Nanomolar VHH binders of PD-L1 and other therapeutic targets from the AbNano™ VHH Naive Library

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Background

VHH domains, derived from the variable heavy chain of the heavy-chain-only IgG2 and IgG3 domains in camelids, represent a small, single-domain antibody fragment. VHH domains have been successfully applied to technologies such as bispecific therapeutic molecules.

Discovering novel VHH domains can take place by multiple workflows, including using B-cell sorting and using display libraries. Within display libraries, the library is generally one of three classes of molecule: synthetic molecules, immune-derived molecules, or naïve germline molecules. Each approach has its own unique advantages and applications.

Here, we present the construction and early validation of a large naïve library from llamas and alpacas. The data suggests that this library, the AbNano™ VHH Naïve Library, may be well-suited for rapid discovery of VHH domains binding to therapeutic targets with varying levels of affinity.

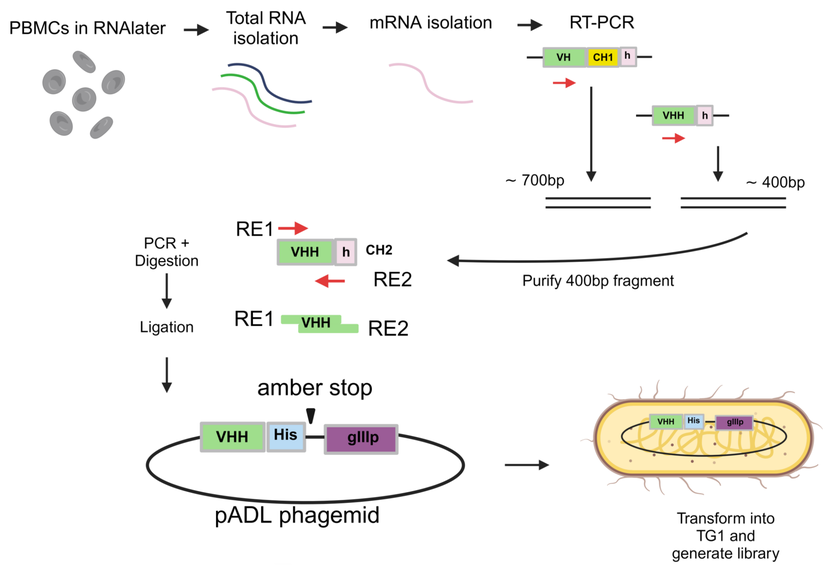

Figure 1: Overview of the library construction process. A total of 1.12 x 10^{10} transformants were collected from 13 sublibraries.

Library Construction

The library was constructed from 103 naïve animals: 77 llamas and 26 alpacas. A total of 1.51 x 10^{10} PBMC cells were collected and used for library construction.

Libraries were constructed using species-specific primers. A total of 13 sublibraries were constructed through transformation. The total number of transformants for the library was 1.12 x 10^{10} cfu. Transformants were analyzed by Sanger and the individual sublibraries were analyzed by next generation sequencing at a total read count of 1.71 M reads using GeneWiz® Amplicon-EZ services from Azenta Life Sciences. NGS reads from each sublibrary were combined and analyzed using Geneious Biologics.

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 x 10^{9} unique clonal VHH.

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.

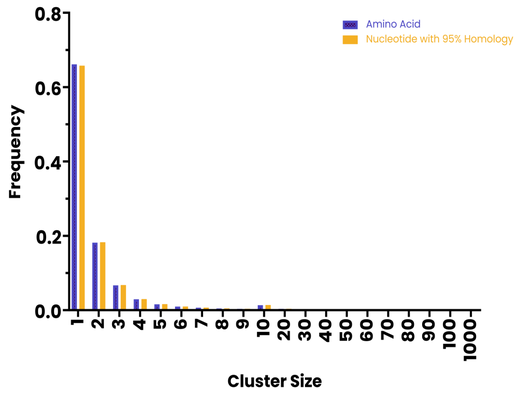

Figure 2: Comparison of cluster diversity for amino acid identity of CDR3 and for 95% homologous nucleotide identity of CDR3 suggests that there is minimal inherent bias in the final constructed library independently of any call or mutation artifacts.

