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Overview

The AbNano™ VHH Naïve Library is a fully natural, single domain naïve library with source material derived from camelid source material and the antibodies displayed on monovalent phage. It is intended for research purposes only to aid in the discovery of VHH single-domain antibodies. VHH single-domain antibodies may be used as therapeutics, in diagnostic applications, and as research tools. This document provides basic information about library construction, physical deliverables, quality control data including pre-selection sequencing and post-selection binder characterization on example antigens, phagemid information, and relevant protocols.

Library Construction

The library was constructed from 103 naïve animals: 77 llamas and 26 alpacas. PBMCs were isolated and preserved in RNeasy Lysis Buffer. A total of 1.51×10^{10} PBMC cells were collected and used for library construction. Libraries were constructed following Abcore's internal workflows for RNA isolation, cDNA synthesis and PCR amplification using species-specific primers. Transformants were analyzed initially by Sanger sequencing. Sanger analysis demonstrated 89% of VHH sequences in-frame. The library was subsequently analyzed by Illumina MiSeq and a total of 1.71 M reads were clustered and analyzed for diversity. Library diversity quantified by MiSeq analysis (CDR3 length distribution, CDR3 cluster density, and CDR composition diversity) meets internal standards for diversity QC (see QC Data section below). The total number of transformants for the library was 1.12×10^{10} cfu. Library bacterial stocks were grown to log phase, infected by M13KO7, PEG precipitated, and the pooled phage was titered. Phage were normalized in PBS + 20% glycerol to 1×10^{11} pfu/mL and aliquoted as 1 mL tubes frozen and stored at -80°C . The library is delivered as 10 x 1 mL aliquots of 1×10^{11} pfu/mL in PBS + 20% glycerol. Each aliquot should be fully thawed prior to use, but we recommend using the entire aliquot the day it is thawed. Avoid freeze-thaw cycles.

Sequencing QC Data

The library was separately sequenced using both Sanger methods (837 total reads) and Illumina MiSeq NGS (1.71 M total reads). The NGS reads for each sublibrary were analyzed as a pool using Geneious Biologics software to generate several QC metrics. The NGS data was analyzed to determine several characteristics of the library, including germline V-gene sampling, diversity of sequences by cluster homology and redundancy, and profiles of CDR regions within the VHH domains.

To analyze V-gene representation, each individual sequence was mapped to IMGT reference sequences in the alpaca germline by using Geneious Biologics. To analyze sequence diversity, we compared the similarity of nucleotide sequences with amino acid sequences in the library to better understand the sequence level differences in the diverse sequences. From this analysis, we would be able to see if individual point mutations on the DNA level were silent, such as from polymerase errors or NGS base call errors, and make sure that diversity in sequence space was conserved and accurately represented between DNA and amino acid levels. We also measured the frequency of individual CDR3 sequences on an amino acid level to demonstrate a lack of PCR bias or non-uniform ligation sampling in the cloning and construction of the libraries.

To characterize the CDR regions within the VHH domains, we performed a position-weight-matrix analysis on the amino acid identity of the most abundant length for each CDR region within the library. CDR3 length sampling is a very important characteristic in VHH libraries, and the length sampling with raw counts is provided below.

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V-gene representation and relative abundance

Variable (V) genes of immunoglobins are responsible for the diversity and eventual affinity that drive immunoglobulin function in immune response. In camelid, different germ lines are responsible for the heavy-chain-only antibodies from which VHH are derived. Differences in the characteristics of VHH on a binding level and their corresponding V-gene is an area of active research, and the germline derivations of libraries is an important consideration for current and future VHH development. V-gene representation from the camelids was analyzed and 99.92% of the NGS reads map to IGHV3. The remaining 0.08% of sequences map to IGHV1 (0.02%) and IGHV4 (0.06%). IGHV3 breakdown is shown, with the top representation coming from IGHV3S53 (32.69%), IGHV3-3 (19.44%), and IGHV3S61 (5.36%). This bias is not unexpected and has been reflected previously in camelid sequencing datasets.^{1,2}

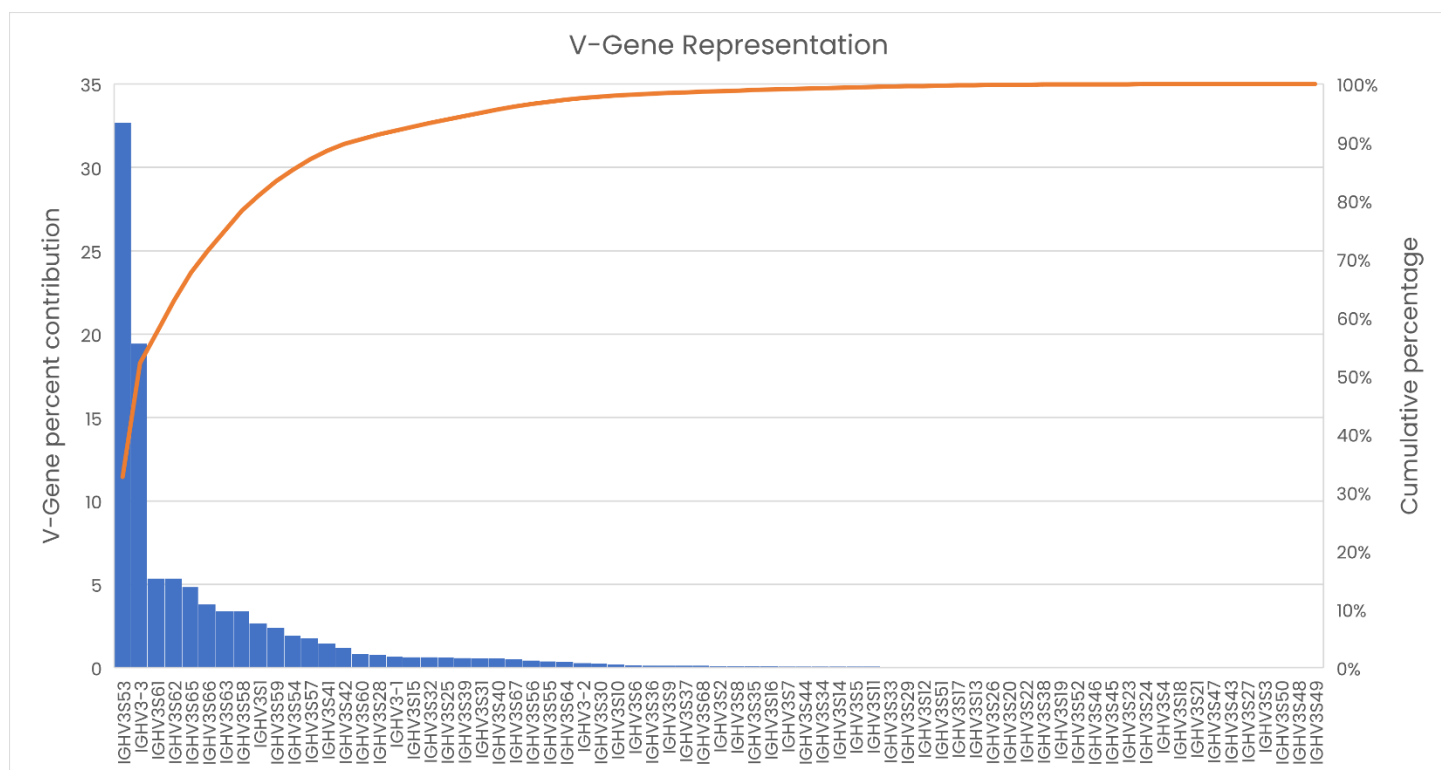


Figure 1. V-gene usage from the NGS dataset analysis, assigned by Geneious Biologics using IMG[®] annotation.

