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# A platform for the early selection of non-competitive antibody-fragments from yeast surface display libraries

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**Abstract:** In this work, we report the development of a platform for the early selection of non-competitive antibody-fragments against cell surface receptors that do not compete for binding of their natural ligand. For the isolation of such subtype of blocking antibody-fragments, we applied special fluorescence-activated cell sorting strategies for antibody fragments isolation from yeast surface display libraries. Given that most of the monoclonal antibodies approved on the market are blocking ligand-receptor interactions often leading to resistance and/or side effects, targeting allosteric sites represents a promising mechanism of action to open new avenues for treatment. To directly identify these antibody-fragments during library screening, we employed immune libraries targeting the epidermal growth factor receptor as proof of concept. Incorporating a labeled orthosteric ligand during library sorting enables the early selection of non-competitive binders and introduces an additional criterion to refine the selection of candidates exhibiting noteworthy properties. Furthermore, after sequencing, more candidates were identified compared to classical sorting based solely on target binding. Hence, this platform can significantly improve the drug discovery process by the early selection of more candidates with desired properties.

**Keywords:** allostery; antibody discovery; competition; library screening; yeast surface display

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## 1 Introduction

Over the last four decades, antibodies have had a transformative impact for therapeutics. Today, antibodies represent one of the predominant classes of treatments with more than 120 approved biologics for a wide range of therapeutic areas including oncology, infectious diseases and immunological disorders (Carter and Lazar 2018; Mullard 2021). Most of the antibody discovery techniques rely on the immunization of an animal (mouse, rat or even camelids) subsequently coupled with the construction of large libraries via a display system (Lu et al. 2020). Well-established technologies for this *in vitro* selection described since 1990's include phage display and yeast surface display (Lu et al. 2020; Sheehan and Marasco 2015). Due to versatility of these systems and their robustness, repertoire sizes of more than $10^9$ variants can be functionally displayed in a single library. These libraries are subsequently screened on a high throughput basis and sequencing allows researchers to identify an enrichment of potential binders that will be further characterized (Lu et al. 2020). A drawback of these methodologies is the long and tedious process of finding a hit candidate with desired properties.

A common mechanism of action (MoA) that is aimed for an antibody is blockade of a receptor or its cognate ligand, meaning competition for the binding site between a ligand and its receptor (Carter and Lazar 2018). This MoA has the advantage of perturbating ligand-associated pathways and therefore dysregulating receptor functions. However, it is also associated with the development of acquired resistance and binding to related off-targets with conserved orthosteric sites (Martinelli et al. 2009). Allosteric antibodies, antibodies that bind to topographically distinct sites from the ligand binding one (Changeux 2013; Monod et al. 1965), can

modulate protein affinity, activity and induce conformational changes (Changeux 2013; Christopoulos et al. 2014; Monod et al. 1965). In addition, allosteric antibodies have the potential to be more specific as they do not recognize the epitope that may cross react with several ligands (Monod et al. 1965). As an example, the epidermal growth factor receptor (EGFR) has eight known characterized ligands and three of them are also binding to the fourth member of the EGFR protein family: HER4 increasing potential off-target effects (Martinelli et al. 2009). Furthermore, as only 15 % of patients are responding to current anti-EGFR approved orthosteric antibodies with a growing number of acquired resistance (Martinelli et al. 2009), it would be beneficial to develop antibodies with a distinct mechanism of action to open new avenues for treatments.

One of the first challenges is to identify these allosteric antibodies. Currently serendipity is the main driver as this MoA is often only characterized after reformatting by performing competition assays with the natural ligand. In 1996, Parsons and colleagues reported a method called competitive deselection of a phage library to specifically select fetal hemoglobin (HbF) binders in the presence of adult hemoglobin (HbA) (Parsons et al. 1996). Scientists at XOMA used a similar method thus preventing the selection of orthosteric antibodies against the insulin receptor from a phage display library (Bhaskar et al. 2012; Corbin et al. 2014a, 2014b). In efforts to treat the second most common neurodegenerative disorder (Parkinson's disease), Singh et al. (2022) raised allosteric activity-modulating nanobodies against LRRK2. They did so by combining different immunization strategies as well as phage panning in the presence of large excess of GTP analog or GDP (Singh et al. 2022). Regarding the yeast surface display platform, a novel approach for the discovery of conformational-specific nanobodies against a G-protein-coupled receptor: the human M2 muscarinic receptor to enrich clones that bind preferentially to agonist-occupied receptor has been described by Kruse et al. (2013). Their methodology has been expanded recently to two proteins of the same target class: $\beta_2$AR and $A_{2A}$R (McMahon et al. 2018). However, even though all these approaches favor allosteric antibodies against different targets, there is no direct identification of their non-competitive binding to their respective targets.

We sought to develop a streamlined approach to discover non-blocking antibody-fragments through direct screening of libraries taking advantage of the accurate control over several selection parameters of fluorescence-activated cell sorting (FACS). We describe here an approach combining yeast surface display libraries and a three-color staining strategy designed to detect the presence of an orthosteric ligand in order to identify allosteric binders that bind to the receptor in the presence of this ligand. As proof of concept we used proprietary anti-EGFR diversities derived from camelid (Sellmann et al. 2020) or cattle (Pekar et al. 2021) immunizations. The use of the labeled orthosteric ligand allows the early selection of non-competitors and introduces an additional criterion to refine the selection of allosteric candidates exhibiting noteworthy properties. Furthermore, after sequencing, more candidates were identified compared to classical sorting based solely on target binding. All of the binders were effectively binding to an allosteric site of EGFR validating thus the staining and sorting approach.

