Antibody discovery and engineering by enhanced CRISPR-Cas9 integration of variable gene cassette libraries in mammalian cells

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ABSTRACT
Antibody engineering in mammalian cells offers the important advantage of expression and screening of libraries in their native conformation, increasing the likelihood of generating candidates with more favorable molecular properties. Major advances in cellular engineering enabled by CRISPR-Cas9 genome editing have made it possible to expand the use of mammalian cells in biotechnological applications. Here, we describe an antibody engineering and screening approach where complete variable light (V\text{L}) and heavy (V\text{H}) chain cassette libraries are stably integrated into the genome of hybridoma cells by enhanced Cas9-driven homology-directed repair (HDR), resulting in their surface display and secretion. By developing an improved HDR donor format that utilizes in situ linearization, we are able to achieve >15-fold improvement of genomic integration, resulting in a screening workflow that only requires a simple plasmid electroporation. This proved suitable for different applications in antibody discovery and engineering. By integrating and screening an immune library obtained from the variable gene repertoire of an immunized mouse, we could isolate a diverse panel of >40 unique antigen-binding variants. Additionally, we successfully performed affinity maturation by directed evolution screening of an antibody library based on random mutagenesis, leading to the isolation of several clones with affinities in the picomolar range.

Introduction
Due to their enormous and versatile targeting potential, monoclonal antibodies play a dominant role in biopharmaceutical drug discovery and development.\cite{1,2} Several platforms exist for antibody discovery and engineering, such as phage\cite{3} and yeast\cite{4} display, which enable high-throughput generation and screening of massively diversified libraries (10^9–10^11).\cite{5,6} However, phage and yeast display typically only allow for expression of antibody fragments (e.g., single-chain variable fragment and fragment antibody binding domains), which in the context of therapeutic antibodies require subsequent conversion to full-length IgGs. Since correct folding, stability and post-translational modifications are all important for drug development properties, the possibility to engineer antibodies in a mammalian host offers a substantial advantage in terms of developability.\cite{7} However, the exponentially lower throughput of mammalian transfection or genomic integration,\cite{8} when compared to microbial transformation, still represents an intrinsic limitation of mammalian screening platforms.

Targeted genome editing using CRISPR-Cas9\cite{9} has recently enabled a plethora of applications in mammalian cell engineering.\cite{10,11} For example, when providing a suitable repair template, double-stranded breaks (DSB) induced by Cas9 can be repaired via homology-directed repair (HDR), resulting in the targeted integration of transgenes.\cite{12} However, the cellular DNA repair machinery is naturally biased toward the alternative and more efficient response to DSB, non-homologous end joining (NHEJ),\cite{13} which results in insertions and deletions (indels). Substantial efforts have therefore been invested to devise strategies that would alter this intrinsic preference, including targeted slencing of fundamental proteins involved in the NHEJ pathway,\cite{14-17} chemical inhibitors\cite{18,19} and cell cycle synchronization.\cite{20,21} Notably, HDR improvement has been obtained by optimizing the parameters for donor DNA design, with linear single-stranded DNA generally outperforming double-stranded DNA donors.\cite{22,23} One of the most noteworthy examples is the use of recombinant adeno-associated viral vectors (rAAV) as HDR donors for therapeutic cell engineering, where targeted knock-in efficiencies up to 40% have been observed.\cite{24,25} The field of directed evolution and protein engineering could also benefit from high-throughput genome editing, as recently high-efficiency HDR (>30%) was achieved using degenerate single-stranded oligonucleotide (ssODN) donors to generate site-directed mutagenesis libraries.\cite{26} Chemical synthesis of ssODNs, however, is typically limited to a maximum of 200 nucleotides, which is not suitable for the integration of antibody variable gene libraries (>400 bp).

We recently developed a mammalian cell platform, referred to as plug-and-(dis)play (PnP) hybridomas, in which the genome of hybridomas is reprogrammed by
Here, we present an innovative workflow for region, which was then cloned and integrated for antibody discovery purposes, we generated homology arm (pPnP-lin3 region was exchanged by HDR with the HDR and V in situ performed more proving to be the most efficiently integrated, with HDR Figure 1c and pPnP-lin3). Because of the substantial improvement in poration), the co-transfected gRNA complex would mediate Cas9 cleavage, and the modified plasmids confirmed this rationale: in the case of pPnP-lin5' and pPnP-lin3', the majority of the plasmid sub-strate was linearized, with the cleaved plasmid producing a band migrating above the supercoiled uncut plasmid DNA. In the case of pPnP-lin5'/3', two different outcomes were observed: while a fraction of the substrate underwent a single cut at either gRNA site (upper band), simultaneous cleavage at both sites could be observed as well, yielding two smaller products of the expected size (Figure 1c).

We next used PnP-mRuby cells to assess the HDR performance of the three plasmid variants compared to the original plasmid and the PCR product. HDR could be quantitatively measured by flow cytometry by detecting the percentage of cells that gained antibody expression while losing mRuby expression. For each sample, we transfected equimolar amounts of DNA (Figure 1d). All three in situ linearized donors (bottom row) performed significantly better than both the original plasmid and the PCR product, with pPnP-lin5' proving to be the most efficiently integrated, with HDR rates >5% (Fig. S1). Despite the fact that both donors are constituted of linear dsDNA, pPnP-lin5' performed more than two-fold better than the PCR product, and more than 15-fold better than the original, non-linearized plasmid (Figure 1e). Because of the substantial improvement in terms of HDR efficiency, we selected pPnP-lin5' as the most suited donor format for subsequent experiments.

Antibody discovery from immune libraries with enhanced Cas9 HDR

In order to test the applicability of the improved PnP-HDR workflow for antibody discovery purposes, we generated a combinatorial immune library by cloning into the PnP-lin5' scaffold V and V genes derived from the bone marrow plasma cells of a mouse immunized with OVA (Figure 2a). The bone marrow plasma cell repertoire of immunized mice has previously been shown to be enriched with antigen-specific variable genes that are expressed at high abundance. We therefore reasoned that generating full-length IgG libraries from this rather small B-cell compartment and displaying them with our system might serve as an ideal

Results

Development of a plasmid donor for enhanced Cas9-driven HDR

We previously established the PnP-mRuby hybridoma cell line, in which expression of the endogenous light chain IgK was abolished by Cas9-induced deletion of the V region, and the endogenous V region was exchanged by HDR with the fluorescent reporter mRuby gene. Reprogramming of these hybridoma cells to express a novel antibody was performed by targeting mRuby for HDR exchange with a synthetic antibody (sAb) construct (Figure 1a), which when integrated correctly splices with the native heavy constant region C exon, thus leading to surface-display and secretion of a full-length antibody molecule. Results obtained in this context showed that linear double-stranded (ds) DNA donors were more efficiently integrated via HDR compared to plasmid DNA. However, donor generation via PCR is laborious, resource-intensive and not always reliable, often yielding an insufficient amount of material; additionally, even with high-fidelity polymerases, the possibility still exists of errors being introduced during amplification. Moreover, when a pool of different genetic elements is to be amplified by PCR (which is the case for variable gene libraries), amplification biases can be introduced, thus altering or reducing the diversity of the starting library. We therefore hypothesized that an efficient alternative way to deliver a linearized template would be to rely on nuclear Cas9 cleavage to produce such a donor in situ.

