AbCore®

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ABNANO® VHH ANTI-B-CELL LIBRARY

L780-300 HANDBOOK

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Overview

The AbNano® VHH Anti-B-Cell Library is a fully natural, single domain 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 in therapeutics, in diagnostic applications, and as research tools.

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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 a single llama. The animal was immunized with human CD19-positive B-cells. PBMCs were isolated and preserved in RNALater. A total of 3.6 x 10^8 PBMCs were collected and used for library construction. The library was constructed following Abcore's VHH library construction workflows. VHH was digested, ligated, and transformed into a single sublibrary with estimated 6.2 x 10^8 colony-forming unit (cfu) transformants. Transformants were initially analyzed by Sanger sequencing. 76.8% of Sanger sequences were in-frame VHH.

The library was subsequently analyzed by Illumina MiSeq and a total of 1 million reads were clustered and analyzed for diversity. Library diversity quantified by MiSeq analysis (CDR3 length distribution, CDR3 cluster density, and CDR composition diversity) met internal standards for diversity QC (see *QC Data* section below).

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 x 10^{11} pfu/mL and aliquoted as 1 mL tubes frozen and stored at -80 ˚C.

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.

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Sequencing QC Data

The library was sequenced using Illumina MiSeq NGS (1.0 million total reads). The NGS reads 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 at the DNA level were silent, such as those arising from polymerase errors or NGS base call errors and ensure 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 raw counts of length sampling distribution are 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 germlines 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, with 99.82% of the NGS reads mapping to IGHV3. The remaining sequences were mapped to IGHV1 (0.04%) and IGHV4 (0.14%). IGHV3 breakdown is shown, with the top representation coming from IGHV3-3 (43.48%), IGHV3S53 (31.46%), and IGHV3S66 (4.71%) (Figure 1). This bias is not unexpected and has been reflected previously in camelid sequencing datasets.[1,2]

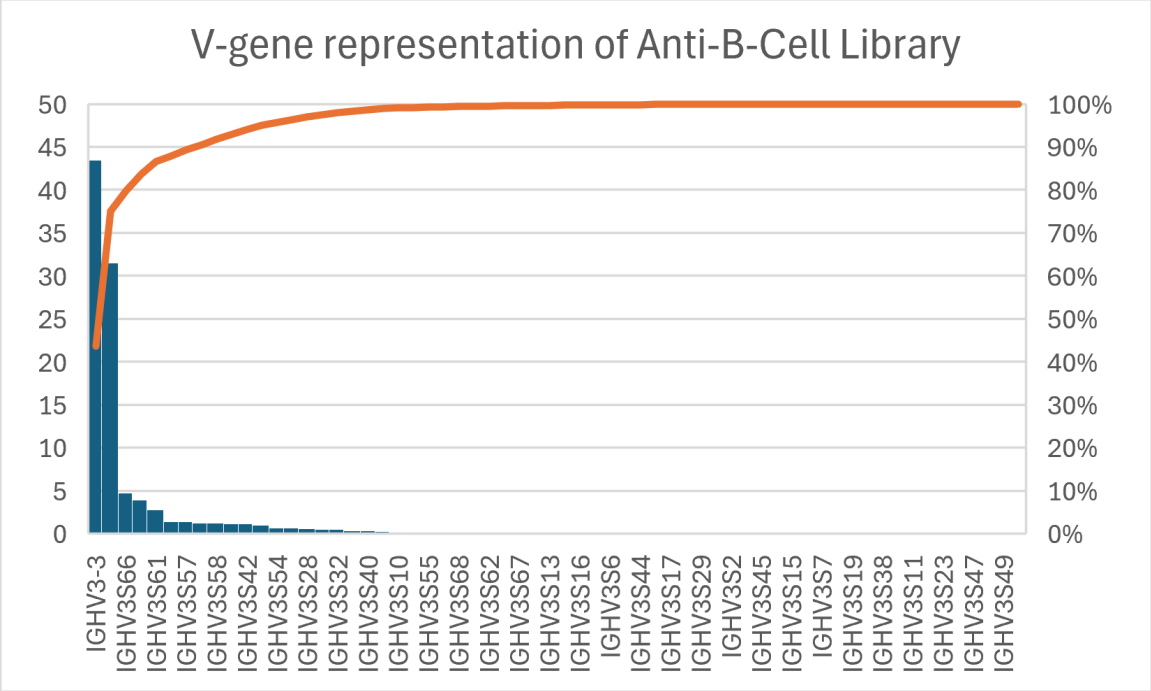

Figure 1. V-gene usage from the NGS dataset analysis, assigned by Geneious Biologics using IMGT® annotation.

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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 Anti-B-Cell Library, each amino acid sequence from the NGS dataset was clustered and the CDR3 region was plotted by length (Figure 2).

CDR3 length distribution of the AbNano VHH Anti-B-Cell Library fits well with existing literature values for libraries from camelid CDR3 and has good representation of CDR3 lengths from 5-25 residues in length.[1] The distribution of these data are similar to representative human CDRH3 length distributions, with minor artifacts of construction and measurement present.[2-4]

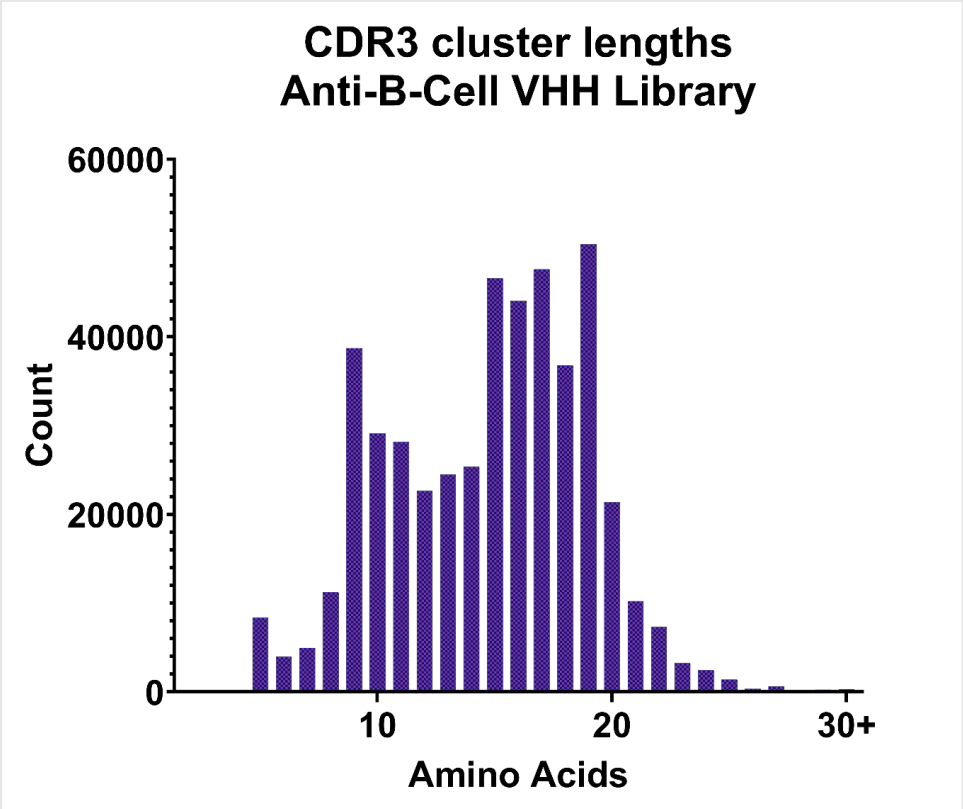

Figure 2. CDR3 amino acid length histogram with raw counts of each length.

