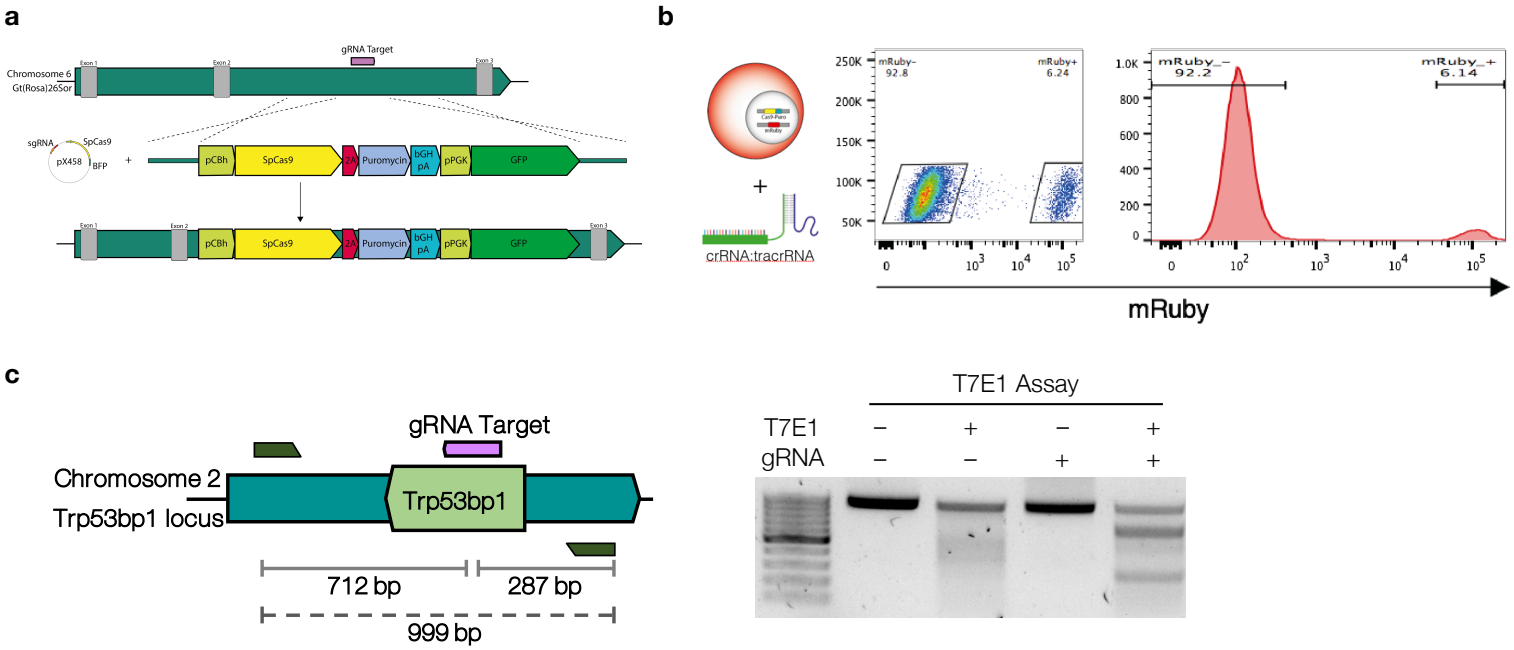
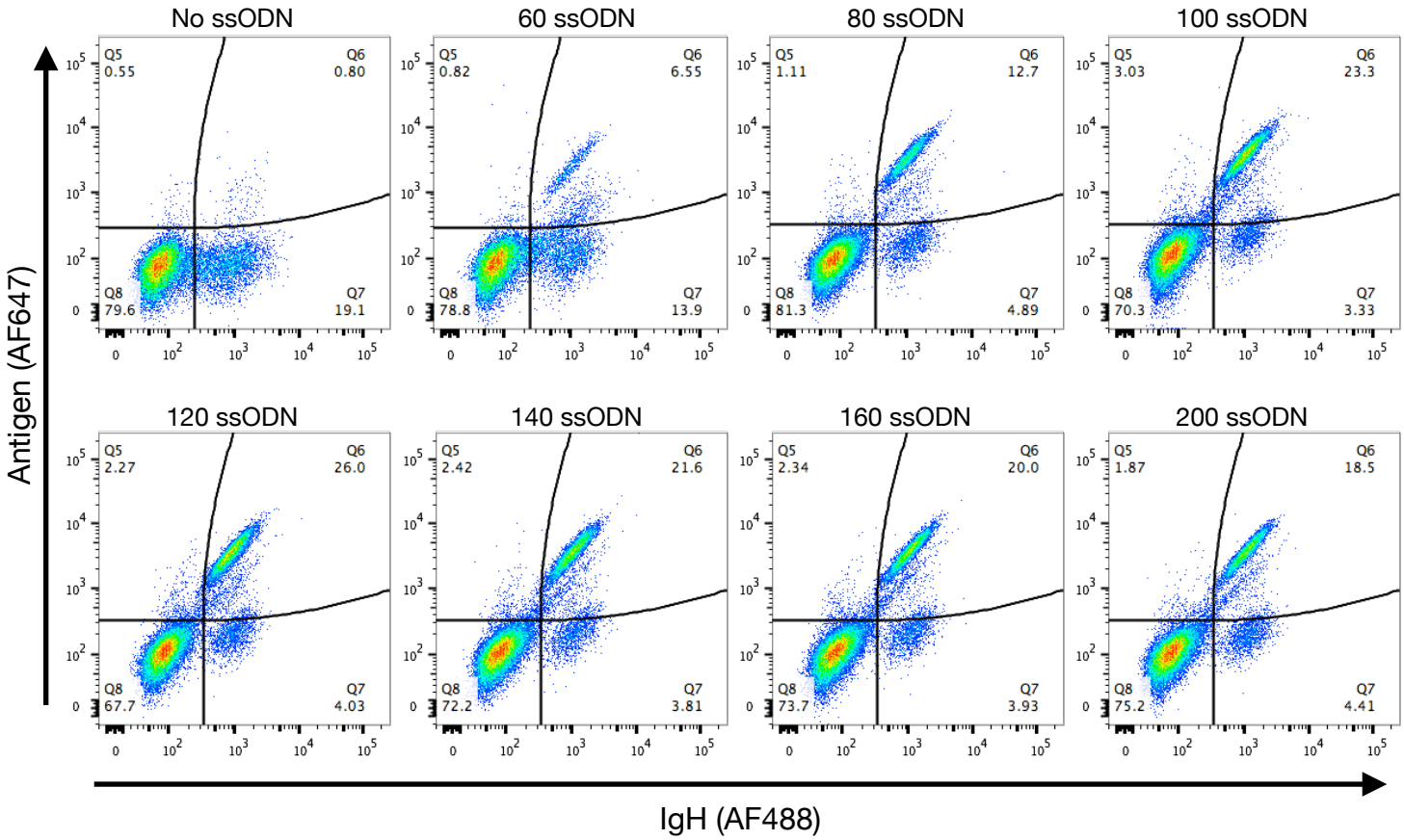