We introduced the same Cas9 guide RNA (gRNA) sequence targeting mRuby into a sAb HDR donor plasmid. The rationale behind this approach is the expected scenario where, upon plasmid entrance into the nucleus of the cell (following electroporation), the co-transfected gRNA complex would mediate Cas9 cleavage and subsequent linearization. Since the same gRNA target site is used for the genomic reprogramming step, Cas9 would in parallel also cleave mRuby without the need to transfact a second gRNA. At this point, a linearized donor would therefore be available for HDR. The use of this HDR donor is particularly beneficial when coupled with a version of the PnP-mRuby cells constitutively expressing Cas9 (Figure 1b). To verify this hypothesis, we started from the original PnP plasmid HDR donor encoding the antibody HEL23, which is specific for the model antigen HEL. We generated three alternative plasmid versions where the Cas9 mRuby target sequence (gRNA-J, including protospacer adjacent motif) is either: 1) immediately upstream of the 5' homology arm (pPnP-lin5'), 2) immediately downstream of the 3' homology arm (pPnP-lin3'), or 3) on both sites (pPnP-lin5'/3') (Figure 1c). After Cas9-mediated cleavage, the first two versions were expected to yield a construct of the same size as the original plasmid, but linear, while the third format would produce a smaller donor, essentially comparable to the PCR product. When incubated in vitro with recombinant Cas9, the modified plasmids confirmed this rationale: in the case of pPnP-lin5' and pPnP-lin3', the majority of the plasmid sub-strate was linearized, with the cleaved plasmid producing a band migrating above the supercoiled uncut plasmid DNA. In the case of pPnP-lin5'/3', two different outcomes were observed: while a fraction of the substrate underwent a single cut at either gRNA site (upper band), simultaneous cleavage at both sites could be observed as well, yielding two smaller products of the expected size (Figure 1c).

Antibody discovery from immune libraries with enhanced Cas9 HDR

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Figure 1. Enhanced Cas9-driven HDR by designing a self-linearized donor plasmid. (a) Schematic shows the PnP workflow to reprogram mRuby hybridomas to express a selected antibody following incorporation of a recombinant synthetic antibody in the V<sub>H</sub> locus. (b) Optimized workflow in which a version of the PnP-mRuby cells constitutively expressing Cas9<sup>26</sup> is used together with an HDR donor plasmid harboring a recognition site for the same Cas9 gRNA that is used to cleave mRuby (protospacer adjacent motif is indicated in red). Following entry of the plasmid and the gRNA complex into the nucleus, Cas9, which is also targeted to the nucleus due to its nuclear localization signal, is recruited to both induce a DSB in the genomic mRuby coding sequence and linearize the plasmid, rendering it more prone to integration by HDR. (c) In vitro testing of plasmid cleavage by recombinant Cas9. Three versions of the self-linearizing plasmid were generated, bearing the cleavage site upstream of the 5'<homology arm (pPnP-lin5') downstram of the 3'<homology arm (pPnP-lin3') and at both sites (pPnP-lin5'/3'). As expected, in the first two cases Cas9 cleavage produced a linearized construct, while the double-cut plasmid underwent both single cleavage at either site or simultaneous cleavage at both, resulting in a shorter construct. (d) Exemplary flow cytometry dot plots show evaluation of HDR integration. The improved plasmids were compared to the unmodified plasmid (pPnP) and to the PCR-linearized donor (PCR). HDR efficiency is evaluated in terms of surface antibody expression 3 d after transfection. (e) Fold improvement of HDR rates of all the linearized donor formats compared to the mean of unmodified plasmid. HDR efficiency is quantified as described in (d). The plot is representative of n = 3 replicates and the error bars indicate standard deviation. Flow cytometry dot plots of all replicates are shown in Figure S1.
test case and, if successful, might offer an alternative to traditional hybridoma generation.

Total RNA was extracted from plasma cells (~2.1 × 10^4 cells) and reverse transcribed to cDNA, followed by multiplex PCR amplification of V<sub>L</sub> and V<sub>H</sub> regions (as described in the Methods section). The V<sub>H</sub> and V<sub>L</sub> gene pools were cloned in the PnP-lin5' scaffold in a stepwise manner: a V<sub>L</sub> library was initially obtained, and subsequently provided the backbone for the introduction of

Figure 2. PnP screening of combinatorial variable gene libraries from an immunized mouse. (a) Schematic shows the workflow to generate and display an immune library from bone marrow plasma cells of an antigen-immunized mouse. (b) Flow cytometry dot plots illustrate the screening and progressive enrichment steps of a full-length combinatorial library obtained from the plasma cells of a mouse immunized with ovalbumin (OVA). From left to right: isolation of all antibody-expressing cells following transfection; detection of OVA-positive clones in the unselected total pool of antibody-positive cells, and the outputs of the first, second and third rounds of OVA enrichment. Representative flow cytometry gates do not correspond to the actual sorting gates but are drawn to illustrate successful enrichment. (c) Flow cytometry dot plots show the surface expression and antigen-binding profile of five representative unique clones, selected among the total pool of 41 according to sequence diversity (see Figure 3). A hybridoma cell line specific for an unrelated antigen was used as negative control.
the V\textsubscript{H} repertoire (Figure 2a) thus creating a combinatorial library of ~5.1 × 10\textsuperscript{6} transformants in \textit{E. coli}. Multiple electro-
porations of HDR donor were pooled together and ~3.6 × 10\textsuperscript{5} cells were initially sorted for positive surface IgG expression (see Methods section); this cell population constitutes the effectively integrated library size. Three subsequent OVA enrichments followed by a de-enrichment step for potentially fluorophore-
specific clones yielded a progressively pure pool of antigen-
positive cells. Closer analysis of flow cytometry data revealed multiple populations had emerged, indicative of the wide vari-
bility in terms of antigen binding. After the third OVA enrich-
ment, the observed positive fraction was higher than 80% (Figure 2b); the “3\textsuperscript{rd} OVA enrichment” plot shows the cells after a final purification step, in which clones potentially specific for the fluorophore conjugated to the antigen were depleted (Fig.
S2a). Finally, as a terminal library screening step, single-cell clones were isolated and expanded for further characterization. The flow cytometry profiles of five representative clones are shown in Figure 2c.

