UPTEC X 07 014
Examensarbete 20 p
Januari 2007

Selection of CEA and VEGFR2 Binding Affibody® Molecules Using Phage Display

Sara Ahlgren

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 07 014	**Date of issue 2007-01**

Author

Sara Ahlgren

Title (English)

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Abstract

Tumours often overexpress surface molecules which can be used as targets for affinity ligands in molecular imaging or targeted therapy. Affibody® molecules are targeting proteins, that can be designed to bind specifically to almost any target. The aim of this study was to isolate Affibody® molecules, through phage display selections, that bind to specific targets, carcinoembryonic antigen (CEA) and vascular endothelial growth factor receptor 2 (VEGFR2), respectively. The possibility to favour alternative epitopes, by blocking VEGFR2 with Affibody® molecules derived from an earlier selection, was also investigated. A number of new candidate binders were identified and it was shown that it is possible to favour the selection of alternative Affibody® molecules through blockage of the target protein.

Keywords

Carcinoembryonic antigen, CEA, Vascular endothelial growth factor receptor 2, VEGFR2, Phage display, Affibody® molecule

Supervisors

Tove Eriksson, PhD
Affibody AB

Scientific reviewer

Mikael Widersten
Department of Biochemistry and Organic Chemistry, Uppsala University

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **62**

Biology Education Centre Biomedical Center Husargatan 3 Uppsala
Box 592 S-75124 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 555217

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Sara Ahlgren

Populärvetenskaplig sammanfattning

Tumörer överuttrycker ofta vissa ytproteiner. Dessa kan fungera som måltavlor för molekyler framtagna att binda specifikt till ett ytprotein. Denna typ av målsökning kan användas vid diagnostisering och behandling av tumörer. Vid diagnostisering får målsökande molekyler som är inmärkta med en ofarlig, diagnostisk radioaktiv molekyl cirkulera i kroppen. Inmärkta molekyler ansamlas vid tumörvävnad och genom imaging kan man med hög upplösning visualisera tumörer. Vid terapi kan samma molekyl istället märkas in med en terapeutisk substans.

Affibody® molekyler är en typ av affinitetsligander vars egenskaper lämpar sig utmärkt för målsökning. Specifika Affibody® molekyler selekteras fram med hjälp av phage display-teknik ur ett bibliotek innehållande $3{,}4 \times 10^9$ olika varianter.

I detta examensarbete var syftet att ta fram Affibody® molekyler mot två olika receptorer som överuttrycks på olika tumörcellers yta, CEA respektive VEGFR2. Dessutom studerades huruvida det är möjligt att främja selektion av alternativa, kanske mindre dominerande, Affibody® molekyler genom blockering av vissa ytor på målproteinet före selektionen.

Resultaten visar att ett antal nya kandidater har identifierats och att det är möjligt att främja selektion av alternativa Affibody® molekyler genom blockering av delar av målproteinet.

Examensarbete 20p
Civilingenjörsprogrammet Molekylär bioteknik
Uppsala universitet, januari 2007

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LIST OF ABBREVIATIONS

aa	Amino acid
ABD	Albumin binding domain
BSA	Bovine serum albumin
CEA	Carcinoembryonic antigen
cfu	Colony forming unit
ELISA	Enzyme-Linked Immunosorbent Assay
Fc	Constant fragment of IgG
HER2	Human epidermal growth factor 2
HSA	Human serum albumin
KDR	Kinase insert domain-containing receptor, other name for VEGFR2
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
mAb	Monoclonal antibody
MW	Molecular weight
OD_{600}	Optical density, measured at 600 nm
PBS	Phosphate buffered saline
PEG/NaCl	20% polyethylene glycol 6000 in 2.5 M NaCl
PBST	PBS with 0.1% Tween-20
RT	Room temperature
TPBSB-3%	3% BSA in PBS with 0.01% Tween-20
TPBSB-5%	5% BSA in PBS with 0.01% Tween-20
TBS	Tryptic soy broth
TYE	TSB with YE
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
YE	Yeast extract
Z_{wt}	Wild-type Z molecule

1 INTRODUCTION

To improve cancer treatments it is very important to be able to make accurate diagnoses with high resolution. This enables individualised, optimised and effective treatment for each patient, which thereby increase the possibilities for full recovery.

It is not uncommon for tumours to overexpress molecules on its cell surfaces. These receptors can be used as targets for affinity ligands in molecular imaging or targeted therapy.

1.1 Affibody AB

Affibody AB is a biotechnology company, whose main focus is on cancer imaging and targeted therapy products. Affibody AB uses innovative protein engineering technologies for the development of their targeting molecule, the Affibody® molecule, which can be designed to bind specifically to almost any target. The same Affibody® molecule, as used for medical imaging, can also be developed to function as a mediator of a therapeutical compound. Affibody AB was founded in 1998 by scientists of the Royal Institute of Technology and Karolinska Institutet. It is a privately held company that is located in Stockholm, Sweden.

1.2 Aim of the Study

The aim of this masters degree project was to isolate Affibody® molecules, through phage display selections, that bind to their specific target, carcinoembryonic antigen (CEA) and vascular endothelial growth factor receptor 2 (VEGFR2) respectively.

The possibility to favour alternative epitopes, by blockage of VEGFR2 with Affibody® molecules derived from an earlier selection, was also investigated. These existing Affibody® molecules contained the amino acid cysteine at certain positions, which led to dimerisation which was unwanted for this application.

2 THEORY AND BACKGROUND

2.1 Carcinoembryonic Antigen (CEA)

The human carcinoembryonic antigen (CEA) family, belonging to the immunoglobulin (Ig) superfamily, has been extensively studied. The genes can be divided into three groups, the CEA subgroup, the pregnancy specific glycoprotein group and a third group of pseudogenes [1]. It is a large trans-membrane glycoprotein with an apparent molecular weight (MW) of 180 kDa of which about 50% comprise carbohydrates [2, 3]. The nature of the CEA subfamily is very complex. Its large number of genes, alternative splicing and posttranslational modifications, such as glycosylation, makes it a complicated marker to study [4].

CEA is an important and well known tumour marker for a majority of adenocarcinomas including colon, breast and lung cancer [5]. Contrary to what its name suggests, CEA is not only expressed during fetal development, but also in normal adult tissue. The large intestine expresses CEA on the surface of the gut lumen [3]. It functions as a receptor for bacteria and viruses and is thought to play a major part in the innate immunity of the colon. The cells on which CEA is expressed on are either secreted, to the intestine, or absorbed by lymphatic tissue upon binding of pathogens [3, 6].

Normally, CEA is only expressed on the apical membrane of the intestinal epithelium, but in cancerous tissue the polarity of the cell is disrupted. The normal polarity of the cell is therefore lost and CEA is expressed around the whole cell surface. This leads not only to overexpression but also to exfoliation of CEA in to the blood and lymphatic system. Figure 1. The levels of CEA in serum have shown to be high in patients with CEA overexpressing carcinomas and the measurement of serum levels is an important way of determining the prognoses for patients at diagnosis as well as monitoring the progression of the disease after surgery [1].

In this study, where the application of an Affibody® molecule binding CEA would be for imaging or therapy it is the membrane bound CEA, consisting of seven domains, which is of interest. The N-terminal domain (N), that comprises 108 amino acids (aa), is homologous to the Ig variable domain but lacks a disulfide bridge. It is followed by six disulfide containing domains, homologous to the Ig constant domain, either being a type A domain that comprise 93 aa, or a type B domain that comprise 85 aa. CEA is anchored to the membrane via a glycosyl phosphatidyl inositol moiety (M). The domain formula for CEA is: N-A1-B1-A2-B2-A3-B3-M. Figure 1 [1].

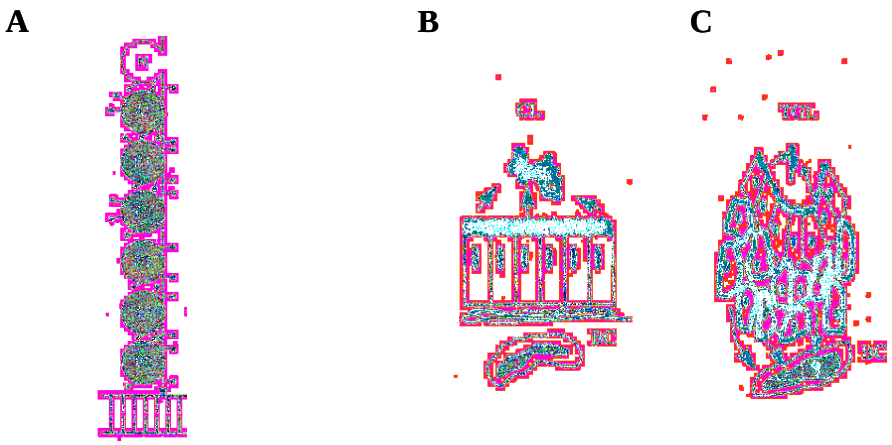


Figure 1. (A) Schematic overview of the CEA domains. (B) Normal cell architecture with CEA (shown as bold) expressed only on the apical membrane of the intestinal epithelium, and exfoliated to the colon lumen (CL). (C) Disrupted cell architecture in cancerous tissue. On cells not facing the tumour gland lumens (TGL), CEA is expressed around the whole cell and is exfoliated into the blood capillaries (BC) [1]. Illustrations used with permission from the author.

2.2 Vascular Endotelial Growth Factor Receptor 2 (VEGFR2)

Vascular endothelial growth factor receptor 2 (VEGFR2) also referred to as kinase insert domain-containing receptor, KDR, belongs to the class III subfamily of receptor tyrosine kinases. It contains seven immunoglobulin-like repeats, the extracellular domains which comprise 745 aa, and kinase insert domains in the intracellular regions consist of 567 aa.

The protein also has a small transmembrane domain of 25 aa. Figure 2. The MW of VEGFR2 is about 150kDa [7]. VEGFR2 is overexpressed on a large number of different tumour types such as colorectal, breast and cervical cancer as well as melanoma [8]. In adults, the expression of VEGFR2 is very low in normal tissue [9].

Compelling evidence suggests that vascular endothelial growth factor (VEGF) and its receptor VEGFR2 are involved in vascularisation, strongly associated with the growth of tumour tissue [10]. Angiogenesis is a critical factor in tumour development and metastasis, since oxygen and nutrients are supplied through blood vessels. It has been shown that molecular blocking of VEGFR2 inhibited a number of critical mechanisms involved in angiogenesis, for example cellular proliferation, cellular migration, cellular differentiation and the formation of capillary networks [11].

Inhibition of VEGFR2 has also shown to increase the response to ionising radiation, resulting in reduced tumour growth in preclinical studies with human tumour xenografts. Even though it is a well known phenomenon that tissue suffering from oxygen deficiency, due to diminished blood supply, shows reduced sensitivity to radiation there are results from studies on VEGFR2 that suggest the opposite [11].

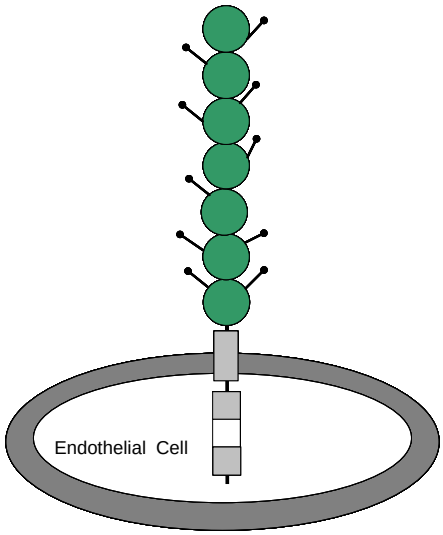


Figure 2. Schematic overview of the structure of glycoprotein VEGFR2 expressed on the surface of a endothelial cell. VEGFR2 is composed of a 745 aa extracellular domain (comprising seven immunoglobulin-like repeats) followed by a 25 aa transmembrane domain and a 567 aa cytoplasmic domain. Lollipops represent glycosylation sites.

2.3 Affibody® Molecules

Affibody® molecules are small and robust affinity proteins derived from the *Staphylococcus aureus* surface protein A. The IgG binding domain Z, consisting of 58 amino acids that form a three-helix bundle with a molecular weight of 6 kDa, was used as a scaffold [12]. Randomisation of 13 selected aa displayed on the surface of Z resulted in a large combinatorial library, where all members have identical backbones but variable surface binding properties. The randomized positions are all on helix one and two, leaving helix three identical for all members in the library, see Figure 3. Due to their great variability, Affibody® molecules can be designed to bind almost any target protein. Even though they have a relatively small size compared to antibodies (~ 150 kDa), the binding area is similar in size to an antibody-antigen interaction [13, 14].

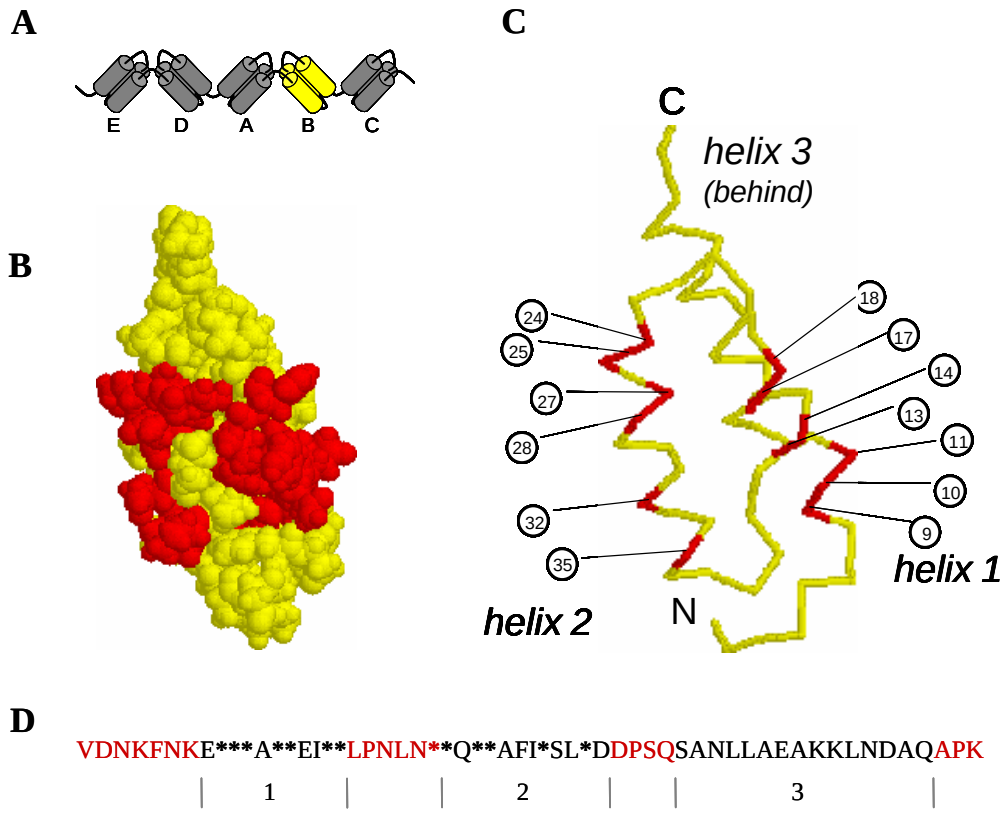


Figure 3. (A) IgG-binding domains of Protein A. Certain modifications were performed on domain B in order to create the Z domain. (B) Z domain with variable residues marked in red. (C) Z domain with variable amino acid positions marked as circled numbers. (D) Amino acid sequence of Affibody® molecule with stars indicating variable positions. Loops are marked in red and helices in black.

Affibody® molecules have many properties which give them advantages over antibodies. A number of them are listed below [15].

