

Glycerol diversifies phage repertoire selections and lowers non-specific phage absorption

Svend Kjær*, Brian Stausbøl-Grøn, Troels Wind, Peter Ravn, Kristian Hobolt Jensen, Liselotte Kahns, Brian F.C. Clark

Institute of Molecular and Structural Biology, University of Aarhus, Gustav Wieds Vej 10C, 8000 Århus C, Denmark

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Abstract Using a semi-synthetic phage displayed antibody repertoire, isoform-specific and cross-reactive phage-antibodies to eukaryotic elongation factor 1A (eEF1A) have been selected. Enrichment of specific antibodies was found to depend on the presence of glycerol. Further selections against lactate dehydrogenase (LDH) revealed that the dominance of a phage-antibody clone to LDH was inhibited by glycerol, a notable feature for selection strategies where a broad variety of binding clones is desired. The impact of glycerol in distinct steps of the selection protocol was examined and glycerol found to affect certain antibody-antigen interactions. Furthermore, the non-specific phage binding was lowered by three orders of magnitude at a 20% (v/v) glycerol concentration.

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Key words: Phage antibody; Selection strategy; Glycerol; Elongation factor 1A; Dominant clone

1. Introduction

Phage displayed repertoires of antibody fragments have gained considerable momentum over the recent years as a source of antibody fragments for use in research and therapy [1,2]. The success of such repertoires is based upon construction of large non-biased repertoires which, in theory, contain antibodies with affinities for an unlimited array of antigens [3–6] including highly interspecies conserved self-antigens where traditional antibody production has proven difficult. One such antigen is the eukaryotic elongation factor 1A (eEF1A) formerly referred to as EF-1 α , which has been the primary target of our studies.

In mammals, eEF1A exists in two distinct isoforms, eEF1A-1 and the muscle specific isoform eEF1A-2 [7–9]. They have similar *in vitro* activities in protein translation assays [9]. The rationale for the existence of two highly homologous, yet conserved isoforms of a centrally important protein remains obscure. However, immunofluorescence studies using isoform-specific antibodies could possibly reveal different sub-cellular localisations of the two isoforms and there-

by potentially different functions. For such studies, isoform-specific antibodies are prerequisites and as phage displayed antibody repertoires offer the possibility of guiding the selection in a given direction by competitive panning schemes [10,11], this technology was applied.

In contrast to a range of antigens examined in our laboratory, it was not possible to enrich specific binders to either eEF1A-1 or eEF1A-2 from a semi-synthetic, medium-size repertoire using standard selection protocols [3]. Eukaryotic EF1A-1 is an unstable protein [12] and a *sine qua non* for the *in vitro* enzymatic activity of the protein is the presence of 15–25% (v/v) glycerol. Therefore, glycerol was included in all phage antibody (phage-ab) selection steps to avoid denaturation and degradation of eEF1A. Glycerol was found to affect positively the enrichment of phage-abs to both eEF1A-1 and eEF1A-2. This intriguing finding led to the investigation of glycerol's effect on selection of phage-abs to a different protein, lactate dehydrogenase (LDH), where selection of phage-abs from the same repertoire has previously been reported [11]. In the presence of glycerol a completely different selection profile was found where a dominant phage-ab clone was inhibited by glycerol, allowing previously 'overtaken' clones to be isolated. This exemplifies the usefulness of glycerol in selection schemes to broaden the access to the clonal diversity of large naive phage-ab repertoires. The effect of glycerol at various levels of the selection protocol was investigated. Glycerol was found to act as an efficient inhibitor of non-specific phage binding to immobilised proteins, which may account for the preferential selection of phage-abs to eEF1A in the presence of glycerol only.

2. Materials and methods

2.1. Strains and phage antibody repertoires

The amber codon suppressing strain *E. coli* strain TG1 was used for production of phage-abs. The phage-ab repertoire was constructed from human germ-line V_H-genes having a randomised third complementarity determining region of the variable heavy chain (HCDR3) region spanning 4–12 residues and an invariant light chain [3]. The phage-ab repertoire and TG1 strain were kindly provided by Dr. Greg Winter, MRC, Cambridge, UK.

