PRODUCTION AND CHARACTERISATION OF scFv BINDERS AGAINST SELECTED ENTEROVIRUSES

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The single chain variable fragment (scFv) is the smallest antibody molecule that contains a full antigen binding site. Antibody consists of two heavy (H) and two light (L) chains which form a Y-shaped molecule. The antibody binding sites are at the tips of the Y. The scFvs are created by genetic technology by cleaving the variable fragments from both H and L chains of antibody molecule and attaching these together with a flexible linker. The scFv can be expressed in a bacteriophage and screened from bacteriophage library with a method called bio-panning. Echovirus 1 (E1) and coxsackievirus B3 (CVB3) are members of the family *picornaviridae* that cause various diseases from common cold to myocarditis.

In this study we screened a chicken antibody based semi-synthetic phage library Nkuku® for scFv for E1 and CVB3. Our findings indicate that the library is a good source of scFv for both of the viruses. The isolated individual clones were enrichened and bound well to the viruses, but when the phages were removed the soluble form of scFv lost their attachment or was disintegrated. Thus, the characterisation experiments were carried out with the phage bound scFv clones.

We showed that the phage bound scFv were able to withstand warmer temperatures relatively well and could be frozen and thawed several times. For long time storage 4°C seemed optimal. The scFv withstood different conditions well, which was to be expected since it has been shown that the phage stabilises the scFv. The ELISA protocol still requires further optimisation to become fully functional in spite of the optimisation experiments already made.

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Nanobodyt (single chain variable fragment, scFv) ovat pienimpiä täyden antigeeninsitoutumispaikan sisältäviä vasta-aineen osia. Vasta-aine on Y:n muotoinen molekyyli, mikä koostuu kahdesta raskaasta (H) ja kahdesta kevyestä (L) ketjusta. Antigeenin sitoutumispaikat sijaitsevat Y:n kärjissä. scFv:t valmistetaan molekyylibiologian keinoin jakamalla H ja L ketjujen vaihtelevat osat vasta-ainemolekyylistä ja liittämällä nämä yhteen joustavan linkkerin avulla. scFv:t voidaan ekspressoida bakteriofaagissa ja seuloa bakteeriofaagikirjastosta bio-panning-nimisellä tekniikalla. Echovirus 1 (E1) ja coxackievirus B3 (CVB3) ovat *picornaviridae* -heimon jäseniä, jotka voivat aiheuttaa useita sairauksia flunssasta sydänlihastulehdukseen.

Me seuloimme Nkuku®-nimisestä semisynteettisestä kanan vasta-aineisiin pohjaavasta scFv kirjastosta vasta-aineita E1:lle ja CVB3:lle. Löytömme osoittavat kirjaston hyväksi scFv-lähteeksi näille viruksille. Kerätyissä yksittäisissä klooneissa oli useita hyvin viruksiin sitoutuvia ja rikastuvia scFv-klooneja. Kun faagit irroitettiin ja scFv:t olivat liuenneessa muodossa, ne menettivät sitoutumisensa tai hajosivat. Tämän vuoksi karakterisointikokeet tehtiin faagiin sitoutuneilla scFv:llä.

Osoitimme karakterisointitesteillä että faagissa kiinni olevat scFv:t kestävät suhteellisen hyvin korkeitakin lämpötiloja ja ne voidaan jäädyttää ja sulattaa useita kertoja. Pidempiaikaiseen säilytykseen 4°C vaikutti optimaalisimmalta lämpötilalta. Ei ole yllättävää että faagin sidotut scFv:t kestävät hyvin eri olosuhteista sillä faagien on todettu vakauttavat scFv:tä. Tehdystä optimoinnista huolimatta ELISA menetelmää ei saatu täysin toimivaksi ja lisäoptimointi on tarpeen.

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ABBREVIATIONS

amp	Ampicillin
BSA	Bovine serum albumin
CAR	Coxsackie and adenovirus receptor
CDR	Complementary-determing-region
cfu	Colony forming unit
CVB3	Coxsackievirus B3
DAF	Decay accelerating factor
E1	Echovirus 1
F(ab´)2 fragment	A fragment of antibody where two antigen binding arms remain linked
Fab	Fragment antigen binding
Ff (M13, fl, Fd and ft)	Filamentous phage particles
H chain	Heavy chain
Ig	Immunoglobulin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kan	Kanamycin
L chain	Light chain
MAb	Monoclonal antibody
MP	Milk powder
OD	Optical density
PAb	Polyclonal antibody
PBS	Phosphate-buffered saline
RT	Room temperature
scFv	Single chain variable fragment, nanobody
TG1	*Escherichia coli* cell strain
TJ	Tight junction

1 INTRODUCTION

1.1 Antibodies

Antibodies, also called as immunoglobulins (Ig), are proteins produced by the B cells of immune system. Their function is to identify pathogens such as bacteria and viruses and disable their function. There are five classes of antibodies, IgM, IgD, IgG, IgA and IgE, and they differentiate by the structure of the constant region. The most common of these is the IgG (Litman et al. 1993, Murphy 2012: 127-130).

Antibody is a Y-shape molecule consisting of three same sized parts that are connected by a flexible tether. There are two heavy (H) and two light (L) chains that are linked by disulfide bonds so that the two heavy chains are linked to each other and the light chains to each heavy chain (figure 1). The light chains are identical so that the molecule has two identical binding sites at the "tips" of the Y-shape. This allows it to bind to two antigen molecules at the same time. The antibodies can also be divided into two regions; constant (C) and variable (V). Main variability of the antibody molecule is limited to the V region of both H and L chains. The genetic variability of the variable region, in both H chain and L chain is concentrated on three regions called the hypervariable regions. When the molecule is assembled, these regions create the very tip of the Y-shape molecule being responsible for the variation in the antigen binding site. This region is called complementary-determing-region (CDR). There are three CDRs in each H and L chain called CDR1, CDR2 and CDR3. These variable CDR domains make the variable region and the rest of the molecule is the constant region (Delves et al. 2011: 53-77, Murphy 2012: 128-135).

An antigen usually has several epitopes that the antibody molecules can bind to. Under normal circumstance several different B lymphocytes activate creating a polyclonal antibody (PAb) response. If response is from a single B lymphocyte clone the response is monoclonal. In research PAb's are developed by injecting an antidote to an animal and collecting the sera, directing PAb's towards a specific antigen. In 1975 Köhler and Milstein developed a hybridoma technique that allowed the production of monoclonal antibodies (MAb's). In comparison to PAb's, MAb's are more specific and produce more targeted response. A limitation with MAb's in their susceptibility to denaturation via conformational change due to multiple reasons such as temperature or pH. Whereas the PAb's are not as sensitive because of the variety of antibodies that can work under different kind of conditions (Lipman et al. 2005, Delves et al. 2011: 142-145).

Antibody molecules can be cleaved into smaller units using proteolytic enzymes called proteases. Limited digestion with a protease called papain cuts the antibody on the aminoterminal side of the disulfide bond that link the H chains. This releases the two arms of the antibody as identical fragments that contain the antigen-binding activity. These fragments are called the Fab (fragment antigen binding) fragments. A fragment where the two antigen binding arms off the antibody remain linked can be produced with another protease called pepsin. This fragment is called F(ab´)2 fragment (Murphy 2012:128-131).

1.2 Single chain variable fragment

The single chain variable fragment (scFv), also called a nanobody, is the smallest immunoglobulin molecule to still contain the full binding site for antigen (Ahmad 2004, Murphy 2012:128-131). It is a truncated Fab fragment that consists of V domain of H chain linked by a stretch of synthetic peptide to a V domain of L chain (figure 1) (Ahmad 2004, Murphy 2012:128-131). Horton et al. (1989) first described a method of creating hybrid genes via polymerase chain reaction (PCR) assembly using complementary regions at the end of the conjoined products. The produced V chains (H and L) can then be combined into plasmid directly after the PCR by *in vitro* recombination (Chaudhary et al. 1990). The PCR method is the most commonly used method to create scFv libraries (Clackson et al. 1991, Marks et al. 1991, van Wyngaard et al. 2004).

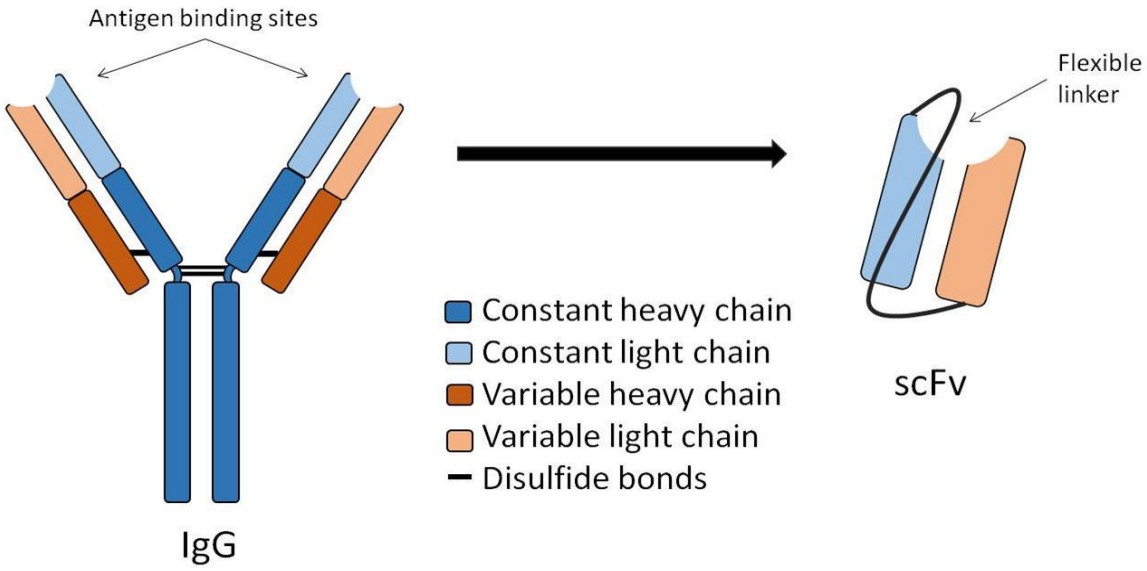


Figure 1: Structure of an IgG molecule and the scFv. Modified from Ahmad 2004 and Murphy 2012.

