

# Isolation of Common Light Chain Antibodies from Immunized Chickens Using Yeast Biopanning and Fluorescence-Activated Cell Sorting

Jan P. Bogen, Juliana Storka, Desislava Yanakieva, David Fiebig, Julius Grzeschik, Björn Hock, and Harald Kolmar\*

The phylogenetic distance between chickens and humans accounts for a strong immune response and a broader epitope coverage compared to rodent immunization approaches. Here the authors report the isolation of common light chain (cLC)-based chicken monoclonal antibodies from an anti-epidermal growth factor receptor (EGFR) immune library utilizing yeast surface display in combination with yeast biopanning and fluorescence-activated cell sorting (FACS). For the selection of high-affinity antibodies, a yeast cell library presenting cLC-comprising fragment antigen binding (Fab) fragments is panned against hEGFR-overexpressing A431 cells. The resulting cell–cell-complexes are sorted by FACS resulting in gradual enrichment of EGFR-binding Fabs in three sorting rounds. The isolated antibodies share the same light chain and show high specificity for EGFR, resulting in selective binding to A431 cells with notable EC50 values. All identified antibodies show very good aggregation propensity profiles and thermostabilities. Additionally, epitope binning demonstrates that these cLC antibodies cover a broad epitope space. Isolation of antibodies from immunized chickens by yeast cell biopanning makes an addition to the repertoire of methods for antibody library screening, paving the way for the generation of cLC-based bispecific antibodies against native mammalian receptors.


## 1. Introduction

Over the last decades, monoclonal antibodies (mAbs) have emerged as one of the most successful classes of biomolecules for therapeutic and diagnostic applications. In May 2020, overall 90 mAbs were approved in the United States, with many more in (pre-)clinical development.<sup>[1]</sup> Their antigen-specific nature allows the isolation of highly affine and selective binders against a large variety of targets, among them diverse cancer-related receptors, such as the epidermal growth factor receptor (EGFR). EGFR is commonly upregulated in metastatic colorectal cancer, non-small-cell lung cancer, pancreatic cancer, glioblastoma, head and neck cancer, and breast cancer.<sup>[2]</sup> Overexpression of EGFR is associated with cancer development, progression, and metastasis as well as poor prognosis.<sup>[3]</sup> Up to date, two EGFR-targeting mAbs (cetuximab and panitumumab) in combination with chemotherapy represent the standard of care for metastatic colorectal cancer.<sup>[4–6]</sup> However, the tumor-specificity of EGFR-targeting

antibodies is low, leading to off-target side effects<sup>[7]</sup>—an issue demanding for new approaches and solutions. Next-generation antibody formats, particularly bispecific antibodies (bsAbs), can overcome classical limitations such as insufficient selectivity by simultaneously targeting two distinct cancer-specific receptors, which in combination exist only on the surface of malignant cells. This strategy could thereby significantly enhance the specific targeting of malignant cells.<sup>[8]</sup> Nevertheless, construction of bsAbs implies two main challenges: Heavy chain heterodimerization and correct light chain pairing. The latter can be overcome using strategies like orthogonal fragment antigen binding (Fab) interfaces<sup>[9]</sup> or cross-mAb<sup>[10]</sup> approaches. However, the most straightforward solution is based on the usage of a common light chain (cLC), where both heavy chains pair with the same light chain. In these cLC antibody formats, the heavy chain variable domains (VHs) are the main driver in antigen recognition.<sup>[8]</sup> A major advantage of this strategy is that it does not require additional engineering and allows for utilization of the cLC in the initial affinity-based screening procedure, where affinity is mainly mediated by the VH domain.

J. P. Bogen, J. Storka, D. Yanakieva, Dr. D. Fiebig, Prof. H. Kolmar  
Institute for Organic Chemistry and Biochemistry  
Technical University of Darmstadt  
Alarich-Weiss-Straße 4, Darmstadt D-64287, Germany  
E-mail: Harald.Kolmar@TU-Darmstadt.de

J. P. Bogen, J. Storka, Dr. D. Fiebig, Dr. J. Grzeschik  
Ferring Darmstadt Laboratory  
Biologics Technology and Development  
Alarich-Weiss-Straße 4 Darmstadt D-64287, Germany  
Dr. B. Hock  
Ferring International Center S.A.  
Chemin de la Vergognausaz 50 Saint-Prex CH-1162, Switzerland

 The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/biot.202000240>

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In this report, chickens were chosen as immunization host due to their phylogenetic distance from humans. This enhances the chance for the isolation of mAbs against conserved mammalian epitopes otherwise not-addressable by immunization of alternative mammalian hosts, like mice and rats.<sup>[11]</sup> These characteristics account for the recent interest in avian-derived mAbs for diagnostic and therapeutic applications.<sup>[12–14]</sup> Additionally, due to the gene diversification in birds, library design is possible with a single set of primers, easing the process compared to rodents.<sup>[15]</sup> To our knowledge, no cLC antibodies were obtained so far upon avian immunization.

Recently, our group established a FACS-based screening technology utilizing yeast surface display (YSD) for the isolation of chicken-derived antibodies.<sup>[13,15,16]</sup> YSD in combination with FACS emerged as a powerful screening strategy, combining the advantages of a eukaryotic expression system with the convenient handling of yeast microorganisms and the online tracking during the ultra-high-throughput selection process.<sup>[17]</sup> However, while YSD in combination with FACS is reported to be efficient for isolating binders of soluble antigens, phage display technology in combination with cell panning is still the selection strategy of choice for membrane-bound proteins.

Screening for yeast displayed antibodies that selectively bind a target protein in the natural environment of a mammalian cell surface would be a preferred route, not only for integral membrane proteins that are difficult to solubilize but also for membrane-anchored proteins, such as EGFR, since it allows for direct screening for specific cancer cell binders.

Up to date, only a few publications reported on YSD antibody library screening via mammalian cell biopanning.<sup>[18–25]</sup> The first study of YSD in combination with cell panning was reported in 2005 when Wang and coworkers demonstrated that a fluorescein-specific single-chain fragment variable (scFv) could be isolated from a pool of unrelated yeast cells in a mixing experiment within three rounds of panning against fluorescein-labeled RBE4 endothelial cells.<sup>[18]</sup> Two years later the same group used yeast biopanning for the isolation of scFvs from a non-immune human single-chain antibody library against brain endothelial cells.<sup>[19]</sup>

This process, however, was not applicable in a high-throughput manner on a single-cell basis. Therefore, yeast biopanning was further optimized by combining it with a flow cytometric sorting process, enabling the isolation of antibodies from a human non-immune library against androgen-dependent prostate cancer cells by Williams et al., in 2014.<sup>[20]</sup> In a recent study by Yang et al., the authors reported a similar approach being used for the generation of affinity matured antibodies against the proton-gated ion channel ASIC1a.<sup>[21]</sup>

In this study, we report the first isolation of cLC antibodies from a chicken-derived immune library using yeast biopanning in combination with FACS, resulting in an enrichment of antibody variants with high affinities, covering a broad epitope space. This strategy might enable the generation of bispecific and biparatopic antibodies directed against challenging membrane-associated target proteins and epitopes in the future.

