Oligovalent Fab display on M13 phage improved by directed evolution

Tuomas Huovinen, Hanna Sanmark, Jani Ylä-Pelto, Markus Vehniäinen and Urpo Lamminmäki[1]

Department of Biochemistry and Food Chemistry, University of Turku, 20520 Turku, Finland

[1]To whom correspondence should be addressed.

E-mail: urpo.lamminmaki@utu.fi. Tel: +358 2 333 8052. Fax: +358 2 333 8050

Abstract

Efficient display of antibody on filamentous phage M13 coat is crucial for successful biopanning selections. We applied a directed evolution strategy to improve the oligovalent display of a poorly behaving Fab fragment fused to phage gene-3 for minor coat protein (g3p). The Fab displaying clones were enriched from a randomly mutated Fab gene library with polyclonal anti-mouse IgG antibodies. Contribution of each mutation to the improved phenotype of one selected mutant was studied. It was found out that two point mutations had significant contribution to the display efficiency of Fab clones superinfected with hyperphage. The most dramatic effect was connected to a start codon mutation, from AUG to GUG, of the PelB signal sequence preceding the heavy chain. The clone carrying this mutation, $FabM_{GUG}$, displayed Fab 19-fold better and yielded 2-fold higher phage titers compared to the original Fab.

Keywords

Fab; Phage display; Hyperphage; PelB signal sequence; Start codon; Evolution

Introduction

Recombinant antibody libraries have facilitated the selection of antibody fragments for diagnostic and therapeutic applications. Filamentous phage display is the most widely used technology to select antibody fragments with desired characteristics from large antibody variant libraries ***[1]***. Antigen binding fragment (Fab) has been claimed to be beneficial in phage display applications over single-chain variable fragment (scFv) ***[2]***. The Fab format shows greater thermodymanical stability ***[3]***, and omits dimer formation observed with scFvs ***[4]***. Fab format also is more amenable for automated screening applications as Fabs are more compatible to established immunological assays than scFv fragments ***[5]***.

Unfortunately, Fabs are generally displayed less efficiently on M13 phage than scFvs, because they are two times larger than scFvs resulting in slower folding kinetics ***[6]***. There should also be a balanced expression between the light and heavy chain as Fabs are heterodimers of two independent polypeptide chains ***[7]***. However, it would be very useful to display several Fab molecules per phage to improve the retrieval of phage during the first selection round(s), and, in some cases, to ensure also the binders that interact weakly with the antigen are rescued by the avidity effect. There are several solutions to increase the valency of displayed Fab ***[8,9]***, and one of the most promising approaches to enforce oligovalent display is a modified helper phage known as hyperphage. The genome of the hyperphage lacks gene-3 for minor coat protein (g3p), thus forcing the phage to display the Fab-g3p fusion gene provided in the phagemid ***[10]***.

We have investigated phage display system in which the g3p was fused to a codon optimized Fab molecule. To our surprise we noticed that display of the Fab was heavily impaired if forced to display with hyperphage. Therefore, we set out to investigate whether the oligovalent display characteristics, *i.e.* phage titer and display efficiency of the Fab fragment per phage, could be increased by mutating the Fab randomly and selecting for mutants with improved display characteristics by using Fab binding antibodies.

Materials and methods

Construction of the pEB30-Fab

Standard molecular biology procedures were used ***[11]*** unless otherwise noted. Minipreps, gel extractions and purifications of both PCR and enzyme reactions were done with Qiagen Kits (Hamburg, Germany) according to manufacturers instructions. Restriction enzymes were from Fermentas (Vilna, Lithuania) or New England Biolabs (Ipswitch, UK). The pEB30 vector was derived from the phagemid vector pAKp3fl ***[12]***. FabD gene was constructed by overlap extension PCR from synthetic codon optimized human V_L and V_H and mouse C_L and C_{H1} gene domains purchased from Entelechon (Regensburg, Germany). C_L domain gene was subsequently replaced by non-optimized mouse C_L domain gene with RsrII and NotI, because all sequenced codon optimized C_L domain genes contained frame-shifts. FabD gene was inserted into pEB30 vector SfiI sites.

pEB30-Fab4D5 was constructed from scFv4D5-8 gene that was received from Dr. Plückthun ***[13]***. Variable domain genes were amplified by PCR and joined in two sequential cloning steps

to mouse C_{H1} (IgG_1) and C_L (κ) genes in pAK100 vector. Fab4D5 gene was cloned from pAK100 to pEB30 vector as SfiI fragment.

Generation of random mutagenesis libraries

FabD gene was mutagenized with GeneMorph II Random mutagenesis kit (Stratagene, La Jolla, CA, USA). 250 ng pEB30-FabD template containing 69 ng of 1752 bp target sequence was amplified in 100 µl reaction containing 500 nM primers W0375 (5´-TCACACAGGAAACAGCTATGAC-3´) and pIIIseq (5´-GATAGCAAGCCCAAT AGGAACC-3´), 0.8 mM dNTP´s and 5 U Mutazyme II Polymerase. Conditions for thermal cycling were initial denaturation 95°C 2 min, denaturation 95°C 30 s, annealing 60°C 1 min, extension 72°C 2 min and final extension 10 min 72°C. After 22 cycles 50 µl was withdrawn from the reaction, set on ice and the program was continued to total 30 cycles.

PCR products were purified with PCR Purification Kit and 1/10 of purified mutagenesis products were taken as templates in 100 µl PCR reactions containing 0.2 mM dNTP´s, 500 nM primers WO375, pIIIseq and 2 U Phusion Polymerase (Finnzymes, Espoo, Finland). Cycling was done according to manufacturers instructions with annealing at 65 °C. Amplified products were purified with PCR Purification Kit, digested with SfiI, gel extracted and ligated into pEB30 vector. Ligation reactions were ethanol precipitated using Pellet Paint (Novagen, Darmstadt, Germany) according to manufacturer's instructions and dissolved in 4 µl H_2O. Ligations were transformed into SS320 *E. coli* strain by electroporation (GenePulser II; Bio-Rad Laboratories, Richmond, CA) and recovered in 2 ml SB (30 g tryptone, 20 g yeast extract, 10 g MOPS (pH

7.0) per litre). After 1 h recovery at 37 °C 10 µl of the cultures were serially diluted and plated on LA supplemented with 35 µg/ml chloramphenicol (cm), 10 µg/ml tetracycline (tet) and 1% glucose (glc) for efficiency calculation. The rest of the recovery cultures were diluted to 20 ml SB (35 µg/ml cm, 10 µg/ml tet, 1% glc) and grown overnight (o/n) at 37 °C and 250 rpm shaking.