Compared to a full IgG 150 kDa or a Fab fragment 50 kDa, the scFv is only 25 kDa in size (Kaiser et al. 2014). The small size allows the molecule to easily penetrate tissues and cells which works as an advantage when using scFv as therapeutic agents (Ahmad 2004, Murphy 2012:128-131). The size of the molecule also brings disadvantages; for example small sized molecules are rapidly eliminated from the body via kidneys (Winthrop et al. 2003). The stability of the scFv molecules is also an issue, since they tend to aggregate under various conditions such as heating or long time storing due to hydrophobic patches in the surface of scFv (Nieba et al. 1997, Wörn & Plückthun 1999). The aggregation of the soluble scFv also prevents the detection of the proteins due to the possible misfolding of the scFv–c-myc fusion proteins. The detection of the soluble scFv is done via linked peptide tag such as c-myc (Hoogenboom et al. 1991). The stability of a scFv can be improved with protein engineering, by finding the problem causing residues and replacing them (Wörn & Plückthun 1999).

1.3 Antibody and scFv applications

Antibodies are used in several ways both in research and medical applications. Immunological detection has been successfully used to detect bacterial cells, spores, viruses and toxins. In immunological detection antibodies are used to identify these antigens from a sample. The used antibody can be polyclonal or monoclonal and it can be truncated such as Fab of a scFv. (Iqbal et al. 2000). The detection is usually done by using a primary antibody that recognises the target antigen, for example a virus capsid protein, and a secondary antibody attached to a detectable component. It is also possible to conjugate the primary antibody to the detection component. The accuracy of the antigen recognition depends on the specificity of the primary antibody (Giepmans et al 2006).

A common detection method is fluorescence microscopy where fluorescent dye is used to label fixed or living cells. This dye can be detected for example by using light microscopy or confocal microscopy. A variety of different colour emitting dyes can be used to detect different proteins at the same time (Giepmans et al. 2006, Cooper & Hausman 2013: 25-26). In immunohistochemistry the labelling is done with enzymes, such as horseradish peroxidase, that can be detected with light microscopy. This works better with tissue staining than cell suspension. In transmission electron microscopy gold nanoparticles are used as an electron dense marker (Delves et al. 2011: 148-160, Murphy 2012: 732-737). Gold nanoclusters for

example have been used to detect hydrophobic pockets in enteroviruses (Martikainen et al. 2015).

Immunoprecipitation and immunoblotting are used in similar ways to detect qualities or if a protein is found in a sample. In immunoprecipitation antibodies are attached on a surface such as agarose beads. This way it is possible to purify an antigen in a mixture and see how it behaves with the other proteins in the mixture. Detection can be done with SDS-PAGE. With immunoblotting it is possible to determine the relative molecular mass of a protein or the presence of a protein in a cell lysate. All the cell proteins are solubilised with a detergent to create lysate and run on SDS-PAGE gel. Detection can be done with antibodies labelled with radioisotopes or an enzyme (Delves et al. 2011: 148-160, Murphy 2012: 732-737).

Both enzyme - linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) use antibodies. In ELISA the antigen is bound to a microtiter plate, recognised with a primary antibody and detected with secondary antibody. ELISA is commonly used in viral diagnostics. RIA is similar to ELISA and it is usually used to detect hormone levels. In RIA assay the detecting antibody is radioactively labelled (Delves et al. 2011: 148-160, Murphy 2012: 732-737).

Antibodies are used in some common diagnostics. The blood group determinations, ABO blood groups and RH blood group, are done with antibodies against the antigens in blood. In testing the ABO blood group the anti-A and anti-B antibodies are induced to blood samples. These cause the blood to precipitate if the antigen is present, anti-A precipitates A blood and both antigens precipitate AB blood. This process is called hemagglutination. RH blood group testing is different since the antibodies against the RH antigen do not cause precipitation. The test is against the antibody IgG molecules that are produces by RH negative blood against RH positive blood. The test has clinical relevance in cases where RH negative mother carries a RH positive child. During birth the mothers' blood can come in contact with the child's blood and cause the antibodies to be created. This can cause haemolytic disease in a second child with RH positive blood, but testing can help prevent that (Murphy 2012: 724-730).

In cancer treatment there are already several antibody drugs in use. These antibodies function in a variety of ways; directly kill cancer cells, activate the immune response against the tumours and affect the tumour vasculature and stroma. All of these ways described above are currently used in cancer medicine (Scott et al. 2012). For example antibody trastuzumab inhibits the function of human epidermal growth factor receptor type 2 (HER2) and limits the survival of breast cancer tumours that depend on it (Hudis 2007). Antibody rituximab activates antibody-dependent cellular cytotoxicity (ADCC) via membrane spanning protein

CD20 (Beers et al. 2010). Antibody bevacizumab inhibits vascular endothelial growth factor (VEGF) and prevents tumour angiogenesis (McCormack & Keam 2008). The use of antibody based therapy in cancer treatment is in growing interest because the side-effects of the drugs are often milder and differ from the traditional chemotherapy (Scott et al. 2012). Antibody based drugs do have side-effects that need to be monitored when used, such as cardiovascular toxicity (reviewed in Strevel & Siu 2009).

The scFv could be used in various ways similarly to the full antibodies (Ahmad et al. 2012). In therapeutic use the small size gives the molecule benefit against full size antibody. For example in cancer treatment, the scFv will penetrate the cancerous tissue more easily and as the clearance is rapid there is less damage to healthy tissue (Yokota et al. 1992, Colcher et al. 1998). Thought the main problem is the rapid elimination via kidneys (Winthrop et al. 2003).

1.4 Phage display library

It was originally found in 1985 that genomes of particles could be inserted into phage genome in a way that they would be displayed on the surface of the phage (Smith 1985). With phage display libraries it is possible to present a large variety of peptides of proteins, such as antibodies and select them with high affinity binding to almost any target (Arap 2005). Libraries have been developed from a variety of animals, such as chicken (Davies et al. 1995), sheep (Li et al. 2000), camel (Arbabi Ghahroudi et al. 1997), and humans (Griffiths et al. 1994).

There are three types of scFv libraries: immunised, naïve and synthetic. In immunised libraries the donor animal, has been immunised by exposing it to the wanted antigen before collecting the immunoglobulins. Immunised libraries produce higher affinity binders. The main problem is that each antigen requires the construction of a new library. Naïve libraries are from non-immunised sources. There is no bias towards any antigen and the library can be used against various antigens, such as toxins where creation of immunised library would be difficult. Main hind side of naïve libraries is low affinity binding due to lack of variety. This variety can be increased by making a synthetic library (Griffiths & Duncan 1998). Synthetic libraries are based on naïve libraries; the natural sequences are enhanced with synthetically randomised CDR's. This will enhance the size and variability of the library and help to create more high affinity antibodies (Griffiths et al. 1994, Ahmad et al. 2012).

The screening process of a phage library is called bio-panning and the technique was originally used with scFv by McCafferty et al. (1990). The bio-panning process starts by creating a library or amplifying an existing library. Next the library particles are exposed to the wanted target proteins, *in vitro* or *in vivo*, and unbound phages are removed. The phage material that is bound to the target protein is then collected. The exposure and collection are repeated several times to enrich the phage population. The bio-panning is easiest to perform *in vitro* where the antigen is bound to a surface, for example to an immunotube (van Wyngaardt et al. 2004), followed by the addition of the phage. The bound phage material can be collected by elution (Koivunen et al. 1999, Arap 2005). Using the *in vitro* technique the library is only interacting with the target antigen. The selection can also be performed *in vitro* in living cells, both as a monolayer and in cell suspension and *in vivo* in living animals. In these more complex biological systems unspecific binding can be a problem and the specific binding needs to be enhanced (Arap 2005) *In vivo* the library is injected intravenously into a living animal. Then the phage material is allowed to locate into tissues and after a chosen time point animal is euthanized and the tissues collected for isolation of the phages. The phages can then be enrichened *in vitro* and then injected again to another animal (Arap 2005, Pasqualini & Ruoslahti 1999).

The bacteriophages used to make phage display libraries are single stranded DNA-viruses that infect gram-negative bacteria, such as *Escherichia coli (E.coli)*. The infection is not lytic, and it causes the bacteria to produce and secrete the phage. Most commonly used phages are filamentous phage particles known as Ff including strains M13, fl, Fd and ft. The library particles are usually expressed in proteins pVIII, encoded by gene VIII or pIII from which pVIII is the main coat protein of the phage. pIII is minor coat protein located at the tip of the phage functioning at the beginning of infection (Webster 2001 cited in Arap 2005, Arap 2005, Ahmad et al. 2012).

1.5 Nkuku® phage display

The Nkuku® phage display library was created by van Wyngaardt et al. in 2004 and it was constructed at the Oderstepoort Veterinary Institute, (OVI), Pretoria, South-Africa. It is a semi-synthetic naïve scFv library. The library is based on immunoglobulin genes of non-immunised chicken immunoglobulin genes (van Wyngaardt et al. 2004).

The Nkuku® is composed of natural L and H chains variable domains from the chicken immunoglobulin repertoire together with a sub-library, in which there is a selection of

synthetically randomised amino acid residues on the H chain (CDR3). The CDR3 is usually the most varying and thus largely responsible for antigen binding, making it an ideal target for synthetic randomisation (Knappik et al. 2000, Ahmad et al. 2012). The randomisation was done to increase the variability in the library. The library is large, with approximately $2 * 10^9$ clones of scFv, and the large size of the library enables it to recognise a large variety of antigens such as haptens, proteins and viruses. The library is constructed mainly for finding antigens for *in vitro* diagnostics, since for use in human therapeutics the avian based antibodies are unlikely to be useful, unless they are humanised; made less immunogenic to humans. For practical use chicken based immunogens can be useful since they function normally in 5°C higher body temperature than humans. Temperature sustainability is a wanted property for example for field analysis in warmer countries (van Wyngaardt et al. 2004). Building the library form chicken immunoglobulins is also practically easier since the all chicken variable regions have essentially identical amino acid sequences at both ends. This enables the use of two sets of PCR primers, one for the L chain and another for the H chain (Davies et al. 1995, van Wyngaardt et al. 2004).

