

Research paper

Parameters affecting the display of antibodies on phage

M. Kirsch, M. Zaman, D. Meier, S. Dübel*, M. Hust

*Institut für Biochemie und Biotechnologie, Abteilung Biotechnologie, Technische Universität Braunschweig,
Spielmannstr. 7, 38106 Braunschweig, Germany*

Received 15 December 2004; received in revised form 27 April 2005; accepted 28 April 2005
Available online 31 May 2005

Abstract

Despite the fact that a multitude of antibody phage display libraries has been built, systematic comparisons of critical design parameters are rare. Here we analysed the impact of various factors on the performance of the phage display system. First, we compared several vector designs for the display of Fab fragments of antibodies. Bicistronic as well as monocistronic expression of the antibody/pIII operon and vectors using fd-pIII as well as LC-pIII fusions were tested. Further, we evaluated the influence of glucose on the promoter induction. We compared monovalent versus oligovalent display of the antibody fragments and we used antibody fragments with different folding efficiency to assess the influence of the individual antibody sequences on the performance of the system. Finally, both phage display efficiency and yield of soluble Fab fragments were analysed. The significant differences found for phage yield, display of Fabs on the phage and expression of soluble Fabs suggest to use a bicistronic vector with an fd-fragment-pIII fusion for the construction of future Fab phage display libraries.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Fab; Phage display; Recombinant antibody; scFv; Vector design

1. Introduction

Within the past decade, antibody phage display has proven its capability to supply human antibody fragments, binders to toxic or non-immunogenic substances and even fragile conformational variants.

However, a broad distribution of this technology as a routine method has been hindered by the still deli-

cate handling of very large antibody repertoires and the extreme sensitivity of the method. It has to be kept in mind that every *E. coli* clone rescued from a phage panning experiment results from a discrete interaction of a single antibody fragment with a single antigen molecule. As a consequence, the use of antibody phage display, particularly in the companies dominating the applications, has been mainly focused to the development of therapeutics, where extensive optimizations in respect of every single application are feasible. In this respect, the vast majority of reports utilise scFv libraries (Hust and

* Corresponding author.

E-mail address: s.duebel@tu-bs.de (S. Dübel).

Dübel, 2004), as the smaller size and the composition from a single polypeptide facilitate production and folding in *E. coli*, in turn allowing a more efficient presentation of the antibody repertoire on the phage surface. Recently, however, novel applications in research, in particular for proteomics and antibody microarrays, induced a re-thinking of this strategy. Here, a robust method adapted to require minimal effort per antigen is necessary. Individual optimizations of clones or antibody format changes would neutralise the benefits of the possible automation. As a result, Fab fragments experience a renaissance as they are much more compatible to established immunological protocols than scFv fragments. However, due to the necessity to produce two different polypeptide chains, Fab expression vectors are more complex than the scFv systems (for review see Hust and Dübel, 2005).

First, the expression of two antibody chains must be well balanced for optimal Fab phage display. Several parameters could affect the optimal expression and display of Fab fragments. Various genetic designs for the expression of two polypeptides have been tried. A monocistronic arrangement of the antibody genes results in two mRNAs. This design was used, e.g., in pComb3 (Barbas et al., 1991), pDH188 (Garrard et al., 1991), pFAB4 and pFAB5c (Ørum et al., 1993). In contrast, in a bicistronic arrangement, both antibody chains will be translated from one mRNA. Examples for bicistronic designs are pEXmide3 (Söderlind et al., 1993), pFAB60 (Johansen et al., 1995), pFAB73h (Engberg et al., 1996) and pCES (de Haard et al., 1999).

Second, either the light chain or the heavy chain can be fused to pIII. Ørum et al. (1993) and Engberg et al. (1996) used an LC–pIII fusion. The HC–pIII fusion was employed by Barbas et al. (1991) and de Haard et al. (1999), among others. As we demonstrate in this study, this orientation can strongly affect the display efficiency.

Third, most phagemids use the lacZ promoter for the antibody expression cassettes. This promoter can be down regulated by glucose in the medium. For example, for the human single fold scFv libraries I+J (Tomlinson I+J), the presence of 0.1% glucose (5.5 mM) gave optimal results (Tomlinson, 2004), whereas removal of glucose was used for induction in other systems (Welschof et al., 1997).

Fourth, the amount of antibody-fragment–pIII fusion protein incorporated into the phage particle, generated with different helper phage, varies by more than two orders of magnitude. The use of helper phage which enforce oligovalent display is frequently used to improve the panning efficiency, but their effect on overall performance in relation to the other factors has not yet been systematically assessed. Several systems for enforced oligovalent display have been reported recently (Rondot et al., 2001; Baek et al., 2002; Soltes et al., 2003). Here we analysed the interference of a well established member of each helper phage type (monovalent VCSM13, oligovalent Hyperphage) with the above factors. Some vectors, e.g., pComb3 (Barbas et al., 1991), utilised an amino terminally truncated form of pIII as a fusion partner for the antibody chain(s). These systems, however, do not allow to benefit from the systems enforcing oligovalent display, as the amino terminal deletion of the pIII would result in phage which are not infective anymore. Therefore, these variants were not included in this study.

Fifth, for convenience, many phage display vectors allow the expression of soluble antibody fragments once the panning process has been finished. Typically, the switch from *E. coli* SupE strains to non-suppressor strains activates a stop codon between the antibody and phage coat protein domains. A desirable feature of a phage display vector optimized for proteomic antibody generation would be a combination of good display yields and compatibility with the oligovalent and monovalent display with a good yield of soluble Fabs without subcloning into an expression vector. For this reason, we have chosen to include the SupE codon into the vectors analysed here.

Sixth, different antibody sequences are known to exhibit large differences in production yield in the *E. coli* periplasm ranging from mg/L to almost marginal yields. We acknowledged a respective influence on the comparison by using antibodies with “bad” and “good” folding abilities. As a representative of the “good” folders we used the D1.3 antibody, binding to lysozyme (Ward et al., 1989). The RNA polymerase II binding antibody ‘215’ represents the “bad” folding species (Liu et al., 1999). This study allowed to conclude on the significance of all these factors and their interactions and resulted in the suggestion of an “optimized” phage display vector.

2. Material and methods

2.1. Construction of the pHAL vector series

Standard cloning procedures were performed according to Sambrook et al. (1989). The pHAL vector series was derived from the phagemid vector pSEX81 (Welschhof et al., 1997). First, the NheI site in pSEX81-phOx(yol) was deleted by site-directed mutagenesis. The next step comprised the introduction of the VH cloning site and deletion of the VH of phOx-scFv gene. To achieve this, human IgG₁ CH1 DNA was amplified from the plasmid pAc-K-CH3-VH(138H11) (Liang et al., 2001) combined with the introduction of a modified pelB sequence, the RBS, the 5' UTR, the *strep*-tag II coding sequence (Voss and Skerra, 1997) and the restriction sites HindIII, PstI and BssHII required for further cloning steps. The vector pHAL1 was constructed by insertion of the LC cloning site leading to deletion of the VL gene of phOx-scFv and modifying the pelB leader to generate a NheI site for LC cloning.

