Engineering Synthetic Multivalent VHH Antibodies at Scale

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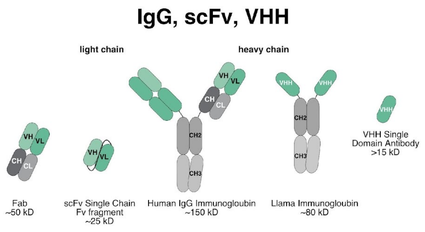
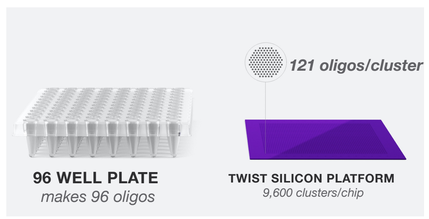
INTRODUCTION

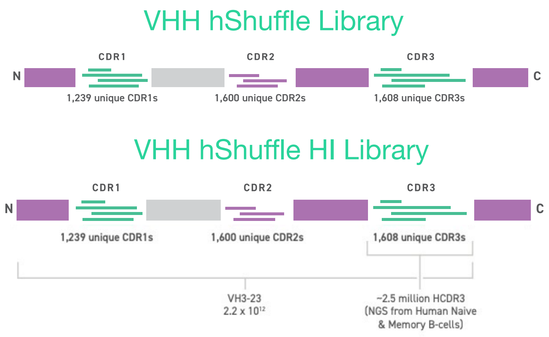
Antibodies must bind their targets with high avidity to effect therapeutic or diagnostic outcomes. The mammalian immune system can produce antibodies with micro- to nanomolar affinity through *in vivo* affinity maturation. *In vitro* display technologies have surpassed this affinity ceiling, enabling discovery of antibodies with picomolar and femtomolar affinities. *In vitro* display technologies offer immediate access to the antibody fragment genes, which can subsequently be multimerized to generate high-avidity antibody fusions.

Among the available antibody formats, single-domain antibodies (VHH) are one of the most engineerable. VHH antibodies have many advantages including their increased thermostability, smaller-molecular weight, lower production cost, and modular format for building multivalent biologics. These advantages have encouraged many to prioritize VHHs when developing diagnostic and therapeutic antibodies against severe acute respiratory coronavirus 2 (SARS-CoV-2), the etiological agent of the coronavirus disease 2019 (COVID-19) pandemic. Multivalent SARS-CoV-2 antibodies have been engineered more recently.`



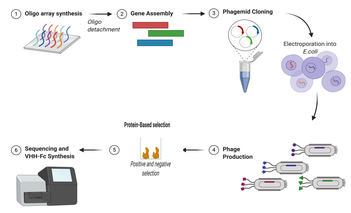
VHH antibodies can be sourced from immune, naïve, or synthetic antibody libraries. Immune and naive libraries require amplification from animal B cells, and are prone to redundancy and bias, which can limit library diversity. Synthetic libraries are based on computational in silico design and use synthetic oligos to introduce diversity. Using Twist's unique silicon-based DNA synthesis platform, we have generated VHH libraries with high diversity (>10^{10}), accuracy (1 error/2 kb), and productivity (> 80%). Recently, we reported the discovery of several high-affinity VHH-Fc antibodies against the SARS-CoV-2 S1 protein subunit from these libraries.





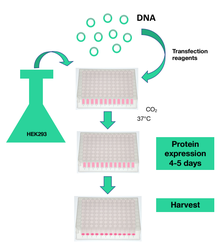
Unlike methods for the construction of synthetic antibody libraries, methods for screening multivalents are comparatively low throughput and ineffective for higher-order multivalent constructs where the number of possible permutations increases exponentially. Beginning with 13 anti-S1 VHH candidates, we constructed multivalent libraries of tandem-assembled VHHs, screened them by phage display, and sequenced the enriched libraries to determine the positional frequencies of each individual VHH fragment Using this pipeline, we engineered mono- and bispecific multivalent VHH-Fc constructs with higher affinity for S1 than their monovalent counterparts.