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Characterization of CDR3 Diversity by Cluster

Diversity of CDR3 amino acids was analyzed by a cluster-size function. The clustering function measures the redundancy or bias of the individual sequences 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 the potential of NGS base-call errors and to measure the distance in sequence space between the genetic diversity and the encoded amino acid diversity, the cluster analysis was repeated on a nucleotide level using a 95% homology filter cutoff (Figure 3). The general trends for both the CDR3 amino acid cluster frequency and the nucleotide homology cluster frequency are not identical, indicating that there is some limit in genetic diversity between unique amino acid clusters. Due to the nature of construction of this library, this is likely demonstrating base-call errors in the NGS reads.

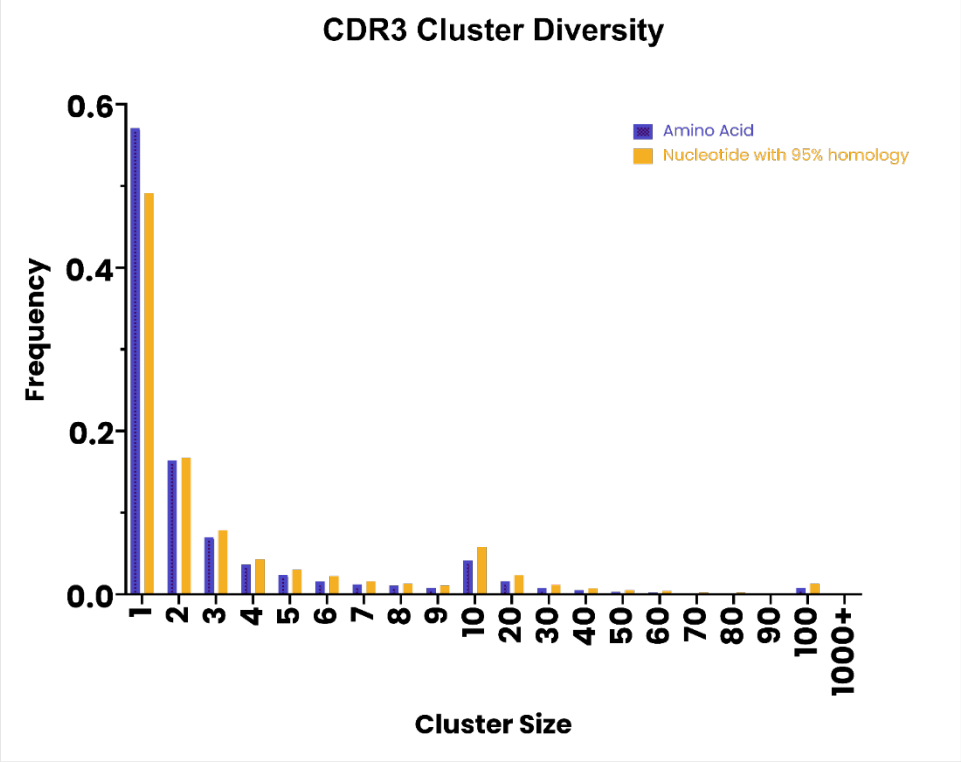

Figure 3. Comparison of cluster densities in amino acid assignment and nucleotide assignment using a 95% homology filter. This comparison helps to better characterize the distance in sequence space between the individual CDR3 clusters in the final library.

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Characterization of CDR3 Diversity by Percent Abundance

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. Here, we see a slight non-uniformity in the distribution (Figure 4).

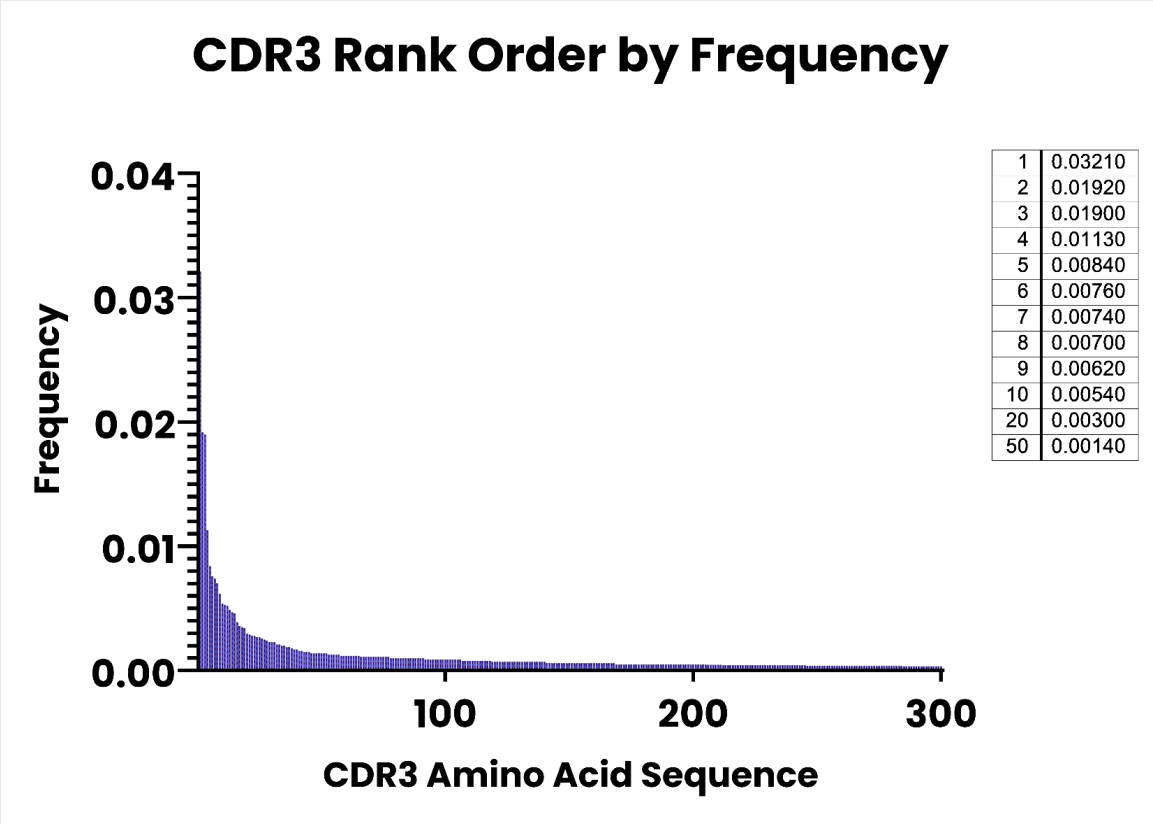

Figure 4. Percent abundance of the top 300 CDR3 amino acid sequences plotted, with specific rank order frequencies identified and given at right.

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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 (Figure 5).