2.2. Selection of phage antibodies

Immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µg/ml of purified rabbit eEF1A-1, eEF1A-2 or rabbit LDH. Eukaryotic EF1A-1 was purified from rabbit reticulocytes and eEF1A-2 from rabbit skeletal muscle according to Kristensen et al. [13]. Both were kind gifts from Ole Kristensen. Rabbit LDH was purchased from Boehringer Mannheim, Germany. Coating was performed in duplicate in 4 ml of 50 mM NaHCO₃, pH 9.1, either with (glycerol-positive) or without (glycerol-negative) 20% (v/v) glycerol (20% Gly). The following day, the tubes were rinsed thrice with PBS and blocked by 2% (w/v) Marvel skimmed milk powder in phos-

*Corresponding author. Fax: (45) (861) 23178.
E-mail: kjaer@biobase.dk

Abbreviations: Ag, antigen; Amp, ampicillin; c.f.u., colony forming units; eEF1A, eukaryotic elongation factor 1A; ELISA, enzyme-linked immunosorbent assay; Glu, glucose; Gly, glycerol; HCDR3, third complementarity determining region of the variable heavy chain; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; OPD, ortho-phenylen diamine; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; Phage-ab, phage displaying antibody fragment; scFv, single-chain fragment variable; V_H, variable region of the heavy chain; V_L, variable region of the light chain

phate-buffered saline (2% MPBS) for 1 h at room temperature (r.t.). For glycerol-positive selections, the 2% MPBS was supplemented with 20% Gly. An input of 10^{12} colony forming units (c.f.u.) phage from the antibody repertoire was incubated for 2 h whilst rotating. In the glycerol-positive selections, the 2% MPBS incubation buffer was supplemented with 20% Gly. The tubes were washed 20 times with PBS+0.05% Tween-20 (PBST) and 20 times with PBS. Bound phages were eluted by incubation with 1 ml 0.1 M triethylamine (TEA) for 10 min rotating. Phages were neutralised with 0.5 ml 1 M Tris-Cl, pH 7.4, and the resulting phage-ab repertoire amplified as previously described [14]. Briefly, 10 ml of TG1, $OD_{600nm} = 0.5$, were infected with eluted phages for 30 min at 37°C. Subsequently 10 ml of 2×TY with 200 µg/ml ampicillin (Amp) and 2% glucose (Glu) were added to the infected cells and the cells grown for 1 h at 37°C with shaking. Helper phages (10^{10} c.f.u. of VCSM13, Stratagene) were added and allowed to superinfect for 15 min at 37°C. The cells were pelleted by centrifugation and resuspended in 10 ml of 2×TY with 100 µg/ml Amp and 25 µg/ml kanamycin and phage-abs produced overnight with shaking at 30°C.

For the subsequent 3–4 rounds of selection, 3 ml of cleared phage-ab supernatant (approximately 3×10^{11} c.f.u.) were used as input. The glycerol-positive selections were supplemented with glycerol to a final concentration of 20% (v/v).

2.3. ELISA

ELISA plates (Greiner GmbH, Germany) were coated with 100 µl antigen at a concentration of 10 µg/ml under conditions identical to those used during selection, i.e. when testing phage-abs from glycerol-positive selections, the coating buffer was supplemented with 20% Gly. Plates were blocked like the immunotubes, rinsed and 50 µl of cleared polyclonal phage supernatants added to 50 µl 4% MPBS which for glycerol-positive selections contained 40% Gly to give overall conditions identical to those applied during selections. Washing was done four times with PBST and four times with PBS and phage detected by a 1:2000 dilution of an HRP-conjugated anti-M13 antibody (Pharmacia, Sweden) in 2% MPBS with or without 20% Gly. The reaction was developed with OPD-tablets (Kem-En-Tec, Denmark) according to the manufacturers instructions and read at 490 nm with an ELISA reader (Bio-Rad, USA). To isolate monoclonal phage-abs, single phage-ab clones from round 4 (eEF1A-2, LDH) and round 5 (eEF1A-1) were screened in monoclonal phage ELISA as described [3] with the alterations indicated above.