## 2. Results

Recently, Yang et al. demonstrated the utility of combining yeast cell panning and fluorescence-activated cell sorting for both

affinity maturation of antibodies and the isolation of antibodies from naïve libraries utilizing stably transfected mammalian cells.<sup>[21]</sup> This enables the isolation of antibodies against native membrane-bound antigens in a high-throughput manner and circumvents time-consuming establishment of appropriate target solubilization and stabilization procedures. However, since the generation of stable cell lines is laborious and many targets with therapeutic relevance, like EGFR, are overexpressed in standard cell lines like A431, we intended to simplify the process by panning viability-stained mammalian cells against immunostained yeast cells displaying a Fab library derived from chicken immunization. Since bsAbs gained increased interest in recent years, we further wanted to investigate whether combined yeast panning and FACS enables isolation of cLC-based antibodies against native mammalian receptors (**Figure 1**).

### 2.1. Library Generation

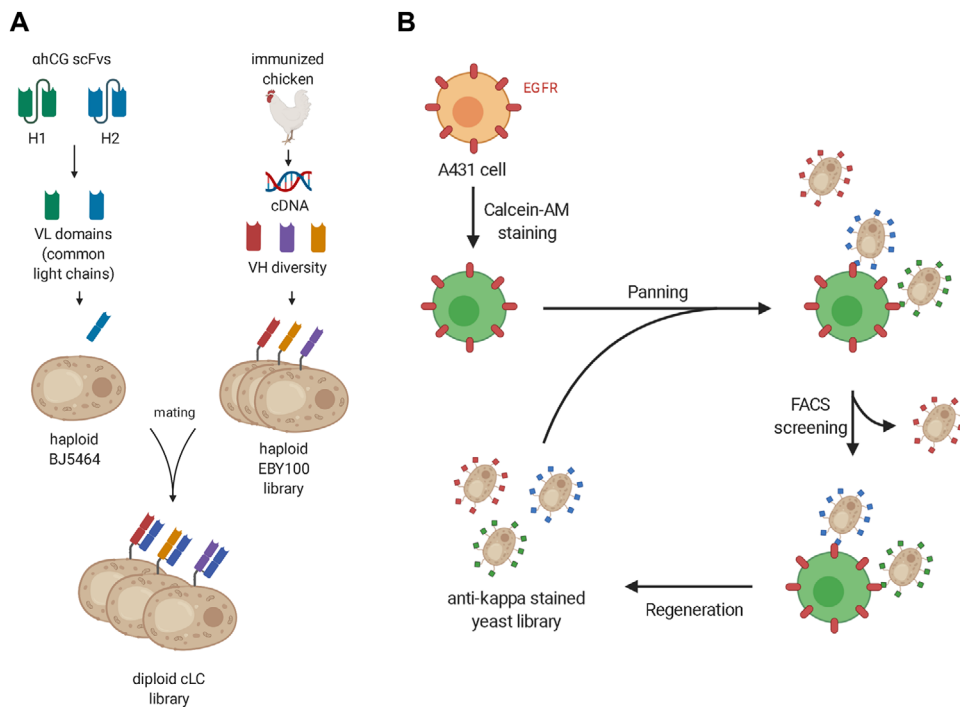
For Fab library generation from chickens, VH genes were amplified from cDNA derived from chicken immunized with the extracellular domain (ECD) of EGFR<sup>[13]</sup> and subsequently cloned into a pYD1-derived vector enabling YSD. To this end, a yeast library utilizing the strain EBY100 MAT $\alpha$  with a size of  $3.48 \times 10^8$  cells was generated, covering the expected heavy chain diversity.

As cLCs, the light chain variable domain (VL) domains of human chorionic gonadotropin (hCG)-specific chicken-derived scFvs were considered, named H1, H2, and H3, since they comprise different complementarity determining region (CDR) length and amino acid compositions.<sup>[13]</sup> To investigate whether these chicken VLs could be converted into a Fab format, corresponding VH and VL gene pairs were subcloned into the corresponding pYD1-derived vector and co-transformed into EBY100. After induction overnight, flow cytometric analysis revealed that all Fabs could be displayed on yeast cells, but only H1 and H2 combined with their corresponding heavy chain were still capable of binding hCG upon format change from scFv to Fab (data not shown). H3 was therefore not further implemented. To assure that H1 and H2 alone do not bind to EGFR, immunostaining using 100 nM EGFR-Fc (fragment crystallizable) chimera (R&D Systems) was performed resulting in no detectable binding (data not shown). Hence, the light chains of H1 and H2 were chosen as cLCs.

The heavy chain diversity in EBY100 MAT $\alpha$  was paired with the light chains of H1 and H2 in separate experiments via mating with yeast strain BJ5464 MAT $\alpha$ <sup>[26]</sup> producing either H1 or H2, respectively, resulting in  $3.85 \times 10^8$  diploid cells for the H1 and  $3.95 \times 10^8$  diploid cells for the H2 library. For sorting, both libraries were combined. A schematic representation of the library generation process is depicted in Figure 1A.

### 2.2. Staining Procedures and Sorting

Due to the high diversity of the initial immune library, the first sorting round was performed utilizing soluble EGFR-Fc chimera to ensure sufficient oversampling, as cell panning would have required a large number of mammalian cells, that are more complex in cultivation and propagation compared to yeast cells.



**Figure 1.** Schematic representation of the library generation and screening process. A) Schematic depiction of the library generation. B) Schematic representation of the library screening. Viable A431 cells were stained with Calcein-AM and were incubated with a pre-stained, Fab-presenting yeast cell library. Cell–cell complexes were isolated using FACS and yeast cells are regenerated for subsequent sorting rounds. Created with BioRender.com.

Additionally, prior studies demonstrated that FACS sorting is most efficient at a higher concentration of a binding population.<sup>[20,27,28]</sup>

For subsequent sorting rounds, EGFR<sup>+++</sup> A431 cells were stained for yeast panning using Calcein-AM, since this viability marker has proven to mediate a strong fluorescence signal in FACS analysis. Additionally, only viable cells are fluorescent and therefore utilized in the screening procedure. This ensures enrichment only of antibodies against the native and correctly folded receptor molecules, enabling discrimination of unspecific binding events elicited by dead cells and cell debris. Calcein-AM titration using the target A431 cells revealed that a concentration of 80 nM mediated the best separation of fluorescence signals between mammalian and yeast cells within the measurement threshold of the FACS device (Figure S1, Supporting Information).

