Identification of "Hot Spots" in the CDRs of a SARS-CoV2 Antibody by Alanine Scanning

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1. What is Alanine Scanning?

Alanine Scanning is a powerful and systematic tool, commonly used to determine the amino acid residues responsible for the function of a protein of interest[1].

An alanine mutant library is created by replacing non-alanine amino acids with alanine one at a time through site-directed mutagenesis within a specific region of the protein, (**Figure 1**). Alanine is used due to its small size, chemical inertness, and structural benefits for mutagenesis studies[2].

A loss of function exhibited by an alanine mutant indicates that the corresponding wild-type residue plays a contributing role to the function of the protein and thus is so-known as a 'hot spot'. It is a simple, effective, and rapid approach to determine the contribution of each amino acid residue to a specific protein function.

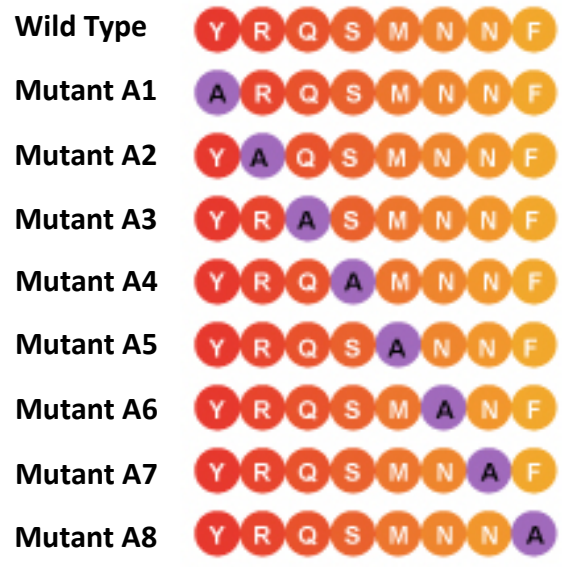

Figure 1. Alanine scanning mutagenesis. Replacement of each wild-type amino acid with an alanine residue within a specific region of a protein of interest, generating a library of 8 mutants.

2. GenScript's Alanine Scanning Libraries

GenScript offers high-quality alanine scanning libraries that can be delivered in two different formats: pooled or arrayed. In the former deliverable format, library mutants are all pooled together and delivered in a single tube; a positive rate or NGS data is provided to indicate the library coverage. In the latter deliverable format, library mutants are individually arrayed and delivered sequence-verified (Figure 2), providing the end users convenience, confidence, and efficiency for their subsequent step in the evaluation process.

GenScript's arrayed alanine scanning libraries enable researchers to rapidly identify the essential amino acid residues responsible for a specific protein function due to their sequence-verified and ready-to-use deliverable format.

3. Utility

In this application note, we harnessed the power of the alanine scanning mutagenesis tool and demonstrated the utility of our arrayed alanine scanning library in an effort to rapidly identify amino-acid residues within the complementarity-determining regions (CDRs) of an antibody of the SARS-CoV2 virus that are essential for binding its target antigen, the receptor-binding domain (RBD) of the viral Spike (S) protein.

The SARS-CoV2 antibody, named BS-R2B12, is one of the resulting antibodies obtained from rabbits immunized with the RBD of the S protein through our B-cell Sorting Platform. This antibody has also been shown to bind both the RBD and the S protein with similar affinities in the order of 10^{-10} M.

4. Experimental Design and Results

An antibody mutant library was created by replacing each non-alanine amino acid residue in the six CDRs of the BS-R2B12 antibody with alanine, while the native alanine sites were each replaced with serine, one at a time through our high-throughput site-directed mutagenesis (HTP SDM), generating a total of 66 mutants. A schematic diagram of alanine replacing each amino acid within the CDR1 of the variable region of the heavy chain of the antibody is shown in **Figure 2** as an example.

