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SELECTION AND SCREENING OF SINGLE CHAIN VARIABLE FRAGMENT ANTIBODIES AGAINST HUMAN PARECHOVIRUS 1

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BACHELOR´S THESIS | ABSTRACT

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SELECTION AND SCREENING OF SINGLE CHAIN VARIABLE FRAGMENT ANTIBODIES AGAINST HUMAN PARECHOVIRUS 1

This Bachelor's Thesis was written in the Department of Virology and Department of Biochemistry/Biotechnology at the University of Turku. The goal of the thesis was to select and screen antibody binders against human parechoviruses from synthetic scFv human antibody libraries. The phage display method was used to express scFv molecules on the surface of phage particles. These were subsequently used in three consecutive panning rounds to identify scFv binders to recombinant viral protein (human parechovirus VP0) that was biotinylated and immobilized onto the bottom of streptavidin coated wells. ScFv phage particles that bound to target protein were inoculated onto bacteria to enrich the phage population and purified, and analyzed further by Eu-Delfia. Increase in specific signals against controls between panning rounds was regarded as a sign of a successful isolation of a phage preparation containing scFv binders specific for viral proteins. The scFv gene was subcloned from phage library to vector pLK06FT, which encodes a scFv-AP-His-FLAG fusion protein. Individual cell clones were identified and scFvs were isolated and screened on pNPP-ELISA. Positive clones were saved for further assay and sequence verification.

KEYWORDS:

Picornavirus, scFv, phage display, parechovirus, ELISA, coat protein

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ABBREVIATIONS

Ags	Antigens
AIROPico	EU-project, abbreviation stands for Academia-Industry Research and Development Opportunities for Picornaviruses
BCA	Bicinchoninic acid assay aka Smith assay
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
Eu-Delfia	Eu labelled Dissociation-Enhanced Lanthanide Fluorescent Immunoassay
GST	Glutathione S-transferase, helper tag in protein purification
HPeV-1	Human parechovirus
PBS	Phosphate-buffered saline
pNPP	para-Nitrophenylphosphate = chromogenic substrate for acid and alkaline phosphatase in ELISA
RNA	Ribonucleic acid
scFv	Single chain variable fragment
VP0	Human parechovirus coat protein 0
scFvP	Synthetic human antibody library for protein selection
scFvM	Multipurpose synthetic human antibody library
TBT-0.05	Panning buffer PBS/BSA with 0.05 % Tween
RT	Room temperature (approximately 20 °C)
AP	Alkaline Phosphatase

1 INTRODUCTION

The practical work was done at the Department of Biotechnology and the Department of Virology at the University of Turku. The research project was part of an EU-funded AIROPico project, which stands for Academic-Industry R&D Opportunities for Picorna-viruses. The goal of the study was to identify antibody binders for different picornavirus types to be used in medicine or in diagnostics. Two phage display antibody libraries were used to identify potential picornaviral binders. The phage display method was used to express scFv molecules on the surface of phage particles. These were subsequently used in three consecutive panning rounds to identify scFv binders against recombinant viral proteins. The pool of phages was inoculated onto Escherichia coli to enrich the phage population. ScFv coding genes were subcloned into a vector, which encodes a scFv-AP-His-FLAG fusion protein. The vector was inoculated onto *E. Coli* and individual cell clones were identified. These cell clones were used to produce mono-specific scFv antibodies against picornaviruses. The scFv antibodies were analyzed by ELISA and plasmids encoding the antibodies were sequenced for the binding region. The process behind the thesis is presented in Figure 1

Figure 1. Process flow chart of the thesis.

1.1 Picornaviruses

Picornaviruses are very small (about 30 nm in diameter), non-enveloped, spherical and positive stranded RNA viruses. Picornaviruses belong to order Picornavirales and to Family Picornaviridae. The family consists of 29 genera (Appendix 1). Picornaviruses

cause a wide range of diseases from asymptomatic or mild flu-like illnesses to serious syndromes such as aseptic meningitis, encephalitis, febrile rash illnesses, conjunctivitis, herpangina, myositis, myocarditis and hepatitis. They are also the most common pathogens in human, which cause respiratory tract illnesses and the hand, foot, and mouth disease (Lutwick 2014). Parechoviruses cause mild gastrointestinal or respiratory illnesses and have been implicated in cases of myocarditis and encephalitis in young children (Wolthers et al. 2008). In one study, a seroprevalence of HPeV-1 was found to be 97%, which indicates that almost all individuals have encountered HPeV-1 infection in the childhood (Joki-Korpela et al. 2001). Picornavirus capsid consists of a tightly packed icosahedral arrangement of 60 protomers. Each protomer consists of four capsid proteins (VP1-4, which all derive from the cleavage of a large viral polyprotein (Figure 2). Unlike other picornaviruses human parechoviruses do not have separate VP4 protein but instead they have VP0, which is a fusion protein containing both VP2 and VP4 (Viralzone.org, 2016).

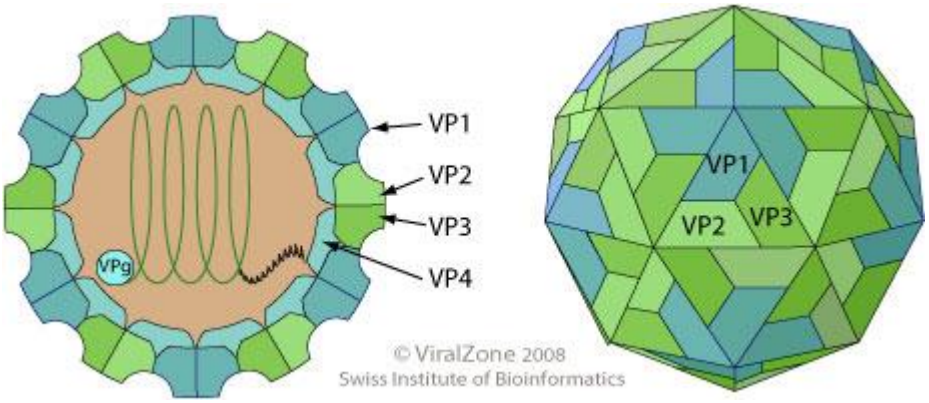

Figure 2 the General structure of picornaviruses (www.expasy.org/viralzone, Swiss Institute of Bioinformatics, 2014).