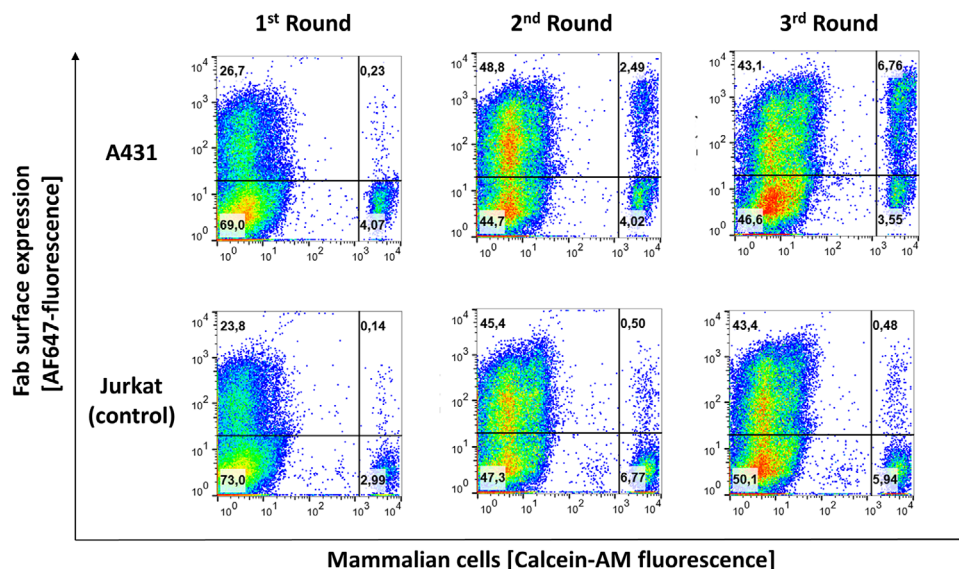
Two rounds of biopanning were performed by mixing yeast cells and A431 cells at a ratio of 20:1 and mammalian–yeast cell complexes (indicated by dual fluorescence signals) were sorted (Figure 1B; Figure S2, Supporting Information). In the sorting process of round three, a significant increase in the portion of double-positive events was observed (0.84% in round two, 8.60% in round three, Figure S2, Supporting Information), indicating enrichment of yeast cells displaying A431-specific Fabs. The population outcomes of the sorting rounds are shown in Figure 2. It is noticeable, that combined biopanning and FACS sorting led to the accumulation of A431-specific yeast cells while binding to EGFR-negative Jurkat cells was negligible and the corresponding number of double-positive events did not exceed 0.5%.

To further verify the accumulation of A431-specific Fab-displaying yeast, cell complexes were analyzed microscopically

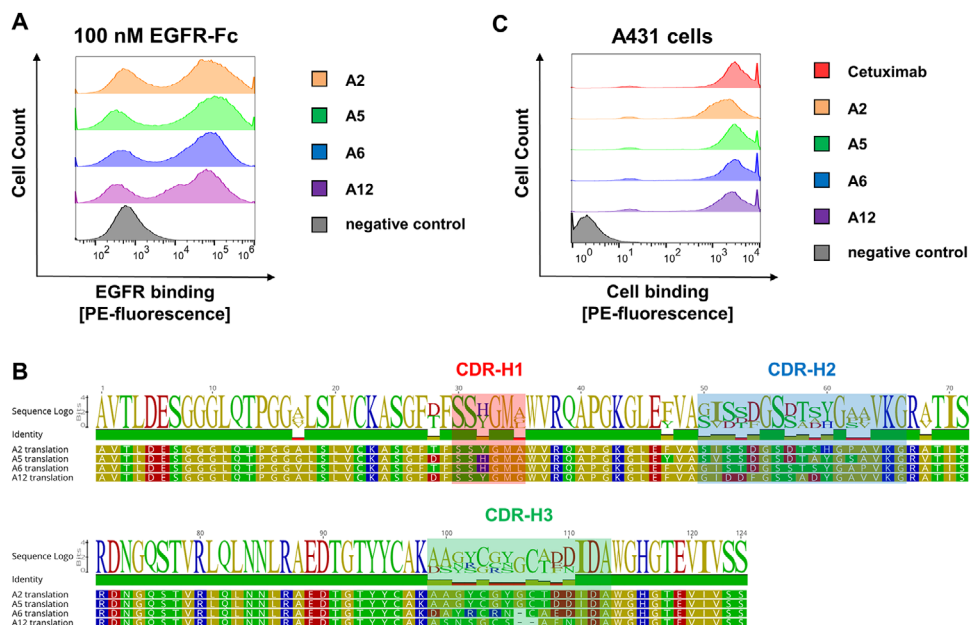
(Figure S3, Supporting Information). While the yeast population resulting from the first sorting round did not significantly form complexes with A431 cells, the population outcomes of the two subsequent rounds showed a substantial increase in yeast–A431 complexes, which is consistent with the observations obtained by FACS analysis. In contrast, the quantity of yeast–Jurkat complexes was very small and did not increase in the iterative sorting rounds. Following the observations made by Yang et al.<sup>[21]</sup> the high receptor density on the A431 cells promoted the interaction of multiple yeast cells with a single A431 cell, resulting in a higher number of sorted yeast cells compared to measured events.

