Supporting Information

Chemical diversification of simple synthetic antibodies

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Supplementary Methods

Materials. The *Saccharomyces cerevisiae* strain RJY100 was constructed as described previously.[1] The ncAA *p*-azido-L-phenylalanine was purchased from Chem-Impex International, Inc., *p*-propargyloxyphenylalanine and H-L-Lys(EO-N3)-OH*HCl were purchased from Iris Biotech GmbH and *p*-azidomethyl-L-phenylalanine purchased from SynChem, Inc. All restriction enzymes used for cloning were from New England Biolabs. Primary and secondary antibodies used for flow cytometry labeling were purchased from Gallus Immunotech (chicken anti-cMyc), BioLegend (mouse anti-HA), Thermo Fisher Scientific (goat anti-chicken Alexa Fluor 647, goat anti-mouse Alexa Fluor 488, streptavidin Alexa Fluor 488 and rabbit anti-donkey DyLight 488), Jackson ImmunoResearch (goat anti-bovine Alexa Fluor 488) and eBioscience (anti-biotin PE). All PCR amplifications were performed with New England Biolabs Q5 DNA polymerase. Synthetic oligonucleotides for cloning and sequencing were purchased from Eurofins Genomics or IDT DNA Technologies. gBlocks encoding the heavy and light chain frameworks used for library construction were synthesized by IDT DNA Technologies. All sequencing in this work was performed by Eurofins Genomics (Louisville, KY) or Quintara Biosciences (Cambridge, MA). Mix and Go! kits from Zymo Research were used to prepare competent *E. coli*. Epoch Life Science GenCatch™ Plasmid DNA Mini-Prep Kits and Macherey-Nagel NucleoBond Xtra Maxi Plus EF kits were used to isolate plasmid DNA from *E. coli*. Frozen-EZ Yeast Transformation II kits and Zymoprep DNA isolation kit from Zymo Research were used to prepare and transform competent yeast, and isolate plasmid DNA from yeast, respectively. Dynabeads™ Biotin Binder from Thermo Fisher Scientific were used for magnetic bead sorting. Penicillin-Streptomycin 100X from Corning was used during propagation of yeast cultures. Chrompure Donkey IgG whole molecule, Chrompure Bovine IgG whole molecule and Chrompure Rabbit IgG whole molecule were purchased in unconjugated forms and in Biotin-SP conjugated forms from Jackson ImmunoResearch. EZ-Link™ NHS-LC-Biotin, Zeba™ Spin Desalting Columns, 7K MWCO, 0.5 mL and Pierce™ Biotin Quantitation Kits were purchased from Thermo Scientific and used for antigen biotinylation. Biotin-PEG_3-Azide, Biotin-PEG_4-Alkyne, Biotin-PEG_4-DBCO and THPTA were purchased from Click Chemistry Tools for use in CuAAC and SPAAC reactions. (+)-Sodium

L-ascorbate, aminoguanidine hydrochloride, copper sulfate pentahydrate, EDTA, dimethylformamide and DMSO were purchased from Sigma Aldrich for use in CuAAC reactions. 4–12% Bis-Tris mini gels, SimplyBlue Safestain and iBlot™ Transfer Stacks were purchased from Thermo Scientific and used for SDS-PAGE and Western Blot analysis.

Media preparation. LB, SD-CAA, SG-CAA, and YPG media were prepared as described in Van Deventer et al.[1, 2] SD-SCAA and SG-SCAA were prepared as described in Stieglitz et al.[3] All SD-SCAA and SG-SCAA media used in this study was prepared without tryptophan, leucine and uracil (–TRP, –LEU, and –URA).

Noncanonical amino acid liquid stock preparation. For all experiments, stocks of noncanonical amino acids were prepared at 50 mM concentration of the L-isomer. NcAAs were dissolved in approximately 90% of the final desired volume of deionized water and vortexed thoroughly. Either 6 N or 1 N sodium hydroxide was used to ensure complete dissolving when needed. Once the stocks were determined to be fully dissolved, deionized water was added to the final volume needed to reach a concentration of 50 mM L-isomer and the stocks were sterile filtered using a 2 µm syringe filter. Stocks were prepared fresh prior to each induction.

Yeast transformations, propagation and induction. For display constructs encoding proteins containing only canonical amino acids, display plasmids (pCTCON2 backbone, TRP marker) were transformed into Zymo competent RJY100 cells, plated on SD-CAA (–TRP –URA) solid media and allowed to grow for 2–3 days at 30 °C, until colonies appeared. For display constructs encoding a TAG codon at L93, H31 and H54 positions, display constructs (pCTCON2 backbone, TRP marker) were transformed alongside aaRS/tRNA suppressor plasmids (pRS315 backbone for LeuOmeRS and LeuRS BH5 T252A, LEU marker; kanamycin resistance modified pRS315 backbone for AcFRS, LEU marker) using Zymo competent RJY100 cells, plated on SD-SCAA –TRP –LEU –URA solid media and allowed to grow for 2–3 days at 30 °C, until colonies appeared. The details of display plasmid and aaRS/tRNA plasmid combinations used in this study are listed in Supplementary Table S7. For propagation, colonies were inoculated in 2–5 mL of the respective liquid media supplemented with penicillin-streptomycin at 1:100 dilution (+ Pen/Strep) and left to grow at 30 °C with shaking at 250–300 RPM until saturation (2–3 days). Saturated cultures were stored at 4 °C for future propagation. To propagate, 100–500 µL of cells from the saturated culture were pelleted and resuspended to an OD600 of 0.5–1 in 5 mL of fresh liquid media + Pen/Strep and grown overnight at 30 °C with shaking at 250–300 RPM. Once saturated, cultures were diluted to an OD600 of 1 and allowed to grow to mid-log phase (OD600 = 2–5; 4–8 hours) and then induced by pelleting and resuspending in the corresponding SG-SCAA media + Pen/Strep to an OD of 1. SG-CAA was used for cultures containing reporter construct plasmids only, SG-SCAA –TRP –LEU –URA was used for cultures containing reporter construct plasmids as well as aaRS/tRNA suppression machinery. To facilitate ncAA incorporation, induction media for cultures with TAG-mutated reporter plasmids were supplemented with ncAAs to a final concentration of 1 mM of the L-isomer (ncAAs used in different experiments are specified in later sections below). Cells were grown in the induction media at 20 °C with shaking at 250–300 RPM for 15–18 hours before further experimentation.

CDR-H3 library construction and sequence characterization. Oligonucleotides were used to amplify the scFv region, which was split into two regions. The gBlock encoding the first region

consisted of the IGKV1-39 light chain and the JK4 J-segment and was amplified with a pCTCON2 overlap region using the primers SynAmpFwd and SidLinkRev (Supplementary Table S3). For the case of the light chain, a DNA sequence encoding the entire IGKV1-39 amino acid sequence was used followed by a sequence encoding the "TFGGGTKVEIK" sequence from JK4. The peptide linker connecting the light and heavy chain variable regions was identical to the linker sequence used in Van Deventer et al[1] and encoded within the SidLinkRev and SidLinkFwd primers. The gBlock encoding the second region contained the sequence for the IGHV3-23 VH region and was amplified with SidLinkFwd and the CDR9–17Rev primers. The CDR9–17Rev primers varied in length according to the number of designed "XYZ" codons encoded in each primer (labeled as the reverse complement z'y'x' in the primer sequence). Additionally, the CDR9–17Rev primers contained an overhang for the first part of the JH4 J-segment sequence. The rest of the JH4 J-segment and the pCTCON2 overlap region were incorporated using SynAmpRev. PCR amplifications of each CDR length were kept separate throughout molecular cloning and yeast electroporation.

The pCTCON2 yeast display vector was linearized based on the method described by Van Deventer and Wittrup 2014.[2] Approximately 30 µg of vector was digested overnight at 37 °C with 9 µL SalI, 30 µL 10× CutSmart Buffer, and 250 µL deionized water. The next day, 9 µL each of BamHI and NheI were added to the reaction and digested overnight at 37 °C. The next morning, 1.5 µL of each enzyme were added to the mix for 1 hour before gel purification. 2 µg of each gel-purified PCR fragment and 1 µg of digested vector mixture were assembled by homologous recombination in electrocompetent RJY100 using previously described methods.[2] PCR fragments of each CDR-H3 length were electroporated in yeast separately to create loop length-based sublibraries. Transformed yeast cultures for each loop length were recovered in SD-CAA (–TRP –URA) + Pen/Strep. Small portions of transformed cells were plated on SD-CAA plates and counted to determine the size of each sublibrary (Table 1). To cryogenically preserve the sublibraries, cultures were expanded in SD-CAA + Pen/Strep, pelleted, resuspended in 15% glycerol and stored in 2 mL aliquots each containing 10^{10} yeast cells. Plasmid DNA was extracted from each sublibrary using the Zymoprep DNA isolation kit. DNA was then transformed into *E. coli* and plated on LB solid media containing ampicillin. The 8–12 *E. coli* colonies per sublibrary were miniprepped and sequenced (Eurofins Genomics) to assess library diversity.

To prepare the full library from the sublibraries, freezer stocks of each sublibrary were thawed at room temperature and a number of cells equal to 20 times the number of transformants were inoculated in 1 L SD-CAA + Pen/Strep at 30 °C. These cells were grown to saturation to create the full CDR-H3 library. Once grown to saturation, 10 times the number of transformants were spun down for 5 minutes at 2,000 rcf and 4 °C, resuspended in 8 L SD-CAA + Pen/Strep, and grown to saturation overnight. The saturated cultures were spun down, resuspended in 15% glycerol, and stored in 2 mL aliquots each containing 10^{10} yeast cells at –80 °C.

Antigen biotinylation. Donkey, bovine and rabbit IgG (Jackson Immuno-Research), and TA99 (prepared according to Zhu et al[4]) were diluted from stock concentration to 1 mg mL^{-1} in ice cold 1× PBS. 100 mM EZ-Link NHS-LC biotin solution was prepared in dimethylformamide and mixed extensively to dissolve. Biotinylation reactions were carried out by incubating 100 µL aliquots of protein with 2–6 fold molar excess of 100 mM EZ-Link NHS-LC biotin solution for IgGs (including TA99) at room temperature for 30 minutes. Reactions were quenched with 10 µL of 0.5 M Tris,

0.02% NaN_3, pH 7.4. Upon completion of the reaction, proteins were desalted twice using Zeba Spin Desalting Columns with 7K MW cutoff according to the manufacturer's specifications (Thermo Scientific, catalog number 89883). The concentration of the resulting protein was measured *via* the absorbance of 280 nm light on a Nano Drop One instrument (Thermo Fisher). The extent of biotinylation was estimated using the HABA-Avidin Premix according to the manufacturer's protocol (Thermo Scientific, catalog number 28005). Clear flat-bottomed 96 well plates were used to blank samples by shaking at 150 RPM for one minute, reading absorbance values at 500 nm and repeating with one-minute shaking intervals until readings were consistent within ± 0.003 AU. Absorbance measurements were similarly recorded after the addition of biotinylated protein until consistent. The extent of biotinylation (moles of biotin per moles of protein) was calculated from the absorbance readings using the online HABA Calculator by Thermo Scientific, based on the Beer Lambert Law (Thermo Scientific, catalog number 28005).

Flow cytometry data collection and analysis. Analytical flow cytometry was performed as described in previous work.[3] To prepare scFvs for flow cytometry analysis, 2 million freshly induced cells of each sample were removed from the culture, washed three times in PBSA (1× PBS, pH 7.4, with 0.1% w/v BSA) and labeled in either 1.7 mL microcentrifuge tubes or 96 well V-bottom plates for flow cytometry. Primary labeling was carried out at room temperature for 30 minutes on a rotary wheel (for microcentrifuge tubes) or on an orbital shaker at 150 RPM (for 96-well plates). Following primary labeling, all subsequent steps were performed on ice or in a centrifuge chilled to 4 °C. Cells were diluted in ice-cold PBSA and washed twice, also with ice-cold PBSA. Secondary labelling was performed on ice for 15 minutes in the dark. Samples were diluted in and washed once with ice-cold PBSA before final resuspension in PBSA for flow cytometry. The labeling reagents and concentrations used for primary and secondary labelling were dependent on the type of experiment and are listed in Supplementary Tables S2, S5–S6 and S8–S9, S11–S15; labeling reagents were prepared in PBSA unless otherwise noted. Flow cytometry was performed on an Attune NxT flow cytometer (Life Technologies) in the Tufts University Science and Technology Center and data was processed using FlowJo™ software Version 10.6.1. Unless otherwise stated, 10,000 events were collected per sample on the flow cytometer.

To prepare sublibraries for flow cytometry characterizations, a volume of culture containing 20 times the number of transformants of each sublibrary was grown separately in either 100 or 150 mL of SD-CAA (–TRP –URA) + Pen/Strep at 30 °C with shaking at 300 RPM (150 mL was used to ensure sufficient coverage of sublibrary CDR14, containing 2.3×10^8 transformants). Once grown to saturation, a volume of cells corresponding to 10 times the number of transformants for each sublibrary was spun down (5 minutes at 2,000 rcf at 4 °C) and resuspended in 100 or 150 mL of SD-CAA + Pen/Strep; based on number of transformants in the sublibrary. After growing the cultures to saturation again, approximately 500 µL of each sublibrary was passaged into 5 mL of SD-CAA + Pen/Strep and grown to mid-log phase for 4–8 hours at 30 °C with shaking at 250–300 RPM. Additionally, 500 µL of the full CDR-H3 library was passaged into 5 mL of SD-CAA + Pen/Strep and grown for 4–8 hours at 30 °C with shaking (250–300 RPM). For induction, cultures were spun down and resuspended at an OD600 of 1 in SG-CAA + Pen/Strep and grown for approximately 16 hours at 20 °C with shaking (250–300 RPM).

Data analysis was performed using FlowJo and Microsoft Excel following these general steps. The data was 1) gated to isolate the single cell populations, 2) gated to isolate induced populations (full-length display events with positive c-Myc detection), 3) median fluorescence intensity (MFI) data was retrieved for c-Myc detection of induced populations and uninduced populations (truncated or no protein display), 4) MFI data of induced and uninduced populations was retrieved for other parameters being detected, and 5) background correction was performed by subtracting MFI values of uninduced cell populations from MFI values of corresponding induced populations. Additionally, quadrants were drawn on dot plots based on the putatively uninduced populations of cells in order to identify positive and negative populations. In most cases, quadrants prepared in this way result in c-Myc-positive events in Q2 and Q3, events positive for a second parameter in Q1 and Q2, and background level detection of each parameter in Q4 (corresponding to uninduced or unlabeled populations).

Truncation analysis samples were gated for 1) the induced full-length population and 2) the induced truncated populations. The full-length induced population was defined as exhibiting fluorescence indicating both HA and c-Myc detection whereas the truncated population was defined as exhibiting fluorescence indicating only HA detection. To obtain the percentage of truncated clones, the percent of the truncated induced population was divided by the total percent of induced population for each CDR sublibrary and the full CDR-H3 library.

Library sorting. Two aliquots of the full, frozen CDR-H3 library were thawed at room temperature and inoculated into a 1 L SD-CAA (–TRP –URA) + Pen/Strep culture. The culture was grown to saturation at 30 °C with shaking (250–300 RPM). The library was spun down and resuspended at an OD600 of 1 and grown again to saturation. The remainder of the culture was placed at 4 °C as a short-term stock. Alternatively, stocks saved at 4 °C were pelleted and resuspended to an OD600 of 1 in 1 L of fresh SD-CAA + Pen/Strep and grown to saturation with shaking (250–300 RPM). Following saturation, the cultures were pelleted and resuspended to an OD600 of 1 in fresh media and grown until mid-log phase (OD600 between 2–5; 4–8 hours). Cells were pelleted (5 minutes at 2,000 rcf and 4 °C) and resuspended to an OD600 of 1 in SG-CAA + Pen/Strep induction media and grown at 20 °C for at least 16 hours with shaking (250–300 RPM) before sorting.

150–250 µL of Dynabeads Biotin Binder were prepared by washing three times in 1 mL ice-cold PBSA. Washes were done by incubation on a DynaMag2 (Thermo Fisher Scientific) for 2 minutes followed by aspiration of supernatant. After washing, the beads were resuspended to the original 150–250 µL volume in ice-cold PBSA. Beads were aliquoted in 50 µL volumes and left alone or incubated with biotinylated TA99 (33 pmol antigen/10 µL beads) or 5.3 µL of 0.01 mg mL^{-1} D-biotin stock solution on a rotary wheel at 4 °C for 2–16 hours.

For the first round of negative sorting, 1.5×10^{10} induced cells were pelleted, washed and resuspended in 5 mL ice-cold PBSA. For each sort, prepared beads were washed and added to the cells, then placed at 4 °C on a rotary wheel for 2 hours. After this incubation, the cells were distributed evenly into five 1.7 mL microcentrifuge tubes and placed on the DynaMag for 5 minutes. After this time, the supernatants were transferred to a new conical tube. This was repeated for each negative sort against the beads, TA99, and biotin. After the final sort, the remaining cells were rescued in 1 L SD-CAA + Pen/Strep for overnight growth until saturation.

Then, cells were induced, beads were prepared, and sorts were performed a second time exactly as stated above.

After the second negative sort, cells were rescued overnight and induced as stated above. After overnight induction, cells were pelleted, washed, and resuspended in 5 mL PBSA as stated above, with beads also washed as stated above. Beads were prepared for positive enrichments by incubating them with either biotinylated donkey, bovine, or rabbit IgG (33 pmol antigen/10 µL beads) on a rotary wheel at 4 °C for 2–16 hours. 50 µL of antigen-coated beads were added to the suspension culture and placed on a rotary wheel at 4 °C for 2 hours. Then the cells were incubated on the DynaMag for 5 minutes and the supernatant was transferred to new tube on the DynaMag to ensure no loss of beads. Remaining beads were washed once in 1 mL ice-cold PBSA and supernatant was discarded after 2 minutes of incubation. Beads and attached cells were rescued in 5 mL SD-CAA + Pen/Strep at 30 °C overnight. The next morning, cells were passaged into 50 mL SD-CAA + Pen/Strep and grown until about OD 3, when cells were pelleted and resuspended in 1 mL induction media. Cells were incubated on the DynaMag to remove beads and then grown in 50 mL induction media for overnight growth at 20 °C to prepare for the fourth round of sorting.

For the fourth round of sorting, 10 µL Dynabeads were prepared with the biotinylated antigens needed for negative and positive selections. After overnight growth, cells were pelleted, washed and resuspended in 1 mL ice-cold PBSA. Cells were subjected to depletions against the Dynabeads, biotin, and TA99 by incubating the cells with beads for 2 hours on a rotary wheel at 4 °C. Cells were then placed on the DynaMag for 5 minutes and supernatants were transferred to a new conical tube for the next sort. For the final positive sort on IgG, the cells were incubated on the DynaMag for 5 minutes and supernatant was removed. The supernatant was transferred to new tube on the DynaMag to ensure no loss of beads. Remaining beads were washed once in 1 mL ice-cold PBSA and supernatant was discarded after 2 minutes of incubation. Cells were rescued in 5 mL SD-CAA + Pen/Strep and grown overnight. This process was repeated two or three times.