1.2 Phage display and panning

Phage display is an in vitro technique where target protein, e.g. an antibody molecule is expressed on the surface of phage particlet in fusion to phage coat protein (Hoogenboom et al. 1998). Phage display can be used as a library, which means that it may contain up to 10E11 different phage particles displaying different target antibodies on the phage surface. Identification of a binding of any displayed antibody to a specific target protein can be done using the phage display panning method. This means a coating of the target antigen onto plate or beads, and then incubating a phage display library in the

wells or with beads. Non-binders can be removed from the mixture allowing only binder phages to be isolated. These phages can be enriched in *E. Coli* to generate a new pool of phage particles to be used in the next round of panning. This time the new pool contains a large number of target-specific antibodies. Typically, three rounds of panning are performed (Figure 3). Compared with standard hybridoma technology for production of target-specific monoclonal antibodies, phage display is a much faster method (Carmen, Jermutus 2002). In addition, it is possible to select antibodies, which would never be produced in immunological reactions in the immunized animal. The panning process can also be altered to direct the selection of specific antibodies and their affinity can be improved by phage display affinity maturation technique.

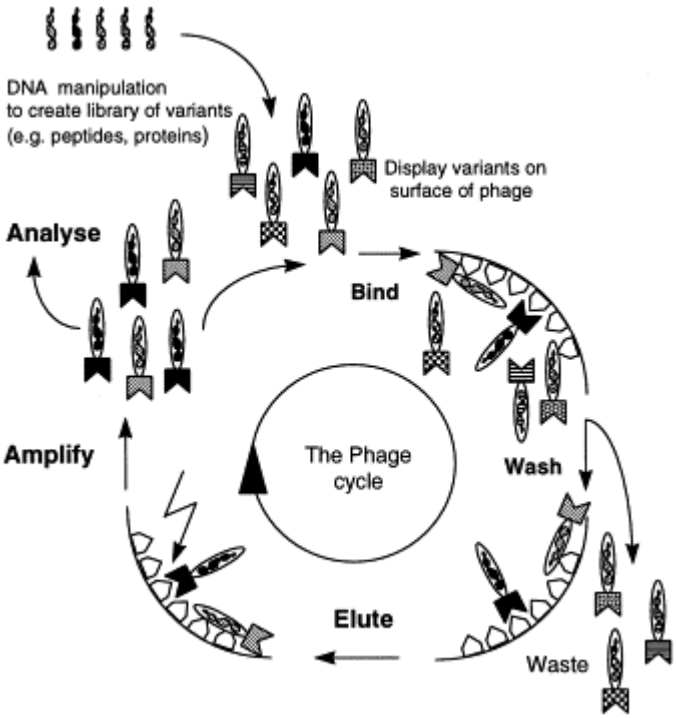

Figure 3 Phage display round (cycle) (Hoogenboom et al. 1998)

2 MATERIALS AND METHODS

Three panning rounds (Figure 4) were conducted to enrich a phage preparation with specific scFv antibodies against recombinant virus coat protein. In each panning round *E. Coli* XL1 Blue cell line was infected with enriched scFv phage preparation, which was subsequently analyzed in Delfia assay using Eu-labelled detector antibodies generated against phage particles. After second and third rounds of panning, scFv genes were isolated from phage DNA and were subcloned into the screening vector, pLK06FT. Individual bacterial cell clones were picked from selection plates, cultivated, and scFv fusion protein production was induced for further analyses.

2.1 Phage display

GST-tagged recombinant virus protein was produced and purified in *E. Coli*. Recombinant HPeV-1-VP0-GST protein was used in phage display to find binders against it. In phage display panning, synthetic human antibody libraries scFvM and scFvP were used. ScFvM with a functional diversity of 6E9 phages and scFvP with a functional diversity of 3E9 phages have phages containing identical antibody VL-VH framework, but they differ in CDR-H3 site from each other (Huovinen et al. 2013). CDR-H3 is the binding site for the target antigen and therefore the most important region in scFv antibodies.

2.1.1 Biotinylation of antigens

In the panning protocol, biotinylated antigens were used. For the elimination of the amino groups which could interact with the biotinylation reaction, the storage buffer (Tris) was changed to PBS-buffer by performing ultrafiltration five times using Amicon Ultra-4 Centrifugal filter with 10 kDa molecular weight cut-off. After the buffer exchange, anti-gens (HPeV-1-VP0-GST and GST control) were biotinylated using Thermo Scientific EZ-Link® Sulfo-NHS-SS-Biotinylation Kit. Biotin concentration used was 0.1 mM. De-salting columns NAP-5 and NAP-10 were used to exchange the buffer. Absorbance with 280 nm wavelength of antigens used were measured before and after biotinylation (Table 1)

2.2 Panning

Phage display selections were carried out in three rounds by using biotinylated antigens and magnetic streptavidin M280 Dynabeads. For negative control, streptavidin coated wells (2) and Neutravidin beads (3) were used.

Panning round 1

In first panning round synthetic human antibody libraries scFvP and scFvM were pooled together.1 mL of each phage library was precipitated by PEG/NaCl to get rid of storage glycerol, pelleted and resuspended in panning buffer TBT-0.05 to reach phage concentration of 5E12 phage particles / mL.

Panning protocol is presented in (Figure 4). Magnetic Streptavidin M280 Dynabeads (Invitrogen) conjugated particles were prewashed and coated with bio-Ags by incubating 30min in rotation at RT. The beads were washed three times with TBT-0.1 and one time with TBS using volume of 1 mL. Diluted phages from passively coated panning were added onto negative selection GST coated beads and incubated in rotation for 1 h at RT. Dynabeads were immobilized with a magnet and phages were transferred onto VP0-GST coated beads, free biotin and GST were added and beads were incubated in rotation over night at 4 °C. Beads were again immobilized with a magnet and supernatant was discarded. Beads were washed three times with TBT-0.1 and one time with TBS to avoid any inhibition in trypsin elution because of BSA in TBT buffer. Sigma Trypsin was added onto beads and incubated 15 – 30 min at RT. Phages were collected from supernatant and Gibco Soybean trypsin inhibitor 10 mg/mL diluted 1:100 in TBS was added onto phages equal volume to eluted phages.

