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ORIGINAL PAPER

A One-Step Process for the Construction of Phage Display scFv and VHH Libraries

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Abstract

In this work we present a one-step cloning approach for the establishment of antibody phage display libraries relying on type IIs restriction enzymes. We show that single chain variable fragment (scFv) libraries with adequate qualities can readily be cloned in a 'scar-less' manner and that the isolation of antigen-specific antibodies from immunized chickens is feasible within three selection rounds. Moreover, we demonstrate the general applicability of this method by rapidly constructing and panning VHH single domain antibody phage display libraries from immunized Llama repertoires.

Keywords Phage display · Golden gate cloning · Type IIs enzyme · Antibody · scFv · VHH · Single domain antibody

Abbreviations

BLI	Bio-layer interferometry
BSA	Bovine serum albumin
CDR	Complementarity-determining region
ECD	Extracellular domain
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-linked immunosorbent assay
Fab	Antigen-binding fragment
GGC	Golden Gate Cloning
IPTG	Isopropyl-beta-D-thiogalacto pyranoside
MTP	Microtiter plate
PBS	Phosphate-buffered saline
RT	Room temperature
ScFv	Single chain variable fragment
TMB	3,3′,5,5′-Tetramethylbenzidne
VH	Variable domain of the heavy chain
VHH	Variable domain of the heavy chain of a heavy chain only antibody
VL	Variable domain of the light chain

Carolin Sellmann and Lukas Pekar contributed equally to this work.

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Background

Monoclonal antibodies emerged as the most important class of biologics on the therapeutic market. The simple fact that over 80 therapeutics have been granted marketing approval as well as that more than 570 antibody-derived candidates are currently being investigated in clinical trials is corroborating their therapeutic and economical potential [1]. The most prominent approach for the generation of antigen-specific antibodies relies on the combination of animal immunization with hybridoma technology, as pioneered by Köhler and Milstein [2]. With the advent of transgenic animals, e.g., rats or mice endowed with partially human antibody gene repertoires [3–7], fully human antibodies can be isolated in this manner. Alternatively, in vitro selection technologies such as phage display, yeast surface display, or ribosomal display enable the isolation of antigen-specific entities from naïve, synthetic as well as immunized repertoires

[8–12]. Here, the key feature that enables the identification of desired candidates from antibody libraries is the linkage between the antibody variant to its coding genetic information. In this respect, phage display is the most established as well as most widely used platform technology. In 2016, six phage-derived antibodies, among them also the best-selling drug Adalimumab (Humira®) [13], were approved for therapy and several further were tested in clinical trials. Only three years later, already 12 antibodies discovered via the phage display platform have reached approval.

In the commonly applied phagemid format [14], the expression of the antibody fragment as fusion with phage envelope protein pIII is uncoupled from phage protein production and phage replication. A morphogenic signal is added for packaging as well as for phage replication. Correct phage assembly thus requires co-expression of other phage proteins, as provided by helper phage [14]. Phage display allows for the generation of huge antibody libraries in different formats such as scFv, Fab as well as single antibody domains [15-19]. However, cloning of libraries comprising heavy and light chain diversities is a tedious and laborious process, for instance, as it requires sequential cloning of the light and heavy chain repertoire into the phagemid [20]. We have previously shown that the process of yeast surface display library generation can be streamlined significantly [21–23]. Utilization of type IIs restriction enzymes allowed for efficient one-step cloning of heavy as well as light chain diversities into a single Fab display plasmid in a directional manner as well as the isolation of high-affinity antibodies. Similarly, it was demonstrated by Nelson and Valadon that this cloning principle, referred to as Golden Gate Cloning (GGC), is also valid for the construction of phage display Fab libraries [24].

In this present work, we set out to broaden the applicability of GGC for library establishment in the context of phage display by the facile generation of phage display chicken (Gallus gallus domesticus) scFv immune libraries as well as camelid (Lama glama) single domain VHH immune libraries. To this end, we analyzed the respective antibody germline repertoires for type IIs restriction enzymes having a minimal or neglectable frequency of recognition sites in antibody variable domain genes. Likewise, PCR-amplified VH-, VL-, and VHH-repertoires, respectively, were scrutinized in this manner, enabling the identification of optimal enzymes for library establishment with minimal restriction enzyme-based bias. A cloning strategy was designed, allowing for one-step library generation (Fig. 1, Fig. S1). Libraries with a high level of insert correctness could readily be constructed using either splenocytes from chicken or PBMCs from llamas immunized with human EGFR antigen (extracellular domain) as starting material, an important target in cancer therapy. Panning of libraries enabled the selection of antigen-specific antibodies with high affinities ultimately giving clear evidence that GGC is a versatile tool for phage display-derived library construction and antibody hit discovery.

Methods

Animal Immunization

One chicken (Gallus gallus domesticus) was immunized with recombinant human EGFR (extracellular domain containing C-terminal hexahistidine-tag, produced, and purified in-house) as described by Grzeschik and colleagues at Davids Biotechnologie (Regensburg, Germany) [25]. Immunization was performed using 150 µg of antigen combined with AddaVax (InvivoGen) adjuvant. After initial administration, the chicken received boosts after 2, 4, 5, and 8 weeks. After 9 weeks, the animal was sacrificed and splenic RNA was isolated. All experimental procedures and animal care were in accordance with local animal welfare protection laws and regulations.

Moreover, two approximately five-year-old llamas (*Lama glama*) were immunized with recombinant human EGFR at preclinics GmbH (Potsdam, Germany). All experimental procedures and animal care were in accordance with local animal welfare protection laws and regulations. In brief, for each immunization 300 µg of EGFR diluted in a volume of 1 ml PBS were emulsified with either 1 ml Complete Freund's Adjuvant (first immunization) or Incomplete Freund's Adjuvant (subsequent immunizations). Injections were administered subcutaneously at three sites. A total of four immunizations were performed over the course of 56 days (day 0, 28, 42, and 56). On day 60, blood (100 ml) was collected and total RNA was extracted. After the study, camelids remained alive. All animals in this study were obtained from the respective service provider.