- small size (6 kDa, 58 aa)
- robust physical properties (pH, temperature, proteases)
- structure without S-S bonds
- flexibility of constructs (multimers, gene fusions)
- fast and cost-effective production in bacteria (easily expressed, secretable)
- possible to produce through peptide synthesis
- high specificity and low unspecific binding

The low molecular weight of Affibody® molecules gives them excellent properties for targeting tumours through their rapid clearance from the bloodstream, favourable biodistribution and tumour penetration.

2.4 Tumour Targeting

An Affibody® molecule used for targeting of tumours can be applied in a number of different ways. Commonly, antibodies are used for targeting as well as two antibody derived fragments, single-chain variable fragment (scFv) and Fab.

Commercially there are two monoclonal antibodies against CEA A3B3 available. CEA-Scan (a ^{99m}Tc-labeled murin Fab) used for imaging and CEA-Cide (a ^{90}Y-labeled humanised mAb) used for therapy [16, 17]. There are a number of radiolabled anti-CEA monoclonal antibodies in clinical trials. A common usage of these antibodies is prior to therapy to facilitate the dose calculations of the therapeutic nuclide or to monitor the treatment process [18, 19]. Another method, tested in clinical trials, is radioimmunoguided surgery (RIGS) where a ^{125}I-labeled antibody is given before or during surgery and a hand-held gamma-detecting probe is used to locate tumour tissue in the operative field [20-22]. Affibody® molecules have properties that could be useful for such an application. There are also a large number of monoclonal antibodies against different epitopes on CEA used as research tools [4, 23].

If the receptor is used in a signalling pathway, as VEGFR2, it can be used to inhibit the natural ligand and thereby prevent vascularisation of tumour tissue [9]. There are two different molecules in clinical trials. A synthetic small molecule, that inhibits the tyrosine kinase domain of VEGFR2, SU5416 (semaxanib), is in phase II trials [24]. Another potential product is a fibronectin derived molecule, Angiocept™, in phase I clinical trials [25].

Imaging

Medical imaging can be used for diagnosis and localisation of tumours and metastasis. Indium-111 (^{111}In), Gallium-68 (^{68}Ga) and Iodine-125 (^{125}I) are examples of nuclides suitable for imaging. Affibody® molecules labelled with radionuclides can accumulate in tumour tissue and be detected using, for example, SPECT (Single Photon Emission Computerised Tomography) or PET (Positron Emission Tomography) [27]. In an imaging application it is favourable to use a molecule that does not have an intrinsic biological activity, i.e. not binding the active site or inhibit binding of the natural ligand to it.

An Affibody® molecule that bind human epidermal growth factor receptor 2 (HER2) has already shown to be excellent for imaging applications. HER2 is overexpressed on 25-30% of all breast cancers, and these are the most serious cases. Z_{HER2} have been clinically tested on breast cancer patients with great success [26].

Therapy

Another potential use for an Affibody® molecule binding specifically to a chosen target is tumour therapy. The Affibody® molecule is used to mediate a pharmaceutical agent to, or into, a receptor expressing cell. This enables selectivity in the destruction of the cells and would thereby allow lower doses and lead to less side effects than conventional therapy, for example external radiation [27].

β-emitting radionuclides suitable for this purpose is Lutetium-177 (^{177}Lu), Yttrium-90 (^{90}Y) and Iodine-131 (^{131}I) [28]. ^{90}Y labelled anti-CEA monoclonal antibodies have been tested in phase I clinical trials on patients with metastatic CEA-producing cancer. Prior to treatment the patients were imaged using the same antibody labelled with ^{111}In [18]. ^{131}I-labelled anti-CEA antibodies have also been tested in phase I/II clinical trials on patients with medullary thyroid cancer [29]. A third possible application is monitoring of the tumour development or treatment progress.

2.5 Selection by Phage Display

Phage display is a bio-panning method that is used to isolate molecules with binding specificity to a specific target protein through a number of selection cycles.

Phage display

The Bacteriophage

A bacteriophage is a virus that can infect bacteria and use them as a host for their replication. The particle's approximate length is 1 µm with a diameter of 6.5 nm. Display of foreign proteins or peptides on the phage surface is possible through a genetic fusion to one of its coat proteins [30]. Exposure to the target makes it available for selection. After selection, the selected phage are amplified by letting them infect bacteria and thereafter reproduce. The bacteriophage used in this study is an Ff class filamentous M13 phage that can only infect bacteria with an F-pilus [31].

Display Formats

There are a number of different display systems used in phage display. They are all based on the use of two different phage coat proteins pIII and pVIII. pIII is expressed in three to five copies at one end of the phage and can express a fused foreign protein larger than six aa. pVIII covers the whole phage surface with about 2700 copies but can only express a fused foreign peptide with a maximum of 6 aa. Therefore, only the pIII protein is possible to use for the display of Affibody® molecules. The selection system used at Affibody AB is the 3+3 type, where the gene coding for the inserted DNA is fused to the gene of the bacteriophage surface protein pIII. This is the same protein that attaches to the tip of the bacterial F-pilus and thereby causes infection [32]. Figure 4 shows different display formats used.

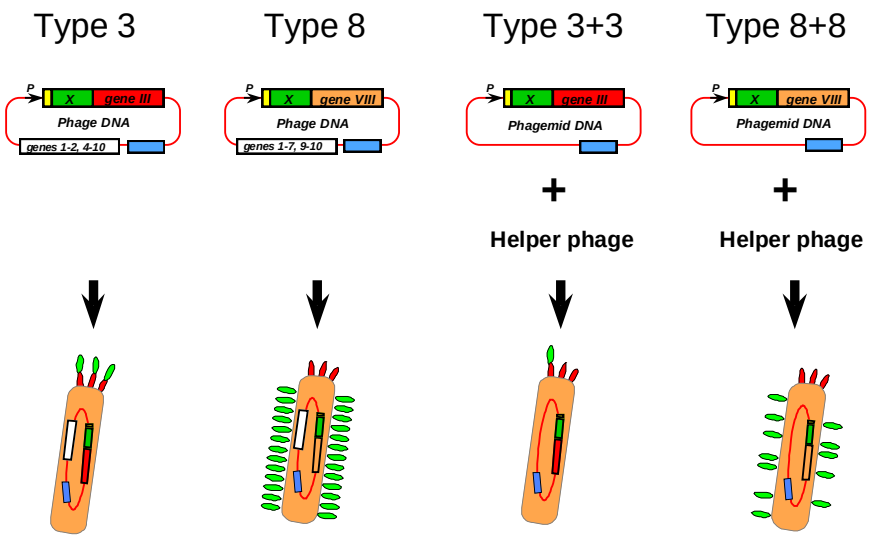


Figure 4. Overview of display formats. Type 3: Foreign gene fused to gene III. The vector also contains genes for other coat proteins. Type 8: Foreign gene fused to gene VIII. The vector also contains genes for other coat proteins. Type 3+3: The display format used at Affibody AB. Foreign gene fused to gene III but the phagemid does not contain genes for other coat proteins. Co-infection with helper phage is necessary for phage production. Type 8+8: Foreign gene fused to gene VIII but the phagemid does not contain genes for other coat proteins. Co-infection with helper phage is necessary for phage production.

The Phagemid

A phagemid is a plasmid that in addition to its *E. coli* origin of replication (ori) contains a phage-derived ori. It contains a gene cassette coding for a foreign peptide fused to the gene for a surface protein, for example pIII, but lacks the genes coding for the machinery of capsid production. A gene coding for antibiotic resistance is also included as a selection marker. The phagemid can, unlike plasmids, be packed into a phage coat and thereby infect bacteria. After infection the phagemid can replicate along with the bacteria but not produce its own capsid. This feature is used to control the amplification of the infecting phage. For phage propagation, the bacteria must be co-infected with wild-type M13 helper phage. The helper phage can contain another antibiotic resistance gene

enabling selection of bacteria that contains the genomes for both the phagemid and the helper phage.[31]

The phagemid used at Affibody AB is called pAffiI. Figure 5 and Appendix 1. The different domains of pIII are encoded partly in the phagemid, aa 249-406, and partly in the helper phage, M13K07. In co-infected bacteria the domains for pIII can be used in combination to propagate the phage particles [33]. The phagemid, pAffiI, consists of the genes for a Z-protein flanked by an upstream signal peptide, OmpA, and a downstream albumin binding domain, ABD (a 5 kDa albumin binding domain from streptococcal protein G). This gene cassette is fused to the pIII protein gene. The phage library currently used at Affibody AB, Zlib2002, contains $3.4x10^9$ different Affibody® molecules [34].

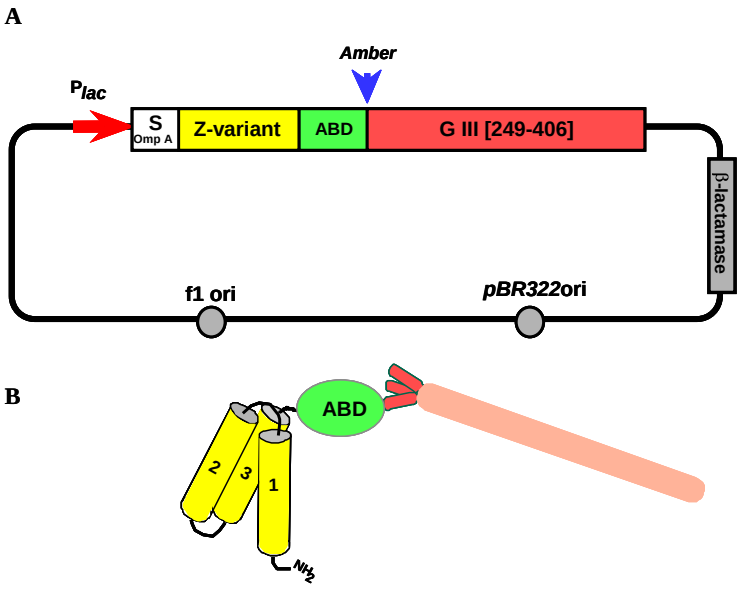


Figure 5. (A) Schematic overview of the pAffiI phagemid. (B) Phage M13 with a displayed Affibody® molecule, connected via ABD.

Selection Phases

There are basically two different approaches used in phage display selections, selection on solid phase or selection in solution. In the solid phase selections, the target protein is attached to a surface, for example the inside of a polystyrene tube or a streptavidin coated magnetic bead. The linkage can be either direct or via an intermediate molecule with favourable binding properties. As an example biotinylated target can bind to streptavidin coated beads. For selection in solution, the target protein is unbound during the selection which enables the phage to bind in a more natural way. At the end of the selection the target protein and bound phage are captured by the addition of magnetic beads coated in a suitable way to bind the target protein. The different selection phases both have their advantages and drawbacks but depending on the desired binding properties for the selected binder, selection on solid phase or in solution is preferred. If high specificity but low affinity is demanded, the solid phase is beneficial due to the possibility of more multivalent bindings. When selecting for high affinity selection in solution is preferred since affinity discrimination is optimised [31].

A Selection Cycle

To avoid selection for affinity to other components present during the selection (for example plastic, streptavidin or BSA) the phage library can be pre-incubated in tubes or solutions that contain these components. After this pre-selection the number of background binders are reduced. The incubation can be repeated in order to pre-select against more than one component [31].

In the selection, the target protein and phage library are exposed to each other allowing phage expressing a peptide with target binding properties to bind the target. When the selection is finished, unbound phage and phage bound loosely are removed by washing several times. The washing conditions are continuously toughened throughout the cycles. Selected phage are eluted using for example low pH (as used in this study) which disrupt the interaction. Eluted phage are amplified by infection of log phase *E. coli* and thereafter expressed through co-infection with helper phage. A new phage stock is prepared by

precipitation and suspension of the expressed phage. After three to five selection cycles phage clones are picked for further analysis [12, 31]. Figure 6.

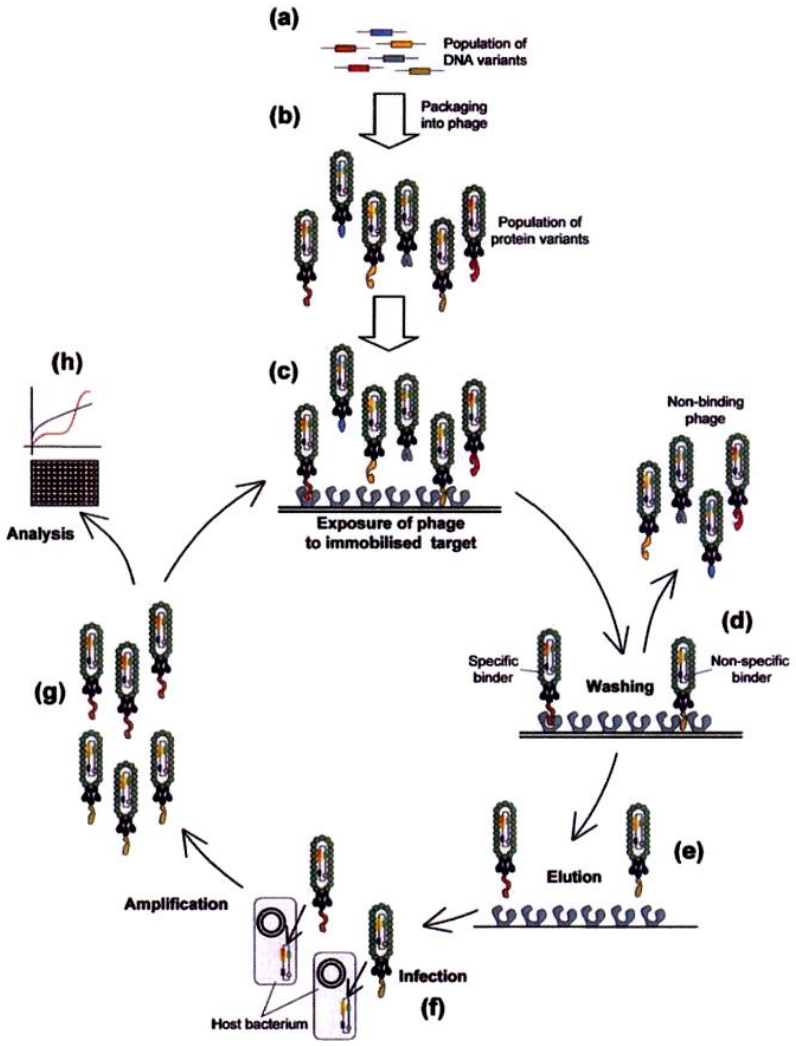


Figure 6. The phage display cycle. (a) A library of variant DNA sequences encoding peptides or proteins is created and (b) cloned into phage or phagemid genomes as fusions to a coat protein gene. (c) The phage library displaying variant peptides or proteins is exposed to target molecules and phage with appropriate specificity are captured. (d) Non binding phage are washed off – although some non-specific binding may occur. (e) Bound phage are eluted by conditions that disrupt the interaction between the displayed peptide or protein and the target. (f) Eluted phage are infected into host bacterial cells and thereby amplified. (g) This amplified phage population is in effect a secondary library that is greatly enriched in phage displaying the peptides or proteins that bind the target. If the bio-panning steps (c) to (f) are repeated, the phage population becomes less and less diverse as the population becomes more and more enriched in the limited number of variants with binding capacity. (h) After several (usually three to five) rounds of bio-panning monoclonal phage populations may be selected and analysed individually. [35] Illustration used with permission from the author. Please note that this is a description of solid phase selection.

3 MATERIALS AND METHODS

3.1 Selection of Affibody® Molecules by Phage Display

Target proteins

CEA A3B3

In the selection against CEA the A3-B3 domains of the seven domain protein was used as a target. The construct was flanked by an upstream FLAG tag and a downstream c-myc tag followed by a 6-His tag. It also contained both an N-terminal 9 aa residue from a removed signal peptide and a number of spacers of 4 aa in between all the tags and the CEA A3B3 peptide. Figure 7. An overview of the construct can be found in Appendix 2. The construct had a theoretical MW of about 24 kDa, which can be compared to the full length MW of 180 kDa. It was produced in house and expressed in mammalian HEK 293 cells [34].