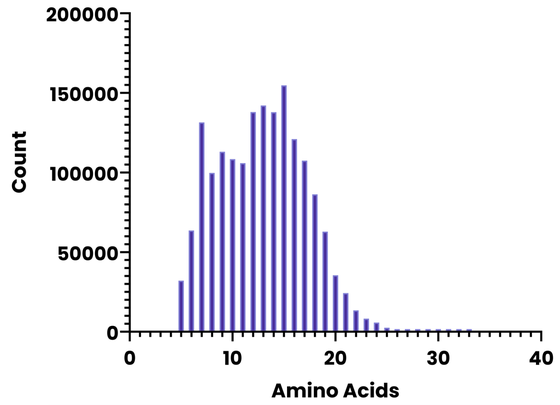

Figure 3: Histogram of length distribution in CDR3 of the VHH domains in the library. CDR3 length distribution of the pooled naïve library fits well with existing literature values for naïve camelid CDR3 and has good representation of CDR3 lengths from 5-22 residues in length.

Panning and Screening

Phage selections were performed from the pooled phage input. Round 1 panning was performed with approximately 2.5 x 10^{10} pfu of phage. Here, we present campaigns against three separate targets of interest: INSRβ ECD, EGFR ECD, and PD-L1 ECD. All antigens were purchased commercially from Acro Biosystems.

For INSRβ panning, the recombinant antigen (residues H28-K944 of accession number P06213-2 with a C-terminal His tag) was adsorbed onto polystyrene plates. Phage was eluted by triethylamine after panning and PEG-precipitated between rounds. Clonal shockate screening generated 47 hits above the threshold of A405 > 0.4, with 29 unique binders by CDR3 analysis. Clone AbN3c-F01 (531 nM EC_{50}, Figure 5) was the most abundant clone in the sequenced pool.

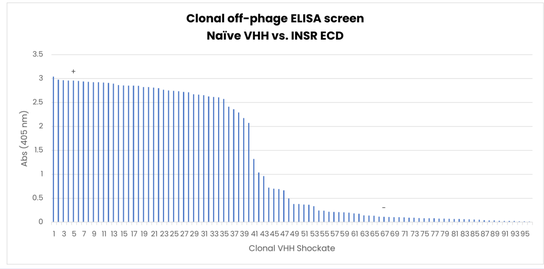

Figure 4. Clonal crude VHH were prepared by osmotic shock and assayed against recombinant INSR adsorbed on polystyrene plates. The single-point binding was detected by HRP-anti-VHH conjugate and ABTS.

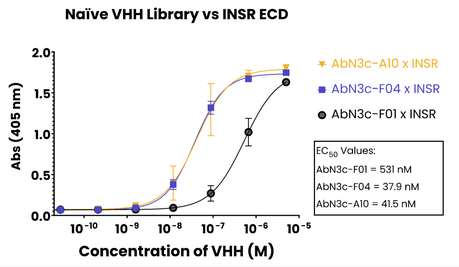

Figure 5. INSR lead VHH identified from clonal screen data were purified by Ni-NTA and assayed in duplicate titration. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

Recombinant human epidermal growth factor (residues A310-C620 of accession number P00533-1 with C-terminal biotinylated Avi tag and poly-His) was pre-bound to streptavidin-coated magnetic beads. Phage was eluted by triethylamine after panning, and unprecipitated supernatant was used for further rounds. Clonal shockate screening generated 34 hits above the threshold of A405 > 0.4, but only 7 unique sequences by CDR3 analysis. AbN16-E07 was a dominant clonotype comprising 14 of the 34 hits.

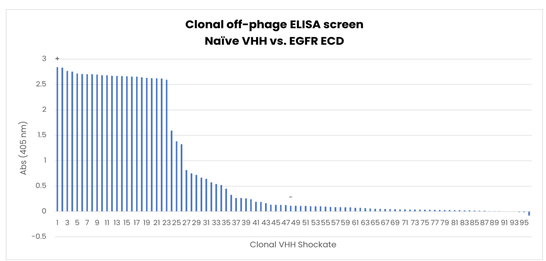

Figure 6. Clonal crude VHH were prepared by osmotic shock and assayed against recombinant EGFR bound to streptavidin plates. The single-point binding was detected by HRP-anti-VHH conjugate and ABTS.