### 2.3. Analysis of Isolated Clones

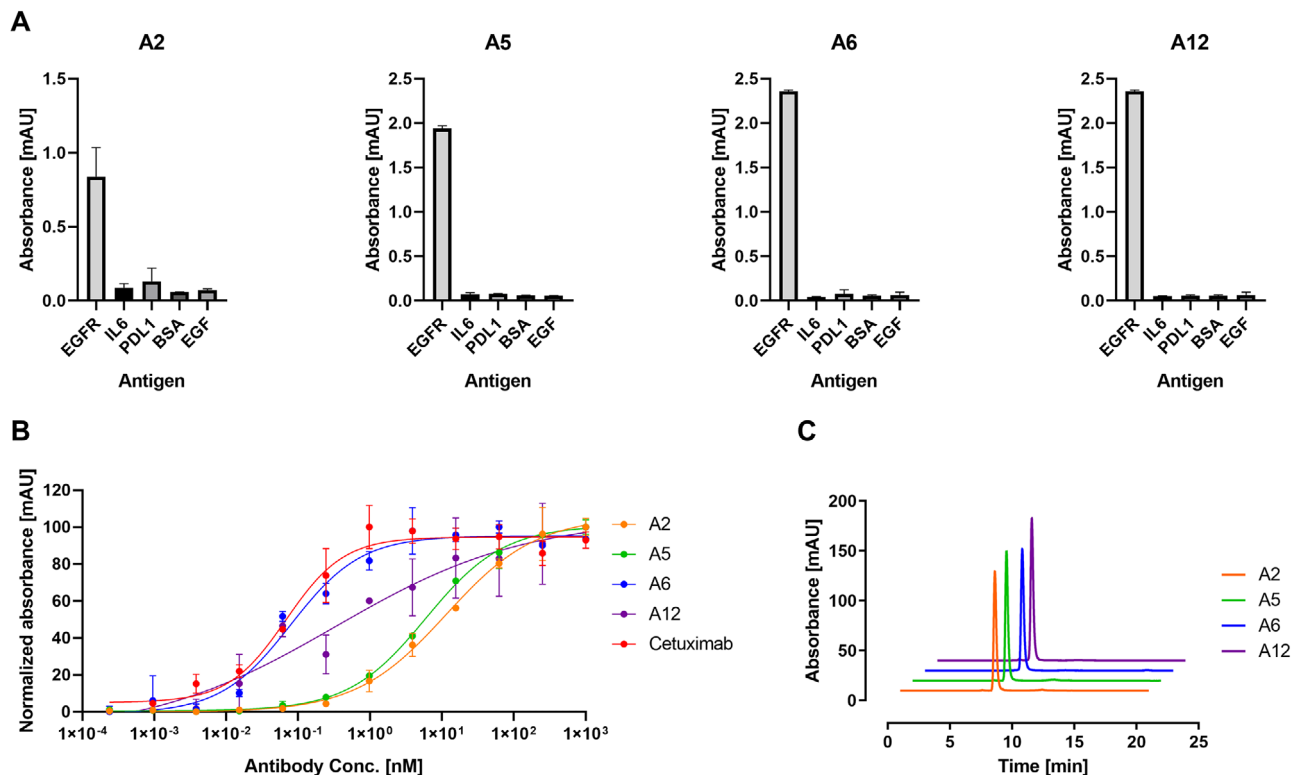
Next, A431-specific antibodies were isolated for further characterization. For this purpose, 20 randomly chosen single clones derived from the third sorting round were analyzed for binding to A431 cells (Figure S4, Supporting Information). Again, Jurkat cells were used as a negative control. Of the 20 clones tested, 12 bound to A431 cells. No binding to Jurkat cells was observed for any of the clones (Figure S5, Supporting Information). To verify the specificity of the enriched antibodies for EGFR, Fab-displaying yeast cells were incubated with 100 nM EGFR-Fc and antigen-binding was analyzed using FACS. All 12 analyzed clones showed strong binding to the EGFR chimera, underscoring EGFR targeting (Figure 3A). Sequencing revealed four unique sequences, named A2, A5, A6, and A12, that share the same cLC derived from H2 (Figure 3B). Aside from different sequences, deviations in CDR-H3 length were also observed. Taken together, these results prove the feasibility of combined



**Figure 2.** The outcome of sorting rounds biopanned against A431 (EGFR<sup>+++</sup>) and test for specificity with Jurkat (EGFR<sup>-</sup>) cells. Surface presentation is displayed on the y-axis. On the x-axis, the fluorescence intensity of Calcein-AM is depicted referring to stained EGFR-positive A431 or EGFR-negative Jurkat cells. In the lower left quadrant, yeast cells are depicted. In the upper left quadrant, yeast cells expressing a Fab-fragment are shown. In the lower right quadrant, Calcein-AM stained mammalian cells are shown. Double positive events (right upper quadrant) represent Fab variants on yeast cells that can bind to viable mammalian target cells. The percentage of events per gate is indicated. 50 000 events are plotted.



**Figure 3.** Binding specificity and sequence alignment of isolated antibodies. A) Binding of yeast-displayed Fabs to EGFR-Fc. For the negative control, EGFR-Fc addition was omitted. FACS analysis was performed using the SH800S Cell Sorter (Sony Biotechnology). Each plot depicts 100 000 events. B) VH domain sequences of single clones and resulting sequence logo. CDR1-3 are marked in red, blue, and green. The figure was created using Geneious Prime 2019. C) Binding of full-length chimeric antibodies to A431 cells. FACS analysis was performed using the BD Influx FACS device. Each histogram depicts 50 000 events.



**Figure 4.** Determination of unspecific binding behavior, EC50 values, and size exclusion chromatography of isolated antibodies. A) EGFR and additional unrelated proteins were coated on a MaxiSorp ELISA plate, followed by binding of respective antibodies and detection utilizing a goat anti-human Fc HRP antibody ( $n = 3$ ). B)  $2.5 \times 10^4$  A431 cells were stained with defined concentrations of isolated full-length chimeric antibodies. The binding was determined using a goat anti-human Fc HRP antibody. Resulting absorbance was plotted against the logarithmic antibody concentrations ( $n = 2$ ). Data points were fitted using GraphPad Prism 8 for determination of EC50 values. C) The aggregation propensity profile was analyzed using size exclusion chromatography in native conditions. Therefore a TSKgel SuperSW3000 column (Tosoh Bioscience) was utilized at a flow rate of  $0.35 \text{ mL min}^{-1}$  and PBS as eluent. A  $10 \mu\text{L}$  sample of a  $1 \text{ mg mL}^{-1}$  antibody solution was applied and protein elution was detected at  $280 \text{ nm}$ .

yeast panning and FACS for the isolation of heavy chain only binders from a chicken-derived immune-library.

#### 2.4. Biochemical and Biophysical Characterization

Besides EGFR, A431 cells exhibit a large number of additional membrane-bound proteins. To assure the binding specificity of the isolated mAbs, A2, A5, A6, and A12 were reformatted into a standard human IgG format and were produced as chimeric full-length antibodies in Expi293F cells. Protein A purified mAbs were used in an ELISA-based unspecific binding assay utilizing antigens that differs in size, folding and glycosylation. While all antibodies showed a strong binding toward immobilized EGFR, no significant binding signal was observed utilizing any other antigen (Figure 4A).