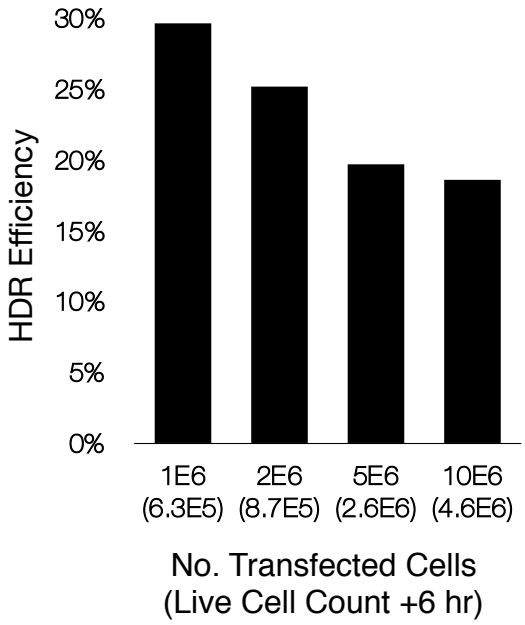
Supplementary Fig. 1: Creation of a stable hybridoma cell line with constitutive Cas9 expression

a, A constitutive Cas9 expression cassette contains two genes under control of separate promoters. The first gene encodes for the Cas9-2A-puromycin gene from the plasmid pSpCas9(BB)-2A-Puro (PX459) and enables expression of the Cas9 protein and the puromycin resistance protein from a single transcript. The second gene encodes for the fluorescent protein eGFP used in selection of successfully integrated cassettes. The cassette is integrated into the Rosa26 safe harbor locus of the murine genome by co-transfection with the plasmid pSpCas9(BB)-2A-GFP (PX458). **b,** Validation of Cas9 activity by transfecting the PnP-mRuby hybridoma with only a gRNA complex targeting the mRuby gene. Results from flow cytometry confirms high levels (>90%) of gene knock-out. **c,** Gene knockout of the 53BP1 located on chromosome 2 of the hybridoma genome. PnP-HEL23.FS cells were transfected with gRNA targeting the gene, generating a polyclonal population of 53BP1+ and 53BP1- cells. T7E1 assay confirms the large majority of cells contain NHEJ/MMEJ frameshift mutations.



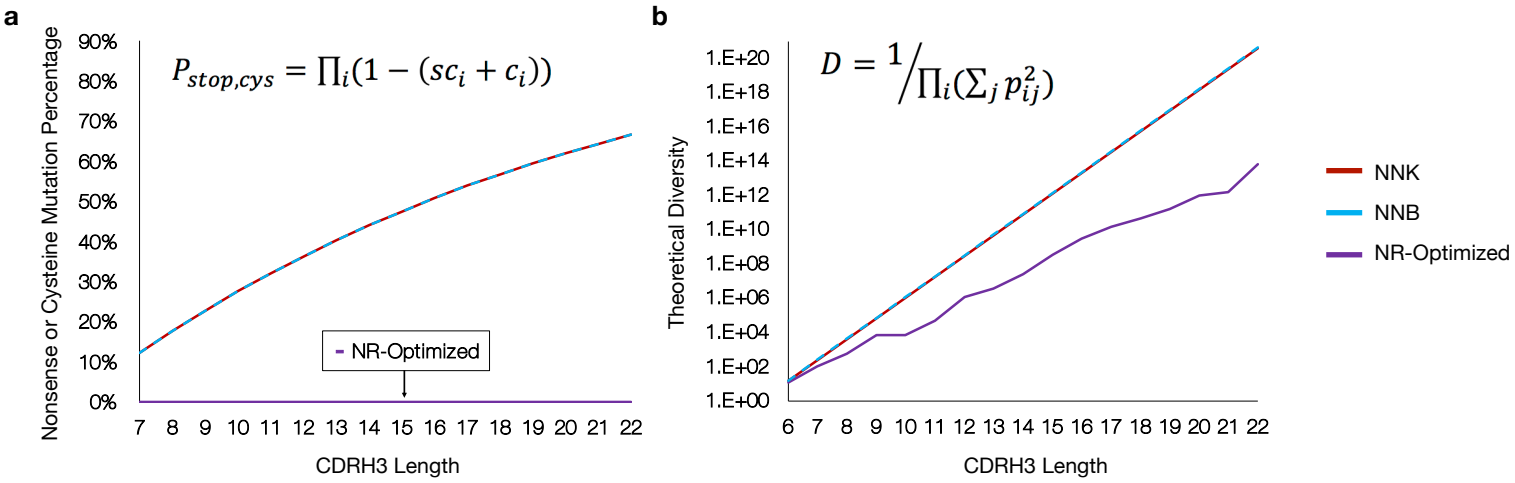
Supplementary Fig. 2: Flow cytometry plots for optimizing HDR parameters

Flow cytometry plots testing HDR integration efficiencies of all ssODN lengths by transfecting gRNA and modified ssODNs into the Cas9-expressing cell line (PnP-HEL23.FI). $2x10^5$ cells were transfected in replicates and cultured for a minimum of 7 days post-transfection. On day 7, cells were labeled for flow cytometry with a fluorescent antibody (AlexaFluor® 488) targeting the constant region of the antibody heavy chain (IgG2c) and a fluorescent antigen (HEL-AlexaFluor® 647). Data presented is representative of 1 of 2 replicates.