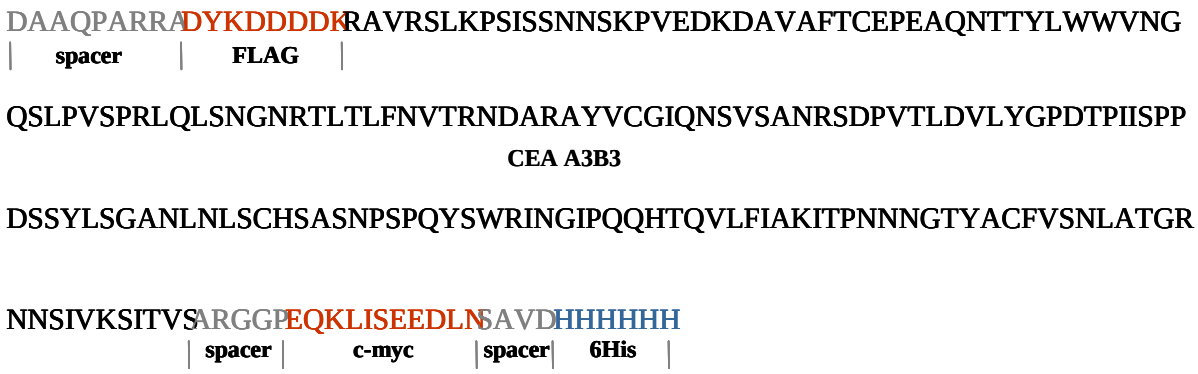


Figure 7. Amino acid sequence of, the in-house produced, CEA A3B3 construct with flanking tags and spacers. The spacer upstream of the FLAG tag is from a removed signal peptide.

VEGFR2-Fc

VEGFR2 used in the selections was expressed in a mouse myeloma cell line, MS0, and was purchased from R&D systems. This VEGFR2 was a homodimer of the extracellular domain of human VEGFR2, consisting of 764 aa, fused with a 6-His tagged Fc of human IgG_1. Each monomer contained 988 aa and had a predicted MW of 110 kDa. As result of glycosylation the dimer had a MW of approximately 330 kDa [36].

Selection Strategy

Affibody® molecules specific for CEA A3B3 and VEGFR2 respectively were selected from the combinatorial phage library, Zlib2002, in four panning cycles.

For CEA A3B3 solid phase selection was used and for VEGFR2 three different selections was performed, one using solid phase and the other two using selection in solution. In one of the selections in solution, VEGFR2 was pre-blocked with two different cysteine containing Affibody® molecules derived from a previous selection on the same target [34]. Figure 8 shows a schematic overview of the different strategies used in the selections.

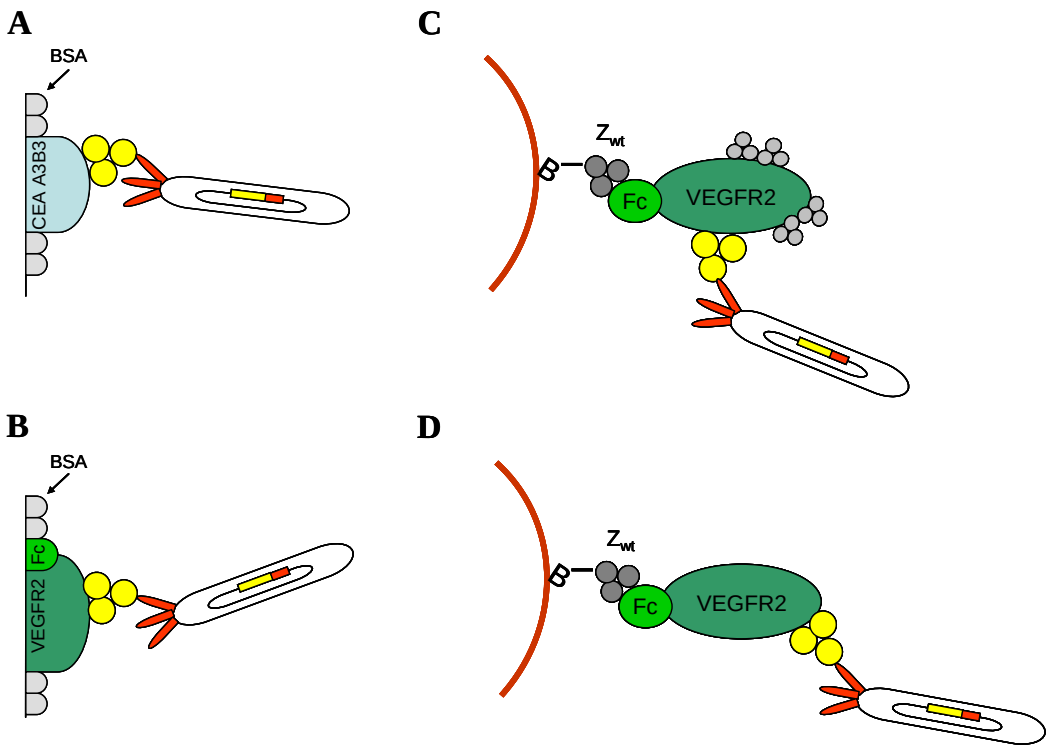


Figure 8. Schematic overview of selection set-ups. (A) Solid phase selection. Affibody® molecule expressing phage bound to CEA A3B3, coated on an immuno tube. (B) Solid phase selection. Affibody® molecule expressing phage bound to VEGFR2-Fc coated on an immuno tube. (C) Selection in solution. Affibody® molecule expressing phage bound to blocked VEGFR2-Fc. The phage/target-complex was captured at the end of selection by Z_{wt} linked by biotin to a streptavidin coated paramagnetic bead. (D) Selection in solution. Affibody® molecule expressing phage bound to VEGFR2-Fc. Capturing as described in (C) above.

At the start of the third cycle, selection A and B were each split into two different variants in order to allow different selection parameters during the last two cycles. Selection C was divided into two variants at the start of cycle four. Figure 9

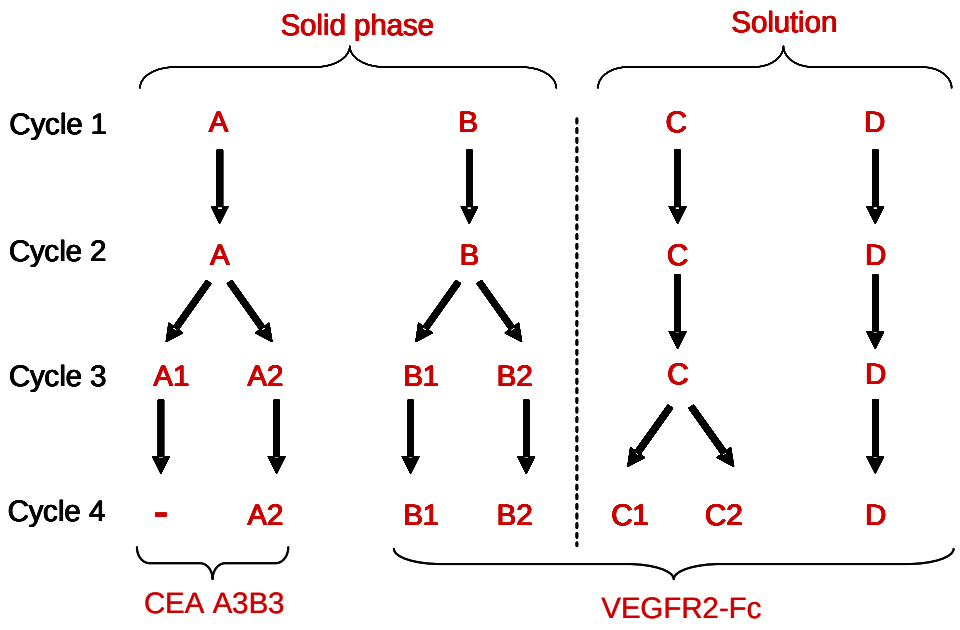


Figure 9. Overview of the selections. Selection A and B were split after cycle 2. Selection C was split after cycle 3. Selection A1 ended after cycle 3 due to low phage yield.

Phage Library

Prior to selections, the phage library Zlib2002 was precipitated using 1/5 volume of 20% polyethylene glycol 6000 (Merck, Hohenbrunn, Germany) in 2.5 M NaCl (PEG/NaCl) and incubated on ice for 1 h. The phage were pelleted at 10700 x g for 30 min and resuspended in PBS-T (0.1%) (PBS with 0.1% Tween-20) + 0.1% gelatine. The precipitation was made to remove any free Affibody® molecules from the phage library.

The phage library was also titrated to determine the concentration. This was made by infecting log phase RR1ΔM13 *E. coli* cells with serial dilutions of the phage library for 5 min at RT and spread onto TYE plates containing 100µg/ml ampicillin and incubating overnight at 37°C.

Coating

Prior To Solid Phase Selection

Coating of proteins to the surface of Nunc-Immuno Tubes, MaxiSorp (Nunc, Roskilde Denmark) used for selection was made with the proteins diluted in ELISA carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) in a total volume of 3.5 ml. The tubes were incubated for > 2 h at RT followed by blocking of unoccupied surfaces using TPBSB-buffer 5% (PBS with 5% BSA, 0.1% Tweeen-20 and 0.02% Na-azid) for > 2 h at RT or overnight at + 4°C. Tubes with proteins for pre-selections were coated as described above except for incubation which was performed end-over-end. Protein concentrations used can be seen in Table 1.

Prior To Selection In Solution

Streptavidin coated paramagnetic beads (Dynabeads® M-280 Streptavidin, Dynal Biotech ASA, Oslo, Norway), pre-washed in PBS-T (0.1%), were coated with biotinylated dimers of wild-type Fc binding Z molecules ($(Z_{wt})_2$ + biotin) by incubation for 30 min end-over-end at RT in a total volume of 1 ml. Unbound $(Z_{wt})_2$ + biotin was removed by washing with PBS-T (0.1%). Unoccupied bead surfaces were blocked with PBS-T (0.1%) + 0.1% gelatine 30 min end-over-end at RT. The fraction of beads to be used for pre-selection against Fc was coated with polyclonal human IgG-Fc (Jackson Immuno Research, West Grove, PA, USA) by incubation for 1 h end-over-end at RT. Unbound Fc was removed through washing with PBS-T (0.1%). Concentrations used can be seen in Table 1.

Pre-selection

Phage stocks used for selection in cycle one through three were pre-incubated according to Table 1 in order to remove phage binding other components used in the selections. In the solid phase selection the phage stocks were diluted in TPBSB-buffer 3% (PBS with 3% BSA, 0.1% Tween-20 and 0.02% Na-azid) to a total volume of 3 ml and incubated in BSA-coated immuno tubes end-over-end 30-60 min. Phage in selection B were diluted in

PBS-T (0.1%) + 0.1% gelatine to a total volume of 0.5 ml and incubated with Z_{wt}-Fc-coated beads, for 30-60 min, before transferring the phage stock solutions to the selection tubes. The pre-selection for the selection in solution was performed in tubes blocked with PBS-T (0.1%) + 0.1% gelatine. Beads and non-binding phage were separated before transferring the phage stock solution to the selection tubes.

Blocking of VEGFR2 with Affibody® molecules

In selection C, VEGFR2-Fc was blocked with 100x molar excess each of two different Affibody® molecules, Z01755 and Z01763, derived from a previous selection on the same target. Both these were monomer Affibody® molecules with C-terminal ABD-fusions.[34] The mixture was incubated for > 1 h at RT or + 4°C.

Selection

The selection was performed using two different strategies for the selection, in solution and solid phase respectively. An overview of the selection parameters can be seen in Table 1.

Table 1. Overview of phage display selection parameters

Selection Cycle	Selection id.	Selection phase	Protein	Pre-selection*	Protein conc. (nM)	Protein amount (µg)**	Selection temperature (ºC)	Number of washes with 3% TPBSB	Number of washes with PBS-T(%)
Cycle 1	A	Solid	CEA A3B3	BSA	–	10	4	1	1x (0.1%)
	B	Solid	VEGFR2	BSA+Fc	–	50	RT	1	1x (0.1%)
	C	Solution	VEGFR2 blocked	Fc	100	–	RT	–	2x (0.1%)
	D	Solution	VEGFR2	Fc	100	–	RT	–	2x (0.1%)
Cycle 2	A	Solid	CEA A3B3	BSA	–	5	4	2	1x (0.2%)
	B	Solid	VEGFR2	BSA+Fc	–	25	4	2	1x (0.2%)
	C	Solution	VEGFR2 blocked	Fc	50	–	4	–	3x (0.2%)
	D	Solution	VEGFR2	Fc	50	–	4	–	3x (0.2%)
Cycle 3	A1	Solid	CEA A3B3	BSA	–	1	37	3	4x (0.3%)
	A2	Solid	CEA A3B3	BSA	–	2.5	37	3	4x (0.3%)
	B1	Solid	VEGFR2	BSA+Fc	–	2.5	37	3	4x (0.3%)
	B2	Solid	VEGFR2	BSA+Fc	–	12.5	37	3	4x (0.3%)
	C	Solution	VEGFR2 blocked	Fc	25	–	37	–	7x (0.3%)
	D	Solution	VEGFR2	Fc	25	–	37	–	7x (0.3%)
Cycle 4	A1***	Solid	CEA A3B3	–	–	–	–	–	–
	A2	Solid	CEA A3B3	–	–	2.5	37	3	4x (0.3%)
	B1	Solid	VEGFR2	–	–	0.5	37	3	7x (0.4%)
	B2	Solid	VEGFR2	–	–	12.5	37	3	7x (0.4%)
	C1	Solution	VEGFR2 blocked	–	1	–	37	–	10x (0.4%)
	C2	Solution	VEGFR2 blocked	–	5	–	37	–	10x (0.4%)
	D	Solution	VEGFR2	–	5	–	37	–	10x (0.4%)

* The pre-selection against Fc was in practice against both Fc and streptavidin beads.

** Maximum theoretical amount

*** Selection A1 cancelled after cycle tree

Solid Phase Selection

Phage stocks diluted in TPBSB-buffer 3% (PBS with 3% BSA, 0.1% Tween-20 and 0.02% Na-azid) to a total volume of 3 ml were incubated in target coated tubes end-over-end for (cycle 1: selection A: over night at +4°C, selection B: 2h at RT, cycle 2: overnight at + 4°C, cycle 3-4: 2h at 37°C). Unbound phage were discarded and the immuno tubes washed according to Table 1 with 3.5 ml TPBSB-buffer 3% and 3.5 ml PBST of varying Tween-20 concentrations by inverting. Bound phage were eluted with

2 ml 50 mM Glycine-HCl (pH 2.1), at RT for 10 min followed by immediate neutralization with 1.6 ml PBS and 400 µl 1 M Tris-HCl (pH 8.0).

Selection In Solution

Target protein and phage stocks diluted in PBS-T (0.1%) + 0.1% gelatine to a total volume of 1 ml were incubated end-over-end for (cycle 1: 2h at RT, cycle 2: overnight at + 4°C, cycle 3-4: 2h at 37°C) in tubes pre-blocked with PBS-T (0.1%) + 0.1% gelatine. After selection Z_{wt}-coated beads were added for capturing of phage/VEGFR2-Fc complexes for 15 min. Unbound phage were discarded and beads washed as described in Table 1 with 1 ml TPBSB-buffer 3% by inverting. Bound phage were eluted with 500 µl 50 mM Glycine-HCl (pH 2.1) at RT for 10 min followed by immediate neutralization with 450 µl PBS and 50 µl 1 M Tris-HCl (pH 8.0).