Preparation of phage library stocks

Next morning the cultures were diluted to OD (600 nm) 0.05 in total volume of 20 ml SB (35 µg/ml cm, 10 µg/ml tet, 1% glc) and grown to OD (600 nm) 0.3. Cultures were infected with helper phage VCS-M13 (Stratagene, La Jolla, CA, USA) 20x MOI. After phage addition cultures were incubated at 37 °C first 30 min without shaking and then 1 h with 250 rpm shaking. Kanamycin was added to 70 µg/ml final concentration and cultures were grown o/n at 37 °C with 250 rpm shaking.

Phage stocks were prepared from the o/n cultures in the following way. 20 ml cell cultures were centrifuged at 12 000 g 10 min 4 °C. Supernatants were transferred to new centrifuge tubes and 1/3 vol 20% PEG/ 1.5 M NaCl solution was added and mixed by vortexing. Phage were let to precipitate on ice for 30 min and centrifuged 10 000 g 20 min 4 °C. Supernatants were discarded, the pellets were resuspended in 1 ml TSA/BSA (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.02% W/V Na-azide and 1% W/V BSA) and spinned 13200 rpm 5 min at 4 °C in tabletop centrifuge. 900 µl of the supernatants were transferred to new tubes and 300 µl PEG/NaCl solution was added. Samples were mixed and incubated on ice for 10 min. Phage precipitates were centrifuged

10 000 rpm 4 min at 4 °C. Supernatant was discarded and the pellet was resuspended in 500 µl TSA/BSA buffer. Phage stocks were stored at 4 ºC for use.

Preparation of phage display stocks

XL1-Blue cells in 20 ml SB (10 µg/ml tet) were infected with library phage stocks 20x MOI at OD(600 nm) 0.3. Cultures were incubated at 37 °C 30 min without stirring, diluted to 100 ml SB (10 µg/ml tet) and incubated 30 min at 37 °C 250 rpm. 35 µg/ml cm was added, and the cultures were grown to OD (600 nm) 0.3 and infected with VCS-M13 20x MOI or with hyperphage 10x MOI for 60 min at 37 °C without stirring. Dilutions were plated on LA (35 µg/ml cm, 10 µg/ml tet, 0.2% glc) for infectivity calculations. Cultures were further incubated 1h at 37 °C 250 rpm, 70 µg/ml kan and 250 µM IPTG were added and grown o/n at 26 °C 250 rpm. The phage stocks were prepared as described above.

Phage panning

For enrichment of efficiently Fab displaying phage 10^{11} cfu were added per well on rabbit anti-mouse IgG (RAM) (Sigma-Aldrich, St. Louis, MO, USA) coated Maxisorp (Nunc Int., Roskilde, Denmark) plate. For unspecific binding control the RAM plates were blocked with 300 ng per well free mouse IgG. The wells were incubated for one hour with slow shake at RT and subsequently washed 12 times with Innotrac Wash Buffer (Turku, Finland) containing 5 mM Tris–HCl, pH 7.75, 150 mM NaCl, 1 g/l Germall II, 0.05 % w/v Tween 20 using 1296-026 Delfia Platewash (Wallac, Turku, Finland). Phage were detached by adding 100 µl 0.1 M glycine HCl pH 2.2 per well and incubating for 20 min at RT with slow shake. Phage were neutralized by adding 2 M Tris-Cl pH 9.0. After brief rigorous shaking the neutralized phage of two replica

wells were combined. For infection 1 ml of XL1 cells at OD (600 nm) 0.3 were added on phage. The cells were incubated for 1 h at 37 °C after which 60 µl of the culture was serially diluted and plated on LA (35 µg/ml cm, 10 µg/ml tet, 0.2% glc) for infectivity calculations and glycerol stocks were prepared from the remainder and stored at -20 °C.

For the next selection round glycerol stocks were inoculated in 20 ml SB (5 mM $MgCl_2$, 35 µg/ml cm, 10 µg/ml tet, 1% glc) and grown o/n at 37 °C 250 rpm. Cultures were diluted to OD (600 nm) 0.1, grown to OD (600 nm) 0.4 and infected with helper or hyper phage as described before. Phage stocks of the infected cultures were prepared as above described. Three panning rounds were done.

Phage titer determination

Phage display stock of Fab4D5 was prepared as described above and infective titer of the Fab4D5 stock was determined in three independent experiments. XL1-Blue cells were infected in mid-log phase with dilutions of the phage stock. After 30 min incubation at 37 °C the infected cells were plated on LA plates (35 µg/ml cm, 10 µg/ml tet, 0.2% glc), incubated o/n at 37 °C and counted.

All other phage titers were determined on streptavidin plates (Kaivogen, Turku, Finland) coated with 100 ng per well biotinylated mouse anti-M13 antibody (Dept. of Biochemistry and Food Chemistry, University of Turku, Finland) using Fab4D5 phage stock with known infectivity as relative phage particle standard and europium labeled rabbit anti-fd (anti-phage) Ab (Sigma-Aldrich, St. Louis, MO, USA) as label. All washing steps in the phage titer immunoassay were

done with 1296-026 Delfia Platewash using Innotrac Wash Buffer and all dilutions were done in Innotrac Assay Buffer containing 50 mM Tris–HCl, pH 7.75, 150 mM NaCl, 0.5 g/l NaN_3, 0.1 g/l Tween 40, 0.5 g/l bovine-γ-globulin, 20 μM DTPA in 1 M NaOH, 5 g/l bovine serum albumin (BSA) and 20 mg/l cherry red. After addition of biotinylated anti-M13 Ab, plate was incubated at RT with slow shaking for 30 min. Plate was washed 4 times, the sample phage dilutions & standard dilutions were added and incubated at RT with slow shaking for 1 h. Plate was washed 4 times and 100µl per well of 130 ng/ml europium labeled anti-phage Ab stock was added. The label was incubated for 1 h at RT with slow shaking and washed 4 times. Delfia Enhancement solution was added (Wallac) and incubated further 15 min. Time-resolved fluorescence signal was measured with Victor 1420 Multilabel Counter (Wallac).