1.6 Picornaviruses

Picornaviruses belong to the family of *Picornaviridae*, which is a large family on small single strand positive sense RNA viruses that cause several serious and mild diseases both in human and animals. Poliovirus and food-and-mouth disease virus are among the most known picornaviruses (Brummer-Korvenkontio 2007 134-137, Tuthill et al. 2010). One species in the picornavirus family are B enteroviruses, which consist of EV-B serotypes, echoviruses, coxsackieviruses B1-B6 and coxsackievirus A9 (Marjomäki et al. 2015).

Echovirus 1 (E1) and coxsackievirus B3 (CVB3) both cause mild enteric diseases and respiratory diseases, typically to enteroviruses. Coxsackie B viruses have been found to be a major cause of myocarditis (Brummer-Korvenkontio 2007 134-137, Fairweather et al. 2012). There is also some evidence of enteroviruses being involved in the onset of type 1 diabetes (reviewed in Alidjinou et al. 2014) Enterovirus structure (figure 2) is an icosahedral capsid formed by four capsid proteins (VP1, VP2, VP3 and VP4). VP1-VP3 form the outer surface and VP4 is in contact with the viral matter inside (Kääriäinen & Peränen 1996, Tuthill et al. 2010).

E1 uses α2β1 integrin as its receptor, attaching to the I-domain of the integrin (Bergelson, et al. 1992, King et al. 1995). The virus binding causes the integrins to cluster (Upla et al.

2004), followed by internalisation of the integrins in to the cell with the virus and further processed in a new pathway that differs from the normal endosomal entry (Rintanen et al. 2012). The early stage of entry occurs in a macropinocytosis like pathway. The endosomal structures containing E1 and α2β1 grow in size and become multivesicular bodies (MVB) from 15 minutes up to three hours post infection (Karjalainen et al. 2008). Lipid domains are involved in the internalisation process making the uptake cholesterol dependent (Upla et al. 2004, Siljamäki et al. 2012). In the later stages of the infection the integrins are degraded by calpain proteases (Karjalainen et al. 2008). The uncoating of the E1 viruses starts 30 minutes post infection and the RNA is released when the MVB become gradually more permeable from two hour post infection (Soonsawad et al. 2014). The detailed uncoating and genome release process is still not fully understood.

CVB3 entry is mediated by decay accelerating factor (DAF) and coxsackie and adenovirus receptor (CAR) functioning as co-receptors in polarised cells (Coyne & Bergelson 2006). In nonpolarised cells DAF does not play such a big role since CAR is freely available to the virus (Patel et al. 2009). CVB3 binds to DAF which clusters and the CVB3/DAF complexes are transported to the tight junction (TJ) about 15 minutes post infection. In the TJ the complex binds to CAR (Coyne & Bergelson 2006), causing an alternation in the virus structure and leading to formation of A-particle (Milstone et al. 2005). When the complex gets to the TJ, the TJ becomes more permeable and is depleted of its internal membrane component occludin. The virus does not directly interact with occludin but the depletion allows the virus internalisation to cell (Coyne et al. 2007). One hour post infection the CVB3 is in caveolin-1 containing vesicular structure in the cytoplasm. Both of the receptors remain in the TJ in polarised cells (Coyne & Bergelson 2006), in nonpolarised cells CAR is internalised with the viruses (Patel et al. 2009). Cholesterol also plays a role in CVB3 infection since DAF associates with lipid-rafts, with depleted cholesterol the virus cannot locate to the TJ (Coyne & Bergelson 2006).

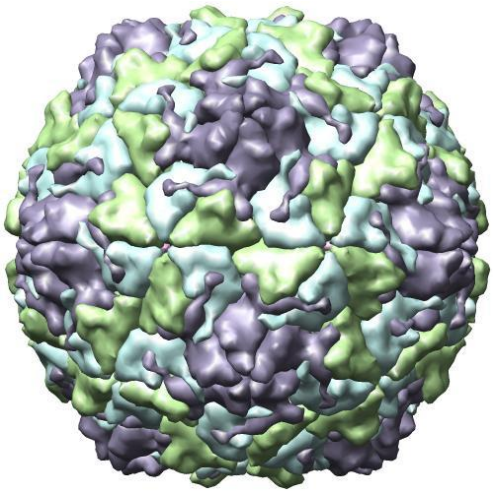


Figure 2: E1 virus structure. Image from the RCSB Protein Data Bank (Berman et al. 2000), PDB ID 1EV1 (Filman et al. 1998).

2 AIM OF THE STUDY

The aim of this study was to produce better binders and potential markers against two enteroviruses, E1 and CVB3, to be used in microscopy studies *in vivo* and *in vitro*. The scFv antibodies were screened from the chicken based scFv phage library Nkuku® using a method called bio-panning. In addition, found scFv clones were characterised for their thermal stability.

3 MATERIALS AND METHODS

3.1 Viruses and antibodies

The used virus strains were Farouk strain (E1) and Nancy strain (CVB3). Used antibodies were mouse monoclonal antibody, B62-FE2 (PROGEN Biotechnik gmbh, Germany) which detects the Fd phage coat protein pVIII. MAb against myc peptide produced in 9E10 hybridoma cells (originally gift from H.Garoff) (Suomalainen & Garoff 1994). Also E1 antibody rabbit anti-E1 (Marjomäki et al. 2002) was used.

3.2 Bio-Panning

The selection of antibodies from the Nkuku® phage display was done in vitro using a method called bio-panning. It was done as described earlier (van Wyngaardt et al. 2004).

Here shortly: Immunotubes (Nunc Maxisorp) were coated with 20 µg/ml of purified virus (E1 & CVB3) diluted in phosphate-buffered saline (PBS) overnight at 4°C. The following steps were done at room temperature (RT). After 16 hours incubation immunotubes were washed 3 times with PBS and then blocked with 2% (w/v) fat free milk powder (MP) (Valio Oy, Finland) in PBS for one hour followed by two PBS washes. Nkuku® library particles (concentration $5x10^{12}$) were incubated in 2% MP in 0.1% Tween 20/PBS. After one hour the prepared library phage particles were added to the immunotubes and rotated 30 minutes and then stationary incubated for 90 minutes.

During the incubation time TG1 *E. coli* cell were grown shaking (Certomat H., B. Braun Biotech International) 200 rpm at 37°C until the cells were exponentially growing with optical density at 600 nanometres (OD600) (Ultraspec10, Amersham Biosciences) in the range of 0.4-0.6. Immunotubes were washed 20 times with 0.1% Tween 20/PBS and 20 times with PBS. The bound phages were eluted from the immunotubes with 10 minutes incubation with 100 mM triethylamine (Sigma-Aldrich, Belgium) (pH 12) and then neutralised with 1M TRIS, pH 7.4. This was the output of the first round of selection and it was stored 4°C.

Part of the output was incubated for 30 minutes at 37°C with exponentially growing TG1 cells 2xTY (tryptone, yeast extract and NaCl in H_2O) allowing infection. The infected TG1 cells were centrifuged (Megafuge 1.0R, Heraeus) at 3300 x g for 10 minutes and re-suspended in 2xTY followed by plating on 16cm petri dishes containing TYE agar with 2% glucose and 100 mg/ml ampicillin (amp). In addition for titration, input and output phages were serially diluted from $1:10^{-7}$-$1:10^{-10}$ (input) and $1:10^{-2}$-$1:10^{-6}$ (output). Each diluted phage was left to infect TG1 cells and plated on 10 cm dishes as described above. All of the plates were then grown over night at 30°C in humid environment.

Next day the phage culture bacteria were scraped from the plates with 2xTY (2% glucose, 100 mg/ml amp). The scraped bacteria was then stored at -80°C in 15% glycerol concentration. From the titration plates the titre of each round was counted using the formula (Eq.3.1.)

$$Number\ of\ colonies/plaques\ x\ dilution\ of the\ plate\ x\ 2\ x\ 10 = cfu/ml \qquad (3.1)$$

In the start of selection round 2, TG1 cells were inoculated to 2xTY (2% glucose, 100 mg/ml amp) and grown at 37°C until OD600 reached 0.5. The cells were then infected with M13-K07 helper phage, to enhance infection, in a ratio of 1:20 (number of bacterial

cells:helper phage particles, Eq.3.2.). 1 OD of bacteria at 600 nm stands for approximately $8*10^8$ bacteria/ml.

$$\frac{OD\ reading\ of\ the\ bacteria\ x\ volume\ of\ phage\ culture\ inoculated\ x\ 8{*}108\ x\ 20}{concentration\ of\ helper\ phage} \quad (3.2)$$

Infection was done for 30 minutes at 37°C then centrifuged at 3300 x g for 10 minutes. The pellet re-suspended in 2xTY (100mg/ml amp, 25mg/ml kanamycin (kan)) and grown for 16 hours at 30°C shaking at 240 rpm. Second round of selection was started with coating of immunotubes as described earlier. Next, grown bacteria was pelleted down at 3100 x g for 20 minutes, and the phages were recovered from the remaining supernatant with 1/5 volume of PEG/NaCl (20% (w/v) PEG-6000, 2.5 M NaCl) incubated on ice for one hour. Then the phages were rescued by centrifuging at 3100 x g for 15 minutes. The pellet was re-suspended in PBS and centrifuged at 13 000 x g for 2 minutes to remove bacterial debris. The supernatant was used as input of the next selection round.

3.3 ELISA

Firstly NUNC Maxisorp 96-well plates were coated with E1 and CVB3 for 16 hours at 4° C. Unbound virus was washed away three times with PBS, 5 minutes each. To prevent unspecific binding 3% (w/v) BSA-PBS (30 minutes at room temperature (RT)) was used for blocking.

Next the produced scFv, diluted in 1% BSA-PBS, were bound to virus for one hour at 37° C. The bound scFvs were detected with detection antibodies (specified for each experiment later), which was bound for one hour at 37° C, and washed three times with 0.05% Tween-TBS, 5 minutes each.

For the OD detection enzyme protein A alkaline phosphatase (Calbiochem, USA) was added. It was diluted 1:1000 in 1% BSA-PBS and the plates were incubated one hour at 37°C followed by 0.05% Tween-TBS washes. Substrate p-nitrophenylphospate (PNPP, Sigma-Aldrich, USA) 1 mg/ml in 1M DEA (dietholamine, H_2O, $MgCl_2$ x 6 H_2O, NaN_3, HCl) was added and incubated at 37°C. The reaction was stopped after 1 hour 15 minutes with 3N NaOH and absorbance was measured at 405 nm with a microplate reader Victor (Perkin Elmer).

