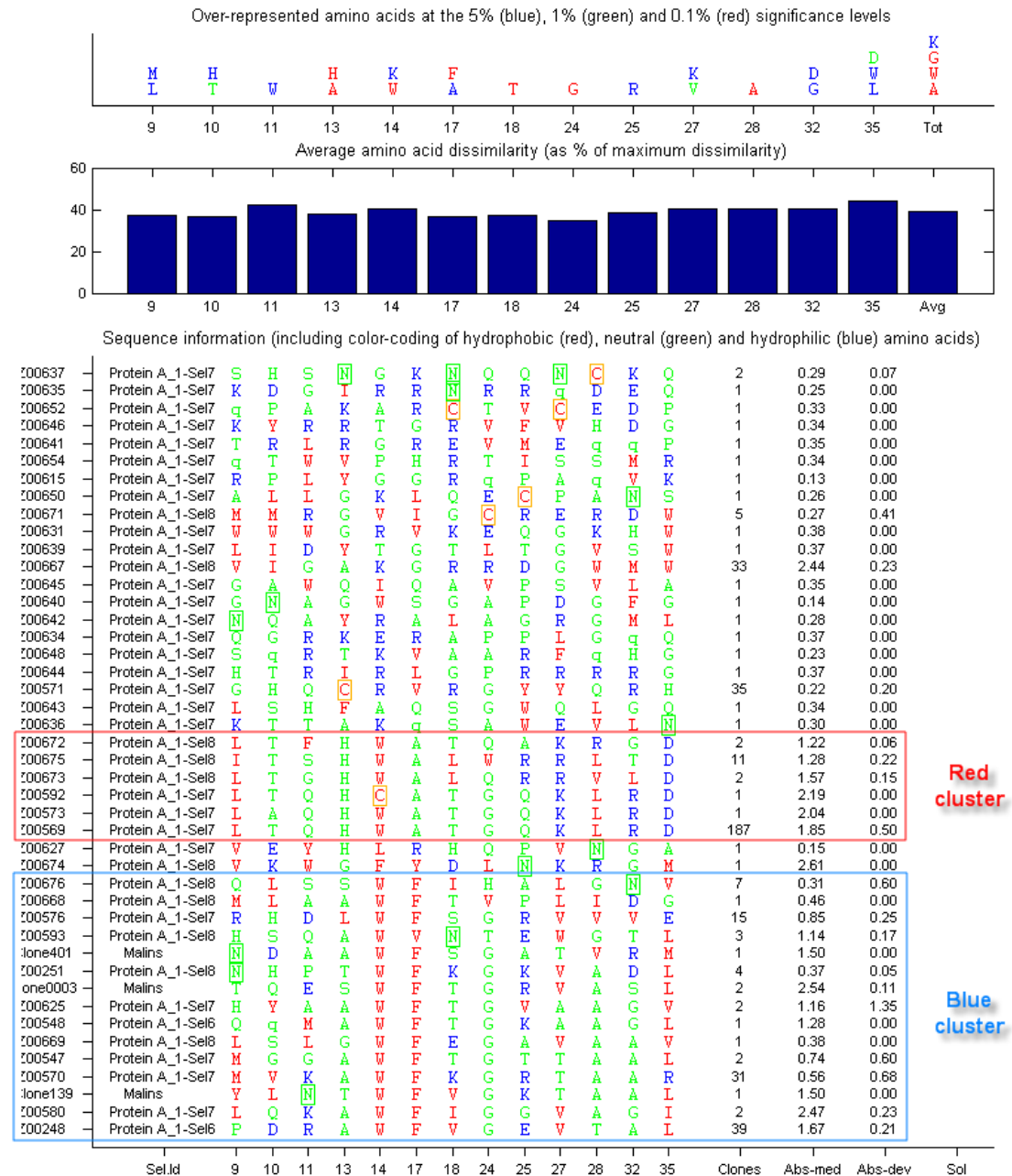
## Appendix A

Data over found protein A binders. Their protein A and NA affinity shown and in which selections they were found and how many clones of each binder it were. In the bottom, data regarding the different selections are found.