# 2 Results

## 2.1 Experimental design

To develop a platform for rapid discovery of non-competitive antibody-fragments, we designed a staining and sorting protocol with immune anti-EGFR libraries as proof of concept. We used proprietary diversities derived from camelid or cattle immunizations (Pekar et al. 2021; Sellmann et al. 2020). These libraries were generated as described elsewhere by homologous recombination (Benatuil et al. 2010; Pekar et al. 2021; Roth et al. 2020). Both libraries were incubated with secondary antibodies to detect the functional surface display (anti-HA for the camelid derived library and anti-lambda for the cattle derived library) and EGFR binding (anti-His APC conjugate). Libraries were subjected to FACS and a classical two-dimensional gate was applied to select binders based on functional display in addition to EGFR binding. The two libraries were enriched for EGFR binders within two rounds (Supplementary data 1).

To identify antibodies that do not bind the orthosteric site of EGFR we wanted to block its access by preincubating EGFR and its orthosteric ligand EGF and by simultaneously detecting the presence of the target and the ligand during the screening of the camelid derived library (Figure 1A) and the cattle derived one (Figure 1B). Antigen-binding was detected by indirect fluorescence: anti-HA Alexa Fluor 488 or anti-light chain FITC for the surface display, anti-His APC for EGFR binding and streptavidin-PE or anti-Fc PE for the binding of the orthosteric ligand. Enriched libraries were subsequently screened by FACS using a three-color sorting strategy. First, a two-dimensional gate was applied to select surface display in addition to EGFR binding positive cells (classical gate), then, a second two-dimensional gate was

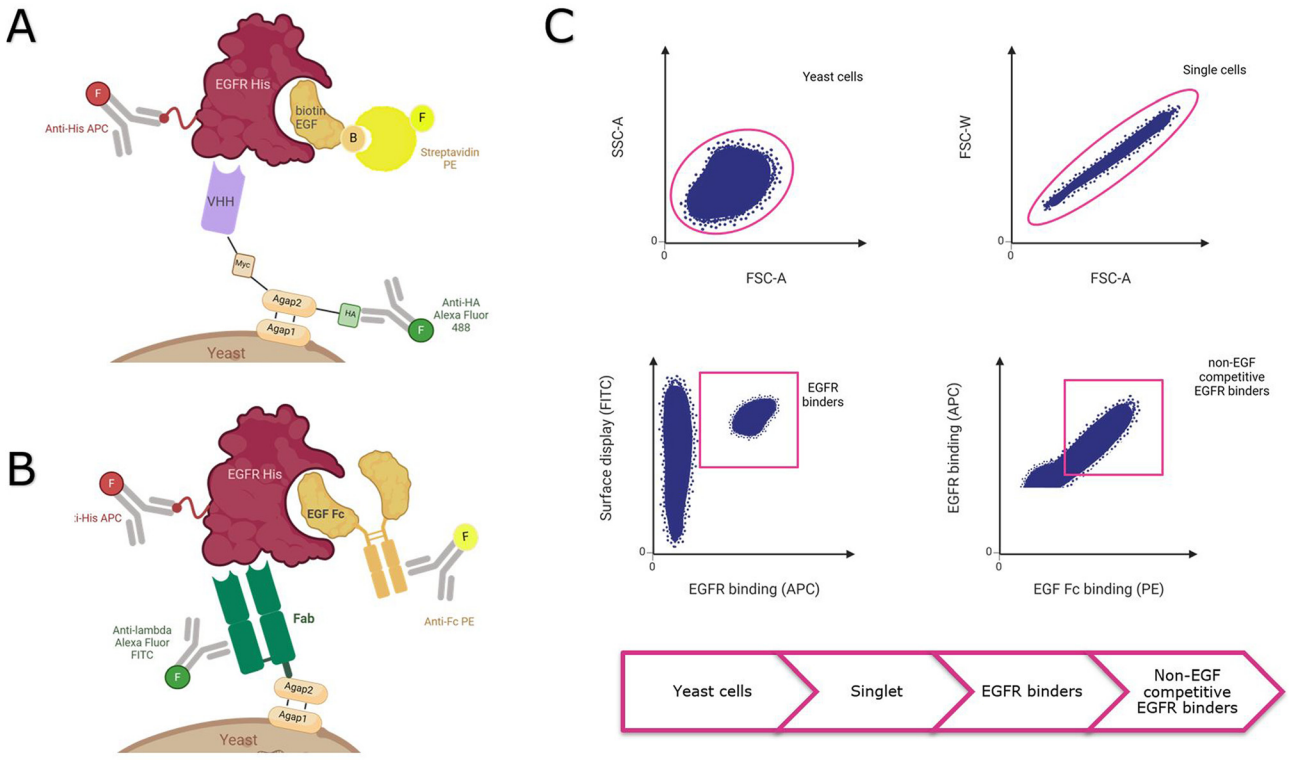


**Figure 1:** Overview of the strategy for the discovery of non-competitive antibodies from yeast surface display libraries. (A) VHH display. Yeast cell (brown) expressing VHH (purple) is incubated with the target (EGFR his tagged protein; in red) and its orthosteric ligand: biotinylated EGF (bEGF, as an example in yellow). Secondary detection is subsequently incubated to detect the VHH surface display (anti-HA AlexaFluor® 488 conjugated antibody), EGFR (anti-His APC conjugated antibody) and biotinylated EGF (Streptavidin PE conjugate). (B) Fab display. Yeast cell (brown) expressing Fab fragment (green) is incubated with the target (EGFR his tagged protein in red) and its orthosteric ligand: EGF Fc fusion protein (as an example, in yellow). Secondary detection antibodies are subsequently incubated to detect the Fab functional surface display (anti-lambda light chain FITC conjugated antibody), EGFR (anti-His APC conjugated antibody) and EGF Fc (anti-Fc PE conjugated antibody). (C) Gating strategy. Created with BioRender.com.