The construction of pHAL2 was done in two steps. In the first step, the fd-fragment expression cassette with pelB and RBS was amplified from pHAL1 and cloned into the EcoRI and HindIII sites, replacing the LC expression cassette. The LC expression cassette with pelB and the RBS was amplified from pHAL1 and cloned into the PstI and BamHI sites, replacing the fd-fragment expression cassette. The vector pHAL5 was constructed by amplifying the lacZ promoter for the HC expression cassette from pHAL2 and introduction of the T7-terminator behind the LC expression cassette, cloning into the HindIII and NcoI sites of pHAL1. To obtain pHAL6, the lacZ promoter from pHAL1 was amplified and the T7-terminator was introduced by cloning into the HindIII and NheI sites of pHAL2.

The mouse antibody D1.3 DNA was cloned into the four vectors pHAL1, pHAL2, pHAL5 and pHAL6 in two steps. The LC DNA was amplified from the plasmid pASK99D1.3 (kindly provided by Arne Skerra, Technische Universität München) and cloned into the NheI and MluI sites. The fd-fragment DNA was amplified and cloned into the NcoI and NotI sites.

The VL gene fragment of mouse antibody '215' was amplified from the plasmid pOPE101-215(yol)

(Schmiedl et al., 2000). Human C kappa fragment DNA was amplified from the plasmid pAc-K-CH3(138H11) (Liang et al., 2001) and linked to the mouse VL sequence by assembly PCR, before being cloned into the NheI and MluI sites of pHAL1. To obtain pHAL5-215, pHAL2-215 and pHAL6-215 the chimeric LC gene was amplified from pHAL1-215 and cloned into the NheI and MluI sites. The '215' VH DNA was amplified from the plasmid pOPE101-215(yol) and cloned into the SfiI and BssHII sites. Transformations of XL1-Blue MRF' (Stratagene, Amsterdam, The Netherlands) with the constructs were done by electroporations according to the manufacturers instructions. All steps in vector construction and antibody cloning were confirmed by sequencing of the affected regions.

2.2. Fab phage production

50 mL 2xTY medium (Sambrook et al., 1989)+100 µg/mL ampicillin+100 mM glucose were inoculated with an overnight culture to O.D.₆₀₀=0.025. The bacteria were grown to O.D.₆₀₀=0.4–0.5 at 37 °C and 250 rpm. 2 mL bacteria (~1 × 10⁹ bacteria) were infected with 2 × 10¹⁰ helper phage VCSM13 (Stratagene, Amsterdam, The Netherlands) or Hyperphage (Rondot et al., 2001), incubated at 37 °C for 30 min without shaking, followed by 30 min at 250 rpm. The infected cells were harvested by centrifugation for 10 min at 3220 × g and the pellet was resuspended in 30 mL 2 × TY+100 µg/mL ampicillin+50 µg/mL kanamycin containing various glucose concentrations. The phage were produced at 30 °C and 250 rpm for 16 h. The cells were pelleted for 10 min at 10,000 × g. The phage in the supernatant were precipitated with 1/5 volume of 20% PEG/2.5 M NaCl solution for 1 h on ice with gentle shaking and pelleted 1 h at 10,000 × g at 4 °C. The precipitated phage were resuspended in 10 mL dH₂O, precipitated again with 1/5 volume of PEG solution as given above for 20 min on ice and pelleted 30 min at 10,000 × g at 4 °C. The precipitated phage were resuspended in 300 µL PBS (phosphate-buffered saline, Sambrook et al., 1989) and cell debris was pelleted by additional centrifugation for 5 min at 15,400 × g at 20 °C. The supernatant containing the Fab phage was stored at 4 °C. Phage titration (cfu) was done according to Koch et al. (2000) with one modification: the infected

bacteria were pipetted directly onto LB agar plates, omitting nitrocellulose sheets.

2.3. Antigen-binding ELISA

Microtitre plates (Costar, Cambridge, USA) were coated with 100 ng antigen in 100 μ L 0.1 M NaHCO₃ pH 9.6 per well over night at 4 °C. For the D1.3 antibody phage, hen egg white lysozyme (Sigma, Taufkirchen, Germany) was used as antigen. For the '215' antibody phage the '215' oligopeptide was employed (Kontermann et al., 1995). Coated wells were washed 3 $\times$ with PBS and blocked with 2% (w/v) skim milk powder in PBS for 1.5 h at RT, followed by 3 $\times$ washing with PBS. Fab phage or periplasmic fractions were diluted in 100 μ L blocking solution and incubated for 1.5 h, followed by 5 $\times$ washing with PBST (PBS+0.1% (v/v) Tween 20). Bound Fab phage were detected with mAb anti-M13 conjugated with HRP (Amersham Biosciences, Freiburg, Germany) (1:5000), soluble Fab fragments were detected with mAb goat anti-mouse conjugated with HRP (1:10,000) and visualised with TMB substrate (Bio-Rad, München, Germany). The staining reaction was stopped by adding 100 μ L 1 N sulphuric acid. The absorbances at 450 nm and 620 nm were recorded using a SUNRISE microtitre plate reader (Tecan, Crailsheim, Germany). The absorbance of scattered light at 620 nm was subtracted from the absorbance at 450 nm.

2.4. SDS-PAGE and immunoblot

Fab phage were separated by SDS-PAGE and blotted onto PVDF. The membrane was blocked with 5% (w/v) skimmed milk powder in PBST for 1 h at RT. The minor coat protein pIII was detected with 1:1000 diluted mouse mAb anti-pIII (Mobitec, Göttingen, Germany) for 1.5 h at RT, followed by 2 $\times$ washing with PBST. Goat anti-mouse (fc specific) (Sigma, Taufkirchen, Germany) conjugated with AP 1:1000 diluted was used for detection and visualised by NBT/BCIP. For the detection of mouse kappa LC the mAb rat anti-mouse kappa LC (1:1000) (Sigma, Taufkirchen, Germany) was used as first antibody and goat anti-rat conjugated with AP (1:1000) (Sigma, Taufkirchen, Germany) was used as second antibody.