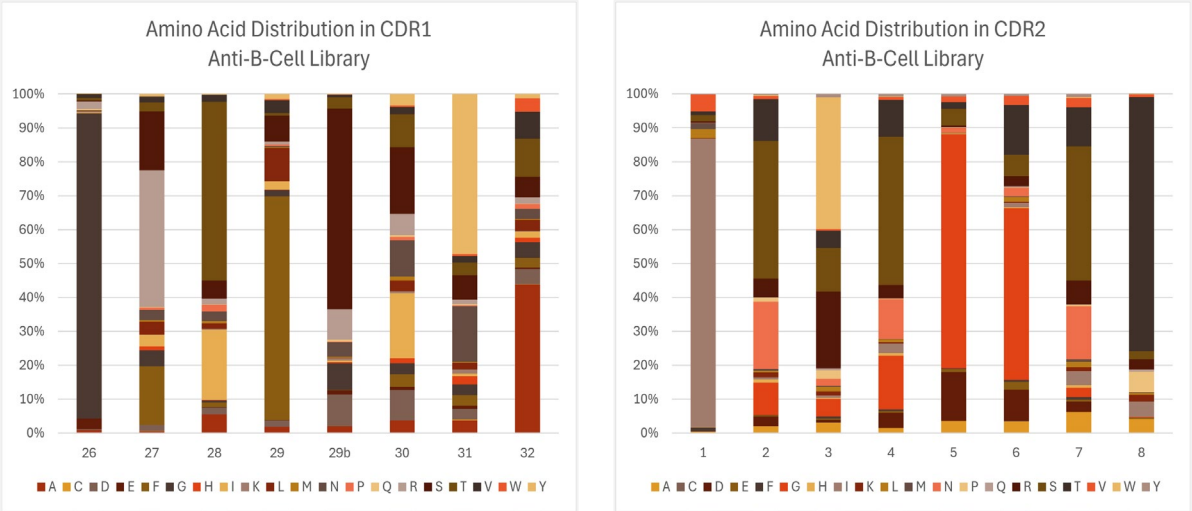

Figure 5. Amino acid distribution for CDR1 and CDR2 by Kabat position number in the VHH library. The individual identities are in alphabetical order from bottom to top, such that alanine appears nearest the baseline and tryptophan at the axis-maximum.

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]

Of note, CDR3 distribution (Figure 6) is reasonably unbiased with good contributions from small, polar, and aromatic residues that are demonstrated to be fundamental in CDR3 interactions.

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

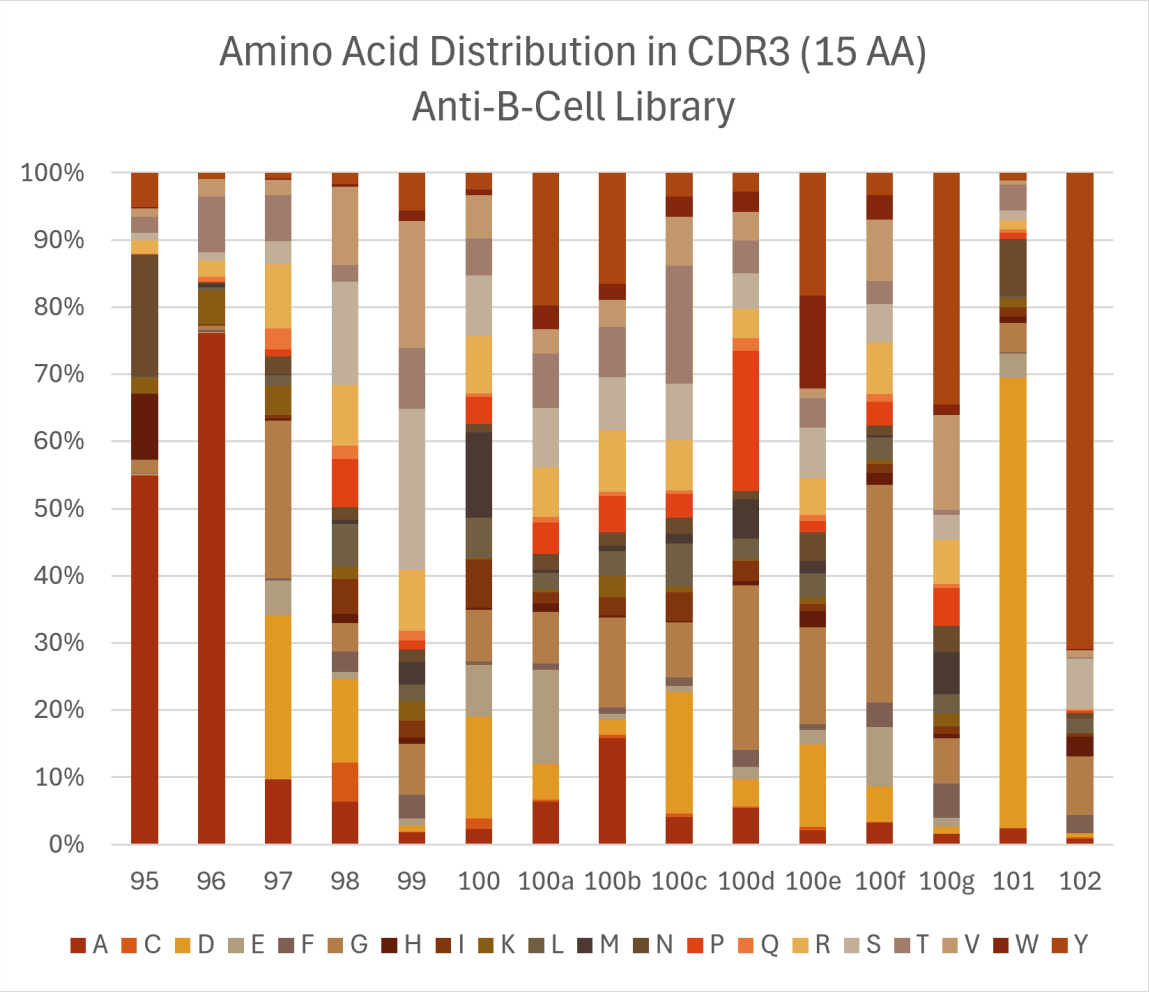

Figure 6. *Amino acid distribution for the 15-amino acid length of CDR3 by Kabat position number in the AbNano VHH Anti-B-Cell Library. The individual identities are in alphabetical order from bottom to top, such that alanine appears nearest the baseline and tryptophan at the axis-maximum.*

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Representative Panning Campaigns with the VHH library

To validate the discovery potential of the AbNano VHH Anti-B-Cell Library, multiple targets were identified for phage display panning affinity selections. Multiple rounds of panning were performed against two recombinant extracellular domain protein targets.

First, we targeted B-cell maturation antigen (BCMA) extracellular domain (ECD) as model antigen. BCMA is present in mature human B-cells and is a meaningful target in CAR-T therapies against blood cancers. Next, the library was panned against CD40, which is a target in B-ALL and other cancers.

Following panning, the phage pool was miniprepped and transformed into the non-amber-suppressor strain of *E. coli* SS320 (MC1061F′). Random clones were picked, and crude periplasmic shockate VHH was assayed using a single point ELISA. Clones of varying signal were chosen from the single-point ELISA data and purified, then titrated against immobilized panning antigen to generate EC_{50} binding curves. Curves were fit using GraphPad Prism® by a sigmoidal nonlinear fit function. Panning and screening antigens were purchased commercially from Acro Biosystems®.

Panning of BCMA

For BCMA panning, the recombinant antigen (residues M1-A54 of accession number Q02223-1 with a C-terminal biotinylated Avi tag and poly-His) was bound to blocked phage, and the complex was captured by streptavidin-coated magnetic beads before washing. Phage was eluted by triethylamine and PEG-precipitated between rounds. 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, Cat. No. 128-035-232).

Clonal shockate screening from 470 clones generated 126 hits above the threshold of $A_{405} > 0.4$, with 49 unique CDR3 amino acid sequences. Five unique clonotypes with varying levels of signal were selected for small-scale protein prep. VHH shockates were purified by nickel resin, dialyzed into PBS, and quantified by A_{280}. Shockate stocks were normalized before serial dilution.

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Recombinant BCMA target was immobilized on streptavidin-coated plates, and the titrated VHH was added to the plate. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson Immunoresearch, Cat. No. 128-035-232) (Figure 7). VHH binders with affinities in the single- and double-digit nanomolar range were identified directly from the AbNano VHH Anti-B-Cell VHH Library.