applied to select from the classical gate yeast cells binding to EGFR in the presence of the orthosteric ligand to screen for non-competitive binders (Figure 1C). The cattle derived library has been described as containing non-EGF competitive EGFR binders (Pekar et al. 2021). As a negative control we also used yeast cells displaying the humanized fab fragment of cetuximab, an approved anti-EGFR antibody that competes with EGF for the binding to EGFR (Supplementary data 2) (Martinelli et al. 2009). Controls also include unspecific binding to the orthosteric ligand only or its detection compounds.

## 2.2 Choice and detection of the orthosteric ligand

We first focused on the choice of the orthosteric ligand and its detection. EGFR is known to be the receptor of numerous ligands (Martinelli et al. 2009), but we decided to focus on EGF, as the main orthosteric ligand. To detect EGF, we either biotinylated it (bEGF, detection via streptavidin-fluorophore conjugate) or alternatively, we used a respective Fc fusion protein (EGF Fc, detection via secondary detection antibody). We first confirmed that bEGF and EGF Fc can bind to EGFR by BLI experiments (Supplementary data 3). We then incubated the cattle- and camelid-derived libraries enriched for EGFR binders with EGFR:bEGF or EGFR:EGF Fc respectively (Figure 2). Interestingly, most of the enriched camelid-derived library was positive for the complex EGFR:EGF Fc but only approximately one third for EGFR:bEGF resulting in a loss of potential candidates by doing the staining strategy with bEGF despite screening the same enriched library. Because the camelid-derived library also showed slight unspecific binding of EGF and its detection, the corresponding gate was adjusted accordingly. Unexpectedly, when we incubated the enriched anti-EGFR library displaying Fab fragment derived from cattle immunization, we could only detect double positive clones by incubation with EGF Fc while usage of bEGF revealed no double positive binders (Figure 2).

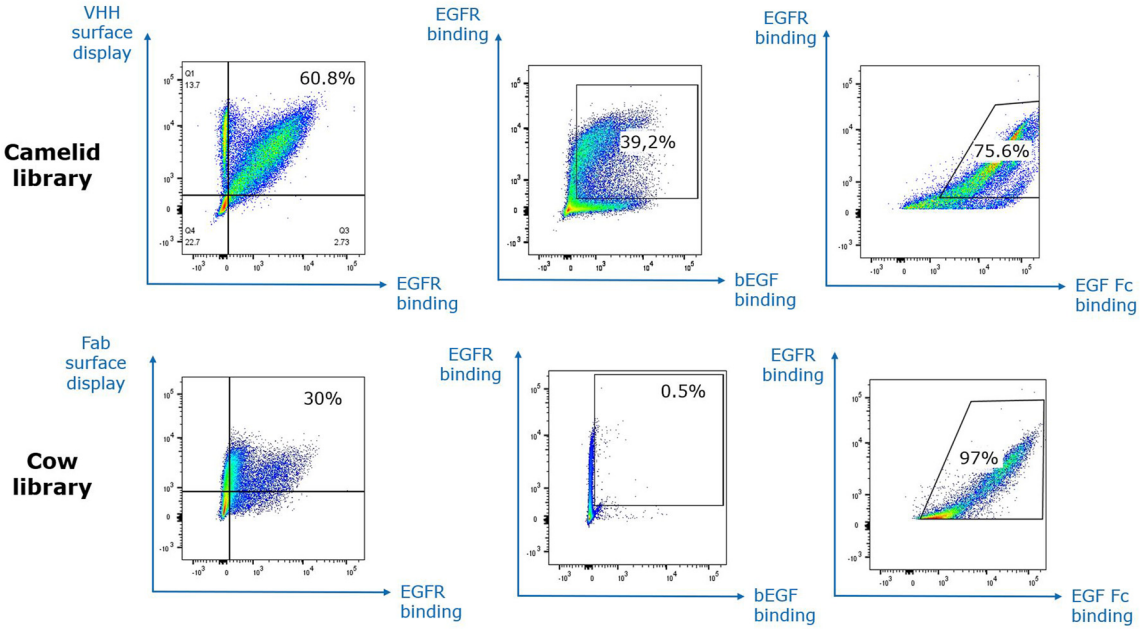


**Figure 2:** Ligand-influence on the staining strategy. Camelid-derived library (upper panel) or cattle-derived library (lower panel) were incubated with EGFR and either bEGF or EGF Fc. Secondary detection is subsequently incubated to detect the surface display (anti-HA AlexaFluor® 488 conjugated antibody for VHH display or anti-lambda light chain FITC conjugated antibody for Fab display), EGFR (anti-His APC conjugated antibody) and biotinylated EGF (Streptavidin PE conjugate) or EGF Fc (anti-Fc PE conjugated antibody). A first two-dimensional gate was applied to select simultaneously functional display and EGFR binding. From this, another two-dimensional gate was applied to select binders that could bind EGFR in the presence of EGF. For the VHH library, EGF was detected for both bEGF and EGF Fc strategies with an improvement for the EGF Fc one. Regarding the Fab library, it was not possible to detect bEGF but detection via EGF Fc resulted in identification of non-EGF-competitive clones. Binding was detected via FACS and applied sorting gates and corresponding cell population (as % of total cells) are shown. Plots were generated with FlowJo.