2.5. Expression of soluble Fab fragments

Preparation of soluble antibody fragments from the periplasm was modified from the method given by Schmiedl and Dübel (2001). *E. coli* HB2151 were infected with one of the four different pHAL phage. 300 mL TB medium (Sambrook et al., 1989)+100 μ g/mL glucose+100 μ g/mL ampicillin were inoculated with an overnight culture yield an O.D.₆₀₀<0.1 and cultured at 22 °C and 160 rpm. The induction was started by adjusting to 1 mM IPTG at O.D.₆₀₀=0.6 and shaken for 20 h. The bacteria were harvested by 10 min at 4400 $\times$ g at RT. The pellets were resuspended in 30 mL ice-cold PE buffer, pH 8 (500 mM sucrose, 100 mM Tris, 1 mM EDTA) and incubated for 20 min on ice, interrupted by short vortexing every 5 min. Subsequently, the bacteria were pelleted for 30 min at 30,000 $\times$ g at 4 °C. The supernatant representing the periplasmic fraction was stored. The pellet was resuspended in 30 mL ice-cold dH₂O and incubated for 20 min on ice, interrupted by short vortexing every 5 min. The spheroblasts were centrifuged for 30 min at 30,000 $\times$ g and 4 °C. The supernatant representing the osmotic shock preparation was combined with the periplasmic fraction and stored at –20 °C.

3. Results

3.1. The pHAL Vector series

Four different vector designs were constructed (Fig. 1). The vectors pHAL1 and pHAL2 lead to a bicistronic expression under the control of lacZ promoter, resulting in a single mRNA with two cistrons. Both leaders were chosen to have the same amino acid sequence but vary on nucleotide level to introduce a NcoI site for VH cloning and a NheI site for LC cloning. The vectors pHAL5 and pHAL6 were designed for a monocistronic expression with each antibody chain gene controlled by an individual lacZ promoter, resulting in two mRNAs. Further, the order of heavy and light chain coding regions was inversed, resulting in either an LC or fd-fragment fusion to pIII. In the vectors pHAL1 and pHAL5, the fd-fragment is fused to gIII, whereas in pHAL2 and pHAL6 the LC is fused to gIII (Fig. 1). Inserts for the production of

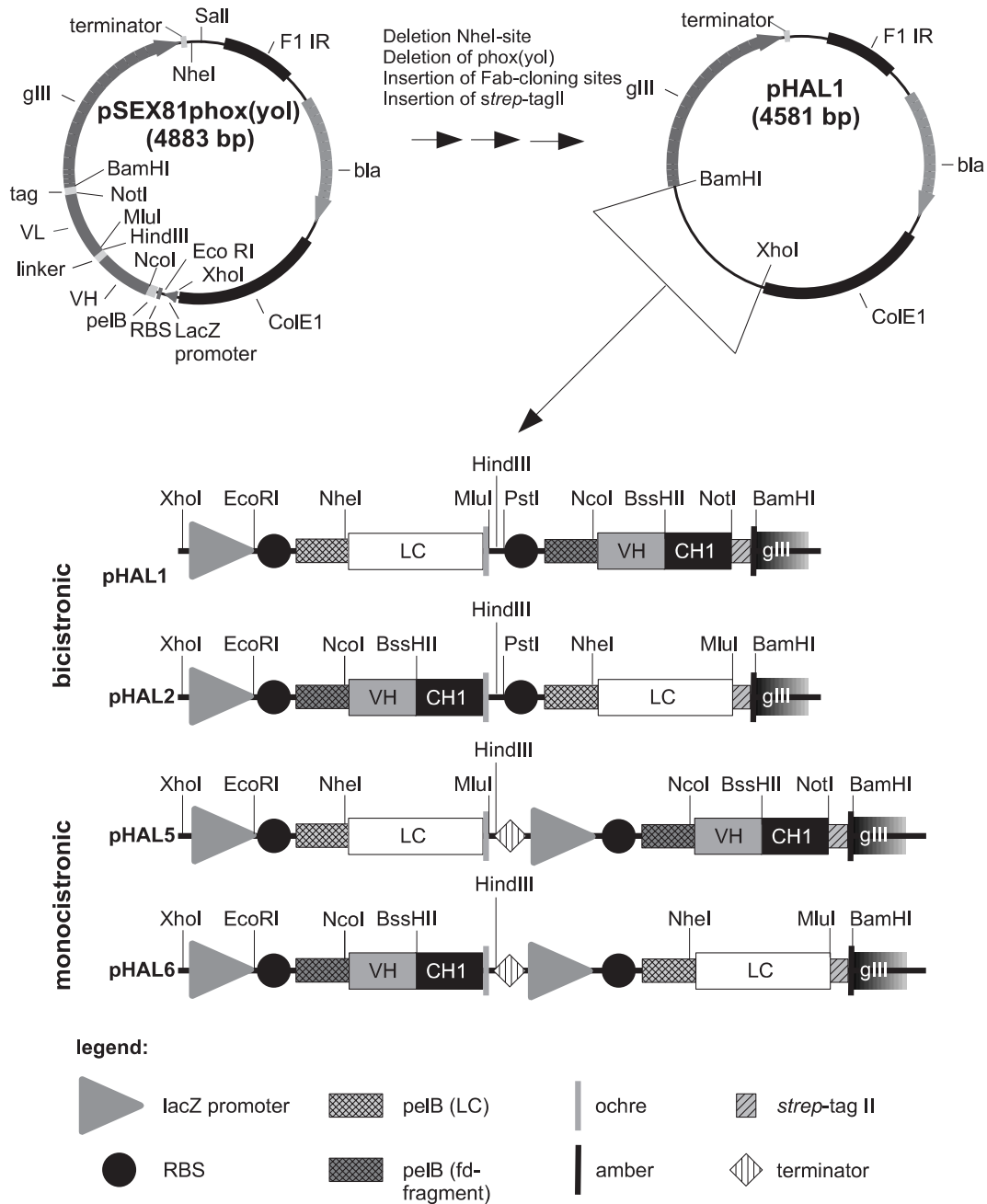


Fig. 1. Vector constructs used in this study. Abbreviations: lacZ promoter—promoter of the bacterial lac operon; RBS—ribosome binding site; pelB—signal peptide sequence of bacterial pectate lyase, mediating secretion into the periplasmic space; VH—variable fragment of the heavy chain; LC—light chain; ochre—ochre stop codon; amber—amber stop codon; strep-tag II—synthetic tag binding to streptactin; terminator—sequence terminating transcription; bla— β -lactamase gene for ampicillin resistance; ColE1—bacterial origin of DNA replication; F1 IR—intergenic region of phage f1. The elements of the inserts are not drawn to scale.

two different Fab fragments were cloned in each of these vectors. Either the mouse anti-hen egg white lysozyme antibody D1.3 (Ward et al., 1989) as the mouse anti-*Drosophila melanogaster* RNA polymerase II antibody '215' (Liu et al., 1999) were used.