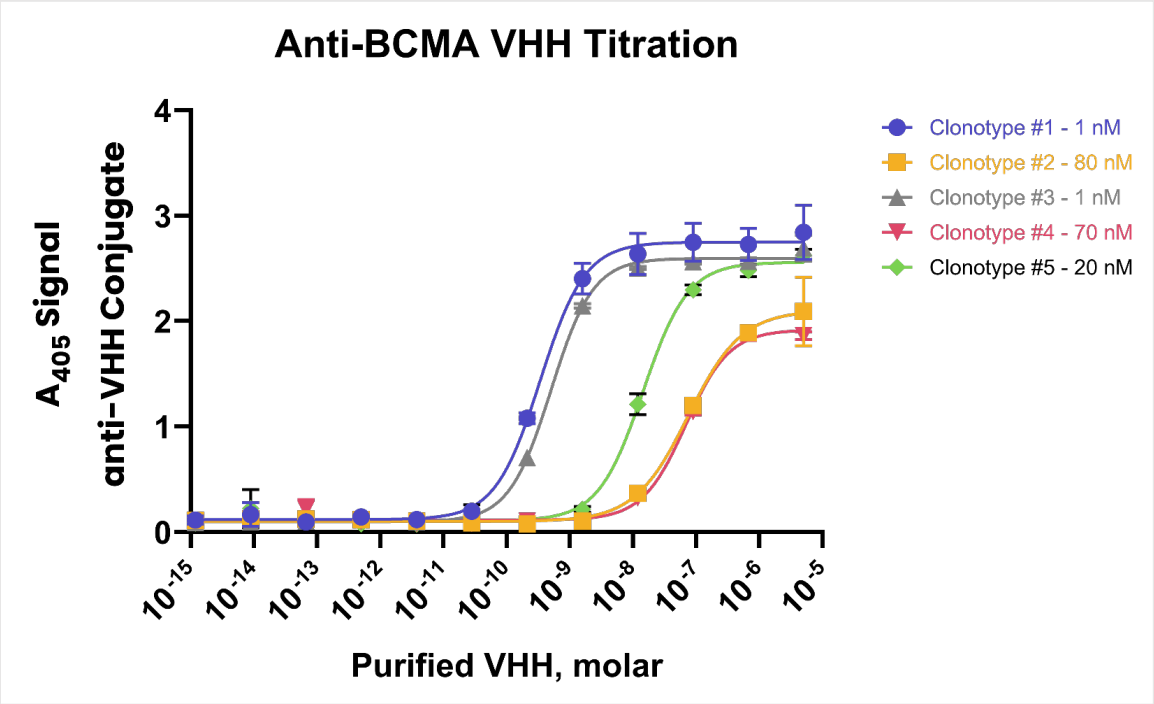

Figure 7. Several BCMA-binding clones from the preliminary screen were purified and titrated against captured BCMA antigen. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

Panning of CD40

For CD40 panning, the recombinant antigen (residues E21-R193 of accession number P25942-1 with a C-terminal biotinylated Avi tag and poly-His) was bound to blocked phage, and the complex was captured by streptavidin-coated magnetic beads before washing. Phage was eluted by triethylamine and PEG-precipitated between rounds.

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, Cat. No. 128-035-232).

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Clonal shockate screening from 470 clones generated 15 hits above the threshold of A_{405} > 0.4, with 14 unique CDR3 amino acid sequences. Three unique clonotypes with varying levels of signal were selected for small-scale protein prep. VHH shockates were purified by nickel resin, dialyzed into PBS, and quantified by A_{280}. Shockate stocks were normalized before serial dilution.

Recombinant CD40 target was immobilized on streptavidin-coated plates, and the titrated VHH was added to the plate. Absorbance was detected using HRP conjugated to anti-VHH antibody (Jackson Immunoresearch, Cat. No. 128-035-232) (Figure 8). VHH binders with affinities in the single- and double-digit nanomolar range were identified directly from the AbNano VHH Anti-B-Cell VHH Library.

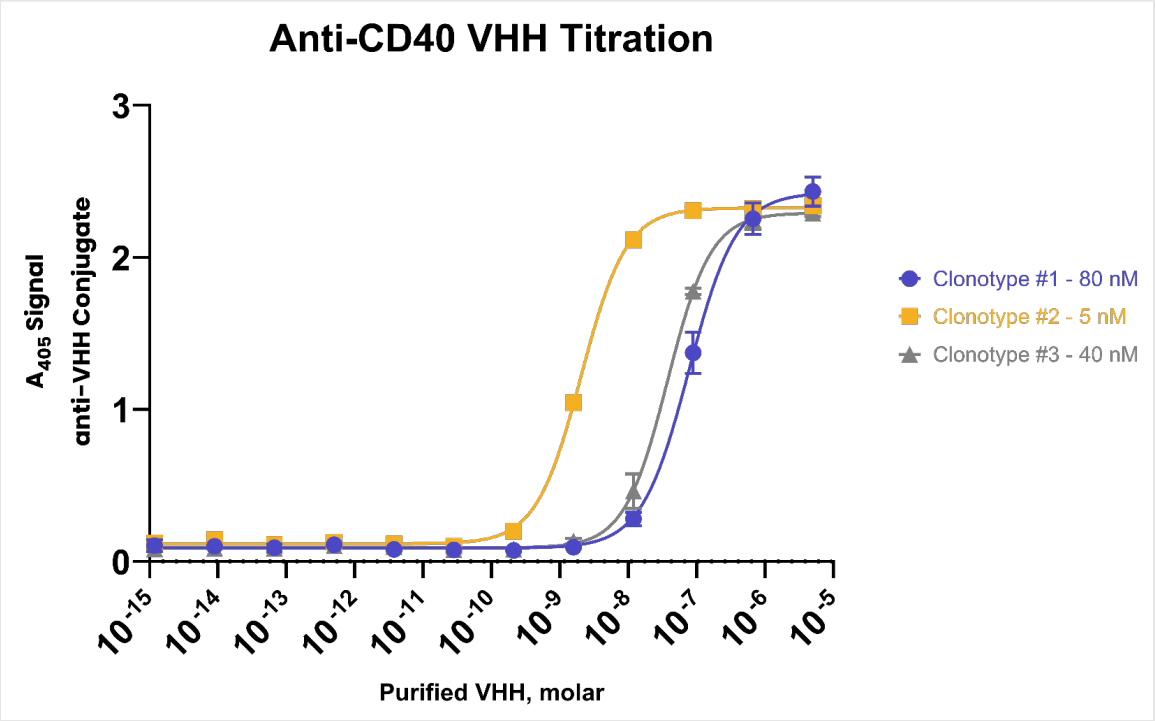

Figure 8. CD40-binding clones from the preliminary screen were purified and titrated against captured CD40 antigen. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

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Conclusions

The AbNano VHH Anti-B-Cell Library represents a high-diversity set of naturally-derived VHH sequences that are capable of binding to multiple protein targets present on healthy human B cells. Phage-displayed VHH are enriched well with multiple binding strengths, including low- to sub-nanomolar interactions, against therapeutically relevant targets.

The theoretical diversity of this library is likely limited by the diversity of the source material. Based on the unique cluster frequency, the in-frame VHH rate, and the number of representative transformants, we can estimate the maximum library size of the AbNano VHH Anti-B-cell VHH Library to be around 1.5 x 10^8, or 150 million, unique clonal VHH.

Additional Product Information

Physical Characteristics

Amount: 1 x 1 mL (L780-300); 10 x 1 mL (L780-300B)
Concentration: 1 x 10^{11} pfu/mL
Volume: 1 mL
Buffer: PBS + 20% glycerol
Storage: -80 ˚C, avoid freeze-thaw cycles

Phagemid Information

AbNano™ 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 (Figure 13).