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Characterization of CDR3 diversity based on length

CDR3 length variability is a fundamental characteristic of VHH domains and allows differentiation in epitope binding as well as potential to access cryptic epitopes that would not be accessible in paired IgG interfaces. To characterize CDR3 length distribution in the AbNano VHH Naïve library, each amino acid sequence from the NGS dataset was clustered and the CDR3 region was plotted by length. CDR3 length distribution of the pooled naïve library fits well with existing literature values for naïve camelid CDR3 and has good representation of CDR3 lengths from 5-22 residues in length.¹ The distribution of this data is similar to representative human CDRH3 length distributions which may improve the potential for therapeutic application.²⁻⁴

CDR3 cluster lengths Naïve VHH Library

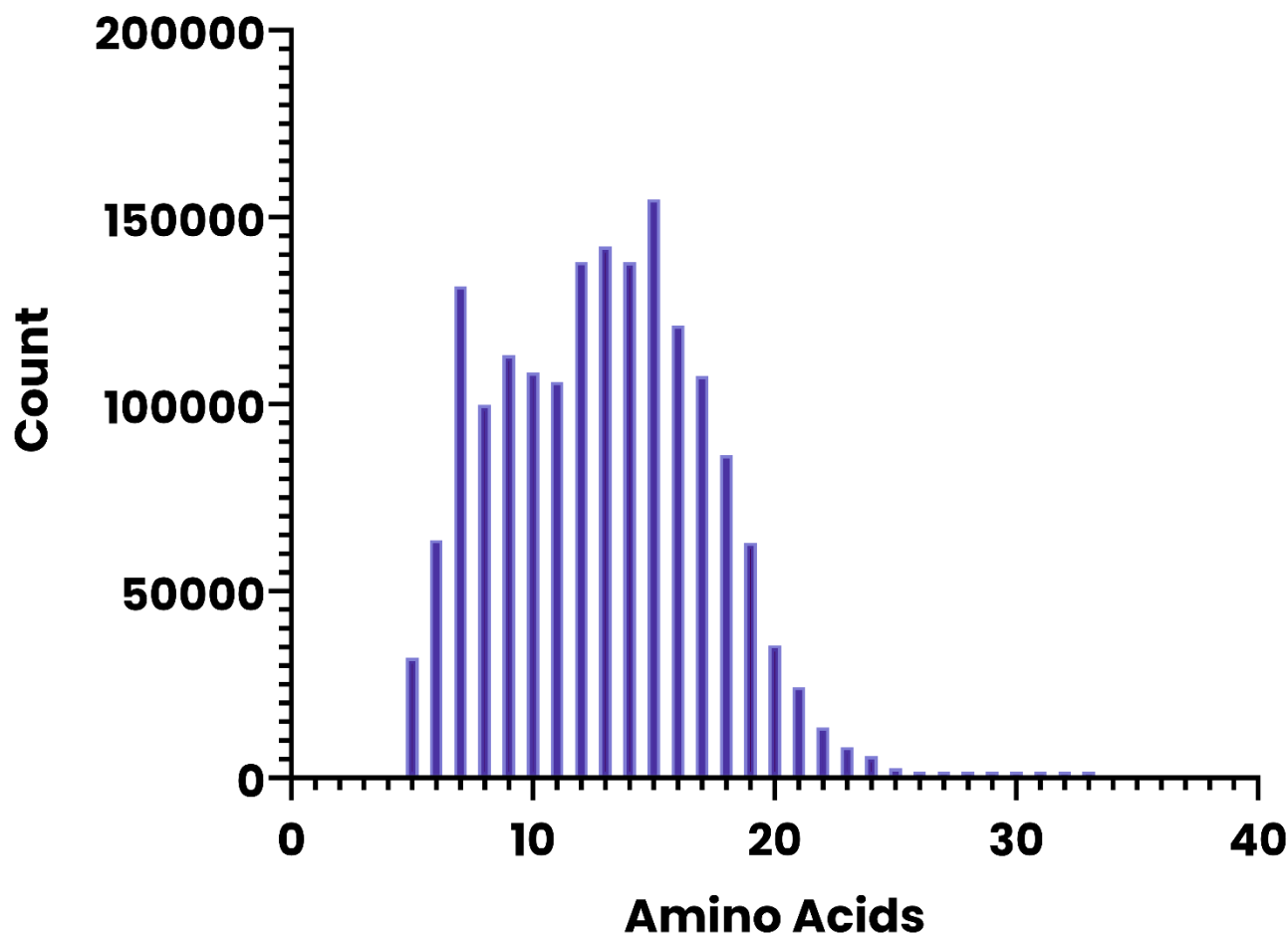


Figure 2. CDR3 amino acid length histogram with raw counts of each length.

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Characterization of CDR3 diversity by cluster density

Diversity of CDR3 amino acids was analyzed by a cluster-density function. The cluster density function measures the redundancy or bias of the individual sublibraries relative to one another in the final pool and may also function as a metric for PCR bias introduced during the library construction or sequencing processes. To control for the potential of NGS base-call errors, the cluster analysis was repeated on a nucleotide level using a 95% homology filter cutoff. The general trends for both the CDR3 amino acid cluster density and the nucleotide homology cluster density are the same, indicating that the CDR3 diversity analysis is not due to experimental artifacts from the NGS read technologies used in these characterizations.

CDR3 Cluster Diversity

Amino Acid and Nucleotide (95% homology)