A single yeast cell can display up to  $10^5$  Fab fragments on its surface.<sup>[17]</sup> In a panning process with EGFR<sup>+++</sup> A431 cells, this could have resulted in a remarkable avidity effect, potentially leading to the isolation of low-affinity binders. To determine their binding characteristics, all soluble produced antibodies were at first verified for binding of A431 cells in a flow cytometric setup using cetuximab as a control (Figure 3C). Since all antibodies exhibited strong binding signals, EC50 values were determined

conducting a cell-based ELISA in duplicates. Soluble antibodies were titrated on a fixed number of A431 cells and stained using an anti-human HRP-conjugated secondary antibody (Figure 4B). The resulting absorbance was plotted against the antibody concentration. As a control, cetuximab was included in the experiment as a reference. The EC50 value for cetuximab on A431 cells was calculated to be  $0.07 \text{ nM}$  being in the range of the published affinity constants between  $0.1$  and  $5.2 \text{ nM}$  indicating the reliability of the experiment.<sup>[29–31]</sup> A6 and A12 showed the highest affinities toward A431-bound EGFR with EC50 values of  $0.08$  and  $0.34 \text{ nM}$ , respectively, while A2 ( $11.50 \text{ nM}$ ) and A5 ( $6.0 \text{ nM}$ ) showed lower EC50 values. Furthermore, we performed affinity titration experiments utilizing flow cytometry (Figure S8, Supporting Information). Obtained EC50 values ranged between  $27.2$  for A6 and  $59.5 \text{ nM}$  for A2, while cetuximab exhibited  $8.9 \text{ nM}$ , which resembles prior reported affinities.<sup>[30,31]</sup>

Recently, Grzeschik et al.<sup>[13]</sup> published chicken-derived scFvs against EGFR with affinities between  $6.8$  and  $42 \text{ nM}$ . Since these scFvs originate from a light chain shuffled repertoire of VHs and VLs from an immunization campaign, the cLC comprising chicken Fabs identified in this work were considered to have a notable affinity.

In the course of the biopanning process, yeast cells forming complexes with A431 cells were enriched. Therefore, we

**Table 1.** Data summaries for isolated anti-EGFR antibodies including EC50 values, stability, and aggregation behavior. EC50 data are derived from the cell-based ELISA and the 95% confidence interval is indicated.

Antibody	EC50 [nM]	$T_M$ [°C]	Aggregates [%]
A2	11.50 ± 4.3	74.6 ± 0.1	0.6
A5	6.0 ± 1.0	70.6 ± 0.1	0.3
A6	0.08 ± 0.04	65.4 ± 0.4	0.2
A12	0.34 ± 0.1	81.9 ± 0.1	0.9
Cetuximab	0.07 ± 0.03	—	—

investigated whether this screening would favor aggregation of isolated antibodies. Size exclusion chromatography (SEC) showed defined peaks for monomeric antibodies at retention times between 7.55 and 7.82 min (Figure 4C). In the case of A2, A5, and A6 there was nearly no aggregation while A12 showed marginal aggregation behavior, proving that this method leads to isolation of antibodies with extremely favorable aggregation propensity.

Subsequent NanoDSF measurements showed, that A12 exhibited the highest thermal stability having a melting temperature ( $T_M$ ) value of 81.9 °C. A2 and A5 exhibited similar melting points of 74.6 and 70.6 °C, respectively. The lowest  $T_M$  was observed for A6, namely 65.4 °C (Table 1; Figure S6, Supporting Information). These experiments demonstrate the high stability of the isolated antibodies resembling those of human or humanized full-length IgGs.<sup>[32]</sup>

## 2.5. Epitope Binning

To examine the EGFR epitope-coverage by the isolated antibody set, FACS-assisted epitope binning in a sandwich setup using YSD was performed. Thus, one antibody was displayed as Fab on the yeast surface (first antibody, capture antibody) and was incubated with His-tagged EGFR-ECD (produced in-house). Afterward, the respective second antibody was added in a soluble IgG format (second antibody, detection antibody) and detected by the anti-human Fc PE-conjugated antibody (Figure 5A). In case that both antibodies recognize orthogonal epitopes, a fluorescence signal can be detected. All isolated antibodies were tested in all combinations for both setups, using cetuximab (C225) as a reference (Figure 5B).

Resulting mean fluorescence was normalized and plotted (Figure 5C). A2, A5, and A12 showed overlapping epitopes with cetuximab and the epitopes of A2 and A5 were overlapping with each other. The determination of epitope overlap was conflicting in the case of A2 and A6. Interestingly, if A6 was used as the first antibody and A2 as the second mAb, binding could be observed, indicating orthogonal epitopes. However, in a switched setup, only weak binding was observed. These results indicate orthogonal epitopes in direct proximity that cannot be targeted in the A2–A6 setup due to potential steric hindrance caused by the yeast surface. The same was true for the A12–A2/A5 interaction (Figure 5C). A network plot combining all epitope-relationships of the tested antibodies was generated, summarizing the results of the epitope binning experiments (Figure 5D).