Single colony screening

Single colonies were picked from biopanned library infection plates to inoculate 200µl SB (35µg/ml cm and 10 µg/ml tet) on 96-well plates. As controls FabD and Fab4D5 colonies were also included. Cultures were allowed to grow for 3 h at 37 °C with 700 rpm shaking. Cultures were infected with helper phage VCS-M13 using 20x MOI. After 1 h of infection without shaking and 1 h recovery at 37 °C with 700 rpm shaking, 70µg/ml kan was added and the cultures were induced with 250 µg/ml IPTG. Cultures were allowed to grow o/n at 26 °C. Plates were centrifuged 10 min 4000 rpm and the supernatants were collected in fresh plates. Supernatants were assayed for Fab displaying phage on Maxisorp plates coated with RAM using europium labeled rabbit anti-phage Ab as label. Immunoassay was performed as described above. Minipreps were prepared and integrity of the Fab gene insert was verified by restriction

digestions. Positive clones were sequenced (Sequencing Service, Center of Biotechnology, Turku, Finland).

Separation of pEB30-FabA1 mutations by cloning

Mutations of the FabA1 gene were separated by inserting C-terminal FabD gene fragments into vector providing N-terminal FabA1 gene fragments with HindIII and the following restriction enzyme sites: FabFM_{GUG}MQT, SacI; FabFM_{GUG}MQ, XhoI; FabFM_{GUG}M, BspEI; FabFM_{GUG}, NotI and FabF, RsrII. Variant FabM_{GUG} was gained by combining RsrII & HindIII fragment of the clone FabFM_{GUG} with RsrII & HindIII digested pEB30-FabD vector. Variant FabFM was obtained by combining C-terminal NotI-HindIII fragment of the clone FabFM_{GUG}M with NotI & HindIII digested vector pEB30-FabF. Variant FabC_{H1}* was cloned by inserting C_{H1} domain of the FabA1 gene into pEB30-FabD with SacI and HindIII sites. Fab genes were cloned as SfiI-fragments into pAK400 vector for soluble expression studies. All display constructs were sequenced (Sequencing service, Center of Biotechnology, Turku, Finland).

Display efficiency assay

Phage display stocks were prepared with hyperphage superinfection from 20ml cultures as described above. Phage samples were diluted in Innotrac Assay Buffer (Innotrac, Turku, Finland) and 10^5 phage particles per well was applied on RAM plate. RAM plate assay was performed as described before. The RAM signal was corrected according to the phage titer determined from the same samples on anti-M13 plates.

For Western blotting analysis $2x10^7$ rcfu PEG/NaCl precipitated phage were boiled for 5 min in SDS PAGE sample buffer (75 mM Tris pH 6.8, 1.2 % SDS, 15 % glycerol, 7.5 % β-mercaptoethanol, 9 µg/ml bromophenol blue) and separated on 10 % SDS-PAGE. Proteins were transferred to PVDF membrane (Hybond-P, Amersham Int., Buckinghamshire, UK) and blotted using 1 mg/ml Anti-M13 pIII Monoclonal Ab (New England Biolabs, Ipswitch, UK) in 1:1000 dilution as primary antibody and HRP conjugated sheep anti-mouse Ig (Amersham Int., Buckinghamshire, UK) in 1:1000 dilution as secondary antibody. The membrane was visualized with CN\DAB Substrate Kit (Thermo Scientific, Rockford, USA) with 5 consecutive substrate exposures.

Growth curves

Three 5 ml precultures in SB (35 µg/ml cm, 10 µg/ml tet, 1% glc) were inoculated from single colonies of freshly streaked LA plates and grown o/n at 30 ºC. Cultures were diluted to OD (600 nm) 0.05 with SB (35µg/ml chloramphenicol, 10 µg/ml tetracycline, 0.05 % glc, ± 100 µM IPTG) and 4 x 200 µl of each culture was applied on 96-well plate. Plates were incubated at 37 ºC, 700 rpm with 70 % humidity. Absorbance at 595 nm of the cultures was measured at 1 h intervals with Victor^2 1420 Multilabel Counter (Wallac, Turku, Finland).

Soluble Fab assay and *in vivo* folding

Main cultures of 20 ml SB (35µg/ml cm, 10µg/ml tet, 0.05% glc) were inoculated with precultures to OD(600 nm) 0.05 and grown at 37 ºC with 250 rpm shaking to OD (600 nm) 0.5. The cultures were induced with 250 µM IPTG and grown further 6 h at 30 ºC with 250 rpm shaking. Cells were harvested by centrifugation 12 000 g for 10 min. 1 ml sample was taken

from the supernatant and the rest was discarded. The pellet was resuspended in 1 ml of spheroblast buffer (200 mM Tris-HCl pH 8.0, 500 mM sucrose, 0.5 mM EDTA, 2 mg/ml lysozyme), incubated 15 min at RT and set on ice. Samples were further sonicated on ice and centrifuged 5 min 13200 rpm on table top centrifuge. The supernatant, soluble fraction, was pipetted to fresh tubes and stored at -20 ºC. The remaining cell debris pellet was resuspended in 1 ml spheroblast buffer and stored at -20 ºC. Fab concentrations of the fractions were determined with RAM coated Maxisorp plates using Fab standard and europium-labeled rabbit anti-mouse IgG Ab (Sigma-Aldrich, St. Louis, MO, USA) as secondary label. Otherwise, same assay protocol was used as earlier described. Western blotting was done by separating 200 ng Fab on 12 % SDS-PAGE. Proteins were transferred to PVDF membrane and both chains were blotted using 1:1000 dilution Zymax Goat anti-mouse IgG (H+L) biotin conjugated Ab (Zymed, San Fransisco, CA, USA) as primary antibody and 1:1000 dilution HRP conjugated streptavidin (Zymed, San Fransisco, CA, USA) as secondary antibody. The his-tagged heavy chain was blotted with PentaHis-HRP conjugate (Qiagen, Hamburg, Germany) in 1:500 dilution. The membranes were visualized with CN\DAB Substrate Kit (Thermo Scientific, Rockford, USA) according to manufacturer's instructions.

Results

Enrichment of Fab displaying phage

We studied a chimeric Fab fragment where the light chain consisted of a codon optimized human variable domain gene (V_L germline family κ3) joined to mouse constant domain (C_L) and the heavy chain was a fusion of human variable domain gene (V_H germline family H3) to mouse IgG_1 constant 1 domain gene (C_{H1}). Codon optimization was done to increase the protein expression in *E. coli*. Mouse constant domains were preferred to human domains, because they are more widely used in antibody based assays facilitating the use of binders and the chimeric Fab produced well in *E. coli* cultures.