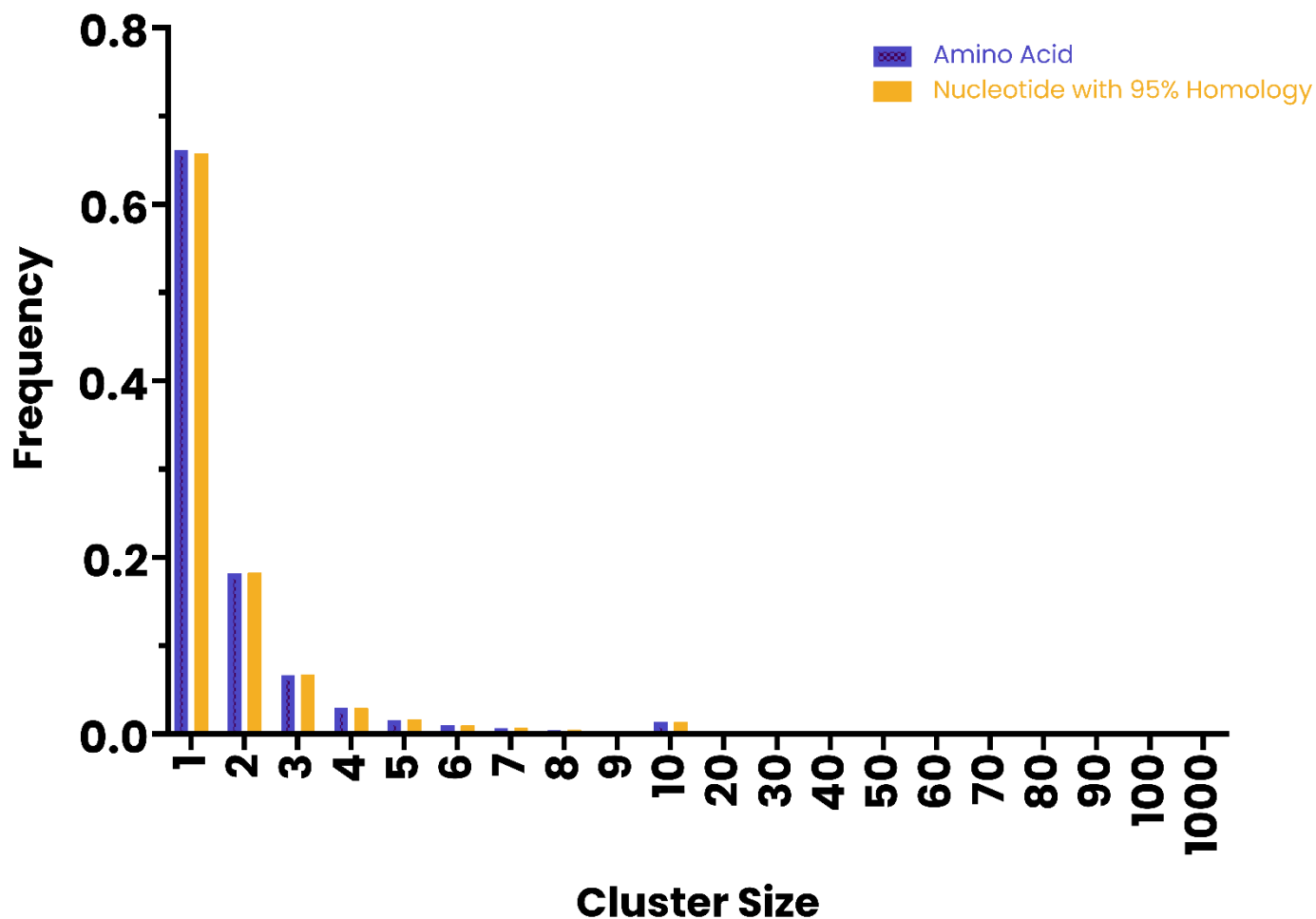


Figure 3. Comparison of cluster densities in amino acid assignment and nucleotide assignment using a 95% homology filter. This comparison helps to better characterize the distance in sequence space between the individual CDR3 clusters in the final library.

Characterization of CDR3 diversity by cluster density

Diversity of the CDR3 amino acids was analyzed by plotting frequency of CDR3 in the final NGS dataset. This metric is indicative of PCR bias in library construction, and multiple high-abundance clones in the final pool will skew the library selection process or may require a greater over-representation of the library in the initial panning rounds to account for a non-uniform representation of rarer clonotypes. This data suggests a very minute bias and are representative of a library that is cloned with minimal artifacts from a PCR cloning bias standpoint.

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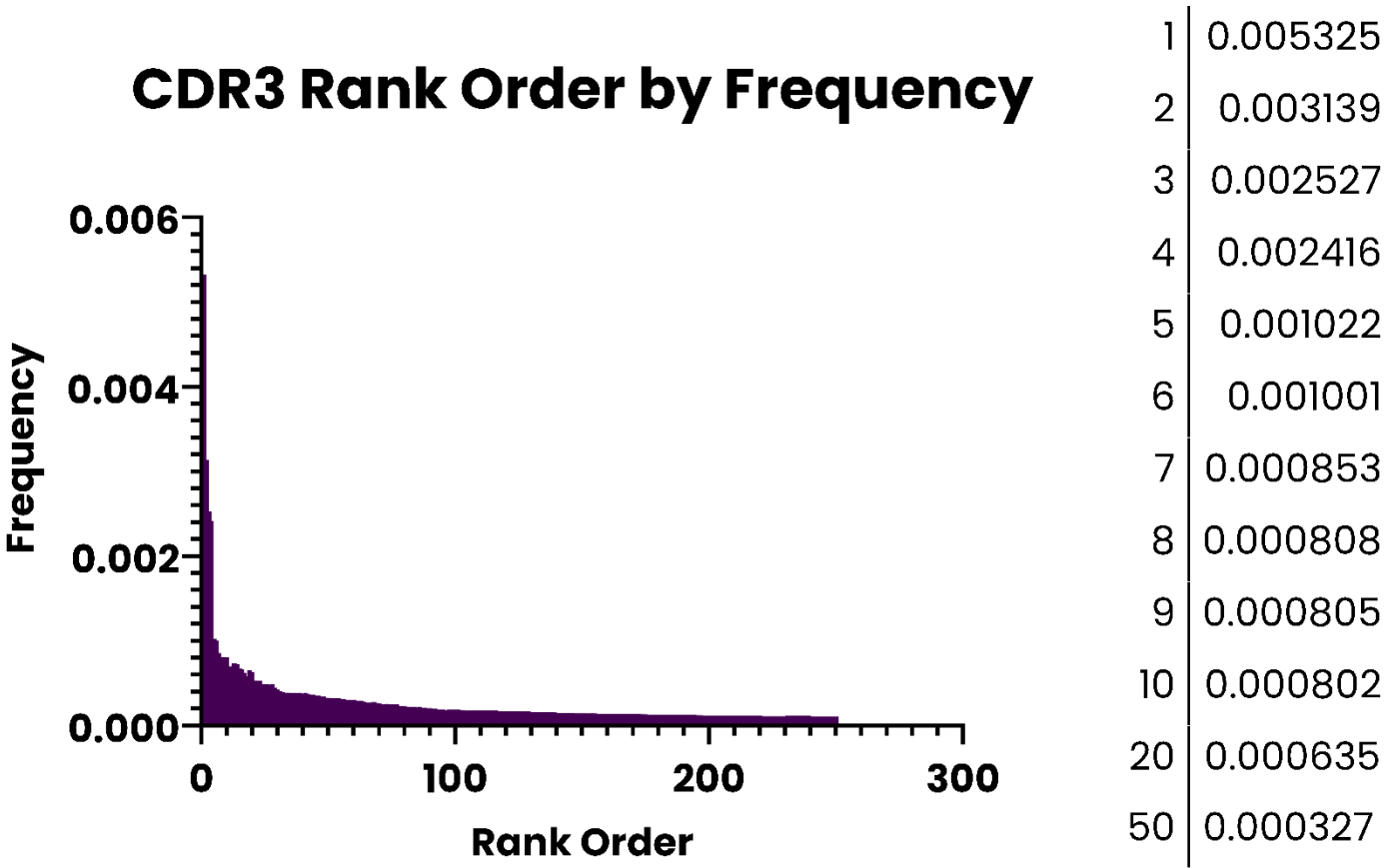


Figure 4. Percent abundance of the top 250 CDR3 amino acid sequences plotted, with specific rank order frequencies identified and given at right.

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Characterization of CDR diversity by position-specific weight matrix

Individual position-specific amino acid distribution was analyzed for each CDR. A representative distribution of each amino acid constructed from position-weight matrices generated during the NGS clustering is provided below with Kabat numbering schema for reference. CDR1 and CDR2 length distributions were minimal, as expected. CDR1 and CDR2 have much less diverse amino acid distribution in literature than CDR3, and the convergence at certain positions is not uncommon. Some evidence exists that these conservations correlate with secondary structural folds to allow the VHH the adaptation of different binding modes.⁵