Plasmids

All plasmids were designed in-house and synthesized at GeneArt (Thermo Fisher Scientific). The plasmid pHAL14 served as template for the Golden Gate Cloning-compatible cloning approach [20]. All destination plasmids were designed to contain an ampicillin resistance cassette, whereas the respective entry plasmids comprised a kanamycin resistance gene.

Library Construction

Following RNA extraction, cDNA was synthesized using 50 µl RNA extract, 10 µl random hexamer primers (50 ng/µl), 10 µl 50 µM dNTPs, 30 µl H_2O, 40 µl 25 mM $MgCl_2$, 20 µl 10 × RT-buffer, 20 µL 0.1 M DTT, 10 µl RNase OUT,

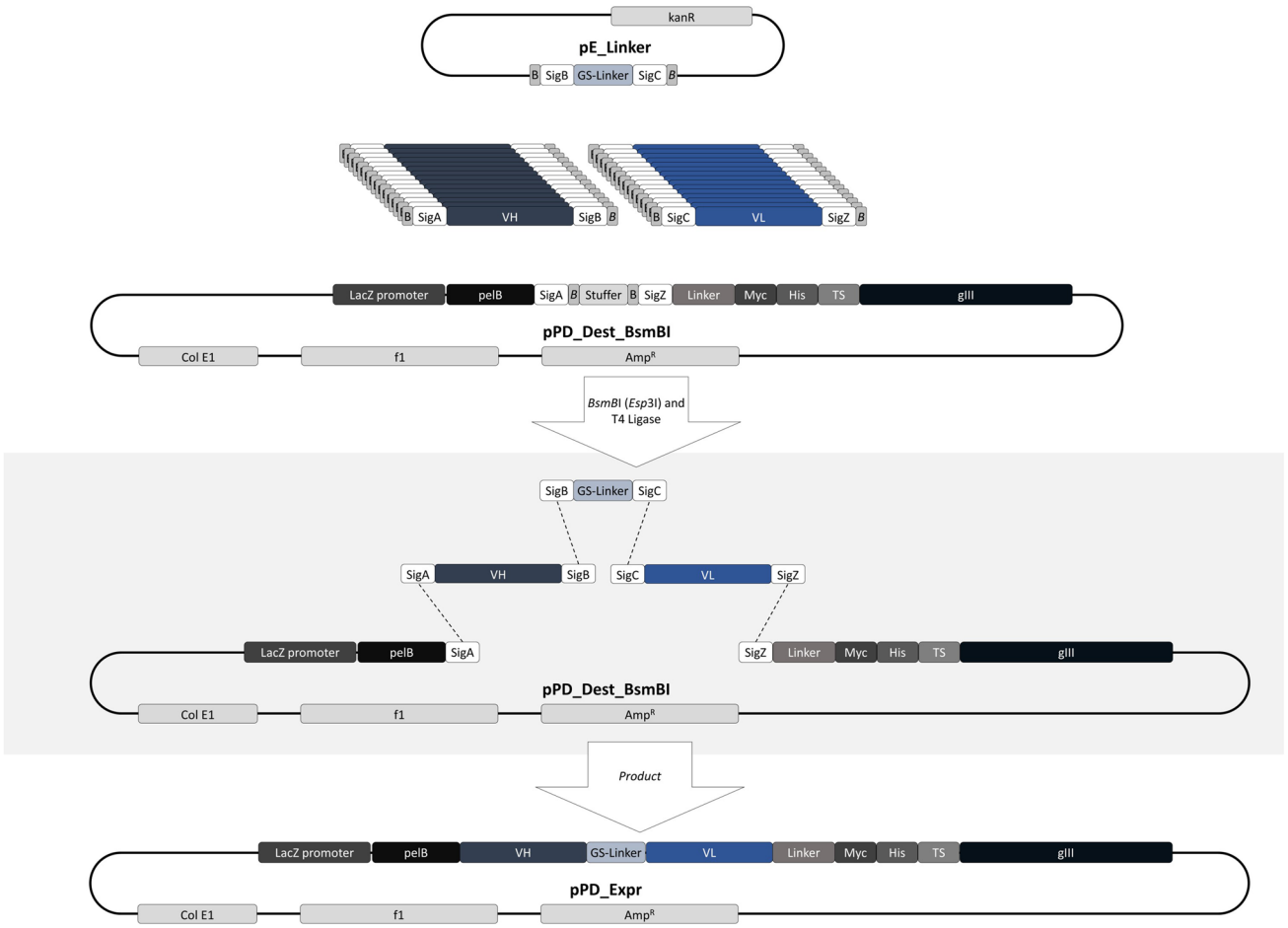

Fig. 1 Schematic overview of the one-step generation process of phage display plasmids for the construction of scFv antibody libraries. The destination plasmid (pPD_Dest_BsmBI) as well as the PCR-amplified VH and VL repertoires contain or are flanked by BsmBI recognition sites in opposite orientations (B: CGTCTCN, *B*: NGAG ACG). Complementary overhangs i.e., signature sequences (SigX) after Esp3I digestion enable the defined one-pot assembly of the four-component system during ligation resulting in the final display plasmid (pPD_Expr). ColE1: origin of replication. f1: origin of replication. Amp^R: Ampicillin resistance gene, pelB: leader sequence. Linker: amino acids AAAGS, Myc: c-Myc epitope EQKLISEEDL, His: hexahistidine-tag, TS: trypsin site KDIR, gIII: gene encoding for phage protein pIII. Note that an amber stop codon is introduced between the hexahistidine-tag and the trypsin site

and 10 µl SuperScript III reverse transcriptase in a volume of 200 µl (SuperScript III First-Strand Kit, Thermo Fisher Scientific). Reaction conditions were as follows: 5 min at 25 °C, 60 min at 50 °C followed by 5 min at 85 °C. 1 µl RNase was added followed by incubation for 20 min at 37 °C.