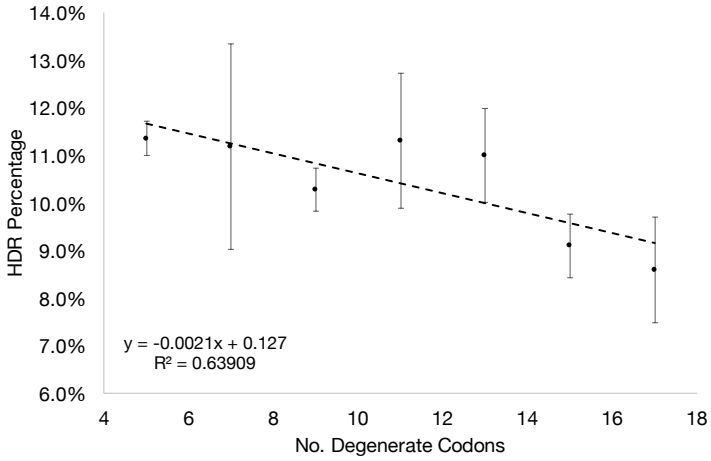
Supplementary Fig. 3: HDR integration efficiencies when scaling up transfection numbers

Bar graphs based on flow cytometry measurements of HDR integration efficiencies after scaling up transfection numbers under optimal parameters (Cas9 cell: PnP-HEL23.FI, ssODN length 120 with PS bonds). Cell counts ranging between 10^6 to 10^7 were transfected by scaling the amount of reagents accordingly up to $5x10^6$ cells (e.g. 10^6 cells, 500 pmol gRNA, 500 pmol ssODN donor). The transfection of 10^7 cells was performed under identical conditions as the transfection for $5x10^6$ cells (e.g. 10^7 cells, 2.5 nmol gRNA, 2.5 nmol ssODN donor). Cell counts were taken 6 hours post-transfection. 3 days post-transfection, cells were labeled for flow cytometry with a fluorescent antibody (AlexaFluor® 488) targeting the constant region of the antibody heavy chain (IgG2c) and a fluorescent antigen (HEL-AlexaFluor® 647).



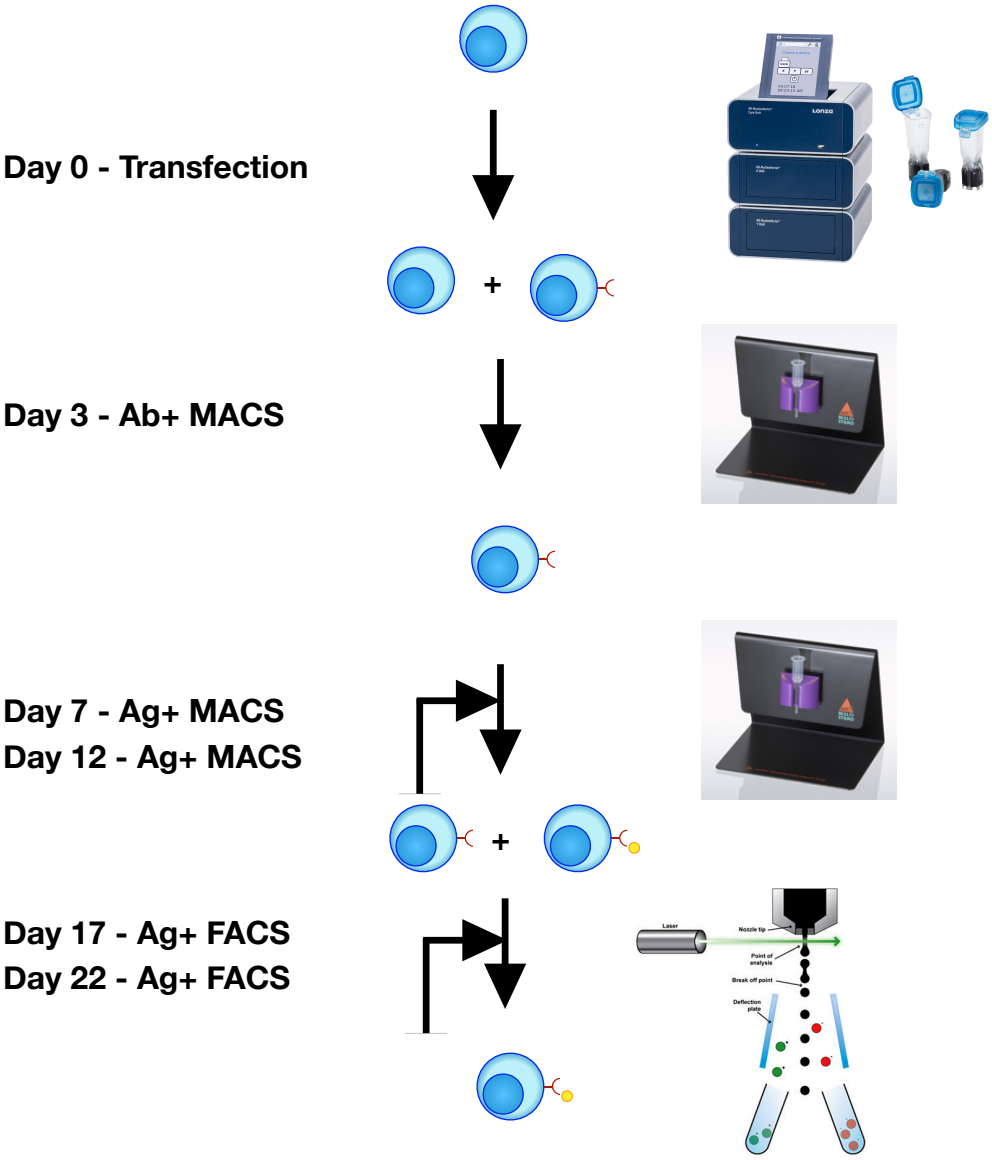
Supplementary Fig. 4: Library design and diversity metrics