Characterization of a panel of antigen-specific antibodies

Antigen-specific clones were defined phenotypically on the basis of antigen binding by ELISA and genotypically by the presence of a unique V\textsubscript{H} amino acid sequence (Figure 3a and Figure S4). Applying these parameters, 41 unique antibodies were identified. The lineage tree in Figure 3a shows a discrete level of V\textsubscript{H} sequence heterogeneity among the clones, which differed substantially on the basis of V-germline gene usage, complementary-determining region (CDR)H3 sequence iden-
tity and length and number of somatic hypermutations, demonstrating that various different clonal lineages are repre-
sented. Additionally, contributing to this high level of sequence heterogeneity, we found 34 different V\textsubscript{L} chains, five of which could pair with more than one specific V\textsubscript{H} (Figure S3). Figure 3b shows the antigen-binding profiles by ELISA of the five representative clones (also displayed in Figure 2c) chosen on the basis of phylogenetic distance (indi-
cative of sequence diversity). Analysis of the remaining pool of 36 clones is provided in Figure S4.

Generation and Cas9-based integration of random mutagenesis libraries

Next, we aimed to determine if enhanced Cas9-driven HDR could also be used for the engineering of antibodies. We generated a random mutagenesis library by performing error-
prone (EP) PCR on the V\textsubscript{H} region of the antibody clone HEL23, specific for the antigen HEL. The starting HEL23 V\textsubscript{H} was amplified from the original PnP-HEL23 donor plas-
mid by EP-PCR, and cloned in the pPnP-lin5’ vector in place of the original V\textsubscript{H} (Figure 4a). Multiple EP-PCR conditions were tested, allowing us to define the desired range of sub-
stitutions for the final library; to maximize the variability, we proceeded with the highest mutation rate, 9.4 mutated nucleo-
tides (nt)/kilobase (kb) (3.3/V\textsubscript{H} gene, Figure 4b). A final library of 8,000 transformants was obtained, with a mutational rate similar to what was previously observed (Figure 4b). Among 30 sequenced transformants, 13.33% encoded for the original V\textsubscript{H} and 16.67% contained loss-of-
function mutations (Fig. S7a). Subtracting the original and dysfunctional genes, the library was calculated to yield 5,600 in-frame, functional variants. In order to ease the evaluation of possible deleterious effects of random mutations, the library was transfected in parallel with the pPnP-HEL23 5’-linearized plasmid (pPnP-lin5’, also referred to as Control library). Figure 4c shows the cells after being transfected and before any selection step. The fact that the same pool of cells, stocks of reagents and amount of donor plasmid were used suggested that similar integration rates between the control and the EP library would be expected. Thus, if we assume that both samples had a ~ 5% integration rate, the 2.86% IgG positive fraction of the EP library would imply that only ~60% of the clones produced a functional IgG (Figure 4c).

Antibody affinity maturation with mammalian display screening

Among the IgG\textsuperscript{+} clones in the EP library, a HEL\textsuperscript{−} population (~10%) emerged (Figure 4d, IgG enrichment plots), likely due to mutated V\textsubscript{H} that lost specificity. Compared to the IgG\textsuperscript{+} enriched fraction of the Control library (Figure 4d, left plot), the HEL\textsuperscript{+} bulk of the EP library showed a broader distribution, indicative of heterogeneity in terms of affinity. We reasoned that potential affinity-matured clones would exhibit improved HEL binding with little variation in IgG expression; this guided our gating strategy for two rounds of fluorescence-activated cell sorting (FACS) enrichment (Figure 4d, first and second HEL enrich-
ment). The enrichment outputs of both HEL sorting steps were used for single-cell sorting in order to recover monoclonal populations. Fifty-three single-cell clones were genotyped, finally yielding five unique amino acid variants, labeled as HEL23 variant (v) 1–5 (Figure S5c). Figure 5a shows a summary of the coding mutations of all variants with regard to the original HEL23 amino acid sequence. All the clones except HEL23v3 had mutations in one CDR, with CDRH2 being the most prominently mutated. Next, the five clones were further characterized; both flow cytometry (Figure 5b) and ELISA (Figure 5c and S5b) demonstrated that all variants exhibiting an improved antigen-binding profile. Finally, a bio-layer inter-
ferometry (BLI) kinetics assay was performed on purified IgG; equilibrium dissociation constants (K\textsubscript{D}) showed that all five clones had improved affinity to the original HEL23, with HEL23v2, v3 and v4 exhibiting the sharpest improvement (4–5 folds, Figure 5d and S6).

Deep sequencing of Cas9-integrated antibody mutagenesis libraries

In order to more comprehensively explore and characterize the diversity of antibody libraries generated by enhanced Cas9 integration, we performed deep sequencing on the V\textsubscript{H} region at the various enrichment steps, including the IgG\textsuperscript{−} control
library. Deep sequencing allowed us to determine how the selected affinity-matured variants were represented in the overall mutagenesis repertoire. As expected, the frequencies of HEL23v1, v2, v4 and v5 were all substantially enriched whereas the original HEL23 was depleted following selection (Fig. S7e). HEL23v3 sequence was not found in IgG+ library.

**Figure 3.** Characterization of the antibody discovered from immune library screening. (a) Lineage tree of the 41 unique clones retrieved after single-cell sorting. Somatic hypermutations (SHM) refer to non-silent amino acid changes to the V-germline gene. The five clones highlighted in blue were selected as representative due to sequence diversity. V- and J-germline gene usage, CDRH3 sequences and amino acid changes were retrieved via IMGT/V-quest (http://www.imgt.org/IMGT-vquest/vquest). (b) Supernatant ELISA of the five clones highlighted in (a) and whose surface expression profile is shown in Figure 2c. Two technical replicates were included for each sample and a five-parameter logistical curve was fitted to the data by nonlinear regression. For each data point, the mean is represented and the error bars indicate standard deviation. PnP-mRuby-Cas9 cell supernatant was used as negative control.
Figure 4. Affinity maturation by PnP screening of a random mutagenesis library. (a) Schematic shows the workflow to generate a random mutagenesis library by error-prone (EP) PCR of the V\textsubscript{H} region of HEL23, expression in hybridoma cells and screening for affinity maturation. (b) Mutation rate varies based on amount of template used in the EP-PCR reaction. 100 ng of plasmid template produced the highest mutation rate and was selected for cloning of final library. (c) Flow cytometry dot plots show the frequency of antibody-expressing cells following transfection with the wild-type HEL23 plasmid (left) and the random mutagenesis library (right). (d) Flow cytometry dot plots show library screening and enrichment. From left to right: control transfection (wild-type HEL23) after enrichment of all antibody-expressing cells; EP-PCR library after enrichment of all antibody-expressing cells, but prior to any antigen enrichment; outputs of the first and second rounds of antigen enrichment. Each of the three screening steps of the EP-PCR library is shown at two different antigen staining concentrations to highlight affinity maturation. Representative flow cytometry gates do not correspond to actual sorting gates but are drawn to illustrate successful enrichment.
Figure 5. Characterization of antibody variants selected for affinity maturation. (a) The table shows the coding mutations of each of the five unique clones (HEL23v1-5) retrieved after single-cell sorting of the antigen-enriched libraries. (b) Flow cytometry dot plots show the surface expression and HEL-binding profile of the five HEL23 variants (green) and wild-type HEL23 (black). The ratio of HEL over antibody signal for each clone is shown in Figure S5a. (c) Supernatant ELISA comparing HEL binding for the five isolated variants (green) with wild-type HEL23 (black). Supernatants were adjusted to equal IgG-concentration (Fig. S5b). Two technical replicates were included for each of the mutated variants and one for the controls (PnP-HEL23 and PnP-mRuby-Cas9), and a five-parameter logistical curve was fitted to the data by nonlinear regression. For each data point, the mean is represented and the error bars indicate standard deviation. PnP-mRuby-Cas9 cell supernatant was used as negative control. (d) Affinity values of wild-type HEL23 and the five isolated variants obtained by bio-layer interferometry (BLI). The curves and fitting values are reported in Figure S6. (e) Heatmap shows enrichment of all possible substitutions for each WT residue (x-axis) in the HEL+ library compared to IgG+ library in a log2-fold change scale. Red substitutions are enriched in the HEL+ library, while blue substitutions are depleted. White squares (log2 ratio = 0) indicate neutral substitutions not impacted by enrichment, or residues in which the mutation rate is negligible. The mutation rate for each position was calculated by summing the mutation frequency of all replicates (n = 3) for each condition. Key residues found in affinity-matured clones are indicated with black boxes.
but was found to be fourth most frequent $V_H$ after HEL$^+$ sorting, further supporting effective enrichment (Fig. S6f). Notably, after HEL$^+$ enrichment, all of the five affinity-matured $V_H$ sequences ranked among the top 10 clones in abundance. Finally, we set out to evaluate the mutational trend across the amino acid sequence of HEL23 $V_H$ by comparing the frequency of all possible substitutions at every position along the amino acid sequence in the IgG$^+$ pool and after the first HEL$^+$ sorting. Mutagenesis and selection did not seem to have preferential targeting, although mutations in framework (FR) 1 region appear to be quite relevant for affinity maturation (Figure 5e). The heatmap illustrates the level of enrichment (red) or depletion (blue), as a consequence of antigen selection. Notably, some of the hallmark mutations of the five affinity-improved clones are among the most represented substitutions for the residue of interest (S91G (HEL23v1), Q5P (HEL23v2), C22S (HEL23v3), G53S (HEL23v4 and v5); Figure 5e).