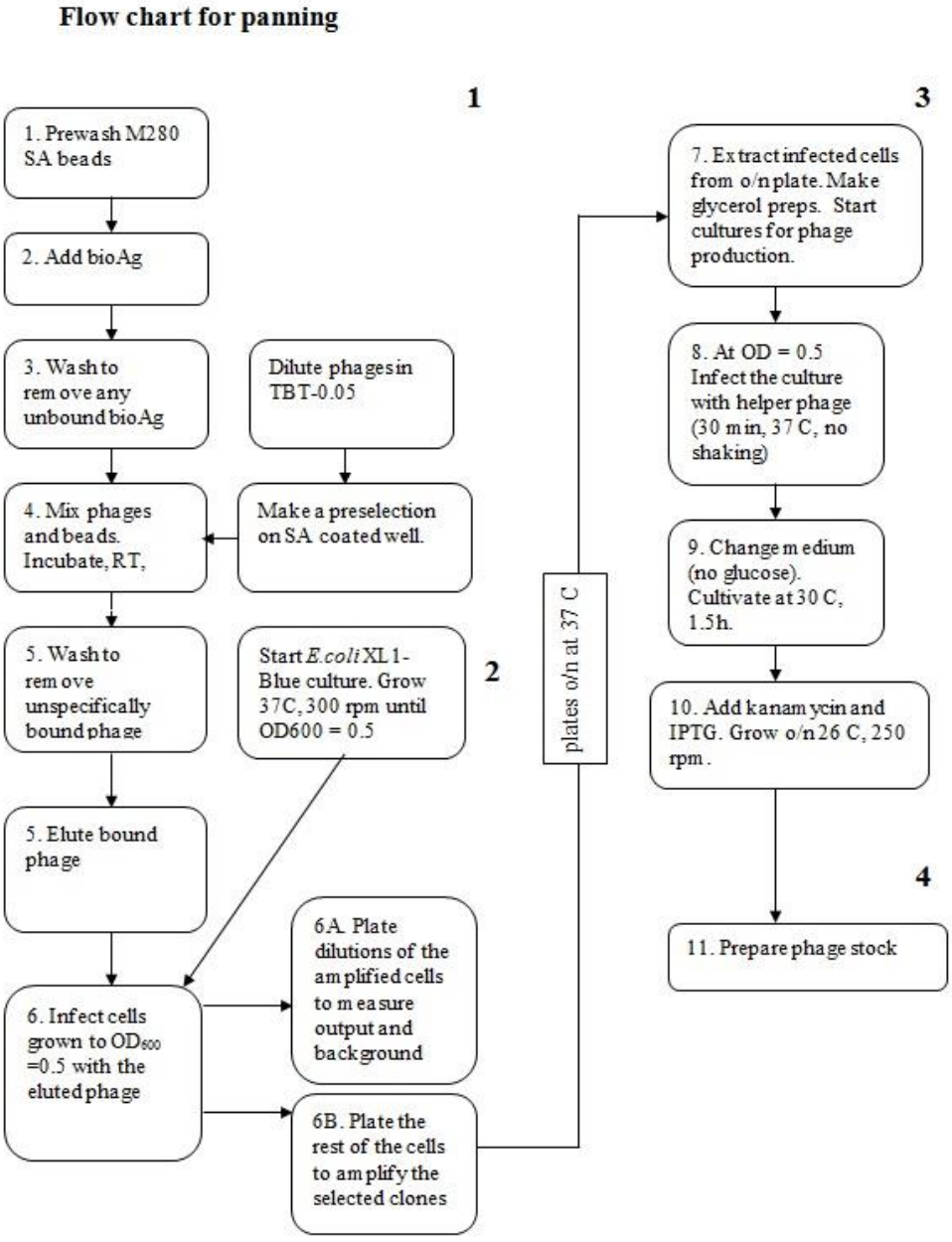

Figure 4. Flow chart for HPeV-1 VP0-GST panning

Cell infection

Escherichia coli XL-1 Blue cells were inoculated into SB medium with 10 µg/mL tetracyclin and 0.2 % glucose. Cells were grown at 37 °C, 300 rpm until OD600 was around 0.5 estimated by a naked eye. *E. Coli* cells were infected with eluted phages and incubated at 37 °C for 30 min to allow infection. Dilution series (10e-2, -3 and -4) were performed to calculate the phage output. XL-1 Blue without infection was plated as a nega-

tive control. 100 µL of dilutions were plated on LA small plates with tetracyclin, chloramphenicol and glucose. Plates were incubated overnight at 37 °C. The rest of undiluted cells were centrifuged at 4000 rpm for 10 min at 4 °C, pellets were resuspended in 5 mL of SB media and all of it was plated on large square LA-Tet-Cm-Glu plates. Plates were incubated overnight at 37 °C.

E. Coli cells were collected from big square plates by adding 15 mL SB medium and gently scraping cells off the plate. 20 % of glycerol was added and glycerol preps were stored in -70 °C. Phage output plates were calculated and finally panning round one phage output was calculated.

For the phage production from glycerol prep, medium of 20 mL SB, 1 % glucose, 5 mM MgCl2, 10 µg/mL tetracyclin and 25 µg/mL chloramphenicol was prepared. The amount of glycerol prep addition was calculated to reach starting OD_{600} 0.05. Cells were cultivated in 37 °C, 300 rpm until OD600 = 0.5. VCS M13 helper phage (Stratagene) were added and carefully mixed avoiding breaking the pilus. Cells and phages were incubated 30 min at 37 °C to infect the cells. Infected cells were pelleted by centrifuging 10 min, 4000 rpm at 4 °C in Eppendorf 5810R swinging bucket centrifuge. Cells were transferred into a fresh batch of medium like above but without glucose. After 1 h growing period, 70 µg/mL of kanamycin and 100 µM of IPTG was added to induce the phage production in *E. Coli*. Cells were grown overnight at 26 °C, 250rpm, pelleted down and discarded. Phages in supernatant were precipitated by adding 1/5 volume of PEG/NaCl solution and incubating on ice for 30 min. Phages were pelleted by centrifuging 10000 g, 20 min, 4 °C and resuspended into 1 mL of TSA/BSA buffer. To make sure that no cells were left, precipitation with PEG/NaCl was performed again until the solution was clear. Phage stock for panning round two was at this point ready. The phage titer was measured by an Absorbance method, which roughly stipulates that 1 OD265 equals 1.23E13 phages / mL. The phage stock was diluted to water in 1:50 dilution and TSA/BSA was used as a blank.