The reduced binding of bEGF compared to EGF-Fc in both experimental settings may be due to avidity effects. In addition, it has been described that the accessibility and detection of a protein displayed on the yeast cell surface might be impacted by the wall glycans (McMahon et al. 2018). We thus sought to increase the distance between the detection moiety and the protein complex. Therefore, we biotinylated EGF using long (EZ-Link™ Sulfo-NHS-LC-Biotin, 22,4 Å) or extra-long (EZ-Link™ Sulfo NHS-LC-LC-Biotin, 30,5 Å) linkers. Confirmation of the successful biotinylations and the fact that the binding of the biotinylated EGF to EGFR was not impacted was done by BLI experiments (Supplementary data 3). Unfortunately, the use of long linkers did not improve the detection of bEGF on yeast cell surface (Supplementary data 4).

In order to investigate whether a lack of signal amplification by using biotin/streptavidin-PE conjugate rather than a secondary detection antibody could be the source of the reduced staining, we incubated both enriched libraries with cetuximab (142 kDa). We detected cetuximab again with either an anti-Fc antibody or after biotinylation via streptavidin PE conjugate. Detection via the Fc portion of cetuximab resulted in the identification of 100 % of binders whereas the detection via biotin labelling generally led to a lower amount of double positive binders, with a bigger extent for the fragment-antigen-binding (Fab) library than for the VHH one (Supplementary data 5).

No double positive with EGFR:bEGF or EGFR:EGF Fc were identified for yeast cells displaying the humanized version of the Fab fragment of cetuximab (negative control, Supplementary data 6).

Taking all these data together, it is very tempting to assume that the differences observed between ligands rely on their detections and subsequent signal amplification. These data indicate that the preferred format for the detection of EGF binding is a Fc fusion protein with detection by a fluorescently labelled anti-Fc antibody. The type of ligand expression and detection strategy may also be considered when expanding the methodology to other target/ligand pairs.

## 2.3 Determination of the optimal conditions for the staining

In order to determine the appropriate incubation time for maximum detection of the orthosteric ligand, we first tried

to determine the equilibrium dissociation constant ($K_D$) values for EGF Fc and bEGF against EGFR. The apparent $K_D$ of the bivalent EGF Fc fusion protein appeared to be in the low pM range and could not be resolved precisely with the utilized assay conditions. The $K_D$ of bEGF was fitted to a 1:1 model and determined at 91.3 nM with a $k_{on}$ of $9.54 \times 10^3$ 1/Ms and $k_{off}$ of $8.71 \times 10^{-4}$ 1/s (Supplementary data 7). Based on these values, and as its association rate constant ($k_{on}$) is relatively slow, bEGF was pre-incubated for 1.5 h with EGFR at room temperature to allow equilibrium as the equilibrium time was estimated to 66 min (Jarmoskaite et al. 2020). Lower incubation time would decrease the correct identification of non-competitive clones. Equilibrium time for EGF Fc could not be calculated, therefore we used the same conditions as for bEGF resulting in maximum detection.

In their approach, researchers at XOMA described the use of a saturating concentration of insulin (10 µM) for phage display panning (Bhaskar et al. 2012; Corbin et al. 2014a, 2014b). In order to save material, we explored lower concentrations of EGF to determine the optimal ratio between EGFR and its ligand EGF for the maximal identification of non-competitive binders. We first tried a 1:1 M ratio between EGFR and EGF at a concentration of 1 µM for which we obtained slightly lower fractions of double-positive candidates especially for the Fab library (Figure 3). Therefore, we concluded that an excess of ligand compared to target would be better for the sorting (1 µM for EGFR and 3 µM for EGF). We tried higher concentrations of EGF (10 µM), this did not result in a larger number of double positive cells nor for the VHH than the Fab displaying library. Indeed, we even noted that a higher EGFR:EGF ratio (1 µM:10 µM) resulted in higher unspecific binding of EGF particularly to the cells of the camelid-derived library (Figure 3).

These findings emphasized the critical nature of conducting experiments under equilibrium conditions. This approach is essential to optimize ligand detection while minimizing potential unspecific discoveries.

## 2.4 Validation of the platform

Based on these previous findings and method optimization, we choose to sort the initial non-enriched camelid-derived library again with either the EGFR:bEGF complex or EGFR:EGF Fc complex without pre-enrichment of EGFR binders to compare the output. To this end, we used a 1:3 M ratio of EGFR:EGF (1 µM:3 µM) followed by 1.5 h of pre-incubation prior to incubation with yeast cells.