a, A comparison between standard randomization schemes, NNK and NNB, and the NRO scheme. For any CDRH3 length (or number of degenerate codons), the NRO scheme has a 0% probability of introducing a nonsense or cysteine mutation. Reducing this probability leads to a higher likelihood of producing a functional antibody sequence following HDM. **b,** Although, there is a reduction in the overall amino acid usage in the NRO scheme, adequate levels of diversity are still maintained, particularly when considering CDRH3 lengths >15, the average length observed in the natural antibody repertoire of mice. Diversity calculations are performed according to the equation above the graph as described by Makowski and Soares (69) where differences in amino acid frequencies are taken into consideration.



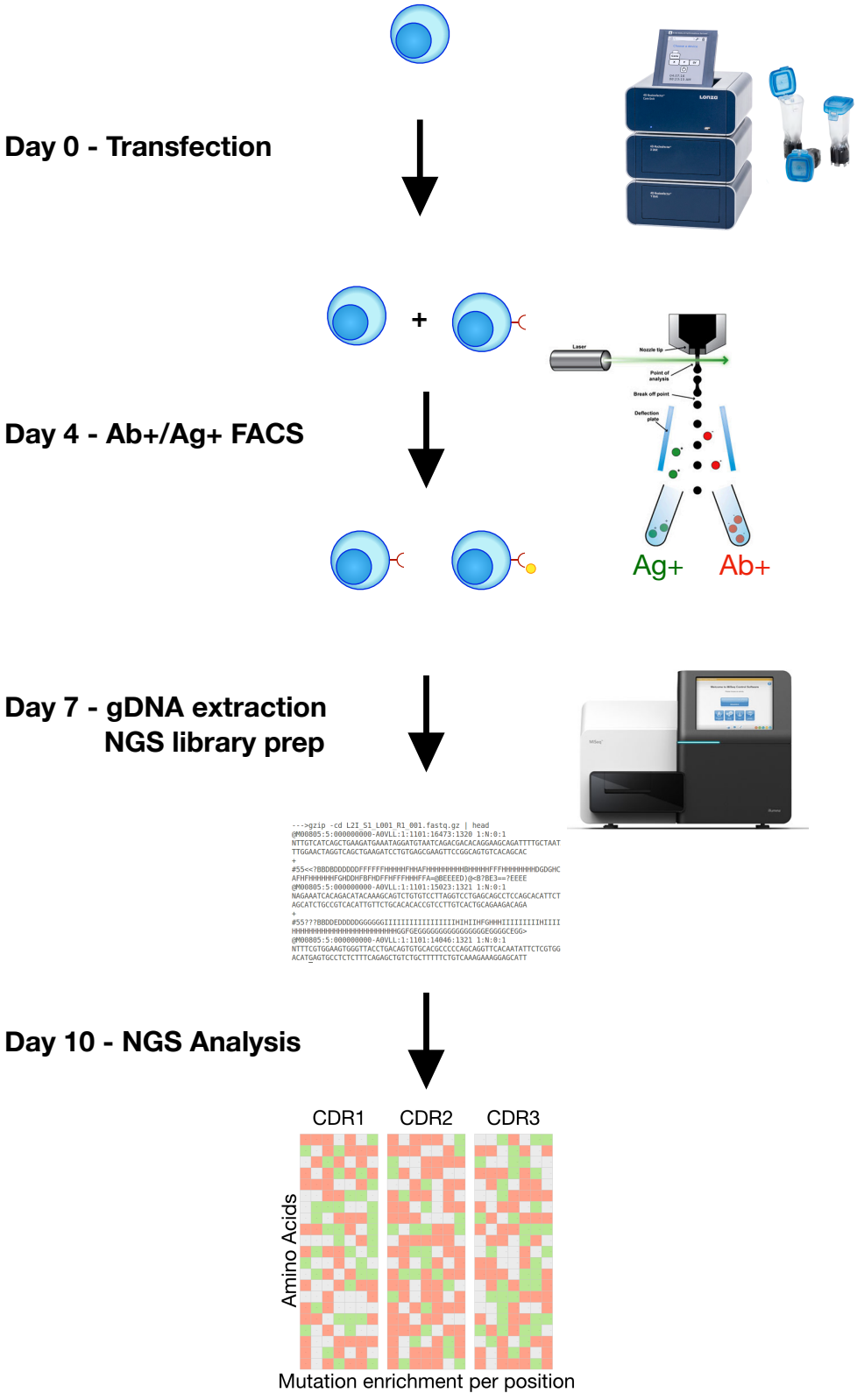
Supplementary Fig. 5: Impact of degeneracy length on HDM efficiency

Flow cytometry results for HDM percentages of ssODN donors containing increasing degeneracy/insertion lengths in order to study its impact on integration efficiencies. $2x10^5$ cells were transfected in replicate and cultured for a minimum of 7 days post-transfection. On day 7, cells were labeled for flow cytometry with a fluorescent antibody (AlexaFluor® 488) targeting the constant region of the antibody heavy chain (IgG2c) and a fluorescent antigen (HEL-AlexaFluor® 647). Data presented (mean±sd) is representative of n = 2.