3.3.1 Polyclonal phage ELISA

Polyclonal phage ELISA was performed to show the accumulation of phage during the five rounds of panning. The inputs of the different panning rounds were added in dilution of 1:200 in 1% BSA-PBS. The detection antibody for the polyclonal phage scFv was mouse monoclonal antibody, B62-FE2 diluted in 1% BSA-PBS, final concentration 0.5µg/ml.

3.3.2 Monoclonal phage ELISA

In order to produce monoclonal phages, single phage clones were collected from the titration plates to 96-well cell culture plates, and grown 16 hours in 2xTY (2% glucose, 100 mg/ml amp). Prepared master plates were then stored at -80°C in 15% glycerol concentration. Small inoculums were collected and grown in 2xTY (2% glucose, 100 mg/ml amp) to exponential growth. Helper phage M13-K07 was added and incubated for 30 minutes at 37°C.

Next centrifuged 10 minutes at 600 x g and supernatant was discarded and pellet was resuspended to 2xTY (100mg/ml amp, 25mg/ml kan) and grown 16 hours at 30°C shaking at 220 rpm. Then centrifuged 10 minutes 600 x g and the supernatants were used, in 1:1 dilution in 1% BSA-PBS, in the ELISA protocol. The detection antibody for the monoclonal phage scFv was mouse monoclonal antibody, B62-FE2 diluted in 1% BSA-PBS final concentration 0.5µg/ml.

3.3.3 Monoclonal soluble antibody ELISA

For monoclonal soluble antibody scFv small inoculums from the master plate were grown overnight in 2xTY with 2% glucose and 100 mg/ml amp shaking in 220 rpm 30°C. Then inoculated into 2xTY with 0.01% glucose and 100 mg/ml amp and grown shaking for 2.5 hours at 37°C. Half of the volume of 2xTY, 100 µg/ml amp and 3 mM IPTG (Sigma-Aldrich, United States) was added and were grown overnight shaking at 30°C. Next day the plates were centrifuged 10 minutes 600 x g and the supernatants were used, in 1:1 dilution in 1% BSA-PBS, in the ELISA protocol. The detection antibody for the monoclonal soluble scFv was the mAb against myc peptide produced in 9E10 hybridoma cells, diluted 1:100 in 1% BSA-PBS.

3.3.4 Characterisation

Characterisation experiments were performed on the highest affinity monoclonal phage scFv. For the characterisation we chose scFv against E1. In the first experiment the phage scFvs were stored overnight at various temperatures; at 4°C, 37°C and 50°C. One set was frozen at -80°C and thawed at room temperature for three times. Secondly, the scFv were stored for 7 days in various temperatures: RT, 4°C, -20°C and -80°C. The effects on thermal stability were analysed with the ELISA protocol described earlier using the scFv in 1:1 dilution. B62-FE2 was used in concentration 0.5µg/ml.

3.4 ELISA optimising

The ELISA protocol was optimised to meet our purposes. In the first optimising experiment two different concentrations (1.5µg/ml and 20µg/ml) of E1 and the scFv library (1:15 and 1:100 dilutions) were tested. In the second experiment the antibody B62-FE2 concentration was optimised, testing different concentrations (0.1µg/ml, 0.2µg/ml and 0.5µg/ml). This experiment was performed without virus proteins by coating the ELISA plate with the phage library.

In addition, we tested various brands of BSA's by their blocking ability. The brands were Albumin fraction V(1,12018,0100 Merck KGaA, Germany), Albumin bovine fraction V (441555J, VWR international, United States), Albumin form bovine serum (A3294 Sigma-Aldrich, United States) and Albumin form bovine serum (A7030 Sigma-Aldrich, United States). Milk powder (2% MP-PBS) was also used in the experiment. MP was also tested as a blocker with an ELISA plate coated with virus.

4 RESULTS

4.1 Selection rounds and characterisation

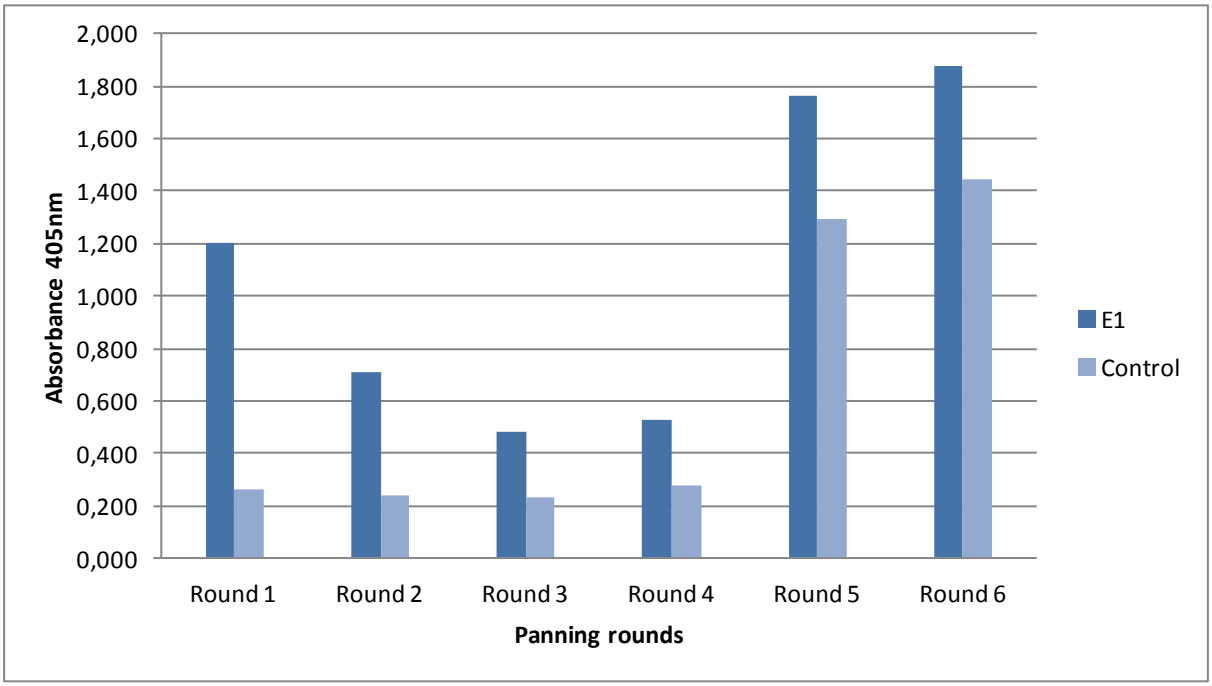
In first part of the study E1 and CVB3 were panned against the Nkuku® library for five rounds, and titres of each round were calculated as colony forming unit (cfu) (table 1). ELISA test (polyclonal phage ELISA) was performed to observe the amount of phage material after each round (figure 3). Both cfu calculations and ELISA experiments showed an increase in virus bound scFv phages.

Table 1: Titres of the panning rounds (cfu/ml). Colony-forming unit (cfu) is an estimate of the amount of viable bacterial cells in a culture counted from a plate.

Round	1.	2.	3.	4.	5.	6.
1. Input	$1.8x10^{13}$					
E1 Input		$1.12x10^{13}$	$2.48x10^{13}$	$2.74x10^{13}$	$1.22x10^{13}$	$6.8x10^{13}$
E1 Output	$1.8x10^{5}$	$2.4x10^{6}$	$1.42x10^{6}$	$5x10^{6}$	$1.2x10^{9}$	
CVB3 Input		$7.6x10^{12}$	$1.98x10^{13}$	$4.26x10^{13}$	$1.58x10^{13}$	$1.4x10^{14}$
CVB3 Output	$8x10^{4}$	$3.6x10^{5}$	$5.8x10^{7}$	$2.5x10^{8}$	$4.2x10^{13}$	

In polyclonal phage ELISA (figure 3) the amount of virus bound phage material was increased from the first selection round to the fifth producing 2.5 fold increase in their absorbance values, thus indicating that the amount of phages elevates with every round of selection. The slight drop from the second round to third round is explained by the increasing specificity. The controls in all ELISA experiments showed high results, similar to the level of the studied samples.