We selected double positive binders for sorting and could enrich within two rounds for the sorting with EGF Fc fusion protein, but three rounds were needed for the procedure with bEGF (Figure 4). These findings indicate that the addition of the orthosteric ligand during the library

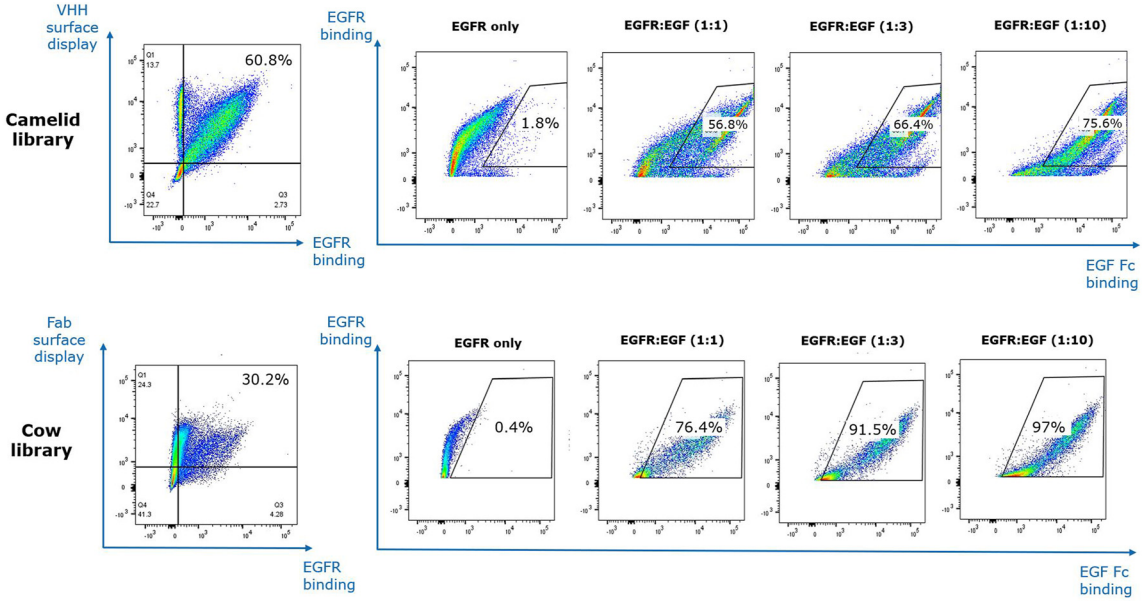


**Figure 3:** Determination of the optimal ratio target protein:ligand. Camelid- and cattle-derived libraries were incubated with EGFR (1 µM) and 1 µM, 3 µM and 10 µM of EGF Fc. Secondary detection antibodies are subsequently incubated to detect the functional surface display anti-HA AlexaFluor® 488 conjugated antibody for VHH display or anti-lambda light chain FITC conjugated antibody for Fab display), EGFR (anti-His APC conjugated antibody) and EGF Fc (anti-Fc PE conjugated antibody). Increasing concentrations of ligand improves its detection but at the highest concentration tested, the camelid library showed unspecific binding of both EGF and its secondary detection antibody, impacting the gating. Binding was detected via FACS and applied sorting gates and corresponding cell population (as % of total cells) are shown. Plots were generated with FlowJo.

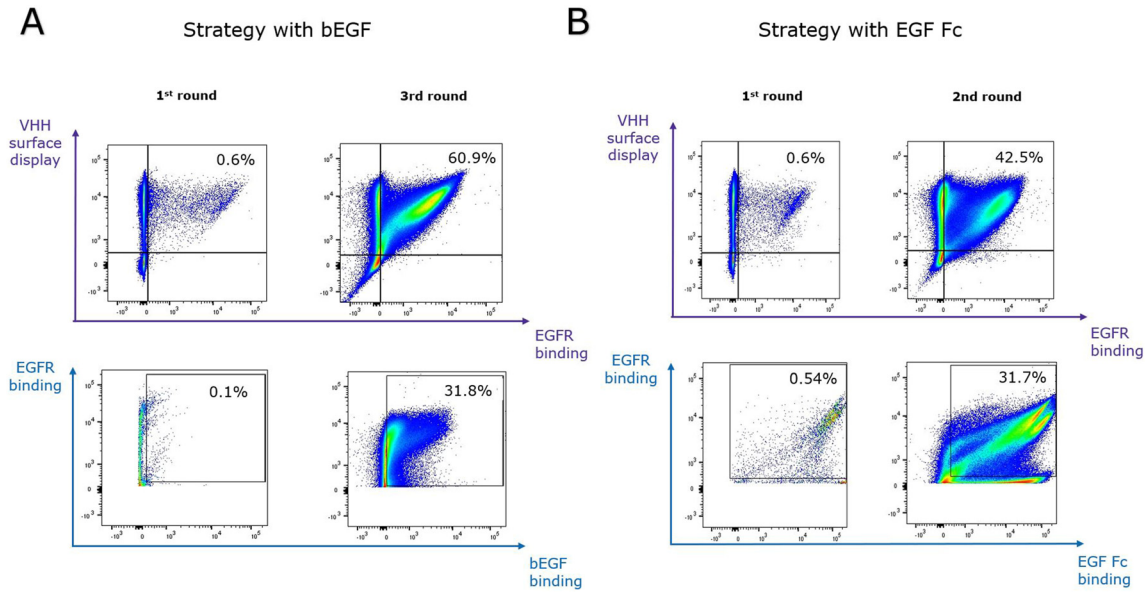


**Figure 4:** New FACS-based sorting of the camelid-derived library based on our findings. (A) Staining and sorting strategy with bEGF. (B) Staining and sorting with EGF Fc. Selection based on functional display and target binding (upper panel) and then based on non-competition with EGF (lower panel). Secondary detection is subsequently incubated to detect VHH surface display (anti-HA AlexaFluor® 488 conjugated antibody), EGFR (anti-His APC conjugated antibody) and biotinylated EGF (Streptavidin PE conjugate) or EGF Fc (anti-Fc PE conjugated antibody). Binding was detected via FACS and applied sorting gates and corresponding cell population (as % of total cells) are shown. Plots were generated with FlowJo.

screening did not significantly impede the duration of the enrichment procedure as two rounds were needed when sorting with EGFR only (Supplementary data 1).