Panning round 2

For panning round two negative selections against SA and GST for VP0-GST were performed by using Streptavidin strips (yellow colored). SA Strips were prewashed with TBT-0.05. Phages from the panning round one were diluted to 10E11 in TBT-0.05 and added onto strips and incubated 1 h at RT in slow shaking (Negative selection against SA). After incubation, phages were transferred onto GST coated streptavidin strips and

incubated for 1 h at RT in a shaker (Negative selection against GST). Negatively selected phages were transferred onto prewashed and bio-VP0-GST coated Dynabeads and incubated 1 h at RT on rotation. Phages were discarded and beads washed three times with 1 mL of TBT-0.1 and one time with TBS. From this point onwards the panning was performed like in the previous round one.

Panning round 3

In the panning round three phage concentration used was 10E11. The final round of panning was performed using HPeV-1-VP0-GST and CsCl-purified HPeV-1 as targets.

6 µg per well of purified HPeV-1 was coated on Nunc Maxisorp 96 well plate in 150 µL per well of 50 mM HCO3-/CO32+ buffer (pH 9.7) and incubated at 4 °C overnight. After overnight incubation, the plate was washed two times with PBS + 0.1 % Tween. Plate was blocked with 2 % BSA in PBS and incubated 1 h in a shaker. Plate was washed four times with PBS + 0.1 % Tween. For the negative selection in non-coated wells, only the wash buffer was added. The background was done without coating and only by blocking with 2 % BSA in PBS. First diluted phages from the panning round two were added onto negative selection wells and incubated 1 h at RT in a shaker. The wells were washed four times with the wash buffer and the phages were transferred to the next row. The plate was incubated 1 h at RT in a shaker and washed four times with a wash buffer. The elution was performed as in panning round two.

HPeV-1-VP0-GST panning was performed as in panning round 2 with two exceptions. Neutravidin beads without any antigen coating as a background were used and infected cells were plated on rounded medium sized LA-plates.

2.2.1 Analysis of binding of phage library to target antigen by Delfia

Time-resolved fluoroimmunoassay was used to measure the binding of phage preparation obtained from panning rounds 2 and 3 to GST, VP0-GST and HPeV-1. Biotinylated antigens were coated onto prewashed streptavidin microtiter plate. 200 ng per well of antigens were added with 200 µL of Kaivogen Red Assay buffer to reach saturation level of 75%. The plate was incubated for 30 min at RT in a shaker. The plate was washed four times with Kaivogen Wash buffer. 125 ng/mL per well of a labelled anti-phage antibody Eu-N1-Mab (N1EuαVCSM13) in Kaivogen Red Buffer was added and the plate was incubated 1 h at RT in a shaker. The plate was washed four times with the wash buffer

and Kaivogen Enhancement solution was added and plate was again incubated for 10 min at RT in a shaker. Time-resolved fluorescence of Europium was measured with Victor multilabel counter.

For analyzing antigens against HPeV-1, the virus was coated onto plate like previously in the panning round three. After the incubation, the plate was washed three times with PBS. Phages were diluted to 10E13 in Red Kaivogen red assay Buffer (AB) and 200 µL per well of diluted phages were added onto plate. The plate was incubated 1 h at RT in a shaker and washed four times with PBS + 0.1 % Tween. Two hundred µL of labelled anti-phage antibody (125 ng/mL Eu-Mab) was added per well and the plate was incubated 1 h at RT in a shaker. The plate was washed four times with the wash buffer and Kaivogen Enhancement solution was added 200 µL per well. The plate was incubated 10 min at RT in a shaker and time-resolved fluorescence of Europium was measured with Victor multilabel counter.

2.3 Cloning the enriched scFv library into screening vector pLK06FT

Plasmid DNA isolation from glycerol preps (PR2-3) was done with Thermo Scientific Miniprep Kit.

Isolation of vector XL1-Blue/pLK06FT was done with Thermo Scientific GeneJET Plasmid Midiprep Kit. Preculture of cells done in 50 mL SB medium with 0.5 % glucose, 100 µg/mL of ampicillin and 10 µg/mL of tetracyclin. Cells were incubated overnight at 37 °C in 300 rpm shaker.

DNA concentration for isolated phage plasmid DNA and vector were measured Nanodrop ND-1000 Spectrophotometer.

SfiI digestions for the vector and plasmids were performed in Tango buffer over weekend at 50 °C. Eco01091 digestions were performed in Tango buffer at 37 °C for 4 h. 0.8 % agarose gel for analyzing the digestions was prepared. The gel was run 45 min with 60 V. Midori green was used as coloring reagent and results were compared to DNA Ladder Thermo Scientific GeneRuler 1kb (Figure 6). A preparative gel for gel extraction of digested vector pLK06FT was prepared. The gel was ran four hours with 50 V and two hours with 60 V. 500 bp band was isolated from the gel using scalpel and GeneJET Gel Extraction Kit was performed. The concentration of gel-purified vector pLK06FT was measured with Nanodrop. Ligations of the vector and scFv inserts were performed using

Fermentas T4DNA ligase buffer and T4 DNA-ligase. Control ligation without insert. Reagents were added and incubated 1 h at RT. The ligase was inactivated by incubating 20 min at 65 °C and then put on ice. New vector pLK06FT-scFv can be seen in Figure 7.

2.3.1 Heat shock transformation with $CaCl_2$ competent cells

Heat shock transformation into $CaCl_2$ competent *E. Coli* cells was performed using 50 µL XL1 $CaCl_2$ competent cells and 5 µL of each ligation reaction. Solutions were incubated 30 min on ice, heat shocked at 42 °C for 90 s. Tubes were then put back on ice for 2 minutes after which 200 µL of SOC medium per tube was added and incubated in 250 rpm at 37 °C for 1 h. Transformants were plated onto two LA plates with 0.5 % glucose and 100 µg/mL ampicillin. Plates were incubated overnight at 37 °C. Number of colonies was calculated. (Table 2)

2.4 Screening of scFv production in microtiter wells

Three plates (PR2, PR3 and PR3-HPeV-1) were prepared. 160 µL of SB medium with 1 % glucose, 10 µg/mL tetracyclin and 100 µg/mL ampicillin was added in each well. Single colonies per every well were picked from transfection plates with sterile toothpicks. The microtiter plates were covered with breathable sealing tape and incubated overnight at 26 °C with 900 rpm and 70 % moisture. The next morning 4 µL of cultures were inoculated into new plates with fresh SB medium with 0.05 % glucose and the same antibiotics. The fresh plates were incubated at 37 °C with 900 rpm and 70 % moisture for 5 h until the cultures looked cloudy. Glycerol preps were made from overnight cultures by adding 40 µL of 80 % glycerol per well and they were stored at -70 °C. After 5 hours of incubation cultures looked cloudy and protein production was induced by adding 20 µL of 2 mM IPTG in SB per well and incubated overnight at 26 °C with 900 rpm. 10x lysis buffer was prepared. 10x lysis buffer contained Alfa Aesar 10 mg/mL Lysozyme from chicken egg white and Merck Benzonase 1x100000U purity grade 2 (>90%) in 10x TBS pH 7,5. The lysis of cells was performed by adding 20 µL of 10x lysis buffer into each well and incubating 30 min at RT. The plates were stored in -70 °C.