The chimeric Fab, called FabD, was expressed from phagemid as g3p fusion under the control of lac operon requiring superinfection with helper phage for display (Fig. 1). We observed that the bacteria harbouring FabD-g3p insert grew very slowly, yielded low phage titers and poor display efficiency. Instead of rational design based troubleshooting we committed to a directed evolution approach to improve the unsatisfying performance of the FabD. We decided to randomly mutate the FabD gene and select for improved variants based on Fab display. Error prone PCR was used to introduce random mutations in the light and heavy chains of the Fab gene including the PelB signal sequence of the heavy chain. Two libraries with different frequency of mutations were done by amplifying template either 30 (library A) or 22 cycles (library B). The transformed libraries were superinfected with helper phage VCS-M13 or hyperphage yielding all together four phage libraries. These four libraries differed by mutation frequency and display format (so called mono- and oligovalent display).

In order to isolate improved variants, the libraries were selected against microtiter wells coated with polyclonal rabbit anti-mouse IgG antibody (RAM). The enrichment was monitored with a time-resolved fluorescence based phage immunoassay where the phage were allowed to react with RAM strips and, after washes, detected using europium labeled anti-M13 antibody. After three rounds of selection RAM binding phage were enriched in all libraries as indicated by the increase of the signal per phage unit in the immunoassay (Fig. 2). The enrichment was more efficient for the library A. After three selection rounds the immunoassay signals from library A were 10.9-fold (hyperphage superinfection) and 10.5-fold (VCS-M13 superinfection) higher than the signal of the unpanned library. Respectively, in the case of the library B the signal increased 3.4- and 5.8-fold. In addition, the library A showed efficient enrichment already at the second selection round, but library B did not. Interestingly, the results suggested that the enrichment of phage binding to the RAM plate correlates more with the mutation frequency in the gene than with the display valency.

Single clone screening & sequencing results

Individual clones from the third selection round were analyzed for the Fab display efficiency by displaying Fab in monovalent format with VCS-M13 and by screening for positive clones on RAM coated microtiter plates. 96 clones were screened from the library A and 60 clones from the library B. Fab displaying phage of the well-characterized humanized anti-HER2 antibody 4D5 carrying mouse constant domains with excellent expression and display properties ***[14]*** was used as a positive control. Signal strengths equivalent or exceeding the signal of the positive control were regarded as positive, resulting in 84/96 positive clones in library A and 8/60

positive clones in library B. RAM plate binding of arbitrarily chosen nine positive clones, 5/9 library A and 4/9 library B, was reconfirmed by phage immunoassay and minipreps were prepared. Restriction digestion revealed deletions in Fab gene in four clones which all originated from the library A. The five remaining clones harbouring full-length Fab gene were sequenced.

All the sequenced clones contained several mutations in the heavy chain but only one mutation was observed in the light chain, namely Y102F in the V_L of the library B originating clone FabA1. Residue numbering is according to IMGT®, the international ImMunoGeneTics information system ***[15, 16]***. Interestingly, the clone FabA1 contained also AUG→GUG mutation in the start codon of the heavy chain PelB signal sequence in addition to several other mutations in the V_H and C_{H1} domains. Three of the sequenced clones had identical sequence and originated from the library B. These clones had a mutation UCG→UAG in the framework 1 region of V_H changing a serine residue into amber stop codon. The amber stop codon is suppressed in the host *E. coli* strain XL1-Blue by suppressor supE activity, which results in restored but reduced read-through ***[17]***. A frameshift mutation was observed in the C_{H1} domain of the fifth clone originating from library A. Thus, only the less mutated library B clones turned out to be a valuable source of candidates for further studies as all selected library A clones had undesired genotypes. Mutations in the PelB signal sequence and the heavy chain of the clone FabA1 and FabC10 representing the three identical clones are shown in Table 1. The clone FabA1 was chosen for further functional analysis, because it did not contain the suppressed amber stop codon in V_H, which would totally abolish the Fab expression in nonsupressor host.

Separation of the mutations by cloning

The effects of the mutations in the clone A1 were studied first by replacing C_{H1} domain of the FabD gene with the mutated gene domain. It was found out that the higher display level was not connected to the C_{H1} domain (Fig. 3). Thus, the remaining mutations in the signal sequence and the variable domains were separated in a cumulative manner to assess their contribution to the observed phenotype. The modular structure of the synthetic V_H and V_L genes containing unique restriction enzyme sites in all framework regions of the variable domain genes made it possible to separate the mutations by combining fragments of the mutated gene to the original FabD gene by simple restriction enzyme based cloning. The constructed genotypes are listed in Table 2.

Display efficiency

Clones harbouring different antibody variants were infected with hyperphage and the Fab expression was induced with IPTG to display antibodies on the M13 coat in oligovalent format. Phage particles were purified by double PEG/NaCl precipitation and the display efficiency was assayed by the phage immunoassay performed on the RAM strips by applying same amount of phage particles (10^6) per well.

We have noticed that measuring phage stock titer by infectivity is time consuming and too inaccurate for normalizing the phage particle amounts for the display assay. Especially, if the displayed moiety on hyperphage affects the infectivity ***[10]*** it is difficult to compare phage particle numbers between samples. One solution to this problem would be to use trypsin to restore the wild-type phenotype ***[18]***, but still the accurate titering of the stocks would require several independent infectivity assays. Instead, the amount of phage particles in the samples were determined by immunoassay where the phage were immobilized on the microtiter strips coated

with monoclonal anti-M13 antibody and detected with europium labeled polyclonal anti-phage antibody. Fab4D5 hyperphage stock with known infectivity and containing same genome size as the sample phage was used as a relative phage particle number standard. Thus, the phage particle numbers are compared to each other as relative colony forming units (rcfu). The data was normalized by dividing the signal counts received from the RAM plate by reconfirmed relative number of phage particles. The data in Figure 3 is shown as relative signal to the RAM signal of the bound FabD phage. To exclude the possibility of non-specific binding the samples were also applied on rabbit anti-mouse IgG plates blocked with free mouse IgG.

The binding of the oligovalently Fab displaying phage to the RAM plate was specific and dependent on the displayed Fab as the binding of the phage could be blocked by mouse IgG antibody (Fig. 3). In addition, VCS-M13 helper phage were not able to bind to the rabbit anti-mouse IgG, as expected, further indicating the displayed Fab dependent binding interaction. Relative signal from Fab4D5 phage, included as a positive control, was 12-fold higher compared to the FabD on the RAM plate. Equal level of binding as for Fab4D5 was seen in the case of the clone FabF containing only the mutation $Y102^{L}F$ and in a clone FabFM which contained $L19^{H}M$ mutation in V_H domain in addition to the $Y102^{L}F$. This indicates that a single mutation in the light chain variable domain framework 3 replacing tyrosine to phenylalanine is sufficient to improve the display of the Fab molecule. All the other descendants of the original clone FabA1 including itself showed significantly higher display efficiency than the positive control. The 18 – 22-fold higher display efficiency compared to FabD phage was connected to the mutated start codon of the heavy chain PelB signal sequence (Fig. 3). As a summary, there are three levels of display based on the immunoassay. The highest level is connected to the $M1^{SS}M_{GUG}$ mutation,

the lowest to the original genotype and $Y102^{L}F$ –genotype represents the medium level of display.