3.2. Phage production using pHAL vectors encoding D1.3 Fab

pHAL-D1.3 phagemids were packaged using either Hyperphage or VCSM13 and at various glucose concentrations. As expected, with VCSM13, phage production showed only a marginal dependency on the glucose concentration, as the vast majority of pIII originates from the glucose-independent genomic source (Fig. 2). The highest titres were achieved at 5–10 mM glucose. For pHAL1, pHAL2 and pHAL6, with Hyperphage, the highest yields were achieved with 5 mM glucose. Increased glucose concentration led to decreased Fab phage titre, when pHAL5 was used (Fig. 2).

About 5×10^{11} Fab phage were produced per 30 mL culture from all constructs when VCSM13 was used. In contrast, the production using Hyperphage yielded different phage titre, as reported before, the total phage yield was significantly lower than the titre obtained with VCSM13. Using Hyperphage, about 10 times more cfu were produced with the vectors pHAL2 and pHAL6 (LC–pIII fusion) compared to

pHAL1 (fd–pIII fusion). The vector pHAL5 yielded the lowest titres.

3.3. Biochemical analysis of phage produced using pHAL vectors encoding D1.3 Fab

D1.3 Fab phage preparations generated with Hyperphage or VCSM13 were separated by 10% SDS–PAGE and blotted onto PVDF membranes. pHAL5 packaged with Hyperphage was not analysed, due to the very low phage titre. pIII was visualised by immunostaining using monoclonal mouse anti-pIII Ab. pIII has a calculated molecular mass of 42.5 kDa but runs at an apparent molecular mass of 65 kDa in SDS–PAGE (Goldsmith and Konigsberg, 1977). As expected, the Fab–pIII fusion is prominent in the phage generated with Hyperphage, with a band with a relative molecular mass of wt pIII present in almost equal amounts (Fig. 3A). With phage produced using VCSM13, only the pIII band was strongly visible, reflecting the low amount of fusion protein usually obtained in monovalent display.

To investigate the possible occurrence of light chain dimers (Bence–Jones proteins), Fab phage were analysed by immunoblot (Fig. 3B). pHAL2 and pHAL6 packaged with Hyperphage showed a weak band corresponding to free LC, indicating the existence of Bence–Jones proteins. Free LC was also detected when VCSM13 was used for packaging of

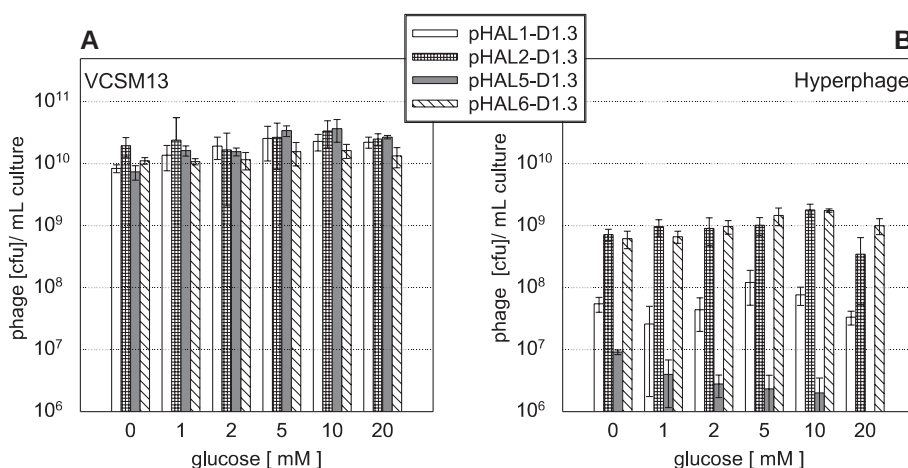


Fig. 2. Comparison of the phage obtained from 30 mL of culture for the four different pHAL constructs encoding D1.3 Fab at different glucose concentrations. The titres are given per milliliter culture volume. The data show the mean value and standard deviations of three completely independent experiments. Packaged (A) with VCSM13, (B) with Hyperphage.

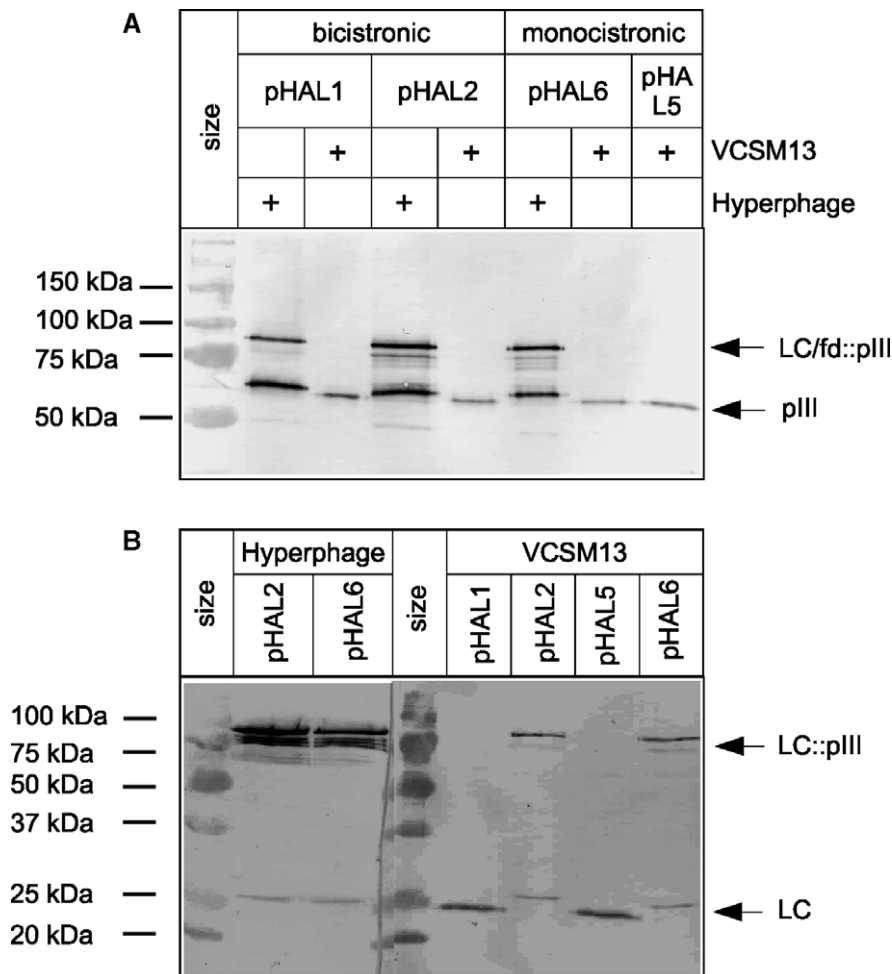


Fig. 3. Biochemical analysis of the phage produced with either Hyperphage or VCSM13. (A) 10^8 D1.3 Fab phage were separated on a reducing 10% SDS–PAGE, pIII was detected using mouse mAb anti-pIII. (B) 2.5×10^9 D1.3 Fab phage produced with Hyperphage or 5×10^{10} D1.3 Fab phage produced with VCSM13 were separated on a reducing 12% SDS–PAGE, D1.3 light chains were detected using rat mAb anti-mouse kappa LC.

these two vectors. As expected, the relative molecular mass of these free LCs was higher as for the free LCs derived from pHAL1 and pHAL5 due to the attached *strep*-tag II.