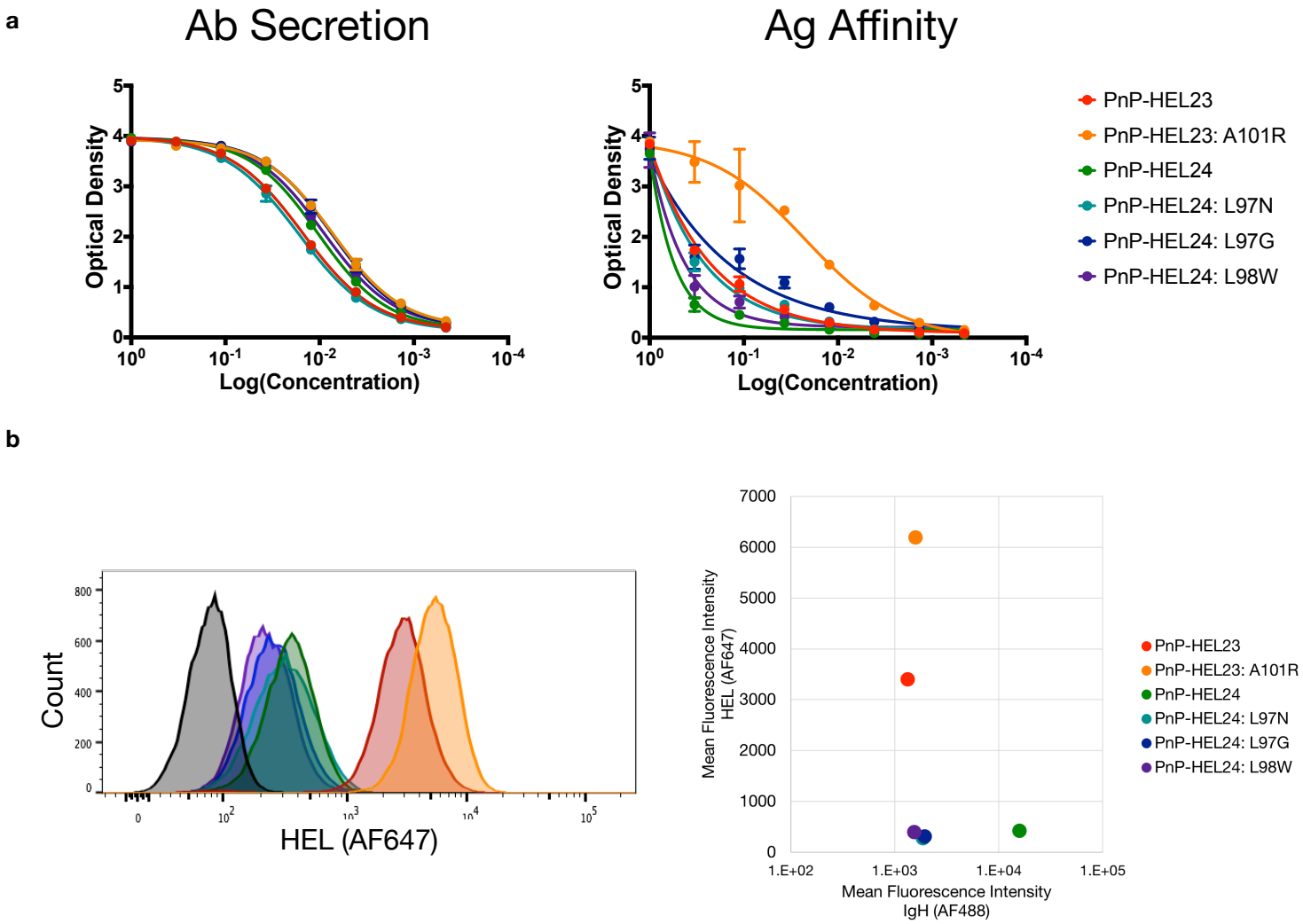
Supplementary Fig. 6: Workflow of library generating for novel antibody discovery by HDM

An example workflow for generating antibody libraries in the PnP-hybridoma cell line and screening it for antigen specific variants by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS). The process workflow can be further optimized by increasing the number of cells subjected to sorting, decreasing the downtime post-sorting where cells require time to recover and proliferate before the subsequent step(s).



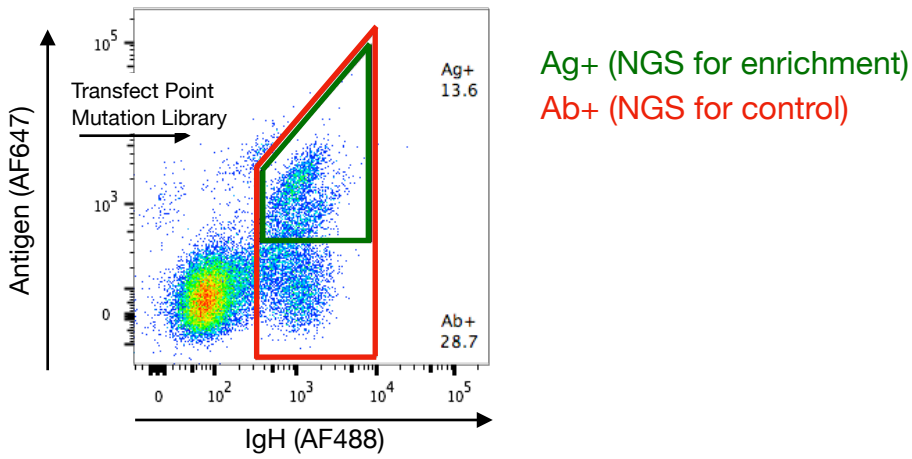
Supplementary Fig. 7: Workflow of library generating for DMS by HDM

An example workflow for generating single-site mutation libraries in the PnP-hybridoma cell line and subjecting it to deep mutational scanning (DMS). The process workflow can be further optimized by increasing the number of cells subjected to sorting, decreasing the downtime post-sorting where cells require time to recover and proliferate before gDNA extraction.