**Discussion**

Antibody discovery in mammalian cells offers the possibility for expression in their native, full-length IgG format, thus providing access to the physiological protein folding and post-translational modification machinery, which in comparison to microbial systems offers a superior approach to assess their drug developability parameters. Several mammalian display platforms have been previously developed, but due to the inherently limited throughput, none of them has achieved the widespread adoption of microbial display systems. In order to overcome limitations in Cas9-driven HDR, we designed an HDR donor architecture that incorporated linearizing motifs in the backbone, similarly to what was reported before, when applied to PnP cells, integration efficiencies of variable gene libraries were observed to be above 5% (Figure 1d), a 15-fold improvement over the yield of the original donor (Figure 1e). Interestingly, the engineered plasmid performed better even when compared with PCR-linearized DNA. It is possible, in this regard, that circularized donors are better protected from the intracellular exonuclease-based degradation machinery, resulting in sustained circular donor levels in the nucleus available for linearization and subsequent integration. Furthermore, from the point of view of practicality, such a linearizing plasmid format is easy to produce and purify from bacterial culture compared to the costly and demanding production and purification steps used for viral (rAAV) constructs.

As a first application, we tested our enhanced Cas9 HDR approach for antibody discovery from a library of combinatorial $V_L$ and $V_H$ genes extracted from the murine bone marrow plasma cell compartment of an immunized mouse (Figure 2). Bone marrow plasma cells of immunized mice have previously been shown to possess highly abundant $V_L$ and $V_H$ genes that correspond to antigen-specific clones. As previously shown, integration by HDR in our hybridoma system occurs in a manner that does not show any substantial bias toward specific templates; thus, the plasmid HDR donor library diversity after cloning should reflect the diversity after HDR. Moreover, due to the requirement of splicing with the endogenous IgG constant region to achieve functional expression, and to the presence of only one mRuby-IgG2c locus in the PnP cells, we expect no aberrant background expression from residual, non-integrated plasmid or random genomic integration. Consequently, each correctly edited cell will express one single antibody.

PnP screening by flow cytometry resulted in a large panel of antigen-specific hybridoma cell lines. The whole process of mouse organ preparation, cloning, library generation and PnP screening was considerably rapid (4–6 weeks). Furthermore, the isolation of 41 unique variants (based on $V_H$ amino acid sequence) across several different lineages (CDRH3 clonotypes) demonstrates that a diverse panel is obtained by PnP library screening. In contrast to traditional hybridoma antibody discovery, which consists of fusing primary memory B cells with a myeloma partner and performing plate-based screening, our approach offers several advantages in terms of speed and ease of screening and selection. Furthermore, antibody genes can be isolated from plasma cells, which may offer a unique compartment of antibody diversity, and, due to their high polarization toward antigen-specific variable genes, it may be possible to recover natural pairings at least for the most frequent clones by combinatorial screening. While the limited number of plasma cells used to generate the library results in a relatively low diversity library, the high enrichment of antigen-specific clones makes it compatible for screening and discovery. In the future, deep sequencing of the input plasmid library, initial cell library as well as sequential enrichment rounds will provide more characterization and quantitation of library diversity. In recent years, novel single-cell methodologies based on droplet microfluidics have been adapted to screen naturally paired $V_L$ and $V_H$ genes by yeast display. A similar approach of cloning natural $V_H$:V$L$ pairs into our synthetic antibody HDR scaffold would offer an attractive way to functionally screen antibody repertoires in mammalian cells.

We used enhanced Cas9-driven HDR in PnP cells for antibody engineering, in particular for affinity maturation of a previously identified antibody (HEL23) that was specific for the HEL antigen (Figure 4). We performed EP-PCR on the $V_H$ region of HEL23 and following Cas9 HDR integration and PnP library screening, we were able to isolate five affinity-matured variants (HEL23v1-5, Figure 5). The C22S mutation in the PnP-HEL23v3 clone was unexpected, since it likely caused a disruption of the disulfide bond in the $V_H$ domain, which would lead to decreased protein stability and expression levels (Figure 5b). However, wide range intrachain contacts could potentially mitigate this effect, which may be the reason why expression of this variant was still observed. Due to the current lack of a co-crystal structure, it remains to be determined how this mutation exactly translates into an increase in affinity. Deep sequencing of our libraries allowed us to more thoroughly interrogate library diversity, selection and enrichment and uncover mutational preferences, which all suggested that PnP screening was a robust approach for antibody engineering. It is reasonable to expect that additional mutagenesis libraries, such as extension of EP-PCR to the $V_L$ variable region shuffling, and combining the beneficial mutations of different clones could also be successfully integrated for PnP screening. Importantly, compared to the most common strategies for affinity maturation using phage and yeast display to express
antibody fragments, our PnP system presents the fundamental advantage of selection in the native mammalian cell IgG context, which may further aid in the selection of candidates with favorable drug development parameters.