Following bead-based enrichments, each of these populations were sorted using fluorescence-activated cell sorting (FACS) performed at the Tufts Laser Cytometry Core on a BD FACSAria following four (donkey IgG), three to four (bovine IgG) or four (rabbit IgG) rounds of bead sorting. To prepare samples for sorting, 2×10^7 cells from each population were pelleted and resuspended in PBSA. During primary labeling, cells were labeled with chicken anti-cMyc at a dilution of 1:250 in PBSA and 50 nM biotinylated antigen. During secondary labeling, cells were labeled with goat anti-chicken Alexa Fluor 647 at a dilution of 1:500 in PBSA and anti-biotin PE added to a dilution of 1:500 in PBSA. For each sort, approximately 50,000 to 1 million events were sorted by FACS and approximately 300–8,000 c-Myc-positive, antigen binding-positive events were collected. Sorted cells were rescued in 3 mL SD-CAA + Pen/Strep at 30 °C with shaking at 300 RPM; populations were grown to saturation prior to subsequent evaluations.

Analytical flow cytometry was used to evaluate the binding properties of enriched populations. Cells from each population were induced and prepared for flow cytometry as described in the section, “Flow cytometry data collection and analysis”. Briefly, for each round of sorting after the first round of positive enrichment, induced cells were evaluated for binding to the

desired antigen and streptavidin by flow cytometry. Cells were subjected to the same wash conditions as in “Flow cytometry data collection and analysis”. Samples were labeled with 50 nM biotinylated antigen and chicken anti-cMyc at a 1:250 dilution in PBSA. For secondary labeling, cells were labeled with goat anti-chicken Alexa Fluor 647 and either streptavidin Alexa Fluor 488 or anti-biotin PE at a 1:500 dilution.

Binder characterizations. Plasmid DNA was isolated from enriched populations sorted against donkey, bovine and rabbit IgG and transformed into *E. coli* to prepare single clones for sequence analysis. 9–12 clones were sequenced from each of the sorted donkey, bovine and rabbit IgG populations. Unique clones were transformed into RJY100, plated on solid SD-CAA (–TRP –URA) media, and grown at 30 °C until colonies appeared (2–3 days). Colonies were inoculated in 5 mL of SD-CAA + Pen/Strep and allowed to grow to saturation at 30 °C with shaking (250–300 RPM) for 2–3 days. Alternatively, 100–200 µL of saturated culture stored at 4 °C was inoculated in 5 mL fresh media and allowed to grow to saturation overnight. Following saturation, the cultures were diluted to an OD600 of 1 in fresh SD-CAA + Pen/Strep and grown at 30 °C until mid-log phase (OD 2–5; 4–8 hours). Cells were pelleted (5 minutes at 2,000 rcf and 4 °C) and resuspended to an OD600 of 1 in SG-CAA. Cells were prepared for flow cytometry analysis as described in the section “Flow cytometry data collection and analysis,” labelling for biotinylated IgG (using either 50 nM or 250 nM biotinylated antigen) and c-Myc according to Supplementary Table S2 (secondary antibodies were used in a 1:500 dilution).

Biotinylated antigen competition. Biotin competition assays were used to determine if clones preferentially recognize biotinylated antigen over unconjugated antigen.[5] To conduct these experiments, induced RJY100 cells displaying unique scFvs isolated from the CDR-H3 library (Supplementary Table S5) were washed three times in PBSA, and first labeled with 1 or 5 µM unconjugated antigen and chicken anti-cMyc at a 1:500 dilution in PBSA. Cells were incubated for 30 minutes at room temperature on an orbital shaker at 150 RPM. After incubation, the plate was kept on ice or at 4 °C for the remainder of the experiment. Biotinylated antigen was added to a final concentration of 50 or 250 nM (for the initial unmodified antigen concentrations of 1 or 5 µM, respectively). Cells were mixed and incubated on an orbital shaker at 150 RPM for 15 minutes at 4 °C. After the incubation, cells were diluted in 200 µL ice cold PBSA and washed three times at 4 °C. Secondary labeling and subsequent data collected was performed as outlined in “Flow cytometry data collection and analysis” and Supplementary Tables S2 and S5.

Introduction of TAG codons into scFvs. Primers encoding a TAG codon at L93, H31 or H54 positions (Supplementary Table S3) were used to introduce mutations to the Donkey1.1, Bovine2.1, Bovine2.2 and Rabbit1.1 scFvs. Two DNA segments were amplified *via* PCR for each scFv, one with the pCTCON2Fwd + ###TAGRev and one with ###TAGFwd + pCTCON2Rev primers (### refers to the mutation sites L93, H31 or H54) to introduce the mutation at the desired position. Gel electrophoresis was performed on the PCR products to extract DNA fragments of the expected insert size, and then recombined with a pCTCON2 vector digested at the NheI and BamHI restriction sites using Gibson Assembly. The assemblies were transformed into chemically competent *E. coli* DH5αZ1, plated on LB plates containing ampicillin and grown at 37 °C overnight. Resulting colonies were picked and grown to saturation in 5 mL LB liquid media containing ampicillin. Plasmid DNA was then isolated from these cultures using GenCatch Plasmid DNA

Mini-Prep kits. The cloned plasmids were digested at the NheI and BamHI sites to confirm the presence of correctly sized inserts by performing gel electrophoresis. Finally, the plasmids were sequence verified from Quintara Biosciences.

RRE and MMF calculations. For relative readthrough efficiency (RRE) and maximum misincorporation frequency (MMF) calculations, all display plasmids (with or without TAG codon mutations) were transformed along with aaRS/tRNA suppressor plasmids using Zymo-competent RJY100 cells and plated on SD-SCAA –TRP –LEU –URA solid media. Colonies were grown in SD-SCAA –TRP –LEU –URA + Pen/Strep liquid media as described above, and induced in SG-SCAA –TRP –LEU –URA + Pen/Strep both in the presence of 1 mM ncAA as well as in the absence of ncAA. Three separate transformants per antibody clone + aaRS/tRNA combination were used to generate the data for RRE and MMF measurements. Data analysis was performed as described previously by Stieglitz et al[3] and Potts et al.[6]

Binding affinity (K_D) data collection and analysis. 1.5 million freshly induced RJY100 cells displaying scFvs were aliquoted in 1.7 mL microcentrifuge tubes, pelleted, washed three times with 1× PBSA, and then resuspended in 1 mL PBSA. 300 µL of the washed cells were then combined with 0.3 µL of chicken anti-cMyc, vortexing gently to mix. To prepare for binding titration, donkey, bovine or rabbit IgG was added to wells of 96-well V-bottom plates starting at 1 µM with seven subsequent 4-fold dilutions and a final PBSA blank (no IgG). To each of these 9 IgG concentrations, 10 µL of the cells incubated with chicken anti-cMyc were added, resulting in approximately 15,000 cells per sample, and incubated on the orbital shaker at 150 RPM at room temperature overnight (final chicken anti-cMyc dilution of 1:1000). Wild-type Donkey1.1 and its ncAA-containing variants (at L93, H31 and H54 positions) were added to wells containing different concentrations of biotinylated donkey IgG; wild-type Bovine2.2 and its ncAA-containing variants, wild-type Bovine2.1 and wild-type Bovine2.3 were added to biotinylated bovine IgG; and finally, wild-type Rabbit1.1 was added to biotinylated rabbit IgG. After the overnight incubation with chicken anti-cMyc, samples were diluted in and washed 3–5 times with ice-cold PBSA. Secondary labeling and flow cytometry data collection was performed as described for scFvs in the "Flow cytometry data collection and analysis" above, with the exception that 3,000 events were collected per sample instead of the usual 10,000 events. Reagents used for secondary labeling are listed in Supplementary Table S11. All titrations were done in technical triplicates.

Data analysis to obtain binding affinities (K_D) were done using steps similar to those outlined in the "Flow cytometry data collection and analysis" section above, with a few modifications. Samples were gated to isolate single cells, and gated on c-Myc detection levels to isolate positive (induced) and negative (uninduced) populations. MFI data for biotinylated antigen detection of induced populations and uninduced populations was retrieved. Background correction was performed by subtracting biotinylated antigen detection MFI values of uninduced cell populations from MFI values of corresponding induced populations. On GraphPad Prism Version 8.3.0, the background corrected data for biotinylated antigen detection were normalized for binding (by converted MFI values to ratios between the highest IgG concentration and all subsequent lower concentrations) and then, using the "Receptor binding – Saturation binding" model and "One site -- Specific binding" equation, the data was fitted to a curve and K_D estimated including standard error and 95% confidence interval.

Click chemistry on the yeast surface. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions were performed as described previously using 100 μM concentration of biotin-alkyne or biotin-azide probes.[3, 7] Strain-promoted azide-alkyne cycloaddition (SPAAC) reactions were carried out essentially as previously described with minor modifications.[7] Induced cells were pelleted, washed with PBSA pH 7.4 three times, and resuspended in 248.75 μL ice-cold PBS pH 7.4. 1.25 μL 2 mM DBCO-PEG_4-Biotin, prepared in DMSO, was added to each sample and vortexed briefly. SPAAC reactions were allowed to run for 2 hours at 4 °C on a rotary wheel, after which the samples were diluted in PBSA, pelleted, and then washed three times with ice-cold PBSA. Following the click chemistry reactions (both CuAAC and SPAAC), each sample was split into two and prepared for flow cytometry analysis. One set of samples were labeled to detect the biotin probes as a measure of click chemistry reactions; the other set labeled to detect IgG treatment after click chemistry reactions. For the sample set detecting IgG binding, Donkey1.1 clones were treated with 200 nM donkey IgG, and Bovine2.2 clones were treated with 200 nM bovine IgG during primary labeling. Both sample sets were also labeled to detect c-Myc (full-length display). Primary and secondary labeling conditions are outlined in the "Flow cytometry data collection and analysis" section above for scFvs, and antibodies used for labeling are reported in Supplementary Table S14. All CuAAC and SPAAC reactions and subsequent labeling experiments were done in technical triplicates.

Evaluation of click chemistry flow cytometry data was performed using FlowJo and Microsoft Excel with steps similar to those described above. Briefly, we 1) gated samples to isolate single cell, c-Myc positive populations, 2) retrieved MFI data for c-Myc detection of induced populations, 3) retrieved MFI data for biotin detection for click chemistry analysis of induced populations and uninduced populations, 4) retrieved MFI data for IgG detection for binding analysis of cMyc-positive (induced) populations and uninduced populations, and 5) performed background correction by subtracting biotin or IgG detection MFI values of uninduced cell populations from MFI values of corresponding induced populations. Background correct IgG detection MFI values of the technical triplicates were averaged and used to evaluate the observed binding of the samples. Standard deviations were also calculated using the STDEV.S function on Microsoft Excel and used as the measure of error.

CuAAC concentration and time-point assays. Copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions were performed as described previously, with modifications to the concentration of biotin-alkyne and reaction time. Induced cells were washed and resuspended in 220 μL PBS. For the 20 μM 1 step reactions: 1) 1.25 μL 4 mM Biotin-PEG_4-Alkyne, 2) 3.8 μL $CuSO_4$/THPTA (1:2 ratio of 20 mM $CuSO_4$:50 mM THPTA), 3) 12.5 μL 100 mM aminoguanidine and 4) 12.5 μL 100 mM sodium ascorbate were added in the given order, vortexing briefly in between each addition and then allowed to run for either 15 minutes or one hour at room temperature. Once the reaction was complete, samples were diluted in ice-cold PBSA, pelleted, and then washed three times with ice-cold PBSA. For the 100 μM 1 step reactions: identical steps as before were followed, with the exception that a higher concentration of Biotin-PEG_4-Alkyne was used (1.25 μL 20 mM). For the 2 step reactions, cells were washed three times with ice-cold PBSA after the first CuAAC reaction, and then a second round of reaction was performed following the same steps as before, using 1.25 μL of either 4 mM or 20 mM Biotin-PEG_4-Alkyne to get to a final concentration of 20 μM or 100 μM respectively. Once samples were washed with PBSA after

the reaction, they were prepared for flow cytometry following the steps outlined for primary and secondary labeling in the “Flow cytometry data collection and analysis” section above. Antibodies used for labeling are reported in Supplementary Table S13. CuAAC concentration and time-point reactions were evaluated in technical triplicates.

SPAAC time-point assays. Strain-promoted azide-alkyne cycloaddition (SPAAC) time-point reactions were carried out essentially as described above. Induced cells were washed and resuspended in 248.75 µL ice-cold PBS. 1.25 µL 2 mM Biotin-PEG_4-DBCO was added to each sample, vortexed briefly, and left to react on a rotary wheel at 4 °C for the duration of the time-point (9 minutes, 15 minutes, 30 minutes, 60 minutes, 90 minutes, 2 hours, 3 hours or 4 hours). Once the reaction durations were complete, samples were diluted in and washed with ice-cold PBSA and prepared for flow cytometry. Steps outlined for primary labeling onwards in the “Flow cytometry data collection and analysis” section above were followed; antibodies used for labeling are reported in Supplementary Table S12. SPAAC time-point reactions were evaluated in single samples.

Photo-crosslinking on the yeast surface. Two million freshly induced RJY100 cells displaying Donkey1.1 or Bovine2.2 scFvs and their ncAA containing variants (see Supplementary Table S15) were transferred to a 96-well V-bottom plate, pelleted, washed three times with PBS, and then subjected to three types of sample preparation: photo-crosslinked, non-photo-crosslinked controls, and binding controls. For the photo-crosslinked sample set, samples were resuspended in 50 µL 200 nM IgG (either donkey or bovine) and incubated for 30 minutes at room temperature with orbital shaking at 150 RPM. Next, samples were transferred to a 96-well clear flat-bottomed plate lined with aluminum foil, shiny side facing the plate (referred to as the “irradiation plate” below), and irradiated without the plate lid using low intensity 365 nm UV radiation from a 8 W hand-held UV lamp positioned 1–2 inches from the samples (Spectroline EN-180) for 6 hours on ice, resuspending once every hour. The samples were then diluted in 50 µL ice-cold PBS, resuspended extensively and transferred to a new 96-well V-bottomed plate. To maximize the recovery of cells after UV exposure, the wells of the irradiation plate were rinsed three more times with 50 µL ice-cold PBS, each time resuspending thoroughly and transferring to the corresponding well on the 96-well plate. The samples were pelleted and washed three times with ice-cold PBS. Following this, samples were resuspended in 50 µL 500 nM biotinylated IgG (donkey IgG for Donkey1.1 clones and bovine IgG for Bovine2.2 clones) containing chicken anti-cMyc and incubated within the range of 2 hours to overnight at room temperature with orbital shaking at 150 RPM. Samples were then diluted in and washed twice with ice-cold PBSA. Subsequent steps starting from secondary labeling were performed according to the “Flow cytometry data collection and analysis” section above. For the non-photo-crosslinked controls, samples were first resuspended in 50 µL 200 nM IgG (donkey IgG for Donkey1.1 clones and bovine IgG for Bovine2.2 clones) and incubated for 30 minutes at room temperature with orbital shaking at 150 RPM, after which the samples were left stationary on ice for 6 hours in the dark, resuspending once every hour. Next, the samples were diluted in 200 µL ice-cold PBS and washed three times with ice-cold PBS. Following this, samples were resuspended in 50 µL 500nM biotinylated IgG (donkey or bovine as required) containing chicken anti-cMyc and incubated within the range of 2 hours to overnight at room temperature with orbital shaking at 150 RPM and then prepared for flow cytometry as described above and in the “Flow cytometry data collection and analysis”

section. For the binding controls, samples were resuspended in 50 µL 500 nM biotinylated IgG (donkey or bovine as required) containing chicken anti-cMyc after the initial washes, incubated within the range of 2 hours to overnight at room temperature with orbital shaking at 150 RPM and then prepared for flow cytometry analysis similarly to the photo-crosslinked and non-photo-crosslinked sample sets. Details of labeling conditions are listed in Supplementary Table S15. Photo-crosslinking, non-photo-crosslinking and binding control conditions were all tested in technical triplicates.

Data from photo-crosslinking flow cytometry experiments was analyzed using a combination of FlowJo and Microsoft Excel as described earlier, with modifications as detailed here. First, samples were gated to include only single cell and c-Myc positive populations. Second, using the quadrant gating from dot plots, the population count of Q2 (full-length scFvs binding to biotinylated IgG) was divided by population count of Q2 + Q3 (full-length scFvs) to calculate the percentage of cells in which binding was detected (referred to as "apparent percentage binding" below and elsewhere in the manuscript). Last, the fraction binding exchanged was calculated by dividing the apparent percentage binding of each sample by the apparent percentage binding of the control (binding only) sample (see equations in Figure 6C). Values calculated for the fraction binding exchanged of the technical triplicates were averaged and used to evaluate photo-crosslinking; standard deviations were obtained using the STDEV.S function on Microsoft Excel and used as the measure of error.

Production and purification of soluble scFv-Fcs. To facilitate ncAA incorporation, the TAG stop codon in a previously established yeast secretion plasmid,[1] pCHA-FcSup-TAG, was first converted to a TAA stop codon. Primers used to encode a TAA codon in place of the TAG codon in the pCHA-FcSup-TAG plasmid backbone are listed in Supplementary Table S3. Two DNA segments were amplified *via* PCR, one with the pCHA_TAAFwd1 + pCHA_TAARev1 and one with pCHA_TAAFwd2 + pCHA_TAARev2 primers, and then recombined with the pCHA-FcSup-TAG vector digested at the XmaI and XhoI restriction sites using Gibson Assembly. In the second step, DNA encoding Donkey1.1 WT, L93TAG, H31TAG and H54TAG scFvs was amplified *via* PCR using the pCHA_scFvFwd and pCHA_scFvRev primers. Each PCR amplified insert was then recombined with the pCHA-FcSup-TAA vector digested at the NheI and XmaI restriction sites using Gibson Assembly. Quality control steps to isolate PCR products of approximately the correct size and successful Gibson Assembly reactions, as well as general transformation and plasmid isolation steps were performed as described in the "Introduction of TAG codons into scFvs" section. All plasmids were sequence verified with sequencing performed at Quintara Biosciences.