Infection of *E. coli* and Phage Titration

After each round of panning, a major part (i.e. 3 ml for selection A and B or 950 µl for selection C and D) of the eluted phage was used to infect early log phase RR1ΔM15 *E. coli* cells (50 ml for cycle 1 or 25 ml for cycle 2-4) with phage for 20 min at 37°C without shaking. Infected cells were centrifuged at 3300 x g for 10 min, the cell pellet resuspended in a smaller volume and plated on to TYE (tryptic yeast extract, Merck) plates with 100 µg/ml ampicillin and incubated overnight at 37°C. Eluted phage were also titrated after each cycle as described in the paragraph 'Phage Library' above.

Preparation of Phage Stocks

Cells from plates were harvested in TBS (tryptic soy broth 30 g/l, Merck, Darmstadt, Germany) medium and cell concentrations determined using optical density measurements at 600 nm assuming that $OD_{600} = 1$ corresponds to $5x10^8$ cfu (colony forming unit)/ml. A fraction of the suspended cells, aiming to be a 1000 fold excess as compared to eluted phage from the previous selection round, were inoculated to a limiting $OD_{600} < 0.1$ in a maximum of 500 ml of TSB medium supplemented with

2% glucose and 100 µg/ml ampicillin. Inoculation excess ratios and culture volumes can be seen in Table 2. The cultures were grown to log phase.

Table 2. Overview of inoculation excess ratios and cultivation volumes for preparation of phage stocks.

Selection Cycle	Selection id.	Protein	Inoculation excess as compared to eluted phage from previous round of selection	Cultivation volume (ml)
Cycle 1	A	CEA A3B3	25 x	500
	B	VEGFR2	20 x	500
	C	VEGFR2 blocked	60 x	500
	D	VEGFR2	35 x	500
Cycle 2	A	CEA A3B3	$1x10^3$ x	65
	B	VEGFR2	$1x10^3$ x	200
	C	VEGFR2 blocked	$1x10^3$ x	20
	D	VEGFR2	$1x10^3$ x	20
Cycle 3	A1	CEA A3B3	$1x10^3$ x	10
	A2	CEA A3B3	$1x10^3$ x	10
	B1	VEGFR2	$1x10^3$ x	10
	B2	VEGFR2	$1x10^3$ x	10
	C	VEGFR2 blocked	$1x10^3$ x	10
	D	VEGFR2	$1x10^3$ x	10

The harvested cells were also used for preparing glycerol stocks from each round by addition of glycerol to a final glycerol concentration of 15%.

A fraction of the culture, corresponding to the same cell number as used for inoculation, was infected with 10 x excess plaque forming units (pfu) of helper phage M13K07 (New England Biolabs, Ipswich, USA) for 30 min at 37°C without shaking. The infected cells were pelleted at 3300 x g for 10 min, resuspended in the original cultivation volume of TSB+YE medium supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and grown overnight at 100 rpm at 27°C.

The overnight cultures were centrifuged at 2500 x g for 10 min and the phage in the supernatant were precipitated using 1/5 volume of PEG/NaCl and incubated on ice for

1 h. The phage were pelleted at 10500 x g for 30 min and resuspended in 10 ml PBS followed by a second precipitation with a 1/5 volume of PEG/NaCl and incubation on ice for 45 min. After a second centrifugation at 10500 x g for 30 min the phage pellet was resuspended in 2 ml of the selection buffer. Cell debris was removed by filtering through a Minisart 0.45µm filter (Sartorius, Goettingen, Germany) and thereafter centrifuged at 13000 x g for 2 min. The concentration of the phage stocks was determined by titration as described in the selection.

3.2 Characterisation of Affibody® Molecules

ELISA-screening

After the fourth round of panning, Affibody® molecules from individual clones were expressed and used for target binding screening using an ELISA. Colonies grown on TYE-plates were randomly picked and inoculated in 1 ml of TSB+YE medium supplemented with 100 µg/ml ampicillin and 0.1 mM IPTG in 96-well deep-well plates (Nunc, Roskilde, Denmark) and grown on a shaker overnight at 37°C.

The cells in the deep well plates for the ELISA-screening were pelleted at 3000 x g for 10 min and resuspended in 400 µl PBS-T (0.05%). The plates were frozen in -80°C for > 1 h to lyse the cells and release the Affibody® molecules. After thawing, the plates were centrifuged at 3700 for > 20 min and the supernatant, containing Affibody® molecules, was used in the ELISA.

A replica of the 96-well deep-well plate was inoculated in 400 µl TSB medium with 100 µg/ml ampicillin using a 96 pin deep well Multi-Blot Replicator (V&P Scientific Inc., San Diego, USA). This plate was grown overnight on a shaking board at 37°C before 50% glycerol was added to a final concentration of 15%. The replica plates were stored in -20°C.

Two different ELISA strategies were used, see Figure 10. Regarding clones derived from the solid phase selections, Corning Costar half area ELISA plates (Corning Incorporated Life Sciences, Acton, USA) were coated with target protein, using 0.15 µg CEA A3B3 or 0.25 µg VEGFR2 in 50 µl 50mM Na_2CO_3 (pH 8.6) per well, through incubation overnight at +4°C. The wells were blocked using 0.5% Casein (Sigma-Aldrich, Steinheim, Germany) in PBS-T (0.05%) (blocking buffer) on a shaker for 1.5 h at RT. Affibody® molecules in the supernatant, prepared as described above, were added by a volume of 50 µl to each well and incubated on a shaker for 1.5 h at RT. Polyclonal rabbit anti-Z-ABD IgG (Turbo, Affibody AB, Bromma, Sweden), diluted 1:1000 in PBS-T (0.05%), was added to each well in a volume of 50 µl and incubated on a shaker for 1.5 h at RT. Polyclonal goat anti-rabbit IgG conjugated with horseradish peroxidise (HRP) (Dako, Glostrup, Denmark), diluted 1:10000 in PBS-T (0.05%), was added using a volume of 50 µl/well as a secondary reagent and incubated for 1 h at RT. The wells were washed with four times with PBS-T (0.05%) before addition of each new reagent. ImmunoPure TMB developing solution (Pierce, Rockford, USA) was added by a volume of 50 µl to each well and incubated for 30 min before 50 µl of 2M H_2SO_4 was added to stop the development process. Absorbance was measured at 450 nm (A_{450}) in ELISA spectrophotometer Tecan Ultra 384 (Tecan Group Ltd., Männedorf, Switzerland) using the software Magellan. Two wells were used as negative controls. One was un-coated, no Z added, no rabbit anti-Z-ABD IgG added, detected with goat anti-rabbit IgG-HRP. In the other control, no target protein was coated, random Z molecule was added and the well analyzed as regular clones. Due to high background signal in this set-up, each clone was measured on a parallel ELISA plate where no target protein was coated but otherwise identical. The absorbance values from each well on these plates were subtracted from the absorbance values measured above.

The second ELISA set-up was used to analyse clones derived from the selections in solution. Half area ELISA plates were coated with 50 µl HSA of a concentration of 6 µg/ml through incubation overnight at +4 C. Blocking and incubation with Affibody® molecules as above. As primary reagent 50 µl VEGFR2-Fc with a

concentration of 4 µg/ml was used and incubated on a shaker at RT for 1.5h. A polyclonal donkey anti-human IgG-Fcγ-HRP (Jackson Immunoresearch, West Grove, PA, USA) diluted 1:20000 in PBS-T (0.05%) was used as a secondary reagent and incubated for 1.5h at RT. The wells were washed, developed and analysed as described above. Three controls, one negative, one positive and one blank, were used. All controls were coated and blocked as the rest of the plate. In the negative control $Z_{insulin}$-ABD (1 µg/ml) was used and detected as the rest of the plate. In the positive control $Z_{insulin}$-ABD (1 µg/ml) was used and detected with biotinylated insulin (10 µg/ml) followed by streptavidin conjugated with HRP (DakoCytomation, Glostrup, Denmark) diluted 1:5000 in PBS-T (0.05%). The blank control was incubated without Z molecule and detected as the rest of the plate.

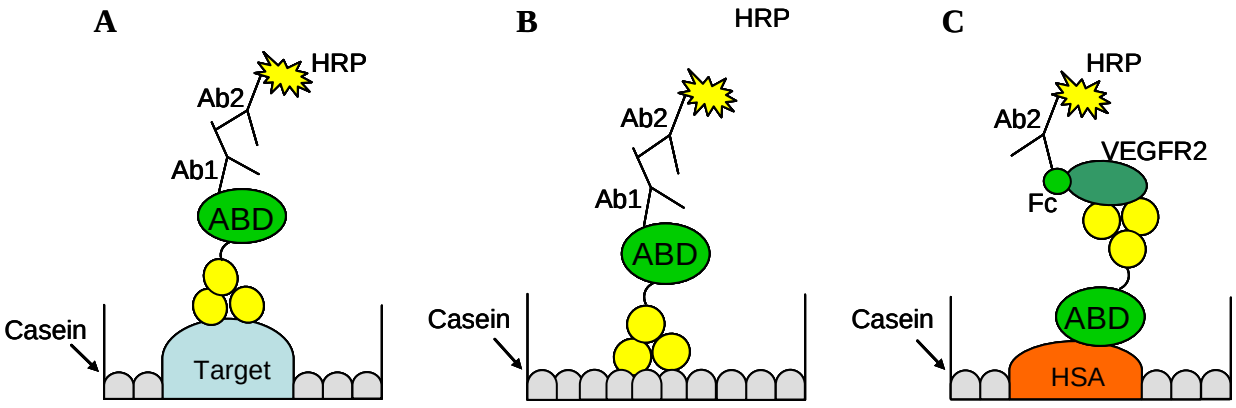


Figure 10. (A) ELISA set up for solid phase selection. (B) Control plate set-up for solid phase selection. Unspecific binding, as in figure, generates background absorbance. (C) ELISA set-up for the selection in solution.

Sequencing

Clones, with an ELISA absorbance value above a certain threshold were chosen for DNA sequencing.

Cells from the clone plates were used to amplify the gene fragment encoding the Affibody® molecule and its ABD fusion. The PCR-amplification was performed on a Mastercycler ep (Eppendorf, Hamburg, Germany) using primers specific for the pAffiI

vector and AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The cycling program consisted of an initial denaturing step (96°C for 10 min) followed by 30 cycles of amplification (96°C for 15 sec, 55°C for 30 sec, 72 C for 90sec) and a final extension step (72 C for 7 min). The primer sequences can be seen in Appendix 3. PCR products were verified using a 96-well 2% agarose E-gel® (Invitrogen) following the manufacturer's instructions.

Clones were sequenced using an ABI PRISM®3100 Genetic analyser (Applied Biosystems, Foster City, USA). Sequence PCR was performed using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using biotinylated primers specific for the pAffiI vector. Primer sequences can be found in Appendix 4. Prior to sequencing, the biotinylated sequence products were purified using the magnetic bead purification system Magnatrix 8000 (Magnetic Biosolutions, Stockholm, Sweden) with streptavidin coated Dynabeads® M-280. Sequences were analysed using an in-house developed analysis software, resulting in identification of new Affibody® molecules.

Clustering

The DNA sequences of identified Affibody® molecules were clustered to show degree of similarity. In short, the analysis was performed the following way. For each Affibody® molecule, the three "nearest neighbors" (i.e. the three least dissimilar molecules) were compared with a library consisting of confirmed streptavidin/plastic binders. The dissimilarities with the nearest neighbors were taken into consideration. The Affibody® molecules derived from the selections against VEGFR2-Fc were also clustered together with the Affibody® molecules derived from the previous selection against VEGFR2-Fc.[34]

DotBlot

Presumptive, sequence verified, binders were chosen for target binding specificity analysis with DotBlot.

Affibody® molecules were expressed by inoculation of clones in 20 ml TSB+YE supplemented with 100 µg/ml ampicillin and 0.1 mM IPTG grown on a shaker overnight at 37°C. Cultures were centrifuged at 3000 x g for 10 min and the pellet resuspended in 6 ml PBS. The cells were frozen in -80°C for > 1 h to lyse the cells and release the Affibody® molecules. After thawing the cell debris was removed by centrifugation at 3500 x g for 15 min.

For each Affibody® molecule to be analysed, a nitrocellulose membrane was prepared. Target protein together with a number of common human serum proteins were placed in individual dots, each containing 0.1 µg, and adsorbed onto the membrane. An additional dot of 0.1 µg full length CEA (Human, native, USBiological, MA, USA) was adsorbed onto the membranes for analysis of CEA A3B3 binding Affibody® molecules. CEA A3B3 was dotted onto individual smaller membranes, with 5 times 2 µl dotted on the same spot, since there was an unknown concentration on the protein solution. The CEA A3B3 membranes were incubated in same way as the other membrane for each binder.

An overview of the standard human serum proteins used for specificity control and their positions can be found in Figure 11.

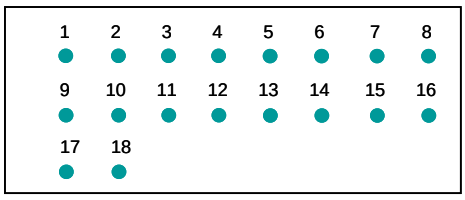


Figure 11. Standard human serum proteins used as control. HSA was used as a positive control, binding the ABD fused to the Z-molecules.

Free surface on the membranes was blocked with PBS-T (0.1%) + 0.05% casein on a shaker at RT for >1 h. The membranes were incubated overnight at +4°C with

periplasmic fraction, containing Affibody® molecules, followed by a 4 x 4-5 min wash with PBS-T (0.1%). Thereafter, the membranes were incubated on a shaking board at RT for 1h in two different antibody solutions each followed by a 4 x 4-5 min wash with PBS-T (0.1%). The first were primary antibody, polyclonal rabbit α-Z-ABD IgG (1:10000 in PBS-T(0.1%)) followed by a secondary antibody, polyclonal goat α-rabbit IgG-HRP (1:10000 in PBS-T(0.1%)).

The signals from the membranes were detected with chemiluminiscense using about 1.5 ml Supersignal® West Dura Extended Duration Substrate (1:1, Pierce, Rockford, USA) per membrane. Chemiluminiscense was registred using a ChemiImager™ (Apha Innotech Corporation, San Leandro, USA) and its software ChemiImager™ 5500. Exposure times were individualised and varied between 1 and 2 min.

Human serum albumin (HSA) was used as a positive control since it binds to the ABD fusion of the Affibody® molecule. As a negative control a vector identical to the pAffiI vector but not encoding an Affibody® molecule was used. Appendix 5.

4 RESULTS

4.1 Selection of Affibody® molecules by phage display

The phage yield was calculated as percentage of eluted phage of the total input of phage used in the selection rounds ((phage out/phage in) x 100). An overview of the selection parameters and numerical results can be seen in Table 3. Figure 12 shows a graphical overview of the phage enrichment during the selection cycles.

Table 3. Variables, titration values and enrichment factors for each round of selection. For selection A-D, please refer to Table 1.