Materials and methods

Hybridoma cell culture conditions

PnP-mRuby-Cas9 cells were obtained as previously described. All hybridoma cell lines were cultivated in high-glucose Dulbecco’s Modified Eagle Medium (DMEM), Thermo Fisher Scientific (Thermo), 61965–026 with GlutaMAX® supplement, supplemented with 10% (v/v) fetal bovine serum (FBS), Thermo, 16000–044), 100 U/ml penicillin/streptomycin (Thermo, 15140–122), 10 mM HEPES buffer (Thermo, 15630–056) and 50 μM 2-mercaptopethanol (Thermo, 31350–010). Hybridoma cells were maintained in incubators at a temperature of 37°C and 5% CO₂, typically in 5 ml of culture in T-25 flasks (TPP, 90026) and passed every 48/72 h. The PnP-mRuby-Cas9 cell line was confirmed annually to be negative for mycoplasma contamination (Universal Mycoplasma Detection Kit, ATCC, 30–1012K).

Preparation of the test HDR donor formats and in vitro testing of Cas9 cleavage

The PnP-lin donor plasmids were generated by adding the CRISPR RNA-J target sequence (including the PAM sequence) by Gibson cloning directly 5’ of the upstream homology arm of the HEL23 HDR vector, 3’ of the downstream homology arm, or in both sites in a stepwise manner. The PCR product was prepared with the primers reported in Table S1 and the following cycling conditions: initial denaturation 3 min at 95°C; 23 cycles of denaturation at 98°C (20 s), annealing at 65°C (15 s), elongation at 72°C (2 min); final elongation at 72°C (3 min). In order to test if PnP-lin donor plasmids were recognized and successfully linearized by the Cas9 nuclease, an in vitro digestion assay was set up in a total reaction volume of 30 μl as follows: 16 μl of nuclelease-free water (Thermo, AM9937) were mixed with 3 μl of NEBuffer 3.1 (NEB, B7203), 6 μl of 300 nM crRNA-J/tracr RNA complex (final concentration of 60 nM) and 2 μl of 1 μM Cas9 nuclease (NEB, M0386; final concentration of 60 nM) and the mixture was incubated for 10 min at 25°C in order to prepare the RNP complex. 3 μl of 30 nM plasmid DNA were added (final concentration of 3 nM) and samples were incubated for 60 min at 37°C. 1 μl of Protease K (NEB, P8107) was added and samples were incubated for another 20 min at room temperature before fragment analysis was performed by agarose-gel electrophoresis on a 1% (w/v) gel using the SDS-containing gel loading dye purple (NEB, B7024S) in order to dissolve residual Cas9-DNA complexes.

Mouse immunization and plasma cell isolation

All mouse experiments were performed under the guidelines and protocols approved by the Basel-Stadt cantonal veterinary office (Basel-Stadt Kantonales Veterinäramt Tierversuchsbevilligung #2582). One female BALB/c mouse (Janvier Laboratories France, 10 weeks old), housed under specific pathogen-free conditions and maintained on a standard chow diet, was repeatedly immunized subcutaneously on day 0, 21 and 49 into the flank with 150 μl of a PBS (Thermo, 10010023)-based solution consisting of 100 μg ovalbumin (Sigma, A5503) and 20 μg monophosphoryl lipid A (MPLA) adjuvant (Sigma, L6895; formulated according to the manufacturer’s instructions in dimethyl sulfoxide (G-Biosciences, 786–1323)). The mouse was sacrificed 9 d after the final boost and in both hind legs (femur and tibia) were isolated and stored in cold RPMI-1640 medium (Sigma, A10491-01) supplemented with 10% (v/v) FBS (Thermo, 16000–044), 100 U/ml penicillin/streptomycin (Thermo,15140–122) and 50 μM 2-mercaptopethanol (Thermo, 31350–010). The bones were flushed with cold 1:20 (v/v) MACS BSA Stock Solution (Miltenyi Biotect, 130-091-376) in autoMACS Rinsing Solution (Miltenyi Biotect, 130-091-222) to extract bone marrow, which was subsequently filtered through a 40 μm nylon cell strainer (Falcon, 352340) in order to obtain a homogenous single-cell suspension. Bone marrow plasma cells were subsequently purified from a total of 7.38 × 10⁶ single cells using the CD138+ Plasma Cell Isolation Kit mouse (Miltenyi Biotect, 130-092-530) according to the manufacturer’s instructions. Buffer volumes used were based on 1 × 10⁸ starting cells. 21060 plasma cells were obtained after purification and cells were frozen in 750 μl TRIzol reagent (Thermo, 15596026) at −80°C until further use.

Generation of immune libraries for HDR donors

Total RNA was extracted using the PureLink RNA Mini Kit (Thermo, 12183025) following the ‘TRIzol Plus RNA Purification Kit’ manual (TRIzol reagent, Thermo, 15596026). Complementary DNA (cDNA) was synthesized via reverse transcription using Maxima Reverse Transcriptase (Thermo, EP0741) and Oligo (dT) as reverse primer (Thermo, SO131). 59.5 ng RNA were used as template in three parallel reactions, which were incubated at 50°C for 30 min and at 85°C for 5 min, following manufacturer’s instructions, and finally pooled. In order to obtain the library scaffold plasmid pPnP-lin5-AarI, two consecutive stop-codons were inserted into the beginning of the HEL23 V₄ gene in order to prevent background expression and an AarI restriction site was cloned in place of the V₄ gene in the HEL23 pPnP-lin5 plasmid using the Gibson Assembly® Master Mix (NEB, E2611S). V₄ genes were amplified using the Kapa HiFi HotStart Ready Mix (Kapa Biosystems, KK2602) using the primer mix reported in Table S2, with the following cycling conditions: initial denaturation 3 min at 95°C; 27 cycles with denaturation at 98°C (20 s), annealing at 50°C (15 s) and final elongation at 72°C (20 s); final elongation at 72°C (1 min). The amplified V₄-repertoire was cloned into the PnP-lin5-AarI scaffold plasmid by Gibson assembly to replace the HEL23 V₄ region and the library quality was assessed by colony PCR (24/24 clones had a new V₄ inserted). The total V₄ transformant library size oversampled the starting plasma cell number by a factor of 4.

In a second step, the V₄-repertoire was amplified with Kapa HiFi HotStart Ready Mix using the primer mix reported in Table S3, with the following conditions: initial denaturation
3 min at 95°C; 27 cycles with denaturation at 98°C (20 s), annealing at 60°C (15 s), elongation at 72°C (30 s); final elongation at 72°C (1 min). The amplified V<sub>H</sub>-repertoire and the previously created PnP-lin5<sup>−</sup>-V<sub>L</sub> library were restriction digested with AarI (Thermo, ER1581) and the backbone was additionally dephosphorylated using Antarctic Phosphatase (NEB, M0289S). Ligation was performed with T4 DNA ligase (NEB, M0202S) and the quality of the final library was assessed by Sanger sequencing (for 28 clones, all of them had unique V<sub>L</sub> and V<sub>H</sub> and no premature stop-codons or frameshift mutations were detected). The final library size was 5.09 × 10<sup>8</sup> transformants. Both the intermediate and final libraries were transformed in electrocompeotent *E. coli* cells and purified by midiprep (Zymo Research, D4200) from liquid cultures.