For the generation of chicken scFv phage display libraries, heavy and light chain antibody variable regions were amplified from cDNA in PCR reactions using germline specific primers annealing either to chicken heavy or light chain variable region framework 1 (Chicken_BsmBI_VH_up, Chicken_BsmBI_VL_up) and to the chicken framework 4 (Chicken_BsmBI_VH_lo, Chicken_BsmBI_VL_lo). Primer sequences are given in Table S1. The reaction was carried out with 1 µl cDNA, the corresponding primer pair for VH or VL amplification (each 1 µl à 10 pmol), and Q5 High-Fidelity 2 × Master Mix (NEB) in a volume of 50 µl using the following parameters: 30 s at 98 °C, 30 cycles of 30 s at 98 °C, 20 s at 60 °C, and 30 s at 72 °C, followed by a final elongation for 2 min at 72 °C. After PCR purification using Wizard® SV Gel and PCR Clean-up System (Promega) according to the manufacturer's instructions, amplified VH and VL regions were subjected to GGC employing 1 µg of destination plasmid pPD_Dest_BsmBI, 1.4 µg entry plasmid including the linker sequence pE_Linker, each 160 ng of VH and VL PCR product together with 400 units Esp3I, 1000 units T4 DNA ligase, and 10 µl T4 Ligase buffer (all NEB) in a final volume of 100 µl (Fig. 1). The reaction mixture was incubated for 40 cycles of 1 min at 37 °C and 1 min at 16 °C followed by a final digestion step for 10 min at 55 °C. Following heat inactivation for 20 min at 80 °C, an additional restriction step with 5 µl HindIII (NEB) was performed to digest residual destination plasmid. After

purification of the Golden Gate Cloning reaction by Wizard® SV Gel and PCR Clean-up System (Promega), 30 µl ligation product was mixed with 50 µl of ER2738 electrocompetent *E. coli* cells (Lucigen) on ice. Electroporation was carried out in prechilled 2 mm cuvettes (Genepulser Xcell™) using a MicroPulser electroporator (BioRad) with 2.5 kV. Immediately after pulsing, electroporated cells were diluted in 920 µl SOC medium (Lucigen) with subsequent incubation at 37 °C at 400 rpm for 1 h. The transformation was then plated out on 245 × 245 mm Petri dishes (Thermo Scientific) with 2xYT-GA agar (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar–agar, 100 nM glucose, 100 µg/ml ampicillin) and incubated overnight at 37 °C. For evaluating the transformation rate, 10^{-6} and 10^{-8} dilutions were prepared for each transformation reaction which were analogously plated out and incubated on small Petri dishes (Greiner). The GGC rate was determined either by colony PCR using primers pHAL30_14_fw and pHAL30_14_rev in RedTaq Mix 1.1x (VWR) to check for the correct insert length or by sequencing (Tab. S1). After overnight incubation, transformants were harvested in 25 mL 2xYT medium. 200 µl glycerol (Roth) was added to 800 µl aliquots of bacteria solution which were subsequently stored at − 80 °C.

For amplification of llama VHH, germline specific primers annealing to VHH framework 1 (VHH_SapI_up) and to the hinge region (short hinge: VHH_SapI_sh_lo, long hinge: VHH_SapI_lh_lo) was performed as described above (Tab. S1). Subsequently, for GGC 500 ng of purified PCR product was incubated with 1 µg destination plasmid pPD_Dest_SapI with 200 units SapI, 1000 units T4 DNA Ligase, and 10 µl 10 × T4 Ligase Buffer (all NEB) using the following reaction parameters: 40 cycles of 1 min at 37 °C and 1 min at 16 °C followed by a final digestion step for 10 min at 65 °C. Plasmid transformation and titration was performed analogously to chicken scFv library construction.

In general, we utilized three different *E. coli* strains for phage display library construction and selections. For library generation, we used ER2738 cells due to very high transformation rates. Because of fast growth rates that allow for performing one round of panning per day, TG1 cells were employed for selections. Finally, XL1 MRF's were used for final infection and plasmid rescue due to adequate plasmid quality after plasmid preparation.

Phage Display Library Packaging

Packaging of each antibody immune library was performed separately by inoculation of the library glycerol stock in 100 ml 2xYT-GA medium (2xTY medium supplemented with 100 µg/ml ampicillin and 100 mM glucose) with a starting OD of ~ 0.1. The cultures were incubated at 37 °C with 200 rpm until reaching an OD of ~ 0.5. Then, 5 ml bacteria culture (~ 2.5×10^9 cfu) were infected with 5×10^{10} M13K07 helper phage (NEB) followed by first 30 min incubation at 37 °C without shaking followed by 30 min incubation at 37 °C at 200 rpm. After centrifugation for 10 min at 3220×*g* at 4 °C, the infected cells were resuspended in 30 ml 2xYT-medium supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin. Phages were produced by incubation at 30 °C at 200 rpm overnight. The next day, the cultures were centrifuged for 15 min at 3220×*g* at 4 °C and the phage containing supernatant was precipitated with 1/5 volume of 20% PEG/2.5 M NaCl solution for 1 h on ice. After centrifugation at 10,000 g for 1 h at 4 °C, the phage pellet was resuspended in 5 ml phage dilution buffer (10 mM Tris, 20 mM NaCl, 2 mM EDTA, pH 7.5). Residual cell debris was removed by further centrifugation at 16,000 g at 4 °C for 15 min and filtrated. After additional precipitation with 1 ml 20% PEG/2.5 M NaCl solution, centrifugation for 45 min at 16,000×*g* at 4 °C, resuspension in 500 µl phage dilution buffer, and final centrifugation for 1 min at 13,000×*g*, the phage eluate was stored at 4 °C. Phages were titrated by incubating 10 µl of phage eluate serial dilution (10^{-2} to 10^{-8}) with 1 µl trypsin (Sigma) and 990 µl PBS pH 7.4 for 15 min at 37 °C and adding 10 µl of trypsinated phage reaction to 50 µl ER2738 cells (Lucigen). Cells were plated on 2xYT-GA agar plates overnight at 37 °C and cfu was counted and the number of infecting phage particles was calculated. Display of antibody fragments on phage was evaluated by 4–12% Bis–Tris-SDS-PAGE (Thermo Fisher) and Western Blot which was developed with mouse anti-pIII antibody (1:1000; MobiTec) and peroxidase conjugated goat anti-mouse antibody (1:1000; Jackson Immuno Research, Fig. S2). For each library, 10^9 of library phage (pfu) were analyzed along with three concentrations of M13K07 helper phage control (10^7, 10^8 ,and 10^9 pfu, NEB).