The results from the immunoassay showing tighter binding of the single point mutant variants of the FabD compared to the original FabD phage were verified by Western blotting (Fig. 4). $2x10^{7}$ rcfu phage particles of FabD, FabF and $FabM_{GUG}$ as representatives of each display level were separated with SDS-PAGE and blotted using mouse anti-g3p. Calculated mass of the M13 virus g3p coat protein is only 42,5kDa, but it runs at 65kDa ***[19]***. The results showed that the clone $FabM_{GUG}$ having the start codon mutation contains more Fd-g3p fusion protein than the clone FabF. For the phage displaying FabD minimal amount of Fd-g3p fusion if any was detected. In the case of FabD and FabF phage there were more wt g3p than g3p-Fd fusion. In contrast, in the $FabM_{GUG}$ displaying phage most of the gene-3 proteins were g3p-Fd fusions and minority as wt g3p.

Phage production

Total phage titers were determined from the o/n culture medium after superinfection with hyperphage. Surprisingly, it was observed that the phage titers do not totally correlate with the display data (Table 2 & Fig. 3). The most effective virus production, $3.7 \pm 0.5\ x10^{9}$ rcfu/ml, was observed in clones containing the combination of mutations $Y102^{L}F$ in the V_L and $M1^{SS}M_{GUG}$ in the signal sequence of the heavy chain. The clones where these two mutations were separated the $Y102^{L}F$ mutation yielded titers of $1.2 \pm 0.1\ x10^{9}$ rcfu/ml and $M1^{SS}M_{GUG}$ $3.0 \pm 0.2\ x10^{8}$ rcfu/ml, respectively. The FabD phage titer was $1.6 \pm 0.1\ x10^{8}$ rcfu/ml, only half less than the titer of the

$FabM_{GUG}$ phage which display Fab at 19-fold higher efficiency. Phage stocks displaying $FabM_{GUG}$ showed higher display efficiency, but lower titers than FabF displaying phage.

Growth curves

The growth of the *E. coli* cells harbouring FabD, FabF and $FabM_{GUG}$ genes were followed by measuring the OD at 605 nm in microtiter well cultures. The clones were grown in the presence or absence of 100 µM IPTG. It was also tested if there was a growth difference between Fab-g3p fusion and soluble Fab expressing clones. The results showed that IPTG induction of the Fab expression systematically inhibited cell growth (Fig. 5). IPTG did not have an effect on the growth of the host XL1-Blue strain. It was observed that FabD as a fusion with g3p caused impaired growth as well in induced as in uninduced conditions implying that the promoter was active enough to produce the toxic protein also without induction. Surprisingly, in the experiment where soluble Fab was produced, $FabM_{GUG}$ clone was the most sensitive to induction with IPTG resulting in earlier growth cessation.

Soluble Fab concentrations and *in vivo* folding assay

Soluble Fab concentrations were determined from the periplasmic fraction and cell culture supernatant of 20 ml cell cultures grown o/n at 30 ºC under 100 µM IPTG induction. The Fab yields in the periplasmic fraction were 3.8 ± 0.2 µg/ml, 2.6 ± 0.7 µg/ml and 1.4 ± 0.3 µg/ml cell culture for FabD, $FabM_{GUG}$ and FabF, respectively (Fig. 6). Interestingly, the total Fab concentration was about 4 µg/ml with all Fab clones, which is adequate expression level for high-throughput screening of antigen binding clones in library applications. The increased leakage of periplasmic proteins to culture supernatant has been associated with cell lysis,

misfolding and aggregation of the recombinant protein ***[20]***. To find out if there were differences between aggregation of the Fab variants, after extracting the periplasmic fraction (soluble) the remaining cell debris (insoluble fraction) was resuspended in the same volume of buffer as the periplasmic fraction. Then, the volume equivalent to 200ng of Fab was run on SDS-PAGE gel and analysed by Western blotting (Fig. 7).

First we blotted the samples with biotinylated polyclonal goat anti-mouse IgG primary antibodies in combination with HRP conjugated streptavidin as secondary label to detect the light and heavy chain of Fab variants. In order to assess the heavy chain expression independently it was blotted with HRP-conjugated anti-pentaHis antibody recognizing the hexahistidine tag present only in the carboxy terminus of the heavy chain. According to the Western blot experiment FabD is located mainly in the soluble periplasmic fraction whereas a majority of the FabF is in the insoluble fraction. This is consistent with the immunoassay results of the leakage of FabF into the culture medium. In the case of $FabM_{GUG}$ the ratio of soluble to insoluble product is approximately 1:1. Surprisingly, there is only very slight difference in the concentration of the heavy chain between the the start codon mutant $FabM_{GUG}$ and FabD in the soluble fraction.

Discussion

In this study we have displayed randomly mutagenized Fab library on M13 phage and selected for phage mutants binding to polyclonal anti-mouse IgG antibodies coated on solid phase. Two parameters determine the success of the clones in this *in vitro* evolution experiment. First, only phage expressing IgG domains are rescued and second, the phage that produce more progeny between the panning rounds are enriched.

In the display system utilizing hyperphage the virus production is directly connected to the expression and folding of the antibody-g3p fusion ***[10]***. In general the slow folding and large size of the Fab has been shown to interfere with phage coat packing resulting in decreased amount of phage produced ***[1]***. Thus, it is anticipated that in-frame deletion mutants that still retain some immunoglobulin domains for selection are enriched during the biopanning of the library. Four of the nine selected positive mutants had deletions in Fab genes based on restriction mapping. One of them was sequenced and turned out to be a clone containing the light chain fused to g3p. The small size of the displayed protein which is adequate to withstand the selection conditions combined to efficient phage coat packing made this mutant very competitive.

Several mutants with improved display characteristics were enriched in the biopanning experiment. One of the mutants was taken into closer examination. The selected clone FabA1 yielded 27-fold higher phage titers and 19-fold higher Fab display level than the template FabD. FabA1 contained altogether seven mutations of which one in V_L, one in PelB signal peptide, three mutations in V_H and two mutations in C_{H1} gene domain. After excluding C_{H1} domain

mutations as insignificant, the contribution of each remaining mutation to the final phenotype was analyzed. The most relevant mutations for the improved display characteristics were $Y102^{L}F$ in the V_L and $M1^{SS}M_{GUG}$ in the PelB signal sequence in front of Fd gene.