**Generation of the EP-PCR V<sub>H</sub> libraries for HDR donors**

To clone the test libraries, 100, 250 or 500 ng of pPnP-HEL23 were used to amplify the V<sub>H</sub> region with the GeneMorph II EZClone Domain Mutagenesis Kit (Agilent, 200552) following manufacturer’s instruction and using the primers reported in Table S4. This kit contains Mutazyme II, an enzyme blend which combines the activity of a novel Taq DNA polymerase and Mutazyme I DNA polymerase; such a mixture is optimized to yield minimal bias toward certain substitutions due to the opposite mutational spectra of the two polymerases. Since the mutational rate is expected to be adjustable by varying the amount of PCR template, we initially cloned three test libraries in order to explore different conditions (Figure 4b). The libraries were generated using 100, 250 or 500 ng of plasmid template. The following cycling conditions were used: initial denaturation 2 min at 95°C; 30 cycles with denaturation at 95°C (30 s), annealing at 65°C (30 s), elongation at 72°C (1 min); final elongation at 72°C (10 min). The product was purified by agarose-gel extraction and 300 ng were used as a template for a second PCR with Kapa HiFi HotStart Ready Mix, with the following cycling conditions: initial denaturation 3 min at 95°C; 20 cycles with denaturation at 98°C (20 s), annealing at 65.6°C (15 s), elongation at 72°C (15 s); final elongation at 72°C (25 s). Each insert library and the pPnP-lin5<sup>−</sup>-Aar scaffold were restriction digested with AarI (Thermo, ER1581) and purified by gel extraction, and the digested backbone was subsequently treated with Antarctic Phosphatase (NEB, M0289S) prior to ligation with T4 DNA ligase (NEB, M0202S).

The test libraries were transformed in electrocompeotent *E. coli* cells and single bacterial colonies were sequenced from each library to estimate the mutational rate. As expected, the lowest amount of template led to the highest mutational frequency. Since the mutational rate is expected to be adjustable by varying the amount of PCR template, we initially cloned three test libraries in order to explore different conditions (Figure 4b).

**Flow cytometry analysis and sorting of hybridomas**

Flow cytometry and sorting experiments were performed, respectively, with the BD LSR Fortessa<sup>™</sup> and BD FACS Aria<sup>™</sup> III (BD Biosciences). For cell staining, 5 × 10<sup>5</sup> cells were isolated by centrifugation, washed with cold PBS (Thermo, 10010–015) and centrifuged again. Labeling mixes were prepared in 50 μl/sample by diluting the staining reagents in PBS at the working concentrations described in Table S6. The cells, resuspended in labeling mix, were incubated for 30 min on ice, protected from light, and finally washed 2–3 times with PBS by centrifugation. All the centrifugation steps in the labeling protocol were performed at 250 xG for 5 min at 4°C. When labeling a higher number of cells, the amount of reagents was adjusted accordingly by maintaining a 100 μl labeling mix/1 × 10<sup>6</sup> cells ratio.

**Supernatant ELISA**

Sandwich ELISAs were used to measure antibody secretion and assess the antigen specificity of the secreted IgG. Plates were coated with the capture reagent (antigen or anti-IgK antibody) at a concentration of 4 μg/ml in PBS overnight.
μ5088). Briefly, 6.25 µg/cells were isolated from antibody region was PCR-isolated from each sample and normalized to a volume of 2 ml for the anti-OVA clones, an equal volume of culture was isolated for each sample, but any further normalization was omitted. The supernatants were serially diluted 1:3 down in PBSMT and incubated for 1 h at room temperature. Anti-IgK-HRP secondary antibody was used for detection in antigen ELISAs, while anti-IgG-Fc was used in antibody secretion ELISAs. The 1-Step Ultra TMB-ELISA Substrate Solution (Thermo, 34028) was used for signal development, and the reaction was stopped with 1 M H₂SO₄. The signal was finally measured by reading the absorbance at 450 nm (Infinite® 200 PRO, Tecan). The working concentrations and other relevant specifications of the ELISA reagents are reported in table S7.

**Genotyping and Sanger sequencing of single-cell hybridoma clones**

Typically, genomic DNA of single-cell hybridoma clones was extracted from 5 x 10⁵ cells using the QuickExtract DNA Extraction Solution (Epicenter, QE09050). Cells were incubated at 68°C for 15 min and 95°C for 8 min and the integrated synthetic V₅-Ck-2A-V₆ antibody region was PCR-amplified with flanking primers sAb_fw and sAb_rev that were specific for the 5’ and 3’ homology arms. From this single amplicon, both V₅ and V₆ regions could be Sanger-sequenced using primers VH-fw and VL-fw, respectively (Microsynth, Balgach). Primers are reported in Table S8.

Sequence analysis was performed after trimming the initial and terminal amino acids, as the length of the trimmed region varied according to the primer set in use (VL or VH) to exclude artifacts due to preferential primer binding during multiplex PCR amplification.

**Affinity measurement by bio-layer interferometry (BLI)**

Total IgG was purified from 2.5 to 4 ml of culture with NAb™ Protein G Spin Columns (Thermo, 89953) and buffer exchanged with PBS with Slide-A-Lyzer™ MINI Dialysis Devices (Thermo, 69560). Kinetics measurements were performed using the Octet RED96e and anti-mouse Fc Capture (AMC) biosensors (Pall FortéBio, 18–5088). Briefly, 6.25 µg/ml of purified antibody were loaded on sensors for 10 min, then underwent association with HEL at decreasing concentrations of 8, 2 and 0.5 nM (10 min) and dissociation (10 min); the assay was performed in Kinetics Buffer (Pall FortéBio, 18–1105). Association and dissociation curves are shown in Figure S6.

**Deep sequencing**

Genomic DNA (gDNA) was isolated from 5 x 10⁶ cells per sample with the PureLink genomic DNA Mini kit (Thermo, K182002). The protocol for library preparation was adapted from previously published work.²⁶,⁵³ For each replicate, 7200 ng of gDNA were amplified with Kapa HiFi HotStart Ready Mix, split in 8 50 µl reactions, with the primers reported in Table S5 (step: PCR1). The following cycling conditions were used: initial denaturation 3 min at 95 °C; 16 cycles with denaturation at 98°C (20 s), annealing at 69°C (15 s), elongation at 72°C (12 s); final elongation at 72°C (25 s). The eight reactions were pooled and underwent a first purification with the DNA Clean & Concentrator kit (Zymo Research, D4033) and an additional clean-up step with SPlisect beads (Beckman Colter, B23319) with a 0.8X beads to sample ratio. The whole purified product was used as template for the second PCR (Illumina indexing) using Kapa HiFi HotStart Ready Mix, split in 8 50 µl reactions, with the primers reported in Table S5 (step: PCR2). The following cycling conditions were used: initial denaturation 3 min at 95 °C; 2 cycles with denaturation at 98°C (20 s), annealing at 40°C (15 s), elongation at 72°C (1 min); 14 cycles with denaturation at 98°C (20 s), annealing at 65°C (15 s), elongation at 72°C (1 min); final elongation at 72 °C (5 min). The eight reactions were pooled, underwent a first purification with the DNA Clean & Concentrator kit (Zymo Research, D4033) and a final clean-up step by agarose-gel extraction with Zymoclean™ Gel DNA Recovery kit (Zymo Research, D4001).