3.4. Antigen binding of phage produced using pHAL vectors encoding D1.3 Fab

Antigen binding of phage preparations using pHALx-D1.3 vectors was determined by ELISA on lysozyme. 10^{10} phage/well were used when VCSM13 was used for phage rescue, whereas 10^7 phage/well

were employed after packaging with Hyperphage to compensate the differences in Fab presentation efficiency of both systems.

The glucose concentration had an effect on antigen binding of the produced D1.3 Fab phage produced with VCSM13. For phage produced with pHAL1, 0–1 mM glucose resulted in slightly better antigen binding, whereas for pHAL2, 2–5 mM glucose gave the best results. When using glucose concentrations of 10 mM and higher, the antigen binding was strongly reduced. As expected, when using Hyperphage, glucose concentrations up to 10 mM had little effect on

antigen binding. Only at 20 mM was antigen binding significantly reduced.

Interestingly, the effect on antigen binding of the various vectors was different for Hyperphage and VCSM13. Using VCSM13, the Fab phage produced with bicistronic vectors showed the best antigen binding. Only weak antigen binding was detected using the monocistronic vectors pHAL5 and pHAL6. In contrast, Hyperphage packaging seems to favour heavy chain–pIII fusions (Fig. 4).

3.5. Phage production and antigen binding using pHAL vectors encoding ‘215’ Fab

To check whether the results obtained for D1.3 Fab phage depend on folding efficiency of the antibody insert, the experiment was repeated using a different antibody fragment (“215”). This antibody is known for low yields when secreted into the *E. coli* periplasm. Therefore, the ‘215’ Fab was produced and checked by antigen ELISA.

Initially, the ‘215’ Fab phage were not able to bind the ‘215’ antigen. This was found to be a result of the introduction of the BssHIII restriction site, which led to an additional amino acid between VH and CH1. The BssHIII site was removed by direct site mutagenesis,

leading to vectors allowing the production of functional Fabs.

The phage were produced without glucose, by using VCSM13 or Hyperphage. Approximately 10^{12} phage were obtained per 30 mL culture using VCSM13 for every vector construct, whereas the titre using Hyperphage was different for the different vector designs. The Fab Hyperphage titre was in the range from 2×10^9 using pHAL5 to 2×10^{11} using pHAL6 per 30 mL culture (Fig. 5A).

The antigen binding of ‘215’ Fab phage produced with VCSM13 and Hyperphage is given in Fig. 5B. With both types of helper phage, the vectors with the fd–pIII fusion performed best.

3.6. Antigen binding of soluble D1.3 antibodies

In order to investigate expression of soluble antibody Fab fragments, periplasmic fractions of *E. coli* strain HB2151, transformed with pHALx-D1.3 constructs, were analysed by antigen ELISA. The best yield of functional Fab fragments was obtained with vectors using fd–pIII fusions, whereas less functional antibodies were produced using the vectors pHAL2 and pHAL6 (with the LC–pIII fusion) (Fig. 6). The bicistronic pHAL1 performed best, yielding three

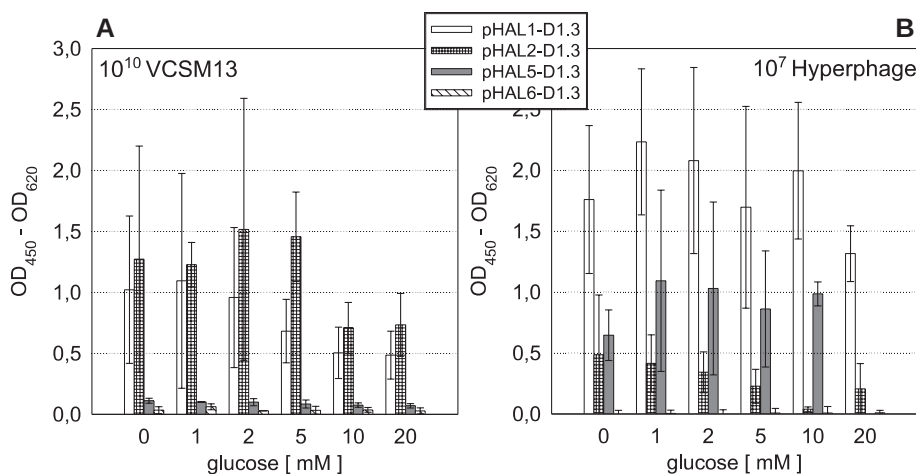


Fig. 4. Antigen-binding phage ELISA of the four pHAL constructs encoding D1.3 Fab. The data show the mean value and standard deviations of three completely independent experiments. The absorbance of scattered light at 620 nm was subtracted from the absorbance at 450 nm. The background signal of the antigen incubated with the detection antibody mAb anti-M13 HRP was subtracted. Antigen: 100 ng/well lysozyme. The D1.3 Fab phage were detected using mAb anti-M13 conjugated with HRP. (A) 10^{10} Phage produced with VCSM13, (B) 10^7 phage produced with Hyperphage.

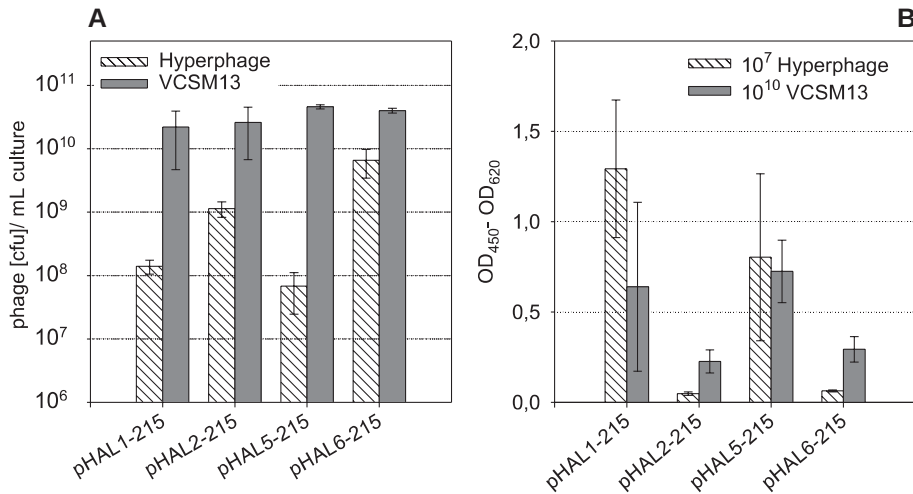


Fig. 5. Comparison of the phage yield obtained from 30 mL of culture, given per milliliter culture volume (A) and antigen binding (B) of phage derived from pHAL constructs encoding the '215' Fab fragment. Fab phage were produced in three independent experiments and every Fab phage production was measured in duplicate in ELISA. Antigen: 100 ng '215' oligopeptide per well. The '215' Fab phage were detected using mAb anti-M13 conjugated with HRP.

times more antigen-binding activity than the other vectors.