Selection Cycle	Selection id.	Selection phase	Protein	Protein conc. (nM)	Protein amount (µg)*	Number of washes	Phage in (cfu)	Phage out (cfu)	Enrichment (%)
Cycle 1	A	Solid	CEA A3B3	–	10	2	$5.0x10^{12}$	$3.2x10^{8}$	$6.4x10^{-3}$
	B	Solid	VEGFR2	–	50	2	$5.0x10^{12}$	$4.8x10^{9}$	$9.6x10^{-2}$
	C	Solution	VEGFR2 blocked	100	–	2	$5.0x10^{12}$	$4.0x10^{8}$	$8.0x10^{-3}$
	D	Solution	VEGFR2	100	–	2	$5.0x10^{12}$	$6.8x10^{8}$	$1.4x10^{-2}$
Cycle 2	A	Solid	CEA A3B3	–	5	3	$1.0x10^{12}$	$1.28x10^{7}$	$1.3x10^{-3}$
	B	Solid	VEGFR2	–	25	3	$1.0x10^{12}$	$4.8x10^{6}$	$4.8x10^{-4}$
	C	Solution	VEGFR2 blocked	50	–	3	$1.0x10^{12}$	$2.0x10^{5}$	$2.0x10^{-5}$
	D	Solution	VEGFR2	50	–	3	$1.0x10^{12}$	$1.2x10^{6}$	$1.2x10^{-4}$
Cycle 3	A1	Solid	CEA A3B3	–	1	7	$3.2x10^{10}$	–	–
	A2	Solid	CEA A3B3	–	2.5	7	$3.2x10^{10}$	$1.6x10^{4}$	$5.0x10^{-5}$
	B1	Solid	VEGFR2	–	2.5	7	$1.2x10^{10}$	$8.0x10^{4}$	$6.7x10^{-4}$
	B2	Solid	VEGFR2	–	12.5	7	$1.2x10^{10}$	$4.8x10^{5}$	$4.0x10^{-3}$
	C	Solution	VEGFR2 blocked	25	–	7	$2.0x10^{9}$	$2.4x10^{5}$	$1.2x10^{-2}$
	D	Solution	VEGFR2	25	–	7	1.2x1010	$1.6x10^{5}$	$1.3x10^{-3}$
Cycle 4	A1**	Solid	CEA A3B3	–	–	–	–	–	–
	A2	Solid	CEA A3B3	–	2.5	7	$4.0x10^{7}$	$1.6x10^{3}$	$4.0x10^{-3}$
	B1	Solid	VEGFR2	–	0.5	10	$2.0x10^{9}$	$8.0x10^{3}$	$4.0x10^{-4}$
	B2	Solid	VEGFR2	–	12.5	10	$1.2x10^{9}$	$4.8x10^{4}$	$4.0x10^{-3}$
	C1	Solution	VEGFR2 blocked	1	–	10	$2.4x10^{9}$	$2.4x10^{5}$	$1.0x10^{-2}$
	C2	Solution	VEGFR2 blocked	5	–	10	$2.4x10^{9}$	$2.4x10^{5}$	$1.0x10^{-2}$
	D	Solution	VEGFR2	5	–	10	$1.6x10^{9}$	$1.6x10^{5}$	$1.0x10^{-2}$

* Maximum theoretical amount

** Selection A1 cancelled after cycle tree

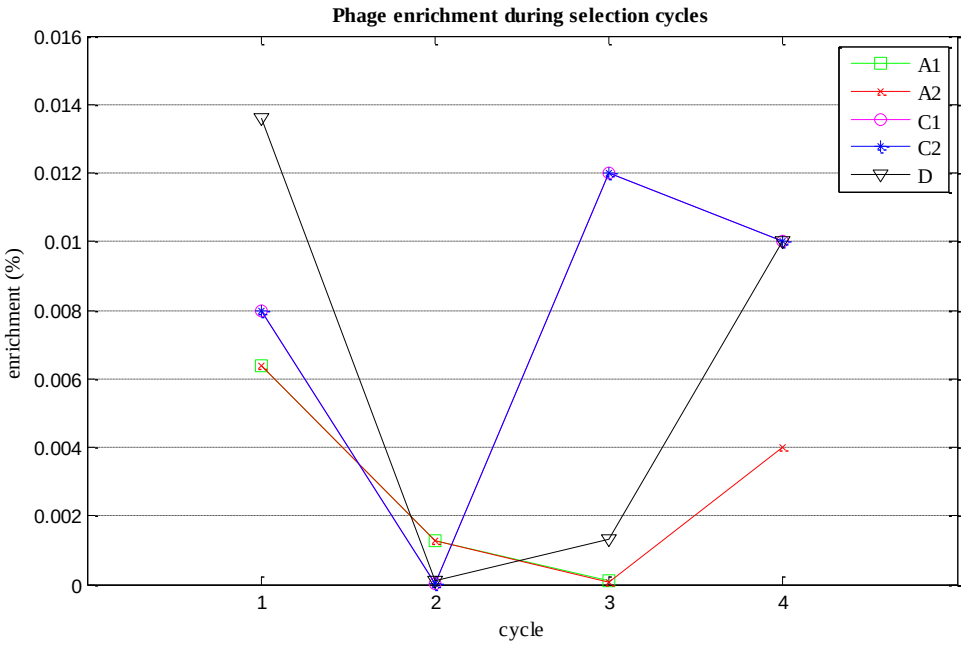


Figure 12. Graphical illustration of the phage enrichment during the selection cycles. Selections B1 and B2 were not included due to outlier values.

Selection A1 was cancelled after cycle three due to low phage yield. This allowed selection C to be divided into two variants at the start of cycle four. The number of washing steps was also modified during the selection, i.e. fewer washes than originally planned were performed when the page yield was lower than expected.

4.2 Characterisation of Affibody® molecules

ELISA screening

Clones from the selections were randomly picked for analysis of their binding activity to CEA A3B3 or VEGFR2-Fc, respectively, using an ELISA. From the CEA A3B3 selection (A2) 93 clones were screened and from the VEGFR2-Fc selections a total number of 435 clones (63 clones from selection B1 and 93 clones each from selection

B2-D) were screened. The clones were tested using two different ELISA set-ups, depending on which selection phase they were derived from.

Clones from the solid phase selection were analysed using a set-up known to yield high background signals. Therefore each clone was run on two parallel ELISA plates. One plate coated with target protein and the other one not coated. Absorbance values (A_{450}) from wells without target protein was compared with the absorbance values measured on the corresponding coated wells. The absorbance measured on the non-coated wells was plotted next to the absorbance values from the corresponding coated wells. Clones with an absorbance value of at least two times the background signals, and two times the absorbance value of the negative control were regarded as positive.

Clones from the selections in solution were tested in a different ELISA set-up which did not, to the same extent, suffer from high background signal problems. Therefore the parallel plate set-up was not applied. Clones with an absorbance value of at least two times the background signals were regarded as positive. ELISA absorbance values can be seen in Figure 13-18. Controls used in the ELISA are described in the individual figure texts.

Positive clones, with an absorbance value above a certain threshold, individual for each plate, were chosen for sequencing.

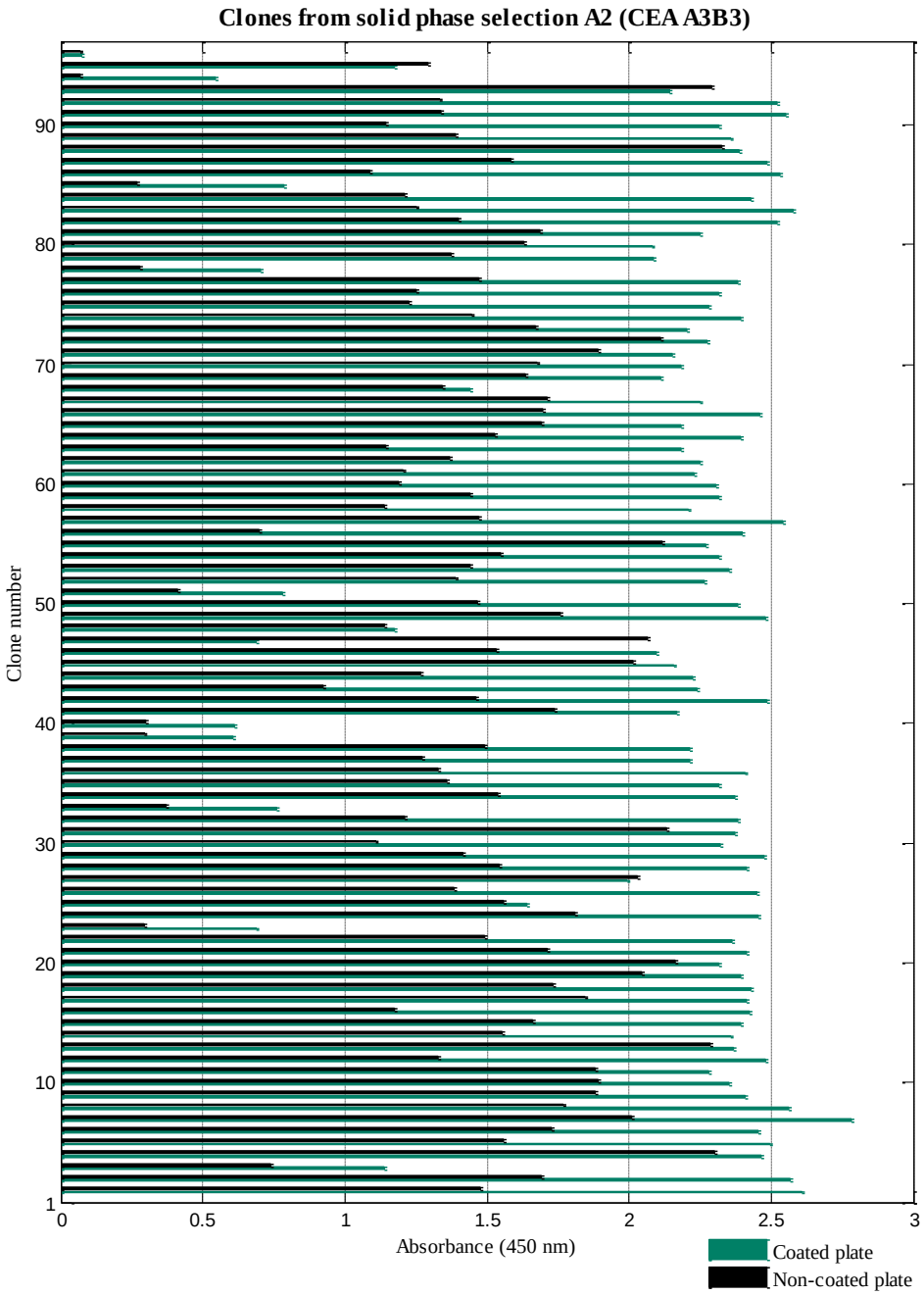


Figure 13. ELISA screening of clones expressing Affibody® molecules with CEA A3B3-binding activity. Positions 1-93 represent randomly picked clones, where each clone was analysed both on a CEA A3B3 coated plate and a plate coated without CEA A3B3; position 94-95 represent negative controls, position 94: coated with CEA A3B3, no Z, no rabbit α-Z-ABD IgG (PBS-T), detected with goat α-rabbit IgG-HRP (PBS-T); position 95: coated without CEA A3B3, random Z, analyzed as for position 1-93.

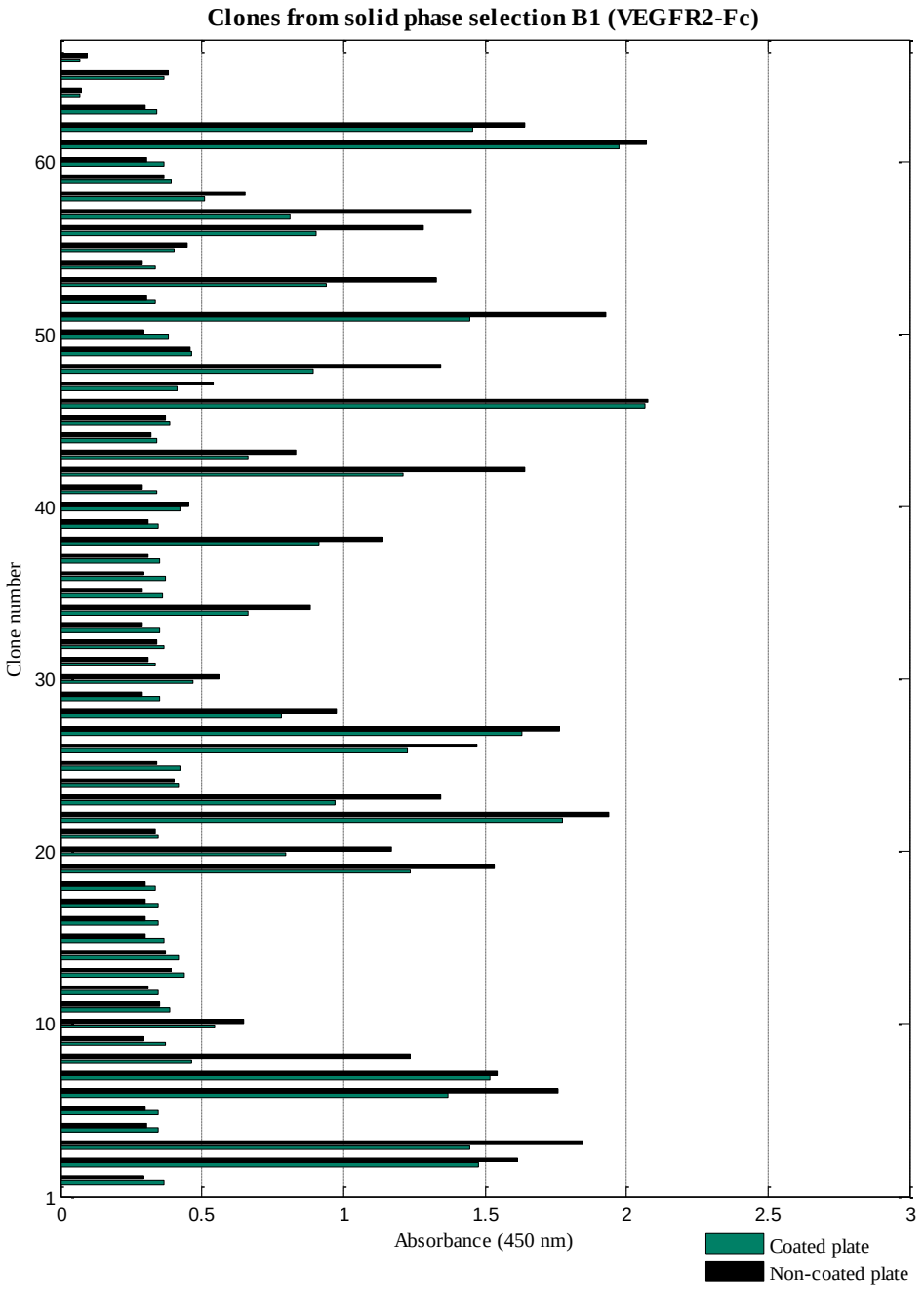


Figure 14. ELISA screening of clones expressing Affibody® molecules with VEGFR2-binding activity. Positions 1-63 represent randomly picked clones, where each clone was analysed both on a VEGFR2 coated plate and a plate coated without VEGFR2; position 64-65 represent negative controls, position 64: coated with VEGFR2, no Z, no rabbit α-Z-ABD IgG (PBS-T), detected with goat α-rabbit IgG-HRP (PBS-T); position 65: coated without VEGFR2, random Z, analyzed as for position 1-63.