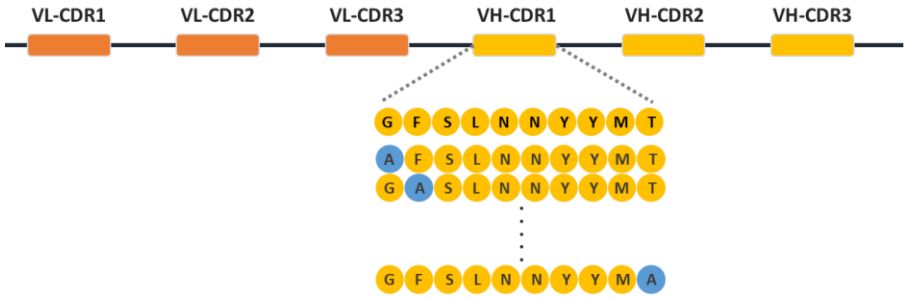

Figure 2. A schematic diagram of alanine replacement in each site within a specific region of an antibody, resulting in a library of corresponding alanine mutants. An example of such alanine replacement is shown within the CDR1 of the variable region of the heavy chain (VH).

Following the library construction, the library mutants were screened, individually sequence-verified, and arrayed. Each of the arrayed, readily-available mutants was then transformed into an expression host. The resulting individual mutant clones were subsequently evaluated via our high-throughput expression and ELISA-based characterization platform, known as FASEBA, for determining the antigen binding activities of these individual mutants. The ELISA data in **Figure 3** showed that there were 13 mutants with reduced binding activities (represented by their corresponding values of <1 and indicated in red dots), when compared to that of the wild type (represented by a value of 1). This result suggested that the corresponding residues in the wild-type sequence may each play a significant role in binding the target antigen.