A



B

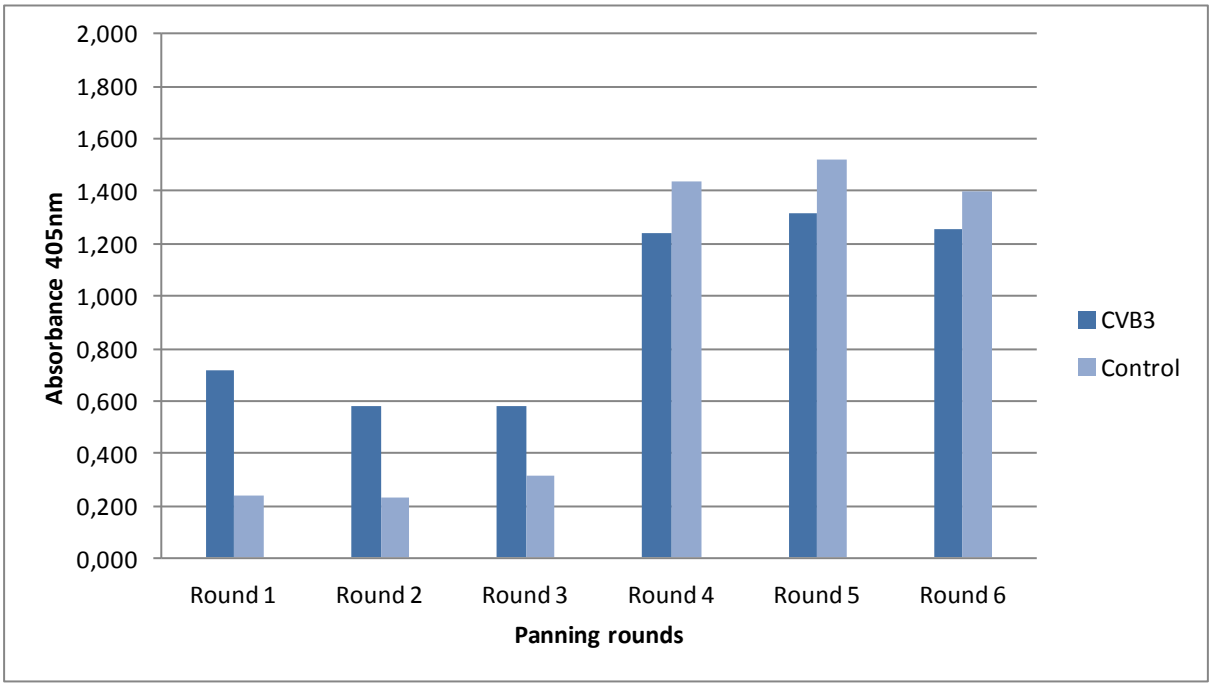
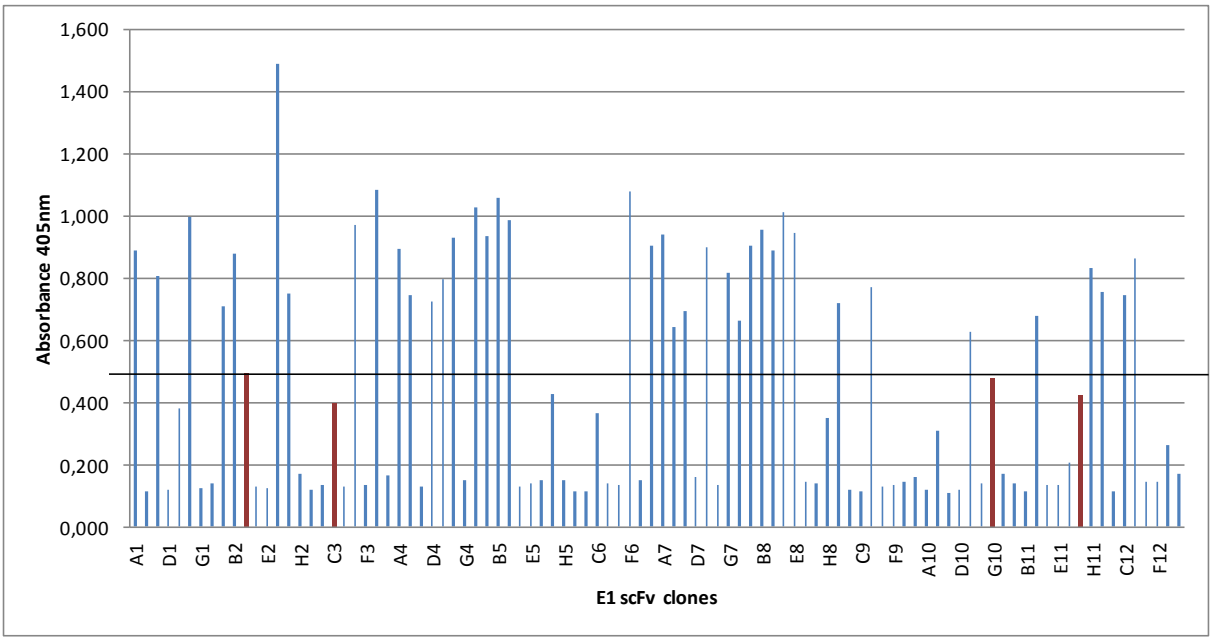


Figure 3: Polyclonal phage ELISA shows the affinity of the phage scFv pool against target virus of the different rounds of panning for (A) E1 and (B) CVB3. 3% BSA-PBS was used as a control.

After collecting randomly 96 individual scFv phage clones, their function to bind target virus was tested with ELISA (monoclonal phage ELISA). The experiments (figure 4) showed several well produced clones towards both E1 and CVB3. The best produced clone F2

showed 3 fold higher absorbance value than the mean absorbance values of phage clones produced against E1. Phage clones against CVB3 showed higher production overall, but did not work as well in individual clones.

A



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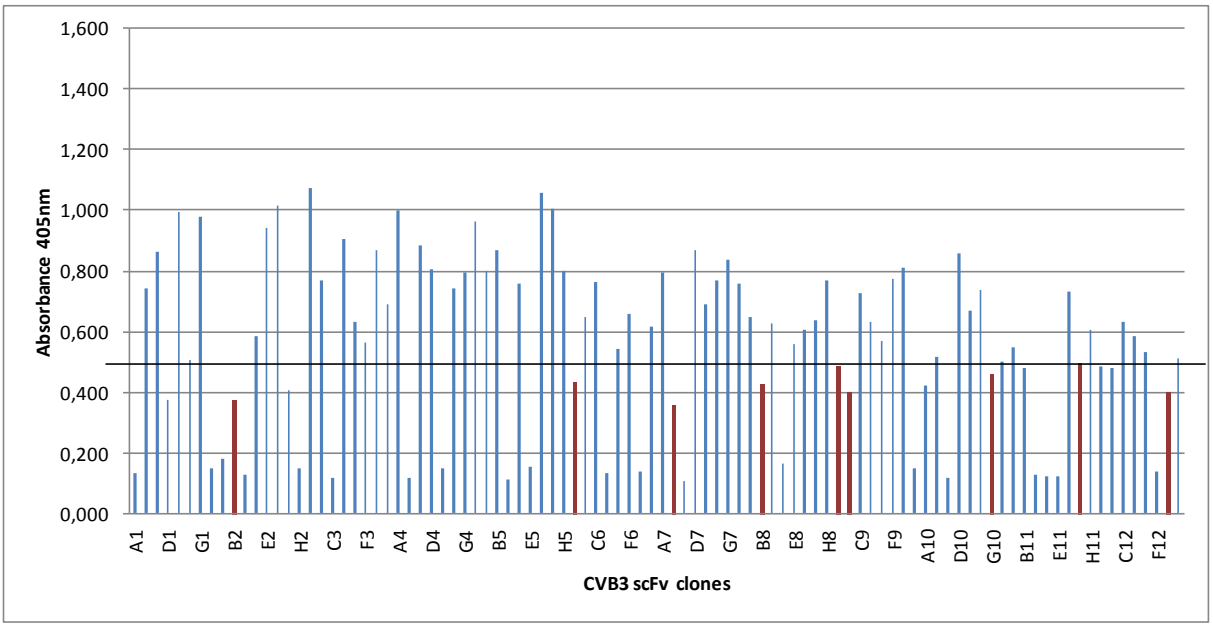
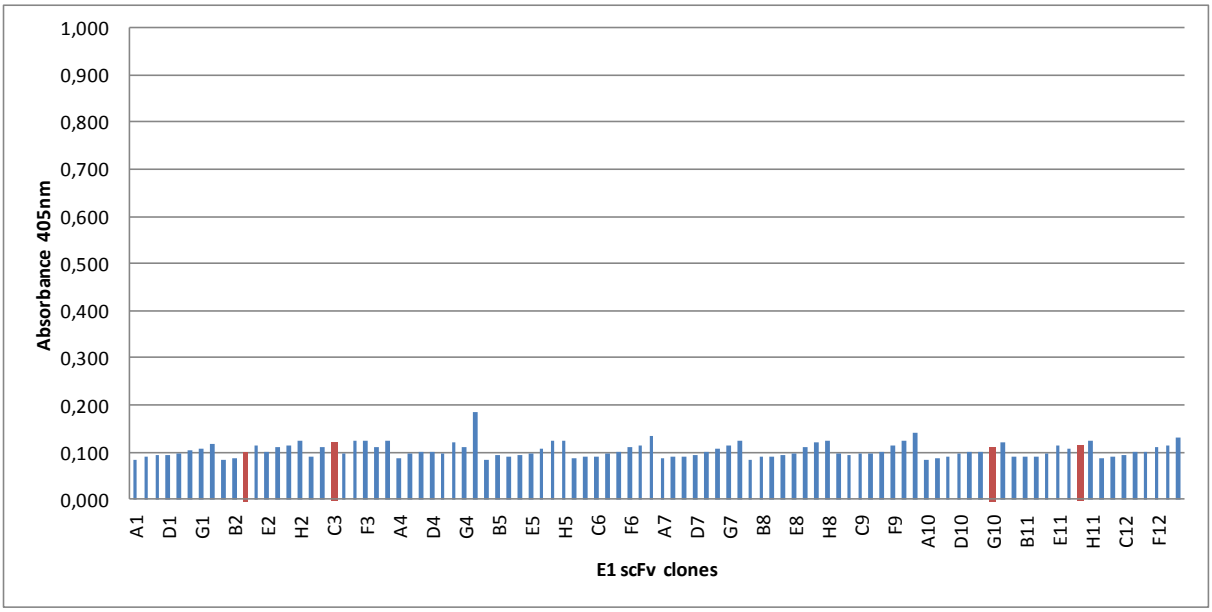


Figure 4: In monoclonal phage ELISA each column represents an individual scFv phage clone collected from a titration plate, (A) for E1 and (B) for CVB3. The columns marked with red did not have visible bacterial matter in them and the line represents the threshold of the results considered reliable.

The phages were removed from the scFv and the soluble scFv ELISA was performed to see whether the selected scFv functioned in solution. The soluble scFvs (figure 5) did not show any binding for either of the viruses. Only one scFv clone (H4) showed a slight affinity against E1.