Interestingly, an expression of soluble D1.3 antibodies using the vector pHAL1 was found to be possible in *E. coli* strain XL1-Blue MRF' which was used for phage display. The ELISA analysis of soluble D1.3 produced in *E. coli* strains HB2151, TB1 and XL1-Blue showed a yield of soluble functional Fab fragments from XL1-Blue and TB1 up to 1.6 times better than that obtained with HB2151. This

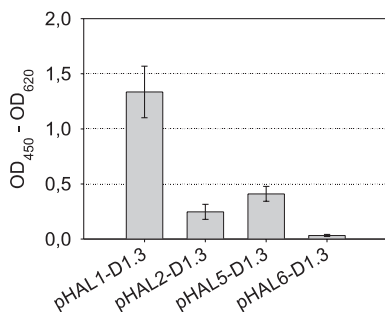


Fig. 6. Comparison of the antigen binding of soluble D1.3 Fab fragments secreted from *E. coli* (HB2151) from 30 mL of culture produced with four different pHAL constructs. ELISA on lysozyme (100 ng per well) was done using the periplasmic fraction. The soluble D1.3 Fab fragments were detected with mAb goat anti-mouse conjugated with HRP. Data represent two independent experiments, every sample was measured in duplicate in ELISA. Errors bars represent standard deviation of the resulting four values.

binding activity is not a result of soluble Fab pIII fusion protein as can be seen on immunoblot (data not shown).

4. Discussion

The aim of the work was the assessment of the influence and interdependence of various factors on antibody phage production. For the optimal phage display packaging system, three output parameters need to be optimized. First, total phage titres should be sufficient to yield enough material for a panning round. When using monovalent display, however, only a small fraction of the titred phage presents antibodies on their surface. Further, the production level of antibody-pIII fusion proteins can influence the phage particle output from the *E. coli* cells. As a consequence, total phage titres do not indicate the amount of functional phage, i.e., those which carry a correctly folded antibody fragment. Therefore, an antigen phage ELISA provides an essential quality benchmark of a phage display system. Finally, when throughput is essential, as in proteomics applications (Hust and Dübel, 2004), the production of soluble antibody fragments is of importance. As no helper phage have to be present for this step, the conditions for the production of Fab fragments may vary from

those when generating antibody phage. In this study, various factors with a possible impact on these three benchmark parameters were analysed. When compared to scFv phage display, the expression of two antibody chains at the Fab fragment must be well balanced for optimal display. Several parameters could affect this balance.

4.1. Relevance of display valency

First, oligovalent and monovalent antibody display has been described for phagemid vectors, allowing to switch between a first (few) panning round(s) using oligovalent display at high antigen concentrations followed by several rounds with monovalent display. This is usually done to avoid loosing any specific binder in the first round when only a few binding phage are diluted in a billion unspecific ones, but later allowing a competition for high affinity clones once the entire specific repertoire has been rescued. Most commonly, this switch is done by employing different helper phage. We used VCSM13 (Stratagene), derived from M13K07 (Vieira and Messing, 1987), as a widely used representative for monovalent display. Hyperphage (Rondot et al., 2001) was employed to enforce oligovalent display.

As expected, the production levels of both D1.3 and '215' Fab phage confirmed the large differences between VCSM13 and Hyperphage. By using the helper phage VCSM13, 10–100 times more phage were produced, depending on the vector design. When using VCSM13, its wildtype pIII compete with the antibody–pIII fusion in phage assembly. As the production and folding of the antibody–pIII fusion is slow, the majority of pIII assembled into phage is wildtype. Hyperphage avoids to introduce wildtype pIII into the packaging process, thus leaving the phagemid as the sole source of pIII and therefore enforcing multivalent display from phagemid vectors (Rondot et al., 2001). It is important to note that in contrast to monovalent display systems, the titre of phage produced with Hyperphage directly reflects the expression and folding of the antibody. Therefore, despite the fact that the overall phage particle yield was lower, much less Fab Hyperphage (10^7) as Fab phage produced with VCSM13 (10^{10}) were needed to detect antigen binding by ELISA. Using Hyperphage for packaging, the same amount of phage particles

offers approximately 1000-fold stronger antigen binding. Both monocistronic and bicistronic vectors using the fd–pIII fusion (pHAL1 and pHAL5) showed better compatibility to Hyperphage in respect of antigen binding, which interestingly was not reflected by the total particle number. Much more Fab phage were produced using the LC–pIII fusion vectors, but the antigen binding was much lower compared to the Fab phage produced with the fd–pIII fusion vectors. This strong difference may be due to the formation of Bence–Jones proteins like LC-dimers by the Fab-portion of the pIII fusion proteins on the phage, which would result in faster assembly but yields inactive antibody fragments.

4.2. Relevance of mono- and bicistronic vector design

Second, mRNAs can be made using mono- or bicistronic vector designs. Both designs are well established for phage display but have rarely been compared systematically. The bicistronic vector design was used, e.g., for the complex human naive Fab library in pCES by de Haard et al. (1999) with one lacZ promoter and two different leaders. Our vectors pHAL1 and pHAL2 resemble this bicistronic design, using one lacZ promoter and two pelB leaders. The two pelB leaders were modified on nucleotide level to minimize internal recombinations but encode for identical amino acid sequences. The pelB leader was used twice to provide comparability. The monocistronic vector design was described, e.g., with two lacZ promoters and two pelB leaders in pComb3 (Barbas et al., 1991). Our vectors pHAL5 and pHAL6 represent this monocistronic design, with two lacZ promoters and two pelB leaders. Here, the lacZ promoter was used twice to provide that different Fab phage production and display is not influenced by the usage of different promoters.