DNA concentration was determined using a Nanodrop2000c spectrophotometer (Thermo) and the quality was confirmed with a fragment analyzer (Advanced Analytical Technologies) using the DNF-473 Standard Sensitivity NGS fragment analysis kit. Samples successfully passing quality control were pooled to reach a final concentration of 4 nM, and sequenced on the Illumina MiSeq platform using the reagent kit v3 (2 x 300 cycles, paired-end) with 10% PhiX control library spiked in. For each sample, raw reads were paired and annotated using the MiXCR platform (https://mixcr.readthedocs.io/en/master/).⁵⁴ Statistical analysis was performed with the software GraphPad Prism and R.

**Informatics and statistical analysis**

ELISA data, HDR improvement rate (Figure 1) and mutation frequency in EP-PCR libraries (Figure 4b) were analyzed with GraphPad Prism. Lineage trees of V₅ and V₆, sequences of the anti-OVA clones (Figure 3a and S3a) were generated using Geneious. Germline gene usage information, CDR3 sequences and amino acid mutations were obtained with the IMGT/ V-quest online tool (http://www.imgt.org/IMGT_vquest/vquest).⁵⁵

**Acknowledgments**

We acknowledge the ETH Zurich D-BSSE Single Cell Unit and the Genomics Facility Basel, in particular, Dr. Aleksandra Gumieny, Dr. Thomas Horn, Elodie Burcklen and Dr. Christian Beisel, for excellent support and assistance. We also thank Dr. Rodrigo Vazquez-Lombardi for assistance with BLI affinity measurements and data analysis. This work was supported by the European Research Council Starting Grant 679403 (to S.T.R.). The professorship of S.T.R. is supported by an endowment from the S. Leslie Misrock Foundation.
Author contributions

C.P., D.N. and S.T.R. developed the methodology and wrote the manuscript; C.P., D.N., L.C. and S.T.R. wrote the manuscript; C.P., D.N. and S.T.R. designed the experiments; C.P. and D.N. performed the experiments and analyzed the data; S.F. analyzed deep sequencing data; L.C. performed mouse immunizations and plasma cell isolation; M.D. T. assisted with flow cytometry sorting procedures; S.F., L.C. and D.M. M. provided technical advice and support for deep sequencing experiments; D.M.M. provided technical advice and support for the BLI affinity measurements and data analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the H2020 European Research Council [679403].

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References

1. Weiner GJ. Building better monoclonal antibody-based therapeutics. Nat Rev Cancer. 2015;15:361–70. doi:10.1038/nrc3930.
2. Walsh G. Biopharmaceutical benchmarks 2018. Nat Biotechnol. 2018;36:1136–45. doi:10.1038/nbt.4046.
3. Marks JD, Hoogenboom HR, Bonnett TP, McCafferty J, Griffiths AD, Winter G. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J Mol Biol. 1991;222:91–7.
4. Feldhaus MJ, Siegel RW, Opresko LR, Coleman JR, Feldhaus JM, Yeung YA, Cochran JR, Heinzelman P, Colby D, Swers J, et al. Flow cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. Nat Biotechnol. 2003;21:163–70. doi:10.1038/nbt785.
5. Hoogenboom HR. Selecting and screening recombinant antibody libraries. Nat Biotechnol. 2005;23:1105–16. doi:10.1038/nbt1126.
6. Bradbury ARM, Sidhu S, Dube S, McCafferty J. Beyond natural antibodies: the power of in vitro display technologies. Nat Biotechnol. 2011;29:245–54. doi:10.1038/nbt.1791.
7. Jain T, Sun T, Durand S, Hall A, Houston NR, Nett JH, Sharkey B, Bobrowicz B, Caffry I, Yu Y, et al. Biophysical properties of the clinical-stage antibody landscape. Proc Natl Acad Sci U S A. 2017;114:9444–49. doi:10.1073/pnas.1614081114.
8. Partibhan K, Perera RL, Sattar M, Huang Y, Mayle S, Masters E, Griffiths D, Surade S, Leah R, Dyson MR, et al. A comprehensive search of functional sequence space using large mammalian display libraries created by gene editing. MABS. 2019;11:884–98. doi:10.1080/19420682.2019.1618673.
9. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337:816–21. doi:10.1126/science.1225829.
10. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. Multiplex genome engineering using CRISPR/Cas systems. Science. 2013;339:819–23. doi:10.1126/science.1231143.
11. Black JB, Perez-Pinera P, Gersbach CA. Mammalian synthetic biology: engineering biological systems. Annu Rev Biomed Eng. 2017;19:249–77. doi:10.1146/annurev-bioeng-071516-044649.
12. Sander JD, Joung JK. CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol. 2014;32:347–55. doi:10.1038/nbt.2842.
13. Pannunzi NR, Watanabe G, Lieber MR. Nonhomologous DNA end-joining for repair of DNA double-strand breaks. J Biol Chem. 2016;291:10512–22. doi:10.1074/jbc.M115.674503.
14. Chu VT, Van Trung C, Weber T, Webers B, Wurst W, Sander R, Rajewsky K, Kühn R. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotechnol. 2015;33:543–48. doi:10.1038/nbt.3198.
15. Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR, Ploegh HL. Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. Nat Biotechnol. 2015;33:538–42. doi:10.1038/nbt.3190.
16. Lin C, Li H, Hao M, Xiong D, Luo Y, Huang C, Yuan Q, Zhang J, Xia N. Increasing the efficiency of CRISPR/Cas9-meditated precise genome editing of HSV-1 virus in human cells. Sci Rep. 2016;6:34531. doi:10.1038/srep34531.
17. Hu Z, Shi Z, Guo X, Jiang B, Wang G, Luo D, Chen Y, Zhu Y-S. Ligase IV inhibitor SCR7 enhances gene editing directed by CRISPR-Cas9 and ssODN in human cancer cells. Cell Biosci. 2018;8. doi:10.1186/s13578-018-0200-z.
18. Pinder J, Salsman J, Dellaire G. Nuclear domain “knock-in” screen for the evaluation and identification of small molecules enhancing CRISPR-based genome editing. Nucleic Acids Res. 2015;43:9379–92. doi:10.1093/nar/gkv993.
19. Song J, Yang D, Xu J, Zhu T, Chen YE, Zhang J. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nat Commun. 2016;7:10548. doi:10.1038/ncomms10548.
20. Lin S, Staahl BT, Alla RK, Doudna J. Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. Elife. 2014;3:e04766. doi:10.7554/elife.04766.
21. Yang D, Scavuzzo MA, Chmielowiec J, Sharp R, Bajic A, Borowiak M. Enrichment of G2/M cell cycle phase in human pluripotent stem cells enhances HDR-mediated gene repair with customizable endonucleases. Sci Rep. 2016;6:21264. doi:10.1038/srep21264.
22. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. Nat Biotechnol. 2016;34:339–44. doi:10.1038/nbt.3481.
23. Li H, Beckman KA, Pessino V, Huang B, Weissman JS, Leonetti MD. Design and specificity of long ssDNA donors for CRISPR-based knock-in. 2017. doi:10.1101/178905.
24. Dever DP, Bak RO, Reinsch A, Camarena J, Washington G, Nicolas CE, Pavel-Dimu M, Saxena N, Wilkins AB, Mantri S, et al. CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. Nature. 2016;539:384–89. doi:10.1038/nature20134.
25. Eyquem J, Mansilla-Soto J, Giavridis T, van der Stegen SJC, Hamieh M, Cunanan KM, Odak A, Gönen M, Sadelain M. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017;543:113–17. doi:10.1038/nature21405.
26. Mason DM, Weber CR, Parola C, Meng SM, Greiff V, Kelton WJ, Reddy ST. High-throughput antibody engineering in mammalian cells by CRISPR/Cas9-mediated homology-directed mutagenesis. Nucleic Acids Res. 2018;46:7436–49. doi:10.1093/nar/gky550.
27. Pogson M, Parola C, Kelton WJ, Heuberger P, Reddy ST. Immunogenic engineering of a plug-and-(dis)play hybridoma platform. Nat Commun. 2016;7:12535. doi:10.1038/ncomms12535.
28. Khan TA, Friedensohn S, Gorter de Vries AR, Straszewski J, Ruscheweyh H-J, Reddy ST. Accurate and predictive antibody repertoire profiling by molecular amplification fingerprinting. Sci Adv. 2016;2:e1501371. doi:10.1126/sciadv.1501371.
29. Reddy ST, Ge X, Miklos AE, Hughes RA, Kang SH, Hoi KH, Chrysostomou C, Hunicke-Smith SP, Ivenson BL, Tucker PW, et al. Monoclonal antibodies isolated without screening by analyzing the variable-gene repertoire of plasma cells. Nat Biotechnol. 2010;28:965–69. doi:10.1038/1665.
30. Ho M, Pastan I. Mammalian cell display for antibody engineering. Ther Antibodies. 2009;5:337–52. doi:10.1007/978-1-59745-524-1_18.