2.4.1 scFv-AP screening assay

In total of 288 clones' proteins were analyzed using scFv-AP assay. The assay was performed for all three protein production plates. Two plates for each, one coated with bio-GST as control and the second coated with bio-VP0-GST. Kaivogen streptavidin microtitration plate "normal" were used for coating. Streptavidin plates were prewashed and antigens for coating were used in a manner to reach saturation level of 25 %. Plates were incubated for 30 min at RT and washed four times with Kaivogen wash solution. 180 µL of Kaivogen Red assay buffer was added in each well. Pelleted and lysed cells supernatants from the previous section were used as samples. 20 µL of samples were added in each well. Samples were incubated in slow shaking for 1 h at RT. Plates were then washed four times with Kaivogen wash buffer and 200 µL of 1 mg/mL pNPP in pNPP buffer was added into each well. Plates were incubated with slow shaking for 1h at RT. After one hour of incubation absorbance at 405 nm was measured with Victor. To clearer results, plates were incubated 2 h and measured absorbance again.

2.4.2 Virus coating for scFv-AP screening

4.5 µg of purified HPeV-1 was added on each well in 200 µL of 50 mM HCO_3^-/CO_3^{2+} buffer (pH 9.7) and the plate was incubated overnight at 4 °C. The plate was prewashed with PBS and blocked with PBS + 1 % BSA. The plate was incubated for 1 h on a shaker at RT and washed three times with PBS 0.1 % Tween. Phages were added onto plate in 1:10 dilution in PBS + 1% BSA and the same buffer was used as background. The plate was incubated for 1 h on a shaker at RT. Wells were washed three times with PBS + 1mM $MgCl_2$. 1 mg/mL of pNPP in pNPP buffer was added onto wells and the plate was incubated for 1 h on a shaker at RT. Absorbance at 405 nm was measured and results corrected with blank.

2.4.3 Secondary culture and secondary scFv-AP screening assay

Eighty-four out of 288 analyzed colonies with signals above cut-off value were picked for further analysis from the primary screening. Secondary screening was performed as the primary screening but in a larger scale (5 mL instead of a microtiter scale).

2.4.4 Sequencing

Plasmid DNA was isolated from 84 colonies with Thermo Scientific Miniprep Kit. Plasmid DNA and primers were pipetted on a plate which was then send to Macrogen for sequencing. Sequences were analyzed for CDR-3 region.

3 RESULTS

3.1 Biotinylation of antigens

To increase the specificity in the panning rounds, antigens were biotinylated. Absorbance at 280 nm was measured to see if biotinylation and buffer exchange had worked (Table 1).

Table 1 Antigen absorbance on wavelength 280 nm before and after biotinylation

Antigen	Original Absorbance (280 nm)	Absorbance (280 nm) after biotinylation
HPeV-1-VP0-GST	0,569	0,127
GST-control	-	0,124

3.2 Panning

After three panning rounds, Eu-labelled Delfia was performed to see whether the VP0 specific phage had been amplified or not.

Figure 5 illustrates that panning rounds have been successful and phage titer specific to VP0 is amplified throughout the panning rounds. The background and GST in illustrate that phages did not bind to streptavidin or GST. After three panning rounds using VP0-GST as antigen, phages that bind to purified HPeV-1 are not amplified but that can be due to the restrictions of assay used or the coating buffer pH being too high

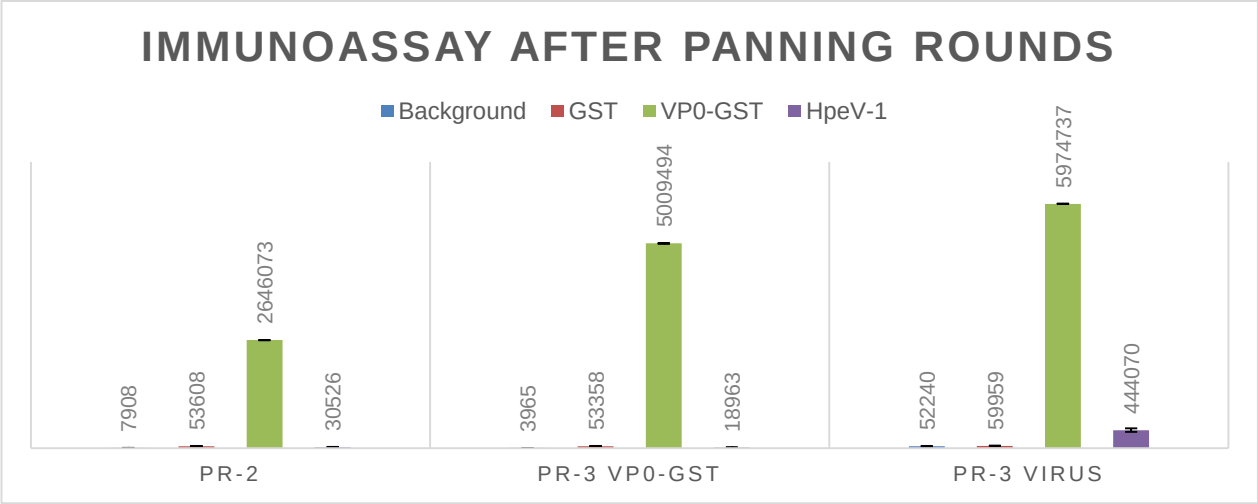

Figure 5 Immunoassay results after panning rounds against SA, GST, VP0-GST and HPeV-1

3.3 Cloning

ScFv fragments were subcloned from scFv phage library to the pLK06FT screening vector. That is, instead of subcloning scFv fragment from individual clones, phage DNA was isolated from phage display library preparation, scFv fragments from all phage particles were ligated into pLK06FT. To see the digestion efficiency an analytical gel was prepared and digested and undigested vectors were added to it with undigested and digested inserts marked with red circles (Figure 6).The picture illustrates the successful digestions of plasmids and vector, and that around 700 bp insert marked with red circle, is cut off from plasmid. After the ligation of the vector and the insert, the new vector should look like in Figure 7. To verify the success of the ligation from plates after the heat shock transformation, a background vector ratio was calculated. The background vector ratio was calculated by comparing control plate readings without the insert to the plate with insert ligated into the vector.