Supplementary Fig. 8: Antibody secretion and antigen affinity measurements

a, ELISA data for antibody secretion and antigen (HEL) affinity for the original CDRH3 sequences (HEL23, HEL24) and the point mutation variants isolated for higher affinity by flow cytometry (**Figure 4c,d**). Similar secretion profiles indicate a comparable amount of secreted full-length IgG, while difference in antigen affinity profiles indicates similar or increased antigen affinity for point mutation variants compared to the parent sequence. **b**, Flow cytometry data for corresponding hybridoma cell lines depicting their relative binding to HEL antigen. The scatter plot displays the mean fluorescences intensities for antigen binding (y-axis) and antibody expression (x-axis).



Supplementary Fig. 9: Flow cytometry gating to perform DMS

Flow cytometry plot displaying an example gating strategy to sort cell populations utilized in DMS experiments. 10^6 cells are transfected with a pool of ssODNs containing point mutations tiling along the entire CDRH3 sequence. Antibody positive (Ab+) cells are isolated as the control library for NGS and antigen positive (Ag+) cells are isolated as the enriched library for NGS. Samples were prepared for sequencing according to the protocol provided in the **Online Methods** section (**Supplementary Table 3**).

Sample	Description	Raw Read count (post-merge)	Aligned Reads	Unique CDRH3s
FI-NNK (1)	Pilot library generated by integrating a NNK degenerate codon into the Frameshift-Indel cell line	577,915	551,591 (95.45%)	13,873
FI-NNK (2)	Pilot library generated by integrating a NNK degenerate codon into the Frameshift-Indel cell line (replicate)	801,603	756,545 (94.38%)	14,842
FI-NNB (1)	Pilot library generated by integrating a NNB degenerate codon into the Frameshift-Indel cell line	847,687	803,802 (94.82%)	13,602
FI-NNB (2)	Pilot library generated by integrating a NNB degenerate codon into the Frameshift-Indel cell line (replicate)	777,070	734,095 (94.47%)	12,773
FS-NNK (1)	Pilot library generated by integrating a NNK degenerate codon into the Frameshift-Stop cell line	822,301	773,378 (94.05%)	10,329
FS-NNK (2)	Pilot library generated by integrating a NNK degenerate codon into the Frameshift-Stop cell line (replicate)	810,508	759,066 (93.65%)	10,100
FS-NNB (1)	Pilot library generated by integrating a NNB degenerate codon into the Frameshift-Stop cell line	780,441	729,344 (93.45%)	8,176
FS-NNB (2)	Pilot library generated by integrating a NNB degenerate codon into the Frameshift-Stop cell line (replicate)	549,813	514,451 (93.57%)	8,054
NR-O Ab+/-	Library generated by transfecting a pool of different length ssODNs containing NR-Optimized codon schemes. Pre-MACS for antibody expressing cells	5,082,357	4,776,253 (93.98%)	99,233
NR-O Ab+	Library generated by transfecting a pool of different length ssODNs containing NR-Optimized codon schemes. Post-MACS for antibody expressing cells	1,444,914	1,364,764 (94.45%)	146,551

Supplementary Table 1: Next generation sequencing (NGS) statistics

NGS statistics for all samples referenced in this study. All sequencing libraries prepared for this study yielded high read counts sufficient for adequate sequencing depth along with a high percentage of alignment (>93%).

Sample	FI-NNK (1)	FI-NNK (2)	FI-NNB (1)	FI-NNB (2)	FS-NNK (1)	FS-NNK (2)	FS-NNB (1)	FS-NNB (2)
FI-NNK (1)	-	0	0	0	0	0	0	0
FI-NNK (2)		-	0	0	0	0	0	0
FI-NNB (1)			-	0	0	0	0	0
FI-NNB (2)				-	0	1	0	0
FS-NNK (1)					-	0	0	0
FS-NNK (2)						-	0	0
FS-NNB (1)							-	0
FS-NNB (2)								-

Supplementary Table 2: Overlap analysis between pilot libraries

Overlap analysis between all 8 NNK/NNB library NGS datasets (**Supplementary Table 1**) indicates that each library generated by HDM produces a unique set of CDRH3 sequences.

Sample	Description	Raw Read count (post-merge)	Aligned Reads	Point Mutation Variants (Observed/Possible)
23-1-Ab	HEL23 CDRH1 DMS - Control Library	655,558	599,806 (91.5%)	109/115
23-1-Ag	HEL23 CDRH1 DMS - Antigen Positive	277.177	251.937 (90.89%)	109/115
23-2-Ab	HEL23 HEL24-3 DMS - Control Library	214,682	202,528 (94.34%)	162/191
23-2-Ag	HEL23 HEL24-3 DMS - Antigen Positive	464,073	427,205 (92.06%)	140/191
23-3-Ab	HEL23 CDRH3 DMS - Control Library	352,683	327,838 (92.96%)	134/134
23-3-Ag	HEL23 CDRH3 DMS - Antigen Positive	784,781	721,275 (91.91%)	130/134
24-1-Ab	HEL24 CDRH1 DMS - Control Library	887,680	843,578 (98.03%)	108/115
24-1-Ag	HEL24 CDRH1 DMS - Antigen Positive	455,792	427,935 (93.89%)	81/115
24-2-Ab	HEL24 HEL24-3 DMS - Control Library	579,301	548,357 (94.66%)	169/191
24-2-Ag	HEL24 HEL24-3 DMS - Antigen Positive	500,914	450,736 (89.98%)	96/191
24-3-Ab	HEL24 CDRH3 DMS - Control Library	622,195	571,173 (91.8%)	190/191
24-3-Ag	HEL24 CDRH3 DMS - Antigen Positive	463,234	427,774 (92.35%)	185/191

Supplementary Table 3: NGS statistics for DMS studies

NGS statistics for all DMS samples referenced in this study. All sequencing libraries prepared for this study yielded high read counts sufficient for adequate sequencing depth along with a high percentage of alignment (>90%).