The sequencing of the sorting output hits was analyzed based on complementarity determining region 3 (CDR3) homology. Based on sequence similarity of the CDR3 those clones were clustered into different clonotypes. All unique sequences were expressed as VHH-Fc fusion protein in Expi293F cells. We then excluded clones that were showing aggregation profiles and tested the remaining ones for their binding to EGFR by bio-layer interferometry (BLI). From the classical approach with EGFR only, three EGFR binders, belonging to three clonotypes were identified (Figure 5). Whereas the approach with EGFR:bEGF revealed eight

A

| Binder | Sorting with EGFR only | Sorting with EGFR:bEGF | Sorting with EGFR:EGF Fc |
|---|---|---|---|
| #1 | found | found | not found |
| #2 | found | found | found |
| #3 | not found | found | found |
| #4 | not found | found | found |
| #5 | found | found | found |
| #6 | not found | found | found |
| #7 | not found | found | found |
| #8 | not found | found | found |
| #9 | not found | not found | found |
| #10 | not found | not found | found |
| #11 | not found | not found | found |

B


**Figure 5:** Sequence analysis. (A) Comparison of the identification of binders for each approach. (B) Sequence alignment of EGFR binders obtained after the sortings. Complementarity-determining regions (CDR) are indicated above the alignment. Amino acid given in 1-letter code and in different color. Alignment generated with Geneious Prime.

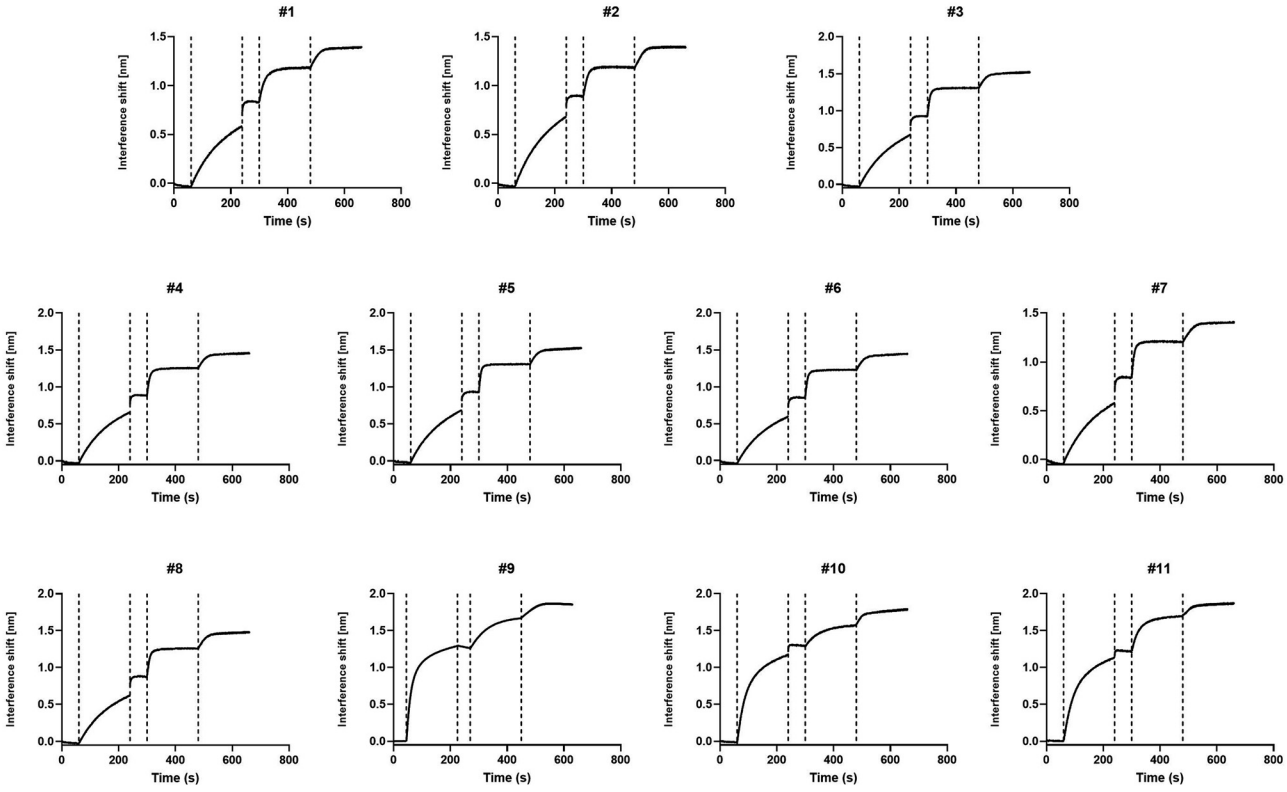


**Figure 6:** Competition assay with EGF for the binding to EGFR determined by BLI. Antibodies were loaded on AHC biosensors, followed by a baseline step before a first association with EGFR and a second association with EGF Fc. BLI sensograms show no competition between the antibodies and EGF.

binders belonging to three clonotypes (Figure 5). The approach with EGFR:EGF Fc led to the discovery of ten binders belonging to five clonotypes (Figure 5). This analysis confirmed that staining and sorting with bEGF instead of EGF Fc led to a slight loss of candidates. Nevertheless, our approach greatly enhances the possibilities of discovering more candidates compared to the classical sorting methodology with EGFR only.