A



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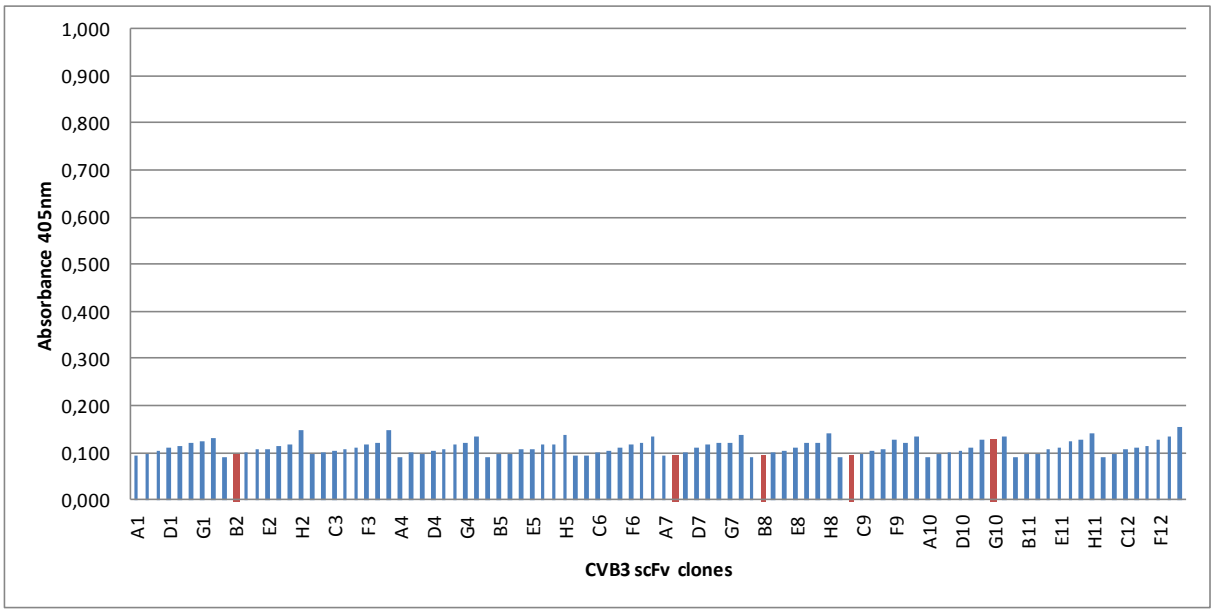
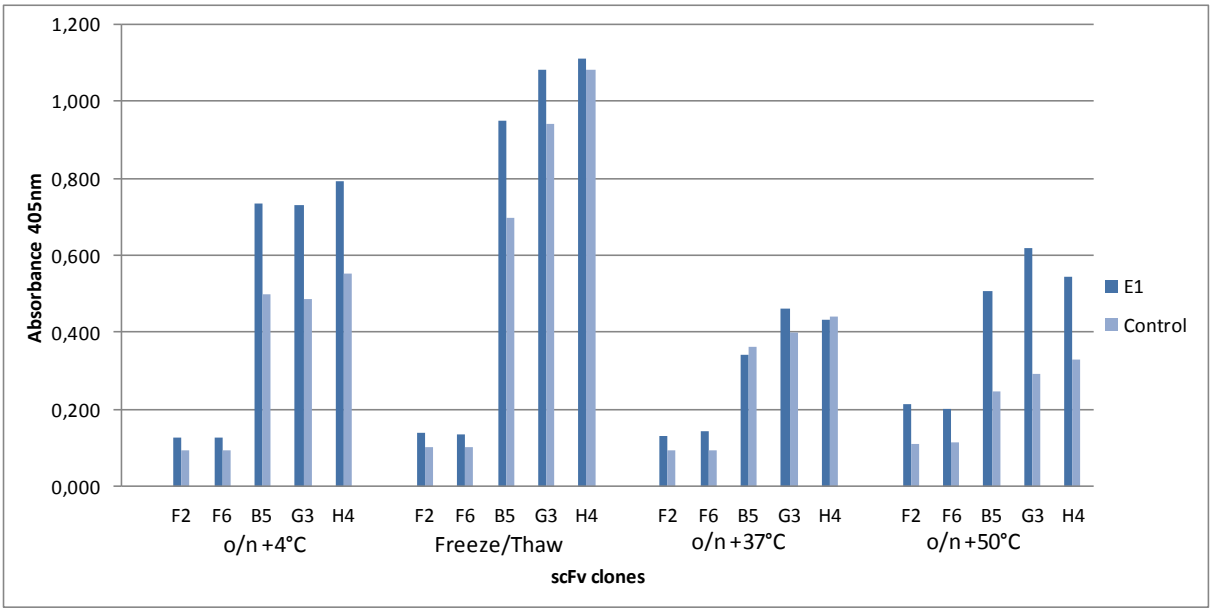


Figure 5: In monoclonal soluble ELISA each column represents and individual scFv clone in a soluble form without the phage, (A) for E1 and (B) for CVB3. The columns marked with red did not have visible bacterial matter in them.

Characterisation experiments were performed with the five scFv clones (in phage displayed form) that showed the highest binding, against E1, producing highest absorbance values in the monoclonal phage ELISA. After exposure to various temperature conditions, the selected scFvs showed some but not total loss of binding to E1 (figure 6). Two of the scFvs, F2 and F6, showed no binding. Storing temperatures lower than RT stabilized the phage displayed scFv better that higher temperatures.

A



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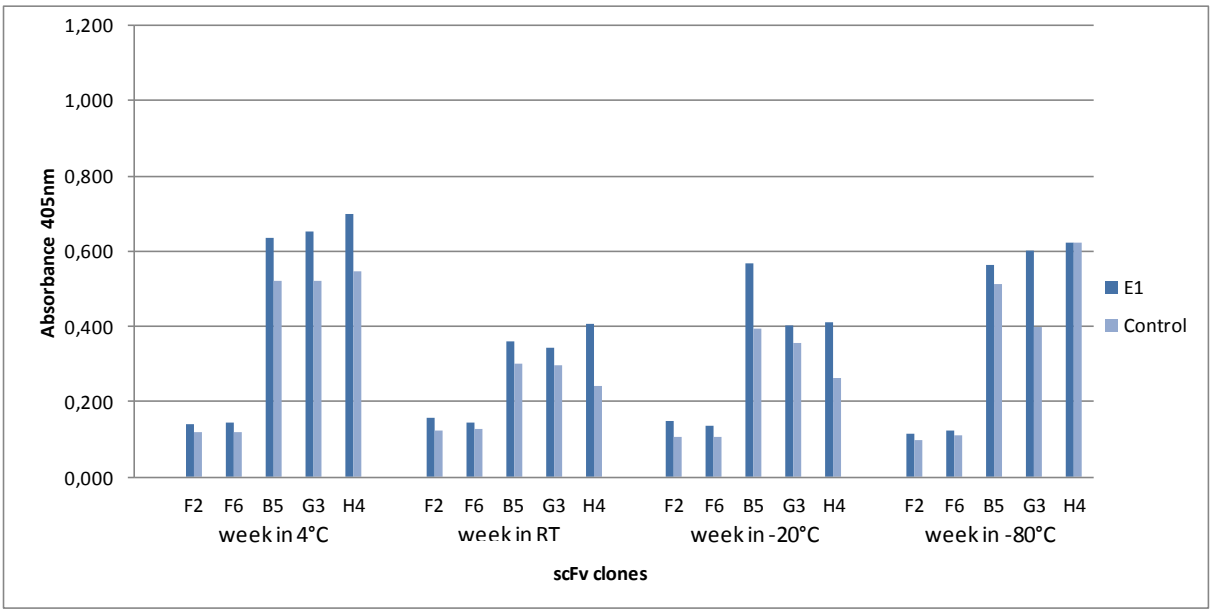
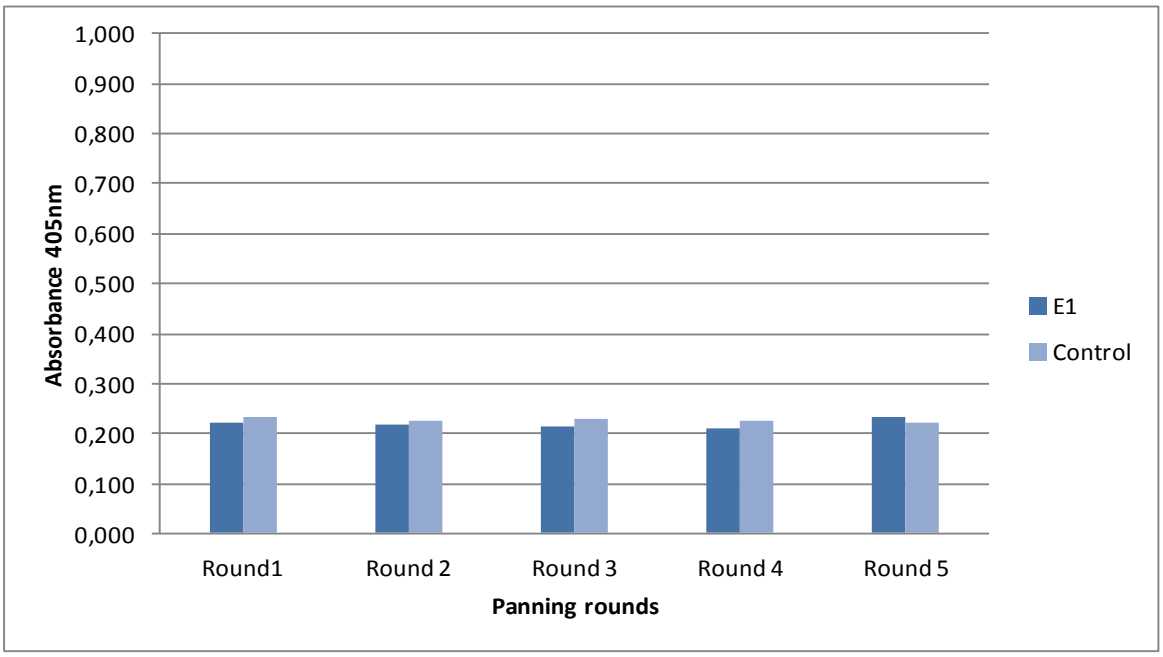


Figure 6: ELISA of the characterisation experiments done with selected E1 scFv monoclonal phage clones, F2, F6, B5, G3 and H4. (A) The overnight preservation experiment and (B) the 7 days preservation experiment. 3% BSA-PBS was used as a control.

4.2 ELISA optimising

The absorbance values from the first polyclonal phage ELISA (figure 7), which was performed with the inputs of different selection rounds, the results were inconclusive showing no difference between the samples and a low overall expression.