2.4. Sequence analysis of phage-ab clones

The scFv encoding inserts were amplified by PCR with the primers *rev* (5'-AAACAGCTATGACCATG-3') and *gIII-fwd* (5'-GACAGC-CCTCATAGTTAGCG-3') for 25 cycles (94°C, 1 min; 55°C, 1 min; and 72°C for 1 min 30 s). The 900-bp fragments were isolated and the V_H sequenced with the *link-seq* primer (5'-CGATCCGCCACCGCC-AGA-3') using an Applied Biosystems 373A sequencer and the ABI Prism Dye terminator cycle sequencing kit (Perkin-Elmer). The HCDR3 and V_H -gene segment were identified by the VBASE directory [15].

2.5. Western blotting analysis

One microgram of either of the proteins eEF1A-1 and eEF1A-2 was run on a 12.5% SDS-PAGE gel and blotted onto nitrocellulose filters (Hybond-C, Amersham, Life Sciences) according to Sambrook et al. [16]. The filter was blocked overnight with 5% MPBS and 10% Gly. One milliliter of cleared phage-ab G3 supernatant was used to probe

the blot. Incubation with phage-abs was performed in a rolling tube for 1 h, whereby the solution was repeatedly passed over the filter. Ten percent glycerol was included in the incubation buffer. The filter was washed 3× 5 min with PBST and 3× 5 min with PBS. Bound phage-abs were detected by a 1:1000 dilution of HRP-conjugated anti-M13 (Pharmacia, Sweden) in 2% MPBS with 10% Gly. After washing, the blot was visualised using a chemiluminescence kit (SuperSignal, Pierce, USA) according to the manufacturer's instructions.

2.6. Analysis of glycerol's effect on phage-ablag interactions

ELISA plates were coated with LDH and eEF1A-1 without glycerol. The plates were blocked with 2% MPBS for 1 h at room temperature (r.t.). Fifty microliters of cleared monoclonal phage-ab A5, A9 and G3 supernatants (isolated by screening) were added in triplicate to wells coated with either eEF1A-1 or LDH containing 50 µl 4% MPBS with glycerol varying from 0 to 40%. Incubation was performed for 2 h at r.t. Upon washing, the bound phage-abs were detected with 1:2000 HRP-conjugated anti-M13 in 2% MPBS and processed as above.

2.7. Effect of glycerol on coating capacity

LDH and eEF1A-1 were immobilised overnight at 4°C in the same concentrations as above in 50 mM NaHCO₃, pH 9.1, supplemented with glycerol varying from 0 to 20%. Further processing of the ELISA was devoid of glycerol and performed as above using the monoclonal phage-abs A5, A9 and G3.

2.8. Non-specific phage binding assay

Phage-abs A9 and G3 (5×10^9 c.f.u.) were added to either non-coated or to wells coated with non-cognate antigen that had been blocked with 2% MPBS. After washing with 5× PBST and 5× PBS, phages were eluted, neutralised as above and titered.

3. Results

3.1. Phage antibody selection and screening

As phage repertoire selection of antibody fragment to eEF1A did not enrich specific binders to the protein, the standard selection protocol was changed to obviate the stability problems of the antigen. Selections were carried out against eEF1A-1, eEF1A-2 and LDH in identical experiments where glycerol was either present in all selection steps, except washing, or completely absent. As seen in Fig. 1, we consistently found that polyclonal phage-ELISA signals to eEF1A-1 and eEF1A-2 were only obtained in the glycerol-positive selections. In contrast, glycerol tended to 'slow down' the enrichment of specific binders to LDH as the positive signal did not appear until round 4 compared to round 2 for the glycerol-negative selection. Individual clones from the different selections were screened by monoclonal ELISA and positive clones subjected to sequence analysis. Three different phage-ab clones (A2, G3 and D1) selected against eEF1A-1 and one (C7) selected against eEF1A-2 were identified as presented in Table 1. Interestingly, two different clones (A9 and C11) were