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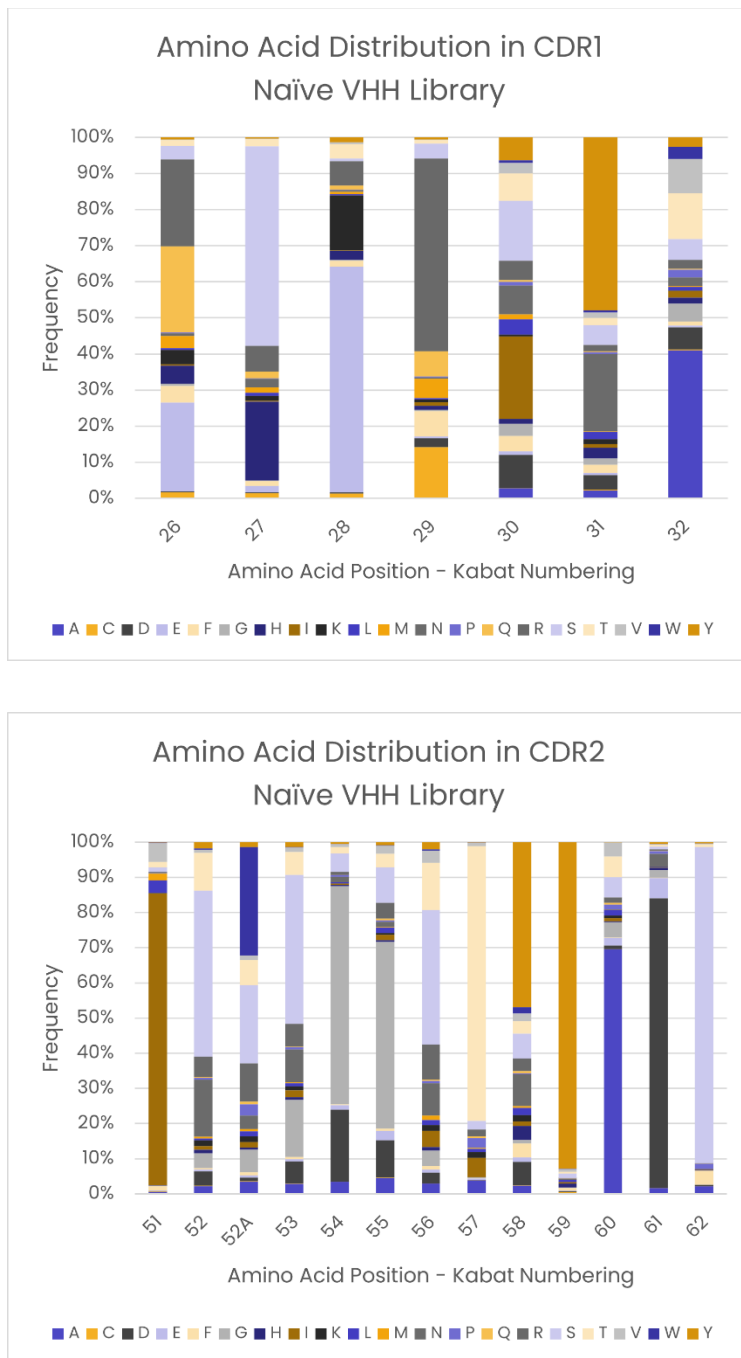


Figure 5. Amino acid distribution for CDR1 and CDR2 by Kabat position number in the VHH library. The individual identities are in alphabetical order from bottom to top, such that alanine appears nearest the baseline and tryptophan at the axis maximum.

Of note, CDR3 distribution is reasonably unbiased with good contributions from small, polar, and aromatic residues that are demonstrated to be fundamental in CDR3 interactions. Additionally, there is a significant cysteine contribution in CDR3 that contributes to a non-canonical second disulfide bond in the VHH architecture. Cysteines are observed in literature to occur in CDR3 both in germline and in functional

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binders.^{6,7} These contributions introduce additional binding modes into the VHH library and expand the potential candidate targets.

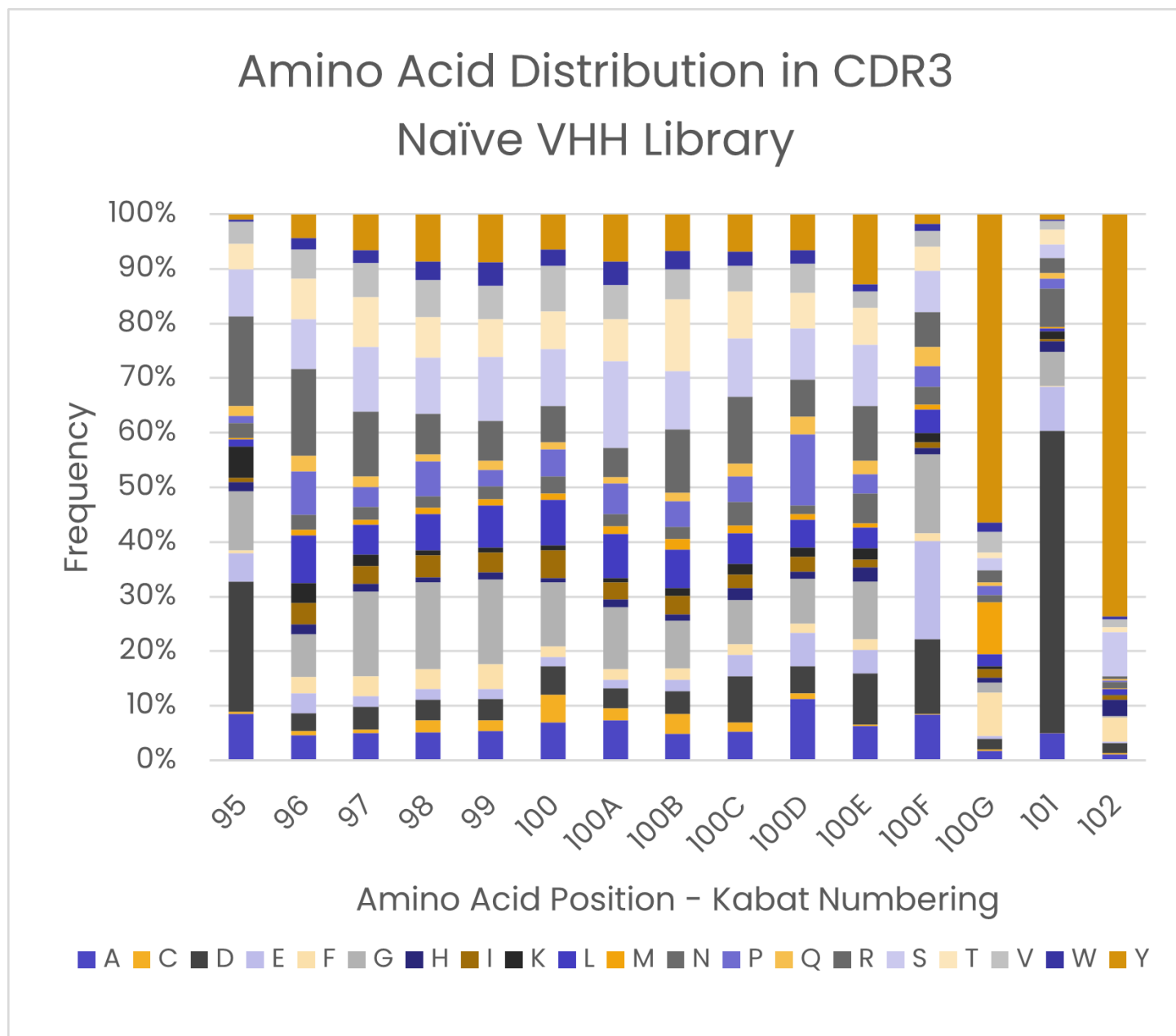


Figure 6. Amino acid distribution for the 15-amino acid length of CDR3 by Kabat position number in the VHH library. The individual identities are in alphabetical order from bottom to top, such that alanine appears nearest the baseline and tryptophan at the axis maximum.

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Representative Panning Campaigns with the naïve VHH library

To validate the discovery potential of the naïve VHH library, multiple targets were identified for phage display panning affinity selections. Three rounds of panning were performed against three recombinant targets. First, we targeted two extracellular domains of receptor tyrosine kinase complexes, the insulin receptor *INSR* β isoform and epidermal growth factor receptor *EGFR* canonical isoform, as potential targets for signaling studies and therapeutic targets in cell growth and metabolism. Separately, we targeted programmed death-ligand 1 *PD-L1* as a signaling molecule in immune response and a therapeutic target of interest as a cancer marker. Following panning, the phage pool was minipreped and transformed into the non-amber-suppressor strain of *E. coli* SS320 (MC1061F'). 94 random clones were picked, and crude periplasmic shockate VHH was assayed using a single point ELISA. High-signal clones were chosen from the single-point ELISA data and purified, then titrated against immobilized panning antigen to generate EC₅₀ binding curves. Curves were fit using GraphPad Prism® by a sigmoidal nonlinear fit function.