ScFv-Fcs were secreted and purified according to Van Deventer et al, 2015,[1] with the following modifications. For the WT antibody (containing only canonical amino acids), the secretion plasmid (pCHA-FcSup-TAA backbone, TRP marker) was transformed into Zymo competent RJY100 cells, inoculated and grown in SD-CAA (–TRP –URA) + Pen/Strep, and induced in 100 mL YPG + Pen/Strep at 20 °C with shaking at 250–300 RPM for 4 days. Following induction, the culture was pelleted for 35 minutes at 3214 rcf, filtered using a 0.2 µM filter and pH adjusted to 7.4 using 10× PBS. The filtrate was passed twice through a protein A column, following which the scFv-Fc containing resin was washed three times with 10 mL PBS. The scFv-Fcs were then eluted using 7 mL 100 mM glycine, pH 3.0 and immediately neutralized with 0.7 mL 1 M Tris,

pH 8.5. The eluant was buffer exchanged using Amicon Ultra-15 centrifugal filter units (30 kDa molecular weight cut-off, Millipore Sigma) into PBS, pH 7.4 and concentrated. Protein concentrations were measured *via* the absorbance of 280 nm light on a Nano Drop One instrument (Thermo Fisher). Protein purity was determined *via* sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) without the use of PNGase F (Supplementary Figure S23). For scFv constructs encoding a TAG codon at L93, H31 or H54, secretion plasmids (pCHA-FcSup-TAA backbone, TRP marker) were co-transformed with the pRS315-KanRmod-AcRFS aaRS/tRNA suppressor plasmid (LEU marker) using Zymo competent RJY100 cells, inoculated and grown in SD-SCAA –TRP –LEU –URA + Pen/Strep, and induced in 1 L YPG + Pen/Strep + 1 mM AzF at 20 °C with shaking at 250–300 RPM for 4 days in the dark. The subsequent purification steps were identical to that of the WT purification, with the additional step of keeping all protein samples putatively containing AzF (supernatant, filtrate, resin and eluate) in the dark. Protein purity was determined *via* SDS-PAGE (Supplementary Figure S23).

Click chemistry on soluble scFv-Fcs. CuAAC click chemistry reactions were performed using 100 μM concentrations of biotin probes based on previous protocols.[8] In microcentrifuge tubes containing 220 μL aliquots of 500 nM scFv-Fc in 1× PBS pH7.4, we added 1) 1.25 μL 20 mM biotin-$(PEG)_4$-alkyne, 2) 3.8 μL $CuSO_4$/THPTA (1:2 ratio of 20 mM $CuSO_4$:50 mM THPTA), 3) 12.5 μL 100 mM aminoguanidine and 4) 12.5 μL 100 mM sodium ascorbate (in the given order), vortexing briefly in between each addition. The reactions were left to proceed for one hour at room temperature, following which they were quenched using 2 μL of 100 mM EDTA. ScFv-Fcs that had undergone reactions with DMSO in place of biotin probes and scFv-Fcs that had not been subjected to click chemistry reactions were used as controls. Western blots were used to analyze click chemistry reactions and verify the presence of AzF in the TAG-mutated scFv-Fcs. SDS-PAGE using 4–12% Bis-Tris gels was performed on all samples in duplicate gels. One gel was stained with coomassie SimplyBlue SafeStain to confirm the presence of protein. Western blots were performed by transferring the second PAGE gel to a nitrocellulose membrane using an iBlot2 Dry Blotting System (Life Technologies). Transferred membranes were blocked overnight with 5% w/v BSA in TBS + 0.1% v/v Tween20, following which they were probed with streptavidin Alexa Fluor 488 in a 1:1000 dilution for the presence of biotin (Figure 6E and Supplementary Figure S24).

Photo-crosslinking of soluble scFv-Fcs. For photo-crosslinked samples, 25 μL of 200 nM donkey IgG was aliquoted to wells of a 96-well clear flat-bottomed plate containing 25 μL of 1 μM Donkey1.1 WT, L93TAG, H31TAG or H54TAG scFv-Fc (for a final concentration of 100 nM donkey IgG and 500 nM scFv-Fc) and incubated for 30 minutes at room temperature with orbital shaking at 150 RPM. Next, the bottom of the plates were lined with aluminum foil, shiny side facing the plate, and samples irradiated without the lid using low intensity 365 nm UV radiation from a 8 W hand-held UV lamp (Spectroline EN-180) for 2 hours on ice, mixing once every hour during irradiation. For non-photo-crosslinked control samples, 25 μL of 200 nM donkey IgG was aliquoted to wells of a 96-well clear flat-bottomed plate containing 25 μL of one of the four 1 μM scFv-Fcs (for a final concentration of 100 nM donkey IgG and 500 nM scFv-Fc) and incubated for 30 minutes at room temperature with orbital shaking at 150 RPM, and then left in the dark for 2 hours on ice, mixing once every hour while on ice. SDS-PAGE using 4–12% Bis-Tris gels was then performed for all samples alongside donkey IgG only and scFv-Fc only controls for

comparison (Figure 6F and Supplementary Figure S25). Gels were stained with coomassie SimplyBlue SafeStain and imaged using the visible settings on a c400 imaging system (Azure Biosystems).

Construction of pRS315-KanRmod-AcFRS suppressor plasmid. The pRS315 vector backbone in the pRS315-KanRmod-AcFRS suppressor plasmid was modified to introduce the kanamycin resistance gene in place of ampicillin resistance, based on the previously reported pRS315-TyrAcFRS construct.[3] First, the NeoR/KanR gene and promoter was amplified *via* PCR from the pREP4 vector (Qiagen) using the pREP4_KanR_XmaIFwd1 and pREP4_KanR_AvrIIRev1 primers. DNA fragments of the expected insert size were isolated from the PCR products using gel electrophoresis and extraction. A second amplification was performed using the pREP4_KanR_XmaIFwd2 and pREP4_KanR_AvrIIRev2 primers; the purified DNA fragments from the first reaction were used as the template, followed by gel electrophoresis and extraction to obtain the correctly sized insert. XmaI and AvrII restriction sites were introduced at the 5' and 3' ends of the NeoR/KanR gene and promoter sequence respectively during this process. Next, a quick change PCR reaction was performed on pRS315-TyrAcFRS to introduce the XmaI restriction site at the 5' end of the AmpR gene and the AvrII restriction site at the 3' end of the AmpR promoter (using pRS315_AmpR_XmaIFwd and pRS315_Ampp_AvrIIRev). PCR products were DpnI digested, transformed into chemically competent *E. coli* DH5αZ1, plated on LB plates containing ampicillin and grown at 37 °C overnight. Resulting colonies were picked and grown to saturation in 5 mL LB liquid media containing ampicillin. Plasmid DNA was then isolated from these cultures using GenCatch Plasmid DNA Mini-Prep kits. Following the quick change reaction, the modified pRS315-TyrAcFRS plasmid and NeoR/KanR insert were digested at the XmaI and AvrII sites and ligated together. The ligations were transformed into chemically competent *E. coli* DH5αZ1, grown to saturation in LB media containing kanamycin, and plasmid DNA isolated as before. The cloned plasmids were digested at the XmaI and AvrII sites to confirm the presence of correctly sized inserts using gel electrophoresis. The plasmid, referred to as pRS315-KanR-AcFRS below, was sequence verified through Sanger sequencing performed at Quintara Biosciences.

Lastly, the NcoI restriction site was removed from the KanR gene of the pRS315-KanR-AcFRS plasmid to obtain the final form of the suppressor plasmid used in this study, referred to as pRS315-KanRmod-AcFRS. To do this, two DNA segments were amplified *via* PCR, one with the pREP4_KanR_XmaIFwd2 + NcoI_RemovalRev and the other with the NcoI_RemovalFwd + pREP4_KanR_AvrIIRev2 primers, and then recombined with the pRS315-KanR-AcFRS vector digested at the XmaI and AvrII restriction sites using Gibson Assembly. Quality control steps to isolate PCR products of approximately the correct size, successful Gibson Assembly reactions, as well as transformation and plasmid isolation steps were performed as described above for kanamycin resistance, and sequence verified from Quintara Biosciences.

Construction of pCTCON2-M0076 display plasmid. Plasmid DNA encoding the MMP-9 non-inhibitory M0076-D03 scFv[9] was amplified *via* PCR using the pCTCON2Fwd and pCTCON2Rev primers and ligated with the pCTCON2 vector digested at the NheI and BamHI restriction sites. Quality control steps to isolate PCR products of approximately the correct size, successful digestion and ligation reactions, as well as general transformation and plasmid isolation steps

were performed as described previously and sequence verified with sequencing performed at Quintara Biosciences.

Supplementary Figures

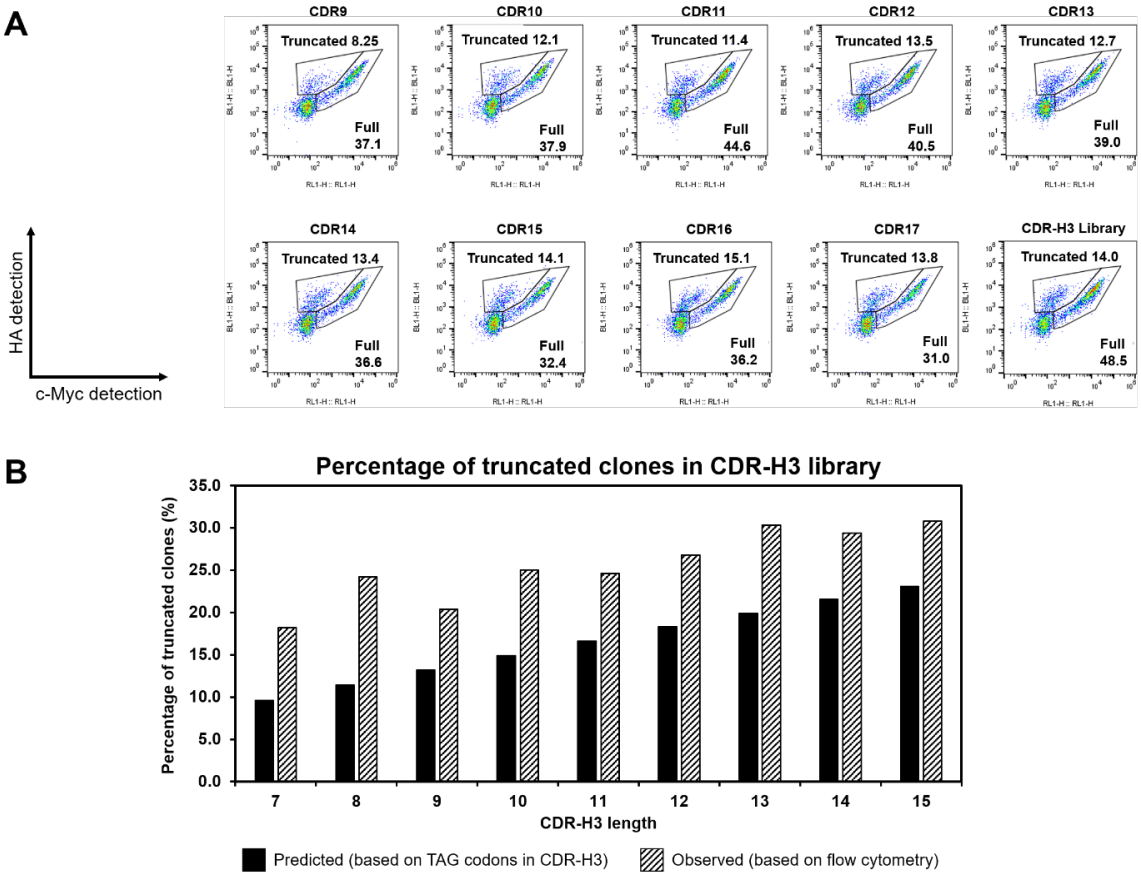

Figure S1. Evaluation of truncation frequency in CDR-H3 library. A) Flow cytometry analysis of displayed proteins and truncation frequency. B) Quantification of truncation analysis and comparison to expected truncation frequency based on library design.

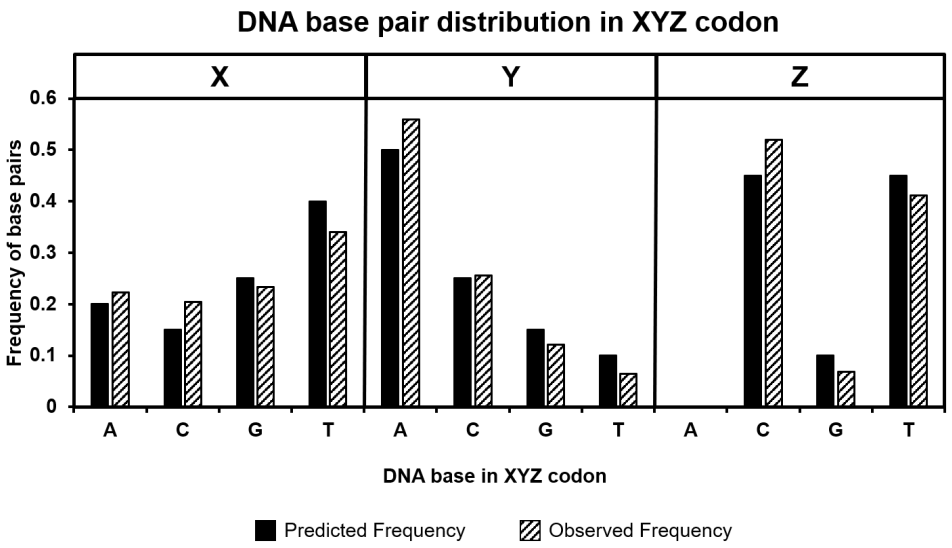

Figure S2. DNA base pair distribution in the designed "XYZ" codon. The predicted frequency is calculated based off the library design;[10] the observed frequency is calculated from sequencing 8–12 clones from each individual sublibrary.

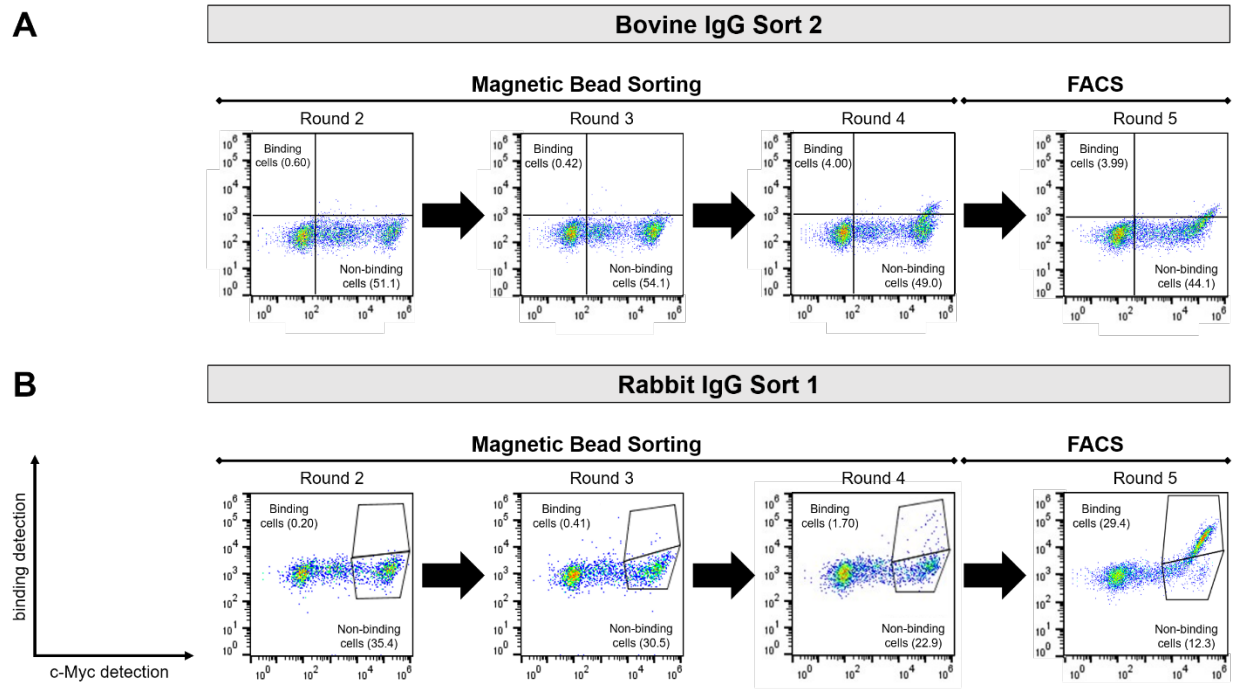

Figure S3. CDR-H3 library sorting against A) biotinylated bovine and B) biotinylated rabbit IgG. In the enrichments shown here, four rounds of magnetic bead sorting (round 1 not shown) were followed by one round of Fluorescence Activated Cell Sorting (FACS). Binding was detected by labeling for biotinylated antigen; full-length display was detected by labeling for c-Myc.