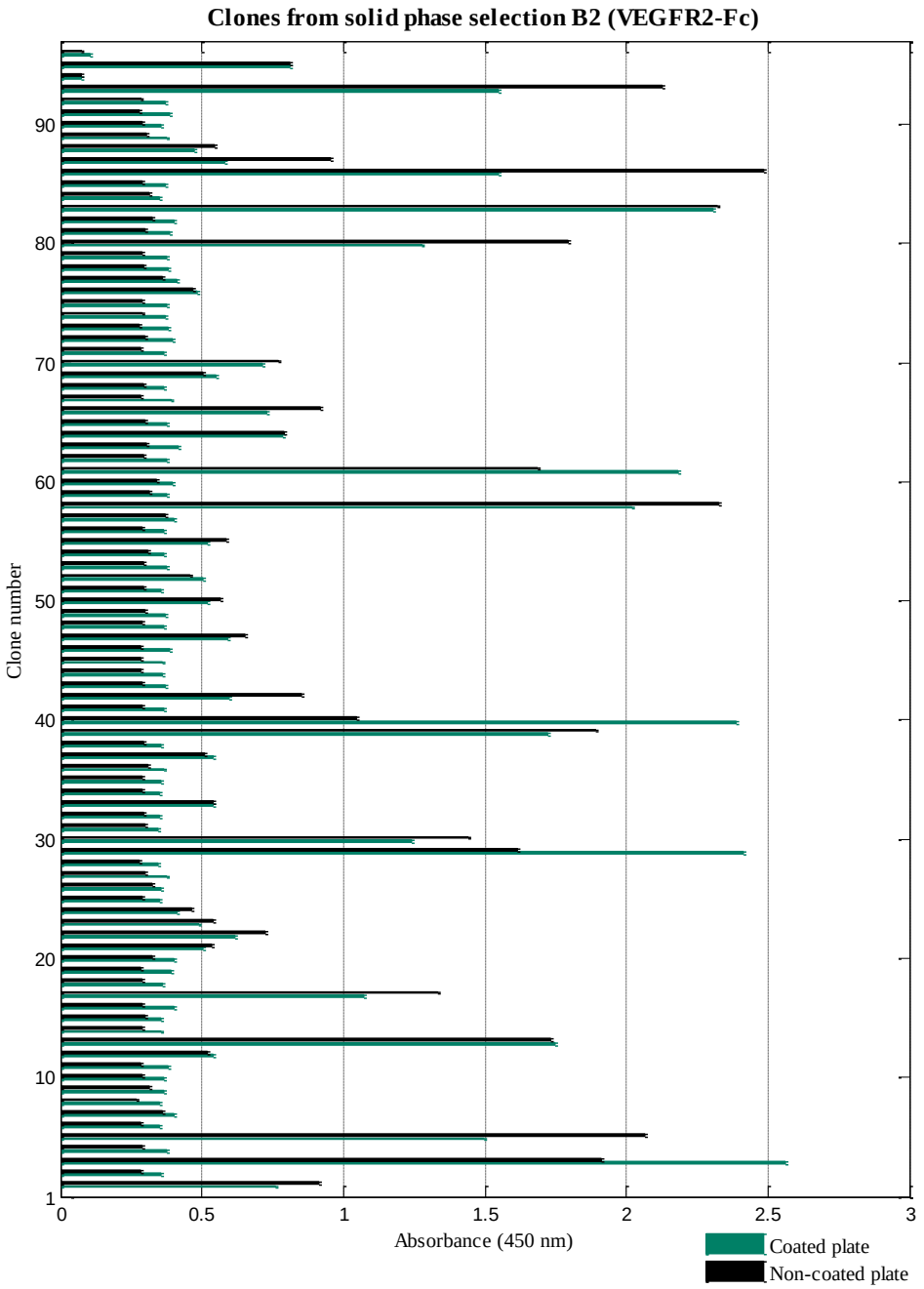


Figure 15. ELISA screening of clones expressing Affibody® molecules with VEGFR2-binding activity. Positions 1-93 represent randomly picked clones, where each clone was analysed both on a VEGFR2 coated plate and a plate coated without VEGFR2; position 94-95 represent negative controls, position 94: coated with VEGFR2, no Z, no rabbit α-Z-ABD IgG (PBS-T), detected with goat α-rabbit IgG-HRP (PBS-T); position 95: coated without VEGFR2, random Z, analyzed as for position 1-93.

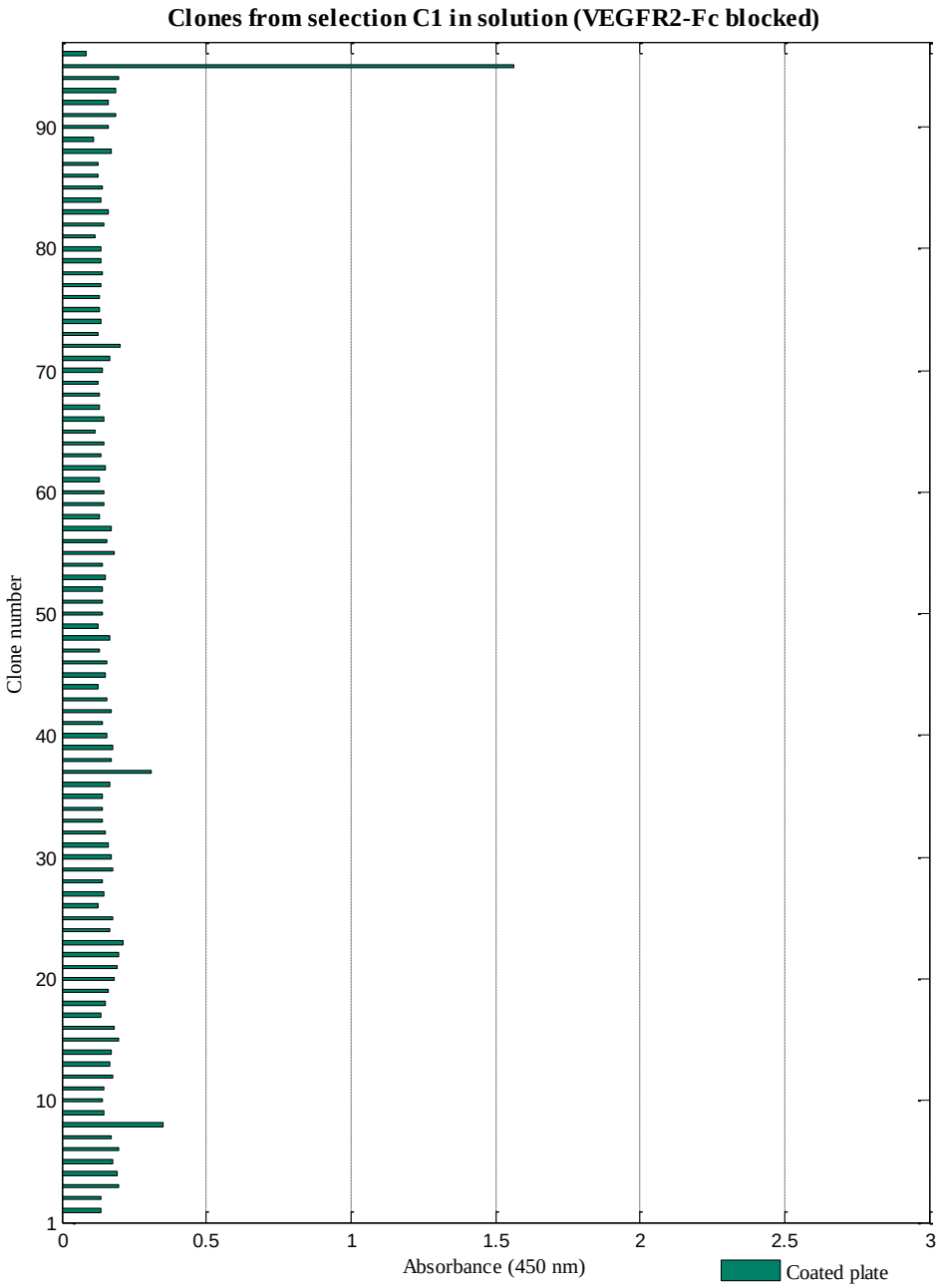


Figure 16. ELISA screening of clones expressing Affibody® molecules with VEGFR2-binding activity. Positions 1-93 represent randomly picked clones, where each clone was analysed both on a VEGFR2 coated plate and a plate coated without VEGFR2; position 94 represents a negative control: coated with HSA, $Z_{insulin}$-ABD (double concentration), otherwise like random clones; position 95 represents a positive control: coated with HSA, $Z_{insulin}$-ABD (double concentration), biotinylated insulin (double concentration), detected with streptavidin-HRP; position 96 represents a blank control: coated with HSA, no Z, detected with donkey α-human IgG Fcγ-HRP.

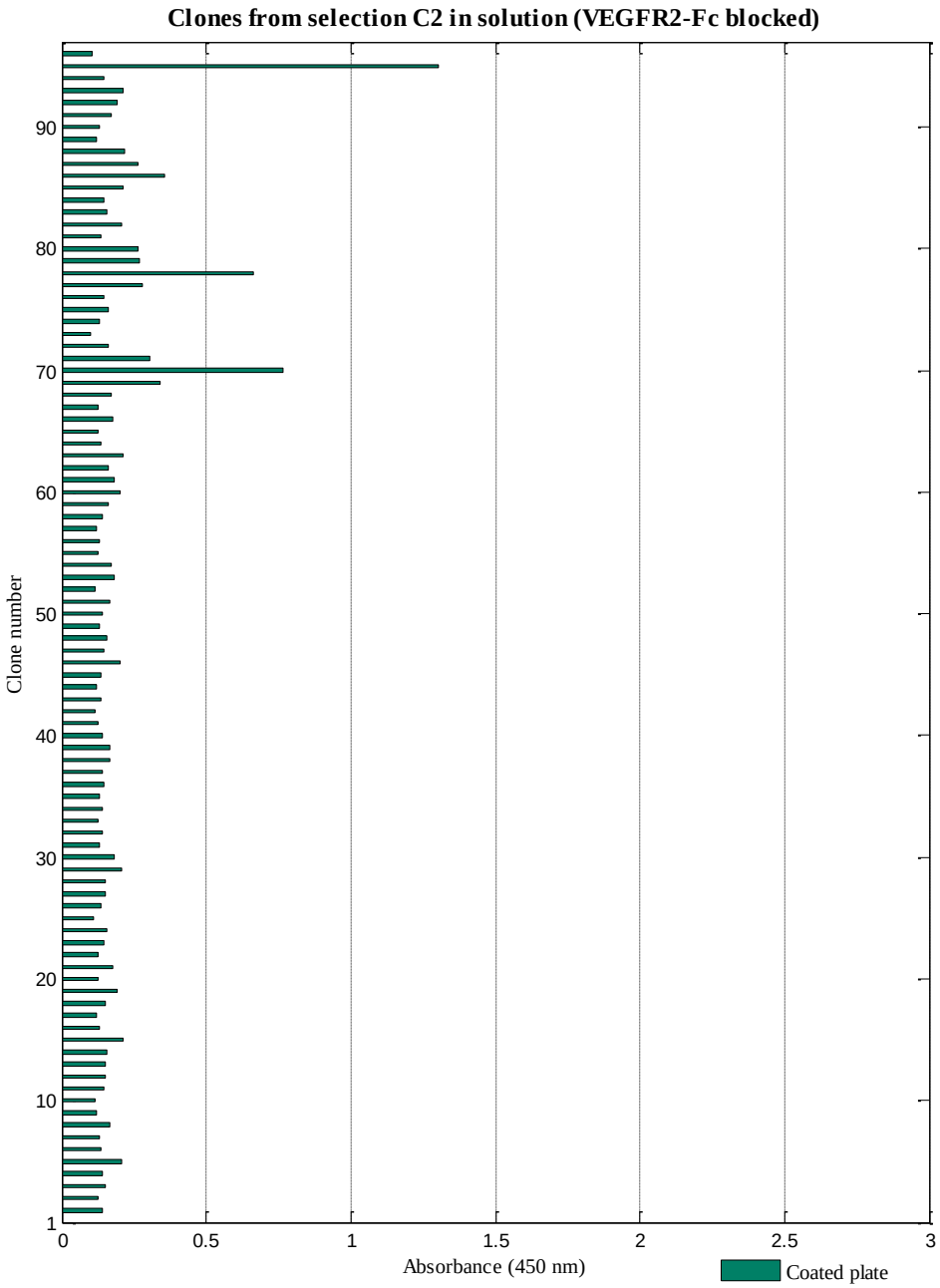


Figure 17. ELISA screening of clones expressing Affibody® molecules with VEGFR2-binding activity. Positions 1-93 represents randomly picked clones, where each clone was analysed both on a VEGFR2 coated plate and a plate coated without VEGFR2; position 94 represents a negative control: coated with HSA, $Z_{insulin}$-ABD (double concentration), otherwise like random clones; position 95 represents a positive control: coated with HSA, $Z_{insulin}$-ABD (double concentration), biotinylated insulin (double concentration), detected with streptavidin-HRP; position 96 represents a blank control: coated with HSA, no Z, detected with donkey α-human IgG Fcγ-HRP

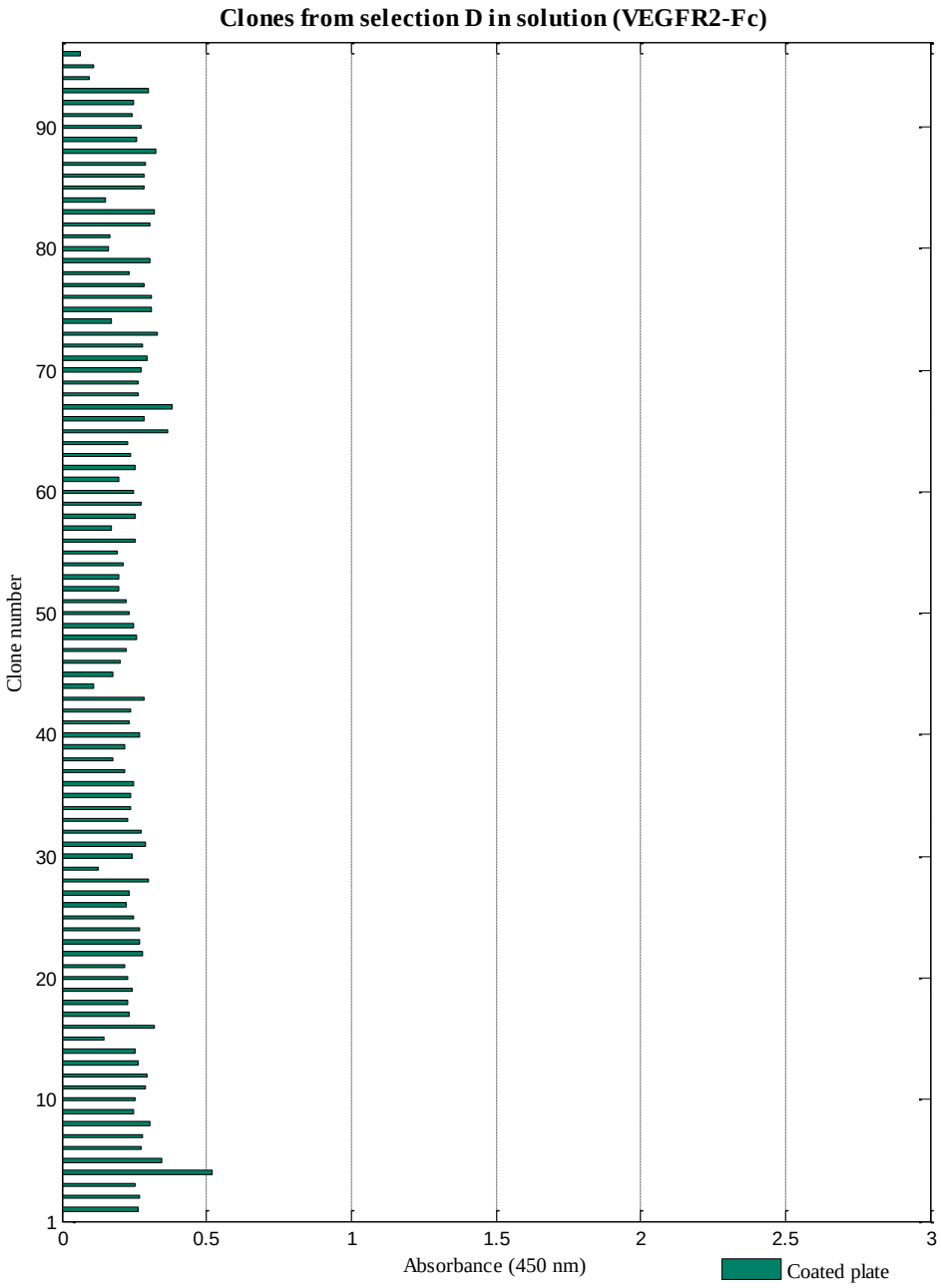


Figure 18. ELISA screening of clones expressing Affibody® molecules with VEGFR2-binding activity. Positions 1-93 represent randomly picked clones, where each clone was analysed both on a VEGFR2 coated plate and a plate coated without VEGFR2; position 94 represents a negative control: coated with HSA, $Z_{insulin}$-ABD, otherwise like random clones; position 95 represents a positive control: coated with HSA, $Z_{insulin}$-ABD, biotinylated insulin, detected with streptavidin-HRP; position 96 represents a blank control: coated with HSA, no Z, detected with donkey α-human IgG Fcγ-HRP.