In contrast to the general idea of forced oligovalent display in which the sole source of the g3p is the Fd-g3p fusion gene in the phagemid, high phage titers are necessarily not connected with high display level of the g3p fusion partner. Higher phage titers were repeatedly gained with the mutant FabF than with $FabM_{GUG}$, although more Fab per phage could be displayed with Fab_{GUG}. A potential explanation for this controversy is the proteolytic degradation of the g3p fusion protein at the periplasmic space of *E.coli*. This claim is supported by the report that even in a system where phagemids have not been used and the display protein is fused to the g3p in the phage genome only 20–30% of the g3p carry intact fusion moiety ***[21]***.

Especially the mutation $Y102^{L}F$ can be considered destabilizing and thus rendering the fusion moiety exposed to proteases. The highly conserved residue $Y102^{L}$ is located in the tightly packed lower hydrophobic core of the V_L domain and speculated to possibly influence the thermodynamic stability of the domain ***[22]***. The destabilizing nature of this mutation was verified by the *in vivo* folding assay results where FabF mutant has remarkable amount of Fab in the insoluble fraction.

The low yield of phage and impaired display ability of the FabD-g3p fusion is partly explained by toxicity. The aberrant cell growth in the presence of the FabD-g3p fusion gene was restored by the single base substitution in the start codon of the heavy chain PelB signal sequence

accompanied by two-fold higher phage titers. According to several reports a change of the start codon from AUG to GUG decreases the expression (two- to five-fold) of the translated gene in *E. coli* ***[23, 24]***. A very slight reduction in the Fd expression of the Fab_{GUG} compared to FabD can be observed in the Western blot of the soluble Fabs. Despite the reduced translation of the Fd-g3p fusion the display of the Fab_{GUG} is outstandingly better than FabD. Jestin et al. ***[25]*** have also successfully increased the display of fusion protein in phage display by mutating only the PelB signal sequence and selecting for improved variants highlighting the importance of proper translocation. In our case, beneficial mutations were also observed in immunoglobulin domains indicating that the improvement might be connected to the *in vivo* folding process in addition to the translocation.

The observations that the original codon optimized Fab performed poorly and that changing the start codon from optimal to suboptimal increased the display have encouraged us to speculate that codon optimization by itself is not necessarily increasing display. In fact, the toxic effect of codon optimized Fab has recently been reported also by others ***[26]***. It seems to be the fine tuned balance of expression between the light chain and Fd-g3p genes and the avoidance of toxic effects that matter more. In general, the directed evolution strategy described here is a valuable and easy to access tool to improve oligovalent display of Fab molecules.

Funding

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Figure legends

Fig. 1. Fab genes and used restriction enzyme sites.

Fig. 2. The enrichment of efficiently Fab displaying phage was analyzed in phage immunoassay. $1x10^7$ rcfu of each panning round were applied to Maxisorp wells coated with rabbit anti-mouse IgG. Bound phage were detected with europium labeled anti-phage antibody. (■) Oligovalent library A, (□) Monovalent library A, (●) Oligovalent library B, (○) Monovalent library B.

Fig. 3. Oligovalent Fab display per phage unit. Black bars represent relative binding of oligovalently Fab displaying phage to microtiter wells coated with rabbit anti-mouse IgG. White bars represent relative binding of oligovalently Fab displaying phage to rabbit anti-mouse IgG coated wells preblocked with mouse IgG. The data presented is the average of three independent experiments ± S.D. Figure legend 1: FabD, 2: Fab4D5, 3: FabA1, 4: FabD C_{H1}*, 5: FabF, 6: $FabM_{GUG}$, 7: $FabFM_{GUG}$, 8: FabFM, 9: $FabFM_{GUG}M$, 10: $FabFM_{GUG}MQ$, 11: $FabFM_{GUG}MQT$ and 12: VCS-M13 helper phage.

Fig. 4. Western blot of the gene-3 coat protein of Fab displaying hyperphage.

Fig. 5. Growth curves of selected clones. (A) Growth curves of clones expressing soluble Fab with 100 µM IPTG (open symbols) and without (filled symbols). (B) Growth curves of clones expressing Fab-g3p fusion with 100 µM IPTG (open symbols) and without (filled symbols). (■,□) FabD; (●,○) FabF; (▲,△) $FabM_{GUG}$; (▼,▽) XL-1 Blue strain without plasmid. Data are expressed as the mean ± standard deviation of three independently grown cultures of each clone.

Fig. 6. Fab yields. Black bar, periplasmic fraction; white bar, culture medium.

Fig. 7. Comparison of soluble (s) and insoluble (i) fractions of sonicated extracts on reducing SDS-PAGE and subsequent Western blotting and immunostaining with (A) Goat anti-mouse IgG antibody and (B) pentaHis antibody.

Figures

Figure 1.

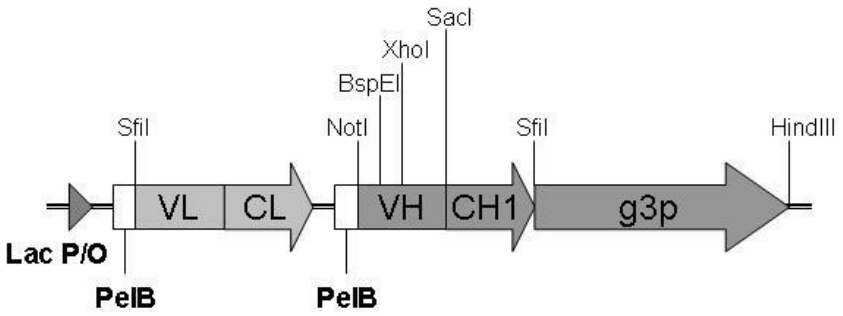


Figure 2.