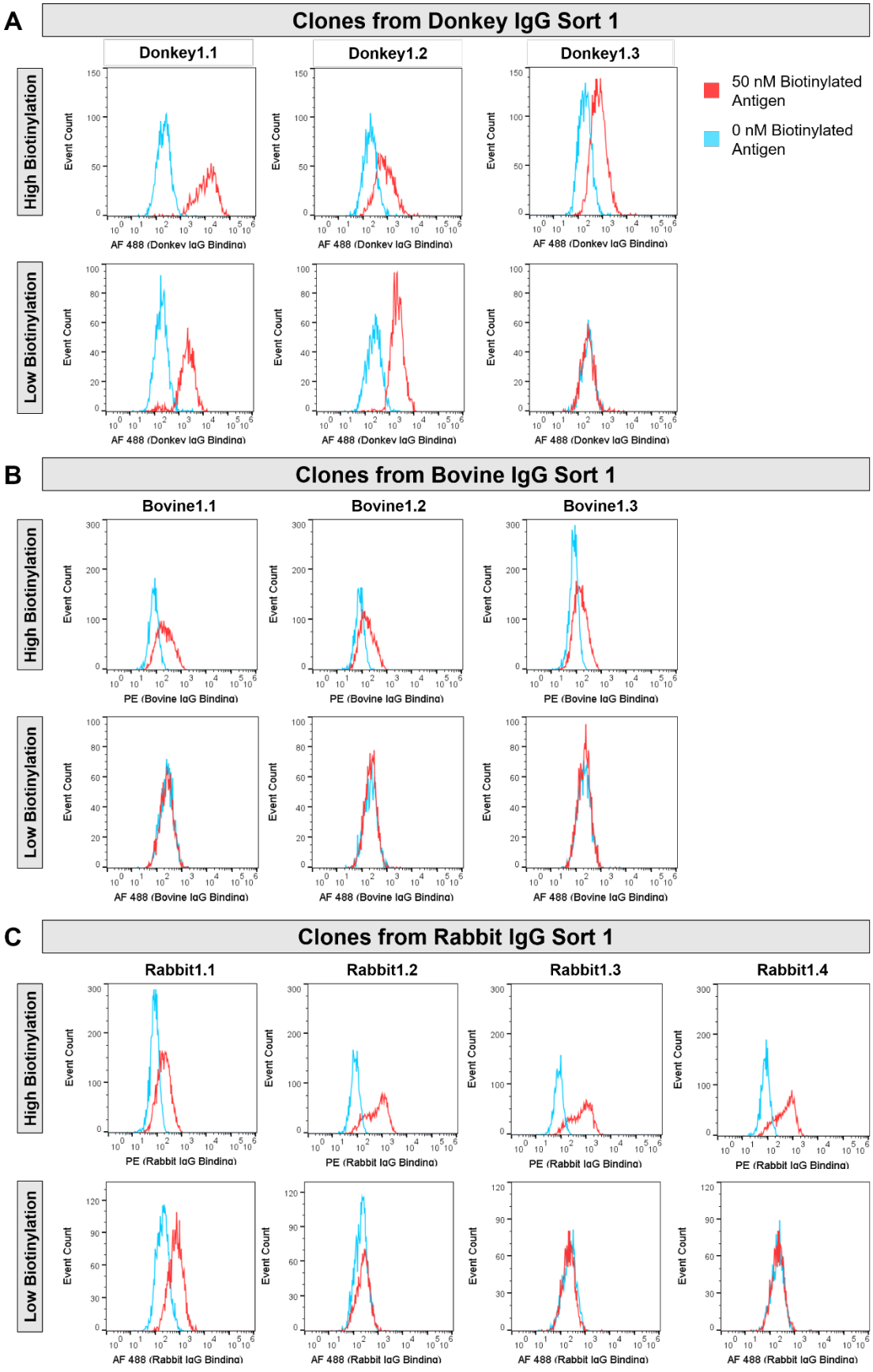
A
Clones from Donkey IgG Sort 1
Donkey1.1
Donkey1.2
Donkey1.3
High Biotinylation
Low Biotinylation
Event Count
AF 488 (Donkey IgG Binding)
50 nM Biotinylated Antigen
0 nM Biotinylated Antigen
B
Clones from Bovine IgG Sort 1
Bovine1.1
Bovine1.2
Bovine1.3
PE (Bovine IgG Binding)
AF 488 (Bovine IgG Binding)
C
Clones from Rabbit IgG Sort 1
Rabbit1.1
Rabbit1.2
Rabbit1.3
Rabbit1.4
PE (Rabbit IgG Binding)
AF 488 (Rabbit IgG Binding)

Figure S4. Binding assays with different batches of biotinylated IgG to identify clones that preferentially bind to native IgGs as opposed to clones that preferentially bind to biotinylated forms of the IgGs. A) Binding of donkey IgG sort 1 clones showing two out of three clones (Donkey1.1 and Donkey1.2) bind preferentially to donkey IgG. B) Binding of bovine IgG sort 1 clones showing none of the three clones bind preferentially to bovine IgG. C) Binding of rabbit IgG sort 1 clones showing one out of four clones (Rabbit1.1) binds preferentially to rabbit IgG. High Biotinylation: > 2 moles of biotin/mole of IgG; Low Biotinylation: 1–2 moles of biotin/mole of IgG.

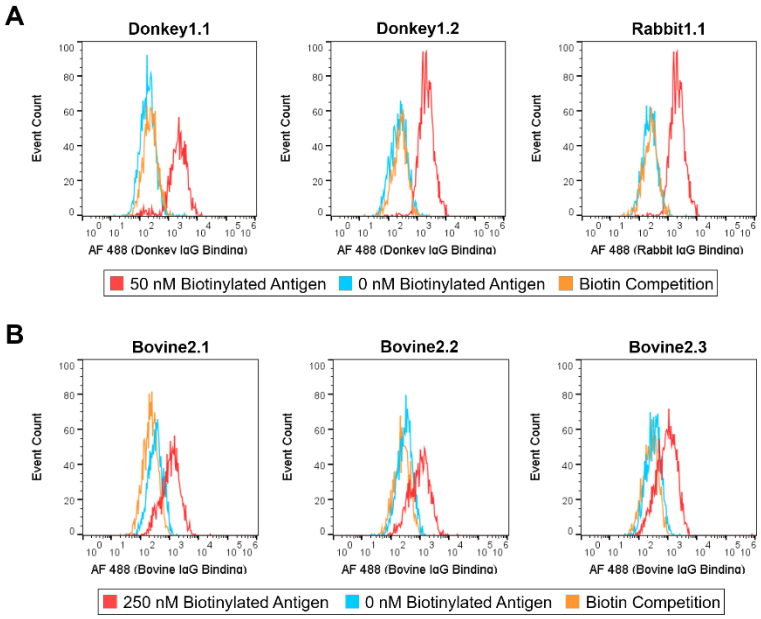

Figure S5. Binding competition assay for Sort 1 and Sort 2 clones that preferentially bind to the target IgG. A) Sort 1 clones treated with biotinylated and unconjugated IgG: “50 nM Biotinylated Antigen” samples were treated with only 50 nM biotinylated IgG. “Biotin Competition” samples were treated with 1 µM unconjugated IgG followed by 50 nM biotinylated IgG. B) Sort 2 clones treated with biotinylated and unconjugated IgG: “250 nM Biotinylated Antigen” samples were treated with only 250 nM biotinylated IgG. “Biotin Competition” samples were treated with 5 µM unconjugated IgG in addition to 250 nM biotinylated IgG.

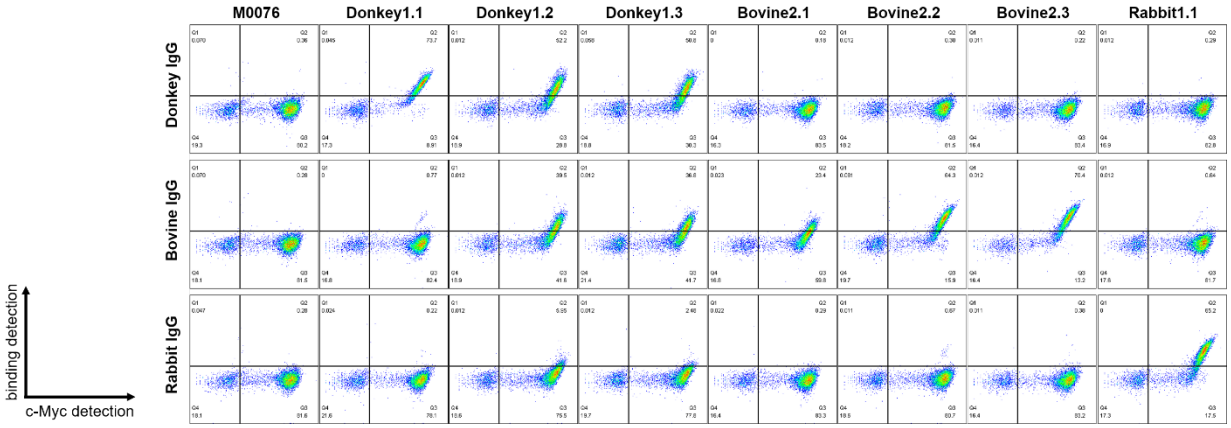

Figure S6. Flow cytometry dot plots to determine antigen specificity of the unique wild-type scFvs isolated from the CDR-H3 library (corresponding to Figure 3A). M0076 is a non-inhibitory binder of MMP-9[9] and serves as the non-binding control against IgGs here. Clones were treated with 200 nM of the different

biotinylated IgGs and analyzed for binding. Binding was detected by labeling for biotinylated antigen. Full-length display was detected by labeling for c-Myc. These plots are representative of technical triplicates performed for each clone.

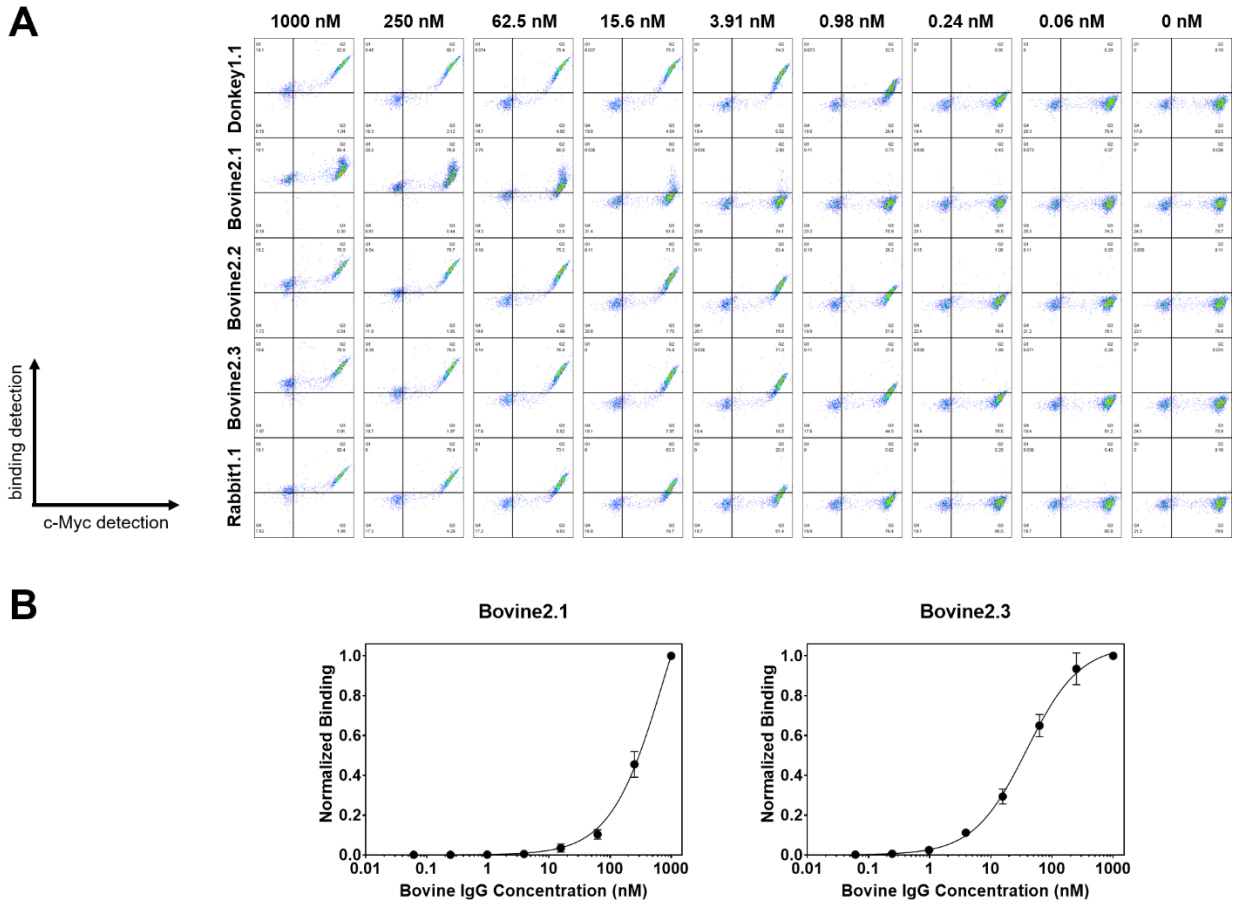

Figure S7. Binding titrations to determine binding affinities (K_D) of unique wild-type scFvs isolated from the CDR-H3 library (corresponding to Table 2 and Figure 3B). A) Flow cytometry dot plots and B) titration curves for scFvs not included in Figure 3B. Clones were treated with different concentrations of the respective biotinylated IgG (starting from 1 μM with seven subsequent 4-fold dilutions and a final, 0 nM IgG concentration) and analyzed for binding. Binding was detected by labeling for biotinylated antigen. Full-length display was detected by labeling for c-Myc. Median Fluorescence Intensity (MFI) data for biotinylated IgG detection was corrected for background detection by subtracting MFI of non-displaying populations from the MFI of displaying populations for each sample. Background subtracted MFI data was then normalized to the highest MFI detection for each scFv (obtained with 1 μM IgG), using GraphPad Prism 8, and plotted as a function of IgG concentration. Each condition was evaluated in technical triplicates with average values and standard errors reported here. Binding affinities (K_D) were calculated using the "Receptor binding – Saturation binding" model and "One site -- Specific binding" equation in GraphPad Prism 8; these results are reported in Table 2.

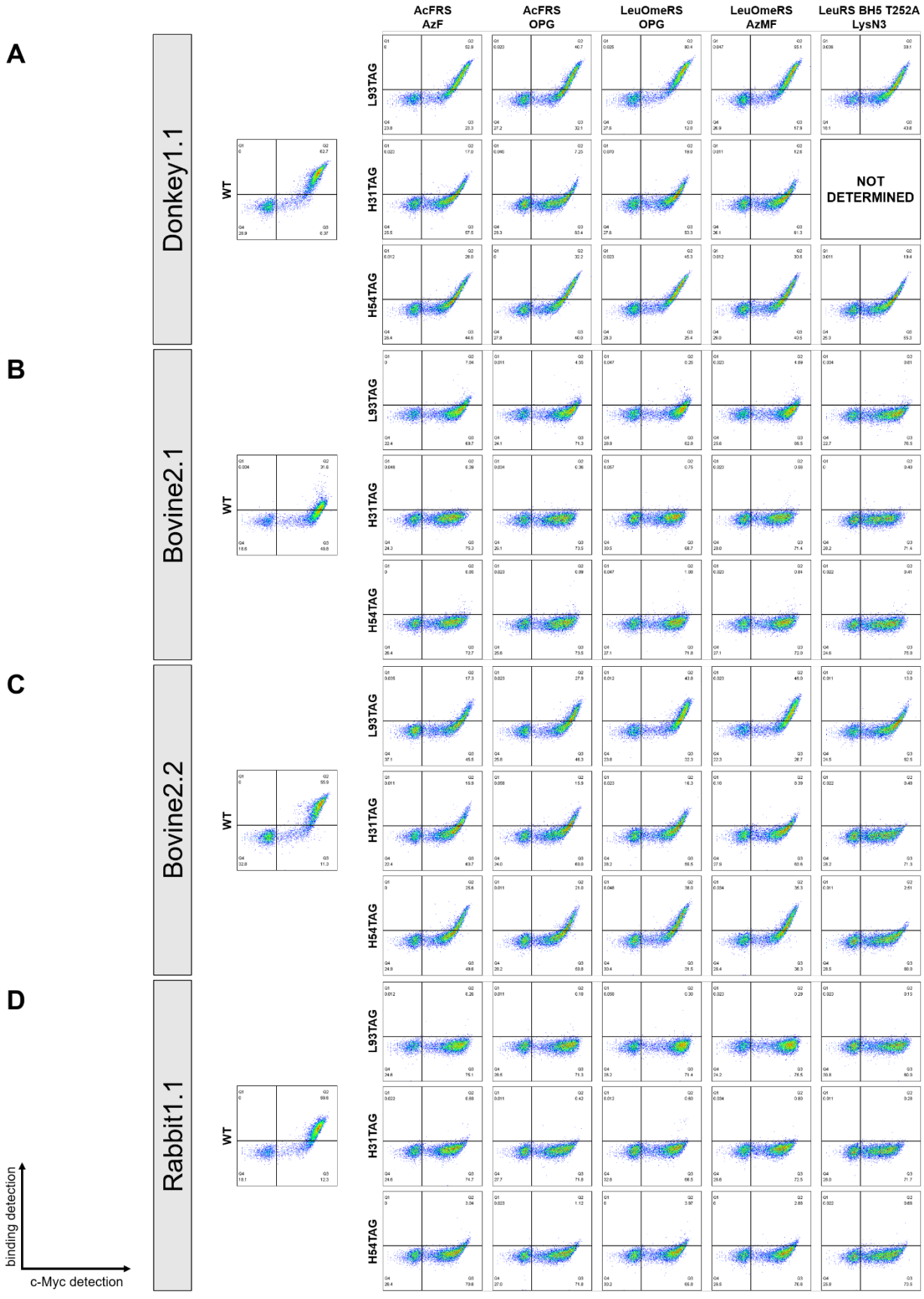
A
B
C
D
Donkey1.1
Bovine2.1
Bovine2.2
Rabbit1.1
AcFRS AzF
AcFRS OPG
LeuOmeRS OPG
LeuOmeRS AzMF
LeuRS BH5 T252A LysN3
WT
L93TAG
H31TAG
H54TAG
NOT DETERMINED
binding detection
c-Myc detection

Figure S8. Flow cytometry dot plots of antigen binding for wild-type and ncAA-substituted clones characterized in this study (corresponding to Figure 4C and D). A) Donkey1.1 wild-type and ncAA-containing variants binding to biotinylated donkey IgG. B) Bovine2.1 wild-type and ncAA-containing variants binding to biotinylated bovine IgG. C) Bovine2.2 wild-type and ncAA-containing variants binding to biotinylated bovine IgG. D) Rabbit1.1 wild-type and ncAA-containing variants binding to biotinylated rabbit IgG. Binding was detected by labeling for biotinylated antigen. Full-length display was detected by labeling for c-Myc. These plots are representative of technical triplicates prepared for each sample.