From the left: GeneRuler 1kb Ladder, digested vector, undigested vector, three digested plasmids, three undigested plasmids, Ladder 1kb

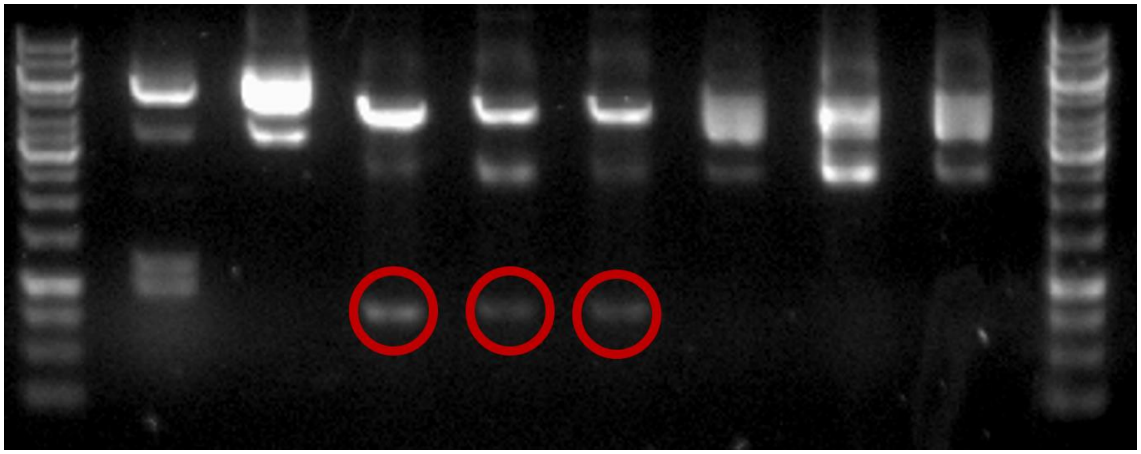

Figure 6 an analytical agarose gel.

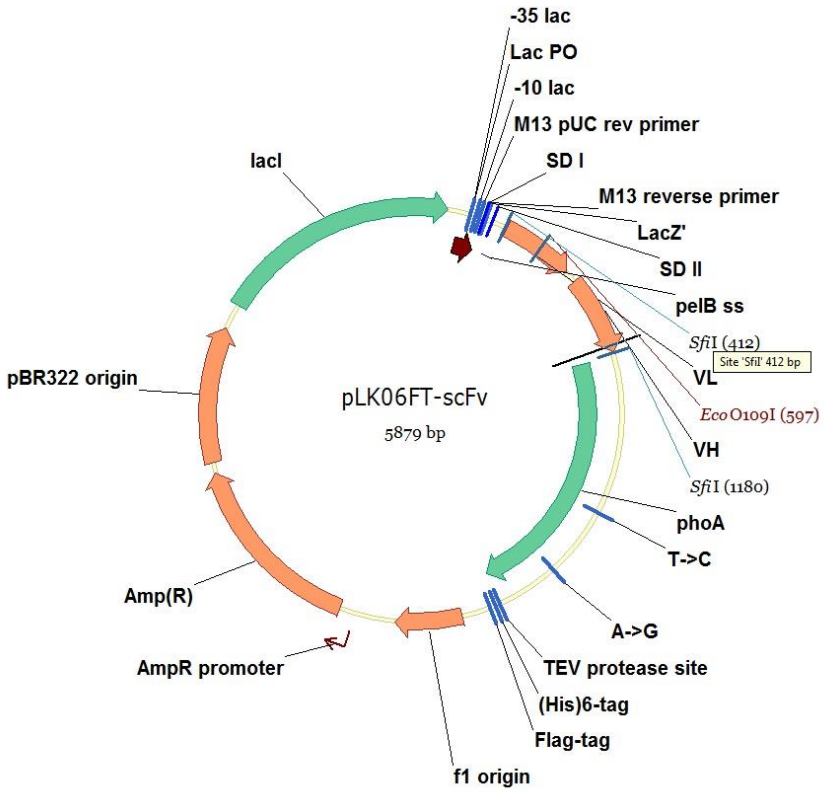

Figure 7 pLK06FT-scFv after ligation.

Table 2 Plate readings after the heat shock transformation.

	pLK06FT-PR2/scFv	pLK06FT-PR3/scFv	pLK06FT-PR3Virus/scFv	pLK06FT Control ligation
25 µL plate	1	26	12	16
The rest plate	753	600	600	5

A background vector =a vector which does not have the insert = $\frac{21}{600} \times 100\% \approx 3.5\ \%$

3.4 Screening

Primary scFv-AP screening was performed to see how many binders against VP0 and HPeV-1 could be found. The assay is based on the fact that pNPP produces a yellow water-soluble reaction product that absorbs light at 405 nm when encountered with alkaline phosphatase which is produced with the scFvs. In total of 288 clones (96 per round) were picked from the transformation plates for the primary screening. Secondary screening was performed to verify the primary screening results and to produce more antigens for further analysis.

From the primary screening (Figure 8), 84 promising clones, marked with a red circle, were found which were further analyzed in the secondary screening (Figure 9). Eighty-four hits out of 288 clones analyzed was a really promising result but it could be said that out of these 288 clones picked from the transformation plates, not nearly all different clones was found ($\frac{84}{288} \times 1991 = 580$). Eighty-four clones was a number that could easily be tested further. The secondary screening results are not that good as primary screening results but it can be seen that the 84 picked clones have properties to bind against VP0 or HPeV-1. In the secondary screening after 3 h incubation graph, the first 31 original absorbance values are picked from the o/n incubation and that is why the absorbance on them is so high. In the secondary screening done against HPeV-1, the results are not optimal even with the pNPP incubation of over weekend. Still from the graph it is visible that phages amplified in panning rounds have properties to bind against purified HPeV-1. The first 31 hits in Figure 9 in HPeV-1 graph are from the panning round three, the next 9 are from the panning round two and the last 44 are from the panning round three done with HPeV-1. The interesting part is that phages from the panning round two

against VP0 had more binders against the HPeV-1 than the panning round three against the VP0.