Solid-phase panning of Human Insulin Receptor (INSR).

Recombinant protein of the extracellular domain of the human insulin receptor (Acro Biosystems #INR-H52Ha), consisting of residues H28-K944 of accession number P06213-2 with a C-terminal His tag, was immobilized by adsorption onto polystyrene plates. Phage was eluted using triethylamine after panning, and each output was amplified overnight with M13KO7 and PEG-precipitated before the next round of panning. The third-round output was transformed into SS320 cells for screening. Clonal cultures were induced and VHH was extracted from cells by osmotic shock. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232).

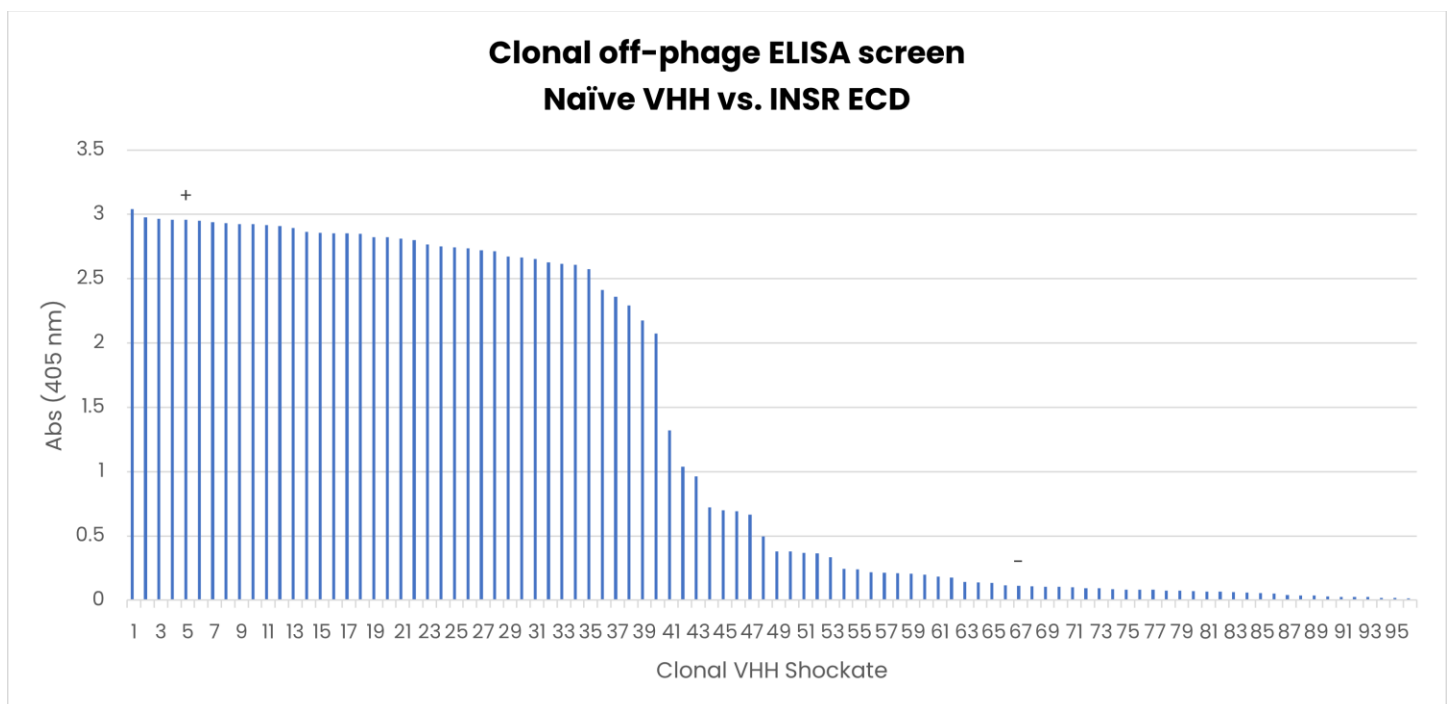


Figure 7. Clonal crude VHH were prepared by osmotic shock on 1 mL scale and assayed against recombinant INSR adsorbed on polystyrene plates. The single-point binding was detected by HRP-anti-VHH

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conjugate and ABTS. (+) indicates adsorbed VHH positive control and (-) indicates VHH-free negative control.

Clonal shockate screening generated 47 hits above the threshold of $A_{405} > 0.4$, with 29 unique binders by CDR3 analysis. Three high-signal wells were sequenced before small-scale protein prep. VHH shockates were purified by nickel resin, dialyzed into PBS, and quantified by A_{280} . Shockate stocks were normalized to 5 μ M before serial dilution. Recombinant INSR target was immobilized on polystyrene plates as before, and the titrated VHH was added to the plate in duplicate. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232). VHH binders with affinities on the order of 5E-07M to 5E-08M were identified directly from the naïve VHH library. The gray curve, clone F01, in the titration experiment is representative of the most abundant sequence from the clonal panning output.

Naïve VHH Library vs INSR ECD

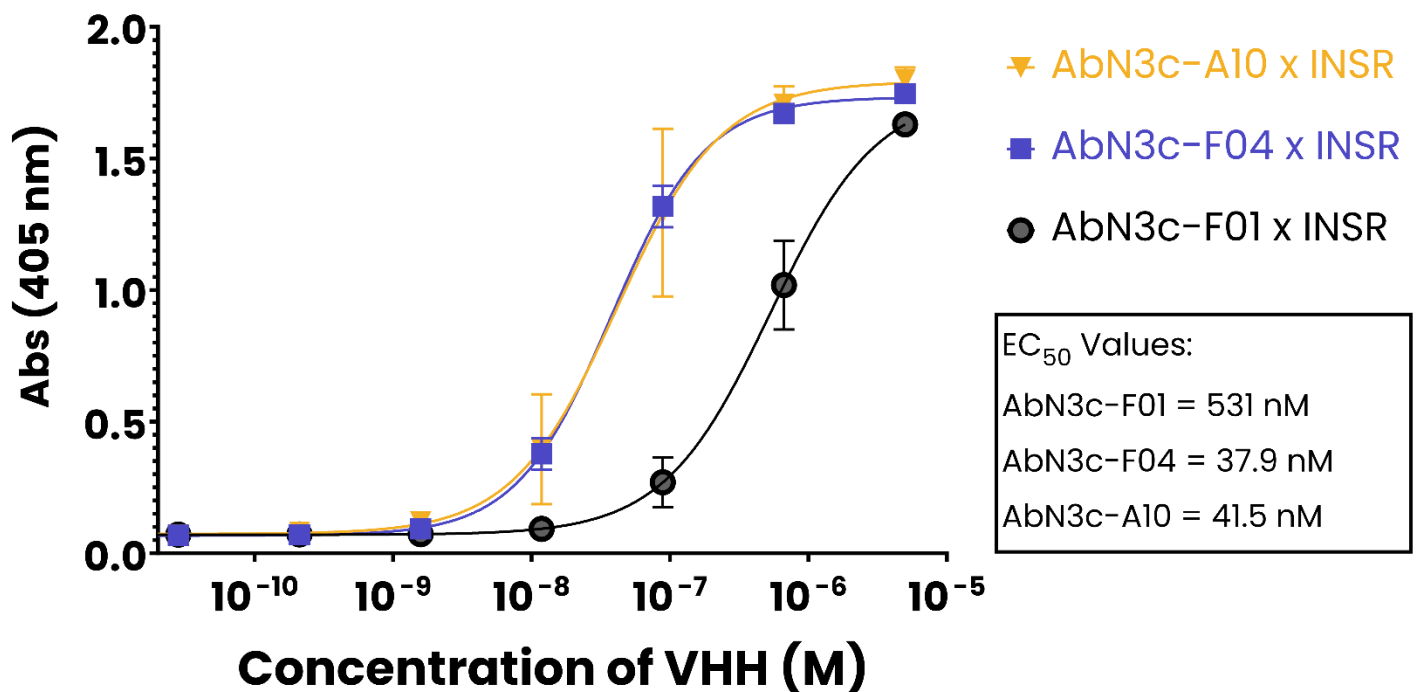


Figure 8. INSR lead VHH identified from clonal screen data were purified by nickel resin and assayed in duplicate titration against INSR antigen adsorbed onto a polystyrene plate. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