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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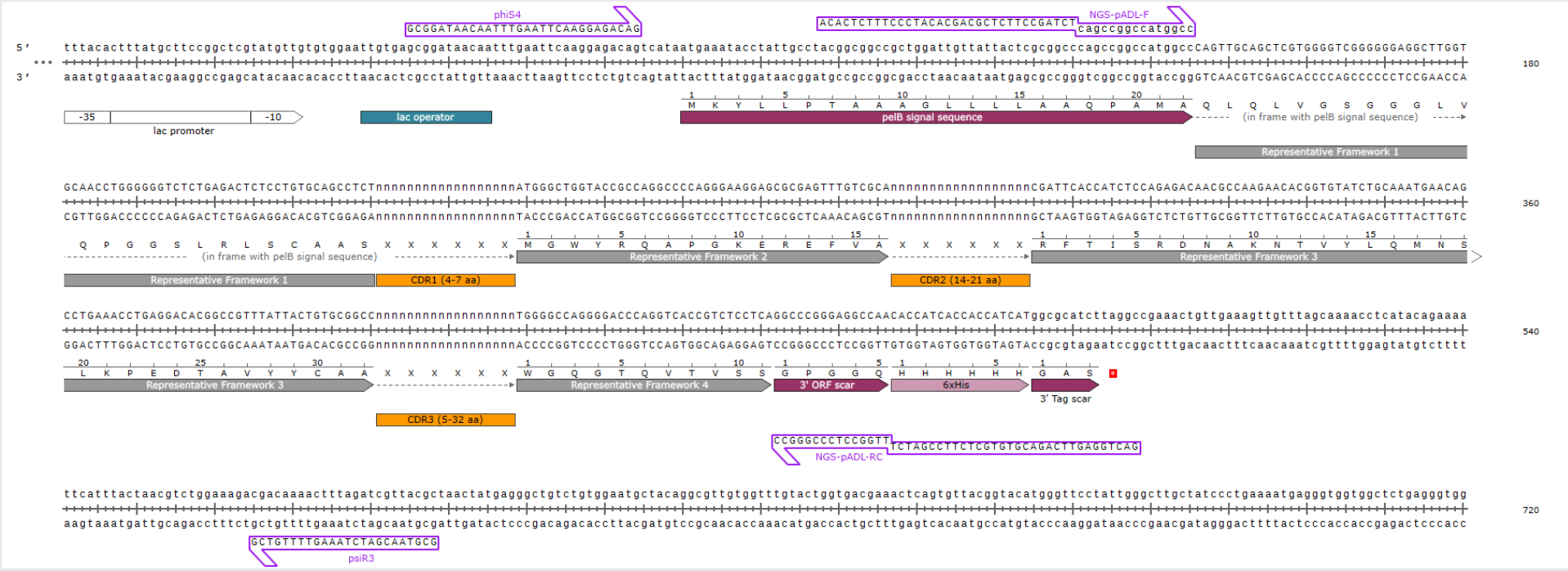

Figure 13. Representative framework and CDR regions of VHH in pADL™-20c. The lac promoter and operator are located 5′ to the pelB secretion signal. Representative framework sequences are given in gray and CDR regions of a typical length are shown in orange. The CDR length variations are shown for each and are representative of naïve and many immunized VHH length distributions. The annealing sites of the two sequencing primer sets provided, for NGS and Sanger workflows respectively, are shown in the context of the VHH. Here, the NGS adapters given are for Azenta's Amplicon-EZ® NGS service.

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Sequencing Workflows for AbNano™ VHH in pADL™-20c

Sanger sequencing for all VHH clones in this vector can be performed using the following primer set:

Primer	Sequence (5′ –> 3′)	Tm (˚C)
Forward (phiS4)	GCGGATAACAATTTGAATTCAAGGAGACAG	60 ˚C
Reverse (psiR3)	GCGTAACGATCTAAAGTTTTGTCG	57 ˚C

PCR reactions can be performed from either bacterial stabs or 1 µL liquid culture following the general protocol:

PCR Reactions Setup (50 µL total volume):

Component	Volume
Taq 2X Master Mix, (e.g., NEB®, Cat. No. M0270L)	25 µL
10 µM phiS4 primer stock	1 µL
10 µM psiR3 primer stock	1 µL
Nuclease-free water	22 µL
Bacterial stab or clonal culture	1 µL

Mix gently by swirling before sealing the tube. Run the reaction on a thermocycler using the program below. The expected product size is ~450–550 single bp fragment.

Thermocycler Program

Step	Temperature (˚C)	Time	Cycles
Initial denaturation	95 ˚C	3 min	1
Denaturation	95 ˚C	30 s	30x
Annealing	55 ˚C	30 s	
Extension	68 ˚C	40 s	
Final Extension	68 ˚C	5 min	1
Hold	4 ˚C	Forever	-

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Next-Generation Sequencing for AbNano VHH Libraries

Two-step NGS library preparation can be performed following a standard Illumina-style workflow. Adapter sequences are shown as placeholders (orange) and annealing sequences as black lowercase letters for clarity.

> *NOTE: Consult with sequencing core or provider before proceeding with adapter sequences.*

Adapter Primer Set

Primer	Sequence (5′ –> 3′)	Tm (˚C)
NGS-pADL-F	Adapter - cagccggccatggcc	61 ˚C
NGS-pADL-RC	Adapter - ttggcctcccgggcc	62 ˚C

> *NOTE: Nextera universal forward adapters may anneal within the pelB leader sequence. Use annealing sequence CTCGCGGCCC instead of the standard Nextera forward sequence.*

Template DNA Preparation

NGS workflows are recommended to start with high-quality miniprepped DNA from output bacteria.

1. Collect 1 mL of overnight culture.
2. Centrifuge at 3,000 × g for 5 min at room temperature (RT).
3. Miniprep using a plasmid DNA kit (e.g., Macherey-Nagel®, Cat. No. 740588.50)
4. Elute DNA in nuclease-free water.
5. Measure concentration and dilute to 5 ng/µL using nuclease-free water.

NGS PCR Setup (50 µL reaction)

For NGS library preparations, a polymerase with excellent fidelity and low template strand displacement is required.

Component	Volume
CloneAmp™ HiFi PCR Premix (Takara, Cat. No. 639298), or equivalent	25 µL
10 µM NGS-forward adapter primer	2 µL
10 µM NGS-reverse adapter primer	2 µL
Nuclease-free water	19 µL
DNA template (10 ng total)	2 µL

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Thermocycler Program

Step	Temperature (˚C)	Time	Cycles
Initial denaturation	95 ˚C	30 s	1
Denaturation	98 ˚C	10 s	10x
Annealing	60 ˚C	10 s	
Extension	72 ˚C	10 s	
Denaturation	98 ˚C	10 s	10x
Annealing	54 ˚C	10 s	
Extension	72 ˚C	10 s	
Final Extension	72 ˚C	1 min	1
Hold	4 ˚C	Forever	-

Post-PCR Cleanup and Quality Control

1. Purify PCR products using EZ Bioresearch columns, (Cat. No. M1001), or magnetic beads (e.g., Omega Biotek, Cat. No. M1378).
2. Elute DNA in nuclease-free water.
3. Verify purity and fragment size using agarose gel (Image 1), SYBR™-stained PAGE, or urea gel, or by TapeStation.
4. If residual plasmid template persists, treat the product with DpnI before indexing PCR. Set up indexing reactions according to the recommendations from your sequencing core or provider.