Cell line	Description
PnP-HEL23	PnP-HEL23 cells are immunogenomically engineered to produce antigen specific antibodies targeting hen egg lysozyme (HEL). The light and heavy chains are expressed from a single transcript containing a 2A, self-cleaving peptide.
PnP-HEL23.FI	PnP-HEL23.FI cells are a monoclonal population containing a random nucleotide insertion in the CDRH3 after targeting PnP-HEL23 with CRISPR/Cas9. The random insertion causes a frameshift mutation leading to dysfunctional antibody expression. Final version of this cell line and subsequent clones includes constitutive Cas9 expression.
PnP-HEL23.FS	PnP-HEL23.FS cells are a monoclonal population containing a designed frameshift mutation sequence. This designed sequence contains in-frame stop codons on both the 5' and 3' sides of the target cleavage site, decreasing the probability of a non-HDR event leading to functional antibody expression. This in turn, results in a more uniformly diverse library. The cell line was generated by knocking in the gRNA target sequence with a ssODN donor by transfecting with a gRNA targeting the CDRH3 of HEL23.FI and then performing a single-cell sort on Ab- cells. Several clones had to be genotyped in order to differentiate between HDR events of the optimized gRNA target sequence and NHEJ/MMEJ events.
PnP-HEL23.FS (53BP1-)	PnP-HEL23.FS (53BP1-) cells are a polyclonal population originating from PnP-HEL23.FS cells. The PnP-HEL23.FS cells were transfected with a gRNA targeting the 53BP1 protein and it was assumed these cells had a high efficiency of gene knockout (T7E1 assay, Supplementary Fig. 1).
PnP-HEL23.A101R	PnP-HEL23.A101R cells contain a point mutation in the CDRH3 compared to the original HEL23 sequencing. The point mutation increases the antibody's affinity for its target antigen, hen egg lysozyme.
PnP-HEL24	PnP-HEL24 cells contain the newly identified CDRH3 sequence after subjecting the generated library to the antibody discovery workflow (Figure 4a). These cells produce antigen specific antibodies targeting hen egg lysozyme.
PnP-HEL24.L98N	PnP-HEL24.L98N cells contain a point mutation in the CDRH3 compared to the original HEL24 sequencing. The point mutation increases the antibody's affinity for its target antigen, hen egg lysozyme.
PnP-HEL24.L98G	PnP-HEL24.L98G cells contain a point mutation in the CDRH3 compared to the original HEL24 sequencing. The point mutation increases the antibody's affinity for its target antigen, hen egg lysozyme.
PnP-HEL24.L99W	PnP-HEL24.L99W cells contain a point mutation in the CDRH3 compared to the original HEL24 sequencing. The point mutation increases the antibody's affinity for its target antigen, hen egg lysozyme.
PnP-mRuby	PnP-mRuby cells are genetically engineered to produce the fluorescent reporter protein, mRuby, from the heavy chain locus of the hybridoma genome.

Supplementary Table 4: Cell lines and descriptions

A summary table providing a brief description of the hybridoma cell lines generated or used in this study.

Description	Sequence
PnP-HEL23 CDRH3 sgRNA target	TGCGCGCGTGATAGCAGCGGCGG
PnP-HEL23.FI CDRH3 sgRNA target	ATTGCGCGCGTGATAGCAGGCGG
PnP-HEL23.FS CDRH3 sgRNA target	TTGTGCACGTTAGAGCCAGGCGG
Hybridoma ROSA26 intron 2 sgRNA target	AAGCATGTATTGCTTTACGTGGG
Hybridoma 53BP1 sgRNA target	GCAGTTGGTGACCACTAACTCGG
PnP-mRuby sgRNA target	GTCATGGAAGGTTCGGTCAACGG
PnP-HEL CDRH1 sgRNA target	GCTATACCTTTAGCAACTATTGG
PnP-HEL CDRH2 sgRNA target	GGCGAAATTCTGCCGGGCAGCGG
PnP-HEL VH forward primer for NGS preparation via primer extension (PCR1)	CCCTCCTTTAATTCCCGAAGTGCAGCTGCAGCAG
PnP-HEL VH reverse primer for NGS preparation via primer extension (PCR1)	GAGGAGAGAGAGAGAGGACCTGGAGAGGCCATTC
TruSeq Universal Adapter forward primer for NGS preparation via primer extension (PCR2)	UNIVERSAL ADAPTER - NNNN CCCTCCTTTAATTCCC
TruSeq Index Adapter reverse primer for NGS preparation via primer extension (PCR2)	ADAPTER INDEX X - NNNN GAGGAGAGAGAGAGAG

Supplementary Table 5: gRNA target and primer sequences

A summary table providing the nucleotide sequences of all relevant primers and gRNA target sites referenced in this study.