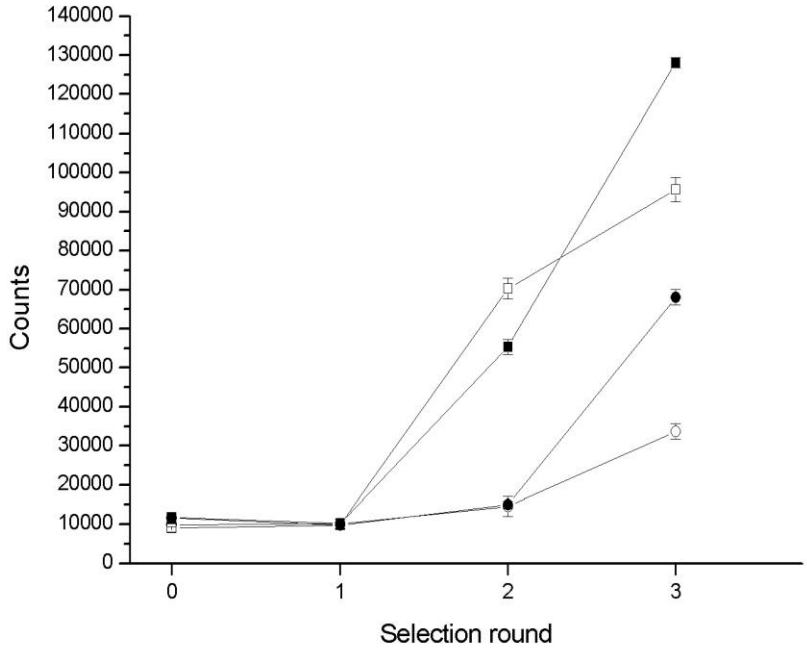


Figure 3.

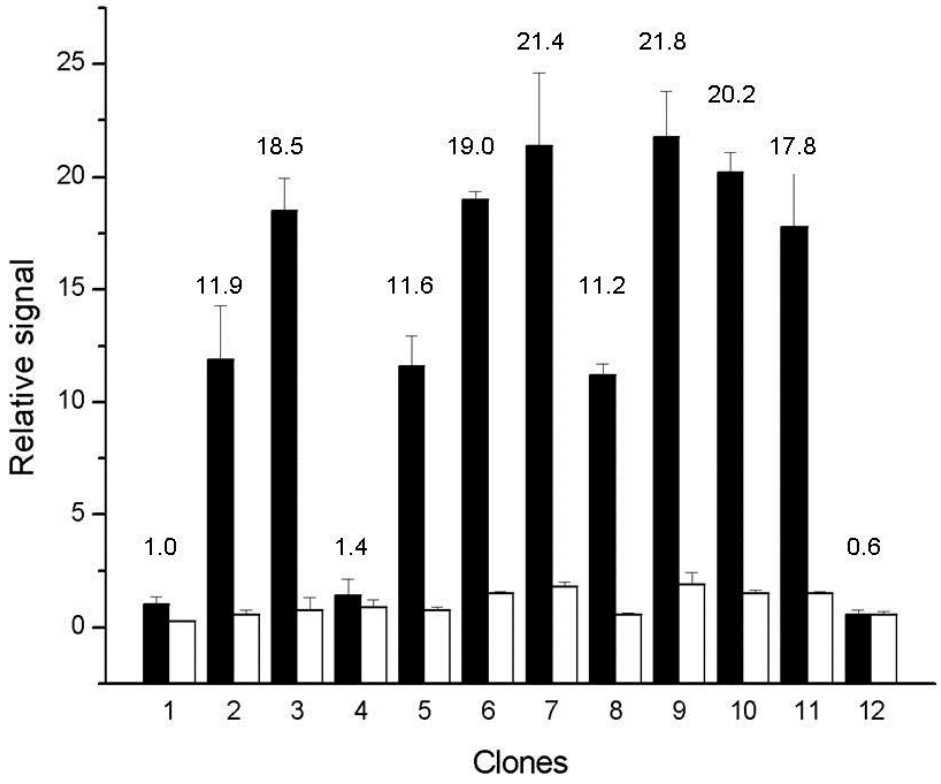


Figure 4.

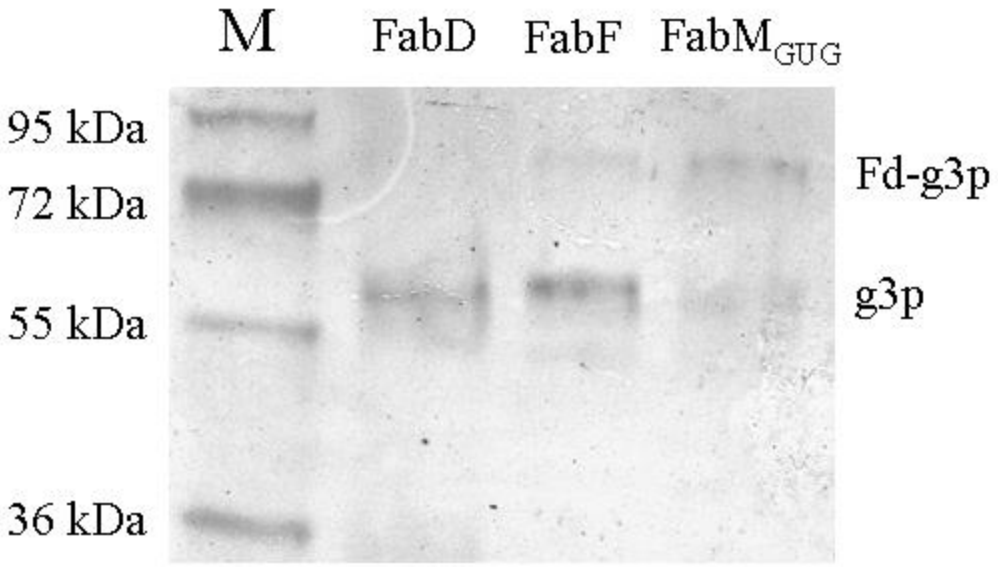


Figure 5A.