Table 1
Overview of the different clones isolated against different antigens with different selection conditions

Target antigen	scFv	V_H -gene segment	HCDR3 sequence	Glycerol
eEF1A-1	A2	DP-51	VGRSSNTF	+
	G3	DP-49	VQRETSE	+
	D1	DP-49	WDATE	+
eEF1A-2	C7	DP-31	GAVL*D	+
LDH	A9	DP-45	L*SPRV	+
	C11	DP-45	*VDEN	+
LDH	A5	DP-3	TNSP	—

The CDR3 sequences are deduced based on DNA sequencing of the insert and the V_H -gene family is established using Vbase [15]. An asterisk indicates that an amber STOP codon was found at this position, which in the TG1 suppressor strain is read as a glutamic acid, E. The glycerol column indicates whether the clone in question was selected in the presence (+) or absence (—) of glycerol.

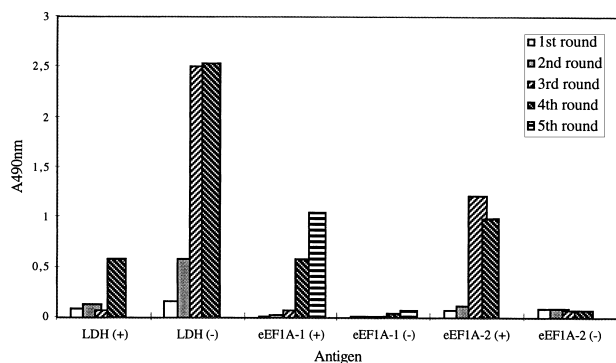


Fig. 1. ELISA data showing signals obtained with polyclonal pools of phage produced from individual selection rounds. The antigen in question is indicated as is the selection condition regarding glycerol; (+) indicates that 20% Gly was present during the selections in both incubation and coating buffer, whereas (-) indicates complete absence of glycerol in the selections.

found in the glycerol-positive LDH selections, which were absent in the glycerol-negative selection. The latter was totally dominated (15/15) by one clone (A5), that had previously been found to have high affinity for LDH [11]. This suggests that in the case of LDH, the selection profile was shifted by glycerol from being dominated by one clone to allow otherwise 'overtaken' clones to prosper.

3.2. Western blotting

The phage-abs were tested in Western blots and the G3 phage-ab displayed isoform-specificity for eEF1A-1 in Western blot as shown in Fig. 2, whereas D1 and A2 cross-reacted to eEF1A-2 as well (data not shown). The phage-ab C7, selected by panning to eEF1A-2, was cross-reactive to eEF1A-1 in ELISA but recognised neither of the denatured proteins (data not shown). Phage-ab A5 was reactive to the denatured protein as previously shown [11] (data not shown). Phage Western blots were carried out with 10% Gly in the incubation buffer. The incubation of phage-ab solution with the nitrocellulose filter was performed in a rolling tube, whereby the phage solution was repeatedly passed over the filter. These combined actions led to a significant increase in the signal-to-noise ratio compared to standard Western blotting procedure using phage-abs (S.K. and P.R., unpublished).

3.3. Phage-ab/ag interactions are differentially affected by glycerol

To elucidate the effect of glycerol during the selection procedure, phage ELISA was carried out with glycerol present solely in the incubation buffer during binding of phage-abs A5, A9 and G3. This set-up allowed detection of the influence of glycerol on the phage-ab/ag interaction. As seen from Fig. 3, glycerol had widely different effects depending on the phage-ab clone, antigen and selection conditions. Whereas phage-ab G3 was largely unaffected, the interaction between the dominant phage-ab A5 and LDH was dramatically weakened with increasing concentrations of glycerol. The positive effect of being selected in the presence of glycerol was clearly seen with the anti-LDH clone A9, where an increase in the ELISA signal was observed with increasing glycerol concentration. These monoclonal LDH data explain the different selection profiles between glycerol-positive and glycerol-negative seen for LDH in Fig. 1. The majority of LDH binding in