Phage Display Selection

In panning round one, approx. 1×10^{11} antibody fragment displaying phages for each library were separately diluted in 2% panning block (2% (w/v) milk powder, 2% (w/v) BSA, 0.05% Tween-20 in PBS pH 7.4), pre-incubated for 1 h in a 96-well microtiter plate well, blocked, and coated with an unrelated protein for negative selection at room temperature (RT). Subsequently, the supernatant was transferred to a well with 250 ng immobilized human EGFR-His (in-house production) for 2 h at RT. Non-bound phages were removed by washing 10-time with PBST (300 µl PBS with 0.05% Tween 20). Target-specific antibody fragment displaying phages were trypsinized for 30 min at 37 °C (10 µg/ml trypsin in 150 µl, Sigma) and were used for infection of 1 ml TG1 cells with OD 0.5 for 30 min without shaking followed by 30 min at 550 rpm at 37 °C. For the output titration, 1 µl of infected TG1 cells was diluted after the first 30 min in 1 ml PBS and

1/10 was plated on 2xYT-GA plates and incubated overnight at 37 °C. To the remaining infected TG1 cells, 3.5 ml 2xYT-GA medium was added for further 30 min incubation. For phage packaging, the TG1 cells were additionally infected with 1×10^{10} M13K07 helper phage (NEB) by 30 min incubation without shaking and 30 min at 550 rpm and 37 °C. After centrifugation at 3220×*g* for 10 min, the pellet was resuspended in 5 ml 2xYT-AK medium and incubated overnight with 550 rpm at 30 °C. The next day, bacteria were pelleted by centrifugation at 3220×*g* for 10 min and phage containing supernatant was either stored at 4 °C or used for the subsequent panning rounds. For panning round two and three, the antigen concentration of negative selection target and human EGFR protein was decreased to 100 ng and 50 ng, respectively, and for higher stringency, the number of washing steps was increased to 20-times and 30-times, respectively. For both round two and three, 100 µl phage supernatant from the respective previous round was mixed with 50 µl 2% panning block as input. After the third panning round, trypsinized phages were used to infect 1 ml XL1 MRF' supercompetent cells (Stratagene) for 30 min at 37 °C without shaking. Bacteria were pelleted at 3200 g for 10 min, resuspended in 250 µl 2xYT medium, and plated on 2xYT-A agar plates. The next day, bacteria were harvested from plates in 25 mL 2xYT medium. Bacterial stocks containing 20% (v/v) glycerol were prepared as described above and stored at − 80 °C.

Antibody Production and Screening in Microtiter Plates

Single bacteria clones carrying antibody fragment phagemids were picked from 2xYT-GA agar plates after selection and used for inoculation of 96-well polypropylene MTPs (Greiner) filled with 2xYT-GA medium. After incubation overnight at 37 °C and 700 rpm with 70% humidity, 10 µl of this pre-culture was transferred to 190 µl fresh phosphate-buffered 2xYT-A medium in a new MTP. After 2 h further incubation, 100 µM isopropyl-beta-D-thiogalacto pyranoside (IPTG) and 50 mM sucrose were added followed by incubation at 30 °C at 800 rpm overnight. Bacteria were pelleted by centrifugation for 10 min at 3220×*g* at 4 °C, supernatants were transferred to 384-well MTPs (Greiner) and diluted 1:1 with 2% BSA-PBS.

For the antigen ELISA, 50 ng positive or negative target protein were immobilized on 384-well MTPs (MaxiSorp, Nunc) in PBS pH 7.4 overnight at 4 °C. The next day, wells were washed three times with 100 µl PBST and blocked with 2% BSA-PBST for 1 h at room temperature (RT). After three times of washing with PBST, the expressed antibody fragments were added for an additional 1 h at RT. Antibody fragments bound to the antigen were detected with mouse anti-c-myc antibody followed by goat-anti-mouse IgG secondary antibody conjugated to peroxidase (Jackson Immuno Research). The colorimetric reaction was performed with the substrate TMB (3,3′,5,5′-tetramethylbenzidne) which was stopped with 1 N sulfuric acid after 2 min. Absorbance was measured with a Paradigm™ MTP reader (Beckman Coulter). A clone was declared as hit when absorbance at 450 nm for EGFR-coated well exceeded 1.5, whereas the ELISA signal for the negative control well was lower than 0.8.

Expression and Purification of Isolated Library Candidates

Isolated VHHs were reformatted into pTT5 plasmid as human Fc-fusions. Isolated chicken scFv regions were reformatted as scFv-Fc antibodies (human) [26]. For antibody production, Expi293 cells were transiently transfected with expression vectors (pTT5) according to the manufacturer's instructions (Thermo Fisher Scientific). Supernatants were harvested by centrifugation five days post transfection and purified via MabSelect antibody purification chromatography resin (GE Healthcare). Finally, buffer was exchanged to PBS, pH 6.8, over 24 h at 4 °C using Pur-A-Lyzer™ Maxi 3500 Dialysis Kit (Sigma Aldrich/Merck KGaA) followed by a sterile filtration step employing Ultrafree®-CL GV 0.22 µm Centrifugal Devices (Merck Millipore).

Bio-layer Interferometry (BLI)

The Octet RED96 system was employed to perform binding kinetic measurements (ForteBio, Pall Life Science). All steps were performed at 30 °C and 1000 rpm agitation. Camelid and chicken-derived antibodies were loaded on anti-human Fc biosensors (AHC) at 5 µg/ml in PBS for 3 min. Subsequently, tips were transferred to kinetics buffer (KB; PBS, 0.1% (v/v) Tween-20 and 1% (w/v) BSA) for 60 s. Association to recombinant human EGFR (concentration range 100–6.25 nM) was measured for 300 s in KB followed by dissociation for 300 s in KB. For each antibody-derivative, a negative control was measured using KB and an unrelated antigen at 100 nM in KB instead of EGFR. Moreover, species cross-reactive binding was assessed at a single concentration of 100 nM recombinant mouse EGFR (ECD, produced in-house). Data fitting and analysis was performed with ForteBio data analysis software 8.0 using a 1:1 binding model after Savitzky–Golay filtering.