Sequencing

From the CEA A3B3 selection (A2) 49 clones were chosen for sequencing and from the VEGFR2 selections a total number of 138 clones (7 clones from selection B1, 39 clones from selection B2, 24 clones from selection C1, 18 clones from selection C2, 50 clones from selection D) were chosen for sequencing. After sequencing the clones with complete sequence were identified. Complete and unique sequences for these selections were given Affibody® molecule identification numbers, Z-numbers, and sequences known from previous selections were identified with their original Z-number. A number of the identified Affibody® molecules were found in more than one clone. Identical clones were grouped. Table 4 shows an overview of the identified Affibody® molecules.

After sequencing there was a rather large group of 42 clones from the selection on CEA A3B3 which had been completely sequenced but had a modification in the constant region. Therefore, they were not identified as proper Affibody® molecules. After further investigation it was shown that all these clones were identical and contained an amino acid substitution in one of the constant parts of helix two due to a single base substitution on DNA level, see Figure 19. Since the clone did not contain the amino acid cysteine and had such a dominant presence among the clones from the selection it was given a Z-number, Z02594, and was joined for clustering.

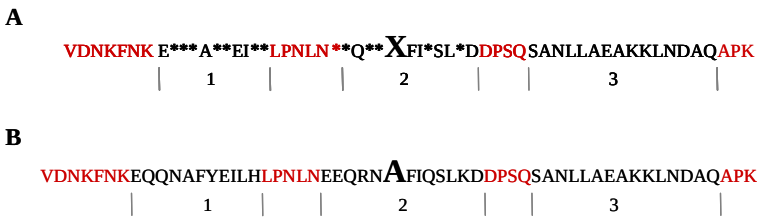


Figure 19. Comparison of (A) the mutated Affibody® molecule, Z02594, and (B) Z_{wt}. The enlarged X in (A) represent the substituted amino acid and the enlarged A shows the corresponding to alanine in the proper backbone.

Table 4. Overview of the identified Affibody® molecules.

Affibody® molecule id.	Target protein	Cysteine containing	Number of clones identified from each selection						
			A2	B1	B2	C1	C2	D	total
Z02327	CEA A3B3	no	1	–	–	–	–	–	1
Z02328	CEA A3B3	no	1	–	–	–	–	–	1
Z02594	CEA A3B3	no	42	–	–	–	–	–	42
Z01100*	VEGFR2-Fc	no	–	–	6	–	–	–	6
Z02320	VEGFR2-Fc	yes	–	–	–	12	1	–	13
Z02321	VEGFR2-Fc	no	–	–	–	1	3	–	4
Z02322	VEGFR2-Fc	no	–	–	–	1	1	–	2
Z02323	VEGFR2-Fc	yes	–	–	–	2	1	–	3
Z02324	VEGFR2-Fc	no	–	–	–	–	1	–	1
Z02325	VEGFR2-Fc	yes	–	–	–	1	–	50	51
Z02326	VEGFR2-Fc	no	–	–	–	3	4	–	7
Z02329	VEGFR2-Fc	yes	–	–	1	–	–	–	1
Z02330	VEGFR2-Fc	yes	–	–	1	–	–	–	1
Z02331	VEGFR2-Fc	no	–	–	–	1	–	–	1

* Z01100 is identical to Z_{wt}, on amino acid, level and and is a known Fc-binder. It has been identified in several previous selections against a variety of different target proteins.

Before clustering, the sequences were compared with sequences for known background binders but no significant similarities were found (data not shown).

Clustering of Amino Acid Sequences

For each target the amino acid sequences from the identified Affibody® molecules were clustered to show degree of similarity in amino acid sequence and properties, see Figure 20 and 21. The identified Affibody® molecules derived from the VEGFR2-Fc selections were also clustered along with the Affibody® molecules derived from the previous selection on the same target, see Figure 22.

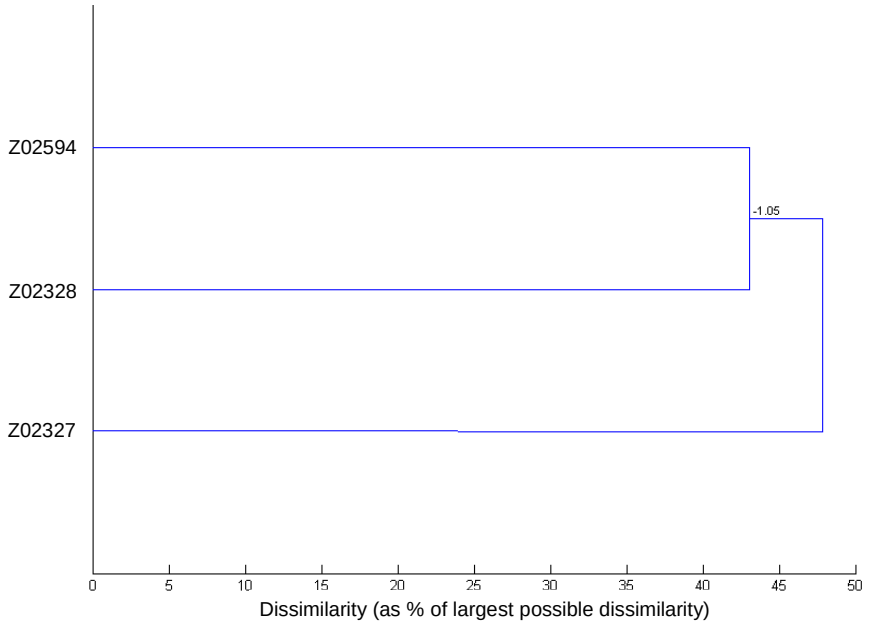


Figure 20. Clustering of clones identified from the selection on CEA A3B3 as percent of largest possible dissimilarity. None of the clones contains the amino acid cysteine in variable positions.

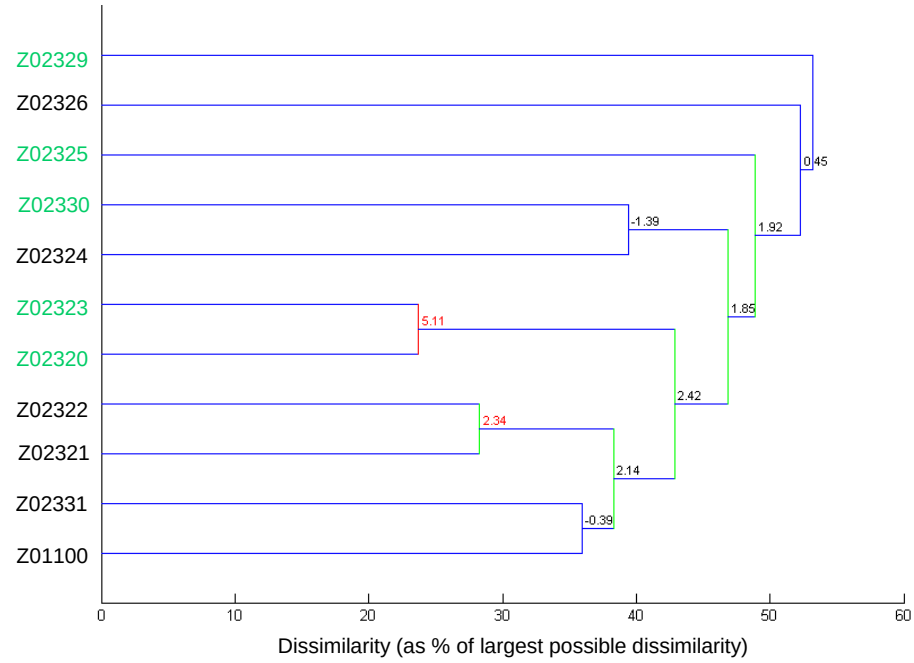


Figure 21. Clustering of clones identified from the selections on VEGFR2-Fc as percent of largest possible dissimilarity. Black labels indicate clones without the amino acid cysteine in variable positions. Green labels indicates clones that contains the amino acid cysteine in variable positions

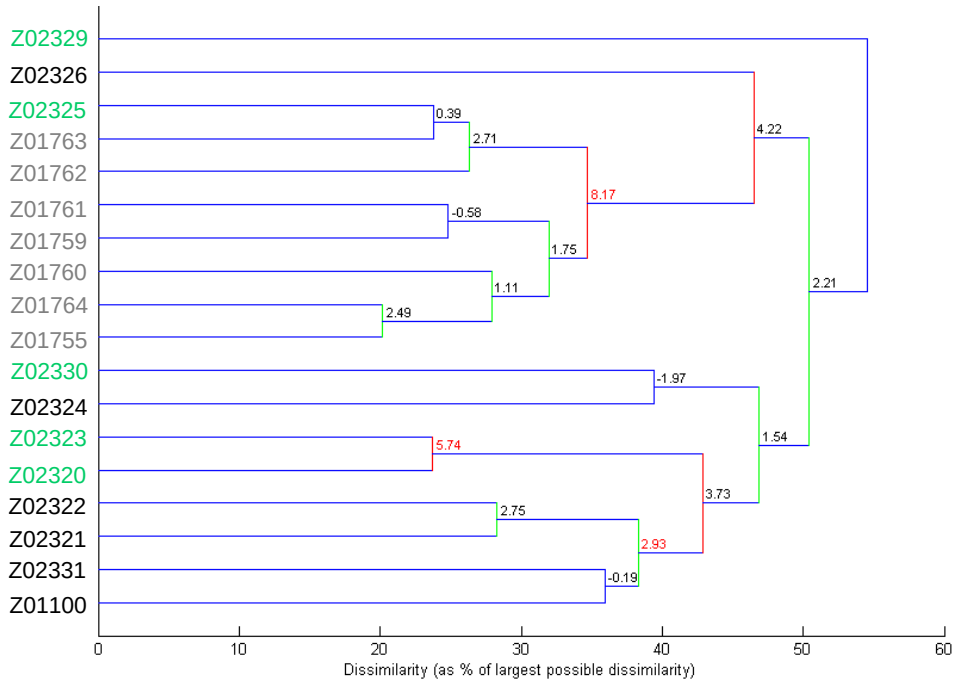


Figure 22 Clustering of clones identified from the selections on VEGFR2-Fc along with the clones identified in the previous selection on the same target as percent of largest possible dissimilarity. Black labels indicate clones without the amino acid cysteine in variable positions. Green labels indicate clones that contain the amino acid cysteine in variable positions. Grey labels (Z01755-Z01764) indicates clones identified in the previous selection whereof all contains the amino acid cysteine.

The sequencing of the Affibody® molecules identified from the selections on VEGFR2-Fc identified six Affibody® molecules free from cysteines, Figure 21. All molecules are different from the molecules identified in the previous selection, Figure 22.

The identified non cysteine containing Affibody® molecules, unique for these selections, were further investigated in a DotBlot experiment. An exception was Z02328, derived from the selection on CEA A3B3, which was not included since it contained proline at two randomised positions.

DotBlot

Two clones from the CEA A3B3 selection and five clones from the VEGFR2-Fc selection were chosen for target binding and specificity analysis with DotBlot. Binding properties was measured with chemiluminescence. Exposure times were individual for each membrane and can be found in the figure texts. Figure 23 shows the developed DotBlot membranes.

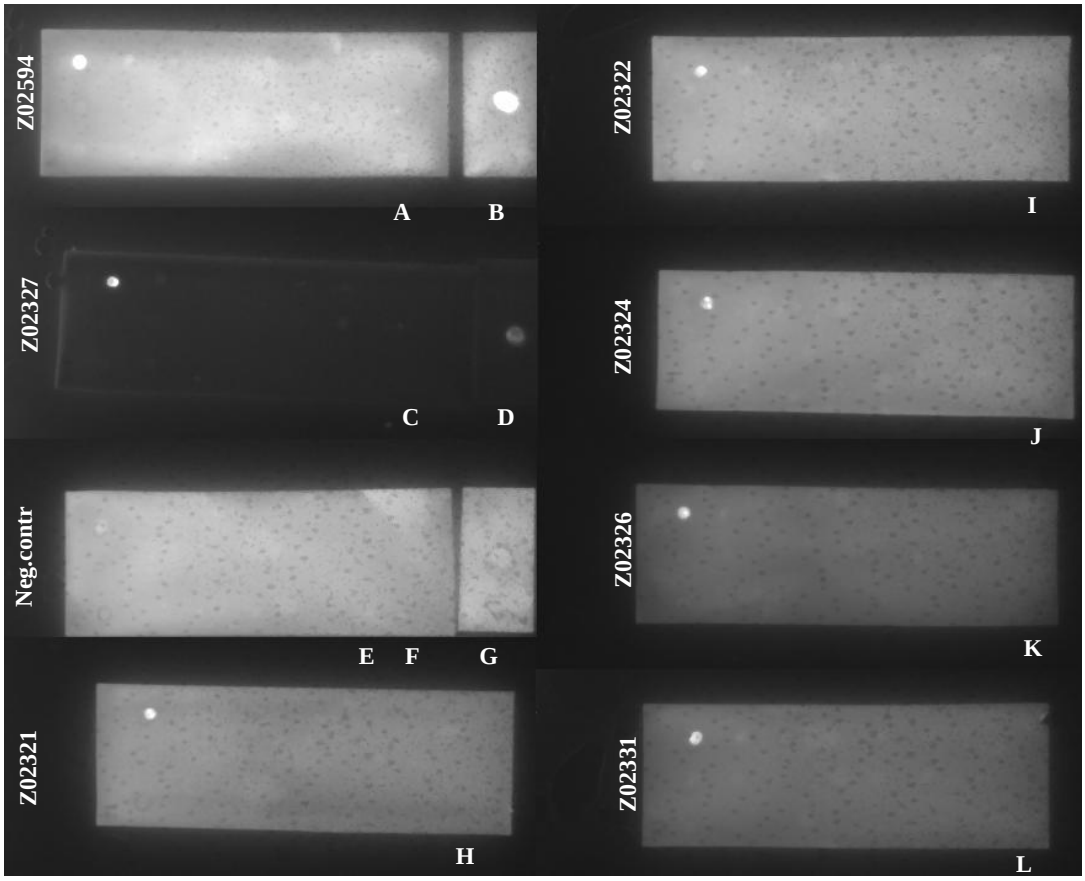


Figure 23. Developed DotBlot membranes. (A,C and E) contains full length CEA. (B,D and G) contains CEA A3B3. (F,H,I,J,K,L) contains VEGFR2-Fc. All dots contained 0.1µg protein except for the dots with CEA A3B3 where 5 x 2 µl, of an uncertain protein concentration, were dotted. Exposure times were 2 min for Z02327 and Z02594 and 1 min for all the other molecules in the test. A chart over the standard proteins, dotted on the membranes, can be found in Figure 11. In the upper left corner of each membrane, HSA were dotted which was used as a positive control.

5 DISCUSSION

The construction of randomised protein libraries displayed on the surface of filamentous phage have become an important technology used to select specific binders to target proteins through repeated cycles of binding and replication [37, 38]. This can be a powerful tool in the development of new drugs and mediators in diagnostic techniques.