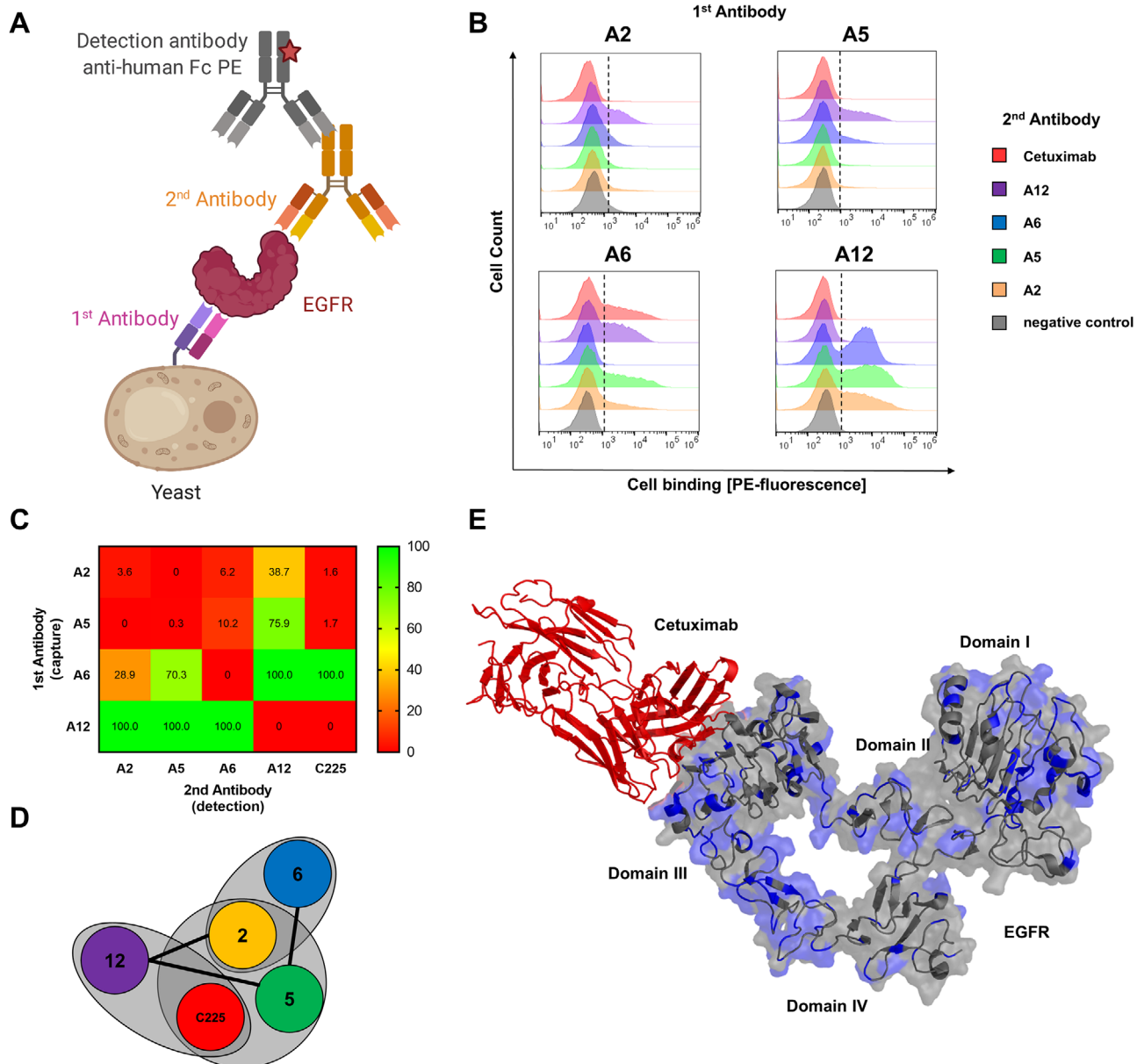
## 3. Discussion

Here we report, for the first time, utilization of FACS-assisted yeast biopanning for the enrichment of chicken-derived cLC antibodies from immune libraries. This strategy combines the high-throughput screening of antibody libraries against native membrane proteins overexpressed in standard cell lines, with the advantages of an immune library, like the reduced screening effort due to clonal expansion of target-specific antibody variants as well as direct isolation of higher-affinity antibodies due to the natural affinity maturation processes. To the best of our knowledge, this is also the first work showing that chicken-derived antibodies can be screened using a cLC approach.

There are only two reported examples of yeast mammalian cell biopanning in combination with FACS sorting: The first is from the Sooter group, where panning of a human non-immune scFv library was performed for three rounds against androgen-dependent prostate cancer cells followed by four additional FACS-assisted screening rounds, resulting in the isolation of binders exhibiting affinities of 27.3 and 33.2 nM, respectively.<sup>[20]</sup> More recently, the Lerner lab reported a FACS-assisted approach for the affinity maturation of an antagonistic antibody specific for the proton-gated ion channel ASIC1a ( $K_D = 0.28$  nM) and yielded antibodies with affinities of 0.10 and 0.12 nM.<sup>[21]</sup> The Lerner group was also able to demonstrate that FACS-assisted panning can be utilized for the isolation of scFvs from naïve libraries against the human mu opioid receptor, resulting in antibody fragments with affinities of 76 and 280 nM. The chicken-derived mAbs in this work were obtained from an immune library and exhibited favorable EC50 values in the range of 0.08 to 11.50 nM. Notably, no light chain diversity was implemented for obtaining these binders. These findings corroborate the suitability of FACS-assisted yeast biopanning for the enrichment of highly affine antibodies. As Yang and coworkers supposed, the formation of stable yeast-mammalian cell complexes most probably requires the presence of Fab molecules on the yeast surface in a sufficient number and with high affinity to persist shearing forces during FACS.<sup>[21]</sup>

Antibodies with double-digit to subnanomolar EC50 values were identified in this work using a cLC approach. Interestingly, EC50 values in the lower double-digit nanomolar range were obtained when flow cytometric affinity titrations were performed. This most probably originates in deviating cell numbers, the prior trypsinization, the measurement of fluorescence instead of absorbance, and the shear forces applied by the flow cytometer. Nevertheless, the obtained EC50 values of the cetuximab control (0.07 and 8.9 nM) closely resembles prior published affinities in the range of 0.1 and 5.2 nM, determined by different methods.<sup>[29–31]</sup>

cLC antibodies, isolated in previous work typically exhibited lower affinities,<sup>[33]</sup> and only recent studies describe the isolation of picomolar binders comprising cLCs.<sup>[8]</sup> Due to the broad immune response of chickens, a large spectrum of binders against the antigen used for immunization was obtained. Among these, a significant number of clones was identified that upon pairing with an unrelated light chain provide several VH sequences of distinct lineages covering several different epitopes, including the therapeutically relevant epitope of cetuximab on EGFR-ECD III, with high affinity. Additionally, besides excellent SEC



**Figure 5.** Epitope binning of anti-EGFR antibodies. A) Schematic overview of FACS-based sandwich epitope binning. Created with BioRender.com. B) Epitope binning for all four antibodies in all combinations and setups including cetuximab as reference. For the negative control, no soluble anti-EGFR antibody was added. FACS analysis was performed using the SH800S cell sorter (Sony Biotechnology). Each histogram depicts 100 000 events. Experiments were performed twice and yielded similar results, representative data are shown.  $n = 2$  C) Heat map of epitope binning. Red represents antibody combinations that cross-block each other, while green combinations represent orthogonal epitope coverage. Yeast bound (first) antibodies are depicted on the left side, applied detection antibodies (second) are shown on the x-axis. D) Network plot deduced from FACS results. Antibodies exhibiting overlapping epitopes are condensed in gray circles, antibodies competing with each other dependent of the staining order are connected via a black line. E) Crystal structure of EGFR extracellular domains in complex with the cetuximab Fab fragment (PDB: 1YY9). Cetuximab and EGFR are indicated in red and gray, respectively. Amino acid differences between the human EGFR and its chicken counterpart are indicated in blue.

profiles, high thermal stabilities were observed. Of the identified antibodies, only A2 and A5 had overlapping epitopes. This was expected, as both antibodies exhibited the same CDR-H3 and the only differences were found within the CDR-H1 and CDR-H2 sequences. Interestingly, the epitopes targeted by A6 and A2 seem to be in proximity, as a ternary complex formation on the surface of yeast cells depended on the order of binding. Since EGFR is

a receptor known for its conformational changes upon binding to ligands or antibodies,<sup>[34]</sup> antibody-induced conformation switches might have led to different binning results, depending on the setup. Additionally, steric hindrances by the yeast cell surface could be the reason for this finding and the lower EC<sub>50</sub> value of A2 might hinder the assay in this setup. Given that A2 and A5 targets the same epitope based on the identical CDR-H3, the

difference in EC50 might be responsible for a good binding signal using A5 and the weak binding signal using A2. Taken together, the four examined antibodies targeted three different epitopes in total, demonstrating that a broad epitope space was covered.