ANTI-S1 VHH CANDIDATE DISCOVERY

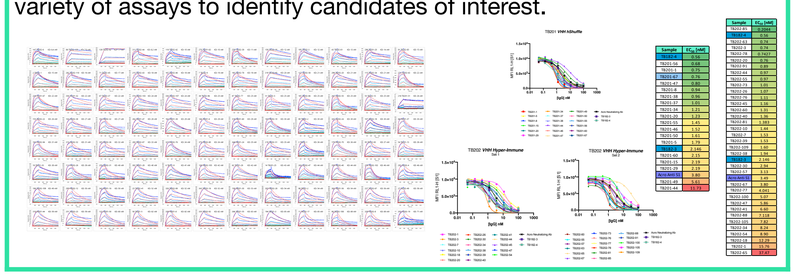


For our anti-S1 campaigns two of our phage libraries VHH hShuffle and VHH hShuffle HI were used (TB201, TB202). Phage ELISAs as well as NGS sequencing was used for candidate identification.

Taking advantage of our HT Antibody Production Platform, over 200 candidates were reformatted into VHH-Fc fusions. The constructs were codon optimized, cloned into custom high-copy mammalian expression vectors, and expressed at the 1 mL scale in HEK293 cells.

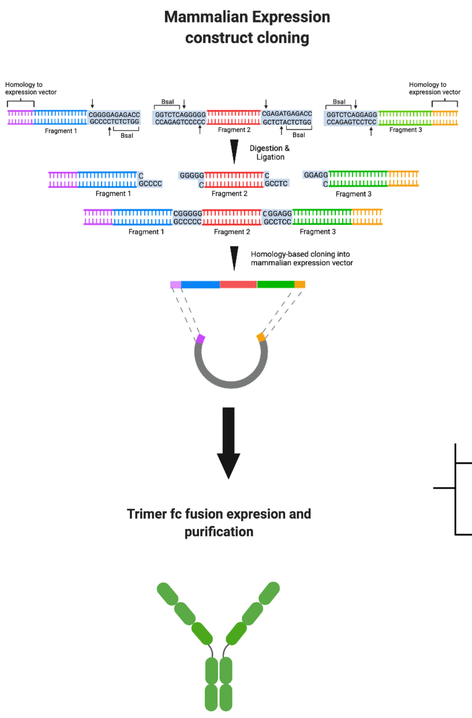


To fully leverage our throughput, the Carterra LSA system was used to rapidly assess the binding of our VHH Fc variants. Scores of TB201 and TB202 VHH-Fcs bound with a range of affinities in the picomolar and nanomolar range to S1 and S trimer. Yield was sufficient for a variety of assays to identify candidates of interest.



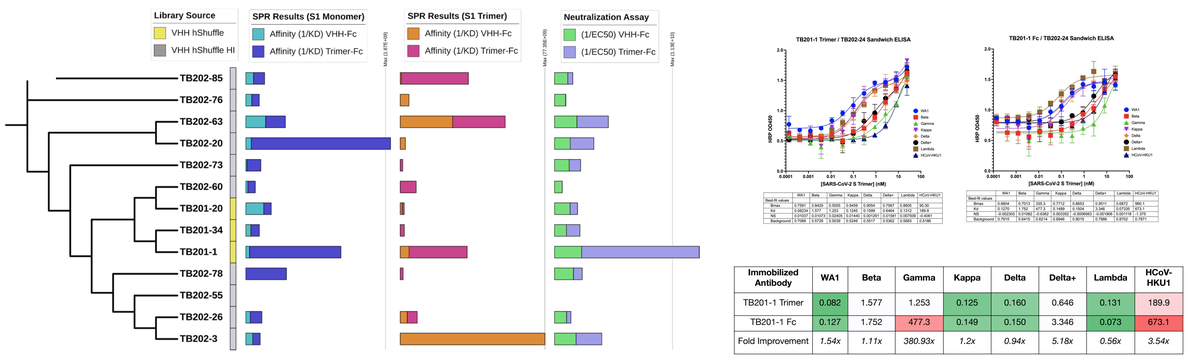
MULTIVALENT WORKFLOW

A. Homotrimer Cloning, Expression, and Assessment



Multivalent constructs have been shown to improve construct affinity to the target of interest. Although we have been successful in generating neutralizing, high-affinity anti-S1 VHH-Fc antibodies from phage-display, we sought to further improve the affinity of 13 candidates by trimerization. We initially constructed homotrimer VHH-Fc antibodies for each candidate by ligating three identical VHH cassettes, separated by 2x4GS-AS linkers, and inserting the resulting construct into an Fc acceptor vector.