Neutravidin		Protein A		Binder	A2	A3	K3	K4	E2	E3	T2	T3	W3	W4	N <sub>3</sub>	N <sub>4</sub>	N <sub>2</sub>	N <sub>3</sub>	S3	S4	Found in earlier selections
Absorbance (AU)	Standard deviation (AU)	Absorbance (AU)	Standard deviation (AU)																		
0,24	0,08	0,37	0,05	Z00251														4			Yes
0,17		0,74	0,60	Z00547			1	1													Yes
		0,12		Z00548				1													Yes
0,30	0,20	1,82	0,43	Z00569		4	6	64							30	88					Yes
0,36	0,18	0,56	0,68	Z00570			18	13													Yes
0,38	0,23	0,22	0,20	Z00571			5	7						17						6	
0,22		2,04		Z00573				1													
0,71	0,56	0,85	0,25	Z00576																15	
0,26	0,09	2,58		Z00580														1		2	Yes
0,46	0,24	2,19		Z00592		3										1					
0,15	0,08	1,14	0,17	Z00593									1	55							
		1,16	1,35	Z00625											2						
0,99		0,38		Z00631											1						
		0,37		Z00634									1								
		0,25		Z00635									1								
		0,31		Z00636									1								
		0,29	0,07	Z00637									2								
		0,37		Z00639									1								
		0,35		Z00641									1								
		0,28		Z00642									1								
		0,34		Z00643									1								
		0,37		Z00644									1								
		0,35		Z00645									1								
		0,34		Z00646									1								
		0,23		Z00648									1								
		0,26		Z00650									1								
		0,33		Z00652									1								
		0,34		Z00654									1								
0,25	0,08	2,44	0,25	Z00667		12			1	21											
0,22		0,46		Z00668		1															
0,34		0,38		Z00669		1															
0,36	0,14	0,87	0,09	Z00670		2															
0,12	0,03	0,27	0,41	Z00671							1	4									Yes
0,15	0,02	1,22	0,06	Z00672								2									Yes
0,24	0,00	1,57	0,15	Z00673						2											Yes
0,28		2,61		Z00674						1											Yes
0,20	0,11	1,31	0,35	Z00675													1	11			Yes
0,21	0,11	0,31	0,60	Z00676														6			
Total # of sequenced protein A-binders					0	23	30	87	1	24	1	6	17	72	33	89	1	22	0	23	
# of unique binders with protein A affinity					0	6	4	6	1	3	1	2	16	2	3	2	1	4	0	3	
# of unique binders with high protein A-affinity					0	4	2	3	1	3	0	1	1	1	2	2	1	2	0	2	
Diversity of binders (# of unique binders / # total binders)					0%	26%	13%	7%	100%	13%	100%	33%	94%	3%	9%	2%	100%	18%	0%	13%	
Diversity of high affinity binders (# of high affinity binders / # total binders)					0%	17%	7%	3%	100%	13%	0%	17%	6%	1%	6%	2%	100%	9%	0%	9%	
Clones in red cluster					2	1	2			1		1			1	2	1	1			
Clones in blue cluster					2	2	3						1		1			3		1	