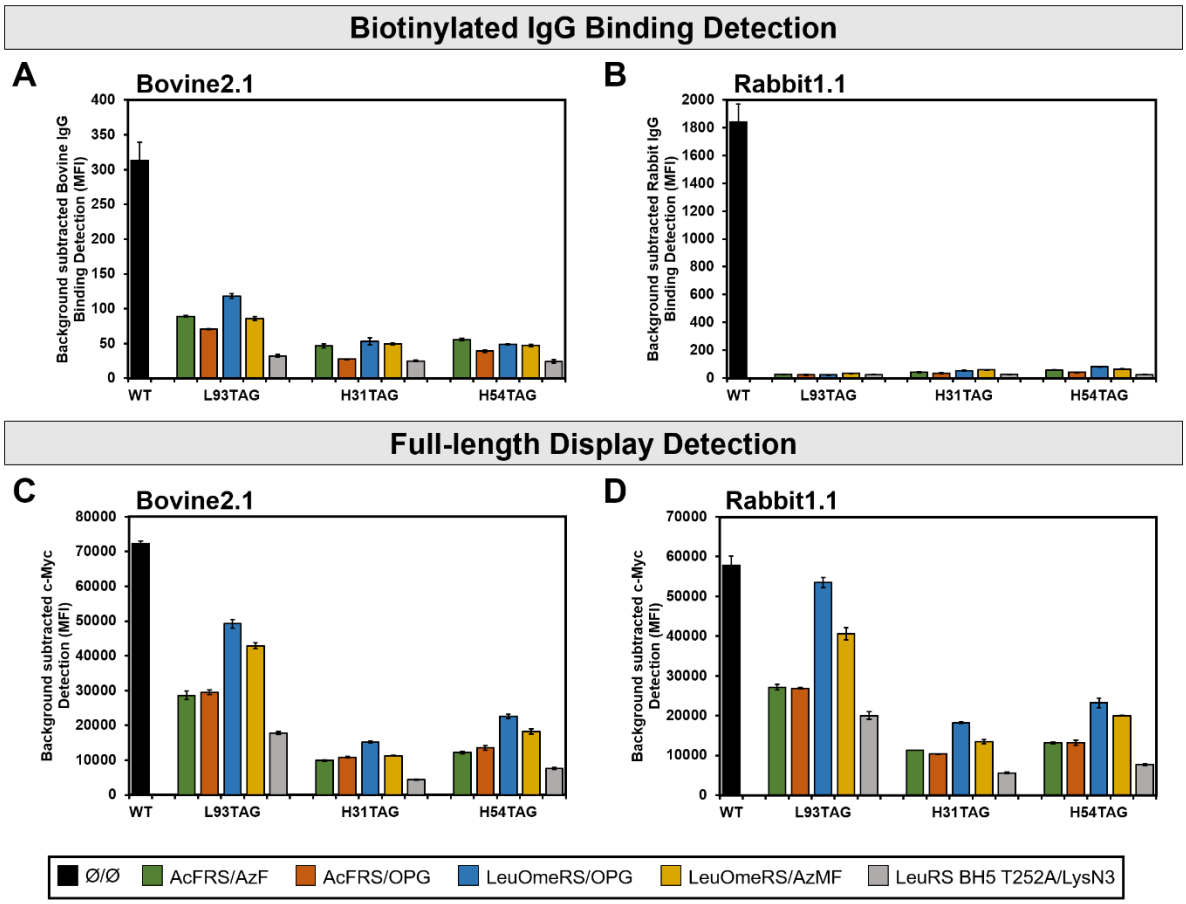

Figure S9. Evaluation of antigen binding and full-length display detection for Bovine2.1 and Rabbit1.1 clones. A) Binding detection of Bovine2.1 wild-type and ncAA-containing clones using 200 nM biotinylated bovine IgG. B) Binding detection of Rabbit1.1 wild-type and ncAA-containing clones using 200 nM biotinylated rabbit IgG. C) Full-length display detection of Bovine2.1 wild-type and ncAA-containing clones. D) Full-length display detection of Rabbit1.1 wild-type and ncAA-containing clones. For all data shown here, background-subtracted median fluorescence intensity (MFI) values of technical triplicates are averaged and reported as a measure of binding or full-length display detection, with error bars representing standard deviations (see *Supplementary Methods* for details).

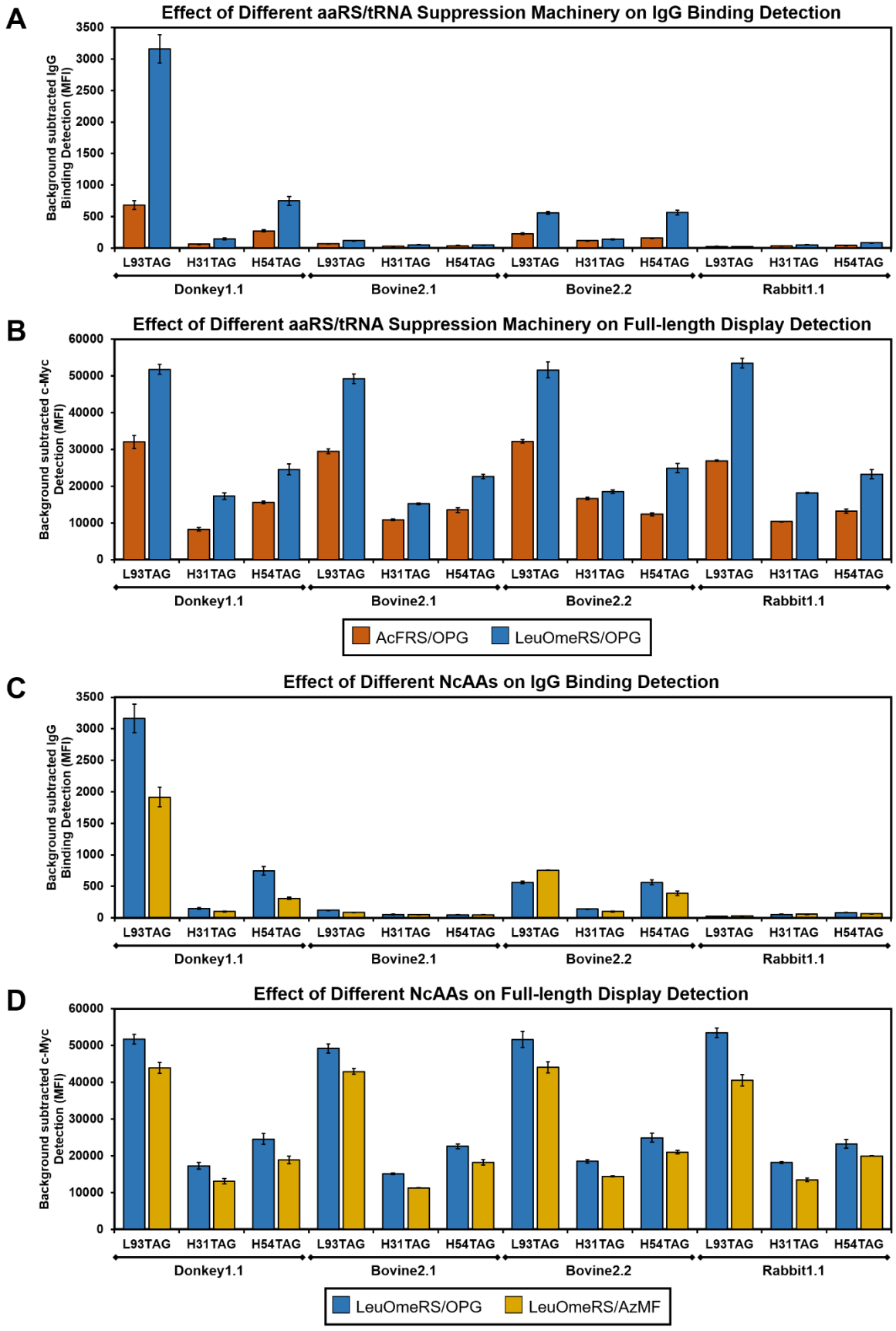
A
Effect of Different aaRS/tRNA Suppression Machinery on IgG Binding Detection
Background subtracted IgG Binding Detection (MFI)
3500
3000
2500
2000
1500
1000
500
0
L93TAG
H31TAG
H54TAG
Donkey1.1
Bovine2.1
Bovine2.2
Rabbit1.1
B
Effect of Different aaRS/tRNA Suppression Machinery on Full-length Display Detection
Background subtracted c-Myc Detection (MFI)
60000
50000
40000
30000
20000
10000
0
AcFRS/OPG
LeuOmeRS/OPG
C
Effect of Different NcAAs on IgG Binding Detection
D
Effect of Different NcAAs on Full-length Display Detection
LeuOmeRS/OPG
LeuOmeRS/AzMF

Figure S10. Evaluation of the effects of aminoacyl tRNA synthetase and tRNA (aaRS/tRNA) pairs and noncanonical amino acids (ncAA) on antigen binding and full-length display. Effect of using distinct aaRS/tRNA pairs to incorporate OPG on A) antigen binding, and B) protein translation. Effect of utilizing the same aaRS/tRNA pair to incorporate two different ncAAs on C) antigen binding, and D) protein translation. Data from Figure 4C and D and Supplementary Figure S9 have been replotted to highlight these effects.

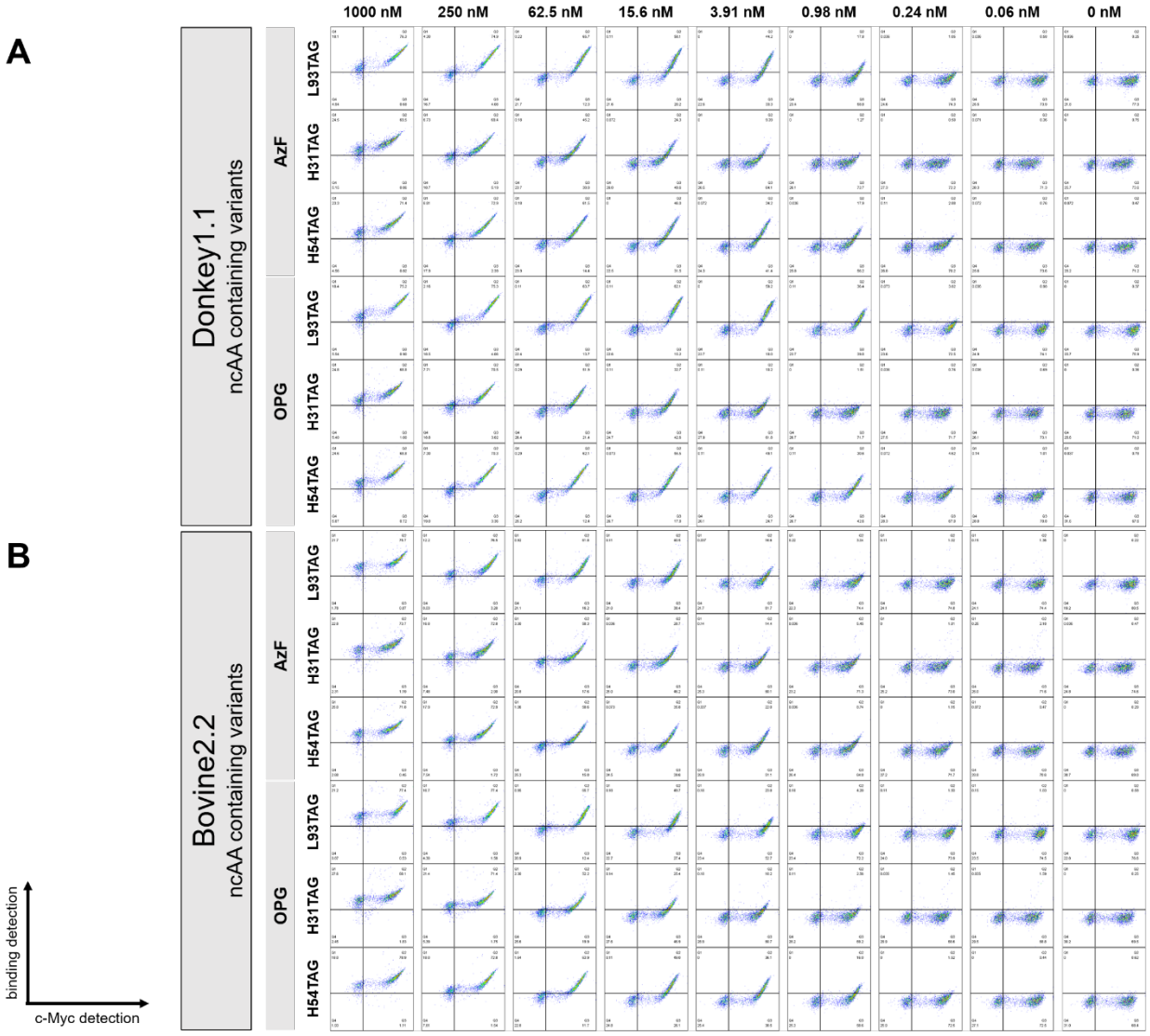

Figure S11. Flow cytometry dot plots of binding titrations used to determine binding affinities (corresponding to Table 3 and Supplementary Figure S12). A) Donkey1.1 ncAA-containing clones and B) Bovine2.2 ncAA-containing clones were treated with different concentrations of respective biotinylated IgG (starting from 1 μM with seven subsequent 4-fold dilutions and a final, 0 nM IgG concentration) and analyzed for binding. Binding was detected by labeling for biotinylated antigen. Full-length display was detected by labeling for c-Myc. Each condition represented here was evaluated in technical triplicates.

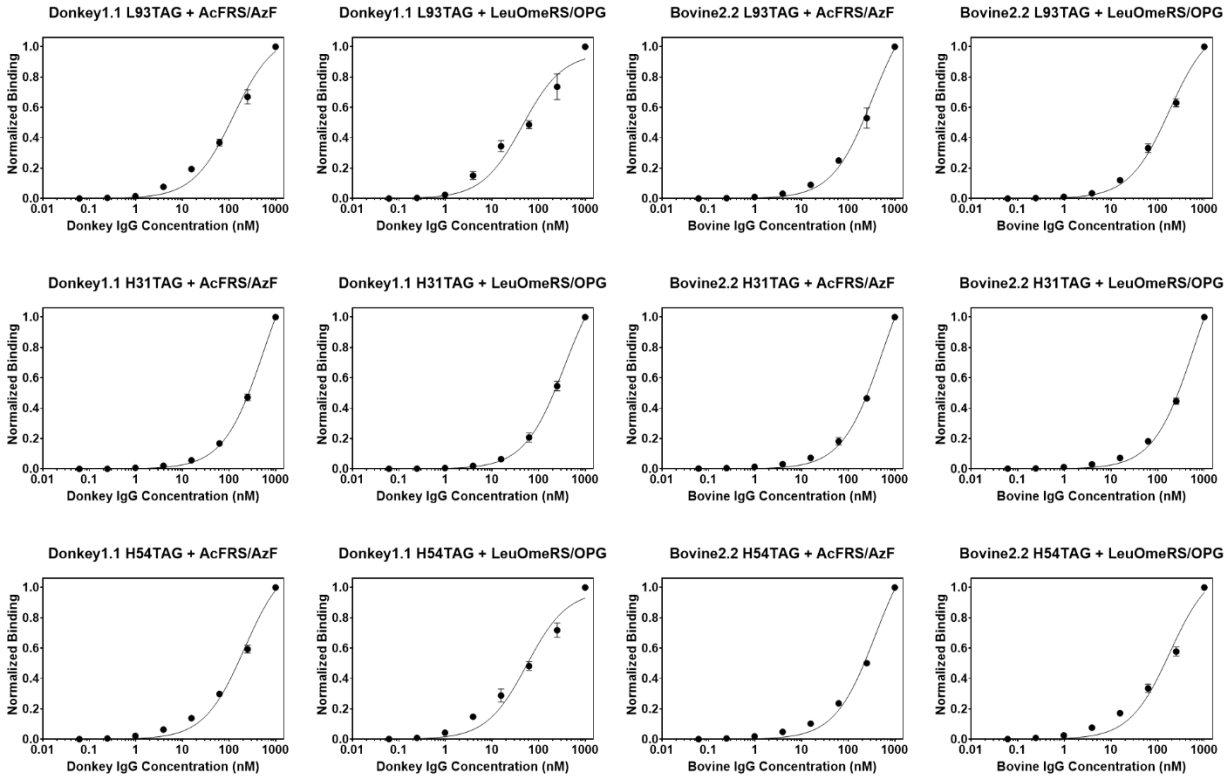

Figure S12. Titration curves for ncAA-containing scFvs studied in this work. Binding assays were performed with different concentrations of biotinylated IgG (starting from 1 μM with subsequent 4-fold dilutions) for each scFv (see also Supplementary Figure S11). Median Fluorescence Intensity (MFI) data for biotinylated IgG detection was corrected for background detection by subtracting MFI of non-displaying populations from the MFI of displaying populations for each sample. Background subtracted MFI data was then normalized to the highest MFI detection for each scFv (obtained with 1 μM IgG), using GraphPad Prism 8, and plotted as a function of IgG concentration. Each condition was tested in technical triplicates with average values and standard error reported here. Binding affinities (K_D) were calculated using the "Receptor binding – Saturation binding" model and "One site -- Specific binding" equation in GraphPad Prism 8; these results are reported in Table 3.

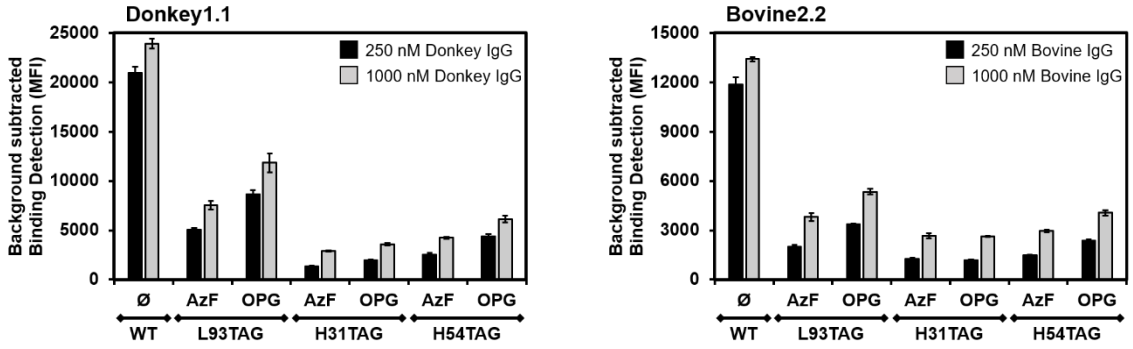

Figure S13. Binding comparison of different scFvs with 250 nM and 1000 nM biotinylated IgG. Wild-type and ncAA-substituted variants for Donkey1.1 and Bovine2.2 clones were treated with either 250 nM or 1000 nM respective biotinylated IgG and analyzed for binding using detection of the biotinylated antigen. Background subtracted median fluorescence intensity data of technical triplicates was averaged and used to analyze binding; error bars represent standard deviation.

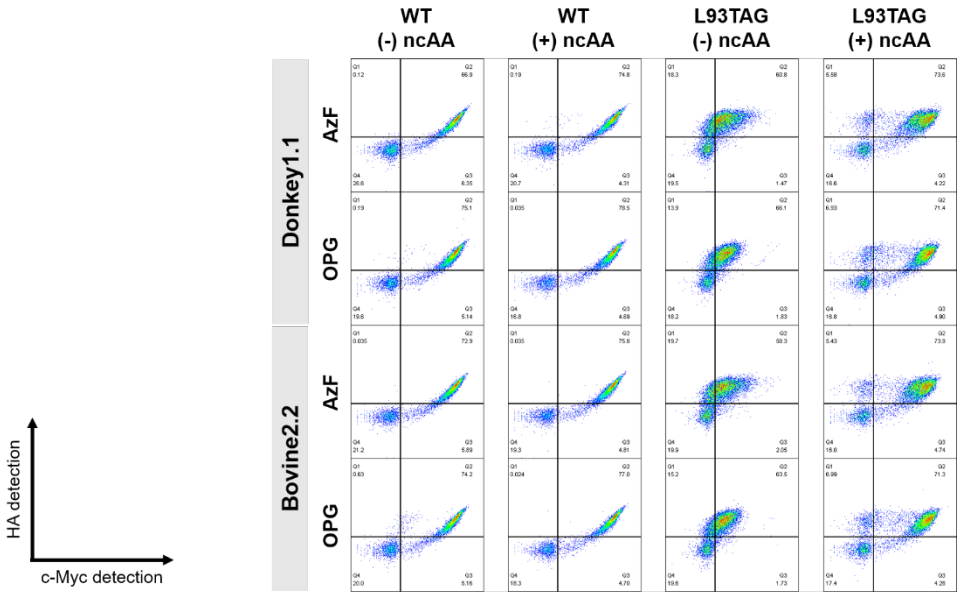

Figure S14. Flow cytometry dot plots of Donkey1.1 (wild-type and L93TAG) and Bovine2.2 (wild-type and L93TAG) clones used to determine relative readthrough efficiency (RRE) and maximum misincorporation frequency (MMF). The AcFRS suppression machinery was used to incorporate AzF and the LeuOmeRS suppression machinery was used to incorporate OPG. N-terminal (HA) and C-terminal (c-Myc) epitope tags were detected in the absence and presence of noncanonical amino acids ((-) ncAA and (+) ncAA, respectively). Median fluorescence intensity data was used to calculate RRE and MMF[3, 6] (Figure 4E and Supplementary Table S10). Each condition represented here was evaluated using biological triplicates.