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Solution-phase panning of Human Epidermal Growth Factor Receptor (EGFR)

A site-specifically biotinylated, recombinant extracellular domain of the human epidermal growth factor receptor (Acro Biosystems #EGR-H82E6), consisting of residues A310-C620 (accession number P00533-1) was immobilized on streptavidin-coated magnetic beads (Promega® #Z5482). Phage was eluted using triethylamine after panning, and each output was amplified overnight with M13KO7. Outputs were centrifuged and the resulting supernatant was used as input for the subsequent round. The third round output was transformed into SS320 cells for screening. Clonal cultures were induced and VHH was extracted from cells by osmotic shock. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232).

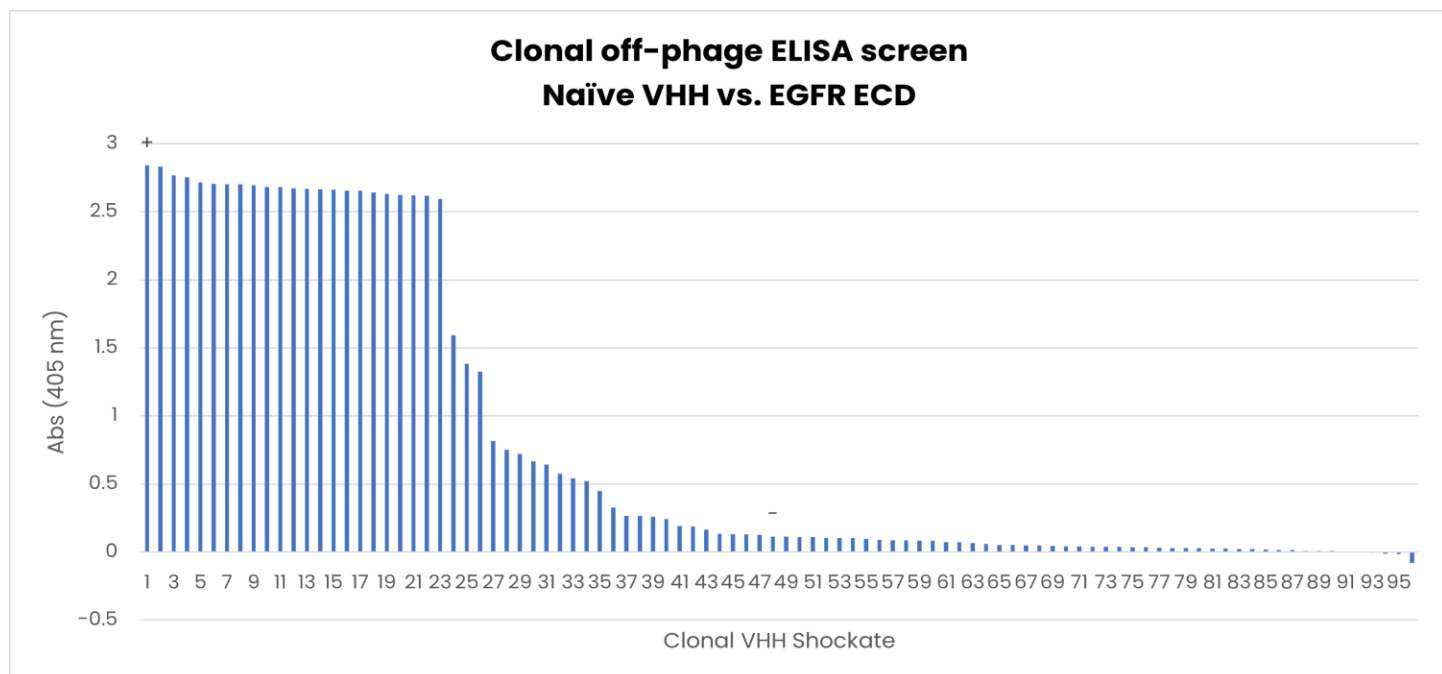


Figure 9. Clonal crude VHH were prepared by osmotic shock on 1 mL scale and assayed against recombinant EGFR bound to streptavidin-coated plates. The single-point binding was detected by HRP-anti-VHH conjugate and ABTS. (+) indicates adsorbed VHH positive control and (-) indicates VHH-free negative control.

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Clonal shockate screening generated 34 hits above the threshold of $A_{405} > 0.4$, but only 7 unique sequences by CDR3 analysis. The most abundant sequence in the clonal hits, comprising 14 of the 34 total hits, was expressed and VHH shockates were purified by nickel resin, dialyzed into PBS, and quantified by A_{280} . Shockate stocks were normalized to 5 μM before serial dilution. Recombinant EGFR target was immobilized on streptavidin plates as before, and the titrated VHH was added to the plate in duplicate. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232). The VHH binder dominating the panning output was determined to have an EC_{50} on the order of $2\text{E}-7$ M.