The trimeric nature of the constructs along with the repetitive and GC-rich nature of the linkers makes full-length DNA construct synthesis challenging. To overcome this challenge, we flanked VHH genes with Type II restriction sites to enable seamless directional assembly of three VHH fragments in tandem and homologous overhangs to enable the insertion of these trimeric constructs into an Fc-fusion acceptor vector. We expressed our homotrimer VHH-Fcs along their VHH-Fc counterparts and compared them side-by-side in affinity and neutralization assays.



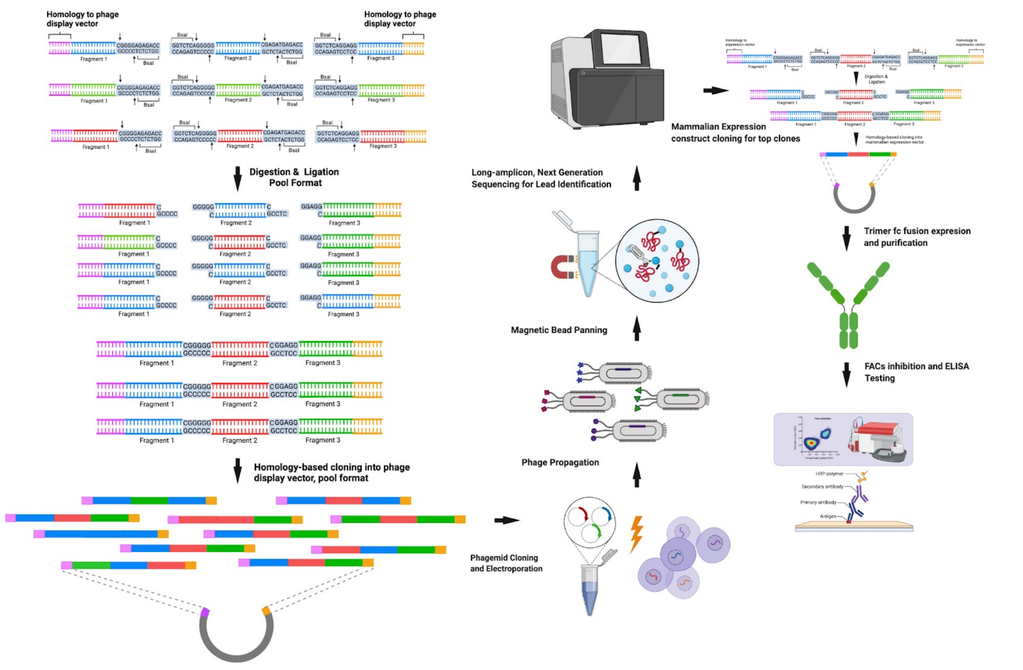
Immobilized Antibody	WA1	Beta	Gamma	Kappa	Delta	Delta+	Lambda	HCoV-HKU1
TB201-1 Trimer	0.082	1.577	1.253	0.125	0.160	0.646	0.131	189.9
TB201-1 Fc	0.127	1.752	477.3	0.149	0.150	3.346	0.073	673.1
Fold Improvement	*1.54x*	*1.11x*	*380.93x*	*1.2x*	*0.94x*	*5.18x*	*0.56x*	*3.54x*

Overall, trimerization of VHH sequences conferred higher binding affinities to the S1 monomer and S1 trimer as well as greater competition with the ACE2 receptor in the S1-ACE2 competition. Trimerization of TB201-1 (1-1-1) resulted in marked improvements across all three measures. To further characterize 1-1-1, we evaluated its ability to detect a wide variety of SARS-CoV-2 S variants when paired with TB202-24 in a sandwich ELISA. In this assay, immobilized 1-1-1 or TB201-1 served as a capture antibody; TB202-24 was used as a detector antibody because it binds a unique S1 epitope not targeted by any other antibody. S trimers from the original WA1/2020 strain and the Beta, Gamma, Kappa, Delta, Delta+, and Lambda variants were assayed. The S trimer of human coronavirus HKU1 (hCoV-HKU1) was used as a negative control. 1-1-1 detected all S variant trimers except the Delta and Lambda S trimers with a higher apparent binding affinity than TB201-1.

B. Trimer Library Approach

Bispecific antibodies possess several advantages over their monospecific counterparts. Bispecificity can make diagnostic antibodies more sensitive and therapeutic antibodies more broadly neutralizing. Since our homotrimeric assembly strategy also lends itself to heterotrimeric assembly, we generated a library of bispecific anti-S1 VHH antibodies for screening and characterization. We generated a phage library to interrogate the 2,197 homo- and heterotrimeric permutations that can be assembled from our initial 13 VHH leads.