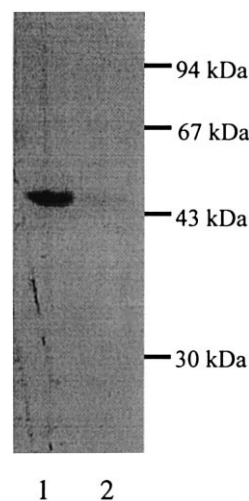


Fig. 2. One microgram of purified eEF1A-1 (lane 1) and eEF1A-2 (lane 2) was blotted onto nitrocellulose filters and probed with phage-ab G3.

the glycerol-negative selection was due to one 'super-binder', A5. The antigen affinity of this clone was reduced by glycerol, which eliminated the clone in the glycerol-positive selection, whereas other binders such as A9 were favoured by higher apparent affinity constants compared to normal buffer conditions. As no binders to eEF1A-1 or eEF1A-2 were found in the absence of glycerol, alternative explanations for their selection profiles were examined.

3.4. Glycerol lowers the coating capacity

The effect of glycerol in the coating buffer was tested as this could explain selection of phage-ab to the unstable eEF1A. As seen from Fig. 4, there was no direct advantage of glycerol in the coating buffer. Estimated from the ELISA signals, the coating capacity was lowered by around 25% with 20% Gly in the coating buffer. However, in this set-up where the initial rounds of selection were carried out with 50 µg/ml antigen, such a reduction is not detrimental to successful enrichment.

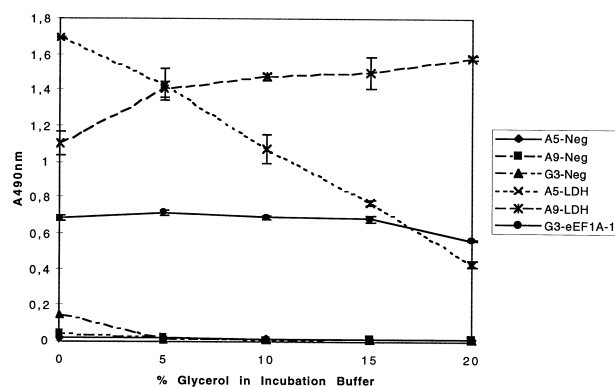


Fig. 3. ELISA data showing different effects of the presence of glycerol during the incubation with phage-ab. Phage-abs A5, A9 (anti-LDH) and G3 (anti-eEF1A-1) were incubated in triplicate with the indicated amount of glycerol in the incubation buffer in ELISA wells coated with cognate antigens. The specificity of the phage-abs was verified by incubation of phage-abs with skimmed milk powder blocked wells (Neg). Bars indicate the standard deviation of the means of triplicate experiments.

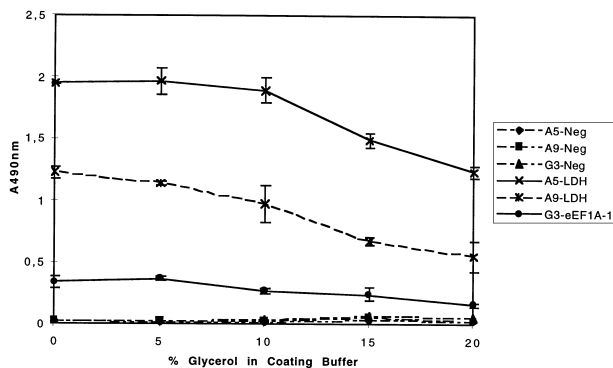


Fig. 4. ELISA data showing the effect of including glycerol in varying concentrations in the coating buffer. Cognate antigens, LDH for A5 and A9 and eEF1A-1 for G3, were coated overnight in 50 mM NaHCO₃, pH 9.1, with the indicated amounts of glycerol. Phage-ab A5, A9 and G3 were added in triplicate to antigen-coated wells and in a single experiment to wells coated with skimmed milk powder (Neg). Bars indicate the standard deviation of the means of triplicate experiments.