Naïve VHH Library vs EGFR ECD

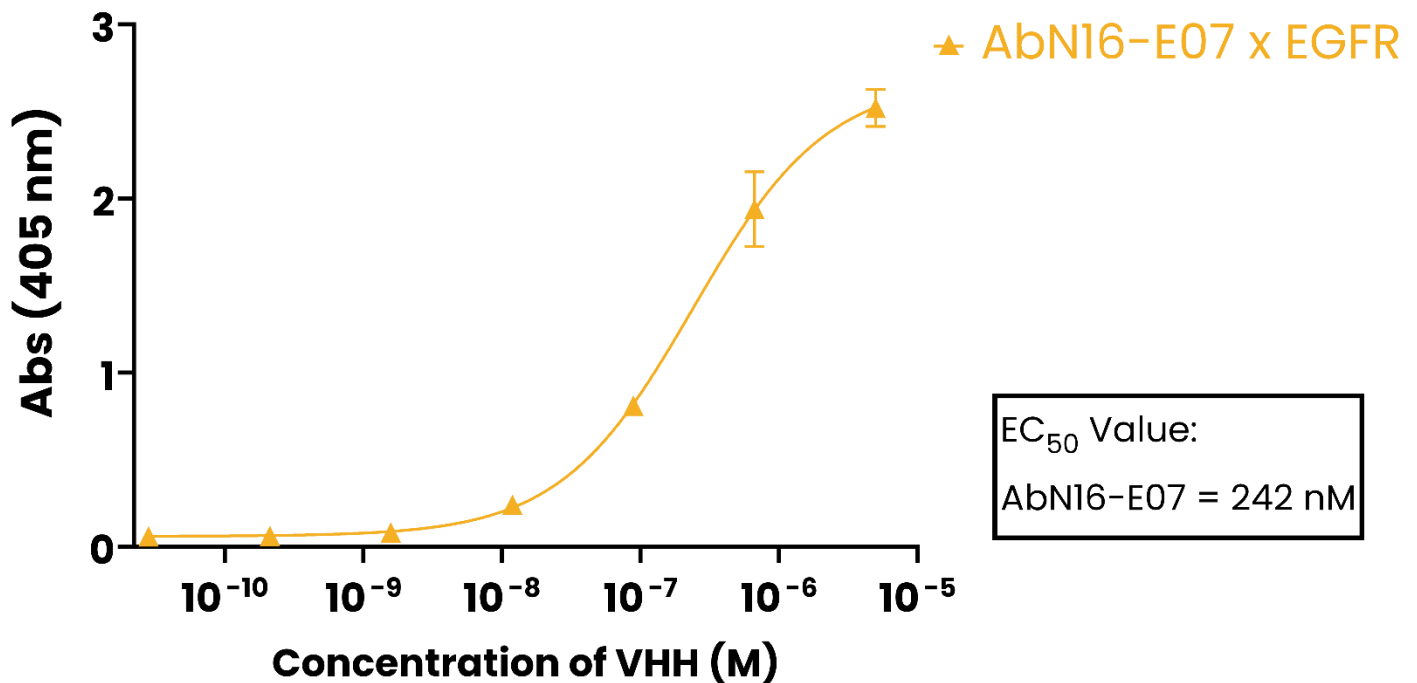


Figure 10. Anti-EGFR VHH identified from clonal screen data was assayed in duplicate titration against EGFR antigen bound to a streptavidin plate. Binding was visualized with HRP-conjugated anti-VHH and ABTS.

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Solution-phase panning of recombinant human PDL-1

Site-specifically biotinylated recombinant protein from the human programmed death ligand 1, PDL-1 (Acro Biosystems # PDL-H82E4), consisting of residues F19-Y134 (accession number Q9NZQ7-1) was immobilized on streptavidin-coated magnetic beads (Promega #Z5482). Phage was eluted using triethylamine after panning, and each output was amplified overnight with M13KO7. Outputs were PEG-precipitated and the concentrated phage stock was used as input for the subsequent round. The third-round output was transformed into SS320 cells for screening. Five plates of 96 wells each were screened. Clonal cultures were induced and VHH was extracted from cells by osmotic shock. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232).

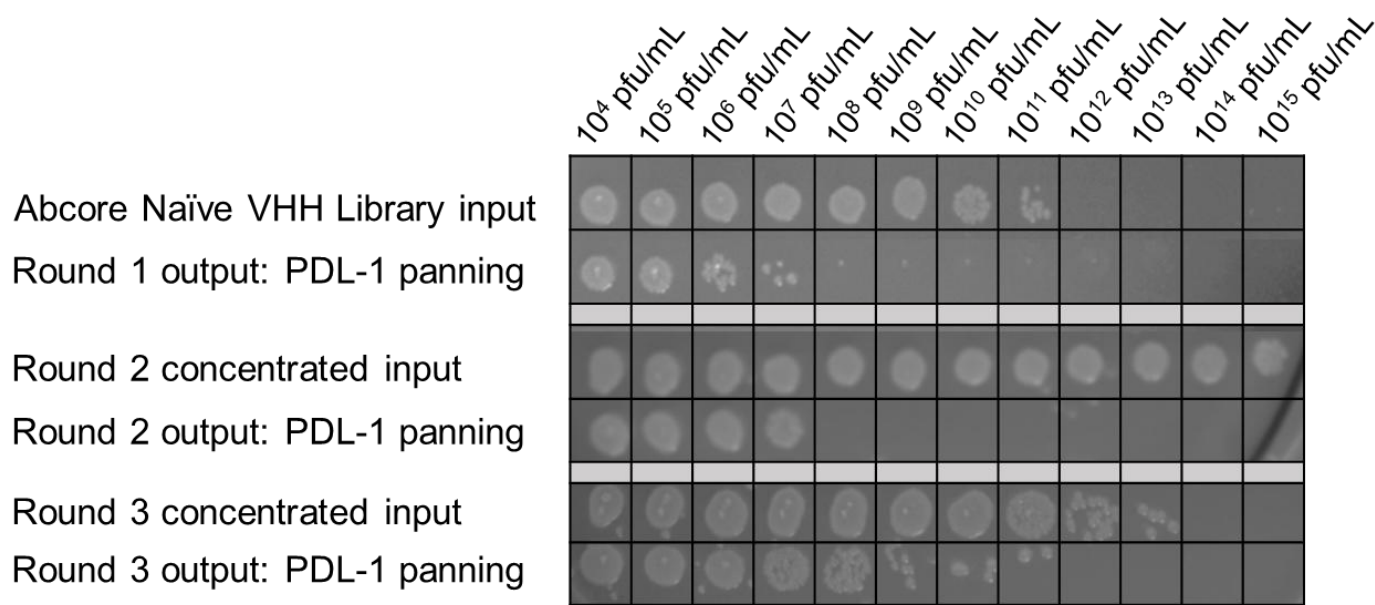


Figure 11. Panning titers from the phage selection rounds of PDL-1 using the naïve library. Abcore VHH Naïve Library was panned against biotinylated PDL-1 antigen. Phage output was infected into TG1 cells and amplified overnight with M13KO7 helper phage. The resulting phage supernatant was PEG-precipitated before being used as the input for subsequent panning selection rounds.

Clonal off-phage ELISA screen Naïve VHH vs. PD-L1 (top 250)