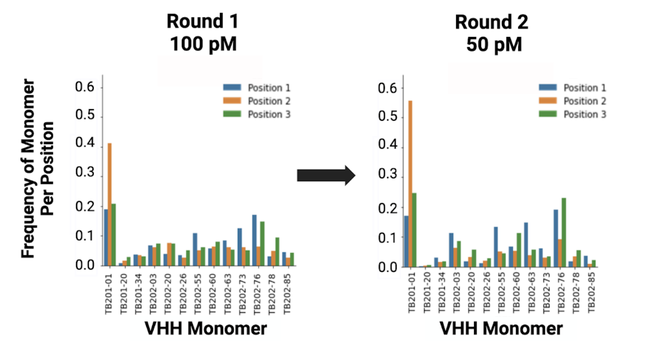
The resulting phage library was biopanned with low antigen concentrations (100 pM, 50 pM) over two rounds to enrich for high-affinity binders and long-read NGS sequencing was performed on the round outputs for candidate selection.

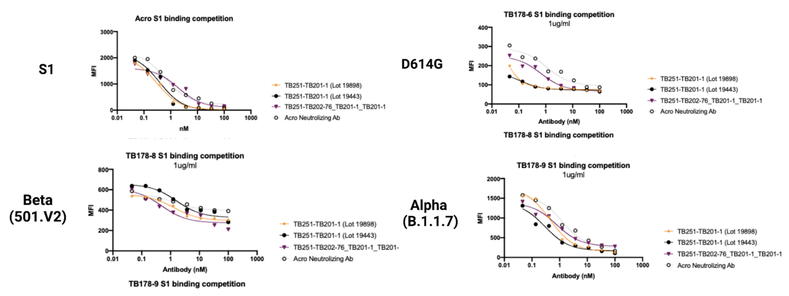


C. Results

Long-read next-generation sequencing was used to assess the frequency of each VHH monomer in each trimeric position (1, 2 ,3). Sequencing revealed TB201-1 to be the most prevalent VHH fragment at the secondary position after enrichment; the most frequent VHH fragments at primary and tertiary positions were TB201-1 and TB202-76. We decided to clone one bispecific trimer construct into a mammalian expression vector for further characterization: 76-1-1.

We evaluated the ability of our lead monospecific (1-1-1) and bispecific (76-1-1) to block S1 binding to cellular ACE2 (receptor through which infection occurs), via a cellular competition assay. In our competition assays we assessed various SARS-CoV-2 variants including: D614G, Alpha and Beta. TB201-1 homotrimer fared better against the wild-type, D614G, and Alpha S1 proteins while (76-1-1) heterotrimer more potently blocked the interaction between ACE2 and the S1 protein of the Beta variant. We also evaluated the construct affinity against S1 in a titration ELISA and compared their performance to their VHH-Fc counterparts.





Format	Construct	Neutralization (1/EC50 nM)				ELISA (1/EC50 nM)
		S1 Antigen	D614G	Beta	Alpha	S1 Trimer
Homotrimer-Fc	1-1-1	2.61	9.93	0.63	3.38	1246.90
VHH-Fc	TB201-1	-				833.33
Heterotrimer-Fc	76-1-1	0.53	1.49	2.03	1.30	1009.30
VHH-Fc	TB202-76	-				190.11
IgG	Acro control	1.55	0.92	0.72	0.81	-

With our pipeline we improved the affinity of anti-S antibodies against SARS-CoV-2 variants by trimerizing the VHH sequences of VHH-Fc antibodies. We also developed a high-throughput approach for screening multivalent permutations of VHH fragments. Panning against specific antigens with more diverse multivalent libraries has great potential.

SUMMARY

High-throughput technologies such as next-generation sequencing, microarray-based gene synthesis, and phage display have empowered the discovery and engineering of precisely defined, synthetic antibodies with high avidity and drug-like features. Here, we describe a scalable process for engineering of homo- and hetero-trimer variable domain of camelid heavy-chain (VHH)-Fc antibodies against the severe acute respiratory coronavirus 2 (SARS-CoV-2) spike (S) protein. Overall, we demonstrate that VHH trimerization is an effective and modular approach for increasing the affinity of anti-S1 VHH-Fc antibodies for the highly mutated S proteins of SARS-CoV-2 variants. This approach could be leveraged to improve existing antibody-based diagnostics and therapeutics targeting SARS-CoV-2 as it evolves and increase affinity to potentially to any target.