Results

Analysis of Type IIs Restriction Sites in Chicken and Camelid Antibody Repertoires

In order to identify suitable type IIs restriction enzymes for Golden Gate Cloning of chicken and camelid antibody

repertoires following immunization, we retrieved IGHV, IgHJ, IgLV, and IgLJ datasets from the IMGT database [27]. Moreover, approximately 100 randomly chosen sequences of PCR-amplified VH and VL genes (*Gallus gallus domesticus*) as well as amplified VHH genes (*Lama glama*) were analyzed in this respect. To this end, we only focused on commonly used type IIs enzyme BsaI as well as on SapI and BsmBI. The two latter enzymes have been proven to be useful for cloning of antibody repertoires [24]. As presented in Table 1, for BsaI, which is one of the most commonly utilized enzymes for GGC-based approaches, a high frequency of restriction sites in chicken IgLV sets was found. This finding was also confirmed for the PCR-amplified VL repertoire. Similarly, BsaI recognition sites were also found frequently in *Lama glama* IgHV sets as well as PCR-amplified VHH genes. Consequently, this enzyme was excluded for further investigations. On the other hand, chicken as well as llama antibody genes exhibited only very few recognition sites for BsmBI (or Esp3I) and SapI. In case of BsmBI, a restriction site was identified in chicken and camelid IgHJ sets. This has also been reported for human, murine, and rabbit IgHJ genes [24]. Likewise, due to its location near the end of the gene, this site can be readily removed during primer design. Due to a lower presence of BsmBI recognition sites in chicken IgHV sets, this enzyme was selected for cloning of chicken scFv libraries, while SapI was chosen for the construction of VHH single domain antibody libraries.

Design of Phage Display Library Components

For the generation of scFv libraries, we designed a four-component system comprising a destination plasmid, the amplified variable region repertoire of the heavy chain, the amplified variable region repertoire of the light chain as well as an entry module for the incorporation of a linker of choice (Fig. 1). The destination plasmid was pHAL14-derived [20] and major elements were the LacZ promoter, the pelB leader followed by a stuffer sequence (containing a HindIII restriction site), and the short linker, as well as a myc-tag, a hexahistidine-tag, a trypsin-site, and phage coat protein gene III. Between the his-tag and the trypsin-cleavage site, we incorporated an amber stop codon. Moreover, the stuffer sequence contained BsmBI recognition sites for seamless cloning of ScFv entities. Resulting overhangs, i.e., signature sequences were part of the pelB leader (SigA) or part of the short linker (SigZ). Besides, the plasmid contained an ampicillin resistance gene cassette as well as the ColE1 and the f1 origins of replication. The entry plasmid contained an 18 amino acid scFv linker, flanked by BsmBI recognition sites. Signature sequences were part of the linker sequence (SigB, SigC). Moreover, this plasmid contained the ColE1 ori for propagation as well as a kanamycin resistance gene. Amplified VH and VL genes were flanked by BsmBI recognition sites as well as corresponding signature sequences (SigA and SigB for VH, SigC and SigZ for VL). Primer sequences for specific chicken antibody variable domain amplification as well as signature sequences can be found in the supplementary material section (Tab. S1, Tab. S2).

For VHH library construction, we designed a two-component system comprising a destination plasmid as well as the amplified VHH repertoire module (Fig. S1). The destination plasmid was designed in a very similar way to the scFv destination module, except for an exchange of the BsmBI recognition sites for SapI recognition sites. Consequently, the amplified VHH repertoire was flanked by SapI sites as well as corresponding signature sequences (SigA, SigZ).

Table 1 Frequency of pre-selected type IIs recognition sites in chicken and llama gene repertoires

		BsaI (%)	BsmBI (%)	SapI (%)
Chicken sets				
Gallus gallus domesticus[a]	IMGT IGH-V	4.90	0	1.60
	IMGT IGH-J	0.00	0[b]	0
	PCR-amplified VH repertoire	0	1.40[b]	1.40
	IMGT IGL-V	41.20	0	1.90
	IMGT IGL-J	0	0	0
	PCR-amplified VL repertoire	71.20	0	0
Camelid sets				
Lama glama[a]	IGH-V3	37.50	12.50	12.50
	for germline genes VHH1-5 [31]	20	0	0
	IgH-J	0	0[b]	0
	PCR-amplified VHH repertoire	51.20	7.10[b]	0

[a]In genes and pseudogenes

[b]After silent mutagenesis of conserved *BsmB*I site at 3′end of IgH-J segment

Library Construction, Selection, and Characterization of EGFR-Specific Chicken-Derived Scfv Fragments

It's been known for quite some time, that chicken antibodies can be readily obtained by combining animal immunization with display technologies [28, 29]. To demonstrate that the designed GGC-based approach for library generation allows for the facile isolation of antigen-specific antibodies following avian immunization, we constructed a scFv library of a recombinant human EGFR (extracellular domain)-immunized chicken. To this end, after cDNA synthesis, the heavy chain variable region repertoire as well as the light chain variable region repertoire was amplified using a single PCR step, respectively. Subsequently, both PCR products served as entry modules, together with the entry module comprising the scFv linker and the destination plasmid in a Golden Gate Cloning reaction, yielding the final display vector (Fig. 1, pPD_Expr). Due to its optimal temperature of activity at 37 °C, we decided to utilize the enzyme Esp3I, which is an isoschizomer of BsmBI instead of the latter enzyme that has a temperature optimum of 55 °C. To enhance the efficacy of the reaction i.e., insert rate, a final restriction step with HindIII was performed in order to digest residual destination plasmid. After clean-up, the resulting product was transformed into ER2738 *E. coli* cells in a single electroporation reaction, yielding a small library with a calculated size of 4×10^6 independent clones (Table 2). Library analysis via colony PCR of 48 clones revealed an GGC rate of 92%. Moreover, sequencing of approximately 100 single clones revealed a high correctness of 84% (data not shown). Successful display was verified by Western Blot (Fig. S2). Subsequently, the library was subjected for panning against EGFR. After three rounds of phage display panning with decreasing antigen concentrations (250, 100, 50 ng/well; Tab. S3) and increasing washing cycles, 192 clones were sent out for sequencing (Fig. S3 and Fig. S4), ultimately resulting in 41 unique VHs and 55 unique VLs. To get a more comprehensive view of the diversity of the library output, a clustering strategy was applied in which clones with less than three amino acid exchanges within CDR-H3 belong to the same family. Moreover, at least three sequences must fulfil this criterion to be considered as cluster. This resulted in the identification of eight sequence clusters.