In Figure 5E the structure of EGFR-ECDs (gray) in complex with the cetuximab Fab (red) is shown. Amino acids that differ between the human and the chicken EGFR are highlighted in blue. Even though these differences are distributed over the whole molecule, all isolated binders target domain III, like cetuximab. The EGF binding site is located on domain I and III and this interaction is blocked by cetuximab. Therefore, mAbs showing overlapping epitopes with cetuximab might block EGF interaction and therefore show biological activity. Notably, chicken-derived VH/VL pairs can easily be humanized without loss of affinity upon CDR loop grafting into a human framework in combination with YSD screening of a library of variants with chicken/human residue combination at key positions for spatial CDR orientation.<sup>[35]</sup> Hence, chicken immunization provides a cost-effective and fast route for the generation of regular<sup>[13]</sup> but also cLC antibodies. Experiments for constructing bispecific humanized cLC antibodies using this strategy are currently underway.

Interestingly, all isolated antibodies in this work were combined with the H2 light chain, while no variants comprising the H1 light chain were identified. The reason for the preference of H2 is currently unclear. It might be related to pairing problems of H1 with a large set of VH domains. It is tempting to speculate that conformational changes imposed by the extremely short CDR1 of H1 and/or differences at four framework positions might account for this (Figure S7, Supporting Information). On the other hand, the H2 light chain functions as a versatile cLC as it seems to be able to pair with a large VH repertoire and to exhibit favorable biophysical properties.

In conclusion, it was demonstrated, that FACS-assisted yeast biopanning can be used for the screening of immune libraries, resulting in the isolation of a diverse set of antibodies covering a broad range of epitopes with good affinities. Here, this technology was applied for the isolation of cLC-based chicken antibodies from avian immune libraries. Concerning the future, this work paves the way for chicken-derived bispecific and biparatopic antibodies against surface proteins, especially in case of challenging targets like GPCRs and ion channels.

#### 4. Experimental Section

**Plasmids:** Plasmids used for YSD were derived from the pYD1 vector (Yeast Display Vector Kit, version D, #V835-01, Thermo Fisher Scientific). The heavy chain plasmid-encoded an ampicillin resistance gene and a tryptophan auxotrophic marker, as well as an Aga2 signal sequence, followed by the gene of the variable heavy chain domain, the constant human CH1 domain and the Aga2p-encoding sequence. The plasmid utilized for light chain secretion carried a kanamycin resistance, a leucine auxotrophic marker, the  $\alpha$ Mfp8 signal sequence followed by the variable light chain domain, and the human CL kappa domain. Gene expression of both plasmids was controlled via the galactose inducible promoter (GAL1). For expression as full-length chimeric IgG1 chimeric antibody, a pTT5-derived vector (Expresso CMV based system, Lucigen) with ampicillin resistance and CMV promoter was utilized as destination vector for Golden Gate subcloning. As entry vectors, pYD1-derived plasmids, encoding the human CL

lambda domain or the human CH1-CH2-CH3 domains, respectively, was utilized.

**Yeast Strains and Media:** The *Saccharomyces cerevisiae* strains EBY100 [MAT $\alpha$  URA3-52 trp1 leu2 $\Delta$ 1 his3 $\Delta$ 200 pep4::HIS3 prb1 $\Delta$ 1.6R can1 GAL (pIU211:URA3)] (Thermo Fisher Scientific) and BJ5464 (MAT $\alpha$  URA3-52 trp1 leu2 $\Delta$ 1his3 $\Delta$ 200 pep4::HIS3 prb1 $\Delta$ 1.6R can1 GAL) (American Type Culture Collection) were transformed with the plasmids harboring the genes for the heavy chain and the light chain for Fab display, respectively. Yeast strains were cultivated in YPD medium composed of 20 g L<sup>-1</sup> peptone/casein (CarlRoth, #8986.2), 20 g L<sup>-1</sup> glucose (CarlRoth, #HN06.3) supplemented with 10 g L<sup>-1</sup> yeast extract (CarlRoth, #2904.3). The cultivation of haploid and diploid yeasts on SD-CAA and SG-CAA media was performed as described before.<sup>[26]</sup>

**Cultivation of A431 Cells:** A431 human epidermoid carcinoma cells (ATCC CRL-1555) were grown in DMEM medium (D6429 Sigma-Aldrich), supplemented with 10% FBS superior (Merck Millipore) and 1% Penicillin-Streptomycin (Sigma-Aldrich P0781) in T75 or T25 cell culture flasks (Sarstedt, Cap/CS300, Cap/CS100) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells were trypsinized (Gibco, 15400054) and passaged every 3–4 days, after reaching  $\approx$ 80% confluence.<sup>[14]</sup>

**Library Construction and Yeast Mating:** VH genes were amplified using cDNA extracted from spleen cells of chickens being immunized with EGFR-ECDs.<sup>[13]</sup> PCRs were performed as described by Grzeschik and coworkers.<sup>[13]</sup> Amplicons were purified using the Promega Wizard SV Gel and PCR Clean-Up System (A9281). The heavy chain pYD1 vector was linearized utilizing NheI-HF and BamHI-HF (NEB) according to the manufacturer's protocol. Homologous recombination of VH genes into the pYD1 vector was conducted in EBY100 yeast cells according to the protocol described by Benatuil et al.<sup>[36]</sup> Electroporations were performed utilizing a Bio-Rad Gene Pulser Xcell Electroporation System (1652660) and Bio-Rad Gene Pulser/MicroPulser Electroporation Cuvettes (0.2 cm gap, 1652082).

The light chains utilized as cLCs (H1 and H2) were recently described by Grzeschik et al.<sup>[13]</sup> For incorporation of amplified VL genes into the light chain pYD1 vector, the latter was linearized utilizing NheI-HF and NcoI-HF (NEB) and homologous recombination was performed in BJ5464 yeast cells. PCRs were either performed utilizing Taq or Q5 polymerase (NEB) according to the manufacturer's instructions. All primers utilized for library generation are listed in Table S1, Supporting Information.