Description	Sequence
PnP-HEL23.FS ssODN repair template	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAGTA- CTAAAACTAGCCGCCTGGCTCTA -ACGTGCACAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
HEL23 CDRH3 ssODN repair template, length 60 nt	TGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGC
HEL23 CDRH3 ssODN repair template, length 80 nt	GTCACCAGGGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGCTATCTTCGCT
HEL23 CDRH3 ssODN repair template, length 100 nt	CGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGCTATCTTCGCTGGTCAGGCTG
HEL23 CDRH3 ssODN repair template, length 120 nt	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
HEL23 CDRH3 ssODN repair template, length 140 nt	CTGGAGAGGCCATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCATATACGCGGT
HEL23 CDRH3 ssODN repair template, length 160 nt	AAATAAAGACCTGGAGAGGCCATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA -CAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCATATACGCGGTGTTGCTGCTG
HEL23 CDRH3 ssODN repair template, length 200 nt	AACTCCATAACAAAGGTTAAAAATAAAGACCTGGAGAGGCCATTCTTACCCGCGCTCACGGTCACCAG GGTGCCCTGGCCCCAATA- GGCGAAACCACCCGACGAGTCCCTGGCA CAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCATATACGCGGTGTTGCTGCTGGTA TCCGCGGTAAAGGTCGC
5’ homology arm- CDRH1 sequence -3’ homology arm	GGCCATGGCCCGGACGCTGTTTCACCCAGCCAATCCA- ATAGTTTGAAAAGGTATA -GCCGGTCGCTTTGCAGCTAATTTTC
Example sequence for single-site mutagenesis. MNN sequence is tiled along the entire CDRH sequence	GGCCATGGCCCGGACGCTGTTTCACCCAGCCAATCCA- **MNN**GTTTGAAAAGGTATA -GCCGGTCGCTTTGCAGCTAATTTTC
5’ homology arm- CDRH2 sequence -3’ homology arm	CTTTGCCTTTAAATTTTTCGTTATA- GTTGGTGCTGCCGGAACCCGGCAGAATTTC -GCCAATCCATTCCAGGCCATGGCCCG
Example sequence for single-site mutagenesis. MNN sequence is tiled along the entire CDRH sequence	CTTTGCCTTTAAATTTTTCGTTATA- **MNN**GGTGCTGCCGGAACCCGGCAGAATTTC -GCCAATCCATTCCAGGCCATGGCCCG
5’ homology arm- HEL23 CDRH3 sequence -3’ homology arm	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- CGCAAAGCCGCCGCTGCTATC -ACGCGCGCAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
Example sequence for single-site mutagenesis. MNN sequence is tiled along the entire CDRH sequence	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAATA- **MNN**AAAGCCGCCGCTGCTATC -ACGCGCGCAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
5’ homology arm- HEL24 CDRH3 sequence -3’ homology arm	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAGTA- GTCAAAGGCGTCGAAGTAGAGGAGAATGGT -ACGTGCACAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
Example sequence for single-site mutagenesis. MNN sequence is tiled along the entire CDRH sequence	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAGTA- **MNN**AAAGGCGTCGAAGTAGAGGAGAATGGT -ACGTGCACAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA

Description	Sequence
5’ homology arm- Replacement sequence -3’ homology arm	CATTCTTACCCGCGCTCACGGTCACCAGGGTGCCCTGGCCCCAGTA- $(XXX)_X$ -ACGTGCACAATAATACACCGCGCTATCTTCGCTGGTCAGGCTGCTCAGCTGCA
CDRH3 length 14 a.a. ssODN repair template, NNK scheme	5’HA- MNNMNNMNNMNNMNNMNNMNNMNNMNN -3’HA
CDRH3 length 14 a.a. ssODN repair template, NNB scheme	5’HA- VNNVNNVNNVNNVNNVNNVNNVNN -3’HA
CDRH3 length 10 a.a. ssODN repair template, NRO scheme	5’HA- GKCVAWVNBVNBVNB -3’HA
CDRH3 length 12 a.a. ssODN repair template, NRO scheme	5’HA- GKCDAAGDVVNBVNBVNBVNB -3’HA
CDRH3 length 14 a.a. ssODN repair template, NRO scheme	5’HA- GKCDAAGKMGDNGDNGDNGDNKYYVNB -3’HA
CDRH3 length 15 a.a. ssODN repair template, NRO scheme	5’HA- GTCDAAGKMGDNGDNGDNGDNGDNVNBVNB -3’HA
CDRH3 length 16 a.a. ssODN repair template, NRO scheme	5’HA- GTCDAAGKMGKNGDVGDNVHYGDHGDNVNBVNB- 3’HA
CDRH3 length 18 a.a. ssODN repair template, NRO scheme	5’HA- GTCVAWGKMGKNGDVGDVVHYVHYGKHGKHGDNNNBVNB -3’HA
CDRH3 length 20 a.a. ssODN repair template, NRO scheme	5’HA- GTCSAWGKMGKMGKVGDNGVBVHYVHYGDHGKHGDDGDNBYYDYB -3’HA
CDRH3 length 22 a.a. ssODN repair template, NRO scheme	5’HA- GTCSAWKKCGTAGTMGDNVNBVNYVNYVNYGDHGDHGDDGDNDNBBYYDYB -3’HA

Supplementary Table 6: ssODN donor sequences

A summary table providing the nucleotide sequences of all relevant ssODN donors referenced in this study.

a

Target/ Antigen	Working conc.	Dilution from stock	Incubation volume	Fluorophore	Product ID
IgG2c	12 µg/ml	1:100	100 µl	AlexaFluor® 488	115-545-208 (Jackson ImmunoResearch)
IgK	2.5 µg/ml	1:80	100 µl	Brilliant Violet 421™	409511 (BioLegend)
Hen egg lysozyme (HEL)	0.99 µg/ml	1:100	100 µl	AlexaFluor® 647	62971-10G-F (Sigma-Aldrich)

B

Flow Cytometry Round	Dilution from stock	HEL conc.	Incubation volume
1	1:100	0.99 µg/ml	1000 µl
2	1:150	0.67 µg/ml	1000 µl
3	1:200	0.50 µg/ml	1000 µl

Supplementary Table 7: Flow cytometry labeling concentrations

a, A summary table providing information on the fluorescently labeled antibodies and antigens referenced in this study along with their working concentrations. **b**, A summary table providing concentrations used for antigen labeling following subsequent rounds of FACS enrichment to screen for increased affinity variants.