In addition, phage-abs recognising conformational epitopes may be selected. Supportive thereof, the phage-abs C7, A9 and C11 that were selected with glycerol all recognised epitopes lost upon SDS-PAGE and blotting, which are therefore likely to be conformational (data not shown).

3.5. Glycerol lowers the non-specific binding of phage

Another parameter of importance for the selection procedure is the level of non-specific background binding. Glycerol decreases hydrophobic and polar interactions and the effect of the non-specific phage binding was investigated as this could explain the eEF1A selection profile. The phage background binding was found to be lowered by three orders of magnitude for phage-ab A9 and G3 by 20% Gly as shown in Fig. 5. This is superior to background lowering obtained by the traditional detergent Tween-20, which in similar experiments at 1% (v/v) only reduced background binding 100-fold (data not shown). Lowered non-specific background binding combined with unaltered phage-ab/ag interaction will overall increase the enrichment rate of specific clones compared to non-specific and thereby accelerate the selection.

4. Discussion

We set out to isolate isoform-specific antibodies to the elon-

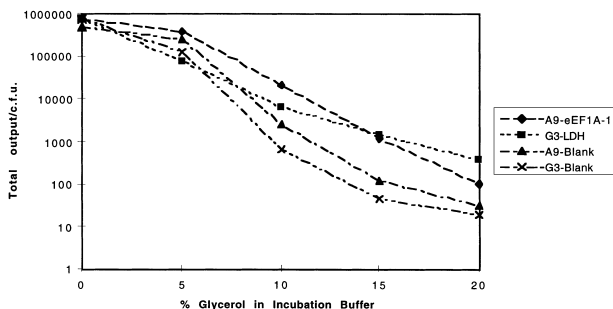


Fig. 5. Background assay to monitor the effect of glycerol on the number of phage particles sticking non-specifically to the antigen. Phage-ab G3 and A9 were added either to blank wells or to wells coated with 2 µg of the non-cognate antigen. Upon washing, phages were eluted and titered.

gation factor-1A. Two forms, eEF1A-1 and eEF1A-2, exist. Their individual expression seems from Northern blot analysis to be linked to the differentiation status of the investigated cells [17,18]. However, by use of isoform-specific monoclonal antibodies to human eEF1A, more information can be gained about the expression and localisation of the proteins in a spatio-temporal manner during differentiation. Large naive antibody repertoires displayed on phages offer a means of isolating new antibodies with desired specificities within a few weeks [1,2]. A traditional enrichment procedure involves: (i) immobilisation of the desired antigen to a plastic surface; (ii) incubation of the phage-ab repertoire; and (iii) washing to remove non-specifically adsorbed phages followed by elution and propagation of specific phage-abs. Initial selection attempts using a semi-synthetic phage-ab repertoire [3] were unsuccessful, which led to examination of the individual parts of the selection procedure and attention was drawn to the instability of eEF1A [12,19]. As glycerol is normally used as a stabiliser of eEF1A, we included this cosolvent in the coating, blocking and incubation buffer. Glycerol is a commonly used protein stabilising agent and has been found to be a very efficient compound in protein refolding [20,21]. Glycerol acts by inducing an ordering of the solvent by preferential hydration of the protein [22]. This makes unfolding of proteins, and hence exposure of hydrophobic domains, in glycerol solutions a thermodynamically less favoured event compared to the aqueous state [22]. Thus exposed hydrophobic regions on a denatured protein pack together, thereby causing increased refolding. The altered selection strategy led to enrichment of specific binders to eEF1A-1 and eEF1A-2. Parallel selection performed with the same material but without any glycerol failed as in preceding experiments. To investigate whether the observed phenomenon was intrinsic to eEF1A and to evaluate the general use of glycerol in phage repertoire selections, identical selections were done against LDH. In this case, binders were found in both glycerol-negative as well as glycerol-positive selections although the enrichment proceeded markedly slower with glycerol compared to the standard glycerol-negative set-up.