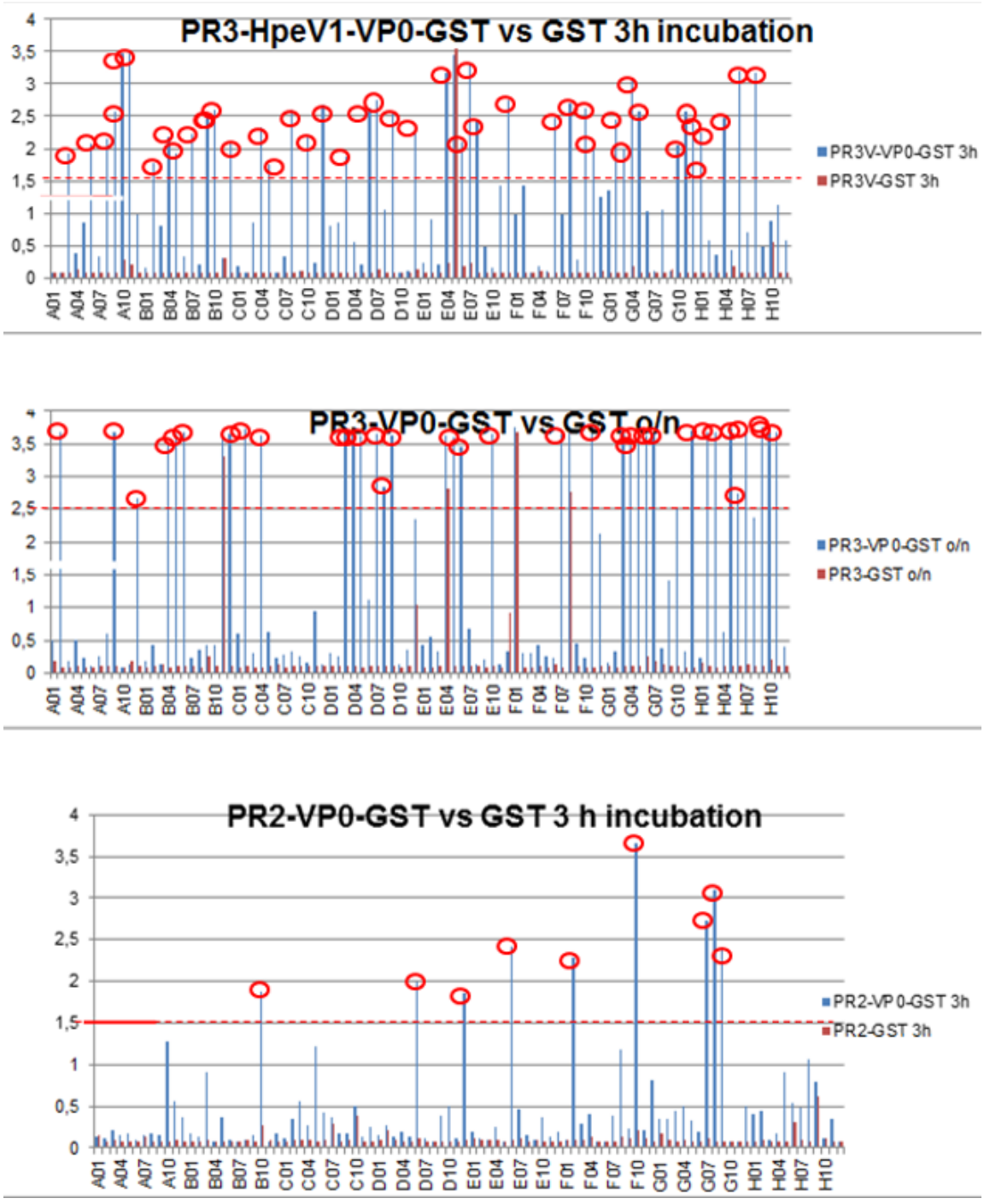

Figure 8 Primary screening results against VP0 and HPeV-1.