To add another layer of quality assessment of the library output, we also screened 188 selected library candidates for EGFR binding via ELISA after microtiter expression in *E. coli* (Fig. 2a, Fig. S5) yielding in a hit rate of approximately 50%.

In order to demonstrate, that the isolated chicken-derived entities also bind EGFR when expressed in mammalian expression systems, based on sequence analysis, eight clones were reformatted as scFv-Fc fragments and produced in Expi293 cells (Fig. 3). After purification via Protein A, binding kinetics to recombinant human EGFR were investigated via BLI (Table 3, Fig. S6). All reformatted and expressed scFv fusion protein showed binding against EGFR in the single digit to double digit nanomolar range. Moreover, species cross-reactivity against the murine counterpart, which shows approximately 90% sequence identity to the human protein, was assessed at 100 nM mouse EGFR (ECD). This revealed that six out of eight clones were also able to bind to recombinant murine EGFR in a comparable manner, demonstrating that those molecules might be beneficial as tools in pre-clinical models.

Library Construction, Selection, and Characterization of EGFR-Specific Camelid-Derived VHH Single Domain Antibodies

Similar to the generation of the scFv immune library, we aimed at constructing camelid-derived VHH single domain antibody libraries following llama immunization. To this end, we used PBMC-derived cDNA from two EGFR-immunized *Lama glama* as starting material. The forward primer was designed to specifically anneal in framework 1, whereas reverse primers were designed to anneal in the hinge region. Since camelids produce different VHH isotypes comprising short hinge regions and a long hinge regions [30, 31], we designed two different oligonucleotides, respectively (Tab. S1). For each immunized animal, two sub-libraries were constructed, yielding a total number of four VHH phage

Table 2 Analysis of constructed EGFR scFv and VHH libraries

Library	Misc	Library size (per electroporation reaction)	GGC rate (%)	Phage titer (total)
Chicken ScFv	–	4×10^6	92	3.5×10^{12}
Llama 1 VHH	Short hinge	1×10^6	83	4.0×10^{12}
	Long hinge	5×10^6	96	4.5×10^{12}
Llama 2 VHH	Short hinge	4×10^6	83	3.0×10^{12}
	Long hinge	5×10^6	75	3.5×10^{12}

Library sizes as calculated by serial dilution plating are given per electroporation reaction. GGC rates were determined by colony PCR. Phage titer was assessed by serial dilution plating