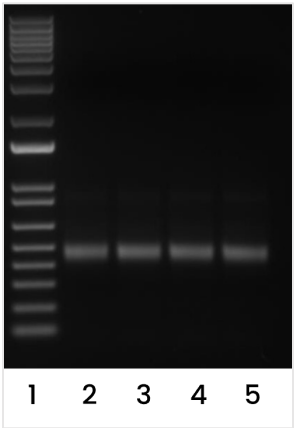

Image 1. *Representative gel image of NGS library preps from AbNano VHH libraries following DpnI digest and PCR cleanup.* ***Lane 1:*** *1 kb+ ladder (Thermo Fisher Scientific, Cat. No. 10787026);* ***Lanes 2-5****: Purified NGS adapter PCR products after DpnI digest and cleanup, normalized in concentration. The DNA band is non-discrete, based primarily on CDR3 length, and varies from 450–500 base pairs.*

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Protocol #1:

Phage Display Selections Using Biotinylated Antigen and Streptavidin Mag-Beads

> *NOTE: Consult MSDS and/or instructions for use for all equipment and/or materials before use.*

1. Consumables

- Magnetic Dynabeads® M-280 Streptavidin (Thermo Fisher Scientific, Cat. No. 11205D)
- Biotinylated antigen of interest (1 µM stock in PBS; use 100 µL per round of panning)
- 1.7 mL low-binding snap-cap tubes (Corning®, Cat. No. 3207)
- 50 mL centrifuge tubes (Corning®, Cat. No. 430290)
- Sterile Erlenmeyer flasks, 125 mL, unbaffled, (Corning®, Cat. No. 431143)
- Sterile water
- M13KO7 helper phage (Antibody Design Labs, Cat. No. PH010S)
- Sterile cell scraper (Corning®, Cat. No. CLS3010)
- Tris (free base), 1M, pH adjusted to 8.0, 0.2 µm sterile filtered
- Glycerol (CAS No. 56-81-5)
- Carbenicillin (RPI Biosolutions, Cat. No. C46000)
- Kanamycin (RPI Biosolutions, Cat. No. K22000)
- Tween-20 (CAS No. 9005-64-5)
- Optional: PEG/NaCl solution (Teknova, Cat. No. P4133) (for phage precipitation)
- Syringes and syringe filters (0.2 µm and 0.45 µm)
- Triethylamine (CAS No. 121-44-8)
- 1X PBS, pH 7.4, sterile
- *E. coli* TG1 cells (F', supE44; Antibody Design Labs, Cat. No. PC001)
- AbNano VHH library stock (Round 1) or amplified phage output (subsequent rounds)

2. Prepared Solutions

2xYT Media Variants, sterile (RPI Biosolutions, Cat. No. X15600):

- 2xYT-G: 2xYT + 1% (w/v) glucose
- 2xYT-GC: 2xYT-G + 100 µg/mL carbenicillin
- 2xYT-CK: 2xYT-C + 50 µg/mL kanamycin

2xYT-GC + Glycerol:

- Autoclave 2xYT (30% water replaced by glycerol); post-cooling add glucose (1% w/v) + carbenicillin (100 µg/mL).

Antibiotic Stocks (1000X, sterile):

- 2xYT-Carbenicillin agar plates (150 mm) for bacterial growth.
- Carbenicillin (1000x): Dissolve 100 mg carbenicillin in 1 mL sterile water, filter (0.22 µm).
- Kanamycin (1000x): Dissolve 50 mg kanamycin in 1 mL sterile water, filter (0.22 µm).

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Additional Solutions:

- Glucose 50% (w/v), 0.2 µm sterile filtered (50X solution).
- Buffer A: 1X PBS, pH 7.4, 0.1% (v/v) Tween-20, 1% (w/v) BSA, filter (0.22 µm); store at 4 ˚C.

3. Equipment

- Magnetic separator (e.g., EpiCypher Cat No. 10-0008)
- Benchtop microcentrifuge
- Benchtop centrifuge for 50 mL tubes
- Incubated orbital shaker
- Water bath

4. Methods: Prepare the Target and Deselection Beads:

4.1 Label two 2 mL snap-cap tubes: '**subtract**' and '**target**'.

4.2 Add 1 mL Buffer A to each tube.

4.3 Vortex M-280 beads container for 30 s until uniform.

4.4 Add 25 µL of M-280 beads to each snap-cap tube.

> ***NOTE:*** *25 µL of M-280 beads will allow for ~5–50 pmol of biotinylated target to be immobilized, depending on antigen size, steric environments, and biotin labelling technique. Different beads have different binding capacities; numbers may vary based on manufacturer or technology used.*

4.5 Place tubes on magnetic separation rack until beads are fully separated (60–90 s). Carefully remove and discard supernatant.

4.6 Briefly pulse-spin tubes in a microcentrifuge to collect residual liquid and beads at bottom of tube.

4.7 Repeat magnetic separation (60–90 s). Remove and discard supernatant.

4.8 Add 300 µL of Buffer A to each tube.

4.9 Vortex briefly to mix. Pulse-spin tubes to collect liquid to bottom of tube.

4.10 Repeat magnetic separation. Remove and discard supernatant.

4.11 Add 950 µL of Buffer A to each tube.

4.12 Add 100 µL of biotinylated antigen to '**target**' tube.

4.13 Incubate both tubes on an orbital shaker or rotisserie rotator at RT for 30 min.

4.14 Wash tubes 3 times as follows:

4.14.1 Add 300 µL of Buffer A to each tube.

4.14.2 Vortex briefly to mix. Pulse-spin tubes to collect liquid.

4.14.3 Repeat magnetic separation (60–90 s). Remove and discard supernatant.

4.15 Add in 750 µL of Buffer A to each tube.

4.16 Store washed beads on ice until ready to begin panning.

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5. Methods: Panning Round 1

Preparation

5.1 Pre-warm 2xYT-G media at 37 ˚C for TG1 rescue. Required volume depends on the OD_{600} of TG1 rescue stock.

5.1.1 For 0.5 mL aliquots of 20 OD_{600}/mL, dilute in 20 mL media to achieve a final OD_{600} of ~0.5.

5.2 Thaw one aliquot of the AbNano VHH library stock.

> *NOTE: A single aliquot can be used to pan up to four targets. Do not re-freeze the phage stock, as this may significantly reduce phage quality.*

Subtraction Step

5.3 Add 250 µL of phage stock to the '**subtract**' tube, (empty beads + blocking buffer).

5.4 Incubate tubes on an orbital shaker or rotisserie rotator at RT for 60 min.

5.5 Vortex briefly to mix, then pulse-spin tubes to collect liquid to bottom of tube.

5.6 Place '**subtract**' tube on magnetic separation rack. ***Do not remove the supernatant*** *(contains subtracted phage).*

Target Binding

5.7 Place '**target**' tube on magnetic separation rack (60—90 s). Remove and discard buffer.

5.8 Transfer the subtracted phage from '**subtract**' tube into '**target**' tube to begin the binding step.

5.9 Incubate tubes on orbital shaker or rotisserie rotator at RT for 60 min.

5.10 Vortex briefly to mix, then pulse-spin tubes briefly to collect liquid to bottom of tube.

5.11 Place '**target**' tube on magnetic separation rack (60–90 s). Remove and discard excess liquid, careful not to disturb the beads.

> *NOTE: This liquid contains all the phage in the pool that did not bind to your fixed target.*

Wash

5.12 Wash the '**target**' beads five times with 500 µL of **Buffer A**:

- 5.12.1 Add Buffer A to beads. Mix gently by pipetting or briefly vortexing.
- 5.12.2 Pulse-spin to collect liquid.
- 5.12.3 Separate on magnetic rack (60–90 s). Remove and discard supernatant.
- 5.12.4 Repeat for a total of five washes.
- 5.12.5 After final wash, remove all liquid before eluting.

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Elution

5.13 Turn on water bath and set to 37 ˚C.
5.14 Prepare fresh diluted triethylamine: Add 14 µL of triethylamine to 1 mL of sterile water.
5.15 Add 300 µL diluted triethylamine to washed beads.
5.16 Incubate for 10 min at RT with rotation.
5.17 Separate on magnetic rack (60–90 s). Transfer the supernatant to a fresh tube labelled (e.g., "Project ## R1 elution").
5.18 Neutralize with 30 µL of 1M Tris pH 7.4.
5.19 Spin elution tubes in a microcentrifuge at 11,000 × g for 1 min.
5.20 Transfer supernatant to a fresh labelled tube.