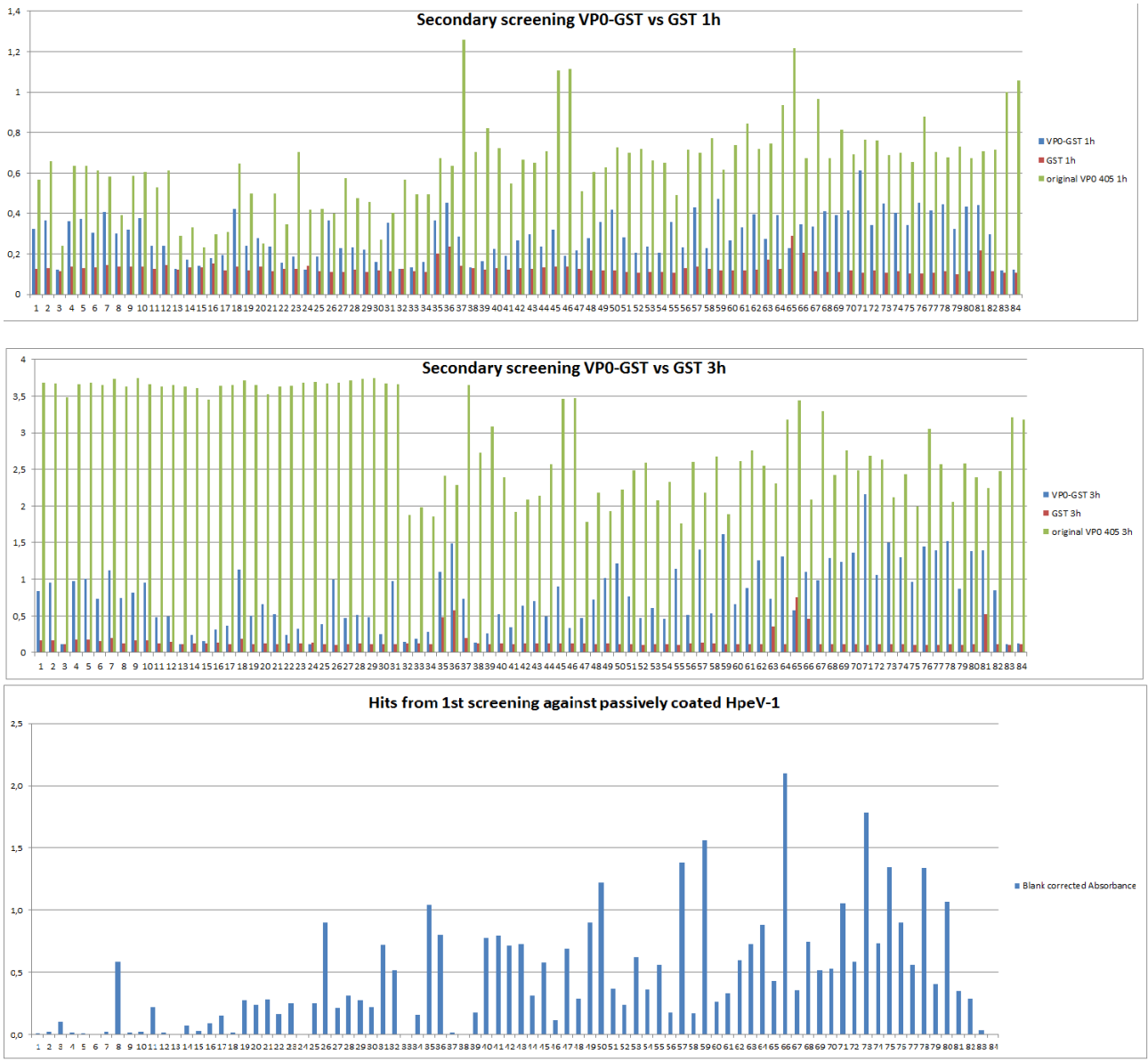

Figure 9 Secondary screening results against VP0-GST and HPeV-1.

3.5 Sequencing

The sequence data was compared with the secondary screening results (Table 3) and 12 different CDR-H3 clone groups, which detected either VP0 or HPeV-1 in secondary screening, were found. From the 84 DNA samples send to sequencing, 16 in total had some errors which made reliable sequencing impossible, eight of them had more than one sequence. Forty out of 84 samples had strong interaction with VP0 in secondary screening. Twenty out of these 40 were from two different CDR-H3 clone group named CTGWAY and CASGAV. Both of them are groups, which have been present in every panning round. The remaining 20 consisted of three main groups CAAESFRGGAY, CASGYI and CATAWLEY and of five individual clones and of one group which recognized only HPeV-1 but not the VP0.

Table 3 Sequencing data against 2nd screening data

CDR Groupname	2nd screening Abs405 - background	CDR Groupname	2nd screening Abs405 - background
CAAESFRGGAY	1,3395	CATAWLEY	0,9465
CAAESFRGGAY	1,1955	CATAWLEY	0,928
CAAESFRGGAY	0,8685	CATAWLEY	0,8325
CAAESFRGGAY	0,7835	CATAWLEY	0,78
CAAESFRGGAY	0,761	CATAWLEY	0,674
CAKTYVDWYLDY	0,865	CATAWLEY	0,5745
CARESWTSLSPDY	1,186	CTGWAY	1,4985
CARGATNRFAY	0,6145	CTGWAY	1,411
CARGIFDL	0,5345	CTGWAY	1,391
CARRYVGNYLDY	0,645	CTGWAY	1,2725
CASGAV	1,269	CTGWAY	0,8955
CASGAV	1,242	CTGWAY	0,8875
CASGAV	1,1845	CTGWAY	0,8625
CASGAV	1,1395	CTGWAY	0,616
CASGAV	1,1205	CTGWAY	0,572
CASGAV	1,0985	CTGWAY	0,514
CASGAV	0,9455	Error	0,9045
CASGAV	0,766	Error	0,865
CASGAV	0,736	Error	0,7915
CASGAV	0,6555	Error	0,781
CASGAY	1,0395	Error	0,647
CASGYI	2,058	Error	0,615
CASGYI	1,282	Error	0,5275
CASGYI	0,536		

4 DISCUSSION

In this study, human parechovirus 1 recombinant protein VP0 and CsCl-purified HPeV-1 particles were used in phage display as a target to find binders against. The first two panning rounds were performed with VP0-GST and the last round with HPeV-1. When comparing results from the panning round three VP0 against the panning round three done with HPeV-1 it seems to be clear that by doing the last round of phage display with the virus one can find more binders which recognize also VP0. Forty-four of 84 clones analyzed in the secondary screening were from the last panning round with the virus. In general, panning rounds were successful, which is evident from the screening results. Signals intensified as the panning rounds went further which supports the fact that phages specific to VP0-GST were amplified (Figure 9).

In the secondary large scale culture of 84 clones, the protein production seemed to have some problems, which can be seen in the graph comparing the primary screening done in 96-well plate results (Figure 8) to 5 mL culture results (Figure 9). Figure 9 illustrates large scale signals being very much lower than in the smaller scale.

At this stage of the project, clones with 12 different CDR-H3 regions were identified. The representative clones should be cultivated in larger volumes, and further analyzed for their functionality in immunofluorescence assay and virus neutralization. Furthermore, binding to different HPeV types as well as HPeV-1 clinical isolates should be examined. The most prominent clones may be subjects of affinity maturation to improve the binding properties.

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Picornavirus family tree

Picornaviridae family (Wikipedia)

Genus	Species
Aphthovirus	Bovine rhinitis A virus
	Bovine rhinitis B virus
	Equine rhinitis A virus
	Foot-and-mouth disease virus
Aquamavirus	Aquamavirus A
Avihepatovirus	Duck hepatitis A virus
Cardiovirus	Encephalomyocarditis virus
	Theilovirus
Cosavirus	Cosavirus A
Dicipivirus	Cadicivirus A
Erbovirus	Equine rhinitis B virus
Hepatovirus	Hepatitis A virus
Kobuvirus	Aichivirus A
	Aichivirus B
	Aichivirus C
Megrivirus	Melegrivirus A
Parechovirus	Human parechovirus
	Ljungan virus
Piscevirus	Fathead minnow picornavirus

Genus	Species
Salivirus	Salivirus A
Sapelovirus	Porcine sapelovirus
	Simian sapelovirus
	Avian sapelovirus
Senecavirus	Seneca Valley virus
Teschovirus	Porcine teschovirus
Tremovirus	Avian encephalomyelitis virus
Enterovirus	Enterovirus A
	Enterovirus B
	Enterovirus C
	Enterovirus D
	Enterovirus E
	Enterovirus F
	Enterovirus G
	Enterovirus H
	Enterovirus J
	Rhinovirus A
	Rhinovirus B
	Rhinovirus C