The choice of mono- or bicistronic vector design had no substantial effect on the phage titre neither produced with Hyperphage nor VCSM13. In the biochemical analysis of Fab phage, no important differences were detected between these both vector designs encoding D1.3. In contrast, the antigen binding of D1.3 and '215' Fab phage, produced with Hyperphage, was better when using bicistronic vectors compared to their monocistronic counterpart with the same pIII fusion. For Fab D1.3, when

using VCSM13 the phage produced with the bicistronic vectors showed better antigen binding than the Fab phage produced with the monocistronic vectors. Interestingly, this effect depends on the Fab folding efficiency as indicated by the differences between D1.3 and '215' Fab phage. For '215' Fab phage, the usage of VCSM13 and Hyperphage led to similar antigen binding in respect to the vector design. Soluble Fabs produced with the bicistronic vectors showed a better antigen binding compared to their monocistronic counterpart with the same pIII fusion, as also seen for antigen binding of '215' Fab phage using Hyperphage or VCSM13 and D1.3 using Hyperphage.

4.3. Relevance of the pIII fusion partner

Another relevant parameter was the choice of the pIII fusion partner, which could be either the fd-fragment or the light chain. Both designs have been used before. The HC-pIII fusion was used by, e.g., [Barbas et al. \(1991\)](#) and [de Haard et al. \(1999\)](#). In our vectors, pHAL1 and pHAL5 represent this variant. The LC-pIII was used by, e.g., [Ørum et al. \(1993\)](#) and [Engberg et al. \(1996\)](#). In our vectors, pHAL2 and pHAL6 represent this variant.

Vectors with the LC-pIII yielded the best titres when packaged with Hyperphage. Ten times less Fab Hyperphage was produced using the vector pHAL1 with fd-fragment-pIII fusion and 100 times less using pHAL5. However, the phage titre for pHAL1 is still good enough for practical application. In the biochemical analysis of phage produced either with Hyperphage or with VCSM13 by SDS-PAGE, for pHAL2 and pHAL6 free LC were detected in immunoblot. The abundance of free light chains may be explained with the appearance of light chain dimers like Bence-Jones proteins ([Nachman et al., 1965](#)). The free light chain may originate from proteolysis. The functional yield of D1.3 and '215' Fab phage produced with Hyperphage was much better for vectors with fd-pIII fusion. The lower functionality of phage produced with the LC-pIII fusion vectors can be explained by the possibility to form Bence-Jones proteins like complexes, as supported by the biochemical analysis. The antigen binding of D1.3 is mainly mediated by the heavy chain ([Ward et al., 1989](#)). Here, no significant impact of the

fusion partner could be observed when vectors encoding D1.3 were packaged with VCSM13. For the '215' antibody, antigen binding was similar for VCSM13 and Hyperphage. The vectors encoding the fd-pIII fusion led to a better Fab phage display and the vectors encoding a LC-pIII fusion. Most strikingly, this effect was also seen for soluble Fab fragments, when pIII was not included. It can be concluded that the LC→fd orientation offers a more balanced production than the fd→LC design, independent from the fusion to pIII.

4.4. Relevance of glucose

A fourth parameter known to influence the Fab phage production and display is the induction level of the promoter(s). The secretion and folding machinery of *E. coli* can easily be impaired by overexpression of recombinant antibodies ([Dübel et al., 1992](#)). Therefore, the controlled repression of the promoter is necessary for the optimal phage display. In the lacZ system, the promoter can be fine tuned via the glucose concentration. The induction of the lacZ system with IPTG has an inhibitory effect on phagemid packaging ([Bass et al., 1990](#); [Breitling et al., 1991](#)). Glucose reduces the transcription of genes under control of a lacZ promoter ([Bujard et al., 1987](#)). For the "Tomlinson I" and "J" scFv libraries, 0.1% (w/v) (5.5 mM) glucose was reported to be optimal for the display of scFvs on the phage surface ([Tomlinson, 2004](#)).

Using Hyperphage, a glucose concentration of 5–10 mM led to best titres with pHAL1, pHAL2 and pHAL6, but the differences were small. With pHAL5, any addition of glucose led to lower phage titre. Significant effects of the glucose concentration on the phage production when using VCSM13 are not expected, because there are two alternative sources of pIII. The glucose concentration only influences the expression of the Fab-pIII fusion protein encoded on the phagemid, but not the expression of the wildtype pIII encoded on the phage genome. The wildtype pIII source is sufficient to produce all the pIII needed for phage assembly. As the concentration of the Fab-pIII fusion protein is neglectable in this particular situation, changes in its concentration cannot be expected to exceed the standard deviation of the titration experiments.

The glucose concentration can be expected to strongly affect antigen binding when two competing pIII sources are present. Accordingly, this is seen for VCSM13 packaged phage at concentrations above 5 mM glucose. In contrast, when using Hyperphage, the antigen binding cannot be changed by variations of the pIII/Fab–pIII ratio, as the phagemid is the sole pIII source. However, fusion protein levels may interfere with other aspects of phage packaging or structure. As only a slight tendency of reduced antigen binding with increasing glucose concentration had been observed. The Hyperphage packaging protocol is quite robust with respect to variation of the glucose concentration, except when using the pHAL5 design. The results emphasize the significance of adjusting the glucose concentration for “monovalent” display.

Very recently, a paper comparing vector designs with a focus on functional expression of antibodies was published by [Corisdeo and Wang \(2004\)](#). They used a tetanus toxoid antibody to compare three antibody designs and used VCSM13 as the helper phage. In all vector designs, the fd-fragment was fused to pIII. LC–pIII fusions were not analysed. However, their experiments nicely complement our work as they compared a monocistronic (pFab1) and bicistronic design (pFab2) where fd-fragment–pIII fusion cassette was upstream of the LC expression cassette and with a bicistronic design (pFab3) with the fd-fragment–pIII fusion cassette downstream the LC expression cassette. Confirming our results, the bicistronic design offered a slightly better display of functional antibodies. The arrangement of the pIII fusion cassette at the 3′ end led to a twofold better display compared to the arrangement with the pIII fusion at the 5′ start. This confirmed our choice to place the pIII fusion protein cassette downstream of the cassette encoding unfused antibody chain. It supports the hypothesis that by weaker expression of a gene at 3′ end of a polycistronic mRNA, due to breakups in the transcription and translation process, excess of the non-pIII fused antibody chain is beneficial for the display of functional Fab on phage surface.

In summary, the analysis of the three benchmark parameters – phage titre, display efficiency and production of soluble antibodies – yielded a clear recommendation for the design of an optimal antibody phage display vector. The monocistronic vectors

were inferior, despite pHAL6 yielded higher phage titre. As these high titres do not reflect functional Fab phage, the bicistronic vectors should be preferred here. The use of pHAL2 offers higher phage titres than pHAL1, but both yields are good enough for doing a panning. Phage made with pHAL1 were best for the display of functional D1.3 Fab when using Hyperphage, and yielded best results with “slow-folding” ‘215’ Fab with both types of helper phage. The yield of functional ‘215’ Fab phage produced with pHAL2 was much lower compared to using pHAL1. This result illustrates the strong influence of folding efficiency on antibody display and further emphasises the importance to include different antibody fragments in any systematic comparison. Finally, the best yields of soluble antibodies were obtained with pHAL1.