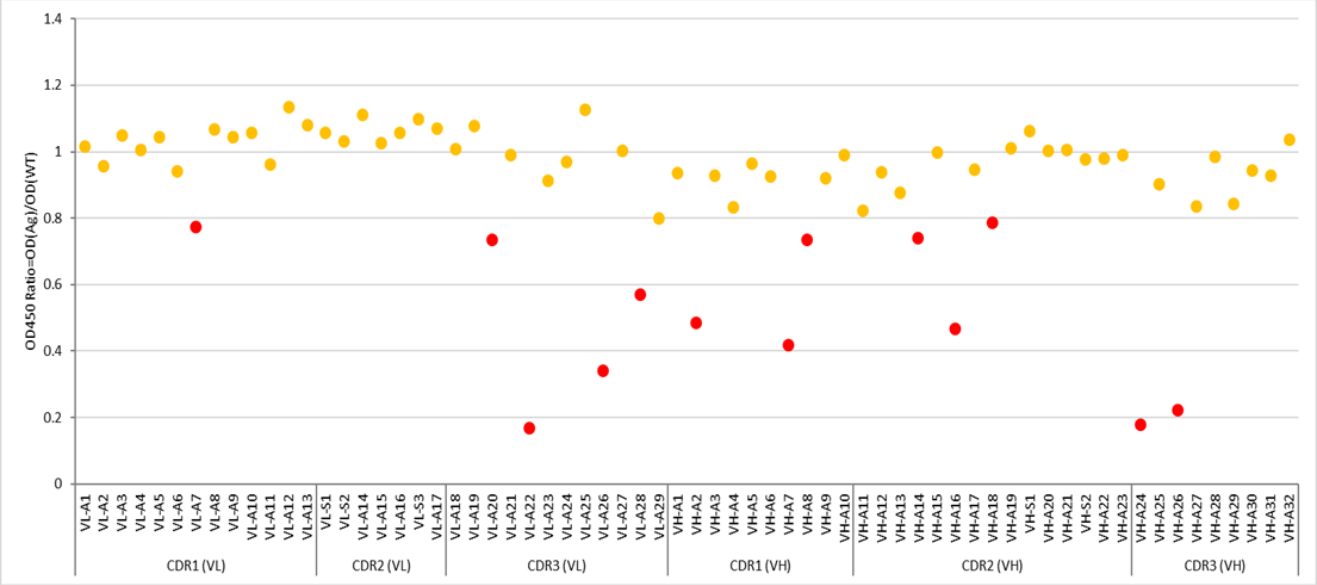

Figure 3. ELISA-based antigen binding activities exhibited by the individual mutants of the arrayed alanine scanning library derived from BS-R2B12. The alanine mutants in yellow dots exhibited binding activities similar to that of the wild type (represented by a value of 1) whereas those mutants in red dots showed binding activities less than that of the wild type. Any value above or below 1 is considered better and worse than the wild type in target binding, respectively.

The 13 mutants with reduced binding activities from the ELISA experiment were further characterized using Surface Plasmon Resonance (SPR) assays to quantify their binding affinities. The resulting binding affinities represented by their corresponding KD values are shown in **Table 1**. Consistent with the ELISA results, all 13 mutants exhibited target binding affinities lower than that of the wild type (highlighted in blue), with a few of them close to an order of magnitude lower (highlighted in orange), further confirming the significance of these corresponding residues in target antigen binding. Consequently, such experimental information allows scientists to take a more rational approach by targeting specific residues/sites in engineering the wild-type antibody to potentially improve its antigen-binding affinity.

Arrayed alanine scanning libraries can help researchers to engineer their proteins of interest more efficiently and effectively through a targeted, data-driven approach.

5. Summary

Alanine Scanning is an effective mutagenesis tool commonly used by various researchers to identify the key residues responsible for the function of a protein of interest. Here, we utilized such a powerful tool in the identification of the amino acid residues within the CDRs of a SARS-CoV2 antibody that potentially have contributing roles in binding its target antigen, by creating an arrayed alanine scanning library, individually expressing the sequence-verified library mutants in a host, and characterizing their binding activities through ELISA and SPR assays. The resulting binding data provided residue/site-specific information that can be used to improve the binding affinity of the antibody in a more targeted approach. Such utilization of arrayed alanine scanning libraries is applicable to any antibodies or proteins, whose functions are desired to be improved through a more rational and targeted approach.

Table 1. Target binding affinities measured by SPR.

Mutant	ka (1/Ms)	kd (1/s)	KD (M)
WT	1.93E+06	2.02E-04	1.05E-10
VL-A7	1.12E+06	7.10E-04	6.31E-10
VL-A20	1.56E+06	5.15E-04	3.30E-10
VL-A22	1.23E+06	5.47E-03	4.45E-09
VL-A26	1.45E+06	4.97E-03	3.42E-09
VL-A28	1.82E+06	1.57E-03	8.67E-10
VH-A2	1.48E+06	1.03E-03	6.97E-10
VH-A7	1.43E+06	1.82E-03	1.28E-09
VH-A8	2.38E+06	9.46E-04	3.97E-10
VH-A14	1.80E+06	7.98E-04	4.43E-10
VH-A16	1.11E+06	1.40E-03	1.27E-09
VH-A18	1.40E+06	5.97E-04	4.26E-10
VH-A24	1.52E+06	4.26E-03	2.80E-09
VH-A26	2.46E+06	9.57E-03	3.89E-09

6. References

1. Berdougo, E. High-Throughput Alanine Scanning: Epitope Mapping and Engineering Complex Membrane Proteins by Comprehensive Mutagenesis. *Genetic Engineering & Biotechnology News*. 32:30-31 (2012).
2. Lefèvre, F. et al. Alanine-stretch scanning mutagenesis: a simple and efficient method to probe protein structure and function. *Nucleic Acids Research.* 25: 447–448 (1997)

7. Notes

For more information about our Alanine Scanning Library Service, please visit:
https://www.genscript.com/alanine_scanning.html

For more information about our B-cell Sorting Platform and the BS-R2B12 antibody, please contact: grace.tan@genscript.com.