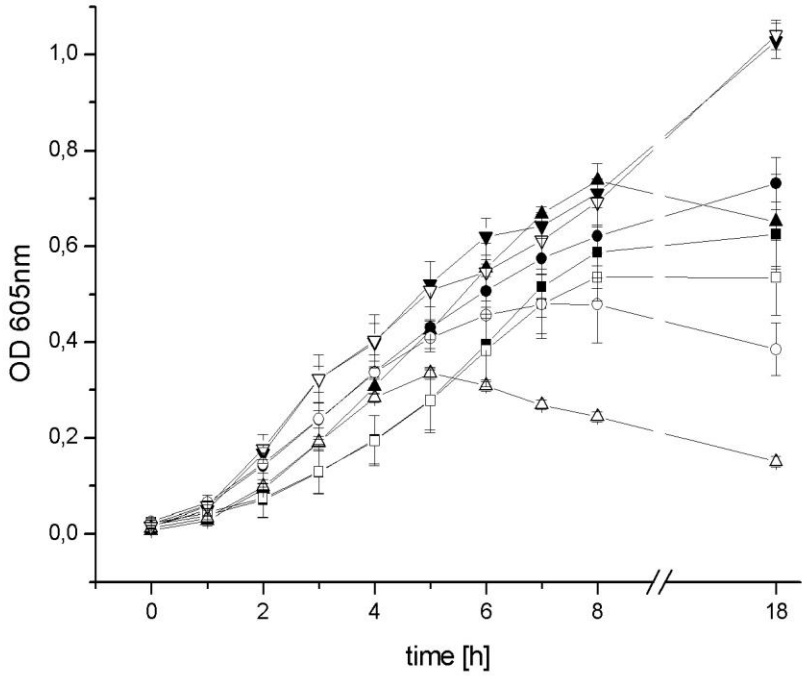


Figure 5B

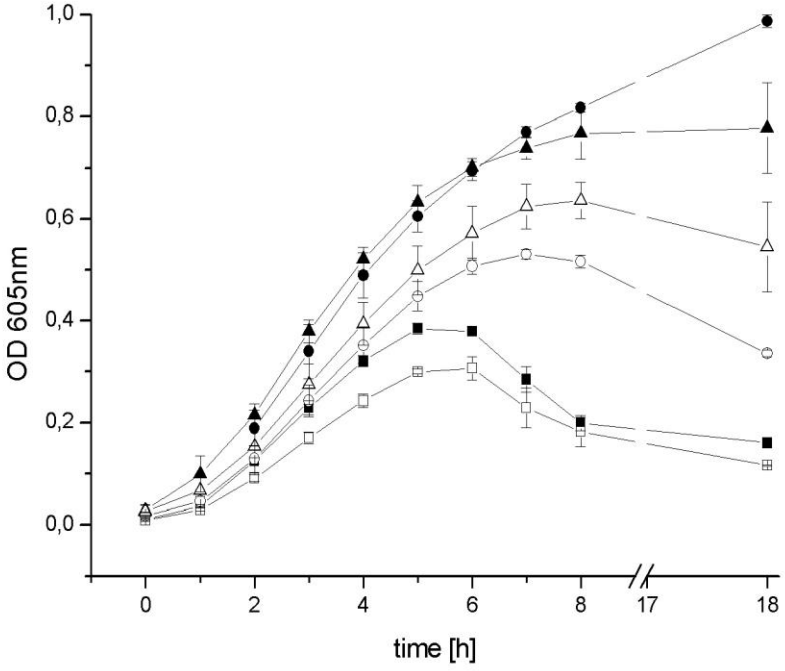
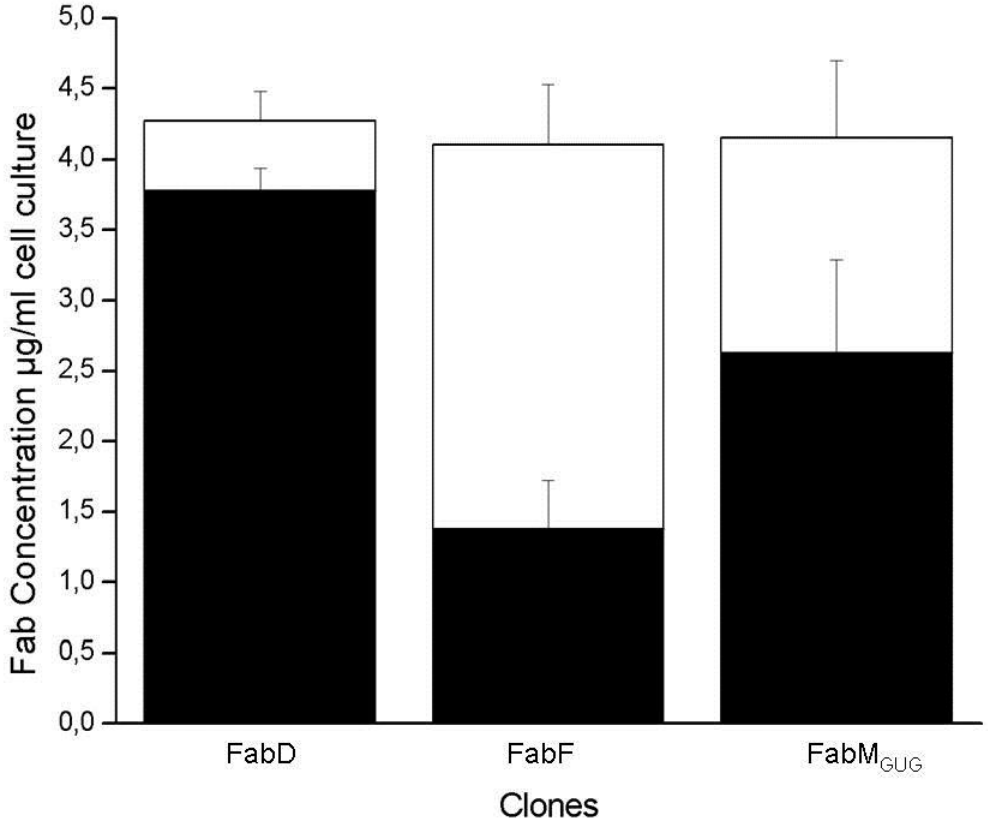


Figure 6.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Fab Concentration µg/ml cell culture
FabD
FabF
FabM$_{GUG}$
Clones

Figure 7A.

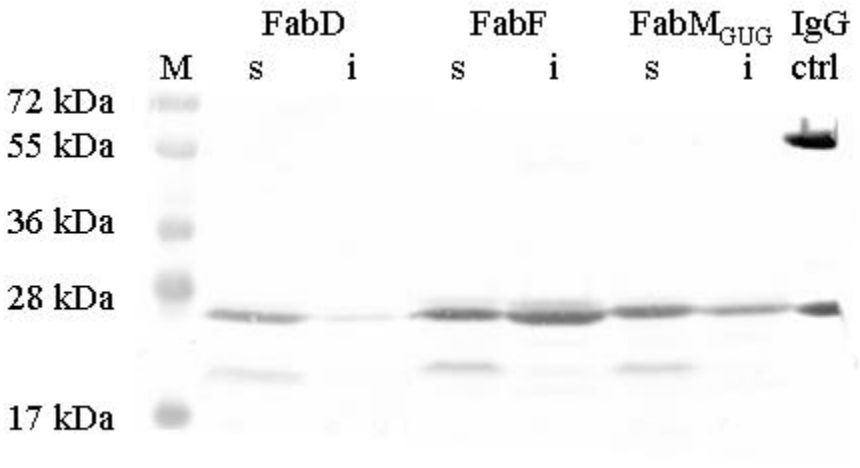

Figure 7B.