Therefore, we suggest for the construction of any future Fab phage display library the use of a bicistronic vector design with a fd-fragment–pIII fusion and an arrangement of the fusion cassette at the 3′ end of the operon.

Acknowledgements

We would like to thank Lars Toleikis for the corrections and discussion on the manuscript, Mifang Liang for the suggestions on restriction sites for antibody gene cloning and Cindy Menzel for the corrections on the manuscript.

We gratefully acknowledge the financial support by the German ministry of education and research (BMBF, SMP “Antibody Factory” in the NGFN2 program) and of the German Research Foundation (DFG, SFB 578).

References

- Baek, H., Suk, K.H., Kim, Y.H., Cha, S., 2002. An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res.* 30, e18.
- Barbas, III, C.F., Kang, A.S., Lerner, R.A., Benkovic, S.J., 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7978.
- Bass, S., Greene, R., Wells, J.A., 1990. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins* 8, 309.

- Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I., Little, M., 1991. A surface expression vector for antibody screening. *Gene* 104, 147.
- Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahim, I., Haepfle, M.T., Dobberstein, B., 1987. A T5 promoter-based transcription-translation system for the analysis of proteins in vitro and in vivo. *Methods Enzymol.* 155, 416.
- Corisdeo, S., Wang, B., 2004. Functional expression and display of an antibody Fab fragment in *Escherichia coli*: study of vector designs and culture conditions. *Protein Expr. Purif.* 34, 270.
- de Haard, H.J., van Neer, N., Reurs, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.-W., Hoogenboom, H.R., 1999. A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J. Biol. Chem.* 274, 18218.
- Dübel, S., Breitling, F., Klewinghaus, I., Little, M., 1992. Regulated secretion and purification of recombinant antibodies in *E. coli*. *Cell Biophys.* 21, 69.
- Engberg, J., Andersen, P.S., Nielsen, L.K., Dziegiel, M., Johansen, L.K., Albrechtsen, B., 1996. Phage-display libraries of murine and human Fab fragments. *Mol. Biotechnol.* 6, 287.
- Garrard, L.J., Yang, M., O'Connell, M.P., Kelley, R.F., Henner, D.J., 1991. Fab assembly and enrichment in a monovalent phage display system. *Bio/Technology* 9, 1373.
- Goldsmith, M.E., Konigsberg, W.H., 1977. Adsorption protein of the bacteriophage fd: isolation, molecular properties, and location in the virus. *Biochemistry* 16, 2686.
- Hust, M., Dübel, S., 2004. Mating antibody phage display with proteomics. *Trends Biotechnol.* 22, 8.
- Hust, M., Dübel, S., 2005. Phage display vectors for the in vitro generation of human antibody fragments. In: Burns, R. (Ed.), *Immunochemical Protocols*, 3rd ed. *Methods Mol. Biol.*, vol. 295. p. 71.
- Johansen, L.K., Albrechtsen, B., Andersen, H.W., Engberg, J., 1995. pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Protein Eng.* 8, 1063.
- Koch, J., Breitling, F., Dübel, S., 2000. Rapid titration of multiple samples of filamentous bacteriophage (M13) on nitrocellulose filters. *BioTechniques* 29, 1196.
- Kontermann, R.E., Liu, Z., Schulze, R.A., Sommer, K.A., Queitsch, I., Dübel, S., Kipriyanov, S.M., Breitling, F., Bautz, E.K.F., 1995. Characterization of the epitope recognized by a monoclonal antibody directed against the largest subunit of *Drosophila* RNA polymerase II. *Biol. Chem. Hoppe-Seyler* 376, 473.
- Liang, M., Dübel, S., Li, D., Queitsch, I., Li, M., Bautz, E.K.F., 2001. Baculovirus expression cassette vectors for rapid production of complete human IgG from phage display selected antibody fragments. *J. Immunol. Methods* 247, 119.
- Liu, Z., Song, D., Kramer, A., Martin, A.C., Dandekar, T., Schneider-Mergener, J., Bautz, E.K.F., Dübel, S., 1999. Fine mapping of the antigen-antibody interaction of scFv'215', a recombinant antibody inhibiting RNA polymerase II from *Drosophila melanogaster*. *J. Mol. Recognit.* 12, 103.
- Nachman, R.L., Engle, R.L., Stein, S., 1965. Subtypes of Bence-Jones proteins. *J. Immunol.* 2, 295.
- Ørum, H., Andersen, P.S., Øster, A., Johansen, L.K., Riise, E., Bjørnevad, M., Svendsen, I., Engberg, J., 1993. Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucleic Acids Res.* 21, 4491.
- Rondot, S., Koch, J., Breitling, F., Dübel, S., 2001. A helper phage to improve single-chain antibody presentation in phage display. *Nat. Biotechnol.* 19, 75.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schmiedl, A., Dübel, S., 2001. Functional minipreps. In: Kontermann, R., Dübel, S. (Eds.), *Antibody Engineering*. Springer Verlag, Heidelberg.
- Schmiedl, A., Breitling, F., Dübel, S., 2000. Expression of a bispecific dsFv-dsFv' antibody fragment in *E. Coli*. *Protein Eng.* 13, 725.
- Söderlind, E., Lagerkvist, A.C.S., Dueñas, M., Malmberg, A.-C., Ayala, M., Danielsson, L., Borrebaeck, C.A.K., 1993. Chaperonin assisted phage display on antibody fragments of filamentous bacteriophages. *Bio/Technology* 11, 503.
- Soltes, G., Barker, H., Marmai, K., Pun, E., Yuen, A., Wiersma, E.J., 2003. A new helper phage and phagemid vector system improves viral display of antibody Fab fragments and avoids propagation of insert-less virions. *J. Immunol. Methods* 274, 233.
- Tomlinson, 2004 (http://www.hgmp.mrc.ac.uk/geneservice/reagents/products/descriptions/scFv_tomlinsonIJ.shtml).
- Vieira, J., Messing, J., 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* 153, 3.
- Voss, S., Skerra, A., 1997. Mutagenesis of a flexible loop in streptavidin leads to higher affinity for the Strep-tag II peptide and improved performance in recombinant protein purification. *Protein Eng.* 10, 975.
- Ward, E.S., Güssow, D., Griffiths, A.D., Jones, P.T., Winter, G., 1989. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature* 341, 544.
- Welschof, M., Terness, P., Kipriyanov, S.M., Stanesco, D., Breitling, F., Dorsam, H., Dübel, S., Little, M., Opelz, G., 1997. The antigen-binding domain of a human IgG-anti-F(ab')₂ autoantibody. *Proc. Natl. Acad. Sci. U. S. A.* 94, 1902.