> *NOTE: There is often no visible pellet.*

TG1 Rescue

5.21 Remove 1 mL tubes of concentrated TG1 cell stock from -80 ˚C.
5.22 Thaw TG1 cells in 37 ˚C water bath for 2 min.
5.23 Transfer entire TG1 volume into warm 2xYT blank and add to a sterile 125 mL unbaffled Erlenmeyer flask.
5.24 Add the entire volume of neutralized phage eluate to the bacterial culture.
5.25 Grow TG1 cells at 37 ˚C, with shaking at 200 rpm on an orbital shaker for 60 min.
5.26 Streak TG1 cell cultures onto 2xYT-Carbenicillin plates. Incubate overnight at 37 ˚C.

6. Methods: Storing the Output Cultures

6.1 Overnight bacterial cultures can be stored in cryovials.
6.1.1 Scrape bacterial cultures from the agar plates into 2xYT-GC + glycerol in a 50 mL falcon, and transfer into cryovials. Invert to mix.
6.1.2 Flash freeze in liquid nitrogen (LN_2). Long-term storage should take place at -80 ˚C.

> *NOTE: We recommend resuspending the output plates in approximately 5 mL of the glycerol media and adjusting the OD_{600} to approximately 25. Measure a 1:10 or 1:100 diluted amount of stock for accurate quantification.*

6.2 Pellet 100 µL of glycerol stock, remove supernatant, and resuspend the cell pellet in the miniprep kit resuspension buffer for NGS analysis.

7. Methods: Panning Additional Rounds

7.1 Inoculate 1 mL OD_{600} = 25.0 stock into 100 mL pre-warmed 2xYT-GC media; grow at 37 ˚C until the OD_{600} reaches approximately 0.5 (mid-log bacterial growth phase).
7.2 Add M13KO7 to 1 x 10^8 pfu/mL final (e.g., 100 µL of a 1 x 10^{11} stock into 100 mL media).
7.3 Incubate 60 min at 37 ˚C **without** shaking.
7.4 Transfer into two 50 mL tubes; centrifuge at 3,000 × g for 10 min at RT.
7.5 Decant supernatant into 10% bleach.

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7.6 Resuspend pellets in 100 mL 2xYT-CK: transfer to sterile 125 mL Erlenmeyer flask.

7.7 Grow overnight at 30 ˚C with shaking, 200 rpm.

7.8 Centrifuge culture at 3,000 × g for 10 min at RT. Retain supernatant as crude phage.

> ***NOTE:*** *The phage is present in the supernatant, do not discard!*

7.9 Optional PEG/NaCl Concentration (enriches rare clones)

- 7.9.1 Filter phage supernatant using a 0.45 µm syringe filter.
- 7.9.2 Mix 2 parts phage supernatant to 1 part PEG/NaCl with PEG/NaCl solution (Teknova, Cat. No. P4133).
- 7.9.3 Incubate on ice for 60 min.
- 7.9.4 Centrifuge 18,000 × g for 1 h at 4 ˚C. Discard supernatant.
- 7.9.5 Resuspend pellet in approximately 5 mL PBS.

> ***NOTE:*** *Centrifugation at 8,000 x g for 1 h at 4 ˚C will produce marginally lower preservation of rare clones if high-speed centrifugation is prohibitive.*

7.10 Optional ultra-concentration

- 7.10.1 Add equal volume PEG/NaCl solution (1:1).
- 7.10.2 Incubate on ice for 60 min.
- 7.10.3 Centrifuge at 18,000 × g for 1 h at 4 ˚C. Discard supernatant.
- 7.10.4 Resuspend final pellet in minimal PBS (approx. 250 µL); store at 4 ˚C. Use 100 µL as input for the next round.

7.11 Prepare beads (see section: Methods: Prepare the Target and Deselection Beads):

- 7.11.1 Vortex and distribute magnetic beads.
- 7.11.2 Wash 3x with Buffer A.
- 7.11.3 Add target antigen and allow to bind 30 min rotating at 4 ˚C. Hold on ice.

> ***NOTE:*** *Target concentration is typically decreased in a round-wise fashion.*

- 7.11.4 Additional panning rounds can be performed either with supernatant or PEG-precipitated phage stocks:
  - 7.11.4.1 Supernatant: 0.5 mL supernatant + 0.5 mL Buffer A.
  - 7.11.4.2 PEG-precipitated phage stocks: 0.1 mL stock + 0.9 mL Buffer A.

7.12 Typical antigens require 3–4 rounds of panning/propagation to isolate binders.

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Protocol 2:

Clonal screening of VHH after phage panning

> *NOTE: Consult MSDS and/or instructions for use on all equipment and/or materials before use.*

1. Consumables

- Phage panning output bacterial culture
- Streptavidin-coated 96-well plates, (e.g., R&D Systems, Cat. No. CP004)
- Biotinylated antigen of interest, 1 µM stock in PBS
- 1.7 mL low-binding snap-cap tubes, (e.g., Corning®, Cat. No. 3207)
- Miniprep DNA kit (e.g., Macherey-Nagel™, Cat. No. 740588.50)
- Sterile water
- IPTG 0.5 M, 0.22 µm sterile filtered, stored at -20 ˚C
- Sterile deep-well 96-well plates, (e.g., Corning®, Cat. No. 3960)
- Breathable plate sealing film, (e.g., Thomas Scientific, Cat. No. BF-400)
- Foil sealing film, for long-term storage of stocks, (e.g., Millipore, Cat. No. Z722642)
- Buffer A:
  - PBS, pH 7.4
  - 0.1% (v/v) Tween-20 (CAS No. 9005-64-5)
  - 1% (w/v) BSA, sterile filtered and stored at 4 ˚C
- PBS, pH 7.4
- *E. coli* cells without *supE44* (TAG codon is encoded as stop). We recommend electrocompetent SS320 cells, (e.g., Lucigen®, Cat. No. 60512-1)
- Anti-VHH antibody with conjugated HRP, (e.g., Jackson ImmunoResearch, Cat. No. 128-035-232)
- Colorimetric substrate, such as ABTS (KPL, Cat. No. 5120-0043 or similar)
- Colorimetric stop solution (e.g., SeraCare, Cat. No. 5150-0017 for ABTS)

2. Prepared solutions, in addition to those from Protocol #1:

- TES buffer:
  - Tris base 200 mM
  - EDTA 0.5 mM
  - Sucrose 500 mM, pH 8.0, sterile filtered and stored at 4 ˚C.
- TES/4 buffer:
  - 250 mL TES buffer
  - 750 mL sterile water, stored at 4 ˚C.

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3. Equipment

- Electroporator and cuvettes
- UV-Vis to measure OD_{600} of cultures and cuvettes
- Benchtop microcentrifuge
- Benchtop centrifuge for 96-well plates
- Incubated orbital shaker
- Plate reader with endpoint colorimetric readout (A_{405} nm for ABTS)

4. Method: Preparing the primary glycerol stocks

4.1 Miniprep 1–3 mL of phage-panning output culture per kit instructions; elute in sterile water.

4.2 Pre-warm 2xYT to 37 ˚C, and pre-warm 6 × 150 mm 2xYT-C agar plates to 37 ˚C.

4.3 Chill electroporation cuvettes on ice for 20 min.

4.4 Transform 200 ng of plasmid DNA following manufacturer's transformation protocol. Rescue with pre-warmed media. Incubate for 15–30 min at 37 ˚C before further handling.