As an ultimate validation of the platform, we performed a competition assay by BLI between the VHH-Fc and EGF Fc for the binding to EGFR. We were able to confirm the non-EGF-competitive binding of all the EGFR binders (Figure 6).

Taken together, these results show that our novel platform enables the identification of a significantly larger number of clones to a given receptor-ligand pair and even has the potential for fine-tuning the selection towards a different pattern of sequences compared to the classical sorting method solely based on target binding.

# 3 Discussion

Classical techniques to discover antibodies rely on the screening of large libraries to enrich potential binders that will be further characterized (Lu et al. 2020). This is a slow, uncertain, and expensive process to identify a hit candidate with desired properties. Our approach here addresses some of these challenges by providing a novel platform for the early selection of non-competitive binders from yeast surface display libraries. Our approach was designed to obtain antibodies that are bound to allosteric sites of EGFR because the latter site is occupied by its ligand, EGF.

While there are many assays to characterize the competitive nature of antibodies upon reformatting, we demonstrate that a three-color based staining and sorting strategy provides a rapid approach for their early discovery. However, FACS-based discovery requires purified and soluble proteins, both ligand and target for our platform which might be a limitation for difficult-to-express membrane proteins like G-protein couple receptors (GPCRs) (Jo and Jung 2016) or proteins for which binding partners are still unknown like HER2 (Hsu and Hung 2016).

An additional interest for this novel platform would be to unveil non-dominant antibodies of yeast surface display libraries as the sequencing output also showed that our approach revealed a different pattern of sequences compared to the classical one with EGFR only. Ditzel and colleagues previously demonstrated that their approach

called ‘epitope masking’ of a phage display library to be successful in rescuing antibodies against a poorly immunogenic epitope of CD4-binding-site on HIV-1 gp120 using dominant clones (Ditzel 2002; Ditzel et al. 1995). Similar findings have been reported for phage libraries by Tsui and colleagues against the respiratory syncytial virus, ‘epitope-blocked panning’ (Tsui et al. 1996) and by Sanna and colleagues against the herpes virus ‘antigen capture’ (Sanna et al. 1995).

Our platform would also be valuable when one wants to design biparatopic antibodies and obtain non-competitive binders from a reference molecule (Bogen et al. 2020; Niquille et al. 2024).

Expansion of this platform toward the discovery of conformational-locking antibodies is also expected. Recently, Davies and colleagues from Genentech reported the discovery via phage display of conformation locking antibodies against KRAS that could enable the discovery of more non-covalent hits binding in this specific conformation (Davies et al. 2022). With our approach, direct identification of conformation-locking antibodies would be possible upon the labeling of a compound inducing the of-interest conformation. Though it is possible that our approach leads to missing of potential candidates because of low signal from the binding partner, given the precision of modern FACS equipment we show that more candidates were identified by adding an extra criterion during the sorting. Parsons and colleagues also obtained comparable findings for their competitive deselection method for phage display library (Parsons et al. 1996).

Taken together, our novel yeast surface display screening platform enables the discovery of more candidates with desired properties even though some precautions are needed regarding the choice and detection of the ligand, particularly for libraries displaying Fab. We envision that early screening of desired properties will be an enabling resource for broadening antibody discovery.

# 4 Materials and methods

## 4.1 Proteins and labeling

Recombinant human Epidermal Growth Factor (rhEGF) was purchased from R&D Systems (236-GMP-01M) and was biotinylated (10-fold molar excess) using EZ-Link Sulfo-NHS-Biotin or EZ-Link™ Sulfo-NHS-LC-Biotin (LC; long linker) or EZ-Link™ Sulfo NHS-LC-LC-Biotin (LC-LC; extra-long linker) kits from ThermoFisher Scientific. Confirmation of the biotinylation and binding capacity of biotinylated rhEGF (bEGF) was done by Bio-Layer Interferometry (BLI) on an Octet RED BLI System (Sartorius) by measuring the binding against Streptavidin biosensors and EGFR, respectively. Recombinant human EGF Fc fusion protein was purchased from Sino Biological (10605-H01H). Recombinant human (rh) his-tagged extra cellular (ECD) domain of the Epidermal Growth Factor Receptor, (rhEGFR, amino acids: 25–642) was produced by Merck Healthcare KGaA. rhEGFR was labeled with Alexa Fluor® 647 using the Alexa Fluor 647 Conjugation kit (Abcam, ab269823) and confirmation of its labeling was confirmed by measuring the absorbance at 650 nm (Nanodrop) and by BLI experiments for binding capacity. Cetuximab (Erbitux) was produced by Merck Healthcare KGaA. Cetuximab was biotinylated (20-fold molar excess) also with EZ-Link Sulfo-NHS-Biotin (ThermoFisher Scientific) and confirmation of its biotinylation and binding capacity tested by BLI experiments.

Fab display was monitored with goat-F(ab2) anti human kappa (chain spec.)-FITC (Southern Biotech) for cetuximab or anti-lambda light chains-FITC (Sigma Aldrich) for the cattle-derived library. VHH surface display was detected using goat-HA Tag Alexa Fluor® 488-conjugated antibody (R&D systems). Detection of rhEGFR His binding was monitored using SureLight® APC Anti-6× His Tag® antibody (Abcam). Biotinylated compounds were detected via PE Streptavidin (Biolegend). R-Phycoerythrin AffiniPure Goat Anti-Human IgG, Fc Fragment specific (Jackson Immunoresearch) was used for the detection of Fc-fusion compounds.