Two different effects of glycerol most likely account for the different selection profiles seen with eEF1A and LDH. In the LDH selection, sequence analysis and ELISA with increasing amounts of glycerol in the incubation buffer suggest that the selection was directed toward a different subset of binding phage-abs by glycerol. Without glycerol one clone, phage-ab A5, completely dominated the selection after 4 rounds. However, the interaction between LDH and phage-ab A5 was shown to be glycerol sensitive, which allowed poorer phage-abs to be selected in the presence of glycerol. One of these, phage-ab A9, showed an apparent increase in affinity by increasing amounts of glycerol, which consequently enhanced the selection shift. This demonstrates that a wider access to the diversity contained in large phage displayed repertoires can be obtained through selection with a cosolvent. This is obtained by promotion of 'glycerol-resistant' phage-abs at the expense of 'glycerol-sensitive' phage-abs in glycerol-positive selections. Evidently, glycerol-negative selections should be performed in parallel to enrich glycerol-sensitive phage-abs.

For eEF1A selections, we hypothesise that the reproducible preferential selection of phage-abs in the presence of glycerol can be explained by a lowered background binding. Eukaryotic EF1A is a basic hydrophobic protein [7], which may

therefore interact with the phage particle in a non-specific manner whereby obscuring of epitopes on eEF1A occurs. This is critical in the first round of selection, where a small number of each phage-ab is present [23]. For thermodynamic reasons, a threshold value of the epitope concentration needed for retention of sufficient numbers of specific clones exists. Glycerol may act by reducing the non-specific interaction between eEF1A and phage. Hereby, an otherwise hidden epitope on eEF1A is rendered accessible which in effect increases the epitope concentration above the threshold value. In addition, the low background will lead to higher enrichment factors for specific phage-abs. It is therefore imaginable that the binders would have emerged had the glycerol-negative selections been continued beyond 5 rounds.

Inclusion of glycerol in the coating buffer did not seem to have any measurable effect in either of the two selections. However, it is conceivable that more conformational epitopes are preserved by coating in glycerol, which may be useful, for instance, when antibodies for intracellular use are required [24]. In this context, selection of the phage-abs in glycerol may also be beneficial, as glycerol may mimic the intracellular environment with lower water activity and hence select phage-abs which perform well intracellularly.

A direct consequence of the low background level using glycerol in incubation is higher signal-to-noise ratio in downstream analysis of phage-abs. We found that the improved signal-to-noise ratio was sustained, when phage-abs were used as reagents in Western blotting. Glycerol most likely reduces the observed phage particle aggregation [25] by decreasing hydrophobic interactions between phage particles. A similar beneficial use of glycerol to reduce background binding has been reported using monoclonal antibodies in a traditional Western blotting set-up [26]. We have exploited this feature to set-up a protocol, that allows specific elution of phage-abs bound to a target of interest immobilised on a nitrocellulose filter. It was demonstrated in a model system that phage-abs specific for a given band on a blot could be enriched from a pool of different phage-abs (P.R., in preparation). This accelerates the screening procedure in situations where selections are carried out against complex mixtures of proteins in search of interesting antibodies to, for instance, proteins differentially expressed between two cell types.

In brief, we have generated isoform and cross-reactive phage-abs to eEF1A, which may prove of great utility in further studies of this intriguing protein. Their affinities, however, were poor (data not shown) but *in vitro* affinity maturation [27,28] has been performed leading to higher affinities, whilst maintaining the specificity profile demonstrated in this study (S.K., in preparation). Furthermore, we have investigated the properties of glycerol in the context of phage repertoire selections and found it a useful agent in a general selection strategy since: (i) certain antigen-antibody interactions can be inhibited at the benefit of others, which is useful if struggling with dominant binders [10]; (ii) a less denatured protein is presumably coated leading to maintenance of the native protein fold thus allowing selection of phage-abs to conformational epitopes; and (iii) the background binding of

the phage particle to matrix and protein is significantly reduced, which is of importance in assays such as phage-ELISA and phage Western blotting but may also increase the efficiency of panning phage-ab repertoires against cells in suspension by reduction of the non-specific interaction between the phage particle and the cell surface.

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