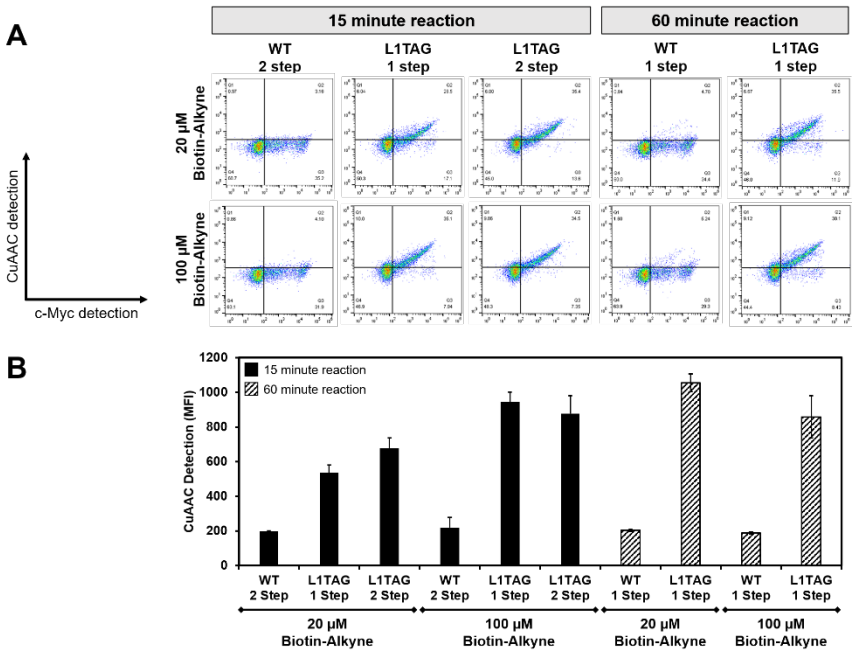

Figure S15. Time point and concentration assays with FAPB2.3.6 (an antibody fragment that recognizes human and murine isoforms of fibroblast activation protein)[3] to determine optimum conditions for copper-catalyzed azide-alkyne cycloaddition (CuAAC) experiments at room temperature. A) Flow cytometry dot plots depicting CuAAC reactivity for two concentrations of Biotin-Alkyne using two reaction times. “1 step”

refers to one round of CuAAC reaction at the given conditions; “2 step” refers to two rounds of reaction (identical conditions in each round). CuAAC was evaluated by labeling for biotin. Full-length display was detected by labeling for c-Myc. Each condition represented here was evaluated in technical triplicates. B) Average Median Fluorescence Intensity (MFI) values of biotin detection represent CuAAC reactivity for each condition; error bars represent the standard deviation of technical triplicates.

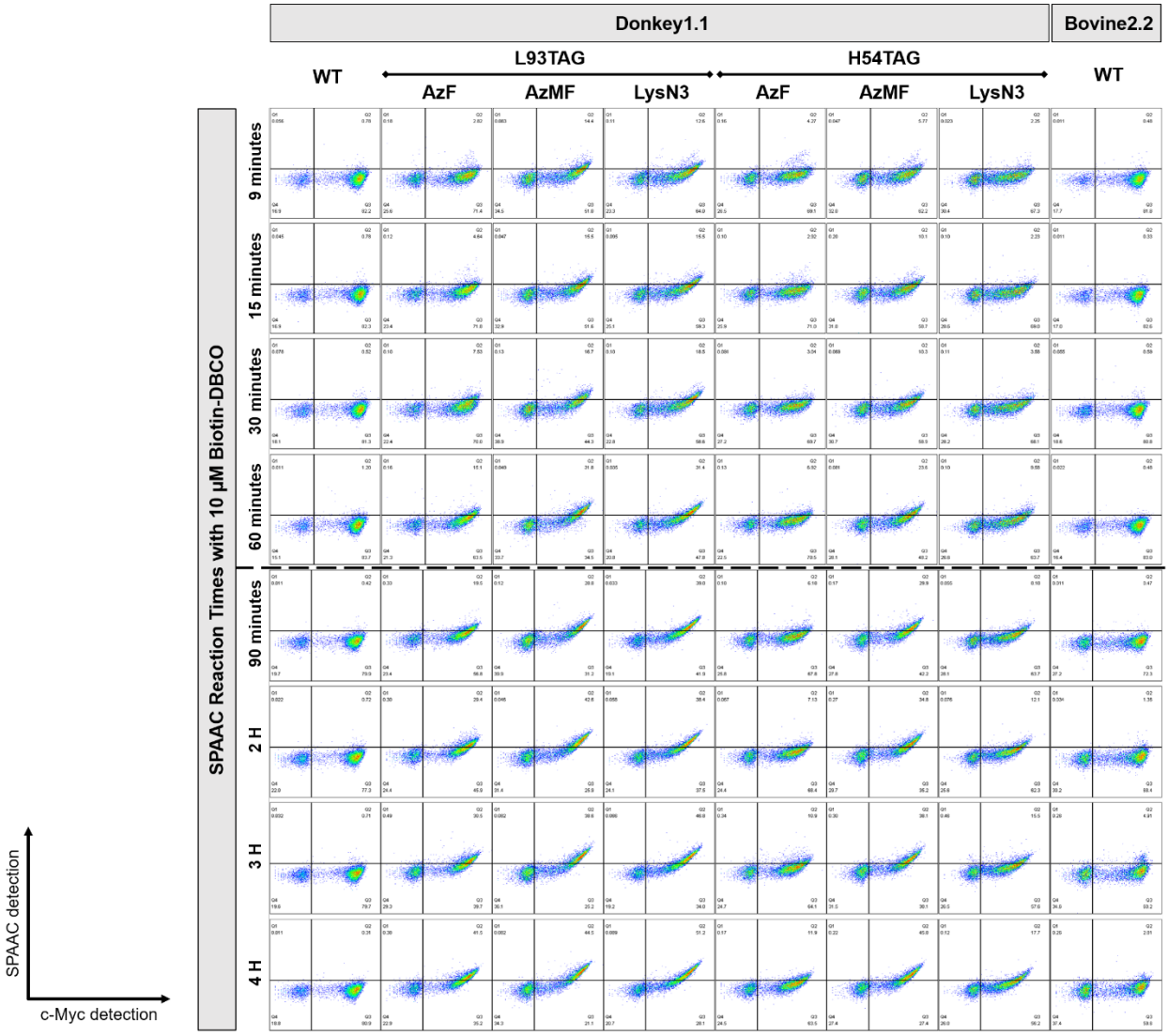

Figure S16. Time point assay to determine conditions for strain-promoted azide-alkyne cycloaddition (SPAAC) reactions. Flow cytometry dot plots depicting effects of different reaction times on SPAAC reactivity for Donkey1.1 wild-type and azide-containing variants and Bovine2.2 wild-type scFvs. Each reaction was carried out at 4 °C and evaluated using single samples. SPAAC was detected by labeling for biotin. Full-length display was detected by labeling for c-Myc. Dashed line separates data collected on different days.

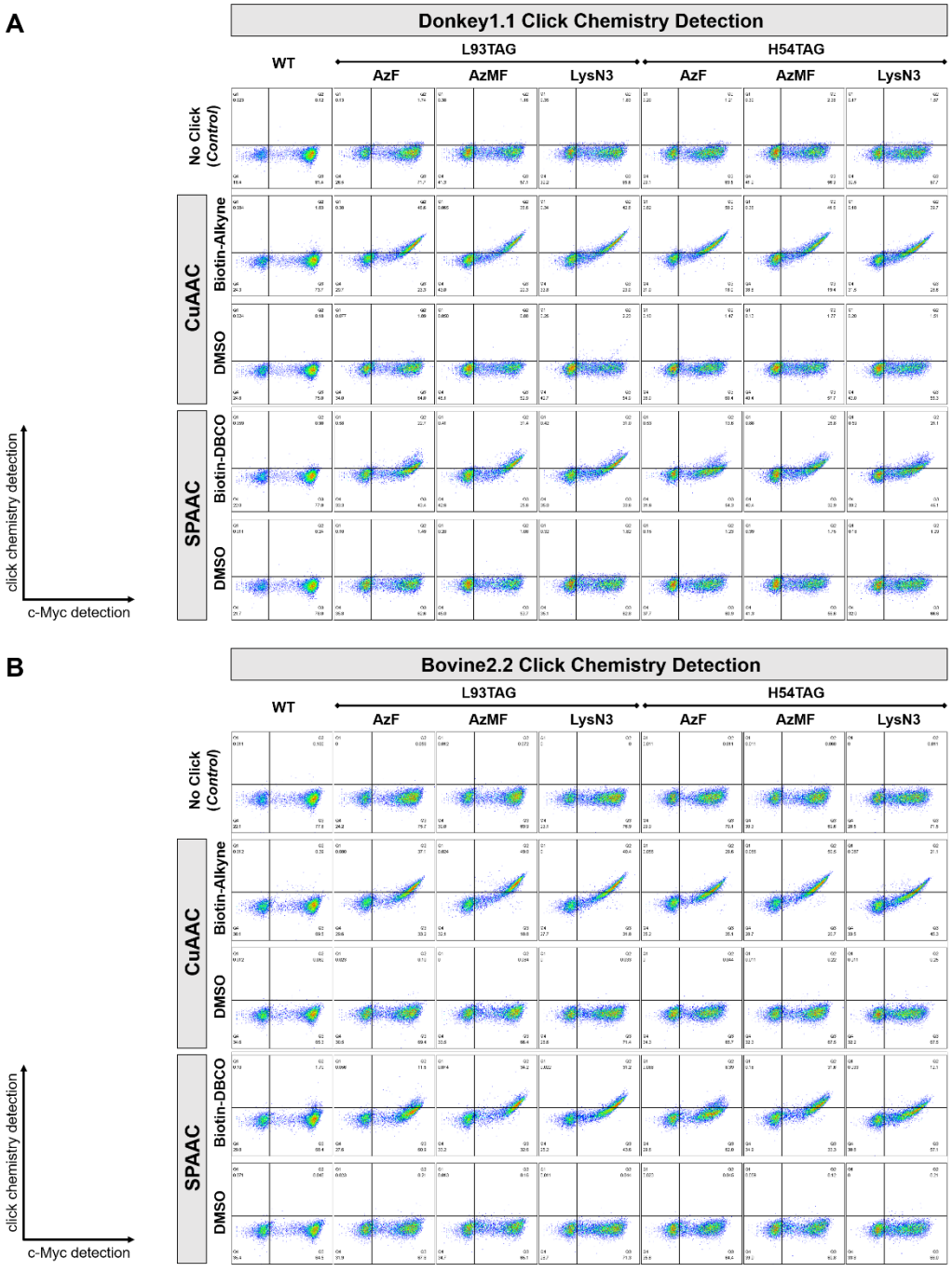

Figure S17. Evaluation of copper-catalyzed azide-alkyne cycloaddition (CuAAC) and strain-promoted azide-alkyne cycloaddition (SPAAC) reactions. A) Reactions performed with Donkey1.1 wild-type and azide-containing clones, corresponding to Figure 5B. B) Reactions performed with Bovine2.2 wild-type and azide-containing clones, corresponding to Supplementary Figure S21A. Reactivity was detected by labeling for biotin. Full-length display was detected by labeling for c-Myc. "No Click (*Control*)" indicates no reaction. CuAAC Biotin-Alkyne and SPAAC Biotin-DBCO represent CuAAC and SPAAC reactions using corresponding biotin probes. CuAAC DMSO and SPAAC DMSO represent reactions using DMSO in place of the respective biotin probes. Each condition represented here was evaluated in technical triplicates.

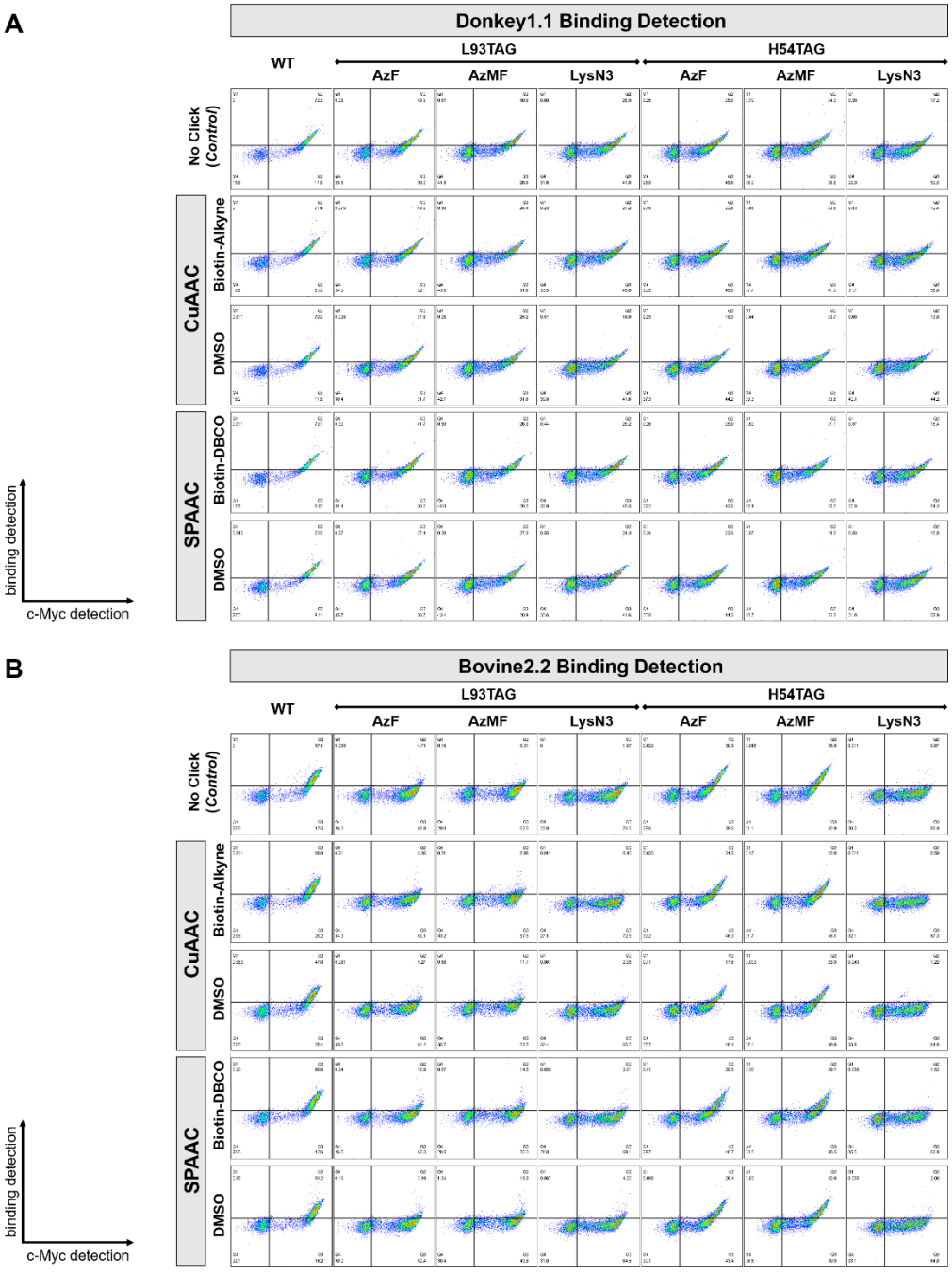

Figure S18. Evaluation of antigen binding after copper-catalyzed azide-alkyne cycloaddition (CuAAC) and strain-promoted azide-alkyne cycloaddition (SPAAC) click chemistry reactions. A) Donkey1.1 wild-type and azide-containing clones treated with unconjugated donkey IgG, corresponding to Figure 5C. B) Bovine2.2 wild-type and azide-containing clones treated with unconjugated bovine IgG, corresponding to Supplementary Figure S21B. Binding was detected by labeling for the respective IgG. Full-length display was detected by labeling for c-Myc. "No Click (*Control*)" represents binding after no click reaction, CuAAC Biotin-Alkyne and SPAAC Biotin-DBCO represent binding after CuAAC and SPAAC reactions using the biotin probes, CuAAC DMSO and SPAAC DMSO represent binding after reactions with DMSO instead of the biotin probes. Each condition represented here was evaluated in technical triplicates.

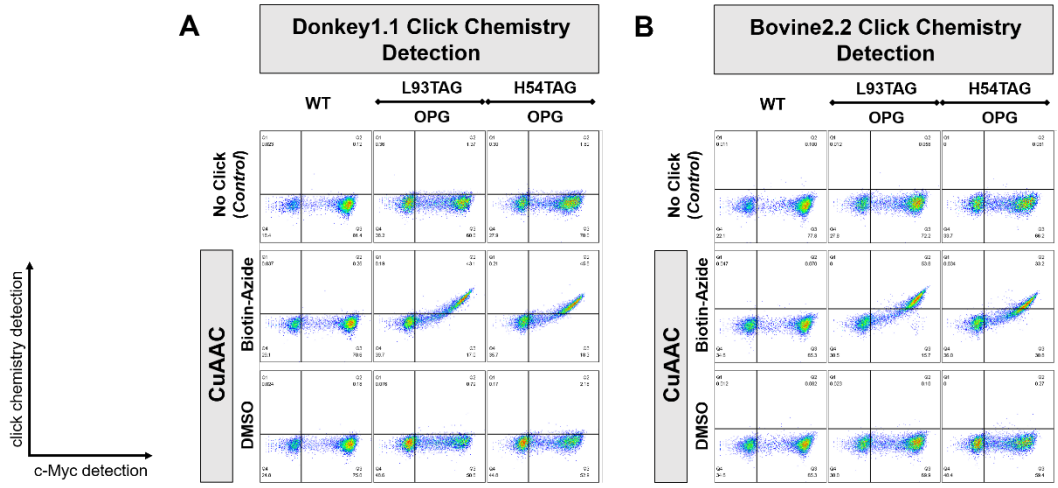

Figure S19. Evaluation of copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. "No Click (*Control*)" represents no reaction, Biotin-Azide represents CuAAC reactions using the biotin-azide probe, DMSO represents CuAAC reactions using DMSO instead of the biotin probe. A) Reactions performed with Donkey1.1 wild-type and alkyne-containing clones, corresponding to Figure 5B. B) Reactions performed with Bovine2.2 wild-type and alkyne-containing clones, corresponding to Supplementary Figure S21A. Reactivity was detected by labeling for biotin. Full-length display was detected by labeling for c-Myc. Each condition represented here was evaluated in technical triplicates.