## 4.2 Negative control

As a negative control, we used yeast cells displaying a humanized version of the cetuximab Fab fragment, an antibody competing with EGF for the binding to EGFR. Variable and first constant regions of the heavy chain (VH and CH1) were fused in a vector with Aga2p carrying a polyhistidine tag (6×His) to enable yeast cell surface presentation and detection. The light chain of cetuximab (kappa type) was also fused into another destination vector. After electroporation, yeast cells harboring heavy and light chains were matted by incubation on plates for 72 h at 30 °C. Confirmation of cells binding capacity to EGFR was tested on an iQue3 (Sartorius) by incubated the yeast cells with Alexa Fluor® 647 EGFR His tagged protein.

## 4.3 Camelid-derived library

The library was generated from B cells of two llamas that were immunized with the extracellular domain of EGFR. Library generation was performed by homologous recombination as

described previously (Benatuil et al. 2010; Pekar et al. 2021). *Saccharomyces cerevisiae* EBY100 was used for yeast surface display library generation. All experimental procedures and animal care were in accordance with European Union's animal welfare protection laws and regulations.

## 4.4 Cattle-derived library

We used a proprietary immune anti-EGFR yeast surface display library from Merck Healthcare KGaA derived from cattle immunization (Pekar et al. 2021). Library generation, sorting and subsequent assays have been described elsewhere (Pekar et al. 2021). All experimental procedures and animal care were in accordance with European Union´s animal welfare protection laws and regulations.

## 4.5 Library sortings

This section describes the enrichment of the aforementioned libraries against EGFR.

Briefly, yeast cells were grown overnight in SD medium with the appropriate drop out mix for 24 h at 30 °C. Afterwards, cells were seeded at an OD (Optical Density) of 1 in SG medium with the appropriate drop out mix for 48 h–72 h at 20 °C. Then, yeast cells were incubated with 1 µM of rhEGFR for 30 min on dark before a second incubation step with the detection antibodies for 30min in the dark. Cells were resuspended in an appropriate volume for sorting on a BD FACSAria™ Fusion cell sorter (BD Biosciences). A two-dimensional gate was applied to simultaneously detect surface display and EGFR binding. EGFR-binders were enriched within two rounds for both libraries. Enriched libraries were cryopreserved.

## 4.6 Library screenings

This part describes the screening of the aforementioned EGFR-binders enriched libraries in order to set up and optimize the platform.

For library screening, enriched libraries were grown overnight in SD medium with the appropriate drop out mix for 24 h at 30 °C. Afterwards, cells were induced in SG medium with the appropriate drop out mix for 48 h–72 h at 20 °C. Prior to the staining procedure, the ECD recombinant human his-tagged EGFR (1 µM) was incubated with the indicated orthosteric ligand molar concentration for 1.5 h at room temperature. All the subsequent labelling steps were performed on ice and in the dark for 30 min. Briefly, cells were first washed with PBS followed by incubation with the pre-formed complex. Cells were washed twice with PBS and then incubated with secondary detection antibodies. After washing twice with PBS cells were resuspended in 300 µL for screening on a BD FACSAria™ Fusion cell sorter (BD Biosciences).

## 4.7 Antibody expression

After sequencing, all the sequences were reformatted in a VHH-Fc fusion format in a pTT5 vector and subsequently expressed in Expi293F cells (ThermoFisher Scientific). After 6 days, cells were harvested, and heavy-chain antibodies purified via MabSelect (GE Healthcare). Purity was determined by size exclusion chromatography (SEC).

## 4.8 Biolayer interferometry

Qualitative binding assays were performed using an Octet RED® BLI (Sartorius) according to the manufacturer´s guidelines at 25 °C with 1000 rpm agitation.

For the confirmation of the biotinylation of EGF and its binding capacity to EGFR, biotinylated EGF (5 µg/mL) was loaded on SA biosensors for 180 s. After a rinsing step in kinetic buffer (KB, PBS + 0.1 % Tween 20 + 1 % Bovine Serum Albumine, BSA) for 60 s, the association with EGFR (100 nM) was tested for 180 s followed by 180 s of dissociation in KB.

For kinetic analysis, bEGF was loaded on SA biosensors alternatively EGF Fc was loaded on AHC biosensors both at 5 µg/mL for 180 s. After a rinsing step in KB for 60 s, the association was tested with various concentrations of EGFR for 300 s followed by a 600-s dissociation step in KB. Data were analyzed using Sartorius analysis software and $K_D$ were determined according to a 1:1 model.

For qualitative binding of the antibodies, these were loaded on AHC biosensors (5 µg/mL) for 180 s and rinsed in KB for 60 s. Association with EGFR (100 nM) was tested for 180 s before a dissociation step in KB. Alternatively for EGFR binders, the dissociation step was substituted for an association step with EGF Fc (100 nM).

Relevant controls were included for each experiment.

## 4.9 Data analysis

FlowJo software was used for plotting FACS data. Sequence alignment was performed using Geneious software.

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**Competing interests:** Except HK all authors are affiliated with Merck Healthcare KGaA. Besides, this work was conducted in the absence of any commercial interest.

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**Data availability:** The raw data can be obtained on request from the corresponding author.

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