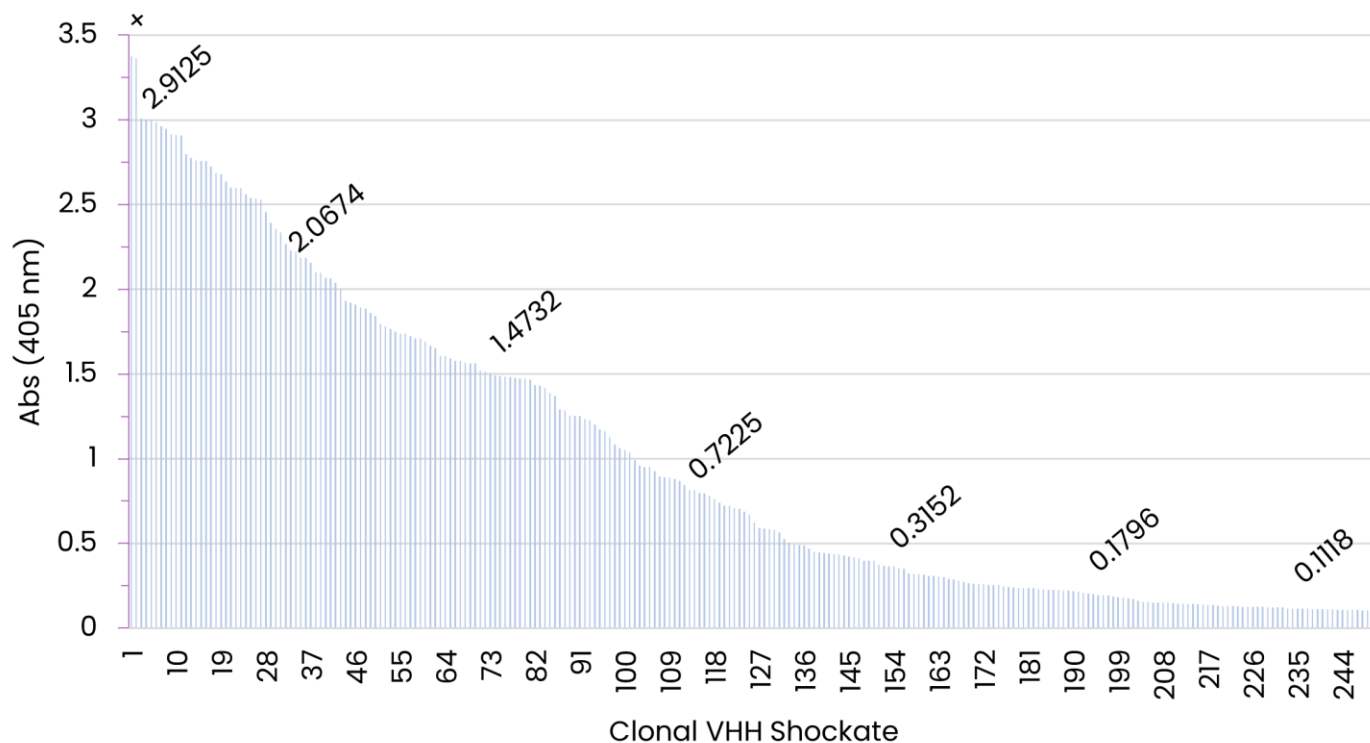


Figure 12. Clonal crude VHH were prepared by osmotic shock on 1 mL scale and assayed against biotinylated PDL-1 bound to streptavidin-coated plates. The single-point binding was detected by HRP-anti-VHH conjugate and ABTS. (+) indicates VHH positive control. The top 250 clones are represented here from the 470 clones screened.

Clonal shockate screening generated 147 hits above the threshold of $A_{405} > 0.4$ with 68 unique sequences identified from the 470 clones screened. This represents a unique hit rate of 14.5%.

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Naïve VHH Library vs PD-L1

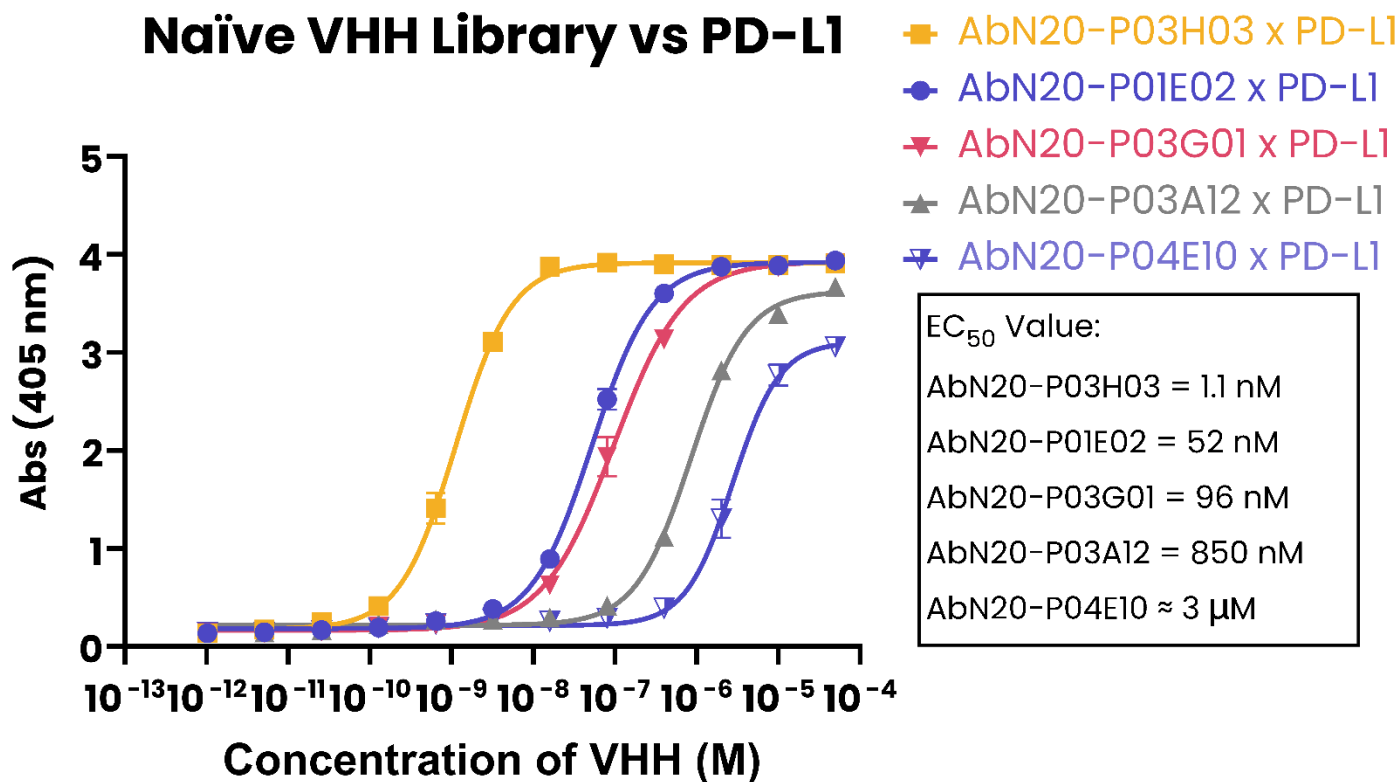


Figure 11. Anti-PD-L1 VHH identified from clonal screen data were assayed in duplicate titration against PD-L1 antigen bound to a streptavidin plate. Binding was visualized with HRP-conjugated anti-VHH and ABTS.

Five clones with varying single-point ELISA signal (P03H03 = 3.38, P01E02 = 3.00, P03G01 = 2.91, P03A12 = 0.62, P04E10 = 1.23) were expressed and purified by nickel resin. Purified shockate stocks were normalized to 50 μM before serial dilution. Recombinant PD-L1 target was immobilized on streptavidin plates as before, and the titrated VHH was added to the plate in duplicate. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson ImmunoResearch # 128-035-232). EC₅₀ curves were plotted using GraphPad Prism®.

Conclusions

The pooled naïve VHH library represents a high-diversity, unbiased, and non-redundant set of germline VHH sequences that are capable of binding to a diverse set of protein targets. Phage-displayed VHH are enriched well with multiple panning conditions, with condition variables including antigen presentation and the phage titer at each round.

The theoretical diversity of this library is difficult to predict due to variations in VHH-presenting heavy-chain-only IgG ratios among PBMCs, but sequencing data suggests that this library is transformation-limited in its diversity. Minimal redundancy was measured, and the library is generally free from bias or other cloning artifacts in its construction. Based on the unique cluster frequency, the in-frame VHH rate, and the number of representative transformants, we estimate the maximum possible library size of the naïve VHH library to be 6.48×10^9 unique clonal VHH.

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Additional Product Information***Physical Characteristics*****Amount:** 10 x 1 mL**Concentration:** 1×10^{11} pfu/mL**Volume:** 1 mL**Buffer:** PBS + 20% glycerol**Storage:** -80 °C, avoid freeze-thaw cycles***Phagemid Information***

Abcore VHH libraries are supplied as packaged monovalent phage from bacterial stocks. The bacterial stocks are ligation products of VHH cloned into the backbone phagemid vector pADL™-20c, a commercially available vector from Antibody Design Labs™. The pADL™ -20c vector has TEM1 beta-lactamase which confers ampicillin/carbenicillin resistance to clones. The vector is under a pMB1 origin of replication, derived from pBR322, conferring high copy number in *E. coli* cells. A map of the vector and a representative VHH framework is shown on the following page, as well as the respective annealing sites for NGS and Sanger primers.

VHH reading frames are cloned as fusions to the *pelB* secretion signal, which causes localization to the periplasmic space and subsequent secretion of the packaged phage protein. After periplasmic localization and processing, the leader sequence is completely removed to yield the native heavy-chain residues.

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