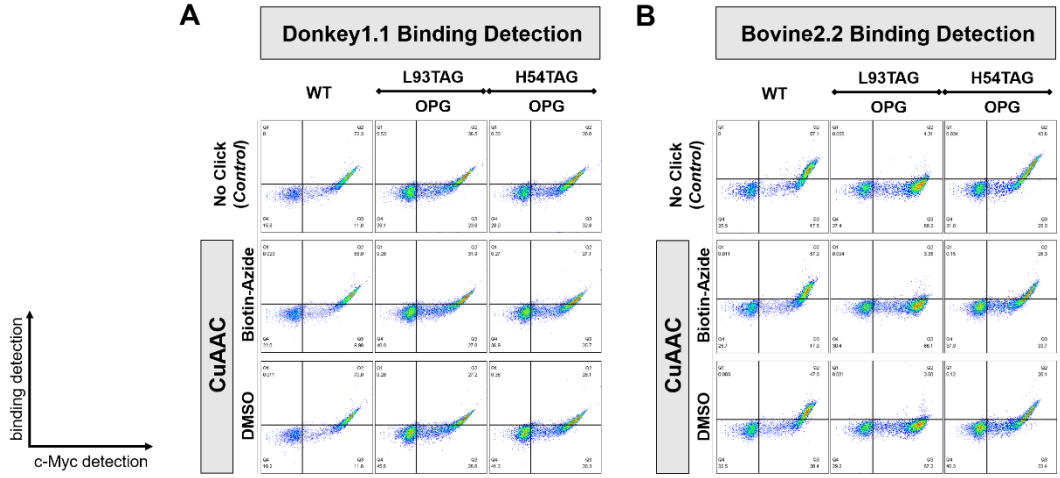

Figure S20. Evaluation of antigen binding after copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. A) Donkey1.1 wild-type and alkyne-containing clones treated with unconjugated donkey IgG, corresponding to Figure 5C. B) Bovine2.2 wild-type and alkyne-containing clones treated with unconjugated bovine IgG, corresponding to Supplementary Figure S21B. Binding was detected by labeling for the respective IgG. Full-length display was detected by labeling for c-Myc. "No Click (*Control*)" represents binding after no click reaction, Biotin-Azide represents binding after CuAAC reactions using the biotin probe, DMSO represents binding after CuAAC reactions with DMSO instead of the biotin probe. Each condition represented here was evaluated in technical triplicates.

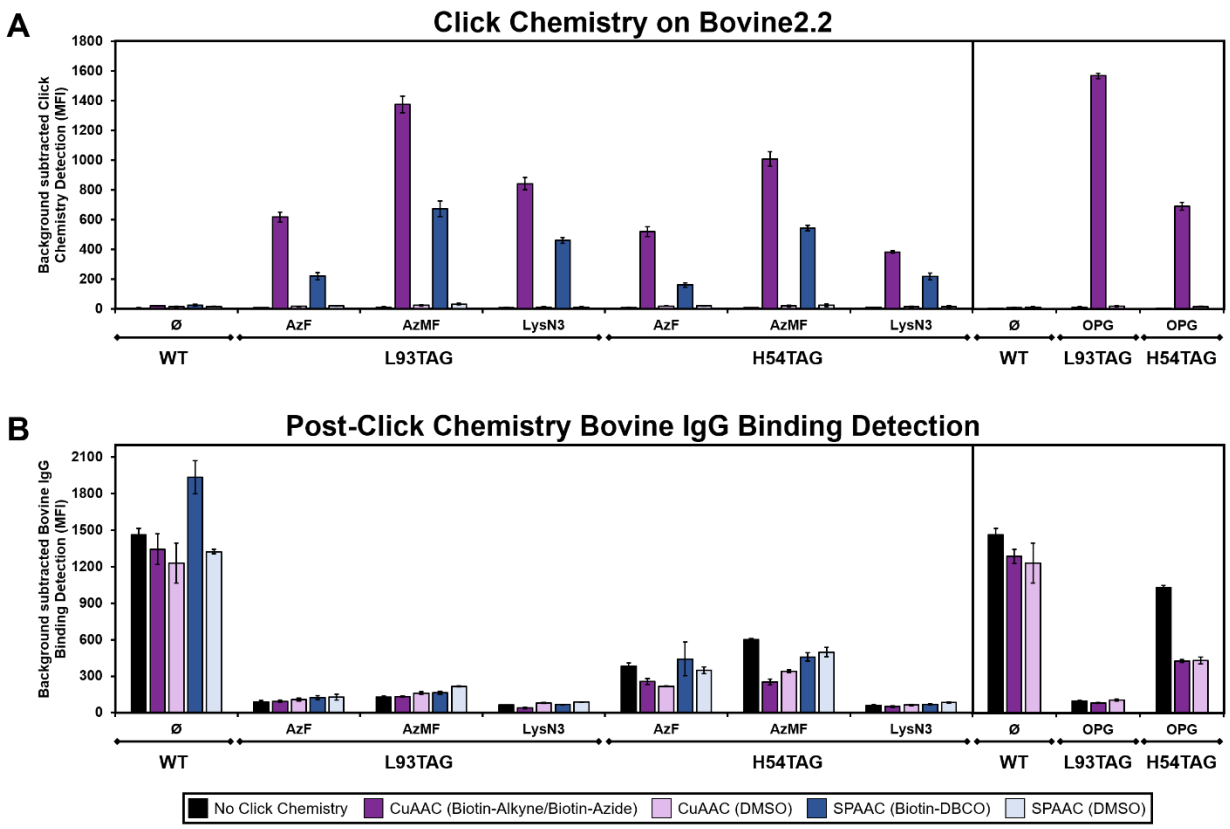

Figure S21. Evaluation of Bovine2.2 copper-catalyzed azide-alkyne cycloaddition (CuAAC) and strain-promoted azide-alkyne cycloaddition (SPAAC) reactivity and subsequent binding detection. A) Background subtracted Median Fluorescence Intensity (MFI) values to determine click chemistry reactivity. B) Background subtracted MFI data for unconjugated bovine IgG detection to determine antigen binding. Values reported here are averages of background subtracted MFI of technical triplicates. Error bars represent the standard deviation.

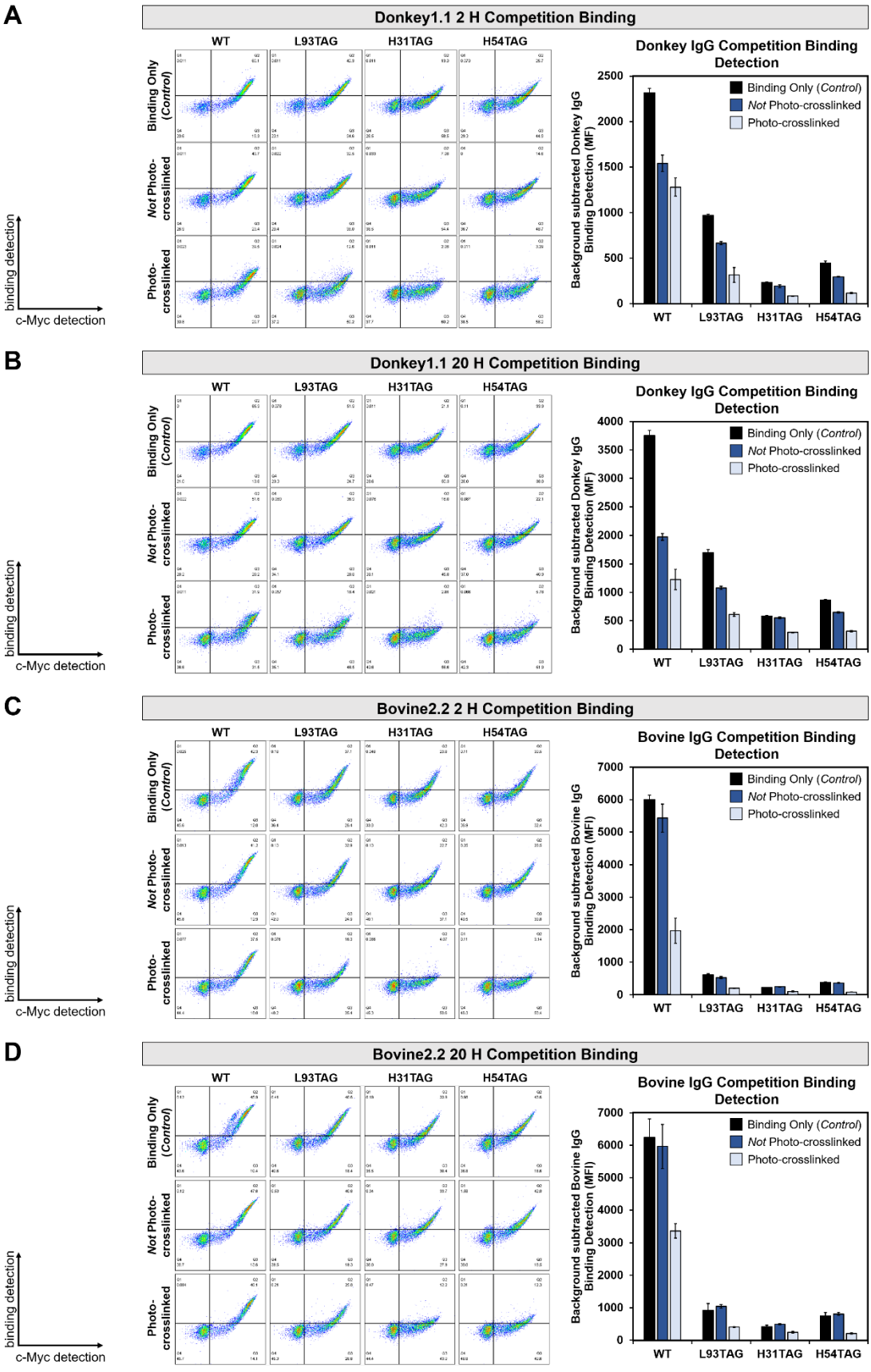
A
Donkey1.1 2 H Competition Binding
WT
L93TAG
H31TAG
H54TAG
Binding Only (Control)
Not Photo-crosslinked
Photo-crosslinked
binding detection
c-Myc detection
Donkey IgG Competition Binding Detection
Background subtracted Donkey IgG Binding Detection (MFI)
Binding Only (Control)
Not Photo-crosslinked
Photo-crosslinked
B
Donkey1.1 20 H Competition Binding
C
Bovine2.2 2 H Competition Binding
Bovine IgG Competition Binding Detection
Background subtracted Bovine IgG Binding Detection (MFI)
D
Bovine2.2 20 H Competition Binding

Figure S22. Photo-crosslinking competition assay with Donkey1.1 and Bovine2.2 wild-type and AzF-containing constructs. A) Donkey1.1 clones incubated with biotinylated donkey IgG for 2 hours following UV irradiation with unconjugated donkey IgG, corresponding to Figure 6B and 6D. B) Donkey1.1 clones incubated with biotinylated donkey IgG for 20 hours following UV irradiation with unconjugated donkey IgG. C) Bovine2.2 clones incubated with biotinylated bovine IgG for 2 hours following UV irradiation with unconjugated bovine IgG, corresponding to Figure 6B and 6D. D) Bovine2.2 clones incubated with biotinylated bovine IgG for 20 hours following UV irradiation with unconjugated bovine IgG. Binding in all cases was detected by labeling for biotinylated antigen. Full-length display was detected by labeling for c-Myc. Binding differences between the control, not photo-crosslinked and photo-crosslinked conditions were used to identify putative photo-crosslinking events. Each condition represented here was evaluated in technical triplicates.

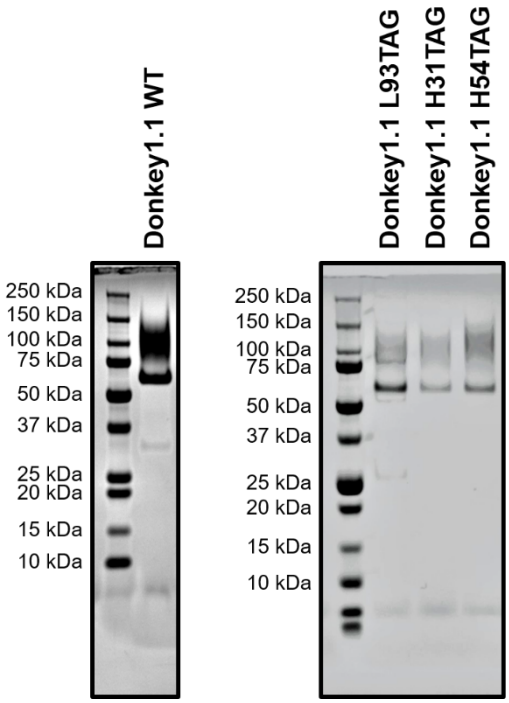

Figure S23. SDS-PAGE analysis of Protein A-purified Donkey1.1 wild-type and azF-containing scFv-Fcs. Gels were loaded under reducing conditions and stained with coomassie SimplyBlue SafeStain.

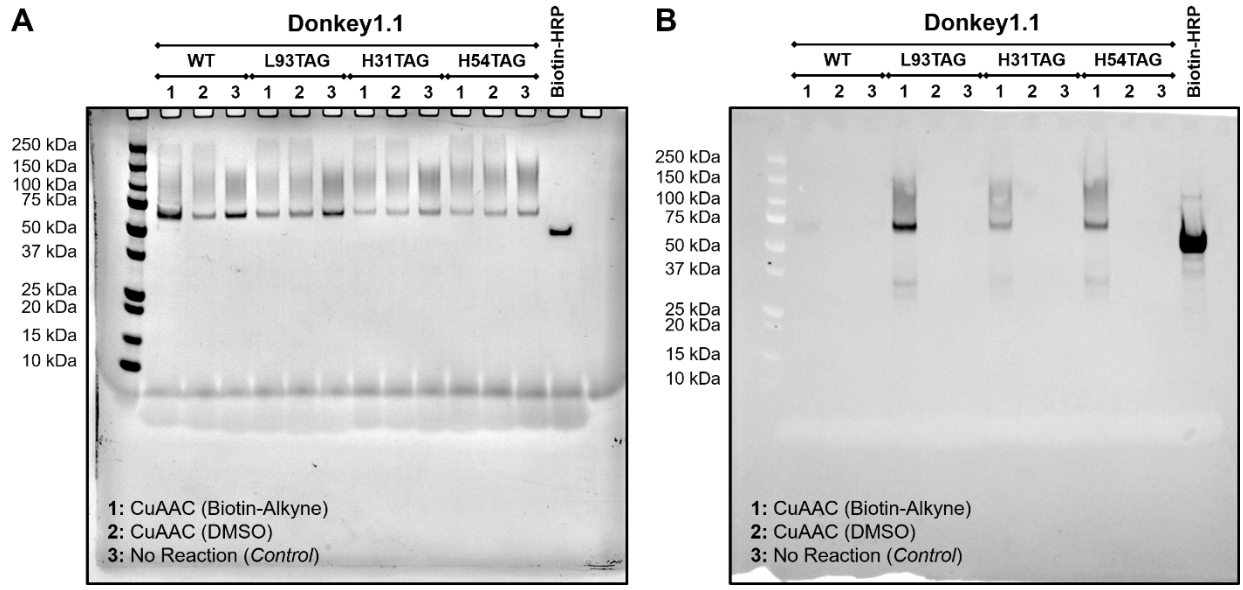

Figure S24. SDS-PAGE and Western Blot analysis of copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions. A) Gel stained with coomassie SimplyBlue SafeStain to evaluate the presence of Donkey1.1 wild-type and AzF-containing scFv-Fcs. B) Western blot analysis of CuAAC reactions. Protein samples transferred to nitrocellulose membranes and then probed for biotin detection.

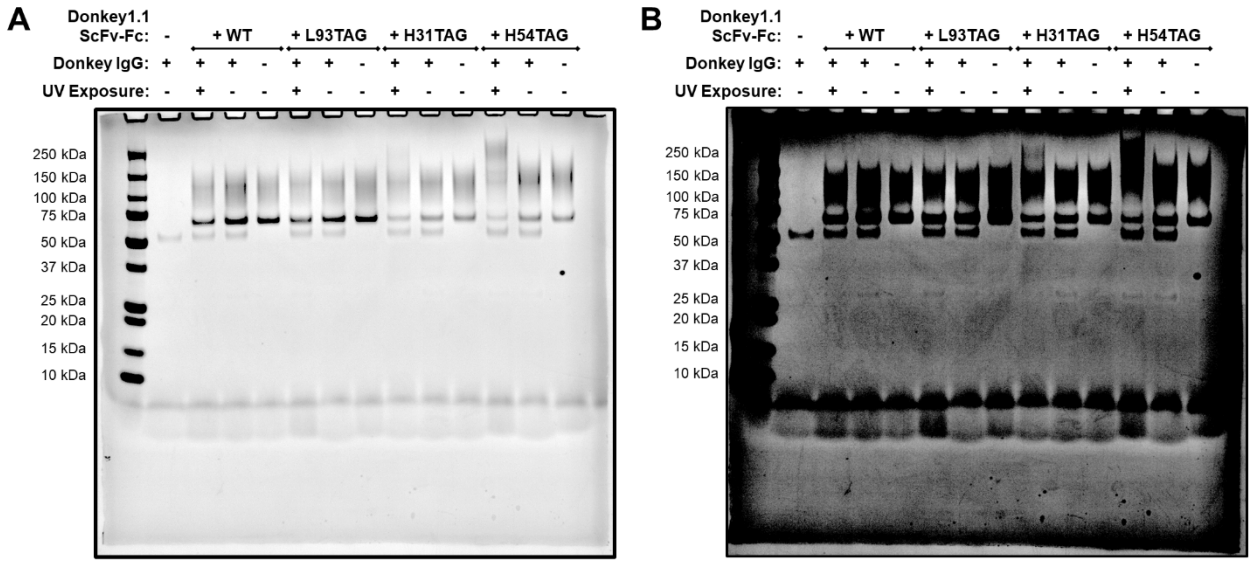

Figure S25. SDS-PAGE analysis of photo-crosslinking. A) Gel stained with coomassie SimplyBlue SafeStain. B) Over-exposed image to confirm the presence of light chain bands (25 kDa).