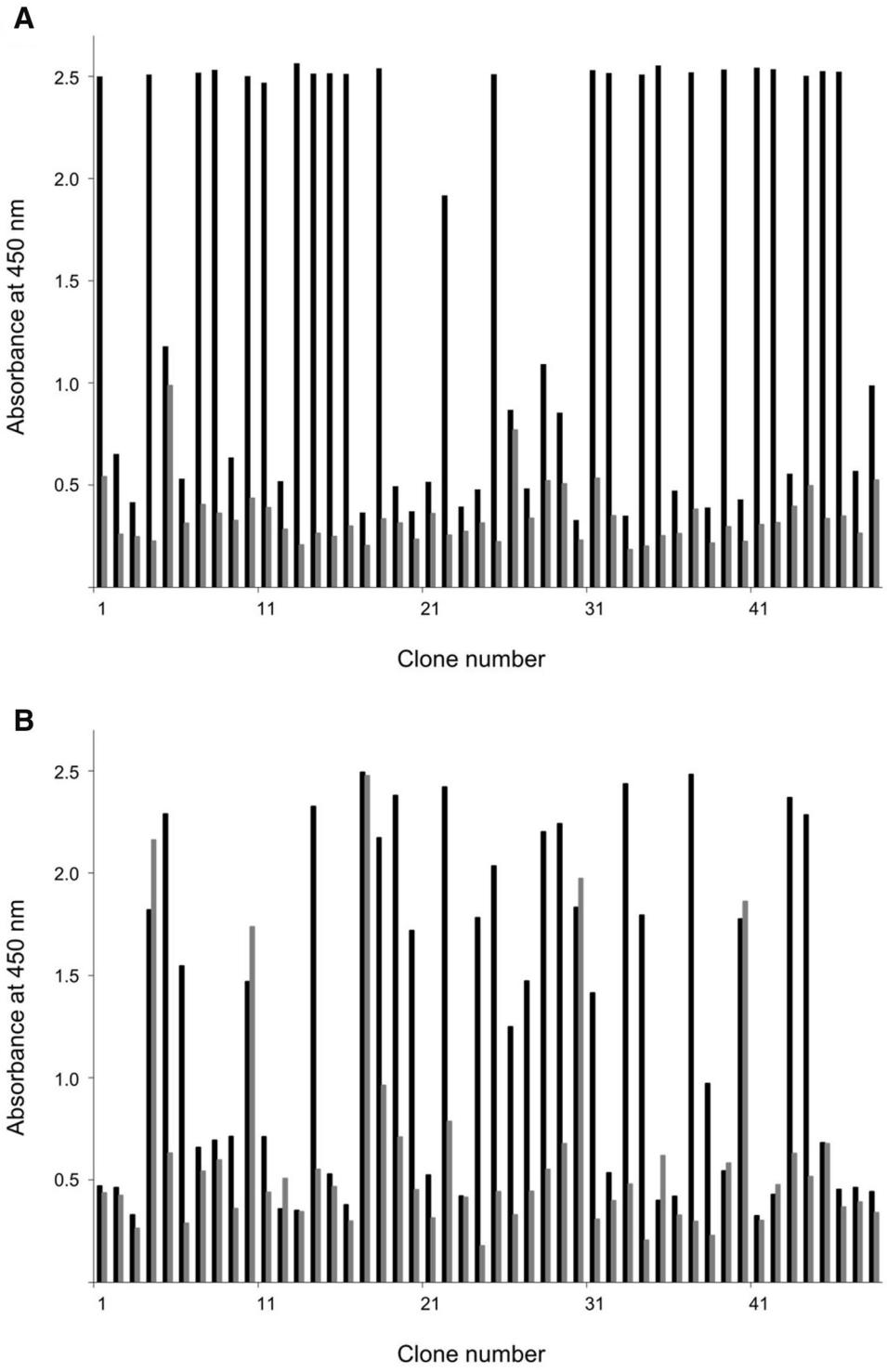

Fig. 2 ELISA screening after microtiter production of selected library candidates in *E. coli*. **a** Screening outcome of EGFR-immunized avian scFv antibody library after three rounds of selection. **b** Screening outcome of EGFR-immunized llama VHH single domain antibody library after three rounds of panning. A representative block of 48 consecutive clones as assessed by ELISA is shown. Black: ELISA signal against EGFR, gray: Absorbance at 450 nm against unrelated negative control antigen

display immune libraries. Consequently, four GGC reactions were performed in total using the respective PCR product as entry module and the destination plasmid (Fig. S1). For each library, only one electroporation reaction was performed into ER2738 cells, yielding library sizes in the range of 1 to 5×10^6. GGC rates as determined by colony PCR were in the range of 75% to 96% (Table 2). All constructed libraries showed display on the surface of phage particles, as demonstrated by Western Blot (Fig. S2). Subsequently, the different VHH libraries were selected against plate-immobilized EGFR in parallel. After three rounds of library panning using step-wise reduced antigen concentrations (Tab. S3), a

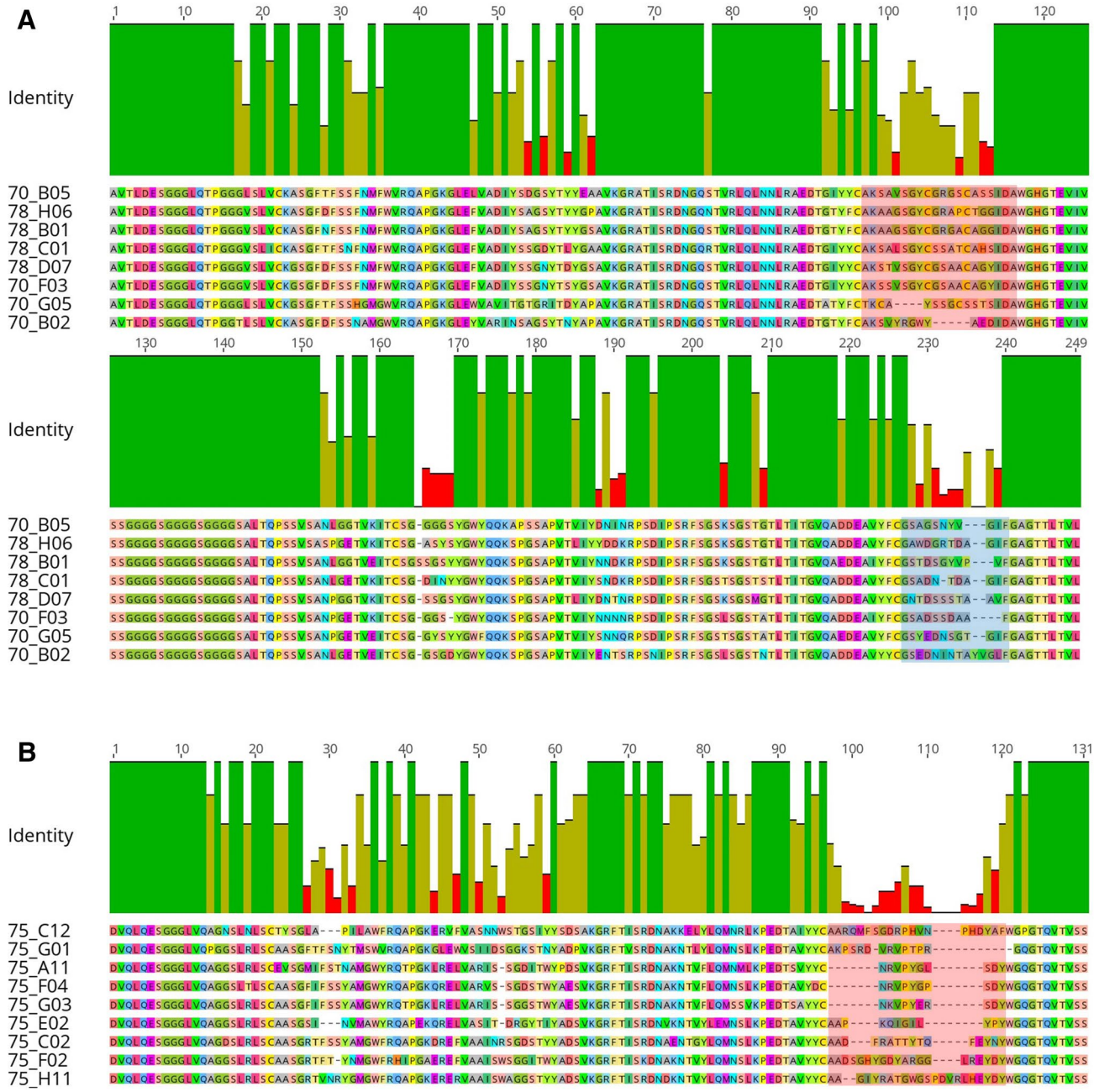

Fig. 3 Multiple sequence alignment of chicken-derived scFv clones (**a**) and llama-derived VHH single domain antibodies (**b**) chosen for expression as Fc-fusion proteins after phage display panning. CDR-H3 is shaded in red, CDR-L3 shown in blue. Alignment was created using MUSCLE Alignment tool within Geneious Prime software (www.geneious.com)

total number of 192 clones (i.e., 48 clones per sub-library) were sent out for sequencing revealing 69 unique sequences (Fig. S7). Moreover, a total number of 752 clones were screened for EGFR binding in an ELISA screening after MTP production, resulting in a hit rate of approximately 20% (Fig. 2b, Fig. S8). Nine clones were reformatted into a VHH-Fc fusion and expressed in Expi293 cells. After purification via protein A, binding kinetics were obtained using BLI (Table 3, Fig. S6). This revealed binding of six VHH-Fc fusion proteins against recombinant human EGFR with a broad spectrum of affinities, ranging from high-affinity binding (picomolar) to moderate binding in the triple digit nanomolar range. Moreover, three clones also showed species cross-reactive binding against recombinant mouse EGFR similar to the human counterpart.