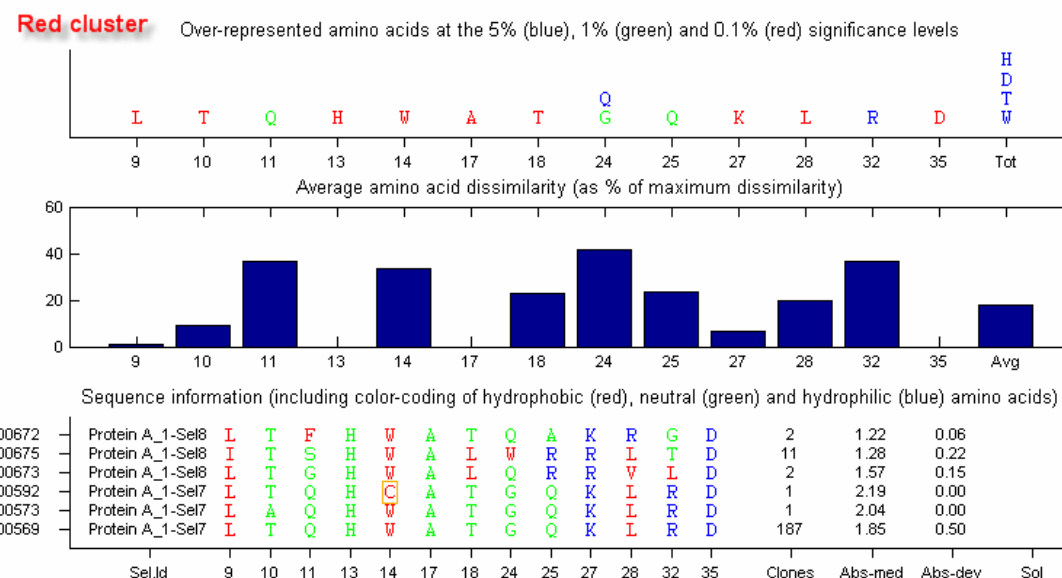
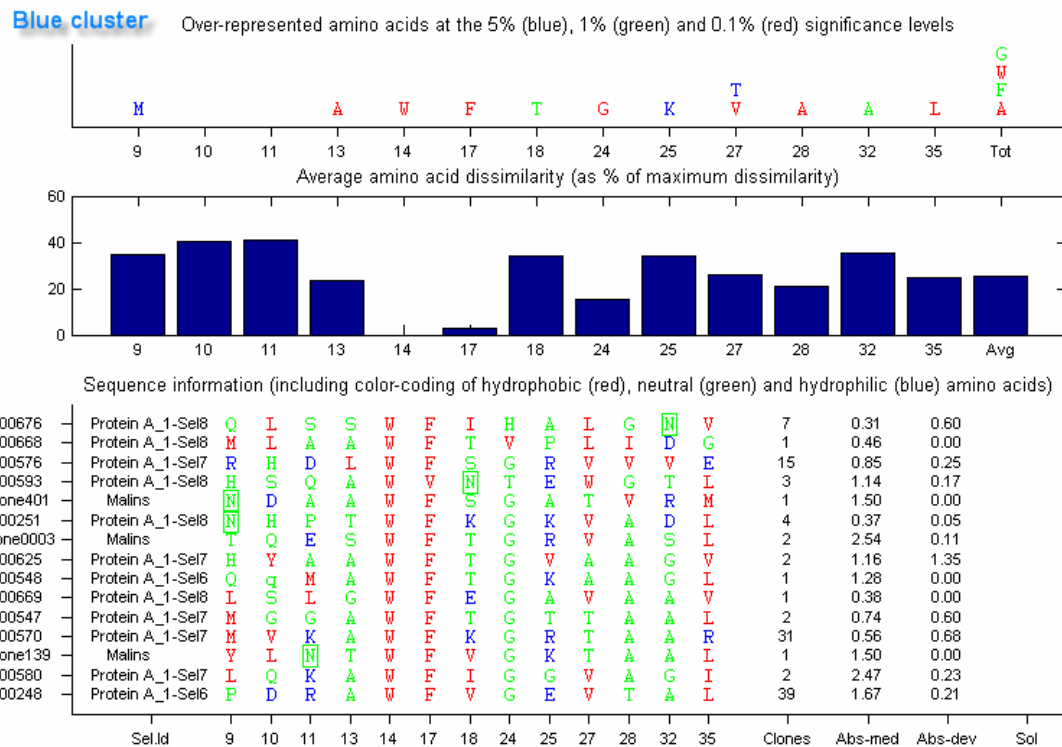
## Appendix B

Sequence data over variable regions in found protein A binders. Color coding of amino acid hydrophilicity is based on the Hopp and Wood hydrophilicity scale [42].



## Appendix C

Sequence data over variable regions in protein A binders belonging to blue respectively red cluster. Color coding of amino acid hydrophilicity is based on the Hopp and Wood hydrophilicity scale [42].



## Appendix D

Number of phage in and out, in each selection round and the ratio between in and out, i. e. exchange.