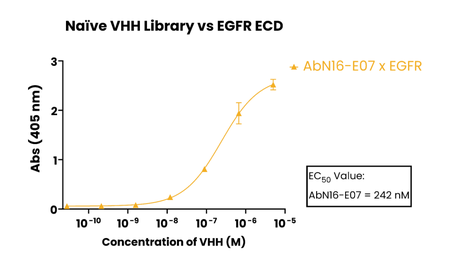

Figure 7. EGFR lead VHH AbN16-E07 identified from clonal screen data was purified by Ni-NTA, then was assayed in duplicate titration. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

Recombinant human PD-L1 (residues F19-Y134 from accession number Q9NZQ7-1 with C-terminal biotinylated Avi tag and poly-His) was incubated with blocked phage before being pulled down by addition of streptavidin-coated magnetic beads. Phage was eluted by triethylamine after panning and PEG-precipitated between rounds. 470 clones were screened by single point ELISA, generating 147 hits above the threshold of A405 > 0.4 with 68 unique sequences identified from the 470 clones screened. This represents a unique hit rate of 14.5%.

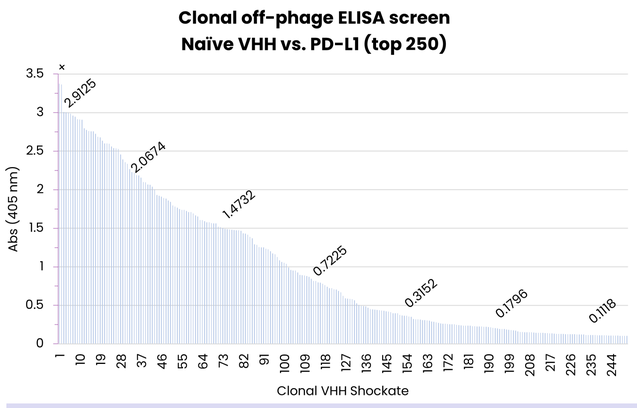

Figure 8. Clonal crude VHH were prepared by osmotic shock and assayed against recombinant PD-L1 bound to streptavidin plates. The single-point binding was detected by HRP-anti-VHH conjugate and ABTS.

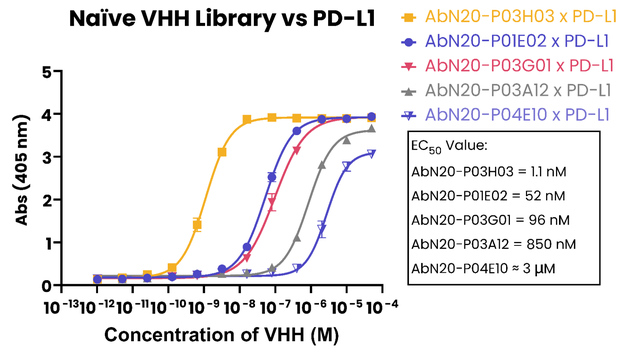

Figure 9. Five clones with varying single-point ELISA signal (P03H03 = 3.38, P01E02 = 3.00, P03G01 = 2.91, P03A12 = 0.62, P04E10 = 1.23) were purified by Ni-NTA then assayed in duplicate titration. Binding was visualized with HRP-anti-VHH conjugate and ABTS.

Conclusions & Future Directions

Early validation of the AbNano™ VHH Naïve Library suggests that the library is diverse and well-suited for rapid discovery of VHH domains binding to protein targets in a range of panning conditions. There is evidence to suggest that PEG precipitation between rounds minimizes the clonotype collapse at round 3. There is evidence to suggest that clones with approximately three orders of magnitude difference in affinity can be recovered side-by-side.

Future directions with this library are abundant. Additional panning verification datasets will yield greater confidence in library quality, especially against atypical antigens or antigen presentations. Semi-synthetic methods to diversify the library pool may be appealing at first, but due to the fully natural framework sampling such methods would inadvertently reduce the diversity in the frameworks. Individual hit and lead molecules are appealing to characterize and develop. Lead molecules derived from this library may also be independently engineered toward therapeutic applications.

Acknowledgements

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