To combine the heavy chain library with the cLCs for subsequent Fab display, mating was performed as described before.<sup>[26]</sup>

**Library Sorting and Yeast Panning:** For sorting rounds performed with soluble EGFR, 5  $\times$  10<sup>8</sup> induced yeast cells were washed once with PBSB (PBS + 0.1% BSA (CarlRoth, #8076.2)) and incubated for 15 min with a goat anti-human Kappa-Alexa Fluor 647 antibody (SouthernBiotech, 2064-31), diluted 1:75 in PBSB to detect surface presentation. After washing once with PBSB, yeast cells were incubated with 250 nM EGFR-Fc (R&D systems, 344-ER-050) for 30 min on ice. Following another washing step, target binding was detected by the addition of a goat anti-human IgG-Fc-PE conjugate (Fisher Scientific, #12-4998-82), diluted 1:50 in PBSB for 15 min. Cells were washed again and subsequently analyzed by flow cytometry using a BD Influx FACS device. Immunostaining of hCG-specific binders was performed analogously utilizing the goat anti-human Kappa-PE antibody (SouthernBiotech, 2060-09, diluted 1:75), 100 nM biotinylated hCG (BBI Solutions P111-0) and streptavidin-APC (Fisher Scientific, SA1005, diluted 1:50).

For yeast panning, A431 cells were detached from the culture flask, washed once with PBS, resuspended at a cell density of 1  $\times$  10<sup>7</sup> cells per mL PBS and stained with 80 nM Calcein-AM (Fisher Scientific, C1430) for 20 min at RT. The cells were centrifuged down and resuspended in pre-warmed standard cultivation medium followed by incubation at 37 °C for 10 min to optimize retention of Calcein-AM. A431 cells were subsequently washed once with PBSB before being prepared for the biopanning experiment. In parallel, the surface presentation of induced yeast cells was marked as described above. Subsequently, treated yeast and A431 cells were mixed in a 20:1 ratio and incubated at 4 °C for 30 min while shaking gently.



Cells were directly used for flow cytometry using either a BD Influx FACS Cell sorter (software 1.0.0.650) or a Sony SH800 (2.1.5).

**Reformatting, Expression, and Purification of Chimeric Full-Length Antibodies:** Plasmids were isolated from yeast cells utilizing the Zymoprep Yeast Plasmid Miniprep kit I (Zymoresearch, D2001) and were used for transformation of *Escherichia coli* DH5 $\alpha$  (Life Technologies Cat.No. 82655A). Subsequently, plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification System (Promega, A1330) and sequenced at Microsynth Seqlab. VH genes, as well as the gene for the H2 VL, were amplified incorporating terminal SapI sites for subsequent Golden Gate cloning. All PCR reactions were performed utilizing Q5 polymerase (NEB) according to the manufacturer's protocol. Amplicons were subcloned into a pTT5-derived destination vector via Golden Gate assembly utilizing the CH1-CH2-CH3 entry vector for VH genes or the Lambda-CL entry vector for VL sequences, respectively. The alterations induced into these vectors to allow Golden Gate subcloning are shown in Figure S9, Supporting Information. Golden Gate reactions were performed using SapI (NEB), following the manufacturer's protocol. All primers utilized for reformatting are listed in Table S1, Supporting Information. After transformation of *E. coli*, DH5 $\alpha$  and sequence validation by Sanger sequencing, plasmids were isolated utilizing the PureYield Plasmid Midiprep System.

Expi293F cells (Thermo Fischer, A14527) were cultivated in Expi293 Expression Medium (ThermoFisher, A1435103) at 37 °C and 8.0% CO<sub>2</sub> at 110 rpm and subsequently transiently transfected according to the manufacturer's protocol (ThermoFisher). 5 days after transfection, sterile-filtered cell culture supernatant was applied to a Protein A HP column (GE Healthcare, 17-0402-01) using an Äkta Start FPLC system according to the manufacturer's instructions. Purified antibodies were subjected to dialysis against PBS and concentrated utilizing Amicon Ultra Centrifuge Filters (Amicon Ultra-15 Centrifugal Filter Units, Merck Millipore, 3 kDa MWCO).

**NanoDSF:** To evaluate the overall thermal stability of the isolated antibodies, melting points were determined utilizing the Prometheus NT.48 Protein Stability Instrument (NanoTemper Technologies). Therefore, tryptophan fluorescence at 350 and 330 nm of a 1 mg mL<sup>-1</sup> sample was measured between 20–90 °C with a heating rate of 1 °C min<sup>-1</sup>.  $T_M$  values were defined as the first maxima of the ratios of the fluorescence's first derivative at 330 and 350 nm.

**Unspecific Binding ELISA:** A Nunc MaxiSorp flat-bottom 96 well plate (ThermoFisher, 44-2404-21) was coated with 50  $\mu$ L of a 650 ng mL<sup>-1</sup> solution of the respective antigen overnight at 4 °C. Subsequently, wells were washed three times using PBS-T (0.05% Tween-20 in PBS) and incubated with 100  $\mu$ L blocking solution (5% BSA in PBS). After 1 h incubation, wells were washed three times, followed by 1 h incubation with 50  $\mu$ L of the respective antibody (200 nM). Following three washing steps, 50  $\mu$ L of a 1:10 000 dilution of goat anti-human Fc HRP in PBS with 2% BSA was applied and incubated for 2 h. Subsequently, the plate was washed and incubated with 50  $\mu$ L TMB One solution (Promega, G7431). The reaction was stopped by adding 25  $\mu$ L of 160 nM sulfuric acid. Absorbance was measured at 450 nm.

**EC50 Determination:** Affinity of isolated antibodies was determined via affinity titration using A431 cells. To this end,  $2.5 \times 10^4$  viable cells were seeded to each well of a 96-well flat-bottom microtiter plate. Cells were grown overnight, followed by washing with PBS once and incubation with 100  $\mu$ L blocking solution (5% BSA in PBS). After washing three times using PBS, 50  $\mu$ L of the respective antibodies were applied in a concentration range of 1  $\mu$ M down to 0.2 nM in a fourfold serial dilution series. Following three washing steps, 50  $\mu$ L of a 1:10 000 dilution of goat anti-human Fc HRP in PBS with 2% BSA was applied and incubated for 2 h. Subsequently, the plate was washed and incubated with 50  $\mu$ L TMB One solution (Promega, G7431). The reaction was stopped by adding 25  $\mu$ L of 160 nM sulfuric acid. Absorbance was measured at 450 nm. The resulting curves were fitted using GraphPad Prism 8 using a variable slope four-parameter fit.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

antibody discovery, cell panning, common light chain, fluorescence-activated cell sorting, yeast display

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