Supplementary Tables

Table S1. DNA encoding antibody frameworks used to construct the CDR-H3 library. The NheI and BamHI restriction sites introduced before and after the framework to allow cloning into a pCTCON2 vector are highlighted. The diversified CDR-H3 sequence is in the heavy chain after the IGHV3-23 and before the JH4 domains.

Domain	Sequence
VL: IGKV1-39-JK4	GCTAGCGACATACAGATGACTCAAAGTCCCAGTTCACTATCTGCGTCTGTTGGTGATAG AGTCACCATTACGTGTAGAGCTTCTCAGTCGATTAGCTCGTACTTGAATTGGTATCAACA GAAACCAGGGAAAGCTCCAAAGTTGCTGATCTATGCAGCATCTAGCTTACAAAGTGGTG TACCTTCCAGGTTTTCAGGCTCAGGATCTGGAACTGATTTCACACTTACCATATCATCCT TACAACCGGAAGATTTCGCCACATATTACTGCCAACAATCCTACTCTACTCCACCTACAT TTGGTGGTGGCACTAAAGTGGAGATTAAGGGTACTACTGCCGCTAGTGGTAGTAGTGG TGGCAGTAGCAGTGGTGCC
VH: IGHV3-23	GGTACTACTGCCGCTAGTGGTAGTAGTGGTGGCAGTAGCAGTGGTGCCGAGGTGCAAT TGCTAGAATCAGGAGGTGGTTTGGTACAACCTGGTGGTAGCTTAAGGTTGTCTTGTGCT GCTAGTGGATTCACGTTTAGTAGCTATGCCATGTCATGGGTTAGACAAGCTCCAGGTAA AGGCTTAGAATGGGTTTCTGCGATATCTGGATCTGGTGGGTCAACTTACTATGCAGATT CCGTCAAAGGCAGATTTACCATTTCCAGAGACAATTCGAAGAATACACTGTACCTTCAGA TGAACTCGTTACGTGCAGAAGATACTGCTGTTTACTACTGTGCTAAG
VH: JH4	GACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAGGATCC

Table S2. Antibody labeling conditions for flow cytometry and FACS sorting

Detection	Primary Label	Secondary Label
HA epitope tag	Mouse anti-HA	Goat anti-mouse Alexa Fluor 488
c-Myc epitope tag	Chicken anti-cMyc	Goat anti-chicken Alexa Fluor 647
Biotin	N/A	Streptavidin Alexa Fluor 488
Biotin-Donkey IgG binding	Biotin-Donkey IgG	Streptavidin Alexa Fluor 488 **OR** Anti-biotin PE
Donkey IgG binding	Donkey IgG	Rabbit anti-donkey DyLight 488
Biotin-Bovine IgG binding	Biotin-Bovine IgG	Streptavidin Alexa Fluor 488 **OR** Anti-biotin PE
Bovine IgG binding	Bovine IgG	Goat anti-bovine Alexa Fluor 488
Biotin-Rabbit IgG binding	Biotin-Rabbit IgG	Streptavidin Alexa Fluor 488 **OR** Anti-biotin PE
Biotin-TA99 binding	Biotin-TA99	Anti-biotin PE
TA99 binding	TA99	Goat anti-mouse Alexa Fluor 488

Table S3. Primers used for cloning in this work.

Primer name	Sequence (5' to 3')
SidLinkAmpFwd	GGTACTACTGCCGCTAGTGGTAGTAGTGGTG
SidLinkAmpRev	GGCACCACTGCTACTGCCACCACTACTACCAC
SynAmpFwd	GGAGGCGGTAGCGGAGGCGGAGGGTCGGCTAGCGACATACAGATGACTCAAA GTCCCAG
SynAmpRev	GTCCTCTTCAGAAATAAGCTTTTGTTCGGATCCTGAGGAGACGGTGACCAGGGT TCCTTGGCCCCAGTAGTC
CDR9–17Rev[a]	CAGGGTTCCTTGGCCCCAGTAGTCSADASC(z'y'x')$_{5\text{-}13}$ CTTAGCACAGTAGTAAACAGCAGTATCTTC

pCTCON2Fwd	GTTCCAGACTACGCTCTGCAG
pCTCON2Rev	GATTTTGTTACATCTACACTGTT
CDRH3_L93TAGFwd	TTCGCCACATATTACTGCCAACAATCCTACTAGACTCCACCTACATTTGGTGGTG GCACTAAA
CDRH3_L93TAGRev	TTTAGTGCCACCACCAAATGTAGGTGGAGTCTAGTAGGATTGTTGGCAGTAATA TGTGGCGAA
CDRH3_H31TAGFwd	TCTTGTGCTGCTAGTGGATTCACGTTTAGTTAGTATGCCATGTCATGGGTTAGAC AAGCTCCAG
CDRH3_H31TAGRev	CTGGAGCTTGTCTAACCCATGACATGGCATACTAACTAAACGTGAATCCACTAG CAGCACAAGA
CDRH3_H54TAGFwd	GGCTTAGAATGGGTTTCTGCGATATCTGGATAGGGTGGGTCAACTTACTATGCA GATTCCGTC
CDRH3_H54TAGRev	GACGGAATCTGCATAGTAAGTTGACCCACCCTATCCAGATATCGCAGAAACCCA TTCTAAGCC
pCHA_TAAFwd1	ACGCTGCTAGCCAGTCGCATATGACTGAGCCCGGG
pCHA_TAARev1	CCTCCACCAGAGCCTCCTCCACCTTAAGATCTCTTACCTGGAGACAAGGATAAT
pCHA_TAAFwd2	ATTATCCTTGTCTCCAGGTAAGAGATCTTAAGGTGGAGGAGGCTCTGGTGGAGG
pCHA_TAARev2	ACATCTACACTGTTGTTATCAGATTTCGCTCGAG
pCHA_scFvFwd	CCATACGACGTTCCAGACTACGCTGCTAGCGACATACAGATGACTCAAAGTCCC
pCHA_scFvRev	TTTGTCGGAACTTTTAGGTTCTACCCCGGGTGAGGAGACGGTGACCAGGGTTC CTTG
pREP4_KanR_XmaIFwd1	GTATATATGAGTAAACCCGGGCTGACAGCCGGAATTGCCAGCTGGGGCGC
pREP4_KanR_XmaIFwd2	TAAATCAATCTAAAGTATATATGAGTAAACCCGGGCTGACAG
pREP4_KanR_AvrIIRev1	CCCTAGGTGTTTATTTTTCTAAATACATCAGAAGAACTCGTCAAGAAGGCG
pREP4_KanR_AvrIIRev2	CTTTTCGGGGAAATGTGCGCGGAACCCCTAGGTGTTTATTTTTCTAAATACA
pRS315_AmpR_XmaIFwd	TAAATCAATCTAAAGTATATATGAGTAAACCCGGGCTGACAGTTACCAATGCTTA ATCAG
pRS315_Ampp_AvrIIRev	GCGCGGAACCCCTATTTGTTTATTTTCCTAGGTACATTCAAATATGTATCCGCTC ATGTA
NcoI_RemovalFwd	CATGCCCGACGGCGAGGATCTCGTCGTGACTCACGGCGATGCCTGCTTGCCGA ATATCAT
NcoI_RemovalRev	ATGATATTCGGCAAGCAGGCATCGCCGTGAGTCACGACGAGATCCTCGCCGTC GGGCATG

[a]The CDR9-17Rev primers only varied in the number of XYZ codons, which are labeled as the reverse complement z'y'x' (these primers are used to add CDR-H3 sequences at the 3' end of PCR products and amplify in the reverse direction).

Table S4. Summary of sequencing data collected by CDR-H3 sublibrary.

Sublibrary	Clones sequenced	Unique clones	Unique clones with designed size	Truncated clones	Clones with indels	Base pair mutations in constant region	Indel mutations in constant region
CDR9	12	12	11	1	1	1	1
CDR10	10	10	10	0	0	2	0
CDR11	10	10	7	2	2	1	0
CDR12	9	9	7	3	2	0	0
CDR13	8	8	8	0	0	1	0
CDR14	10	10	9	2	1	0	1
CDR15	12	12	10	2	2	1	1

CDR16	12	12	10	3	2	0	1
CDR17	12	12	10	5	2	1	0
Total	95	95	82	18	12	7	4

Table S5. Experimental conditions for binding characterizations of clones isolated from the library, corresponding to Supplementary Figure S4 and S5.

Display Plasmids			**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.2 WT	pCTCON2-Donkey1.3 WT	Donkey IgG
pCTCON2-Bovine1.1 WT	pCTCON2-Bovine1.2 WT	pCTCON2-Bovine1.3 WT	Bovine IgG
pCTCON2-Bovine2.1 WT	pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.3 WT	
pCTCON2-Rabbit1.1 WT	pCTCON2-Rabbit1.2 WT	pCTCON2-Rabbit1.3 WT	Rabbit IgG
pCTCON2-Rabbit1.4 WT	–	–	

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:250)
Donkey IgG binding	Biotin-Donkey IgG (50 nM)	Streptavidin Alexa Fluor 488 (1:250) **OR** Anti-biotin PE (1:250)
Bovine IgG binding	Biotin-Bovine IgG (50 OR 250 nM)	Streptavidin Alexa Fluor 488 (1:250) **OR** Anti-biotin PE (1:250)
Rabbit IgG binding	Biotin-Rabbit IgG (50 nM)	Streptavidin Alexa Fluor 488 (1:250) **OR** Anti-biotin PE (1:250)

Table S6. Experimental conditions for binding crossreactivity assay corresponding to Figure 3A and Supplementary Figure S6.

Display Plasmids			**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.2 WT	pCTCON2-Donkey1.3 WT	Donkey IgG
			Bovine IgG
			Rabbit IgG
pCTCON2-Bovine2.1 WT	pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.3 WT	Donkey IgG
			Bovine IgG
			Rabbit IgG
pCTCON2-Rabbit1.1 WT	pCTCON2-M0076 WT	–	Donkey IgG
			Bovine IgG
			Rabbit IgG

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)

Donkey IgG binding	Biotin-Donkey IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)
Bovine IgG binding	Biotin-Bovine IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)
Rabbit IgG binding	Biotin-Rabbit IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)

Table S7. Aminoacyl tRNA synthetase/tRNA and corresponding noncanonical amino acid combinations used in this study.

Clone	NcAA Incorporation Site	aaRS/tRNA	NcAA in Induction Media
Donkey1.1	L93	AcFRS	*p*-azido-L-phenylalanine (AzF)
			p-propargyloxyphenylalanine (OPG)
		LeuOmeRS	*p*-azidomethyl-L-phenylalanine (AzMF)
			p-propargyloxyphenylalanine (OPG)
		LeuRS BH5 T252A	H-L-Lys(EO-N3)-OH (LysN3)
	H31	*Same as above*	
	H54	*Same as above*	
Bovine2.1	*Same as above*		
Bovine2.2	*Same as above*		
Rabbit1.1	*Same as above*		

Table S8. Experimental conditions for binding and full-length display analysis of all ncAA containing and corresponding wild-type scFvs (Figure 4C and D, Supplementary Figures S8–S10).

Display Plasmids				**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	pCTCON2-Donkey1.1 H31TAG	pCTCON2-Donkey1.1 H54TAG	Donkey IgG
pCTCON2-Bovine2.1 WT	pCTCON2-Bovine2.1 L93TAG	pCTCON2-Bovine2.1 H31TAG	pCTCON2-Bovine2.1 H54TAG	Bovine IgG
pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.2 L93TAG	pCTCON2-Bovine2.2 H31TAG	pCTCON2-Bovine2.2 H54TAG	
pCTCON2-Rabbit1.1 WT	pCTCON2-Rabbit1.1 L93TAG	pCTCON2-Rabbit1.1 H31TAG	pCTCON2-Rabbit1.1 H54TAG	Rabbit IgG

Noncanonical Amino Acid Incorporation Strategy					
Suppressor Plasmid	pRS315-KanRmod-AcFRS		pRS315-LeuOmeRS		pRS315-LeuRS BH5 T252A
NcAA used in Induction Media (1 mM of L-isomer)	AzF	OPG	AzMF	OPG	LysN3

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)
Donkey IgG binding	Biotin-Donkey IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)

Bovine IgG binding	Biotin-Bovine IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)
Rabbit IgG binding	Biotin-Rabbit IgG (200 nM)	Streptavidin Alexa Fluor 488 (1:500)

Table S9. Experimental conditions for relative readthrough efficiency (RRE) and maximum misincorporation frequency (MMF) analysis corresponding to Figure 4E and Supplementary Figure S14.

Display Plasmids		
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	
pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.2 L93TAG	
Noncanonical Amino Acid Incorporation Strategy		
Suppressor Plasmid	pRS315-KanRmod-AcFRS	pRS315-LeuOmeRS
NcAA used in Induction Media (1 mM of L-isomer)	AzF	OPG

Antibody Labeling Conditions		
Detection	**Primary Label (dilution)**	**Secondary Label (dilution)**
HA epitope tag	Mouse anti-HA (1:500)	Goat anti-mouse Alexa Fluor 488 (1:500)
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)

Table S10. Relative readthrough efficiency (RRE) and maximum misincorporation frequency (MMF) values corresponding to the bar graph in Figure 4E and Supplementary Figure S14.

Sample	**RRE**				**RRE STDEV**			
	AcFRS		LeuOmeRS		AcFRS		LeuOmeRS	
	(-)	AzF	(-)	OPG	(-)	AzF	(-)	OPG
Donkey1.1 L93TAG	0.006095	0.500977	0.006547	0.710824	0.000809	0.073941	0.001133	0.067267
Bovine2.2 L93TAG	0.005890	0.465266	0.006804	0.770934	0.001264	0.102367	0.000676	0.042327

Sample	**MMF**		**MMF STDEV**	
	AcFRS	LeuOmeRS	AcFRS	LeuOmeRS
	AzF	OPG	AzF	OPG
Donkey1.1 L93TAG	0.012167	0.009211	0.002414	0.001816
Bovine2.2 L93TAG	0.012658	0.008825	0.003890	0.001002

Table S11. Experimental conditions for binding titration analysis corresponding to Tables 2 and 3, Figure 3B, and Supplementary Figures S7, S11 and S12.

Display Plasmids				**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	pCTCON2-Donkey1.1 H31TAG	pCTCON2-Donkey1.1 H54TAG	Donkey IgG

pCTCON2-Bovine2.1 WT	pCTCON2-Bovine2.3 WT	–	–	Bovine IgG
pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.2 L93TAG	pCTCON2-Bovine2.2 H31TAG	pCTCON2-Bovine2.2 H54TAG	
pCTCON2-Rabbit1.1 WT	–	–	–	Rabbit IgG

Noncanonical Amino Acid Incorporation Strategy		
Suppressor Plasmid	pRS315-KanRmod-AcFRS	pRS315-LeuOmeRS
NcAA used in Induction Media (1 mM of L-isomer)	AzF	OPG

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)
Donkey IgG binding	Biotin-Donkey IgG ([a])	Streptavidin Alexa Fluor 488 (1:500)
Bovine IgG binding	Biotin-Bovine IgG ([a])	Streptavidin Alexa Fluor 488 (1:500)
Rabbit IgG binding	Biotin-Rabbit IgG ([a])	Streptavidin Alexa Fluor 488 (1:500)

[a]Various concentrations starting from 1 μM with subsequent 4-fold dilutions

Table S12. Experimental conditions for copper-catalyzed azide-alkyne cycloaddition (CuAAC) time point and concentration assays (Supplementary Figure S15).

Display Plasmids	
pCTCON2-FAPB2.3.6 WT	pCTCON2-FAPB2.3.6 L1TAG
Noncanonical Amino Acid Incorporation Strategy	
Suppressor Plasmid	pRS315-AcFRS
NcAA used in Induction Media (1 mM of L-isomer)	AzF

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:250)
Biotin	N/A	Streptavidin Alexa Fluor 488 (1:250)

Table S13. Experimental conditions for strain-promoted azide-alkyne cycloaddition (SPAAC) time point assays (Supplementary Figure S16).

Display Plasmids		
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	pCTCON2-Donkey1.1 H31TAG
pCTCON2-Donkey1.1 H54TAG	pCTCON2-Bovine2.2 WT	–
Noncanonical Amino Acid Incorporation Strategy		

Suppressor Plasmid	pRS315-KanRmod-AcFRS	
NcAA used in Induction Media (1 mM of L-isomer)	AzF	
Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)
Biotin	N/A	Streptavidin Alexa Fluor 488 (1:500)

Table S14. Experimental conditions for click chemistry analysis on the yeast surface corresponding to Figure 5 and Supplementary Figures S17–S21.

Display Plasmids			**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	pCTCON2-Donkey1.1 H54TAG	Donkey IgG
pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.2 L93TAG	pCTCON2-Bovine2.2 H54TAG	Bovine IgG

Noncanonical Amino Acid Incorporation Strategy				
Suppressor Plasmid	pRS315-KanRmod-AcFRS	pRS315-LeuOmeRS		pRS315-LeuRS BH5 T252A
NcAA used in Induction Media (1 mM of L-isomer)	AzF	AzMF	OPG	LysN3

Antibody Labeling Conditions		
Detection	**Primary Label (dilution/concentration)**	**Secondary Label (dilution)**
c-Myc epitope tag	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)
Donkey IgG binding	Donkey IgG (200 nM)	Rabbit anti-donkey DyLight 488 (1:500)
Bovine IgG binding	Bovine IgG (200 nM)	Goat anti-bovine Alexa Fluor 488 (1:500)
Biotin	N/A	Streptavidin Alexa Fluor 488 (1:500)

Table S15. Experimental conditions for photo-crosslinking analysis on the yeast surface corresponding to Figure 6 and Supplementary Figure S22.

Display Plasmids				**Target Antigen**
pCTCON2-Donkey1.1 WT	pCTCON2-Donkey1.1 L93TAG	pCTCON2-Donkey1.1 H31TAG	pCTCON2-Donkey1.1 H54TAG	Donkey IgG
pCTCON2-Bovine2.2 WT	pCTCON2-Bovine2.2 L93TAG	pCTCON2-Bovine2.2 H31TAG	pCTCON2-Bovine2.2 H54TAG	Bovine IgG

Noncanonical Amino Acid Incorporation Strategy	
Suppressor Plasmid	pRS315-KanRmod-AcFRS
NcAA used in Induction Media (1 mM of L-isomer)	AzF
Antibody Labeling Conditions	

Detection	Initial Binding (concentration)	Primary Label (dilution/concentration)	Secondary Label (dilution)
c-Myc epitope tag	N/A	Chicken anti-cMyc (1:500)	Goat anti-chicken Alexa Fluor 647 (1:500)
Donkey IgG binding	Donkey IgG (200 nM)	Biotin-Donkey IgG (500 nM)	Streptavidin Alexa Fluor 488 (1:500)
Bovine IgG binding	Bovine IgG (200 nM)	Biotin-Bovine IgG (500 nM)	Streptavidin Alexa Fluor 488 (1:500)

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