A



B

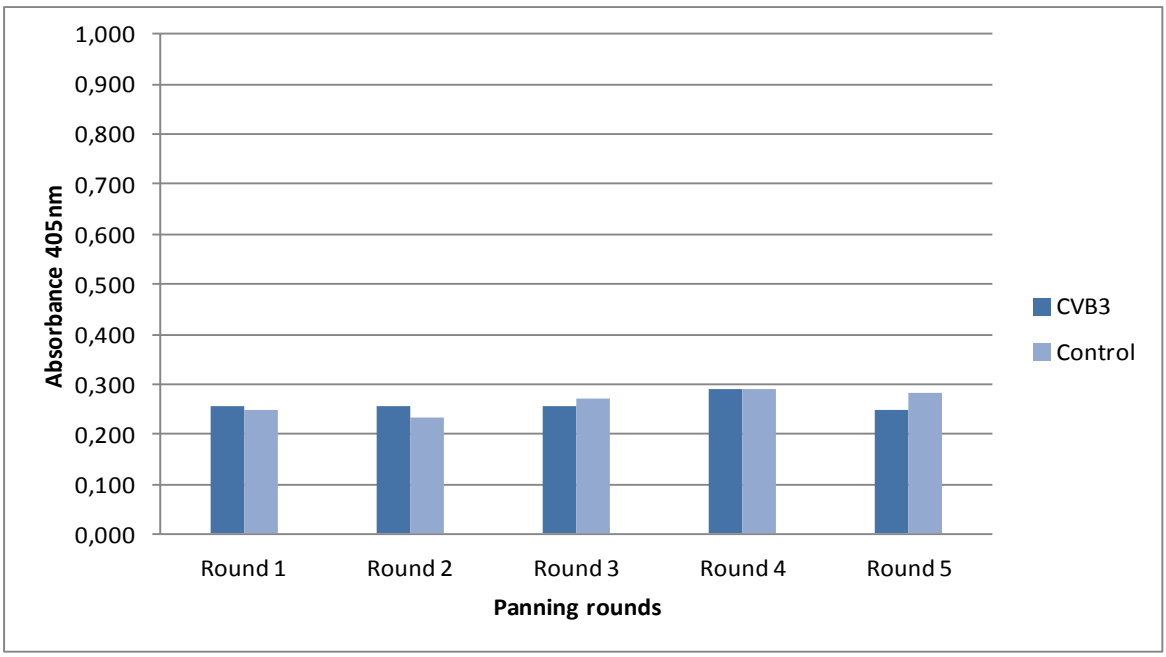


Figure 7: The first polyclonal phage ELISA results showing the binding of the phage scFv pool of the different rounds of panning for (A) E1 and (B) CVB3. 3% BSA-PBS was used as a control.

First we optimised the virus concentration (1.5µg/ml and 20µg/ml) used with anti-E1 antibody and the scFv phage dilution (1:100 and 1:15) (figure 8). Higher virus concentration produced approximately 35% higher absorbance values. The different dilution of the phages showed no difference.

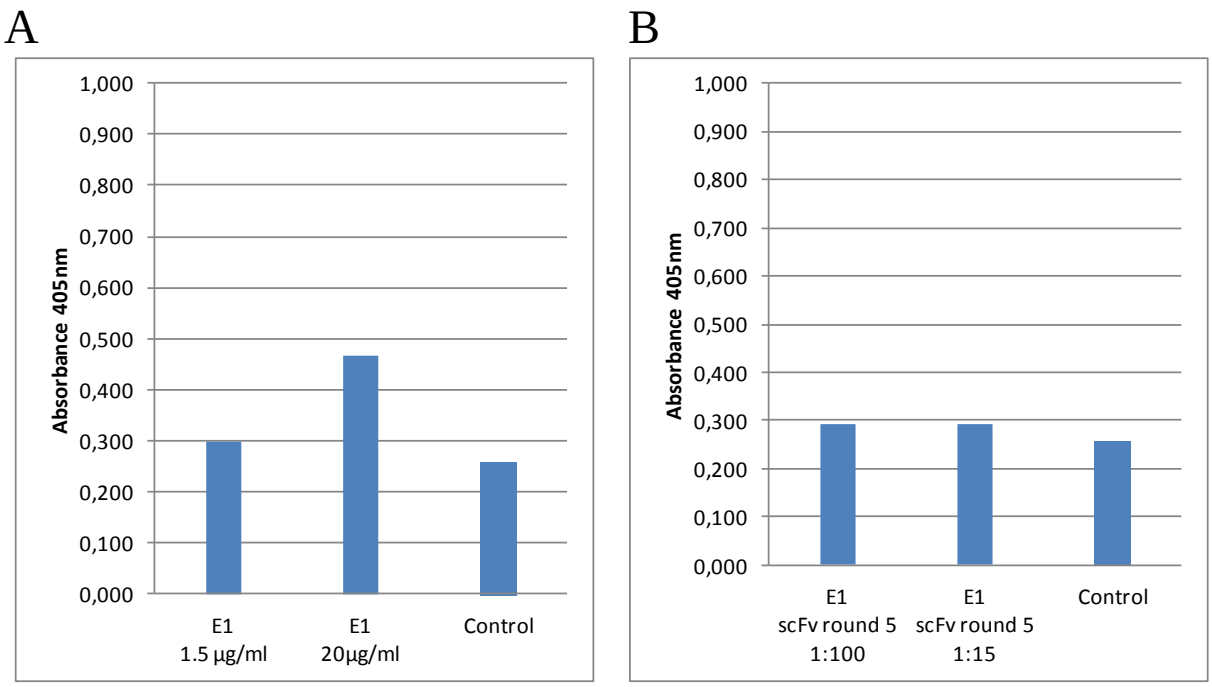


Figure 8: Testing of the different variables in the ELISA protocol. (A) Shows the difference of the E1 virus concentration, (B) is the difference of the phage concentrations. 3% BSA-PBS was used as a control.

The increased phage antibody (B62-FE2) concentrations (figure 9) from 0.1µg/ml to 0.5µg/ml showed an approximately 60% increase in absorbance indicating that higher B62-FE2 concentration was needed. The different scFv phage dilutions showed no difference.

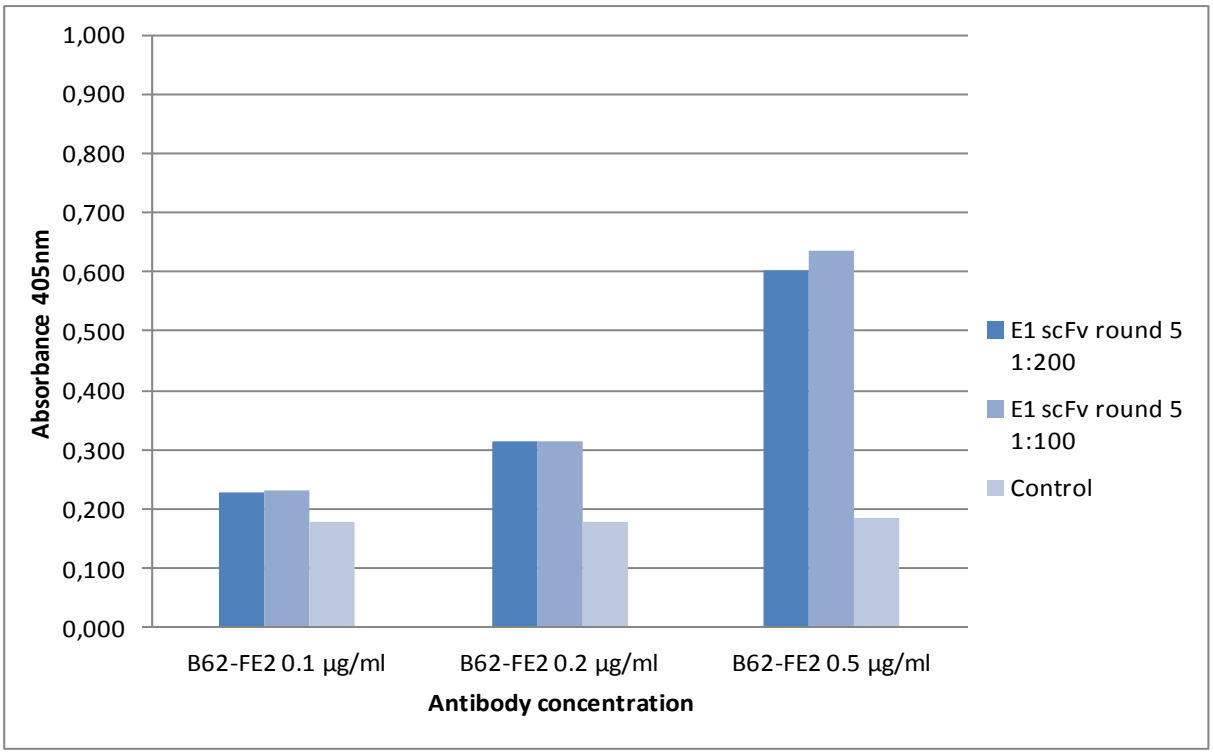


Figure 9: ELISA of the different antibody B62-FE2 concentrations tested on different scFv polyclonal phage panning round 5 dilutions. 3% BSA-PBS was used as control.

Although we were able to optimize the virus and antibody concentrations, we were not able to show specific binding using the polyclonal phage ELISA (data not shown). Later it was discovered that the used detection solution was incorrectly prepared causing a decrease in the absorbance values. All of the selection and characterisation results (figures 3-6) were obtained with the correctly prepared detection solution.

High unspecific binding was a constant problem in the ELISAs. An experiment with various brands BSA's and milk powder showed only minor difference between the BSA's. Highest control value was in the earlier used Merck KGaA and lowest in VWR International (figure 10). Milk powder gave low values in the experiment where the ELISA plate was coated with the blocker. When another experiment was performed coating the plate with virus and blocking with milk powder the reaction was blocked entirely showing no binding (figure 11).