Table 3 Binding kinetics of selected and recombinantly expressed scFv-Fc and VHH-Fc fragments against rhEGFR as assessed by BLI

Name	Format	KD (M)	k_{on} (1/Ms)	k_{off} (1/s)	Cross-reactivity (murine EGFR)
70_B02	scFv Fc-fusion	7.91E−09	4.85E+05	3.84E−03	No
70_B05	scFv Fc-fusion	2.86E−08	1.20E+05	3.45E−03	Yes
70_F03	scFv Fc-fusion	3.66E−08	4.45E+04	1.63E−03	Yes
70_G05	scFv Fc-fusion	3.08E−09	9.71E+04	2.99E−04	No
78_B01	scFv Fc-fusion	1.90E−08	1.26E+05	2.39E−03	Yes
78_C01	scFv Fc-fusion	1.35E−08	1.85E+05	2.50E−03	Yes
78_D07	scFv Fc-fusion	6.62E−08	6.86E+04	4.54E−03	Yes
78_H06	scFv Fc-fusion	4.43E−09	9.44E+04	4.18E−04	Yes
75_H11	VHH Fc-fusion	1.27E−07	3.16E+04	4.03E−03	No
75_G03	VHH Fc-fusion	4.91E−10	2.20E+05	1.08E−04	Yes
75_G01	VHH Fc-fusion	–	–	–	No
75_F04	VHH Fc-fusion	5.11E−09	1.97E+05	1.00E−03	Yes
75_F02	VHH Fc-fusion	–	–	–	No
75_E02	VHH Fc-fusion	–	–	–	No
75_C12	VHH Fc-fusion	1.11E−08	3.50E+05	3.90E−03	No
75_C02	VHH Fc-fusion	2.40E−08	1.23E+05	2.95E−03	No
75_A11	VHH Fc-fusion	1.46E−09	2.36E+05	3.45E−04	Yes

Cross-reactive binding against rmEGFR at 100 nM is also indicated

Discussion

Phage display is the most frequently used platform technology for antibody hit discovery and has delivered several therapeutic entities that have been granted marketing approval [13]. It was demonstrated that therapeutic entities can be successfully generated from synthetic or naïve as well as from immunized repertoires [32-36]. Canonical antibodies i.e., antibodies comprising heavy as well as light chain diversities, are typically displayed either as Fab- or scFv fragments on the surface of phage particles [15, 16]. Either way, the construction of such libraries is typically tedious, involving multiple consecutive steps [16, 20]. Our group has recently demonstrated that the construction of antibody Fab libraries for Yeast Surface Display can be significantly simplified by employing a one-step, one-pot type IIs restriction enzyme-based process referred to as Golden Gate Cloning [21, 22]. Likewise, it was shown by Nelson and Valadon that GGC also enables the facile generation of Fab antibody libraries for phage display [24]. Moreover, the authors elegantly analyzed the occurrence of type IIs restriction sites in human, mouse as well as rabbit antibody gene repertoires. In this present work, we set out to adapt a similar strategy for the construction of scFv antibody libraries as well as camelid single domain antibody libraries for phage display. To this end, we first analyzed the frequency of three different type II restriction sites in chicken and llama antibody repertoires. Essentially, we focused on BsaI as one as the most commonly used enzyme for GGC approaches as well as on BsmBI and SapI, since those enzymes were found to be suitable for the construction of human, mouse, and rabbit antibody libraries [24]. Essentially, this revealed optimal enzymes for the construction of chicken scFv as well as camelid VHH single domain libraries. We demonstrate that the GGC approach enables the generation of libraries in the range of 5×10^6 unique clones per electroporation reaction and that the constructed libraries exhibited high functionalities i.e., insert rates. Consequently, libraries with diversities in the range of 10^8 clones can be readily constructed in a convenient way which should be sufficient to adequately sample immune repertoires following immunization. However, for the generation of naïve or synthetic libraries, one typically aims for diversities in the range of 10^{10}–10^{11} in order to be generally applicable [37]. In this respect, a lot of optimization would be required. Nonetheless, even with the constructed proof-of-concept libraries we were able to select for a diverse panel of antigen-binding antibodies from both, the EGFR-immunized avian scFv as well as from the EGFR-immunized camelid VHH libraries. Recombinantly expressed entities showed a broad spectrum of different affinities against EGFR in the single to double digit nanomolar range for chicken-derived scFv-Fc proteins and triple digit picomolar to triple digit nanomolar range for camelid-derived VHH-Fc fusions.

Conclusion

Besides being the most successfully applied platform technology for the generation of therapeutic antibodies, the construction of phage display library remains challenging and cumbersome. Recently, a significantly simplified process

of library construction was presented by Nelson and Valadon [24]. In their work, the authors describe the generation of phage display Fab libraries using a type IIs restriction enzyme-based process. Herein, we adopted a similar strategy for the establishment of scFv and single domain antibody libraries. We demonstrate that libraries with adequate qualities can be readily cloned using this one-step approach enabling the rapid isolation of antigen-specific antibodies from immunized animals.

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Author contributions SZ, CS and LP designed the experiments. LP, CB, EC, BV, LT, SB and SK performed in silico analysis and experiments. JK, AF and MH gave scientific advice and guidance on overall strategy. CS, LP and SZ wrote the manuscript.

Compliance with Ethical Standards

Conflict of interests All authors are either affiliated with Merck Healthcare KGaA or Yumab GmbH.

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