4.5 Prepare 3 dilutions (10X, 100X, 1,000X); streak 100 µL of one dilution on two corresponding agar plates. Streak each remaining dilution on two of the four remaining plates.

4.6 Incubate the agar plates overnight at 37 ˚C.

4.7 The next day, prepare 100 mL of 2xYT-C per deep well plate of stocks.

4.8 Aliquot 1 mL of 2xYT-C per well in a sterile deep well 96-well plate.

> ***NOTE:*** *We prefer to run a positive and negative control on each plate, and so we omit the wells G12 and H12 on a standard plate of cultures.*

4.9 Pick 1 colony for each well from the most disperse agar plate.

4.10 Cover with a breathable plate-cover and grow overnight at 37 ˚C shaking at 200–800 rpm.

> ***NOTE:*** *Optimize shaking speed for your shaker's arm motion and plate stability.*

4.11 The next day, add 1,000 µL of sterile 2xYT-GC+glycerol to each well and shake for 30 min. Seal the plate with a foil seal and store at -80 ˚C for Long-term preservation.

> ***NOTE:*** *1 µL culture volumes of this stock work well for Sanger sequencing reactions.*

5. Method: Preparing the Shockates

5.1 Add 1 mL 2xYT-C medium to each well of a fresh 96-well deep-well plate for the shockate culture.

5.2 Thaw the primary glycerol stock plate completely and transfer 100 µL of the stock volume to the corresponding shockate plate wells.

5.3 Shake the culture at 37 ˚C until average well reaches OD_{600} of 0.3–0.5 (~2.5–4 h).

5.4 Add IPTG to a final concentration of 1 mM.

5.4.1 Add 250 µL of 0.5 M IPTG stock to 5 mL of sterile water to make a working IPTG solution.

5.4.2 Add 50 µL of working IPTG solution to each well of bacterial culture.

5.5 Cover with a breathable plate-cover and grow overnight at 30 ˚C shaking at 200–800 rpm.

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5.6 The next day, spin down the deep well 96-well plates in a centrifuge.

NOTE: Follow the plate manufacturer's RCF limits. Centrifuge 800 × g for 20 min (low ramp speed).

5.7 Confirm that culture is pelleted, and discard supernatants.

5.8 Resuspend pellets in 200 µL of ice-cold TES buffer.

5.9 Cover and incubate with shaking (200–400 rpm) at 4 ˚C or on ice for 30 min.

5.10 Add 400 µL of ice-cold TES/4 buffer.

5.11 Re-cover and incubate with shaking (200–400 rpm) at 4 ˚C or on ice for 30 min.

5.12 Centrifuge 96-well deep-well plates at 4 ˚C, (800 × g for 30 min), within manufacturer's RCF limit.

5.13 Transfer 500 µL supernatant to a sterile deep-well plate without disturbing cell pellet.

5.14 Store covered at 4 ˚C until ready to perform the binding ELISA assay.

6 Method: Anti-VHH single-point ELISA screening

6.1 Coat the streptavidin plates following manufacturer's protocols. Typical coating conditions would be 100 µL of a 100 pmol/mL stock in PBS, coated for 1 h at RT or 4 ˚C overnight.

> *NOTE: We typically prepare a plate with target antigen and a separate plate with off-target antigen or BSA to identify non-specific or 'sticky' binders.*

6.2 Flick and blot dry the ELISA plates to remove antigen.

6.3 Block by adding 100 µL of Buffer A to each well and incubating at RT for 60 min.

6.4 Flick and blot dry the ELISA plates to remove blocking buffer.

6.5 Add 50 µL of the crude VHH shockate per well and 50 µL PBS. Incubate at RT for 60 min.

6.6 Flick and blot dry to remove the VHH shockate.

6.7 Wash 3x with 250 µL/well of Buffer A, flicking and blotting to dry between.

6.8 Prepare a 1:1,000 dilution of anti-VHH-HRP antibody in Buffer A. Confirm with manufacturer's lot recommendation if using alternative antibodies.

6.9 Add 100 µL/well of the anti-VHH solution and incubate at RT for 60 min.

6.10 Flick and blot dry to remove the anti-VHH-HRP.

6.11 Wash 3x with 250 µL/well of Buffer A, flicking and blotting to dry between.

6.12 Wash 1x with 250 µL/well of PBS, then flick and blot to dry plate.

6.13 Add 100 µL/well of ABTS substrate (or suitable alternative).

6.14 Incubate 30 min at RT.

6.15 After 30 min, stop the reaction with 100 µL/well of stop solution.

6.16 Read absorbance at A_{405} (endpoint settings). For this assay setup, a typical 'positive' threshold is A_{405} above roughly 0.5.

> *NOTE: 1) Signal does not scale linearly with affinity. 2) Shockate expression can vary by more than tenfold and is not controlled. 3) Anti-VHH conjugates may favor specific frameworks, so top signals may not indicate the strongest binders.*

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Protocol 3:

Large-scale shockate preparation and purification of VHH-6His construct

> *NOTE: Consult MSDS and/or instructions for use on all equipment and/or materials before use.*

1 Consumables, in Addition to those from Protocol #2:

- 14 mL snap-cap culture tube (e.g., Celltreat®, Cat. No. 230439)
- Nickel-NTA resin (e.g., Macherey-Nagel™, Cat. No. 745400)
- Gravity column (e.g., Genesee, Cat. No. 20-591G12)
- Sterile Erlenmeyer flasks and centrifuge tubes of appropriate scale
- Sterile prepared TB media (e.g., RPI #T15000) or similar
- Optional: Dialysis tubing or cassettes. buffer exchange MWCO column, BCA assay kit.

2 Method: Protein Expression and Purification by Periplasmic Shock

2.1 Inoculate clone(s) of interest from the primary glycerol stock plate (SS320 cells), into 5 mL 2xYT-C in a 14 mL snap-cap tube. Grow overnight at 37 ˚C shaking.

2.2 Prepare TB media with carbenicillin at desired culture volume (typically 50–500 mL) *using a baffled flask with 3X capacity of culture volume for adequate aeration.*

> *NOTE: TB yielded over twice as much as 2xYT, though the benefit was less clear at smaller scales due to proportionally more linear-phase growth.*

2.3 Reserve 1 mL of TB media from the flask to blank the spectrophotometer.

2.4 Dilute the overnight culture 1:100 into fresh TB medium.

2.5 Grow the TB culture with shaking at 37 ˚C until OD_{600} = 0.4.

2.6 Induce with IPTG to a final concentration of 1 mM.

2.7 Continue growth for ~16 h at 30 ˚C.

2.8 The next day, pellet the cells by spinning at 3,000 × g, RT, for 10–20 min.

2.9 Cool centrifuge to 4 ˚C.

2.10 Resuspend pellet in 8 mL ice-cold TES (use 16 mL for 500 mL culture).

2.11 Incubate resuspension with gentle shaking (~200 rpm) for 30 min at 4 ˚C, or on ice.

2.12 Add ice-cold TES/4 buffer at 2x volume (16 mL for small cultures, 32 mL for 500 mL cultures). Incubate suspension with gentle shaking for 30 min at 4 ˚C or on ice.

2.13 Centrifuge cultures at 4 ˚C, 3,000 × g for 10–20 min.

2.14 Transfer supernatant (periplasmic shockate) to a new tube and hold on ice.

2.15 Equilibrate Ni-NTA resin per manufacturer's instructions. Shockate yield is typically 3–10 mg/L; scale resin accordingly.

2.16 Purify using manufacturer's Ni-NTA protocol.

2.17 Dialyze or buffer exchange if needed for downstream work.

2.18 Quantify by A_{280} or BCA assay. Typical VHH extinction coefficients are 20,000–30,000 M^{-1} cm^{-1}; use sequence-specific values for highest accuracy.

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