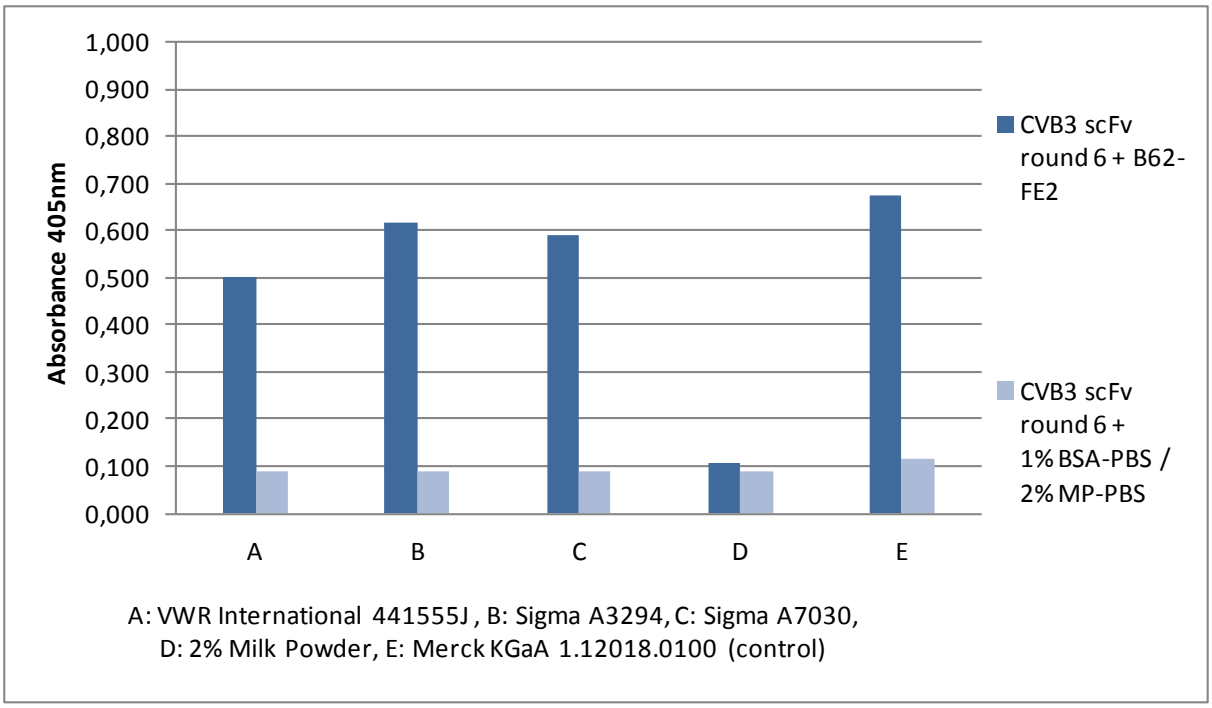


Figure 10: Experiment on the blocking capability of BSA's of different brands (3% solution) and 2% milk powder tested with CVB3 polyclonal phage scFv from panning round 6.

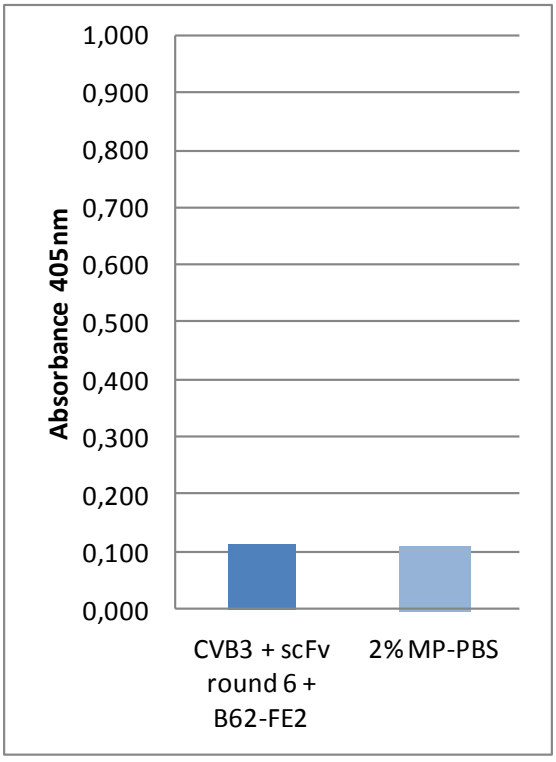


Figure 11: The blocking efficiency of 2% milk powder solution tested with coating the plate with CVB3 and using the polyclonal scFv phages from round 6.

5 DISCUSSION

Enteroviruses, such as E1 and CVB3, are responsible for several diseases including respiratory and enteric disease and myocarditis (Brummer-Korvenkontio 2007: 134-137). Currently there are no proper monoclonal virus-specific antibodies for E1 or CVB3. One used antibody is an enterovirus antibody developed by Yousef et al. (1987) and provided by DAKO (Monoclonal Mouse Anti-Enterovirus Clone 5-D8/1). Also polyclonal antibodies are used (Marjomäki et al. 2002). Polyclonal antibodies have a risk of cross reactivity with, for example, the infected cells. Monoclonal antibodies have a low cross reaction risk which allows the use of them under various conditions. The group-specific antibody, such as the antibody toward the whole enterovirus group, can vary in detection efficiency towards the different individual virus types (Yousef et al. 1987).

The scFv are promising in their use as markers especially when considering their use in cell experiments. The small size allows easy penetration through the cell membrane, but presents a problem in low stability. When removed from the phages the scFv tend to disintegrate. Though the qualities of the scFv can be improved and modified (Wörn & Plückthun 1999).

The bio-panning technique is a simple and inexpensive way to search and produce monoclonal scFv from a large source material. When done in a test tube it can be performed with basic techniques and equipment. With the several types of libraries it is possible to find one that suits for the wanted purpose. The Nkuku® library is suitable to search for antibodies to research use. Since it is made from chicken immunoglobulins, the size of the library is more manageable. With large libraries there is a lot of binding and a possibility to miss some possibly good antibodies. Since the Nkuku® library is naïve it allows to find antibodies for different viruses, but usually the affinity is not as high as with immunised libraries (van Wyngaard et al. 2004). The Nkuku® library has been used to search antibodies for a variety of viruses. Opperman et al. (2012) found good antibodies to SAT2 food-and-mouth disease virus. van Wyngaard et al. (2004) and Rakabe (2008) used the library on African horsesicness and bluetongue viruses. Wemmer et al. (2010) used the library to find scFv against a heat-shock protein in *Mycobacterium bovis* that causes bovine tuberculosis. These results further indicate that the Nkuku® library is a good source of scFv to viruses. Main intend of these studies was to use the scFv in creating reliable and efficient diagnostic tests, as was the main purpose intended for the library originally (van Wyngaard et al. 2004). For example

Opperman et al. (2013) intended to use the scFvs in an ELISA based test to match vaccines based on a serotype of the virus since the scFvs that they found were serotype specific.

As is shown by the titres of the panning rounds and the polyclonal ELISA, the Nkuku® library seems to be a good source of scFv to E1 and CVB3. An increase in phage material shows that the amount and specificity of the scFv increase with every round. These results were similar to other panning results from the Nkuku® library (Rakabe 2008, Opperman et al. 2012) indicating a good output with several different clones.

When the scFvs were selected as individual clones, several of them were promising with high output. This shows that the clones have been produced well and have strong binding to the virus, which is required for the use in cellular contexts. A great variety in binding shows that some individual clones are clearly better than others providing good material for further studying of their properties.

When the phages were removed leaving the scFv in a soluble form, the binding activity decreased greatly. This is not unexpected since some of the particles may not function in the environment outside the phage. Other studies done on scFv have had similar problems with the lack of binding in the soluble form (van Wyngaard et al. 2004, Opperman et al. 2012). The scFv may lose some of the intrinsic affinity when removed from the phage. Expression levels are often also greatly lower and possibly too low to detect. Other possible reasons for lack of binding are in the procedure. There could have possibly been problem with the detachment of the phages with the scFv being also removed from the solution. Although this is not likely since the used protocol was the same as in other studies. Another possible problem is with the anti-c-myc antibody that detects the soluble scFv. Besides the scFv we did not have a proper antigen to use in the testing the self-produced antibody.

Due to the lack of binding in the soluble scFv the characterisation experiments were performed with individual clones of scFv in phage displayed form. Temperature was chosen as the measure of stability. It has been shown that the stability in warm temperatures correlates well with the general stability of the scFv (Wörn & Plückthun 1999). For practical use in cell experiments it is vital that the scFv functions in a temperature suitable for the cells. It is important to know the best possible conditions for the long time storing of the scFv.

The chosen phage clones handled different conditions quite well and there were no great differences between different conditions. This could be expected since the phage strongly stabilises the scFv. The phage particles can cope with severe conditions such as low pH and low temperatures (Arap 2005). If the experiment would have been performed with soluble

scFv the results would have probably been different since the scFv tend to aggregate and lose their binding properties in heat and long-time storage (Wörn & Plückthun 1999).

The results indicate that preservation in 4°C is suitable condition for the scFv, this is similar to an earlier study on scFv preservation which showed that the phage libraries can be stored at 4°C for several years (Smith & Scott 1993). The first experiment had the highest result in the frozen/thawed samples but the week preservation in -80°C gives a slightly lower signal. This could mean that long time preservation in -80°C may not be the most suitable option for the scFv. For more accurate results a longer time exposure experiments are required.

Since the phage bound scFv molecules are stable and easy to detect, they are promising as antibody molecules. The size of the phage is a problem with most methods, taking away the main benefit of using the scFv, but some are still available. The phage bound scFv could possibly be used in virus detection as a secondary antibody in ELISA (van Wyngaard et al. 2004). Willis et al. (1993) used phage bound scFv directly to produce antibodies without use of adjuvants since the phage particles are good immunogens.

There were some considerable problems with the ELISA protocol chosen for this study and a lot of optimising was required for the proper function of the method. The original ELISA results were inconclusive showing no increase in the phage amount. First tested were different concentrations of virus and scFv. Since increased scFv amount showed no increase in binding the secondary antibody (B62-FE2), concentration was thought to be too low. When a higher concentration of B62-FE2 was shown to improve the results it was used. The results seemed to improve after the change of antibody dilution (data not shown) but still were not what was expected. While doing the experiments it was realised that the used detection solution (DEA media) was wrongly prepared and thus did not provide proper results. With properly prepared DEA good results were obtained, except for the high controls.

During this study we were not able to find proper blocking solution, which yielded in the results as high control values. The blocking problem is most likely due to the small size of the scFv. Since BSA is quite large molecule it cannot block all of the smallest binding areas in an ELISA well. When the scFv is added it can bind directly to the wall of the well and then be detected with the secondary antibody.

Studies have shown that different blocking substances can have drastic effect on the result. Xiao and Isaacs (2012) tested different kind of BSA's in ELISA and found that only one type of the tested provided good results. On this account we tested BSA's from different manufactures to see if any of them provided better results. Unfortunately, none of the

available BSA's was able to block the unspecific binding. In addition to BSA, 2% fat free milk powder was studied, which in turn showed to be too potent blocker, blocking the absorbance entirely. For the rest of the experiments, it was decided to use the BSA that provided best blocking in our optimizing studies (Albumin bovine fraction V, 441555J, VWR international, United States).

6 CONCLUSIONS

In conclusion, despite the problems with the protocol optimising, the results indicated that the used Nkuku® library is suitable for finding binders for the two enteroviruses. The monoclonal scFv, while attached to the phage, showed high binding to the virus. In addition, selected individual clones were able to withstand the studied temperature conditions rather well. Further research is required to optimise the protocol and to remove the scFv from the phage particles to enable better characterisation and after that, usage.

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