31. Ho M, Nagata S, Pastan I. Isolation of anti-CD22 Fv with high affinity by Fv display on human cells. Proc Natl Acad Sci U S A. 2006;103:9637–42. doi:10.1073/pnas.0603653103.

32. Higuchi K, Araki T, Matsuuzaki O, Sato A, Kanno K, Kitaguchi N, Ito H. Cell display library for gene cloning of variable regions of human antibodies to hepatitis B surface antigen. J Immunol Methods. 1997;202:193–204. doi:10.1016/s0022-1759(97)00010-0.

33. Zhang J, Zhang XA, Liu Q, Li M, Gao L, Gao X, Xiang S, Wu L, Fu J, Song H. Mammalian cell display for rapid screening scFv antibody therapy. Acta Biochim Biophys Sin. 2014;46:859–66. doi:10.1093/abbs/gmu092.

34. Akamatsu Y, Pakabunto K, Xu Z, Zhang Y, Tsurushita N. Whole IgG surface display on mammalian cells: application to isolation of neutralizing chicken monoclonal anti-IL-12 antibodies. J Immunol Methods. 2007;327:40–52. doi:10.1016/j.jim.2007.07.007.

35. Zhou C, Jacobsen FW, Cai L, Chen Q, Shen WD. Development of a novel mammalian cell surface antibody display platform. MAbs. 2010;2:508–18. doi:10.4161/mbas.2.5.13089.

36. Tomimatsu K, Matsumoto S-E, Tanaka H, Yamashita M, Nakanishi H, Teruya K, Kazuno S, Kinjo H, Hamasaki T, Kusumoto K-I, et al. A rapid screening and production method using a novel mammalian cell display to isolate human monoclonal antibodies. Biochem Biophys Res Commun. 2013;441:59–64. doi:10.1016/j.bbrc.2013.10.007.

37. King DJ, Bowers PM, Kehry MR, Horlick RA. Mammalian cell display and somatic hypermutation in vitro for human antibody discovery. Curr Drug Discov Technol. 2014;11:56–64. doi:10.2174/15701638113109990037.

38. Beerli RR, Bauer M, Buser RB, Gwerder M, Muntwiler S, Maurer P, Saudan P, Bachmann MF. Isolation of human monoclonal antibodies using a novel mammalian cell surface display to isolate human monoclonal antibodies. Biochem Biophys Res Commun. 2010;392:111–22. doi:10.1016/j.bbrc.2010.06.009.

39. Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Araoka T, Li Z, et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. Nature. 2016;540:144–49. doi:10.1038/nature20555.

40. Abe T, Harashima A, Xia T, Konno H, Konno K, Morales A, Ahn J, Gutman D, Barber GN. STING recognition of cytoplasmic RNA by upon viral infection. Nature. 2015;525:337–38. doi:10.1038/nature15263.

41. Wang B, Kluwe CA, DeKosky BJ, Kerr SA, Johnson EL, Tanno H, Lee C-H, Jung J, Rezigh AB, et al. Facile discovery of 15-fold higher affinity of a Fusarium-specific single-chain antibody by directed molecular evolution coupled to phage display. Mol Biotechnol. 2012;52:111–22. doi:10.1007/s12033-011-9478-3.

42. Steimer WP. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc Natl Acad Sci U S A. 1994;91:10747–51. doi:10.1073/pnas.91.22.10747.

43. Vanhercke T, Ampe C, Tiry L, Denolf P. Reducing mutational bias in random protein libraries. Anal Biochem. 2005;339:9–14. doi:10.1016/j.ab.2004.11.032.

44. Hendl A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol. 2015;33:985–98. doi:10.1038/nbt.3290.

45. Menzel U, Greiff V, Khan TA, Haessler U, Hellmann I, Friedensohn S, Cook SC, Pogson M, Reddy ST. Comprehensive evaluation and optimization of ampiclon library preparation methods for high-throughput antibody sequencing. PLoS One. 2014;9:e96727. doi:10.1371/journal.pone.0096727.

46. Bolotin DA, Poslavsky S, Mitropanov I, Shugay M, Mamedov IZ, Putintseva EV, Chudakov DM. MiXCR: software for comprehensive adaptive immunity profiling. Nat Methods. 2015;12:380–81. doi:10.1038/nmeth.3364.

47. Brochet X, Lefranc MP, Giudicelli V. IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res. 2008;36:W503–8. doi:10.1093/nar/gkn316.