Phage display libraries have been developed for selection of CEA binding scFv fragments and VEGFR2 bindning Fab fragments showing promising potential in therapy and imaging applications [10, 37]. Antibody derived fragments, like scFv and Fab, have along with Affibody® molecules targeting properties favourable to the ones shown by full size antibodies. Low molecular weight, effective penetration as well as other characteristics makes them well suited for molecular imaging.

In this study phage display technique was used to generate Affibody® molecules with specific binding properties to CEA and VEGFR2 respectively. The proteins used in this study were expressed in mammalian cell lines. These cell lines express the proteins with their natural glycosylation which increase the chances to generate Affibody® molecules actually binding to the target proteins *in vivo*.

Due to low phage yield, selection A1 was cancelled after cycle three, and the number of washes in selection A2 was changed from ten to seven, in order to relieve the selection conditions in the fourth cycle. CEA was known to be a difficult target to find binders for. This might be due to its heavy glycosylation which could be an explanation for the poor phage yield during the selections. However, VEGFR2 is also a glycosylated protein but gave good yields, wherefore this can not be considered to be the only explanation. Another explanation for the poor yield, from the selections on CEA A3B3, could be if less protein than planned had been used in the selections, due to the uncertainty in protein concentration. The actual concentration appeared to be lower than stated on the label when the protein was run on a gel parallel to the selection start. An alternative could have

been to do the selection on CEA A3B3 in solution. To do that the protein would have had to be labelled, for example with biotin, to enable capturing using for example streptavidin coated beads after the selection. In this case this was not possible since it would have required larger amount of the protein than was at hand.

The graphic overview of the phage enrichment ((phage out/phage in) x 100) throughout the selection cycles, Figure 12, shows a pattern that indicates a successful selection. A rough analysis of the pattern shows that in the first cycle the enrichment was high due to the presence of a high number of phage binding unspecifically. In the second cycle the enrichment was much lower due to reduction of unspecific binders. In the last two cycles the enrichment was enhanced due to amplification of phage binding specifically to the target. Selection B1 and B2 was not included in the graphic overview due to outlier values.

The reason for using two different ELISA set-ups was to mimic the conditions used in the selections. The ELISA screens gave rather high background signal which can be the result of unspecific binding and uneven washing. The time frame did not allow optimisation of the assays which could have given better signal to background ratios. One of the ELISA set-ups were known to yield high background signal, wherefore a parallel non-coated plate was used.

The study identified several Affibody® molecules, two from the CEA A3B3 selection and five from the VEGFR2-Fc selections, that were regarded as interesting. These candidates did not contain the amino acid cysteine, at randomised positions, which is a fundamental criterion to avoid dimerisation.

The different selections gave rather varying results. Selection against CEA A3B3 was performed only on solid phase with two different variants of target protein concentrations and resulted in two Affibody® molecules, Z02327 and Z02328 represented only once. Z02328 probably have a disrupted structure on helix two due to the presence of two prolines at randomised positions. Therefore, Z02328 was not further investigated. In

addition a molecule with a mutation in the constant region, Z02594, occurred 42 times in the screen and was very dominating from this selection. However, the three molecules derived from the selection on CEA A3B3 (A2) did not show any significant similarities when clustered.

For the selection rounds on VEGFR2 the outcome varied significantly with the phase used. This was not seen in the titres, during the selections, or in the characterisation analysis with ELISA, but on the results from the sequencing. From the solid phase selections very few clones were successfully identified and all of them either contained the amino acid cysteine or was a known Fc-binder, Z01100, identical to Z_{wt} on amino acid level. The selections in solution on VEGFR2 gave, on the other hand, rather promising results. Regarding the selections where epitopes on the target protein were blocked with Affibody® molecules, resulted in five interesting molecules that did not contain cysteine and whose sequences were significantly different from the ones derived from the previous selection, see Figure 22. Two of them, Z02321 and Z02322, forms a cluster, i.e. shows less than 30% dissimilarity. They have five out of 13 variable amino acids in common and another tree amino acids share the same properties. Z02331 shows some similarities with the Z02321/Z02322-cluster. Z02331 is branched together with Z01100, which is a known Fc-binder, but the dissimilarity is above 35% and it remains to be investigated if Z02331 is specific for VEGFR2 or bind other proteins such as Fc. The DotBlot did not show any binding to IgG, among the serum proteins, wherefore there is reason to believe that Z02331 do not bind Fc. The other two, of the five candidates, Z02324 and Z02326 are not similar to any of the other candidates from this selection. However, it can be mentioned that sequence similarities and binding activity do not necessary have a correlation since the candidates can bind different epitopes on VEGFR2.

Z02321, Z02322 and Z02326 have been derived from both of the blocked selections in solution (C1 and C2), which may indicate that the selections have been successful.

The selection in solution with un-blocked VEGFR2 resulted in only one, but rather dominant Affibody® molecule, Z02325, which was identified 51 times. It clusters very well with the Affibody® molecule Z01763 from the previous VEGFR2-Fc selection, which was used for blocking, but also with another molecule Z01762 from the same selection. In fact Z02325 is more similar to all the molecules from the previous VEGFR2-Fc selection than any of the molecules from the new selections. It also shares a highly conserved cysteine with all molecules from the previous selection, not appearing in any of the other molecules derived from the present selections. Z02325 also shares two other amino acids with all Affibody® molecules from the previous selection (with the exception that Z01763 only have one of these amino acids conserved). Z02325 was also identified in the blocked selection, C1, but only with one clone, compared to the 50 clones from the un-blocked selection D. Since it is not very likely to block 100% of the epitopes it is of course possible for this molecule to be derived from the blocked selection by chance. Furthermore, one can not know whether or not Z02325 binds to the epitope to be blocked. Z01755 and Z01763, from the previous VEGFR2-Fc selection, were chosen for blocking since both contained cysteines at the same position but had different aa at certain positions. See Figure 22 to follow the discussion.

A pre-selection performed prior to the selection can never be considered to completely eliminate phage binding to components present during the selection. The pre-selection is rather a way to reduce the number of background binders in the phage library. In the selections performed the pre-selections seemed to have been efficient, though. Known background binders have only been identified in the solid phase selection for VEGFR2-Fc, selection B2, where the known Fc-binder Z01100 appeared. In these selections there is reason to believe that the protein was not suited to be adsorbed directly onto a plastic surface and the Fc domain fused to VEGFR2 might well have been the only epitope available in the selection. This could have led to a strong selection pressure for the low number of background binders still present in the phage library. For completeness, it can be mentioned that the pre-selection method used prior to the selections in solution was a variant of solid phase pre-selection, since the Fc domain was attached to the bead during the whole incubation.

The different selection systems used in this study (solid phase and solution) display the protein epitopes in different ways. It is individual for each protein which method is best suited for selection procedures. Selection in solution allows the protein to have a more natural shape and display a larger number of epitopes but a membrane bound protein can also have hydrophobic domains that dislike being in solution. An alternative set-up for the solid phase selection of VEGFR2-Fc could have been to coat Z_{wt} on the immuno tube surface and, after blockage of free surface, allow VEGFR2 to bind to Z_{wt} via the Fc fusion. This could have displayed the target protein in a more natural way but would have been more time consuming and, along with the five other parallel selections, outside the manageable limits for a phage display novice.

The DotBlot used for target binding specificity gave ambiguous but promising results. Z02327 and Z02594, from the CEA A3B3 selection, indicated binding activity to CEA A3B3. However, the reason for this could be that the concentration of CEA A3B3 was not fully known and the amount of protein in the dot might have been much higher than 0.1 µg, as in the other dots. This might also explain the weak binding of the negative control to CEA A3B3. An alternative could have been to dot a series of CEA A3B3 with different amounts of the protein. Regarding the full length CEA, these Affibody® molecules did not show the same binding activity. Only Z02594 showed binding activity for the full length CEA, very weak though. An explanation for the low binding could be that the full length CEA, at hand, had been stored at +4ºC for quite some time and the quality of it was not checked prior to the DotBlot test.

The DotBlot on VEGFR2 selected Affibody® molecules was not successful. The reason for this could be the same as mentioned for the solid phase selection, that direct adsorption of VEGFR2 to a solid support was not favourable. An alternative set-up is to use a Z_{wt} linker to attach the target protein, but this demands a more complicated procedure in solution. However, there was no indication that any of the Affibody® molecules derived from the selection on VEGFR2-Fc bound unspecifically to human serum proteins. Further investigation will be necessary to reveal the target binding

activity. This can be performed e.g. through surface plasmon resonance analysis using Biacore (Biacore AB, Uppsala, Sweden) which requires sub-cloning and expression of each Affibody® molecule which could not be done in the time frame of this project.

Specific binding to the target protein would be a milestone in the development of new Affibody® molecules but to be sure about the molecules' binding properties they must be tested on target protein expressing cells.

6 CONCLUSIONS

The selections performed resulted in two new candidates from the selections on CEA A3B3 and five new candidates on the selections on VEGFR2-Fc. None of these candidates had cysteines in variable positions.

The candidates from the CEA A3B3 selection, Z02327 and Z02594, indicated binding activity to CEA A3B3 and, for the case of Z02594, to the full length protein as well. The candidates did not bind unspecific to the human serum proteins included in the DotBlot analysis. A third candidate, Z02328, probably had a disrupted helix structure due to the presence of two prolines in variable helix positions. Therefore this molecule was not further investigated.

Regarding the candidates from the VEGFR2-Fc selections they were distinguished from all the Affibody® molecules from the previous selection on the same target. The blocking of the protein, prior to selection, was successful since all candidates were derived from these selections. It also shows that it is possible to favour the selection of other Affibody® molecules since none of the molecules from the previous selection was identified in any if the selections. This holds true even when all eleven identified Affibody® molecules were taken into consideration. However, in the selection on un-blocked VEGFR2-Fc one Affibody® molecule, Z02325, with similar sequence to the present ones were identified. Z02325 also contained a strongly conserved cysteine shared by all Affibody® molecules from the previous selection.

The solid phase selections on VEGFR2-Fc was not very successful and resulted only in two cysteine containing Affibody® molecules and a known Fc binder, Z01100. This indicated that VEGFR2-Fc was not suited for direct adsorption onto a plastic surface.

The DotBlot analysis did not work for the Affibody® molecules derived from the selections on VEGFR2-Fc. None of the candidates did show any binding activity to

VEGFR2-Fc. The only conclusion that could be drawn was that none of the candidates did bind un-specifically to human serum proteins.

The time frame of this project did not allow further investigations on target binding specificity but so far the results have been promising. If future studies show specific binding activity to target expressing cells, the research towards new products will continue with a second selection using a maturated library to identify Affibody® molecules that bind the target protein both specifically and with high affinity.

7 ACKNOWLEDGEMENTS

I would like to thank my supervisor, Tove Eriksson, for all help, enthusiasm and positive support during this project.

I would also like to thank:
Fredrik Nilsson - for giving me the opportunity to perform my Master's degree project at Affibody AB

Anders Blomqvist, Gunilla Fant, Joakim Galli and Anna Jarelöv-Palm – for all practical and theoretical help.

Anders Persson - for all help with the matlab figures.

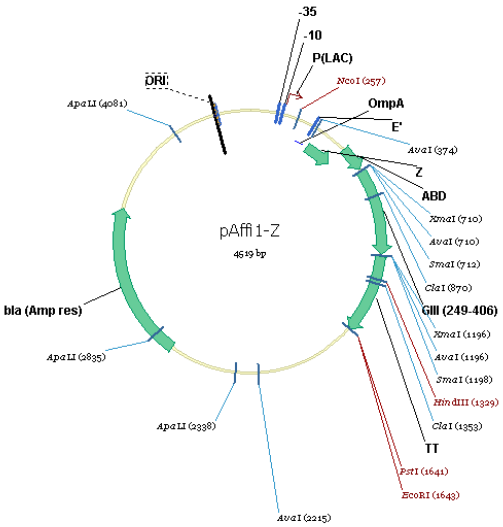
"Team Uppsala": Anders Blomqvist, Mikaela Magnusson and Sara Nystedt – for nice company and excellent driving in the car pool.

Malin Gustafsson and Helena Wållberg – for making all hours at my desk so enjoyable.

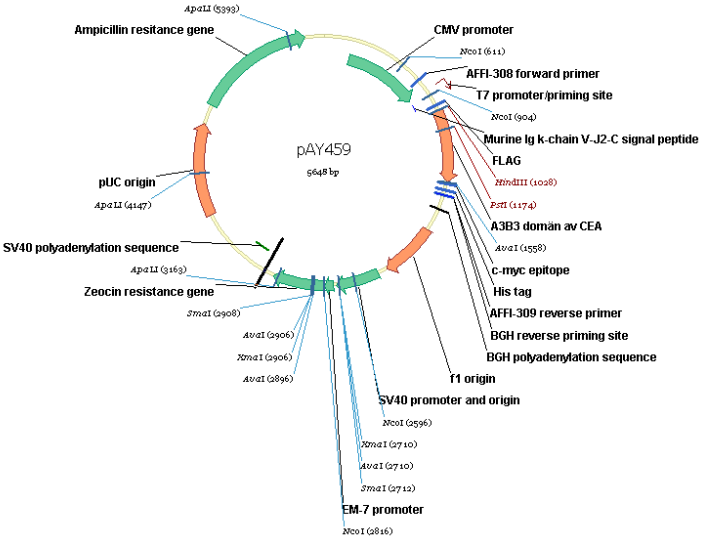
All others at Affibody AB - for all help and the welcoming atmosphere.

8 APPENDICES

Appendix 1. pAffiI – Phagemid with a Z-variant fused to ABD and geneIII.



Appendix 2. pAY459 – Eukaryotic expression vector used for in house expression of CEA A3B3. Originating from an eukaryotic expression vector from Invitrogen,with a N-terminal secretion signal peptide followed by a FLAG tag and a C-terminal c-myc epitope followed by a 6His-tag.



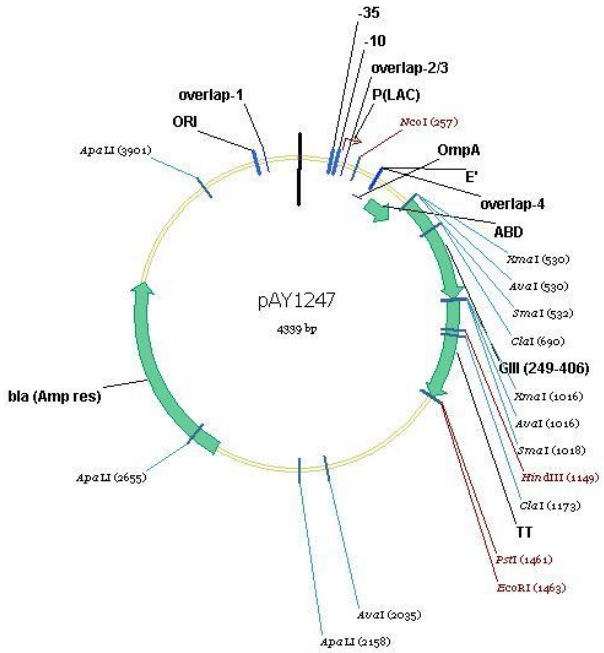
Appendix 3. Primers for PCR-screen prior to sequencing. Amplifies a DNA fragment containing genes coding for a variant of an Affibody® molecule and its fusion protein ABD.

AFFI-21: (forward) 5'-TGC TTC CGG CTC GTA TGT TGT GTG-3'
AFFI-22: (reverse) 5'- CGG AAC CAG AGC CAC CAC CGG-3'

Appendix 4. Primer for sequence PCR reaction, biotinylated in 5' end.

AFFI-72: reverse 5'-CGG AAC CAG AGC CAC CAC CGG-3'

Appendix 5. pAY1247 - pAffiI vector without Z insert. Used as negativecontrol in DotBlot.



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