Sel.	Round	Phages IN (cfu/ml)	Phages OUT (cfu/ml)	Exchange (%)
W	1	7,50E+12	4,80E+07	6,4E-04
W	2	1,00E+12	8,60E+05	8,6E-05
W	3	1,24E+11	4,20E+04	3,4E-05
W	4	2,90E+11	1,72E+06	5,9E-04
S	1	7,50E+12	2,28E+07	3,0E-04
S	2	1,00E+12	4,00E+06	4,0E-04
S	3	3,60E+10	3,60E+04	1,0E-04
S	4	3,04E+11	6,80E+05	2,2E-04
K	1	7,50E+12	2,26E+06	3,0E-05
K	2	1,00E+12	9,20E+05	9,2E-05
K	3	1,04E+11	1,18E+05	1,1E-04
K	4	3,39E+11	4,40E+07	1,3E-02
N <sub>1</sub>	1	7,50E+12	3,20E+06	4,3E-05
N <sub>1</sub>	2	1,00E+12	2,28E+05	2,3E-05
N <sub>1</sub>	3	5,60E+10	1,30E+05	2,3E-04
N <sub>1</sub>	4	2,36E+11	1,68E+08	7,1E-02
T	1	7,50E+12	1,26E+07	1,7E-04
T	2	1,40E+11	1,22E+04	8,7E-06
T	3	8,00E+10	2,40E+05	3,0E-04
E	1	7,50E+12	1,92E+06	2,6E-05
E	2	4,20E+11	8,00E+04	1,9E-05
E	3	4,40E+10	5,60E+07	1,3E-01
N <sub>2</sub>	1	7,50E+12	8,00E+05	1,1E-05
N <sub>2</sub>	2	3,60E+11	4,40E+05	1,2E-04
N <sub>2</sub>	3	8,20E+10	8,80E+04	1,1E-04
A	1	7,50E+12	1,32E+06	1,8E-05
A	2	2,80E+11	5,20E+05	1,9E-04
A	3	6,00E+10	6,60E+05	1,1E-03

## Appendix E

Clustering of Affibody molecules calculates dissimilarities by the formula:

$$L_p(X, Y) = \sum_{i=1}^{13} D(x_i, y_i)^p$$

There  $x_i$  and  $y_i$  denote the residues in the  $i$ :th variable position of sequences X and Y, respectively,  $p = 0.8$  and  $D(x, y)$  is the dissimilarity between the amino acids  $x$  and  $y$  as defined by Grantham's *amino acid dissimilarity matrix* (table F). Grantham matrix is based on the following formula for the difference between amino acids:

$$D_{ij} = \sqrt{\mathbf{a}(c_i - c_j)^2 + \mathbf{b}(p_i - p_j)^2 + \mathbf{g}(v_i - v_j)^2},$$

where  $c$ ,  $p$  and  $v$  refer to composition, polarity and molecular volume, respectively, and  $\mathbf{a}$ ,  $\mathbf{b}$  and  $\mathbf{g}$  are weighting factors. The program also computes cluster quality indexes by a Nearest-Exterior-Neighbor/Furthest-Interior-Neighbor cluster validation method.

**Table F. Grantham's amino acid dissimilarity matrix**

A	V	L	I	P	F	W	M	G	S	T	C	Y	N	Q	D	E	K	R	H
0	64	96	94	27	113	148	84	60	99	58	195	112	111	91	126	107	106	112	86
64	0	32	29	68	50	88	21	109	124	69	192	55	133	96	152	121	97	96	84
96	32	0	5	98	22	61	15	138	145	92	198	36	153	113	172	138	107	102	99
94	29	5	0	95	21	61	10	135	142	89	198	33	149	109	168	134	102	97	94
27	68	98	95	0	114	147	87	42	74	38	169	110	91	76	108	93	103	103	77
113	50	22	21	114	0	40	28	153	155	103	205	22	158	116	177	140	102	97	100
148	88	61	61	147	40	0	67	184	177	128	215	37	174	130	181	152	110	101	115
84	21	15	10	87	28	67	0	127	135	81	196	36	142	101	160	126	95	91	87
60	109	138	135	42	153	184	127	0	56	59	159	147	80	87	94	98	127	125	98
99	124	145	142	74	155	177	135	56	0	58	112	144	46	68	65	80	121	110	89
58	69	92	89	38	103	128	81	59	58	0	149	92	65	42	85	65	78	71	47
195	192	198	198	169	205	215	196	159	112	149	0	194	139	154	154	170	202	180	174
112	55	36	33	110	22	37	36	147	144	92	194	0	143	99	160	122	85	77	83
111	133	153	149	91	158	174	142	80	46	65	139	143	0	46	23	42	94	86	68
91	96	113	109	76	116	130	101	87	68	42	154	99	46	0	61	29	53	43	24
126	152	172	168	108	177	181	160	94	65	85	154	160	23	61	0	45	101	96	81
107	121	138	134	93	140	152	126	98	80	65	170	122	42	29	45	0	56	54	40
106	97	107	102	103	102	110	95	127	121	78	202	85	94	53	101	56	0	26	32
112	96	102	97	103	97	101	91	125	110	71	180	77	86	43	96	54	26	0	29
86	84	99	94	77	100	115	87	98	89	47	174	83	68	24	81	40	32	29	0